High-Amplitude Circadian Rhythms in *Drosophila* Driven by Calcineurin-Mediated Post-translational Control of *sarah*

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ABSTRACT Post-translational control is a crucial mechanism for circadian timekeeping. Evolutionarily conserved kinases and phosphatases have been implicated in circadian phosphorylation and the degradation of clock-relevant proteins, which sustain high-amplitude rhythms with 24-hr periodicity in animal behaviors and physiology. Here, we report a novel clock function of the heterodimeric Ca^{2+}/calmodulin-dependent phosphatase calcineurin and its regulator *sarah* (*sra*) in *Drosophila*. Genomic deletion of the *sra* locus dampened circadian locomotor activity rhythms in free-running constant dark after entrainment in light–dark cycles. Poor rhythms in *sra* mutant behaviors were accompanied by lower expression of two oscillating clock proteins, PERIOD (PER) and TIMELESS (TIM), at the post-transcriptional level. RNA interference-mediated *sra* depletion in circadian pacemaker neurons was sufficient to phenocopy loss-of-function mutation in *sra*. On the other hand, a constitutively active form of the catalytic calcineurin subunit, *Pp2B-14D*^{ACT}, shortened circadian periodicity in locomotor behaviors and phase-advanced PER and TIM rhythms when overexpressed in clock neurons. Heterozygous *sra* deletion induced behavioral arrhythmicity in *Pp2B-14D*^{ACT} flies, whereas *sra* overexpression rescued short periods in these animals. Finally, pharmacological inhibition of calcineurin in either wild-type flies or clock-less S2 cells decreased the levels of PER and TIM, likely by facilitating their proteasomal degradation. Taken together, these data suggest that *sra* negatively regulates calcineurin by cell-autonomously titrating calcineurin-dependent stabilization of PER and TIM proteins, thereby sustaining high-amplitude behavioral rhythms in *Drosophila*.

KEYWORDS *Drosophila*; circadian rhythms; calcineurin; *sarah*; post-translational regulation

CIRCADIAN rhythms have evolved as endogenous clock mechanisms to adaptively align behavioral and physiological processes to daily environmental changes. Circadian clocks involve three genetic components: (1) central clocks that cell-autonomously generate 24-hr rhythmicity in molecular and/or neural forms, such as daily oscillations in relative expression of a dedicated set of clock-relevant genes; (2) an input pathway that receives timing cues from the environment and entrains the endogenous clocks; and (3) an output pathway that transmits timing information in the brain to peripheral tissues to synchronize their clock phase and execute circadian physiology (Allada and Chung 2010; Bass and Takahashi 2010; Dubowy and Sehgal 2017).

The core molecular clock consists of daily rhythms in the transcriptional activation of gene promoters containing E-box sequences (CACGTG) (Hardin 2011; Zheng and Sehgal 2012). In *Drosophila*, a heterodimeric CLOCK/CYCLE (CLK/CYC) activator binds the E-box and induces the transcriptional expression of its target genes, such as *period* (*per*) and *timeless* (*tim*), in the early evening. *per* and *tim* transcription reaches its highest levels at midnight. Meanwhile, the cytoplasmic PER–TIM protein complex accumulates and translocates into the nucleus late at night, suppressing its own transcription by inhibiting CLK/CYC-dependent transcriptional activation. Two additional feedback loops involving the circadian transcription factors *vrille*, *PAR-domain protein 1*, and

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clockwork orange further support high-amplitude circadian transcription with precise 24-hr periodicity (Cyran *et al.* 2003; Kadener *et al.* 2007; Lim *et al.* 2007a; Matsumoto *et al.* 2007; Richier *et al.* 2008). This molecular framework of transcriptional–translational feedback loops is highly conserved among *Drosophila* and mammalian clocks (Hardin and Panda 2013; Lim and Allada 2013; Takahashi 2017).

On top of transcriptional control, post-translational modifications of clock proteins play crucial roles in circadian timekeeping (Tataroglu and Emery 2015; Hirano *et al.* 2016). In the early morning, degradation of PER and TIM derepresses CLK/CYC-dependent transcription in *Drosophila*, initiating another cycle of daily rhythmic expression. Several kinases and phosphatases modulate the post-translational stability, nuclear entry, and transcriptional activity of PER proteins. The DOUBLETIME (DBT)/casein kinase I ε (CKIε) and NEMO kinases phosphorylate PER and control its degradation (Kloss *et al.* 1998; Price *et al.* 1998; Ko *et al.* 2002; Chiu *et al.* 2011). DBT-dependent phosphorylation of PER also potentiates its transcriptional repression (Nawathean and Rosbash 2004; Kivimäe *et al.* 2008), whereas PER phosphorylation by kinases such as casein kinase II and GSK3β/SHAGGY regulates its nuclear entry (Martinek *et al.* 2001; Lin *et al.* 2002; Akten *et al.* 2003; Ko *et al.* 2010). On the other hand, protein phosphatase 1 and protein phosphatase 2A stabilize PER via TIM-dependent and TIM-independent dephosphorylation of PER, respectively (Sathyanarayanan *et al.* 2004; Fang *et al.* 2007).

In a previous study (Lim *et al.* 2011), we performed a reverse genetic screen of ∼4000 transgenic fly lines to discover new clock genes that regulate circadian locomotor behavior. We identified *sarah* (*sra*), a *Drosophila* homolog of Down syndrome critical region gene 1 (DSCR1)/regulator of calcineurin 1 (RCAN1), as a candidate gene implicated in clock function. *sra* was previously identified as a regulator of the Ca^{2+}/Calmodulin-dependent serine/threonine phosphatase calcineurin (see below). Heterodimeric calcineurin consists of catalytic and regulatory subunits, which are evolutionarily conserved (Rusnak and Mertz 2000). In *Drosophila*, *sra*- and calcineurin-dependent regulation is involved in mitochondrial function (Chang and Min 2005); axonal transport (Shaw and Chang 2013); female reproductive activities such as ovulation, egg activation, and meiosis (Ejima *et al.* 2004; Horner *et al.* 2006; Takeo *et al.* 2010); olfactory associative learning and long-term memory (Chang *et al.* 2003); and courtship behaviors (Ejima *et al.* 2004; Sakai and Aigaki 2010). Moreover, both neuroprotective and neurotoxic effects of *sra* have been reported in *Drosophila* models of Alzheimer's disease (Shaw and Chang 2013; Lee *et al.* 2016).

To date, genetic studies on *Drosophila* sleep have revealed the strongest association between the calcineurin–*sra* pathway and circadian rhythms (Nakai *et al.* 2011; Tomita *et al.* 2011). Genomic deletion of *sra* or a specific subunit of calcineurin (*e.g.*, *CanA-14F* or *CanB*) causes hyperactivity and sleep loss. In addition, pan-neuronal expression of either constitutively active calcineurin subunits or transgenic calcineurin RNA interference (RNAi) modulates sleep. However, the mechanism underlying sleep regulation and its possible link to circadian clock function remains elusive.

In this study, we demonstrate that *Drosophila* clocks are sensitive to calcineurin activity in circadian pacemaker neurons, and that the balance of activity between calcineurin and *sra* drives high-amplitude behavioral rhythms. Furthermore, we provide molecular evidence that calcineurin-mediated suppression of the proteasomal degradation of PER and TIM proteins is involved in clock regulation.

Materials and Methods

Fly stocks

Flies were raised on cornmeal–yeast–agar medium at 25°C and 60% humidity in a 12 hr light/12 hr dark (LD) cycle. *CanA1*^{KO}, *CanA-14F*^{KO}, *Pp2B-14D*^{KO}, *sra*^{KO} (Takeo *et al.* 2006), UAS-*sra* (Ejima *et al.* 2004), and UAS-*Pp2B-14D*^{ACT} flies were generously provided by Toshiro Aigaki (Tokyo Metropolitan University, Japan). UAS-*sra*^{FLAG}, UAS-*sra*^{WT}, UAS-*Pp2B-14D*^{ACT}, UAS-*Pp2B-14D*^{WT}, and UAS-*Pp2B-14D*^{H217Q} flies were obtained from the Kyoto Stock Center (*Drosophila* Genomics and Genetics Resources). UAS-*sra*^{RNAi} [transformant identifier (ID): 107373] and *DH44*-Gal4 (transformant ID:207474) were obtained from the Vienna *Drosophila* RNAi Center. c305-Gal4, MB247-Gal4, 30y-Gal4, c309-Gal4, 201y-Gal4, OK107-Gal4, *Dilp2*-Gal4, UAS-mCD8:GFP, and *Df*(3R) SBD45 flies were obtained from the Bloomington *Drosophila* Stock Center (BDSC). *pdf*-Gal4, *tim*-Gal4, *cry*-Gal4-16, mz520-Gal4, c929-Gal4, mai179-Gal4, and *pdf*-Gal4^{GeneSwitch} lines were described previously (Kaneko and Hall 2000; Zhao *et al.* 2003; Grima *et al.* 2004; Depetris-Chauvin *et al.* 2011). To exclude possible genetic background issues in circadian behaviors, all mutants were isogenized by outcrossing five times to w^{1118} control flies (BDSC #5905). Stable mutant stocks were then reestablished by crossing to isogenic balancer lines.

Behavior analysis

Male flies (5–7 days old) were loaded individually into glass tubes (length, 6.5 cm; inner diameter, 3 mm) containing 2 cm of behavior food (5% sucrose and 2% agar) at one end, and a cotton stopper was placed on the other end. Locomotor activity was measured using a *Drosophila* activity monitor (Trikinetics), which counts the infrared beam crossings of individual flies in each tube every 30 min. Flies were entrained for 3 days in the LD cycle (under 400 lx of light) at 25°C, and then transferred to constant dark (DD) for 7 days at 25°C. To induce transgenic expression by *pdf*-Gal4^{GeneSwitch}, two groups of transgenic flies were directly loaded onto behavior food containing either 500 μM RU486 or 1% ethanol (vehicle control) at the beginning of the first LD cycle in our behavioral runs, and their free-running behaviors were monitored on each type of food. For oral administration of a calcineurin inhibitor, wild-type flies were loaded onto behavior food containing either 1 μM cyclosporin A (CsA; Sigma [Sigma Chemical],

St. Louis, MO) or 1 mM DMSO (vehicle control), and their circadian behaviors were measured as described above. Behavioral data were continuously recorded throughout behavioral runs, and then analyzed using the ClockLab software in MATLAB (Actimetrics). Circadian periods and the power of rhythmicity (power – significance) were calculated in individual flies using the χ^2 periodogram, with the significance level set to an α value of 0.05). Flies with a power of rhythmicity value < 10 were defined as arrhythmic. Locomotor activity profiles were analyzed using Microsoft Excel. Morning anticipation index was calculated in individual flies by the ratio of total activity count for the last 3 hr prior to lights-on (*i.e.*, from ZT21 to ZT0) to total activity count for the last 6 hr prior to lights-on (*i.e.*, from ZT18 to ZT0) in the LD cycle (Seluzicki *et al.* 2014). Morning anticipation index in the second DD cycle was similarly calculated based on the subjective lights-on time (*i.e.*, CT0). Behavioral data were computed as averages from three or more independent behavioral tests. Dead flies were manually scored at the end of behavioral testing, and their raw data were excluded from further analyses.

Western blotting

Male flies were entrained in LD cycles, and then transferred to DD. Groups of flies were harvested at the indicated time points during the LD cycle or the first DD cycle, and then stored at −70° prior to protein analyses. Thirty fly heads were homogenized in 120 μl of lysis buffer containing 25 mM Tris-Cl (pH 7.5), 300 mM NaCl, 10% glycerol, 1 mM EDTA, 1 μM dithiothreitol (DTT), 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (ThermoFisher Scientific) on ice. Homogenization was performed for 1 min for each fly head sample using a motorized pellet pestle (Kimble Kontes). After centrifugation, the supernatant was mixed with 1/6 vol of 6× SDS sample buffer and then incubated at 95°C for 10 min. Protein samples were resolved by SDS-PAGE analysis, and then transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). After blocking with 3% nonfat dry milk in Tris-buffered saline (TBS)-Tween 20 for 1 hr at room temperature, protein blots were incubated overnight with primary rabbit anti-PER (serum or purified, 1:3000), guinea pig anti-TIM (1:3000), mouse anti-FLAG (1:5000; Sigma), or rabbit anti-ACTIN antibody (1:5000; Sigma). Secondary antibodies used were HRP-linked anti-guinea pig IgG, anti-mouse IgG, or anti-rabbit IgG (Cell Signaling Technology). Chemiluminescence signals from immunoblots were generated using Pierce ECL Plus reagent (ThermoFisher Scientific) and visualized on a LAS3000 or LAS4000 mini (FUJI).

Quantitative transcript analysis

Flies were entrained in LD cycles and harvested as described above. Total RNA was purified from 40 fly heads using TRIzol (Invitrogen, Carlsbad, CA). After the removal of contaminating genomic DNAs by RQ I DNase digestion, 2 μg of total RNA was reverse-transcribed using M-MuLV reverse transcriptase with random hexamers (Promega, Madison, WI). Quantitative real-time PCR was performed using SYBR reaction mix (Enzynomics, Daejeon, Korea) and the following sets of gene-specific primers: *sra*, forward: 5′-TAC TGG TGC AGC TTG ATT CG-3′ and reverse: 5′-CCT ACC CAC CTC AAT CAT CG-3′; *per*, forward: 5′-CTC AAC AGG TAA CTT CAC CTG C-3′ and reverse: 5′-ACG GGA AGA AAA AGC GCG AGA A-3′; *tim*, forward: 5′-TGC TCC TCC TGG GGG CAG CAA-3′ and reverse: 5′-AGA GAG CTA GCG AAA GTA TTT AC-3′; glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) (as a control), forward: 5′-CTA CCT GTT CAA GTT CGA TTC GAC-3′ and reverse: 5′-AGT GGA CTC CAC GAT GTA TTC G-3′.

Immunofluorescence assay

Adult flies were fixed in phosphate-buffered saline (PBS) containing 4% formaldehyde for 2.5 hr, and subsequently whole brains were dissected out in PBS. Alternatively, adult brains were first dissected out in PBS, transferred to PBS containing 4% formaldehyde, and incubated for 30 min at room temperature. After permeabilization in PBS containing 0.3% Triton X-100 (PBS-T), brain samples were blocked in PBS-T containing 5% normal goat serum (The Jackson Laboratory) for 30 min at room temperature. After washing in PBS-T, brain samples were incubated with primary antibodies diluted in blocking solution at 4°C for 2 days. After repeated washes, samples were incubated for 1 day with secondary antibodies diluted in PBS-T, washed intensively, and then mounted using VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA). Immunofluorescence images of brain were acquired on a BX51 fluorescence microscope (Olympus) or a confocal laser scanning microscope (LSM780; Zeiss [Carl Zeiss], Thornwood, NY). Quantification of PER and TIM levels in individual clock neurons was performed as described previously (Lee *et al.* 2017). The primary antibodies used were mouse anti-PIGMENT-DISPERSING FACTOR (PDF) (Development Studies Hybridoma Bank), rabbit anti-PER, and guinea pig anti-TIM (Choi *et al.* 2009). The secondary antibodies used were Alexa Fluor 594-conjugated AffiniPure F(ab')2 fragment goat anti-guinea pig, anti-rabbit, and anti-mouse, and fluorescein isothiocyanate (FITC)-conjugated AffiniPure F(ab')2 fragment goat anti-rabbit, anti-guinea pig, and anti-mouse (Jackson ImmunoResearch Laboratories).

S2 cell culture and transient transfection

Drosophila S2 cells were maintained in Shields and Sang M3 insect medium (Sigma) supplemented with 10% fetal bovine serum (GIBCO [Grand Island Biological], Grand Island, NY) and 1% penicillin–streptomycin (Invitrogen). Expression vectors for V5-tagged PER, TIM, or CLK proteins (pAc-PER-V5, pAc-TIM-V5, and pAc-CLK-V5, respectively) (Lim *et al.* 2007b) were transiently transfected into S2 cells using Effectene transfection reagent (QIAGEN, Valencia, CA). Where indicated, 20 μM CsA (Sigma), 20 μM tacrolimus (Sigma), and/or 50 μM MG132 (Sigma) were added to the transfected cells and incubated for 2 hr before protein analyses.

Data availability

The authors state that all data necessary for confirming the conclusions of the article are represented fully within the article. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.6216251.

Results

sra mutants exhibit arrhythmic circadian behaviors

To investigate the possible role of *sra* in sustaining circadian rhythms, we examined circadian locomotor behaviors in individual sra^{KO} mutant flies. Control flies exhibited robust locomotor rhythms with bimodal activity peaks in LD cycles (*i.e.*, morning and evening peaks), and their rhythmic behaviors persisted in free-running DD cycles (Figure 1A). On the other hand, locomotor activity peaks in sra^{KO} homozygous mutants were less evident, and their locomotor rhythms dampened rapidly in DD (Figure 1A). Quantification of free-running circadian behaviors confirmed poor rhythmicity of sra^{KO} mutants in comparison with wild-type and heterozygous controls (Figure 1, B and C and Supplemental Material, Table S1), indicating the absence of a functional clock in sra^{KO} mutants. In addition, the circadian periods in $sra^{KO}/+$ heterozygous flies were modestly shortened (by ~0.6 hr) (Figure 1, D and E and Table S1).

Because sra^{KO} mutants have short baseline sleep (Nakai *et al.* 2011), hyperactivity might explain their loss of behavioral rhythmicity. However, previous studies have shown that short-sleep mutants can have intact circadian clocks (Liu *et al.* 2014; Shi *et al.* 2014), indicating that hyperactivity in such mutants is, in principle, separable from their circadian phenotypes. In fact, sra^{KO} mutants exhibited lower activity in DD, when their behavioral arrhythmicity was most evident (Figure 1F). Moreover, *trans*-heterozygosity of the sra^{KO} allele over a larger genomic deletion of the *sra* locus (*i.e.*, a deficiency chromosome) did not significantly affect locomotor activity in LD, whereas the *trans*-heterozygous mutants still exhibited arrhythmic behaviors in DD (Figure 1, A–C and Table S1). Nonetheless, the weaker circadian phenotypes in *sra trans*-heterozygotes suggest that the effects of *sra* on circadian behaviors may be modulated by genetic modifiers present in the sra^{KO} mutants or deficiency line.

Two groups of circadian pacemaker neurons, the small and large lateral ventral neurons (s-LN_Vs and l-LN_Vs), sustain behavioral rhythmicity by expressing a neuropeptide called PDF (Renn *et al.* 1999). Immunostaining of whole-mount fly brains with anti-PDF antibody revealed that the cell bodies and axonal projections in PDF-expressing clock neurons were similar between control and sra^{KO} mutants (Figure S1). Accordingly, abnormal development of PDF neurons is not likely to be responsible for the arrhythmic behaviors of sra^{KO} mutants. Together, these data suggest that *sra* is necessary for sustaining high-amplitude rhythms in free-running circadian behaviors.

sra mutation post-transcriptionally dampens circadian expression of PER and TIM

Given that *sra* acts as a regulator of Ca^{2+}-dependent protein phosphatase calcineurin, we hypothesized that *sra* might control the post-transcriptional expression of circadian clock genes to maintain rhythmic behaviors. Accordingly, we collected control and sra^{KO} flies every 4 hr during the first DD cycle after LD entrainment, and then quantified the relative expression of PER and TIM proteins in head extracts. This analysis revealed that circadian expression levels of both clock proteins were reduced in sra^{KO} flies compared to the wild type (Figure 2A). Relative quantification of *per* and *tim* mRNA levels in head extracts revealed no significant difference between control and sra^{KO} flies (Figure 2B), implying that the effects of *sra* on the rhythmic expression of clock proteins are likely to be post-transcriptional.

Circadian expression of clock genes in head extracts reflects the pace of the molecular clock in peripheral tissues, but it is not necessarily coupled to rhythmic behaviors in DD. In fact, modest effects of *sra* on the protein oscillations in head extracts do not convincingly explain stronger behavioral phenotypes in *sra* mutants. Therefore, we assessed circadian expression of PER and TIM proteins in behaviorally relevant PDF neurons by immunostaining adult fly brains. PDF-expressing s-LN_Vs play pivotal roles in multiple aspects of circadian behaviors, including morning anticipation and free-running rhythms in DD (Grima *et al.* 2004; Stoleru *et al.* 2004). Consistent with this, PER and TIM levels in PDF neurons were significantly lower in *sra* mutant flies than in controls at the peak time point in LD. The effects of *sra* on PER and TIM levels were less evident in dorsal lateral neurons (LN_ds), another group of circadian pacemaker neurons that express PER and TIM, but not PDF (Figure S2), although it is possible that lack of counterstaining of all LN_ds in our experimental conditions might have underestimated *sra* effects in these clock neurons. We reason that our protein analyses using polyclonal antibodies could not have revealed more subtle, qualitative defects in the molecular oscillations that are actually responsible for *sra* mutant behaviors. Nonetheless, these data support the idea that *sra*-dependent post-transcriptional control may contribute to the robust expression of circadian clock proteins, and likely, high-amplitude rhythms in locomotor behaviors.

sra depletion in circadian pacemaker neurons phenocopies *sra* mutation

A transgenic reporter harboring the *sra* promoter (*sra*-Gal4) (Lee *et al.* 2016) is broadly expressed in the adult fly brain (Figure S3), suggesting that *sra* functions in multiple brain regions including circadian clock neurons, the mushroom body, and the pars intercerebralis. To determine whether a specific neural locus is important for *sra* function in circadian behaviors, we constitutively overexpressed an RNAi transgene against *sra* (sra^{RNAi}) along with the RNAi-enhancing *Dicer2* (DCR2) transgene in different sets of brain neurons and

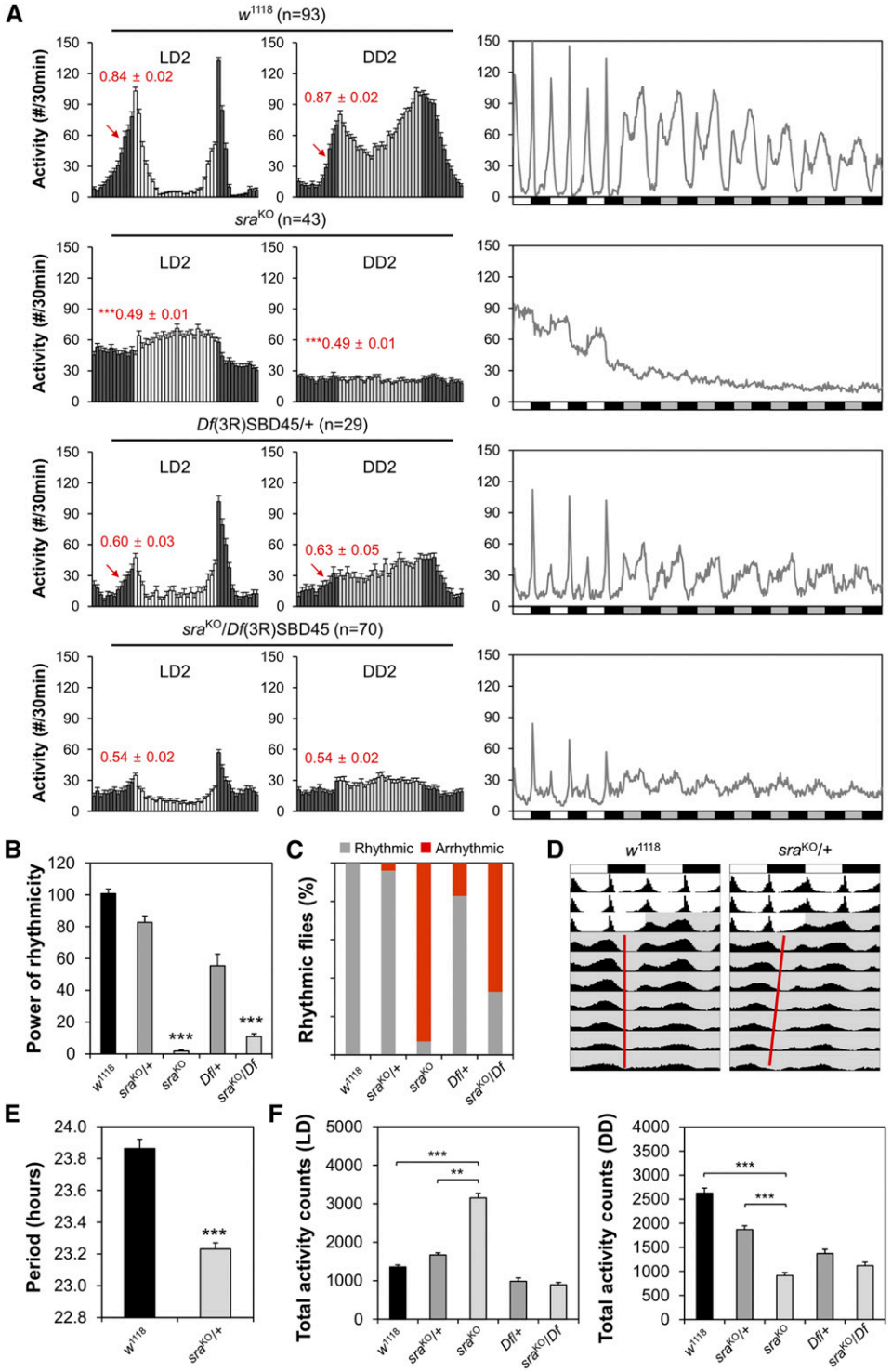

Figure 1 Genomic deletion of *sra* locus impairs circadian locomotor behaviors. (A) Male flies homozygous or *trans*-heterozygous for *sra* mutant alleles show weaker anticipatory activity to lights-on (red arrows) and poor rhythmicity in free-running locomotor behaviors in DD. Activity profiles on the second day of LD cycles (LD2, left), on the second day of DD cycles (DD2, middle), or throughout the behavioral analyses (right) were averaged from individual flies. Averaged morning anticipation index values ± SEM are shown in red. *** $P < 0.001$ *vs.* wild-type as determined by Student's *t*-test. *n*, the number of flies analyzed; white/black bars, LD cycles; and gray/black bars, DD cycles. (B) Rhythmicity in free-running locomotor behaviors was determined by measuring power (*P*) − significance (*S*) values from the χ^2 periodograms of individual flies, and averaging over each genotype. *** $P < 0.001$ *vs.* wild-type or heterozygous control, as determined by one-way ANOVA with Tukey's *post hoc* test. Error bars indicate SEM (n = 29–98). (C) Percentage of flies with detectable rhythmicity ($P - S > 10$) was calculated per each genotype. (D) Averaged actograms of wild-type or sra^{KO} heterozygous flies were double-plotted (n = 93–98). Gray shade indicates constant dark. (E) Circadian periods in DD were averaged from rhythmic flies per each genotype. *** $P < 0.001$ *vs.* wild-type, as determined by Student's *t*-test. Error bars indicate SEM (n = 93–94). (F) Daily total activity counts were averaged from individual flies per each genotype in LD (left) or DD (right) cycles. ** $P < 0.01$ and *** $P < 0.001$ as determined by one-way ANOVA with Tukey's *post hoc* test. Error bars indicate SEM (n = 29–98). DD, constant dark; LD, 12 hr light/12 hr dark.

examined their behavioral effects. Expression of sra^{RNAi} in *tim*-expressing clock cells under the control of the *tim*-Gal4 driver (*tim*-Gal4/UAS-sra^{RNAi}; DCR2/+) was sufficient to mimic most of the circadian phenotypes of sra^{KO} flies (Figure 3 and Table S2). *sra*-depleted flies lost free-running behavioral rhythms in DD (Figure 3, A and B and Table S2). Nonetheless, sra^{RNAi} flies did not exhibit hyperactivity but their locomotor activity was rather reduced in DD (Figure 3C). In addition, *sra* depletion post-transcriptionally impaired PER and TIM rhythms in fly heads (Figure 3, D and E), to a similar extent as the sra^{KO} allele.

Because the *tim*-Gal4 transgene is expressed in all clock-relevant cells, including glia (Suh and Jackson 2007), we tested additional Gal4 drivers to narrow down the neural

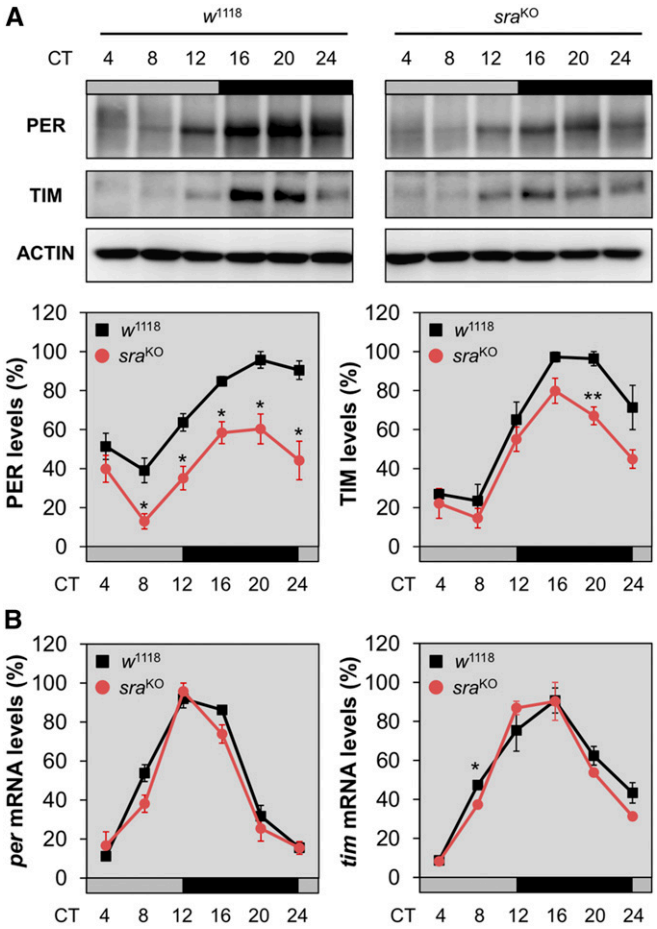

Figure 2 *sra*^{KO} mutation post-transcriptionally dampens circadian expression of PER and TIM proteins in adult fly heads. (A) Adult male flies were harvested at the indicated time points during the first DD cycle following the LD entrainment. Head extracts were immunoblotted with anti-PER, anti-TIM, and anti-actin (loading control) antibodies. Representative blot images from three independent experiments are shown. Protein band intensities in each lane were quantified using ImageJ software and normalized against the corresponding level of actin protein. The *y*-axis indicates % relative expression levels of PER and TIM, calculated by normalizing to the peak value in w^{1118} control flies (set as 100). Data represent average ± SEM ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ *vs.* controls at the same time point, as determined by Student's *t*-test. Gray/black bars, DD cycles; black lines, w^{1118} control flies; and red lines, *sra*^{KO} flies. (B) Flies were harvested during the first DD cycle following the LD entrainment. Total RNAs were purified from head extracts, and relative levels of *per*, *tim*, and *gapdh* (normalizing control) mRNAs were quantitatively analyzed by real-time PCR. The *y*-axis indicates % relative expression levels of *per* and *tim* mRNAs at each time point, calculated by normalizing to the peak value in w^{1118} control flies (set as 100). Data represent average ± SEM ($n = 3$). * $P < 0.05$ *vs.* control at the same time point, as determined by Student's *t*-test. CT, circadian time; DD, constant dark; LD, 12 hr light/12 hr dark.

substrate of *sra*-dependent clocks. Although the majority of Gal4 drivers expressed in circadian pacemaker neurons affected free-running circadian behaviors, *sra* depletion in PDF-expressing LN$_V$s was sufficient to dampen free-running behavioral rhythms (Figure S4 and Table S2). However, the behavioral phenotypes by the LN$_V$-specific *pdf*-Gal4 or mz520-Gal4 driver were relatively modest. One possible explanation for this observation is that *sra* depletion in PDF neurons by these LN-specific Gal4 drivers might not be as efficient as depletion by *tim*-Gal4. In addition, we cannot exclude the possibility that non-PDF clock neurons positive for the *tim*-Gal4 or *cryptochrome*-Gal4 (*cry*-Gal4) transgene could make additional contributions to *sra*-dependent behavioral rhythms.

To determine whether *sra* effects on circadian behaviors are specific to the adult stage, we conditionally depleted *sra* expression in PDF neurons of adult fly brains using a geneswitch transgene (Depetris-Chauvin *et al.* 2011). Although baseline expression of the *sra*^{RNAi} transgene by the leaky *pdf*-Gal4^{GeneSwitch} decreased behavioral rhythmicity in control flies fed vehicle (ethanol)-containing food, we observed that the RU486-induced depletion of *sra* in PDF neurons eventually led to arrhythmic behaviors in subsequent DD cycles (Figure S5 and Table S3). Taken together, these data support the idea that *sra* functions in adult PDF neurons to sustain locomotor rhythms, excluding the possibility that *sra* affects circadian behaviors by influencing the development of PDF neurons.

sra overexpression in circadian pacemaker neurons disrupts circadian rhythms

Circadian rhythms could be sensitized to a specific dosage of a clock-relevant gene. To explore this idea, we investigated whether *sra* overexpression could modulate circadian behaviors in wild-type flies. For this purpose, we employed three *sra* transgenes, encoding either wild-type or epitope-tagged *sra* cDNAs, independently established in previous studies (Ejima *et al.* 2004; Takeo *et al.* 2012). Each *sra* transgene was genetically combined with either *tim*-Gal4 or LN-specific Gal4 drivers to induce *sra* overexpression in all clock cells or PDF neurons, respectively. The strongest effects of *sra* overexpression were observed with the UAS-*sra* transgene: its expression in PDF neurons was sufficient to cause longer periodicity, with lower-amplitude rhythms in free-running behaviors, than in heterozygous controls (Table S4). However, modest dampening of behavioral rhythms with longer periods was consistently observed when *sra* overexpression in *tim*-expressing cells was genetically induced using the other *sra* transgenes (UAS-*sra*^{FLAG} and UAS-*sra*^{WT}). Different levels of *sra* overexpression in PDF neurons under the control of *tim*-Gal4 or LN-specific Gal4 drivers might explain the phenotypic differences among *sra* transgenic lines. An alternative (but not mutually exclusive) explanation is that overexpression or ectopic expression of *sra* in non-PDF neurons under the control of *tim*-Gal4 might have contributed to the deficient rhythms in these transgenic flies. Nonetheless, *sra* overexpression driven by *tim*-Gal4 partially rescued the disrupted behavioral rhythms in *sra*^{KO} flies, and also elevated the levels of PER and TIM proteins in head extracts of *sra* mutants (Figure 4 and Table S4). Taken together, these results suggest that the robustness of 24-hr behavioral rhythms requires a specific dosage window of *sra* expression in circadian pacemaker neurons.

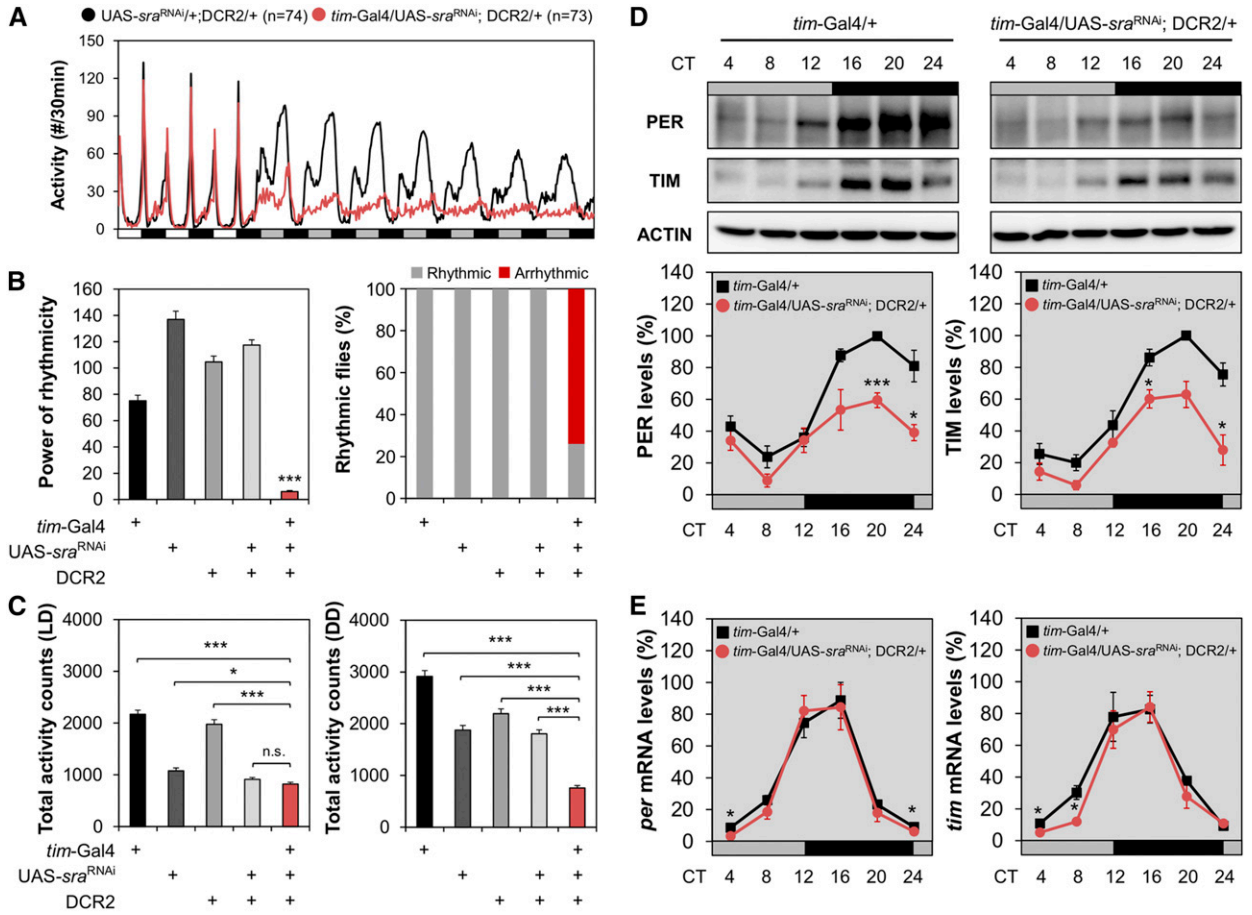

Figure 3 *sra* depletion in *tim*-expressing circadian clock cells is sufficient to phenocopy sra^{KO} mutation. (A) Activity profiles were averaged from individual flies in LD cycles followed by DD. (B and C) Rhythmicity in free-running locomotor behaviors [power (*P*) − significance (*S*)], percentage of flies with detectable rhythmicity ($P - S > 10$), and daily total activity counts were analyzed similarly as in Figure 1. * $P < 0.05$ and *** $P < 0.001$ *vs.* other transgenic controls, as determined by one-way ANOVA with Tukey's *post hoc* test. Error bars indicate SEM ($n = 31–73$). (D and E) Transgenic flies were harvested at the indicated time points during the first DD cycle following the LD entrainment. Relative expression levels (%)of PER and TIM proteins (D), or those of *per* and *tim* mRNAs (E) were quantified in head extracts similarly as in Figure 2. Data represent average ± SEM ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ *vs.* controls at the same time point, as determined by Student's *t*-test. CT, circadian time; DD, constant dark; LD, 12 hr light/12 hr dark; n.s., not significant; UAS: upstream activating sequence.

Genetic manipulation of calcineurin expression or activity affects circadian rhythms

The *Drosophila* calcineurin contains one of three catalytic subunits: *CanA1*, *CanA-14F*, or *Pp2B-14D* (Takeo *et al.* 2006). Nonetheless, increased locomotor activity and short-sleep phenotypes have been reported most evidently in $CanA\text{-}14F^{KO}$ flies (Nakai *et al.* 2011). To determine whether *sra* collaborates with a specific calcineurin to sustain circadian rhythms, we assessed circadian behaviors in individual calcineurin mutant flies. Genomic deletion of either the *CanA-14F* or *Pp2B-14D* locus modestly dampened rhythm amplitude in free-running DD behaviors (Figure S6 and Table S5). However, the percentage of rhythmic flies was much higher than in *sra* mutants. This observation supports the idea that circadian phenotypes and hyperactivity are, at least in part, dissociable in calcineurin mutants. We also reasoned that functional redundancy among the three catalytic calcineurin subunits might explain their relatively weak individual phenotypes in comparison with *sra* mutants.

On the other hand, we observed that transgenic overexpression of constitutively active *Pp2B-14D* ($Pp2B\text{-}14D^{ACT}$), but neither a wild-type ($Pp2B\text{-}14D^{WT}$) nor dominant-negative mutant ($Pp2B\text{-}14D^{H217Q}$), in *tim*-expressing clock cells shortened circadian periods in free-running locomotor behaviors (Figure 5, A and B and Table S6). Similar to the effects of *sra*, $Pp2B\text{-}14D^{ACT}$ overexpression in PDF-expressing clock neurons was sufficient to cause short-period phenotypes in DD rhythms. Circadian expression of PER and TIM proteins (as reflected by protein levels in head extracts) was accordingly phase-advanced by $Pp2B\text{-}14D^{ACT}$ (Figure 5C). Moreover, transgenic flies overexpressing $Pp2B\text{-}14D^{ACT}$ from two copies of the transgene were strongly arrhythmic in DD (Figure 5, A and B and Table S6), and displayed lower levels of PER and TIM proteins in head extracts (Figure 5D). Taken together,

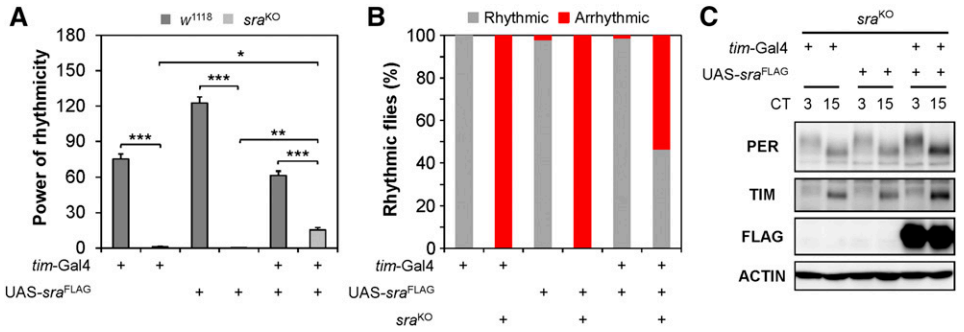

Figure 4 *sra* overexpression in *tim*-expressing clock cells partially rescues circadian phenotypes in sra^{KO} mutation. (A and B) Overexpression of a transgene encoding the FLAG-tagged *sra* cDNA was driven by a *tim*-Gal4 driver in a w^{1118} control or sra^{KO} mutant background. Rhythmicity in free-running locomotor behaviors [power (*P*) − significance (*S*)] and percentage of transgenic flies with detectable rhythmicity ($P - S > 10$) were analyzed similarly as in Figure 1. Data represent average ± SEM (n = 47–75). Two-way ANOVA detected significant interaction between *sra* overexpression and sra^{KO} mutation on power of rhythmicity [F (2, 369) = 71.67, $P < 0.0001$], validating the transgenic rescue. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as determined by Tukey's *post hoc* test. (C) Flies harboring the indicated transgenes in sra^{KO} mutant background were harvested at CT3 and CT15 during the first DD cycle following the LD entrainment. Head extracts were immunoblotted with anti-PER, anti-TIM, anti-FLAG, and anti-actin (loading control) antibodies. CT, circadian time; DD, constant dark; LD, 12 hr light/12 hr dark; UAS: upstream activating sequence.

these data further support the idea that circadian clocks are sensitive to overall calcineurin activity in circadian pacemaker neurons, consistent with the effects of *sra* on circadian behaviors.

sra genetically titrates calcineurin activity to sustain circadian rhythms

sra and *Pp2B-14D* interact genetically and biochemically in *Drosophila* models (Takeo *et al.* 2006; Nakai *et al.* 2011). To determine whether both genes function in the same genetic pathway related to circadian clocks, we tested the effects of constitutively active *Pp2B-14D*^{ACT} on free-running locomotor behaviors in genetic backgrounds with different dosages of *sra* expression. Heterozygous sra^{KO} negligibly affected circadian behaviors in control genetic backgrounds (Figure 6 and Table S7), but substantially dampened behavioral rhythmicity in transgenic flies overexpressing *Pp2B-14D*^{ACT} in *tim*-expressing clock cells. Given that higher overexpression from two copies of the *Pp2B-14D*^{ACT} transgene caused arrhythmic behaviors, the genetic interaction between sra^{KO} and *Pp2B-14D*^{ACT} supports the model that *sra* normally suppresses *Pp2B-14D*^{ACT}. Furthermore, we found that overexpression of wild-type *sra* masked short-period rhythms in *Pp2B-14D*^{ACT}-overexpressing flies (Figure 6 and Table S7). Moreover, *Pp2B-14D*^{ACT} overexpression rescued the poor rhythmicity caused by *sra* overexpression. Taken together, these data provide compelling genetic evidence that *sra* acts as a negative regulator of calcineurin to titrate its overall activity, and thereby sustains 24-hr rhythms in free-running locomotor behaviors.

Pharmacological inhibition of calcineurin promotes proteasomal degradation of PER and TIM proteins

We next took a pharmacological approach to examine the behavioral and molecular consequences of calcineurin inhibition in circadian rhythms. Oral administration of a calcineurin inhibitor, CsA, to wild-type flies did not affect their viability during our tests of circadian behaviors (*i.e.*, 3 days in LD cycle followed by 7 days in DD). However, CsA-fed flies exhibited weaker activity peaks during the subjective day in DD, as well as longer circadian periods in free-running behaviors than vehicle (DMSO)-fed flies (Figure 7A and Table S8). Circadian expression of PER and TIM proteins (as reflected by protein levels in head extracts) was also compromised in CsA-fed flies (Figure 7B).

To determine whether the observed CsA effects on PER and TIM rhythms were driven either by behavioral dampening or by cell-autonomous effects of calcineurin inhibition, we investigated the effects of CsA on PER and TIM expression in clock-less *Drosophila* S2 cells. Because S2 cells barely express the endogenous clock proteins, we transiently transfected S2 cells with expression vectors encoding epitope-tagged PER or TIM, and then monitored changes in their steady-state levels in response to pharmacological treatments. CsA treatment decreased PER and TIM levels in a dose-dependent manner (Figure S7A). The proteasome inhibitor MG132 suppressed the effects of CsA on PER and TIM levels (Figure 7, C and D), indicating that CsA treatment promoted proteasomal degradation of the two proteins. Similar results were obtained using another calcineurin inhibitor tacrolimus (FK506). By contrast, neither CsA nor FK506 treatment affected steady-state levels of CLK proteins in transfected S2 cells (Figure S7B). Together, these data suggest that calcineurin specifically protects PER and TIM proteins from proteasomal degradation in a cell-autonomous manner. In addition, we reason that calcineurin-dependent stabilization of PER or TIM proteins is unlikely to be mediated by the formation of a PER–TIM complex.

Discussion

The phosphorylation status of specific residues in clock proteins changes temporally in a well-coordinated manner. Consequently, the subcellular localization, transcriptional activity, and stability of *Drosophila* PER and TIM proteins are collaboratively regulated by multiple protein kinases and phosphatases, thereby sustaining overt rhythms in circadian gene expression and behaviors. Previous studies in mammals

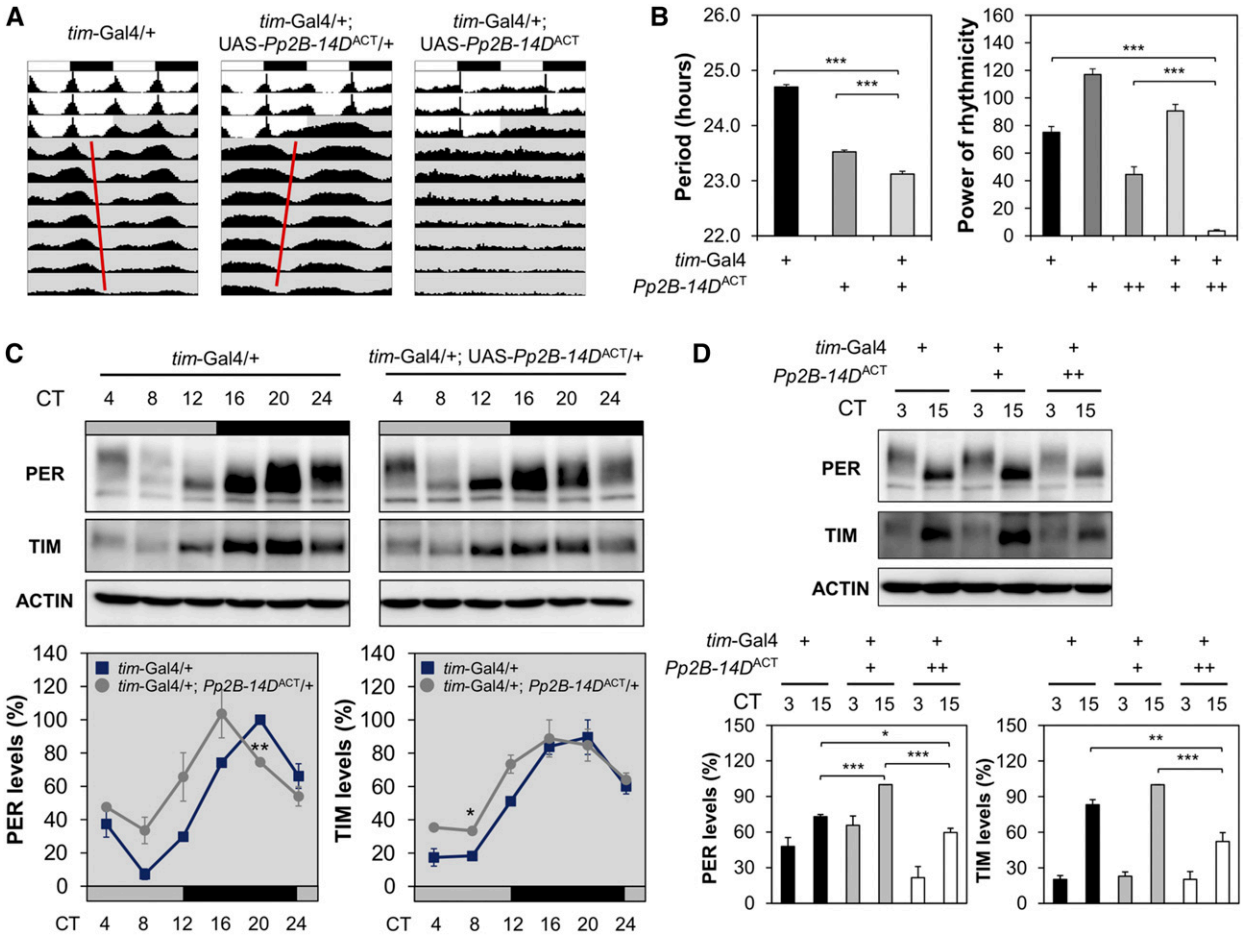

Figure 5 Constitutively active form of *Pp2B-14D* disrupts circadian behaviors in a dose-dependent manner when overexpressed in *tim*-expressing clock cells. (A) Averaged actograms of flies overexpressing the constitutively active *Pp2B-14D*^{ACT} from a single or two copies of the transgene were double-plotted (n = 32–136). (B) Circadian periods in rhythmic flies [power (P) − significance (S) > 10] and rhythmicity in free-running locomotor behaviors ($P - S$) were analyzed similarly as in Figure 1. *** $P < 0.001$ as determined by one-way ANOVA with Tukey's *post hoc* test. Error bars indicate SEM (n = 31–136). (C and D) Transgenic flies were harvested at the indicated time points during the first DD cycle following the LD entrainment. Relative expression levels (%) of PER and TIM proteins were quantified in head extracts similarly as in Figure 2. Data represent average ± SEM (n = 3). * $P < 0.05$ and ** $P < 0.01$ *vs.* Gal4 heterozygous controls at the same time point, as determined by Student's t-test (C); * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as determined by one-way ANOVA with Tukey's *post hoc* test (D). CT, circadian time; DD, constant dark; LD, 12 hr light/12 hr dark; UAS: upstream activating sequence.

showed that chemical inhibitors of the Ca^{2+}-dependent protein phosphatase calcineurin impose their effects on a wide range of circadian physiology, including daily changes in blood pressure, phase shift by light entrainment, and locomotor rhythms (van den Dorpel *et al.* 1996; Katz *et al.* 2008). Here, we provide compelling genetic evidence that balancing the activity of calcineurin and its negative regulator *sra* in *Drosophila* circadian pacemaker neurons is crucial for high-amplitude circadian behaviors. The effects of calcineurin on *Drosophila* clocks are likely mediated by the proteasomal degradation of the clock proteins PER and TIM, but not by their circadian transcription. Thus, our data support the idea that *Drosophila* circadian rhythms are post-translationally controlled by the calcineurin–*sra* pathway.

The intimate interaction between circadian clocks and sleep suggests that a common pathway is responsible for the behavioral phenotypes observed in *Drosophila* models lacking *sra* and individual calcineurin genes. However, arguing against this idea, we observed clear differences between their circadian and sleep phenotypes. First, mutant flies homozygous for genomic deletion of the *sra* locus exhibited hyperactivity (*i.e.*, short sleep) in LD cycles (Nakai *et al.* 2011), but exhibited lower locomotor activity than wild-type or heterozygous controls in subsequent DD cycles, during which their free-running behaviors actually became arrhythmic. Second, *sra trans*-heterozygous mutants exhibited poor rhythmicity in DD locomotor behaviors, whereas their total activity counts were comparable to those of heterozygous controls. Accordingly, circadian phenotypes in this *sra* mutant background were dissociable from hyperactivity or short-sleep phenotypes. Third, genomic deletion of either *sra* or *CanA-14F* shortened baseline sleep to comparable extents (Nakai *et al.* 2011). By contrast, we observed much severer rhythmicity phenotypes (*i.e.*, lower percentages of rhythmic

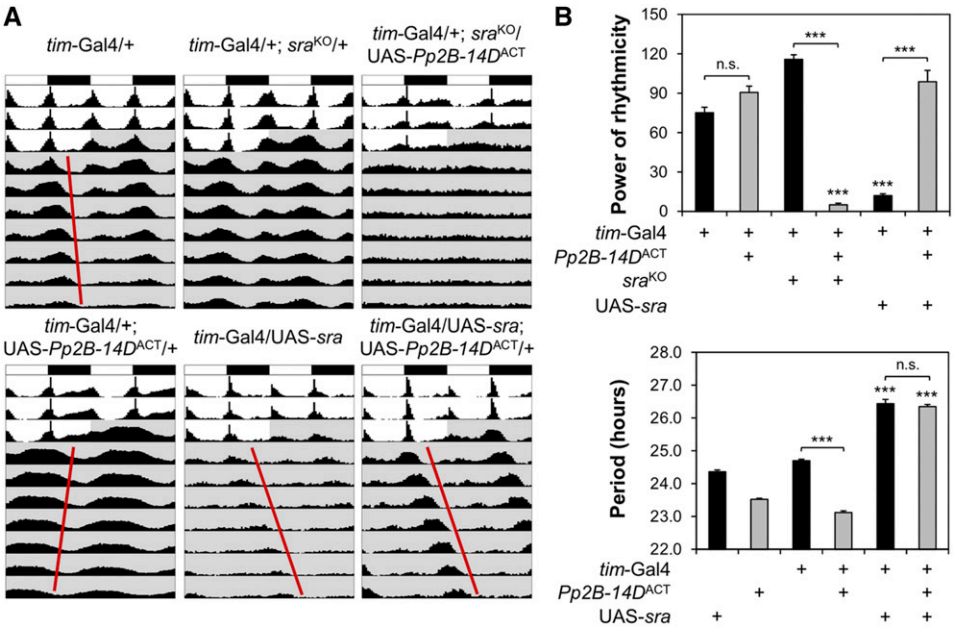

Figure 6 *sra* genetically suppresses *Pp2B-14D*^{ACT} to sustain circadian locomotor rhythms. (A) Averaged actograms of transgenic flies overexpressing the constitutively active *Pp2B-14D*^{ACT} in wild-type, *sra*^{KO} heterozygous, or *sra*-overexpressing background were double-plotted (n = 29–136). (B) Rhythmicity in free-running locomotor behaviors (top) and circadian periods in rhythmic flies (bottom) were analyzed similarly as in Figure 1. Data represent average ± SEM (n = 29–136). Two-way ANOVA detected significant interaction of *Pp2B-14D*^{ACT} with *sra*^{KO} [F (1, 344) = 185.3, $P < 0.0001$ for power of rhythmicity] and *sra* overexpression [F (1, 297) = 36.24, $P < 0.0001$ for power of rhythmicity; F (1, 249) = 38.52, $P < 0.0001$ for circadian periods], validating the genetic interaction between *sra* and *Pp2B-14D*^{ACT} on free-running locomotor behaviors in DD. *** $P < 0.001$ *vs.* the same combination of *tim*-Gal4 or *Pp2B-14D*^{ACT} transgene in wild-type, *sra* background as determined by Tukey's *post hoc* test. CT, circadian time; DD, constant dark; n.s., not significant UAS: upstream activating sequence.

flies) in *sra* mutants. Finally, overexpression of a constitutively active calcineurin catalytic subunit (*Pp2B-14D*^{ACT}) caused shorter circadian periods or arrhythmic clocks, but had no detectable effects on baseline sleep under particular experimental conditions (Tomita *et al.* 2011). Together, these observations suggest sleep-independent clock regulation by the calcineurin–*sra* pathway.

One puzzling observation regarding *Drosophila* mutants in the calcineurin–*sra* pathway is that single deletions of either *sra* or the *CanA-14F* locus led to behavioral defects (*i.e.*, hyperactivity, short sleep, and arrhythmic clocks) in the same direction. These data argue against the idea that *sra* negatively regulates calcineurin, and instead favor a model in which these factors act cooperatively or at least independently. In fact, *sra* and calcineurin mutants exhibit similar phenotypes in other genetic models of *Drosophila* and mammals, defining *sra* as a calcineurin activator (Vega *et al.* 2003; Takeo *et al.* 2010). Another complication comes from the observations that either loss-of-function or overexpression of *sra* can impair learning in olfactory conditioning (Chang *et al.* 2003) or behavioral rhythmicity in DD, indicating that an optimal *sra* dosage is required to control these behaviors.

Nonetheless, the sleep-promoting effects of *sra* require *CanA-14F* or *Pp2B-14D* (Nakai *et al.* 2011), and these non-additive effects of *sra* and calcineurin mutations on the total amount of sleep support the idea that these factors participate in a common genetic pathway involved in sleep regulation. Further, we showed that strong overexpression of constitutively active *Pp2B-14D*^{ACT} dampened PER and TIM rhythms, and caused arrhythmic circadian behaviors, phenotypically mimicking loss of *sra* function. Moreover, abnormal circadian behaviors in *Pp2B-14D*^{ACT}-overexpressing flies were exaggerated or suppressed by genomic deletion or overexpression of *sra*, respectively. These pieces of genetic evidence convincingly demonstrate that *sra* is a negative regulator of calcineurin in the genetic pathway that regulates circadian rhythms, as exemplified in other *Drosophila* models (Takeo *et al.* 2006; Shaw and Chang 2013; Lee *et al.* 2016). However, it remains possible, that *sra* might have a stronger preference for a specific calcineurin subtype (*e.g.*, calcineurin harboring a catalytic subunit *Pp2B-14D*) in regard to its clock-regulatory function. Thus, the molecular phenotypes in *sra*^{KO} mutants may largely reflect the consequences of derepression of a specific calcineurin activity. On the other hand, in wild-type flies and *Drosophila* S2 cells, the proteasomal degradation of PER and TIM proteins could have been promoted by the overall suppression of both *sra*-dependent and *sra*-independent calcineurin activity. Another possibility is that the PER- and TIM-stabilizing effects of calcineurin might be gated by circadian clocks due to the time-dependent shuttling of these clock proteins between the nucleus and cytoplasm, or by the rhythmic changes in their phosphorylation status.

Calcineurin dephosphorylates nuclear factor of activated T-cells (NFAT) and promotes its nuclear entry to support its transcriptional regulatory activity (Hogan *et al.* 2003). In mammals, NFAT undergoes daily rhythmic nuclear translocation in skeletal muscle and heart, and mediates activity-dependent circadian gene expression (Sachan *et al.* 2011; Dyar *et al.* 2015). In fact, DSCR1/RCAN1 (a mammalian homolog of *sra*) is a transcriptional target of NFAT (Yang *et al.* 2000). Daily circadian oscillation in DSCR1/RCAN1 occurs in the liver and heart (Storch *et al.* 2002; Bray *et al.* 2008; Sachan *et al.* 2011). Because DSCR1 associates with the catalytic subunit of calcineurin and inhibits its enzymatic activity

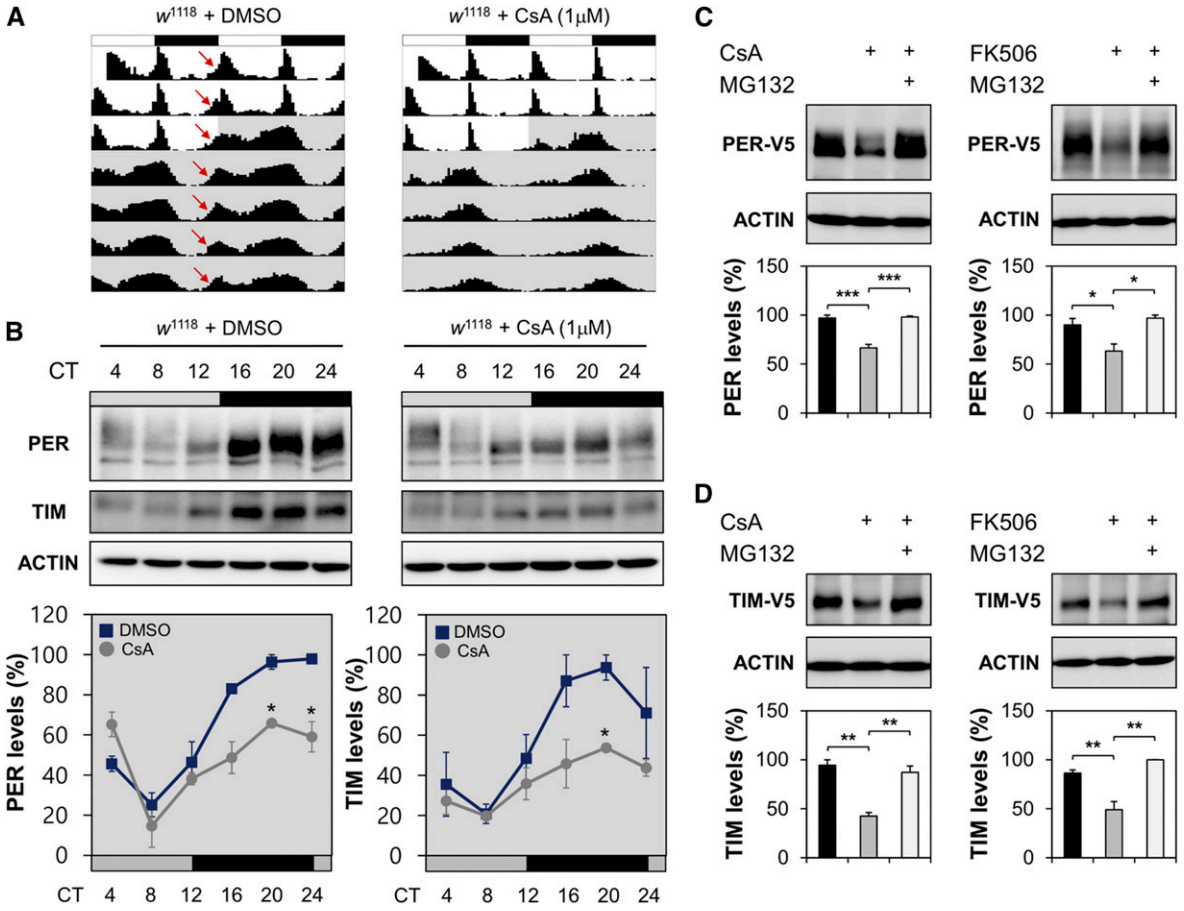

Figure 7 Pharmacological inhibition of calcineurin dampens circadian expression of PER and TIM proteins in wild-type fly heads and enhances their proteasomal degradation in cell culture. (A) Wild-type flies were fed on behavior food (5% sucrose and 2% agar) containing either 1 mM DMSO (vehicle control) or 1 μM CsA throughout the behavioral test, and their averaged actograms were double-plotted (n = 45–57). Locomotor activity peaks anticipatory to lights-on were indicated by red arrows. (B) Wild-type flies were entrained by three LD cycles on the behavior food containing either 1 mM DMSO or 1 μM CsA, transferred to DD, and then harvested at the indicated time points during the first DD cycle. Relative expression levels (%) of PER and TIM proteins were quantified in head extracts similarly as in Figure 2. Data represent average ± SEM (n = 2). * $P < 0.05$ *vs.* DMSO controls at the same time point as determined by Student's *t*-test. (C and D) *Drosophila* S2 cells were transfected with the expression vector for V5-tagged PER or TIM proteins. Where indicated, 20 μM CsA, 20 μM tacrolimus (FK506), or 50 μM MG132 were incubated with transfected cells for 2 hr before harvest. Relative expression levels (%) of V5-tagged PER or TIM proteins were quantified in transfected cell extracts similarly as in Figure 2. Data represent average ± SEM (n = 3). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as determined by one-way ANOVA with Tukey's *post hoc* test. CsA, cyclosporin A; CT, circadian time; DD, constant dark; LD, 12 hr light/12 hr dark.

(Fuentes *et al.* 2000; Rothermel *et al.* 2000; Chan *et al.* 2005), the DSCR1/RCAN1–calcineurin–NFAT pathway possesses an intrinsic auto-inhibitory mechanism (Yang *et al.* 2000; Minami 2014). This additional layer of transcriptional feedback might supplement circadian gene expression in a Ca^{2+}- and/or activity-dependent manner.

Conservation of circadian clock function among DSCR1/RCAN1–calcineurin homologs, and their functional interactions with other clock-regulatory factors such as cAMP response element-binding protein (Kingsbury *et al.* 2007; Kim and Seo 2011), CKIε/DBT (Liu *et al.* 2002), GSK3β/SHAGGY (Kim *et al.* 2009; Takeo *et al.* 2012), and FMRP (Wang *et al.* 2012), indicate that DSCR1/RCAN1–calcineurin-dependent signaling is integral to the core clock mechanism and suggest that it is derived from ancestral clocks. Future studies should seek to elucidate how the transcriptional feedback loop of the DSCR1/RCAN1–calcineurin–NFAT pathway shapes circadian transcription, as well as how post-translational regulation by DSCR1/RCAN1–calcineurin signaling elaborately contributes to high-amplitude 24-hr rhythms.

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