Circadian pacemaker neurons display co-phasic rhythms in basal calcium level and in fast calcium fluctuations

Authors: Xitong Liang[1#], Timothy E. Holy[1], & Paul H. Taghert[1]*

Affiliations:

1. Department of Neuroscience, Washington University in St. Louis, MO 63110, USA

[#]Present address: Max Planck Institute for Brain Research, 60438 Frankfurt am Main, Germany

*Lead contact: taghertp@wustl.edu

Abstract

Circadian pacemaker neurons in the *Drosophila* brain display daily rhythms in the levels of intracellular calcium. These calcium rhythms are driven by molecular clocks and are required for normal circadian behavior. To study their biological basis, we employed genetic manipulations in conjunction with in vivo light-sheet microscopy to measure calcium dynamics in individual pacemaker neurons over complete 24-hour periods. We found co-phasic daily rhythms in basal calcium levels and in high frequency calcium fluctuations. Further we found that the rhythms of basal calcium levels require the activity of the *IP3R*, a channel that mediates calcium fluxes from internal endoplasmic reticulum (ER) calcium stores. Independently, the rhythms of fast calcium fluctuations required the T-type voltage-gated calcium channel, a conductance that mediates extracellular calcium influx. These results suggest that *Drosophila* molecular clocks regulate *IP3R* and T-type channels to generate coupled rhythms in basal calcium and in fast calcium fluctuations, respectively. We propose that both internal and external calcium fluxes are essential for circadian pacemaker neurons to provide rhythmic outputs, and thereby regulate the activities of downstream brain centers.

Introduction

Circadian rhythms in multiple aspects of cellular physiology help organisms across taxa, from unicellular cyanobacteria to multicellular animals, adapt to environmental day-night changes (Dunlap 1999; Herzog 2007). In mammals, neurons in the hypothalamic suprachiasmatic nucleus (SCN) show circadian rhythms in gene expression, intracellular calcium, neural activity, and other cellular properties (Welsh et al 2010). Circadian rhythms in SCN neuronal outputs coordinate circadian rhythms in other cells throughout the body and generate behavioral rhythms (Mohawk et al 2012). The rhythms of SCN neuronal outputs can be generated cell-intrinsically by the negative transcription/translation feedback loop of core clock genes, as a molecular clock, which then generates 24-hour oscillations in a series of genes (Welsh et al 1995; Dunlap 1999; Panda et al 2002; Colwell 2011). These gene oscillations then regulate different aspects of membrane physiology, such as the expression levels of channels for potassium (K^+), sodium (Na^+), and calcium (Ca^{2+}) (Pennartz et al 2002; Itri et al 2005; Pitts et al 2006; Meredith et al 2006; Flourakis et al 2015). The mechanisms by which the molecular clockworks coordinate complex membrane physiology to generate neural activity rhythms within individual circadian pacemakers remain to be defined.

Calcium signaling regulates many cellular processes, such as neural excitability, neurotransmitter release, and gene expression (Berridge 1998). Cytoplasmic calcium can be regulated from extracellular calcium influx, as well as from intracellular calcium stored in the endoplasmic reticulum (ER) and mitochondria (Chorna and Hasan 2012). Studies on SCN neurons *in vitro* (Colwell 2000; Ikeda et al 2003) and recently *in vivo* (Jones et al 2018) measured circadian calcium rhythms in SCN neurons. Some studies suggested that calcium rhythms were driven by neuronal firing and voltage-gated calcium channels (Colwell 2000;

48 Enoki et al 2017), while others suggested they were driven by intracellular stores, via the ER
49 channels ryanodine receptor (Ikeda et al 2003). These alternative hypotheses may derive from
50 the technical differences in the various studies, including the details of *in vitro* preparations, but
51 also due to a lack of single-cell resolution in the calcium measurements.

52 In *Drosophila*, circadian pacemaker neurons also show clock-driven circadian calcium
53 rhythms (Liang et al 2016). The dynamics can be resolved across all five major pacemaker
54 groups, s-LNv, l-LNv, LNd, DN1, and DN3, and each group exhibits distinct and sequential
55 daily peak phases. Within such groups, the rhythms can be measured in single identified cells
56 (Liang et al., 2017). The multi-hour phase diversity exhibited by this network requires a series
57 of delays effected by environmental light and by non-cell-autonomous modulation mediated by
58 different neuropeptides (Liang et al 2017). Precisely how neuropeptide signaling regulates
59 calcium activity in pacemaker neurons over long (many-hour) durations is unknown. To begin to
60 understand these critical mechanisms of pacemaker modulation, we begin by addressing the
61 cellular and molecular basis of pacemaker calcium rhythms with physiological, genetic and
62 behavioral measures.

63 In this study, we again used *in vivo* calcium imaging at single-cell resolution, here using a
64 high-speed light sheet microscope (Greer and Holy 2019). We simultaneously measured both
65 basal calcium levels and fast calcium fluctuations over entire 24-hr periods. We found circadian
66 rhythmicity in both basal calcium levels and in the frequency of fast calcium fluctuations. We
67 consider the fast fluctuations to represent events closely coupled to neuronal firing, as have
68 previous, related studies (Pologruto et al 2004, Yaksi and Friedrich 2006, Chen et al., 2013,
69 Streit et al. 2016, Greenberg et al 2018). In all pacemaker neurons studied, these two layers of
70 calcium rhythms shared the same daily temporal pattern (i.e., they were co-phasic). To gain

71 insights into the mechanism of these patterns, we exploited the fact that in *Drosophila* many
72 calcium channels are encoded by single genes (Chorna and Hasan 2012), and used genetics to
73 study the roles of individual channels in generating daily pacemaker calcium rhythms. Here, we
74 present results of experiments in which we knocked down RNAs encoding different calcium
75 channels selectively in all or a subset of pacemakers. We evaluated the impact of individual
76 channels in setting both slow daily changes in basal calcium levels and in fast fluctuations.
77 Finally, we measured PERIOD staining levels and behavior to determine which channels provide
78 feedback to the molecular clock and which are required for normal circadian output from the
79 pacemaker network.
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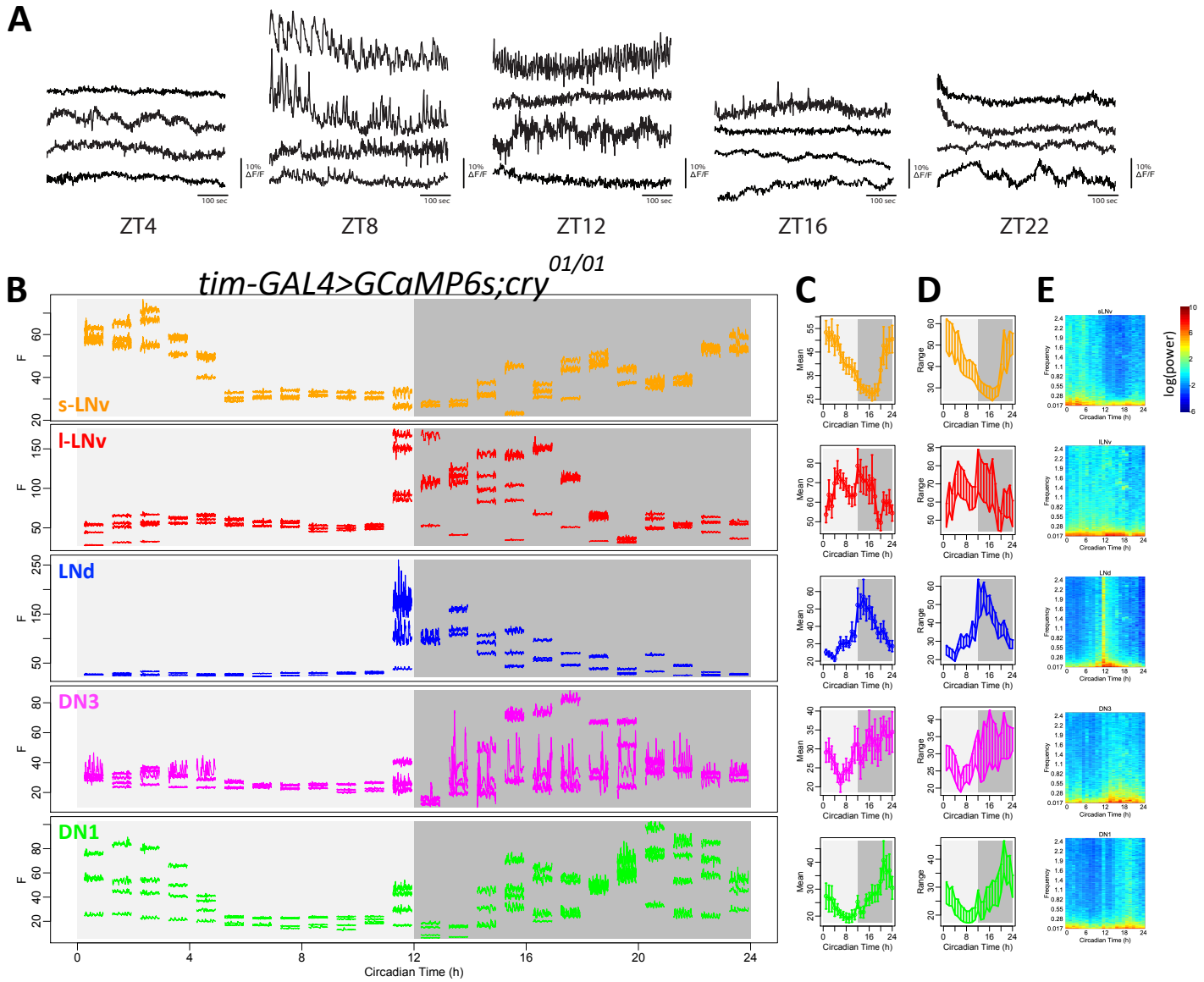

Figure 1. Daily pattern of fast calcium activity in circadian pacemaker neurons.
(A) Representative calcium activity traces of LNd recorded at 1Hz from five different times of day: ZT4, ZT8, ZT12, ZT16, and ZT22. For each timepoint, the fly's brains are acutely exposed for short-term imaging. **(B)** Raw calcium activity traces from one representative fly. Each segmented trace is 1-min activity of single neuron recorded at 5Hz. 24 segments are recorded over 24 hours with 1-hour intervals. Activity traces of five circadian pacemaker groups are plotted in separate panels and color-coded. For each group, only cells that can be tracked throughout all 24 recording sessions are showed. In this specific fly, 3 s-LNv, 4 l-LNv, 3 LNd, 4 DN3, and 4 DN1 can be reliably tracked across 24 recording sessions. Circadian pacemaker neurons exhibit daily modulation in basal calcium level and in the frequency of fast calcium spikes. **(C)** Daily pattern of mean calcium intensity over the 1-min recording session at each of the 24 timepoints, averaged across all 6 flies studied. Error bars denote SEM. **(D)** Daily patterns of the range of calcium transients over the 1-min recording session at each of the 24 timepoints, averaged across all 6 flies studied. **(E)** Daily pattern of the power spectrum over the 1-min recording session at each of 24 timepoints, averaged across all 6 flies studied.

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Results

The rhythms of slow and fast calcium activity changes show similar daily patterns

Previously we reported that five major groups of circadian pacemaker neurons each exhibit daily calcium rhythms with distinct phases (Liang et al 2016). These results stand in apparent contrast to descriptions of synchronous daily electrical activity rhythms among three of these groups, the s-LNv, l-LNv, and DN1 (Cao and Nitabach 2008; Sheeba et al 2008; Flourakis et al 2015). The electrical activity rhythms were recorded *ex vivo* from different brains isolated at four to six different time points of the day. In contrast, we measured calcium rhythms *in vivo* by scanning individual flies every 10 min for 24 hours. Because of the close peak phases of calcium rhythms in s-LNv, l-LNv, and DN1 (between late night to mid-morning - Liang et al 2016), and because of the coarse sampling of electrophysiological studies, it is not certain whether calcium and electrical activity patterns are in fact distinct. To help clarify apparent differences in results derived from the two sets of studies, we began by performing short-term, continuous *in vivo* calcium imaging (1 Hz volumetric rate) on fly brains that were exposed acutely before each imaging experiment at five different times of day. We focused on the LNd because this group has a phase of calcium rhythms most distinct from those of the s-LNv, l-LNv, and DN1. In addition, the daily electrophysiological activity pattern of LNd has not previously been reported. We found that ~0.1 Hz calcium fluctuations peaked at around the same ZT8 - ZT10 at which this pacemaker group shows peak intensity in its daily calcium rhythm (Figure 1A and Figure S1). The time course of "fast" (by circadian standards) calcium fluctuations in the evening suggests they might be caused by the calcium influx during single action potentials or bursts of them (Figure 1A, Yaksi and Friedrich 2006, Chen et al., 2013, Greenberg et al 2018). This result

104 suggested that one or more LNd pacemakers exhibit a daily rhythm in electrical neural activity
105 that is roughly co-phasic with this pacemaker group's slow daily calcium rhythm.
106 Because the slow and fast calcium LNd rhythms are synchronous as measured, it is
107 formally possible that one rhythm is downstream of the other: for example, the slow calcium
108 rhythm could be the consequence of a rhythm in the fast. Alternatively, these two processes
109 could be completely distinct. To better understand the relationships between the two, and better
110 describe their phases across the entire network, we performed a series of short-term (1 min) high-
111 frequency (5 Hz) *in vivo* calcium imaging episodes at 1-hr intervals using the light-sheet
112 microscope OCPI-II (Greer and Holy 2019). In so doing, we tracked both slow basal calcium
113 level and fast calcium fluctuations in the same individual neurons, from all five major circadian
114 pacemaker groups: we collected these data consecutively from single brains for entire 24 hr
115 periods (Figure 1B). To ensure minimal disruption to the circadian clocks due to repeated optical
116 scanning, we used cry^{01} flies for these experiments which are null for the internal photoreceptor
117 CRYPTOCHROME. On average, all circadian neuron groups displayed slow calcium rhythms
118 comparable to those we previously reported (Liang et al 2016), except for the l-LNv, which
119 showed a second daily calcium activation right after the time of lights off. Nevertheless, all
120 pacemaker groups displayed daily changes in the minimal calcium level, demonstrating that their
121 basal calcium levels cycle with a daily rhythm (Figure 1CD). We found that within all five
122 pacemaker groups, changes in basal calcium levels and in fast calcium fluctuations shared
123 similar daily patterns: when basal calcium levels were high within a single pacemaker group, that
124 group also exhibited larger-amplitude fast calcium fluctuations. Power spectrum analysis clearly
125 revealed that, for individual neurons within each pacemaker group, calcium activity at all
126 frequency domains increased when the basal calcium level was high (Figure 1E). We asked

127 whether the change in the incidence of high frequency GCaMP6 fluctuations could have a
128 technical basis: specifically, whether it derives from a higher level of photon shot-noise due to
129 the higher baseline intensity. In order to normalize the effect of shot noise, we also calculated the
130 intensity of the calcium signal as the square root of photon number collected from an individual
131 region of interest (ROI). In this analysis, we still found daily rhythms in fast calcium fluctuations
132 (Figure S2). These results support the hypothesis that, in each circadian pacemaker group, fast
133 calcium fluctuations exhibit a daily rhythmic pattern that is co-phasic with a slow daily rhythm
134 in basal calcium levels.
135

136 **An RNAi screen to identify potential contributions of different calcium channels**

137 The observations described above support the conclusion that for individual pacemakers,
138 slow and fast calcium activities co-vary across the day. Yet, these observations do not reveal
139 whether the two rhythms are mechanistically linked, or represent independent functions. To

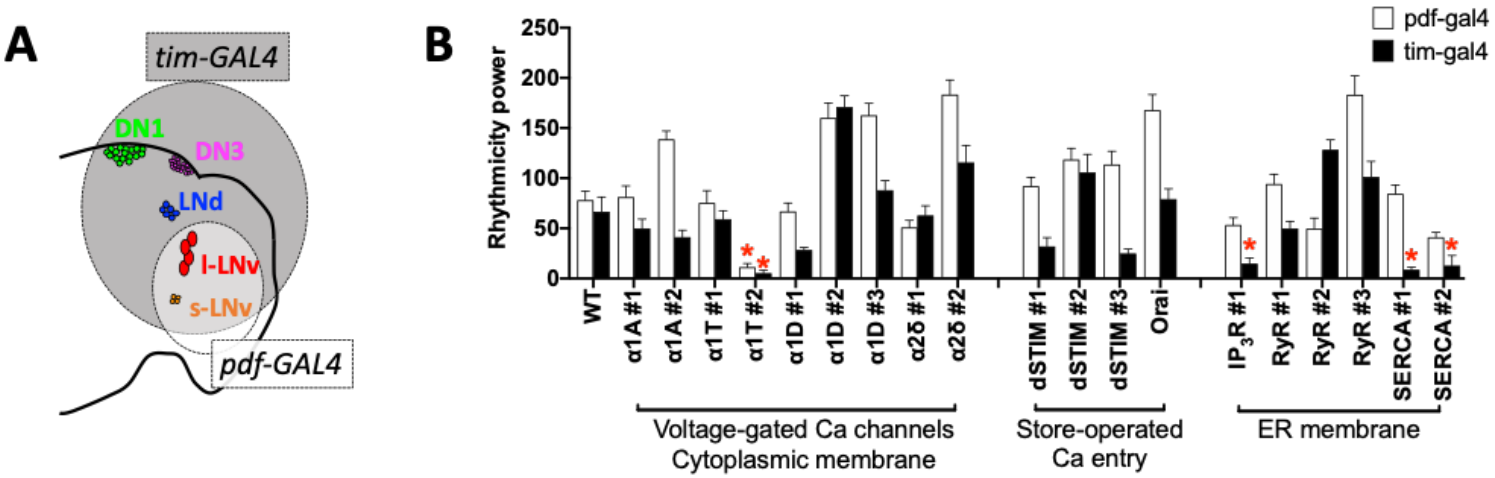

Figure 2. RNAi-screening for calcium channels required for circadian rhythms.
(A) Schematic of the five pacemaker groups in the brain superimposed with a Venn diagram of expression driven by tim-GAL4 (all groups) and pdf-GAL4 (s- and l-LNV groups). **(B)** Summary of behavioral screening for calcium-channel knockdowns that reduced average rhythm strength in locomotor activity under DD (*p<0.05, two-way ANOVA followed by Dunnett's multiple comparisons test; also see Table 1).

140 identify the sources for different calcium rhythms, and ask about their relatedness, we used
141 RNAi to knockdown different calcium channels. We performed a limited screen for calcium
142 channels, including three subtypes of α1 subunits and one type of α2δ subunits of voltage-gated
143 calcium channels; in addition, we tested two types of store-operated calcium entry (SOCE),
144 *dSTIM* and *dOrai*, and two types of calcium channels on the endoplasmic reticulum (ER):
145 ryanodine receptor (*RyR*) and inositol trisphosphate receptor (*IP3R*); finally, we included the
146 sarco/endoplasmic reticulum calcium-ATPase (*SERCA*). By knocking down these genes
147 selectively in circadian pacemaker neurons, using *tim-GAL4*, or in a subset of eight PDF-positive
148 pacemaker neurons using *pdf-GAL4*, we first tested whether any of these genes are required for
149 normal circadian behavioral rhythms. We found evidence for the involvement of three (Figure 2
150 and Table 1) as indicated by increases in the percentage of arrhythmic (%AR) flies tested under
151 constant darkness (DD). Reduced expression of a channel on the cytoplasmic membrane, *α1T,*
152 which encodes the α1 subunit for T-type voltage-gated calcium channel, caused the strongest
153 behavioral arrhythmicity when driven by either *pdf-GAL4* or by *tim-GAL4* (65% and 83%) with
154 one of the two RNAi lines tested (KK100082). Likewise, knockdown of expression of the
155 *SERCA* calcium pump, caused strong arrhythmicity in two different RNAi lines. Yet knocking
156 down *SERCA* with the stronger RNAi line in all circadian pacemakers by *tim-GAL4* also
157 shortened the flies' lifespans: 69% flies died during behavioral experiments. Knockdown of
158 another calcium channel on the ER membrane, *IP3R,* also affected the circadian rhythm in
159 behavior when driven by *tim-GAL4*. These behavioral deficits suggested that *α1T*, *SERCA*, and
160 *IP3R* might be involved in the regulation of calcium rhythms in circadian pacemaker neurons.
161

162 Table 1. The rhythm strength and period of locomotor activity under constant darkness of flies expressing calcium channel RNAi transgenes in PDF neurons or all clock neurons.

GENE	CG#	Genotype*	N#	%AR	Period	pwr	wid	SNR	ACT-day	ACT-night	ACT-cycle
Control	-	*pdf>dcr2*	24	4%	24.8	81.3	6.0	1.6	23.7	27.9	25.8
		tim>dcr2	14	21%	24.2	66.3	5.1	1.6	20.4	10.2	15.3
α1A cac	43368	*tim>JF02572*	16	0%	24.1	49.8	4.5	0.5	19.9	10.7	15.3
		pdf>JF02572	16	6%	24.5	81.0	4.1	0.7	16.4	16.1	16.2
		pdf>kk104178	16	0%	25.4	138.5	8.6	3.1	13.9	28.7	21.3
		tim>kk104178	14	7%	24.4	41.1	4.9	1.0	14.8	21.0	17.9
α1T	15899	*pdf>JF02150*	14	7%	24.5	75.1	4.9	1.5	22.1	15.6	18.8
		tim>JF02150	16	0%	23.6	59.0	4.4	1.1	14.3	2.4	8.4
		pdf>KK100082	23	65%	24.4	21.4	3.0	0.7	14.4	11.9	13.2
		tim>KK100082	23	83%	24.2	13.2	2.0	0.4	13.5	12.5	13.0
α1D	4894	*pdf>JF01848*	14	0%	24.3	66.7	4.4	0.9	18.7	13.4	16.1
		tim>JF01848	16	0%	24.3	28.1	4.1	0.5	14.3	5.7	10.0
		pdf>GD1737	16	0%	24.7	159.8	6.4	2.4	19.8	10.2	15.0
		tim>GD1737	23	0%	24.4	170.8	6.3	2.3	18.4	14.8	16.6
		pdf>HMS00294	16	0%	24.5	162.5	5.4	1.9	25.7	30.2	27.9
		tim>HMS00294	16	0%	24.0	87.9	4.1	0.8	19.7	7.9	13.8
α2δ	12295	*pdf>JF01825*	16	6%	24.3	50.8	4.6	0.7	27.1	18.0	22.6
		tim>JF01825	16	13%	23.9	62.7	5.9	1.0	25.0	13.7	19.4
		pdf>KK101267	16	0%	24.7	183.1	6.2	2.6	17.0	21.3	19.1
		tim>KK101267	15	0%	24.0	115.5	4.9	1.4	20.7	11.8	16.2
Orai	11430	*pdf>HMC03562*	15	0%	24.6	167.6	6.3	2.5	10.4	14.2	12.3
		tim>HMC03562	20	10%	23.6	78.9	4.1	0.8	10.6	4.2	7.4
		pdf>uas-dOrai	13	0%	24.9	108.9	5.6	1.0	24.8	26.2	25.5
		tim>uas-dOrai	15	0%	24.3	115.4	5.7	1.2	16.1	8.8	12.4
dSTIM	9126	*pdf>GLC01785*	16	6%	24.2	91.8	7.4	2.0	21.4	18.6	20.0
		tim>GLC01785	15	27%	23.7	31.9	3.6	0.7	13.3	6.0	9.7
		pdf>KK102366	23	0%	25.4	118.3	6.2	1.2	11.4	12.6	12.0
		tim>KK102366	22	5%	23.9	105.5	4.8	1.6	11.9	12.6	12.2
		pdf>JF02567	16	0%	24.5	113.5	5.3	1.3	23.5	24.5	24.0
		tim>JF02567	12	25%	23.8	24.7	3.3	0.6	7.3	3.4	5.3
		pdf>uas-dSTIM	15	0%	24.3	129.4	5.8	1.2	23.6	11.5	17.5
		tim>uas-dSTIM	14	0%	23.7	137.1	5.3	1.4	21.1	6.2	13.7
SERCA	3725	*pdf>JF01948*	14	0%	25.2	84.1	6.4	1.3	33.6	35.2	34.4
		tim>JF01948	12	50%	25.3	12.7	2.8	1.4	13.1	10.1	11.6
		pdf>KK107371	16	13%	24.5	40.9	4.4	0.7	28.3	29.1	28.7
		*tim>KK107371***	15	73%	23.7	65.4	3.4	0.7	14.9	8.7	11.8
RyR	10844	*pdf>HM05130*	15	0%	24.7	94.0	6.2	1.7	19.0	19.1	19.0
		tim>HM05130	16	6%	24.0	49.8	4.6	0.8	17.4	6.7	12.0
		pdf>JF03381	39	28%	24.4	55.2	3.4	0.7	16.5	11.7	14.1
		tim>JF03381	28	7%	23.9	128.1	5.3	1.4	19.1	10.1	14.6
		pdf>KK101716	13	8%	24.7	165.1	6.6	2.5	15.1	17.1	16.1
		tim>KK101716	16	0%	24.8	101.1	4.9	1.1	12.1	17.6	14.8
IP3R	1063	*pdf>ip3rRNAi#1*	36	11%	24.2	51.7	4.0	0.8	18.9	18.1	19.4
		tim>ip3rRNAi#1	20	50%	24.4	14.7	2.8	0.9	9.0	5.6	9.7

*All genotypes include *UAS-dcr2* if it is not mentioned.
N = flies alive at the end of the testing period.
**In this genotype, 69% flies died during the behavioral experiment.

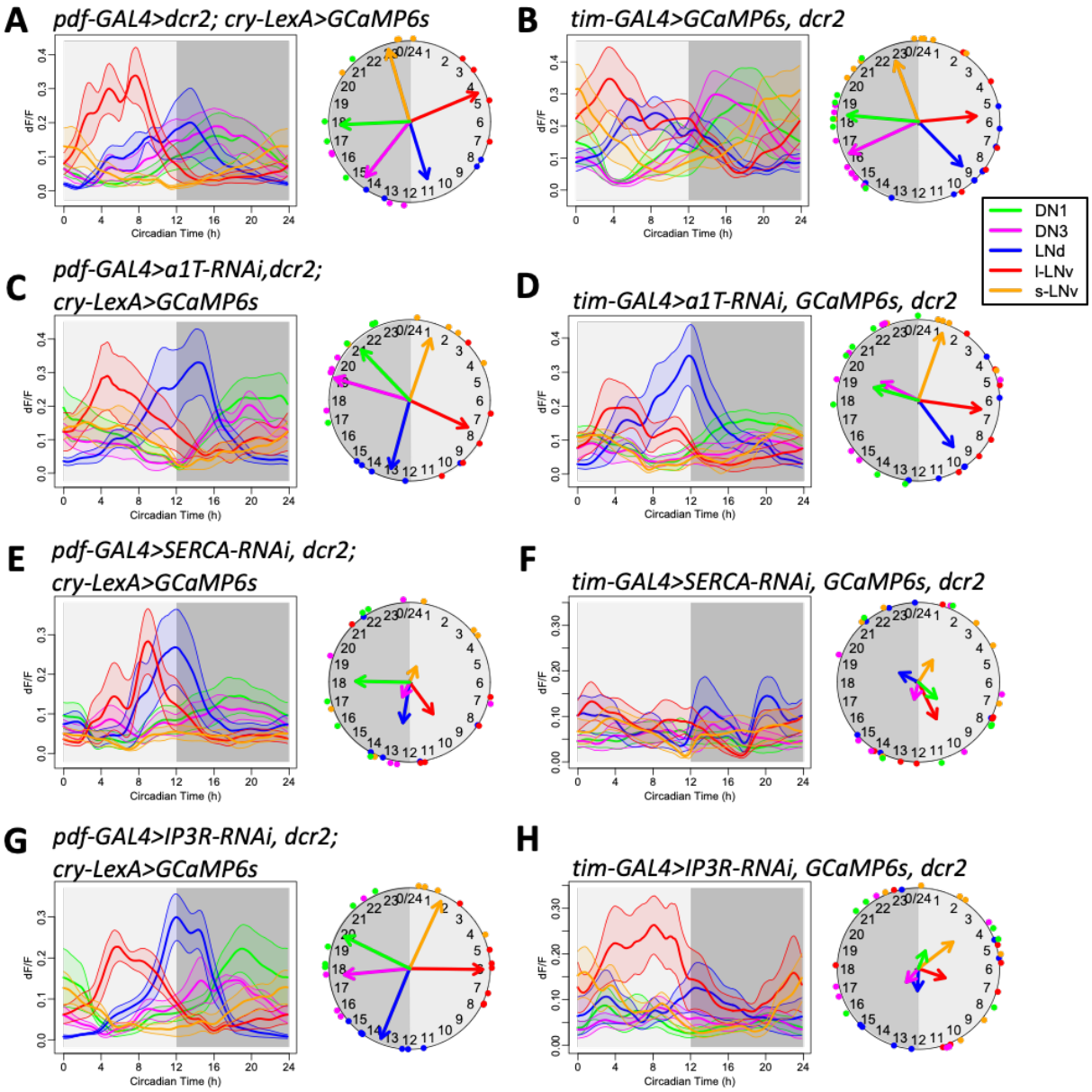

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Figure 3. RNAi-screening for calcium channels required for circadian calcium rhythms.
(A-B) Daily calcium activity rhythms of five major circadian pacemaker groups in control flies for (A) *pdf-GAL4*-driven knockdown (n = 4 flies) and (B) *tim-GAL4*-driven knockdown (n = 12 flies) in first day under constant darkness (DD1). **(C-D)** Daily calcium activity rhythms are normal in (C) *pdf-GAL4*-driven *a1T* knockdown (KK100082) flies (n = 5 flies) and (D) *tim-GAL4*-driven *a1T* knockdown flies (n = 7 flies). **(E-F)** Daily calcium activity rhythms are partially impaired in (E) *pdf-GAL4*-driven *SERCA* knockdown (KK107371) flies (n = 5 flies) and completely impaired in (F) *tim-GAL4*-driven *SERCA* knockdown flies (n = 6 flies). **(G-H)** Daily calcium activity rhythms are normal in (G) *pdf-GAL4*-driven *ip3r* knockdown flies (n = 7 flies) and impaired in (H) *tim-GAL4*-driven *ip3r* knockdown flies (n = 7 flies).

165 **Slow calcium rhythms require IP3R**

166 We then asked whether the *α1T*, *SERCA*, and *IP3R* regulating circadian behavior also
167 influence calcium rhythms. We measured GCaMP6 fluorescence during *in vivo* 24-hr recordings
168 in *Drosophila* knock-downs in all, or in just the subset of PDF-positive, circadian neurons
169 (Figure 3A-H). Although knocking down *α1T* caused the strongest behavioral deficits, the slow
170 calcium rhythms of all pacemaker neuron groups in these flies were similar to those in the
171 control genotypes (Figure 3A-D). The amplitude of calcium rhythms in flies with *α1T* knocked
172 down in all pacemaker neurons showed a non-significant trend of decrease to 59.3% on average,
173 while their activity phases were still normal (Figure S3). In contrast, when *SERCA* was knocked
174 down in PDF neurons (Figure 3E), or in all circadian neurons (Figure 3F, using the stronger
175 RNAi line KK107371), the slow calcium activities of these neurons were largely
176 arrhythmic. The amplitudes of calcium fluctuations were decreased to 37.8% on average (Figure
177 S3), and the coherence was lost within groups (Rayleigh test, $P > 0.2$). Likewise, the calcium
178 rhythms were still normal when *IP3R* was knocked down in PDF neurons (Figure 3G) but
179 became largely arrhythmic when *IP3R* was knocked down in all circadian neurons (Figure 3H).
180 In the latter case, the coherence of peak phase was lost within groups (Rayleigh test, $P > 0.1$),
181 consistent with the behavioral phenotypes of the two manipulations for *IP3R* RNAi. Knocking
182 down *IP3R* in all circadian neurons caused stronger deficits in the amplitude of calcium rhythms
183 of non-PDF-positive neurons (LNd, DN1, and DN3) than in those of PDF-positive neurons (s-
184 LNv and l-LNv) (Figure S3A). The difference in the vulnerability to IP3R disruption between

185 PDF-negative and PDF-positive neurons might
186 explain why the *PDF-GAL4*-driven knockdown
187 of *IP3R* affected neither calcium rhythms nor
188 behavior. Together, these results implicate
189 SERCA and IP3R channel activities as essential
190 for slow calcium rhythms, and suggest the ER
191 may be a key calcium source for the daily
192 fluctuations of the basal calcium levels in
193 circadian pacemaker neurons.

194 Because the slow calcium rhythms are
195 driven by molecular clock gene oscillations
196 (Liang et al 2016), we asked whether the
197 molecular clock generates the slow calcium
198 rhythms by regulating *SERCA* and *IP3R*. If
199 *SERCA* and *IP3R* are downstream of the
200 molecular clock, knocking down these genes
201 would affect calcium rhythms and behavior but
202 not affect the molecular clock itself. We
203 examined PER protein levels in all five
204 circadian pacemaker groups at four LD time points and found that PER cycling appeared robust
205 in *IP3R*-knockdown flies, but was clearly diminished in *SERCA*-knockdown flies (Figure 4).
206 Therefore, in this system, only *IP3R* appears to operate downstream of the molecular clock and
207 is necessary to generate daily rhythms in basal calcium levels.

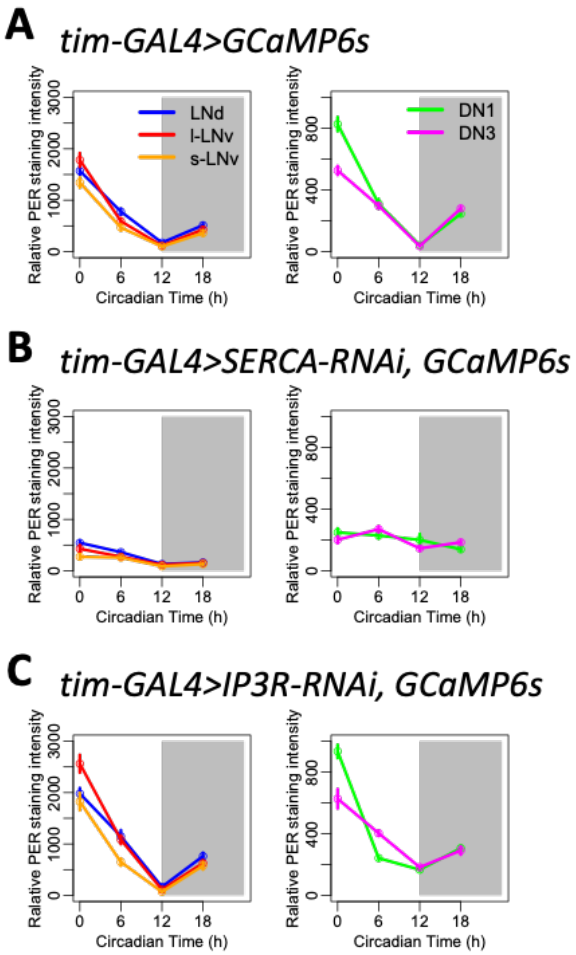

Figure 4. PER protein rhythms of control flies and flies with *SERCA* or *ip3r* knocked down in all pacemaker neurons.
(A) Averaged PER protein staining intensity at four different time points (ZT0, ZT6, ZT12, and ZT18) in five groups of circadian pacemaker neurons from control flies. **(B)** PER protein rhythms are diminished when knocking down

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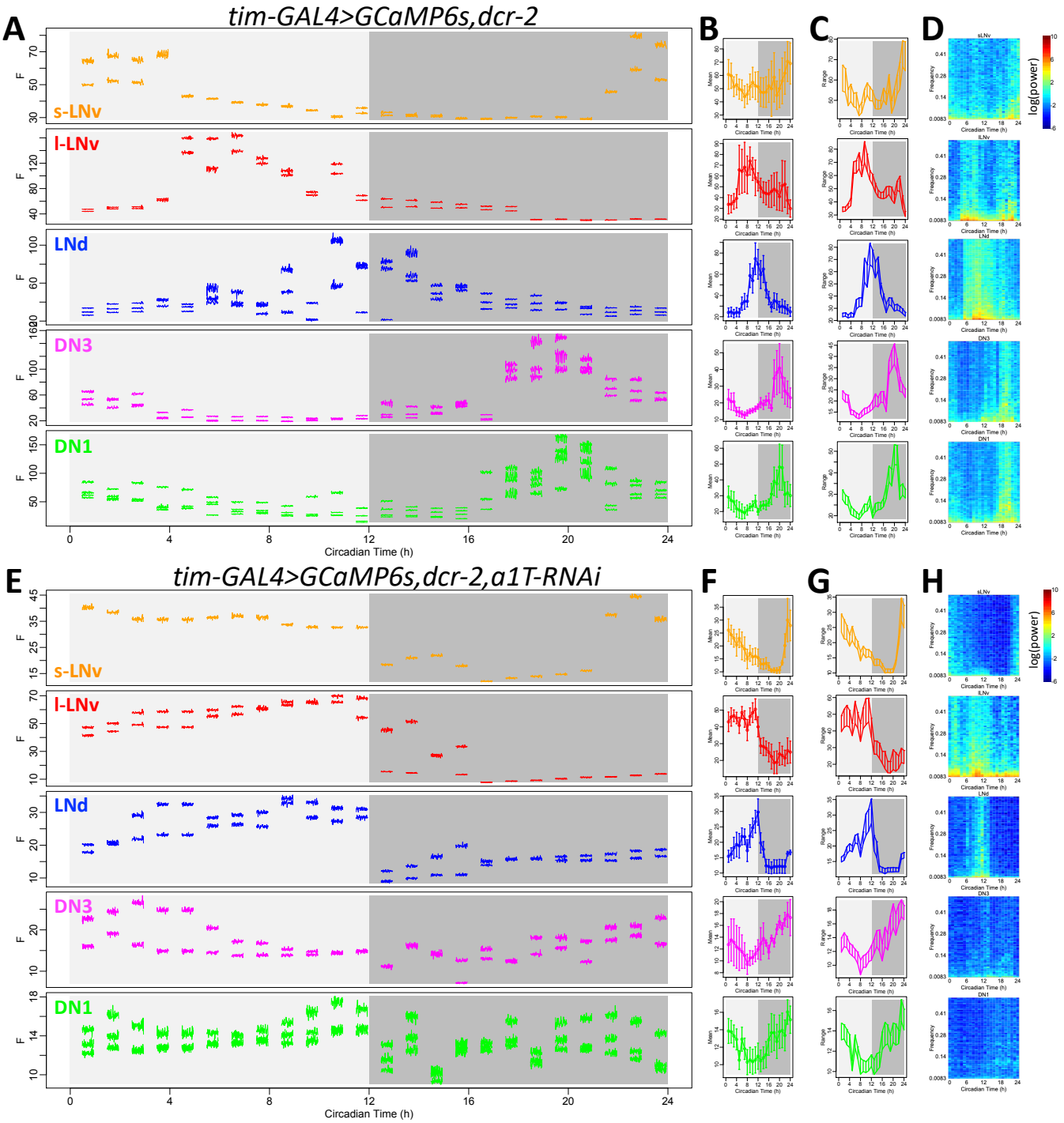
A
tim-GAL4>GCaMP6s,dcr-2
s-LNv
l-LNv
LNd
DN3
DN1
F
Circadian Time (h)
B
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E
tim-GAL4>GCaMP6s,dcr-2,a1T-RNAi
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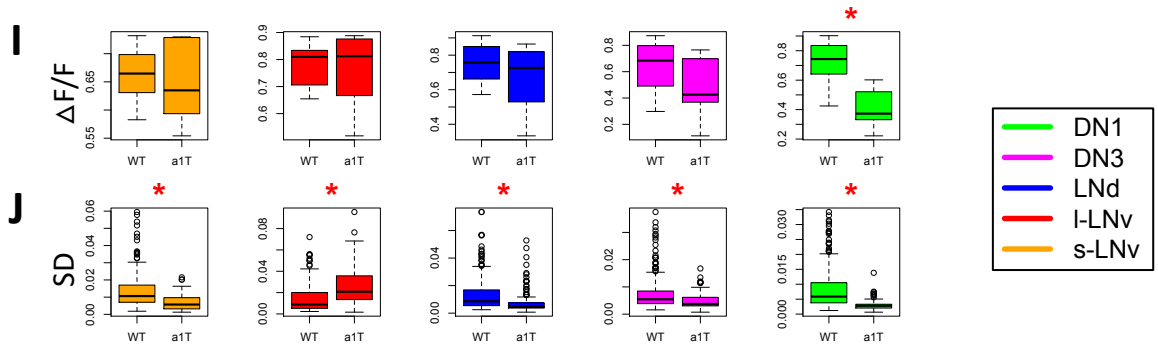
I
ΔF/F
J
SD
WT
a1T
*
DN1
DN3
LNd
l-LNv
s-LNv

Figure 5. *α1T* knockdown reduces fast calcium fluctuations.
(A-D) As Figure 1A-D, (A) raw calcium activity traces from one representative control fly. Each segmented trace is 2-min activity recorded at 1Hz. Averaged daily patterns of (B) mean calcium intensity, (C) the range of calcium transient, and (D) the power spectrum (n = 4 flies). **(E-H)** As in (A-D), raw calcium activity traces from one representative fly with *α1T* knockdown (KK100082) in all pacemaker neurons and averaged daily patterns of mean, range, and power spectrum in this genotype (n = 4 flies). **(I)** Box plot of daily range of calcium signal for individual neurons of five pacemaker groups between control flies and *α1T*-knockdown flies. The daily variation of DN1 calcium reduces in *α1T*-knockdown flies (t-test, *P < 0.05). **(J)** Box plot of standard deviations of calcium signal in each recording session for individual neurons of five pacemaker groups between control flies and *α1T*-knockdown flies. The standard deviations of fast calcium fluctuations in s-LNv, LNd, DN3, and DN1 are smaller, while that in l-LNv is larger in *α1T*-knockdown flies than that in control (t-test, *P < 0.05).

209

210 **Fast calcium fluctuations require *α1T* calcium channels and *IP3R***

211 Knocking down the RNA for *α1T* voltage-gated calcium channels in pacemaker neurons
212 impaired circadian rhythms in behavior, but did not affect circadian rhythmicity in basal calcium
213 levels within those neurons. Therefore, we next asked whether *α1T* may underlie the circadian
214 rhythm of fast calcium fluctuations in pacemakers. To test this, we again performed imaging
215 across a series of short-term (2 min) high-frequency (1 Hz) calcium measurements on the same
216 flies for a 24-hour day with 1-hour intervals (similar to Figure 1, yet with a slightly lower
217 sampling rate and using flies that were WT for *cry*). By comparing control *Drosophila* to those
218 with *α1T* knocked down in all circadian pacemaker neurons by *tim-GAL4,* we found that
219 knocking down *α1T* did not affect the daily rhythms in the basal (slow) calcium level in any
220 pacemaker group except for the DN1, which showed a reduction in the day-night difference of
221 basal calcium level (Figure 5A-I and Figure S4). These high-frequency measures of slow basal
222 calcium levels largely conform with those obtained with the slow-frequency (every 10 m)
223 recording sessions (cf. Figure 3D). However, the high-frequency recording revealed that fast
224 calcium fluctuations were significantly reduced in all circadian pacemaker neurons of the *α1T*-

225 knockdown flies, except for the l-LNv, (Figure 5J and Figure S5). That specific pacemaker group
226 instead displayed higher levels of fast calcium fluctuations. These results indicated that, at least
227 in the majority of circadian pacemaker neurons, *α1T* is required for strong daily rhythms in fast
228 calcium fluctuations and that the rhythm of fast calcium fluctuations can be selectively impaired.
229
230 We then asked whether the fast calcium fluctuations are also affected in flies with *IP3R*
231 knocked down in all pacemaker neurons. We performed the same high-frequency calcium
232 imaging and analysis as in *α1T*-knockdown flies (Figure 6). The daily variation of pacemaker
233 calcium was greatly reduced in *IP3R* -knockdown flies (Figure 6E), which largely recapitulated
234 previous observations with less frequent sampling (cf. Figure 3H). In addition, fast calcium
235 fluctuations were significantly reduced in all circadian pacemaker neurons of the *IP3R*-
236 knockdown flies (Figure 6F and Figure S5). These results suggested that rhythms in basal
237 calcium levels, regulated by *IP3R*, may be necessary for rhythms in fast calcium fluctuations.

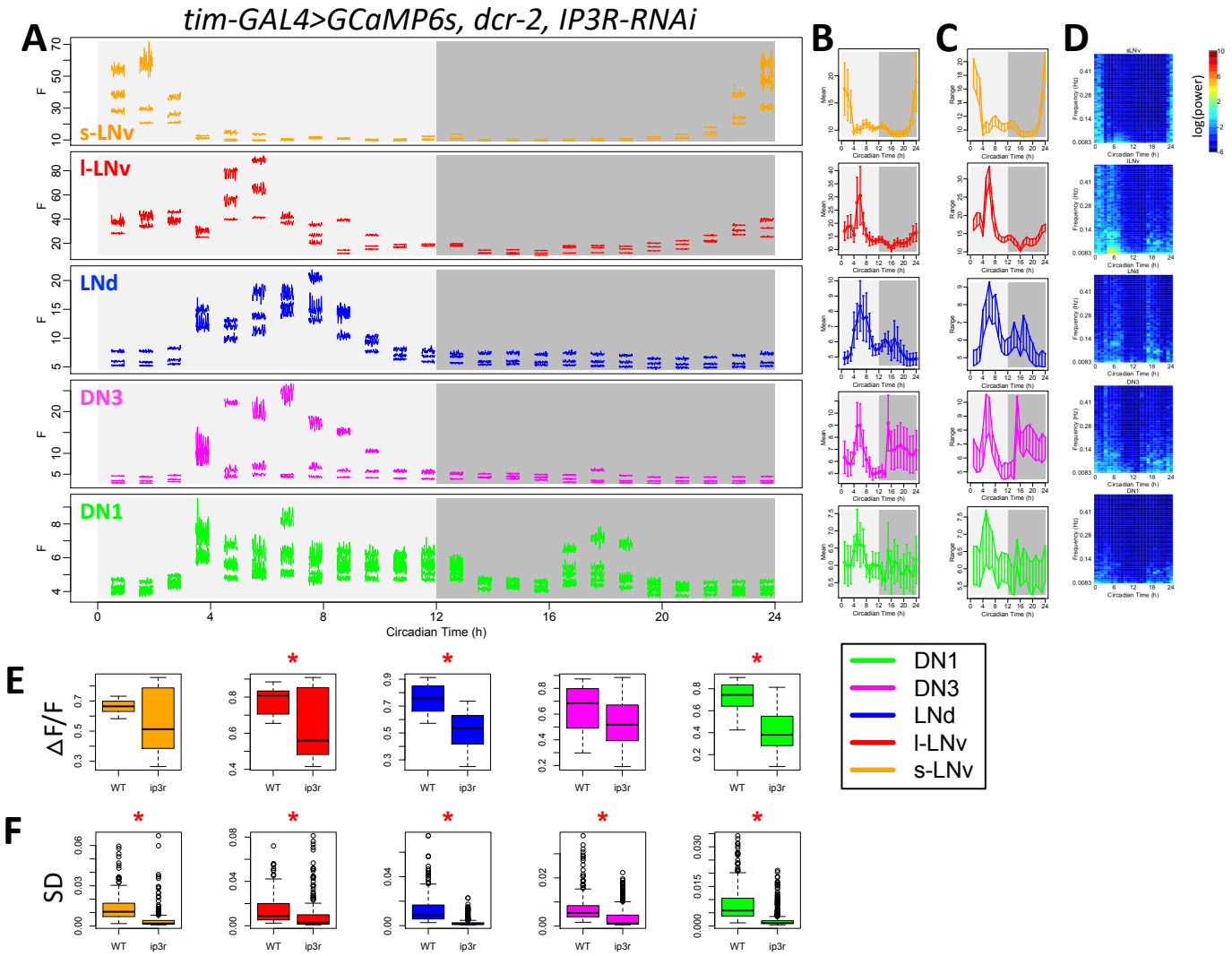

Figure 6. *IP3R* knockdown reduces fast calcium fluctuations.
(A-D) As Figure 1A-D, (A) raw calcium activity traces from one representative fly with *a1T* knockdown in all pacemaker neurons. Each segmented trace is 2-min activity recorded at 1Hz. Averaged daily patterns of (B) mean calcium intensity, (C) the range of calcium transient, and (D) the power spectrum (n = 5 flies). **(E)** Box plot of daily range of calcium signal for individual neurons of five pacemaker groups between control flies and *IP3R*-knockdown flies. The daily variation of l-LNv, LNd, and DN1 calcium reduces in *IP3R*-knockdown flies (t-test, *P < 0.05). **(F)** Box plot of standard deviations of calcium signal in each recording session for individual neurons of five pacemaker groups between control flies and *IP3R*-knockdown flies. The standard deviations of fast calcium fluctuations in all circadian pacemaker neuron groups are smaller in *IP3R*-knockdown flies than that in control (t-test, *P < 0.05).

238

239 **Discussion**

240 In this study, we used *in vivo* 24-hr high-frequency calcium imaging and genetic
241 screening to study the cellular biology of daily calcium rhythms in circadian pacemaker neurons
242 of *Drosophila*. We found that the calcium rhythm is in fact a composite: it reflects daily
243 fluctuations in both a slow component (basal levels) and a fast one (high frequency fluctuations).
244 We interpret fast calcium fluctuations as representations of calcium dynamics that occur as
245 neurons fire single action potentials or bursts of them. While it is not sufficient to resolve single
246 action potentials, GCaMP6-induced fluorescence is a good index of neuronal electrical activity
247 (e.g. Chen et al., 2013; Greenberg et al 2018). For individual identified pacemakers, these two
248 calcium rhythms share the same daily pattern, yet distinct calcium sources appear to contribute
249 differentially to these two rhythms. An extracellular calcium influx, through plasma membrane
250 calcium channels that include the *α1T* subunit, is critical for the fast calcium fluctuations. In
251 contrast, calcium fluxes from the ER via the channel *IP3R* are required for both the slow rhythms
252 in the basal calcium levels and the fast ones. Importantly, both channels are essential for normal
253 circadian behavior. Thus, the molecular clocks may drive circadian rhythms in pacemaker
254 neuron output by regulating different calcium sources to generate coordinate, but distinct
255 rhythms in its calcium activities.

256 Circadian calcium rhythms (CCR) are widespread across taxa (Knight et al 1991; Colwell
257 2000). Calcium rhythms are required for circadian pacemaker functions in both rodents and
258 *Drosophila* (Lundkvist et al 2005; Harrisingh et al 2007). Studies on mammalian circadian
259 pacemakers in the suprachiasmatic nucleus (SCN) are controversial regarding the temporal
260 relationship between the CCR and rhythms in electrical activity, such as in spontaneous firing
261 rate (SFR) and in resting membrane potential (RMP). Recordings from SCN slice cultures

262 showed that the phases of CCR in individual pacemakers are diverse and could be different from
263 the populational phase of SFR rhythms (Ikeda et al 2003; Enoki et al 2012). However, the
264 populational SFR phase is composed of many diverse phases of SFR rhythms on the individual
265 cell level (Vanderleest et al, 2007); it is unclear whether SFR phases align with the phases of
266 CCR. Imaging with both voltage sensor and calcium sensors in SCN slices, Brancaccio et al
267 (2017) concluded that RMP rhythms and CCR were in phase, yet Enoki et al (2017) concluded
268 that RMP rhythms and CCR were in phase in the ventral SCN, but in dorsal SCN, the CCR
269 phase-led the RMP rhythms by about 2 hours. Because the voltage sensor signal measured from
270 dorsal SCN may derive from the neural processes of ventral SCN neurons, the cellular
271 interpretation of these results is unclear. Another source for the inconsistency might be the
272 culture conditions: when SCN neurons were recorded *in vivo* by photometry, the rhythms in fast
273 calcium activity were in phase with slow calcium rhythms (Jones et al 2018). In general,
274 comparisons of population rhythms and rhythms in single cells are not easily reconciled. Our
275 recordings that tracked pacemaker neurons from different identified groups *in vivo* for 24 hours
276 showed that at the single cell level, slow calcium rhythms (CCR) were in phase with rhythms in
277 fast calcium fluctuations; the latter likely reflect rhythms in SFR (Figure 1).
278 To measure the fast calcium fluctuations, we employed high-frequency light-sheet
279 scanning with the wavelength of light that may activate pacemaker neurons and alter molecular
280 clocks (Fogle et al 2011). We used cry^{01} flies to avoid the direct light responses of pacemaker
281 neurons and found that all pacemaker groups displayed slow calcium rhythms, comparable to
282 those we previously reported (Liang et al 2016), except for the l-LNv (Figure 1) which showed
283 an additional calcium peak in the early evening. Because l-LNv innervate the optic lobes and
284 receive large-scale visual inputs (Ashmore and Sehgal 2003), we speculate that the repeated

285 optical scanning might activate l-LNv in the evening via visual systems. In the later experiments,
286 when we reduced the illumination duration per hour from 31.5 sec (Figure 1, 7 ms exposure
287 time, 15 frames per stack, 5Hz for 1min) to 2.4 sec (Figure 5 & 6, 1 ms exposure time, 20 frames
288 per stack, 1Hz for 2 min), even in *cry* wild-type flies l-LNv did not show the evening peak. All
289 pacemaker groups displayed slow calcium rhythms (Figure 5A), with the same phases as those
290 obtained with the slow-frequency (every 10 min) recording sessions (cf. Figure 4D and Liang et
291 al 2016). Therefore, by carefully tuning the illumination intensity for calcium imaging, we could
292 monitor normal slow calcium rhythms and fast calcium fluctuations from the same individual
293 pacemaker neurons.

294 The causal relationships between clock gene rhythms, calcium rhythms, and electrical
295 activity rhythms in the SCN remain generally unresolved. Treating SCN slices with TTX (to
296 block Na-dependent action potentials) diminished SFR rhythms (Ikeda et al 2003), partially
297 affected RMP rhythms and CCRs (Hong et al 2012; Enoki et al 2012; Enoki et al 2017), and
298 slowly affected clock gene rhythms over several days (Yamaguchi et al 2003). Dispersed SCN
299 cells *in vitro* showed a TTX-resistant CCR, suggesting that CCR is driven by clock gene rhythms
300 (Noguchi et al 2017). Thus, the variation in CCR sensitivity to TTX treatment might be caused
301 by the degree to which clock gene rhythms *in vitro* become progressively dysfunctional. In
302 *Drosophila*, our findings suggested that clock gene rhythms drive two components of the CCR -
303 both basal calcium levels and fast calcium fluctuations - via circadian regulation of the ER
304 channel *IP3R* and membrane voltage-gated calcium channel *α1T*. Both channels might then
305 contribute to SFR and RMP rhythms. Similarly, in SCN pacemakers, pharmacologically
306 blocking another ER channel *RyR* affected both CCR and SFR rhythms (Ikeda et al 2003),
307 suggesting that rhythms in basal calcium levels are regulated by calcium from ER and are

308 required for fast electric activity rhythms. In addition, SCN pacemakers also showed a circadian
309 rhythm in fast calcium activity mediated by L-type voltage-gated calcium channels (Pennartz et
310 al 2002). Pharmacologically blocking these membrane channels affected SFR rhythms and in
311 some case affected CCR (Ikeda et al 2003; Enoki et al 2017). In our studies, manipulating a
312 membrane voltage-gated calcium channel in all or a subset of pacemakers selectively affected
313 rhythms in fast calcium fluctuations, which likely reflected SFR rhythms and thus impaired
314 circadian outputs; however, it did not significantly affect the slow rhythms in basal calcium
315 levels (Figure 5). Manipulating the ER calcium channel IP3R in all pacemakers affected rhythms
316 in both slow and fast calcium rhythms. Therefore, in parallel to mammalian SCN neurons,
317 *Drosophila* circadian pacemakers generate calcium rhythms by regulating both ER and
318 extracellular calcium sources. Since our results suggest little or no role for the RyR channel
319 (Figure 2B), the daily rhythmic regulation in fly pacemakers acts on a different set of ER and
320 cytoplasmic membrane channels from those in mammalian pacemakers.
321 The RNAi knockdown experiments indicate a role for the ER calcium channel SERCA in
322 supporting slow calcium rhythms in *Drosophila* pacemakers and behavioral rhythms. However,
323 the high degree of lethality and the strong effects of SERCA-knockdown on the PER molecular
324 oscillation precluded an assessment of its precise role. We conclude that *SERCA*, which
325 maintains the ER-cytoplasmic calcium gradient, is essential for the normal physiology of the
326 cells. In contrast to *SERCA*, our results support a hypothesis that *IP3R* is crucial actuator of the
327 molecular clock. Previous transcriptomic analysis also supports that possibility: in circadian
328 neurons, *IP3R* displays rhythmic expression, while *SERCA* does not (Figure S7; Abruzzi et al
329 2017).

330 Finally, the RNAi knockdown of plasma membrane calcium channel α1T indicated a role
331 for voltage-gated T-type calcium channels in the final rhythmic output of the pacemakers.
332 Consistent with a role in the presumed output pathway, impairing the rhythm of fast calcium
333 activity strongly affected circadian behavior but did not affect the molecular clock or the slow
334 calcium rhythms. T-type channels play a crucial role in other pacemakers such as the SA node of
335 the mammalian heart (cite). Their conduction in the hyperpolarized state, and closure at more
336 depolarized potentials, is central to their role in generating bursting dynamics with periods much
337 longer than the membrane time constant (cite). Given the power spectrum of the fluctuations we
338 observed, it seems possible they play a similar role in the "fast" (~0.1Hz) fluctuations of
339 Drosophila circadian neurons. Remarkably, the expression of *α1T* also displays a circadian
340 rhythm, with distinct phases in different groups of pacemaker neurons (Figure S7; Abruzzi et al
341 2017). Collectively, these results suggest that *IP3R* and *α1T* channel activity are together critical
342 to produce clock regulation of rhythms in both slow and fast calcium activities of critical
343 pacemaker neurons.
344

345 **Methods**

346 **<u>Fly stocks.</u>**

347 Flies were reared on standard yeast-supplemented cornmeal/agar food at room temperature. After
348 eclosion, male flies were entrained under 12 h light: 12 h dark (LD) cycles at 25°C for at least 3
349 days. The *tim>GCaMPS6s; cry*$^{01/01}$ flies were entrained under LD for more than 6 days.
350 The following fly lines were previously described: *tim(UAS)-GAL4* (Blau & Young 1999), *pdf-*
351 *GAL4* (Renn et al 1999), *cry-LexA* (Liang *et al.,* 2017), *UAS-GCaMP6s* and *LexAop-GCaMP6s*
352 (Chen *et al.,* 2013). *UAS-dSTIM* and *UAS-dOrai* (Agrawal et al 2010) were gifts from Dr. G
353 Hasan (NCBS, India). *UAS-ip3rRNAi* (Liu et al 2016) was a gift from Dr. M Wu (Johns Hopkins
354 U.). Stable line: *UAS-dcr2; tim(UAS)-GAL4; UAS-GCaMP6s* and *pdf-GAL4;UAS-dcr2;cry-*
355 *LexA,LexAop-GCaMP6s* were created for RNAi screening of calcium channels. The *cry-LexA*
356 line was a gift from Dr. F Rouyer (CNRS Gyf, Paris).
357 RNAi lines were obtained from Bloomington Stock Center, Vienna Drosophila Resource Center,
358 and Tokyo Stock Center. Two lines for *cac* (CG43368): *UAS-KK101478-RNAi* (VDRC 104168)
359 and *UAS-JF02572-RNAi* (BDSC 27244). Two lines for *α1T* (CG15899): *UAS-KK100082-RNAi*
360 (VDRC 108827) and *UAS-JF02150-RNAi* (BDSC 26251). Three lines for *α1D* (CG4894): *UAS-*
361 *GD1737-RNAi* (VDRC 51491), *UAS-JF01848-RNAi* (BDSC 25830), and *UAS- HMS00294-*
362 *RNAi* (BDSC 33413). Two lines for *α2δ* (CG12295): *UAS-KK101267-RNAi* (VDRC 18569) and
363 *UAS-JF01825-RNAi* (BDSC 25807). One line for *Orai* (CG11430) *UAS-HMC03562-RNAi*
364 (BDSC 53333). Three lines for *dSTIM* (CG9126): *UAS-KK102366-RNAi* (VDRC 106256), *UAS-*
365 *GLC01785-RNAi* (BDSC 51685), and *UAS-JF02567-RNAi* (BDSC 27263). Two lines for
366 *SERCA* (CG3725): *UAS-KK107371-RNAi* (VDRC 107446) and *UAS- JF01948-RNAi* (BDSC

367 25928). Three lines for *RyR* (CG19844): *UAS-KK101716-RNAi* (VDRC 109631), *UAS-*
368 *HM05130-RNAi* (BDSC 28919), and *UAS-JF03381-RNAi* (BDSC 29445).
369
370 ***In vivo* fly preparations and calcium imaging.**
371 The fly surgery followed procedures previously described (Liang et al 2016; 2017). Flies were
372 first anesthetized by CO_2 and immobilized by inserting the neck into a narrow cut in an
373 aluminum foil base. A portion of the dorso-anterior cuticle on one side of the head, an antenna,
374 and a small part of one compound eye were then removed. For slow calcium rhythm
375 measurements, imaging was conducted with a custom horizontal-scanning Objective Coupled
376 Planar Illumination (hsOCPI) microscope (Holekamp *et al.,* 2008). The scanning was done by
377 moving the stage horizontally every 10 min for 24 hours. Each scan contained 20-40 separate
378 frames with a step size of 5 to 10 microns. For fast calcium rhythm measurements, imaging was
379 conducted with a custom high-speed dual-channel Objective Coupled Planar Illumination (OCPI-
380 II) microscope (Greer and Holy 2019). Each scanning session involved moving the objective
381 using a piezo motor at 1-5 Hz for 1-2 min. The same scans were then repeated on the same
382 specimens every hour, for 24 hrs. During both slow and fast imaging modes, HL3 saline was
383 continuously perfused (0.1-0.2 mL/min).
384
385 **Locomotor activity rhythm.**
386 Trikinetics *Drosophila* Activity Monitor (DAM) system was used to monitor the locomotor
387 activity rhythms of individual flies. 4-6-day-old male flies were monitored for 6 days under
388 light-dark (LD) cycles and then for 9 days under constant darkness (DD) condition. The
389 circadian rhythmicity and periodicity were measured by χ^2 periodogram with a 95% confidence

390 cutoff and SNR analysis (Levine et al 2002). Arrhythmicity were defined by a power value (χ^2
391 power at best period) less than 10, width lower than 1, a period less than 18 hours or more than
392 30 hours.
393
394
395 **Immunocytochemistry.**
396 The flies were entrained for 6 days under LD and dissected at ZT0, ZT6, ZT12, and ZT18. After
397 dissected in ice-cold, calcium-free saline, fly brains were fixed for 15 min in 4%
398 paraformaldehyde containing 7% picric acid (v/v) in PBS. Primary antibodies were rabbit anti-
399 PER (1:5000; kindly provided by Dr. M. Rosbash, Brandeis Univ.; Stanewsky *et al.,* 1997).
400 Secondary antisera were Cy3-conjugated (1:1000; Jackson Immunoresearch, West Grove, PA).
401 Images were taken on the Olympus FV1200 confocal microscope. PER protein immunostaining
402 intensity was measured in ImageJ-based Fiji (Schindelin *et al.,* 2012).
403
404 **Imaging data analysis.**
405 Calcium imaging data was acquired by a custom software, Imagine (Holekamp *et al.,* 2008) and
406 pre-processed using custom scripts in Julia 0.6 to produce non-rigid registration, alignment and
407 maximal projection along z-axis. The images were then visualized and analyzed in ImageJ-based
408 Fiji by manually selecting regions of interest (ROIs) over individual cells or groups of cells and
409 measuring the intensity of ROIs over time. Slow calcium activity was analyzed as described
410 previously (Liang *et al.,* 2016, 2017). Fast calcium activity in each scanning session was
411 analyzed similarly. Between sequential scanning sessions, the ROIs for individual neurons were
412 manually corrected for position drifts. For the calcium signal of each ROI in each session, the

413 mean of calcium intensity and the range of calcium intensity change was measured, and the
414 power spectrum was generated by fast Fourier transform. Then the calcium signal was filtered by
415 a high-pass filter (1/15 Hz) and the standard deviation of calcium changes was measured.
416 Calcium activity trace analysis and statistics were performed using R 3.3.3 and Prism 8
417 (GraphPad, San Diego CA).

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426

427 **Author contributions**

428 X.L., T.H.E., and P.H.T. conceived the experiments; X.L. performed and analyzed all
429 experiments; X.L., P.H.T. and T.H.E. wrote the manuscript.

430

431 **Declaration of Interests**

432 The authors have no financial interests or positions to declare. T.E.H. has a patent on OCPI
433 microscopy.

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Supplemental Materials

Figure S1

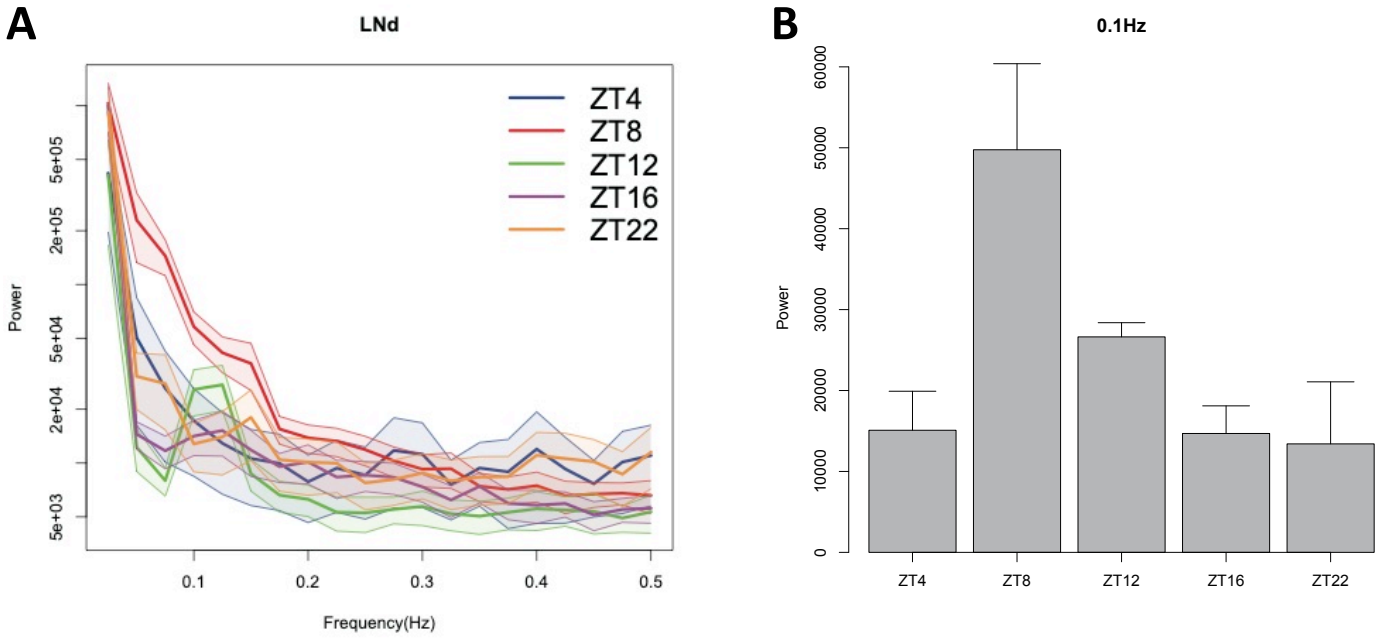

Figure S1. Daily pattern of fast calcium activity in LNd.
(A) Averaged power spectrums for five different timepoints (n > 6 flies for each timepoint). **(B)** Averaged power at 0.1Hz for five different timepoints shows a similar daily pattern as LNd slow calcium rhythms.

Figure S2

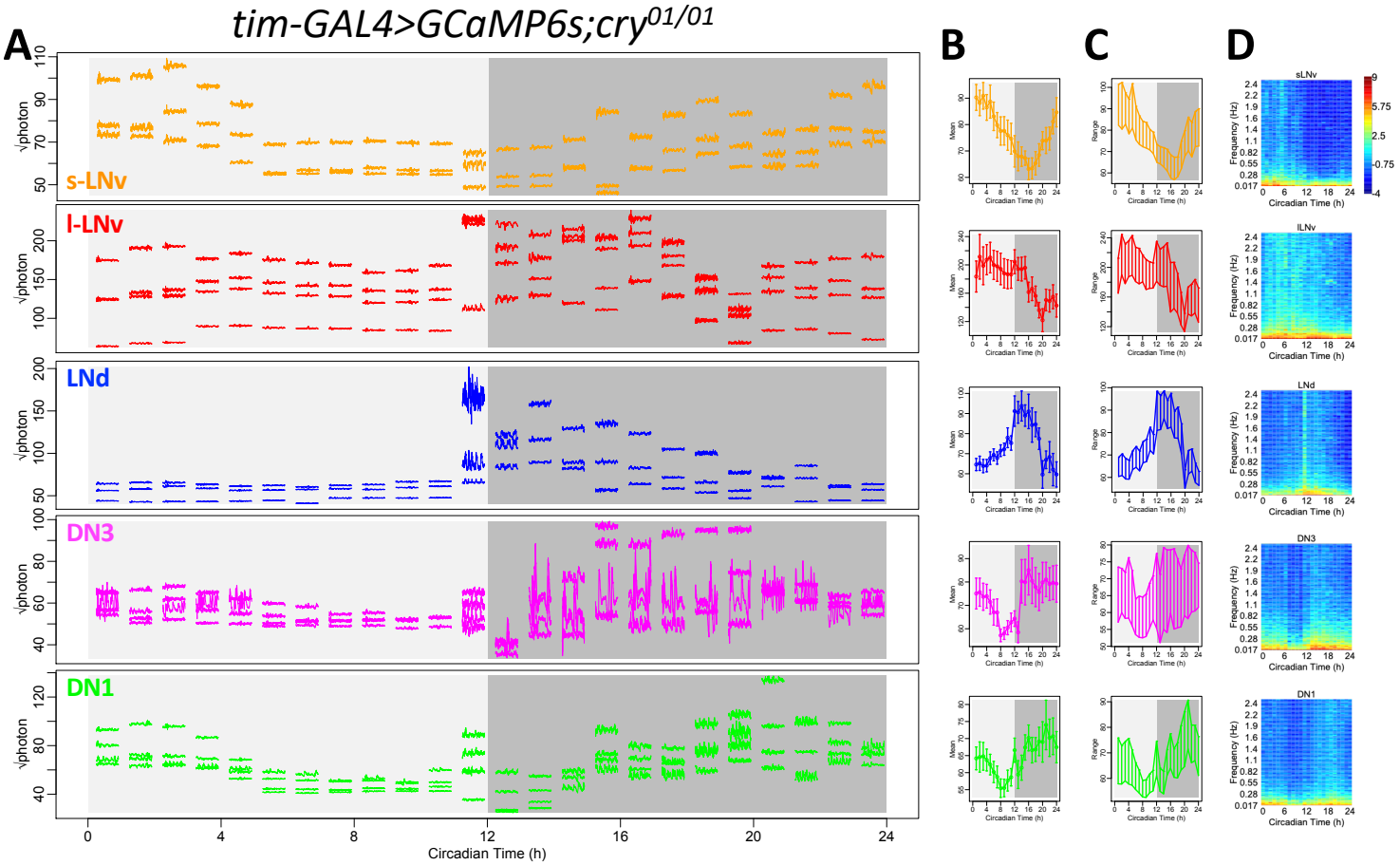

Figure S2. Daily pattern of fast calcium activity in circadian pacemaker neurons normalized for shot noise. (A) Raw calcium activity traces from the representative fly shown in Figure 1A. The intensity of calcium signal was calculated as the square root of photon number collected from individual region of interest (ROI), in order to normalize the effect of shot noise. **(B)** The mean of the photon number square root over the 1-min recording session at each of the 24 timepoints, averaged across all 6 flies studied. Error bars denote SEM. **(C)** Daily patterns of the range of calcium transients based on the square root of photon number collected from individual ROI, over the 1-min recording session at each of the 24 timepoints, averaged across all 6 flies studied. **(D)** Daily pattern of the power spectrum based on the square root of photon number collected from individual ROI, over the 1-min recording session at each of 24 timepoints, averaged across all 6 flies studied.

Figure S3

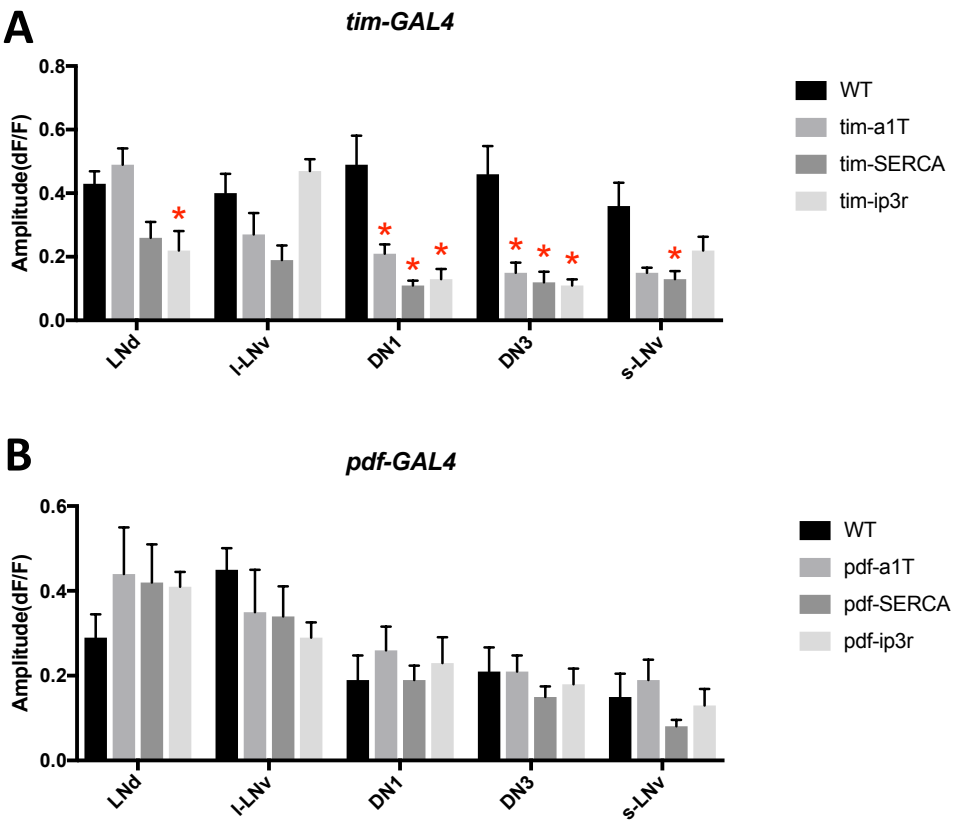

Figure S3. Amplitude of daily calcium peaks in calcium-channel-knockdown flies.
(A) The averaged amplitude of daily calcium peaks of five pacemaker groups in flies measured in Figure 3B, 3D, 3F, and 3H, wild type (*tim-GAL4>dcr2*) or with calcium channels knockdown by *tim-GAL4* (two-way ANOVA followed by Dunnett's multiple comparisons test, *P < 0.05). **(B)** The averaged amplitude of daily calcium peaks of five pacemaker groups in flies measured in Figure 3A, 3C, 3E, and 3G, wild type (*pdf-GAL4>dcr2*) or with calcium channels knockdown by *pdf-GAL4*.

Figure S4

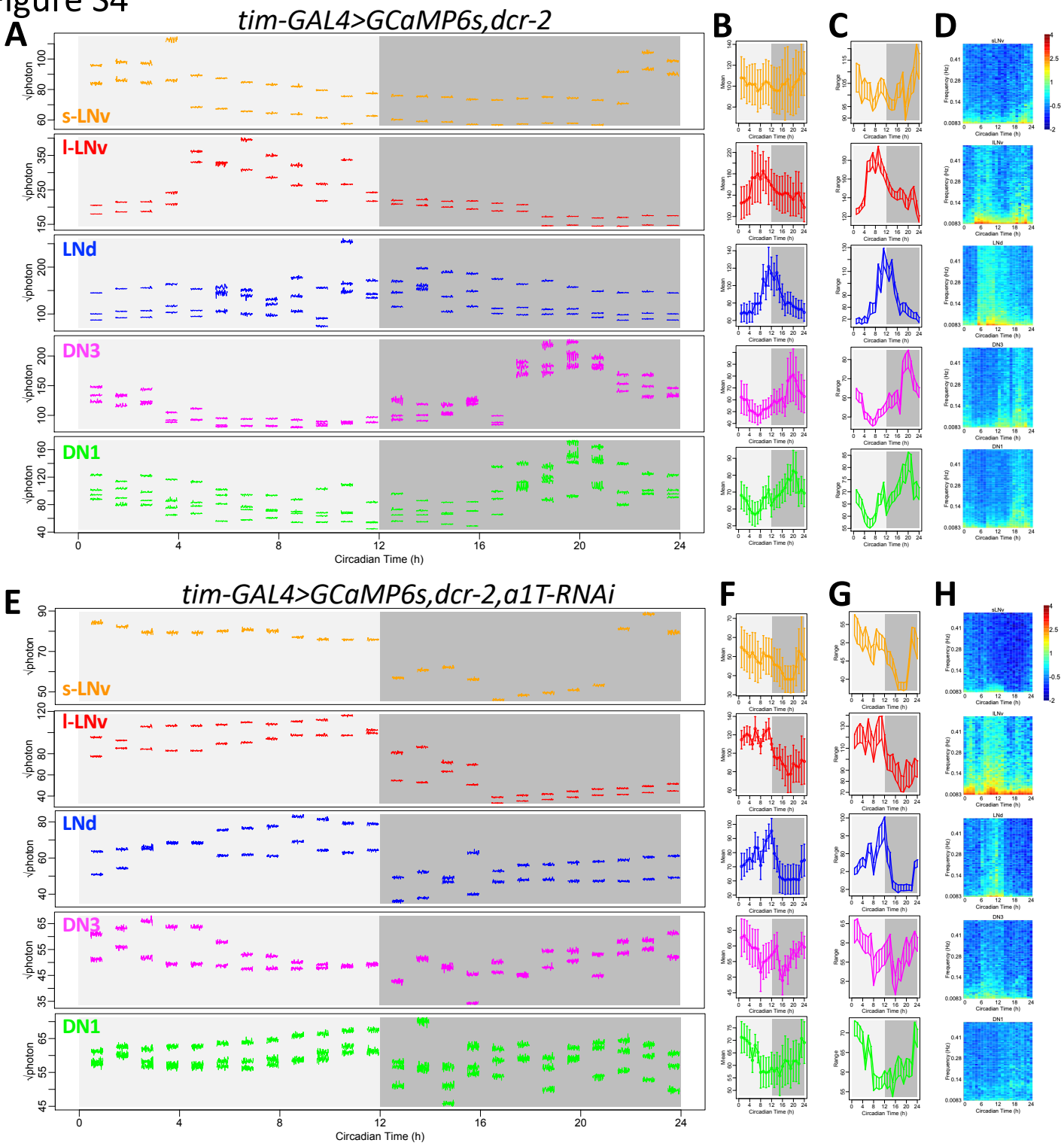

Figure S4. Changes in shot noise levels do not explain reductions in fast calcium fluctuations by *a1T* knockdown.

(A-D) As Figure S2A-D, (A) raw calcium activity traces from the representative fly shown in Figure 5A. The intensity of calcium signal was calculated as the square root of photon number collected from individual region of interest (ROI), in order to normalize the effect of shot noise. Averaged daily patterns of (B) mean calcium intensity, (C) the range of calcium transient, and (D) the power spectra were calculated based on the square root of photon number collected from individual ROIs (n = 4 flies). **(E-H)** As in (A-D), raw calcium activity traces from one representative fly shown in Figure 5E with *a1T* knockdown in all pacemaker neurons and averaged daily patterns of mean, range, and power spectrum in this genotype calculated based on the square root of photon number collected from individual ROIs (n = 4 flies).

Figure S5

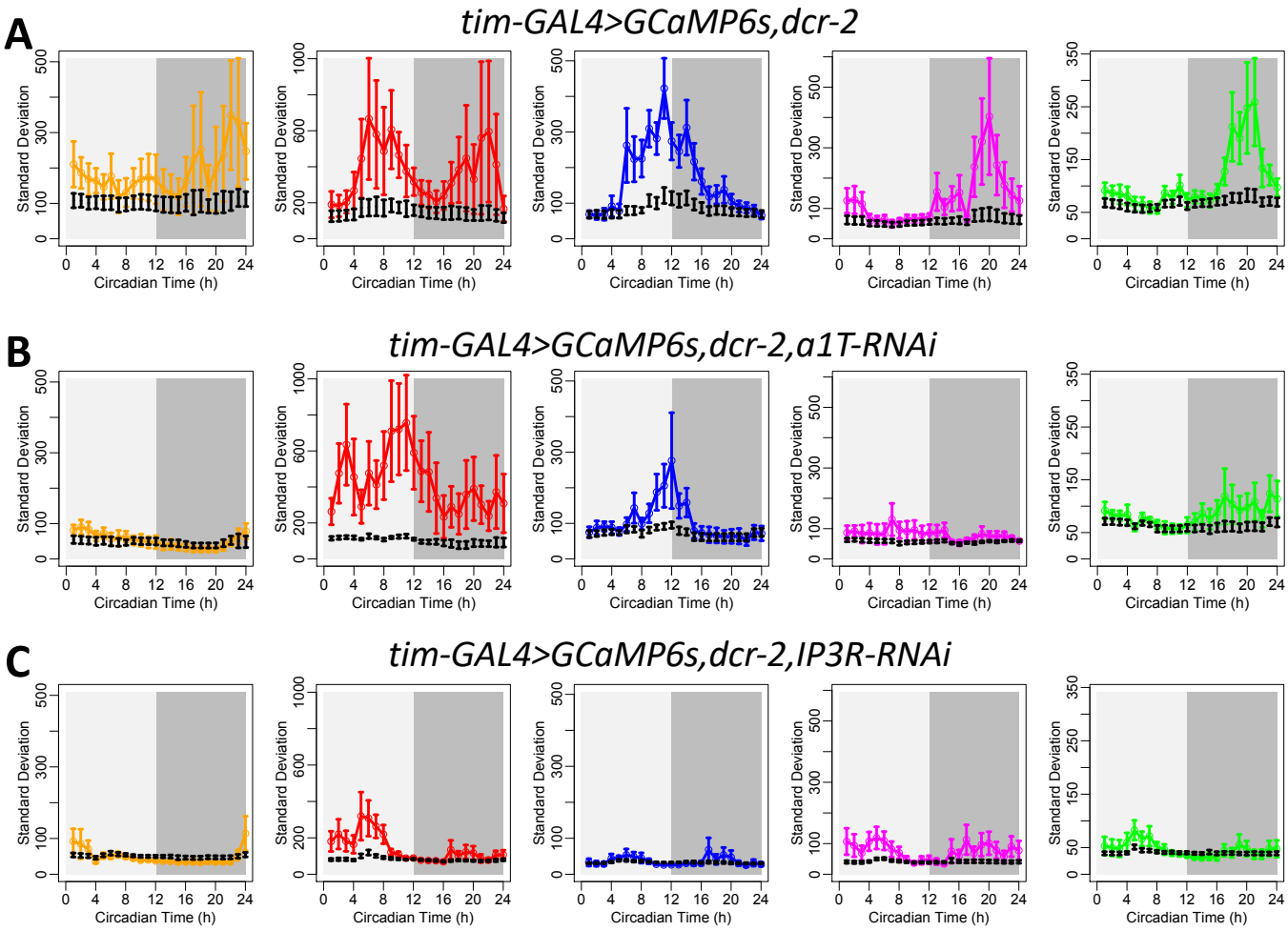

Figure S5. Fast calcium fluctuations of *a1T* or *IP3R* knockdown were below the range of shot noise. **(A)** Comparison of standard deviations between calcium activity and shot noise in wild type flies. Color lines, daily pattern of the standard deviations of calcium intensity (calculated as the collected photon number from individual ROIs) over the 1-min recording session at each of the 24 timepoints, averaged across all wide-type 4 flies studied in Figure 5A-D. Error bars denote SEM. For each timepoint, black error bars were the standard deviations of shot noise estimated by the square root of photon number collected from individual ROIs. At those time of day when mean calcium signal was high (shown in Figure 5B), the standard deviations of calcium signal were significantly higher than the standard deviations generated by shot noise , suggesting that the calcium fluctuation was generated by physiologically-relevant events. **(B)** Comparison of standard deviations between calcium activity and shot noise in flies shown in Figure 5E-H with *a1T* knockdown. Except for LNd and l-LNv, the standard deviations of calcium signal were not significantly different from the standard deviations generated by shot noise at any time of day. **(C)** Comparison of standard deviations between calcium activity and shot noise in flies shown in Figure 6 with *IP3R* knockdown.

Figure S6

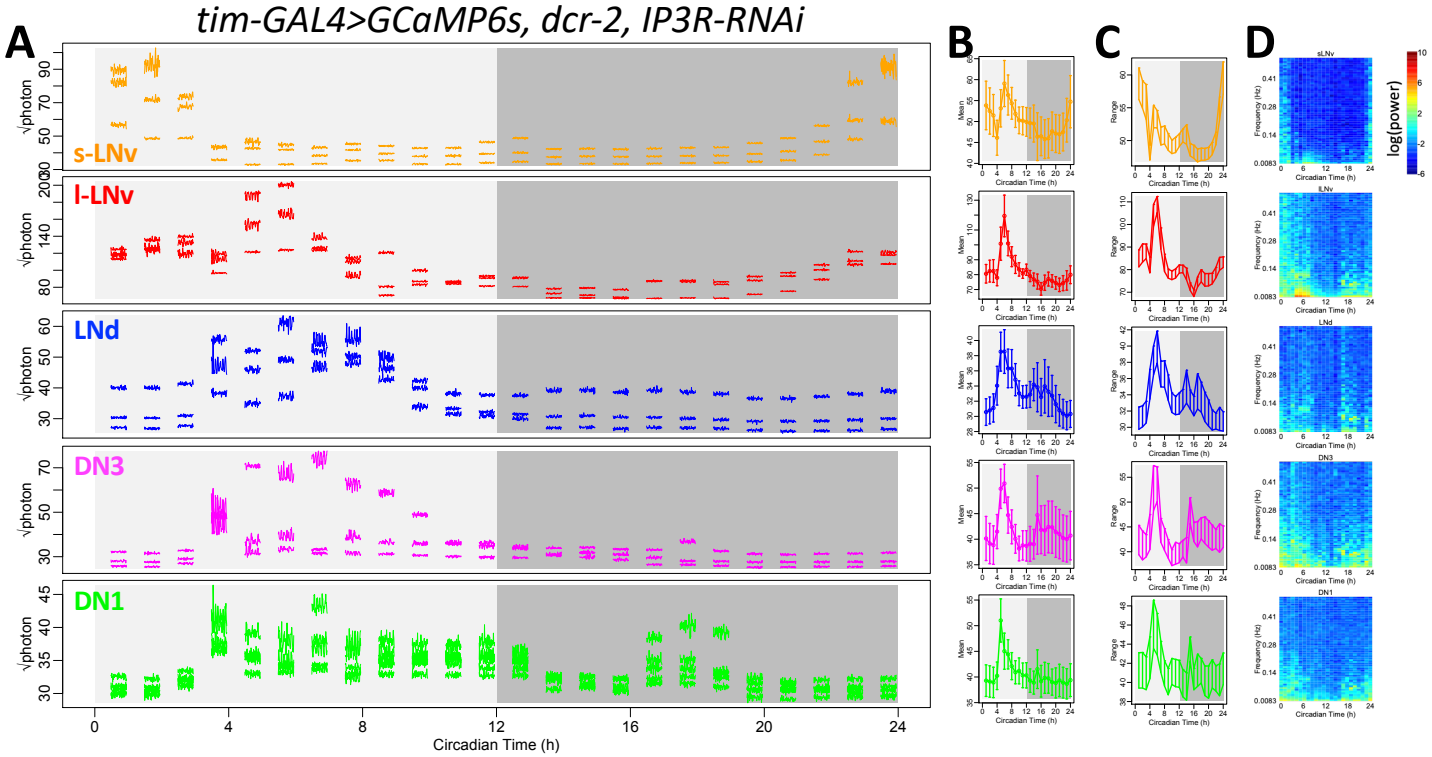

Figure S6. Changes in shot noise levels do not explain reductions in fast calcium fluctuations by *IP3R* knockdown.

(A-D) As Figure S2A-D, (A) raw calcium activity traces from the representative fly shown in Figure 6A. The intensity of calcium signal was calculated as the square root of photon number collected from individual region of interest (ROI), in order to normalize the effect of shot noise. Averaged daily patterns of (B) mean calcium intensity, (C) the range of calcium transient, and (D) the power spectrum was calculated based on the square root of photon number collected from individual ROIs (n = 5 flies).

Figure S7

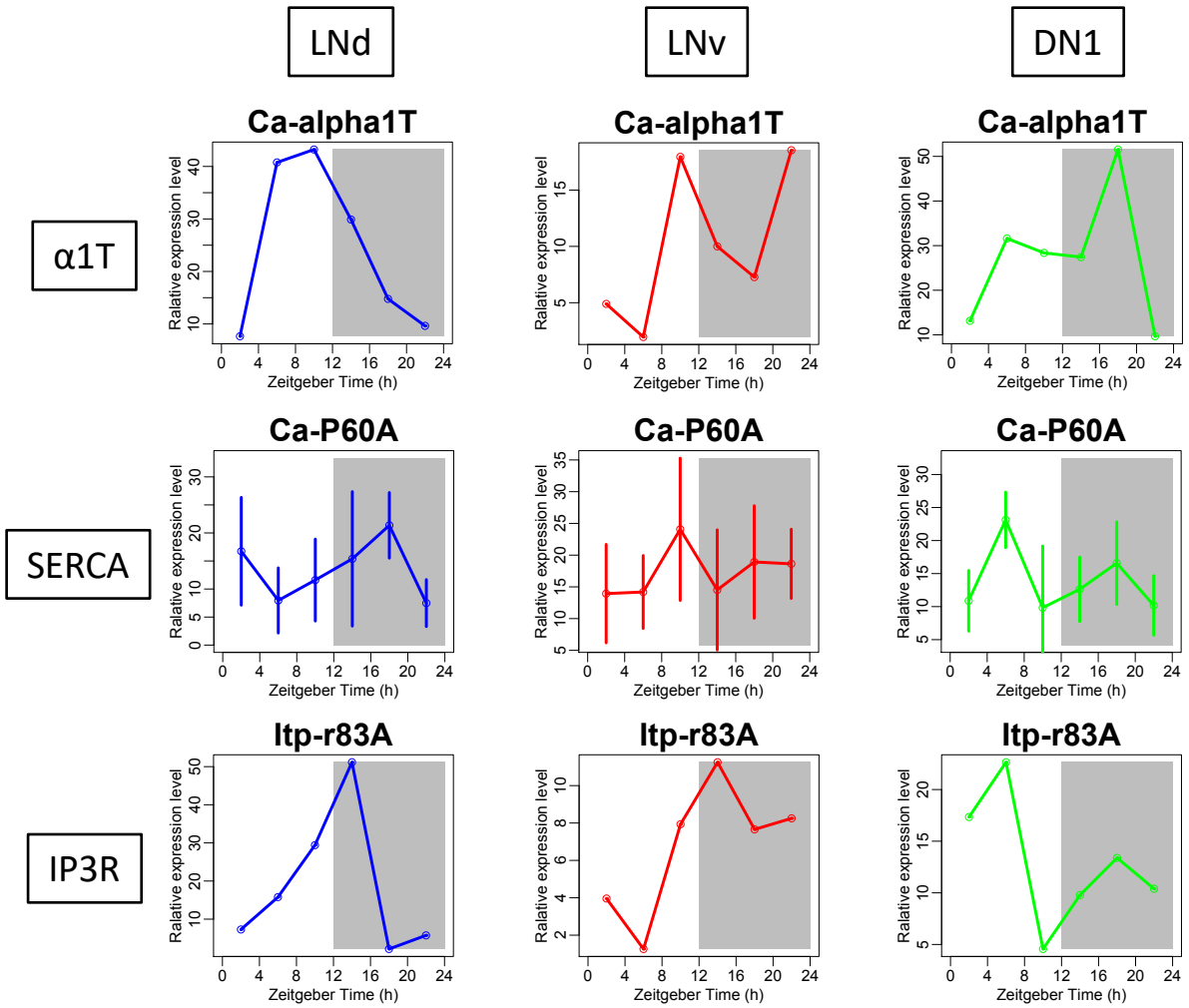

Figure S7. Daily rhythms in expression level of different calcium channels.
The daily expression pattern of *a1T*, *SERCA,* and *ip3r* in three different pacemaker groups LNd, PDF-positive LNv neurons, and DN1. Data is obtained from Abruzzi et al 2017, in which each pacemaker group was sorted and analyzed by RNA sequencing for two continuous days with 4-hour intervals.