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.

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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. In recent years, evidence emerged that there is a bidirectional relationship between circadian disruptions and AUD. AUD patients often suffer from circadian dysregulation, which, in turn, increases the risk for developing AUD and alcohol relapses during withdrawal.

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. 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. 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, and circadian gene expression is reduced in AUD patients compared with healthy controls. In mice, mutations of Per1, Per2, and Clock result in enhanced alcohol consumption behaviour and increased sensitizations to reward. 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. In mice, however, repeated LD phase shifts and constant darkness (DD) or constant light (LL) often decrease alcohol consumption. 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. Similarly, the neuropeptide orexin has been associated with increased alcohol consumption. 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 altered 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. 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.

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 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, often result in reduced alcohol intake in mice. 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. 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−/−;Per2Luc (henceforth referred to as Cry1/2−/−) and Cry1/2−/−;Per2Luc mice (henceforth referred to as wild-type; WT) on C57BL/6J background were obtained as described previously. 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 24 weeks of age at the beginning of 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 = \(5 \times e^{0.2 \times \text{stage number}}\) − 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 cocaine43,44 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 PER2Luc 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 Per2Luc 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 described procedures 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 RNALater™ 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 $\beta$-actin (Fw-CCCTGAGTACCCTCATTGAA, Rev-AGGTTGTGTTGCCAGATCTTC) and PPO (Fw-TTGGACCAC TGCACTGAAG, Rev-CCCCAGGGAACCTTTGTAGAAG).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 (2–12%) 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$^{Lc}$ 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$^{119}$ 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
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...
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 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,
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. 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. 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 solution alone. Under alternating “shift work” light conditions, WT 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. 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

Table S5-1). Consistent with previous results, the latter two behaviours are severely abnormal in Cry1/2−/− mice at baseline. 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. 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
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 behaviors 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 Per2Luc 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, ClockΔ19 and Per2Brdm/Brdm mice, show increased preference for alcohol.16,17 Per2Brdm/Brdm mice show increased consumption and preference for EtOH as well as elevated motivation to receive EtOH in a progressive ratio paradigm. Similarly, ClockΔ19 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

**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 shift work 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.
intake of drugs and alcohol in rodents.\textsuperscript{25,53} However, orexin levels are decreased in both Cry1/2\textsuperscript{−/−} and Clock\textsuperscript{−/−} mice,\textsuperscript{56} suggesting that altered alcohol preferences cannot be explained by orexin changes alone. Yet, Cry1/2\textsuperscript{−/−} mice also display significantly increased CORT levels and CRY proteins are known to interact with glucocorticoid receptors.\textsuperscript{52} Although stress is often a trigger for increased alcohol consumption in humans,\textsuperscript{30} in rodents, increased stress and CORT levels often lead to decreased alcohol consumption and reward seeking.\textsuperscript{31,32} Interestingly, in contrast to those of Cry1/2\textsuperscript{−/−} mice, CORT levels of Clock\textsuperscript{−/−} and Per2\textsuperscript{Brdm/Brdm} mice are permanently suppressed,\textsuperscript{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\textsuperscript{−/−} and Per2\textsuperscript{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.\textsuperscript{58,59} While the Per2\textsuperscript{Brdm/Brdm} mice have a deficit in the removal of glutamate from the synaptic cleft, the Clock\textsuperscript{−/−} mice have a reduced glutamate uptake, both leading to a hyperglutamatergic state. Whether Cry1/2\textsuperscript{−/−} 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\textsuperscript{−/−} mice exhibit other characteristics of AUD such as higher motivation to obtain alcohol. Cry1/2\textsuperscript{−/−} mice exhibit the same behaviour when sucrose is used instead of alcohol\textsuperscript{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.\textsuperscript{60–62} The latter two being hallmarks of Cry1/2\textsuperscript{−/−} mice.\textsuperscript{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.\textsuperscript{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\textsuperscript{−/−} mice\textsuperscript{63} and the presence of CRYs suppresses dopamine signaling.\textsuperscript{64} These findings suggest that dopamine signalling may be a mechanism for altered drinking behaviour in Cry1/2\textsuperscript{−/−} mice which will be subject of future investigation.

In conclusion, this study shows that WT mice under stressful withdrawal conditions approximate anxiety-like behaviours, which Cry1/2\textsuperscript{−/−} mice show independently of withdrawal already at baseline.

Together, since Cry1/2\textsuperscript{−/−} 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.\textsuperscript{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,\textsuperscript{69,70} while mice are more likely to drink less alcohol under alternating light conditions.\textsuperscript{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\textsuperscript{−/−} mice “shift work” lighting conditions. Interestingly, the differences in alcohol preference between WT and Cry1/2\textsuperscript{−/−} 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\textsuperscript{−/−} female mice have an increased preference for some alcohol concentrations. Nevertheless, in both WT and Cry1/2\textsuperscript{−/−} 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\textsuperscript{−/−} 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\textsuperscript{−/−} 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\textsuperscript{−/−} 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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