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# The first-line cluster headache medication verapamil alters the circadian period and elicits sex-specific sleep changes in mice

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## ABSTRACT

Verapamil is the first-line preventive medication for cluster headache, an excruciating disorder with strong circadian features. Whereas second- and third-line preventives include known circadian modulators, such as melatonin, corticosteroids, and lithium, the circadian effects of verapamil are poorly understood. Here, we characterize the circadian features of verapamil using both in vitro and in vivo models. In *Per2::LucSV* reporter fibroblasts, treatment with verapamil (0.03–10 μM) showed a dose-dependent period shortening of the reporter rhythm which reached a nadir at 1 μM, and altered core clock gene expression at 10 μM. Mouse wheel-running activity with verapamil (1 mg/mL added to the drinking water) also resulted in significant period shortening and activity reduction in both male and female free-running wild-type C57BL6/J mice. The temporal patterns of activity reduction, however, differ between the two sexes. Importantly, piezo sleep recording revealed sexual dimorphism in the effects of verapamil on sleep timing and bout duration, with more pronounced adverse effects in female mice. We also found altered circadian clock gene expression in the cerebellum, hypothalamus, and trigeminal ganglion of verapamil-treated mice. Verapamil did not affect reporter rhythms in ex vivo suprachiasmatic nucleus (SCN) slices from *Per2:Luc* reporter mice, perhaps due to the exceptionally tight coupling in the SCN. Thus, verapamil affects both peripheral (trigeminal ganglion) and central (hypothalamus and cerebellum) nervous system structures involved in cluster headache pathophysiology, possibly with network effects instead of isolated SCN effects. These studies suggest that verapamil is a circadian modulator in laboratory models at both molecular and behavioral levels, and sex is an important biological variable for cluster headache medications. These observations highlight the circadian system as a potential convergent target for cluster headache medications with different primary mechanisms of action.

## Introduction

Cluster headache (CH) is a headache disorder widely regarded as one of the most painful human experiences (Burish et al. 2020) with a high rate of suicidal thoughts and attempts (Ji Lee et al. 2019; Rozen and Fishman 2012; Trejo-Gabriel-Galan et al. 2018). The diagnosis of cluster headache is made by defining criteria: 1–8 headache attacks per day of one side of the face, with each attack lasting between 15 min and 3 h, associated with restlessness and/or cranial autonomic features, such as a bloodshot eye and nasal congestion on the same side as the pain (Headache Classification Committee of the International Headache Society (IHS) 2018).

In addition to these defining criteria, CH has remarkable circadian features. A total of 82% of the patients have headaches at the same time each day (Rozen and Fishman 2012); while there is inter-individual variability, the most common attack in day-active persons time is 02:00 h, regardless of time zone (Barloese et al. 2015; Rozen and Fishman 2012; Steinberg et al. 2018). Anatomical imaging studies in CH have shown enlargement of the anterior hypothalamus, the location of the central pacemaker, the suprachiasmatic nuclei (Arkink et al. 2017). Physiology studies have shown alterations in two circadian-related hormones, melatonin and corticosteroids, in CH patients (Bruera et al. 2008; Chazot et al. 1984; Leone et al. 1998; Waldenlind et al. 1987). Of note, melatonin and corticosteroids are also effective treatments for CH (May et al. 2006).

Molecularly, the circadian system consists of cell-autonomous molecular oscillators which drive a cycle of activation and inhibition of gene expression lasting approximately 24 h. The oscillators can be modulated by

external stimuli, including light (Albrecht 2012; Hughes et al. 2015; LeGates et al. 2014), food (Edmonds and Adler 1977; Lewis et al. 2020), and medications (Chen et al. 2018; Tamai et al. 2018). These core oscillators are present in most cell types in the body and are regulated by the suprachiasmatic nucleus (SCN) in the anterior hypothalamus. Lymphoblasts from CH patients show reduced expression of the circadian gene *Nr1d1* (encoding the nuclear receptor REV-ERBα) compared to control subjects (Costa et al. 2015).

Unfortunately, current CH treatments, especially preventive medications, are not particularly effective for many patients: based on patient reports, the most effective preventive treatments are corticosteroids and verapamil, but they are highly effective in only about 50% of the patients (Lademann et al. 2015). Due in part to serious side effects of corticosteroids with long-term use, verapamil is considered the first-line preventive medication (May et al. 2018; McGeeney 2018). Verapamil is a canonical L-type calcium channel antagonist, though it also affects other calcium channels, sodium channels, potassium channels, and *p*-glycoprotein (Lemma et al. 2006; Tfelt-Hansen and Tfelt-Hansen 2009). Although the transport of verapamil across the blood–brain barrier is limited by *p*-glycoprotein (Luurtsema et al. 2005; Petersen et al. 2019; Römermann et al. 2013), verapamil has known binding targets in the SCN (Nahm et al. 2005). Verapamil's mechanism of action in CH is not fully understood (Petersen et al. 2019): many second- and third-line CH preventives do not have strong calcium channel inhibition, including melatonin, corticosteroids, lithium, and valproate (Burish et al. 2019). However, many second- and third-line CH medications, including all those listed here, have putative effects on the molecular circadian system (Dickmeis et al. 2013; Johansson et al. 2011; Kandalepas et al. 2016; Li et al. 2012; Meneses-Santos and Buonfiglio 2018; Yin et al. 2006). The molecular circadian effects of verapamil are not known, but clinical observations have noted that CH patients taking verapamil have headaches approximately 1 h later than CH patients not taking verapamil (Barloese et al. 2018).

Given the lack of a clear common mechanism for preventive medications in CH and the multiple lines of evidence for circadian effects of CH medications and CH, itself, we sought to investigate the effects of the first-line medication verapamil on molecular and behavioral circadian rhythms. To address this, we analyzed the effects of verapamil on circadian reporter cells (*Per2::LucSV* mouse fibroblasts) and mouse wheel-running and sleep behaviors. We show that verapamil has circadian effects both *in vitro* and *in vivo*, shortening the period of PER2 protein oscillation in cells, shortening the period of mice on running wheels, and altering the expression of core circadian genes in reporter cells as well as the hypothalamus, cerebellum, and trigeminal ganglion. Furthermore, verapamil displays sexual dimorphic effects on sleep timing (daytime vs. nighttime) and sleep bout length. These studies demonstrate a clear circadian effect of verapamil on core oscillators and behavioral rhythms.

## Materials and methods

### Animals

Animal husbandry and experiments were carried out under international ethical standards (Portaluppi et al. 2010), ARRIVE guidelines (Percie Du Sert et al. 2020), and local IACUC guidelines in an animal protocol approved by the University of Texas Health Science Center at Houston (UTHSC-H). A total of 129 mice were used in this study.

### Cell culture studies

Adult mouse ear fibroblast cells isolated from *Per2::LucSV* knock-in mice by replacement of the 3′-UTR with an SV40 late poly(A) sequence (Yoo et al. 2017) were used for real-time bioluminescence monitoring. Cells were grown to confluency on 35 mm plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Cells were synchronized with 200 nM dexamethasone (Sigma–Aldrich) for 1 h, and verapamil (Sigma-Aldrich) was then added at concentrations of 0 (nothing added), 0.03, 0.1, 0.3, 1, 3, and 10 μM, along with luciferin-containing recording media (Yoo et al. 2004). The plates were then tightly sealed with vacuum grease and placed in a luminometer (LumiCycle 32, Actimetrics) for continuous bioluminescence monitoring over 6d. The data were detrended using a first-order polynomial, and then best-fit to a sine wave estimated by a Levenberg–Marquardt algorithm for measurement of circadian parameters in the LumiCycle data analysis program (Actimetrics). For real-time qPCR analysis of core clock genes, cells were synchronized with 200 nM dexamethasone (Sigma–Aldrich). Verapamil (Sigma-Aldrich) was then added at concentrations of 3 and 10 μM in recording media. Cells were harvested every 4 h for 28 h (8 time points) and total RNA were extracted.

### RNA extraction and real-time RT-PCR analysis

RNA extraction and real-time RT-PCR analysis were carried out as previously described (Nohara et al.

2020). Expression levels of 10 genes were analyzed using real-time RT-PCR. Total RNA from *Per2::LucSV* mouse fibroblasts, cerebellum, hypothalamus, and trigeminal ganglion was isolated using PureXtract RNAsol reagent (GenDEPOT, TX, USA) as indicated by the manufacturer's protocol. Reverse transcription was performed by cDNA synthesis kit (GenDEPOT, TX, USA). All real-time RT-PCR reactions were performed with SYBR Green PCR Master Mix kits (GenDEPOT, TX, USA) on QuantStudio 7 Flex system (Applied Biosystems). Data were analyzed using Prism 8 software (GraphPad Software, Inc.). *Gapdh* and *beta-actin* were used as the housekeeping gene for controls. The primer sets used are shown in Table 1.

### Mouse wheel-running behavioral study

C57BL6/J mice (Jackson Laboratory) at 9 weeks of age were transferred into individual cages equipped with running wheels. Mice were acclimated for 2 weeks in a 12 h light/12 h dark cycle (LD, light levels 300 lux, room temperature and relative humidity were maintained at 22.6–24.1°C and 38–42%, respectively), followed by 2 weeks in constant darkness (DD) to measure baseline free-running periods in DD. Once a circadian period was established in DD (the free-running period), we measured the effects of verapamil on the free-running period by changing regular water to verapamil (1 mg/mL)-containing water and monitoring mouse activities for an additional 2 weeks. Water intake was measured once per week for measurements of verapamil intake in the first 29 mice. Activity data were recorded continuously by a PC system (Chronobiology Kit, Stanford Software Systems) and analyzed using CLOCKLAB software (Actimetrics). Free-running period was calculated using a periodogram with 6 min resolution (CLOCKLAB). Wheel-running activity level was quantified as a summation every 20 min for each mouse and averaged during the 14d data collection.

### Piezo sleep recording

Sleep/wake recording was performed with a noninvasive piezoelectric transducer sleep/wake recording system (Signal Solutions, Inc.) as previously described (Nohara et al. 2019). The initial 48 h acclimation period was followed by data recording for 2d. Data were extracted and analyzed by using the Sleepstats software (Signal solutions, Inc).

### Real-time bioluminescence measurement from SCN ex vivo cultures

Circadian bioluminescence measurement was performed as previously described (Yoo et al. 2004). Briefly, SCNs were dissected and sliced with oscillating tissue slicer (OTS-5000) into 300 um thickness. SCN slices were cultured on Millicell culture membranes (PICMORG50, Millipore) in 35 mm tissue culture dishes containing 2 mL DMEM media (Invitrogen) supplemented with 352.5 μg/ml sodium bicarbonate, 10 mM HEPES (Invitrogen), 2 mM L-Glutamine, 2% B-27 Serum-free supplement (Invitrogen), 25 units/ml penicillin, 25 μg/ml streptomycin (Invitrogen), and 0.1 mM luciferin potassium salt (L-8240, Biosynth AG). DMSO or verapamil (10 uM) was added to the recording media. Bioluminescence was recorded continuously using the LumiCycle luminometer (Actimetrics). Data were analyzed using LumiCycle data analysis program (Actimetrics).

### Statistical analysis

Sample size was based on previous studies (Duong et al. 2011; Nohara et al. 2019). No data were excluded, and no randomization or blinding was performed. Strategies were put in place to minimize confounders, included isolating animals in individual cages and placing all animals in the same cabinet of recording boxes. Data are presented as mean ± standard deviation (cell culture data) or mean ± standard error of the mean (wheel-

**Table 1.** qPCR primer sequences.

| Gene | Forward (5′-3′) | Reverse (5′-3′) |
|------|-----------------|------------------|
| *Clock* | CCTTCAGCAGTCAGTCCATAAAC | AGACATCGCTGGCTGTGTTAA |
| *Bmal1* | CCACCTCAGAGCCATTGATACA | GAGCAGGTTTAGTTCCACTTTGTCT |
| *Per1* | CCCAGCTTTACCTGCAGAAG | ATGGTCGAA AGG AAGCCTCT |
| *Per2* | TGTGCGATGATGATTCGTGA | GGTGAAGGTACGTTTGGTTTGC |
| *Per3* | GTGATTGTTCACGCGTCT GT | CACTGCCATCTCGAGTTCAA |
| *Cry1* | TGA GGC AAG CAG ACT GAA TAT TG | CCT CTG TAC CGG GAA AGC TG |
| *Cry2* | CTG GCG AGA AGG TAG AGT GG | GACGCAGAATTA GCCTTTGC |
| *Rev-erba* | CATGGTGCTACTGTGTAAGGTGTGT | CACAGGCGTGCACTCCATAG |
| *Rev-erbβ* | TGAACGCAGGAGGTGTGATTG | GAGGACTGGAAGCTATTCTCAGA |
| *Dbp* | CTGGCCCGAGTCTTTTTGC | CCAGGTCCACGTATTCCACG |
| *Gapdh* | CAAGGTCATCCATGACAACTTTG | GGCCATCCACAGTCTTCTGG |
| *beta-actin* | TTGTCCCCCCAACTTGATG | CCTGGCTGCCTCAACACCT |

running and sleep data). Statistical significance was determined by two-tailed Student's t-test (Excel, Microsoft Office Professional Plus version 2016) and two-way ANOVA with Sidak's multiple comparison test (GraphPad Prism version 8.20). $P < .05$ was considered statistically significant.

## Results

### Verapamil shortens the reporter period of Per2:: LucSV mouse fibroblasts

For cell culture dose determinations, the dose was based on the effective oral dose of verapamil in CH patients of 200–960 mg/d (Blau and Engel 2004; A May et al. 2006; Petersen et al. 2019; Tfelt-Hansen and Tfelt-Hansen 2009). We used data from the following studies to help determine our dose range. A single oral dose of verapamil 80 mg in humans results in peak serum plasma levels of 38.4 ng/mL (McAllister and Kirsten 1982) or 0.08 µM, while a single oral dose of verapamil 160 mg results in a peak plasma level of 90.2 ng/mL (McAllister and Kirsten 1982) or 0.20 µM. Verapamil extended release 240 mg results in a peak serum plasma level between 80 and 164 ng/mL (fda.gov 2019) or 0.19–0.36 µM with a nonlinear correlation between dose and plasma level. A chronic 480 mg total daily dose of verapamil (120 mg four times daily) results in plasma levels between 125 and 400 ng/mL (fda.gov 2019) or 0.27–0.88 µM. Taken together, we chose doses between 0.03 and 10 µM.

To investigate the effect of the first-line CH medication verapamil on molecular circadian rhythms, we used *Per2::LucSV* fibroblast cells, which express PER2::LUC fusion proteins from the endogenous *Per2* gene promoter to report circadian molecular rhythms with high sensitivity (Chen et al. 2012; Yoo et al. 2017). We performed whole-field real-time bioluminescence recordings of *Per2::LucSV* fibroblast cell cultures. Verapamil significantly shortened the period at most concentrations between 0.03 and 10 µM (Figure 1a,b). There was a dose-dependent period shortening from 0.03 to 1 µM, and the effect reached a nadir of 23.4 h at 1 µM (the period was 24.0 h in controls). Verapamil did not significantly change the reporter amplitude of *Per2::LucSV* fibroblast cell cultures (Figure 1c). These results provide evidence that verapamil specifically alters circadian periodicity at the oscillator level, without changing its robustness.

To investigate the molecular basis of verapamil's effects on the circadian oscillator, we measured clock gene expression in *Per2::LucSV* fibroblast cells. At 10 µM verapamil concentration, we observed significant effects of verapamil on mRNA expression of *Clock, Bmal1,*

*Per1, Per3, Cry2,* and *Dbp* (Figure 2). There was no significant change in *Per2, Cry1, Rev-erbα,* and *Rev-erbβ*. At a lower verapamil concentration of 3 µM, only *Clock* displayed altered mRNA expression, while all other genes were not significantly different from control (Supplemental Figure 1).

### Verapamil shortens the period of free-running C57BL6/J mice

We next studied the effects of verapamil in an animal behavioral model. For mouse behavioral assay dose determinations, the dose of 1 mg/mL verapamil was chosen based on the conversion from human to mouse dosing from the FDA's "Guidance for Industry for Estimating Safe Starting Doses for Clinical Trials" (DHHS et al. 2005). The initial conversion of dosage (mg/kg) to drinking water (mg/mL) assumed a daily water intake of 4 mL and weight of approximately 25 g/20 g (male/female) in 12-week C57BL6/J mice. This dose corresponds to 768 mg (male) and 960 mg (female) daily doses in a 60 kg human. The dose of 1 mg/ mL verapamil in the drinking water has also been used in prior mouse studies (Abais et al. 2014; Chandra et al. 2002; Dong et al. 1992; Morris et al. 1989).

C57BL6/J wild-type mice were individually housed in cages with running wheels. To establish baseline circadian rhythms, mice were given food and water ad libitum for 2 weeks in a 12 h light/12 h dark cycle (LD), followed for up to 4 weeks in constant darkness (DD). Once a circadian period was established in DD (the free-running period), we measured the effects of verapamil on the free-running period by changing regular water to 1 mg/mL verapamil-containing water. The mice continued in DD and had access to food and verapamil water *ad libitum* for an additional 2 weeks. Water intake averages (across the first 29 mice) were 7.4 ml (LD), 7.9 ml (DD), and 6.5 ml (DD/Verapamil water) per day, which are within the normal range (Bachmanov et al. 2002). The free-running period was found to be significantly shortened after treatment with verapamil in both male (23.75 ± 0.03 h vs. 23.63 ± 0.04 h, $p < .0001$) and female mice (23.78 ± 0.01 h vs. 23.70 ± 0.02 h, $p < .001$) (Figure 3a,b for males and E and F for females). Likewise, verapamil significantly reduced activity levels in both sexes (males: 30,162 ± 1425 counts/20 min vs. 20,071 ± 1191 counts/ 20 min; $p < .0001$; females: 43,295 ± 1991 counts/20 min vs. 26,695 ± 1215 counts/20 min; $p < .0001$) (Figure 3c,d for males and G and H for females). In male mice, verapamil did not affect the activity onset (which occurred around Circadian Time 12), but it decreased the subsequent activity. Interestingly, however, activities
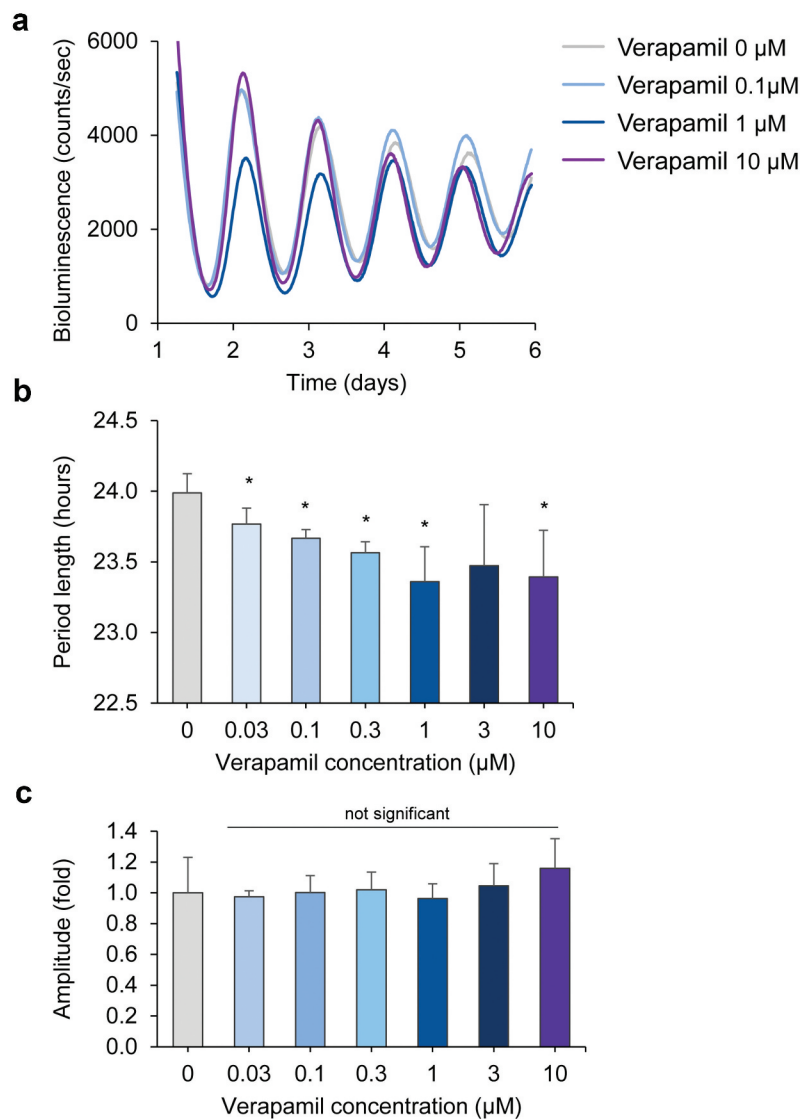
**Figure 1.** Verapamil shortens the circadian period of mouse *Per2::lucSV* reporter fibroblast cells. (a) Representative PER2::LUC bioluminescence recording of *Per2::LucSV* fibroblast cells. PER2::LUC fusion protein oscillations show a period shortening effect with verapamil compared to controls. (b) Average circadian period lengths for reporter fibroblast cells treated with increasing concentrations of verapamil (n = 4 for each concentration). Error bars represent standard deviation, * represents a significant ($p < .05$) change compared to control ("Verapamil 0"). (c) Verapamil does not cause amplitude changes in *Per2::LucSV* fibroblast cells. Average amplitude values for fibroblasts treated with increasing concentrations of verapamil (n = 4 for each concentration) are shown. Error bars represent standard deviation, $p < .05$ indicates significance.

of female mice were reduced throughout the active period to a greater extent, suggesting a sexual dimorphic effect on the temporal pattern of activity between males and females.

## Verapamil displays sex-specific effects on sleep

Next, we employed a piezo noninvasive sleep assay (Nohara et al. 2019) to investigate whether verapamil affects sleep. Male and female mice were pretreated with verapamil for two to three weeks prior to sleep measurements and compared to control mice receiving regular

water. Although the total amount of sleep was not altered by verapamil (Figure 4a,d), male and female mice exhibited distinct sleep changes in response to verapamil exposure (Figure 4b – dsss). Whereas verapamil-treated male mice showed significantly more daytime sleep (58.6 ± 1.2% vs. 63.0 ± 1.6%, $p < .05$), in female mice, verapamil increased sleep amount during the nighttime (22.1 ± 1.7% vs. 27.8 ± 1.2%, $p < .01$), the active phase for mice. Mean sleep bout duration time was significantly reduced by verapamil treatment only in female mice (Figure 4e). Furthermore, histogram analysis of sleep bout distribution revealed a trend toward
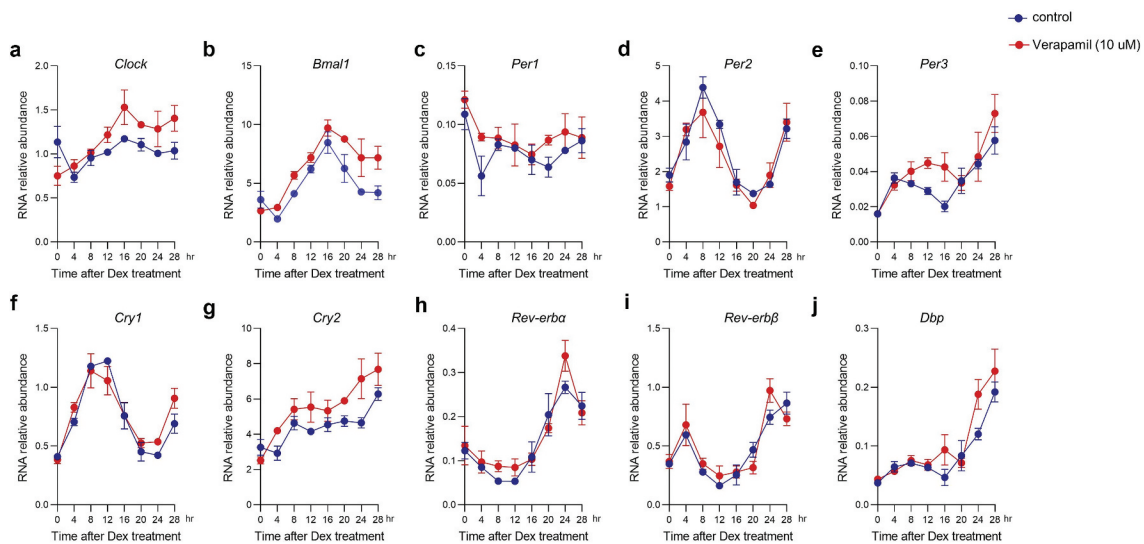
**Figure 2.** Real time qPCR analysis of clock gene expression. (A-J) Circadian expression of *Clock, Bmal1, Per1, Per2, Per3, Cry1, Cry2, Rev-erba, Rev-erbβ*, and *Dbp* in *Per2::lucSV* reporter cells quantified by qRT-PCR for control (blue) and verapamil 10 uM (red). Data are shown as mean ± SEM every 4 h for 28 h (n = 3). Two-way ANOVA with Sidak's multiple comparison test showed significant differences between control and *Clock* (p = .076), *Bmal1* (p = .0001), *Per1* (p = .0315), *Per3* (p = .0199), *Cry2* (p = .0004), *and Dbp* (p = .0345). The other clock genes examined were not significantly different from control.
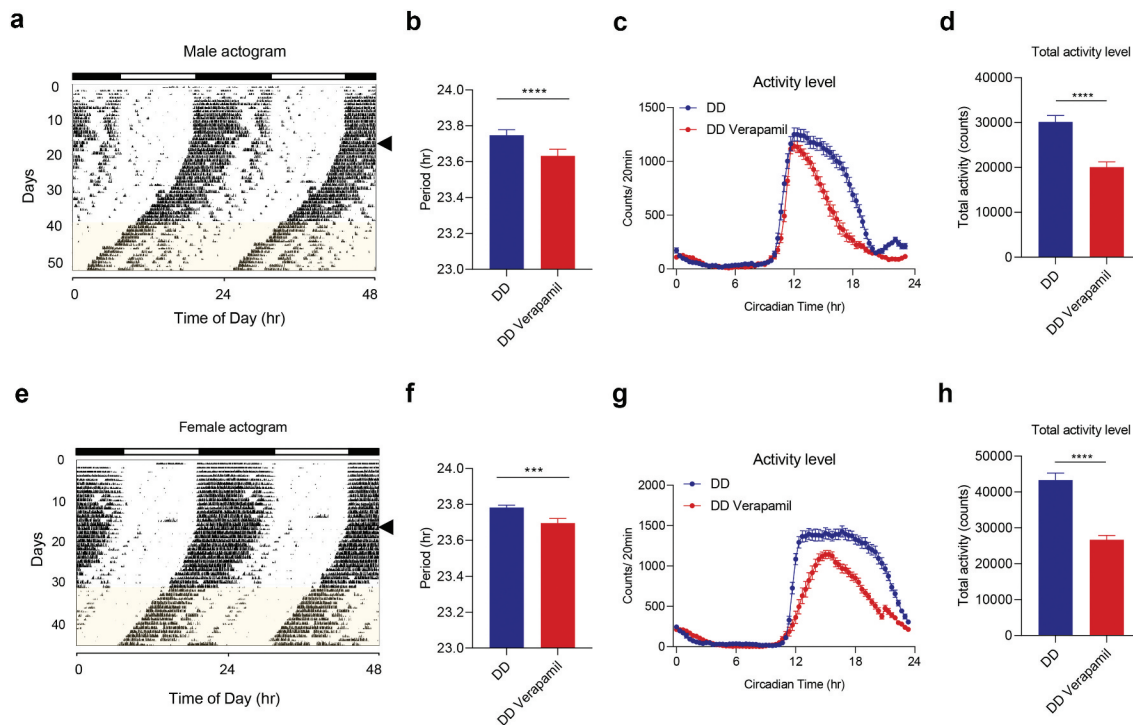


**Figure 3.** Verapamil treatment shortens circadian wheel-running periods in mice. (a–d) Male mice (n = 29). (e–h) Female mice (n = 30). (a and e) Representative actograms are shown for C56BL6/J male and female mice. Arrowheads indicate the LD (light:dark) to DD (dark: dark) transition. Water containing Verapamil (1 mg/ml) was administered during the interval indicated by yellow shading on the actogram. (b and f) Free-running period of C56BL6/J male and female mice under DD (constant darkness) for normal water. Error bars represent ± SEM. (c and g) Average wave plots summarizing wheel-running activity during DD for normal water, and verapamil water. (d and h) Daily total wheel-running activity during DD for normal water and verapamil water. Data are presented as mean ± SEM. T-test shows the significant statistical differences between normal water and verapamil water (***, p < .001; ****, p < .0001).
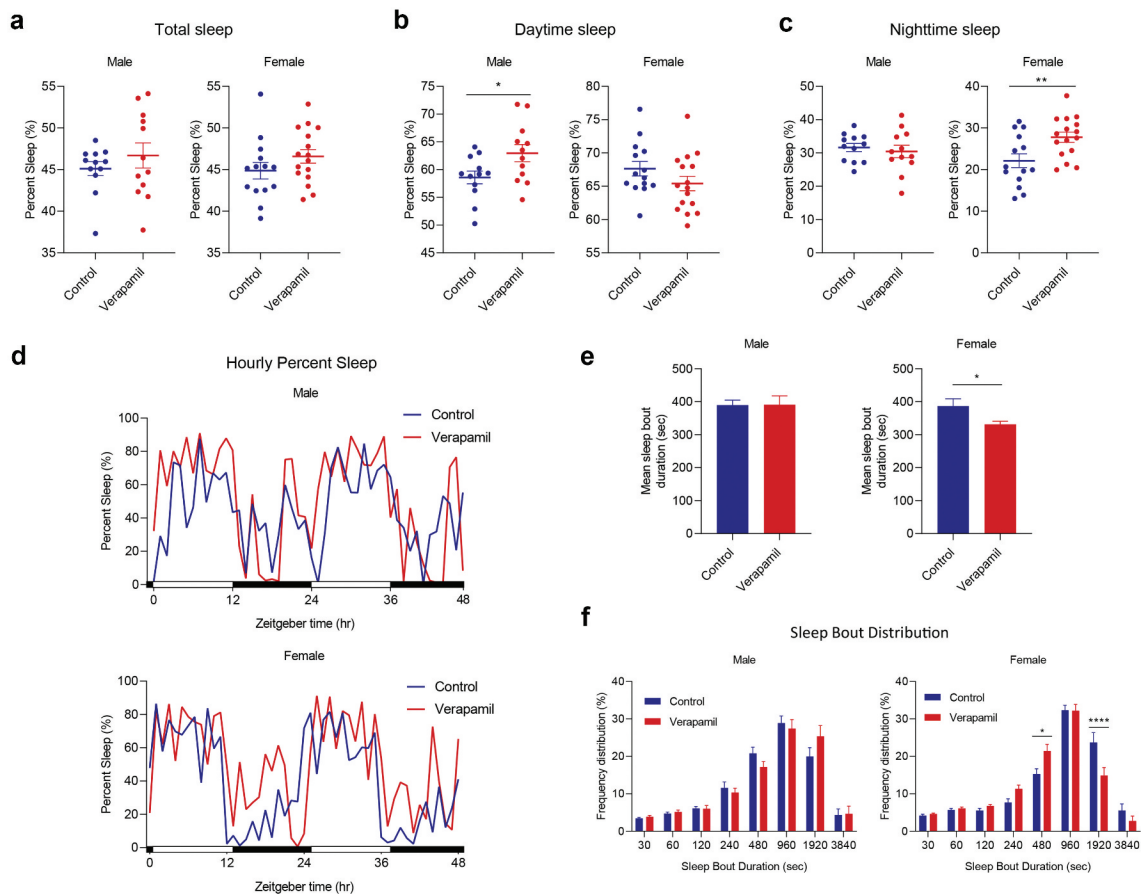
**Figure 4.** Verapamil shows sex-specific effects on sleep timing and quality in C57BL6/J mice. (a) Total percent sleep time of male and female mice with normal water (blue) and verapamil water (red). (b) Percent daytime sleep of male and female mice with normal water (blue; male n = 12, female n = 14) and verapamil water (red; male n = 12, female n = 16). (c) Percent nighttime sleep of male and female mice with normal water (blue) and verapamil water (red). T-test shows the significant statistical difference in percent sleep time between normal and verapamil water (*, $p < .05$; **, $p < .01$). (d) Representative sleep recording for hourly sleep percentage male and female mice with normal water (blue) and verapamil water (red). (e) Mean sleep bout duration for male and female mice with normal water (blue) and verapamil water (red). Data are presented as mean ± SEM. (f) Sleep bout length distribution of male and female mice with normal water (blue) and verapamil water (red). Data are presented as mean ± SEM. Two-way ANOVA with Sidak's multiple comparison test shows the significant statistical difference between percent sleep of normal and verapamil water (*, $p < .05$; ****, $p < .0001$).

shorter bouts in female mice, while male mice did not exhibit significant changes (Figure 4f). These observations indicate strong sexual dimorphic effects of verapamil on mouse sleep, with adverse effects in female mice, including increased nighttime sleep and shorter bout duration.

### *Verapamil alters clock gene expression in central and peripheral regions relevant for CH*

The hypothalamus and cerebellum are central nervous system structures known to have pronounced circadian oscillations and may be important structures in the pathophysiology of cluster headache (Arkink et al. 2017; Clelland et al. 2014; May et al. 1998; Naegel et al. 2014; Teepker et al. 2012; Yang et al. 2015). The trigeminal ganglion is an important peripheral nervous system structure for cluster headache pain (Jarrar et al. 2003; May et al. 2018; McGeeney 2018), though its circadian oscillations are not well studied. To examine whether verapamil treatment can affect the expression of clock genes in these areas, we measured mRNA expression levels of 10 core clock genes collected at ZT6 and ZT18 from verapamil-treated and control mice (Figure 5). Verapamil altered circadian expression of *Clock, Bmal1, Per1, Rev-erbα*, and *Dbp* at ZT6 significantly in the cerebellum from verapamil-treated mice compared to control mice. In the hypothalamus, verapamil altered expression of *Bmal1, Per1*, and *Cry2* at both time points, changed
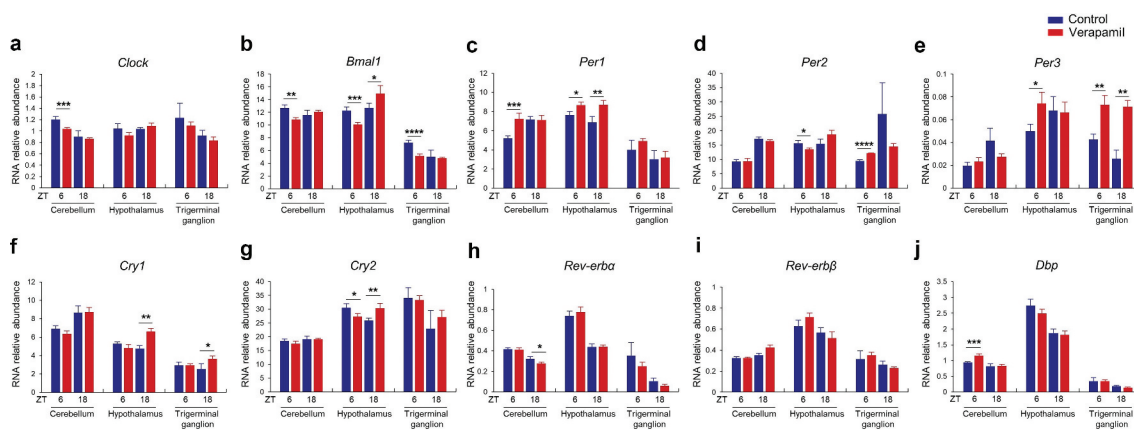
**Figure 5.** Verapamil alters circadian expression of clock genes. (a–j) Circadian expression of *Clock, Bmal1, Per1, Per2, Per3, Cry1, Cry2, Rev-erba, Rev-erbβ,* and *Dbp* in the cerebellum, hypothalamus, and trigeminal ganglion of control (blue bar, n = 4 for ZT 6, n = 3 for ZT18) and verapamil treated male mice (red bar, n = 4 for ZT6, n = 3 for ZT18). The data are shown as mean ± SEM (*, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$). Two-tailed unpaired t-test, $p < .05$ was considered significant.

expression of *Per2* and *Per3* at ZT6, and changed *Cry1* expression at ZT18. Furthermore, in the trigeminal ganglion, we found altered mRNA expression of *Per3* at both time points, altered mRNA expression of *Bmal1* and *Per2* at ZT6, and altered mRNA expression of *Cry1* at ZT18. Taken together, these results show that verapamil can alter circadian clock gene expression in brain regions and in the trigeminal ganglion. Interestingly, *Bmal1* mRNA expression was consistently downregulated by verapamil at ZT6 in all tissues as compared to control.

We next sought to understand the effect of verapamil on the central clock of the circadian system, using SCN ex vivo cultures from *Per2::Luc* mice. Compared with control (DMSO), SCN cultures treated with verapamil 10 µM did not show a significant difference in PER2:: LUC oscillation patterns (Supplemental Figure 2A). Accompanying analyses of circadian parameters revealed that the period length (Supplemental Figure 2B; Mean value, 24.6 ± 0.15 h for DMSO vs. 24.67 ± 0.38 h for 10 µM verapamil) and fold amplitudes (Supplemental Figure 2C; Mean value, 1.15 ± 0.2 for DMSO vs 1.09 ± 0.12 for 10 µM verapamil) of the bioluminescence oscillations were not significantly altered in SCN cultures treated with verapamil compared with control.

## Discussion

In this study, we show that verapamil, the first-line preventive medication for CH, has circadian effects at both the cellular and behavioral levels. Specifically, verapamil altered the core circadian oscillators by shortening the period in *Per2::LucSV* fibroblasts. This core circadian alteration also had a behavioral correlate, as

verapamil also shortened the free-running period of C57BL6/J wild-type mice. Importantly, verapamil displayed sex-specific effects, including differential wheel-running activities and sleep patterns, between male and female mice. The exaggerated nighttime sleep and reduced sleep bout lengths, indicative of dysregulated sleep timing and consolidation, suggest sleep perturbation by verapamil in female mice.

As the central pacemaker is believed to be primarily responsible for driving behavioral rhythms (Moore and Eichler 1972; Ralph et al. 1990; Stephan and Zucker 1972), our results suggest a central action of verapamil consistent with our current understanding of CH as a central nervous system disorder (May et al. 2018). The hypothalamus is considered a potential site for the initiation of a cluster headache attack (May et al. 2018; McGeeney 2018), and verapamil alters core circadian genes in the hypothalamus (specifically *Bmal1, Per1, Per2, Per3, Cry1, and Cry2* in our experiments). In SCN ex vivo slices, however, there was no change in PER2:: LUC reporter oscillations, perhaps due to the exceptionally tight coupling of the SCN clock known to be resistant to genetic and pharmacological manipulation (Chen et al. 2012; Liu et al. 2007). Of note, when verapamil was administered to mice systemically via drinking water, *Bmal1* expression was broadly altered by verapamil in all tissues tested. *Bmal1* was also altered by verapamil 10 uM in our experiments on fibroblasts cultures. These findings suggest general effects of verapamil on *Bmal1*, including structures relevant to cluster headache.

While our data suggest a period-shortening effect, a human observational study suggests that CH patients display a 1 h phase delay in the timing of attacks if they take verapamil (Barloese et al. 2018). Typically, phase delays in humans are associated with period lengthening

(not shortening) in rodent models, as is seen in delayed sleep phase disorder and mutations in CRY*1* (Patke et al. 2017). To our knowledge, no circadian free-running experiments in humans have been performed with verapamil to reconcile the human phase delay with our findings of period-shortening. With our current lack of information on the molecular circadian alterations of cluster headache patients, it is difficult to directly compare the findings of clinical and laboratory studies. Furthermore, there is currently no animal model of CH that includes a circadian timing of attacks (only animal models that explore the trigeminal pain system (Harriott et al. 2019) or the autonomic system (Akerman et al. 2009)). However, verapamil's circadian effects appear to extend to humans, and additional studies are warranted to investigate the specific circadian effect of verapamil in humans.

In humans, CH is more common in males at a ratio of 4.3:1 (Fischera et al. 2008). It has been suggested that verapamil is less effective in women, though no systematic studies have been performed (Petersen et al. 2019). In our study, we found that verapamil caused more pronounced disturbances in female sleep, specifically sleep timing (more sleep during the active phase) and quality (decreased sleep bout length). In addition, verapamil also altered the temporal pattern of wheel-running activity in a sex-specific manner, again with more significant changes in female mice. Sleep disturbances are a rare but occasionally documented side effect of verapamil in humans (PDR 2020), though sex-specific differences in sleep disturbances are not clear. Future studies are needed to investigate whether this sexual dimorphism is related to sex-dependent CH disease manifestations and treatment efficacy in humans.

In the treatment of CH, one issue limiting drug development is the lack of a common mechanism amongst preventive medications. Our study reveals an interesting circadian modulatory mechanism shared by verapamil, corticosteroids, melatonin, lithium, and valproate because of the prominent circadian features of this disease. Additional studies are needed to determine the importance of the circadian effects of these medications in CH and to investigate the specific shared mechanism: these medications might have a common circadian target or might have different targets that result in a convergent effect on the core circadian oscillators.

In conclusion, our work reveals verapamil as a clock-altering drug, shortening the circadian period at both the molecular and behavioral levels. Importantly, we observed significant sex-specific effects of verapamil on sleep and wheel-running behaviors, consistent with the notion that sex is an important biological variable for CH and its medications. Verapamil is the first-line preventive medication for CH, a disorder with strong circadian features, and several CH medications share a circadian-altering effect but are otherwise unrelated mechanistically. Additional studies are needed to understand whether these medications share an ability to modulate circadian rhythms, which will facilitate future chronotherapeutic developments against CH.

## Declaration of Conflicting Interests

The authors declare no conflicting interests.

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