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Archival Report

Metabolic Psychiatry Special Section

Rescue of Comorbid Behavioral and Metabolic Phenotypes of Arrhythmic Mice by Restoring Circadian *Cry1/2* Expression in the Suprachiasmatic Nucleus

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ABSTRACT

BACKGROUND: Psychiatric and metabolic disorders occur disproportionately often comorbidly, which poses particular hurdles for patients and therapists. However, the mechanisms that promote such comorbidities are largely unknown and therefore cannot yet be therapeutically targeted for the simultaneous treatment of both conditions. Because circadian clocks regulate most physiological processes and their disruption is a risk factor for both psychiatric and metabolic disorders, they may be considered as a potential mechanism for the development of comorbidities and a therapeutic target. In the current study, we investigated the latter assumption in *Cry1/2*$^{-/-}$ mice, which exhibit substantially disrupted endogenous circadian rhythms and marked metabolic and behavioral deficits.

METHODS: By targeted virus-induced restoration of circadian rhythms in their suprachiasmatic nucleus, we can restore behavioral as well as several metabolic processes of these animals to near-normal circadian rhythmicity.

RESULTS: Importantly, by rescuing suprachiasmatic nucleus rhythms, several of their anxiety-like behavioral as well as diabetes- and energy homeostasis–related deficits were significantly improved. Interestingly, however, this did not affect all deficits typical of *Cry1/2*$^{-/-}$ mice; for example, restlessness and body weight remained unaffected.

CONCLUSIONS: Taken together, the results of this study demonstrate, on the one hand, that restoration of disturbed circadian rhythms can be used to simultaneously treat psychiatric and metabolic deficits. On the other hand, the results also allow us to distinguish processes that depend more on local canonical clocks from those that depend more on suprachiasmatic nucleus rhythms.

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Psychiatric and metabolic disorders are often associated with each other (i.e., they are highly comorbid) and mutually influence each other. This means that individuals with a history of psychiatric disorder or metabolic disease have a higher prevalence of the other condition than the general population (1,2). The co-occurrence of both types of disorders usually imposes a severe burden on affected individuals and often presents challenges to therapists treating both conditions; for example, psychotropic drugs often have metabolic side effects and can only be used with caution in the presence of preexisting metabolic conditions (3,4). The phenomenon of psychiatric and metabolic comorbidity is so common that the existence of a diagnostically distinct metabolic-mood syndrome is discussed, proposing the existence of biological mechanisms underlying both conditions (5,6). However, the nature of such biological mechanisms remains poorly understood (7).

The circadian clock is a system that is present in nearly all cells throughout the body and imposes 24-hour oscillations on virtually all body functions. Its main pacemaker is the suprachiasmatic nucleus (SCN), which coordinates rhythms in the rest of the body and, if present, harmonizes them with light-dark rhythms from the environment. In each cell, the molecular clock is regulated by autonomous rhythmic expression of so-called clock genes, whose expression products in turn serve as transcription factors of about two-thirds of genes across all organs (8).

Because many genes are rhythmically expressed in multiple brain areas and body tissues, we hypothesized that disruption of circadian rhythmicity would affect more than one body function and therefore should be a risk factor and biological mechanism for developing comorbidity of multiple disorders, including combinations of psychiatric and metabolic pathologies. Accordingly, in a previous mouse study, we demonstrated that disruption of SCN rhythms by targeted knockdown of the essential clock gene *Bmal1* was sufficient to elicit both depression-like behavior and excessive weight gain (9).

In the current study, we aimed to investigate the reverse case, meaning to which extent genetic induction of SCN circadian rhythms in otherwise arrhythmic mice leads to improvements in behavioral and metabolic phenotypes. For this

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purpose, we chose *Cry1/2*$^{-/-}$ mice that are unable to produce endogenous rhythms (10) and exhibit pronounced behavioral (11,12) as well as metabolic (13,14) conditions. With AAVs (adeno-associated viruses), we restored rhythmic expression of the 2 missing clock genes, Cryptochrome 1 (*Cry1*) and Cryptochrome 2 (*Cry2*), specifically in the SCN as previously published (15–18) and then examined behavior and metabolism of the mice, using the same animals for both to address the character of comorbidity. Using this approach, we can answer whether restoration of circadian rhythms has, in principle, concomitant effects on behavioral and metabolic deficits in mice. In addition, because circadian clock gene rhythms are only restored in the SCN, this approach allows us to decipher which specific deficits in mice are predominantly dependent on SCN rhythms and, conversely, which seem to be more dependent on local rhythms in other regions of the body. Furthermore, we investigated to what extent potential improvements of deficits approached the values of untreated wild-type mice. With regard to patients with comorbid psychiatric and metabolic disorders, this animal study represents an important step toward understanding whether enhancement of circadian rhythms can serve as a potential adjuvant therapeutic tool to simultaneously treat both aspects of their illness. Furthermore, the results of the animal study provide a basis to better assess which aspects of the comorbid conditions may be predominantly improved by enhancing SCN rhythms.

METHODS AND MATERIALS

Animals and Housing

All experiments were performed in male *Cry1/2*$^{-/-}$; *Per2*^{Luc} (henceforth referred to as *Cry1/2*$^{-/-}$) and *Cry1/2*$^{+/+}$;*Per2*^{Luc} mice (henceforth referred to as wild-type) (10,19), both on C57BL/6J background (11). Mice were divided into 3 groups that were compared: 1) SCN-*Cry1/2*-Rescue (*Cry1/2*$^{-/-}$ mice injected with a 1:1 mixture of *pCry1-Cry1*-EGFP and *pCry1-Cry2*-EGFP AAVs into their SCN), 2) *Cry1/2*$^{-/-}$ (*Cry1/2*$^{-/-}$ mice injected with a control EF1α-EGFP AAV into their SCN), and 3) wild-type (no injection).

Molecular Cloning

Two vectors, *pCry1-Cry1*-EGFP and *pCry1-Cry2*-EGFP, were prepared. The complementary DNA of *Cry1* and *Cry2* were each coupled with the minimal *Cry1* promoter (*pCry1*) (20) and with EGFP (enhanced green fluorescent protein) complementary DNA (15–17). These sequences were used to later produce capsid serotype 1 AAV. For the control plasmid, an AAV vector encoding EGFP under the control of an EF1α promoter (pEF1α) was used. All AAVs ($\sim 5 \times 10^{13}$ GC/mL) were manufactured by Penn Vector Core (University of Pennsylvania).

Stereotaxic Injection of AAV

Stereotactic injections were performed as previously described with modifications described in the Supplement (9).

Behavioral Tests

Mice were subjected to the open field test, Y-maze test, and sucrose preference test (IntelliCage System) as previously described (11) (for more details, see the Supplement).

Indirect Calorimetric Measurements

Locomotor activity, O_2 consumption, and CO_2 production were continuously monitored by the calorimetry system (TSE Pheno-Master Systems).

More detailed information on all experiments and possible modifications to the previously published cited protocols are included in the Supplement.

RESULTS

Restoration of Circadian Rhythms in the SCN of Arrhythmic *Cry1/2*$^{-/-}$ Mice

The primary technical goal of our study was to restore circadian rhythms in the SCN of arrhythmic *Cry1/2*$^{-/-}$ mice. To this end, we developed viral vectors that, after stereotactic injection into the SCN, lead to rhythmic expression of *Cry1* and *Cry2* under control of the minimal *Cry1* promoter. We tested the efficacy of this approach on the basis of locomotor activity under dark-dark (DD) conditions in which circadian clocks are free running. As expected, wild-type, *Cry1/2*$^{-/-}$, and SCN-*Cry1/2*-Rescue mice showed rhythmic behavior under light-dark (LD) 12:12 conditions that was barely distinguishable from each other (Figure 1A). However, under DD, the circadian rhythmic behavior of *Cry1/2*$^{-/-}$ mice was immediately lost, indicating that their behavioral rhythms in LD are due to masking alone. Importantly, in contrast, SCN-*Cry1/2*-Rescue mice remained stably rhythmic under DD, and these free-running rhythms were comparable to those of wild-type animals in terms of amplitude, period, and regularity (Figure 1B, C; Figures S1 and S2; Table S1). After the completion of all experiments, to characterize the rhythmicity of the SCN in the animals used for this study, we prepared organotypic SCN explants of the animals for luminometric measurements of PER2::LUC signal, followed by microscopic detection of GFP signal from the injected AAVs. Although some of the explants died during cultivation and consequently did not give a usable luminescent signal, the results of the remaining explants confirmed the observations of the locomotor activity measurements because the SCN explants of the SCN-*Cry1/2*-Rescue animals showed comparable rhythms to those of the wild-type mice, whereas the SCNs of the *Cry1/2*$^{-/-}$ animals were arrhythmic (Figure S3). Because the explants had already been in culture for several days by the time of GFP imaging and some of them had already died, we could not detect GFP signal in explants of SCN-*Cry1/2*-Rescue animals, in which GFP is driven by the rather weak *Cry1* promoter. However, GFP was clearly localizable in the SCN in all control animals injected with virus containing GFP under the control of the stronger EF1α promoter, even in already dead explants, although the GFP signal in these explants was weak (Figure S4). Nevertheless, this confirms that we were able to reliably target the SCN during the stereotactic injections.

Thus, by our approach of rhythmic expression of CRY1 and CRY2 in the SCN of *Cry1/2*$^{-/-}$ mice, we have generated a genetically arrhythmic mouse model with a nearly complete restoration of endogenous circadian locomotor rhythms, which we can use to investigate the efficacy of restoring

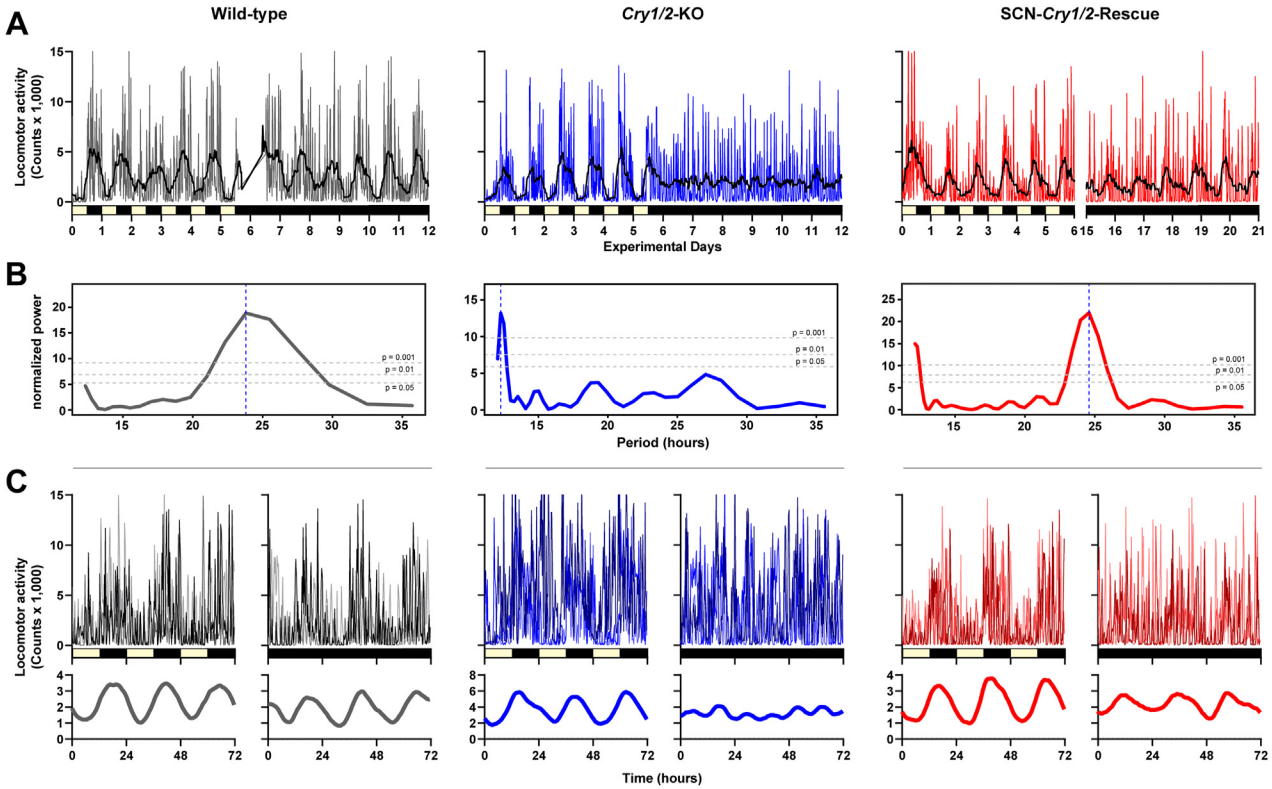

Figure 1. Rescue of rhythmic Cryptochrome expression in the SCN of arrhythmic *Cry1/2*$^{-/-}$ mice induces restoration of endogenous 24-hour activity rhythms. **(A)** Representative locomotor activity data from wild-type (black), *Cry1/2*$^{-/-}$ (blue), and SCN-*Cry1/2*-Rescue (red) mice in light-dark 12:12 and dark-dark light conditions. Activity was recorded continuously using infrared sensors. When measuring the activity, the sensors failed between days 5.5 and 6.5 for the wild-type mice and between days 6 and 15 for the SCN-*Cry1/2*-Rescue animals. Shown are the raw data in color as well as a curve of the smoothed data in black to permit better visualization of patterns of activity rhythms. Light and dark phases are shown as yellow and black bars, respectively. **(B)** Lomb-Scargle periodograms of representative wild-type, *Cry1/2*$^{-/-}$, and SCN-*Cry1/2*-Rescue mice in dark-dark conditions shown in **(A)**. Whereas *Cry1/2*$^{-/-}$ mice do not show significant circadian free-running rhythms of their locomotor activity, wild-type and SCN-*Cry1/2*-Rescue mice show similar significant free-running rhythms at approximately 24 hours. **(C)** Activity of all measured wild-type, *Cry1/2*$^{-/-}$, and SCN-*Cry1/2*-Rescue mice in representative 72-hour time windows of light-dark 12:12 and dark-dark phases. (Top) Raw data of each mouse are plotted on top of each other in different color intensities. (Bottom) Averaged and smoothed activity data of all animals in each group. Wild-type: $n = 3$, *Cry1/2*$^{-/-}$: $n = 3$, SCN-*Cry1/2*-Rescue: $n = 3$. KO, knockout; SCN, suprachiasmatic nucleus.

disrupted circadian rhythms on aspects of mental and metabolic health.

Circadian Rhythm Restoration Improves Anxiety-like Behavior in *Cry1/2*$^{-/-}$ Mice

In a previous study, we showed that *Cry1/2*$^{-/-}$ mice exhibited pronounced anxiety-like behavior, as evidenced by a preference for more protected places, reduced exploration of new areas, and increased restlessness, among others. To investigate whether restoration of endogenous circadian rhythms in the SCN can ameliorate parts of this phenotype, we retested the behavior of wild-type, *Cry1/2*$^{-/-}$, and SCN-*Cry1/2*-Rescue mice in the open field test and Y-maze test. In addition, in the current study, *Cry1/2*$^{-/-}$ mice avoided spending time in the center of the open field and spent correspondingly more time in the periphery (Figure 2A; Table S2). Interestingly, in SCN-*Cry1/2*-Rescue mice, this behavior was entirely normalized, with the consequence that they spent the same amount of time in the center and periphery of the open field as wild-type mice. Furthermore, this study confirmed a lack of willingness on the part of *Cry1/2*$^{-/-}$ mice to explore new areas in the open field test and Y-maze test, as evidenced by significantly reduced entry into each area of the open field (Figure 2A; Table S2) and smaller distance traveled and increased time of immobility in both the open field test and Y-maze test (Figure 2B; Table S2). In SCN-*Cry1/2*-Rescue mice, these behaviors also approximated those of wild-type mice, although they were not entirely normalized. In the open field test, SCN-*Cry1/2*-Rescue mice entered the center significantly more frequently than *Cry1/2*$^{-/-}$ mice (Figure 2A; Table S2). In the Y-maze test, although the distance traveled by SCN-*Cry1/2*-Rescue mice was still significantly smaller than that of wild-type mice, it tended to increase compared with that of *Cry1/2*$^{-/-}$ animals (Figure 2B and Table S2). At the same time, the immobility time of SCN-*Cry1/2*-Rescue mice significantly converged with that of wild-type mice. In the open field test, however, these variables remained unaffected by restoration of SCN rhythms. Likewise, restoration of SCN rhythms in the open field test and the Y-maze test did not affect the restlessness

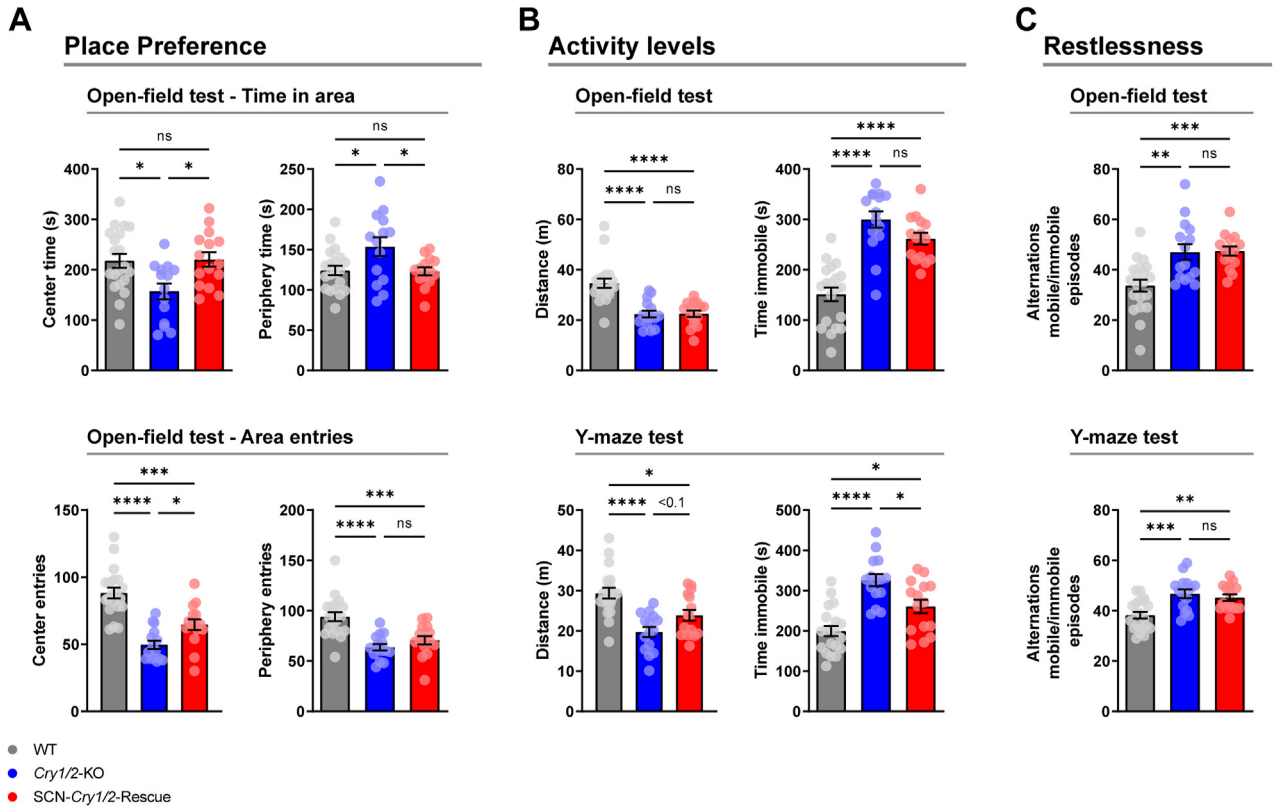

Figure 2. Restoration of rhythms in the SCN of arrhythmic *Cry1/2*$^{-/-}$mice reduces large parts of their anxiety-like phenotype. **(A)** In the open field test, restoration of SCN rhythms in SCN-*Cry1/2*-Rescue mice (red) causes their center time, periphery time, and center entries to closely approximate those of WT mice (gray), resulting in a significant improvement in behavioral deficits of *Cry1/2*$^{-/-}$ mice (blue). Periphery entries are not improved in SCN-*Cry1/2*-Rescue mice. **(B)** In both the open field test and the Y-maze test, *Cry1/2*$^{-/-}$ mice cover reduced distances and spend more time immobile. In the Y-maze test, the immobility time of SCN-*Cry1/2*-Rescue animals significantly approximates that of WT animals. **(C)** Increased restlessness of *Cry1/2*$^{-/-}$ animals, i.e., increased frequency of alternations between mobile and immobile phases during the open field test and the Y-maze test, is not improved in SCN-*Cry1/2*-Rescue mice. Data in **(A–C)** were analyzed with a one-way analysis of variance followed by Bonferroni's multiple comparison test (*$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$). All data are shown as individual values and columns with mean ± SEM. WT: $n = 20$, *Cry1/2*$^{-/-}$: $n = 15$, SCN-*Cry1/2*-Rescue: $n = 15$. KO, knockout; ns, not significant; SCN, suprachiasmatic nucleus; WT, wild-type.

typical of *Cry1/2*$^{-/-}$ mice, which was manifested by an increased number of switches between mobile and immobile episodes (Figure 2C; Table S2). Thus, in summary, restoration of SCN rhythms improved anxiety-like behavior in arrhythmic *Cry1/2*$^{-/-}$ mice, and this mainly involved aspects of place preference and, to some extent, exploration of new areas, but not restlessness.

Changes in Reward Perception and Motivation to Obtain Reward by Restoring SCN Rhythms

When *Cry1/2*$^{-/-}$ mice were given a choice between water and sugar solution in the sucrose preference test, they drank significantly more sugar solution than wild-type mice (Figure 3A; Table S3). Because they continued to ingest the same amount of pure water as wild-type mice, the elevated intake of sugar water on the one hand resulted in significantly increased intake of total fluid and on the other hand resulted in a pronounced preference for sugar solution over plain water (0.87 ± 0.14 SD), although not statistically significantly different from that of wild-type mice (0.51 ± 0.53 SD). Interestingly, in SCN-*Cry1/2*-Rescue mice, all these behaviors were normalized and corresponded to wild-type values. SCN-*Cry1/2*-Rescue mice drank more water than *Cry1/2*$^{-/-}$ mice, but—similar to wild-type mice—they drank significantly less sugar solution, which completely normalized the total amount of fluid ingested. This also means that the restoration of SCN rhythms significantly reduced the preference for sugar solution (0.19 ± 0.64 SD) compared with *Cry1/2*$^{-/-}$ mice but not wild-type mice.

The fact that *Cry1/2*$^{-/-}$ mice perceived sugar water as a strong reward is also evident in the progressive ratio paradigm of reinforcement, in which motivation to reach the sugar solution is measured. In the current experiment, performing a successively increasing number of nosepokes was necessary for the mice to reach the sugar water. The motivation is derived from the maximum number of nosepokes that the mice were willing to perform. As shown in our previous study (11), *Cry1/2*$^{-/-}$ mice were willing to perform a significantly higher number of nosepokes and were also much more goal directed than wild-type mice, requiring fewer attempts to reach the next higher stage of required nosepokes (Figure 3B; Table S3). Interestingly, despite greater efforts to access the sugar solution, they did not drink more of it than wild-type mice. Measured

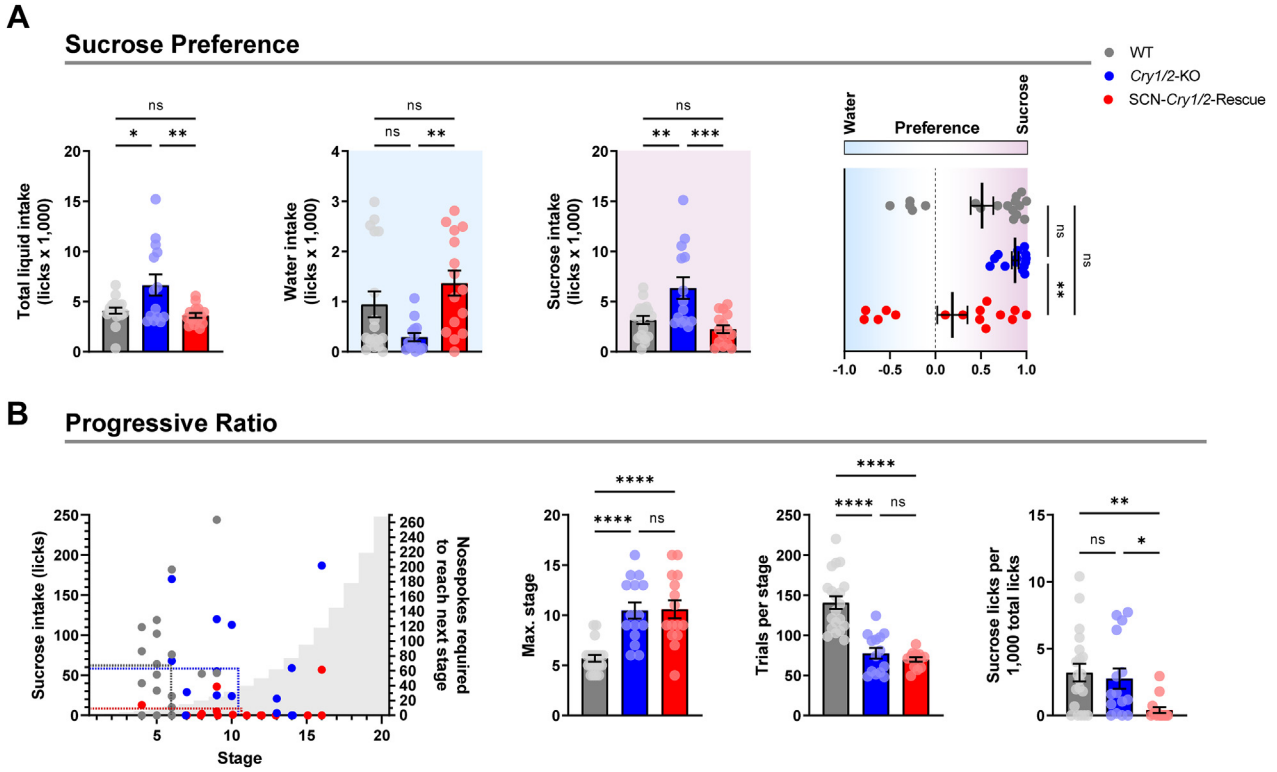

Figure 3. Changes in reward perception in SCN-*Cry1/2*-Rescue mice. **(A)** The increased sucrose preference of *Cry1/2*$^{-/-}$ mice is completely normalized by rescue of SCN rhythms. Deficits typical of *Cry1/2*$^{-/-}$ mice (blue) in the sucrose preference test, namely increased fluid intake, decreased intake of pure water, and increased intake of sucrose solution and the sucrose preference calculated from these are significantly altered in SCN-*Cry1/2*-Rescue mice (red), making them indistinguishable from those of WT mice (gray). **(B)** In the progressive ratio paradigm, motivation to reach a rewarding reinforcer (here, sucrose solution) is measured by requiring animals to perform progressively increasing stages of tasks (here, nosepokes, shown as light gray columns) to reach the reinforcer. Motivation is operationalized by the highest stage reached and the number of attempts needed to reach the next higher stage. This is contrasted with how much the animals consume from the reinforcer after successfully reaching the next higher stage. WT mice reach an average of 6 stages (equivalent to 12 nosepokes) after an average of ∼140 trials per stage and then lick the sugar solution an average of 62 times or 3 times per 1000 total licks (water + sucrose solution). *Cry1/2*$^{-/-}$ animals and SCN-*Cry1/2*-Rescue animals, on the other hand, reach an average of more than 10 stages (equivalent to 32 nosepokes) and require only 77 and 69 attempts, respectively. *Cry1/2*$^{-/-}$ animals drink about the same amount of the sucrose solution after reaching the stages as the WT animals (58 licks or 3 sucrose licks/1000 total licks). However, SCN-*Cry1/2*-Rescue animals drink significantly less (8 licks or 0.4 sucrose licks/1000 total licks). Data in **(A)** and **(B)** were analyzed with a one-way analysis of variance followed by Bonferroni's multiple comparison test (*$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$). All data are shown as individual values and columns with mean ± SEM. WT: $n = 20$, *Cry1/2*$^{-/-}$: $n = 15$, SCN-*Cry1/2*-Rescue: $n = 15$. KO, knockout; max, maximum; ns, not significant; SCN, suprachiasmatic nucleus; WT, wild-type.

by maximum stage reached and the number of attempts to reach the next higher stage, the rhythmic SCN-*Cry1/2*-Rescue mice showed the same increased motivation to reach the sugar solution as the arrhythmic *Cry1/2*$^{-/-}$ mice. However, despite exerting great effort, they drank significantly less of the sugar solution than wild-type and *Cry1/2*$^{-/-}$ mice. This means that the increased motivation of *Cry1/2*$^{-/-}$ mice to perform nosepokes was not normalized by the restoration of endogenous rhythms in SCN-*Cry1/2*-Rescue mice. In contrast, the motivation of SCN-*Cry1/2*-Rescue mice seemed to be detached from the rewarding effect of the sugar water because after performing high numbers of nosepokes, they hardly consumed it.

Effects of Restoration of SCN Rhythms on Energy Metabolism

Our basic assumption is that the circadian system ensures the proper coordination of all bodily functions and therefore has an influence on several aspects of health. For this reason, in the same animals in which we performed the behavioral tests described above, we also performed metabolic tests to verify whether restoration of disturbed circadian rhythms can lead to the simultaneous improvement of comorbid mental and metabolic impairments. At first, we performed indirect calorimetric measurements with the animals under LD 12:12 and DD conditions to investigate, first, to what extent restoration of SCN rhythms can restore rhythms of respiratory exchange ratio (RER) and energy expenditure (EE) and second, to investigate whether RER and EE levels are generally influenced by the absence or presence of endogenous circadian rhythms.

In wild-type mice, both the RER and EE showed distinct 24-hour oscillations, which continued in free-running conditions, i.e., DD (Figure 4A; Table S4). As expected, these oscillations were not observed in *Cry1/2*$^{-/-}$ mice in DD. However, RER and EE of SCN-*Cry1/2*-Rescue animals also exhibited distinct 24-hour rhythms in DD that were not markedly different from

those of wild-type animals, indicating that restoration of SCN rhythms was sufficient to induce diurnal rhythms of central energy metabolic functions.

To test whether the loss or restoration of circadian rhythms affected baseline RER and EE levels, we performed calorimetric measurements under conditions of a normal-chow diet and a high-fat diet. Under a normal-chow diet, RER and EE did not differ across any of the 3 animal groups and were approximately 0.8 and 7 kcal/hour/kg, respectively (Figure 4B; Table S4). However, when the metabolism of the animals was burdened with a high-fat diet, differences between wild-type and *Cry1/2*$^{-/-}$ mice became apparent, which can be partially reversed by restoring circadian rhythms in the SCN. In general, the high-fat diet attenuated diurnal rhythms of RER and EE very strongly in all 3 animal groups, even under LD 12:12 (Figure 4C). This effect was particularly pronounced in *Cry1/2*$^{-/-}$ animals and was also apparent in their locomotor rhythms in LD 12:12. However, in SCN-*Cry1/2*-Rescue animals, the effect of a high-fat diet on RER, EE, and locomotor rhythms was less pronounced and was similarly strong as that in wild-type animals.

Under a high-fat diet, the RER in wild-type mice decreased to about 0.6, indicating that the mice on this diet

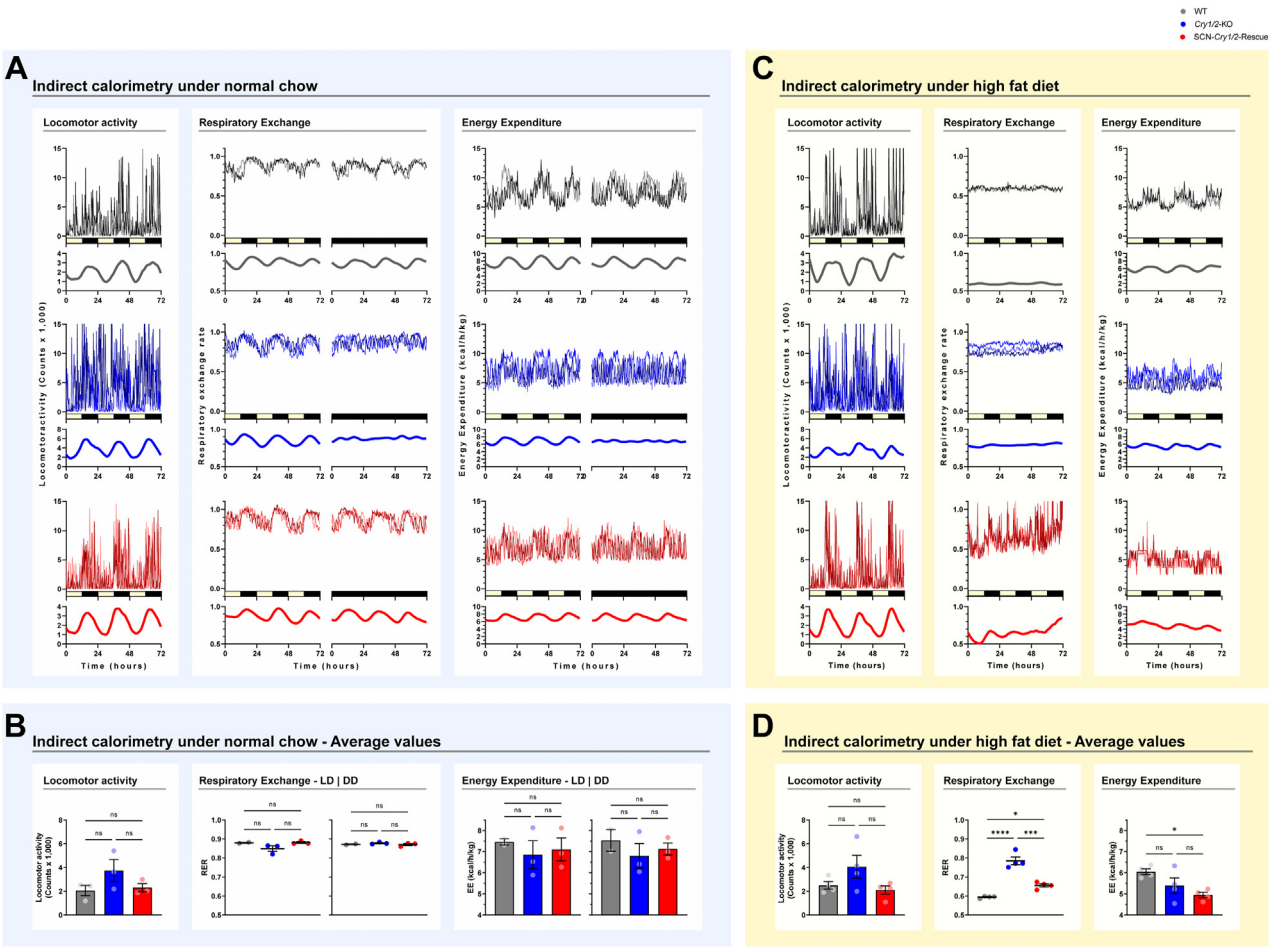

Figure 4. Rescue of circadian SCN rhythms in *Cry1/2*$^{-/-}$ mice normalizes the RER under high-fat diet. **(A)** Circadian rhythms of locomotor activity (see also Figure 1), RER, and EE were measured from WT (gray), *Cry1/2*$^{-/-}$ (blue), and SCN-*Cry1/2*-Rescue (red) mice in LD 12:12 and DD. Data of representative 72-hour time windows are shown. (Top) Raw data of each mouse are plotted on top of each other in different color intensities. (Bottom) Averaged and smoothed activity data of all animals in each group. Light and dark phases are shown as yellow and black bars, respectively. Restoration of SCN rhythms in SCN-*Cry1/2*-Rescue mice results in near-complete restitution of endogenous rhythms of RER and EE lost in *Cry1/2*$^{-/-}$ mice in DD. **(B)** Average levels of locomotor activity, RER, and EE under normal chow. In both LD 12:12 and DD, these variables were not significantly different between the 3 groups. **(C)** The same variables in LD 12:12 under high-fat diet. The high-fat diet causes an attenuation of circadian rhythms of RER and EE in all 3 groups. The particularly strong expression of this effect in *Cry1/2*$^{-/-}$ mice and the impairment of their locomotor activity rhythms are reversed in SCN-*Cry1/2*-Rescue mice. **(D)** Average levels of locomotor activity, RER, and EE under high-fat diet. While overall activity levels are the same in all 3 groups also under high-fat diet, *Cry1/2*$^{-/-}$ mice show a strongly increased RER compared with WT animals. This is almost completely normalized in SCN-*Cry1/2*-Rescue animals. However, SCN-*Cry1/2*-Rescue animals show significantly lower EE than WT animals, whereas *Cry1/2*$^{-/-}$ mice show no deficit. Data in **(A)** and **(C)** were analyzed with a one-way analysis of variance followed by Bonferroni's multiple comparison test (*$p < .05$, ***$p < .001$, ****$p < .0001$). All data are shown as individual values and columns with mean ± SEM. Normal chow: WT: $n = 2/3$, *Cry1/2*$^{-/-}$: $n = 3$, SCN-*Cry1/2*-Rescue: $n = 3$. High-fat diet: WT: $n = 4$, *Cry1/2*$^{-/-}$: $n = 4$, SCN-*Cry1/2*-Rescue: $n = 4$. DD, dark-dark; EE, energy expenditure; KO, knockout; LD, light-dark; ns, not significant; RER, respiratory exchange ratio; SCN, suprachiasmatic nucleus; WT, wild-type.

predominantly used fat as an energy source (Figure 4D). In contrast, in *Cry1/2*$^{-/-}$ mice, the RER did not change and was at approximately 0.8 even under a high-fat diet, indicating a combination of fat and carbohydrate as energy source. In SCN-*Cry1/2*-Rescue mice, the RER was 0.6, which corresponds to the normal state under a high-fat diet based on the results of the wild-type animals. EE was lower in wild-type mice at approximately 6 kcal/hour/kg under a high-fat diet than under a normal-fat diet and was even lower in SCN-*Cry1/2*-Rescue mice. However, in *Cry1/2*$^{-/-}$ mice, the EE was similar to that in wild-type mice (Figure 4D). Physical activity has a strong influence on RER and EE, but its influence on differences in RER and EE between the 3 groups of mice can be excluded because activity levels were the same in all 3 groups of mice. This suggests that loss and restoration of circadian rhythm affects energy metabolism directly, rather than indirectly by altering locomotor activity.

Restoration of Circadian SCN Rhythms Prevents Diabetes in *Cry1/2*$^{-/-}$ Mice

To examine whether the metabolic changes caused by the loss or restoration of circadian rhythms in *Cry1/2*$^{-/-}$ mice affected their physical health, we continuously recorded the body weight of the mice during the 10-week course of the high-fat diet and subsequently used a glucose tolerance test and insulin tolerance test to investigate whether the mice had deficits in their glucose metabolism. From birth, the *Cry1/2*$^{-/-}$ mice were slightly smaller and lighter than the wild-type mice (Figure 5A; Tables S5 and S6). During the high-fat diet, mice in all 3 groups gained significant weight; however, the weight of the *Cry1/2*$^{-/-}$ mice always remained below the weight of the wild-type mice. The rescue of circadian rhythms in the SCN of *Cry1/2*$^{-/-}$ mice had no influence on this because *Cry1/2*$^{-/-}$ and SCN-*Cry1/2*-Rescue mice showed very similar weight development at all times. Nevertheless, *Cry1/2*$^{-/-}$ mice showed pronounced glucose metabolism deficits, which were completely prevented in SCN-*Cry1/2*-Rescue mice until the end of measurement. In the glucose tolerance test, mice in all 3 groups were equally capable of clearing the injected glucose and returning to fasting glucose levels equally rapidly (Figure 5B; Tables S7 and S8). However, during the insulin tolerance test, *Cry1/2*$^{-/-}$ mice displayed marked insulin resistance, as evidenced by lower glucose response to injected insulin. Due to the stress of intraperitoneal injection, blood glucose levels in wild-type mice rose briefly after 15 minutes but dropped back to fasting glucose levels after 30 minutes. The initial rise in blood glucose was significantly more pronounced in *Cry1/2*$^{-/-}$ mice and remained very high until it returned to blood glucose levels of wild-type animals only at the end of the test. Interestingly, this deficit was entirely prevented in SCN-*Cry1/2*-Rescue mice. The responses of their blood glucose to the injection of insulin were indistinguishable from those of the wild-type animals. Taken together, this evidence demonstrates that the rescue of rhythms in the SCN of endogenously arrhythmic *Cry1/2*$^{-/-}$ mice, in addition to leading to improvements in their behavioral deficits, can also improve metabolic deficits, including energy and glucose metabolism (Figure 5C).

DISCUSSION

Psychiatric and metabolic disorders frequently occur comorbidly (1,2,7). However, it is largely unknown which biological factors play a role or whether they can be considered as targets for the simultaneous treatment of comorbidities. We assume that circadian clocks constitute such a factor because they regulate virtually all functions of the body, and a disturbance can have a simultaneous impact on several aspects of health. Such effects have been confirmed by numerous previous studies showing that disruption of circadian rhythms can cause both behavioral and metabolic abnormalities in animals. For example, *Clock*$^{\Delta 19}$ mice develop obesity and metabolic syndrome even under a normal diet and have been described in studies independent of this as an animal model of mania-like behavior (21,22). In a similar way, the *Cry1/2*$^{-/-}$ mice used in this study are known for their anxiety-like phenotype and hyperinsulinemia (11,13). In addition, comorbidity of behavioral and metabolic deficits can be observed in animals in which circadian rhythms of the SCN are disrupted (9).

In the current study, the reverse case was investigated, namely whether restoring disturbed circadian rhythms in the SCN simultaneously improves both behavioral and metabolic deficits in the same animals. We achieved this by using endogenously arrhythmic *Cry1/2*$^{-/-}$ mice and restoring circadian rhythms specifically in their SCN using viral stereotactic injections and performing various behavioral and metabolic tests. Thus, the resulting animal model has the unique property of having a functional SCN but no functional endogenous clocks in the rest of the body. Thus, with this model, we can investigate which behavioral and metabolic aspects of mice depend primarily on SCN rhythms and the peripheral rhythms that result from them or, conversely, which of these aspects are still deficient in SCN-*Cry1/2*-Rescue animals and therefore depend more on local canonical clocks in tissues outside the SCN.

To restore circadian rhythms specifically in the SCN of otherwise arrhythmic *Cry1/2*$^{-/-}$ mice, we chose stereotactic injection of AAVs rhythmically expressing Cryptochromes under control of the *mCry1* promoter, which have already been used successfully in the same form for the same purpose in the studies of Edwards *et al.*, Brancaccio *et al.*, and Maywood *et al.* (15–18). Using this approach, our data correspond to their results that restoration of SCN rhythms caused *Cry1/2*$^{-/-}$ animals to exhibit wild-type–like approximately 24-hour locomotor activity rhythms, which are endogenous because they persist under constant environmental conditions. Moreover, after completion of all experiments, we confirmed the restoration of rhythms of PER2::LUC expression in organotypic SCN explants. However, we only succeeded in doing so in a subset of animals (10 of 15), while the explants of other animals died in culture. For this reason, we cannot state with certainty whether these 5 SCN-*Cry1/2*-Rescue animals became rhythmic. However, we also assume a successful intervention in these animals because their behavior and insulin tolerance test values also improved to the same extent as those of the 10 animals in which we could clearly show the restoration of rhythms.

The results of the current study show that key aspects of the anxiety behavior typical of *Cry1/2*$^{-/-}$ mice (11) are significantly

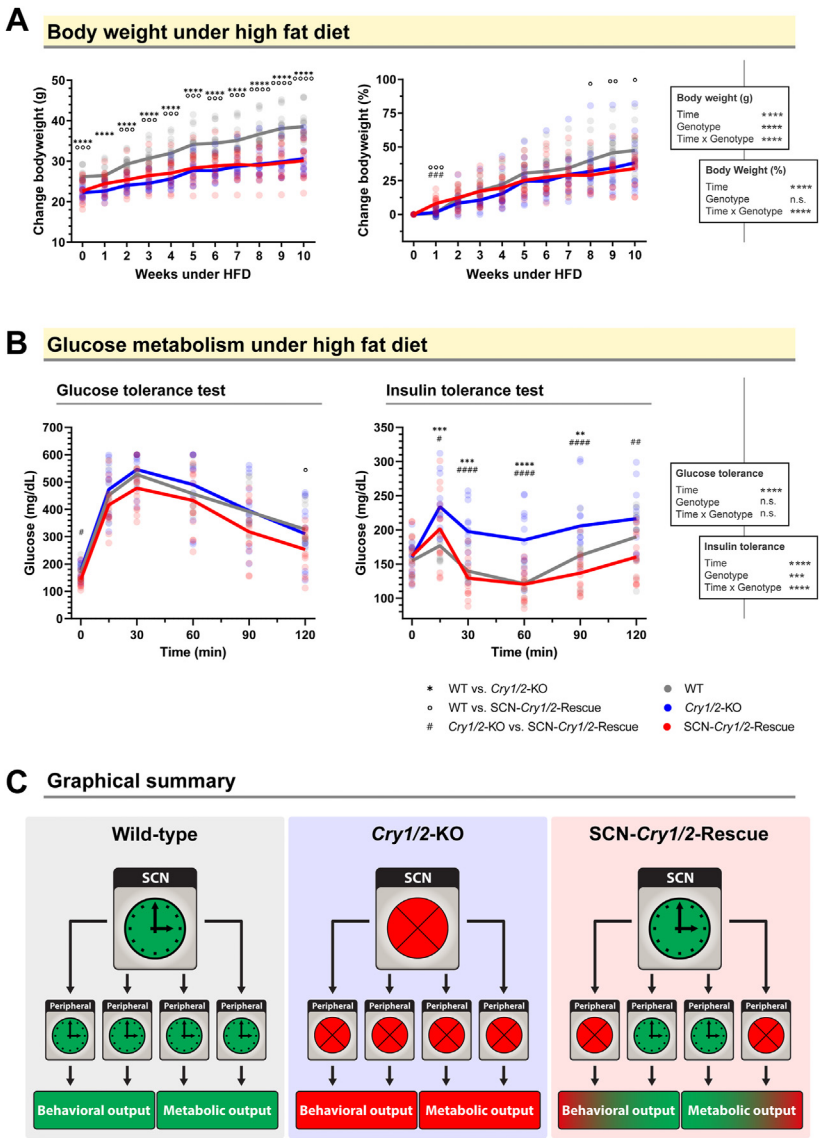

Figure 5. Prevention of a prediabetic state by restoration of endogenous SCN rhythms. **(A)** Body weight development of WT (gray), *Cry1/2*$^{-/-}$ (blue), and SCN-*Cry1/2*-Rescue (red) mice during a 10-week HFD. *Cry1/2*$^{-/-}$ and SCN-*Cry1/2*-Rescue mice are significantly lighter compared with WT mice, and relative weight gain is very similar in all 3 groups, with WT animals gaining slightly more relative weight than Rescue animals during the last 3 weeks of the experiment. **(B)** In the glucose tolerance test, blood glucose increases to the same degree after injection of glucose in all 3 groups and is also cleared to the same degree. However, in the insulin tolerance test, *Cry1/2*$^{-/-}$ mice show significantly lower insulin sensitivity than WT mice, resulting in an increased blood glucose value after insulin injection. This effect is entirely prevented in SCN-*Cry1/2*-Rescue mice. Data in **(A)** and **(B)** were analyzed with a mixed-effects model (due to some single missing values of mice that became ill or died during the experiment) followed by Bonferroni's multiple comparison test. Each replicate is shown with connecting lines of the mean. WT: $n = 20$, *Cry1/2*$^{-/-}$: $n = 14$, SCN-*Cry1/2*-Rescue: $n = 15$. **(C)** Graphical summary of results. WT mice have both intact SCN and peripheral circadian canonical clocks. Their behavioral and metabolic phenotype shows no deficits and is taken as a baseline state for mouse health. *Cry1/2*$^{-/-}$ mice have no functioning canonical circadian clock in any tissue of the body. They show marked deficits at both behavioral and metabolic levels. In SCN-*Cry1/2*-Rescue mice, the canonical clock is restored in the SCN. This leads to restoration of a number of downstream rhythms, for example, locomotor activity, respiratory exchange rate, and energy expenditure. In these mice, restoration of the endogenous circadian rhythmicity of the SCN results in improvement of many, but not all, deficits typical of *Cry1/2*$^{-/-}$ mice. WT vs. *Cry1/2*$^{-/-}$: $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$; WT vs. SCN-*Cry1/2*-Rescue; $^{\circ}p < .05$, $^{\circ\circ}p < .01$, $^{\circ\circ\circ}p < .001$, $^{\circ\circ\circ\circ}p < .0001$; *Cry1/2*$^{-/-}$ vs. SCN-*Cry1/2*-Rescue: $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$, $^{\#\#\#\#}p < .0001$. HFD, high-fat diet; KO, knockout; n.s., not significant; SCN, suprachiasmatic nucleus; WT, wild-type.

improved by the rescue of SCN rhythms and in some cases reach near–wild-type levels. This mainly concerns place preference and novelty-induced locomotor activity in the open field test and the Y-maze test. Moreover, in the sucrose preference test, the restoration of SCN rhythms resulted in a separation of mice strongly preferring sucrose and mice preferring water, which corresponds to the preference of wild-type mice. Accordingly, SCN-*Cry1/2*-Rescue animals drank less sucrose on average than *Cry1/2*$^{-/-}$ animals and thus the same amount as wild-type animals. In addition to increased preference, the progressive ratio experiment also shows that *Cry1/2*$^{-/-}$ mice were significantly more willing to perform operant responses to obtain the sucrose solution. This behavior remained unaffected by the rescue of SCN rhythms. From the perspective of the incentive sensitization theory of the development of addiction to substances, the rescue of circadian SCN rhythms thus causes a reduction in excessive "liking" (normalization of consumption in the sucrose preference test), but not in the compulsive "wanting" of substance-related rewards (no normalization of pursuit of sucrose in the progressive ratio test) (23,24).

Comorbidity is characterized by the presence of multiple disorders in the same individual. For this reason, we also performed metabolic measurements in the same mice with which we conducted the behavioral experiments. Importantly, in addition to the behavioral improvements, the rescue of circadian SCN rhythms also improved metabolic deficits in the *Cry1/2*$^{-/-}$ mice. The previously described obesity of *Cry1/2*$^{-/-}$ mice under a high-fat diet (13) was not replicated in our study; accordingly, no improvement could be expected in this regard.

On the contrary, our *Cry*-deficient mice were slightly smaller and lighter than wild-type animals throughout the experiment, and SCN rescue did not change this. Interestingly, our *Cry1/2*$^{-/-}$ mice on a high-fat diet nevertheless ultimately gained weight in a manner similar to that described previously; however, in the former study, the wild-type animals on high-fat diets surprisingly had not experienced any excessive weight gain, which is why the *Cry1/2*$^{-/-}$ mice appeared overweight by comparison (13). Nevertheless, our data show that *Cry1/2*$^{-/-}$ mice suffered from a prediabetic state after 10 weeks of a high-fat diet. This was manifested on the one hand by reduced insulin sensitivity in the insulin tolerance test and on the other hand possibly by the fact that *Cry*-deficient mice consumed more fluid. This so-called polydipsia represents a typical consequence of diabetes also in rodents (25,26). Impressively, restoration of SCN rhythms completely prevented both the decreasing insulin sensitivity and the increased fluid consumption by the time of measurement. In addition, our calorimetry data show that SCN-*Cry1/2*-Rescue animals on a high-fat diet derived most of their energy from lipids (27), similar to wild-type animals at the same physical activity level, whereas the RER of *Cry1/2*$^{-/-}$ animals indicates increased utilization of sugars. Thus, in addition to reductions in anxiety-like behavior, there were also effective metabolic improvements in SCN-*Cry1/2*-Rescue mice.

The SCN has several ways of transmitting its own time signal to other parts of the body (28). These include direct as well as indirect pathways. Direct pathways of this relatively small brain structure are neuronal connections and humoral signals that can ultimately reach other parts of the body by diffusion. Indirectly, the SCN reaches other parts of the body by determining sleep-wake rhythms and thus restricting food intake and exposure to other zeitgebers, such as light, to certain times of the day. The resulting zeitgeber rhythms serve as a time signal to tissues outside the SCN.

Regarding the transmission of SCN timing signaling to a variety of bodily functions, it is important to note that some of these physiological rhythms can be established and maintained by such direct and indirect SCN timing signals alone, but others depend on local, canonical clocks in the respective tissues. For example, metabolic deficits induced by high-fat diets in *Cry1/2*$^{-/-}$ mice and mice with liver-specific deletion of circadian rhythms can be largely prevented by temporal restriction of the zeitgeber food alone, showing that the rhythmic presence of the zeitgeber food is sufficient to prevent health deficits even in the absence of molecular local clocks (29). On the other hand, mice with tissue-specific knockout of circadian rhythms show deficits in both behavior and metabolism despite exposure to rhythmic zeitgebers (30–36).

Consistent with this and with our expectations, our data show that rescue of circadian SCN rhythms ameliorated many of the behavioral and metabolic deficits typical of *Cry1/2*$^{-/-}$ mice, but not all. Deficits in place preference, novelty-induced locomotor activity, "liking" in sucrose preference, substrate metabolism, and insulin sensitivity can be significantly improved or prevented by rescuing the SCN clock. However, SCN-*Cry1/2*-Rescue mice showed nearly unchanged deficits in, for example, their restlessness, sucrose "wanting," and EE, suggesting that these domains of health are more dependent on local, canonical clocks in peripheral tissues.

Nevertheless, it is of the utmost importance that restoration of SCN rhythms is sufficient to significantly improve or prevent a variety of behavioral and metabolic deficits in *Cry1/2*$^{-/-}$mice simultaneously. This study again emphasizes that circadian rhythms influence a variety of different body functions. In addition, these results demonstrate that this influence may be translated into therapeutic benefits because stabilization of circadian rhythms may also be used in patients to support treatment of comorbid psychiatric and metabolic disorders or to prevent such comorbidities. Our study is limited to the SCN, and we showed that many but not all deficits in *Cry1/2*$^{-/-}$ mice were improved by restoring SCN rhythms. In terms of translating the results into a therapy, it would therefore probably be reasonable to aim at stabilizing as many other rhythms in the body as possible to positively affect other deficits that are unaffected by stable SCN rhythms. This would create the possibility of a therapy with a low risk of side effects for effective additional treatment of comorbid psychiatric and metabolic diseases or their prevention.

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