Cyclin-dependent Kinase 5 (Cdk5) Regulates the Function of CLOCK Protein by Direct Phosphorylation

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Background: Cdk5 is a kinase involved in the various neuronal processes.
Results: Cdk5 directly phosphorylates CLOCK and regulates its transcriptional activity in association with altered stability and subcellular distribution.
Conclusion: Cdk5 functions as a regulator of CLOCK protein.
Significance: Our findings may provide mechanistic links between diverse Cdk5-mediated brain functions and the molecular clock.

Circadian rhythm is a biological rhythm governing physiology and behavior with a period of ~24 h. At the molecular level, circadian output is controlled by a molecular clock composed of positive and negative feedback loops in transcriptional and post-translational processes. CLOCK is a transcription factor known as a central component of the molecular clock feedback loops generating circadian oscillation. Although CLOCK is known to undergo multiple post-translational modifications, the knowledge of their entities remains limited. Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine-threonine kinase that is involved in various neuronal processes. Here, we report that Cdk5 is a novel regulator of CLOCK protein. Cdk5 phosphorylates CLOCK at the Thr-451 and Thr-461 residues in association with transcriptional activation of CLOCK. The Cdk5-dependent regulation of CLOCK function is mediated by alterations of its stability and subcellular distribution. These results suggest that Cdk5 is a novel regulatory component of the core molecular clock machinery.

Circadian rhythm is an internally generated biological rhythm with a period of ~24 h under control of day/night cycle. Circadian rhythm enables our body to adapt to the environmental changes by optimizing a wide variety of physiological processes such as the sleep/wake cycle, hormonal response, and feeding behaviors (1–3). This biological rhythm is found in most tissues, including brain (4, 5). The rhythm is generated by transcriptional and post-translational feedback loops that are composed of networks of clock proteins at the cellular level (1–3, 6). This cell-autonomous event is initiated by the positive limb of feedback loops comprised of the CLOCK/BMAL1 heterodimer. The CLOCK/BMAL1 complex enters nucleus by BMAL1-dependent shuttling and binds to E-box enhancers to drive the transcription of clock-controlled genes, including Per and Cry (7, 8). Newly synthesized PER and CRY proteins heterodimerize, translocate into the nucleus, and repress the transcriptional activity of the CLOCK/BMAL1 complex, forming the core part of the negative feedback loop.

Various clock components undergo post-translational modifications, such as phosphorylation (1, 3, 9–11) and acetylation (12, 13), which are critical for their stability, intracellular localization, and transcriptional activity. Interestingly, CLOCK has its own histone acetyltransferase activity; thus, it acetylates both histone and non-histone proteins, including BMAL1 and PER2 (12, 13). In addition to its histone acetyltransferase enzymatic activity, a CLOCK-dependent phosphorylation of BMAL1 has also been reported (8, 14, 15). CLOCK itself is known to be regulated by cGMP-dependent protein kinase (PKG) and PKC phosphorylations that are important for temporal progression into the circadian daytime and resetting of the molecular clock (16, 17). Recently, glycogen synthase kinase 3β (GSK3β)2 has also been reported as a kinase that phosphorylates CLOCK in a BMAL1-dependent manner, thereby regulating degradation and activation of CLOCK (18). Moreover, it has also been reported that dominant negative CLOCK (CLOCKΔ19) lacking the CLOCK-interacting protein, circadian (CIPC)-binding domain shows less phosphorylation and more stability than wild-type CLOCK does (14, 19). Therefore,

2 The abbreviations used are: GSK3β, glycogen synthase kinase 3β; ANOVA, analysis of variance; IP, immunoprecipitation.
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it appears that post-translational modifications widely occur in clock components and play crucial roles in maintaining the circadian feedback loop, but additional post-translational modification-mediated regulation of the molecular clock remains unidentified.

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine-threonine kinase that is controlled by the neural specific activators, p35 and p39. Cdk5 controls various neuronal processes such as neurogenesis, neuronal migration, and axon guidance (20–22). It has also been proposed that Cdk5 acts as a modulator of the brain reward system, mediating the response to various drugs including psychostimulants (23, 24). Some reports suggest that Cdk5 activities in the brain are linked to various psychiatric diseases related conditions (25, 26), in which CLOCK also has been reportedly associated (27–29).

In this study, we demonstrated that Cdk5 can directly phosphorylate CLOCK, thereby modulating the robustness of the positive limb of the molecular clock. This finding refines the current model for the molecular basis of circadian rhythm by placing Cdk5 as a novel regulatory component in the molecular clock.

EXPERIMENTAL PROCEDURES

Animals—Mice were housed at a constant temperature and maintained in a 12-h light/12-h dark cycle with food and water available ad libitum. Institute for Cancer Research (CrljBgi: CD-1) mice were purchased from Taconic. Genotype of p35 mice (C57BL/6 × 129/Sv) was analyzed as described previously (22). At subjective night, mice were killed, and their brains were obtained under dim red light. All procedures were approved by the Pohang University of Science and Technology Institutional Animal Care and Use Committee. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and all efforts were made to minimize suffering.

Plasmid Constructs and Primers—Human CLOCK cDNA cloned in pEGFP-C3 vector and human BMAL1 cDNA in pEGFP-C3, pCDNA 3.1 Myc-His, and mRFP-N1 plasmid vector were used. Site-directed mutagenesis of CLOCK was performed using PCR-based methods, and the PCR primers used were as follows: 5’-gaaaggtctctgactttcctgtgctcagtaagaggaatgt-3’ for T181F; 5’-gggctgcttgttctctgtgctcagtaagaggaatgt-3’ for T259F; 5’-gaaaggtctctgactttcctgtgctcagtaagaggaatgtg-3’ for T451F; 5’-gggctgcttgttctctgtgctcagtaagaggaatgtg-3’ for T451F; 5’-gggctgcttgttctctgtgctcagtaagaggaatgtg-3’ for T451F; 5’-gtttgcgcctcctctgctcagtaagaggaatgtg-3’ for T451F; 5’-gggctgcttgttctctgtgctcagtaagaggaatgtg-3’ for T451F; 5’-gggctgcttgttctctgtgctcagtaagaggaatgtg-3’ for T451F; 5’-gtttgcgcctcctctgctcagtaagaggaatgtg-3’ for T451F; 5’-gtttgcgcctcctctgctcagtaagaggaatgtg-3’ for T451F; 5’-gtttgcgcctcctctgctcagtaagaggaatgtg-3’ for T451F.

In Vitro Kinase Assay—Recombinant GST-fused CLOCK protein fragments were expressed and purified from the BL21 strain of *Escherichia coli* using glutathione-Sepharose affinity chromatography (GE Healthcare). Each purified protein was incubated in the presence or absence of immunoprecipitates from mouse whole brain extracts using an anti-p35 antibody (Santa Cruz Biotechnology). Reactions were carried out in a reaction buffer (30 mM HEPES, pH 7.2, 10 mM MgCl₂, and 1 mM DTT) containing [γ-³²P]ATP (10 μCi) at room temperature for 1 h and then terminated by adding SDS sample buffer and boiling for 10 min. Samples were subjected to SDS-PAGE, stained with Coomassie Brilliant Blue, and dried, and then phosphorylated CLOCK fragments were detected by autoradiography. The purified recombinant N-terminal His₆-tagged human Cdk5 (14–516) and N-terminal GST-tagged human p25 (14–516) were purchased from Millipore.

Cell Culture and Transfection—HEK293 and NIH3T3 cells were cultured in DMEM (Hyclone) supplemented with 10% fetal bovine serum and antibiotics in 5% CO₂ at 37 °C. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. NIH3T3 cells were treated with 100 nm dexamethasone (Sigma) for 2 h in culture medium. For stable cell line, transiently transfected cells were selected using G418 (Geneticin, Invitrogen). For neuronal cultures, primitive cortices were dissected from E15 mouse embryos in Hank’s balanced salt solution (Invitrogen).

Cells were dissociated by treating with DNase I (0.1%) and trypsin (0.25%) for 5 min at 37 °C. The dissociated cells were diluted in plating medium (Neurobasal, Invitrogen) with 1 mM HEPES (pH 7.4) and 10% horse serum and then plated onto 12-well plates coated with poly-d-lysine and laminin. After 3 h from plating, plating medium was replaced with culture medium (Neurobasal supplemented with 0.02% B27, 10% glutamine, and antibiotics).

Antibodies and Immunoblot Analyses—Anti-CLOCK (H-276), anti-BMAL1 (N-20), anti-α-tubulin (DM1A), anti-Cdk5 (DC17), anti-p35 (C-19), anti-Lamin-B (G-14), anti-GAPDH (6C5), anti-GFP (B-2), and anti-Myc (9E10) antibodies were purchased from Santa Cruz Biotechnology. Anti-pThr-Pro (9391) antibodies were purchased from Cell Signaling Technology. Cell and brain extracts were prepared with the ELB lysis buffer (250 mM NaCl, 50 mM Tris-HCl, pH 8.0, 15 mM EDTA, 0.1% Nonidet P-40, 1× protease inhibitor mixture (Roche Diagnostics), 5 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10 mM NaF, 2 mM Na₃VO₄, and 1 mM DTT) and centrifuged for 10 min at 13,000 rpm. For immunoblot analyses, protein samples in 2× SDS sample buffer were boiled for 10 min and subjected to SDS-PAGE and immunoblot analyses. Densitometric analyses were carried out using NIH ImageJ software.

Immunoprecipitation and Immunofluorescence Microscopy—Immunoprecipitation was performed on cell and mouse forebrain lysates in ELB lysis buffer. 1 μg of anti-CLOCK or anti-p35 antibodies was added to the lysates and incubated for 3 h at 4 °C. Immunocomplexes were purified using protein A-agarose. After 2 h, supernatants were carefully discarded and washed three times with lysis buffer, and SDS sample buffer was added for subsequent SDS-PAGE and immunoblot analyses. For immunofluorescence imaging, cells were fixed for 10 min with 4% paraformaldehyde in PBS and blocked with PBS containing 2% goat serum and 1% Triton X-100. Cells were incubated with primary antibodies as indicated and visualized with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 568, and Fluor Alexa 647 (Invitrogen), respectively. Hoechst dye was used for nuclear staining. Images were captured using confocal microscopy (Olympus, FluoView-1000).
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Subcellular Fractionation—Cells were trypsinized and harvested in PBS and then resuspended in ice-cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 1 × protease inhibitor mixture (Roche Applied Science), 10 mM KCl, 0.5 mM DTT). After homogenization with 40 strokes of a dounce homogenizer and a brief centrifugation (228 × g, 5 min, 4 °C), the supernatant was collected as a "cytoplasmic fraction." The pellet was resuspended in buffer B (250 μM sucrose, 10 mM MgCl₂, 1 × protease inhibitor mixture), on which buffer C (880 μM sucrose, 500 μM MgCl₂, 1 × protease inhibitor mixture) was carefully layered. The pellet from centrifugation (2800 × g, 10 min, 4 °C) was used as the "nuclear fraction."

Dual-Luciferase Assay—Cells were plated in 12-well plates 24 h before transfection. They were transiently transfected with pGL3 backbone constructs encoding hPer1 or mPer1-promoter (2.2 kb, containing three E-box elements) fused with a destabilized firefly luciferase and pRL-β-actin constructs (to normalize the efficiency of transfection) with the indicated plasmid DNA. Luciferase activity was measured 36 h later using the Dual-Luciferase reporter assay system (Promega) following the manufacturer's instructions.

RNA Isolation and Quantification of mRNA Levels—Total RNA of mouse brain and cultured cells was isolated using TRIzol reagent (Invitrogen). SuperScript III (Invitrogen) or manufacturer's instructions. RNA of mouse brain and cultured cells was isolated using TRIzol reagent (Invitrogen). SuperScript III (Invitrogen) or manufacturer's instructions. Reverse transcription was performed using the ImProm-II reverse transcription system (Promega) was used for reverse transcription. Synthesized cDNA was subjected to quantitative PCR with SYBR Premix Ex Taq (TaKaRa) using gene-specific primers. The primers were 5’-gcaagaagacgtcgcctagc-3’ and 5’-tcagtcaggtcctgacgact-3’ for per1, 5’-agattggccagtaacag-3’ and 5’-atgctccaaaccacgtaagg-3’ for mper1, and 5’-cactagagccagcataag-3’ and 5’-ttactctttgagcgcctc-3’ for GAPDH. The levels of mper1 and mper2 mRNA were normalized to those of GAPDH mRNA.

Statistical Analysis—All data are presented as mean ± S.E. Statistical analyses were conducted using one-way ANOVA followed by a Bonferroni post hoc test or a two-tailed t test. All data were analyzed using the GraphPad Prism software for Windows (version 5). Statistical significance is indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001; and ns, not significant.

RESULTS

Cdk5 Phosphorylates Thr-451 and Thr-461 of CLOCK—From a bioinformatics screening using the consensus sequence for Cdk5 phosphorylation, (S/T)P(X/K/H/R), we detected CLOCK as a potential substrate for Cdk5. Five threonines and one serine residue of CLOCK protein are highly conserved across various vertebrate species (Fig. 1A). To confirm whether Cdk5 can phosphorylate CLOCK, we carried out an immunoprecipitation (IP)-linked in vitro kinase assay (Fig. 1C, panel i) (22, 30, 31). CLOCK protein was separated into two fragments based on the potential phosphorylation sites and the predicted secondary structure (Fig. 1B), and the residues were mutated to Ala or Phe by site-directed mutagenesis. In the in vitro kinase assay, both two fragments showed phosphorylation signals, and the purified recombinant CLOCK fragment, GST-CLOCK238-477, showed a stronger phosphorylation signal than GST-CLOCK23-244 (Fig. 1C, panel ii). Treatment of roscovitine, a Cdk5 inhibitor, significantly decreased phosphorylation signal (Fig. 1D, panel i). We also tested LiCl, an inhibitor of GSK3 because an interaction between GSK3 and Cdk5 has been reported (32). LiCl did not affect the phosphorylation signal, supporting a relative specificity of Cdk5/p35-mediated in vitro phosphorylation of CLOCK (Fig. 1D, panel ii). To remove possible contamination of unknown kinase pulled down in the p35 IP process, we also tried in vitro kinase assay using purified recombinant Cdk5 and could observe consistent result with IP-linked in vitro kinase assay (Fig. 1D, panel ii). Remarkably, substitutions of the Thr-451 and Thr-461 residues with Phe caused dramatic decreases in the phosphorylation signal, whereas the other residues did not show significant changes, indicating that Thr-451 and Thr-461 are major target residues for the Cdk5-mediated phosphorylation (Fig. 1E).

CLOCK Is a Physiological Substrate of Cdk5—As a next step, we tested whether CLOCK is phosphorylated by Cdk5 in more physiological settings. Consistent with the result of in vitro kinase assays, co-expression of Cdk5/p35 significantly increased proline-directed phospho-Thr (pThr-Pro) signal of wild-type CLOCK, whereas T451F/T461F mutation abolished the effect of Cdk5/p35 (Fig. 2A). Moreover, in synchronized cells, the phosphorylation of proline-directed Thr on endogenous CLOCK occurred rhythmically (Fig. 2B), and roscovitine treatment significantly decreased pThr-Pro signal at peak time point (Fig. 2C). To further confirm the phosphorylation of CLOCK protein by Cdk5 in more physiological conditions, we used heterozygote p35 KO mice (22). Consistently, pThr-Pro signal of CLOCK was significantly decreased in the heterozygote p35 KO mouse brain (Fig. 2D). Taken together, these results indicate that CLOCK can be phosphorylated by Cdk5 in physiological conditions.

Cdk5 Physically Interacts with CLOCK—To further examine the functional link between Cdk5 and CLOCK, we investigated whether CLOCK forms a complex with Cdk5/p35 because many of the Cdk5 substrates show physical interactions (30, 33, 34). In co-immunoprecipitation (co-IP) experiments, Cdk5 and p35 were detected in the CLOCK immunoprecipitates, suggesting their physical interaction in the cellular environment (Fig. 3A). Interestingly, co-expression of all of these proteins remarkably decreased CLOCK protein. Furthermore, treatment of MG132, a 26S proteasome inhibitor, significantly increased Cdk5 and p35 levels in co-immunoprecipitates, suggesting that the interaction is likely to be mediated by p35 and is susceptible to Cdk5 activity-dependent proteasome-mediated degradation (20). Moreover, significant co-localization of CLOCK, Cdk5, and p35 was observed, further supporting their physical interaction (Fig. 3B). Consistently, the association of CLOCK and Cdk5/p35 in the mouse brain extract was also observed by the co-IP experiment (Fig. 3C). Collectively, these results indicate that Cdk5 directly interacts with and phosphorylates CLOCK.

Cdk5 Regulates Transcriptional Activity of CLOCK—As a way to assess the roles for the Cdk5-mediated phosphorylation of CLOCK, we tested its impact on transcriptional activity of CLOCK. We analyzed CLOCK/BMAL1-dependent transcriptional activation by using the luciferase reporter construct carrying the hPer1 promoter (35). CLOCK and BMAL1 caused a
significant increase in transcriptional activity of the hPer1 promoter and the co-expression of Cdk5/p35 further enhanced the effect (Fig. 4A). Importantly, the substitution of Thr-451/461 residues with Phe in CLOCK effectively abolished the effect of Cdk5/p35 co-expression. On the other hand, the phosphorylation mimic mutant CLOCK, T451/461E, showed significantly increased hPer1 promoter-dependent luciferase activity even without the co-expression of Cdk5/p35. We further tested whether endogenous mPer1 mRNA expression is altered by expression or inhibition of Cdk5/p35. The overexpression of

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**FIGURE 1. Cdk5 phosphorylates Thr-451/461 of CLOCK.**

A. sequence alignment of CLOCK proteins from various species. Potential Cdk5 phosphorylation sites are indicated in boldface type. B, a schematic diagram of CLOCK protein. Predicted functional domains are indicated by a shaded box, and two fragments of CLOCK protein used for GST fusion protein are indicated as lines. C. panel i, validation of p35 IP-linked in vitro kinase assay. Histone was used for positive control substrate. IP was performed with control IgG or anti-p35 antibody. Arrows and asterisk indicate Histone and phosphorylated p35, respectively. C. panel ii, IP-linked in vitro phosphorylation of CLOCK fragments. IP was performed with control IgG or anti-p35 antibody. Arrows and asterisks indicate GST-fusion CLOCK fragments and phosphorylated p35, respectively. D, panel i, in vitro kinase assay upon treatment of kinase inhibitors. Kinase reactions were carried out with p35 immunoprecipitates preincubated with LiCl (10 and 50 mM) or roscovitine (Rosc.; 20 μM) for 1 h. Arrows and an asterisk indicate the GST-CLOCK258-477 fragment and phosphorylated p35, respectively. D. panel ii, in vitro kinase assay using purified recombinant Cdk5. Kinase reactions with GST-CLOCK fragments were carried out with the purified recombinant His6-Cdk5/GST-p25. Arrows and an asterisk indicate GST-CLOCK258-477 and phosphorylated GST-p25, respectively. E, IP-linked in vitro kinase assay with mutant CLOCK fragments. The gel was stained with Coomassie Brilliant Blue (top) and applied to autoradiography (bottom). Arrows and asterisks indicate GST-fused CLOCK fragments and phosphorylated p35, respectively. Band intensities were measured using ImageJ software and statistical significance was determined by one-way ANOVA (n = 3). Error bars indicate mean ± S.E. ***, p < 0.001; ns, not significant.
Cdk5/p35 significantly increased endogenous mPer1 mRNA, presumably reflecting enhanced transcriptional activity of endogenous CLOCK, and the treatment with roscovitine effectively abrogated the effect (Fig. 4B). Next, we tested to see whether this phenomenon is recapitulated in cortical neurons where indispensable roles for Cdk5 was defined (20) and a robust CLOCK/BMAL1-dependent circadian rhythmicity has been observed (36, 37). Consistently, treatment with roscovitine significantly decreased mPer1 mRNA levels in cultured cortical neurons in a dose-dependent manner (Fig. 4C). Moreover, cortical neurons cultured from p35 knock-out mice embryos also showed significantly lower mPer1 mRNA levels than wild-type neurons (Fig. 4D). Collectively, these results indicate that Cdk5/p35 enhances transcriptional activity of CLOCK/BMAL1 complex likely by phosphorylation of Thr-451 and Thr-461. Next, we examined the role of Cdk5-mediated CLOCK phosphorylation in circadian rhythmicity by monitoring the oscillatory expression of mPer1 in NIH3T3 cells upon dexamethasone treatment. As shown in Fig. 4E, the amplitude of mPer1 mRNA expression significantly increased in cells expressing Cdk5 and p35 compared with the control group. Moreover, the expression of phosphomimic mutant, T451E/T461E, showed the effects similar to the Cdk5/p35 expression (Fig. 4F). We also tested oscillatory expression of CLOCK target genes using heterozygote p35 knock-out mice, which show reduced Cdk5 activity without developmental abnormalities (22, 25, 38). We observed a drastically dampened peak of mPer1 mRNA oscillation (Fig. 4G). In addition, another CLOCK target gene, mPer2, also showed decreased amplitude in mRNA expression (Fig. 4H). These results further confirm that Cdk5 participates in the regulation of the molecular clock by modulating the function of CLOCK protein.

Cdk5 Regulates the Stability of CLOCK—Regulation of the stability of various clock proteins is a key control point for the accuracy and precision of the molecular clock (11, 39). As CLOCK is also known to be regulated by phosphorylation-dependent proteolysis (14, 18), we examined the effect of Cdk5/p35 expression on CLOCK stability. Indeed, co-expression of Cdk5/p35 decreased CLOCK protein levels, but not BMAL1 (Fig. 5A). Consistently, inhibition of Cdk5 activity with roscovitine significantly decreased mPer1 mRNA levels (Fig. 4C). Furthermore, the expression of phosphomimic mutant, T451E/T461E, showed the effects similar to the Cdk5/p35 expression (Fig. 4F). We also tested oscillatory expression of CLOCK target genes using heterozygote p35 knock-out mice, which show reduced Cdk5 activity without developmental abnormalities (22, 25, 38). We observed a drastically dampened peak of mPer1 mRNA oscillation (Fig. 4G). In addition, another CLOCK target gene, mPer2, also showed decreased amplitude in mRNA expression (Fig. 4H). These results further confirm that Cdk5 participates in the regulation of the molecular clock by modulating the function of CLOCK protein.
vitine treatment blocked this effect (Fig. 5B). This phenomenon is likely due to decreased CLOCK stability because co-expression of Cdk5 significantly increase the decay rate of CLOCK protein upon cycloheximide treatment, which was effectively abolished by roscovitine pretreatment (Fig. 5C). Importantly, the T451F/T461F mutant was relatively stable upon the co-expression of Cdk5/p35 complex and T451E/T461E mutant CLOCK showed a half-life similar to that of wild-type CLOCK upon Cdk5/p35 co-expression (Fig. 5D). These results indicate that Cdk5-mediated phosphorylation of the Thr-451/461 residues of CLOCK is critical for regulating CLOCK stability.

Next, we tested whether Cdk5 co-expression affects CLOCK/BMAL complex formation because the translocation and activation of CLOCK are dependent on the BMAL1 (8, 15). Interestingly, the co-IP of CLOCK and BMAL1 was readily detectable upon Cdk5/p35 co-expression or T451F/T461F mutation, although CLOCK and BMAL1 protein levels were significantly decreased (Fig. 5E). Moreover, Cdk5-mediated diminishment of CLOCK and BMAL1 was effectively abolished by either treatment of roscovitine or mutations of Thr-451/461 on CLOCK (Fig. 5F and G). These results suggest that the phosphorylation of CLOCK by Cdk5 accelerates the destabilization of CLOCK/BMAL1.

**Altered Cellular Distribution of CLOCK by Cdk5 and Mutation of Thr-451/461 on CLOCK**—As nuclear translocation of CLOCK is essential for its roles as a transcription factor (7, 8), we investigated the potential impact on subcellular distribution of CLOCK. In the subcellular fractionation assays, cytoplasmic CLOCK level was reduced by co-expression of Cdk5/p35 compared with nuclear CLOCK (Fig. 6A). To confirm this observation, we visualized altered cellular CLOCK distribution by enhanced Cdk5 activity using immunocytochemistry. Consistently, co-expression of Cdk5/p35 significantly altered cellular distribution of CLOCK; however, replacement of Thr-451/461 with Phe effectively blocked this phenomenon (Fig. 6B). When comparing mutant CLOCKs with wild-type CLOCK, phospho-mimic CLOCK (T451E/T461E) showed a relatively increased ratio of nuclear CLOCK to total CLOCK and decreased ratio of cytoplasmic CLOCK to total CLOCK (Fig. 6C). Interestingly, T451F/T461F mutant CLOCK showed strikingly reversed cellular distribution with CLOCK primarily in the cytoplasm, despite having BMAL1 expression (Fig. 6C). Moreover, T451F/T461F mutant CLOCK was not affected by treatment of leptomycin B, a nuclear export inhibitor, whereas wild-type CLOCK showed a remarkable accumulation in the nucleus (Fig. 6D). These results suggest that the Thr-451 and -461 residues participate in the regulation of CLOCK nuclear translocation.

**DISCUSSION**

It is known that CLOCK undergoes rhythmic phosphorylation, which has been proposed as an important regulatory mechanism for the molecular clock (10, 14). Indeed, abnormal function of a mutant clock protein, CLOCKΔ19, has been associated with reduced phosphorylation (14). Moreover, cGMP-
dependent protein kinase and PKC phosphorylate CLOCK and activate the positive arm of the molecular clock feedback loop and thus promote the initiation of the subjective day phase (16, 17). GSK3 has also been identified as a kinase for phosphorylation of CLOCK at Ser-427 in a BMAL1-dependent manner (18). Ser-38 and Ser-42 residues of CLOCK have also been reported to be phosphorylated by unknown kinases, thereby affecting nuclear localization and DNA binding (14). In the present study, we report that Cdk5 directly phosphorylates CLOCK and alters its transcriptional activity. Moreover, reduced Cdk5

FIGURE 4. Cdk5 regulates transcriptional activity of CLOCK. A, luciferase assay to assess hPer1 promoter activity. HEK293 cells were transfected as indicated and hPer1 luciferase reporter construct followed by luciferase activity measurement (n = 4). B, increased mPer1 mRNA level by expression of Cdk5. Cdk5/p35 constructs were stably expressed in NIH3T3 cells and incubated with roscovitine (Rosc.; 20 μM) or dimethyl sulfoxide (vehicle) for 12 h and applied to qRT-PCR (n = 3). C, decreased mPer1 mRNA level in cultured cortical neurons by roscovitine treatment. Cultured cortical neurons (days in vitro 7) were incubated with roscovitine for 12 h and subjected to analysis (n = 3). D, decreased mPer1 mRNA level in p35 knock-out neurons. Cortical neurons (days in vitro 7) cultured from wild-type and p35 knock-out mouse embryos were analyzed (n = 6). E, enhanced amplitude of mPer1 mRNA expression by Cdk5. NIH3T3 cells stably expressing Cdk5/p35 were synchronized by dexamethasone (Dex.) and subjected to qRT-PCR at each time point (n = 3). F, increased amplitude of mPer1 mRNA oscillation by phospho-mimic mutant CLOCK (n = 3). G and H, dampened amplitude of mPer1 and mPer2 mRNA expression level in heterozygote p35 knock-out mouse brain. Wild-type and p35 heterozygote knock-out mice were entrained in 12-h light/12-h dark cycles for 7 days, and cerebral cortices isolated at each time points were subjected to quantitative RT-PCR analysis. Experiments were performed in duplicate. Each mPer1 or mPer2 mRNA value was normalized to GAPDH. Statistical significance was determined by one-way ANOVA or two-tailed t test. Error bars indicate mean ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001. ns, not significant.
activity significantly decreased oscillatory power in the mRNA level of CLOCK target genes. Therefore, phosphorylation of CLOCK by CdK5 can serve as a novel regulatory component for a robust activation of molecular clock. Notably, the Thr-451/461 residues are in close proximity to the phospho-degron regulated by GSK3 (18), which implies the possibility that multiple kinases and phosphorylation sites are functionally interlinked, for the regulation of CLOCK protein function. Collectively, we believe that current findings and further studies may provide important clues for understanding the roles of CLOCK modifications in the molecular clock and possibly linked cellular system.

Although, roscovitine is widely used as an inhibitor of Cdk5, its specificity is not restricted to Cdk5. For example, roscovitine is known to inhibit other Cdks and MAP kinase in a concentration-dependent manner (40–42). In our results, in NIH3T3 cells where p35 is not readily detectible, roscovitine treatment displayed a significant impact on functionalities of CLOCK especially when Cdk5/p35 was co-expressed. Moreover, unlike cell division-related Cdks, Cdk5 is known to be predominantly

FIGURE 5. Cdk5 regulates the stability of CLOCK. A, decreased CLOCK protein level by co-expression of Cdk5. CLOCK or BMAL1 were expressed with Cdk5/p35 in HEK293 cells, and cell lysates were analyzed by immunoblotting. Desitomtric quantification is also shown (n = 3). B, recovery of CLOCK protein level by inhibition of Cdk5. Transfected cells were incubated with roscovitine (Rosc.; 20 μM) for 12 h and applied to immunoblotting (n = 4). C, enhanced CLOCK degradation by Cdk5 co-expression. The transfected cells were incubated with roscovitine (20 μM) for 12 h followed by 40 μg/ml cycloheximide (CHX) treatment for indicated periods. D, altered CLOCK degradation kinetics by the Thr-451/461 mutations. Band intensities were determined using ImageJ software and normalized to α-tubulin, and statistical significance was determined by one-way ANOVA. Error bars indicate mean ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001. ns, not significant. E, co-IP of CLOCK and BMAL1 upon co-expression of Cdk5/p35 or mutation of Thr-451/461. Anti-CLOCK immunoprecipitates from transfected HEK293 cells were analyzed by immunoblotting. F and G, CLOCK-dependent degradation of BMAL1 upon co-expression of Cdk5/p35. HEK293 cells were transfected and treated as indicated. Cell lysates were analyzed by immunoblotting (n = 3). Band intensities were determined using ImageJ software and normalized to α-tubulin. Statistical significance was determined by one-way ANOVA. Error bars indicate mean ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001. ns, not significant.
active in post-mitotic neurons (20, 43), where roscovitine showed significant effect. These indicate that the roscovitine effect in our experimental setting is likely to be mediated by Cdk5 inhibition. However, it is still possible that other Cdk5s may also affect the function of CLOCK, as the cell cycle-related Cdk5s and Cdk5 share consensus substrate motifs. Actually, the
link between circadian clock and cell-cycle system has been proposed (44). Obviously, more detailed mechanistic analyses on the interface between circadian clock and cell cycle are in demand.

In mammals, resetting the circadian rhythm is mainly accomplished by the master clock located in the hypothalamic suprachiasmatic nucleus. However, robust circadian rhythmicity of the clock genes that are expressed in other brain regions has also been discovered (45–47). Notably, although the master clock is mainly governed by light stimulation, the peripheral clock, which is located outside of the suprachiasmatic nucleus, cannot only be affected by the suprachiasmatic nucleus-dependent resetting signal but also affected by non-photic stimulation, such as food intake or psycho-active drugs (36, 48). Considering previous reports that demonstrated enhanced Cdk5 activity from a chronic psychostimulant administration and increased food reinforcement upon loss of Cdk5 function (24, 49), Cdk5 activity may be involved with non-photic stimuli in the brain. Thus, it is intriguing to speculate that Cdk5 might be a link between the circadian system and non-photic inputs that are mostly related to the peripheral clock, demanding further studies at the organism level.

Previously, it has been reported that transcriptional activation of CLOCK is tightly coupled with its nuclear translocation and degradation (8). For example, GSK3-mediated phosphorylation of CLOCK regulates transcriptional activity and degradation simultaneously (18). In addition CLOCKΔ19, a mutant CLOCK that displays reduced phosphorylation and transcriptional activity, is resistant to degradation compared with wild-type CLOCK (14, 50). These molecular events are consistent with the “black widow model”; the stability and transcriptional activity of certain transcription factors, including Jun, Fos, Myc, p53, and HIF1-α, exhibit a negative correlation that is critical for temporal fine tuning of gene expression (8, 47, 51). In the present study, we showed that the stability of CLOCK was decreased by the expression of Cdk5. It was noteworthy that Cdk5 promoted the degradation of BMAL1 only under co-expression of CLOCK, indicating that the Cdk5-mediated destabilization of CLOCK induces the degradation of BMAL1. Our results also showed that Cdk5 elicited transcriptional activation and altered translocation of CLOCK. Interestingly, CLOCK-dependent degradation of BMAL1 is known to be coupled with translocation of CLOCK/BMAL1 complex in a good correlation with enhanced transcriptional activity (8, 10, 15, 47). Thus, our results suggest that Cdk5-mediated phosphorylation of CLOCK affects molecular events following dimerization of CLOCK and BMAL1, thereby triggering translocation, transcriptional activation, and eventually degradation of CLOCK/BMAL1 complex, which appears to fit to the “black widow model.” Thus, the functional relationship between CLOCK and Cdk5 identified in this study may provide an additional example of this model.

A potential link between the circadian system and mood disorders has been suggested. For example, an insufficient length of light phase has been linked to seasonal affective disorder by affecting entrainment of the molecular clock (52, 53). In addition, abnormalities of the sleep/wake cycle, hormonal function, and daily activity are prominent symptoms of mood disorders, and normalization of daily cycles is mostly associated with mood stabilization (54, 55). Moreover, therapies controlling light stimulation effectively reverse the symptoms of mood disorders (56–59). Recently, it has been reported that the CLOCKΔ19 mouse displays phenotypes strikingly similar to human mania in terms of their hyperactive ventral tegmental area dopaminergic neurons and manic-like behaviors (28). Moreover, decreased CLOCK expression of CLOCK in the ventral tegmental area elicits a mixed state of mania and depression-like behaviors (29). These reports indicate that abnormal CLOCK may be related to an abnormal dopaminergic system and mood conditions. Intriguingly, Cdk5 is well known to regulate dopamine signaling by regulating key players such as tyrosine hydroxylase and DARPP-32 in both the presynaptic and postsynaptic parts (20, 21), and increased Cdk5/p35 activity was associated with depressive-like behaviors in rats (26). To this end, our results may provide a mechanistic link between dopamine system-related mood conditions and the molecular clock, which has long been postulated.

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**FIGURE 6. Altered subcellular distribution of CLOCK by Cdk5 and mutation of Thr-451/461.** A, altered cytoplasmic and nuclear distribution of CLOCK by Cdk5. Transfected cells were subjected to nuclear fractionation analysis. Densitometric quantification is shown on the right (n = 3). B, Immunocytochemistry of increased nuclear CLOCK by Cdk5. Wild-type or mutant CLOCK (green) was co-expressed with RFP-BMAL1 (red) and Cdk5 (cyan) in HEK293 cells, and fluorescence intensity was analyzed. Nuclei were stained with Hoechst dye (n > 10). White arrows indicate the cells expressing CLOCK without Cdk5/p35, and yellow arrows indicate the cells expressing CLOCK with Cdk5/p35. C, altered subcellular distribution of Thr-451/451 mutant CLOCKS. Wild-type, T451F/T461F, and T451E/T461E mutant CLOCK proteins were expressed with RFP-BMAL1. Relative (Rel.) fluorescence intensity in individual cells was analyzed across the longest axis of the cell (yellow line). Green indicates GFP-CLOCK, and nuclei stained by Hoechst dye were outlined by white dashed lines (n > 18). D, T451F/461F mutant fails to accumulate in the nucleus under leptin B treatment. HEK293 cells were transfected as indicated and leptin B (LMB; 10 ng/ml) was treated for 5 h. Images were captured using confocal microscopy (n > 25). Measurement of fluorescence intensities was carried out using ImageJ software. Statistical significance was determined by the two-tailed t test or one-way ANOVA. *Error bars* indicate mean ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
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