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

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 \( \approx 21^\circ \text{C} \). 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 \( \approx 200 \) 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 sacrifice (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 \(-20^\circ \text{C} \) until used for RNA isolation or immediately immersed in liquid nitrogen and stored at \(-80^\circ \text{C} \) 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 I 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 Na4O2P7, 10 mM Na3VO4 and protease inhibitor cocktail (1\( \times \), Roche Diagnostics, Mannheim, Germany). After sonication for approximately 5 cycles \( \times 5 \)s \( \times 5 \), the suspension...
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 0.1% Tween 20 (TTBS) for 1 h. Membranes were then incubated for 1 hour and 45 minutes. 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 densitometry of target proteins normalized to those of the housekeeping protein β-tubulin for the same samples.

**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 ultradian 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 ultradian rhythms in the HAB model (Figure 3A). This observation was further investigated by analysis of activity bouts and indeed revealed fragmented ultradian 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
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
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-morbir 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...
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