Vasopressin receptor V1a regulates circadian rhythms of locomotor activity and expression of clock-controlled genes in the suprachiasmatic nuclei

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Abstract The suprachiasmatic nuclei (SCN) serve as the principal circadian pacemaker that coordinate daily cycles of behavior and physiology for mammals. A network of transcriptional and translational feedback loops underlies the operating molecular mechanism for circadian oscillation within the SCN neurons. It remains unclear how timing information is transmitted from SCN neurons to eventually evoke circadian rhythms. Intercellular communication between the SCN and its target neurons is critical for the generation of coherent circadian rhythms. At the molecular level, neuropeptides encoded by clock-controlled genes have been indicated as important output mediators. Arginine-vasopressin (AVP) is the product of one such clock-controlled gene. Previous studies have demonstrated a circadian rhythm of AVP levels in the cerebrospinal fluid and the SCN. The physiological effects of AVP are mediated by three types of AVP receptors, designated as V1a, V1b and V2. Here, we report that V1a mRNA levels displayed a circadian rhythm in the SCN, peaking during night hours. The circadian rhythmicity of locomotor activities was significantly reduced in V1a-deficient (V1a−/−) mice (50-75% reduction in the power of Fast Fourier Transformation). However, the light masking and light-induced phase shift effects are intact in V1a−/− mice. Whereas the expression of clock core genes was unaltered, the circadian amplitude of prokineticin 2 (PK2) mRNA oscillation was attenuated in the SCN of V1a−/− mice (≈50% reduction in the peak levels). In vitro experiments demonstrated that AVP, acting through V1a receptor, was able to enhance the transcriptional activity of the PK2 promoter. These studies thus indicate that AVP-V1a signaling plays an important role in the generation of overt circadian rhythms.

Keywords Circadian, Vasopressin, V1a receptor, Suprachiasmatic nuclei, Prokineticin
**INTRODUCTION**

Almost all organisms from bacteria to mammals exhibit circadian (~ 24 hr) physiology and behavior to adapt to the environmental changes imposed by the daily revolutions of the earth planet. In mammals, the endogenous pacemaker that drives circadian rhythms resides in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The SCN coordinates daily cycles of behavior and physiology, such as the rhythm of activity and rest (23, 31, 32). A network of transcriptional and translational feedback loops has been demonstrated as the operating machinery for circadian oscillation within the SCN neurons (31, 32). Some so-called clock controlled genes (CCGs) are also rhythmically regulated by using the same transcriptional machinery that controls the clock feedback loops. The peptides encoded by three CCGs (prokineticin 2, cardiotrophin-like cytokine and arginine-vasopressin) have been particularly implicated as critical SCN output molecules that link the SCN and its efferent targets (6, 17, 19).

Arginine-vasopressin (AVP) is one of the first neurotransmitters discovered in the SCN (5, 37, 41). The concentrations of AVP in the cerebrospinal fluid (CSF) vary in a circadian cycle, with morning levels approximately five times higher than that of night hours (30). Previous studies have demonstrated that the circadian variation of AVP levels in the CSF originates from the AVP content in the SCN (16, 34, 38, 40). Recent molecular biology studies revealed that the transcription of AVP gene in the SCN is rhythmically regulated by the same positive and negative elements that control the core molecular loops (17). The CLOCK/BMAL1 complex positively activates the expression of AVP gene, which is suppressed by periods (Pers) and cryptochromes.
(Crys). The vasopressinergic fibers are observed in the SCN as well as some of the SCN targets, including the paraventricular nuclei (PVN) and the dorsomedial hypothalamic nuclei (DMH) (11). Thus, AVP was thought to not only function as an output factor, but also regulate the activity of SCN neurons.

There are three types of AVP receptors, designated as V1a, V1b and V2 (9, 22, 25). Among them, only V1a and V1b receptor subtypes are expressed in the central nervous system. The V1b receptor is located primarily in the pituitary and discrete areas of the brain, including the amygdale, while V1a receptor is expressed in many areas throughout of the brain. Importantly, V1a receptor is expressed in the SCN and its target area, indicating that it may be the major mediator for the circadian function of AVP. To understand the role of V1a receptor in the circadian regulation, we examined the circadian locomotor activity and the expression of clock genes in V1a-/- mice.

MATERIALS AND METHODS

Animals

The generation of V1a-/- mice by homologous recombination has been previously described (12). V1a-/- mice and their littermate wild-type (WT) mice with 50% C57BL/6 × 50% 129/Sv mix background or 94% C57BL/6 background were used. Mixed female and male mice (approximately 1:1) from 3-5 months of age were used. Before behavioral experiments, mice of the same sex were group-housed (3–5 animals per cage) under controlled conditions (temperature
20 ± 2°C; relative humidity 50–60%; 12h light-dark cycle, lights on 7:00 a.m. and lights off 7:00 p.m.) and had free access to food and water. All procedures regarding the care and use of animals have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Irvine.

**Locomotor behavior**

Mice (16 WT and 16 V1a−/− mice at mixed background; 6 WT and 7 V1a−/− mice at 94% C57BL/6 background) were individually housed within cages equipped with running wheels or infrared motion sensors and were allowed free access to food and water. Their locomotor activities were recorded as revolutions per 5-min interval. Mice were entrained to an initial 12h Light: 12h Dark (LD) cycle (light intensity ~150 lux, lights on 7:00 a.m., and lights off 7:00 p.m.). After 2-3 weeks of activity recording in LD conditions, the mice were placed in constant darkness (DD) with a dim red light (< 5 lux) for ~4 weeks. Another group of mice (6 WT and 6 V1a−/− mice at mix background) in LD were also exposed to a light treatment (white light, 60 min, ~150 lux) during their activity period [zeitgeber time (ZT) 15, zeitgeber time is measured in hours after the light has been turned on in a LD cycle] to measure negative masking behavior, i.e., light-induced suppression of activity. The number of revolutions during this light treatment was compared with the number recorded during the same phase on the previous day. The light-induced phase shift was also performed on this group of mice at the 15th day in DD. Animals in their home cages were moved to another room and exposed to a 10 min pulse of white light (~150 lux) at circadian times (CT) 16, at which CT12 was designated as activity onset. Light-induced phase shift amplitude was derived from regression lines drawn through the activity onset of at least 7 d immediately before
the day of stimulation and 7 d after reestablishment of steady-state circadian period after
stimulation.

The free-run period and Fast Fourier Transformation (FFT) were analyzed using ClockLab
software (Actimetrics, Evanston, IL) in the MatLab environment. The free-run period was
measured by a χ² periodogram from day 10 through 25 under DD. The daily revolution and FFT
was determined by analyzing the activity of the last 10 days under LD and day 10 to 25 under DD.
FFT circadian amplitude values represent the peak relative amplitude in the circadian range
(18–30 hr) normalized to a total variance of 100%. To estimate the cycle-to-cycle variability in
activity onset, a linear regression to 15 cycles (day 10 to 25 under DD) of activity onset was
calculated. The onset of activity for each cycle was defined as the occurrence of the first
concentrated bout of activity after an extended period of rest. The divergence between the
measured and predicted onsets was then determined, and the average difference was calculated for
each animal (7). The duration of each cycle (α) was calculated as described (7).

In situ hybridization

Mice were sacrificed via cervical dislocation and brains were quickly frozen and stored at -80 ºC
until use. In situ hybridization was carried out on coronal sections (16-μm) as described by
Winzer-Serhan et al (43). Antisense and sense cRNA probes were generated by in vitro
transcription in the presence of ³⁵S-UTP (1200 Ci/mmol). The following probes were used: mouse
vasopressin, nucleotides 19–254 (GenBank accession number BC051997); mPer1, nucleotides
340–761 (GenBank accession number AF022992); mPer2, nucleotides 9–489 (GenBank accession
number AF035830); *mRev-erb-a*, nucleotides 957-2108 (Genbank access number NM_145434); *mBmal1*, nucleotides 84–1282 (GenBank accession number AF015953); *mPK2*, nucleotides 1–528 (GenBank accession number AF487280) and *mV1a*, nucleotides 1779-2068 (Genbank accession number BC024149). The slides were hybridized with corresponding probes (100 μl/slide at 10^7 cpm/ml) overnight at 60°C. Following completion of the wash steps, slides were air dried and apposed to Kodak BioMax MR films for 4 days. The specific hybridization signals were quantified by using a video-based computer image analysis system (MCID, Imaging Research, UK). A calibration curve of optical density versus radioactivity [disintergations per minute (d.p.m.) per mg tissue wet weight] was constructed using ^14^C standards. Specific hybridization signals in the SCN were obtained by subtracting background values obtained from adjacent brain areas that have no hybridization signal. Data were normalized with respect to the differences between signal intensities in equal areas of SCN. The detection threshold for the *in situ* hybridization was at the range of 10-20 copies mRNA per cell (43).

**Luciferase assay**

A 2.8-kb 5'-flanking region of the mouse *PK2* gene was sub-cloned into a pGL3-Basic vector (Promega, WI) to generate the PK2.8-Luc plasmid (6). PK2.8-Luc and V1a-expressing plasmids were transfected into human embryonic kidney (HEK293) cells using lipofectamine (Invitrogen, CA). At 24 hours after transfection, cells were treated with various concentrations of AVP (0 - 50 nM) in DMEM media supplemented with 0.5% fetal bovine serum and incubated for another 24 hours. Cells were lysed and the firefly luciferase activity was assayed with a Sirius luminometer (Berthold, Germany). Luciferase activities were normalized to protein concentration. Protein
concentration was determined by a Bio-Rad protein assay, with a detection level of 1 μg protein.

**Statistical analyses**

A repeated-measures ANOVA followed by unpaired t test was used to analyze the data for differences between genotypes and time points. All statistical analysis was performed using Prism 4.4 (GraphPad Software, CA).

**RESULTS**

**Circadian rhythm of $V1\alpha$ mRNA in wild-type and $Bmal1^{-/-}$ mice**

We quantified the $V1\alpha$ transcript level in the SCN under constant darkness through in situ hybridization. As shown in Fig. 1A, $V1\alpha$ mRNA in the SCN displayed a robust circadian rhythm under constant darkness [$F = 16.93$, $P < 0.0001$, one-way ANOVA], consistent with previous reports (28, 39). Peak level at CT17 was about 2.4 times that of the nadir level at CT 5. In the $V1\alpha^{-/-}$ mice, no $V1\alpha$ transcript was detected in the SCN at any time point examined, confirming the targeted allele as a null mutation (Fig. 1A, B). Furthermore, under a light-dark condition, the rhythm of $V1\alpha$ mRNA was significantly attenuated in mice deficient in a clock core gene, $Bmal1$ [$F (1) = 8.92$, $P < 0.02$, genotype × time, two-way ANOVA] (Fig. 1C). Thus, the oscillation of $V1\alpha$ mRNA in the SCN seems under the control of clockwork oscillator. As BMAL1/CLOCK heterodimer had no effect on the transcriptional activity of $V1\alpha$ promoter (data not shown),
BMAL1 may regulate the transcription of V1a gene in the SCN indirectly. Apparently, the light-dark cycle also affects the oscillation of V1a expression in the SCN as it was reduced, but not abolished in the SCN of Bmal1−/− mice.

**V1a−/− mice showed attenuated circadian rhythms in locomotor activities**

To address the function of V1a in the regulation of circadian rhythms, we first monitored the wheel-running activity of V1a−/− mice and their wild-type (WT) littermate controls. Animals were housed individually in cages equipped with running wheels. After continuous monitoring of wheel-running activities for 2-3 weeks under a 12h Light: 12h Dark (LD) schedule, mice were switched to constant darkness (DD) for 4 weeks. Under LD conditions, both WT and V1a−/− mice entrained to LD cycles and showed no significant difference in daily counts or amplitude of locomotor rhythmicity shown by Fast Fourier Transformation (FFT) power (Fig. 2A and Table1). However, the FFT power of V1a−/− mice under DD was significantly attenuated as compared to the WT controls (Fig. 2A and Table1). V1a−/− mice exhibited an expansion in duration of their daily activity bout (α). There was a significant genotypic difference on the duration of activity (α) after 25 days under DD (Table 1). As a result, four out of the 16 V1a−/− mice became arrhythmic after 4 weeks under DD. Moreover, the cycle-to-cycle variability in the onset of the daily activity bout was significantly increased in the V1a−/− mice. The V1a−/− mice showed about three-fold higher cycle-to-cycle variability than WT controls in the phase of activity onset (Table 1). However, there were no significant differences in the daily counts, free-running period or phase angle (Table 1).

Home-cage activity of the mice was also monitored by infrared motion sensors. Consistent
with the wheel-running activity, no significant differences were observed in LD conditions (Fig. 2B and Table 1). However, the daily activity periods expanded gradually when \( V1a^{-/-} \) mice were subjected to DD conditions, whereas WT mice still displayed consolidated activity patterns. At day 25 under DD, the activity periods of \( V1a^{-/-} \) mice were significantly longer than WT mice (Table 1). Some of the \( V1a^{-/-} \) mice (4/16) became arrhythmic gradually (FFT power <0.01) as the activity distributed uniformly during the 24 hour span (Fig. 2B). As a result, the mean FFT power of \( V1a^{-/-} \) mice under DD was only 30% of that for WT mice (Table 1). Interestingly, the daily activity of \( V1a^{-/-} \) mice under DD was also significantly lower than WT (Table 1), while the daily activity under LD did not show a marked genotypic difference.

It is known that the genetic background affects the circadian behavior of mice. To rule out this possible confounding effect, we backcrossed \( V1a^{+/+} \) mice with C57BL/6 mice for 4 sequential generations (N4). And N4 \( V1a^{+/+} \) mice were mated to obtain N4F1 \( V1a^{-/-} \) and their WT controls. These N4F1 \( V1a^{-/-} \) mice are predicted to have a ~94% C57BL/6 background. We measured the circadian locomotor phenotypes of N4F1 \( V1a^{-/-} \) mice. The defect of N4F1 \( V1a^{-/-} \) mice in circadian locomotor rhythm was quite similar to that obtained from the mixed C57/129 background. As shown in Table 1, N4F1 \( V1a^{-/-} \) mice were normal under entrained LD conditions, but displayed significantly damped circadian rhythms and a longer free-running period under DD. Two of 7 N4F1 \( V1a^{-/-} \) mice gradually became arrhythmic under DD. Taken together, these data unequivocally indicate that a deficiency in the V1a gene results in attenuation of circadian locomotor rhythms.
**Light masking and light-induced phase shift**

Since the *V1a*−/− mice behave normally under LD but not DD conditions, we next examined the direct influence of light on locomotor activity in these mice. When mice were maintained in an LD cycle, we found no differences in the ability of light [white light, 1h, zeitgeber time (ZT) 15] to acutely suppress (eg. masking) wheel-running activity during an animal's activity period (WT, 99.84 ± 0.07 %, *V1a*−/−, 99.97 ± 0.02%, *P* = 0.165, *n* = 6 mice/genotype).

We also compared the phase shifts generated by the exposure of a brief light pulse to WT and *V1a*−/− littermates at CT16 (white light, ~150 lux, 10 min). In this treatment, we did not see any significant differences between WT and *V1a*−/− mice (WT, 1.23 ± 0.44 h, *n* = 6; *V1a*−/−, 1.25 ± 0.21 h, *n* = 4, *P* = 0.97). However, this result should be interpreted with caution as *V1a*−/− mice showed greater cycle-to-cycle variability than WT mice under DD conditions (Table 1).

**Altered circadian rhythms of clock-controlled gene expression in the SCN**

To determine whether attenuated activity rhythms were caused by altered clock gene expression, we quantified gene expression in the SCN by *in situ* hybridization on brain sections of WT and *V1a*−/− mice. Samples were collected every 4 hr after 49 hr under DD conditions. As shown in Fig. 3, *Per1*, *Per2* and *Rev-erb-a* showed similar patterns in WT and *V1a*−/− mice. *Bmal1* expression was slightly shifted in *V1a*−/− mice and vasopressin peak levels were slightly attenuated in *V1a*−/− mice. However, these differences were not significant. Interestingly, the circadian amplitude of *PK2* mRNA was significantly attenuated in *V1a*−/− mice [*F* (1) = 12.72, *P* < 0.01, two-way ANOVA]. Particularly, the values of *PK2* mRNA at CT1 and CT5 were about half in *V1a*−/− mice as compared to WT controls (Fig. 3).
Vasopressin enhanced PK2 promoter activity through V1a in vitro

We have previously shown that activation of PKR2 by PK2 dose-dependently stimulated the CLOCK/BMAL1-mediated transcription of the 2.8-kb PK2 promoter (6). Since the signaling of PKR2 and V1a are similar (3, 20), the attenuated circadian amplitude of PK2 expression in the SCN of V1a-/- mice prompted us to investigate the effects of V1a receptor signaling on the activity of PK2 promoter. As shown in Fig. 4, vasopressin enhanced the luciferase activity in a dose-dependent manner when V1a and PK2.8-luc were co-transfected into HEK293 cells [F = 31.68, P < 0.0001, one-way ANOVA].

DISCUSSION

The role of AVP signaling in the regulation of circadian rhythms has been studied with a variety of approaches, including behavior models, electrophysiology, histology and pharmacology (4, 8, 10, 14-16, 18, 27, 33, 35, 42). However, the involvement of the AVP receptor subtypes in the regulation of circadian rhythms is still poorly understood. In this report, we examined the role of V1a receptor in the regulation of circadian rhythm. V1a receptor deficiency led to attenuation in circadian locomotor rhythms. Furthermore, our results revealed that V1a signaling is important in the maintenance of high amplitude of PK2 mRNA oscillation in the SCN, indicating that SCN output molecules also appear to function within the SCN to coordinate the expression of clock-controlled genes.
AVP-deficient Brattleboro rats serve as excellent models for studying the role of AVP in circadian rhythms. These rats display attenuated rhythms in a variety of circadian parameters, including body temperature, hormone synthesis, SCN neural firing and sleep-wakefulness (8, 14, 26, 27, 29, 33, 42). Recent studies from common voles further support an important role of AVP in circadian locomotor rhythm. Arrhythmic voles exhibited constantly high populations of AVP immunoreactive neurons in the SCN (16). Conversely, AVP release in cultured SCN neurons is attenuated in the arrhythmic voles (15). However, contradictory results also exist that AVP is not essential for circadian locomotor behaviors. For instance, central infusion of either AVP or V1 receptor antagonist failed to produce significant effects on the patterns of wheel-running activity (1, 36). In this report, we presented evidence that deficiency in $V1a$ receptor results in attenuated oscillation of $PK2$ mRNA in the SCN as well as locomotor rhythms in mice. The interrupted communication between the SCN and its vasopressingeric efferent targets through V1a receptor may contribute to the damped locomotor rhythms in $V1a^{-/-}$ mice. However, as PK2 has been proposed to be an inhibitory factor for locomotor activity in rats (6), the reduced and compacted oscillation of $PK2$ expression in the SCN of $V1a^{-/-}$ mice may contribute mainly to the expansion of the activity period and attenuation in circadian rhythms.

It is estimated that nearly one third of SCN neurons synthesize AVP and a high proportion of the neurons (> 40%) can be excited by AVP through the V1 receptors, suggesting that AVP may act as a feedback regulator within the nucleus (13, 14). Indeed, AVP-deficient Brattleboro rats showed a dampened amplitude of firing in the SCN during the subjective day when AVP levels are high (14). The effect of AVP on the SCN gene expression is reported, yet not pursued in detail, as
AVP peak levels are attenuated (20%–34%) in Brattleboro rats (40, 44). Here, we show that AVP, via the V1a receptor, positively regulates the expression of a clock-controlled output gene, PK2. 

V1a−/− mice displayed reduced PK2 mRNA levels during the subjective day, when AVP levels are high. Conversely, AVP, acting through the V1a receptor, is able to enhance the PK2 promoter activity in an in vitro luciferase assay. Interestingly, the peak value of AVP also showed a trend of reduction, yet not significant, in V1a−/− mice. These data suggest that AVP may specifically enhance the output signals in the SCN. The exact molecular mechanism how AVP signaling may regulate the oscillatory amplitude of PK2 mRNA is still unclear. It is possible that AVP released locally within SCN or from the collateral output projections may regulate the expression of certain clock-related genes, such as PK2. Alternatively, it is also possible that the damped PK2 rhythm in the SCN of V1a−/− mice is due to the reduced electrophysiological activity of SCN neurons. AVP is known to positively regulate the firing activity of SCN neurons (21, 24). Recently, tetrodotoxin, an Na+-channel blocker, has been shown to suppress the oscillation of PK2 in cultured SCN without affecting the oscillation of all clock core genes (2). The absence of AVP-V1a signaling may thus lead to reduced neuronal activity in the SCN that eventually results in attenuated peak PK2 expression levels in the SCN. Nevertheless, we cannot exclude the possibility that the attenuated PK2 expression amplitude in the SCN is caused by the absence of AVP-V1a signaling in some brain areas afferent to the SCN.

**Perspective and Significance:**

In mammals, almost all physiological parameters display circadian rhythms, which is under the control of the master clock in the SCN. The SCN is enriched with a variety of
neuropeptides. Several secreted factors encoded by clock-controlled genes (PK2, AVP and
cardiotrophin-like cytokine) have been implicated as output molecules that transmit circadian
information from the SCN to its target areas. We report here that disruption of AVP-V1a signaling
results in damped circadian rhythms in locomotor activity. We also demonstrate that AVP as an
output signal may also function within the SCN regulating certain clock gene expression.
Importantly, our data reveals an interaction between two putative output molecules, AVP and PK2.
Nevertheless, animals with disruption in single output pathway still display residual circadian
rhythms, suggesting these output factors may play some redundant or synergistic functions in
eliciting the overall circadian rhythms. It will be of interest to decipher the interactions between
these output pathways by analyzing double or triple knockout mice.

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20. **Lin DC, Bullock CM, Ehlert FJ, Chen JL, Tian H, and Zhou QY.** Identification and


Figure Legends:

Figure 1. V1a mRNA level in the SCN showed a circadian rhythm. (A) Quantitative analysis of V1a mRNA signals in the SCN of wild-type and V1a−/− mice sampled every 4 h beginning at 49 h under constant darkness (n = 3 mice; mean ± SEM). *, P < 0.05; **, P < 0.01; post-hoc Dunnett’s t test. (B) Representative in situ hybridization images of V1a mRNA signals from WT or V1a−/− mice sacrificed at circadian time (CT) 17. (C) The circadian rhythm of V1a mRNA in the SCN was attenuated in Bmal1−/− mice (n = 3 mice/genotype/time point). *, P < 0.05; post-hoc Bonferroni t test.

Figure 2. The wheel-running (A) and home-cage activity (B) of wild-type and V1a−/− mice. Forty-eight hour double plots are shown, in which the recordings of each day are replicated and appear under those of the previous day. Animals were initially housed in LD and then transferred to DD. The shift from LD to DD was indicated. The timing of the LD cycle is indicated by the bar above the record; dark bars, lights off; open bars, lights on. Note that the home-cage activity of some V1a−/− mice gradually became arrhythmic after an extended DD time period (B, right panel).

Figure 3. Analysis of clock genes in the SCN. The V1a−/− mice (open circles) and wild-type controls (closed circles) were sacrificed every 4 hr beginning at 49 hr under constant darkness. In situ hybridization on coronal brain sections was performed with corresponding 35S-labeled probes. The expression of PK2 in the SCN displayed significant genotypic differences [F (1) = 12.72, P < 0.01]. Each data represents mean ± SEM of 3 mice. *, P < 0.05; **, P < 0.01; post-hoc Bonferroni
Figure 4. AVP, acting through V1a receptor, enhanced *PK2* promoter activity *in vitro*. PK2.8-Luc and V1a-expressing plasmids were transfected into HEK293 cells. Cells were treated with various concentrations of AVP and incubated for another 24 hours. Luciferase activity of PK2.8-Luc in the absence of AVP treatment was designated as 1. Each value is the mean ± SEM of three replicates from a single assay. The results shown were representative of at least three independent experiments. *, \( P < 0.05 \); **, \( P < 0.01 \); *post-hoc* Dunnett’s *t* test.
Table 1. Summary of locomotor activity obtained from wild-type and \( V{1a}^{-} \) mice

<table>
<thead>
<tr>
<th>Behavioral measurements</th>
<th>50% C57BL/6 × 50% 129/Sv</th>
<th>94% C57BL/6 ×6% 129/Sv</th>
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<tbody>
<tr>
<td></td>
<td>WT wheel ((n = 16))</td>
<td>( V{1a}^{-} ) wheel ((n = 16))</td>
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<tr>
<td>Light-Dark (LD)</td>
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<td>FFT circadian amplitude</td>
<td>0.22 ± 0.02</td>
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<td>Total activity per 24 h</td>
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<td>17662 ± 2707</td>
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<td>Constant Darkness (DD)</td>
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<tr>
<td>FFT circadian amplitude</td>
<td>0.14 ± 0.02</td>
<td>0.07 ± 0.02 ( * )</td>
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<tr>
<td>Total activity per 24 h</td>
<td>20483 ± 3620</td>
<td>19442 ± 2366</td>
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<td>Free-running period (h)</td>
<td>23.62 ± 0.16</td>
<td>23.51 ± 0.20</td>
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<td>( \alpha ) (h)</td>
<td>18.5 ± 3.1</td>
<td>48.70 ± 11.05 ( * )</td>
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<tr>
<td>Phase angle (min)</td>
<td>29.90 ± 15.72</td>
<td>28.14. ± 20.28</td>
</tr>
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Data are presented as mean ± SEM. \( *, P < 0.05; **, P <0.001, \) Student’s \( t \) test. ND, not determined.