Mice lacking circadian clock components display different mood-related behaviors and do not respond uniformly to chronic lithium treatment

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Genomic studies suggest an association of circadian clock genes with bipolar disorder (BD) and lithium response in humans. Therefore, we tested mice mutant in various clock genes before and after lithium treatment in the forced swim test (FST), a rodent behavioral test used for evaluation of depressive-like states. We find that expression of circadian clock components, including \( \text{Per}_2 \), \( \text{Cry}_1 \) and \( \text{Rev-erb}_a \), is affected by lithium treatment, and thus, these clock components may contribute to the beneficial effects of lithium therapy. In particular, we observed that \( \text{Cry}_1 \) is important at specific times of the day to transmit lithium-mediated effects. Interestingly, the pathways involving \( \text{Per}_2 \) and \( \text{Cry}_1 \), which regulate the behavior in the FST and the response to lithium, are distinct as evidenced by the phosphorylation of GSK3β after lithium treatment and the modulation of dopamine levels in the striatum. Furthermore, we observed the co-existence of depressive and mania-like symptoms in \( \text{Cry}_1 \) knock-out mice, which resembles the so-called mixed state seen in BD patients. Taken together our results strengthen the concept that a defective circadian timing system may impact directly or indirectly on mood-related behaviors.

Keywords: Bipolar disorder, \( \text{Cry}_1 \), cryptochrome, depression, mixed state, neurogenesis

INTRODUCTION

The importance of genetic components in mood disorders has been demonstrated in many different studies, including analyses on single-nucleotide polymorphisms and genome-wide association studies (GWAS) (Lau & Eley, 2010; Wittchen et al., 2011). Although such studies did not point to a main role of clock genes, a number of candidate clock gene studies have identified variants associated with mood-related phenotypes including depression (Kripke et al., 2009; Mansour et al., 2009; McGrath et al., 2009; Nievergelt et al., 2006; Partonen et al., 2007; Shi et al., 2008; Soria et al., 2010). Accordingly, disruptions of circadian patterns of gene expression in human brains with major depressive disorder have been observed (Li et al., 2013).

The molecular circadian clock is an auto-regulatory network of transcription factors orchestrating behavioral and metabolic pathways with a period of about 24 h, approximately reflecting the natural 24 h light-dark cycle (Takahashi et al., 2008). The activators BMAL1 and CLOCK or NPAS2 heterodimerize, bind to E-boxes, and regulate the transcription of a wide variety of genes, including period (\( \text{Per}_1-3 \)), cryptochrome (\( \text{Cry}_1 \) and 2) and \( \text{Rev-erb}_a \). PER and CRY heterodimerize and inhibit the transcriptional activity of the BMAL1:CLOCK/NPAS2 heterodimers, whereas \( \text{Rev-erb}_a \) represses transcription of \( \text{Bmal}_1 \), \( \text{Clock} \) and \( \text{Npas}_2 \) genes (Takahashi et al., 2008).

Studies using mice mutant in clock genes show behavioral abnormalities similar to those observed in human mood disorders. For example a mutation in \( \text{Clock} \) causes lithium-sensitive, mania-like behavioral abnormalities (Mukherjee et al., 2010; Roybal et al., 2007) and a mutation of the \( \text{Per}_2 \) gene leads to reduced
monoamine oxidase A levels, thereby increasing dopamine in the striatum leading to mania-like behavior (Hampp et al., 2008). Additionally, midbrain dopamine production is modulated by Rev-Erbα, leading to mania-like behavior in Rev-Erbα knock-out mice (Chung et al., 2014). Mice lacking both Cry1 and 2 genes show altered anxiety-like behavior (De Bundel et al., 2013). Moreover, the mood-stabilizing drug lithium, a mainstay of bipolar disorder (BD) treatment, alters clock gene expression (McQuillin et al., 2007) and delays circadian rhythms in various species (Kafka et al., 1982; Kripke & Wyborsky, 1980; Kripke et al., 1978, 1979; Stewart et al., 1991; Welsh & Moore-Ede, 1990) and lengthens clock period in cell culture (Li et al., 2012). Lithium treatment inhibits GSK3β activity (Klein & Melton, 1996), which is a kinase that phosphorylates many clock proteins, including PER2, CRY2, BMAL1, CLOCK and REV-ERBα, thereby regulating their stability and as a consequence modulates circadian period length and phase (Klein & Melton, 1996; Ko et al., 2010; Kurabayashi et al., 2010; Sahar et al., 2010; Spengler et al., 2009; Yin et al., 2006).

A growing body of evidence suggests that mood-disorders are associated with disturbed adult neurogenesis (Benes et al., 1998; Rajkowska, 2000), which appears to be crucial for proper mood control and efficient antidepressant therapy (Petrik et al., 2012; Samuels & Hen, 2011). Interestingly, lithium increases adult hippocampal neurogenesis (Chen et al., 2000) and mice mutant in the clock genes Per2 or Rev-erbα display increased adult hippocampal neurogenesis (Borgs et al., 2009; Schnell et al., 2014).

In order to examine the role of clock genes in mood-related behaviors and in lithium treatment, we investigated various clock mutant mice, including Cry1 knock-out animals, in the forced swim test (FST) and evaluated their response to lithium. In wild-type, Per2 and Cry1 mutants we additionally investigated lithium effects on clock gene expression, GSK3β phosphorylation, dopamine accumulation and adult hippocampal neurogenesis.

MATERIALS AND METHODS

Animal experiments

Animal handling and care was performed in accordance with the guidelines of the Schweizer Tierschutzgesetz (TSchG, SR455) and the declaration of Helsinki. Lithium experiments were approved by the veterinarian of the Canton of Fribourg (permit nr. 22174). Breeding of single clock gene knock-out/mutant mice in the animal facility in Fribourg was started in 2001 and continued for over 20 generations. Cry1 and Cry2 knock-out mice originally provided by Prof. van der Horst, Rotterdam (van der Horst et al., 1999), Per2Brdm1 mutant mice (Zheng et al., 1999) and Per1Brdm1 mutant mice (Zheng et al., 2001) all originated from a C57BL6/129SV background. Double knockouts were generated by intersecting homozygous single knockout animals. Rev-Erbα knock-out mice (Preitner et al., 2002) were obtained from heterozygous Rev-Erbα breeding pairs originally provided by Prof. U. Schibler, Geneva. Two-to-six-month-old animals were used for experiments and wild-type mice served as controls. Animals were kept under 12 h light and 12 h dark (LD 12:12) with food and water ad libitum.

Lithium treatment

Regular rodent chow containing 0.4% lithium carbonate (Harlan) was fed ad libitum during 10–14 days. Experimental scheme of lithium treatment and forced swim test (FST) sessions before and after lithium treatment is displayed in Supplementary Figure S1. The drinking water was supplemented with an additional drinking flask containing saline (0.9% NaCl) to prevent sodium depletion, which leads to lithium nephrotoxicity. During the phase of treatment, overall health state was checked daily and body weight was recorded weekly.

Treatment efficiency was verified by quantifying lithium concentration in blood plasma by flame photometry (hôpital fribourgeois HFR Riazi in Bulle, Switzerland). Blood plasma was prepared by centrifugation of the blood sample at 5400 g and 4°C for 10 min. The supernatant plasma was stored at −20°C until analyzed.

Antibody characterization

Fragments of PER1 (amino acids 937 to 1290), PER2 (amino acids 691 to 1029), CRY1 (amino acids 470 to 606) and CRY2 (amino acids 416 to 592) were produced and purified from bacteria using the expression vector pET15b system and the instructions of the manufacturer (Merck Millipore, Billerica, MA). Purified protein was used to immunize rabbits. Antiserum against PER1 were pre-absorbed against PER2, and vice versa, and subsequently affinity purified. The antiserum against CRY1 was pre-absorbed against CRY2 before affinity purification. The purified antibodies detect specific bands at ~140 kDa (PER1), ~150 kDa (PER2) and ~65 kDa (CRY1), which are not found in the corresponding knockout animals (Supplementary Figure S2) (Please see Supplementary Table 1 for a list of all antibodies used).

The phospho-GSK-3β (Ser9) (5B3) Rabbit mAb detects endogenous levels of GSK-3β only when phosphorylated at Ser9. The antibody recognizes a band (46 kDa) on western blot of mouse brain, and these specific bands are absent in GSK-3β (−/−) mouse embryonic fibroblasts (MEFs) or after phosphatase treatment of cell extract (manufacturer’s datasheet). Due to high-sequence homology, the antibody may cross-react weakly with the phosphorylated form of GSK-3β. The GSK-3β rabbit mAb recognizes a single band (46 kDa). It detects endogenous levels of total GSK-3β protein and was used to quantify the amount of phosphorylated GSK-3β.

The rabbit GAPDH pAb recognizes an epitope located in the region encoded by aa 150/200 of GAPDH (36 kDa). GAPDH is localized in both, the nucleus and the...
cytoplasm were used as loading control for total protein extract.

The doublecortin antibody is targeted against two doublecortin domains and is widely used as a marker of neuroblasts, which are expressed only in distinct regions of the adult mouse brain (forebrain, SGZ of the DG, olfactory bulb). The Antibody stains the cytoplasm of cells with dendrite morphology (neuroblasts) (Gleave et al., 2013). In our staining of PFA-fixed frozen tissue, the antibody recognized the characteristic dendritic morphology of the neuronal precursors in the mouse DG.

The mouse neuronal nuclei (NeuN) mAb was targeted against the DNA-binding, neuron-specific protein NeuN, only one clone exists (A60), which reacted with an uncharacterized nuclear protein. In our study, the antibody strongly labeled nuclei throughout the mouse brain, but only mature neurons showed immunoreactivity. Cells located in proliferative zones, such as the SGZ of the DG, showed no staining, whereas immunoreactivity for doublecortin was observed in these cells. This antibody has been widely used and validated (Magavi et al., 2000).

The anti-BrdU mAb [BU1/75 (ICR1)] reacts against BrdU, but no other nucleotides, in single-stranded DNA. The antibody marks nucleated cells that incorporated BrdU into their DNA during S-phase, and provides, therefore, a mean to estimate cell proliferation in the brain. To separate the DNA strands we performed acid denaturation with HCl by harshening conditions step-wise (1 M HCl on ice, 2 M HCl at RT and 2 M HCl at 37 °C). Immunoreactivity and specificity of the antibody has been verified by immunohistochemistry and flow cytometry (Borgs et al., 2009).

Western blot analysis
For protein extraction, flash frozen striatal tissue was disrupted with a motor-driven, disposable plastic pestle in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % Triton X-100, 0.5 % sodium deoxycholate) containing 1 X protease inhibitor cocktail, 0.2 mM NaV, 5 mM NaF, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM PMSF (approx. 300 μl buffer for 30 mg tissue). Proteins were separated on 8.5 % SDS-PAGE and transferred to Hybond™ ECL nitrocellulose membranes (GE healthcare, Chalfont St Giles, UK). Primary antibodies were incubated over night at 4 °C, Anti-PER2 1:1000, Anti-PER1 1:2000, Anti-CRY1 1:2000 (Preitner et al., 2002), Anti-phosphoGSK3β (Ser9) 1:1000 (Cell Signaling Technology Cat# 9323P) and Anti-GSK3β 1:5000 (27C10) (Cell Signaling Technology 9315, Danvers, MA). Detection of the immune complexes was performed using Western Bright Quantum system (Advanta, Menlo Park, CA) and quantification was done with the Quantity One analysis software (Bio-Rad Laboratories AG, Cressier, Schweiz). Anti-GADPH 1:2000 (Abcam ab36840, Cambridge, UK) was used for normalization and relative protein levels were calculated by defining maximal protein levels as 100%.

Behavioral studies
Porsolt Forced swim tests were performed using a cylindrical tank (35 cm height, 25 cm diameter) filled with water to a height of 20 cm. The water temperature was maintained at 27 ± 2 °C. An initial period of 2 min was given for habituation and then immobility time was recorded during 4 min using a stopwatch. Mice were considered immobile, when no obvious limb movements were observed. After a total session of 6 min, mice were warmed up on a heating pad, and then placed back into their home cage. Mice were tested at the same time of day (ZT6 and ZT18) at three subsequent days and mean values were plotted as cumulative immobility time in seconds.

For sucrose preference mice were placed in single cages with two water bottles at least one day before testing to acclimate them to the bottles and to record their normal drinking behavior during 24 h. Sucrose preference was evaluated testing three different sucrose concentrations (1, 2.5 or 5 %), each over eight consecutive days. For all test sessions, mice had access to one bottle containing sucrose solution and to one containing tap water. Every 24 h, the position of the sucrose and water bottles was switched to prevent effects of side preference in drinking behavior. Every 48 h, the amount of water or sucrose solution intake was calculated by recording the bottle weight differences (at ZT6). Sucrose consumption was determined dividing the ingested amount of sucrose solution by the total amount of liquid consumed (water and sucrose) over 48 h, and expressed as percentage. Results from eight days (4 × 2 days) were taken into account for calculating sucrose preference. Drinking behavior and body weight were compared between the different genotypes as control values.

The elevated O-maze consisted of an elevated (42 cm above the floor) annular runway (outer diameter 46 cm and 5.5 cm with) divided into four sectors. The two opposing 90° closed sectors were protected by 11 cm high inner and outer walls, while the remaining two open sectors had a border of 3 mm only. Animals were released at the interface of closed and open area and observed for 5 min using a video camera. Number of entries and time spent in the open sector was recorded. In order to avoid habituation to the maze, the mice were tested once for a total test session of 5 min.

RT-PCR
Total RNA was extracted from snap-frozen striatal tissue using RNA-Bee (AMS Biotechnology (Europe) Limited, Abingdon UK). RNA was treated with RNase-free DNase I (Roche, Basel, Switzerland), precipitated in 4 M LiCl and purified further by phenol: chloroform extraction and ethanol precipitation. ssDNA complementary to the RNA starting from hybridized random hexamer primers was synthesized with SuperScript II (Invitrogen, Life
Assessment of cell proliferation and neurogenesis

Mice aged 2–3 months were used for the assessment of neurogenesis. To assess the total amount of newborn cells in the adult dentate gyrus, bromodesoxyuridin (BrdU) was administered by intraperitoneal injection at 100 mg/kg body weight and the mice (three per genotype) were sacrificed four days later. The tissue was fixed by cardiovascular perfusion, cryopreserved and sections of 40 μm were cut using a cryostat. For immunohistochemical detection of BrdU streptavidin-biotin detection was chosen. Free-floating sections were incubated in 1 M HCl on ice for 10 min, then in 2 M HCl at RT for 10 min and finally in 2 M HCl at 37 °C for 20 min. Incubation in 0.1 M boric acid at pH 8.5 for 12 min was performed for neutralization. Sections were blocked for 1 h in 10% FBS / 0.1% Triton X-100 / 1 × TBS at RT, followed by specific blocking of streptavidin and biotin binding sites in the tissue (Streptavidin-Biotin blocking kit Vector labs). Primary antibodies diluted in 1% FBS / 0.1% Triton X-100 / 1 × TBS were added to the sections and incubated overnight at 4 °C. Antibodies were Anti-DCX (Abcam ab18723), Anti-BrdU [BU1/75 (ICR1)] (Abcam ab6326, Cambridge, UK) and Anti-NeuN (Merck Millipore Q4 MAB377, Darmstadt, Germany). Secondary antibodies were biotinylated Anti-rat (Vector Laboratories BA9400, RRID, Burlingame, CA), Anti-mouse Cy5 and Anti-rabbit Cy3 (Jackson Immuno Research Q4 715-605-150, and 711-165-152, West Grove, PA) for 3 h at RT and subsequently Streptavidin-FITC conjugate (Vector Laboratories SA5001, Burlingame, CA) 2 h at RT. Mounted tissue sections were analyzed with a confocal microscope (Leica TCS SP5). To estimate the number of immunolabeled BrdU+ cells in the dentate gyrus (DG), systematic random sampling of every sixth 40-μm coronal section along the rostro-caudal axis of the DG (~1.06 mm to ~3.80 mm from bregma) was chosen and performed according to Borgs et al. (2009). Fluorescent images covering the DG region were taken with 40 × magnification and Z-stacks of 1.5 μm through the entire coronal section with frame average three. Images were processed with LAS AF software from LEICA (Leica Microsystems (Schweiz) AG, Heerbrug, Schweiz). Immunopositive cells were counted and the total amount of cells per DG was calculated by multiplying the results by six (based on the one on six section sampling).

Determination of dopamine and serotonin

The determination of both target compounds, namely dopamine and serotonin, was based on the utilization of isotopically labeled internal standards prepared according to a procedure described by Ji et al. (2008). Their separation from flash-frozen striatum samples was carried out using multistep solid – liquid extraction performed as follows: volume of 10 μl × mg⁻¹ of extraction solvent (acetonitrile: acetic acid, 0.98:0.02, v/v), containing as-prepared heavy internal standards of dopamine and serotonin, was mixed with each previously weighted sample. Total concentration of each internal standard was 50 nmol × L⁻¹. Mixtures were homogenized for 3 × 30 s using a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The homogenates were centrifuged at 22 000 g for 60 min at 4 °C. Supernatant was completely removed by evaporation using a SpeedVac system for 40 min at 35 °C; the resulting volume was 5 μl. Samples were afterwards reconstituted in 100 μl of deionized water. The isotopic labeling of both compounds was performed using the following procedure: 15 μl of 4% formaldehyde and 15 μl of NaBH₃CN were added to 100 μl of reconstituted sample, which led to a highly efficient di-methylation of presented dopamine and serotonin. The reaction time was set to 90 min at 25 °C to obtain >99% yield. The reaction was afterwards stopped by an addition of 10 μl of 1% ammonia solution and 1 μl of formic acid.

The chemical analysis was carried out using LTQ-Orbitrap Discovery (Thermo Fisher Scientific, Bremen, Germany) hyphenated to a 2D NanoLC (Eksigent Technologies, Dublin, CA). Spray voltage was set to 2.2 kV, cone voltage to 45 V, all remaining experimental parameters were tuned automatically using a build-in software-based apparatus. Chromatographic separation was performed as follows: 1 μl of sample was injected directly on a home-packed column with ID = 200 μm, packed with C18 AQ particles (5 μm, 100 Å). The separation was based on a gradients elution, where solvent A consisted of 0.2% formic acid in water; solvent B was acetonitrile. Initial conditions were 97% A, composition was changed to 10% of solvent A after 12 min, and kept there for 5 min. Finally, initial conditions were restored during the last 5 min.

Statistical analysis

Statistical evaluation of all experiments was performed using GraphPad Prism4 software (GraphPad Software, Inc., La Jolla, CA). Depending on the type of data, either unpaired t-test, one- or two-way ANOVA with Bonferroni post-test was performed. Values were considered significantly different with p < 0.05 (*), p < 0.01 (**), or p < 0.001 (**).
Cry1 double mutant, Cry1 knock-out, Cry2 knock-out and Rev-erbα knock-out mice in the forced swim test (FST) (Figure 1, left panel), a rodent behavioral assay commonly used for the evaluation of anti-depressant drugs and experimental manipulations that are aimed at rendering or preventing depressive-like states (Can et al., 2012). The experiments were performed 6 h after lights on (Zeitgebertime (ZT6)) with mice held in a 12 h light / 12 h dark cycle. We observed that immobility time in the FST was similar among wild-type, Per2/Cry1 double mutant and Cry2 knock-out mice. In contrast, Per2 mutant, Per1/Per2 double mutant and Rev-erbα knock-out mice displayed significantly lower immobility times as previously observed (Chung et al., 2014; Hampp et al., 2008; Schnell et al., 2014), indicating that mice of these genotypes may be in a manic-like state. Interestingly, Cry1 knock-out mice were the only animals showing longer immobility times indicative of a more depressive-like state.

Subsequently, the effects of a chronic (two weeks) treatment with a 0.4% lithium carbonate diet on the FST responses of all genotypes have been evaluated (Figure 1, right panel). As expected, immobility time decreased strongly in wild-type animals and a similar reduction was observed in Cry2 knock-out mice. Per2 mutant, and Per1/Per2 double mutant animals showed a decrease in immobility time as well, although to a lower extent, probably because they already display lower immobility times before lithium treatment. Rev-erbα knock-out mice showed very-short immobility under normal conditions and lithium treatment was not able to shorten this even further. Interestingly, the genotypes lacking the Cry1 gene poorly reacted to the lithium treatment, indicating that Cry1 may be at least partially involved in the process of mediating lithium effects.

To corroborate our findings we measured the concentration of lithium in the plasma of all genotypes before and after lithium treatment (Figure 1B). We observed that all genotypes displayed comparable amounts of lithium in the plasma. This suggests that the behavioral differences observed among the genotypes in the FST, are not due to variability in uptake of lithium from the diet.

Lithium differentially affected clock gene mRNA expression in the striatum of clock mutant mice

As a next step we tested the influence of lithium on clock gene expression at ZT6 in the striatum, a brain region
involved in the regulation of mood-related behaviors. As the Cry1 gene seemed to be important for mediating the effects of lithium (Figure 1), we focused our analyses on Per2/Cry1 double mutant and Cry1 knock-out mice and compared them with wild-type and Per2 mutants used as controls (Figure 2). We observed that Cry1 mRNA expression was induced upon lithium treatment in wild-type mice but not in Per2 mutants (Figure 1A), which indicates that lithium affects Cry1 gene expression involving directly or indirectly Per2. In contrast Cry2 mRNA expression was not significantly altered in all genotypes tested (Figure 2B). However, it appears that in contrast to wild-type and the two genotypes lacking Cry1, in which Cry2 seemed to be slightly increased, the Per2 mutants displayed a slight reduction in Cry2 expression upon lithium treatment (Figure 2B). Per1 expression appeared to be significantly reduced after lithium treatment in Cry1 knock-out mice only, although a similar but not significant decrement in Per1 mRNA was detected in the other genotypes (Figure 2C). Expression of Rev-erbα was significantly reduced after lithium treatment only in wild-type and Cry1 knock-out...
mice whereas in the Per2 and Per2/Cry1 double mutants no changes were observed (Figure 2D). This indicated that Per2 may be involved in lithium mediated down-regulation of Rev-erβ. In contrast, no effect of lithium on the Rorα expression was observed. Nevertheless, in the two genotypes lacking Cry1 a tendency for increase of Rorα expression was seen (Figure 2E). Since Per2 appears to be involved in the effects of lithium on the Cry1 and Rev-erβ mRNA expression we tested the effects of lithium on the Per2 gene expression in wild-type and Cry1 knock-out mice (Figure 2F). In wild-type mice Per2 was induced after lithium treatment, whereas in Cry1 knock-out animals Per2 was constitutively high before lithium uptake and was not increased further by the treatment. Hence, the levels of Per2 mRNA and its induction by lithium appeared to depend on Cry1. Interestingly, Bmal1 expression appeared not to be affected by lithium in wild-type as well as in Cry1 knock-out animals (Figure 2G), although the mRNA levels after lithium treatment were higher in wild-type compared to Cry1 knock-out mice.

Lithium treatment affected the phase of Cry1 protein expression
To examine the dynamics of Cry1, PER1 and PER2 expression in the mouse striatum after chronic lithium treatment, we analyzed diurnal protein levels by Western blotting (Figure 3). Wild-type animals showed significant changes in the daily Cry1 protein expression profile after lithium treatment (Two-way ANOVA, \( p = 0.013 \)), with a shift in the peak of expression from ZT22 to ZT6 (Figure 3A). In contrast, expression of PER1 and PER2 proteins was unaffected in wild-type animals. In Cry1 knock-out mice, no lithium-mediated changes in the diurnal expression profile of PER1 and PER2 proteins were observed (Figure 3B). In Per2 mutant animals, however, the daily expression pattern of Cry1 was significantly altered upon lithium treatment (Two-way ANOVA, \( p = 0.026 \)), with a shift of the maximal expression from ZT2 to ZT22 (Figure 3C). In contrast, PER1 expression was not affected by lithium in these animals. Finally, comparing the Cry1 protein expression patterns in treated and untreated wild-type and Per2 mutant mice, it is interesting to note that the Cry1 profile of treated Per2 mice matched with that of untreated wild-type animals, and vice versa. This indicates that Per2 is important for setting the phase in the control animals but is not involved in the phase shifting action of lithium on Cry1.

Lithium differentially affected GSK3β in Cry1 and Per2 clock mutants
One of the molecular targets of lithium action is GSK3β (Klein & Melton, 1996) (Stambolic et al., 1996), which is phosphorylated and thereby inactivated upon lithium treatment. Therefore, we tested the diurnal GSK3β phosphorylation (pGSK3β) pattern in the striatum of wild-type, Per2 mutant, and Cry1 knock-out mice before and after lithium treatment (Figure 4). We found that in both wild-type (Figure 4A left panel) and Per2 mutant mice (Figure 4A right panel) the diurnal phosphorylation pattern of GSK3β was not strongly affected with the exception of ZT2 in the wild-type animals (Figure 4A left panel). However, the phase of diurnal pGSK3β was not altered. In contrast, the 24 h phosphorylation pattern was inverted in Cry1 knock-out mice after lithium treatment (Figure 4A, middle panel), with significantly increased levels of pGSK3β at ZT6, the time point in which the Cry1 knock-out animals did not respond to lithium in the FST (Figures 1A and 5). Taken together, we observed a change in the pGSK3β phase in Cry1 knock-out mice after lithium treatment and this phase modification appeared not to involve Per2 gene activity. This is consistent with our observation that Per2 seemed not to have a role in setting the phase of the diurnal Cry1 protein expression profile after lithium treatment (Figure 3). Furthermore, our results suggest a direct or indirect involvement of Cry1 protein on the phosphorylation of GSK3β in response to lithium.

The response of Cry1 knock-out mice to lithium was time of day-dependent in the FST
In order to extend our findings on the depressive state of Cry1 knock-out animals, we investigated their responses in the FST at the two opposite time points ZT6 and ZT18, in comparison to those of wild-type mice. We observed that at both time points Cry1 knock-out mice displayed longer immobility times as compared to wild-type animals (Figure 5). In response to lithium treatment, wild-type animals showed decreased immobility time at both ZTs. In contrast, Cry1 knock-out mice reduced their immobility only at ZT18 but not at ZT6 (Figure 5). This indicates that the behavioral response to lithium treatment in the FST involving Cry1 is time of day-dependent and the pathways involved in the lithium response vary over the day.

To assess another core symptom of depression, we performed a sucrose-preference test to assess anhedonia (Crawley, 2000; Pollak et al., 2010). Interestingly, Cry1 knock-out animals did not differ in this test from wild-type (Figure 5B). Similarly, the weight (Figure 5C) as well as the drinking behavior (Figure 5D) were not different between the two genotypes. Taken together, these results indicate that Cry1 knock-out animals do not show any difference in anhedonia, but can display other mood-related phenotypes, which resembles a mixed-state seen in bipolar-disorder patients (Lee et al., 2013).

Cry1 knock-out mice were less anxious in the O-maze test
Besides reward (sucrose) and despair-based behavior (FST), anxiety-like phenomena can occur in rodent models of depression (Nestler & Hyman, 2010). Therefore, we performed the elevated O-maze test, which assesses the innate conflict in mice between
curiosity to explore a novel environment and fear to be exposed to a possible predator in a brightly lit open space (Crawley, 2000). At ZT6 Cry1 knock-out mice showed strikingly more number of entries into the closed area of the O-maze as compared to wild-type and this number of entries was reduced in Cry1 knock-out but not wild-type animals after lithium treatment (Figure 5E). In addition, Cry1 knock-out mice spent less time in the closed area, but this time was increased after lithium treatment, whereas wild-type animals did...
not respond to lithium (Figure 5F). These observations suggested that Cry1 knock-out mice can respond at ZT6 to lithium in the O-maze test in contrast to the FST (Figures 1A and 5A). Furthermore, it appeared that these animals are less anxious than wild-type, which is counterintuitive given the results of the FST.

Striatal dopamine levels were differentially affected in Cry1 and Per2 mutant mice

In a previous study, we showed that the behavioral response in the FST correlated with dopamine levels in the striatum of wild-type and Per2 mutant mice (Hampp et al., 2008). In order to investigate whether dopamine levels correlate with the FST behavioral response in the Cry1 knock-out animals, we measured striatal dopamine levels of wild-type, Per2 mutant, and Cry1 knock-out mice before and after lithium treatment by mass spectrometry at ZT6 (Figure 6A). Consistent with our previous findings, Per2 mutants showed increased dopamine levels compared to wild-type (Figure 6A). In contrast, Cry1 knock-out animals displayed significantly lower dopamine levels, which correlated with their longer immobility time in the FST (1A). Lithium treatment had no effect on dopamine accumulation in wild-type animals, but in Cry1 knock-outs dopamine levels significantly increased whereas in Per2 mutants they significantly decreased. Taken together, it appeared that lithium had opposite effects on striatal dopamine levels in Cry1 knock-out compared to Per2 mutant animals. Serotonin, another neurotransmitter believed to be involved in the regulation of mood related behaviors, was decreased in Cry1 knock-out but increased in Per2 mutant mice compared to wild-type (Figure 6B). However, no significant changes after lithium treatment were observed (Figure 6B). These results indicate that lithium modifies neither dopamine levels nor serotonin levels in the striatum of wild-type mice. In contrast, however, lithium affects dopamine levels in an opposite manner in Cry1 knock-out and Per2 mutants. This indicates that, in wild-type conditions the activity of these two genes is important to keep the dopamine balance in the murine striatum.

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Lithium increased adult neurogenesis in wild-type but not Cry1 knock-out mice

Memory and cognitive dysfunctions have been reported for Cry1 knock-out animals (De Bundel et al., 2013; Van der Zee et al., 2008). Furthermore, adult hippocampal neurogenesis has been reported to affect mood-related behavior (Snyder et al., 2011) and it is altered in Per2 mutant and Rev-erbs knock-out mice (Borgs et al., 2009; Schnell et al., 2014). Therefore, we tested adult hippocampal neurogenesis in Cry1 knock-out animals and whether lithium had an effect on this process. We observed that lithium increased the pool of proliferating...
neural precursor cells (NPCs) in the hippocampal subgranular zone (SGZ) of wild-type animals (Figure 7A and C), as observed previously (Chen et al., 2000; Riadh et al., 2011; Yoneyama et al., 2014). The majority of BrdU positive cells (BrdU*) were also positive for doublecortin (Dcx), a marker of immature neurons, indicating that proliferating cells differentiate into neurons (Figure 7A and B). In contrast, there was no effect of lithium in the Cry1 knock-out mice. However, there were more BrdU* cells in this region before lithium treatment if compared to the SGZ of wild-type mice (Figure 7C). Taken together, it appears that adult hippocampal neurogenesis in the SGZ of Cry1 knock-out mice is increased as observed previously in Per2 mutant and Rev-erbα knockout mice (Borgs et al., 2009; Schnell et al., 2014). However, Cry1 knock-out animals did not respond to lithium with an increase of neurogenesis in contrast to wild-type mice.

**DISCUSSION**

This study provides evidence that the Cry1 gene is important to regulate mood-related behaviors (Figure 1). We found that Cry1 is important at specific times of the day to transmit lithium-mediated effects (Figure 5A). In the molecular clockwork, Cry1 and Per2 play a role in the same negative feedback loop (Takahashi et al., 2008), although Per2 may under certain circumstances act positively (Akashi et al., 2014). Our data suggest that the Cry1 and Per2 pathways regulating the behavior in the FST and the response to lithium are distinct (Figures 2 and 3). This is also evidenced by the differences in the GSK3β phosphorylation profiles of the Cry1 and Per2 mutants, both before and after lithium administration (Figure 4). That mice lacking functional Per2 or Cry1 can display opposite phenotypes is not unusual. For example, the response to a nocturnal light pulse at ZT14 elicits a phase delay in wild type and Cry1 knock-out mice (Spoelstra et al., 2004), whereas Per2 mutant mice do not delay clock phase (Albrecht et al., 2001). Similarly, dopamine levels in the striatum are regulated in opposite manner by the Per2 and Cry1 genes (Figure 6A). Furthermore, we observed the co-existence of depressive and mania-like symptoms in the Cry1 knock-out mice (Figure 5), which resembles the so-called mixed state seen in bipolar disorder subjects.

Mixed states may occur at the transition from depression to mania, when lithium treatment is often less efficient and treatment outcome varies greatly between different subjects (Kruger et al., 2005; Muzina, 2009). Defects in the circadian-clock mechanism at the cellular level cause alterations in the synchronization between cellular clocks leading to a change in coherence and phasing of the circadian network (Figures 2–4) (Welsh et al., 2010). As a consequence this may change the response to treatments, such as exemplified here by lithium, leading to the observed phenomena of mixed features (Lee et al., 2013). We do not know, however, whether Per2 and Cry1 affect mood related behaviors through the clock or indirectly.

Our results are in agreement with the widely accepted view that GSK3β activity is a pivotal factor in the etiology of bipolar disorder. GSK3β is able to phosphorylate CRY1 leading to degradation of the protein (Kurabayashi et al., 2010). Lithium treatment decreases GSK3β activity and consequently CRY1 protein dynamics is changed (Figure 3A). This leads to a shortening of the immobility time in the FST (Figure 5A). In Cry1 knock-out mice no change in immobility time in the FST would be expected (Figure 5A, ZT6). However, GSK3β most likely affects additional proteins as evidenced by the response of Cry1 knock-out animals at ZT18 (Figure 5A). This is in agreement with a recent study showing that circadian rhythmicity of active GSK isoforms modulates clock gene rhythms (Besing et al., 2015).
FIGURE 7. Immunohistochemistry and BrdU labeling in the dentate gyrus (DG). (A) DG sections of wild-type control (left panels) and lithium treated (right panels) mice. (B) DG sections of Cry1 knock-out control (left panels) and lithium treated (right panels) mice. The upper panels in (A) and (B) show visualization of cell division in the SGZ of the DG at ZT6 using bromodeoxyuridine (BrdU). Higher magnification of the boxed regions is depicted on the lower panels. Antibodies recognizing NeuN mark nuclei of mature neurons (blue), antibodies recognizing Dcx are in red and antibodies against BrdU are in green. Scale bars: 50 μm. (C) Quantification of the BrdU+ cells represented by mean ± SEM of three animals per group. Asterisks in the graph show significant changes revealed by Two-way ANOVA and Bonferroni post-tests (* p < 0.05, ** p < 0.01).
Our experiments indicate that GSK3β activity and its phase are altered in Cry1 knock-out mice (Figure 4), and this may contribute to the abnormal mood-related phenotypes observed in these animals (Figure 5). Furthermore, our results suggest an involvement of GSK3β in the therapeutic effects of lithium that may be related to the less distinct behavioral responses in Cry1 knock-out mice to lithium treatment. Interestingly, fibroblasts from bipolar disorder patients were less sensitive to lithium than cells from healthy subjects (McCarthy et al., 2013), similar to the response observed in Cry1 knock-out mice. Previous studies described a lengthening of circadian period after lithium treatment in vivo (Kafka et al., 1982; Kripke & Wyborney, 1980; Kripke et al., 1978, 1979; Stewart et al., 1991; Welsh & Moore-Ede, 1990) and in vitro (Li et al., 2012) establishing a relationship between the effects of lithium and the circadian clock. In agreement with this, we describe here a correlative relationship between clock gene mutant mice and lithium treatment. However, we cannot decipher from our observations how lithium is mechanistically related to circadian clock components.

Inhibition of GSK3β has been suggested to promote adult hippocampal neurogenesis in vitro and in vivo (Morales-Garcia et al., 2012). Lithium promotes phosphorylation and inactivation of GSK3β (Klein & Melton, 1996; Stambolic et al., 1996). From these findings the prediction is that lithium treatment promotes adult hippocampal neurogenesis in wild-type mice. Indeed, we observed that lithium treatment increased adult hippocampal neurogenesis in wild-type animals (Figure 7), supporting this notion. Although we do not have direct evidence that in the hippocampus GSK3β phosphorylation is modified by lithium, we demonstrated that in the striatum lithium affects this process (Figure 4A). Furthermore, our results indicate that Cry1 plays a role in the lithium-mediated phosphorylation of GSK3β (Figures 3 and 4). In Cry1 knock-out animals, the absence of Cry1 alters both the basal GSK3β phosphorylation profile and its modification in response to lithium treatment. This is consistent with our observation that Cry1 knock-out mice did not increase adult hippocampal neurogenesis in response to lithium (Figure 7). Nevertheless, these animals did show a high basal level of neurogenesis before lithium treatment, which may be due to alterations in the GSK3β signaling pathway due to lack of Cry1.

Mood-related behaviors, such as bipolar disorder, are influenced by at least three systems: the HPA-axis, monoamine signaling, and the circadian system (Schnell et al., 2014). In the experiments presented in this study we observed that changes in the circadian clock are accompanied by alteration in dopamine levels and time of day-dependent responses to lithium. Furthermore, Cry1 knock-out mice exhibit increased corticosterone levels and hence display alterations in the HPA-axis (Lamia et al., 2011). These data support the view that Cry1 knock-out animals are in a mixed mood state (Figure 8). However, the link between Cry1 and BP in human case-control studies is unclear. Several analyses reported a link between BP and the chromosomal region 12q23-q24, which contains Cry1 (Curtis et al., 2003; Degn et al., 2001; Morisette et al., 1999). The association studies of BP with Cry1 single nucleotide polymorphisms (SNPs) revealed only a nominal significant association with a common intergenic variant (rs2287161), not confirmed after correction for multiple tests (Soria et al., 2010). Since BP is a multifactorial disease in which genetic and environmental factors might exert additive roles, the contribution of a single candidate gene is difficult to determine from human population genetic studies. Therefore, data obtained from knock-out animal models represent an informative tool to help to clarify the influence of a single gene. In addition to Cry1 knock-out animals, which display a mixed-mood state, mice with a knockdown of the Clock gene in the ventral tegmental area (Mukherjee et al., 2010) and in olfactory bulbectomized rats (Morales–Medina et al., 2012; Song & Leonard, 2005) show similar features.
In summary, our study shows intricate relationships between lithium treatment and the circadian system, dopamine signaling and neurogenesis. Mood state and the circadian system are network-regulated properties of an organism. The interaction of these two networks generates second-order properties that are difficult to define by single molecular pathways, and hence, challenges of the system may provoke unexpected phenomena. Overall, we show that circadian clock components are affected by lithium treatment and may contribute to the beneficial effects of lithium therapy.

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DECLARATION OF INTEREST

The authors declare they have no competing financial interests.

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: AS, FS, VR, LA, UA. Acquisition of data: AS, FS, VR, JAR, EB. Analysis and interpretation of data: AS, FS, LA, VR, GR, UA. Writing of the manuscript: AS, UA. Obtained funding: UA. Support from the Swiss National Science Foundation, the Velux Foundation and the State of Fribourg is gratefully acknowledged.

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