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CELL-SPECIFIC EXPRESSION OF THE LARK RNA-BINDING PROTEIN IN *DROSOPHILA* RESULTS IN MORPHOLOGICAL AND CIRCADIAN BEHAVIORAL PHENOTYPES

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Past studies have implicated the *Drosophila* LARK protein in the circadian control of adult eclosion behavior. LARK has a broad tissue pattern of distribution, and is pan-neuronal in the differentiated brain. In certain peptidergic neurons, LARK abundance changes in a circadian manner. However, the precise cellular requirement for LARK, with respect to circadian behavior, is still not

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known. To explore this issue, we employed the GAL4/UAS binary expression system to increase LARK abundance in defined neuronal cell types. Interestingly, LARK expression in Crustacean Cardioactive Peptide (CCAP) neurons caused an early-eclosion phenotype, whereas a similar perturbation in the Eclosion Hormone (EH) cells resulted in abnormally late peaks of eclosion. Surprisingly, LARK expression in Pigment Dispersing Factor (PDF)- or TIMELESS (TIM)-containing clock neurons caused behavioral arrhythmicity, even though clock protein cycling was found to be normal in these flies. Although the observed effects of LARK expression mirrored those seen with genetic ablation of the relevant peptidergic populations, there was no evidence of defective cell development or morphology. This suggests that an alteration of cell function rather than cell death is the cause of the aberrant phenotypes. Diminished PDF immunoreactivity in flies expressing LARK in the PDF neurons suggests that an effect on neuropeptide synthesis, transport, or release may contribute to the observed arrhythmicity. Importantly, the expression of LARK in several other cell populations did not have detectable effects on development, viability or behavior, indicating a specificity of action within certain cell types.

Keywords: *Drosophila*; circadin; LARK; activity; eclosion; peptidergic

INTRODUCTION

Circadian clocks regulate the timing of physiological and behavioral processes that occur rhythmically with an approximate 24-h cycle. The molecular mechanisms comprising the circadian system are similar between vertebrates and invertebrates, and they have been described in great detail in several model organisms (Young & Kay, 2001; Panda et al., 2002; Stanewsky, 2003; Hall, 2003). Such clock analyses have stimulated molecular investigations of the intercellular signaling (output) pathways through which the clock regulates circadian behaviors. These output pathways include factors released in rhythmic fashion from clock neurons (Park et al., 2000; Kramer et al., 2001; Cheng et al., 2002) as well as neural targets of these factors, such as downstream groups of neurons that mediate specific behaviors.

In *Drosophila*, a neural clock controls a circadian rhythm in adult ecdysis (eclosion) (Jackson et al., 2001; Ewer & Reynolds, 2002). From studies in *Drosophila* and other insects, it is known that ecdysis is

regulated by the actions of at least three neuropeptides: ecdysis-triggering hormone (ETH), eclosion hormone (EH) and crustacean cardioactive peptide (CCAP). Physiological studies suggest that these peptides may act through a pathway in which ETH and EH regulate each other's release, and EH positively regulates CCAP cell function (Ewer & Reynolds, 2002). Recent genetic studies of ETH, EH, and CCAP in *Drosophila* have demonstrated the complex nature of the pathways regulating the various ecdyses in this species. Animals bearing null alleles of the *eth* gene die during larval ecdyses, indicating an essential role for ETH at these stages (Park et al., 2002). Mutations do not exist for the *EH* or *CCAP* genes; however, flies lacking EH- or CCAP-containing neurons are viable and show defects in ecdysis behavior (McNabb et al., 1997; Park et al., 2003). It has been reported that ablation of the EH neurons does not eliminate circadian eclosion rhythms, but the prominent burst of emergence seen in wild-type populations in response to the "lights-on" signal is absent in EH-less flies (McNabb et al., 1997). Adult eclosion is rhythmic in populations lacking the CCAP neurons, but the circadian gate of eclosion is broader than normal and the lights-on response is diminished (Park et al., 2003).

Previous work has implicated an essential RNA-binding protein called LARK in the circadian control of eclosion (Newby & Jackson, 1993, 1996). LARK is localized in neurons of the developing and differentiated *Drosophila* nervous system (McNeil et al., 1999) and exhibits circadian cycles in abundance within the CCAP cell population (Zhang et al., 2000). LARK has a nuclear localization in most or all differentiated neurons, with the exception of the CCAP cells; in those, the protein has a prominent cytoplasmic pattern of localization (McNeil et al., 1998; Zhang et al., 2000). It has been postulated that LARK cycling within the CCAP neurons contributes to the circadian gating of eclosion (Zhang et al., 2000), perhaps by modulating CCAP cell excitability or peptide release.

Several different peptidergic neuronal populations, including the CCAP neurons, function in the circadian regulation of *Drosophila* behavior. For example, brain neurons containing Pigment-Dispersing Factor (PDF) are important for behavioral rhythmicity (Renn et al., 1999); consistent with this function, PDF immunoreactivity within these neurons (called LN_v cells) oscillate in a circadian manner (Park et al., 2000), suggesting that this neuropeptide could be released in a rhythmic manner. As previously mentioned, EH and CCAP probably play modulatory roles in the circadian regulation of the adult ecdysis, although eclosion rhythms persist in populations lacking EH or CCAP. Given the postulated modulatory role of LARK in the CCAP cells, we wondered whether this RNA-binding protein might have a regulatory function in other peptidergic neurons. In this report, we show that increased expression of LARK in three different peptidergic populations is associated with alterations of circadian behavior.

MATERIALS AND METHODS

Drosophila Strains, Growth Conditions, and Crosses

A *Drosophila* culture medium like that typically used was employed in crosses and to maintain stocks (Newby et al., 1991). Stocks were maintained and crosses performed at 25°C and 60% humidity in a 12h:12h light:dark (LD) cycle (LD 12:12) unless otherwise noted. The pan-neural *elav-gal4* driver (a.k.a. enhancer trap strain c155) was obtained from the Bloomington, Indiana, stock center. The neurosecretory-cell drivers c929-*gal4* and 386Y-*gal4* (Taghert et al., 2001), and the C21 *EH-gal4* driver (McNabb et al., 1997) were obtained in transgenic strains reported in the publications just cited. The *CCAP-gal4*-16 and *Pdf-gal4* drivers were obtained in strains reported by Park et al. (2003) and Renn et al. (1999), respectively; the *tim-gal4* driver was strain number 62 as reported by Kaneko & Hall (2000); the UAS-*rpr* strain contains this transgene inserted into the 2nd chromosome (as used by Park et al., 2003); and the "outer-photoreceptor" driver (in which *gal4* is expressed in such R1-6 cells within a given compound-eye facet), here named *Rh-gal4*, was provided by J.E. O'Tousa (a personal communication from whom described the *gal4* driver properties just described). The generation and initial characterization of the three UAS-*lark* strains (94A, 9A, and 23A) used in the present study were previously published (Schroeder & Jackson, 2002). A *lark* transgene carrying mutations in the RNP2 coding region of both RRM1 (F10A) and RRM2 (F89A) was previously described (McNeil et al., 2001). To generate a mutant UAS-containing transgene for the present study, a DNA fragment containing both RNP2 mutations (within the *lark* ORF) was subcloned from the pW8 vector into pUAST. The protein encoded by the UAS-*lark* transgene—within a strain called 16A—contains single amino acid changes (F to A) at positions 10 and 89 of the 352-residue LARK sequence. Transgenic flies carrying a genomic insertion of 16 A were generated using standard techniques (Spradling & Rubin, 1982); a resulting strain carrying one such insert into the X chromosome was used in the current study.

In order to produce flies heterozygous for a GAL4 driver and the appropriate balancer chromosome, flies homozygous for a GAL4 driver were crossed to w^{1118}; *Bl/In(2LR)O, Cy* or w^{1118}; $lark^{1}$/*In(3LR)TM3, Sb* flies for 2nd and 3rd chromosomal inserts, respectively. Male and virgin female progeny of the appropriate genotypes were collected and crossed with homozygous UAS-*lark* flies. Crosses were set up in 3–5 vials with 7–10 male and female parents per vial.

Monitoring Eclosion Rhythms

Bottles containing medium and developing *Drosophila* were reared at 25°C for 5 days and then transferred to 18°C for the rest of a given experiment; such cultures were entrained for at least 4 days in a 12-h light: 12-h dark (LD) cycle (whereby lights-on is defined as Zeitgeber Time 0 and lights-off ZT 12). Bottles were cleared every 2 h starting at ZT 16 and numbers of flies that emerged in the previous 2 h were recorded (via emptying cultures and counting numbers of emergents by hand). In most experiments, collections were continued for 2 complete cycles in LD. The lights were switched off during the night of the second day of collection and flies were allowed to free run in constant darkness (DD). On day 3, 4, or 5 of DD, flies were collected and scored every 2 h for one or 2 circadian cycles. For the experiment involving *tim-gal4*/UAS-*lark* flies (Figure 6), eclosion was monitored at 25°C, instead of 18°C.

Monitoring and Analysis of Locomotor Activity

Such behavior was recorded for a given series of individual flies at 24–25°C using monitors from Trikinetics and *Drosophila* Activity Monitoring (DAM) software (Waltham, MA) as previously described (Levine et al., 1994). Activity events were collected in 30-min bins and rhythmicity was analyzed using a MATLAB-based software package that was developed, applied, and reported by Levine et al. (2002). The Rhythmicity Index (RI), defined as the amplitude of the third peak of an autocorrelogram (Levine et al., 2002), was used to determine the presence and strength of rhythms. As we knew that differences in genetic background influence the consolidation and robustness of locomotor activity rhythms, we carefully selected RI value cutoffs (below which flies were assigned as arrhythmic) with respect to behavior of flies carrying the different GAL4-encoding transgenes. For the experiments using the *Pdf-gal4* driver line, visual inspection of actograms, followed by consideration of RI, values led us to set a cutoff RI value of 0.2, below which a record was designated arrhythmic. For those experiments employing the *CCAP-gal4, EH-gal4* or *tim-gal4* lines, flies with an RI < 0.1 were considered arrhythmic. For all experiments, records were considered strongly rhythmic if the RI value was ≥ 0.3. Circadian period estimates were made using both χ^2-periodogram analysis (Sokolove & Bushell, 1978) and the autocorrelation function (Levine et al., 2002). Results of applying the two methods were in agreement to within 0.1 h for (inter-fly) average period values; such values, as reported in Table III, are from the autocorrelation analyses.

Fast Phototaxis Assay

Light-induced fast phototaxis was assayed in a dark room using a countercurrent apparatus as described (Benzer, 1967; Simon et al., 2003). The relevant countercurrent apparatus (reported as just cited) was situated immediately in front of a fluorescent light source of 2000 lux. Approximately 60–90 flies were assayed for each countercurrent distribution, and genotypes were assayed 3 or 4 times to obtain the results shown in Figure 7.

Protein Extraction, Immunoblotting, and Immunostaining

After entrainment of adults for 3–5 days in LD 12:12 cycles, flies were collected every 4 h over the course of a single day; then the lights were turned off and collection continued for an additional 24 h in DD. Collected flies were frozen immediately in liquid nitrogen and stored at −80°C. Heads were isolated from other body parts by sieving, and protein was extracted as previously described (Edery et al., 1994). Approximately 50 μg of protein were loaded per lane; proteins were separated on 5.7% polyacrylamide/SDS gels and transferred to a nylon membrane. PER was detected using a 1:3000 dilution of an anti-PER antibody (a gift of Isaac Edery), followed by an anti-guinea pig HRP-conjugated second antibody diluted 1:1000. Signals were detected using ECL reagents (Amersham) according to the manufacturer's instructions. Immunostaining experiments were performed in whole mounts of pharate-adult or adult brains using materials reported previously (Atken et al., 2003) and the following primary antibodies (all produced in rabbits): anti-EH (diluted 1:200), anti-CCAP (1:1000), and anti-PDF (1:1200). An anti-rabbit secondary antibody coupled to Cy3 was used in all immunostainings (dilution 1:400). Fluorescent signals were visualized by confocal microscopy (Leica TCS SP2).

RESULTS

In the studies reported here, we were primarily interested in the effects of LARK expression on general viability and circadian phenomena. Previous studies have indicated a role for LARK in both processes (Newby & Jackson, 1993). In the first two subsections of RESULTS, we examine the cell types required for LARK-induced effects on viability and development. The remainder of the RESULTS section documents circadian phenotypes that resulted from LARK expression.

Gal4-mediated Lark Expression in Restricted Peptidergic Neuronal Populations Had Minimal Effects on Viability

We previously reported that ubiquitous expression of LARK using an actin GAL4 driver (Act5C-GAL4) and 3 independent UAS-*lark* insertions (94A, 9A and 23A) caused lethality, with most animals dying early in pupal development or prior to pupation (Schroeder & Jackson, 2002). Similarly, LARK expression, driven by an *elav-gal4* transgene, caused lethality or semi-lethality depending on the UAS-*lark* line employed in crosses (Table I); but in this case—whereby *lark* is expressed pan-neuronally under the control of *elav* sequences—animals died as differentiated adults still in the pupal case (pharate adults). The previous studies also showed that all three of the UAS-*lark* transgenic insertions

TABLE I LARK-induced Lethality with Several Different GAL4 Driver Strains

	UAS-*lark* transgenic strain Number of progeny			
gal4 driver[a]	UAS-*lark* (94A)	UAS-*lark* (9A)	UAS-*lark* (23A)	No UAS-*lark*
elav-gal4[b]	24	293	0	155
+[c]	348	472	418	165
386Y	117	85	5	343
+	358	347	324	306
c929	355	295	256	308
+	468	431	425	343
EH-gal4	285	439	327	318
+	323	456	394	275
CCAP-gal4	416	230	535	319
+	358	212	477	345

[a]Flies homozygous for the UAS-*lark* transgene were crossed to flies heterozygous for the indicated GAL4-encoding driver and balancer chromosome (indicated by a + in this table; cf. Materials and Methods). Information regarding the *gal4* drivers is given in the text. Numbers of adults carrying either the driver or balancer chromosome (+) are shown.

[b]Numbers of adult female *elav-gal4*/UAS-*lark* flies resulting from crosses of *elav-gal4* males to females homozygous for indicated homozygous UAS-*lark* (strain) type. These data were reported previously in Schroeder and Jackson (2002) and are shown again for comparison.

[c]Indicates the number of male UAS-lark flies emerging from the cross with *elav-gal4*.

expressed a LARK protein of the predicted size class (Schroeder & Jackson, 2002). Importantly, lethality associated with transgene expression was dependent on an intact LARK protein. Pan-neuronal expression of a truncated form of LARK lacking the carboxy-terminal half of the protein had no obvious phenotypic effects; adult flies emerged in the expected ratios and were morphologically and behaviorally normal (data not shown). Consistent with such a lack of effect, it had been shown previously (McNeil et al., 2001) that transgenes expressing a similarly truncated LARK protein were not able to rescue the embryonic lethality of *lark* mutants.

As a preface to analyzing the effects of LARK expression on behavior, we examined whether ectopic expression of the protein in clock neurons or neurosecretory cells would result in decreased viability or morphological phenotypes. To examine the consequences of LARK expression in peptidergic neuronal populations, we employed several GAL4-encoding transgenes that drive expression in different subsets of these cell types and in non-neural (peripheral) secretory tissues. The 386Y-*gal4* insertion, for example, is located within the 3′ region of the *amontillado* gene, and drives reporter expression in a few hundred secretory cells of the nervous system and periphery in a pattern that is largely overlapping with that of peptide-amidating enzymes (Taghert et al., 2001). GAL4-mediated LARK expression using 386Y caused reduced adult viability relative to siblings that carried only the 386Y transgene. Viability was dependent on the UAS-*lark* line employed in crosses, with 33%, 24% and 2% of GAL4/94A, GAL4; 9A and GAL4; 23A adults surviving, respectively (Table I). In each cross, more than 50% of the non-surviving animals developed to the late pupal (pharate adult) stage but failed to eclose (data not shown).

Compared with 386Y, the c929-*gal4* driver provides expression in a smaller but overlapping pattern of neurosecretory neurons (Taghert et al., 2001). LARK expression using this driver also resulted in reduced viability but to a lesser extent than with 386Y. With c929, 73%, 68% and 60% of the expected number of GAL4-UAS-lark adults emerged using UAS-*lark* lines 94A, 9A and 23A, respectively. We note, however, that the c929-*gal4* insertion itself appears to have a minor effect on viability ($\sim$90% of expected adults emerge; Table I). LARK expression mediated by the c929 drive caused lethality at the pharate adult stage, similar to that observed with 386Y.

The so-called neurosecretory-cell drivers described above have relatively wide patterns of expression, and GAL4 can be detected in non-neural secretory cells of the ring gland, gut and peritracheal organ. To determine whether lethality observed with these drivers might be due to LARK expression in peptidergic neurons previously implicated in ecdysis, we employed GAL4-encoding transgenes that provide expression in the eclosion hormone (EH)-containing (McNabb et al., 1997) and

CCAP-containing neurons (Park et al., 2003). Although *Eh-gal4*/UAS-*lark* flies emerged in slightly reduced numbers, the only statistically significant reduction in viability was with the UAS-*lark* transgene in line 23A (Table I). Flies expressing LARK in the CCAP neurons developed as well or better than siblings lacking the *CCAP-gal4* driver (Table I). Therefore, the expression of LARK in a small (and relevant) subset of neurosecretory cells does not lead to high levels of lethality, although the progeny of these crosses exhibited behavioral and post-ecdysis morphological defects (see below). Finally, we examined *per-gal4, tim-gal4* and *Pdf-gal4*, as all of these drivers target expression to so-called clock cells. None of these drivers affected viability when combined with UAS-*lark* transgenes, and all *gal4*/UAS-*lark* combinations produced phenotypically normal adults in the expected ratios (data not shown).

Lark Expression in Specific Peptidergic Neurons Produces Post-ecdysis Defects

We previously reported several types of defects in flies that express LARK pan-neurally—specifically, a failure to eclose along with defective wing expansion and cuticle tanning (Schroeder & Jackson, 2002). Table II shows that the vast majority of eclosed adults in these populations failed to expand their wings and had a significant delay in cuticle hardening. These phenotypes might be a consequence of effects on the tanning hormone bursicon or other peptide hormones. We observed the same morphological phenotypes with expression of LARK in several different (but overlapping) populations of peptidergic neurons (Table II). For example, flies carrying the 386Y-*gal4* driver along with either the 94A or 9A UAS-*lark* transgene exhibited the wing-expansion and tanning phenotypes (38% of flies for 94A, 56% for 9A). In contrast to 386Y-*gal4*, the majority of adults from crosses using the c929-*gal4* driver were morphologically normal (Table II). This difference might reflect the broader pattern of 386Y transgene expression. Alternatively, 386Y could promote a higher level of GAL4 expression in the relevant cell population.

GAL4-driven LARK expression in the EH cells (an ecdysis-relevant population) also resulted in tanning and wing expansion defects. Expression in the two EH-containing neurons of the brain, while not dramatically affecting viability, caused such morphological defects in a fraction of the population (Table II). As the EH neurons likely communicate with CCAP cells (reviewed by Ewer & Reynolds, 2002), the morphological phenotypes of *EH-gal4*/UAS-*lark* flies might be a consequence of abnormal CCAP cell regulation. Consistent with this hypothesis, a more substantial effect was observed in flies expressing LARK within the CCAP neurons: $\sim$90–100% of adults exhibited the morphological defects, regardless of the UAS-*lark* insertion employed (Table II).

TABLE II Effects of LARK Expression on Cuticle Tanning and Wing Expansion

		Wing and Cuticle phenotype		
GAL4-encoding driver	UAS-*lark*	Normal	Partial[a]	Complete[b]
elav-gal4	94A	0	0	100
	9A	2	4	94
	23A	n.a.	n.a.	n.a.
	none	100	0	0
386Y	94A	62	0	38
	9A	44	0	56
	23A	100	0	0
	none	100	0	0
c929	94A	99	<1	<1
	9A	99	<1	<1
	23A	98	1	1
	none	100	0	0
EH-gal4	94A	67	5	28
	9A	56	5	38
	23A	67	2	31
	none	>99	0	<1
CCAP-gal4	94A	7	6	87
	9A	5	4	91
	23A	<1	0	99
	none	100	0	0

[a]Partial refers to partial but incomplete wing expansion.

[b]Complete indicates that flies have completely unexpanded wings and unhardened cuticle.

[c]n.a. indicates not applicable, because these three types of animals died before eclosion.

Lark Expression Within CCAP Neurons Alters the Daily Timing of Eclosion Events

Given that LARK plays a role in circadian processes and cycles in abundance within CCAP neurons, we examined circadian behaviors using *gal4* drivers that target expression to relevant cell populations (i.e., clock neurons or peptidergic neurons implicated in clock control). LARK is likely to be present in all of these cell types, as the protein has an apparent pan-neuronal distribution (McNeil et al., 2001). As a result of the highly penetrant morphological phenotypes caused by LARK expression within CCAP-containing neurons, we assayed the circadian rhythm of eclosion in populations of *CCAP-gal4*/UAS-*lark* flies. Interestingly, an altered distribution of eclosion events was observed using the *CCAP-gal4* (line 16) driver and any of the UAS-*lark* transgenes (Figure 1).

Although *CCAP-gal4*/UAS-2 *lark* cultures (a.k.a. "populations") exhibited rhythmic eclosion, two aspects of the rhythm were different compared to control populations. First, during a given light/dark (LD) cycle, significantly more adults eclosed in the 2-to-4 h interval before lights-on compared with control populations. When several days of LD data were averaged, a statistically significant shift in the percentage of flies emerging prior to "lights on" was observed for all three *CCAP-gal4*/UAS-*lark* combinations (Figure 1C). This trend persisted in constant darkness, although the difference between pre- and post-peak emergence of controls and experimental populations was less pronounced (Figure 1D), perhaps because of the expected broadening of the circadian gate during constant darkness.

TABLE III Effects of LARK Expression on Locomotor-activity Rhythms

Genotype	N	Rhythmic (%)[a]	Strongly Rhythmic (%)[a]	τ ($\pm$SD)
Pdf-gal4	54	89	81	24.2 (0.3)
UAS-*lark*(94A)	44	91	70	24.2 (0.3)
UAS-*lark*(9A)	32	97	97	24.4 (0.3)
UAS-*lark*(23A)	30	87	63	24.3 (0.3)
Pdf-gal4/UAS-*lark*(94A)	50	34	16	24.3 (0.5)
Pdf-gal4/UAS-*lark*(9A)	52	63	48	24.6 (0.5)
Pdf-gal4/UAS-*lark*(23A)	54	31	19	23.7 (0.4)
CCAP-gal4	47	98	81	23.9 (0.2)
CCAP-gal4/UAS-*lark*(23A)[b]	16	81	38	24.0 (0.3)
EH-gal4	26	96	73	23.9 (0.2)
EH-gal4/UAS-*lark*(23A)[c]	22	91	50	23.9 (0.2)
tim-gal4	28	93	64	24.1 (0.3)
tim-gal4/UAS-*lark*(23A)	29[d]	0	0	–
tim-gal4/UAS-*lark*(16A)[e]	27	93	48	24.1 (0.4)

[a]The % rhythmicity and % strongly rhythmic are based on RI values of individual activity records as described in Materials and Methods; such rhythmic individuals contributed to calculations of the average behavioral cycles durations (free-running periods, or τ values).

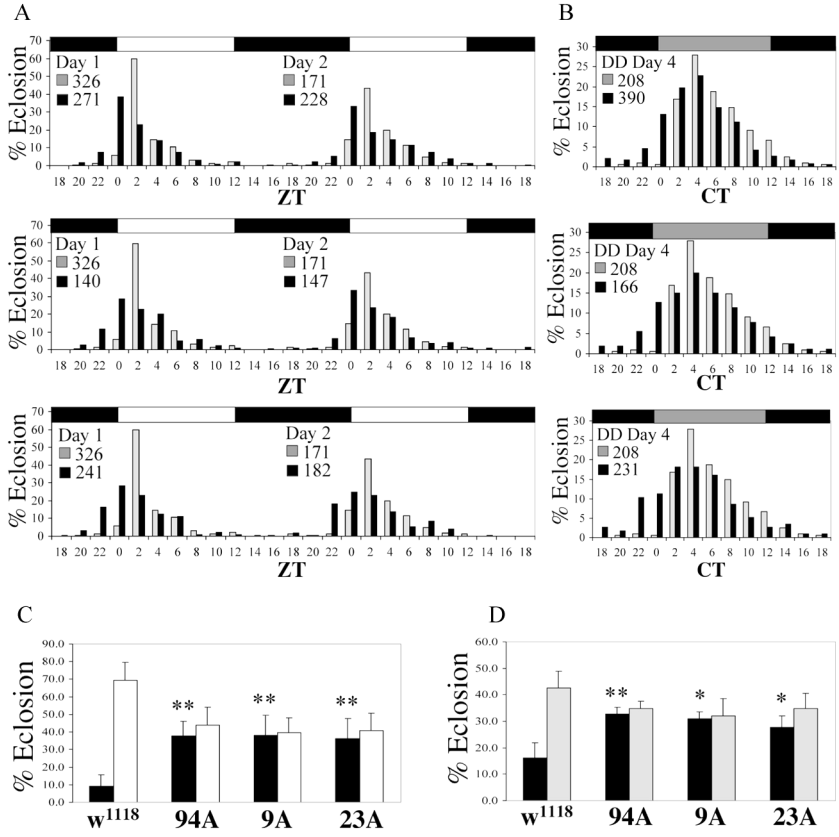
[b]All of these flies exhibit the cuticle tanning and wing expansion phenotypes.

[c]This population included 18 flies with normal wings and cuticle and 4 morphologically abnormal flies.

[d]Some of these flies (11/29) were monitored at 20°C, rather than the standard temperature of 25°C.

[e]These flies carried a UAS-*lark* transgene with site-directed mutations in the RRM1 (F10A) and RRM2 (F89A) domains of LARK protein (see Materials and Methods, and McNeil et al., 2001).

Compared with control flies, *CCAP-gal4*/UAS-*lark* populations also showed a less pronounced response to the lights-on signal at the beginning of the photoperiod. Normal populations show a distinct response to light, which results in increased eclosion in the first bin after the lights-on signal. For example, on day one of the experiment shown in Figure 1, flies from control crosses were five times more likely to emerge in the 2-hour interval (i.e., bin) following lights-on than in other bins of the cycle. This response was less apparent on day 2, although the number of adults emerging in the bin after lights on was still two-fold greater than emergence in any other bin that day. In contrast to the control case, peak emergence for *CCAP-gal4*/UAS-*lark* populations was actually in the bin just prior to lights on, and the number of flies emerging during the subsequent bin was slightly reduced compared to the peak. This dimin-



ished lights-on response is phenotypically similar to the behavior of flies lacking the CCAP or EH cell populations (see Discussion).

The phenotypes of CCAP-GAL4 UAS-lark flies do not result from non-specific effects of high-level protein expression in the CCAP neurons. When β-galactosidase (encoded by *E. coli lacZ*), for example, was expressed in CCAP neurons, under the control of *CCAP-gal4*, the phase of eclosion and the lights-on response were indistinguishable from those of control populations (data not shown). The observed phenotypes are also not due to the UAS-*lark* transgene insertions or the genetic background of transgenic strains: populations resulting from crosses between any of the UAS-lark strains and the w^{1118} (marker-bearing) strain had eclosion profiles similar to those of the control populations depicted in Figure 1 (data not shown).

Although we observed obvious morphological and eclosion phenotypes in *CCAP-gal4*/UAS-*lark* populations, the locomotor-activity rhythms of individuals were relatively normal (Table III). More than

FIGURE 1 GAL4-mediated expression of LARK in CCAP neurons alters the daily profile of eclosion. In (A) and (B), eclosion profiles are shown for the progeny of *CCAP-gal4* crossed with w^{1118} (marker-bearing control flies, gray bars) and UAS-*lark* lines (black bars). The latter were transgenic strains 94A, 9A, and 23A–in the top, middle, and bottom panels, respectively. Two consecutive days of eclosion are shown for populations in LD 12:12 (A), and a single day (DD Day 4) in constant darkness is shown (B). The total number of flies that emerged over the course of each day is indicated for each genotype. The horizontal rectangles above the histograms indicate the light/dark cycle during entrainment; the open and closed bars indicate light and darkness respectively, while the gray bar in the bottom panel shows the light period prior to the shift to DD. Collections were performed at the Zeitgeiber (ZT) or Circadian (CT) Times (respectively referring to LD and DD conditions) indicated on the x-axes. Data are normalized for each time point as the percentage of the total number of flies that emerged over the course of the day. (C) The average percentage of flies that eclosed in the 4 h prior to (black bars) or after (white bars) lights-on are shown for the progeny of *CCAP-gal4*(16) crossed with w^{1118} and each of the UAS-*lark* lines. The histograms were generated from 11 days of LD data for w^{1118} and UAS-*lark*(23A), and from 9 days of LD data for UAS-*lark*'s 9A and 94A. (D) The average percentage of flies that eclosed in the 4 hours prior to (black bars) or after (grey bars) the peak of eclosion for control strains are shown for the progeny of *CCAP-gal4*(16) crossed with w^{1118} and each of the UAS-*lark* lines. The histograms were generated from 4 days of DD day 4 data for w^{1118} and UAS-lark(23A) and from 3 days of DD data for UAS-*lark*(9A or 94A) ** indicates $p < 0.01$, * indicates $0.01 < p < 0.05$, based on unpaired 2-tailed t-tests between pre-peak values for control and *gal4*/UAS-*lark* flies.

80% of the *CCAP-gal4*/UAS-*lark*(23A) individuals were rhythmic, similar to controls that carried only the *CCAP-gal4* driver (Table III); but the robustness of rhythmicity for the doubly-transgenic flies was slightly reduced compared with controls. As all *CCAP-gal4*/UAS-*lark* individuals exhibit a cuticle tanning defect (Table II) that affects viability (perhaps by making them more susceptible to desiccation), the decreased strength is probably simply a consequence of reduced adult viability. Nonetheless, the phasing of activity and circadian periods were normal in *CCAP-gal4*/UAS-*lark* flies.

Expression of Lark in EH Cells Delays the Circadian Gate for Eclosion

In contrast to the early eclosion exhibited by *CCAP-gal4*/UAS-*lark* populations, flies expressing LARK in EH-containing neurons exhibited

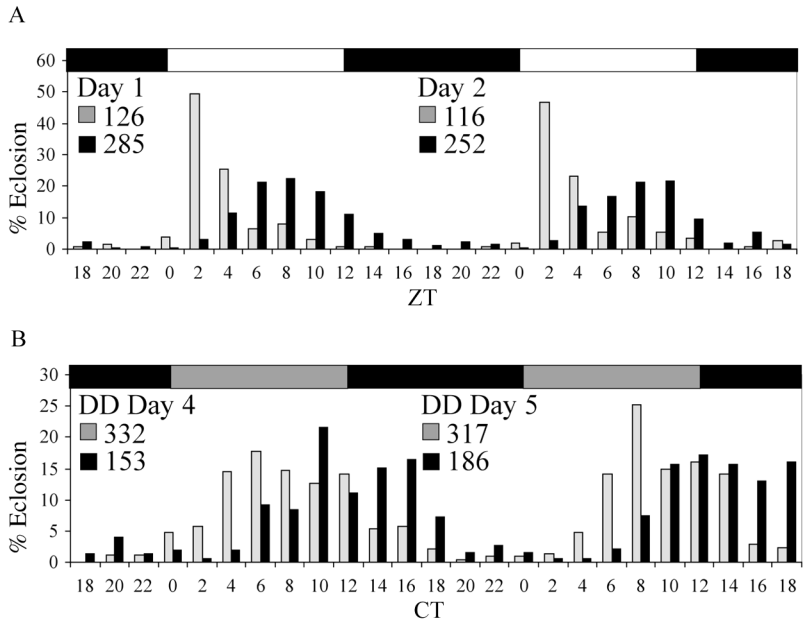


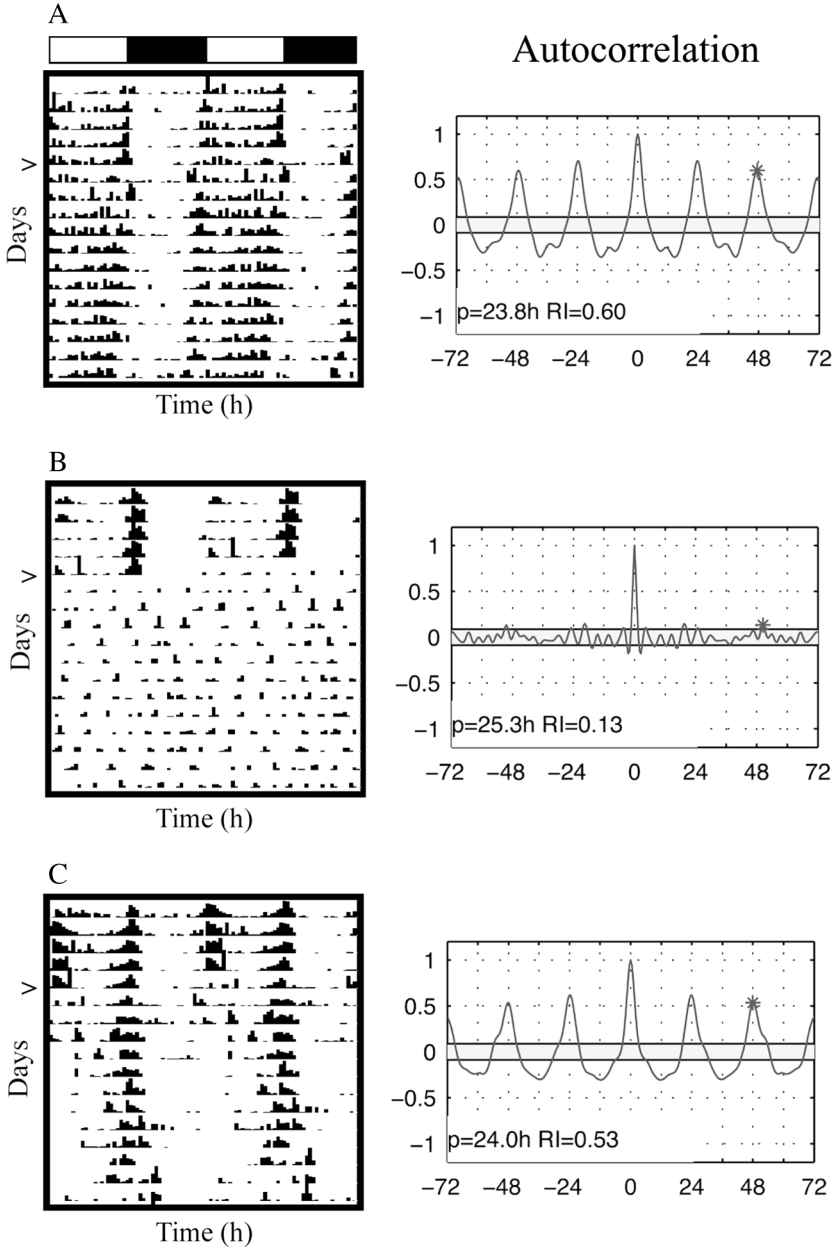
FIGURE 2 GAL4-mediated LARK expression in Eclosion Hormone-containing cells causes delayed adult emergence. Eclosion profiles are shown for the progeny of *EH-gal4* crossed separately with w^{1118} (gray bars) and UAS-*lark*(23A) (black bars). Two consecutive days of eclosion are shown for populations in LD 12:12 (A) or DD (B) conditions. Two independent experiments gave similar results. Shading and notation are as in Figure 1.

a late-eclosion phenotype (Figure 2). Populations carrying an *EH-gal4* driver with 23A UAS-*lark* showed daily peaks of eclosion that were delayed by 6-to-8 h relative to sibling *EH-gal4* flies (Figure 2A). The late phase of eclosion persisted in constant darkness (Figure 2B), with the peak of eclosion for *EH-gal4*/UAS-*lark* flies occurring at least 4 hours after that of control flies. This late-eclosion phenotype was also observed in a single experiment that employed the 94A or 9A UAS-*lark* lines in crosses with *EH-gal4* (data not shown). Importantly, the phenotype is not a consequence GAL4-mediated protein expression in the EH cells, because *EH-gal4*/UAS-*lacZ* flies exhibited normal eclosion rhythms (data not shown). The late-eclosion phenotype was observed in flies with normal morphology and those with a fully or partially penetrant morphological phenotype (i.e., tanning or wing expansion), suggesting that the eclosion phenotype is independent of the wing expansion and cuticle defects.

Similar to *CCAP-gal4*/UAS-*lark*flies, *EH-gal4*/UAS-*lark*(23A) individuals had normal locomotor- activity rhythms (Table II). The phasing of activity, strength of rhythmicity, percentage of individuals rhythmic, and circadian period were similar to those observed in control flies. Thus, there is no evidence for a role of the EH cells in adult circadian processes. Given that the EH cells die in the adult (J. Ewer, personnel communication), it is not surprising that LARK expression in this class of cells has no effect on locomotor rhythms.

Gal4-Mediated Lark Expression Causes Arrhythmic Behavior

Pigment Dispersing Factor (PDF)-containing cells are located in the accessory medulla of the *Drosophila* brain and are known to be cellular components of the clock controlling locomotor activity; furthermore, PDF's presence in (and inferentially release from) these cells is an important feature of clock-controlled rhythmic locomotion (Renn et al., 1999). To determine whether LARK expression can influence a circadian behavior other than eclosion, we analyzed activity rhythms in individual flies expressing LARK in the PDF peptidergic neurons. We found that such flies had a markedly increased tendency to exhibit behavioral arrhythmicity (Figure 3, Table III). Whereas control flies (*Pdf-gal4* or UAS-*lark* alone) showed robust activity rhythms (Figure 3A) with percent rhythmic ranging from 87–97%, flies carrying both transgenes were mostly arrhythmic (Figure 3B) or weakly rhythmic (% rhythmic = 31% for line 23A, 34% for line 94A, and 63% for line 9A). For the *lark* transgenes in two of the UAS-containing lines (94A and 23A), a small percentage of the *Pdf-gal4*/UAS-*lark* flies had strong rhythmicity (Figure 3C; Table III). In these individuals, both the phasing of activity and circadian period were normal.

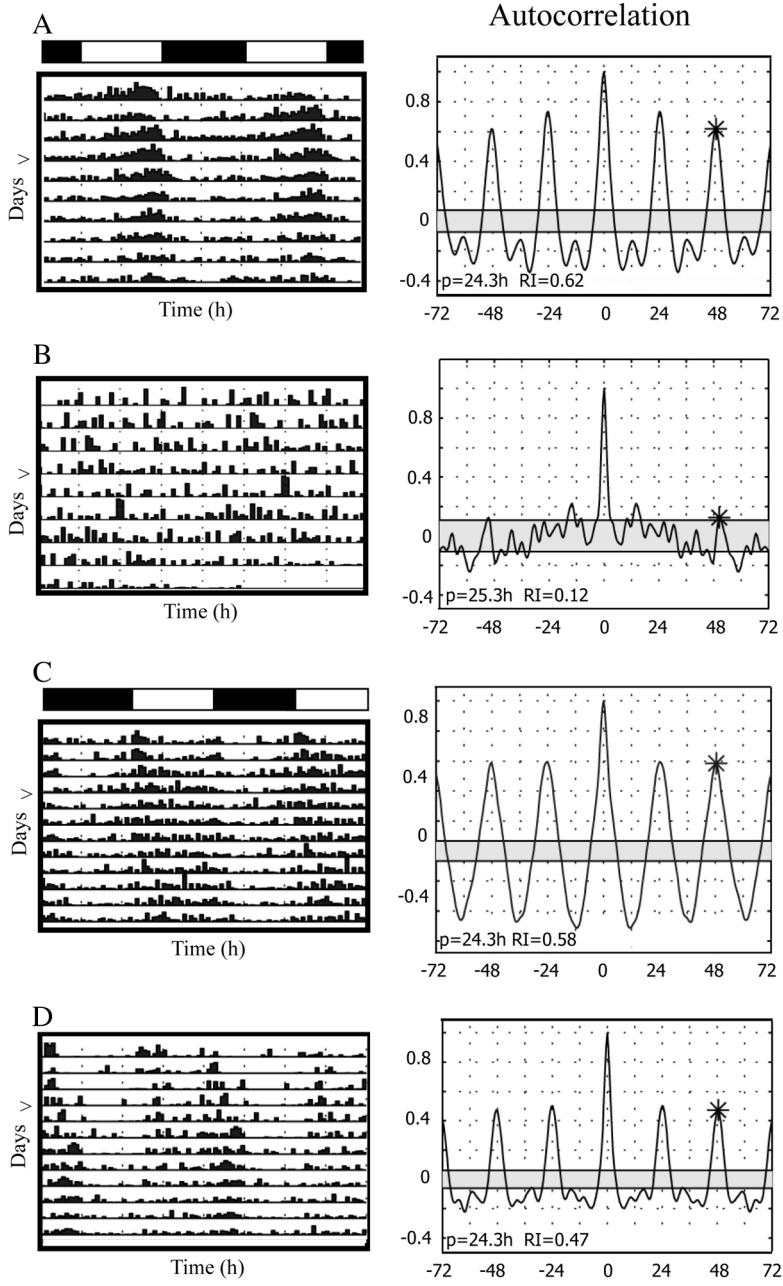

A
Autocorrelation
Days
Time (h)
p=23.8h RI=0.60
B
Days
Time (h)
p=25.3h RI=0.13
C
Days
Time (h)
p=24.0h RI=0.53
1
0.5
0
−0.5
−1
−72 −48 −24 0 24 48 72

The PDF cells represent only a fraction of all PER/TIM-containing clock cells (reviewed by Hall, 2003). To determine how a broader expression of LARK might influence behavior, we utilized a *tim-gal4* strain to drive expression of LARK in all TIM-containing cells. Remarkably, this manipulation completely eliminated rhythmicity in both LD and DD for all individuals tested (Figure 4B, Table III; the table shows pooled data for *tim-gal4*/UAS-*lark* flies characterized in two independent experiments). In contrast, control flies carrying only the *tim-gal4* transgene displayed normal rhythmicity (Figure 4A, Table III).

LARK-Induced Effects are Dependent on a Functional RNA-Binding Domain

We previously demonstrated that *lark* transgenes are inactive (i.e., not able to rescue the recessive lethality caused by *lark* mutations) if they encode a protein with single amino-acid changes affecting critical residues of the RNP2 domains of the two RNA Recognition Motifs (RRMs; McNeil et al., 2001). To test the behavioral effects of such a LARK mutant protein, we expressed it in the TIM-containing cell population using the *tim-gal4* driver and an X-linked UAS-*lark* insertion (16A) encoding the mutant protein (see Materials and Methods). Importantly, we determined that the mutant UAS-*lark*(16A) and wild-type UAS-*lark*(23A) transgenes expressed LARK protein at comparable levels, when driven by GAL4 (by Western blotting of protein extracts; data not shown). As illustrated in Figure 4D and summarized in Table III, *tim-gal4*/UAS-*lark*(16A) flies exhibited activity rhythms that were

FIGURE 3 Expression of LARK in PDF-containing neurons alters activity rhythms. Representative actograms are shown on the left and correlograms on the right for a control [UAS-*lark*(23A)] individual (A), an arrhythmic *Pdf-gal4*/UAS-*lark*(23A) individual (B) and a strongly rhythmic *Pdf-gal4*/UAS-*lark*(23A) individual (C). The fly shown in panel C was one of the most robustly rhythmic *Pdf-gal4*/UAS-*lark*(23A) flies. The actograms show double plotted activity records with the first day of LD shown at the top and subsequent days plotted sequentially. Black histograms represent periods of activity. The arrowhead indicates the day on which the lights were turned off (entry into DD). Horizontal rectangles above the actograms show the light/dark schedule during entrainment. The correlograms are representations of the application of an autocorrelation function and distinct peaks indicate a robust rhythmicity. The circadian period estimated from the autocorrelation function is indicated as *p*. The Rhythmicity Index (RI) is an indicator of the strength of the rhythm (see Materials and Methods).


A
Autocorrelation
Days
Time (h)
0.8
0.4
0
-0.4
p=24.3h RI=0.62
-72 -48 -24 0 24 48 72
B
Days
Time (h)
0.8
0.4
0
-0.4
p=25.3h RI=0.12
-72 -48 -24 0 24 48 72
C
Days
Time (h)
0.8
0.4
0
-0.4
p=24.3h RI=0.58
-72 -48 -24 0 24 48 72
D
Days
Time (h)
0.8
0.4
0
-0.4
p=24.3h RI=0.47
-72 -48 -24 0 24 48 72

indistinguishable from those of control flies (compare to Figure 4C), demonstrating that the behavioral effects observed with wild-type LARK expression are dependent on a functional RRM domain.

Eclosion is Arrhythmic in TIM-GAL/UAS-*lark* populations

To determine whether periodic eclosion would also be affected by increased LARK abundance, we examined daily eclosion profiles in *tim-gal4*/UAS-*lark* and control UAS-*lark* populations. As shown in Figure 5, the eclosion of *tim-gal4*/UAS-*lark* flies was modestly rhythmic, at best, when populations were entrained to a 12:12 LD cycle (black histograms in Figure 5A); but eclosion became completely aperiodic in constant darkness (Figure 5B). In contrast, the eclosion of control UAS-*lark* populations was rhythmic in LD and DD conditions (gray histograms in Figure 5A, B).

PER Cycling in head extracts from TIM-GAL4/UAS-*lark* flies

To examine whether clock protein cycling might be altered in TIM-GAL4/UAS-*lark* flies, we assayed circadian cycling of the Period (PER) clock protein in head extracts prepared from such flies. As shown in Figure 6, rhythmic changes in PER abundance were detected in both LD and DD conditions, and the phase of peak protein abundance (late night/early morning) was the same as has been observed for wild-type protein extracts in many previous such immunochemical assessments (reviewed by Hall, 2003). Thus, at least for this adult tissue, PER cycling is apparently normal in the *tim-gal4*/UAS-*lark* flies. Since we have not examined PER cycling *in situ* in the lateral neuron cell population, we cannot exclude the possibility that cycling is specifically altered in clock neurons. However, there is no evidence for a role of LARK in clock

FIGURE 4 *tim-gal4*-mediated LARK expression eliminates rhythmicity. (A) a rhythmic *tim-gal4* control fly. (B) An arrhythmic *tim-gal4*/UAS-*lark*(23A) fly. (C) A w^{1118} control fly of similar genetic background as in UAS-*lark* strains. (D) A rhythmic *tim-gal4*/UAS-*lark* (16A) fly (expressing an RRM-mutated LARK protein). Plots and conventions are the same as in Figure 3. The light/dark schedule shown above panel A indicates entrainment for the flies depicted in panels A and B, whereas the schedule shown above panel C indicates entrainment for flies C and D.

function per se, and it seems likely that the behavioral effects observed in *tim-gal4*/UAS-*lark* flies are the consequence of a "clock-output" defect.

LARK Expression in Non-Secretory cells has no Detectable Phenotypic Effects

Notwithstanding the lack of effect on clock protein cycling, we wondered whether LARK expression resulted in a general cell toxicity that caused the morphological and behavioral phenotypes observed in the

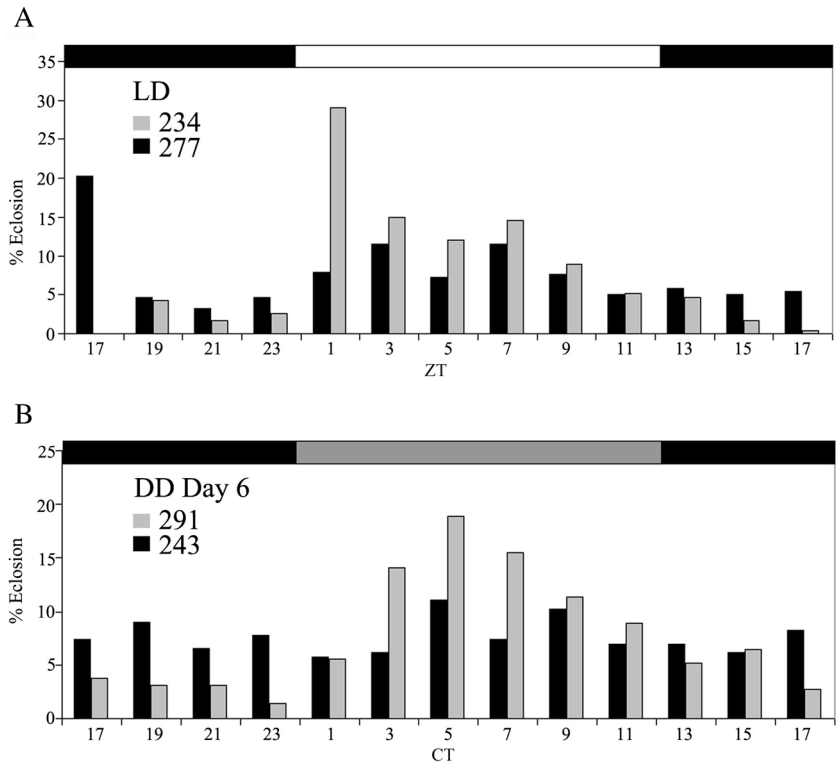


FIGURE 5 Eclosion profiles for *tim-gal4*/UAS-*lark*(23A) (black histograms) and UAS-*lark*(23A) control (gray histograms) populations. (A) Eclosion profiles in LD 12:12. (B) Eclosion profiles on day 6 of constant darkness (DD). The numbers below the LD and DD designations indicate the total number of flies that emerged in the experiment. The control population was generated by outcrossing flies from a homozygous UAS-*lark*(23A) strain to flies of the w^{1118} strain (the strain used to generate UAS-*lark* transgenic flies).

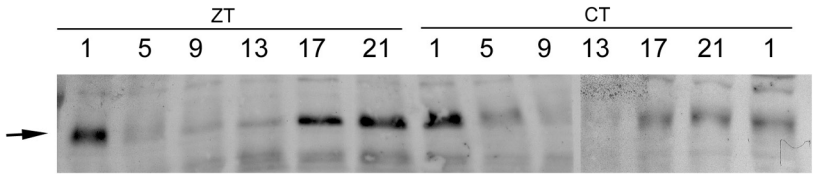


FIGURE 6 Cycling of the PER clock protein in head extracts of *tim-gal4*/UAS-*lark*(23A) flies. Normal cycling of PER abundance in LD (ZT) and DD (CT). Samples ZT 1 to CT 9 are from one blot, whereas CT 13 to CT 1 are from a separate blot. The arrow indicates the position of PER protein, as determined by the position to which it ran on the gel and its abundance oscillation (cf. Edery et al., 1994, Atken et al., 2003, and other such studies reviewed by Hall, 2003).

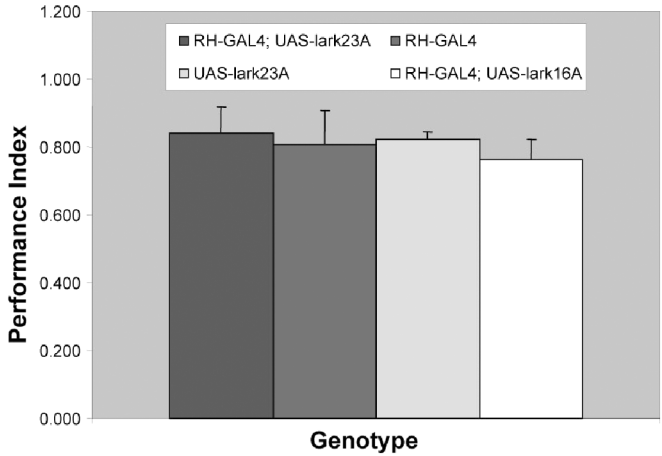
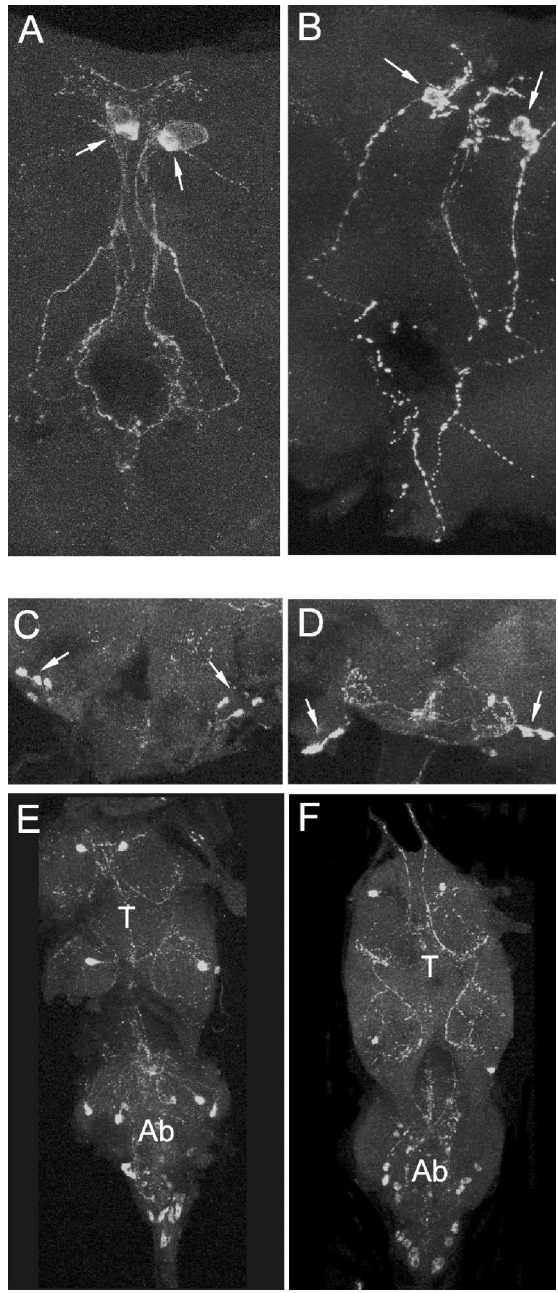


FIGURE 7 Fast phototaxis in *Rh-gal4*/UAS-*lark* flies. Neither wild-type LARK [produced by UAS-*lark*(23A)] nor an inactive mutant form of the protein [UAS-*lark*(16A)], when either was overexpressed in the eyes, affected this light-induced behavior (photophilic locomotion, following agitation of fly groups). Expression was driven by a GAL4-encoding driver (see above) that targets the six outer-retinal cells within all compound-eye ommatidia. Fast phototaxis was determined according to Simon et al. (2003). The performance index is the number of positive responses (flies that ran to the light) divided by the total. Histograms represent the average ($\pm$ standard deviation) for 3 or 4 independent fast-phototaxis. There were no statistical differences for any pair-wise comparison of the performance index. Data are shown for males, but similar results were obtained for females carrying *Rh-gal4* only, UAS-*lark* only, or both transgenes (data not shown) (See Color Plate VI at the end of this issue).


A
B
C
D
E
T
Ab
F
T
Ab

present study. To test this idea, we used unrelated GAL4 drivers to promote the expression of LARK in non-secretory cell types. *TH-gal4* (Friggi-Grelin et al., 2003) and *Rh-gal4* (J.E. O'Tousa, personal communication) drive expression in tyrosine hydroxylase-containing and compound-eye R1-6 photoreceptors, respectively.

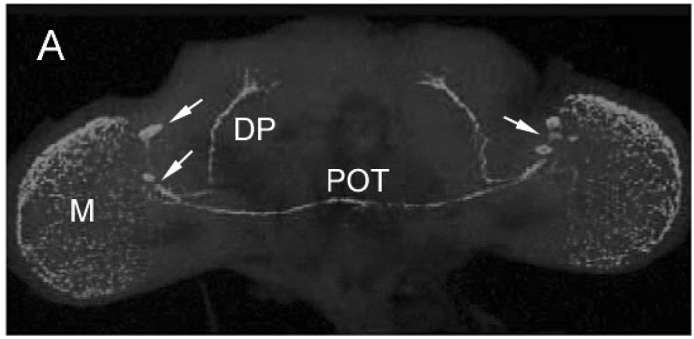
It is known from previous studies that null *pale* mutations, which eliminate TH activity, are associated with lethality and defects in cuticle formation (Wright, 1987). Furthermore, inhibition of TH activity during larval development results in defects in adult behavior, probably due to perturbed sensory function (Neckameyer et al., 2001). To ask whether LARK expression can cause lethality, we examined the development of *TH-gal4*/UAS-*lark*(23A) flies. These flies developed normally, emerged in the expected numbers and were morphologically normal (data not shown). In contrast, when the *TH-gal4* driver was used to promote expression of the cell death gene *reaper* (*rpr*, e.g., Park et al., 2003), all resulting *TH-gal4*/UAS-*rpr* flies died prior to adulthood (not shown), similar to the lethality observed for *pale* null mutants. Thus, LARK expression in TH-containing cells is not associated with toxicity.

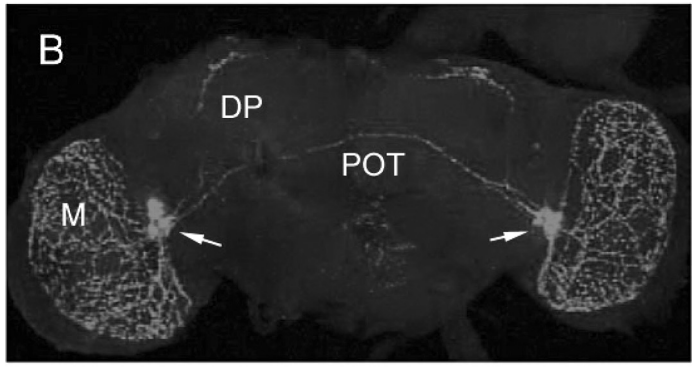
As a second test of toxicity, we expressed LARK in the R1-6 retinal cells using the *Rh-gal4* driver. *Rh-gal4*/UAS-lark(23A) adults had normal eye morphology (data not shown) and responded to light in a manner comparable to control siblings in a fast phototaxis assay (Figure 7). Together with the lack of effects observed in *TH-gal4*/UAS-*lark* flies, these results suggest that LARK expression does not perturb function in all cell types.

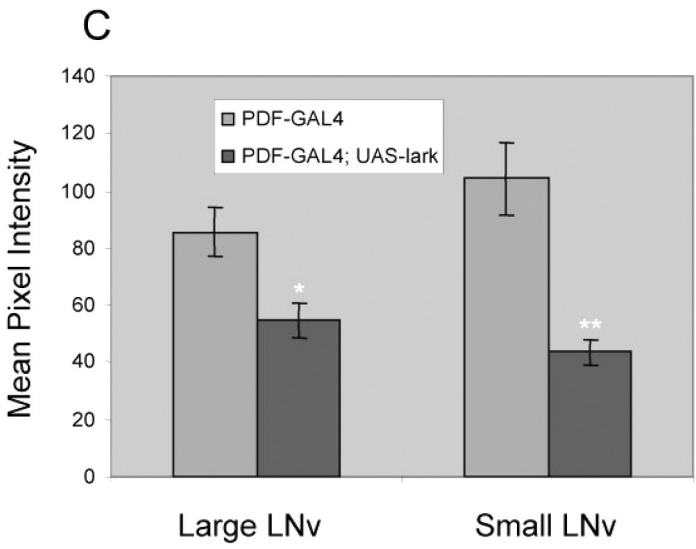
LARK Expression Results in Diminished PDF Immunoreactivity

We considered the possibility that LARK-mediated effects on behavioral rhythmicity were a consequence of altered peptidergic neuronal development. Therefore, we examined the morphology of PDF, CCAP, and EH neurons in flies expressing LARK in each of these cell types. In

FIGURE 8 Presence and morphology of EH and CCAP cells in *gal4*/UAS-*lark* flies. (A-B) High magnification pictures of the EH neurons and their projections from UAS-*lark* control and *EH-gal4*/UAS-*lark* pharate adults, respectively. The EH cells are indicated by arrows. (C-D) Brains from UAS-*lark* and *CCAP-gal4*/UAS-*lark* pharate adults, respectively. Arrows indicate the CCAP cell bodies within the subesophageal ganglion. (E-F) CCAP cells and their processes in the ventral nerve cords of UAS-*lark* and *CCAP-gal4*/UAS-*lark* pharate adults, respectively. Immunostaining can be visualized within CCAP cell bodies and processes of the thoracic (T) and abdominal (Ab) ganglia in both types of flies (See Color Plate VII at the end of this issue).


A
DP
POT
M


B
DP
POT
M


C
Mean Pixel Intensity
140
120
100
80
60
40
20
0
PDF-GAL4
PDF-GAL4; UAS-lark
*
**
Large LNv
Small LNv

all flies examined, the numbers and morphologies of the different cell types appeared normal, relative to control flies carrying only the *gal4* driver (Figures 8 and 9). We carefully examined the architecture of the PDF-containing small and large ventro-lateral neurons (LN_v's in Figure 9), as these cells have well-characterized projection patterns (reviewed by Hall, 2003) that cross the midline (large) or extend to the dorsal brain (small). Visual inspection did not reveal dramatic differences in the number or projection patterns of PDF neurons between *Pdf-gal4*/UAS-*lark* and control flies (data not shown). Although we did not examine neuronal architecture in *tim-gal4*/UAS-*lark* flies, there is no evidence from the analysis of other transgenic types in which *lark* sequences are driven by *gal4* that LARK expression is associated with perturbations of neural development.

Consistently, however, we observed reduced PDF immunoreactivity in *Pdf-gal4*/UAS-*lark* flies relative to control individuals (Figure 9). We further examined the decrement in PDF immunoreactivity using quantitative confocal microscopy. In those studies, we calculated the total pixel intensities for the small and large LNv cell projections of control (*Pdf-gal4* alone) and experimental [*Pdf-gal4*/UAS-*lark*(23A)] populations in samples collected at the same time of day (late subjective morning of a LD 12:12 cycle). Decreased PDF immunoreactivity was observed for both cell types of experimental flies in three independent experiments (Figure 9C), and the decrement in staining intensity was most apparent in the dorsal projections of the small LNv cells (projections labeled DP in Figure 9). These neurons are postulated to be particularly relevant cellular substrates of behavioral rhythmicity (e.g., Helfrich-Förster et al.,

FIGURE 9 PDF cells and projections in *Pdf-gal4*/UAS-*lark* and control flies. (A) UAS-*lark*. (B)*Pdf-gal4*/UAS-*lark*(23A). Clusters of PDF-containing cells are indicated by arrows. The orientations of the brains in panels A and B are not precisely the same, and therefore the positions of the large and small PDF cells and the optic-lobe (M, Medulla) arborization patterns appear slightly different. Note that the inspection of 14 experimental and 14 control brains did not reveal differences in cell position nor optic lobe staining pattern (only the apparent intensity of immunostaining in the PDF axonal projections differed between the two types of brains). POT, projections of the large PDF-containing LN_v cells through the Posterior Optic Tract. DP, dorsal projections of the small PDF-containing LN_v neurons. (C) Quantitation of PDF staining (pixel intensity) in the projections of the large and small PDF-containing neurons in control (*Pdf-gal4*) and experimental (*Pdf-gal4*/UAS-*lark*) brains. Samples sizes were 14 brains for each genotype, and means $\pm$ standard deviation are shown in panel C. Asterisks indicate statistical differences between experimental and control populations; *, $p < 0.05$; **, $p < 0.01$ (unpaired 2-tailed t-tests) (See Color Plate VIII at the end of this issue).

2000; Park et al., 2000; Yang & Sehgal, 2001; Akten et al., 2003). We conclude that high-level expression of LARK within the PDF neurons has effects on PDF synthesis, processing, transport or release. It seems likely that an effect on PDF contributes to the arrhythmicity of *Pdf-gal4*/UAS-*lark* flies.

DISCUSSION

Broad Neuronal Expression of LARK Causes Lethality

In this study, we documented effects of LARK expression on viability, adult morphology, and circadian behavior. We show that there are significant effects on development and viability with broad expression of LARK in secretory cell populations, but minimal effects with a targeted expression in specific neurosecretory cell types (those containing the EH, CCAP, or PDF peptides). A lethal phenotype is observed with neuronal-specific expression of LARK (driven by an *elav-gal4* transgene), indicating that death is not simply due to peripheral expression of the protein (e.g., in peripheral secretory cells, cf. Schoeder & Jackson, 2002). These results indicate that the observed lethality is due to effects in neuronal populations other than the EH, CCAP, or PDF cells; or that lethality is a consequence of LARK expression, simultaneously, in several different cell types.

Targeted Expression of LARK in Clock or Peptidergic Neurons Alters Circadian Behavior

Using GAL4-encoding transgene drivers expressed in defined anatomical patterns, we have shown that the expression of LARK in each of three different types of peptidergic neurons (CCAP, EH, and PDF) is associated with predictable effects on the circadian control of behavior. In each case, the behavioral phenotypes are similar to those observed with genetic (*rpr*-mediated) ablation of the particular peptidergic population (McNabb et al., 1997; Renn et al., 1999; Park et al., 2003): early eclosion for CCAP, late-eclosion and diminished light response for EH (see below), and arrhythmicity for PDF cells. Given the ubiquitous neural expression pattern of LARK, it is likely that all these cell types normally contain the protein and that the observed behavioral phenotypes are due to increased protein abundance rather than ectopic expression of LARK. In the case of CCAP neurons, it is possible that increased LARK abundance perturbs the molecular rhythm that is normally observed for LARK in this cell population.

We note that a rhythm phenotype has not previously been reported for EH-less flies, but we recently found that *EH-gal4*/UAS-*rpr* populations

exhibit a late-eclosion phenotype identical to that observed in *EH-gal4*/UAS-*lark* populations (G. Genova & F.R. Jackson, in preparation). Interestingly, this phenotype is most apparent when populations are entrained to a temperature rather than a light/dark cycle. Although there are obvious phenotypes associated with GAL4-driven LARK expression in clock and peptidergic neurons, a lack of phenotype with certain *gal4* drivers, such as *TH-gal4* and *Rh-gal4*, suggests that the effects of LARK may be restricted to particular cell types.

Our studies also show that a broad expression of LARK in cells relevant to the clock system dramatically alters circadian behavior. Adult flies expressing LARK in PDF- or TIMELESS (TIM)-containing cells, for example, exhibited arrhythmic locomotor activity behavior. Similarly, *tim-gal4*/UAS-*lark* populations showed aperiodic eclosion profiles in constant darkness. The observation that PER protein cycling was found to be normal in head extracts of *tim-gal4*/UAS-*lark* flies suggests that the "molecular oscillator" is functional. However, we realize that most PER protein in such extracts originates from the eyes (reviewed by Hall, 2003), allowing for the possibility that our experiments missed a specific effect on cycling within the population of PER-containing lateral-brain neurons. Yet, because there is no evidence suggesting a role for LARK in circadian-oscillator function (cf. Newby & Jackson, 1993, 1996), we believe it is more likely that LARK expression alters a clock-controlled component of *Drosophila*'s circadian system. In this regard, arrhythmic behavior for both locomotor-activity and eclosion in the *tim-gal4*/UAS-*lark* animals suggests that there is an effect on a "proximal" component of clock output (that is, one functioning above the level of far-downstream factors and processes that would be devoted solely to one rhythmic phenotype or the other).

It is worth contrasting our results with those of previous studies that used genetic manipulations to directly interfere with oscillator function. Those studies used targeted over-expression of clock proteins (PER or TIM: Yang & Sehgal, 2001; Kaneko et al., 2000) or electrical silencing of clock cells (Nitabach et al., 2002) to disrupt oscillator function. In contrast, we demonstrate that oscillator function is normal (as assessed by PER cycling) in the presence of high LARK protein levels (i.e., in *tim-gal4*/UAS-*lark* flies). Also of interest, the LARK-induced arrhythmicity we document is more severe with *tim-gal4*-than with *Pdf-gal4*-mediated expression of LARK. Normal PER cycling in such flies indicates that the clock has not stopped, and thus the behavioral effects must be due to a perturbation of "output" mechanisms in other TIM-containing neurons. These may include the dorsal neuronal populations (or so-called DN cells) in which clock protein cycling has been observed (Kaneko & Hall, 2000), or other peptidergic neurons that might function as part of the circadian system (Taghert et al., 2001).

RNA-Recognition Motifs are Required for LARK Activity

At least for the flies' locomotor-activity rhythm, effects of LARK expression are ameliorated by mutation of residues known to be critical for the RNA-binding function of this class of proteins (cf. McNeil et al., 2001). This indicates that normal RNA-binding function is required for the observed LARK-induced arrhythmicity, and that such an effect is probably mediated by binding of the protein to certain target mRNA(s) (rhythm-related ones as opposed to very broad categories of such targets). Thus, a reduction in the abundance or activity of a relevant LARK mRNA target might be expected to suppress phenotypes associated with GAL4-driven LARK expression. This suggests a novel behavioral screen for genetic modifiers of LARK-associated phenotypes. Such a screen may lead to the identification of mRNA targets or interacting proteins that are important for the behavioral functions of LARK.

Increased LARK Expression did not Cause Cell Death

Although LARK expression seems to mimic a cell ablation phenotype for peptidergic neurons, this manipulation does not have obvious effects on neuronal development. For each *gal4*/UAS-*lark* combination, the relevant neuronal type (CCAP, EH, or PDF) was found to be present in the differentiated nervous system and exhibited the pertinent peptide immunoreactivity. The analysis of *Pdf-gal4*/UAS-*lark* individuals, however, revealed a decrement in PDF immunoreactivity in the projections of the LN_v neurons, but we attribute this result to an abnormal regulation of PDF levels rather than a developmental defect. Consistent with this interpretation, the architecture and projections of these neurons were found to be normal. We conclude that LARK expression results in a functional alteration of neuronal properties, rather than cell death, for all three types of peptidergic cells.

A Role for LARK in Peptidergic Neuronal Regulation?

What is the function of the LARK RNA-binding protein within the nervous system, and how does such a function explain the LARK-mediated phenotypes observed in the present study? Although the RNA targets of LARK have not yet been identified, previous studies have documented a pan-neuronal distribution for the protein (Zhang et al., 2000). Interestingly, in all neurons except the CCAP cells the protein has a nuclear intracellular distribution. In contrast, within the CCAP neurons LARK has a distinctive cytoplasmic localization (suggestive of a role in translational regulation), and within these cells exhibits a circadian rhythm in abundance. Zhang et al., 2000 as RNA-binding proteins are

capable of transient "shuttling" between the nuclear and cytoplasmic compartments, the importance of these differences in the intracellular distribution of LARK remains to be determined. Notwithstanding the apparently different localization patterns, the behavioral effects of LARK expression suggest that the protein may have a general role in the regulation of peptidergic neuronal physiology. Indeed, we observed reduced peptide immunoreactivity in PDF-containing neurons, as a consequence of GAL4-mediated LARK expression. Together with the finding that LARK expression mimics a cell ablation phenotype, this result suggests that LARK is capable of behaving as a repressor molecule within diverse types of peptidergic neurons.

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