


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

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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 depression¹³ 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*^{Δ19} 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/SvlmJ 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 nose pokes. Visits in corners, number of nose pokes, 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 nosepekes inconsecutive stages. The increase of required nosepekes 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 nosepekes 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 nosepekes 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 nosepekes 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 $\Delta\Delta\text{CT}$ calculation was performed as described.³⁵ Primer sequences were: *β -act*: for. 5'-CCCTGAAGTACCCATTGAA-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 sensorimotor 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

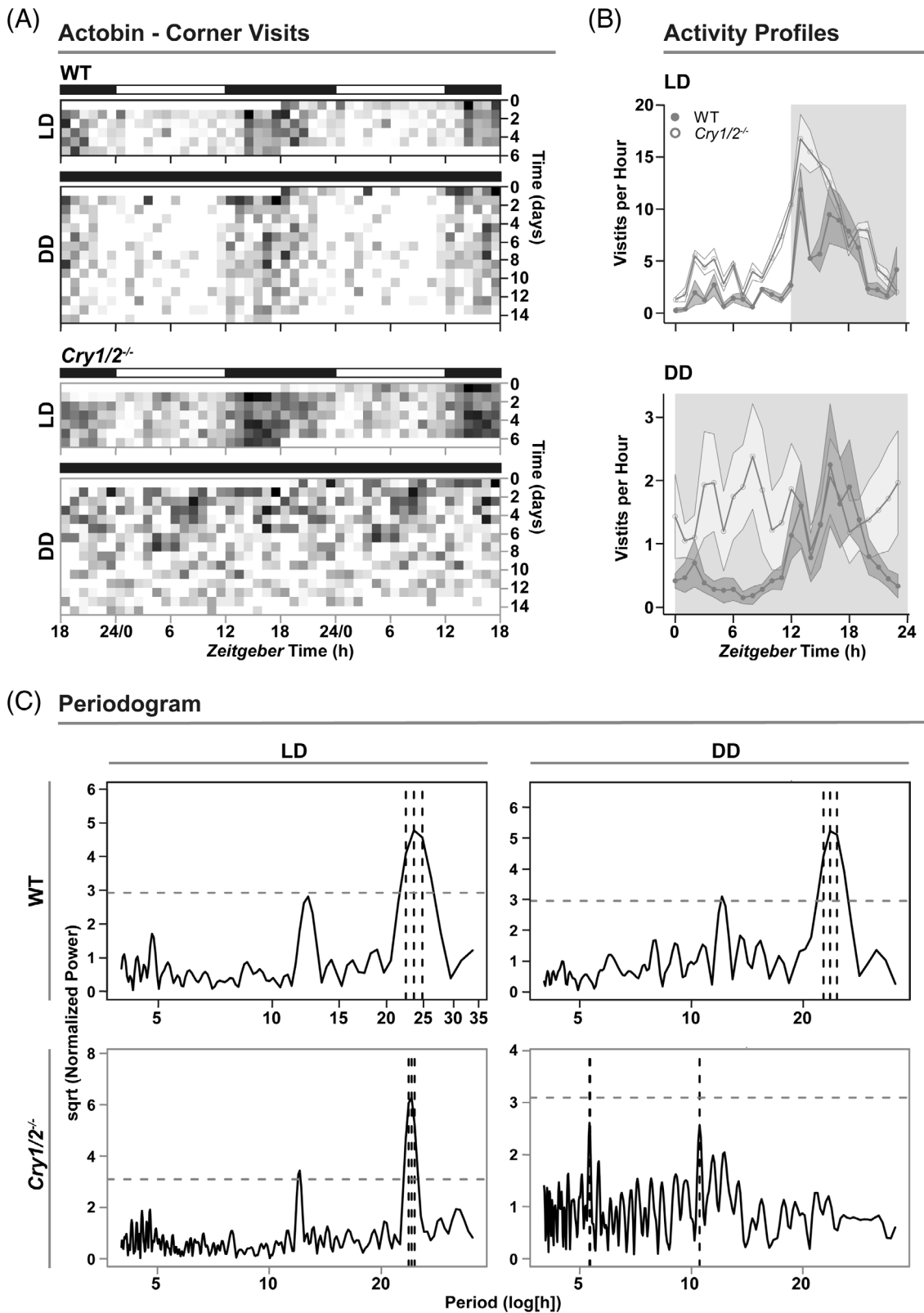


FIGURE 1 Endogenous circadian rhythms are lost in *Cryptochrome*-deficient mice. Day-night activity patterns were determined based on corner visits in IntelliCages. A, Average activity patterns under LD and DD conditions show that *Cry1/2^{-/-}* mice do not express endogenous circadian rhythms in constant 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

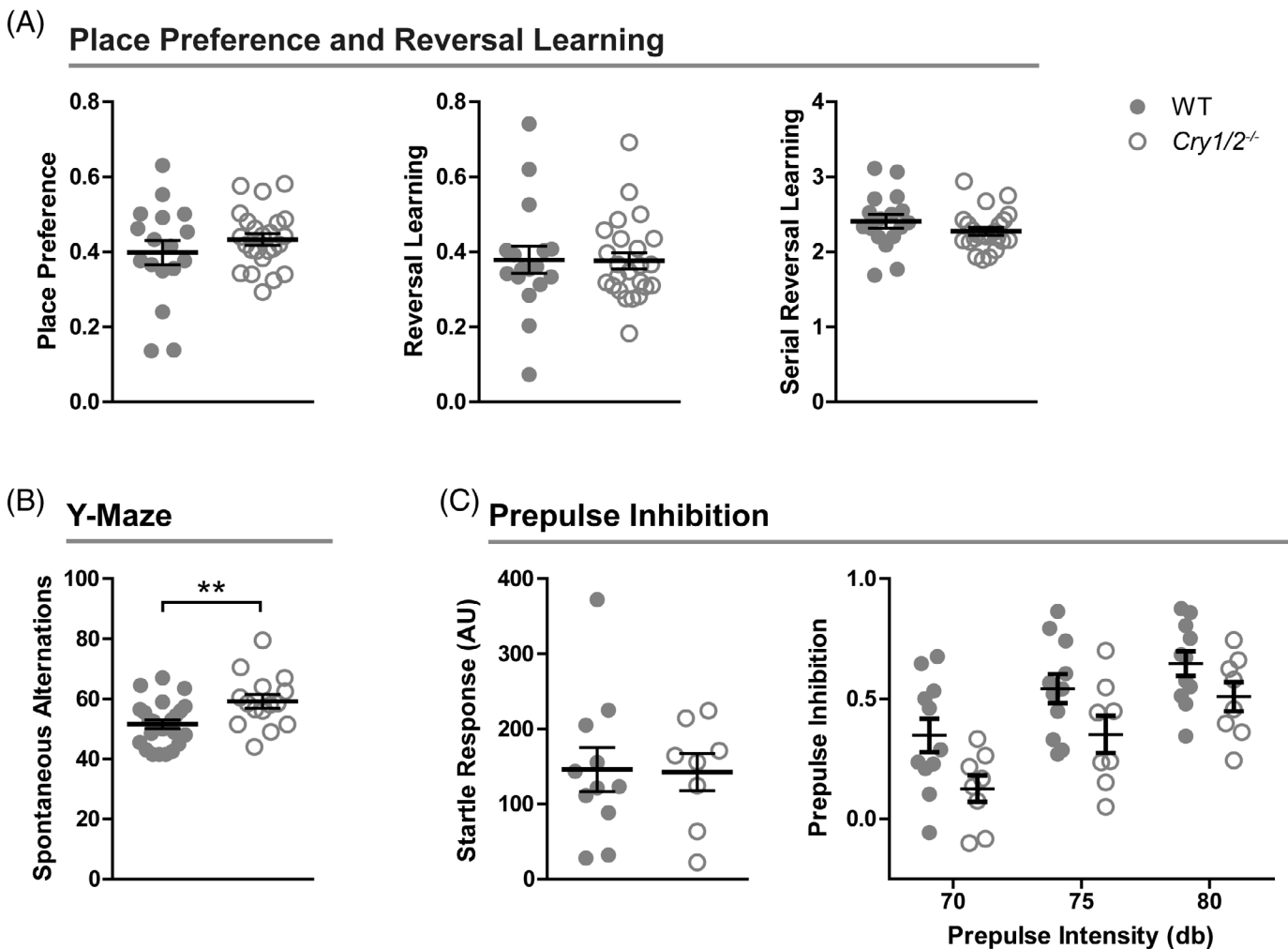


FIGURE 2 *Cryptochrome*-deficient mice show mild cognitive abnormalities. A, Reversal learning is not affected in *Cry1/2^{-/-}* mice. Place preference for water bottles, single and reversal learning of water bottle position changes were tested in IntelliCages. Data are shown as dot plot with mean \pm 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 \pm 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 \pm 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

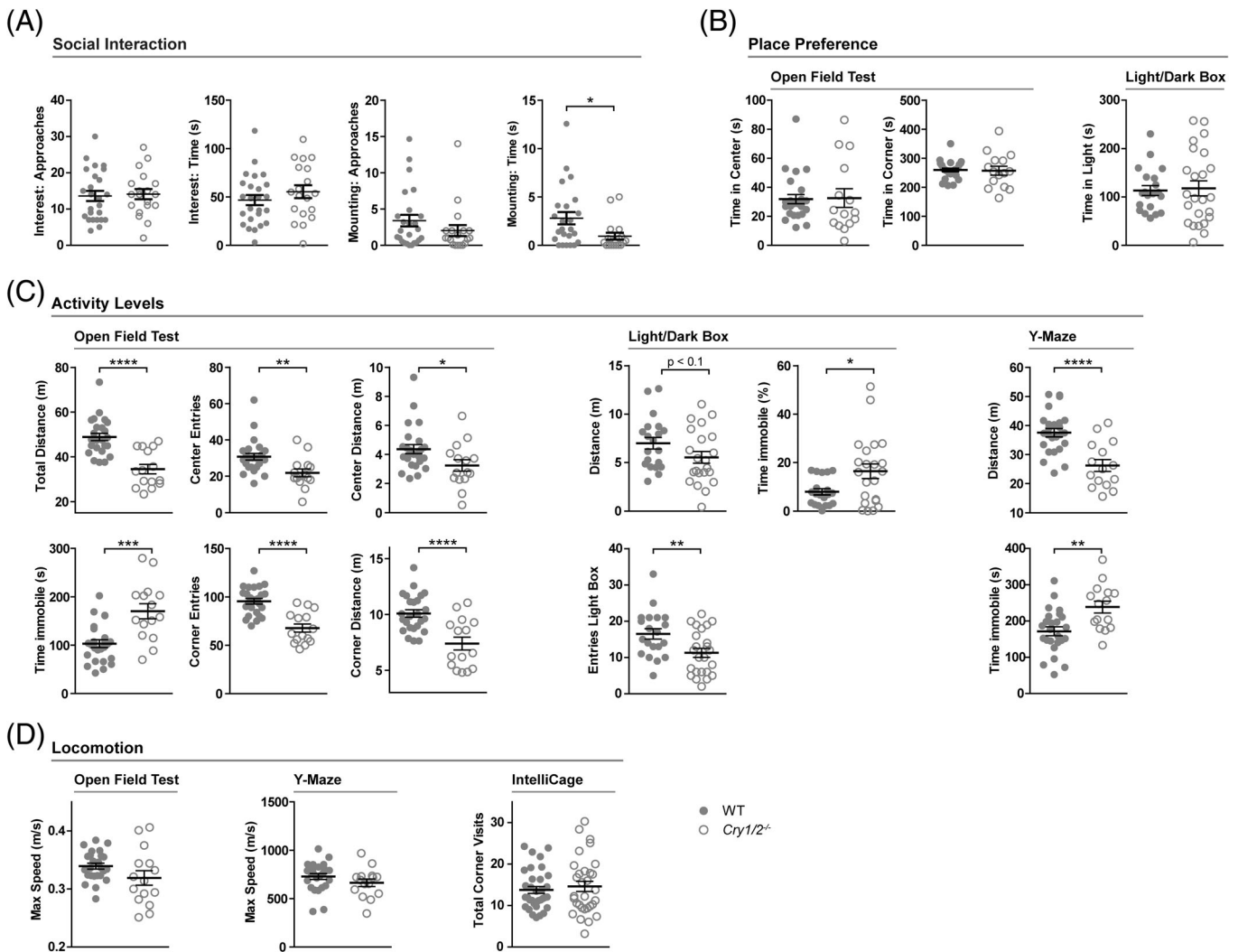
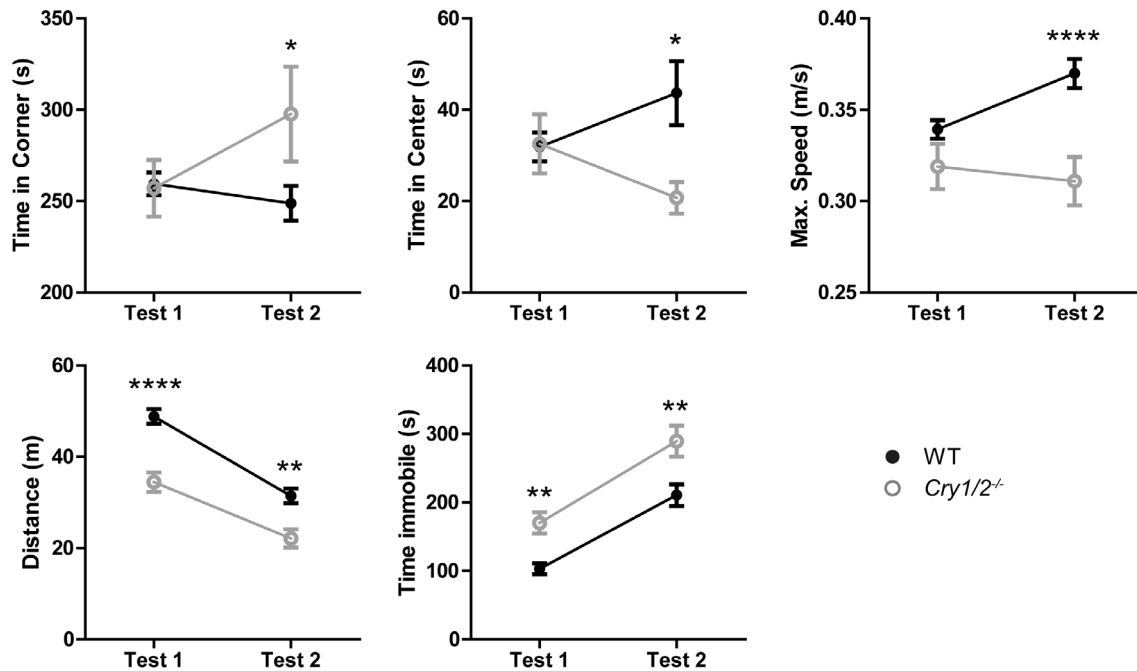


FIGURE 3 *Cryptochrome*-deficient mice exhibit hypoactivity in unfamiliar environments and other signs of elevated anxiety. A, In the social interaction test, *Cry1/2^{-/-}* mice spend less time mounting an ovariectomized female mouse but show no differences in the number of mounting approaches and in interest time and approaches. Data are shown as dot plot with mean \pm 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 \pm 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 \pm 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 \pm 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

(A) Adaptive Behavior in Open Field Test



(B) Adaptive Behavior in Y-Maze Test

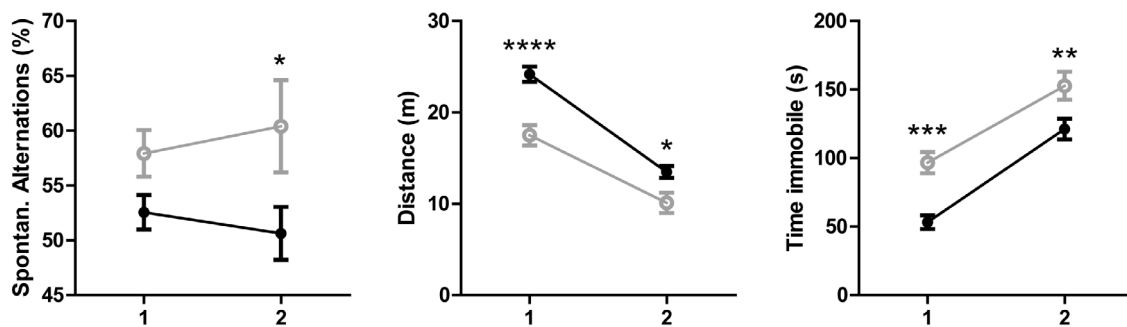


FIGURE 4 *Cryptochrome*-deficient mice have a limited habituation to adapt to new environments. A, In a second open field test, *Cry1/2^{-/-}* mice spend more time in the corner and less time in the center than in first test and as WT mice. Also, WT mice increase movement speed while *Cry1/2^{-/-}* mice 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 \pm 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 \pm 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

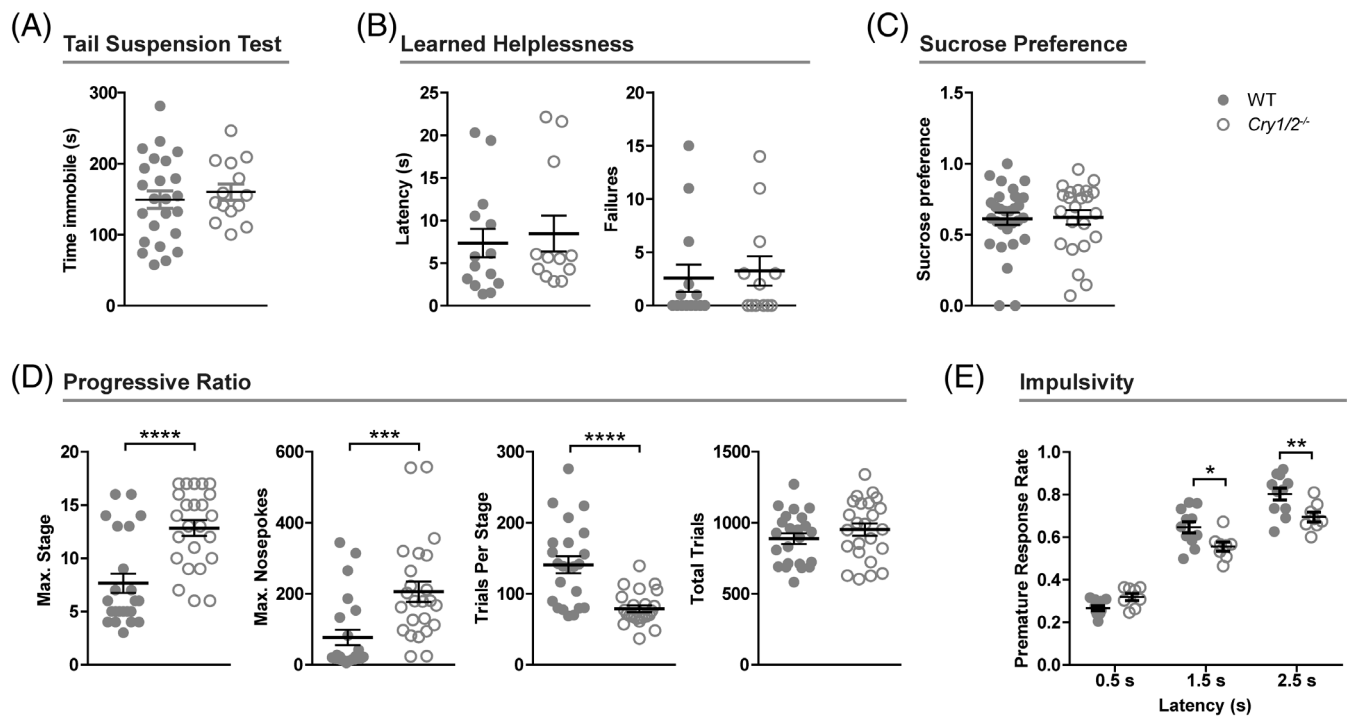


FIGURE 5 Depression-like behavior is not induced by loss of *Cryptochromes*. A, In the tail suspension test, *Cry1/2^{-/-}* mice do not show significant differences in immobility time. Data are shown as dot plot with mean \pm SEM; (Student *t* test); $n = 24/14$. B, Escape latency and number of failures in the learned helplessness paradigm are not different in WT and *Cry1/2^{-/-}* mice. Data are shown as dot plot with mean \pm 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 \pm 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 \pm SEM; **** $P < .0001$ (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 \pm 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

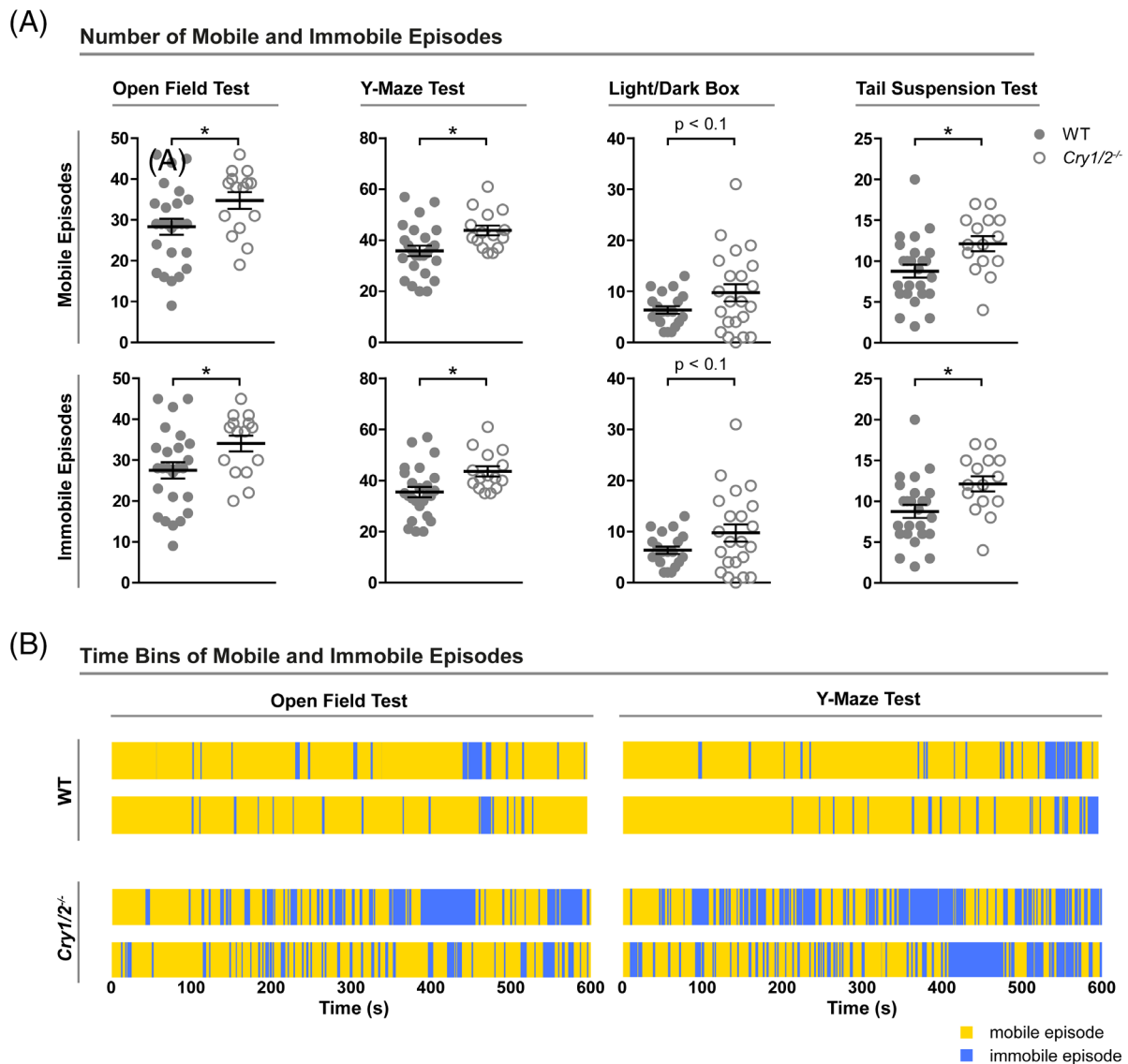


FIGURE 6 *Cryptochrome*-deficient mice display restless behavior across various tests. A, In the open field test, Y-maze test, light/dark box test, and tail suspension test, *Cry1/2^{-/-}* mice switch significantly more frequently between mobile and immobile states, which is indicated by similarly increased numbers of both episodes. Data are shown as dot plot with mean \pm 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 humans³⁶ 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.

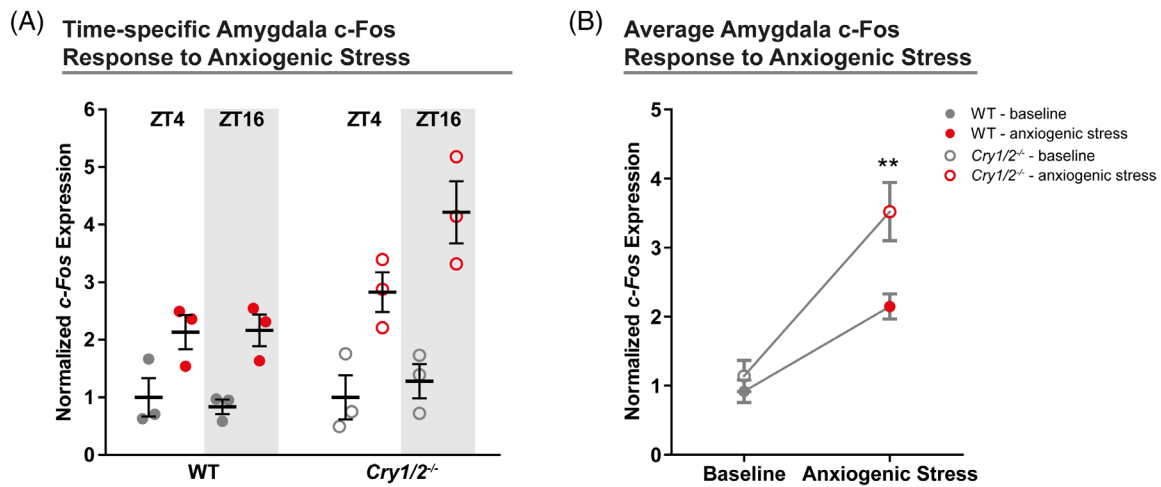


FIGURE 7 The amygdala responsiveness to an anxiogenic stressor is increased in *Cryptochrome*-deficient mice. A, To induce anxiety, WT and *Cry1/2^{-/-}* mice were transferred to an open field arena at ZT4 and ZT16. Subsequently, *c-Fos* induction in the amygdala was measured. As controls, baseline *c-Fos* 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^{Δ19}* 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 nosepekes of *Cry1/2^{-/-}* mice in the progressive ratio task without the purpose of drinking more sucrose solution. As the increased number of nosepekes 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 nosepekes 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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