



**HAL**  
open science

## Gene expression of circadian genes and CIART in bipolar disorder: A preliminary case-control study

Cindie Courtin, Cynthia Marie-Claire, Gregory Gross, Vincent Hennion, Emeline Mundwiller, Justine Guégan, Manon Meyrel, Frank Bellivier, Bruno Etain

### ► To cite this version:

Cindie Courtin, Cynthia Marie-Claire, Gregory Gross, Vincent Hennion, Emeline Mundwiller, et al.. Gene expression of circadian genes and CIART in bipolar disorder: A preliminary case-control study. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2022, 122, pp.110691. 10.1016/j.pnpbp.2022.110691 . inserm-03914243

**HAL Id: inserm-03914243**

**<https://inserm.hal.science/inserm-03914243>**

Submitted on 28 Dec 2022

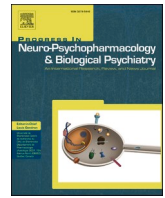
**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Contents lists available at ScienceDirect

# Progress in Neuropsychopharmacology & Biological Psychiatry

journal homepage: [www.elsevier.com/locate/pnp](http://www.elsevier.com/locate/pnp)

## Gene expression of circadian genes and *CIART* in bipolar disorder: A preliminary case-control study

Cindie Courtin<sup>a</sup>, Cynthia Marie-Claire<sup>a,\*</sup>, Gregory Gross<sup>b,c</sup>, Vincent Hennion<sup>a,d</sup>,  
Emeline Mundwiller<sup>e</sup>, Justine Guégan<sup>f</sup>, Manon Meyrel<sup>d</sup>, Frank Bellivier<sup>a,d</sup>, Bruno Etain<sup>a,d</sup>

<sup>a</sup> Université de Paris Cité, INSERM UMR-S 1144, Optimisation Thérapeutique en Neuropsychopharmacologie, OTeN, Paris, France

<sup>b</sup> Pôle Hospitalo-Universitaire de Psychiatrie d'Adultes et d'Addictologie du Grand Nancy, Centre Psychothérapeutique de Nancy, Laxou, France

<sup>c</sup> Université de Lorraine, Vandoeuvre-lès-Nancy, France

<sup>d</sup> Département de Psychiatrie et de Médecine Addictologique, Hôpitaux Lariboisière-Fernand Widal, GHU APHP.Nord – Université de Paris, Paris, France

<sup>e</sup> JGenSeq, Institut du Cerveau, Paris, France

<sup>f</sup> Data Analysis Core platform, Institut du Cerveau - Paris Brain Institute – ICM, Sorbonne Université, Inserm, CNRS, APHP, Hôpital de la Pitié Salpêtrière, Paris, France

### ARTICLE INFO

#### Keywords:

Bipolar disorder  
Gene expression  
Circadian  
*CIART*  
RNA sequencing  
Polymerase chain reaction

### ABSTRACT

Based on the observed circadian rhythms disruptions and sleep abnormalities in bipolar disorders (BD), a chronobiological model has been proposed suggesting that core clock genes play a central role in the vulnerability to the disorder. In this context, the analysis of circadian genes expression levels is particularly relevant, however studies focused on the whole set of core clock genes are scarce. We compared the levels of expression of 19 circadian genes (including the recently described circadian repressor (*CIART*)) in 37 euthymic individuals with BD and 20 healthy controls (HC), using data obtained by RNA sequencing of lymphoblastoid cell lines and validated the results using RT-qPCR. RNA sequencing data showed that *CIART* gene expression was correlated with those of *ARNTL*, *ARNTL2*, *DBP*, *PER2* and *TIMELESS*. Data from RNA sequencing showed that the level of expression of four circadian genes (*ARNTL*, *ARNTL2*, *BHLHE41* and *CIART*) discriminated individuals with BD from HC. We replicated this result using RT-qPCR for *ARNTL* and *CIART*. This study suggests that an imbalance between activation/repression of the transcription within the circadian system in individuals with BD as compared to HC and as such opens avenues for further research in larger independent samples combining both expression and epigenetic analyses.

### 1. Introduction

Bipolar Disorders (BD) affect 1–2% of the adult population and are characterized by recurrent episodes of major depression and (hypo)mania interspaced by periods of remission (i.e. euthymia). Sleep abnormalities and circadian rhythms (CR) disruptions have been identified as predisposing factors to BD (Murray et al., 2020). First, meta-analyses suggested that these altered patterns pre-exist to the onset of BD (Scott et al., 2021; Scott et al., 2022). Second, meta-analyses of studies using self- or clinician-based questionnaires demonstrated that, as compared to healthy controls (HC), euthymic individuals with BD have an evening chronotype (preference for activities in the evening) and lower amplitude, lower flexibility and less synchronization of CR (Meyrel et al., 2021). Studies using actigraphy recordings also suggested abnormalities in timing, amplitude and stability of CR (Krane-Gartiser et al., 2019).

Altogether, these observations have served as bases for a chronobiological model of BD. According to this model, underlying abnormalities of the biological clock lead to greater CR irregularities and sleep disturbances, then to alterations in mood regulation and further to mood episodes, especially when individuals are exposed to life events that can desynchronize CR or disrupt sleep (Harvey, 2008).

This chronobiological model mainly relies on the assumption that some abnormalities of the circadian genes may predispose individuals to altered circadian phenotypes. Core-clock circadian genes (i.e. those expressed in the suprachiasmatic nuclei) interact through complex positive and negative loops to control CR. Genetic variants of these genes have been studied in association with BD, however with inconsistent or un-replicated results (Milhiet et al., 2011; Etain et al., 2014; McCarthy et al., 2021). In this context, the study of the level of expression of circadian genes may offer a complementary approach to

\* Corresponding author.

E-mail address: [cynthia.marie-claire@u-paris.fr](mailto:cynthia.marie-claire@u-paris.fr) (C. Marie-Claire).

<https://doi.org/10.1016/j.pnpbp.2022.110691>

Received 25 August 2022; Received in revised form 18 November 2022; Accepted 30 November 2022

Available online 5 December 2022

0278-5846/© 2022 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

explore the contribution of this particular biological pathway to the susceptibility of BD. Some studies have investigated such an issue, mainly focusing on only one circadian gene (Bengesser et al., 2021), but extremely few have offered a comprehensive study of the whole set of genes (Yang et al., 2009; Zhang et al., 2022). For instance, differential gene expressions have been reported in individuals with BD as compared to HC for *BMAL1*, *NR1D1*, *DBP*, *ARNTL2*, *CRY1*, *CRY2*, *PER2*, or *PER3*, however among non-euthymic participants (mostly during depression). Moreover, the expression levels of certain genes such as *CIART* (Circadian Associated Repressor Of Transcription), recently identified as a component of the circadian clock (Goriki et al., 2014) have not been studied yet in BD.

Gene expression levels are generally analyzed in peripheral blood samples of individuals with BD. However, in the specific case of circadian genes, this requires a very rigorous sample collection conditions and schedules to take into account the circadian cycle of the expression of these genes at the peripheral level. Lymphoblastoid cell lines (LCLs), derived from lymphocytes of patients, are much easier to obtain and to grow in standardized and controlled conditions thus reducing variability (Viswanath et al., 2015). This model has been successfully used for the investigation of core clock genes expression in BD (McCarthy et al., 2011; Etain et al., 2012; Kittel-Schneider et al., 2015; Geoffroy et al., 2018) and therefore represent a useful model to explore the contribution of this set of genes in association with BD.

Therefore, the aim of this study was to compare the levels of expression of 19 circadian genes between euthymic individuals with BD and HC, using data obtained by RNA sequencing of LCLs and validated using RT-qPCR.

## 2. Methods

### 2.1. Sample

The present study is an ancillary analysis of the research protocol (Clinical Trials Number NCT02627404) that investigated the genetic and environmental factors of vulnerability to BD. The sample consisted of Caucasian euthymic individuals with a diagnosis of BD type 1 according to DSM-IV criteria. Euthymia at inclusion was confirmed by scores below 8 for both the Montgomery Asberg Depression rating Scale (MADRS) and the Young mania rating Scale (Young et al., 1978; Montgomery and Asberg, 1979). Caucasian HC were recruited from the general population and had no personal history of mood disorders, schizophrenia, substance use (except nicotine use) and suicidal attempts. The appropriate ethical committee approved the study. All participants provided written informed consent prior to inclusion. Details on inclusion and exclusion criteria have been described previously (Etain et al., 2012).

### 2.2. Cell culture and RNA isolation

A blood sample was drawn from all participants. LCLs (Lymphoblastoid Cell Lines) were established from fresh blood by transforming lymphocytes using standard with Epstein-Barr virus transformation, as previously described (Neitzel, 1986). LCLs were cultured in RPMI-1640 glutamax medium containing 10% fetal bovine serum and 1% gentamycin (Life Technologies, France) in a 5% CO<sub>2</sub> humidified incubator at 37 °C. All procedures were standardized and LCLs from individuals with BD and HC were seeded and collected at the same time of day in parallel. LCLs were seeded at  $2 \times 10^5$  cells/mL. After 4 days, cells were harvested and washed twice with PBS before RNA extraction. Total RNA was extracted, for individuals with BD and HC, from  $2 \times 10^6$  cells pellets using the miRNeasy Mini Kit according to the manufacturer's protocol (QIAGEN, France) and quantified with a NanoDrop One ND-1000 spectrophotometer (ThermoFisher Scientific, Ozyme, France). Total RNAs were stored at -80 °C until processing.

### 2.3. RNA sequencing

RNA sequencing was performed at the Institut du Cerveau (IGenSeq platform - Paris). Samples were randomized and libraries were generated from total RNA using TruSeq Stranded mRNA Kit (Illumina, USA). Paired-end sequencing ( $2 \times 75$  bp) was performed on a NextSeq500 instrument (Illumina, USA). Quality of raw reads was assessed with FastQC49, trimming was done with Trimmomatic39 to cut adapters and exclude reads <40 bp. The processed reads were aligned on human reference genome hg19 with Star v 2.5.3a (Dobin et al., 2013). RSEM (Li and Dewey, 2011) was used to obtain expected read counts and FPKM (Fragments Per Kilobase of transcript per Million mapped reads), which eliminated the influence of different gene length and discrepancy of the library size. Samples types, RNA isolation and sequencing methods were identical for samples from individuals with BD and HC, therefore these data were used for the following analyses (Zhao et al., 2020).

### 2.4. Genes selection

FPKM data for the following circadian genes were selected from the dataset: *ARNTL1* and *ARNTL2* (Aryl hydrocarbon receptor nuclear translocator-like protein), *BHLHE40* and *BHLHE41* (Basic helix-loop-helix family, member E), *CLOCK* (Circadian Locomotor Output Cycles Protein Kaput), *CRY1*, *CRY2* (Cryptochrome Circadian Regulator), *CSNK1D*, *CSNK1E* (Casein Kinase 1 Delta and Epsilon), *DBP* (D-Box Binding PAR BZIP Transcription Factor), *GSK3B* (Glycogen Synthase Kinase 3 Beta), *NR1D1* (Nuclear Receptor Subfamily 1 Group D Member 1), *PER1*, *PER2*, *PER3* (Period Circadian Regulator), *RORA* (RAR Related Orphan Receptor A), *NPAS1* (Neuronal PAS Domain Protein 1) and *TIMELESS* (Timeless Circadian Regulator) (Kavakli et al., 2022). We added to this list *CIART* (Circadian-Associated Transcriptional Repressor) because of its recent characterization as a potential contributor to CR alterations (Yang et al., 2020).

*RORB* (RAR Related Orphan Receptor B), *NPAS2* (Neuronal PAS Domain Protein 2) and *PPARGC1A* (Peroxisome Proliferator Activated Receptor Gamma Coactivator 1 Alpha) were excluded from the analyses because of low expression levels.

### 2.5. Validation using PCR

In order to validate the differential expression of genes identified in the RNA-seq analyses, RT-qPCR were performed on the same samples. One µg of total RNA was reverse transcribed using the iScript™ Reverse Transcription Supermix following the manufacturer's protocol (Bio-Rad laboratories, France). One patient and one HC with no RNA left could not be included in the PCR experiments. Commercially available Prime PCR primers targeting identified genes from RNAseq data analyses were purchased from BioRad laboratories (France). Based on previous results, prime PCR primers targeting *GUSB* and *TBP* were used as reference genes (Curis et al., 2019), as described in the GeNorm method the geometric mean of the two reference genes was used as a normalization factor. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad laboratories) was used for amplification following the manufacturer's instructions. The specificity of PCR products was verified using a melting curve analysis step. Amplifications were performed, in triplicate, on a CFX384 instrument and gene expression levels ( $\Delta\Delta C_q$ ) were calculated using the built-in gene expression mode of the CFX Maestro software (Bio-Rad laboratories).

### 2.6. Statistical analysis

Some of the studied genes had observed distributions in the whole sample (cases and controls) that did not fit a normal distribution. This was the case for *ARNTL*, *BHLHE40*, *BHLHE41*, *CSNK1E*, *DBP*, *GSK3B*, *NR1D1*, *PER1*, *PER3*, and *RORA*. Therefore, we mainly used non-parametric tests for the analyses. Qualitative data were described by

numbers and percentages and quantitative data by medians and interquartiles (IQR). Correlations between variables were investigated using Spearman correlation tests. Comparisons between groups for quantitative data were performed using Mann-Whitney tests. For this main analysis we used a False Discovery rate (FDR) correction for multiple testing. Spearman correlations were performed to analyze covariations between circadian genes and results were visualized as a network. The association between clinical status and gene expression levels was tested using logistic regression, and further adjusted for age, sex, BMI (Body mass Index) and tobacco use. ROC analyses and AUC were generated to investigate the accuracy of the model.

### 3. Results

#### 3.1. Comparison between BD and HC

The sample consisted in 57 individuals (37 with BD type 1 and 20 HC). The groups were similar for sex, age at inclusion, BMI, and current tobacco use (see Table 1). The mean age at onset of BD was around 27 years old. The mean number of lifetime mood episodes was 7.6 (+/- 4.6). At inclusion, most individuals with BD were treated with lithium ( $n = 28$ , 75%), around one third were on anticonvulsants ( $n = 12$ , 32%), and around one quarter were on atypical antipsychotics ( $n = 10$ , 27%). None of the individuals with BD was drug naive.

#### 3.2. Correlation between genes expression

We constructed a network plot in the whole sample ( $N = 57$ ) to visualize the correlations of expression of the 19 genes (threshold for edge = 0.3). As seen in Fig. 1, the network is dominated by two main groups of genes linked with a large number of edges: *DBP*, *ARNTL2*, *GSK3b*, *CLOCK*, *CRY2*, *PER1*, *NR1D1*, *BHLHE40*, *CSNK1E* and *CSNK1D* on the one part and *ARNTL*, *BHLHE41*, *PER2*, *NPAS1*, *CRY1* and *TIMELESS* on the other. The expression level of *CIART* is correlated with both groups. The main correlations between the level of expression of *CIART* and those of other circadian genes were found with *ARNTL* ( $\rho = -0.39$   $p = 0.003$ ) and *DBP* ( $\rho = 0.33$   $p = 0.01$ ). *PER3* and *RORA* were not connected to the two main groups of genes with correlated expression levels.

#### 3.3. Comparison between groups for gene expression

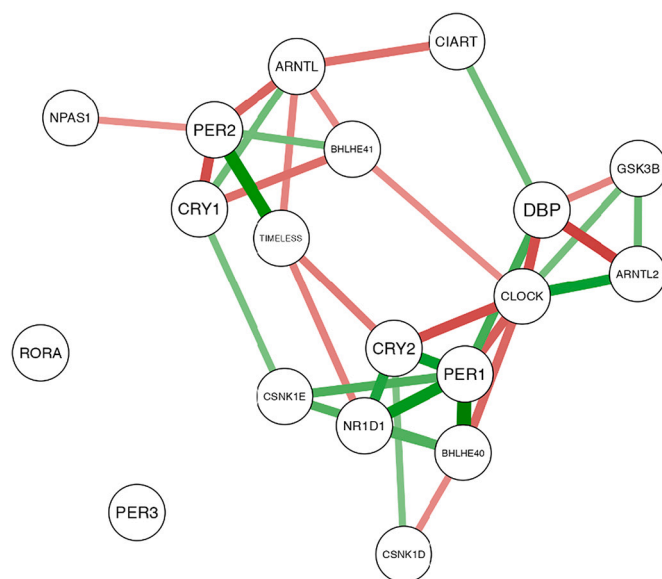
As compared to HC, individuals with BD had significantly lower levels of expression of *ARNTL* ( $p = 0.00006$ ) and of *ARNTL2* ( $p = 0.0003$ ) and higher levels of expression of *CIART* ( $p = 0.00009$ ) and of *BHLHE41* ( $p = 0.003$ ). These differences resisted to the FDR correction. Other associations (not significant after the correction) were found with *BHLHE40* ( $p = 0.015$ ), *RORA* ( $p = 0.038$ ) and *TIMELESS* ( $p = 0.023$ ) (see Table 2).

We used a logistic regression with clinical status as the dependent variable, and the following independent variables: *ARNTL*, *ARNTL2*, *CIART* and *BHLHE41* (model 1 with a stepwise option), then adjusted for covariates (model 2 with a forced entry). Clinical status was associated with *ARNTL*, *ARNTL2*, *CIART* but not with *BHLHE41* (see Table 3), which remained significant after adjustment. We used ROC analyses to

**Table 1**  
group comparison for age, sex, BMI and tobacco use.

Variables	BD group	HC group	p values
Females N (%)	21 (57%)	9 (45%)	0.39
Age at inclusion (median IQR)	47 (42–54)	44 (42–45)	0.37
BMI (median IQR)	26 (23–28)	25 (23–26)	0.45
Tobacco current use N (%)	17 (46%)	5 (28%)*	0.19

\* Two missing variables. BD: Bipolar disorder, HC: healthy control, N: number, IQR: interquartile, BMI: body mass index (kg/m<sup>2</sup>).



**Fig. 1.** Correlation network of levels of expression of circadian genes. The edges of the network refer to Spearman correlation coefficients between gene expression. Only edges with a threshold of 0.30 are drawn.

explore whether each single gene, and then the combination of the three significantly differentially expressed genes, better discriminated BD from HC (see Fig. 2). The AUC was 0.79 for *ARNTL2* ( $p = 3.10^{-4}$ ), 0.82 for *CIART* ( $p = 9.10^{-5}$ ), 0.82 for *ARNTL* ( $p = 6.10^{-5}$ ) and was the highest when combining the three genes (AUC = 0.95  $p = 3.10^{-8}$ ). The increment in the AUC obtained when combining the three genes was significant as compared to the AUC obtained with each single gene ( $p$  values between 0.03 and 0.01 for the increments) (see Fig. 2).

#### 3.4. Validation using PCR

RT-qPCR experiments were performed in the same samples to validate the results observed with RNA-seq. Based on the FDR corrected  $p$ -values obtained with the RNA-seq data, only *ARNTL*, *ARNTL2*, *CIART* and *BHLHE41* were tested. (Table 4). In individuals with BD as compared to HC, we confirmed lower levels of expression of *ARNTL* ( $p = 0.002$ ) and higher levels of expression of *CIART* ( $p < 0.001$ ) and of *BHLHE41* ( $p < 0.001$ ). As seen in Table 4, the log<sub>2</sub> fold change were similar to the ones observed with the RNA Seq data. However, expression levels of *ARNTL2* were similar in patients with BD and HC (0.95 fold change,  $p = 0.55$ ).

We used the logistic regression model adjusted for covariates (Model 2) using the RT-qPCR data. Both *ARNTL* and *CIART* levels of expression were found associated with diagnosis (Table 5). The AUC for this model was 0.97 (0.93–0.99).

### 4. Discussion

This is the first study that explored the level of expression of a comprehensive set of 19 circadian genes, also including the recently described circadian repressor (*CIART*), in individuals with BD as compared to controls. First, we suggest for the first time that *CIART* is co-expressed with other circadian genes in humans. Second, using RNAseq data, we show that the level of expression of four circadian genes (*ARNTL*, *ARNTL2*, *BHLHE41* and *CIART*) discriminated individuals with BD from healthy controls. Third, we replicated the results using RT-qPCR for two of these genes: *ARNTL* and *CIART*.

We constructed a correlation network of 19 circadian genes based on their expression levels in LCLs from 57 individuals. Indeed, the complex interplay between circadian genes at the transcriptional and functional

**Table 2**  
group comparison for the level of expression of 19 circadian genes.

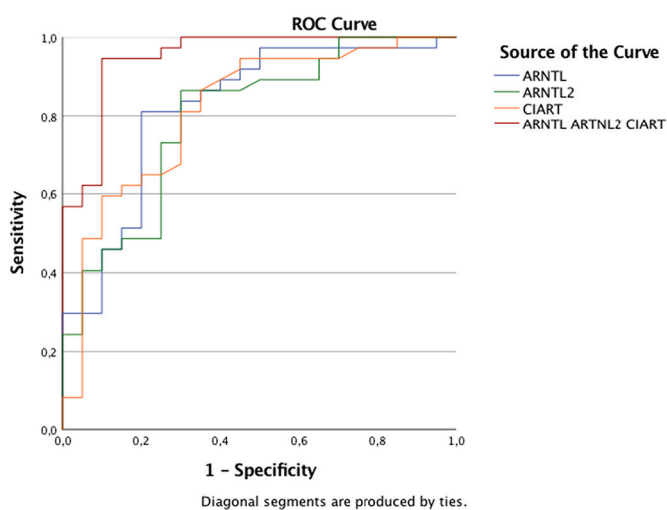
Status	HC		BD		log2 fold change	p values
	Median	IQR	Median	IQR		
ARNTL	10.14	9.04–12.59	7.67	7.16–8.74	−0.40	<b>0.00006</b>
CIART	0.99	0.72–1.47	1.64	1.34–1.92	0.73	<b>0.00009</b>
ARNTL2	36.01	30.77–42.68	30.06	23.97–33.22	−0.26	<b>0.0003</b>
BHLHE41	2.31	1.42–4.71	4.54	2.46–7.18	0.97	<b>0.003</b>
BHLHE40	46.49	29.80–66.81	62.55	51.62–72.26	0.43	0.015
TIMELESS	14.77	12.45–17.65	16.79	14.95–17.75	0.18	0.023
RORA	4.30	2.67–6.58	5.76	4.32–7.13	0.42	0.038
PER2	4.80	4.26–5.39	5.21	4.86–5.54	0.12	0.070
CSNK1E	28.94	25.63–35	32.04	29.91–34.71	0.15	0.074
DBP	5.41	4.72–6.81	6.18	5.61–7.13	0.19	0.075
CSNK1D	30.97	29.6–33.31	30.03	29.07–31.33	−0.04	0.084
PER1	11.08	8.5–15.73	12.98	10.68–15.66	0.23	0.095
CRY1	3.35	2.22–4.76	2.75	1.67–3.47	−0.28	0.098
PER3	0.74	0.55–2.58	1.51	0.68–2.34	1.03	0.173
NR1D1	4.53	3.57–6.17	4.16	3.74–5.04	−0.12	0.259
CLOCK	4.39	4.14–4.62	4.14	3.91–4.76	−0.08	0.266
NPAS1	0.29	0.22–0.44	0.29	0.20–0.39		0.598
GSK3B	21.42	20.88–23.31	21.74	20.66–22.24	0.02	0.738
CRY2	7.63	7.04–8.43	7.77	7.05–8.41	0.03	0.874

In bold, p values that remained significant after a FDR correction.  
Genes are ordered by p values.

**Table 3**  
Comparisons between BD and HC for ARNTL, ARNTL2, CIART without and with adjustment.

Variables	MODEL 1 *					MODEL 2 *				
	B	S.E.	Wald	df	p	B	S.E.	Wald	df	p
ARNTL	−0.72	0.25	8.32	1	<b>0.004</b>	−1.29	0.52	6.29	1	<b>0.01</b>
ARNTL2	−0.29	0.11	6.88	1	<b>0.009</b>	−0.38	0.15	6.45	1	<b>0.01</b>
CIART	3.51	1.38	6.46	1	<b>0.01</b>	6.32	2.45	6.65	1	<b>0.01</b>
Age	−	−	−	−	−	0.21	0.12	3.33	1	0.07
Sex	−	−	−	−	−	−0.27	1.33	0.04	1	0.84
Tobacco	−	−	−	−	−	−2.67	1.80	2.18	1	0.14
BMI	−	−	−	−	−	0.17	0.22	0.59	1	0.44

\* Intercept is not shown. B: beta, SE: standard error, df: degree of freedom.



**Fig. 2.** Prediction of BD status for ARNTL, ARNTL2 and CIART alone, then combined.

levels depends on numerous factors and varies along the nycthemere (Rijo-Ferreira and Takahashi, 2019; Sun et al., 2020). However, we were able to identify two main groups of inter-correlated genes and show that the expression level of *CIART*, a recently described circadian repressor, is correlated to these two groups in this cellular pathway. The study of circadian gene expression over 24 h under controlled conditions should

allow to describe more precisely the genes correlated with *CIART* and better understand its role.

Our study suggests that *ARNTL* and *CIART* may be relevant candidate circadian genes whose levels of expression may discriminate BD from HC. *ARNTL* is an Aryl Hydrocarbon Receptor Nuclear Translocator-Like Proteins (also known as *Bmal1* for Brain and Muscle ARNT-Like 1) that dimerizes with *CLOCK*. This heterodimer is able to bind DNA and activate transcription from an *E-box* element, thus participating in the activation of the transcription of core clock genes such as *PER1–3* and *CRY1–2*. The loss of *Bmal1* in mice results in immediate and complete loss of circadian rhythmicity in constant darkness and *Bmal1* knockout is the only single gene deletion that fully eliminates circadian clock function in the suprachiasmatic nucleus and in peripheral tissues (Bunger et al., 2000; Tamaru and Takamatsu, 2018; Haque et al., 2019). *CIART* (Circadian-Associated Transcriptional Repressor) also known as *CHRONO* (computationally highlighted repressor of the network oscillator) or *Gm129* (Gene Model 129) encodes a nuclear-localized protein that directly interacts with *BMAL1* and represses *CLOCK-BMAL1* activity (Annayev et al., 2014). Overexpression of *CHRONO* leads to suppression of *BMAL1-CLOCK* activity (Goriki et al., 2014) and *Chrono* knockout mice display a prolonged free-running circadian period (Anafi et al., 2014). Given the physiological functions of these two genes, we suggest that, as compared to controls, individuals with BD might display a lower expression of one circadian transcription driver (*ARNTL*) and a higher expression of one circadian repressor of transcription (*CIART*). If replicated, these results lead to the hypothesis that BD might be characterized by an imbalance between activation/repression of the transcription in the biological clock.

**Table 4**  
group comparison for the level of expression of ARNTL, CIART, ARNTL2 and BHLHE41 using RT-qPCR.

Status	HC		BD		log2 fold change	p values
	Median	IQR	Median	IQR		
CIART	0.50	0.33–0.58	0.82	0.68–1.04	0.71	<0.001
BHLHE41	0.24	0.16–0.48	0.7	0.37–1.05	1.54	<0.001
ARNTL	0.79	0.66–1.07	0.62	0.49–0.78	−0.35	<b>0.001</b>
ARNTL2	0.92	0.79–1.05	0.89	0.76–0.97	−0.05	0.557

In bold, p values <0.05.

Genes are ordered by p values.

**Table 5**  
Comparisons between BD and HC for ARNTL, BHLHE41 and CIART with adjustment.

Variables	MODEL 2 *				
	B	SE	Wald	df	p
ARNTL qPCR	−7.01	3.35	4.38	1	<b>0.04</b>
BHEHL41 qPCR	3.04	2.51	1.47	1	0.23
CIART qPCR	11.29	5.47	4.25	1	<b>0.04</b>
Age	0.25	0.14	3.22	1	0.07
Sex	0.42	1.11	0.14	1	0.71
Tobacco	−3.26	2.04	2.55	1	0.11
Body Mass Index	0.08	0.19	0.18	1	0.67

\* Intercept is not shown. B: beta, SE: standard error, df: degree of freedom.

Several studies have explored genetic variants of circadian genes as vulnerability factors to BD. Even though some studies provided replication in independent samples (Etain et al., 2014), for example with *TIMELESS* and *RORA*, it cannot be concluded that one particular circadian gene may represent a risk factor to BD based on genetic approaches. Of note, while most genome-wide association studies (GWAS) failed to demonstrate any robust associations between genetic variants in circadian genes and BD, a recent one nevertheless suggested an association with *ARNTL* ( $p = 1.8 \cdot 10^{-6}$ ) using gene-based tests (Mullins et al., 2021). In this context, a gene expression study may represent a complementary approach. Since we found associations between BD and levels of expression for some circadian genes, this may suggest that the abnormalities leading to alterations in gene expression might be linked to epigenetic modifications rather than to abnormalities at the level of genetic variants. Further studies should then explore epigenetic marks of these circadian genes (DNA methylation levels, histone modifications and/or targeted miRNAs expression) that may differ between individuals with BD and controls in order to disentangle the mechanisms at stake. Interestingly, methylation of *ARNTL* has been suggested to differ significantly between BD and controls (Bengesser et al., 2018), which may be one possible explanation of the lower *ARNTL* gene expression in BD observed in our study.

Several limitations of this study should be discussed. First, the sample size is relatively limited, although standard for a gene expression study. This does not preclude some false negative findings, especially regarding genes that we did not include in the multivariable model after the correction using FDR. Replications in samples of larger size are thus deserved. Second, this study was performed on RNA from LCLs. Although this model has been shown to retain characteristics of patients with BD and to express most of the circadian genes (McCarthy et al., 2011; Viswanath et al., 2015), we cannot exclude that potential artefacts might have been caused by the transformation of the lymphocytes. In addition, LCLs have repeatedly been shown to capture differences in circadian gene expressions in individuals with BD (Kato et al., 2011; Geoffroy et al., 2014; Kittel-Schneider et al., 2019; Grillault Laroche et al., 2021). These results need to be replicated using RNA samples directly extracted from whole blood. Third, we focused on genes expressed in the suprachiasmatic nuclei. We did not explore melatoninergic genes that may also display some abnormalities in terms of gene

expression between cases and controls (Etain et al., 2012). This analysis may also be extended to upstream clock modulator genes (genes that modulate circadian rhythms) and downstream clock-controlled genes (McCarthy et al., 2012), however this would massively increase the number of genes to be investigated. We did not provide any methylation analysis to further explore potential underlying epigenetic mechanisms. Finally, all individuals with BD were currently treated by mood stabilizers and a complementary study should be proposed ideally in drug-naïve participants (which may be impossible in BD).

In conclusion, this study suggested abnormalities in two circadian genes (*ARNTL*, and *CIART*) in individuals with BD as compared to controls. We reported for the first time that a recently characterized circadian repressor (*CIART*) may contribute to the vulnerability to BD. Our results suggest that BD might be characterized by an imbalance between activation/repression of the transcription within the circadian system. Replications in larger independent samples that would combine both expression and epigenetic analyses are required.

#### Statement of ethics

This research protocol (Clinical Trials Number NCT02627404) was approved by the French medical ethics committee (Comité de Protection des Personnes (CPP) – IDRCB2008\_AO1465\_50 VI – Pitié-Salpêtrière 118–08) and carried out according to the approved guidelines. Written informed consent was obtained from all participants.

#### Funding

This research was supported by Institut National de la Santé et de la Recherche Médicale (INSERM), and Assistance Publique des Hôpitaux de Paris (AP-HP). CMC is supported by the Centre National pour la Recherche Scientifique (CNRS). These funding sources had no role in the study design, data collection, analysis, preparation of the manuscript, or decision to submit the manuscript for publication.

#### Author contribution

BE is the principal investigator of this study and obtained the funding. EM performed the RNA-seq experiments. JG performed the QC and extraction of the RNA-seq data. CC performed the cellular experiments, RNA isolation and qPCR validation. BE and CMC performed the first exploratory statistical analyses and wrote the first draft of the manuscript. BE, CMC and GG reviewed the statistical analyses and wrote the final draft of the manuscript. BE, GG, VH and MM contributed to the recruitment of participants. FB critically reviewed the final version of the manuscript. All authors approved the submitted version of the manuscript.

#### Declaration of Competing Interest

None in relation to this article.

## Data availability

Data will be made available on request. Due to ethical and legal restrictions, data involving clinical participants cannot be made publicly available. All relevant data are available upon request to the authors for researchers who meet the criteria for access to confidential data.

## Acknowledgements

We express our thanks to the individuals who participated to the present study. Lymphoblastoid cell lines were established at the Centre de Ressources Biologiques Site Cochin in Paris.

## References

- Anafi, R.C., Lee, Y., Sato, T.K., Venkataraman, A., Ramanathan, C., Kavakli, I.H., Hughes, M.E., Baggs, J.E., Growe, J., Liu, A.C., Kim, J., Hogenesch, J.B., 2014. Machine learning helps identify CHRONO as a circadian clock component. *PLoS Biol.* 12, e1001840.
- Annavey, Y., Adar, S., Chiou, Y.Y., Lieb, J.D., Sancar, A., Ye, R., 2014. Gene model 129 (Gm129) encodes a novel transcriptional repressor that modulates circadian gene expression. *J. Biol. Chem.* 289, 5013–5024.
- Bengesser, S.A., Reininghaus, E.Z., Lackner, N., Birner, A., Fellendorf, F.T., Platzer, M., Kainzbauer, N., Tropper, B., Hormanseder, C., Queissner, R., Kapfhammer, H.P., Wallner-Liebmann, S.J., Fuchs, R., Petek, E., Windpassinger, C., Schnalzenberger, M., Reininghaus, B., Evert, B., Waha, A., 2018. Is the molecular clock ticking differently in bipolar disorder? Methylation analysis of the clock gene ARNTL. *World J. Biol. Psychiatry.* 19, S21–S29.
- Bengesser, S.A., Hohenberger, H., Tropper, B., Dalkner, N., Birner, A., Fellendorf, F.T., Platzer, M., Rieger, A., Maget, A., Hamm, C., Queissner, R., Pilz, R., Bauer, K., Lenger, M., Morkl, S., Wagner-Skacel, J., Kapfhammer, H.P., Meier-Allard, N., Stracke, A., Holasek, S.J., Murphy, L., Reininghaus, E.Z., 2021. Gene expression analysis of MAOA and the clock gene ARNTL in individuals with bipolar disorder compared to healthy controls. *World J. Biol. Psychiatry.* 1–8.
- Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J. B., Simon, M.C., Takahashi, J.S., Bradfield, C.A., 2000. Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell.* 103, 1009–1017.
- Curis, E., Nepost, C., Grillault Laroche, D., Courtin, C., Laplanche, J.L., Etain, B., Marie-Claire, C., 2019. Selecting reference genes in RT-qPCR based on equivalence tests: a network based approach. *Sci. Rep.* 9, 16231.
- Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics.* 29, 15–21.
- Etain, B., Dumaine, A., Bellivier, F., Pagan, C., Francelle, L., Goubran-Botros, H., Moreno, S., Deshommes, J., Moustafa, K., Le Dudal, K., Mathieu, F., Henry, C., Kahn, J.P., Launay, J.M., Muhleisen, T.W., Cichon, S., Bourgeron, T., Leboyer, M., Jamain, S., 2012. Genetic and functional abnormalities of the melatonin biosynthesis pathway in patients with bipolar disorder. *Hum. Mol. Genet.* 21, 4030–4037.
- Etain, B., Jamain, S., Milhiet, V., Lajnef, M., Boudebasse, C., Dumaine, A., Mathieu, F., Gombert, A., Ledudal, K., Gard, S., Kahn, J.P., Henry, C., Boland, A., Zelenika, D., Lechner, D., Lathrop, M., Leboyer, M., Bellivier, F., 2014. Association between circadian genes, bipolar disorders and chronotypes. *Chronobiol. Int.* 31, 807–814.
- Geoffroy, P.A., Boudebasse, C., Henrion, A., Jamain, S., Henry, C., Leboyer, M., Bellivier, F., Etain, B., 2014. An ASMT variant associated with bipolar disorder influences sleep and circadian rhythms: a pilot study. *Genes Brain Behav.* 13, 299–304.
- Geoffroy, P.A., Curis, E., Courtin, C., Moreira, J., Morvillers, T., Etain, B., Laplanche, J.L., Bellivier, F., Marie-Claire, C., 2018. Lithium response in bipolar disorders and core clock genes expression. *World J. Biol. Psychiatry.* 19, 619–632.
- Goriki, A., Hatanaka, F., Myung, J., Kim, J.K., Yoritaka, T., Tanoue, S., Abe, T., Kiyonari, H., Fujimoto, K., Kato, Y., Todo, T., Matsubara, A., Forger, D., Takumi, T., 2014. A novel protein, CHRONO, functions as a core component of the mammalian circadian clock. *PLoS Biol.* 12, e1001839.
- Grillault Laroche, D., Curis, E., Bellivier, F., Nepost, C., Gross, G., Etain, B., Marie-Claire, C., 2021. Network of co-expressed circadian genes, childhood maltreatment and sleep quality in bipolar disorders. *Chronobiol. Int.* 38, 986–993.
- Haque, S.N., Booreddy, S.R., Welsh, D.K., 2019. Effects of BMAL1 manipulation on the brain's master circadian clock and behavior. *Yale J. Biol. Med.* 92, 251–258.
- Harvey, A.G., 2008. Sleep and circadian rhythms in bipolar disorder: seeking synchrony, harmony, and regulation. *Am. J. Psychiatry* 165, 820–829.
- Kato, T., Hayashi-Takagi, A., Toyota, T., Yoshikawa, T., Iwamoto, K., 2011. Gene expression analysis in lymphoblastoid cells as a potential biomarker of bipolar disorder. *J. Hum. Genet.* 56, 779–783.
- Kavakli, I.H., Ozturk, N., Baris, I., 2022. Protein interaction networks of the mammalian core clock proteins. *Adv. Protein Chem. Struct. Biol.* 131, 207–233.
- Kittel-Schneider, S., Schreck, S., Ziegler, C., Weissflog, L., Hilscher, M., Schwarz, R., Schmetzler, L., Neuner, M., Reif, A., 2015. Lithium-induced clock gene expression in Lymphoblastoid cells of bipolar affective patients. *Pharmacopsychiatry.* 48, 145–149.
- Kittel-Schneider, S., Hilscher, M., Scholz, C.J., Weber, H., Grunewald, L., Schwarz, R., Chiochetti, A.G., Reif, A., 2019. Lithium-induced gene expression alterations in two peripheral cell models of bipolar disorder. *World J. Biol. Psychiatry.* 20, 462–475.
- Krane-Gartiser, K., Scott, J., Nevoret, C., Benard, V., Benizri, C., Brochard, H., Geoffroy, P.A., Katsahian, S., Maruani, J., Yeim, S., Leboyer, M., Bellivier, F., Etain, B., 2019. Which actigraphic variables optimally characterize the sleep-wake cycle of individuals with bipolar disorders? *Acta Psychiatr. Scand.* 139, 269–279.
- Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics.* 12, 323.
- McCarthy, M.J., Nievergelt, C.M., Shekhtman, T., Kripke, D.F., Welsh, D.K., Kelsoe, J.R., 2011. Functional genetic variation in the rev-ErbA pathway and lithium response in the treatment of bipolar disorder. *Genes Brain Behav.* 10, 852–861.
- McCarthy, M.J., Nievergelt, C.M., Kelsoe, J.R., Welsh, D.K., 2012. A survey of genomic studies supports association of circadian clock genes with bipolar disorder spectrum illnesses and lithium response. *PLoS One* 7, e32091.
- McCarthy, M.J., Gottlieb, J.F., Gonzalez, R., McClung, C.A., Alloy, L.B., Cain, S., Dulcis, D., Etain, B., Frey, B.N., Garbaza, C., Ketchesin, K.D., Landgraf, D., Lee, H.J., Marie-Claire, C., Nusslock, R., Porcu, A., Porter, R., Ritter, P., Scott, J., Smith, D., Swartz, H.A., Murray, G., 2021. Neurobiological and behavioral mechanisms of circadian rhythm disruption in bipolar disorder: a critical multi-disciplinary literature review and agenda for future research from the ISBD task force on chronobiology. *Bipolar Disord.* 24, 232–263.
- Meyrel, M., Scott, J., Etain, B., 2021. Chronotypes and circadian rest-activity rhythms in bipolar disorders: a meta-analysis of self- and observer rating scales. *Bipolar Disord.* 24, 286–297.
- Milhiet, V., Etain, B., Boudebasse, C., Bellivier, F., 2011. Circadian biomarkers, circadian genes and bipolar disorders. *J. Physiol. Paris* 105, 183–189.
- Montgomery, S.A., Asberg, M., 1979. A new depression scale designed to be sensitive to change. *Br. J. Psychiatry* 134, 382–389.
- Mullins, N., Forstner, A.J., O'Connell, K.S., Coombes, B., Coleman, J.R.I., Qiao, Z., Als, T. D., Bigdeli, T.B., Borte, S., Bryois, J., Charney, A.W., Drange, O.K., Gandal, M.J., Hagenaars, S.P., Ikeda, M., Kamitaki, N., Kim, M., Krebs, K., Panagiotaropoulou, G., Schilder, B.M., Sloofman, L.G., Steinberg, S., Trubetskoy, V., Winsvold, B.S., Won, H. H., Abramova, L., Adorjan, K., Agerbo, E., Al Eissa, M., Albani, D., Allieu-Rodriguez, N., Anjorin, A., Antilla, V., Antoniou, A., Awasthi, S., Baek, J.H., Baekvad-Hansen, M., Bass, N., Bauer, M., Beins, E.C., Bergen, S.E., Birner, A., Bocker Pedersen, C., Boen, E., Boks, M.P., Bosch, R., Brum, M., Brumpton, B.M., Brunkhorst-Kanaan, N., Budde, M., Bybjerg-Grauholm, J., Byerley, W., Cairns, M., Casas, M., Cervantes, P., Clarke, T.K., Cruceanu, C., Cuellar-Barboza, A., Cunningham, J., Curtis, D., Czerski, P.M., Dale, A.M., Dalkner, N., David, F.S., Degenhardt, F., Djurovic, S., Dobbyn, A.L., Douzenis, A., Elvsashagen, T., Escott-Price, V., Ferrier, I. N., Fiorentino, A., Foroud, T.M., Forty, L., Frank, J., Frei, O., Freimer, N.B., Frisen, L., Gade, K., Garnham, J., Gelernter, J., Giortz Pedersen, M., Gizer, I.R., Gordon, S.D., Gordon-Smith, K., Greenwood, T.A., Grove, J., Guzman-Parra, J., Ha, K., Haraldsson, M., Hautzinger, M., Heilbronner, U., Hellgren, D., Herms, S., Hoffmann, P., Holmans, P.A., Huckins, L., Jamain, S., Johnson, J.S., Kalmann, J.L., Kamatani, Y., Kennedy, J.L., Kittel-Schneider, S., Knowles, J.A., Kogevinas, M., Koromina, M., Kranz, T.M., Kranzler, H.R., Kubo, M., Kupka, R., Kushner, S.A., Lavebratt, C., Lawrence, J., Leber, M., Lee, H.J., Lee, P.H., Levy, S.E., Lewis, C., Liao, C., Lucae, S., Lundberg, M., MacIntyre, D.J., Magnusson, S.H., Maier, W., Maihofer, A., Malaspina, D., Maratou, E., Martinson, L., Mattheisen, M., McCarroll, S.A., McGregor, N.W., McGuffin, P., McKay, J.D., Medeiros, H., Medland, S.E., Millischer, V., Montgomery, G.W., Moran, J.L., Morris, D.W., Muhleisen, T.W., O'Brien, N., O'Donovan, C., Olde Loohuis, L.M., Oruc, L., Papiol, S., Pardinas, A.F., Perry, A., Pfennig, A., Porichi, E., Potash, J.B., Quedest, D., Raj, T., Rapaport, M.H., DePaulo, J.R., Regeer, E.J., Rice, J.P., Rivas, F., Rivera, M., Roth, J., Roussos, P., Ruderfer, D.M., Sanchez-Mora, C., Schulte, E.C., Senner, F., Sharp, S., Shilling, P.D., Sigurdsson, E., Sirignano, L., Slaney, C., Smeland, O.B., Smith, D.J., Sobell, J.L., Soholm Hansen, C., Soler Artigas, M., Spijker, A.T., Stein, D. J., Strauss, J.S., Swiatkowska, B., Terao, C., Thorgerisson, T.E., Toma, C., Tooney, P., Tsermpini, E.E., Vawter, M.P., Vedder, H., Walters, J.T.R., Witt, S.H., Xi, S., Xu, W., Yang, J.M.K., Young, A.H., Young, H., Zandi, P.P., Zhou, H., Zillich, L., Psychiatry, H. A.-I., Adolfsson, R., Agartz, I., Alda, M., Alfredsson, L., Babadjanova, G., Backlund, L., Baune, B.T., Bellivier, F., Bengesser, S., Berrettini, W.H., Blackwood, D. H.R., Boehnke, M., Borglum, A.D., Breen, G., Carr, V.J., Catts, S., Corvin, A., Craddock, N., Dannlowski, U., Dikeos, D., Esko, T., Etain, B., Ferentinos, P., Frye, M., Fullerton, J.M., Gawlik, M., Gershon, E.S., Goes, F.S., Green, M.J., Grigoriou-Serbanescu, M., Hauser, J., Henskens, F., Hillert, J., Hong, K.S., Hougaard, D.M., Hultman, C.M., Hveem, K., Iwata, N., Jablensky, A.V., Jones, I., Jones, L.A., Kahn, R. S., Kelsoe, J.R., Kirov, G., Landen, M., Leboyer, M., Lewis, C.M., Li, Q.S., Lissowska, J., Lochner, C., Loughland, C., Martin, N.G., Mathews, C.A., Mayoral, F., McElroy, S.L., McIntosh, A.M., McMahon, F.J., Melle, I., Michie, P., Milani, L., Mitchell, P.B., Morken, G., Mors, O., Mortensen, P.B., Mowry, B., Muller-Myhsok, B., Myers, R.M., Neale, B.M., Nievergelt, C.M., Nordentoft, M., Nothen, M.M., O'Donovan, M.C., Oedegaard, K.J., Olsson, T., Owen, M.J., Paciga, S.A., Pantelis, C., Pato, C., Pato, M.T., Patrinos, G.P., Perlis, R.H., Posthuma, D., Ramos-Quiroga, J.A., Reif, A., Reininghaus, E.Z., Ribases, M., Rietschel, M., Ripke, S., Rouleau, G.A., Saito, T., Schall, U., Schalling, M., Schofield, P.R., Schulze, T.G., Scott, L.J., Scott, R. J., Serretti, A., Shannon Weickert, C., Smoller, J.W., Stefansson, H., Stefansson, K., Stordal, E., Streit, F., Sullivan, P.F., Turecki, G., Vaaler, A.E., Vieta, E., Vincent, J.B., Waldman, I.D., Weickert, T.W., Werge, T., Wray, N.R., Zwart, J.A., Biernacka, J.M., Nurnberger, J.I., Cichon, S., Edenberg, H.J., Stahl, E.A., McQuillin, A., Di Florio, A., Ophoff, R.A., Andreassen, O.A., 2021. Genome-wide association study of more than 40,000 bipolar disorder cases provides new insights into the underlying biology. *Nat. Genet.* 53, 817–829.
- Murray, G., Gottlieb, J., Hidalgo, M.P., Etain, B., Ritter, P., Skene, D.J., Garbaza, C., Bullock, B., Merikangas, K., Zippunikov, V., Shou, H., Gonzalez, R., Scott, J., Geoffroy, P.A., Frey, B.N., 2020. Measuring circadian function in bipolar disorders:

- empirical and conceptual review of physiological, actigraphic, and self-report approaches. *Bipolar Disord.* 22, 693–710.
- Neitzel, H., 1986. A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Hum. Genet.* 73, 320–326.
- Rijo-Ferreira, F., Takahashi, J.S., 2019. Genomics of circadian rhythms in health and disease. *Genome Med.* 11, 82.
- Scott, J., Kallestad, H., Vedaa, O., Sivertsen, B., Etain, B., 2021. Sleep disturbances and first onset of major mental disorders in adolescence and early adulthood: a systematic review and meta-analysis. *Sleep Med. Rev.* 57, 101429.
- Scott, J., Etain, B., Miklowitz, D., Crouse, J.J., Carpenter, J., Marwaha, S., Smith, D., Merikangas, K., Hickie, I., 2022. A systematic review and meta-analysis of sleep and circadian rhythms disturbances in individuals at high-risk of developing or with early onset of bipolar disorders. *Neurosci. Biobehav. Rev.* 135, 104585.
- Sun, L., Ma, J., Turck, C.W., Xu, P., Wang, G.Z., 2020. Genome-wide circadian regulation: a unique system for computational biology. *Comput. Struct. Biotechnol. J.* 18, 1914–1924.
- Tamaru, T., Takamatsu, K., 2018. Circadian modification network of a core clock driver BMAL1 to harmonize physiology from brain to peripheral tissues. *Neurochem. Int.* 119, 11–16.
- Viswanath, B., Jose, S.P., Squassina, A., Thirthalli, J., Purushottam, M., Mukherjee, O., Vladimirov, V., Patrinos, G.P., Del Zompo, M., Jain, S., 2015. Cellular models to study bipolar disorder: a systematic review. *J. Affect. Disord.* 184, 36–50.
- Yang, S., Van Dongen, H.P., Wang, K., Berrettini, W., Bucan, M., 2009. Assessment of circadian function in fibroblasts of patients with bipolar disorder. *Mol. Psychiatry* 14, 143–155.
- Yang, Y., Li, N., Qiu, J., Ge, H., Qin, X., 2020. Identification of the repressive domain of the negative circadian clock component CHRONO. *Int. J. Mol. Sci.* 21.
- Young, R.C., Biggs, J.T., Ziegler, V.E., Meyer, D.A., 1978. A rating scale for mania: reliability, validity and sensitivity. *Br. J. Psychiatry* 133, 429–435.
- Zhang, C., Ni, P., Liang, S., Li, X., Tian, Y., Du, X., Wei, W., Meng, Y., Wei, J., Ma, X., Deng, W., Guo, W., Li, M., Yu, H., Zhao, L., Wang, Q., Pak, S.C., Li, T., 2022. Alterations in CRY2 and PER3 gene expression associated with thalamic-limbic community structural abnormalities in patients with bipolar depression or unipolar depression. *J. Affect. Disord.* 298, 472–480.
- Zhao, S., Ye, Z., Stanton, R., 2020. Misuse of RPKM or TPM normalization when comparing across samples and sequencing protocols. *RNA.* 26, 903–909.