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A Translational Approach of Mouse and Human Studies to Integrate Chronobiology into Therapies for Psychiatric Disorders:

From Bedside to Bench… and Back.

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„Das Leben ist wert, gelebt zu werden, sagt die Kunst, die schönste Verführerin; das Leben ist wert, erkannt zu werden, sagt die Wissenschaft“.

— Friedrich Nietzsche

Abstract

Background: Circadian rhythms are endogenous manifestations of the external 24-hour light-dark cycle that allow the organism to adapt and to anticipate daily temporal changes in the environment. These ~24-hour rhythms, also called circadian clocks, are driven by clock genes in almost every cell throughout our body and are set to 24 hours each day by the external light and dark cycle. Because circadian clocks regulate virtually all of our physiology and behavior, organisms may be susceptible to various types of disorders when circadian rhythms are disrupted. Thus, there is, for example, a bidirectional relationship between the disturbance of circadian clocks and the development of psychiatric disorders, such as anxiety disorders, and alcohol use disorder (AUD).

Results: We show that *Cryptochrome 1* and *2* double knockout mice (*Cry1/2*$^{-/-}$), which do not express endogenous circadian rhythms, exhibit a pronounced anxiety-like phenotype and are more sensitive to stressful situations. These behavioral effects are confirmed by increased neuronal activity (*c-Fos*) in the basolateral amygdala. Furthermore, we show that the *Cry1/2*$^{-/-}$ mice exhibit distinct traits that predispose humans to an increased risk of problematic alcohol consumption. *Cry1/2*$^{-/-}$ mice show lower alcohol consumption behavior (*liking*) concomitant with higher motivation to acquire the substance (*wanting*), a finding that is consistent with the incentive sensitization theory of addiction. These phenotypes are also supported by molecular analyses: In the absence of the *Cry* genes, the stress hormone corticosterone is continuously elevated, and the level of the orexin precursor prepro-orexin is persistently low, which together represent explanatory factors for an overall altered alcohol preference. In terms of gene-environment interaction, the phenotype of altered alcohol drinking behavior of *Cry1/2*$^{-/-}$ mice, was enhanced by additional environmental circadian perturbations (shift work model).

Outlook: Our results underline the importance of stable endogenous and environmental circadian rhythms as well as their interaction for mental health. From our findings, we assume that patients suffering from anxiety disorders, AUD, or both, regardless of whether underlying circadian rhythm disturbances are genetically or environmentally induced, may benefit from chronotherapies. This is why, based on our results, we developed a new adjunctive chronotherapeutic treatment for AUD patients.

Zusammenfassung

Hintergrund: Zirkadiane Rhythmen sind endogene Manifestationen des äußeren 24-Stunden-Hell-Dunkel-Zyklus, die es dem Organismus ermöglichen, sich an täglich wiederkehrende Veränderungen in der Umwelt anzupassen und diese zu antizipieren. Diese ~24-Stunden-Rhythmen, auch zirkadiane Uhren genannt, werden durch Uhrengene in unserem gesamten Körper gesteuert und durch Licht- und Dunkelheit auf die äußere Zeit der Umwelt eingestellt. Da zirkadiane Uhren praktisch unsere gesamte Physiologie und unser Verhalten regulieren, können Organismen, bei möglichen Störungen der circadianen Rhythmen, anfällig für verschiedene Arten von Krankheiten sein. So besteht beispielsweise ein bidirektionaler Zusammenhang zwischen der Störung der zirkadianen Uhren und der Entwicklung psychiatrischer Erkrankungen wie Angststörungen und Alkoholabhängigkeit (AUD).

Ergebnisse: Bei *Cryptochrome 1* und *2* Doppelknockout-Mäusen (*Cry1/2*$^{-/-}$), die keine endogenen zirkadianen Rhythmen exprimieren können, zeigen wir, dass diese einen ausgeprägten angstähnlichen und unruhigen Phänotyp aufweisen und empfindlicher auf Stresssituationen reagieren. Diese Verhaltenseffekte werden durch eine erhöhte neuronale Aktivität (*c-Fos*) in der basolateralen Amygdala bestätigt. Darüber hinaus zeigen wir, dass dieselben Mäuse bestimmte Merkmale aufweisen, die beim Menschen zu einem erhöhten Risiko für problematischen Alkoholkonsum führen. *Cry1/2*$^{-/-}$ Mäuse zeigen ein geringeres Alkoholkonsumverhalten (*liking*) bei gleichzeitig höherer Motivation, die Substanz zu erwerben (*wanting*), ein Befund, der mit der Anreizsensibilisierungstheorie der Sucht in Einklang steht. Diese Phänotypen werden auch durch molekulare Analysen gestützt: In Abwesenheit der *Cry*-Gene ist das Stresshormon Kortikosteron kontinuierlich erhöht und der Spiegel des Orexin-Vorläufers Prepro-Orexin ist konstant niedrig, was zusammen Erklärungsfaktoren für eine insgesamt veränderte Alkoholpräferenz darstellt. Im Sinne einer Gen-Umwelt-Interaktion, wurde der Phänotyp des veränderten Alkoholtrinkverhaltens der *Cry1/2*$^{-/-}$ Mäuse, durch zusätzliche umweltbedingte zirkadiane Störungen (Schichtarbeitsmodell), verstärkt.

Ausblick: Unsere Ergebnisse unterstreichen die Bedeutung von stabilen endogenen und umweltbedingten zirkadianen Rhythmen sowie deren Interaktion für psychische Gesundheit. Aufgrund unserer Ergebnisse gehen wir davon aus, dass Patienten, die an Angst, AUD oder beidem leiden, unabhängig davon, ob die zugrundeliegenden zirkadianen Rhythmusstörungen genetisch oder umweltbedingt sind, von

Chronotherapien profitieren können. Aus diesem Grund haben wir auf der Grundlage unserer Ergebnisse eine neue chronotherapeutische Zusatzbehandlung für AUD-Patienten entwickelt.

Table of Contents

List of publications

This thesis is based on the following publications, referred to in the text by their Roman numerals.

Paper I (Original research paper)

Hühne, A., Volkmann, P., Stephan, M., Rossner, M., & Landgraf, D. (2020). **An in-depth neurobehavioral characterization shows anxiety-like traits, impaired habituation behavior, and restlessness in male *Cryptochrome*-deficient mice.** *Genes, brain, and behavior, 19*(8), e12661.

Paper II (Original research paper)

Hühne, A., Echtler, L., Kling, C., Stephan, M., Schmidt, V. M., Rossner M., & Landgraf, D. (2021). **Circadian gene × environment perturbations influence alcohol drinking in *Cryptochrome*-deficient mice.** *Addiction Biology,* e13105.

Additional Paper III (Study protocol paper)

Hühne, A., Hoch, E., & Landgraf, D. (2021). **DAILY - A Personalized Circadian *Zeitgeber* Therapy as an Adjunctive Treatment for Alcohol Use Disorder Patients: Study Protocol for a Randomized Controlled Trial.** *Frontiers in psychiatry*, *11*, 569864.

Additional Paper IV (Review paper)

Hühne, A., Welsh, D. K., & Landgraf, D. (2018). **Prospects for circadian treatment of mood disorders.** *Annals of medicine*, *50*(8), 637–654.

List of abbreviations

AAV	adeno-associated viruses
AUD	alcohol use disorder
BD	bipolar disorder
BMAL1	brain and muscle arnt-like protein 1
Casein kinase	casein kinase 1ε/δ (CK1ε/δ)
CBSRT	cognitive behavioral social rhythm therapy
CBT-I	cognitive behavioral therapy for insomnia
CLOCK	circadian locomotor output cycles kaput
CORT	corticosterone
CRT	circadian reinforcement therapy
Cry1/2	cryptochromes1/2
EEG	Electroencephalography
GWAS	Genome wide association study
HPA	hypothalamic-pituitary-adrenal
H	hour
IPSRT	interpersonal and social rhythm therapy
MDD	major depressive disorder
Per1/2/3	period1/2/3
PFC	prefrontal cortex
PPO	prepro-orexin
PTSD	post-traumatic stress disorder
QPCR	quantitative real-time polymerase chain reaction
REM	rapid eye movement
SCN	suprachiasmatic nucleus
SNRI	selective serotonin-noradrenalin-reuptake-inhibitors
SNP	single nucleotide polymorphism
SSRI	selective serotonin reuptake inhibitors
SUD	substance use disorder
TTL	transcriptional-translational feedback loop
VTA	ventral tegmental area

Aims of the thesis

A Translational Approach of Mouse and Human Studies to Integrate Chronobiology into Therapies for Psychiatric Disorders: From Bedside to Bench… and Back.

During my PhD, I wanted to mechanistically study observations I made in psychiatric patients in the clinic in mouse models in order to use these results to develop new therapeutic approaches.

a. We know from clinical studies that psychiatric disorders such as affective disorders, anxiety disorders as well as AUD are often associated with circadian dysregulations.

b. In mouse models that have either genetically or environmentally disturbed circadian clocks or both, I investigated mechanisms linking circadian clock disturbances with the development of abnormal anxiety-like and alcohol drinking behavior.

c. Utilizing the knowledge that I obtained from these animal studies, I was able to develop a novel, complementary therapy for AUD patients with comorbid depression and anxiety disorders.

1. Introductory summary

1.1 The circadian system

Most organisms generate endogenous daily oscillations of gene expression which ultimately drive rhythms of their behavior and physiology [1]. They enable organisms to anticipate daily recurring environmental changes such as light, temperature, and food availability to ensure optimal fitness [2-5]. These rhythms are termed circadian, which is derived from the Latin words “circa diem” and means “approximately a day”, thus ~24 hours [6]. The circadian clock is based on an endogenous, cell-autonomous, and self-sustaining timekeeping system consisting of interlocking transcriptional-translational feedback loops (TTL) [7]. In the TTL, the positive elements that drive molecular rhythms are heterodimers of the transcription factors BRAIN AND MUSCLE ARNT-LIKE PROTEIN 1 (BMAL1) and CIRCADIAN LOCOMOTOR OUTPUT CYCLES KAPUT (CLOCK). The complex of BMAL1 and CLOCK bind to so-called E-box elements in the promoter regions of the other core clock genes *Period1/2/3* (*Per1/2/3*) and *Cryptochromes1/2* (*Cry1/2*). About 12 hours later, the PER1/2/3 and CRY1/2 proteins also build complexes which represent the negative limb of the TTL as they bind to and inhibit BMAL1 and CLOCK and thus their own transcription. Another 12 hours later, PER and CRY proteins are degraded and the BMAL1/CLOCK-mediated transcription can resume its activity. The rhythms of these clock genes are transferred to a large part of the genome as E-boxes and other circadian regulatory elements are present in promotors of the majority of all genes [8]. This leads to the fact that more than 80% of protein-coding genes show a 24-hour rhythm [9].

On the anatomical level of mammals, the circadian system is organized as a hierarchical network consisting of peripheral oscillators and one master pacemaker, the suprachiasmatic nuclei (SCN), which reside in the hypothalamus dorsal of the optic chiasm [10, 11]. Thus, the SCN receives information about environmental light and darkness from the retinohypothalamic tract and synchronizes central as well as peripheral clocks throughout the body accordingly [12]. As light adjusts the clock, it is called a *Zeitgeber* (German: time giver). *Zeitgebers* are environmental stimuli that determine the phase of the endogenous circadian clock in a process called entrainment, i.e., they adjust the internal timing to the environmental time. Other prominent *Zeitgebers* are food, physical activity, and temperature. The responsiveness of the circadian system to *Zeitgebers* is dependent on how strong they are and at what time of day they occur. Together, they ensure synchronization with environmental day/night rhythms and optimization of the synchrony among cellular, physiological, and behavioral processes

of the body [13, 14]. Peripheral clocks are entrained and synchronized by time information from the SCN, which is transmitted humorally (including melatonin and cortisol) and neuronally [12]. In addition, since most peripheral clocks do not receive direct light/dark information, they are sensitive to other *Zeitgebers* such as physical activity and especially food intake. Food intake as a *Zeitgeber* is important for clocks in peripheral organs such as liver, pancreas, muscle, and adipose tissue as well as non-SCN brain regions, such as the amygdala or the hippocampus, which use metabolic signals from food intake to achieve entrainment with the environment [15-17].

In summary, with a majority of genes showing a 24-hour rhythm in their expression patterns, circadian rhythms control virtually all physiological and behavioral processes, including the activity of the central nervous system as well as peripheral processes, which in turn influence basic daily routines such as sleep and feeding patterns [9]. Importantly, these rhythms are driven by endogenous networks of clock genes but can be entrained by environmental stimuli, such as sunlight and food to the external time.

1.2 Etiology of circadian disruptions

Like with all biological processes, it can occur that the circadian system gets disturbed. Many common and current chronic disorders, such as type 2 diabetes, major depression, or sleep disorders, are facilitated by chronodisruption, i.e., the disturbance of internal clock functions and their interplay as well as the perturbation of these clocks with the external time [15]. Circadian rhythm abnormalities can be caused by both environmental as well as genetic factors [18]. Polymorphisms in circadian clock genes have been, for example, associated with morningness–eveningness preference, familial advanced sleep phase, and delayed sleep phase type [19]. A prominent example of how the circadian system can be disrupted by environmental factors is irregular lighting conditions such as during shift work, which is known to cause severe circadian disruption [20]. Individuals working in shifts, e.g., night shifts, rotating shifts, and afternoon and early morning shifts, often report insomnia, daytime sleepiness, and problems falling asleep [18, 21]. Moreover, many of them suffer affective symptoms including irritability and depression [22]. Trans-meridian travelling is another well-known example of how circadian disruption can arise. It shifts the sleep-wake cycle rapidly and causes the so-called jet lag. During jet lag, synchrony of endogenous clocks with the time of the environment gets lost and clocks throughout the body become desynchronized among each other [23, 24].

However, even more relevant than jet lag caused by air travelling might be the phenomenon of the so-called social jet lag since most people experience this form of

circadian disruption in everyday life [25]. It refers to the discrepancy between the social and one's own biological time, such as the conflict between school/work schedules and individual preferences for when to be awake and to sleep [26]. Most people have clear differences in timing and duration of sleep during work and work-free days, which can lead to temporary circadian desynchronization, which is often characterized by impaired sleep at night, daytime sleepiness, and considerable sleep debt during workdays [25, 27]. Lastly, irregular exposure to *Zeitgeber* often leads to circadian rhythm disruption. Ideally, the strongest *Zeitgebers*, such as light and food, should occur every day at approximately the same time in order to keep the organism permanently synchronized with the environment and to coordinate physiological processes in the best possible way [28]. For example, exposure to light at night is strongly perturbing the circadian system as light signals to the SCN that daytime has dawned, and it mistakenly adjusts the body, all physiological processes, and behavior accordingly [29]. Taken together, these studies show that circadian disturbances can be both genetic, environmental, or a combination of both factors.

1.3 Physiological consequences and pathogenesis of circadian rhythm disruptions

As described above, there are various ways in which the circadian system can become disturbed, either by genetic factors or by environmental changes. Such circadian disturbances can contribute to the development of a variety of disorders. In this chapter, it will first be described which underlying consequences on the physiological level can be caused by circadian disturbances, which can then ultimately lead to pathological conditions. In a review paper, we elaborated on the four basic mechanisms of how circadian disruption can ultimately contribute to organism dysfunction and illness [30]:

1. External desynchronization between the organism and the environment,
2. Internal desynchronization among cellular and tissue clocks within the organism,
3. Low amplitude of circadian oscillations, and
4. Alterations in sleep architecture.

First, external desynchronization is characterized by a mismatch of endogenous circadian time and the external light-dark cycle [8]. This is often a result of frequently changing light-dark conditions or light deprivation, which in turn, for example, can lead to humoral or neurotransmitter changes [31-34]. After exposure to light at night, humans show a differently pronounced melatonin and cortisol secretion. While the change in

melatonin levels is very rapid and persistent, the changes in cortisol depend on the duration of light exposure [35]. In rats, it could be shown that neurotransmitters switch between dopamine and somatostatin depending on short- and long-day photoperiods [31]. Furthermore, it was found that keeping rats in constant darkness for several weeks resulted in neuronal damage in monoaminergic brain circuits, which in turn was associated with depression-like phenotypes [36]. To apply this result to humans, continuous darkness conditions in animal experiments can serve as an extreme model for light deprivation, which concerns late chronotypes, whose sleep phase often extends until well after sunrise and thus their exposure time to daylight is shortened.

Second, internal desynchronization refers to differing periods and changes in the phase relationship between the central pacemaker and peripheral clocks as well as among themselves [37]. This may provoke inadequate coordination of neural, hormonal, and behavioral processes, which can make the organism more susceptible to adverse health effects. For example, many hormones that control food intake, including insulin, corticosterone, leptin, and ghrelin, are secreted in a tissue-specific circadian manner, requiring coordinated sequential processes. In the event of loss of internal synchrony, this may then put the individual at risk of metabolic dysfunction [38, 39]. In the brain, the release of neurotransmitters and the expression of receptors are also under circadian control and therefore occur only at certain periods of the day [40]. Importantly, synchrony occurs when neurons have the same circadian period, but not necessarily the same phase [41]. For example, after a shifted light pulse, the dorsal SCN resynchronizes much more slowly than the ventral SCN, resulting in a state of internal desynchrony [42, 43].

The third type of circadian perturbations is a reduced differentiation between diurnal and nocturnal processes, i.e., reduced amplitude of circadian rhythms. A strong amplitude is important for sufficient activity and also inactivity of various biological functions, which are necessary to adequately initiate, execute, and complete cellular, molecular, and behavioral processes at the right time of the day. Consequently, if this is not the case, the separation of opposing biological processes cannot occur properly [44]. For example, high cortisol levels are associated with activity and wakefulness and elevated melatonin levels are associated with sleep, so expression of both should occur separately and at different times [45, 46]. Reduced amplitudes of melatonin and cortisol are found in patients with psychiatric disorders, such as depression, which then can lead to nocturnal wakefulness due to lack of high melatonin levels and daytime sleepiness due to lowered cortisol levels [47, 48]. A post-mortem study in humans has confirmed reduced amplitude of circadian rhythmicity in brains of MDD patients, as rhythms of core clock genes and clock-controlled genes in various mood-controlling brain regions are drastically blunted in affected subjects [49].

The final physiological characteristic that is almost always associated with circadian disruption are altered sleep patterns. Current evidence is insufficient to determine conclusively whether disrupted circadian processes affect the integrity of sleep or whether sleep is affected first and then disrupts circadian processes or both. Nevertheless, it should be noted that disturbed circadian rhythms are most often associated with altered sleep, which in turn increases the risk of developing various disorders, including breast cancer, metabolic syndrome, or mental illnesses such as affective, anxiety, or substance use disorders [50-52].

These four basic mechanisms show that disruption of circadian clocks can have multiple negative effects on the functionality of physiological systems resulting from disrupted coordination of different processes, incorrect regulation of expression, and disturbed sleep. When these processes are disrupted, various physiological functions can become dysregulated. Pathologies that are particularly closely associated with disturbed circadian rhythms include cancer [53, 54], metabolic [55, 56] and endocrine disorders [57], neurological diseases such as dementia or Parkinson's disease [58], as well as stress-related [59] and psychiatric disorders [18].

1.4 Circadian disruptions in psychiatric disorders

1.4.1 Affective disorders: major depressive disorder

1.4.1.1 Definition and etiology

Major Depressive Disorder (MDD) is characterized by symptoms in the affective state with increased sadness, lack of motivation, and inability to experience positive emotions such as pleasure [60]. In addition, patients often suffer from psychophysiological changes, such as sleep and appetite disturbances, and all symptoms together can lead to suicidal thoughts [61]. Disturbed circadian rhythms are a hallmark of MDD [18]; patients usually suffer from fragmented sleep (e.g. increased rapid eye movement (REM) and reduced deep sleep phases) and live unstructured daily life routines [62, 63].

1.4.1.2 Circadian patterns of MDD

Interestingly, the severeness of MDD symptoms shows diurnal variations itself. Patients often report worse symptomatology either in the morning or in the evening while a more serious form of MDD is often associated with symptoms that are more pronounced in the morning [64].

1.4.1.3 Effects of MDD on circadian clocks

As mentioned above, MDD patients often display weakened rhythmic melatonin and cortisol expression as well as reduced amplitudes in body temperature [65] and in a post-mortem study of brains from MDD patients, reduced amplitudes and altered phase-relationship in circadian core clock genes were described [49]. Despite many studies in humans and animals, it cannot be disentangled whether, for instance, the dampened physiological rhythms are a consequence of MDD or represent a predisposing risk factor. However, in the last years evidence has emerged that circadian rhythm disruptions are sufficient to induce depression [66].

1.4.1.4 Effects of disrupted clocks on mood

Causality between circadian disruption and depression symptoms was first demonstrated in a mouse study showing that disruption of circadian rhythms exclusively in the SCN in adulthood without affecting development, brain anatomy or environmental conditions induced depression-like behavior in mice [67]. Furthermore, compared to dayworkers, night-shift-workers have a 40% higher risk of developing MDD [68]. Data from human studies revealed that the extent of circadian misalignment is associated with symptom severity in MDD [69, 70]. Conversely, therapies that directly influence and stabilize circadian rhythms, such as bright light therapy, social rhythm therapy and antidepressants such as Selective Serotonin Reuptake Inhibitors (SSRIs), Selective Serotonin-Noradrenalin-Reuptake-Inhibitors (SNRIs) and agomelatine, help MDD patients with great success [30, 70], indicating that circadian clock disturbances are a significant dysfunctional process in MDD.

1.4.2 Affective disorders: bipolar disorder

In the spectrum of affective disorders, circadian rhythm disruptions are also prevalent in bipolar disorder (BD), which is characterized by extreme alterations in mood states that range from depression to mania. So far, it is known that circadian clock dysregulations can induce or predispose an individual to BD although it is still unclear if states of circadian desynchrony represent cause or consequence of BD [71, 72]. Furthermore, *ClockΔ19* mutant mice, which are not able to express circadian rhythms show mania-like behaviors which could be improved by the functional restoration of rhythms in their ventral tegmental area (VTA) [73]. In addition, data from human studies provides evidence that depressive and manic episodes can be both successfully treated by the targeted use of *Zeitgebers*, in particular light. For example, one therapeutic approach used for depressive episodes is morning or midday light therapy, which has been shown

to be successful for bipolar depression in clinical randomized controlled trials [74-76]. In manic episodes, the opposite of light therapy is attempted, i.e., reduction of daily light exposure, for which prolonged stays in darkness or bed rest or wearing blue light blocking glasses are prescribed [77-79].

1.4.3 Anxiety and anxiety-related disorders

1.4.3.1 Definition and etiology

Anxiety and anxiety-related disorders such as post-traumatic stress disorder (PTSD) and obsessive-compulsive disorder are characterized by intense, excessive, and persistent feelings of fear and anxiety in relation to everyday situations. Such episodes of perceived fear often lead to avoidance of subjectively threatening situations, which, in turn, can easily interfere with usual daily activities.

In addition to the intense feelings of fear or anxiety, common symptoms of anxiety disorders include restlessness, tension, physiological changes such as increased heart rate, hyperventilation, and sweating, as well as disturbances in sleep and circadian rhythms, which are considered core symptoms of anxiety [80].

1.4.3.2 Circadian patterns of anxiety disorders

Similar to MDD, anxiety symptoms also seem to follow a 24h circadian pattern [81]. While depression symptoms are often worst in the morning, anxiety symptoms usually peak in the late afternoon or early evening [82].

1.4.3.3 Effects of anxiety on circadian clocks

Rodent studies could display the effects of anxiety on the circadian clock system and sleep. For example, mice selectively bred for high anxiety-related traits show longer free-running periods, have fragmented ultradian rhythms, and a blunted phase shift response [83]. Further, they have an increased demand for sleep with more REM sleep proportions [84]. Moreover, mice that were subjected to anxiety-inducing experiments showed lower expression of the clock genes *Per1* and *Per2* in the nucleus accumbens [85].

In humans, main characteristics of anxiety or fear are hypervigilance and permanent rumination, which often result in increased heart rate and blood pressure. This, in turn, often leads to sleep problems, such as fragmented sleep [86, 87], and to disturbed circadian rhythms of, for example, the secretion of glucocorticoids [88]. Further on, severe symptoms of generalized anxiety disorder and obsessive-compulsive disorder are associated with delayed sleep phase disorder [89]. Also, PTSD, a mental illness triggered by trauma, often leads to sleep disturbances and the loss of a stable sleep-

wake rhythm as the disorder progresses. Since circadian symptoms are very common in PTSD patients, the term post-traumatic chronodisruption has been coined [90-92].

1.4.3.4 Effects of disrupted clocks on anxiety disorders

Mice lacking the *Per1* and *Per2* genes show increased anxiety-like behavior [85]. Furthermore, our own [i]data (*Paper I*) show that mice lacking the *Cry1* and *Cry2* genes have a distinct anxiety phenotype, which was particularly manifested by restlessness and compulsive-like behavior as well as amygdala hypersensitivity towards threatening and stressful environmental events [93].

In humans, anxiety and circadian disturbances build a vicious cycle, as affected persons who suffer from insomnia at the same time show more pronounced anxiety symptoms than persons without sleep and circadian rhythm disturbances [94]. Also, for jet lag and shift work conditions, it has been shown that anxiety levels increase with the extent of circadian dysregulation [95-97]. The circadian profile of patients with panic disorder shows phase shifts or delays in various behaviors such as falling asleep, waking, hunger, and even cognitive functions compared to healthy controls. This is true not only at the time of acute episodes, but also in the years preceding onset [98]. Interestingly, children who suffer from sleep disturbances, which is often associated with disrupted circadian rhythms, are at higher risk for anxiety disorders during adolescence as well as adulthood [99-101] and sleep disturbances in early adulthood predict anxiety in middle age [102].

1.4.4 Addiction disorders

1.4.4.1 Definition and etiology

Addiction or substance use disorders (SUD) are disorders leading to an inability to control consumption of legal or illegal drugs [103]. Factors that contribute to the development of SUDs, including AUD, are multifactorial due to an interaction of genes, environment, and the properties of the drug itself [104]. Often, impulsive behaviors trigger initial drug use that then becomes associated with both environmental, contextual factors (conditioned stimuli) and the rewarding effects of the drug (reinforcing stimuli). This process primarily involves brain areas such as the amygdala and the hippocampus [105, 106]. With dysfunctional inhibitory processes originating mainly in prefrontal cortex areas, drug users may more easily lose control over their drug use [107, 108]. Drug-seeking behavior is often driven by a combination of decreased reward functions and increased brain stress responses [109]. The so-called *incentive sensitization theory* claims that two different underlying processes control the motivation to use the drug namely "*wanting*" and "*liking*". These two psychological and neural processes and their

associated brain structures typically work together, but often disconnect in the course of addictive disorders [106]. Repeated use of addictive drugs sensitizes the mesolimbic dopamine system (incentive sensitization), the major component of the *wanting* system, leading to excessive *wanting* for drugs and their stimuli [107]. This process is independent of the *liking* of the drug, which either remains unaltered or even decreases during the course of addictive disorders [106]. Thus, finally, the consumption of the drug in progressed addiction disorders is characterized by excessive use despite minimal enjoyment [110].

Besides the above-mentioned processes contributing to the development of addiction disorders, circadian rhythms play an important role in in drug addiction, such as for substances as morphine, methamphetamine, cocaine, and alcohol [73, 111-114]. Particularly AUD patients often display circadian disturbances as described in more detail below, which, in turn, increases the vulnerability to develop or worsen AUD symptomatology [115-117]. Interestingly, although SUD is characterized by circadian disruptions, drug addiction and consumption often follow a very specific and reliably precise diurnal rhythm.

1.4.4.2 Circadian patterns of drug taking behavior

The dopaminergic mesolimbic brain circuit is under control of the circadian system [118] and it is postulated that the development and chronification of drug addiction is mainly driven by glutamatergic and dopaminergic neurocircuitries. Importantly, these are known to be regulated by clock genes [104]. Thus, reward-related, or motivated behaviors, such as drug taking, can vary widely over the course of 24 hours [119]. Interestingly, this leads to the fact that although patients suffer from circadian misalignment, the time at which they are seeking for alcohol drinking is very precise and the first drink of the day often occurs at a specific hour each day [120, 121]. Similarly, abstinent smokers show a diurnal rhythm in nicotine craving and a daily rhythm in cue-induced craving has even been found in abstinent heroin users [122, 123].

1.4.4.3 Effects of alcohol on the circadian clock

Human and animal research have shown that increase in alcohol drinking are correlated with disruptions in sleep patterns and that drugs and alcohol can affect endogenous circadian rhythms [124-128]. Acute and chronic alcohol consumption as well as withdrawals from alcohol can lead to alterations and disruptions in the circadian system with effects on biological parameters such as clock gene expression, hormone secretion or body temperature as well as on sleep and affective states [129-134]. For example, studies in AUD patients assessing sleep abnormalities describe disrupted sleep cycles

including problems in particular with falling asleep and sleeping through, with 36–72% of AUD patients suffering from sleep abnormalities [129, 135]. Furthermore, changes in biological rhythms, e.g. cortisol and body temperature rhythms, can be already observed after a single acute alcohol intake [133]. Interestingly, opposite patterns of melatonin ratio between day and night secretion have been observed in AUD, in particular in patients with chronic daily alcohol abuse [133, 136, 137]. Interestingly, desynchronization of circadian rhythms increases with the severity and chronification of alcohol drinking and is associated with the intensity of withdrawal symptoms [133]. Vice versa, circadian rhythms of, for example, cortisol secretion, and core body temperature resynchronize within two to three weeks of alcohol abstinence [133] suggesting that circadian disturbances are a consequence of increased alcohol consumption.

1.4.4.4 Effects of disrupted circadian rhythms on the development of AUD

However, abstinent AUD patients display markedly lower baseline mRNA levels of circadian clock genes even after prolonged periods of abstinence suggesting that genetically reduced circadian rhythms are not just a consequence of increased alcohol consumption but also constitute a risk factor for AUD [134]. In line with this, circadian rhythm disturbances increase the risk of developing AUD and can modulate alcohol seeking behavior. For example, shift work or jet lag were shown to elevate alcohol consumption [125, 138, 139]. In humans, variations in the *PER2* gene are associated with an increased risk of developing pathological alcohol drinking patterns. Furthermore, genome wide association study (GWAS) results indicate that single nucleotide polymorphisms (SNPs) in the human *PER2* gene moderate alcohol consumption in individuals with severe life stress [140], and that variations in the *PER1* and *PER3* genes are associated with stress-induced alcohol consumption [124, 141]. Similarly, mice lacking the *Per2* gene (*Per2*$^{Brdm1/Brdm1}$ mice*)* also show increased alcohol consumption associated with a strong increase in glutamate levels [113, 142]. In line with this, it is known that individuals suffering from AUD often display particularly high levels of glutamate. For this reason, the drug *Acamprosate*, a glutamate antagonist, is used in AUD therapy to reduce craving for alcohol.

Beyond the *PER* genes, another important component of the circadian molecular clockwork, casein kinase 1ε/δ (CK1ε/δ), is thought to be involved in the etiology of AUD. In a study in rats, inhibition of CK1ε/δ was shown to attenuate the high daytime alcohol intake typically observed after withdrawal and upon re-exposure to alcohol. Thus, relapse-like drinking was prevented in animals in which CK1ε/δ was inhibited, highlighting CK1 as another candidate for the treatment of AUD [143].

Together, these studies show that, on the one hand, both endogenous circadian rhythm disturbances such as sleep irregularities, and reduced amplitudes of hormone oscillations as well as environmental disturbances of circadian rhythms caused by, for example, shift work and jet lag, constitute a risk factor for alcohol abuse and alcohol addiction [144]. On the other hand, particularly chronic alcohol abuse disrupts circadian rhythms as it impacts on a variety of physiological and behavioral rhythms, such as sleep cycles, body temperature or eating habits [145, 146]. Hence, it appears that alcohol abuse and circadian disorders are mutually beneficial and that it is important to break this cycle. Because of this bidirectional relationship, abstinent AUD patients, AUD patients under acute withdrawal as well as subjects who are at risk of developing an alcohol dependence might benefit from therapies aiming specifically at stabilizing circadian rhythms. However, chronotherapies are not available for any kind of SUD patients by now.

1.5 Chronotherapies in psychiatric disorders

1.5.1 Light manipulation therapies

As described above, psychiatric disorders in the spectrum of mood and addiction are often characterized by irregular circadian behavior patterns. So-called chronotherapies are therapy approaches, that specifically aim at stabilizing the circadian clock. The most common chronotherapy for psychiatric disorders is the bright light therapy as light efficiently entrains the SCN. Interestingly, bright light has been shown to be as effective or even more effective than antidepressants in treating depression [147-149]. Standard treatment uses light sources of about 10,000 lux for 30 minutes with an application that usually lasts for a few weeks [150]. The therapeutic success of light therapy for MDD or seasonal affective disorder (SAD) can be explained by several underlying mechanisms. First, it was shown that the expression of neurotransmitters can vary depending on whether an organism receives high or low levels of light. In adult rats, interneurons in the hypothalamus were found to switch between dopamine and somatostatin expression depending on whether the animals were exposed to a long or short photoperiod [31]. Interestingly, in humans an association was found between the photoperiod, i.e., whether it was summer or winter, and the number of dopamine neurons in the midbrain, again suggesting neurotransmitter switching as an underlying mechanism [151].

Second, exposure to light in the morning shifts the melatonin rhythm in a way that causes a phase shift with earlier waking and sleeping times [152], which is particularly helpful for MDD patients since these patients are most often late chronotypes and the severity

of depression correlates with their eveningness [153]. Not only does phase shifting improve synchronization of endogenous and environmental time, but also results in patients getting more natural daylight by waking up earlier in the day [20]. Third, it is assumed that daily recurrent light exposure, which is a powerful *Zeitgeber*, increases the amplitude of signals from SCN neurons, which in turn relay their strong signals to other brain regions [70]. This in turn could lead to improved internal synchronization of communication between neurons and between brain regions [14, 154]. Fourth, it increases the proportion of slow-wave sleep and reduces REM sleep [155]. Along these lines, a recent study in a diurnal mammal has shown that higher light intensity during the day leads to greater robustness of behavioral (waking and sleeping) and physiological (temperature) rhythms [156]. Furthermore, this study shows that light intensity increases the amplitude of circadian rhythms of electrical activity in the SCN, which not only supports the therapeutic efficacy of light therapy, but overall underscores the importance of daily light exposure for circadian health [156, 157].

1.5.2 Agomelatine and melatonin therapies

In addition to therapies acting on the circadian system by manipulating light conditions, there are several other chronotherapeutic possibilities to realign and stabilize the circadian clock. For example, the pharmacological agent agomelatine, which is mainly used as an antidepressant, acts as an agonist at the melatonin receptors MT1 and MT2 and as an antagonist at the serotonin 5-HT2C receptor. In addition to treating depression, it has been shown to be a successful agent in various psychiatric disorders due to its ability to adjust a variety of circadian rhythms including melatonin, cortisol, temperature, and activity rhythms [158-161].

Melatonin is one of the two most prominent humoral manifestations of circadian rhythms. It is secreted at night by the pineal gland, which receives time-of-day information from the SCN [162]. As it circulates in the body and reaches both central and peripheral tissues, it has the ability to synchronize the endogenous timekeeping system among itself and with the external time [163]. Pharmacologically administered melatonin has similar chronobiological effects and is able to improve the stabilization of 24-hour rhythms, reduces latency to slow-wave sleep and increases sleep duration [164, 165]. It is suggested to be useful in psychiatric disorders with comorbid sleep disorders; both in patients with stabilized mood disorders to prevent relapses, and as an adjunct treatment in acute phases of mood disorders, but also in attention deficit hyperactivity disorder, and schizophrenia [166].

1.5.3 Cognitive behavioral therapies with chronotherapeutical components

1.5.3.1 Cognitive behavioral therapy for insomnia

In addition to light therapy and pharmacological approaches described above, there are behavioral therapy approaches that either directly aim to stabilize the circadian clock or at least indirectly influence it positively through interventions that stabilize everyday rhythms.

The focus of cognitive behavioral therapy for insomnia (CBT-I) is on identifying and replacing thoughts and behaviors that lead to sleep problems [167]. CBT-I uses interventions such as establishing a regular sleep schedule, sleep restriction, and relaxation techniques, and integrates psychoeducation about improved sleep hygiene. However, CBT-I does not primarily aim to improve circadian rhythms since, besides sleep, it does not address regularity of daily routines, which include mealtimes and other activities. In the long term, however, by creating more stable daily rhythms of sleep and wakefulness, CBT-I has the potential to strengthen the circadian clock system [168, 169]

1.5.3.2 Social rhythm therapy

In the 1990s, the so-called *Interpersonal and Social Rhythm Therapy (IPSRT)* was developed for patients with bipolar disorder. It aims at the implementation of regular, daily recurring patterns of activity in order to stabilize circadian biological processes. The focus of the therapeutic interventions is on strengthening social rhythms, such as shared meals, social activities, interactions, and relationships [170]. Its effectiveness in bipolar patients has been proven several times [171, 172]. In recent years, the therapy has again gained more attention [173-175] and was expanded in 2016 as *Cognitive Behavioral Social Rhythm Therapy (CBSRT)* for patients with PTSD. It was also proven successful in improving depressive symptoms as well as the quality of sleep in this patient group [176]. Finally, the effectiveness of the so-called *Circadian Reinforcement Therapy (CRT)* is currently being studied in MDD [177]. This therapy concept is similar to IPSRT but focuses less on reinforcing social rhythms and more on reinforcing all kind of *Zeitgebers*, such as daylight exposure, exercise, eating and other social activities. Data from this randomized-controlled trial are still pending, but if the therapy proves to be successful, it would be a further step towards bringing chronotherapeutic interventions into everyday clinical psychiatric practice.

In summary, all the interventions described - from light therapy to pharmacological agents to cognitive behavioral therapies - directly or indirectly target the stabilization and realignment of circadian rhythms to improve symptoms of various psychiatric disorders.

This is achieved by targeting underlying physiological mechanisms (described in more detail in section 1.3), such as by increasing the amplitude and stabilizing the period of biological processes close to 24 hours as well as by establishing a stable phase relationship between internal and external time with a minimal phase variability from day to day. Moreover, the successful treatment of psychiatric disorders with chronotherapies is further evidence that circadian disturbances play a crucial factor in these disorders.

2. Summary of scope and methodological approach of my Ph.D.

I started my Ph.D. by writing a review paper (*Paper IV*), the content of which was intended to cover the overall goal and scope of my Ph.D. project. It defined and described mechanisms by which disturbed circadian clocks can lead to mental illness, especially mood disorders [14]. This review further attempted to demonstrate ways in which this knowledge could be used to easily apply chronotherapies in the clinic that precisely target and achieve improvement in these dysfunctional mechanisms.

I then began working with a mouse model deficient in the two central clock genes, *Cry1* and *Cry2*, to investigate the effects of a disrupted circadian clock on psychiatric phenotypes. *Cry* genes are directly related to and influence functional stress regulation and mechanisms of action of the hypothalamic-pituitary-adrenal (HPA) axis [178]. Conversely, a dysfunctional HPA axis plays a crucial role in the etiology and course in almost all mental illnesses, and in turn can profoundly exacerbate their progression [179-181]. In *Paper I*, I was able to demonstrate through extensive behavioral phenotyping that the *Cry1/2*$^{-/-}$ mice consistently exhibit anxiety-like behavior across various tests. This was particularly evident by decreased mobility in unfamiliar environments, reduced ability to habituate to new environments, and most importantly, restless behavior, which was exhibited independently of various tests. The anxiety-like phenotype was additionally confirmed at the molecular level by increased neuronal activity in one of the brain's major anxiety centers, the amygdala. In contrast to the anxiety-like behavior, the animals do not show depression-like behavior, and at the level of cognitive and social behavior, they demonstrate only minor abnormalities compared with WT [182].

Furthermore, in *Paper II*, I showed that animals of the same mouse line exhibit significantly altered alcohol drinking behavior which in many ways resemble properties of AUD. For example, *Cry1/2*$^{-/-}$ mice exert significantly more effort to obtain alcohol even though their alcohol preference is reduced, consistent with the incentive sensitization theory of addiction [107, 183, 184]. In addition, I exposed the same mice to an environmental disruption of circadian rhythms similar to shift work conditions, which represents a significant risk factor for increased alcohol consumption in humans [130] . Interestingly, the alcohol preference of *Cry1/2*$^{-/-}$ mice was particularly low under these shift work-like lighting conditions. However, the direction of alcohol consumption, i.e., whether it is particularly low or high, depends on the species. For example, rats drink more alcohol under changing light conditions [185, 186], whereas mice tend to drink less [187] , which is also consistent with our study. Interestingly, however, *Cry1/2*$^{-/-}$ mice reject

already low alcohol concentrations under shift-work-like conditions, whereas WT mice under these conditions only drink less under high ethanol concentrations. This illustrates that the interaction, i.e., when genetic and environmental disturbances of the circadian rhythm coincide, has a particularly strong influence on alcohol drinking behavior.

Furthermore, by determining the stress hormone corticosterone, I was able to show in this study that these mice exhibited continuously elevated stress levels over 24 h compared to wild-type mice. Both elevated anxiety and stress levels are significant risk factors for the development of AUD in humans [188]. Overall, this study provides evidence that both endogenous and environmental perturbations of circadian rhythms, as well as resulting hormonal and neuronal changes that are known risk factors for the development of AUD in humans, significantly alter alcohol drinking behavior in mice.

To make these findings relevant and translational for clinical psychiatric work, I developed a new personalized circadian adjunctive therapy for AUD patients, called DAILY, which stands for Depression Alcohol Illness Therapy (*Paper III*). DAILY attempts to expose patients to recurring and very regular daily *Zeitgeber* such as sleep, activity, and meals. The hypothesis is that this will stabilize behavioral and physiological circadian rhythms by improving synchronization between them and with the environment which is expected to reduce the risk of potential relapse and facilitate physical recovery during the first weeks of withdrawal therapy. Whereas the study protocol has already been published [62], the associated randomized controlled trial in AUD patients to test treatment efficacy is still ongoing and not yet completed (see section 4).

According to the title of this thesis "*A Translational Approach of Mouse and Human Studies to Integrate Chronobiology into Alcohol Use Disorder Therapy: From Bedside to Bench… and Back.*", I have attempted during my Ph.D. to use observations from my own clinical work as well as existing knowledge from the literature to systematically gain new insights through animal research and translate them into clinical psychiatric practice through the development of a new adjunctive therapy for AUD patients.

3. Own contribution to the original research papers

3.1 Contribution to Paper I

Hühne, A., Volkmann, P., Stephan, M., Rossner, M., & Landgraf, D. (2020). **An in-depth neurobehavioral characterization shows anxiety-like traits, impaired habituation behavior, and restlessness in male *Cryptochrome*-deficient mice.** *Genes, brain, and behavior*, *19*(8), e12661.

For this study, I performed all behavioral experiments and data analysis. Paul Volkmann assisted me in conducting those behavioral experiments which required two experimenters. While Dominic Landgraf had the principal project idea, namely the extensive behavioral characterization of *Cry1/2*$^{-/-}$ mice, I planned the concrete experimental procedure of the study. It was also my idea to measure *c-fos* in the amygdala of our experimental mice using qPCR method. Thereby, Dominic Landgraf and Paul Volkmann helped me with the tissue sampling. The processing of the raw behavioral data from the IntelliCages using FlowR software was performed by Marius Stephan, Paul Volkmann, and myself. I decided which data should be evaluated and Marius Stephan generated raw data accordingly which I have used for further analyses. After I had finished the data analysis, Dominic Landgraf, Paul Volkmann, and I contributed to the interpretation of the data. I wrote the initial draft of the manuscript. This draft was revised and finalized by Dominic Landgraf, Paul Volkmann, and me. Dominic Landgraf guided the study and provided the funding.

3.2 Contribution to Paper II

Hühne, A., Echtler, L., Kling, C., Stephan, M., Schmidt, V. M., Rossner, M. & Landgraf, D. (2021). **Circadian gene × environment perturbations influence alcohol drinking in Cryptochrome-deficient mice.** *Addiction Biology,* e13105.

Since I had the original idea for this study, I also did the planning and design of this project. I also trained Lisa Echtler in the behavioral experiments, with whom I share first authorship. Under my supervision, Lisa Echtler performed the majority of the behavioral experiments. I performed the *in vitro* experiment (measuring PER2::LUC rhythms in the SCN in the LumiCycle). Processing of the raw behavioral data from the IntelliCages using FlowR software was performed by me with the help of Marius Stephan. I decided which data should be evaluated and Marius Stephan generated raw data accordingly. Charlotte Kling carried out the sampling of the lateral hypothalamus for the qPCR experiment for prepro-orexin quantification. Blood for corticosterone measurements was collected by Lisa Echtler and Mathias Schmidt from the Max Planck Institute in Munich performed the measurement of corticosterone by radioimmunoassay. Data analysis was performed by me. Dominic Landgraf and I performed the data interpretation. I wrote the first draft of the manuscript. This draft was revised and finalized by Dominic Landgraf and Lisa Echtler. With support from Dominic Landgraf, I directed the study to a large extent myself. Dominic Landgraf provided the funding.

Paper I

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Genes, Brain and Behavior

ORIGINAL ARTICLE

An in-depth neurobehavioral characterization shows anxiety-like traits, impaired habituation behavior, and restlessness in male *Cryptochrome*-deficient mice

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Abstract

Many psychiatric disorders, for example, anxiety, are accompanied by disturbances of circadian rhythms, including disturbed sleep/wake cycles, changes in locomotor activity, and abnormal endocrine function. Conversely, alternations of circadian rhythms are a risk factor for the development of psychiatric disorders. This assumption is supported by animals with clock gene mutations which often display behaviors that resemble human psychiatric disorders. In this study, we performed an in-depth behavioral analysis with male mice lacking the central clock genes *Cryptochrome 1* and *2* (*Cry1/2*$^{-/-}$), which are thus unable to express endogenous circadian rhythms. With wild-type and *Cry1/2*$^{-/-}$ mice, we performed an extensive behavioral analysis to study their cognitive abilities, social behavior, and their expression of depression-like and anxiety-like behavior. While *Cry1/2*$^{-/-}$ mice showed only mild abnormalities at cognitive and social behavioral levels, they were consistently more anxious than wildtype mice. Anxiety-like behavior was particularly evident in reduced mobility in new environments, altered ability to habituate, compensatory behavior, and consistent restless behavior across many behavioral tests. In line with their anxiety-like behavioral phenotype, *Cry1/2*$^{-/-}$ mice have higher *c-Fos* activity in the amygdala after exposure to an anxiogenic stressor than wild-type mice. In our study, we identified *Cry1/2*$^{-/-}$ mice as animals that qualify as a translational mouse model for anxiety disorder in humans because of its consistent behavior of restlessness, increased immobility, and dysfunctional habituation in new environments.

KEYWORDS

amygdala, anxiety, *c-Fos*, circadian clock, cryptochrome, habituation, neurobehavioral characterization, restlessness

1 | INTRODUCTION

1.1 | The burden of anxiety and other psychiatric disorders

Psychiatric disorders are a profound societal burden making up nearly a quarter of worldwide disability.[1] Focusing on the characterization of genotypes and phenotypes as well as on molecular mechanisms of these disorders becomes extremely important in order to be able to make diagnoses more precisely and develop new therapies. Thus, the treatment of psychiatric disorders is a considerable challenge not only for clinical practice but also for research. Anxiety disorders are one of the most common psychiatric disorders, with a 12-month prevalence of 18.1% in the U.S. adult population and 31.1% of U.S. adults experiencing anxiety disorders at least once in their lifetime.[2,3] In addition to the core symptom of feeling uncontrollable and inordinate fear, sleep disturbances are highly prevalent in anxiety disorders.[4] Neurobiologically, a core element of the anxiety network in the brain is the amygdala, which serves as a relay station for information from cortical and thalamic areas to generate behavioral states of fear and anxiety.

1.2 | Circadian clocks

The sleep-wake cycle and other daily oscillations in physiologic and behavioral processes are controlled by the mammalian circadian system, which evolved to anticipate daily recurring events resulting from the 24-hours rotation of the earth. The master pacemaker of the mammalian circadian system is located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus. The SCN is entrained to environmental rhythms by direct light and dark signals through the retinohypothalamic tract and distributes this time information to other clocks throughout the rest of the brain and peripheral tissues. The expression of circadian clock genes determines cellular circadian rhythms approximating 24 hours by generating transcriptional-translational feedback loops (TTL) with positive and negative components. The positive arm of the mammalian core TTL consists of the transcription factors *CIRCADIAN LOCOMOTOR OUTPUT CYCLES KAPUT* (*CLOCK*) and *BRAIN AND MUSCLE ARNT-LIKE PROTEIN 1* (*BMAL1* or *ARNTL*). These proteins dimerize and bind to E-box *cis*-elements on target promoters. The CLOCK:BMAL1 protein complex drives the expression of PERIOD 1-3 (PER1-3) and CRYPTOCHROME 1 and 2 (CRY1 and 2) proteins, which in turn represent the negative limb of the TTL, inhibiting CLOCK and BMAL1, and thus their own transcription.[5] Components of the TTL serve as transcription factors for so-called clock-controlled genes and by that induce rhythmic expression of about half of all genes in one tissue or another. Thereby, the circadian clock sets the timing and synchronizes virtually all molecular and physiological processes of the body.

1.3 | Disturbed circadian clocks and psychiatric disorders

The role of disturbed circadian rhythms in psychiatric disorders has been object of manifold studies.[6] Although concrete molecular mechanisms are not well understood, lost synchronization between the organism and the environment and between different physiological processes, reduced increase and decrease of endocrine signals and processes, and altered sleep architecture provide hypothetical explanations of how disturbances of the circadian system and altered regulation of emotions and behavior may be related.[7] It is known from human and animal research that disturbances of circadian clocks are particularly associated with the development of mood and anxiety disorders.[8-11] For instance, disruption of the sleep–wake cycle is a hallmark of affective as well as of anxiety disorders.[12] Shifted or disrupted behavioral and endocrinological rhythms have been found in patients with depression[13] and anxiety.[14] Importantly, this relationship can be regarded as bidirectional. Environmental disruptions of circadian rhythms, such as shift work, increases the risk of mental disorders.[15] Furthermore, subjects with extreme chronotypes are at higher risk to develop psychiatric disorders.[16] Interestingly, patients suffering from mood or anxiety disorders profit from chronotherapeutic interventions,[17,18] demonstrating that therapy approaches normalizing or strengthening circadian rhythms improve symptoms of mental disorders. In addition to human data, animal models were used to support the link between circadian rhythms and psychiatric endophenotypes. For instance, the $Clock^{\Delta 19}$ mutant mouse has a long endogenous circadian period of ~27 hours with arrhythmic behavioral patterns when kept in constant darkness.[19] These mice mimic mania symptoms being hyperactive, showing reduced anxiety and increased seeking for drugs of abuse.[10,20,21] Contrarily, $Per2^{Brdm1}$ mice have short free-running periods before they turn arrhythmic in constant darkness, but display mania-like behavior as well.[22,23] Other clock gene mutations cause depression- or anxiety-like behaviors. For instance, $Per1/2^{ldc}$ mice display anxiety-like behavior.[24] Importantly, behavioral consequences may depend on the background strain and the type of circadian gene mutation.[25] Also, $Cry1/2^{-/-}$ mice, which are not able to express circadian rhythms, were shown to have cognitive dysfunctions and anxiety-like behavior, which was attributed to dysregulation of striatal extracellular signal-regulated kinase (ERK).[26]

However, the previous behavioral characterization of $Cry1/2^{-/-}$ mice only covered a few behavioral aspects. Therefore, in the present study, we intended to conduct an in-depth neurobehavioral analysis in $Cry1/2^{-/-}$ mice and further mechanistic explanations for altered behavior to gain more insights into the relationship of dysfunctional circadian clock mechanisms and psychiatric phenotypes.

2 | MATERIAL AND METHODS

2.1 | Animals

$Cry1/2^{-/-}$; $Per2^{Luc}$ mice[27,28] with C57BL/6J background were kindly provided by Michael Hastings, MRC Laboratory of Molecular Biology,

Cambridge, UK and backcrossed to same C57BL/6J background mice from our stock. To maintain congenic strains, the mutant and the WT strains are backcrossed every 5 to 10 generations to refresh the background. Littermates were paired with each other in order to eventually receive two separate *Cry1/2*$^{-/-}$; *Per2*^{Luc} (henceforth referred to as *Cry1/2*$^{-/-}$) and *Cry1/2*$^{+/+}$; *Per2*^{Luc} (henceforth referred to as WT) lines. All experiments were carried out in male WT and *Cry1/2*$^{-/-}$ mice at the age of 8 to 15 weeks. Mice were group housed and maintained in 12:12 light/dark (LD) cycles with lights turned on at 7 AM If not otherwise stated, water and food were provided ad libitum. Mouse studies were conducted in accordance with regulation of German Animal Protection Law. In all behavioral experiments, animals were brought to the experimental room 10 minutes in advance to the start of the actual test for habituation. An attempt was made to keep the number of animals low by using animal cohorts for several tests, starting with the least stressful tests and finishing with the most stressful tests. In total, there were four different cohorts with which tests were performed in the following order: (a) cohort: open field test, Y-maze test, social interaction, tail suspension test, and learned helplessness. (b) cohort: IntelliCage: place preference, reverse learning, serial reverse learning, sucrose preference, progressive ratio, followed by light-dark box. (c) cohort: IntelliCage: impulsivity, followed by prepulse inhibition test, and (d) cohort: learned helplessness only.

2.2 | Open field test

Open Field Test I: Novelty-induced and spontaneous exploratory behavior was monitored at ZT3 in an open field area (50 cm × 50 cm × 50 cm) for 10 minutes. Mice were video-recorded and distance, speed, time spent in predefined areas, number of im/mobile episodes, and time im/mobile were assessed with the behavioral tracking software ANY-maze, Stoelting, IL. Illumination during the test was set to 1600 lx.

Open Field Test II: For assessment of the same parameters in a familiar environment, a second identical open field test (OFT II) was performed 60 minutes after the first open field test.

2.3 | Y-maze test

Working memory capacity was assessed by quantification of spontaneous alternations in the Y-maze at ZT6. The Y-maze consists of three identical arms (A, B, C) in the shape of a "Y" and was conducted at 50 to 70 lx for 10 minutes. Spontaneous alternations describe the number of full sequences of visits to each arm of the arena without repetition (eg, A-B-C, B-A-C or B-C-A, but not A-B-A, C-B-C, or B-A-B). Mice were tracked and analyses were conducted using ANY-maze.

2.4 | Light-dark box

The light-dark boxes consist of two compartments connected by a small open gate. One compartment is open, illuminated (1600 lx) and has clear walls, while the other compartment consists of non-transparent, black walls and a black lid keeping the inside dark (<10 lx). At ZT8, anxiety-like behavior was measured as time spent in the illuminated compartment and the number of entries to that compartment.

2.5 | Social interaction test

Social interaction was assessed at ZT2 by the social interaction test as previously described.[29] The time of active social interaction with a so-called stimulus mouse (ovariectomized 129S1/SvImJ female mice) was measured. Stimulus mice were transferred to individual cages 1 hour before starting the test session for habituation. Illumination during that time and the following testing was set to 50 to 70 lx. After 1 hour, the test mouse was placed into the cage and the frequency and duration the test mouse spent in active social interaction (interest and mounting) was measured manually for 4 minutes.

2.6 | IntelliCage system

The IntelliCage system and software (TSE-Systems GmbH, Bad Homburg, Germany) has been described in detail previously.[30,31] The system consists of cages with four corners, in which the mice can open gates for access to drinking bottles by doing nosepokes. Visits in corners, number of nosepokes, successful opening of gates, and number of licks on the bottles are measured automatically.

At least 5 days before the start of experiments, transponders for radiofrequency identification (PlanetID GmbH, Essen, Germany) were implanted subcutaneously in the dorsocervical region under isoflurane inhalation anesthesia. Then, mice were transferred and adapted in groups to the IntelliCages. *Days 1 to 2, free adaptation*: all gates were open to provide unlimited access to drinking bottles. *Day 3, corner visit adaptation*: gates remained closed until a mouse visited the corner. *Days 4 to 5, nosepoke adaptation*: gates remained closed until mice performed a nosepoke. After the adaptation phase, either learning ability and flexibility, sucrose preference and the willingness to work for it (progressive ratio), waiting impulsivity, or circadian rest/activity behavior were assessed.

Place Learning and Cognitive Flexibility: For the assessment of place learning abilities, mice were given two days to learn that water could only be accessed in one specific corner of the cage. Corner assignment was randomized. Learning success was measured with a preference score in an (A − B)/A + B) design. Positive values signify preference for the assigned corner, while negative values show avoidance. Afterwards, cognitive flexibility was tested using a reversal learning protocol for seven consecutive days, during which the mice had to learn that water was now available at a different corner every 24 hours. The sequence of corners was again randomized. Flexibility was measured as area under the preference score-derived learning curve.

Sucrose Preference and Operant Conditioning: One of the two water bottles in each corner was filled with 1% sucrose solution, whereas the other bottle contained autoclaved tap water. Preference of mice to drink sucrose solution was recorded for a period of 24 hours. Subsequently, mice were trained in a progressive ratio paradigm. For the following 6 days, the sucrose solution was only accessible after executing an increasing number of nosepokes inconsecutive stages. The increase of required nosepokes per trial to obtain access to the sucrose solution and to obtain the next stage was calculated according to the formula: Response ratio = $(5e^{[0.2 * \text{reward number}]}) - 5$, resulting in a rise of nosepokes as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, and so forth. The next stage was reached when the mouse completed the required number of nosepokes within one trial. A trial is defined as a visit of a corner with a minimum of one nosepoke. Leaving the corner before reaching the required number of nosepokes terminates and restarts the trial and the mouse must repeat it until the next stage is reached. During the test, mice had free access to tap water bottles.

Waiting Impulsivity: Assessment of waiting impulsivity has been described previously.[32] Mice had to perform a first nosepoke and then wait either 0.5, 1.5, or 2.5 seconds (assigned randomly) to obtain access to water bottles. A trial in this paradigm follows the same definition as described for *Sucrose Preference and Operant* Conditioning. If a mouse would make a second nosepoke within the waiting time (premature response), the trial was counted as a failure and the mouse was punished with no-access to water during this visit. Based on the number of failures and successful trials a premature response rate (PRR) was calculated, where a high PRR indicates more impulsive behavior.

Circadian Rest/Activity Behavior: Rest/activity cycles were measured based on corner visit frequency throughout the day as an indirect measure of general locomotor activity. First, mice were entrained to an LD 12:12 cycle and corner visits were recorded for 5 days. Then, the IntelliCages were transferred to ventilated, light- and soundproof boxes with DD conditions for 14 days.

2.7 | Prepulse inhibition (PPI)

Startle response was automatically measured via movement-induced vibration of the base plate of startle-response-enclosures (SR-LAB, San Diego Instruments, San Diego) at ZT2. The background noise in the boxes was set to a constant level of 65 dBA. For short term habituation the main 40 ms 115 dBA pulse was presented 10 times before the actual test sequence (baseline startle response). To test the baseline startle response, a 40 ms 115 dBA pulse was presented 10 times before the actual test sequence. Then, a non-startling 20 ms prepulse of an intensity of 70, 75, or 80 dBA was presented, which was followed by a pulse of 115 dBA occurring 100 ms after the start of the prepulse. Each condition was repeated in 10 trials. All trials were presented in pseudorandomized order with inter-trial intervals ranging from 8 to 22 seconds.

2.8 | Tail suspension test

Lack of active avoidance of an aversive situation was measured in the tail suspension test at ZT4 as described previously.[33] Briefly, adhesive tape was used to attach mice to a bar located 30 cm above a flat surface for 6 minutes. Plastic tubes were put over the tail to prevent mice from climbing up the tail. Immobility was quantified by measuring the amount of time when no whole-body movement was observed. Whole-body movement was defined as movement of the center of the body. Flailing with the front limbs was not counted as movement. Mice were video-recorded and whole-body movements were quantified (ANY-maze).

2.9 | Learned helplessness

The learned helplessness study paradigm consisted of two training days (ZT3) and one testing day (ZT8) as described previously.[8,33,34] On both training days mice were restrained and received 120 electric tail shocks, lasting 5 seconds each, within 60 minutes. Shock intensity was gradually increased in 0.05 mA steps from 0.25 to 0.60 mA: every 15 shocks. On the testing day, mice were placed into shuttle boxes (Panlab Harvard Apparatus, Spain) and received 30 electric shocks (0.10 mA) to their feet through the grid floor. During each test shock (maximum duration 30 seconds), the gate remained open, and mice could escape the shock by crossing the gate to the adjacent compartment. The schedule in trials #1-5 was fixed ratio (FR) 1 (crossing the gate once to escape the shock). In the remaining trials #6-30, the schedule was changed to FR-2 (crossing the gate twice to escape the shock). Escape latency and number of escape failures were recorded automatically.

2.10 | Quantitative real-time PCR

c-Fos levels in the prefrontal cortex and the amygdala were quantified in stressed and non-stressed WT and *Cry1/2*$^{-/-}$ mice at ZT4 and ZT16. To induce stress, an open field experiment was performed in animals that never had encountered this setup before for 10 minutes as described above. After the test, the stressed mice were returned to their home cages for 30 to 60 minutes to allow sufficient time for *c-Fos* to increase. Then, brain areas were prepared, harvested and snap frozen on dry ice instantly. During the 30 to 60 minutes waiting period, non-stressed mice were transferred to the procedure room and brain areas were prepared, harvested, and snap frozen immediately. Illumination during the habituation, the stress procedure, and the brain harvest was set to 1600 lx. Quantitative real-time PCR (qPCR) was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems, CA) with GoTaq SYBR Master Mix (Promega, WI). Relative quantification of expression levels by a modified ΔΔCT calculation was performed as described.[35] Primer sequences were: *β-act*: for. 5′-CCCTGAAGTACCCCATTGAA-3′, rev. 5′-AGGTGTGGTGCCAGATCTTC-3′; *c-Fos*: for. 5′-TCGACCTAGGGAGGACCTTACC-3′, rev. 5′-TCGACCTAGGGAGGACCTTACC-3′.

2.11 | Statistical analysis

Data analyses were performed, statistical tests were calculated, and graphs plotted using GraphPad Prism (GraphPad Software, CA) and RStudio (RStudio, MA). The automated user interface FlowR (XBehavior, Dägerlen, Switzerland) was used for behavioral data that has been assessed in the IntelliCage Setup. Details about statistical tests used for specific experiments are indicated in the corresponding figure legends.

3 | RESULTS

3.1 | Endogenous circadian rhythms are lost in *Cryptochrome*-deficient mice

To assess circadian activity patterns, corner visits were measured over time in IntelliCages. In LD conditions, both, WT and *Cry1/2*$^{-/-}$ mice show rhythmic patterns in their visits in the corners of the cages. Under DD conditions, however, the behavior of *Cry1/2*$^{-/-}$ mice is not rhythmic. This shows that their rhythmic behavior in LD is because of so-called masking effects, but that they are not capable of producing endogenous circadian rhythms (Figure 1A). Importantly, their average total activity level under constant conditions is similar to that of WT mice, indicating that they are not restricted in their ability to move because of the loss of *Cryptochrome* genes. (Figure 1B). In line with these results, *Cry1/2*$^{-/-}$ mice only reach significant circadian periods of ~24 hours under LD conditions, whereas under DD they do not display any significant rhythm in corner visits.

3.2 | *Cryptochrome*-deficient mice show mild cognitive abnormalities

Learning abilities and cognitive flexibility, measured in the IntelliCages by tasks of place preference as well as reversal and serial reversal learning, are at the same level in *Cry1/2*$^{-/-}$ mice compared with WT mice (Figure 2A; *Place Pref.*: t_{39} = 1.037, P = .3061; *Rev. Learning*: t_{39} = 0.074, P = .9413; *Serial Rev. Learning*: t_{39} = 1.342, P = .1873 [Student t test]). However, in the Y-maze test, in comparison to WT mice, *Cry1/2*$^{-/-}$ mice show increased spontaneous alternations, which means that they examined the arms of the Y-maze more often in a regular sequence (Figure 2B; t_{38} = 2.919, P = .0059 [Student t test]). To test whether *Cry1/2*$^{-/-}$ mice suffer neurodevelopmental deficits that contribute to impairments of sensomotoric gating, a prepulse inhibition experiment was conducted. In unaffected animals, a prepulse stimulus reduces startle responses to a second, more intense stimulus. Interestingly, in *Cry1/2*$^{-/-}$ mice, this effect is reduced as the prepulse inhibition is lower at all tested prepulse intensities (Figure 2C; *Interaction*, $F_{2,34}$ = .7584, P = .4762; *Prepulse Intensity*, $F_{2,34}$ = 48.93, P < .0001; *Genotype*, $F_{1,34}$ = 5.074, P = .0378 [two-way repeated measures analysis of variance]).

3.3 | *Cryptochrome*-deficient mice exhibit hypoactivity in unfamiliar environments and other signs of elevated anxiety

Next, to test whether *Cry1/2*$^{-/-}$ mice display anxiety-like behavior, social interaction, open field, and light/dark box tests were conducted. In the social interaction test, WT mice and *Cry1/2*$^{-/-}$ mice show the same interest in female ovariectomized stimulus mice. This is reflected in the same number of approaches and the same time that the two genotypes show interest in the stimulus mouse. Furthermore, WT and *Cry1/2*$^{-/-}$ mice mount the stimulus mice equally often. However, the duration of mounting approaches is significantly shorter in *Cry1/2*$^{-/-}$ mice (Figure 3A; *Interest Approaches*: t_{42} = .251, P = .8032; *Time Interest*: t_{42} = 1.035, P = .3065; *Mounting Approaches*: t_{42} = 1.219, P = .2295; *Time Mounting*: t_{42} = 2.323, P = .0254 [Student t test]). Next, place preference and mobility were measured in the open field test and the light/dark box test. WT mice and *Cry1/2*$^{-/-}$ mice have a similar preference for staying in the center and in the corners of the open field arena as well as in the light compartment of the light/dark boxes (Figure 3B; OFT Center: t_{38} = 0.1037, P = .9179; OFT Corner: t_{38} = 0.1659, P = .8692; *LDB Time in Light*: t_{42} = 0.2382, P = .8129 [Student t test]). However, in both tests, *Cry1/2*$^{-/-}$ mice travel less distance, irrespective of center or corner and spend more time immobile (Figure 3C), which becomes also evident from the low number of entries into the center and the corner of the open field and into the light compartment of the light-dark box (Figure 3C; *OFT Total Distance*: t_{38} = 5.375, P < .0001; *OFT Time Immobile*: t_{38} = 4.227, P = .0001; *OFT Center Entries*: t_{38} = 3.061, P = .004; *OFT Corner Entries*: t_{38} = 5.610, P < .0001; *OFT Center Distance*: t_{38} = 2.228, P = .0319; *OFT Corner Distance*: t_{38} = 4.363, P < .0001; *LDB Distance*: t_{40} = 1.705, P = .0959; *LDB Time Immobile*: t_{40} = 2.521, P = .0158; *LDB Entries Light*: t_{40} = 2.750, P = .0088; *YM Distance*: t_{38} = 4.637, P < .0001; *YM Time Immobile*: t_{38} = 3.305, P = .0021 (Student t test)). Similar observations were also made in the Y-maze test, where *Cry1/2*$^{-/-}$ mice also cover less distance and spend more time immobile (Figure 3C). Notably, based on the results of maximum speed in the open field arena as well as in the Y-maze and total corner visits in IntelliCages (see also Figure 1B), *Cry1/2*$^{-/-}$ mice do not suffer from limitations in locomotion or their general ability to move (Figure 3D; *OFT*: t_{38} = 1.748, P = .0885; *YM*: t_{38} = 1.326, P = .1927; *Total Corner Visits*: t_{62} = 0.5867, P = .5595 (Student t test)).

3.4 | *Cryptochrome*-deficient mice have a limited ability to habituate to new environments

To test further whether *Cry1/2*$^{-/-}$ mice show more parallelism to anxiety, habituation behavior was measured in two consecutive open field tests. In the second open field test, WT mice show habituation to the familiar environment as they increase exploratory behavior by visiting the center and disregarding the supposedly safe corner for a longer period. Moreover, they increase their locomotion speed. Contrarily, *Cry1/2*$^{-/-}$ mice prefer corner as much as in the first open

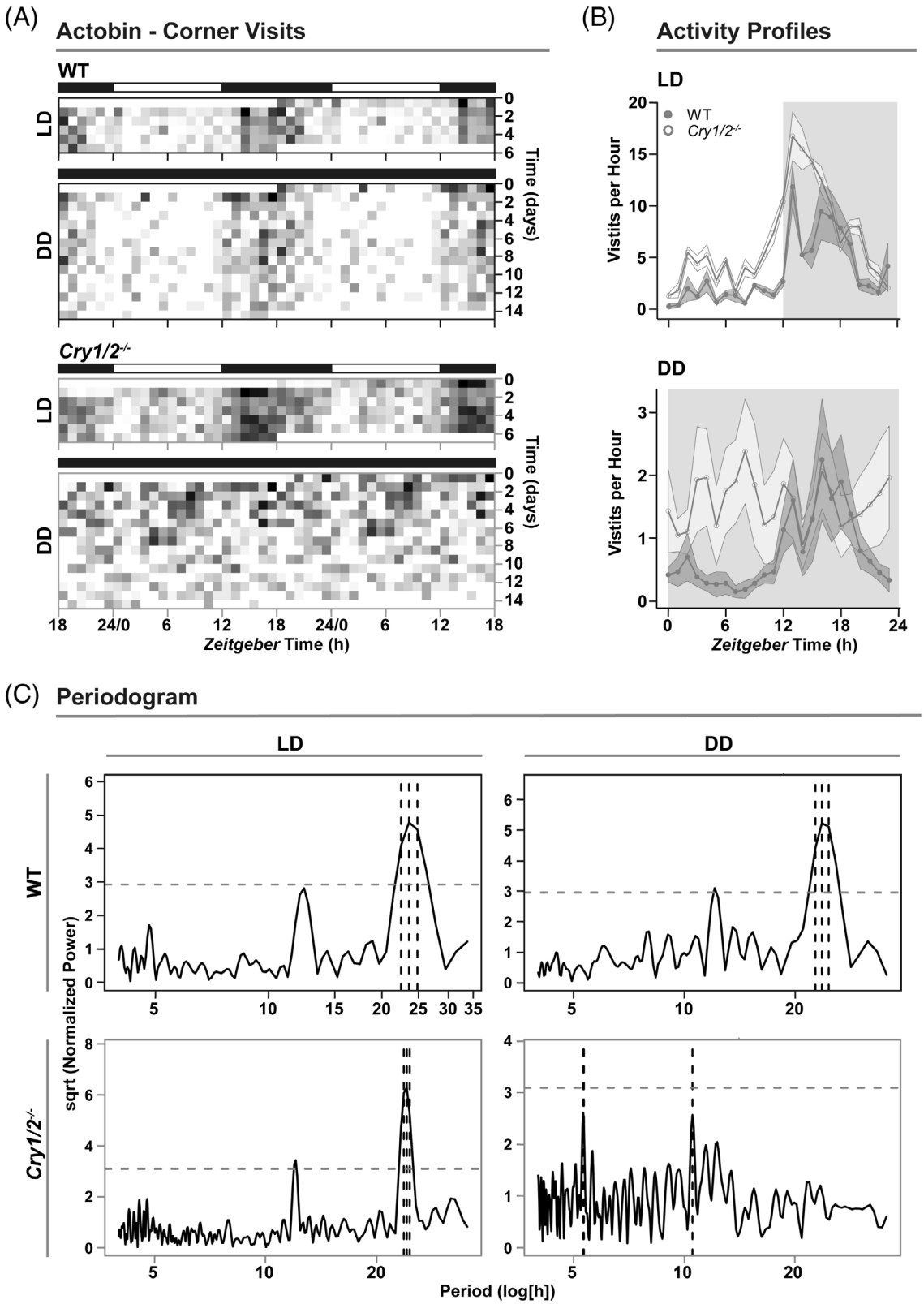

FIGURE 1 Endogenous circadian rhythms are lost in *Cryptochrome*-deficient mice. Day-night activity patterns were determined based on corner visits in IntelliCages. A, Average activity patterns under LD and DD conditions show that *Cry1/2*$^{-/-}$ mice do not express endogenous circadian rhythms in constant conditions but show masking in a rhythmic environment. Data are shown as double plots. Darker tones of gray represent higher numbers of corner visits within 60 minutes; n = 4. B, Accumulated activity profiles of WT and *Cry1/2*$^{-/-}$ mice under LD and DD conditions; n = 4. C, Lomb-Scargle Periodograms of WT and *Cry1/2*$^{-/-}$ mice under LD and DD conditions; n = 4. Light gray dashed lines show thresholds for significantly rhythmic periods. Dark dashed lines show range of significant periods

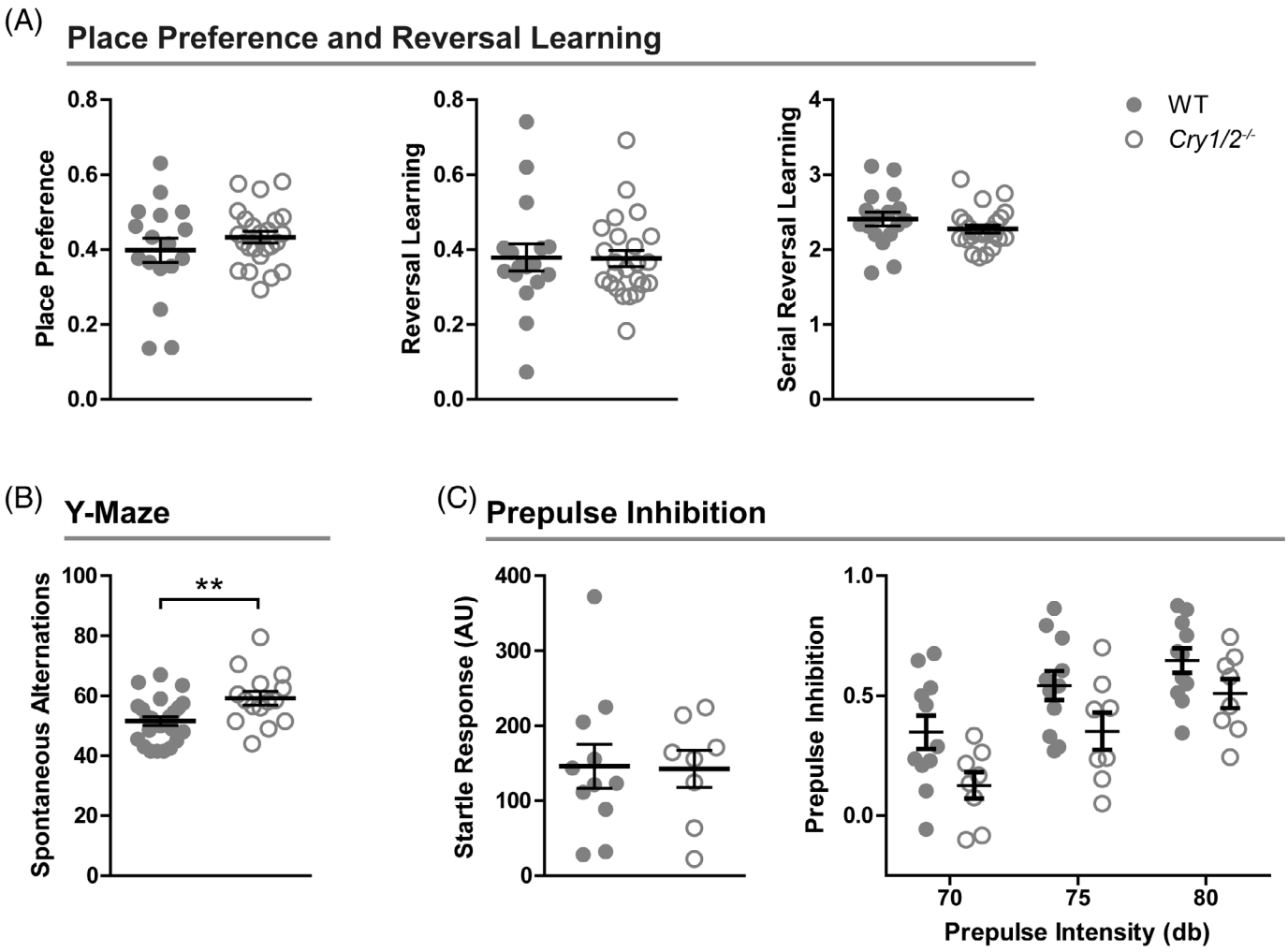

FIGURE 2 *Cryptochrome*-deficient mice show mild cognitive abnormalities. A, Reversal learning is not affected in *Cry1/2*$^{-/-}$ mice. Place preference for water bottles, single and reversal learning of water bottle position changes were tested in IntelliCages. Data are shown as dot plot with mean ± SEM; (Student *t* test); n = 17/24. B, *Cry1/2*$^{-/-}$ mice show more spontaneous alternations in the Y-maze test. Data are shown as dot plot with mean ± SEM; $^{**}P < .01$ (Student *t* test); n = 25/15. C, *Cry1/2*$^{-/-}$ mice display reduced prepulse inhibition. Prepulse inhibition was tested at baseline with a pulse of 115 dB and with prepulses set at 70, 75, and 80 dB. Data are shown as dot plot with mean ± SEM; post hoc test: not significant (two-way repeated measures analysis of variance with Bonferroni posttest); n = 11/8

field test and avoid center even more than during the first test. In both genotypes, walking distance decreases and immobility time increases, although the decrease of walking distance is more pronounced in WT mice (Figure 4A; *CORNER*: *Interaction*, $F_{1,38} = 4.906$, $P = .0328$; *Time*, $F_{1,38} = 1.676$, $P = .2033$; *Genotype*, $F_{1,38} = 2.215$, $P = .1449$; *CENTER*: *Interaction*, $F_{1,38} = 5.075$, $P = .0301$; *Time*, $F_{1,38} < 0.001$, $P = .9975$; *Genotype*, $F_{1,38} = 3.209$, $P = .0812$; *MAX. SPEED*: *Interaction*, $F_{1,38} = 6.731$, $P = .0134$; *Time*, $F_{1,38} = 2.286$, $P = .1388$; *Genotype*, $F_{1,38} = 13.38$, $P = .0008$; *DISTANCE*: *Interaction*, $F_{1,38} = 4.463$, $P = .0413$; *Time*, $F_{1,38} = 153.0$, $P < .0001$; *Genotype*, $F_{1,38} = 25.42$, $p = <.0001$; *IMMOBILITY TIME*: *Interaction*, $F_{1,38} = 0.35$, $P = .5576$; *Time*, $F_{1,38} = 131.0$, $p = <.0001$; *Genotype*, $F_{1,38} = 13.58$, $P = .0007$ (two-way repeated measures analysis of variance)). Similar observations can be made in the Y-maze test. In the first half of the test, WT and *Cry1/2*$^{-/-}$ mice make a comparable number of spontaneous alternations. However, in the second half of the test, WT mice make fewer spontaneous alternations, while *Cry1/2*$^{-/-}$ mice slightly increase the number. Like in the open field test, walking distance and immobility time decrease or increase, respectively, but the decrease of walking distance is more pronounced in WT mice also in the Y-maze test (Figure 4B; *SPONTANEOUS ALTERNATIONS*: *Interaction*, $F_{1,38} = 0.7018$, $P = .4074$; *Time*, $F_{1,38} = 0.0109$, $P = .9174$; *Genotype*, $F_{1,38} = 8.522$, $P = .0059$; *DISTANCE*: *Interaction*, $F_{1,38} = 9.302$, $P = .0042$; *Time*, $F_{1,38} = 279.5$, $P < .0001$; *Genotype*, $F_{1,38} = 18.46$, $P = .0001$; *IMMOBILITY TIME*: *Interaction*, $F_{1,38} = 1.226$, $P = .2754$; *Time*, $F_{1,38} = 135.3$, $P < .0001$; *Genotype*, $F_{1,38} = 15.65$, $P = .0003$ (two-way repeated measures analysis of variance)).

3.5 | Depression-like behavior is not induced by loss of *Cryptochromes*

In order to assess whether *Cry1/2*$^{-/-}$ mice additionally show depression-like characteristics, their behavior was measured in the tail

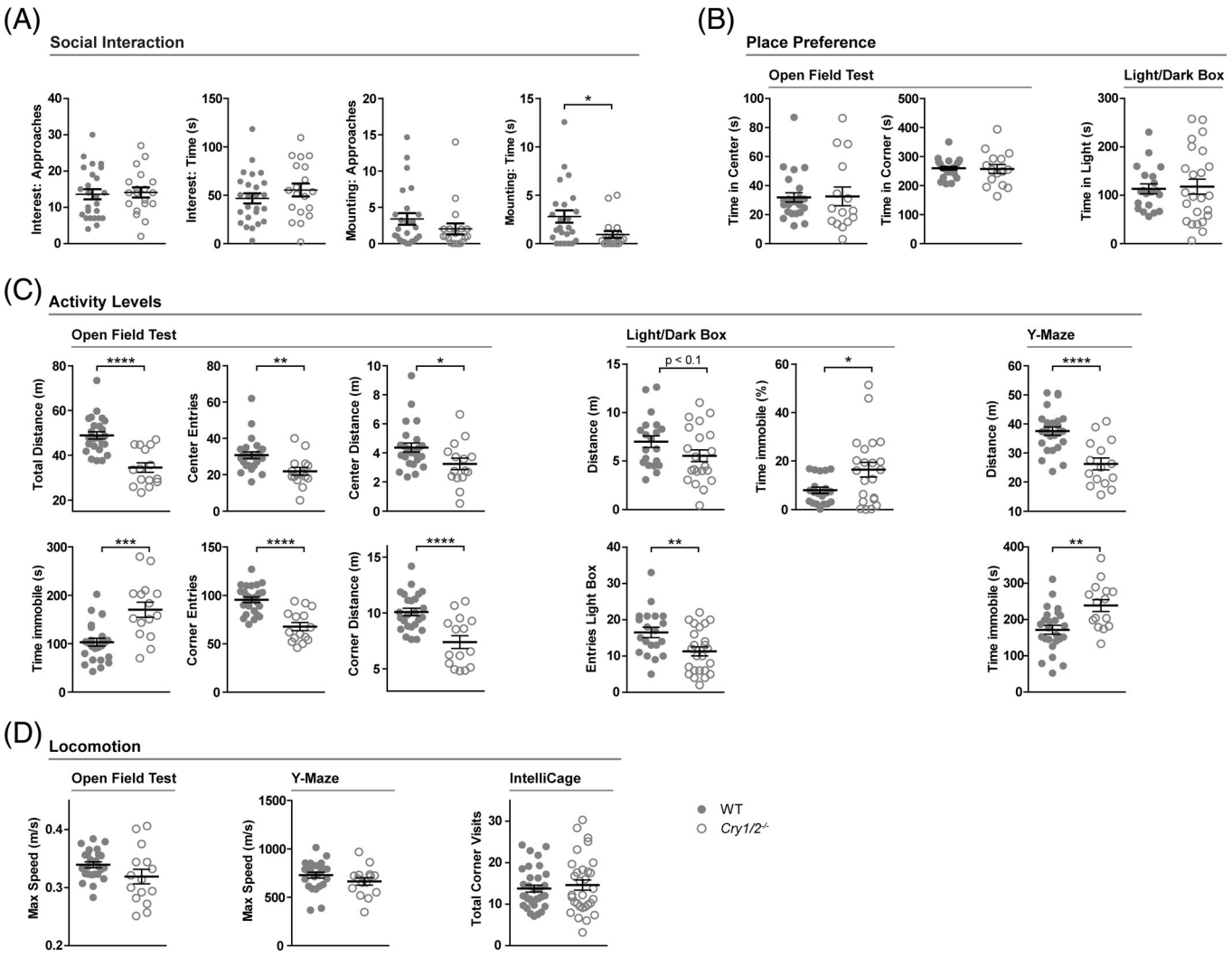

FIGURE 3 *Cryptochrome*-deficient mice exhibit hypoactivity in unfamiliar environments and other signs of elevated anxiety. A, In the social interaction test, *Cry1/2*$^{-/-}$ mice spend less time mounting an ovariectomized female mouse but show no differences in the number of mounting approaches and in interest time and approaches. Data are shown as dot plot with mean ± SEM; **P* < .05 (Student *t* test); n = 24/18. B, In the open field test and the light/dark box test, WT and *Cry1/2*$^{-/-}$ mice display similar place preferences. Data are shown as dot plot with mean ± SEM; (Student *t* test); *OFT*: n = 25/15; *LDB*: n = 20/24. C, *Cry1/2*$^{-/-}$ mice are hypoactive over various tests when exposed to unknown environments. In the open field test, the light/dark box test, and the Y-maze test, they cover less distance, enter specific areas less frequently, and are immobile longer. Data are shown as dot plot with mean ± SEM; **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 (Student *t* test); *OFT*: n = 25/15; *LDB*: n = 20/24; *YM*: n = 25/15. D, Based on the maximum speed in the open field and the Y-maze test as well as the total corner visits in the IntelliCage, the mobility of *Cry1/2*$^{-/-}$ mice is not restricted. Data are shown as dot plot with mean ± SEM; (Student *t* test); *OFT*: n = 25/15; *YM*: n = 25/15; *IC*: n = 33/31

suspension test and their susceptibility to the development of learned helplessness and their preference for sucrose was determined. In the tail suspension test, their immobility time does not differ from WT mice (Figure 5A; t_{36} = 0.5905, *P* = .5586 (Student *t* test)). Moreover, *Cry1/2*$^{-/-}$ mice are no more susceptible to helplessness than WT mice after being exposed to uncontrollable stress in the learned helplessness paradigm, as they have similar escape latencies and a similar number of escape failures (Figure 5B; *LATENCY*: t_{24} = 0.4179, *P* = .6797, *FAILURES*: t_{24} = 0.3624, *P* = .7202 (Student *t* test)). Additionally, in the sucrose preference test, *Cry1/2*$^{-/-}$ mice do not prefer sucrose solution more than WT mice do (Figure 5C: t_{50} = 0.1555, *P* = .8771 (Student *t* test). Interestingly, however, their willingness to work for the sucrose solution is higher on a progressive ratio schedule. *Cry1/2*$^{-/-}$ mice reach higher stages than WT mice, which means that they are willing to do more nosepokes in order to access the sucrose. Compared with WT mice, *Cry1/2*$^{-/-}$ mice also need fewer trials to reach the next stage of the paradigm, indicating that they abort the attempts to reach the sucrose less often (Figure 5D; *MAX. STAGE*: t_{45} = 4.481, *P* < .0001, *MAX. NOSEPOKES*: t_{45} = 3.551, *P* = .0009, *TRIALS PER STAGE*: t_{45} = 4.931, *P* < .0001, *TOTAL TRIALS*: t_{45} = 1.108, *P* = .274 (Student *t* test)). Because the general preference for sucrose was not increased, but the determination to reach the sugar solution

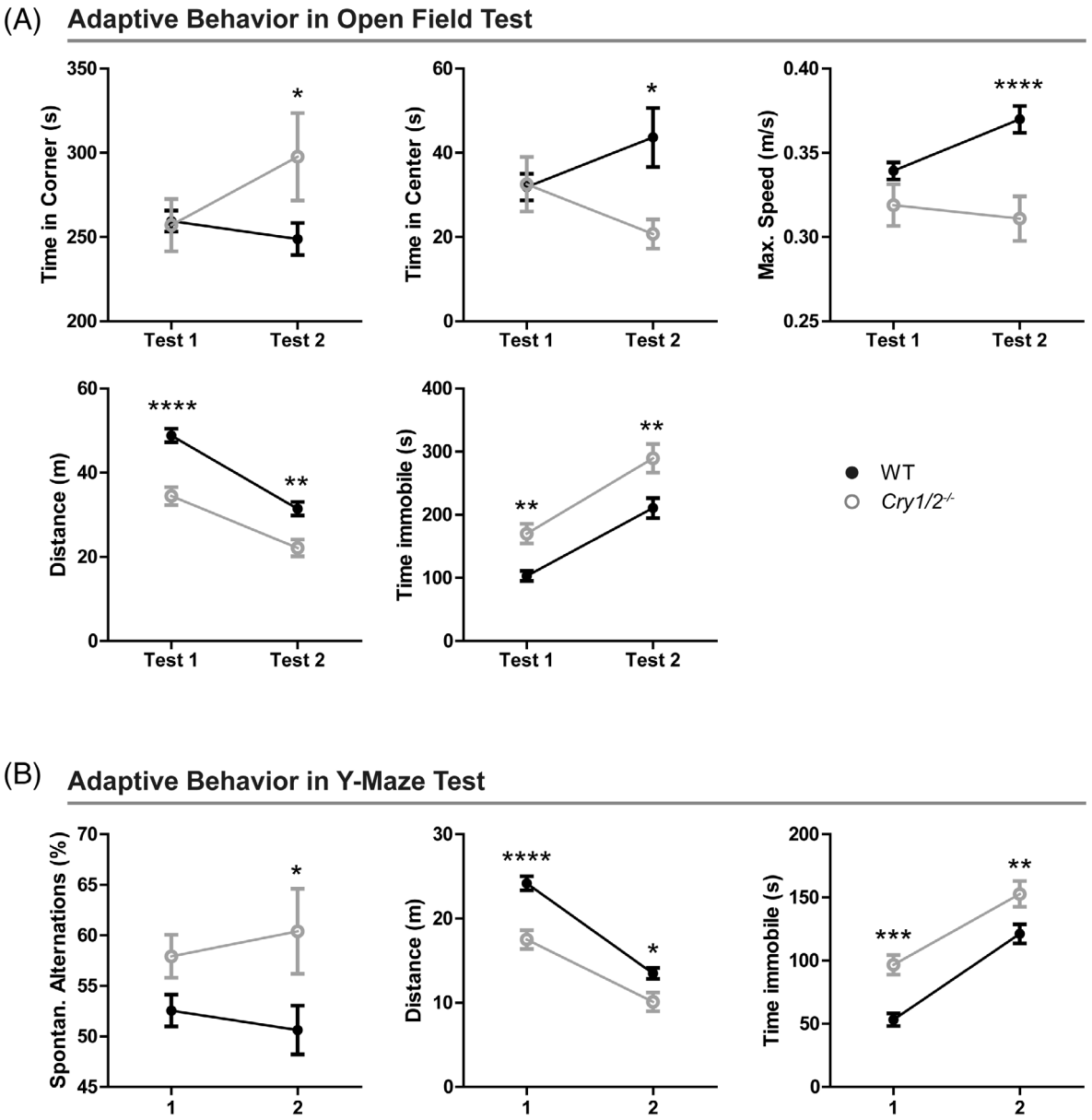

FIGURE 4 *Cryptochrome*-deficient mice have a limited habituation to adapt to new environments. A, In a second open field test, *Cry1/2*$^{-/-}$ mice spend more time in the corner and less time in the center than in first test and as WT mice. Also, WT mice increase movement speed while *Cry1/2*$^{-/-}$ mice do not. Distance decreases and time immobile increases in both genotypes, although distance decrease is more pronounced in WT mice. Data are shown as dot plot with mean ± SEM; **P* < .05, ***P* < .01, *****P* < .0001 (two-way repeated measures analysis of variance with Bonferroni posttest); n = 25/15. B, In the second half of the Y-maze test, *Cry1/2*$^{-/-}$ mice show a similar number of spontaneous alternations as in the first half, while WT mice reduce spontaneous alternations. Distance decreases and time immobile increases in both genotypes, although distance decrease is more pronounced in WT mice. Data are shown as dot plot with mean ± SEM; **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 (two-way repeated measures analysis of variance with Bonferroni posttest); n = 25/15

seemed higher in *Cry1/2*$^{-/-}$ mice, their impulsive behavior was examined. This result should provide information about the capability for operant learning of the mice. In contrast to WT mice, *Cry1/2*$^{-/-}$ mice are more successful in waiting an appropriate time to obtain access to the reward, thus being less impulsive (Figure 5E; *Interaction*, $F_{2,34}$ = 16.44, P < .0001; *Delay Time*, $F_{2,34}$ = 455.8, P < .0001; *Genotype*, $F_{1,34}$ = 3.212, P = .0909 (two-way repeated measures analysis of variance with Bonferroni posttest)).

3.6 | *Cryptochrome*-deficient mice display restless behavior across various tests

In all behavioral experiments in which the overall activity of the mice was measured, it was found that *Cry1/2*$^{-/-}$ mice were restless in these situations. Their restlessness is expressed by significantly increased transitions between mobile and immobile episodes in the open field test, Y-maze test, tail suspension test, and, with a statistical

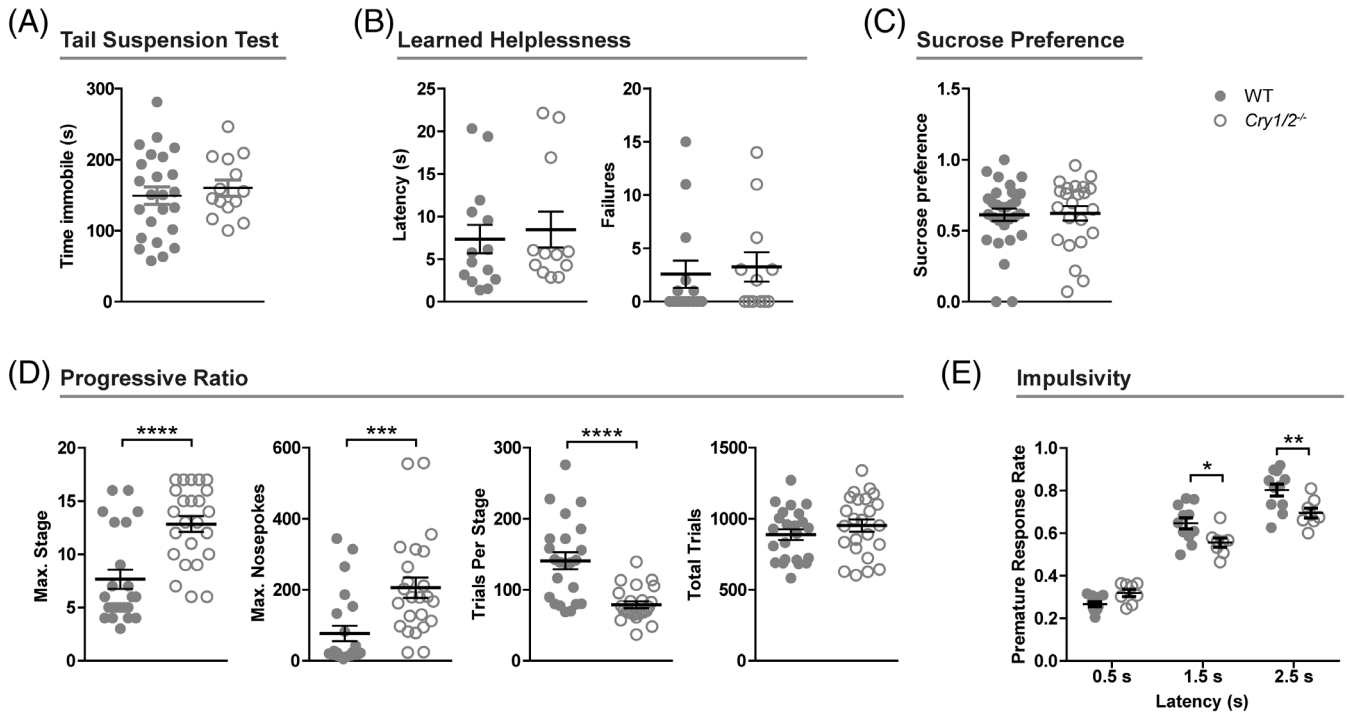

FIGURE 5 Depression-like behavior is not induced by loss of *Cryptochromes*. A, In the tail suspension test, *Cry1/2*$^{-/-}$ mice do not show significant differences in immobility time. Data are shown as dot plot with mean ± SEM; (Student *t* test); n = 24/14. B, Escape latency and number of failures in the learned helplessness paradigm are not different in WT and *Cry1/2*$^{-/-}$ mice. Data are shown as dot plot with mean ± SEM; (Student *t* test); n = 14/12. C, The preference for sucrose is not different in *Cry1/2*$^{-/-}$ and WT mice. Data are shown as dot plot with mean ± SEM; (Student *t* test); n = 29/23. D, Although their preference for sucrose is unchanged, the progressive ratio breakpoint when no higher stage is reached is increased in *Cry1/2*$^{-/-}$ mice. They make more nosepokes overall and need less trials per stage to reach the next one. The number of overall trials is not different in *Cry1/2*$^{-/-}$ and WT mice. Data are shown as dot plot with mean ± SEM; ***P < .001, ****P < .0001 (Student *t* test); n = 23/24. E, Impulsivity is decreased in *Cry1/2*$^{-/-}$ mice. If it is required to endure 1.5 or 2.5 seconds with the second nosepoke to obtain a reward in the form of water, *Cry1/2*$^{-/-}$ mice make less premature nosepokes. Data are shown as dot plot with mean ± SEM; *P < .05, **P < .01 (two-way repeated measures analysis of variance with Bonferroni posttest); n = 11/8

trend, in the light dark box (Figure 6A; *MOBILITY*: *OFT*: t_{38} = 2.251, P = .0306; *YM*: t_{38} = 2.659, P = .0114; *LDB*: t_{40} = 1.773, P = .0839; *TST*: t_{38} = 2.680, P = .0108; *IMMOBILITY*: *OFT*: t_{38} = 2.219, P = .0325; *YM*: t_{38} = 2.682, P = .0108; *LDB*: t_{40} = 1.773, P = .0839; *TST*: t_{38} = 2.680, P = .0108 (Student *t* test)). Higher numbers of mobile and immobile episodes are equivalent to more frequent switching between one episode and the other (Figure 6B).

3.7 | The amygdala responsiveness to an anxiogenic stressor is increased in *Cryptochrome*-deficient mice

To confirm that the behavioral phenotype of *Cry1/2*$^{-/-}$ mice is related to increased anxiety, *c-Fos* levels in the amygdala were measured at ZT4 and ZT16 after the animals were exposed to an open field arena, a new and potentially unsecure environment. While *c-Fos* in the amygdala of WT animals increased only moderately, the increase was more pronounced in the amygdala of *Cry1/2*$^{-/-}$ mice at both times, ZT4 and ZT16 (Figure 7A). Overall, the amygdala of *Cry1/2*$^{-/-}$ mice is significantly more responsive to an anxiogenic stressor than the amygdala of WT mice (Figure 7B; *Interaction*, $F_{1,20}$ = 4.589, P = .447; *Genotype*, $F_{1,20}$ = 8.819, P = .0076; *Anxiogenic Stressor*, $F_{1,20}$ = 45.12, P < .0001 (two-way repeated measures analysis of variance)).

4 | DISCUSSION

In this study, we performed an in-depth characterization of *Cry1/2*$^{-/-}$ mice, which lack the central clock genes *Cry1* and *2*, and are thus unable to express endogenous circadian rhythms. Our results show that while *Cry1/2*$^{-/-}$ mice show only mild abnormalities at cognitive and social behavioral levels, they are more anxious than wildtype mice. Anxiety-like behavior is consistently evident in increased immobility in new environments, altered ability to habituate, compensatory behavior, and restless behavior across different behavioral tests. The relation between the behavior of *Cry1/2*$^{-/-}$ mice and anxiety was confirmed by an increased responsiveness of *c-Fos* in the amygdala after exposure to an anxiogenic stressor.

The experiments shown here were performed under LD conditions and in the inactive phase of the animals. Both can be limitations, because *Cry1/2*$^{-/-}$ mice show masking under LD conditions and are

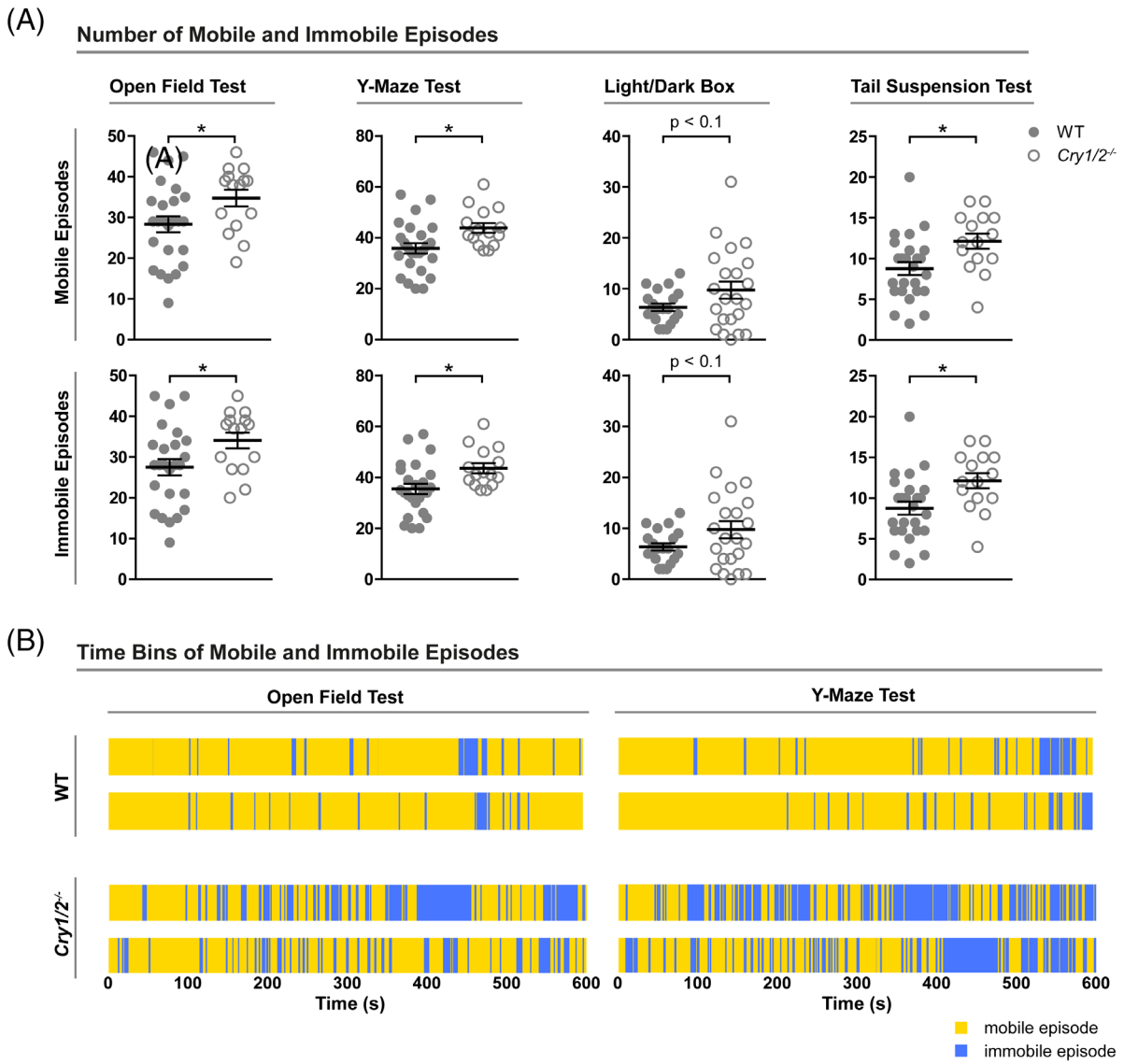

FIGURE 6 *Cryptochrome*-deficient mice display restless behavior across various tests. A, In the open field test, Y-maze test, light/dark box test, and tail suspension test, *Cry1/2*$^{-/-}$ mice switch significantly more frequently between mobile and immobile states, which is indicated by similarly increased numbers of both episodes. Data are shown as dot plot with mean ± SEM; (Student *t* test); *OFT*: n = 24/14, *YM*: n = 25/15, *LDB*: n = 20/22, *TST*: n = 25/15; **P* < .05, ***P* < .01 (Student *t* test). B, Increased switches between mobile and immobile episodes over the course of the full 600 seconds of the open field and the Y-maze tests are illustrated by the use of representative time bins

therefore not completely arrhythmic, and behavioral changes may have a different expression in their active phase. Thus, our results only allow the conclusion that the animals show significant behavioral differences at least at this time of day. Generalized anxiety is a trait rather than a state, and there is only little indication that the time of day plays a role in the expression of symptoms in humans[36] and in mice.[37,38] However, whether the observed differences between knockout and control animals are more or less pronounced in the inactive phase may be the goal of future experiments. In LD, *Cry1/2*$^{-/-}$ mice show rhythmic behavior, which raises the question to what extent their circadian core deficit, namely arrhythmicity, ultimately contributes to their behavioral phenotype. Importantly, masking is not equivalent to entrainment. Entrainment represents synchronization of an organism's endogenous clock with the external environment, which is not possible when the TTL is not functional. In other words, despite an underlying defect in the TTL, masking may occur, but not true entrainment. Our model therefore represents a situation in the real world in which an individual's clock could be disrupted while living in a rhythmic environment.

In our experiments, we confirmed previous results showing that *Cry1/2*$^{-/-}$ mice exhibit rhythmic masking behavior under LD conditions, but display an instantaneous loss of rhythms when transferred to constant darkness.[27] We found that under DD, the total activity levels of *Cry1/2*$^{-/-}$ mice are not different to those of WT mice, but that activity is equally distributed throughout the 24-hours period.

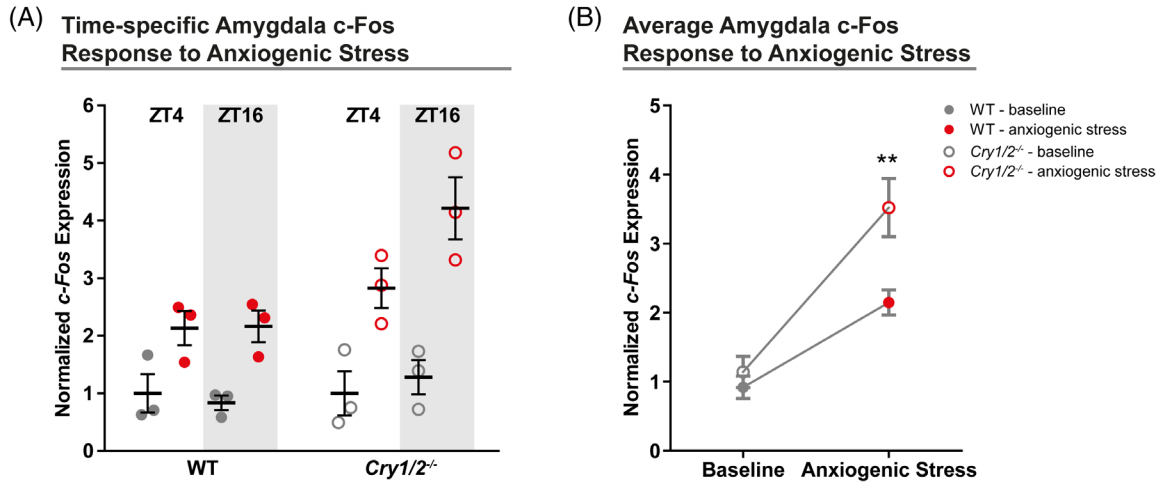

FIGURE 7 The amygdala responsiveness to an anxiogenic stressor is increased in *Cryptochrome*-deficient mice. A, To induce anxiety, WT and *Cry1/2*$^{-/-}$ mice were transferred to an open field arena at ZT4 and ZT16. Subsequently, *c-Fos* induction in the amygdala was measured. As controls, baseline *c-Fos* levels were measured of undisturbed animals at the same time-points. Data are shown as dot plot with mean ± SEM; n = 3/3. B, Overall, the response of *c-Fos* after triggering anxiety in the open field test is more pronounced in *Cry1/2*$^{-/-}$ mice than in WT mice. Data are shown as dot plot with mean ± SEM; $^{**}P < .01$ (two-way repeated measures analysis of variance with Bonferroni posttest); n = 6/6

In our study, we aimed to investigate to what extent the loss of *Cryptochromes* affects different levels of psychiatric and cognitive endophenotypes of mice. *Cry1/2*$^{-/-}$ mice were previously shown to have deficits in 24-hours object memory.[26] To complement this finding, we also tested whether the mice also had limitations in working, spatial and regulatory memory. In line with a former study which tested spatial learning in *Cry1/2*$^{-/-}$ mice,[39] we found that place preference and reversal learning are not altered in *Cry*-deficient mice when tested in IntelliCages. In addition, their working memory in the Y-maze test appears slightly improved. Together, these results show that although long-term memory is impaired, the working memory of *Cry1/2*$^{-/-}$ mice is fully functional. Furthermore, in our experiments we could not find abnormalities in affective states of *Cry1/2*$^{-/-}$ mice. Neither in the tail-suspension test and the sucrose preference test, which measures anhedonia-like behavior, nor in the learned helplessness paradigm, which tests for the susceptibility to develop helplessness, *Cry1/2*$^{-/-}$ mice display depression-like behavior. Similar results have been shown for the forced swim test.[26]

However, our data strongly suggest that the lack of *Cry1* and *2* increases the risk to develop anxiety-related behavior in mice. Across several tests, *Cry1/2*$^{-/-}$ mice exhibit higher levels of anxiety. For instance, *Cry1/2*$^{-/-}$ mice displayed hypoactivity in the open field test, the light/dark box test, and the Y-maze test, and lack of appropriate habituation to new environments in the open field and the Y-maze test. Both, reduced activity levels and failure to habituate are common signs of anxiety in rodents and patients.[40-42] Importantly, *Cry1/2*$^{-/-}$ mice did not show any locomotion disabilities because they are able to reach the same maximum running speed in several tests and are similarly active in familiar environments, for example, in the IntelliCage, as WT mice. Thus, decreased activity in new environments is most likely not a sign of physical disability, but rather reflects a state of behavioral inhibition.

Interestingly, although their total levels of immobility are increased in new environments, *Cry1/2*$^{-/-}$ mice consistently show restless behavior in the same tests, that is, they switch significantly more frequently between mobile and immobile states than WT mice. Interestingly, *Clock*$^{\Delta 19}$ mice also show restlessness, but in the form of increased overall activity, which is interpreted as mania-like behavior.[10] *Cry1/2*$^{-/-}$ mice, on the other hand, show restlessness in the form of increased changes between active and inactive phases without being hyperactive overall. Together with the other results, this suggests anxiety-like behavior rather than mania-like behavior. This assumption is also supported by the fact that they do not show mania-like behavior in any of the other tests performed. Restlessness is a direct consequence of fight or flight responses in anxiety-provoking situations.[43] Restlessness is strongly linked to anxiety disorders and is therefore included in the diagnostic criteria of the DSM-V as one of the core symptoms of generalized anxiety disorders.[44] Thereby, psychomotor agitation is seen as the physical expression of anxious restlessness and mental tension often implying repetitive and purposeless movements.[45] In line with this, we observed significantly increased numbers of repetitive nosepokes of *Cry1/2*$^{-/-}$ mice in the progressive ratio task without the purpose of drinking more sucrose solution. As the increased number of nosepokes seems not to be the result of preference for sucrose, it might be taken into account that the progressive ratio test is not only a test for reward motivation, but also for compulsive-like behavior,[46] which is also associated with anxiety disorders. Compulsions are excessively repeated behaviors that are engaged without obvious reasons, and are believed to be performed to reduce anxiety.[47,48]

Consequently, the high number of nosepokes in *Cry1/2*$^{-/-}$ mice might not be related to anhedonia, but in view of their other behavioral abnormalities might rather be a strategy to cope with a constantly elevated feeling of anxiety and threat.

Impulsivity is a core symptom of numerous psychiatric disorders, including anxiety.[41,49] Therefore, we tested whether *Cry1/2*$^{-/-}$ mice are more impulsive. However, our results indicate that impulsivity is less pronounced in *Cry1/2*$^{-/-}$ mice compared with WT mice. The relationship between anxiety and impulsivity is still controversial.[50] On the one hand, impulsive behavior may be inconsistent with characteristic features of anxiety disorder such as safety-seeking and reduction of risky behaviors.[51,52] On the other hand, impulsive reactions in a novel and potentially anxiety-inducing environment can result from hyperarousal caused by fear.[53] In that respect, impulsivity can present a form of behavioral disinhibition with an anxiolytic function.[54] Notably, *Cry1/2*$^{-/-}$ mice are restless in unfamiliar environments, whereas on the other hand, impulsivity in home cages is significantly reduced. In a translational aspect, this would be consistent with human studies reporting a strong association of anxiety and impulsivity.[49] For instance, patients with high levels of anxiety often react impulsively and irritable in stressful situations, whereas in known and safe environments they behave more carefully and thoughtfully.

In order to confirm our assumption that *Cry1/2*$^{-/-}$ mice display an anxiety-like phenotype, we investigated whether their basolateral amygdala is hypersensitive to an anxiogenic stressor. The basolateral amygdala is a core structure in the brain network processing anxiety-related information in rodents and humans.[55-57] For instance, anxiety patients often show a hyperexcitability in the basolateral amygdala as a response to negative stimuli.[58,59] The exposure to an open field arena can trigger the expression of the neuronal activity marker gene in the anterior part of the BLA in mice.[60,61] Our experiments confirm that *c-Fos* is increased after the open field test, and that this reaction is significantly more pronounced in *Cry1/2*$^{-/-}$ mice than in WT mice. Interestingly, the increase of c-Fos was independent of the time of the day (ZT4 and ZT16) in both genotypes. This data is consistent with the assumption that anxiogenic stimuli are more strongly perceived and possibly less downregulated in *Cry1/2*$^{-/-}$ mice and support our hypothesis of anxiety-like behavior in *Cry1/2*$^{-/-}$ mice.

Interestingly, *Cryptochromes* may play crucial mechanistic roles in the control of systems regulating mood and behavior, such as the hypothalamic-pituitary-adrenal (HPA) axis or the monoaminergic system.[62] For instance, *Cryptochromes* counteract the activation of glucocorticoid receptors, as it was shown that the reaction to glucocorticoids is significantly enhanced when *Cryptochromes* are not present. Moreover, the loss of *Cry1* and *2* results in constitutively high levels of corticosterone in rodents, suggesting a decreased suppression of the HPA axis,[63] which in turn is highly associated with anxiety and mood disorders.[64,65] Moreover, lack of *Cry2* leads to lowered dopamine levels in the striatum of mice.[66] Furthermore, higher traits of anxiety- and depression-like behavior correlate with lower levels of *Cry2* in the hippocampus of mice.[67,68] Besides, rhythmic expression of *Cry2* in the amygdala is disrupted in animals showing anhedonic behavior.[69] *Cry1/2*$^{-/-}$ mice are also sensitive to metabolic challenges and develop signs of the metabolic syndrome more frequently than WT mice.[70] Psychiatric, metabolic, and circadian disorders are often comorbid.[71] Therefore, *Cry1/2*$^{-/-}$ mice may constitute a valuable animal model for psychiatric and metabolic comorbidity.

In summary, our behavioral and physiological data indicate that *Cryptochrome*-deficient mice have a pronounced anxiety-like phenotype, which is manifested by highly increased restlessness and lack of habituation in anxiogenic environments, an increase of repetitive and purposeless movements, reduced impulsivity, and hypersensitivity of their amygdala. These findings also confirm the manifold functions the circadian system unfolds within the brain and call for further research into the mechanistic correlates. Our results further support the assumption that disturbances of circadian rhythms play a causal role in the development of psychiatric disorders. Thus, it stands to reason that anxiety disorder patients might benefit from chronotherapeutic interventions, which help aligning their circadian system and increase amplitude of circadian rhythms.

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DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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REFERENCES

1. Whiteford HA, Degenhardt L, Rehm J, et al. Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. *Lancet*. 2013;382(9904):1575-1586.
2. Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE. Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry*. 2005;62(6):617-627.
3. Merikangas KR, He JP, Burstein M, et al. Lifetime prevalence of mental disorders in U.S. adolescents: results from the National Comorbidity Survey Replication—Adolescent Supplement (NCS-A). *J Am Acad Child Adolesc Psychiatry*. 2010;49(10):980-989.
4. Staner L. Sleep and anxiety disorders. *Dialogues Clin Neurosci*. 2003;5(3):249-258.
5. Takahashi JS, Hong HK, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet*. 2008;9(10):764-775.
6. Walker WH 2nd, Walton JC, Courtney DeVries A, Nelson RJ. Circadian rhythm disruption and mental health. *Transl Psychiatry*. 2020;10(1):28.
7. Hühne A, Welsh DK, Landgraf D. Prospects for circadian treatment of mood disorders. *Ann Med*. 2018;50(8):1-35.
8. Landgraf D, Long JE, Proulx CD, Barandas R, Malinow R, Welsh DK. Genetic disruption of circadian rhythms in the suprachiasmatic nucleus causes helplessness, behavioral despair, and anxiety-like behavior in mice. *Biol Psychiatry*. 2016;80(11):827-835.
9. Kalmbach DA, Pillai V, Cheng P, Arnedt JT, Drake CL. Shift work disorder, depression, and anxiety in the transition to rotating shifts: the role of sleep reactivity. *Sleep Med*. 2015;16(12):1532-1538.

10. Roybal K, Theobold D, Graham A, et al. Mania-like behavior induced by disruption of CLOCK. *Proc Natl Acad Sci U S A*. 2007;104(15):6406-6411.
11. Germain A, Kupfer DJ. Circadian rhythm disturbances in depression. *Hum Psychopharmacol*. 2008;23(7):571-585.
12. Oh CM, Kim HY, Na HK, Cho KH, Chu MK. The effect of anxiety and depression on sleep quality of individuals with high risk for insomnia: a population-based study. *Front Neurol*. 2019;10:849.
13. Hori H, Koga N, Hidese S, et al. 24-h activity rhythm and sleep in depressed outpatients. *J Psychiatr Res*. 2016;77:27-34.
14. Robillard R, Hermens D, Naismith S, et al. Ambulatory sleep-wake patterns and variability in young people with emerging mental disorders. *J Psychiatry Neurosci*. 2015;40(1):28-37.
15. Torquati L, Mielke GI, Brown WJ, Burton NW, Kolbe-Alexander TL. Shift work and poor mental health: a meta-analysis of longitudinal studies. *Am J Public Health*. 2019;109(11):e13-e20.
16. Kivela L, Papadopoulos MR, Antypa N. Chronotype and psychiatric disorders. *Curr Sleep Med Rep*. 2018;4(2):94-103.
17. Janku K, Šmotek M, Fárková E, Kopřivová J. Block the light and sleep well: Evening blue light filtration as a part of cognitive behavioral therapy for insomnia. *Chronobiol Int*. 2019;37(2):1-12.
18. Hurd D, Herrera M, Brant JM, Coombs NC, Arzubi E. Prospective, open trial of adjunctive triple chronotherapy for the acute treatment of depression in adolescent inpatients. *J Child Adolesc Psychopharmacol*. 2019;29(1):20-27.
19. Vitaterna M, King D, Chang A, et al. Mutagenesis and mapping of a mouse gene, clock, essential for circadian behavior. *Science*. 1994; 264:719-725.
20. McClung CA, Sidiropoulou K, Vitaterna M, et al. Regulation of dopaminergic transmission and cocaine reward by the clock gene. *Proc Natl Acad Sci*. 2005;102:9377-9381.
21. Ozburn AR, Larson EB, Self DW, McClung CA. Cocaine self-administration behaviors in ClockDelta19 mice. *Psychopharmacology (Berl)*. 2012;223:169-177.
22. Zheng B, Albrecht U, Kaasik K, et al. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell*. 2001; 105(5):683-694.
23. Abarca C, Albrecht U, Spanagel R. Cocaine sensitization and reward are under the influence of circadian genes and rhythm. *Proc Natl Acad Sci U S A*. 2002;99(13):9026-9030.
24. Spencer S, Falcon E, Kumar J, et al. Circadian genes period 1 and period 2 in the nucleus accumbens regulate anxiety-related behavior. *Eur J Neurosci*. 2013;37:242-250.
25. Landgraf D, McCarthy MJ, Welsh DK. The role of the circadian clock in animal models of mood disorders. *Behav Neurosci*. 2014;128:344-359.
26. De Bundel D, Gangarossa G, Biever A, Bonnefont X, Valjent E. Cognitive dysfunction, elevated anxiety, and reduced cocaine response in circadian clock-deficient cryptochrome knockout mice. *Front Behav Neurosci*. 2013;7:152.
27. van der Horst GT, Muijtjens M, Kobayashi K, et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature*. 1999;398(6728):627-630.
28. Yoo SH, Yamazaki S, Lowrey PL, et al. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A*. 2004;101(15):5339-5346.
29. Holter SM, Garrett L, Einicke J, et al. Assessing cognition in mice. *Curr Protoc Mouse Biol*. 2015;5(4):331-358.
30. Voikar V, Colacicco G, Gruber O, Vannoni E, Lipp HP, Wolfer DP. Conditioned response suppression in the IntelliCage: assessment of mouse strain differences and effects of hippocampal and striatal lesions on acquisition and retention of memory. *Behav Brain Res*. 2010;213(2):304-312.
31. Krackow S, Vannoni E, Codita A, et al. Consistent behavioral phenotype differences between inbred mouse strains in the IntelliCage. *Genes Brain Behav*. 2010;9(7):722-731.
32. Kobayashi Y, Sano Y, Vannoni E, et al. Genetic dissection of medial habenula-interpeduncular nucleus pathway function in mice. *Front Behav Neurosci*. 2013;7:17.
33. Landgraf D, Long J, Der-Avakian A, Streets M, Welsh DK. Dissociation of learned helplessness and fear conditioning in mice: a mouse model of depression. *PLoS One*. 2015;10:1-17.
34. Landgraf D, Long JE, Welsh DK. Depression-like behaviour in mice is associated with disrupted circadian rhythms in nucleus accumbens and periaqueductal grey. *Eur J Neurosci*. 2016;43(10):1309-1320.
35. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29(9):e45.
36. Cameron OG, Lee MA, Kotun J, McPhee KM. Circadian symptom fluctuations in people with anxiety disorders. *J Affect Disord*. 1986;11 (3):213-218.
37. Bedrosian TA, Herring KL, Weil ZM, Nelson RJ. Altered temporal patterns of anxiety in aged and amyloid precursor protein (APP) transgenic mice. *Proc Natl Acad Sci U S A*. 2011;108(28):11686-11691.
38. Albrecht A, Thiere M, Bergado-Acosta JR, Poranzke J, Müller B, Stork O. Circadian modulation of anxiety: a role for somatostatin in the amygdala. *PLoS One*. 2013;8(12):e84668.
39. Van der Zee EA, Havekes R, Barf RP, et al. Circadian time-place learning in mice depends on cry genes. *Curr Biol*. 2008;18(11):844-848.
40. Leussis MP, Bolivar VJ. Habituation in rodents: a review of behavior, neurobiology, and genetics. *Neurosci Biobehav Rev*. 2006;30(7):1045-1064.
41. Avery SN, Blackford JU. Slow to warm up: the role of habituation in social fear. *Soc Cogn Affect Neurosci*. 2016;11(11):1832-1840.
42. Difrancesco S, Lamers F, Riese H, et al. Sleep, circadian rhythm, and physical activity patterns in depressive and anxiety disorders: a 2-week ambulatory assessment study. *Depress Anxiety*. 2019;36(10): 975-986.
43. Godoy LD, Rossignoli MT, Delfino-Pereira P, Garcia-Cairasco N, de Lima Umeoka EH. A comprehensive overview on stress neurobiology: basic concepts and clinical implications. *Front Behav Neurosci*. 2018; 12:127.
44. Tyrer P, Baldwin D. Generalised anxiety disorder. *Lancet*. 2006;368 (9553):2156-2166.
45. Vieta E, Garriga M, Cardete L, et al. Protocol for the management of psychiatric patients with psychomotor agitation. *BMC Psychiatry*. 2017;17(1):328.
46. Harkness KL. *The Oxford Handbook of Stress and Mental Health. Oxford Library of Psychology*. New York, NY: Oxford University Press; 2020.
47. Van Ameringen M, Patterson B, Simpson W. DSM-5 obsessive-compulsive and related disorders: clinical implications of new criteria. *Depress Anxiety*. 2014;31(6):487-493.
48. Stein DJ, Craske MA, Friedman MJ, Phillips KA. Anxiety disorders, obsessive-compulsive and related disorders, trauma- and stressor-related disorders, and dissociative disorders in DSM-5. *Am J Psychiatry*. 2014;171(6):611-613.
49. Jakuszkowiak-Wojten K, Landowski J, Wiglusz MS, Cubała WJ. Impulsivity in anxiety disorders. A critical review. *Psychiatr Danub*. 2015;27(Suppl 1):S452-S455.
50. Xia L, Gu R, Zhang D, Luo Y. Anxious individuals are impulsive decision-makers in the delay discounting task: an ERP study. *Front Behav Neurosci*. 2017;11:5.
51. Winstanley CA, Eagle DM, Robbins TW. Behavioral models of impulsivity in relation to ADHD: translation between clinical and preclinical studies. *Clin Psychol Rev*. 2006;26(4):379-395.
52. Taylor CT, Hirshfeld-Becker DR, Ostacher MJ, et al. Anxiety is associated with impulsivity in bipolar disorder. *J Anxiety Disord*. 2008;22(5): 868-876.
53. Del Carlo A, Benvenuti M, Fornaro M, et al. Different measures of impulsivity in patients with anxiety disorders: a case control study. *Psychiatry Res*. 2012;197(3):231-236.

54. Harro J. Long-term partial 5-HT depletion: interference of anxiety and impulsivity?*Psychopharmacology (Berl)*. 2002;164(4):433-434.
55. Babaev O, Piletti Chatain C, Krueger-Burg D. Inhibition in the amygdala anxiety circuitry. *Exp Mol Med*. 2018;50(4):18.
56. Etkin A, Klemenhagen KC, Dudman JT, et al. Individual differences in trait anxiety predict the response of the basolateral amygdala to unconsciously processed fearful faces. *Neuron*. 2004;44(6):1043-1055.
57. Davis M. Are different parts of the extended amygdala involved in fear versus anxiety?*Biol Psychiatry*. 1998;44(12):1239-1247.
58. Janak PH, Tye KM. From circuits to behaviour in the amygdala. *Nature*. 2015;517(7534):284-292.
59. Inman CS, Manns JR, Bijanki KR, et al. Direct electrical stimulation of the amygdala enhances declarative memory in humans. *Proc Natl Acad Sci U S A*. 2018;115(1):98-103.
60. Hale MW, Hay-Schmidt A, Mikkelsen JD, Poulsen B, Shekhar A, Lowry CA. Exposure to an open-field arena increases c-Fos expression in a distributed anxiety-related system projecting to the basolateral amygdaloid complex. *Neuroscience*. 2008;155(3):659-672.
61. Wang DV, Wang F, Liu J, Zhang L, Wang Z, Lin L. Neurons in the amygdala with response-selectivity for anxiety in two ethologically based tests. *PLoS One*. 2011;6(4):e18739.
62. Albrecht U. Molecular mechanisms in mood regulation involving the circadian clock. *Front Neurol*. 2017;8:30.
63. Lamia KA, Papp SJ, Yu RT, et al. Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature*. 2011;480(7378):552-556.
64. Faravelli C, Lo Sauro C, Godini L, et al. Childhood stressful events, HPA axis and anxiety disorders. *World J Psychiatry*. 2012;2(1):13-25.
65. Bao AM, Swaab DF. The human hypothalamus in mood disorders: the HPA axis in the center. *IBRO Rep*. 2019;6:45-53.
66. Schnell A, Sandrelli F, Ranc V, et al. Mice lacking circadian clock components display different mood-related behaviors and do not respond uniformly to chronic lithium treatment. *Chronobiol Int*. 2015;32(8):1075-1089.
67. Sah A, Schmuckermair C, Sartori SB, et al. Anxiety- rather than depression-like behavior is associated with adult neurogenesis in a female mouse model of higher trait anxiety- and comorbid depression-like behavior. *Transl Psychiatry*. 2012;2:e171.
68. Griesauer I, Diao W, Ronovsky M, et al. Circadian abnormalities in a mouse model of high trait anxiety and depression. *Ann Med*. 2014;46(3):148-154.
69. Savalli G, Diao W, Schulz S, Todtova K, Pollak DD. Diurnal oscillation of amygdala clock gene expression and loss of synchrony in a mouse model of depression. *Int J Neuropsychopharmacol*. 2014;18(5).
70. Barclay JL, Shostak A, Leliavski A, et al. High-fat diet-induced hyperinsulinemia and tissue-specific insulin resistance in cry-deficient mice. *Am J Physiol Endocrinol Metab*. 2013;304(10):E1053-E1063.
71. Barandas R, Landgraf D, McCarthy MJ, Welsh DK. Circadian clocks as modulators of metabolic comorbidity in psychiatric disorders. *Curr Psychiatry Rep*. 2015;17(12):98.

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ORIGINAL ARTICLE

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Circadian gene × environment perturbations influence alcohol drinking in *Cryptochrome*-deficient mice

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Abstract

Alcohol use disorder (AUD) is a widespread addiction disorder with severe consequences for health. AUD patients often suffer from sleep disturbances and irregular daily patterns. Conversely, disruptions of circadian rhythms are considered a risk factor for AUD and alcohol relapses. In this study, we investigated the extent to which circadian genetic and environmental disruptions and their interaction alter alcohol drinking behaviour in mice. As a model of genetic circadian disruption, we used *Cryptochrome1/2*-deficient (*Cry1/2*$^{-/-}$) mice with strongly suppressed circadian rhythms and found that they exhibit significantly reduced preference for alcohol but increased incentive motivation to obtain it. Similarly, we found that low circadian SCN amplitude correlates with reduced alcohol preference in WT mice. Moreover, we show that the low alcohol preference of *Cry1/2*$^{-/-}$ mice concurs with high corticosterone and low levels of the orexin precursor *prepro-orexin* and that WT and *Cry1/2*$^{-/-}$ mice respond differently to alcohol withdrawal. As a model of environmentally induced disruption of circadian rhythms, we exposed mice to a "shift work" light/dark regimen, which also leads to a reduction in their alcohol preference. Interestingly, this effect is even more pronounced when genetic and environmental circadian perturbations interact in *Cry1/2*$^{-/-}$ mice under "shift work" conditions. In conclusion, our study demonstrates that in mice, disturbances in circadian rhythms have pronounced effects on alcohol consumption as well as on physiological factors and other behaviours associated with AUD and that the interaction between circadian genetic and environmental disturbances further alters alcohol consumption behaviour.

KEYWORDS
alcohol, circadian, circadian disruption, corticosterone, cryptochrome, orexin, shift work model

Anisja Hühne and Lisa Echtler contributed equally to the study.

1 | INTRODUCTION

Harmful use of alcohol and alcohol use disorder (AUD) lead to increased mortality with 5.3% of all deaths worldwide being attributable to alcohol consumption.[1] In recent years, evidence emerged that there is a bidirectional relationship between circadian disruptions and AUD.[2,3] AUD patients often suffer from circadian dysregulation, which, in turn, increases the risk for developing AUD and alcohol relapses during withdrawal.[2–6]

Circadian rhythms arise from the rhythmic interplay of so-called clock genes and can be found in almost each cell of the body. In the course of 24 h, the transcription factors *Circadian Locomotor Output Cycles Kaput* (*CLOCK*) and *Brain And Muscle ARNT-like 1* (*BMAL1*) heterodimerize and activate transcription of *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes, which later inhibit *CLOCK* and *BMAL1*, and thus their own transcription.[7] As clock genes also serve as transcription factors for other genes, more than half of the genome and thus virtually all physiological and behavioural processes are under circadian control.[8,9] Therefore, disruptions of circadian rhythms can have significant negative effects on health in general, but also on alcohol consumption behaviour.

Circadian disruption is a perturbation in the biological timing of or between rhythmic molecular, physiological, or behavioural cycles that occur on a daily basis. It can be caused by both genetic and environmental factors, and both factors have been associated with altered alcohol drinking behaviour in humans and in animals. For example, in humans, late chronotypes are known to have an increased prevalence of harmful use of alcohol or AUD,[10,11] and circadian gene expression is reduced in AUD patients compared with healthy controls.[12] In mice, mutations of *Per1*, *Per2*, and *Clock* result in enhanced alcohol consumption behaviour and increased sensitizations to reward.[13–17] Additionally, environmental rhythm disruptions also have significant influence on alcohol consumption in humans and rodents. In humans, air travel increases alcohol consumption, and shift work, especially working on night and rotating shifts, is associated with binge-drinking disorder.[18,19] In mice, however, repeated LD phase shifts and constant darkness (DD) or constant light (LL) often decrease alcohol consumption.[18,20–22] Furthermore, many physiological and neurophysiological systems that influence alcohol consumption are under circadian control, such as the stress system and parts of the reward system including orexin. A dysregulation of the stress system with increased cortisol levels is related to altered alcohol consumption.[23] Similarly, the neuropeptide orexin has been associated with increased alcohol consumption.[24,25] Hence, circadian disruption may have an impact on physiological systems that ultimately lead to altered alcohol drinking behaviour. Together, these results indicate that, taken separately, both genetic as well as environmental disruptions of circadian rhythms can increase the susceptibility to alter alcohol drinking behaviour, which is a risk factor for the development of addiction disorders in humans. However, there is now a large body of scientific evidence demonstrating a dynamic interplay between genetic and environmental variations in the development of individual differences in behaviour and health.[26] Hence, we believe that the investigation of alcohol drinking behaviour in an animal model exposed to genetic and environmental circadian disruptions simultaneously is of high translational relevance, as it is very likely that subjects with genetic circadian burdens are at particularly high risk for developing a substance abuse disorder if they are also exposed to exogenous irregularities such as unstructured daily schedules.[27]

Based on these considerations, we tested the hypotheses that alcohol drinking behaviour of mice is related to their constitution of endogenous circadian clocks and that an interplay between genetic and environmental circadian disruptions produces abnormal alcohol drinking behaviour. To test this, we compared alcohol drinking behaviour between mice with either intact circadian clocks or with significantly inhibited circadian rhythms due to knockout of *Cry1* and *Cry2*[28,29] under either standard 12:12 light/dark (LD) conditions or LD conditions mimicking rotating shift work.

Factors that predispose humans to increased alcohol consumption, like increased stress levels and shift work conditions,[18,30] often result in reduced alcohol intake in mice.[21,31,32] Accordingly, our results show that loss of reduction of endogenous circadian rhythms and shift work LD conditions reduce alcohol preference in mice and that this effect becomes even stronger when both factors are combined. Despite reduced preference for EtOH, the knockout of *Cry* genes leads to a significant increase in operant responses in the progressive ratio reinforcement paradigm, which is consistent with the so-called the incentive sensitization theory of addiction. According to this theory, a common attribute of addiction is that, despite possibly diminished pleasure from a drug (*liking*), incentive motivation to obtain it (*wanting*) increases.[33–35] Furthermore, our data show that in the absence of *CRYS*, CORT levels are increased, and levels of the orexin precursor *prepro-orexin* (*PPO*) are decreased, which may together contribute to reduced alcohol preference. Because circadian perturbations may affect the success of withdrawal, we also examined whether the expression of withdrawal symptoms is different in mice with and without endogenous clocks. Interestingly, WT mice develop behaviours under withdrawal that *Cry*-deficient mice already exhibit at baseline and that do not significantly worsen under withdrawal.

2 | MATERIALS AND METHODS

2.1 | Animals

Cry1/2$^{-/-}$;*Per2*^{Luc} (henceforth referred to as *Cry1/2*$^{-/-}$) and *Cry1/2*$^{+/+}$;*Per2*^{Luc} mice (henceforth referred to as wild-type; WT)[28,36] on C57BL/6J background were obtained as described previously.[37] Mice were housed in groups and maintained on a 12:12 light/dark (LD) cycle, with lights turned on at 7 AM unless otherwise stated. Water and food were provided ad libitum. Mouse studies were conducted in accordance with regulation of German Animal Protection Law. An attempt was made to keep the number of animals low by using animal cohorts for several tests, starting with the least stressful tests, and finishing with the most stressful tests.

2.2 | Experimental design

Behavioural experiments were divided between three different cohorts of animals. While the first cohort was tested for the genetic influence of missing *Cry* genes on alcohol preference, the second cohort was used to examine gene × environment interaction (missing *Cry* genes and "shift work" conditions) on alcohol drinking behaviour (Figure 1A,B). For analyses of possible underlying molecular mechanisms, blood and brain samples were collected in the third cohort to determine both corticosterone (CORT) and *PPO*. Cohort 1 (Figure 1A) consisted of male and female WT ($n = 16$ male/15 female) and *Cry1/2*$^{-/-}$ ($n = 8$ male/16 female) mice aged of 7 to 10 weeks at the start of the experiments. Cohort 2 (Figure 1B) was composed only of female WT ($n = 32$) and *Cry1/2*$^{-/-}$ ($n = 25$) mice between 10 and 18 weeks of age at the beginning of the experiment. Cohort 2 consisted of female mice only, because some aggressive behaviour occurred in the large IntelliCage groups of male mice in cohort 1, which was intended to be avoided in the longer shift work experiment of the second cohort. The temporal order of the experiments of the two cohorts corresponds to the described order in the text below. Cohort 3 (Figure 1C) consisted of a total of 40 animals, of which 16 mice (WT: $n = 8$ male and *Cry1/2*$^{-/-}$: $n = 8$ male) between 11 and

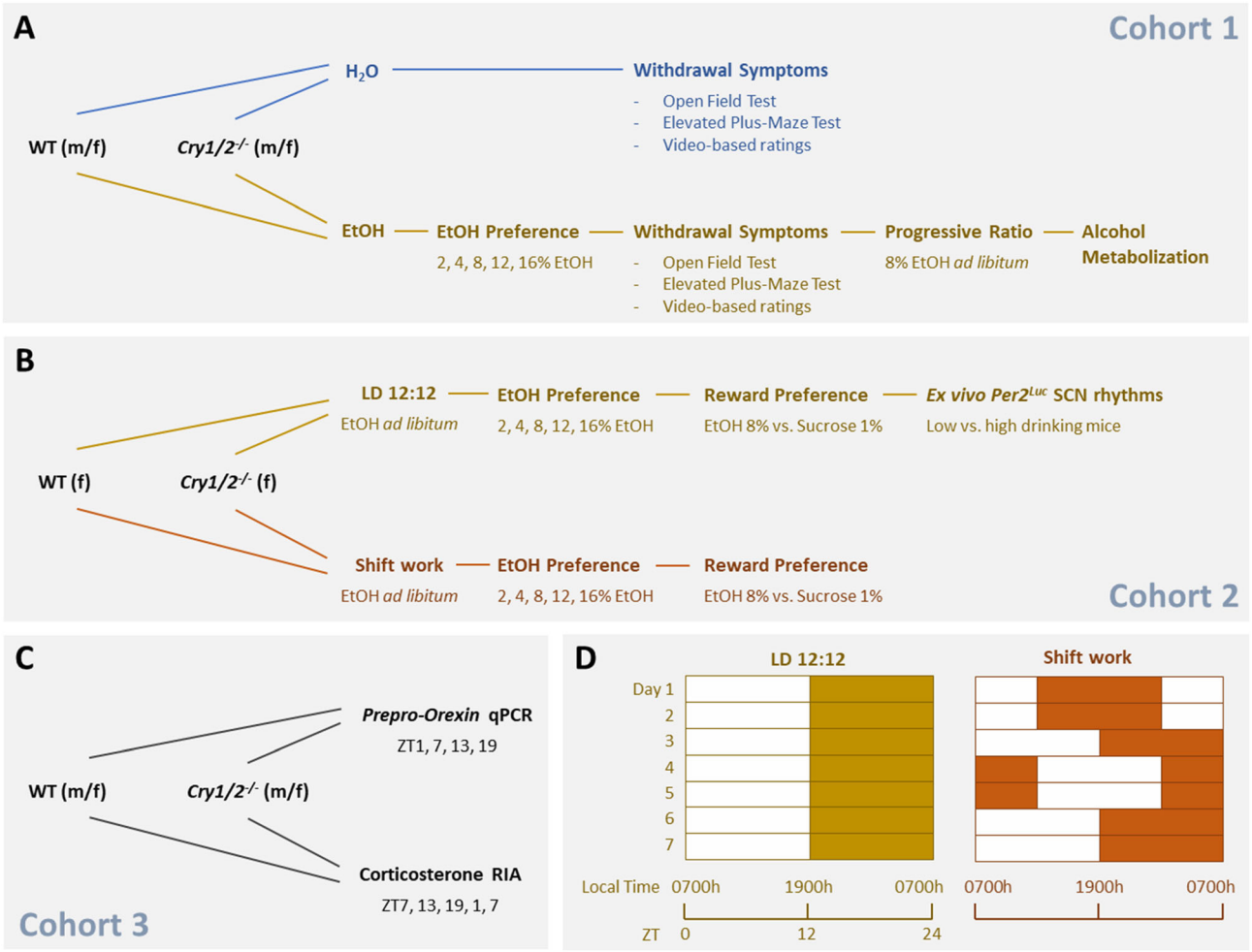

FIGURE 1 Timelines of experimental procedures of Cohort 1 and Cohort 2. (A) Cohort 1: Female and male WT and *Cry1/2*$^{-/-}$ mice were assigned to either the H_2O control group or the EtOH group. The preference for different EtOH concentrations was assessed in mice of the EtOH group. Thereupon, 16% EtOH was provided for 9 days and different withdrawal symptoms were evaluated when the mice were deprived of alcohol for 6 h. After completion of all withdrawal tests, the animals' motivation to obtain 8% EtOH was tested under a progressive ratio paradigm. Finally, the capability to metabolize alcohol was measured. (B) Cohort 2: Female WT and *Cry1/2*$^{-/-}$ mice were assigned to either the shift work paradigm or regular 12:12 LD condition. Under these two conditions, their preference for different EtOH concentrations was assessed. Thereafter, their preference for 8% EtOH was measured under the additional option of choosing a 1% sucrose solution. After behavioural experiments were finished, the SCN of mice with different preferences for 12% EtOH was cultured ex vivo and used for bioluminescence measurements. (C) Cohort 3: The lateral hypothalamus of female and male WT and *Cry1/2*$^{-/-}$ mice was collected every 6 h at a total of four time points for *prepro-orexin* quantitative PCR (qPCR) measurements. Blood was repeatedly collected from the tail of additional male WT and *Cry1/2*$^{-/-}$ mice every 6 h at a total of five time points for plasma corticosterone measurements by radioimmune assay (RIA). (D) The LD 12:12 and shift work light conditions for animals in Cohort 2. Dark blocks represent dark phase, white blocks represent light phase. Light cycles repeated every 7 days during the experiments

18 weeks of age were used for repeated CORT measurements. The remaining 24 mice (WT: $n = 6$ male/6 female *Cry1/2*$^{-/-}$: $n = 6$ male/6 female; 3 mice per time point) were 8–15 weeks old and were used for *PPO* measurements.

2.2.1 | Cohort 1

EtOH preference

Preference for alcohol was assessed in the IntelliCage system (TSE-Systems GmbH, Bad Homburg, Germany).[38,39] This system consists of four-cornered cages with two drinking bottles per corner located behind a gate. According to the experimental paradigm, gates can open and close. In case they are open, mice can access the bottles directly. If gates are closed, they must perform nosepokes at the gate, thereby disrupting a light-barrier which opens the gate. For each mouse, visits in corners, number of nosepokes, and number of licks on each bottle are measured automatically by implanted RFID transponders. Transponders are implanted subcutaneously in the neck region 1 week before mice are transferred into the IntelliCages. Each IntelliCage housed 12–16 mice and provide ad libitum access to water and food. For the alcohol preference assessments, one of the two bottles in each corner was replaced by alcohol solutions with increasing EtOH concentrations (*v*/*v*) for different number of days: 2% for 3 days, 4% for 3 days, 8% for 9 days, 12% for 9 days, and 16% for 10 days.[17,40] The preference was quantified by using a preference score $(A - B)/(A + B)$, where A equals the number of correct trials, that is, the number of licks at a bottle of alcohol solution, and B equals the incorrect trials, that is, the licks at a bottle with water. Since alcohol preferences were measured over several days for differing concentrations, at the end, the preference scores of each EtOH concentration were averaged over the number of days that concentration was provided.

Withdrawal symptoms

Animals had free access to water and 16% EtOH for 10 days. Thereafter, they were withdrawn from alcohol and assessment of anxiety- and ataxia-like behaviours began after 6 h. Anxiety-like behaviours were measured in an open field arena (50 cm × 50 cm × 50 cm) and in an Elevated Plus-Maze test[37,41] Movement profiles of both tests were assessed with the behavioural tracking software ANY-maze (Stoelting, IL). Additionally, ataxia-like behaviours in the open field test were obtained by manual video analysis and included wall rearing, rotations, climbing attempts, and slipping.

Progressive ratio self-administration paradigm

To evaluate alcohol craving after withdrawal, mice were subjected to a progressive ratio paradigm for 6 days during which an 8% EtOH solution was only accessible after executing an increasing number of nosepokes in consecutive stages. The increase of required nosepokes per trial to obtain access to the EtOH was calculated according to the formula: Response ratio $= (5 * e^{[0.2 * \text{stage number}]}) - 5$, resulting in a rise of nosepokes as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, and so forth. During this paradigm, mice had free access to water bottles. The experiment was carried out as described in more detail previously, but with EtOH instead of sugar solution.[37]

Alcohol metabolism

Finally, 8 weeks after completion of the progressive ratio experiment, the ability of the animals to metabolize EtOH was investigated. The mice were injected *i.p.* with EtOH (3.0 g/kg) at *Zeitgeber* time 7 (ZT7, 7 h after lights on); 50-µl blood were taken from the tail vein shortly before the EtOH injection (time 0) and 60, 160, and 240 min afterwards.[42] Blood was immediately processed, and alcohol content was evaluated in plasma with an ethanol assay kit (Megazyme, Ireland).

2.2.2 | Cohort 2

Shift work conditions

The second cohort (Figure 1B) was used to investigate the influence of the interaction between genetic and environmental circadian disruptions on alcohol drinking behaviour in mice. Mice were divided into four parallel groups, WT and *Cry1/2*$^{-/-}$ were each divided into a "shift work" group and a control group with regular 12:12 LD conditions with lights on from 7:00 AM to 7:00 PM. To simulate a three-shift work schedule for the mice, IntelliCages were placed in light-tight, ventilated boxes with adjusted light/dark cycles to simulate 7-day "work weeks" (Figure 1D):

- Days 1 + 2 "Early shift": Light phase 1:00 AM–1:00 PM.
- Day 3 "Day shift": Light phase 7:00 AM–7:00 PM.
- Days 4 + 5 "Night shift": Light phase 01:00 PM–1:00 AM.
- Days 6 + 7 "Weekend": Light phase 7:00 AM–7:00 PM.

EtOH preference and reward preference

After 5 days of adaption, under the two different lighting conditions, alcohol preference was tested as described above. As a measure for reward preference, this was followed by an exclusive-choice experiment in which the mice could choose between 8% EtOH and 1% sucrose for 2 days, as previously described for cocaine[43,44] and for EtOH self-administration.[45]

Brain slice culture and PER2::LUC luminometry

After the behavioural tests were completed, WT mice of the control lighting condition were divided into low, neutral, and high drinking mice depending on their alcohol drinking behaviour under 12% alcohol. At ZT8, brains of two mice of each group were taken for the preparation of organotypic SCN cultures to assess amplitudes of their *PER2*^{Luc} expression patterns with a luminometer.[46] Amplitude was normalized to the brightness of each cultured brain explants to calculate the average single-cell amplitude of those cells that emitted *Per2*^{Luc} signal.

2.2.3 | Cohort 3

Corticosterone radioimmunoassay

Blood was collected from the tail at five time points every 6 h (ZT7, 13, 19, 1, and 7) according to previously the described procedure in Landgraf et al.,[42] except that the animals remained in the normal LD 12:12 cycle.

Plasma CORT levels were subsequently determined using a commercially available radioimmunoassay kit with ^{125}I-labeled anti-CORT antibody (MP Biomedicals, USA).

Prepro-orexin quantitative PCR

Animals were sacrificed at four time points (ZT1, 7, 13, and 19), and the lateral hypothalamus was dissected between Bregma at −1.24 and −1.82.[47] Tissue was stored overnight in RNA*later*™ solution from ThermoFisher Scientific and then transferred to −80°C. RNA was isolated from frozen tissue using QIAzol Lysis Reagent and RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol including DNAse treatment. For cDNA synthesis, the high-capacity RNA-to-cDNA™ kit (Thermo Fisher Scientific) was used. qPCRs were performed using primers for β-act (Fw-CCCTGAAGTACCCCATTGAA, Rev-AGGTGTGGTGCCAGATCTTC) and *PPO* (Fw-TTGGACCAC TGCACTGAAGA, Rev-CCCAGGGAACCTTTGTAGAAG).[48] Fold changes were calculated as described previously.[49]

2.3 | Statistical analyses

Statistical analyses were carried out with SPSS Statistics 27 (IBM, NY) and GraphPad Prism 9.0 (GraphPad Software, CA). The automated user interface FlowR (XBehavior, Dägerlen, Switzerland) was used for IntelliCage-obtained behavioural data. Results are presented as mean ± SEM. Details about statistical tests used are indicated in the figure legends.

3 | RESULTS

3.1 | Genetic disruption of circadian rhythms is related to reduced alcohol preference

A central question of our study was whether a genetic disruption of the circadian system in mice affects their alcohol drinking behaviour. To test this, we compared the preferences for alcohol solutions of different concentrations (2–16%) of male and female WT mice with a functional circadian system and of *Cry1/2*$^{-/-}$ mice with a dysfunctional circadian clock. Towards all EtOH concentrations of 2–12%, WT mice show a significant preference, with the highest preference for 8% EtOH. In contrast, *Cry1/2*$^{-/-}$ mice show significantly lower preferences for EtOH and drink EtOH solutions and water almost equally. At the highest tested concentration of 16% EtOH, both genotypes switch predominantly to water, indicating that such a high EtOH concentration is generally not preferred by mice (Figures 2A and S1, Table S2-1). In a further analysis, we found a significant interaction between EtOH concentration and sex (significant EtOH × sex interaction), presumably due to an increase in EtOH preference of female WT and *Cry1/2*$^{-/-}$ mice at lower EtOH concentrations (Figure 2B, Table S2-1). Together, these results indicate that an extreme suppression of endogenous circadian rhythms due to the loss of *CRYs* significantly reduces the preference for in EtOH in mice.

However, despite generally increased EtOH preference, some WT mice also have decreased EtOH preference. This variance is particularly pronounced at an EtOH concentration of 12%, which is strongly preferred by some WT mice and almost entirely rejected by others. To preliminary investigate whether reduced EtOH preference is also related to low endogenous circadian rhythms in WT mice, we examined the ex vivo SCN *Per2*^{Luc} amplitude of six WT mice from the second cohort that had either high, low, or intermediate preference for 12% EtOH. Interestingly, we found that EtOH preference also decreased in WT mice with decreasing circadian amplitude (Figures 2C and S2, Table S2-2).

3.2 | Possible mechanisms for decreased alcohol preference in *Cry1/2*$^{-/-}$ mice

Our results show that *Cry1/2*$^{-/-}$ mice have a decreased preference for EtOH. However, other clock gene mutations with similar circadian phenotypes, such as *Clock*$^{\Delta 19}$ and *Per2*$^{Brdm/Brdm}$, cause increased EtOH preference. Various reasons are conceivable why *Cry1/2*$^{-/-}$ mice, unlike other clock gene mutant mice, drink less EtOH compared with water.

Consistent with their lower preference for 2–12% EtOH, *Cry1/2*$^{-/-}$ mice also consume about 20% less of these EtOH solutions in absolute amounts per day and per gram of body weight than WT (Figure 3A, Table S3-1). Processes to metabolize EtOH have been shown to be under circadian control.[50,51] These may be disrupted in the *Cry1/2*$^{-/-}$ mice, requiring them to drink less EtOH to achieve similar blood alcohol levels as WT mice. However, blood alcohol clearance is similar in both genotypes (Figure 3B, Table S3-2), suggesting that the reduced alcohol intake in *Cry1/2*$^{-/-}$ mice is not related to retention of blood alcohol levels.

Cry1/2$^{-/-}$ mice are known for their elevated CORT levels and CRY proteins interact with glucocorticoid receptors.[52] In humans, chronic stress is a risk factor for AUD,[30] whereas in mice, chronic stress usually reduces EtOH preference.[31,32] Our results confirm that *Cry1/2*$^{-/-}$ mice show increased baseline CORT over 24 h (average over all measured time points: WT: 66.89 ± 8.02 ng/ml vs. *Cry1/2*$^{-/-}$: 102.2 ± 8.86 ng/ml, mean ± SEM), which may contribute to low EtOH preference (Figure 3C, Table S3-2).

The reward system, including orexin which represents a central factor in reward and motivation, is also under circadian control.[24] It has been shown that blocking orexin signalling leads to decreased craving and intake of alcohol.[25,53] Interestingly, *Cry1/2*$^{-/-}$ mice show decreased expression of *PPO*, the precursor molecule of orexin, over the course of 24 h (average relative expression over all measured time

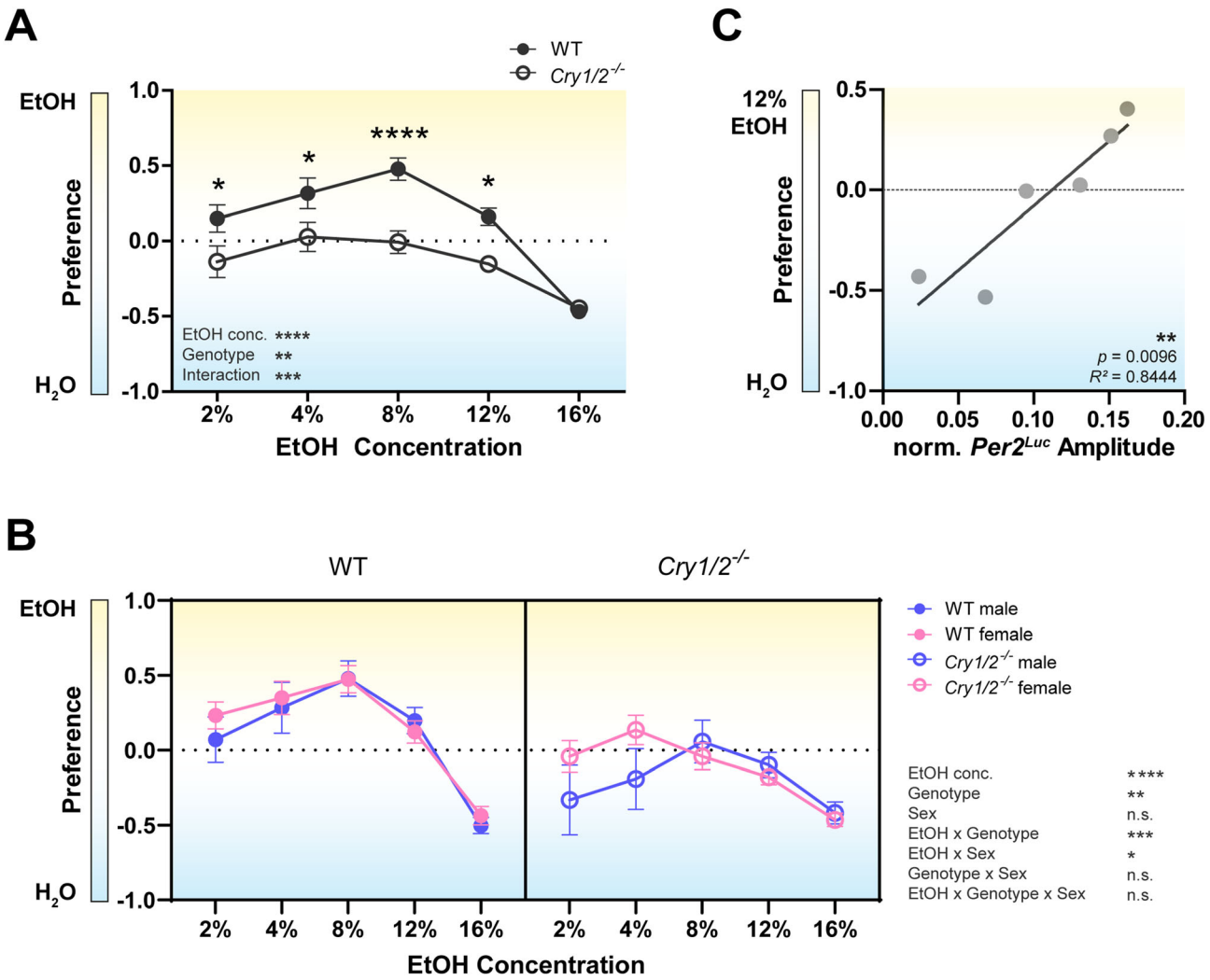

FIGURE 2 Influence of endogenous circadian rhythms on alcohol drinking behaviour in mice. (A) *Cry1/2*$^{-/-}$mice do not discriminate between EtOH and water when drinking, whereas WT mice prefer EtOH. Results presented as mean ± SEM; two-way repeated measures ANOVA with Bonferroni post hoc test; n = 31/24 (WT/*Cry1/2*$^{-/-}$). (B) Drinking behaviour of male and female WT and *Cry1/2*$^{-/-}$ mice is similar. Female mice of both genotypes prefer lower EtOH concentrations slightly more than males, leading to a significant interaction between EtOH concentration and sex. Results presented as mean ± SEM; three-way repeated measures ANOVA with Bonferroni post hoc test restricted to comparisons of genotypes and light conditions at only the respective EtOH concentration; WT: $n = 16/15$, *Cry1/2*$^{-/-}$ $n = 8/16$ (male/female). (C) The endogenous circadian amplitude of organotypic cultured WT SCN explants correlates with the alcohol preference of the donor animal the higher the amplitude, the higher the preference for alcohol. Linear regression, $n = 6$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; n.s. or no symbol = not significant

points: WT: 1.702 ± 0.172 vs. *Cry1/2*$^{-/-}$: 1.173 ± 0.059, mean ± SEM). This, in addition to increased CORT may lead to decreased alcohol intake and preference in *Cry1/2*$^{-/-}$ mice (Figure 3D, Table S3-2).

3.3 | Increased reinforcing effects of alcohol in *Cry1/2*$^{-/-}$ mice

In the next step, we investigated whether *Cry1/2*$^{-/-}$ mice show other characteristics of AUD, such as increased wanting of alcohol despite reduced alcohol preference according to the incentive sensitization theory of addiction. To measure the incentive value of alcohol, a progressive ratio paradigm was performed with 8% EtOH, which WT mice prefer most. Intriguingly, despite significantly lower preference for alcohol, *Cry1/2*$^{-/-}$ mice make significantly more effort to access the EtOH solution than WT mice, i.e., they are disposed to perform a higher number of nosepokes to open the access to the alcohol bottles. *Cry1/2*$^{-/-}$ mice reach up to 18 stages and thus perform up to 178 nosepokes. In contrast, WT reach a maximum of only 10 stages, showing that they are not even willing to perform 40 nosepokes in a row to obtain alcohol. In addition, *Cry1/2*$^{-/-}$ mice require an average of only 46 attempts to reach the next stage, whereas WT mice need an average of 88 trials. However, once the access to the alcohol

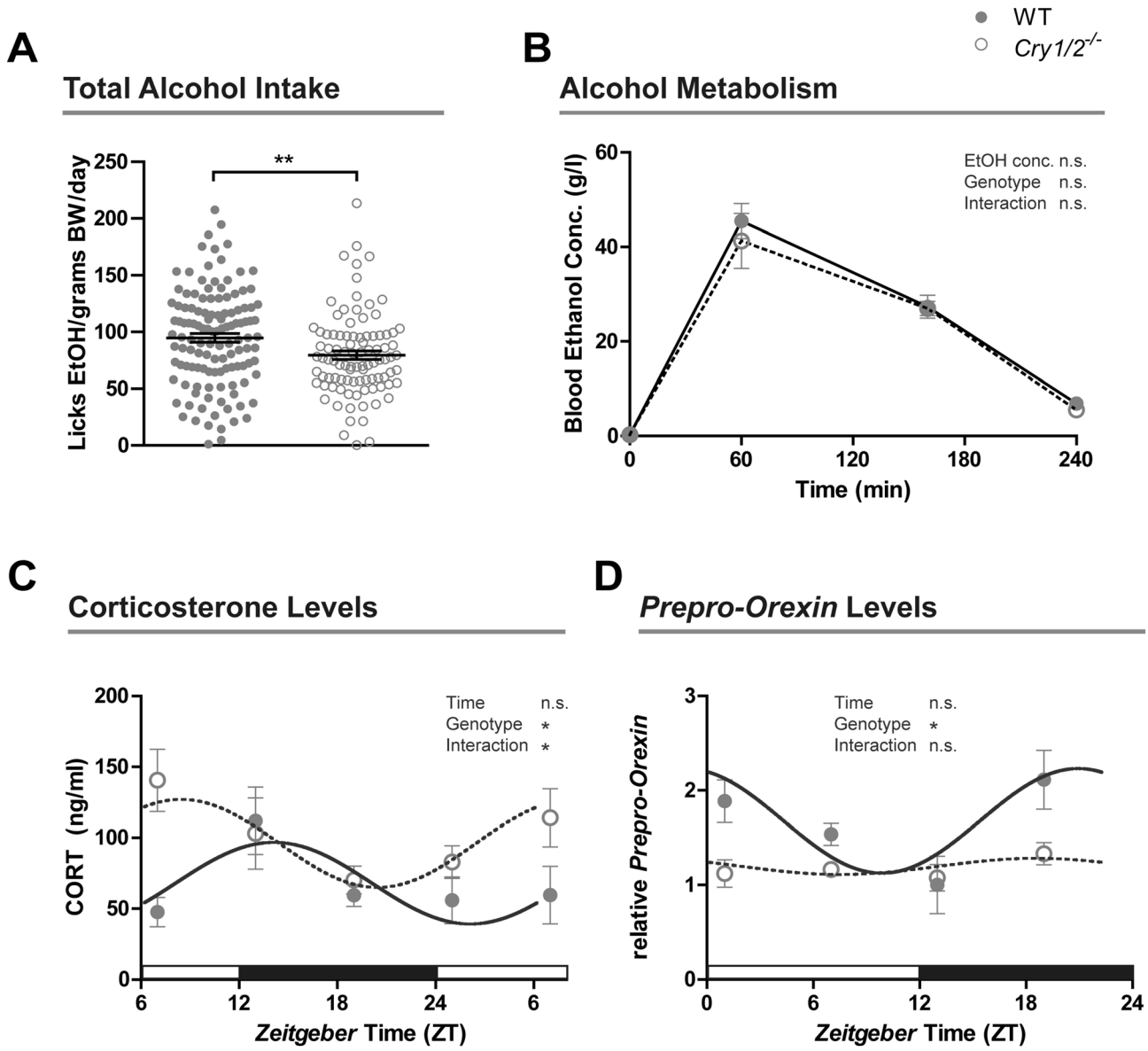

FIGURE 3 Possible mechanisms for decreased alcohol preference in *Cry1/2*$^{-/-}$ mice. (A) *Cry1/2*$^{-/-}$ mice drink lower absolute amounts of 2–12% alcohol solutions. Data are shown as dot plots with mean ± SEM; Student's *t* test; $n = 31/24$ (WT/*Cry1/2*$^{-/-}$). (B) Blood alcohol clearance is similar in WT and *Cry1/2*$^{-/-}$ mice. Results presented as mean ± SEM; two-way repeated measures ANOVA with Bonferroni post hoc test); $n = 16/13$ (WT/*Cry1/2*$^{-/-}$). (C) Time course of CORT plasma levels with significantly increased levels in *Cry1/2*$^{-/-}$ mice. Results presented as mean ± SEM; two-way repeated measures ANOVA; $n = 8/8$ (WT/*Cry1/2*$^{-/-}$). (D) Time course of hypothalamic PPO expression levels with significantly decreased levels in *Cry1/2*$^{-/-}$ mice. Results presented as mean ± SEM; two-way ANOVA; $n = 3/3$–5 per time-point (WT/*Cry1/2*$^{-/-}$). The white and black bars along the x-axis in (C) and (D) represent light and dark phases. $^{*}p < 0.05$, $^{**}p < 0.01$; n.s. = not significant

bottles is free, *Cry1/2*$^{-/-}$ mice lick less often and are about five times more likely than WT to access to the bottles without subsequently licking at it at all (Figure 4, Table S4-1).

3.4 | Withdrawal symptoms

Because it is known from AUD patients that chronobiological disruptions could induce relapses to alcohol drinking,[6] we investigated whether *Cry1/2*$^{-/-}$ mice with disrupted endogenous clocks respond differently to the removal of alcohol than WT mice with functional clocks. For this purpose, we removed alcohol after 10 days of preference measurement of the 16% EtOH solution, which is equally preferred by both genotypes, and then measured different withdrawal-related behaviours, such as anxiety-related behaviour and ataxia.[54,55] In the open field test, mice of both genotypes spend about 50% more time in the centre of the open-field arena under withdrawal. However, WT mice under alcohol withdrawal cover less distance and become more restless, as reflected by increased alternations between mobile and immobile episodes (Figure S3A,

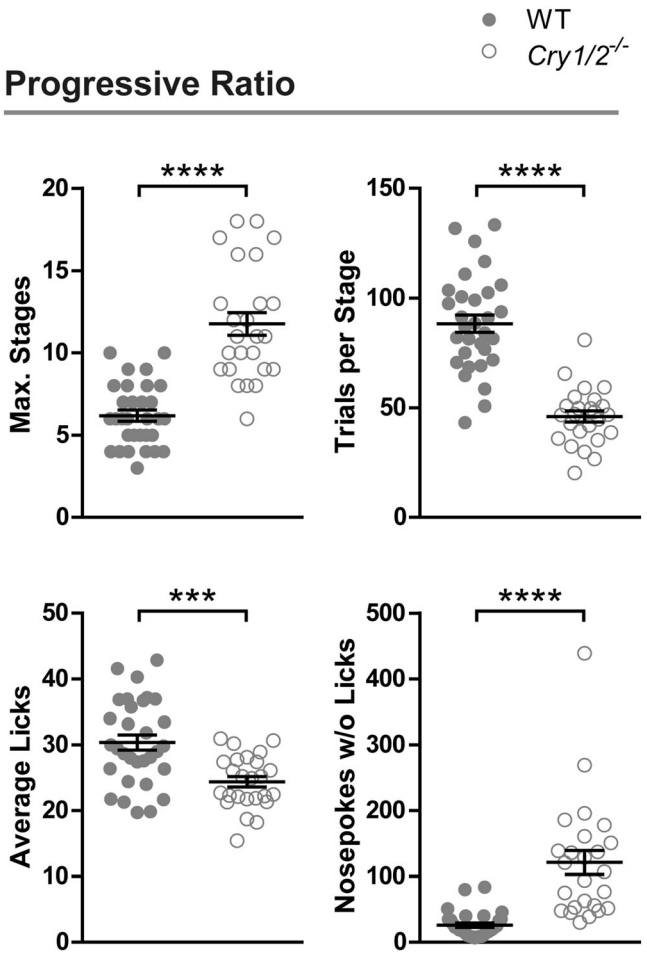

FIGURE 4 Increased reinforcing effects of alcohol in *Cry1/2*$^{-/-}$ mice. The motivation of *Cry1/2*$^{-/-}$ mice to access alcohol in a progressive ratio self-administration paradigm is increased (higher maximum stages and fewer trials per stage); however, they drink less of it once they have achieved access (fewer licks and more nosepokes without licks). Data are shown as dot plots with mean ± SEM, Student's *t* test; $n = 31/24$ (WT/*Cry1/2*$^{-/-}$). ***$p < 0.001$, ****$p < 0.0001$

Table S5-1). Consistent with previous results, the latter two behaviours are severely abnormal in *Cry1/2*$^{-/-}$ mice at baseline.[37] However, they do not worsen under withdrawal. In the elevated plus maze, the time spent in the open arm is not affected by genotype or withdrawal, but distance covered is also greatly reduced in this test under withdrawal in WT animals and moderately reduced in *Cry1/2*$^{-/-}$ mice (Figure S3B, Table S5-1). Other typical alcohol withdrawal symptoms of mice in the open field arena are altered wall rearing, rotations, climbing attempts, and slipping.[54,55] Interestingly, *Cry1/2*$^{-/-}$ mice show very few of these behaviours overall, regardless of whether they were previously given water or alcohol. However, withdrawal significantly reduces wall rearing and climbing attempts in WT mice but causes no further changes in *Cry1/2*$^{-/-}$ mice (Figure S3C, Table S5-1). The number of rotations was similarly increased under withdrawal in both genotypes and only slipping was more pronounced in *Cry1/2*$^{-/-}$ mice than in WT mice under withdrawal. Taken together, it appears that alcohol withdrawal triggers behaviours in WT mice that *Cry1/2*$^{-/-}$ mice already exhibit at baseline and that are barely enhanced in them during withdrawal.

3.5 | Influence of the interaction between genetic and environmental circadian disruptions on alcohol drinking

Both genetic and environmental circadian disruptions are known to be risk factors for impaired health. Therefore, in a second cohort of mice, we examined the consequences of a light cycle mimicking shift work and an interaction between this environmental and a genetic disruption of circadian rhythms on alcohol preference. Detailed analyses of behavioural rhythms of mice of the same strains were carried out earlier in our laboratory using similar methods.[37] Additional experiments of the present study demonstrate that WT and *Cry1/2*$^{-/-}$ mice react differently to shift work conditions. Under normal 12:12 LD conditions, both genotypes show regular activity rhythms with a period of 24 h (Figure 5A,B), which in *Cry1/2*$^{-/-}$ mice is due to so-called masking, in which phases of activity and inactivity are independent of endogenous circadian rhythms and are induced by light and darkness alone.[28] Under alternating "shift work" lighting conditions, WT mice still show significant 24-h rhythms, albeit with lower power than under 12:12 LD conditions (Figure 5B), as they attempt to entrain to the constantly changing light rhythm, resulting in shifting activity onsets and offsets (Figure 5A). In contrast, *Cry1/2*$^{-/-}$ mice lose all rhythmic behaviour and do not even show masking anymore (Figure 5A,B). Interestingly, we found a significant interaction between EtOH concentration, genotype, and light cycle: In WT mice, shift work light conditions reduce the preference for alcohol only at high EtOH concentrations (12% and 16%), whereas in *Cry1/2*$^{-/-}$ mice, shift work conditions reduce preference for alcohol already at low concentrations of 4% and 8% (Figure 5C, Table S6-1). To further test the influence of genetic and environmental circadian disruptions on alcohol drinking in mice, we performed an exclusive choice paradigm in which WT and *Cry1/2*$^{-/-}$ mice under normal LD and shift work conditions in which the mice had the choice between an 8% EtOH and an 1% sucrose solution. *Cry1/2*$^{-/-}$ mice prefer sugar over alcohol significantly more than WT mice, however the shift work light schedule has no additional significant effect neither in WT nor in *Cry1/2*$^{-/-}$ mice (Figure 5D, Table S6-1).

4 | DISCUSSION

Many AUD patients suffer from irregular diurnal patterns and low circadian amplitudes and circadian disruptions are considered a risk factor for the development of AUD.[12,27] In this study, we provide evidence that disruption of endogenous circadian rhythms, environmental rhythms, and their interplay significantly reduce EtOH preference in mice. Furthermore, our data show that genetic disruption of circadian rhythms due to the absence of *CRYs* cause CORT and *PPO*

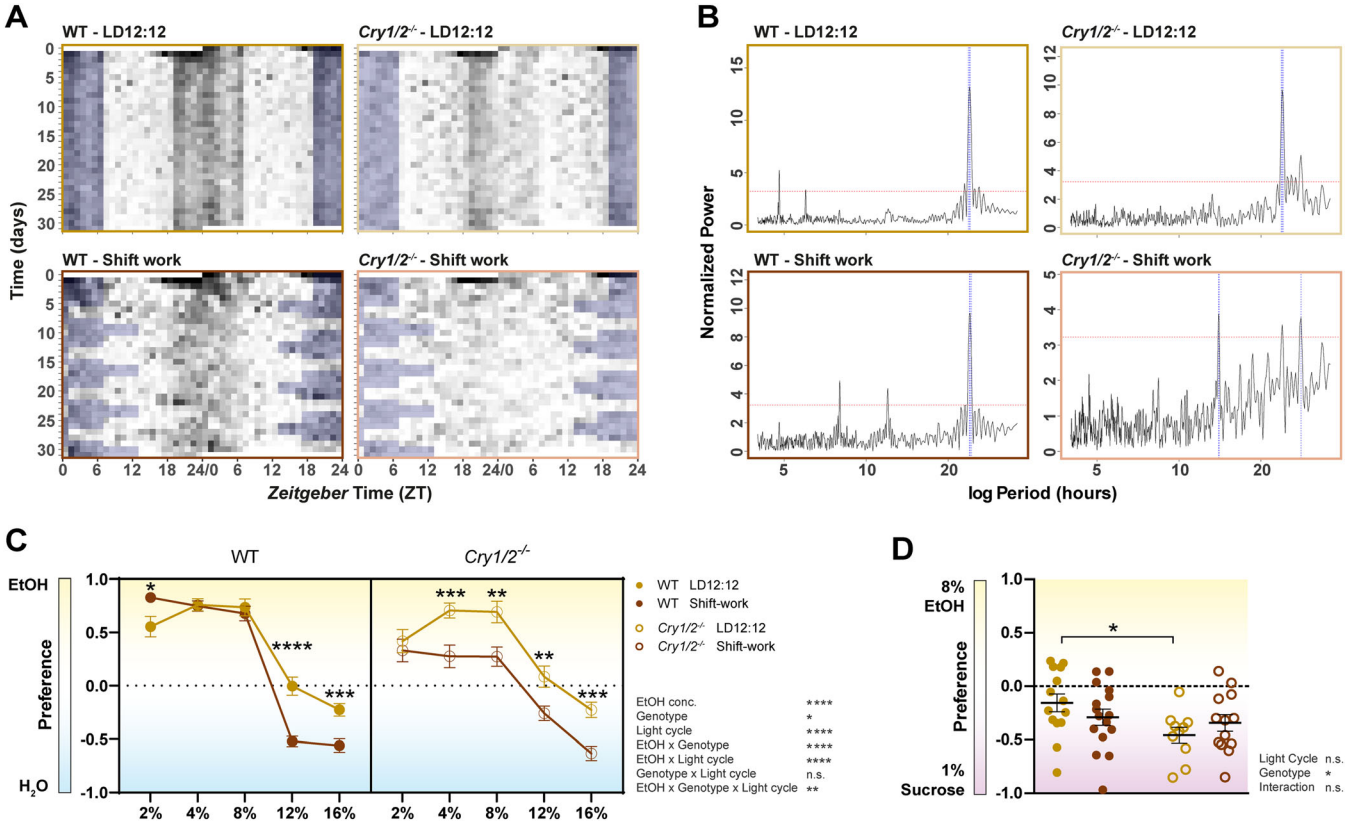

FIGURE 5 Influence of the interaction between genetic and environmental circadian disruptions on alcohol drinking. (A) Average activity patterns of WT and *Cry1/2*$^{-/-}$ mice under regular LD and "shift work" conditions. Data are shown as double plots, times of darkness are shown as blue shades for one of the two plotted days. Darker grey tones of activity bouts represent higher numbers of corner visits within 60 min. (B) Lomb-Scargle Periodograms to detect periodic behaviour in WT and Cry1/2$^{-/-}$ mice under LD12:12 and shiftwork lighting conditions. The normalized power shows that WT LD12:12, WT shift work, and Cry1/2$^{-/-}$ LD12:12 mice show similarly strong circadian locomotor rhythms, but that Cry1/2$^{-/-}$ shift work mice lose circadian rhythmicity. (C) The light cycle influences alcohol preference differently in WT and *Cry1/2*$^{-/-}$ mice. Results presented as mean ± SEM; three-way repeated measures ANOVA with Bonferroni post hoc test restricted to comparisons of genotypes and light conditions at only the respective EtOH concentration; WT: $n = 16/16$, *Cry1/2*$^{-/-}$ $n = 12/13$ (LD12:12/shift work). (C) In an exclusive choice paradigm, where mice have the choice between 8% EtOH and 1% sucrose, *Cry1/2*$^{-/-}$ mice prefer sucrose to EtOH. However, this effect is not enhanced by shift work conditions. Data are shown as dot plots with mean ± SEM; two-way ANOVA with Bonferroni post hoc test; WT: $n = 16/16$, *Cry1/2*$^{-/-}$ $n = 12/13$ (LD12:12/shift work). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; n.s. or no symbol = not significant

alterations that may contribute to lower EtOH preference in these mice. Despite lower EtOH preference, *Cry1/2*$^{-/-}$ mice display increased motivation to obtain EtOH, which is a hallmark of AUD in humans. Furthermore, our results show that WT mice develop behaviours upon alcohol withdrawal that *Cry1/2*$^{-/-}$ mice exhibit already at baseline and therefore do not substantially increase.

As a model for genetic disruption circadian rhythms, we used *Cry1/2*$^{-/-}$ mice, which show roughly normal sleep–wake rhythms due to masking in LD but cannot express intrinsic circadian rhythms.[28,37] In contrast to increased alcohol consumption in AUD, these mice show substantially lower EtOH preference and intake than WT mice with functional circadian clocks. However, our preliminary data show that also in WT mice the amplitude of endogenous *Per2*^{Luc} rhythms of the SCN might predict whether an animal will prefer or reject a rather high alcohol concentration: the lower the amplitude of endogenous SCN rhythms, the lower the alcohol preference. Hence, together with the reduced EtOH preference of arrhythmic *Cry1/2*$^{-/-}$ mice it may seem that diminishing endogenous circadian rhythms leads to a reduction in alcohol preference. Interestingly, however, other arrhythmic mouse mutants, *Clock*$^{\Delta 19}$ and *Per2*$^{Brdm/Brdm}$ mice, show increased preference for alcohol.[16,17] *Per2*$^{Brdm/Brdm}$ mice show increased consumption and preference for EtOH as well as elevated motivation to receive EtOH in a progressive ratio paradigm. Similarly, *Clock*$^{\Delta 19}$ mice display increased consumption and preference for EtOH, however, this concerns mainly higher EtOH concentration of 18–21%. To explore the reasons why *Cry1/2*$^{-/-}$ mice, in contrast to these mice, show decreased alcohol preference, we first measured whether alcohol metabolism, which is under circadian control,[50,51] is impaired. However, the ability to metabolize alcohol is the same in both genotypes. Thus, both genotypes must ingest the same amount of EtOH to achieve comparable blood alcohol levels. Additionally, we measured the expression of the orexin precursor *PPO* as part of the reward system. Our results show significantly decreased orexin levels in *Cry1/2*$^{-/-}$ mice, which has previously been associated with decreased

intake of drugs and alcohol in rodents.[25,53] However, orexin levels are decreased in both *Cry1/2*$^{-/-}$ and *Clock*$^{\Delta 19}$ mice,[56] suggesting that altered alcohol preferences cannot be explained by orexin changes alone. Yet, *Cry1/2*$^{-/-}$ mice also display significantly increased CORT levels and *CRY* proteins are known to interact with glucocorticoid receptors.[52] Although stress is often a trigger for increased alcohol consumption in humans,[30] in rodents, increased stress and CORT levels often lead to decreased alcohol consumption and reward seeking.[31,32] Interestingly, in contrast to those of *Cry1/2*$^{-/-}$ mice, CORT levels of *Clock*$^{\Delta 19}$ and *Per2*$^{Brdm/Brdm}$ mice are permanently suppressed,[56,57] which is consistent with their opposite alcohol preference. Accordingly, the stress system could be a central contributor to whether a mouse drinks much or little alcohol, and depending on the clock gene mutation, CORT levels are persistently high or low.

Interestingly, both the *Clock*$^{\Delta 19}$ and *Per2*$^{Brdm/Brdm}$ exhibit an increased glutamatergic tone, which could contribute to increased alcohol consumption and a hyperglutamatergic state in humans is associated with the aetiology of alcohol dependence.[58,59] While the *Per2*$^{Brdm/Brdm}$ mice have a deficit in the removal of glutamate from the synaptic cleft, the *Clock*$^{\Delta 19}$ mice have a reduced glutamate uptake, both leading to a hyperglutamatergic state. Whether *Cry1/2*$^{-/-}$ mice, which prefer EtOH less, are hypoglutamatergic in contrast will be an interesting part of future studies.

Despite lower alcohol preference, our data also show that *Cry1/2*$^{-/-}$ mice exhibit other characteristics of AUD such as higher motivation to obtain alcohol. *Cry1/2*$^{-/-}$ mice exhibit the same behaviour when sucrose is used instead of alcohol[37] showing that it is not specific to the substance used. The increased willingness to perform operant responses in the progressive ratio paradigm despite lower *liking* of the final EtOH solution resembles compulsive behaviour in humans which has been associated with AUD, anxiety disorders, and increased stress levels,[60–62] the latter two being hallmarks of *Cry1/2*$^{-/-}$ mice.[37,52] This behaviour is also consistent with the incentive sensitization theory, which postulates the existence of two separate neurobiological systems for *wanting* and *liking* a drug.[33–35] The *wanting* system is responsible for compulsive drug use (here: higher number of nosepokes) and can act independently of the system that regulates whether the substance is liked at all (here: lower intake of alcohol). Interestingly, hyperdopaminergic mice show similar *wanting* and *liking* characteristics to *Cry1/2*$^{-/-}$ mice[63] and the presence of *CRYs* suppresses dopamine signaling.[64] These findings suggest that dopamine signalling may be a mechanism for altered drinking behaviour in *Cry1/2*$^{-/-}$ mice which will be subject of future investigation.

In addition, *Cry1/2*$^{-/-}$ mice show a strong anxiety-like phenotype,[27,65] which is also associated with AUD.[66] Interestingly, *Cry1/2*$^{-/-}$ mice exhibit a priori many behaviours that WT mice exhibit only under withdrawal. Since these behaviours are already strongly expressed in *Cry1/2*$^{-/-}$ mice under normal circumstances, they hardly become more pronounced under withdrawal, and it is difficult to conclude whether their circadian disruption makes withdrawal from alcohol more distressful for them than for rhythmic WT mice. Withdrawal is associated with increased stress, anxiety,[67,68] which *Cry1/2*$^{-/-}$ mice already show under normal conditions.[37,52] Thus, we conclude that WT mice under stressful withdrawal conditions approximate anxiety-like behaviours, which *Cry1/2*$^{-/-}$ mice show independently of withdrawal already at baseline.

Together, since *Cry1/2*$^{-/-}$ mice display anxiety-like behaviour and increased *wanting* along with elevated CORT levels, which represent major risk factors for the development of AUD in humans, we consider these mice a suitable model to study the impact of genetic circadian disruption on alcohol drinking behaviour, regardless of the direction of alcohol preference.

To test the effect of environmentally induced disruption of circadian rhythms, we exposed mice to "shift work" lighting conditions which constitutes a risk factor for increased alcohol consumption in humans.[6] In rodents, the effect of shift work conditions on alcohol consumption depends on the species. For example, rats often drink more alcohol under shift work lighting conditions,[69,70] while mice are more likely to drink less alcohol under alternating light conditions.[71,72] The interaction between genes and environment often has considerable influence on the response to environmental factors that contribute to psychiatric disorders. Therefore, we also examined alcohol preference of endogenously arrhythmic *Cry1/2*$^{-/-}$ mice "shift work" lighting conditions. Interestingly, the differences in alcohol preference between WT and *Cry1/2*$^{-/-}$ mice in the second cohort under 12:12 LD conditions were less pronounced under LD conditions than in the first cohort. This may be since only females were used in the second cohort as the results of the first cohort indicate that *Cry1/2*$^{-/-}$ female mice have an increased preference for some alcohol concentrations. Nevertheless, in both WT and *Cry1/2*$^{-/-}$ mice, we were able to show that with increasing alcohol concentrations, mice drank less alcohol under "shift work" lighting regimen. However, this effect only occurred at higher ethanol concentrations in WT mice, whereas *Cry1/2*$^{-/-}$ mice already reject low alcohol concentrations, indicating that the interaction between genetic and environmental circadian disruption has particularly strong influence on alcohol drinking behaviour.

In conclusion, this study shows that both endogenous and environmental rhythms and their interplay have a substantial effect on alcohol drinking behaviour in mice. *Cry1/2*$^{-/-}$ mice show that loss of endogenous circadian rhythmicity can elicit anxiety-like behaviour, stress, and increased *wanting*, while their alcohol preference and intake is reduced. Environmental disruptions of circadian rhythms alter drinking behaviour in a similar manner, and the combination of genetic and environmental disruptions amplifies this effect. Increased stress, elevated anxiety, increased *wanting*, and exposure to irregular daily patterns are known risk factors for AUD in humans. Although the animals in this study drank less alcohol, the same predispositions would be expected to lead to increased alcohol consumption in humans. However, whether these risk factors indeed have opposite effects on alcohol drinking behaviour depending on the species—mouse or human—remains speculation, especially because some other mouse lines with genetically altered circadian rhythms drink more alcohol. A systematic study of physiological factors that influence alcohol consumption in these and in *Cry1/2*$^{-/-}$ mice would provide a better understanding of mechanisms that ultimately link circadian

rhythms to alcohol consumption. A characterization of these mechanisms may provide future insight into the feasibility of using chronotherapies in AUD and which type of chronotherapy may be most appropriate for AUD patients.

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CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

AUTHOR CONTRIBUTIONS

A. H. performed experiments, data analysis and wrote the manuscript. L. E. performed the majority of the experiments. C. K. performed orexin quantification. M. S. performed processing of IntelliCage data. M. V. S. performed measurement of corticosterone. M. J. R. edited the manuscript. D. L. supervised experiments, performed data analysis and wrote the manuscript. All authors were involved in manuscript editing and approving the final version.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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REFERENCES

1. World Health Organization. *Global Status Report on Alcohol and Health 2018*. In: Poznyak V, Rekve DM, eds. Geneva: World Health Organization; 2018.
2. Davis BTT, Voigt RM, Shaikh M, Forsyth CB, Keshavarzian A. Circadian mechanisms in alcohol use disorder and tissue injury. *Alcoholism: Clin Exp Res*. 2018;42(4):668-677.
3. Webb IC. Circadian Rhythms and Substance Abuse: Chronobiological considerations for the treatment of addiction. *Curr Psychiatry Rep*. 2017;19(2):12-20.
4. Hasler BP, Bruce S, Scharf D, Ngari W, Clark DB. Circadian misalignment and weekend alcohol use in late adolescent drinkers: preliminary evidence. *Chronobiol Int*. 2019;36(6):796-810.
5. Perreau-Lenz S, Spanagel R. Clock genes x stress x reward interactions in alcohol and substance use disorders. *Alcohol*. 2015;49(4):351-357.
6. Spanagel R, Rosenwasser AM, Schumann G, Sarkar DK. Alcohol consumption and the body's biological clock. *Alcohol Clin Exp Res*. 2005;29(8):1550-1557.
7. Takahashi JS, Hong HK, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet*. 2008;9(10):764-775.
8. Mure LS, Le HD, Benegiamo G, et al. Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. *Science*. 2018;359(6381):eaao0318.
9. Ruben MD, Wu G, Smith DF, Schmidt RE, Francey LJ, Lee YY, Anafi RC, Hogenesch JB A database of tissue-specific rhythmically expressed human genes has potential applications in circadian medicine. *Sci Transl Med* 2018;10(458): eaat8806).
10. Adan A. Chronotype and personality factors in the daily consumption of alcohol and psychostimulants. *Addiction*. 1994;89(4):455-462.
11. Gulick D, Gamsby JJ. Racing the clock: The role of circadian rhythmicity in addiction across the lifespan. *Pharmacol Ther*. 2018;188:124-139.
12. Huang MC, Ho CW, Chen CH, Liu SC, Chen CC, Leu SJ. Reduced expression of circadian clock genes in male alcoholic patients. *Alcohol Clin Exp Res*. 2010;34(11):1899-1904.
13. Dong L, Bilbao A, Laucht M, et al. Effects of the circadian rhythm gene period 1 (Per1) on psychosocial stress-induced alcohol drinking. *Am J Psychiatry*. 2011;168(10):1090-1098.
14. Falcon E, McClung CA. A role for the circadian genes in drug addiction. *Neuropharmacology*. 2009;56(Suppl 1):91-96.
15. Gamsby JJ, Templeton EL, Bonvini LA, et al. The circadian Per1 and Per2 genes influence alcohol intake, reinforcement, and blood alcohol levels. *Behav Brain Res*. 2013;249:15-21.
16. Ozburn AR, Falcon E, Mukherjee S, et al. The role of clock in ethanol-related behaviors. *Neuropsychopharmacol Off Publi Am Coll Neuropsychopharmacol*. 2013;38(12):2393-2400.
17. Spanagel R, Pendyala G, Abarca C, et al. The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nat Med*. 2005;11(1):35-42.
18. Richter K, Peter L, Rodenbeck A, et al. Shiftwork and alcohol consumption: A systematic review of the literature. *Eur Addict Res*. 2020;1-7.
19. Rogers HL, Reilly SM. A survey of the health experiences of international business travelers. Part One--Physiological aspects. *AAOHN J*. 2002;50(10):449-459.
20. Clark JW, Fixaris MC, Belanger GV, Rosenwasser AM. Repeated light-dark phase shifts modulate voluntary ethanol intake in male and female high alcohol-drinking (HAD1) rats. *Alcohol Clin Exp Res*. 2007;31(10):1699-1706.
21. Rosenwasser AM. Chronobiology of ethanol: animal models. *Alcohol*. 2015;49(4):311-319.
22. Rosenwasser AM, McCulley WD 3rd, Hartmann MC, Fixaris MC, Crabbe JC. Suppression of voluntary ethanol intake in mice under constant light and constant darkness. *Alcohol*. 2020;83:37-46.
23. Boschloo L, Vogelzangs N, Licht CM, Vreeburg SA, Smit JH, et al. Heavy alcohol use, rather than alcohol dependence, is associated with dysregulation of the hypothalamic-pituitary-adrenal axis and the autonomic nervous system. *Drug Alcohol Depend*. 2011;116(1-3):170-176.
24. Azeez IA, Del Gallo F, Cristino L, Bentivoglio M. Daily fluctuation of orexin neuron activity and wiring: The challenge of "chronoconnectivity". *Front Pharmacol*. 2018;9:1061-1077.
25. Moorman DE. The hypocretin/orexin system as a target for excessive motivation in alcohol use disorders. *Psychopharmacology (Berl)*. 2018;235(6):1663-1680.
26. Caspi A, Moffitt TE. Gene-environment interactions in psychiatry: joining forces with neuroscience. *Nat Rev Neurosci*. 2006;7(7):583-590.
27. Hühne A, Hoch E, Landgraf D. DAILY—a personalized circadian zeitgeber therapy as an adjunctive treatment for alcohol use disorder patients: Study protocol for a randomized controlled trial. Front. *Psychiatry*. 2021;11(1554).
28. van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature*. 1999;398(6728):627-630.

29. Putker M, Wong DCS, Seinkmane E, et al. CRYPTOCHROMES confer robustness, not rhythmicity, to circadian timekeeping. *EMBO J*. 2021; 40(7):e106745.
30. Stephens MA, Wand G. Stress and the HPA axis: role of glucocorticoids in alcohol dependence. *Alcohol Res*. 2012;34(4):468-483.
31. Becker HC, Lopez MF, Doremus-Fitzwater TL. Effects of stress on alcohol drinking: a review of animal studies. *Psychopharmacology (Berl)*. 2011;218(1):131-156.
32. Delis F, Thanos PK, Rombola C, et al. Chronic mild stress increases alcohol intake in mice with low dopamine D2 receptor levels. *Behav Neurosci*. 2013;127(1):95-105.
33. Cofresi RU, Bartholow BD, Piasecki TM. Evidence for incentive salience sensitization as a pathway to alcohol use disorder. *Neurosci Biobehav Rev*. 2019;107:897-926.
34. Robinson TE, Berridge KC. The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev*. 1993;18(3):247-291.
35. Robinson TE, Berridge KC. The incentive sensitization theory of addiction: some current issues. *Philos Trans Royal Soc London Ser B Biol Sci*. 2008;363(1507):3137-3146.
36. Yoo SH, Yamazaki S, Lowrey PL, et al. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A*. 2004;101(15):5339-5346.
37. Hühne A, Volkmann P, Stephan M, Rossner M, Landgraf D. An in-depth neurobehavioral characterization shows anxiety-like traits, impaired habituation behavior, and restlessness in male Cryptochrome-deficient mice. *Genes Brain Behav*. 2020;e12661.
38. Krackow S, Vannoni E, Codita A, et al. Consistent behavioral phenotype differences between inbred mouse strains in the IntelliCage. *Genes Brain Behav*. 2010;9(7):722-731.
39. Voikar V, Colacicco G, Gruber O, Vannoni E, Lipp HP, Wolfer DP. Conditioned response suppression in the IntelliCage: assessment of mouse strain differences and effects of hippocampal and striatal lesions on acquisition and retention of memory. *Behav Brain Res*. 2010;213(2):304-312.
40. Eisenhardt M, Leixner S, Spanagel R, Bilbao A. Quantification of alcohol drinking patterns in mice. *Addict Biol*. 2015;20(6):1001-1011.
41. Volkmann P, Stephan M, Krackow S, Jensen N, Rossner MJ. PsyCoP - a platform for systematic semi-automated behavioral and cognitive profiling reveals gene and environment dependent impairments of Tcf4 transgenic mice subjected to social defeat. Front. *Behav Neurosci*. 2020;14:618180.
42. Landgraf D, Long JE, Proulx CD, Barandas R, Malinow R, Welsh DK. Genetic disruption of circadian rhythms in the suprachiasmatic nucleus causes helplessness, behavioral despair, and anxiety-like behavior in mice. *Biol Psychiatry*. 2016;80(11):827-835.
43. Cantin L, Lenoir M, Augier E, et al. Cocaine is low on the value ladder of rats: possible evidence for resilience to addiction. *PloS one*. 2010; 5(7):e11592.
44. Lenoir M, Serre F, Cantin L, Ahmed SH. Intense sweetness surpasses cocaine reward. *PloS one*. 2007;2(8):e698.
45. Augier E, Barbier E, Dulman RS, et al. A molecular mechanism for choosing alcohol over an alternative reward. *Science*. 2018; 360(6395):1321-1326.
46. Landgraf D, Long JE, Welsh DK. Depression-like behaviour in mice is associated with disrupted circadian rhythms in nucleus accumbens and periaqueductal grey. *Eur J Neurosci*. 2016;43(10):1309-1320.
47. Mickelsen LE, Kolling FWT, Chimileski BR, Fujita A, Norris C, et al. Neurochemical heterogeneity among lateral hypothalamic hypocretin/orexin and melanin-concentrating hormone neurons identified through single-cell gene expression analysis. *Eneuro*. 2017;4(5): 13-17.
48. Kim TK, Kim JE, Park JY, et al. Antidepressant effects of exercise are produced via suppression of hypocretin/orexin and melanin-concentrating hormone in the basolateral amygdala. *Neurobiol Dis*. 2015;79:59-69.
49. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29(9): 45e-445.
50. Salsano F, Maly IP, Sasse D. The circadian rhythm of intra-acinar profiles of alcohol dehydrogenase activity in rat liver: a microquantitative study. *Histochem J*. 1990;22(8):395-400.
51. Sturtevant RP, Garber SL. Circadian rhythms of alcohol dehydrogenase and MEOS in the rat. *Proc Soc Exp Biol Med*. 1984;175(3): 299-303.
52. Lamia KA, Papp SJ, Yu RT, et al. Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature*. 2011;480(7378): 552-556.
53. Anderson RI, Becker HC, Adams BL, Jesudason CD, Rorick-Kehn LM. Orexin-1 and orexin-2 receptor antagonists reduce ethanol self-administration in high-drinking rodent models. *Front Neurosci*. 2014; 8:33.
54. Becker HC. Animal models of alcohol withdrawal. *Alcohol Res Health*. 2000;24(2):105-113.
55. Metten P, Schlumbohm JP, Huang LC, et al. An alcohol withdrawal test battery measuring multiple behavioral symptoms in mice. *Alcohol*. 2018;68:19-35.
56. Turek FW, Joshu C, Kohsaka A, et al. Obesity and metabolic syndrome in circadian Clock mutant mice. *Science*. 2005;308(5724): 1043-1045.
57. Russell AL, Miller L, Yi H, Keil R, Handa RJ, Wu TJ. Knockout of the circadian gene, Per2, disrupts corticosterone secretion and results in depressive-like behaviors and deficits in startle responses. *BMC Neurosci*. 2021;22(5).
58. Pulvirenti L, Diana M. Drug dependence as a disorder of neural plasticity: focus on dopamine and glutamate. *Rev Neurosci*. 2001;12(2): 141-158.
59. Tsai G, Coyle JT. The role of glutamatergic neurotransmission in the pathophysiology of alcoholism. *Annu Rev Med*. 1998;49(1): 173-184.
60. Langen M, Kas MJ, Staal WG, van Engeland H, Durston S. The neurobiology of repetitive behavior: of mice. *Neurosci Biobehav Rev*. 2011; 35(3):345-355.
61. Stein DJ, Craske MA, Friedman MJ, Phillips KA. Anxiety disorders, obsessive-compulsive and related disorders, trauma- and stressor-related disorders, and dissociative disorders in DSM-5. *Am J Psychiatry*. 2014;171(6):611-613.
62. Van Ameringen M, Patterson B, Simpson W. DSM-5 obsessive-compulsive and related disorders: clinical implications of new criteria. *Depress Anxiety*. 2014;31(6):487-493.
63. Pecina S, Cagniard B, Berridge KC, Aldridge JW, Zhuang X. Hyperdopaminergic mutant mice have higher "wanting" but not "liking" for sweet rewards. *J Neurosci*. 2003;23(28):9395-9402.
64. Porcu A, Vaughan M, Nilsson A, Arimoto N, Lamia K, Welsh DK. Vulnerability to helpless behavior is regulated by the circadian clock component CRYPTOCHROME in the mouse nucleus accumbens. *Proc Natl Acad Sci U S A*. 2020;117(24):13771-13782.
65. De Bundel D, Gangarossa G, Biever A, Bonnefont X, Valjent E. Cognitive dysfunction, elevated anxiety, and reduced cocaine response in circadian clock-deficient cryptochrome knockout mice. *Front Behav Neurosci*. 2013;7(152).
66. Anker JJ, Kushner MG. Co-occurring alcohol use disorder and anxiety: Bridging psychiatric, psychological, and neurobiological perspectives. *Alcohol Res*. 2019;40(1).
67. Koob G, Kreek MJ. Stress, dysregulation of drug reward pathways, and the transition to drug dependence. *Am J Psychiatry*. 2007;164(8): 1149-1159.
68. Zhou Y, Kreek MJ. Involvement of activated brain stress responsive systems in excessive and "Relapse" alcohol drinking in rodent models: Implications for therapeutics. *J Pharmacol Exp Ther*. 2018;366(1):9-20.

69. Gauvin DV, Baird TJ, Vanecek SA, Briscoe RJ, Vallett M, Holloway FA. Effects of time-of-day and photoperiod phase shifts on voluntary ethanol consumption in rats. *Alcohol Clin Exp Res*. 1997; 21(5):817-825.
70. Resendiz-Flores M, Escobar C. Circadian disruption favors alcohol consumption and differential DeltaFosB accumulation in Corticolimbic structures. *Addict Biol*. 2019;24(6):1179-1190.
71. Abarca C, Albrecht U, Spanagel R. Cocaine sensitization and reward are under the influence of circadian genes and rhythm. *Proc Natl Acad Sci U S A*. 2002;99(13):9026-9030.
72. Rosenwasser AM, Fixaris MC. Chronobiology of alcohol: studies in C57BL/6J and DBA/2J inbred mice. *Physiol Behav*. 2013;110-111: 140-147.

SUPPORTING INFORMATION

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Table S2-1: Statistical data on alcohol preference of different EtOH concentrations in WT and *Cry1/2*$^{-/-}$ mice of both genders together or separately for males and females. The data in the table refers to the data shown in Fig. 2A and 2B.

Fig.	n-value				Statistical test	Source of variation	d.f.	F-value	% of total variation	Significance	
	WT		*Cry1/2*$^{-/-}$							p-value	Summary
	m	f	m	f							
2A	31		24		2-way repeated measure ANOVA	EtOH conc. x Genotype	4, 212	5.048	2.690	**.0007**	***
						EtOH conc.	4, 212	44.59	23.77	**<.0001**	****
						Genotype	1, 53	10.99	7.369	**.0017**	**
2B	16	15	8	16	3-way ANOVA	EtOH conc.	4, 204	41.48	21.52	**<.0001**	****
						Genotype	1, 51	11.01	7.598	**.0017**	**
						Gender	1, 51	.4990	.3443	.4831	n.s.
						EtOH conc. x Genotype	4, 204	5.010	2.598	**.0007**	***
						EtOH conc. x Gender	4, 204	2.922	1.516	**.0222**	*
						Gender x Genotype	1, 51	.04329	.02987	.8360	n.s.
						EtOH conc. x Genotype x Gender	4, 204	.09098	.4591	.4591	n.s.

m = male
f = female
d.f. = degree of freedom

Table S2-2: Statistical data from the correlation between preference for 12% EtOH and *Per2*^{Luc} amplitude of the SCN of WT mice. The data in the table refers to the data shown in Fig. 2C.

Fig.	Variables	n-value	Statistical test	Slope	R^2	Is the slope significantly non-zero?			
						F	d.f.	p-value	Summary
2C	EtOH preference vs. *Per2*^{Luc} SCN amplitude	6	Linear Regression	6.432 ± 1.380	.8444	27.71	1, 4	**.0096**	**

d.f. = degree of freedom

Table S3-1: Statistical data from the comparison between WT and *Cry1/2*$^{-/-}$ mice in regard to total alcohol intake. The data in the table refers to the data shown in Fig. 3A.

Fig.	Variables	Values (mean ± SD)		n-value		Statistical test	d.f.	t-value	Significance	
		WT	*Cry1/2*$^{-/-}$	WT	*Cry1/2*$^{-/-}$				p-value	Summary
3A	Total alcohol intake	94.77 ± 3.827	79.44 ± 3.768	124	95	paired t-test	217	2.800	**.0056**	**

SD = Standard deviation

d.f. = degree of freedom

Table S3-2: Statistical data on EtOH metabolism and CORT and PPO time courses in WT and *Cry1/2*$^{-/-}$ mice. The data in the table refers to the data shown in Fig. 3B-D.

Fig.	Variables	n-value		Statistical test	Source of variation	d.f.	F-value	% of total variation	Significance	
		WT	*Cry1/2*$^{-/-}$						p-value	Summary
3B	EtOH Metabolism	16	13	2-way repeated measure ANOVA	Time x Genotype	3, 83	.2307	.17	.8747	n.s.
					Genotype	1, 27	.3590	.09	.5507	n.s.
					Time	3, 83	108.3	79.20	**<.0001**	****
3C	CORT	16	8	2-way repeated measure ANOVA	Time x Genotype	4, 56	2.787	10.18	**.0350**	*
					Genotype	1, 14	6.811	10.04	**.0206**	*
					Time	4, 56	2.194	8.01	.0813	n.s.
3D	*PPO*	3-5	3	2-way ANOVA	Time x Genotype	3, 18	1.445	10.15	.2629	n.s.
					Genotype	1, 18	7.474	17.50	**.0136**	*
					Time	3, 18	3.031	21.29	.0562	n.s.

CORT = corticosterone

PPO = *prepro-orexin* expression

d.f. = degree of freedom

Table S4-1: Statistical data from the comparison between WT and *Cry1/2*$^{-/-}$ mice in regard to total alcohol intake and progressive ratio. The data in the table refers to the data shown in Fig. 4.

Fig.	Variables		Values (mean ± SD)		n-value		Statistical test	d.f.	t-value	Significance	
			WT	*Cry1/2*$^{-/-}$	WT	*Cry1/2*$^{-/-}$				p-value	Summary
4	Progressive Ratio	Max. Stage	6.194 ± 0.3389	11.76 ± 0.6959	31	25	paired t-test	54	7.633	**<.0001**	****
		Trials per Stage	88.27 ± 3.959	46.06 ± 2.573					8.474	**<.0001**	****
		Average Licks	30.35 ± 1.161	24.38 ± 0.8071					4.029	**.0002**	***
		Nosepokes w/o licks	25.97 ± 3.450	121.2 ± 17.93					5.762	**<.0001**	****

SD = Standard deviation

d.f. = degree of freedom

Table S5-1: Statistical data on behaviors typical for alcohol withdrawal in WT and *Cry1/2*$^{-/-}$ mice. The data in the table refers to the data shown in Fig. S3.

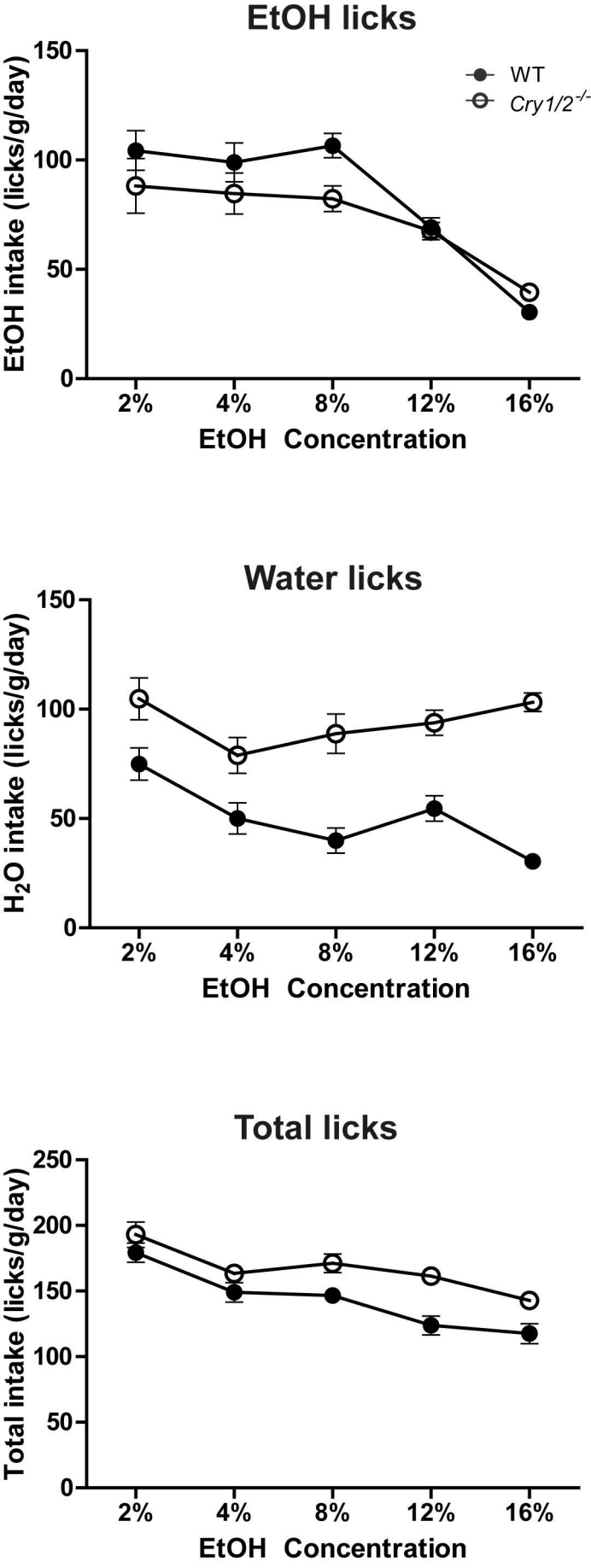
Fig.	Variables	n-value				Statistical test	Source of variation	d.f.	F-value	% of total variation	Significance	
		WT		*Cry1/2*$^{-/-}$							p-value	Summary
		H_2O	EtOH	H_2O	EtOH							
S3A	Centre Time	15	31	19	25	2-way ANOVA	EtOH x Genotype	1, 86	4.329	3.47	**.0404**	*
							Genotype	1, 86	33.61	26.97	**<.0001**	****
							EtOH	1, 86	.0016	.00	.9679	n.s.
	Distance						EtOH x Genotype	1, 86	12.31	5.96	**.0007**	***
							Genotype	1, 86	21.72	10.52	**<.0001**	****
							EtOH	1, 86	107.2	51.94	**<.0001**	****
	Im/mobile episodes						EtOH x Genotype	1, 86	4.784	3.17	**.0314**	*
							Genotype	1, 86	.4946	.33	.4838	n.s.
							EtOH	1, 86	65.01	43.03	**<.0001**	****
S3B	Open arm time	15	31	19	25	2-way ANOVA	EtOH x Genotype	1, 86	3.482	3.90	.0655	n.s.
							Genotype	1, 86	.1292	.14	.7202	n.s.
							EtOH	1, 86	1.216	1.36	.2733	n.s.
	Distance						EtOH x Genotype	1, 86	2.390	1.50	.1259	n.s.
							Genotype	1, 86	35.16	22.11	**<.0001**	****
							EtOH	1, 86	47.73	30.01	**<.0001**	****
S3C	Wall rearing	15	29	19	25	2-way ANOVA	EtOH x Genotype	1, 84	5.388	3.33	.0227	*
							Genotype	1, 84	6.794	4.19	.0108	*
							EtOH	1, 84	74.86	46.21	<.0001	****
	Climbing attempts						EtOH x Genotype	1, 84	4.542	4.55	.0361	*
							Genotype	1, 84	3.440	3.45	.0672	n.s.
							EtOH	1, 84	13.82	13.84	.0004	***
	Rotations						EtOH x Genotype	1, 84	.2431	.19	.6232	n.s.
							Genotype	1, 84	5.146	4.11	.0259	*
							EtOH	1, 84	32.55	25.97	<.0001	****
	Slipping						EtOH x Genotype	1, 84	2.013	2.20	0.1596	n.s.
							Genotype	1, 84	4.122	4.50	0.0454	*
							EtOH	1, 84	0.2354	0.26	0.6288	n.s.

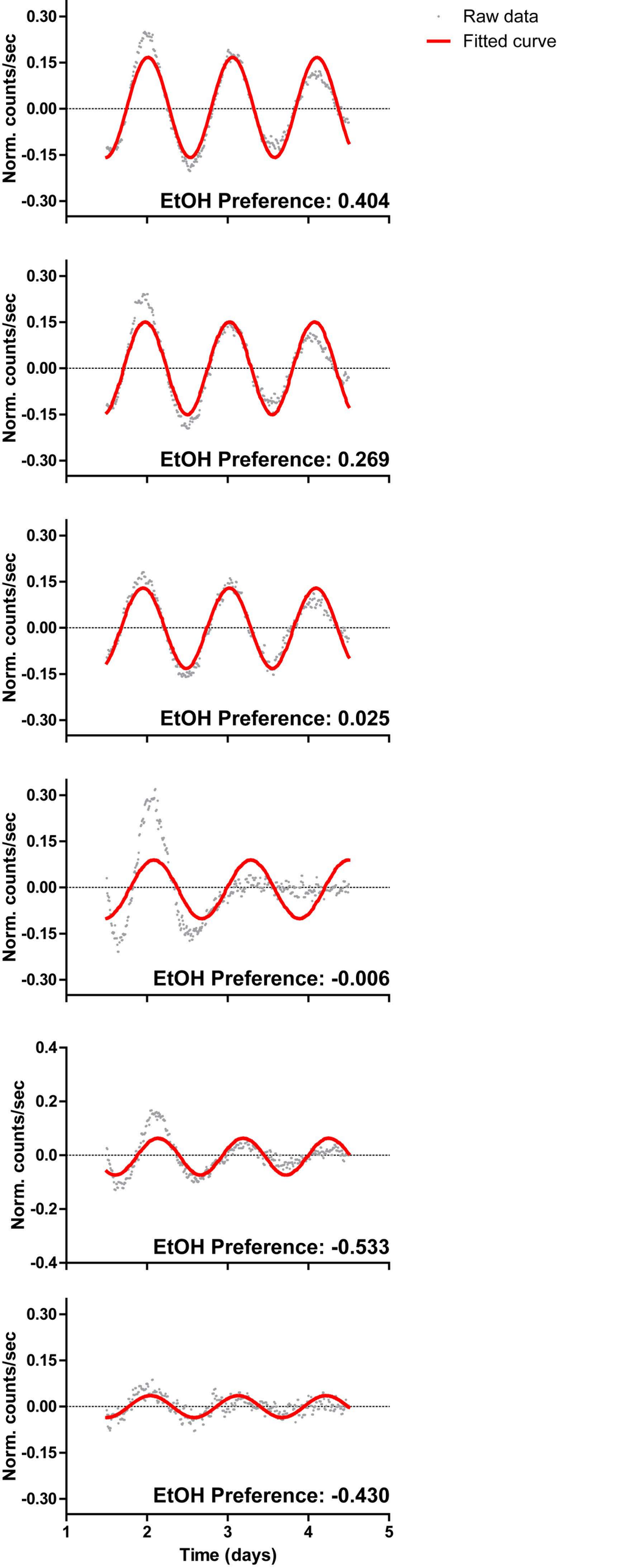
d.f. = degree of freedom

Table S6-1: Statistical data on alcohol preference of different EtOH concentrations in WT and *Cry1/2*$^{-/-}$ mice under regular and irregular lighting conditions. The data in the table refers to the data shown in Fig. 5C and 5D.

Fig.	n-value				Statistical test	Source of variation	d.f.	F-value	% of total variation	Significance	
	WT		*Cry1/2*$^{-/-}$							p-value	Summary
	reg.	shift	reg.	shift							
5C	16	15	8	16	3-way ANOVA	EtOH conc.	4, 212	265.6	59.28	**<.0001**	****
						Genotype	1, 53	5.754	1.377	**.0200**	*
						Light cycle	1, 53	17.74	4.245	**<.0001**	****
						EtOH conc. x Genotype	4, 212	11.38	2.540	**<.0001**	****
						EtOH conc. x Light cycle	4, 212	11.44	2.554	**<.0001**	****
						Genotype x Light cycle	1, 53	3.519	.8420	.0662	n.s.
						EtOH conc. x Genotype x Light cycle	4, 212	4.438	.9905	**.0018**	**
5D	14	16	10	13	2-way ANOVA	Genotype x Light cycle	1, 53	3.509	5.578	.0666	n.s.
						Genotype	1, 53	6.589	10.47	**.0131**	*
						Light cycle	1, 53	.0172	.02734	.8962	n.s.

reg. = regular lighting conditions
shift = shift work
d.f. = degree of freedom


EtOH licks
150
100
50
0
EtOH intake (licks/g/day)
WT
Cry1/2-/-
2%
4%
8%
12%
16%
EtOH Concentration
Water licks
150
100
50
0
H2O intake (licks/g/day)
2%
4%
8%
12%
16%
EtOH Concentration
Total licks
250
200
150
100
50
0
Total intake (licks/g/day)
2%
4%
8%
12%
16%
EtOH Concentration


Raw data
Fitted curve
Norm. counts/sec
0.30
0.15
0.00
-0.15
-0.30
EtOH Preference: 0.404
EtOH Preference: 0.269
EtOH Preference: 0.025
EtOH Preference: -0.006
0.4
0.2
0.0
-0.2
-0.4
EtOH Preference: -0.533
EtOH Preference: -0.430
1
2
3
4
5
Time (days)

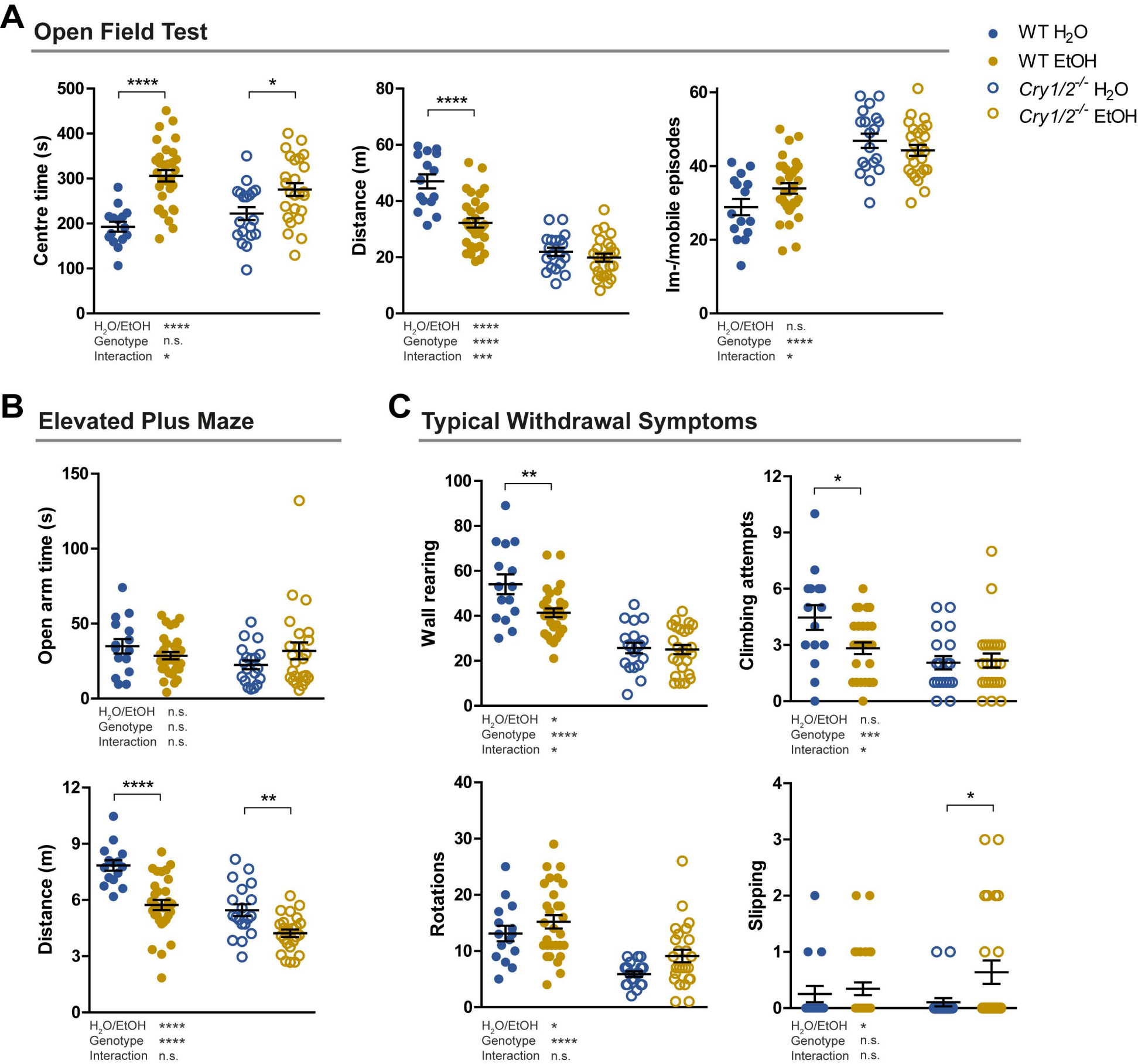

A
Open Field Test
WT H2O
WT EtOH
Cry1/2-/- H2O
Cry1/2-/- EtOH
Centre time (s)
****
*
H2O/EtOH ****
Genotype n.s.
Interaction *
Distance (m)
****
H2O/EtOH ****
Genotype ****
Interaction ***
Im-/mobile episodes
H2O/EtOH n.s.
Genotype ****
Interaction *
B
Elevated Plus Maze
Open arm time (s)
H2O/EtOH n.s.
Genotype n.s.
Interaction n.s.
Distance (m)
****
**
H2O/EtOH ****
Genotype ****
Interaction n.s.
C
Typical Withdrawal Symptoms
Wall rearing
**
H2O/EtOH *
Genotype ****
Interaction *
Climbing attempts
*
H2O/EtOH n.s.
Genotype ***
Interaction *
Rotations
H2O/EtOH *
Genotype ****
Interaction n.s.
Slipping
*
H2O/EtOH *
Genotype n.s.
Interaction n.s.

Table S2-1: Statistical data on alcohol preference of different EtOH concentrations in WT and *Cry1/2*$^{-/-}$ mice of both genders together or separately for males and females. The data in the table refers to the data shown in Figure 2A and B.

Table S2–2: Statistical data from the correlation between preference for 12% EtOH and *Per2*^{Luc} amplitude of the SCN of WT mice. The data in the table refers to the data shown in Figure 2C.

Table S3–1: Statistical data from the comparison between WT and *Cry1/2*$^{-/-}$ mice in regard to total alcohol intake. The data in the table refers to the data shown in Figure 3A.

Table S3–2: Statistical data on EtOH metabolism and CORT and PPO time courses in WT and *Cry1/2*$^{-/-}$ mice. The data in the table refers to the data shown in Figure 3B-D.

Table S4–1: Statistical data from the comparison between WT and *Cry1/2*$^{-/-}$ mice in regard to total alcohol intake and progressive ratio. The data in the table refers to the data shown in Figure 4.

Table S5–1: Statistical data on behaviors typical for alcohol withdrawal in WT and *Cry1/2*$^{-/-}$ mice. The data in the table refers to the data shown in Figure S3.

Table S6–1: Statistical data on alcohol preference of different EtOH concentrations in WT and *Cry1/2*$^{-/-}$ mice under regular and irregular lighting conditions. The data in the table refers to the data shown in Figure 5C and D.

Fig. S1: Number of licks of EtOH and water bottles on which the preference calculations of Figure 2A are based. Licks per day per gram of body weight of mice are shown.

Fig. S2: Bioluminescence data of SCN explant cultures. Shown are baseline subtracted raw data normalized to brightness (gray) and the curve fit (red) used to determine the amplitude. The data are sorted from top to bottom by descending amplitude. In each graph, the EtOH preference of the corresponding mouse is inserted to assign the SCN amplitudes to the preferences.

Fig. S3: Determination of alcohol withdrawal symptoms. (A, B) Anxiety-related behavior of WT and *Cry1/2*$^{-/-}$ mice under control and acute alcohol withdrawal conditions measured in the open field test (A) and in the elevated plus maze (B). (C) During the open field test, mice were video recorded and additional typical withdrawal symptoms were assessed. Data are shown as dot plots with mean ± SEM, two-way ANOVA with Bonferroni post hoc test; WT: n = 16/16, *Cry1/2*$^{-/-}$ n = 12/13 (LD12:12/shift work), n-numbers may differ by one due to possible exclusion of outliers according to Grubb's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

4. Discussion and perspective

At the beginning of my Ph.D., I developed theoretical constructs within the framework of a review paper (*Paper IV*) about possible mechanisms by which disrupted clocks may contribute to the development of, in this review, specifically psychiatric disorders. However, these mechanisms may also be considered as underlying factors in other disorders, such as metabolic or endocrinological disorders, whose etiology is at least in part due to the disturbance of circadian clocks.

These theoretical mechanisms are:

1. *External desynchronization between the organism and the environment,*
2. *Internal desynchronization within the organism*,
3. *Low amplitude of circadian oscillations*, and
4. *Alterations in sleep architecture*.

These mechanisms served as the basis for each of my Ph.D. projects. They formed the starting point for our experiments, either by experimentally confirming their actual occurrence or by showing consequences of their existence for mental health. In *Paper I*, I was able to show that *Cry1/2*$^{-/-}$ double knockout mice with no ability to express endogenous circadian rhythms show a pronounced anxiety-like phenotype with high levels of restlessness and increased sensitivity to stress. These effects have also been confirmed by increased neuronal activity in the amygdala [189]. Moreover, in *Paper II*, I have shown that the same mice display marked characteristics that, importantly, predispose humans to increased alcohol consumption and risk of addiction [190]. *Cry1/2*$^{-/-}$ mice show a reduced alcohol drinking behavior with a simultaneously increased willingness to obtain the substance. A result that we place within the framework of the incentive sensitization theory of addictive disorders, which states that *liking* and *wanting* a drug in addiction disorders are two separate processes. The actual *liking* of the drug itself is often very weak, whereas *wanting* is very strong [106, 183, 191]. We were also able to support these phenotypes by molecular biological analyses: In the absence of CRYs, CORT levels are persistently elevated, and levels of the orexin precursor PPO are decreased, which together represent explanatory factors for reduced alcohol preference [188, 192-194]. Interestingly, the alcohol drinking-related phenotype of *Cry1/2*$^{-/-}$ mice was amplified by additional external circadian disturbances caused by a shift work model. This finding illustrates a gene x environment interaction of circadian rhythm disorders on alcohol consumption. This is of great importance for the clinic, as

patients with problematic alcohol consumption patterns with both types of circadian rhythm disturbances, i.e., genetic and environmental in origin, are likely to benefit from chronotherapy.

Beyond the studies that form the basis of this Ph.D., I am simultaneously working on additional related projects that are ongoing and are expected to be completed over the next year. As described above, we found an anxiety-like phenotype with high levels of restlessness and increased stress levels in the *Cry1/2*$^{-/-}$ animals. Since it is relevant to investigate the direction of causality between disturbed clock and the development of anxiety-like behavior, I started to restore functional circadian rhythmicity in the SCN in these animals by stereotactic injection of adeno-associated viruses (AAV) carrying a vector encoding the *Cry1* and *Cry2* gene. This is to investigate the extent to which restoring rhythmicity in the SCN alone is sufficient to attenuate the anxiety phenotype in these mice. After successful cloning, virus production and stereotactic injection, I was already able to collect behavioral and metabolic data from these animals, which impressively shows that after the rescue of central circadian rhythms in the SCN, the animals lose large parts of their anxiety phenotype (Hühne et al., *under preparation*). This result is of high relevance for the general understanding of how important functional circadian clocks are for psychiatric integrity, and on the other hand represent a milestone in circadian research on the eminent role of stable rhythms in the SCN for mental health.

Further on, the last project I started during my Ph.D., is a study in which I attempt to measure the theoretical construct of internal desynchronization (*Paper IV*) in living animals. Using deep brain electroencephalography (EEG) in two different brain regions, I am recording neuronal activity in *Cry1/2*$^{-/-}$ and WT mice under different light conditions over several days. This will hopefully reveal how endogenous and exogenous rhythms contribute to internal synchronization processes of two different brain regions. This study could be particularly important in a translational sense, as it may shed light on the extent to which external therapeutic interventions, such as light therapy, could be used to interfere with and improve the process of internal synchronization. Our preliminary data show that stable environmental conditions (12 h light and 12 h darkness) have only little effect on the strength of circadian neuronal activity. This is unexpected for us, since light therapy is one of the most frequently used tools for stabilizing sleep-wake rhythms in the setting of chronotherapeutic interventions. The mechanisms underlying the success of light therapy might then need to be re-examined if our preliminary results are further confirmed.

Based on our basic research findings and clinical observations, we have developed a new adjunctive therapy for AUD patients. This is based on strengthening the patients' endogenous circadian system through so-called circadian *Zeitgeber* such as sleep,

meal, and activity times. We hope that this will lead to a strengthening of both the internal synchronization of physiological processes and the synchronization with the environment, which in the long run should lead to an improvement in mental health and in this particular case to a lower risk of relapse in AUD patients. While I developed and published the therapy protocol during my Ph.D., I started in parallel a randomized controlled pilot trial at the addiction ward of our clinic to test its effectiveness. Final conclusions about its efficacy cannot be made at this point, but preliminary data already shows that the more regular the patients' wake-up, get-up, and have breakfast, the weaker the craving for alcohol is on those days. This suggests that a high regularity especially of morning activities has a positive effect on craving during the day. Furthermore, patients of the experimental group have significantly lower alcohol drinking relapses than patients of the control group, further indicating the positive impact of the DAILY therapy on harmful alcohol drinking.

On the one hand, due to the indispensable importance of circadian rhythms for a holistic functional physiology, and on the other hand, due to the lack of new therapeutic interventions in recent years that directly target the circadian system, I think that our newly developed personalized circadian therapy is an extraordinary opportunity to bring cutting-edge research from clinically oriented neuroscience directly into medical practice. If the study proves successful in AUD patients, it should not be limited to them, but could be extended to other patient groups, which could bring the circadian clock a bit further (back) into everyday psychiatric and clinical psychotherapeutic practice.

In summary, during my PhD, I completed two studies in mice that underpinned the importance of circadian rhythms in anxiety disorders, stress, and alcohol consumption behavior. I further used this knowledge to develop a complementary therapy for patients with AUD. In addition, I have started and nearly finished projects that seek to provide causal explanations for the role of circadian rhythms in anxiety disorders in a mouse model, while in parallel I am testing the previously developed adjunctive therapy for AUD patients in a randomized controlled trial with, so far, promising results. When completed all together, these projects will reach from the bedside to the bench and back, representing a translational approach of mouse and human studies to the integration of chronobiology into therapy options, especially for patients with alcohol use disorder.

References

1. Reppert, S.M. and D.R. Weaver, *Coordination of circadian timing in mammals.* Nature, 2002. **418**: p. 935-941.
2. Hurd, M.W. and M.R. Ralph, *The significance of circadian organization for longevity in the golden hamster.* J Biol Rhythms, 1998. **13**(5): p. 430-6.
3. Pittendrigh, C.S. and D.H. Minis, *Circadian systems: longevity as a function of circadian resonance in Drosophila melanogaster.* Proc Natl Acad Sci U S A, 1972. **69**(6): p. 1537-9.
4. Ouyang, Y., et al., *Resonating circadian clocks enhance fitness in cyanobacteria.* Proc Natl Acad Sci U S A, 1998. **95**(15): p. 8660-4.
5. Vaze, K.M. and V.K. Sharma, *On the adaptive significance of circadian clocks for their owners.* Chronobiol Int, 2013. **30**(4): p. 413-33.
6. Sehgal, A., *Physiology Flies with Time.* Cell, 2017. **171**(6): p. 1232-1235.
7. Reppert, S.M. and D.R. Weaver, *Molecular analysis of mammalian circadian rhythms.* Annu Rev Physiol, 2001. **63**: p. 647-76.
8. Patke, A., M.W. Young, and S. Axelrod, *Molecular mechanisms and physiological importance of circadian rhythms.* Nat Rev Mol Cell Biol, 2020. **21**(2): p. 67-84.
9. Mure, L.S., et al., *Diurnal transcriptome atlas of a primate across major neural and peripheral tissues.* Science, 2018. **359**(6381).
10. Ralph, M.R., et al., *Transplanted suprachiasmatic nucleus determines circadian period.* Science, 1990. **247**(4945): p. 975-8.
11. Silver, R., et al., *A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms.* Nature, 1996. **382**(6594): p. 810-3.
12. Schibler, U., et al., *Clock-Talk: Interactions between Central and Peripheral Circadian Oscillators in Mammals.* Cold Spring Harb Symp Quant Biol, 2015. **80**: p. 223-32.
13. Merrow, M., K. Spoelstra, and T. Roenneberg, *The circadian cycle: daily rhythms from behaviour to genes.* EMBO Rep, 2005. **6**(10): p. 930-5.
14. Hühne, A., D.K. Welsh, and D. Landgraf, *Prospects for circadian treatment of mood disorders.* Annals of Medicine, 2018: p. 1-18.
15. Dibner, C., U. Schibler, and U. Albrecht, *The Mammalian Circadian Timing System: Organization and Coordination of Central and Peripheral Clocks.* Annual Review of Physiology, 2010. **72**: p. 517-549.
16. Stenvers, D.J., et al., *Nutrition and the circadian timing system.* Prog Brain Res, 2012. **199**: p. 359-376.
17. Begemann, K., A.M. Neumann, and H. Oster, *Regulation and function of extra-SCN circadian oscillators in the brain.* Acta Physiol (Oxf), 2020. **229**(1): p. e13446.
18. Walker, W.H., 2nd, et al., *Circadian rhythm disruption and mental health.* Transl Psychiatry, 2020. **10**(1): p. 28.
19. Hida, A., et al., *Screening of clock gene polymorphisms demonstrates association of a PER3 polymorphism with morningness-eveningness preference and circadian rhythm sleep disorder.* Sci Rep, 2014. **4**: p. 6309.
20. Bedrosian, T.A. and R.J. Nelson, *Timing of light exposure affects mood and brain circuits.* Translational Psychiatry, 2017. **7**: p. e1017.
21. Sack, R.L., et al., *Circadian rhythm sleep disorders: part I, basic principles, shift work and jet lag disorders. An American Academy of Sleep Medicine review.* Sleep, 2007. **30**(11): p. 1460-83.
22. Reid, K.J. and S.M. Abbott, *Jet Lag and Shift Work Disorder.* Sleep Med Clin, 2015. **10**(4): p. 523-35.
23. Choy, M. and R.L. Salbu, *Jet lag: current and potential therapies.* P T, 2011. **36**(4): p. 221-31.
24. Kiessling, S., G. Eichele, and H. Oster, *Adrenal glucocorticoids have a key role in circadian resynchronization in a mouse model of jet lag.* Journal of Clinical Investigation, 2010. **120**: p. 2600-2609.
25. Wittmann, M., et al., *Social jetlag: misalignment of biological and social time.* Chronobiol Int, 2006. **23**(1-2): p. 497-509.
26. Parsons, M.J., et al., *Social jetlag, obesity and metabolic disorder: investigation in a cohort study.* Int J Obes (Lond), 2015. **39**(5): p. 842-8.

27. Rutters, F., et al., *Is social jetlag associated with an adverse endocrine, behavioral, and cardiovascular risk profile?* J Biol Rhythms, 2014. **29**(5): p. 377-83.
28. Roenneberg, T. and M. Merrow, *The Circadian Clock and Human Health.* Curr Biol, 2016. **26**(10): p. R432-43.
29. Rajaratnam, S.M. and J. Arendt, *Health in a 24-h society.* Lancet, 2001. **358**(9286): p. 999-1005.
30. Huhne, A., D.K. Welsh, and D. Landgraf, *Prospects for circadian treatment of mood disorders.* Ann Med, 2018. **50**(8): p. 637-654.
31. Dulcis, D., et al., *Neurotransmitter switching in the adult brain regulates behavior.* Science, 2013. **340**: p. 449-453.
32. Fonken, L.K. and R.J. Nelson, *Dim light at night increases depressive-like responses in male C3H/HeNHsd mice.* Behav Brain Res, 2013. **243**: p. 74-8.
33. Castaneda, T.R., et al., *Circadian rhythms of dopamine, glutamate and GABA in the striatum and nucleus accumbens of the awake rat: modulation by light.* J Pineal Res, 2004. **36**(3): p. 177-85.
34. Ikeno, T. and L. Yan, *Chronic Light Exposure in the Middle of the Night Disturbs the Circadian System and Emotional Regulation.* J Biol Rhythms, 2016. **31**(4): p. 352-64.
35. Rahman, S.A., et al., *Characterizing the temporal Dynamics of Melatonin and Cortisol Changes in Response to Nocturnal Light Exposure.* Sci Rep, 2019. **9**(1): p. 19720.
36. Gonzalez, M.M.C. and G. Aston-Jones, *Light deprivation damages monoamine neurons and produces a depressive behavioral phenotype in rats.* Proceedings of the National Academy of Sciences, 2008. **105**: p. 4898-4903.
37. Haus, E. and M. Smolensky, *Biological clocks and shift work: circadian dysregulation and potential long-term effects.* Cancer Causes Control, 2006. **17**(4): p. 489-500.
38. Albrecht, U., *Timing to perfection: the biology of central and peripheral circadian clocks.* Neuron, 2012. **74**(2): p. 246-60.
39. Eckel-Mahan, K. and P. Sassone-Corsi, *Metabolism and the circadian clock converge.* Physiol Rev, 2013. **93**(1): p. 107-35.
40. Aton, S.J. and E.D. Herzog, *Come together, right...now: synchronization of rhythms in a mammalian circadian clock.* Neuron, 2005. **48**(4): p. 531-4.
41. Yamaguchi, S., et al., *Synchronization of cellular clocks in the suprachiasmatic nucleus.* Science, 2003. **302**(5649): p. 1408-12.
42. Nagano, M., et al., *An abrupt shift in the day/night cycle causes desynchrony in the mammalian circadian center.* J Neurosci, 2003. **23**(14): p. 6141-51.
43. Albus, H., et al., *A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock.* Curr Biol, 2005. **15**(10): p. 886-93.
44. Takahashi, J.S., et al., *The genetics of mammalian circadian order and disorder: implications for physiology and disease.* Nat Rev Genet, 2008. **9**(10): p. 764-75.
45. Dijk, D.J., et al., *Amplitude reduction and phase shifts of melatonin, cortisol and other circadian rhythms after a gradual advance of sleep and light exposure in humans.* PLoS One, 2012. **7**(2): p. e30037.
46. Crowley, S.J. and C.I. Eastman, *Phase advancing human circadian rhythms with morning bright light, afternoon melatonin, and gradually shifted sleep: can we reduce morning bright-light duration?* Sleep Med, 2015. **16**(2): p. 288-97.
47. Souêtre, E., et al., *Circadian rhythms in depression and recovery: Evidence for blunted amplitude as the main chronobiological abnormality.* Psychiatry Research, 1989. **28**: p. 263-278.
48. Linkowski, P., *Neuroendocrine profiles in mood disorders*, in *International Journal of Neuropsychopharmacology*. 2003. p. 191-197.
49. Li, J.Z., et al., *Circadian patterns of gene expression in the human brain and disruption in major depressive disorder.* Pnas, 2013. **110**: p. 9950-9955.
50. Wang, F., et al., *Meta-analysis on night shift work and risk of metabolic syndrome.* Obes Rev, 2014. **15**(9): p. 709-20.
51. Wang, F., et al., *A meta-analysis on dose-response relationship between night shift work and the risk of breast cancer.* Ann Oncol, 2013. **24**(11): p. 2724-32.
52. Abbott, S.M., K.J. Reid, and P.C. Zee, *Circadian Rhythm Sleep-Wake Disorders.* Psychiatr Clin North Am, 2015. **38**(4): p. 805-23.
53. Masri, S., K. Kinouchi, and P. Sassone-Corsi, *Circadian clocks, epigenetics, and cancer.* Curr Opin Oncol, 2015. **27**(1): p. 50-6.
54. Sotak, M., A. Sumova, and J. Pacha, *Cross-talk between the circadian clock and the cell cycle in cancer.* Ann Med, 2014. **46**(4): p. 221-32.

55. Dabke, K., G. Hendrick, and S. Devkota, *The gut microbiome and metabolic syndrome.* J Clin Invest, 2019. **129**(10): p. 4050-4057.
56. Depner, C.M., E.R. Stothard, and K.P. Wright, Jr., *Metabolic consequences of sleep and circadian disorders.* Curr Diab Rep, 2014. **14**(7): p. 507.
57. Bedrosian, T.A., L.K. Fonken, and R.J. Nelson, *Endocrine Effects of Circadian Disruption.* Annu Rev Physiol, 2016. **78**: p. 109-31.
58. Carter, B., et al., *The Molecular Clock and Neurodegenerative Disease: A Stressful Time.* Front Mol Biosci, 2021. **8**: p. 644747.
59. Oster, H., et al., *The Functional and Clinical Significance of the 24-Hour Rhythm of Circulating Glucocorticoids.* Endocr Rev, 2017. **38**(1): p. 3-45.
60. Otte, C., et al., *Major depressive disorder.* Nat Rev Dis Primers, 2016. **2**: p. 16065.
61. Belmaker, R.H. and G. Agam, *Major depressive disorder.* N Engl J Med, 2008. **358**(1): p. 55-68.
62. Hühne, A., E. Hoch, and D. Landgraf, *DAILY—A Personalized Circadian Zeitgeber Therapy as an Adjunctive Treatment for Alcohol Use Disorder Patients: Study Protocol for a Randomized Controlled Trial.* Frontiers in Psychiatry, 2021. **11**(1554).
63. Palagini, L., et al., *REM sleep dysregulation in depression: state of the art.* Sleep Med Rev, 2013. **17**(5): p. 377-90.
64. Rusting, C.L. and R.J. Larsen, *Diurnal patterns of unpleasant mood: associations with neuroticism, depression, and anxiety.* J Pers, 1998. **66**(1): p. 85-103.
65. Vadnie, C.A. and C.A. McClung, *Circadian Rhythm Disturbances in Mood Disorders: Insights into the Role of the Suprachiasmatic Nucleus.* Neural Plast, 2017. **2017**: p. 1504507.
66. Salgado-Delgado, R., et al., *Disruption of circadian rhythms: A crucial factor in the etiology of depression*, in *Depression Research and Treatment*. 2011.
67. Landgraf, D., et al., *Genetic Disruption of Circadian Rhythms in the Suprachiasmatic Nucleus Causes Helplessness, Behavioral Despair, and Anxiety-like Behavior in Mice.* Biological Psychiatry, 2016. **80**: p. 827-835.
68. Lee, A., et al., *Night Shift Work and Risk of Depression: Meta-analysis of Observational Studies.* J Korean Med Sci, 2017. **32**(7): p. 1091-1096.
69. Chellappa, S.L., C.J. Morris, and F. Scheer, *Circadian misalignment increases mood vulnerability in simulated shift work.* Sci Rep, 2020. **10**(1): p. 18614.
70. Baron, K.G. and K.J. Reid, *Circadian misalignment and health.* Int Rev Psychiatry, 2014. **26**(2): p. 139-54.
71. Melo, M.C.A., et al., *Chronotype and circadian rhythm in bipolar disorder: A systematic review.* Sleep Med Rev, 2017. **34**: p. 46-58.
72. Bellivier, F., et al., *Sleep- and circadian rhythm-associated pathways as therapeutic targets in bipolar disorder.* Expert Opin Ther Targets, 2015. **19**(6): p. 747-63.
73. McClung, C.A., et al., *Regulation of dopaminergic transmission and cocaine reward by the Clock gene.* Proceedings of the National Academy of Sciences, 2005. **102**: p. 9377-9381.
74. Sit, D.K., et al., *Adjunctive Bright Light Therapy for Bipolar Depression: A Randomized Double-Blind Placebo-Controlled Trial.* Am J Psychiatry, 2018. **175**(2): p. 131-139.
75. Zhou, T.H., et al., *Clinical efficacy, onset time and safety of bright light therapy in acute bipolar depression as an adjunctive therapy: A randomized controlled trial.* J Affect Disord, 2018. **227**: p. 90-96.
76. Sit, D. and S. Haigh, *Use of "Lights" for Bipolar Depression.* Curr Psychiatry Rep, 2019. **21**(6): p. 45.
77. Barbini, B., et al., *Dark therapy for mania: a pilot study.* Bipolar Disord, 2005. **7**(1): p. 98-101.
78. Henriksen, T.E., et al., *Blue-blocking glasses as additive treatment for mania: a randomized placebo-controlled trial.* Bipolar Disord, 2016. **18**(3): p. 221-32.
79. Crowe, M. and R. Porter, *Inpatient treatment for mania: A review and rationale for adjunctive interventions.* Aust N Z J Psychiatry, 2014. **48**(8): p. 716-21.
80. Difrancesco, S., et al., *Sleep, circadian rhythm, and physical activity patterns in depressive and anxiety disorders: A 2-week ambulatory assessment study.* Depress Anxiety, 2019. **36**(10): p. 975-986.
81. Geraci, M.F. and T.W. Uhde, *Diurnal rhythms and symptom severity in panic disorder. A preliminary study of 24-hour changes in panic attacks, generalised anxiety, and avoidance behaviour.* Br J Psychiatry, 1992. **161**: p. 512-6.
82. Cameron, O.G., et al., *Circadian symptom fluctuations in people with anxiety disorders.* J Affect Disord, 1986. **11**(3): p. 213-8.

83. Griesauer, I., et al., *Circadian abnormalities in a mouse model of high trait anxiety and depression.* Ann Med, 2014. **46**(3): p. 148-54.
84. Jakubcakova, V., et al., *Sleep phenotyping in a mouse model of extreme trait anxiety.* PLoS One, 2012. **7**(7): p. e40625.
85. Spencer, S., et al., *Circadian genes Period 1 and Period 2 in the nucleus accumbens regulate anxiety-related behavior.* European Journal of Neuroscience, 2013. **37**: p. 242-250.
86. Uhde, T.W., B.M. Cortese, and A. Vedeniapin, *Anxiety and sleep problems: emerging concepts and theoretical treatment implications.* Curr Psychiatry Rep, 2009. **11**(4): p. 269-76.
87. Sysoeva, Y.Y. and E.V. Verbitsky, *Characteristics of sleep-related arousals depend on trait anxiety.* Dokl Biol Sci, 2015. **461**: p. 73-5.
88. Chung, S., G.H. Son, and K. Kim, *Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications.* Biochim Biophys Acta, 2011. **1812**(5): p. 581-91.
89. Schubert, J.R. and M.E. Coles, *Obsessive-compulsive symptoms and characteristics in individuals with delayed sleep phase disorder.* J Nerv Ment Dis, 2013. **201**(10): p. 877-84.
90. Maher, M.J., S.A. Rego, and G.M. Asnis, *Sleep disturbances in patients with post-traumatic stress disorder: epidemiology, impact and approaches to management.* CNS Drugs, 2006. **20**(7): p. 567-90.
91. Agorastos, A. and M. Olff, *Traumatic stress and the circadian system: neurobiology, timing and treatment of posttraumatic chronodisruption.* Eur J Psychotraumatol, 2020. **11**(1): p. 1833644.
92. Landgraf, D., M.J. McCarthy, and D.K. Welsh, *Circadian clock and stress interactions in the molecular biology of psychiatric disorders.* Current psychiatry reports, 2014. **16**: p. 483.
93. Hühne, A., et al., *An in-depth neurobehavioral characterization shows anxiety-like traits, impaired habituation behavior, and restlessness in male Cryptochrome-deficient mice.* Genes Brain Behav, 2020. **19**(8): p. e12661.
94. Coles, M.E., J.R. Schubert, and J.A. Nota, *Sleep, Circadian Rhythms, and Anxious Traits.* Curr Psychiatry Rep, 2015. **17**(9): p. 73.
95. Waage, S., et al., *Predictors of shift work disorder among nurses: a longitudinal study.* Sleep Med, 2014. **15**(12): p. 1449-55.
96. Kalmbach, D.A., et al., *Shift work disorder, depression, and anxiety in the transition to rotating shifts: the role of sleep reactivity.* Sleep Med, 2015. **16**(12): p. 1532-8.
97. Eldevik, M.F., et al., *Insomnia, excessive sleepiness, excessive fatigue, anxiety, depression and shift work disorder in nurses having less than 11 hours in-between shifts.* PLoS One, 2013. **8**(8): p. e70882.
98. Bersani, G., et al., *Premorbid circadian profile of patients with major depression and panic disorder.* Riv Psichiatr, 2012. **47**(5): p. 407-12.
99. Armstrong, J.M., et al., *Associations of child insomnia, sleep movement, and their persistence with mental health symptoms in childhood and adolescence.* Sleep, 2014. **37**(5): p. 901-9.
100. Shanahan, L., et al., *Sleep problems predict and are predicted by generalized anxiety/depression and oppositional defiant disorder.* J Am Acad Child Adolesc Psychiatry, 2014. **53**(5): p. 550-8.
101. Gregory, A.M., et al., *Prospective longitudinal associations between persistent sleep problems in childhood and anxiety and depression disorders in adulthood.* J Abnorm Child Psychol, 2005. **33**(2): p. 157-63.
102. Jackson, M.L., et al., *Sleep difficulties and the development of depression and anxiety: a longitudinal study of young Australian women.* Arch Womens Ment Health, 2014. **17**(3): p. 189-98.
103. McLellan, A.T., *Substance Misuse and Substance use Disorders: Why do they Matter in Healthcare?* Trans Am Clin Climatol Assoc, 2017. **128**: p. 112-130.
104. Tamura, E.K., et al., *Circadian rhythms and substance use disorders: A bidirectional relationship.* Pharmacol Biochem Behav, 2021. **201**: p. 173105.
105. Wise, R.A., *The neurobiology of craving: implications for the understanding and treatment of addiction.* J Abnorm Psychol, 1988. **97**(2): p. 118-32.
106. Berridge, K.C., T.E. Robinson, and J.W. Aldridge, *Dissecting components of reward: 'liking', 'wanting', and learning*, in *Current Opinion in Pharmacology*. 2009. p. 65-73.
107. Robinson, T.E. and K.C. Berridge, *The neural basis of drug craving: an incentive-sensitization theory of addiction.* Brain Res Brain Res Rev, 1993. **18**(3): p. 247-91.
108. Goldstein, R.Z. and N.D. Volkow, *Dysfunction of the prefrontal cortex in addiction: neuroimaging findings and clinical implications.* Nat Rev Neurosci, 2011. **12**(11): p. 652-69.

109. Koob, G.F., *Hedonic Homeostatic Dysregulation as a Driver of Drug-Seeking Behavior.* Drug Discov Today Dis Models, 2008. **5**(4): p. 207-215.
110. Nguyen, D., E.E. Naffziger, and K.C. Berridge, *Positive Affect: Nature and brain bases of liking and wanting.* Curr Opin Behav Sci, 2021. **39**: p. 72-78.
111. Abarca, C., U. Albrecht, and R. Spanagel, *Cocaine sensitization and reward are under the influence of circadian genes and rhythm.* Proc Natl Acad Sci U S A, 2002. **99**(13): p. 9026-30.
112. Liu, Y., et al., *The role of mPer1 in morphine dependence in mice.* Neuroscience, 2005. **130**(2): p. 383-8.
113. Spanagel, R., et al., *The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption.* Nat Med, 2005. **11**(1): p. 35-42.
114. Nikaido, T., et al., *Sensitized increase of period gene expression in the mouse caudate/putamen caused by repeated injection of methamphetamine.* Mol Pharmacol, 2001. **59**(4): p. 894-900.
115. Perreau-Lenz, S. and R. Spanagel, *Clock genes x stress x reward interactions in alcohol and substance use disorders.* Alcohol, 2015. **49**(4): p. 351-7.
116. Hasler, B.P., et al., *Circadian misalignment and weekend alcohol use in late adolescent drinkers: preliminary evidence.* Chronobiol Int, 2019. **36**(6): p. 796-810.
117. Davis, B.T.t., et al., *Circadian Mechanisms in Alcohol Use Disorder and Tissue Injury.* Alcohol Clin Exp Res, 2018. **42**(4): p. 668-677.
118. McClung, C.A., *Circadian rhythms, the mesolimbic dopaminergic circuit, and drug addiction.* ScientificWorldJournal, 2007. **7**: p. 194-202.
119. Webb, I.C., *Circadian Rhythms and Substance Abuse: Chronobiological Considerations for the Treatment of Addiction.* Curr Psychiatry Rep, 2017. **19**(2): p. 12.
120. Danel, T., R. Jeanson, and Y. Touitou, *Temporal pattern in consumption of the first drink of the day in alcohol-dependent persons.* Chronobiol Int, 2003. **20**(6): p. 1093-102.
121. Hisler, G.C., et al., *Is there a 24-hour rhythm in alcohol craving and does it vary by sleep/circadian timing?* Chronobiol Int, 2021. **38**(1): p. 109-121.
122. in *Sleep Disorders and Sleep Deprivation: An Unmet Public Health Problem*, H.R. Colten and B.M. Altevogt, Editors. 2006: Washington (DC).
123. Teneggi, V., et al., *Smokers deprived of cigarettes for 72 h: effect of nicotine patches on craving and withdrawal.* Psychopharmacology (Berl), 2002. **164**(2): p. 177-87.
124. Dong, L., et al., *Effects of the circadian rhythm gene period 1 (Per1) on psychosocial stress-induced alcohol drinking.* American Journal of Psychiatry, 2011. **168**: p. 1090-1098.
125. Gulick, D. and J.J. Gamsby, *Racing the clock: The role of circadian rhythmicity in addiction across the lifespan.* Pharmacol Ther, 2018. **188**: p. 124-139.
126. Nascimento, N.F., et al., *Alcohol and lithium have opposing effects on the period and phase of the behavioral free-running activity rhythm.* Alcohol, 2015. **49**(4): p. 367-76.
127. Olsen, R.H., et al., *Impaired memory and reduced sensitivity to the circadian period lengthening effects of methamphetamine in mice selected for high methamphetamine consumption.* Behav Brain Res, 2013. **256**: p. 197-204.
128. Seggio, J.A., et al., *Chronic ethanol intake alters circadian phase shifting and free-running period in mice.* J Biol Rhythms, 2009. **24**(4): p. 304-12.
129. Brower, K.J., *Alcohol's effects on sleep in alcoholics.* Alcohol Res Health, 2001. **25**(2): p. 110-25.
130. Spanagel, R., et al., *Alcohol consumption and the body's biological clock.* Alcohol Clin Exp Res, 2005. **29**(8): p. 1550-7.
131. Danel, T., C. Libersa, and Y. Touitou, *The effect of alcohol consumption on the circadian control of human core body temperature is time dependent.* Am J Physiol Regul Integr Comp Physiol, 2001. **281**(1): p. R52-5.
132. Wasielewski, J.A. and F.A. Holloway, *Alcohol's interactions with circadian rhythms. A focus on body temperature.* Alcohol Res Health, 2001. **25**(2): p. 94-100.
133. Meyrel, M., B. Rolland, and P.A. Geoffroy, *Alterations in circadian rhythms following alcohol use: A systematic review.* Prog Neuropsychopharmacol Biol Psychiatry, 2020. **99**: p. 109831.
134. Huang, M.C., et al., *Reduced expression of circadian clock genes in male alcoholic patients.* Alcohol Clin Exp Res, 2010. **34**(11): p. 1899-904.
135. Hasler, B.P., et al., *Circadian rhythms, sleep, and substance abuse.* Sleep Med Rev, 2012. **16**(1): p. 67-81.
136. Danel, T., et al., *Inversion of melatonin circadian rhythm in chronic alcoholic patients during withdrawal: preliminary study on seven patients.* Alcohol Alcohol, 2009. **44**(1): p. 42-5.

137. Murialdo, G., et al., *Urine melatonin in alcoholic patients: a marker of alcohol abuse?* J Endocrinol Invest, 1991. **14**(6): p. 503-7.
138. Rogers, H.L. and S.M. Reilly, *A survey of the health experiences of international business travelers. Part One--Physiological aspects.* AAOHN J, 2002. **50**(10): p. 449-59.
139. Trinkoff, A.M. and C.L. Storr, *Work schedule characteristics and substance use in nurses.* Am J Ind Med, 1998. **34**(3): p. 266-71.
140. Blomeyer, D., et al., *Association of PER2 genotype and stressful life events with alcohol drinking in young adults.* PLoS One, 2013. **8**(3): p. e59136.
141. Wang, X., et al., *A promoter polymorphism in the Per3 gene is associated with alcohol and stress response.* Transl Psychiatry, 2012. **2**: p. e73.
142. Perreau-Lenz, S., et al., *Circadian regulation of central ethanol sensitivity by the mPer2 gene.* Addict Biol, 2009. **14**(3): p. 253-9.
143. Perreau-Lenz, S., et al., *Inhibition of the casein-kinase-1-epsilon/delta/ prevents relapse-like alcohol drinking.* Neuropsychopharmacology, 2012. **37**(9): p. 2121-31.
144. Morikawa, Y., et al., *Correlation between shift-work-related sleep problems and heavy drinking in Japanese male factory workers.* Alcohol Alcohol, 2013. **48**(2): p. 202-6.
145. Rosenwasser, A.M., *Alcohol, antidepressants, and circadian rhythms. Human and animal models.* Alcohol Res Health, 2001. **25**(2): p. 126-35.
146. Swanson, G.R., et al., *Night workers with circadian misalignment are susceptible to alcohol-induced intestinal hyperpermeability with social drinking.* Am J Physiol Gastrointest Liver Physiol, 2016. **311**(1): p. G192-201.
147. Pail, G., et al., *Bright-light therapy in the treatment of mood disorders.* Neuropsychobiology, 2011. **64**(3): p. 152-62.
148. Golden, R.N., et al., *The efficacy of light therapy in the treatment of mood disorders: a review and meta-analysis of the evidence.* Am J Psychiatry, 2005. **162**(4): p. 656-62.
149. Lieverse, R., et al., *Bright light treatment in elderly patients with nonseasonal major depressive disorder: a randomized placebo-controlled trial.* Arch Gen Psychiatry, 2011. **68**(1): p. 61-70.
150. Campbell, P.D., A.M. Miller, and M.E. Woesner, *Bright Light Therapy: Seasonal Affective Disorder and Beyond.* Einstein J Biol Med, 2017. **32**: p. E13-E25.
151. Aumann, T.D., et al., *Differences in Number of Midbrain Dopamine Neurons Associated with Summer and Winter Photoperiods in Humans.* PLoS One, 2016. **11**(7): p. e0158847.
152. Takeshima, M., et al., *Inpatient phase-advance therapy for delayed sleep-wake phase disorder: a retrospective study.* Nat Sci Sleep, 2018. **10**: p. 327-333.
153. Drennan, M.D., et al., *The effects of depression and age on the Horne-Ostberg morningness-eveningness score.* J Affect Disord, 1991. **23**(2): p. 93-8.
154. Ohta, H., S. Yamazaki, and D.G. McMahon, *Constant light desynchronizes mammalian clock neurons.* Nat Neurosci, 2005. **8**(3): p. 267-9.
155. Berger, M., D. van Calker, and D. Riemann, *Sleep and manipulations of the sleep-wake rhythm in depression.* Acta Psychiatr Scand Suppl, 2003(418): p. 83-91.
156. Bano-Otalora, B., et al., *Bright daytime light enhances circadian amplitude in a diurnal mammal.* Proc Natl Acad Sci U S A, 2021. **118**(22).
157. Terman, J.S., et al., *Circadian time of morning light administration and therapeutic response in winter depression.* Arch Gen Psychiatry, 2001. **58**(1): p. 69-75.
158. Leproult, R., et al., *Phase-shifts of 24-h rhythms of hormonal release and body temperature following early evening administration of the melatonin agonist agomelatine in healthy older men.* Clin Endocrinol (Oxf), 2005. **63**(3): p. 298-304.
159. Mairesse, J., et al., *Chronic agomelatine treatment corrects the abnormalities in the circadian rhythm of motor activity and sleep/wake cycle induced by prenatal restraint stress in adult rats.* Int J Neuropsychopharmacol, 2013. **16**(2): p. 323-38.
160. Krauchi, K., et al., *Early evening melatonin and S-20098 advance circadian phase and nocturnal regulation of core body temperature.* Am J Physiol, 1997. **272**(4 Pt 2): p. R1178-88.
161. Robillard, R., et al., *Parallel Changes in Mood and Melatonin Rhythm Following an Adjunctive Multimodal Chronobiological Intervention With Agomelatine in People With Depression: A Proof of Concept Open Label Study.* Front Psychiatry, 2018. **9**: p. 624.
162. Moller, M. and F.M. Baeres, *The anatomy and innervation of the mammalian pineal gland.* Cell Tissue Res, 2002. **309**(1): p. 139-50.
163. Hardeland, R., et al., *Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling.* J Pineal Res, 2012. **52**(2): p. 139-66.

164. Liira, J., et al., *Pharmacological interventions for sleepiness and sleep disturbances caused by shift work.* Cochrane Database Syst Rev, 2014(8): p. CD009776.
165. Leonardo-Mendonca, R.C., et al., *The benefits of four weeks of melatonin treatment on circadian patterns in resistance-trained athletes.* Chronobiol Int, 2015. **32**(8): p. 1125-34.
166. Geoffroy, P.A., et al., *The use of melatonin in adult psychiatric disorders: Expert recommendations by the French institute of medical research on sleep (SFRMS).* Encephale, 2019. **45**(5): p. 413-423.
167. Parsons, C.E., et al., *How does cognitive behavioural therapy for insomnia work? A systematic review and meta-analysis of mediators of change.* Clin Psychol Rev, 2021. **86**: p. 102027.
168. Jansson-Frojmark, M. and A. Norell-Clarke, *Cognitive Behavioural Therapy for Insomnia in Psychiatric Disorders.* Curr Sleep Med Rep, 2016. **2**(4): p. 233-240.
169. Smith, M.T. and M.L. Perlis, *Who is a candidate for cognitive-behavioral therapy for insomnia?* Health Psychol, 2006. **25**(1): p. 15-9.
170. Frank, E., H.A. Swartz, and D.J. Kupfer, *Interpersonal and social rhythm therapy: managing the chaos of bipolar disorder.* Biol Psychiatry, 2000. **48**(6): p. 593-604.
171. Frank, E., et al., *Two-year outcomes for interpersonal and social rhythm therapy in individuals with bipolar I disorder.* Archives of General Psychiatry, 2005. **62**: p. 996-1004.
172. Frank, E., H.A. Swartz, and E. Boland, *Interpersonal and social rhythm therapy: an intervention addressing rhythm dysregulation in bipolar disorder.* Dialogues Clin Neurosci, 2007. **9**(3): p. 325-32.
173. Steardo, L., Jr., et al., *Efficacy of the interpersonal and social rhythm therapy (IPSRT) in patients with bipolar disorder: results from a real-world, controlled trial.* Ann Gen Psychiatry, 2020. **19**: p. 15.
174. Goldstein, T.R., et al., *Early intervention for adolescents at-risk for bipolar disorder: A pilot randomized trial of Interpersonal and Social Rhythm Therapy (IPSRT).* J Affect Disord, 2018. **235**: p. 348-356.
175. Geoffroy, P.A. and L. Palagini, *Biological rhythms and chronotherapeutics in depression.* Prog Neuropsychopharmacol Biol Psychiatry, 2021. **106**: p. 110158.
176. Haynes, P.L., et al., *Cognitive Behavioral Social Rhythm Group Therapy for Veterans with posttraumatic stress disorder, depression, and sleep disturbance: Results from an open trial.* J Affect Disord, 2016. **192**: p. 234-43.
177. Dunker Svendsen, S., et al., *Circadian reinforcement therapy in combination with electronic self-monitoring to facilitate a safe post-discharge period of patients with depression by stabilizing sleep: protocol of a randomized controlled trial.* BMC Psychiatry, 2019. **19**(1): p. 124.
178. Lamia, K.A., et al., *Cryptochromes mediate rhythmic repression of the glucocorticoid receptor.* Nature, 2011. **480**(7378): p. 552-6.
179. Naughton, M., T.G. Dinan, and L.V. Scott, *Corticotropin-releasing hormone and the hypothalamic-pituitary-adrenal axis in psychiatric disease.* Handb Clin Neurol, 2014. **124**: p. 69-91.
180. Hori, H., et al., *Relationships between psychological distress, coping styles, and HPA axis reactivity in healthy adults.* J Psychiatr Res, 2010. **44**(14): p. 865-73.
181. Ceruso, A., et al., *Alterations of the HPA Axis Observed in Patients with Major Depressive Disorder and Their Relation to Early Life Stress: A Systematic Review.* Neuropsychobiology, 2020. **79**(6): p. 417-427.
182. Huhne, A., et al., *An in-depth neurobehavioral characterization shows anxiety-like traits, impaired habituation behavior, and restlessness in male Cryptochrome-deficient mice.* Genes Brain Behav, 2020. **19**(8): p. e12661.
183. Cofresi, R.U., B.D. Bartholow, and T.M. Piasecki, *Evidence for incentive salience sensitization as a pathway to alcohol use disorder.* Neurosci Biobehav Rev, 2019. **107**: p. 897-926.
184. Robinson, T.E. and K.C. Berridge, *Review. The incentive sensitization theory of addiction: some current issues.* Philos Trans R Soc Lond B Biol Sci, 2008. **363**(1507): p. 3137-46.
185. Gauvin, D.V., et al., *Effects of time-of-day and photoperiod phase shifts on voluntary ethanol consumption in rats.* Alcohol Clin Exp Res, 1997. **21**(5): p. 817-25.
186. Resendiz-Flores, M. and C. Escobar, *Circadian disruption favors alcohol consumption and differential DeltaFosB accumulation in Corticolimbic structures.* Addict Biol, 2019. **24**(6): p. 1179-1190.
187. Rosenwasser, A.M. and M.C. Fixaris, *Chronobiology of alcohol: studies in C57BL/6J and DBA/2J inbred mice.* Physiol Behav, 2013. **110-111**: p. 140-7.
188. Stephens, M.A. and G. Wand, *Stress and the HPA axis: role of glucocorticoids in alcohol dependence.* Alcohol Res, 2012. **34**(4): p. 468-83.

189. Janak, P.H. and K.M. Tye, *From circuits to behaviour in the amygdala.* Nature, 2015. **517**(7534): p. 284-92.
190. Rosenwasser, A.M., *Chronobiology of ethanol: animal models.* Alcohol, 2015. **49**(4): p. 311-9.
191. Castro, D.C. and K.C. Berridge, *Advances in the neurobiological bases for food 'liking' versus 'wanting'.* Physiology and Behavior, 2014. **136**: p. 22-30.
192. Anderson, R.I., et al., *Orexin-1 and orexin-2 receptor antagonists reduce ethanol self-administration in high-drinking rodent models.* Front Neurosci, 2014. **8**: p. 33.
193. Moorman, D.E., *The hypocretin/orexin system as a target for excessive motivation in alcohol use disorders.* Psychopharmacology (Berl), 2018. **235**(6): p. 1663-1680.
194. Hopf, F.W., *Recent perspectives on orexin/hypocretin promotion of addiction-related behaviors.* Neuropharmacology, 2020. **168**: p. 108013.

Appendix Paper III

frontiers in Psychiatry

STUDY PROTOCOL
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DAILY—A Personalized Circadian *Zeitgeber* Therapy as an Adjunctive Treatment for Alcohol Use Disorder Patients: Study Protocol for a Randomized Controlled Trial

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Background: Hallmarks of alcohol use disorder (AUD) are disturbances of circadian rhythms and everyday structures. While circadian rhythms dictate the timing of daily recurring activities such as sleep, activity, and meals, conversely, these activities represent time cues, so called *Zeitgebers*, that the circadian system uses to synchronize with the environment. Here we present a study protocol for our newly developed therapy approach for AUD patients, in which we take advantage of this mutual influence and stabilize and strengthen their circadian system by creating strict daily schedules for daily *Zeitgeber* activities. Since every person has a circadian system with its own characteristics and is subject to social obligations, the daily plans are personalized for each test person. Our hypothesis is that a regular exposure to *Zeitgebers* stabilizes behavioral and physiological circadian rhythms and thereby reduces the risk of alcohol relapses and depressive symptoms and facilitates physical recovery in AUD patients during the 1st weeks of their addiction therapy.

Methods/design: The study is a 6-weeks single site trial with a controlled, randomized, single-blinded, parallel-group design including patients with a diagnosis of AUD. The study runs parallel to the standard addiction therapy of the clinic. Patients are randomly assigned to either an intervention group (DAILY) or a sham control group (placebo treatment). Questionnaires and physiological assessments of both groups are conducted before and immediately after the intervention or control treatment. According to our hypothesis, the primary outcomes of this study are improvements of regularity, alcohol consumption, and relapse rate in AUD patients compared to AUD patients receiving control treatment. Secondary outcomes are reduced depressive symptoms and increased physical recovery.

Discussion: This study is a randomized controlled trial to investigate the efficacy of a personalized circadian *Zeitgeber* therapy as an adjunctive treatment for alcohol use disorder patients. The overall goal of this and more extended future studies is the

development of an adjunctive therapy for AUD patients that is uncomplicated in its use and easy to implement in the clinical and everyday routine.

Trial registration: This study is registered at the German Clinical Trial Register with the trial number DRKS00019093 on November 28, 2019.

Keywords: circadian, alcohol use disorder (AUD), *Zeitgeber*, daily structure, addiction, chronotherapy, depression, personalized therapeutic approach

BACKGROUND

Harmful Use of Alcohol and Alcohol Use Disorder

Harmful use of alcohol and alcohol use disorder (AUD) lead to increased morbidity and mortality. According to the World Health Organization (WHO), 5.1% of all disorders, diseases, injuries, and 5.3% of all deaths are attributable to alcohol consumption (1). Typical disorders and diseases associated with harmful alcohol consumption and AUD include cardiovascular, infectious, neurological diseases, cancer, and psychological disorders such as depression and anxiety (2, 3). Impressively, as a result, mortality due to alcohol consumption is higher than for other serious diseases such as HIV or tuberculosis (1). In addition to the adverse health effects of those concerned, this also means that, at the societal level, harmful alcohol consumption behavior creates a significant burden on the economy and health systems (4, 5).

The Circadian System

Endogenous circadian clocks (from Latin *circa diem*, about a day) have evolved to prepare organisms to daily recurring environmental changes caused by the rotation of the earth around its axis. Circadian clocks are found in almost all cells of the body and since they regulate the expression of about 50% of our genes (6), there is almost no process in the body that is not under their influence. In humans, the master circadian clock is located in the suprachiasmatic nucleus (SCN) in the anterior part of the hypothalamus (7–9). The SCN acts like a conductor of an orchestra and sets all other cellular clocks in the rest of the body to an appropriate period and phase (10). Synchronization of endogenous circadian rhythms with environmental 24-h cycles is achieved through so called *Zeitgebers* (German: time givers) (11). The strongest stimuli that serve as *Zeitgebers* are light and food, whereby light acts directly on the SCN. In a simplified model, the SCN, in turn, determines sleep and wake times and when hunger arises. The ingested food then entrains peripheral clocks. Physical activity (exercise) and social contact are also regarded as *Zeitgebers*, but weaker ones (12). In an optimal situation, molecular, physiological, and behavioral rhythms are synchronized among each other and with the environment (13). Since the circadian clock is involved in virtually all bodily processes, their perturbation is strongly associated with a high number of disorders, including mental illnesses such as AUD or major depressive disorder (MDD) (13, 14).

Association of Circadian Clocks and Alcoholism

In recent years evidence emerged that there is a bidirectional relationship between circadian rhythms and alcohol consumption (15). AUD patients often suffer from circadian misalignment, which, in turn, is known to increase the risk of developing AUD (15–17).

On the one hand, individuals with AUD almost always suffer from disorders of the circadian system, such as disturbed sleep times, impaired sleep quality, altered molecular rhythms, and altered daily rhythms of neuroendocrinological functions in different regions of the brain (18). Even acute alcohol drinking can cause spontaneous alterations in circadian rhythms (19), and circadian rhythms are also affected during alcohol withdrawal (20, 21). Importantly, alcohol-dependent patients have a higher risk of relapse during withdrawal if their internal clock is disturbed (18). On the other hand, disrupted circadian rhythms are considered a strong risk factor for the development of AUD, as disturbances of circadian sleep-wake cycles are frequently observed in subjects with a late chronotype and shift workers show an increased prevalence of harmful use of alcohol and AUD (19). Moreover, single nucleotide polymorphisms in the clock genes Clock, Per2, and Per3 genes have been associated with alcohol consumption behavior (22, 23).

Curiously, although AUD patients generally suffer disturbances of molecular and behavioral rhythms, their need for alcohol often follows circadian patterns with around 80% of alcohol addicts having their first drink at a specific hour with low variability (17, 24–27). Accordingly, in rodents, the regular administration of drugs can trigger anticipatory locomotor activity before the drug is actually provided, which suggests the existence of a drug-entrainable oscillator (24).

In summary, AUD patients have disturbed circadian rhythms in daily sleep and neuroendocrinological rhythms on the one hand, but on the other hand are strongly entrained by alcohol. Together, these data highly suggest an imbalance of *Zeitgeber* signals in AUD patients. While light, food, and physical activity are usually the strongest *Zeitgebers*, in AUD patients these may lose influence and be replaced by alcohol. Thus, a targeted amplification of the other *Zeitgebers* can possibly reduce the influence of the *Zeitgeber* alcohol and thereby reduce symptoms of AUD patients during withdrawal and help to reduce relapses.

Biological Mechanisms Targeted With a Circadian *Zeitgeber* Therapy in AUD Patients

In recent years, there has been growing evidence that circadian rhythms modulate reward circuits and behavior (28–31). For instance, the activation of reward processing is subject to circadian control (32), the release of neurotransmitters, such as dopamine and serotonin, varies over the course of the day, and dopamine signaling in the SCN has strong impact on its rhythms and processing responses to hedonic stimuli (33, 34). Thus, in animals and humans, depending on the time of day, rewards are valued differently (35) with the consequence that sensitization and motivation toward rewarding stimuli change over the course of 24 h (32, 36–38). Moreover, in humans, under constant conditions, it was shown that rhythms of the diurnal positive affect parallels rhythms of core body temperature, further suggesting endogenous circadian control of reward motivation (39).

Because of the close connection to the reward system, it is not surprising that circadian clocks have also been associated with addiction and alcohol use (16, 40, 41). Circadian misalignment in humans can be proven and quantified by the discrepancy between the phase angles of the dim light melatonin onset (DLMO) and the sleep offset. Interestingly, the more pronounced the circadian misalignment is, the greater the severity of substance abuse and dependence (42). It is also remarkable that melatonin levels, which are usually high at night, are inverted in AUD patients under the influence of alcohol and during acute withdrawal, which further indicates severe circadian disturbances in AUD patients (43). Moreover, circadian misalignment due to a late chronotype is also associated with increased impulsivity, which is a risk factor for the development of AUD (44, 45). Furthermore, it was shown that at certain times of the day, craving is particularly pronounced, since our reward system follows a circadian pattern (27).

In rodents, drug reward displays daily rhythms (46). These rhythms are associated with oscillating expression of the dopamine transporter and are directly related to clock gene expression in the medial prefrontal cortex and mesolimbic dopaminergic system (47). Mice exposed to chronic circadian disruption display reduced neuronal complexity in prelimbic prefrontal brain areas and reduced behavioral inhibition, which again is associated with increased alcohol consumption (48).

The above described studies indicate the existence of a neurobiological circadian-reward system, which in case of disruption can contribute to the development and maintenance of addiction disorders (40). Therefore, our therapeutic approach aims to synchronize circadian rhythms of the entire body among themselves and with the environment by a very regular exposure to *Zeitgebers* (light, dark, food, and if applicable activity). In a stable light-dark cycle, the SCN entrains robustly to it, and can reliably transmit these time signals to the rest of the body. Additionally, food is a very potent *Zeitgeber*, and regular mealtimes can entrain clocks of most peripheral organs as well as many brain oscillators (49, 50). Among those are brain areas that are involved in motivational responses to food and the SCN (51, 52). Consequently, strictly timed food has already been suggested as chronotherapeutic approach to improve circadian disruptions in populations such as shift workers (53, 54).

Circadian *Zeitgeber* Therapy for AUD Patients in the Transition From Inpatient to Outpatient Care

For AUD patients, the transition phase between the inpatient stay and discharge from the clinic to outpatient care or home often represents a stressful and thus susceptible time for alcohol relapses and a worsening of the general psychiatric health situation (55). For patients the sudden change from the clinical environment back to autonomous self-organized daily routines is often very difficult (56). During this transition phase, daily rhythms such as sleep-wake cycles and mealtimes become irregular and often drift into a later phase, which has been associated with a worsening of psychiatric symptoms, including alcohol relapses and depression (57, 58).

Within the framework of psychotherapy the suggestion of structuring the everyday life in an orderly manner to psychiatric patients, especially AUD patients is not new (59). This therapy component of self-management has positive psychological effects such as reducing stressful situations and enhancing the feeling of self-efficacy. However, we have designed a study that, in addition to previous therapy programs, has a special focus on the transition time between inpatient and outpatient care or home, and which focuses on the targeted use of strong *Zeitgebers* to restore and stabilize physiological circadian rhythms in AUD patients. Similar circadian reinforcement therapies have been proven for bipolar disorder (60, 61) and are currently tested in a study for MDD (62). However, the development and systematic investigation of a circadian structure therapy for AUD patients has not yet been published and was recently highlighted as a necessity (63). Thus, with DAILY we develop a new treatment approach for AUD patients, partly based on established concepts of chronotherapies for other mental disorders and implement it alongside standard AUD therapies.

We assume that subjects in our study benefit from the reduction of physical conditions that promote and maintain alcohol consumption behavior. We further believe that the strengthening of circadian rhythms has not only positive effects on alcohol craving, but also on often accompanying symptoms of AUD, such as depressive mood. Therefore, we have called this newly proposed intervention for AUD patients DAILY, which stands for **D**epression **A**lcohol **Ill**ness Therap**y**.

STUDY HYPOTHESES AND OBJECTIVES

Our primary hypothesis is that a regular exposure to *Zeitgebers* stabilizes circadian behavioral rhythms and thereby reduces alcohol consumption and the risk of alcohol relapses in AUD patients. Accordingly, the primary objectives of the study are to determine whether the intervention program DAILY decreases day-to-day variations in behavior and reduces alcohol consumption and the relapse rate in AUD patients compared to those receiving control treatment. Furthermore, we

hypothesize that the regular exposure to *Zeitgebers* contributes to the reduction of depressive symptoms and a subjective and physiological well-being in AUD patients, which in turn positively affects the primary objectives of the study. Accordingly, the secondary objective of the study is to ascertain whether the DAILY program supports the surrogate criterions of increased sleep quality, reduced depressive symptoms, increased self-efficacy expectations, and improved blood-values that are associated with harmful alcohol consumption. We also hypothesize, that additional psychoeducation about biological mechanisms in the development of AUD helps participants to complete their current therapy and maintain compliance. Therefore, as exploratory outcomes, we will investigate whether more participants of the intervention group accomplish the DAILY therapy than participants of the control group. Finally, we will assess whether the DAILY intervention has impact on the chronotype of the participants, although it is not a specific aim of our treatment to change it.

STUDY DESIGN

Design

The study is a 6-weeks trial with a controlled, randomized, single-blinded, parallel-group design including patients with a diagnosis of AUD. Patients are randomly assigned to either an intervention group (DAILY) or a sham control group (placebo treatment). As this study represents a pilot randomized trial, which does not primarily focus on proving treatment successes but more on ascertaining the best options for the future main trial, we will include each eligible patient within this year instead of using formal power calculations (64).

Setting

DAILY is a single site study conducted at the specialist ward for addiction disorders and the outpatient clinic for substance use disorder of the Clinic for Psychiatry and Psychotherapy at the University Hospital of Munich. There are 24 patients with a minimum age of 18 years on the ward, with men and women admitted equally. The day clinic treats 15 patients with a minimum age of 18 years simultaneously. On the ward and in the day clinic there are fixed, individually determined schedules for therapy sessions from which patients are not allowed to deviate. Meals are offered by the clinic at fixed times of the day, but do not have to be taken. Patients are free to leave the ward and the day clinic between therapy sessions in order to take meals outside or to pursue other activities such as walks or sports. The ward is open in principle, but care is taken to ensure that patients do not leave it between 10 pm and 8 am. There are no fixed sleeping hours on the ward, except that patients must arrive on time for their therapy sessions, which usually start between 08:30 and 11:00 am. On the weekend, patients of the ward have no regular appointments. Patients of the Day Clinic leave the clinic in the afternoon and arrange the rest of the day and their weekends independently. In addition, food can be brought to the ward and the day clinic and snacks can be eaten at any time. Both clinics are interdisciplinary staffed by medical doctors, psychologists, nurses, and social workers, all of whom have broad experience in the treatment of substance use disorders, including AUD. About half of the patients on both sites are diagnosed with AUD, the other half suffers from multidrug dependency. On the ward a qualitative detoxification program with psychotherapeutic services is offered to AUD patients and patients with multidrug use. In the day clinic the same psychotherapeutic services are offered as on the ward, but the patients are already detoxified, and the therapy takes place on a day-care basis.

Standard Therapeutic Services on the Ward and the Day Clinic

In parallel to the DAILY or the sham treatment, all patients receive the standard clinical treatment consisting of medical and psychological consultation, individual and group therapy (combining Motivational Enhancement Therapy and Cognitive Behavioral Therapy), and medication if necessary. The psychotherapeutic treatment of AUD and multiple substance use patients is the same but is conducted separately. The standard psychotherapy concept of the ward and the day clinic includes motivation work in which the incentive to abstinence and the decision to change consumption behavior is increased. In addition, situation analyses, the identification of risk factors, and the development of alternative actions are used to work with patients on relapse prevention. In addition, all patients receive psychoeducational therapy sessions to provide theoretical knowledge about the definition of addiction and its development on a neurobiological level. The treatment of AUD patients on the ward is scheduled for 14 days. In the day clinic, the treatment time ranges from 2 to 4 weeks and the concepts described above are elaborated in more detail and sociotherapy is also offered. In none of these standard therapies the advantages of daytime structures and regular sleep times are discussed.

Study Population

Men and women, unemployed and employed subjects are equally included in the study. Depending on whether the subjects are patients of the ward or day clinic, they are in detoxification or post-detoxification treatment. Study enrollment of subjects who are newly admitted to the detoxification program of the ward will take place at the earliest after 5 days of detoxification, to exclude cognitive and physical limitations caused by acute intoxication and its after-effects. Study participants who are discharged from the ward either go home, receive further treatment in the day clinic, or go to other institutions for long-term therapy. Study participants who are discharged from the day clinic either go home after discharge or go to other institutions for further treatment. The health conditions of all test persons allow them to perform their daily tasks without external assistance. Approximately half of the study subjects have a regular job, while the others are unemployed or retired. Usually 3–5 subjects are involved in the study simultaneously.

Eligibility Criteria

All persons are considered eligible on whom the below exclusion criteria do not apply. Subjects are only included in the study if they can assess the nature and scope of the investigations. Written consent is required for inclusion. The study is conducted

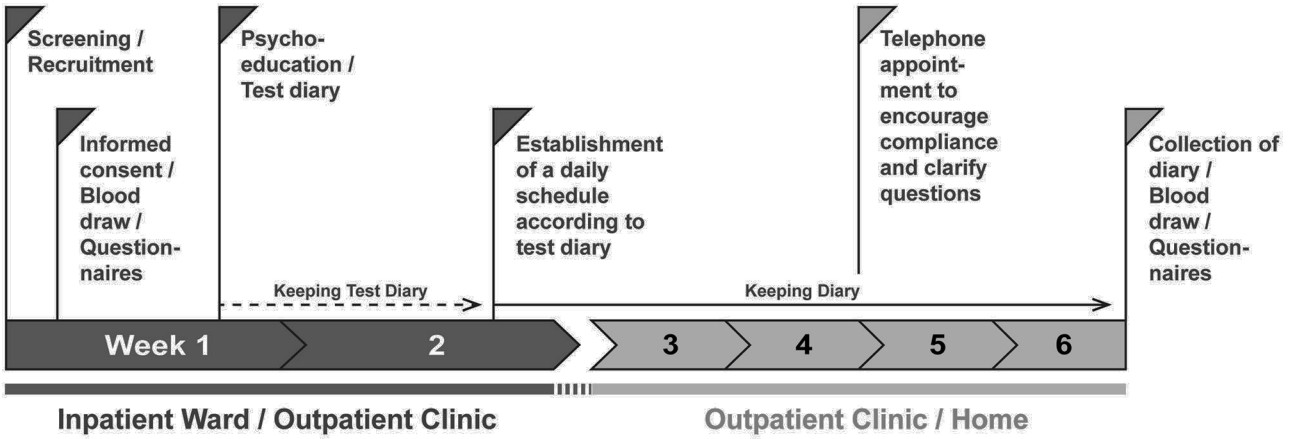

FIGURE 1 | Procedure of the DAILY study. Patients are screened and recruited by a member of the study staff. After 1 day at the earliest the participants sign the consent form. On this day, they are informed about the contents of the study; the contents discussed differ between intervention and control group. In addition, questionnaires are filled out and blood is drawn. During the week, a psychoeducation session takes place in which the contents and the aims of the study are deepened, further questionnaires (**Table 1**) are filled in, and a test diary is handed out to record times of daily actions which act as *Zeitgebers*. After at least 1 week, the diary is evaluated and a daily structure plan for regular exposure to *Zeitgebers* is worked out together with the test persons, to which they should adhere for the next 4 weeks. Subjects in the control group are not given instructions on how to structure their daily routine but are still asked to complete the diary. At least one telephone appointment is made for the time the subjects fill out the diaries to maintain compliance. After 4 weeks, there will be a final personal appointment where the diaries will be collected, all questionnaires will be repeated, and blood will be drawn again.

in accordance with the principles of the Declaration of Helsinki with its amendments of Tokyo, 1975, Hong Kong, 1989, Somerset West, 1996, Seoul, 2008, and Fortaleza, 2013. The subjects must fulfill diagnostic criteria for AUD according to ICD-10 F10.2 and be of age 18–75 years. Smokers are eligible for this study. In case of dependency on other substances, the subjects must have been abstinent from these substances for at least 12 months at the time of study enrollment. One outcome of this study is the change in depressive symptoms in the subjects. For this reason, AUD patients with concomitant diagnosed MDD as well as substance-induced depression are included. However, a diagnosed depression is not a prerequisite for inclusion in the study.

Exclusion Criteria

Excluded are subjects who are unable to give their consent, are pregnant, or are currently working in shifts. In addition, intellectual, neurological, or physical impairment that lead to the inability to independently perform tasks of daily life lead to exclusion of the study. In general, dependence on help with eating or going to bed (e.g., bedridden patients) precludes participation in the study. However, patients with disorders like liver damage, tremors, digestive problems, and blood abnormalities, which are common in AUD patients but do not lead to constant dependence on support from others, are not excluded. Blind subjects and patients with diagnosed mental illnesses other than AUD and MDD are generally excluded from the study. Patients with a dependence on other substances (except alcohol and nicotine) which they have consumed within the last 12 months are also not ineligible. The use of benzodiazepines, agomelatine, or medically prescribed cannabinoids will result in exclusion from the study, the use of other medications will not affect eligibility. Although patients with depressive symptoms are generally eligible for the study, no psychotic symptoms or acute suicidal ideation should be present. Subjects must not participate in other studies that may interfere therapeutically with the DAILY study.

STUDY PROCEDURE

The study procedure for the study participants is shown in **Figure 1**. The basic procedure is the same for participants of the intervention and control group.

Recruitment

In order to facilitate recruitment, a logo was designed for the DAILY study, which has a high recognition value for the test persons and underlines the official character of the study (**Figure 2**). After screening for eligibility, participants are recruited during their clinical stay at either the specialist ward or the outpatient clinic for addiction disorders. A member of the study staff addresses the candidates personally and finds out whether there is in principle interest in participating in a concomitant therapy study for AUD patients. Candidates are given at least 1 day to consider. In the event of positive feedback, they are allocated to either the intervention or control group.

Allocation

Participants are allocated to either the intervention or control group by a computer-generated randomization list. Each test person is assigned an individual study code, which contains information about the current ward and gender, and otherwise consists of a sequential number. Personal data that could reveal identity and participation in the intervention or control group are not part of the code. The decoding of the code is only allowed

FIGURE 2 | Logo of the DAILY therapy study. The logo represents a clock in which the letters act as hands. The clock is set at a full hour, which should underline the desired regularity in everyday life. The color green is synonymous with life and health.

for study staff. Until the end of the study, the participants will not be informed about their assignment to the intervention or control group. However, after the end of the study, participants will be informed about their group affiliation and participants of the control group will be given the opportunity to learn about the circadian system and the importance of a structured daily routine.

Informed Consent and Baseline Assessments

To participate in the study, all participants need to sign an informed consent in the 1st week (**Figure 1**). Afterwards, irrespective of the group allocation, a semi-structured interview is conducted to assess demographical data: age, gender, employment status, occupation, and marital status. Medical data are evaluated as well: size, bodyweight, blood pressure, current medications, and information about previous alcohol problems and possible depressive episodes. In addition, the questionnaires described below (**Table 1**) are filled out together with the participants. Lastly, blood samples are collected. This appointment is also used to prepare the test persons for the topics and contents of their respective groups.

The further course of the study and the contents of the following appointments are described separately for the intervention group and the control group.

Intervention Group

Week 1: Psychoeducation and Test Diary

After baseline assessments, a few days later in the same week, the test persons participate in individual 1-h psychoeducation sessions about circadian clocks and their effects on health and psychiatric disorders, such as addiction and depression. The scientific content is communicated in simple language. Firstly, the participants get explained that circadian rhythms can be found virtually everywhere in nature and that they have impact on essentially all physiological processes, including sleep-wake cycles, hunger, digestion, wound healing, cellular functions, and molecular processes. A focus is on their involvement in neurobiological systems, such as reward, addiction networks, and behaviors. During the session, the test subjects are led to understand how important regular daily routines are for mental and physical health. Therefore, the study assistant emphasizes the importance of certain environmental cues that can serve as *Zeitgebers,* such as light and food. Together with the participants, the impact of light on sleep is discussed and how light can be avoided at late hours (e.g., by stopping the use of mobile phones, watching TV, or playing computer games after sunset or by using blue-light filters). Furthermore, the importance of regular mealtimes is discussed as food is an important *Zeitgeber* for many organs, including brain areas involved in the development of addiction. At the end of this session, the study assistant hands out a circadian diary (**Supplementary Table 1**), which the participant is asked to fill out for 1 week. With the help of the diary, data on times of meals, snacks, activity times, sleep duration, sleep quality, mood, and alcohol craving are collected on a daily basis. At this point in time, the test persons do not yet receive any concrete instructions as to when they should execute activities. Rather, they are instructed to use the following days to see if they can recognize certain temporal patterns in their own behavior and to consciously follow these patterns. Filling out the test diary serves on the one hand to create an awareness of their own daily rhythms. Besides, it gives the test persons the opportunity to familiarize themselves with the filling out of the diary. On the other hand, the data obtained on eating and sleeping times can be used in the next session as a basis for drafting a daily structure plan.

Week 2: Establishment of a Daily Structure Plan

After 1 week, participants are invited for another individual session. During this session, the entries of the test diary are discussed and a personalized daily structure plan for eating and sleeping times and, if desired by the test subjects, times for leisure activities such as sports is created together with the participants. For each subject, a balance is attempted between endogenous circadian preferences and time constraints imposed by the environment, which the subjects may not be able to influence. Thus, it is sought to adapt all possible freely selectable times to the personal preferences given by individual circadian characteristics. However, some test persons are embedded in certain rigid daily structures on some days (e.g., due to working hours). These restrictive environmental factors are also taken into account when creating the daily structure plan in order to ensure their realistic implementation in everyday life and to avoid possible sleep deprivation due to late bed and early work hours. If this is the case, it is recommended to the test persons to strictly adhere to the daily schedule of working days even on non-working days in order to avoid repeated shifts in the circadian system and to maintain a maximum of

TABLE 1 | Overview of questionnaires, physiological assessments, and time points.

Assessment category	Assessment instruments	First session	Last session	Between sessions
Baseline	Demographics	x		
Circadian	Sleep-Food-Diary			x
	Munich ChronoType Questionnaire (MCTQ)	x	x	
	Pittsburgh Sleep Quality Index (PSQI)	x	x	
Alcohol use	Alcohol use disorders identification test (AUDIT)	x	x	
	European Addiction Severity Index (EuropASI)	x	x	
	Timeline Followback 30 (TLFB)	x	x	
Depression	Hamilton Depression Scale (HAMD)	x	x	
	Inventory of Depressive Symptomatology (IDS-SR)	x	x	
Self-efficacy	Self-Efficacy Questionnaire (SWE)	x	x	
Blood values	Blood draws	x	x	

regularity, and therefore endogenous synchronization. However, for each individual subject, it must be checked whether the daily schedule created for them deviates significantly from their endogenous circadian preferences and thus leads to a permanent misalignment between endogenous and exogenous rhythms or sleep deprivation. If the discrepancy is larger than 2 h, a specific adjustment of the lighting conditions is recommended, e.g., for late chronotypes with early work hours, increasing the light in the morning (bright light therapy) to advance the circadian system and to promote wakefulness, and decreasing it in the evening to further support the phase advance and promote tiredness.

Furthermore, at this meeting the test persons are again made aware of the importance of very regular eating and sleeping times. If the test diary shows that the test person has lived rather irregularly, solutions are developed for maintaining more daily regularity. This session also serves as an opportunity to answer potential questions from the study participants and to remind them of the importance of adhering to the daily structure plan that has been drawn up. Participants receive new blank diaries which they are asked to fill in for the next 4 weeks. The times of the designed daily structure plan are handwritten in the empty diary pages as a visual support for precise adherence to the times.

Weeks 3–6: Self-Employed Application of the Daily Structure Plans, Telephone Appointment, and Final Assessment

Starting from this point in the study, the test persons are asked to independently implement the previously developed daily plan for the next 4 weeks and to continue filling out their diaries. The implementation of the daily structure plan can already start at times when the patients are still on the ward or in the day clinic, as patients are allowed to choose the times of all activities (going to bed, eating, leisure activities) freely, with the exception of the fixed therapy sessions on weekdays (see above). However, many patients are discharged or transferred to another institution at different times during this phase of the study. Patients who are discharged home will be asked to follow up the daily schedule outside the clinical setting. Patients who continue their treatment in other institutions will be asked to maintain their daily schedule as much as possible in their new environment. During this time, they can contact the study staff for further assistance (i.e., to adjust the daily structure plan). After 2 weeks, participants are contacted by the study staff by telephone. The telephone call follows the rules of motivational interviewing (65) in order to motivate and remind the participants to further keep to the agreed daily structure and to continue to fill in the diaries thoroughly. If desired, patients are asked to send already completed diaries by fax, mail, or email. The continuous completion of the diary during the application phase of the study serves not only for continuous data collection but also for self-monitoring and increasing compliance. Another 2 weeks later, a last individual appointment is arranged. During this session, diaries will be collected, the questionnaires from the first session are repeated and another blood sample is taken (**Table 1**).

Control Group

The control group follows the same conceptual procedure and sequence as the intervention group (**Figure 1**). Thus, they have the same number of individual sessions and accordingly receive same attention from the study staff. They also fill out all the questionnaires, give blood, and fill out the diary for the same amount of time. However, there are differences in the content of the sessions, in that the control group will not receive information about the circadian clock, its connection to addiction, and the importance of having a structured daily life. Instead, participants of the control group are given the opportunity to discuss, for example, the role of legal drugs, such as alcohol, in society and the effects of alcohol advertising and its impact on both individual and societal consumption behavior. Since the test persons of the control group do not receive any further instructions for the diary and are only asked to fill it in carefully, the evaluation of the study data provides the possibility to compare Routine Variability Scores of test persons of the intervention group and the control group.

MEASURED OUTCOMES

Our primary hypothesis is that a regular exposure to *Zeitgebers* stabilizes circadian behavioral rhythms and thereby reduces the risk of alcohol relapses in AUD patients. Therefore, the

TABLE 2 | Overview of blood parameters that will be assessed at baseline and at 6-weeks follow-up.

Liver values		Blood cells and other AUD related values	
Abbreviation	**Full name**	**Abbreviation**	**Full name**
GOT	Glutamic Oxaloacetic Transaminase	MCV	Mean Corpuscular Volume
GPT	Glutamate-Pyruvate-Transaminase	Hb	Hemoglobin
GGT	Gamma GT	Vit B12	Vitamin B12
aP	Alkaline Phosphatase	CDT	Carbohydrate-Deficient Transferrin

first primary outcomes of the study are the Routine Variability Score and drinking behavior of each individual test person. For generation of the Routine Variability Scores, first, day-to-day variability will be calculated from the following parameters: bedtime, wake-up time, sleep quality, time of getting-up in the morning, time to go to bed in the evening, lights-off time, breakfast time, lunch time and dinner time. The individual Routine Variability Score is comparable to the validated social rhythm metric scale (SRMS) (66) and will be calculated as follows (example for bedtime): For the period of 1 week, the average bedtime will be calculated. Then the deviation from this average bedtime is calculated in minutes for each day. These deviations are summed up and the mean value is calculated. This mean value will be coded according to the magnitude of time variability: 1: 0–15 min, 2: 16–30 min, 3: 31–45 min, 4: 46–60 min, 5: 61–75 min, 6: 76–90 min, 7: 91–105 min, 8: 106–120 min, 9: 2–3 h, 10: 3–4 h, 11: over 4 h (67). This way a separate score can be created for each parameter collected in the diary. These scores can be calculated on a weekly basis to examine whether relapses or deterioration in well-being are related to irregularities in the daily routine during that week, or over the duration of the entire survey phase to check how regularity over a longer period is related to drinking behavior and well-being. In addition, the scores for sleep and meals, for example, can be calculated individually to examine their influence on drinking behavior and well-being separately.

Additionally, a questionnaire specially designed for this study with a total of 30 questions and a 4-step ordinal scale for the answers to assess the rating of the participants' subjective structuredness in their everyday life will be filled out. This questionnaire includes, for example, questions on the subjective assessment of whether activities such as eating, sleeping and sport are carried out regularly, whether relapses are associated with irregular daily routines, and whether the craving for alcohol usually occurs at the same time of day. To give the subjects of the control group no indication of the actual aim of the study, the questions that aim at the circadian daily structure are mixed with neutral questions.

Drinking behavior and daily craving will be assessed with questionnaires and the diary, respectively (**Table 1**). General drinking habits of the last few weeks are surveyed using the AUDIT questionnaire. In addition, the European Addiction Severity Index (EuropASI) is used, which covers not only alcohol and drug consumption but also physical, psychological, financial, and social problem areas of addicted patients. The number of alcoholic drinks and the number of alcohol-free days within the past 30 days is assessed by means of the Timeline Followback 30 (TLFB-30). The diary, which the subjects fill out to collect the Routine Variability Scores, additionally records the highest craving for alcohol on a scale of 0–10 of each day.

According to our secondary hypothesis that the stabilization of circadian rhythms contributes to the reduction of depressive symptoms and increased well-being, changes in the affective state, with a focus on depressive symptoms, are measured with the external assessment questionnaire Hamilton Depression Scale (HAMD) and with the self-reporting questionnaire Inventory of Depressive Symptomatology (IDS-SR). Sleep quantity and quality will be assessed by Pittsburgh Sleep Quality Index (PSQI). Furthermore, for measuring if the DAILY structure program will influence the feeling of self-efficacy, we will employ the Generalized Self-Efficacy Scale (GSES).

As exploratory outcomes, it will be documented how many subjects in the DAILY intervention group and the control groups discontinue the therapy. Furthermore, possible changes of the chronotypes in the course of the study will be defined by the Munich ChronoType Questionnaire (MCTQ).

As alcohol has various negative effects on different blood cells and their functions, we will also take blood samples and assess liver and blood parameters (see **Table 2**).

DATA MANAGEMENT

During the study, all data assessed will be electronically saved. The saved data will be securely stored by password and pseudo-randomization. All data will be subjected to medical confidentiality and general data protection regulations.

DATA ANALYSIS

All statistical analyses will be performed using SPSS software version 25 (SPSS INC., Chicago, IL, USA). Sociodemographic data, chronotype, diary data and blood parameters will be reported in form of descriptive statistics. Continuous variables will be presented as mean $\pm$ *SD* and normality of continuous data will be measured by using the Kolmogorov-Smirnov-Test. Accordingly, we will use parametric and non-parametric analyses. For all calculations, the significance level will be determined at $\alpha < 0.05$.

Data analysis will be performed according to intention-to-treat analysis (ITT) rules. In particular, this means that all

subjects who completed the questionnaires of the first session and the 1st week of the diaries are included in the analysis, regardless of whether their subsequent data sets are complete or whether they have dropped out of the study. This is done by imputing missing data with the help of all available data that was assessed until then. Participants who withdraw their consent and were not assessed with baseline measurements will be treated as dropouts. All other subjects regardless from the study condition will be analyzed according to ITT.

The data will be evaluated in three different ways: First, outcomes of the endpoint of the study will be compared between the intervention and control groups using unpaired group analyses (e.g., Student's *t*-test, Mann-Whitney *U*-test, Kruskal-Wallis test, one-way ANOVA). Secondly, within-subject analyses of the intervention and control groups are performed, comparing the changes between the start and end of the study of the two groups (e.g., two-way ANOVA). Thirdly, the data are calculated independently of group membership in two-dimensional linear regression using the Routine Variability Scores as an independent variant.

Additionally, statistical differences in the frequency of dropouts between the two groups are calculated using chi-square tests and survival analyses. Multiple regression analyses will be applied to investigate the degree to which predictor variables (i.e., Routine Variability Scores, depression level, severity of alcohol consumption behavior, and self-efficacy score) contribute to outcomes of primary and secondary measures.

DISCUSSION

The DAILY intervention is a new concomitant treatment approach for patients suffering from AUD, which is based on the strengthening of the patients' circadian system with the targeted use of *Zeitgebers* such as sleep, meal, and activity times. The timing for the regular use of the *Zeitgebers* is personalized and tailored to the individual patients including both their genetically imprinted circadian characteristics as well as individual social obligations, such as working hours. Hence, the personalized design should therefore make the strengthening of physiological rhythms as effective as possible by taking individual characteristics into account on the one hand, and on the other hand as easy to implement as possible by including environmental constraints. Each individual is determined by a genetic constellation that defines optimal times for sleeping, eating and physical exertion (e.g., sports). In turn, these specific actions serve as *Zeitgebers* for the circadian system. Consequently, the circadian clock and the *Zeitgebers* form a positive feedback system, which leads to mutual reinforcement. Thus, this bidirectional influence can be used to strengthen and stabilize circadian clocks. Our therapy makes sure that actions serving as *Zeitgebers* are performed at the same time on all days. This preserves the circadian system from repeated shifts and ensures that a fixed phase relationship among physiological processes, and thereby endogenous synchronization, can be established. However, there are many individuals who are subject to social times that they cannot completely elude in everyday life and which may change between work and work-free days. The primary focus of our study is to test whether AUD patients benefit from increased regularity of *Zeitgeber* exposure which requires adherence to the same schedule on workdays and workfree days. Thus, our therapy bears the risk of a permanent misalignment or sleep deprivation for some participants. Therefore, if the endogenous rhythms of the test subjects, who are not in the position to choose their sleeping times on most days of the week, deviate more than 2 h from constrained social times, they are advised to adjust their biological time using light therapy to avoid chronic misalignment with the environment. Thus, our therapy ensures endogenous alignment of rhythms and additionally counteracts the so-called social jetlag, which is also associated with increased alcohol consumption (68, 69). However, if internal and external rhythms are found to be extremely different, subjects may not be suitable for this study.

As described above, the brain's reward system and the development and maintenance of substance use disorders are particularly closely related to the circadian clock. These mechanistic explanations will also be discussed with the participants of DAILY. We believe that psychoeducation offers the patients the opportunity to learn about tangible reasons for the importance of regular daily structures, increases their awareness of the timing of their daily activities and enhances the general compliance during therapy. Therefore, DAILY consists of a unique and balanced concept that builds on three main components:

1. psychoeducation about sleep hygiene and the circadian system, its connection to AUD, and the physiological mechanisms through which a regular daily structure may improve therapeutic success,
2. strengthening of the circadian system and thereby optimizing physiological processes and neuronal communication,
3. additional positive psychological effects through the feeling of stability, control, and self-management in the often-difficult transition period between inpatient and outpatient care.

In the past, different chronotherapeutic treatments with different therapy aims have been described and clinically tested in mood disorders, but, to our knowledge, never in addiction disorders. Furthermore, in our opinion, personalization, which aims at balancing genetically and socially determined rhythms, has not been sufficiently incorporated in previous chronotherapeutic approaches. For example, the most common chrono-treatment is light therapy, which has two goals, increasing the daily light dose and shifting the chronotype from late to early. Light therapy is widely applied and highly effective as it can reach similar positive effects on mood as antidepressant drugs (70). Light therapy is known to be effective for the treatment of seasonal affective disorder, MDD and was shown to be effective in bipolar disorder patients (71, 72). Similarly, sleep advance therapies are used to shift late chronotypes to earlier hours, which has been shown to have positive effects on mood. The effect, however, is primarily related to the patient's mood and is not of long duration. The therapeutic effects of total or partial sleep deprivation on mood are even shorter and a connection with the circadian system

is not proven (73). These approaches have the only aim of adapting the chronotype to environmental rhythms and often follow uniform protocols. However, trying to adapt patients to the same early chronotype can be very difficult for those subjects whose endogenous chronotype differs widely.

Contrarily, already in the late 90's, an approach that considered individual circadian traits, the so-called interpersonal social rhythm therapy (IPSRT), has been tested successfully in bipolar patients (60, 74), but, to our knowledge, never for AUD patients. IPSRT focuses primarily on stabilizing social rhythms such as eating regularly with other people and thus improving social interactions and relationships (75). Its efficacy has been proven multiple times (61, 76). Interestingly, there is a renewing increase in awareness and clinical interest for therapy approaches taking the effects of regular daily structures and sleep hygiene into account (19, 63, 77). In accordance with IPSRT, we believe that a more ideal form of chronotherapy is not necessarily the attempt to change endogenous circadian rhythms (e.g., chronotypes), but to exploit and strengthen them as much as possible in order to achieve greater robustness of the individual circadian clock.

Cognitive behavioral therapy for insomnia (CBT-I) focuses, like parts of DAILY, on a regular sleep schedule, stimulus control, and improved sleep hygiene. Other core components of CBT-I are sleep restriction and relaxation techniques, which are not used in DAILY. CBT-I treatment in AUD patients effectively reduces insomnia, associated negative cognitions, and improves sleep hygiene, but, however, is not successful in changing drinking behavior (78, 79). Apart from sleep problems, AUD patients often suffer from irregular routines throughout the day, including the timing of meals. Thus, in contrast to the focus of CBT-I on sleep improvement, DAILY aims to stabilize the circadian system of the entire body, including clocks in peripheral organs and reward-regulating brain areas, which in our opinion is more likely to improve alcohol outcomes.

In summary, in DAILY we aim to increase stability and regularity of circadian rhythms of AUD patients by creating daily routine schedules which will consider as many endogenous circadian preferences as possible, but also social obligations. Actions whose timing is determined by social constraints will be integrated in the daily routine schedules to make their execution feasible, and their timing is kept the same on all days to create endogenous synchronization. In subjects whose daily routine schedule deviates more than 2 h from endogenous rhythms, light therapy will be used to adjust their chronotype to avoid social jetlag. In this way we obtain a high degree of personalization and can try to create the best possible daily profile for each subject.

Limitations

At this state, however, DAILY has some limitations as it is designed as a pilot study and so, it aims primarily at proofing the principle study concept and the overall impact of a daily structure therapy in AUD patients. A limitation of the current design is that it does not include objective physiological and behavioral measures of circadian rhythm improvement such as (e.g., melatonin rhythms or actigraphy, but only focuses on more subjective behavioral rhythms based on diary entries). However, the primary aim of this pilot study is the proof whether a therapeutic approach to improve sleep hygiene and regularity in daily routine can generally improve AUD symptoms.

Furthermore, inpatients and semi-inpatients are included into our study. Inpatients and semi-inpatients usually already have a more structured daily routine than outpatients since they are given a regulated daily structure by the clinical routines and the fixed therapy hours. Therefore, the determination of individual circadian preferences and characteristics might be limited. However, in our clinic, where the study is currently taking place, patients have no mandatory eating or sleeping hours and can leave the ward and day clinic outside therapy hours. Thus, the test persons can in principle follow their own rhythms, which is often taken advantage of, especially for food and snacks in the surrounding areas. Nevertheless, many test persons report that they generally have more structured daily routines in the clinic than at home, as they adapt to the rhythms of their fellow patients as well as the rhythms of the clinic meals offered.

Furthermore, there are also test persons who, after detoxification or after their stay in the day clinic, go for further treatment to other clinics where they are integrated into a daily structure. Since this daily structure does not necessarily correspond to the subject's circadian characteristics, the analysis of pilot data must consider that these test persons cannot always independently follow the daily schedule during the study. Accordingly, it is possible that the implementation of the DAILY program is more suitable for clinics from which patients are usually discharged home.

Additionally, inpatients and semi-inpatients often have a more severe symptomatology than outpatients. Thus, results of this pilot study may not be completely representative for all patients suffering from AUD. However, it might be possible, that the therapeutic effects of the DAILY treatment will be even more pronounced in outpatients. AUD outpatients often suffer from less severe symptoms and might particularly benefit from a daily structure therapy as they are usually at higher risk to have a more disorganized and unstructured daily life. However, this is not tested in the current study. Generally, it is expected that DAILY therapy will be particularly suitable for patients who have severe irregularities in their everyday life. AUD patients, however, who have a very regular daily routine of their own accord, will probably not benefit to the same extent from the therapy presented here.

Another limitation of the current study design is that no long-term follow-up is scheduled. However, this limitation will be addressed in a future study with a more longitudinal perspective.

Outlook

In future studies, the determination of individual circadian traits will be optimized in two approaches. First, outpatients will be included because these patients most likely organize their day following primarily their endogenous clock compared to inpatients who are integrated into the daily structures of the clinic. Second, we will also use actigraphy on the one hand to receive a more objective measure of regularity of rest-activity rhythms and on the other hand because filling out diaries carries a high risk of non-compliance and therefore a high risk of data gaps.

In recent years, great progress has been made in the development of machine learning methods for the evaluation of circadian variables of actimeter data, with which for example the circadian phase can be determined (80–83). In this regard, a newly developed DLMO prediction model based on actimetry data, which can be used to quantify circadian misalignment, seems particularly useful to quantify irregularities in circadian rhythms and behavior (84). Therefore, the application of actimeters and the use of mathematical algorithms for analysis will be part of future and more comprehensive studies. The results obtained can in turn be used to adjust and refine daily structure plans of the individual participants of our second study cohort.

Beyond that, we will try to identify times of the day when alcohol cravings are particularly high. Knowing about these times, we will encourage the patients to use these hours to have recourse on learned skills (doing sports, contacting close persons etc.) in order to overcome this period more successfully.

The overall goal of this and more extended future studies is the development of an accompanying therapy for AUD patients that is uncomplicated in its use and easy to implement in the clinical routine. This therapy should enable AUD patients to bring more regularity and structure into their everyday life and thus to strengthen their circadian system. This, in turn, may be a critical component in overcoming alcohol withdrawals and in preventing relapses more successfully.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local Ethics Committee of the Ludwig Maximilian University Munich. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AH and DL wrote the manuscript. All authors were involved in the design, conceptualization of the study, editing the manuscript, and approving the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.569864/full#supplementary-material

REFERENCES

1. World Health Organization. *Global Status Report on Alcohol and Health* 2018. In: Poznyak V, Rekve D, editors. (2018). Geneva: World Health Organization.
2. Fein G. Psychiatric comorbidity in alcohol dependence. *Neuropsychol Rev.* (2015) 25:456–75. doi: 10.1007/s11065-015-9304-y
3. Shield KD, Parry C, Rehm J. Chronic diseases and conditions related to alcohol use. *Alcohol Res.* (2013) 35:155–73.
4. Laramee P, Kusel J, Leonard S, Aubin HJ, Francois C, Daeppen JB. The economic burden of alcohol dependence in Europe. *Alcohol Alcohol.* (2013) 48:259–69. doi: 10.1093/alcalc/agt004
5. Degenhardt L, Whiteford HA, Ferrari AJ, Baxter AJ, Charlson FJ, Hall WD, et al. Global burden of disease attributable to illicit drug use and dependence: findings from the global burden of disease study 2010. *Lancet.* (2013) 382:1564–74. doi: 10.1016/S0140-6736(13)61530-5
6. Ruben MD, Wu G, Smith DF, Schmidt RE, Francey LJ, Lee YY, et al. A database of tissue-specific rhythmically expressed human genes has potential applications in circadian medicine. *Sci Transl Med.* (2018) 10:458. doi: 10.1126/scitranslmed.aat8806
7. Stetson MH, Watson-Whitmyre M. Nucleus suprachiasmaticus: the biological clock in the hamster? *Science.* (1976) 191:197–9. doi: 10.1126/science.942799
8. Inouye ST, Kawamura H. Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proc Natl Acad Sci USA.* (1979) 76:5962–6. doi: 10.1073/pnas.76.11.5962
9. Stephan FK, Zucker I. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci USA.* (1972) 69:1583–6. doi: 10.1073/pnas.69.6.1583
10. Davidson AJ, Yamazaki S, Menaker M. *SCN: ringmaster of the circadian circus or conductor of the circadian orchestra*? *Novartis Found Symp.* (2003) 253:110–21. doi: 10.1002/0470090839.ch9
11. Harrisingh MC, Nitabach MN. Circadian rhythms. Integrating circadian timekeeping with cellular physiology. *Science.* (2008) 320:879–80. doi: 10.1126/science.1158619
12. Mistlberger RE, Skene DJ. Social influences on mammalian circadian rhythms: animal and human studies. *Biol Rev Camb Philos Soc.* (2004) 79:533–56. doi: 10.1017/S1464793103006353
13. Hühne A, Welsh DK, Landgraf D. Prospects for circadian treatment of mood disorders. *Ann Med.* (2018) 50:637–54. doi: 10.1080/07853890.2018.1530449
14. Landgraf D, McCarthy MJ, Welsh DK. Circadian clock and stress interactions in the molecular biology of psychiatric disorders. *Curr Psychiatry Rep.* (2014) 16:483. doi: 10.1007/s11920-014-0483-7
15. Perreau-Lenz S, Spanagel R. Clock genes x stress x reward interactions in alcohol and substance use disorders. *Alcohol.* (2015) 49:351–7. doi: 10.1016/j.alcohol.2015.04.003
16. Hasler BP, Bruce S, Scharf D, Ngari W, Clark DB. Circadian misalignment and weekend alcohol use in late adolescent drinkers: preliminary evidence. *Chronobiol Int.* (2019) 36:796–810. doi: 10.1080/07420528.2019.1586720

17. Davis BTt, Voigt RM, Shaikh M, Forsyth CB, Keshavarzian A. Circadian mechanisms in alcohol use disorder and tissue injury. *Alcohol Clin Exp Res.* (2018) 42:668–77. doi: 10.1111/acer.13612
18. Spanagel R, Rosenwasser AM, Schumann G, Sarkar DK. Alcohol consumption and the body's biological clock. *Alcohol Clin Exp Res.* (2005) 29:1550–7. doi: 10.1097/01.alc.0000175074.70807.fd
19. Gulick D, Gamsby JJ. Racing the clock: the role of circadian rhythmicity in addiction across the lifespan. *Pharmacol Ther.* (2018) 188:124–39. doi: 10.1016/j.pharmthera.2018.03.003
20. Mukai M, Uchimura N, Hirano T, Ohshima H, Ohshima M, Nakamura J. Circadian rhythms of hormone concentrations in alcohol withdrawal. *Psychiatry Clin Neurosci.* (1998) 52:238–40. doi: 10.1111/j.1440-1819.1998.tb01051.x
21. Rosenwasser AM. Alcohol antidepressants, circadian rhythms. Human and animal models. *Alcohol Res Health.* (2001) 25:126–35.
22. Sjoholm LK, Kovanen L, Saarikoski ST, Schalling M, Lavebratt C, Partonen T. *CLOCK is suggested to associate with comorbid alcohol use and depressive disorders. J Circadian Rhythms.* (2010) 8:1. doi: 10.1186/1740-3391-8-1
23. Spanagel R, Pendyala G, Abarca C, Zghoul T, Sanchis-Segura C, Magnone MC, et al. The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nat Med.* (2005) 11:35–42. doi: 10.1038/nm1163
24. Gillman AG, Rebec GV, Pecoraro NC, Kosobud AEK. Circadian entrainment by food and drugs of abuse. *Behav Processes.* (2019) 165:23–8. doi: 10.1016/j.beproc.2019.05.017
25. Hyman SE, Malenka RC, Nestler EJ. Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu Rev Neurosci.* (2006) 29:565–98. doi: 10.1146/annurev.neuro.29.051605.113009
26. Brick J, Pohorecky LA, Faulkner W, Adams MN. Circadian variations in behavioral and biological sensitivity to ethanol. *Alcohol Clin Exp Res.* (1984) 8:204–11. doi: 10.1111/j.1530-0277.1984.tb05840.x
27. Danel T, Jeanson R, Touitou Y. Temporal pattern in consumption of the first drink of the day in alcohol-dependent persons. *Chronobiol Int.* (2003) 20:1093–102. doi: 10.1081/CBI-120025533
28. Mistlberger RE. Food as circadian time cue for appetitive behavior. *F1000Res.* (2020) 9:F1000. doi: 10.12688/f1000research.20829.1
29. Webb IC, Baltazar RM, Lehman MN, Coolen LM. Bidirectional interactions between the circadian and reward systems: is restricted food access a unique zeitgeber? *Eur J Neurosci.* (2009) 30:1739–48. doi: 10.1111/j.1460-9568.2009.06966.x
30. Mendoza J, Challet E. Circadian insights into dopamine mechanisms. *Neuroscience.* (2014) 282C:230–42. doi: 10.1016/j.neuroscience.2014.07.081
31. Forbes EE, Dahl RE, Almeida JR, Ferrell RE, Nimgaonkar VL, Mansour H, et al. PER2 rs2304672 polymorphism moderates circadian-relevant reward circuitry activity in adolescents. *Biol Psychiatry.* (2012) 71:451–7. doi: 10.1016/j.biopsych.2011.10.012
32. Murray G, Nicholas CL, Kleiman J, Dwyer R, Carrington MJ, Allen NB, et al. Nature's clocks and human mood: the circadian system modulates reward motivation. *Emotion.* (2009) 9:705–16. doi: 10.1037/a0017080
33. Grippo RM, Purohit AM, Zhang Q, Zweifel LS, Guler AD. Direct midbrain dopamine input to the suprachiasmatic nucleus accelerates circadian entrainment. *Curr Biol.* (2017) 27:2465–75.e3. doi: 10.1016/j.cub.2017.06.084
34. Grippo RM, Tang Q, Zhang Q, Chadwick SR, Gao Y, Altherr EB, et al. Dopamine signaling in the suprachiasmatic nucleus enables weight gain associated with hedonic feeding. *Curr Biol.* (2020) 30:196–208 e8. doi: 10.1016/j.cub.2019.11.029
35. Byrne JEM, Hughes ME, Rossell SL, Johnson SL, Murray G. Time of day differences in neural reward functioning in healthy young men. *J Neurosci.* (2017) 37:8895–900. doi: 10.1523/JNEUROSCI.0918-17.2017
36. Sleipness EP, Sorg BA, Jansen HT. Time of day alters long-term sensitization to cocaine in rats. *Brain Res.* (2005) 1065:132–7. doi: 10.1016/j.brainres.2005.10.017
37. Gillman AG, Leffel JK, Kosobud AE, Timberlake W. Behavioral characteristics and pharmacological manipulations of a nicotine-entrainable circadian oscillator. *Chronobiol Int.* (2013) 30:855–69. doi: 10.3109/07420528.2013.782314
38. Sleipness EP, Jansen HT, Schenk JO, Sorg BA. Time-of-day differences in dopamine clearance in the rat medial prefrontal cortex and nucleus accumbens. *Synapse.* (2008) 62:877–85. doi: 10.1002/syn.20552
39. Murray G, Allen NB, Trinder J. Mood and the circadian system: investigation of a circadian component in positive affect. *Chronobiol Int.* (2002) 19:1151–69. doi: 10.1081/CBI-120015956
40. Logan RW, Williams WP 3rd, McClung CA. Circadian rhythms and addiction: mechanistic insights and future directions. *Behav Neurosci.* (2014) 128:387–412. doi: 10.1037/a0036268
41. Ozburn AR, Falcon E, Mukherjee S, Gillman A, Arey R, Spencer S, et al. The role of clock in ethanol-related behaviors. *Neuropsychopharmacology.* (2013) 38:2393–400. doi: 10.1038/npp.2013.138
42. Hasler BP, Bootzin RR, Cousins JC, Fridel K, Wenk GL. Circadian phase in sleep-disturbed adolescents with a history of substance abuse: a pilot study. *Behav Sleep Med.* (2008) 6:55–73. doi: 10.1080/15402000701796049
43. Danel T, Cottencin O, Tisserand L, Touitou Y. *Inversion of melatonin circadian rhythm in chronic alcoholic patients during withdrawal: preliminary study on seven patients. Alcohol Alcohol.* (2009) 44:42–5. doi: 10.1093/alcalc/agn091
44. Hasler BP, Franzen PL, de Zambotti M, Prouty D, Brown SA, Tapert SF, et al. Eveningness and later sleep timing are associated with greater risk for alcohol and marijuana use in adolescence: initial findings from the national consortium on alcohol and neurodevelopment in adolescence study. *Alcohol Clin Exp Res.* (2017) 41:1154–65. doi: 10.1111/acer.13401
45. Kang JI, Park CI, Sohn SY, Kim HW, Namkoong K, Kim SJ. Circadian preference and trait impulsivity, sensation-seeking and response inhibition in healthy young adults. *Chronobiol Int.* (2015) 32:235–41. doi: 10.3109/07420528.2014.965313
46. Webb IC, Baltazar RM, Wang X, Pitchers KK, Coolen LM, Lehman MN. Diurnal variations in natural and drug reward, mesolimbic tyrosine hydroxylase, and clock gene expression in the male rat. *J Biol Rhythms.* (2009) 24:465–76. doi: 10.1177/0748730409346657
47. Sleipness EP, Sorg BA, Jansen HT. Diurnal differences in dopamine transporter and tyrosine hydroxylase levels in rat brain: dependence on the suprachiasmatic nucleus. *Brain Res.* (2007) 1129:34–42. doi: 10.1016/j.brainres.2006.10.063
48. Karatsoreos IN, Bhagat S, Bloss EB, Morrison JH, McEwen BS. Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. *Proc Natl Acad Sci USA.* (2011) 108:1657–62. doi: 10.1073/pnas.1018375108
49. Ekmekcioglu C, Touitou Y. Chronobiological aspects of food intake and metabolism and their relevance on energy balance and weight regulation. *Obes Rev.* (2011) 12:14–25. doi: 10.1111/j.1467-789X.2010.00716.x
50. Wu T, Jin Y, Ni Y, Zhang D, Kato H, Fu Z. Effects of light cues on re-entrainment of the food-dominated peripheral clocks in mammals. *Gene.* (2008) 419:27–34. doi: 10.1016/j.gene.2008.04.010
51. Escobar C, Espitia-Bautista E, Guzman-Ruiz MA, Guerrero-Vargas NN, Hernandez-Navarrete MA, Angeles-Castellanos M, et al. Chocolate for breakfast prevents circadian desynchrony in experimental models of jet-lag and shift-work. *Sci Rep.* (2020) 10:6243. doi: 10.1038/s41598-020-63227-w
52. Tognini P, Samad M, Kinouchi K, Liu Y, Helbling JC, Moisan MP, et al. Reshaping circadian metabolism in the suprachiasmatic nucleus and prefrontal cortex by nutritional challenge. *Proc Natl Acad Sci USA.* (2020) 117:29904–13. doi: 10.1073/pnas.2016589117
53. Ubaldo-Reyes LM, Buijs RM, Escobar C, Angeles-Castellanos M. Scheduled meal accelerates entrainment to a 6-h phase advance by shifting central and peripheral oscillations in rats. *Eur J Neurosci.* (2017) 46:1875–86. doi: 10.1111/ejn.13633
54. Potter GDM, Wood TR. The future of shift work: circadian biology meets personalised medicine and behavioural science. *Front Nutr.* (2020) 7:116. doi: 10.3389/fnut.2020.00116
55. Sinha, R. How does stress lead to risk of alcohol relapse? *Alcohol Res.* (2012) 34:432–40.
56. Josephson SA. Focusing on transitions of care: a change is here. *Neurol Clin Pract.* (2016) 6:183–9. doi: 10.1212/CPJ.0000000000000207
57. Prat G, Adan A. Influence of circadian typology on drug consumption, hazardous alcohol use, hangover symptoms. *Chronobiol Int.* (2011) 28:248–57. doi: 10.3109/07420528.2011.553018
58. Hidalgo MP, Caumo W, Posser M, Coccaro SB, Camozzato AL, Chaves ML. Relationship between depressive mood and

chronotype in healthy subjects. *Psychiatry Clin Neurosci.* (2009) 63:283–90. doi: 10.1111/j.1440-1819.2009.01965.x
59. Kanfer FH, Reinecker H, Schmelzer D. *Selbstmanagement-Therapie, 5 edn*. Ein Lehrbuch für die klinische Praxis: Springer-Verlag GmbH (2011).
60. Frank E, Swartz HA, Kupfer DJ. Interpersonal and social rhythm therapy: managing the chaos of bipolar disorder. *Biol Psychiatry.* (2000) 48:593–604. doi: 10.1016/S0006-3223(00)00969-0
61. Frank E, Kupfer DJ, Thase ME, Mallinger AG, Swartz HA, Fagiolini AM, et al. Two-year outcomes for interpersonal and social rhythm therapy in individuals with bipolar I disorder. *Arch Gen Psychiatry.* (2005) 62:996–1004. doi: 10.1001/archpsyc.62.9.996
62. Dunker Svendsen S, Aggestrup AS, Norregaard LB, Loventoft P, Praestegaard A, Danilenko KV, et al. Circadian reinforcement therapy in combination with electronic self-monitoring to facilitate a safe post-discharge period of patients with depression by stabilizing sleep: protocol of a randomized controlled trial. *BMC Psychiatry.* (2019) 19:124. doi: 10.1186/s12888-019-2101-z
63. Meyrel M, Rolland B, Geoffroy PA. Alterations in circadian rhythms following alcohol use: a systematic review. *Prog Neuropsychopharmacol Biol Psychiatry.* (2020) 99:109831. doi: 10.1016/j.pnpbp.2019.109831
64. Whitehead AL, Julious SA, Cooper CL, Campbell MJ. Estimating the sample size for a pilot randomised trial to minimise the overall trial sample size for the external pilot and main trial for a continuous outcome variable. *Stat Methods Med Res.* (2016) 25:1057–73. doi: 10.1177/0962280215588241
65. Spaeth M, Bleich S, Hillemacher T. Motivational interviewing with alcohol-dependent patients. *Fortschr Neurol Psychiatr.* (2017) 85:549–65. doi: 10.1055/s-0043-115216
66. Monk TH, Frank E, Potts JM, Kupfer DJ. A simple way to measure daily lifestyle regularity. *J Sleep Res.* (2002) 11:183–90. doi: 10.1046/j.1365-2869.2002.00300.x
67. Soehner AM, Kennedy KS, Monk TH. Circadian preference and sleep-wake regularity: associations with self-report sleep parameters in daytime-working adults. *Chronobiol Int.* (2011) 28:802–9. doi: 10.3109/07420528.2011.613137
68. Wittmann M, Dinich J, Merrow M, Roenneberg T. Social jetlag: misalignment of biological and social time. *Chronobiol Int.* (2006) 23:497–509. doi: 10.1080/07420520500545979
69. Wittmann M, Paulus M, Roenneberg T. Decreased psychological well-being in late "chronotypes" is mediated by smoking and alcohol consumption. *Subst Use Misuse.* (2010) 45:15–30. doi: 10.3109/10826080903498952
70. Pail G, Huf W, Pjrek E, Winkler D, Willeit M, Praschak-Rieder N, et al. Bright-light therapy in the treatment of mood disorders. *Neuropsychobiology.* (2011) 64:152–62. doi: 10.1159/000328950
71. Golden RN, Gaynes BN, Ekstrom RD, Hamer RM, Jacobsen FM, Suppes T, et al. The efficacy of light therapy in the treatment of mood disorders: a review and meta-analysis of the evidence. *Am J Psychiatry.* (2005) 162:656–62. doi: 10.1176/appi.ajp.162.4.656
72. Sit DK, McGowan J, Wiltrout C, Diler RS, Dills JJ, Luther J, et al. Adjunctive bright light therapy for bipolar depression: a randomized double-blind placebo-controlled trial. *Am J Psychiatry.* (2018) 175:131–9. doi: 10.1176/appi.ajp.2017.16101200
73. Landgraf D, McCarthy MJ, Welsh DK. The role of the circadian clock in animal models of mood disorders. *Behav Neurosci.* (2014) 128:344–59. doi: 10.1037/a0036029
74. Frank E, Swartz HA, Boland E. Interpersonal and social rhythm therapy: an intervention addressing rhythm dysregulation in bipolar disorder. *Dialogues Clin Neurosci.* (2007) 9:325–32. doi: 10.31887/DCNS.2007.9.3/efrank
75. Haynes PL, Gengler D, Kelly M. Social rhythm therapies for mood disorders: an update. *Curr Psychiatry Rep.* (2016) 18:75. doi: 10.1007/s11920-016-0712-3
76. Steardo L Jr, Luciano M, Sampogna G, Zinno F, Saviano P, de Fazio Fiorillo A. Efficacy of the interpersonal and social rhythm therapy (IPSRT) in patients with bipolar disorder: results from a real-world, controlled trial. *Ann Gen Psychiatry.* (2020) 19:15. doi: 10.1186/s12991-020-00278-3
77. Lamont EW, Legault-Coutu D, Cermakian N, Boivin DB. The role of circadian clock genes in mental disorders. *Dialogues Clin Neurosci.* (2007) 9:333–42. doi: 10.31887/DCNS.2007.9.3/elamont
78. Chakravorty S, Morales KH, Arnedt JT, Perlis ML, Oslin DW, Findley JC, et al. Cognitive behavioral therapy for insomnia in alcohol-dependent veterans: a randomized, controlled pilot study. *Alcohol Clin Exp Res.* (2019) 43:1244–53. doi: 10.1111/acer.14030
79. Arnedt JT, Conroy DA, Brower KJ. Treatment options for sleep disturbances during alcohol recovery. *J Addict Dis.* (2007) 26:41–54. doi: 10.1300/J069v26n04_06
80. Reid KJ. Assessment of circadian rhythms. *Neurol Clin.* (2019) 37:505–26. doi: 10.1016/j.ncl.2019.05.001
81. Wittenbrink N, Ananthasubramaniam B, Munch M, Koller B, Maier B, Weschke C, et al. High-accuracy determination of internal circadian time from a single blood sample. *J Clin Invest.* (2018) 128:3826–39. doi: 10.1172/JCI120874
82. Braun R, Kath WL, Iwanaszko M, Kula-Eversole E, Abbott SM, Reid KJ, et al. Universal method for robust detection of circadian state from gene expression. *Proc Natl Acad Sci USA.* (2018) 115:E9247–E9256. doi: 10.1073/pnas.1800314115
83. Dijk DJ, Duffy JF. Novel approaches for assessing circadian rhythmicity in humans: a review. *J Biol Rhythms.* (2020) 35:421–38. doi: 10.1177/0748730420940483
84. Cheng P, Walch O, Huang Y, Mayer C, Sagong C, Cuamatzi Castelan A, et al. Predicting circadian misalignment with wearable technology: validation of wrist-worn actigraphy and photometry in night shift workers. *Sleep*. (2020). doi: 10.1093/sleep/zsaa180. [Epub ahead of print].

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.

Diary

Week __

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	Today´s date							
Morning	Time of waking up							
	Time of getting up							
	Alarm clock (yes/no)							
	Sleep quality (min **0** - max **10**)							
	Working day (yes/no)							
Evening	Time of going to bed							
	Time of lights off							
	Minutes between lights off and sleep							
	Today´s mood (min **0** - max **10**)							
	Time of max. craving							
	Intensity of max. craving (min **0** - max **10**)							
	00:00							
	00:30							
	01:00							
	01:30							
	02:00							
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To fill in:

"X" = Main meal

"Sn" = Snack

"Dc" = Drink containing caffeine

"Ds" = Drink containing sugar or fruit juice

"Mi" = Milk or dairy product

"Sp" = Sports

"Na" = Nap

Table S1: Diary for recording sleeping, eating, and craving times. In the upper part of the diary, data on sleep times and sleep quality are collected. Sleep times are divided into the times of going to bed or getting up and the times of actual falling asleep or waking up, as the latter allow more reliable conclusions to be drawn about endogenous circadian rhythms of the test persons. It also notes whether the current day is a working day or a non-working day and whether an alarm clock was used to wake up. Furthermore, the average mood state, the highest intensity of craving for alcohol, and the time of the strongest craving of each day are recorded. In the lower part of the diary, all meals are entered in a table with a resolution of 30 min. When entering meals, a rough distinction is made between different foods and the size of the meal. Times of possible sports activities and naps are also entered in the table. At the bottom of each day there is space for possible comments, e.g. special events of the day which might have influenced the daily structure. The diary is a modification of an unpublished template, which we have been kindly provided by the laboratory of Prof. Till Roenneberg of the Institute of Medical Psychology at the Ludwig Maximilian University of Munich.

Appendix Paper IV

ANNALS OF MEDICINE
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REVIEW ARTICLE

Prospects for circadian treatment of mood disorders

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ABSTRACT

Disruption of circadian clocks is strongly associated with mood disorders. Chronotherapies targeting circadian rhythms have been shown to be very effective treatments of mood disorders, but still are not widely used in clinical practice. The mechanisms by which circadian disruption leads to mood disorders are poorly characterized and, therefore, may not convince clinicians to apply chronotherapies. Hence, in this review, we describe specific potential mechanisms, in order to make this connection more credible to clinicians. We believe that four major features of disrupted clocks may contribute to the development of mood disorders: (1) loss of synchronization to environmental 24-h rhythms, (2) internal desynchronization among body clocks, (3) low rhythm amplitude, and (4) changes in sleep architecture. Discussing these attributes and giving plausible examples, we will discuss prospects for relatively simple chronotherapies addressing these features that are easy to implement in clinical practice.

KEY MESSAGES

- In this review, we describe specific potential mechanisms by which disrupted clocks may contribute to the development of mood disorders: (1) loss of synchronization to environmental 24-h rhythms, (2) internal desynchronization among body clocks, (3) low rhythm amplitude, and (4) changes in sleep architecture.
- We provide prospects for relatively simple chronotherapies addressing these features that are easy to implement in clinical practice.

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Circadian clock; mood disorders; depression; mania; bipolar disorder; chronotherapy

1. Introduction

The potential role of circadian clocks in mood regulation has been extensively discussed. There is strong evidence from animal and human studies that disturbances of circadian clocks are associated with the development of mood disorders like major depressive disorder (MDD), bipolar disorder (BD), and seasonal affective disorder (SAD) [1]. For instance, disruption of the sleep–wake cycle is such a characteristic feature of MDD and is one of the core symptoms that define the disorder [2]. Furthermore, patients with mood disorders strongly benefit from so-called chronotherapies targeting the circadian system. However, whereas there is considerable evidence that circadian clocks play a role in mood control and in the pathophysiology of mood disorders, therapeutic interventions targeting circadian clocks of patients are not yet routine in clinical practice.

We believe that one reason for the under-representation of the chronotherapies in medical practice is the lack of discussion of causal chains plausibly linking circadian rhythmicity and brain functions. Circadian intervention may become a more attractive therapeutic option for clinicians if they are provided with concrete explanations how disrupted circadian rhythms can contribute to mood disorders.

Accordingly, this review presents plausible and tangible mechanistic connections between circadian disturbances and mood disorders, thereby helping therapists and patients to understand why aligned and stable circadian clocks might be important. McClung has recently discussed various molecular connections between circadian clocks and mood regulation [3]. Expanding on this, we will discuss more general mechanistic links within the framework of fundamental circadian principles, forming a sound

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conceptual basis for implementing chronotherapies and circadian principles into clinical practice.

2. General information about circadian clocks

Life has adapted to recurring daily changes caused by the Earth's rotation. To optimally anticipate these daily changes, most living organisms evolved "circadian" (ca. 24 h) clocks that control almost all biological processes. To synchronize internal circadian rhythms to environmental fluctuations, circadian clocks are permanently reset by external cues, the so-called *Zeitgebers* (time givers, e.g. light/darkness, meal times, activity patterns). In response to these *Zeitgebers*, individuals can adopt very different timing preferences ("chronotypes") for daily routines and sleep, i.e. a tendency to "eveningness" with a delayed sleep time, or "morningness" with an early sleep time [4]. In mammals, the internal circadian timekeeping system is organized hierarchically, with the master circadian clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and subsidiary clocks in almost every cell of the body [5]. Rhythms in SCN neurons are mainly synchronized by light signals perceived by melanopsin-expressing retinal ganglion cells [6,7].

At the molecular level, the circadian clock mechanism is based on a transcriptional–translational feedback loop comprising "clock proteins" which regulate their own biosynthesis in a rhythm approximating 24 h. Central clock genes include *Brain Muscle Arnt-like Protein 1* (*Bmal1*), *Circadian Locomotor Output Cycles Kaput* (*CLOCK*), *Neuronal PAS Domain Protein 2* (NPAS2), *Period* (*PER1, PER2, and PER3*), and *Cryptochrome* (*CRY1* and *CRY2*). BMAL1 and CLOCK (or NPAS2) dimerize and bind to E-box elements in the promoters of target genes, including *Per* and *Cry*, thereby inducing transcription of those genes. In the cytoplasm, PER and CRY proteins form complexes which translocate back to the nucleus and repress BMAL1/CLOCK(NPAS2)-dependent transcriptional activity, thus inhibiting transcription of their own genes. Gradual degradation of PER/CRY complexes eventually diminishes this negative feedback, allowing a new molecular cycle to start [8]. An additional loop including both activating and inhibiting regulatory elements is formed by retinoic acid-related orphan receptors ROR (α, β, and γ) and nuclear receptors REV-ERB (α and β). Controlled by the rhythmic activity of these clock genes, many physiological and behavioural processes display circadian oscillations, e.g. in body temperature, hormones, and rest/activity, as well as affective and cognitive functions. In mammals, for example, the SCN orchestrates rhythmic release of the hormone melatonin from the pineal gland at night [9].

3. Connections between circadian clocks and mood regulation

3.1. Humans

Although there is strong evidence that mood disorders are associated with both genetic and environmental disturbances of circadian clocks, causality remains to be determined. Environmental disruptions of circadian rhythms, such as shift work, also increase the risk of mood disorders [10]. In patients with SAD, seasonal changes in photoperiod provoke relapse into a depressive episode [11]. SAD may be caused by SCN-mediated alterations of circadian rhythmicity which perturb the affective state [12]. Although not detected by GWAS after strict correction for multiple comparisons, associations between individual clock gene polymorphisms and mood disorders have been found in a number of targeted genotyping studies, e.g. sequence variations in *PER2*, *ARNTL*, and *NPAS2* increase the risk of developing SAD [13,14]. Similarly, BD has been linked to polymorphisms of clock genes [15], including *ARNTL* and *PER3* [16]. In an alternative approach providing greater sensitivity than GWAS, considering all clock genes together as a system has tended to confirm an association between clock genes and mood disorders [17]. Additionally, it may be that only a subgroup of MDD patients develops depression because of disrupted circadian rhythms; accordingly, significant associations with clock genes may be present only in this subgroup.

Beyond genetic and environmental associations, the therapeutic efficacy of chronotherapies targeting circadian rhythms in mood disorders patients provides further evidence for a strong connection between circadian clocks and mood regulation. For example, social rhythm therapy aims to stabilize and regularize daily rhythms of mood disorder patients and was proven to have high efficacy [18]. Some components of this therapy are described below in more detail. Moreover, even standard pharmacological treatment of depressive symptoms often improves circadian function of patients [1,19]. This suggests a bidirectional relationship between circadian rhythm abnormalities and mood disturbances, encouraging the development and implementation of new therapeutic approaches for mood disorders by normalization of circadian rhythms.

3.2. Animals

Further support for a relationship between circadian rhythms and mood dysregulation arises from animal models in which circadian rhythms can be manipulated more freely, and mood-related behaviour and its brain mechanisms studied more intensively [20].

3.2.1. Abolishing rhythms changes mood

The *Clock*$^{\Delta 19}$ mutant mouse has a long endogenous circadian period (~27 h) with eventual arrhythmia if kept in constant darkness [21]. These mice display a behavioural phenotype closely resembling bipolar mania in humans, with hyperactivity, reduced anxiety- and depression-related behaviour, and an enhanced preference for the consumption of drugs of abuse [22–24]. However, other symptoms comprising the rapid alternation between mania and depressive-like behaviour, sleep abnormalities, or grandiosity are not covered by this mouse model. In contrast, *Per2*$^{Brdm1-/-}$ mice have short period free-running rhythms that ultimately become arrhythmic in a constant environment [25] but also display mania-like behaviour.

3.2.2. Genetic manipulations of rhythmicity influence mood

Various other clock gene mutant mice also show altered affective behaviour. For instance, *Per1*$^{Brdm1-/-}$ mice have a short circadian period and show an enhanced preference for alcohol consumption after stressful events [26,27]. Mice deficient in D-box binding protein (DBP), which also have a shorter period, show depressive-like behaviour at baseline but mania-like behaviour under stress conditions, which might make this mouse a promising model for BD [28]. The *after hours (Afh)* mutant mouse, carrying a mutation of *Fbxl3,* exhibits a long free-running period (~26.5 h) [29] and mania-like behaviour [30]. Knockout of CK1δ/ε induces a long circadian period whereas a mutation of CK1ε (tau) causes a short period [31,32]. Whereas forebrain overexpression of CK1δ leads to a decrease in reward-seeking behaviour [33], genetic deletion of CK1ε leads to enhanced reward-seeking behaviour [34]. Finally, mutations in the GSK3αSK genes, which phosphorylate multiple clock genes and regulate circadian rhythms, are also associated with changes in mood-related behaviour [35–37].

3.2.3. SCN-specific reduction of rhythms induces depression-like behaviour

Although studies of circadian mutant mice suggest a causal role for circadian clocks in mood regulation, clock genes have pleiotropic effects, so behavioural changes in these mice may not necessarily be due to changes in clock function. Circumventing this issue, we showed that a reduction of circadian rhythm amplitude specifically in the SCN is sufficient to evoke depression-like behaviour in mice [38]. This mouse provides a model with intact clock genes in all non-SCN brain regions and peripheral organs, as well as normal brain development and anatomy. Therefore, abnormalities in affective state can be attributed solely to changes of circadian oscillation output signals from the SCN central pacemaker.

3.2.4. Changes in light-dark cycles influence mood

Manipulations of external light/dark conditions are widely used to modify circadian rhythms in animals. Such an experimental approach is especially useful to mimic seasonal changes in day length, providing animal models for SAD. In particular, diurnal rodents often exhibit depression-like behaviour in short days [39]. Exposure to constant light (LL) both disturbs circadian clocks and induces depression-like behaviour, implying a connection between circadian rhythms and mood regulation [40]. However, rodents exposed to constant darkness (DD) also show depression-like behaviour [41,42]. As circadian rhythms are not disrupted in DD, it is possible that mood-related abnormalities in response to altered lighting may (at least in part) depend on non-circadian factors.

4. How disturbed clocks may result in mood disorders

As outlined above, data from animal and human studies provide ample evidence that disturbed circadian clocks play a role in the development of mood disorders. However, mechanistic explanations of exactly how disorganized circadian rhythms can lead to mood disorders have not been widely discussed. Here, we extract common elements that are shared by different animal models and human patients that might serve as tangible links between circadian rhythm abnormalities and mood disorders. Identifying concrete and fundamental physiological changes resulting from disrupted circadian rhythms may help clinicians to appreciate the importance of stable and aligned circadian clocks in their patients.

The consequences of clock manipulations in all of the various animal models and human studies can be ascribed to four main possible mechanisms: “external desynchronization” between the animal and the environment, “internal desynchronization” within an animal, low amplitude of circadian oscillations, and alterations

in sleep architecture. Although each of these hypothetical mechanisms is based on experimental data, direct evidence favouring one mechanism over another is lacking. This is largely due to the difficulty of investigating one such circadian mechanism isolated from the others. For example, while many studies indicate a relationship between external desynchronization and mood regulation based on investigations of chronotypes and shift work, desynchronization with the environment in such studies is invariably accompanied by internal desynchronization, amplitude effects, and altered sleep patterns. Whereas some studies have begun to use clever experimental paradigms to manipulate external synchronization, amplitude, and sleep architecture independently, so far only few studies have examined the role of internal desynchronization. This may be at least partly due to the difficulty of creating animal models or identifying human markers to monitor misalignment of tissue oscillators within one subject. In this chapter, we will evaluate the presence of these individual mechanisms in different animal models and human studies and explain why they constitute health risks and might contribute to mood disorders.

4.1. External desynchronization

A desynchronization of the individual's endogenous circadian rhythms (e.g. sleepiness) with the external environment (e.g. sunrise) can produce adverse or pathological states. Animals with clock gene mutations often show changes in period and phase of rhythms, and some lose the ability to synchronize to external oscillations. Other studies use repeated light phase shifts so that animals never fully entrain to daily environmental rhythms. Humans with extreme chronotypes or subject to shift work also experience external desynchronization. And many patients with mood disorders show circadian phase abnormalities, e.g. advanced sleep–wake cycles in mania and delays in bipolar depression [43].

How could external desynchronization and altered light exposure result in depression? In humans, a late chronotype often leads to a lack of morning light exposure due to waking up late in the morning. Light deprivation leads to depression-like behaviour and a reduction of monoamine neurons in the brains of the animals [42]. Apoptosis of neurons is particularly pronounced in the nucleus accumbens and also occurs in the raphe nucleus and the ventral tegmental area, brain areas associated with mood regulation. In addition, Dulcis et al. demonstrated that neurons in the hypothalamus of rats switch between dopamine and somatostatin expression in response to exposure to short- and long-day photoperiods, respectively [44]. Furthermore, the reduction of dopamine in the hypothalamus in long days is accompanied by depression-like behaviour of these nocturnal animals. Interestingly, long days seem to have effects in nocturnal animals (e.g. mice) that are similar to those of short days in diurnal (day-active) animals (e.g. humans; see above). These rodent studies impressively demonstrate a negative impact of altered light exposure on brain structures, which ultimately leads to depression-like behaviour.

Lack of morning light due to a late chronotype may also result in other physiological changes associated with mood disorders. For instance, reduced sunlight exposure can lead to low levels of vitamin D, which is synthesized in the skin upon UV radiation. Some studies have demonstrated a correlation between low levels of vitamin D and MDD (or the severity of depression symptoms). However, no improvement of symptoms could be demonstrated with vitamin D supplementation, so the causality of the proposed relationship is uncertain [45–47].

Desynchronization with the environment may also lead to lack of social interaction in patients with mood disorders. Being desynchronized with the environment implies, for example, sleep–wake cycles and meal times that strongly differ from patterns of family and friends. Similarly, extreme chronotypes lead to difficulties in adapting to a common work schedule and impair participation in free-time activities. Although mood disorders patients can experience either phase advances or delays, phase delays are most concerning, as sleeping during the early day greatly reduces both light exposure and social contact.

In our view, changes in brain structures caused by lack of light constitute the most plausible mechanism for how a late chronotype can contribute to depression. As described, short winter days (Figure 1(A)) represent a risk factor for the development of SAD [48], and in rodents alterations in the light/dark cycle are associated with depression-like behaviour [44]. Of note, humans with very late chronotypes may be exposed to short photoperiods throughout the entire year (Figure 1(B)). Thus, depressive states in SAD or in MDD with delayed circadian rhythms might be largely attributable to insufficient light exposure during the day and the consequent lack of photic inputs relayed by the SCN to other brain regions that are involved in mood and motivational processes.

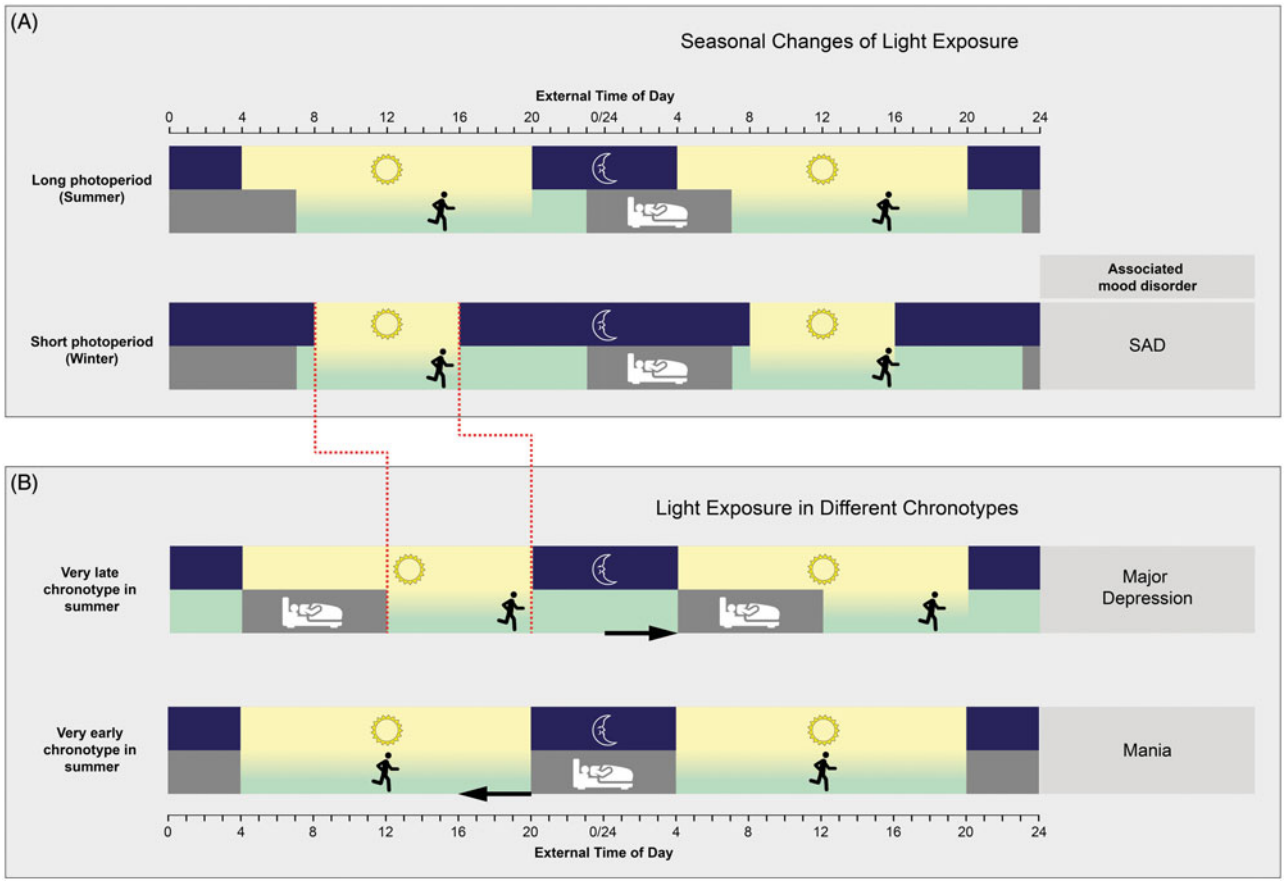

Figure 1. Light exposure during summer and winter months as well as in late and early chronotypes and associations with mood disorders. (A) Natural light exposure of summer and winter is compared to sleep–wake behaviour of a human with an average chronotype. In summer, long photoperiods ensure relatively long exposure to natural light during the activity phase. Contrarily, short photoperiods in winter have the effect that humans are exposed to only short periods of natural light during their activity phase. This condition facilitates the development of SAD during winter. (B) Humans with very late chronotypes are so delayed (arrow) that they miss a lot of daylight and may only receive as much natural light in summer like a person with a normal chronotype receives in winter (the orange dashed line demonstrates similar lengths of light exposure in winter of a normal chronotype and in summer of a late chronotype). In contrast, humans with an extreme early chronotype get a lot of daylight during their activity phase. The chronic lack of light may explain why usually late chronotype strongly correlates with MDD, and not early chronotypes. The lighting conditions approximate seasonal conditions in central Europe. Icons were taken from https://www.flaticon.com/.

Conversely, as mania is more typically associated with early chronotype, excessive daylight exposure in early morning may trigger manic switches in bipolar patients (Figure 1(B)). Increasing morning light exposure does not always induce manic switches [49], but dark therapy or use of blue-blocking glasses (which reduce activation of the blue-sensitive retinal photoreceptor responsible for light input to the SCN and other non-visual brain areas), have been found to reduce manic symptoms [50,51]. Interestingly, exposure to long photoperiod reduces synchrony of SCN neurons and leads to reduced rhythm amplitude in non-SCN brain regions of mice [52], which may contribute to the behavioural changes in mood disorders.

Together, these studies suggest that exposure to extreme photoperiods alters brain circuits and cause amplitude changes and/or disruption of circadian rhythms, including "internal desynchronization" of circadian oscillators. In the next sections, we will explore how internal desynchronization or reduced amplitude might facilitate mood disorders.

4.2. Internal desynchronization

Prominent human circadian rhythms include sleep–wake behaviour, hunger and satiety states, and body temperature rhythms, as well as many molecular oscillations, e.g. in neurotransmitter release and hormone levels. Naturally, these fluctuations peak at different times of day and night. Maintenance of optimal temporal relationships among these many rhythms in various organs, tissues, cells, and even

within one cell can be characterized as a state of internal synchronization. Internal synchrony helps the organism to coordinate compatible physiological processes and avoid coincidence of incompatible processes. However, animal and human studies show that temporal synchrony among different tissues and cells is rather fragile and can be manipulated easily by environmental factors [53–55].

Both animal and human studies have substantiated an association between inner circadian misalignment and mood disorders (and other major health problems) and suggest that internal temporal desynchrony might be part of the aetiology of MDD [56–58]. For instance, mutations of clock genes in animals disrupt phase relationships among molecules, cells, and tissues [59–61], and often lead to metabolic dysfunctions and pronounced changes in behaviour [20,62]. After light/dark shifts simulating jet lag, different organs and tissues adjust their phase at different rates, leading to transient internal desynchrony [63]. Such disruption of circadian rhythms has severe consequences on health and survival of rodents [64]. Similar considerations apply to human shift workers, who are at elevated risk of mood disorders and other diseases. Due to rapidly changing shift schedules, the clocks of shift workers can presumably never adjust to a stable phase relationship and are in a state of perpetual circadian misalignment. Also, because food is a strong Zeitgeber for non-SCN circadian clocks [53,65], as many people eat irregularly, their clocks may also be perpetually misaligned. Importantly, restricting meals to certain hours per day improves body weight and subjective well-being of subjects previously eating irregularly [66]. Interestingly, subjecting rodents to chronic stress also induces circadian misalignment among tissues [67], which may contribute to depression. Indeed, patients with MDD exhibit an altered phase relationship between sleep and body temperature rhythms [68].

Genetically determined properties of the circadian clock may increase vulnerability to internal desynchronization in certain people. Humans only entrain to a relatively small range of *Zeitgeber* periods [69–71] and entrainment to cycles very different from the endogenous period require stronger *Zeitgebers* [72]. Consequently, individuals with a very long or short endogenous period have more difficulty entraining to environmental rhythms, and may also be more vulnerable to internal desynchronization, as some tissues may be more sensitive to *Zeitgebers* than others. In particular, it is possible that some tissues may be well entrained to the 24-h environment while others mainly follow their own internal clocks, leading to a situation where different oscillators drift apart in phase.

There are different possible mechanisms by which temporal desynchronization among molecules, cells, and tissues may induce diseases and mood disorders. Desynchronization of processes on the neural, hormonal, and behavioural level can lead to insufficient coordination of recurring events of daily life, a cardinal function of the circadian system. Loss of normal daily synchronization might demand instead a more energetically costly and less effective ad hoc regulation of physiology and behaviour. In patients suffering from mood disorders, their daily routines are often disrupted, probably making unexpected situations even more stressful and demanding, which may then reinforce fatigue and loss of motivation [73].

Temporal desynchronization may also impair communication among different tissues drastically. Under normal circumstances, phases of distinct tissues are aligned such that one tissue is prepared to receive and process signals from other tissues at a particular time of day. If, however, two tissues are out of phase, it is possible that the receptor for a crucial signal is not present at a time when the signal arises, or vice versa. In the context of mood disorders, this may apply to insufficient communication among different brain areas implicated in affective functions (Figure 2(A)). Only proper synchronization of daily signals allows a smooth flow of multi-step processes in the body. For example, if the timing of multiple signals leading to the secretion of a neurotransmitter proceeds in the wrong order, the processes that depend on this neurotransmitter secretion may be impaired (Figure 2(B)).

4.3. Reduced amplitude

Altered circadian rhythm amplitudes (mostly decreases) have frequently been observed in mood disorders. A landmark study of postmortem brains from MDD patients and healthy controls showed that clock genes such as *BMAL1*, *PER1-3*, *DEC1/2*, *REV-ERBα*, and *DBP* are expressed with clear circadian rhythms in mood-related brain regions, but that these rhythms are much less prominent in brains from MDD patients [74]. Importantly, this study demonstrated dysregulation of circadian rhythmicity at a molecular level in brains of MDD patients. However, as only one time point could be measured for each brain, the weaker circadian rhythms observed in the depressed population might be due to either poor synchronization of

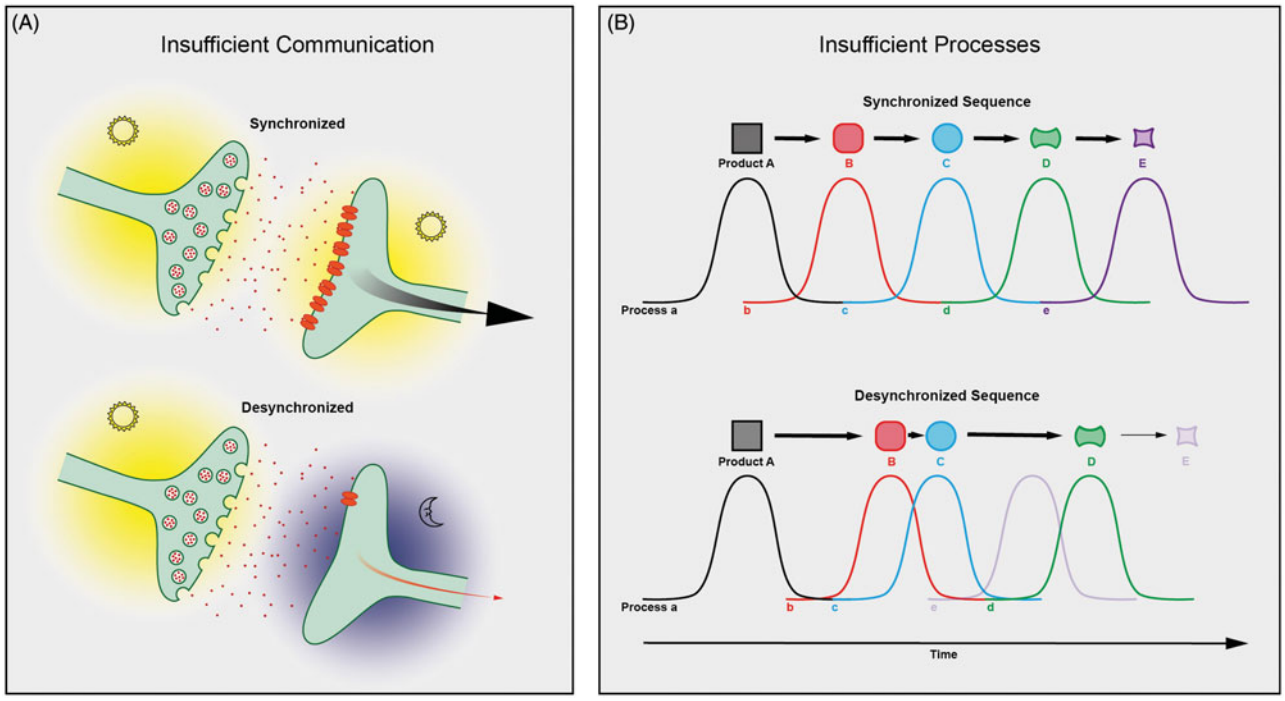

Figure 2. Possible complications induced by internal desynchronization. (A) Internal desynchronization may promote insufficient communication among different tissues. As an example, two neurons of different brain areas are shown. The release of most neurotransmitters is under control of circadian clocks. Similarly, the expression of their receptors is often adjusted accordingly. Consequently, under synchronized conditions, at times of high neurotransmitter release the receptor is most prevalent ensuring optimal communication among the brain areas. In contrast, when brain areas are not synchronized, the release of a neurotransmitter may be ineffective as the receptor is not yet fully expressed or the peak of expression is already over. (B) Internal desynchronization might affect the flow of various physiological processes in the body. Shown is a hypothetical sequence of processes a–e leading to product E. The processes may occur within one cell type/tissue or in different tissues. The circadian clock determines the timing of processes a–e and ensures the fluent synthesis of product E. In case circadian clocks are not synchronized and, therefore, the sequence of processes a–e is disturbed, the production of intermediate products B–D may be affected. In the worst case, when the timing of subsequent processes is extremely misaligned, the synthesis of product E is strongly impaired.

the depressed individuals with external *Zeitgebers* or lower rhythm amplitude in individual subjects. The interpretation of lower rhythm amplitude within one subject is consistent with our mouse study of the relationship of depression-like behaviour and circadian oscillations in mood-regulating brain areas observed over the course of several days within the same animal. Mice susceptible to induction of depression-like behaviour by a learned helplessness training procedure were more likely to have weaker rhythms in the nucleus accumbens and the periaqueductal grey, two brain regions crucially involved in mood regulation [75]. Furthermore, chronically elevated stress in mice alters circadian amplitude in the SCN and mood regulating brain areas, including the nucleus accumbens [76]. Conversely, experimentally reducing amplitude of SCN circadian rhythms by SCN-specific knockdown of *Bmal1* elicited depression-like behaviour in mice [38].

Circadian rhythm amplitude in a tissue is influenced by the strength of the individual cellular circadian oscillators within the tissue as well as by coupling among those cellular oscillators [77]. In either case, low circadian rhythm amplitude at the tissue level can contribute to an unstable circadian system and adversely affect mood regulation. Under normal circumstances, circadian clocks are responsible for ensuring proper timing of physiological processes in the body, particularly separating opposing processes in time, e.g. anabolic versus catabolic pathways or wakefulness versus sleep. With reduced circadian amplitude, the separation of such opposing processes cannot take place efficiently, resulting in simultaneous occurrence of conflicting processes or conditions (Figure 3(A)). For instance, more than 80% of mood disorders patients suffer from sleep problems during the night and fatigue during the day [78]. High cortisol levels are associated with wakefulness and elevated melatonin with sleep, so their rise and fall need to be separated to avoid inappropriately timed wakefulness during the night or sleepiness during the day. Consistent with this idea, in depressed patients, the rhythm amplitude of these hormones is decreased in

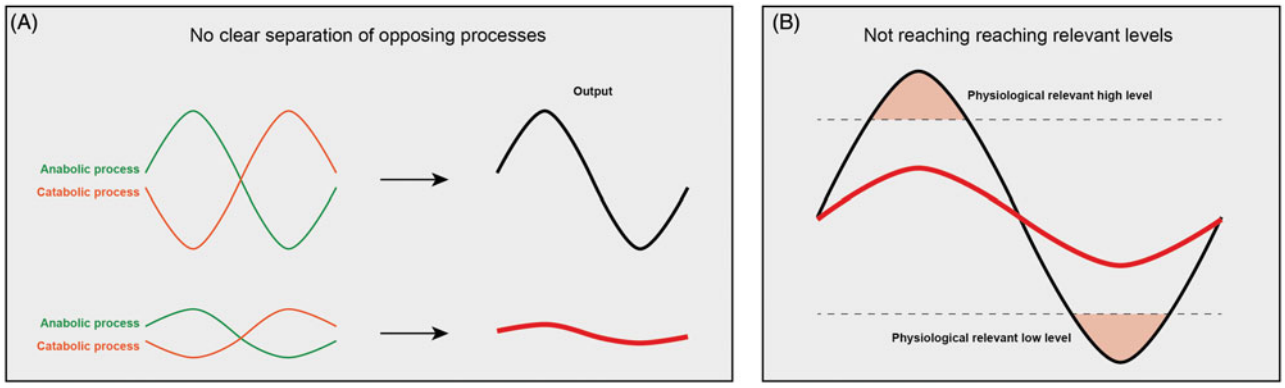

Figure 3. Consequences of decreased circadian rhythm amplitude. (A) Circadian clocks ensure the proper timing and separation of opposing processes in the body, e.g. anabolic versus catabolic pathways, such that the aggregate output of the two processes shows strong circadian rhythms. With low amplitude rhythms, the separation of opposing processes is not very pronounced, and their output shows much weaker rhythmicity. (B) Many physiological processes require a threshold level of activators/inhibitors in order to be induced or interrupted. When circadian rhythm amplitude is low, these levels may never be reached, and the process cannot take place.

depression [79–82], resulting in a less pronounced contrast between nocturnal sleepiness and diurnal wakefulness.

Second, precisely timed initiation or termination of many physiologically relevant processes is dependent on high amplitude circadian rhythms. Such physiological transitions often require certain threshold levels of activators/inhibitors (e.g. neurotransmitters). Thus, in the event of low rhythm amplitude, threshold levels might not be reached, and the physiological process cannot properly start or stop (Figure 3(B)). As mentioned above, cortisol and melatonin rhythms show reductions of amplitude in depression, with relatively constant high levels of cortisol and low levels of melatonin [79,80]. Thus, due to reduced circadian amplitude, cortisol levels never drop low enough and melatonin levels never get high enough to permit efficient sleep.

Lastly, altered amplitudes may also encourage internal desynchronization among tissue clocks due to differential sensitivity to *Zeitgebers*. Decreased amplitude of a circadian pacemaker increases its sensitivity to resetting [83–85]. Thus, the phase relationships among oscillators of different tissues are changed more easily in response to *Zeitgeber* modulations when rhythm amplitude is low. Conversely, increasing the amplitude of circadian rhythms in patients lowers the risk of both internal and external desynchronization.

In summary, both hypothetical assumptions and empirical data suggest strongly that altered circadian rhythm amplitudes, especially decreased amplitudes, can lead to profound circadian disruption in mammalian tissues. Conversely, increasing the amplitude of circadian oscillators in patients suffering from mood disorders might reduce internal and external desynchronization processes, leading to a stabilization of the circadian system.

4.4. Changes in sleep architecture

Sleep architecture refers to the temporal organization of sleep such as number, duration, and timing of rapid eye movement (REM) and non-REM (NREM) sleep episodes. Reduced sleep quality as attributed to abnormal sleep architecture is strongly associated with mood disorders [86]. Current evidence is insufficient to determine conclusively whether the disruption of sleep–wake cycles in MDD patients is a circadian or sleep related phenomenon. Because sleep and circadian clocks influence each other strongly, it may be impossible to separate them definitively. However, it is known that circadian clocks influence sleep architecture [87]. Thus, changes in circadian rhythmicity could lead indirectly to abnormal mood regulation in patients, through their impact on sleep. For instance, human polymorphisms of the clock gene *PER3* have been associated with altered sleep timing and structure [88,89]. Interestingly, *Per3*$^{-/-}$ mice and mice carrying a human variant of the *PER3* gene exhibit both altered sleep architecture and depression-like behaviour [90]. Thus, *PER3* may represent a connecting link between circadian clocks, sleep, and mood regulation. *Clock*$^{\Delta 19}$ mice exhibit severe mania-like behaviour [22] and also reduced sleep [91]. *Cry1/2* double-deficient mice show anxiety-like behaviour [92] as well as increased sleep [93]. Interestingly, however, mice lacking *Per1/2* also exhibit anxiety-like behaviour, similar to *Cry1/2* double-deficient mice, but their sleep is reduced rather than elevated [93].

A vast clinical literature shows that mood disorders are commonly accompanied by sleep disturbances. For example, 50–90% of depressed patients suffer sleep abnormalities [94]. The nature of sleep problems in patients with mood disorders is manifold [95]. Many depressed patients as well as manic patients suffer from insomnia, whereas a decreased need for sleep is a trademark of manic patients. Also common in depression are longer sleep onset latency and early morning awakenings. Changes in REM and NREM sleep architecture are frequently observed, with REM sleep typically peaking earlier in the night in depressed patients. Such disruptions of sleep timing suggest that some of the sleep problems seen in mood disorders may in part be attributable to abnormalities in circadian rhythms. Although an association between sleep disturbances and abnormal mood regulation is well established, the direction of causality remains unclear. While the above-mentioned impairments of sleep quality can be regarded as symptoms of depression, there is also considerable evidence that sleep directly impacts affective functions. For instance, sleep positively influences the function of many mood-regulating brain areas, including prefrontal cortex, amygdala, and locus coeruleus [96]. Furthermore, it is believed that the improvement of sleep by antidepressants contributes to their therapeutic efficacy [97].

Thus, it is likely that sleep is one direct mechanistic link between circadian clocks and mood regulation: disturbing circadian rhythms leads to sleep abnormalities, which in turn adversely impact mood regulation. Depending on the disposition and resilience of the individual, these clock-induced alterations of sleep may contribute greatly to development of a mood disorder.

5. Clinical implementation

As mentioned above, episodes of depression and mania in MDD and BD are often characterized by irregular circadian patterns of behaviour. Critically, patients are at increased risk for mood episodes after drastic life events that lead to severe changes of daily routines (e.g. sudden loss of partner, giving birth) [18]. Furthermore, rotating work shifts frequently precipitate episodes or exacerbate mood symptoms. Physicians have several therapeutic options to help patients achieve stable circadian rhythms. Essentially, these strategies aim for structuring the daily routines of patients as much as possible.

5.1. Regular sleep

The timing of sleep and wakefulness is largely controlled by circadian clocks, but sleep and wakefulness also directly or indirectly alter circadian rhythms. Indeed, sleep can act as a *Zeitgeber* for circadian rhythms. Depending on the time of day, naps can cause phase advances or phase delays of melatonin and other hormone rhythms [98–100]. Whether these effects are mediated directly by sleep or indirectly by reduction of light, activity, social contacts, or changes of posture during naps is not clear [100]. Insufficient sleep also alters the temporal organization of the human blood transcriptome, reducing circadian rhythm amplitude [101].

Many mood disorder patients have poor quality sleep and highly irregular sleep times [102,103], so clinicians should help patients to regularize their sleep as much as possible. Importantly, schedules for sleep and wake-up times should to be tailored to the individual chronotype of each patient, as far as possible. Unfortunately, most people have to get up early on work days, interrupting their natural sleep, and then make up for this disruption by sleeping late on weekends, resulting in repeated back-and-forth phase shifts every week, a phenomenon commonly known as "social jet lag" [104]. But while the longer and delayed sleep on work-free days might feel refreshing for healthy people, the frequent switch between early and late days may become problematic for patients with mood disorders. For these patients, to promote internal synchrony, it is crucial to reduce the schedule shifts between work and work-free days as much as possible. Regular sleep times are also likely to improve sleep quality. Recording baseline sleep/wake routines of patients by wristband actimetres or sleep diaries can help therapists and patients to develop an individualized ideal sleep schedules. On the basis of these measures, irregularities and anomalies can be identified and improved. Notably, cognitive behavioural therapy for insomnia (CBT-I) also improves depressive symptoms [105].

5.2. Regular meals

The circadian clocks of many tissues outside the SCN are very sensitive to meal times [53,65]. To anticipate meal times and to optimize nutrient utilization, organs involved in digestion uncouple from the SCN and gradually adjust their phase to a new meal time over the course of several days [106]. Importantly, the adjustment of tissue clocks takes multiple days, and this process is only beneficial for the organism when

the timing of meals settles stably at the new time. Many people, however, eat at remarkably variable times [66]. If meal times vary greatly from day to day, the clocks in digestive organs never have enough time to adjust completely. Consequently, the clocks of their digestive organs are probably in a perpetual state of disequilibrium, constantly adjusting to new meal times. This phenomenon may lead to significant health problems. Moreover, peripheral and brain clocks are closely linked to each other through hormones [107], and food intake promotes changes in glucocorticoids, body temperature, neuronal activity, and other factors that have impact on brain clocks. Thus, food-induced desynchronization of peripheral organs may also affect synchronization among clocks of various brain regions, thereby contributing to development of mood disorders.

Since the impact of meals on peripheral clocks is so strong, clinicians should encourage their patients to maintain a fixed meal time schedule. Patients should be encouraged to avoid snacks and caloric drinks between scheduled principal meals, as any food intake has an immediate impact on peripheral clocks. Obesity is associated with late meal times [108], so breakfast should be encouraged, and late evening meals or snacks discouraged. But it must be acknowledged that, as for sleep timing, ideal meal schedules may vary from person to person, depending on chronotype, personal preference, and constraints imposed by social or work demands. Nevertheless, it still may be very valuable for patients to minimize day-to-day variability in whatever meal schedule they are willing to adopt.

5.3. Increase of daytime physical activity

Physical activity alleviates depression, and depressed patients are often encouraged to exercise [109]. Possible mechanisms for this therapeutic effect of exercise on mood include augmentation of endogenous opioids [110], normalization of brain structure abnormalities [111], and improving sleep quality [112].

But another possible mechanism is through positive effects of exercise on the circadian system. In mice, exercise enhances the amplitude of circadian rhythms of body temperature, food uptake, and corticosterone secretion [113], three factors involved in synchronizing body clocks. Indeed, physical exercise can alter the phase of clock gene rhythms in peripheral tissues of mice [114]. Importantly, providing mice with access to running wheels significantly decreases age-related desynchrony in SCN and peripheral tissues [115]. In addition, physical activity increases amplitude of SCN and behavioural rhythms, and accelerates phase adjustment after experimental jet lag [115]. Furthermore, access to running wheels enhances amplitude of activity rhythms and reduces depression- and anxiety-like behaviour in rats [116], and protects against the development of depression-like behaviour in mice [117].

In humans, physical activity also has an impact on the circadian system. Depending on the time of day, exercise alters temperature, heart rate, and melatonin rhythms and changes sleep architecture [118]. As in mice, exercise facilitates re-entrainment after phase shifts [119], arguing for positive synchronizing effects of exercise in humans. Additionally, in young men multivariate regression analysis reveals that physical activity has by far the strongest influence on the amplitude and stability of body temperature oscillations of any parameter studied [120]. However, clinicians should make their patients aware that the timing of physical exercise is critical, e.g. nocturnal exercise can phase shift behavioural and hormonal rhythms in a manner that might be unfavourable for the patient [121,122]. Together, these results suggest that regular exercise during the day supports the stabilization of circadian oscillations in patients with weak or irregular daily rhythms.

5.4. Light therapies

In the face of overwhelming competitive advertising by the pharmaceutical industry, light therapy for depression has been slow to achieve widespread adoption in clinical practice, but it is now clear that it can be as effective as antidepressant medicines [123], and is finally becoming more popular [124]. Twenty years ago, a series of studies established that bright light (especially in the morning) is an effective treatment for SAD [125–127]. More recently, a number of studies and meta-analyses have suggested that light is also effective for MDD [128–130]. In particular, a recent randomized, controlled 8 week trial of daily 10,000 lux bright light for 30 min “as early as possible” after awakening, showed that light can be more effective than fluoxetine (Prozac) for non-seasonal MDD [131]. A few studies indicate that bright light may even be therapeutic for the depression of BD, which is often unresponsive to common antidepressant medicines. For example, a recent randomized, controlled 6 week trial of 7000 lux bright light in midday improved sleep and mood in BD, with no manic switches [132]. Conversely, darkness can be

therapeutic for mania [51], and a recent study showed promising antimanic effects of blue-blocking glasses [50]. Thus, light/dark therapies for mood disorders show great clinical promise.

It is not known precisely how light/dark therapies work in mood disorders patients, but animal studies suggest some likely neurobiological mechanisms. First of all, it seems probable that the therapeutic effects of light are mediated not by the conventional image-forming visual system, but rather by the more recently described non-image-forming visual system, involving melanopsin-containing, intrinsically photosensitive retinal ganglion cells (ipRGCs) [133–135]. The ipRGCs project to the SCN and are responsible for circadian photoentrainment, so the effects of light/dark on mood could involve adjustment of brain circadian clocks. The finding that early morning timing of light makes it more effective in SAD suggests that the mechanism may involve circadian phase-dependent shifting of clock phase and improved alignment of brain clocks. It is known, for example, that seasonal changes in day length produce substantial changes in relative phasing of individual SCN neurons in rodents [136]. On the other hand, the fact that some clinical studies (e.g. [132]) find light effective at mid-day, when no phase shifting of circadian clocks would be expected, suggests that enhancement of brain clock amplitude may be a more important mechanism. This is consistent with the findings of a postmortem human brain study that brain clocks of MDD patients have reduced amplitude [74], and with our work in a mouse model [38].

5.5. Wake therapy

It is well documented that sleep deprivation has strong and immediate antidepressant effects, alleviating symptoms of MDD in 40–60% of patients. Improvements in mood are often already apparent even during the night of sleep deprivation and are sustained on the following day. After recovery sleep, most patients (50–80%) suffer from a relapse of depression [137]. Interestingly, keeping patients awake in the second half of the night appears to be sufficient to improve depressive symptoms [124]. Since sleep and circadian clocks are tightly intertwined, the effects of wakefulness at night on mood are often attributed to changes of circadian rhythms. However, even short daytime naps which have only little impact on circadian rhythms cause a relapse of symptoms, raising doubt about how much circadian changes contribute to the antidepressant effects of sleep deprivation. On the other hand, some have hypothesized that MDD patients have abnormal clock gene machinery which is reset by sleep deprivation, thereby improving their affective state [138]. However, a combination of chronotherapeutic treatments such as bright light, sleep phase advance and pharmacotherapy (lithium or antidepressants) has been proven to sustain the antidepressant responses and prevent relapse [139]. Also, the rapid-acting antidepressant ketamine and sleep deprivation produce similar changes in expression patterns of some clock genes [140]. Thus, a combination of neurotransmitter changes during sleep deprivation and adjustment of circadian rhythms during phase advance therapy may underlie the efficacy of this treatment.

Even though the exact mechanisms are not known, wake therapy is a first line of treatment for depression in many European countries, although rarely used in the United States [141].

5.6. Pharmaceuticals to manipulate rhythms

5.6.1. Agomelatine

Agomelatine is a recently developed antidepressant drug available for use in Europe. It acts as an agonist at melatonin receptors MT_1 and MT_2 and an antagonist at the serotonin 5-HT_{2C} receptor. Antidepressant actions of agomelatine have been attributed to various mechanisms including adjustment of circadian rhythms. In diurnal and nocturnal rodents, agomelatine expedites resynchronization of behaviour after shifts in the light-dark cycle [142,143]. Furthermore, it is able to adjust the phase of sleep–wake cycles in rodents under entrained conditions [144], increases the amplitude of melatonin and body temperature rhythms [145], and can synchronize rats to a 24 h schedule when administered in daily doses under constant environmental conditions [146]. Thus, agomelatine has a strong impact on the circadian system.

Importantly, agomelatine normalizes behavioural rhythms, sleep architecture, and neuronal activity in a rat model of depression, which suggests the potential for positive effects on clocks in depressed patients [147]. In humans, early evening administration of agomelatine (like melatonin) phase advances two central synchronizers of peripheral rhythms, core body temperature [148], and cortisol rhythms [149], which might benefit depressed patients with delayed phases. Because of these effects, administration of agomelatine has been suggested as a therapy for several psychiatric disorders that are associated with circadian disturbances [150]. Besides adjustment of circadian

rhythms, agomelatine promotes neurogenesis, cell survival, and expression of brain-derived neurotrophic factor (BDNF), which are other possible mechanisms of antidepressant action. Of note, however, a recent study showed that stress-induced changes of circadian rhythms in mood-regulating brain areas of rats were not normalized by agomelatine administration. Moreover, melatonin itself was not found to have an antidepressant effect [151], raising questions about whether agomelatine's circadian effects underlie its antidepressant action [152]. On the other hand, melatonin could still be therapeutic if its administration is properly timed and dosed to adjust the misaligned circadian phases of certain patients with mood disorders [153].

5.6.2. Lithium

Lithium is a mood stabilizer widely used in the treatment of BD, and some have speculated that its therapeutic efficacy could be related to its prominent effects on circadian rhythms [154]. Lithium lengthens circadian period and potently increases amplitude of circadian rhythms [155]. These circadian effects of lithium may be related to its inhibition of glycogen synthase kinase 3 (GSK3), a protein kinase that regulates the clock by phosphorylating multiple clock proteins: PER2, REV-ERBα, and CRY2. Lithium also affects the inositol signalling pathway, which is also involved in amplitude and period regulation of circadian rhythms [156]. It is likely that lithium contributes to the stabilization of circadian clocks in BD patients. However, since lithium has many side effects and regulates a large number of processes in the body, better options are needed for primary targeting of circadian rhythms.

5.6.3. Nobiletin

It has been discovered recently that the flavonoid nobiletin is capable of regulating circadian rhythms. Flavonoids are plant pigments that are ubiquitous natural components of human foods. In mice, nobiletin substantially increases the amplitude of clock gene rhythms in cells and behavioural wheel-running activity rhythms [157,158]. Interestingly, mice exposed to a high fat diet are protected from metabolic phenotypes when treated with nobiletin [157]. Furthermore, nobiletin resets the liver clock and defends against metabolic abnormalities in cultured cells [159]. Thus, stabilizing body clocks using nobiletin or similar compounds could potentially be useful in mood disorder treatment.

5.6.4. Rev-Erb agonists

The experimental compounds SR9009 (Stenabolic) and SR9011 are agonists of the circadian clock proteins REV-ERBα and REV-ERBβ; they alter amplitude of clock gene expression rhythms and change circadian behaviour in mice [160]. Interestingly, in rodents, these drugs also reduce anxiety-like behaviour, change sleep architecture [161,162], and prevent the development of obesity, which is often related to mood disorders [160]. As the effects of these REV-ERB agonists on circadian rhythms are pronounced but short-term, more research is required to investigate the potential usefulness of such drugs in mood disorder therapy.

6. Conclusion

Many mood disorder patients suffer irregularities in their daily rhythms. These irregularities (e.g. insomnia) may in themselves be unpleasant and uncomfortable for patients. But the disruptions may also trigger or worsen mood episodes. For both these reasons, therapies aiming to improve circadian organization and stability may be beneficial. The circadian clock evolved to help organisms anticipate daily recurring events. If this ability is lost, the body is no longer capable of preparing physiological processes in advance of an event, but rather must react spontaneously to incidents, which may be less efficient and more stressful. Thus, therapies that reduce the day-to-day variability of circadian rhythms are likely to be helpful. However, more evidence is required to establish a direct causal connection between the disruption of circadian rhythms and mood disorders, especially in humans. To prove such causality, more knowledge about underlying mechanisms is needed. In particular, more research is needed to show specific mechanisms by which existing chronotherapies could improve mood by modulating circadian rhythms.

In this review paper, we suggested several concrete mechanisms by which circadian disruptions may contribute to mood disorders: external desynchronization, internal desynchronization, reduced amplitude, and impaired sleep. Importantly, the separation of these mechanisms remains hypothetical since they inevitably influence each other. Therapies to stabilize and strengthen circadian clocks would be expected to normalize all these pathological mechanisms at once. Therapeutic manipulations may variously affect clock phase, period, or amplitude, but in practice these clock parameters are usually intimately related. Thus, the aim is usually to reach a high amplitude, a stable period close to 24 h, a phase compatible with

environmental conditions, and a small phase variability from day to day. Because of the ubiquitous role of circadian clocks in physiology, adjusting circadian properties to such desired values can solve many medical problems at once, including mood disorders.

Fortunately, clinicians and patients have relatively simple therapeutic options available to improve circadian health. The list of options includes regular sleep, meals, and exercise, light therapy, and a few pharmaceuticals such as melatonin. Even though the strategies were discussed separately in this paper, it is advisable to administer several strategies simultaneously. Patients who suffer severe circadian disruptions will gain the greatest benefit if they structure sleep, light, and meal times, and increase amounts of physical activity in parallel. Conveniently, these treatments can target several circadian conditions concurrently. For example, patients committed to (daytime outdoor) physical exercise automatically increase the amount of sunlight. In turn, the increase of sunlight helps to improve rhythm amplitude and, if applied at the right time, shifts the clock to a desired phase. Furthermore, eating and sleeping at regular times helps structuring other daily routines and promotes social synchronization, and will likely also have beneficial effects for metabolic disorders, which are quite frequently comorbid with mood disorders.

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References

[1] Bechtel W. Circadian rhythms and mood disorders: are the phenomena and mechanisms causally related? Front Psychiatry. 2015;6:118.
[2] Kronfeld-Schor N, Einat H. Circadian rhythms and depression: human psychopathology and animal models. Neuropharmacology. 2012;62:101–114.
[3] McClung CA. How might circadian rhythms control mood? Let me count the ways. Biol Psychiatry. 2013; 74:242–249.
[4] Roenneberg T, Kuehnle T, Juda M, et al. Epidemiology of the human circadian clock. Sleep Med Rev. 2007;11:429–438.
[5] Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol. 2010;72:517–549.
[6] Berson DM, Dunn FA, Takao M. Phototransduction by retinal ganglion cells that set the circadian clock. Science. 2002;295:1070–1073.
[7] Hattar S, Liao HW, Takao M, et al. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science. 2002; 295:1065–1070.
[8] Koike N, Yoo S-H, Huang H-C, et al. Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science. 2012;338: 349–354.
[9] Goldman BD. Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms. 2001;16:283–301.
[10] Rosenberg R, Doghramji PP. Is shift work making your patient sick? Emerging theories and therapies for treating shift work disorder. Postgrad Med. 2011; 123:106–115.
[11] Lewy AJ, Lefler BJ, Emens JS, et al. The circadian basis of winter depression. Proc Natl Acad Sci USA. 2006;103:7414–7419.
[12] Porcu A, Riddle M, Dulcis D, et al. Photoperiod-induced neuroplasticity in the circadian system. Neural Plasticity. 2018;2018:5147585.
[13] Etain B, Milhiet V, Bellivier F, et al. Genetics of circadian rhythms and mood spectrum disorders. Eur Neuropsychopharmacol. 2011;21:S676–S682.
[14] Partonen T, Treutlein J, Alpman A, et al. Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression. Ann Med. 2007;39:229–238.
[15] Milhiet V, Etain B, Boudebesse C, et al. Circadian biomarkers, circadian genes and bipolar disorders. J Physiol Paris. 2011;105:183–189.
[16] Nievergelt CM, Kripke DF, Barrett TB, et al. Suggestive evidence for association of the circadian genes PERIOD3 and ARNTL with bipolar disorder. Am J Med Genet. 2006;141B:234–241.
[17] McCarthy MJ, Nievergelt CM, Kelsoe JR, et al. A survey of genomic studies supports association of circadian clock genes with bipolar disorder spectrum illnesses and lithium response. PLoS One. 2012;7: e32091.

[18] Frank E, Swartz HA, Kupfer DJ. Interpersonal and social rhythm therapy: managing the chaos of bipolar disorder. Biol Psychiatry. 2000;48:593–604.
[19] Monteleone P, Martiadis V, Maj M. Circadian rhythms and treatment implications in depression. Prog Neuro-Psychopharmacol Biol Psychiatry. 2011;35: 1569–1574.
[20] Landgraf D, McCarthy MJ, Welsh DK. The role of the circadian clock in animal models of mood disorders. Behav Neurosci. 2014;128:344–359.
[21] Vitaterna MH, King DP, Chang AM, et al. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science. 1994;264: 719–725.
[22] Roybal K, Theobold D, Graham A, et al. Mania-like behavior induced by disruption of CLOCK. Proc Natl Acad Sci USA. 2007;104:6406–6411.
[23] Ozburn AR, Larson EB, Self DW, et al. Cocaine self-administration behaviors in ClockΔ19 mice. Psychopharmacology (Berl). 2012;223:169–177.
[24] McClung CA, Sidiropoulou K, Vitaterna M, et al. Regulation of dopaminergic transmission and cocaine reward by the Clock gene. Proc Natl Acad Sci USA. 2005;102:9377–9381.
[25] Zheng B, Larkin DW, Albrecht U, et al. The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature. 1999;400:169–173.
[26] Dong L, Bilbao A, Laucht M, et al. Effects of the circadian rhythm gene period 1 (per1) on psychosocial stress-induced alcohol drinking. Am J Psychiatry. 2011;168:1090–1098.
[27] Zheng B, Albrecht U, Kaasik K, et al. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell. 2001;105:683–694.
[28] Le-Niculescu H, McFarland MJ, Ogden CA, et al. Phenomic, convergent functional genomic, and biomarker studies in a stress-reactive genetic animal model of bipolar disorder and co-morbid alcoholism. Am J Med Genet. 2008;147B:134–166.
[29] Godinho SIH, Maywood ES, Shaw L, et al. The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. Science. 2007;316: 897–900.
[30] Keers R, Pedroso I, Breen G, et al. Reduced anxiety and depression-like behaviours in the circadian period mutant mouse afterhours. PLoS One. 2012;7: e38263.
[31] Etchegaray J-P, Machida KK, Noton E, et al. Casein kinase 1 delta regulates the pace of the mammalian circadian clock. Mol Cell Biol. 2009;29:3853–3866.
[32] Loudon ASI, Meng QJ, Maywood ES, et al. The biology of the circadian Ck1epsilon tau mutation in mice and Syrian hamsters: a tale of two species. Cold Spring Harb Symp Quant Biol. 2007;72:261–271.
[33] Zhou M, Rebholz H, Brocia C, et al. Forebrain overexpression of CK1delta leads to down-regulation of dopamine receptors and altered locomotor activity reminiscent of ADHD. Proc Natl Acad Sci USA. 2010; 107:4401–4406.
[34] Bryant CD, Parker CC, Zhou L, et al. Csnk1e is a genetic regulator of sensitivity to psychostimulants and opioids. Neuropsychopharmacology. 2012;37: 1026–1035.
[35] Ackermann TF, Kempe DS, Lang F, et al. Hyperactivity and enhanced curiosity of mice expressing PKB/SGK-resistant glycogen synthase kinase-3 (GSK-3). Cell Physiol Biochem. 2010;25: 775–786.
[36] Paul JR, Johnson RL, Jope RS, et al. Disruption of circadian rhythmicity and suprachiasmatic action potential frequency in a mouse model with constitutive activation of glycogen synthase kinase 3. Neuroscience. 2012;226:1–9.
[37] Polter A, Beurel E, Yang S, et al. Deficiency in the inhibitory serine-phosphorylation of glycogen synthase kinase-3 increases sensitivity to mood disturbances. Neuropsychopharmacology. 2010;35: 1761–1774.
[38] Landgraf D, Long JE, Proulx CD, et al. Genetic disruption of circadian rhythms in the suprachiasmatic nucleus causes helplessness, behavioral despair, and anxiety-like behavior in mice. Biol Psychiatry. 2016; 80:827–835.
[39] Leach G, Adidharma W, Yan L. Depression-like responses induced by daytime light deficiency in the diurnal grass rat (Arvicanthis niloticus). PLoS One. 2013;8:e57115.
[40] Tapia-Osorio A, Salgado-Delgado R, Angeles-Castellanos M, et al. Disruption of circadian rhythms due to chronic constant light leads to depressive and anxiety-like behaviors in the rat. Behav Brain Res. 2013;252:1–9.
[41] Monje FJ, Cabatic M, Divisch I, et al. Constant darkness induces IL-6-dependent depression-like behavior through the NF-kappaB signaling pathway. J Neurosci. 2011;31:9075–9083.
[42] Gonzalez MM, Aston-Jones G. Light deprivation damages monoamine neurons and produces a depressive behavioral phenotype in rats. Proc Natl Acad Sci U S A. 2008;105:4898–4903.
[43] Moon J-H, Cho C-H, Son GH, et al. Advanced circadian phase in mania and delayed circadian phase in mixed mania and depression returned to normal after treatment of bipolar disorder. EBioMedicine. 2016;11:285–295.
[44] Dulcis D, Jamshidi P, Leutgeb S, et al. Neurotransmitter switching in the adult brain regulates behavior. Science. 2013;340:449–453.
[45] Anglin RES, Samaan Z, Walter SD, et al. Vitamin D deficiency and depression in adults: systematic review and meta-analysis. Br J Psychiatry. 2013;202: 100–107.
[46] Jorde R, Sneve M, Figenschau Y, et al. Effects of vitamin D supplementation on symptoms of depression in overweight and obese subjects: randomized double blind trial. J Intern Med. 2008;264:599–609.
[47] Kjaergaard M, Waterloo K, Wang CEA, et al. Effect of vitamin D supplement on depression scores in people with low levels of serum 25-hydroxyvitamin D: nested case–control study and randomised clinical trial. Br J Psychiatry. 2012;201:360–368.

[48] Lam RW, Levitan RD. Pathophysiology of seasonal affective disorder: a review. J Psychiatry Neurosci. 2000;25:469–480.
[49] Benedetti F. Rate of switch from bipolar depression into mania after morning light therapy: a historical review. Psychiatry Res. 2018;261:351–356.
[50] Henriksen TEG, Skrede S, Fasmer OB, et al. Blue-blocking glasses as additive treatment for mania: a randomized placebo-controlled trial. Bipolar Disord. 2016;18:221–232.
[51] Barbini B, Benedetti F, Colombo C, et al. Dark therapy for mania: a pilot study. Bipolar Disord. 2005;7: 98–101.
[52] Evans JA, Suen TC, Callif BL, et al. Shell neurons of the master circadian clock coordinate the phase of tissue clocks throughout the brain and body. BMC Biol. 2015;13:43.
[53] Damiola F. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 2000;14:2950–2961.
[54] Archer SN, Oster H. How sleep and wakefulness influence circadian rhythmicity: effects of insufficient and mistimed sleep on the animal and human transcriptome. J Sleep Res. 2015;24:476–493.
[55] West AC, Bechtold DA. The cost of circadian desynchrony: evidence, insights and open questions. Bioessays. 2015;37:777–788.
[56] Barclay JL, Tsang AH, Oster H. Interaction of central and peripheral clocks in physiological regulation. Prog Brain Res. 2012;199:163–181.
[57] Wirz-Justice A. Biological rhythm disturbances in mood disorders. Int Clin Psychopharmacol. 2006;21: S11–S15.
[58] Wulff K, Gatti S, Wettstein JG, et al. Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. Nat Rev Neurosci. 2010;11: 589–599.
[59] DeBruyne JP, Noton E, Lambert CM, et al. A clock shock: mouse CLOCK is not required for circadian oscillator function. Neuron. 2006;50:465–477.
[60] Sujino M, Asakawa T, Nagano M, et al. CLOCKDelta19 mutation modifies the manner of synchrony among oscillation neurons in the suprachiasmatic nucleus. Sci Rep. 2018;8:854.
[61] Landgraf D, Wang LL, Diemer T, et al. NPAS2 compensates for loss of CLOCK in peripheral circadian oscillators. PLoS Genet. 2016;12:e1005882.
[62] Barandas R, Landgraf D, McCarthy MJ, et al. Circadian clocks as modulators of metabolic comorbidity in psychiatric disorders. Curr Psychiatry Rep. 2015;17:98.
[63] Kiessling S, Eichele G, Oster H. Adrenal glucocorticoids have a key role in circadian resynchronization in a mouse model of jet lag. J Clin Invest. 2010;120: 2600–2609.
[64] Davidson AJ, Sellix MT, Daniel J, et al. Chronic jet-lag increases mortality in aged mice. Curr Biol. 2006;16: R914–R916.
[65] Wehrens SMT, Christou S, Isherwood C, et al. Meal timing regulates the human circadian system. Curr Biol. 2017;27:1768–1775 e3.
[66] Gill S, Panda S. A smartphone app reveals erratic diurnal eating patterns in humans that can be modulated for health benefits. Cell Metab. 2015;22: 789–798.
[67] Tahara Y, Shiraishi T, Kikuchi Y, et al. Entrainment of the mouse circadian clock by sub-acute physical and psychological stress. Sci Rep. 2015;5:11417.
[68] Hasler BP, Buysse DJ, Kupfer DJ, et al. Phase relationships between core body temperature, melatonin, and sleep are associated with depression severity: further evidence for circadian misalignment in non-seasonal depression. Psychiatry Res. 2010;178: 205–207.
[69] Erzberger A, Hampp G, Granada AE, et al. Genetic redundancy strengthens the circadian clock leading to a narrow entrainment range. J R Soc Interface. 2013;10:20130221.
[70] Wever RA. Fractional desynchronization of human circadian rhythms. Pflugers Arch. 1983;396:128–137.
[71] Aschoff J, Pohl H. Phase relations between a circadian rhythm and its zeitgeber within the range of entrainment. Naturwissenschaften. 1978;65:80–84.
[72] Gronfier C, Wright KP, Kronauer RE, et al. Entrainment of the human circadian pacemaker to longer-than-24-h days. Proc Natl Acad Sci USA. 2007; 104:9081–9086.
[73] Fried EI, Nesse RM, Guille C, et al. The differential influence of life stress on individual symptoms of depression. Acta Psychiatr Scand. 2015;131:465–471.
[74] Li JZ, Bunney BG, Meng F, et al. Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. Proc Natl Acad Sci USA. 2013;110:9950–9955.
[75] Landgraf D, Long JE, Welsh DK. Depression-like behaviour in mice is associated with disrupted circadian rhythms in nucleus accumbens and periaqueductal grey. Eur J Neurosci. 2016;43:1309–1320.
[76] Logan RW, Edgar N, Gillman AG, et al. Chronic stress induces brain region-specific alterations of molecular rhythms that correlate with depression-like behavior in mice. Biol Psychiatry. 2015;78:249–258.
[77] Duncan WC. Circadian rhythms and the pharmacology of affective illness. Pharmacol Ther. 1996;71: 253–312.
[78] Argyropoulos SV, Wilson SJ. Sleep disturbances in depression and the effects of antidepressants. Int Rev Psychiatry (Abingdon, England). 2005;17: 237–245.
[79] Linkowski P. Neuroendocrine profiles in mood disorders. Int J Neuropsychopharm. 2003;6:191–197.
[80] Souêtre E, Salvati E, Belugou J-L, et al. Circadian rhythms in depression and recovery: evidence for blunted amplitude as the main chronobiological abnormality. Psychiatry Res. 1989;28:263–278.
[81] Emens J, Lewy A, Kinzie JM, et al. Circadian misalignment in major depressive disorder. Psychiatry Res. 2009;168:259–261.
[82] Salgado-Delgado R, Tapia Osorio A, Saderi N, et al. Disruption of circadian rhythms: a crucial factor in the etiology of depression. Depress Res Treat. 2011; 2011:839743.

[83] Pittendrigh CS, Kyner WT, Takamura T. The amplitude of circadian oscillations: temperature dependence, latitudinal clines, and the photoperiodic time measurement. J Biol Rhythms. 1991;6:299–313.
[84] Brown SA, Kunz D, Dumas A, et al. Molecular insights into human daily behavior. Proc Natl Acad Sci USA. 2008;105:1602–1607.
[85] Vitaterna MH, Ko CH, Chang A-M, et al. The mouse Clock mutation reduces circadian pacemaker amplitude and enhances efficacy of resetting stimuli and phase-response curve amplitude. Proc Natl Acad Sci. 2006;103:9327–9332.
[86] Strine TW, Chapman DP. Associations of frequent sleep insufficiency with health-related quality of life and health behaviors. Sleep Med. 2005;6:23–27.
[87] Landgraf D, Shostak A, Oster H. Clock genes and sleep. Pflugers Arch. 2012;463:3–14.
[88] Archer SN, Robilliard DL, Skene DJ, et al. A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference. Sleep. 2003;26:413–415.
[89] Viola AU, Archer SN, James LM, et al. PER3 polymorphism predicts sleep structure and waking performance. Curr Biol. 2007;17:613–618.
[90] Zhang L, Hirano A, Hsu P-K, et al. A PERIOD3 variant causes a circadian phenotype and is associated with a seasonal mood trait. Proc Natl Acad Sci USA. 2016; 113:E1536–E1544.
[91] Naylor E, Bergmann BM, Krauski K, et al. The circadian clock mutation alters sleep homeostasis in the mouse. J Neurosci. 2000;20:8138–8143.
[92] De Bundel D, Gangarossa G, Biever A, et al. Cognitive dysfunction, elevated anxiety, and reduced cocaine response in circadian clock-deficient cryptochrome knockout mice. Front Behav Neurosci. 2013; 7:152.
[93] Wisor JP, O'Hara BF, Terao A, et al. A role for cryptochromes in sleep regulation. BMC Neurosci. 2002;3:20.
[94] Tsuno N, Besset A, Ritchie K. Sleep and depression. J Clin Psychiatry. 2005;66:1254–1269.
[95] Rumble ME, White KH, Benca RM. Sleep disturbances in mood disorders. Psychiatr Clin North Am. 2015;38: 743–759.
[96] Goldstein AN, Walker MP. The role of sleep in emotional brain function. Annu Rev Clin Psychol. 2014; 10:679–708.
[97] Nutt D, Wilson S, Paterson L. Sleep disorders as core symptoms of depression. Dialogues Clin Neurosci. 2008;10:329–336.
[98] Buxton OM, L'Hermite-Balériaux M, Turek FW, et al. Daytime naps in darkness phase shift the human circadian rhythms of melatonin and thyrotropin secretion. Am J Physiol Regul Integr Comp Physiol. 2000; 278:R373–R382.
[99] Akacem LD, Simpkin CT, Carskadon MA, et al. The timing of the circadian clock and sleep differ between napping and non-napping toddlers. PLoS One. 2015;10:e0125181.
[100] Danilenko KV, Cajochen C, Wirz-Justice A. Is sleep per se a zeitgeber in humans?. J Biol Rhythms. 2003; 18:170–178.
[101] Moller-Levet CS, Archer SN, Bucca G, et al. Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. Proc Natl Acad Sci USA. 2013;110: E1132–E1141.
[102] Scott J, Naismith S, Grierson A, et al. Sleep–wake cycle phenotypes in young people with familial and non-familial mood disorders. Bipolar Disord. 2016;18: 642–649.
[103] Robillard R, Hermens D, Naismith S, et al. Ambulatory sleep–wake patterns and variability in young people with emerging mental disorders. J Psychiatry Neurosci. 2015;40:28–37.
[104] Roenneberg T, Kantermann T, Juda M, et al. Light and the human circadian clock. Handb Exp Pharmacol. 2013;217:311–331.
[105] van der Zweerde T, van Straten A, Effting M, et al. Does online insomnia treatment reduce depressive symptoms? A randomized controlled trial in individuals with both insomnia and depressive symptoms. Psychol Med. 2018;1–9.
[106] Stokkan KA, Yamazaki S, Tei H, et al. Entrainment of the circadian clock in the liver by feeding. Science. 2001;291:490–493.
[107] Landgraf D, Neumann AM, Oster H. Circadian clock-gastrointestinal peptide interaction in peripheral tissues and the brain. Best Pract Res Clin Endocrinol Metab. 2017;31:561–571.
[108] McHill AW, Phillips AJ, Czeisler CA, et al. Later circadian timing of food intake is associated with increased body fat. Am J Clin Nutr. 2017;106: 1213–1219.
[109] Wegner M, Helmich I, Machado S, et al. Effects of exercise on anxiety and depression disorders: review of meta-analyses and neurobiological mechanisms. CNS Neurol Disord Drug Targets. 2014;13:1002–1014.
[110] Dinas PC, Koutedakis Y, Flouris AD. Effects of exercise and physical activity on depression. Ir J Med Sci. 2011;180:319–325.
[111] Gujral S, Aizenstein H, Reynolds CF, et al. Exercise effects on depression: possible neural mechanisms. Gen Hosp Psychiatry. 2017;49:2–10.
[112] Kovacevic A, Mavros Y, Heisz JJ, et al. The effect of resistance exercise on sleep: a systematic review of randomized controlled trials. Sleep Med Rev. 2018; 39:52–68.
[113] Yasumoto Y, Nakao R, Oishi K. Free access to a running-wheel advances the phase of behavioral and physiological circadian rhythms and peripheral molecular clocks in mice. PLoS One. 2015;10: e0116476.
[114] Wolff G, Esser KA. Scheduled exercise phase shifts the circadian clock in skeletal muscle. Med Sci Sports Exerc. 2012;44:1663–1670.
[115] Leise TL, Harrington ME, Molyneux PC, et al. Voluntary exercise can strengthen the circadian system in aged mice. Age (Dordr). 2013;35:2137–2152.
[116] Tal-Krivisky K, Kronfeld-Schor N, Einat H. Voluntary exercise enhances activity rhythms and ameliorates anxiety- and depression-like behaviors in the sand rat model of circadian rhythm-related mood changes. Physiol Behav. 2015;151:441–447.

[117] Solberg LC, Horton TH, Turek FW. Circadian rhythms and depression: effects of exercise in an animal model. Am J Physiol. 1999;276:R152–R161.
[118] Yamanaka Y, Hashimoto S, Takasu NN, et al. Morning and evening physical exercise differentially regulate the autonomic nervous system during nocturnal sleep in humans. Am J Physiol Regul Integr Comp Physiol. 2015;309:R1112–R1121.
[119] Miyazaki T, Hashimoto S, Masubuchi S, et al. Phase-advance shifts of human circadian pacemaker are accelerated by daytime physical exercise. Am J Physiol Regul Integr Comp Physiol. 2001;281: R197–R205.
[120] Tranel HR, Schroder EA, England J, et al. Physical activity, and not fat mass is a primary predictor of circadian parameters in young men. Chronobiol Int. 2015;32:832–841.
[121] Van Reeth O, Sturis J, Byrne MM, et al. Nocturnal exercise phase delays circadian rhythms of melatonin and thyrotropin secretion in normal men. Am J Physiol. 1994;266:E964–E974.
[122] Eastman CI. Phase-shifting human circadian rhythms with exercise during the night shift. Physiol Behav. 1995;58:1287–1291.
[123] Pail G, Huf W, Pjrek E, et al. Bright-light therapy in the treatment of mood disorders. Neuropsychobiology. 2011;64:152–162.
[124] Wirz-Justice A, Benedetti F, Terman M. Chronotherapeutics for affective disorders: a clinician's manual for light and wake therapy. 2nd ed. Basel (Switzerland): Karger; 2013.
[125] Terman M, Terman JS, Ross DC. A controlled trial of timed bright light and negative air ionization for treatment of winter depression. Arch Gen Psychiatry. 1998;55:875–882.
[126] Eastman CI, Young MA, Fogg LF, et al. Bright light treatment of winter depression: a placebo-controlled trial. Arch Gen Psychiatry. 1998;55:883–889.
[127] Lewy AJ, Bauer VK, Cutler NL, et al. Morning vs evening light treatment of patients with winter depression. Arch Gen Psychiatry. 1998;55:890–896.
[128] Tuunainen A, Kripke DF, Endo T. Light therapy for non-seasonal depression. Cochrane Database Syst Rev. 2004;2:CD004050.
[129] Golden RN, Gaynes BN, Ekstrom RD, et al. The efficacy of light therapy in the treatment of mood disorders: a review and meta-analysis of the evidence. Am J Psychiatry. 2005;162:656–662.
[130] Lieverse R, Nielen MM, Veltman DJ, et al. Bright light in elderly subjects with nonseasonal major depressive disorder: a double blind randomised clinical trial using early morning bright blue light comparing dim red light treatment. Trials. 2008;9:48.
[131] Lam RW, Levitt AJ, Levitan RD, et al. Efficacy of bright light treatment, fluoxetine, and the combination in patients with nonseasonal major depressive disorder. A randomized clinical trial. JAMA Psychiatry. 2016;73:56–63.
[132] Sit DK, McGowan J, Wiltrout C, et al. Adjunctive bright light therapy for bipolar depression: a randomized double-blind placebo-controlled trial. Ajp. 2018;175:131–139.
[133] Stephenson KM, Schroder CM, Bertschy G, et al. Complex interaction of circadian and non-circadian effects of light on mood: shedding new light on an old story. Sleep Med Rev. 2012;16:445–454.
[134] Roecklein KA, Wong PM, Miller MA, et al. Melanopsin, photosensitive ganglion cells, and seasonal affective disorder. Neurosci Biobehav Rev. 2013;37:229–239.
[135] LeGates TA, Fernandez DC, Hattar S. Light as a central modulator of circadian rhythms, sleep and affect. Nat Rev Neurosci. 2014;15:443–454.
[136] Coomans CP, Ramkisoensing A, Meijer JH. The suprachiasmatic nuclei as a seasonal clock. Front Neuroendocrinol. 2015;37:29–42.
[137] Giedke H, Schwarzler F. Therapeutic use of sleep deprivation in depression. Sleep Med Rev. 2002;6: 361–377.
[138] Bunney BG, Bunney WE. Mechanisms of rapid antidepressant effects of sleep deprivation therapy: clock genes and circadian rhythms. Biol Psychiatry. 2013;73:1164–1171.
[139] Wu JC, Kelsoe JR, Schachat C, et al. Rapid and sustained antidepressant response with sleep deprivation and chronotherapy in bipolar disorder. Biol Psychiatry. 2009;66:298–301.
[140] Orozco-Solis R, Montellier E, Aguilar-Arnal L, et al. A circadian genomic signature common to ketamine and sleep deprivation in the anterior cingulate cortex. Biol Psychiatry. 2017;82:351–360.
[141] Winkler D, Spies M, Al-Resheg Y, et al. Usage of therapeutic sleep deprivation: a survey in psychiatric hospitals in Austria, Germany, and Switzerland. Behav Sleep Med. 2018;1–8.
[142] Van Reeth O, Olivares E, Turek FW, et al. Resynchronisation of a diurnal rodent circadian clock accelerated by a melatonin agonist. Neuroreport. 1998;9:1901–1905.
[143] Weibel L, Turek FW, Mocaer E, et al. A melatonin agonist facilitates circadian resynchronization in old hamsters after abrupt shifts in the light-dark cycle. Brain Res. 2000;880:207–211.
[144] Armstrong SM, McNulty OM, Guardiola-Lemaitre B, et al. Successful use of S20098 and melatonin in an animal model of delayed sleep-phase syndrome (DSPS). Pharmacol Biochem Behav. 1993;46:45–49.
[145] Castanho A, Bothorel B, Seguin L, et al. Like melatonin, agomelatine (S20098) increases the amplitude of oscillations of two clock outputs: melatonin and temperature rhythms. Chronobiol Int. 2014;31: 371–381.
[146] Redman JR, Francis AJ. Entrainment of rat circadian rhythms by the melatonin agonist S-20098 requires intact suprachiasmatic nuclei but not the pineal. J Biol Rhythms. 1998;13:39–51.
[147] Mairesse J, Silletti V, Laloux C, et al. Chronic agomelatine treatment corrects the abnormalities in the circadian rhythm of motor activity and sleep/wake cycle induced by prenatal restraint stress in adult rats. Int J Neuropsychopharm. 2013;16:323–338.
[148] Krauchi K, Cajochen C, Mori D, et al. Early evening melatonin and S-20098 advance circadian phase and

nocturnal regulation of core body temperature. Am J Physiol. 1997;272:R1178–R1188.
[149] Leproult R, Van Onderbergen A, L'Hermite-Baleriaux M, et al. Phase-shifts of 24-h rhythms of hormonal release and body temperature following early evening administration of the melatonin agonist agomelatine in healthy older men. Clin Endocrinol. 2005;63: 298–304.
[150] De Berardis D, Fornaro M, Serroni N, et al. Agomelatine beyond borders: current evidences of its efficacy in disorders other than major depression. IJMS. 2015;16:1111–1130.
[151] Pringle A, Bogdanovskaya M, Waskett P, et al. Does melatonin treatment change emotional processing? Implications for understanding the antidepressant mechanism of agomelatine. J Psychopharmacol. 2015;29:1129–1132.
[152] Hojgaard K, Christiansen SL, Bouzinova EV, et al. Disturbances of diurnal phase markers, behavior, and clock genes in a rat model of depression; modulatory effects of agomelatine treatment. Psychopharmacology (Berl). 2018;235:627–640.
[153] Lewy AJ. Circadian misalignment in mood disturbances. Curr Psychiatry Rep. 2009;11:459–465.
[154] Kripke DF, Mullaney DJ, Atkinson M, et al. Circadian rhythm disorders in manic-depressives. Biol Psychiatry. 1978;13:335–351.
[155] Li J, Lu W-Q, Beesley S, et al. Lithium impacts on the amplitude and period of the molecular circadian clockwork. PLoS One. 2012;7:e33292.
[156] Wei H, Landgraf D, Wang G, et al. Inositol polyphosphates contribute to cellular circadian rhythms: implications for understanding lithium's molecular mechanism. Cell Signal. 2018;44:82–91.
[157] He B, Nohara K, Park N, et al. The small molecule nobiletin targets the molecular oscillator to enhance circadian rhythms and protect against metabolic syndrome. Cell Metab. 2016; 23:610–621.
[158] Shinozaki A, Misawa K, Ikeda Y, et al. Potent effects of flavonoid nobiletin on amplitude, period, and phase of the circadian clock rhythm in PER2::LUCIFERASE mouse embryonic fibroblasts. PLoS One. 2017;12:e0170904.
[159] Qi G, Guo R, Tian H, et al. Nobiletin protects against insulin resistance and disorders of lipid metabolism by reprogramming of circadian clock in hepatocytes. Biochim Biophys Acta. 2018;1863: 549–562.
[160] Solt LA, Wang Y, Banerjee S, et al. Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists. Nature. 2012;485:62–68.
[161] Banerjee S, Wang Y, Solt LA, et al. Pharmacological targeting of the mammalian clock regulates sleep architecture and emotional behaviour. Nat Commun. 2014;5:5759.
[162] Amador A, Huitron-Resendiz S, Roberts AJ, et al. Pharmacological targeting the REV-ERBs in sleep/wake regulation. PLoS One. 2016;11: e0162452.

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Curriculum Vitae

Education and Work Experience

since 01/2019	**Trainee as Psychological Psychotherapist (CBT)** in Cognitive Behavioral Therapy, Rhein-Eifel Institute, Andernach, Germany
since 03/2018	**Structured Ph.D. program** “Medical Research” of the Munich Medical Research School (MMRS)
since 11/2017	**Ph.D. student** in the field of Circadian Biology, Affective and Metabolic Disorders, Department of Molecular and Behavioural Neurobiology, Research Group Circadian Biology, Clinic for Psychiatry and Psychotherapy, Ludwig Maximilian University Munich, Germany
11/2020 - 08/2021	**Scientific stay abroad**, funded by the European Union Horizon 2020 Programme; Marie Skłodowska-Curie Grant, Center for Social and Affective Neuroscience (CSAN; Leah Mayo), University of Linköping, Sweden
03/2019 – 10/2020	**Clinical Psychologist**, Specialist Ward for Addiction Disorders, Clinic for Psychiatry and Psychotherapy, Ludwig Maximilian University Munich, Germany
03/2017 – 09/2017	**Master thesis** “Role of enhanced 2-AG signaling on food intake in mice deficient in cannabinoid CB1 receptors in different neuronal subpopulations”, Department Stress, Neurobiology and Neurogenetics, Max Planck Institute of Psychiatry, Munich, Germany
2015 – 2017	**Graduate studies (Master of Science)** in an International Program in Neurosciences, University Bremen, Germany
01/2015 – 06/2015	**Bachelor thesis** “Effectiveness of orthographic trainings in children and adolescents with reading and spelling disabilities – a meta-analysis“, Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, University Hospital, Ludwig Maximilian University Munich, Germany
08/2014 – 10/2014	**Summer session** in Psychology, University of California Los Angeles (UCLA), USA

10/2014 – 07/2015	**Research assistant**, Max Planck Institute of Psychiatry in Munich, Department Clinical Research, Research Group Molecular Psychology
02/2014 – 03/2015	**Research assistant**, Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, University Hospital, Ludwig Maximilian University Munich, Research group Reading and Spelling Disabilities
2012 - 2015	**Undergraduate studies (Bachelor of Science)** in Psychology, University Ulm, Germany

Research and Clinical Experience

Cell and tissue culture: Tissue culture, primary cell culture, bioluminescence measurements from cultured tissues and single cells

Molecular biology: Western Blot, polymerase chain reaction (PCR), quantitative PCR, DNA-, RNA- and Protein-isolation, cloning, production of AAVs, virus-induced gene expression in cells and tissues (*in vitro* and *in vivo*), immunohistochemistry, histology, microscopy, radioactive-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA)

Animal work and behavioral studies: Mouse and rat handling, husbandry, behavioral testing (open-field, y-maze. elevated plus maze, light-dark-box, social interaction test, object-recognition test, skinner box, touchscreen system, IntelliCage System®, metabolic cages, sucrose-preference test, tail-suspension-test, learned-helplessness test, catatonia-test, stereotactic surgery, EEG-implantation, injections (*i.p.*, *i.c.*, *s.c.*, *t.v.*), blood sampling, tissue harvest

Computational: Microsoft Office, SPSS Statistics, Matlab, GraphPad Prism, R

Neuro-clinical: fEMG, MRI and fMRI (basic), EEG (basic)

Psychological: Cognitive-based therapy for individuals and groups, crisis-intervention, psychiatric and neuropsychological diagnostics, motivational interviewing, relaxation techniques

Publications

Hühne A, Echtler L, Kling C, Stephan M, Rossner M, Landgraf D. **Circadian gene × environment perturbations influence alcohol drinking in *Cryptochrome*-deficient mice.** Addiction Biology, e13105.

Hühne A, Hoch E, & Landgraf D. **DAILY-A Personalized Circadian Zeitgeber Therapy as an Adjunctive Treatment for Alcohol Use Disorder Patients: Study Protocol for a Randomized Controlled Trial.** Frontiers in psychiatry (2020), 11, 569864. PMID: 33519541.

Hühne A, Volkmann P, Stephan M, Rossner M, Landgraf D. **An in-depth neurobehavioral characterization shows anxiety-like traits, impaired habituation behavior, and restlessness in male Cryptochrome -deficient mice**. Genes, Brain and Behavior (2020), e12661. PMID: 32348614.

Hühne A, Welsh DK, Landgraf D. **Prospects for circadian treatment of mood disorders**. Ann Med. 2018;50(8):637-654, PMID: 30265156.

Platform Presentations

March 2021 — Reverse Translation: Role of Genetic and Environmental Circadian Disturbances on Alcohol Drinking Behavior – From Bedside to Bench… and Back. European Behavioural Pharmacology Society (ebps), Toronto, Canada (online).

Sept. 2021 — Eine Personlisierte Ziradiane *Zeitgeber* Therapie als Zusatztherapie für Patienten mit Alkoholabhängigkeit. Deutscher Suchtkongress, Berlin, Germany (online).

Poster Presentations

June 2020 — **DAILY - a RCT Pilot Study: Development of an Individualized Circadian Daily Structure Therapy for Patients with Alcohol Addiction and Comorbid Depression,** Society for Research on Biological Rhythms, Florida, USA

July 2019 — **Different Levels of Circadian Clock Disturbances in Affective Disorders**, Clinic for Psychiatry and Psychotherapy, Ludwig Maximilian University Munich, Germany

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Sept. 2018 — **Disturbance of Circadian Clocks and Disturbance of Psychiatric and Metabolic Disorders**, Chronobiology Summer School, Oxford

March 2017 — **Molecular and biophysical effects of in vitro and ex vivo transcranial direct current stimulation (tDCS)**, Max-Planck Institute of Psychiatry, Munich

March 2015 — **Die Förderung orthographischer Fähigkeiten bei Kindern und Jugendlichen mit Lese- und/oder Rechtschreibstörung – eine Meta-Analyse**, Kongress DGKJP, München

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Teaching Experience

WS 2019/2020	Lecturer: Psychobiological Medicine for Medical Students, LMU Munich

Supervisory Activities

since SS 2020	Supervision of a medical student (mouse work and human study)
since WS 2019/20	Supervision of a student assistant (mouse work, molecular laboratory work)
WS 2019/20 – SS 2020	Supervision of a medical student (human study)
WS 2018/19	Supervision of a laboratory internship of a Master student (cell and tissue culture)

Voluntary Activity

07/2017 - 05/2018	**Mentorship for an underage unaccompanied refugee**, münchner mentoren e.V., Munich

Language Skills

Mother-tongue:	**German**
Other languages:	**English** (C2) TOEFL ibt Test (score: 98) and certified foreign language correspondence clerk (final grade A) **French** (C1) Diplôme approfondi de langue française DALF C1 (80,50/100) **Spanish** (B1) 3-years in high school