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ORIGINAL ARTICLE

Circadian abnormalities in a mouse model of high trait anxiety and depression

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Introduction. **Dysregulation of circadian rhythms is a key symptom of mood disorders, including anxiety disorders and depression. Whether the circadian abnormalities observed in depressed patients are cause or consequence of the disease remains elusive. Here we aimed to explore potential disturbances of circadian rhythms in a validated genetic animal model of high trait anxiety and co-morbid depression and examine its molecular correlates.**
Materials and methods. **Mice selectively bred for high (HAB) and normal (NAB) anxiety- and co-segregating depression-like behavior were subjected to analysis of circadian wheel-running activity to determine light-entrained (LD) and free-running circadian (DD) rhythms and a light-induced phase shift. Clock gene expression in HAB/NAB hippocampal tissue was analyzed by qRT-PCR and verified by Western blotting.**
Results. **Compared to NABs, HAB mice were found to present with altered DD length of daily cycle, fragmented ultradiem rhythms, and a blunted phase shift response. Clock gene expression analysis revealed a selective reduction of *Cry2* expression in hippocampal tissue of HAB mice.**
Discussion. **We provide first evidence for a dysregulation of circadian rhythms in a mouse model of anxiety and co-morbid depression which suggests an association between depression and altered circadian rhythms at the genetic level and points towards a role for *Cry2*.**

Key words: Circadian rhythm, clock gene, depression, hippocampus, mouse model

Key messages

- Aberrant display of circadian rhythms is observed for the first time in a selectively bred mouse line with high trait anxiety and co-morbid depression (HAB mice).
- The circadian behavioral phenotype of HAB mice is associated with specific alterations in clock gene expression in the hippocampus.
- Data obtained from this mouse model provide evidence for an association between depression and altered circadian rhythms at the genetic level and point towards a role for *Cry2*.

Introduction

The etiology of mood disorders, like most neuropsychiatric disorders, is most likely a multifactorial process, involving complex gene–environment interactions, and its molecular mechanisms still remain to be fully elucidated. One key symptom associated with mood disorders, including anxiety disorders and depression, is a dysregulation of biological rhythms manifested as sleep disturbances, alterations in the diurnal patterns of core body temperature, and hormonal secretion, such as cortisol (1). Conversely, chronic disruption of sleep patterns is known as risk factor for the development of several neuropsychiatric illnesses, among those mood disorders (2,3).

Recently, several lines of evidence, mainly based upon genetic association studies, suggest that those deficiencies in rhythmic regulations observed in anxiety disorders and depression may not only result from the disease state but could even be relevant for the underlying pathophysiology (4–6). However, a direct causal link between development of mood disorders and alterations in the circadian system is still elusive, mainly due to the lack of appropriate (animal) model systems.

Here we aimed to explore experimentally, in a genetic mouse model of high trait anxiety and co-morbid depression, a possible association between emotional and circadian dysregulation at the behavioral and molecular level. A mouse line, resulting from selective in-breeding of CD1 mice for high anxiety-related behavior (HAB) displayed on the elevated plus maze (7) (for review see (8,9)) for more than 30 generations, is also characterized by high depression-like behavior as compared to normal anxiety-related behavior (NAB) controls. This was demonstrated in HAB versus NAB mice by the preference of immobility/passive stress-coping strategies in paradigms including forced swim and tail suspension test as well as clear signs of anhedonia assessed by the sucrose preference test (10,11). The high anxiety- and/or depression-related behavior of HAB mice can be normalized by diverse

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pharmacotherapeutic and invasive interventions including selective serotonin re-uptake inhibitors (10), benzodiazepines (12), and deep brain stimulation (11). Interestingly, it has been recently described that HAB mice also present with reduced hippocampal neurogenesis (9), generally accepted as one of the cellular correlates of depression-like behavior and deficient functional integration of the newly born cells (for review see (13)). Stimulated by a study reporting that HAB mice display aberrant sleep patterns and alterations in EEG activities (14), we assessed potential disturbances of circadian rhythms and finally aimed at examining their molecular correlates by analyzing the expression of core and tightly associated clock genes in the hippocampus, a brain region central to the neural circuitry of stress-related psychopathologies, including depressive disorders.

Materials and methods

Subjects

Female high (HAB) and normal (NAB) anxiety mice (12 weeks at the start of the experiments) were obtained from breeding colonies at the Department of Pharmacology and Toxicology, University of Innsbruck, Austria. Their anxious phenotype was confirmed by an elevated plus maze test at 7 weeks of age as previously described by Krömer et al. (7).

All experiments were designed to reduce animal suffering and keep the number of animals used at the minimum level. Animal experiments described in this study were approved by the national ethical committee on animal care and use (Bundesministerium für Wissenschaft und Forschung) and carried out according to international laws and policies.

Housing

Animals were housed individually in Nalgene cages equipped with running wheels (15 cm in diameter; Actimetrics, Evanston, IL, USA) with food and water available *ad libitum* in a sound-attenuated room with constant temperature of ≈ 21°C. Animals were kept on a 12 h:12 h light:dark (LD12:12) cycle before experimental manipulations described below. During the light phase, light intensity at the level of the animals' cages was ≈ 200 lux. During conditions of constant darkness (DD) cage cleaning and animal care taking was carried out under dim red light (15 W).

Locomotor activity assessment

Acquisition

Wheel revolutions were recorded with the ClockLab computer software, with 1-min sampling epochs (Actimetrics). Mice were initially placed in LD12:12 (lights on at 7 a.m.) for 13 days. On the 14th day, conditions were changed to 24 hours darkness (DD), and data acquisition was resumed for 10 days. On day 25, animals were exposed to a brief light pulse (30 minutes, 300 lux) at circadian time (CT) 16 (4 h after activity onset) for induction of a phase shift response. Consecutively, mice were maintained at DD for 7 more days before being switched back to LD for another 7 days prior to sacrification (Figure 1). Brain dissections were carried out between 9 a.m. and 11 a.m.

Analysis

Activity was assessed and evaluated using the ClockLab software package (Actimetrics). Activity records were double-plotted in threshold format for 6-min bins. Activity onsets were determined using the default window settings of 6 h off and 6 h on. If the automatic detection selected as an onset a time clearly outside of the expected range and manual inspection identified an unambiguous onset bout, the onset time for that day was edited to an activity bout. Period measures were derived from regression lines fit to the activity onsets and used for calculation of chi-square periodograms. The free-running period for each animal was calculated from the days under DD prior to the light-pulse treatment. Phase shifts responses were evaluated by comparing the predicted activity onset for the day after the light pulse from extrapolated lines of the activity onsets of the days preceding the light pulse and the days after the pulse starting. All calculations and figures were derived from ClockLab software.

Gene expression analysis

Brain dissection

Subjects were sacrificed by neck dislocation, and brains were rapidly dissected over ice. Isolated hippocampal tissues were stored in RNA later (Ambion, Austria, Austin, TX, USA) at −20°C until used for RNA isolation or immediately immersed in liquid nitrogen and stored at −80°C for protein isolation.

Real time polymerase chain reaction (qRT-PCR)

Hippocampal RNA was isolated using miRNeasy kit (Qiagen®, USA, Hilden, Germany) according to the manufacturer's instructions. A 900 ng of total RNA was used for cDNA synthesis following manufacturer instructions provided with MMLV reverse transcriptase first-strand cDNA synthesis kit, G1 (Biozym®, Hessisch Oldendorf, Germany). A 1:5 dilution of cDNA reaction was used for PCR amplification using the Fast SYBR Green Mastermix (Applied Biosystems, Foster City, CA, USA) on a StepOnePlus realtime PCR system (serial no. 271000455; Applied Biosystems). Target genes were normalized to beta-actin. All primer sequences are listed in Supplementary Table 1 available online at http://informahealthcare.com/doi/abs/10.3109/07853890.2013.866440.

Protein isolation/protein quantification

Hippocampal tissue was powderized in liquid nitrogen and homogenized in a protein lysis buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% SDS, 0.5% Triton X100, 1 mM EDTA, 10 mM NaF, 5 mM $Na_4O_2P_7$, 10 mM Na_3VO_4 and protease inhibitor cocktail (1×, Roche Diagnostics, Mannheim, Germany). After sonication for approximately 5 cycles × 5s × 5, the suspension

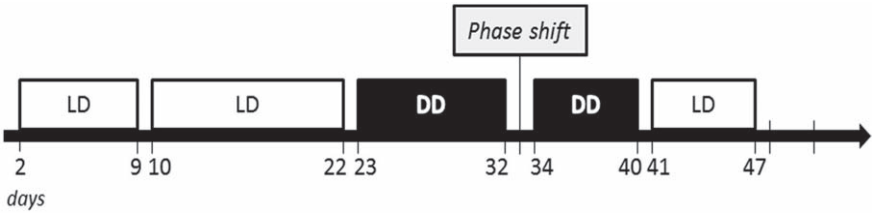

Figure 1. Study design for the analysis of the circadian behavioral phenotype in high and normal anxiety-like and depression-like mice. Light protocol used for the assessment of circadian wheel-running activity in selectively bred high and normal anxiety- and depression-like mice (HAB/NAB).

was left at 4°C on a rotator for 30 minutes and centrifuged at 14,000 *g* for 30 min at 4°C. The supernatant was immediately transferred and was quantified using Pierce BCA assay Kit (Thermo Scientific, Rockford, IL, USA). The standard curve was generated using bovine serum albumin ampules with a concentration of 2 mg/mL. The samples were analyzed in triplicate (microplate procedure: 25 µL sample + 200 µL BCA working reagent and incubated at 37°C for 30 minutes), and concentration was determined by absorbance reading at 595 nm using Synergy H4 Hybrid Reader spectrophotometer (Szabo-Scandic HandelsgmbH & Co KG, Vienna, Austria).

Western blotting

Samples (25 µg protein) were analyzed and loaded on a 10% sodium dodecyl sulfate (SDS) mini-gel (0.75 mm × 6.8 cm × 8.6 cm) and 5% stacking gel and then subjected to electrophoresis at 80 V for 1 hour and 45 min. Electrophoresis was performed with a Mini-Protean System (Bio-Rad Laboratories Inc., Vienna, Austria). Proteins from the gel were transferred onto PVDF membranes (Millipore, Billerica, MA, USA) and were run at 250 mA for 1 h 30 minutes. Membranes were blocked by incubating with 5% non-fat dry milk in 100 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 (TTBS) for 1 h. Membranes were then incubated with diluted primary antibody (Rabbit Polyclonal to *Cry2* (1:500); Abcam PLC, Cambridge, UK) overnight at 4°C, rinsed three times with TTBS, and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Goat Anti-rabbit HRP-linked IgG (1:3000); Cell Signaling Technology, Inc., Danvers, MA, USA). Immunoreactivity was visualized by enhanced chemiluminescence Pierce ECL substrate (Thermo Scientific). Detectable molecular masses were determined by running standard protein markers (Thermo Scientific) ranging from 10 to 250 kDa. Quantification was performed by chemiluminescent imaging with a FluorChem HD2 (Alpha Innotech, San Leandro, CA, USA) using the respective software. Values obtained from densitometry of target proteins were normalized to those of the housekeeping protein β-tubulin for the same samples.

Statistical analysis

For comparisons of behavioral data and gene expression results between HAB and NAB mice, Student *t* tests were carried out. Results were considered significant when *P* values were lower than 0.05. All statistical analyses were performed using BioStat software (AnalystSoft Inc., Alexandria, VA, USA).

Results

HAB mice show abnormal circadian period (*tau*) under free-running conditions

In order to examine whether selective breeding for high anxiety and co-morbid depression may in parallel lead to *in vivo* consequences in alterations in circadian behavior, the wheel-running rhythms of HAB mice were compared to those of NAB mice.

In free-running conditions (constant darkness, DD), HAB mice displayed a significantly longer free-running period (*tau*) than NAB females ($P < 0.05$) (Figure 2A), while no differences were observed under LD conditions (Figure 2B). However, no differences in total wheel revolutions per day, nor individually in the *rho*- or *alpha*-phase, respectively, were observed under either LD or DD conditions (Figure 2C and D), suggesting that alterations in *tau* during DD in HAB mice do not result from effects on overall locomotor activity.

Fragmented ultradiem rhythms under LD and DD conditions

In HAB mice the actograms generated from wheel-running behavior appeared to be fragmented as compared with those derived from NAB mice, suggesting potential alterations of ultradiem rhythms in the HAB model (Figure 3A). This observation was further investigated by analysis of activity bouts and indeed revealed fragmented ultradiem rhythms as manifested by a significantly higher number of activity bouts in HAB females under LD and DD conditions ($P < 0.05$) (Figure 3B and C).

HAB mice display deficient entrainment to light

We next examined light-induced clock entrainment in HAB mice using light-induced phase shift as paradigm assessing the responsiveness of the endogenous circadian rhythms to exogenous *zeitgeber*. To this end, mice were exposed to a brief light pulse (30 minutes, 300 lux) in the early night (CT 16) for induction of a phase shift response. A dramatic and significant reduction ($P < 0.001$) of the mean phase delay induced by this light treatment was observed in HAB mice (Figure 4A).

Hippocampal levels of *Cry2* are altered in HAB mice

We further aimed to investigate the potential mechanisms underlying the observed circadian behavioral phenotype at the molecular level by analyzing clock gene expression in hippocampal tissue of HAB and NAB mice. A qRT-PCR analysis of *Clock*, *Per1–3*, *Bmal*, *Npas2*, *Cry1–2*, *Rev-erb* α-β, *Ror* α-β-γ, *Dec1/2*, *E4bp4*, *NeuroD1*, *CycloB*, and *Dbp* revealed a selective reduction of *Cry2* mRNA levels in the hippocampus of HAB compared to NAB mice ($P < 0.05$) (Figure 4B), which was further verified at the protein level using Western blot ($P < 0.01$) (Figure 4C). In order to examine whether the observed changes in *Cry2* expression were specific to the hippocampus or also occurred in another brain region relevant to the neural circuitry associated with mood and affective disorders, *Cry2* levels were also compared in frontal cortical tissue of HAB and NAB mice. No significant expressional differences between the two mouse lines were observed ($P > 0.05$).

Discussion

We here show for the first time disturbances of the circadian rhythm in a genetic mouse model of high trait anxiety and co-morbid depression and propose derangement of hippocampal clock gene expression as molecular correlate.

Mood disorders, including major depression, are tightly associated with alterations in the circadian rhythm, including disturbances of sleep which are even listed as diagnostic criteria for depression in the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (15). Moreover, a genetic basis for the involvement of the circadian system in mood disorders is suggested by a series of association studies identifying polymorphisms in clock genes in patients suffering from bipolar disorder, seasonal affective disorder (SAD), and major depression (5,16–25). These findings are now complemented with the result of the present study, firstly using a mouse model, indicating that innate high levels of trait anxiety and depression-like behavior may lead to and/or share a common genetic basis with disturbed circadian rhythms.

The lengthened free-running circadian period *tau* in HAB mice indicates that the derangement of the circadian behavior is likely due to a dysfunction of the endogenous circadian rhythm in these animals, most likely originating from a compromise in the molecular circadian machinery orchestrating the circadian rhythm under DD conditions. Modulation of the free-running

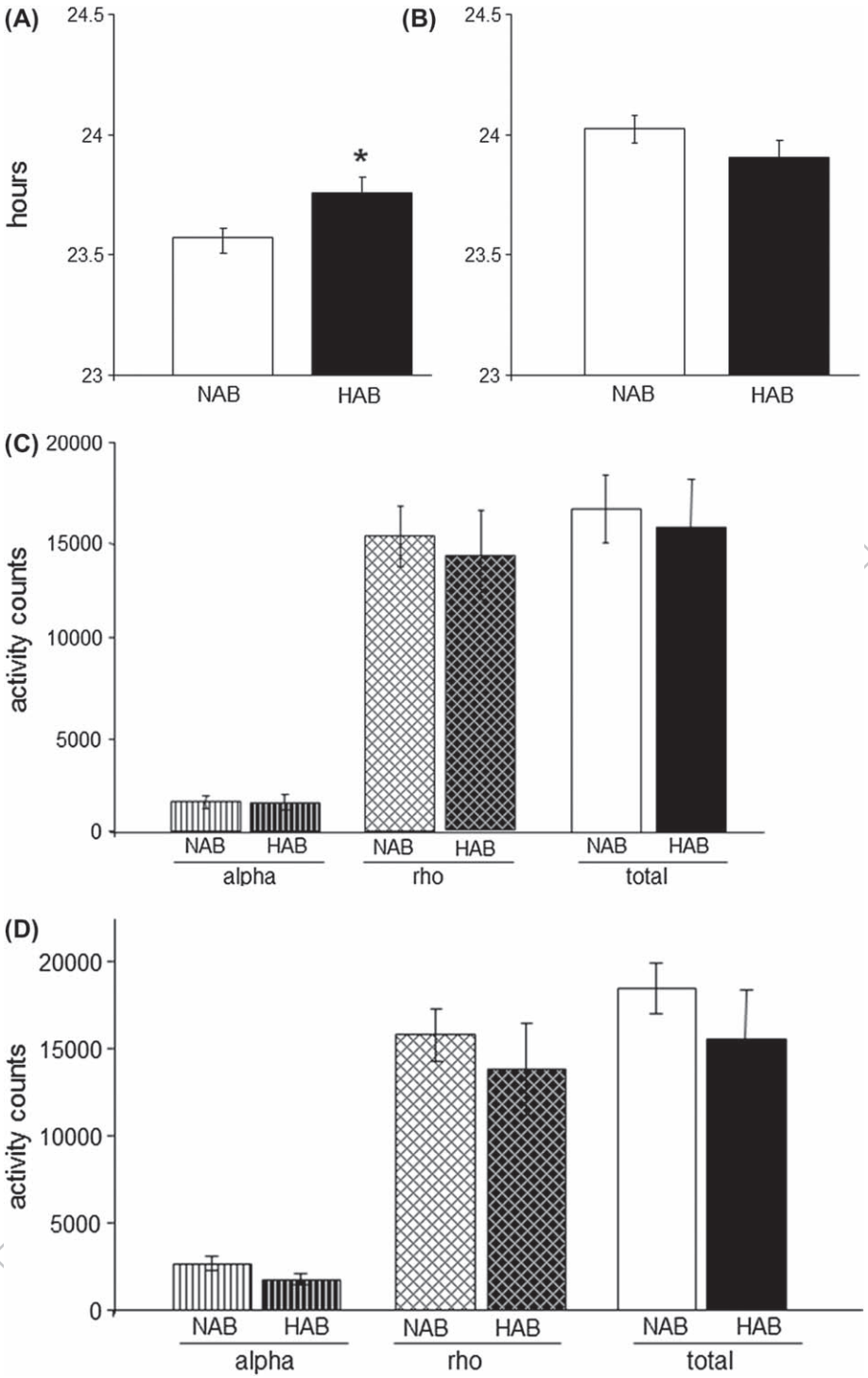

Figure 2. Alterations of the free-running circadian rhythm observed in HAB mice. (A) HAB mice display a significantly longer circadian period under free-running conditions (DD) than NAB mice. (B) No differences are observed under light-entrained conditions (LD). (C) and (D) HAB and NAB mice show comparable amounts of wheel running activity under DD (C) and LD (D) conditions during both their active (*alpha*) and inactive (*rho*) phases. $^{*}P < 0.05$; data displayed as mean ± SEM.

circadian period in mice and other laboratory rodents has been reported under several experimental conditions, including ageing (26), exposure to ethanol (27–29) and selective breeding for ethanol-related traits (30), various knock-out mouse models for core clock genes (31–33), as well as candidate genes related to psychiatric disorders. Interestingly, we had previously observed that long-term exposure to constant darkness in mice also lengthens the circadian period without affecting total activity levels and that this modulation of *tau* is paralleled by depression-like behavior (34).

The increase in the number of activity bouts in HAB mice revealed in the present study complements previous findings characterizing the sleep phenotype of this mouse line (14). Paralleling our own results, it is reported therein that HAB mice exhibited an increase in the number of bouts of wakefulness with recurrent entries to non-REM and REM sleep episodes and shorter

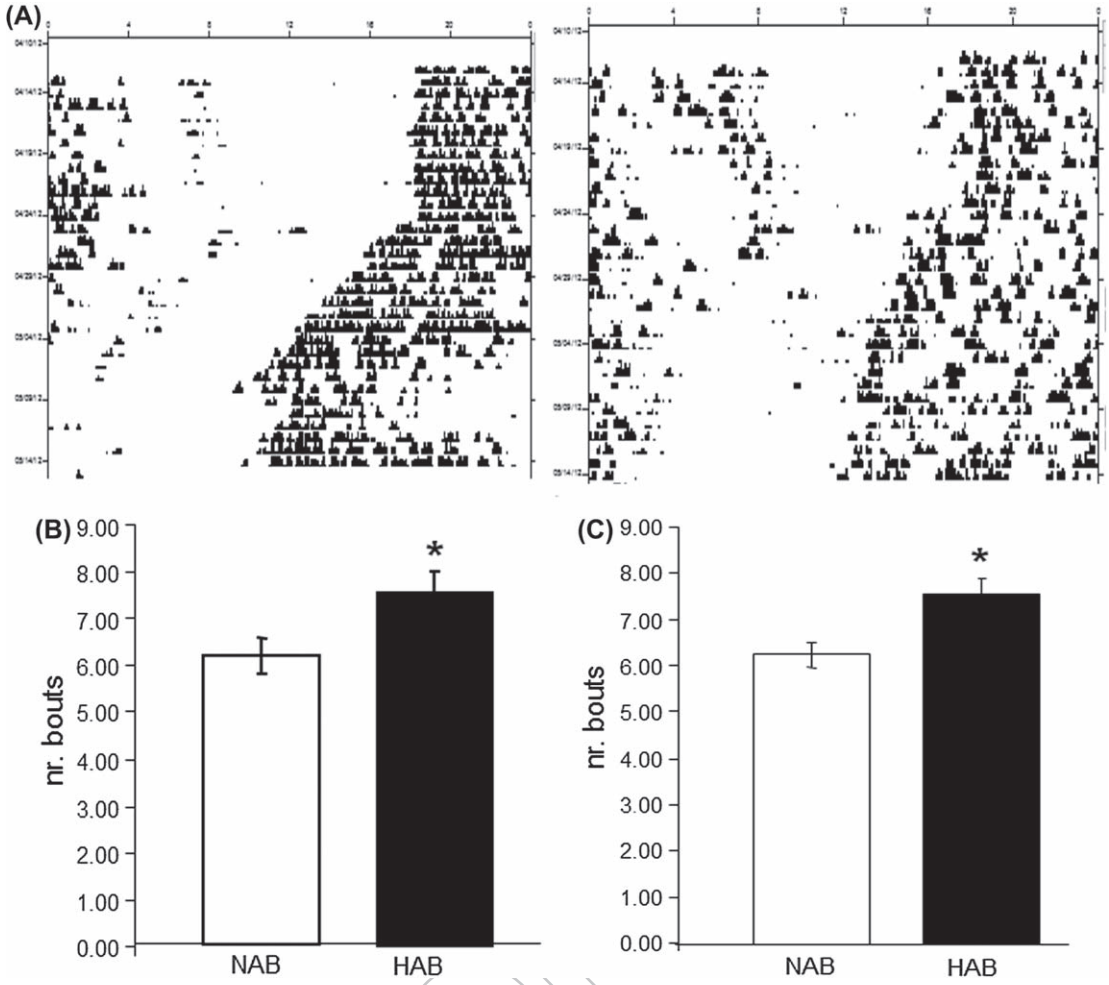

Figure 3. HAB mice display fragmented ultradiem rhythms. (A) Sample double-plotted actograms depicting wheel-running activity in NAB (left) and HAB (right) mice. Individual days are represented by horizontal rows with black vertical bars indicating locomotor activity (wheel revolutions). (B) and (C) HAB mice show significantly more activity bouts under both LD (B) and DD (C) conditions. * $P < 0.05$; data displayed as mean ± SEM.

episodes of non-REM and REM sleep and an enhancement of all state transitions characterized as sleep fragmentation (14).

Our observation of an attenuated phase shift response induced by a light pulse in the early subject night (CT 16) – known to induce phase delays (35) – suggests a deficiency of the endogenous circadian machinery to the entrainment by external stimuli in HAB mice. However, given a stable circadian period under LD conditions, there is no indication for a general light insensitivity in HAB mice. The molecular mechanisms involved in light-induced clock resetting, as experimental paradigm assessing the critical capability of the endogenous clock to be entrained by external stimuli, thus responding to changing environmental settings, still remain poorly understood (36). While a role for several clock genes, including *per1*, *per2*, and *cry2* have been described, also several non-clock genes have been lately implicated in mediating light-entrainment (37).

Trying to elucidate the molecular mechanisms potentially underlying the observed circadian phenotype in HAB mice, we focused on investigating the expression of the elements of the endogenous circadian machinery in the hippocampus. While the suprachiasmatic nucleus (SCN) is the locus of central circadian orchestration, other areas of the brain, including some highly implicated in mood disorders such as the hippocampus, also display clock gene expression (38). It can be speculated that clock gene dysfunction in these extra-SCN sites may directly relate to the pathomechanisms of mood disorders. When analyzing the expression of 21 molecules forming part of the cellular clock we had found a selective reduction of *Cry2* expression in hippocampal tissue of HAB mice, both at the mRNA and protein level. Interestingly, no differences have been observed between HAB and NAB mice in the expression of *Cry2* in the frontal cortex, another brain region forming part of the neural network whose dysfunctionality relates to mood disorders (see for review (39)). This observation suggests that the results obtained from the hippocampus present a region-specific finding and points toward a role of *Cry2* in the regulation of selective functions of the hippocampus potentially altered in mood and anxiety disorders and respective animal models. Previously, a role of *Cry2* in the pathophysiology of depression has been proposed based upon the identification of four CRY2 SNPs identified from the human genome and their association with mood disorders (23,24,40) as well as findings from a pharmacogenomic mouse model (41). However, to the best of our knowledge, the present study is the first to reveal specific expressional changes of *Cry2* in a mouse model of anxiety and co-morbid depression also displaying alterations of the behavioral circadian rhythm. A potential behavioral phenotype related to depression and anxiety still remains to be tested in *Cry2*-KO to be able to assign a direct causal relationship between *Cry2* and depression, and potential *Cry2* SNPs in HAB mice should be analyzed in future studies.

The present study has several limitations: First, the use of bidirectionally bred mouse lines possesses inherent conceptual

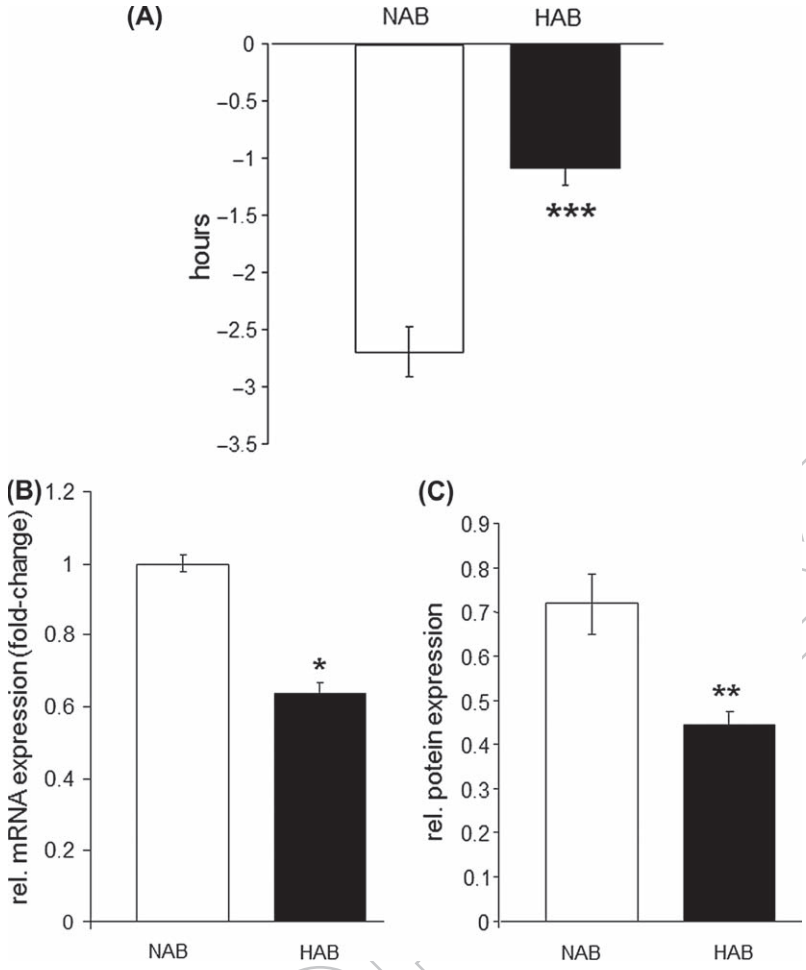

Figure 4. Phase shift response is blunted in HAB mice. (A) A light-induced phase shift at CT 16 reveals a significantly diminished response in HAB mice as evidenced by a more than 50% reduction in the light-induced phase delay (hours). (B) and (C) Expression of *Cry2* is significantly reduced at the mRNA level (B) and the protein level (C) in hippocampal tissue of HAB compared to NAB mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; data displayed as mean ± SEM.

restrictions, limiting the interpretation of results obtained using these animal models. As such, the possibility that altered *Cry2* levels in HAB mice represent an incidental molecular phenotype resulting from co-selection with the phenotype of interest cannot be excluded (e.g. (42)). Second, clock gene expression has been analyzed at a single time point; thus it remains to be investigated whether *Cry2* overall levels are down-regulated in HAB mice or whether the peak of the *Cry2* expression is shifted. Third, the results of hippocampal clock gene expression have not yet been related to those of the SCN which would allow the nature of circadian dysregulation in HAB mice to be understood in further detail.

In summary, we here provide experimental evidence that genetic selection for high anxiety-like and depression-like behavior alters the behavioral circadian rhythm and compromises the expression of a core element of the molecular circadian machinery. These results propose a potential linkage between emotional and circadian dysregulation at the genetic level and provide support for the hypothesis that circadian abnormalities observed in patients suffering from affective disorders may not be a consequence of the disease but rather suggest a potential involvement in the underlying pathomechanisms. Moreover, results of this study recommend the HAB mouse line as novel animal model for investigating circadian abnormalities in mood and anxiety disorders.

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Supplementary material available online

Supplementary Table 1

Supplementary material for Griesauer I, et al. Circadian abnormalities in a mouse model of high trait anxiety and depression, Annals of Medicine, 2013; doi: 10.3109/07853890.2013.866440.

Supplementary Table 1. Sequences of primers used.

Primer Name	Primer length (bp)	sequence(5′ to 3′)
mus_Bmal1_fwd	20	AAC CTT CCC GCA GCT AAC AG
mus_Bmal1_rev	20	AGT CCT CTT TGG GCC ACC TT
mus_Clock_fwd	21	GGC GTT GTT GAT TGG ACT AGG
mus_Clock_rev	21	GAA TGG AGT CTC CAA CAC CCA
mus_Cry1_fwd	21	AGG AGG ACA GAT CCC AAT GGA
mus_Cry1_rev	21	GCA ACC TTC TGG ATG CCT TCT
mus_Cry2_fwd	21	AGC TGA TGT GTT CCC AAG GCT
mus_Cry2_rev	20	CAT AAT GGC TGC ATC CCG TT
mus_Cyclo B_fwd	21	GGT GGA GAG CAC CAA GAC AGA
mus_Cyclo B_rev	19	GCC GGA GTC GAC AAT GAT G
mus_Dbp_fwd	22	GGA ACT GAA GCC TCA ACC AAT C
mus_Dbp_rev	21	CTC CGG CTC CAG TAC TTC TCA
mus_E4bp4_fwd	23	AGA ACC ACG ATA ACC CAT GAA AG
mus_E4bp4_rev	23	GAC TTC AGC CTC TCA TCC ATC AA
mus_Id2_fwd	22	AGG CAT CTG AAT TCC CTT CTG A
mus_Id2_rev	24	AGT CCC CAA ATG CCA TTT ATT TAG
mus_Npas2_fwd	21	ACG CAG ATG TTC GAG TGG AAA
mus_Npas2_rev	19	CGC CCA TGT CAA GTG CAT T
mus_Per1_fwd	24	CCA GAT TGG TGG AGG TTA CTG AGT
mus_Per1_rev	24	GCG AGA GTC TTC TTG GAG CAG TAG
mus_Per2_fwd	21	AGA ACG CGG ATA TGT TTG CTG
mus_Per2_rev	21	ATC TAA GCC GCT GCA CAC ACT
mus_Per3_fwd	20	CCG CCC CTA CAG TCA GAA AG
mus_Per3_rev	20	GCC CCA CGT GCT TAA ATC CT
mus_Rev-erbalpha_fwd	23	CCC TGG ACT CCA ATA ACA ACA CA
mus_Rev-erbalpha_rev	22	GCC ATT GGA GCT GTC ACT GTA G
mus_Rev-erbbeta_fwd	19	GGA ACG GAC CGT CAC CTT T
mus_Rev-erbbeta_rev	19	TCC CCT GCT CCC ATT GAG T