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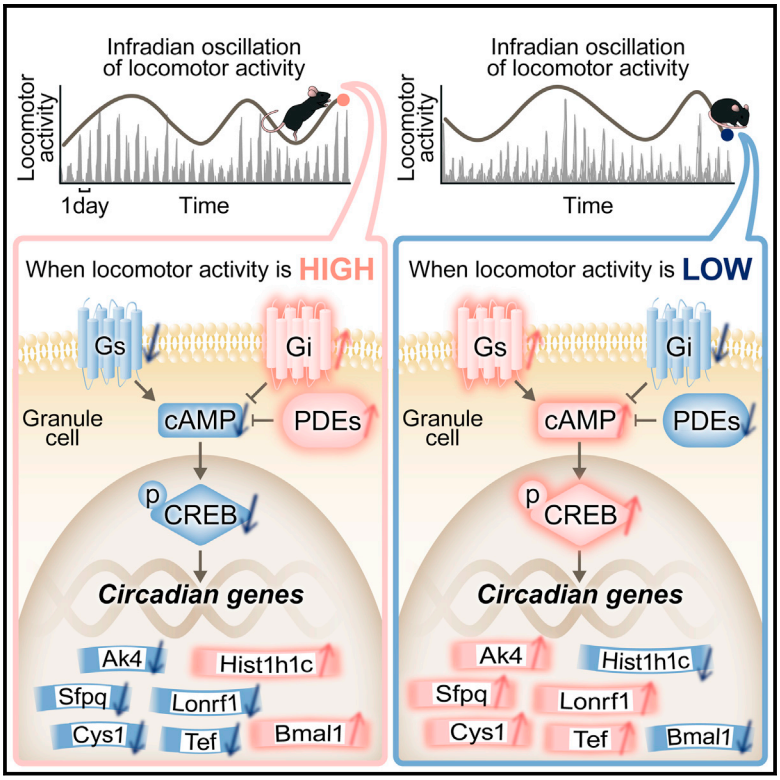
Circadian Gene Circuitry Predicts Hyperactive Behavior in a Mood Disorder Mouse Model

Graphical Abstract



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In Brief

Mood disorders are characterized by large shifts in emotional states and activity levels, but the molecular basis for such irregular mood changes remains unknown. Hagihara et al. report that hippocampal expression patterns of circadian genes and cAMP/CREB pathway-related molecules in a mouse model of bipolar disorder are predictive of whether the mice are in a state of high or low locomotor activity.

Highlights

- Gene expressions of αCaMKII (*Camk2a*) mutant mice, a bipolar disorder model, are analyzed
- Gene expression patterns in the mouse brain retrospectively predict behavioral state
- Expression of many circadian genes correlates with infradian rhythm behavior
- Expression of molecules in the cAMP/CREB pathway also correlates with the behavior

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Circadian Gene Circuitry Predicts Hyperactive Behavior in a Mood Disorder Mouse Model

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SUMMARY

Bipolar disorder, also known as manic-depressive illness, causes swings in mood and activity levels at irregular intervals. Such changes are difficult to predict, and their molecular basis remains unknown. Here, we use infradian (longer than a day) cyclic activity levels in αCaMKII (*Camk2a*) mutant mice as a proxy for such mood-associated changes. We report that gene-expression patterns in the hippocampal dentate gyrus could retrospectively predict whether the mice were in a state of high or low locomotor activity (LA). Expression of a subset of circadian genes, as well as levels of cAMP and pCREB, possible upstream regulators of circadian genes, were correlated with LA states, suggesting that the intrinsic molecular circuitry changes concomitant with infradian oscillatory LA. Taken together, these findings shed light onto the molecular basis of how irregular biological rhythms and behavior are controlled by the brain.

INTRODUCTION

Mood changes occur in an infradian rhythm (Bryson and Martin, 1954; Eastwood et al., 1985) and affect a variety of biological functions, including neuronal and motor activities (Chen et al., 2010; Wolff et al., 1985). However, elucidation of the molecular basis of such changes has been hampered by the lack of animal models exhibiting spontaneous behavioral changes related to the infradian oscillation of mood. In our previous work, we found that mice with heterozygous knockout of the alpha-isoform of calcium/calmodulin-dependent protein kinase II (*Camk2a*-HKO mice) show various dysregulated behaviors, including infradian oscillation of locomotor activity (LA; Hagihara et al., 2013; Shin et al., 2013; Yamasaki et al., 2008), suggesting that *Camk2a*-HKO mice may serve as an animal model showing infradian oscillation of mood, which is substantially found in patients with bipolar disorder (Belmaker, 2004). *CAMK2* has been implicated in mood disorders, including bipolar disorder and depression (Robison, 2014; Xing et al., 2002), as well as autism (Lanz et al., 2013), which is highly comorbid with mood disorders (Mazefsky et al., 2008). A recent study revealed genetic associations of CAMK genes, including *CAMK2A*, with bipolar disorder (Ament et al., 2015). A nonsense mutation and a splice-donor mutation were reported in *CAMK2A* and *CAMK2B*, respectively, in schizophrenia patients in an exome-sequencing study (Purcell et al., 2014). A meta-analysis integrating genetics and genomics of human and animal model data also identified *Camk2a* as one of the "top candidate genes" for bipolar disorder (Le-Niculescu et al., 2009). Further evidence supports that the *Camk2a*-HKO mice have construct and face validity as a model of mood disorders, including bipolar disorder. At the cellular level, these mutant mice exhibit maturation abnormalities in the granule cells of the hippocampal dentate gyrus (DG), in which the molecular and physiological properties are similar to those of normal immature granule cells (Yamasaki et al., 2008). The maturation abnormality of DG has been found in the postmortem brain of patients with bipolar disorder and schizophrenia (Walton et al., 2012). In addition, neuronal hyperexcitability, which we previously detected in the hippocampal DG of *Camk2a*-HKO mice (Hagihara et al., 2013; Yamasaki et al., 2008), was also found in the DG granule cell-like neurons differentiated from induced pluripotent stem cells (iPSCs) of patients with bipolar disorder (Mertens et al., 2015). At a behavioral level, *Camk2a*-HKO mice exhibit additional abnormal behaviors, such as deficits in social activity and working memory, which are analogous to those in patients with bipolar disorder and schizophrenia (Yamasaki et al., 2008). Here, we focused on the unique behavioral phenotype of infradian oscillatory LA exhibited by this mouse model with high validity for mood disorders.

Because the DG is thought to be involved in the regulation of mood (David et al., 2010; Samuels and Hen, 2011), we hypothesized that the gene expression patterns in the DG may retain information about infradian oscillatory LA. In the present study, we examined whether gene expression patterns in the DG could predict LA in *Camk2a*-HKO mice by applying a statistical learning algorithm, which could discover intrinsic links between

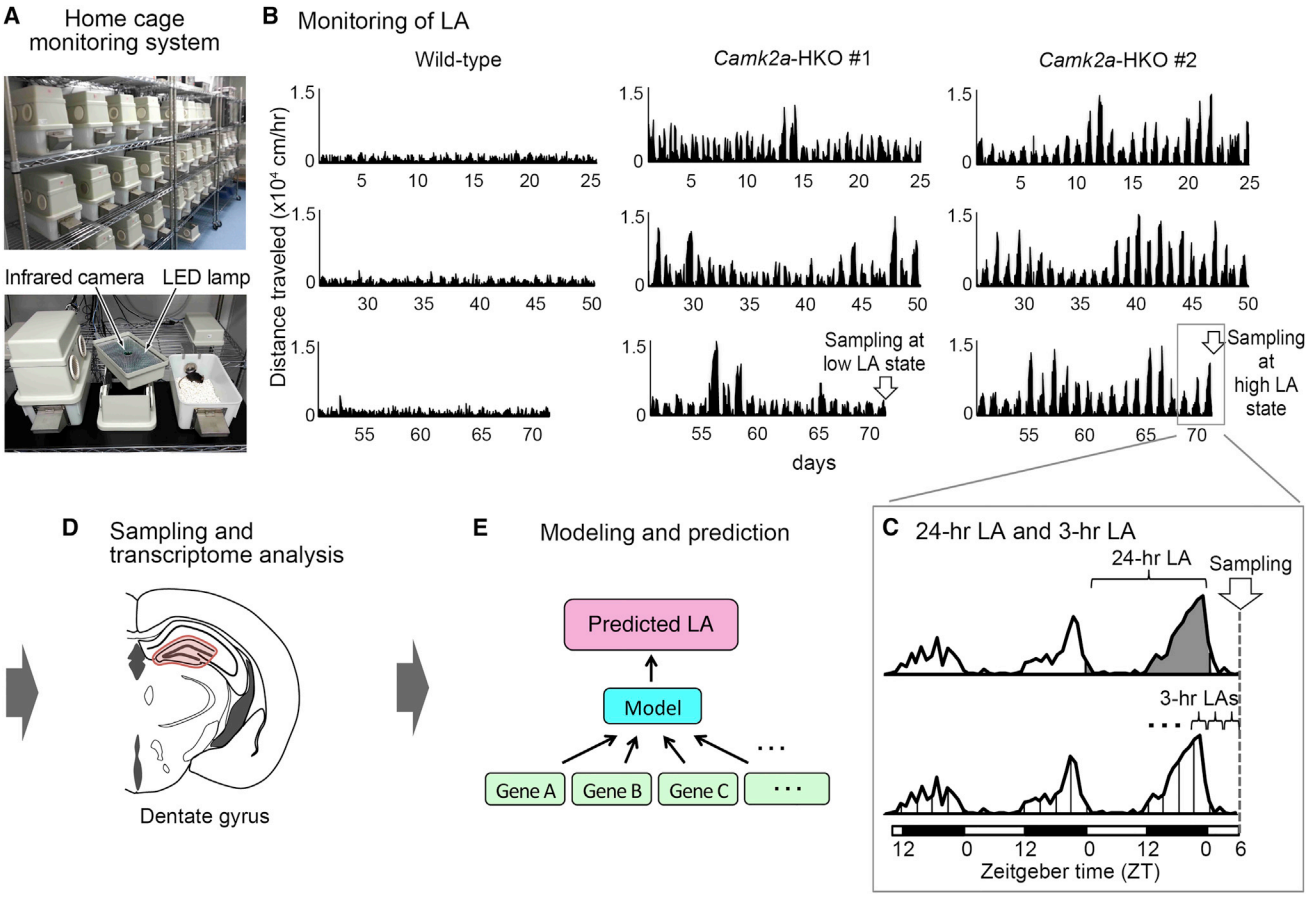


Figure 1. Experimental Overview for Predicting LA from Gene Expression Patterns

(A) LA data were acquired with a home cage monitoring system by measuring the distance traveled in the cage.

(B and C) LA was monitored. (B) Following the monitoring of LA, the DG was taken from each mouse ZT6–ZT7. (C) 24-hr LA, distance traveled during the 24 hr before ZT0 on the sampling day; 3-hr LAs, LAs of every 3-hr window before sampling (ZT6).

(D) The DG was processed for microarray analysis.

(E) Statistical learning models were built using gene expression profiles to predict LA during the time windows of interest.

See also Figure S1 and Table S1.

these molecular signatures and LA. This approach was inspired by the analogy of neural decoding methods, in which statistical learning models predict specific mental contents from human functional MRI patterns (Horikawa et al., 2013; Miyawaki et al., 2008). Statistical learning methods using microarray data have been employed for class predictions (Michiels et al., 2005; Reis-Filho and Pusztai, 2011). Regarding class prediction of behavior, gene expression patterns in the brain can discriminate between two classes of behavior in honeybees (Whitfield et al., 2003). Unlike such class predictions, we sought to conduct quantitative predictions of LA from gene expression patterns in the brain.

RESULTS

LA in the Home Cage Is Correlated with Anxiety- and Depression-like Behaviors in *Camk2a*-HKO Mice

To examine whether infradian oscillatory LA in the home cages of *Camk2a*-HKO mice is associated with traditional measures of anxiety- and depression-like behaviors, we conducted an open field test and the Porsolt forced swim test following measurement of home cage LA. The behavioral tests were performed from zeitgeber time (ZT) 6 (ZT0, lights on; ZT12, lights off). The total distance traveled in the open field was not correlated with the level of home cage LA, as represented by 24-hr LA (distance traveled during the 24 hr between ZT0 of the day before the tests and ZT0 of the day of the tests; Figures 1B and 1C; Figure S1A). This indicated that changes in home cage LA were not caused by general problems in physical activity (Figure S1B). Time spent in the center of the open field apparatus, which is considered an index of anxiety-like behavior, positively correlated with 24-hr LA in the home cage (Figure S1C). In the forced swim test, the percentage of immobility time was negatively correlated with 24-hr LA in the home cage (Figures S1D and S1E). These results suggested that the LA state in the home cage was correlated with anxiety- and depression-like behaviors, potentially reflecting a certain state of mood in *Camk2a*-HKO mice.

LA of Mice Can Be Retrospectively Predicted from Gene Expression Profiles in the Hippocampal DG

An experimental overview for predicting LA from gene expression patterns in the DG is depicted in Figure 1. We monitored LA in the home cage (Figure 1A) for 72–82 days using 37 *Camk2a*-HKO mice (Figure 1B). Following longitudinal monitoring, the DG was sampled ZT6–ZT7 (Figure 1C) and was processed for transcriptomic analysis (Figure 1D). Mice were selected for the sampling such that their 24-hr LA levels varied among the 37 mice (Figures 1C and 2A). Of 45,037 transcripts tested, the expression levels of 864 transcripts (817 genes) were correlated with the 24-hr LA ($p < 0.01$, Pearson's correlation coefficient), and 60 transcripts survived false discovery rate correction for multiple tests (q value < 0.1; Table S1). Pathway analysis using KeyMolnet, a literature-based knowledgebase containing highly reliable information on a range of human proteins, small molecules, molecular relations, and diseases (Satoh and Tabunoki, 2011), revealed that bipolar disorder was the disease most relevant to the genes exhibiting high correlations with 24-hr LA ($p < 0.0005$, 111 transcripts; Table 1). To determine whether gene expression patterns in the DG could retrospectively predict 24-hr LA of individual mice, we performed an out-of-sample prediction test, in which independent sets of mice were used for training (including feature selection from the entire set of 45,037 transcripts) and testing a linear regression model that predicted 24-hr LA from transcriptome data (Figures 1E and 2B). Correlations between the actual and the predicted 24-hr LA were significant (Figure 2C), revealing that gene expression patterns in the DG could accurately predict 24-hr LA.

To investigate whether gene expression patterns in the DG could predict the LA of the past several days, we constructed gene expression-based models for predicting the LA of every 3-hr window (3-hr LA) within the 5 days before sampling (yielding 40 time windows). The actual and the predicted 3-hr LAs were similar to each other for the past 3–4 days in almost all mice; however, differences gradually appeared at 4 or more days before sampling (Figure 2D; Figure S2). Statistical evaluation of prediction accuracy of the models detected significant correlations between the actual and the predicted 3-hr LAs at 6 of 40 time windows after Bonferroni correction for multiple tests (Figures 2E–2K). The oldest time window with significant correlation was from 117 to 114 hr before sampling. Thus, the gene expression-based models can successfully predict 3-hr LAs of the past 5 days, suggesting that the gene expression in the DG would hold the information about LA for the past several days. Notably, three of the six time windows were within 24 hr before sampling (Figures 2F–2H). However, gene expression patterns in the DG could not predict 3-hr LA immediately before sampling (Figure 2E). The expression levels of some major neuronal activity-regulated genes were correlated with the 3-hr LA, as well as LA during 60 min immediately before sampling, but not with 24-hr LA (Table 2), suggesting that expression patterns of genes correlated with LA immediately before sampling, including the known neuronal activity-regulated genes, do not have prediction ability for infradian states of LA.

Focusing on the six previously mentioned time windows (Figure 2E), we examined differences between the gene sets used in those prediction models. At three time windows within the 24 hr before sampling, similar genes were used in the prediction models (Table S2). However, genes used in the prediction models of 3-hr LAs 3–5 days before sampling generally differed (Table S2), suggesting that the expression of distinct sets of genes in the DG may retain information about LA during different and specific time windows.

Expression Levels of Circadian Genes in the DG Are Correlated with LA

Prediction analysis of microarray data was implemented to find which genes are most useful to characterize the behavioral state of individual mice (Chemello et al., 2011; Kittleson and Hare, 2005; Whitfield et al., 2003). The prediction algorithm we used identified gene signatures related to infradian oscillatory LA by weighting genes according to their individual predictive strength. Thus, we tried to find molecular alterations accompanying changes in LA by examining the weighted genes used for 24-hr LA prediction at least one time in linear regression (Figure 3A). Our preliminary survey on the genes exhibiting correlation with 24-hr LA (Table S1) using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics tool showed significant enrichment for pathways or biogroups, such as immunoglobulin I set, steroid biosynthesis, and biological rhythms (Benjamini-adjusted $p < 0.05$). This result led us to notice that some well-known circadian genes, such as *Arntl* (*Bmal1*) and *Tef*, are in the predictive gene list (Figure 3A). The reliability of the prediction analysis of the microarray data was supported by the presence of genes known to be associated with mood disorder, such as bipolar disorder (*Grm4* and *Bmal1*, Kato, 2007; *Fam107a*, Nakajima and Koizumi, 2014) and depression (*Tef*, Hua et al., 2012, 2014). In addition, dysregulated expression of *Sfpq* (Kubota et al., 2010; Nakatani et al., 2006) was reported in the brain of patients with bipolar disorder. Then, we determined how many circadian genes were included in the list by querying the Photoperiodism database (http://photoperiodism.brainstars.org/rhythmic/), which describes genes showing a 24-hr rhythm in the mouse pars tuberalis (Masumoto et al., 2010). Of the 29 transcripts, 7 were circadian genes (Figure 3A); this was significantly higher than the chance level ($\chi^2(1) = 56.85$, $p = 4.70 \times 10^{-14}$), which was determined as the percentage of transcripts present in the Photoperiodism database (1,107) among the transcripts examined in the DG of *Camk2a*-HKO mice (45,037). When applying the same analysis to the list of genes exhibiting correlations with 24-hr LA ($p < 0.01$), we found a significantly larger number of circadian genes than chance level in the list (55 of 864 transcripts, $\chi^2(1) = 55.03$, $p = 1.19 \times 10^{-13}$; Table S1). In the gene sets used in successful predictions of 3-hr LA, circadian genes were found in three time windows within the 24 hr before sampling but were not found in three earlier time windows (Table S2). These findings suggested that circadian genes may specifically correlate with LA within the past 24 hr in the DG.

For the respective top ten circadian genes showing positive and negative correlations with 24-hr LA, the direction of correlation between circadian gene expression and 24-hr LA matched their circadian phase (Figures 3B and 3C). The circadian phase represents the peak time of diurnal oscillation of gene expression (Figure 3B; Masumoto et al., 2010). Genes whose expression

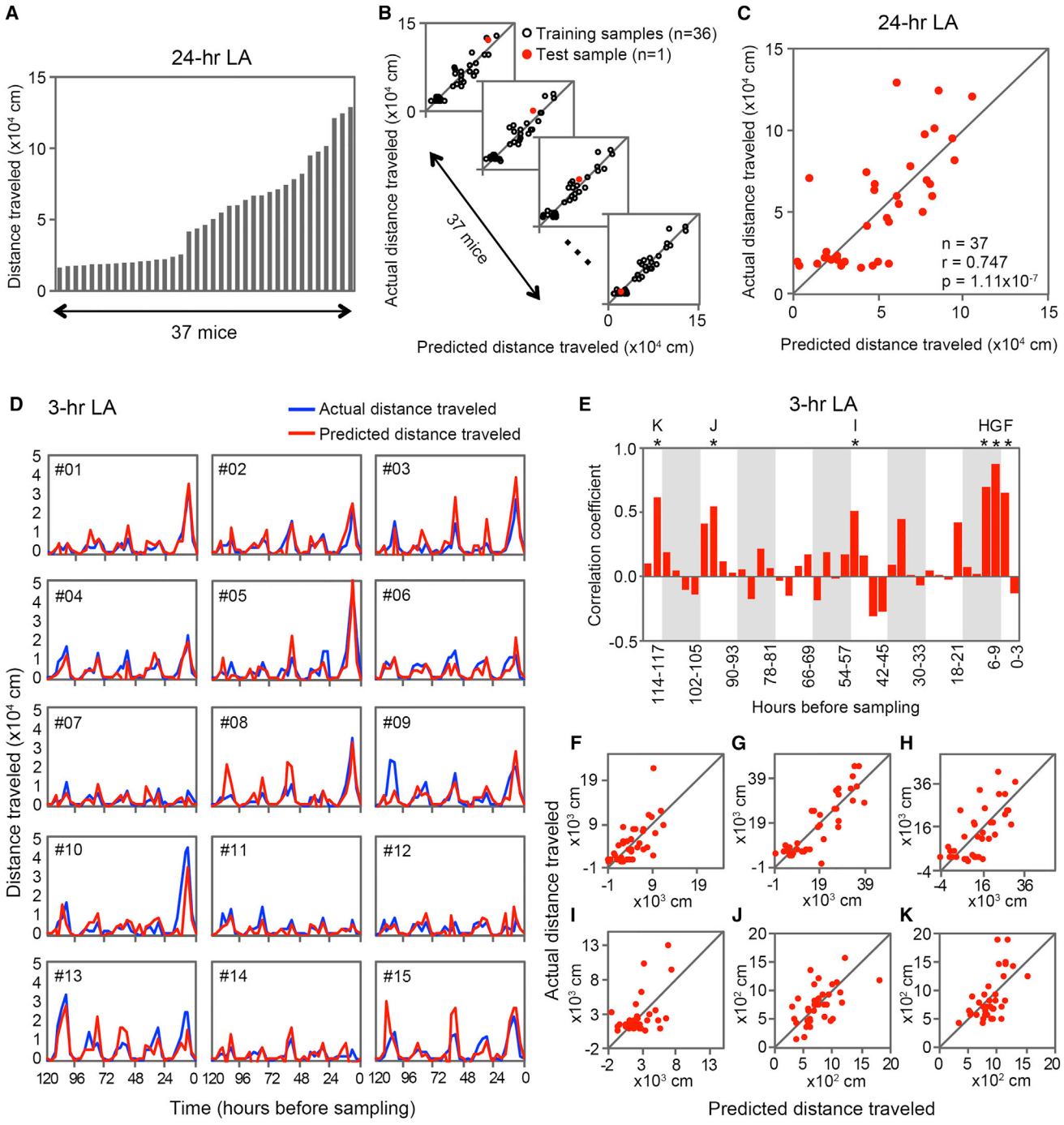


Figure 2. LA of Mice Could Be Retrospectively Predicted from Gene Expression Profiles in the DG

(A) Distribution of 24-hr LA for 37 *Camk2a*-HKO mice.

(B) Illustration of an out-of-sample prediction test.

(C) Prediction of 24-hr LA from gene expression patterns in the DG of *Camk2a*-HKO mice. The scatter plot shows significant correlations between predicted and actual LAs (n = 37 mice, r = 0.747, p = 1.11 × 10^{-7}).

(D) Prediction of 3-hr LAs during the 5 days before sampling. The prediction results of 15 mice are shown; the results of the remaining 22 mice are shown in Figure S1.

(E) Correlation coefficients between the actual and the predicted 3-hr LAs at each time window. Asterisks indicate statistically significant correlations between actual and predicted 3-hr LAs after Bonferroni correction ($p < 0.00125 = 0.05/40$).

(F–K) Scatter plots of predicted and actual 3-hr LAs of each mouse at the time windows indicated in (E).

See also Figure S2 and Table S2.

Table 1. Diseases and Pathways Relevant to the Genes Exhibiting High Correlations with 24-hr LA

Rank	Name	Score	Score (p)
Disease			
1	bipolar disorder	49.60	1.17×10^{-15}
2	chronic myelogenous leukemia	38.60	2.40×10^{-12}
3	viral myocarditis	37.11	6.74×10^{-12}
4	mantle-cell lymphoma	33.45	8.53×10^{-11}
5	neuroblastoma	31.51	3.28×10^{-10}
6	metabolic syndrome	30.92	4.92×10^{-10}
7	adult T cell lymphoma/leukemia	30.76	5.51×10^{-10}
8	prostate cancer	26.08	1.41×10^{-8}
9	colorectal cancer	25.44	2.20×10^{-8}
10	viral hepatitis	22.82	1.35×10^{-7}
11	bladder cancer	22.71	1.46×10^{-7}
12	ovarian cancer	21.62	3.11×10^{-7}
13	depression	21.17	4.25×10^{-7}
14	autism	20.93	5.01×10^{-7}
15	small cell lung cancer	20.64	6.13×10^{-7}
16	hepatitis	20.60	6.27×10^{-7}
17	amyotrophic lateral sclerosis	17.41	5.76×10^{-6}
18	osteoporosis	16.97	7.77×10^{-6}
19	hypergranular promyelocytic leukemia	16.37	1.18×10^{-5}
20	pancreatic cancer	15.55	2.09×10^{-5}
20	non-small cell lung cancer	15.55	2.09×10^{-5}
Pathway Based on Molecule			
1	p160 SRC signaling pathway	78.40	2.51×10^{-24}
2	Transcriptional regulation by SMAD	69.56	1.15×10^{-21}
3	ING signaling pathway	59.53	1.20×10^{-18}
4	Transcriptional regulation by androgen receptor	57.59	4.62×10^{-18}
5	Mst (Hippo) signaling pathway	51.38	3.41×10^{-16}
6	Bcl-2 family signaling pathway	50.63	5.75×10^{-16}
7	Arrestin signaling pathway	50.38	6.81×10^{-16}
8	Transcriptional regulation by CREB	50.06	8.52×10^{-16}
9	HSP90 signaling pathway	49.52	1.24×10^{-15}
10	MAPK signaling pathway	48.27	2.95×10^{-15}
11	Transcriptional regulation by NFAT	47.67	4.45×10^{-15}
12	Transcriptional regulation by high mobility group protein	46.49	1.01×10^{-14}
13	AKT signaling pathway	46.16	1.27×10^{-14}
14	PIN1 signaling pathway	44.61	3.72×10^{-14}
15	Huntingtin signaling pathway	44.45	4.16×10^{-14}
16	Transcriptional regulation by STAT	42.80	1.31×10^{-13}
17	Nucleophosmin signaling pathway	41.76	2.69×10^{-13}
18	Transcriptional regulation by p53	39.87	9.95×10^{-13}
19	CDK inhibitor signaling pathway	37.14	6.61×10^{-12}
20	Transcriptional regulation by FOXO	35.47	2.11×10^{-11}

levels were positively correlated with 24-hr LA had circadian phase values less than 12, during which the peak time of diurnal oscillation of gene expression appeared less than 12 hr after lights on, and genes with negative correlation had circadian phase values greater than 12, with the exception of Acat2, in which the peak time appeared more than 12 hr after lights on (Figure 3C). Moreover, the correlation between expression levels of some circadian genes and 3-hr LA showed circadian-like oscillation (Figures S3A–S3D). The correlation curves were similar to the circadian oscillations of these gene expression patterns in the mouse brain and pituitary (Figures S3E–S3H; http://bioinf.itmat.upenn.edu/circa/). These findings suggested that the transcriptional regulatory machineries underlying variations in the expression levels of these genes at the same time each day among mutant mice may be partially shared with those generating the circadian rhythm of these genes. These findings led us to postulate that transcriptional regulatory machineries underlying the infradian rhythm of these genes' expression may be partially shared with those underlying the circadian rhythm.

cAMP and pCREB Levels in the DG Are Correlated with LA during Infradian Oscillation

Intracellular cyclic AMP (cAMP) in the mouse suprachiasmatic nuclei shows circadian oscillation and regulates clock gene transcription via activation of the cAMP response element-binding protein (CREB; Harrisingh and Nitabach, 2008; O'Neill et al., 2008). Abnormalities in CREB signaling in neurons have been suggested to be involved in bipolar disorder according to a meta-analysis of genome-wide association study (GWAS) datasets (Nurnberger et al., 2014). Our bioinformatics analysis using KeyMolnet for the genes exhibiting high correlations with 24-hr LA showed significant enrichment in the transcriptional regulation by the CREB pathway (Table 1). Levels of cAMP in the DG were negatively correlated with 24-hr LA (Figure 3D). In the mouse hippocampus, cAMP exhibits circadian oscillation, with lower levels at night and higher levels during the day (Eckel-Mahan et al., 2008). Extrapolating the findings to infradian oscillatory LA in *Camk2a*-HKO mice supports the idea that cAMP in the hippocampal DG shows infradian oscillations in these mutants, that is, high or low cAMP in mice at low or high LA states, respectively.

Next, we examined the correlations between the expression levels of genes related to regulation of cAMP synthesis and 24-hr LA. G protein-coupled receptors (GPCRs) modulate cAMP synthesis, and G protein-linked signaling has been implicated in mood regulation in the human brain (Tomita et al., 2013). Therefore, we searched for GPCR genes in the list of genes exhibiting correlations with 24-hr LA by querying GPCR gene lists (Hinckley et al., 2005; Wong, 2003) and found 12 GPCR genes (13 transcripts) in our gene list. Of the 13 transcripts, 6 and 7 were encoded by Gs- and Gi-coupled GPCR genes, respectively (Table S3). Of the six transcripts encoded by Gs-coupled GPCR genes, five were negatively correlated with 24-hr LA, which was significantly more than expected ($\chi^2(1) = 4.27$, $p = 0.039$). In addition, the list of genes exhibiting correlations with 24-hr LA ($p < 0.05$) included three members of the phosphodiesterase (PDE) family (*Pde1c*, 1444067_at, $r = 0.376$; *Pde10a*, 1419390_at, $r = 0.363$; and *Pde11a*,

Table 2. IEGs Whose Expression Levels Were Correlated with LA during the Time Windows of Interest at a Significance Level of $p < 0.05$

	Correlation with 24-hr LA			Correlation with 3-hr LA Immediately before Sampling			Correlation with LA during 60 min Immediately before Sampling		
Gene Symbol	Probe ID	C.C.	p Value	Probe ID	C.C.	p Value	Probe ID	C.C.	p Value
Arc	NA			NA			1418687_at	0.648	0.000014
Bdnf	NA			NA			NA		
Egr1	NA			NA			NA		
Egr2	NA			1427682_a_at	0.392	0.0165	NA		
Fos	NA			1423100_at	0.461	0.0041	1423100_at	0.629	0.00003
Homer1	NA			NA			1425710_a_at	−0.367	0.0254
Homer2	NA			NA			NA		
Jun	1417409_at	−0.409	0.012	NA			NA		
Junb	NA			NA			1415899_at	0.566	0.0003
Narp	NA			NA			1420720_at	0.498	0.0017
Npas4	NA			NA			NA		
Nr4a1	NA			NA			1416505_at	0.396	0.0152
Nr4a2	NA			1450749_a_at	0.325	0.0494	NA		
Nrn1	NA			NA			NA		
Rgs2	NA			NA			1447830_s_at	−0.431	0.0077

C.C., correlation coefficient; p value, p value of correlation; NA, not applicable.

1432999_at, r = 0.371; Table S1), which can downregulate intracellular cAMP (Bender and Beavo, 2006; Soderling and Beavo, 2000; Wayman et al., 2005). The expression levels of these three PDE genes were positively correlated with 24-hr LA, suggesting that relatively high or low expression of these PDE genes may result in low or high cAMP levels during high or low LA states, respectively.

We also found that CREB phosphorylation levels in the DG were negatively correlated with 24-hr LA (Figure 3E), with high or low levels in the dentate granule cells of mice at low or high LA states, respectively (immunoreactivity of phosphorylated cAMP response element-binding protein, or pCREB, normalized to CREB in the granule cell layer, a.u.: 0.61 ± 0.058 in the low LA group, 0.43 ± 0.033 in the high LA group, means ± SEMs, n = 4 mice in each group, p = 0.038; Figure 3F). The pCREB is predominantly expressed in NeuN-positive granule cells in the DG (Figure 3G). Considering that transcriptions of some circadian genes are directly or indirectly regulated by cAMP and CREB (O'Neill et al., 2008; Valor et al., 2010; Zhang et al., 2005), our results suggested that alterations in the expression of the cAMP/CREB pathway-related molecules could orchestrate the expression of some circadian genes during infradian oscillation of LA in DG granule cells. Previous findings showing that genetic and pharmacological manipulations, which could decrease CREB phosphorylation, result in hyperlocomotion in rodents (Einat et al., 2003; Prickaerts et al., 2006) are consistent with this idea.

Chronic Treatment of *Camk2a*-HKO Mice with a Mood Stabilizer Alters Their LAs with Concomitant Changes in pCREB Expression Levels in the DG

To examine whether infradian oscillatory LA and pCREB expression in the DG respond to mood stabilizer treatment, we treated *Camk2a*-HKO mice with lamotrigine and carbamazepine, which exert antidepressive effects (Calabrese et al., 1996; Kusumakar and Yatham, 1997) and antimanic effects (Okuma et al., 1979; Weisler et al., 2005), respectively, in the context of bipolar disorder. Lamotrigine has been reported to inhibit glutamate release (Lee et al., 2008) and attenuate CREB phosphorylation (Ginty et al., 1993) in the rodent brain. Subchronic carbamazepine treatment has been shown to increase pCREB levels in neuronal cell culture (Mai et al., 2002). In *Camk2a*-HKO mice, chronic lamotrigine treatment was found to increase home cage LA (Figures 4A and 4B) with a concomitant trend toward decreased pCREB expression in the DG (Figure 4C) compared to controls. Chronic carbamazepine also decreased home cage LA (Figures 4D and 4E), with a concomitant increase in pCREB expression in the DG (Figure 4F) compared to controls. Our findings were consistent with previous observations showing the effects of mood stabilizers on pCREB (Lee et al., 2008; Ginty et al., 1993; Mai et al., 2002). These results, showing that LAs of *Camk2a*-HKO mice responded to mood stabilizer treatment at clinically relevant doses, support the predictive validity of these mutant mice as a model of mood disorders. Furthermore, our results showing that drugs affecting pCREB expression resulted in changes in LA support the idea that pCREB levels in the brain may modulate LA.

Correlations between Home Cage LA and the Expression of Circadian Genes in the DG of *Schnurri-2*-KO Mice

Finally, we examined whether the correlations between gene expression patterns in the DG and LA states are specific to *Camk2a*-HKO mice. To address the issue, we evaluated correlations between these parameters using *schnurri-2* knockout

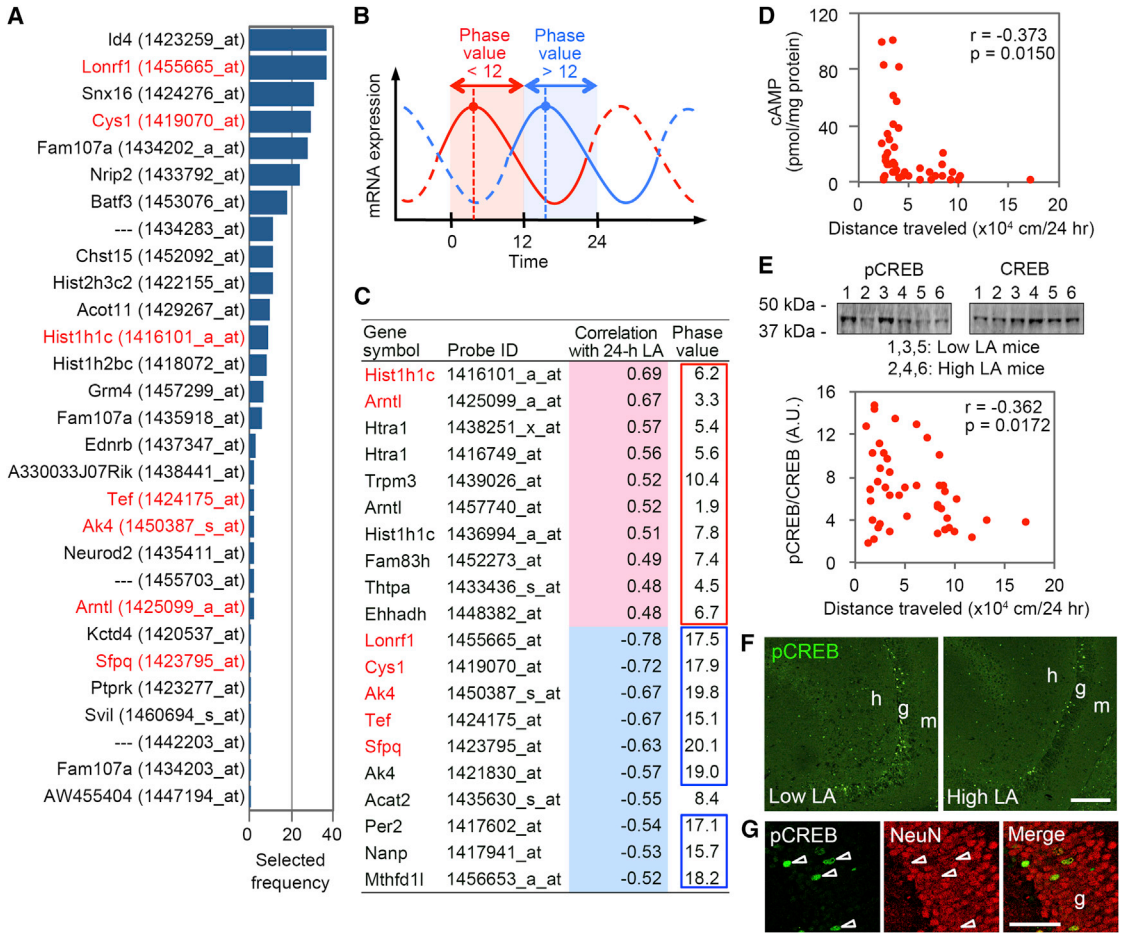


Figure 3. Expression Levels of Circadian Genes and cAMP/CREB Pathway Were Altered with Changes in LA during Infradian Oscillation

(A) Genes used to predict 24-hr LA. Genes shown in red represent circadian or circadian-regulated genes presented in the Photoperiodism database.

(B) Phase values in Figure 2C represent the peak times of circadian gene expression. The red and blue lines show the expression patterns of genes with circadian phase values less than and greater than 12, respectively.

(C) Circadian genes in the list of genes exhibiting correlations with 24-hr LA ($p < 0.01$). Circadian phase values less than and greater than 12 are outlined with red and blue lines, respectively.

(D) Scatter plot showing correlations between 24-hr LA and cAMP concentrations in the DG. The y axis is the cAMP concentration, obtained by ELISA analysis and normalized to total protein amount.

(E) Top: representative blot images for pCREB and CREB from mice at low LA status (distance traveled: approximately 1.8×10^4 to 2.0×10^4 cm/24 hr) and high LA status (distance traveled: approximately 9.5×10^4 to 1.3×10^5 cm/24 hr). Bottom: scatter plot showing correlations between 24-hr LA and pCREB level in the DG. The y axis is the pCREB level normalized to total CREB expression, obtained by immunoblot analysis.

(F) Distribution of pCREB-positive cells in the DG of mice with low and high LA (see Experimental Procedures for details). g, granule cell layer; h, hilus; m, molecular layer. Scale bar, 200 μm.

(G) Double immunostaining for pCREB, along with NeuN, in the granule cell layer. Arrowheads indicate examples of double-positive cells. Scale bar, 50 μm.

See also Figure S3 and Table S3.

(*Shn2*-KO) mice and wild-type mice (Figure 5). We previously reported that *Shn2*-KO mice show increased LA in the home cage (but without clear infradian oscillation; Takao et al., 2013). In *Shn2*-KO mice, the expression levels of 1,388 transcripts (1,250 genes) were correlated with 24-hr LA ($p < 0.01$; Figure 5B; Table S4). Genes exhibiting correlations with 24-hr LA ($p < 0.01$) included a significantly larger number of circadian genes than chance level (57 of 1,388 transcripts, $\chi^2(1) = 15.73$, $p = 7.29 \times 10^{-5}$; Table S4), although the gene expression patterns did not accurately predict 24-hr LA with the method used for predicting that of *Camk2a*-HKO mice (data not shown). Similarly, in wild-type mice, we found that expression levels of 285 transcripts (283 genes) were correlated with 24-hr LA ($p < 0.01$; Figure 5B; Table S4); however, the gene expression patterns could not predict 24-hr LA. In contrast to *Shn2*-KO mice, genes exhibiting correlations with 24-hr LA ($p < 0.01$) did not include a significantly larger number of circadian genes than chance level in wild-type mice (9 of 285 transcripts, $\chi^2(1) = 0.58$, $p = 0.45$; Table S4). A meta-analysis of pathway enrichment analysis using NextBio (Hagihara et al., 2014; Takao and Miyakawa, 2015), in which two gene lists of 24-hr LA-correlated genes in *Camk2a*-HKO mice and *Shn2*-KO mice

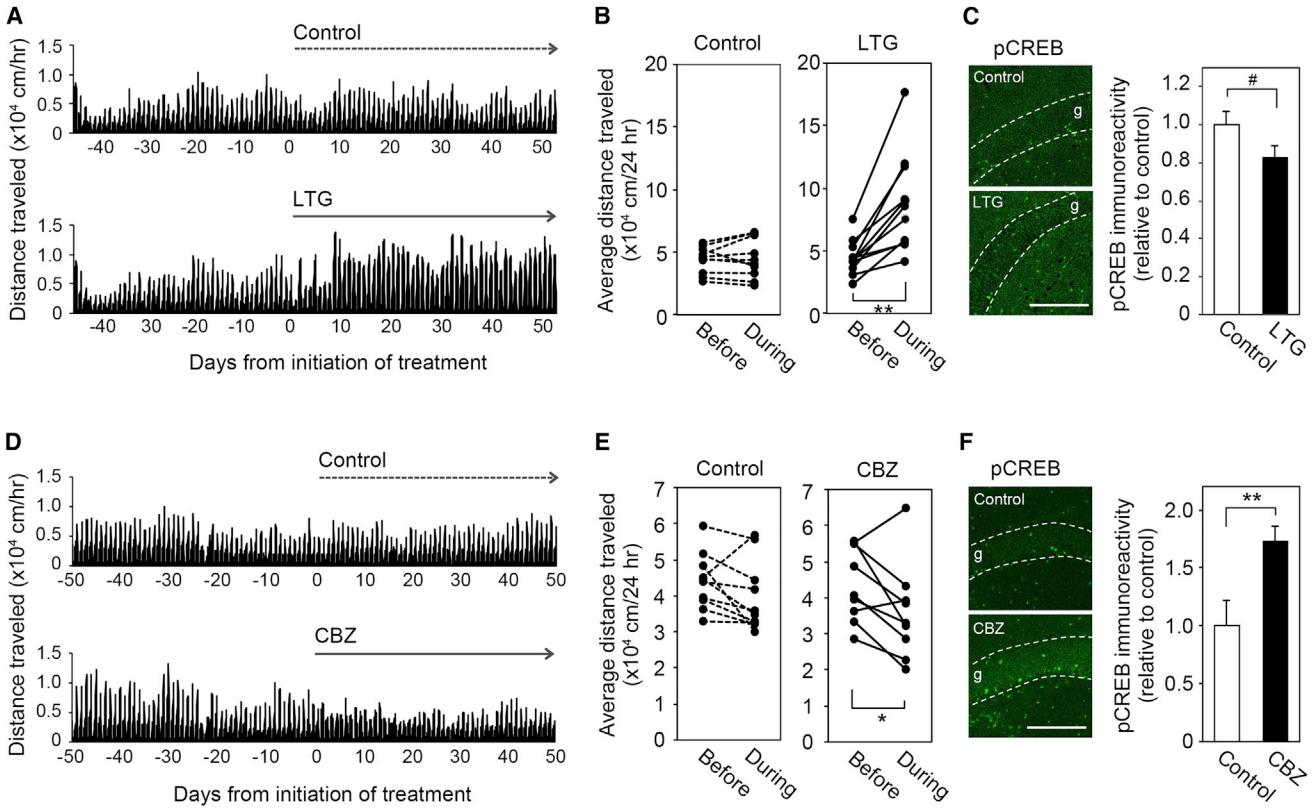


Figure 4. Chronic Treatment of *Camk2a*-HKO with Mood Stabilizers Altered Their LAs with Concomitant Changes in pCREB Expression in the DG

(A) Home cage LAs of control (an average of 10 mice) and lamotrigine (LTG)-treated mice (an average of 11 mice).

(B) Effects of LTG treatment on LA. The average LAs during the period 45 days before and after the initiation of treatment were compared within individual mice. **$p < 0.01$, two-tailed paired t test.

(C) Effects of LTG treatment on pCREB levels in the DG. g, granule cell layer. Scale bar, 200 μm. Bars represent mean ± SEM. #$p < 0.1$, Student's t test.

(D) Home cage LAs of control (an average of ten mice) and carbamazepine (CBZ)-treated mice (an average of nine mice).

(E) Effects of CBZ treatment on LA. The average LAs during the period 50 days before and after the initiation of treatment were compared within individual mice. *$p < 0.05$, two-tailed paired t test.

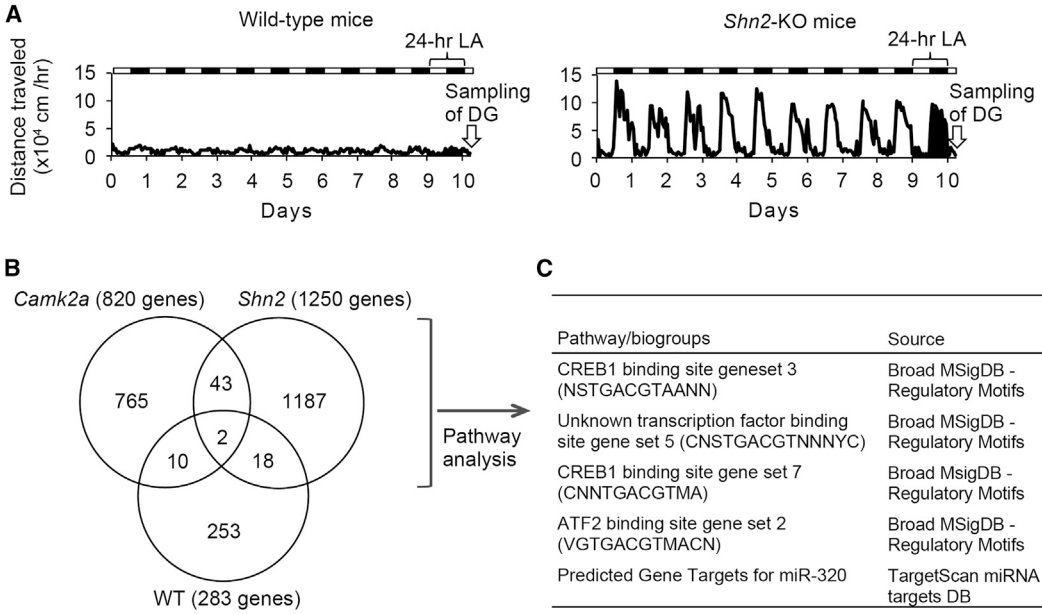
(F) Effects of CBZ treatment on pCREB levels in the DG. g, granule cell layer. Scale bar, 200 μm. Bars represent mean ± SEM. **$p < 0.01$, Student's t test.

were integrated, again identified CREB-related signaling pathways (Figure 5C).

DISCUSSION

In this study, we demonstrated that gene expression patterns in the DG can retrospectively predict states of infradian rhythm behavior, which were represented by 24-hr LA (LA of the time window from 6 to 30 hr before sampling; Figure 2C). One might expect that this result simply relies on the gene expressions affected by LA immediately before sampling. However, the expressions of some major immediate-early genes (IEGs; e.g., *Fos*, *Egr2*, and *Nr4a2*; McNulty et al., 2012; VanElzakker et al., 2008) were correlated with the 3-hr LA immediately before sampling but not with the 24-hr LA (Table 2). In addition, none of these IEGs were used in the 24-hr LA prediction model (Figure 3A). Moreover, in the prediction analysis of 3-hr LA, gene expression patterns in the DG could not predict LA during the 3 hr immediately before sampling (Figure 2E), indicating that LA immediately before sampling is not a major factor determining the gene expression patterns in this brain region. These findings indicate that our microarray data used for LA predictions do not reflect LA immediately before sampling. Thus, gene expression patterns in the DG also hold information about behavioral states during specific time windows that are several hours or days apart from the sampling time.

Another main finding in this study is that circadian genes exhibit infradian expression changes in the brain of *Camk2a*-HKO mice. A recent study demonstrated that expressions of a substantial number of genes, including circadian genes, display annual periodicities in human white blood cells (Dopico et al., 2015), demonstrating a long infradian periodicity of circadian gene expressions. This circannual rhythm of gene expressions was suggested to be human environmental adaptation, because such gene expression levels were correlated with temperature and sunlight hours (Dopico et al., 2015). In the present study, however, mice were maintained in the condition of constant temperature and light-and-dark hours, suggestive of internally generated rhythm of gene expressions in an infradian manner



Pathway/biogroups	Source	Overall Biogroup score
CREB1 binding site geneset 3 (NSTGACGTAANN)	Broad MSigDB - Regulatory Motifs	36.1
Unknown transcription factor binding site gene set 5 (CNSTGACGTNNNYC)	Broad MSigDB - Regulatory Motifs	34.8
CREB1 binding site gene set 7 (CNNTGACGTMA)	Broad MsigDB - Regulatory Motifs	32.0
ATF2 binding site gene set 2 (VGTGACGTMACN)	Broad MSigDB - Regulatory Motifs	28.5
Predicted Gene Targets for miR-320	TargetScan miRNA targets DB	28.4

Figure 5. Correlations between Home Cage LA and Gene Expression Patterns in the DG of *Shn2*-KO Mice

(A) After monitoring home cage LA, the DG of wild-type mice (n = 9) and *Shn2*-KO mice (n = 6) sampled ZT6–ZT7 were processed for microarray analysis.

(B) Genes exhibiting significant correlations with 24-hr LA ($p < 0.01$) were partly overlapped among the three strains of mice. WT, wild-type.

(C) Meta-analysis identified the canonical pathways or biogroups over-represented in the genes exhibiting significant correlations with 24-hr LA ($p < 0.01$) in *Camk2a*-HKO and *Shn2*-KO mice using NextBio. The top five pathways or biogroups are shown.

See also Table S4.

in these mice. Among the circadian genes Dopico et al. (2015) examined, circannual expression rhythm of *ARNTL* (*BMAL1*) in human white blood cells was replicated in all five independent cohorts they examined, in which *BMAL1* expression was higher in summer than in winter. Considering that physical activity of humans is higher in summer than in winter (Hesketh et al., 2014), *BMAL1* expression in human white blood cells may be positively correlated with physical activity during a long seasonal infradian rhythm, which is in accordance with our result showing positive correlation between *Bmal1* expression in the DG and 24-hr LA (Figure 3C; Table S1). Regarding the relationships between Bmal1 expression in the CNS and LA, mice lacking *Bmal1* specifically in the CNS showed lower home cage LA compared with control mice (Mieda and Sakurai, 2011). This was also consistent with our results.

BMAL1 has been shown to be associated with bipolar disorder (Mansour et al., 2006; Nievergelt et al., 2006). Deficiencies in *Bmal1* and *Clock*, a circadian gene regulated by Bmal1 protein (Kondratov et al., 2003), alter sleep patterns in mice (Laposky et al., 2005; Naylor et al., 2000), and sleep disturbances are considered a symptom of the disorder (Jackson et al., 2003). *CLOCK* has also been suggested as one of the susceptibility genes for bipolar disorders from a multi-locus interaction analysis (Shi et al., 2008). The expression of *Bmal1* and *Clock* in the DG of *Camk2a*-HKO mice was correlated with 24-hr LA with the same direction of correlation (Table S1), suggested that sleep patterns may be altered with changes in LA states in these mice.

We also found that cAMP and pCREB levels in the DG were negatively correlated with LA. It has been suggested that manipulations leading to downregulation of CREB activation cause increased LA in rodents (Einat et al., 2003; Prickaerts et al., 2006). Rats treated with an inhibitor of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (Einat et al., 2003), a positive regulator of CREB, and transgenic mice overexpressing *Gsk3* (Prickaerts et al., 2006), which inhibits CREB (Salas et al., 2003), exhibited elevated LA compared to control animals. In addition, it has been reported that deficiency of *Pde10a*, a molecule that can downregulate intracellular cAMP (Soderling and Beavo, 2000) and whose expression is positively correlated with 24-hr LA (Table S1), causes reduced LA (Sano et al., 2008; Siuciak et al., 2006). These findings are consistent with our results showing negative correlations between cAMP and pCREB levels and LA during infradian rhythm behavior.

Abnormalities in CREB-related pathways have been implicated in mood disorder (Coyle and Duman, 2003; Nestler et al., 2002; Nurnberger et al., 2014). A meta-analysis integrating four independent datasets of GWASs also identified signaling pathways related to protein kinase A (PKA), a cAMP-dependent protein kinase that phosphorylates CREB, and CREB as the over-represented pathways for bipolar disorder (Nurnberger et al., 2014). In addition to the cAMP/PKA-mediated CREB pathway, the protein kinase B (AKT) pathway, and the ras-MAPK pathway (including ERK), other signaling pathways upstream of CREB are thought to be involved in mood disorders, as shown by pharmacological studies on mood stabilizers (Coyle and Duman, 2003). Our bioinformatics analysis also showed that genes exhibiting high correlations with 24-hr LA were involved in the MAPK and AKT signaling pathways (Table 1), suggesting that

such pathways and the cAMP-mediated pathway may be involved in mood change-like behaviors in *Camk2a*-HKO mice. Downstream of CREB, although interleukin-6 and FOSB have been implicated in mood disorders (Krishnan and Nestler, 2008), such genes were not found in the results of our analyses. Instead, a signaling pathway related to the CREB target gene *Bcl2*, which is also affected by mood stabilizers (Coyle and Duman, 2003), was enriched in the genes exhibiting high correlations with 24-hr LA (Table 1). These results suggested that some upstream and downstream pathways of CREB implicated in mood disorders were related to infradian oscillatory LA in *Camk2a*-HKO mice.

Previous studies on human and animal models indicated an association of CAMK2 genes with mood disorders, including bipolar disorder (Ament et al., 2015; Le-Niculescu et al., 2009; Robison, 2014; Shin et al., 2013; Xing et al., 2002; Yamasaki et al., 2008). Decreased expression of *Camk2a* mRNA has been reported in postmortem analysis of the brain from patients with bipolar disorder (Xing et al., 2002). Hyperexcitability of DG neurons, which we identified in *Camk2a*-HKO mice in previous studies (Hagihara et al., 2013; Yamasaki et al., 2008), has been suggested in patients with bipolar disorder by using iPSC technology (Mertens et al., 2015). Moreover, in the present study, we found that infradian oscillatory LA of *Camk2a*-HKO mice responded to mood stabilizer treatment (Figure 4). Thus, these previous reports, together with the present study, indicated that *Camk2a*-HKO mice displayed construct, face, and predictive validity and may be useful in elucidating the pathogenesis and pathophysiology of bipolar disorder.

In addition to *Camk2a*-HKO mice, we found correlations between LA and gene expression in the DG by simple correlation analysis in *Shn2*-KO mice, which showed hyperactivity in the home cage. Knockdown of *Shn2*, a major histocompatibility complex enhancer binding protein, causes atypical inflammation and associated hippocampal and cortical abnormalities, which may induce a series of schizophrenia-related behavioral abnormalities in mice (Takao et al., 2013). As observed in *Camk2a*-HKO mice, circadian genes were concentrated in the genes exhibiting significant correlations with 24-hr LAs in *Shn2*-KO mice. These results suggested that LA-related gene regulation in the DG may be partly common among these mice.

Accumulating evidence from human studies, including volumetric and functional evaluations using MRI, has suggested that the hippocampus is a core region for mood disorders (Bertolino et al., 2003; Brown et al., 1999; Campbell et al., 2004; Chen et al., 2010; Femenía et al., 2012; Nestler et al., 2002). In addition, adult neurogenesis in the hippocampal DG has been suggested to have an important role in the pathophysiology of mood disorders and in mediating the response to antidepressants (Duman et al., 2001; Samuels and Hen, 2011). In this context, we examined the relationships between gene expression patterns in the DG and infradian oscillatory LA. We found significant correlations between expression of some circadian genes and behaviors in the present study. However, it will be interesting to examine whether the expression of circadian genes shows infradian rhythms in other brain regions, such as cornu ammonis (CA) areas in the hippocampus and the bed nucleus of the stria terminalis, in which, similar to the DG, abnormal cellular activities have been found in *Camk2a*-HKO mice from an in vivo manganese-enhanced MRI study (Hattori et al., 2013).

In conclusion, our results showed that statistical learning models using gene expression patterns in the DG can predict the behavioral state of individual mice showing exaggerated infradian rhythm. This is the first demonstration, to our knowledge, of successful quantitative predictions of the individual behavioral state from molecular expression patterns in the brain. Moreover, informatics analyses of genes used in the prediction models showed concomitant changes in the expression levels of multiple circadian genes in the brain and states of infradian rhythm behavior, providing the evidence for a novel concept that some circadian genes may be involved in the generation of infradian rhythm behavior. Further studies are needed to evaluate relationships between these molecules and infradian rhythm behavior.

EXPERIMENTAL PROCEDURES

Animals

We used *Camk2a*-HKO mice (Yamasaki et al., 2008), *Shn2*-KO mice (Takao et al., 2013), and wild-type mice. All animal experiments were approved by the Institutional Animal Care and Use Committee of Fujita Health University, based on the Law for the Humane Treatment and Management of Animals and the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain. Every effort was made to minimize the number of animals used.

LA Monitoring in the Home Cage and Sampling

LA monitoring in the home cage was performed with adult male mice over 8 weeks of age as previously described (Yamasaki et al., 2008). In *Camk2a*-HKO mice, LAs in their home cage gradually and greatly change over time, with an approximate cycle length of 10–20 days (Yamasaki et al., 2008). To ensure variations in LAs of the sampled mice, up to eight mice with short or long distances traveled (assessed by distance traveled during the 24 hr before ZT0 on the sampling day) were preferentially sampled ZT6–ZT7 of the sampling day. This process was repeated as needed. To minimize the influence of individual differences in LAs immediately before sampling on the gene expression patterns in the DG, we sampled ZT6–ZT7, before which the mutant mice showed relatively similar LAs.

Open Field Test and Porsolt Forced Swim Test

An open field test and the Porsolt forced swim test were conducted as previously described (Takao et al., 2013; Yamasaki et al., 2008). See the Supplemental Information for details.

Lamotrigine and Carbamazepine Treatment

Lamotrigine was administered in drinking water to *Camk2a*-HKO mice, aiming at 30 mg/kg/day. Carbamazepine was administered in the diet to *Camk2a*-HKO mice at dietary levels of 0.75%. See the Supplemental Information for details.

Expression Microarray Analysis

Dissection of mice DG (Hagihara et al., 2009) and microarray experiments (Takao et al., 2013; Yamasaki et al., 2008) were performed as described previously. See the Supplemental Information for details. The microarray data were deposited in the GEO database under accession number GEO: GSE68293.

Cross-validated LA Prediction

Leave-two-out cross-validation prediction of LA (24- and 3-hr LAs) was performed with MATLAB using $\log_2$-transformed gene expression values for individual mice. See the Supplemental Information for details.

Bioinformatics Analysis of Gene Expression Data

Bioinformatics analyses of the lists of genes exhibiting correlations with 24-hr LAs were performed using KeyMolnet (KM Data), DAVID (https://david.ncifcrf.gov/), or NextBio (Illumina). See the Supplemental Information for details.

Querying the Database for Circadian Gene Expression Patterns

To determine how many circadian genes were included in our gene list, we first queried the Photoperiodism database, which presents genes showing circadian expression patterns within a 24-hr period in short-day and long-day conditions in the adult mouse pars tuberalis (Masumoto et al., 2010). We considered the genes in our lists to be circadian genes when we found the genes in the database by searching with the Affymetrix probe set IDs (Figure 3; Tables S1, S2, and S4). Phase values in Figure 3C referred to those from the short-day condition. Expression patterns of the circadian genes were obtained from the CircaDB database. We searched the database with gene symbols and used data describing circadian expression patterns in the mouse brain and pituitary (Figure S3).

Measurement of cAMP Concentrations

Measurement of cAMP concentrations in the DG was performed with another set of mice (n = 42) that was used for microarray analysis. The isolated DG (Hagihara et al., 2009) was sonicated in 50 μl of 0.1 M HCl, and the suspension was centrifuged at 1,000 × *g*. The supernatant was diluted at 1:500 and used to measure cAMP levels according to the manufacturer's instructions (Cayman Scientific). Protein concentrations were measured using Bradford assays (Thermo Scientific). Data obtained from two independent experiments were combined and shown in Figure 3D.

Immunological Detection of pCREB

The isolated DG (Hagihara et al., 2009) was homogenized and separated by gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with anti-pCREB antibody (Cell Signaling Technology, 9198, 1:1,000), rabbit anti-CREB antibody (Cell Signaling Technology, 9197, 1:1,000), or mouse anti-β-actin antibody (Sigma-Aldrich, A5316, 1:30,000). Immunoreactivity was quantified using ImageMaster software (GE Healthcare). This analysis was performed with a set of mice (n = 43) that was partially overlapped with the set used in cAMP measurement analysis. Data obtained from three independent experiments were combined and shown in Figure 3E.

A separate set of mice was used for immunohistochemical analyses (Figures 3 and 4). Mice with high LA (distance traveled: approximately 9.0×10^4 to 1.2×10^5 cm/24 hr; n = 4) and low LA (distance traveled: approximately 1.5×10^4 to 2.7×10^4 cm/24 hr; n = 4) were processed for immunohistochemical analyses (Figure 3F). Frozen sections were probed with rabbit anti-pCREB antibody (1:100), anti-CREB antibody (1:2,000), or mouse anti-NeuN antibody (Millipore, MAB377, 1:100). Quantification of the immunofluorescence intensities of CREB and pCREB was performed using ZEN software (Zeiss). See the Supplemental Information for details.

Statistical Analysis

Chi-square values were calculated from the differences between the observed and the expected number of genes. Significance was defined as a p value of less than 0.05. Immunohistochemical data were analyzed with unpaired Student's t test, and differences were considered significant when the p value was less than 0.05.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO: GSE68293.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.067.

AUTHOR CONTRIBUTIONS

H.H., H.K.N., J.U., and H.S. performed behavioral experiments and sampling of brain. H.H., H.K.N., and J.U. performed molecular experiments. T.H., H.K.N., and Y.K. performed prediction analyses. H.H., T.H., H.K.N., H.S., Y.K., and T.M. wrote and edited the manuscript. H.H. and T.M. designed the study.

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