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Role of Activating Transcription Factor-4 in 24-Hour Rhythm of Serotonin Transporter Expression in the Mouse Midbrain[S]

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ABSTRACT

Serotonin (5-HT) transporter (5-HTT) plays a key role in the control of 5-HT neuronal activity by reuptaking extracellular 5-HT from the synapse cleft. We have previously demonstrated that *5-HTT* mRNA expression levels and its uptake activity in the mouse midbrain are significantly higher in the dark phase than those in the light phase. However, the molecular mechanisms of time-dependent expression of *5-HTT* have not been clarified. In this study, expression of *5-HTT* mRNA in the mouse midbrain showed a significant 24-h rhythm and was higher in the dark phase. Although such an oscillation was eliminated by a *Clock* gene mutation, CLOCK and BMAL1 did not activate *5-HTT* transcription in the luciferase reporter assay. Activating transcription factor-4 (ATF4), a member of the ATF/cAMP response element (CRE)-binding protein family, is a component responsible for sustaining circadian oscillations of CRE-mediated gene expression. ATF4 significantly activated *5-HTT* transcription in vitro and time dependently bound to the CRE site in the *5-HTT* promoter in the mouse midbrain. In addition, mutation of the *Clock* gene disrupted temporal binding of ATF4 to the CRE site in the *5-HTT* promoter. These results indicated that the circuit of circadian-basis molecular regulation between the clockwork system and mouse *5-HTT* gene was connected by the ATF4 signaling pathway.

Introduction

Serotonin (5-HT) is widely distributed in the central nervous system and its behavioral effects include feeding, sexual behavior, and circadian rhythms (Saller and Stricker, 1976; Hull et al., 1999; Mistlberger et al., 2000; Portas et al., 2000). Raphe nuclei located in the midbrain are the origin of serotonergic fibers that innervate many regions of the brain (Jacobs and Azmitia, 1992). 5-HT transporter (5-HTT) is located at the presynaptic membrane and plays a key role in the control of 5-HT neuronal activity by reuptaking extracellular 5-HT from the synapse cleft (Borowsky and Hoffman, 1995). In a previous study, we demonstrated that *5-HTT* mRNA expression levels and its uptake activity in the mouse midbrain are significantly higher in the dark phase than those in the light phase, which would contribute to the dosing time-dependent effect of antidepressants (Ushijima et al., 2005).

In mammals, 24-h rhythmicity is under the control of a molecular pacemaker that is composed of clock gene products (Reppert and Weaver, 2002; Lowrey and Takahashi, 2004). These gene products constitute an oscillatory mechanism that is based on self-sustained transcriptional/translational feedback loops. This oscillatory transcriptional system not only resides in the hypothalamic suprachiasmatic nucleus (SCN), the center of the mammalian circadian clock, but is also in almost all peripheral tissues (Yoo et al., 2004). Gene products of *Clock* and *Bmal1* form a heterodimer that activates transcription of period (*Per*) and cryptochrome (*Cry*) genes. Once PER and CRY proteins have reached a critical concentration, they attenuate CLOCK/BMAL1 transactivation, thereby gener-

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ABBREVIATIONS: 5-HT, serotonin; 5-HTT, serotonin transporter; SCN, suprachiasmatic nucleus; CRE, cAMP response element; ATF, activating transcription factor; CREB, cAMP response element-binding protein; PCR, polymerase chain reaction; ZT, zeitgeber time; NIH, National Institutes of Health; MOPS, 4-morpholinepropanesulfonic acid; TEF, thyrotroph embryonic factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; shRNA, short hairpin RNA; ANOVA, analysis of variance; PPARE, PAR-bZip protein response element.

ating circadian oscillations in their own transcription (Gekakis et al., 1998; Kume et al., 1999).

It has been reported that cAMP-dependent signaling is involved in circadian output pathways, but cAMP signaling subsequently sustains core oscillation loops in the SCN (Obrietan et al., 1999; O'Neill et al., 2008). Intracellular accumulation of cAMP induces CRE-mediated gene expression via ATF/CREB activation. ATF/CREB proteins belong to the bZIP transcription factor superfamily and are characterized by a conserved domain including highly charged basic amino acids that are required for DNA recognition at the TGACGT[C/A][G/A] sequence. We have demonstrated that ATF4 periodically activated transcription of the *Per2* gene; furthermore, loss of ATF4 disrupted expression rhythms of clock genes (Koyanagi et al., 2011). On the basis of these findings, ATF4 is a component responsible for sustaining circadian oscillations of CRE-mediated gene expression.

It is anticipated that time-dependent expression of *5-HTT* mRNA in the mouse midbrain would be controlled by the circadian clockwork system. However, molecular mechanisms underlying this periodical expression have not yet been clarified. In this study, we found that the 24-h profile of *5-HTT* mRNA expression in the midbrain was eliminated by a mutation of the *Clock* gene in mice. In addition, ATF4 acted as a regulator of circadian *5-HTT* gene expression in the mouse midbrain.

Materials and Methods

Animals. Male ICR mice (5 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). *Clock* mutant (*Clk/Clk*) mice (C57BL/6J-*Clock*mlJt/J) were purchased from The Jackson Laboratory (Bar Harbor, ME). We placed mice in an ICR genetic background to enhance the robustness of the breeding and care of the young. Mice were backcrossed using a Jcl:ICR background more than eight generations. *Clock* mutant mice were heterozygous, and genotypes were determined using PCR methods. These studies were performed using wild-type ICR mice (control) or homogeneous *Clock* mutation mice. Mice were housed in a light-controlled room (light on at 07:00 AM [zeitgeber time (ZT) 0] and off at 7:00 PM [ZT 12]) at room temperature of 24 ± 1°C and a humidity of 60 ± 10% with food and water ad libitum. All mice were adapted to the light/dark cycle for 2 weeks before experiments. All animal procedures were performed in accordance with the Guidelines for Animal Research at Kyushu University (Fukuoka, Japan) and approved by the Use and Care of Experimental Animals Committee.

Western Blotting Analysis. Whole brains were removed from wild-type and *Clk/Clk* mice, and the midbrain was separated at ZT 2, 6, 10, 14, 18, and 22. For preparation of membrane proteins, midbrain samples were homogenized in Krebs-Ringer buffer (128.9 mM NaCl, 4.2 mM KCl, 1.5 mM $CaCl_2$, 22.4 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, and 10 mM D-glucose) containing proteinase inhibitors (100 μM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, and 2 mg/ml aprotinin) on ice. After centrifugation at 8000*g* for 15 min, supernatants were further centrifuged for 60 min at 105,000*g*. After removal of the soluble fraction, pellets obtained were resuspended in membrane preparation buffer [20 mM MOPS-Tris (pH 7.0), 0.3 M sucrose, 5 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, and 2 mg/ml aprotinin]. Resuspensions were centrifuged for 60 min at 105,000*g*, and the resulting pellets were resuspended in membrane preparation buffer and used as cellular membrane fractions. For detection of ATF4 and ACTIN proteins, midbrain samples were homogenized in CelLytic M-Cell Lysis buffer (Sigma-Aldrich, St. Louis, MO). Homogenate was centrifuged, and supernatant was collected as a protein sample. Protein concentrations were determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA). Lysate samples were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were reacted with antibodies against 5-HTT (Alpha Diagnostic International Inc., San Antonio, TX), ATF4, and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunocomplexes were further reacted with horseradish peroxidase-conjugated secondary antibodies and visualized using SuperSignal Chemiluminescent Substrate (Thermo Fisher Scientific). Membranes were photographed using Polaroid-type film, and the density of each band was analyzed using NIH Image software on a Macintosh computer.

Preparation of Crude Synaptosomes and 5-HT Uptake Assay. Midbrain samples were homogenized in 0.32 M sucrose and centrifuged at 100*g* at 4°C for 10 min. The pellet was discarded, and supernatant was centrifuged at 12,000*g* at 4°C for 20 min. The supernatant was discarded, and the pellet was resuspended in the original volume of 0.32 M sucrose used as crude synaptosomes. Crude synaptosomes (final concentration: 0.25 mg of protein) were preincubated at 37°C for 5 min with Krebs-HEPES buffer (127 mM NaCl, 5 mM KCl, 1.3 mM NaH_2SO_4, 15 mM HEPES, 10 mM glucose, and 1.2 mM $MgSO_4$). The buffer was gassed with oxygen for 30 min before use. ^{3}H-labeled 5-HT (final concentration 50 nM; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was added and incubated at 37°C for 5 min, and the reaction mixture was rapidly filtered under a vacuum (GF/B filters; Whatman, Maidstone, UK). Filters were washed three times with Krebs-HEPES buffer, dried, and placed in an ACSII Scintillation Cocktail (GE Healthcare). Nonspecific uptake was calculated from data obtained at incubation at 0°C.

RNA Extraction and PCR Analysis. Midbrain samples were homogenized by a BioMasher (Assist, Tokyo, Japan). Total RNA was extracted using RNAiso (TaKaRa Bio, Shiga, Japan), and cDNAs were synthesized using PrimeScript Reverse Transcriptase (TaKaRa Bio). For analysis of clock genes and *Atf4* mRNA expressions, synthesized cDNAs were amplified using a THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and the 7500 real-time PCR system (Life Technologies, Carlsbad, CA). To control variations in the amount of cDNA available for real-time PCR in different samples, mRNA expression levels of target sequences were normalized to the expression of an endogenous control, β-actin. Sequences of primers for amplification are described in Supplemental Table 1. Data were analyzed using the comparative threshold cycle method. For analysis of *5-HTT* exon1a expression, synthesized cDNAs were amplified using GoTaq Green Master Mix (Promega, Madison, WI). PCR products were run on 2% agarose gels. After staining with ethidium bromide, the gel was photographed using Polaroid-type film. The density of each band was analyzed using NIH Image software on a Macintosh computer. To evaluate the quantitative reliability of reverse transcriptase-PCR, kinetic analysis of the amplified products was performed to ensure that signals were derived only from the exponential phase of amplification, as described previously (Ohdo et al., 2001).

Construction of Reporter and Expression Vectors. The 5′-flanking region of the mouse *5-HTT* (from base pairs −2132 to +97; +1 indicates the transcription start site) gene was amplified using an Elongase Enzyme Mix (Life Technologies). Template DNA was extracted from mice using the Wizard SV Genomic DNA Purification System (Promega), and PCR was performed using the forward primer 5′-AAA CCT CCC CTC CAT CCA AGT CTC CTC AGC-3′ and reverse primer 5′-AAA GGG AAA GCG ACC CAC CTG CGG GA-3′. PCR products were purified and ligated into a pCR-XL-TOPO vector (Life Technologies). A fragment of the target DNA region was separated from the TOPO vector using a M13 primer, and religated into a pGL4 Basic vector (Promega) using Nhe1 and EcoRV enzymes (*5-HTT* exon1a-LUC). CRE in the *5-HTT* exon1a-LUC was mutated from TGACGCCA to TGAATTCA using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Expression vectors for mouse CLOCK, BMAL1, PER2, CRY1, TEF, and ATF4 were

constructed using cDNAs derived from mouse liver RNA. All coding regions were ligated into the pcDNA3.1(+) vector (Life Technologies) as described previously (Koyanagi et al., 2003).

Luciferase Reporter Assay. NIH 3T3 cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Bioscience, Kansas City, MO) at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded at 3×10^5 cells per well in six-well culture plates (BD, Franklin Lakes, NJ). After 18 h, cells were transfected with 100 ng per well of reporter vector and 1 μg per well (total) of expression vector using Lipofectamine LTX reagent (Life Technologies). A 0.5 ng per well sample of phRL-TK vector (Promega) was also cotransfected as an internal control reporter. The total amount of DNA per well was adjusted by addition of pcDNA3.1 vector (Life Technologies). At 24 h after transfection, cells were harvested, and the lysate was analyzed using a dual-luciferase reporter assay system (Promega). The ratio of firefly luciferase activity to *Renilla* luciferase activity in each sample served as a measure of normalized luciferase activity.

Serum Shock. Mouse C-1300 neuroblastoma (C-1300N) cells were purchased from the RIKEN Cell Bank (Kobe, Japan). Cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml insulin. To synchronize circadian clocks in cultured C-1300N cells, serum shock was performed as follows. Cells were grown to semiconfluence in DMEM supplemented with 10% FBS. On the day of serum shock, 50% FBS was added for 2 h, and then cells were incubated in DMEM supplemented with 2% FBS. Cells were harvested for RNA or protein extraction at the indicated times after serum treatment. To explore the role of ATF4 in the regulation of mouse 5-HTT exon1a mRNA expression, cells were transfected with retrovirus vector expression shRNA targeting *Atf4* and thereafter treated with 50% FBS to synchronize their circadian clocks.

Construction of Retrovirus Vectors Encoding shRNA Targeting *Atf4*. Specific silencing of endogenous *Atf4* in C-1300N cells was achieved using a shRNA-expressing retrovirus vector. Nucleotides 1114 to 1132 base pairs of the mouse *Atf4* (GenBank accession number NM_009716) coding sequence were chosen as a target for shRNA. *Atf4* shRNA-encoding oligonucleotides were created as indicated below, each containing the 19-nucleotide target sequence of *Atf4*, followed by a short spacer and an antisense sequence of the target: 5′-GAGCATTCCTTTAGTTTAGAGAGCTCACCCTAAACTAAAGGAATGCTC-3′. The *Atf4* shRNA-encoding sequence was cloned into the BamHI and BglII sites of the pDON-AI2 vector (TaKaRa Bio) and transfected into G3T-hi packaging cells. All infected cells were cultured in a medium containing appropriate antibiotics. The control shRNA-expressing retrovirus vector was made using the same procedure with the oligonucleotide sequence 5′-GCAAGCTGACCCTGAAGTTCAGAGCTCACCGAACTTCAGGGTCAGCTTGC-3′.

Chromatin Immunoprecipitation Assays. Midbrains were prepared at ZT 6 or 18. Samples were excised and treated with 1% formaldehyde for 10 min at room temperature to cross-link chromatin, and the reaction was stopped by addition of glycine to a final concentration of 0.125 M. Each cross-linked sample was sonicated on ice and then incubated with an antibody against ATF4 (Santa Cruz Biotechnology, Inc.). Chromatin/antibody complexes were extracted using a protein G agarose kit (Roche Diagnostics, Indianapolis, IN). DNA was isolated using the Wizard SV Genomic DNA Purification System and subjected to PCR amplification using CRE and negative primers (Supplemental Table 2). PCR products were run on an agarose (3%) gel, including 0.2 μg/ml ethidium bromide, and analyzed using NIH Image software.

Statistical Analysis. A one-way analysis of variance (ANOVA) was used for multiple comparisons, and a Bonferroni-Dunn test was used for comparison between two groups. $P < 0.05$ was considered to be significant.

Results

Twenty-Four-Hour Rhythms of 5-HTT Protein Expression and Transport Activity in the Mouse Midbrain. We investigated the temporal profile of 5-HTT protein abundance in the mouse midbrain. As shown in Fig. 1A, 5-HTT protein levels showed a time-dependent change with higher levels in the dark phase. Similar to time-dependent changes in protein expression, uptake activity of 5-HTT in the mouse midbrain showed a significant 24-h rhythm with higher activity in the dark phase ($F = 4.68$, $P < 0.01$ by ANOVA) (Fig. 1B). However, time-dependent alterations in 5-HTT protein levels and uptake activity were not observed in *Clk/Clk*.

Expression Rhythms of Clock Genes and *Atf4* mRNA in the Mouse Midbrain. Expression levels of *Bmal1*, *Per2*, and *Cry1* mRNA showed significant 24-h rhythms in the mouse midbrain ($F = 5.42$, $P < 0.01$ by ANOVA for *Bmal1*; $F = 12.38$, $P < 0.01$ by ANOVA for *Per2*; $F = 6.28$, $P < 0.01$

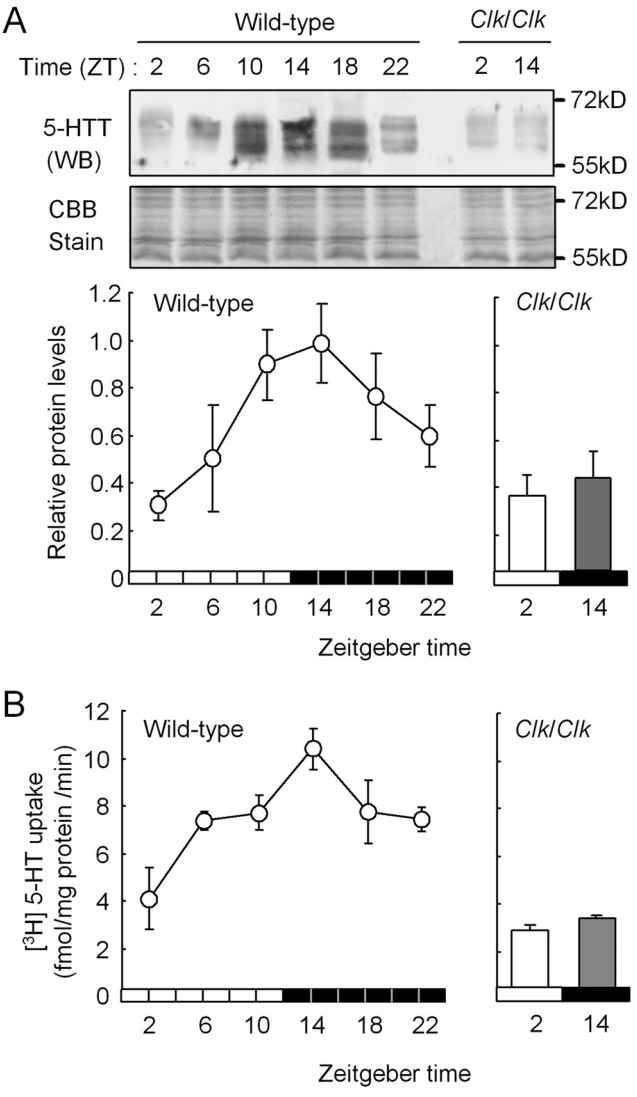

Fig. 1. Twenty-four-hour rhythms of 5-HTT protein expression level (A) and 5-HT uptake activity (B) in the mouse midbrain. Representative images of Western blot (WB) and staining with Coomassie Brilliant Blue (CBB) are shown. For protein expression levels, mean values at the highest observation point in the wild-type were set to 1.0. Each value represents the mean ± S.E.M. ($n = 3$–4).

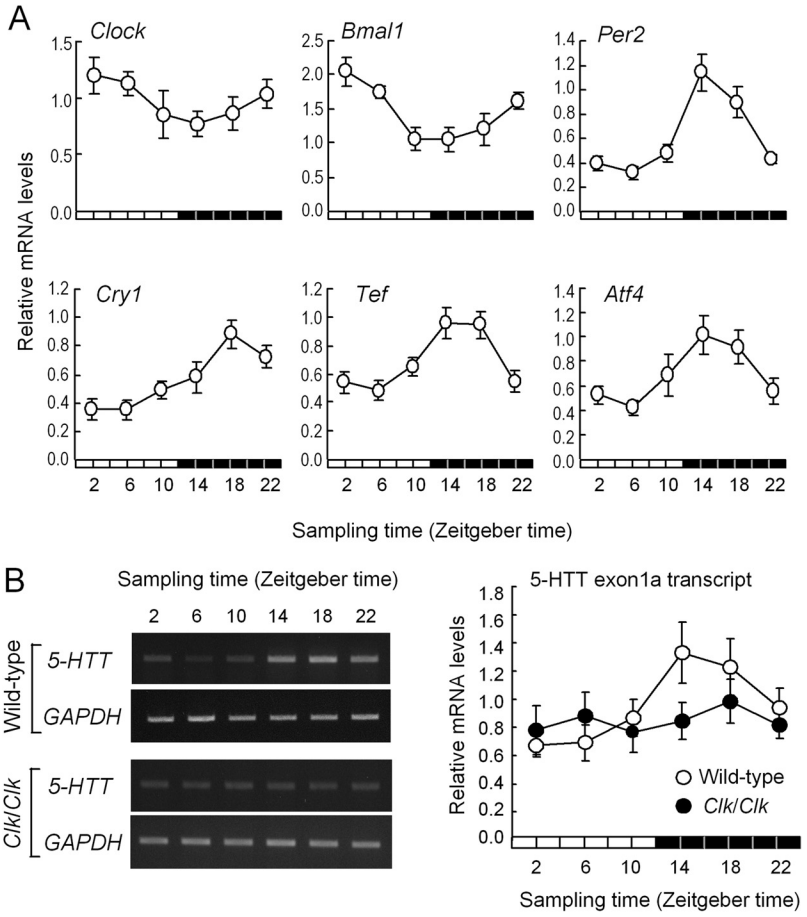

Fig. 2. A, 24-h rhythms of clock genes and *Atf4* mRNA expression in the mouse midbrain. Each value represents the mean ± S.E.M. (n = 4–5). B, 24-h rhythms of *5-HTT* mRNA expression in the midbrain of wild-type and *Clk/Clk* mice. Representative electrophoretic images of reverse transcriptase-PCR products are shown in the left panel, and quantitative analysis data are described in the right panel. Each value represents the mean ± S.E.M. (n = 6). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

by ANOVA for *Cry1*) (Fig. 2A). The phase in 24-h expression of *Bmal1* was higher at early in the light phase, and those of *Per2* and *Cry1* were higher in the dark phase. *Atf4* mRNA expression also showed a significant 24-h rhythm peaking at ZT 14 in the mouse midbrain (F = 3.05, $P < 0.05$ by ANOVA). Expression profiles of *Clock* and *Tef* mRNA seemed to be altered time dependently, but these did not reach significance.

Influence of the *Clock* Gene Mutation on 24-H Rhythm of *5-HTT* mRNA Expressions in the Mouse Midbrain. The rodent *5-HTT* gene has three transcriptional variants, which consist of a different first exon (exon1a, exon1b, or exon1c) and the same exon 2 to exon 5. It was reported that two of the variants (exon1a and exon1a + exon1b) were expressed in the rat brain, but the distribution of these variants was not equally expressed (Ozsarac et al., 2002). Therefore, we investigated which variant was mainly expressed in the mouse midbrain. As described in Supplemental Figure S1, the variant including only exon1a was detected in the mouse midbrain. In wild-type mice, mRNA expression levels of the *5-HTT exon1a* transcript in the midbrain showed a significant 24-h rhythm with higher levels during the dark phase and lower levels during the light phase (F = 3.04, $P < 0.05$ by ANOVA) (Fig. 2B). However, a 24-h expression rhythm of the *5-HTT* exon1a transcript was not observed in *Clk/Clk* mice.

Influence of Clock Genes and ATF4 on Transcriptional Activity of the Mouse *5-HTT* Gene. Nucleotide sequences of the 5′-flanking region of the mouse *5-HTT* gene were analyzed up to 2.1 kilobase pairs in the present study. By analysis of the transcriptional elements under circadian clock regulation, one E-box, one PAR-bZip protein response element (PPARE), and one CRE site were detected in this region (Fig. 3A).

To investigate which transcriptional element was responsible for the rhythmic expression of mouse *5-HTT* transcription, a luciferase reporter gene assay was performed. ATF4 induced significantly higher transcriptional activity of *5-HTT* exon1a-LUC than that of the control vector, whereas neither CLOCK/BMAL1 nor TEF showed an effect (Fig. 3B). In addition, mutation of the CRE site dampened activation of the *5-HTT* exon1a-LUC transcript by ATF4. Suppressive circadian clock genes such as PER2 and CRY1 did not affect the *5-HTT* exon1a-LUC transcription.

To explore the influence of ATF4 on the oscillation of *5-HTT* transcription, mouse neuroblastoma (C-1300N) cells were treated with 50% serum to synchronize the circadian clock. Expression of the *5-HTT* exon1a transcript showed a time-dependent change with higher levels at 24 and 48 h after serum treatment (Fig. 3C). Knocking down of *Atf4* by shRNA decreased *5-HTT* exon1a mRNA levels and diminished its oscillation in serum shocked cells.

Temporal Profiles of ATF4 Protein Abundance in the Mouse Midbrain. Similar to mRNA expression, the profile of ATF4 protein abundance in the midbrain of wild-type mice showed 24-h changes with higher levels in the dark phase (Fig. 4A). However, time-dependent differences

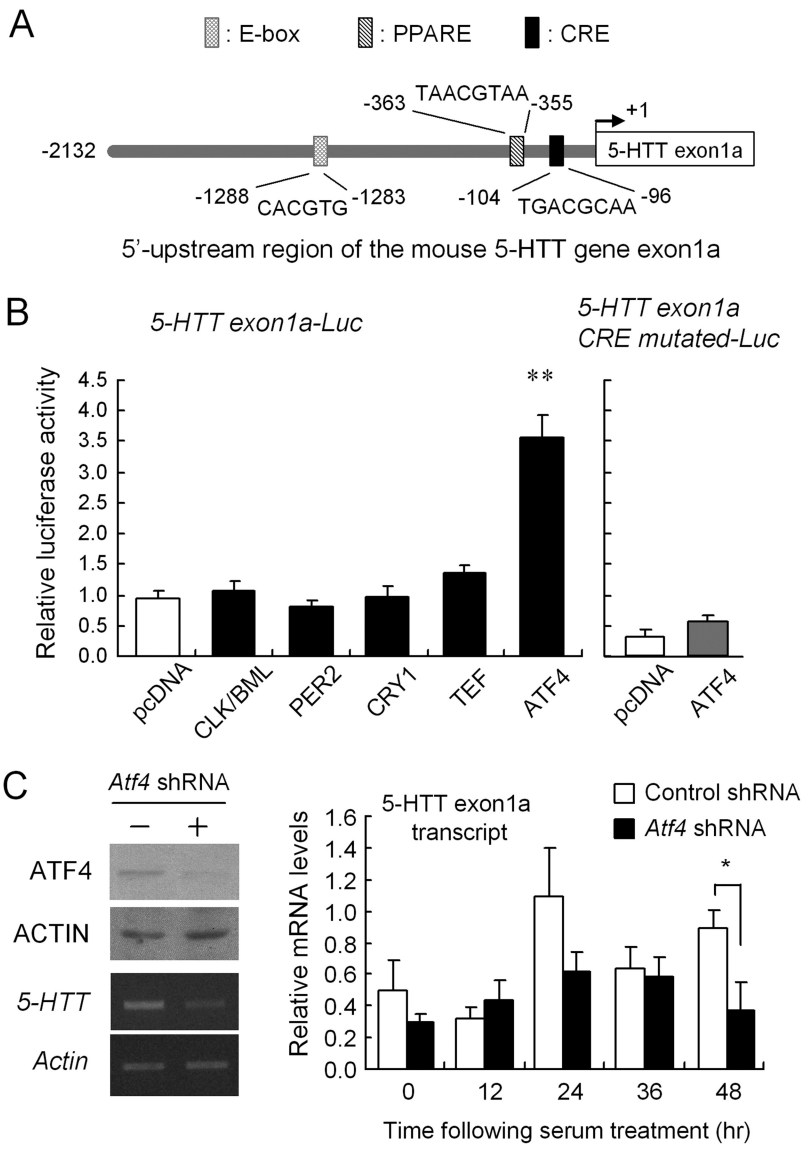

Fig. 3. Influence of clock gene products and ATF4 on transcript activity of the *5-HTT* gene. A, schematic analysis of transcript element in the mouse *5-HTT* promoter region. The numbers listed on the left side of the sequence indicate the distance (base pairs) from the putative transcriptional start site (+1). B, NIH 3T3 cells were cotransfected with the *5-HTT* reporter vector and expression constructs. Values are shown as fold changes from controls (pcDNA3.1). Each value represents the mean ± S.E.M. ($n = 4$). **, $P < 0.01$ versus control. CLK, CLOCK; BML, BMAL1. C, left, Western blot and electrophoretic image of PCR products. Right, expression of *5-HTT* exon1a transcript in 50% serum shocked C-1300N cells. Each value represents the mean ± S.E.M. ($n = 3$). *, $P < 0.05$.

were not observed in *Atf4* mRNA or protein levels of *Clk/Clk* mice.

To investigate whether the binding of ATF4 to the *5-HTT* promoter region changed depending on the time of day, we performed chromatin immunoprecipitation assays. ATF4 time dependently bound to the CRE site at the *5-HTT* promoter region with higher binding at ZT 18 and lower binding at ZT 6 in wild-type mice (Fig. 4B). However, binding of ATF4 to this region did not differ between ZT 6 and 18 in *Clk/Clk* mice.

Discussion

In this study, we showed 24-h rhythms of 5-HTT protein expression and 5-HT uptake activity in the mouse midbrain. We have already reported that the uptake activity of 5-HTT in the mouse midbrain was significantly higher at the dark phase than that at the light phase (Ushijima et al., 2005). The present results were in agreement with our previous findings. Rhythmic expressions of clock genes are reported in many brain regions outside of the SCN (Guilding and Piggins, 2007). In this study, mRNA expressions of many clock genes showed significant 24-h rhythms in the mouse midbrain. Therefore, the oscillation of 5-HTT expression in the mouse midbrain was supposed to be controlled by the circadian clockwork system.

To confirm the role of circadian clockwork systems on the 24-h rhythm of *5-HTT* mRNA expression, we investigated the midbrain of *Clk/Clk* mice. *Clk/Clk* mice have a point mutation causing the deletion of exon 19 of the *Clock* gene and synthesize the mutant CLOCK protein (CLOCKΔ19), which lacks transcriptional activity. Many previous studies reported that these mice exhibit low-amplitude rhythms in various gene expressions, including PAR-bZIP proteins (Ripperger et al., 2000; Oishi et al., 2003; Murakami et al., 2008). In this study, 24-h rhythmicity of *5-HTT* exon1a mRNA expression in the mouse midbrain was eliminated by a *Clock* mutation. In addition, 5-HTT protein levels and its activity did not show time-dependent alterations in *Clk/Clk* mice, therefore suggesting that the 24-h expression rhythm of 5-HTT transcription in the mouse midbrain was sustained by clock gene products.

The transcriptional circuit underlying mammalian cir-

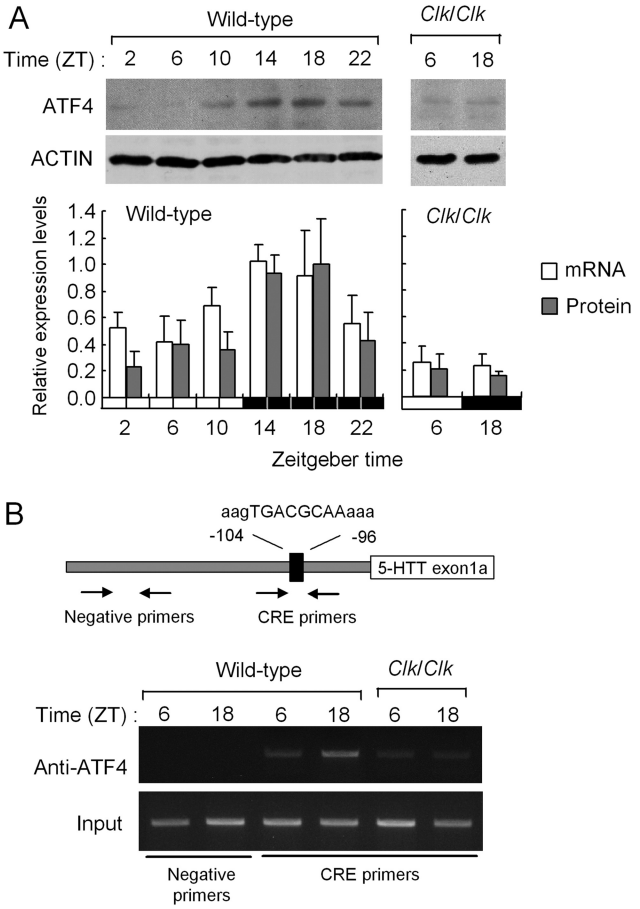

Fig. 4. Chromatin immunoprecipitation analyses at the mouse *5-HTT* gene promoter. A, temporal profiles of ATF4 protein expression in the mouse midbrain. Top, representative images of Western blots. Data for *Atf4* mRNA were the same as in Fig. 2A. Bottom. for protein expression levels, mean values at the highest observation point in the wild-type were set to 1.0. Each value represents the mean ± S.E.M. (n = 3). B, top, schematic image of target regions for PCR amplification in chromatin immunoprecipitation analysis. Arrows represent the regions of primer setting for amplification analysis. Bottom, an electrophoretic image of PCR amplification.

cadian clocks consists of at least three clock-controlled DNA elements: E-box/E′-box (CACGT[G/T]), PPARE (TTA[T/C]GTAA),and Rrev-erbα/ROR binding element ([A/T]A[A/T]NT[A/G]GGTCA) (Gekakis et al., 1998; Preitner et al., 2002; Ueda et al., 2005). Analysis of the nucleotide sequences found the clock-controlled transcript elements, E-box and PPARE, and a CRE site at the mouse *5-HTT* promoter region. To explore which element contributes to transcription of the *5-HTT* gene, we performed a luciferase reporter gene assay. Contrary to our expectations, transcript activity of *5-HTT* exon1a-LUC was not increased by CLOCL/BMAL1 or TEF (transcript activator through PPARE). On the other hand, ATF4 significantly activated the *5-HTT* exon1a-LUC transcript, which was abrogated by mutation of the CRE site. In addition, oscillation of the *5-HTT* exon1a transcript in serum-shocked cells was eliminated by knocking down of *Atf4*. On the basis of these findings, it was indicated that 24-h rhythmicity of the *5-HTT* gene transcript was directly controlled by ATF4/CRE signaling.

Finally, we investigated whether ATF4 time dependently bound to the *5-HTT* promoter region in the mouse midbrain. In wild-type mice, a profile of ATF4 protein abundance in the midbrain showed a time-dependent alteration, and subsequently binding of ATF4 to the CRE site at the *5-HTT* promoter was increased in the dark phase. On the other hand, mutation of the *Clock* gene decreased ATF4 protein levels and diminished time-dependent properties of protein expression. It was suggested that such a lowering of ATF4 protein expression in *Clk/Clk* mice would decrease binding of ATF4 to the *5-HTT* promoter in the midbrain. We previously reported that transcription of the *Atf4* gene was regulated by CLOCK and BMAL1 (Koyanagi et al., 2011). These data led us to confirm that the circadian clockwork system affected the oscillation of mouse *5-HTT* transcription and its transporting activity in the midbrain. Furthermore, ATF4 linked the circadian clock system to 5-HTT.

Circadian-related abnormalities are present in virtually all subtypes of depression including seasonal affective disorder, major depression, and bipolar disorder (Duncan, 1996; Szuba et al., 1997). A recent genomic analysis study revealed interesting clues to implicate abnormal clock gene function in mood disorders (Bunney and Potkin, 2008). In addition, it was reported that *Clk/Clk* mice displayed increases in dopamine cell firing in the ventral tegmental area and induced mania-like behavior (McClung et al., 2005; Roybal et al., 2007). However, the molecular influence of abnormalities in clock gene function on the 5-HT neuron system has not been explored well yet. In the present study, we showed that mutation of the *Clock* gene dampened rhythmic expression of *5-HTT* mRNA with decreases during the dark phase in the mouse midbrain. In two decades, many genomic association studies indicated that the 5-HTT linked polymorphic region short variant, which results in lower expression of 5-HTT, was associated with anxiety-related and negative personality traits in humans (Lesch et al., 1996; Caspi et al., 2003). Furthermore, deficiencies in the *5-HTT* gene in mice exhibited increased anxiety-like behaviors (Holmes et al., 2003; Lira et al., 2003). These findings led us to speculate that abnormalities in the circadian clockwork system would cause a depressive- and anxiety-related mood disorder through alterations in *5-HTT* gene expression.

In this study, we demonstrated a role of ATF4 in 24-h rhythmic expression of the mouse *5-HTT* gene in the midbrain. In addition, the dysfunction of the clockwork system disrupted time-dependent expression of ATF4, which led to the lack of circadian 5-HTT expression and its function in the mouse midbrain. Because 5-HTT is one of the important etiological elements in depressive disorders, further experiments are required to clarify the physiological significance of 24-h rhythmic expression of 5-HTT in the midbrain and to investigate the impact of disturbances in circadian clock regulation in the midbrain on anxiety- and depressive-related behaviors.

Authorship Contributions

Participated in research design: Ushijima, Koyanagi, Matsunaga, and Ohdo.

Conducted experiments: Ushijima, Koyanagi, Sato, and Ogata.

Contributed new reagents or analytic tools: Koyanagi and Ogata.

Performed data analysis: Ushijima.

Wrote or contributed to the writing of the manuscript: Ushijima, Koyanagi, Fujimura, and Ohdo.

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