

## Report

# Circadian Time-Place Learning in Mice Depends on *Cry* Genes

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## Summary

Endogenous biological clocks allow organisms to anticipate daily environmental cycles [1–3]. The ability to achieve time-place associations is key to the survival and reproductive success of animals. The ability to link the location of a stimulus (usually food) with time of day has been coined time-place learning [4–11], but its circadian nature was only shown in honeybees [1] and birds [5–7]. So far, an unambiguous circadian time-place-learning paradigm for mammals is lacking. We studied whether expression of the clock gene *Cryptochrome* (*Cry*), crucial for circadian timing, is a prerequisite for time-place learning. Time-place learning in mice was achieved by developing a novel paradigm in which food reward at specific times of day was counterbalanced by the penalty of receiving a mild footshock. Mice lacking the core clock genes *Cry1* and *Cry2* (*Cry* double knockout mice; *Cry1*<sup>-/-</sup> *Cry2*<sup>-/-</sup>) learned to avoid unpleasant sensory experiences (mild footshock) and could locate a food reward in a spatial learning task (place preference). These mice failed, however, to learn time-place associations. This specific learning and memory deficit shows that a *Cry*-gene dependent circadian timing system underlies the utilization of time of day information. These results reveal a new functional role of the mammalian circadian timing system.

## Results

### Time-Place Learning in Wild-Type Mice

We developed a paradigm for mice in order to study the role of the circadian timing system in time-place learning at a genetic and molecular level in mammals. Mice (C57BL6/J) were challenged in a symmetric three-arm maze with a balanced approach of a positive reinforcer (food gathered at the end of the arm) and a negative reinforcer (a mild but aversive footshock; delivered via a grid floor at the end of the arm; Figure 1A;

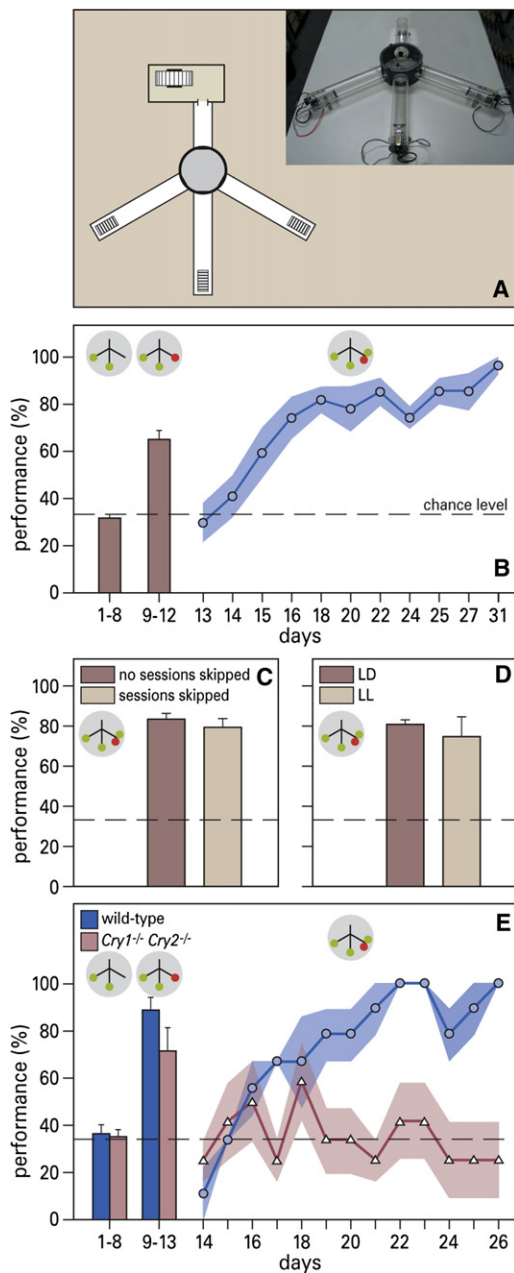
see the [Supplemental Data](#) available online and [Figure S1](#) for the detailed protocol of the experimental procedure). Mice had to learn and remember, depending on the time of day, in which of the three arms the positive reinforcer was present and which arm should be avoided because of the negative reinforcer. Mice ( $n = 9$ ), housed under 12 hr:12 hr light:dark (LD) conditions, received three daily sessions during the light period, each 3 hr apart. They rapidly learned to avoid the negatively reinforced arm at the correct time of day (days 9–12 in [Figure 1B](#)). We used a rigorous criterion: A session was considered correct if the mouse visited the two baited arms first, irrespective in which order and thus avoiding the negatively reinforced arm at this stage. Hence, correct performance refers to avoiding the negatively reinforced arm while visiting the other two arms. Mice perform above chance level significantly (two-sample  $t$  test;  $p = 0.0001$ ). However, as soon as all arms were baited (i.e., a conflict was introduced with both positive and negative reinforcers in a single arm), the performance of the mice dropped back to chance level (day 13 in [Figure 1B](#)). We supposed that at this stage of the protocol, visual and olfactory cues (i.e., mice can see and smell the food crumbs in the baited arms) were used to discriminate the negatively reinforced arm from the safe (and baited) arms instead of forming a time-place association. With evenly spread olfactory cues, however, mice gradually acquired and finally mastered the time-place learning task (i.e., correctly avoiding the negatively reinforced arm at the correct time of day), reaching a correct performance of at least 80% (see learning curve over experimental days 13–31 in [Figure 1B](#); performance significantly increased over time [logistic regression,  $F_{9,80} = 4.54$ ,  $p < 0.0001$ ]). No differences were found in performance for the three time points; morning, noon, and afternoon sessions were all performed at a level of at least 80% at the end of the time-place association protocol. In line with this observation is that skipping of sessions (see the [Supplemental Data](#)) still resulted in a correct performance of at least 80% ([Figure 1C](#)).

Time-place learning can be based on the sequence of events (ordinal timer), on a stopwatch-like mechanism (interval timer), or on knowledge of (circadian) time of day. To discriminate between these, we first tested how mice responded to skipping sessions (morning session, noon session, or a combination of morning and noon session). With the skipping of sessions, the sequence of events is altered, and stopwatch-like mechanisms become unreliable. If mice perform time-place associations on the basis of these mechanisms, they will visit the wrong arms, resulting in a significant drop in correct performance. At days with omitted sessions, however, performance was as good as in days without skipped sessions ([Figure 1C](#), performance  $\geq 80\%$ ; Wilcoxon signed rank test  $p > 0.80$ ), ruling out the use of such timers. Second, to further test whether an interval timer could have been used, with the transition of lights on and/or off as a starting point under 12 hr:12 hr LD conditions, we tested time-place learning under constant-light conditions ([Figure 1D](#)). Mice performed equally well under constant-light and LD conditions (Wilcoxon signed rank test  $p > 0.43$ ), rendering it unlikely that an interval timer was used. By deduction, all these results are in line with the

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**Figure 1. Time-Place Learning and Memory Depends on Intact Circadian Systems**

(A) Schematic representation of the maze (insert: photographic representation).

(B) Time-place learning in C57BL6/J mice ( $n = 9$ ). At experimental days 1–8 (test situation with food in two arms, no footshock delivery), performance is at chance level. At experimental days 9–12 (test situation with food in two arms and footshock delivery [0.09 mA, < 1 s] in the nonbaited arm), mice reach a high level of performance (mice perform significantly above chance level;  $p = 0.0001$ ). At experimental days 13–31 (test situation with food reward in all arms and footshock delivery in one arm), mice gradually master the task and display time-place learning ( $p < 0.0001$ ).

(C) Skipping of sessions (on days omitted on the x axis of Figure 1B) does not impair performance ( $p > 0.80$ ), indicating that mice cannot rely on an ordinal timer for time-place learning.

(D) Testing of mice under constant-light conditions does not impair performance ( $p > 0.43$ ).

(E) Like (B), but now with  $Cry1^{-/-}Cry2^{-/-}$  ( $n = 4$ ) and  $Cry1^{+/+}Cry2^{+/+}$  ( $n = 3$ ) control mice.  $Cry1^{-/-}Cry2^{-/-}$  mice fail to display time-place learning

interpretation of the use of a circadian-clock system and time of day in this type of learning.

#### Time-Place Learning in $Cry1^{-/-}Cry2^{-/-}$ Mice

To show that the circadian system is involved in time-place learning, we selected mice lacking functional cryptochrome proteins mCRY1 and mCRY2 as a critical clock gene knockout model. These  $Cry1^{-/-}Cry2^{-/-}$  mice are arrhythmic under constant-light or -dark conditions but show masking under LD [12, 13].  $Cry1^{-/-}Cry2^{-/-}$  mice ( $n = 4$ ) and their specific C57BL6/J wild-type controls ( $n = 3$ ) were tested in the time-place-learning paradigm. No apparent differences in overt behavior of the  $Cry1^{-/-}Cry2^{-/-}$  mice were observed during the habituation phase (experimental days –6–0). During experimental days 1–8, both lines of mice performed again at chance level (Figure 1E). During experimental days 9–13, both lines learned to avoid the negatively reinforced, nonbaited arm (Figure 1E; both wild-types and  $Cry1^{-/-}Cry2^{-/-}$  mice perform above chance level; one-sample t test: wild-types  $p = 0.01$ ,  $Cry1^{-/-}Cry2^{-/-}$  mice  $p = 0.003$ ). Thereafter, testing was performed with food in all arms and footshock delivery in one of the three arms (depending on time of day). Wild-type control mice performed similarly to the mice in the first experiment (Figure 1B). In contrast,  $Cry1^{-/-}Cry2^{-/-}$  mice failed to master the task (experimental days 14–26, Figure 1E; learning curves differed significantly between  $Cry1^{-/-}Cry2^{-/-}$  mice and wild-type controls [logistic regression slope,  $T = 4.76$ ,  $df = 5$ ;  $p \leq 0.006$ ]).  $Cry1^{-/-}Cry2^{-/-}$  performance never exceeded chance level (one-sample t test,  $p > 0.05$ ), even after 8 days of testing (averaged over the last five testing days:  $Cry1^{-/-}Cry2^{-/-}$   $32\% \pm 0.11\%$  standard error of the mean [SEM]; wild-types  $93\% \pm 0.04\%$  SEM;  $T = 4.59$ ,  $p < 0.006$ ). When a less rigorous criterion was used for a correct session (only avoiding the negatively reinforced arm during the first visit),  $Cry1^{-/-}Cry2^{-/-}$  mice still performed at chance level (binominal test;  $p > 0.23$ ; controls performed above chance level;  $p < 0.04$ ). These results unambiguously demonstrate that *Cry* genes are necessary for time-place learning and therefore that an intact molecular circadian (clock) system is required for this type of time memory.

Behavioral activity patterns did not differ between  $Cry1^{-/-}Cry2^{-/-}$  and control mice, indicating that the difference in capability of time-place learning between  $Cry1^{-/-}Cry2^{-/-}$  mice and control mice was not due to differences in behavioral organization (Figures 2 and 3). Increased activity was observed at the end of the light phase for all mice. Some form of food-anticipatory activity occurred in all mice, including the  $Cry1^{-/-}Cry2^{-/-}$  mice. Deletion of the two *Cry* genes seemed not to interfere with this type of overt behavior. It should be noted, however, that the activity of the  $Cry1^{-/-}Cry2^{-/-}$  mice was suppressed in the dark phase (Figures 2 and 3). After the testing, all mice received food ad libitum again (Figure 2, posttest). Behavioral activity shifted back completely to the dark phase after several transient days, as was seen both in  $Cry1^{-/-}Cry2^{-/-}$  mice and control mice (Figures 2 and 3).

(learning curves differed significantly between  $Cry1^{-/-}Cry2^{-/-}$  mice and wild-type controls [ $p \leq 0.006$ ]).

Symbols: green indicates a baited arm, and red indicates arm with footshock delivery. Note that the symbols represent one particular configuration of reward and penalty during one of the three daily training sessions. This configuration shifts predictably according to time of day of the training session (see Figure S1 for additional experimental details). Error bars and shaded areas indicate the SEM.

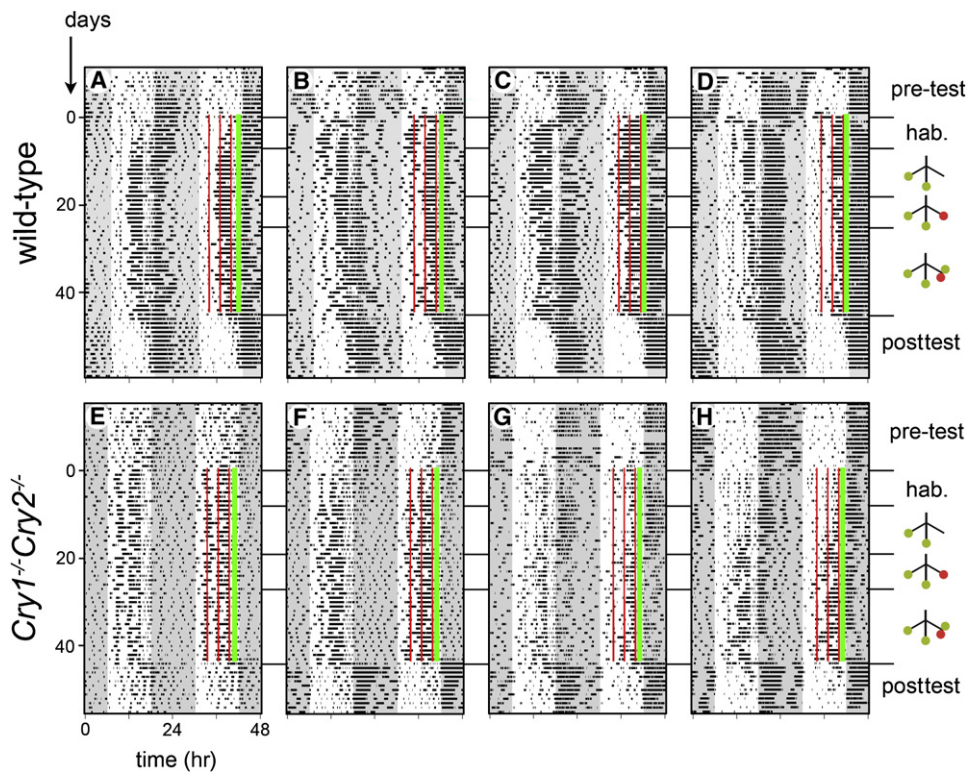


Figure 2. Activity Records of *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* Mice and C57BL6/J Controls Show No Distinct Differences Related to the Time-Place Learning Task

Double-plotted actograms of running wheel activity of four (out of nine) wild-type control mice (upper panels, [A]–[D]) and four *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice (lower panels, [E]–[H]) under 12 hr:12 hr light:dark (dark is period depicted as the gray zone). Before the start of the experiment, mice were acclimatized to their home cage with a running wheel and free access to food for 10 days (pretest). *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice reveal masking, with most activity in the dark period. Mice were then food deprived and habituated (hab) to the test apparatus for 8 days. Thereafter, experimental days started with test conditions indicated by symbols as explained in Figure 1 and Figure S1. Both *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice and controls shifted their locomotor activity toward the end of the light period because of testing and feeding. At the end of the experiment (posttest), mice were put back on free access to food. Red lines indicate the training sessions (3 hr apart for each individual). Green lines indicate feeding times in the home cage.

### Learning Performance of *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* Mice in Other Learning Tasks

To test whether *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice were not deficient for place association per se, we tested mice in a Y maze reference task for spatial learning and reversal learning (Figure 4A). Both *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* ( $n = 7$ ) and control mice ( $n = 9$ ) gradually learned to discriminate the baited arm from the nonbaited arm ( $F_{7,98} = 6.589$ ;  $p < 0.001$ ; repeated-measures analysis of variance [ANOVA]). *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* and control mice did not differ from each other ( $F_{1,14} = 0.001$ ;  $p = 0.98$ ). Reversal training, reflecting behavioral flexibility and the ability to adapt to a novel situation, showed that both types of mice gradually learned to discriminate the baited arm from the nonbaited arm in the new situation ( $F_{5,70} = 26.658$ ;  $p < 0.001$ ) with similar acquisition rate ( $F_{1,14} = 0.026$ ;  $p = 0.87$ ).

To exclude the possibility that *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice were unable to form an association between obtaining a footshock and the environment where the footshock was received, we tested the *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice in a contextual fear conditioning task. *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* ( $n = 8$ ) and control mice ( $n = 8$ ) explored the fear conditioning box during the habituation phase with equal activity ( $F_{1,12} = 0.877$ ;  $p = 0.37$ ; Figure 4B). The memory retention test 24 hr later revealed that both *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* and control mice associated the box with the footshock (0.7 mA, 2 s, constant current) to a similar level (percent of time spent freezing was  $38.2\% \pm 8.0\%$  SEM and  $52.0\% \pm 11.0\%$  SEM) for *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice and controls, respectively;  $F_{1,12} = 1.058$ ;

$p = 0.32$ ; Figure 4C). These results clearly show that the *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice were not deficient in either associative learning or place association in a nontemporal context (when time of day is no issue) and that mice lacking *Cry1* and *Cry2* behaved normally for essential aspects of learning and memory.

### Discussion

The data unambiguously demonstrate that *Cry* genes are necessary for time-place learning, suggesting that intact circadian (clock) systems are required for this type of time memory and the temporal aspects of a learned behavior. A critical element in this paradigm is the reward-penalty aspect. If only a reward is used, mice do not perform time-place association. Clearly, they are not motivated to take time of day into account and, being opportunistic feeders, explore the arms randomly in search for food. Only when a risk assessment has to be made (entering an arm to obtain the food reward while having the chance of receiving the aversive footshock) does time-place association become apparent at the behavioral level. What actually is learned in this task is at what time of day which arm should be avoided, or, in other words, the association of time of day with a particular spatial location. From an ecological point of view, this could, for example, be considered equal to associating time of day with the occurrence of a predator at a specific location in a given environment. This ability is key to the survival and reproductive success of an individual.

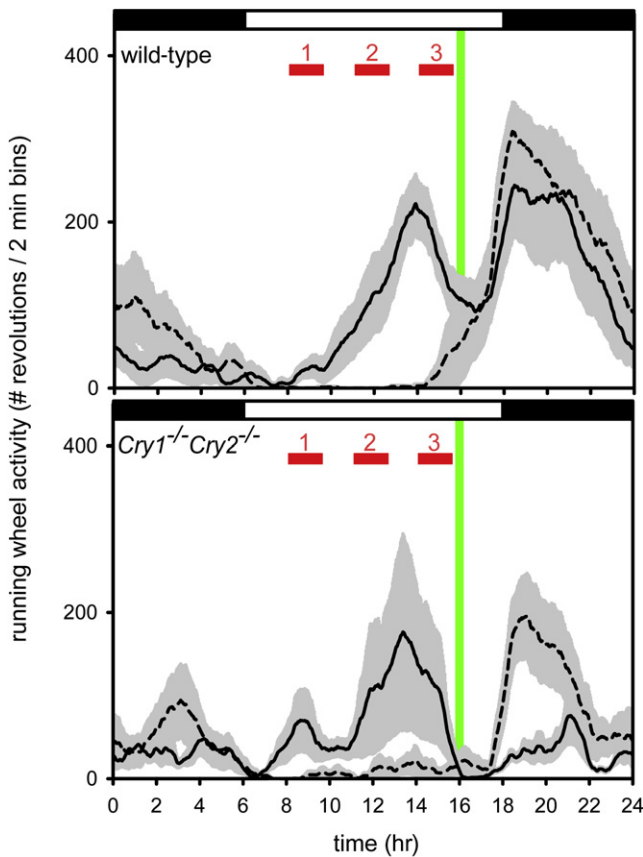


Figure 3. Analysis of Running Wheel Activity of *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* Mice and C57BL/6/J Control Mice in Relation to Time-Place Learning

Activity profiles of *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice ( $n = 4$ ) and wild-type controls ( $n = 4$  out of 9) during the last 5 days of testing (continuous line; Figure 1 days 22–26; Figure 2 days 41–45) versus the last 5 days of the posttesting phase (dashed line; Figure 2 posttest days 9–13). The testing procedure elicited increased diurnal activity in both *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* and wild-type mice with additional suppressed activity levels in *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice during the first 3 hr of the dark phase. Lines indicate 40 min running means, and gray areas indicate variation between individuals as SEM. Red bars indicate training session 1, 2, and 3; the green line indicates feeding time in the home cage during the testing phase.

Although a small number of *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice were used ( $n = 4$ ), all four mice dramatically and significantly ( $p < 0.006$  over the last five testing days) failed to master the time-place association task (in sharp contrast to all other mice). The presence of a stable light:dark cycle, although inducing synchronization of activity patterns was not sufficient to recall information of time of day.

At present, it is unclear which brain region(s) is (are) critically involved in time memory, but the suprachiasmatic nucleus as a *Cry* gene-dependent circadian pacemaker [14], involved in memory function [15–19], is an important candidate. Other brain regions well known to be involved in learning and memory (e.g., the cerebral cortex and hippocampus) could also play a role in time-place learning because *Cry1* and *Cry2* are expressed at these sites (see [www.brain-map.org](http://www.brain-map.org) and [www.genepaint.org](http://www.genepaint.org)). For this issue to be addressed, region-specific *Cry* knock-out mice need to be generated.

Most likely, *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice failed to master the time-place task as a consequence of lacking an intact circadian system. Hence, acquisition and transmission of time-of-day

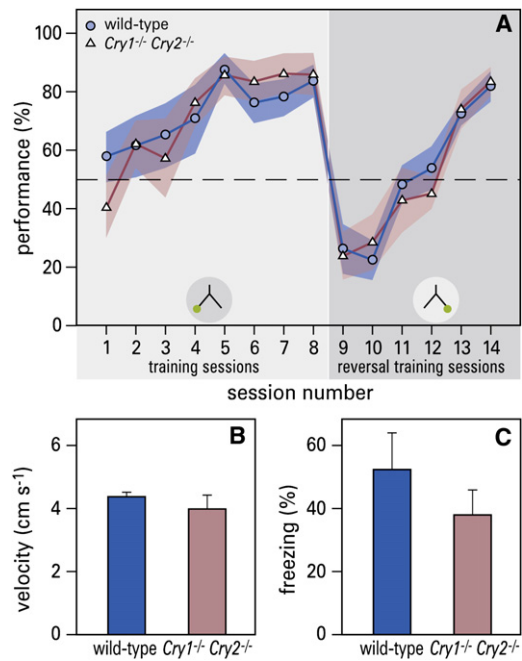


Figure 4. *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* Mice Are Not Deficient in Time-Independent Learning Tasks

(A) Behavioral performance of *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* and control mice does not differ during training (food reward in one arm;  $p = 0.98$ ) and reversal training (food reward in the other arm;  $p = 0.87$ ) in the Y maze. The percentage of correct trials ( $\pm$  SEM) per session (six trials per session, one session per day) is shown. (Controls,  $n = 9$ ; *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice,  $n = 7$ .)

(B and C) To test whether the loss of *Cry* genes affected the perception of a footshock and the formation of an association with the environment where the footshock was received, we tested *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* ( $n = 8$ ) and wild-type mice ( $n = 8$ ) in a contextual fear paradigm. *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice showed similar activity levels as wild-types during the habituation phase of contextual fear conditioning ([B];  $p = 0.37$ ). Twenty-four hours after the conditioning, mice were re-exposed to the conditioning box. Both *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* and wild-type mice showed similar levels of freezing behavior ([C];  $p = 0.32$ ), indicating that loss of *Cry* genes did not affect formation of contextual memories.

Error bars and shaded areas indicate the SEM.

information (either directly or indirectly via *Cry* genes) to brain regions and mechanisms underlying the formation of time-place associations is hampered. This implies that various times of day cannot be distinguished, rendering it impossible to form time-place associations. Alternatively, but more unlikely, these mice may fail to associate different times of day with different spatial locations as a consequence of the loss of *Cry* genes. It could also be argued that *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice failed this task in contrast to the other learning tasks because it is more difficult to achieve. The rate of acquisition for time-place learning and Y maze spatial learning are, however, comparable (see learning curves in Figures 1B and 4A, respectively). This renders it unlikely that the failure of the *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice to perform time-place learning is due to a higher cognitive demand of this task, although our data cannot completely rule out the possibility that *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice have a more general deficit to perform three-way conditional spatial discriminations (not just those in which time of day acts as the conditional cue). No differences in overt behavior were observed in the various steps of the experimental procedure, and the *Cry1<sup>-/-</sup>Cry2<sup>-/-</sup>* mice could obviously smell and locate a food reward and were responsive to the applied

footshocks. Taken together, we consider it unlikely that other (minor and more generalized) deficits in *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> mice not related to the circadian system are responsible for the specific failure of performing time-place learning.

*Cry* genes are considered clock genes, but it cannot be excluded that they serve other functions. *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> mice have disturbed sleep patterns, which indicate that CRY proteins are functionally implicated in the homeostatic regulation of sleep [20]. Sleep may be important for consolidation of time-place learning, because sleep is supposed to be of general relevance for the processing of information acquired while awake [20, 21]. However, it is unlikely that such a functional aspect of *Cry* genes is responsible for the lack of time-place learning in *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> mice, because they exhibit increased non-rapid eye movement (non-REM) sleep features, spending more time in non-REM sleep with higher EEG delta power [20]. Higher non-REM sleep in *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> mice would rather have facilitated time-place memory formation and consolidation instead of hindering the formation of time-place learning.

We showed that mice are capable of assessing time-place associations and that the lack of *Cry* genes leads to a specific deficit in this type but not in other types of associations. Whether the loss of time-place learning is specific for *Cry* genes or true for any clock gene will be addressed in future experiments with other clock-gene mutants. Deficits in temporal organization of memory are observed in a variety of neuropsychiatric diseases and during the early stages of Alzheimer's disease [22, 23]. The establishment of this mammalian model of time-place learning and its dependence on *Cry* genes opens new avenues to test the contribution of circadian system components in learning and memory function. Its scope may encompass neurodegenerative-related studies in aging and dementia.

#### Supplemental Data

Experimental Procedures and one figure are available at <http://www.current-biology.com/cgi/content/full/18/11/844/DC1/>.

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