The harmala alkaloid harmine is a modulator of circadian \textit{Bmal1} transcription

Yoshiaki ONISHI\(^1\), Katsutaka OISHI, Yasuhiro KAWANO and Yoshimitsu YAMAZAKI

Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Higashi 1-1-1, Tsukuba 305-8566, Japan

Synopsis

Biological rhythms are orchestrated by a cell-autonomous clock system that drives the rhythmic cascade of clock genes. We established an assay system using NIH 3T3 cells stably expressing the \textit{Bmal1} promoter-driven luciferase reporter gene and used it to analyse circadian oscillation of the gene. Modulators of PKC (protein kinase C) revealed that an activator and an inhibitor represented short- and long-period phenotypes respectively which were consistent with reported effects of PKC on the circadian clock and validated the assay system. We examined the effects of the alkaloid harmine, contained in \textit{Hoasca}, which has a wide spectrum of pharmacological actions, on circadian rhythms using the validated assay system. Harmine dose dependently elongated the period. Furthermore, EMSA (electrophoretic mobility-shift assay) and Western-blot analysis showed that harmine enhanced the transactivating function of ROR\(\alpha\) (retinoid-related orphan receptor \(\alpha\)), probably by increasing its nuclear translocation. Exogenous expression of ROR\(\alpha\) also caused a long period, confirming the phenotype indicated by harmine. These results suggest that harmine extends the circadian period by enhancing ROR\(\alpha\) function and that harmine is a new candidate that contributes to the control of period length in mammalian cells.

Key words: alkaloid, circadian rhythm, harmine, nutrition, transcription

INTRODUCTION

Circadian rhythms in behaviour and physiology have an adaptive significance in living organisms from bacteria to humans and reflect the existence of an underlying intrinsic circadian oscillator. The master clock that generates circadian rhythms in mammals is located in the SCN (suprachiasmatic nucleus) of the hypothalamus, where it controls all aspects of physiology such as sleep–wake cycles, body temperature, hormone secretion, blood pressure and metabolism. Co-ordination among such aspects of physiology by the circadian clock is essential to optimize metabolic responses and strengthen the inherent homoeostatic regulatory mechanisms [1]. The circadian clock generates robust and relevant rhythms even in the absence of external inputs such as light. Thus circadian dysfunction is considered to be a contributory factor to the incidence and severity of a wide range of clinical and pathological conditions, including sleep disorders, cancer, depression, metabolic syndrome and inflammation [2].

The principle of circadian oscillation is strikingly similar across species, and the molecular mechanism of the circadian oscillator consists of autoregulatory transcriptional and translational feedback loops that have both positive and negative elements [3]. The key transcription factors, CLOCK and BMAL1, form heterodimers that bind to E-box enhancer sequences and activate transcription of the \textit{Per} and \textit{Cry} genes. The \textit{PER} and \textit{CRY} proteins subsequently repress transcription at their own promoters through negative feedback resulting from action on the CLOCK–BMAL1 complex [4]. This feedback loop system not only controls the central clock in the SCN and the peripheral clocks in most peripheral tissues, but is also involved in the transcription of thousands of output genes [5]. Among the core clock genes, \textit{Bmal1} is apparently essential and non-redundant in the mammalian clock and its expression level robustly oscillates in the SCN and in peripheral clock cells [6]. The \textit{Bmal1} promoter contains two recognition motifs for ROR (retinoid-related orphan receptor) and REV-ERB orphan nuclear receptors [ROREs (retinoic acid-receptor-related orphan receptor \(\alpha\)-subunit (ROR\(\alpha\))-binding element)] and a unique chromatin structure that is required for its intracellular circadian transcription [7]. REV-ERB\(\alpha\) represses \textit{Bmal1} expression with the nuclear receptor co-repressor [8] and ROR\(\alpha\) activates \textit{Bmal1} transcription [9].

Abbreviations used: DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; NE, nuclear extract; PKC, protein kinase C; ROR, retinoid-related orphan receptor; RORE, retinoic acid receptor-related orphan receptor \(\alpha\)-subunit (ROR\(\alpha\))-binding element; RT–PCR, reverse transcription–PCR; SCN, suprachiasmatic nucleus.

\(^1\)To whom correspondence should be addressed (email y-onishi@aist.go.jp).
opposing activities of these receptors are important in the mainte-
nance of the circadian clock function [10], but RORα does not 
seem to antagonize REV-ERBα in some types of peripheral cells 
such as salivary gland cells [11].

As the molecular clock machinery described above resides at 
the cellular level, cell-based assay systems have been developed 
to study the role of the clock–gene network in regulating circa-
dian amplitude, resistance to perturbation and the modulation of 
period length. Phase shifts and amplitude changes in the mam-
malian clock have been analysed in fibroblasts from Per2lac 
knock-in mice [12], and modifiers of the circadian clock have 
been screened using U2OS cells stably expressing the Bmal1-
dluc reporter gene [13,14] and in Rat-1 cells stably expressing the mPer2-dluc reporter gene [15]. These cell-based assay sys-
tems have enabled determination of the direct effects of chemical 
ons on the circadian clock in the absence of metabolism and can 
overcome lethality in vivo. As circadian dysfuncion is closely 
associated with pathological and clinical conditions [2], to identify 
compounds that potently affect circadian clock function is an im-
portant topic. Synthetic chemicals and drugs that can modulate 
circadian clocks have mainly been identified in the screens de-
scribed above [13,15]. However, food constituents that potently affect the circadian clock function are preferable when considering applying circadian modulators to humans.

Various species of Passiflora have been extensively used for 
centuries, for example, as a remedy for insomnia in the traditional 
therapeutic systems of various countries. However, the phyto-
constituents that are medicinally important remain unclear. Ac-
cumulating evidence indicate that the major phyto-constituents of 
the Passiflora species are alkaloids, flavonoids, glycosides, 
phenolic and volatile compounds and that the harmala alkaloid, 
harmine, is one principle constituent that has variable pharma-
cutical effects [16]. Harmine is a β-carboline alkaloid that was 
originally isolated in 1847 from seeds of Peganum harmala and 
Banisteropsis caapi, both of which have traditionally been used 
in ritual and medicinal preparations of the Middle East, Central 
Asia and South America [17]. Harmine is also found in common 
plant-derived foods and in animal tissues [18], and it has a wide 
spectrum of pharmacological actions, including antiplasmodial 
activity, antioxidative action, antimutagenic and antigenotoxic 
properties, and it interacts with monoamine oxidase A and se-
veral cell-surface receptors [19]. However, the effect of harmine 
on circadian rhythms has not yet been elucidated.

Here, we established an assay system based on NIH 3T3 cells 
combined with the Bmal1 promoter-driven luciferase gene. We 
then investigated the effect of harmine on circadian rhythms using 
this system.

**MATERIALS AND METHODS**

**Cell culture**

To establish stable cell lines containing the luciferase reporter 
gene driven by the Bmal1 promoter region –197 to +27

![Figure 1](image-url)  
**Figure 1 Establishment of stable clones to monitor rhythmic Bmal1 gene transcription**  
(A) Map of Bmal1 promoter region. Arrows and open boxes indicate transcription start sites and two recognition motifs for ROR and ROREs respectively. Arrow size corresponds to relative amounts of transcript [7]. The promoter region (–197 to +27) was used as a promoter for the reporter gene. (B) Genomic Southern blots. Genomic DNA prepared from established clones was digested with HindIII and analysed by Southern blotting. DNA fragments of the luciferase served as the probe. Clone number was indicated above (1–11). (C) Analysis of the selected clones by a real-time reporter gene assay. Selected clones 1 (black squares), 2 (dark grey squares), 6 (light grey), 8 (open circles) and 10 (closed circles) were stimulated with dexamethasone, and then biolu-

inescence was analysed. Detrended results are the representative of duplicate experiments. (D) Real-time reporter gene assay of stable clone 10. Detrended results, grey; fit results, black.

(Figure 1A), NIH 3T3 cells (5 × 10⁵) were transfected with 10 μg of the luciferase reporter gene construct and with 1 μg of pTracer-CMV (Invitrogen). Cells were selected in Zeocin (10 μg/ml) and clones were established as described previously [7]. RPMI 8402-based stable reporter cells were also established in the same way. All cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FBS (fetal bovine serum) and a mixture of penicillin and streptomycin in a humidified incubator at 37°C under a 5% CO₂ atmosphere.

**Southern blotting**

Genomic DNA prepared from stable clones was digested with HindIII. After electrophoretic resolution on 1% agarose gels, DNA was transferred on to a membrane, hybridized and detected as described previously [20]. Probes labelled with ³²P were
generated from cDNA fragments of the luciferase (region 53–751 of pGL3-Basic; Promega).

**Real-time reporter gene assay**

Real-time reporter gene assays proceeded as described previously [7]. Stable clones established from NIH 3T3 cells were stimulated with 100 nM dexamethasone for 2 h and then incubated with DMEM containing 0.1 mM luciferin (Promega), 25 mM Hepes (pH 7.2) and 10% FBS. Bioluminescence was measured and integrated for 1 min at intervals of 10 min using a Kronos AB-2500 luminometer (ATTO). In brief, results were detrended by subtraction of a best-fit line and, subsequently, were fitted to a sine wave to obtain circadian period length as described previously [21].

**Transient reporter gene assay**

The luciferase reporter gene plasmid pGL3-Basic (Promega) containing the Bmal1 promoter −197 to +27 or the pGL3-Promoter (Promega) and the internal control plasmid, pRL-CMV (Promega) were transfected into NIH 3T3 cells. Luciferase was assayed using a Dual Luciferase Reporter Assay System (Promega) and the internal control plasmid, pRL-CMV (Promega) as described previously [20]. Transcriptional activities were normalized relative to the Renilla luciferase activities.

**Real-time quantitative RT–PCR (reverse transcription–PCR)**

Real-time quantitative RT–PCR proceeded using a Light Cycler (Roche) with a Light Cycler-FastStart DNA Master SYBR Green I kit (Roche) as described previously [7]. The primer sequences are as follows: Actin, 5′-TACGCACACCAGTGCTGTCG-3′ and 5′-TTTTTCTGGCAAGTTAGGTTGTC-3′; RORα, 5′-CCAACCGTCTCACAGGAC-3′ and 5′-GCACAGCTGCAACATCC-3′; Bmal1, 5′-GGACTTCCCTCTACCTGTTCCA-3′ and 5′-AACCATGCGAGTGACGGCG-3′. The authentic template comprised PCR products cloned into the pGEM-T Easy vector (Promega). Relative expression levels were evaluated using Light Cycler software, version 3.5.

**EMSA (electrophoretic mobility-shift assay)**

EMSA proceeded as described previously [22]. Briefly, the DNA probe (5′-GATGGTTGGAAGTGGTATTGC-3′ was end-labelled with [γ-32P]ATP using T4 polynucleotide kinase (New England BioLabs). Portions (10 μl) of the DNA probe were suspended in 20 μl of 16 mM Hepes (pH 7.5), 150 mM KCl, 16% (v/v) glycerol, 1.6 mM MgCl2, 0.8 mM DTT (dithiothreitol), 0.4 mM PMSF, 1 mM EDTA, 0.8 mg/ml BSA, 0.06 mg/ml poly(dI-dC) and 0.01% Nonidet P40, and incubated with proteins. NEs (nuclear extracts) were prepared according to the method of Dyer and Herzog [23]. The anti-RORα antibody (Santa Cruz Biotechnology) was added for super-shift assays. These mixtures were resolved by electrophoresis on 4% polyacrylamide gels in 40 mM Tris/acetate, 1 mM EDTA and 5% glycerol.

**Western blotting**

SDS/PAGE and Western blotting proceeded as described previously [24]. Briefly, proteins were resolved by SDS/PAGE (9% gel) and transferred to PVDF membranes (Amersham). Non-specific binding was blocked with 5% dried milk powder in PBS. Proteins were probed with anti-RORα antibody (Santa Cruz Biotechnology) or anti-actin antibody (Millipore) or anti-lamin A/C antibody (Santa Cruz Biotechnology) and then incubated with horseradish-peroxidase-conjugated anti-mouse or anti-goat IgG (Upstate). Immunoreactive proteins were visualized using ECL® (Amersham) according to the manufacturer’s instructions.

**RESULTS**

**Establishment and characterization of stable cell lines expressing the bmal1 reporter**

Conventional real-time reporter assays represent a convenient method of monitoring effects on circadian rhythm, and many genes that are expressed in a circadian fashion were originally identified using such assays. We analysed the circadian transcription of the Bmal1 gene and found a unique chromatin structure [7]. Because the Bmal1 gene has multiple transcription start sites, we designed a minimal promoter region (−197 to +27) to observe a clear transcriptional oscillation in NIH 3T3 cells (Figure 1A). To establish a stable clone containing the reporter gene driven by the Bmal1 promoter, we characterized 11 clones with higher resistance to the selective marker, zeocin, among established 20 clones by Southern blotting. Figure 1(B) shows that the established clones have been classified into several groups on a Southern-blot profile, and one from each group was analysed by real-time reporter-gene assays. Clones 1 (black squares) and 2 (dark grey squares) had neither luciferase activity nor transcriptional oscillation of the reporter gene, and clone 6 (light grey) had luciferase activity but no transcriptional oscillation (Figure 1C). The reporter gene oscillated in a circadian manner in clones 8 (open circles) and 10 (closed circles) (Figure 1C). In particular, the oscillation in clone 10 persisted beyond 7 days (Figure 1D) and thus was selected for further study.

To evaluate the reporter gene as an indicator of circadian rhythm, we examined the effect of PKC (protein kinase C) on circadian rhythms using the established stable clone 10. The PKC activator, PMA and the PKC inhibitor dequalinium analogue C14 linker, have short- and long-period phenotypes respectively in the U2OS cells stably expressing the Bmal1-dluc reporter [14]. Although NIH 3T3 cells used in our system, instead of U2OS cells as parental cells for the stable line, showed a clear circadian period (26.3 ± 0.72 h, dark grey in Figure 2), PMA, an activator of PKC, also had a short-period phenotype (25.0 ± 1.06 h, light grey in Figure 2). On the other hand, G6850, a PKC-specific
Figure 2 Effect of PKC on circadian Bmal1 gene transcription
Stable clone 10 was stimulated with dexamethasone, and then bioluminescence was measured in the presence of 50 nM PMA (light grey) or 2 μM G6850 (black) or without chemicals (dark grey). Results are representative of triplicate experiments.

Analysis of harmine using the assay system
Harmine is a harmala alkaloid with variable pharmaceutical effects [16]. We investigated whether or not harmine affects the circadian rhythm using our established reporter cell line. Figure 3(A) shows that the period length of the cells treated without (grey) and with 20 μM harmine (black) were 25.8 ± 0.95 and 30.9 ± 0.99 h respectively and 20 μM harmine extended the circadian period by approx. 5 h in a dose-dependent manner (Figure 3B). These results indicated that harmine dose-dependently extends the circadian period.

Harmine enhances RORα function
The harmine-induced period extension of the Bmal1 promoter-driven reporter gene in the stable cells suggests that Bmal1 transcription is altered. To avoid the side effects of the circadian feedback system on the harmine-induced period extension, we analysed transcriptional effects using RPMI 8402-based stable reporter cells, in which the most critical gene for the circadian rhythm, Bmal1, is silenced by promoter CpG island hypermethylation (see Supplementary Figure S1 at http://www.bioscirep.org/bsr/032/bsr0320045add.htm). Acute transcriptional activation of the reporter gene was observed in RPMI 8402-based stable reporter cells in spite of silence of Bmal1 expression, suggesting that harmine directly activated the reporter gene promoter (Figure 4A). At 3 h after incubation with 20 μM harmine, the Bmal1 promoter in NIH 3T3 cells was activated, whereas the SV40 (simian virus 40) promoter was not, suggesting that the harmine activation was Bmal1 promoter specific (Figure 4B). Thereafter, Bmal1 transcripts started to accumulate in the cells (Figure 4C). Harmine activation was also evident in vivo. Expression levels of the Bmal1 gene in the liver increased at 3 h after an intraperitoneal injection of harmine (30 mg/kg of body weight), even though it did not reach statistical significance (P = 0.056). Nevertheless, the trend suggested that harmine activates Bmal1 transcription in peripheral tissues (see Supplementary Figure S2 at http://www.bioscirep.org/bsr/032/bsr0320045add.htm).

Figure 3 Harmine extends period length
(A) Oscillation profiles induced by harmine. Stable clone 10 was stimulated with dexamethasone, and then bioluminescence was measured with (black) or without 20 μM harmine (grey). The detrended results are the representative of triplicate experiments. (B) Dose-dependent effect of harmine on period length. Stable clone 10 was analysed by real-time reporter-gene assays with various concentrations of harmine and period parameters obtained by curve fitting were plotted against final harmine concentration. Values are means ± S.E.M. for triplicate assays.

We then examined how harmine activates the Bmal1 promoter. The Bmal1 promoter region used for the reporter gene contains only ROREs as major regulatory elements. One possibility is that the function of a positive factor(s) such as RORα, which interacts with the ROREs, increases. Incubating a DNA probe containing the ROREs with NE generated two retarded bands (triangles) that disappeared on the addition of an excess of unlabelled probe, indicating specific DNA–protein complexes (Figure 5A). The faster migrating band (closed triangle) disappeared after incubation with anti-RORα antibody, whereas the slower migrating band (open triangle) did not, indicating that the former is a DNA–protein complex containing RORα. When the NE from harmine-treated NIH 3T3 cells was analysed by EMSA, the DNA–RORα complex temporally increased 3 h later (Figure 5B). An increase in the amount of the complex is consistent with activation of the Bmal1 promoter. RT–PCR revealed that harmine reduced, rather than enhanced, RORα transcripts (Figure 6A). On the other hand, nuclear RORα increased 3 h after incubation with harmine (Figure 6B), and the profile of nuclear RORα protein was similar to that of the DNA–RORα complex. Total RORα protein calculated as the amount of cytosolic and nuclear RORαs did not significantly differ among the samples incubated with harmine, suggesting...
that harmine enhances the nuclear localization of RORα. Taken together, these results suggest that harmine induces RORα accumulation in nuclei, presumably by enhancing its nuclear transport and then activates Bmal1 transcription.

Enhancement of RORα expression extends the circadian period

To evaluate the effect of RORα accumulation on the circadian period, a RORα expression plasmid [7] was transfected into the stable reporter cells, and the circadian period was analysed using a real-time reporter assay. Figure 7 shows that the circadian period of the cells transfected with the RORα expression vector (black) was 25.1 ± 1.03 h, whereas that of the cells transfected with control vector (grey) was 24.1 ± 0.95 h. These results indicate that up-regulation of the RORα gene extended the circadian period of the reporter gene by approx. 1 h.

In summary, these lines of evidence suggest that harmine induces nuclear RORα accumulation and then the circadian period is extended.

DISCUSSION

Analysis methods for modulators of the circadian rhythm

Circadian rhythm is a fundamental mechanism in all organisms, as it generates daily fluctuations, for example, in behaviour, physiology and metabolic processes. As it is directly or indirectly involved in many diseases, it is significant to control circadian rhythm [2]. Methods of analysing circadian rhythms include the direct measurement of organism activities and analyses of clock components. Cell-based assay systems, such as real-time reporter-gene assays based on a cell-autonomous clock have recently become popular [25] and they have been applied to screen circadian clock modulators [13,15]. The molecular clock machinery functions at the cellular level, where it regulates clock-controlled genes in a cell-specific manner. Therefore the cells and the promoter region selected for the reporter gene are important in real-time reporter gene assays. The cells included in a real-time reporter assays should have a functional clock system and therefore U2OS [13] and Rat-1 [15] cells are popular. The NIH 3T3 cell line is one of the most popular and has the best-understood circadian system, and we also elucidated the transcriptional mechanisms of clock genes using a real-time reporter-gene assay and NIH 3T3 cells [7,26,27]. Here, we attempted to establish a stable reporter cell line using NIH 3T3 cells because sustained stable oscillation of the reporter gene transcription was required. The promoter region used for the assay should have transcriptional activity with circadian oscillation that corresponds not only to the promoter of the clock genes, such as Bmal1, Per1 and Per2 but also to that of the clock-controlled genes such as Cyp7a (cholesterol 7α-hydroxylase) [28]. Among these promoters, Bmal1 is the most applicable to the assay because the Bmal1 gene is not only a functional and essential gene of the circadian pacemaker [6], but...
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Figure 6  Harmine enhances nuclear translocation of RORα
(A) Harmine does not increase amount of RORα mRNA amount. NIH 3T3 cells were cultured with 20 μM harmine for indicated periods, and then transcripts were analysed by real-time quantitative RT–PCR. Levels of RNA were normalized to actin expression, and the value at zero time was set at 1. Values are means ± S.E.M. for triplicate assays. (B) Harmine increases the amount of nuclear RORα. NIH 3T3 cells were cultured with 20 μM harmine for indicated periods, and then nuclear and cytosolic fractions were prepared as described previously [23] and analysed by Western blotting. RORα, with anti-RORα antibody; Actin, with anti-actin antibody; Lamin, with anti-lamin A/C antibody. Levels of RORα protein were normalized to actin or lamin protein, and the value at zero time was set to 1. Closed and open bars indicate nuclear and cytosolic RORα proteins respectively. Values are means for triplicate assays. *P < 0.05 (Student’s t test).

Figure 7  Exogenous RORα expression indicates long-period phenotype
The RORα expression plasmid [7] was transfected into the stable clone 10, and then the circadian period was analysed by real-time reporter gene assay. Results are the representative of triplicate experiments. Control, grey line; RORα, black line.

it is also abundantly expressed in most cell types [29]. We discovered a unique chromatin structure within the Bmal1 promoter and suggested that it is involved in circadian transcriptional regulation [7]. We also analysed the circadian profile of each Bmal1 transcript and determined the minimal promoter region for circadian Bmal1 transcription. We then designed a promoter region for the reporter gene based on these findings and established several clonal NIH 3T3 lines stably expressing the Bmal1-luc reporter gene (Figure 1). Stable clones 8 and 10 with rhythmic transcription of the reporter gene harboured multi-copies of the reporter gene, but had different profiles on genomic Southern blots (Figure 1B) and a similar period and amplitude of circadian oscillation (Figure 1C), suggesting that major transcriptional elements for circadian transcription of the reporter gene (probably the ROReS) were located in our selected region of the Bmal1 promoter. On the other hand, the oscillatory phase and a damping degree of oscillation were different between these clones, which had individual integrated positions and copy numbers of the reporter gene (Figure 1C). These individual phenotypes might be caused by the chromatin structure integrated around the promoter because the chromatin structure of the Bmal1 promoter is involved in its circadian transcription [7]. Besides, PARP-1 [poly (ADP-ribose) polymerase-1] catalyses poly(ADP-ribosyl)ation to proteins including core histones, indicating that altering the chromatin structure modulates the circadian phase [30].

Analysis using the NIH 3T3 stable reporter cell line revealed that PKC inhibition increases the period length of circadian oscillations and PKC activation results in a short-period length (Figure 2), which is consistent with reported findings using an U2OS reporter clone [14]. These results demonstrate the validity of the assay and suggest that PKC exerts a universal effect on the circadian period in cells.

Period elongation by harmine
One traditional and ethno-pharmacological use of Passiflora is as a remedy for insomnia, and among the major phyto-constituents of the Passiflora species, harmine, a harmala alkaloid has traditionally been included in ritual and medicinal preparations [16]. Harmine has a wide spectrum of pharmacological actions, including antiplasmodial activity, antioxidative action, antimutagenic and antigonotoxic activities, and it also interacts with monoamine oxidase A and several cell-surface receptors [19]. Then, known pharmacological effects of harmine and β-carboline alkaloids include hallucinogenesis, convulsive or anticonvulsive actions and tremorgenesis [31]. These results indicated a need to determine the effect of harmine on circadian rhythm. Our findings using a NIH 3T3 stable reporter cell line showed that harmine dose dependently extended the circadian period (Figure 3). In addition, harmine activated the Bmal1 promoter after 3 h (Figure 4B) in an NIH 3T3 stable reporter cell line and in a lymphoma cell line (Figure 4A). Harmine also activated the Bmal1 promoter in the mouse liver at 3 h after an intraperitoneal injection (Figure S2). Pharmacokinetics have revealed that the harmine concentration in plasma peaks at 102 min after the administration of Hoasca alkaloids and returns to the basal level at 300 min [31], which supports its rapid temporal activation of Bmal1. On the other hand, we could not find any effects of harmine on locomotor activity (results not shown) such as those of Fortunato et al. [19], who reported that acute exposure to harmine does not affect spontaneous locomotor activity in open-field tests, but decreases immobility time and increases both swimming and climbing in the forced swimming test. These results suggest that harmine affects the cell-autonomous clock in peripheral tissues such as the liver, but not the master clock in the SCN of the hypothalamus.
Harmine modulates RORα function

The Bmal1 promoter region of the reporter gene contains ROREs as the main cis-elements, where ROR and REV-ERB families can bind. The amount of the RORα–DNA complex in EMSA was proportional to the levels of transcriptional activation induced by harmine (Figure 5), whereas harmine reduced, rather than enhanced, RORα transcription itself (Figure 6A). Western blotting revealed that nuclear RORα protein accumulated in proportion to both RORα–DNA complex formation and transcriptional activation by harmine (Figure 6B), suggesting that harmine induces nuclear RORα accumulation presumably by enhancing its nuclear transport and activates Bmal1 transcription. The transcriptional activating function of RORα is regulated not only by its natural ligand, cholesterol and cholesterol derivatives [32] but also by its intracellular localization [33], supporting the notion that Bmal1 transcription was activated by harmine-enhanced RORα nuclear transport.

Harmine mimics the effects of PPARγ (peroxisome-proliferator-activated receptor γ; a member of a nuclear receptor superfamily) ligand [34], and its transcriptional activation of RORα. RORα is a member of ROR family that shares functions [35] and cofactors that interact with RORα and are involved in RORα function [36]. Involvement of other ROR family members and cofactors to the effect by harmine will be evaluated in the future.

RORα in the circadian mechanism

Enhanced RORα expression in the NIH 3T3 stable reporter line represents the long-period phenotype (Figure 7), which is consistent with the effect of harmine. Mice that were deficient in RORα have short-period locomotor activity [9,10] and knockdown of RORα by siRNA (small interfering RNA) seems to result in the short-period phenotype being manifested in real-time reporter-gene assays using NIH 3T3 cells that are transiently transfected with Bmal1 reporter gene [9]. These results support our notion that the RORα function enhanced by harmine extends the circadian period. The function of RORα is inhibited via phosphorylation by PKC [37], and this could explain the effects of PKC on the circadian period (Figure 2) through RORα function. Thus, activation of PKC with PMA induces RORα phosphorylation, reduces RORα activity and finally results in the expression of a short-period phenotype.

On the other hand, harmine (20 μM) extended the period length by approx. 5 h (Figure 3), whereas transfection with RORα expression plasmids extended the length by only approx. 1 h (Figure 7). One explanation for this is that RORα is a clock-controlled gene and its expression level is strictly regulated by the feedback system [10] even when the RORα expression plasmid is transfected. For instance, temporal exogenous RORα expression up-regulates Rev-erbα, a negative regulator of Bmal1 expression, and Bmal1 expression is reduced by REV-ERBα, which finally down-regulates endogenous RORα expression. Another explanation is that the long-period phenotype is induced by harmine via a mode of action other than RORα activation because harmine is a harmala alkaloid with multi-pharmaceutical activities [38]. These issues still remain to be elucidated.

In the present study, we established and validated a specific assay system that includes the NIH 3T3 cell line stably expressing the Bmal1 reporter gene to analyse the circadian rhythms. We analysed the food-derived harmala alkaloid, harmine using the assay system and found that it extended the circadian period through enhanced RORα activity, suggesting that harmine could play a role as a circadian modulator and that the dietary intake of harmine probably modulates the endogenous periods of different peripheral oscillator cells directly in a dose-dependent manner.

AUTHOR CONTRIBUTION
Yoshiaki Onishi, Katsutaka Oishi and Yoshimitsu Yamazaki were involved in the design of the study; Yoshiaki Onishi, Katsutaka Oishi, Yasuhiro Kawano and Yoshimitsu Yamazaki performed the research; and Yoshiaki Onishi wrote the paper.

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SUPPLEMENTARY ONLINE DATA

The harmala alkaloid harmine is a modulator of circadian Bmal1 transcription

Yoshiaki ONISHI1, Katsutaka OISHI, Yasuhiro KAWANO and Yoshimitsu YAMAZAKI

Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Higashi 1-1-1, Tsukuba 305-8566, Japan

MATERIALS AND METHODS

CpG methylation analysis

Methylation was analysed as a bisulfite modification using Epitect Bisulfite (Qiagen) according to the manufacturer’s instructions, followed by PCR cloning and sequence analysis as described previously [1].

Figure S1

Transcriptional silence of the Bmal1 gene in RPMI 8402-based stable reporter cells

(A) Hypermethylated CpG island of the Bmal1 promoter in RPMI 8402-based stable reporter cells. Genomic sequence of RPMI 8402-based stable reporter cells analysed after bisulfite modification. Vertical lines indicate CpG sites in the Bmal1 promoter region. Filled and open circles indicate methylated and non-methylated CpG sites respectively. (B) Rescue of Bmal1 transcription by 5-aza-2′-deoxycytidine (aza-dCTP). RPMI 8402-based stable reporter cells were treated with (+) or without (−) 2 mM 5-aza-2′-deoxycytidine for 2 days and then Bmal1 expression was analysed by RT-PCR.

Figure S2

Expression of Bmal1 gene determined by real-time quantitative RT–PCR in livers of mice infused with harmine (30 mg/kg of body weight) or saline for 3 h

Levels of RNA were normalized to those of actin expression, and control value was set at 1. Values are means ± S.E.M., n = 4–6 per group.

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1 To whom correspondence should be addressed (email y-onishi@aist.go.jp).