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Cellular Requirements for LARK in the *Drosophila* Circadian System

Vasudha Sundram,*[,1] Fanny S. Ng,*[,1] Mary A. Roberts,* Carola Millán,[†,‡] John Ewer,[†] and F. Rob Jackson*[,2]

*Department of Neuroscience, Center for Neuroscience Research, Tufts University School of Medicine, Boston, MA, USA, †Interdisciplinary Neuroscience Center of Valparaiso, University of Valparaiso, Valparaíso, Chile, and ‡Department of Science, Facultad de Artes Liberales y Facultad de Ingeniería, Universidad Adolfo Ibañez, Viña del Mar, Chile

Abstract RNA-binding proteins mediate posttranscriptional functions in the circadian systems of multiple species. A conserved RNA recognition motif (RRM) protein encoded by the *lark* gene is postulated to serve circadian output and molecular oscillator functions in *Drosophila* and mammals, respectively. In no species, however, has LARK been eliminated, *in vivo*, to determine the consequences for circadian timing. The present study utilized RNA interference (RNAi) techniques in *Drosophila* to decrease LARK levels in clock neurons and other cell types in order to evaluate the circadian functions of the protein. Knockdown of LARK in *timeless* (TIM)– or pigment dispersing factor (PDF)–containing clock cells caused a significant number of flies to exhibit arrhythmic locomotor activity, demonstrating a requirement for the protein in pacemaker cells. There was no obvious effect on PER protein cycling in *lark* interference (RNAi) flies, but a knockdown within the PDF neurons was associated with increased PDF immunoreactivity at the dorsal termini of the small ventral lateral neuronal (s-LNv) projections, suggesting an effect on neuropeptide release. The expression of *lark* RNAi in multiple neurosecretory cell populations demonstrated that LARK is required within pacemaker and nonpacemaker cells for the manifestation of normal locomotor activity rhythms. Interestingly, decreased LARK function in the prothoracic gland (PG), a peripheral organ containing a clock required for the circadian control of eclosion, was associated with weak population eclosion rhythms or arrhythmicity.

Key words clock output, posttranscriptional, RNA binding, locomotor activity, eclosion

In both prokaryotic and eukaryotic species, circadian rhythms in biochemistry, physiology, and behavior are governed by endogenous cellular clocks. Feedback loops that drive rhythmic changes in gene transcription are important components of the clocks governing circadian behavior (Hardin, 2005; Kadener et al., 2008). However, there is evidence in several species that certain types of circadian oscillators can function in the complete absence of rhythmic clock gene transcription (Lakin-Thomas, 2006;

1. These authors contributed equally to this work.

2. To whom all correspondence should be addressed: F. Rob Jackson, Department of Neuroscience, Center for Neuroscience Research, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA; e-mail: rob.jackson@tufts.edu.

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O'Neill et al., 2011; O'Neill and Reddy, 2011). In multicellular organisms, circadian clocks are connected, via biochemical and cellular output pathways, to rhythmic physiological processes, and there is evidence in many species that both transcriptional and posttranscriptional mechanisms function in such output pathways (Jackson et al., 2005; Taghert and Shafer, 2006; Benito et al., 2007; Garbarino-Pico and Green, 2007; Kojima et al., 2010).

Perhaps the best studied clock output factor is *Drosophila* pigment dispersing factor (PDF), a neuropeptide essential for circadian behavior that is rhythmically released from a ventral subset (the ventral lateral neuron [LNv] cells) of approximately 150 neurons comprising the fly clock neuronal circuit (Renn et al., 1999; Park et al., 2000; Myers et al., 2003; Shafer et al., 2006). PDF acts through a class II G protein–coupled receptor (PDFR) (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005) to stimulate intracellular cAMP signaling (Mertens et al., 2005); recent studies indicate that most fly clock neurons respond to PDF and that this coordinates the circadian network (Shafer et al., 2008; Im and Taghert, 2010).

The intracellular pathways regulating neuropeptide release from fly clock neurons have not been well defined, although there is evidence that the CLOCK and CYCLE clock proteins might regulate *pdf* mRNA expression in the LNv (Park et al., 2000), even though the gene does not exhibit transcriptional rhythms (Park and Hall, 1998). In addition, it is likely that posttranscriptional mechanisms contribute to PDF synthesis, storage, or release, and possible candidate regulatory factors include RNA-binding proteins (RBPs), several of which have been implicated in circadian control in various organisms (Heintzen et al., 1997; McNeil et al., 1998; Morales et al., 2002; Dockendorff et al., 2002; Baggs and Green, 2003; Iliev et al., 2006; Hassidim et al., 2007; Green et al., 2007). Our previous work characterized a *Drosophila* RBP of the RNA recognition motif (RRM) class called LARK that functions in the control of circadian behavior (Newby and Jackson, 1993; McNeil et al., 1998; Schroeder et al., 2003; Huang et al., 2007). LARK is an evolutionarily conserved RBP (Jackson et al., 1997) that exhibits circadian rhythms in abundance in the nervous systems of both flies and mice (McNeil et al., 1998; Zhang et al., 2000; Kojima et al., 2007). In flies, LARK posttranscriptionally regulates RNA targets thought to function in clock output (Huang et al., 2007), whereas the mouse homolog translationally regulates expression of the PERIOD1 clock protein (Kojima et al., 2007). The fly protein has pan-neuronal distribution and nuclear localization in the nervous system (McNeil et al., 2001). Previous studies show that overexpression of LARK in *Drosophila* clock neurons causes arrhythmic adult eclosion and locomotor activity (Schroeder et al., 2003; Huang et al., 2009). This phenotype depends on the presence of functional RNA-binding (RRM) domains within LARK, suggesting it is due to misregulated expression of a target RNA (Huang et al., 2007).

Because LARK is essential for development, at least in *Drosophila*, it has not been possible to generate null mutants for behavioral studies. In this report, we employ RNA interference (RNAi) technology to knock down *Drosophila* LARK in a cell-specific manner in order to examine the effects of reduced abundance on circadian rhythmicity.

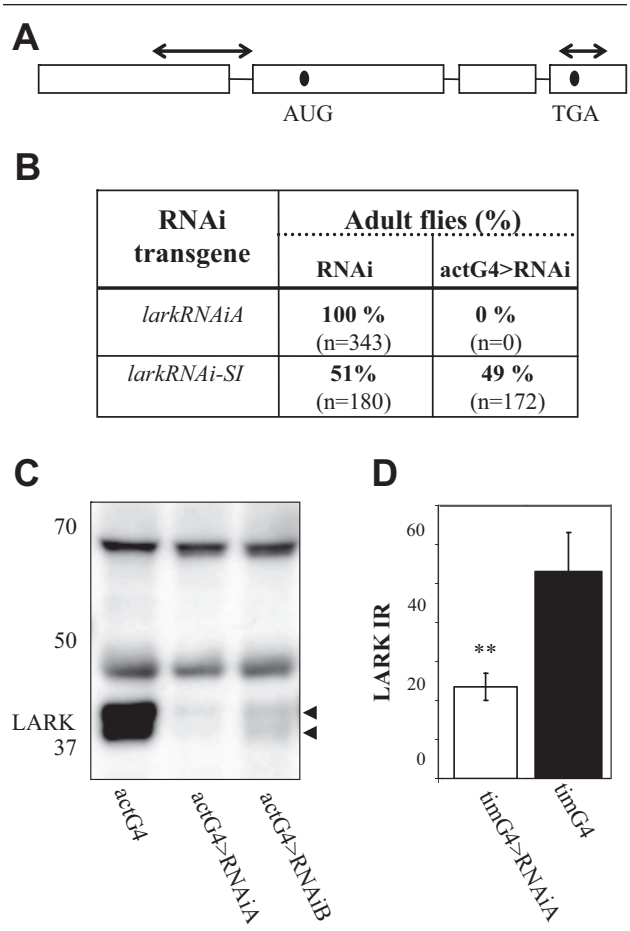
MATERIALS AND METHODS

Construction of *lark* RNAi Transgenic Flies

Two different types of *lark* RNAi constructs were generated (Fig. 1A): one (*RNAi-SI*) contains an inverted repeat that targets the 5′UTR (bp 611-1490 as the sense strand, and bp 837-1234 as the complement). PCR-generated clones containing the 2 sequences were ligated into the pUAST transformation vector to form an inverted repeat. The second RNAi transgene (called *RNAi*) utilized a sequence from the 3′ end of the *lark* gene (313 bp that includes 76 bp of coding sequence and the 3′UTR). This segment was cloned as an inverted repeat into the pWIZ *P*-element vector. Both the *RNAi-SI* and *RNAi* constructs were sequenced prior to production of transgenic strains using standard procedures. Two independent genomic insertions of the *RNAi* transgene were obtained: *RNAiA* and *RNAiB*.

Fly Stocks and Growth Conditions

Various Gal4 and UAS strains were obtained from the Indiana University Stock Center or individual investigators (see Acknowledgments). Strains were maintained and crosses carried out using a standard culture medium (Newby and Jackson, 1991). Crosses were reared in a cycle consisting of 12 hours of light and 12 hours of dark (LD 12:12) with constant



RNAi transgene	Adult flies (%)	
	RNAi	actG4>RNAi
larkRNAiA	**100 %** (n=343)	**0 %** (n=0)
larkRNAi-SI	**51%** (n=180)	**49 %** (n=172)

Figure 1. Generation of *lark* RNAi transgenes and confirmation of their effectiveness. (A) Schematic representation of the *lark* gene. Arrows above the diagram indicate the regions targeted for knockdown by the *RNAi-SI* (5′) or *RNAiA* transgenes. (B) Ubiquitous knockdown of LARK using the *RNAiA* transgene results in lethality at pupal stages. The *actG4* driver was used to drive ubiquitous expression of *RNAi-SI* or *RNAi*. The embedded table shows the percentage of eclosed LARK knockdown flies and sibling controls. The experiment was carried out at 25 °C. Four independent *RNAi-SI* and 2 independent *RNAi* strains (A, B) were tested with similar results. The *RNAiA* transgene was most effective and was used for subsequent experiments. (C) Ubiquitous knockdown of LARK using the *RNAi* transgene results in reduced LARK levels. Protein lysates from larvae expressing either of 2 independent LARK RNAi transgenes, *actG4>RNAiA* and *actG4>RNAiB*, were probed with affinity-purified LARK antibody (McNeil et al., 1998). Similar results were obtained in 2 independent experiments. Note that 2 LARK bands are always observed by Western blotting, and these are thought to represent distinct isoforms of the protein (McNeil et al., 1998). (D) An approximately 2- to 3-fold reduction in LARK-IR was observed in PDF cells, using *tim-uasG4* for a clock cell knockdown of the protein. Histograms show quantification of LARK-IR within the large PDF (l-LNv) cells of *tim-uasG4>RNAiA* (n = 8) or control *tim-uasG4* (n = 12) brains. Control *RNAiA* and *tim-uasG4* brains were similar. The y-axis shows arbitrary units of fluorescence intensity. Error bars represent SEM. **Different from control at $p < 0.01$ (Student t test).

humidity (~60%) at 23 °C to 29 °C, depending on the experiment. Standard meiotic recombination was used to generate a third chromosome containing the $lark^1$ mutation, a null allele, and the *RNAiA* transgene. This chromosome is designated *RNAiA*, $lark^1$ in the text.

Locomotor Activity Assays

Locomotor activity was monitored at 30 °C or 25 °C unless otherwise indicated. Flies were collected under CO_2 anesthesia, aged for 3 to 4 days, and then placed in Trikinetics activity monitors (Waltham, MA). They were entrained to LD 12:12 for 4 days, followed by transfer to constant darkness for approximately 2 weeks. Activity data were analyzed for periodicity using the MatLab-based fly toolbox package (Levine et al., 2002).

Eclosion Assays

Crosses of 30 females and 20 males were reared at 29 °C with entrainment to LD 12:12 for at least 5 days prior to the experiment. Newly emerged flies were counted by hand at the same temperature at 2-hour intervals under LD 12:12 or constant dark (DD) conditions (in red light using a 7.5-W bulb and a Kodak GBX-2 filter [Rochester, NY]). Assays of eclosion in *phmN1G4>lark RNAi* and control populations were performed at 20 °C in DD, following entrainment to LD 12:12 for 3 days, using Trikinetics automated eclosion monitors. Data were analyzed using the Maximum Entropy Spectral Analysis (MESA) function of the fly toolbox software package (Levine et al., 2002).

Immunostaining and Western Blotting

Standard laboratory immunostaining procedures were employed with whole mounts of the fly brain (Ng et al., 2011). Primary antibody dilutions were the following: rabbit anti-PER (1:300 preabsorbed against per^0 embryos; from R. Stanewsky), rabbit anti-PDF (1:20,000; from K. Rao), guinea pig anti-PAP (1:2000; from P. Taghert), mouse anti-PDF (1:100; from the University of Iowa Hybridoma Bank), and rabbit anti-LARK (1:2000; from G. McNeil). The following secondary antibodies, all from Molecular Probes (Eugene, OR), were used at a 1:1000 dilution: goat anti-rabbit, Alexa-488 or Cy3 conjugated; goat anti-guinea

pig, Alexa-488 or Cy3 conjugated; and donkey anti-mouse, Alexa-488 conjugated. Confocal images were acquired using a Leica TCS SP2 confocal microscope (Wetzlar, Germany). Image intensities (PER and PDF) were assessed using ImageJ 1.33 (National Institutes of Health, Bethesda, MD). Statistical significance of PER and PDF immunoreactive signal intensities was determined using either an ANOVA or *t* test (InStat, GraphPad Software, La Jolla, CA).

Standard procedures were used to prepare and analyze larval protein extracts (McNeil et al., 2001). Blocking of blots was performed using 5% skim milk in Tris-buffered saline, pH 8.0, containing 0.1% Tween-20 (TBST). They were incubated overnight with anti-LARK antibody (1:15,000) diluted in blocking solution, washed with TBST (4 × 20 minutes), and then incubated with HRP-tagged donkey anti-rabbit antibody (1:3000 in TBST) prior to chemiluminescence detection (GE Healthcare, Little Chalfont, UK). Protein band intensities were quantified using Kodak 1D Image Analysis Software and normalized against the intensity of a nonspecific band or MAP kinase (detected using an antibody dilution of 1:25,000; Sigma, St. Louis, MO).

RESULTS

Ubiquitous Expression of *lark* RNAi Causes Lethality

We employed the Gal4/UAS binary expression system and RNAi transgenes to study the functions of LARK in specific cell populations. As *lark* RNAi transgenes were not available from public resources at the time these experiments were initiated, we generated 2 different types for our studies (Fig. 1A): one called *RNAi-SI*, and another called *RNAi* (see Materials and Methods). Two independent insertions of the second transgene were named *RNAiA* and *RNAiB*.

To test the efficacy of our several different RNAi transgenes, ubiquitous knockdowns of LARK were performed using the *act5C-Gal4* (*actG4*) driver, which is expressed in all cell types. We predicted that effective RNAi would result in a substantial decrease in LARK levels and perhaps mimic the *lark* null phenotype that is associated with pupal lethality. Indeed, *actG4*-driven expression of either *RNAiA* or *RNAiB* (i.e., *actG4>RNAi*) was associated with pupal lethality in pilot crosses (no RNAi-expressing adults observed) (Fig. 1B), although a few escaper flies were observed using the *B* transgene. In contrast, flies expressing the *RNAi-SI* transgene had normal viability perhaps because it is less effective than the other *RNAi* transgenes.

Expression of *lark* RNAi Decreases LARK Abundance

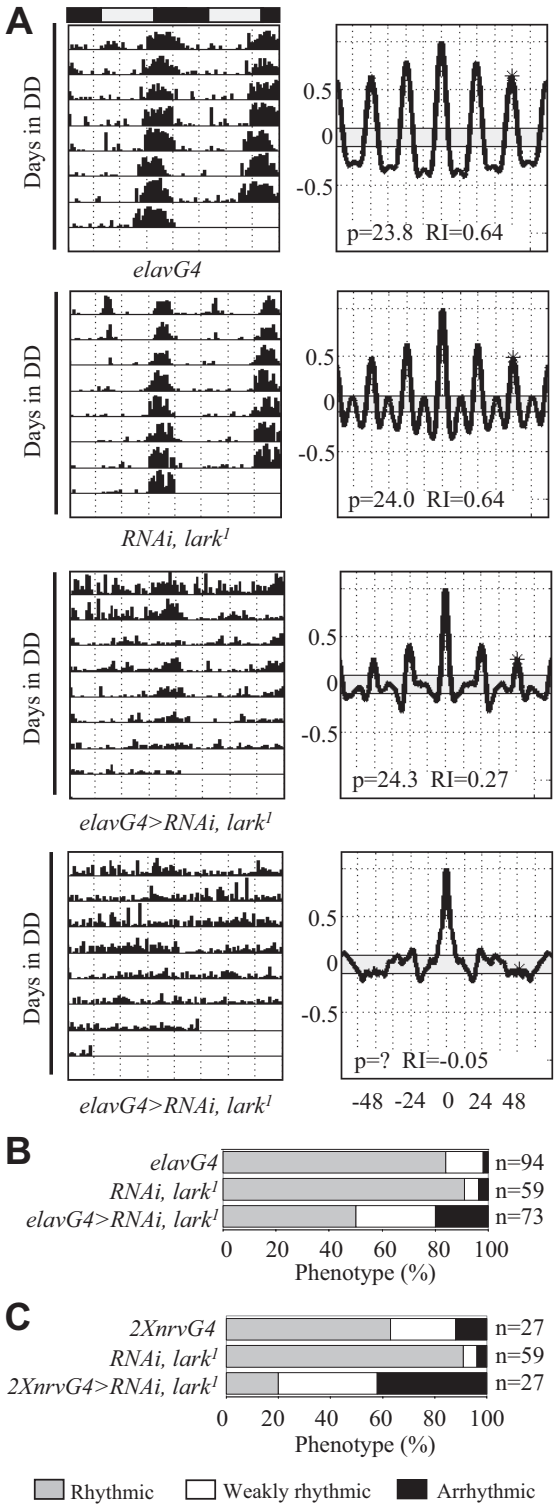
The analysis of larval protein lysates, using an anti-LARK antibody (Zhang et al., 2000), demonstrated *RNAiA*- and *RNAiB*-mediated knockdowns of LARK and indicated that the knockdown is more extreme with *RNAiA* (>5-fold reduced with *RNAiA* v. ~3-fold with *RNAiB*) (Fig. 1C). Immunostaining of adult brains indicated that expression of the *RNAiA* transgene in clock cells, using *tim-uasG4*, resulted in decreased amounts of LARK in the PDF neurons (~2- to 3-fold reduced) (Fig. 1D).

Pan-Neuronal Knockdown of LARK Decreases Robustness of the Locomotor Activity Rhythm

We initially examined adult locomotor activity in flies with a pan-neuronal knockdown of LARK using the *elavG4* driver (*elavG4>RNAiA*). Data were analyzed for changes in the patterning of activity, period length, and robustness of rhythmicity using the Rhythmicity Index (RI) (Levine et al., 2002); experimental and control flies were categorized as rhythmic (RI ≥ 0.3), weakly rhythmic (RI = 0.1-0.3), or arrhythmic (RI ≤ 0.1 and obvious aperiodic activity). All behavioral results were verified in 2 or more independent experiments.

When flies were reared and assayed at 25 °C, there were no significant differences observed between *elavG4>RNAiA* and control flies (data not shown). However, when reared at 29 °C and assayed at 29 °C to 30 °C, 21% of *elavG4>RNAiA* flies were arrhythmic, and 16% were weakly rhythmic, whereas only 0% to 2% of control flies were arrhythmic (Fig. 2A and 2B; see Table 1 for statistics for this and all other genotypes). We attribute the effects on rhythmicity at higher temperatures to increased Gal4 activity and/or a more efficacious RNAi process (Fortier and Belote, 2000), although we have not examined LARK knockdown at the 2 different temperatures.

We examined the locomotor activity of flies carrying another pan-neuronal driver, *NirvanaGal4* (*nrvG4*). Similar to results using *elavG4*, these *nrvG4; nrvG4>RNAiA* flies, which carried 2 copies of the



driver, exhibited less robust rhythmicity when assayed at 30 °C (Table 1 and Fig. 2C). Indeed, when flies of a similar genotype were heterozygous for *lark*[1], 42% of them were arrhythmic, whereas controls for this genotype (*nrvG4; nrvG4* and *RNAiA, lark*[1]) were mostly rhythmic (only 4%-12% arrhythmicity) (Fig. 2C). A more severe phenotype when flies were heterozygous for *lark*[1] suggests specificity for the observed RNAi-mediated knockdown (compare similar genotypes with or without *lark*[1] in Figs. 2 and 3 and Table 1).

Knockdown of LARK in Clock Neurons Causes Arrhythmicity

A group of approximately 150 clock neurons comprises the pacemaker driving behavioral rhythmicity (Nitabach and Taghert, 2008). To determine whether LARK is required within clock neurons, we expressed the *RNAiA* transgene in all such neurons (alone or in a *lark*[1] background) using either the *perG4* or the *tim-uasG4* driver. When flies were behaviorally assayed at 29 °C, we observed altered rhythmicity in *tim-uasG4>RNAiA* flies (Fig. 3A and 3B and Table 1). The phenotype was more severe in *tim-uasG4>RNAiA/RNAiA* flies, which carry 2 copies of the RNAi transgene: only 21% were strongly rhythmic versus 57% for *tim-uasG4>RNAiA* flies. We note that little or no effect was seen using the *per-Gal4* or *Cry-G4* drivers (data not shown) perhaps because these are weaker drivers compared to *tim-uasG4*, which is expressed at high levels due to the presence of UAS elements in the construct that provide a positive feedback of Gal4 to the *tim* promoter (Blau and Young, 1999). To determine if further reductions in LARK resulted in a more extreme phenotype, we assayed *tim-uasG4>RNAiA/RNAiA, lark*[1] flies (2 copies of *RNAiA* with *lark*[1]). That genotype exhibited an extreme phenotype (51% arrhythmic and 29% weakly rhythmic) compared to other RNAi-expressing flies or control genotypes (*RNAiA/RNAiA, lark*[1]) (Fig. 3B and Table 1). Similarly, more extreme phenotypes were observed in flies carrying recombinant *RNAiB, lark*[1] or both the

Figure 2. A neuron-specific knockdown of LARK alters the locomotor activity rhythm. Pan-neuronal knockdown of LARK was carried out in flies heterozygous for *lark*[1] using either the *elavG4* or *nrvG4* driver to express *RNAiA*. Unless otherwise indicated, in this and all subsequent activity experiments, flies were reared at 29 °C, entrained to LD 12:12 for 4 days at 30 °C, and then tested for locomotor activity at the same temperature. (A) Representative actograms and correlograms for control *elavG4*, control *RNAiA, lark*[1] (flies carrying *RNAiA* in a *lark*[1]/+ background), weakly rhythmic *elavG4>RNAiA, lark*[1], and arrhythmic *elavG4>RNAiA, lark*[1] flies. (B) Categorization of flies based on the robustness of activity rhythms: rhythmic (gray), weakly rhythmic (white), and arrhythmic (black). (C) Effects of LARK knockdown using 2 copies (2×) of *nrvG4* driving expression of *RNAiA* in *lark*[1] heterozygotes.

Table 1. Locomotor activity rhythm phenotypes resulting from decreased LARK function.

GAL4 Type	*N*	R, %	WR, %	AR, %	RI ± SEM	τ ± SEM
elavG4>RNAiA	57	63	16	21	0.39 ± 0.02	23.8 ± 0.06
elavG4>RNAiA, lark^1	73	50	30	20	0.26 ± 0.02[a]	23.5 ± 0.08
elavG4	94	84	14	2	0.39 ± 0.03	23.8 ± 0.08
nrvG4; nrvG4>RNAiA	20	45	35	20	0.19 ± 0.03[b]	24.0 ± 0.10
nrvG4; nrvG4>RNAiA, lark^1	27	20	38	42	0.19 ± 0.04[b]	23.7 ± 0.06
nrvG4; nrvG4	27	63	25	12	0.37 ± 0.02	23.5 ± 0.05
tim-uasG4>RNAiA	21	57	25	18	0.30 ± 0.03[c]	23.9 ± 0.13
tim-uasG4>RNAiA (replicate)	42	55	51	24	0.31 ± 0.03[c]	23.9 ± 0.12
tim-uasG4>RNAiA, lark^1	32	20	33	29	0.18 ± 0.02[d]	23.7 ± 0.10
tim-uasG4>RNAiA/RNAiA, lark^1	41	20	29	51	0.15 ± 0.03	24.1 ± 0.07
tim-uasG4>RNAiA/RNAiA	43	21	60	19	0.20 ± 0.03	24.0 ± 0.05
tim-uasG4	23	91	4.5	4.5	0.39 ± 0.03	23.8 ± 0.07
RNAiA	21	86	14	0	0.45 ± 0.02	23.7 ± 0.09
RNAiA, lark^1	27	70	26	4	0.34 ± 0.02	23.5 ± 0.07
RNAiA/RNAiA, lark^1	14	36	50	14	0.20 ± 0.03	23.7 ± 0.34
RNAiA/RNAiA	28	61	36	4	0.29 ± 0.02	23.7 ± 0.08
pdfG4>RNAiA	59	95	5	0	0.54 ± 0.04	23.8 ± 0.02
pdfG4>RNAiA, lark^1	21	86	14	0	0.52 ± 0.03	24.0 ± 0.05
pdfG4>RNAiA/RNAiA, lark^1	38	78	21	0	0.36 ± 0.03	23.1 ± 0.07
pdfG4>dcr2; RNAiA/RNAiA, lark^1	113	12	51	37	0.18 ± 0.01	23.5 ± 0.05
pdfG4>dcr2	31	97	0	3	0.46 ± 0.02	23.9 ± 0.05
RNAiA	23	96	4	0	0.56 ± 0.04	23.8 ± 0.10
pdfG4	46	96	4	0	0.56 ± 0.03	24.1 ± 0.06
RNAiA, lark^1	27	93	4	4	0.63 ± 0.03	23.7 ± 0.05
c929G4>RNAiA	27	48	33	19	0.24 ± 0.02[e]	23.3 ± 0.08
c929G4>RNAiA, lark^1	58	50	21	29	0.23 ± 0.06	24.0 ± 0.05
pdfGal80; c929>RNAiA, lark^1	48	52	17	31	0.38 ± 0.02[f]	23.4 ± 0.05
c929G4	26	88	13	0	0.38 ± 0.02	23.8 ± 0.07
pdfGal80; RNAiA, lark^1	51	98	2	0	0.47 ± 0.02	23.4 ± 0.03
mai179G4>RNAiA	15	40	47	13	0.30 ± 0.03[g]	23.1 ± 0.06
mai179G4>RNAiA, lark^1	49	41	35	24	0.17 ± 0.03[h]	23.5 ± 0.12
pdfGal80; mai179>RNAiA, lark^1	34	56	21	24	0.42 ± 0.02[f]	23.3 ± 0.05
mai179G4	15	81	19	0	0.38 ± 0.03	23.5 ± 0.09
RNAiA	15	75	19	6	0.36 ± 0.04	23.3 ± 0.05
RNAiA, lark^1	16	81	19	0	0.36 ± 0.03	23.2 ± 0.06
phmN1G4>RNAiA	26	88	8	4	0.23 ± 0.02	24.1 ± 0.10
phmN1G4	23	91	4	4	0.26 ± 0.03	23.5 ± 0.12
RNAi	29	97	0	3	0.57 ± 0.02	24.1 ± 0.04
RNAiA, lark^1	16	100	0	0	0.53 ± 0.02	23.8 ± 0.04

R = rhythmic; WR = weakly rhythmic; AR = arrhythmic; RI = Rhythmicity Index; τ = circadian period.
a. $p < 0.001$ compared to *elavG4* or *elavG4>RNAiA*.
b. $p < 0.002$ compared to *nrvG4; nrvG4*.
c. $p < 0.006$ compared to control *RNAiA*, *tim-uasG4>RNAiA, lark*^{1}, or *tim-uasG4>RNAiA/RNAiA, lark*^{1}.
d. $p < 0.001$ compared to *RNAiA, lark*^{1} control.
e. $p < 0.001$ compared to *c929G4*.
f. $p < 0.001$ compared to *pdfGal80; RNAiA, lark*^{1}.
g. $p < 0.05$ compared to *mai179G4* or *mai179G4; RNAiA, lark*^{1}.
h. $p < 0.002$ compared to *RNAiA, lark*^{1}.

RNAiA and RNAiB transgenes, relative to *RNAiB* or either transgene alone, respectively (data not shown). Flies carrying 2 copies of *tim-uasG4* were not analyzed because such flies were arrhythmic even without the *RNAi* transgene, presumably due to a genomic insertion effect (data not shown). Altogether, our results demonstrate a dosage dependency for the observed effects and verify specificity of the *RNAi* transgenes. Although *tim-uasG4* drives expression in both neurons and glia, LARK cannot be detected in glial cells (McNeil et al., 2001; Huang et al., 2009); thus, we interpret our results to indicate a requirement for the RBP within clock neurons.

Among the approximately 150 clock neurons, there are 16 PDF-containing LNv, which serve as pacemakers for the regulation of free-running rhythms in constant dark conditions (Nitabach and Taghert, 2008). To refine the spatial requirement for LARK, we selectively knocked down the RBP in PDF neurons using the *pdfG4* driver. Surprisingly, there was little or no effect on the percentage of rhythmicity even in *pdfG4>RNAiA/RNAiA, lark*^{1} flies, which carry multiple copies of the RNAi transgene (Table 1). However, upon examination of LARK immunoreactivity in the RNAi flies, we did not detect a knockdown of the protein (Suppl. Fig. S1B). Thus, we included a *UAS-dicer2* (*dcr2*) transgene in the background of RNAi flies (*pdfG4>dcr2; RNAiA/RNAiA, lark*^{1} in Table 1), which is known to enhance RNAi effects (Dietzl et al., 2007). In flies expressing *dcr2*, there was an obvious knockdown of LARK in the PDF neurons compared to controls lacking the *pdfG4* driver (Fig. 4A and Suppl. Fig. S1). Importantly, there was no obvious effect on cell viability or neuronal projections with knockdown of LARK in the PDF cells (data not shown). As shown in Figure 4B and 4C, *pdfG4>dcr2; RNAiA/RNAiA, lark*^{1} flies exhibited

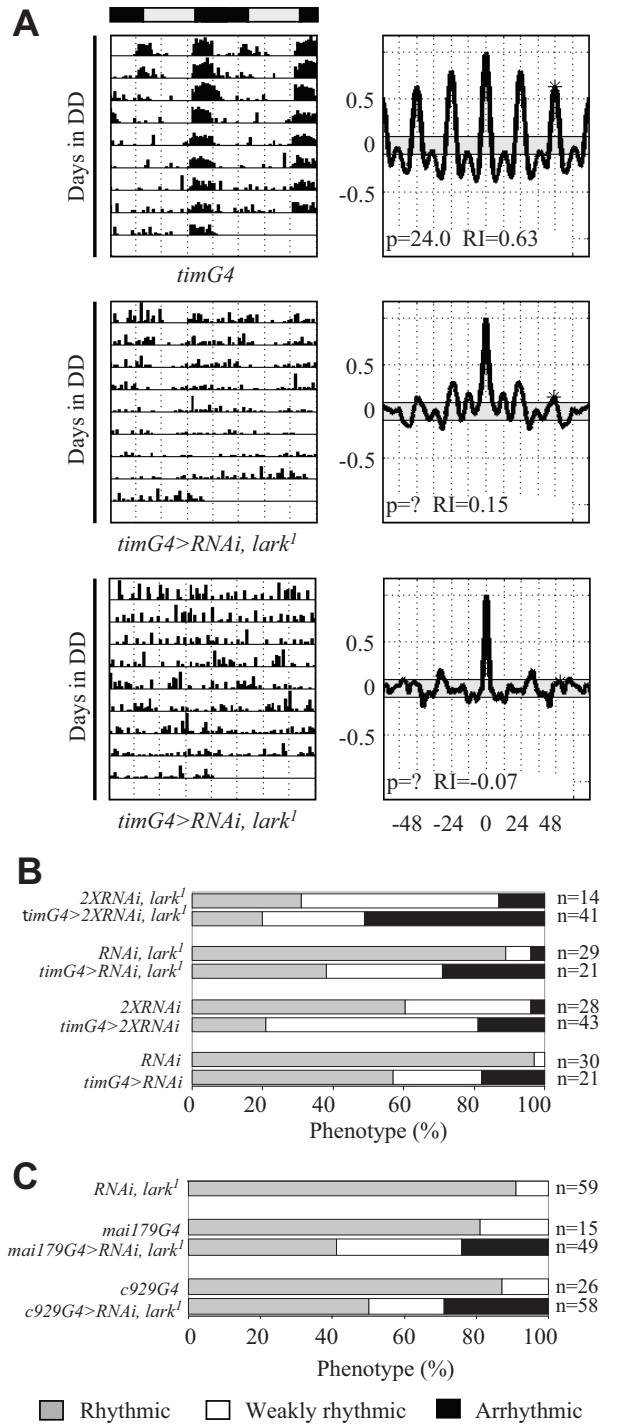


Figure 3. Knockdown of LARK using *tim-uasG4* results in weak rhythms or arrhythmic behavior. (A) Representative actograms and correlograms for *tim-uasG4>RNAiA, lark*^{1} and control flies (top panels). The middle panels show a weakly rhythmic fly, whereas the bottom panels show an arrhythmic fly. (B) Percentages of rhythmic, weakly rhythmic, or arrhythmic flies in various *tim-uasG4* knockdown and control genotypes. (C) Behavior with knockdown of LARK using *c929G4* or *mai179G4*, which drive expression in clock and other neurosecretory cells. Activity was examined at 30 °C.

altered behavior: only 12% of the flies were strongly rhythmic, and 37% were arrhythmic, whereas 97% of controls (run at the same time) were strongly rhythmic. Correspondingly, the average RI value for the knockdown population was only 0.18 versus 0.46 for the *pdfG4>dcr2* controls (Table 1). We conclude that LARK is required within PDF neurons for robust rhythmicity.

We examined 2 additional Gal4 drivers, *c929* and *mai179*, that express in the PDF neurons as well as other neurosecretory cells. The *c929* driver expresses in approximately 100 peptidergic neurons, including the PDF-containing l-LNv cells (the large PDF neurons) (Taghert et al., 2001). It has been shown (Grima et al., 2004) that *mai179* is expressed in adult clock neurons including some of the l-LNv, most of the LNd (which are PDF negative), and all s-LNv (4/5 being PDF positive). Interestingly, knockdown of LARK using *c929G4* or *mai179G4* produced similar results, with 50% or fewer of the *G4>RNAiA* flies possessing robust rhythms (Fig. 3C and Table 1). As expected, the percentage of such flies showing arrhythmic behavior increased when the knockdown was performed in a *lark*^{1} heterozygous background (Table 1).

With regard to the clock neuronal population, the overlap of the *c929* and *mai179* expression patterns is restricted to the l-LNv cells (the only clock neurons in which *c929* expresses). Thus, either the l-LNv or a nonclock population of neurons requires LARK for normal behavioral rhythms. In contrast to *pdfG4*, however, we did not observe a significant knockdown of LARK in the PDF neurons using either the *mai179* or *c929* driver (Suppl. Fig. S2). In fact, there seemed to be an increase in LARK amounts in the l-LNv cells of *c929G4>RNAiA* flies. These results suggest a requirement for LARK in non-PDF cell types in which *c929G4* or *mai179G4* is expressed. To confirm such a requirement, we examined *pdfGal80; c929G4>RNAiA, lark*^{1} and *pdfGal80; mai179G4>RNAiA, lark*^{1} flies, which express the *lark* knockdown transgene in PDF neurons but also express an inhibitor of Gal4 (Gal80) in the same cells (effectively turning off Gal4 activity in PDF cells but allowing RNAi effects in other neurosecretory cells). These populations were arrhythmic to a similar extent as *c929G4>RNAiA, lark*^{1} and *mai179G4>RNAiA, lark*^{1} flies (Table 1), indicating that there is a neuronal requirement for LARK outside of the PDF population.

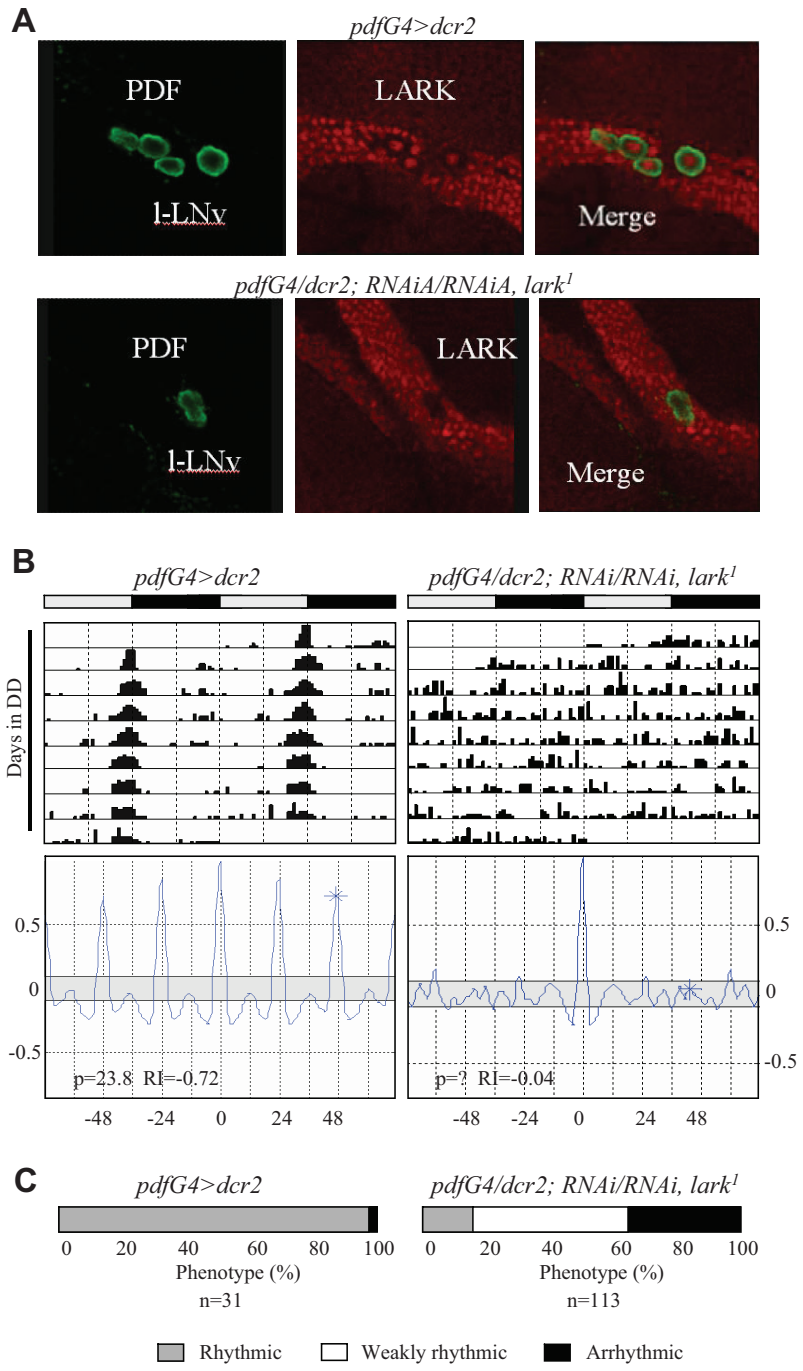


Figure 4. A strong knockdown of LARK in PDF neurons causes arrhythmicity. (A) *Dicer* (*dcr2*) expression greatly increases effectiveness of knockdown of LARK in the PDF neurons. High-magnification optical sections for control (upper panels) and LARK (lower panels) knockdown brains. Note the "hole" where LARK nuclear staining ought to be in the PDF cells of knockdown brains (lower right panel). Only 2 large PDF cells (l-LNv), in close proximity, are shown for the LARK knockdown in this optical section, but l-LNv cells in other optical sections also exhibited a severe knockdown of the protein; similarly, there was decreased LARK staining in the s-LNv cells. Green = PDF; red = nuclear LARK. (B) Actograms and correlograms for control (left) and knockdown (right) flies. (C) Percentages of rhythmic, weakly rhythmic, and arrhythmic flies for control and knockdown populations. Flies employed in these experiments were reared and tested at 30 °C.

PDF Immunoreactivity (PDF-IR) Is Altered by LARK Knockdown

We examined PER cycling in the adult brains of *tim-uasG4>RNAiA/RNAiA, lark*^{1} and *pdfG4>dcr2; RNAiA/RNAiA, lark*^{1} flies to determine if the observed arrhythmicity was caused by altered molecular oscillator function. However, both genotypes exhibited neuronal PER abundance and nuclear entry rhythms on DD day 2 that were indistinguishable from those observed in control brains (Suppl. Fig. S3; data not shown). We next examined PDF-IR in *pdfG4>dcr2; RNAiA/RNAiA, lark*^{1} knockdown brains based on our previous finding that increased LARK expression within PDF cells, which also causes arrhythmicity, resulted in decreased neuropeptide signaling in LNv projections (Schroeder et al., 2003). Whereas PDF-IR in the LNv cell bodies of knockdown flies was similar to that of controls (Suppl. Fig. S3B), immunosignals for the peptide were increased in the dorsal projections of the s-LNv (Fig. 5), an effect opposite to the decrease observed in LARK-overexpressing flies. Although PDF-IR continued to cycle, as expected, in knockdown brains, with a high at CT0 and a low at CT12, the signal at the high point was increased relative to controls in 4 independent experiments. On average, it was approximately 55% stronger at the termini of the s-LNv projections than that observed in control brains (Fig. 5B). To determine if peak PDF-IR was shifted in RNAi flies relative to controls, we examined signals at CT21, CT0, and CT3. As shown in Supplementary Figure S4, RNAi flies exhibited a significant increase in PDF-IR at CT21 and CT0 relative to controls, but signals were similar in the 2 genotypes by CT3 (see Discussion). As decreased PDF signals in the s-LNv projections at the beginning of the day are thought to represent neuropeptide release (Park et al., 2000), which drives

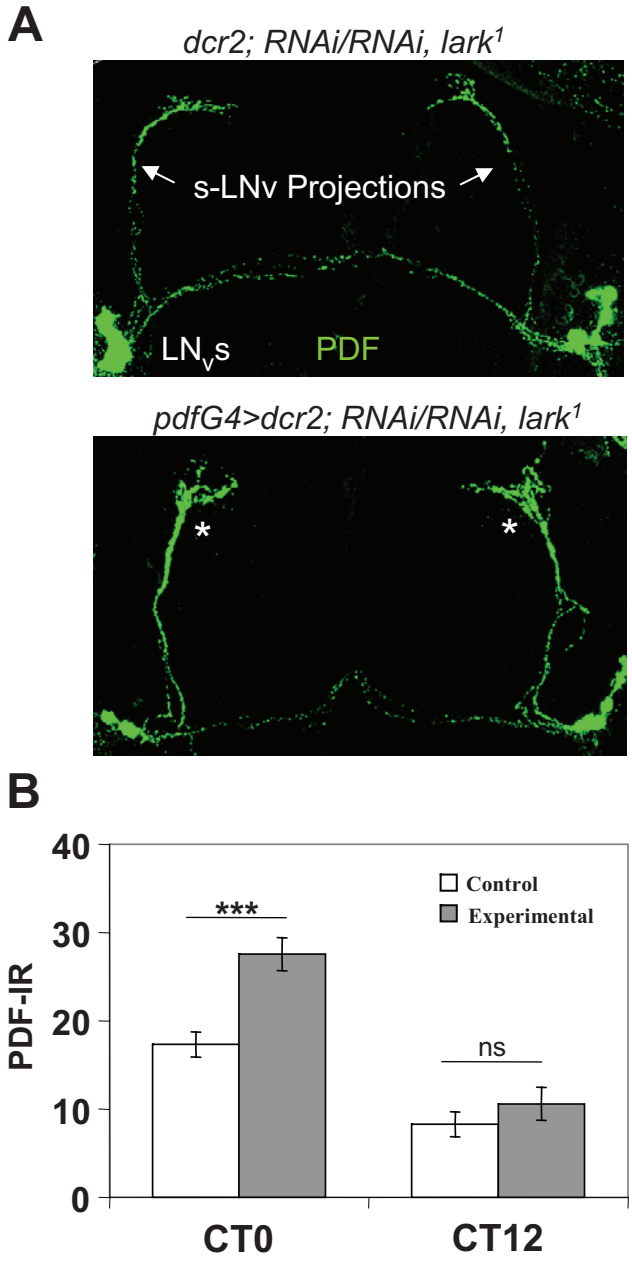


Figure 5. PDF-IR is abnormally high in s-LNv projections at CT0 with *pdfG4*-driven LARK knockdown. (A) Representative brain hemispheres showing PDF-IR in control and experimental (*pdfG4>dcr2; RNAiA/RNAiA, lark*^{1}) brains showing abnormally high signals in the s-LNv dorsal projections. (B) Quantification of PDF-IR signals in the 2 types of brains on DD day 2. ***Difference from the control: $p < 0.001$ (ANOVA with Tukey-Kramer multiple comparisons).

rhythmic locomotor activity, our results suggest that LARK knockdown flies may be defective in this process.

LARK Is Required in the Prothoracic Gland for Normal Rhythmicity in Adult Eclosion

LARK was discovered in a *P*-element screen for mutants with defects in the daily timing of population eclosion (Newby and Jackson, 1993). The *lark*^{1} allele is lethal, but we found in those earlier studies that *lark*^{1}/+ heterozygotes emerged earlier in the cycle than did control populations, indicating a role for LARK in the temporal gating of eclosion. We note that this early eclosion phenotype has not been reproducibly observed in more recent studies (using stocks maintained for many generations in the laboratory). Nevertheless, other studies suggest that LARK functions in the clock regulation of eclosion (Schroeder et al., 2003; Park et al., 2003).

To determine the effects of LARK knockdown on the timing of eclosion, we examined eclosion profiles for several different *Gal4>RNAi* and control populations. In experiments performed at 29 °C, LARK knockdowns with the *elav*, *crustacean cardioactive peptide (ccap)*, *eclosion hormone (eh)*, *c929*, *pdf*, or *tim-uasG4* drivers did not cause significant arrhythmicity in LD or DD, although we observed elevated night-time eclosion with *tim-uasG4*–driven *lark RNAi* (data not shown), reminiscent of that previously reported for *lark*/+ heterozygotes (Newby and Jackson, 1993).

The *tim* gene is expressed in the *Drosophila* nervous system and peripheral tissues including the prothoracic gland (PG), which synthesizes the insect molting hormone ecdysone. Furthermore, it is known that a PER/TIM-based oscillator exists in the PG (Emery et al., 1997), and it is thought to be important for the clock control of eclosion (Myers et al., 2003). We previously showed that LARK can be detected in the PG (Zhang et al., 2000). Thus, it is possible that the observed effects on the eclosion rhythm with *tim-uasG4* result from decreased LARK in this tissue. To test this idea, we employed 2 different PG drivers, *mai60G4* and *phmN1G4*, to restrict knockdown of LARK to the PG. Both drivers have been described as PG specific in pupae (Myers et al., 2003; Mirth et al., 2005). In particular, *phmN1G4* reflects expression of the *phantom* gene, which is involved in steroidogenesis and is exclusively expressed in the PG during postembryonic stages. Whereas *mai60G4>RNAiA* populations showed normal daily eclosion profiles (data not shown), *phmN1G4>RNAi* populations, expressing any 1 of 3 different *lark RNAi* transgenes (RNAiA and 2 others from the Japanese National Institute of Genetics), exhibited a less robust eclosion

rhythm in DD. However, there was variability among experiments, which might reflect a hypomorphic effect of the LARK knockdown. With *phmN1G4>RNAiA* populations, for example, eclosion profiles were arrhythmic in 5 experiments, weakly rhythmic in 5 others, and rhythmic in 4 experiments (as assessed using the MESA statistic; see Materials and Methods). In contrast, control populations (n = 3 experiments) uniformly showed robust rhythms. Inclusion of *dcr2* in the genetic background seemed to reduce variability. In 3 experiments, *phmN1G4>dcr2; RNAiA* populations were weakly rhythmic and significantly different from controls, which were robustly rhythmic (Fig. 6A and 6B; note scale differences between control and RNAi MESA plots in panel A). Thus, we conclude that LARK is required within the PG for robust free-running rhythms of population eclosion. We note that adult locomotor activity rhythms were completely normal in *phmN1G4>RNAiA* flies (Table 1), consistent with degeneration of the PG after metamorphosis.

To determine why *phmN1G4*, and not *mai60G4*, was associated with altered eclosion rhythms, we re-examined the expression patterns of both drivers, using *UAS-GFP* as a reporter. Surprisingly, we did not detect fluorescence in the PG of *mai60G4>GFP* pharate adults, although intense fluorescence was observed in the salivary glands of these animals (Suppl. Fig. S5). The previous study reporting the *mai60G4* expression pattern utilized a *UAS-lacZ* transgene and β-galactosidase staining to visualize expression of the driver (Myers et al., 2003), and the increased sensitivity associated with this assay may explain the discrepancy between our results and that study. In contrast, *phmN1G4>GFP* pharate adults exhibited an intense fluorescence specifically in the PG (Suppl. Fig. S5). Considered with the *lark* RNAi results using this driver, we conclude that LARK is required in the PG for normal eclosion rhythms.

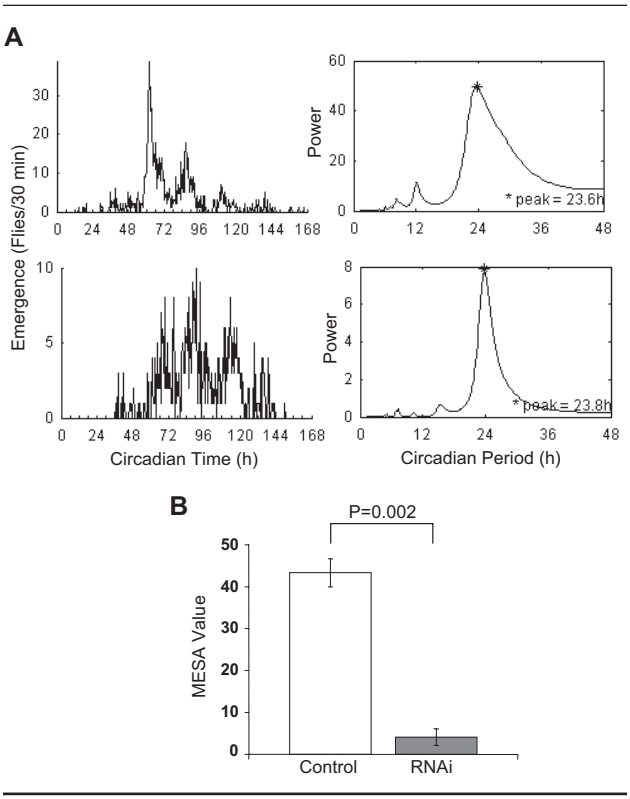


Figure 6. LARK is required in the PG for normal free-running eclosion rhythms. (A) Plots of adult eclosion and MESA statistics for *phmN1G4>dcr2/RNAiA* (lower) and control *phmN1G4>dcr2* (upper) populations (note different scales for MESA plots). Eclosion was assayed at 20 °C using an automated (Trikinetics) monitor (see Materials and Methods). (B) Average MESA values for *phmG4>dcr2/RNAiA* and control *phmN1G4>dcr2* populations (n = 3 experiments for both genotypes).

DISCUSSION

LARK Is Required in Clock Neurons for Robust Activity Rhythms

This report documents a requirement for LARK in circadian pacemaker neurons. Behavioral arrhythmicity was observed with knockdown of the RBP in either PDF- or TIM-containing neurons, although RNAi flies appeared to entrain normally to LD 12:12. With knockdown of LARK in PDF cells, there was a corresponding effect on PDF-IR; immunoreactive signaling was significantly increased in the termini of the s-LNv projections at the time of peak neuropeptide release. A decrease in PDF-IR in the projections of the s-LNv has previously been interpreted as neuropeptide release (Park et al., 2000); thus, it is possible that increased immunoreactive signals with LARK knockdown reflect an inhibition of peptide release. Examination of PDF-IR at several different circadian times showed that signaling is significantly higher in RNAi versus control flies at both CT21 and CT0, but similar in the 2 genotypes by CT3, that is, not significantly delayed. Given the arrhythmic phenotype observed in many *lark* knockdown flies, we favor the idea that there is decreased PDF release in such individuals, perhaps with a subsequent intracellular degradation of the peptide (because the immunosignal is normal by CT3). That all flies with decreased LARK function are not arrhythmic (i.e., the phenotype is not completely penetrant) is presumably due to the inherently "hypomorphic" nature of RNAi-mediated knockdowns. Together with previous results showing that LARK overexpression is associated with decreased PDF-IR within s-LNv projections

(Schroeder et al., 2003), our findings suggest that LARK may regulate release of the neuropeptide. In this regard, it is of interest that putative target RNAs have been identified for LARK (Huang et al., 2007), and many of them encode proteins predicted to function in synaptic transmission (Huang et al., 2007).

LARK Is Required in Nonclock Cells for Normal Activity Rhythms

We observed arrhythmicity with LARK knockdown by *mai179G4* or *c929G4*, drivers that express broadly in neurosecretory cells of the adult brain including neurons of the clock circuitry. Arrhythmicity was also observed with these drivers even when flies carried *pdfGal80*, which is predicted to specifically inhibit RNAi expression in the PDF neurons. Thus, LARK is required in PDF and non-PDF neurosecretory cells for normal circadian behavior. Perhaps the RBP regulates a posttranscriptional event within neurons downstream of the clock circuitry to mediate clock output. A requirement for LARK in other neurosecretory cells also suggests that the RBP may generally regulate neuropeptide secretion.

LARK Expression within the PG Is Required for Robust Adult Eclosion Rhythms

Although the *lark*^{1} mutant was isolated on the basis of an eclosion rhythm phenotype (Newby and Jackson, 1993), it has never been determined which tissues require the RBP for normal daily eclosion rhythms. A complication with this determination is the ubiquitous expression of LARK and its vital role during oogenesis and zygotic development. The present study indicates that LARK knockdown with either of 2 different Gal4 drivers (*tim* and *phmN1*) results in abnormal population eclosion rhythms, although effects are more prominent using *phmN1*. Importantly, *phmN1* is exclusively expressed in the PG of developing adults, indicating a requirement for LARK within that tissue. Differences in phenotypic severity for the 2 drivers may reflect the strength of expression within the PG or modification of the phenotype by expression within the nervous system (*tim*).

Previous studies have shown that a PER-based oscillator exists in the PG and that this peripheral oscillator may interact with the neuronal pacemaker to regulate the timing of eclosion (Emery et al., 1997; Myers et al., 2003). The only defined function of the PG is the synthesis of ecdysteroid (Riddiford, 1993), and the hormone is known to be constitutively released from that tissue. Thus, our results, surprisingly, suggest that LARK may have a role in ecdysteroid biosynthesis perhaps by posttranscriptional regulation of a factor required for this process. Synthesis of the hormone is known to be rhythmic in the PG of certain insects (Vafopoulou and Steel, 1991), but it is unknown whether such a rhythm exists in *Drosophila*. Furthermore, it has not been determined whether LARK oscillates in abundance within the PG, although the RBP shows circadian changes in the nervous system (McNeil et al., 1998). It will be of interest to examine the hypothesis that LARK participates in the interactions between central and peripheral oscillators (Myers et al., 2003) that govern the daily timing of eclosion events.

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CONFLICT OF INTEREST STATEMENT

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

NOTE

Supplementary material is available on the journal's website at http://jbr.sagepub.com/supplemental.

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