DOI: 10.1534/genetics.107.076513

Rhythm Defects Caused by Newly Engineered Null Mutations in Drosophila's *cryptochrome* Gene

Eva Dolezelova,[1] David Dolezel[1,2] and Jeffrey C. Hall[3]

Department of Biology, Brandeis University, Waltham, Massachusetts 02454

Manuscript received May 24, 2007
Accepted for publication July 3, 2007

ABSTRACT

Much of the knowledge about cryptochrome function in Drosophila stems from analyzing the cry^b mutant. Several features of this variant's light responsiveness imply either that CRY^b retains circadian-photoreceptive capacities or that additional CRY-independent light-input routes subserve these processes. Potentially to resolve these issues, we generated *cry* knock-out mutants (cry^0's) by gene replacement. They behaved in an anomalously rhythmic manner in constant light (LL). However, cry^0 flies frequently exhibited two separate circadian components in LL, not observed in most previous cry^b analyses. Temperature-dependent circadian phenotypes exhibited by cry^0 flies suggest that CRY is involved in core pacemaking. Further locomotor experiments combined cry^0 with an externally blinding mutation ($norpA^{P24}$), which caused the most severe decrements of circadian photoreception observed so far. cry^b cultures were shown previously to exhibit either aperiodic or rhythmic eclosion in separate studies. We found cry^0 to eclose in a solidly periodic manner in light:dark cycles or constant darkness. Furthermore, both cry^0 and cry^b eclosed rhythmically in LL. These findings indicate that the novel cry^0 type causes more profound defects than does the cry^b mutation, implying that CRY^b retains residual activity. Because some $norpA^{P24}$ cry^0 individuals can resynchronize to novel photic regimes, an as-yet undetermined light-input route exists in Drosophila.

CIRCADIAN clocks evolved in most organisms in part to mediate the internal "temporal order" that is associated with various biological phenomena, and, furthermore, such that an animal, plant, or microbe can anticipate everyday periodic changes of light and temperature. Light comprises the principal clock resetting cue, which keeps the process running at a 24.0-hr pace in natural conditions (Dunlap *et al.* 2004). Thermal cycles can also "entrain" circadian clocks; and another aspect of how these processes respond to temperature changes is to compensate such that the pacemaker operates at the same velocity in different thermal conditions (Dunlap *et al.* 2004).

One way by which *Drosophila melanogaster*'s version of a cryptochrome (CRY) protein was implicated in circadian rhythms was by screening for mutations that would perturb luciferase (*luc*)-reported expression of a canonical clock gene called *period* (*per*). The seminal cry^b mutation was thus recognized because it eliminated the normal daily cycles of luminescence mediated by a *per-luc* transgene in real-time monitorings of whole flies (Stanewsky *et al.* 1998). This mutation entails an amino-acid substitution within the fly's CRY at a site believed to be involved in binding a flavin moiety that participates in the protein's capacity to absorb blue light (Stanewsky *et al.* 1998; *cf.* Sancar 2000).

Most of the "glow cycling" emanating from cry^+ (and otherwise rhythm-normal) Drosophila comes from peripheral tissues (Stanewsky *et al.* 1997), including several appendages projecting from the adult animal (Plautz *et al.* 1997; Levine *et al.* 2002b). The effects of the cry^b mutation on such molecular rhythmicity implied that the normal gene is expressed in the periphery, but cry^+ was found also to make its products in the central nervous system (Emery *et al.* 2000b; Klarsfeld *et al.* 2004). In one test of cry^b's effects on whole-animal rhythm-related phenotypes, mutant individuals resynchronized their behavior in a largely normal manner to shifted light:dark (LD) cycles; "L" in the postshift regime was dim blue light (Stanewsky *et al.* 1998). However this mutant type required longer-than-normal times to resynchronize (Emery *et al.* 2000b). Much poorer photic sensitivity was observed when a *no-receptor-potential-A* mutation ($norpA^{P41}$), which probably causes blindness at the level of all external photoreceptors, was added to a cry^b genetic background. However, appreciable proportions of the $norpA^{P41}cry^b$ double mutants were capable of re-entraining their locomotor cycles to shifted photic regimes that involved dim blue light (Stanewsky *et al.*

[1]*Present address:* Institute of Entomology, Biology Centre, ASCR, Ceske Budejovice 37005, Czech Republic.

[2]*Present address:* Faculty of Biological Sciences, University of South Bohemia, Ceske Budejovice 37005, Czech Republic.

[3]*Corresponding author:* Department of Biology, MS-008, Brandeis University, Waltham, MA 02454-9110. E-mail: hall@brandeis.edu

1998; see also Helfrich-Förster *et al.* 2001; Mealey-Ferrara *et al.* 2003). More severe entrainment problems were observed when cry^b was combined with a *glass* eye mutation (gl^{60j}), which eliminates external photoreceptive structures *and* a putative "extra-ocular" photoreceptor called the Hofbauer–Buchner (H–B) eyelet (Helfrich-Förster *et al.* 2001; Mealey-Ferrara *et al.* 2003). The H–B structure is located underneath the compound eye at the distal extremity of the CNS's optic lobes (Hofbauer and Buchner 1989).

Issues revolving round a circadian-photoreceptive role played by the H–B eyelet connect with chronogenetic variants produced for or applied in the current study: Consider that the normal form of the aforementioned *norpA* gene is expressed not only in the compound eyes and ocelli, but also within H–B eyelet photoreceptors—along with *Rhodopsin* gene products that are possessed by such extra-ocular photoreceptive cells (Malpel *et al.* 2002). Therefore, one could predict that all photic input routes to the clock should be blinded in a *norpA* cry^b double mutant, with the proviso that both variants are null. But Drosophila suffering the simultaneous effects of these mutations were re-entrainable (see above), and a *norpA*-independent function has recently been re-argued as to how the H–B structure operates within the fly's circadian system (Veleri *et al.* 2007). With regard to the eyelet-eliminating effect of gl^{60j}, the enhanced degradation of rhythm-related photoreception caused by combining that mutation with cry^b (see above) involved an overinterpretation of the extent to which that doubly mutant type is circadian blind (see Mealey-Ferrara *et al.* 2003 and further discussions of this matter within the current article). More generally, one can denigrate the *glass* mutation as a dull experimental tool: The gl^{60j} mutant is likely to possess pleiotropic abnormalities beyond its lack of eyes and H–B eyelets; that this genetic variant could be brain damaged in only partly appreciated ways is implied by elements of the findings in Helfrich-Förster *et al.* (2001) and Klarsfeld *et al.* (2004).

In any case, these mutational effects indicated that the entrainment pathway that keeps Drosophila behaving in synchrony with LD cycles entails multiple input routes. That one of them includes photoreception by CRY in the head was signified by two further tests (Stanewsky *et al.* 1998; Emery *et al.* 2000a): (i) cry^b individuals were found not to exhibit locomotor phase shifts after relatively brief light *pulses* were delivered to flies otherwise maintained in constant darkness (DD) and (ii) cry^b behaved in an anomalously *rhythmic* manner in LL. Genetically normal Drosophila behave arrhythmically when the LL intensity is rather high but exhibit longer than normal (compared with DD) locomotor periods in dim light (Konopka *et al.* 1989). By comparison, cry^b individuals have been reported either to display periodicities in LL that are either equivalent to the free-running behavior of wild-type Drosophila in DD (Emery *et al.* 2000a) or entail slightly lengthened cycle durations (Helfrich-Förster *et al.* 2001; Mealey-Ferrara *et al.* 2003). If the latter sets of results are accurate, the suggestion arises that this mutant is not completely "circadian-blind" in this kind of photic situation: cry^b flies would be perceiving an ordinary level of LL as dim light.

"Deep brain" expression of the normal *cry* coding sequence has been deduced to connect with elements of these behavioral phenomena. Driving cry^+ with a *Pdf-gal4* transgene, which is expressed in the brain only within ~10 pairs of so-called ventro-lateral neurons (LN_v's) and in the posterior-most ganglion of the ventral nerve cord (Park *et al.* 2000), resulted in partial "rescue" of cry^b-induced insensitivity to light pulses and abnormal rhythmicity in LL (Emery *et al.* 2000a,b). Rescue of the mutant's light responsiveness was better (Stoleru *et al.* 2007) when cry^+ was transgenically restored to dorsal LN's, which are also among the brain cells expressing clock genes.

In adult appendages, cry^b caused poor cycling of *per* and *timeless (tim)* and essentially quashed a daily cycle of odor sensitivity that normally operates in the antenna (*cf.* Krishnan *et al.* 1999); these results led to the suggestion that CRY functions as a "clock protein" in the periphery (Krishnan *et al.* 2001; *cf.* Ivanchenko *et al.* 2001; Collins *et al.* 2006). This supposition was considered further in the context of cry^b's (partial) disruption of molecular rhythms in isolated peripheral-tissue specimens (Levine *et al.* 2002a,b). However, monitoring luciferase-reported cyclings of clock genes in cultured appendages implied that residual *per* and *tim* gene-expression rhythmicities may still be running in cry^b specimens (Krishnan *et al.* 2001; Levine *et al.* 2002b). In contrast, clock function in the CNS remains robust under the influence of this mutation. For example, free-running behavioral rhythmicity was found to be unimpaired in cry^b flies in DD (*e.g.*, Stanewsky *et al.* 1998; Mealey-Ferrara *et al.* 2003). However, when CRY was caused to be "constitutively active," longer-than-normal behavioral cycles were observed (Busza *et al.* 2004; Dissel *et al.* 2004); and when cry^b was added to a genotype that causes overexpression of a "clock kinase" and shortened free-running periods, the locomotor cycle durations were pushed closer to the normal range (Stoleru *et al.* 2007).

The cry^b mutation causes a lowering of CRY levels sufficient to reveal the participation of this factor in circadian photoreception and in peripheral timekeeping. However, residual CRY activity associated with both such processes suggests that cry^b is not a loss-of-function mutant. Initially, CRY immunoreactivity was undetectable in homogenates of this mutant type (Stanewsky *et al.* 1998). However, that antibody is no longer available, and applications of an anti-CRY subsequently produced by Busza *et al.* (2004) indicated that cry^b "leaks out" a low level of its encoded protein; additionally, these

investigators generated a new cryptochrome mutant (cry^m), which also is hypomorphic. Key features of these findings prompt the hypothesis that the existing mutants *retain enough functional CRY* to allow for the various rhythmic attributes now being surveyed. Alternatively, cry^b is a null mutant; and *alternative input routes* that do not use CRY would mediate light-to-clock signaling in flies suffering the effects of an extant *cry* mutant, including in those that carry an additional visual-system mutation (*norpA* or *glass*).

Therefore, we felt that an expansion of the allelic series for *cryptochrome* in *D. melanogaster* was warranted. We set out to target this autosomal locus by the transposon-based tactics that allow for exogenous DNA sequences to be engineered into a specific Drosophila gene (reviewed in Bi and Rong 2003; Venken and Bellen 2005). We then analyzed rhythms of behavior and of *luc*-reported clock-gene expression in *cry*-null flies and their peripheral tissues.

One more reason for creating a new *cry* mutant connects with whether or not CRY-depleting genetic variants affect the circadian rhythm accompanying eclosion (emergence of adults from metamorphosis). It was assumed that the compound eyes are not involved in transmitting light inputs toward the clock that underlies periodic eclosion, because *D. pseudoobscura* cultures dramatically depleted of retinal (required for the function of compound-eye rhodopsins) still can be synchronized by photic inputs to exhibit rhythmic eclosion with undiminished sensitivity (Zimmerman and Goldsmith 1971). In contrast, dietary depletion of retinal causes markedly subnormal sensitivity of the fly's circadian system insofar as light-induced synchronization of adult locomotion is concerned (Ohata *et al.* 1998). Furthermore, the most efficacious light subserving synchrony of developing Drosophila is in the blue range (Frank and Zimmerman 1969; Klemm and Ninnemann 1976), where CRY absorbs maximally (Sancar 2000). Therefore, one could readily predict that cry^b cultures would eclose aperiodiocially—a result that was reported in one study (Myers *et al.* 2003); but dramatically different effects stemmed from another one (Mealey-Ferrara *et al.* 2003). We surmised that testing effects on eclosion of a novel, even more severely mutated, *cry* allele could resolve this conundrum.

MATERIALS AND METHODS

Construct for homologous recombination into the *cry* locus: A plasmid derived from a vector *pW25* (Gong and Golic 2003) was modified to contain separate pieces of third chromosomal DNA that closely flank the *cry* locus. This vector otherwise contains a shortened version of the *white* gene (intronless mini-$white^+$, hereafter called w^+) and two FRT sequences that are substrates for flipase (FLP) recombinase (see Figure 1A). The "near-*cry*" sequences inserted into *pW25* were two genomic fragments, one containing a 5′-flanking and the other containing a 3′-flanking sequence. They were amplified by PCR from DNA extracted from Canton-S wild type (stock no. 1 at the Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN), using the following primers: (1) to produce the 5′ locus-flanking fragment (consisting of 2944 bp): CRYf-Acc65I, 5′-CAGGTACCGTTTTATTTGGTTTCGTTTTAC; CRYr-SphI, 5′-CAGCATGCCGCGGTCCCCTGGTG; and (2) the 3′ fragment (2826 bp): CRY2f-BsiWI, 5′-CACGTACGCAGGCGAGGAGACAAC; CRY2r-AscI, 5′-CAGGCGCGCCATGCGAAATTTGAGTGCTGAGAA. The two amplicons were ligated into *pW25* restriction sites (for the 2.9-kb fragment: *Acc*65I/*Sph*I; for the 2.8-kb one: *Bsi*WI/*Asc*I). Thus, the *pW25/cry* vector carries w^+ flanked by the two pieces of near-*cry* DNA; located outside of those 2.9- and 2.8-kb fragments are the FRTs and two I-*Sce*I sites (Figure 1).

Molecular procedures and genetic crosses for creation of transgenic lines: The *pW25/cry* construct aimed at mediating HR was injected into *y w* embryos whose third chromosome contained the transposase-encoding *Δ2-3* variant (Robertson *et al.* 1988). Otherwise, the procedure for producing germline *P*-element transformants was conventional (*e.g.*, Rubin and Spradling 1982; Park and Lim 1995). Flies developing from the injected embryos were crossed individually to *y w In(2LR)O,* Cy/*Bl*, and also to *y w In(3LR)TM3, Sb Ser/H* or *w In(3LR)TM3, Sb Ser/In(3LR)TM6B, Hu* (as an alternative for tracking the inheritance of third chromosomes). Backcrossing non-*w* progeny (each of which carried a given second or third chromosome dominant marker) to the *In*-containing balancer types disclosed the chromosomal location of a given w^+ insert and produced balanced transgenic stocks. (The second chromosome balancer specified above is hereafter called *CyO*; and the third chromosome balancers are called *TM3* and *TM6*.)

To induce HR, transgenic lines carrying the *pW25/cry* insert on the *X* or second chromosome were employed. Such transgenics were crossed to flies carrying *FLP recombinase* and I-*SceI endonuclease* (both under control of a *heat shock protein* promoter) on the second chromosome (using the transgenic types and the scheme reported in Rong and Golic 2000). {This strain is formally designated *y w (v) P[ry*$^+$*, 70FLP]4 P[v*$^+$*, 70I-SceI]2B Sco/S 2 CyO.*} Progeny were heat shocked for 3 days at 37° for 1 hr per day to induce FLP and I-SceI production. Among the resulting adults females were selected, because targeting-recombination frequencies are much higher in the germline of such Drosophila compared with males (Rong and Golic 2000). Females without *CyO* (therefore carrying *FLP recombinase* and I-*SceI endonuclease*) potentially carry gametes in which HR occurred between the *pW25/cry* insert and the *cry* locus. Certain such females, whose eyes were either mosaic for w^+ expression or were completely *w* (due to the high rate of induced somatic excision and thus loss of the transgene), were selected for crossing to males carrying constitutively active *FLP recombinase* homozygous on the second chromosome (w^{1118} *P[ry*$^+$*, 70FLP]10*). From among the progeny of this cross, flies with non-mosaic w^+ eye color were selected, signifying HR involving the *cry* insert (the pertinent excised and then re-inserted DNA fragment lacks FRT sites and therefore cannot be excised by FLP). The chromosomal locations of such new insert sites were determined by crossing the w^+ flies just indicated to *y w CyO/Bl* and *y w TM3/H* or *w TM3/TM6*. As above, the logic of balancer/dominant inheritance revealed which of these final stocks entailed re-insertion into chromosome *3*, where the normal *cry* gene is located (Stanewsky *et al.* 1998).

Total replacement of cry^+ in the four recombinant lines obtained (see RESULTS) was tested by PCR, using primers corresponding to intragenic *cry* sequences. One primer pair amplified part of the second *cry* exon: CRYf-2, 5′-TGGACTCGTTGCAGGACATC; CRYr-2, 5′-AGGAACATTTGGTAGGTC

AGC. The second primer pair amplified a DNA fragment near the 3′ end of the third exon: CRYf-3, 5′-GTGTGCGGG GCGTTCAGATG; CRYr-3, 5′-GCCAGCGCAGACCGACCAAT. To gauge the integrity of these PCRs, control primers were also applied that corresponded to the third chromosomal *Clock* gene of (*cf.* Allada *et al.* 1998): CLKf, 5′-ATGATATTTTACT GCGTGAGGAT and CLKr, 3′-TCGCTGATGCCCATGTGAA AGT.

Additional genetic variants: A deletion of cry^+ and neighboring third chromosomal genes—*Df(3R)Dl-Bx12* (Stanewsky *et al.* 1998)—was applied in certain tests by placing it in heterozygous conditions with a cry^0 mutation. The "eye-blinding" $norpA^{P24}$-null mutation (Pearn *et al.* 1996) was applied in singly mutant flies and by combining this *X*-chromosomal variant with three of the novel cry^0 mutations. Flies carrying the latter along with the gl^{60j} null allele, both of which are located within the right arm of the third chromosome, were generated by producing females homozygous for *w* and heterozygous in *trans* for the two mutations. These were mated to *w* gl^{60j} males, and the progeny from 100 crosses were inspected for *glass* eyes and patches of red eye color; the latter marked the presence of w^+ in the cry^0 variants. These gl^{60j} cry^0 third chromosomes were extracted by crossing non-*w, gl* individuals to *y w TM3/H*, which led to the establishment of *glass*-eye CRY-less stocks.

To cope with genetic-background effects on rhythmic characters, material from the w^{1118} strain (Bloomington no. 6326, Hoskins *et al.* 2001), used as the source of control flies in several such phenotypic assessments, was introduced into cry^0-containing flies. cry^0 strains were crossed to w^{1118} followed by tracking w^+ among the offspring; this outcrossing and re-extraction entailed six generations' worth of females heterozygous for cry^0 and (w^{1118}-derived) cry^+. The resulting cry^0 lines (called isoW) carry the w^{1118} mutation on the *X* chromosome, so their eye pigmentation is influenced only by mini-$white^+$ presence (in this case the eye color is close to wild-type brick-red). Viability tests of the novel cry^0 alleles were affected by performing the crosses specified in supplemental Table 1 at http://www.genetics.org/supplemental/.

Two extant transgenic strains were applied in neuroanatomical tests of a cry^0 mutation's (possible) effects on certain clock neurons in the brain and the neurites that extend from them. For this, a *Pdf-gal4* driver (Park *et al.* 2000) was combined with a UAS-*mCD8gfp* drivee that encodes a membrane-bound derivative of green fluorescent protein (Lee and Luo 1999).

Genotyping elements of cry^0's genetic background: The rhythm-related *timeless* (*tim*) and *jetlag* (*jet*) genes exist in variant forms among certain strains of *D. melanogaster* (Koh *et al.* 2006; Peschel *et al.* 2006). Certain combinations of *tim* and *jet* variants can influence the flies' light responsive in chronobiological contexts (Peschel *et al.* 2006). Against this background, PCR was used to determine the *tim* alleles harbored by a cry^0 stock used in phenotype analyses. Amplification of the requisite *tim* fragment—corresponding to the sites of alternative translation-initiating codons (Rosato *et al.* 1997; Tauber *et al.* 2007)—used these primers: 5′, 5′-AGA TACCGCGCAAATGGCTAAGAAG and 3′, 5′-GGTGCGCAG TGTTTGGTCCTCATA. For amplification of a *jet* fragment, which includes the sites where mutations have been recognized at this locus (Koh *et al.* 2006; Peschel *et al.* 2006), the primers were: 5′, 5′-CACTGCTGGCCAACAACAAGAAAC and 3′, 5′-TGCACGCCATAGTCGGAGATAGC.

The H–B eyelet in *D. melanogaster* (see Introduction) expresses *Rhodopsin* genes called *Rh5* and *Rh6* (Malpel *et al.* 2002). In some strains of this species, *Rh6* is a null variant: a 19-bp deletion that causes a stop codon to come in frame, within a portion of the coding sequence corresponding to the fifth transmembrane domain of the Rh6 polypeptide (Cook *et al.* 2003). Therefore, an *Rh6* fragment, encompassing the region where this intragenic deletion lies, was amplified using these primers: 5′, 5′-CAAGGACTGGTGGAACAGGT; and 3′, 5′-GTTCATCTTCTTCGCCTGCT. It is notable that this *Rh6*-null mutation is harbored by certain w^{1118} stocks, which are used in many bio-genetic studies of *D. melanogaster*; this is also the case for a *y cn bw sp* stock from which DNA was taken to sequence the genome of this species (see the relevant BDGP subsite at http://flybase.bio.indiana.edu/). The expressible version of this *Rhodopsin* gene's sequence was initially reported by Huber *et al.* (1997).

The *period* gene has long been known to vary according to a Thr-Gly-repeat-encoding sequence in the middle of the gene. Such repeat-length variants are segregating in laboratory stocks (*e.g.*, Yu *et al.* 1987) and in natural populations (*e.g.*, Costa *et al.* 1992). Variation of the latter kind prompted tests of temperature compensation of the different *per*-isoallelic types (Sawyer *et al.* 1997). Because some of the phenotyping of currently applied strains involved varying the thermal condition, amplification of the relevant *per* fragment (taken from cry^0-containing flies) was performed using these primers: 5′, 5′-GAGGGCAGCGGGGGCAGTG and 3′, 5′-GAGCAGGG ATTCGGTCAGC.

The various amplicons produced as described in the foregoing passages were gel purified and directly sequenced.

Monitorings and analyses of whole-animal rhythmicities: *D. melanogaster* were raised on a cornmeal/sugar/yeast/agar medium (supplemented with the mold inhibitor Tegosept) at 25° in 12:12 LD cycles and collected as 2–5-day-old adults. Locomotor activity of males was recorded using the Drosophila Activity Monitor system (DAM 5, Trikinetics, Waltham, MA). Flies had their locomotor movements recorded automatically for 5 days in LD (light intensity 800–1000 lux) and, in some tests, were switched to conditions of either LL or DD for at least 10 further days in a given run. In an LD-shift experiment, flies were entrained for 5 days in 12:12 LD, during the last one of which the onset of L was delayed by 8 hr, followed by 8 days of locomotor monitoring postshift (as in Helfrich-Förster *et al.* 2001). These data were plotted as actograms and average-activity histograms, as in Stanewsky *et al.* (1998). In another kind of re-entrainment experiment, flies were monitored for 5 days in the usual LD condition, then switched to a regime that entailed 9:9, 13:13, or 14:14 LD cycles (using equipment described in Mealey-Ferrara *et al.* 2003 *re* 12:12 to 13:13 shifts) for 7–8 days; at the end of the final (new, non-24-hr) LD cycle, free-running behavior was monitored for 4–6 days in DD. In certain photic conditions to which flies homozygous for cry^0 were exposed, the ambient (constant) temperature was 18° or 29° instead of 25°.

Most of the locomotor analyses were performed using MATLAB-based software, which was developed for chronobiological purposes by Levine *et al.* (2002a,b). For flies that displayed two separate rhythmic components, by inspection of "actogram" plots of their locomotor cycles, autocorrelation analyses and MESA's failed to extract the "biperiodic" nature of these behaviors (see Figure 3 in results for an example). Therefore, each of the period values was determined by placing a ruler across the locomotor "onsets" that occurred on successive days. The slope defined by optimal placement of the straight-edge specified the free-running period. These semi-subjective "analog" determinations of periodicity were effected in an observer-blind manner, vis-à-vis the fly's genotype.

Eclosion monitoring was performed as in Konopka *et al.* (1994), as modified slightly by Mealey-Ferrara *et al.* (2003). Key features of the procedures: The animals were raised in a 12:12 LD regime (throughout development); numbers of adult

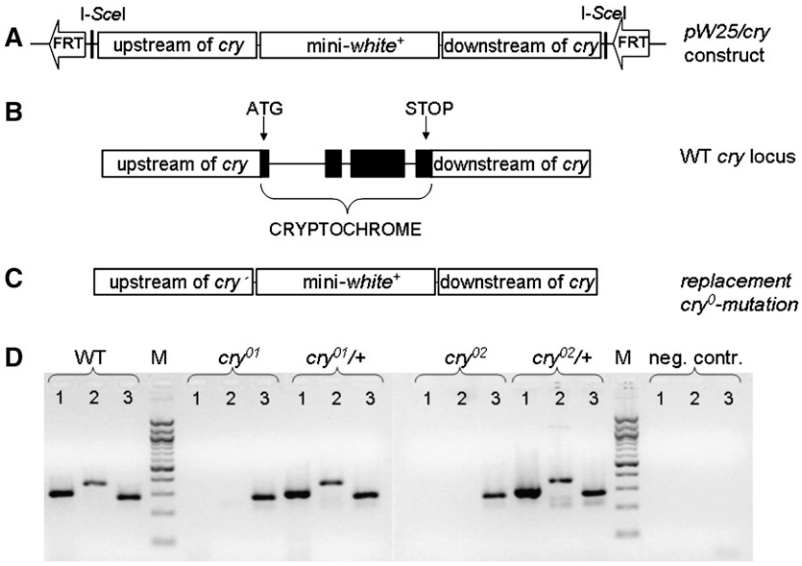

FIGURE 1.—HR into the *cry* locus to knock it out. (A) Design of *pW25/cry* transformation vector. This plasmid carries mini-*white*$^+$ marker gene surrounded by segments of third-chromosomal DNA flanking the *cry* gene on either side (upstream and downstream of *cry* coding sequence), along with two FRT sequences and two recognition sites for the I-*Sce*I endonuclease. (B) Genomic organization of the wild-type (WT) *cry*$^+$ gene with translation start and stop sites indicated. (C) Genomic organization of newly created *cry* locus after HR. The entire coding sequence of *cry* was replaced by mini-*white*$^+$. (D) PCR analysis of different *cry*-locus forms. Genomic DNA was isolated from adult flies, homozygous or heterozygous for the third chromosome carrying the putative targeted HR event. PCR was also carried out on DNA from control WT flies and without any DNA. Flies heterozygous for a given *cry*^{0} mutation (*cry*^{01} or *cry*^{02}) carried the normal allele in a third-chromosomal balancer called *TM3* (see MATERIALS AND METHODS). Three sets of primers were used for each DNA sample (see MATERIALS AND METHODS for such sequences). Lane 1, amplification product of the second *cry* exon. Lane 2, amplification of a DNA fragment near the 3′ end of *cry*'s third exon. Lane 3, amplification of *Clock* gene sequence, to gauge the integrity of these PCRs. Lane M, 100-bp DNA ladder (New England BioLabs, Ipswich, MA), applied to calibrate PCR product lengths.

flies emerging per time unit were automatically counted, first for 1–2 days in 12:12 LD, then continuing in that condition or shifted to DD or to LL (light intensity at the level of the eclosion disc: 200 lux).

Luciferase reporting of clock-gene cycling: The transgene called *BG-luc*, which encodes the N-terminal 2/3 of PER fused in frame to a firefly luciferase-encoding sequence (STANEWSKY *et al.* 1997, 1998), was applied in combination with two of the newly created *cry*^{0} mutations. To obtain meiotic recombinants, *cry*^{0} flies were crossed to *BG-luc* ones, and the resulting trans-heterozygous females were mated to *y w TM3/H* males. Progeny from 40 crosses were visually inspected for eye color, whereby the *w*$^+$ markers contained in both *BG-luc* and the *cry*^{0} allele combine their effects to produce a darker eye color than that exhibited by either single genotype. Recombinants recognized accordingly were crossed to *y w TM3/H* to establish balanced *BG-luc cry*^{0} stocks. These lines were verified by PCR for the presence of *cry*^{0} (primers: CRYf-2 and CRYr-2; CRYf-3 and CRYr-3, as above). Flies homozygous for both variants, along with *BG-luc cry*$^+$ controls, were raised at 25° in 12:12 LD cycles and collected as 2–5-day-old adults. To assess LUC cycling in isolated tissue specimens, antennae or wings were pulled off adults (as in PLAUTZ *et al.* 1997 and LEVINE *et al.* 2002b). Antennae and wings were chosen because *BG-luc* cycling was observed in these tissues homozygous for *cry*^{0} (LEVINE *et al.* 2002b). Immediately postsurgery, specimens were placed in a luciferin-containing solution [insect tissue-culture medium (HyClone, Logan, UT) with 10% FBS (Invitrogen, Carlsbad, CA), 1% luciferin (Promega, Madison, WI), 0.5% insulin (Sigma, St. Louis, MO), and 1% antibiotic–antimycotic solution (GIBCO/Invitrogen)]. This material was distributed in 96-well plates, which were inserted into a Packard-Topcount Multiplate Scintillation counter (Perkin-Elmer, Waltham, MA). A given luminescent-monitoring run (*cf.* KRISHNAN *et al.* 2001; LEVINE *et al.* 2002b) proceeded for 5–7 days in 12:12 LD cycles (25°), over the course of which luminescence-based counts were accumulated every 30 min. The resulting data sets were analyzed as in LEVINE *et al.* (2002a,b).

Histology: To visualize the axons projecting from "clock neurons" (known as LN$_v$ cells) (HALL 2003), brains from *y w Pdf-gal4*/UAS-*mCD8gfp cry*$^{01\text{ or }02}$, in parallel with *cry*$^+$ controls, were dissected in PBS and immediately imaged on a Leica laser scanning confocal microscope TCS SP2 (Leica, Wetzlar, Germany).

RESULTS

Isolation of *cry*^{0} mutants: To target the *cryptochrome* locus of *D. melanogaster* we choose an ends-out homologous recombination (HR) targeting strategy (GONG and GOLIC 2003). The construct for HR was designed such that the entire coding sequence of the *cry*$^+$ allele would be replaced by mini-*white*$^+$ (Figure 1). The *pW25/cry* vector contains two ~3-kb fragments of third chromosomal DNA flanking the *cry* locus, along with *w*$^+$ (between the segments of near-*cry* DNA) and FRT sequences (Figure 1). Transgenic lines were generated that contain this construct inserted at arbitrary genomic locations. To induce HR, whereby the *cry*-flanking/*w*$^+$/*cry*-flanking DNA would be released from a "starting" transgene position and be able to replace the *cry* locus on chromosome 3, crosses were performed to elicit transgenically encoded FLP recombinase and I-*Sce*I endonuclease activity in the female germline (see MATERIALS AND METHODS). Approximately 700 food vials, each containing four of the relevant females and three males (carrying constitutively active FLP), were established; this led to the recovery of 55 potential HR lines. For these, chromosomal locations of the newly inserted *w*$^+$ markers were determined, revealing that the eye-color marker had recombined into the third chromosome in four lines.

Focusing on the four candidates, PCRs were performed using primers derived from intra-*cry* locus sequences. Characterization of these amplicons revealed complete replacement of cry^+ DNA in these four lines (Figure 1D). One line also contained another transposon-derived insert elsewhere on the third chromosome and therefore was not used further. The three variants for which cry^+ was replaced by exogenous sequences were called *cry–zero* (cry^0) mutants (cry^{01} through cry^{03}). Several of the phenotypic tests of how these mutations influence rhythmic characters entailed usage of more than one cry^0 strain (*e.g.*, Tables 1 and 4). Drosophila homozygous for a cry^0 mutation exhibited normal external morphology as well as robust male or female fertility. From viability-determining crosses the two kinds of tests showed emergence probabilities for cry^0 flies to be normal (supplemental Table 1 at http://www.genetics.org/supplemental/).

Background genotypes of cry^0 strains: Several spontaneously occurring alleles of rhythm-related genes have been recognized in laboratory strains and in natural populations; these allelic types are not rarely encountered variants. They can influence chronobiological characters (Sawyer *et al.* 1997; Koh *et al.* 2006; Peschel *et al.* 2006), or one suspects that they would (*cf.* Malpel *et al.* 2002; Cook *et al.* 2003). Therefore, the relevant DNA sequences were characterized in our newly created cry^0 lines. We focused on the four genes specified in materials and methods. To exclude possible interference of *jetlag* variants, we sequenced the pertinent part of the *jet* gene; none of the novel *cry*-null lines was found to harbor a "light-insensitive" *jet* mutation (*viz.* jet^c or jet^r, *cf.* Koh *et al.* 2006; Peschel *et al.* 2006). With regard to *tim* iso-alleles that produce either (relatively) short TIM protein or long-plus-short TIM in a given fly (Rosato *et al.* 1997; Tauber *et al.* 2007), we found that the Canton-S strain used in the current study carried *ls-tim* (encoding *long* and *short*-forms of TIM); and the w^{1118} strain harbors *s-tim*. But *tim* variation detected among the currently applied strains should not be problematic, because all of them are jet^+ (Peschel *et al.* 2006). In this regard, cry^0 combined with *ls-tim* did not promote behavior different from cry^0 flies with the *s-tim* variant in LL (Table 1). Because expression of the *Rhodopsin6* gene in the H–B eyelet is likely to influence circadian photosensitivity (see Introduction), we analyzed our stocks to ask whether any of them contain the *Rh6*-null mutation (*cf.* Cook *et al.* 2003). All the cry^0 lines were found to be capable of encoding full-length Rh6. Given the temperature-dependent phenotype of cry^0 flies (see Table 1), we sequenced the *period* gene with respect to a Thr-Gly (TG) repeat polymorphism. These numbers of TG pairs were found to be present in the cry^0 strains: 17 TGs; the $norpA^{P24}$ $cry^{01\text{-}03}$ double mutants (whose *X* chromosome carries the *per* gene as well as the *norpA* one) and 20 TGs. Alleles of *period* that produce 17 or 20 TG pairs are among the commonly encountered

TABLE 1

Free-running locomotion, in DD or LL, at different temperatures

Photic condition	Genotype	18°			25°			29°		
		N	*N* rhy (%)	Mean hr ± SEM	*N*	*N* rhy (%)	Mean hr ± SEM	*N*	*N* rhy (%)	Mean hr ± SEM
DD	*s-tim* $cry^{01\text{ or }02}$	82	51 (62)	23.7 ± 0.1	51	50 (98)	23.7 ± 0.1 (82% of rhy) or 22.9 ± 0.1 (18% of rhy)	55	52 (95)	23.4 ± 0.1
DD	*s-tim* cry^+	16	15 (94)	23.9 ± 0.1	14	14 (100)	23.9 ± 0.0	29	29 (100)	23.4 ± 0.1
LL	*s-tim* $cry^{01\text{ or }02}$	89	55 (62)	24.2 ± 0.1	74	70 (95)	21.4 ± 0.0 and 25.4 ± 0.1	85	48 (57)	20.1 ± 0.1 and 23.8 ± 0.2
LL	*ls-tim* $cry^{01\text{ or }02}$		ND		67	57 (85)	21.7 ± 0.0 and 25.4 ± 0.1		ND	
LL	$Df/cry^{01,\ 02,\text{ or }03}$		ND		63	58 (92)	23.6 ± 0.1 (38% of rhy) 21.9 ± 0.2 and 25.0 ± 0.1 (62% of rhy)		ND	

Flies were entrained under 12:12 hr light-dark cycling conditions for 5–6 days then released into constant conditions for 7 days of locomotor monitoring. *s-tim* and *ls-tim*, naturally occurring *tim* iso-alleles (Rosato *et al.* 1997; Peschel *et al.* 2006); *Df*, deletion of the *cry* locus and of neighboring third-chromosomal genes; *N*, numbers of tested flies or number rhythmic (rhy); Mean, average period determined by periodogram and verified by the straight-edge/slope procedure (see materials and methods); SEM, standard error of the mean; ND, not done (meaning that the LL monitorings of flies with genotypes in the bottom two rows were tested only at 25°). No differential effects on these characters were discernible with regard to cry^0 derived from the separately numbered such strains (second column from the left).

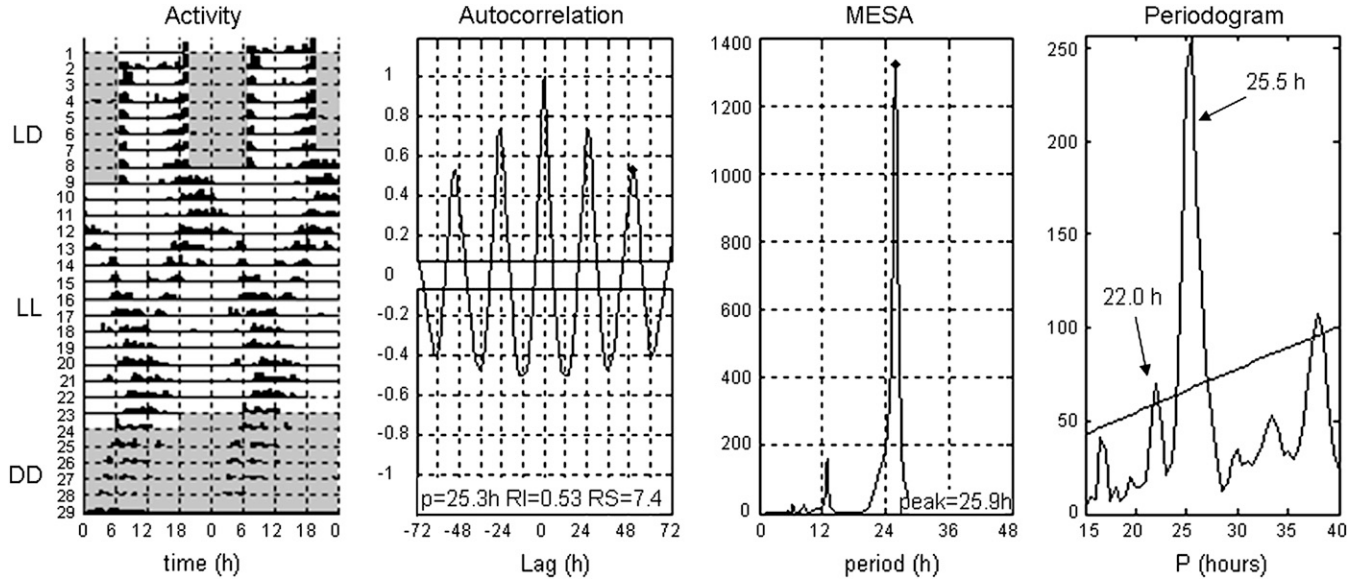

FIGURE 2.—Locomotor behavior of *cry*^{0} in LD → LL → DD monitorings. The actogram (left) shows rhythmic behavior (Activity) in all three of the light regimes just indicated (respectively: LD cycles, LL, and DD). This *cry*^{0} fly displayed two rhythmic components during the LL component of the test, which disappeared after changing the conditions to DD. Autocorrelation, MESA, and Periodogram results were computed from the LL portion of the locomotor monitoring. The former two analyses revealed only one period >25 hr. (A given Lag in the correlogram is a value in hours that represents testing such numbers with respect to a "perfect" correlation between the behavioral data that were not lagged with respect to each other, which leads to a maximal peak height; in the MESA plot, the ordinate indicates spectral density, in arbitrary units, with regard to extraction of appreciably periodic components corresponding to hour values on the abscissa.) The Periodogram in the right-hand plot teased out the two free-running components (P on the abscissa = period) that are pointed to by arrows; each such hour value involved a significantly periodic component. [The Chi-square significance line crossing the periodogram, with respect to varying ordinate values in arbitrary units, corresponds to $\alpha = 0.05$ (SOKOLOVE and BUSHELL 1978).] Other *cry*^{0}-derived actograms manifested two periodic components by inspection, but not all such behavioral records had the corresponding hour values extracted by Chi-square Periodogram analysis.

types (COSTA *et al.* 1992), bearing in mind the *per*^{17TG} and *per*^{20TG} types exhibit better temperature compensation of the clock's pace than do Drosophila containing other repeat-length PER protein types (SAWYER *et al.* 1997).

Locomotor rhythmicity in constant photic conditions affected by *cry*^{0}: Periodic locomotion of the new *cry*-mutant was robust in two free-running conditions: DD, in which nearly all wild-type Drosophila exhibit solid ~24-hr periodicities (HALL 2003; PRICE 2005); and in LL, a condition that causes genetically normal flies to go arrhythmic (see Introduction). In DD, *cry*^{0} flies showed solidly rhythmic locomotion in almost all cases at 25° or 29°. Most period values were in the normal range, except that ~20% of the *cry*^{0} individuals tested at 25° gave shorter than normal values (Table 1). When this mutant type was cooled to 18°, weak rhythmicity in DD was the norm, although the rhythmic individuals (60% of the total) gave normal period values (Table 1).

In LL, the great majority of *cry*^{0} individuals behaved rhythmically at 25°, a distinctly non-wild-type phenotype. This anomalous rhythmicity is similar to the behavior of the original *cry*^{b} mutant in LL. However, that mutation, in some studies of it, led to simple outcomes of ~24- or ~25-hr free-running periods in LL (see Introduction). Here, most of the rhythmic individuals with *cry*-null genotype displayed two rhythmic components within a given behavioral record in LL (YOSHII *et al.* 2004). The average for the relatively short free-running periods (among these *cry*^{0} flies) was ~21–22 hr; that of the longer was ~25–26 hr (Table 1, example in Figure 2). A minority of the LL-rhythmic *cry*-null flies showed one periodic component per fly, ~21–22 hr or ~25–26 hr (legend to Table 1). Two split-rhythmic components had also been reported for *cry*^{b} mutants in certain earlier studies involving locomotion in LL, but not in the majority of them (see DISCUSSION).

Results of testing flies heterozygous for a *cry*^{0} mutation and a deletion of the gene bear comment: Nearly all such *Df/ cry*^{0} individuals (92%) behaved rhythmically in LL at 25°; ~38% of rhythmic flies exhibited one periodic component (~24 hr); whereas 62% gave two periodic components per individual (~22 and ~25 hr). Therefore, less splitting occurred when a *cry*-null mutation had its effects "uncovered" by the deletion, compared with the LL behavior of homozygotes; this could be explained in part by genetic-background differences. In this regard, the rhythmic characters displayed by *Df/ cry*^{0} flies entailed much "noisier" periodic

TABLE 2

Re-entrainment to delayed LD cycles

Genotype	*N*	Response categories %		
		In synchrony with orig cycle	Resynchronized	Partially resynchronized
$norpA^{P24}$	45	0	89	0
$cry^{01,02, \text{ or } 03}$	21	0	95	0
$norpA^{P24}cry^{01,02, \text{ or } 03}$	115	37	15	43

Flies were entrained in 12:12 LD cycles then exposed to 8-hr delayed LD cycles. The ability of a fly to resynchronize to the new LD regime after 6 days was judged by inspecting that individual's actogram. Outcomes in the first data column here refer to flies whose locomotor components remained ostensibly in synchrony with the original (orig) LD regime (which could mean "no entrainment," *i.e.*, free-running behavior with ~24-hr periodicity). The second data column documents flies whose activity was changed to conform with the postshift LD cycle, during at least 3 days after the 8-hr delay. Partially resynchronized: flies whose daily peaks of locomotion were judged to have conformed to neither the old nor the new LD regime, by 6 days after the shift. 11% of the $norpA^{P24}$ flies and 5% of each of the $norpA^{P24}$ cry^0 and cry^0 types became arrhythmic after phase shift (so the values in a given row do not sum to 100%). *N*, total number of flies rhythmic in initial LD. Doubly mutant individuals whose cry^0 knock-out was derived from strain *01* exhibited the worst ability to resynchronize their behavior (postshift); but the cry^{02}- or cry^{03}-including flies of this type were only slightly better off.

components compared with those influenced by homozygosity for cry^0 mutations (Table 1). Therefore, specifying the precise proportions of *Df/* cry^0's exhibiting a given single- *vs.* split-periodic attribute was problematical. (One reason for this could be result of the overall poor health of flies carrying the *cry Df*, as noted within supplemental Table 1 at http://www.genetics.org/supplemental/.)

We assessed effects of temperature changes on the bicomponent periodicity caused by a loss of *cry* function, because this gene has been previously connected with thermally varying situations in which two pacemaking functions operate within in a given animal (Rosato *et al.* 2001; Yoshii *et al.* 2004; Rieger *et al.* 2006). In LL at 29°, cry^0 flies exhibited relatively large degrees of arrhythmicity (Table 1). When periodic components could be reliably analyzed, for ~60% of the mutant types free-running in this heated condition, two periodicities were observed whose average values were ~20 and ~24 hr (Table 1). When LL behavior was monitored at 18°, again a higher percentage of arrhythmic behavior was observed (40%) compared with the 25° tests; and in this relatively cool condition, the rhythmic *cry*-null individuals yielded only one periodic component, ~24 hr (Table 1).

***cry*^{0} flies display normal axonal projections from clock neurons that interconnect the brain hemispheres:** Splitting of free-running locomotor rhythmicity has been observed in other genetically defective circumstances. One such situation involves the effect of an eye-removing *sine oculis* (*so*) mutation, for which behavior in DD involved the frequent occurrence of two separate period values per mutant individual (Helfrich 1986). In a fair proportion of *so* brains, axons of certain "clock neurons" that project across the brain midline were found to be absent after an antibody against the *Pdf* gene product was applied (Helfrich-Förster and Homberg 1993). It was thus important to inspect the brain anatomy of cry^0 adults. Furthermore, so-called "rhythm-functional" mutants can exhibit anomalies of clock-neuron axonal anatomy, as documented in Park *et al.* (2000). Examining whole-mounted brains homozygous for cry^0, in a situation where homozygosity for cry^0 was combined with a *Pdf-gal4* transgene and UAS-*mCD8gfp*, disclosed no cry^0-induced anatomical abnormalities ($N = 40$). The GFP signals projecting from LN_v cells appeared in the typical wild-type pattern (*cf.* Hall 2003), which we also observed in control specimens (20 doubly transgenic brains carrying cry^+).

***norpA cry* doubly null mutants exhibit severely defective resynchronization to altered LD cycles:** To create potentially circadian-blind Drosophila, we combined the $norpA^{P24}$-null mutation with a cry^0 one. The simultaneous effects of these variants were tested in two types of experiments. In one, we examined the ability of flies to resynchronize to new LD cycles after they were delayed by 8 hr. Singly mutant $norpA^{P24}$ or cry^0 individuals nicely re-entrained to the new light regime. But the great majority of double mutants failed to do so, in that their daily peaks of locomotion (typically occurring around lights-off and lights-on, respectively) continued to occur at the time of the photic transitions that were in operation before the 8-hr shift. In some cases the flies "partially entrained:" Six days after the photic shift, daily peaks of locomotion fell in between the phases expected for continuing entrainment to the old LD regime *vs.* re-entrainment to the new one (Table 2, Figure 3). It is likely that these doubly mutant individuals would have re-entrained if given enough time postshift; longer than normal times for such resynchronization of *norpA* cry^b individuals were previously reported by Emery *et al.* (2000b) and Veleri *et al.* (2007).

In a second set of experiments, flies that had been put through 12:12-hr LD cycles were shifted to 9:9, 13:13,

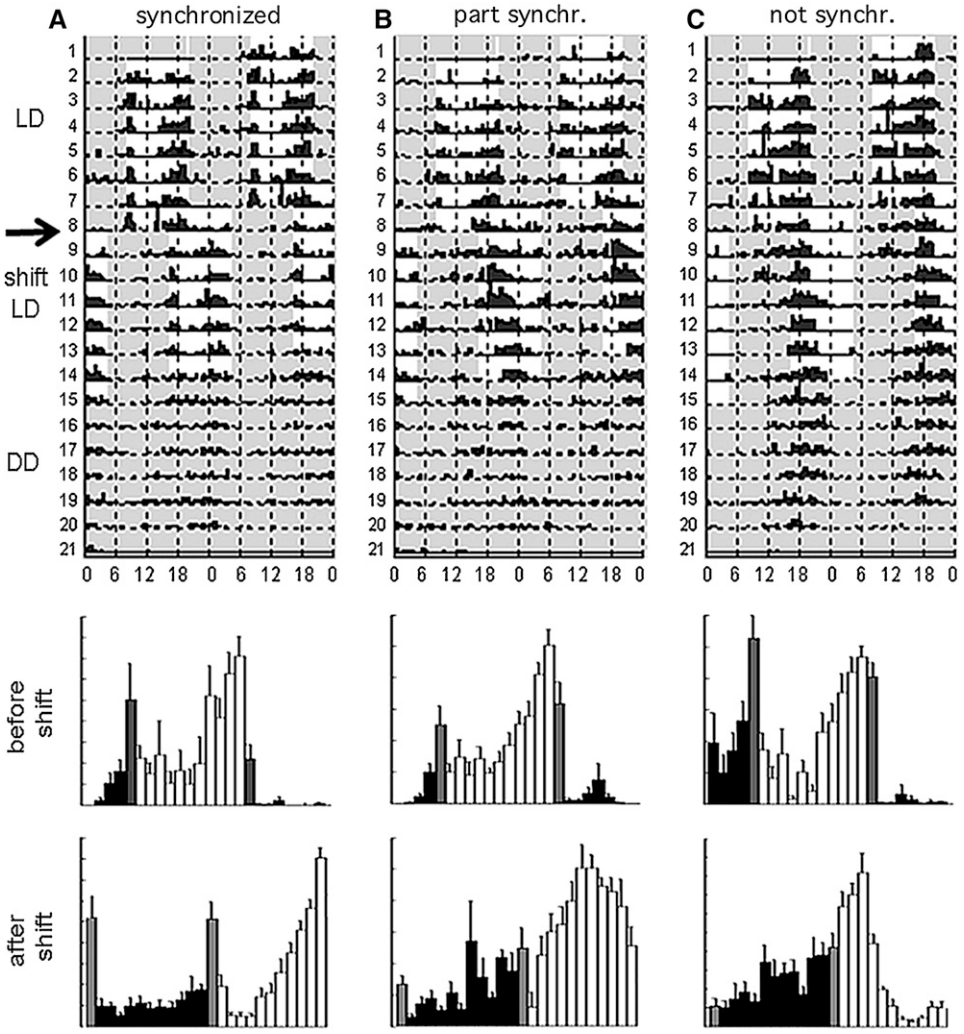

Figure 3.—Locomotor behavior of *norpA*^{P24} *cry*^{0} flies after an abrupt LD shift. Top-row data: double-plotted actograms of representative flies behaving in 12-hr:12-hr LD cycles, then responding or not (as the case may be) to an 8-hr-delayed LD regime; the final portion of each behavioral record plots locomotor events occurring after the flies proceeded into DD (after the postshift LD regime). The arrow indicates when the LD-cycles were shifted. Plots at the bottom: "average activity" of flies before and after the photic shift, depicted in histograms that display the LD data only (white bars, locomotion in L; black, in D; gray, time bins during which the photic regime was changed from L to D or vice versa); a given bar shows the mean number of locomotor events (± SEM), per time bin per day for each of the three individuals. (A) A doubly mutant fly that adjusted its locomotion to behave in synchrony with the postshift LD cycle; note that there are clear behavioral "anticipations" of the L-to-D transitions (pre- and postshift), visible in the histograms. (B) A partially resynchronized double mutant whose daily peaks of locomotion got partly resynchronized (part synchr.), such that the phase of postshift locomotion was between the *environmental* phases of the old and the new LD regime 6 days after the shift. (C) A *norpA*^{P24} *cry*^{0} fly whose locomotor components did not get resynchronized (not synchr.), remaining in phase with original LD regime; note that the evening locomotor peak in new LD regime is similar to the preshift one (in the histograms), except that flies are active mostly in afternoon in the first LD regime, while this activity happened to occur in the "morning" in the new LD cycles.

or 14:14 ones, to ask whether they would re-entrain to exhibit the appropriate 18, 26, or 28-hr periodicities. Examples from the 12:12 to14:14 test are in Figure 4. The singly mutant types routinely re-entrained to behave in synchrony with the novel non-24-hr environmental cycles (Table 3). *norpA*^{P24} *cry*^{0} flies, however, responded much less well: only 3–18% of the double mutants behaved in some synchrony with the new LD regime, depending on its duration. It is crucial to register that various proportions of the flies that shifted away from 12:12 cycles gave "ambiguous" results (see the right-hand portion of Table 3). Some individuals in this category yielded such sloppy behavioral records that no particular periodic component could be discerned. But several other of the ambiguously behaving *norpA*^{P24}*cry*^{0} flies were solidly periodic and in no way had maintained close-to-24-hr cycle durations. Yet—as is detailed in the legend to Table 3–these double mutants manifested periods that differed from the new LD cycle durations by 1 to 4.5 hr (*e.g.*, 43% of flies in the 12:12 to 9:9 experiment behaved with 22.5-hr periodicities in the 9:9 condition). Strikingly, ~15% of *norpA*^{P2} *cry*^{0} flies in the 12:12 to 9:9 experiment yielded a roughly re-entrained periodic component *together* with ~22–24-hr component (Table 3), within a given individual activity record of this type.

Against a backdrop of the modest ability of *gl*^{60j} *cry*^{b} flies to synchronize their behavior to 26-hr LD cycles (Mealey-Ferrara *et al.* 2003), our tests of the newly created *gl*^{60j} *cry*^{0} double mutant showed that it exhibited weak rhythmicity in general terms; several such individuals were arrhythmic altogether (data not shown). Among the numerical read-outs of these locomotor tests was an indication of poorly periodic behavior in 12:12 LD—signifying that these double mutants could not entrain to such "normal" cycles or that they were exhibiting sloppy free-run rhythmically in that condition. Thus it was not possible to assess whether re-entrainment to the newly imposed LD cycles was

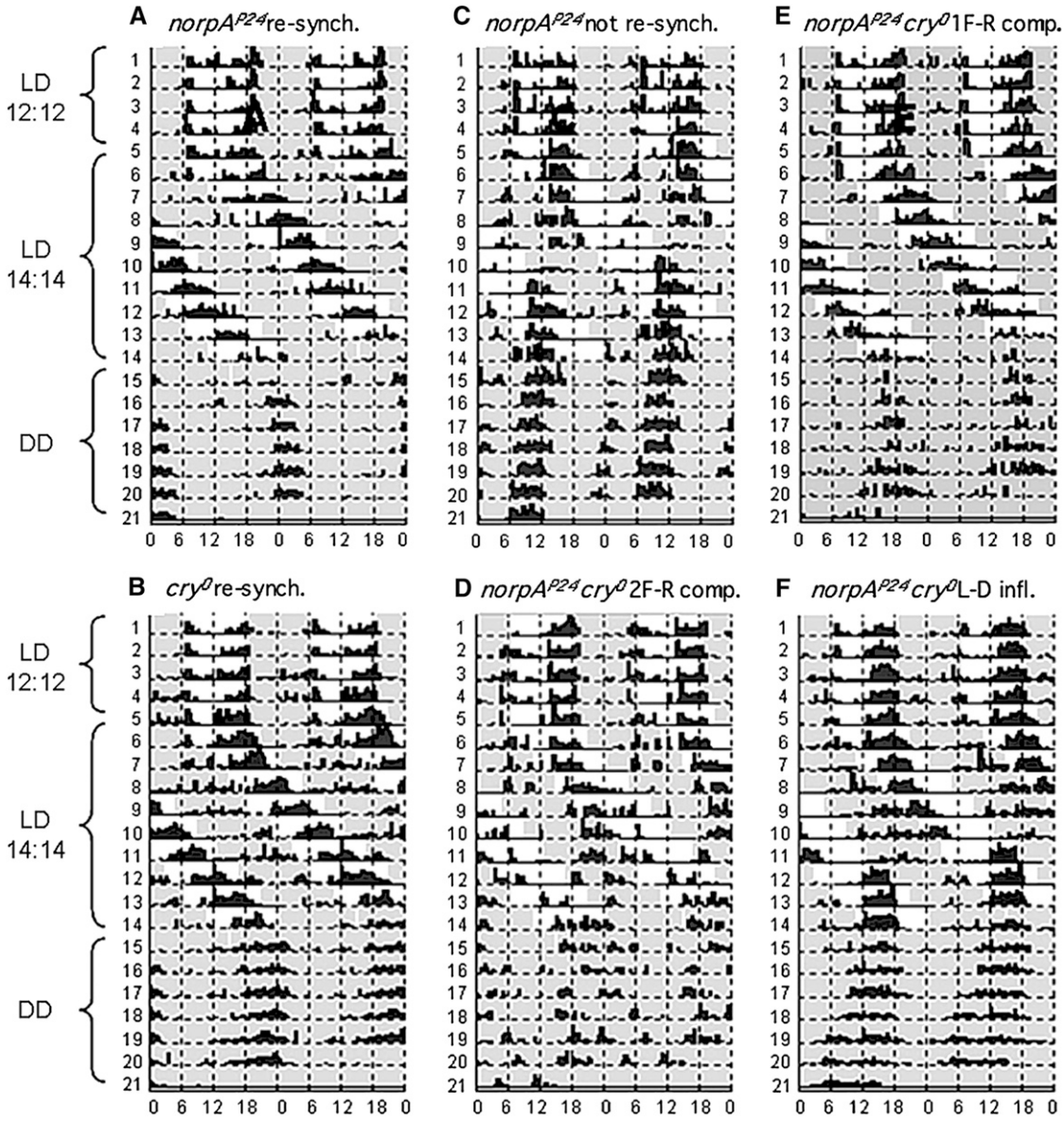

Figure 4.—Locomotor behavior of singly-mutant cry^0 and $norpA^{P24}$ flies and of doubly mutant $norpA^{P24}$ cry^0 ones after a shift from 12:12 LD to a novel cycle duration. Each animal was released from the second L:D conditions to DD. (A and B) Singly mutant $norpA^{P24}$ and cry^0 individuals that resynchronized (resynchron.) to the new light regime, exhibiting ~28-hr periods during 14:14 LD; signified by the upper-left to lower-right slant of the locomotor events during this photic condition (this being a typical long-period feature of an actogram component, vis-à-vis the two successive 12-hr days indicated on the abscissa). (C) A $norpA^{P24}cry^0$ fly that failed to resynchronize to 14:14 LD, maintaining free-running periodicity (~24 hr) in both photic conditions. (D) A doubly mutant individual for which two rhythmic free-running (F-R) components (comp.) appeared after it was shifted to 14:14 LD; one component possessed a period <24 hr; the second component was ~26 hr. (E) A double mutant that behaved with a single free-running component of ~26 hr. (F) Example of a locomotor result influenced by the two mutations and (environmentally) by the "lights-on" transitions; this $norpA^{P24}cry^0$ individual displayed its highest activity during the light part of LD cycles but failed to re-entrain to 14:14 LD; a feature of the behavior plotted in this panel is "disconnected" locomotor during days 10–11 (arrow); for the remainder of the behavior occurring in 14:14 LD, this fly behaved with an ~24-hr period.

occurring in flies suffering the simultaneous effects of a *cry*-null and a *glass* mutation.

***cry* mutants display periodic eclosion in all photic conditions, including LL:** Assays of eclosion rhythmicity affected by the novel *cry* mutations (Figure 5) showed that, after cultures were entrained by LD cycles, cry^{01} and cry^{02} adults emerged rhythmically in DD with periodicities similar to control values (Table 4). In LL, which causes eclosion of genetically normal Drosophila to be arrhythmic (Chandrashekaran and Loher 1969; Winfree 1974; Sheeba *et al.* 1999), we encountered a novel chrono-genetic phenomenon: cry^0 cultures eclosed rhythmically; these LL period values were similar to those derived from cry^0 or cry^+ animals emerging in DD (Figure 5, Table 4). Because no rhythm mutants have been studied for eclosion occurring in LL, we tested cry^b in that

TABLE 3

Locomotor responses to shifts from 12:12 LD cycles to <24- or >24-hr cycles

Genotype	*N* rhy/*N* tested (% rhy)	Behavioral categories after shift to 9:9 LD, mean period(s) ± SEM: One periodic component: ~24hr	One periodic component: ~8 hr	Two periodic components	Ambiguous
$norpA^{P24}$	39/44 (89)	NA	18.1 ± 0.1	NA	NA
$cry^{01,02,\text{ or }03}$	54/60 (90)	23.5 ± 0.1 (11% of rhy)	18.4 ± 0.2 (63% of rhy)	18.0 ± 0.0 and 22.6 ± 0.3 (19% of rhy)	NA (7% of rhy)
$norpA^{P24}$ $cry^{01,02,\text{ or }03}$	104/107 (97)	23.6 ± 0.0 (69% of rhy)	18.0 ± 0.9 (4% of rhy)	19.8 ± 0.2 and 23.6 ± 0.1 (15% of rhy)	NA (12% of rhy)

Genotype	*N* rhy/*N* tested (% rhy)	Behavioral categories after shift to 13:13 LD, mean period(s) ± SEM: One periodic component: ~24 hr	One periodic component: ~26 hr	Two periodic components	Ambiguous
$norpA^{P24}$	20/21 (95)	NA	26.0 ± 0.0 (100% of rhy)	NA	NA
$cry^{01\text{ or }02}$	43/43 (100)	NA	25.8 ± 0.0 (100% of rhy)	NA	NA
$norpA^{P24}$ $cry^{01\text{ or }02}$	38/38 (100)	24.2 ± 0.1 (42% of rhy)	25.6 ± 0.1 (18% of rhy)	22.4 ± 0.1 and 25.1 ± 0.2 (13% of rhy)	NA (26% of rhy)

Genotype	*N* rhy/*N* tested (% rhy)	Behavioral categories after shift to 14:14 LD, mean period(s) ± SEM: One periodic component ~24 hr	One periodic component ~28 hr	Two periodic components	One periodic component with phase shift	Ambiguous
$norpA^{P24}$	19/21 (91)	NA	27.8 ± 0.1 (100% of rhy)	NA	NA	NA
$cry^{01,02,\text{ or }03}$	22/23 (96)	NA	27.7 ± 0.1 (100% of rhy)	NA	NA	NA
$norpA^{P24}$ $cry^{01,02,\text{ or }03}$	122/129 (95)	24.2 ± 0.1 (20% of rhy)	27.0 ± 0.0 (3% of rhy)	23.5 ± 0.1 and 26.1 ± 0.1 (28% of rhy)	25.7 ± 0.1 (13% of rhy)	NA (36% of rhy)

Flies were exposed to 12:12 LD cycles for 5 days, then shifted to the novel LD cycles indicated for 7–8 days, followed by monitoring locomotion in DD (data from the latter condition not tabulated, but see Figure 4). Each postshift locomotor record was subjected to observer-blind analysis to evaluate its periodic component(s), if any, including whether or not the fly had resynchronized to the new cycle duration. Periods were determined by the straight-edge slope procedure noted in MATERIALS AND METHODS.

Examples of behavioral responses in the 12:12 →14:14 test are in Figure 4. The "one periodic component with phase shift" outcome refers to the following: flies that displayed one periodic component, running with a certain period (always <28 hr) until the time when such individual flies "should" be active in the dark if their behavior continued with the same period; but starting at that point, an individual behaving in this category was not active during the entire 14 hr of DD; after lights-on, locomotion commenced again and continued to be periodic, although this new component was shifted relatively to the first such component (see Figure 4F).

Ambiguous, referring either to (i) locomotor patterns that were so messy (involving, for example, three putatively separate rhythmic components per actogram) that no proper categorization was possible; or (ii) to solid and simple periodic postshift outcomes that were judged not to be near enough to either 24 hr *or* to the novel LD cycle durations; specifically, in the 9:9 test, 3 of the 12 NA flies gave 22.5-hr periods; in 13:13, 7 of 10 NA's gave 25-hr periods; in 14:14 out of 44 NA's 1 fly gave a 25-hr period, 3 gave a 25.5-hr period, 19 gave a 26-hr period, and 12 gave a 26.5-hr period; NA, not applicable (meaning either that no behavioral outcomes fell into that category or that some did, but a particular period value could not be specified because of ambiguities among individuals); rhy, rhythmic; SEM, standard error of the mean.

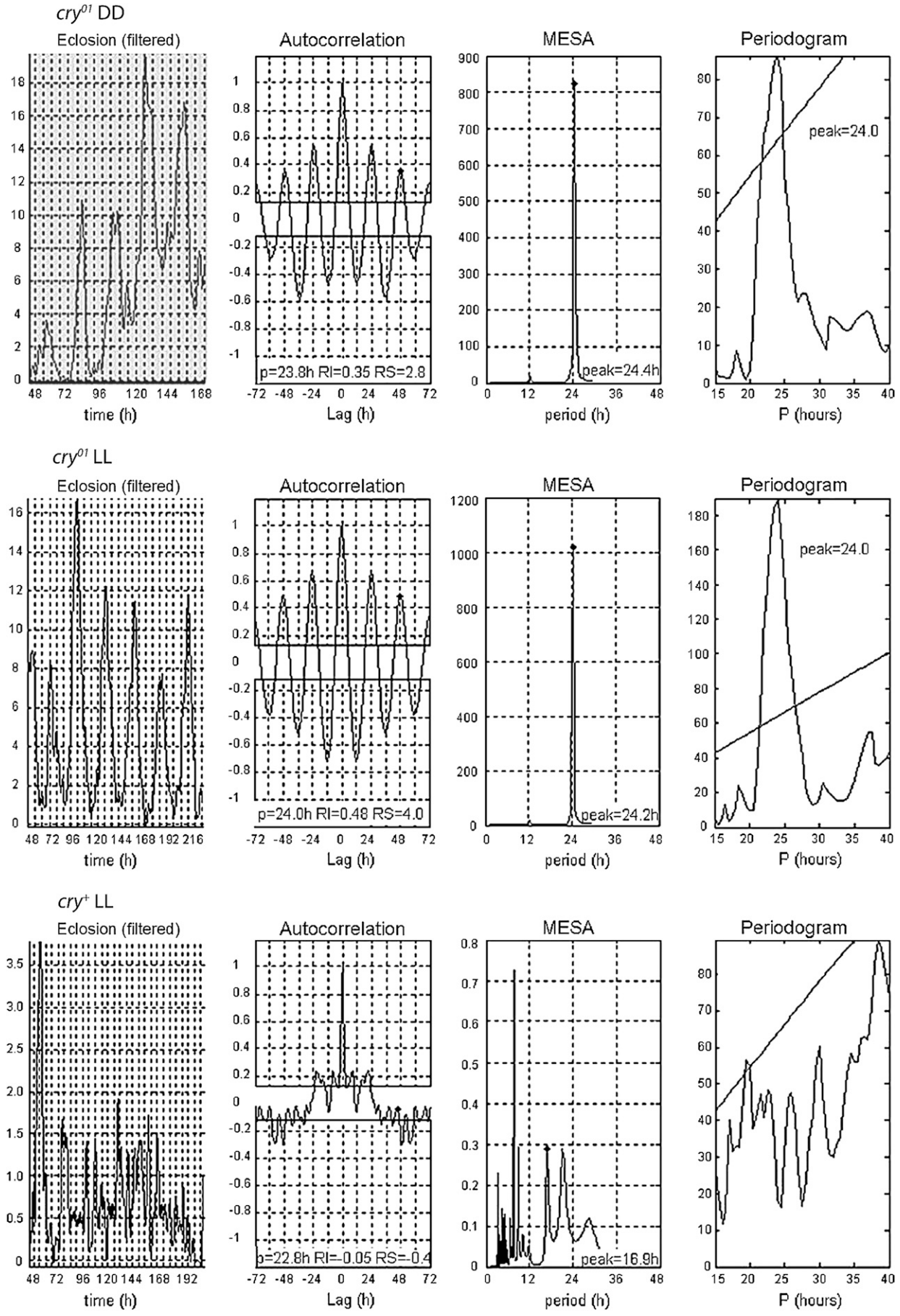
cry01 DD
Eclosion (filtered)
Autocorrelation
MESA
Periodogram
time (h)
Lag (h)
period (h)
P (hours)
p=23.8h RI=0.35 RS=2.8
peak=24.4h
peak=24.0
cry01 LL
Eclosion (filtered)
Autocorrelation
MESA
Periodogram
time (h)
Lag (h)
period (h)
P (hours)
p=24.0h RI=0.48 RS=4.0
peak=24.2h
peak=24.0
cry+ LL
Eclosion (filtered)
Autocorrelation
MESA
Periodogram
time (h)
Lag (h)
period (h)
P (hours)
p=22.8h RI=-0.05 RS=-0.4
peak=16.9h

TABLE 4

Adult emergence of *cry*-mutant and *cry*$^+$ flies in DD or LL

	DD			LL		
Genotype	*N*	Autocorr τ	MESA τ	*N*	Autocorr τ	MESA τ
cry^{02}	3295	23.9	23.9	5121	23.9	23.5
cry^{b}	ND	ND	ND	8050	24.6	24.8
cry^{+}	1488	23.0	23.6	6470	AR	AR

Eclosion profiles of cry^{0}, cry^{b}, and cry^{+} cultures were obtained from three independent monitorings, each of which encompassed two eclosion-monitor "discs" (*cf.* Konopka *et al.* 1994) for cry^{0} and cry^{b}, and one for cry^{+} pupae. Data from all tests were pooled and analyzed together for a given genotype to estimate a combined period value (τ in hours), using autocorrelation (Autocorr) and MESA. AR, arrhythmic; *N*, total numbers of emergents; ND, not done.

condition. This mutant type also exhibited anomalously periodic adult-emergence in LL (Table 4).

A previous test of cry^{b} eclosion in DD showed such cultures to emerge rhythmically in that condition (Mealey-Ferrara *et al.* 2003), whereas this mutant was reported to eclose arrhythmically in LD (Myers *et al.* 2003). In the current study, monitoring cry^{0} eclosion in the latter condition led to vigorously periodic adult emergence, although the average peak time (per LD day) was slightly before dawn, as opposed to slightly after that time for cry^{+} control cultures or cry^{b} mutant ones (supplemental Figure 1 at http://www.genetics.org/supplemental/).

***cry*^{0}'s effect on luciferase reporting of clock-gene cycling in peripheral tissues:** Krishnan *et al.* (2001) and Levine *et al.* (2002b) previously reported the effects of the cry^{b} mutation on *period* and *timeless* gene expression within isolated peripheral *Drosophila* tissues. That seminal mutation significantly decreased proportions of rhythmic specimens in LD and DD (compared with the usual values given by various tissue types), with these exceptions: Antennal rhythmicity was only partially degraded, and wing rhythmicity was barely affected by cry^{b} (Levine *et al.* 2002b). To ask whether elimination of CRY would lead to more severe arrhythmia in isolated tissues, we monitored *per-luc* luminescence fluctuations in wings and antennae under LD conditions. Luciferase-reported cycling was reduced by the effects of cry^{0} on both appendage types, but an appreciable proportion of specimens displayed rhythmicity: approximately two-thirds of the wings and approximately one-third of the antennae were rhythmic (supplemental Table 2 at http://www.genetics.org/supplemental/).

DISCUSSION

The aim of this work was to generate a true *cryptochrome*-null mutation in Drosophila and assess the spectrum of rhythm-related abnormalities that could be caused by an absence of CRY protein. These cry^{0} mutants were generated by an HR tactic that removed the entire CRY-encoding region of chromosome *3*; this resulted in a handful of knock-out lines, all of them in-principle identical. Three of these strains and their derivatives were studied in detail.

Two-component free-running rhythms induced by *cry* mutations: One striking phenotype of the original *cry* mutant is behavioral rhythmicity in LL (*e.g.*, Emery *et al.* 2000a), a condition that causes wild-type flies to exhibit aperiodic locomotion (Konopka *et al.* 1989). When cry^{0} flies were exposed to this photic condition (LL), they also displayed rhythmic behavior, albeit with properties different from those usually described for cry^{b} (the initially isolated mutant). Nearly all cry^{0} individuals displayed two free-running periodic components in LL. A similar phenotype was described for cry^{b} by Yoshii *et al.* (2004) and by Rieger *et al.* (2006), with the following provisos: When the photic conditions entailed very dim LL (<1 lux in Yoshii *et al.* 2004), a single free-running locomotor component was observed, whereas two such components were displayed by this mutant as the intensity of LL was raised (>10 lux). Such cry^{b}-induced "rhythm dissociation" was *not* observed by other investigators (Emery *et al.* 2000a; Helfrich-Förster *et al.* 2001; Mealey-Ferrara *et al.* 2003), even though they applied high light intensities for which behavioral "splitting" was observed by, for example, Yoshii *et al.* (2004). A mnemonic device for the ensemble of these

Figure 5.—Free-running periodic emergence of cry^{0} flies into adulthood. Late-developing cry^{0} cultures displayed periodic adult emergence in both DD and LL. The left-hand plots show adult-emergence profiles after application of a bandpass filter, which removed periodic components <4 and >40 hr (*cf.* Levine *et al.* 2002a). The other plots show analytical outcomes, the conventions for which (*e.g.*, ordinate labels) are as in Figure 2. Wild-type cry^{+} cultures, which routinely eclose periodically in DD (*e.g.*, Table 4), gave an eclosion profile in LL for which daily peaks damped out after ~48 hr of being shifted to this condition.

results is that cry^b flies exhibit splitting when LL is in the range of ~10–400 lux, whereas cry^0 ones give this response even at a higher light intensity.

What causes a CRY-less fly, and a CRY-depleted one in certain circumstances, to exhibit bicomponent periodicities? Such splitting could have to do with separate free running-periods by which the clockworks might be operating in different brain cells, or even arise from two rhythm components being present in one such cell, as occurs in unicellular Gonyaulax (Roenneberg and Morse 1993). A related possibility is that the locomotor splittings in question are not *per se* the result of removing or depleting CRY; instead, these complex rhythmic components could be part of Drosophila's "basic" chronobiological capacity (*cf.* Helfrich-Förster 2001) and are uncovered by the effects of *cry* mutations. In other words, relatively fast- and slow-running clocks would (also) be harbored within wild-type brains, but these different paces are ordinarily "processed" to result in overall ~24-hr periodicities. One speculative function for the naturally occurring two-pacemaker situation is that the <24-hr and >24-hr components are involved in temperature compensation: Relatively cool or warm thermal conditions would have the opposite effect on each component (speeding up one while slowing down the other), resulting in a final read-out near 24 hr. Our temperature-varied experiments, however, do not support this notion, at least not in the case of 29° LL locomotor monitorings, for which both free-running components manifested the apparent effects of fast-running circadian pacemakers.

The abovementioned CNS defect induced by *sine oculis* (*so*) mutations, which can cause individual Drosophila to display more than one circadian component in DD, leads to the idea that an inter-pacemaker communication defect is among cry^0's rhythm-related defects. That is, the insensitivity of deep-brain photoreceptors (*cf.* Emery *et al.* 2000b) to LL in a *cry* mutant could be connected with a failure of these CRY-less neurons to mediate synchrony between bilaterally symmetrical pacemaker cells, by analogy to the correlation between *so*-induced behavioral splitting (Helfrich 1986) and the frequent absence of an inter-pacemaker anatomical connection in this mutant type (Helfrich-Förster and Homberg 1993). Wild-type flies in DD could not, of course, enjoy any photically controlled communication between such neurons in each of the two brain hemispheres. But when such cry^+ individuals get exposed to LD cycles (or at least LD transitions) prior to proceeding into DD, operations of these circadian neurons may gear-up (thanks to CRY-mediated photoreception within the brain), to sustain intra-brain synchrony-related functions. Squarely in this regard, some genetically normal Drosophila exhibit the behavioral effects of (apparent) internal desynchronization after *lengthy sessions in DD*, similar to *so*-induced splitting in that condition or *cry*-induced splitting in LL (Helfrich-Förster 2000).

Entrainment to LD cycles and resynchronization to shifted photic conditions: The cry^b mutation causes rhythm-related entrainment problems, especially in combination with a given visual-system mutation: *no-receptor-potential-A* or *glass* (Stanewsky *et al.* 1998; Helfrich-Förster *et al.* 2001). When a probable *norpA*-null mutation (allele *P41*) was added to a cry^b-homozygous genetic background, certain proportions of the doubly mutant flies were capable of re-entraining their locomotor cycles. For example, $norpA^{P41}$ cry^b individuals were asked to resynchronize from LD cycles ($L = 650$ lux white light) to 4-hr-shifted ones ($L = 16$ lux blue); 27% of the flies readjusted their locomotor cycles accordingly (Stanewsky *et al.* 1998). Our findings, involving a confirmed *norpA*-null allele (*P24*) combined with cry^0, revealed that only 15% of the double mutants re-entrained fully to an 8-hr LD shift (both L's = 850 lux white); this lower proportion (compared with 27% in Stanewsky *et al.* 1998) may have occurred because of the more abrupt LD shift employed in the current experiments. The same kind of test was performed by Helfrich-Förster *et al.* (2001) on *glass*-null cry^b double mutants, 0% of which individuals resynchronized their behavior after 8-hr alterations of LD cycles. The *gl*-with-*cry* case will resurface shortly below.

Another prior related study asked $norpA^{P41}$ cry^b double mutants to adjust their locomotion away from being synchronized with 12:12 LD ($L = 850$ lux white), such that they would operate on the same time scale of 13:13 cycles. Approximately 40% of the individuals ended up entraining to the latter cycle, shifting from displays of ~24-hr periods to ~26-hr ones (Mealey-Ferrara *et al.* 2003). Less impressively, at most 18% of $norpA^{P24}cry^0$ flies exhibited resynchronization to the second kind of cycle in our corresponding experiment, but <5% did so in the 12:12 → 9:9 or the 12:12 → 14:14 tests (Table 3). This means that this double mutant type readjusted its behavior most readily when such correction involved only a 2-hr stretch (12:12 → 13:13). Combining a *glass* mutation with the latter kind of *cry* was once said to create the most severe circadian-blinding genotype, soley on the basis of experiments involving abrupt 8-hr shifts of LD cycles (Helfrich-Förster *et al.* 2001). However, in conditions comparable to our current 12:12 → novel-cycle tests (whereby L within a given LD period was 850 lux) ~55% of gl^{60j} cry^b individuals resynchronized to the new environmental cycle (Mealey-Ferrara *et al.* 2003).

Overall, then, mutant Drosophila in which no light-induced responsiveness of external photoreceptors is possible and who are (at last) thoroughly devoid of CRY protein exhibit among the poorest instances of entrainment to photic cycles and resynchronization to adjusted LD cycles. However, behavioral readjustments do occur for varying proportions of the double mutants (as summarized in this subsection). Nonetheless, the simultaneously *norpA*- and *cry*-null genotype has more

severe consequences than does a *norpA*-mutated, *cry*-missense combination.

Temperature responsiveness of the novel *cry*-null mutant: Although cry^0 flies displayed normal behavior in DD at 25°, their rhythmicity was affected when locomotor cycles were running at 18° or 29° (Table 1, which documents reduced percentages of rhythmic individuals in relatively cool or warm conditions). A similar effect was observed in LL, again at temperatures lower or higher than the apparent optimal one (25°). These data suggest further that, in addition to its role in photic entrainment, CRY is also involved in central pacemaking as such (*cf.* Ivanchenko *et al.* 2001; Krishnan *et al.* 2001; Collins *et al.* 2006; Stoleru *et al.* 2007). In other words, cry^+ seems to be important for the most favorably temperature-compensated behavior, although not in this case speaking to the maintenance of ~24-hr periodicities in different thermal conditions (Sawyer *et al.* 1997); instead, for *basic rhythm maintenance* when the temperature strays rather far from 25°.

Synchronization of Drosophila late in development for periodic eclosion: It is necessary to synchronize fruit-fly cultures with environmental inputs if pharate adults are to emerge rhythmically, because this circadian periodic phenotype is a population phenomenon. A corollary, in a way, is that eclosion occurs arrhythmically if pupae are exposed to LL (Chandrashekaran and Loher 1969). And because that condition militates against CRY accumulation (Emery *et al.* 1998; Lin *et al.* 2001), one would imagine that *cry*-depleting mutations should induce aperiodic eclosion, if this protein is essential for its photic entrainment. Fulfillment of this tacit prediction was reported by Myers *et al.* (2003) for cry^b eclosion, which was said to be arrhythmic even in LD. In contrast, Mealey-Ferrara *et al.* (2003) showed that that mutation allows for LD entrainment of cultures such that they exhibited solidly periodic eclosion in (subsequent) DD. Another matter addressed by the study just cited: the fact that $norpA^+$ function contributes to synchronization of clock-protein cycling in developing Drosophila; this phenomenon operates in larval brain neurons (Kaneko *et al.* 2000). But the simultaneous presence of a *norpA* mutation and cry^b left developing animals synchronizable for periodic eclosion if they were exposed to LD *after* the larval stage before proceeding into DD (Mealey-Ferrara *et al.* (2003). The same photic regime was applied in elements of the current eclosion tests. Their results imply that what is necessary, or at least sufficient, for LD synchronization of pupae entails a mysterious photoreceptive process that is both NORPA- and CRY-independent (see *Conclusions*).

Elements of the current findings shed further light on CRY's contribution to the control of periodic eclosion, because adult emergence of cry^0 flies was found to be clearly rhythmic in LD (supplemental Figure 1 at http://www.genetics.org/supplemental/). This could imply that CRY has *no* influence of synchronization of late-developing Drosophila. But that supposition is belied by a newly uncovered effect of *cry* mutations—which is that depletion or elimination of the encoded protein renders the animal insensitive to the arrhythmia-inducing effects of LL (Figure 5).

Conclusions: Previous studies of *cryptochrome* mutants revealed a role for the light-absorbing substance it encodes in photic-input and peripheral clock mechanisms but left many questions unanswered. To address these issues, which mostly involve ambiguous effects of the cry^b mutation, we generated the first true *cry*-null flies and subjected them to the requisite array of chronobiological tests. Along the way, we accounted for possibly confounding genetic-background effects and neurodevelopmental defects (none and none). We found that cry^0 flies displayed more severely abnormal light-response phenotypes than do cry^b ones, bolstering the frequently discussed supposition that residual CRY function lurks within chronobiologically meaningful tissues of cry^b animals.

Our analysis also lends credence to the existence of CRY-independent pathways that are responsible for the residual photoreceptive capacities previously observed in $norpA^{P41}$ cry^b flies (or gl^{60j} cry^b ones) and the maintenance of peripheral-clock functioning in certain appendage types set up to express the effects of cry^b. The "extra" pathway in question, the one functioning somewhere within the intact animal, may be headed-up by photoreceptive CNS neurons (N's) located in the dorsal (D) brain (see Helfrich-Förster *et al.* 2001 and Veleri *et al.* 2003 for the relevant results and discussions). A seemingly remarkable implication of this presumption is that the dorsal neurons in question (called neuronal cluster DN1) would possess photoreceptive capacities that are dependent neither on CRY (this report), nor on any known form of Drosophila rhodopsin. This notion should be considered in context of the conventional wisdom about the latter such substances, whereby the *norpA* gene product would transduce the results of all rhodopsin-mediated light absorption (Pak and Leung 2003). However, recall our mentioning the discussion of rhodopsin-containing H–B eyelet cells functioning independently of *norpA* function insofar as circadian photoreception is concerned; although this supposition was based mainly on the fact that a *norpA cry* double mutant, for which neither mutation was known to be a null, can "still entrain" (Veleri *et al.* 2007; *cf.* Stanewsky *et al.* 1998). Nevertheless, and thus assuming that circadian photoreception in Drosophila can occur in a manner that is both NORPA- and CRY-independent (this report), what if the abovementioned DN1 cells indeed are CNS photoreceptors? Their position is not so deep into the brain, with reference to the CRY-containing lateral neurons (Emery *et al.* 2000b). The dorsally located neurons could possess an opsin that does *not need the*

norpA-*encoded enzyme* for the photransduction process in question (Chang and Reddy 2000).

As a final point, we have obtained novel results suggesting that CRY plays a role in the maintenance of central clock function at temperature extremes, and that hypomorphic or null alleles of *cry* permit synchronized periodic eclosion of flies even in LL. The ensemble of these experiments advance our understanding of roles played by cryptochrome on behalf of circadian inputs and of pacemaker mechanisms, as well as establishing an important new genetic variant for future research.

We thank Yikang Rong and Kent Golic for providing strains that mediate homologous recombination of transgenes and the Bloomington Indiana Stock Center for supplying some of the basic stocks (genetically normal and mutant strains). We appreciate the assistance with Matlab applications furnished by Pablo Funes and the confocal help provided by Edward Dougherty. We are grateful for comments on the manuscript from Dennis C. Chang. This work was supported by grants from the National Institutes of Health (NS-44232 and GM-66778).

LITERATURE CITED

Allada, R., N. E. White, W. W. So, J. C. Hall and M. Rosbash, 1998 A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell **93:** 791–804.

Bi, X., and Y. S. Rong, 2003 Genome manipulation by homologous recombination in *Drosophila*. Brief Funct. Genomic Proteomic **2:** 142–146.

Busza, A., M. Emery-Le, M. Rosbash and P. Emery, 2004 Roles of the two *Drosophila* CRYPTOCHROME structural domains in circadian photoreception. Science **304:** 1503–1506.

Chandrashekaran, M. K., and W. Loher, 1969 The effect of light intensity on the circadian rhythm of eclosion in *Drosophila pseudoobscura*. Z. Vergl. Physiol. **62:** 337–347.

Chang, H. Y., and D. F. Reddy, 2000 Rescue of photoreceptor degeneration in rhodopsin-null *Drosophila* mutants by activated Rac1. Science **290:** 1978–1980.

Collins, B., E. O. Mazzoni, R. Stanewsky and J. Blau, 2006 *Drosophila* CRYPTOCHROME is a circadian transcriptional repressor. Curr. Biol. **16:** 441–449.

Cook, T., F. Pichaud, R. Sonneville, D. Papatsenko and C. Desplan, 2003 Distinction between color photoreceptor cell fates is controlled by Prospero in *Drosophila*. Devel. Cell **4:** 853–864.

Costa, R., A. A. Peixoto, G. Barbujani and C. P. Kyriacou, 1992 A latitudinal cline in a *Drosophila* clock gene. Proc. R. Soc. Lond. Ser. B **250:** 43–49.

Dissel, S., V. Codd, R. Fedic, K. J. Garner, R. Costa *et al.*, 2004 A constitutively active cryptochrome in *Drosophila melanogaster*. Nat. Neurosci. **7:** 834–840.

Dunlap, J. C., J. J. Loros and P. P. Decoursey, 2004 *Chronobiology: Biological Timekeeping*. Sinauer Associates, Sunderland, MA.

Emery, P., W. V. So, M. Kaneko, J. C. Hall and M. Rosbash, 1998 CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell **95:** 669–679.

Emery, P., R. Stanewsky, J. C. Hall and M. Rosbash, 2000a *Drosophila* cryptochrome - a unique circadian-rhythm photoreceptor. Nature **404:** 456–457.

Emery, P., R. Stanewsky, C. Helfrich-Förster, M. Emery-Le, J. C. Hall *et al.*, 2000b *Drosophila* CRY is a deep brain circadian photoreceptor. Neuron **26:** 493–504.

Frank, K. D., and W. F. Zimmerman, 1969 Action spectra for phase shifts of a circadian rhythm in Drosophila. Science **163:** 688–689.

Gong, W. J., and K. G. Golic, 2003 Ends-out, or replacement, gene targeting in *Drosophila*. Proc. Natl. Acad. Sci. USA **100:** 2556–2561.

Hall, J. C., 2003 Genetics and molecular biology of rhythms in *Drosophila* and other insects. Adv. Genet. **48:** 1–280.

Helfrich, C., 1986 Role of the optic lobes in the regulation of the locomotor activity rhythms in *Drosophila melanogaster*: behavioral analysis of neural mutants. J. Neurogenet. **3:** 321–343.

Helfrich-Förster, C., 2000 Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster*–sex-specific differences suggest a different quality of activity. J. Biol. Rhythms **15:** 135–154.

Helfrich-Förster, C., 2001 The activity rhythm of *Drosophila melanogaster* is controlled by a dual oscillator system. J. Insect Physiol. **47:** 877–887.

Helfrich-Förster, C., and U. Homberg, 1993 Pigment-dispersing hormone-immunoreactive neurons in the nervous system of wild-type *Drosophila melanogaster* and of several mutants with altered circadian rhythmicity. J. Comp. Neurol. **337:** 177–190.

Helfrich-Förster, C., C. Winter, A. Hofbauer, J. C. Hall and R. Stanewsky, 2001 The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron **30:** 249–261.

Hofbauer, A., and E. Buchner, 1989 Does *Drosophila* have seven eyes? Naturwissenschaften **76:** 335–336.

Hoskins, R. A., A. C. Phan, M. Naeemuddin, A. F. Mapa, D. A. Ruddy *et al.*, 2001 Single nucleotide polymorphism markers for genetic mapping in *Drosophila melanogaster*. Genome Res. **11:** 1100–1113.

Huber, A., S. Schulz, J. Bentrop, C. Groell, U. Wolfrum *et al.*, 1997 Molecular cloning of *Drosophila* Rh6 rhodopsin: the visual pigment of a subset of R8 photoreceptor cells. FEBS Lett. **406:** 6–10.

Ivanchenko, M, R. Stanewsky and J. M. Giebultowicz, 2001 Circadian photoreception in *Drosophila*: functions of cryptochrome in peripheral and central clocks. J. Biol. Rhythms **16:** 205–215.

Kaneko, M., M. J. Hamblen and J. C. Hall, 2000 Involvement of the *period* gene in developmental time-memory: effect of the *per^{Short}* mutation on phase shifts induced by light pulses delivered to *Drosophila* larvae. J. Biol. Rhythms **15:** 13–30.

Klarsfeld, A., S. Malpel, C. Michard-Vanhee, M. Picot, E. Chelot *et al.*, 2004 Novel features of cryptochrome-mediated photoreception in the brain circadian clock of *Drosophila*. J. Neurosci. **24:** 1468–1477.

Klemm, E., and H. Ninnemann, 1976 Detailed action spectrum for the delay shift in pupae emergence of *Drosophila pseudoobscura*. Photochem. Photobiol. **24:** 369–371.

Koh, K., X. Zheng and A. Sehgal, 2006 JETLAG resets the *Drosophila* circadian clock by promoting light-induced degradation of TIMELESS. Science **312:** 1809–1812.

Konopka, R. J., C. Pittendrigh and D. Orr, 1989 Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. J. Neurogenet. **6:** 1–10.

Konopka, R. J., M. J. Hamblen-Coyle, C. F. Jamison and J. C. Hall, 1994 An ultrashort clock mutation at the *period* locus of *Drosophila melanogaster* that reveals some new features of the fly's circadian system. J. Biol. Rhythms **9:** 189–216.

Krishnan, B., S. E. Dryer and P. E. Hardin, 1999 Circadian rhythms in olfactory responses of *Drosophila melanogaster*. Nature **400:** 375–378.

Krishnan, B., J. D. Levine, K. S. Lynch, H. B. Dowse, P. Funes *et al.*, 2001 A novel role for cryptochrome in a *Drosophila* circadian oscillator. Nature **411:** 313–317.

Lee, T., and L. Luo, 1999 Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron **22:** 451–461.

Levine, J. D., P. Funes, H. B. Dowse and J. C. Hall, 2002a Signal analysis of behavioral and molecular cycles. BMC Neurosci. **3**: 1.

Levine, J. D., P. Funes, H. B. Dowse and J. C. Hall, 2002b Advanced analysis of a cryptochrome mutation's effects on the robustness and phase of molecular cycles in isolated peripheral tissues of *Drosophila*. BMC Neurosci. **3**: 5.

Lin, F.-J., W. Song, E. Meyer-Bernstein, N. Naidoo and A. Sehgal, 2001 Photic signaling by cryptochrome in the *Drosophila* circadian system. Molec. Cell. Biol. **21:** 7287–7294.

Malpel, S., A. Klarsfeld and F. Rouyer, 2002 Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with

clock neurons during *Drosophila* brain development. Development **129:** 1443–1453.

Mealey-Ferrara, M. L., A. G. Montalvo and J. C. Hall, 2003 Effects of combining a cryptochrome mutation with other visual-system variants on entrainment of locomotor and adult-emergence rhythms in *Drosophila*. J. Neurogenet. **17:** 171–221.

Myers, E. M., J. Yu and A. Sehgal, 2003 Circadian control of eclosion: interaction between a central and peripheral clock in *Drosophila melanogaster*. Curr. Biol. **13:** 526–533.

Ohata, K., H. Nishiyama and Y. Tsukahara, 1998 Action spectrum of the circadian clock photoreceptor in *Drosophila melanogaster*, pp. 167–170, in *Biological Clocks: Mechanisms and Applications*, edited by Y. Touitou. Elsevier Science B. V., Amsterdam.

Pak, W. L., and H. T. Leung, 2003 Genetic approaches to visual transduction in *Drosophila melanogaster*. Recept. Channels **9:** 149–167.

Park, J. H., C. Helfrich-Förster, G. Lee, L. Liu, M. Rosbash *et al.*, 2000 Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. Proc. Natl. Acad. Sci. USA **97:** 3608–3613.

Park, S., and J. K. Lim, 1995 A microinjection technique for ethanol-treated eggs and a mating scheme for detection of germline transformants. Drosoph. Inf. Serv. **76:** 197–199.

Pearn, M. T., L. L. Randall, R. D. Shortridge, M. G. Burg and W. L. Pak, 1996 Molecular, biochemical, and electrophysiological characterization of *Drosophila norpA* mutants. J. Biol. Chem. **271:** 4937–4945.

Peschel, N., S. Veleri and R. Stanewsky, 2006 *Veela* defines a molecular link between Cryptochrome and Timeless in the light-input pathway to *Drosophila*'s circadian clock. Proc. Natl. Acad. Sci. USA **103:** 17313–17318.

Plautz, J. D., M. Kaneko, J. C. Hall and S. A. Kay, 1997 Independent photoreceptive circadian clocks throughout *Drosophila*. Science **278:** 1632–1635.

Price, J. L., 2005 Genetic screens for clock mutants in *Drosophila*. Meth. Enzymol. **393:** 35–60.

Rieger, D., O. T. Shafer, K. Tomioka and C. Helfrich-Forster, 2006 Functional analysis of circadian pacemaker neurons in *Drosophila melanogaster*. J. Neurosci. **26:** 2531–2543.

Robertson, H. M., C. R. Preston, R. W. Phillis, D. M. Johnson-Schlitz, W. K. Benz *et al.*, 1988 A stable genomic source of *P* element transposase in *Drosophila melanogaster*. Genetics **118:** 461–470.

Roenneberg, T., and D. Morse, 1993 Two circadian oscillators in one cell. Nature **362:** 362–364.

Rong, Y. S., and K. G. Golic, 2000 Gene targeting by homologous recombination in *Drosophila*. Science **288:** 2013–2018.

Rosato, E., A. Trevisan, F. Sandrelli, M. Zordan, C. P. Kyriacou *et al.*, 1997 Conceptual translation of *timeless* reveals alternative initiating methionines in *Drosophila*. Nucleic Acids Res. **25:** 455–457.

Rosato, E., V. Codd, G. Mazzotta, A. Piccin, M. Zordan *et al.*, 2001 Light-dependent interactions between *Drosophila* CRY and the clock protein PER mediated by the carboxy terminus of CRY. Curr. Biol. **11:** 909–917.

Rubin, G. M., and A. C. Spradling, 1982 Genetic transformation of *Drosophila* with transposable element vectors. Science **218:** 348–353.

Sancar, A., 2000 Cryptochrome: the second photoactive pigment in the eye and its role in circadian photoreception. Ann. Rev. Biochem. **69:** 31–67.

Sawyer, L. A., M. J. Hennessey, A. A. Peixoto, E. Rosato, H. Parkinson *et al.*, 1997 Natural variation in *Drosophila* clock gene and temperature compensation. Science **278:** 2117–2120.

Sheeba, V., V. K. Sharma, M. K. Chandrashekaran and A. Joshi, 1999 Persistence of eclosion rhythm in *Drosophila melanogaster* after 600 generations in an aperiodic environment. Naturwissenschaften **86:** 448–449.

Sokolove, P. G., and W. N. Bushell, 1978 The chi square periodogram: its utility for analysis of circadian rhythms. J. Theoret. Biol. **72:** 131–160.

Stanewsky, R., C. F. Jamison, J. D. Plautz, S. A. Kay and J. C. Hall, 1997 Multiple circadian-regulated elements contribute to cycling period gene expression in *Drosophila*. EMBO J. **16:** 5006–5018.

Stanewsky, R., M. Kaneko, P. Emery, B. Beretta, K. Wager-Smith *et al.*, 1998 The *cry*^{b} mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell **95:** 681–692.

Stoleru, D., P. Nawathean, Mde. L. Fernandez, J. S. Menet, M. F. Ceriani *et al.*, 2007 The *Drosophila* circadian network is a seasonal timer. Cell **129:** 207–219.

Tauber, E., M. Zordan, F. Sandrelli, M. Pergoraro, N. Osterwalder *et al.*, 2007 Natural selection favors a newly derived *timeless* allele in *Drosophila melanogaster*. Science **316:** 1895–1898.

Veleri, S., C. Brandes, C. Helfrich-Förster, J. C. Hall and R. Stanewsky, 2003 A self-sustaining, light-entrainable circadian oscillator in the brain of *Drosophila*. Curr. Biol. **13:** 1758–1767.

Veleri, S., D. Rieger, C. Helfrich-Förster and R. Stanewsky, 2007 Hofbauer-Buchner eyelet affects circadian photosensitivity and coordinates PER and TIM expression in *Drosophila* clock neurons. J. Biol. Rhythms **22:** 29–42.

Venken, K. J. T., and H. J. Bellen, 2005 Emerging technologies for gene manipulation in *Drosophila melanogaster*. Nat. Rev. Genet. **6:** 167–178 (erratum: Nat. Rev. Genet. **6:** 340).

Winfree, A. T., 1974 Suppressing Drosophila's circadian rhythm with dim light. Science **183:** 970–972.

Yoshii, T, Y. Funada, T. Ibuki-Ishibashi, A. Matsumoto, T. Tanimura *et al.*, 2004 *Drosophila cry*^{b} mutation reveals two circadian clocks that drive locomotor rhythm and have different responsiveness to light. J. Insect Physiol. **50:** 478–488.

Yu, Q., H. V. Colot, C. P. Kyriacou, J. C. Hall and M. Rosbash, 1987 Behaviour modification by *in vitro* mutagenesis of a variable region within the *period* gene of *Drosophila*. Nature **326:** 765–769.

Zimmerman, W. F, and T. H. Goldsmith, 1971 Photosensitivity of the circadian rhythm and of visual receptors in carotenoid-depleted Drosophila. Science **171:** 1167–1169.

Communicating editor: J. J. Loros