Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.
Molecular Pharmacology Fast Forward. Published on May 9, 2012 as doi: 10.1124/mol.112.079079

Role of activating transcription factor-4 in 24-hour rhythm of serotonin transporter expression in the mouse midbrain

Kentarou Ushijima, Satoru Koyanagi, Yuuki Sato, Takamitsu Ogata, Naoya Matsunaga, Akio Fujimura, Shigehiro Ohdo

Department of Pharmaceutics (S.K., T.O., N.M. and S.O.), and Department of Clinical Pharmacokinetics (Y.S.), Graduate School of Pharmaceutical Sciences, Kyushu University, and Division of Clinical Pharmacology, Department of Pharmacology, Jichi Medical University (K.U. and A.F.)

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Copyright 2012 by the American Society for Pharmacology and Experimental Therapeutics.

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

a) Running title: Circadian regulation of serotonin transporter by ATF4

b) Corresponding author: Shigehiro Ohdo, Ph.D.

3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

Phone: +81 92 642 6610; Fax: +81 92 642 6614; e-mail: ohdo@phar.kyushu-u.ac.jp

c) The number of text pages; 25

The number of figures; 4

The number of references; 33

Abstract; 205 words

Introduction; 457 words

Discussion; 871 words

d) Abbreviations: 5-HT, serotonin; 5-HTT, serotonin transporter; ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE-biding protein

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Serotonin (5-HT) transporter (5-HTT) plays a key role in the control of 5-HT neuronal activity by re-uptaking 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 that 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-hr rhythm and was higher in the dark phase. While 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 (CREB) 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 clock-work system and mouse *5-HTT* gene was connected by the ATF4 signaling pathway.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Serotonin (5-HT) is widely distributed in the central nervous system and its behavioral effects include feeding, sexual behavior, and circadian rhythms (Hull et al., 1999; Mistlberger et al., 2000; Portas et al., 2000; Saller and Stricker, 1976). 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 pre-synaptic membrane and plays a key role in the control of 5-HT neuronal activity by re-uptaking 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 that in the light phase, which would contribute to the dosing time-dependent effect of anti-depressants (Ushijima et al., 2005).

In mammals, 24-hr rhythmicity is under the control of a molecular pacemaker that is composed of clock gene products (Lowrey and Takahashi, 2004; Reppert and Weaver, 2002). These gene products constitute an oscillatory mechanism that is based on self-sustained transcriptional/translational feedback loops. This oscillatory transcriptional system resides not only 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 generating circadian oscillations in their own transcription (Gekakis et al., 1998; Kume et al., 1999).

Recently, 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

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

(O'Neill et al., 2008; Obrietan et al., 1999). 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). Based on 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 clock-work system. However, molecular mechanisms underlying this periodical expression have not yet been clarified. In this study, we found that the 24-hr 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.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

Materials & 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 [zeitgeber time, ZT 0] and off at 19:00 [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, 1.5mM $CaCl_2$, 22.4 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 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 8,000g for 15 min, supernatants were further centrifuged for 60 min at 105,000g. After removal of the soluble fraction, obtained pellets

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

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 105,000g, 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, Rockford, IL). 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, Santa Cruz, CA). Immunocomplexes were further reacted with horseradish peroxidase–conjugated secondary antibodies and visualized using Super Signal 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.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

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 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

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

1.2 mM $MgSO_4$). The buffer was gassed with oxygen for 30 min prior to use. [3H]-labeled 5-HT (final concentration 50 nM; GE Healthcare, 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 cold 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 were described in Supplementary 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 RT-PCR, kinetic analysis of the amplified products was performed to ensure that signals were derived only from the exponential phase of amplification, as previously described (Ohdo et al., 2001).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

Construction of reporter and expression vectors

The 5'-flanking region of the mouse *5-HTT* (from bp -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 re-ligated 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 (thyrotroph embryonic factor), and ATF4 were constructed using cDNAs derived from mouse liver RNA. All coding regions were ligated into the pcDNA3.1 (+) vector (Life Technologies), as previously described (Koyanagi et al., 2003).

Luciferase reporter assay

NIH3T3 cells were maintained in DMEM (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Bioscience, Kansas City, MI) 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 Technologies, Franklin Lakes, NJ). After 18-hr, cells were transfected with 100 ng per well of reporter vector and 1 μg per well (total) of expression vector using Lipofectamine LTX

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

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 adding pcDNA3.1 vector (Life Technologies). At 24-hr posttransfection, 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 semi-confluence in DMEM supplemented with 10% FBS. On the day of serum shock, 50% FBS was added for 2-hr 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 1114–1132 bp of the mouse *Atf4* (Genbank Accession# NM_009716) coding sequence were chosen as a target for shRNA. *Atf4* shRNA-encoding oligonucleotides were created as indicated below, each containing the

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

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 following oligonucleotide sequence: 5'-GCAAGCTGACCCTGAAGTTCAGAGCTCACCGAACTTCAGGGTCAGCTTGC-3'

Chromatin immunoprecipitation (ChIP) assays

Midbrains were prepared at ZT 6 or 18. Samples were excised and treated with 1% formaldehyde for 10 minutes at room temperature to cross-link chromatin, and the reaction was stopped by adding 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). Chromatin/antibody complexes were extracted using a protein G agarose kit (Roche Diagnostics). DNA was isolated using the Wizard SV Genomic DNA Purification System (Promega) 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 the 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.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

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 Figure 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-hr rhythm with higher activity in the dark phase ($F=4.68$, $P<0.01$ by ANOVA, Figure 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-hr 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*; $F=6.28$, $P<0.01$ by ANOVA for *Cry1*, Figure 2A). The phase in 24-hr 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-hr 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-hr 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

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

exon (exon1a, exon1b or exon1c) and the same exon-two to exon-five. 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-hr rhythm with higher levels during the dark phase and lower levels during the light phase ($F=3.04$, $P<0.05$ by ANOVA, Figure 2B). However, a 24-hr 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 was analyzed up to 2.1 kbp in the present study. By analysis of the transcriptional elements under circadian clock regulation, one E-box, one PPARE, and one CRE-site were detected in this region (Figure 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 (Figure 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 *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

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

clock. Expression of the *5-HTT exon1a* transcript showed a time-dependent change with higher levels at 24 and 48 hr after serum treatment (Figure 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-hr changes with higher levels in the dark phase (Figure 4A). However, time-dependent differences 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 ChIP 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 (Figure 4B). However, binding of ATF4 to this region did not differ between ZT 6 and 18 in *Clk/Clk* mice.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

In this study, we showed 24-hr 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 to 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-hr rhythms in the mouse midbrain. Therefore, the oscillation of 5-HTT expression in the mouse midbrain was supposed to be controlled by the circadian clock-work system.

To confirm the role of circadian clock-work systems on the 24-hr rhythm of *5-HTT* mRNA expression, we investigated the midbrain of *Clk*/*Clk* mice. *Clk*/*Clk* mice have a point mutation at exon 19 of the C*lock* 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 (Murakami et al., 2008; Oishi et al., 2003; Ripperger et al., 2000). In this study, 24-hr 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, this suggests that the 24-hr expression rhythm of 5-HTT transcription in the mouse midbrain was sustained by clock gene products.

The transcriptional circuit underlying mammalian circadian clocks consists of at least three clock-controlled DNA elements; E-box/E'-box (CACGT[G/T]), PAR-bZip protein response

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

element (PPARE, TTA[T/C]GTAA), and Rrev-erbα/ROR binding element (RORE, [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 element, E-box and PPARE, and 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 inhibited 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*. Based on these findings, it was indicated that 24-hr 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). This data led us to confirm that the circadian clock-work 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

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

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 (5-HTTLPR) short variant, which results in lower expression of 5-HTT, was associated with anxiety-related and negative personality traits in humans (Caspi et al., 2003; Lesch et al., 1996). 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 clock-work 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-hr rhythmic expression of the mouse *5-HTT* gene in the midbrain. In addition, the dysfunction of the clock-work system disrupted time-dependent expression of ATF4, which led to the lack of circadian 5-HTT expression and its function in the mouse midbrain. Since 5-HTT is one of the important etiologic elements in depressive disorders, further experiments are required to clarify the physiological significance of 24-hr 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.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

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

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

References

Borowsky B and Hoffman BJ (1995) Neurotransmitter transporters: molecular biology, function, and regulation. *Int Rev Neurobiol* **38**:139-199.

Bunney JN and Potkin SG (2008) Circadian abnormalities, molecular clock genes and chronobiological treatments in depression. *Br Med Bull* **86**:23-32.

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, McClay J, Mill J, Martin J, Braithwaite A and Poulton R (2003) Influence of life stress on depression: Moderation by a polymorphism in the 5-HTT gene. *Science* **301**(5631):386-389.

Duncan WC, Jr. (1996) Circadian rhythms and the pharmacology of affective illness. *Pharmacol Ther* **71**(3):253-312.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS and Weitz CJ (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280**(5369):1564-1569.

Guilding C and Piggins HD (2007) Challenging the omnipotence of the suprachiasmatic timekeeper: are circadian oscillators present throughout the mammalian brain? *Eur J Neurosci* **25**(11):3195-3216.

Holmes A, Yang RJ, Lesch KP, Crawley JN and Murphy DL (2003) Mice lacking the serotonin transporter exhibit 5-HT(1A) receptor-mediated abnormalities in tests for anxiety-like behavior. *Neuropsychopharmacology* **28**(12):2077-2088.

Hull EM, Lorrain DS, Du J, Matuszewich L, Lumley LA, Putnam SK and Moses J (1999) Hormone-neurotransmitter interactions in the control of sexual behavior. *Behav Brain Res* **105**(1):105-116.

Jacobs BL and Azmitia EC (1992) Structure and function of the brain serotonin system. *Physiol Rev* **72**(1):165-229.

Koyanagi S, Hamdan AM, Horiguchi M, Kusunose N, Okamoto A, Matsunaga N and Ohdo S (2011) cAMP-response Element (CRE)-mediated Transcription by Activating

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

Transcription Factor-4 (ATF4) Is Essential for Circadian Expression of the Period2 Gene. *J Biol Chem* **286**(37):32416-32423.

Koyanagi S, Kuramoto Y, Nakagawa H, Aramaki H, Ohdo S, Soeda S and Shimeno H (2003) A molecular mechanism regulating circadian expression of vascular endothelial growth factor in tumor cells. *Cancer Res* **63**(21):7277-7283.

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH and Reppert SM (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* **98**(2):193-205.

Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, Benjamin J, Muller CR, Hamer DH and Murphy DL (1996) Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* **274**(5292):1527-1531.

Lira A, Zhou M, Castanon N, Ansorge MS, Gordon JA, Francis JH, Bradley-Moore M, Lira J, Underwood MD, Arango V, Kung HF, Hofer MA, Hen R and Gingrich JA (2003) Altered depression-related behaviors and functional changes in the dorsal raphe nucleus of serotonin transporter-deficient mice. *Biol Psychiatry* **54**(10):960-971.

Lowrey PL and Takahashi JS (2004) Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu Rev Genomics Hum Genet* **5**:407-441.

McClung CA, Sidiropoulou K, Vitaterna M, Takahashi JS, White FJ, Cooper DC and Nestler EJ (2005) Regulation of dopaminergic transmission and cocaine reward by the Clock gene. *Proc Natl Acad Sci U S A* **102**(26):9377-9381.

Mistlberger RE, Antle MC, Glass JD and Miller JD (2000) Behavioral and serotonergic regulation of circadian rhythms. *Biological Rhythm Research* **31**(3):240-283.

Murakami Y, Higashi Y, Matsunaga N, Koyanagi S and Ohdo S (2008) Circadian clock-controlled intestinal expression of the multidrug-resistance gene mdr1a in mice. *Gastroenterology* **135**(5):1636-1644 e1633.

O'Neill JS, Maywood ES, Chesham JE, Takahashi JS and Hastings MH (2008)

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. *Science* **320**(5878):949-953.

Obrietan K, Impey S, Smith D, Athos J and Storm DR (1999) Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nuclei. *J Biol Chem* **274**(25):17748-17756.

Ohdo S, Koyanagi S, Suyama H, Higuchi S and Aramaki H (2001) Changing the dosing schedule minimizes the disruptive effects of interferon on clock function. *Nat Med* **7**(3):356-360.

Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi G, Ohkura N, Azama T, Mesaki M, Yukimasa S, Kobayashi H, Iitaka C, Umehara T, Horikoshi M, Kudo T, Shimizu Y, Yano M, Monden M, Machida K, Matsuda J, Horie S, Todo T and Ishida N (2003) Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. *J Biol Chem* **278**(42):41519-41527.

Ozsarac N, Santha E and Hoffman BJ (2002) Alternative non-coding exons support serotonin transporter mRNA expression in the brain and gut. *J Neurochem* **82**(2):336-344.

Portas CM, Bjorvatn B and Ursin R (2000) Serotonin and the sleep/wake cycle: special emphasis on microdialysis studies. *Prog Neurobiol* **60**(1):13-35.

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U and Schibler U (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**(2):251-260.

Reppert SM and Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* **418**(6901):935-941.

Ripperger JA, Shearman LP, Reppert SM and Schibler U (2000) CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. *Genes Dev* **14**(6):679-689.

Roybal K, Theobold D, Graham A, DiNieri JA, Russo SJ, Krishnan V, Chakravarty S, Peevey J, Oehrlein N, Birnbaum S, Vitaterna MH, Orsulak P, Takahashi JS, Nestler EJ,

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

Carlezon WA, Jr. and McClung CA (2007) Mania-like behavior induced by disruption of CLOCK. *Proc Natl Acad Sci U S A* **104**(15):6406-6411.

Saller CF and Stricker EM (1976) Hyperphagia and increased growth in rats after intraventricular injection of 5,7-dihydroxytryptamine. *Science* **192**(4237):385-387.

Szuba MP, Guze BH and Baxter LR, Jr. (1997) Electroconvulsive therapy increases circadian amplitude and lowers core body temperature in depressed subjects. *Biol Psychiatry* **42**(12):1130-1137.

Ueda HR, Hayashi S, Chen W, Sano M, Machida M, Shigeyoshi Y, Iino M and Hashimoto S (2005) System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat Genet* **37**(2):187-192.

Ushijima K, Sakaguchi H, Sato Y, To H, Koyanagi S, Higuchi S and Ohdo S (2005) Chronopharmacological study of antidepressants in forced swimming test of mice. *J Pharmacol Exp Ther* **315**(2):764-770.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M and Takahashi JS (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A* **101**(15):5339-5346.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This study was supported by a Grant-in-Aid for Young Scientists (B) [K.U. 22790166].

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Fig. 1 Twenty-four-hr 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) were shown. For protein expression levels, mean values at the highest observation point in the wild-type was set to 1.0. Each value represents the mean ± SEM (n=3-4).

Fig. 2 (A) Twenty-four-hr rhythms of clock genes and *ATF4* mRNA expression in the mouse midbrain

Each value represents the mean ± SEM (n=4-5).

(B) Twenty-four-hr rhythms of *5-HTT* mRNA expression in the midbrain of wild-type and *Clk/Clk* mice

Representative electrophoretic images of RT-PCR products were shown in the left panel, and quantitative analysis data was described in the right panel. Each value represents the mean ± SEM (n=6).

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 was shown. The numbers listed on the left side of the sequence indicate the distance (base pair) from the putative transcriptional start site (+1).

(B) NIH3T3 cells were co-transfected with the *5-HTT* reporter vector and expression constructs. Values are shown as fold changes from controls (pcDNA3.1). Each value represents the mean ± SEM (n=4). CLK, CLOCK; BML, BMAL1; **, P <0.01 vs. control

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

(C) Western blot and electrophoretic image of PCR products were shown in the left panel. Expression of *5-HTT exon1a* transcript in 50% serum shocked C-1300N cells was described in the right panel. Each value represents the mean ± SEM (n=3). *, $P<0.05$

Fig. 4 Chromatin immunoprecipitation (ChIP) analyses at the mouse *5-HTT* gene promoter

(A) Temporal profiles of ATF4 protein expression in the mouse midbrain

Representative images of western blots were shown in the upper panel. Data of *ATF4* mRNA was the same as Figure 2A. For protein expression levels, mean values at the highest observation point in the wild-type was set to 1.0. Each value represents the mean ± SEM (n=3).

(B) The upper panel shows a schematic image of target regions for PCR amplification in ChIP analysis. Arrows represent the regions of primer setting for amplification analysis. The lower panel described an electrophoretic image of PCR amplification.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

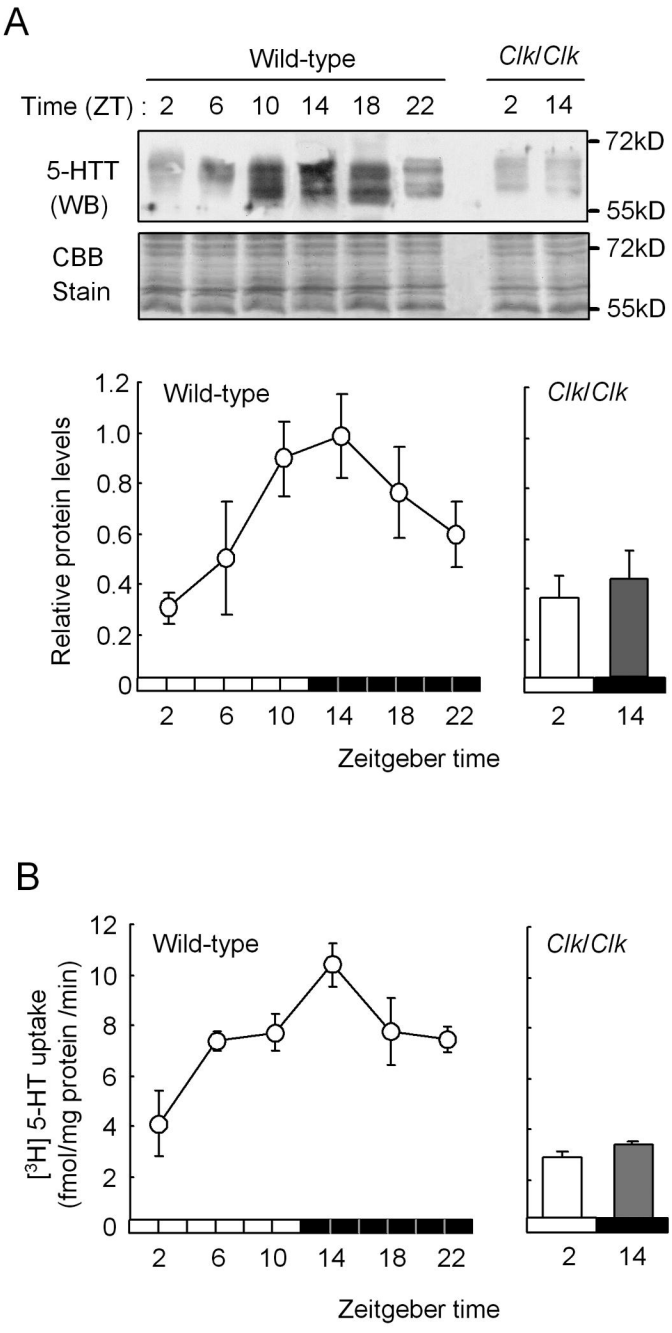


Figure 1

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.

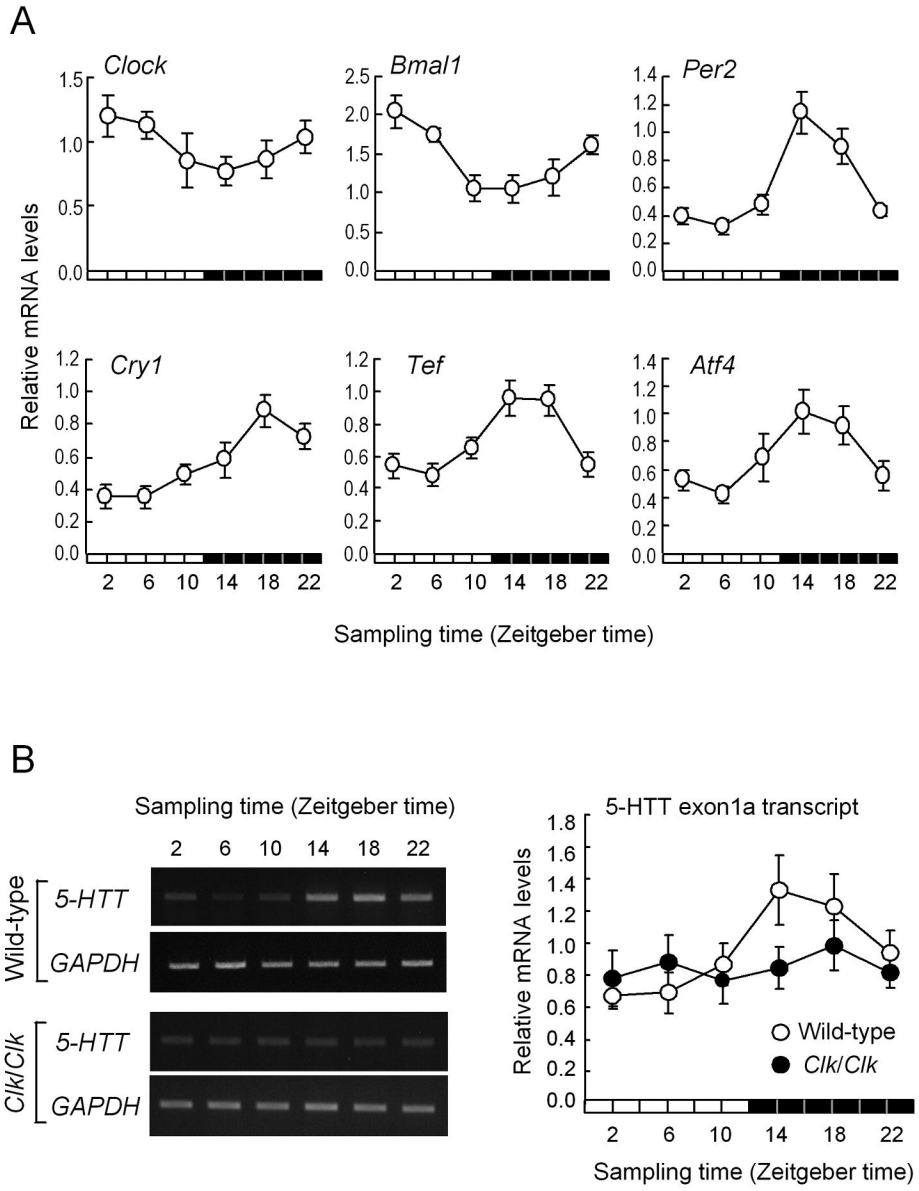
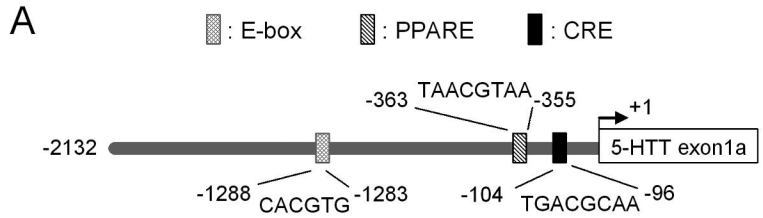


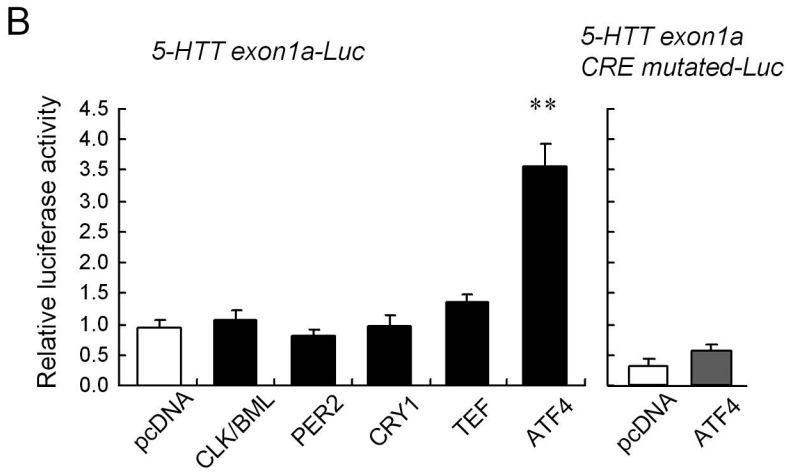
Figure 2

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.



5'-upstream region of the mouse 5-HTT gene exon1a



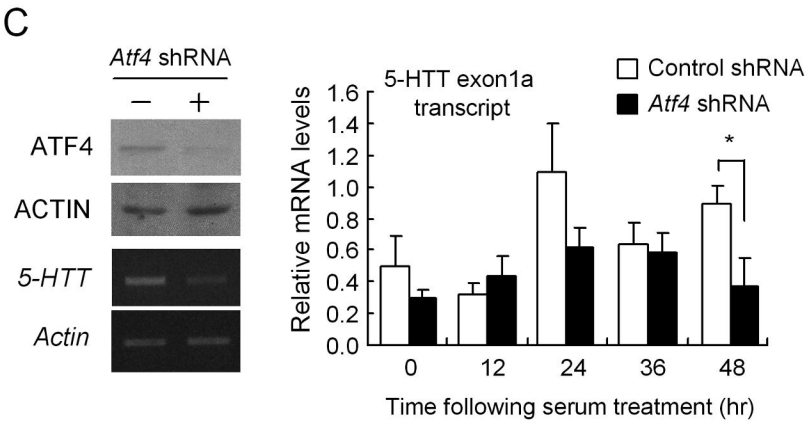
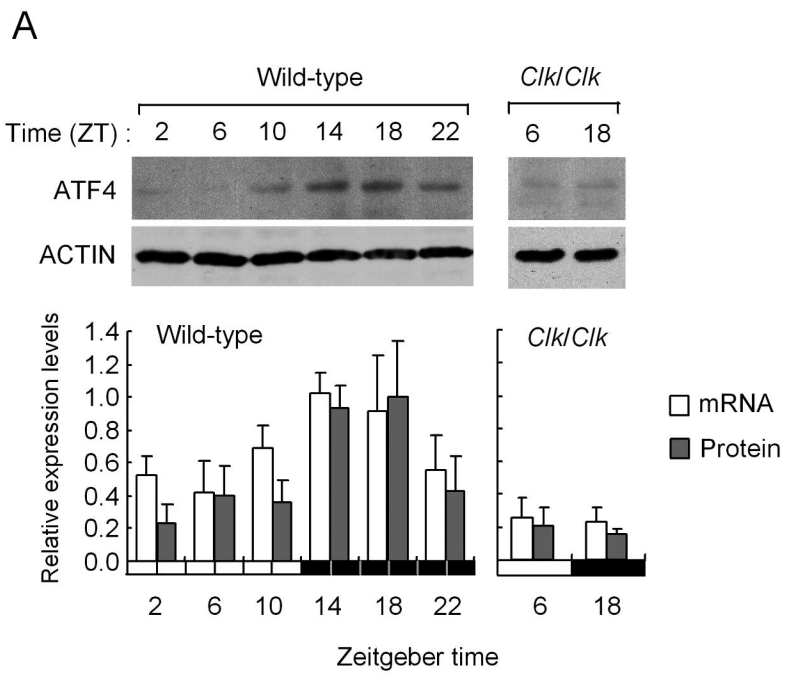


Figure 3

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Molecular Pharmacology Fast Forward. Published on May 9, 2012 as DOI: 10.1124/mol.112.079079
This article has not been copyedited and formatted. The final version may differ from this version.



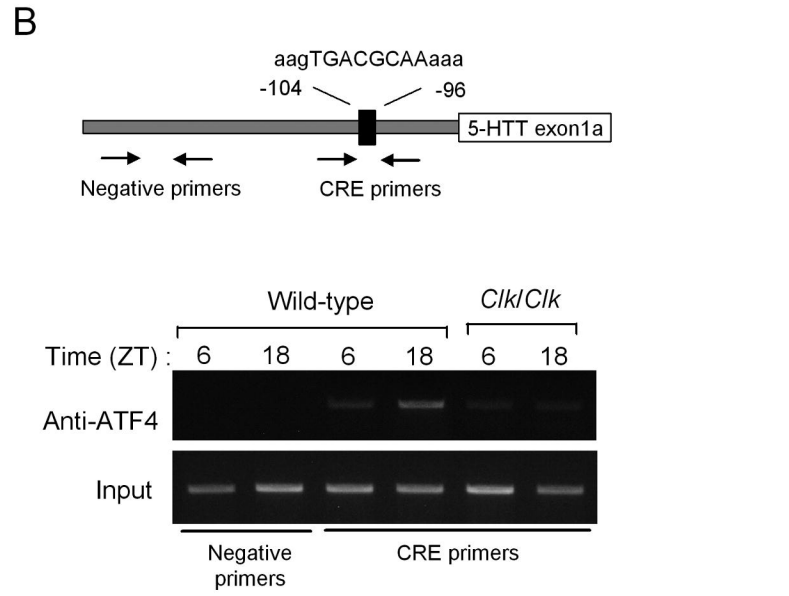


Figure 4

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 18, 2024

Supplemental Data
Molecular Pharmacology

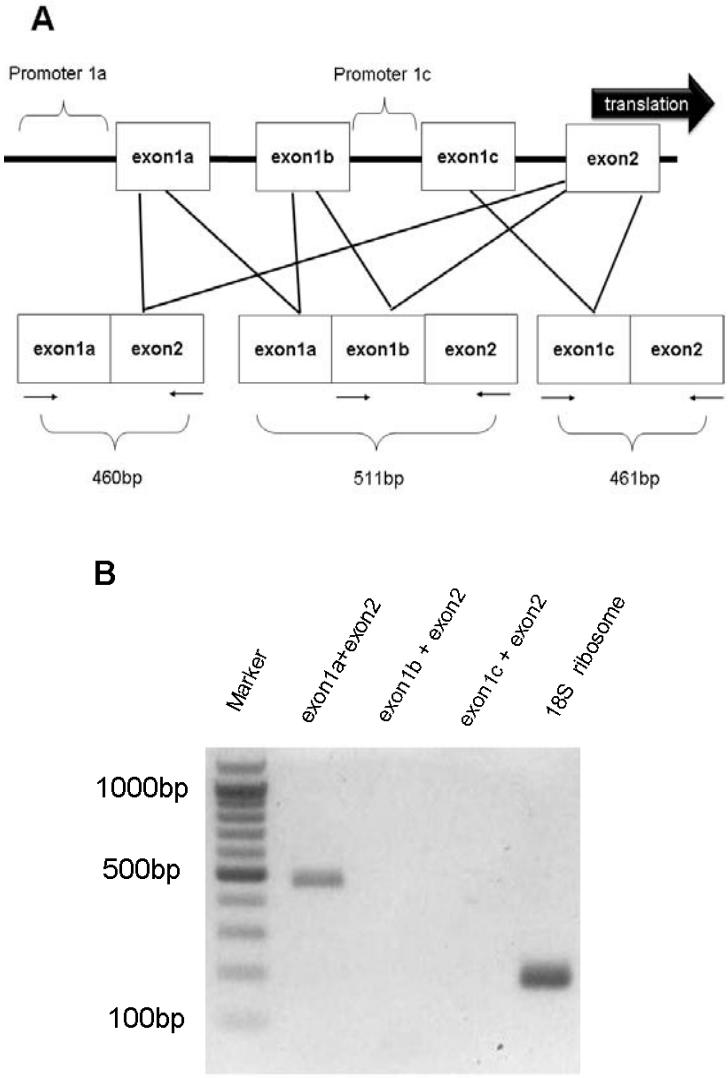
Role of activating transcription factor-4 in 24-hour rhythm of serotonin transporter expression in the mouse midbrain

Kentarou Ushijima, Satoru Koyanagi, Yuuki Sato, Takamitsu Ogata, Naoya Matsunaga, Akio Fujimura, Shigehiro Ohdo

Supplemental method

Total RNA extraction from the midbrain sample, the synthesis of cDNA and PCR analysis were performed as described in the manuscript. Sequences of primers used were described as follows.

Target gene	Primer sequence	Tm(^{o}C)
5-HTT		
Forward (exon1a)	5’-TCG AGG GCG CGA GGG-3’	54.0
Forward (exon1b)	5’-AAG CTG GCA GCT CCC AA-3’	60.4
Forward (exon1c)	5’-CTC AGT CCA GAA GAG AGA G-3’	56.1
Reverse	5’-CCA CGG CAT AGC CAA TGA C-3’	58.2
18S ribosome		
Forward	5’-TCA AGA ACG AAA GTC GGA GG-3’	56.3
Reverse	5’-GGA CAT CTA AGG GCA TCA CA-3’	56.3



Supplemental Fig S1

(A) Genomic structure of the mouse *5-HTT* gene

The *serotonin transporter* gene has three exon1s. Primer sets, indicated by a pair of solid line arrows, were designed for amplification of each variant.

(B) *Serotonin transporter* mRNA expression in the mouse midbrain

The transcript variant including only exon1a was detected in the mouse midbrain. Ribosomal protein 18S was used as internal control.

Supplemental Tables

Molecular Pharmacology

Role of activating transcription factor-4 in 24-hour rhythm of serotonin transporter expression in the mouse midbrain

Kentarou Ushijima, Satoru Koyanagi, Yuuki Sato, Takamitsu Ogata, Naoya Matsunaga, Akio Fujimura, Shigehiro Ohdo

Supplementary Table 1. Sequences of primers for real-time PCR amplification

Gene	Primer sequence (5'-3')
CLOCK	(forward) TTGCTCCACGGGAATCCTT
	(reverse) GGAGGGAAAGTGCTCTGTTGTAG
Bmal1	(forward) CAGATGATGAACTGAAACACCT
	(reverse) CGCAGTGTCCGAGGAAGATAGC
Per2	(forward) CCAGAGGAACTAGCCTATAAGAACC
	(reverse) GAACTCGCACTTCCTTTTCAGG
Cry1	(forward) AGGGAACCCCATCTGTGTTC
	(reverse) TGGTGCATTCCAAGGATCGT
Tef	(forward) CGAGTACATGGACCTGGATGAGT
	(reverse) GTCAGGGTTGAAGTTCACATCGACC
Atf4	(forward) CGAATGGATGACCTGGAAAC
	(reverse) GGCTGCAAGAATGTAAAGGG
β-actin	(forward) CACACCTTCTACAATGAGCTGC
	(reverse) CATGATCTGGGTCATCTTTTCA

Supplementary Table 2. Sequences of primers for PCR amplification in ChIP assay

	Primer sequence (5'-3')
CRE-primers	(forward) TAGGGCAGATTTGGTAAGGG
	(reverse) CCCTCACATGGTCTGATCTC
Negative-primers	(forward) GTGGCTAAGTAGCDACTCTCA
	(reverse) GCTGTACAAAACGAGGTTCA