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Original Article

WNK3-PER1 interactions regulate the circadian rhythm in the suprachiasmatic nucleus in rats

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Abstract: PER1 is a core component of the internal time-keeping system. In the suprachiasmatic nucleus, it serves as the primary circadian pacemaker in mammalian brains. PER1 functions with other clock components to generate a feedback loop involving the transcriptional repression of gene expression to produce a circadian rhythm with an approximately 24-hour cycle. Post-transcriptional modifications (PTMs) are a basic regulatory mechanism that both perpetuate self-sustained oscillations and interpret metabolic input into circadian physiology by affecting factors such as protein stability, interactions, localization, and activity. Here we examined whether the serine/threonine protein kinase WNK3, which is expressed in a circadian rhythm, can interact and colocalize with PER1 in the SCN. In rats, WNK3 knockdown in the SCN is associated with altered sleep patterns. Moreover, WNK3 can phosphorylate PER1 to promote its degradation and is associated with circadian oscillations when PER1 is expressed in vitro.

Keywords: Circadian rhythm, WNK3, PER1, sleep disorder, SCN, phosphorylation

Introduction

Most biological behaviors and physiology are shaped by circadian rhythms. The negative feedback loop in clock genes is thought to control the circadian oscillations of all organisms. Disruption of the circadian rhythm can cause tremendous physical stress in night shift workers and contribute to the development of bipolar disorder [1]. Sleep disorders and circadian rhythm disturbances are also associated with several neuro-psychiatric diseases, such as Smith-Magenis Syndrome (SMS), Prader-Willi syndrome (PWS), Attention Deficit Hyperactivity Disorder (ADHD), and Autism Spectrum Disorder (ASD) [2].

The suprachiasmatic nucleus (SCN) is a pacemaker of mammalian circadian rhythms [3]. Previous studies involving animals showed that damage to the bilateral SCN results in the complete loss of many physiological and behavioral circadian rhythms, such as sleeping, waking up, drinking, hormone release, and feeding [2, 4]. Measurement of the membrane potential in cultured SCN neurons *in vitro* indicates that the SCN can autonomously maintain a stable circadian rhythm for several weeks. The circadian rhythm is controlled by the biological clock, which is roughly comprised of an input path, a central oscillator, and an output path. The central oscillator can generate rhythms autonomously through the Transcription Translation Feedback Loop (TTFL) [5]. The working model of the circadian molecular clock is complex and has multiple additional components that function together in the circadian cycle. Given the rapidity of transcription and translation reactions, a significant delay must be imposed on the core TTFL mechanism in order to generate a 24-hour oscillation. This delay is achieved through a complex network involving the regulation of protein phosphorylation and other post-translational modifications as well as pro-

tein complex assembly, nuclear translocation, and the degradation of TTFL components.

PER1 is highly expressed in the SCN and is an important circadian rhythm gene. The main function of PER1 is to maintain the accuracy and stability of the circadian rhythm and to adjust the cycle length [6]. A recent study showed that the serine/threonine kinases CK1α and DBT target PER in *Drosophila*. CK1α enhances the phosphorylation and degradation of PER in the nucleus via DBT and induces further effects on the PER-dependent timing of the circadian transcriptome [7]. In addition, the serine/threonine protein kinase salt-inducible kinase 3 was found to play a role in mammalian circadian clock regulation through PER2 protein destabilization [8]. The phosphorylation of PER1 determines its nucleoplasm distribution, accumulation, and degradation, which in turn affects the robustness, speed, and phase of the circadian oscillator. These occurrences translate into clear circadian changes [9]. Here we examined whether other serine/threonine protein kinases in mammals can also bind PER1 to regulate circadian rhythms.

In *Arabidopsis*, WNK1 regulates circadian rhythm through the phosphorylation of the clock-associated protein APRR3 [10]. In mouse kidneys, WNK4 regulates the salt balance and the blood pressure through the phosphorylation of the OSR1/SPAK-NaCl cotransporter (NCC), which translates to a circadian rhythm for blood pressure and renal salt excretion [11]. WNK1 and WNK4 are members of the with-no-lysine (K) (WNK) kinase family, which includes WNK1, 2, 3, and 4. WNK1 and WNK4 are key regulators of renal ion transport in mammals. The inactivation of WNK1 or WNK4 can cause hypertension and hyperkalemia [12]. The specific physiological functions of WNK2 and WNK3 are not fully understood, but WNK2 is mainly expressed in the fetal heart, and WNK3 is widely expressed in the nervous system [13, 14]. Our previous results demonstrated that WNK3 may be related to neuronal apoptosis after CNS injury [15], although its physiological function in the mammalian CNS remains unclear. The amino acid sequence of the *Arabidopsis* WNK1 kinase site is highly similar to that of human WNK3 [16]. The WNK3 gene is 165 kb and can be expressed as two different transcripts based on exons 18 and 22; only WNK3 having exon 18 is detectable in the brain tissue of rats, particularly in the hippocampus, the SCN region, and the hypothalamus [16, 17]. Taken together, these results suggest that WNK3 could have important functions in regulating the circadian rhythms in mammals.

Here we examined whether WNK3 can interact with PER1 in the SCN and whether WNK3 affects PER1 expression to regulate circadian rhythms.

Materials and methods

Animals

Male Sprague-Dawley (SD) rats (10-12 weeks-old) in this study were obtained from JointVentures Sipper BK Experimental Animal (Shanghai, China) or Shanghai Jiesijie Laboratory Animal Co., Ltd. For circadian rhythm setup and maintenance, the rats were housed individually in a temperature-controlled room on 12 h daily photoperiods (12:12 hour light/dark cycle) for at least 7 days before and during all the circadian experimental periods. The animals were given free access to a regular rodent chow (RC) diet and water *ad libitum*. All the animal experiments were approved by the Animal Care and Use Committee of Shanghai University (SHUACUC. 20181210R60).

SCN microinjection

The SD rats were anesthetized with ketamine/xylazine (60/5 mg/kg body weight, i.p.) and secured on a stereotaxic apparatus. The microinjection of 1 ul lentivirus with a glass electrode (20-25 μm in inner tip diameter) was driven by a Nanoject injector (Drummond Scientific Company, USA) into the SCN area (1.3 mm posterior to the Bregma, 0.2 mm from the midline, and 9.3 mm below the skull).

Rat sleep pattern records

The SD rats' (220-250 g) circadian rhythms were established under 12 h daily photoperiods. The animals were allowed to feed *ad libitum* for at least 1 week before the experiments. To study their daytime sleep latency differences, electrophysiology recordings were conducted 48-72 h after the lentivirus micro-injections

and also after the basal neuronal activity had stabilized.

Plasmid constructs

The pEGFP-WNK3 (49-436) and the mutant pEGFP-WNK3 (49-436) K159M plasmids were constructed as previously described [15]. The most effective WNK3si-3 sequence was inserted into the lentiviral vector using the BglII and XhoI sites (Nuobai Biotech Ltd). The Per1-dLUC plasmids were a kind gift from Prof. Tsuyoshi Hirota. All the constructs were then validated using DNA sequencing.

WNK3 RNAi

The three siRNA candidates were designed from the rat WNK3 DNA sequences: WNK3si-1, CCAACAGGCTCTAAGATTC; WNK3si-2, GCAGGCATGTTCATACCTA; and WNK3si-3, CCTCCAAGTTAGATGGTAA. The WNK3si-3 sequence was identified as the most effective. The WNK3si-3 corresponding shRNAs were cloned into the pSuper shRNA expression vector and the lentivirus shRNA expression vector. To test the effectiveness of the shRNA constructs, the WNK3 levels in the SCN were measured using immunoblotting 48 h after the transfection with the WNK3si lentivirus. Empty lentivirus was used as a control for the immunoblotting and functional analyses.

Immunoprecipitation and immunoblotting

Tissue samples and cultured cells were collected to extract the total protein using a RIPA lysis buffer containing protease inhibitors (Roche Applied Science) supplemented with PMSF. The lysates were then centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was carefully collected (500 µl) and incubated with 5 µg of the first antibody for 2 h at 4°C. Agarose beads (Protein A+G, Santa Cruz Biotechnology) were added for an additional 12 h with rotation at 4°C. The IP product was washed three times with a lysis buffer, and then boiled in a loading buffer for 5 minutes. The protein bands were separated using SDS-PAGE electrophoresis, transferred to a membrane, and measured using western blot. The antibodies required for the experiment were as follows: rabbit anti-WNK3 (1:500, Alpha Diagnostics), monoclonal rabbit anti-PER1 (1:500), monoclonal rabbit anti-BMAL1 (1:200), and rabbit anti-phospho-Cry1 (1:200) (Cell Signaling Technology), HRP-conjugated anti-Actin (1:10,000, Kangcheng, Shanghai, China), and HRP-conjugated secondary antibodies (1:10,000; Santa Cruz Biotechnology).

Phosphorylation analysis

For the phosphorylation analysis, 8% SDS-PAGE gels containing 20 mM Phosbind acrylamide (F4002, ApexBio) and 40 mM MnCl2 were prepared. The electrophoresis and western blots were performed according to the manufacturer's guidelines [18].

Immunofluorescence

The tissue slices from the rat SCN were washed with 0.01 M PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. The samples were then treated with 0.1% Triton X-100 for 30 min and subsequently blocked with 1% bovine serum albumin (BSA) in 0.01 M PBS. The slices were incubated overnight at 4°C with the first antibody [rabbit anti-WNK3 (1:100) or mouse anti-Per1 (1:100) and subsequently quantified with species-specific FITC- or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). A Leica SP5 confocal microscope was used to observe and collect the immunofluorescence images.

Statistical analysis

GraphPad 5 software was used to analyze the data and draw the images. Data were expressed in the form of the mean ± SEM, and independent sample t-tests were used for the intergroup comparisons. The comparisons among more than two groups were done using one-way analyses of variance (ANOVA). For the comparisons of the treatment groups undergoing repeated measurements at different time points, we used two-way repeated measures ANOVA. When the results of the overall analysis of variance were statistically significant, a post-hoc Dunnett's test was performed to determine the locations of these differences. A statistical value of $P<0.05$ was considered statistically significant. Tukey's test was used to assess the statistical significance of the multiple comparisons in a small sample.

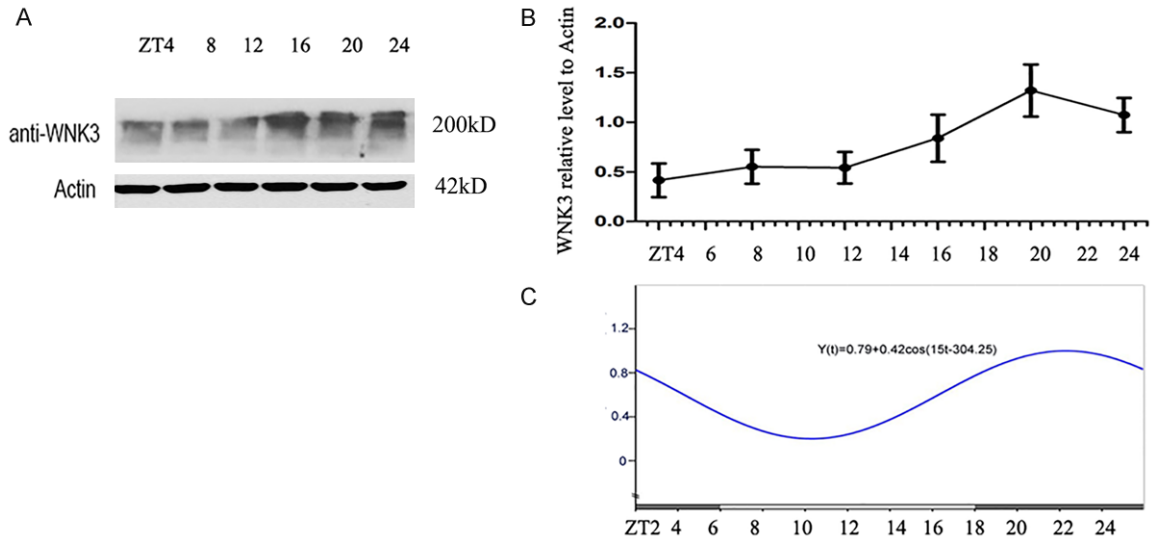

Figure 1. The WNK3 protein expression shows circadian rhythm fluctuation in the rat SCN. A. The Sprague-Dawley rat littermates were maintained under a standard 12 hr photoperiod [light/dark cycle of 12:12 hr (LD 12:12) for 2 weeks before being sacrificed at 4-hr intervals during exposure to constant darkness, and the SCN region was isolated for immunoblotting with anti-WNK3. B. A representative immunoblotting shows the WNK3 protein expression in a circadian oscillation. B. The quantification of the WNK3 relative expression to actin is shown as the mean ± SEM values from at least three independent experiments. C. The cosine curve fitting of the B graph shows that the WNK3 expression presents the circadian rhythm.

Results

WNK3 circadian rhythm expression in rat SCN

The Sprague-Dawley rat littermates were maintained for at least 2 weeks under a light/dark cycle of 12:12 h (LD 12:12). The animals were sacrificed at 4 hr intervals during their exposure to constant darkness, and the SCN region was isolated for immunoblotting with anti-WNK3 antibodies. The Western blot bands were analyzed using Image J software, and the WNK3 expression levels were normalized relative to actin as an internal loading control (**Figure 1A**, **1B**). The changes in the WNK3 expression levels over 24 h showed that the WNK3 protein expressions exhibited circadian oscillations (**Figure 1C**).

WNK3 knockdown in the SCN led to altered sleep patterns in the rats

We next examined whether the WNK3 expression knockdown in rat SCN affects physiological rhythmicity. A WNK3si lentivirus was microinjected into the rat SCN to specifically knockdown the WNK3 expression (**Figure 2C**). The WNK3 knockdown efficiency was confirmed through the western blotting of the SCN tissues (**Figure 2A**). The EEGs of the treated and untreated rats were continuously monitored to detect any changes in their sleep patterns after the SCN WNK3 knockdown. The average sleep time of the WNK3 knockdown rats as a reflection of the circadian rhythmicity of the sleep-wake cycle showed a significant phase delay of approximately 4 h compared to the control rats (**Figure 2D**). However, there was no significant difference in the total sleep duration over 24 h between the control group and the WNK3 knockdown group (**Figure 2E**).

WNK3 is associated with PER1 in vitro and in vivo

To determine whether WNK3 binds circadian rhythm proteins in the rat SCN, we immunoprecipitated rat SCN lysates with an anti-WNK3 antibody, and immunoblotted them with anti-BMAL1, anti-Cry1, and anti-PER1 antibodies (data not shown). Only PER1 immunoprecipitated with WNK3 in the rat SCN (**Figure 3C**). To further verify this interaction, we co-transfected 293 cells with pEGFP-WNK3 and FLAG-PER1, prepared cell lysates, and immunoprecipitated them with an anti-FLAG antibody

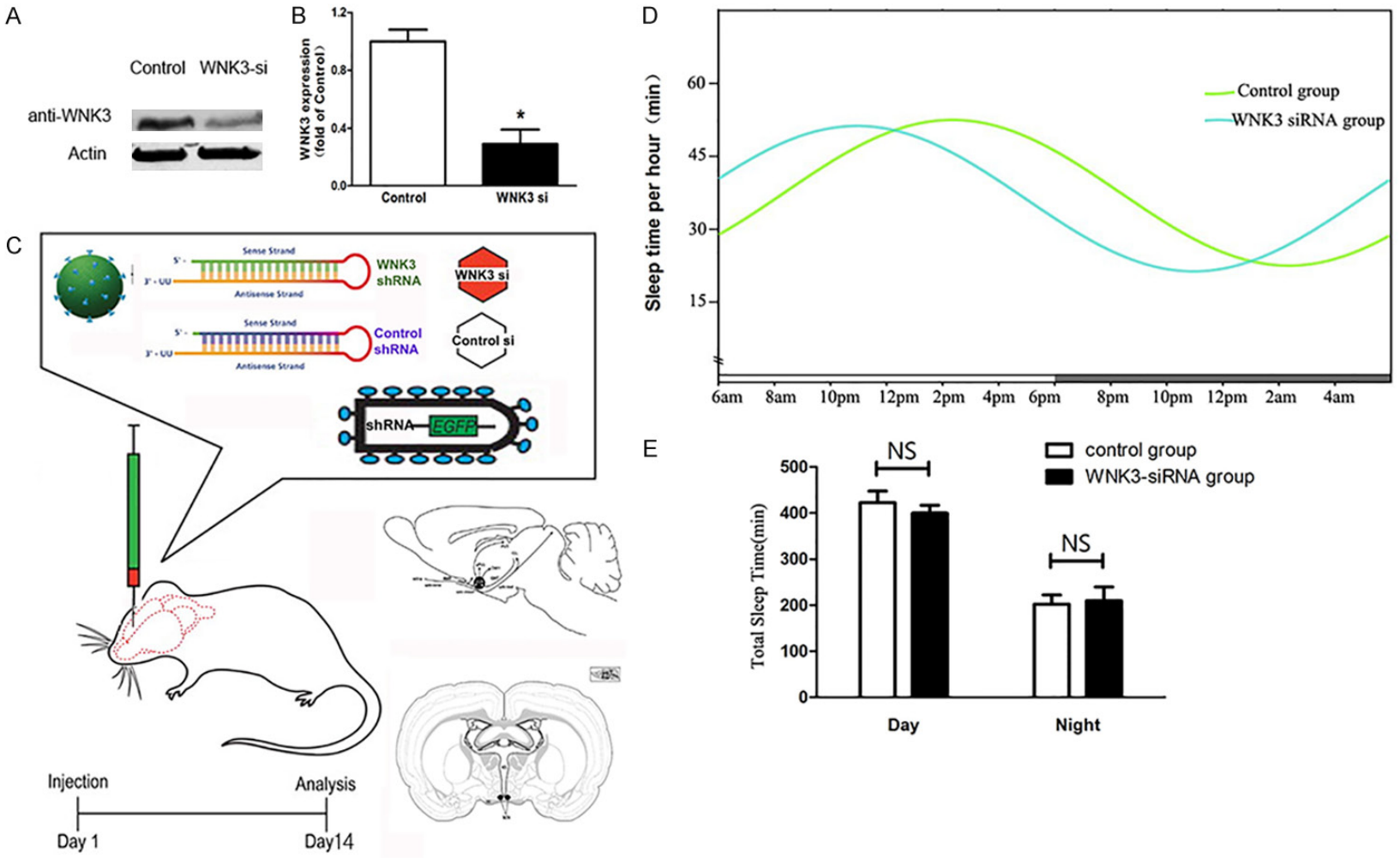

Figure 2. The WNK3 knockdown in the SCN led to sleep pattern changes in the rats. A. Immunoblotting shows a significant decrease in the WNK3 expression levels in the rat brain slices infected with Lv-WNK3 shRNA, compared with those transfected with the Lv-scramble control shRNA; B. Quantification of the expression of the WNK3 as a fold of the control. C. A schematic map showing the strategies for the sleep EEG analysis after the injection of the virus to mediate the WNK3 knock-down. D. The amount of time spent continuously in each sleep state over the entire recording period was recorded using EEG and EMG, and we further analyzed by the methods of the cosinor-rhythmometry with Origin 6.0 software. E. The total sleep times during the day (light on) and night (light off) show the total time spent in sleep is not significantly changed. n=8 in each group from three independent experiments.

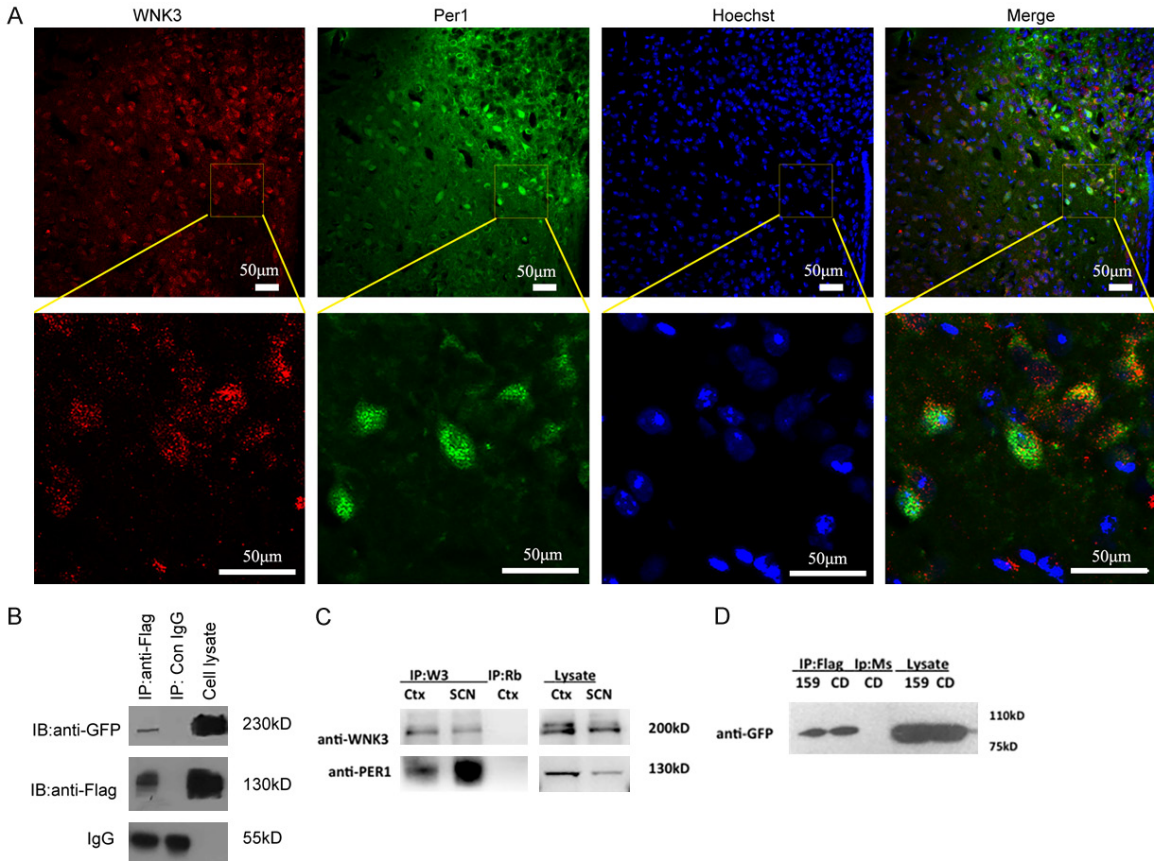

Figure 3. WNK3 is associated with PER1 in vitro and in vivo. A. The immunofluorescence images show the co-localization of WNK3 and PER1 in the adult rat SCN, Bar, 50 μm. B. 293 cells co-transfected with pEGFP-WNK3 and Flag-PER1 were lysed and immunoprecipitated with the anti-FLAG antibodies, and the control Mouse IgG (Con IgG) was set as the immunoprecipitation control, followed by immunoblotting with anti-GFP and anti-FLAG antibodies at 250 kD and 100 kD respectively. The cell lysate was set as the input control. C. The Rat cortexes (Ctx) and the SCN (SCN) lysate were immunoprecipitated with anti-WNK3 antibodies and blotted with anti-WNK3 and anti-PER1 antibodies at 200 kD and 100 kD, respectively, and Rb IgG was set as an immunoprecipitation control and the total lysates were set as input controls. D. pEGFPN1-WNK3 (49-436) (CD) or its kinase-dead mutant pEGFPN1-WNK3 (49-436)-K159M (159) was co-transfected with Flag-PER1 into 293 cells and the cell lysate was immunoprecipitated with anti-FLAG antibodies, with the mouse IgG set as an immunoprecipitation control. The immunoblotting of anti-GFP shows that both WNK3 (49-436) and its kinase-dead mutant WNK3 (49-436) K159M could interact with PER1 *in vitro*. The total cell lysate was set in parallel as an input control.

followed by immunoblotting with anti-GFP or anti-FLAG antibodies. The immunofluorescence analysis showed that WNK3 and PER1 co-localize in adult rat SCN neurons (**Figure 3A**). Taken together, these results indicate that WNK3 and PER1 can bind each other (**Figure 3B**). To assess whether WNK3 binding with PER1 affects the PER1 phosphorylation levels, we used phosphate affinity SDS-PAGE, which is an electrophoresis method that incorporates Phosbind Acrylamide to identify protein phosphorylation [18]. The dinuclear metal complex of the Mn^{2+}-Phosbind selectively binds to phosphorylated proteins and retards their gel shifts; thus, the phosphorylated proteins can be separated from the non-phosphorylated proteins. Immunoblotting with an anti-PER1 antibody identified bands that were shifted, indicating phosphorylated PER1. As shown in **Figure 4**, both the proportion of phosphorylated PER1 and the total PER1 expression were significantly affected by the WNK3 kinase truncated mutants (the WNK3 CD, WNK3 catalytic domains are a kinase active truncation and K159M is a kinase-dead mutant), demonstrating that the WNK3 kinase can indeed promote

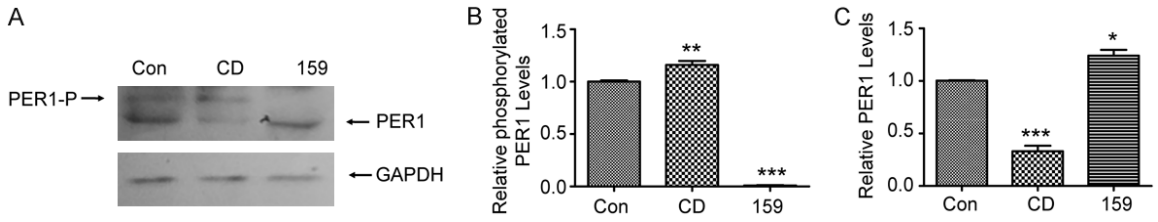

Figure 4. WNK3 affected the expression level and phosphorylation status of the PER1 protein. A. Neuro 2a cells were transfected with WNK3 expressing constructs, and their effects on the expression level and phosphorylation status of PER1 were examined using Western blotting. The overexpression of pEGFPN1-WNK3 (49-436) (CD) in the neuro 2a cells but not its kinase-dead mutant pEGFPN1-WNK3 (49-436)-K159M (159) significantly reduced the PER1 protein levels and increased the PER1 phosphorylation states. B, C. The quantification of the PER1 expression and phosphorylated PER1 using Image J software was standardized by GAPDH and then normalized as a fold change according to the expression levels in the control groups. (n=3, P<0.001 using Tukey's test).

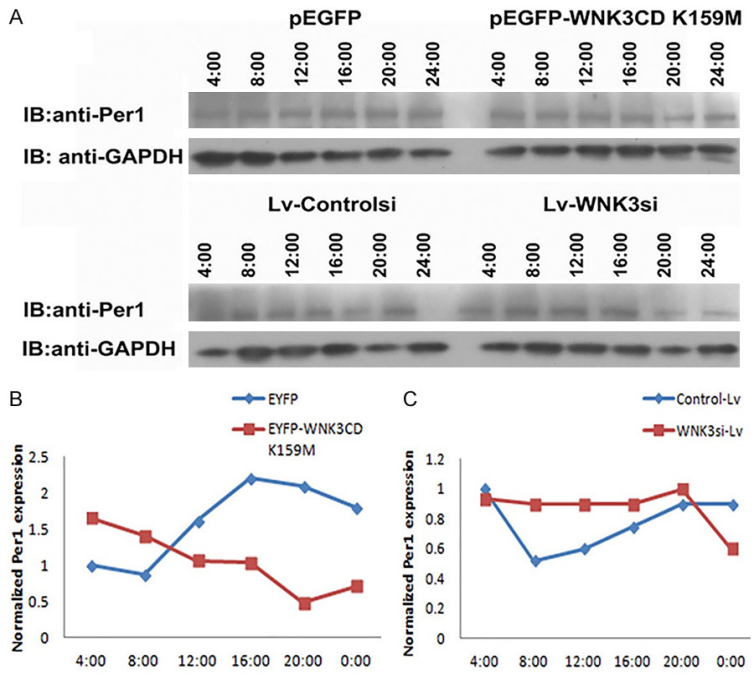

Figure 5. The WNK3 expression and its kinase activity affected the PER1 circadian oscillation in vitro. A. Neuro 2a cells transfected with pEGFP-WNK3 (49-436)-K159M, or with the EGFP vector as a control, (upper panel), as well as the Neuro 2a cells infected with lentivirus containing WNK3 shRNA (Lv-WNK3si) or a scrambled control shRNA (Lv-Control si) (lower panel) were processed using the serum-shocked procedure, and the cells were lysed at different time points post serum-shock and were immunoblotted with anti-PER1 antibodies and anti-actin blotting for the input control. B, C. The quantification of the PER1 expression using Image J software was standardized using actin and then normalized as a fold change according to the expression level at 4 pm in the control groups.

PER1 phosphorylation and the subsequent degradation.

The WNK3 expression and the kinase activity affected the PER1 circadian oscillation in vitro

To determine if the changes in the sleep patterns observed following the WNK3 knockdown are also associated with the changes in PER1 levels, we evaluated the rhythmic patterns of the PER1 protein expression. The neuro 2a cells infected with lentivirus containing WNK3 shRNA (Lv-WNK3si) or a scrambled control shRNA (Lv-Control si) were lysed at different time points after the serum shock, and the lysates were immunoblotted with an anti-PER1 antibody (**Figure 5A**, lower panel). The band intensity of PER1 was quantified using ImageJ, and anti-actin blotting was used as an input control (**Figure 5C**). As WNK3 is a Ser/Thr kinase, we also wanted to determine if the kinase activity of WNK3 affects the PER1 expression patterns. Thus, we transfected Neuro 2a cells with a WNK3 kinase-inactive mutant (K159M) or a vector control and used the abovementioned procedure to evaluate the changes in the PER1 protein levels (**Figure 5A**, upper panel, **Figure 5B**). We found that the PER1 expression pattern was affected by the WNK3 expression and the kinase activity, which in turn affected the circadian oscillation in vitro.

Discussion

Molecular and cellular oscillations constitute fundamental cellular timing mechanisms that regulate important and complex physiological

processes requiring precise adjustments. As the central players within the circadian clock gene-regulatory network, the transcriptional activators CLOCK, Cry1, PER1, and BMAL1 modulate the functions of the interacting proteins and thus are likely to be highly relevant for the maintenance of normal circadian rhythms [19]. Ser/Thr kinases, such as CKI, PKCα, and AKT, bind and modify the expressions and activity of these and other circadian rhythm proteins to affect the circadian rhythms [20-22].

In this study, we demonstrated that the Ser/Thr protein kinase WNK3 interacted and co-localized with PER1 in the rat SCN. The WNK3 protein expression exhibited a circadian rhythm in the rat SCN, and the WNK3 knockdown altered the animals' sleep patterns. Moreover, the WNK3 expression and its kinase activity affected the PER1 circadian oscillations in vitro.

This study is the first to demonstrate that WNK3 and PER1 functionally interact in the SCN to regulate circadian rhythms. However, results from earlier studies have suggested that WNK3 can play an important role in circadian regulation. In *Arabidopsis*, the gene expression of the WNK3 homolog apWNK1 is consistent with the expression rhythm of the clock gene APRR3. Furthermore, the phosphorylation of APRR3 by apWNK1 is likely involved in the production and regulation of the circadian rhythm in *Arabidopsis* [10].

Given that PER1 is a key protein involved in the circadian clock gene-regulatory network, we hypothesized that its expression level could be affected by WNK3 binding, which in turn may affect sleep patterns. Indeed, we found that the rats with the SCN WNK3 knockdown showed a significant phase delay of approximately 4 hrs compared with the untreated control rats.

Sleep disorder symptoms are considered to be common comorbidities in patients with autism spectrum disorder (ASD) [2, 23]. It has been shown that these patients exhibit WNK3 deletion mutations [24]. Moreover, ASD patients often exhibit sleep difficulties, including poor sleep efficiency, prolonged sleep latency, and increased frequency and length of night awakenings [23, 24]. Some of these symptoms are consistent with our WNK3 knockdown rat models. Thus, future studies using genetic sequencing could examine whether WNK3 mutations are associated with sleep problems.

The internal circadian rhythms of the SCN can be regulated by external light. Changes in light and dark in the external environment are detected in the retina, where they stimulate the activity of the melanopsins to allow the transmission of relevant information from the retinal ganglion cells to the SCN through the retinohypothalamic tract. Most neurons in the SCN are gamma-aminobutyric acid (GABA) neurons [25, 26]. Although the role of GABA action is largely unclear, GABA is known to promote chloride influx by activating the $GABA_A$ receptors [25]. Notably, WNK3 has been reported to act as a neuron-specific, chloride-sensitive, Ser-Thr kinase in the SCN [27]. Thus, activated WNK3 can regulate circadian rhythms through the phosphorylation and degradation of proteins, such as PER1. Our findings provide new clues for further exploring the role of the WNK3 and PER1 association in the regulation of circadian rhythms within the SCN.

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Disclosure of conflict of interest

None.

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