TIME, GENETICS AND COMPLEX DISEASE

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TIME, GENETICS AND COMPLEX DISEASE

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Editorial: Time, genetics, and complex disease

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Editorial on the Research Topic
Time, genetics and complex disease

Decoding the genetic mechanisms of complex diseases is difficult since they typically involve hundreds of genes (Gibson, 2009; Hu et al., 2016). Additionally complicating the task is the fact that these genes can form complex interactions with each other (Gibson, 2009). These interactions change over time, further increasing the complexity of the entire system (Hu et al., 2016). The circadian clock is genetically based on a relatively simple regulatory system that can generate complex behaviors and impact health and disease in a dynamic manner (Takahashi, 2017; Rijo-Ferreira and Takahashi, 2019). Circadian regulation has been shown to be associated with several complex diseases including, but not limited, to neurodegenerative disorders (Nassan and Videnovic, 2022), chronic diseases (Chaix et al., 2019), and various aging-related disease (Rijo-Ferreira and Takahashi, 2019; Acosta-Rodríguez et al., 2021). Modeling the complex interactions of disease-related genes and other genes involved in the physiology of an organism from a circadian perspective is still in its early stages (Sun et al., 2020; Li et al., 2022).

This Research Topic aims to gather information gained from research concerned with advancing the understanding of complex diseases through time related mechanisms, especially those related to circadian regulation. In the following we provide a brief thematic overview of the diverse content of the Research Topic.

We first present research work on the regulation of circadian behavior and its effect on multiple layers. For the central clock, Lu et al. examined the effects of electroacupuncture on the phosphoproteome of the mammalian pacemaker, the suprachiasmatic nucleus, at different circadian times. More than 5,000 distinct phosphosites were quantified by mass spectrometry–based analysis, with many of

them contributing to the phase shifts induced by electroacupuncture. In a mechanistic study, Wu et al. discovered that *PCBP1*, a circadian expressed gene, acts as a regulator of period length in human U2OS cells, possibly through enhancing the link between CRY1 and the CLOCK/BMAL1 complex. Xing et al. found that sleep deprivation reduces the expression of the core circadian clock gene NR1D1 in mice, which leads to a down-regulation of CYP7A1, the enzyme responsible for the conversion of cholesterol into bile acids. This in turn results in cholesterol accumulation. Lu et al. focused on the effect of acute sleep deprivation on the circadian transcriptome in mouse lung. By subjecting mice to sleep deprivation, thousands of genes were identified that showed an altered rhythmic expression pattern in the lung, which may directly or indirectly impact COVID-19 infection. In another approach, a video recording-based analytical method was developed by Niu et al. It can be used to monitor the daily feeding rhythm in fruit flies and enables the stable and reliable analysis of long-term feeding behavior in great detail.

Next, we included three review manuscripts discussing the linkage between complex diseases and circadian regulation. Li et al. reviewed the pathogenesis of circadian-related complex diseases by focusing on the liver, heart, skeletal muscle, blood and other peripheral organs. The paper highlights the notion that different circadian regulatory molecules or checkpoints are involved in different diseases. From the circadian and developmental disorders perspective, Lorsung et al. explored the link between autism spectrum disorder (ASD) and circadian regulation. Specifically, four circadian physiological factors that are disrupted in ASD (sleep–wake cycle, melatonin, cortisol, and serotonin) are elaborated upon in detail. Peripheral clocks are important components of the circadian clock that function even when the master clock is decoupled. Lee and Hong summarized the progress of utilizing 3D organoids to investigate the property of circadian peripheral clocks and their relevance for complex diseases.

Last, but not least, we included contributions on several other complex diseases. For Netherton syndrome, Wang et al. revealed a new frameshift mutation, which is located in the *SPINK5* gene, by utilizing whole exome sequencing. They also found that N-terminal and C-terminal mutations of the protein LEKT1 cause distinct disease phenotypes. For intervertebral disc degeneration, integrative data analysis by Jiang et al. revealed a gene co-expression network composed of long non-coding RNAs, miRNA, and protein-coding RNAs, all with functions that may be related to cytokine secretion and immune cells. For chronic kidney disease, Gao et al. found that mortality of this disease can be better predicted with the help of the nomogram model based standard deviation of the normal-to-normal (SDNN) R-R intervals and other clinical factors.

In summary, based on genetic data, an increasing number of complex diseases have been found to be closely linked to time, specifically to circadian-related regulation. The availability of public omics data and computational toolsets make the examination and statistical detection of linkages more straightforward. However, additional efforts and high-quality datasets are still required to fully understand the temporal dynamics of complex diseases. We hope the readers will find this Research Topic (and e-book) to be a good resource for exploring the linkage between complex diseases and time. Finally, we acknowledge all the experts that have contributed to this Research Topic and the reviewers' excellent comments and valuable suggestions.

Author contributions

All authors listed have made substantial and intellectual contributions to the work and have approved publication.

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Conflict of interest

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Sleep Disturbance Induces Increased Cholesterol Level by NR1D1 Mediated CYP7A1 Inhibition

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Disturbed sleep is closely associated with an increased risk of metabolic diseases. However, the underlying mechanisms of circadian clock genes linking sleep and lipid profile abnormalities have not been fully elucidated. This study aimed to explore the important role of the circadian clock in regulating impaired cholesterol metabolism at an early stage of sleep deprivation (SD). Sleep disturbance was conducted using an SD instrument. Our results showed that SD increased the serum cholesterol levels. Concentrations of serum leptin and resistin were much lower after SD, but other metabolic hormone concentrations (adiponectin, glucagon, insulin, thyroxine, norepinephrine, and epinephrine) were unchanged before and after SD. Warning signs of cardiovascular diseases [decreased high density lipoprotein (HDL)-cholesterol and increased corticosterone and 8-hydroxyguanosine levels] and hepatic cholestasis (elevated total bile acids and bilirubin levels) were observed after SD. Cholesterol accumulation was also observed in the liver after SD. The expression levels of HMGCR, the critical enzyme for cholesterol synthesis, remained unchanged in the liver. However, the expression levels of liver CYP7A1, the enzyme responsible for the conversion of cholesterol into bile acids, significantly reduced after SD. Furthermore, expression of NR1D1, a circadian oscillator and transcriptional regulator of CYP7A1, strikingly decreased after SD. Moreover, NR1D1 deficiency decreased liver CYP7A1 levels, and SD could exacerbate the reduction of CYP7A1 expression in *NR1D1–/–* mouse livers. Additionally, NR1D1 deficiency could further increase serum cholesterol levels under SD. These results suggest that sleep disturbance can induce increased serum cholesterol levels and liver cholesterol accumulation by NR1D1 mediated CYP7A1 inhibition.

Keywords: CYP7A1, circadian clock, cholesterol, sleep disturbance, NR1D1

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INTRODUCTION

Maintenance of sleep homoeostasis regulated by the homoeostatic and circadian process plays important roles in the balance of psychological and physical health (Saper et al., 2005; Kon et al., 2017). However, short sleep duration or disturbed sleep due to shift work, jet lag, and insomnia is becoming a widely social and medical problem in modern society

(Kecklund and Axelsson, 2016; Bertisch et al., 2018). Numerous epidemiological studies have suggested that adverse effects of sleep loss are closely associated with increased risk of obesity, type 2 diabetes, and cardiovascular diseases (CVDs; Reutrakul and Van Cauter, 2018; Liu and Chen, 2019). The association of metabolic disorders with sleep loss caused by total sleep deprivation (SD), chronic sleep restriction, or sleep fragmentation has gained attention (Killick et al., 2012; Nedeltcheva and Scheer, 2014). It has been suggested that the development of metabolic disorders or CVDs may be associated with long term or immediate effects of sleep loss or disruption. Thus, it is important to explore the potential mechanism underlying this association.

The circadian clock is regarded as the endogenous timekeeper of many day to day biological and physiological processes, including the sleep-wake cycle and cyclic metabolic changes (Panda, 2016; Kon et al., 2017). The circadian clock is located in the suprachiasmatic nucleus and peripheral tissues, and it synchronizes physiology and behavior throughout the 24-h day-night cycle (Takahashi, 2017). It has been suggested by some experimental and epidemiological reports that misalignment or disruption of the circadian clock can increase the risk of metabolic diseases. For example, the genetic model of clock mutant mice is susceptible to obesity, hyperlipidaemia, hyperglycaemia, and hepatic steatosis (Turek et al., 2005). Additionally, knockout of clock genes, such as aryl hydrocarbon receptor nuclear translocator-like (ARNTL), PERIOD (PER2), and cryptochrome (CRY1/CRY2), increased their susceptibility to obesity and metabolic disorders (Yang et al., 2009; Marcheva et al., 2010; Hemmeryckx et al., 2011; Shimba et al., 2011; Barclay et al., 2013). Polymorphisms of human clock genes, such as ARNTL, circadian locomotor output cycles kaput (CLOCK), CRY2, and nuclear receptor subfamily 1, group D, member 1 (NR1D1), have been linked to obesity or some other features of metabolic syndromes (Woon et al., 2007; Sookoian et al., 2008; Ruano et al., 2014; Machicao et al., 2016). Clinical studies on chronic circadian misalignment due to shift work reported an increased incidence of diabetes, obesity, and CVDs in the study participants (Khan et al., 2018; Torquati et al., 2018; Chellappa et al., 2019). Thus, disruption of the circadian clock may contribute to the development of obesity and metabolic diseases.

Emerging evidence has revealed that total sleep deprivation or sleep restriction influences concentrations of lipid profiles in plasma (e.g., triglyceride, cholesterol, and lipoproteins), suggesting the role of sleep in regulating lipid metabolism (Chua et al., 2015; Brianza-Padilla et al., 2016). Additionally, it has been reported that insufficient or disturbed sleep can lead to an impairment of rhythmic transcriptomes of central and peripheral tissues, including the genes associated with circadian rhythm and metabolism (Archer and Oster, 2015). However, the underlying mechanisms of circadian clock genes linked to sleep duration and lipid profile abnormalities have not been fully understood.

Therefore, this study aimed to explore the role of the circadian clock in regulating impaired lipid metabolism under disturbed sleep conditions. Sleep disturbance measurement was conducted by a sleep deprivation instrument. Total cholesterol, triglycerides, free fatty acid, some risk factors for hepatic cholestasis, and CVDs were detected first. Pathological staining of the liver, cholesterol synthesis, and conversion of cholesterol to bile acids were also assessed during the study. Finally, the NR1D1 function in liver cholesterol accumulation induced by sleep disturbance was analyzed.

Abbreviations: CVDs, Cardiovascular diseases; SD, Sleep deprivation; ARNTL, Aryl hydrocarbon receptor nuclear translocator-like; PER2, PERIOD2; CRY1/CRY2, Cryptochrom1/2; NR1D1, Nuclear receptor subfamily 1, group D, member 1; HDL, High density lipoprotein; VLDL, Very low density lipoprotein; 8-OHdG, 8-Hydroxyguanosine; ALP, Alkaline phosphatase; HMGCR, 3-Hydroxy-3-methylglutaryl-CoA reductase; CYP7A1, Cytochrome P450 family 7 subfamily A member 1; FXR, Farnesoid-X-receptor; RXRa, Retinoid X receptor alpha; LRH1, Liver receptor homolog 1; HNF4a, Hepatocyte nuclear receptor 4 alpha; C/EBPa, CCAAT enhancer binding protein α; FOXO1, Forkhead box O1; E4BP4, E4, promoter binding protein 4.

MATERIALS AND METHODS

Animals and Sleep Deprivation

The male Sprague-Dawley rats and C57BL/6J mice aged at 6–8 weeks were purchased from Charles River Laboratory Animal Technology Co. (Beijing, China). The NR1D1$^{-/-}$ mice were purchased from Cyagen Technology Co. (Guangzhou, China). The rats and mice were bred in animal facilities under specific pathogen-free conditions with a 12:12 h light-dark cycle (light on at 06:00 AM and light off at 18:00 PM). Morning 06:00 (zeitgeber time) was considered as the ZT 0. Food and water were available ad libitum. The rats and mice were randomly divided into experimental and control groups. Animals in the experimental group were subjected to the sleep deprivation instrument (DB036, BEIJING ZHISHU DUOBAO BIOLOGICAL TECHNOLOGY CO., LTD), which used a randomly moving platform to keep animals awake. The platform provides separate cages to allow animals free access to food and water. Parameters for the moving platform can be set by the touch screen of the controller. Additionally, the parameters can be changed by programming to reduce the adaptation of sleep deprived animals to the environment. After the parameters are set, sleep deprivation can be carried out in rats or mice without human intervention. Sleep deprivation was carried out at ZT 0, and animals were sacrificed after SD for 72 h. Then fresh blood samples and liver tissues were obtained for further analysis. The instrument does not need to train animals and can limit sleep in a mild way, which could greatly reduce the damage caused by the traditional water environment sleep deprivation method. Care, use, and treatment of rats and mice in this study were in strict agreement with international guidelines for the care and use of laboratory animals. This study was approved by the Animal Ethics Committee of the Beijing Institute of Basic Medical Sciences.

Serum Lipids and Metabolic Related Hormones Assay

Fresh blood samples were obtained and then centrifuged at 2000 *g* at 4°C for 20 min. The serum was transferred into a new tube and stored at −80°C until use. The serum levels of

cholesterol (CEB701Ge), triglycerides (CEB687Ge), leptin (SEA084Ra), resistin (RETN) (SEA847Ra), adiponectin (ADP) (SEA605Ra), thyroxine (CEA452Ge), epinephrine (CEA858Ge), norepinephrine (CEA907Ge), insulin (CEA448Ra), glucagon (CEB266Ra), corticosterone (CEA540Ge), 8-hydroxyguanosine (8-OHdG) (CEA660Ge), alkaline phosphatase (ALP) (SEB472Ra), and bilirubin (CEK522Ge) were measured using the corresponding ELISA kits (CLOUD-CLONE CORP, China). Concentration of high density lipoprotein (HDL)-cholesterol (HDL-C; ab65390, Abcam), free fatty acid (ab65341, Abcam), and total bile acids (ab239702, Abcam) were analyzed with the corresponding assay kits. All the assay procedures were performed according to the protocols provided by the manufacturer.

Hepatic Lipids Assay

Rat hepatic lipids were determined by the method as described previously (Becares et al., 2019). Briefly, liver tissues were homogenized in 10 mM Tris buffer with 250 mM sucrose and 2 mM EDTA. Hepatic lipids in the homogenates were extracted with chloroform:methanol solution (1:1) by shaking for 3–4 h at room temperature. The homogenates were vortexed and centrifuged at 15000 *g* to obtain the organic phase. The organic phase could be dried with nitrogen and dissolved in 0.01 M phosphate buffer saline (PBS, with 1% Triton X-100). Then, further analyses of cholesterol (CEB701Ge), HDL (SEB006Ra), triglycerides (CEB687Ge), and very low density lipoprotein (VLDL; E-EL-R1203c, Elabscience) were performed according to the protocols provided by the manufacturer.

Oil Red O Staining

The rats were anesthetized and then transcardially perfused with normal saline, 4% paraformaldehyde in 0.01 M PBS. Liver tissues were dissected out, fixed in 4% PFA in 0.01 M PBS at 4°C overnight, transferred into 30% (w/v) sucrose in PBS for 24 h, embedded in tissue freezing medium (JUNG, Leica Biosystems), and stored at −80°C. Livers samples were cryosectioned into 10 μm thick sections and mounted on glass slides. The sections were stained with 0.3% Oil red O solution, counterstained with hematoxylin, rinsed under running tap water, and coverslipped with aqueous mountant.

Hematoxylin-Eosin Staining

Hematoxylin-eosin (HE) staining was conducted according to routine protocols. Liver tissues of six rats from each group were fixed in 10% neutral buffered formalin for 48 h, and then embedded in paraffin and sectioned into 3–5 μm thick sections. After deparaffinization and rehydration, the sections were stained with hematoxylin, dipped in 1% acid ethanol, and rinsed in distilled water. Then, the sections were stained with eosin solution and followed by with graded alcohol dehydration. The mounted slides were photographed by an Olympus BX53 fluorescence microscope (Tokyo, Japan).

Western Blot Analysis

Fresh liver samples were harvested, washed with cold PBS, and lysed with ice-cold lysis buffer supplemented with protease inhibitors. Cell lysate was run on 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (PVDF; ISEQ00010, Millipore), blocked with 5% skim milk, and incubated with primary antibodies at 4°C overnight. The primary antibodies against 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR; sc-271595, Santa Cruz Biotechnology), cytochrome P450 family 7 subfamily A member 1 (CYP7A1; sc-518007, Santa Cruz Biotechnology), NR1D1 (sc-323,915, Santa Cruz Biotechnology), and β-actin (sc-8432, Santa Cruz Biotechnology) were used. Then, the membrane was incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. After extensive washing three times, the bands were detected using an ECL detection system (4600SF, Tanon Science & Technology Co., Ltd).

RT-PCR Analysis

Total RNA was isolated from fresh liver tissues using Trizol reagent (T9424, Sigma Aldrich) according to the manufacturer's instructions. After concentration determination, the cDNA products were obtained using All-in-One cDNA Synthesis SuperMix (B24403, Biotool) for reverse transcription. The amplification of target genes was performed using the standard protocol by Bio-Rad T100TM Thermal Cycler (1861096, Bio-Rad). The rat primers used were as follows: HMGCR-F: 5'-gcctccattgagtc cggagg-3', HMGCR-R: 5'-gcaccgggttatcgtgagga-3'; CYP7A1-F: 5'-gaccaagtctttccggcac-3', CYP7A1-R: 5'-cagagaatagcgaggtgcgt-3'; FXR-F: 5'-aggaagtgcagagagatggga-3', FXR-R: 5'-agcttggtcgtggag gtcac-3'; RXRa-F: 5'-cgctcctcaggcaaacacta-3', RXRa-R: 5'-ggagg atgccgtctttcaca-3'; SHP-F: 5'-aggctcactgggcattgtgt-3', SHP-R 5'-ac atctccgatgacagggcg-3'; HNF4a-F: 5'-aaaaccctcgccgacatgga-3', HNF4a-R: 5'-ctgacacccaggctgttgga-3'; C/EBPa-R: 5'-tggagggttctta gcccctt-3', C/EBPa-F: 5'ggctggcgacatacagtaca-3'; NR1D1-F: 5'-tac aagtggccatggaagaca-3', NR1D1-R: 5'-ttggccaagttcatggcgttc-3'; E4BP4-F: 5'-ctgatgcagctgagaaaaatgc-3', E4BP4-R: 5'-gggagagcagct cagctttta-3'; R-GAPDH-R: 5'-cagttccagcctcgtctcat-3', GAPDH-F: 5'-actgtgccgttgaacttgcc-3'. The mouse primers used were as follows: NR1D1-F: 5'-acgaccctggactccaataa-3', NR1D1-R: 5'-ccattggagctgtcactgtaga-3'; β-actin-F: 5'-ttgccctagacttcgagcaa-3', β-actin-R: 5'-caggaaggaaggctggaaga-3'. Gene relative expression was normalized to the mRNA levels of β-actin.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, United States). Data are presented as the mean ± SE and analyzed using the two-tailed, nonpaired Student's *t*-test for comparison of two groups. The significant difference was set at $p < 0.05$.

RESULTS

SD Induced Increased Cholesterol Concentration in Serum Lipid Profiles

Rat serum samples were analyzed to evaluate the metabolic effects induced by SD. SD for 72 h induced significant increasing of cholesterol level, while triglycerides and free fatty acids levels kept unchanged compared with the control group (**Figures 1A–C**).

Hypercholesterolemia is a metabolic disorder characterized by increased cholesterol levels in circulation. The above results indicated that the tendency of hypercholesterolemia occurrence was much earlier than hypertriglyceridemia induced by SD. Some hormones secreted during the sleep-wake cycle contribute to the metabolism and energy balance of the body. Therefore, concentrations of some hormones involved in metabolism regulation and appetite control were determined. The results showed that concentration of serum leptin and resistin in the SD group were much lower than that in the control group (**Figures 1D,E**). However, serum levels of some other hormones, including adiponectin, glucagon, insulin, thyroxine, norepinephrine, and epinephrine, slightly differed in both groups (**Figures 1F–H**). The above results suggested that high cholesterol level in serum lipid profiles was induced by disturbed sleep stress.

SD Induced the Warning Signs of an Increased Risk of Cardiovascular and Hepatic Diseases

A high level of serum cholesterol or hypercholesterolemia is closely associated with increased risk of CVDs and hepatic diseases due to the induction of various stress pressures. In the experimental group, the total serum bile acid and bilirubin levels significantly increased after SD, but alkaline phosphatase (ALP) concentration remained unchanged in serum (**Figures 2A–C**). The results suggest the risk of hepatic cholestasis caused by SD. Simultaneously, the level of some risk factors for CVDs such as corticosterone and 8-OHdG in serum were found to be notably increased, while the concentration of protective component predicting cardiovascular risk HDL-C was markedly decreased in the SD group (**Figures 2D–F**). The above results together suggested the warning signs of future hepatic diseases and CVDs.

SD Induced Accumulation of Cholesterol in the Liver

Synthesis and degradation of cholesterol mainly occurs in the liver. It was found that cholesterol and HDL levels in rat liver were significantly increased after SD (**Figure 3A**). Meanwhile, triglycerides and VLDL levels in liver remained unchanged by SD (**Figure 3A**). Moreover, no obvious lipid droplets were observed in the pathological results of oil red O staining, but

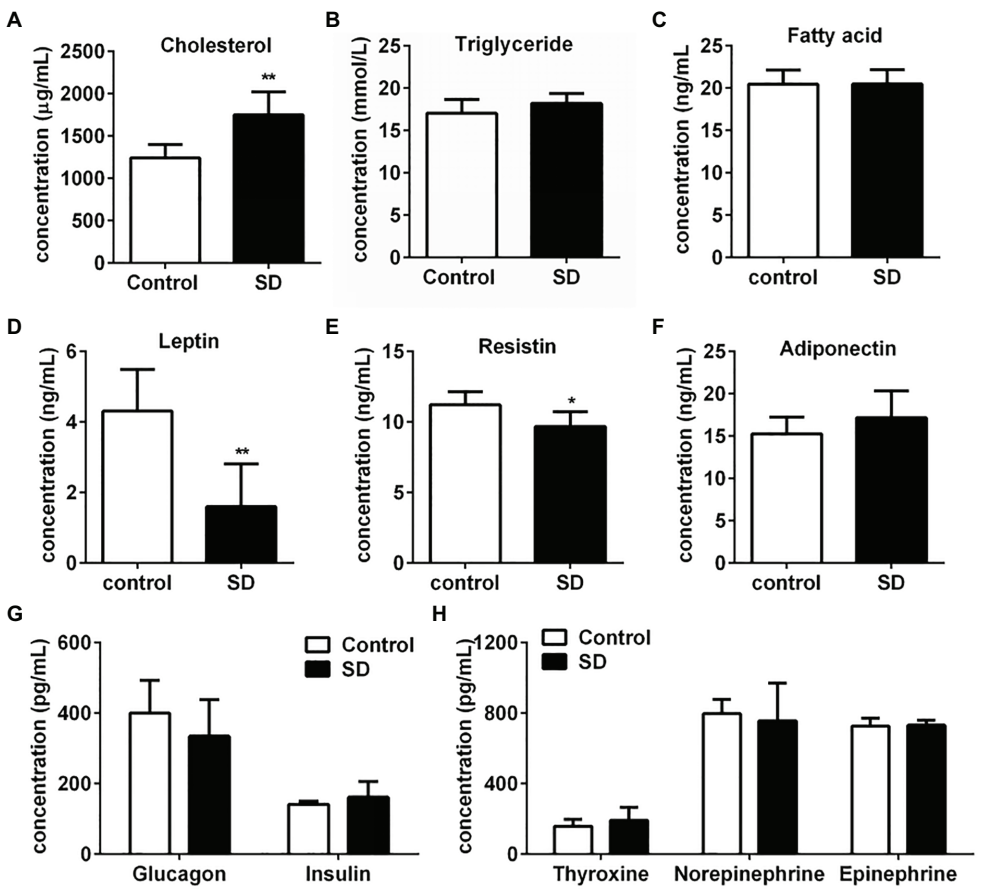

FIGURE 1 | Sleep deprivation (SD) induced increased cholesterol concentration in serum lipid profiles. **(A)** Cholesterol, **(B)** triglyceride, and **(C)** free fatty acids concentrations in rat serums were analyzed under disturbed sleep condition for 72 h. Metabolism related hormones including **(D)** leptin, **(E)** resistin (RETN), **(F)** adiponectin (ADP), **(G)** glucagon and insulin, **(H)** thyroxine, norepinephrine, and epinephrine concentration in rat serums were detected. $^{**}p < 0.01$; $^{*}p < 0.05$.

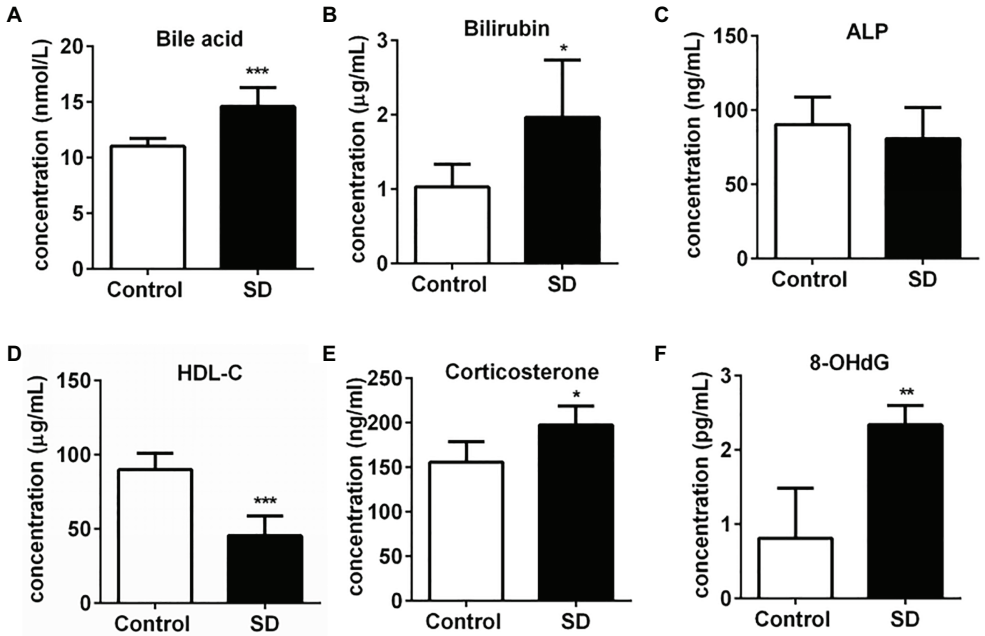

FIGURE 2 | Sleep deprivation induced the warning signs of an increased risk of cardiovascular and hepatic diseases. **(A)** Total bile acids, **(B)** bilirubin, and **(C)** alkaline phosphatase (ALP) concentrations in rat serums increased after sleep disturbance for 72 h. The decreased concentration of **(D)** high density lipoprotein (HDL)-cholesterol (HDL-C) and increased concentration of **(E)** corticosterone and **(F)** 8-hydroxyguanosine (8-OHdG) in rat serums were induced by SD. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

the coloring degree of staining was seemingly slightly enhanced after SD (**Figure 3B**), and there was no distinct increasing of inflammatory cells invasion by HE staining after SD (**Figure 3B**). The above results indicate that sleep disturbance induced an abnormal cholesterol metabolism in the liver before the occurrence of other pathological changes.

Bile Acid Synthesis Was More Vulnerable to SD Than Cholesterol Synthesis

To determine the reason behind high serum cholesterol levels, the synthesis and degradation of cholesterol in the liver were analyzed. Cholesterol synthesis and its conversion to bile acids are necessary to ensure cholesterol homoeostasis. The two rate-controlling enzymes involved in cholesterol and bile acid synthesis are HMGCR and CYP7A1, respectively. The expression of CYP7A1 mRNA and protein in rat livers was downregulated but not HMGCR expression after SD (**Figures 4A,B**). In addition, liver CYP8B1 expression, a downstream enzyme of CYP7A1, was also weakened under the SD condition. However, expression of CYP27A1, a critical enzyme in the alternative pathway of bile acid synthesis, remained unchanged after SD (**Supplementary Figure 1**). The results indicate that bile acid synthesis was more vulnerable to SD than cholesterol synthesis. Additionally, bile acid synthesis regulators were analyzed. The results showed that expression levels of farnesoid-X-receptor (FXR), retinoid X receptor alpha (RXRa), liver receptor homolog 1 (LRH1), hepatocyte nuclear receptor 4 alpha (HNF4a), CCAAT enhancer binding protein α (C/EBPa), and forkhead box O1 (FOXO1) at transcription level were not strikingly affected by SD (**Figure 4C**). The above results suggest that bile acid synthesis was partially inhibited under disturbed sleep conditions, which indicates that the conversion of cholesterol to bile acids is more susceptible to SD than cholesterol synthesis.

Correlation of NR1D1 With Bile Acid Synthesis Under Disturbed Sleep Conditions

Hepatic cholesterol and bile acid synthesis are controlled by the circadian clock. We found that SD for 24 h could induce obvious increasing of cholesterol level in rat serum (**Figure 5A**). The rapid increase in cholesterol levels induced by an acute SD of 24 h suggests the potential role of circadian clock in regulating cholesterol metabolism. The rapid increase in cholesterol level induced by an acute SD in 24 h suggests the potential role of the circadian clock in regulating cholesterol metabolism. NR1D1 and E4 promoter binding protein 4 (E4BP4) have been reported to participate in regulation of CYP7A1 expression. It was found that NR1D1 expression was downregulated after SD for 72 h but not E4BP4 expression, which remained unchanged (**Figure 5B**). The expression of CYP7A1 in liver displayed diurnal oscillation variation (data not shown). Further analyses demonstrated that amplitudes of NR1D1 expression were partially decreased by acute SD of 24 h. NR1D1 expression could exhibit a rapid response to SD at ZT4, but the pattern of E4BP4 expression remained unchanged by an acute SD of 24 h (**Figures 5C,D**). The above results suggested the role of NR1D1 in regulating CYP7A1 expression under disturbed sleep conditions.

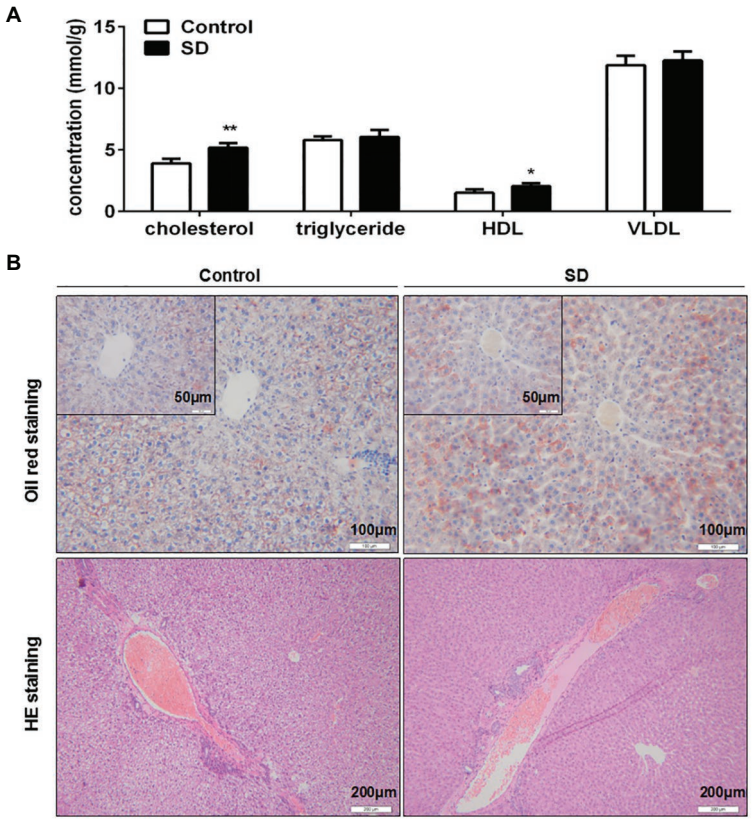

FIGURE 3 | Sleep deprivation induced accumulation of cholesterol in liver. **(A)** The level of cholesterol, triglyceride, HDL, and very low density lipoprotein (VLDL) in rat liver lipid profiles were analyzed. **(B)** Pathological staining of rat livers was assessed by oil red O staining and hematoxylin-eosin (HE) staining. $^{**}p < 0.01$; $^{*}p < 0.05$.

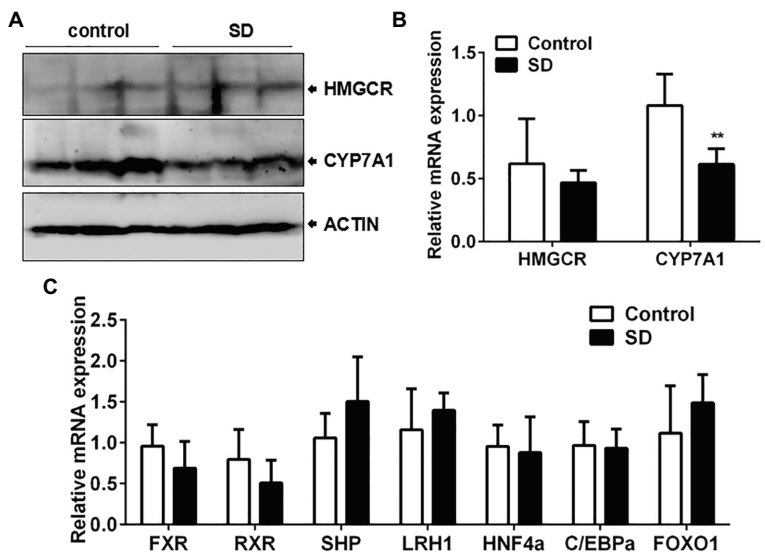

FIGURE 4 | Conversion of cholesterol to bile acids was affected by SD. **(A)** The protein and **(B)** relative mRNA expression of cytochrome P450 family 7 subfamily A member 1 (CYP7A1) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) in rat livers. **(C)** Expression of some reported bile acid synthesis regulators including farnesoid-X-receptor (FXR), retinoid X receptor alpha (RXRa), liver receptor homolog 1 (LRH1), hepatocyte nuclear receptor 4 alpha (HNF4a), CCAAT enhancer binding protein α (C/EBPa), and forkhead box O1 (FOXO1) were not strikingly affected by SD. $^{**}p < 0.01$.

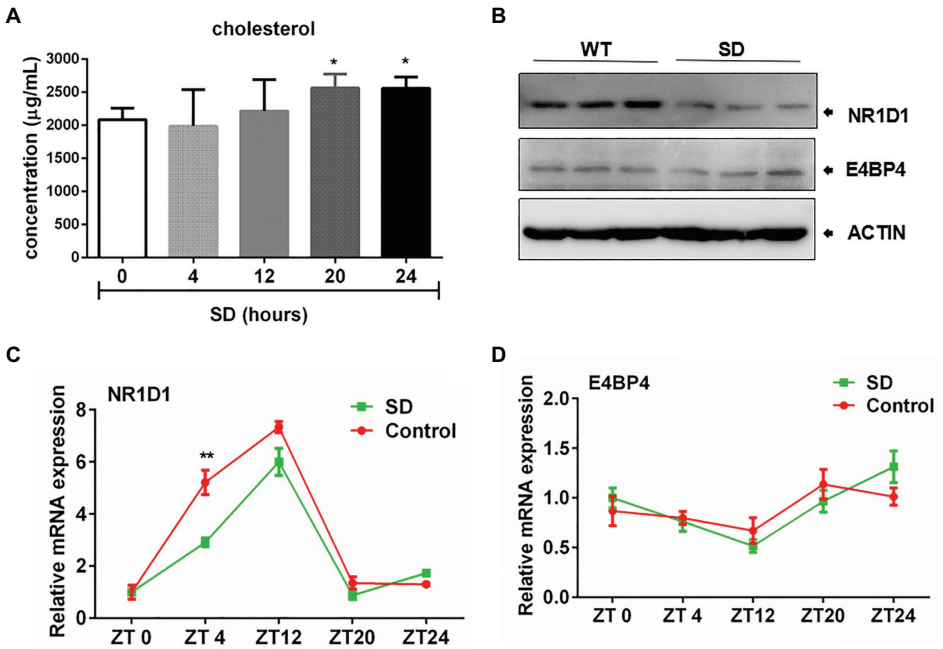

FIGURE 5 | Correlation of nuclear receptor subfamily 1, group D, member 1 (NR1D1) with bile acid synthesis under sleep disturbance condition. **(A)** Increasing of cholesterol level in rat serum induced by acute SD in 24 h. **(B)** NR1D1 and E4, promoter binding protein 4 (E4BP4) expression in rat liver after SD for 72 h. Oscillation expressions of **(C)** NR1D1 and **(D)** E4BP4 in rat liver under acute SD stress. $^{**}p < 0.01$; $^{*}p < 0.05$.

High Cholesterol Level Induced by SD Correlated With Downregulation of CYP7A1 Expression by NR1D1

In order to figure out the potential role of NR1D1 in CYP7A1 expression, we compared the responses of wild type (WT) and NR1D1$^{-/-}$ mice after SD. Similarly, SD could induce an obvious increase in serum cholesterol levels along with downregulation of NR1D1 and CYP7A1 expression in the livers of WT mice (**Figures 6A–C**). Furthermore, NR1D1 deficiency downregulated the expression levels of CYP7A1, and sleep disturbance could exacerbate the decreased CYP7A1 expression (**Figure 6D**). Additionally, NR1D1 deficiency could also further increase serum cholesterol level and the tendency of cholesterol accumulation in the liver under sleep disturbance conditions (**Figures 6E,F**). The above data indicated that downregulation of NR1D1 expression by the SD-mediated transcriptional inhibition of CYP7A1, leading to the block of cholesterol degradation and high level cholesterol accumulation in serum.

DISCUSSION

Accumulating evidence from the epidemiologic and laboratory studies have suggested the role of reduced or disturbed sleep in the metabolic dysfunction, leading to an increased risk of type 2 diabetes, obesity, or weight gain. Considering the early metabolic alterations caused by sleep disturbance, our results showed that a high cholesterol level in serum lipid profiles was rapidly induced by sleep deprivation exposure (24 and 72 h) in both rat and mice. Therefore, influences on cholesterol homeostasis regulation should be the early metabolic related events urged by sleep disturbance. Long term and uncontrolled exposure to hypercholesterolemia can lead to a slow and progressive occurrence of cardiovascular events (Jeong et al., 2018). In our study, sleep disturbance induced some early signs of future CVDs. Plasma HDL-C was decreased under disturbed sleep stress. The cholesterol contained in HDL is verified to be inversely associated with the risk of coronary heart disease and a predictor of cardiovascular risk for its central role in reverse cholesterol transport (Rader and Hovingh, 2014; Ouimet et al., 2019). Besides, HDL has also been proved to exert inhibitory roles on oxidative pathways in CVDs (Kontush and Chapman, 2010; Brites et al., 2017). High levels of 8-OHdG, a biomarker of oxidative stress, have been observed in patients with severe atherosclerotic lesions and hypertension (Martinet et al., 2002; Xiang et al., 2011; Rosello-Lleti et al., 2012). Therefore, the elevated 8-OHdG level observed in this study reminds the future rising oxidative damage induced by SD. Accordingly, the above risk factors together suggest that hypercholesterolemia induced by SD might predict the warning sign of CVDs by affecting multiple blood parameters related to the cardiovascular system.

High plasma cholesterol can contribute to the development of liver disorders, such as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatosis hepatitis (NASH) (Kerr and Davidson, 2012; Ioannou, 2016). Our results found the elevated level of bilirubin, bile acids, and cholesterol in serum under disturbed sleep stress. Bilirubin, bile acids, and cholesterol are important components of bile, and impairment in their formation or

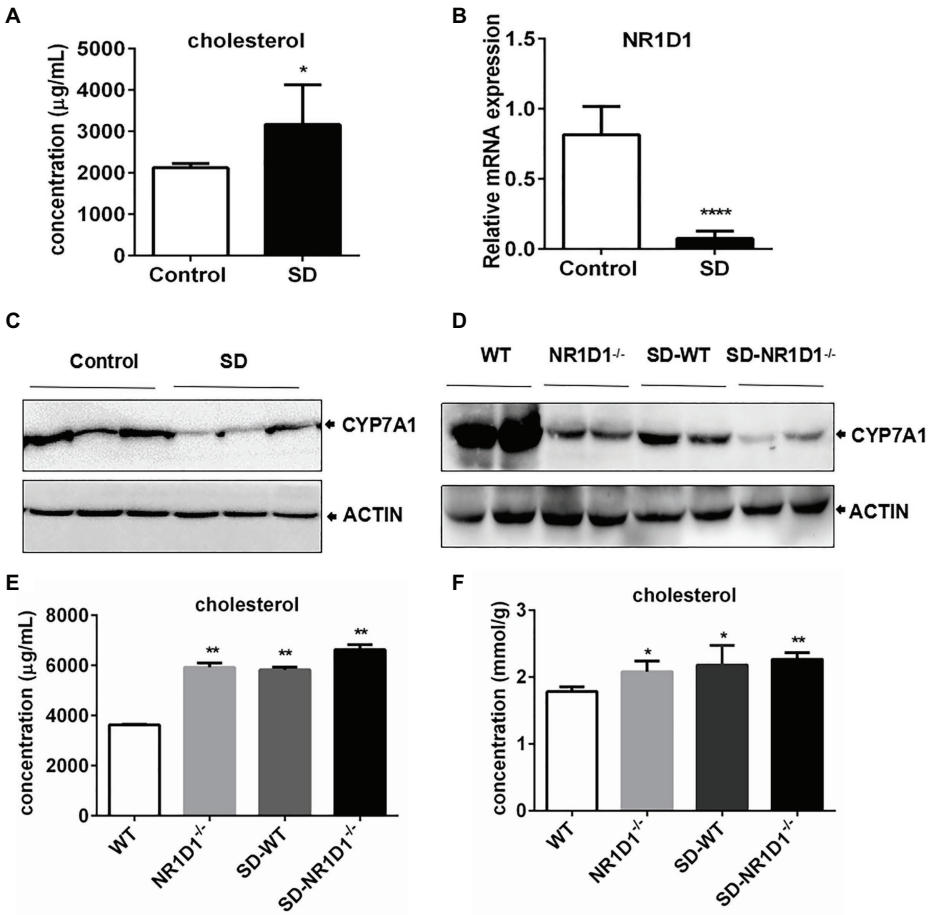

FIGURE 6 | High level cholesterol induced by SD correlated with downregulation of CYP7A1 expression by NR1D1. **(A)** Increasing of cholesterol concentration in mice serums induced by SD. **(B)** Relative mRNA expression of NR1D1 in mice livers after SD. **(C)** Inhibition of CYP7A1 expression in mice livers after SD. **(D)** Reduction of CYP7A1 expression in sleep disturbed NR1D1$^{-/-}$ mice. **(E)** Serum cholesterol level in sleep disturbed NR1D1$^{-/-}$ mice. **(F)** liver cholesterol level in sleep disturbed NR1D1$^{-/-}$ mice. ****$p < 0.0001$; **$p < 0.01$; *$p < 0.05$.

excretion can lead to cholestasis (Paumgartner, 2010). Particularly, the accumulation of bile acids in the serum and hepatocytes suggests the increased risk of cholestasis occurrence at early stage of liver diseases under disturbed sleep stress. Cholestasis is usually caused by alterations of hepatobiliary transport systems in hereditary cholestasis or acquired cholestatic liver diseases (Lee and Boyer, 2000; Sticova et al., 2018). Apart from mutations in transporter genes, pro-inflammatory cytokines, hormones, and drugs are major pathogenic factors for acquired forms of cholestasis (Stieger et al., 2000; Zollner and Trauner, 2006). Thus, the results of our study indicate that short sleep duration or sleep disturbance also should be a risk factor for cholestatic liver diseases.

Various epidemiological studies have reported that short sleep duration is associated with an elevated weight and body mass index (BMI; Kobayashi et al., 2012; Bayon et al., 2014). Being overweight is associated with greater energy intake than expenditure. Though some reported hormones involved in regulation of lipid and glucose metabolism and triglycerides levels kept unchanged, leptin concentration was found to be decreased under disturbed sleep stress for 72 h in this study. Leptin plays a major role in energy homoeostasis through appetite control and food intake limitation. It has been reported that insufficient sleep promotes dietary intake and contributes to the development of obesity and metabolic alteration (Prinz, 2004; Taheri et al., 2004). Thus, the decline in serum leptin levels induced by SD is in agreement with earlier reports on the links between short sleep duration and metabolic alterations by regulation on dietary intake. Therefore, exogenous dietary intake by appetite regulation is likely to be an important source of high cholesterol level in serum induced by sleep disturbance.

Regulation of hepatic cholesterol metabolism is controlled by the circadian clock (Pan et al., 2013; Ma et al., 2015). Oscillatory transcription of genes encoding key enzymes regulating cholesterol homoeostasis is tightly controlled during day and night. The results in this study showed that CYP7A1 expression was inhibited but with HMGCR expression unchanged induced by sleep disturbance. Therefore, conversion of cholesterol to bile acids rather than cholesterol synthesis was more susceptible

to disturbed sleep stress. CYP7A1 expression exhibits a strong circadian rhythm, which is negatively regulated by peroxisome proliferator-activated receptor a, DEC1/2, and E4BP4, and positively regulated by NR1D1 and DBP (Noshiro et al., 2007; Duez et al., 2008). In this study, expressions NR1D1 were downregulated, and exhibit rapid response to SD at ZT4. Moreover, sleep disturbance could exacerbate the reduction of NR1D1 mediated CYP7A1 expression. Accordingly, the results provided a way to understand how circadian clocks link sleep disturbance to cholesterol metabolism by NR1D1. Additionally, the results partially explained the endogenous regulatory mechanism of liver cholesterol accumulation. However, NR1D1 acts as a transcriptional repressor in the inhibition of BMAL1 transcription and regulation of biological processes, such as adipocyte differentiation and lipid metabolism (Wang and Lazar, 2008; Le Martelot et al., 2009). Since both NR1D1 and CYP7A1 expression were downregulated in this study, regulation of CYP7A1 expression should be an indirect mechanism mediated by NR1D1 through negative regulators of CYP7A1 rather than positive regulators. The involvements of negative regulators of CYP7A1 in this study remains to be explored in our future work.

In summary, our study has demonstrated that an increased serum and liver cholesterol level induced by sleep disturbance is an early pathological event, which closely correlates with a decreased conversion of cholesterol to bile acids by NR1D1 mediated CYP7A1 inhibition. Thus, our finding explained the important role of the circadian clock in linking sleep loss and lipid profile abnormality.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material** and further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of the Beijing Institute of Basic Medical Sciences.

AUTHOR CONTRIBUTIONS

XH and YZ were the co-first authors who performed most of the experiments and data analysis. WW, CZ, and LW performed the animal experiments. MD and MZ contributed to animal breeding. CX designed, performed, and supervised the study and wrote the paper. LS provided suggestions and guidance of this study and revised the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.610496/full#supplementary-material

Supplementary Figure 1 | CYP8B1 and CYP27A1 expression under sleep deprivation (SD) condition. **(A)** The protein expression of CYP8B1 and CYP27A1 in liver under SD condition. **(B)** Relative mRNA expression of CYP8B1 in livers after SD.

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frontiers in Neuroscience

REVIEW
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Biological Timing and Neurodevelopmental Disorders: A Role for Circadian Dysfunction in Autism Spectrum Disorders

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Autism spectrum disorders (ASDs) are a spectrum of neurodevelopmental disorders characterized by impaired social interaction and communication, as well as stereotyped and repetitive behaviors. ASDs affect nearly 2% of the United States child population and the worldwide prevalence has dramatically increased in recent years. The etiology is not clear but ASD is thought to be caused by a combination of intrinsic and extrinsic factors. Circadian rhythms are the ∼24 h rhythms driven by the endogenous biological clock, and they are found in a variety of physiological processes. Growing evidence from basic and clinical studies suggest that the dysfunction of the circadian timing system may be associated with ASD and its pathogenesis. Here we review the findings that link circadian dysfunctions to ASD in both experimental and clinical studies. We first introduce the organization of the circadian system and ASD. Next, we review physiological indicators of circadian rhythms that are found disrupted in ASD individuals, including sleep–wake cycles, melatonin, cortisol, and serotonin. Finally, we review evidence in epidemiology, human genetics, and biochemistry that indicates underlying associations between circadian regulation and the pathogenesis of ASD. In conclusion, we propose that understanding the functional importance of the circadian clock in normal and aberrant neurodevelopmental processes may provide a novel perspective to tackle ASD, and clinical treatments for ASD individuals should comprise an integrative approach considering the dynamics of daily rhythms in physical, mental, and social processes.

Keywords: circadian rhythms, autism spectrum disorders, melatonin, cortisol, serotonin, clock genes, mTOR, sleep

INTRODUCTION

Circadian rhythms are evolved as a result of the axial rotation of the earth and have been observed in almost all living organisms including human beings. The approximately 24 h rhythms are intrinsically driven by circadian clocks but are entrained by environmental cues such as light (Reppert and Weaver, 2002). Many neurophysiological processes exhibit robust daily fluctuations in their functional states. In humans, language, learning, memory, and social behavior adapt to the

sleep–wake cycles, and the performance in all these activities exhibits daily fluctuations (Amir and Stewart, 2009). The functional significance of the circadian clock is being increasingly appreciated as circadian dysfunctions have been linked to an increasing number of human diseases including metabolic syndromes, cardiovascular diseases, diabetes, and cancer (Rijo-Ferreira and Takahashi, 2019). Anomalies in timing have been observed in neurological and psychiatric diseases including seasonal affective disorders, bipolar disorder, and schizophrenia, etc. (Wehr et al., 2001; Wulff et al., 2012; Logan and McClung, 2019). In neurodegenerative diseases such as Alzheimer's disease, the disruption of daily activity rhythms is often associated with or even precedes underlying pathophysiological changes in the brain (Duncan, 2020). In fact, disruption of daily rhythms is the leading cause of institutionalization of individuals with Alzheimer's disease (Musiek et al., 2015). Thus, a key role for circadian regulation/deregulation in neurological and psychiatric disorders is emerging in recent decades.

Autism spectrum disorders (ASDs) are a compilation of neurodevelopmental disorders defined by behavioral abnormalities (Mughal et al., 2020; American Psychiatric Association DSM-5). Growing evidence indicates dysfunction of the endogenous circadian system is associated with the neural dysfunctions prevalent in the development of ASD. Studies on the circadian clock and sleep in ASD improve our understanding of its pathogenesis and inspire potential chronotherapeutic strategies to treat or prevent the diseases. In this review, we discuss the involvement of the circadian timekeeping system in the development and functionality of the nervous system, and summarize evidence indicating underlying links between the circadian clock and ASD. We first introduce the organization of the circadian system and ASD. Next, we review physiological parameters of endogenous rhythms that are found disrupted in ASD patients, including the sleep/wake cycle, and the daily oscillations of the circadian biomarkers melatonin, cortisol and serotonin. Finally, we review evidence indicating underlying links between circadian dysfunction and ASD pathogenesis, including epidemiology, human genetics, and the mTOR pathway.

The Circadian Timekeeping System

The term "circadian" was originally coined by Franz Halberg from the Latin root *circa* meaning "around" and *diem* meaning "day." Circadian rhythms refer to the approximately 24 h rhythms that are found in a variety of physical, mental, or behavioral processes (Halberg, 1969; Pittendrigh, 1993). The rhythms are endogenously driven by circadian clocks, which are oscillating proteins in cells that are found in nearly all living organisms (Rosbash, 2009). A variety of physiological events are regulated by circadian clocks and exhibit circadian rhythms, including the sleep–wake cycles, core body temperature, blood pressure, hormone secretion, and cognition (Patke et al., 2020). Circadian rhythms are found at every level of the organization of life: cellular, tissue, organ, and organismal level (Takahashi et al., 2008).

The oscillations of the cellular clock are driven by transcriptional-translational feedback loops (TTFLs) (Rosbash, 2009). In mammals, TTFLs are driven by rhythmic oscillations of about a dozen clock genes and their protein products (**Figure 1**), including two *Period* genes (*Per1* and *Per2*), two *Cryptochrome* genes (*Cry1* and *Cry2*), *Clock, Bmal1, Rev-erbα/β*, *Rorα/β/γ,* and *CkIε/δ* (Takahashi et al., 2008). The CLOCK and BMAL1 proteins are activators and form a heterodimer to bind E-box enhancers in the promoters of *Per* and *Cry* genes. PER and CRY proteins are synthesized during the day and form a protein complex which accumulates in the cytoplasm during the afternoon and evening. Upon reaching a certain level, the PER-CRY complexes translocate into the cell nucleus during the nighttime and block the activities of the CLOCK: BMAL1 heterodimer to inhibit their own gene transcription (Shearman et al., 2000; Ramanathan et al., 2006; Yan et al., 2020). In addition, the CLOCK: BMAL1 complex also promotes the transcription of *Rev-erbα/β* and *Rorα/β/γ*. REV-ERBα/β in turn inhibits *Bmal1* transcription whereas RORα/β/γ promotes *Bmal1* transcription (Preitner et al., 2002). In this way, the CLOCK: BMAL1 heterodimer is a self-regulator.

The abundance of PER proteins is also controlled at the level of mRNA translation by an eIF4E-dependent mechanism. Rhythmic phosphorylation of eIF4E by the mitogen-activated protein kinase-interacting kinases (MNKs) promotes mRNA translation of *Per1* and *Per2* (Cao et al., 2015). At the posttranslational level, levels of PER and CRY proteins are regulated by phosphorylation and ubiquitination-mediated protein degradation (Hirano et al., 2013; Yoo et al., 2013). CKIε and CKIδ phosphorylate PER (Lee et al., 2001; Meng et al., 2008; Etchegaray et al., 2009; Lee et al., 2011), whereas AMPK phosphorylates CRY (Lamia et al., 2009). Phosphorylation of PER and CRY proteins promotes their degradation and speeds up the clock. Although intracellular clock mechanisms are thought to be conserved in different cells, intercellular coupling mechanisms are unique between neurons and glial cells in the suprachiasmatic nucleus (SCN) and confer robustness and precision to the SCN clock (Aton and Herzog, 2005; Hastings et al., 2018). When SCN cells are isolated, the cell autonomous oscillations are poorly organized (Welsh et al., 1995; Herzog et al., 1998; Patton et al., 2016). Numerous body clocks are orchestrated by the SCN pacemaker in the hypothalamus, which is a pair of tear-drop-like structures in the inferior portion of the brain composed of ∼20,000 neurons (Moore et al., 2002; Hastings et al., 2018). The neurons express the neuropeptide vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) in the core (ventral) region of the SCN, and arginine vasopressin (AVP) in the shell (dorsal) region. The astrocytes are regulatory cells to the neurons, and primarily utilize the neuro-excitatory molecule glutamate at night to inhibit SCN neuron activity (Brancaccio et al., 2017).

The circadian clocks are entrained by external signals called zeitgebers to synchronize themselves with the ever-changing environment (**Figure 2**). The SCN utilizes light as its primary zeitgeber. SCN receives photic information from the intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina (Berson et al., 2002). The ipRGCs express the photopigment melanopsin and their axons form the retinohypothalamic tract (RHT) that terminates in the SCN. The RHT pathway is separated

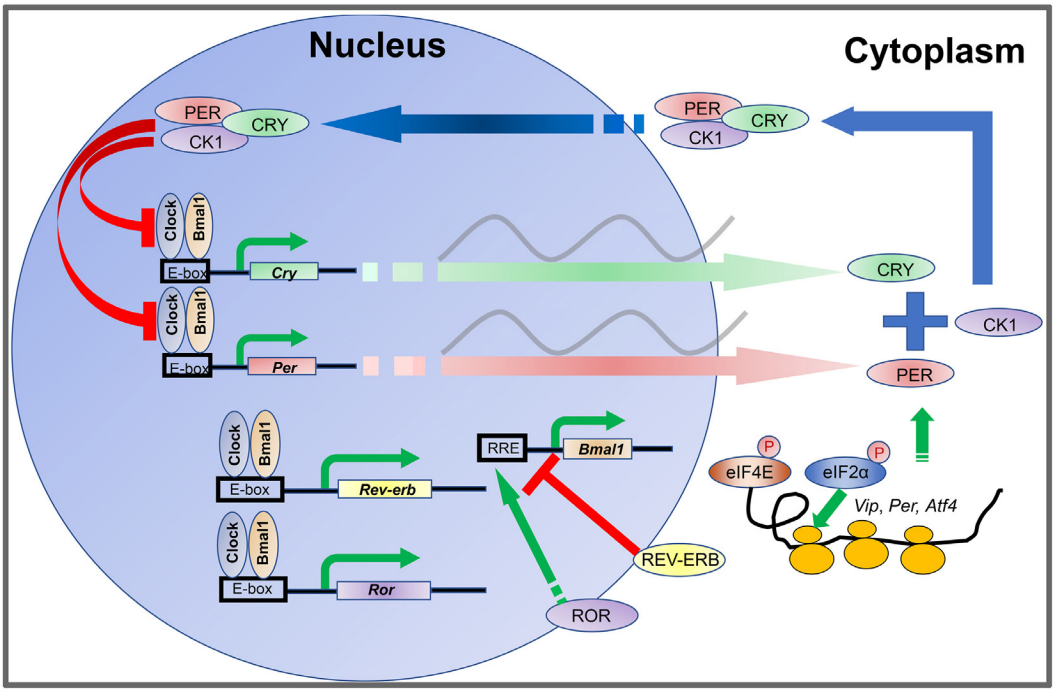

FIGURE 1 | Transcription-translation feedback loops (TTFLs) in the mammalian circadian clock. The CLOCK and BMAL1 proteins are activators and form a heterodimer to bind to E-box enhancers in the promoters of *Per* and *Cry* genes. PER and CRY proteins are synthesized during the day and form a protein complex which accumulates in the cytoplasm during the afternoon and evening. Upon reaching certain level, the PER-CRY complexes translocate into the cell nucleus during the nighttime and block the activities of the CLOCK: BMAL1 heterodimer to inhibit their own gene transcription. In addition, the CLOCK: BMAL1 complex also promotes the transcription of *Rev-erb* and *Ror*. REV-ERB in turn inhibits *Bmal1* transcription whereas ROR promotes *Bmal1* transcription. The abundance of PER proteins is controlled at the level of mRNA translation by rhythmic phosphorylation of eIF4E. Phosphorylation of eIF2α promotes translation of *Atf4*. ATF4 directly activates *Per2* transcription. At the posttranslational level, levels of PER and CRY protein are regulated by phosphorylation and ubiquitination-mediated protein degradation CKI phosphorylates PER. Phosphorylation of PER and CRY proteins promotes their degradation and speeds up the clock.

from the image forming visual pathway (Provencio et al., 2002; Peirson and Foster, 2006). The RHT terminals form direct synaptic connections with the core SCN neurons that express the neuropeptides VIP or GRP. Upon photic stimulation at night, RHT terminals release glutamate and the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) that are the neurotransmitters functioning to evoke clock gene expression and reset the SCN clock by regulating intracellular signaling pathways (Obrietan et al., 1998; Butcher et al., 2002; Hannibal, 2002).

Besides light, non-photic inputs (electrical stimulation, odor, etc.) can also influence the SCN through two brain regions, the intergeniculate leaflets (IGL) and the dorsal/median raphe nucleus (DRN/MRN) (Rusak et al., 1989; Meyer-Bernstein et al., 1997). The afferent pathway from the IGL is the geniculohypothalamic tract (GHT), and the DRN/MRN communicates with the SCN through serotonergic neurons (Meyer-Bernstein and Morin, 1996; Moga and Moore, 1997). Besides these inputs, other external cues such as social activities, exercise, and temperature have been examined as zeitgebers to the sleep-wake cycle in adult humans, but there are critiques of the role for social zeitgebers beyond their role of light regulation (Korczak et al., 2008). The SCN communicates internally with peripheral tissues through neural and endocrine outputs, i.e., electrical signals, neurotransmitters, and hormones (Kalsbeek et al., 2006). The SCN resets the peripheral clocks via these output signals. The peripheral clocks can also be reset by extrinsic and intrinsic cues that are relevant to their physiological functions. For example, adrenal hormones and feeding schedule are of notable importance to liver clock gene rhythms (Su et al., 2016). The timing of physical activities is a cue for the skeletal muscle clock (Wolff and Esser, 2012). The peripheral clocks regulate local physiology and help to orchestrate the organismal function by synchronizing rhythms in various systems. Thus, by synchronizing with the environmental light-dark cycles, the SCN clock orchestrates rhythms in different systems and coordinates various physiological processes and systemic well-being.

Autism Spectrum Disorders (ASDs)

Autism spectrum disorders are a group of developmental disabilities diagnosed by core behavioral symptoms including trouble with social interaction, abnormal communication skills, and atypically restricted, stereotyped, repetitive behaviors (American Psychiatric Association DSM-5, 2013). Children are now commonly diagnosed by 3 years of age, which is earlier than in the past (Mazurek et al., 2014). Clinical symptoms exhibited by autistic children are not uniform. Children with autism have social developmental problems and exhibit interest

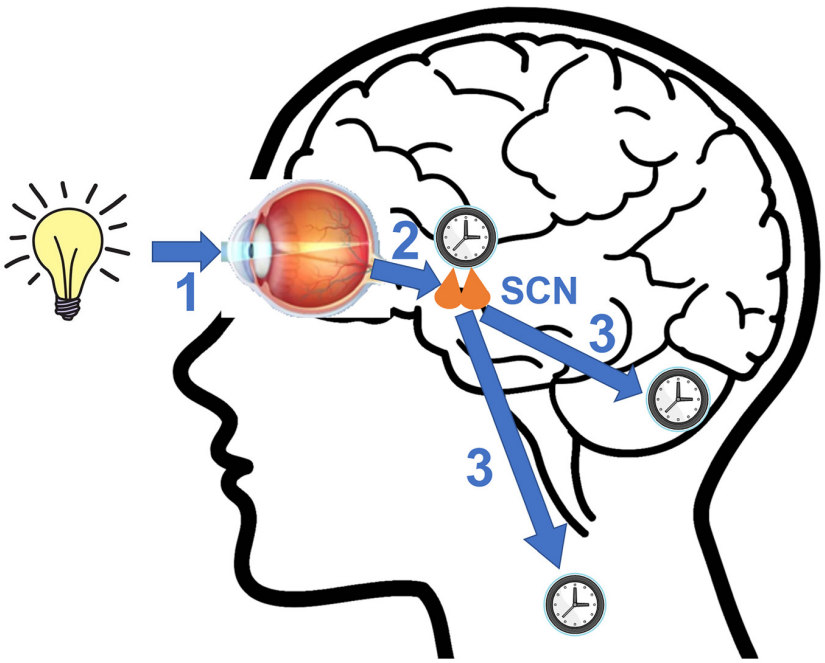

FIGURE 2 | A diagram illustrating key steps involved in photic entrainment of the circadian system. **(1)** Ambient light stimulates intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina. **(2)** The axons of ipRGCs travel via the retinohypothalamic tract (RHT) to form synaptic connections with the core neurons of hypothalamic suprachiasmatic nucleus (SCN). Glutamate and pituitary adenylate cyclase activating polypeptide (PACAP), among other neurotransmitters are released at the synapses of the RHT terminals to the SCN neurons. Synaptic activities induce clock gene expression and reset the SCN clock. **(3)** SCN sends rhythmic outputs to other brain regions and peripheral oscillators to reset their rhythms.

toward repetitive behavioral processes (Bodfish et al., 2000). Developmental deficits in ASDs have been confirmed by studies finding abnormalities in both prenatal and postnatal brain development (Carper et al., 2002; Hazlett et al., 2005; Bonnet-Brilhault et al., 2018). Their delay in development of communication and restricted interests is thought to be correlated to the severity of the anomalies in the brain (Anderson et al., 2009). ASD is often accompanied by intellectual disability and hyperactivity. In addition, children with ASD commonly exhibit comorbid medical conditions, including abnormal tactile sensation, food selectivity, and sleep disruption (Baranek et al., 2006; Ben-Sasson et al., 2007; Souders et al., 2009). Currently there is no unified theory to explain all core and comorbid abnormalities in ASD children.

The incidence of ASD has dramatically increased around the globe in the past 50 years (World Health Organization, 2017). For example, according to a British study, autism incidence rate was 4.5 per 10,000 children in the 1960s (Lotter, 1966). In the 1980s, the prevalence of autism was between 5 and 12 in 10,000 persons (Gillberg et al., 1991). In the U.S., the frequency of the autism has risen from 3 per 10,000 individuals in 1991–1992 to 53 per 10,000 children in 2003–2004 (Gurney et al., 2006). According to estimates from CDC's Autism and Developmental Disabilities Monitoring Network, about 1 in 54 children are diagnosed with ASDs in 2014[1]. Notably, the diagnostic criteria for ASD was changed in the DSM-5 published in 2013, and an expanded group of disorders are now classified as ASDs (American Psychiatric Association DSM-5, 2013). ASDs now include several conditions that used to be diagnosed separately including autistic disorder, pervasive developmental disorder not otherwise specified (PDD-NOS), and Asperger syndrome. The high prevalence of ASDs is accompanied by unprecedented social and economic burdens on the affected families and society (Newschaffer et al., 2007). The yearly total costs for children with ASD were estimated to be between $11.5 and $60.9 billion in the U.S. The clinical expenses of autism children are comparatively higher than normal children and are ten times greater than the costs of normal children's medical expenditure (Mandell et al., 2006). Children with ASD cost more than those without ASD by $4,110–$6,200 per year. Thus, there is an urgent need to find novel therapeutic strategies to tackle these diseases.

The etiology of ASD remains elusive, but it is thought to be a combination of extrinsic and intrinsic factors (**Figure 3**). Less than 20% of ASDs have an identifiable genetic origin, whereas over 75% of cases are idiopathic, suggesting a multifactorial etiology (Abrahams and Geschwind, 2008). The discovery of single nucleotide variants (SNVs) and copy number variants (CNVs) in genes associated with ASD supports the claim for a genetic basis of ASD etiology and over 1200 risk genes have been identified (Xiong et al., 2019[2]). In addition, epigenetic and immunological factors are also speculated as possible causes of autism (Lee et al., 2015; Sun et al., 2016). An increased risk

[1]www.cdc.gov

[2]https://gene.sfari.org/

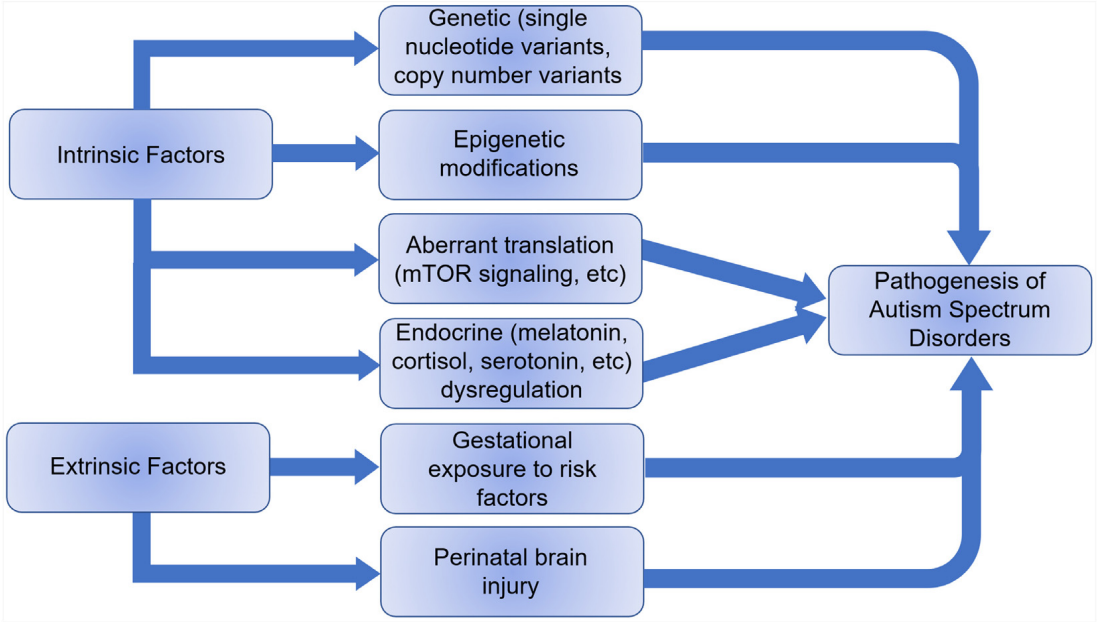

FIGURE 3 | Intrinsic and extrinsic factors that can lead to the pathogenesis of autism spectrum disorders.

of ASD with advanced paternal age coupled to an increased rate of DNA methylation abnormalities in older fathers at multiple imprinted gene loci suggests an epigenetic association (Kong et al., 2012; Smith et al., 2013). Animal models have shown transgenerational aberrant DNA methylation and histone modifications with abnormal neurodevelopment as a result of abnormal nutrition, stress and drugs, as well as transplacental psychiatric medication affecting GABAergic, dopaminergic, serotonergic, and glutamatergic pathways (Franklin et al., 2010; Morgan and Bale, 2011). Besides genetic and epigenetic factors, development of ASD can be influenced by embryonic exposure to detrimental environmental factors including pollution, maternal stressors, etc. (Becerra et al., 2013; Volk et al., 2013; Walder et al., 2014). Perinatal brain injury, especially cerebellar injury, can also contribute to autism development (Singh et al., 2016).

Multiple theories have been proposed regarding the neural mechanisms underlying ASD pathogenesis, but no theory has convincingly integrated the diverse behavioral dysfunctions in autism. Aberrant neurotransmission of dopamine, glutamate, serotonin, oxytocin/vasopressin and GABA have all been implicated in the development of ASD (Modahl et al., 1998; Blatt et al., 2001; Chandana et al., 2005). A number of studies suggest that glutamate systems are dysfunctional in ASD (Purcell et al., 2001; Shinohe et al., 2006; Rojas, 2014). The dysfunction of the GABAergic system, synthesized from glutamate, has been suggested to result in impaired cognitive and motor function as well as seizure disorder, a comorbidity of autism (Russo, 2013; Rojas, 2014). Excessive dopamine receptor DRD1a activation has been shown to elicit autistic behaviors in mouse models (Lee et al., 2018). The increased prevalence of ASD in recent decades cannot be simply explained by reclassification and increased diagnosis. Neither can it be completely ascribed to genetic factors that cause the disease. Apparently, a combination of extrinsic and intrinsic factors should be investigated in the development of autism.

DISRUPTION OF SLEEP AND DAILY RHYTHMS IN ASD

Sleep Problems in ASD

Sleep is a conserved physiological process in animals that is critical for brain development and maturation. Suboptimal sleep can have adverse effects on children's cognitive functions including attention, memory, mood regulation, and behavior (Pilcher and Huffcutt, 1996; Belenky et al., 2003). Children with ASD exhibit sleep problems at a higher rate than children with other developmental disorders as well as typically developing children (Owens et al., 2000; Cotton and Richdale, 2006; Johnson and Zarrinnegar, 2021). Repeated sleep disruption adversely affects the process of neural development in ASD children, whereas impaired neurodevelopment further exacerbates the sleep problem in ASD.

It is estimated that 50∼80% ASD children have sleep problems, compared to less than 30% in the general children population (Souders et al., 2017). Prolonged sleep latency, frequent waking at night, alterations in sleep architecture, unusual morning arousal and reduction in total sleep duration are commonly found in children with autism (Richdale, 1999; Polimeni et al., 2005). A reduced percentage of REM sleep and higher percentage of slow-wave sleep has been observed in association with ASD (Buckley et al., 2010). There is also a report of a higher rate of REM sleep behavior disorder in autistic children (Thirumalai et al., 2002). Atypical REM sleep patterns indicate disruption in central nervous system maturation and

neuronal network organization, as well as atypical synapse homeostasis involved in sleep–wake function (Buckley et al., 2010). Notably, sleep problems may differ between different types of ASD patients. In a study by Richdale and Prior (1995), it was found that low functioning (IQ < 55) ASD individuals showed increased naps, earlier time going to sleep, increased sleep latency, increased sleeping time at night, and increased total sleeping time over 24 h compared to controls. By contrast, high functioning (IQ > 55) ASD individuals exhibited increased sleep latency, decreased total night sleep, increased length of waking episodes at night, and earlier wake time. A more detailed review on sleep problems in ASD has been published recently (Karthikeyan et al., 2020).

The possible causes for the sleep problems in ASD can be classified into four categories. (1) Synaptic protein abnormalities. Sleep and synaptic functions are tightly interconnected. Sleep relies on normal functionality of complex neural circuitries with synapses as the connectors between neurons (Scammell et al., 2017). Proper sleep is essential for synaptogenesis and synaptic plasticity (Cirelli and Tononi, 2019). The disruption of the Neurexin/Neuroligin/Shank synaptic protein complex has been found to be involved in ASD (Jamain et al., 2003; Durand et al., 2007). Neuroligins and Neurexins are synaptic proteins of excitatory glutamatergic and inhibitory GABAergic synapses. Neuroligin-1/3/4 are confined to glutamatergic synapses whereas neuroligin-2 is specific to GABAergic synapses (Graf et al., 2004; Varoqueaux et al., 2004). Neurexins, encoded by *Nrxn 1/2/3*, are a family of presynaptic cell adhesion proteins that interact with Neuroligins to connect neurons at the synapse. Mutation of Shank3, a gene encoding a scaffolding protein on the postsynaptic membrane that tethers Neuroligins and regulates dendritic organization, has been shown to be associated with autism (Durand et al., 2007). Furthermore, a *de novo* deletion on chromosome 2p16 encoding Neurexin-1 was identified in ASD (Szatmari et al., 2007). Interestingly, Neuroligins, Neurexin, and Shank3 have all been shown to regulate the sleep architecture and clock gene expression in mouse models (El Helou et al., 2013; Tong et al., 2016; Seok et al., 2018; Ingiosi et al., 2019). It is possible that dysregulated synaptic proteins link sleep disorders to the development of autism. (2) Sensory dysregulation and increased arousal (Wiggs and Stores, 2004; Souders et al., 2009). This can be explained by two hypotheses, cognitive arousal and physiological arousal. Cognitive arousal, which is caused by increased cognitive activities due to increased anxiety in ASD, can increase the sleep latency. Physiological arousal is caused by increased responses to environmental stimuli due to low sensory thresholds in children with ASD, and can result in difficulty falling or staying asleep (Mazurek and Petroski, 2015). (3) Abnormal sleep-regulating hormones. Abnormal levels of hormones such as melatonin in ASD will be discussed in "Disruption of Circadian Biomarkers in ASD." (4) Circadian sleep disruptions. Mutations in genes that regulate circadian timing can also cause changes in the timing and duration of sleep in ASD and will be discussed in "Clock Gene Polymorphisms in ASD." It is important to recognize the possible overlaps in causes, and their additive or amplified effects on the complex sleep problems in ASD.

Disruption of Circadian Biomarkers in ASD

In clinical studies, levels of traditional circadian biomarkers including melatonin and cortisol are measured from biological specimens such as blood, urine, and saliva at different times of day in order to assess the functions of the body clock. Melatonin is a pineal hormone with daily rhythmic synthesis that peaks at night and is suppressed by light during the day (Socaciu et al., 2020). Cortisol is a sterol hormone that peaks in the early morning and falls throughout the day (Russell and Lightman, 2019). Serotonin is a monoamine neurotransmitter and also the intermediate product to synthesize melatonin (Comai et al., 2020). Here we discuss how levels and daily rhythms of these biomarkers are changed in ASD, and how abnormalities in these hormones may in turn contribute to neural dysfunctions in ASD individuals. The abnormalities of these biomarkers have been summarized in **Table 1**.

Melatonin

Melatonin is a neurohormone synthesized in the pineal gland. Melatonin levels are normally higher at night than during the day in both nocturnal and diurnal animals. Melatonin induces sleep and resets the SCN circadian clock (McArthur et al., 1991; Zhao et al., 2019). Sleep phase and duration is determined by the phase of the melatonin cycle suggesting a key role for melatonin in regulating the sleep–wake cycle (Lockley et al., 1997). Melatonin has direct effects on the SCN circadian clock through its two G protein coupled receptors MT1 and MT2. MT1 is the high affinity receptor responsible for acute suppression of neuronal firing and MT2 is the low affinity receptor required for efficient phase-shifts (Liu et al., 1997; Jin et al., 2003). Binding of melatonin to MT1 and MT2 indirectly regulates clock gene expression by inhibition of adenylate cyclase, and inhibition of PKA due to reduction of cAMP. The G protein coupled receptors also directly inhibit phosphorylation of cAMP response element-binding protein (CREB) (Ross et al., 1996; von Gall et al., 2002). CREB inhibition causes decreased expression of clock proteins PER1 and PER2 and attenuates photic entrainment of the circadian clock (Lee et al., 2010). Melatonin signaling disruption has been linked to sleep disorders such as insomnia, and has been reported in neurological and psychiatric conditions such as Parkinson's disease and depression (Claustrat et al., 1984; Adi et al., 2010). There is also evidence that melatonin is involved in neural differentiation, and its dysfunction in ASD individuals could contribute to their non-typical development (Shu et al., 2016).

Melatonin is the most well documented circadian biomarker associated with ASD. Lower levels of melatonin and its major metabolite, urinary 6-sulfatoxymelatonin, have been found in the urine, serum, and plasma of ASD individuals (Nir et al., 1995; Tordjman et al., 2005; Melke et al., 2008). Low melatonin amplitude and a delayed melatonin rhythm have been associated with increased sleep problems in ASD children (Kulman et al., 2000; Melke et al., 2008). Excretory levels of the metabolite 6-sulphatoxymelatonin were decreased in a group of 50 autistic children and these decreased concentrations were associated with

TABLE 1 | Circadian dysfunctions in ASD.

ASD patients and control	Circadian biomarkers	Findings	References
Age: Mean = 9 years **Number(sex):** 19 (M), 3 (F) **Control:** Six adults (mean age = 30 years), 5 (M) and 1 (F); 27 children (mean age = 9 years), 15 (M) and 12 (F) **Other factors:** 15 highly developed and 7 poorly developed ASD cases based on IQ 60	Cortisol in saliva and blood	Abnormal diurnal rhythm of salivary cortisol (higher peak in the morning) and lower response in dexamethasone suppression test in ASD vs. control, especially in poorly developed cases	Hoshino et al., 1987
Age: 4–19 years, mean = 10.2 years **Number(sex):** 30 in total, no sex data **Control:** 106 children, aged 1–19 years, mean = 9.7 years; 17 adults, aged 20–55 years, mean = 35.5 years	Serotonin in blood	(1) Summer serotonin levels in ASD significantly are lower compared to other seasons. (2) Average serotonin level in ASD is significantly higher than controls.	Badcock et al., 1987
Age: 4–14 years, mean = 8.3 years, **Number(sex):** 14 (M), 4 (F) **Control:** 16 (M), 3 (F)	Cortisol in urine	Increased cortisol levels at all times of day, particularly morning to mid-afternoon	Richdale and Prior, 1992
Age: Mean = 18 years **Number(sex):** 10 in total, no sex data **Control:** 15 parents, 1 grandparent, 9 siblings, and 10 unrelated healthy individuals **Other factors:** Control were significantly older than autism group	Melatonin in urine	Increased daytime melatonin level and ratio of daytime/nighttime melatonin levels compared to controls	Ritvo et al., 1993
Age: 16–30 years **Number(sex):** 10 (M) **Control:** 5 matched in age and weight	Melatonin in blood	(1) Melatonin levels in ASD higher during the day and lower at night vs. controls (2) No differences in cortisol levels	Nir et al., 1995
Age: 3–23 years, mean = 9.2 years **Number(sex):** 42 (M), 20 (F) **Control:** 91 in total, aged 2–16 years, age and sex matched **Other factors:** Relatives of autism patients were also examined for serotonin levels	Serotonin in blood	(1) Higher serotonin levels in ASD vs. control above age 16 (2) No difference in serotonin levels between ASD and control below age 16 (3) Distribution of serotonin levels significantly more variable in ASD than control (4) Serotonin levels in control decrease with age, while serotonin levels in ASD is independent of age	Leboyer et al., 1999
Age: Mean = 8.5 years **Number(sex):** 12(M) **Control:** 10 (M), mean age = 9.2 years **Other factors:** Groups were matched on age and gender but not on IQ. Mean IQ of autism group = 77, and mean IQ of normal group = 114	Cortisol in saliva	(1) No significant difference in mean cortisol daily variation between children with autism and typically developing children (2) Children with autism showed significantly increased response to a non-social stressor (mock MRI), while typically developing children showed no response in cortisol level	Corbett et al., 2006
Age: 14.8 $\pm$ 7 years **Number(sex):** 29 (M), 14 (F) **Control:** 45 (M), 30 (F), sex and age matched. Thirty four parents of ASD patients were also examined.	Asmt mutations, melatonin and serotonin in blood and platelets	(1) Non-conservative variations of Asmt (the gene encoding the last enzyme of melatonin synthesis) identified in ASD families but not in controls. Two polymorphisms located in the promoter were more frequent in ASD compared to controls associated with a decrease in ASMT transcripts in blood cell lines (2) Decreased in ASMT activity and melatonin levels in individuals with ASD and damped melatonin daily rhythms in ASD (3) Increased serotonin levels in ASD and their parents compared to controls (4) Poor sleep efficiency and higher arousal index but normal REM and slow wave sleep in patients with ASMT mutations	Melke et al., 2008
Age: Mean = 9.08 years, Range = 6.5–12 years **Number(sex):** 21(M), 1(F) **Control:** 19(M), 3(F) **Other factors:** Cortisol levels were measured in anticipation and response to a stressful event (mock-MRI)	Cortisol in saliva	(1) Children with autism showed consistently higher cortisol levels in the evening (2) Diurnal variations of cortisol are more inconsistent in autism individuals	Corbett et al., 2008
Age: Mean = 9.1 years **Number(sex):** 13(M), 2(F) **Control:** 21(M), 4(F), aged 6–12 years	Cortisol in saliva	No significant difference in the cortisol awakening response between individuals with high functioning autism and controls	Zinke et al., 2010
Age: 2–5 years, mean = 3.75 years **Number(sex):** 22(M), 4(F) **Control:** 23(M), 3(F), mean age = 3.3 years	Cortisol in saliva	(1) Moderately increased mean cortisol secretion levels in autism children upon waking compared to controls (not statistically significant $p > 0.05$) (2) Mildly increased mean cortisol in autism children during daytime and evening compared to controls (not statistically significant $p > 0.05$)	Kidd et al., 2012
Age: Mean = 10.3 years **Number(sex):** 47 in total, 35 autistic disorder, 10 Asperger syndrome, five pervasive development, no sex data included **Control:** 50 in total, mean = 9.9 years	Cortisol in saliva	No differences in cortisol levels at any given time point for ASD children when compared with controls	Corbett and Schupp, 2014

(Continued)

TABLE 1 | Continued

ASD patients and control	Circadian biomarkers	Findings	References
Age: Mean = 10.2 years **Number(sex):** 30(M), 6(F) **Control:** 23(M), 4(F), mean = 9.71 years	Cortisol in saliva	(1) Higher overall cortisol levels in ASD than control (2) Higher cortisol levels in ASD in the evening compared to controls (3) Flatter diurnal cortisol rhythm in some ASD children	Tomarken et al., 2015
Age: LFASD mean = 9.23 years, HFASD mean = 9.38 years **Number(sex):** LFASD 13(M), HFASD 16(M) **Control:** 14(M), mean age = 9.36 years	Cortisol in saliva	(1) Children with low functioning ASD (LFASD) demonstrated higher cortisol levels at morning, afternoon, and evening compared with children with high functioning ASD (HFASD) and normal children (2) Lower cortisol levels in HFASD individuals in the morning than typically developing individuals	Putnam et al., 2015
Age: Mean = 7.51 years **Number(sex):** 35(M), 8(F) **Control:** 30(M), 10(F), mean = 7.83 years	Cortisol in saliva and serotonin in blood	(1) Elevated cortisol levels in ASD compared with control (2) Elevated serotonin levels in ASD compared with control (3) Flattened cortisol diurnal rhythms in ASD compared with control	Yang et al., 2015

their verbal and play abilities (Tordjman et al., 2005). Seizure comorbidities and electroencephalogram (EEG) discrepancies in autism individuals have been associated with the aberrant phase cycles of melatonin (Nir et al., 1995). Increased levels of melatonin have been found during the daytime in small samples of autistic children, whereas no significant difference was reported in nighttime concentration (Ritvo et al., 1993; Kulman et al., 2000). Low overall levels of melatonin and a sharp increase in concentration during the daytime was reported in a study of 14 autistic children (Kulman et al., 2000). Interestingly, 10 of the 14 autistic individuals in the study exhibited no observable daytime rhythmic changes in their blood melatonin levels (Kulman et al., 2000). Unusual patterns of melatonin in both amplitude and phase suggests fundamental impairments of the body circadian clock. As the level of melatonin is in general decreased in ASD individuals, melatonin supplements at night before bedtime may help with the sleep problems in ASD. In one study, melatonin administered 30 min before bedtime improved sleep latency in ASD children (Malow et al., 2012).

The mechanisms underlying melatonin abnormalities in ASD remain elusive and is a topic still under investigation. It is unclear whether the total amount of melatonin is reduced in a circadian period or if the phase has been altered by the abnormal circadian clock (Tordjman et al., 2005). There may also be abnormalities in melatonin synthesis, regulation, or receptor binding and efficacy in ASD. There are three main G-protein coupled receptors (GPCR) receptors involved in melatonin signaling: *MNTR1A* (MT1), *MNTR1B* (MT2), and the orphan receptor *GPR50*, which has no affinity for melatonin, but inhibits melatonin signaling when bound to MT1 (Chaste et al., 2010). When individuals with ASD were screened for mutations in the genes encoding melatonin receptors, no significant difference was found compared to controls, indicating that abnormal melatonin production rather than abnormal receptor function may be involved in ASD. Thus, rectifying melatonin levels using exogenous melatonin is a plausible therapeutic strategy. Indeed, clinical evidence exists demonstrating high efficacy of melatonin treatment for ASD individuals with sleep disruption (Chaste et al., 2010; Malow et al., 2012).

It is likely that melatonin disruption in a significant number of ASD individuals is due to dysfunction of melatonin synthesis. There are two enzymatic steps in the conversion of serotonin into melatonin: the conversion of serotonin to *N*-acetylserotonin by the enzyme Serotonin *N*-acetyl transferase (SNAT), and the conversion of *N*-acetylserotonin into melatonin by the enzyme Acetylserotonin *O*-methyltransferase (ASMT) (**Figure 4**). 14-3-3 is a family of conserved regulatory proteins that bind to a variety of signaling proteins. It has been proposed interaction of the protein 14-3-3 with SNAT, and more importantly 14-3-3 with ASMT, is necessary for melatonin synthesis (Obsil et al., 2001; Maronde et al., 2011). The protein miR-451, a known suppressor of 14-3-3, was elevated in ASD individuals (Pagan et al., 2017). An increasing body of evidence supports a disruption of the 14-3-3/ASMT/SNAT 'melatoninosome' in ASD individuals (Obsil et al., 2001; Maronde et al., 2011). Slow metabolization of melatonin may lead to accumulation of melatonin in the body and cause sleep problems. The liver cytochrome P450 enzyme, CYP1A2, has been demonstrated to be the primary metabolizing enzyme of melatonin in the liver (Facciolá et al., 2001). In the small number of ASD individuals where exogenous treatment with melatonin loses effectiveness, high level of melatonin was found around noon. It has been hypothesized this is due to a single nucleotide polymorphism (SNP) in CYP1A2 (Braam et al., 2013). While melatonin treatment has been shown to be effective in treating sleep–wake difficulties in ASD individuals, more research is necessary to elucidate the precise role of melatonin and its dysregulation in ASD.

Cortisol

Cortisol production is a result of a cascade response along the hypothalamic-pituitary-adrenocortical (HPA) axis in three major steps with negative feedback at each step: (1) Corticotropin releasing hormone (CRH) is released by the paraventricular nucleus (PVN) of the hypothalamus; (2) Adrenocorticotropic hormone (ACTH) is released from the anterior pituitary; and (3) Cortisol is released from the adrenal cortex (**Figure 5**). Each hormone is released in response to the action of the preceding hormones action on its respective target tissue. The HPA axis regulates hormonal stress response. Daily cortisol levels exhibit robust oscillations as PVN is innervated by the SCN. Here we discuss circadian rhythm abnormalities of cortisol in ASD individuals and whether these abnormalities are associated with

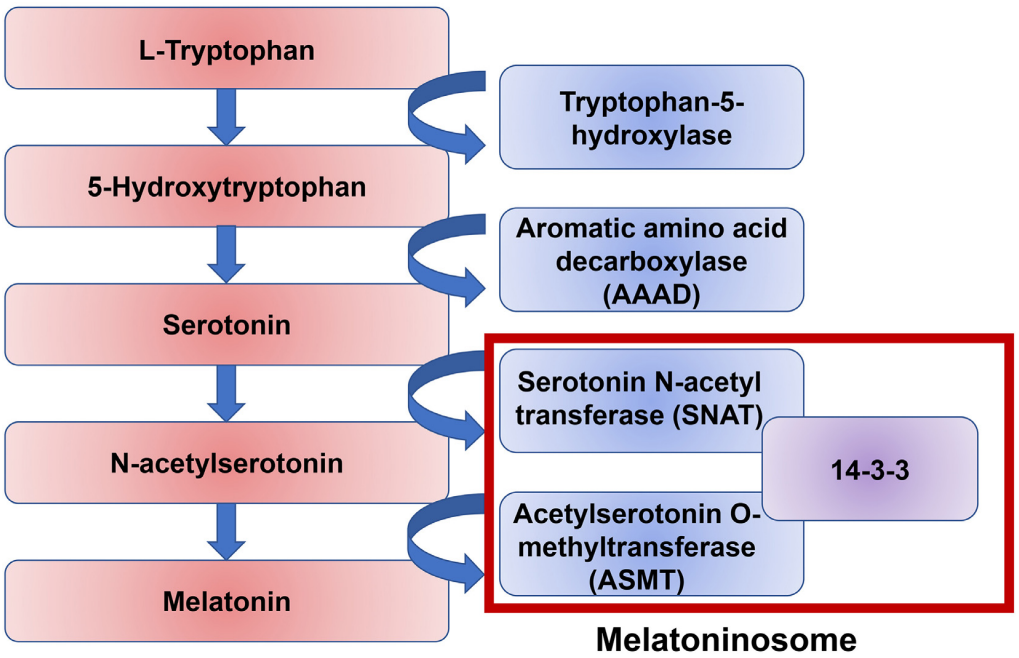

FIGURE 4 | A pathway of melatonin biosynthesis. Melatonin synthesis is the result of the amino acid tryptophan through the intermediate neurotransmitter serotonin. The conversion of serotonin to melatonin is mediated by two enzymes, serotonin *N*-acetyl transferase (SNAT) and acetylserotonin *O*-methyltransferase (ASMT). The protein 14-3-3 mediates both of these stepwise interactions.

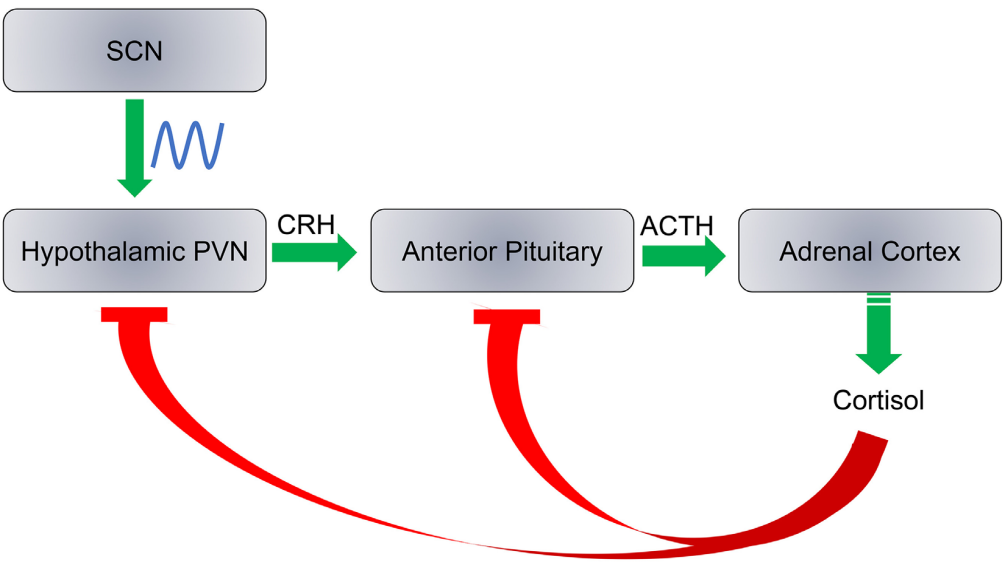

FIGURE 5 | SCN regulates the hypothalamic-pituitary-adrenocortical axis. Cortisol production is regulated by three steps: (1) Release of corticotropin releasing hormone (CRH) by the hypothalamic paraventricular nucleus (PVN); (2) the adrenocorticotropic hormone (ACTH) is released from the anterior pituitary; and (3) Cortisol is synthesized and released from the adrenal cortex. Cortisol inhibits CRH and ACTH release at a feedback mechanism. The SCN has direct neural inputs into PVN and controls the daily rhythms of cortisol levels.

ASD as a causative factor that may exacerbate traits of the disorder, or simply a result of ASD symptoms.

The PVN receives direct neural input from the hippocampus, amygdala, prefrontal cortex, and SCN (Gray et al., 1989; Boudaba et al., 1996; Li and Kirouac, 2012). Cortisol has ubiquitous physiological effects throughout the body, and has been proposed to play a key role in daily cognitive and behavioral functions. Disruption of its rhythms have been implicated in the etiology of a variety of physical and mental health disorders (Adam et al., 2017). The significance of adrenal glucocorticoids to peripheral

circadian rhythms have been demonstrated, as elimination of the adrenal glands in rats caused disruption of clock gene expression in the kidneys and corneas (Pezük et al., 2012). Interestingly, the adrenalectomy did not have a significant impact on the SCN, pituitary gland, or lungs of the rats, but introduction of hydrocortisone following adrenalectomy did have a significant impact on all circadian gene expression in each of these tissues (Pezük et al., 2012).

The blood concentration of cortisol, the human glucocorticoid stress hormone, varies across the 24 h day and possesses significant diurnal rhythms. It reaches its peak during the early morning, decreases during the day, and starts to rise at late night (Hoshino et al., 1987; Van Cauter et al., 1996). The phase and levels of cortisol can be used as reliable indicators for the endogenous circadian clock (Désir et al., 1980; James et al., 2007). The circadian clock could also be responsible for regulating the cortisol awakening response, an increase of cortisol within the first hour after awakening that is separate from the cortisol increase during the second half of the night (Vargas and Lopez-Duran, 2020). Circadian misalignment can cause deregulated cortisol production in neurotypical individuals. Even one night of sleep loss can elevate cortisol concentration, notably during the early morning and evening hours (Wright et al., 2015). Interestingly, there is some evidence for the absence of feedback on the SCN from the HPA axis (Oster et al., 2006). An abnormal central pacemaker stimulating the HPA axis at abnormal intervals with no feedback may cause abnormal cortisol profiles.

A number of studies suggest abnormalities in circadian rhythms of cortisol in ASD individuals, but the severity and type vary greatly (Yamazaki et al., 1975; Hill et al., 1977; Corbett et al., 2006). Aberrant rhythmic patterns of cortisol were found to be associated with lower functioning autistic children (Jensen et al., 1985; Hoshino et al., 1987). Additionally, one study found significantly decreased cortisol in ASD children, but elevated ACTH levels compared to typically developing subjects (Curin et al., 2003). Subsequently, the same researchers found a delayed cortisol response to artificial ACTH stimulation in ASD children compared to controls (Marinović-Curin et al., 2008). Another investigation found elevated ACTH and β-Endorphin (a hormone released concurrently with ACTH) in ASD individuals, but no difference in cortisol levels (Tordjman et al., 1997). On the contrary, one study measuring total daily urinary cortisol secretion found no abnormalities between typically developing individuals and ASD individuals (Marinović-Curin et al., 2008). This suggests that while measured cortisol levels and rhythms may be abnormal in ASD individuals, total cortisol output may be similar to typically developing individuals. The variable results across studies could be caused by the differences in investigation methods, size of the sample groups, and variation in determined cognitive function (low functioning vs. high functioning). A comprehensive future examination of the cortisol circadian rhythm in ASD individuals with larger sample sizes, standardized measurement methods, controlled environments, and age/gender diversification is warranted.

There is also evidence of abnormal sensitivity to stress in ASD individuals related to increased variability of cortisol rhythms (Corbett et al., 2009). One study examined cortisol response to amicable social interaction with a confederate among younger and older ASD children compared to neurotypical children. The investigators found a significant increase in cortisol levels for older ASD children compared to younger ASD children; this significant difference was not present in neurotypical individuals (Corbett et al., 2010). This difference could be explained by an awareness of social limitations among older ASD individuals, or a learned negative threat response to what would commonly be perceived as a neutral or positive social stimulus, leading to an increased cortisol response. Regardless of the etiology for the heightened response, the findings suggest increased susceptibility of the cortisol rhythm to external social zeitgebers in ASD children. Given the evidence for ASD symptoms impacting the cortisol circadian rhythm, the question remains if this relationship is unilateral, or can an aberrant circadian system in ASD individuals exert an atypical influence on cortisol levels and exacerbate negative symptoms. There is some intriguing evidence that abnormalities of cortisol circadian rhythms may be a function of ASD symptoms. A study in 2006 investigated salivary cortisol response to a disturbing non-social stimuli (mock MRI) in ASD individuals (IQ mean = 77) and neurotypical individuals, and found a strikingly significant cortisol increase in relation to the controls that exhibited a mean decrease in cortisol levels (Corbett et al., 2006). The finding of increased HPA response to a non-social stressor (blood draw) in ASD individuals compared to matched controls was replicated across variable measurements of cortisol including salivary, urinary, and serum (Spratt et al., 2012). A follow up to Corbett et al. (2006) examined cortisol response to a stressor as well as a subsequent exposure to the same stressor, and found increased circadian variability of cortisol in ASD individuals as well as increased cortisol levels in the evening following stressor exposure. This suggests the aberrant cortisol rhythm of ASD individuals may be more susceptible to entrainment by external non-social zeitgebers (Corbett et al., 2009). While there is evidence of abnormal circadian cortisol profiles in ASD individuals, the extent of the relationship and underlying mechanisms remains unclear and warrants further study.

Serotonin

Serotonin is produced in the central nervous system and duodenum. As serotonin cannot cross the blood-brain barrier, central and peripheral serotonergic systems are thought to be anatomically ad functionally separated (Hery et al., 1977; Ebert-Zavos et al., 2013). Serum serotonin levels exhibit diurnal variations, with a peak early in the morning and a trough in the midafternoon and during sleep (Wirz-Justice et al., 1977; Kwon et al., 2018). The diurnal oscillations of serotonin are affected by meal intake or fasting and are blunted in obese individuals (Ebert-Zavos et al., 2013; Kwon et al., 2018). An earlier study detected another peak of serotonin in the early evening (Sarai and Kayano, 1968).

In the brain, serotonin (5-hydroxytryptamine/5-HT) is synthesized in a stepwise manner from the amino acid tryptophan with two enzymes, tryptophan hydroxylase and aromatic amino acid decarboxylase (AAAD) respectively, through the intermediate, 5-hydroxytryptophan. Following

release into the synaptic cleft, serotonin is retaken back into the neuron by its transporter, 5-hydroxytryptamine transporter (5HTT/SERT), or signals via one of 15 known receptors. Serotonin can also signal through monoaminylation that has been described in the monoamines 5-HT, histamine, dopamine, and norepinephrine (Walther et al., 2003; Farrelly et al., 2019). 5-HT neurons innervate into and have regulatory control on both the SCN and the intergeniculate leaflets (IGL) (Meyer-Bernstein and Morin, 1996; Glass et al., 2000, 2003). Serotonin is integral to the regulation and development of neural systems of the brain; including neural cell growth, differentiation and development of synaptic processes (D'Amato et al., 1987; Cases et al., 1995). An imbalance of serotonin negatively affects the neocortical excitation/inhibition balance, sensory stimulus perception and social communication. Abnormal serotonin levels seem to affect the synaptic processes in the sensory cortices during the developmental period (Bennett-Clarke et al., 1994; Cases et al., 1996). Abnormalities in the 5-HT system have also been associated with disruption of the mammalian circadian system and sleep–wake cycles during development (Paulus and Mintz, 2012).

The primary resource of serotonin required for development of the forebrain in the fetus is the tryptophan concentration in the placenta of the mother (Bonnin et al., 2011). Sufficient concentrations of serotonin during the prenatal and perinatal period is a determining factor for the normal regulation of the neural system, and abnormal serotonin concentration might be integral to development of ASD. Differential levels of serotonin synthesis during the stages of development underscores the importance of serotonin in the structural development of the brain in ASD individuals (Chugani et al., 1999). Human and animal studies have found that disruption of the 5-HT system during development is particularly catastrophic to phenotypic behavioral function (Sundström et al., 1993; Anderson et al., 2002; Chen et al., 2015). Proponents of the 5-HT/brainstem theory in ASD pathogenesis have proposed manifestation of ASD as a cascade of events with multiple entry points, rather than a singular devastating event. A stepwise mechanism of this cataclysmic cascade has been proposed, with particular emphasis on a mutual disturbance of the 5-HT system and the mammalian circadian system causing downstream ASD behavioral manifestation and comorbid impairments (Takumi et al., 2020).

Studies using various methodologies from biochemical analysis, genetics, neuroimaging and pharmacology have established the abnormalities of the serotonin system in ASD (Muller et al., 2016). It is well established that serotonin levels are elevated in ASD individuals (Hoshino et al., 1984; Cook et al., 1990; Gabriele et al., 2014). One of the first markers observed in autistic children was excessive levels of serotonin present in the blood plasma and more than 25% of autistic children exhibit this aberrant level of serotonin (Gabriele et al., 2014). In children diagnosed with autism, serotonin secretion is unusual, its synthesis was significantly elevated throughout late development, and the levels of serotonin correlated with the severity of autistic symptoms (Chugani et al., 1999; Abdulamir et al., 2018). However, in another study, seven ASD boys exhibited reduction in their serotonin production in left frontal cortex and thalamus, whereas one girl was unaffected. High levels of serotonin were found in the contralateral dentate nucleus of all the autistic boys (Chugani et al., 1997). High blood serotonin was found in an analysis of studies comparing ASD children to typically developing children (Gabriele et al., 2014). This further reinstates the notion of excessive serotonin in ASD children. There is some evidence of serotonin treatment returning behavior and brain function to a more typical state in an ASD mouse model (Nakai et al., 2017). Maintaining the right concentration of serotonin rescued normal conditions from autistic symptoms in mice (Nakai et al., 2017). Furthermore, a polymorphic variant identified at the site of serotonin transporter gene could result in aberrant serotonin concentration in thalamo-cortical projections (Tordjman et al., 2001).

The serotonin synthesis pathway, its molecular interactions, and the genes responsible for these interactions have been investigated in relation to the development of ASD. There is some evidence of serotonin synthesis capacity abnormalities in developing ASD individuals (Chugani et al., 1997, 1999). Decreased transporter binding of serotonin has also been demonstrated in both ASD children and adults (Makkonen et al., 2008; Nakamura et al., 2010), but there is also contradictory evidence of no significant reduction in individuals with Asperger's Disorder (Girgis et al., 2011). Receptor binding has also been found to be reduced in ASD individuals (Murphy et al., 2006; Beversdorf et al., 2012), again with contradictory evidence among Asperger's individuals (Girgis et al., 2011). The genes for the serotonin pathway enzymes (tryptophan hydroxylase and AAAD), its transporter (5HTT/SERT), and its receptors have all been studied as candidates for ASD pathogenesis (Makkonen et al., 2008; Nakamura et al., 2010). The gene *SLC6A4* encodes the serotonin transporter (SERT), and significant variation in allele transmission to progeny of the locus HTTLPR within *SLC6A4* has been examined in ASD; particularly an increased rate of the short allele relay compared to its long form (Devlin et al., 2005). An interesting amino acid substitution in *SLC6A4*, Gly56Ala, has been associated with certain behaviors in ASD, including compulsiveness and increased sensory aversion (Sutcliffe et al., 2005). In mice, the Gly56Ala mutation caused inhibition of social behaviors and impaired multisensory processing (Veenstra-VanderWeele et al., 2012; Siemann et al., 2017). Furthermore, there is evidence of an association between high-expressing SERT genotypes and tactile hypersensitivity in ASD individuals (Schauder et al., 2015). Regarding the enzymes responsible for 5-HT synthesis, the brain specific gene for tryptophan hydroxylase, *TPH2*, has been manipulated in mouse models extensively. When knocked out, *TPH2* null mice showed decreased vocalizations and interactions with social odors, deficits in social memory, impaired motor control, and cognitive inflexibility (Alenina et al., 2009; Del'Guidice et al., 2014; Mosienko et al., 2015). Concerning receptors, there is little evidence of malfunction in ASD. However, a few mouse models have found significant social deficits with manipulation of 5-HT1a, 5-HT1b, and 5-HT3a (Saudou et al., 1994; Smit-Rigter et al., 2010). Beyond the enzymes of the 5-HT pathway, its transporter, and its receptors, there is some evidence of malfunction in regulatory molecules

and their interaction with serotonin; particularly monoamine oxidase A, the protein responsible for metabolizing 5-HT, and integrin B3 (Carneiro et al., 2008; Bortolato et al., 2013; Whyte et al., 2014). Despite intensive investigation, the mechanism of serotonin pathology in ASD remains unclear and warrants further investigations.

CIRCADIAN DYSFUNCTION AND ASD PATHOGENESIS

It has been long recognized that deficits in temporal processing are fundamental in autism (Hermelin, 1972; Ornitz et al., 1972). Individuals with autism have trouble perceiving the passage of time (Martin et al., 2010; Brenner et al., 2015). Even high-functioning autism patients have a poor intuitive sense of time, and temporal information processing is disrupted (Boucher, 2001; Doenyas et al., 2019). The "weak coherence" hypothesis proposes there is a deficit or alternate pathway for neural information processing in ASD children. In typical brain development, coherence is present in the timing system, whereas in autism, coherence is out of phase and possibly responsible for social behavior deficits (Happé and Frith, 2006). The "temporal binding deficit" hypothesis proposes abnormal visual processing in ASD is due to the aberrant pattern of gamma waves which could partially explain abnormal neurobehavioral function (Brock et al., 2002). The "social timing hypothesis" proposes that biological oscillators are essential for neural information processing, and impairments in any of these oscillators would have physiological and psychological consequences. The timing deficits in ASD could be derived from pathological variations in the structure and function of clock-related genes (Wimpory et al., 2002). In support of this hypothesis, several lines of evidence indicate the dysfunction of circadian timing is associated with ASD. As aforementioned, abnormal diurnal profiles of cortisol, melatonin and abnormal sleep–wake cycles indicate underlying impairments of the circadian system in the ASD patients (Geoffray et al., 2016). In addition, here we discuss epidemiological studies linking the incidence of autism to birth seasons, clock gene polymorphisms in ASD and the role of the mTOR pathway as a common regulator of circadian rhythms and ASD pathogenesis.

Epidemiological Evidence Indicating the Involvement of Birth Timing Factors in ASD

Epidemiological studies have linked the incidence of autism to birth seasons. In Canada, children born in spring and summer are more susceptible to development of autism than children born in winter (Konstantareas et al., 1986). In Israel, higher frequencies of ASD are found in babies born in March (18%) and August (20.2%) than babies born in February (7.6%) (Barak et al., 1995). In a more recent study in Israel, the highest incidence of ASD was found in children born during the month of May (10.3%) (Shalev et al., 2017). Another study in Italy found that some ASD children exhibited a higher degree of sleep problems when the season changed from winter to spring (Giannotti et al., 2006). The correlation between birth months and the development of autism may indicate a role for photoperiod in determining the ontogeny of the individual (Castrogiovanni et al., 1998). Many factors change with seasons. For example, the changing weather (temperature) in different seasons may be associated with different incidence of viral infection during pregnancy.

Among the many factors changing with seasons, a major factor is photoperiod (day length), which has a significant impact on the circadian clocks (Porcu et al., 2018). The durations of light and darkness in a 24-h cycle significantly influence the dynamics of circadian gene expression in different systems (Sumová et al., 2002). Rhythmic gene expression in the SCN are sculpted by the length of photoperiods. Significant differences in synchronization of clock cells and patterns of spatial clock gene expression are found between longer and shorter photoperiods (Sumová et al., 1995; Evans et al., 2013). The secretion of neuroendocrine hormones according to the biological day and night is aligned by the circadian oscillator to the changes in the environmental photoperiod (Wehr, 1998). In addition to fine-tuned circadian output by the SCN, the photoperiod also regulates the phase and levels of cortisol, melatonin and prolactin. Prolonged duration of nighttime is characterized by increased synthesis of cortisol, melatonin and prolactin and shorter nighttime periods are characterized by decreased synthesis (Wehr, 1998). Duration of the photoperiod also affects neural development and functions of offspring. Variation in the photoperiod moderates the function and electrical properties of the serotonin neurons present in the dorsal raphe nuclei of the mouse brain (Green et al., 2015). Functional properties of serotonin neurons are regulated by melatonin signaling. Firing rate and levels of the neurotransmitters serotonin and norepinephrine are altered by the duration of the light/dark cycle (Green et al., 2015). Environmental signaling of light dictates the synthesis of glucocorticoids, and timing of light exposure influences functioning of the HPA axis as well as subsequent levels of stress (Dijk et al., 2012).

Availability of nutrition, inadequate vitamin supply and infection rates vary between seasons, and could also be partially responsible for the seasonal discrepancies in ASD birth rates (Gillberg, 1990). Low birth weight, a risk factor of ASD, has been associated with season of birth (Losh et al., 2012). The birth weight of infants varies according to the season (Doblhammer and Vaupel, 2001; Day et al., 2015). Individuals born in summer had higher mean birth weight, later pubertal development and taller adult height compared to those born in all other seasons. Concordantly, those born in winter showed directionally opposite differences in these outcomes. One interesting hypothesis proposed to explain the variation in ASD rates is the availability of vitamin D to the mother (Grant and Soles, 2009). The photoperiod during the post-natal period mediates the metabolic profile and increases body weight of adults in rat models (Uchiwa et al., 2016). Regarding light-exposure, the percentage of prevalence of autism is higher in congenitally blind children (more than 30%) (Jure et al., 2016) than children with auditory impairments (1 in 59) (Szymanski et al., 2012). These findings demonstrate the association between

light and its timing with the development of the neural communication system.

Clock Gene Polymorphisms in ASD

As aforementioned, the molecular circadian clock is driven by TTFLs consisting of about a dozen clock genes in mammals (**Figure 1**). These clock genes are increasingly found to play fundamental roles in different physiological systems beyond their timing functions. In a mouse model, *Npas2* (–/–) caused impairments in complex emotional memory, but not non-emotional memory (Garcia et al., 2000). Another *Npas2* (–/–) mouse model showed NPAS2 is critical for non-REM sleep homeostasis and caused a reduction in total sleep time in male mice, an interesting comorbidity noted in ASD investigations (Franken et al., 2006). In humans, the protein variant NPAS2 471 Leu/Ser has been implicated in seasonal affective disorder (SAD) and diurnal preference (Johansson et al., 2003). In the repressing limb of the TTFL, PER, CRY, and CK1e form a complex in the cytoplasm, translocate across the nucleus to inhibit binding of CLOCK:BMAL1 or NPAS2:BMAL1, and downregulate transcription of both the *period* and *cryptochrome* genes (Ye et al., 2014). PER1 has been shown to have an instrumental role in cell growth and DNA damage control in human cancer cells (Gery et al., 2006). The PER1 protein also interacts with the checkpoint proteins, ATM and CHK2, regulating DNA repair and cellular apoptosis (Gery et al., 2006). Two rare variants in PER3 in humans with familial advanced sleep phase are associated with seasonal depressive traits (Zhang et al., 2016).

Increasing evidence supports the association between clock gene variants and ASD. Evidence for a genetic basis of timing in communication was originally provided by *Drosophila* studies. The *Drosophila Per* gene was the first identified clock gene and *Per* mutations disrupt the fly's circadian rhythms (Konopka and Benzer, 1971). In addition to circadian disruption, *Per* mutations also affect the rate of sound production of the male fly's courtship song, a primary way of communication that leads to mating (Konopka et al., 1996). In recent decades, human genetic studies of autism have identified single-nucleotide polymorphisms and *de novo* loss-of-function variants of multiple clock genes, indicating functional abnormality of these genes (**Table 2**). There is evidence that genes with direct influence on the mammalian circadian rhythm are highly variable in ASD individuals (Yang et al., 2016). A number of polymorphisms located within *Npas2* were identified in ASD individuals; however, only a cytosine/thymine SNP in intron 3 (NPAS2_X3_C_T) remained significant following statistical analysis (Nicholas et al., 2007). A few mutations in *Per1* were identified in ASD individuals; however, only a cytosine → guanine SNP (Per1_rs885747), and a cytosine/adenine SNP (*Per1*_rs6416892), remained significant following statistical analysis (Nicholas et al., 2007). A proline/alanine substitution at amino acid 1228 in PER2 and an arginine/glutamine substitution at amino acid 366 in PER3 were shown to negatively affect gene function; implicating PER2 and PER3 in the pathogenesis of ASD via gene expression control through the E-box (Yang et al., 2016). Also, in the repressing limb of the TTFL, REV-ERBα/β (NR1D1/2), encoded from *Nr1d1/2*, respectively, inhibits transcription of the activating genes *Bmal1* and *Nfil3*. *Nfil3* encodes NFIL3, a protein that upregulates production of RORα/β, and in turn RORα/β activates transcription of *Bmal1* and *Nfil3* (Preitner et al., 2002; Ueda et al., 2005). Aberrant function of RORα, possibly as a result of mutations in *Nr1d1*, has been implicated in abnormal ASD brain development (Goto et al., 2017). While the above findings are interesting and warrant investigation, polymorphisms in clock genes can only explain a small number of the abnormalities found concurrently between dysfunctional circadian related proteins and ASD phenotypes.

A Role for the mTOR Pathway in Circadian Regulation and ASD Pathogenesis

The mTOR (mammalian target of rapamycin) signaling cascade integrates various intracellular signals to regulate cell growth and metabolism (Wullschleger et al., 2006). mTOR is a serine/threonine protein kinase that forms two multiprotein complexes in cells, mTORC1 and mTORC2. mTORC1 is composed of six components, mTOR, PRAS40, DEPTOR, mLST8 (Mammalian lethal with sec13 protein 8), Raptor, and the Tti1/Tel2 complex (Brown et al., 1994). mTORC2 is composed of seven components, four of which are shared with mTORC1: mTOR, DEPTOR, mLST8, and the Tti1/Tel2 complex. The other three, Rictor (rapamycin insensitive companion of mTOR), mSin1 (mammalian stress-activated map kinase-interacting protein 1), and Proctor are unique for mTORC2 (Jacinto et al., 2006; Pearce et al., 2007). The upstream regulators of mTORC1 are diverse, but can be generally grouped into four activators, which are oxygen, growth factors (e.g., insulin), amino acids (e.g., leucine and arginine), and energy (e.g., ATP), and one inhibitor, which is stress (Laplante and Sabatini, 2012). Notably, the GTPase activating protein Tuberous Sclerosis Complex (TSC) is the key negative regulator of mTORC1 by an intermediary effect on the GTPase, Rheb, which directly binds and activates mTORC1. The downstream effects of mTORC1 are also diverse but can be generally grouped into three categories, regulation of protein synthesis, regulation of lipid and nucleotide synthesis, and inhibition of autophagy. The regulatory and signaling pathways of mTORC2 are not as well defined, but generally it is regulated by growth factors (e.g., insulin or insulin-like growth factor-1) and has the downstream effect of cell survival and proliferation (Saxton and Sabatini, 2017). mTOR signaling regulates a variety of fundamental biological processes. During brain development, it regulates cell growth and differentiation, neuronal migration and differentiation, axonogenesis, axonal navigation and regeneration, dendrite growth and spine development, myelination by oligodendrocytes and Schwann cells, and autophagy (Cao et al., 2009). In the mature brain, it regulates synaptic plasticity, learning, memory, and feeding (Lipton and Sahin, 2014). Disruption of mTOR signaling has been implicated in a number of human brain diseases (Costa-Mattioli and Monteggia, 2013).

TABLE 2 | Clock gene polymorphisms in ASD.

Clock genes	Chr	Location	SFARI gene and score	Findings	References
NPAS2	2	NC_000002.12 (100820139..100996829)	Yes, Score 3	Association analysis in an AGRE cohort revealed two Npas2 significant selected markers. Rs1811399 C > A (p = 0.018), and NPAS2-X3-C-T T > C (p = 0.028)	Nicholas et al., 2007
PER1	17	NC_000017.11 (8140470..8156360, complement)	Yes, Score 3	Association analysis in an AGRE cohort revealed two Per1 significant selected markers. Rs885747 C > G (p = 0.047), and rs6416892 C > A (p = 0.042)	Nicholas et al., 2007
PER2	2	NC_000002.12 (238244038..238290102, complement)	Yes, Score 2	A *de novo* loss-of-function variant in the PER2 gene was observed in an ASD proband from the Simons Simplex Collection in Iossifov et al. (2014). Yuen et al. (2017) identified additional PER2 variants by whole genome sequencing in four ASD families, including a de novo LoF variant in a simplex family from the ASD: Genomes to Outcome Study cohort.	Iossifov et al., 2014 Yuen et al., 2017
PER3	1	NC_000001.11 (7784285..7845181)	No	Base change c.1361G > A causing amino acid change p.R366Q considered disease causing in 1/28 ASD individuals with sleep disturbance.	Yang et al., 2016
CIOCK	4	Chromosome 4, NC_000004.12 (55427903..55547138, complement)	No	Base change c.2551A > G causing amino acid change p.H542R considered disease causing in 1/28 ASD individuals with sleep disturbance. SNP number = rs3762836	Yang et al., 2016
ARNTL	11	NC_000011.10 (13276552..13387268)	No	Base change c.38G > C causing amino acid change p.S13T considered disease causing in 1/28 ASD individuals without sleep disturbance	Yang et al., 2016
ARNTL2	12	NC_000012.12 (27332836..27425813)	No	Base change c.1418T > C causing amino acid change p.L473S considered disease causing in 1/28 ASD individuals without sleep disturbance	Yang et al., 2016
NR1D1	17	NC_000017.11 (40092793..40100589, complement)	Yes, Score 3	Base change c.58A > C, c.1031 A > C, c.1499G > A causing amino acid change p.S20R, p.N344T, p.R500H, respectively, considered disease causing in ASD individuals	Yang et al., 2016; Goto et al., 2017
RORA	15	NC_000015.10 (60488284..61229302, complement)	Yes, Score S	Allele frequencies of rs4774388 showed significant overrepresentation of T allele in patients compared with controls in Sayad et al. (2017). Increased DNA methylation and decreased gene expression of Rora in autistic co-twin than undiagnosed co-twin and unaffected controls in Nguyen et al.	Sayad et al., 2017 Nguyen et al., 2010
RORB	9	NC_000009.12 (74497335..74693177)	Yes, Score 1	A de novo missense variant in the RORB gene has been identified in an ASD proband from the Simons Simplex Collection by Iossifov et al. (2014) Rudolf et al. (2016) found that two individuals from patients with de novo mutations involving RORB also presented with autism spectrum disorder. Boudry-Labis et al. (2013) found that RORB was one of four genes within the minimal region of overlap in 9q21.13 microdeletion syndrome, a disorder characterized by autistic features	Iossifov et al., 2014 Rudolf et al., 2016 Boudry-Labis et al., 2013
CSNK1E	22	NC_000022.11 (38290691..38318084, complement)	Yes, Score 3	Two *de novo* missense variants that were predicted *in silico* to be damaging were identified in the CSNK1E gene in ASD probands from the Autism Sequencing Consortium in De Rubeis et al. (2014). Base change c.2551A > G causing amino acid change p.H542R considered disease causing by Mutation Taster analysis in three ASD individuals. SNP number = rs77945315 TADA-Denovo analysis using a combined dataset of previously published cohorts from the Simons Simplex Collection and the Autism Sequencing Consortium, as well as a novel cohort of 262 Japanese ASD trios, in Takata et al. (2018) identified CSNK1E as a gene significantly enriched in damaging *de novo* mutations in ASD cases	De Rubeis et al., 2014; Yang et al., 2016; Takata et al., 2018
TIMELESS	12	NC_000012.12 (56416363..56449426, complement)	No	Base changes c.1493T > C causing amino acid changes p.F498S considered disease causing in 1/28 ASD individuals with sleep disturbance	Yang et al., 2016

mTOR is emerging as a conserved circadian regulator (Cao, 2018). The mTORC1/eIF4E (eukaryotic translation initiation factor 4E) pathway regulates fundamental functions of the circadian clock such as entrainment, synchrony, and timing (Cao et al., 2013, 2015; Liu et al., 2018). In mammals, mTOR regulates the SCN circadian clock in three facets. First, mTORC1 signaling is part of the photic entrainment pathway in the SCN. In the SCN, light activates S6K1 by phosphorylating Thr389. S6K1 then phosphorylates ribosomal protein S6, a component of the 40S ribosomal subunit and regulates mRNA translation (Cao et al., 2010). S6K1 also phosphorylates the clock protein BMAL1 and activates translation (Lipton et al., 2015). On another branch, light-induced mTORC1 activation increases phosphorylation of eIF4E-binding proteins (4E-BPs) in the SCN, causing disinhibition of eIF4E-dependent translational initiation (Cao et al., 2008). Phosphorylation of both S6K1 and 4E-BP1 is mTORC1-dependent, because rapamycin eliminates the phosphorylation of both these targets in the SCN and regulates photic entrainment of the clock in animals (Cao and Obrietan, 2010; Cao et al., 2010). Second, mTORC1 regulates

network properties of coupled circadian oscillators in the SCN by translational control of *Vip* (Vasoactive intestinal peptide). By phosphorylating and inhibiting the eIF4E repressor protein 4E-BP1, mTORC1 upregulates mRNA translation of *Vip* (Cao et al., 2013). VIP is synthesized by core SCN neurons, and following their photic input and entrainment, entrain and reset the shell SCN neurons that typically express arginine vasopressin (AVP). VIP signaling promotes synchrony of SCN cells, and increases the robustness of clock gene oscillations and clock functionality (Harmar et al., 2002; Aton and Herzog, 2005; Maywood et al., 2006). Conditional mTOR deletion in VIP neurons disrupts SCN cell synchrony and impairs circadian rhythms in mice, in a way largely similar to *Vip* mutation (Liu et al., 2018). Third, mTOR regulates autonomous clock properties in a variety of cellular circadian oscillators. Effects of pharmacological and genetic mTOR manipulation on autonomous circadian clock properties have been examined in various cellular and tissue oscillators including the SCN, fibroblasts, hepatocytes, and adipocytes. mTOR inhibition reduces amplitudes of oscillation and increases circadian period of the clock gene *Per2* expression, whereas mTOR activation shortens circadian period and augments amplitudes (Ramanathan et al., 2018), indicating the mTOR pathway regulates both central and peripheral clock properties.

Abnormal mTOR activities have been associated with several genetic forms of ASDs, including Tuberous Sclerosis Complex (TSC), Phosphatase and tensin homolog (PTEN), Hamartoma Tumor syndrome, Fragile X syndrome, RASopathies, Angelman Syndrome, Rett Syndrome, and Phelan-McDermid syndrome (Bhattacharya et al., 2012; Costa-Mattioli and Monteggia, 2013; Jülich and Sahin, 2014; Winden et al., 2018). Mutations in negative regulators of mTORC1, such as *TSC1*, *TSC2,* and *PTEN* are found in monogenic ASD (Buxbaum et al., 2007; O'Roak et al., 2012; Lipton and Sahin, 2014). In laboratory studies, mTOR dysregulation has been found in ASD derived neural progenitor cells (Alsaqati et al., 2020). Deletion of the *Tsc1* gene in Purkinje cells leads to mTORC1 hyperactivation and autism-like behaviors in mice (Tsai et al., 2012). Mice lacking the repressor of eIF4E, 4E-BP2, demonstrate increased translation of neuroligins, which are causally linked to ASD (Gkogkas et al., 2013). The increased levels of eIF4E also increase the ratio of excitatory: inhibitory synaptic inputs, social interaction deficits, and repetitive/stereotyped behaviors (Santini et al., 2013). A model has been proposed describing the relationship between synaptic proteins and translational control in ASD. The model includes proteins and protein complexes implicated in circadian control discussed in this paper such as Neurexins, Neuroligins, Shank, mTOR/4E-BP, and eIF4E (Santini and Klann, 2014). Thus, the circadian clock and autism are both regulated by mTOR signaling pathways. Dysregulation of the mTORC1/eIF4E axis disrupts the circadian clock and engenders ASD-like phenotypes in animal models, indicating potential crosstalk between the circadian clock and ASD via the mTORC1/eIF4E axis.

CONCLUSION

In an era of rapidly increased prevalence of ASD, there is an urgent need to understand the mechanisms underlying ASD pathogenesis and develop new therapeutic strategies. Various physiological parameters such as circadian biomarkers, sleep/wake rhythms, neurotransmitters, language and communication, information processing and brain rhythms are associated with circadian clock function and are altered in ASD patients. Mounting evidence exists demonstrating malfunctions of the endogenous circadian timing system in ASD. Correlations exist between clock gene polymorphisms, seasonal discrepancies, and ASD. Understanding the functional importance of the circadian clock in neurodevelopment and its dysregulation in neurodevelopmental disorders may provide a novel approach to tackle ASD. Clinical treatments for ASD children can comprise an integrated approach considering physical, mental and social strategies based on highly dynamic daily rhythms in neurophysiology and behavior. The associations between circadian dysfunction and ASD can be bidirectional. Circadian clock malfunctions may be one of the many pathophysiological aspects underlying ASD pathogenesis, whereas experimental evidence demonstrating that circadian disruption can lead to neurodevelopmental disorders is still lacking. We propose it is necessary to comprehensively investigate the altered circadian patterns of the sleep/wake cycle, cortisol, melatonin and clock gene polymorphisms in ASD. The findings would not only reveal intrinsic connections between aberrant circadian timing and ASD development, but also be instrumental for applying chronotherapy-based strategies to treat the diseases.

AUTHOR CONTRIBUTIONS

EL and RK created the figures and tables. EL, RK, and RC wrote the manuscript. All the authors contributed to the article and approved the submitted version.

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Identification of PCBP1 as a Novel Modulator of Mammalian Circadian Clock

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The circadian clock governs our daily cycle of behavior and physiology. Previous studies have identified a handful of core clock components and hundreds of circadian modifiers. Here, we report the discovery that poly(C)-binding protein 1 (PCBP1), displaying a circadian expression pattern, was a novel circadian clock regulator. We found that knocking down *PCBP1* resulted in period shortening in human U2OS cells, and that manipulations of *PCBP1* expression altered the activity of CLOCK/BMAL1 in an E-box-based reporter assay. Further mechanistic study demonstrated that this clock function of PCBP1 appears to work by enhancing the association of Cryptochrome 1 (CRY1) with the CLOCK/BMAL1 complex, thereby negatively regulating the latter's activation. Co-immunoprecipitation of PCBP1 and core clock molecules confirmed the interactions between PCBP1 and CRY1, and a time-course qPCR assay revealed the rhythmic expression of *PCBP1* in mouse hearts *in vivo*. Given that the RNA interference of *mushroom-body expressed* (*mub*), the poly(rC) binding protein (PCBP) homolog of *Drosophila*, in the clock neurons also led to a circadian phenotype in the locomotor assay, our study deemed PCBP1 a novel clock modifier whose circadian regulatory mechanism is conserved during evolution.

Keywords: circadian clock, period shortening, clock modifier, PCBP1, mechanism

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INTRODUCTION

Poly(rC) binding proteins (PCBPs) are generally known as RNA-binding proteins. These proteins comprise two subsets: PCBPs (1–4; also referred to as α-CPs or hnRNP E 1–4) and hnRNPs K/J in mammalian cells, the homolog of which is *mushroom-body expressed* (*mub*) in *Drosophila*. The PCBP family is related evolutionarily by the common feature of poly(C)-binding specificity and three highly conserved hnRNP K homology (KH) domains (Makeyev and Liebhaber, 2002). PCBPs perform multiple biological processes through the conserved characteristics, including mRNA stabilization, transcriptional regulation, and translational silencing (Huo and Zhong, 2008; Zhang et al., 2020a). At present, poly(C)-binding protein 1 (PCBP1), which exhibits 82%

similarity at amino acid level to poly(C)-binding protein 2 (PCBP2) (Guo and Jia, 2018), has been studied in the greatest detail. The ability of PCBP1 to recognize and bind poly(C) DNA and RNA sequences *via* KH domains is critical for their function in mammalian cells. As such, PCBP1 binds to various proteins, including iron-containing polyamine pathway dioxygenase ADI1 and signal transducer and activator of transcription 3 (STAT3), and then participates in iron homeostasis, tumorigenesis, and metastasis (Yanatori et al., 2020; Zhang et al., 2020b). Together, these studies describe the complexity of distinct interacting factors that are mediated by PCBP1, which revealed that PCBP1 participate in multiple biological processes.

In the dynamic homeostasis of living organisms, the circadian clock drives endogenous rhythms of multiple physiological processes (Zhang and Kay, 2010). In addition, disruption of the circadian rhythm plays a key role in numerous chronic diseases, such as cancer and metabolic syndrome (Roenneberg and Merrow, 2016; Jagannath et al., 2017). The core circadian oscillator in mammals is composed of a transcription-translation feedback loop (TTFL) with CLOCK and BMAL1 as positive factors and PER and CRY as negative regulators. An additional feedback loop exits with the ROR transcriptional activators and the dimeric REV–ERB repressors regulating the Bmal1 gene, the expression of which, in turn, controls the transcription of ROR and REV–ERB. These feedback loops work in concert to generate a period of 24 h. A highly similar central oscillator exists in *Drosophila melanogaster*, one associate loop stabilizes the primary loop, consisting of CLOCK (CLK)–cycle (CYC) and PER–timeless (TIM; Zhang and Kay, 2010). The core circadian clock genes have been reported to participate in tumorigenesis and metabolic disorders. Early landmark studies have shown that mice carrying mutations of *Per2*$^{m/m}$ had reduced apoptotic responses in thymocytes and increased lymphoma occurrence when irradiated (Fu et al., 2002). Similar experiments have been performed in Cry1$^{-/-}$ mice, which exhibits resistance to obesity induced by a high-fat diet (Griebel et al., 2014). Recent studies have shown that BMAL1 can promote tumorigenesis and chemoresistance with MiR-135b (Jiang et al., 2016, 2018). Altogether these studies have supported that circadian clocks are associated with various physiological processes and pathological events. The multiple functions of core circadian oscillator and PCBPs suggests that these two pathways engage in crosstalk with each other.

Our previous screen identified PCBP1, a member of the PCBPs family, as a period-short candidate (Zhang et al., 2009). The CircaDB online database predicts that most PCBP genes are under daily cycling in mouse tissues (Hughes et al., 2009). According to these observations, we hypothesize that PCBP1 plays important role in circadian rhythm and studied the role of PCBPs in circadian machinery.

MATERIALS AND METHODS

Plasmid DNA and Antibodies

Transient expression vectors were generated by inserting cDNA into the multi-cloning site of pcDNA3.1(+)/hygro. Small interfering RNA (siRNA) of PCBP1 and PCBP2 were synthesized as follows: siPCBP1-1, 5'-GCUCCUCUGGUAGGCAGGUUAC UAU-3' (forward) and 5'-AUAGUAACCUGCCUACCAGAGG AGC-3' (reverse); siPCBP1-2, 5'-CCGGUGUGACUGAAAGUG GACUAAA-3' (forward) and 5'-UUUAGUCCACUUUCAGUCA CACCGG-3' (reverse); siPCBP2, 5'-CCGGUGUGAUUGAAGGU GGAUUAAA-3' (forward) and 5'-UUUAAUCCACCUUCAAUC ACACCGG-3' (reverse). The short hairpin RNA (shRNA) expression vectors were obtained by subcloning short DNA sequences into pLKO.1 as follows: shPCBP1, 5'- TAAGAGTGG AATGTTAATAAACTCGAGTTTATTAACATTCCACTCTTA-3'; shPCBP2, 5'-CTAGAGGCCTATACCATTCAACTCGAGTTG AATGGTATAGGCCTCTAG-3'. Antibodies used for co-immunoprecipitation included anti-Flag (Cat# F3165, sigma) and anti-HA (Cat# ab9110, Abcam).

Cell Culture and Transfection

U2OS cells and HEK293T cells were cultured at 37°C in a 5% CO_2 atmosphere in DMEM supplemented with 10% fetal bovine serum and a mixture of penicillin and streptomycin. Stable shPCBP1 or shPCBP2 cell lines were generated following a previously described protocol (Zhang et al., 2009). Cells were transfected with siRNA and plasmids using RNAi MAX and Lipofectamine 2000 transfection reagent, respectively, according to the manufacturer's instructions (#13778150, #11668019, Life Technologies).

Quantitative Real-Time PCR

RNA was extracted from cells and mouse hearts with Trizol reagent according to the manufacturer's instructions (#15596, Life Technologies). cDNA was generated from RNA using a PrimeScript RT Master Mix Real-time RT-PCR Kit (#RR036A, Takara). Gene expression was analyzed *via* quantitative real-time PCR (qRT-PCR) with a KAPA SYBR FAST qPCR Kit (#KP-KK4601, Kapa Biosystems). The reactions were first incubated at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s. Gene-specific primer sequences are as follows: hTBP, 5'-GAACCACGGCACTGATTTTC-3' and 5'-CCCCACCATGTTCTGAATCT-3', hPCBP1, 5'-GGACAACA CACCATTTCTCCGC-3' and 5'-AGCCTTTCACCTCTGGAG AGCT-3'; hPCBP2, 5'-ATCTGCTGCCAGCATTAGCCTG-3' and 5'-GGTGGTGAACAGCAGAAAGGGA-3'; mTbp, 5'-CAA ACCCAGAATTGTTCTCCTT-3' and 5'-ATGTGGTCTTCCT GAATCCCT-3'; mPcbp1, 5'-GGACAACACACCATTTCTCC GC-3' and 5'-AGCCTTTCACCTCTGGAGAGCT-3'; mBmal1, 5'-CTCGACACGCAATAGATGGGA-3' and 5'-CTTCCTTGGTC CACGGGTT-3'; mPer2, 5'-GAAAGCTGTCACCACCATAG AA-3' and 5'-AACTCGCACTTCCTTTTCAGG-3'; mCry1, 5'-GGTTGCCTGTTTCCTGACTCGT-3' and 5'-GACAGCCA CATCCAACTTCCAG-3'.

Luminometry and Luciferase Repression Assays

Human U2OS cells harboring *Per2-dLuc* were grown to confluence in 3.5 cm dishes and were then placed in XM medium and sealed (Zhang et al., 2009). Data were collected in a LumiCycle luminometer at 36°C for 5–7 days; data excluding the first

24-h cycle were analyzed with LumiCycle Analysis software (Actimetrics, Inc.; Liu et al., 2007).

Luciferase repression assays were performed in Corning 96-well white bioassay plates with HEK293T cells as described previously (Liu and Zhang, 2016). The luciferase reporter with tandem E-boxes derived from the Dbp gene was used in the experiments as described previously (Wu et al., 2017).

Luciferase Complementation Assay

Luciferase complementation assay was used to determine the interaction of proteins as previously described (Liu and Zhang, 2016). The N-terminal and C-terminal luciferase fragment were fused to two different proteins, such as CLOCK and BMAL1, and were then co-expressed as fusion proteins with luciferase fragments in HEK293T cells. Twenty-four hour after transfection, cells were prepared for the Dual-Luciferase Reporter Assay System (Promega).

Co-immunoprecipitation Assay

HEK293T cells were transfected with Lipofectamine 2000 according to the manufacturer's instructions (#11668019, Life Technologies). At 24 h after transfection, the cells were washed with PBS buffer and harvested with lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40) containing protease inhibitor. The lysates were immunoprecipitated with HA or FLAG-agarose beads and the complexes were washed three times with lysis buffer. The immunoprecipitated proteins were separated using 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Non-specific binding was blocked with 5% skimmed milk in Tris buffer, then the proteins were probed with anti-Flag antibody and anti-HA antibody. Antibody binding was subsequently detected by incubation with secondary antibodies linked to horseradish peroxidase (HRP), and immunoreactive proteins were visualized using an ECL detection kit (Thermo).

Behavior Measurements

The fly strains Cry16-Gal4 were gifts from Luoying Zhang, and UAS-shMub (THU02931) were from the Tsinghua University Stock Center. All the flies were raised on a standard cornmeal-yeast-sucrose medium and a 12-h light/12-h dark (LD) cycle at 25°C. Male flies (2–3 days after eclosion) were used to monitor locomotor activity levels using the *Drosophila* Activity Monitor system (Trikinetics, Waltham, MA). Individual flies were placed in a glass tube with food, and their activity were recorded. Flies were subjected to an LD cycle with light intensity of 200 lux for 7 days and then 7 days of constant darkness (DD) condition. Data of behavior rhythmicity during the DD condition were analyzed with ClockLab software (Actimetrics, Inc.; Bu et al., 2019).

Statistical Analysis

In all experiments, unless noted, error bars represent SEM. Statistical analyses were performed using Graph-Pad PRISM (version 7.00, GraphPad Software, Inc.) and JTK_Cycle software (Hughes et al., 2010). Two-sided Student's *t*-tests were used when only two groups were analyzed. One-way ANOVA with Dunnett's multiple comparisons test was used when more than two groups were being analyzed, otherwise we used two-way ANOVA with Bonferroni's multiple comparisons test in some experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Ethics Approval Statement

Mice were housed in a specific-pathogen-free (SPF) environment under a 12 h light/dark photoperiod with food/water. All experiments with mice were performed following the guidelines of the Institutional Animal Care and Use Committee (IACUC) at NIBS.

RESULTS

PCBP1 Is a Period-Short Modifier for the Mammalian Clock

To investigate whether PCBPs regulate the circadian clock or not, we first knocked down the expression of PCBP1 in U2OS cells containing Per2-dLuc using siRNA. Our results showed that the knockdown efficiency of PCBP1 was 93.4%, and that the clock of PCBP1 knockdown cells had a shorter period (−0.8 h) than scramble control cells (**Figures 1A,B**), suggesting that PCBPs could modify the mammalian clock. Considering that PCBP1 and PCBP2 share a high level of amino acid sequence similarity (Makeyev and Liebhaber, 2002), we further used a shRNA lentivirus system to knock down the expression of PCBP1 or PCBP2 (**Figures 1C,D**). The knockdown efficiency of PCBP1 and PCBP2 were 70.8 and 87.6%, respectively (**Figure 1D**). Interestingly, the circadian period was short in shPCBP1 cells (−1.0 h) like siPCBP1 cells but did not have larger changes in scramble control and shPCBP2 cells (**Figure 1C**), indicating that PCBP1 but not PCBP2 is a period-short modifier for the mammalian clock in U2OS cells.

To further investigate the roles of PCBP1 and PCBP2 in the circadian clock, we detected the activity of CLOCK/BMAL1 using E-box-based luciferase reporter assay when manipulating the expression of PCBP1 or PCBP2 in HEK293T cells. We looked for associations of transcriptional activity and repression activity with response to PCBP1 by two-way ANOVA including factor interaction. Our results showed that the transcriptional activity of CLOCK/BMAL1 was reduced in PCBP1 overexpressed cells (**Figure 1E**) but induced in shPCBP1 cells of which the knockdown efficiency was 75.7% (**Figures 1F,G**). In addition, the transcriptional repression activity of CRY1 was enhanced and weakened in PCBP1 overexpressed cells and knockdown cells, respectively, although with no significance. Two-way ANOVA test showed a significant *Interaction* effect between PCBP1 and CRY1 for the transcriptional activity of CLOCK/BMAL1 ($p < 0.001$). However, the activity of CLOCK/BMAL1 and CRY1 did not have obvious change in shPCBP2 cells (**Figure 1F**), and the knockdown efficiency of PCBP2 was 84.4% (**Figure 1G**). Altogether these results showed that PCBP1 was a period-short modifier that appears to be mediated by its impact on the CLOCK/BMAL1 activation.

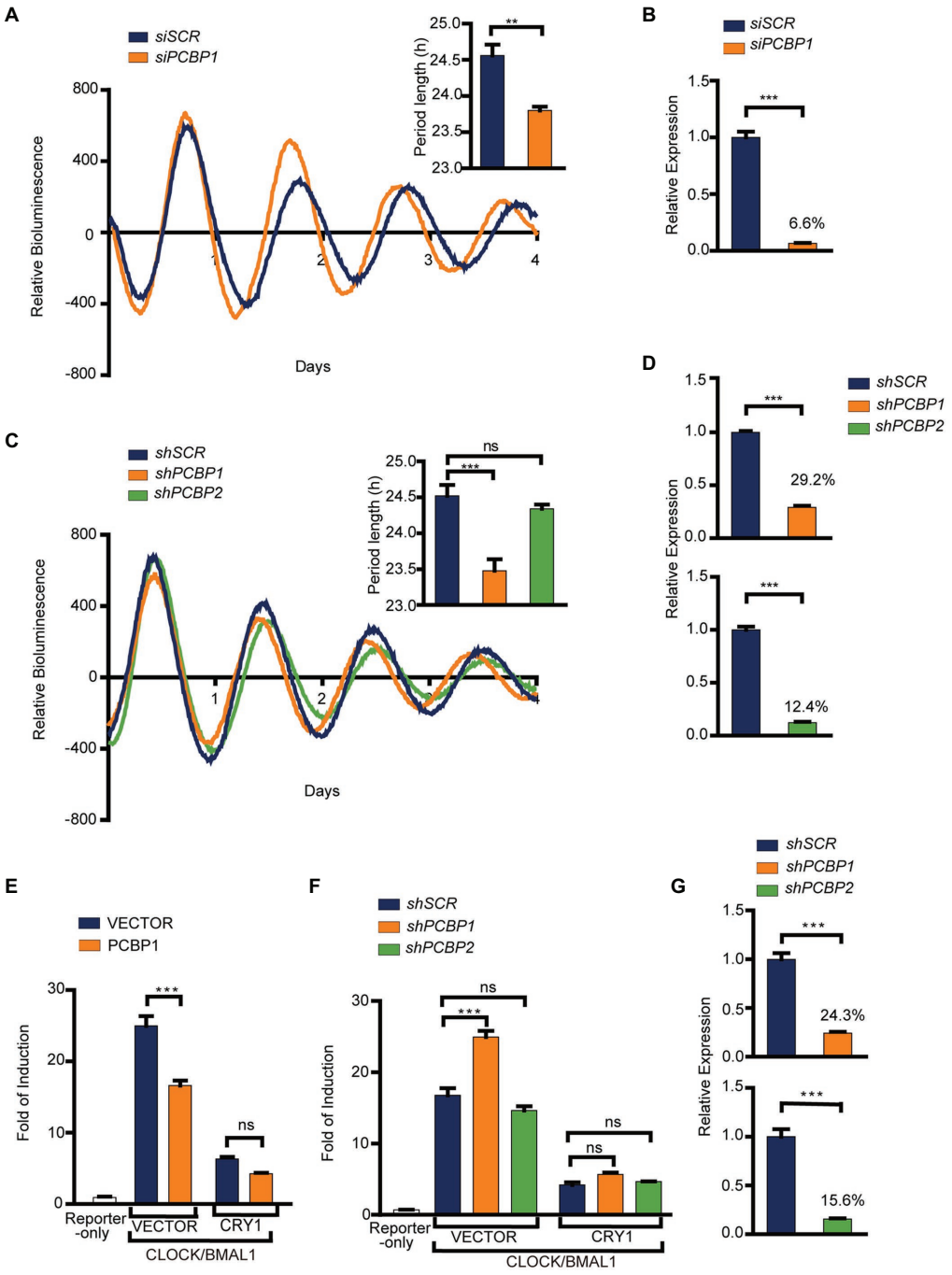

FIGURE 1 | PCBP1 is a period-short modifier for mammalian clocks. **(A)** Knockdown of PCBP1 results in a short period of circadian rhythm in U2OS cells harboring Per2-luc. Top right: Bars show quantitative period change values. **(B)** The knockdown efficiency of siPCBP1. The expression level of PCBP1 was knocked down to 6.6% compared to scramble. **(C)** Knockdown of PCBP1 using short hairpin RNA (shRNA) results in a short period of circadian rhythm in U2OS cells harboring Per2-luc, and the period had no obvious change in shPCBP2 cells. Top right: Bars show quantitative period change values. **(D)** The knockdown efficiency of shPCBP1 and shPCBP2 in U2OS cells harboring Per2-luc. The expression levels of PCBP1 and PCBP2 were 29.2 and 12.4% compared to scramble, respectively. **(E)** A transient transfection luciferase assay was conducted in 293 T cells to show that over-expressed PCBP1 decreased the transcriptional activity of CLOCK/BMAL1. **(F)** A transient transfection luciferase assay was conducted in shPCBP1 and shPCBP2 cells to show the difference in the transcription or repression activity. **(G)** The knockdown efficiency of shPCBP1 and shPCBP2 in 293 T cells. The bioluminescence traces in **(A,C)** were representative of five independent experiments, and the four additional experiments gave similar results. Mean and error bars (SEM) of five independent transfections are shown. Statistical significance was determined using Student's *t*-tests **(C)**, one-way ANOVA **(A,B,D,G)** or two-way ANOVA followed by Bonferroni's multiple comparisons test **(E,F)**. ($n = 5$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

PCBP1 Enhances the Interaction of CRY1 With the CLOCK/BMAL1 Complex

To investigate how PCBP1 regulates the activity of CLOCK/BMAL1 and CRY1, we conducted a luciferase complementation assay to determine whether PCBP1 affects the interactions between CRY1 and the CLCOK/BMAL1 complex (**Figure 2A**). CLOCK (or BMAL1) and CRY1 (or BMAL1) were co-expressed as fusion proteins with N- and C-terminal luciferase fragments in HEK293T cells, as previously described (Liu and Zhang, 2016). The formation of complexes could produce functional luciferase, the activity of which could be recorded in luciferin-containing medium. Data showed that, compared to the CLOCK/BMAL1 complex and BMAL1/CRY1 complex, the interaction between CLOCK and CRY1 was weakest, although it was enhanced by BMAL1 significantly (**Figure 2B**). The results indicated that CRY1 could interact with BMAL1 to form the CLOCK-BMAL1-CRY1 complex, which was consistent with a previous report that CRY1 regulated the circadian clock through dynamic interactions with BMAL1 (Xu et al., 2015). Based on this result, we next determined the effect of PCBP1 on the CLOCK-BMAL1 and CLOCK-BMAL1-CRY1 complex. Our results showed that PCBP1 could not enhance the formation of the CLOCK-CRY1 or CLOCK-BMAL1 complex but the formation of the CLOCK-BMAL1-CRY1 complex (**Figure 2C**), which indicated that the transcriptional activity of CLCOK/BMAL1 was reduced by PCBP1 associated with endogenous CRY1 (**Figure 1E**).

In addition, we further detected the interaction between CRY1 and the CLOCK-BMAL1 complex in two different siPCBP1 cells in which the expression of PCBP1 was knocked down to 13.6 and 18.8%, respectively, and our results showed that the knockdown of PCBP1 could attenuate the signal of bioluminescence, similar to the knockdown of CRY1 or BMAL1 (**Figures 2D,E**). All these results suggested that PCBP1 mediated the circadian clock by enhancing the association of CRY1 with the CLOCK/BMAL1 complex, thereby negatively regulating the latter's activation.

PCBP1 Interacts With CRY1 *in vitro*

We next conducted co-immunoprecipitation and luciferase complementation experiments to study whether PCBP1 could interact with each core clock molecule. The whole cell lysates containing over-expressed PCBP1 with HA-tag and CRY1 (or CRY2, BMAL1) with FLAG-tag were subjected to affinity chromatography on HA or FLAG-agarose beads. The PCBP1 protein complexes were then analyzed by Western Blot (**Figure 3A**). To avoid non-physiological interaction in over-expressed system in cells, we generated negative control: GFP with FLAG-tag co-expressed with PCBP1-HA. As expected, CRY1 was a major component of the protein complex, although BMAL1 and CRY2 could also copurify with PCBP1. In accordance, the luciferase complementation assay showed that the interaction between PCBP1 and CRY1 was stronger than PCBP1 and PER2 or BMAL1, which was similar with CRY1 and PER2 (**Figure 3B**). Identification of CRY1, CRY2, and PER2 in the PCBP1 protein complex further confirmed that PCBP1 plays an important role in circadian rhythm modulation.

PCBP1 Regulating Circadian Rhythm Is Conserved in Evolution

As the CircaDB online database predicts that most PCBP genes are under daily cycling in mouse tissues (Hughes et al., 2009), we determined that the mRNA levels of PCBP1 in mouse hearts exhibited significant circadian oscillation (**Figure 4A**). The peak mRNA expression level of PCBP1 was at night, which was in accordance with CRY1, PER2 and contrary to BMAL1, which could explain that PCBP1 tended more to interact with CRY1. We further demonstrated that *mub* gene, the homolog of PCBP1 in *Drosophila*, controlled the circadian behavior of fruit flies. We knocked down *mub* in the circadian neurons of *Drosophila via* RNAi interference and recorded the locomotor activity using the *Drosophila* activity monitoring system (**Figure 4B**). The cry16-GAL4UAS-shMub *Drosophila* showed a significant period lengthening phenotype compared to UAS-shMub *Drosophila*, which suggested that the circadian regulatory mechanism of this post-transcriptional factor is conserved during evolution, although the phenotype was different between mammalian cells and fruit flies.

DISCUSSION

From the basic TTFL model, both positive and negative elements are important for generating the auto-regulatory feedback loops that drive the circadian rhythm and regulate circadian clock outputs. It has been reported that between 2 and 45% of mouse tissue's transcriptome display a circadian rhythm (Duffield et al., 2002; Panda et al., 2002; Kornmann et al., 2007; Perelis et al., 2015), and that these clock-controlled genes (CCGs) contribute to multiple physiological and pathological processes including signal transduction, cancer and immune system disorders, and so on (Jagannath et al., 2017). In this study, we found that PCBP1, displaying a circadian expression pattern, was a novel circadian clock regulator, whose function was conserved in evolution.

Poly(rC) binding proteins, known as RNA binding proteins, have multiple functions in biological processes. However, the functions of PCBPs in circadian clock modulation have rarely been reported. Previous genome-wide siRNA screening have showed that PCBPs play important roles in maintaining the period and amplitude in U2OS cells, such as short periods in siPCBP1 or siPCBP3 cells and low amplitudes in siHNRNPK cells (Zhang et al., 2009). In accordance with these findings, our results showed that the circadian period was short in PCBP1 knockdown cells *via* siRNA or shRNA interference and that PCBP2 knockdown cells exhibited no obvious change (**Figures 1A,B**). Although PCBP1 exhibits the greatest sequence similarity to PCBP2, they may possess non-redundant functions in circadian clock. Immunofluorescence studies revealed that

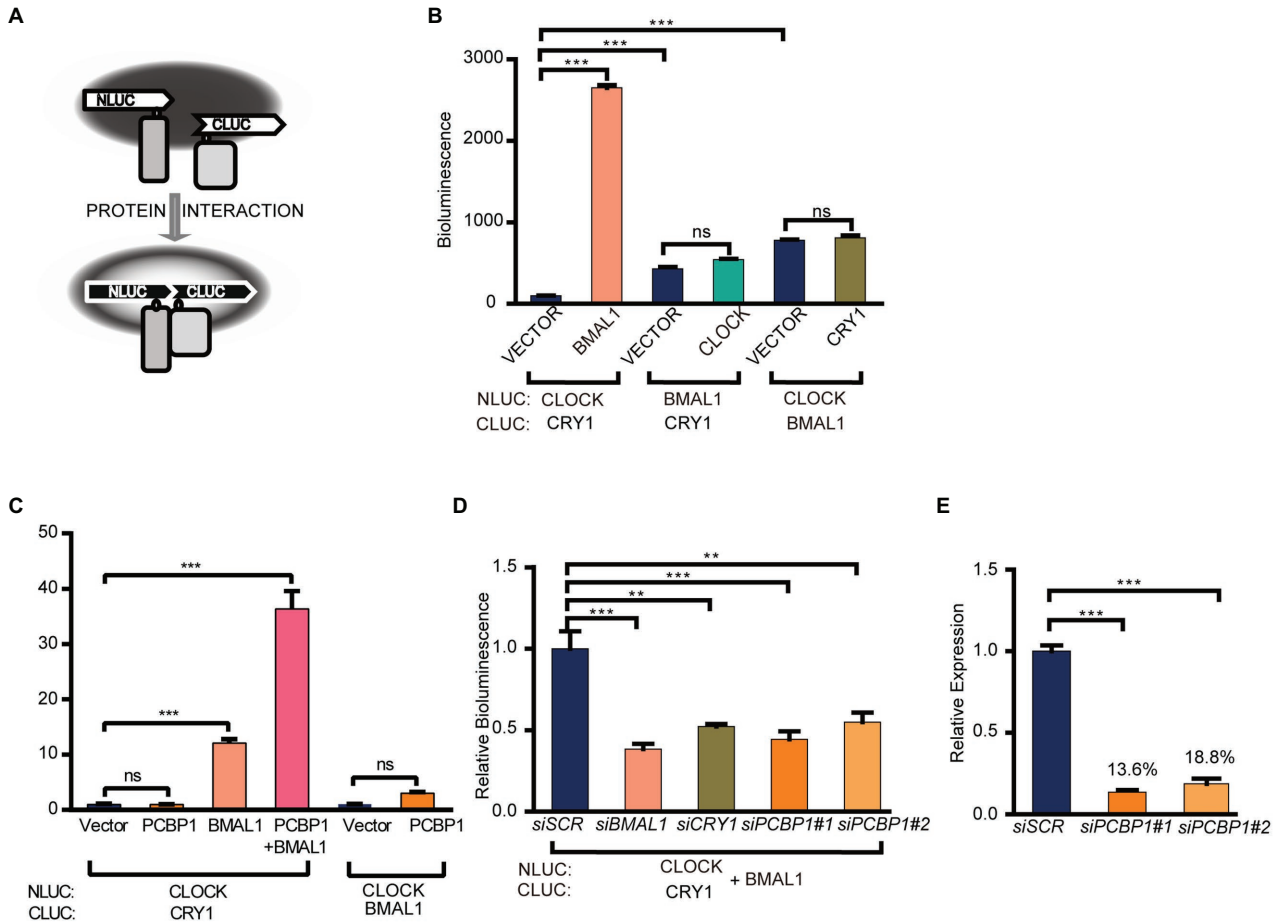

FIGURE 2 | PCBP1 enhances the interaction of CRY1 with the CLOCK/BMAL1 complex. **(A)** schematic diagram of luciferase complementation assay. **(B)** Luciferase complementation assay was used to determine the interaction among CLOCK, BMAL1, and CRY1. **(C)** PCBP1 could not enhance the complex formation of CLOCK-BMAL1 or CLOCK-CRY1, but the formation of CLOCK-BMAL1-CRY1. **(D)** The complex formation of CLOCK-BMAL1-CRY1 in siBMAL1, siCRY1, or siPCBP1 cells. **(E)** The knockdown efficiency of siPCBP1#1 and #2 in 293 T cells. All data are presented as the mean ± SEM from at least three independent experiments. Statistical significance was determined using one-way ANOVA **(C–E)** or two-way ANOVA followed by Bonferroni's multiple comparisons test **(B)**. ($n \geq 3$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

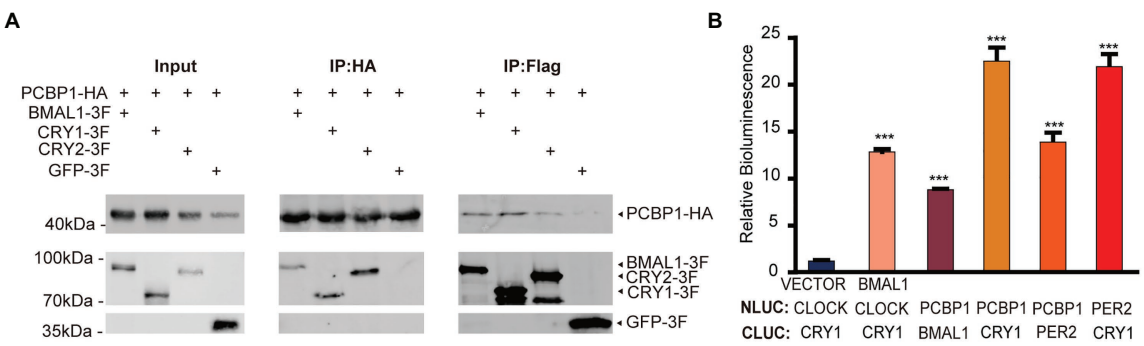

FIGURE 3 | PCBP1 interacts with core clock molecules. **(A)** Co-immunoprecipitation assay shows the interaction between PCBP1 and core clock molecules (CYR1, CRY2, and BMAL1). **(B)** Luciferase complementation assay validates the interaction between PCBP1 and core clock molecules (CRY1, PER2, and BMAL1). All data are presented as the mean ± SEM from three independent experiments. Statistical significance was determined using one-way ANOVA ($n = 3$, $^{***}p < 0.001$).

PCBP1 is predominantly in the nucleus (Berry et al., 2006), suggesting PCBP1 works on the circadian clock through transcriptional activation or a repression mechanism. The decreased CLOCK/BMAL1 transcriptional activity and increased CRY1 repression activity by PCBP1 over-expression (**Figure 1E**) implied that PCBP1 regulates the core circadian transcription-translation negative feedback loop, which validated our hypothesis.

It has been reported that PCBP1 is relevant to tumorigenesis in diverse human cancers (Choi et al., 2009; Shi et al., 2018; Zhang et al., 2020b), and circadian clocks have close interactions with cancer metabolism (Roenneberg and Merrow, 2016; Masri and Sassone-Corsi, 2018). However, the mechanism between these is still unclear. To investigate the underlying mechanism, we further conducted luciferase complementation and co-immunoprecipitation experiments to determine the interaction between PCBP1 and core circadian clock genes. Our results showed that PCBP1 had strong interaction with CRY1 and weak interaction with BMAL1 (**Figures 3A,B**), because of which PCBP1 could enhance the formation of the CLOCK-BMAL1-CRY1 complex (**Figure 2D**). In addition, PCBP1 could interact with CRY2 and PER2, which were negative regulators like CRY1 (**Figures 3A,B**). The mRNA expression level of PCBP1, as expected, showed a circadian pattern consistent with CRY1 and PER2 (**Figure 4A**), which provide new theoretical basis for chronotherapy (Ju and Zhang, 2020). All these results provided evidence that PCBP1 and circadian clock cross talk with each other, which might be the key to regulate the development of tumorigenesis.

Finally, to define the function of PCBP1 as a novel clock modifier *in vivo*, we recorded the locomotor activity of *Drosophila* with knockdown of *mub* gene, which is the homolog gene of PCBP. Interestingly, we found that *Drosophila* with knockdown of *mub* in circadian neurons showed a circadian rhythm with period-lengthening, the phenotype of which was different from mammalian cells. We considered the difference for two reasons. First, *Drosophila* CRY function is distinct from that of its ortholog in mammals, the major role of which is to repress the transcriptional activity of CLCOK/BMAL1 complex. By contrast, *Drosophila* CRY is a photoreceptor and is responsible for transducing the light input signal to the core repressors PER and TIM (Zhang and Kay, 2010). As such, in *Drosophila*, knockdown of *mub* results in a different phenotype from mammalian cells. Second, in mammalian cells, the PCBP protein family contains five components, PCBP1–4 and HNRNPK, which may interact with each other to affect the circadian period. However, *mub* in *Drosophila* is the only one PCBP homolog gene, *thereby* manipulating circadian rhythm by a different mechanism.

In summary, we identified PCBP1 as a novel circadian clock modifier through interaction with core clock molecules, whose function was conserved in evolution, although the mechanism might be different between mammalian cells and *Drosophila*.

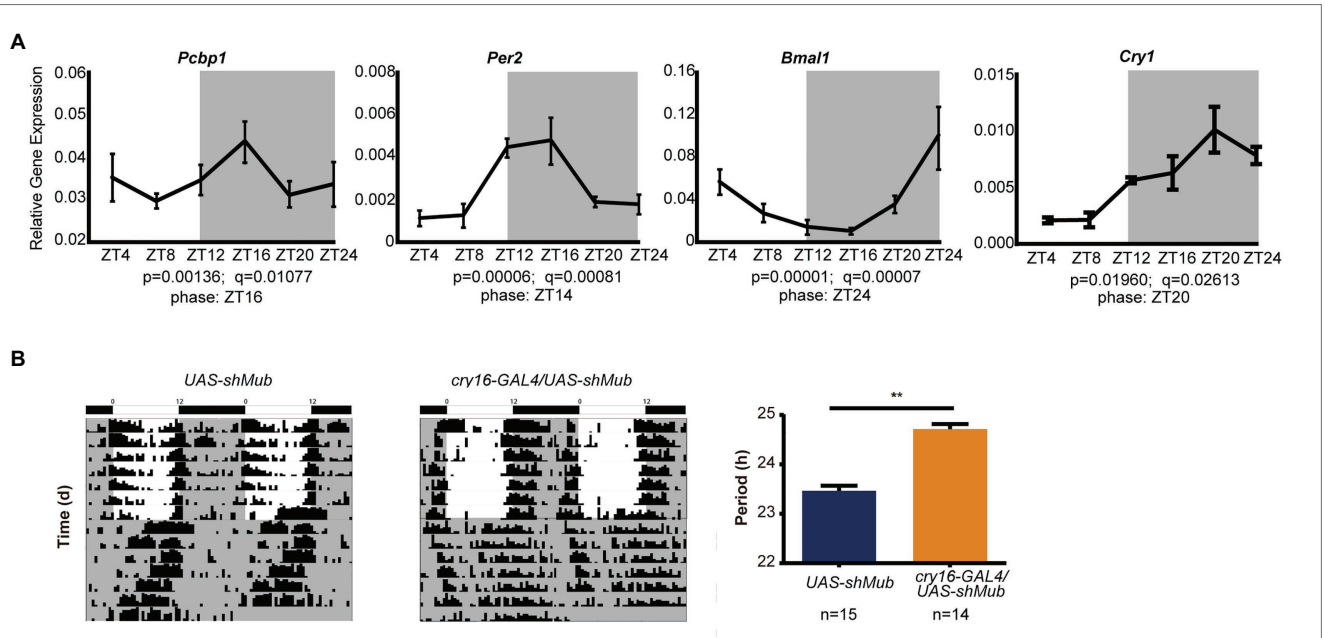

FIGURE 4 | The ability of PCBP1 to regulate circadian rhythm is conserved in evolution. **(A)** The expression of the PCBP1 gene is rhythmic in the mouse heart. The phase of PCBP1 is ZT16, which is similar with PER2 (ZT14) and CRY1 (ZT20), but contrary to BMAL1 (ZT24). All data are presented as the mean ± SEM from at least four independent experiments. The qPCR data were analyzed by JTK_Cycle analysis to determine the rhythmicity and the phases of the peaks (time course threads with both *p* and *q* values less than 0.05, were considered as rhythmic). **(B)** *Drosophila* with conditional knockdown of *mushroom-body expressed* (*mub*), the homolog gene of PCBPs in *Drosophila*, in circadian neurons show a period-lengthening circadian rhythm (middle) compared to wild-type (left). Right: Bars show quantitative period change values Statistical significance was determined using Student's *t*-tests ($n \geq 14$, $^{**}p < 0.01$).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at NIBS.

AUTHOR CONTRIBUTIONS

YW and NL conceived the study. YW, HZ, and NL carried out the experiments and analyzed the data. EZ provided guidance. NL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Genetics

ORIGINAL RESEARCH
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Disruption of Circadian Transcriptome in Lung by Acute Sleep Deprivation

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Inadequate sleep prevails in modern society and it impairs the circadian transcriptome. However, to what extent acute sleep deprivation (SD) has impact on the circadian rhythms of peripheral tissues is not clear. Here, we show that in mouse lung, a 10-h acute sleep deprivation can alter the circadian expression of approximately 3,000 genes. We found that circadian rhythm disappears in genes related to metabolism and signaling pathways regulating protein phosphorylation after acute sleep deprivation, while the core circadian regulators do not change much in rhythmicity. Importantly, the strong positive correlation between mean expression and amplitude (E-A correlation) of cycling genes has been validated in both control and sleep deprivation conditions, supporting the energetic cost optimization model of circadian gene expression. Thus, we reveal that acute sleep deprivation leads to a profound change in the circadian gene transcription that influences the biological functions in lung.

Keywords: circadian clock, lung, circadian transcriptome, sleep deprivation, energetic cost

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INTRODUCTION

Lack of sleep is a commonplace in modern society. Inadequate sleep leads to decreased performance and deterioration in health. Sleep gives our body a chance to repair itself, so the lack of sleep can have harmful health effects. Circadian rhythms are tightly related with the cycle of sleep and wakefulness (Medic et al., 2017). Inadequate or irregular sleep can disrupt the circadian rhythms not only in the brain, but also in peripheral tissues (Huang et al., 2011; Moller-Levet et al., 2013; Archer and Oster, 2015). It has bad consequences on the outcome of health and is associated with several medical conditions, including diabetes, heart disease, stroke, high blood pressure, kidney disease, and mood disorders (Goel et al., 2013; Touitou et al., 2017). Particularly, in the pulmonary system, disruption of circadian rhythm accelerates lung cancer due to the enhanced cell proliferation and the metabolic deregulation (Papagiannakopoulos et al., 2016).

The circadian clock in animals orchestrates genome-wide oscillatory gene expression in a roughly 24-h rhythmicity manner. The molecular mechanism of the circadian oscillator as a transcriptional-translational feedback loop has been identified in multiple species including

insects and mammals (King and Takahashi, 2000; Young and Kay, 2001; Hurley et al., 2016). In mouse, two transcriptional activators (*CLOCK* and *BMAL1*), together with their inhibitors (*PER1*, *PER2*, *CRY1*, and *CRY2*) consist of a circadian oscillation transcriptional network that bridge the cycling gene expression and the circadian control of organ's function. Circadian clocks work in a cell-autonomous manner across all major organs and tissues of the body (Schibler, 2005). In the hierarchical organization of circadian gene transcription, the hypothalamic suprachiasmatic nucleus (SCN) acts as a master pacemaker to synchronize or entrain peripheral clocks throughout the body (Husse et al., 2015). Nevertheless, it is thought that proximally 5–20% of genes expressed in any particular tissue including lung undergo circadian oscillations at the mRNA level (Sun et al., 2020).

Understanding the circadian gene transcription regulation bears significant indication of the biology of lung function, especially while we are facing the situations of the prevailing coronavirus pandemic and the increased incidence of lung cancers nowadays. Reports have shown that many people are facing sleep problems [sleep deprivation (SD)] due to the stress and anxiety caused by COVID-19 (Morin and Carrier, 2020). Thus, it is of importance to know how lack of sleep can have effects on the peripheral clocks especially the regulation of circadian gene transcription in lung. In this study, we found that the oscillation pattern of ~3,000 genes has been changed, and the phase of rhythmic mRNA expression in lung was dramatically shifted after a 10-h sleep deprivation. The genes that lost their circadian expression rhythm after acute sleep deprivation are enriched in biological pathways including metabolism and protein phosphorylation, while the genes that obtained rhythmicity after sleep deprivation are mainly related to cell morphogenesis. Here, we demonstrate that sleep debt effects gene expression rhythmicity with significant implication for its effect on health.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the Institutional Animal Care and Use Committee at Shanghai Jiaotong University School of Medicine. Six- to eight-week-old male C57BL/6 mice housed in a 12 h-light:12 h-darkness (light on at 7:00 AM and light off at 19:00 PM) schedule with *ad libitum* water and mouse chow supply.

Sleep Deprivation

The mice were randomly divided into experimental and control group (12 mice for each group). Animals in the experimental group were subjected to the sleep deprivation instrument, in which animals were kept awake *via* forced locomotion through a slowly rotating drum (40 cm in diameter, 0.4 m per min). Mice had free access to food and water in the drum. All 12 mice in experimental group were placed in the sleep deprivation device for 10 h (6:00 AM–4:00 PM) during their normal resting phase while the control animals were undisturbed. Then fresh lung tissues were obtained at next six circadian time (CT) points with an interval of 4 h (CT4, CT8, CT12, CT16, CT20, and CT24).

RNA-seq

The lung samples from 24 mice were sent to Beijing Novogene Co., Ltd. (Beijing, China) for RNA sequencing, using Illumina Novaseq 6000. The samples were firstly qualified using 1% agarose gel electrophoresis for possible contamination and degradation. RNA integrity and quantity were finally measured using RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Masotti and Preckel, 2006). RNA libraries were prepared as described previously (Takahashi et al., 2015). Raw reads were tested for quality using FastQC. The resulting reads (FASTQ files) were aligned to the mm10 annotation from UCSC using Hisat2 (Pertea et al., 2016). The average fragments per kilobase of transcripts per million reads (FPKM) value for each gene was calculated separately for each circadian time point of the two conditions.

Analysis of RNA-seq Data

To identify periodically expressed genes, the list was further filtered based on expressional level, with a cutoff of FPKM > 1 in 80% samples (n = 16,505 genes left in the final dataset). After the filterion, MetaCycle, an R package that incorporates ARSER, JTK_CYCLE, and Lomb-Scargle to conveniently evaluate periodicity in time-series data, was performed to identify oscillation genes (Wu et al., 2016). The output result of MetaCycle contained p value, period, and revised amplitude (Wu et al., 2016). Circadian transcripts were defined based on the cutoff of adjusted $p < 0.05$.

Frequency histogram of the difference between the phase of the 318 R-R genes in SD and control conditions was performed by ggplot package in R (**Figure 1E**), in which the values showed in the x-axis equaled the phase of SD minus the phase of control. Heatmaps were generated using the pheatmap package in R to display the oscillated genes based on z-score (Kolde, 2018). The gene expression levels were normalized by $\log_2$ (FPKM + 1) in heatmaps. The Venn Diagram Plotter generated by VennDiagram package was used to compare the rhythmic genes under control and SD conditions (Chen and Boutros, 2011). Gene ontology (GO) analysis on circadian disrupted genes was carried out using GO over-representation test in clusterProfiler package (Yu et al., 2012). A value of $p < 0.05$ was used as a cutoff for significantly enriched terms, and the graphs were drawn by R package enrichplot. Scatter plots were conducted based on log transformed amplitude and FPKM value, and the linear regressions were implemented using the "lm" function in R.

Weighted Gene Co-expression Network Analysis

Weighted gene co-expression network analysis (WGCNA) was performed using WGCNA package (Langfelder and Horvath, 2008). Only genes for which the FPKM value was greater than 1 in

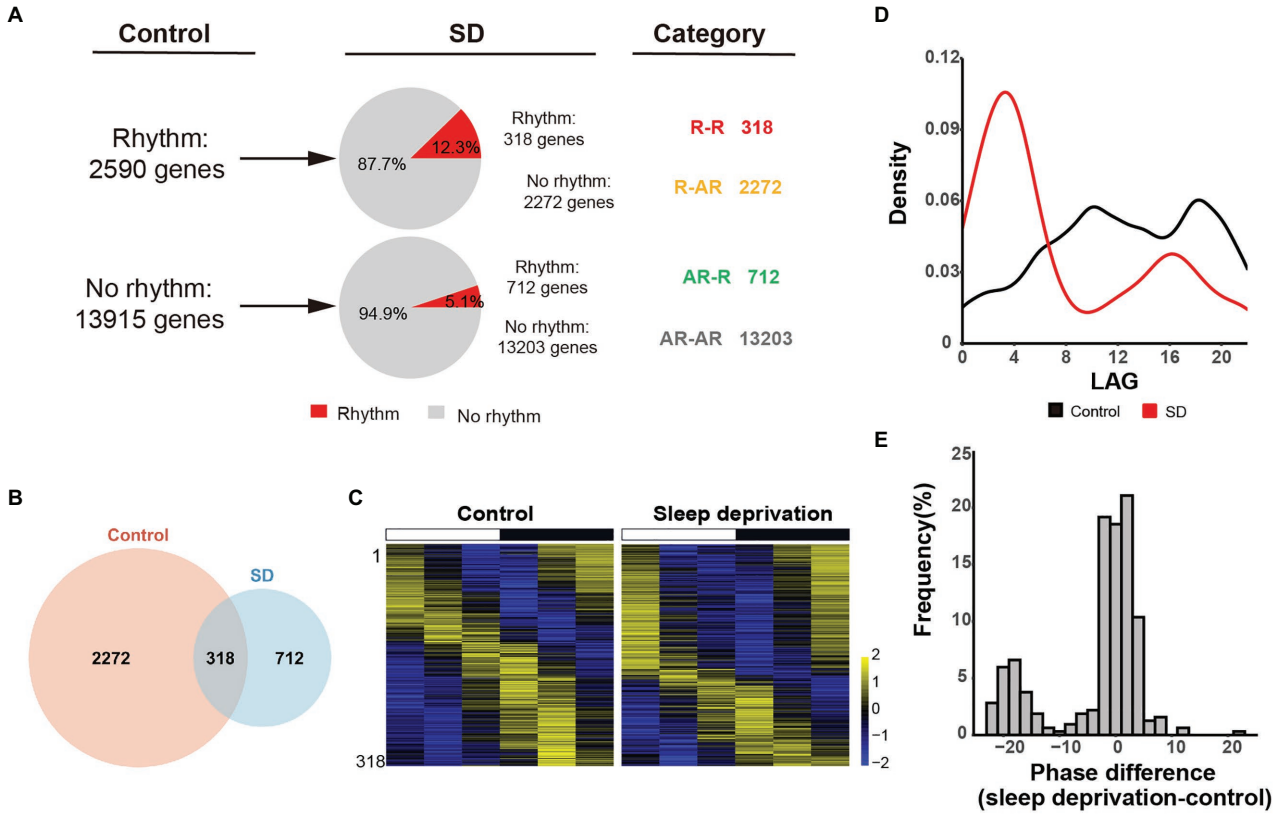

FIGURE 1 | Sleep deprivation alters the circadian gene transcription in lung. **(A)** Quantification of the number of genes that are rhythmically transcribed in the mouse lung. The rhythmic signals are detected by JTK_CYCLE from time-series datasets. Four categories of rhythmically expressed genes were determined by comparing the control and sleep deprivation conditions: rhythmic genes in both conditions (R-R), rhythmic genes only in control condition (R-AR), rhythmic genes only in SD condition (AR-R), and arrhythmic genes in both conditions (AR-AR). **(B)** Area-proportional Venn diagram comparing rhythmic genes identified in the two conditions. **(C)** Heatmap representation of R-R category for the 318 genes that are rhythmically transcribed in the mouse lung. Classification is based on the phase of mRNA oscillations, and each lane corresponds to one gene. High expression is displayed in yellow (*z*-score > 1) while low expression in blue (*z*-score < 1). **(D)** Phase distribution for 3,620 rhythmic genes in two conditions. **(E)** Distribution of the difference between the phase of cycling gene expression in control and SD for the 318 R-R genes.

80% samples were used (n = 16,505). Pearson's correlations between each gene pair were calculated to build an adjacency matrix. A soft-threshold power was automatically calculated to achieve approximate scale-free topology ($R^2 > 0.85$). Then, the topological overlap measure (TOM) and corresponding dissimilarity (1-TOM) was calculated using adjacency matrix. 1-TOM was used as a distance for gene hierarchical cluster, and then DynamicTree Cut algorithm (Langfelder et al., 2008) was used to identify the modules (defined as clusters of highly interconnected genes). We generated 22 modules in control condition. Each module was named following a different color. Gene set enrichment applied for modules was performed using a Fisher's exact test in R. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Kanehisa and Goto, 2000) analysis and was conducted to identify circadian disrupted genes at the biologically functional level. The Database for Annotation, Visualization, and Integrated Discovery (DAVID; David.abcc.ncifcrf.gov) was used to integrate functional genomic annotations (Dennis et al., 2003). Value of $p < 0.05$ was considered to indicate a statistically significant difference (Huang et al., 2008).

RESULTS

Sleep Deprivation Alters the Circadian Gene Transcription Profile in Lung

To assess the effect of acute sleep deprivation on circadian rhythm of gene expression in lung, we deprived sleep from the mice for 10 h in 1 day. The animals in the control group were housed under 12 h: 12 h light/dark cycle, while the animals in the acute sleep deprivation group (SD) were kept awake for 10 h from 6 AM. to 4 PM according to a sleep deprivation paradigm. After 10-h sleep deprivation, we harvested the lung tissue at six time points with 4-h intervals during the following 24 h. Transcriptome analyses were performed by total RNA-sequencing (whole-transcriptome sequencing). The comparison between control and SD conditions was restricted

to genes that were sufficiently expressed in both datasets, with a cutoff of FPKM > 1 in at least 80% samples (16,505 genes left in the final dataset).

To identify oscillated gene expression, we performed MetaCycle analysis and found poor overlap exist between control and SD rhythmic gene sets (adjusted $p < 0.05$, Benjamini and Hochberg's method). We found that in both conditions, the expression of a large group of genes have exhibited robust oscillation signals (**Figure 1A**), implicating that cycling transcriptome is a basic feature of gene transcription. Specifically, we identified 2,590 rhythmically expressed genes in control group. Only 12.3% of them (318 out of 2,590 genes) manifested maintained rhythmicity in SD group. The rhythmically expressed genes that maintained rhythmicity after sleep deprivation were referred to as "R-R" gene set (**Figures 1A,B**). The other 87.7% of rhythmically expressed genes in control group (2,272 out of 2,590 genes) failed to keep rhythmicity after acute sleep deprivation. The rhythmically expressed genes that lost rhythmicity after sleep deprivation were referred to as "R-AR" gene set (**Figures 1A,B**). In contrast, 712 genes gained rhythmicity after sleep restriction. The *de novo* rhythmically expressed genes after sleep restriction were referred to as "AR-R" gene set (**Figures 1A,B**).

We found that there were 318 genes in R-R data set that kept rhythmicity after acute sleep deprivation (**Figure 1C**). However, we found that oscillation phase was dramatically altered after sleep deprivation, when we analyzed the phase of circadian transcripts in both control and SD conditions. The peaks at circadian time 10 (CT10) and circadian time 18 (CT18) in control condition coincided with the trough in SD condition, while the peaks at circadian time 14 (CT4) and circadian time 16 (CT16) in the SD condition concurred with the trough of control (**Figure 1D**), suggesting that most oscillation genes in lung exhibited a time-dependent transcription profile after acute sleep deprivation. Further, we wanted to know whether the genes in R-R data set kept the phase of rhythmicity. We performed the phase difference calculation (phase of sleep deprivation – phase of control) and found that there was a significant phase shift in rhythmicity between the control and SD conditions (**Figure 1E**).

Circadian Rhythm Disappears in Genes Related to Metabolism and Signaling Pathway Regulating Protein Phosphorylation After Sleep Deprivation

We found the majority of rhythmically expressed genes (2,272 "R-AR" genes) in control condition lost oscillation in SD condition (**Figure 2A**). To further understand the functional and biological pathways of those genes, we performed GO analysis on biological processes. We found that those genes were significantly enriched in functional classes, including regulation of catabolic process (p = 0.001407), regulation of protein phosphorylation (p = 0.001407), ameboidal-type cell migration (p = 0.001407), and phospholipid metabolic process (p = 0.001479; **Figure 2C**). In contrast, the 712 "AR-R" genes that gained circadian expression after sleep deprivation were enriched in processes, such as epithelial tube morphogenesis (p = 7.63E-06), vasculature development (p = 0.000375), morphogenesis of a branching structure (p = 6.89E-05), and morphogenesis of a branching epithelium (p = 6.89E-05; **Figures 2B,C**). In addition, we observed a strong positive correlation between mean expression and amplitude (E-A correlation) of periodically expressed genes in both control and SD conditions ($R^2 > 0.75$, **Figure 2D**), supporting the energetic optimization models of the transcriptome in the two conditions (Wang et al., 2015; Cheng et al., 2019; Sun et al., 2020). Notably, the E-A correlation in the two conditions was comparable, implicating that a similar level of optimization on the transcriptome was achieved.

Core Circadian Regulators Maintain Rhythmicity After Sleep Deprivation

As more than 2,200 genes lost rhythmicity in their gene expression after a 10-h sleep deprivation, we next examined whether there was a significantly oscillation behavior changes in the core circadian regulatory network. We found that the overall circadian behavior does not change much in the core transcriptional factors. The six core circadian regulators (*Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1*, and *Cry2*) still exhibited strong oscillation signal after acute sleep deprivation (**Figure 3A**). However, we found that the amplitude of *Per1* and *Cry1* was slightly decreased in SD condition (**Figure 3A**). The detailed effects of the changing amplitude of core circadian regulators need more sophisticated modeling. Further, compared with the effects of insufficient sleep on human blood samples, it seems that *Camk2d*, which is related to sleep homeostasis, and *SLC2A3* and *ABCA1*, which are related to metabolism, changed their oscillation behavior in SD condition (**Figure 3B**).

Large-Scale Co-expressed Gene Networks Are Associated With Period Heterogeneity

Because functionally related genes are usually co-expressed (Heyer et al., 1999), we further characterized the circadian disrupted genes by examining their co-expression patterns. Using WGCNA, we generated 22 modules from 16,505 genes in control condition (**Figure 4A**). The soft-threshold for network construction was selected as 12 to make a scale-free network (**Figure 4A**). Several modules exhibited significant enrichment for circadian disrupted genes (**Figure 4B**). Royal blue, purple, midnight blue, light green, and cyan modules were enriched in R-AR gene set, while salmon, magenta, and black modules were enriched for AR-R gene set (**Figure 4B**). In this WGCNA analysis, the genes that were not clustered in any module were in grouped as the gray module, so the subsequent analysis was no longer performed on this module.

To further screen the specific biological functions or pathways of each module, we performed KEGG analysis (**Figures 4C–G**). Within the R-AR-enriched modules, the "midnight blue" module was enriched in several signaling pathways including the Hypoxia-inducible factor 1 (HIF-1) signaling pathway (**Figure 4C**; p = 2.95E-04). This pathway is known to trigger adaptive responses of cells under hypoxic stress through transcriptional activation of hundreds of downstream genes involved in cancer development (Lu et al., 2016). The "purple" module was enriched in the function of circadian rhythms (p = 0.039131), in addition to

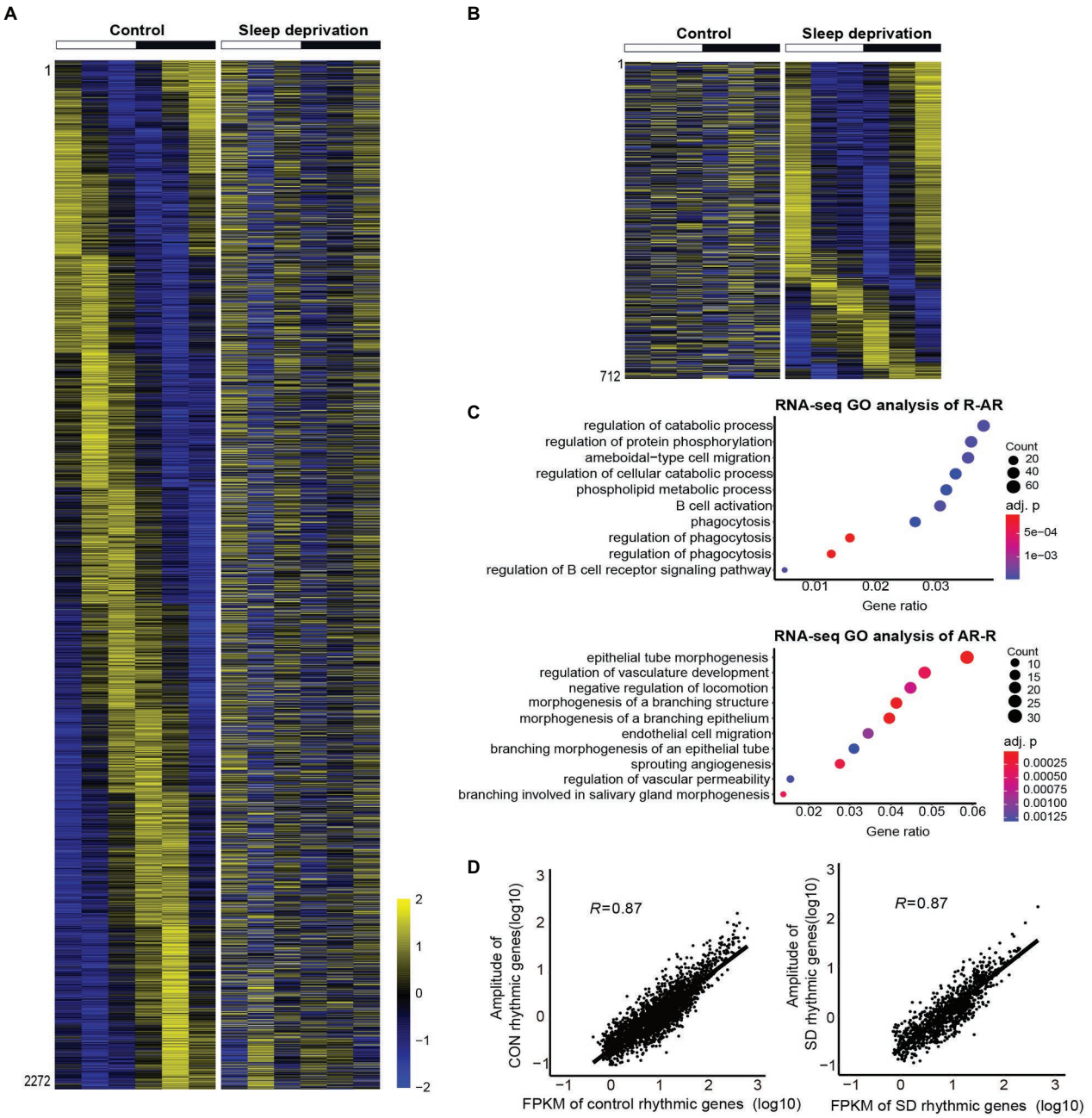

FIGURE 2 | Gene ontology (GO) analyses on the genes that altered rhythmic expression after sleep deprivation. **(A,B)** Heatmap of cyclic mRNA expression in the mouse lung. The majority of rhythmically expressed genes in control condition lost oscillation after sleep deprivation (**A**, n = 2,272, R-AR category in **Figure 1A**). There were many genes that gained circadian oscillation expression after sleep deprivation (**B**, n = 712, AR-R category in **Figure 1A**). High expression is displayed in yellow (z-score > 1) and low expression in blue in the heatmaps (z-score < 1). **(C)** GO analysis for functional classes in which circadian disrupted genes in the control and SD conditions were enriched. **(D)** Amplitude of rhythmic genes was correlated with mean expression in both control (n = 2,590, R = 0.87) and SD (n = 1,030, R = 0.87) conditions.

MAPK signaling pathway (p = 0.043868), Hippo signaling pathway (p = 0.011556), and GnRH signaling pathway (p = 0.020401; **Figure 4D**). The "blue" module was shown to be functional in metabolic-related processes, further confirming the importance of the circadian-disrupted gene networks (**Figure 4E**). Given that the HIF-1, MAPK, and Hippo signaling pathways all involve biological processes of protein phosphorylation, the KEGG analyses of R-AR-related modules (**Figure 4**), together with the GO analyses of R-AR gene set (**Figure 2**), indicated that sleep deprivation impaired signaling pathways regulating

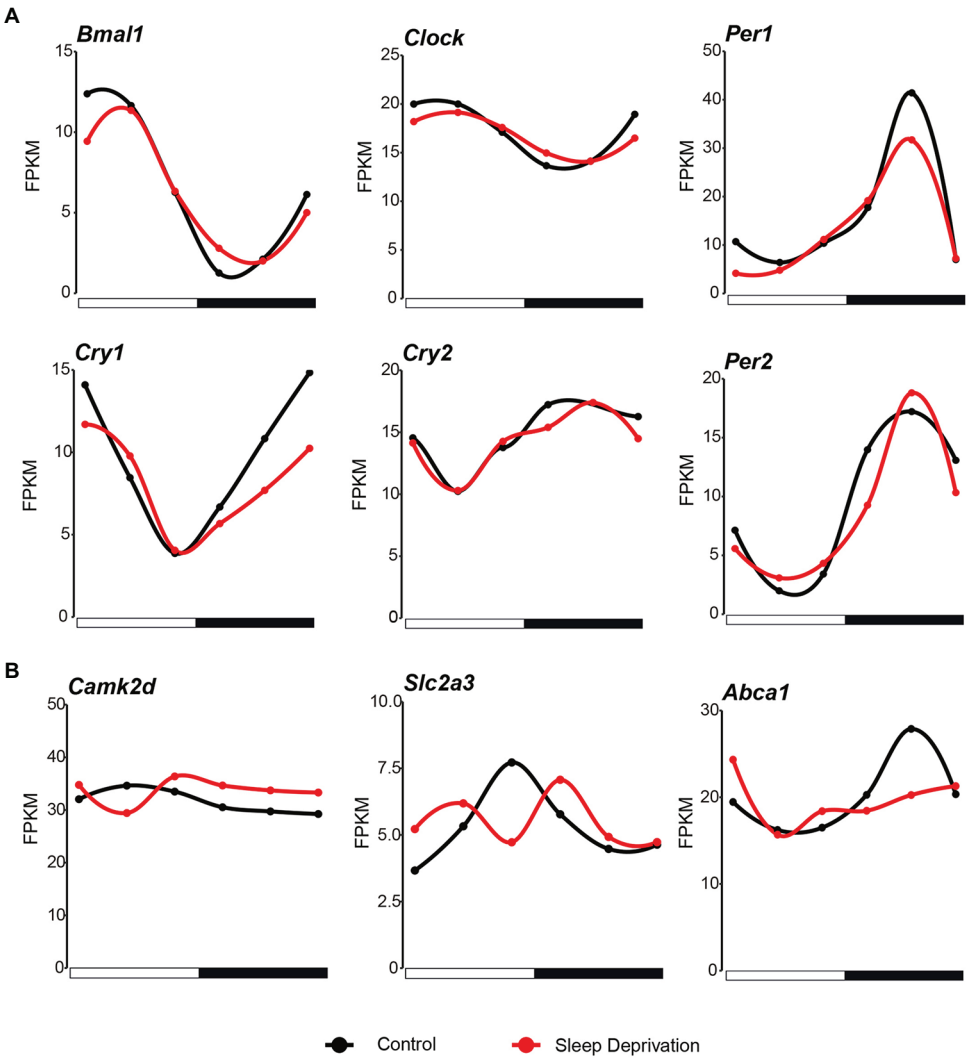

FIGURE 3 | Core circadian regulators maintain rhythmicity after sleep deprivation. **(A)** The circadian expression of core clock genes in the mouse lung during the 24 h in both control (black lines; time points every 4 h starting at CT4) and SD (red lines; time points every 4 h starting at CT4) conditions. **(B)** The mRNA expression of genes that changed the circadian behavior compared with human after sleep deprivation (black lines; time points every 4 h starting at CT4) and SD (red lines; time points every 4 h starting at CT4) conditions.

protein phosphorylation. Within the AR-R enriched modules, the "black" module was enriched in cAMP signaling pathway (p = 0.0025) (**Figure 4F**). The "magenta" module was enriched in MAPK signaling pathway (p = 0.001295), aminoacyl-tRNA biosynthesis (p = 0.005513), and NF-kappa B signaling pathway (p = 0.026075; **Figure 4G**). The 712 genes in AR-R gene set were mainly associated with the regulation of intercellular communication, transcription, and protein synthesis, suggesting that they contributed to gene overexpression and cell proliferation. In addition, the 318 genes that maintained rhythmicity after acute sleep deprivation (the R-R gene set) were mainly associated with the regulation of circadian rhythm (**Supplementary Figure S2**). Taken together, these results suggested that sleep deprivation resulted in profound changes on several biological processes related to adverse health outcomes.

DISCUSSION

By utilizing a 10-h sleep deprivation paradigm, we found that >2,500 genes displayed periodically expression signal in the normal light-dark condition, while only ~1,000 genes have oscillated gene expression in the sleep deprivation condition. The gain and loss of rhythmicity of gene expression were associated with widely distributed biological functions. The reason why the "AR-R" genes gained rhythmicity might be due to the altered expression of several transcription factors and cofactor after acute sleep deprivation (**Supplementary Figure S1**). Although the outcome of the circadian regulatory network dramatically changed, the core circadian transcriptional factors maintained their rhythmicity in this short-period sleep disruption. Finally, the circadian oscillation of gene expression in both

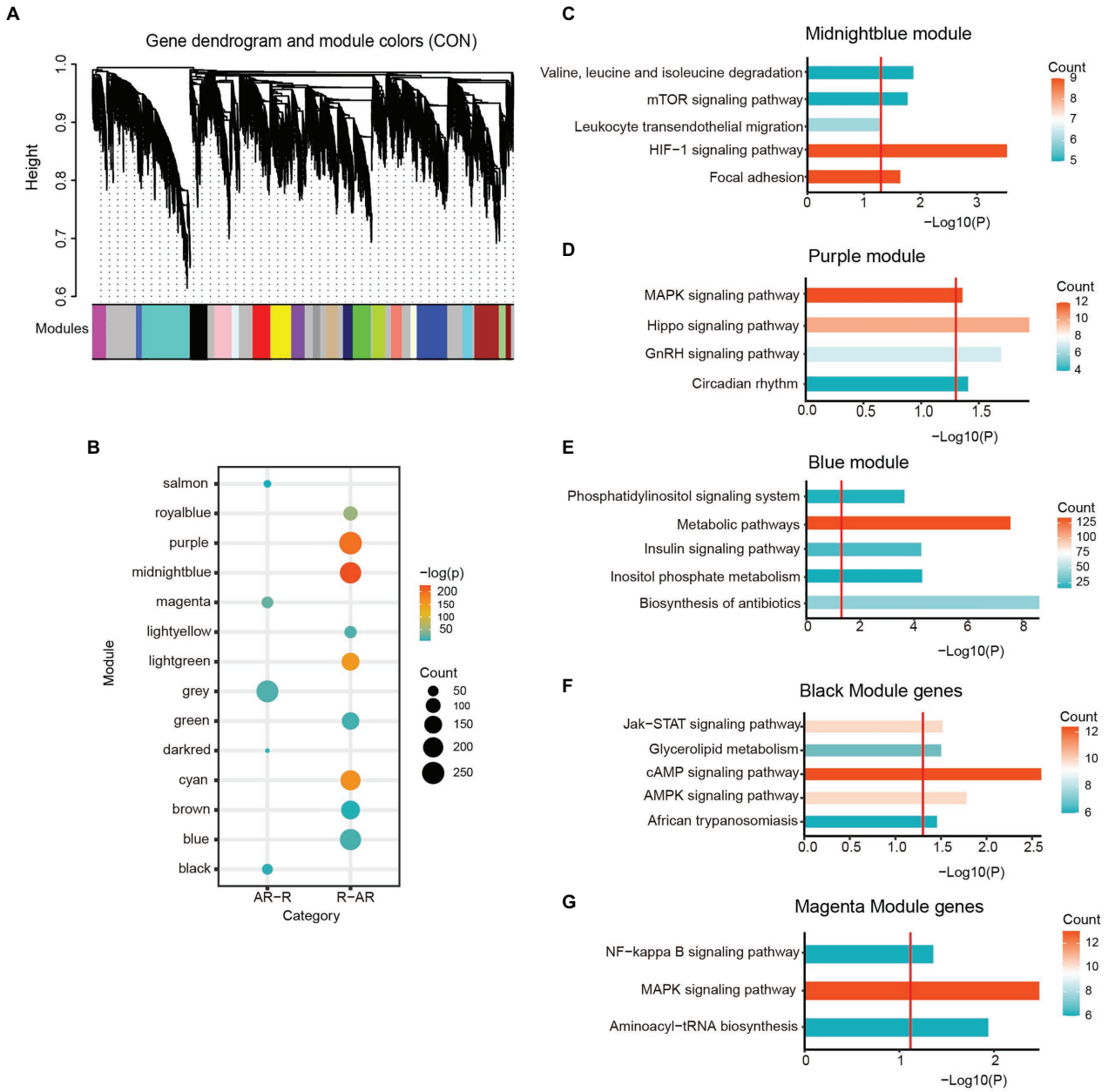

FIGURE 4 | Weighted gene co-expression network analysis (WGCNA) analyses on the circadian disrupted genes. **(A)** Hierarchical cluster analysis was conducted to detect co-expression clusters with corresponding color assignments. Each color represented a module in the constructed gene co-expression network by WGCNA. Twenty-two modules were identified and marked by colors on the horizontal bar. **(B)** Bubble plot showing enrichment of AR-R (n = 712) and R-AR (n = 2,272) genes in the main modules. Values of p were calculated using the Hypergeometric test. **(C–G)** Top ingenuity pathways associated with the midnight blue module **(C)**, the purple module **(D)**, the blue module **(E)**, the black module **(F),** and the magenta module **(G)**. Red threshold lines refer to p = 0.05.

normal and SD conditions support the energetic cost optimization model of circadian gene expression.

Although chronic sleep restriction has been proved to show profound impacts on transcription regulation in many peripheral tissues including the liver and adipose tissue (Barclay et al., 2012; Husse et al., 2012), acute sleep deprivation has also been demonstrated to raise risk of developing acute sleep loss-associated adverse outcomes in several peripheral tissues such as adipose tissue (Wilms et al., 2019), and skeletal muscle (Lamon et al., 2021). The RNA-seq data from adipose tissue revealed that acute sleep loss could up-regulate oxidative phosphorylation- and ribosome-related signaling pathways (Cedernaes et al., 2018). Lamon et al. (2021) showed that acute sleep deprivation blunted the muscle protein synthesis that affected the muscle protein turnover in skeletal muscle tissue. Taken together, these studies, as well as our current

study, indicated that acute sleep deprivation affected gene expression in a tissue-specific manner by targeting different signaling and biological pathways.

Previously, it was reported that only several hundreds of genes have lost rhythmicity signals in insufficient sleep, such as in human blood (Moller-Levet et al., 2013). There are several reasons why a larger number of genes have been detected in our study. Firstly, we have densely sampled the lung for two replicates with an interval of 4 h, and the sequencing depth for each sample is relatively high (approximately 50 million reads *per* sample); secondly, the peripheral tissue (lung) that we focused is the organ that contains one of the highest number of cycling genes among other organs; thirdly, there are less genetic heterogeneity in the laboratory mouse (C57/BL6 strain) samples than in human samples collected before (Moller-Levet et al., 2013).

We found that circadian rhythm disappears in genes related to metabolism and signaling pathway regulating protein phosphorylation after sleep deprivation. Putative mechanisms linking sleep deprivation and metabolic disorders have been uncovered for both animals (Opperhuizen et al., 2015) and humans (Nedeltcheva and Scheer, 2014). Studies have provided convincing evidence that even short-term decreases in sleep quantity or quality can have deleterious effects on glucose regulation, interfere with the secretion of anabolic hormones including growth hormones and prolactin, and alter the amount and timing of catabolic hormones including glucocorticoids and catecholamines (Spiegel et al., 1999; Penev et al., 2005). Also, the signaling pathway of insulin (phosphorylation of Akt) was disturbed along with abnormal glucose control by sleep restriction (Broussard et al., 2012).

Phosphorylation is one of the most important modalities in the regulation of protein activity and it is used to control the basic function of cells (Cohen, 2002; Nishi et al., 2014). In the nervous system, the global phosphorylation statuses in neurons appear to be controlled by sleep-wake cycles (Wang et al., 2018; Bruning et al., 2019). It is thought that protein phosphorylation and sleep-wake cycles have bidirectional mutual effects on each other. CaMKIIα/CaMKIIβ, SIK3 and ERK1/ERK2 have been identified as sleep-promoting kinases (Ode and Ueda, 2020). Phosphoproteomic studies indicated that SIK3 appears to induce a comparable phosphoproteomic profile to that caused by sleep deprivation, and the dynamics of CaMKII activation correlate well with the expected accumulation of sleep need. These previous studies support our major finding that circadian rhythm disappears in genes related to metabolism and signaling pathway regulating protein phosphorylation after sleep deprivation.

The disappearance of a large number of oscillated genes in lung after a short insufficient sleep has many biomedical implications, especially in the period of COVID-19 pandemic. Patients who have been infected by COVID-19 may face sleep problems due to anxiety and symptoms, such as fever, headache, and difficulty breathing. The short of sleep may influence the circadian regulation of lung itself, aggravating the symptoms of the infection. Thus, COVID-19 patient may be highly advised to have high quality sleep, especially during the infection and recovery period.

Taken together, in this research, we revealed that the plasticity of lung transcriptome was higher than previous thought. A short-term sleep disruption may have a strong effect on the circadian gene expression. Further studies may focus on the consequences of the dramatic expression changes, especially on the direction of the connection between transcriptome plasticity and lung cancer etiology.

DATA AVAILABILITY STATEMENT

The data files for RNA-seq reported in this article have been deposited in the Gene Expression Omnibus database under GSE166335.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Shanghai Jiaotong University School of Medicine.

AUTHOR CONTRIBUTIONS

JH and YL designed and supervised the project. BL and JM performed bioinformatics analyses. JH and BL wrote the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.664334/full#supplementary-material

Supplementary Figure S1 | Transcription factors gain rhythmicity after sleep deprivation. The expression of some transcription factors in the mouse lung during the 24 h in both control (black lines; time points every 4 h starting at CT4) and sleep deprivation (red lines; time points every 4 h starting at CT4) conditions.

Supplementary Figure S2 | KEGG analyses on the genes that maintained rhythmicity after sleep deprivation. Top ingenuity pathways associated with the R-R gene set. Red threshold lines refer to $p = 0.05$.

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Circadian Clock-Controlled Checkpoints in the Pathogenesis of Complex Disease

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The circadian clock coordinates physiology, metabolism, and behavior with the 24-h cycles of environmental light. Fundamental mechanisms of how the circadian clock regulates organ physiology and metabolism have been elucidated at a rapid speed in the past two decades. Here we review circadian networks in more than six organ systems associated with complex disease, which cluster around metabolic disorders, and seek to propose critical regulatory molecules controlled by the circadian clock (named clock-controlled checkpoints) in the pathogenesis of complex disease. These include clock-controlled checkpoints such as circadian nuclear receptors in liver and muscle tissues, chemokines and adhesion molecules in the vasculature. Although the progress is encouraging, many gaps in the mechanisms remain unaddressed. Future studies should focus on devising time-dependent strategies for drug delivery and engagement in well-characterized organs such as the liver, and elucidating fundamental circadian biology in so far less characterized organ systems, including the heart, blood, peripheral neurons, and reproductive systems.

Keywords: circadian clock, complex disease, checkpoint, Nr1d1/Rev-erbα, melatonin, circadian rhythm, diabetes complications, systems biology

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INTRODUCTION

To align with the daily cycling of environmental conditions associated with the Earth's rotation, terranean and subterranean organisms have evolved an internal circadian (Latin words: circa, about; dies, day) clock system to organize metabolism, physiology, and behavior (Bass and Lazar, 2016; Zhang et al., 2020b; López-Otín and Kroemer, 2021). The circadian clock manifests as internal and resettable (known as entrainable in the field) circadian rhythms at the molecular, physiological, and organismal levels. The circadian clock system in mammals is organized in a hierarchical manner, orchestrated by a master pacemaker in the hypothalamus. The circadian clock in individual cells is genetically similar and composed of transcriptional-(post)translational feedback loops.

In a time when fundamental and preclinical research on circadian rhythms is progressing at an unprecedented speed, the concept of time medicine (or chronomedicine, circadian medicine) is emerging as a new dimension in medicine (Cederroth et al., 2019; Panda, 2019; Allada and Bass, 2021). Here, we focused on research in the role of the circadian clock in major organs/tissues related to the pathogenesis of complex diseases, particularly those with metabolic disorders, and summarized tissue-specific circadian clock-controlled checkpoints as genes, proteins, and biological pathways that would be valuable for time medicine.

The Architecture of the Circadian System in Mammals

The architecture of the mammalian circadian clock system is established by a pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Allada and Bass, 2021). Light activates the non-cone non-rod melanopsin 4-expressing retinal ganglion neurons in the retina, transmitting signals to the SCN through a retinohypothalamic tract. The phase of the SCN is readily entrained by light, but seems refractory to other time signals associated with feeding and body temperature (Buhr et al., 2010; Reinke and Asher, 2019). The SCN coordinates clocks in non-SCN cells and tissues via neuronal or humoral connections and metabolic signaling associated with feeding rhythm. Feeding rhythm is crucial in clock synchronization in the body (Reinke and Asher, 2019), yet different peripheral clocks seem to respond with a broad spectrum of kinetics (Xin et al., 2021). Inverted feeding readily reverses circadian rhythms in liver and adipose tissue (Manella et al., 2021; Xin et al., 2021). Recently, a multi-omics study indicated that diurnal rhythms of transcriptomes, metabolomes, and clocks in the heart and kidney are resistant to circadian entrainment by feeding rhythm (Xin et al., 2021). The tissue-specific response to inverted feeding is gated by the temporal signaling from the SCN clock and the liver-clock (Manella et al., 2021; Xin et al., 2021). Thus, the coordinated actions of the SCN-peripheral clock axes orchestrate the harmony of mammalian physiological activities throughout the day (**Figure 1**), including sleep-wake cycle, endocrine secretion, blood pressure, energy metabolism, and urine production (Bass and Lazar, 2016).

Clocks in individual neurons of the SCN pacemaker are coupled to establish a robust circadian rhythm, which can last for weeks *in vitro* without dampening (Welsh et al., 2009). This strong intercellular coupling property is crucial for organizing the body's clock. Both gap junctions and paracrine neurotransmitter signaling contribute to intercellular coupling. The SCN pacemaker is made of a pair of neuronal lobes containing ∼10,000 neurons, which are divided into a ventral core region and a dorsal shell region. The core region sits above the retinohypothalamic tract and receives input signals from environmental light. The shell region receives signals from the core region through neuronal projections and gap junctions. These two regions can be distinguished by the concentration of different neuropeptides, represented by vasoactive intestinal polypeptide (VIP) in the core neurons and arginine vasopressin (AVP) in the shell neurons. Upon light entrainment, VIP is released from the core neurons, depolarizing shell regions, leading to the resetting of the SCN clock. Recently, Shan et al. (2020) devised a color-switch dual-color imaging system in mice and showed that AVP-expressing shell neurons are crucial for maintaining cell-autonomous circadian rhythm in the SCN.

Molecular Mechanisms of the Circadian Clock

In mammals, the circadian clock is mainly composed of three transcriptional-(post)translational feedback loops (**Figure 1**). Transcription factors BMAL1 and CLOCK heterodimerize and activate transcription of the clock genes *Period (PER1, PER2)* and *Cryptochrome (CRY1, CRY2)*. As they increasingly accumulate in the cytosol, PER and CRY heterodimerize, translocate into the nucleus, and shut down the expression of their own genes by inhibiting BMAL1-CLOCK. The turnover and activity of these core clock proteins are controlled by post-translational modifications, including phosphorylation, ubiquitination, acetylation, and O-linked β-*N*-acetylglucosaminylation (*O*-GlcNAcylation) (Panda, 2016; Takahashi, 2017). Phosphorylation of PER2 serine 662 is a key regulatory node in setting the speed of the clock, initiating the casein kinase 1δ/ε-controlled phosphorylation events and ubiquitin-mediated protein degradation.

BMAL1-CLOCK also activates the expression of *REV-ERBα (NR1D1)* and *REV-ERBβ (NR1D2)*, which repress expression of *BMAL1* and *NFIL3* (nuclear factor, interleukin 3-regulated, also known as E4BP4). Retinoic acid-related orphan receptor (ROR) and REV-ERB competitively bind to RORE *cis*-elements, resulting in the regulation of one stabilizing loop of the circadian clock. The other stabilizing loop is composed of the PAR-bZIP (proline and acidic amino acid-rich basic leucine zipper) factors DBP (D-box binding protein), TEF (thyrotroph embryonic factor), and HLF (hepatic leukemia factor), whose expression is controlled by BMAL1-CLOCK. REV-ERB/ROR-controlled repressor NFIL3 and the PAR-bZIP transcription factors regulate expression of genes containing D-box *cis*-elements. Together, these three interlocking feedback loops generate robust circadian rhythms of the clock genes and output genes, whose phase

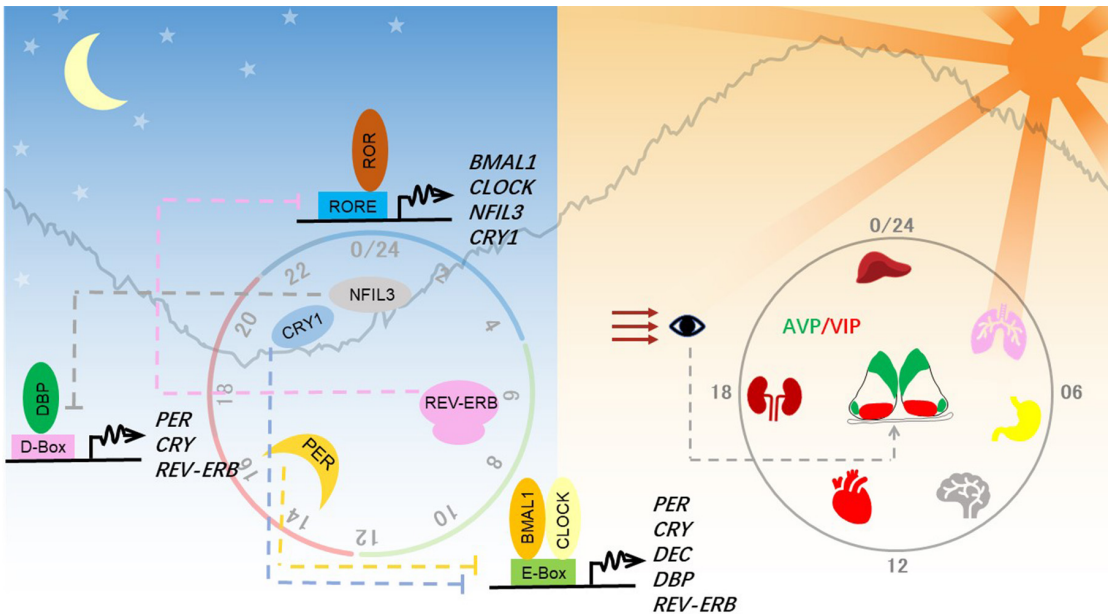

FIGURE 1 | Molecular and anatomical structure of the circadian clock system. **Left panel:** Molecular mechanisms of the circadian clock mainly consist of three interlocked transcriptional-translational feedback loops. Transcriptional factors activate the transcription of repressor genes via binding to promoter regions containing different combinations of three *cis*-elements and thereby generating a different phase. These protein products of the repressor genes then enter the nucleus and feed back to repress their own transcription to close the circadian loop. **Right panel:** Circadian clocks exist in almost all tissues in mammals. The hypothalamic suprachiasmatic nucleus (SCN) in the brain serves as the master clock or the central clock, which is entrained by light through the retinohypothalamic tract pathway. Neuropeptides AVP and VIP maintain the robust cell-autonomous rhythmicity and light entrainment, respectively, in the SCN. The SCN clock synchronizes slave clocks in peripheral tissues through neuronal and humoral pathways throughout the body.

and amplitude are determined by combinations of circadian *cis*-regulatory elements in the promoter and enhancer regions.

CIRCADIAN PHYSIOLOGY IN MAJOR ORGANS ASSOCIATED WITH COMPLEX DISEASE

Liver

The liver is a prototypical peripheral organ that is deeply regulated by the circadian clock system. Both transcriptional and organelle-level mechanisms orchestrate diurnal rhythms of liver physiology, ranging from the well-characterized metabolic functions (including metabolism of glucose, glycogen, lipids, and amino acids) to cell growth and xenobiotic responses. For example, the circadian clock drives diurnal expression of glycogen synthase 2 (GYS2) to modulate glycogen synthesis, phosphoenolpyruvate carboxykinase 1 (PEPCK1) and glucose-6-phosphatase, catalytic (G6PC) for gluconeogenesis; and insulin induced gene 2 (INSIG2) for cholesterol and bile acid synthesis (Bass and Lazar, 2016; Panda, 2016; Reinke and Asher, 2019; Zhang et al., 2020b). PER2 and CRY1 bind to nuclear hormone receptors, such as REV-ERBα/β, PPARα, and GR to output the diurnal rhythms to glucose and lipid metabolism (Lamia et al., 2008; Schmutz et al., 2010; Zhang et al., 2010). Emerging evidence suggests a SCN-liver axis in the control of diurnal rhythm in hepatic glucose metabolism. REV-ERBα/β in SCN GABAergic neurons are required for rhythmic insulin-mediated suppression of glucose production in the liver (Ding et al., 2021). In addition to cell-autonomous effects, the hepatocyte clock can modulate the circadian rhythm of local microenvironment. Hepatic REV-ERBα/β is required for the circadian rhythmicity of transcriptomes and metabolomes in local endothelial cells and Kupffer cells in the liver, which is associated with circadian rhythms of glutathione metabolism, glycolysis, and hexosamine pathway (Guan et al., 2020).

It has become apparent that clock proteins can execute temporal order through crosstalk to organelle structure and dynamics. To meet the daily demand of cellular energy and stress adaptation, mitochondrial structure and organization are regulated by the circadian clock. Hepatic BMAL1 is crucial for circadian rhythm of the mitochondrial fission, fusion, and turnover by autophagy, in part through its transcriptional target genes involved in mitochondrial fission and fusion (Jacobi et al., 2015). Nutrient-sensing pathways such as AMPK and Sirtuins drive circadian rhythm of phosphorylation of mitochondrial fission executor, dynamin-related protein 1 (DRP1, peak at CT 12 h). This in turn leads to diurnal oscillations of mitochondrial morphology between fission and fusion and cellular energy (Schmitt et al., 2018). In synchrony with the cycling of mitochondrial structure, mitochondrial fatty acid beta-oxidation (peak at ZT0) and protein abundance of mitochondrial rate-limiting enzymes (CPT1A peak at ZT 0 h and PDHB peak at ZT 3 h)

exhibit diurnal rhythms, which are all controlled by PER1/PER2 (Neufeld-Cohen et al., 2016).

Lysosomes exhibit diurnal activities in the liver. Autophagy promotes lysosomal degradation of CRY1, contributing to clock regulation (Toledo et al., 2018). Using p62/SQSTM1 as a marker, Ryzhikov et al. (2019) found that basal autophagosome formation (peak at ZT 8 h) is coupled to proteasomal protein degradation (peak at ZT 10 h) during the resting phase. Interestingly, proinflammatory signals such as lipopolysaccharide (LPS) alter the substrate preference of autophagy from cytosolic proteins to mitochondrial proteins (Ryzhikov et al., 2019). Diurnal expression of autophagic genes is conferred in part through diurnal transcriptional activity of CCAAT enhancer binding protein beta (C/EBPβ) (Ma et al., 2011). Emerging evidence supports the strong circadian regulation of autophagy at the proteomic and post-translational levels. Wang Y. et al. (2018) showed that lysosomal transport signature is enriched in diurnal proteome in liver, however, there is no circadian rhythm of the transcripts encoding these proteins. Autophagic proteins and their regulator, mammalian target of rapamycin complex 1 (mTORC1), which is among the 25% of the hepatic circadian phospho-proteome, exhibit circadian rhythm in liver (Robles et al., 2017). Cytosolic PER2 tethers tuberous sclerosis 1 protein (TSC1) to suppress the activity of mTORC1, contributing to diurnal rhythms of protein synthesis and autophagy in liver (Wu et al., 2019). To date, the majority of evidence suggesting clock-output pathways to lysosomes and autophagosomes is based on biochemical markers. Compelling evidence from microscopy of subcellular structure would help establish the links between lysosome/autophagy and circadian clocks in the liver and other tissues.

Heart and Skeletal Muscle

Heart and the Cardiovascular System

The heart and cardiovascular system exhibit circadian rhythm of genes and functions from the heart tissue to various types of blood vessels (Crnko et al., 2019). Heart tissue is a specialized mechanic pump in the body, which creates a unique organ system where two physiological cycles interact like a Russian doll. Namely, the second-scale cardiac cycle is gauged by the circadian clock. It is estimated that 13% of gene transcripts and 8% of proteins in the mouse heart are diurnal (Martino et al., 2004; Podobed et al., 2014). Under constant darkness, a lower percentage (6–8%) of gene transcripts oscillate in a circadian manner (Storch et al., 2002; Zhang et al., 2014). In the aorta, 4–5% of genes oscillate in a circadian manner (Rudic et al., 2005; Zhang et al., 2014). These circadian pathways span from cellular energy metabolism and sarcolemma calcium signaling, to cellular signaling pathways.

The cardiomyocyte clock is crucial in driving the cell-autonomous oscillation of heart function (Durgan et al., 2005; Bray et al., 2008). Mitochondrial function and dynamics sit at the hub of this regulation (Zhang et al., 2020a). Intervening in CLOCK function by overexpressing a dominant-negative mutant abolishes diurnal variation of mitochondrial oxidative metabolism (peak ZT 18h) and tolerance to ischemic-reperfusion injury (peak ZT 0 h) (Durgan et al., 2005, 2010; Bray et al., 2008). These phenotypes are associated with the abolished oscillation of PDK4, a metabolic gauge between glycolysis and oxidative metabolism. Genetic deletion of the *Bmal1* gene in mouse cardiomyocytes results in dilated cardiomyopathy, decreased heart rate and increased arrhythmias (Lefta et al., 2012; Schroder et al., 2013). In human embryonic stem cell-derived cardiomyocytes, loss of BMAL1 inhibits mitochondrial fission and mitophagy, impairs oxidative metabolism, and leads to disorganized sarcolemmal structure, decreased contractility, and dilated cardiomyopathy (Li E. et al., 2020). Mechanistically, BMAL1/CLOCK bipartite TF trans-activates expression of the mitophagy receptor gene BNIP3 (Li E. et al., 2020). Provided that BNIP3 is not a robust diurnal gene in the mouse heart (source: CirGRDB, CircaMetDB), it remains open to post-translational or organelle-level regulation of mitochondria which contributes to diurnal oscillation of the cardiac cycle and metabolism in the heart.

Moreover, clock-controlled transcription factors orchestrate the cardiac cycle and metabolism (**Figure 2**). Krüppel-like factor 15 (KLF15), a BMAL1-controlled output TF, determines diurnal oscillation of the cardiac cycle (peak ZT 6 h) and susceptibility to ischemic-reperfusion injury to the heart (Jeyaraj et al., 2012; Li L. et al., 2020). The cardiac arrhythmogenesis in KLF15 transgenic animals phenocopies that in an inducible cardiomyocyte-specific *Bmal1* knockout model (Schroder et al., 2013). Though initially thought to act through diurnal expression of ion channels, transcriptome profiling demonstrates that KLF15 controls the diurnal oscillation of cellular energy metabolism (peak ZT 14–20 h) and tissue remodeling and repair (peak ZT 0–6 h) (Zhang et al., 2015). Compelling evidence links the activity of oxygen-sensing HIF-1α transcription factor to the heart-clock, which outputs the diurnal control of glycolysis and the hypoxia response (Eckle et al., 2012; Wu et al., 2017). Chemical activation of REV-ERBα protects against heart failure, which is partially linked to transcription repression (Zhang et al., 2017). While it is intriguing to propose TF-specific labor division in organizing various aspects of the cardiac cycle, contractility, and metabolism throughout the day, it remains unsolved how these clock-output TFs forge a network to coordinate diurnal oscillation of heart physiology and metabolism.

Skeletal Muscle

Skeletal muscle is the most abundant tissue in mammals, comprising approximately 40% of body mass. Similar to other tissues, skeletal muscle also possesses its own self-sustaining endogenous molecular clock. Approximately 20% of genes (>2300 genes) show 24 h oscillations in skeletal muscle, and these genes participate in a wide range of functions, including myogenesis, transcription and metabolism (Chatterjee and Ma, 2016). Recently, the importance of circadian rhythms for skeletal muscle has been made more evident by the skeletal muscle phenotype observed in models of molecular clock disruption (**Figure 3**).

Firstly, the circadian clock network plays a prominent role in muscle differentiation and maintenance. A study of *Bmal1*-null mice clearly shows atrophy and tissue damage in skeletal

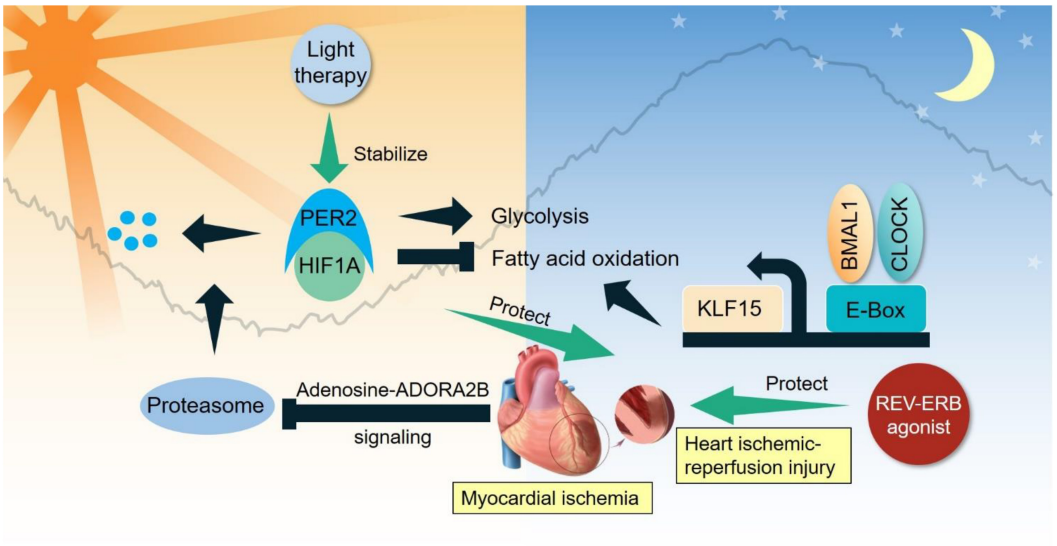

FIGURE 2 | Clock-controlled checkpoints in the heart. Myocardial ischemia stabilizes PER2 to promote glycolysis and suppress fatty acid oxidation. BMAL1/CLOCK bipartite TF modulate the diurnal oscillation of fatty acid oxidation partly through KLF15. Light therapy stabilizes PER2 to benefit ischemic-reperfusion injury, while REV-ERB agonist also protects against ischemic-reperfusion injury.

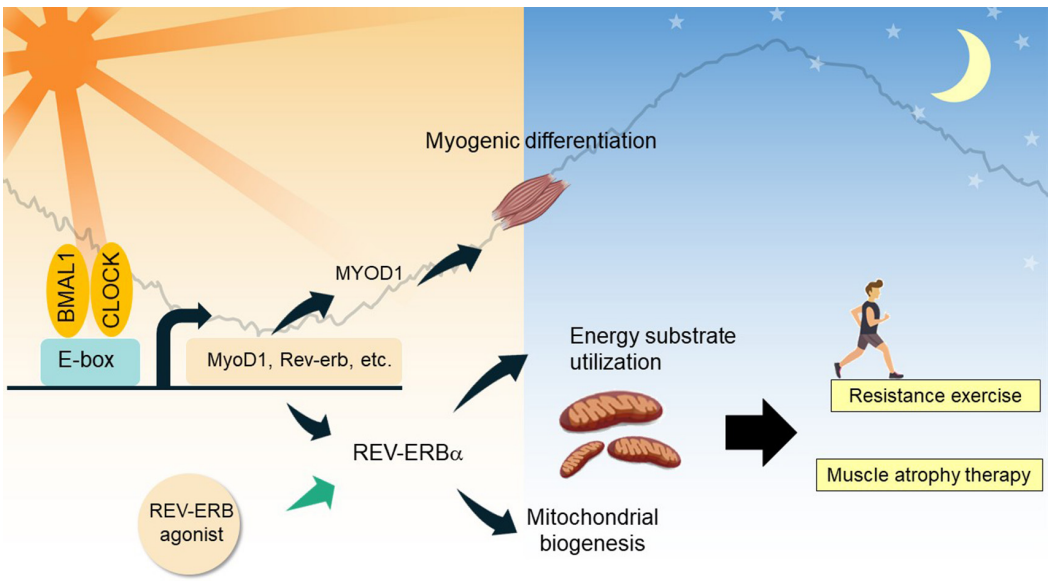

FIGURE 3 | Clock-controlled checkpoints in skeletal muscle. In myocytes, BMAL1 and CLOCK regulate diurnal expression of genes involved in myogenic differentiation and energy substrate utilization. REV-ERBα and its interactome regulate expression of mitochondrial biogenic genes and energy substrate utilization, contributing to myofiber type switch. As demonstrated in chemical biology studies, REV-ERBα agonism holds promise to treat skeletal muscle atrophy, and improve resistance exercise performance.

muscle, which could be attributed to the blunted circadian expression of MyoD1 (myogenic differentiation 1) and a defect in the regenerative myogenic process accompanied by lowered repair and an altered satellite cell expansion process (Schiaffino et al., 2016; Vitale et al., 2019). Clock nuclear receptors REV-ERBα/β are highly expressed in proliferating myoblast cells and repress key genes involved in myotube differentiation (Everett and Lazar, 2014; Chatterjee and Ma, 2016). Schroder et al. (2015) investigated a *Bmal1* conditional knockout line in adult skeletal muscle, and showed that remarkably, this model mimics the advanced aging phenotype in the musculoskeletal system in Bmal1-null mice, characterized by increased fibrosis, bone calcification, and decreased joint collagen. Skeletal muscle BMAL1 exclusively controls diurnal expression of core clock

genes and nuclear receptor genes (Dyar et al., 2014; Schroder et al., 2015). CRY2 regulates the circadian rhythm of myogenic differentiation through stabilization of Cyclin D1 and Tmem176b transcripts (Lowe et al., 2018).

Secondly, the circadian clock participates in the temporal control of energy metabolism in skeletal muscle and contributes to systemic metabolism. Early studies indicate that metabolic genes involved in substrate utilization and energy storage are expressed in a temporally segregated manner over a 24-h period, suggesting a clock-controlled orchestration of distinct catabolic and anabolic metabolic pathways in skeletal muscle (Chatterjee and Ma, 2016; Gabriel and Zierath, 2019). Further, a number of studies have demonstrated the mechanisms underlying the interplay between the molecular clock and fuel preference in cellular energy metabolism (Dyar et al., 2014; Mayeuf-Louchart et al., 2015; Perrin et al., 2018). Muscle-specific *Bmal1* knockout resulted in a clear up-regulation of genes involved in lipid metabolism concomitant with the down-regulation of circadian genes involved in glucose utilization, indicating muscle fiber type switches to a slow oxidative fiber-type, together with a substrate shift from carbohydrate to lipid utilization (Schiaffino et al., 2016). BMAL1 also controls HIF-1α activity in myocytes, contributing to the regulation of anaerobic glycolysis (Peek et al., 2017). REV-ERBα is highly expressed in oxidative fiber types, which promotes oxidative metabolism and ultimately exercise performance, in part through the nutrient-sensing LKB-AMPK-SIRT1-PGC1α signaling complex (Woldt et al., 2013). Increased fatty acid utilization, hyperglycemia, and a clear fast-to-slow MyHC in soleus muscle were observed in *Rev-Erb*α-deficient mice (Woldt et al., 2013), whereas activation of REV-ERBα by synthetic agonists resulted in lipid lowering and anti-obesity effects (Cho et al., 2012).

Thirdly, the circadian clock has strong control of muscle strength and locomotor function. Results from circadian transcriptomics studies indicate that many essential functions and physiological processes in skeletal muscle are influenced by the transcriptional output of the clock (Dyar et al., 2018a; Perrin et al., 2018). As observed in mice, a high proportion of cycling transcripts peak midway through the dark phase, coinciding with the peak period of physical activity and feeding in nocturnal species. Clock genes in the negative limb such as CRY and PER, control the diurnal rhythm of exercise performance through regulation of energy substrate utilization (Jordan et al., 2017; Ezagouri et al., 2019). Histone deacetylase 3 (HDAC3), the interacting partner of clock proteins including REV-ERB, also governs exercise performance (Hong et al., 2017). Meanwhile, a chronotype effect on the circadian expression of many types of physical performance has also been observed (Chatterjee and Ma, 2016; Vitale et al., 2019). For example, optimal human muscle torque, strength and power are generally displayed in the late afternoon but not in the morning, suggesting that locomotor activity may coordinate the phase of the intrinsic rhythmic expression of genes in skeletal muscle.

Besides the above mentioned circadian regulation on skeletal muscle, physical activity could function as a strong clock entrainment signal, particularly for the skeletal muscle clock (Sato et al., 2019). Resistance exercise is capable of shifting the expression of diurnally regulated genes in human skeletal muscle (Zambon et al., 2003). Loss of muscle activity leads to marked muscle atrophy and reduced expression of core clock genes in mouse skeletal muscle (Zambon et al., 2003). Overall, recent findings demonstrate the intimate interplay between the cell-autonomous circadian clock and muscle physiology.

Blood

Many parameters in blood exhibit circadian rhythmicity, including leukocytes, erythrocytes, chemokines (e.g., CCL2, CCL5), cytokines (e.g., TNFα, IL-6), and hormones (Schilperoort et al., 2020). The most apparent oscillation in blood is observed in the number and type of circulating leukocytes, which peak in the resting phase and reach a trough in the activity phase during 24 h in humans and rodents (He et al., 2018). This time-dependent alteration of leukocytes reflects a rhythmic mobilization from hematopoietic organs and the recruitment process to tissue/organs (Méndez-Ferrer et al., 2008; Scheiermann et al., 2012). For example, the mobilization of leukocytes from the bone marrow is regulated by photic cues which are transmitted to the SCN and modulate the microenvironment of the bone marrow through adrenergic signals (Méndez-Ferrer et al., 2008).

Leukocytes exit the blood by a series of interactions with the endothelium, which involves various adhesion molecules, chemokines and chemokine receptors (Vestweber, 2015). Using a screening approach, He et al. (2018) depicted the time-dependent expression profile of the pro-migratory molecules on different endothelial cells and leukocyte subsets. Specific inhibition of the promigratory molecule or depletion of Bmal1 in leukocyte subsets or endothelial cells can diminish the rhythmic recruitment of the leukocyte subset to tissues/organs, indicating that the spatiotemporal emigration of leukocytes is highly dependent on the tissue context and cell-autonomous rhythms (Scheiermann et al., 2012; He et al., 2018). Cell-autonomous clocks also control diurnal migration of neutrophils (Adrover et al., 2019), Ly6C-high inflammatory monocytes (Nguyen et al., 2013) in the blood and leukocyte trafficking in the lymph nodes (Druzd et al., 2017).

Furthermore, the circadian recruitment process of leukocytes was not only found in the steady state but also in some pathologic states, such as natural aging (Adrover et al., 2019), the LPS-induced inflammatory scenario (He et al., 2018), and parasite infections (Hopwood et al., 2018). These findings suggest that leukocyte migration retains a circadian rhythmicity in response to pathogenic insults. Although mammalian erythrocytes lack the genetic oscillator, the peroxiredoxin system in erythrocytes has been shown to follow 24-h redox cycles (O'Neill and Reddy, 2011). Moreover, the membrane conductance and cytoplasmic conductivity of erythrocytes exhibit circadian rhythmicity depending on cellular K++ levels (Henslee et al., 2017). These observations indicate that non-transcriptional oscillators can regulate the circadian rhythms in denucleated cells. In addition to leukocytes and erythrocytes, other parameters in blood like chemokines and cytokines also exhibit a circadian rhythmicity (Schilperoort et al., 2020). Together, emerging evidence shows that the circadian rhythm can be easily found in blood elements

which are essential contributors to the maintenance of circadian physiology (**Figure 4A**).

Peripheral and Central Nervous System

Circadian clock directs multiple metabolic and physiological functions in both the peripheral and central nervous system (**Figure 5**). In the central nervous system, many physiological processes controlled by extra-SCN hypothalamic nuclei display diurnal rhythms, such as those involved in energy and temperature regulation, glucose and lipid metabolism (Paul et al., 2020). Clocks in the forebrain, arcuate nucleus and dorsomedial hypothalamus can integrate external cues including temperature and nutrition cycles. Complete loss of circadian behavior was found in forebrain/SCN-specific Bmal1 knockout mice, and the related circadian rhythms in peripheral tissues was differentially affected by light/dark cycles and feeding (Izumo et al., 2014). Time-restricted feeding in mice has been shown to impair the body temperature homeostasis (Zhang et al., 2020c). Circadian gene expression analysis in the dorsomedial hypothalamus revealed that rhythmically reprogramming of thermoregulation gene expression is involved in the impairment of body temperature regulation (Zhang et al., 2020c). Integrative cistromic and transcriptomic analysis showed that REV-ERB-dependent leptin signaling in the arcuate nucleus plays an important role in the control of diurnal leptin sensitivity and food intake in diet-induced obesity (Adlanmerini et al., 2021). With more and more neuronal circadian oscillators uncovered, circadian rhythms of the circuit-level communication, organization, and physiological functions need to be explored.

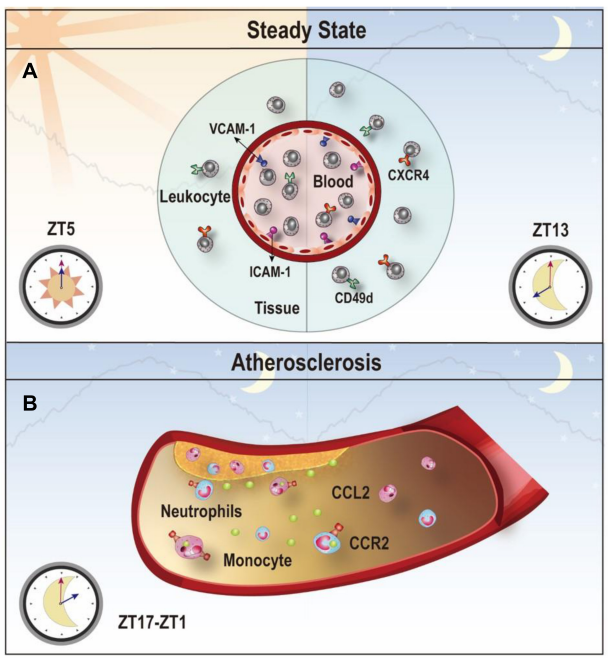

FIGURE 4 | Clock-controlled checkpoints in leukocyte migration. **(A)** Leukocyte migration is controlled by the circadian clock. Rhythmic expression of promigratory molecules, such as ICAM-1, VCAM-1, CD49d, and CXCR4, promotes migration and retention of leukocytes to tissues, which peaks at ZT5 in the steady state. **(B)** Chemokine CCL2-CCR2 signaling is a clock-controlled checkpoint in leukocyte migration under atherosclerosis. Myeloid cells adhere to atherosclerotic lesions in a rhythmic manner with a peak between ZT17-ZT1 because of the diurnal expression of the CCL2-CCR2 axis. Targeting the CCL2-CCR2 axis in this time period may reduce inflammation during atherogenesis.

The hypothalamus-pituitary-adrenal (HPA) axis is a major neuroendocrine pathway involved in stress response, metabolism, and circadian rhythm. HPA is regulated in a circadian manner, and peaks in the dawn in humans, or in the onset of the dark phase for nocturnal animals (Oster et al., 2017). Rhythmic release of the inhibitory neuropeptide, vasopressin, from the SCN periodically inhibits corticotrophin-releasing hormone (CRH)-neurons in the paraventricular nucleus (PVN) of hypothalamus. Vasopressin may reach the CRH neurons of PVN via either extracellular space/fluid or direct neuronal projection (Vrang et al., 1995; Tousson and Meissl, 2004). Release of CRH into the anterior pituitary promotes the release of adrenocorticotrophic hormone (ACTH) into the blood. Plasma ACTH is circadian, however, ACTH precursor Pomc does not oscillate at the transcript level in the anterior pituitary (Oster et al., 2017). ACTH reaches the adrenal gland and promotes secretion of glucocorticoids to the blood. The adrenal-clock controls circadian and acute secretion of glucocorticoids (Leliavski et al., 2014). *Bmal1*-deficiency decreases the diurnal rise of plasma glucocorticoids around the onset of the dark phase. *Bmal1*-decificency abolishes stress-induced rise of plasma glucocorticoids, but does not affect stress-induced rise of plasma ACTH. PER2 governs the diurnal rise in plasma glucocorticoid at the onset of the dark phase (Yang et al., 2009; Russell et al., 2021). PER2 does not regulate glucocorticoid secretion towards major stressors, such as hypoglycemia, ACTH, and physical restraint, neither does PER2 regulate ACTH peptide rhythm in the hypothalamus (Yang et al., 2009). Together, these findings demonstrate that both the SCN-clock and the adrenal-clock control the circadian activity of the HPA axis.

The peripheral nervous system mainly consists of sensory neurons, and their primary function is to transmit the sensory signals such as pain, touch, itch, and temperature sensation (Niesler et al., 2021). Pain sensitivity follows a daily cycle in many clinical conditions, and there is strong evidence to support the rhythmicity in response to nociceptive stimuli (Bruguerolle and Labrecque, 2007). The processing of painful stimulation occurs in the dorsal horn (DH), an area of the spinal cord that receives noxious stimulation signals from peripheral tissues via several types of primary afferent nerve fibers (Crodelle et al., 2019). Pain perception exhibits a 24-h rhythm regardless of whether pain threshold is objectively or subjectively assessed (Burish et al., 2019). Mechanistically, BMAL1, CLOCK, PER1, and REV-ERBα contribute to neuropathic pain at evening and cluster headache at midnight (Burish et al., 2019). Core clock genes are rhythmically expressed in neurons of the dorsal root ganglion (Kim H. K. et al., 2020). A Per2 mutation abolishes the circadian rhythm of the inflammatory pain response (peak ZT4) (Zhang et al., 2012b). Together, these studies provide evidence to support a potential strategy for improvement of pain treatment based on

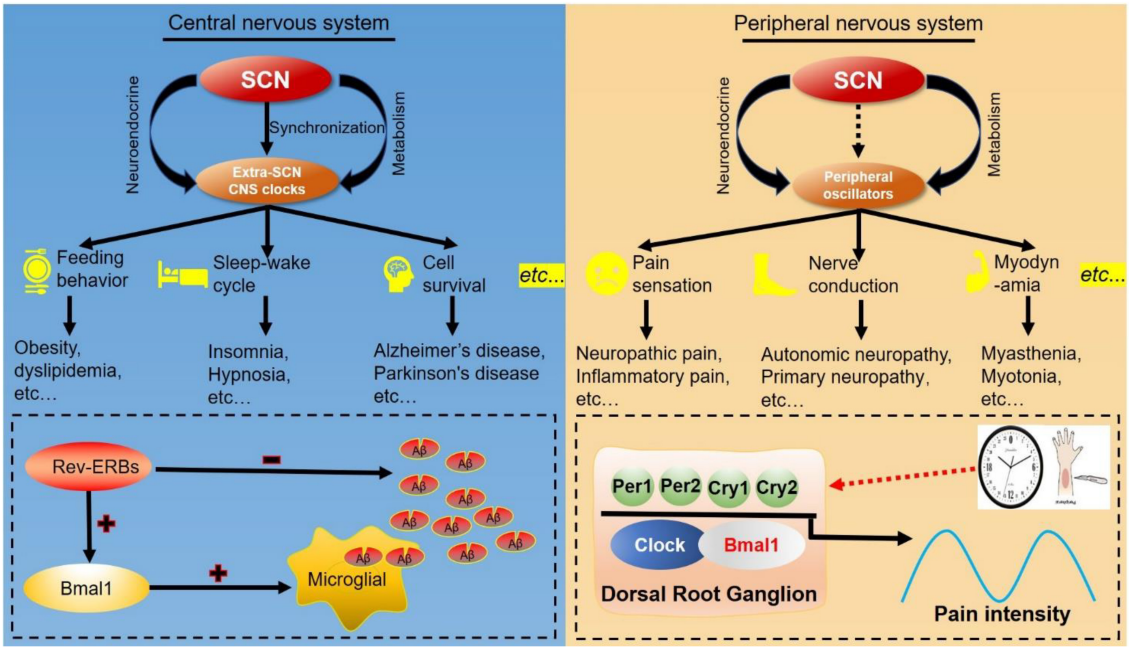

FIGURE 5 | Clock-controlled checkpoints in neurons. The suprachiasmatic nucleus (SCN) synchronizes extra-SCN clocks and peripheral oscillators in the central nervous system (CNS) and peripheral nervous system. **Left:** The SCN master oscillator and its output extra-SCN CNS clocks control diverse functions in the central nervous system, including feeding behavior, sleep–wake cycles and cell survival. For example, REV-ERB decreases the number of amyloid-beta (Aβ) and increases BMAL1 transcription to accelerate microglial uptake of Aβ. **Right:** Peripheral oscillators can regulate functions and diseases of the peripheral nervous system, such as pathological pain. Rhythmic expression of BMAL1 and PER2 in dorsal root ganglions modulate the response to noxious stimulation, and establish the circadian rhythm of pathological pain.

the circadian rhythm. More detailed studies are required to explore this phenomenon and more clinical trials needed to validate these findings.

Male Reproductive System

Diurnal rhythms of sperm count, sperm motility, and sperm chromatin integrity have been found in rodents. There are also several studies which have investigated the diurnal variation of semen parameters in humans (Ni et al., 2019; Sati, 2020). Variations in semen parameters were found across different time points in most of the studies, although a high-resolution sampling study is required to confidently profile the circadian pattern. It is also well known that semen parameters have circannual variation both in men and male animals (Chemineau et al., 2008; Xie et al., 2018). These studies suggest that the circadian clock and its regulatory mechanisms may play an important role in the regulation of the male reproductive system.

Clock genes are expressed in different parts of the male reproductive system, including extra-testicular ducts and accessary organs. However, the presence of a cell-autonomous clock in testes remains controversial (**Figure 6**; Alvarez et al., 2003; Mazzoccoli et al., 2012). In insects, *per* mRNA in testes oscillate under light-dark conditions, but the diurnal rhythm is not self-sustaining under constant darkness (Gvakharia et al., 2000). Leydig cells, the primary androgen-secreting cells in testes, express BMAL1 in a circadian manner, however, the mRNA levels of clock genes are not diurnal in testes (Chen et al., 2017). Dexamethasone synchronizes the expression of several circadian clock genes and steroidogenic-related genes in Leydig cells *in vitro* (Chen et al., 2017). Expression of PER1 protein is strictly isolated to certain stages of spermatogenesis, i.e., spermatogonia and condensing spermatids, while the expression of CLOCK is restricted to round spermatids (Alvarez et al., 2003). In the diurnal transcriptome atlas for major neural and peripheral tissues of the Papio anubis (baboon), 1672 cycling genes were identified in testes (Mure et al., 2018). However, the core clock genes such as *CLOCK, BMAL1, PER1-3, CRY1-2*, and *RORA* were not in the list. It is likely that a cell-autonomous clock is restricted to specific cell types such as the Leydig cell. The molecular analysis using bulk tissues masks the rhythmicity since clock genes may be expressed ubiquitously in different types of cells including the clock-less cells. The use of single-cell omics in circadian studies would resolve this issue since these techniques have already been applied in the testes (Guo et al., 2018; Lau et al., 2020; Shami et al., 2020).

Clock genes are essential for the development of the male reproductive system. Knockout of the Bmal1 gene induces infertility and decreases the production of testosterone in mice (Alvarez et al., 2008). Bmal1-deficient male mice seem to have defects in copulatory behaviors (Schoeller et al., 2016). Interestingly, if Bmal1 was conditionally knocked out during adult life, the fertility of the mice was conserved (Yang et al., 2016). Circadian clock becomes functional at around embryonic days 13–18 (Umemura and Yagita, 2020). It has

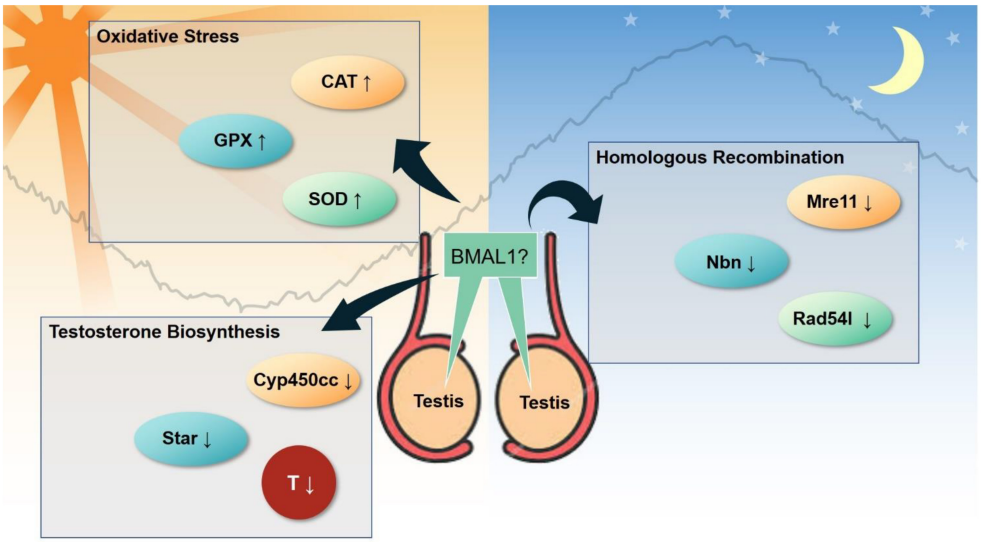

FIGURE 6 | Emerging clock-controlled checkpoints in the testis. Emerging evidence suggests the role of the core clock protein BMAL1 in the regulation of oxidative stress, homologous recombination and testosterone biosynthesis in testis, which may potentially contribute to the pathogenesis of circadian-related disorders. CAT, catalase. GPx, glutathione peroxidase. SOD, superoxide dismutase. Cyp450cc, cytochrome P450 side-chain cleavage. Star, steroidogenic acute regulatory protein. T, testosterone. Mre11, double strand break repair nuclease. Nbn, nibrin. Rad54l, RAD54 like.

to be determined whether Bmal1 has a non-clock function on the male reproduction or that the testes-clock is required for the male reproduction before sex maturity. If induced deletion of Bmal1 on embryonic days 13–18 still preserved fertility in males, it would clearly support the non-clock function of BMAL1 in male fertility. Knockdown of Clock in mouse testes leads to reproductive damages, such as small litter size, decreased *in vitro* fertility rate, blastula formation rate, and acrosin activity of the sperm (Liang et al., 2013). Mechanistically, BMAL1 and CLOCK regulate the assembly of chromatoid bodies through interaction with MVH, a key component of the sperm chromatoid body (Peruquetti et al., 2012). A deficiency of Cry1 was found to increase apoptosis of mouse testicular germ cells and decrease sperm count (Li C. et al., 2018). Sertoli cells provide a nurturing and supportive niche for spermatogenesis. The specific knockdown of Rora in Sertoli cells at puberty leads to declined sperm count in rats (Mandal et al., 2018). In *C. elegans*, NHR-23/NR1F1 (RORA) depletion causes infertility due to the arrest of primary spermatocytes rather than haploid sperm (Ragle et al., 2020).

Disruption of circadian cycles is associated with high activity of the hypothalamic-pituitary-adrenal (HPA) axis. Lassi et al. (2021) showed that inverted feeding (night restricted feeding, of which night refers to the light phase) leads to dampened rhythm and high circulating levels of corticosterone in male founder mice. By carefully controlling the mating and breeding procedure, Lassi et al. (2021) demonstrated that paternal circadian disruption by inverted feeding can be transmitted to male offspring through hyper-corticosteronemia at conception. Hyper-corticosteronemia at conception resulted in a plethora of metabolic abnormalities in the male offspring, such as hyperphagia, hyper-metabolism, hyperglycemia, and hyper-corticosteronemia (Lassi et al., 2021). This trans-generational transmission of paternal circadian disruption does not seem to be pathogenic, as body weight, glucose tolerance, and insulin levels remained normal. Interestingly, wildtype male offspring of Clock mutant males recapitulate the high energy metabolism, indicating that inverted feeding imprints the high HPA activity in male offspring in part through the circadian clock (Lassi et al., 2021). Thus, paternal glucocorticoid signaling at conception may transmit the effects of circadian disruption to the offspring.

In humans, genetic polymorphisms of circadian genes have been linked to fertility and semen quality. Three single nucleotide polymorphisms (SNPs) of the Clock gene (rs11932595, rs6811520, and rs6850524) were associated with the risk of infertility in a case-control study of 961 Slovenian and Serbian Caucasian men (Hodžić et al., 2013). Another two Clock SNPs (rs1801260 and rs3817444) were found to correlate with infertility risk in 672 Chinese men (Shen et al., 2015). Several SNPs were also associated with semen volume, sperm count, sperm motility, as well as the levels of testosterone and follicle-stimulating hormone (Zhang et al., 2012a; Shen et al., 2015). In an observational study of 106 university students, levels of testosterone correlated with scores on the Composite Scale of Morningness, an indicator of chronotype, but not with sleep duration (Randler et al., 2012). However, it is not clear whether there is substantial damage to male fertility in syndromes related to clock gene mutations, such as Familial Advanced Sleep Phase Syndrome.

Female Reproductive System: Placenta

The placenta is an organ that functions to exchange nutrients, gasses, wastes, and hormones between the mother and fetus.

Growing evidence suggests the presence of a circadian clock in the placenta (**Figure 7**). Hypoxia has been shown to induce circadian expression of *PER2* and *DEC1* genes in human placental cells (Frigato et al., 2009). Transcript levels of clock genes *BMAL1*, *CLOCK*, *CRY1-2*, and *PER1-2* oscillate in the murine gravid uterus and placenta during late gestation (Ratajczak et al., 2010). Placental function also expresses a daily rhythm, as observed in the maternal plasma human chorionic gonadotropin (hCG) levels during early pregnancy, at the time of maximum placental hCG expression (Díaz-Cueto et al., 1994). Pérez et al. (2015) revealed the first piece of evidence demonstrating circadian expression of *CLOCK*, *BMAL1*, *PER2*, and *CRY1* genes in the human full-term placenta. Clarkson-Townsend et al. (2020) found seasonal rhythmicity of *DEC1* in human full-term placentas, adding evidence for the placenta as a peripheral clock. While clock mutant mice exhibit defects in female fertility and growth retardation associated with the placenta, the standard transcriptional/translation feedback loops of the oscillator mechanism in placenta appear less coordinated and robust. Rather, one study showed *BMAL1* and *PER* rhythms are of low amplitude and not anti-phasic, suggesting a weak, if any, functioning of the core clock in the placenta (Wharfe et al., 2011). As of now, it is premature to conclude that a self-sustaining circadian clock is present in the placenta. It is likely that only certain regions of the placenta have a functional clock. Demarez et al. (2021) showed that the trophoblast layer of the labyrinth zone has a functional clock by late gestation, which controls diurnal expression and activity of ABCB1, a xenobiotic efflux pump. A simple bioinformatics survey in the Human Protein Atlas database revealed strikingly high levels of core clock proteins in the placenta, such as *PER2*, *CRY1*, *BMAL1*. Together, these studies provide justification for the need to explore cell type-specific clocks in the placenta and link the circadian clock to the nexus of maternal-fetal communication.

Furthermore, melatonin and steroid hormones are known to follow circadian pattern due to the diurnal activity of the pineal gland and the hypothalamus-pituitary axis (Urlep and Rozman, 2013). Melatonin, a pineal gland secreted factor, is secreted mainly during the nighttime of the diurnal cycle. Melatonin plays an essential role in synchronizing the peripheral tissues to the 24-h circadian rhythms and likely provides feedback to the hypothalamus-pituitary-adrenal axis and the hypothalamus-pituitary-gonad axis (Shi L. et al., 2013; Huang et al., 2020). Melatonin can regulate the expression of clock genes in the placenta via its melatonin receptors (Lanoix et al., 2006), and this may contribute to adverse pregnancy outcomes (Lanoix et al., 2012; Olcese et al., 2013; Shimada et al., 2016).

CIRCADIAN PATHOPHYSIOLOGY IN COMPLEX DISEASES

Metabolic Associated Fatty Liver Disease

Metabolic associated fatty liver disease (MAFLD), formerly known as non-alcoholic fatty liver disease, is the liver manifestation of metabolic syndrome, and affects 25% of the global population (Bayoumi et al., 2020). MAFLD is the beginning stage of a continuum of chronic liver diseases, which ends with cirrhosis, liver failure, or hepatocellular carcinoma (Friedman et al., 2018). MAFLD is characterized by fat-loaded hepatocytes without signs of liver injury. Recent epidemiological studies have revealed a clear association between circadian rhythm disorder and the incidence of MAFLD (Peng et al., 2017;

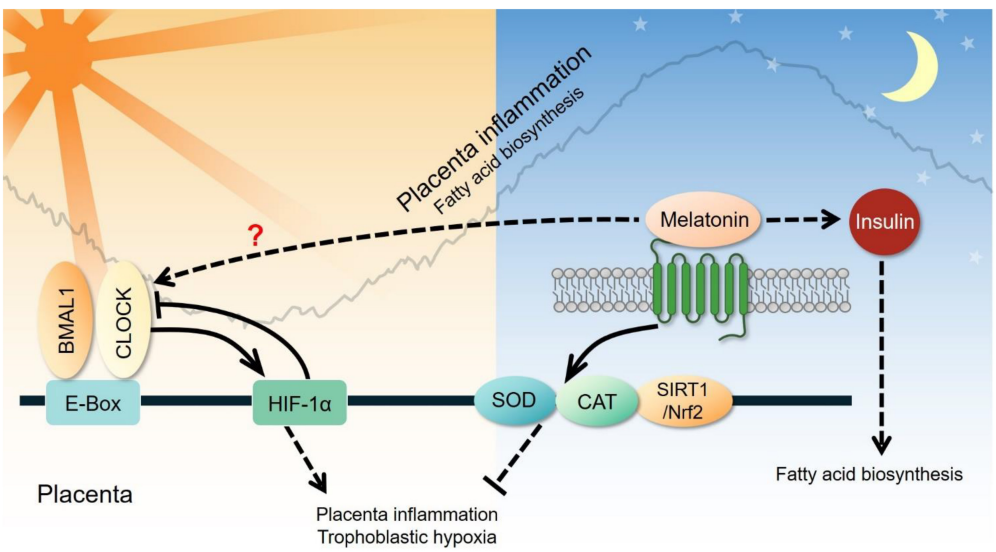

FIGURE 7 | Clock-controlled checkpoints in the placenta. Placental inflammation and trophoblastic hypoxia are promoted by the CLOCK-HIF-1α signaling pathway during the active phase and suppressed through the melatonin-superoxide dismutase (SOD)-catalase (CAT) signaling pathway during the rest phase. However, the exact relationship between circadian genes and melatonin remains unclear. Melatonin can also elevate plasma insulin and subsequently increase fatty acid biosynthesis in the placenta.

Wang H. et al., 2018; Hu et al., 2020). Clock mutant mice are prone to liver steatosis when fed a high-fat diet, which was able to be restored to homeostasis by time-restricted feeding (Chaix et al., 2019; Saran et al., 2020; Zhang et al., 2020b). The vast majority of hepatic triglycerides in the human liver come from adipose-released fatty acids (59%), followed by de novo lipogenesis (26%), and the diet (15%) (Francque et al., 2020). Studies of germline, liver-specific, and adipose-specific deletion of core clock genes indicate that core clocks contribute to liver steatosis mainly through extra-hepatic organs, such as the hypothalamus and adipose (Turek et al., 2005; Paschos et al., 2012; Zhang et al., 2020b), and may be dependent on exposure to circadian insult in early life (Yang et al., 2016).

Nuclear receptors and other non-clock transcription factors are emerging as regulators of diurnal lipid metabolism involved in the pathogenesis of MAFLD. Twenty of the 49 nuclear receptor genes exhibit a diurnal rhythm in the liver, providing the first line of transcription factors linking metabolism and clock (Yang et al., 2006). It has been well-established that REV-ERB and the co-repressor HDAC3 orchestrate diurnal rhythms of lipogenesis, and bile acid production (Bass and Lazar, 2016; Panda, 2016). Peroxisome-proliferating activator receptors (PPARα/β/γ) promote remodeling of the diurnal lipid metabolism in liver. A high-fat diet induces de novo oscillation of thousands of transcripts through PPARγ (peak ZT 8 h) in the liver, and contributes to diurnal rhythms of genes involved in lipid storage and gluconeogenesis (Eckel-Mahan et al., 2013). PPARδ is required for the diurnal rhythm of hepatic lipogenesis, and release of a diurnal lipid phosphatidylcholine 18:0/18:1 (peak at ZT 18 h) that promotes muscle fatty acid uptake (Liu et al., 2013). A high-fat diet enhances the daily cycling of enhancer activities associated with two opposing transcription factors, i.e., PPARα for fatty acid oxidation and SREBP1c for de novo lipogenesis (Guan et al., 2018). PPARs promote resolution of inflammation in immune cells through tethering apart the pro-inflammatory signaling complex, such as nuclear factor-kappa B and AP-1 (Li and Yang, 2011). Myeloid cell-specific disruption of Per1/Per2 results in hepatic steatosis, inflammation, and liver injury (Xu et al., 2014). Introducing PPARγ2 transgene back to clock-less macrophages helps to resolve the exacerbation of inflammation (Xu et al., 2014). Currently, dual-, and pan-PPAR agonists are intensively investigated as potential therapeutics for chronic liver diseases (Francque et al., 2020).

Cardiovascular Disease

Heart Disease

Heart diseases, including ischemic heart disease, diabetic cardiomyopathy, and heart failure, have a relationship to circadian clocks throughout their pathogenesis and prognosis (Crnko et al., 2019; Zhang et al., 2020a,b). Experimental models of heart disease, such as cardiac ischemia-reperfusion, pressure overload-induced cardiac hypertrophy, and diabetic cardiomyopathy, show clear disturbance of circadian clock oscillation in the heart (Young et al., 2001, 2002; Kung et al., 2007). Shift work increases the susceptibility to ischemic heart disease (Knutsson et al., 1986; Vetter et al., 2016), as does circadian disruption of behavioral rhythm in mice (Martino et al., 2007).

Ischemic heart disease is initiated by insufficient supply of blood (ischemia) to heart tissue due to obstruction of coronary arteries. Adaptive remodeling of heart metabolism is key to recovery and survival after ischemia (Sedej, 2018). Compelling evidence demonstrates several key clock-controlled checkpoints in heart metabolism that are crucial for treating ischemic heart disease. Myocardial ischemia induces adenosine-ADORA2B signaling that stabilizes PER2 through inhibition of proteasomal degradation (Eckle et al., 2012). PER2 promotes glycolysis and suppresses fatty acid oxidation in a HIF-1α-dependent manner, leading to reduced myocardial infarction. Interestingly, strong light exposure (10,000 lux) in the subjective day time stabilizes PER2 and protects the heart from ischemic-reperfusion injury. As reviewed in a previous section, BMAL1/CLOCK bipartite TF can modulate the diurnal oscillation of fatty acid oxidation, in part through transcriptional activity of a clock-output protein KLF15. REV-ERBα agonism protects against ischemic-reperfusion injury in the heart, though the detailed clock-controlled mechanism is not fully characterized (Stujanna et al., 2017). A transcriptional network including PER2-HIF1α and BMAL1/CLOCK-KLF15 is emerging as a clock-controlled checkpoint that licenses diurnal oscillation of cellular energy metabolism for metabolic reprogramming in ischemic heart disease (**Figure 2**).

Atherosclerosis

Atherosclerosis is a chronic process of plaque build-up in the vessel wall driven by lipid deposition and leukocyte infiltration to the subendothelial space (Wolf and Ley, 2019; Libby, 2021). The stenosis and restriction of the blood flow caused by the plaque make atherosclerosis the main cause of cardiovascular disease (Swirski and Nahrendorf, 2013). Epidemiological studies have demonstrated a strong connection between the disruption of circadian rhythms and atherogenic risk factors, such as lipid disorder and impaired glucose tolerance (Gooley, 2016; Poggiogalle et al., 2018). Leukocyte recruitment is greatly involved in the development of atherosclerosis (Swirski and Nahrendorf, 2013). In murine models of induced atherosclerosis using $ApoE^{-/-}$ mice on a high-fat diet, neutrophils and monocytes were recruited to the atherosclerotic lesions rhythmically due to a morning peak of the CCL2 rhythm on the endothelium and the CCR2 rhythm on neutrophils and monocytes (Winter et al., 2018). Targeting the CCR2-CCL2 axis at a specific time achieved better attenuation of myeloid cell adhesion (Winter et al., 2018). Disturbing the rhythmicity of the SCN clock may be sufficient to promote atherosclerosis. For example, feeding low-fat diet to ApoE–/– mice generated more atherosclerotic lesions in aortic roots under constant light exposure, compared to feeding the same diet under normal light-dark cycles (Chalfant et al., 2020). Another mouse model using APOE*3-Leiden mice with alternating light/dark cycles also exhibited more severe atherosclerosis with more macrophages in the lesion due to increased expression of ICAM-1 and CCL2 in the lesions (Schilperoort et al., 2020). The findings from these studies suggest that disturbance of the circadian rhythm can aggravate atherosclerosis. The rhythmic recruitment of myeloid

cells to the atherosclerotic lesions driven by the circadian expression of the CCL2-CCR2 axis provides a mechanistic basis for chronotherapeutic treatments (**Figure 4B**).

Neurodegenerative Diseases and Diabetic Neuropathy

Diabetic neuropathy is associated with a markedly reduced quality of life and poor prognosis (Callaghan et al., 2012). Impairment of cognitive function is increasingly being considered as an inevitable complication of type 2 diabetes mellitus (T2DM), and it has been associated with the risk of Alzheimer's disease (AD) and vascular dementia. Considerable overlap has also been identified in the pathophysiology of T2DM and AD (Biessels and Despa, 2018). Sundown syndrome or sundowning, a well-known rhythmical neuropsychiatric phenomenon taking place in the late afternoon or early evening, is commonly observed in AD patients (Khachiyants et al., 2011). Another neuropsychiatric phenomenon is that pain intensity is often higher during night compared to day time. In addition, bright light exposure or exogenous melatonin supplementation has been found to improve sundowning symptoms, cognitive deficits, and sleep quality in AD patients (Roccaro and Smirni, 2020).

Emerging evidence supports the role of the circadian clock in the pathogenesis and progression of these neurological diseases. Pharmacological inhibition or genetic knockdown of REV-ERB was found to increase *Bmal1* transcription and to accelerate microglial uptake of amyloid-beta (Aβ) (Lee et al., 2020; Liu W. W. et al., 2020). Deletion of *Rev-Erbα* in an AD model was shown to decrease Aβ number and prevent the increase in AD-associated microglia markers such as TREM2 and CD45 (Lee et al., 2020). Exogenous light can inhibit the synthesis and secretion of melatonin, while the absence of light can promote production and secretion of melatonin. Melatonin can exhibit its effects regarding alleviating circadian disruption through regulation of related clock genes including PER1 and BMAL1 (Yamanaka et al., 2010), which have been confirmed to be associated with the development of neurodegenerative diseases. Deletion of *Per1/Per2* has been found to increase Chi3l1 expression in cerebrospinal fluid, which is a widely biomarker that is increased with the development of AD (Lananna et al., 2020). Loss of *Bmal1* in astrocytes was demonstrated to promote neuronal death due to aging and neurodegenerative diseases through the regulation of astrogliosis in a synergistic manner by a cell-autonomous mechanism and a lesser non-cell-autonomous signal from neurons (Lananna et al., 2018). These studies further confirmed that circadian clock-controlled checkpoints such as REV-ERBα play an important role in the pathogenesis of neurological diseases (**Figure 5**).

At present, accumulated β-amyloid (Aβ) in extracellular senile plaques and abnormally hyperphosphorylated tau in intracellular neurofibrillary tangles have been identified as the pathological features of AD (Polanco et al., 2018). Melatonin, produced by the pineal gland and associated with circadian rhythms, has been demonstrated to play an important role in the interaction with Aβ and tau pathology. Melatonin has been found to suppress the reduction of PI3K activity, pSer473 on Akt and pSer9 on GSK-3β, and this can lead to the reduction of Aβ aggregation in AD (Ali and Kim, 2015). Additionally, melatonin can effectively attenuate tau pathology in AD through regulation of kinases including death-associated protein kinase 1 (DAPK1) (Chen et al., 2020). In a post-transcriptional manner melatonin can significantly decrease DAPK1 expression in mouse primary cortical neurons and human neuronal cell lines. Moreover, melatonin directly binds to DAPK1 to increase DAPK1 degradation resulting in decreased tau phosphorylation at multiple sites related to AD (Chen et al., 2020). Based on these studies, it is clear that melatonin signaling is a promising clock-controlled checkpoint in the pathogenesis of AD and further exploration of effective therapeutic targets based on these findings is warranted.

Neuropathic pain is another important symptom in diabetic neuropathy, and this symptom can severely decrease the patients' quality of life. One previous human study has suggested that patients' pain intensity of diabetic neuropathy is often exacerbated at night (Gilron et al., 2013), indicating that pain intensity in diabetic neuropathy follows a circadian rhythm. However, the related mechanism has not yet been elucidated. One study attempted to characterize the circadian properties of neuropathic pain hypersensitivity in animal models of type I and type II diabetes (Akamine et al., 2018). Using a diabetic mouse model, they found that accumulation of sorbitol in the sciatic nerve may modulate the circadian properties of diabetes-induced neuropathic pain hypersensitivity. However, as described above, various forms of neuropathic pain share similar circadian properties. The special mechanism underlying the circadian rhythm of diabetes-induced neuropathic pain requires further research.

Male Reproductive Disorder

The 24/7 modern lifestyle and social system has resulted in substantial changes in circadian behaviors of humans. Approximately 20 percent of the population are shift workers (Rajaratnam and Arendt, 2001). The social jetlag between work days and weekends is even more prevalent (Leypunskiy et al., 2018; Zhang et al., 2019). The effect of circadian disruption on the male reproductive system deserves attention. Several studies have analyzed the association between shift work and infertility risk, semen quality and/or reproductive hormones, as summarized by Caetano et al. (2020). Nevertheless, the results of these studies did not reach a consensus. One of the major reasons for this controversy may be that shift work consists of different work schedules, e.g., permanent night work and rotating shift work, which may lead to different health effects. A study of 1346 men in the community showed that rotating shift workers, but not permanent night workers, had lower sperm count compared to day workers (Liu K. et al., 2020). In the same publication, social jetlag between work days and free days was found to be quantitatively associated with decreased sperm count in 796 male university students.

Circadian disorder may contribute to the pathogenesis of male reproductive disorder in rodents. Male Sprague-Dawley rats with prolonged light exposure (20-h light:4 h-dark) were found to have increased sperm count and sperm motility and lower proportion

of sperm abnormalities, while the rats with prolonged exposure to darkness (4-h light : 20-h dark) only showed a decreased proportion of sperm abnormalities (Moustafa, 2020). In contrast, another study reported that photoperiod changes only induced alterations in testosterone levels but had no effect on other characteristics such as semen quality and pregnancy rate after mating (Majrashi et al., 2017). Moreover, when pregnant rats were exposed to constant light, their male offspring showed a decrease in numerous indicators, including testosterone levels, number of Sertoli cells, sperm count and normal sperm count (Ogo et al., 2020). To simulate exposure to rotating shift work, a recent study put male mice into rotating light/dark cycles (Liu K. et al., 2020). The authors found that the sperm count of the male mice decreased, while the circadian oscillation of sperm count was not affected. In addition, sleep deprivation or sleep restriction is also hazardous to the male reproductive system (Chen et al., 2016). Despite numerous studies indicate the essential role of circadian disorder on the pathogenesis of male infertility, the underlying mechanism remains to be clarified (**Figure 6**).

In regards to the clock-controlled checkpoints in male reproductive system, several studies have suggested that reactive oxygen species (ROS) are activated in part through glutathione peroxidase, and superoxide dismutase (Torres et al., 2014; Alvarenga et al., 2015; Moustafa, 2020). Alteration of the testosterone levels were also observed in some circadian misalignment models (Qin et al., 2014; Arrebola and Abecia, 2017; Majrashi et al., 2017; Moustafa, 2020). The current knowledge around specific details of molecular events following the input of circadian misalignment is limited. Liu K. et al. (2020) suggested that the homologous recombination pathway was disrupted following circadian desynchrony in a rodent model. It remains unknown whether any of these molecular events are induced by unique signals. We speculate that BMAL1 may be a master regulator. BMAL1 is well known to govern the synthesis of testosterone (Alvarez et al., 2003), and regulates oxidative stress in other organ systems such as the vasculature and liver (Jacobi et al., 2015; Xie et al., 2020), although no such evidence is available for the testis. It is not clear whether disruption of the homologous recombination can also be induced by BMAL1, but the expression of core clock genes including BMAL1 is altered in the mouse testis (Liu K. et al., 2020). Twelve micro (mi)RNA are altered in the testes of Cry1 mutant mice, all of which are predicted to target the components of the core clock (Li C. et al., 2018). These findings deserve further confirmation under different exposure conditions. In addition, emerging evidence suggests that retinoic acid and its nuclear receptors are controlled by the clock during spermatogenesis (Bittman, 2016).

Diabetic Complications Related to the Female Reproductive System

Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is a complication of pregnancy that has similar characteristics as type 2 diabetes mellitus (T2D), such as glucose intolerance, insulin resistance, and impaired insulin secretion (Catalano et al., 1999). The pathogenesis of GDM is associated with abnormal expression of circadian-related genes. In a cohort of 40 Greek pregnant women with GDM, four with T2D and 20 healthy pregnant women, significant reductions in the peripheral blood *BMAL1*, *PER3*, *PPARD*, and *CRY2* transcript levels were found in the GDM group, supporting the view that disorders of clock gene expression may play a pathogenic role in GDM (Ratajczak et al., 2010). Pappa et al. (2013a) studied *BMAL1* polymorphisms in GDM women and healthy controls and showed that the rs7950226 (G > A) and rs11022775 (T > C) polymorphisms of the *BMAL1* gene, combined with the rs7950226A/rs11022775C haplotypes were able to increase the susceptibility to GDM. In addition, the expression level of *BMAL1* mRNA from peripheral blood was significantly decreased in GDM patients compared to healthy controls (Pappa et al., 2013b).

Circadian neuroendocrine factors, such as glucocorticoids and melatonin, are altered in GDM (Pilorz et al., 2009; Sen and Hoffmann, 2020). Fabiś et al. (2002) found that melatonin increases blood insulin levels and decreases the synthesis of free fatty acids in experiments conducted on rats. In pancreatic β-cells, a genome-wide association analysis of 18,236 type 2 diabetic subjects demonstrated that mutations in melatonin receptor 2 (MT2) inhibits their response to melatonin, blocking the release of insulin from pancreatic β-cells (Prokopenko et al., 2009). Tuomi et al. (2016) also demonstrated that melatonin treatment reduced insulin secretion in risk G-allele carriers, which suggests that enhanced melatonin signaling decreases insulin secretion in the pancreas. More recently, genotyping of 1,025 Chinese women with a history of GDM showed that gestational weight gain may alter the effect of circadian-associated melatonin receptor 1B (MTNR1B) gene variants on long-term glucose changes (Nisa et al., 2018). GDM is known to be associated with chronic inflammation and the accumulation of oxidative damage in the placenta without affecting placental anatomy and their vascular structure in the majority of cases. Nevertheless, little has been studied on the distribution and pathophysiology of core clock genes in the GDM placenta.

Preeclampsia

Preeclampsia (PE) is characterized by hypertension and proteinuria after 20 weeks of gestation. The most accepted mechanism leading to the etiology of PE is shallow trophoblast invasion and abolished spiral artery remodeling that leads to placental hypoxia and oxidative stress. The most well-established link between circadian rhythm and PE is melatonin. Nakamura et al. (2001) reported that nocturnal melatonin levels in pregnant women with PE are significantly lower than those in normal pregnant women. Lanoix et al. (2012) also demonstrated reduced blood levels of melatonin in pregnant women with PE, compared to those with normal pregnancies. These results indicate that melatonin may be involved in the pathogenesis of PE. Melatonin is a potent antioxidant and may reduce the oxidative damage caused by ROS in the placenta (Manda et al., 2007; Tan et al., 2007; de la Sierra et al., 2009). Reiter et al. (2014) reported that melatonin protects cells against oxidative stress in the ovary and placenta. Another study has shown that melatonin prevents oxidative stress by inducing the expression and activity of catalase and superoxide dismutase, and inhibiting the expression

of vascular endothelial growth factor (Valenzuela et al., 2015). In a mouse model, Lee et al. (2019a) found that melatonin reduces placental oxidative stress associated with intrauterine inflammation, which is capable of causing maternal placental malperfusion. A recent study demonstrated that the *CLOCK* gene may participate in the pathogenesis of PE via hypoxia. They found that the oscillation of *CLOCK* mRNA and protein levels are abnormal in the placenta of human patients and in rodent models of PE (Li Y. et al., 2020). The impairment of trophoblast proliferation, migration, and invasion under hypoxic conditions is able to be reversed by silencing the *CLOCK* gene in trophoblast cells (Li Y. et al., 2020). Therefore, studies on placental clock-controlled checkpoints in oxidative stress and the response to hypoxia may provide mechanistic insights into the pathogenesis of PE (**Figure 7**).

Preterm Birth

Preterm birth is defined as birth at a gestational age of fewer than 37 weeks and is one of the major causes of neonatal death worldwide (Kumar et al., 2017). Many studies have indicated that maternal shift work is related to preterm birth. In a study of occupationally exposed pregnancy cohorts, McDonald et al. (1988) found that a long work duration and shift changes were correlated with preterm birth. A cohort study of 845 female textile workers in China in 1992 showed that shift work increased the risk of preterm birth (Xu et al., 1994). Findings from a prospective cohort study of 1,908 pregnant women indicated that women who worked night shifts during pregnancy had a 50% increased risk of preterm delivery (Pompeii et al., 2005). Prospective cohort studies in the Nurses' Health Study have suggested that night shift work is associated with an increased risk of early preterm birth (Whelan et al., 2007). In a study of 673 pregnant women from Singapore, they found that women with night-eating had a higher risk of preterm delivery and speculated that this may be due to the discrepancy between the timing of eating and circadian rhythms (Loy et al., 2020). Interestingly, one study used full-spectrum light at night to suppress maternal melatonin secretion which resulted in lower serum melatonin concentrations and fewer contractions in full-term pregnant women. The authors suggested that light therapy or melatonin therapy may have the ability to delay labor and overcome preterm birth (Olcese et al., 2013). Furthermore, Lee et al. (2019b) indicated that melatonin treatment could alleviate LPS-induced intrauterine/placenta inflammation and reduce preterm birth in mice by activating the silent information regulator transcript-1/nuclear factor-erythroid 2-related factor 2 signaling pathway. It is clear that further investigations should be conducted into the links between clock-dependent maternal inflammation in the placenta and placenta. However, meta-analyses studies showed that there is no association between shift work and preterm birth (Bonzini et al., 2011; van Melick et al., 2014). Shift work can dramatically alter sleep/wake rhythms and meal timing, which may also drive preterm birth. Therefore, studies eliminating these confounding factors should be conducted in order to unravel the underlying dysregulation of circadian oscillation caused by shift work and its implication for preterm birth.

Obesity-Associated Circadian Regulation of Inter-Organ Communication

Obesity is a common comorbid risk factor in the pathogenesis of the complex disease, which is mechanistically linked to ectopic lipid deposition (Roden and Shulman, 2019) and metabolic-associated inflammation (metaflammation) (Hotamisligil, 2017). Mounting evidence supports the close links between circadian dysfunction and obesity, which can be referred to in previous reviews (Bass and Lazar, 2016; Panda, 2016; Reinke and Asher, 2019). Briefly, both irregular behavioral rhythms and clock dysfunction lead to obesity in rodents. Jet lag or constant light exposure contributes to leptin resistance (a hallmark of obesity), increased adiposity and weight gain (Shi S. Q. et al., 2013; Kettner et al., 2015). A high caloric diet increases food intake in the sleep phase, and results in a dampened daily rhythm of food intake (Kohsaka et al., 2007). Similarly, genetic mutation of core clock genes such as Bmal1, Clock, and Per2 leads to a dampened rhythm of food intake, and profound susceptibility to diet-induced obesity (Turek et al., 2005; Yang et al., 2009; Paschos et al., 2012).

Obesity is not merely a problem of overnutrition, but also a dysfunction of inter-organ communication, particularly in the adipocyte-brain axis. Adipocyte BMAL1 controls diurnal rhythms of de novo synthesis of polyunsaturated fatty acids (PUFA) through periodic expression of stearoyl-CoA desaturase 1 (SCD1) and long-chain fatty-acid elongase ELOVL6 (Paschos et al., 2012). The release of PUFA to the circulation may engage the hypothalamic circuit and inhibit food intake. However, the circadian rhythm of adipose SCD1 transcript is not found in major omics studies (source: CircaDB, CircaMetDB, CirGRDB). Instead, the SCD2 transcript oscillates robustly in adipose tissue. Adipocyte *O*-GlcNAc transferase promotes SCD1/2-controlled fatty acid desaturation and tissue accumulation of anandamide, which activates local cannabinoid receptor signaling and promotes diet-induced hyperphagia and obesity (Li M.-D. et al., 2018). Adipocyte *O*-GlcNAcylation may promote obesity through its well-established target clock proteins, such as BMAL1, CLOCK and PER2 (Durgan et al., 2011; Kim et al., 2012; Kaasik et al., 2013; Li et al., 2013, 2019; Liu et al., 2021). Adipocyte REV-ERBα limits adipocyte expansion under a high caloric diet (Hunter et al., 2021). Deletion of *Rev-Erbα* in adipocytes results in profound obesity without adipose inflammation and fibrosis. It appears that REV-ERBα controls SCD1 expression and fatty acid desaturation in adipose tissue (Hunter et al., 2021).

Despite these findings, the receiving end in the brain is not fully characterized. Recently, Cedernaes et al. (2019) have shown that clocks in AgRP neurons (hunger neurons) of hypothalamus govern circadian transcriptional response to leptin, a critical adipose-secreted endocrine factor (Cedernaes et al., 2019). AgRP neurons project to the paraventricular nucleus (PVN) of hypothalamus, which coordinates neuronal inputs to elicit feeding and satiety. Kim E. R. et al. (2020) reported that the PVN-clock determines the diurnal rhythm of energy metabolism through rhythmic sensitivity to GABAergic inputs. Ablation of the PVN-clock results in obesity. Thus, clock-controlled checkpoints involved in the metabolism and signaling of PUFA,

anandamide, and leptin may coordinately link adipocytes and hypothalamic neurons, which maintain energy homeostasis through inter-organ communication.

The hypothalamic-pituitary-adrenal (HPA) axis provides another potent means to mediate inter-organ crosstalk during obesity. Cushing syndrome presents extreme obesity when HPA goes wrong. Both hyper-cortisolism and low-amplitude rhythm of plasma glucocorticoids are well-known indicators of natural aging from rodents to nonhuman primates and humans (Thompson et al., 2020). However, it is still unsolved whether and how the circadian rhythm of HPA is involved in the pathogenesis of the complex disease. Adrenal gland-specific manipulation of the core clock genes would provide insights into this question.

In addition, obesity-associated inter-organ communication can take place between peripheral tissues. Metabolite profiling of diet-induced obese mice reveals that obesity decreases the temporal correlation of metabolites among seven organs and serum (Dyar et al., 2018b). Comparing metabolites in alanine metabolism and gluconeogenesis among organs and serum suggests that the glucose-alanine cycle, which transports amino acids from the muscle to liver for glucose production, is highly active in obesity (Dyar et al., 2018b). Though still in the early stage, the application of multi-omics, and single-cell omics would facilitate the elucidation of clock-controlled checkpoints in the obesity-associated inter-organ communication, which holds promise for diagnosis, prevention, and treatment of complex disease.

TIME MEDICINE

As we reviewed above, the circadian clock is widely involved in the pathophysiology of complex diseases, particularly metabolic diseases. Time medicine is emerging as an interdisciplinary field to understand and target clock-controlled checkpoints for the improvement of well-being and health (Cederroth et al., 2019; Ruben et al., 2019; Allada and Bass, 2021; López-Otín and Kroemer, 2021). Time medicine includes two major parts, namely, clinical treatment based on the internal time, and drugging the clock.

Clinical Treatment Based on the Internal Time

For optimizing the time of clinical treatment, the time-of-day effect of both drug treatments and surgeries needs to be extensively determined in future clinical studies. Proof-of-principle examples are bedtime dosing of the anti-dyslipidemia drug statins and afternoon heart surgeries (Cederroth et al., 2019). It is reported that more than 75% of drugs from 106 clinical trials have shown obvious time-of-day effects or toxicity (Ruben et al., 2019), corroborating the idea that circadian time should be taken into consideration at both the pre-clinical and clinical stages (Cederroth et al., 2019). The onsets of complex diseases show a diurnal pattern. For example, cancer pain erupts at 10 a.m., stroke and sudden cardiac death usually occurs between 6 and 12 a.m., and asthma occurs at 4 a.m. Therefore, detailed clinical logs are also required for time-optimization of clinical treatment.

While hormones such as melatonin and glucocorticoids are well-established biomarkers of the circadian rhythm, tissue-specific biomarkers are largely unexplored. The circadian transcriptome is usually the starting point to identify tissue-specific biomarkers for chronotherapy. However, it is impractical to obtain clinical samples (especially for the brain) suitable for circadian studies, which normally require frequent sampling around the clock with decent temporal resolution. To fill the gap, an unsupervised algorithm called cyclic ordering by periodic structure (CYCLOPS) was developed to order clinical samples into circadian structure without time indication (Anafi et al., 2017). CYCLOPS analysis of RNA-seq data in 13 human tissues indicates that nearly half of the protein coding transcriptome is rhythmic in at least one tissue (Ruben et al., 2018). Most excitingly, they found that nearly a thousand of these cycling genes, which are involved in drug delivery and metabolism, or as drug targets, may mediate time-of-day drug efficacy. Empirical validation of these findings and large-scale input of data into CYCLOPS would improve the precision and accuracy of circadian biomarkers.

Prediction of the circadian phase in vivo is another important aspect for optimizing the time of clinical treatment. Due to the dynamic nature, profiling the circadian transcriptome atlas of human tissues is not enough but the phase information by itself is equally important for optimization of the time of clinical treatment. The prediction of the circadian phase of an individual's drug target tissue(s) is a hot topic. In order to achieve this, several algorithms were invented, including Molecular-Timetable, ZeitZeiger, BIO-CLOCK, PLSR, and Time-Signature (Naef and Talamanca, 2020). The phase prediction process mainly includes four steps: training algorithms with time-indicated RNA seq data to extract biomarkers, building low-dimensional circadian trajectory, cross validation with known time labeled sample, and finally inferring the unknown sample's phase. Using dim light melatonin onset (DLMO) as an SCN phase indicator, the accuracy of these algorithms was verified through inferring the phase of SCN, with a maximum prediction error of approximately 3 h. Besides SCN, more druggable tissues' specific biomarkers and clinical feasible phase prediction methods need to be developed in the future.

Drugging the Clock

Dysregulation of the circadian rhythm is a hallmark of complex diseases (López-Otín and Kroemer, 2021). Alteration of the period length results in abnormal sleep-wake patterns (Ashbrook et al., 2020). Circadian amplitude damping always precedes neurodegenerative disorders and accelerates aging related disorders (Abbott et al., 2020). The promising efficacy of time-restricted feeding in preclinical anti-obesity and anti-cardiometabolic disease studies indicates a robust phase misalignment in these complex diseases related to life style (Panda, 2016). The scheduling of light exposure and diet quality represents a handy approach to restore circadian rhythms and health. For example, short-term exposure to bright light shifts the phase of SCN and alleviates jet-lag or circadian related mood disorders (Blume et al., 2019). Time-restricted eating improves the metabolic profile in cardiometabolic

diseases (Panda, 2016). Future work targeting clock-controlled checkpoints hold great promise for translating these mechanisms into clinical practice and devising small chemicals for applications in people that have compliance issues with these cues.

Clock-modulating compounds represent a highly valuable approach to reset the clock. Several small molecules have been identified from chemical screening (Ruan et al., 2021). GSK3-β and CKIε/δ inhibitors were discovered from the LOPAC library (Library of Pharmacologically Active Compounds) to shorten or lengthen the circadian period. CRY1 stabilizer KL001 increases the circadian period and inhibits hepatic glucose production in vitro. SR9009/SR9011 and Nobiletin regulate circadian amplitude through REV-ERB/ROR agonism (He et al., 2016; Nohara et al., 2019). Notably, cordycepin shifts circadian phase in both central and peripheral clocks through RUVBL2-mediated circadian chromatin remodeling (Ju et al., 2020). Structure-based virtual screen is an alternative method, which is time and labor saving. Encouragingly, a recent virtual screen based on the crystal structure of melatonin receptor 1 uncovered several bioactive molecules from 150 million 'lead-like' molecules, which may have potentially therapeutics significance (Stein et al., 2020). Another study using a molecular-docking approach also found small molecules which have circadian amplitude-modulating activity, binding to CLOCK and disrupting its interaction with BMAL1 (Doruk et al., 2020). In light of these advances, more clock-modulating compounds targeting clock-controlled checkpoints would likely be available in the near future and their clinical efficacy would be tested for better care and treatment of complex diseases.

CONCLUSION

Over the past two decades, circadian regulation of physiology and metabolism has been the key direction of circadian rhythm research. Substantial progress has been made in elucidating circadian clock-controlled pathways and checkpoints in peripheral tissues and their relevance in complex diseases. Despite these achievements, the majority of the research has been focused on a few prototypical organs, such as the liver, and to a lesser degree, the heart and muscle. We anticipate that the next phase of this research would lead to translational medicine in liver disease, deep mechanistic insights in circadian biology and medicine related to the heart and muscle, and further findings in less characterized tissues and organs relevant to complex diseases.

AUTHOR CONTRIBUTIONS

M-DL: conceptualization, investigation (intro and liver), and supervision. HX: investigation (liver) and visualization. YY and WH: investigation (blood). XY and GD: investigation (neuron). HL, HZ, and T-LH: investigation (female reproduction). DT, FD and ZZ: investigation (heart). T-LH: investigation (female reproduction). QC: investigation (male reproduction). DJ: investigation (intro and time medicine). KC: investigation (muscle). M-DL, HX, YY, XY, HL, DT, HZ, ZZ, T-LH, QC, GD, DJ, KC, FD, and WH: writing. All authors contributed to the article and approved the submitted version.

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METHODS
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dFRAME: A Video Recording-Based Analytical Method for Studying Feeding Rhythm in *Drosophila*

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Animals, from insects to humans, exhibit obvious diurnal rhythmicity of feeding behavior. Serving as a genetic animal model, *Drosophila* has been reported to display feeding rhythms; however, related investigations are limited due to the lack of suitable and practical methods. Here, we present a video recording-based analytical method, namely, *Drosophila* Feeding Rhythm Analysis Method (dFRAME). Using our newly developed computer program, FlyFeeding, we extracted the movement track of individual flies and characterized their food-approaching behavior. To distinguish feeding and no-feeding events, we utilized high-magnification video recording to optimize our method by setting cut-off thresholds to eliminate the interference of no-feeding events. Furthermore, we verified that this method is applicable to both female and male flies and for all periods of the day. Using this method, we analyzed long-term feeding status of wild-type and *period* mutant flies. The results recaptured previously reported feeding rhythms and revealed detailed profiles of feeding patterns in these flies under either light/dark cycles or constant dark environments. Together, our dFRAME method enables a long-term, stable, reliable, and subtle analysis of feeding behavior in *Drosophila*. High-throughput studies in this powerful genetic animal model will gain great insights into the molecular and neural mechanisms of feeding rhythms.

Keywords: feeding rhythms, *Drosophila*, video recording, food approaching events, residence time, *period* mutant fly

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INTRODUCTION

Feeding rhythm has recently been found to play a critical role in animal health. It is vital for animals to synchronize their feeding behavior to both internal biological clock and external environmental conditions, while the underlying mechanisms remain to be elucidated. With powerful tools for genetic and neural manipulation, *Drosophila melanogaster* serves as an excellent model for investigating the molecular and neural mechanisms underlying circadian rhythms (Franco et al., 2018). Earlier studies in *Drosophila* uncovered the molecular mechanisms controlling internal clock, which are evolutionarily conserved from insects to mammals (Yu and Hardin, 2006). Among the various environmental cues, light and food are the two major zeitgebers (Tomioka and Matsumoto, 2010; Barber et al., 2016; Challet, 2019; Helfrich-Forster, 2020). The sleep rhythm and the role of light entrainment have been well studied in flies (Shafer and Keene, 2021). In contrast, studies on feeding rhythms remain limited, largely because of the limitation of available analytical methods of fly feeding.

Various assays have previously been developed to study feeding behavior in fruit flies. One traditional category utilizes different types of tracers, such as non-digestible dyes (Edgecomb et al., 1994; Xu et al., 2008), radioactive substances (Thompson et al., 1991; Carvalho et al., 2005; Carvalho et al., 2006; Deshpande et al., 2014), and DNA oligomers in the BARCODE assay (Park et al., 2018; Huang et al., 2020). To study feeding rhythms using this approach, different groups of flies are transferred into food with tracers at different time points of the day. After a short period, usually less than 0.5 h to avoid excretion, the flies are quickly frozen, and the tracers in fly abdomens are quantified as the amount of food consumption. In these assays, normal laboratory food is used, and flies consume food in a natural manner. However, feeding behavior is inevitably being interfered by frequent manipulation, especially at night, and measurements are inevitably discontinuous.

The Capillary Feeder (CAFE) assay constitutes a practical way to continuously monitor food intake for several days (Ja et al., 2007; Diegelmann et al., 2017). In the CAFE assay, a small group of flies are put into a breeding vial, and they descend along a capillary and suck liquid food from it. The declining surface measured in the capillary represents the food amount consumed by the flies. Murphy et al. (2017) introduced an infrared monitor to the CAFE system, namely, Activity Recording Capillary Feeder (ARC), so that the food intake of single flies can be continuously recorded with a greatly improved sample rate of 1 min (Murphy et al., 2017; Li et al., 2021). Nevertheless, it is still difficult to capture single feeding events at this temporal resolution, therefore missing detailed information of feeding behavior. Moreover, the volatilization, stickiness, and potential leakage of liquid foods require strict control of the recording environment.

Proboscis extension response (PER) assay is utilized to study taste behavior in fruit flies, which is related to eating motivation (Wong et al., 2009). In the traditional setup, flies are fixed in a pipette tip, and upon food touching, their PER are monitored, however, for no longer than half an hour. To record naturally feeding flies, the Fly Proboscis and Activity Detector (fly PAD) was developed, which detects a capacitance signal whenever the proboscis of a free-moving fly touches food (Itskov et al., 2014). A similar assay called the Fly Liquid-Food Interaction Counter (FLIC) uses liquid food, and an analog electrical signal is measured (Ro et al., 2014). These two methods allow for continuous monitoring of feeding behavior in a high temporal resolution. However, the limited food amount used in these systems and the susceptibility to electromagnetic disturbances render long-term operation a considerable challenge.

Drosophila video recording system is a stable and easy-operating system, and it has been widely used to continuously monitor locomotor activity and study circadian rhythms of sleep (Zimmerman et al., 2008; Gilestro and Cirelli, 2009; Pfeiffenberger et al., 2010). Based on the image data obtained from this system, we designed a computer program called FlyFeeding to analyze food-approaching behavior. By setting filters, we distinguished food-approaching events (FAE) with feeding from those with no-feeding and determined three indexes, food-approaching events (FAEn), residence time of FAE (FAErt), and FAErt per event (FAErt/n). Three wild-type strains and a clock gene mutant were examined under fed/starvation and light–dark (LD)/dark–dark (DD) conditions using this analytical method. The results recaptured earlier reported features of feeding rhythms and, moreover, revealed new profiles of feeding patterns of these flies. Thus, our method, *Drosophila* Feeding Rhythm Analysis Method (dFRAME), allows for a stable, reliable, and extensive study of feeding rhythm in *Drosophila*.

MATERIALS AND EQUIPMENT

Fly Strains and Rearing

Fly strains were obtained from the Bloomington *Drosophila* Center, including the three wild-type strains w^{1118}, Canton S (CS), and *w*CS and a *period* mutant strain $period^{01}$ (per^{01}). Flies were reared on standard corn meal food (Bloomington recipe) and maintained at 25°C with 60% humidity on a 12-h/12-h LD cycle or constant darkness (DD). Wild-type w^{1118} male flies were used for experiments unless otherwise specified.

Experimental Setup and Video Recording

The *Drosophila* video recording system was set up according to previous reports (Pfeiffenberger et al., 2010; Gilestro, 2012). Briefly, individual flies at age of 3–5 days were transferred into a thin and straight glass tube with 5-mm diameter and 65-mm length. At one end of the tube, 2% agar with or without 5% sucrose was supplied as food. Flies were allowed to adapt to the environment for at least 12 h, and data collected thereafter were used for analysis. Images were recorded at one frame per second (fps) for 2–5 days, and the raw image data were processed to obtain the coordinate information of fly barycenter in each frame using pySolo (Gilestro and Cirelli, 2009). If the individual fly was not moving at the beginning of a day, the coordinate value of the first second of ZT 0 was automatically set to 0 (at the cotton side), and these traces were removed from the movement tracks.

High-Magnification Video Recording

A high-magnification video recording system was constructed to characterize the food-approaching behavior. Individual flies in glass tubes were arranged under a Nikon SMZ25 stereomicroscope equipped with an ANDOR CCD camera. The area close to the food surface was video recorded at 5 fps for 30 min using the ANDOR Solis software. Videos were taken at five time-points of the day; for each time-point, the recording was repeated for at least four times. Data were exported in MP4 format prior to manual inspection.

Statistical Analysis

All experimental data were analyzed and plotted with SPSS Statistics, GraphPad Prism, and Adobe Illustrator. *T* test (two-tailed, independent samples) was used to compare the two groups. One-way ANOVA and two-way ANOVA with

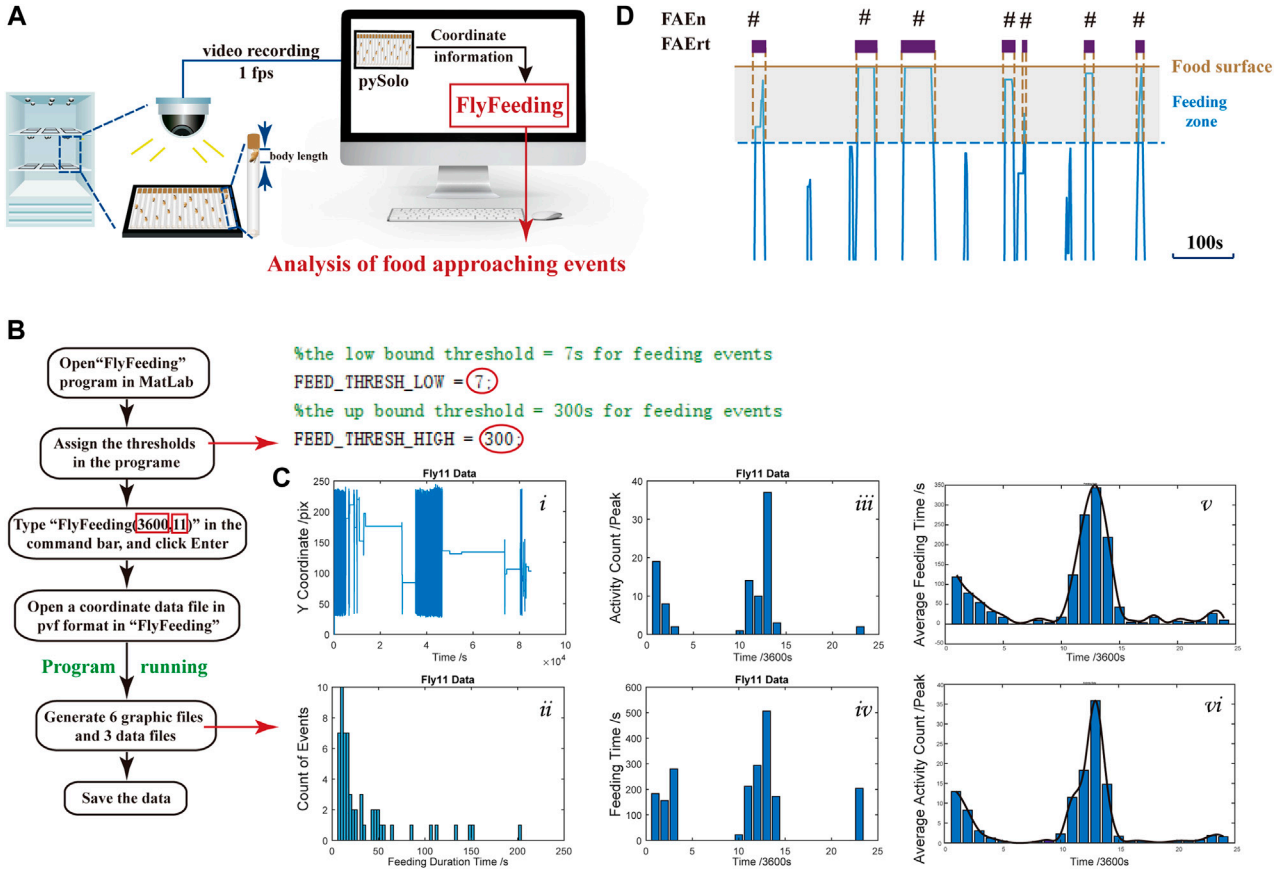

FIGURE 1 | Experimental setup, computer program, and data output **(A)** Experimental setup for dFRAME method. A group of 18 flies were monitored individually and the coordinate data are used for analysis **(B)** Operational flowchart of the "FlyFeeding" program. The right part represents a screenshot of the program start; the cut-off thresholds can be assigned by users (red circles). The two numbers in red rectangles indicate the analyzing period (3,600 s) and fly body length (11 mm) **(C)** "FlyFeeding" simultaneously analyzes a group of 18 flies, generating a set of six graphs and three data flies. Graphs i–iv show the analytical results of a representative fly, including the movement tracks (i), the FAEn distribution according to either the residence time (ii) or in every hour of the day (iii), and the FAErt distribution in every hour of the day (iv). The averaged distribution of FAEn and FAErt of the 18 flies are shown in every hour of the day (v and vi, respectively). In addition, the program generates three data files, "Activity.csv" for FAEn every hour, "Feeding.csv" for total FAErt every hour, and "FeedingPDF.csv" for FAErt of each time. All data are listed for individual flies **(D)** Schematic diagram of food-approaching events (FAE). The brown line indicates the food surface. The gray area represents a fly body length from the food surface, which is determined as feeding zone. A short period of movement track was clipped out, and those close to the food end are shown in blue lines. Once the fly enters the zone, the FAEn counts 1, and the duration that the fly stays in the zone is recorded as the FAErt.

Tukey's honestly significant difference (Tukey HSD) *post hoc* test were applied to determine the difference among groups. Significance levels were set at $p < 0.05$ for all comparisons. Data were presented as mean ± standard error of mean (SEM). The following levels of significance were used: * or #, $p < 0.05$; ** or ##, $p < 0.01$; *** or ###, $p < 0.001$; **** or ####, $p < 0.0001$. n. s. indicates no significant difference. *n* represents the repetition of independent samples.

METHODS

Food-Approaching Behavior Observed in the Video Recording System

To monitor feeding behavior at a long-term range, we utilized the video recording system, and on each recording plate, 18 flies in 18 glass tubes were recorded. The coordinate data of each fly were obtained using pySolo for further analysis (**Figure 1A**). To characterize the feeding behavior, we developed a MATLAB program, FlyFeeding (see **Figure 1B** for the analysis flow chart), allowing synchronous data processing for 18 flies. As shown in **Figure 1C**i), the movement track of one representative fly exhibited conspicuous rhythmicity of locomotor activity, shown as intensive track lines around light–dark and dark–light switch periods. Notably, flies did not stay on food for a long period. Instead, flies usually approached the food, ate, left, and returned (**Figure 1D**), namely, food-approaching behavior.

To quantify the food-approaching behavior, we defined a feeding zone as the distance of one fly body length from the food surface (**Figure 1D**). In our system, one body length of female flies is approximate 12 pixels and 11 pixels for males

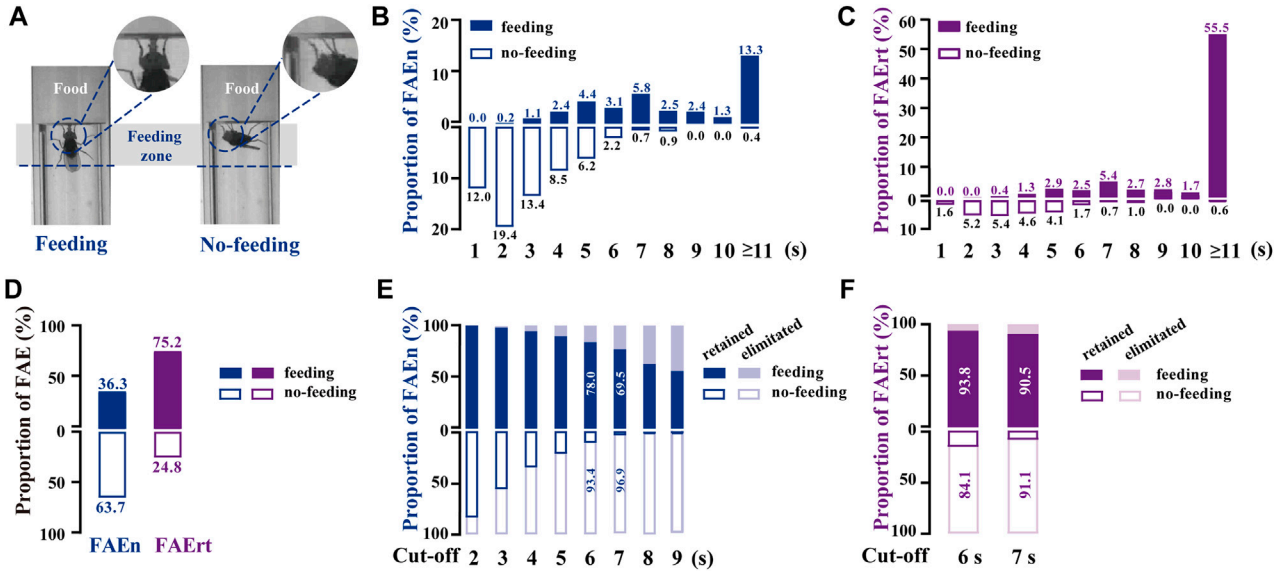

FIGURE 2 | Analyzing food-approaching behavior using high-magnification recording **(A)** Two video snapshot images showing FAE under feeding **(left)** and no-feeding conditions **(right) (B)** Proportional distribution of FAEn. The numbers on each column indicate the percentage of the event numbers with or without feeding in the total event number (551) **(C)** Proportional distribution of FAErt. The numbers on each column indicate the percentage of the residence time with or without feeding in the total residence time (4,132 s) **(D)** The proportion of total feeding and no-feeding events (FAEn) and time (FAErt) **(E)** As the cut-off threshold increases from 2 to 9 s, the ratio of retained feeding events to all feeding events (200) gradually decreases, while the ratio of retained no-feeding events to all no-feeding events (351) decreases rapidly **(F)** The proportion of retained FAErt with the cut-off threshold set to either 6 or 7 s.

(**Supplementary Table S1**). For each tube, the food surface is automatically detected by the find-peaks module in FlyFeeding, defined as the farthest coordinate position of the fly in every 24-h recording. As shown in **Figure 1D**, once the fly enters the zone, the FAEn counts 1, and the duration that the fly stays in the zone is recorded as the FAErt.

FlyFeeding provided the distribution of food-approaching events according to their time duration for individual flies, and we found that most events were short in duration (**Figure 1C**ii). In addition, both numbers and residence time of FAE were high in the morning and the evening periods (**Figure 1C**iii-iv). Flies showed high levels of both feeding and locomotor activities in these two periods; thus, we speculated that the FAE were closely related to feeding behavior and also included no-feeding activities.

Setting Cut-Off Filters to Minimize the Inference of No-Feeding FAE

To distinguish FAE with feeding and with no-feeding, we established a high-magnification video recording system. Similar to our analysis of the regular video recordings, we marked a feeding zone of one body length from the food surface in these high-magnification videos (**Figure 2A**). Feeding behavior was distinguished manually when flies extended proboscis twice or more times in one FAE. All recorded FAE were classified into two types, namely, food approaching with feeding or with no-feeding (**Figure 2A**; **Supplementary Movie S1, S2**).

We found that among 551 FAE collected from 45 male flies, most no-feeding events occurred when the residence time was shorter than 7 s, whereas most feeding events happened when the residence time was longer than 3 s (**Figures 2B,C**). Notably, food-approaching events lasting longer than 10 s contributed more than half of the residence time, and most of them were accompanied with feeding behavior. Overall, approximately 64% of FAEn and 25% of FAErt were not related to feeding (**Figure 2D**). We next recorded 634 FAE from 58 female flies. The proportion of feeding and no-feeding FAE exhibited identical profiles to those in males (**Supplementary Figure S1A, B**). In addition, approximately 65% of events and 30% of residence time were not related to feeding in females (**Supplementary Figure S1C**).

To minimize the inference of no-feeding FAE, the low cut-off filter was set with the criteria of eliminating the no-feeding events and retaining the feeding events. As shown in **Figure 2E**, the ratio of retained no-feeding events rapidly declined when the cut-off threshold increased, while the ratio of the retained feeding events decreased at a slower rate. When the low cut-off threshold was set as 7 s, approximately 97% of events and 91% of residence time with no-feeding were not included for calculation, while 70% of events and 91% of residence time with feeding were retained (**Figures 2E, F**). We next tested the 7-s cut-off in female flies. Approximately 95% of events and 86% of the residence time with no-feeding were rejected, while 77% of the events, together with 93% of residence time with feeding, were retained (**Supplementary Figure S1D, E**). Therefore, the FAErt with

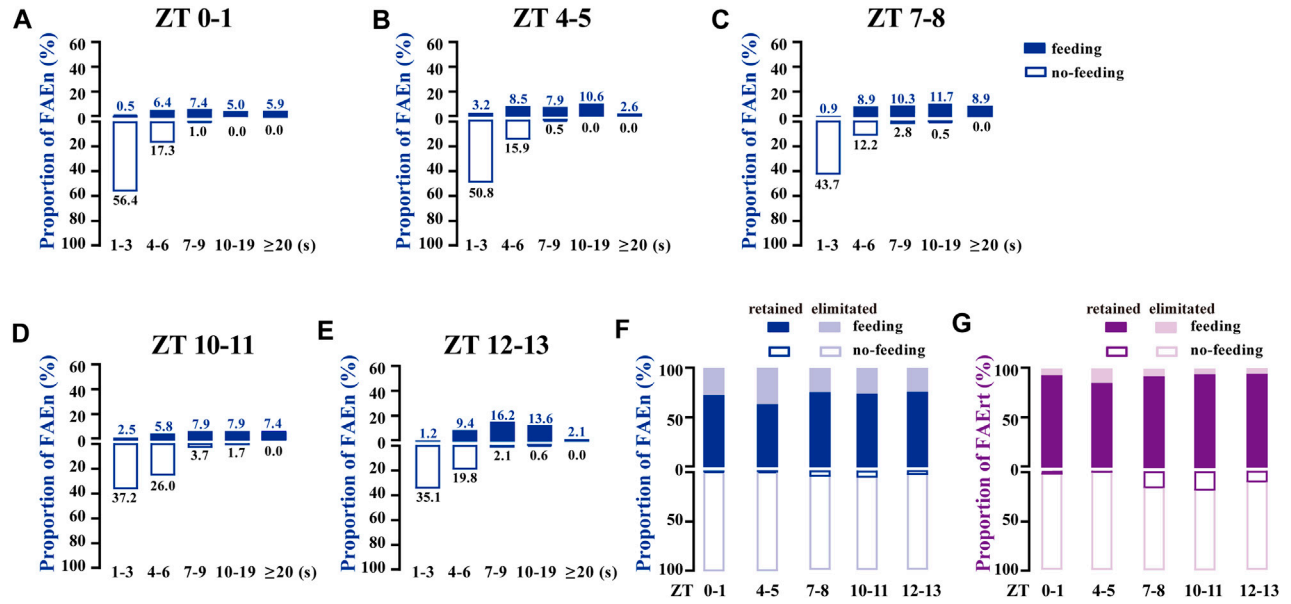

FIGURE 3 | FAE exhibits similar distributions of feeding and no-feeding behaviors at different time-points of the day **(A–E)** The proportion distribution of FAE at ZT0–1 (n = 202), ZT4–5 (n = 189), ZT7–8 (n = 213), ZT10–11 (n = 242), and ZT12–13 (n = 339) **(F–G)** With the cut-off threshold of 7 s, the proportion of retained and eliminated FAEn **(F)** and FAErt **(G)** at different times of the day. Data are shown for both sexes, with a total of 1,185 events and a total time of 8,603 s used for analysis.

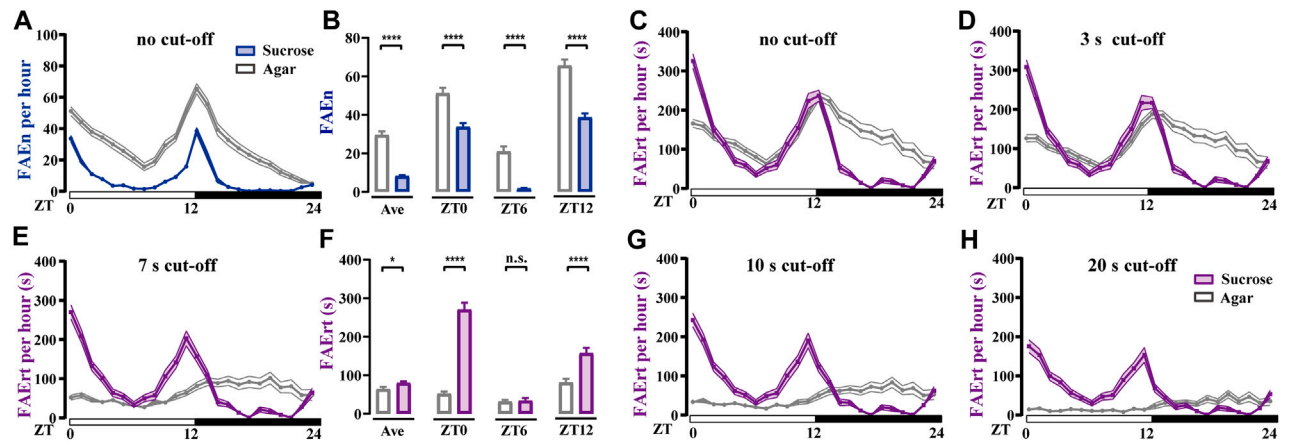

FIGURE 4 | Food-approaching behavior exhibits different profiles in wild-type w^{1118} male flies **(A)** Flies in the agar group display higher levels of FAEn compared to FAEn in flies in the sucrose group. The numbers of FAE were merged every hour and are shown in curves **(B)** The average FAEn per hour of the whole day and the FAEn in ZT 0, 6, and 12 **(C–E, G–H)** The curves of FAErt with either no cut-off **(C)** or with cut-off as indicated **(F)** The average FAErt per hour of the whole day and the FAErt in ZT 0, 6, and 12. Data are shown as means ± SEM. $^{*}p < 0.05$; $^{****}p < 0.0001$. n. s. indicates no significant difference. n = 78 in the agar group and 86 in the sucrose group.

the 7-s cut-off commendably represents feeding status in both female and male flies.

To determine whether the 7-s cut-off is suitable to process the data from different time-points of the day, the proportional distribution of a total of 1,185 FAE was analyzed according to the five recording periods. As shown in **Figures 3A–E**, most of the no-feeding FAE were shorter (1–3 s) in the morning (ZT 0–1 and 4–5), while the proportion of 4–6 s or longer time increased in late afternoon and evening (ZT 10–11 and 12–13). Nevertheless, these subtle differences failed to affect the choice of the 7-s cut-off. For all five sets of data at different time points, at least 92% of FAEn with no-feeding were eliminated, and 85% of FAErt with feeding were retained with the 7-s cut-off (**Figures 3F,G**). Together, these results indicated that data collected at different time-points were suitable for processing using the same low cut-off threshold.

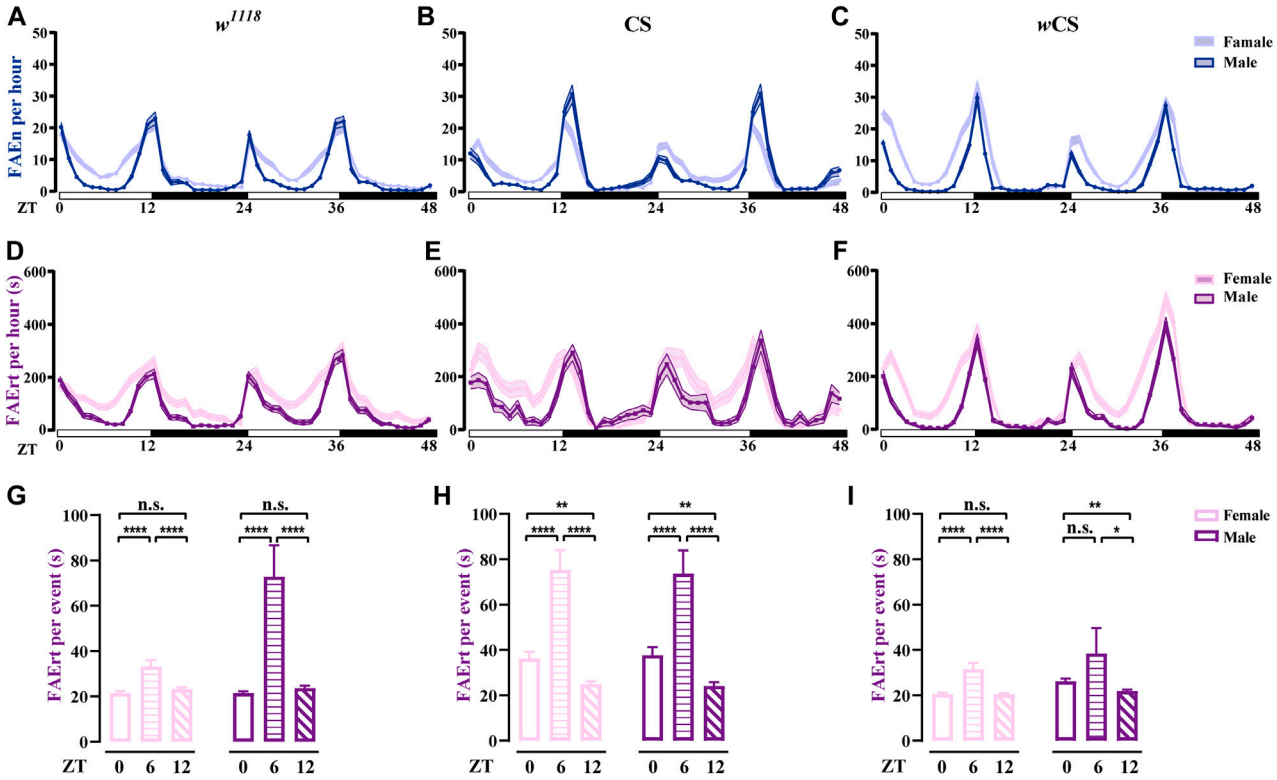

FIGURE 5 | Analysis of feeding rhythms of wild-type flies using dFRAME **(A–C)** The FAEn with no cut-off of w^{1118} (n = 88–89), CS (n = 34–36), and *w*CS (n = 72–85) flies **(D–F)** The FAErt showing the feeding rhythms of three types of flies. The cut-off threshold is 7–300 s **(G–I)** The FAErt/n showing the average residence time per event of three types of flies. Data are shown as mean ± SEM. *p < 0.05; **p < 0.01; ****p < 0.0001. n. s indicates no significant difference.

When we analyzed the high-magnification video data, we observed six long-lasting FAE events, which were longer than 120 s, with the longest event lasting 250 s. In addition, we identified a small number of FAE with durations longer than 300 s in regular video recording. Because 5 min or more of quiescence is defined as sleep in flies (Shaw et al., 2000; Zimmerman et al., 2008), we set 300 s as the high cut-off threshold. On the basis of these results, we set the band filter of 7–300 s for all further analysis of data obtained from regular video recording for all time-points of the day, for both females and males.

Validation of the Cut-Off Thresholds in the Regular Video Recording System

Fruit flies display significant daily rhythms, showing as a typical double-peak curve with high levels of locomotor activity in the morning and evening (Helfrich-Forster, 2000; Hall, 2003; Schabler et al., 2020; Cascallares et al., 2018). Earlier studies also reported that flies consume food mostly during these time periods (Ro et al., 2014). To test whether our method captures the rhythm of feeding rather than locomotor activity, we video-recorded wild-type w^{1118} male flies using two types of foods, either agar alone or agar containing sucrose. As shown in **Figure 4A**, FAEn exhibited the typical double-peak curve in both groups, indicating that flies approach food more frequently in the mornings and evenings. In addition, we detected significantly more FAE in the agar group compared to events recorded for the sucrose group throughout the day (**Figures 4A,B**). This result is in agreement with previous findings that hungry flies exhibit a higher locomotor activity (Yang et al., 2015).

We next analyzed the index of FAErt with different cut-off thresholds. We found that the sucrose group exhibited typical double-peak curves regardless of the thresholds (**Figures 4C–H**). In contrast, the FAErt curves of the agar group turned flatter as the threshold increased, and the peak shape was completely lost when the threshold was 7 s or higher (**Figures 4E–H**). Moreover, the average FAErt per hour of the agar group was significantly lower than that of the sucrose group, which was more evident in the morning and evening (ZT 0 and ZT 12, **Figure 4F**). Together with the FAEn results (**Figures 4A, B**), these findings indicated that when provided with food lacking nutrients, flies display more short-time food-approaching behavior, presumably representing the processes of exploring and foraging. Therefore, the influence of these no-feeding events on the FAErt is effectively eliminated with the 7-s cut-off filter, and the filtered FAErt reliably represents the feeding status of flies.

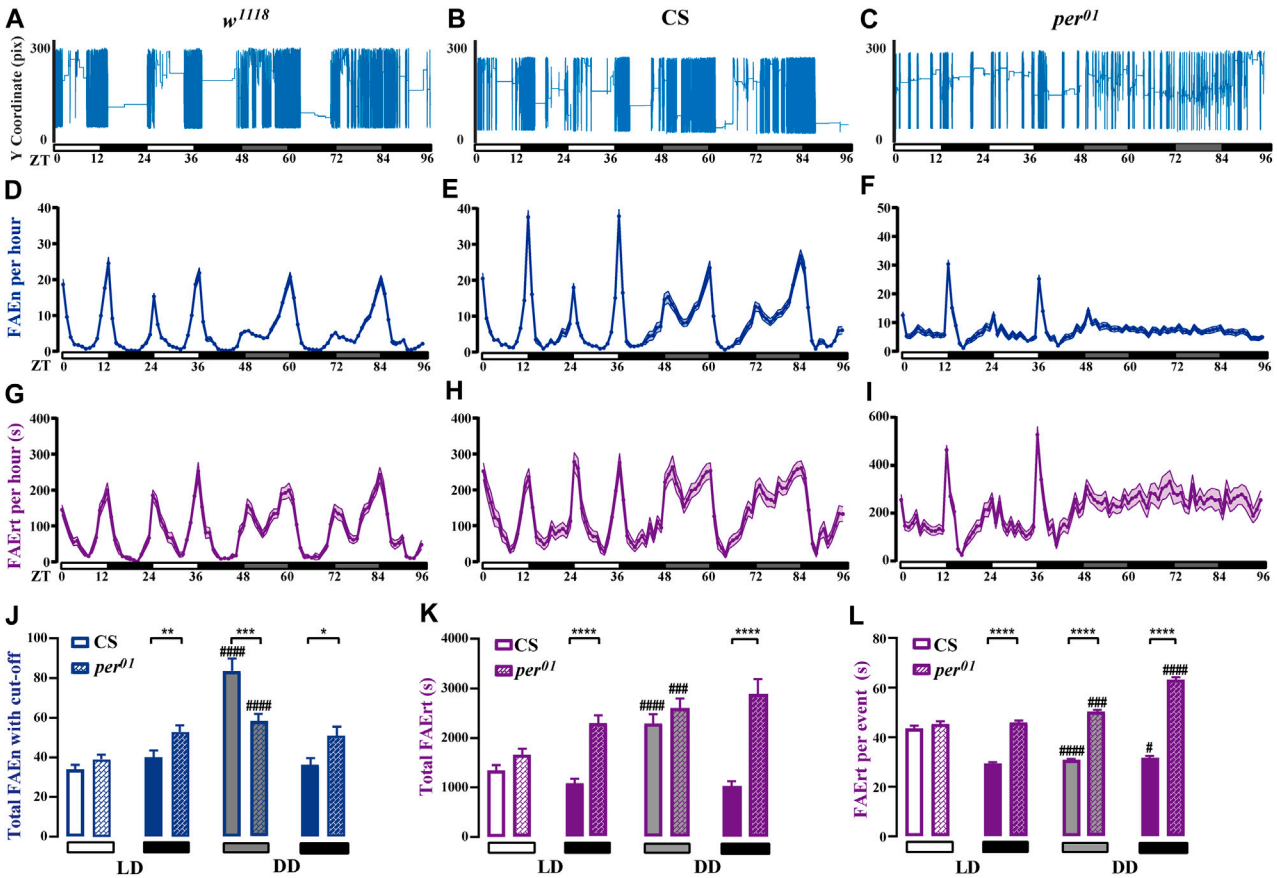

FIGURE 6 | Feeding rhythm analysis under LD and DD conditions of wild-type and *per*^{01} mutant flies **(A–C)** The movement track of a representative male fly of wild-type w^{1118} and CS, as well as *per*^{01} mutant **(D–I)** dFRAME analysis of FAEn (**D–F**, with no cut-off) and FAErt **(G–I)** for three types of flies under continuous LD and DD cycles for 4 days **(J–L)** The day time and night time of total FAEn (**J**, with 7-s cut-off), total FAErt **(K)**, and FAErt/n **(L)** under LD and DD conditions. Symbols: #, ##, ###, and #### indicate significance when comparing DD conditions to LD conditions of corresponding genotype and corresponding day or night time. w^{1118}, n = 88; CS, n = 66; and *per*^{01}, n = 72. Data are shown as mean ± SEM. #p < 0.05; ** or ##p < 0.01; ###p < 0.001; **** or ####p < 0.0001. No label means no significant difference.

RESULTS

Analysis of Feeding Rhythms in Wild-type Flies

Using the dFRAME method with the 7–300-s cut-off, we examined the FAErt of three types of wild-type flies, w^{1118}, CS, and *w*CS for both female and male flies. As shown in **Figures 5A–F**, the FAErt of these three strains exhibited the double-peak curve in both sexes, which is in agreement with the results obtained earlier using the FLIC assay (Ro et al., 2014). In addition, our results showed that the FAErt curves of female flies in all three stains were wider in comparison with those in males, indicating longer meal times of female flies (**Figures 5D–F**). We calculated the FAErt for four time periods, ZT 0–2 (morning peak), 3–11 (day time), 12–14 (evening peak), and 15–23 (night time). Our results supported the observation that compared to male flies, females displayed increased levels of FAErt mostly during day time (**Supplementary Figure S2**).

Notably, different wild-type fly strains exhibited some unique features. For example, compared to w^{1118} and CS, *w*CS displayed sharper peaks in the FAErt curves, especially for males (**Figure 5F**), indicating that the feeding pattern of *w*CS flies was more concentrated. In addition, CS male flies displayed one- to two-fold more FAEn in the evening compared with that in the morning (**Figure 5B**); however, the levels of FAErt were close between these two peaks (**Figure 5E**), indicating that CS flies displayed more short-time no-feeding visiting in the evening. Moreover, the FAErt curves showed a small noon peak in CS male flies, while it did not exist in the FAEn curves.

To better characterize the food-approaching behavior, we analyzed a new parameter, residence time per visit, namely, FAErt/n. Interestingly, although the FAErt levels were lowest in the noon time, the FAErt/n levels were significantly higher than the other two time points in all three fly strains, which was the most evident in CS flies (**Figures 5G–I**), suggesting that flies take food in a more relaxed manner during lunch time. Notably, the average FAErt/n of female flies were comparable or lower than

males for all three time-points, dispelling the concern that the FAErt levels of females were inflated by the egg-laying behavior. Taken together, our dFRAME analytical method characterizes the feeding rhythm of wild-type flies and reveals detailed information about their feeding patterns, thereby allowing cross-comparison among different fly strains.

Analysis of Feeding Rhythm Under Constant Darkness Condition

Light is a strong zeitgeber for the circadian rhythm. To study the effects of light on feeding rhythm, we examined w^{1118} and CS male flies under LD and DD cycles using our dFRAME method. Compared to LD condition, both wild-type flies displayed higher locomotor activities in the subjective day time under DD condition (**Figures 6A, B**). Consistently, the FAEn and FAErt also significantly increased in day time, however, not in night time (**Supplementary Figure S3A, B, D, E**). As shown in **Figures 6D, E, G, H**, the FAEn and FAErt curves remained in double-peak shape under DD condition, while some changes were evident. For example, the morning peak of the FAEn curve in w^{1118} and the evening peak in CS decreased, whereas the FAErt peak values remained stable; moreover, the peak width in both FAEn and FAErt curves increased under DD condition. These findings indicated that in the absence of light, wild-type flies are able to maintain the 24-h cycle of feeding rhythm, as well as the levels of feeding behavior at morning and evening meal times; however, they elicit more food-approaching behavior in non-meal day time, so that the concentrated feeding pattern that is observed under LD condition is weakened.

It has been reported that per^{01} mutant flies display rhythmic feeding patterns in LD cycles; however, this pattern is lost under a DD condition (Ro et al., 2014; Schabler et al., 2020). In agreement with these observations, we found that per^{01} flies displayed daily feeding rhythms under LD condition. However, our dFRAME method showed that both the FAEn and the FAErt curves of per^{01} flies exhibited only one peak in the evening, with the morning peak inapparent, which was different from the typical double-peak curves observed in wild-type flies (**Figures 6D–I**). In addition, wild-type flies started to approach food more frequently before light was off, whereas the food-approaching behavior of per^{01} flies did not increase until the light was off, suggesting that loss-of-function of *period* impairs the organization of morning feeding and the prediction of light off.

When the light condition was switched to DD, per^{01} flies completely lost their feeding rhythm. As shown in **Figures 6F, I**, both the FAEn and FAErt curves remained flat throughout the day. Similar to the genetic control CS flies, the FAEn in the subjective day time also increased in per^{01} flies (**Supplementary Figure S3B, C**); however, the increase occurred at a significantly lower level compared with CS flies (**Figure 6J**). In contrast, we found that the FAErt levels in subjective day time of per^{01} flies increased to a similar level of those in CS flies (**Figure 6K**). Two-way ANOVA showed that the DD condition affected the day time FAEn differently between these two fly strains ($p < 0.001$) but at similar levels on FAErt ($p > 0.05$). We thus calculated the average food residence time per event (FAErt/n). Intriguingly, when switched to the DD condition, the day time FAErt/n of CS flies significantly decreased, whereas it increased in per^{01} flies (**Figure 6L**), with a highly significant interaction between genotypes and light conditions ($p < 0.0001$, two-way ANOVA).

For the night time, the FAEn levels of per^{01} flies were comparable between the LD and DD conditions, and they were modestly higher than those of CS flies (**Figure 6J**). Two-way ANOVA indicated that the p value was greater than 0.05. However, per^{01} flies displayed significantly higher FAErt levels than CS flies in night time of LD cycles (**Figure 6K**), and the difference was enlarged under the DD condition ($p < 0.05$, two-way ANOVA). Consequently, the average FAErt/n during night time were significantly longer in per^{01} flies than those in CS flies under both LD and DD conditions (**Figure 6L**), and the difference was more evident under the DD condition ($p < 0.0001$, two-way ANOVA).

Taken together, our findings indicate that under the LD condition, per^{01} mutant flies respond to the lights off to maintain daily feeding rhythms. They visit food sites (FAEn) at comparable levels to CS control flies, however, stay longer (FAErt/n and FAErt) in the night than CS flies. Under the DD condition, both wild-type and mutant flies perform more food-approaching behavior during the subjective day time but with different feeding patterns. CS flies visit food more often with shorter residence time per stay, whereas mutant flies prolong their residence time every visit, similar to that in night time under the LD condition.

DISCUSSION

Here, we report a video recording-based analytic method called dFRAME for studying the feeding rhythm in *Drosophila*. Utilizing the high-magnification video recording system, we determined a cut-off range of 7–300 s and three indexes, the numbers of FAE (FAEn), the residence time (FAErt), and the average residence time per event (FAErt/n), to analyze feeding patterns. Our method recaptured the feeding rhythms earlier found in these flies and, moreover, revealed new profiles of feeding behavior.

Drosophila is a powerful genetic animal model to investigate the molecular and neural mechanisms underlying behaviors. Several assays have been developed to analyze feeding behavior at different time ranges, including PER for testing immediate response (within 10 min), tracer-based methods for short-term examination (0.5–1 h), electric signal-based methods (Fly Pad and FLIC) for medium-term recording (1–3 days), and the video-based method (ARC) for long-term monitoring (up to 7 days). Our dFRAME method is also a video-based method, and the recording is even longer (up to 1–2 weeks) and more stable than ARC, because no liquid food is used in our system. The advantage of ARC is that it reports the precise levels of food intake; however, the temporal resolution of 1 min makes it difficult to capture the detailed information of feeding behaviors. In contrast, the data obtained by dFRAME are less correlated to food consumed; instead, they characterize feeding-related behavioral features in a highly reproducible manner. For

studying feeding rhythms in a long term, dFRAME is ideal to perform high-throughput screen and explore new behavioral patterns, and ARC can be used to determine the amount of food intake.

dFRAME is built on the basis of video-recording system that has been used for analyzing sleep. Thus, we are able to analyze locomotor activity, foraging, feeding, and sleep from the same set of video data. In addition, since the infrared light is used for video recording, the light conditions (e.g., LD, DD, and LL) can be designed and conducted without interfering the recording. Similarly, genetical methods for manipulating neural activity, including heat and optogenetics controls of target neurons, can also be incorporated into the system. Moreover, the fly chamber can be modified according to experimental requirements. In this study, we use standard straight glass tubes with food in one end. It is also applicable to two-end or multi-end chambers by adding modules in the FlyFeeding program. Electrically operated gates can also be integrated. Therefore, taking the advantage of powerful genetic manipulation and rich behavior of *Drosophila*, dFRAME enables a comprehensive investigation of feeding rhythms.

In this study, we tested per^{01} mutant flies, which has been reported to exhibit feeding rhythms under the LD condition (Ro et al., 2014; Schabler et al., 2020). Consistently, we found that these flies display 24-h rhythms; however, our results indicated that they fail to predict the timing for the evening meal and almost completely lose the breakfast even under LD cycles. Intriguingly, during the subjective day time under the DD condition, both per^{01} flies and the genetic control CS flies increase their total food residence time to a similar level; however, they achieve this using two different approaches. Specifically, the per^{01} mutant flies considerably prolong the residence time per stay (FAErt/n) to the similar levels observed during the night. In comparison, CS flies dramatically decrease the residence time per stay and instead revisit the food more frequently. These findings suggest that CS flies are aware of the day-time period in DD cycles and the cognitive dissonance results in anxiety-like behavior. This is in agreement with a previous finding that CS flies display decreased day-time sleep under DD conditions (Parisky et al., 2016). In contrast, lacking of the internal clock, per^{01} mutant flies exhibit same feeding patterns in the subjective day time to that observed during the night. These interesting profiles identified through our dFRAME analysis await further mechanistic investigation in future.

SUMMARY STATEMENT

We developed a new analytical method, dFRAME, which is simple, stable, robust, and reliable for investigating the feeding rhythms of fruit flies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YL and JW conceived the project and designed the experiments. MN and XZ performed the experiments, and MN analyzed the data. WL wrote the program of FlyFeeding. MN and YL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.763200/full#supplementary-material

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Synaptic Protein Phosphorylation Networks Are Associated With Electroacupuncture-Induced Circadian Control in the Suprachiasmatic Nucleus

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Phosphorylation is one of the most important posttranslational modifications and regulates the physiological process. While recent studies highlight a major role of phosphorylation in the regulation of sleep–wake cycles to a lesser extent, the phosphoproteome in the suprachiasmatic nucleus (SCN) is not well-understood. Herein, we reported that the EA treatment elicits partial reparation of circadian rhythmicity when mice were exposure to constant darkness for long time. We investigated the effects of EA on circadian rhythms in constant darkness between EA stimulation and free-running control. Next, mass spectrometry–based phosphoproteome was utilized to explore the molecular characteristics of EA-induced phosphorylation modification in the SCN. A total of 6,192 distinct phosphosites on 2,488 proteins were quantified. Functional annotation analysis and protein–protein interaction networks demonstrated the most significant enriched phosphor-proteins and phosphosites involved in postsynapse and glutamatergic synapse. The current data indicated that most of the altered molecules are structural proteins. The target proteins, NMDAR and CAMK2, were selected for verification, consistent with the results of LC–MS/MS. These findings revealed a complete profile of phosphorylation modification in response to EA.

Keywords: circadian rhythm, phosphorylation, mass spectrometry, suprachiasmatic nucleus, electroacupuncture

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INTRODUCTION

The suprachiasmatic nucleus (SCN) is a mammalian master clock that regulates various circadian rhythms, such as physiology and behavior, to adapt the internal environment to changes in the external environment. The SCN can be entrained by zeitgeber to regulate the biological rhythms. Several studies have been conducted to examine the proteomics of the SCN to elucidate the circadian rhythm (Deery et al., 2009; Chiang et al., 2014). The posttranslational modifications (PTMs) can alter the activity of proteins involved in cell signaling. Phosphorylation is a major PTM (Robles et al., 2017). Two recent reports highlighted the role of phosphorylation in the regulation of sleep–wake cycles by allowing prompt modulation of protein activity (Wang et al., 2018; Brüning et al., 2019). Despite extensive research on phosphorylation in the liver, little is known about its role in the regulation of the SCN circadian rhythm.

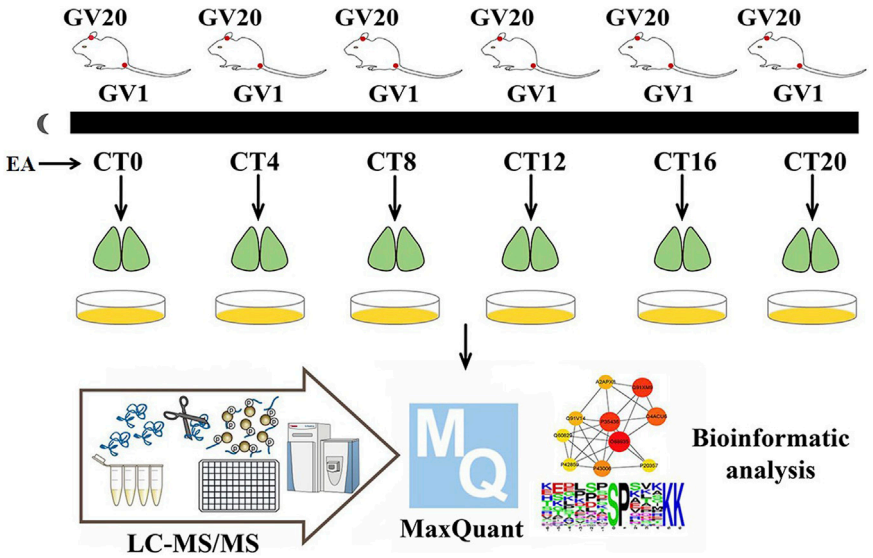

FIGURE 1 | Experimental workflow. On day 10 of constant darkness, the EA group mice were treated by EA at CT0, CT4, CT8, CT12, CT16, and CT20, respectively. The phase shift value was tested by ClockLab. Two hours post-EA stimulation, the SCN tissues were collected and iced in a quickly. Then mass spectrum data were collected, and MaxQuant was used for protein estimation.

Acupuncture is an ancient practice to treat sleep disturbances (Huang et al., 2018). Laser acupuncture can help patients with circadian rhythm disorders (Wu et al., 2009). Reportedly, electroacupuncture (EA) regulates the expression of circadian rhythm genes (*Per1* and *Per2*) in SCN under the pathological condition of morphine tolerance (Hou et al., 2018). It has been shown acupuncture treatment could improve the circadian rhythm of blood pressure (Kim et al., 2012; Yang et al., 2016). EA also decreases phosphorylation of the N-methyl-D-aspartate (NMDA) receptor subunit GluN1 of the spinal cord in a persistent pain model (Zhang et al., 2014). Traditional Chinese medicine has emphasized the circadian rhythm of diseases for a millennium. This information was used to arrange the acupuncture treatment at appropriate hours of the day (Samuels 2000). A previous study described evidence that acupuncture has different effects on fibroblast cytoskeleton remodeling at different time points (Liu et al., 2020). It could be a critical first step in chronotherapy, and analyzing the impact of acupuncture on phase synchronization within the circadian network would provide the chronotherapy tool. Therefore, in this study, we investigated the effect of EA on circadian rhythm at different circadian time points. Also, multiplex tandem mass tag (TMT)–labeling coupled with liquid chromatography–mass spectrometry (LC-MS) was used to study the rhythmic regulatory effects of EA at different time points and the mechanism of EA-associated SCN protein phosphorylation. To the best of our knowledge, this is the first phosphoproteomic study of the SCN to reveal circadian control of EA that highlights the EA contributions to phosphorylation in SCN on a system level.

MATERIALS AND METHODS

Animals and Housing

Eight-week-old male Balb/c mice were used in this study because female mice have periodical estrum cycles that show irregular activity levels. Each animal was housed in a standard cage (40 × 22 × 20 cm) with a running wheel (10 cm diameter). The wheel running data of each mouse were recorded throughout the process using ClockLab analysis software (version 3.208, Actimetrics).

Electroacupuncture Treatment

The EA group mice received EA at the corresponding circadian time points (CT0, CT4, CT8, CT12, CT16, and CT20). Acupoints GV1 (Changqiang) and GV20 (Baihui) were selected in this study (**Figure 1**). The research showed that electroacupuncture at GV1 and GV20 can restore the rats' circadian rhythm of heart rate back to normal quickly, caused by a reversed light/dark cycle (Xue et al., 2010). GV-1 is an acupuncture point located at the midpoint of the line between the tailbone and the anus (Cerreta et al., 2018). GV20 is located above the apex auriculate, in the midline of the head. After piercing, the needles were connected to the Hans acupoint nerve stimulator that sent an electric current frequency of 2/15 Hz, sparse-dense wave, and a current of 0.5 mA for 15 min. As described by Zhang et al (Zhang et al., 2020), EA mice immobilized by two Velcro brand hooks and loop fasteners; additional tapes fixed the animal to a wooden block only for the duration of EA, and the control group also received immobilization at the same period. Similar to previous proteomics studies on acupuncture (Xu et al., 2020), since there was not a disease model group, only the control group and EA group were set up. We want to investigate if EA

entrainment only once is sufficient to trigger phase shift compared to the control group under constant dark background.

Experimental Schedule

Balb/c mice were housed in an environment with free access to get food and water and 12-h/12-h light–dark cycle (7 a.m. lights on) for 10 days, constant temperature (23 ± 1°C), and humidity. A total of 72 mice were randomly divided into six time point groups (CT0, CT4, CT8, CT12, CT16, and CT20); each CT group was divided into EA and control (non-EA) groups. After 10 days of light–dark acclimatization, the animals were kept in constant darkness (dark–dark, DD) for 10 days. After all the animals were acclimatized for 10 days, those not synchronized in the light–dark cycle were eliminated. The phasing of the EA group was examined according to the Aschoff type I protocol (Aschoff 1984). All manipulations of DD mice were performed under red light (5 Lux). On day 10 of constant darkness, the EA group was treated with EA at CT0, CT4, CT8, CT12, CT16, and CT20, respectively, with six mice for each time point. ClockLab software was used to calculate the predicted activity's onset time; the actual activity onset time was day 10, following which the CT time was determined. The predicted initial activity time of each animal was determined by the linear regression equation based on the actual activity onset time. Then the phase shift value of each animal was calculated. Similar to previous studies, mice were killed by acute cervical dislocation after 2 h post-EA (Sundquist and Nisenbaum 2005; Chen et al., 2017). The brains were dissected and cut into 800-μm-thick coronal sections containing the SCN in cooled oxygenated media. The SCN region was isolated using microsurgery forceps and knives according to the Allen Mouse Brain Atlas (Chiang et al., 2014). TMT-labeled phosphorylation was applied. The protein extracts from the three SCN were mixed in equivalent amounts for two samples per time point for each time point. A total number of 24 samples were used for peptide extraction.

Protein Extraction

Mouse SCN tissues were quickly frozen in liquid nitrogen. The sample was ground into a cell powder and resuspended in four volumes of lysis buffer (8 M urea, 1% phosphorylase inhibitor cocktails, 1% protease inhibitor), followed by sonication three times on ice. The supernatant was collected by centrifugation at 12,000 *g*, 4°C for 10 min, and the protein concentration was determined using the BCA kit.

Phosphoproteome Sample Preparation

The protein solution was reduced with 5 mM dithiothreitol at 56°C for 30 min, followed by the addition of iodoacetamide to a final concentration of 11 mM and incubation in the dark for 15 min. Subsequently, the protein was digested with trypsin at 1:50 and 1:100, respectively (Huang et al., 2020; Feb 1). The peptide was solubilized in 0.5 M TEAB and labeled according to the TMT kit protocol method. One unit of the TMT labeling reagent was dissolved in acetonitrile, mixed with the peptide, and incubated at room temperature for 2 h. The Strata X C18 SPE column (Phenomenex) was used for desalting and lyophilizing peptides in vacuo. The peptide mixtures were solubilized in the buffer (50% acetonitrile/6% trifluoroacetic acid), and the supernatant was transferred to the IMAC microspheres and mixed with gentle shaking by a shaker. The buffer of 50% acetonitrile/6% trifluoroacetic acid and 30% acetonitrile/0.1% trifluoroacetic acid was used sequentially to wash the mixtures three times. Finally, the phosphopeptide was eluted in 10% NH_4OH, frozen, drained, and desalted. The reagents used in the experiment were HPLC grade.

LC-MS/MS Analysis

The peptides were solubilized in 0.1% formic acid solution and separated by the EASY-nLC 1000 UPLC system (Thermo Fisher Scientific) at a flow rate of 700 nL/min. Buffer A was composed of 0.1% formic acid and 2% acetonitrile, and buffer B was composed of 0.1% formic acid and 90% acetonitrile. The gradient was set at 4–80% for 60 min. The peptides were introduced into the NSI source for ionization and analyzed by Q-Exactive™ Plus (Thermo Fisher Scientific) mass spectrometry. The voltage was set to 2.0 kV. Both the peptide and its secondary fragments were detected using Orbitrap. The range for the mass spectrometer was 350–1800 m/z, and the secondary scan resolution was set to 35,000. The top 10 peptide ions were isolated with the highest signal intensity to enter a high-energy collisional dissociation (automatic gain control 5E4, signal threshold 5E3, maximum injection time 200 m, 28% collision energy, and dynamic exclusion time 15 s).

Database Search

Raw MS files were processed using MaxQuant software (version 1.5.2.8) on the SwissProt mouse database of 16,964 sequences with a false discovery rate (FDR). The FDR of protein identification and peptide-spectrum matches was set to <1%. The number of trypsin/P missing cleavages was set to 2, and seven amino acid residues were defined as the minimum length of the peptide. Cysteine alkylation is set as fixed modification, and variable modification is the oxidation of methionine, acetylation of the N-terminus of the protein, and the phosphorylation of serine, threonine, and tyrosine. Data are available via ProteomeXchange with identifier ProteomeXchange: PXD029450.

QC Validation of MS Data

Quality control results: The mass error of the center shaft was 0 and was found to be <10 ppm, indicating that the quality error was within the normal range. The length of the peptide was controlled at 8–20 amino acid residues, according to the law of trypsin digestion (**Supplementary Material Figure S1**).**Supplementary Figure S1** Quality of mass spectrometry data: (A) Mass error distribution of all identified peptides. (B) Length distribution of peptides.

Bioinformatics and Statistical Analysis

At least two independent biological replicates were run for each experiment. UniProt ID was used for annotation analysis. Metascape protein function and pathway annotation were applied through Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome and WikiPathways

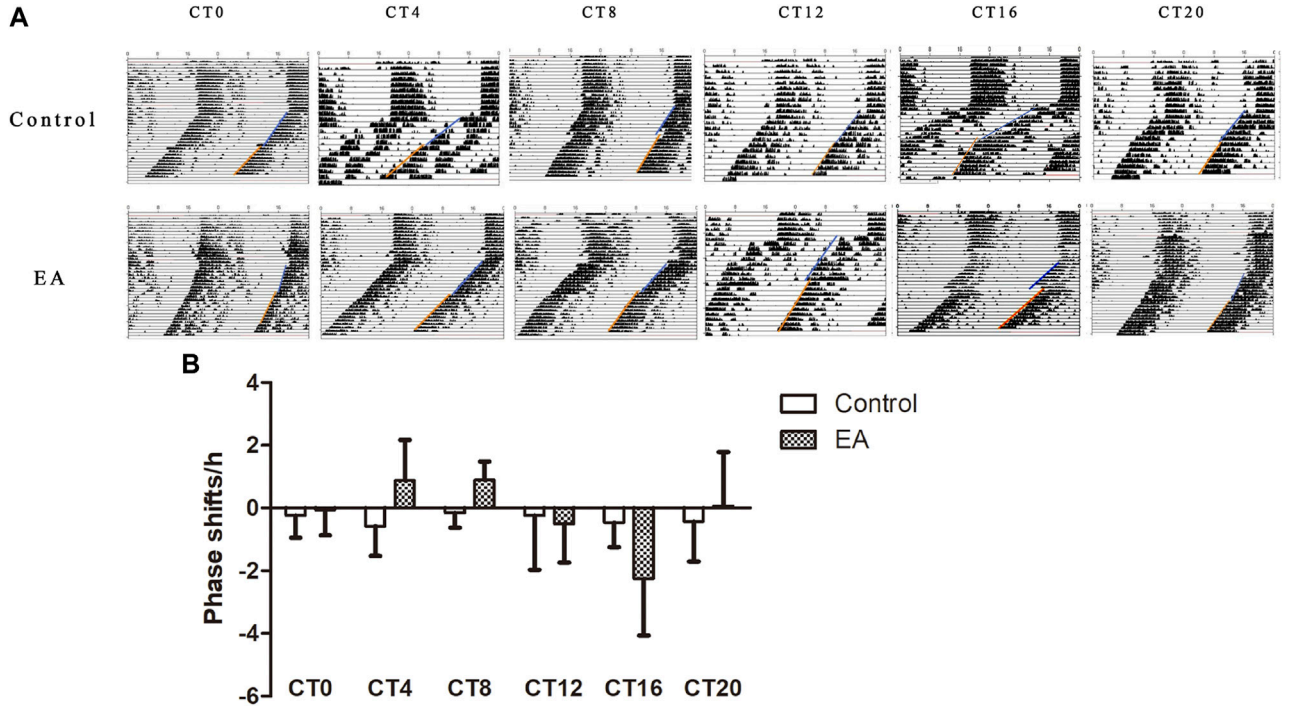

FIGURE 2 | On day 10 of constant darkness, the EA group mice received electroacupuncture at CT0, CT4, CT8, CT12, CT16, and CT20, respectively. Two acupoints, GV1 (Changqiang) and GV20 (Baihui), were adopted using standard acupuncture needles connected with HANS. Phase shifts were examined following EA. **(A)** Representative double-plotted actograms of phase shifts. **(B)** Comparison of phase shifts between control *vs*. EA at the same circadian time. Phase advances at CT4 and CT8 in the EA group (CT4 EA group $p = 0.031$, $n = 6$ mice. CT8 EA group $p = 0.007$, $n = 6$ mice) and phase delays at CT16 in the EA group ($p = 0.022$, $n = 6$ mice). One-way ANOVA data are shown as mean ± SEM, $^{*}p < 0.05$.

(Zhou et al., 2019). Enrichment analysis was performed using Fisher's exact test. Protein–protein interaction (PPI) networks were generated from the STRING database (Szklarczyk et al., 2019) and visualized with Cytoscape 3.8.0. (Shannon et al., 2003).

Immunohistochemistry (IHC)

IHC was performed as described previously (Mori and Cardiff 2016). Tissues are loaded into embedding cassettes. Then the paraffin sections were deparaffinized and rehydrated. The antigen retrieval was performed by immersing the slides in sodium citrate buffer (10 mM, pH = 6.0) and heating in a microwave oven for 10 min. After cooling, the slides were washed three times with phosphate-buffered saline (PBS) for 5 min. Endogenous peroxidase was blocked using 3% hydrogen peroxide at room temperature for 10 min, followed by washing with PBS. Then the slides were blocked with normal goat serum at room temperature for 20 min. Primary antibodies were added to each section and incubated at 4°C overnight, followed by incubation with secondary antibody at 37°C for 30 min. The tissue slide was then immersed in the DAB solution and rinsed with distilled water. IHC antibodies were obtained from Abcam. Image data were analyzed using Indica Labs (United States). All statistical analyses were performed using SPSS software 23.0 (IBM Corporation, United States). The differences in immunopositivity and staining were assessed using Student's *t*-test. All IHC tests were carried out blind.

RESULTS

Phase Shifts

Light is the strongest stimulus to regulate the circadian phase (Julius et al., 2019). To avoid potential photic confounds, the experiments were performed in the absence of light cycles to examine the impact of EA on the circadian phase. First, we determined the circadian phase shifts using the wheel running activity. It is a widely used behavioral assay with a stable phenotype (Meijer and Robbers 2014). On day 10 of constant darkness, the EA group mice were treated by EA at CT0, CT4, CT8, CT12, CT16, and CT20, respectively. During DD, the control group showed a free-running rhythm from the phase of the previous light–dark cycle (**Figure 2A**). EA causes a phase shift, and large phase advances at CT4 and CT8 in the EA group; while phase delays occur at CT16 in the EA group. No differences were observed in phase data among other groups (**Figure 2B**).

Influence of EA on Mouse SCN Phosphoproteomic Profile

In the phosphoproteomic analysis of the SCN samples, 7,605 phosphosites and 2,829 phosphoproteins were identified (**Figure 3A**). To ensure the credibility of the results, we used the standard of localization probability >0.75 to filter the identification data (Lundby et al., 2012); consequently, 6,192

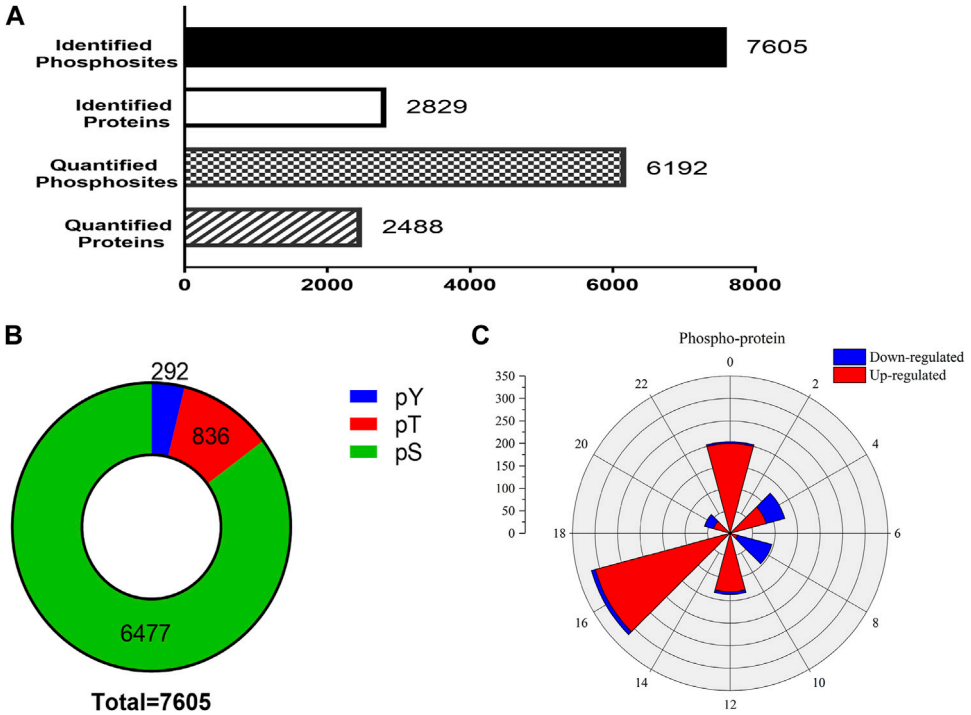

FIGURE 3 | Outline of the phosphoproteomic data. **(A)** The number of identified phosphosites and the quantification of phosphosites and phosphoproteins. **(B)** The proportions of phosphoserine (green), phosphothreonine (red), phosphotyrosine (yellow). The 7,605 phosphosites included 6,477 phosphoserine sites (85.2%), 836 phosphothreonine sites (11%), and 292 pY sites (3.8%). **(C)** Rose plots represent the phase distribution of global analysis for all quantified phosphoproteins. Red, upregulated (>1.2-fold) or blue, downregulated (<1/1.2-fold).

distinct phosphosites and 2,488 phosphoproteins were quantified (**Figure 3A**). **Supplementary Material Table S1** summarizes all identified phosphosites. Interestingly, 7,605 phosphosites included 6,477 phosphoserine (pS) sites (85.2%), 836 phosphothreonine (pT) sites (11%), and 292 phosphotyrosine (pY) sites (3.8%), which is consistent with the general pattern of phosphorylation sites in the mouse tissue (Huttlin et al., 2010). We also identified Tpd52 in samples, which were substantiated as a new synaptic protein (Biesemann et al., 2014). These results enabled further high-resolution biochemical analyses.

To check whether EA-induced phase shifts could modify the protein level of SCN, the phosphoproteins that exhibited significant differences between the control and EA groups at the same CT point were selected. Phosphorylation sites with upregulation or downregulation in two biological replicates were arranged. The differences in phosphosites were examined at a 1.2-fold change quantitative ratio as the threshold (Li et al., 2020). The up- and downregulated phosphosites in the EA were compared to the control at different time points. During EA entrainment, the mice showed different resetting patterns of activity initiation between various groups. Interestingly, scanning of the phosphorylation properties in the SCN revealed a significantly different number of proteins grouped into two main segments. We found that most downregulated proteins clustered in CT4 and CT8, while CT16 showed that upregulated proteins were dominant (**Figure 3C**). These results suggested that EA has significant effects on the phosphorylation pattern of SCN proteins.

Analysis of Functional Annotations Illustrated that EA Group Proteins Participate in Postsynaptic and Glutamatergic Synapses and have High Levels of Phosphorylation

To identify the SCN-related molecules post-EA, we assessed the unique features defining CT4, CT8, and CT16 groups because CT4, CT8, and CT16 electroacupuncture induced phase shift. Next, the phosphoprotein that exhibited significant differences between the EA and control SCN tissues was subjected to the GO, KEGG, Reactome, and WikiPathways analyses presented in the heat map format. Fisher's exact test was used to assess CT4, CT8, and CT16 differentially modified proteins based on the quantified outcome. The phosphoproteins upregulated in the SCN of EA mice were enriched in pathways, GO cellular components, GO molecular function, and GO biological process with respect to post- and glutamatergic synapses. Typically, the proteins including Grin2a, Map1a, and Vps35, with >1.5-fold change in the SCN of EA mice, are postsynaptic (**Supplementary Material Table S2**). Most postsynaptic proteins such as NMDAR, mGluR (Grm1), CAMK2 (Camk2b and Camk2d), Shank (Shank1 and Shank3), SynGAP (Syngap1), and nNOS (Nos1) showed upregulated expression between EA and control. The

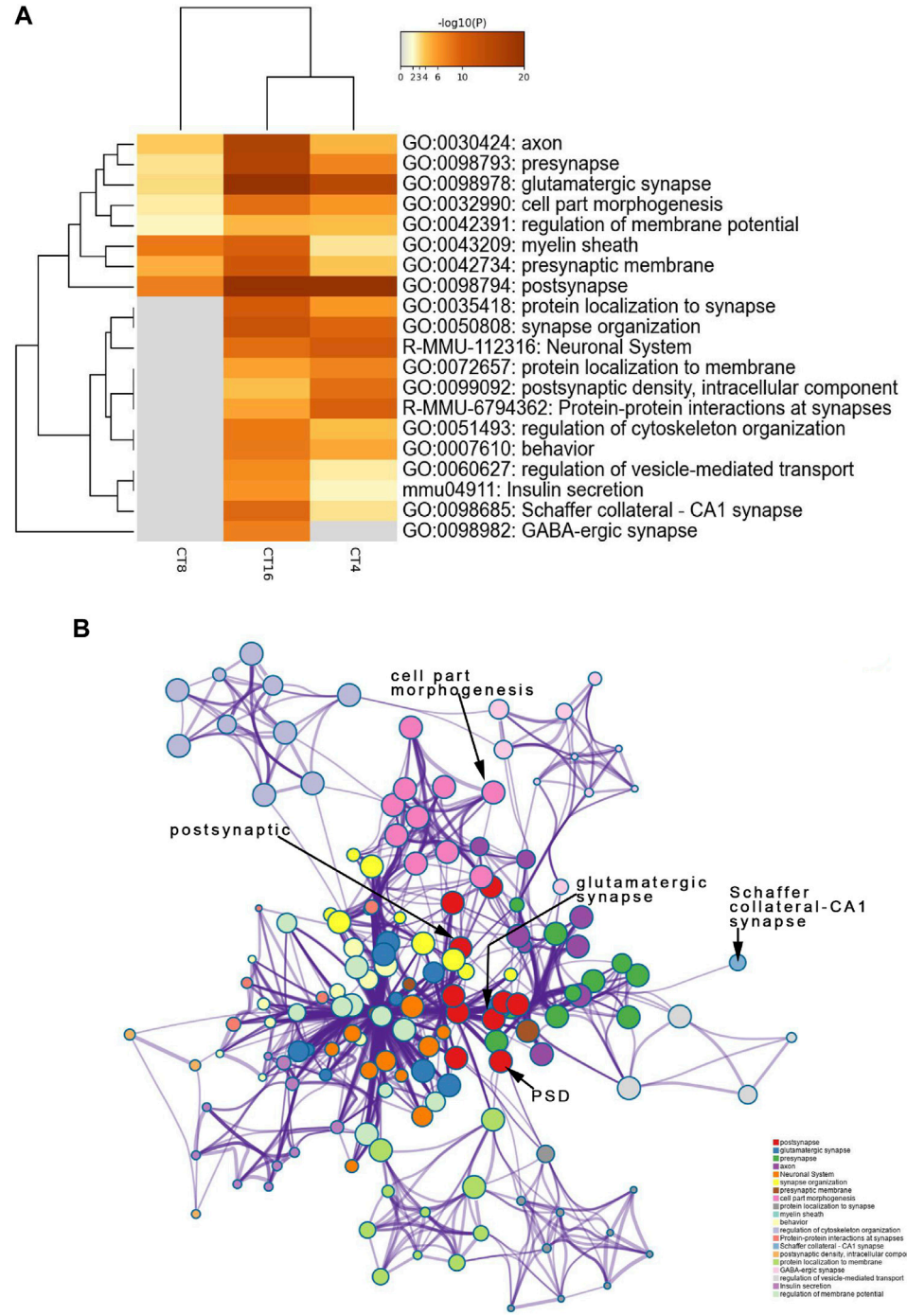

FIGURE 4 | Functional annotation and pathway enrichment analysis of proteins present significantly higher levels in the SCN of the EA group than the mice of the control group. The results are from annotation analysis by Metascape. Metascape selects a subset of representative terms from the full cluster and converts them into a network layout. **(A)** Top 20 items of KEGG pathways, reactome pathway, and GO terms are at a high level in EA. A high -log10 (*p*-value) indicates enrichment. The darker the color, the statistically significant will be the enrichment. **(B)** A higher expression level and an abundance of GO terms in the EA group are represented as networks. Similar terms are organized into groups and colors according to the representative functions of the group. The higher the similarity, the thicker will be the line between the circles. The more similar the function, the tighter will be the connection between the circles. See **Supplementary Table S3** for complete list of enriched GO terms.

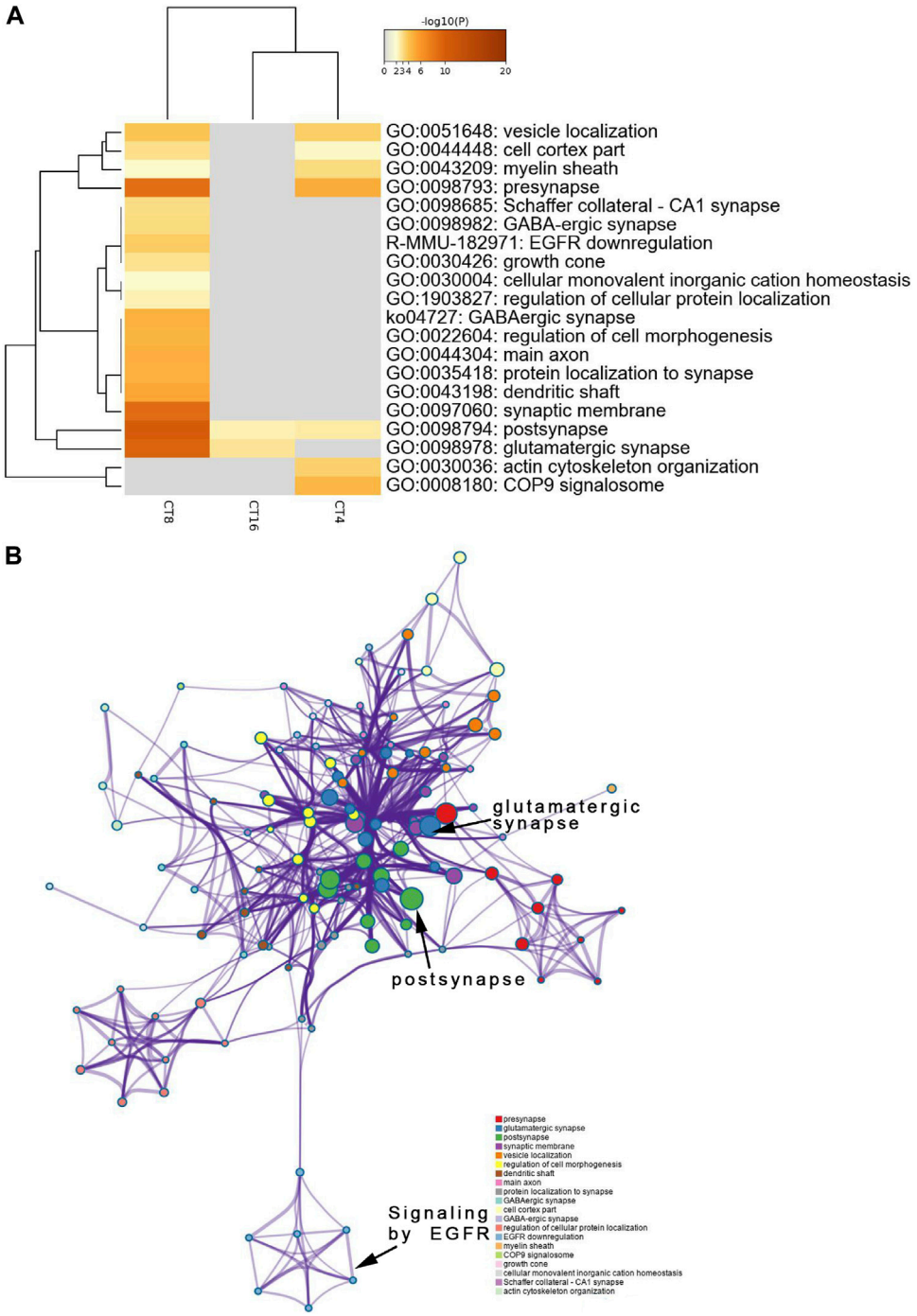

FIGURE 5 | Functional annotation and pathway enrichment analysis of proteins present significantly lower levels in the SCN of the EA group than the mice of the control group. The results are from annotation analysis by Metascape. Metascape selects a subset of representative terms from the full cluster and converts them into a network layout. **(A)** A low expression level and abundance of GO terms in the EA group are represented as networks. Similar terms are organized into groups, and the color is according to the representative functions of the group. The higher the similarity, the thicker will be the line between the circles. The similar the function, the tighter will be the connection between circles. A higher -log10 (*p*-value) indicates more enrichment (Fisher's exact test). The darker the color, the more statistically significant will be the enrichment. See Supplementary Table S4 for complete list of enriched GO terms.

differentially expressed phosphoproteins focused on postsynaptic plasticity. The NMDA receptor and CAMK2 are strongly implicated in mediating synaptic plasticity (Bayer et al., 2006). CAMK2/NR2B interaction effect is postsynaptic (Barcomb et al., 2016). Only CAMK2β has the morphogenic activity that regulates the extension and fine dendrites and the number of synapses

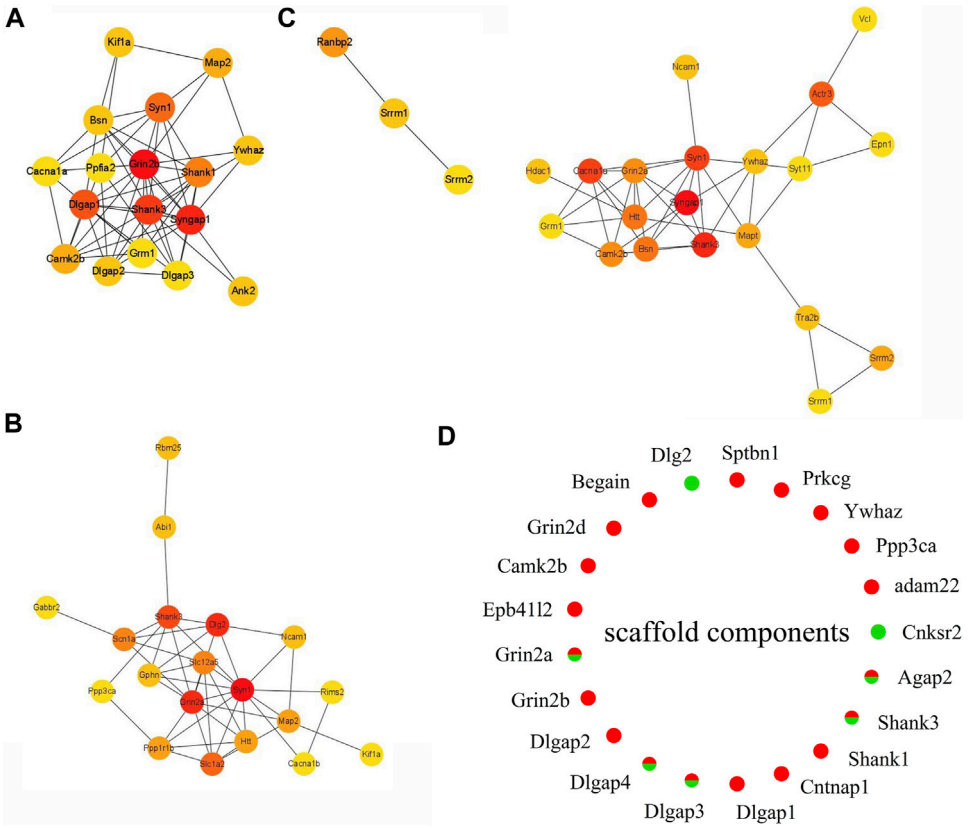

FIGURE 6 | Expression heat map of top 20 hub proteins. **(A)** EA-CT4 *vs* Con-CT4. **(B)** EA-CT8 *vs*. Con-CT8. **(C)** EA-CT16 *vs*. Con-CT16. **(D)** Extracted and assessed upregulation and downregulation status of the phosphorylation of the core scaffold proteins. Red, upregulated (>1.2-fold); green, downregulated (<1/1.2-fold).

(Fink et al., 2003). The phosphorylation of CAMK2β in S367 is correlated to the increased phosphorylation of Grin2b, also known as NR2B, which is the substrate of CAMK2 (Omkumar et al., 1996). The phosphorylation level of proteins, associated with Schaffer collateral–CA1 synapse and postsynaptic density (PSD), was also increased in CT4 and CT16. Consistent with the upregulation of proteins involved in excitatory synapses, the long-term potentiation (LTP) was also higher in SCN (**Supplementary Material Table S3**). The best example of plasticity is LTP, which requires the action of glutamate receptor NMDAR to be enriched in the Schaffer collateral (Lüscher and Malenka 2012). The changes in the PSD of excitatory synapses are essential for LTP (Li et al., 2016). Interestingly, this function of mediating the PPIs at synapses suggested that EA at CT4 and CT16 induces LTP for network clusters (**Figure 4B**).

Downregulated Phosphorylation Proteins Involved in Postsynapses, Dendrites, and Neuronal Systems

The decreased phosphosites were subjected to pathway analyses. Proteins in postsynapse categories including Cnksr2, PCLO, Ccm2, and Ppfia2 were present at > 1.5-fold levels (**Supplementary Material Table S2**) and known as scaffold proteins (Lim et al., 2014; Miura et al., 2017). The decrease in PCLO corresponds to the downregulation of EGFR in EA compared to the control (**Figure 5B**). PCLO promotes EGFR-dependent signaling (Zhang et al., 2017). Interestingly, several sites in the CT8 group showed decreased phosphorylation after EA compared to the CT4 and CT16 groups. Glutamatergic synapse was significantly downregulated in CT8, which included Abi1 (S225), Cacna1b (S2221), Grin2a (S929), Ncam1 (T1030), Slc1a2 (S542), Dlg2 (Y364), Shank3 (S995), Rims2 (S965), and Gabbr2 (T762). These proteins exhibited maximal connectivity at CT8 in PPI analysis (**Figure 6B**). Other proteins at CT8 present at significantly lower levels were involved in the γ-aminobutyric acid (GABA)ergic synapse pathway, including Cacna1b, Gng12, Slc12a5, Gabbr2, and Gephyrin. Gephyrin proteins are located in the GABAergic synapse. Moreover, Gephyrin is a major factor that anchors and stabilizes GABA receptors on the synapses of the brain (Choii and Ko 2015). In addition, Slc4a4 was identified in only CT8 at > 1.5-fold lower levels in SCN (**Supplementary Material Table S2**). Also, S257 phosphorylation of Slc4a4 was crucial for transport activity (Khakipoor et al., 2019). The upregulated and downregulated phosphor-proteins were

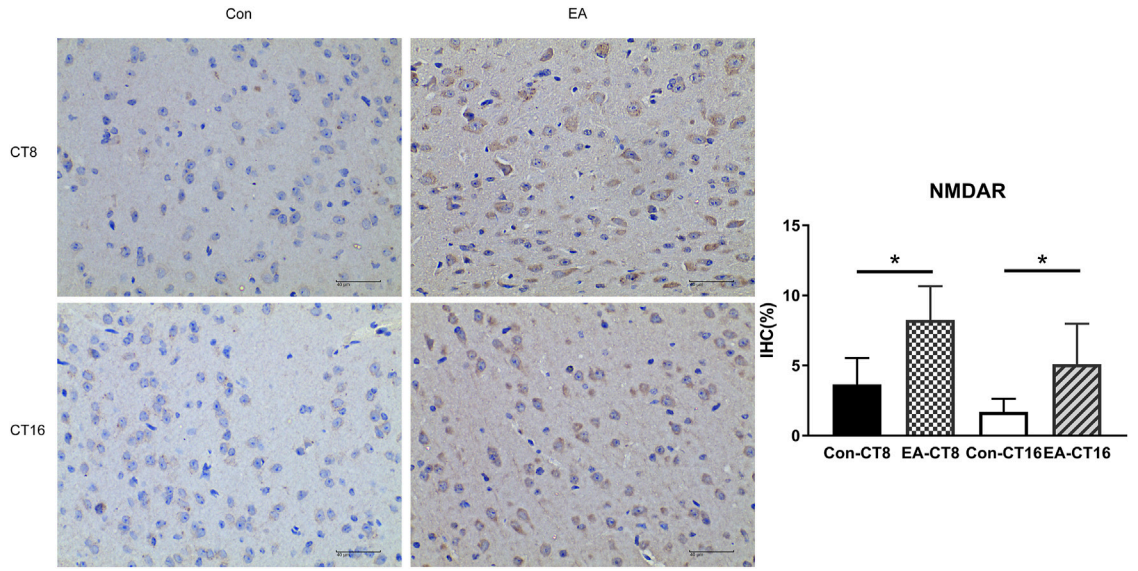

FIGURE 7 | Representative IHC images of NMDAR. All the tissues were observed at ×400. Halo 101 data analysis system was applied to calculate the percentage of positive area of each image. The positive expression was brown. Compared to the control group, the NMDAR in the SCN of the EA group was significantly increased (CT8 EA group $p = 0.01$, CT16 EA group $p = 0.037$); $n = 5$ mice each group, Student's t-test, $^*p < 0.05$.

consistent in many terms in the enrichment analysis such as post- and glutamatergic synapses (**Figure 4A** and **Figure 5A**).

PPI analysis shows that the phosphorylation of high–connectivity degree core scaffolding proteins indicate synaptic plasticity. In the PPI networks, the most enriched three-node proteins with high connectivity degree between EA-CT4 and Con-CT4 groups are shown as red nodes (**Figure 6A**): Grin2b, Syngap1, Shank3, Syngap1, and Neurabin-II (PPP1R9B) (**Supplementary Material Table S1**) show increased phosphorylation; both play critical roles in synaptic plasticity, LTP, and learning (Siino et al., 2018). SynGAP is abundant in the PSD and is also known to be a synaptic substrate for CAMK2 (Kennedy 2000). Syn1, Grin2a, and Dlg2 are the three most enriched proteins with the highest contact between the EA-CT8 and Con-CT8 groups (**Figure 6B**). Syngap1, Shank3, and Syn1 exhibited maximal connectivity between the EA-CT16 and Con-CT16 groups (**Figure 6C**). Synapsin 1 is widely used as a sensitive indicator of synaptogenesis and is the highly connected hub node in PPI networks (Mundy et al., 2008). We found that Synapsin 1 was upregulated in CT4 and CT16 and downregulated in CT8, which is consistent with the results of functional annotation analysis.

According to PPI analysis, structural proteins including cytoskeletal (microtubule-associated protein 2, MAP2), scaffold (Shank1, Shank3, Dlg2, Dlgap1, Dlgap2, Dlgap3, Dlgap4), cell adhesion (Ncam1), and 14-3-3 molecules (Ywhaz) (**Figures 6A–C**) are common factors (Li, Wilkinson, Clementel, Hou, O'Dell and Coba 2016). DLGs, DLGAPs, SHANKs, and SynGAP1 are considered PSD core scaffold components that interact with various PSD scaffold proteins. To evaluate the changes in PSD, we extracted the phosphorylated scaffold proteins in SCN and marked them in a circle map (**Figure 6D**). The most identified scaffold proteins (19/21) are upregulated, while five are downregulated (Grin2a, Dlgap3, Dlgap4, Shank3, and Agap2). The phosphorylation levels of Grin2a, Shank3, Dlgap3, Dlgap4, and Dlg2 are decreased at CT8. We also found that the most closely linked proteins in the PPI networks were the core scaffold proteins. Taken together, these data indicated that compared to control, EA is associated with the phosphorylation of core scaffold proteins, and the expression changes are primarily postsynaptic. Furthermore, these phosphoproteins are correlated with synaptic plasticity.

Validation of TMT-Based Results

The target proteins, NMDAR and CaMK2, were selected for verification using IHC. Compared to the control and the EA groups at the same CT time point, IHC revealed that the changes in NMDAR and CAMK2 were significant. Significant differences were noted in NMDAR (**Figure 7**) and CAMK2 (**Figure 8**) between the EA-CT8 and Con-CT8 groups and between EA-CT16 and Con-CT16 groups. IHC also showed that the two candidate proteins had trends similar to those observed in the TMT results, supporting the proteomics data.

DISCUSSION

To the best of our knowledge, these findings are novel. The current study provides a glimpse at the effects of EA-induced phase shift on the phosphoproteomic patterns in constant darkness in mice SCN. Most studies on proteomics responses to acupuncture have focused on analgesia and

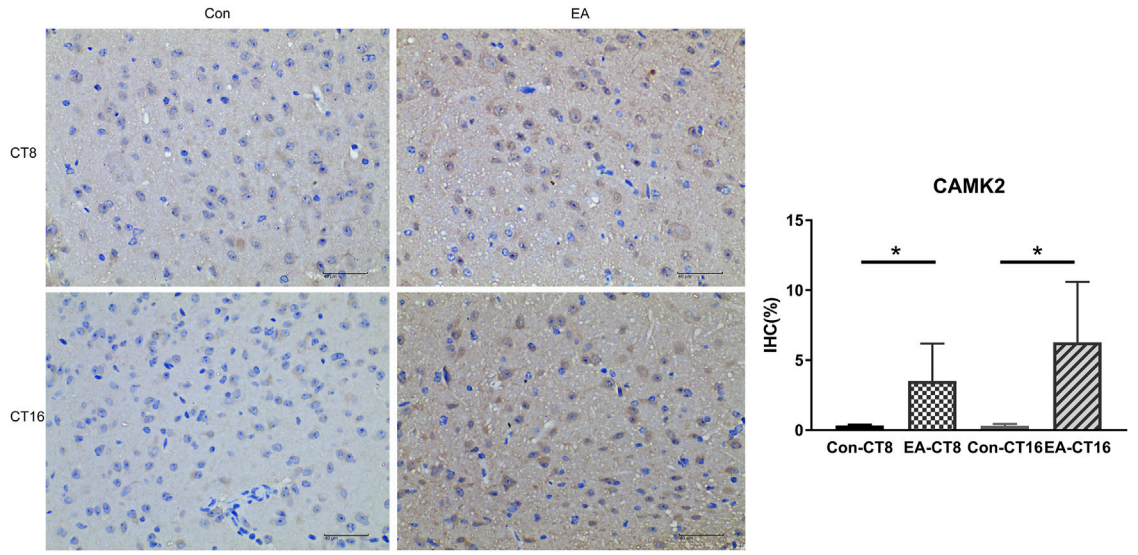

FIGURE 8 | Representative IHC images of CAMK2. All the tissues were observed at ×400. The positive expression was brown. Compared to the control group, the CAMK2 in the SCN of the EA group was significantly increased (CT8 EA group $p = 0.029$, CT16 EA group $p = 0.036$); $n = 5$ mice each group, Student's t-test, $^*p < 0.05$.

neuroprotective mechanisms (Sung et al., 2004; Yang et al., 2018). Only one study has attempted to identify the phosphoproteins of EA in an inflammatory pain model (Lee et al., 2012 Jul). In this study, we investigated the effect of EA at various time points on the characteristics of mice circadian rhythm in the absence of an external light–dark cycle. EA causes phase advances during the subjective day and delays during the subjective night (**Figure 2B**). Our data demonstrated that EA treatment elicits partial reparation of circadian rhythm exposed to constant darkness. The phase of EA changes shows a bidirectional effect in mice under constant darkness.

Reportedly, EA regulates the expression of circadian rhythm genes (*Per1* and *Per2*) in SCN under the pathological condition of morphine tolerance. However, no differences were observed in the expression levels of *Per1* and *Per2* between the wild-type (WT) control group and EA group (Chen et al., 2017; Hong et al., 2018), so we performed further analysis. Phosphorylation events correspond to phase shifts of circadian rhythms, with both advances and delay (Golombek et al., 2004). Here, phosphoproteomics and PPI analysis showed complicated changes in proteins critical to synaptic integrity and function in the SCN. The comparison of individual phosphor-protein changes at each time point revealed that distinct phosphorylation sites were up- or downregulated in SCN at the different time points (**Figure 3C**). These data indicated that phosphorylation might be a key mechanism for EA-induced resynchronization patterns. The GO analysis showed that the term "postsynaps" in all CT groups was significantly enriched in cellular components, which might be the general efficacy of EA. Since CT4 and CT8 found that the biological process (BP) term was related to cell morphogenesis, and the BP term of CT16 involved regulation of neurotransmitter transport, and these three unique enrichment terms were not found in other groups without phase shift, which may indicate the specific role of phase regulation in EA. The synaptic molecular functions related to sleep–wake cycles are mainly enriched in glutamatergic and GABAergic synapses (**Figure 4A** and **Figure 5A**) (Brüning et al., 2019). Glutamate and GABA levels play a notable role in the adjustment of the sleep–wake cycle in SCN, thereby confirming that the glutamatergic and GABAergic synapse pathways are involved in EA.

Furthermore, we determined that differential phosphorylation of proteins involved in postsynapse in the SCN of EA compared to the control mice. The most enriched functional groups represented structural proteins involved in the regulation of cell morphogenesis and synaptic plasticity (**Figure 4A** and **Figure 5A**). PPI analysis showed that the candidate proteins, NR2A, NR2B, Shanks, and Syngap1 exhibited the highest connectivity degree between the EA and control groups. The NMDA receptor and CAMK2 may serve as signal transduction molecules and synaptic structural components in PSD (Chen et al., 1998; Ehlers 1999). PSD plays a critical role during synaptic plasticity. After 2 weeks of acupuncture, PSD was significantly increased, and the synapse cleft width was narrowed in ischemia/reperfusion rats (Xia et al., 2017 Apr). Conversely, our phosphoproteomics data demonstrated that EA entrainment only once is sufficient to trigger changes in the phosphorylation of the component proteins of the postsynaptic density in SCN. Next, we extracted the scaffold proteins in SCN to explore the changes of proteins in PSD. Similar to the results of function annotation analysis, most identified scaffold proteins were upregulated. As evidence for

synaptic plasticity, hub proteins are termed "scaffold interactors."

Although the neural and the molecular basis of mammalian rhythms have been elucidated, the practical tools to rapidly adjust the clock to accommodate shift work and trans-meridian jet travel are still lacking. Reportedly, the effect of EA in the phase shifts would provide the chronotherapy tool. The deficiency of this study lies in that no corresponding dose-related research is carried out. For the parameter setting of EA, it refers to previous research. In future studies, we will focus on dose-related responses to different EA intensity research and the role of glutamatergic and GABAergic synapses and neurotransmitters in EA-induced transmission and synaptic plasticity. Therefore, the current study would provide new insights to clarify the role of these proteins and phosphosites in SCN, especially related to synaptic plasticity.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data presented in the study are publicly available in ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) *via* the iProX partner repository with the dataset identifier PXD029450.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare Officer of the Chengdu University of Traditional Chinese Medicine.

AUTHOR CONTRIBUTIONS

XL drafted the manuscript and analyzed the data. MZ performed most of the experiments. NL performed animal breeding and part of the animal experiments. CZ performed and supervised the study. DC and ZZ, provided suggestions on this study, and revised the article. All authors contributed to the study and approved the submitted manuscript version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.762557/full#supplementary-material

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Organoids as Model Systems to Investigate Circadian Clock-Related Diseases and Treatments

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Circadian rhythms exist in most cell types in mammals regulating temporal organization of numerous cellular and physiological processes ranging from cell cycle to metabolism. The master clock, suprachiasmatic nucleus (SCN) in the hypothalamus, processes light input and coordinates peripheral clocks optimizing organisms' survival and functions aligning with external conditions. Intriguingly, it was demonstrated that circadian rhythms in the mouse liver can be decoupled from the master clock under time-restricted feeding regimen when food was provided during their inactive phase. Furthermore, mouse liver showed clock-controlled gene expression even in the absence of the master clock demonstrating independent functions of peripheral clocks apart from the SCN. These findings suggest a dynamic relationship between the master and peripheral clocks and highlight potential functions of peripheral clocks independent of the master clock. Importantly, disruption of circadian rhythms correlates with numerous human ailments including cancer and metabolic diseases, suggesting that diseases may be exacerbated by disruption of circadian rhythms in the SCN and/or peripheral clocks. However, molecular mechanisms providing causative links between circadian rhythms and human diseases remain largely unknown. Recent technical advances highlighted PCS- and tissue-derived 3-dimensional organoids as *in vitro* organs that possess numerous applications ranging from disease modeling to drug screening. In this mini-review, we highlight recent findings on the importance and contributions of peripheral clocks and potential uses of 3D organoids investigating complex circadian clock-related diseases.

Keywords: circadian rhythms, organoids, circadian medicine, chronotherapy, circadian drug screening

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INTRODUCTION

Circadian rhythms are periodic events that occur with a period of approximately 24 h regulating temporal organizations of diverse cellular and physiological processes such as metabolism, immune response, and sleep/wake cycles. In the past four decades, extensive research uncovered detailed molecular mechanisms of circadian rhythms in several eukaryotic model organisms including *Neurospora crassa, Arabidopsis Thaliana*, *Drosophila melanogaster*, and *Mus musculus* (Dunlap and Loros, 2017; Cox and Takahashi, 2019; Creux and Harmer, 2019; Patke et al., 2020), and demonstrated that eukaryotic systems possess conserved molecular blueprint that consist of time-delayed transcriptional-translational feedback loop (TTFL) (Hurley et al., 2016), which generates autonomous circadian oscillations even at single cell level (Nagoshi et al., 2004). The endogenous circadian clocks enable organisms to process external time cues such as light/dark cycles, and coordinate temporal organization of physiological processes anticipating recurring daily events.

Comparative analyses of circadian clock mechanisms in different organisms have been previously reviewed (Hurley et al., 2016; Saini et al., 2019).

Briefly, molecular mechanisms of mammalian circadian machinery involve heterodimeric basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) domain containing transcription factors, CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle Arnt-like protein-1), which serve as positive elements activating negative elements, *Period* (*Per1, Per2, Per3*) and *Cryptochrome* (*Cry1, Cry2*) genes. CLOCK/BMAL1 binds to E-box motifs on target gene promoters including other transcription factors, which regulate a large number of clock-controlled genes (CCGs) (Takahashi, 2017). PER and CRY proteins form complexes, and translocate into the nucleus where they inhibit CLOCK/BMAL1 forming a time-delayed negative feedback loop. PER and CRY undergo progressive phosphorylations and subsequent ubiquitination and degradation (Reischl et al., 2007; Siepka et al., 2007; Yoo et al., 2013), which relieves the repression on CLOCK/BMAL1. Nuclear receptors, RORs (RORα, RORβ, RORγ) and REV-ERBs (REV-ERBα, REV-ERBβ), form additional feedback loops with BMAL1 regulating circadian rhythms and metabolism (Preitner et al., 2002; Cho et al., 2012). Molecular mechanisms of mammalian circadian rhythms have been extensively reviewed previously (Partch et al., 2014; Cox and Takahashi, 2019; Rosensweig and Green, 2020).

In mammals, the suprachiasmatic nucleus (SCN) within the hypothalamus serves as the master clock coordinating peripheral clocks in different parts of the body (Ralph et al., 1990). The SCN contains approximately 20,000 neurons that generate synchronized oscillations *via* intercellular signaling and orchestrate rhythmic physiological activities. Light is one of the strongest external time cues that travels through rods, cones, and intrinsically photosensitive retinal ganglion cells (ipRGCs) to the SCN *via* retinal innervation inducing phase shift and entrainment of circadian rhythms (Lazzerini Ospri et al., 2017). Either genetic or physical ablation of circadian rhythms in the SCN abolishes rhythmic behavioral activities and disrupts tissue-specific clock functions in peripheral organs (Ralph et al., 1990). Intriguingly, mice that are forced to eat only during their inactive phase results in food-induced phase resetting of circadian rhythms in peripheral organs (e.g., liver, kidney, heart, etc.) decoupling the phase relationship between the master and peripheral clocks (Damiola et al., 2000). Current understanding of the SCN such as intercellular coupling, light input signaling pathways, and hierarchical regulation of peripheral clocks by the SCN have been extensively reviewed elsewhere (Lazzerini Ospri et al., 2017; Hastings et al., 2019). In this mini-review, we focus on the importance of peripheral clocks and potential uses of 3-dimensional organoids addressing roles of circadian rhythms in complex human diseases.

PERIPHERAL CLOCKS

Genome-wide analysis of mouse and human gene expression profiles demonstrated peripheral organ-specific CCGs uncovering organ-specific functions of circadian rhythms. Specifically, the transcriptome of 12 mouse organs showed that up to approximately 40% of protein coding genes are CCGs (Zhang et al., 2014). And more recently, gene expression analysis of 13 human tissues using the cyclic ordering by periodic structure (CYCLOPS) algorithm uncovered tissue-specific regulation of CCGs including numerous drug target genes, which highlighted the importance of circadian rhythms in drug treatment and response (Ruben et al., 2018). Tissue-specific regulations of CCGs involve interactions of circadian clock transcription factors with tissue-specific transcription factors (Meireles-Filho et al., 2014) and tissue-specific chromatin loops enabling tissue-specific rhythmic gene expression profiles (Yeung et al., 2018). It is important to note that the above data is a result of an integrated output from both systemic and peripheral clocks. Peripheral organs receive temporal cues from the SCN *via* humoral and neuronal signals, which enables them to align and coordinate tissue-specific functions with the master clock.

Surprisingly, it was discovered that peripheral tissues can directly process external time cues such as light, temperature, and nutrient information regulating entrainment of tissue-specific circadian rhythms and CCGs. Specifically, temperature cycles entrain circadian rhythms in the liver and cultured fibroblasts *via* heat-shock factor 1 (HSF1) (Brown et al., 2002; Saini et al., 2012). Low-amplitude endogenous body temperature cycles were sufficient to induce temperature-dependent alternative splicing that could potentially regulate a large number of gene expression in the mouse liver (Preussner et al., 2017). Intriguingly, transcriptome profiles in the epidermis and the liver show CCGs in the absence of circadian rhythms in other tissues including the SCN under light-dark cycle (Koronowski et al., 2019; Welz et al., 2019), which suggest light entrainment of peripheral clocks independent of the SCN. Furthermore, the mouse liver clock maintains synchronized oscillations in the SCN-lesioned mice and "hepatocyte clock-only mice" even in the absence of external time cues, which indicates that peripheral clocks could function independent of the SCN (Sinturel et al., 2021). In addition, neuropsin (OPN5) and encephalopsin (OPN3) regulate entrainment of murine skin (Buhr et al., 2019) and light sensing of adipocytes (Nayak et al., 2020) providing evidence of direct photosensing pathways in peripheral systems.

Nutrient is another strong external cue that could alter the period and phase of canonical clock genes and the composition of CCGs in peripheral organs. High-fat diet (HFD) induces diet-induced obesity and disrupts circadian rhythms in mice. HFD lengthens the free running period of circadian rhythms, reduces amplitude of canonical clock genes in fat and liver, and changes diurnal profiles of key metabolic genes in hypothalamus, fat, and liver (Kohsaka et al., 2007). Furthermore, HFD induces reorganization of CCGs in the mouse liver resulting in both loss and gain of oscillating transcripts compared to normal chow diet indicating that the composition of CCGs is dynamic (Eckel-Mahan et al., 2013). Intriguingly, mice that are fed HFD for 8 h during their active phase, time-restricted feeding (TRF), did not experience obesity nor other metabolic conditions that are commonly observed in mice that are fed HFD ad libitum

(Hatori et al., 2012). It is important to note that TRF entrained the liver clock and metabolic components to provide protection against HFD highlighting the importance of peripheral clock function (Hatori et al., 2012). The above supporting data underscores the importance of peripheral clocks, dynamic relationship between the master and peripheral clocks, and potential impact of disrupted peripheral clocks in human diseases.

Aforementioned mouse models that enable tissue-specific rescue of circadian rhythms will be instrumental to uncover peripheral organ-specific functions of circadian rhythms. However, it is still difficult to determine functions of peripheral clocks in human subjects and how those functions may change as human diseases emerge with conventional experimental models. Recent advancements of patient-derived 3-dimensional organoids indicate that human organoid models will play a critical role determining functions of peripheral clocks, remodeling of clock-controlled processes in different diseases, and circadian target genes for potential chronotherapy.

ORGANOIDS TO INVESTIGATE CLOCK-RELATED COMPLEX HUMAN DISEASES

Previous findings indicated correlation between cancer and expression of core clock genes in breast, prostate, pancreatic, liver, colorectal, and lung cancer. For example, reduced expression of *Period* (i.e., *Per1*, *Per2*, and/or *Per3*) genes have been observed in sporadic breast tumors (Winter et al., 2007), hepatocellular carcinoma tissues (Lin et al., 2008), and colon cancer cells of primary colorectal tumors (Mostafaie et al., 2009). On the other hand, overexpression of *Per2* and downregulation of *Bmal1* has been shown to inhibit growth of human pancreatic cancer cells and glioblastoma stem cells, respectively (Oda et al., 2009). Furthermore, human cancers show altered circadian rhythms (Shilts et al., 2018), but consequences of human diseases impacting the core circadian machinery and clock-controlled signaling pathways remain largely unknown.

One of the limitations of investigating human disease-specific mechanisms of circadian rhythms is the lack of human model systems that enable diseased tissue-specific investigations of altered circadian rhythms and clock-controlled signaling pathways. Transformed homogenous 2D human cell lines such as HeLa and U2OS provide cost effective access to human cells but do not possess multicellular characteristics of *in vivo* organs and cannot model/represent disease-specific alterations. Furthermore, previous studies using NIH3T3 and U2OS cells demonstrated that they possess less than a dozen rhythmic genes (Hughes et al., 2009), which suggested that transformed cell lines are not ideal systems investigating disruption of clock-controlled signaling pathways. Recently, human gene expression data from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) have been used to extract rhythmic signatures using cyclic ordering by periodic structure (CYCLOPS) algorithm (Anafi et al., 2017). However, CYCLOPS requires a minimum of 250 samples to identify rhythmic genes, and still requires human *in vitro* systems to test potential chronotherapeutic regimens for applications of translational circadian medicine. In this regard, patient tissue-derived organoids present as attractive models that enable disease-specific interrogation of remodeling of clock-controlled genes and signaling pathways and *in vitro* validations of chronotherapeutic regimens.

Organoids are *in vitro* multicellular culture systems mimicking cellular and physiological functions of specific organs depending on the source and development of those 3D cultures. Organoids can be derived from either pluripotent stem cells (PSCs) or adult stem cells (ASCs). Human PSCs can be used to derive different types of human organoids including nerve/sensory (brain, optic/retina) (Meyer et al., 2011; Lancaster et al., 2013), endocrine/gland (thyroid, mammary gland, prostate, fallopian tube, endometrium) (Ma et al., 2015; Qu et al., 2017; Yucer et al., 2017; Hepburn et al., 2020; Cheung et al., 2021), respiratory/cardiovascular (lung, heart, blood vessel) (Dye et al., 2015; Wimmer et al., 2019; Drakhlis et al., 2021), and gastrointestinal tract/digestive (stomach, liver, pancreas, small intestine, colon, kidney) (Spence et al., 2011; Mccracken et al., 2014; Takebe et al., 2014; Boj et al., 2015; Morizane et al., 2015; Munera et al., 2017) *via* directed differentiation. However, PSC-derived organoids demonstrate immature, fetal-like, characteristics, which require additional developmental processes to obtain mature organoids. For example, human intestinal organoids (HIOs) derived from PSCs do not possess a full spectrum of markers for mature epithelial cell types, and these HIOs can be matured by surgical implantation into the kidney capsule of immunocompromised non-obese diabetic/*scid*/IL2R γ^{null} (NSG) mice (Watson et al., 2014; Finkbeiner et al., 2015). After 3 months of maturation in mice, human intestinal crypts can be isolated and cultured to derive mouse kidney capsule-matured human intestinal enteroids (kcHIEs).

Alternatively, mouse or human tissue can be used to generate organoids from ASCs that recapitulate organotypic characteristics corresponding to the tissue that was used to derive organoids. For example, mouse intestinal organoids or enteroids can be derived from the leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) positive intestinal stem cells. Lgr5+ stem cells isolated from the mouse small intestine can proliferate, differentiate, and organize into 3D enteroids that consist of a single intestinal epithelial cell layer with distinct regions of crypt and villus domains. Analogous to mouse enteroids, ASC-containing patient biopsy-derived human intestinal enteroids (bHIEs) possess a wide spectrum of differentiated cells that reflects the maturity of enteroids derived from ASCs in contrast to fetal-like PSC-derived HIOs. Reviews on PSC- and ASC-derived organoids have been extensively reviewed elsewhere (Rookmaaker et al., 2015; Bartfeld and Clevers, 2017; Schutgens and Clevers, 2020; Lesavage et al., 2022).

Both PSC- and ASC-derived human organoids provide attractive platforms revealing patient- and/or disease subtype-specific differences for disease modeling and drug screening for personalized precision medicine. Broutier et al. (2017) established cancer organoids derived from primary liver cancer (PLC) tissue

and demonstrated the heterologous expression of cancer markers depending on PLC subtypes including hepatocellular carcinoma, cholangiocarcinoma and the combined tumor. Despite gene mutations commonly found in tumor tissue, gene set enrichment analysis (GSEA) and next-generation sequencing (NGS) analysis revealed that each subtype of PLC exhibited transcriptomic alterations in gene mutations. Accordingly, they identified candidates of biomarkers for drug screening tests as therapeutic agents for PLC subtypes. Seidlitz et al. (2019) used normal and tumorigenic gastric organoids from patients, and analyzed whole genome sequencing to investigate a broad mutational spectrum from different types of cancer (Seidlitz et al., 2019). The analysis demonstrated significantly high variance in the mutation rate per megabase pair among the patient-derived organoids (PDOs) implying the diversity of pathways and response to treatments. Sachs et al. (2019) established biopsy-derived airway organoid from lung cancer patients and the sequencing analysis determined mutations of *ALK*, *EMR1*, *MDN1*, *STK11*, *TP53*, *KRAS*, and *ERBB2*, which are tumor-related genes (Sachs et al., 2019). Importantly, they also examined differential response to anti-cancer drugs in the airway organoids possessing different mutations, implying that different strategies of treatment are necessary depending on the lung cancer subtypes. Hubert et al. (2016) established the protocol of patient-derived glioblastoma cancer stem cell organoids, and subsequently demonstrated that the alteration of lipid metabolism by upregulation of fatty acid desaturase in glioblastoma organoids, implying that metabolism may vary among different subtypes (Hubert et al., 2016). Additionally, Lee S. H. et al. (2018) used bladder cancer organoids to discover that epigenetic regulators such as *ARID1A*, *KMT2C*, *KMT2D*, and *KDM6A* are mutated as in the bladder cancer tissue, suggesting the differential epigenetics in human cancer (Lee S. H. et al., 2018). They also examined the differential responses to cancer treatment agents among different tumor subtypes from the subjects. Together, these studies using tumorigenic PDOs uncovered fundamental information that could be utilized for *in vitro* drug screening and potential translational applications. However, dysregulation of circadian rhythms, alterations of clock-controlled genes, and potential usages of circadian rhythms for disease treatments have not been thoroughly investigated using organoid models.

Do organoids possess circadian rhythms? We discovered that PSC-derived immature human intestinal organoids (HIOs) do not possess robust circadian rhythms (Rosselot et al., 2021). In contrast, mouse kidney capsule-matured human intestinal enteroids (kcHIEs) show robust circadian rhythms that are comparable to biopsy-derived human intestinal enteroids (bHIEs) based on the expression of a bioluminescence *Bmal1-luciferase* reporter (Rosselot et al., 2021). RNA-Seq analysis of both mouse and human enteroids show that between 3 and 10% of genes are under the control of the intestinal circadian clock (Rosselot et al., 2021). Importantly, we observe appropriate phase relationships between canonical clock genes and eight conserved rhythmic signaling pathways in mouse and human enteroids including circadian clock, cell cycle, and metabolism of carbohydrates (Rosselot et al., 2021). Intriguingly, we observe

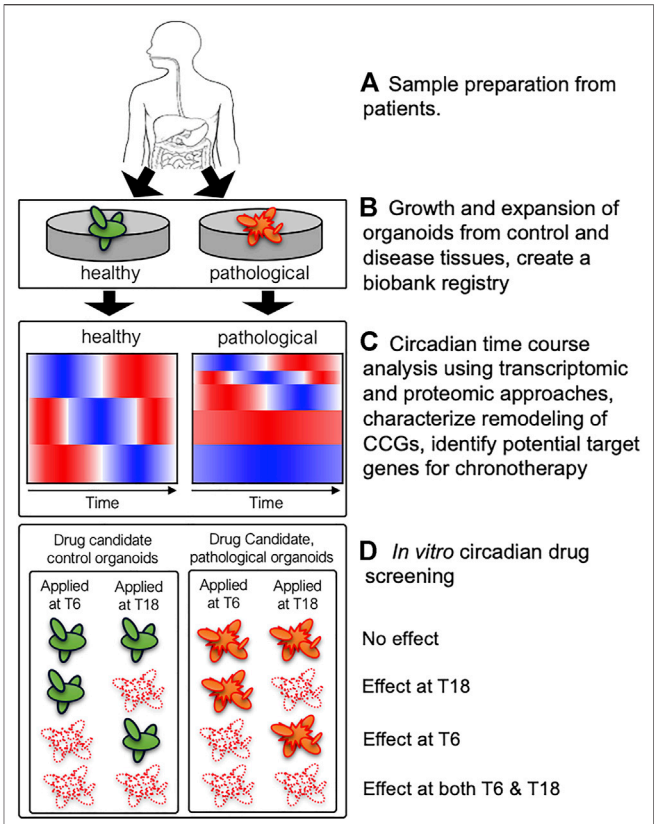

FIGURE 1 | A schematic diagram describing utilization of patient-derived organoids (PDOs) to uncover remodeling of clock-controlled genes (CCGs) and potential circadian drug screening. **(A)** Healthy and pathological tissues (containing stem-cell niche) from biopsies can be used to derive paired control (green) and pathological (orange) organoids from the same patient. **(B)** PDOs are expanded to establish organoid cultures and a subsequent biobank registry containing a library of PDOs from multiple subjects. **(C)** Circadian transcriptomic and proteomic analyses will uncover remodeling of CCGs in control vs. pathological organoids and identify potential target genes for chronotherapy. Cartoons of heatmaps illustrate a potential reduction of rhythmic genes in pathological organoids compared to control organoids. **(D)** Systematic *in vitro* circadian drug screening may reveal different phenotypes between control and pathological organoids with respect to efficacy and circadian timing of drug efficacy, which will provide critical information designing chronotherapeutic regimens. Four potential scenarios (no effect, effect at T6 or T18, and effect at both T6 and T18) are shown for control and pathological organoids. If pathological organoids (orange) show similar cell death (white) at both time points (effects at both T6 and T18) while control organoids (green) showing time-dependent efficacy (effect at T6 or T18) to candidate drugs, then one could design a chronotherapeutic regimen to target pathological organoids while minimizing toxicity to control organoids.

conserved phases for six signaling pathways including the circadian clock, but two pathways, cell-cell communication and neurotrophin signaling pathways, show dramatically different phases between mouse and human enteroids. Notably, we discovered that *Rac1*, a small GTPase directly inactivated by *Clostridium difficile* toxin B (TcdB), shows anti-phasic gene expression profile between mouse and human enteroids determining anti-phasic necrotic cell death phenotypes against TcdB (Rosselot et al., 2021). The above data demonstrate that PDOs could be ideal *in vitro* model

systems investigating disease-specific alterations of circadian rhythms and clock-controlled signaling pathways.

As discussed above, remodeling of clock-controlled genes and signaling pathways has been previously observed in mouse liver in different genetic and external perturbation conditions resulting in different metabolic phenotypes (Hatori et al., 2012; Eckel-Mahan et al., 2013; Zarrinpar et al., 2016; Manella et al., 2021). Hence, we hypothesize that pathological tissues possess altered circadian rhythms and/or clock-controlled genes and signaling pathways exacerbating disease progression. However, it is not feasible to collect human tissues over circadian cycle with fine time resolution (e.g., every 2 h for 48 h). Therefore, paired PDOs derived from control and pathological tissues (e.g., colorectal cancer) from the same patient will provide unique platforms to characterize alterations of clock-controlled genes and signaling pathways using high-throughput transcriptomics and proteomics (**Figure 1**). This process will uncover remodeling of CCGs in the organoids derived from pathological tissues highlighting altered rhythmicity and/or phase information between control and pathological organoids, which will identify potential circadian target genes for chronotherapeutic interventions. Importantly, this process will also determine whether existing drug target genes show altered temporal patterns between control and pathological organoids. If there exist distinct temporal patterns of existing drug target genes between control and pathological organoids, then this information can be used to design chronotherapeutic regimens maximizing the efficacy of drugs to pathological organoids while minimizing side effects to control organoids. Collected high-throughput time course data from both control and pathological organoids will enable scientists to rationally design potential chronotherapeutic regimens and validate them using PDOs in a disease-specific manner.

CONCLUDING REMARKS

In this minireview, we highlighted the importance of peripheral clocks and potential uses of human organoids to investigate peripheral organ-specific clock functions and remodeling of clock-controlled genes and signaling pathways in pathological tissue-derived organoids using high-throughput transcriptomics and proteomics. It is important to emphasize that mature organoids derived from patients' tissue biopsy are ideal to generate such data, because the maturity of organoids impacts robustness and function of circadian rhythms. The collected transcriptomic and proteomic data will uncover distinct temporal signatures between control and pathological tissue-derived organoids, which will lead to novel chronotherapeutic regimens in a disease-specific manner. However, it will not be trivial to identify optimal chronotherapeutic design for several drug combinations and numerous target genes. Therefore, it will be necessary to construct mathematical models based on temporal information of key drug target genes and employ computational methods to narrow down testable chronotherapeutic regimens. Rationally designed chronotherapeutic regimens can be validated *in vitro* using PDOs from numerous patients stored in a biobank registry. In particular, utilization PDOs derived from treatment resistant and responsive patients may uncover distinct temporal profiles of CCGs and signaling pathways identifying distinct timing of treatments and/or novel circadian target genes for the treatment resistant group.

It is important to note that the collected transcriptomic and proteomic data using PDOs reflect tissue- and organ-specific CCGs and signaling pathways in the absence of systemic and entrainment cues. Devoid of systemic and entrainment cues in organoid cultures pose critical challenges of organoid usages for potential translational applications of chronotherapy, because patients' responses to chronotherapy will depend on integrated systemic and peripheral clocks under different entrainment conditions. On the other hand, these challenges present opportunities to further utilize PDOs to determine organ-specific entrainment to different external conditions. For example, microfluidic devices that enable luminal flow could be utilized to test chronotherapeutic regimens using organoids under different nutrient entrainment conditions (Lee K. K. et al., 2018; Sidar et al., 2019). In addition, it would be interesting to test potential bidirectional relationship between the SCN and peripheral clocks by co-culturing mouse SCN slices and organoids. PDOs can be also tested for their light responses for potential light/dark entrainment studies in organ-specific manner, because different Opsins are expressed in different peripheral tissues and organs. For example, Opn3 is expressed in murine skin, adipocytes, and HIEs (Buhr et al., 2019; Nayak et al., 2020; Rosselot et al., 2021). Finally, it is necessary to further validate the identified chronotherapeutic regimens *in vivo*, because it is critical to make sure that the uncovered temporal information in PDOs persist when they are in communication with the rest of the body. As previous research demonstrated that PDOs can be subcutaneously xenografted into NSG mice and measure their growth and toxicity in the NSG mice (Vlachogiannis et al., 2018; Steele et al., 2019), the identified chronotherapeutic regimens may be tested using NSG mice. Synergistic integration of computational models based on *in vitro* and *in vivo* validations using PDOs and mouse models, respectively, will move us forward to potential translational applications of circadian medicine in a disease specific manner.

AUTHOR CONTRIBUTIONS

SL and CH wrote the manuscript and approved the submitted version.

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Nomogram Model Based on Clinical Risk Factors and Heart Rate Variability for Predicting All-Cause Mortality in Stage 5 CKD Patients

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Background: Heart rate variability (HRV), reflecting circadian rhythm of heart rate, is reported to be associated with clinical outcomes in stage 5 chronic kidney disease (CKD5) patients. Whether CKD related factors combined with HRV can improve the predictive ability for their death remains uncertain. Here we evaluated the prognosis value of nomogram model based on HRV and clinical risk factors for all-cause mortality in CKD5 patients.

Methods: CKD5 patients were enrolled from multicenter between 2011 and 2019 in China. HRV parameters based on 24-h Holter and clinical risk factors associated with all-cause mortality were analyzed by multivariate Cox regression. The relationships between HRV and all-cause mortality were displayed by restricted cubic spline graphs. The predictive ability of nomogram model based on clinical risk factors and HRV were evaluated for survival rate.

Results: CKD5 patients included survival subgroup (n = 155) and all-cause mortality subgroup (n = 45), with the median follow-up time of 48 months. Logarithm of standard deviation of all sinus R-R intervals (lnSDNN) (4.40 ± 0.39 *vs*. 4.32 ± 0.42; $p = 0.007$) and logarithm of standard deviation of average NN intervals for each 5 min (lnSDANN) (4.27 ± 0.41 *vs*. 4.17 ± 0.41; $p = 0.008$) were significantly higher in survival subgroup than all-cause mortality subgroup. On the basis of multivariate Cox regression analysis, the lnSDNN (HR = 0.35, 95%CI: 0.17–0.73, $p = 0.01$) and lnSDANN (HR = 0.36, 95% CI: 0.17–0.77, $p = 0.01$) were associated with all-cause mortality, their relationships were

negative linear. Spearman's correlation analysis showed that lnSDNN and lnSDANN were highly correlated, so we chose lnSDNN, sex, age, BMI, diabetic mellitus (DM), β-receptor blocker, blood glucose, phosphorus and ln intact parathyroid hormone (iPTH) levels to build the nomogram model. The area under the curve (AUC) values based on lnSDNN nomogram model for predicting 3-year and 5-year survival rates were 79.44% and 81.27%, respectively.

Conclusion: In CKD5 patients decreased SDNN and SDANN measured by HRV were related with their all-cause mortality, meanwhile, SDNN and SDANN were highly correlated. Nomogram model integrated SDNN and clinical risk factors are promising for evaluating their prognosis.

Keywords: chronic kidney disease, circadian rhythm, heart rate, heart rate variability, all-cause mortality, nomogram model

INTRODUCTION

Chronic kidney disease (CKD) is a global disease with high incidence and brings heavy social and economic burden. The United States Renal Data System (USRDS, 2021) reported that 14.90% of the U.S. adult population were diagnosed as CKD, which was associated with increased risks of cardiovascular diseases (CVD) and case fatality ("USRDS," 2021), (Tonelli, et al., 2018). Traditional risk factors associated with CVD include hypertension, smoking, diabetic mellitus (DM) and anemia, etc (Deli, et al., 2013). Furthermore, the important role of abnormal heart rate circadian rhythm in CVD and all-cause mortality have been revealed in patients suffering from myocardial infarction or type 2 DM (La Rovere, et al., 1998; Bosone, et al., 2017).

Heart rate variability (HRV) parameters reflect the circadian rhythm of heart rate. Recorded by a non-invasive, repeatable and convenient clinical 24-h Holter examination, HRV parameters include SD of the normal-to-normal R-R intervals (SDNN), SD of 5-min average of normal R-R intervals (SDANN), root mean square of differences between adjacent normal R-R intervals (rMSSD), proportion of adjacent R-R intervals differing by>50 ms over 24 h (pNN50%), very low frequency (VLF), low frequency (LF), high frequency (HF), LF/HF ratio, et al., which are regulated by sympathetic and parasympathetic activities (Chou, et al., 2016). The frequency domain analysis showed that reduced HRV are associated with poorly controlled type 2 DM (Nganou-Gnindjio, et al., 2018). Five-minute HRV, particularly LF, are suggested to be promising risk predictors for coronary heart disease (Kotecha, et al., 2012). Compared with healthy controls, HRV parameters such as SDNN, SDANN, pNN50%, LF/HF, LF are lower in CKD patients (Cui, et al., 2021; J.; Zhang, et al., 2013), and related with their all-cause mortality and long-term prognosis (Mylonopoulou, et al., 2010; Chandra, et al., 2012; Kouidi, et al., 2013; Chang, et al., 2020). However, which HRV parameters are associated with all-cause mortality in CKD are controversial. Whether CKD related clinical risk factors combined with HRV can improve the predictive ability for their death remains uncertain.

Nomogram is a number of rulers where variables are listed separately, with a number of points assigned to a given magnitude of the variable. The scores obtained by the sum of all variables are matched to a scale of outcome (Balachandran, et al., 2015). Nomogram is used widely in clinical practice, especially in Oncology and medical outcomes, can make individualized estimates of prognosis for specific patients. In CKD patients, relevant clinical prognostic factors include body mass index (BMI), DM, mineral and bone metabolism disorders and medication history, etc. However, there are currently no study including above factors with HRV to investigate the predictive value for all-cause mortality in CKD patients.

Taking advantage of well-established CKD5 study cohort that initiated at Nanjing China, we screened HRV parameters which were correlated with all-cause mortality. Furthermore, their predictive value combined with clinical risk factors was analyzed by nomogram model in order to facilitate clinical utility.

MATERIALS AND METHODS

Study Population

In this retrospective cohort study, 231 CKD5 patients were enrolled from the First Affiliated Hospital of Nanjing Medical University and the Affiliated BenQ Hospital of Nanjing Medical University in China from March 2011 to December 2019.

The enrolled patients were aged 18–75 years and had an estimated glomerular filtration rate (CKD-EPI equation) < 15 ml/min/1.73 m^2. The dry weight of the dialysis patients was stable for at least 1 month to avoid the effects of overhydration on heart rate rhythm. The exclusion criteria were as follows: 1) undergoing maintenance dialysis between 0 and 3 months; 2) history of parathyroidectomy (PTX); 3) history of kidney transplantation; 4) fasting blood glucose on the day of evaluation≥200 mg/dl; 5) presence of fever, infection, pregnancy or lactating women; 6) severe congenital heart disease, atrial fibrillation or flutter, high-grade atrioventricular block, or permanent pacemaker implantation; 7) severe hepatic disease, chronic obstructive lung disease, malignant tumors, or severe mental disorders; 8) episodes of acute myocardial

infarction, stroke, or a major surgical procedure within the past 2 months; 9) use of immunosuppressive medications, calcitonin, or bisphosphonates.

Data Collection

The baseline characteristics of the patients were collected as follows: demographic information, dialysis mode, co-morbidities, causes of end stage kidney disease (ESKD) and history of CKD treatment. The primary end point was all-cause mortality. We followed up the patients by a phone interview or reviewing their medical records up to August 2019. The primary outcome was censored on the date of the last follow-up, and the patients who did not experience the primary outcome had their survival times censored. A further 31 (13.42%) participants who were uncontactable were not followed up.

Measurements of Blood Parameters

Venous blood samples were tested in the morning after overnight fasting. For hemodialysis patients, blood samples were tested before dialysis. Routine blood tests were performed using an LH 750 Hematology Analyzer (Beckman Coulter). Biochemical indicators were measured by an automated biochemical analyzer (AU5400; Olympus Corporation). Serum intact parathyroid hormone (iPTH) levels were measured with second-generation iPTH assay kits using a UniCel DxI 800 Access Immunoassay System (Beckman Coulter).

HRV Measurements

Each patient underwent 24-h Holter electrocardiogram (MARS Ambulatory ECG Analysis System; GE Healthcare). For hemodialysis patients, the dynamic electrocardiogram was analyzed on the nondialysis days.

HRV can be quantified using time-domain analysis and frequency-domain analysis, time-domain analysis consisting of MEANNN, SDNN, SDANN, rMSSD, pNN50%. Frequency-domain analysis consists of HF, VLF, LF, LF/HF.

Statistical Analysis

Normally distributed data were described as mean ± standard deviation, the *t*-test and non-parametric test were used to compare continuous variables between groups. Categorized variables were expressed as frequency and analyzed by Chi-square test. Baseline characteristics of preterm cases and controls were compared using the *t*-test or Mann-Whitney *U* test for continuous variables depending on the data distribution, and chi-square test was used for categorical variables. Baseline factors that were differentially distributed in the survival and dead groups, including age, sex, BMI, DM, ln alkaline phosphatase (lnALP), lniPTH, phosphorus, β-receptor blocker were considered covariates of the statistical models. Log-transformed *t*-test was performed to compare HRV parameters between survival and dead group. ALP, iPTH and HRV risk factors were $\log_e$ transformed before statistical analysis as they had left-skewed distributions.

Multivariable Cox hazard proportional regression was performed to evaluate the associations between HRV parameters and all-cause mortality. To further explore nonlinear relationships between the base level of lnSDNN, lnSDANN and risk of all-cause mortality, restricted cubic spline[10] was used with four knots at the 20th, 40th, 60th, and 80th percentiles. The 10th percentile of the predictor was the reference in corresponding curve by the R package of "rms" along with "ggplot2". We also applied the multivariable Cox regression to predict the all-cause mortality using the HRV parameters. Spearman's correlation analyzed the relationships between lnSDNN and lnSDANN. A nomogram for predicting all-cause mortality of these patients was developed on the basis of multivariable Cox regression analysis results. The sensitivity and specificity in predicting the long-term prognosis of CKD patients, were evaluated by the area under the curve (AUC) value of the receiver operating characteristic (ROC) curve by using the "timeROC" package in R.

All the statistical analyses were performed using R Software Version 3.6.2 (The R Foundation for Statistical Computing), and two-sided $p < 0.05$ was considered statistically significant unless stated otherwise.

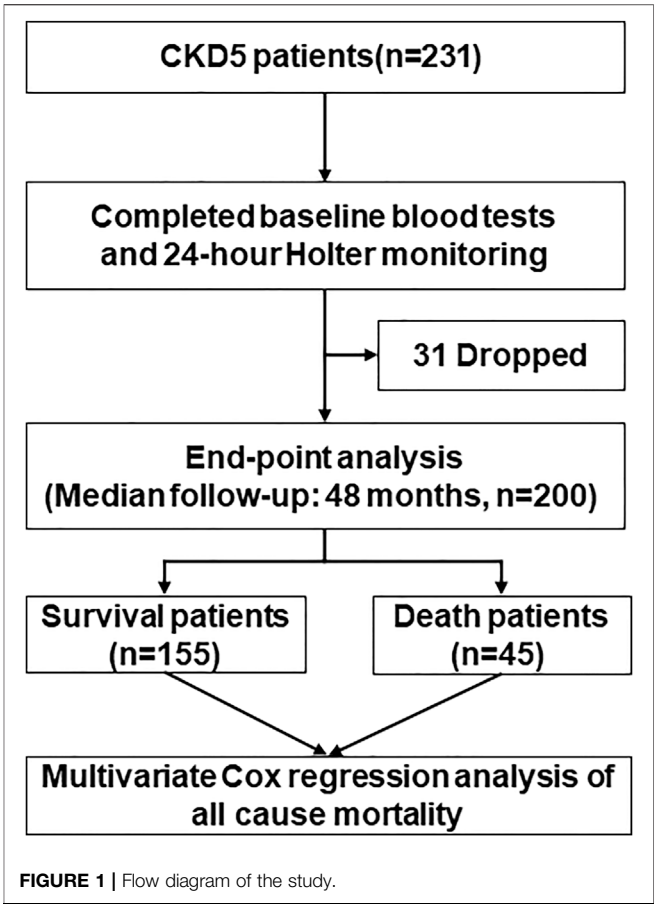

FIGURE 1 | Flow diagram of the study.

Ethics Statement

This study was approved by the Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, Nanjing, China (ethics approval numbers: 2011-SR-072 and 2019-SR-368). All participants provided the informed consent.

TABLE 1 | Baseline demographics, clinical characteristics and laboratory results in survival and all-cause mortality subgroups of CKD5 patients.

Clinical characteristics	Overall (n = 200)	Survival subgroup (n = 155)	All-Cause Mortality subgroup (n = 45)	*p*-Value
Demographics				
Female/Male	90/110 (45)	70 (45.16)	20 (44.44)	1.000
Age (years)	52.26 ± 13.25	49.85 ± 13.13	61 ± 9.98	<0.001
BMI (kg/m^2)	22.36 ± 3.43	22.56 ± 3.52	21.22 ± 3.06	0.125
Systolic BP (mmHg)	148.98 ± 25.67	148.45 ± 24.60	145 ± 29.29	0.590
Diastolic BP (mmHg)	85.62 ± 13.46	85.96 ± 14.06	81 ± 11.20	0.507
Dialysis mode, n (%)				
Predialysis	53 (26.50)	47 (30.32)	6 (13.33)	0.037
Hemodialysis	121 (60.50)	89 (57.42)	32 (71.11)	0.138
Peritoneal dialysis	27 (13.50)	20 (12.90)	7 (15.56)	0.833
Dialysis vintage(months)	12 (0-40.25)	9 (0-36)	24 (8-54)	0.006
Comorbidities, n (%)				
Diabetic mellitus	41 (20.50)	26 (16.77)	15 (33.33)	0.027
Hypertension	161 (80.50)	126 (81.29)	35 (77.78)	0.757
Cause of ESKD, n (%)				
Glomerulonephritis	132 (66)	109 (70.32)	23 (51.11)	0.027
Diabetic nephropathy	31 (15.50)	18 (11.61)	13 (28.89)	0.010
Hypertensive nephropathy	23 (11.50)	20 (12.90)	3 (6.67)	0.374
Polycystic kidney disease	14 (7)	13 (8.39)	1 (2.22)	0.274
Others	19 (9.50)	11 (7.10)	8 (17.78)	0.063
Medication history, n (%)				
Dihydropyridine CCBs	132 (66)	104 (67.10)	28 (62.22)	0.668
ACEI/ARB	44 (22)	33 (21.29)	11 (24.44)	0.806
β-receptor blocker	67 (33.50)	53 (34.19)	14 (31.11)	0.837
Phosphate binders	62 (43.36)	53 (43.80)	9 (40.91)	0.986
Active vitamin D sterols	59 (41.26)	53 (43.80)	6 (27.27)	0.225
Cinacalcet	8 (5.59)	8 (6.61)	0	0.461
Laboratory values				
Hemoglobin (g/L)	96.18 ± 22.39	96.67 ± 23.17	96 ± 19.59	0.566
Glucose (mmol/L)	5.44 ± 2.25	5.25 ± 1.84	4.88 ± 3.23	0.024
Creatinine (μmol/L)	868.35 ± 351.42	890.07 ± 363.04	747.70 ± 299.73	0.105
Urea (mmol/L)	25.51 ± 9.51	26.11 ± 9.60	21.95 ± 9.02	0.102
Total cholesterol (mmol/L)	4.44 ± 1.26	4.37 ± 1.21	4.41 ± 1.39	0.135
Triglyceride (mmol/L)	1.66 ± 1.03	1.61 ± 0.94	1.41 ± 1.27	0.223
Albumin (g/L)	37.50 ± 5.79	37.78 ± 5.52	38 ± 6.62	0.193
Calcium (mmol/L)	2.22 ± 0.28	2.21 ± 0.26	2.23 ± 0.34	0.608
Adjust Calcium (mmol/L)	2.27 ± 0.26	2.26 ± 0.23	2.31 ± 0.35	0.264
Phosphorus (mmol/L)	1.92 ± 0.64	1.93 ± 0.61	1.77 ± 0.76	0.621
ALP(U/L)	81.80 (63.62-109.22)	78.70 (61.45-106.00)	92.20 (77.10-113.10)	0.549
ln [ALP] (U/L)	4.48 ± 0.51	4.45 ± 0.54	4.52 ± 0.41	0.060
iPTH (pg/ml)	198.85 (96.13-470.70)	193.70 (88.40-468.25)	219.10 (148-479)	0.963
ln [iPTH] (pg/ml)	5.24 ± 1.27	5.23 ± 1.24	5.39 ± 1.36	0.821

Abbreviations: CKD, chronic kidney disease; BMI, body mass index; BP, blood pressure; ESKD, end-stage kidney disease; CCB, calcium channel blocker; ACEI/ARB, angiotensin-converting enzyme inhibitors/angiotensin receptor blocker; ALP, alkaline phosphatase; iPTH, intact parathyroid hormone; ln, the natural logarithm. Data are presented as mean ± SD, numbers and percentages, as appropriate. Other causes of ESKD, included obstructive nephropathy, interstitial nephritis, renovascular disease, lupus nephritis, and some unknown causes.

This research was designed in accordance with the Declaration of Helsinki.

RESULTS

Baseline Characteristics of Participants

A total of 200 CKD5 patients were divided into two subgroups: 45 patients with all-cause mortality and 155 patients with survival. Baseline data, hematology and HRV examinations were completed (**Figure 1**). There were 90 women and 110 men enrolled in our study, the average age was 52.26 ± 13.25 years old and median dialysis vintage was 12.0 months. Compared with survival group, the patients with all-cause mortality had older age and lower blood glucose levels, furthermore, their dialysis vintage was longer. The all-cause mortality group had more patients with diabetic nephropathy and fewer patients with chronic glomerulonephritis (CGN). The baseline demographics, clinical characteristics and laboratory results in survival and all-cause mortality subgroup of CKD5 patients are summarized in **Table 1**.

Baseline HRV Parameters in CKD5 Patients

Compared with all-cause mortality subgroup, SDNN [82 (67-112) *vs.* 75 (52-84); $p = 0.010$] and SDANN [71 (56-97.50) *vs.* 65 (45-76); $p = 0.006$] were significantly higher in survival patients.

TABLE 2 | Baseline HRV indexes in survival and all-cause mortality subgroups of CKD5 patients.

Heart rate variability	Overall (n = 200)	Survival Subgroup (n = 155)	All-Cause mortality Subgroup (n = 45)	*p*-Value
MHR (bpm)	80.06 ± 11.98	80.08 ± 11.87	79 ± 12.47	0.970
Time domain measures				
MEANNN (ms)	763 (684-835)	757.50 (680.75-835)	781 (704-848)	0.237
SDNN (ms)	80 (62.25-106)	82 (67-112)	75 (52-84)	0.010
SDANN (ms)	70 (54-91)	71 (56-97.50)	65 (45-76)	0.006
rMSSD (ms)	17 (13-23)	18 (14-23)	15 (12-23)	0.467
PNN50(%)	3.85 ± 6.42	3.64 ± 5.80	1.50 ± 8.24	0.389
lnMEANNN (ms)	6.63 ± 0.15	6.63 ± 0.15	6.66 ± 0.16	0.266
lnSDNN (ms)	4.36 ± 0.40	4.40 ± 0.39	4.32 ± 0.42	0.007
lnSDANN(ms)	4.23 ± 0.42	4.27 ± 0.41	4.17 ± 0.41	0.008
lnrMSSD (ms)	2.90 ± 0.46	2.90 ± 0.44	2.71 ± 0.56	0.915
Frequency domain measures				
VLF	96.86 (18.37-321.80)	30.30 (17.40-332.50)	134.38 (46.75-292.67)	0.217
LF	14.02 (8.34-55.76)	13.04 (8.98-53.61)	19.28 (7.02-62.95)	0.153
HF	7.98 (4.50-27.51)	7.84 (4.62-24.67)	14.36 (3-29.79)	0.188
LF/HF	1.75 (1.27-3.90)	1.75 (1.31-2.94)	1.91 (1-5.45)	0.969
lnVLF	4.42 ± 1.66	4.31 ± 1.71	4.90 ± 1.47	0.078
lnLF	3.11 ± 1.42	3.07 ± 1.38	2.96 ± 1.57	0.529
lnHF	2.22 ± 1.76	2.20 ± 1.65	2.66 ± 2.10	0.760
lnLF/HF	0.88 ± 1.08	0.87 ± 1.04	0.65 ± 1.19	0.758

Abbreviations: HRV, heart rate variability; MHR, mean heart rate; MEANNN, mean normal-to-normal R–R intervals; SDNN, SD of normal-to-normal R–R intervals; SDANN, SD of 5-minute average of normal R–R intervals; rMSSD, root mean square of differences between adjacent normal R–R intervals; pNN50%, proportion of adjacent R–R intervals differing by 50 ms over 24 h; VLF, very low frequency; LF, low frequency; HF, high frequency. ln, the natural logarithm based on e. Data are presented as mean ± SD, numbers and percentages, as appropriate.

LnSDNN (4.40 ± 0.39 *vs*. 4.32 ± 0.42; $p = 0.007$) and lnSDANN (4.27 ± 0.41 *vs*. 4.17 ± 0.41; $p = 0.008$) were also higher in survival group than all-cause mortality group. There was no significant difference between two subgroups in other time-domain and all of frequency-domain parameters (**Table 2**).

Associations between Clinical Characteristics and Risks for All-Cause Mortality in CKD5 Patients

After putting all clinical characteristics data in **Supplementary Table S1** into the multivariate Cox regression model, we found that age (HR = 1.09, 95%CI:1.06–1.11, $p < 0.001$), DM (HR = 3.63, 95%CI:2.05–6.42, $p < 0.001$), β-receptor blocker (HR = 0.54, 95%CI:0.31–0.95, $p = 0.032$), blood glucose levels (HR = 0.54, 95%CI:0.31–0.95, $p = 0.032$), blood phosphorus levels (HR = 0.58, 95%CI:0.36–0.93, $p = 0.024$), and lniPTH (HR = 0.74, 95%CI:0.63–0.86, $p < 0.001$) were demonstrated as independent predictors for all-cause mortality in CKD5 patients. Sex (HR = 0.97, 95%CI: 0.59–1.62, $p = 0.920$) and BMI (HR = 0.94, 95%CI:0.87–1.02, $p = 0.118$) were not associated with all-cause mortality (**Table 3**).

Then we put all HRV parameters to determine the independent predictors for all-cause mortality in CKD5 patients. Cox regression analysis revealed that lnSDNN (HR = 0.35, 95% CI:0.17–0.73, $p = 0.005$) and lnSDANN (HR = 0.36, 95% CI:0.17–0.77, $p = 0.008$) are independent risk factors for all-cause mortality with adjustment for age, sex (male, 0; female, 1), BMI, diabetic mellitus (yes or no), ln (iPTH), phosphorus and drug intaking of β-receptor blocker (**Figure 2**).

Non-Linear Relationships between LnSDNN/LnSDANN and All-Cause Mortality in CKD5 Patients

The restricted cubic spline regression analysis between lnSDNN/lnSDANN and the risks of all-cause mortality in CKD5 patients was established (**Figure 3**). There was a negative correlation between lnSDNN and the risk of all-cause mortality (p for non-linearity = 0.452), the same outcome had been found between lnSDANN and the risk of all-cause mortality (p for non-linearity = 0.899) in 200 CKD5 patients. Restricted cubic spline analysis revealed linear relationships between lnSDNN/lnSDANN and all-cause mortality. Indicating that as the increase of lnSDNN and lnSDANN, all-cause mortality of CKD5 patients decreased. Spearman's correlation analysis proved that lnSDNN and lnSDANN were highly correlated (r = 0.97, $p < 0.001$) (**Figure 4**).

Nomogram Model Based on Clinical Risk Factors and LnSDNN for Predicting All-Cause Mortality in CKD5 Patients

Multivariate Cox regression analysis proved that the risk factors for all-cause mortality in CKD5 patients included age, DM, β-receptor blocker, blood glucose, phosphorus and lniPTH levels. Spearman's correlation analysis indicated that lnSDNN and lnSDANN were highly correlated, thus lnSDNN, above clinical factors, sex and BMI were implemented by nomogram model.

The prediction performance of the nomogram was further confirmed by AUC analysis. The AUC of lnSDNN for 3-year mortality was 0.79 (95% CI: 0.69-0.90) with sensitivity of

TABLE 3 | Multivariate cox regression of clinical characteristics for all-cause mortality in CKD5 patients.

Clinical characteristics	HR (95%CI)	*p*-Value
Demographics		
Female/Male	0.97 (0.59,1.62)	0.920
Age (years)	1.09 (1.06,1.11)	<0.001
BMI (kg/m^2)	0.94 (0.87,1.02)	0.118
Systolic BP (mmHg)	1.00 (0.99,1.01)	0.448
Diastolic BP (mmHg)	0.98 (0.96,1.00)	0.016
Dialysis mode, n (%)		
Predialysis	0.98 (0.42,2.28)	0.969
Hemodialysis	0.73 (0.41,1.31)	0.288
Peritoneal dialysis	1.63 (0.80,3.30)	0.178
Dialysis vintage(months)	1.00 (0.99,1.00)	0.264
Comorbidities, n (%)		
Diabetic mellitus	3.63 (2.05,6.42)	<0.001
Hypertension	0.86 (0.49,1.52)	0.606
Cause of ESKD, n (%)		
Glomerulonephritis	0.38 (0.23,0.63)	0.000
Diabetic nephropathy	4.24 (2.29,7.82)	<0.001
Hypertensive nephropathy	0.37 (0.05,2.65)	0.320
Polycystic kidney disease	0.62 (0.15,2.55)	0.512
Others	1.86 (0.89,3.92)	0.100
Medication history, n (%)		
Dihydropyridine CCBs	0.95 (0.57,1.57)	0.838
ACEI/ARB	0.79 (0.42,1.46)	0.456
β-receptor blocker	0.54 (0.31,0.95)	0.032
Phosphate binders	0.76 (0.38,1.53)	0.442
Active vitamin D sterols	0.87 (0.43,1.78)	0.708
Cinacalcet	0.32 (0.08,1.33)	0.118
Laboratory values		
Hemoglobin (g/L)	0.99 (0.98,1.00)	0.060
Glucose (mmol/L)	1.20 (1.11,1.31)	<0.001
Creatinine (μmol/L)	0.9986 (0.9977,0.9996)	0.004
Urea (mmol/L)	0.99 (0.96,1.03)	0.688
Total cholesterol (mmol/L)	1.20 (0.97,1.48)	0.091
Triglyceride (mmol/L)	1.00 (0.84,1.20)	0.967
Albumin (g/L)	0.91 (0.87,0.96)	0.000
Adjust calcium (mmol/L)	0.48 (0.20,1.18)	0.110
Phosphorus (mmol/L)	0.58 (0.36,0.93)	0.024
ln [ALP] (U/L)	0.9987 (0.9977,0.9998)	0.019
ln [iPTH] (pg/ml)	0.74 (0.63,0.86)	<0.001

Abbreviations: CKD, chronic kidney disease; BMI, body mass index; BP, blood pressure; ESKD, end-stage kidney disease; CCB, calcium channel blocker; ACEI/ARB, angiotensin-converting enzyme inhibitors/angiotensin receptor blocker; ALP, alkaline phosphatase; iPTH, intact parathyroid hormone; ln, the natural logarithm. Other causes of ESKD included obstructive nephropathy, interstitial nephritis, renovascular disease, lupus nephritis, and some unknown causes. HR, hazard ratio; CI, confidence interva.

88.24% and specificity of 53.93%, and for 5-year mortality was 0.81 (95% CI: 0.74-0.89) with sensitivity of 90.00% and specificity of 57.58%, respectively. These results demonstrated that the nomogram based on clinical risk factors and lnSDNN had a great discriminatory ability in predicting all-cause mortality for CKD5 patients. Moreover, AUC showed good predictive power for both 3-year and 5-year mortality (**Figure 5A,B**,).

DISCUSSION

Appropriate screening, classification and management for CKD5 patients are of great importance to prevent poor prognosis of CKD, including CVD and mortality. It has been suggested that autonomic dysfunctions indicated by lower HRV are associated with higher all-cause mortality and CVD, especially in patients with advanced CKD (Chandra, et al., 2012; "Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology," (Author Anonymous, 1996; Koomans, et al., 2004).

HRV parameters include Time-domain measures (Cygankiewicz, et al., 2013). SDNN, the SD of NN intervals, is the most common assessment. Both sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) activities contribute to SDNN, which is highly correlated with Ultra-Low Frequency (ULF), VLF, LF band power, and total power (Umetani, et al., 1998). SDNN, recommended by the European Society of Cardiology (ESC) task force, is reported to be the "gold standard" for medical stratification of cardiac risk when recorded over a 24 h period ("Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology," 1996). Nolan revealed that SDNN was the powerful contributor for the risks of death in heart failure patients (Nolan, et al., 1998). When stratified analysis was performed, Agata found that SDNN <96 ms in CKD patients was an independent predictor for CVD with 2 years follow-up (Buonacera, et al., 2016). In hemodialysis patients, SDNN<75 ms was a strong predictor for all-cause mortality (Chou, et al., 2016).

SDANN, the SD of average NN intervals, is not a surrogate for SDNN since it is calculated using 5 min segments instead of an entire 24 h series (Kuusela, 2012). European Society of Cardiology and the North American Society of Pacing and Electrophysiology (ESC/NASPE) Task Force on HRV analysis illustrated that decreased SDANN was verified to predict both all-cause mortality and CVD ("Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology," 1996). However, Shaffer F reported that SDANN did not provide additional meaningful information (Shaffer, et al., 2014). Letian Yang confirmed that decreased SDANN and LF/HF were associated with all-cause death and CVD, and decreased SDNN was the only identified predictor for CVD in maintenance hemodialysis patients (Yang, et al., 2021). In non-diabetic HD patients, lower SDNN or SDANN group developed more major adverse cardiac and cerebrovascular event (MACCE) than higher groups (Kida, et al., 2017).

Here we confirmed that compared with survival subgroup, SDNN [75 (52-84) *vs.* 82 (67-112); $p = 0.010$] and SDANN [65 (45-76) *vs.* 71 (56-97.50); $p = 0.006$] were lower in all-cause mortality subgroup. Our team also reported that SDNN [82.7 ± 48.4 *vs.* 140.9 ± 34.9); $p < 0.001$] and SDANN [74.7 ± 61.5 *vs.* 128.4 ± 34.2; $p < 0.001$] in CKD5 patients were significantly

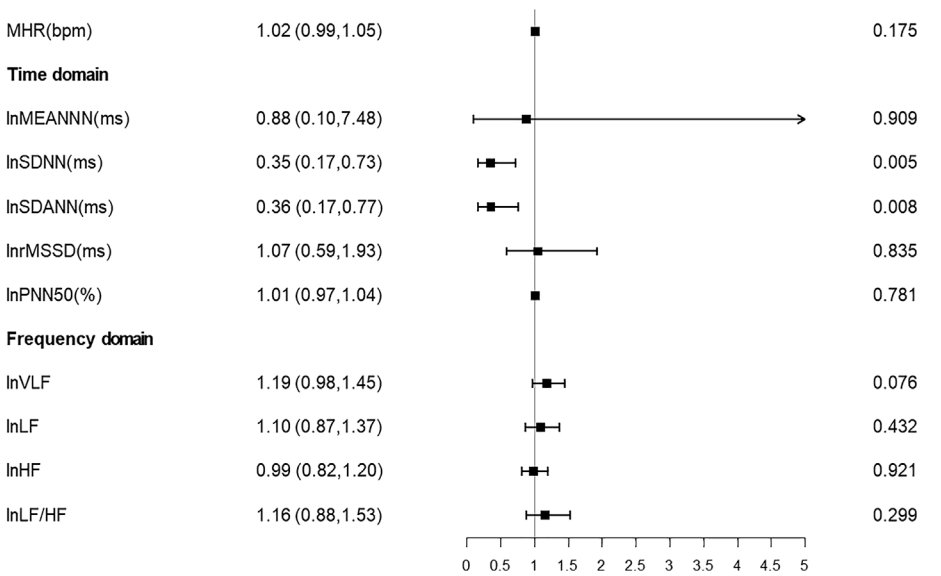

FIGURE 2 | Forest plots of associations between HRV parameters and all-cause mortality. Present the estimates with a horizontal line representing 95% confidence intervals (CIs). Abbreviations: HRV, heart rate variability; SDNN, SD of normal-to-normal R-R intervals; SDANN, SD of 5-minute average of normal R-R intervals.

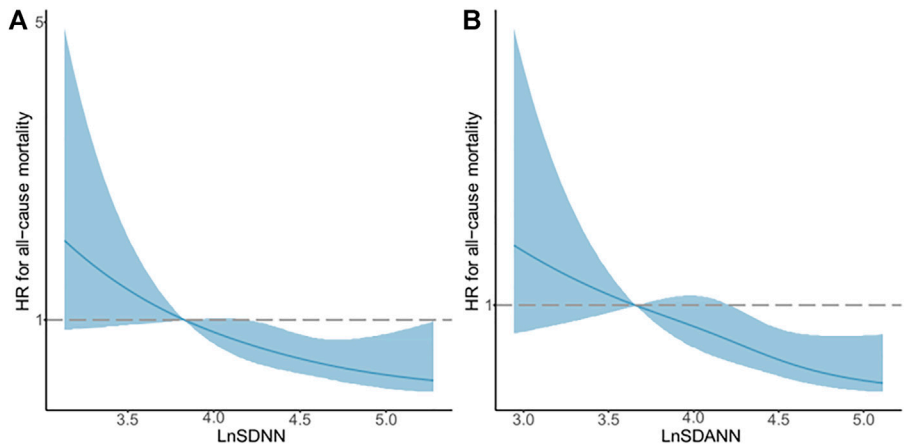

FIGURE 3 | Associations between LnSDNN/LnSDANN and All-cause Mortality Displayed by Restricted Cubic Spline Analysis in CKD5 Patients. **(A)** lnSDNN and all-cause mortality. **(B)** lnSDANN and all-cause mortality. Hazard ratios are indicated by solid blue lines and 95% confidence intervals by shaded areas. Reference lines for no association are indicated by dashed lines at a hazard ratio of 1.0. Abbreviations: SDNN, SD of normal-to-normal R-R intervals; SDANN, SD of 5-minute average of normal R-R intervals. CKD, chronic kidney disease.

lower when compared with healthy populations (J. Zhang, et al., 2013).

In a recent study from Chinese population, Zhang et al. (2022) identified functional diurnal patterns and parameters by monitoring personalized, heart rate based diurnal changes. These findings have important implications for understanding how a regular heart diurnal pattern benefits cardiac function and raising the possibility of non-pharmacological intervention against circadian related CVD. Consistent with above and previous studies, we proved that both SDNN and SDANN were independent risk factors for all-cause mortality in CKD5 patients. Furthermore, we firstly used the restricted cubic spline regression models to show the negative linear relationships between them, with the increase of lnSDNN/lnSDANN and decrease of all-cause mortality in CKD5 patients.

There is a dearth of literature in the area of exact mechanisms of decreased HRV. Abnormal cardiac autonomic modulations are reported to be linked with increased serum phosphate levels and secondary hyperparathyroidism (SHPT), which contributes to a higher risk of sudden cardiac death (Poulikakos, et al., 2014). Our

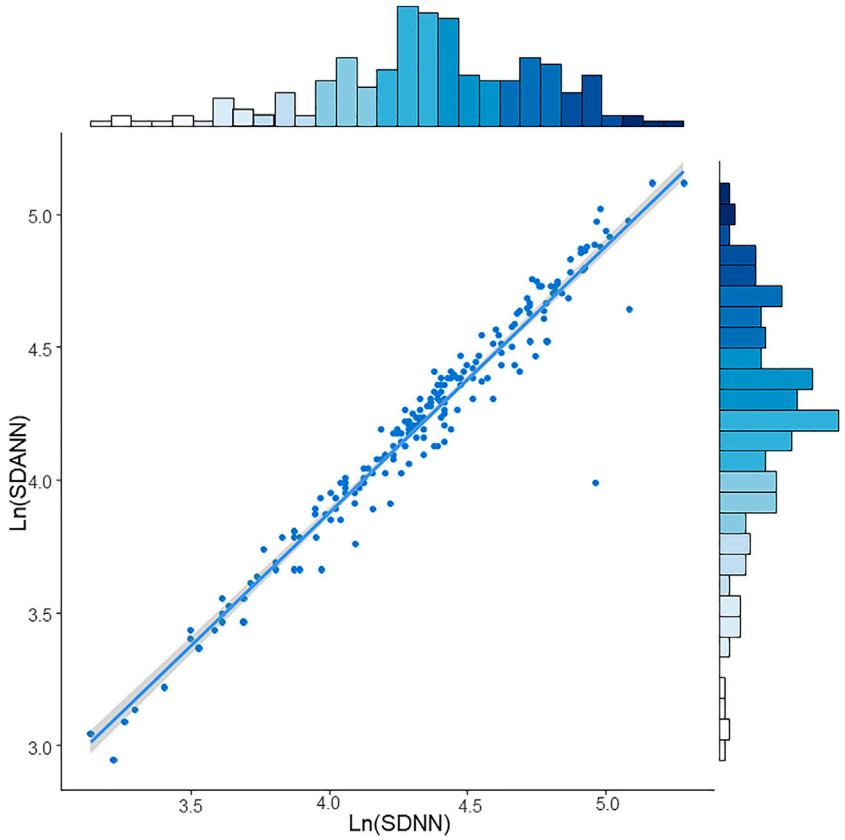

FIGURE 4 | Spearman's correlation between LnSDNN and LnSDANN in CKD5 patients. Abbreviations: SDNN, SD of normal-to-normal R-R intervals; SDANN, SD of 5-minute average of normal R-R intervals. CKD, chronic kidney disease.

team found that successful PTX in severe SHPT patients may contribute to reverse the high CVD risks by blunting sympathetic hyperactivity and enhancing parasympathetic activity as indicated by normalized HRV parameters (J. Zhang, et al., 2013). Ng et al. (2017) reported that calcification of hand artery (HA) was associated with autonomic dysfunction, the patients with lower autonomic tone and severe HA calcification had the highest mortality rate. Fadaee et al. (2017) found that oxidative stress is significantly and independently associated with decreased HRV in patients with CKD.

The emerging research has been proved that the predictive power of HRV parameters was limited by low sensitivity (Nganou-Gnindjio, et al., 2018), and also influenced by variables such as age, sex, glucose, uremia toxin and drugs intake, etc (Kotecha, et al., 2012; Sarnak, et al., 2019). Among them β-receptor blocker has been reported to play an important role in improving HRV parameters (Mortara, et al., 2000; Kubo, et al., 2005).

Nomogram has been emerged as a simple tool with numerous advantages and estimated individualized risks based on the characteristics of patients and disease (Grimes, 2008). As a quantitative analysis tool, nomogram overcomes the shortcomings of hierarchical analysis. In order to improve the predictive value of HRV parameters and considering various clinical related factors, we firstly applied nomogram constructed by sex, age, BMI, DM, β-receptor blocker, glucose, phosphorus, lniPTH, lnSDNN to predict the risks of all-cause mortality in CKD5 patients.

To our knowledge, there is no study demonstrated a nomogram based on HRV to predict all-cause mortality for CKD5 patients. We developed simple and easy-to-use prognostic model integrating clinical parameters for CKD5 patients from the perspective of heart rate circadian rhythm, and the nomogram model is promising for evaluating their all-cause mortality. Moreover, we discovered that there is collinearity between SDANN and SDNN. This finding is consistent with Shaffer F's study which confirmed that SDANN did not provide additional useful information than SDNN (Shaffer, et al., 2014).

Our research has several potential limitations. The lack of external validation studies, the rather long recruitment period and a low rate of in-hospital limited our study by reducing the power to detect possible associations between HRV and all-cause mortality for CKD5 patients. In the future, we will continue to incorporate more data of CKD5 patients, and conduct external validation of nomograms to confirm its sensitivity and specificity, so as to facilitate clinical application.

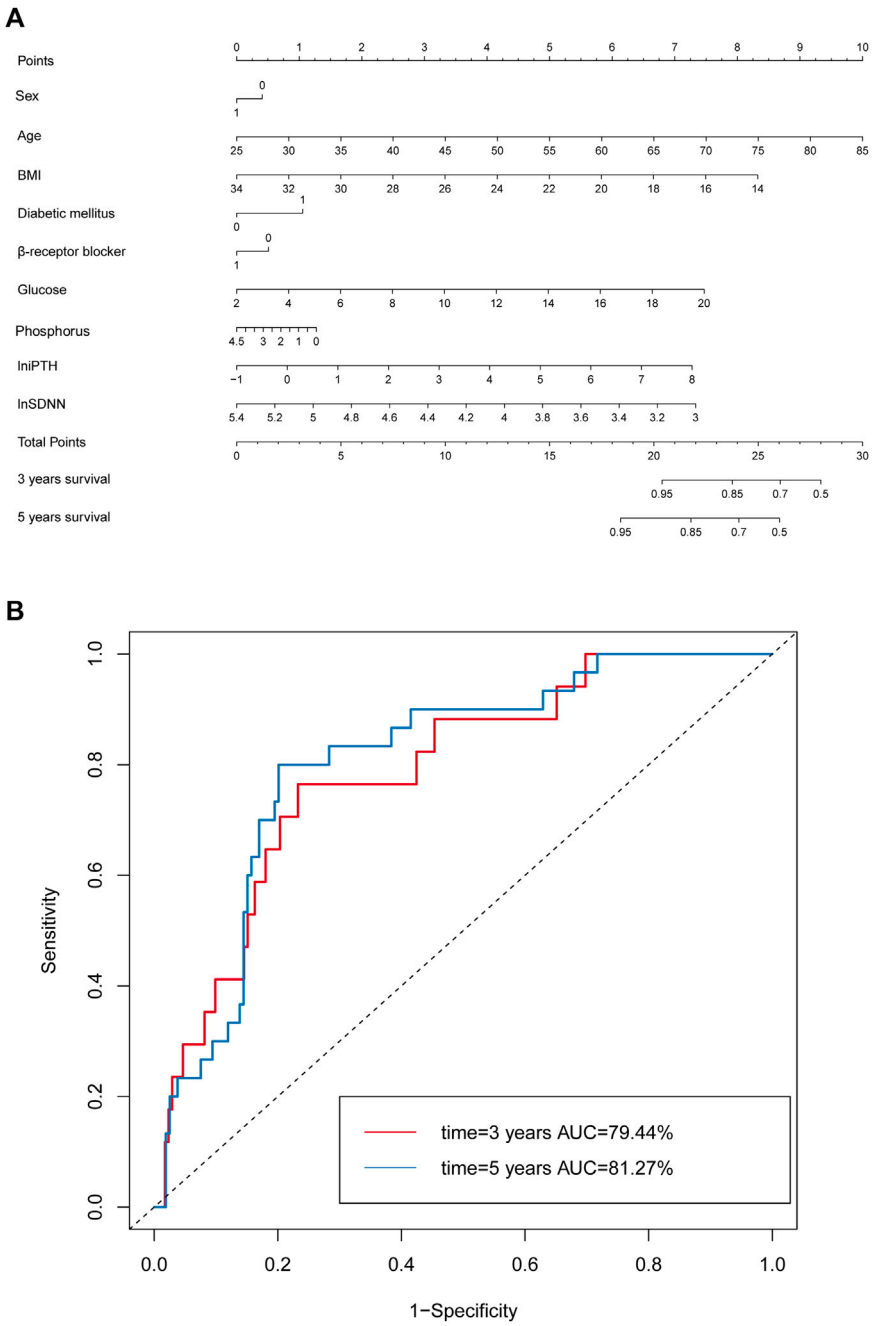

FIGURE 5 | Nomogram model based on clinical risk factors and HRV to predict all-cause mortality in CKD5 patients. **(A)** Nomogram model based on clinical risk factors and lnSDNN for all-cause mortality. **(B)** ROC validating the discriminatory power of the nomogram model for 3-year and 5-year survival rates in CKD5 patients. Abbreviations: HRV, heart rate variability; SDNN, SD of normal-to-normal R-R intervals; CKD, chronic kidney disease; ROC, receiver operator characteristic curve; AUC, area under ROC.

CONCLUSION

Here we proved that decreased heart rate variability parameters SDNN and SDANN were negatively correlated with all-cause mortality in CKD5 patients. Spearman's analysis showed that lnSDNN and lnSDANN were highly correlated. Based on SDNN and clinical risk factors, we established the first practical nomogram that can predict individualized prognosis of CKD5 patients from the perspective of heart rate circadian rhythm, the nomogram is promising for evaluating their all-cause mortality with high accuracy and reliability. Our study may provide new insight for improved

decisions and treatments of CKD patients, in order to decrease their all-cause mortality.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XG, JW, and HH contributed equally to this work. NW devised the concept and designed the study. CX supervised the study. NW, ZG, LZ, JZ, YC, FX, HQ, and WR enrolled the healthy controls. CX, NW, MZ, GY, ZG, YH, and JW were responsible for the management of stage 5 chronic kidney disease patients. XG, YC, ZG, FX, HQ, WR, LZ, YJ, YX, AB, FL, XY, and JZ finished the follow-up study. XG, JW, HH, WR, YC, YW, ST, and XY carried out the statistical analyses and reported the results. NW, XG, JW, HH, YC, WR, and XY contributed to manuscript writing. All authors reviewed the manuscript and signed off on its accuracy.

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SUPPLEMENTARY MATERIAL

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Key LncRNAs Associated With Oxidative Stress Were Identified by GEO Database Data and Whole Blood Analysis of Intervertebral Disc Degeneration Patients

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Background: Intervertebral disc degeneration (IDD) is a major cause of low back pain, but the onset and progression of IDD are unknown. Long non-coding RNA (lncRNA) has been validated to play a critical role in IDD, while an increasing number of studies have linked oxidative stress (OS) to the initiation and progression of IDD. We aim to investigate key lncRNAs in IDD through a comprehensive network of competing endogenous RNA (ceRNA) and to identify possible underlying mechanisms.

Methods: We downloaded IDD-related gene expression data from the Gene Expression Omnibus (GEO) database and obtained differentially expressed-lncRNAs (DE-lncRNA), -microRNAs (DE-miRNA), and -messenger RNAs (DE-mRNA) by bioinformatics analysis. The OS-related lncRNA-miRNA-mRNA ceRNA interaction axis was constructed and key lncRNAs were identified based on ceRNA theory. We performed gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses on mRNAs regulated by lncRNAs in the ceRNA network. Single sample gene set enrichment analysis (ssGSEA) was used to reveal the immune landscape. Expression of key lncRNAs in IDD was assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Results: In this study, 111 DE-mRNAs, 20 DE-lncRNAs, and 502 DE-miRNAs were identified between IDD patients and controls, and 16 OS-related DE-lncRNAs were also identified. The resulting lncRNA-miRNA-mRNA network consisted of eight OS-related DE-lncRNA nodes, 24 DE-miRNA nodes, 70 DE-mRNA nodes, and 183 edges. Functional enrichment analysis suggested that the ceRNA network may be involved in regulating biological processes related to cytokine secretion, lipid, and angiogenesis. We also identified four key lncRNAs, namely lncRNA GNAS-AS1, lncRNA MIR100HG, lncRNA LINC01359, and lncRNA LUCAT1, which were also found to be significantly associated with immune cells.

Conclusion: These results provide novel insights into the potential applications of OS-related lncRNAs in patients with IDD.

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INTRODUCTION

Intervertebral Disc degeneration (IDD) is a common pathologic change in the intervertebral disc. It is directly related to lower back pain, which causes great pain to patients and great financial pressure on society (Risbud and Shapiro, 2014). In China, low back pain is gradually becoming an epidemic. Statistics show that the number of patients with low back pain has exceeded 200 million, accounting for about 16% of the country's population.

The underlying pathogenesis of disc degeneration is not fully understood. We know that it is not only related to external factors such as obesity and fatigue, but also to genetic factors. These factors can lead to changes in cell morphology, apoptosis, senescence, inflammation and oxidative stress (Roh et al., 2021). However, these currently known mechanisms do not provide good results for the treatment of IDD, so further research is needed to develop more effective prevention and treatment strategies.

Noncoding RNAs include long non-coding RNAs (lncRNAs), mirco RNAs (miRNAs) and circular RNAs (circRNAs). LncRNAs, with a length of over 200 nucleotides, can't encode proteins, but it plays a regulatory role in biological processes such as proliferation, invasion and apoptosis and deregulates inflammation and infection (Hausser and Zavolan, 2014). LncRNA may act as competing endogenous RNAs (ceRNAs) to regulate the expression of target mRNAs through competitively binding to mRNAs. It's pivotal to illustrate interactions of lncRNAs, miRNAs and mRNAs for treatment of IDD (Salmena et al., 2011). With the development of the society, more and more studies have been conducted to verify the hypothesis. A recent study shows that (Ma et al., 2021) MIR155 host gene (MIR155HG), a long non-coding RNA, promotes cell pyroptosis. In human degenerative NP tissue samples, MIR155HG expression was significantly increased and positively correlated with the Pfirrmann score. Excessive expression of MIR155HG reduces the miR-223-3p level, upregulates NLRP3, and promotes cell pyroptosis in human degenerative NP cells. The interaction of MIR155HG, miR-223-3p, and NLRP3 is like the mode of ceRNA (Yang et al., 2022). We know that lncRNA HLA complex group 18 (HCG18) also has its own ceRNA network to induce the development of IDD (Xi et al., 2017). The regulatory function of ceRNA provides a new perspective for elucidating the gene expression regulatory network constructed by transcriptome, and provides more dimensions for analyzing the molecular mechanisms of important biological processes. Intervertebral disc degeneration is closely related to intervertebral disc senescence, and oxidative stress is the main factor causing cell senescence. Nucleus pulposus cells are one of the main sources of reactive oxygen species (ROS), and ROS levels in intervertebral discs increase with the progression of intervertebral disc degeneration (Brandl et al., 2011). Hydrogen peroxide can activate the senescence signal transduction pathway and induce cell cycle stagnation in G0/G1 phase of nucleus pulposus cells. These studies indicate that oxidative stress is an important inducement of intervertebral disc degeneration (Yudoh et al., 2005; Wang et al., 2021a).

We aimed to identify DE-lncRNA, DE-miRNA, and DE-mRNA between an IDD group and a control group by bioinformatics analysis, then a ceRNA network was made, and through GO and KEGG pathway analyses, the underlying function was revealed. The present study researched the ceRNA network and how to regulate biological processes associated with cytokine secretion, lipid, and angiogenesis and found four key lncRNAs, lncRNA GNAS-AS1, lncRNA MIR100HG, lncRNA LINC01359, and lncRNA LUCAT1, which were also found to be significantly associated with immune cells. QRT-PCR was used to certify it. This study may provide new therapeutic strategies and novel insights into the potential applications of OS-related lncRNAs for IDD.

MATERIALS AND METHODS

Data Source

IDD-related gene expression profile data were obtained from the GEO database. mRNA and lncRNA expression profiles were obtained from the GES124272 dataset (platform:GPL21185; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124272), which contains eight patients with MRI-confirmed IDD and eight volunteers (no clinical evidence of low back pain or sciatica) of fasting whole blood (collected from the left median cubital vein of each participant) samples (Wang et al., 2019; Wang et al., 2021b). The miRNA microarray of three IDD patients [nucleus pulposus (NP) tissues] and three controls (normal NP tissues from fresh traumatic lumbar fracture patients) was obtained from the GSE116726 dataset (platform: GPL20712; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE116726) (Ji et al., 2018).

GeneCards online tool (https://www.genecards.org/) was utilized to obtain oxidative stress-related genes (OSRG). Briefly, using the keyword "oxidative stress" and setting the "Category" to "Protein Coding", filtered genes based on Relevance score ≥ 7, and finally, 2,129 OSRGs were obtained (**Supplementary Table S1**).

Differential Expression Analysis

The differential expression analysis was performed by R package limma. The significance threshold was set at $|\log_2$ fold change (FC)$| \geq 1$ and $p < 0.01$. The basis of DE-mRNAs and DE-lncRNAs analysis was from the GSE124272 dataset and DE-miRNAs were identified in the GSE116726 dataset. Volcano plots were drawn using the R package ggplot2 to demonstrate the expression distribution of DE-mRNAs, -miRNAs, and -lncRNAs.

Identification of Oxidative Stress-Related Differentially Expressed-lncRNAs

After matching the expression profiles of 2,129 OSRGs in the GSE124272 dataset, the relationships between all lncRNAs in the GSE124272 dataset and OSRGs were calculated based on the expression values. OS-related lncRNAs were identified based on Spearman correlation coefficients with absolute values >0.8 and $p < 0.01$. Subsequently, an overlap analysis was performed using the R package VennDiagram for DE-lncRNAs and OS-related lncRNAs, whose common elements were labeled as OS-related DE-lncRNAs.

Prediction of Target miRNAs and mRNAs of Oxidative Stress-Related Differentially Expressed-lncRNAs

Prediction of target miRNAs for OS-related DE-lncRNAs using Starbase and LncBase. Starbase (http://starbase.sysu.edu.cn/) is a friendly database for the prediction of target miRNAs by high-throughput CLIP-Seq experimental data and degradome experimental data. LncBase database (www.microrna.gr/LncBase) is a specialized database for recording lncRNA-miRNA interactions. The screening criteria of Starbase were as follows: low stringency ≥1; the screening criteria of LncBase was set to miTG-score ≥ 0.95; other parameters used the default parameters provided by the website. The final screening criteria for miRNAs were the miRNAs predicted by the combined Starbase and LncBase databases. we also used the miRWalk (http://mirwalk.umm.uni-heidelberg.de/) database to predict the target mRNAs of the predicted target miRNAs, which is a comprehensive database for predicting miRNA target genes (Dweep et al., 2011).

Construction of the LncRNA-miRNA-mRNA Network

According to ceRNA regulation theory, lncRNAs are expressed in the same direction as mRNAs, while miRNAs and mRNAs & lncRNAs are expressed in the opposite direction. lncRNA-miRNA networks were identified using intersection analysis to identify common miRNAs between predicted targeting miRNAs and DE-miRNAs. In the same way, we obtained common mRNAs. After collating lncRNA-miRNA and miRNA-mRNA relationship pairs, the lncRNA-miRNA-mRNA networks were embellished using Cytoscape software, which is an open-source network visualization software platform mainly used for the analysis of complex biological networks. It can generate network structures and hierarchies for gene expression regulatory networks, protein interaction networks, and others.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Analysis of the mRNAs in the CeRNA Network

To predict the biological function of the ceRNA network of IDD, we performed GO analysis and KEGG pathway analysis on the mRNAs in the ceRNA network using ClusterProfiler (Yu et al., 2012). The screening conditions of GO analysis and KEGG pathway analysis were $p < 0.05$ and count ≥ 2.

TABLE 1 | Basic information of the specimens.

Sample	Age	Gender	MRI grade
Control 1	25	M	II
Control 2	24	F	I
Control 3	29	M	I
Control 4	27	M	I
Control 5	30	F	II
Control 6	26	M	I
Control 7	28	M	I
IDD 1	53	F	IV
IDD 2	54	M	V
IDD 3	71	M	V
IDD 4	49	F	IV
IDD 5	80	F	V
IDD 6	28	M	IV
IDD 7	36	M	V
IDD 8	38	M	V
IDD 9	75	M	V
IDD 10	63	M	IV
IDD 11	59	F	V

M, male; F, male; MRI, magnetic resonance imaging.

Protein-Protein Interaction Network Construction

The PPI information among all mRNAs in the ceRNA networks was identified using the search tool for the retrieval of interacting genes/proteins (STRING) online platform (http://www.string-db.org/) (Szklarczyk et al., 2019), and subsequently, their interactions were imported into the Cytoscape software to construct and visualize a PPI network. Then, the MCODE analysis was used to screen the top PPI networks.

Identification of Key LncRNAs

We extracted all the lncRNA nodes and calculated their degrees in the ceRNA network. Candidate lncRNAs were selected based on degree >2. We also calculated the number of first relationship lncRNA-miRNA pairs and secondary relationship miRNA-mRNA pairs. The top five lncRNAs were candidate lncRNAs according to the descending order of the total number of first and second relationships. The common lncRNAs screened by both methods were identified as key lncRNAs. ceRNA sub-networks of each key lncRNA were extracted from the ceRNA network, and their relationships were demonstrated by the Sankey diagram plotted in the R package Ggalluvial. Moreover, in order to evaluate the diagnostic value of these four key lncRNAs, we constructed a nomogram and evaluated its diagnostic value using the ROC curve.

Patient Preparation

Eleven patients (mean 55.1 age) with MRI-confirmed IDD and seven volunteers (mean 25.7 age, no clinical evidence of low

TABLE 2 | The primers for qRT-PCR.

Genes	Sequence (5′-3′)
MIR100HG	F: ATTTGGTGTATCGCTTCC
	R: CCCCTTTCTTTTCTCTT
LINC01359	F: TGAAGAGGTAGCAAGAGAGC
	R: GAGGATGGAAGGATAGATGG
LUCAT1	F: GTGCTCGCTCTTGGTGA
	R: GGGGGGGAGTATGAAAC
GNAS-AS1	F: AGACCACAAAAGCATCCA
	R: GACCCAGCACAAAAACGG
GAPDH	F: CCCATCACCATCTTCCAGG
	R: CATCACGCCACAGTTTCCC

back pain or sciatica) of fasting whole blood (collected from the left median cubital vein of each participant) samples were stored in the refrigerator at minus 80°C and collected to conduct the experiment (**Table 1**). Magnetic resonance imaging scans were performed to assess the extent of disc degeneration according to the Pfirrmann classification (Castro-Mateos et al., 2016). All IDD patients underwent surgery in the Second Affiliated Hospital of Shanxi Medical University, Taiyuan, Shanxi, China. The study was approved by the hospital Ethics Review Committee. In the study, informed consent was exempted. The ethical approval number of the clinical protocol is 2022YXNO.055.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

A total of 18 whole blood samples were lysed with TRIzol Reagent (Life Technologies-Invitrogen, Carlsbad, CA, United States), and the total RNA was isolated following the manufacturer's instructions. Then, the concentration and purity of the RNA solution were quantified using a NanoDrop 2000FC-3100 nucleic acid protein quantifier (Thermo Fisher Scientific, Waltham, MA, USALife Real). The extracted RNA was reverse-transcribed to cDNA using the SureScript-First-strand-cDNA-synthesis-kit (Genecopoeia, Guangzhou, China) prior to qRT-PCR. The qRT-PCR reaction consisted of 3 μl of reverse transcription product, 5 μl of 5 × BlazeTaq qPCR Mix (Genecopoeia, Guangzhou, China), and 1 μl each of forward and reverse primer. PCR was performed in a BIO-RAD CFX96 Touch TM PCR detection system (Bio-Rad Laboratories, Inc., United States) under the following conditions: initial denaturation at 95°C for 1 min, followed by 40 cycles that each involved incubation at 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s. All primers (**Table 2**) were synthesized by Servicebio (Servicebio, Wuhan, China). The GAPDH gene served as an internal control, and the relative expression of four key lncRNAs was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The experiment was repeated in triplicate on independent occasions. Statistical differences of four key lncRNAs between normal and IDD samples were detected by paired t-tests, using GraphPad Prism V6 (GraphPad Software, La Jolla, CA, United States), and the level of statistical significance was tested and represented as * for $p < 0.05$ and ** for $p < 0.01$.

Analysis of Immune Cell Characteristics

We further evaluated the infiltrating scores of 24 immune cells with the single-sample gene set enrichment analysis (ssGSEA) in the gsva R package (Rooney et al., 2015). Moreover, the relationships between immune cells and key lncRNAs were explored through Pearson correlation analysis.

Statistical Analysis

All the statistics were done using the R software (version 4.0.2) and GraphPad Prism V6 (GraphPad Software, La Jolla, CA, United States). $p < 0.05$ was set as statistically significant for all the analyses.

RESULTS

Identification of Intervertebral Disc Degeneration-Related Aberrantly Expressed Genes

Differential expression analysis was performed using the R package limma based on transcriptomic data from the GSE124272 and GSE116726 datasets, with the significance threshold set at $|\log_2 FC| \geq 1$ and $p < 0.01$. A total of 111 DE-mRNAs (**Figure 1A**, **Supplementary Table S2**) and 20 DE-lncRNAs were identified in the GSE124272 dataset (**Figure 1B**, **Supplementary Table S3**), with 52 mRNAs and 16 lncRNAs upregulated in the IDD group ($n = 8$) and 59 mRNAs and four lncRNAs downregulated in the IDD group ($n = 8$) compared with the control group ($n = 8$). miRNAs expression profiles were obtained from the GSE116726 dataset, containing three cases each of IDD and normal samples. A total of 502 DE-miRNAs were identified, of which, 402 miRNAs were expressed up-regulated in IDD samples and 100 miRNAs were expressed downregulated in IDD samples (**Figure 1C**, **Supplementary Table S4**).

Analysis of Oxidative Stress-Related Differentially Expressed-LncRNAs-Mediated CeRNA Networks

After calculating the Spearman correlation of 2,129 OSGs with 43596 lncRNAs (from the GSE124272 dataset), a total of 1565 OS-related lncRNAs were identified based on a significance threshold of $|cor| > 0.8$ and $p < 0.01$ (**Supplementary Table S5**). Subsequently, an intersection analysis was performed using the R package VennDiagram to identify the common lncRNAs between OS-related lncRNAs and DE-lncRNAs. The results are shown in **Figure 2**, and a total of 16 OS-related DE-lncRNAs were identified, of which 14 were up-regulated and two were down-regulated in the IDD (**Table 3**).

Based on the prediction results from Starbase, LncBase, and miRWalk databases, after combining DE-miRNAs and DE-mRNAs according to ceRNA regulation theory, we finally visualized a ceRNA network containing eight OS-related DE-

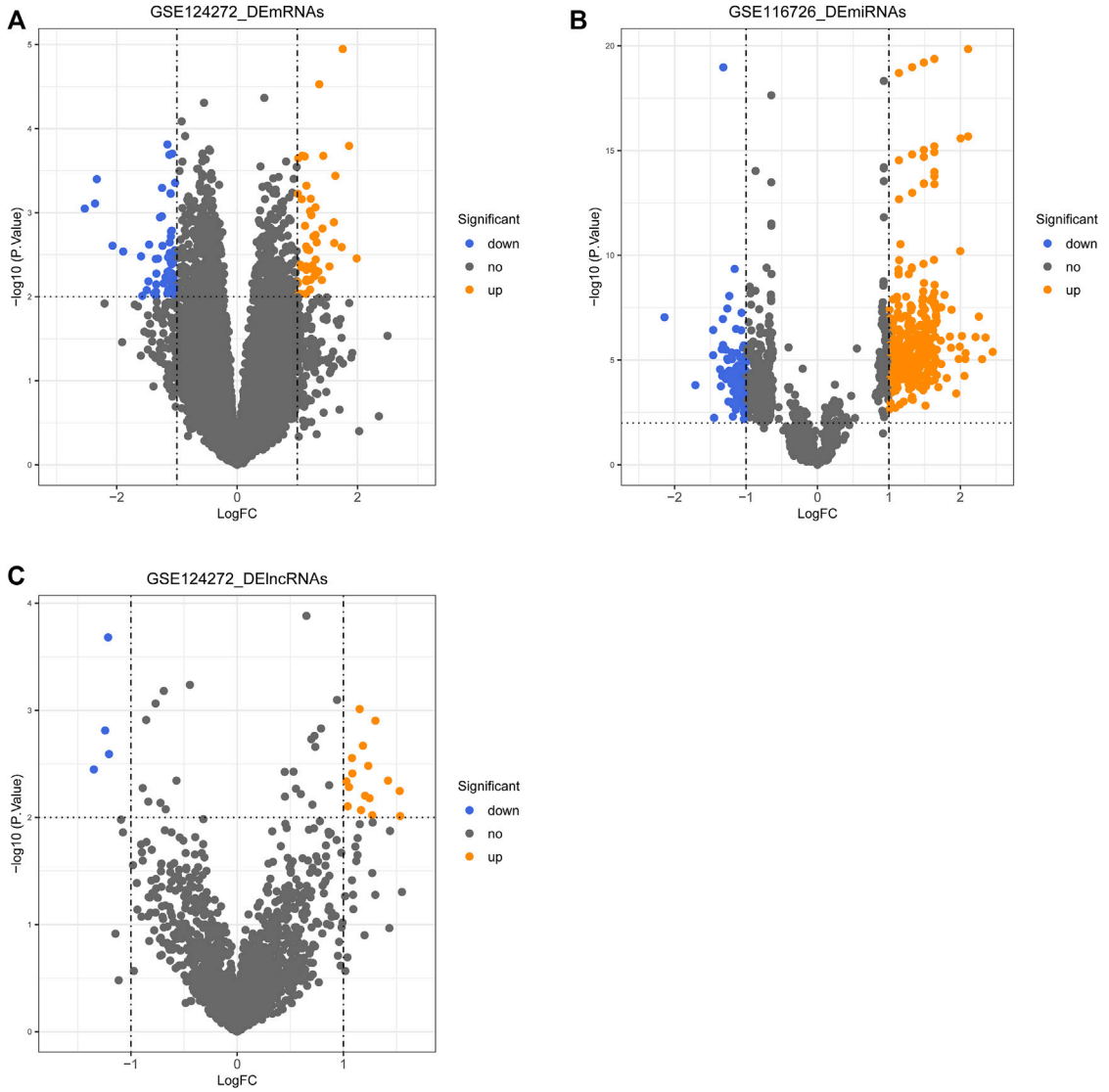

FIGURE 1 | Volcano plot of differentially expressed genes (DEGs), lncRNAs (DE-lncRNAs), and miRNAs (DE-miRNAs) in the IDD group compared with the control group. **(A)** Volcano plot of DE-mRNAs. **(B)** Volcano plot of DE-lncRNAs. **(C)** Volcano plot of DE-miRNAs.

lncRNAs, 24 DE-miRNAs, and 70 DE-mRNAs by Cytoscape (**Figure 3**), which featured 183 edges (**Supplementary Table S6**) and a total of 159 ceRNA regulatory mechanisms (**Supplementary Table S7**). Specifically, lncRNA GNAS-AS1 (downregulated) could downregulate the expression of 35 mRNAs by competitive binding to seven DE-miRNAs (up-regulated); lncRNA RNF157-AS1 (downregulated) could simultaneously regulate the expression of 16 DE-mRNAs (downregulated) by competitive binding to hsa-miR-7152-3p (upregulated); lncRNA AADACL2-AS1 (upregulated) could act as a sponge for hsa-miR-127-3p (downregulated) to up-regulate the expression of nine mRNAs; lncRNA LINC01359 (upregulated) could regulate the expression of 16 DE-mRNAs (upregulated) by competitive binding with 3 DE-miRNAs (downregulated); lncRNA LUCAT1 (upregulated) regulated 8 DE-mRNAs (upregulated) through competitive binding to hsa-miR-181c-5p (downregulated) and hsa-miR-6815-5p (downregulated); lncRNA MIR100HG (upregulated) with 8 DE-miRNAs (downregulated) and 25 DE-mRNAs (upregulated) formed 38 ceRNA regulatory mechanisms; lncRNA STARD7-AS1 (upregulated) regulated the expression of three DE-mRNAs (upregulated) when acting as a sponge for hsa-miR-151a-5p (downregulated) and hsa-miR-151b (downregulated).

Subsequently, we performed functional enrichment analysis for 70 DE-mRNAs in the ceRNA network to initially explore the regulatory mechanisms of the eight OS-related DE-lncRNAs-mediated ceRNA networks. A total of 30 BP terms, 2 CC terms, and 11 MF terms were enriched by the GO system (**Supplementary Table S8**). In this study, which focused on

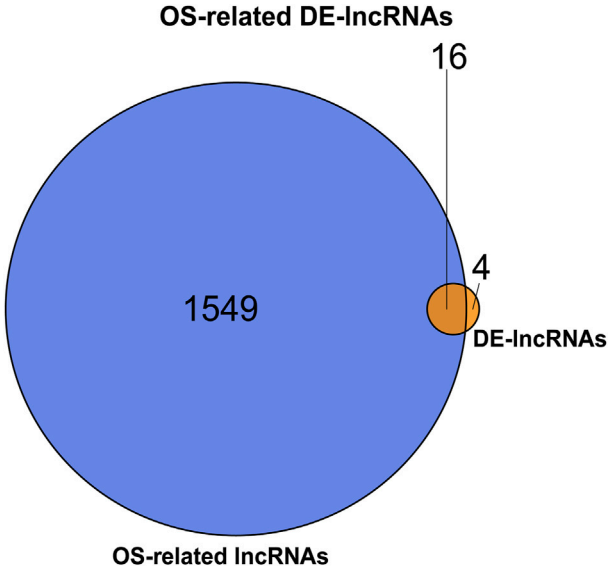

FIGURE 2 | Venn diagram of common lncRNAs between OS-related lncRNAs and DE-lncRNAs.

BP categories, we found that these DE-mRNAs were closely related to the secretion of cytokines and their regulatory processes. Meanwhile, neuron-related terms such as ensheathment of neurons, axon ensheathment, and negative regulation of vascular permeability were significantly enriched, suggesting that ceRNA networks may be involved in regulating the pain or other proprioceptive sensations in IDD patients' transmission. In addition, these DE-mRNAs were enriched in lipid-related biological processes, suggesting crosstalk between obesity and IDD. Moreover, angiogenic biological processes, which are considered to be the main cause of disc-related diseases, were significantly enriched. **Figure 4A** shows the top 10 terms in the GO-BP category. KEGG analysis revealed that these DE-mRNAs were significantly involved in the PPAR signaling pathway, fatty acid biosynthesis, degradation, and metabolic pathway, and adipocytokine signaling pathway (**Figure 4B**, **Supplementary Table S9**). Additionally, we explored the interaction relationships of 70 DE-mRNAs by the STRING tool. The confidence was set to 0.15 and after removing discrete proteins, finally, we could visualize a PPI network containing 63 nodes and 108 edges (**Figure 5A**). Moreover, we screened the top two clusters with the highest clustering scores (**Figure 5B**).

Screening of Key Oxidative Stress-Related Differentially Expressed-LncRNAs

To select key lncRNAs from the ceRNA network, we performed the following analysis. The degree values of each lncRNAs in the ceRNA network were obtained by the NetworkAnalyst tool of Cytoscape software. The results are shown in **Table 4**, and we identified five nodes as candidate key lncRNAs (degree >2), including lncRNA MIR100HG, lncRNA GNAS-AS1, lncRNA LINC01359, lncRNA STARF7-AS1, and lncRNA LUCAT1 (all degree = 2). Meanwhile, we counted the number of first-relationship lncRNA-miRNA pairs and second-relationship miRNA-mRNA pairs. The results showed that lncRNA GNAS-AS1, MIR100HG, LINC01359, RNF157-AS1, and LUCAT1 were the top five lncRNAs (**Table 5**). Taken together, the four lncRNAs had higher degree values and more lncRNA-miRNA and miRNA-mRNA pairs, implying that these four lncRNAs were highly associated with the occurrence of IDD. The four lncRNAs were lncRNA GNAS-AS1 (GNAS Antisense RNA 1), lncRNA MIR100HG (Mir-100-Let-7a-2-Mir-125b-1 Cluster Host Gene), lncRNA LINC01359 (Long Intergenic Non-Protein Coding RNA 1359), lncRNA LUCAT1 (Lung Cancer Associated Transcript 1), which were defined as key lncRNAs.

In order to evaluate the diagnostic value of these four key lncRNAs, we constructed a nomogram and evaluated its diagnostic value using the ROC curve. We found that the AUC value of these four key lncRNAs and nomogram was greater than 0.8, indicating that these four key lncRNAs had high diagnostic value (**Figure 6**).

TABLE 3 | Differential expression of 16 OS-related DE-lncRNAs between normal and IDD groups.

Symbol	ID	logFC	*p*-value	Type
GRIK1-AS1	ENSG00000174680	1.533548116	0.009711464	Up
AADACL2-AS1	ENSG00000242908	1.529591952	0.005669244	Up
KCNJ2-AS1	ENSG00000267365	1.419842136	0.004527719	Up
NFE4	ENSG00000230257	1.271172161	0.009540417	Up
COL4A2-AS1	ENSG00000232814	1.245683747	0.006615357	Up
JMJD1C-AS1	ENSG00000272767	1.203987822	0.006267746	Up
MIR100HG	ENSG00000255248	1.183718415	0.002141863	Up
LINC00029	ENSG00000125514	1.165817974	0.008548733	Up
SYP-AS1	ENSG00000237341	1.152784528	0.000973094	Up
LINC01359	ENSG00000226891	1.084122661	0.003884443	Up
LUCAT1	ENSG00000248323	1.080241162	0.002788182	Up
MIR646HG	ENSG00000228340	1.052595815	0.005208917	Up
LINC00906	ENSG00000267339	1.040172614	0.007876342	Up
STARD7-AS1	ENSG00000204685	1.02798182	0.004603308	Up
RNF157-AS1	ENSG00000267128	−1.206265373	0.002561201	Down
GNAS-AS1	ENSG00000235590	−1.214634107	0.000208454	Down

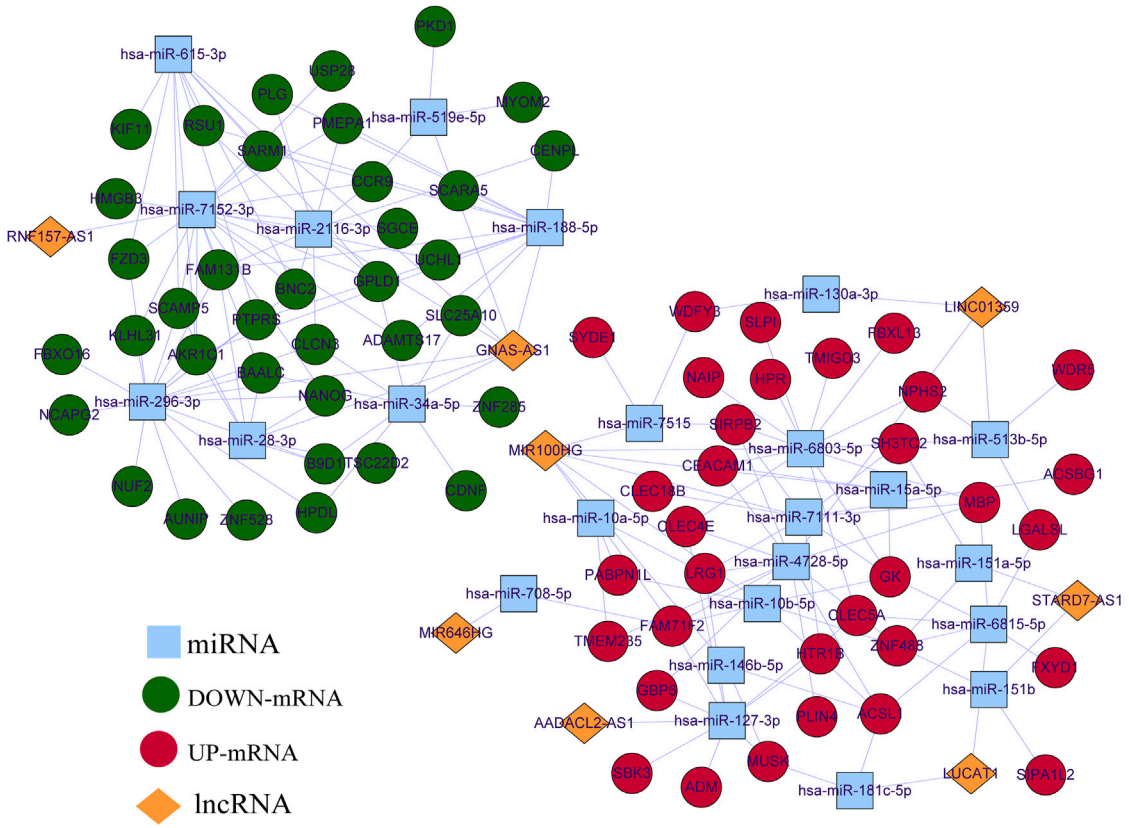

FIGURE 3 | CeRNA network of OS-related DE-lncRNAs, DE-miRNA, and DE-mRNA. Red circle nodes represent up-regulated expression of mRNAs. Green circle nodes represent down-regulated expression of mRNAs. Yellow rhombus nodes represent upregulated expression of lncRNAs. Purple rhombus nodes represent downregulated expression of lncRNAs.Sky-blue square nodes represent DE-miRNAs.

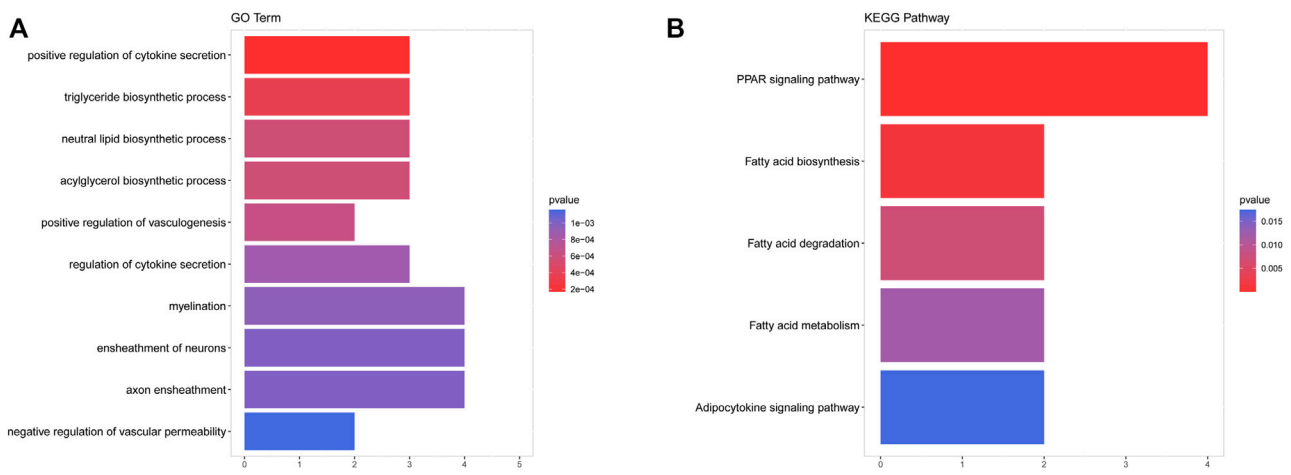

FIGURE 4 | Functional enrichment of analysis of DE-mRNAs based on GO and KEGG. **(A)** The top 10 terms in the GO-BP category of GO analyses DE-mRNAs. **(B)** KEGG pathway analysis of DE-mRNAs.

Construction of LncRNA-miRNA-mRNA Subnetworks

We selected four key lncRNAs related to IDD, extracted the miRNAs and mRNAs related to them, and reconstructed the corresponding lncRNA-miRNA-mRNA subnetwork. The lncRNA GNAS-AS1-miRNA-mRNA network was composed of one lncRNA node, seven miRNA nodes, 35 mRNA nodes, and 67 edges (**Figure 7A**). The lncRNA MIR100HG-miRNA-mRNA subnetwork consisted of

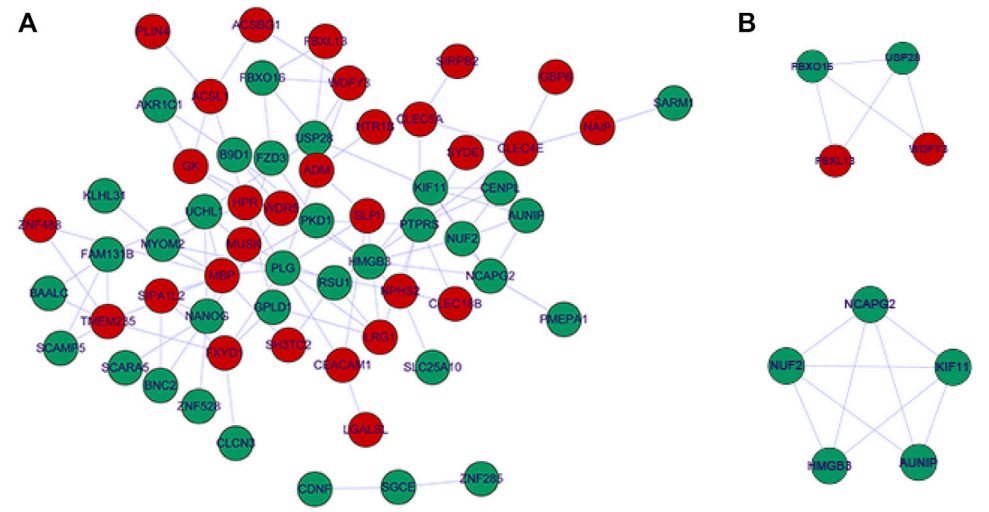

FIGURE 5 | Protein-protein interaction network complex. **(A)** The protein-protein interaction network visualized by Cytoscape. Red nodes represent up-regulated expression, Green nodes represent up-regulated expression. **(B)** Top two PPI networks in MCODE analysis.

TABLE 4 | The degree values of each lncRNAs in the ceRNA network.

Rank	Gene name	Degree	Gene type
1	MIR100HG	7	lncRNA
2	GNAS-AS1	7	lncRNA
3	LINC01359	3	lncRNA
4	STARD7-AS1	2	lncRNA
5	LUCAT1	2	lncRNA
6	AADACL2-AS1	1	lncRNA
7	MIR646HG	1	lncRNA
8	RNF157-AS1	1	lncRNA

one lncRNA node, seven miRNA nodes, 25 mRNAs, and 37 edges (**Figure 7B**). The lncRNA LINC01359-miRNA-mRNA subnetwork consisted of one lncRNA node, three miRNA nodes, 16 mRNA nodes, and 16 edges (**Figure 7C**). The lncRNA LUCAT1-miRNA-mRNA subnetwork consisted of one lncRNA node, two miRNA nodes, eight mRNA nodes, and nine edges (**Figure 7D**).

Verification of Four Key LncRNAs

To verify the authenticity of the key lncRNAs we identified, we selected eleven blood samples from IDD patients and seven blood samples from healthy control subjects for qRT-PCR molecular validation. The results have shown significant differences ($p < 0.05$) in the relative expression of all key lncRNAs between IDD patients and healthy control subjects, as shown in **Figure 8**. Compared with healthy subjects, lncRNAs MIR100HG, LINC01359, and LUCAT1 were significantly upregulated in the IDD group (**Figures 8A–C**), whereas the expression level of lncRNA GNAS-AS1 was significantly downregulated in the IDD group (**Figure 7D**), and these results were consistent with bioinformatic analysis (**Table 3**).

Association of Key LncRNAs With Immune Infiltrating Cells

We assessed the abundance of immune infiltrating cells in IDD ($n = 8$) and normal ($n = 8$) samples from the GSE124272 dataset using ssGSEA. By Wilcoxon rank-sum test, we found that the abundance of neutrophils, Tem, and TReg was significantly higher in the IDD group than in the normal group; whereas CD8 T cells, cytotoxic cells, NK cells, T cells, T helper cells, Tgd, and Th2 cells in the normal group were accounted for a higher percentage (**Figure 9A**). Subsequently, we calculated the Pearson correlation of four key lncRNAs with the above 10 differentially abundant immune infiltrating cells. The results were shown in **Figure 9B**. Specifically, lncRNA GNA-AS1 was moderately negatively correlated with Neutrophils (cor = −0.68) and TReg (cor = −0.61), while moderately or strongly positively correlated with T helper cells (cor = 0.59), T cells (cor = 0.65), CD8 T cells (cor = 0.65), and Th2 cells (cor = 0.78); lncRNA LINC01359 was moderately or strongly negatively correlated with Th2 cells (cor = −0.54), T cells (cor = −0.67), and CD8 T cells (cor = −0.78), and with Neutrophils (cor = 0.52) and Tem (cor = 0.66) showed moderate positive relationship; lncRNA LUCAT1 was associated with Th2 cells (cor = −0.80), T cells (cor = −0.76), NK cells (cor = −0.63), T helper cells (cor = −0.61), and CD8 T cells (cor = −0.61) showed moderate or strong negative correlation and moderate or strong positive correlation with Neutrophils (cor = 0.84) and Tem (cor = 0.62); lncRNA MIR100HG correlated with Th2 cells (cor = −0.74), NK cells (cor = −0.60), and Cytotoxic cells (cor = −0.55), Tgd (cor = −0.53), and T cells (cor = −0.52) with moderate or strong negative correlations, and with Neutrophils (cor = 0.54) with moderate positive correlations. The research process of this study is as follows (**Figure 10**).

TABLE 5 | The number of first-relationship lncRNA-miRNA pairs and second-relationship miRNA-mRNA pairs.

Rank	Gene name	lncRNA-miRNA pairs	miRNA-mRNA pairs	Total number
1	GNAS-AS1	7	67	74
2	MIR100HG	7	37	44
3	LINC01359	3	16	19
4	RNF157-AS1	1	16	17
5	LUCAT1	2	9	11
6	AADACL2-AS1	1	9	10
7	STARD7-AS1	2	4	6
8	MIR646HG	1	1	2

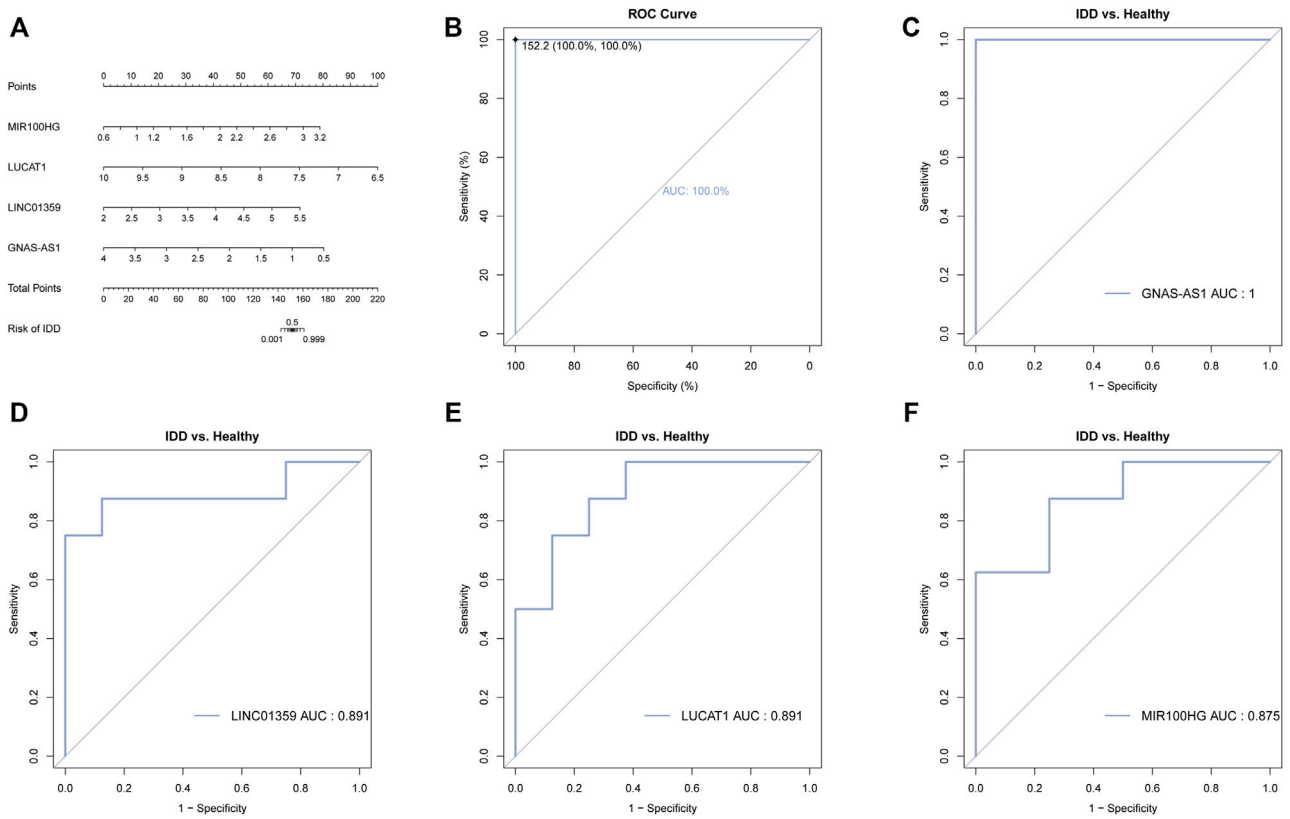

FIGURE 6 | Diagnostic and prognostic value of the four key lncRNAs. **(A)** Nomogram of the four key lncRNAs. The value of each lncRNA was given a score on the point scale axis. A total score could be easily calculated by adding every single score and, by projecting the total score to the lower total point scale, we were able to estimate the probability of IDD. **(B)** The calibration curve for the nomogram. **(C)** ROC analysis of GNAS-AS1. **(D)** ROC analysis of LINC01359. **(E)** ROC analysis of LUCAT1. **(F)** ROC analysis of MIR100HG.

DISCUSSION

As we know, a serious imbalance between endogenous and exogenous reactive oxygen species and the body's antioxidant defense system can lead to oxidative stress (OS).OS plays an important role in disc degeneration, causing disc cell death and extracellular matrix degeneration (Zhang et al., 2020). An increasing number of studies have demonstrated that some non-coding RNAs, such as circRNAs, LncRNAs and micro RNAs (miRNAs), serve as ceRNAsthat regulate IDD initiation and progression (Li et al., 2018; Zhan et al., 2019). Analysis of the characteristics of OSRG associated with IDD, as well as the regulatory mechanisms of ceRNA and immune infiltration analysis, may help researchers to distinguish IDD and reveal the underlying mechanisms (Gámez-Valero et al., 2020).

In this study, we conducted functional enrichment analysis of 70 DE-mRNAs in the ceRNA network, and preliminarily explored the regulatory mechanism of eight OS-related DE-lncRNAS mediated ceRNA networks. GO enrichment analyses were conducted to find these DE-mRNAs were closely related to the secretion of cytokines and their regulatory processes. In IDD, cytokines production is increased. Production of cytokines is

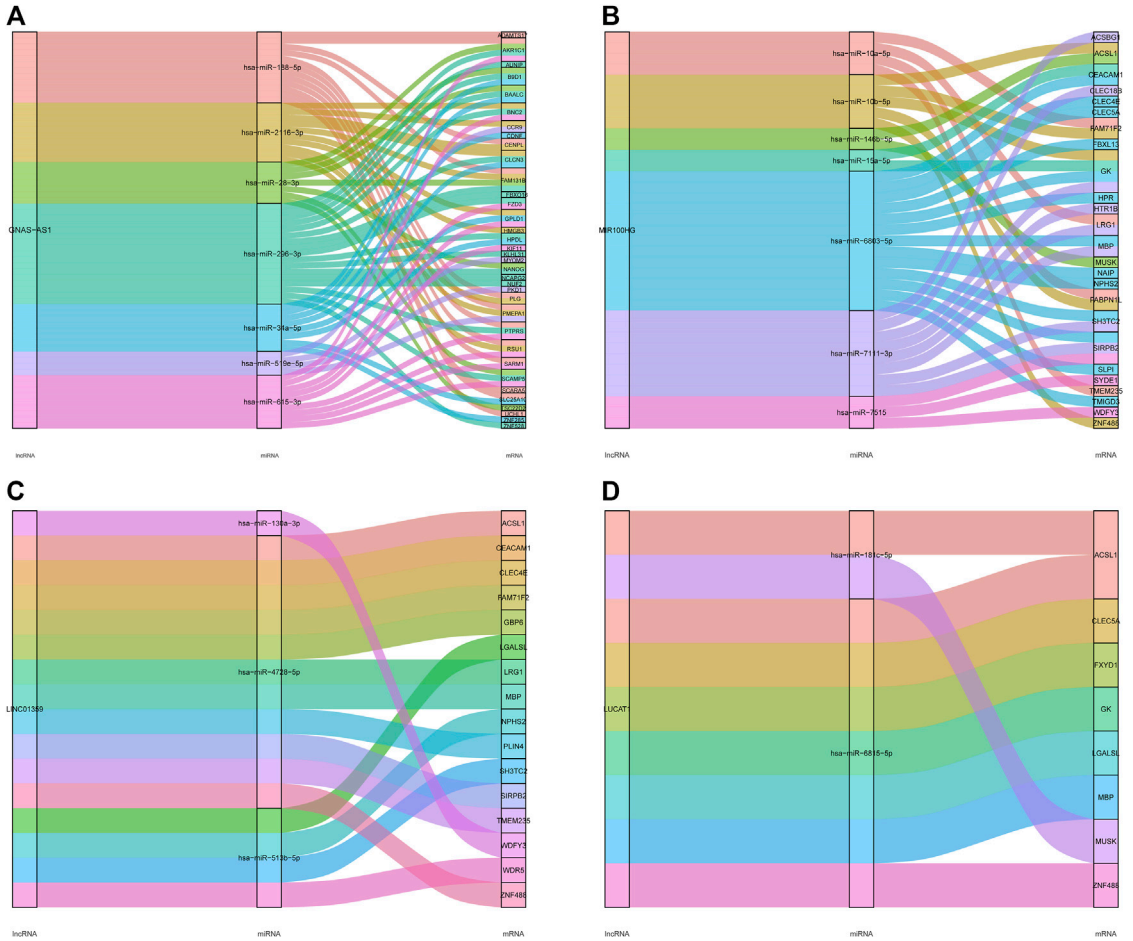

FIGURE 7 | LncRNA-miRNA-mRNA subnetwork of the four key lncRNAs. **(A)** The lncRNA GNAS-AS1-miRNA-mRNA network. **(B)** The lncRNA MIR 100HG-miRNA-mRNA network. **(C)** The lncRNA LINC01359-miRNA-mRNA network. **(D)** The lncRNA LUCAT1-miRNA-mRNA network.

more common in degenerative discs than in normal discs (Roberts et al., 2006). There is also evidence that cytokines may be the cell nucleus pulposus-induced nerve lesions of factors, such as inflammatory cytokines IL-1β and TNF-α, are considered to be the key mediators of IDD that can effectively accelerate the IDD progress, and they also upregulated in the IDD. They are closely related to the various pathological processes of IDD, including inflammation, matrix damage, cell aging, autophagy, and apoptosis (Battié et al., 2009; Yang et al., 2020). Meanwhile, neuron-related terms such as ensheathment of neurons, axon ensheathment, and negative regulation of vascular permeability were significantly enriched. Some studies suggest that the growth of sensory afferent fibers into the disc may lead to discgenic pain after IDD (Leimer et al., 2019; Zhang et al., 2021). Fiber wrapping, changes in axon distance, and loss of myelin sheath can lead to neural structural abnormalities that may lead to the onset of pain and the exacerbation of chronic pain (Brifault et al., 2020). In addition, these DE-mRNAs were enriched in lipid-related biological processes, suggesting crosstalk between obesity and IDD. In addition to mechanical effects, obesity has important metabolic and inflammatory effects on the homeostasis of the intervertebral disc environment, which are mediated by adipokines (Ruiz-Fernández et al., 2019). Leptin, the most common adipokines, and adiponectin have a significant effect on the internal environment of the intervertebral disc. Adipose tissue can cause a systemic inflammatory state through secretion of pro-inflammatory adipocytokine, leptin (Sharma, 2018). Leptin can regulate proteomes through different signal transduction pathways. In contrast, adiponectin has been reported to play an anti-inflammatory role in diseased disc tissue (Sharma, 2018; Brifault et al., 2020). Moreover, angiogenic biological processes, which are considered to be the main cause of disc-related diseases. Disc degeneration is associated with angiogenesis. Degenerative disc disease is thought to be characterized by angiogenesis and increased expression of vascular endothelial growth factor (VEGF). Angiogenesis affects the pain intensity of disc herniation and negatively affects postoperative pain improvement, mobility, and overall quality of life (Lee et al., 2011; Sheng et al., 2018). KEGG analysis revealed that these DE-mRNAs were significantly involved in the PPAR signaling pathway, fatty acid biosynthesis, degradation, and metabolic pathway, and

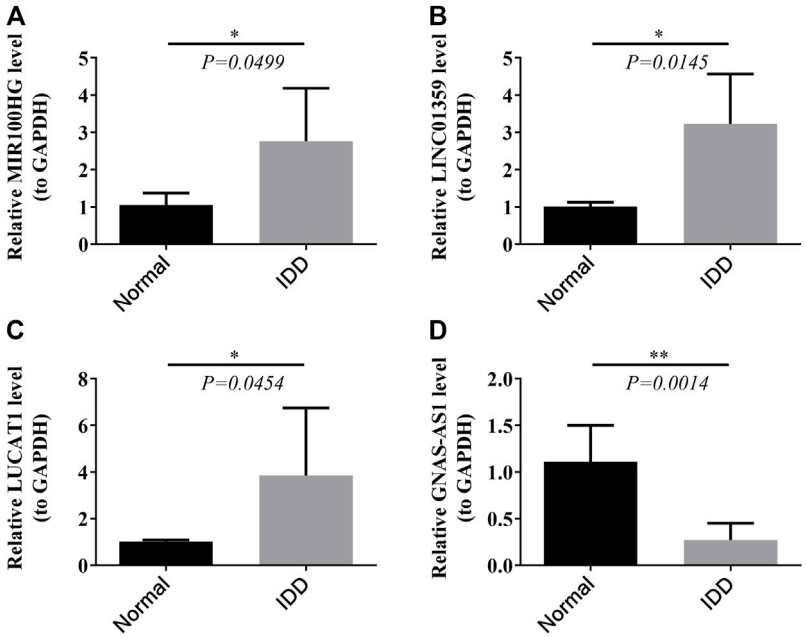

FIGURE 8 | Relative expression of four key lncRNAs MIR 100HG **(A)**, LINC01359 **(B)**, LUCAT1 **(C)**, and GNAS-AS1 **(D)** in clinical samples by RT-PCR.

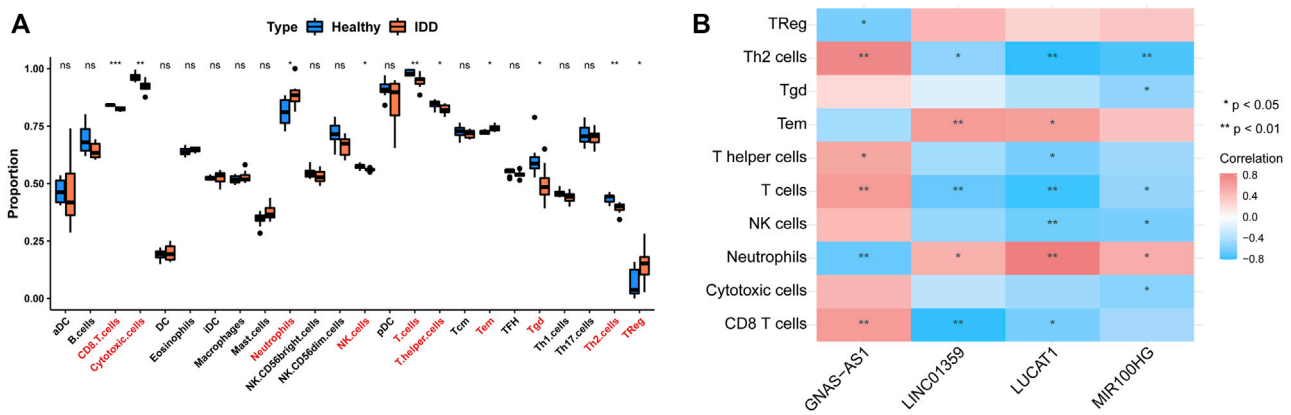

FIGURE 9 | Single sample GSEA and Pearson correlation analysis of immune infiltrating cells. **(A)** The proportion of immune infiltrating cells in IDD and healthy group. **(B)** The correlation of the above 10 differentially abundant immune infiltrating cells with four key lncRNAs.

adipocytokine signaling pathway. These pathways are associated with obesity (Mao et al., 2021).

In this study, we identified four differentially expressed lncRNAs associated with oxidative stress. These are lncRNA GNAS-AS1, lncRNA MIR100HG, lncRNA LINC01359, lncRNA LUCAT1. GNAS-AS1 has been reported to promote tumor progression in non-small cell lung cancer (NSCLC) by altering macrophage polarization via the GNAS-AS1/mir-4319/NECAB3 axis (Li et al., 2020b). GNAS-AS1 plays a carcinogenic role by mediating β-catenin expression and may be an important gene involved in the formation and progression of nasopharyngeal carcinoma (Wang et al., 2020). Overexpression of Mir-185 Inhibits disc degeneration by inactivating the Wnt/β -catenin signaling pathway and galactose agglutinase-3 (Yun et al., 2020). Although there are no studies on the relationship between GNAS-AS1 and Wnt/β -catenin signaling Pathway, we have reason to believe that GNAS-AS1 can affect the process and development of IDD by regulating Wnt/β -catenin Signaling Pathway. GNAS-AS1 has also been reported as a prognostic indicator of osteosarcoma (Mi et al., 2021). MIR100HG promotes the development of triple-negative breast cancer (TNBC) by regulating the P27 gene and sponging mir-5590-3p. MIR100HG regulates p27 gene to control cell cycle. In G1 phase, knockdown of MIR100HG reduced cell proliferation

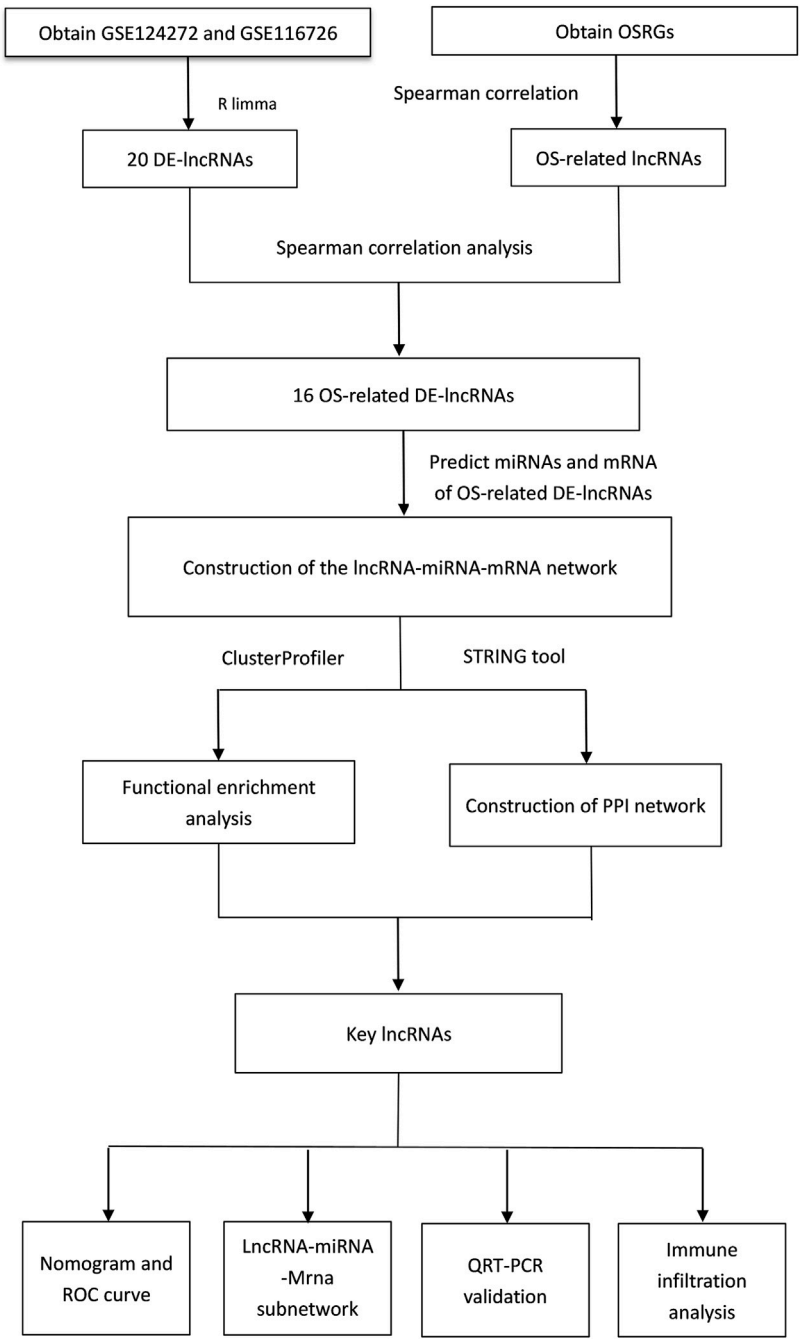

FIGURE 10 | Flowchart of key lncRNAs associated with oxidative stress were identified by GEO database data and whole blood analysis of intervertebral disc degeneration patients.

and induced cell stagnation. Overexpression of MIR100HG significantly increased cell proliferation, and mir-5590-3p expressed the opposite effect (Wang et al., 2018b; Chen et al., 2020). The lack of the P27 gene activates the expression of Shh signaling pathway and promotes the proliferation of osteoblasts, thus playing a role in promoting IDD (Liu et al., 2017). MIR100HG may promote the proliferation, migration, and invasion of laryngeal squamous cell carcinoma (LSCC) through down-regulation of mir-204-5p (Huang et al., 2019). It is also associated with ductal adenocarcinoma of the pancreas and colorectal cancer (Ottaviani and Castellano, 2018; Peng et al., 2022). LINC01359 may be linked to hepatocellular carcinoma (HCC), that's all we got (Tan et al., 2019). LUCAT1 is not available in PubMed, but the silencing of hsa-miR-181c-5p can inhibit the proliferation and promote the apoptosis of nucleus pulposus cells (Meng and Xu, 2021). We revealed a direct

association between these four key lncRNAs and IDD, it may be important for the pathogenesis and clinical treatment exploration of IDD, which will be our focus in the future.

In addition to the above regulatory mechanisms, patterns of immune infiltration are also very important. Neutrophils, a type of white blood cell, are the most abundant in white blood cells, accounting for about 50%–70%, and are an important part of innate immunity. It has been suggested that neutrophils accumulate in IDD tissues and directly act on degenerative intervertebral discs by releasing relevant inflammatory factors, forming a vicious cycle. Neutrophils were significantly associated with IDD progression (Li et al., 2022). This was also consistent with our results that neutrophils were significantly higher in the IDD group than in the normal group. Effector memory T cell (Tem) is a type of T cell that performs immune protection by rapidly producing effector cytokines. NK cells and Tem secrete IL-4 and IFN-γ to play an immune role. Regulatory T cells (Tregs) are a subgroup of immunosuppressive T cells that secrete anti-inflammatory cytokines such as IL-4 and IL-10 to suppress inflammatory responses. It has been shown that both IFN-γ and IL-4 are elevated in degenerative intervertebral discs, and IFN-γ affects IDD by inducing the release of inflammatory cytokines and increasing ICAM-1 expression (Gabr et al., 2011; Risbud and Shapiro, 2014). T cell is the main component of lymphocytes and is a very important group in human immunity. There are a lot of different types of T cells, based on different criteria. Helper T cells are the largest subgroup of T cells, with many types and different functions. Th2 is one of the helper T cells, and the effector molecules are IL-4, IL-5, IL-10, and IL-13. Studies have shown that IL-10 reduction can accelerate disc degeneration in animal models (Wang et al., 2018a). A series of studies have shown that intervertebral disc degeneration can be alleviated by inhibiting IL-13, as well as blocking the associated cellular signaling pathways and inhibiting fibrosis in tissues (Van Dyken and Locksley, 2013; Li et al., 2019). This is different from our experimental results, which may be related to the number of different effect factors. CD8 was expressed in 30%–50% T cells and differentiated into cytotoxic T cells (CTL) after activation. Usually called CD8+T cells are CTLs, its main function is to kill target cells. Recent studies have indicated that CD8 T cells in rat IDD models are more prone to apoptosis, and CD8 T cells play a role in the pathogenesis of IDD and apoptosis (Li et al., 2020a; Cao et al., 2021). T cells gamma delta (Tgd) is a unique group of lymphocytes that are often enriched on the surface of epithelial cells. It regulates not only inflammation, but also autoimmune diseases (Yeung et al., 2000; Paul et al., 2014). It may play a role in IDD by modulating immune and inflammatory responses (Ma et al., 2022).

There are some limitations to this experiment. The sample size of qRT-PCR is insufficient, so the sample size should be increased for further study. None of the four key lncRNAs we obtained has been reported in IDD.

CONCLUSION

In this study, DE-lncRNAs, DE-miRNAs, DE-mRNAs, and OS-related DE-lncRNAs in the IDD group and control group were obtained through the GEO database and GeneCards database. The new four key lncRNAs were screened by constructing a ceRNA network, namely lncRNA GNAS-AS1, lncRNA MIR100HG, lncRNA LINC01359, and lncRNA LUCAT1, and further validated by qRT-PCR. We also identified 10 immune infiltrating cells associated with key lncRNAs, suggesting that extensive infiltration of neutrophils, Tem, and Tregs may be associated with the development of IDD. The four new lncRNAs identified by us can provide help for the early diagnosis and treatment of IDD. We will continue to pay attention to the role of these lncRNAs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Second Hospital of Shanxi Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

WHS and XLJ designed and directed the project; XLJ, JFW, and CHG conducted the experiments and analyzed the data; XLJ wrote the manuscript. WHS supervised the study and revised the manuscript. All authors read and approved the final manuscript.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.929843/full#supplementary-material

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A novel mutation in *SPINK5* gene underlies a case of atypical Netherton syndrome

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Netherton syndrome (NS, OMIM #256500) is a rare autosomal recessive disease characterized by a triad of congenital ichthyosiform erythroderma (CIE) or ichthyosis linearis circumflexa (ILC), trichorrhexis invaginata (TI), and atopic predisposition. The disease is caused by a mutation in the *SPINK5* gene (serine protease inhibitor of Kazal type 5) encoding LEKTI (lymphoepithelial Kazal type-related inhibitor). We performed whole-exome sequencing on one Chinese NS family and made genotype–phenotype correlation analysis on the patients clinically diagnosed with NS or congenital ichthyosis erythroderma. We identified a novel frameshift mutation c.2474_2475del (p.Glu825Glyfs*2) in the *SPINK5* gene. The N-terminal mutations of LEKTI cause a severer phenotype, while the C-terminal mutations of LEKT1 are related to a milder phenotype. Our findings suggest that Netherton syndrome may be underestimated clinically, and our findings further expand the reservoir of *SPINK5* mutations in Netherton syndrome.

KEYWORDS

Netherton syndrome, *SPINK5*, congenital ichthyosiform erythroderma, mutation, phenotype

Introduction

Netherton syndrome (NS, OMIM #256500) was first described in 1958 by Earl Netherton. This disorder was characterized by a classical triad of congenital ichthyosiform erythroderma (CIE) or ichthyosis linearis circumflexa (ILC), trichorrhexis invaginata (TI), and atopic diathesis. The exact incidence of NS is unknown, but estimates range from 1:100,000 to 1:200,000 live births (Abdalrheem et al., 2020). Congenital ichthyosiform erythroderma is usually present at birth or shortly thereafter and manifests as generalized erythroderma with desquamation. Ichthyosis linearis circumflexa is characterized by migratory, serpiginous, erythematous patches with double-edged scales at the periphery. ILC is usually not permanent and can flare up after an interval (Hovnanian, 2013). The other common features of NS are recurrent infections, abnormal IgG level, bowel disease,

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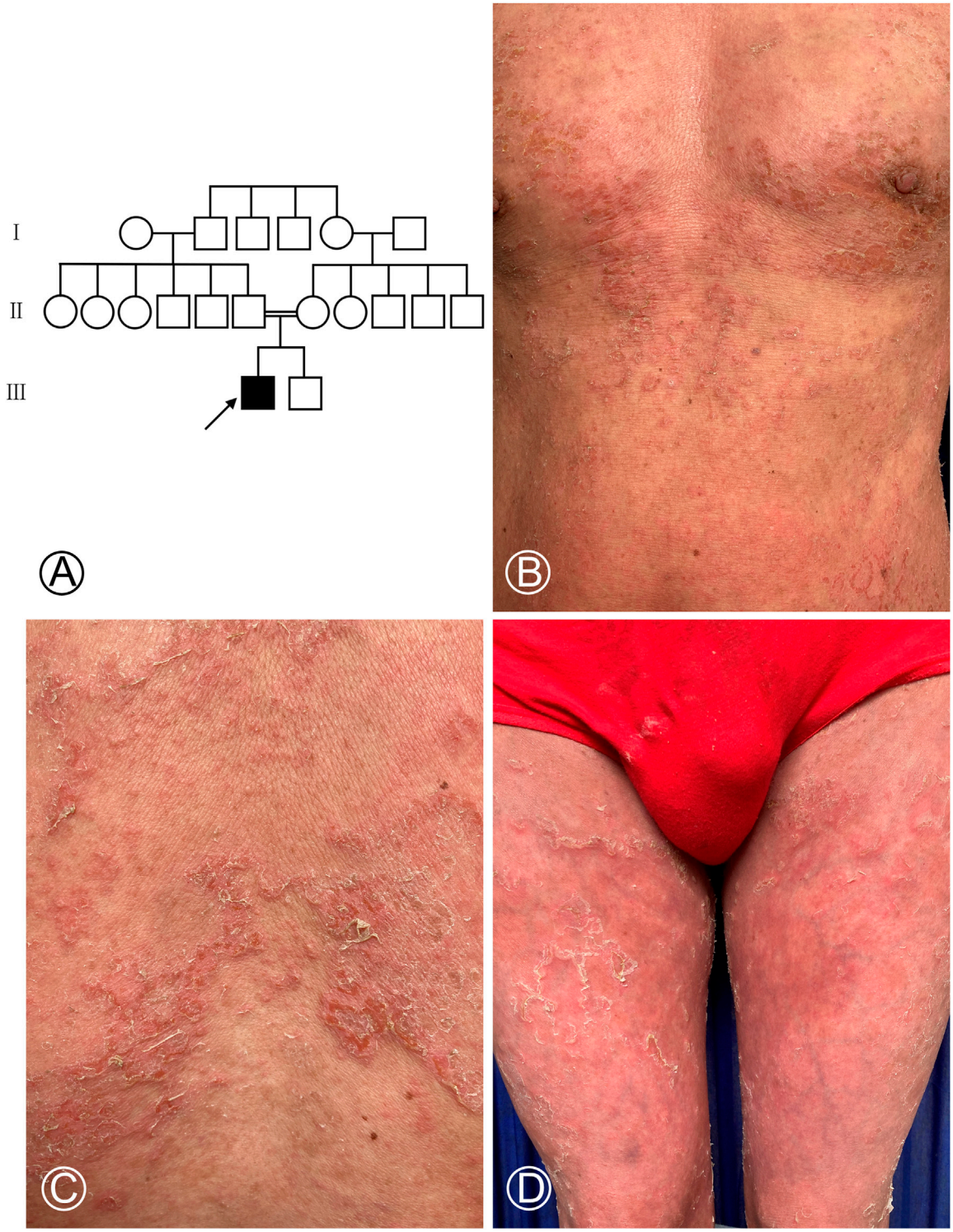

FIGURE 1
Pedigree chart of the NS-affected family and cutaneous manifestations of the proband. **(A)** Proband is marked with an arrow. Females were indicated by circles, while males were indicated by squares. The blackened symbol represented a diseased member. **(B–D)** Diffuse erythema covered with greyish white scales on the chest, abdomen, and both thighs and scattered multiple blisters in the chest.

hypernatremia, elevated IgE level, and low albuminemia (Śmigiel et al., 2017). NS is caused by a loss-of-function mutation of *SPINK5* (serine protease inhibitor of Kazal type 5) on chromosome 5q31-32 that encodes a lymphoepithelial Kazal-type related inhibitor (LEKTI) (Sarri et al., 2017; Herz-Ruelas et al., 2021). The LEKTI plays a critical role in maintaining skin barrier function and regulating the desquamation of keratinocytes (Chavanas et al., 2000; Flora and Smith, 2020).

Currently, there is no cure or satisfactory treatments for NS. Some modalities are used to relieve the disease, such as topical emollients, topical calcineurin inhibitors, narrowband ultraviolet B, psoralen plus UVA, topical or systemic corticosteroids, and topical or systemic acitretin (Lazaridou et al., 2009; Maatouk et al., 2012). Intravenous immunoglobulin and infliximab were attempted to treat a few NS patients with severe illness (Small and Cordoro, 2016; Roda et al., 2017).

Clinically, it is very difficult to distinguish atypical NS from congenital ichthyosiform erythroderma or other types of ichthyosis. Therefore, the mutation analysis of *SPINK5* gene is an important method in the diagnosis of atypical NS cases.

Case presentation

Ethical compliance

Following written informed consent, skin biopsies and hair samples were collected from the patient for histopathological examination and scanning electron microscope study at the Dermatology Clinic of The First Affiliated Hospital of Anhui Medical University, and blood samples were obtained from the patient and his family members for genetic analysis. This study was approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University, and all procedures adhered to the principles of the Declaration of Helsinki.

Report

A 35-year-old male was admitted to our outpatient department for generalized erythema on his body with pruritus. The marriage of his parents was consanguineous. Some erythematous skin lesions were noted on his body 3 months after birth and gradually spread throughout the body with age. Sometimes, some blisters occurred. He denied a history of atopic dermatitis, asthma, or allergic rhinitis. Dermatological examination showed diffuse erythema with desquamation, and multiple flaccid vesicles over his whole body (Figure 1). His palms, soles, and oral mucosa were spared. His scalp hairs looked yellowish and lusterless and were easily plucked. His parents and younger brother lacked cutaneous abnormalities. However, his younger brother suffered from deafness since birth. Laboratory examination showed normal peripheral eosinophil counts and a significant increase in the serum IgE level with 749.95 IU/mL (normal range <200 IU/mL). Biochemical tests and serum levels of IgG, IgA, and IgM were all within normal limits. The histopathology of a biopsy revealed remarkable hyperkeratosis, parakeratosis, hypergranulosis, and acanthosis, as well as dermal perivascular lymphocytic infiltrates (Figure 2). Under the scanning electron microscope, his hairs showed the absence of trichorrhexis invaginata/trichorrhexis nodosa. Some hair cuticles were damaged, deformed, and exfoliated on two-thirds of the lower part of the hair shaft, while the hair cuticles were normal on the upper one-third of the hair shaft (Figure 2). Initially, we considered the diagnosis as bullous congenital ichthyosiform erythroderma (BCIE, OMIM #113800) based on the patient's history and clinical and histopathological findings. None of the mutations in the related genes (*KRT1*, *KRT10*, and *KRT2e*) was found by Sanger sequencing in this family. Subsequently, whole-exome sequencing was performed to screen out a homozygous frameshift mutation in *SPINK5* gene, which was verified by Sanger sequencing. Although there was absence of trichorrhexis invaginata, the serum IgE level was significantly elevated, so this patient was finally diagnosed as affected by NS. According to the European guidelines for the management of congenital ichthyoses (Mazereeuw-Hautier et al., 2019), we recommended this patient be treated with oral acitretin, topical emollients, and topical corticosteroid ointment. Because the patient worried about adverse reactions to acitretin, he chose self-administered oral prednisone (10–15 mg/d) combined with topical emollients and topical corticosteroid ointment and maintained treatment for 6 months. The skin lesions on his chest and back improved significantly after 6 months of follow-up.

Methods

Peripheral blood collection and DNA extraction

After obtaining informed consent from all participants (including the proband, his parents, and his younger brother), EDTA-anticoagulated venous blood samples were collected from the patient and his family members for DNA analysis. DNA was extracted from peripheral blood with the use of the QIAamp DNA Mini kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. The DNA concentration was measured using the Qubit 3.0 Fluorometer (Invitrogen, Life Technologies, Van Allen Way, Carlsbad, CA, United States), and DNA that met the criteria was selected and stored at −80°C.

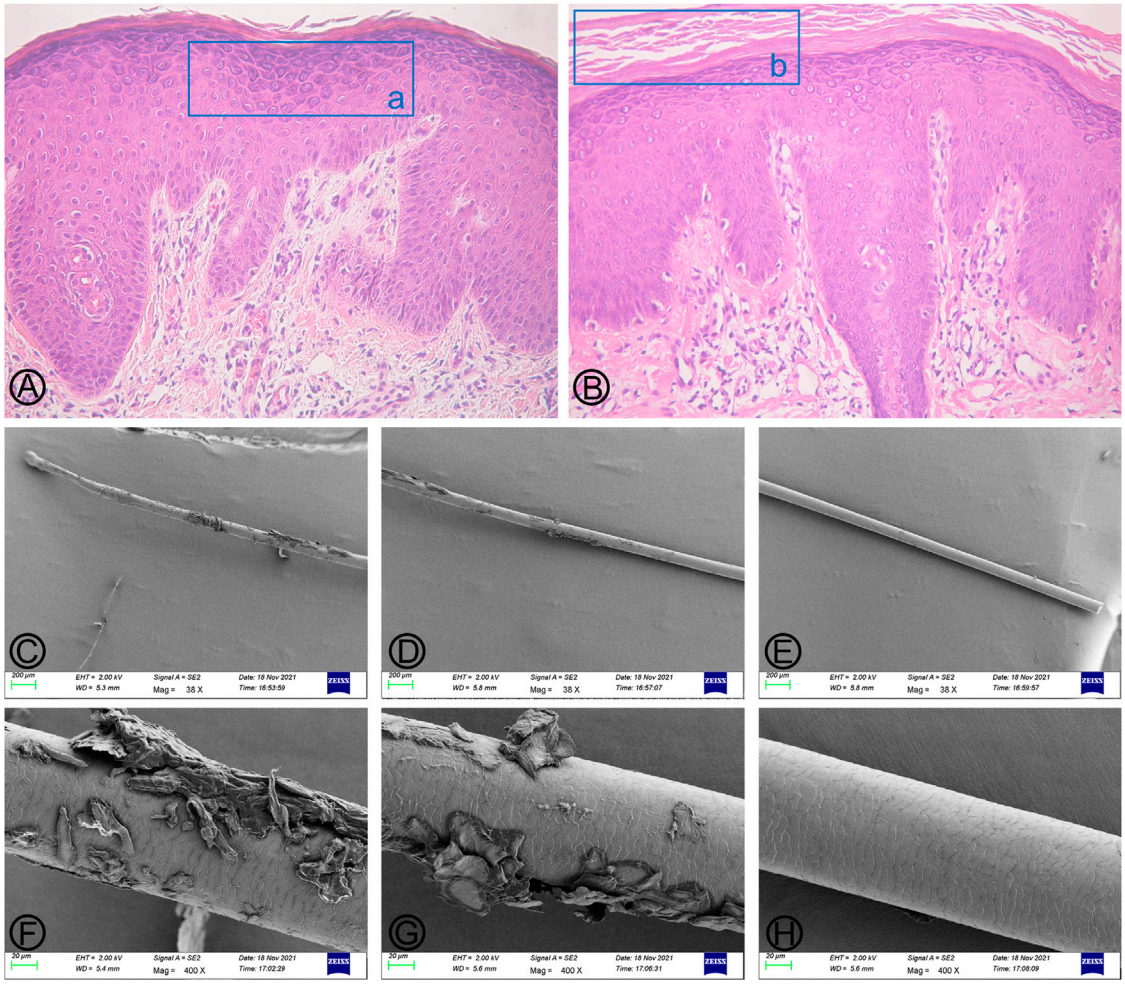

FIGURE 2
Findings of histopathological and hair scanning electron microscopy (SEM). **(A,B)** (HEx100) Marked hyperkeratosis, parakeratosis, hypergranulosis, and acanthosis, as well as dermal perivascular lymphocytic infiltrates. The blue box (a) indicates hypergranulosis, and the blue box (b) indicates hyperkeratosis. **(C–E)** (Mag×38); **(F–H)** (Mag×400) No characteristic bamboo hair was found under SEM. Some hair cuticles were damaged, deformed, and exfoliated in the two-thirds of the lower part of the hair shaft, while the hair cuticles were normal on the upper one-third of the hair shaft.

Mutation analysis of *KRT1*, *KRT10*, and *KRT2e* genes by Sanger sequencing

The mutations of *KRT1*, *KRT10*, and *KRT2e* genes in the proband, his parents, and his younger brother were analyzed by Sanger sequencing. Primers flanking all coding regions in *KRT1*, *KRT10*, *and KRT2e* were designed using Primer Premier 5.0 software (Primer Biosystems, Foster City, CA, United States). Primer sequences were provided in the supplementary data. The PCR system was configured, and the Veriti (ABI, Foster City, CA, United States) was set according to the standardized operation process, and the amplification was carried out according to the following conditions: 35 cycles of 30 s at 95°C; 30 s at 57°C; 120 s at 72°C. The reaction was ended with 20 min incubation at 72°C. PCR products were detected by 1.5% agarose gel electrophoresis. The Gel & PCR Clean-Up Kit D2000 (Omega Bio-Tek, Norcross, GA, United States) operation instructions were followed to purify and recycle the product. PCR products purified from genomic DNA were sequenced using an ABI 3730XL DNA Analyzer (ABI, Foster City, CA, United States). The sequencing results were analyzed using Finch TV (Version 1.5).

Whole-exome sequencing

Since none of the mutations in *KRT1*, *KRT10*, or *KRT2e* genes was detected in the proband, his parents, or his younger brother, whole-exome sequencing (WES) was then performed on

this family to screen for the deleterious mutation of the pathogenic gene. The patient and his younger brother were selected for WES. Genomic DNA was randomly broken into DNA fragments of 150–250 bp using a Covaris ultrasonic disintegrator (Covaris, Inc., Woburn, Massachusetts, United States). DNA libraries were prepared by repairing the ends and adding the Y-junction at both ends of the connecting fragment after the A-tail. Liquid phase hybridization was performed by pooling DNA libraries and whole-exome probes. All exons were captured through NimbleGen (Roche NimbleGen, Inc., Basel, Switzerland) with streptavidin-coated magnetic beads. The library quality was tested after linear PCR amplification, and then qualified libraries were used for high-throughput sequencing. High-throughput sequencing was performed on the Illumina HiseqXTen platform (Illumina, San Diego, CA, United States) using 2 × 100 bp paired-end (Diociaiuti et al., 2016; Nijman et al., 2014).

Data analysis and interpretation

BWA-MEM was applied to compare the WES data with the HG19 version of the human genome. Quality control, variation comparison, variation identification, and annotation were performed on the raw data, and all possible pathogenic mutations were screened by comparison with allele frequency population databases such as ExAC database (http://exac.broad.insti.tute.org/dbsnp), 1000 Genomes database (http://www.inter.nationalge.nome.org/data-portal/sample), and KEGG database (https://www.kegg.jp/). The frequency of the identified variant in the Asian population has to be equal to zero or less than 1% in all the databases used. The identified variation was assessed by browsing databases including NCBI dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), OMIM (http://www.omim.org/), HGMD (http://www.hgmd.cf.ac.uk/ac/index.php), and NCBI ClinVar (https://www.ncbi.nlm.nih.gov/ clinvar/). Combined with the phenotype of the proband, mutation interpretation was conducted according to the ACMG genetic variation classification criteria and guidelines.

Verification by Sanger sequencing

All reported mutations should be confirmed by Sanger sequencing, as false-positive results may occur with WES. When a mutation in the *SPINK5* gene was found by WES analysis, Sanger sequencing of *SPINK5* exons was performed in the proband, his parents, his younger brother, and 10 healthy controls independent of this family. PCR and Sanger sequencing of the *SPINK5* gene were performed using the aforementioned method to verify the genetic segregation pattern of this family. The sequences of primers used for validation sequencing were *SPINK5*-E25-26-Forward primer: GCCTGACTCTTGGAAAGAAA and *SPINK5*-E25-26-Reverse primer: CAGTTGTCACTG

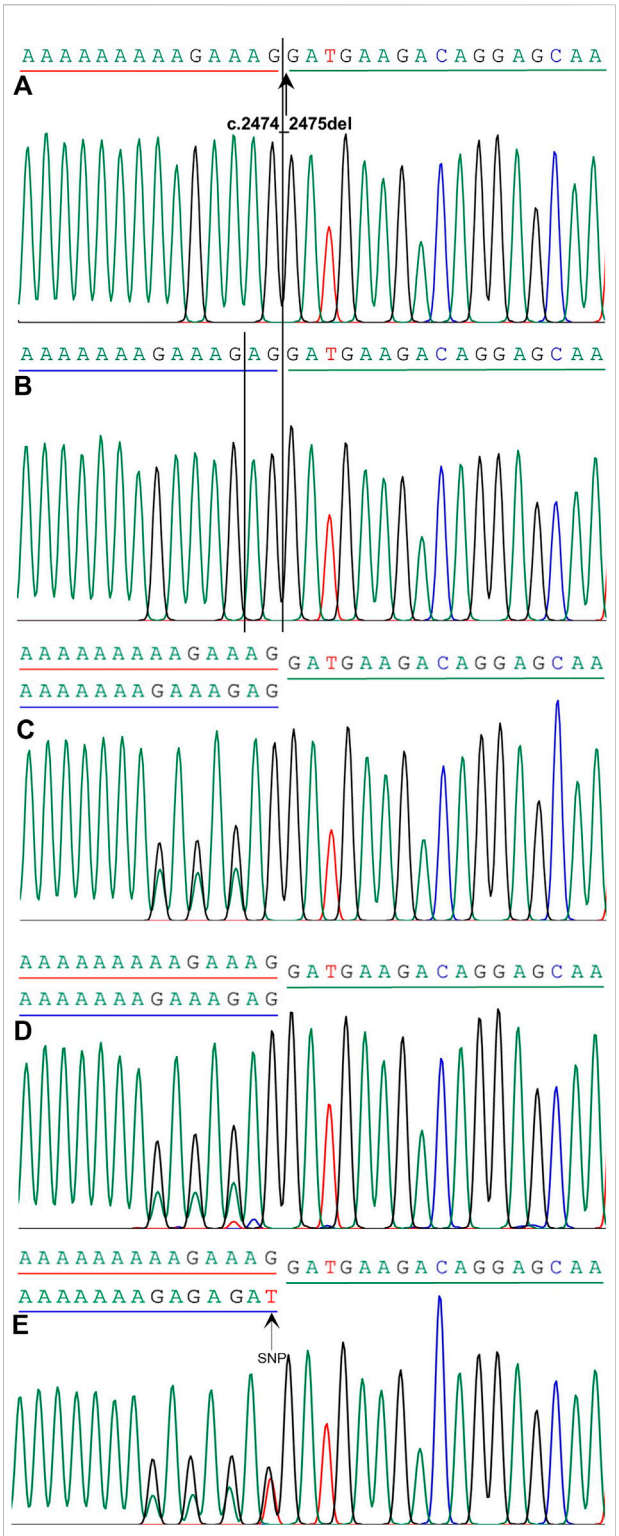

FIGURE 3
Mutation analysis of *SPINK5* gene in this family. **(A)** Homozygous frameshift mutation c.2474_2475del in *SPINK5* gene was identified in the proband. Reference sequencing of *SPINK5* gene in normal controls **(B)**, proband's younger brother **(C)**, his father **(D)**, and his mother **(E)**. Also, single nucleotide polymorphism (SNP) in the DNA sequence of the proband's mother was observed.

GTTCTACA. The variation was identified by comparing with the reported cDNA reference sequences (GenBank Accession Number: NM_00112698, https://www.ncbi.nlm.nih.gov/genbank/). Also, Chromas (version 2.6.5) and PowerPoint were used to draw the sequence diagram of mutant genes (Figure 3).

Clinical and genetic data collection

To further analyze the genotype–phenotype correlation of NS syndrome, we searched "Netherton syndrome" and "mutation" from the PubMed\Embase\MEDLINE\LILACS\Cochrane database and collected 91 relevant literature works in the last decade. By deleting duplicate literature works, removing review articles, and reading titles, abstracts, and full texts, 23 literature works with definite mutation sites in NS patients were obtained. A total of 25 NS patients were reported in detail. We summarized the clinical and genetic data of patients with Netherton syndrome.

Results

Mutation analysis of *KRT1*, *KRT10*, and *KRT2e* genes

The Sanger sequencing showed none of the mutations in *KRT1*, *KRT10*, *or KRT2e* gene in the patient, his younger brother, and his parents.

WES and Sanger sequencing

We considered only substitutions, insertions, and deletions in the coding region, variants at canonical splicing sites in the noncoding region, and variations at canonical splicing sites, excluding variations with a minor allele frequency greater than 0.01 in different common and local resources. A homozygous frameshift mutation in the *SPINK5* gene (NM_001127698), c.2474_2475del (p.Glu825Glyfs*2) in exon 26, was identified after integrative analysis (Figure 3). The mutation was not present in ExAC, 1000 Genomes databases, KEGG databases, and others and leads to a frameshift and a premature stop codon. Sanger sequencing showed that this variation was not observed in other family members with normal skin phenotypes or in 10 healthy controls independent of this family. His parents and his younger brother were the heterozygous carriers of this mutation (Figure 3).

Clinical and genetic data analysis

The clinical and genetic data of 25 individuals with NS were summarized (Table 1). Only 12 (48%) patients exhibited the classical triad of congenital ichthyosiform erythroderma (CIE) or ichthyosis linearis circumflexa (ILC), trichorrhexis invaginata (TI), and atopic diathesis. All patients showed scaly erythroderma. However, this condition was absent at birth in six patients. About half of the patients were presented with ILC. TI, atopic diathesis, and high IgE levels were observed in 20 (80%), 15 (60%), and 13 (52%) patients, respectively. Two patients died of skin barrier destruction and septicemia among 25 NS patients. However, their IgE levels were normal or mildly elevated, suggesting that the IgE levels are not correlated with the severity of the disease.

Genotype–phenotype correlation analysis

So far, more than 80 different mutations in the *SPINK5* gene have been reported. Most of the mutations in *SPINK5* responsible for the NS phenotype introduce a premature termination codon (PTC), resulting in a lack of detectable LEKTI expression (Zelieskova et al., 2020; Hovnanian, 2013). The phenotypes of NS were variable with manifestations from mild clinical signs to life-threatening complications, especially during the neonatal period. Some patients with truncated mutations located early in the coding sequence have a more severe phenotype, while the mutations in *SPINK5* gene that interrupt coding frames near the C-terminus may allow the retention of functional LEKTI fragments, leading to a milder phenotype.

Discussion

Netherton syndrome is a rare autosomal recessive disease with skin, hair, and immune abnormalities. The anomaly of the hair shaft is highly specific in NS patients, but the absence of this condition does not rule out the diagnosis of NS (Torchia and Schachner, 2011). The scalp hairs, eyebrows, or eyelashes may be lusterless, sparse, and brittle. Light microscopy examination almost always shows characteristic bamboo hair or trichorrhexis invaginata (TI), which refers to the cup-shaped protrusion of the distal part of the hair shaft toward the proximal part (Hovnanian, 2013). Hair examination is sometimes difficult because not every hair is affected. The eyebrows are considered a prior choice to visualize this abnormality (Bittencourt et al., 2015). In addition, a few patients with NS only have ichthyosis linearis circumflexa (ILC) but do not have the changes of trichorrhexis invaginata (TI) (Guerra et al., 2015; Özyurt et al., 2019).

Two-thirds of NS patients suffer from allergic diseases including atopic dermatitis, allergic asthma, allergic rhinitis, anaphylactic reactions to food, urticaria, and angioedema, as well as elevated serum IgE level and blood hypereosinophilia (Erden et al., 2020). Some severe comorbidities were also seen in a

TABLE 1 Summary of the clinical phenotype and genotype from patients with Netherton syndrome.

	Age[1]	Sex	SE	Diagnostic parameters				LEKTI detection	SPINK5 mutation				Treatment
				ILE/CIE	TI	Atopic manifestation	Other systemic finding		Location (exon)	Pathogenic variant	Nucleotide change	Reference	
1	3 years	M	+	ILC	+	E, EO, and H-IgE: 35,200 U/ml	–	NM	Exon 2	c.80A > G	p.Gln27Arg	Zhang et al. (2021)	IVIG therapy
2	1 month	ND	+	CIE	ND	–	HN	NM	Exon 3	c.153delT	p.Gln52LysfsTer6	Tiryakioğlu et al. (2017)	Specially prepared ointments
3	13 years	M	+	CIE	+	NM	NM	NM	Exon 4	c.238_239insG	p.Ala80fs	Schepis et al. (2019)	NM
4	20 years	F	+	CIE	+	NM	NM	NM	Exon 4	c.238_239insG	p.Ala80fs	Schepis et al. (2019)	NM
5	23 years	F	+	ILC	+	A, AR, FA, and H-IgE: 1,373 U/ml	–	NM	Exon 5 Exon 26	c.316_317delGA c.2468dupA	p.Asp106TrpfsTer7 p.Les824GlufsTer3	Kogut et al. (2015)	Topical calcineurin inhibitors and moisturizers
6	18 years	F	+	ILC	+	H-IgE: 2,176 U/ml	–	Negative	Exon 5	c.318G > A	p.Asp106Ter	Xi-Bao et al. (2012)	NM
7	6 months	F	+	CIE	+	FA and H-IgE: 708 U/ml	HN	NM	Exon 5 Exon 25	c.377_378delAT c.2368C > T	p.Tyr126Ter p.Arg790Ter	Konishi et al. (2014)	NM
8	2 years	M	+	ILC	+	H-IgE: 528 U/ml	–	Negative	Exon 6 Exon 19	c.474G > A c.1732C > T	p.Gln158 = p.Arg578Ter	Numata et al. (2016)	NM
9	6 years	F	-	CIE	+	FA	GR	NM	Exon 7	c.581_82delGT	p.Cys194fsTer4	Aydın et al. (2014)	Growth hormone therapy
10	22 years	M	–	ILC	+	FA and H-IgE: 1,878 U/ml	–	NM	Exon 8 Exon 11	c.649C > T c.957_960dupTGGT	p.Arg217Ter p.Pro321TrpfsTer23	Alpigiani et al. (2012)	NM
11	10 months	M	+	CIE	+	H-IgE: 951 U/ml	GR, HP, and IN	Negative	Exon 8	c.652C > T	p.Arg218Ter	Wang et al. (2021)	IVIG therapy, topical emollients, corticosteroid cream, and antibiotic cream
12	11 months	F	–	CIE	+	–	GR, HN, IA, and IN	NM	Exon 11	c.995delT	p.Met332SerfsX43	Nevet et al. (2017)	Acitretin 1 mg/kg and topical humectants (death)
13	10 years	M	+	ILC	+	E and H-IgE: >10,000U/ml	IN	NM	Exon 11	c.997C > T	p.Gln333Ter	Fong et al. (2011)	Topical emollients and corticosteroid cream
14	5 years	F	–	ILC	+	–	IN	NM	Exon 13 Exon 25	c.1099dupT c.2368C > T	p.Cys367Leufs*3 p.Arg790Ter	Konishi et al. (2014)	NM
15	6 months	M	+	CIE	+	–	AN, HN, HP, IN, and SZ	Negative	Exon 13	c.1111C > T	p.Arg371X	Diociaiuti et al. (2013)	(Death)
16	2 years	M	–	ILC	–	NM	NM	Negative	Exon 15 Intron 14	c.1258G > A c.1302+4A > T	p.Glu420Lys	Guerra et al. (2015)	Topical steroid

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TABLE 1 (*Continued*) Summary of the clinical phenotype and genotype from patients with Netherton syndrome.

	Age[1]	Sex	SE	Diagnostic parameters				LEKTI detection	SPINK5 mutation				Treatment
				ILE/ CIE	TI	Atopic manifestation	Other systemic finding		Location (exon)	Pathogenic variant	Nucleotide change	Reference	
17	15 years	M	+	CIE	ND	H-IgE: 10,700 U/ml	ALL and IN	NM	Exon 17	c.1530C > A	p.Cys510Ter	Skoczen et al. (2020)	Chemotherapy, topical emollients, and corticosteroid cream
18	2 years	M	+	ILC	+	–	GR, HN, HO, HP, and IN	NM	Exon 17	c.1530C > A	p.Cys510Ter	Zelieskova et al. (2020)	IVIG therapy
19	50 months	M	+	ILC	+	AO and H-IgE: 11,425 U/ml	–	Negative	Exon 19	c.1772delT	p.Leu591GlnfsTer124	Hannula-Jouppi et al. (2016)	Acitretin treatment showed no benefit
20	42 years	M	+	ILC	ND	E	IN and IVL	Negative	Exon 26 Intron 4	c.2468dupA c.283-2A > T	p.Les824GlufsTer3	Albluwi et al. (2020)	Topical emollients, antibiotic cream, and oral antibiotics
21	6 moths	M	–	ILC	+	E, EO, and H-IgE: 453 U/ml	–	NM	Intron 5	c.410 +1G > A		Cicek et al. (2021)	Infliximab therapy
22	8 months	M	+	CIE	+	–	BH, HN, HO, and IN	NM	Intron 15 Intron 19	c.1431-12G > A c.1816-1820 + 21delinsCT		Śmigiel et al. (2017)	NM
23	5 months	M	+	CIE	+	–	HN and HO	NM	Intron 17	c.1608-1G>a		Okulu et al. (2018)	ECMO support, topical Aquaphor, and bronchodilators
24	20 years	M	+	CIE	+	H-IgE: >2,000 U/ml	–	NM	Intron 17	c.1608-1G>a		Erden et al. (2020)	NM
25	4 years	M	+	ILC	–	E, AO, and H-IgE: 3,420 U/ml	–	NM	Intron 22	c.2112+2T > A		Özyurt et al. (2019)	Acitretin 0.5 mg/kg and topical moisturizers

Abbreviations: A, asthma; ALL, acute lymphoblastic leukemia; AN, anemia; AO, angioedema; AR, allergic rhinitis; BH, bilateral hypoacusia; CIE, congenital ichthyosiform erythroderma; E, eczematous-like rashes; ECMO, extracorporeal membrane oxygenation; EO, eosinophilia; F, female; FA, food allergy; GR, growth retardation; H-IgE, hyper-IgE; HN, hypernatronemia; HO, hypoxemia; HP, hypoproteinemia; IA, intestinal atresia; ILC, ichthyosis linearis circumflexa; IN, infection; IVIG, intravenous immunoglobulin; IVL, inflammatory vegetative lesions; M, male; NM, not mentioned; SE, scaly erythroderma at birth; SZ, seizure; TI, trichorrhexis invaginata.

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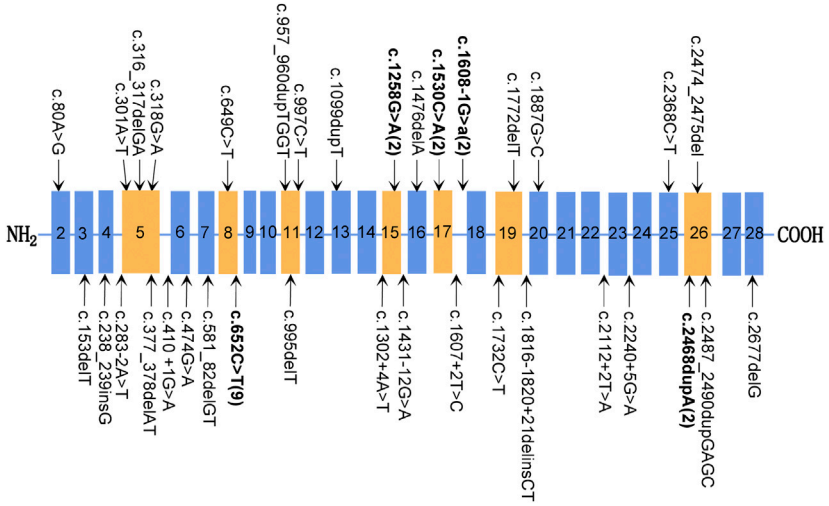

FIGURE 4
Mutations of the *SPINK5* gene. Typical exon domains are represented in orange, and other exon domains are depicted in blue. Mutations occurring more than once are shown in bold, and the number of unrelated families carrying this mutation is shown in parentheses.

minority of NS neonates, such as skin infection, enteropathy, hypernatremia, hypoproteinemia, sepsis, and growth retardation (Hernández-Martín and González-Sarmiento, 2015).

The diagnosis of NS is straightforward in the presence of characteristic cutaneous manifestations, trichorrhexis invaginata, atopic diathesis, and a positive family history (Leung et al., 2018). However, it is still challenging to make an accurate diagnosis for the NS cases that lack a typical clinical phenotype. Some powerful diagnostic tests have facilitated the recognition of these cases of NS, such as LEKTI immunodetection in the skin and mutation analysis of *SPINK5* gene (Lacroix et al., 2012).

The *SPINK5* gene contains 33 exons spanning 61 kb and encodes LEKTI expressing in the skin, mucosa, and thymic epithelium (Hovnanian, 2013). The pathogenic mutations in the *SPINK5* gene result in a truncation of LEKTI, which reduce LEKTI's ability to inhibit skin serine proteases (Kasparek et al., 2017; Tiryakioğlu et al., 2017). The deficiency of LEKTI leads to hyperactivation of skin kallikreins (KLK) and increased degradation of desmosine and corneodesmosomal cadherins. Consequently, the defect of the skin barrier occurs following abnormal skin homeostasis and detachment of the stratum corneum. KLK5 can activate protease-activated receptor 2 (PAR-2) and lead to increased expression of thymic stromal lymphopoietin (TSLP), TNF-α, IL-8, and ICAM-1 through the KLK5-PAR2 cascade, thereby enhancing the inflammatory process (Stuvel et al., 2020). The loss of LEKTI expression in the trachea can lead to the destruction of the airway epithelial barrier, further making NS patients more susceptible to inhaled allergens (Berthold et al., 2016). LEKTI deficiency leads to increased cleavage of cross-linkage in hair keratin structures, local defect of the inner root sheath, and morphological change of trichorrhexis invaginata (Bittencourt et al., 2015). The decreased expression of LEKTI in the sinonasal epithelium is associated with allergic rhinitis (Hannula-Jouppi et al., 2014).

To date, more than 80 kinds of mutations have been identified in intronic and exonic regions of the *SPINK5* gene. Most of the *SPINK5* mutations causing NS introduce a premature termination codon (PTC) which results in a lack of detectable LEKTI expression (Furio et al., 2014). The primary structure of LEKTI consists of 15 serine protease inhibitory domains (Śmigiel et al., 2017), in which the D6–D9 domains of LEKTI possess the most effective inhibiting activity on KLK (Guerra et al., 2015). A more severe phenotype was observed in some patients with truncated mutations located early in the coding sequence, which may be due to a substantial reduction or complete lack of LEKTI expression (Diociaiuti et al., 2013; Nevet et al., 2017), whereas a milder phenotype was related to the *SPINK5* mutations interrupting coding frames near the C-terminus, which allow the retention of functional LEKTI fragments (Guerra et al., 2015). In addition, some deep intronic mutations activate the hidden splicing sites and cause a milder phenotype (Śmigiel et al., 2017).

Genotype–phenotype correlation analysis shows that more severe phenotypes were associated with the mutations of exons 1–25 in the *SPINK5* gene (Table 1; Figure 4). None of the mutations in the downstream of exon 28 was detected in the NS patients. Mostly, the mutations of exon 26 were compound heterozygous mutations [c.283-2A > T in intron 4 and c.2468dupA in exon 26 (Albluwi et al., 2020); c.316_317delGA in exon 5 and c.2468dupA in exon 26 (Kogut et al., 2015); and c.2260A > T in exon 24 and c.2468delA in exon 26 (Chao et al., 2003)]. In our study, the patient carried a homozygous mutation c.2474_2475del on exon 26 and displayed a milder phenotype,

which is probably related to the mutation site near the C-terminal of LEKTI. The number of mutations in exons 1–17 of *SPINK5* gene was significantly more than that in exons 18–33 (Figure 4), which may be related to neglecting of those patients with the milder phenotype. The prevalence of NS is likely underestimated. Therefore, it is recommended to make gene panel (*KRT1*, *KRT10*, *KRT2e*, and *SPINK5*) testing for those patients with congenital ichthyosiform erythroderma (CIE) or ichthyosis linearis circumflexa (ILC). The method is beneficial to improve the diagnosis of NS. The diagnosis of this patient was demonstrated by mutation analysis of the *SPINK5* gene.

In conclusion, we describe a case of Netherton syndrome without the appearance of trichorrhexis invaginata and a novel homozygous *SPINK5* frameshift mutation. Our findings further expand the spectrum of both clinical phenotypes of NS and mutations of *SPINK5* gene.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YW conducted Sanger sequencing and wrote the manuscript. HS, XZ, and BL performed whole-exome sequencing and wrote the manuscript. NW and LY collected clinical data and blood samples and performed DNA extraction. PW was responsible for the study design and guiding the study implementation and revising the manuscript. All authors contributed to the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.943264/full#supplementary-material

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