Check for updates

Received: 12 May 2021 | Accepted: 3 June 2021

DOI: 10.1111/jpi.12751

ORIGINAL ARTICLE

Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

WILEY

Clock-controlled arylalkylamine *N*-acetyltransferase (*aaNAT*) regulates circadian rhythms of locomotor activity in the American cockroach, *Periplaneta americana,* via melatonin/MT2-like receptor

A. S. M. Kamruzzaman[1] | **Susumu Hiragaki**[1] | **Yasuhiko Watari**[2] | **Takashi Natsukawa**[1] | **Akie Yasuhara**[1‡] | **Naoyuki Ichihara**[1‡] | **Amr A. Mohamed**[3] | **Azza M. Elgendy**[1,3] | **Makio Takeda**[1]

[1]Graduate School of Natural Science and Technology, Graduate School of Agricultural Science, Kobe University, Kobe, Japan

[2]Faculty of Clinical Education, Ashiya University, Ashiya, Japan

[3]Department of Entomology, Faculty of Science, Cairo University, Giza, Egypt

Correspondence
Makio Takeda, Graduate School of Natural Science and Technology, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan.
Email: mtakeda@kobe-u.ac.jp

Amr A. Mohamed, Department of Entomology, Faculty of Science, Cairo University, Giza, PO Box 12613, Egypt.
Email: mamr@sci.cu.edu.eg

Present address
A. S. M. Kamruzzaman, Entomology Department, Pest Management Division, Bangladesh Jute Research Institute, Dhaka, Bangladesh

Funding information
Japan Society for the Promotion of Science; Ministry of Education, Culture, Sports, Science and Technology

Abstract

Melatonin (MEL) orchestrates daily and seasonal rhythms (eg, locomotion, sleep/wake cycles, and migration among other rhythms) in diverse organisms. We investigated the effects of pharmacological doses (0.03-1 mM) of exogenous MEL intake in the cockroach, *Periplaneta americana,* on locomotor activity. As *per os* MEL concentration increased, cockroach locomotor rhythm in light-dark (LD) cycles became more synchronized. The ratio of night activity to 24-h activity increased and the acrophase (peak) slightly advanced. MEL application also influenced total activity bouts in the free-running rhythm. Since MEL slightly influenced τ in the free-running rhythms, it is not a central element of the circadian pacemaker but must influence mutual coupling of multi-oscillatory system components. Arylalkylamine *N*-acetyltransferase (aaNAT) regulates enzymatic production of MEL. aaNAT activities vary in circadian rhythms, and the immunoreactive aaNAT (aaNAT-ir) is colocalized with the key clock proteins cycle (CYC)-ir and pigment-dispersing factor (PDF)-ir These are elements of the central pacemaker and its output pathway as well as other circadian landmarks such as the anterior and posterior optic commissures (AOC and POC, respectively). It also partially shares immunohistochemical reactivity with PER-ir and DBT-ir neurons. We analyzed the role of *Pamericana* aaNAT1 (*PaaaNAT1*) (AB106562.1) by injecting dsRNA^{aaNAT1}. qPCR showed a decrease in accumulations of mRNAs encoding PaaaNAT1. The injections led to arrhythmicity in LD cycles and the arrhythmicity persisted in constant dark (DD). Continuous administration of MEL resynchronized the rhythm after arrhythmicity was induced by dsRNA^{aaNAT1} injection, suggesting that *PaaaNAT* is the key regulator of the circadian system in the cockroach via MEL production. *PaaaNAT1* contains putative E−box regions which may explain its tight circadian control. The receptor that mediates MEL

A. S. M. Kamruzzaman and Susumu Hiragaki should be considered a joint first author.

‡Deceased.

© 2021 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

function is most likely similar to the mammalian MT2, because injecting the competitive MT2 antagonist luzindole blocked MEL function, and MEL injection after luzindole treatment restored MT function. Human MT2-ir was localized in the circadian neurons in the cockroach brain and subesophageal ganglion. We infer that MEL and its synthesizing enzyme, aaNAT, constitute at least one circadian output pathway of locomotor activity either as a distinct route or in association with PDF system.

KEYWORDS

immunohistochemistry, insect arylalkylamine *N*-acetyltransferase 1, locomotor rhythm, luzindole, melatonin, RNA interference

1 | INTRODUCTION

McCord and Allen first observed that bovine pineal extracts injected into *Rana pipiens* tadpoles caused lightening of their skin color.[1] Lerner and his coworkers[2] isolated and identified the bioactive factor as melatonin (*N*-acetyl-5-methoxytryptamine; MEL) from bovine pineals, initiating the modern era of pineal research. The pineal organ in some avian species contains a circadian pacemaker, because the surgical removal renders the normal rhythms arrhythmic; however, rhythmicity was restored by transplanting a donor pineal into the pinealectomized bird, which drove the phase of the donor bird.[3] The pineal gland secretes MEL both in vivo and in culture, either from q dispersed cell culture or organ culture of chicken pineal, exhibiting a circadian rhythm.[4] Exogenous MEL administered periodically restores locomotor rhythmicity to pinealectomized birds.[5]

Arylalkylamine *N*-acetyltransferase (aaNAT) is the final step in MEL biosynthesis, and it regulates seasonal reproduction in hibernating mammals, reptiles, and songbirds, and it controls migration behavior.[6,7] aaNAT in vertebrates catalyzes the *N*-acetylation of serotonin (5-hydroxytryptamine; 5-HT) to produce *N*-acetyl serotonin (NAS) for the synthesis of MEL by hydroxy indole *O*-methyltransferase (HIOMT). aaNAT is the key enzyme to generate a MEL rhythm[8,9] and a rate-limiting step in circulating MEL levels.[10] MEL is a chemical token for night, and the accumulation of MEL depends on the length of the dark period aaNAT is exposed to. The latter is very sensitive to light,[11,12] which delayed its chromatographic isolation for a long time.

The suprachiasmatic nucleus (SCN) serves as the circadian pacemaker (CPM) in mammalian and some avian systems. SCN receives photoperiodic signals from the retina along the retinohypothalamic tract via glutamate and sympathetically regulates pineal MEL biosynthesis via the supercervical ganglion.[13] Both avian and mammalian *aaNATs* are clock-controlled genes (CCGs).[14,15] The rhythmic activity in the MEL metabolic pathway is highly conserved among vertebrates. MEL shows a stable rhythmicity of about 24 h, with the peak at night. MEL is a neurohormone that regulates many physiological processes including locomotory activity, sleep/wake rhythms, body temperature, blood pressure, feeding, and oncogenesis.[16] MEL also has been reported in various nonvertebrate taxa, including insects.[17-19]

The aaNAT family has been found in Gram-positive bacteria, fungi, algae, placozoans, annelids, cephalochordates, and vertebrates[9,20] as well as in higher plants.[21] Based on sequence similarity, it can be classified as either vertebrate (VT-aaNAT) or nonvertebrate (NV-aaNAT).[20] Structural and functional aspects of insect aaNAT (insect-type iaaNAT) have been reviewed in Hiragaki et al[22] iaaNAT occurrence is not limited to arthropods but it occurs also in lower deuterostomes. Evolutionary lineage analyses indicate that iaaNAT-type is more basally placed, that is, apomorphic, and that VT-type show more recent derivation, since some protochordates such as tunicates and hemichordates had insect-type enzyme.[22]

iaaNAT is involved in coloration and sclerotization of cuticles and suppression of melanin pigmentation.[23] This is the first documented role of aaNAT in insects, and it was conventionally called dopamine *N*-acetyltransferase (DAT). aaNAT loss of function by mutation causes possible buildup of excess dopamine that is then diverted for melanization in *Bombyx mori*.[24] The aaNAT enzymes also control the inactivation and metabolic clearance of a number of neurotransmitter monoamines as insects do not express or have very low monoamine oxidase (MAO) activities.[25,26] This stands in contrast to mammals where MAOs are the major detoxification enzymes.[27] MEL has a neurohormonal releasing effect in the insect nervous system and induces the release of the prothoracicotropic hormone (PTTH) from the brain in the American cockroach, *P americana*.[28] Orally administrated MEL synchronized both entrained and free-running rhythms in locomotor activity of the house cricket, *Acheta domesticus*.[29]

Both MEL content in the hemolymph and aaNAT enzymatic activity in the brain-subesophageal ganglion complex showed a circadian fluctuation in *P americana*,[30] *B mori*,[31]

 Journal of Pineal Research Molecular, Biological, Physiological and Clinical Aspects of Melatonin —WILEY

and *A pernyi*.[32,33] We purified the enzymes from various tissues of *P americana* and obtained calculated amino acid sequences.[30,34] The sequences are true aaNAT, since the proteins expressed showed *N*-acetylation capabilities.[31,32] iaaNAT is the critical link between the circadian system and photoperiodism in *A pernyi*.[33] After injecting dsRNA^{iaaNAT} into diapause pupae, the pupae failed to exit diapause under long days. aaNAT was indispensable for PTTH secretion. Immunohistochemical localization of various clock proteins indicates that PER-ir, Cyc-ir, etc, are colocalized with aaNAT-ir, MT-ir, and HIOMT-ir, while hMT2-ir and PTTH-ir are colocalized. Further, both groups of neurons are situated side by side.[33] Injection of dsRNA^{iaaNAT} induced a large decline of MEL content over 48 h. In contrast, after knockdown of *per*, MEL levels were significantly higher than controls after only 24 h. We infer that pupal diapause of *A pernyi* is terminated by MEL via aaNAT activation and the *iaaNAT* is a circadian-controlled gene (CCG).[33] Our next goal was to clarify the possible role of *iaaNAT* in circadian rhythms in locomotor activity, using *P americana* as a model insect.

In this study, we posed the hypothesis that aaNAT functions as an essential connection between the circadian system and behavioral rhythm. Here, we report on the outcomes of experiments designed to test our hypothesis.

2 | MATERIALS AND METHODS

2.1 | Experimental insect

Stock colonies of American cockroaches (*Periplaneta americana* L.) were maintained in a walk-in constant temperature room set at 25°C and 60% relative humidity (RH) under 12:12 (LD) and provided with water and artificial diet (MF, Oriental Yeast Corp., Tokyo, Japan) ad libitum. Newly emerged white male adults were collected immediately and kept in a transparent plastic box (14 × 14 × 20 cm) to ensure the ages.

2.2 | Application of exogenous melatonin and related pharmaceuticals

Pharmaceutical grade MEL (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in a minimal volume of ethanol and then diluted with Gibco distilled water (Thermo Fisher Scientific, MA, USA), 0, 0.03, 0.1, 0.3, and 1 mM MEL concentrations, with less than 0.01% EtOH. Cockroaches were treated with MEL *per os*.[29] The EtOH did not influence the amplitude, acrophase, and daily activity of the locomotor rhythm (data not shown). Luzindole, a competitive antagonist of MEL receptor type 2 (MT_2), was purchased from Sigma-Aldrich (St. Louis, USA). It was dissolved in 10 μl phosphate-buffered saline (PBS) at 100 nM (=30 pg). Luzindole was injected into each cockroach with a Hamilton 701 syringe equipped with a 26s gauge needle (Hamilton Co., Reno, Nevada, USA). The injury to the cuticle was sealed with an instant adhesive, Aron-alpha (Toagosei Co. Ltd., Tokyo, Japan). Locomotor activity was examined in 10 male cockroaches injected with luzindole, and an equivalent control group was injected with MEL at concentrations reported in Results. Locomotor activity in LD 12:12 was recorded for 10 days before injection and for 10 days after injection. The activity was recorded for a further 10 days in DD from the last lights-off.

2.3 | Behavioral analysis

Locomotor rhythms were recorded in a monitoring chamber (plastic box, 193 × 104 × 27 mm) with a shelter (U-shaped plastic of 50 × 40 × 20 mm upside down) flanked by an infrared light emitter and detector (GT2; Takenaka Electronic Industrial, Kyoto, Japan).[35] Water was provided from a bottle (35 mm D × 50 mm H) with a wire-meshed opening. Artificial diet was supplied as food. When a cockroach crossed the infrared beam, a signal was sent a computer (PC98; NEC, Tokyo, Japan) for a 6-min bin. Locomotor rhythms of individuals were recorded at 25°C under LD 12:12; light source was a 10 W fluorescent lamp providing more than 400 lux. Adult males aged 7 days were kept in LD 12:12 at 25°C and 60% RH for 10 days for entrainment and then were allowed to free-run in DD. Ten insects were used for each group. Statistics of *t* and *F* were used for the differences in activity level, acrophase, τ, and amplitude between the group drinking water control and that drinking different concentrations of MEL. Amplitudes and acrophases were analyzed by cosinor analysis and τ by chi-square periodogram analysis.

2.4 | Preparation and injection of dsRNA

The brain-subesophageal ganglion complex (Br-SOG) of *P americana* was dissected and immediately transferred to liquid N_2. The total RNA was extracted by using the RNAiso Plus reagent (Takara Bio Inc, Kusatsu, Japan). Two hundred fifty ng samples of total RNA and ReverTra Ace®qPCR master mix (Toyobo Co. Ltd., Osaka, Japan) were used to synthesize cDNA. Double-stranded DNA with 282-base pair (bp) fragments of *aaNAT* (GenBank accession number: AB106562.1, now designated as *PaaaNAT1*) was amplified by PCR. The sequence AB106562.1 used here for RNAi corresponds to the encoded $aaNAT_A$ that showed circadian fluctuation in the activity and mRNA content.[34] The primers used to generate templates for in vitro transcription contained a T7 promoter (TAATACGACTCACTATAGGGAGA) at each end. The primers for amplification were as follows: F: 5´-TAATACGACTCACTATAGGGAGATAATGGCAG TATCCAGAA-3′; and R: 5′-TAATACGAC TCACTATAG GGAGAGATATTATGCGCACTTCTAC-3′. The PCR mixture (50 µL) included 4µL of 50X diluted cDNA template, 5 µL (10 pmol) of each primer, 5µL of 2 mM dNTPs, 25 µL of 2x buffer, and 1 µL of KOD FX Neo (Toyobo Co. Ltd.). PCR (35 cycles) was performed with denaturation at 98°C for 10 sec, annealing at 65°C for 30 sec, and extension at 68°C for 5 min. The *aaNAT* double-stranded RNA ($dsRNA^{aaNAT1}$) was synthesized from a purified PCR product by using a MEGAscript T7 Transcription Kit (Thermo Fisher Scientific, MA, USA), following the manufacturer's protocol. For the experimental group, 32 µg of $dsRNA^{aaNAT1}$ was injected by Hamilton syringe into the abdominal cavity of each cockroach. The control groups were injected with an equal volume of H_2O.[36] We injected $dsRNA^{GFP}$ into cockroaches and observed no effect on the expression of *iaaNAT* transcription. After injection, each cockroach was returned immediately to the rearing container or placed in a locomotion monitoring chamber.

2.5 | Measurement of *iaaNAT* expression by qPCR

Total RNA was extracted and purified from individual adult male Br-SOGs with RNAiso Plus. Total RNA was treated with DNase I (Takara Bio Inc) followed by cDNA synthesis using Rever Tra Ace® qPCR master mix. The qRT-PCR was performed with the SYBR® Green and THUNDERBIRD™ qPCR Mix (Toyobo Co. Ltd.), with the forward and reverse primers 5´-TGTGTTTCAACCAGCTCTGC-3´ and 5´-AACTTCCACTCGTAGTGGTTCC-3´ for *iaaNAT*; and 5´-TGAATCCTAAGGCCAACAGG-3´ and 5´-AC CGGAATCCAGCACAATAC-3´ for *actin* (GenBank: AY116670.1). Cycling parameters were 95°C for 1 min to activate DNA polymerase and then 40-cycle amplification programmed for 95°C for 15 sec and 60°C for 2 min. The acquisition of fluorescence data was performed at the end of the elongation step using thermal cycler dice real-time system software (Takara Bio Inc). An initial amount of template was calculated from cDNA, and then, a standard curve was generated for each PCR run. For expression levels of each transcript, *actin* mRNA was used as the internal control.[33] The primers used in qPCR were designed to correspond to outside the region of the dsRNA construct. The sizes of the PCR products were 130 bp for *iaaNAT* and 140 bp for *actin*.

2.6 | Immunohistochemistry

Specificities of all antibodies used in this study have been described.[33,37] The Br-SOGs were isolated at ZT6 and fixed in Bouin's solution for 24 h at 4°C. Artificial Zeitgeber time (ZT) is defined as the time after the light-on under LD (light-dark) cycle = 12:12. Standard histochemical methods were employed for tissue dehydration, embedding in Paraplast Plus® (Sigma-Aldrich, MO, USA), sectioning (12 µm), deparaffinization, and rehydration. The sections were washed in distilled water and Tris-buffered saline (TBS; 135 mM NaCl, 2.6 mM KCl, 25 mM Tris-HCl, pH 7.6) containing 0.1% Tween 20 (TBS-Tw) at RT, blocked with antibody dilution buffer (TBS-Tw containing 1% BSA) for 30 min at RT. Double labeling followed using anti-*Ap* iaaNAT (rabbit) and anti-CYC (rat). The sections were incubated with a cocktail of both primary antibodies containing anti-iaaNAT (1:1000) and anti-CYC (1:200), rinsed 3 times in TBS-Tw, and then incubated with 7.5 µg/ml of biotinylated goat anti-rabbit IgG (Vector Laboratories, CA, US). After rinsing in TBS-Tw, the iaaNAT-like immunohistochemical reactivity (-ir) was visualized with green fluorophore using a TSA Labeling Kit #22 (Thermo Fisher Scientific). The sections were rinsed 3 times in TBS-Tw, treated with 1% H_2O_2 in TBS for 30 min at RT to inactivate residual HRP, and washed again three times with TBS-Tw. Incubation with 0.025% (w/v) avidin in TBS for 30 min at RT was followed by rinsing 3 times with TBS-Tw. Subsequently, incubation with 0.001% (w/v) biotin in TBS for 15 min at RT was conducted to block reactive biotin and streptavidin. The slides were incubated with 7.5 µg/ml of biotinylated goat anti-rat IgG (1:2000) (Vector Laboratories). CYC-ir was visualized with red fluorophore using a TSA Labeling Kit #42 (Thermo Fisher Scientific).

Double labeling was performed using antibodies derived from the same animal donor according to Hiragaki et al.[38] In detail, anti-aaNAT (Rabbit) with anti-PDF (Rabbit) was used as follows. The slides were incubated with the antibody solution (anti-iaaNAT1:1000) overnight at 4°C. After rinsing with TBS-Tw, the slides were incubated with 7.5 µg/ml of biotinylated goat anti-rabbit IgG for 60 minutes in RT. After rinsing with TBS-Tw, the sections were treated with TSA Biotin System (PerkinElmer, MA, USA). After labeling, anti-aaNAT was stripped off the sections for 24 h at RT in stripping buffer (100 mM 2-mercaptoethanol, 50 mM glycine-HCl, pH2.2) in a 40 V horizontal electric field. The sections were then incubated with anti-PDF (1:2000). iaaNAT-ir was visualized with green fluorophore using a TSA Labeling Kit #22. Sections were treated with H_2O_2, avidin, and biotin, incubated with biotinylated goat anti-rabbit IgG. PDF-ir was visualized with red fluorophore using a TSA Labeling Kit #42.

The stained sections were washed in TBS and mounted in Aqua-Poly/Mount medium (Polysciences Inc, PA, USA) and examined using a BX50 microscope (Olympus, Tokyo, Japan) equipped with BX-FLA reflected light fluorescence, WIG and NIBA mirror/filter units for detection of Alexa Fluor® 555 and Alexa Fluor® 488, respectively and a CCD camera. Immunohistochemical specificities of all antibodies used in this study were confirmed previously.[33,39]

The MEL-ir was investigated by employing the primary antibody Rabbit anti-MEL serum (Immunotech IM0615, purchased from MBL) that was used for immunohistochemical staining. Cockroach was preinjected with 10 µl of 1 mM colchicine in PBS to block neurosecretion and dissected 2 days later. A second anti-melatonin serum purchased from Stockgrand Ltd, product number AB/S/01, previously HP/S/704-6483 (also used for the Radioimmunoassay (RIA) procedure), had weaker reactivity in immunohistochemistry (IHC).

Colocalization of aaNAT-ir with clock proteins, PER and DBT, was made using antisera we produced against PamPER and BmDBT of that specificities were checked in Sehadova et al[37] Adjacent sections of 7 µm were mounted onto two sets of slides, and each set was exposed to either anti-PER or anti-DBT as primary antibody at 1:1000.

2.7 | Identification of putative *PaaaNAT1 cis*-regulatory motifs related to circadian control

Periplaneta americana genome assembly sequence ASM293952v1 was retrieved from NCBI and blasted against the *PaaaNAT1* cDNA sequence AB106562.1. The resulted blast hits were converted into exon and intron coordinates. The *PaaaNAT* 3kb upstream genomic sequence (from the transcription start side) was then submitted to JASPAR database (available at http://jaspar.genereg.net) for identifying the putative transcription factors binding sites (TFBS). The resulted output was explored manually to identify a targeted set of conserved *cis*-regulatory motifs linked to circadian control that were subsequently highlighted as sequence annotations in Geneious 7.1.7 (Biomatters Ltd., Auckland, New Zealand). Corresponding figure was manually redrawn in Adobe Illustrator CS5 (Adobe Inc CA, USA), and the obtained putative elements are coded.

2.8 | Statistical analysis

Activity rhythm was analyzed by chi-square periodogram[35] at the significance level of 0.05. Mean differences were analyzed by one-way ANOVA followed by Tukey's HSD (Honestly significant difference) test using SPSS ver. 22 (IBM, NY, US). Differences were accepted as significant for $P < .05$. Significance of mRNA level was examined each time point by *t* test.

3 | RESULTS

3.1 | Effects of exogenous melatonin on circadian locomotor activity

Figure 1 shows representative double-plotted actograms of insects drinking 0, 0.03, 0.1, 0.3, and 1.0 mM MEL, respectively, under LD12:12 at 25°C. The activity of 10 individuals per group was recorded in LD12:12 for 10 days and then for another 10 days in DD following the last lights-off. Figure 2 summarizes the compiled data in LD12:12. When exogenous MEL was applied, there was a ca. 10% arrhythmic pattern, in comparison to the control, which showed a ca. 30% arrhythmic pattern. The daily activity, acrophase, ratios of nighttime activity, N to entire activity, T (N/T, %), and amplitude were analyzed for each concentration of MEL (Table 1).

The acrophase of the rhythm in individuals drinking MEL was slightly phase-lagged, and the circadian period was slightly lengthened by increased MEL, compared with the control ($F = 3.61$, $P < .01$), but the difference was not significant as shown in Table 2. Both the activity level ($F = 3.76$, $P < .01$) and amplitude ($F = 3.03$, $P < .001$) increased as MEL concentrations increased up to 1 mM. The daytime activity was suppressed with increased concentration of MEL-drinking groups under LD cycle. Therefore, the N/T values were changed to square root of percentages and ANOVA statistical analyses were performed (Table 1). The effect was highly significant ($F = 20.1$, $P < .0001$). A complete description of each circadian parameter is detailed in Table 1. MEL imbibing/drinking had clear effects on the circadian parameters.

As shown in Figure 3 and Table 2, MEL had no significant effects on acrophase ($F = 0.29$, $P < .897$) and τ ($F = 1.44$, $P < .242$), but activity levels ($F = 3.77$, $P < .01$) and amplitude ($F = 2.75$, $P < .05$) significantly increased. MEL synchronizes free-running rhythms in a dose-dependent manner up to 1 mM.

3.2 | Effect of dsRNA^{aaNAT} injection on the *PaaaNAT* expression

To examine the effect of injection of dsRNA^{aaNAT1} on the *PaaaNAT* transcript levels, we measured the levels of *PaaaNAT* mRNA in the Br-SOG by qPCR (Figure 4). Br-SOGs were collected at ZT6, 7, 14, 21, and 28 days after injection. In dsRNA^{aaNAT1}-injected cockroaches, the *PaaaNAT* mRNA level was reduced significantly, by about 89% ($F = 10.5$, $P < .01$) from control cockroaches at each time point, suggesting that injection of dsRNA^{aaNAT1} suppressed the expression of *PaaaNAT* mRNA throughout the experimental period of 28 days.

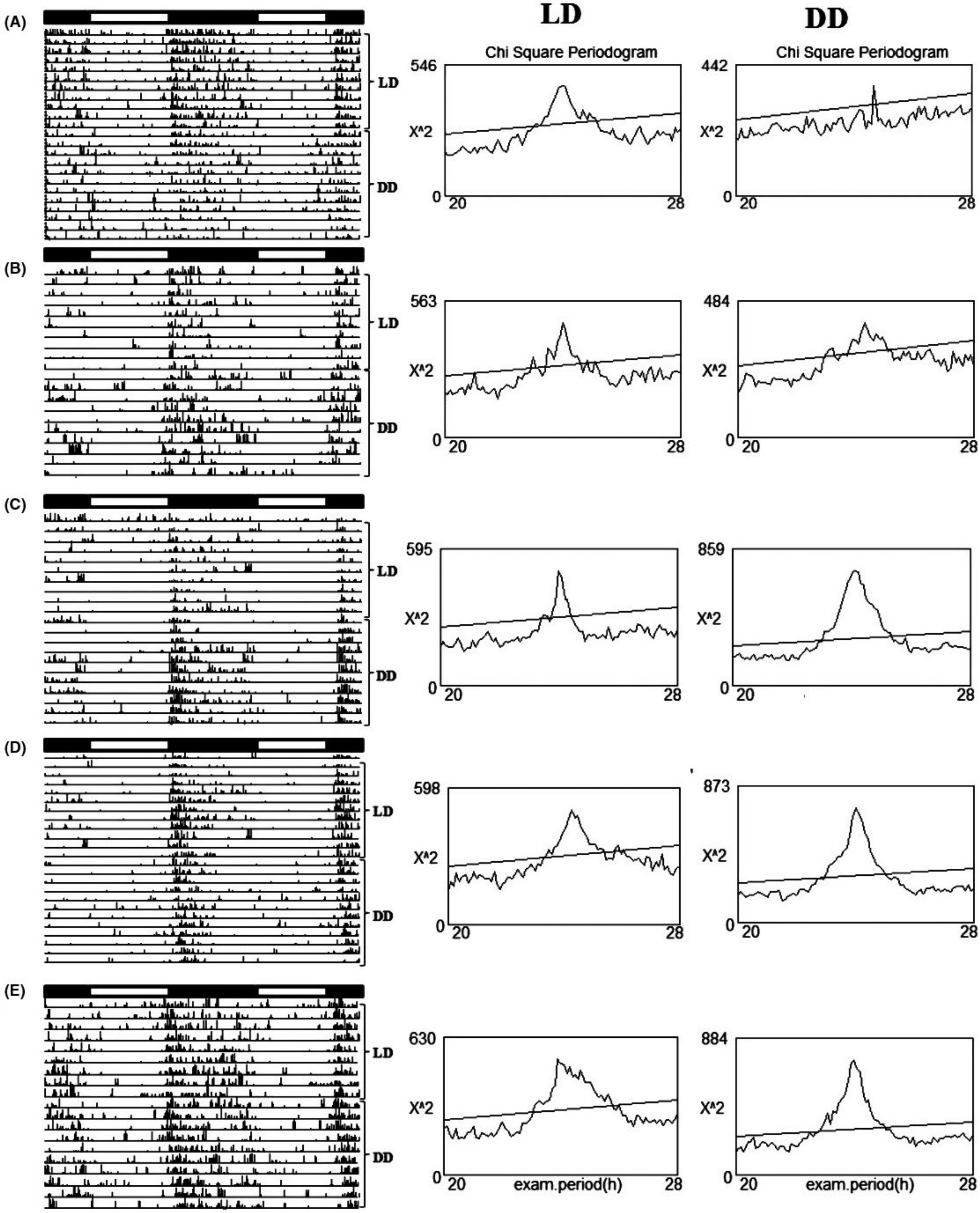

FIGURE 1 Typical double-plotted actograms of male cockroaches, *P americana*, when drinking 0 (A), 0.03 (B), 0.1 (C), 0.3 (D), and 1 (E) mM MEL at 25°C. The activities were recorded under LD 12:12, with the activity double plotted for 10 days. The activities in DD were then recorded for a further 10 days following the last lights-off. The white and black bars indicate the light and dark periods. The chi-square periodogram analyses (right) show the free-running activity in DD became significantly more rhythmic (peaks above the lines; *P* <*.01* under DD conditions) with the higher MEL levels. Lines indicate significance level at 0.05

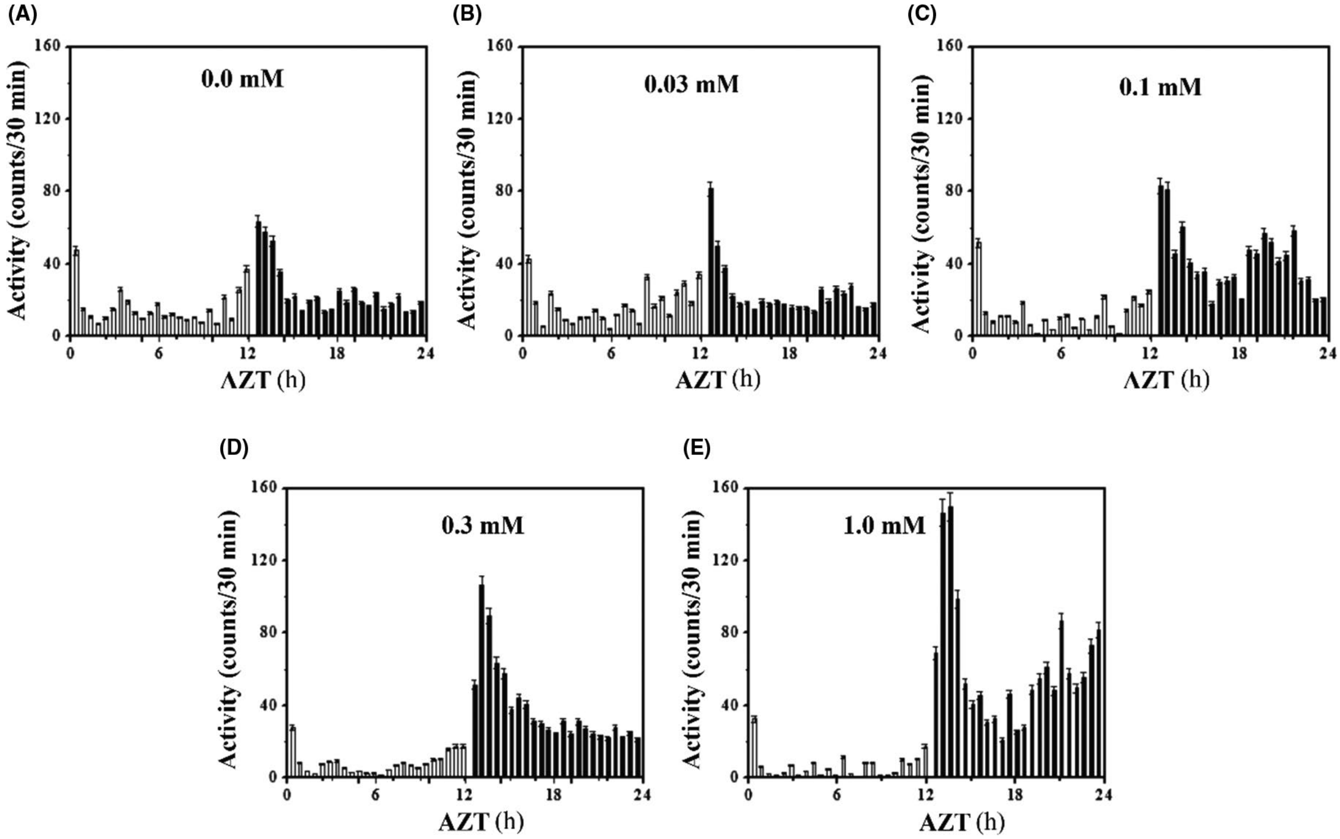

FIGURE 2 Activities compiled for all individuals (N = 10) and all cycles. AZT, (artificial Zeitgeber time) 0-12 is day and AZT 12-24 is night in LD 12:12. White bars represent activities in light phase. Dark bars those in night phase. The inlet numbers denote the concentrations of MEL in the drinking water

TABLE 1 Acrophase, activity level, and amplitude of the locomotor rhythm entrained to LD 12:12. N, activity at night; T, activity in day and night

Treatments (log mM Melatonin)	Acrophase (h)	Activity bouts/24 h	N/T (%)	Amplitude (%)
0 (control)	1.19 ± 0.5[d]	97.11 ± 34.7[c]	61.75 ± 8.9[b]	29.00 ± 6.1[c]
0.03	1.39 ± 0.4[c]	101.63 ± 16.3[c]	63.46 ± 2.4[b]	31.25 ± 9.8[c]
0.1	1.93 ± 0.4[b]	129.49 ± 71.5[c]	75.22 ± 6.9[b]	32.57 ± 5.6[c]
0.3	2.05 ± 0.9[b]	151.83 ± 78.9[b]	79.57 ± 7.0[b]	40.33 ± 7.1[b]
1	2.46 ± 1.2[a]	202.66 ± 59.4[a]	95.96 ± 4.1[a]	46.29 ± 8.8[a]

Note: Means (±SD) compared by Tukey's test. In the same column, means followed by unlike letters are significantly different ($P < .05$).

TABLE 2 Acrophase, activity level, amplitude, and τ of the free-running rhythm in DD

Treatments (log mM Melatonin)	Acrophase (h)	τ	Bouts/τ	Amplitude (%)
0 (control)	1.93 ± 0.7[a]	24.10 ± 0.3[a]	117.14 ± 12.7[c]	25.86 ± 2.9[b]
0.03	2.04 ± 0.6[a]	24.29 ± 0.3[a]	181.75 ± 55.1[c]	30.13 ± 7.2[b]
0.1	2.09 ± 0.6[a]	24.31 ± 0.4[a]	203.86 ± 59.9[c]	30.29 ± 5.6[b]
0.3	2.17 ± 0.4[a]	24.56 ± 0.3[a]	212.67 ± 79.7[b]	37.50 ± 17.3[a]
1	2.23 ± 1.9[a]	24.69 ± 0.9[a]	399.57 ± 59.9[a]	38.86 ± 4.6[a]

Note: Means (±SD) compared by Tukey's test. In the same column, means followed by unlike letters are significantly different ($P < .05$).

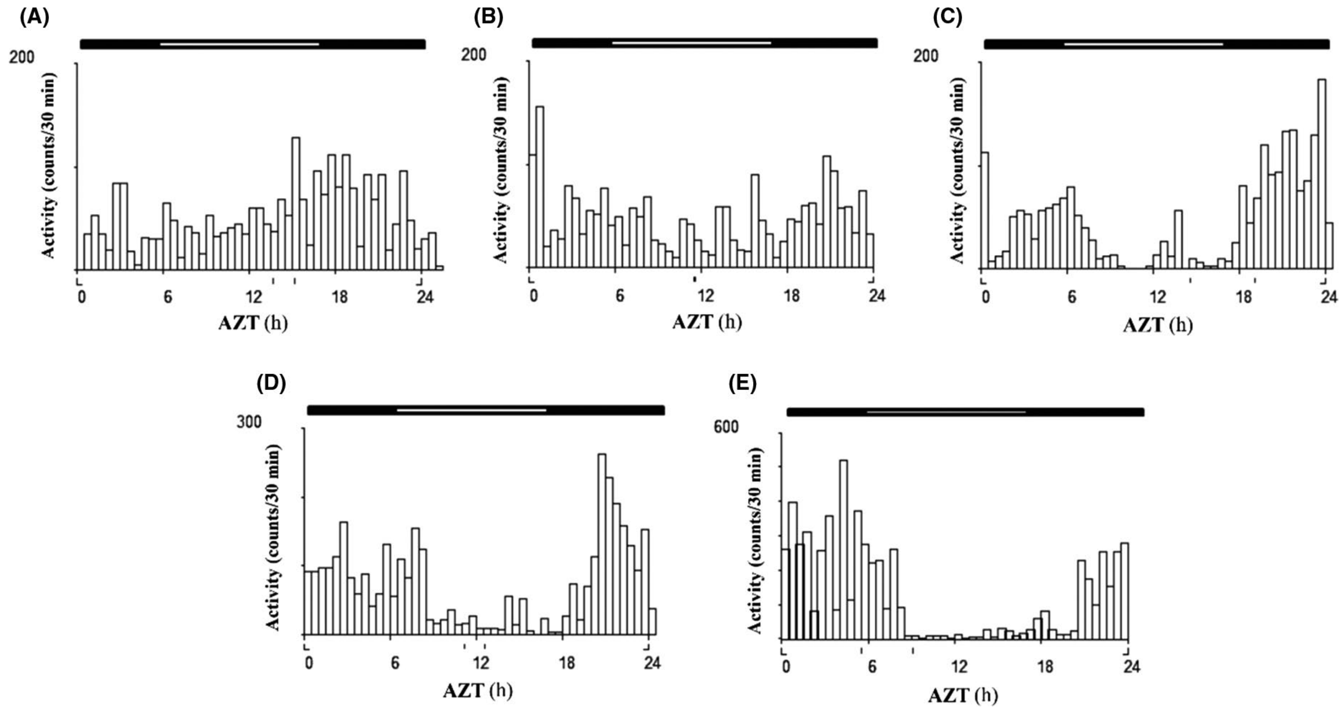

FIGURE 3 The effect of MEL intake on the locomotor rhythm in *P americana*. Free-running activities compiled for 10 days after DD transition in a 0 (A), 0.03 (B), 0.1 (C), 0.3 (D), and 1 mM MEL (E) (CT scale). τ for each treatment were 24.1, 24.3, 24.3, 24.6, 24.7 h, respectively. AZT stands for artificial or arbitrary Zeitgeber time

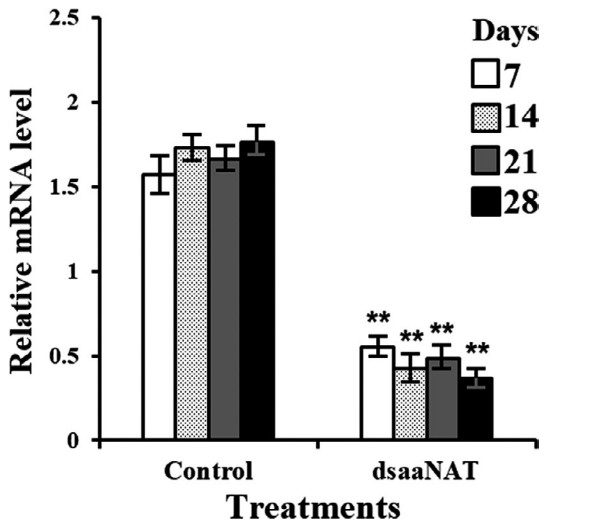

FIGURE 4 Relative abundance of Pa^{aaNAT1} mRNA in heads of intact adult male and of Pa^{aaNAT1} dsRNA-injected adult male. Ordinate indicates relative abundance of Pa^{aaNAT1} mRNA. Samples were collected at ZT6 in LD 12:12, 25°C. The Br-SOGs were collected 7, 14, 21, and 28 days after injection of $dsRNA^{aaNAT1}$. The abundance of Pa^{aaNAT1} mRNA was measured by qPCR. The data collected from three independent experiments were averaged and plotted as mean ± SEM. against water injected (control) and $dsRNA^{aaNAT1}$-injected insect ($dsRNA^{aaNAT1}$). The abundance of *actin* was used as reference. **: highly significant difference ($P < .01$), as compared to the control group, using Student's *t* test

3.3 | $dsRNA^{aaNAT1}$ injection desynchronized locomotor rhythm

To investigate the effects of $dsRNA^{aaNAT1}$ injection on the locomotor rhythm, we measured the locomotor activity in 20 male cockroaches injected with $dsRNA^{aaNAT1}$. We also used 10 intact and 10 water-injected cockroaches as controls. Locomotor activity in LD 12:12 was recorded for 10 days before injection and 10 days after injection. It was then recorded for 10 days in DD following the last lights-off. The representative actogram records of locomotor activity are shown in Figure 5. Injection of $dsRNA^{aaNAT1}$ significantly reduced locomotor activity at night (Figure 6 and Table 3). Seventy percent of the treated cockroaches (n = 20) became arrhythmic, in comparison to the control cockroaches (n = 20), which remained rhythmic (Figure 5) after injection in the LD cycle. The result indicates the treated cockroaches lost their ability to synchronize with the Zeitgeber due to silencing of their *PaaaNAT1* gene.

Under DD conditions, 70% of $dsRNA^{aaNAT1}$-injected insects expressed arrhythmia with suppressed activity over a 24-hour period, while only 10% insects in the control lost their free-running rhythm (Figure 6). $DsRNA^{aaNAT1}$-injected cockroaches showed significantly shorter τ compared with control (Table 3). $DsRNA^{aaNAT1}$ injection disrupted not only phases of circadian rhythm and synchrony in locomotor

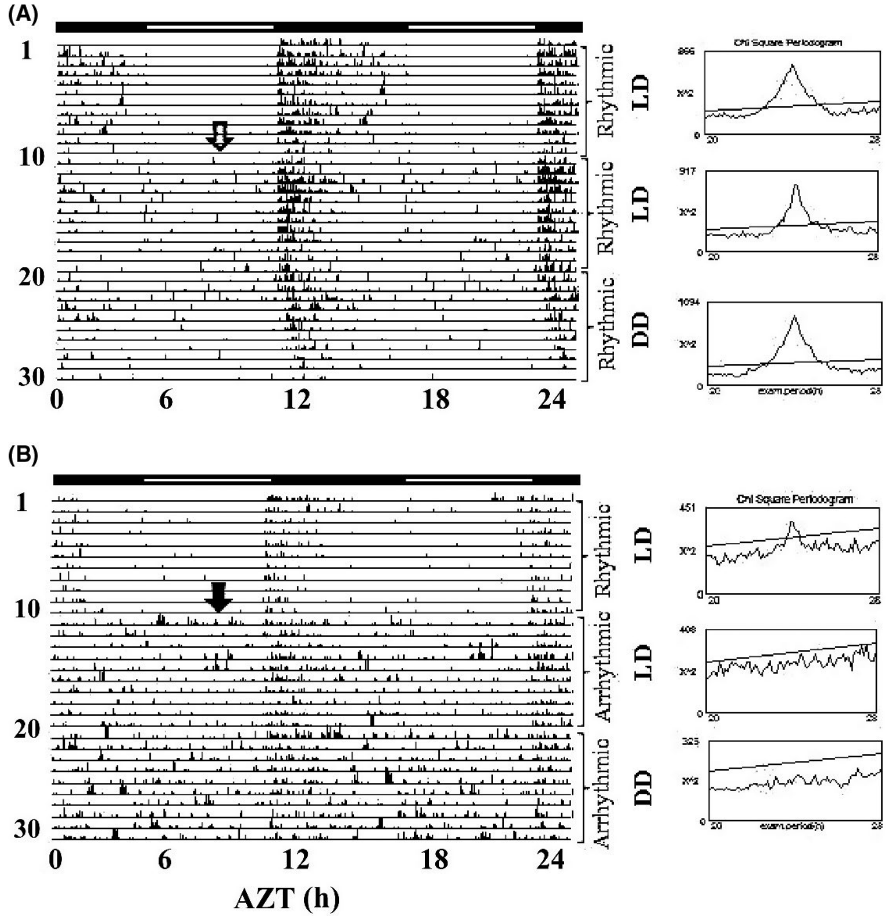

FIGURE 5 Effect of RNA interference against *aaNAT1*. Double-plotted actograms (left) and χ^2-periodograms (right) of representative cockroaches injected with dsRNA^{aaNAT} (B). White and black bars above actograms indicate light and dark phases, respectively. The solid arrow indicates the time of dsRNA^{aaNAT} injection. The open arrow in the actograms indicates the time of water injection (A). Rhythmicity was suppressed by the injection of the dsRNA^{aaNAT}

rhythm, but also the activity level significantly (Figure 6 and Table 3). Activities in 20 days of postinjection became less than a half that of control.

3.4 | Reestablishment of locomotor rhythm by melatonin following dsRNA^{aaNAT1} injection

Resynchronization of locomotor rhythm was observed in dsRNA^{aaNAT1}-injected cockroaches when MEL (1.0 mM) was supplied in the drinking water. DsRNA^{aaNAT1}-injected cockroaches were examined 10 days before MEL administration under LD cycle. Ten days after injection, the cockroaches were given MEL-infused water to drink ad libitum and then transferred into DD. The representative actogram records of locomotor activity are shown in Figure 7. Under LD conditions, 76% (n = 10) of the water-only insects were arrhythmic, whereas only 28.6% (n = 10) of MEL-supplied insects showed an arrhythmic pattern on the *PaaaNAT*-silencing background. The re-entrained rhythm suggested that MEL enhanced night phase activity, resulting in increased N/T ratio (Figure 8E) against control group (Figure 8B). Under DD, MEL supply significantly increased the amplitude (Table 4).

3.5 | Colocalization of aaNAT-ir, CYC-ir, and PDF-ir in the Br-SOGs of *P americana*

Involvement of aaNAT in the regulation of the circadian system was investigated by double labeling for aaNAT-ir and CYC-ir and PDF-ir (Figure 9A-l). NAT-ir disappeared when aaNAT was reabsorbed with 1 μg/ml of antigen (data not shown). Both small and large aaNAT-ir neurons in the proximal frontoventral (Pfv) cluster in the optic lobe (OL) shared CYC-ir (Figure 9A-F). aaNAT-ir and PDF-ir in this region partially overlapped: PDF-ir occurred at least in several small NAT-ir neurons while the large aaNAT-ir neuron was not PDF-positive (Figure 9G-I). The colocalization of aaNAT-ir and PDF-ir was also observed in some of the landmarks for circadian structures such as the posterior optic commissure (POC) and anterior commissures (AOC) (Figure 9J-l). Hence, the aaNAT system is located on the circadian neural system.

Figure 10A-D shows MEL-ir in the protocerebrum of *P americana*. Prominent large neurons in the pars intercerebralis showed MEL-ir and axonal branch was traced to the ocellus (Figure 10E 2-8). Figure 10F,G shows PER-ir and DBT-ir in adjacent sections of 7 μm. These reactivities are at identical neurons at the apical optic chiasma. The same neurons at the dorsolateral protocerebrum showed no change in PER

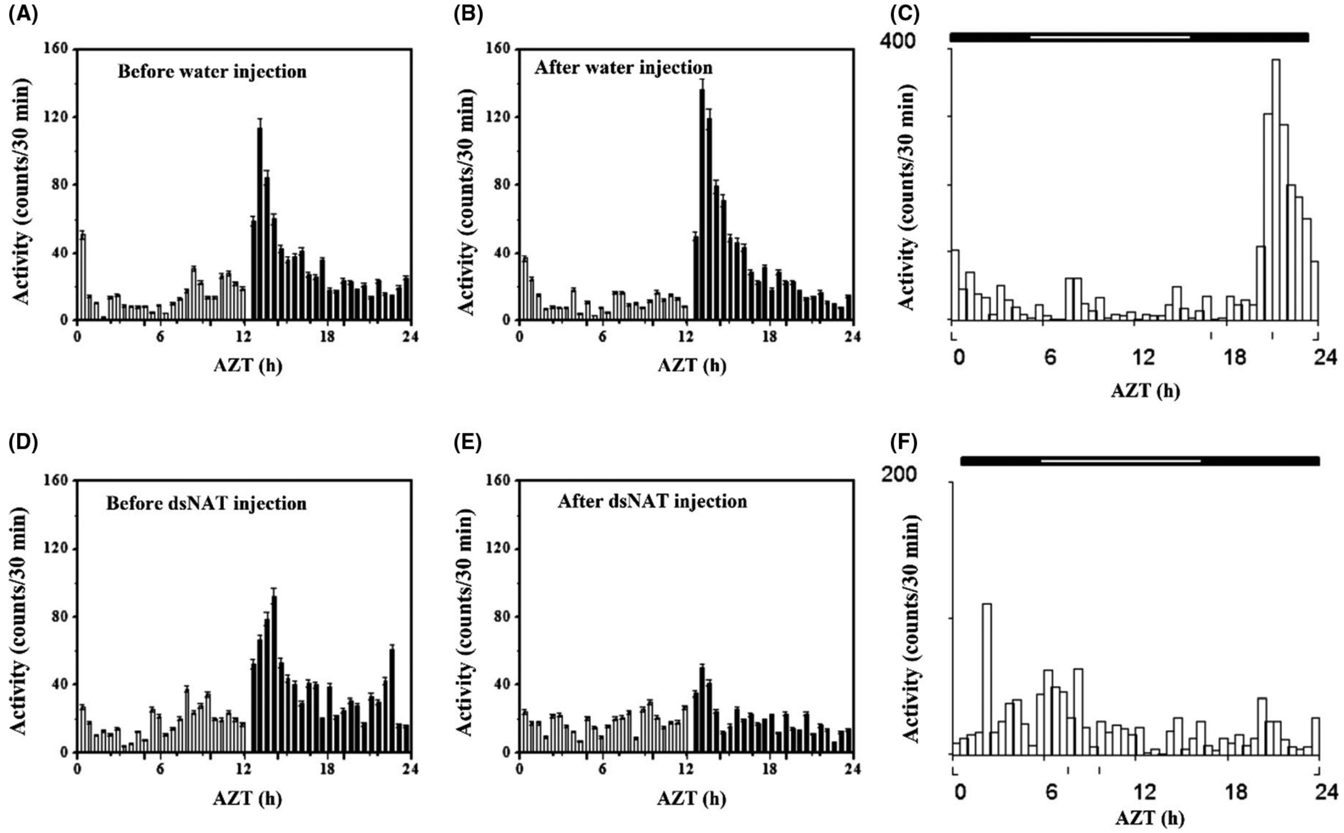

FIGURE 6 Effect of dsRNA^{aaNAT1} on locomotor activity per 30 min is shown as average of daily total activity. Black and white columns indicate activity during dark and light periods, respectively, and vertical lines indicate SEM. (A, B, C) water-injected control group [before injection (A), after (B), under LD cycles and free-running cycle in DD (C)] and (D, E, F) dsRNA^{aaNAT1}-injected group [before injection (D), after (E) under LD cycles and free-running cycle in DD (F)]

TABLE 3 The effects of dsRNA^{aaNAT1} injection on locomotor rhythm in male *Periplaneta americana*

	N/T (%) (mean ± SE)		Circadian period (mean ± SE)	Daily Locomotor Activity (mean ± SE)		
	LD	LD	DD	LD	LD	DD
Injected with	10 days before injection	10 days after injection	20 days after injection	10 days before injection	10 days after injection	20 days after injection
Control (H_2O)	81.05 ± 12.9^a	78.71 ± 12.3^a	24.2 ± 0.2^a	192.81 ± 24.5^a	144.63 ± 59.6^a	208.88 ± 49.4^a
dsRNA^{aaNAT1}	78.92 ± 14.2^a	55.24 ± 15.2^b	23.7 ± 0.3^b	171.08 ± 38.9^a	91.61 ± 35.6^b	87.13 ± 26.9^b

Note: Student' *t* test was used to compare the daily activity before and after the injection ($P < .05$).

antigen localization between cytoplasm and nucleus, unlike *D melanogaster*.[40] aaNAT-ir was not completely observed in the same neurons as clock proteins-ir but it occurred at the close vicinities to PER-ir and DBT-ir as well as MEL-ir particularly in the protocerebrum.

3.6 | Effect of luzindole on locomotor rhythm

Whether the MEL action on locomotor activity is receptor-mediated or not was investigated by using luzindole, a competitive antagonist of MT_2 receptor. Representative double-plotted actogram records of locomotor activity are shown in Figure 11. After injection, 90% of the luzindole-treated cockroaches (n = 10) became arrhythmic in LD (Figure 11B), while all the control cockroaches (n = 10) remained rhythmic (Figure 11A). The luzindole injection significantly reduced locomotor activity during the night (Figures 12 and 13). In DD, 80% of the luzindole-injected insects showed no rhythmicity, with continuing reduced activity. Only 10% of the control insects lost their rhythm. The luzindole injection disrupted the circadian rhythm of locomotor rhythm and the activity level (Figures 12 and 13).

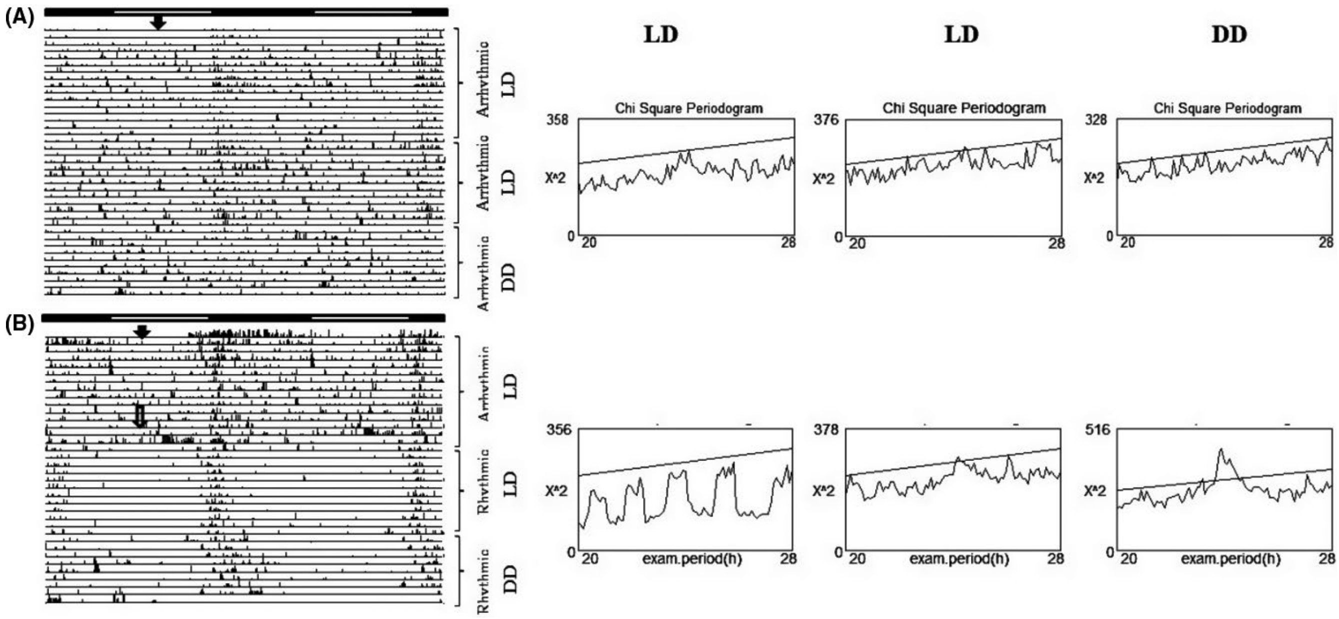

FIGURE 7 Effect of MEL on arrhythmicity caused by dsRNA^{aaNAT1}. Double-plotted actograms (left) and χ^2-periodograms (right) of representative cockroaches. The solid arrow indicates the time of dsRNA^{aaNAT1} injection (A and B), and the open arrow indicates the time of 1.0 mM MEL supply (B). The control group was supplied with water only (A)

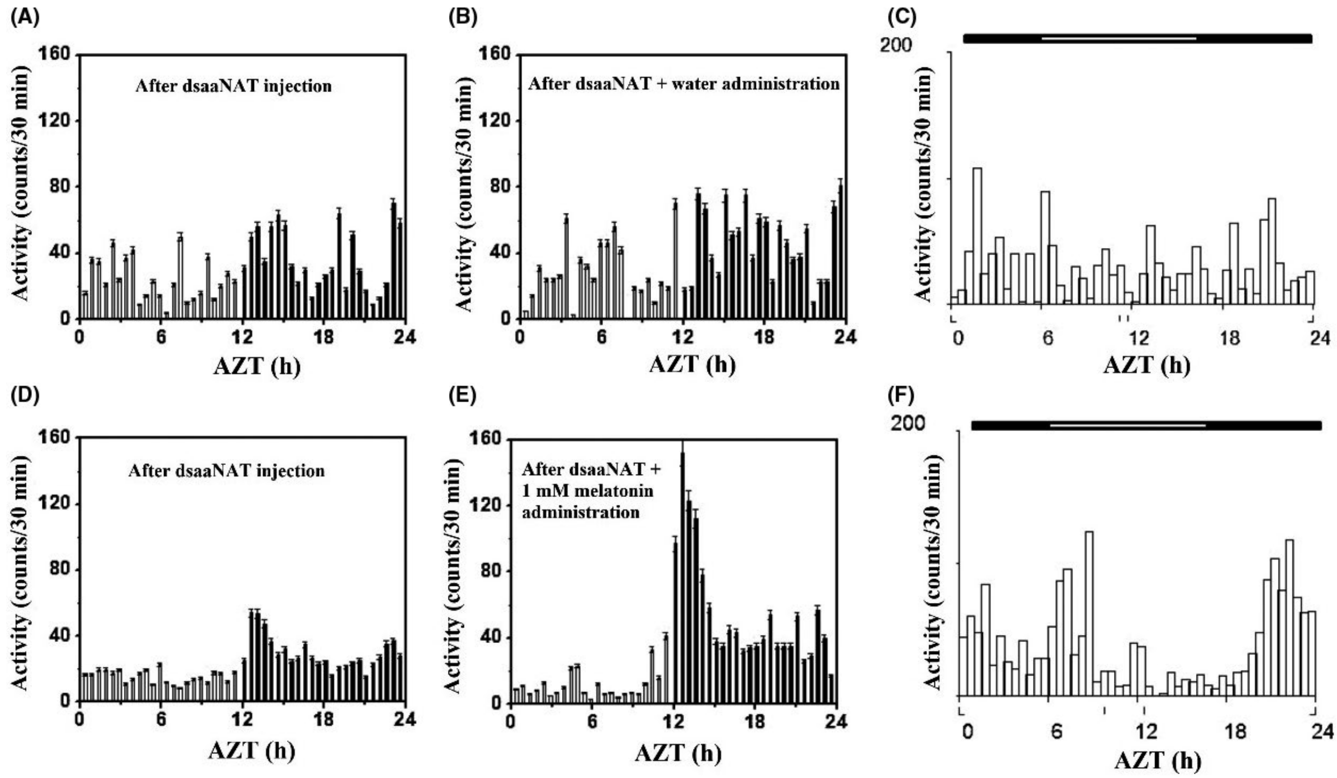

FIGURE 8 Effect of dsRNA^{aaNAT1} on locomotor activity. Activity per 30 min is shown as average of daily total activity. The black and white bars indicate activity during dark and light periods, respectively, and the vertical bars indicate SEM. (A, B, C) control group (dsRNA^{aaNAT1} injection) and (D, E, F) experimental group (dsRNA^{aaNAT1} then 1 mM MEL supply)

TABLE 4 The apparent resynchronization of the locomotor rhythm by melatonin administration following injection of dsRNA^{aaNAT1} in male *Periplaneta americana*

	N/T (%) (mean ±SE)		Circadian period (mean ±SE)	Daily Locomotor Activity (mean ±SE)		
	LD	LD	DD	LD	LD	DD
Injected with	Before treatment	10 days after treatment	20 days after treatment	Before treatment	10 days after treatment	20 days after treatment
dsRNA^{aaNAT1}+ Control (H_2O)	58.67 ± 7.8[a]	53.06 ± 18.1[b]	23.3 ± 1.7[b]	6.25 ± 3.1[a]	7.50 ± 2.6[b]	6.75 ± 2.1[b]
dsRNA^{aaNAT1}+ Melatonin	60.68 ± 11.1[a]	70.96 ± 9.9[a]	24.3 ± 0.2[a]	4.62 ± 2.4[a]	24.75 ± 3.4[a]	37.87 ± 10.7[a]

Note: Student' *t* test was used to comparison ($P < .05$).

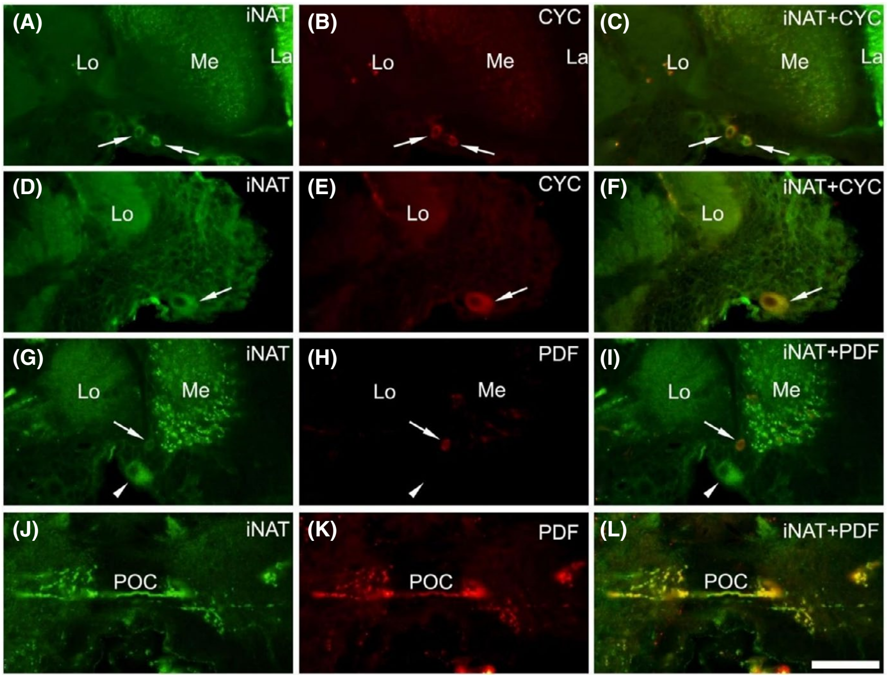

FIGURE 9 Colocalization of immunoreactivities of aaNAT and CYC in the brain including one of the circadian landmark structure, posterior optic commissure (POC) in *P americana*. iaaNAT-ir and immunoreactivities of circadian clock-related gene products were visualized using Alexa Fluor® 488 (green fluorophore) and Alexa Fluor® 555 (red fluorophore), respectively. Pictures on the right show merged images. (A-F) Colocalization of iaaNAT-ir and CYC-ir in the Pfv. CYC-ir occurred in all the small aaNAT-positive cells (arrows). (G-I) Colocalization of aaNAT-ir and PDF-ir in the Pfv. PDF-ir occurred in small aaNAT-ir cells (arrow) but did not occur in a very large cell (arrowhead). Not all aaNAT-ir arborizations in the Me were PDF positive, but some showed colocalization. (J-L) Colocalization of aaNAT-ir and PDF-ir in the POC. aaNAT-ir occurred PDF-ir neural bundle in this region. Pfv = proximal frontoventral; Me = medulla; La = lamina; Lo = lobula; do = dorsal; me = medial. Scale bar = 100 μm

3.7 | *Cis*-acting elements of *aaNAT* for circadian regulation

Putative *Cis*-acting elements involved in circadian regulation and metamorphic influence were explored within the genomic sequence of *PaaaNAT1* available through analyzing the publicly accessible draft genome of *P americana*. The analyzed 3kb region upstream from *PaaaNAT1* transcription start site (TSS), following Zhang et al,[41] contains numerous instances of the putative circadian E-box motif CANNTG,[42] PER repeat (PERR), and Pdp1- and D-boxes (Figure 14; Table 5). Nine putative E-box motifs, with the first two, CAGCTG and AACGTG, are present within the first 200 bp of the TSS (Table 5). Four transcription binding sites for ecdysone receptor (ECR) are also detected.

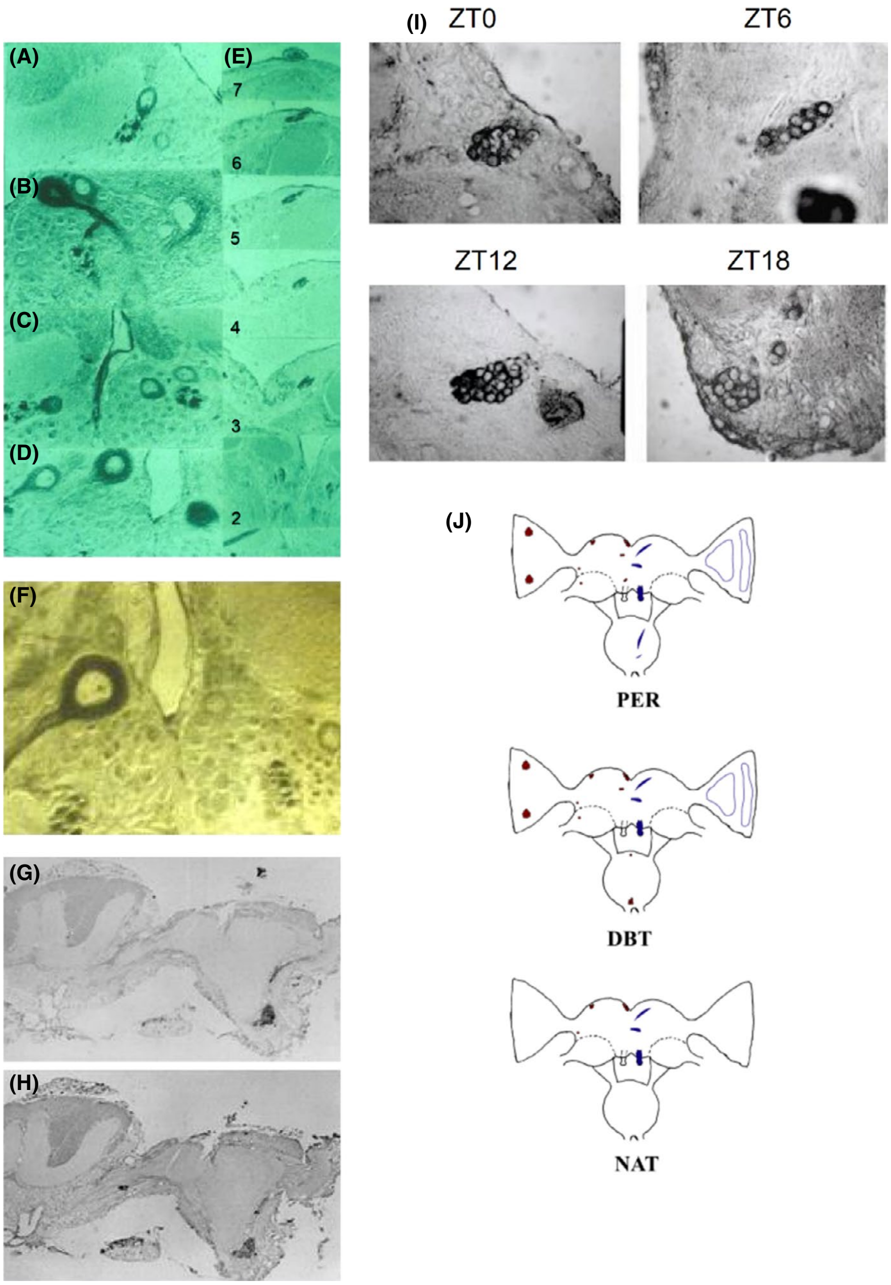

FIGURE 10 MEL-ir in the protocerebrum of *P americana* (A, B, C,D, F) and the tract leading to the ocellus (E2-8) and colocalization of clock proteins-ir, PER-ir (G) and DBT-ir (H). PER-ir in the same locus at different ZTs (I). aaNAT-ir is overlapped with the clock proteins-ir (J) in the protocerebrum

4 | DISCUSSION

The data put forth in this paper strongly support our hypothesis that aaNAT functions to integrate the circadian system and a behavioral rhythm via MEL. Several points are germane. First, MEL drinking synchronized locomotor rhythm both in LD and DD, enhancing the total bouts, amplitude, and nocturnality. Second, knocking down of *aaNAT1* blocked the rhythmicity which was restored by MEL drinking. Similarly, the injection of a competitive MT2 antagonist, luzindole, abolished the rhythmicity but the injection of MEL after luzindole injection restored the rhythmicity. We infer that these points make up a convincing argument supporting that circadian system controls locomotor activity in *P americana* via MEL receptor 2 (MT2). As in *A permyi*,[33] *PaaaNAT* probably is a CCG. Transcriptional rhythmicity probably most strongly contributes to the generation of overt rhythm in *P americana*.

Pigment-dispersing factor is a strong candidate as an output messenger of the circadian pacemaker in *D melanogaster*[43,44] and *L maderae*.[45,46] PDF peaks early in the day and troughs at early night in axon terminals in *D melanogaster*.[47] When examined in a *per*^{0} and *tim*^{0} mutant background, the expression of PDF was detected at constant high levels at axon terminals.[47] Although the molecular gating mechanism of the neurosecretion of PDF is not fully understood, we can pull in guidance from other works. Accumulations of mRNA encoding pdf occurred in *D melanogaster* heads[48] and were recorded by in situ hybridization in the CPM[47] and they remained constant in LD cycles. We suggest that PDF was

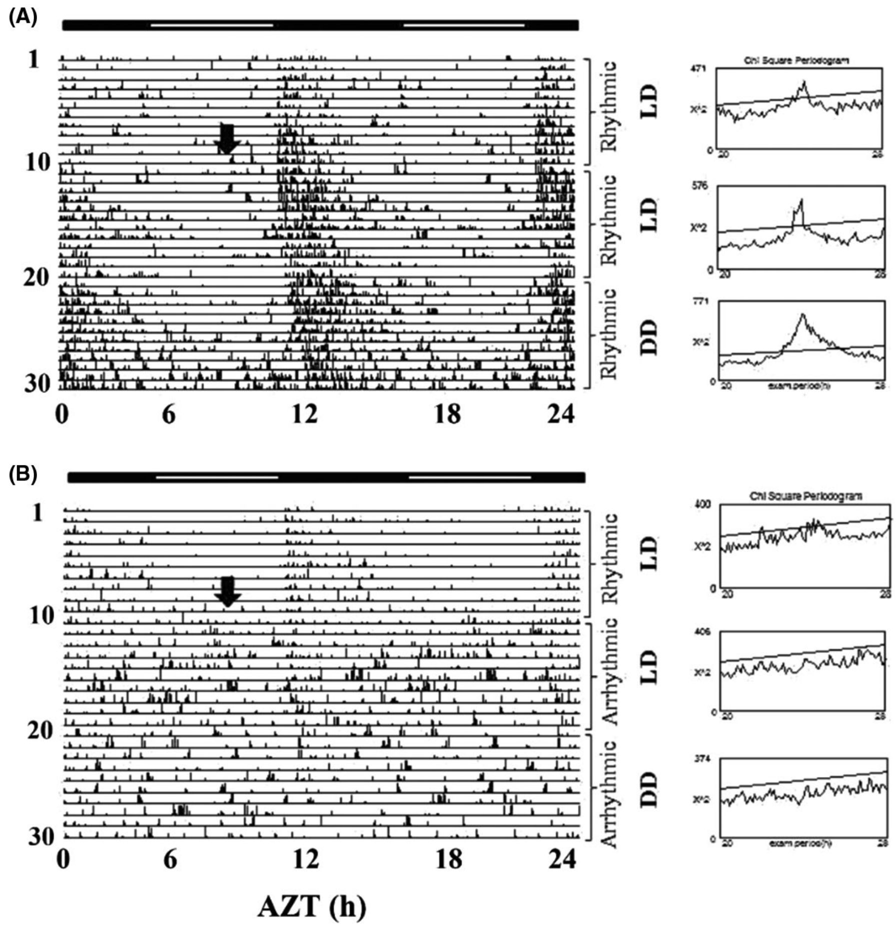

FIGURE 11 Actogram records of locomotor activity after luzindole injection. Double-plotted actograms (left) and χ^{2-}periodograms (right) of representative cockroaches injected with 100 nM (30 pg) melatonin (A) and 100 nM (30 pg) luzindole (B). White and black bars above actograms indicate light and dark phases. The solid arrow indicates the time of injection

not solely involved in mutual synchronization among multiple circadian neurons in this species because RNAi against *aaNAT1* alone desynchronized the locomotor rhythm and could be resynchronized by MEL administration alone.

Unlike PDF, MEL levels show a circadian rhythm with a peak at night in *P americana*.[30] Some other insect species also show rhythmic fluctuation in the hemolymph MEL concentrations, recorded in the cricket, *Gryllus bimaculatus*[49] and the damselfly, *Ischnura verticalis*.[50] RNAi knockdown studies showed that *aaNAT1* is controlled by a negative feedback loop via regulatory binding of the E-box elements in *A pernyi*.[33] Hemolymph MEL concentrations and accumulations of mRNAs encoding iaaNAT1 occur in rhythms, as does iaaNAT1 enzyme activity occurred in rhythmicity in the Br-SOG of *P americana*[30] and *A pernyi*.[33] aaNAT-ir, HIOMT-I, and MEL-ir were colocalized with PER-ir and Cyc-ir (or Bmal1-ir) while PTTH-ir and MT2-ir colocalized in the brain of *A pernyi*.[33] Coincubation of the brain-suboesophageal ganglion-prothoracic gland complex with MEL led to release of PTTH.[28] The same neural network was confirmed here. aaNAT-ir was colocalized in Cyc-r(Bmal1-ir). Colocalization of aaNAT-ir occurred not only with CYC-ir (Bmal1-ir) but also with PDF-ir

In *L maderae*, dissevering the connection between the accessory medulla (AMe) of the optic lobe (OL) and the central brain-induced arrhythmic locomotor activity, but subsequent regeneration of PDF-neural fibers between the Pfv cluster of the OL and median protocerebrum restored rhythmicity.[45] Similarly, transplantation of PDF-positive neurons in the Pfv of the OL into the AMe of an arrhythmic individual restored rhythmicity after restoration of the neural network from the transplant was attained.[51] Thus, PDF-neural fibers projected from the Pfv cluster connect the CPM and motor controller. These results demonstrate the occurrence of aaNAT-ir in the PDF-positive neural structure in the Pfv region of *P americana* and colocalization of aaNAT-ir with CYC-ir This supports the hypothesis that aaNAT underlies the output pathway connecting CPM with the motor controller.

There is evidence that the mutually coupled CMPs regulate the overt circadian rhythm, through either dawn/dusk oscillator coupling or bilateral coupling.[52-54] In digital assistant simulations, the coupled oscillator process is affected by the output of the rhythm.[55] Our observations revealed that MEL strengthens the coupling between oscillators because as the concentration of MEL increased, the amplitude increased.

The neural commissure coupling of bilateral CPMs has been demonstrated by rhodamine backfill into the AMe, immunostaining using positive fibers running through the anterior optic commissure (AOC), and the POC in *L maderae*.[56] These commissures are PDF-ir[46] but aaNAT-ir also occurs here.

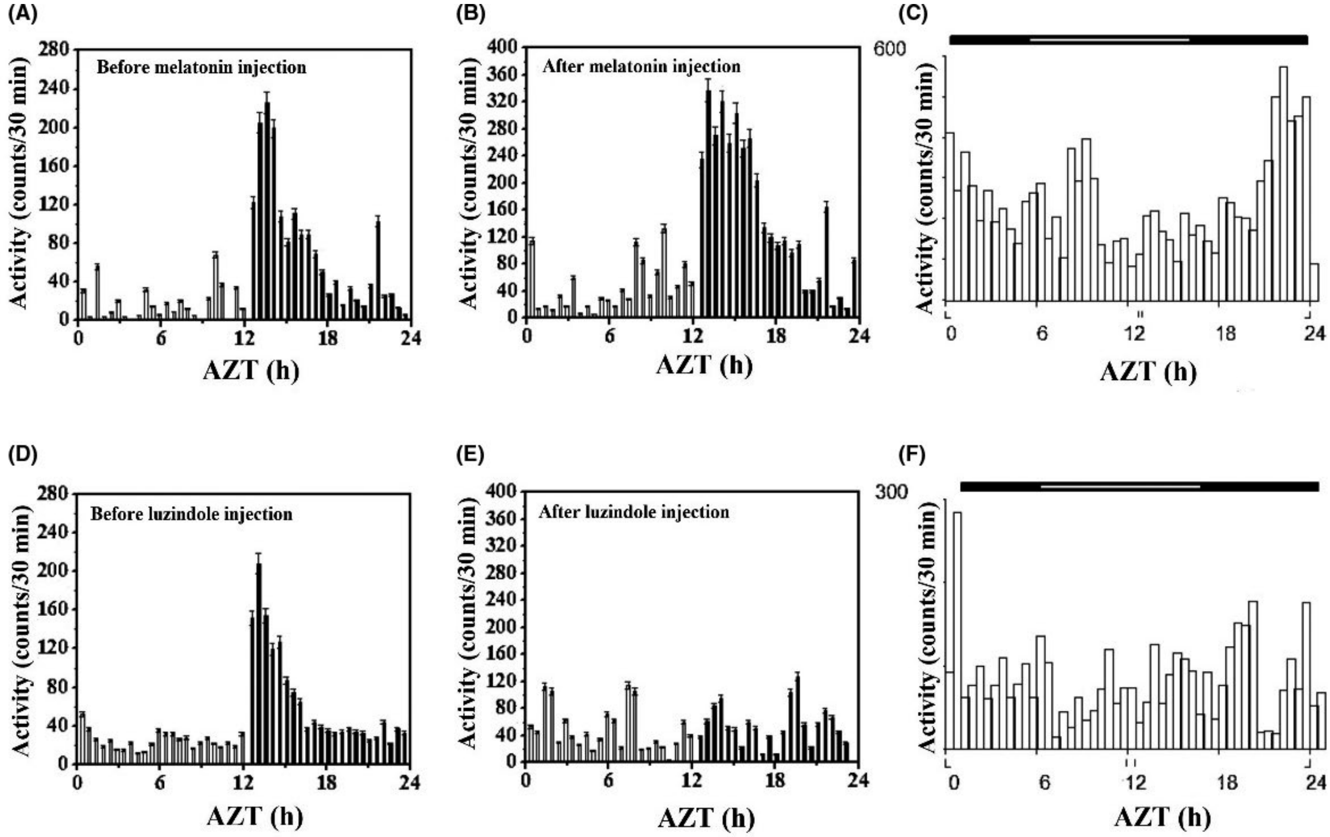

FIGURE 12 Effect of luzindole injection on locomotor activity. Activity per 30 min is shown as average of daily total activity. Black and white bars indicate activities in dark and light periods, respectively, and vertical lines indicate SEM. The melatonin control group is plotted before injection (A), after (B), under LD cycles and in DD (C); and the luzindole group is plotted before injection (D), after (E), under LD and in DD (F)

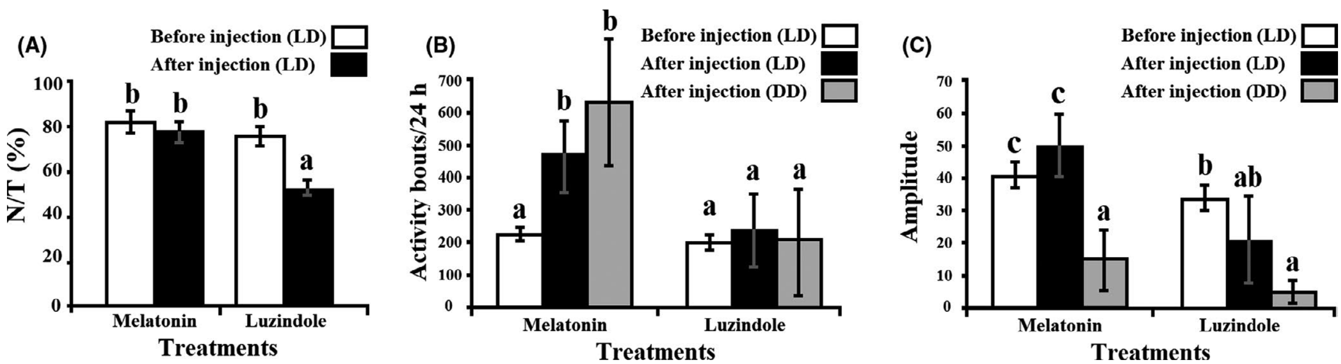

FIGURE 13 Ratio of night activity (A) to total activity; activity level (B) and amplitude (C) of the locomotor rhythm entrained to LD 12:12 before and after injection of 100 nM (30 pg) MEL and luzindole. N, activity at night; T, activity in day and night. Bars (mean ± SD) with different letters differ significantly ($P < .05$, Tukey's test)

The effects of MEL on circadian rhythms of locomotor activity in *P americana* were observed in distinct ways: (1) exogenous MEL synchronized locomotor activity under both LD cycle and DD as MEL affected the locomotor rhythm in *A domesticus*[29] ; (2) rhythmicity in locomotor activity was disrupted by injection of dsRNA^{aaNAT1}; (3) arrhythmia was reduced by *per os* MEL treatments after dsRNA^{aaNAT1} injections, confirming that the enzyme is an endogenous MEL synthesizing enzyme; (4) the enzyme activity is controlled by the circadian pacemaker, because aaNAT-ir and CYC-ir are colocalized in the same neurons; and (5) the N-terminal promotor sequence, the 3kb region upstream from *PaaaNAT1* TSS, contains *cis*-acting elements, notably E-boxes, Pdp1, D-boxes, and PERR which may enhance the modulation of circadian system for transcription.

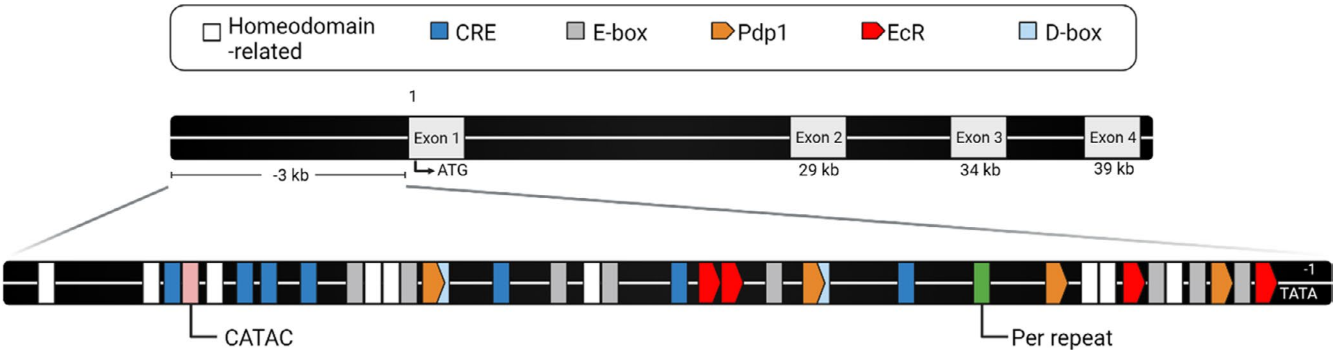

FIGURE 14 Upstream location of *cis*-regulatory motifs annotated in 3 kb region from the transcription start site of the genomic *PaaaNAT1*. Exon structure (middle) and c-terminal promotor sequence of *PaaaNAT1* (bottom) are shown

TABLE 5 Upstream location of *cis*-regulatory motifs annotated in 3 kb region from the transcription start site of the genomic *PaaaNAT1*

	Position		
Binding factor	Start	End	Sequence
E-box	−2137	−2132	3 (CACGTT)
	−2074	−2069	
	−1230	−1225	
	−1724	−1719	2 (AACGTG)
	−106	−101	
	−1632	−1627	CACATG
	−341	−336	CAAGTG
	−268	−263	CAGTTG
	+110	+105	CAGCTG
CRE	−2648	−2643	CATTGT
	−2479	−2472	2 (TGACGGCA)
	−984	−977	
	−1872	−1867	ATGAAT
	−1410	−1405	TGACGC
	−2430	−2424	GCATCTT
	−2352	−2347	ATTTAT
Per repeat (PERR)	−757	−748	GTTCGCTAAA
CATAC	−2625	−2614	AGTTACGCAATG
ECR	−1357	−1345	GGGTCTTTGAAAA
	−1322	−1310	AGGTCAATAACTA
	−373	−361	AGGTAATTGTGAT
	−82	−70	ACGTCATTAACCT
TATA-box	−41	−35	TATATAT
D−box (V/P box)	−2063 −1133	−2056 −1126	TTATGTAA TTATGCAC
Pdp1	−2065	−2056	ATTTATGTAA
	−1136	−1127	GTTTTATGCA
	−591	−582	ATTATCAAAT
	−132	−123	AATATGTAAA
Homeodomain related	−2962	−2957	(2) AATTAA
	−2669	−2664	
	−2127	−2122	(4) AGAAAA
	−2083	−2078	
	−1648	−1643	
	−321	−316	
	−411	−406	ATAAAA
	−2542	−2537	TTTCCT
	−489	−484	TTTCCA

In the latter context, direct coupling of the circadian processes to the E-boxes is an essential feature of rhythmic transcription. For example, the noncanonical E-box enhancer CACGTT drives mouse Period2 circadian oscillations *in vivo*.[57] The PER repeat (PERR) plays a role in transcriptional activation of the *Drosophila period*.[58] Other detected elements include homeobox transcription factors that contribute to intracellular timekeeping mechanisms responsible for daily rhythms in *Drosophila*[59] and cAMP response elements (CRE) which may function for a feedback regulation by MEL as in vertebrate systems.[60] *Cis*-regulatory elements linked to metamorphic influence like EcR are also detected. This is reasonable since aaNAT is mobilized upon metamorphosis and cuticle formation occurs with a circadian periodicity.[24]

Two possible mechanisms for MEL's action enhancing synchronization exist. (1) MEL synchronizes rhythms via suppressing light phase activity through the photo-receptor pathway, or (2) MEL enhances coupling among constituent oscillators of the circadian system. The first possibility is however refuted, because MEL synchronized free-running rhythm.

The MEL-ir was located in the protocerebrum and ocellar nerve in *P americana* (Figure 10). In most insect species, the ocelli often suppress nocturnal activity.[61] 5-HT-ir, HIOMT-ir, and iaaNAT enzyme activity have been characterized in the *P americana* brain.[62,63]

The NAT activity occurs in the brains of fruit flies and cockroaches[30,64] and the head of the fruit fly synthesizes MEL.[65] We speculate that changes in NAT activity in the cockroach brain may regulate the cycle of MEL synthesis. aaNAT is a multi-substrate enzyme but the destruction and subsequent restoration of rhythmicity after dsRNA^{aaNAT1} injection and MEL drinking suggest to us that this enzyme regulates locomotor activity rhythm via MEL. We suggest that MEL in the cockroach may function as a circadian regulator. Evidence of MEL affecting τ has come from the work of Diez-Noguera,[66] who presented a model showing that τ depends both on "neutral elements" (NEs) and internal coupling. With substantial values of NEs, strong coupling tends to increase τ. The observed longer τ after dsRNA^{aaNAT1} injection and drinking higher MEL concentrations in *P americana* may reflect this situation. Higher levels of MEL in the brain may be responsible for rhythmic regulation of circadian activity. A competitive presynaptic MT antagonist, luzindole disrupts circadian rhythms in vertebrates,[67,68] and here we showed that luzindole inhibits the presynaptic receptor to control locomotor activity.

In conclusion, our results appear to provide the first evidence for the receptor-mediated effect of MEL action on circadian rhythms of locomotor activity in the insect brain. The insect aaNAT/MEL pathway appears to be similar to the CPM output pathway found in vertebrates and merits further study.

ACKNOWLEDGMENTS

Prof. Steven M. Reppert (Department of Neurobiology, University of Massachusetts Medical School, MA, USA) kindly supplied us with plasmid containing *Pa*PER, from that we raised antiPaPER antibodies by expressing polypeptides via His-tag cassette. We thank Prof. David Stanley (USDA ARS, MO, USA), Dr Danielle Goodspeed (Baylor College of Medicine, TX, USA), and Dr Brian Taylor (FRES and a Fellow of the Society of Biology, Nottingham, UK) for critically reading the manuscript. Christa Heryanto and Ioannis Eleftherianos (Department of Biological Sciences, Institute for Biomedical Sciences, The George Washington University, Washington DC, USA) is much thanked for technical help with figures. This research was partially supported by a MEXT funding (No. 91306004362) and JSPS grants-in-aid (No. 15K18809 & No. 18380043).

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTION

MT, SH, TN, and AAM designed the study. MT, SH, YW, TN, AY, NI, AAM, AME, and ASMK developed methods and collected data. ASMK, SH, AAM, TN, AY, NI, YW, AME, and MT analyzed and interpreted the data. ASMK, SH, AAM, and MT prepared the original draft. MT and AAM edited and reviewed the final manuscript. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article.

ORCID

Amr A. Mohamed https://orcid.org/0000-0003-2788-5534
Makio Takeda https://orcid.org/0000-0001-6768-6682

REFERENCES

1. McCord CP, Allen FP. Evidences associating pineal gland function with alterations in pigmentation. *J Exp Zool*. 1917;23(1):207-224. https://doi.org/10.1002/jez.1400230108
2. Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. Isolation of melatonin, the pineal gland factor that lightens melanocytes. *J Am Chem Soc*. 1958;80(10):2587. https://doi.org/10.1021/ja01543a060
3. Zimmerman NH, Menaker M. The pineal gland: a pacemaker within the circadian system of the house sparrow. *Proc Natl Acad Sci USA*. 1979;76(2):999-1003. https://doi.org/10.1073/pnas.76.2.999
4. Deguchi T. A circadian oscillator in cultured cells of chicken pineal gland. *Nature*. 1979;282(5734):94-96. https://doi.org/10.1038/282094a0
5. Chabot CC, Menaker M. Effects of physiological cycles of infused melatonin on circadian rhythmicity in pigeons. *J Comp Physiol A*. 1992;170(5):615-622. https://doi.org/10.1007/BF00199337
6. Kennaway DJ. The role of circadian rhythmicity in reproduction. *Hum Reprod Update*. 2005;11(1):91-101. https://doi.org/10.1093/humupd/dmh054
7. Pan X, Taylor MJ, Cohen E, Hanna N, Mota S. Circadian clock, time-restricted feeding and reproduction. *Int J Mol Sci*. 2020;21(3):831. https://doi.org/10.3390/ijms21030831
8. Klein DC. Arylalkylamine *N*-acetyltransferase: "the Timezyme". *J Biol Chem*. 2007;282(7):4233-4237. https://doi.org/10.1074/jbc.R600036200
9. Falcón J, Coon SL, Besseau L, et al. Drastic neofunctionalization associated with evolution of the timezyme AANAT 500 Mya. *Proc Natl Acad Sci USA*. 2014;111(1):314-319. https://doi.org/10.1073/pnas.1312634110
10. De Angelis J, Gastel J, Klein DC, Cole PA. Kinetic analysis of the catalytic mechanism of serotonin *N*-acetyltransferase (EC 2.3.1.87). *J Biol Chem*. 1998;273(5):3045-3050. https://doi.org/10.1074/jbc.273.5.3045
11. Reiter RJ, Tan DX, Sharma R. Historical perspective and evaluation of the mechanisms by which melatonin mediates seasonal reproduction in mammals. *Melaton Res*. 2018;1:58-76. https://doi.org/10.32794/mr11250004
12. Zhao D, Yu Y, Shen Y, et al. Melatonin synthesis and function: Evolutionary history in animals and plants. *Front Endocrinol*. 2019;10:249. https://doi.org/10.3389/fendo.2019.00249

13. Bell-Pedersen D, Cassone VM, Earnest DJ, et al. Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nat Rev Genet*. 2005;6(7):544-556. https://doi.org/10.1038/nrg1633
14. Borjigin J, Wang MM, Snyder SH. Diurnal variation in mRNA encoding serotonin N-acetyltransferase in pineal gland. *Nature*. 1995;378(6559):783-785. https://doi.org/10.1038/378783a0
15. Coon SL, Roseboom PH, Baler R, et al. Pineal serotonin *N*-acetyltransferase: expression cloning and molecular analysis. *Science*. 1995;270(5242):1681-1683. https://doi.org/10.1126/science.270.5242.1681
16. Altun A, Ugur-Altun B. Melatonin: therapeutic and clinical utilization. *Int J Clin Pract*. 2007;61(5):835-845. https://doi.org/10.1111/j.1742-1241.2006.01191.x
17. Vivien-Roels B, Pévet P. Melatonin: presence and formation in invertebrates. *Experientia*. 1993;49:642-647. https://doi.org/10.1007/BF01923945
18. Hardeland R, Poeggeler B. Non-vertebrate melatonin. *J Pineal Res*. 2003;34(4):233-241. https://doi.org/10.1034/j.1600-079x.2003.00040.x
19. Sainath SB, Swetha CH, Reddy PS. What do we (need to) know about the melatonin in crustaceans? *J Exp Zool A Ecol Genet Physiol*. 2013;319(7):365-377. https://doi.org/10.1002/jez.1800
20. Coon SL, Klein DC. Evolution of arylalkylamine *N*-acetyltransferase: emergence and divergence. *Mol Cell Endocrinol*. 2006;252(1–2):2-10. https://doi.org/10.1016/j.mce.2006.03.039
21. Arnao MB, Hernández-Ruiz J. Melatonin: A new plant hormone and/or a plant master regulator? *Trends Plant Sci*. 2019;24(1):38-48. https://doi.org/10.1016/j.tplants.2018.10.010
22. Hiragaki S, Suzuki T, Mohamed AA, Takeda M. Structures and functions of insect arylalkylamine *N*-acetyltransferase (iaaNAT); a key enzyme for physiological and behavioral switch in arthropods. *Front Physiol*. 2015;6:113. https://doi.org/10.3389/fphys.2015.00113
23. Karlson P, Sekeris C *N*-Acetyl-dopamine as sclerotizing agent of the insect cuticle. *Nature*. 1962;195:183-184. https://doi.org/10.1038/195183a0
24. Dai FY, Qiao L, Tong XL, et al. Mutations of an arylalkylamine-*N*-acetyltransferase, Bm-iAANAT, are responsible for silkworm melanism mutant. *J Biol Chem*. 2010;285(25):19553-19560. https://doi.org/10.1074/jbc.M109.096743
25. Matsumoto M, Takeda M. Changes in brain monoamine contents in diapause pupae of *Antheraea pernyi* when activated under long-day and by chilling. *J Insect Physiol*. 2002;48(8):765-771. https://doi.org/10.1016/s0022-1910(02)00102-6
26. Sloley BD. Metabolism of monoamines in invertebrates: the relative importance of monoamine oxidase in different phyla. *Neurotoxicology*. 2004;25(1–2):175-183. https://doi.org/10.1016/S0161-813X(03)00096-2
27. Bortolato M, Chen K, Shih JC. Monoamine oxidase inactivation: from pathophysiology to therapeutics. *Adv Drug Deliv Rev*. 2008;60(13–14):1527-1533. https://doi.org/10.1016/j.addr.2008.06.002
28. Richter K, Peschke E, Peschke D. A neuroendocrine releasing effect of melatonin in the brain of an insect, *Periplaneta americana* (L.). *J Pineal Res*. 2000;28(3):129-135. https://doi.org/10.1034/j.1600-079x.2001.280301.x
29. Yamano H, Watari Y, Arai T, Takeda M. Melatonin in drinking water influences a circadian rhythm of locomotor activity in the house cricket. *Acheta domesticus. J Insect Physiol*. 2001;47(8):943-949. https://doi.org/10.1016/S0022-1910(01)00067-1
30. Bembenek J, Sehadova H, Ichihara N, Takeda M. Day/night fluctuations in melatonin content, arylalkylamine *N*-acetyltransferase activity and NAT mRNA expression in the CNS, peripheral tissues and hemolymph of the cockroach, *Periplaneta americana*. *Comp Biochem Physiol B Biochem Mol Biol*. 2005;140(1):27-36. https://doi.org/10.1016/j.cbpc.2004.03.017
31. Tsugehara T, Iwai S, Fujiwara Y, Mita K, Takeda M. Cloning and characterization of insect arylalkylamine *N*-acetyltransferase from *Bombyx mori*. *Comp Biochem Physiol B Biochem Mol Biol*. 2007;147(3):358-366. https://doi.org/10.1016/j.cbpb.2006.10.112
32. Tsugehara T, Imai T, Takeda M. Characterization of arylalkylamine N-acetyltransferase from silkmoth (*Antheraea pernyi*) and pesticidal drug design based on the baculovirus-expressed enzyme. *Comp Biochem Physiol C Toxicol Pharmacol*. 2013;157(1):93-102. https://doi.org/10.1016/j.cbpc.2012.10.003
33. Mohamed AA, Wang Q, Bembenek J, et al. *N*-acetyltransferase (nat) is a critical conjunct of photoperiodism between the circadian system and endocrine axis in *Antheraea pernyi*. *PLoS One*. 2014;9(3):e92680. https://doi.org/10.1371/journal.pone.0092680
34. Ichihara N, Okada M, Takeda M. Characterization and purification of polymorphic arylalkylamine *N*-acetyltransferase from the American cockroach. *Periplaneta americana. Insect Biochem Mol Biol*. 2001;32(1):15-22. https://doi.org/10.1016/s0965-1748(01)00075-3
35. Arai T, Watari Y. Effects of photoperiod and aging on locomotor activity rhythms in the onion fly. *Delia antiqua. J Insect Physiol*. 1997;43(6):567-576. https://doi.org/10.1016/s0022-1910(97)00002-4
36. Kamruzzaman ASM, Mikani A, Mohamed AA, Elgendy AM, Takeda M. Crosstalk among indoleamines, neuropeptides and JH/20E in regulation of reproduction in the American Cockroach, *Periplaneta americana*. *Insects*. 2020;11(3):155. https://doi.org/10.3390/insects11030155
37. Sehadová H, Markova EP, Sehnal F, Takeda M. Distribution of circadian clock-related proteins in the cephalic nervous system of the silkworm. *Bombyx mori. J Biol Rhythms*. 2004;19(6):466-482. https://doi.org/10.1177/0748730404269153
38. Hiragaki S, Uno T, Takeda M. Putative regulatory mechanism of prothoracicotropic hormone (PTTH) secretion in the American cockroach, *Periplaneta americana* as inferred from co-localization of Rab8, PTTH, and protein kinase C in neurosecretory cells. *Cell Tissue Res*. 2009;335(3):607-615. https://doi.org/10.1007/s00441-008-0747-9
39. Shao QM, Hiragaki S, Takeda M. Co-localization and unique distributions of two clock proteins CYCLE and CLOCK in the cephalic ganglia of the ground cricket. *Allonemobius allardi. Cell Tissue Res*. 2008;331(2):435-446. https://doi.org/10.1007/s00441-007-0534-z
40. Saez L, Young MW. Regulation of nuclear entry of the *Drosophila* clock proteins period and timeless. *Neuron*. 1996;17(5):911-920. https://doi.org/10.1016/s0896-6273(00)80222-6
41. Zhang J, Li S, Li W, et al. Circadian regulation of night feeding and daytime detoxification in a formidable Asian pest *Spodoptera litura*. *Commun Biol*. 2021;4(1):286. https://doi.org/10.1038/s42003-021-01816-9
42. Ripperger JA, Shearman LP, Reppert SM, Schibler U. CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. *Genes Dev*. 2000;14(6):679-689. https://doi.org/10.1101/gad.14.6.679
43. Helfrich-Förster C. The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide

that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. *Proc Natl Acad Sci USA*. 1995;92(2):612-616. https://doi.org/10.1073/pnas.92.2.612
44. Helfrich-Förster C. Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons: a brain-behavioral study of disconnected mutants. *J Comp Physiol A*. 1998;182(4):435-453. https://doi.org/10.1007/s003590050192
45. Stengl M, Homberg U. Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. *J Comp Physiol A*. 1994;175(2):203-213. https://doi.org/10.1007/BF00215116
46. Reischig T, Stengl M. Ultrastructure of pigment-dispersing hormone-immunoreactive neurons in a three-dimensional model of the accessory medulla of the cockroach *Leucophaea maderae*. *Cell Tissue Res*. 2003;314(3):421-435. https://doi.org/10.1007/s00441-003-0772-7
47. Park JH, Helfrich-Förster C, Lee G, Liu L, Rosbash M, Hall JC. Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc Natl Acad Sci USA*. 2000;97(7):3608-3613. https://doi.org/10.1073/pnas.070036197
48. Park JH, Hall JC. Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. *J Biol Rhythms*. 1998;13(3):219-228. https://doi.org/10.1177/074873098129000066
49. Itoh MT, Hattori A, Nomura T, Sumi Y, Suzuki T. Melatonin and arylalkylamine *N*-acetyltransferase activity in the silkworm. *Bombyx mori*. *Mol Cell Endocrinol*. 1995;115(1):59-64. https://doi.org/10.1016/0303-7207(95)03670-3
50. Tilden AR, Anderson WJ, Hutchison VH. Melatonin in two species of damselfly, *Ischnura verticalis* and *Enallagma civile*. *J Insect Physiol*. 1994;40(9):775-780. https://doi.org/10.1016/0022-1910(94)90006-X
51. Reischig T, Stengl M. Ectopic transplantation of the accessory medulla restores circadian locomotor rhythms in arrhythmic cockroaches (*Leucophaea maderae*). *J Exp Biol*. 2003a;206(Pt 11):1877-1886. https://doi.org/10.1242/jeb.00373
52. Pittendrigh CS, Daan S. A Functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: Pacemaker as clock. *J Comp Physiol A*. 1976;106(3):291-331. https://doi.org/10.1007/BF01417859
53. Page TL, Caldarola PC, Pittendrigh CS. Mutual entrainment of bilaterally distributed circadian pacemaker. *Proc Natl Acad Sci USA*. 1977;74(3):1277-1281. https://doi.org/10.1073/pnas.74.3.1277
54. Page TL. Effects of localized low-temperature pulses on the cockroach circadian pacemaker. *Am J Physiol*. 1981;240(3):R144-R150. https://doi.org/10.1152/ajpregu.1981.240.3.R144
55. Pittendrigh CS. Circadian systems: entrainment. In: Aschoff J, ed. *Biological Rhythms (Handbook of Behavioral Neurobiology*, vol. 4. New York: Plenum Press; 1981:95-124.
56. Reischig T, Petri B, Stengl M. Pigment-dispersing hormone (PDH)-immunoreactive neurons form a direct coupling pathway between the bilaterally symmetric circadian pacemakers of the cockroach *Leucophaea maderae*. *Cell Tissue Res*. 2004;318(3):553-564. https://doi.org/10.1007/s00441-004-0927-1
57. Yoo SH, Ko CH, Lowrey PL, et al. A noncanonical E-box enhancer drives mouse Period2 circadian oscillations *in vivo*. *Proc Natl Acad Sci USA*. 2005;102(7):2608-2613. https://doi.org/10.1073/pnas.0409763102
58. McDonald MJ, Rosbash M, Emery P. Wild-type circadian rhythmicity is dependent on closely spaced E boxes in the *Drosophila* timeless promoter. *Mol Cell Biol*. 2001;21(4):1207-1217. https://doi.org/10.1128/MCB.21.4.1207-1217.2001
59. Nair S, Bahn JH, Lee G, Yoo S, Park JH. A Homeobox transcription factor Scarecrow (SCRO) negatively regulates Pdf neuropeptide expression through binding an identified cis-acting element in *Drosophila melanogaster*. *Mol Neurobiol*. 2020;57(4):2115-2130. https://doi.org/10.1007/s12035-020-01874-w
60. Foulkes NS, Whitmore D, Sassone-Corsi P. Rhythmic transcription: the molecular basis of circadian melatonin synthesis. *Biol Cell*. 1997;89(8):487-494. https://doi.org/10.1016/s0248-4900(98)80004-x
61. Mizunami M. Gain control of synaptic transfer from second- to third-order neurons of cockroach ocelli. *J Gen Physiol*. 1996;107(1):121-131. https://doi.org/10.1085/jgp.107.1.121
62. Nishiitsutsuji-Uwo J, Takeda M, Saito H. The production of an antiserum to serotonin and serotonin-like immunoreactivity in the cockroach brain–midgut system. *Biomed Res*. 1984;5(2):211-224. https://doi.org/10.2220/biomedres.5.211
63. Takeda M, Endo Y, Saito H, Nishimura M, Nishitsutsuji-Uwo J. Neuropeptide and monoamine immunoreactivity of the circadian pacemaker in *Periplaneta*. *Biomed Res*. 1985;6(6):395-406. https://doi.org/10.2220/biomedres.6.395
64. Dewhurst SA, Croker SG, Ikeda K, McCaman RE. Metabolism of biogenic amines in *Drosophila* nervous tissue. *Comp Biochem Physiol B*. 1972;43(4):975-981. https://doi.org/10.1016/0305-0491(72)90241-6
65. Finocchiaro L, Callebert J, Launay JM, Jallon JM. Melatonin biosynthesis in *Drosophila*: its nature and its effects. *J Neurochem*. 1988;50(2):382-387. https://doi.org/10.1111/j.1471-4159.1988.tb02923.x
66. Díez-Noguera A. A functional model of the circadian system based on the degree of intercommunication in a complex system. *Am J Physiol*. 1994;267(4 Pt 2):R1118-1135. https://doi.org/10.1152/ajpregu.1994.267.4.R1118
67. Dubocovich ML. Luzindole (N-0774): a novel melatonin receptor antagonist. *J Pharmacol Exp Ther*. 1988;246(3):902-910. https://jpet.aspetjournals.org/content/246/3/902.long
68. Sugden D. Effect of putative melatonin receptor antagonists on melatonin-induced pigment aggregation in isolated *Xenopus laevis* melanophores. *Eur J Pharmacol*. 1992;213(3):405-408. https://doi.org/10.1016/0014-2999(92)90629-i

How to cite this article: Kamruzzaman ASM, Hiragaki S, Watari Y, et al. Clock-controlled arylalkylamine *N*-acetyltransferase (*aaNAT*) regulates circadian rhythms of locomotor activity in the American cockroach, *Periplaneta americana,* via melatonin/MT2-like receptor. *J Pineal Res*. 2021;00:e12751. https://doi.org/10.1111/jpi.12751