The role of CRYPTOCHROME in the *Drosophila* circadian clock

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Abstract

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The circadian clock of *Drosophila melanogaster* consists of at least two interlocked feedback loops. In the first, the *period* and *timeless* gene products negatively regulate their own transcription. Although this mechanism persists without any environmental cue, the molecular mechanism underlying circadian clocks can be synchronized by the daily oscillation of light and dark. CRYPTOCHROME (CRY) is the dedicated circadian photoreceptor, and flies carrying a strong hypomorphic mutation in the *cry* gene have severely blunted circadian photoresponses. CRY physically interacts with the core components of the clock, PERIOD (PER) and TIMELESS (TIM) in a light-dependent manner. Previous work carried out in the laboratory showed that removing 20 amino acids at the C-terminus of CRY to create CRYΔ results in the loss of light-dependency of CRY interactions in yeast two-hybrid assays. Based on this work, the aim of my project was to study the role of the CRY C-terminus *in vivo* by clock neurons targeted overexpression of CRYΔ with the hypothesis that it should behave as a constitutively active form of the protein. CRYΔ flies have long period of locomotor activity in constant darkness, show abnormal responses to light and exhibit altered oscillation of the PER and TIM proteins in central and peripheral clocks. These phenotypes are reminiscent of responses observed when wild-type flies are kept under continuous low-light intensity. Therefore, this study provides strong behavioural, molecular and immunohistochemical evidence confirming that CRYΔ is constitutively active, and elicits continuous light responses. Moreover, previous work demonstrated that CRY role in the *Drosophila* clock exclusively involves light signalling to the core components of the clock. This study identified a potential new light-independent function for CRY *in vivo*.

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Abbreviations.

PER= PERIOD
MP= Morning Peak
EP= Evening Peak
LD= Light-Dark
DD= Dark-Dark
EMS= Ethyl-Methane-Sulfonate
NLS= Nuclear Localisation Signal
CLD= Cytoplasmic Localisation Domain
CCID= Clock-Cycle Inhibition Domain
ZT= Zeitgeber Time
TIM= TIMELESS
LNs= Lateral Neurons
LN_ds= Lateral Neurons Dorsal
s-LN_vs= Small Lateral Neurons Ventral
l-LN_vs= Large Lateral Neurons Ventral
DNs= Dorsal Neurons
DN1s= Dorsal Neurons 1
DN2s= Dorsal Neurons 2
DN3s= Dorsal Neurons 3
PDF= PIGMENT DISPERSING FACTOR
bHLH= Basic Helix-Loop-Helix
CLK= CLOCK
CYC= CYCLE
DBT= DOUBLETIME
$CK1_\varepsilon$= CASEIN KINASE 1_ε
CK2= CASEIN KINASE 2
Tik= Timekeeper
SGG= SHAGGY
PRC= Phase Response Curve
GSK3= Glycogen Synthase Kinase-3

PP2A= Protein Phosphatase 2A
tws= twins
wdb= widerborst
mts= mutagenic star
VRI= VRILLE
CRY= CRYPTOCHROME
PDP1= PAR DOMAIN PROTEIN 1
HB= Hofbauer-Buchner
FAD= Flavin Adenin Dinucleotide
SCN= Suprachiasmatic Nucleus
HA= hemagglutinin
LL= Light-Light
CT= Circadian Time
ICC= Immunocytochemistry

Chapter 1: Introduction

1.1 Biological rhythms

Rhythmicity is a pervasive feature of nature and almost every organism on the planet has to adapt to repetitive periodic events. There are many biological clocks, which are able to control cycling biological phenomena across an impressive range of periodicities. The time domain they operate with varies from one minute (i.e. the *Drosophila melanogaster* song cycle), (Kyriacou and Hall, 1990), to yearly cycles (i.e. circannual rhythm of migrations displayed by some species of birds) (Gwinner, 2003). Because of the available combination of automated behavioural techniques, genetics and molecular biology, the most extensively studied biological oscillation is the circadian (about 24h) cycle, imposed by the Earth's rotation. Across taxa, the clock genes are not always the same, but they generate the same type molecular mechanism, namely an interlocked transcription/translation feedback loop (Dunlap and Loros, 2004; Hardin, 2005; Iwasaki and Kondo, 2004; Salome and Mc Clung, 2004; Stanewsky, 2003).

1.2 The circadian clock

Circadian clocks have been found in almost every organism, ranging from prokaryotic and eukaryotic microbes, to plants, insects and mammals (Stanewsky, 2003; Young, 1999). Their widespread occurrence probably reflects the fact that they provide an advantage to organisms in a cyclic environment such as the one imposed by the daily light-dark oscillations. For example, the loss of circadian

clock function in *Drosophila melanogaster* results in reduced reproductive fitness (Beaver et al., 2002). Recent work also suggests that mutations in clock genes cause shorter lifespan in fruitflies (Kumar et al., 2005). In insects, the most common manifestations of the circadian clock are adult locomotor activity and eclosion rhythms (the emergence of adult flies from their pupal cases). Leaf movements, photosynthesis and flowering time are among the circadian phenomena observed in plants. In humans, the sleep/wake cycle and the daily oscillation in body temperature are circadian regulated processes. The most perceptible effect of the circadian clock that humans experience is when the endogenous clock is being brought out of phase with the external daily oscillations of light and dark. This is happening for example when we travel across different time zones, an effect known as 'jet lag'. A defective circadian clock is involved in sleep disorders in humans, such as the familial advanced sleep phase syndrome (FASPS, (Toh et al., 2001)) and delayed sleep phase syndrome (Archer et al., 2003). More recent work has revealed a link between the circadian clock and the control of the cell cycle in mice (Matsuo et al., 2003). Furthermore, mutations in the mouse *period2* gene increase the occurrence of cancer (Fu et al., 2002). Finally, the recently occurring field of chronotherapy, which advocates the importance of delivering drugs according to the circadian cycle in order to maximize efficiency (Green, 2005), furthermore underlines the importance of understanding how circadian clocks work.

The fruitfly *Drosophila melanogaster* has been, and still is, pivotal in unravelling the molecular cogs of the clock. The ease of genetic manipulation and the simpler organisation of the clock ensure that *Drosophila* leads the way for new discoveries, which are then investigated in mammals. I shall start with the description of the circadian clock of *Drosophila melanogaster*, which was brought

into the realms of genetics in 1971 by the discovery of the *period* gene by Konopka and Benzer. The discovery of *per*, the first behavioural gene to be identified, also marks the start of modern 'neurogenetics'.

1.3 The Drosophila circadian clock

Figure 1.1 depicts the locomotor activity pattern of *Drosophila melanogaster* flies in alternating conditions of light and dark. It can be seen that the activity chart displays two peaks of activity. The morning peak (MP on Figure 1.1) is centred on the dark to light transition and the evening peak (EP on Figure 1.1) on the light to dark transition in cyclic conditions of LD 12:12 (12h of light followed by 12h of darkness). This bimodal pattern of locomotor activity is typical of flies kept in LD cycles and is repeated on a daily basis with a periodicity of 24h.

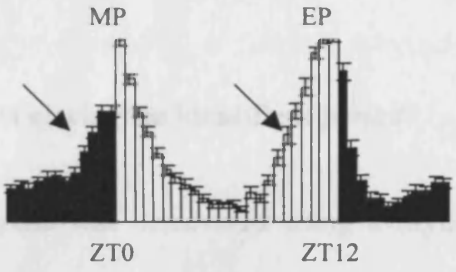


Figure 1.1: *Drosophila melanogaster* LD locomotor activity pattern. Average locomotor activity profile of wild-type *Drosophila melanogaster* male flies at 25 °C in 12:12 LD cycles (12h of light followed by 12h of darkness). The black and white boxes represent night and day activity respectively. ZT0, by convention, is corresponding to lights on and ZT12 to lights off. MP, morning peak of activity, EP, evening peak.

It is important to note that the two peaks of activity are not the result of a simple reaction to lights on and off (a phenomenon known as 'masking') but are the products of an endogenous timekeeping mechanism. This is illustrated by the fact that the flies anticipate the dark/light and light/dark transitions by starting to be active beforehand (as indicated by the arrows on Figure 1.1). One of the properties of the circadian clock is that it is synchronised (or entrained) to the environment by a *Zeitgeber* (from German, readily meaning 'time giver'). Several stimuli can act as *Zeitgeber*, but the most important one is the natural cycle of daily light. Another important property of the circadian timekeeping mechanism is that it is sustained in constant conditions (with no external entraining cues). As a result, the rhythmic locomotor activity pattern is kept when flies are transferred in constant darkness (DD). Finally, circadian rhythms are temperature compensated, so that the period of locomotor activity is stable over a wide range of temperature (Konopka et al., 1989).

1.3.1 The first clock gene identified: *period*

The *period* gene was discovered using a rhythm showed by *Drosophila* populations: the emergence of adult flies from the pupal case. This so-called eclosion process is a rhythmic phenomenon displaying peaks that are separated by ca. 24h (in constant darkness) in wild-type populations. In 1971 Konopka and Benzer mutagenised flies with EMS and identified three mutant lines with altered eclosion rhythmicities. The periodicity was of 19h for the first line and 29h for the second while the last one showed no rhythmicity at all. These mutations were found to map the same locus on the X-chromosome and were called $period^{short}$ (per^{s}), $period^{long}$ (per^{l}) and per^{0}, respectively (Konopka and Benzer, 1971). When tested,

the locomotor activity pattern of individual flies from these mutant strains was also affected in a similar way by the different *per* mutations (Konopka and Benzer, 1971). Interestingly, the *per* mutations also alter the male courtship song cycle, an ultradian rhythm of 60s in wild-type flies. This rhythm is reduced to 40s in $per^s$and increased to 75s in per^l while being almost abolished in per^0 flies (Kyriacou and Hall, 1980). Each of these mutations consists of a single nucleotide substitution, which results in a serine to asparagine replacement at position 589 in per^s flies, a valine to aspartic acid change at position 243 in per^l and the introduction of a stop codon at position 464 in per^0 (Baylies et al., 1987; Yu et al., 1987).

The *period* gene was cloned (Bargiello et al., 1984; Reddy et al., 1984; Zehring et al., 1984) and it was shown that by P-element transformation, genomic fragments from the *per* locus restored rhythmicity to per^0 mutants (Bargiello et al., 1984; Zehring et al., 1984). The *period* gene encodes a 1218 amino acid protein (Citri et al., 1987) containing several regulatory domains (Saez and Young, 1996). Figure 1.2 shows the position of these different domains. In the N-terminal, there is a nuclear localisation signal (NLS) and the PAS protein-protein interaction module. PAS is divided into PAS-A and PAS-B and was first identified within PER, and the transcription factors ARNT and SIM (Huang et al., 1993), and is now widely recognised in many proteins involved in signal reception and transduction, including clock components in prokaryotes and eukaryotes (Gu et al., 2000). Interestingly, the per^l mutation is located in the PAS A region. The cytoplasmic localisation domain (CLD) keeps PER in the cytoplasm in the absence of TIMELESS (TIM), a partner of PER (Saez and Young, 1996) and is the location of the per^0 mutation. Overlapping it is the C-domain, which can interact either via inter or intra-molecular reaction to the first of the two PAS regions, PAS-A (Huang et al., 1995). The

original per^s mutation lies in the C-domain. In the middle of the sequence, there is a region made of about 20 Threonine-Glycine repeats (TG domain), which is polymorphic in length (Costa et al., 1991). This region is involved in temperature compensation of the circadian clock (Sawyer et al., 1997) and is also implicated in the male courtship song cycle (Wheeler et al., 1991). Finally, the most recently found CCID (clock-cycle inhibition domain) lies in the C-terminus of the protein, downstream of the TG region, and harbours a novel NLS (Chang and Reppert, 2003).

Figure 1.2: Schematic representation of PERIOD. NLS= Nuclear Localisation Signal, CLD= Cytoplasmic Localisation Domain, TG= TG repeats, CCID= Clock-Cycle Inhibition Domain.

The expression of *per* mRNA in fly heads cycles with a 24h period (Hardin et al., 1990). The mRNA level peaks during the early night around ZT16 and reaches its trough during the early day at ZT4. Lack of *per* mRNA oscillations in per^0 mutants suggested a feedback of the PER protein on the transcription of the *per* gene (Hardin et al., 1990). Regarding the PER protein, there is also a corresponding cycle with maximum level being reached late at night, around ZT22 and minimum level occurring at the end of the day at ZT8-ZT10 (Edery et al., 1994). Importantly, not only is the overall level of PER fluctuating during a circadian cycle, but the apparent size of the protein is also changing throughout the day, with heavier forms

accumulating late at night when protein levels increase. This change in protein mobility reflects the different phosphorylation status of PER (Edery et al., 1994).

1.3.2 The molecular partner of *per*: *timeless*

A second major mutagenesis screening, this time with P-elements, led to the identification of a second clock gene, *timeless* (*tim*) (Sehgal et al., 1994; Vosshall et al., 1994). The *tim* gene is located on the second chromosome (Sehgal et al., 1994) and was cloned (Gekakis et al., 1995; Myers et al., 1995), revealing that the P-element induced tim^{01} mutation consisted of a deletion of 64bp that leads to a truncated TIMELESS (TIM) protein (Myers et al., 1995). Like per^{0} mutants, tim^{01} flies show arrhythmic locomotor activity and eclosion rhythms when analysed in DD conditions (Sehgal et al., 1994). In addition, the oscillation of *per* mRNA level is abolished by the tim^{01} mutation (Sehgal et al., 1994). Interestingly, PER cellular localisation is limited to the cytoplasm in tim^{01} flies and PER protein levels are very low and do not oscillate (Price et al., 1995; Vosshall et al., 1994). The remaining PER proteins are also constitutively hyperphosphorylated (Price et al., 1995).

The expression pattern of the *tim* gene is very similar to that of *per*, *tim* mRNA level is cycling with maximum and minimum occurring at ZT16 and ZT4 respectively (Sehgal et al., 1995). The *tim* mRNA cycling is abolished by the tim^{01} mutation suggesting feedback of the gene product on its own transcription (Sehgal et al., 1995). Moreover, in per^{0} mutants, *tim* mRNA is non cycling and TIM cannot localise in the nucleus suggesting that PER and TIM are acting together (HunterEnsor et al., 1996; Sehgal et al., 1995). The *tim* gene encodes a 1398 amino acid protein (Myers et al., 1997; Myers et al., 1995; Ousley et al., 1998; Rosato et

al., 1997). TIM protein levels oscillate in phase with PER (HunterEnsor et al., 1996) and like PER, TIM is subjected to phosphorylation (Marrus et al., 1996; Zeng et al., 1996) as hyperphosphorylated forms of the protein can be seen late at night on western blots. TIM is degraded very quickly in response to light whether it is in a LD cycle or in light pulse experiments (HunterEnsor et al., 1996; Lee et al., 1996; Marrus et al., 1996; Myers et al., 1996; Zeng et al., 1996). Zeng et al. (1996) proposed that TIM degradation might be the initial response of the clock to light. Indeed, light was shown to be responsible for the degradation of TIM via the ubiquitine-proteasome pathway (HunterEnsor et al., 1996; Myers et al., 1996; Naidoo et al., 1999). In addition, phosphorylation of TIM on tyrosine residues is implicated in the proteasome degradation (Naidoo et al., 1999). The protein sequence of TIM has a NLS between amino acids 446 and 577, and a C-terminal cytoplasmic localisation domain between amino acids 1228 and 1389 (Saez and Young, 1996). *In vitro* protein binding studies revealed that TIM binds the PER CLD (Saez and Young, 1996),but there is another site of interaction between PER and TIM involving the TIM NLS and the PER PAS-A region (Gekakis et al., 1995; Saez and Young, 1996).

1.3.3 Neurons expressing PER and TIM

The expression pattern of the *per* and *tim* genes as well as immunocytological localisations of the corresponding clock proteins give essential information uncovering the anatomical and functional organization of the pacemaker cells that control rhythmic behaviour. *per* expression is the most extensively studied and is widespread in brain as well as in many different tissues in

adult flies (Ewer et al., 1992; Frisch et al., 1994; Kaneko and Hall, 2000; Kaneko et al., 1997; Liu et al., 1988; Rachidi et al., 1997; Saez and Young, 1988; Siwicki et al., 1988; Stanewsky et al., 1997; Zerr et al., 1990). *tim* expression pattern is also well known (HunterEnsor et al., 1996; Kaneko and Hall, 2000; Kaneko et al., 1997; Myers et al., 1996; Yang et al., 1998), and *per* and *tim* expression patterns in the adult brain are almost identical (Helfrich-Forster, 2003). Figure 1.3 illustrates the clock gene expressing neurons and their arborisation in adult brain. These include the photoreceptor cells of the compound eyes, the ocelli, and an extraretinal eyelet as well as many glial cells and six distinct clusters of neurons that have been classified according to size and position (Helfrich-Forster, 2003). The lateral neurons (LNs) are located in the anterior brain and can be subdivided into three cell clusters. The dorsal-lateral neurons (LN_ds) constitute a cluster of 5-8 cells located in a more dorsal position. There are two ventrally located clusters of LNs that are differing in size and called the small ventral-lateral neurons (s-LN_vs, 5 cells) and large ventral-lateral neurons (l-LN_vs, 4-6 cells). The remaining three clusters are found posterior in the dorsal superior brain and are called dorsal neurons (DNs). The DNs are divided in ca 15 DN1s, 2 DN2s and ca 40 DN3s. In the larval brain, the expression of PER and TIM is restricted to only the 5 s-LN_vs, the two DN2s and two DN1s (Kaneko et al., 1997). Although PER and TIM are expressed together in the majority of clock neurons, there are three cells in the adult lateral posterior brain that express TIM but not PER (Kaneko and Hall, 2000).

The s-LN_vs project into the dorsal central brain where they terminate close to the DN1s and DN2s. The l-LN_vs project onto the surface of the medulla and also send fibers in the posterior optic tract thus connecting l-LN_vs and s-LN_vs from opposite brain hemisphere. The LN_ds, DN1s, DN2s and DN3s all project towards

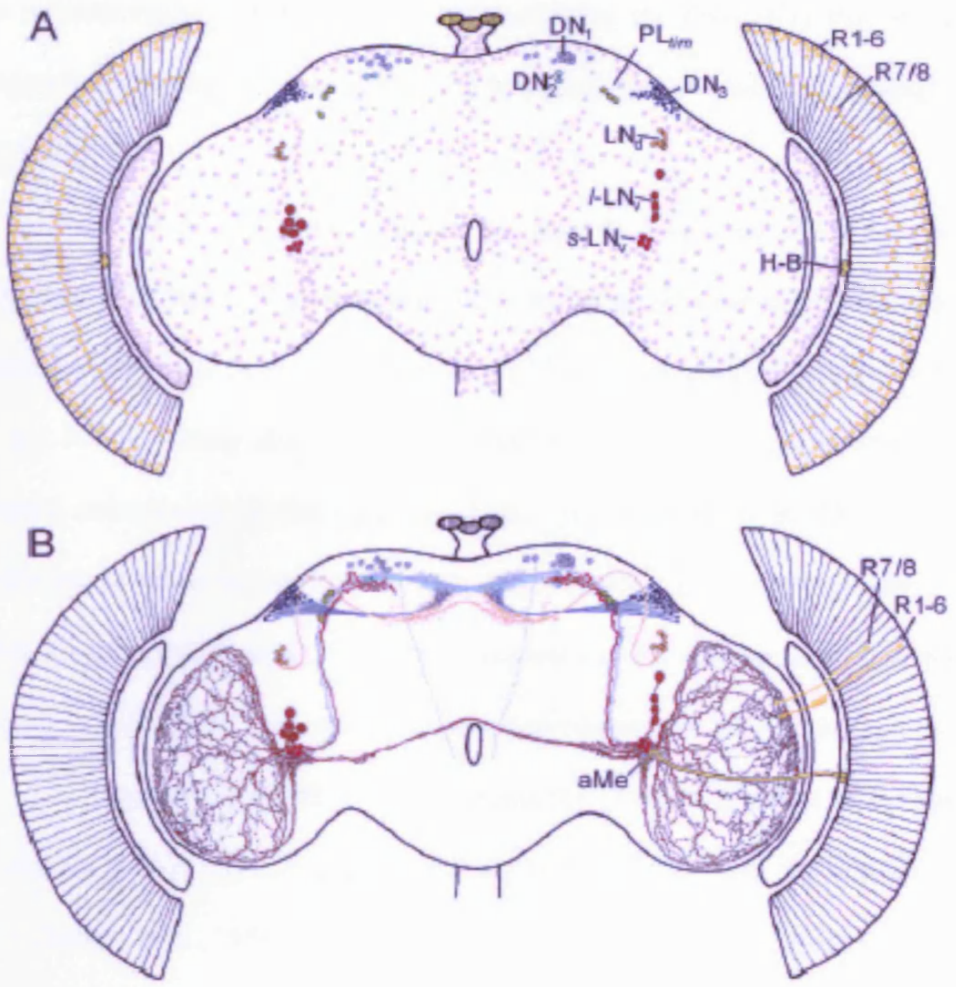


Figure 1.3: (Helfrich-Forster, 2003). The clock gene expressing cells in the *Drosophila* adult brain. **A**: the lateral neurons (LN_ds, s-LN_vs and l-LN_vs) are shown in red/orange and the dorsal neurons (DN1s, DN2s and DN3s) in blue. In green are three cells that express TIM but not PER (Pl_{tim}). Glial cells are in pink while photoreceptor nuclei are in yellow (including compound eyes, ocelli and HB eyelet). **B**: projections from the clock gene expressing neurons. The light input pathways from photoreceptor nuclei are shown in the right hemisphere. AMe= accessory medulla.

the dorsal brain in a region known as the dorsal protocerebrum that houses much of the neurosecretory system of the fly. In addition the two DN1s that are already present in larva also project towards the accessory medulla where they may contact the LN_vs.

Not all of the clock neurons are equally important for the control of behavioural rhythms. Photoreceptors cells and glial cells are not implicated in the control of rhythmic behaviour (Ewer et al., 1992; Vosshall and Young, 1995; Zeng et al., 1994). Many studies demonstrated that the most important neurons that control behavioural rhythms are the LNs. *per* expression in the LNs only is sufficient to drive rhythmic locomotor activity while restricted expression in the DNs is not (Frisch et al., 1994). *disconnected* (*disco*) mutants that have no functional LNs but keep the DNs are arrhythmic for locomotor and eclosion rhythms (Dushay et al., 1989). Nevertheless the DNs are involved in the circadian system as residual rhythmic activity is seen in *disco* flies during the first few days in DD (Dushay et al., 1989; Hardin et al., 1992; Helfrich-Forster, 1998).

The LN_vs express the neuropeptide PIGMENT DISPERSING FACTOR (PDF) that is involved in mediating circadian signals to downstream neurons (Helfrich-Forster et al., 2000; Park et al., 2000; Renn et al., 1999). Among its functions, PDF is required to coordinate the phase and amplitude of circadian protein oscillations among the different clock neurons (Lin et al., 2004). Flies carrying the Pdf^0 null mutation show arrhythmic locomotor activity after a few days in DD furthermore underlying the role of the LN_vs (Renn et al., 1999). In addition, flies where the LN_vs are killed by targeted expression of cell death or apoptosis genes are also arrhythmic (Blanchardon et al., 2001; Renn et al., 1999). Nevertheless, Pdf^0 and LN_vs ablated flies show short period rhythm for the first few

days in DD illustrating the involvement of other clock neurons in the control of locomotor rhythm (Blanchardon et al., 2001; Renn et al., 1999). Comparison between Pdf^0 and *disco* mutants implicates the LN_ds in control of behavioural rhythms.

Recent work shows that the LN_ds control the evening peak of activity while the s-LN_vs are responsible for the morning peak in LD conditions and that the s-LN_vs but not the LN_ds are able to sustain locomotor activity rhythm in constant conditions (Grima et al., 2004; Stoleru et al., 2004). This is an important result as it shows that different parts of the neuronal circadian network control different aspects of the circadian program, like in mammals (Jagota et al., 2000; Lee et al., 2003) and suggests an anatomical substrate to the morning (M) and evening (E) oscillator hypothesis (Daan and Berde, 1978; Pittendrigh and Daan, 1976).

1.3.4 The transcription factors CLOCK and CYCLE

The finding that PER and TIM protein and mRNA levels cycle out of phase suggested that PER and TIM were negative regulator of the transcription of the *per* and *tim* genes. However, PER and TIM do not contain DNA binding domains (Huang et al., 1993), making a direct interaction of PER and TIM with DNA difficult to conceive. Nevertheless, PER contains a PAS region, which is known to mediate interactions between basic helix-loop-helix (bHLH) transcription factors (Huang et al., 1993). The promoter region of the *per* gene was examined and revealed the presence of a regulatory elements called an E-box *(CACGTG),* which is known to be a binding site for bHLH transcription factors (Darlington et al., 1998; Hao et al., 1997).

An EMS mutagenesis led to the finding of a new mutant with altered locomotor activity rhythms and low levels of *per* and *tim* transcription (Allada et al., 1998). The gene affected by the mutation maps to chromosome 3 and was identified as a homologue of *mClock* (Vitaterna et al., 1994) and therefore named *dClock* (Allada et al., 1998). The *dClock* gene encodes a 1016 amino acids long bHLH transcription factor that also harbours a PAS protein-protein interaction domain and several polyglutamine repeats which are known transcriptional activators (Allada et al., 1998; Mitchell and Tjian, 1989). The $dClock^{Jrk}$ mutation resulting from the mutagenesis screen replaces a glutamine by a stop codon at amino acid 776, removing part of the transcriptional activation domain (Allada et al., 1998). The mutation is semi-dominant, showing rhythmic locomotor activity with longer periods in $dClock^{Jrk}$ heterozygous as opposed to completely arrhythmic behaviour in homozygotes (Allada et al., 1998). *dClock* shows rhythmic expression at both the mRNA (Bae et al., 1998) and protein levels (Lee et al., 1998). However, unlike *per* and *tim*, there is no delay between maximum levels of accumulation of mRNA and protein, both of them peaking from ZT23 to ZT4 (Bae et al., 1998; Lee et al., 1998). PER and TIM are necessary for rhythmic transcription of *dClock* as per^{0} and tim^{01} flies show constant low levels of *dClock* mRNA (Bae et al., 1998). Like PER and TIM, CLK shows different state of phoshorylation during a circadian cycle (Lee et al., 1998) and this is important in controlling the function of CLK (Kim et al., 2002). Besides being a transcriptional activator, CLK also seems to be involved in mediating light responses as flies with increased expression of the protein show differences in their locomotor activity pattern in response to lights-on in a LD cycle or as a light pulse (Kim et al., 2002).

CYCLE (CYC), a second bHLH-PAS transcription factor was identified through the cyc^0 nonsense mutation (Rutila et al., 1998). The cyc^0 mutation results in a stop codon at amino acid 159 shortening CYC from the wild-type 412 amino acid long protein and eliminating most of the C-terminus of the protein including the PAS B domain (Rutila et al., 1998). The transcription of *per* and *tim* is very low in homozygous cyc^0 flies (Rutila et al., 1998) as are PER and TIM levels (Rutila et al., 1998). Homozygous cyc^0 mutants have arrhythmic locomotor behaviour while heterozygotes show a rhythmic phenotype with long period (Rutila et al., 1998). The *cyc* gene maps to chromosome 3 (Rutila et al., 1998) and is homologous to mammalian *Bmal1* (Hogenesch et al., 1998; Ikeda and Nomura, 1997; Rutila et al., 1998). *cycle* expression is constitutive, with levels of mRNA and protein remaining high and non-cycling (Rutila et al., 1998).

1.3.5 Phosphorylation of PER and TIM

The phosphorylation of PER and TIM plays an important role in controlling the stability and cellular localization of the clock proteins (Akten et al., 2003; Grima et al., 2002; Kloss et al., 1998; Ko et al., 2002; Lin et al., 2002; Martinek et al., 2001; Price et al., 1998), and several genes and their products have been involved in phosphorylation of clock proteins.

Doubletime

The first identified *Drosophila* gene affecting phosphorylation of clock components was *doubletime* (*dbt*) (Kloss et al., 1998; Price et al., 1998). Mutants of *dbt* that have short, long or arrhythmic locomotor activity periods have been isolated

(Kloss et al., 1998; Price et al., 1998; Rothenfluh et al., 2000a; Suri et al., 2000). *dbt* maps to chromosome 3 and encodes a 440 amino acids long protein (DBT) closely related to the human Casein Kinase 1_ε (Kloss et al., 1998). *In situ* hybridisation and immunocytological experiments show that *per*, *tim* and *dbt* are expressed in the same cell types of the adult brain (Kloss et al., 1998; Kloss et al., 2001). Moreover, DBT interacts physically with PER *in vitro* and *in vivo* (Kloss et al., 1998; Kloss et al., 2001; Preuss et al., 2004) and DBT promotes the phosphorylation of PER (Ko et al., 2002; Price et al., 1998). The circadian clock does not control either the transcription of *dbt* or the stability of the DBT protein (Kloss et al., 1998; Kloss et al., 2001). Nevertheless, DBT subcellular localisation is influenced by PER, and DBT, PER and TIM are found as a complex (Kloss et al., 2001). Removing TIM from nuclear PER-TIM-DBT complexes results in enhanced phosphorylation of PER (Kloss et al., 2001). In addition, in per^0 flies, DBT is always nuclear suggesting that when PER is lacking, the default localisation for DBT involves nuclear accumulation (Kloss et al., 2001).

dbt was identified through a screen for circadian rhythm mutants, in which two variants were isolated. Homozygous dbt^S and dbt^L mutations have 18h and 27h locomotor activity and eclosion rhythms respectively (Price et al., 1998). The mutations are semi-dominant as $dbt^S/+$ and $dbt^L/+$ heterozygous have 22h and 25h periods of locomotor activity respectively (Price et al., 1998). The dbt^S and dbt^L mutations affect the phosphorylation status of PER, suggesting a role for DBT in the stability of PER (Price et al., 1998). The transition from hyperphosphorylated to hypophosphorylated PER is faster in dbt^S and slower in dbt^L when compared to wild-type (Price et al., 1998). The dbt^S and dbt^L mutations are due to single amino acid substitutions in a highly conserved domain of the protein (Kloss et al., 1998).

A light pulse phase response curve for dbt^S mutants is very similar to that of per^S flies (Bao et al., 2001; Saunders et al., 1994). Phase changes are larger than those of wild-type flies and the transition period from delays to advances is shorter (Bao et al., 2001). Immunocytochemistry (ICC) reveals that PER accumulates later and declines to lower levels in photoreceptor nuclei of dbt^S flies compared with wild-type or per^S flies while overall PER protein accumulation, assayed by Western blot, is neither reduced nor delayed (Bao et al., 2001). On the contrary, in dbt^S flies, *per* mRNA accumulates later and declines sooner than in wild-type suggesting that DBT can regulate the negative feedback by delaying PER nuclear entry and affecting PER nuclear stability (Bao et al., 2001). Recent work showed that there is no difference of expression of DBT in *wt*, dbt^S or dbt^L mutants. Moreover, DBT, DBTS and DBTL proteins can bind to PER equivalently. The phenotypes observed with these mutants are explained by a reduced enzymatic activity of the DBTS and DBTL proteins (Preuss et al., 2004).

A P-element insertion line, dbt^P, that failed to complement the dbt^S and dbt^L mutations was also identified (Price et al., 1998) and homozygous mutants are adult lethal. Larvae, however, survive until the third instar larval stage allowing the study of PER phosphorylation and cellular localisation in the larval lateral neurons (lLN_vs) (Price et al., 1998). dbt^P homozygotes larvae show constitutively high levels of nuclear PER in clock cells even in the absence of TIM suggesting a role for DBT in the control of the subcellular localisation of PER (Cyran et al., 2005; Price et al., 1998). Besides, the PER proteins appear to be hypophosphorylated (Price et al., 1998). As transcription of the *per* gene is unaffected in dbt^P larvae, increased stability is the likeliest explanation for the high level of PER protein (Price et al., 1998).

The identification of another *dbt* mutation (dbt^{ar}) that gives arrhythmic behaviour in homozygotes and long period in heterozygotes as well as constitutive high levels of PER also supports the view that DBT regulates the stability of PER (Rothenfluh et al., 2000a). The DBT^{AR} protein is expressed at similar levels as wild-type DBT and can bind to PER in a similar way but is defective in phosphorylating PER (Cyran et al., 2005). PER is constitutively nuclear in the photoreceptor cells and s-LN_vs of dbt^{ar} mutants (Cyran et al., 2005; Rothenfluh et al., 2000a). Moreover, PER is nuclear in tim^{01}; dbt^{ar} double-mutants, repeating the results obtained with the dbt^{P} mutation (Cyran et al., 2005). Besides, this TIM-free nuclear PER is able to repress the activity of the CLK-CYC heterodimer (Cyran et al., 2005).

In vitro experiments failed to show that DBT directly phosphorylates PER (Kloss et al., 2001; Preuss et al., 2004; Suri et al., 2000), however two *dbt* mutations that produce long periods of locomotor activity, dbt^{g} and dbt^{h} seriously reduce the kinase activity of the highly similar yeast casein kinase1 HRR25 (Demaggio et al., 1992) on peptide substrates (Suri et al., 2000). Wild-type HRR25 is on the other hand able to phosphorylate PER *in vitro* (Suri et al., 2000). In addition, mammalian $CK1_{\varepsilon}$ phosphorylates *Drosophila* and human PER *in vitro* (Kloss et al., 2001; Toh et al., 2001) and the dbt^{S} and dbt^{L} mutations reduce the kinase activity of *Xenopus laevis* CKIδ on PER *in vitro* (Preuss et al., 2004).

CK2

The serine-threonine kinase casein kinase 2 (CK2) was shown to be able to phosphorylate PER and TIM *in vitro* (Zeng et al., 1996), but more recent work emphasizes the role of CK2 in the circadian clock (Akten et al., 2003; Blau, 2003;

Lin et al., 2002). The CK2 holoenzyme is tetrameric α2:β2 and mutations affecting circadian rhythms have been identified in the α and β subunits. The *Timekeeper (Tik)* mutant was identified through an EMS screen for circadian behavioural defects (Lin et al., 2002). *Tik* affects the gene encoding the catalytic α-subunit of CK2 that maps on chromosome 3 (Lin et al., 2002). CK2α is able to phosphorylate PER and to a lesser extent TIM *in vitro* (Lin et al., 2002) and the $CK2\alpha^{Tik}$ mutation results in a strong reduction of CK2 activity *in vivo* and *in vitro* (Lin et al., 2002). $CK2\alpha^{Tik}$ homozygotes flies do not survive to adulthood but heterozygotes have a 26.4h period of locomotor activity (Lin et al., 2002). In agreement with the behavioural defect, PER and TIM molecular oscillations are altered in $CK2\alpha^{Tik}$ heterozygotes. The levels of the clock proteins are increased in the early subjective day of a DD cycle indicative of a faulty degradation of PER and TIM. An increase presence of hypophosphorylated forms of PER is also observed (Lin et al., 2002). CK2α is not circadianly regulated as levels of the protein are constant throughout a LD cycle (Akten et al., 2003). CK2α is expressed in the adult ventral-lateral neurons where it seems to be constitutively cytoplasmic (Lin et al., 2002). The subcellular localization of PER is strongly affected in $CK2\alpha^{Tik}$ third-instar larval brains as nuclear entry of the clock protein is significantly delayed (Lin et al., 2002).

Andante is a previously described clock mutant that has a lengthened period of locomotor activity and eclosion rhythm (Konopka et al., 1991; Newby et al., 1991). Recent work shows that *Andante* is a mutation in the gene encoding the β regulatory subunit of CK2 that maps on the X chromosome (Akten et al., 2003). The mutation is thought to affect β subunit dimerization and α:β interactions (Akten et al., 2003). CK2β subunits levels are not circadianly controlled (Akten et al.,

2003). CK2β is localized in the adult lateral neurons where, like CK2α, it seems restricted to the cytoplasm (Akten et al., 2003). *Andante* results in reduced β subunit levels and higher levels of PER and TIM than wild-type indicative of a diminished degradation of the clock proteins (Akten et al., 2003). Moreover, the nuclear accumulation of these two clock proteins is delayed in the s-LN_vs of *Andante* flies (Akten et al., 2003). Interestingly, in the l-LN_vs PER and TIM nuclear accumulation is unaffected (Akten et al., 2003).

Altogether, these data implicate CK2 as a major contributor to the *Drosophila* circadian clock system. CK2 possible mode of action is that it phosphorylates PER and TIM in the cytoplasm, thereby promoting nuclear entry of the clock proteins. The higher levels of PER and TIM observed at times when the proteins are nuclear also suggests that cytoplasmic CK2 phosphorylation of PER and TIM somehow influence their nuclear degradation (Akten et al., 2003; Lin et al., 2002).

SHAGGY

TIMELESS also undergoes rhythmic changes in its phosphorylation status (Marrus et al., 1996; Zeng et al., 1996). A tyrosine-linked phosphorylation is implicated in the degradation of TIM by the ubiquitin-proteasome pathway (Naidoo et al., 1999). The X-chromosome located gene *shaggy* (*sgg*) encodes the *Drosophila* orthologue of glycogen synthase kinase-3 (Bourouis et al., 1990; Martinek et al., 2001; Siegfried et al., 1990). The *sgg* gene is essential for development and is involved in the *wingless* signalling pathway where it regulates the subcellular localization of ARMADILLO (Peifer et al., 1994a; Peifer et al., 1994b; Siegfried et al., 1992; Siegfried et al., 1994). Overexpressing *sgg* in clock

cells results in a shortening of the period of locomotor activity period to 21h in adult flies, an increased phosphorylation of TIM *in vivo* and an advanced nuclear entry of PER and TIM in the s-LN_vs of third-instar larval brains (Martinek et al., 2001). In addition, the PRC of flies overexpressing *sgg* differs from wild-type, mostly in the delay portion which is shortened by 4h. This suggests that SGG affects a cytoplasmic event of the molecular cycle (Martinek et al., 2001). On the other hand, hypomorphic mutations of *sgg* have longer period of locomotor activity (25.2 to 26.2h depending on the mutation), reduced TIM phosphorylation and increased levels of PER and TIM (Martinek et al., 2001). *In vitro* studies show that GSK3β can phosphorylate TIM. GSK3β and SGG have a 85% amino acid identity in the kinase domain and GSK3β can rescue the viability of *sgg* mutants (Martinek et al., 2001; Ruel et al., 1993; Siegfried et al., 1992). Altogether these data suggest that SGG phosphorylates TIM promoting nuclear entry of the PER/TIM complex (Martinek et al., 2001).

SLIMB

The 3rd chromosome located *Drosophila* gene *supernumerary limbs* (*slimb*) encodes an F-box/WD40 protein that regulates transcription factor levels in the *wingless* and *hedgehog* signalling pathways (Jiang and Struhl, 1998). SLIMB is a member of the ubiquitin ligase SCF complex that targets phosphorylated proteins for degradation (Margottin et al., 1998; Skowyra et al., 1997; Spencer et al., 1999; Winston et al., 1999). The expression of *slimb* is not circadianly regulated as levels of *slimb* mRNA and SLIMB protein are constant (Grima et al., 2002). However, phoshorylation of SLIMB is observed on western blots which could regulate its affinity towards target proteins (Ko et al., 2002). Overexpressing *slimb* in clock

cells results in 26.7h free-running period of locomotor activity and PER oscillations are delayed accordingly in the lateral neurons (Grima et al., 2002). Constitutive overexpression of *slimb* results in a majority of flies being arrhythmic in DD conditions (Ko et al., 2002). Strong hypomorphic mutants of *slimb* which normally die as larvae can be brought to adulthood by providing the *slimb* gene product throughout development under the control of a heat-shock promoter (Grima et al., 2002). These *slimb* mutants flies are severely affected in their locomotor activity pattern as they are arrhythmic in DD conditions and fail to show the light-off anticipation in LD typically observed in wild-type flies (Grima et al., 2002). In agreement with the behavioural data, PER and TIM molecular oscillations are strongly altered in *slimb* mutants as levels of the clock proteins are almost constant with highly phosphorylated forms present at all times throughout a DD cycle (Grima et al., 2002). In addition, PER oscillations in the lateral neurons are also abolished in adult *slimb* mutants kept in DD conditions (Grima et al., 2002). Interestingly and in contrast to DD, robust PER and TIM oscillations are seen in LD conditions indicating that light-induced and clock-induced degradation of the clock proteins use different mechanisms (Grima et al., 2002). The phosphorylation pattern of PER is nevertheless altered as hyperphosphorylated forms of the protein are present as early as ZT15 in LD (Grima et al., 2002).

SLIMB binds PER and DBT *in vivo* suggesting that these proteins constitute a complex (Grima et al., 2002). Coimmunoprecipitation assays in S2 cells indicate that SLIMB binds preferentially phosphorylated DBT (Ko et al., 2002). In S2 cells, RNAi interference on *slimb* prevents the degradation of hyperphosphorylated PER (Ko et al., 2002). These data suggest that SLIMB targets phosphorylated PER and

maybe also phosphorylated TIM for degradation by the ubiquitin-proteasome pathway (Grima et al., 2002; Ko et al., 2002).

PP2A

Recent work illustrates the involvement of the serine-threonine phosphatase, protein phosphatase 2A (PP2A) in the circadian clock (Sathyanarayanan et al., 2004). PP2A is a heterotrimeric enzyme composed of a highly conserved catalytic subunit (C), a variable regulatory subunit (B) and a structural subunit (A). The regulatory B subunit targets the phosphatase to distinct substrates and intracellular locations (Janssens and Goris, 2001; Virshup, 2000). *In vitro* assays show that PP2A dephosphorylates PER and experiments carried out in S2 cells demonstrate that PP2A stabilizes PER (Sathyanarayanan et al., 2004). The regulatory B subunits encoded by the *twins* (*tws*) and *widerborst* (*wdb*) genes target PP2A to PER (Sathyanarayanan et al., 2004). *tws* and *wdb* mRNA cycle in wild-type flies under control of the circadian clock (Sathyanarayanan et al., 2004). *tws* mRNA level is maximum at ZT12 and minimum at ZT0 while *wdb* oscillation is antiphase (maximum at ZT0 and minimum at ZT12) (Sathyanarayanan et al., 2004). In S2 cells, TWS is a cytoplasmic protein while WDB has a predominantly nuclear subcellular localization (Sathyanarayanan et al., 2004). Besides, TWS is expressed in third-instar larval lateral neurons (Sathyanarayanan et al., 2004).

Overexpression of *wdb* in clock cells lengthens the period of locomotor activity and this is associated with a dampened oscillation of PER showing higher levels and increased presence of hypophosphorylated forms (Sathyanarayanan et al., 2004). Surprisingly, in the larval lateral neurons, PER nuclear accumulation is advanced by *wdb* overexpression (Sathyanarayanan et al., 2004). The regulation of

tws levels is critical for the maintenance of behavioural rhythm as overexpression of *tws* in adult lateral neurons results in short period of locomotor activity that degenerates into arrhythmic behaviour after a few days in DD conditions (Sathyanarayanan et al., 2004). A strong hypomorphic mutation of *tws* that is lethal in early pupal stage when homozygotes shows reduced PER levels and delayed nuclear accumulation of the protein in third-instar larval lateral neurons (Sathyanarayanan et al., 2004).

Overexpression in adult lateral neurons of the gene *mutagenic star* (*mts*) that encodes the catalytic subunit (C) of PP2A gives short and weak to arrhythmic behaviour (Sathyanarayanan et al., 2004). In addition, PER oscillations are abolished and the protein subcellular localization is always nuclear in third-instar larval lateral neurons (Sathyanarayanan et al., 2004). Overexpressing *mts* also results in an increase of hypophosphorylated forms and a decrease of hyperphosphorylated forms of PER.

Altogether, these data implicate PP2A as an important player of the *Drosophila* circadian system. Its function involves the stabilisation of the PER protein by dephosphorylation. As a result, PP2A influences nuclear accumulation of PER as well as overall level of this protein. PP2A seems to act in opposite direction as the previously described kinases DBT, CK2 and SGG.

1.3.6 The first feedback loop

The functioning of circadian clocks is based upon interlocked negative feedback loops. In *Drosophila*, two interconnected regulatory feedbacks are known thus far (Glossop et al., 1999). The first negative feedback loop of *Drosophila* is

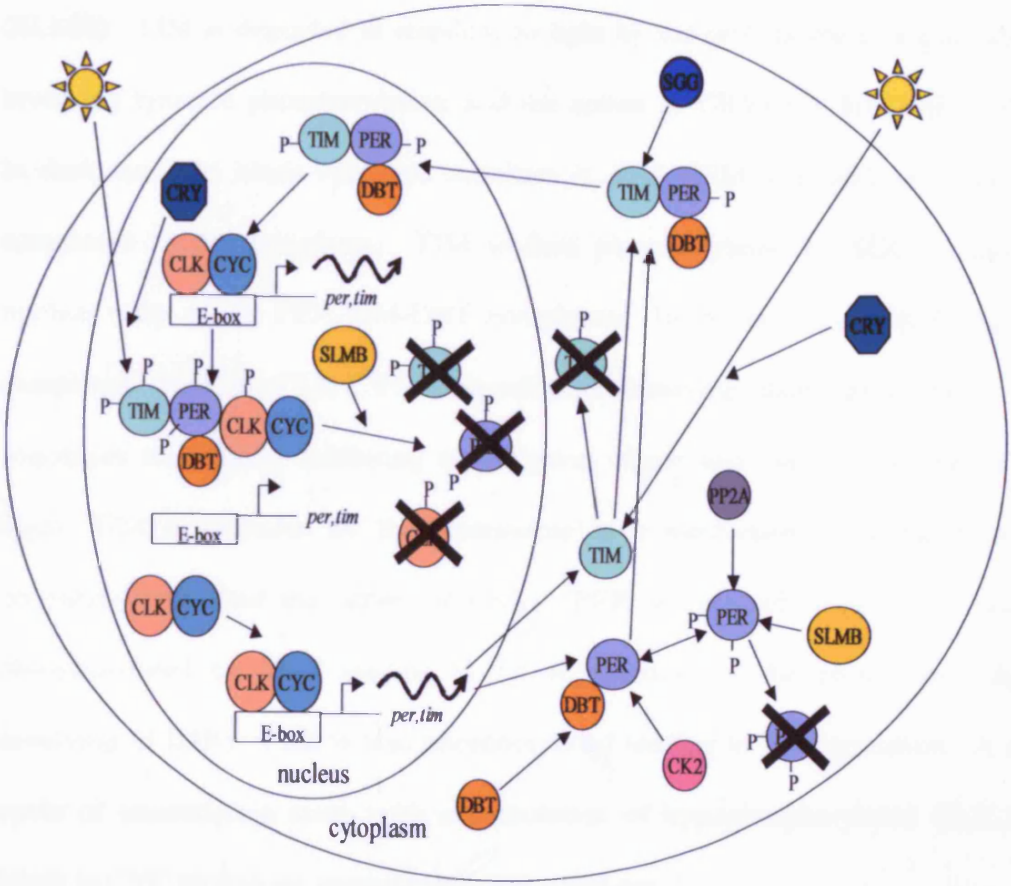

SGG
TIM
PER
P
DBT
CRY
CLK
CYC
E-box
per,tim
SLMB
PP2A
CK2
nucleus
cytoplasm

Figure 1.4: The first feedback loop of *Drosophila*. Adapted from Hardin 2005. CLK and CYC heterodimers bind to E-box sequences in the promoter of the *per* and *tim* genes activating their transcription. In the cytoplasm, PER is subjected to DBT and CK2 phosphorylation, which destabilises it and leads to degradation of phosphorylated PER by the proteasome. This degradation involves SLIMB (SLMB). TIM is degraded in response to light by the proteasome in a mechanism involving tyrosine phosphorylation and the action of CRYPTOCHROME (CRY). In darkness TIM binds PER and stabilises it. PER, TIM and DBT are found as complexes in the cytoplasm. TIM is then phosphorylated by SGG promoting nuclear entry of the PER-TIM-DBT complexes. In the nucleus, PER-TIM-DBT complexes bind to CLK-CYC heterodimers removing them from the E-box sequences and thereby inhibiting transcription of *per* and *tim*. At the end of the night, TIM is degraded by the proteasome in a mechanism involving tyrosine phosphorylation and the action of CRY. PER is then left unprotected and is phosphorylated by DBT leading to its degradation by the proteasome (again involving SLIMB). CLK is also phosphorylated leading to its degradation. A new cycle of transcription starts with accumulation of hypophosphorylated CLK that binds to CYC to activate transcription of *per* and *tim*.

illustrated in Figure 1.4. The key components of the first loop are PER, TIM, CLK and CYC (Allada et al., 1998; Konopka and Benzer, 1971; Rutila et al., 1998; Sehgal et al., 1994). *per* and *tim* are rhythmically transcribed with a 24h periodicity (Hardin et al., 1990; Sehgal et al., 1995). The fact that *per* and *tim* mRNA levels do not oscillate in per^0 and tim^0 led to the model that PER and TIM are repressors of their own transcription (Hardin et al., 1990; Sehgal et al., 1995). In order to repress transcription, a crucial step consists in the ability of the *per* and *tim* gene products to enter the nucleus (Curtin et al., 1995). It is known that PER and TIM can bind to each other forming heterodimers (Gekakis et al., 1995; Saez and Young, 1996; Zeng et al., 1996). Formation of PER-TIM complexes were long thought to be an essential step that regulates the nuclear translocation of these proteins (Saez and Young, 1996). That is because PER is restricted in the cytoplasm in tim^0 flies (Vosshall et al., 1994) and reciprocally TIM is cytoplasmic in per^0 mutants (HunterEnsor et al., 1996).

The transcription of *per* and *tim* is driven by the two bHLH-PAS transcription factors dCLK and CYC (Allada et al., 1998; Rutila et al., 1998). CLK and CYC bind each other to form heterodimers (Darlington et al., 1998; Lee et al., 1999). The CLK/CYC complex bind E-box sequences in the *per* and *tim* promoters to activate transcription (Darlington et al., 1998; Hao et al., 1997; Hao et al., 1999). The *per* and *tim* mRNAs are then transported to the cytoplasm and translated. Nevertheless, there is a 6h delay between maximal accumulation of mRNA and protein (Edery et al., 1994; Hardin et al., 1990; HunterEnsor et al., 1996; Sehgal et al., 1995). This delay is thought to be generated mainly by the action of several kinases and phosphatase (Akten et al., 2003; Kloss et al., 1998; Lin et al., 2002; Martinek et al., 2001; Price et al., 1998; Sathyanarayanan et al., 2004). DBT

promotes PER phosphorylation (Ko et al., 2002; Price et al., 1998) which leads to PER degradation by the ubiquitin proteasome pathway (Ko et al., 2002). CK2 is also involved in PER phosphorylation which influences PER stability as well as PER subcellular localization (Akten et al., 2003; Lin et al., 2002). PP2A appears to have opposite effects as DBT and CK2, dephosphorylating PER and by doing so increasing its stability as well as affecting its nuclear entry (Sathyanarayanan et al., 2004). Finally, SGG phosphorylates TIM in the cytoplasm promoting nuclear entry of the PER/TIM complex (Martinek et al., 2001).

Once in the nucleus PER-TIM complexes directly repress the transcription of their own genes by physical interaction with the CLK/CYC activator dimer (Darlington et al., 1998; Lee et al., 1999; Lee et al., 1998). PER is essential and sufficient for the repression (Cyran et al., 2005). It has been shown that when TIM is removed from the nucleus, at the end of the night, PER is still able to repress CLK-CYC activation. Furthermore, the repression is higher when PER is on its own (Rothenfluh et al., 2000b), whereas TIM on its own cannot repress *per* and *tim* transcription (Ashmore et al., 2003). The region of PER responsible for the repression of CLK/CYC activation has been identified and named CCID (clock-cycle inhibition domain) (Chang and Reppert, 2003) and is located in the C-terminus of the PER protein between residues 764 and 1034 (Chang and Reppert, 2003). At the end of the night/beginning of the day TIM is actively degraded upon light exposure (HunterEnsor et al., 1996; Myers et al., 1996; Zeng et al., 1996). The disappearance of TIM allows DBT to phosphorylate PER leading to its degradation and finally resulting in de-repression of CLK/CYC mediated transcription. This start a new molecular cycle with *per* and *tim* mRNA building up again.

The obligate binding of PER and TIM in order to gain access to the nucleus has been challenged by recent work (Ashmore et al., 2003; Chang and Reppert, 2003; Cyran et al., 2005; Nawathean and Rosbash, 2004; Shafer et al., 2004; Shafer et al., 2002; Weber and Kay, 2003). Firstly, PER is clearly nuclear before TIM in the adult lateral neurons (Shafer et al., 2004; Shafer et al., 2002). Furthermore, in some S2 cell studies, transfected PER represses CLK/CYC activity without cotransfected TIM (Chang and Reppert, 2003; Nawathean and Rosbash, 2004; Weber and Kay, 2003). Even more significant is the discovery that in very strong hypomorphic or null mutations of *dbt* PER is constitutively localized in the nucleus of the lateral neurons in the absence of TIM (Cyran et al., 2005). Finally, TIM was shown to be able to shuttle between cytoplasm and nucleus without PER. TIM sequence harbours six potential nuclear export signals which are recognised by CRM1-exportin (Ashmore et al., 2003). These findings indicate that PER nuclear accumulation may not simply depend on heterodimerization with TIM.

1.3.7 The second feedback loop

Another feedback loop, interconnected to the PER/TIM one has been identified in *Drosophila*. This loop controls the cyclic expression of the *Clk* gene (Allada et al., 1998; Bae et al., 1998; Blau and Young, 1999; Cyran et al., 2003; Glossop et al., 2003; Glossop et al., 1999; Lee et al., 1998). The *vrille* (*vri*) gene encodes a basic zipper (bZip) transcription factor that has a role in the *decapentaplegic* signalling pathway during development (George and Terracol, 1997). *vri* mRNA levels oscillate in LD and DD conditions with maximum and minimum being reached at ZT(CT)14 and ZT(CT)2 respectively (Blau and Young,

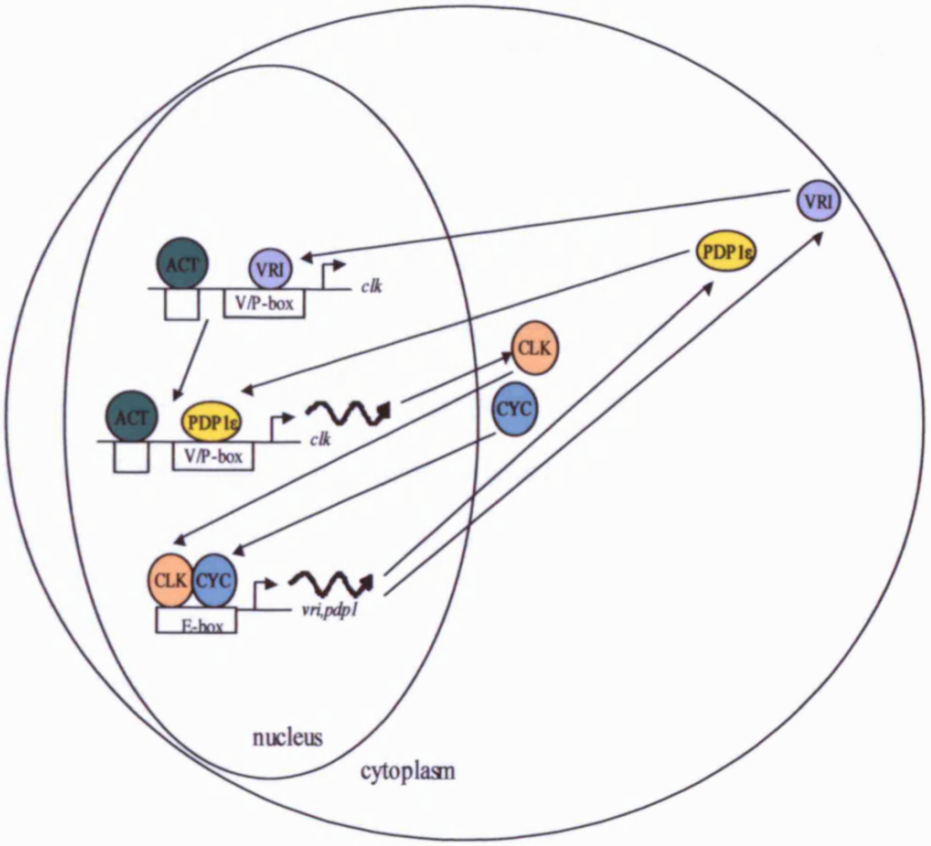


Figure 1.5: The second feedback loop of *Drosophila*. Adapted from Hardin 2005. CLK/CYC heterodimers bind to E-box sequences in the promoter of the *vri* and *pdp1* genes activating their transcription. VRI accumulates earlier tan PDP1ε and bind to V/P boxes in the *Clk* promoter inhibiting its transcription. When PDP1ε accumulates, it out competes VRI for the binding of the V/P boxes and activates transcription of *Clk*. The fact that levels of *Clk* mRNA are constitutively high in the absence of CLK or CYC suggests the existence of a clock independent activator of *Clk* transcription in the absence of VRI.

1999). Oscillations of *vri* mRNA are abolished in per^0 mutant flies indicating that *vri* is a clock-controlled gene (Blau and Young, 1999). Transcription of *vri* is activated by CLK/CYC heterodimers via E box sequences in its promoter (Blau and Young, 1999). *In situ* hybridisation and immunocytochemistry reveals that *vri* is expressed in the lateral neurons (Blau and Young, 1999). VRILLE (VRI) protein levels peak in the early night and reach trough levels during the early day (Cyran et al., 2003; Glossop et al., 2003). VRI also appears to be phosphorylated which might be important for its function (Cyran et al., 2003; Glossop et al., 2003). Because *vri* null mutations are homozygous lethal (George and Terracol, 1997) it is impossible to monitor activity rhythm of flies depleted of VRI. However heterozygous null mutations of *vri* shortens the period of locomotor activity by 0.4 to 0.8h indicating a role for *vri* in the control of 24h rhythmic behaviour (Blau and Young, 1999). Overexpressing *vri* in clock cells results in period lengthening or arrhythmicity and low levels of PER and TIM that do not oscillate in larval lateral neurons (Blau and Young, 1999). Interestingly, PDF levels are also very low in *vri* overexpressing flies but *Pdf* mRNA levels are unaffected (Blau and Young, 1999). More importantly, *Clk* mRNA levels are affected by *vri* expression, being reduced or increased by upregulation or downregulation of *vri* respectively (Cyran et al., 2003; Glossop et al., 2003). This result, in addition tothe finding that VRI binds E4BP4 consensus sequences in the *Clk* gene promoter *in vitro,* suggests that VRI is a repressor of *Clk* transcription (Glossop et al., 2003). Besides, VRI also binds similar sequences in the *cry* promoter and overexpression of *vri* reduces *cry* mRNA cycling indicating that VRI is a repressor of *cry* transcription (Glossop et al., 2003).

The gene *PAR domain protein 1* (*Pdp1*) encodes a basic zipper transcription factor that has a DNA binding domain very similar to that of VRI (Cyran et al.,

2003). *Pdp1* is expressed cyclically in fly heads and its transcription is controlled by CLK/CYC heterodimers via E box sequences in its promoter (Cyran et al., 2003; McDonald and Rosbach, 2001; Ueda et al., 2002). Out of the six possible *Pdp1* isoforms, only *Pdp1ε* mRNA levels oscillate in fly heads with maximum reached at ZT18 and minimum at ZT3-6 (Cyran et al., 2003). PDP1ε protein levels cycle with a phase reflecting its mRNA oscillation (Cyran et al., 2003), and is expressed in larval lateral neurons where it is a nuclear protein (Cyran et al., 2003). Downregulation of *Pdp1ε* lengthens the period of locomotor activity by 0.5h and reduces *Clk* mRNA levels (Cyran et al., 2003). These effects are opposite to the ones triggered by *vri* downregulation suggesting that *Pdp1ε* is an activator of *Clk* transcription (Cyran et al., 2003; Glossop et al., 2003). In addition, molecular oscillations of *per* and *tim* expression are abolished in larval brains of homozygous null mutation of *Pdp1* (Cyran et al., 2003).

Mechanistically, VRI and PDP1 compete for the same binding site in the *Clk* promoter and regulate its expression (Cyran et al., 2003). The delay between maximum levels of VRI and PDP1 explains the accumulation profile of *Clk* mRNA i.e. repression during the early night, followed by activation later at night (Cyran et al., 2003). The second feedback loop is illustrated in Figure 1.5.

1.3.8 Entrainment of the circadian clock

To be efficient, the molecular loops underlying circadian rhythm need to be synchronised to the external world. Although several *Zeitgebers* can be used by the circadian system, the most important is light. In *Drosophila*, the presence of the eyes is not necessary for entrainment of circadian rhythms (Wheeler et al., 1993;

Yang et al., 1998). However, flies with genetically ablated eyes or mutations in the visual phototransduction pathway are less sensitive to light mediated entrainment of circadian rhythms indicating that the eyes are involved in this mechanism (Helfrich-Forster et al., 2001; Rieger et al., 2003; Stanewsky et al., 1998). There are at least 6 different pathways involved in circadian photoreception: the compound eyes, the ocelli, a structure underneath the retina called the Hofbauer-Buchner (HB) eyelet, unknown photoreceptors in DN1s and DN3s and CRYPTOCHROME (CRY) (Helfrich-Forster et al., 2001; Rieger et al., 2003).

The *Drosophila* gene *cryptochrome* (*cry*) encodes a protein of 542 amino acids that has homology with 6-4 photolyases (Emery et al., 1998). Photolyases are enzymes that directly repair lethal and carcinogenic UV-light induced DNA lesions in the genome of many organisms, in a reaction dependent on near UV to blue light (350-450 nm)(Carell et al., 2001). CRYPTOCHROMES are found in a variety of organisms including plants and mammals (Sancar, 2000). However, their functions are markedly different as *Arabidopsis* CRYs act only as photoreceptors when mammalian CRYs are core component of the negative feedback loop (Sancar, 2000). CRYPTOCHROMES from different organisms and PHOTOLYASES are highly conserved especially in the N-terminal portion of the proteins (Emery et al., 1998), they bind two chromophores, the first one is a pterin or deazaflavin, and the second one is Flavin Adenin Dinucleotide (FAD). Although PHOTOLYASES can bind DNA, CRYs cannot, despite the fact that the putative DNA binding domains are well conserved between CRYs and PHOTOLYASES (Emery et al., 1998). The most important difference between CRYs and PHOTOLYASES is that CRYs have extended C-termini, which are highly different between species (Green, 2004). Figure 1.6 illustrates the similarities and differences between CRYs and

PHOTOLYASES. The extremely different nature of CRYs C-termini might be of special relevance to the markedly different way in which CRYs from different species operate. Evidences supporting this come from *Arabidospis*, as transgenic plants expressing the C-terminal domain (CCT) of *Arabidopsis* CRYs mediate a constitutive light response indicative of a functional role of the C-terminal extensions of *Arabidopsis* CRY1 and CRY2 (Yang et al., 2000).

The *Drosophila cry* gene has been identified through the cry^b mutation, which results in an Asp to Asn substitution at position 410, a highly conserved residue between *Drosophila* CRY and other related proteins (Stanewsky et al., 1998). It is believed that this amino acid is located in an important domain for the binding of FAD. In cry^b flies, there is no oscillation of *per* and *tim* products in peripheral clocks such as the eyes (Stanewsky et al., 1998). Overexpression of *cry* in all clock cells or just in the LN_vs results in hypersensitive responses to light (Emery et al., 1998; Emery et al., 2000b) while cry^b mutant flies display aberrant responses to photic stimuli (Emery et al., 2000a; Stanewsky et al., 1998). cry^b flies are unable to phase-shift their locomotor activity when short pulses of light are given at night while wild-type do (Stanewsky et al., 1998). In agreement with this, TIM is not degraded in response to light in S2 cells, larval lateral neurons and adult Malpighian tubules of cry^b flies (Ivanchenko et al., 2001; Lin et al., 2001). Furthermore, cry^b flies show rhythmic locomotor activity in constant light, a condition that normally triggers arrhythmicity in wild-type (Emery et al., 2000a). Targeted overexpression of *cry* in the LN_vs of cry^b flies partially restores arrhythmic behaviour under constant light and responses to pulses of light given at night (Emery et al., 2000b). Nevertheless, cry^b flies still entrain to changes in their LD regimes and still show rhythmic expression of PER and TIM in the LNs and DN1s

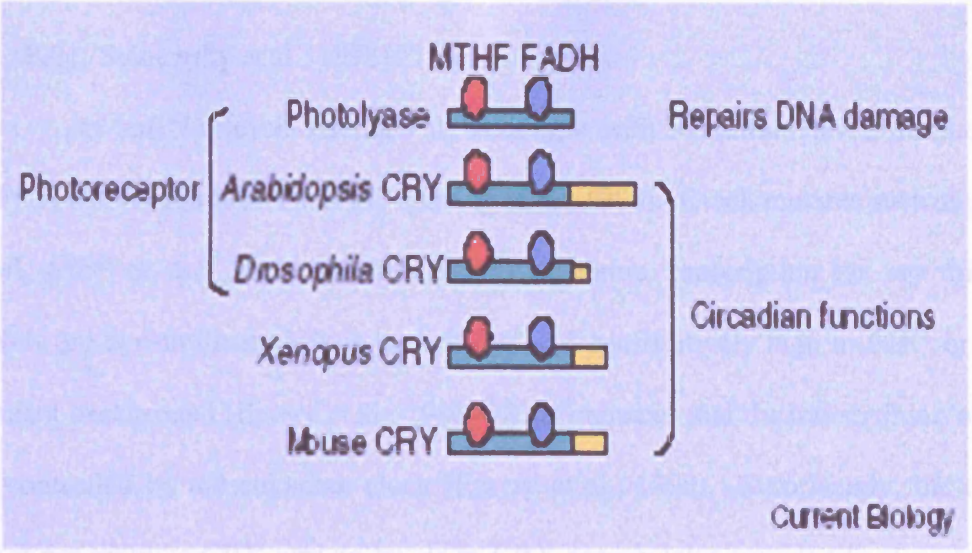


Figure 1.6: Structure of CRYs from different species. Taken from Green, 2004. Cryptochromes from all organisms contain a 'core' domain (green) with high sequence similarity to DNA photolyases. This domain contains binding sites for two cofactors, a flavin (FADH, blue) and a pterin (MTHF, red). Most residues known to have critical roles in photolyase function are also conserved in cryptochromes. Unlike photolyases, cryptochromes harbour carboxy-terminal 'tails' of varying length and sequence composition (yellow). *Arabidopsis* and *Drosophila* CRYs, like photolyases, are blue-light photoreceptors, while direct evidence for photoreceptor function in vertebrate CRYs is still lacking.

which are highly important for the control of locomotor activity (Helfrich-Forster et al., 2001; Stanewsky et al., 1998).

cry mRNA levels oscillate in wild-type with maximum levels reached at ZT1-5, and trough levels at ZT17 (Emery et al., 1998). Clock mutants such as per^0, tim^0, Clk^{jrk} or cyc^0 show abolition of *cry* rhythmic transcription i.e. *cry* mRNA levels are constitutively low in per^0 or tim^0 and constitutively high in Clk^{jrk} or cyc^0 mutant background (Emery et al., 1998). This indicates that the transcription of *cry* is controlled by the circadian clock (Emery et al., 1998). Surprisingly, the clock does not regulate CRY protein levels as CRY accumulates continually at night and is degraded by the proteasome pathway upon light exposure (Emery et al., 1998; Lin et al., 2001). CRY mode of action is believed to involve electron transfer between several tryptophan residues (Froy et al., 2002). Interestingly, blocking electron transport from the flavin reduces CRY degradation in S2 cells (Lin et al., 2001).

CRY is expressed in larval and adult clock neurons (Emery et al., 2000b; Klarsfeld et al., 2004). In larvae, CRY is found in the lateral neurons and in the only two DN1s present at this stage, but not in the two DN2s (Emery et al., 2000b; Klarsfeld et al., 2004). Interestingly, PER oscillations in the larval DN2s are in antiphase compared with the other larval clock cells (Kaneko et al., 1997). Forced expression of CRY in larval DN2 brings PER oscillations in phase with other clock neurons (Klarsfeld et al., 2004). In adults, CRY is expressed in the two clusters of LNvs, in the LNds, in most of the DN1s, and weakly in the two DN2s and in 25% of the DN3s (Klarsfeld et al., 2004).

The clock protein TIM is rapidly degraded by light (HunterEnsor et al., 1996; Myers et al., 1996; Zeng et al., 1996). It was hypothesized that the degradation of this protein by the proteasome is the first step of the response to light

by the circadian clock. In agreement with a role as a circadian photoreceptor CRY interacts with TIM (Ceriani et al., 1999) and PER (Rosato et al., 2001) in a light dependent manner in a yeast two hybrid assay and in cell culture system (S2 cells). Removal of 20 amino acids (residues 522-541) at the C-terminus to create CRYΔ abolishes the light dependence of these interactions (Rosato et al., 2001). This result, in addition to the studies on *Arabidopsis* CRYs, points to an important role of the *Drosophila* CRY C-terminus for the regulation of the protein function. Analysis of the C-terminus sequence of CRY that is deleted in CRYΔ reveals a PKA phosphorylation site, a CK2 phosphorylation site and a PDZ motif involved in protein-protein interactions (Figure 1.7).

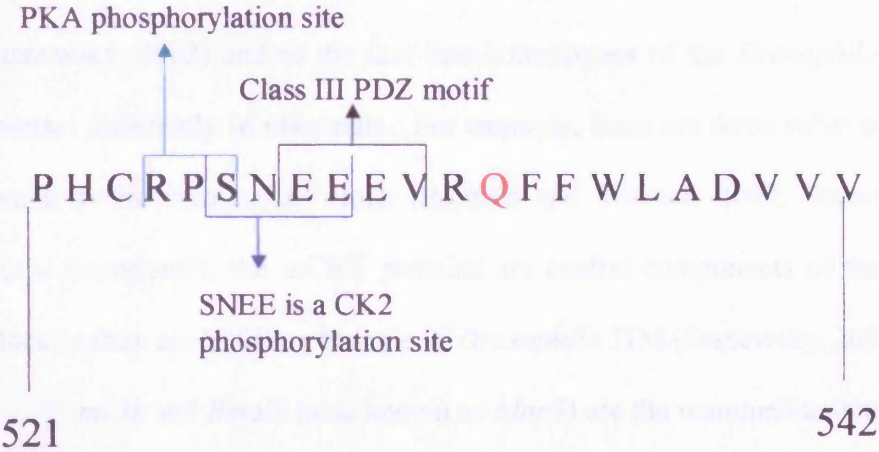


Figure 1.7: Sequence analysis of the *Drosophila* CRY C-terminus. The 20 amino acids that are deleted in CRYΔ (residues 522-541) harbour two phosphorylation sites, one for PKA and one for CK2 as well as a class III PDZ motif involved in protein-protein interactions.

1.4 The mammalian clock

The central pacemaker of the mammalian circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Weaver, 1998). The mammalian circadian clock is similar to the *Drosophila* system in many aspects. The basic molecular mechanism is the same, namely an interlocked transcription/translation feedback loop. Moreover, most of the *Drosophila* clock genes have been identified in mammals (Reppert and Weaver, 2000; Stanewsky, 2003). However, the mammalian clock is more complex, this is due in part to genome duplication events (Clayton et al., 2001) which resulted in two or more copies of the core components of the *Drosophila* clock (Reppert and Weaver, 2000; Stanewsky, 2003) and to the fact that homologues of the *Drosophila* components interact differently in mammals. For example, there are three *mPer* and two *mCry* genes in the mammalian clock (Reppert and Weaver, 2000; Stanewsky, 2003). More importantly, the mCRY proteins are central components of the mammalian clock as they are fulfilling the role of *Drosophila* TIM (Stanewsky, 2003).

mClk and *Bmal1* (also known as *Mop3*) are the mammalian homologs of *Clk* and *cyc* and have similar functions. mCLK and BMAL1 form heterodimers that initiate transcription of the *mPer* and *mCry* genes in a rhythmic manner through binding E-box elements in their promoters (Gekakis et al., 1998; Reppert and Weaver, 2002; Yamaguchi et al., 2000). *Bmal1* deficient mice are arrhythmic (Bunger et al., 2000), whilst *mClk* deficient mice exhibit long period rhythm that becomes arrhythmic after a few days in DD (Jin et al., 1999). In contrast to *Drosophila*, BMAL1 cycles in abundance whilst mCLK is constitutively expressed (Maywood et al., 2003; Shearman et al., 2000b).

The *mCry* and *mPer* genes are rhythmically expressed in the SCN with a phase that is opposite to that of *Bmal1* (Kume et al., 1999; Miyamoto and Sancar, 1999). Mice with knockout mutations in both *mCry* genes simultaneously are behaviourally arrhythmic while single knockout have longer or shorter than 24h period in DD. Null mutations in *mPer2* cause arrhythmia, whilst *mPer1* deficient mice display circadian rhythm with a shorter period of 23h (Zheng et al., 2001). Interestingly, *mPer3* deficient mice show only a marginal effect in period indicating that mPER3 is not necessary for circadian rhythms in mice (Shearman et al., 2000a). Moreover, *mPer1/ mPer2* deficient mice are arrhythmic showing that mPER3 is unable to sustain circadian rhythms independently (Zheng et al., 2001).

mCRY1 and mCRY2 proteins levels are maximum at times of maximal inhibition of mCLK-BMAL1 mediated transcription suggesting feedback of the mCRY proteins on the transcription of their genes (Kume et al., 1999). Moreover, each of the mCRY alone is able to inhibit mCLK-BMAL1 induced transcription *in vitro*, underlying the role of mCRY proteins as negative regulators in the mammalian clock (Kume et al., 1999; Shearman et al., 2000b). However, both mCRY proteins need to enter the nucleus to inhibit transcription. mCRY1 and mCRY2 are found in complexes with mPER1 and mPER2 in nucleus (Lee et al., 2001) and the formation of these complexes is essential for nuclear localization of mCRY and mPER proteins (Kume et al., 1999; Lee et al., 2001). In addition, mCRY proteins stabilize mPER2 (Lee et al., 2001; Shearman et al., 2000b; Yagita et al., 2000)

The mammalian homologue to *Drosophila* DBT, casein kinase Iε (CKIε) and the mammalian casein kinase Iδ (CKIδ) are able to bind and phosphorylate the mPER proteins (Akashi et al., 2002; Keesler et al., 2000; Lowrey et al., 2000;

Vielhaber et al., 2000). This phosphorylation destabilizes the mPER proteins leading to their degradation by the ubiquitin-proteasome pathway (Akashi et al., 2002).

Recent work involves the mammalian homologue of *Drosophila* SGG, glycogen synthase kinase-3 beta (GSK-3beta) in the circadian clock (Iitaka et al., 2005). GSK-3beta binds mPER2 and phoshorylates it promoting nuclear translocation of mPER2 (Iitaka et al., 2005).

As in *Drosophila*, a second loop exists in mammals, controlling the rhythmic transcription of *Bmal1*. This loop involves two members of the retinoic orphan nuclear receptor family, REV-ERBα, which causes a rhythmic inhibition of *Bmal1* transcription (Preitner et al., 2002) and RORa that is a positive regulator of *Bmal1* transcription (Sato et al., 2004).

It was revealed that *mTim* showed higher homology with a more recently identified second *Drosophila tim* gene called *timeout* (*tim2*) that is not known to be involved in controlling circadian rhythmicity (Benna et al., 2000). A knockdown of rat mTIM decreases levels of mPER1, mPER2 and mPER3, and increases the levels of mCRY1 and mCRY2 (Barnes et al., 2003). Coimmunoprecipitation experiments showed that mTIM is able to interact with all three mPERs (Barnes et al., 2003) and with the two mCRYs (Field et al., 2000; Griffin et al., 1999; Kume et al., 1999). Therefore it appears that mTIM is required for normal mammalian circadian rhythmicity (Barnes et al., 2003). More recent work identified mTIM as an important factor linking human circadian and cell cycles (Unsal-Kacmaz et al., 2005).

1.5 Aims of the project

CRY has been identified as the main circadian photoreceptor in *Drosophila* (Emery et al., 1998; Stanewsky et al., 1998). It was shown that CRY binds PER and TIM in a light dependent manner (Ceriani et al., 1999; Rosato et al., 2001). Previous work carried in the laboratory showed that the removal of 20 amino-acids at the C-terminus of CRY resulted in the loss of light dependency of CRY interactions with PER and TIM in yeast two-hybrid assays (Rosato et al., 2001). The truncated protein (named CRYΔ) therefore seemed to be constitutively active. The aim of my study was to assess the role of the CRY C-terminus *in vivo*. To do so, CRY and CRYΔ targetted overexpression in clock neurons was achieved by means of the GAL4/ *UAS* system (Brand and Perrimon, 1993). On the basis of previous work (Rosato et al., 2001), it was predicted that CRYΔ (but not CRY) would behave as a constitutively active form of the molecule and show a dominant effect upon the endogenous protein, eliciting a constitutive light response in *Drosophila*. In order to investigate whether or not this hypothesis is correct, I used two previously known properties of the *Drosophila* clock. Firstly, flies exposed to constant light are either arrhythmic or show long period of locomotor behaviour, depending on the intensity of the stimulus (Konopka et al., 1989). Moreover, constant light also reduces the amplitude of PER and TIM molecular cycling and attenuates their apparent phosphorylation status (Marrus et al., 1996).

The role of CRY in central and peripheral clocks (such as the eyes and antennae) seems different as CRY looks to be a core component of the peripheral oscillator while only being a photoreceptor in the central clock controlling locomotor behaviour (Ivanchenko et al., 2001; Krishnan et al., 2001; Stanewsky et

al., 1998). Previous studies showed that overexpression of *cry* in clock neurons only results in hypersensitive responses to light (Emery et al., 1998; Emery et al., 2000b). I wanted to repeat these experiments and in an attempt to identify new functions for CRY *in vivo* I also checked whether other changes in locomotor behaviour and molecular oscillations were triggered by CRY overexpression. The overexpression of CRY and CRYΔ was performed in a wild-type cry^{+} background and also in the strong hypomorphic cry^{b} context (Stanewsky et al., 1998).

Chapter 2: Materials and methods

2.1 Fly stocks maintenance

Fly stocks are kept in glass vials on standard yeast/sucrose/agar medium (46.5g yeast, 46.5g sucrose, 15g agar, 2.25g nipagin/1L). Flies are reared at 25°C or 18°C under a regime of alternating conditions of 12h of light and 12h darkness (12:12 LD) in temperature and light controlled rooms. Light is on between 8.00 and 20.00 (winter time) or between 9.00 and 21.00 (British summer time). For this work the following *Drosophila melanogaster* strains are used.

- w^{1118}: this is the standard strain used for the production of transgenics flies. This stock carries a mutation in the *white* gene, giving white coloured eyes. This strain is generally used as a wild-type control in the different experiments.

- cry^{b} (Stanewsky et al., 1998): carries a strong hypomorphic mutation in the *cry* gene.

- *yw; tim-GAL4* (Emery et al., 1998): carries a P-element inserted on chromosome 2 in which sequences from the *timeless* promoter drive the expression of the yeast protein GAL4.

- *yw; Pdf-GAL4* (Renn et al., 1999): carries a P element inserted on chromosome 2 in which sequences from the *Pigment dispersing factor (Pdf)* promoter drive the expression of the yeast protein GAL4.

- *w;; gal1118* (Blanchardon et al., 2001): an enhancer trap line that drives expression of GAL4 in the lateral neurons of the brain.

- *yw; UAS-cry24B* (Emery et al., 1998): carries a P element inserted on chromosome 2 in which yeast *UAS* sequences that are activated by GAL4 drive the expression of the *cry* gene.

- *w;; UAS-HAcry16.1*: carries a P element inserted on chromosome 3 in which yeast *UAS* sequences that are activated by GAL4 drive the expression of a N-terminal fusion protein between an hemagglutinin (HA) tag and CRY.

- *w, UAS-cryΔ14.6*: carries a P element inserted on the X chromosome in which yeast *UAS* sequences that are activated by GAL4 drive the expression of the CRYΔ protein.

- *w;; UAS-cryΔ4.1*: carries a P element inserted on chromosome 3 in which yeast *UAS* sequences that are activated by GAL4 drive the expression of the CRYΔ protein.

- *w, UAS-HAcryΔ15.3*: carries a P element inserted on the X chromosome in which yeast *UAS* sequences that are activated by GAL4 drive the expression of a N-terminal fusion protein between an HA tag and the CRYΔ protein.

- *FM7; Cyo/Sco*: chromosome balancer lines carrying useful visible markers. This line is used in crosses schemes.

- *yw;; TM38/dbtP*: this line is used in crosses scheme as a source of the useful TM38 chromosome balancer.

The transgenic lines that I have used were either obtained from other groups or made before I started my studies in the laboratory by Ezio Rosato, Veryan Codd, Karen Garner and Robert Fedic. Their chromosomal location is known however their exact site of insertion has not been mapped. Figure 2.1 shows a schematic diagram of the three constructs that were made, cloned in the pUAST vector (Brand and Perrimon, 1993) (appendix 1) and injected in the laboratory.

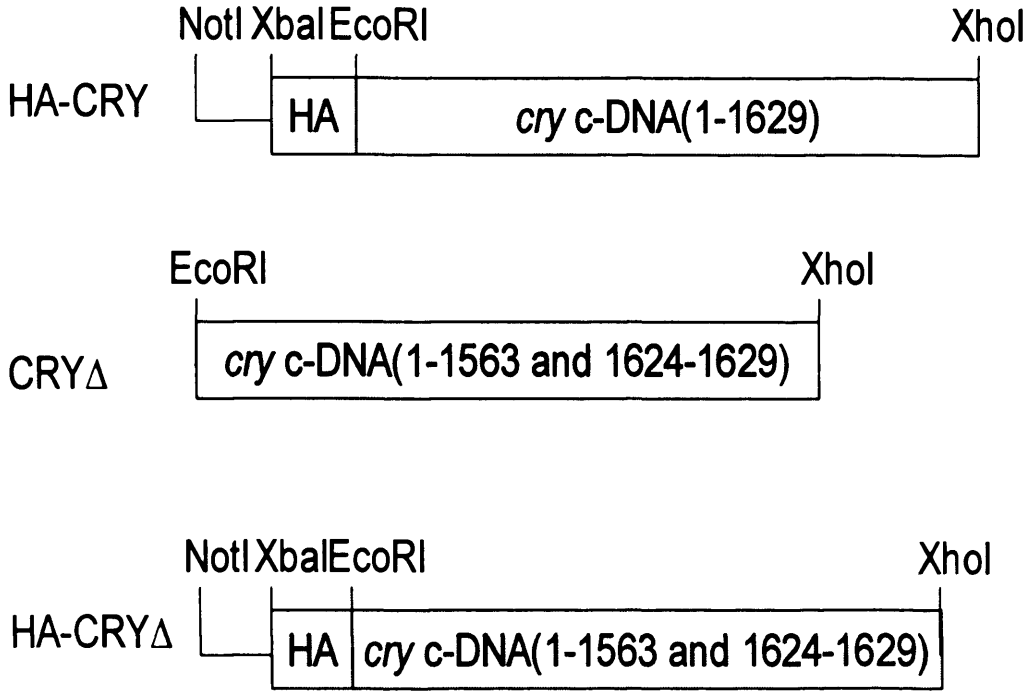


Figure 2.1: Schematic diagram of the HA-CRY, CRYΔ and HA-CRYΔ constructs. Restriction sites that were used in the cloning of these constructs are indicated. The CRYΔ and HA-CRYΔ constructs have a deletion of the *cry* c-DNA from nucleotide 1564 to nucleotide 1623 resulting in the absence of amino acids 522 to 541. These three fragments were cloned in the pUAST vector (Brand and Perrimon, 1993) (appendix 1) and injected in w^{1118} genetic background. HA-CRY and HA-CRYΔ were cloned NotI-XhoI and CRYΔ was cloned EcoRI-XhoI. HA stands for the hemagglutinin tag that was positioned upstream of the *cry* c-DNA in the two HA constructs.

2.2 Locomotor activity experiments

The locomotor activity is monitored using an activity event recorder produced by PIXEL Srl. (Padova, Italy). Cylindrical glass tubes (80x3mm) are filled with approximately 20mm of yeast/sucrose/agar medium at one end, which is then sealed with wax to prevent desiccation. Single male flies are placed individually into each tube and the open end is sealed with cotton wool. The tubes are then loaded into individual slots in the activity monitoring machine, where two diodes of a photocell detect every time each fly disrupts the infra-red beam, counting it as an activity event. The number of events for each fly is recorded in 30min time windows (bins). Data are sent and stored in an attached PC. Infra-red light is used as flies are not sensitive to this wavelength. All locomotor activity experiments are carried at 25°C in the same two incubators to allow comparison of the results. The light intensity used is 40 μEinsteins/m^2/sec.

Flies used for the experiments are all raised in 12:12 LD light regimes at 25°C. When analysing 12:12 LD locomotor activity behaviour, the incubators housing the monitoring machinery are set to the same light phase as the flies have previously been experiencing. When the analysis is performed under DD conditions the flies are loaded into the activity recorder during the light phase of the 12:12 LD regime and the LD conditions are maintained for at least three days. During the last night of the LD conditions, lights are switched off for the remaining of the experiment. When a LL experiment is studied, flies are loaded during the light phase of the 12:12 LD regime and the LD conditions are maintained for at least three days. During the last day of the LD conditions, lights are switched on for the remaining of the experiment.

Flies that have died during the course of the experiment are not included in any subsequent analysis. The data are analysed in several different ways. Firstly, flies of the same genotype under the same condition (LD, DD or LL) are pooled together. An average activity is calculated for each 30 min bin. The average activity level for each bin is then used to build an average locomotor activity histogram for each genotype under a given condition. This enables one to see if flies show rhythmic behaviour but in order to have a more accurate idea of the periodicity of any shown rhythm, spectral (Fourier) analysis is used. The Fourier analysis is based on the CLEAN algorithm (Kyriacou and Hall, 1989; Roberts et al., 1987). Fourier analysis decomposes the string of bin values of each individual fly into a linear combination of sinusoids of different periods and identifies the curves that more closely match the data (several periodicities of different amplitude can be identified). These are then displayed as a spectrogram in which the X-axis represents the period and the Y-axis the amplitude. The 95% and 99% confidence limits are then calculated for every given periodicity using a Monte Carlo simulation that randomly shuffles the bins and repeats the spectral analysis on these new strings of data. This simulation is repeated 100 times. The 100 amplitudes obtained are ordered and the 95th and 99th in ascending order are plotted on the spectrogram to give the 95% and 99% confidence levels. The period corresponding to the highest peak is taken as the period of locomotor activity of the fly. If none of the spectral peaks are above the 95% confidence level, the fly is judged as arrhythmic. ANOVA (SPSS) is used to determine statistical differences in period length between genotypes.

2.3 Average daily activity

To gain confidence in the analysis of locomotor activity patterns, several days of activity for a single fly are superimposed (wrapped) obtaining an 'average' day representative of the whole length of the experiment. This is further averaged across all flies of the same genotype. Under LD 12:12, this procedure is easily performed as each fly has a period of 24h, as imposed by the 12:12 LD cycles. Under DD, first the period of locomotor activity is calculated by Fourier analysis for each fly. Then, taking into account the period, the raw activity data of each fly are resampled in 24h intervals for each day and the average is calculated. ANOVA (SPSS) is used to determine statistical differences between genotypes.

2.4 Phase response curves experiments

Phase response curves (PRC) are used to study the circadian photoresponsiveness of flies. They are based on the fact that when flies are subjected to a short pulse of light during the last dark phase of a 12:12 LD condition, they phase-shift their locomotor activity in subsequent days in DD conditions. Practically, flies from the same genotype are split in two groups, which are loaded into two separate incubators. The experiment starts with at least 3 days in a 12:12 LD condition in both incubators (light intensity of 40 μEinsteins/m^2/sec). During the night of the last LD cycle, light is switched on for 5 minutes at a given ZT in one of the incubators. When the pulse of light is finished, both incubators are put in DD conditions for at least 5 days. The data are then processed by examining

the phase of activity of individual flies. All flies from a given genotype that have been in the incubator where the light was switched on constitute the pulsed flies. The second group makes the unpulsed control flies. The phase of activity is measured by looking at the third evening peak in DD conditions. As a reference point, the bin number when the activity level falls below 50% of the peak value is taken. This is repeated for each individual flies in pulsed and unpulsed pools. The bin numbers of the unpulsed flies are averaged and the individual bin numbers of the pulsed flies are subtracted from the unpulsed average. Finally, these subtracted values are averaged to give the amplitude of shift for a given genotype at a particular ZT. A positive value corresponds to a phase-advance while a negative value indicates a phase-delay. ANOVA (SPSS) is used to determine statistical differences in phase-shifts amplitude between genotypes.

2.5 Western blotting.

Prior to collections, flies are entrained in 12:12 LD conditions at 25°C in two identical boxes that have antiphase LD cycles (one box has light on between 8.00 and 20.00 while the other is on between 20.00 and 8.00). This is to avoid having to collect samples throughout the day and night. For LD collections flies from each box are collected every 4h starting at ZT0 and ZT12 (which correspond to light-on and light-off respectively). For DD collections, after initial entrainment in 12:12 LD regime, the flies are collected on the first day of the DD conditions starting at CT0 and CT12. Flies are collected by freezing them in liquid nitrogen. Heads are separated from bodies by vortexing frozen flies and then collected with a sieve on dry ice. Proteins are extracted with 2 times heads volumes of BW buffer (0.9575M

HEMG buffer, 0.1% Triton X-100, 0.01M EDTA, 0.001M DTT, 0.0005M PMSF, 0.01mg/mL aprotinin, 0.005mg/mL leupeptin and 0.005mg/mL pepstatin). HEMG buffer is made of 0.1M KCL, 0.02M HEPES pH 7.5 and 2mL of glycerin in a final volume of 40mL. The OD at 595nm is measured for each sample using the Bradford reagent and the concentration of proteins equalised (800µl H_2O, 200µl Bradford reagent, 1µl of sample; incubate between 5 to 30 minutes). 3x protein loading buffer (188mM Tris pH6.8, 6% (v/v) SDS, 30% (v/v) glycerol, 15% (v/v) β–mercaptoethanol, 0.03% (w/v) bromophenol blue) is added to each sample. The samples are then boiled, spun down and loaded in a 6% (PER and TIM) or 10% (HA-CRY and HA-CRYΔ) SDS-polyacrylamide gel. 6% resolving gel is made of 11.6mL H_2O, 4mL Tris 2M pH8.8, 200µl 10% SDS, 4mL 30% acrylamide, 160µl Ammonium persulphate and 24µl TEMED for a final volume of 20mL. 10% resolving gel is made of 8.6mL H_2O, 3.7mL Tris 2M pH8.8, 200µl 10% SDS, 6.6mL 30% acrylamide, 80µl 25% Ammonium persulphate and 12µl TEMED for a final volume of 20mL. Stacking gel is made of 7.3mL H_2O, 1mL Tris 1M pH6.8, 1.5mL 30% acrylamide, 100µl 10% SDS, 20µl 25% Ammonium persulphate and 10µl TEMED for a final volume of 10mL. The gels are run overnight using a HOEFER system. The gel running buffer consists of 2.5mM Tris, 0.25M Glycine, 0.1% (v/v) SDS.

Once the samples have run to the bottom of the gel, they are electro-blotted onto a nitrocellulose membrane (PROTRAN, Schleicher and Schuell, the blotting buffer is: 40mM Tris, 40mM glycine, 0.0375% (v/v) SDS, 20% MetOH) for 3h at 400mA.

Membranes are blocked for 1h at room temperature in TBST (10mM Tris-Cl (pH7.5), 150mM NaCl, 0.05% (w/v) Tween-20) containing 5% milk. They are then

incubated with the primary antibody diluted in TBST containing 5% milk for 2h at 4°C with agitation. Primary antibodies used are a rabbit anti-PER antibody (Stanewsky et al., 1997)(dilution 1:15000), a rat anti-TIM (Myers et al., 1996) (dilution 1:1000), a mouse anti-HA (Sigma, dilution 1:10000) and a mouse anti-HSP70 (Sigma, dilution 1:50000). After this, three 5min washes in TBST are carried out at room temperature before incubation with a secondary horseradish peroxidase-conjugated antibody for 1h at 4°C. Secondary antibodies used are goat anti-rat (Sigma, dilution 1:8000), goat anti-rabbit (Sigma, dilution 1:6000) and goat anti-mouse (Sigma, dilution 1:6000). A further three 5min washes are then carried out in TBST. The membranes are then incubated with 0.1M Tris-Cl (pH 8.5), 6.25μM Luminol, 6.38μM p-Coumaric acid, 2.7mM H_2O_2 for 1min to create a luminescent signal. This signal is then detected on an auto-radiograph film (Fuji). Developed films are scanned into a PC and quantified with scion-image analysis software (Scion corporation).

2.6 Immunohistochemistry

For each time point, control and *CRYΔ* flies are processed in parallel in a 30 min window centered on each reported ZT. The procedure up to the end of fixation is carried out in a dark room under dim red light. Flies are anaesthetised and placed on ice. Brains are partially dissected with a pair of fine forceps in ice cold PBS, and stored in it until 5 brains for each genotype are processed (ca 30 min). Fixation is then carried out at room temperature (RT) for 2h with 4% formaldehyde in PBS. After, the brains are washed with PBS for 15 min and the dissection completed. Brains are permeabilised 3 x 20 min with PBS, 1% Triton-X. Blocking is

performed in PBS, 0.5% Triton-X, 10% goat serum for 1h. Primary antibodies are diluted in fresh PBS, 0.5% Triton-X, 10% goat serum and incubated for 72h at 4 °C. Unbound antibodies are washed 3 x 20 min with PBS, 0.5% Triton-X. Secondary fluorescent antibodies are diluted in PBS, 0.5% Triton-X and incubated (in darkness) for 3h at RT. After washes (3x 20 min, PBS, 0.5% Triton-X) brains are rinsed in distilled water and mounted in 20% PBS (pH = 8.5), 80% Glycerol + 3% n-propylgallate. Primary antibodies are as follows: rat anti-TIM(UPR41) (Yang and Sehgal, 2001) dilution 1:1000, rabbit anti-PER (Stanewsky et al., 1997) dilution 1:15000, rabbit anti-cPDH (Dircksen et al., 1987) dilution 1:2500 and rat anti-PDF (Park et al., 2000) dilution 1:400. Fluorescent secondary antibodies are as follows: Goat anti-rat IgG-Cy3 (Jackson Laboratories, 1:400), goat anti-rabbit IgG-Cy3 (Jackson Laboratories, 1:200), goat anti-rabbit IgG-Cy2 (Jackson Laboratories, 1:400). Optical sections are imaged on a Zeiss LMS510 confocal microscope and exported as tiff files. Scion image analysis software (Scion corporation) is used to quantify the mean pixel intensity of cytoplasm, nucleus and background of inverted imported images. Two-way ANOVA (SPSS) is used to find statistical differences between genotypes.

Chapter 3: Overexpression of CRY in a *cry*$^{+}$ background

3.1 Introduction

The *Drosophila* circadian clock consists of two interlocked negative autoregulatory feedback loops (Glossop et al., 1999). The *period* (*per*) and *timeless* (*tim*) genes and their products are central components of this mechanism, and their levels oscillate with a 24h pattern. One of the most important functions of the clock is the ability to react to and predict environmental cues, light being the most important, resetting the subjective time in phase with the cycling of the external variables; we refer to this phenomenon as 'entrainment'. Several light input pathways contribute to entrainment in *Drosophila*. The most obvious are photoreceptive structures, such as eyes and ocelli (Helfrich-Forster et al., 2001) and the Hofbauer-Buchner eyelets (Hofbauer and Buchner, 1989). The latter are a small bilateral group of photoreceptive cells that lies underneath the retina and have only recently been convincingly shown to be involved in circadian photoreception (Helfrich-Forster et al., 2002; Malpel et al., 2002). In addition, the presence of circadian relevant photopigments in Dorsal Neurons 1 and 3 has been proposed (Veleri and Wulbeck, 2004). However, in *Drosophila melanogaster*, CRYPTOCHROME (CRY) is key to entrainment and it is expressed in clock neurons, bringing light information directly into the cogs of the clock. Cryptochromes are blue-light sensitive proteins, related to photolyases (a family of light-activated DNA repair enzymes), which employ light energy for signalling rather than repair. In particular, CRY is thought to play an important role in the rapid light-induced degradation of TIM, which is believed to be one of the primary

responses of the clock to photic cues (Lin et al., 2001). In a yeast two hybrid assay, CRY binds PER and TIM in a light dependent manner (Ceriani et al., 1999; Rosato et al., 2001). In this chapter I shall present data regarding the overexpression of CRY in flies to see if it affects the entrainment ability, free-running locomotor activity and circadian photosensitivity of the *Drosophila* clock.

The targeted overexpression of CRY in clock cells is achieved by using the GAL4/ *UAS* binary system (Brand and Perrimon, 1993) whose principle is as follows. One transgenic line (driver) carries the coding sequence of the yeast transcription factor GAL4 under control of the *timeless* promoter. The other transgenic line (reporter) carries the coding sequence of the *cry* gene or a hemagglutinin tag version of it under control of the *UAS* (upstream activating sequences) element, which are activated by GAL4. By crossing these two lines, GAL4 and *UAS* are brought together, eventually leading to the expression of the reporter gene in cells and times dictated by the specific promoter (Brand and Perrimon, 1993). It should be noted that the expression of *cry* does not 100% overlap the expression of *tim.* It would have been adequate to use a *cry-GAL4* driver, however at the time I started my experiments there was no such driver available. As a consequence, *tim-GAL4* was used because it is expressed in all 6 groups of clock neurons including *cry* expressing cells. The downside of this driver is that it may express high levels of CRY in cells that do not or express little amount of this protein. CRY overexpression has been performed in two different genetic backgrounds, first in a cry^{+} context (this chapter) and then in the strong hypomorphic cry^{b} background (chapter 4).

3.2 Results and discussion

3.2.1 Entrainment

Figure 3.1 shows the average locomotor activity pattern of male flies overexpressing CRY (*yw*; *tim-GAL4/UAS-cry24B*; +/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *yw*; *UAS-cry24B*/+; +/+) during 5 days under a 12:12 Light-Dark condition (LD, alternating conditions of 12h of light followed by 12h of darkness). This is best suited to give information about the entrainment ability of the flies, ie how the internal clock interacts with the external cues (*Zeitgeber*). Under LD, both wild-type controls show a typical bimodal pattern of locomotor activity with a peak around the dark to light transition (the morning peak of activity, MP in Figure 3.1, left-hand panel) and another around the light to dark transition (evening peak, EP). It is important to realize that these activity peaks are the products of an endogenous timekeeping mechanism rather than a direct response of the flies to the external light condition (a phenomenon called masking). This is illustrated by the fact that the flies anticipate the dark to light and light to dark transitions. In between these two bouts of activity, control flies have very little activity during the middle of the light phase and during the dark phase. Flies overexpressing CRY (thereafter named CRY flies, Figure 3.1, right-hand panel) do not show major variations from the two control lines. They have a bimodal pattern of activity with both peaks being anticipated and the overall activity shape and phase are very similar indicating that CRY flies entrain normally under parametric (LD) conditions. The entrained periodicities of CRY and control flies were calculated by spectral analysis (Fourier) and are given in table 3.1 (rows 1, 2 and 4). There are no differences between CRY and controls with each of these

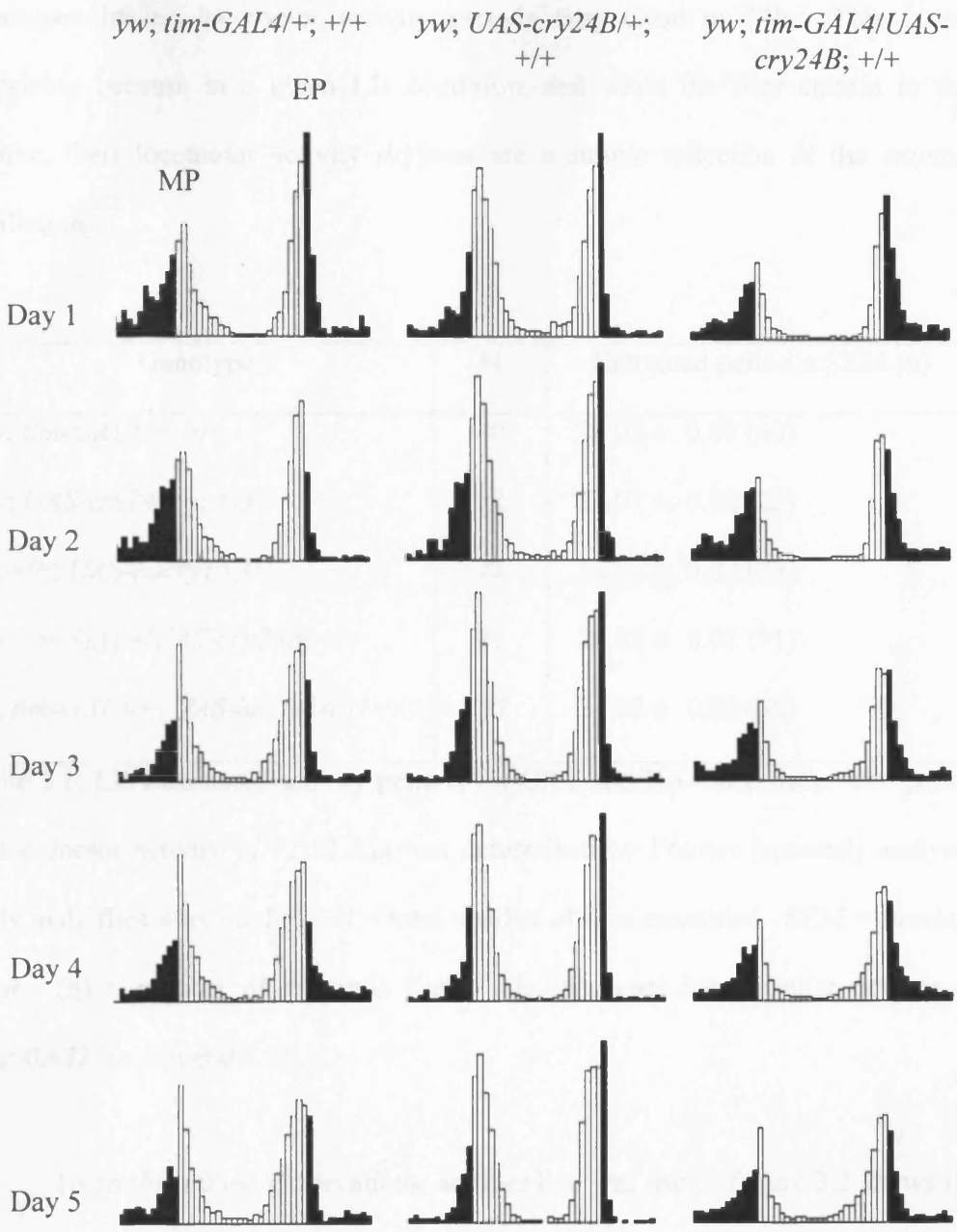


Figure 3.1: LD behaviour of CRY flies. Average locomotor activity pattern of male flies overexpressing CRY (*yw*; *tim-GAL4/UAS-cry24B*; +/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *yw*; *UAS-cry24B*/+; +/+) during 5 days under a 12:12 Light-Dark condition (LD, alternating conditions of 12h of light followed by 12h of darkness). Open bars= light period, filled bars= dark period. MP= morning peak, EP= evening peak.

genotypes having locomotor activity periods very close to 24h. This is not surprising because in a given LD condition, and when the flies entrain to this regime, their locomotor activity rhythms are a simple reflection of the external oscillation.

Genotype	N	Entrained period ± SEM (n)
yw; *tim-GAL4*/+; +/+	40	24.03 ± 0.03 (40)
yw; *UAS-cry24B*/+; +/+	22	23.97 ± 0.02 (22)
w; +/+; *UAS-hacry16.1*/+	23	24.03 ± 0.02 (23)
yw; *tim-GAL4*/*UAS-cry24B*; +/+	91	24.02 ± 0.02 (91)
w; *tim-GAL4*/+; *UAS-hacry16.1*/+	35	24.02 ± 0.02 (35)

Table 3.1: LD locomotor activity periods for CRY and HA-CRY flies. The period of locomotor activity in 12:12 LD was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies. All genotypes have similar periods ($F_{4,206}$=0.473, ns, appendix 2).

To confirm these observations, another line was used. Figure 3.2 shows the average locomotor activity pattern of males flies overexpressing HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) as well as the relevant controls (*yw*; *tim-GAL4*/+; +/+ and *w*; +/+; *UAS-hacry16.1*/+) during 5 days under a 12:12 LD condition. Here again, controls behave as expected, namely they display bimodal activity with peaks centred on the dark/light and light/dark transitions (Figure 3.2, left-hand and middle panels). Flies overexpressing HA-CRY (thereafter named HA-CRY flies, Figure 3.2, right-hand panel) are able to entrain to the LD cycle with no obvious

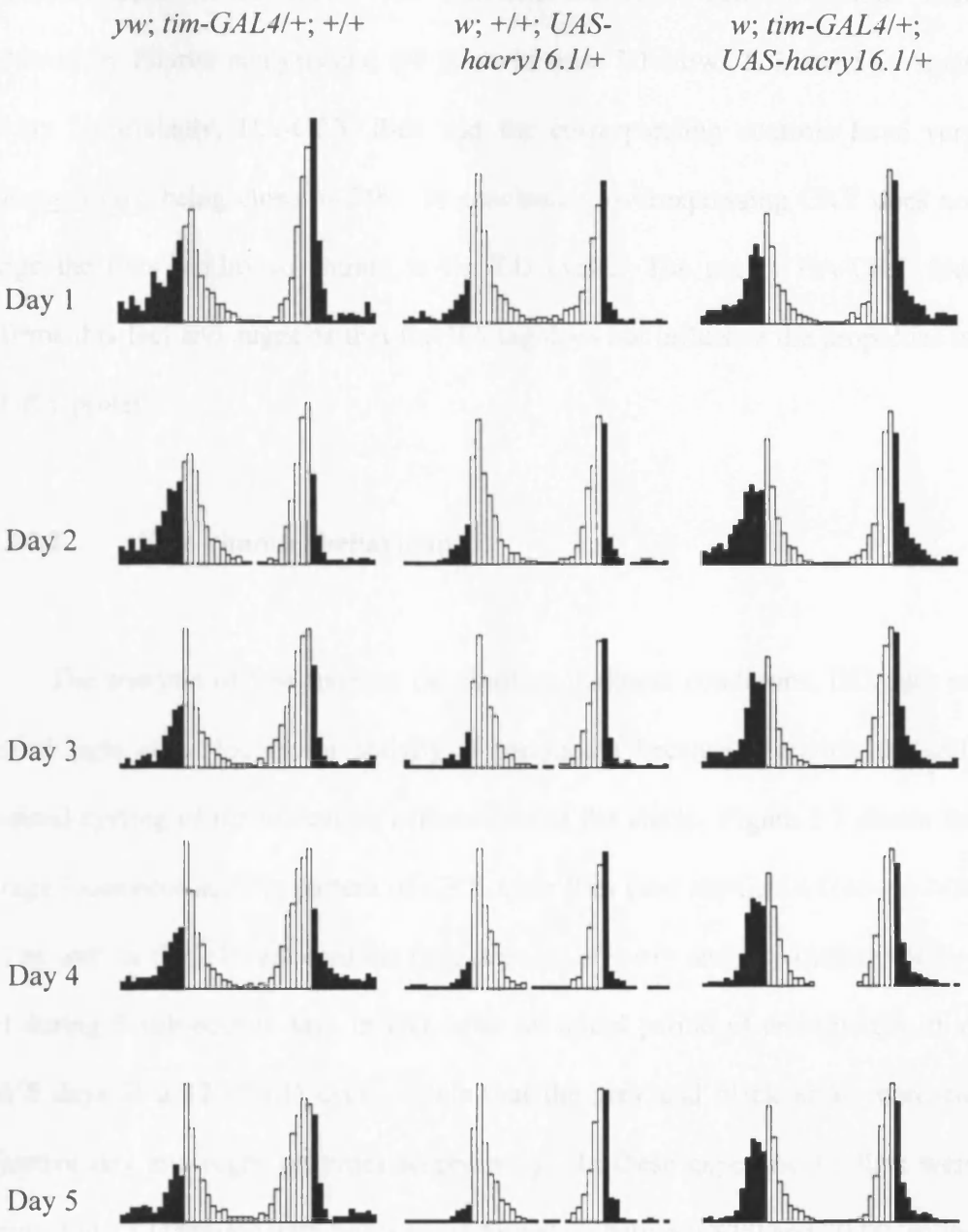


Figure 3.2: LD behaviour of HA-CRY flies. Average locomotor activity pattern of male flies overexpressing HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) as well as the relevant controls (*yw*; *tim-GAL4*/+; +/+ and *w*; +/+; *UAS-hacry16.1*/+) during 5 days under a 12:12 LD condition. Open bars= light period, filled bars= dark period.

differences from the controls. The entrained locomotor activity periods were calculated by Fourier analysis and are given in table 3.1 (rows 1, 3 and 5). Again and not surprisingly, HA-CRY flies and the corresponding controls have very similar periods, being close to 24h. In conclusion, overexpressing CRY does not change the flies' ability to entrain to the LD cycle. The use of HA-CRY flies confirms this fact and suggests that the HA tag does not influence the properties of the CRY protein.

3.2.2 Free-running behaviour

The analysis of free-running (in constant darkness conditions, DD with no external light cues) locomotor activity is paramount because it reports the self-sustained cycling of the molecular components of the clock. Figure 3.3 shows the average locomotor activity pattern of CRY male flies (*yw*; *tim-GAL4/UAS-cry24B*; +/+) as well as the relevant controls (*yw*; *tim-GAL4*/+; +/+ and *yw*; *UAS-cry24B*/+; +/+) during 5 subsequent days in DD, after an initial period of entrainment of at least 5 days in a 12:12 LD cycle. Note that the grey and black areas represent subjective day and night activities respectively. In these experiments, flies were entrained in a LD regime with lights being turned on between 8.00 and 20.00, so the subjective day period will be from 8.00 to 20.00 on each subsequent day in DD. In DD conditions, control flies conserve a bimodal pattern of activity (Figure 3.3, left-hand and middle panels). However, as the time spent in DD increased, the morning component appears to have lower amplitude from one day to the other. The evening component amplitude stays fairly similar throughout the experiment. As a result, after 5 days in DD, the evening component has higher amplitude. This is in

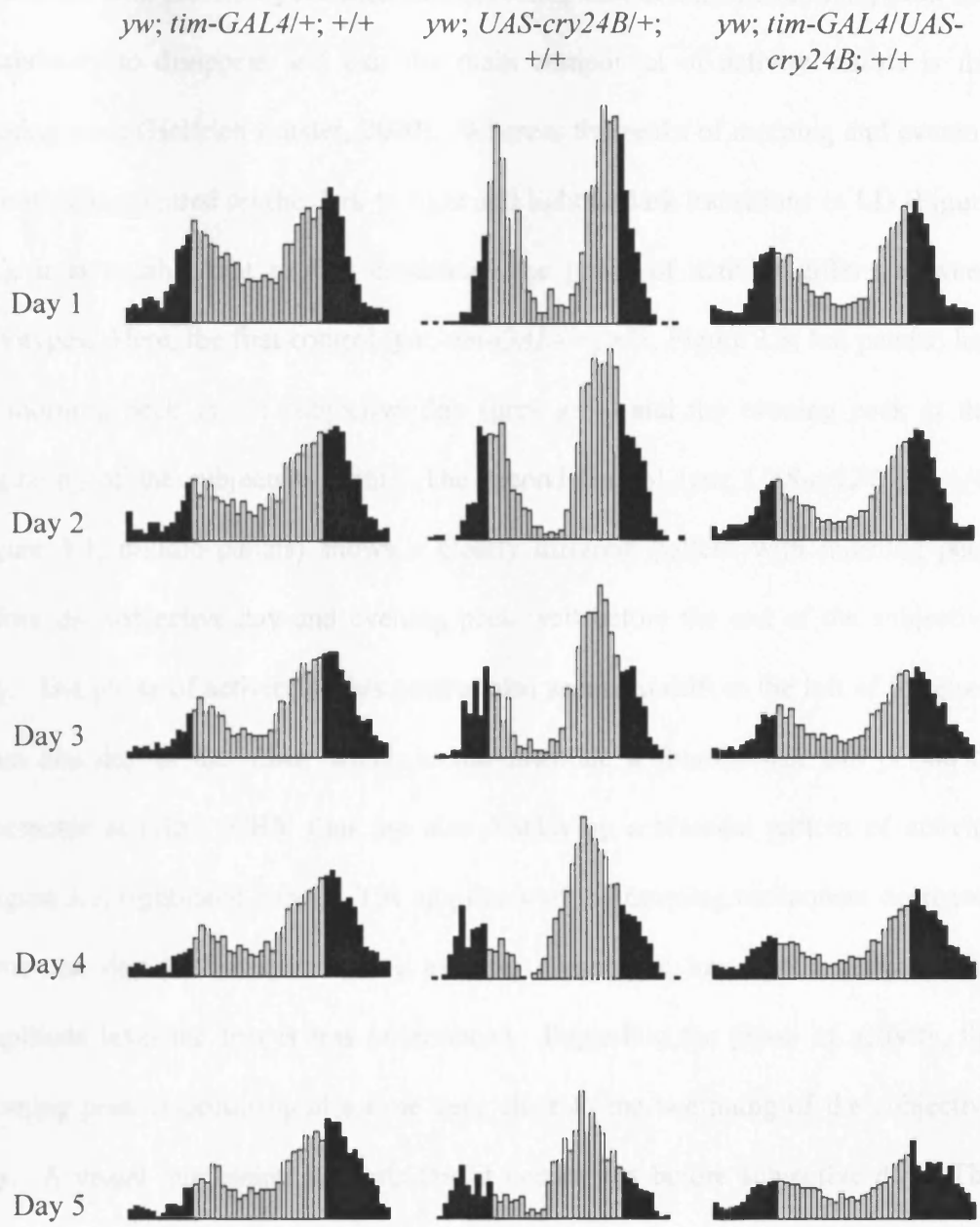


Figure 3.3: DD behaviour of CRY flies. Average locomotor activity pattern of male flies overexpressing CRY (*yw*; *tim-GAL4/UAS-cry24B*; +/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *yw*; *UAS-cry24B*/+; +/+) during 5 days under DD conditions. Grey bars= subjective day period, filled bars= subjective night period.

agreement with previous published data revealing that in DD, the morning peak has a tendency to disappear and that the main component of activity visible is the evening peak (Helfrich-Forster, 2000). Whereas the peaks of morning and evening activities are centred on the dark to light and light to dark transitions in LD (Figure 3.1), it is notable that in DD conditions the phase of activity differs between genotypes. Here, the first control (*yw*; *tim-GAL4*/+; +/+, Figure 3.3, left panels) has its morning peak in the subjective day (grey area) and the evening peak at the beginning of the subjective night. The second control (*yw*; *UAS-cry24B*/+; +/+, Figure 3.3, middle panels) shows a clearly different pattern with morning peak before the subjective day and evening peak well before the end of the subjective day. The phase of activity of this control also seems to drift to the left of the chart from one day to the other, which would illustrate a shorter than 24h period of locomotor activity. CRY flies are also displaying a bimodal pattern of activity (Figure 3.3, right-hand panel). The amplitude of the morning component decreases from one day to the other. The evening component has also a reduction in amplitude level but this is less pronounced. Regarding the phase of activity, the morning peak is occurring at a time very close to the beginning of the subjective day. A visual impression suggests that it occurs just before subjective day. The evening peak is centred on the subjective day/subjective night transition and again visual impression suggests it is just after the start of the subjective night.

Whereas locomotor activity periods are close to 24h in LD, the free-running periods reflect the state of the endogenous circadian clock. Fourier analysis was used to calculate the free-running periods of these flies and the results are given in table 3.2.

Genotype	N	Free-running period ± SEM (n)
yw; *tim-GAL4*/+; +/+	55	24.44 ± 0.08 (55)
yw; *UAS-cry24B*/+; +/+	22	23.86 ± 0.10 (22)
w; +/+; *UAS-hacry16.1*/+	23	24.30 ± 0.09 (23)
yw; *tim-GAL4*/*UAS-cry24B*; +/+	107	24.36 ± 0.04 (107)
w; *tim-GAL4*/+; *UAS-hacry16.1*/+	35	24.09 ± 0.04 (35)

Table 3.2: DD locomotor activity periods for CRY and HA-CRY flies. The period of locomotor activity in DD was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

Examination of table 3.2 reveals that the periods of locomotor activity are virtually indistinguishable between CRY (24.36±0.04h) flies and the *yw*; *tim-GAL4*/+; +/+ control (24.44±0.08h) ($F_{1,159}$=0.8, ns, appendix 3). The other control (*yw*; *UAS-cry24B*/+; +/+) has a significantly shorter period than the other two lines (23.86±0.10h) ($F_{1,181}$=21.69, P<0.01, appendix 3), this could give a partial explanation for the altered phase of activity of this genotype (Figure 3.3, middle panel). This marginally shorter period is however not enough to generate such a phase change, which is probably due to a line effect.

Figure 3.4 illustrates the average locomotor activity pattern of HA-CRY flies (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) as well as the controls (*yw*; *tim-GAL4*/+; +/+ and *w*; +/+; *UAS-hacry16.1*/+) for 5 days in DD following an entrainment period in LD 12:12. The control (*w*; +/+; *UAS-hacry16.1*/+, middle panel) shows bimodal activity for the first 4 days, but the morning peak has weaker amplitude from one day to the other before finally disappearing at day 5. When the morning

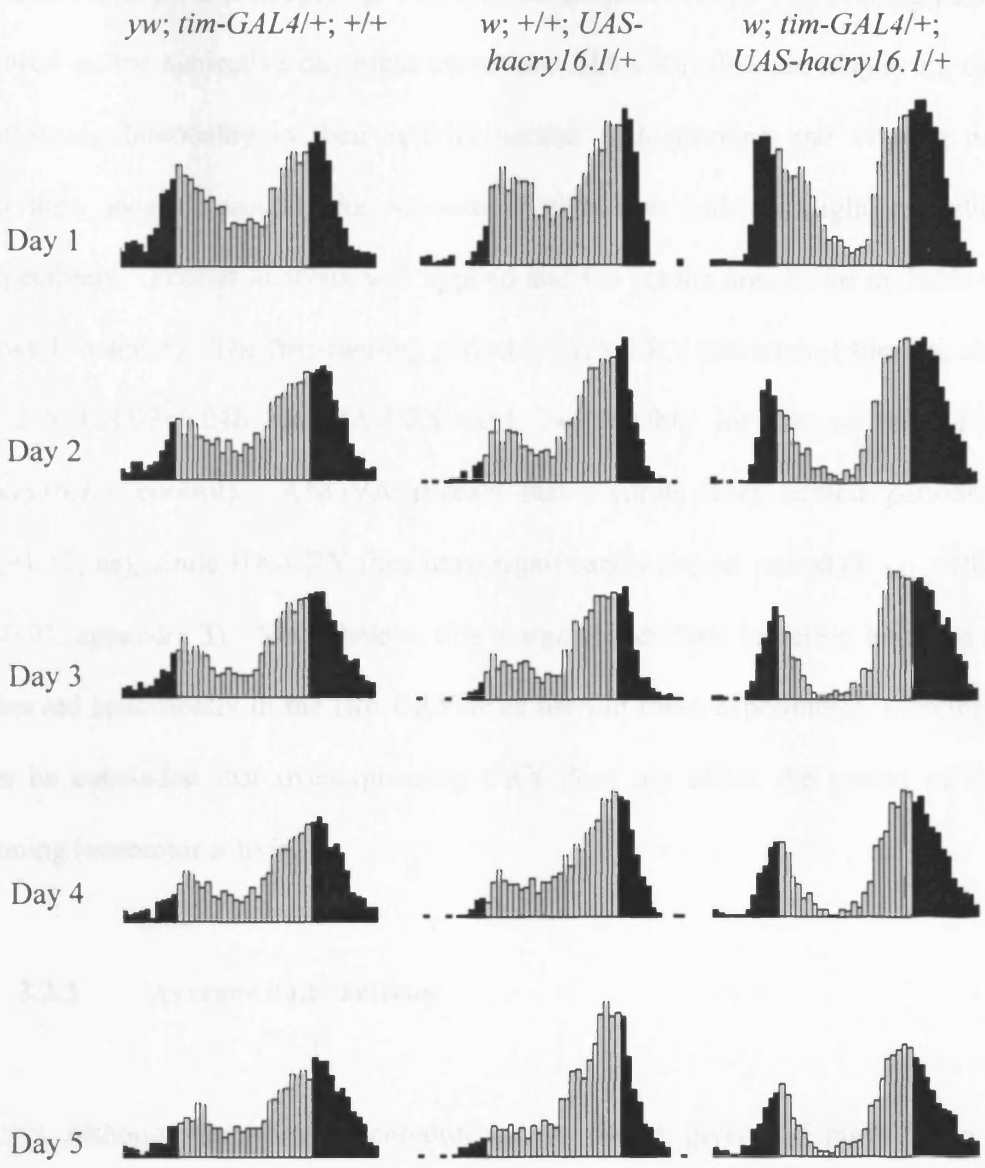


Figure 3.4: DD behaviour of HA-CRY flies. Average locomotor activity pattern of male flies overexpressing HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) as well as the relevant controls (*yw*; *tim-GAL4*/+; +/+ and *w*; +/+; *UAS-hacry16.1*/+) during 5 days under DD conditions. Grey bars= subjective day period, filled bars= subjective night period.

peak is observed, it is happening well into the subjective day. The evening peak is centred on the subjective day/night transition. HA-CRY flies are displaying clear and strong bimodality in their activity pattern with morning and evening peak activities located around the subjective night/day and day/night transitions respectively. Fourier analysis was applied and the results are shown in Table 3.2 (rows 1, 3 and 5). The free-running periods of HA-CRY and control flies are close to 24h (24.09±0.04h for HA-CRY and 24.30±0.09h for the *w*; +/+; *UAS-hacry16.1*/+ control). ANOVA reveals that controls have similar periods ($F_{1,76}$=1.15, ns), while HA-CRY flies have significantly shorter period ($F_{1,111}$=10.26, P<0.01, appendix 3). Nevertheless, this marginal reduction in period length is not observed consistently in the two CRY lines used in these experiments, therefore it can be concluded that overexpressing CRY does not affect the period of free-running locomotor activity.

3.2.3 Average daily activity

Although the average locomotor activity pattern given in Figures 3.1 to 3.4 are useful to see if flies entrain in LD, or to check the rhythmicity in DD, they are a rough representation and do not provide enough accuracy to analyse the behaviour of each individual fly in a given condition. In order to overcome this fact, individual flies' average day were built using computer software. Similar analysis have been done by others (Helfrich-Forster, 2000; Helfrich-Forster et al., 2001). This was done in both LD and DD conditions. In LD, the 5 days activity of each individual fly is wrapped using a 24h period (imposed by the 12:12 LD cycle) and redistributed in a 24h day. In DD, the 5 days activity of each individual fly is

wrapped using the value of its free-running period (as given by Fourier analysis) and redistributed in a 24h day. Finally, an average daily activity was calculated for each genotype by averaging individual flies' average day.

Figure 3.5 shows the average daily activity in LD for CRY (*yw*; *tim-GAL4/UAS-cry24B*; +/+), HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) flies and the relevant controls (*yw*; *tim-GAL4*/+; +/+, *yw*; *UAS-cry24B*/+; +/+ and *w*; +/+; *UAS-hacry16.1*/+). Inspection of these suggests that CRY and HA-CRY flies start their morning activity earlier than the controls and that they have an increased period of "rest-like state" (what I shall refer to as "siesta") in the middle of the day. However, to confirm the visual impression, each fly from a given genotype was analysed individually. Several points were used as reference, namely the onset, the peak and the offset of morning and evening activity components. The "siesta" time was defined as the difference between onset of evening activity and offset of morning component. Finally, the proportion of activity located in the light and dark phase was calculated for each fly. Averaged numbers could then be calculated for each genotype and the results are given in table 3.3. For the line 24B, controls only differ for the timing of evening peak while onset and offset of both activity components, timing of morning peak, "siesta" time and distribution of activity are similar (ANOVA, appendix 4). For line 16.1, controls again are very similar except for the timing of morning and evening peaks (ANOVA, appendix 4). Table 3.3 reveals that CRY and HA-CRY flies start their morning activity before the controls. The onset is at ZT21.71±0.12 for CRY and ZT21.08±0.17 for HA-CRY flies, when the controls only start their activity after ZT22 (ZT0 corresponds to lights on and ZT12 to lights off). The peaks of activity are also occurring earlier and the offset of morning activity happen roughly an hour earlier in CRY and HA-CRY flies.

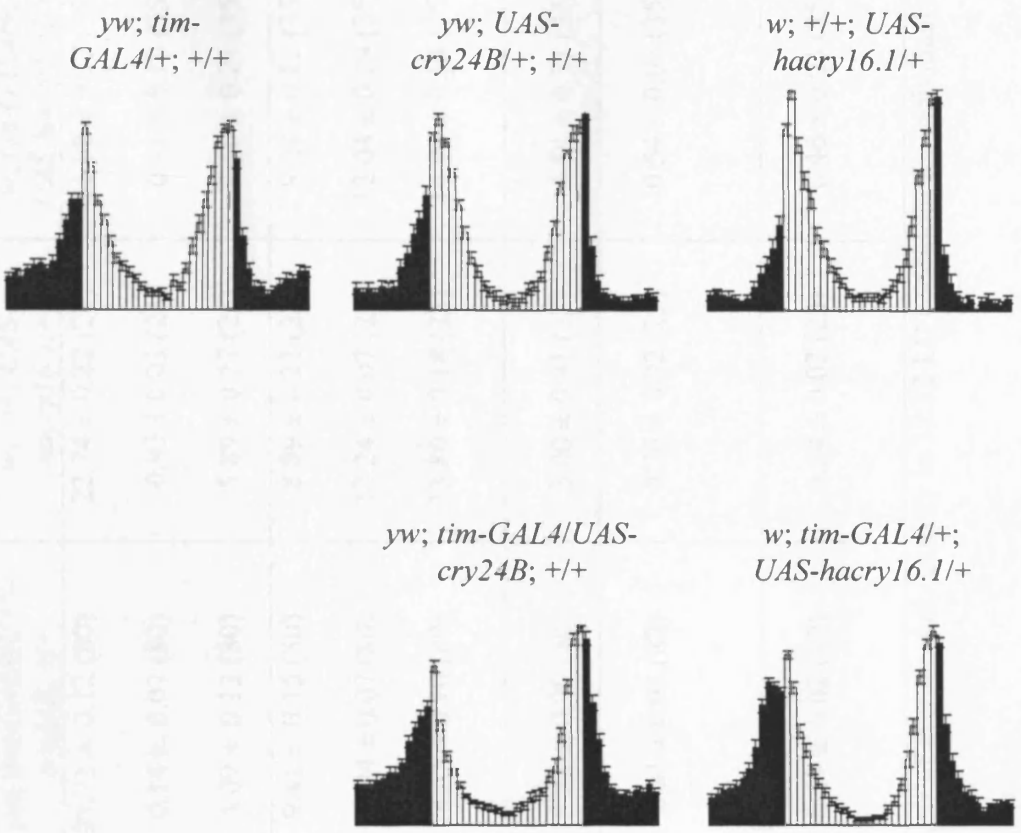


Figure 3.5: LD average daily activity for CRY and HA-CRY flies. Average daily activity in 12:12 LD conditions for CRY (*yw*; *tim-GAL4/UAS-cry24B*; +/+), HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) flies and the relevant controls (*yw*; *tim-GAL4*/+; +/+, *yw*; *UAS-cry24B*/+; +/+, and *w*; +/+; *UAS-hacry16.1*/+). Open bars= light phase, filled bars= dark phase.

Genotype		*yw*; *tim-GAL4*/+; +/+	*yw*; *UAS-cry24B*/+; +/+	*yw*; *tim-GAL4*/*UAS-cry24B*; +/+	*w*; +/+; *UAS-hacry16.1*/+	*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+
Morning activity	Onset	22.29 ± 0.13 (40)	22.36 ± 0.19 (22)	21.71 ± 0.12 (80)	22.74 ± 0.22 (23)	21.08 ± 0.17 (35)
	Peak	0.60 ± 0.04 (40)	0.75 ± 0.07 (22)	0.14 ± 0.07 (80)	0.93 ± 0.03 (23)	0.41 ± 0.10 (35)
	Offset	5.56 ± 0.17 (40)	5.11 ± 0.17 (22)	4.09 ± 0.13 (80)	5.89 ± 0.27 (23)	4.70 ± 0.24 (35)
Evening activity	Onset	8.42 ± 0.16 (40)	8.5 ± 0.15 (22)	9.41 ± 0.15 (80)	8.89 ± 0.23 (23)	9.24 ± 0.12 (35)
	Peak	11.64 ± 0.12 (40)	12.23 ± 0.09 (22)	11.94 ± 0.07 (80)	12.24 ± 0.07 (23)	12.04 ± 0.09 (35)
	Offset	14.05 ± 0.11 (40)	14.20 ± 0.12 (22)	14.55 ± 0.10 (80)	13.80 ± 0.18 (23)	14.90 ± 0.18 (35)
Siesta		2.86 ± 0.21 (40)	3.39 ± 0.24 (22)	5.32 ± 0.20 (80)	3.00 ± 0.41 (23)	4.54 ± 0.31 (35)
Proportion of activity during light phase		0.69 ± 0.02 (40)	0.73 ± 0.02 (22)	0.51 ± 0.02 (80)	0.76 ± 0.02 (23)	0.54 ± 0.03 (35)
Proportion of activity during dark phase		0.31 ± 0.02 (40)	0.27 ± 0.02 (22)	0.49 ± 0.02 (80)	0.24 ± 0.02 (23)	0.46 ± 0.03 (35)

Table 3.3: LD average daily activity for CRY and HA-CRY flies. Analysis of average daily activity in 12:12 LD conditions for individual male flies overexpressing CRY and HA-CRY as well as the relevant controls.

Regarding the evening component, the onset is occuring slightly later in CRY and HA-CRY flies. The evening peak timings are almost indistinguishable from the controls. The offset of evening activity is again slightly delayed. As a result, the siesta time is increased for CRY and HA-CRY flies. Finally, CRY and HA-CRY flies have a different distribution of activity. When the controls have roughly 70% of their activity during the light phase, the CRY overexpressing flies have only 50% of activity in the same period. This fits well with an advanced morning activity and a delayed evening activity in these flies resulting in the activity being pushed towards the dark phase. ANOVA analysis confirms that CRY and HA-CRY flies differ from controls regarding onset and offset of both activity components, "siesta" time and distribution of activity (appendix 4).

The average daily activity provides a more accurate insight into the behaviour of CRY flies and allows the identification of subtle differences that could not be seen in the average activity pattern. In conclusion, overexpressing CRY does not change the ability of the flies to entrain to LD cycles, or the period of locomotor activity. However, it triggers a subtle change in the distribution of locomotor activity, pushing the morning and evening activity towards the dark phase.

Figure 3.6 shows the average daily activity in DD for CRY (*yw*; *tim-GAL4/UAS-cry24B*; +/+), HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) flies and the relevant controls (*yw*; *tim-GAL4*/+; +/+, *yw*; *UAS-cry24B*/+; +/+ and *w*; +/+; *UAS-hacry16.1*/+). The same fly by fly analysis was done for DD behaviour but in this case the focus was on the presence of the morning peak and its timing if present, the timing of the evening peak and the proportion of activity in subjective day and night. In this case the phase difference was defined as the interval between morning and evening peaks. Table 3.4 shows that CRY and HA-CRY flies have

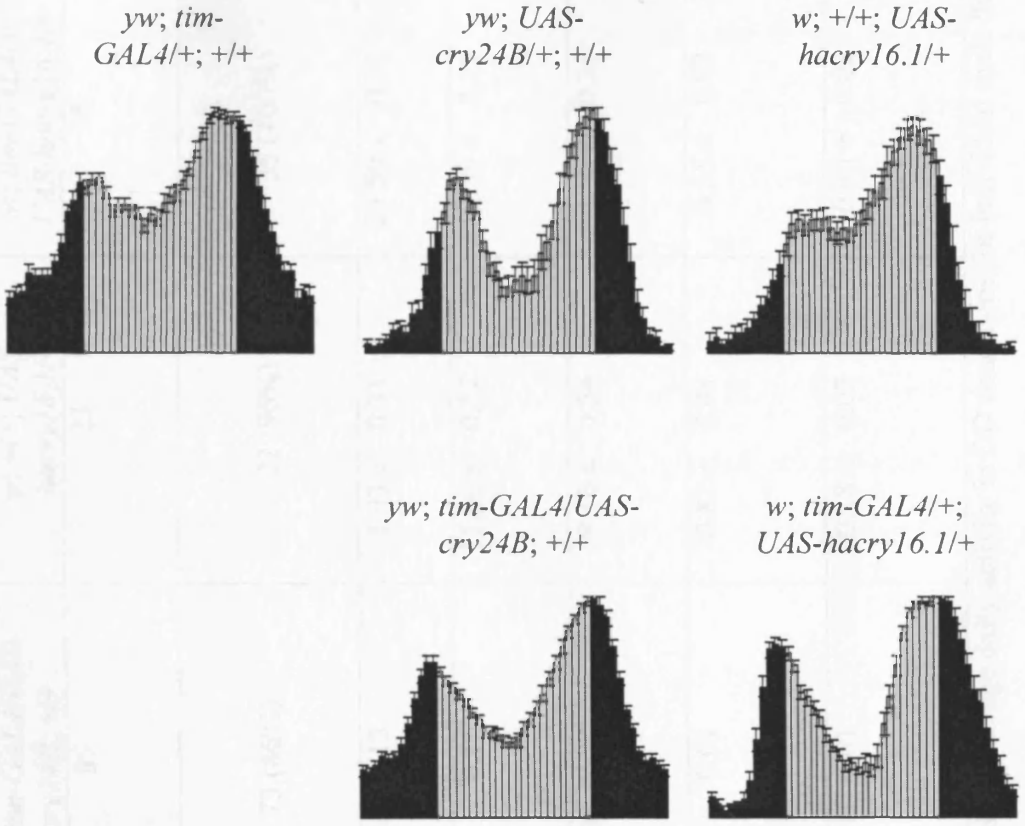


Figure 3.6: DD average daily activity for CRY and HA-CRY flies. Average daily activity in DD conditions for CRY (*yw*; *tim-GAL4/UAS-cry24B*; +/+), HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) flies and the relevant controls (*yw*; *tim-GAL4*/+; +/+, *yw*; *UAS-cry24B*/+; +/+, and *w*; +/+; *UAS-hacry16.1*/+). Grey bars= subjective day phase, filled bars= subjective night phase.

Genotype	*yw*; *tim-GAL4*/+; +/+	*yw*; *UAS-cry24B*/+; +/+	*yw*; *tim-GAL4*/*UAS-cry24B*; +/+	*w*; +/+; *UAS-hacry16.1*/+	*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+
Number of flies examined	54	22	85	23	35
Number of flies where morning peak was present	50 (93%)	22 (100%)	73 (86%)	22 (96%)	35 (100%)
Morning peak	0.87 ± 0.20	1.09 ± 0.22	23.71 ± 0.13	1.93 ± 0.31	23.59 ± 0.15
Evening peak	11.76 ± 0.20	11.75 ± 0.33	11.98 ± 0.16	11.09 ± 0.37	12.39 ± 0.29
Phase difference	10.89 ± 0.23	10.66 ± 0.27	12.27 ± 0.20	9.16 ± 0.54	12.8 ± 0.26
Proportion of activity during subjective day	0.65 ± 0.01	0.67 ± 0.02	0.55 ± 0.01	0.82 ± 0.03	0.55 ± 0.02
Proportion of activity during subjective night	0.35 ± 0.01	0.33 ± 0.02	0.45 ± 0.01	0.18 ± 0.03	0.45 ± 0.02

Table 3.4: DD average daily activity for CRY and HA-CRY flies. Analysis of average daily activity in DD conditions for individual male flies overexpressing CRY and HA-CRY as well as the relevant controls.

their morning peak at the end of the subjective night (at CT23.71±0.13 for CRY and CT23.59±0.15 for HA-CRY flies, where CT0 and CT12 are the beginning and end of subjective day, respectively). For controls the morning peak occurs at CT0.87±0.2 (*yw*; *tim-GAL4*/+; +/+), CT1.09±0.22 (*yw*; *UAS-cry24B*/+; +/+) and CT1.93±0.31 (*w*; +/+; *UAS-hacry16.1*/+). For line 24B, controls are similar (appendix 5) while for line 16.1 differences are seen between the two controls. CRY flies have a significantly advanced MP ($F_{1,143}=36.28$, $P<0.01$), an increased phase difference ($F_{1,143}=29.15$, $P<0.01$), and an increased proportion of activity during subjective night ($F_{1,143}=38.70$, $P<0.01$) but a similar timing of EP ($F_{1,143}=0.93$, ns, appendix 5). Similarly, HA-CRY flies show an advanced MP ($F_{1,83}=22.52$, $P<0.01$ and $F_{1,55}=57.69$, $P<0.01$), an increased phase difference ($F_{1,83}=30.15$, $P<0.01$ and $F_{1,55}=46.07$, $P<0.01$), an increased proportion of activity during subjective night ($F_{1,83}=20.08$, $P<0.01$ and $F_{1,55}=55.66$, $P<0.01$) but a similar timing of EP ($F_{1,83}=3.43$, ns and $F_{1,55}=7.48$, $P<0.01$, appendix 5). The increase of the phase difference in CRY and HA-CRY flies is therefore mainly due to the advanced morning activity. The change in distribution of activity is also probably reflecting the advanced morning component, which pushes activity towards the subjective night phase. Finally, regarding the presence of the morning peak, it is observed that a vast majority of flies display bimodal activity in DD (from 86% to 100% depending on the genotype). In conclusion, overexpressing CRY does not alter the free-running periodicities of flies but changes the distribution of activity mainly by advancing the time of the morning component. This result is repeating the advanced morning activity in LD and also suggests a possible role for CRY in darkness, something that has not yet been proposed.

3.2.4 Response of CRY flies to constant light exposure

When transferred into constant high intensity light conditions (LL), wild-type flies desynchronise, becoming arrhythmic, after one or two days (Konopka et al., 1989). It is known that CRY is required for this function, as cry^b flies (a strong hypomorphic mutant) keep rhythmic activity in LL (Emery et al., 2000). It has been previously reported that overexpressing CRY increases light sensitivity (Emery et al., 1998). I wanted to check whether CRY flies would prove more sensitive to constant light of high intensity (40 μEinsteins/m^2/s). Figure 3.7 shows the average locomotor activity pattern of CRY male flies (*yw*; *tim-GAL4/UAS-cry24B*; +/+) as well as the relevant controls (*yw*; *tim-GAL4*/+; +/+ and *yw*; *UAS-cry24B*/+; +/+) during 6 subsequent days, the first one being in normal LD until lights did not go off at ZT12, releasing the flies in LL. Note that the flies were initially submitted to a period of entrainment of at least 5 days in a 12:12 LD cycle. The grey area corresponds to the period of darkness in the previous LD entraining condition. The *yw*; *tim-GAL4*/+; +/+ controls manifest very weak rhythmicity for the first two days in LL, as indicated by the arrow (Figure 3.7, left-hand panel) but after this the activity profile becomes messy with no signs of rhythm. The *yw*; *UAS-cry24B*/+; +/+ control does not display any rhythm at all. Rhythmicity could also not be seen in CRY flies. Fourier analysis was used to check for rhythmic components in each individual fly and the results are given in table 3.5.

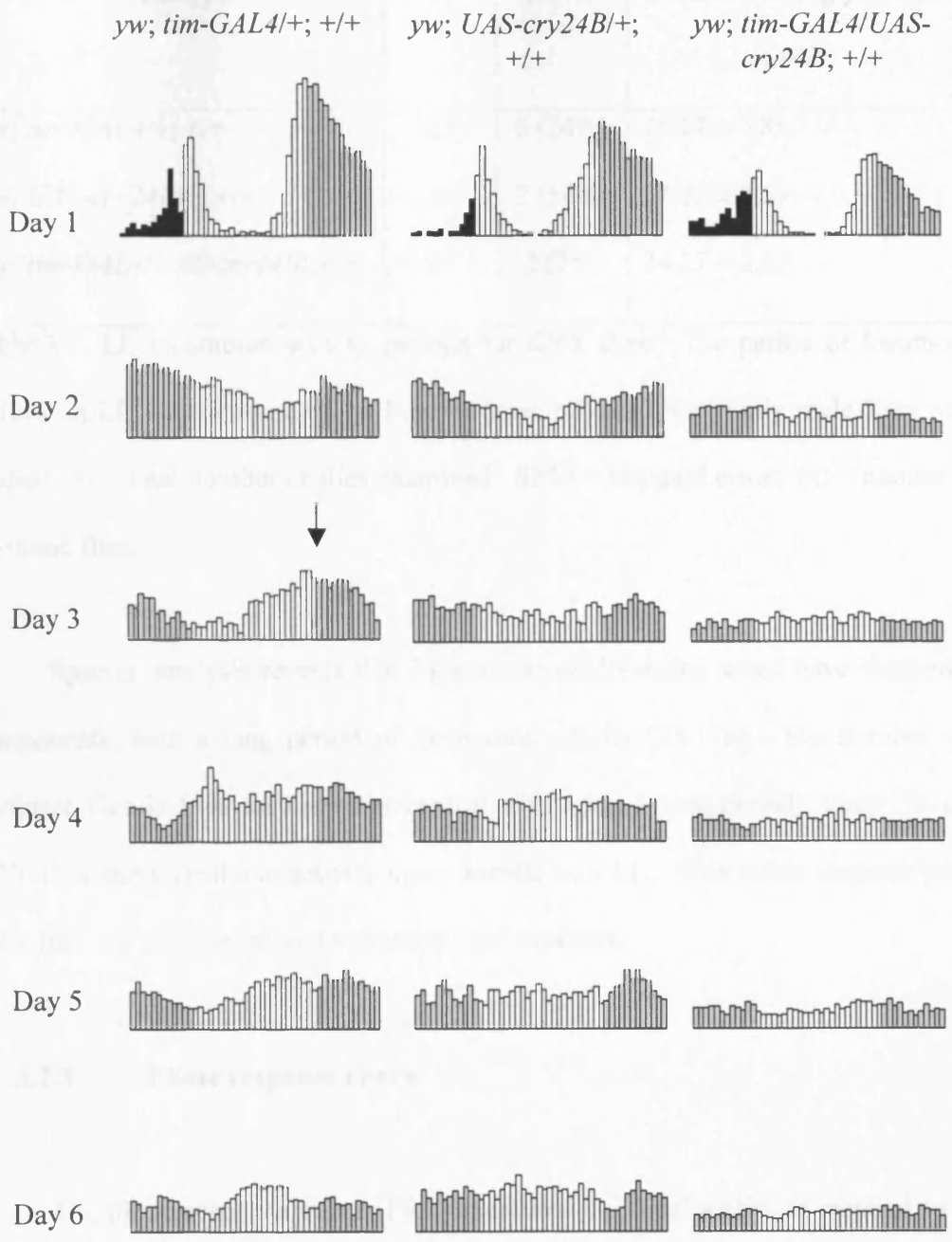


Figure 3.7: LL behaviour of CRY flies. Average locomotor activity pattern of male flies overexpressing CRY (*yw*; *tim-GAL4/UAS-cry24B*; +/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *yw*; *UAS-cry24B*/+; +/+) during 1 day in 12:12 LD cycle followed by 5 days under Light-Light conditions (LL). Open bars= subjective day period, grey bars= subjective night period. The arrow indicates residual rhythmicity in the *yw*; *tim-GAL4*/+; +/+ control.

Genotype	N	n (%)	LL free-running period ± SEM
yw; *tim-GAL4*/+; +/+	25	6 (24%)	25.17 ± 0.86
yw; *UAS-cry24B*/+; +/+	14	2 (14%)	27.25 ± 2.69
yw; *tim-GAL4*/*UAS-cry24B*; +/+	28	2 (7%)	24.17 ± 2.25

Table 3.5: LL locomotor activity periods for CRY flies. The period of locomotor activity in LL was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

Spectral analysis reveals that 24% of *tim-GAL4* males tested have rhythmic components, with a long period of locomotor activity (25.17h). The number of rhythmic flies is 14% for the other control with again a long period. Only 7% of CRY flies show rhythmic activity upon transfer into LL. This result suggests that CRY flies are more sensitive to constant light exposure.

3.2.5 Phase response curve

The phase response curve (PRC) measures the flies' ability to respond to a short saturating light pulse. When wild-type flies are subjected to pulses of light in the night, they shift the phase of their activity depending on when the pulse is given. If a pulse is administered early at night, a phase-delay is observed. When the pulse happens late at night, a phase-advance is seen. This property is known to involve CRY, as the strong hypomorphic cry^b mutant fails to respond to such pulses (Stanewsky et al., 1998). Previous work suggested that CRY overexpression

increases light sensitivity, for instance CRY flies are more sensitive to lower levels of light (Emery et al., 1998). To investigate this further, control (*yw*; *tim-GAL4*/+; +/+) and CRY flies (*yw*; *tim-GAL4*/*UAS-cry24B*; +/+) were subjected to 5 minutes pulses at ZT15, ZT17, ZT19, ZT21 and ZT23 and the phase response curve (PRC, Figure 3.8) was calculated (see also table 3.6).

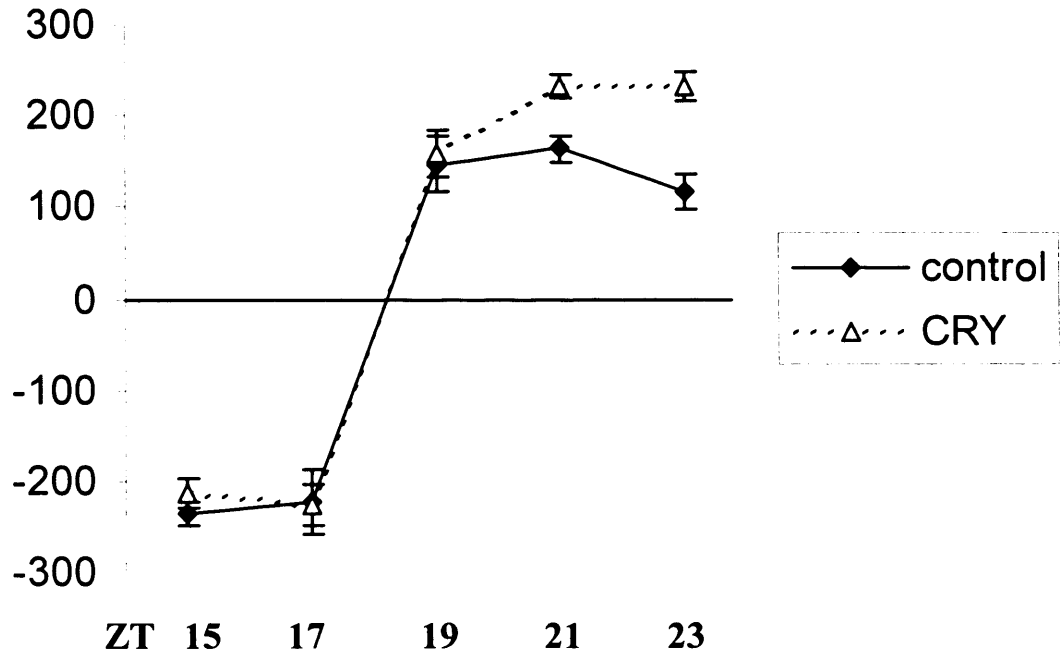


Figure 3.8: PRC of CRY flies. Phase response curve (PRC) for controls and CRY flies. Amplitude of shift obtained in minutes with a 5 minutes light pulse given at the indicated ZT. Numbers of pulsed flies at each time points are indicated in table 3.6. Numbers of unpulsed control flies were 19 for *yw*; *tim-GAL4*/+; +/+ and 14 for *yw*; *tim-GAL4*/*UAS-cry24B*; +/+. Error bars represent standard errors.

Genotype		*yw*; *tim-GAL4*/+; +/+	*yw*; *tim-GAL4/UAS-cry24B*; +/+
Amplitude of shift in minutes at ZT	15 (n)	-235.71± 12.97 (35)	-214.09± 15.86 (22)
	17 (n)	-223.33± 36.09 (9)	-225.83± 22.80 (12)
	19 (n)	146.67± 30.60 (9)	158.33± 25.87 (9)
	21 (n)	163.75± 14.33 (24)	233.00± 13.56 (15)
	23 (n)	118.24± 19.16 (17)	232.50± 15.85 (16)

Table 3.6: PRC of CRY flies. Amplitude of shift obtained in minutes ± SEM with a 5 minutes light pulse given at different ZTs. (n) = number of flies pulsed. Number of unpulsed control flies were 19 for *yw*; *tim-GAL4*/+; +/+ and 14 for *yw*; *tim-GAL4/UAS-cry24B*; +/+.

Examination of Figure 3.8 and table 3.6 reveals that the control behaves according to published data. When the pulse is given at ZT15, a roughly 4h phase-delay is obtained. At ZT17, the control is still showing a 3.5h delay. However, at ZT19, a 2.5h phase-advance is observed. At ZT21, the advance is slightly bigger, being almost 3h. Finally at ZT23, the phase-advance has a value of 2h. CRY flies do not show any significant differences in the phase-delay portion of the PRC ($F_{1,55}$=1.10, ns at ZT15 and $F_{1,19}$=0.04, ns at ZT17, appendix 6) (there is a 21 and 2 minutes difference at ZT15 and ZT17, respectively). At ZT19, the situation is very similar to the control ($F_{1,16}$=0.085, ns, appendix 6) with a 2.5h phase-advance. Remarkably, the PRCs of both genotypes are almost indistinguishable at ZT15, ZT17 and ZT19 (Figure 3.8). At ZT21, CRY flies display an advance of almost 4h, which constitutes a more than one-hour increase to the control. This difference is

statistically significant ($F_{1,37}$=10.77, P<0.01, appendix 6). And finally at ZT23, CRY flies advance their phase of activity by almost 4h; a significant ($F_{1,31}$=20.83, P<0.01, appendix 6) 2h increase relative to the control. In conclusion, CRY flies behave similarly to the control in the phase-delay portion and at ZT19 but when the pulse is given in the late night (ZT21 and ZT23) they show a more pronounced response revealing an increased sensitivity to light in the late night.

3.2.6 Molecular cycling of PER and TIM in CRY flies

Control, CRY and HA-CRY flies were used to perform western-blot analysis on whole head protein extracts. However, as the head mainly comprises peripheral clocks (eyes, fat bodies, glia), it is important to note that molecular observations mainly reflect the status of peripheral tissues and might not be fully extrapolated to pacemaker neurons. Molecular cycles for PER and TIM are seen both in LD and DD conditions (Edery et al., 1994; HunterEnsor et al., 1996). Western-blots were performed for CRY flies under LD and DD. For LD samples, flies were entrained for at least 5 days under a 12:12 LD cycle, they were then collected on the next day every 4h starting at ZT0 and up to ZT20. For DD experiments, the same period of entrainment was applied but at ZT12 of the last day of entrainment, the flies were transferred to DD and then collected every 4h starting at CT0 and up to CT20. Figure 3.9 shows PER and TIM molecular oscillations for control (*yw*; *tim-GAL4*/+; +/+), CRY (*yw*; *tim-GAL4*/*UAS-cry24B*; +/+) and HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) flies in LD (Figure 3.9a) and DD conditions (Figure 3.9b).

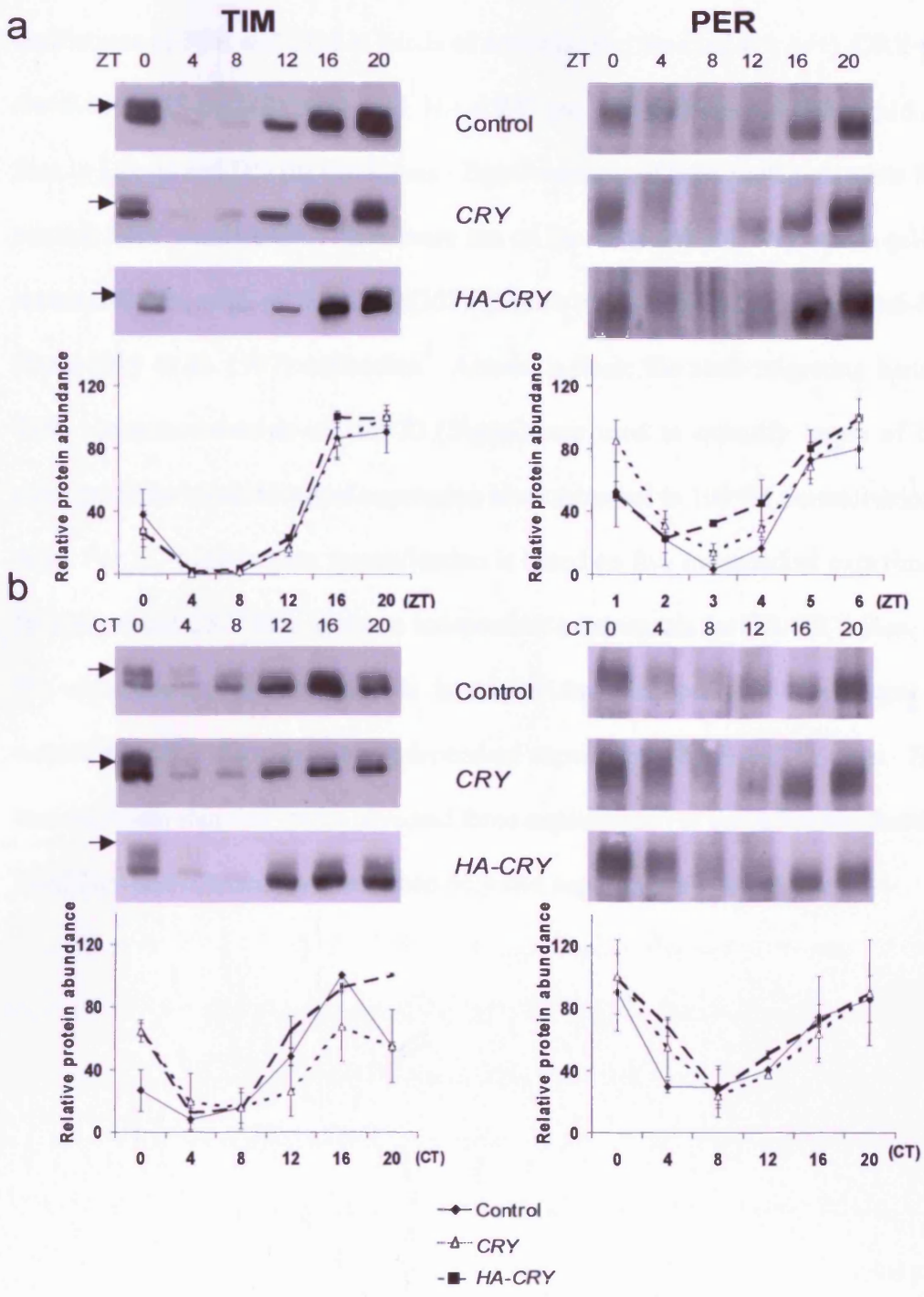

a
TIM
PER
ZT 0 4 8 12 16 20
Control
CRY
HA-CRY
Relative protein abundance
120
80
40
0
0 4 8 12 16 20 (ZT)
1 2 3 4 5 6 (ZT)
b
CT 0 4 8 12 16 20
0 4 8 12 16 20 (CT)
Control
CRY
HA-CRY

Figure 3.9: PER and TIM oscillations in CRY and HA-CRY flies. Molecular oscillations of PER and TIM in heads of controls (*yw*; *tim-GAL4*/+; +/+), CRY (*yw*; *tim-GAL4*/*UAS-cry24B*; +/+) and HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) flies in LD (a) and DD (b) conditions. Equal amounts of head protein extracts from control, CRY and HA-CRY flies, were run on the same 6% polyacrylamide gel and immunoblotted with rat anti-TIM(307) (Myers et al. 1996) and rabbit anti-PER (Stanewsky et al. 1997) antibodies. Arrows indicate the slow migrating band of TIM. Immunodetection of HSP70 (Sigma) was used to quantify levels of both clock proteins. Peak levels of expression were set equal to 100 for normalisation of data. For LD experiments, quantification is based on five independent experiments for control and CRY flies, and two independent experiments for HA-CRY flies. For DD experiments, quantification is based on three independent experiments for control and CRY flies, and two independent experiments for HA-CRY flies. Error bars represent standard errors (five and three experiments) or the difference between maximum and minimum values when only two experiments were available.

In LD, TIM is cycling in control flies in a way that protein levels are maximal at the end of the night (at ZT20) and minimal during the day; this underlies the important light sensitivity of the TIM protein. The amplitude of the oscillation is very important; levels of TIM are very high at night (ZT0, ZT16 and ZT20) whereas at ZT4 and ZT8, there is almost no TIM. TIM starts to accumulate again at ZT12. An important characteristic of TIM protein accumulation profile is that at night, TIM appears in two different migrating forms. This is especially evident at ZT0, and to a lesser extent at ZT20. These two forms of protein are known to reflect different phoshorylation status of TIM (Marrus et al., 1996), with the upper slow migrating band representing hyperphoshorylated forms of TIM and the lower fast migrating band accounting for hypophosphorylated TIM. CRY flies have a very similar pattern of TIM accumulation in LD. The quantification presented in Figure 3.9a left-panel, reveals that TIM oscillations are almost identical in CRY and control flies. Levels of TIM are maximal at ZT20 and minimal at ZT4 and ZT8. The two phosphorylation forms of TIM are also found at the end of the night (especially at ZT0). Overall there are no significant differences between CRY and control flies regarding the accumulation of TIM in LD. The profile of accumulation of TIM in HA-CRY flies is not different from CRY and control flies.

PER is also cycling in LD in controls, the phase of the oscillation is very similar with high levels of PER seen at night and lower levels during the day. The maximum is found at ZT20 and the minimum at ZT8. PER is also undergoing oscillation in its phosphorylation status with highly phosphorylated forms seen at ZT4 and ZT8 (these are represented by slower migrating forms of the protein). The overall oscillation is less sharp for PER than for TIM, because PER could be seen at all time points in the light phase, rather than being absent as TIM. CRY flies show

a very similar pattern of PER accumulation in LD as indicated by the quantification on Figure 3.9a right-hand panel. Levels are high at night and lower during the day with maximum and minimum reached at ZT20 and ZT8 respectively. PER is also seen as hyperphosphorylated forms at ZT4 and ZT8. HA-CRY flies show cycling levels of PER with maximum and minimum reached at ZT20 and ZT4 respectively. The overall oscillation is not very different from CRY and control flies. In conclusion, there are no differences in PER and TIM oscillations in LD between control, CRY and HA-CRY flies.

In DD, control flies show cycling accumulation of TIM (Figure 3.9b, left-hand panel). Maximal levels are reached at CT16 and minimal at CT4. Levels are higher during the subjective night (CT16, CT20 and CT0) and lower during the subjective day. The amplitude of the oscillation is not as large as in LD, because TIM could be seen at all time points in DD. This difference again underlies the important light-sensitivity of the TIM protein. TIM is seen as two different phosphorylated forms at all time points in DD, in sharp contrast with the absence of hyperphosphorylated TIM in the light phase of LD condition, highlighting the light sensitivity of hyperphosphorylated TIM. CRY flies also show cycling TIM accumulation in DD with maximal and minimal levels being reached at CT20 and CT8 respectively. Levels are also higher during the subjective night phase, which results in dampened overall amplitude of the oscillation compared to LD. Hyperphosphorylated forms of TIM are visible, especially during subjective night (CT20 and CT0). HA-CRY flies show a similar pattern to CRY flies. On overall, the profiles of accumulation of TIM are very similar in DD between CRY, HA-CRY and control flies. This is also true for PER oscillation, which does not display

significant differences in DD as indicated by the quantification given in Figure 3.9b, right-hand panel.

3.2.7 Molecular oscillation of overexpressed HA-CRY

CRY undergoes daily oscillation in level with high levels during the night and low levels during the day, illustrating the light sensitivity of the photoreceptor (Emery et al., 1998). In order to check the pattern of overexpression of HA-CRY (flies overexpressing HA-CRY were used in this experiment because of the lack of a CRY antibody), western blots were performed on flies overexpressing HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) in LD and DD conditions (Figure 3.10). Analysis of Figure 3.10 reveals that HA-CRY levels are low during the light phase and high during the dark phase of the LD cycle. Moreover, in DD, HA-CRY levels are constantly high. This profile of protein accumulation is consistent with published data on the accumulation profile of the endogenous CRY protein (Emery et al., 1998). In conclusion, overexpressed HA-CRY behaves as endogenous CRY in terms of light induced degradation of the protein.

3.3 Summary

Overexpressing CRY in a cry^{+} background does not have an effect on the period of locomotor activity in both LD and DD conditions. The molecular oscillations of PER and TIM are also unaffected by the upregulation of CRY. Nevertheless subtle differences are observed in the light sensitivity of CRY flies in LL and PRC experiments. In the PRC, this is especially the case for the advance

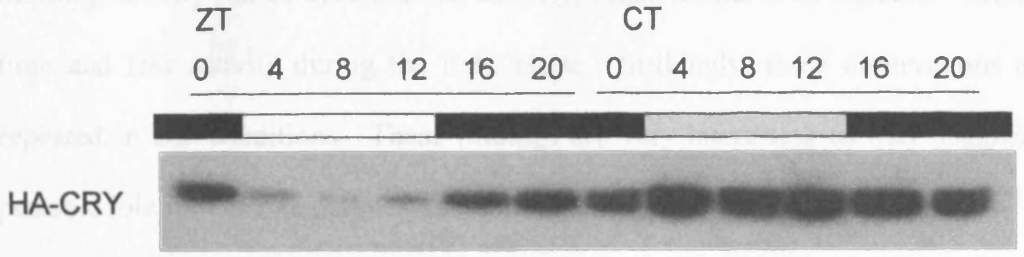


Figure 3.10: HA-CRY molecular oscillations. Molecular oscillations of overexpressed HA-CRY in heads of HA-CRY flies (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) in LD (ZT 0-20) and DD (CT 0-20) conditions. Equal amounts of head protein extracts from HA-CRY flies, were run on a 10% polyacrylamide gel and immunoblotted with mouse anti-HA antibody (Sigma). Filled bars= night or subjective night period, open bars= light period and grey bars= subjective day period. Similar experiments were repeated independently at least 3 times. A representative blot is shown.

portion of the curve. However, the most interesting differences are found in the analysis of the phase and distribution of activity. In LD, CRY flies show advanced morning activity and delayed evening activity, which results in an increased "siesta" time and less activity during the light phase. Strikingly, these observations are repeated in DD conditions. These findings are very interesting as they suggest a possible role for CRY in darkness, something that has not yet been proposed.

Chapter 4: Overexpression of CRY in a cry^b background

4.1 Introduction

Overexpressing CRY in a cry^+ genetic background does not change the flies' ability to entrain to LD cycles or alter the 24h free-running locomotor activity period. Western blot analysis show that PER and TIM oscillations are not affected in peripheral clocks. Nevertheless, subtle differences are observed in the distribution of activity in LD conditions. CRY flies have less activity during the light period because the two major components of activity are pushed towards the night phase; this is especially true for the morning peak. Changes in distribution of activity also occur in DD, with less activity during the subjective day, a result of an advanced timing of the morning component. Finally the PRC suggests that CRY flies are more responsive to pulses of light given in the late night. To investigate whether the above results are influenced by endogenous CRY, I repeated the experiments under cry^b background (the cry^b mutation is a strong hypomorph). Previous studies have shown that cry^b flies are severely blunted in all their responses to light. For instance, they show entrainment defects when subjected to a change in the phase of the LD cycle (Stanewsky et al., 1998; Helfrich-Forster et al., 2001) and behave as almost "blind" in PRC experiments with almost no phase-shift at all (Stanewsky et al., 1998). The most striking phenotype is the rhythmicity displayed by cry^b flies in LL, a condition that triggers arrhythmia in wild-type (Emery et al., 2000). Western blot analysis show that PER and TIM cycles are obliterated in peripheral tissues, with hyperphosphorylated forms of these proteins being constantly at a high level (Stanewsky et al., 1998). However, PER and TIM

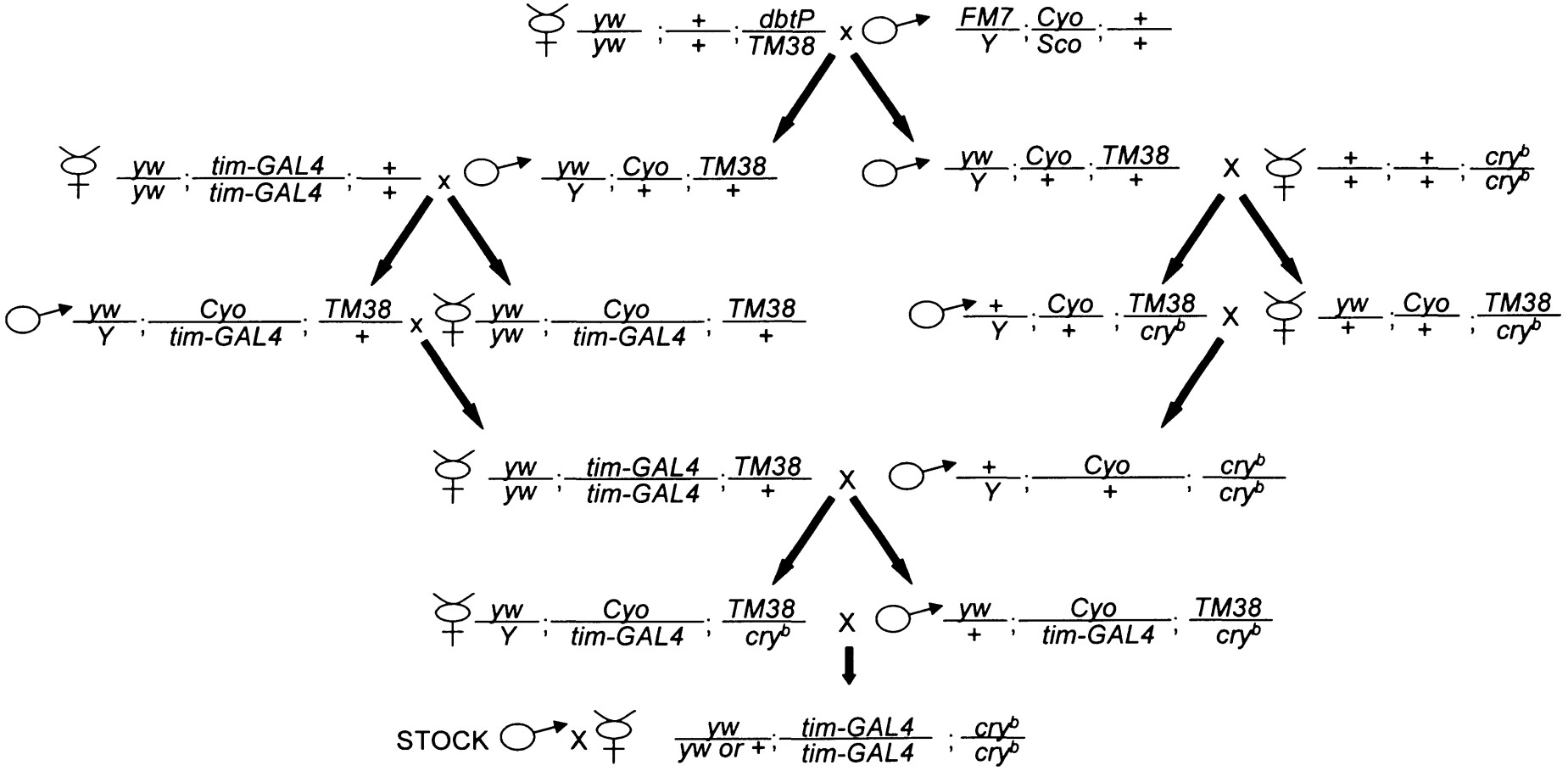


Figure 4.1: Scheme used to cross *tim-GAL4* in the *cry*^{b} mutant background. FM7, Cyo and TM38 are chromosome balancers for the X, second and third chromosome respectively. They prevent recombination and also carry a visible marker making them easy to identify.

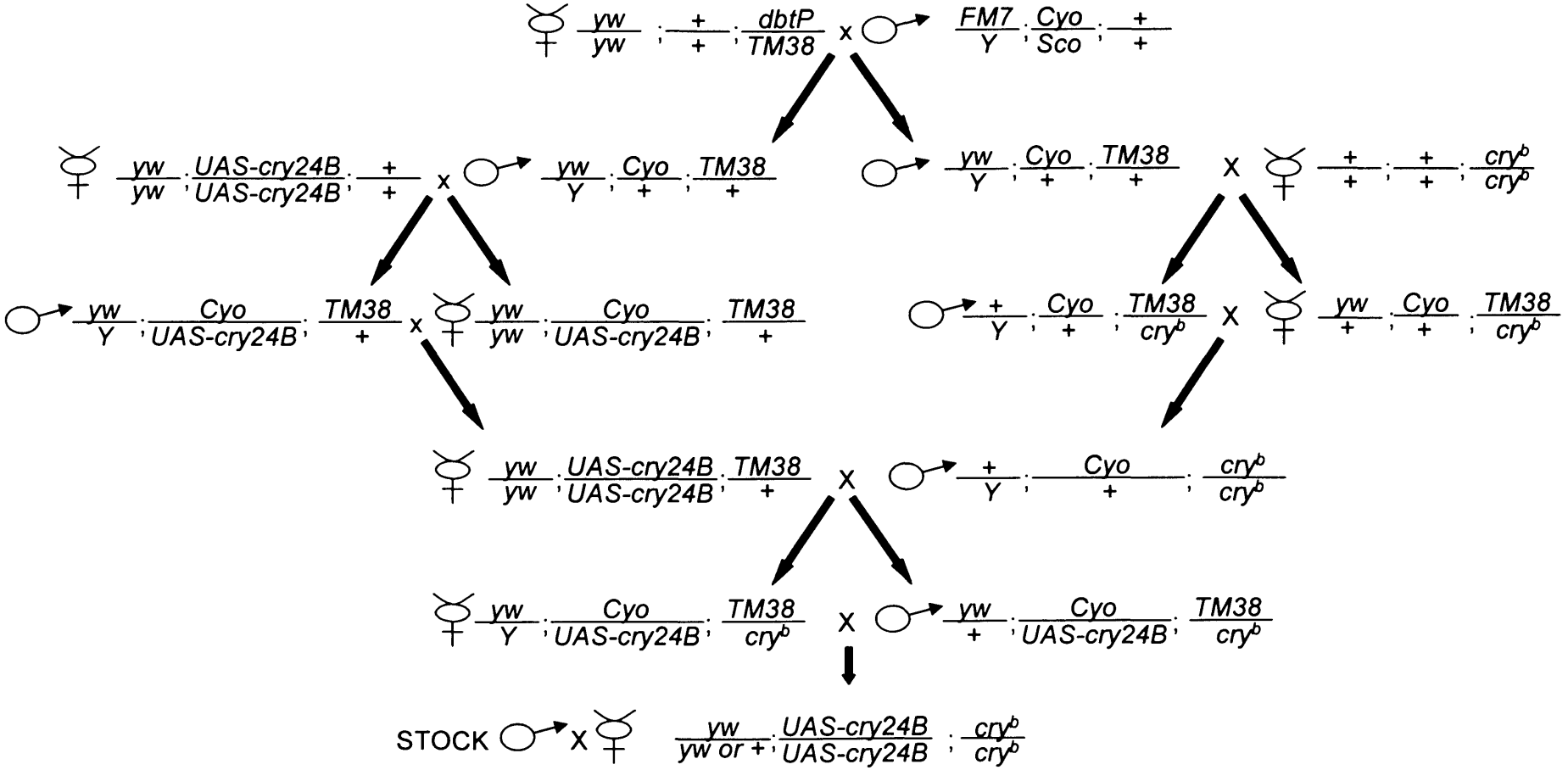


Figure 4.2: Scheme used to cross *UAS-cry24B* in the cry^b mutant background. FM7, Cyo and TM38 are chromosome balancers for the X, second and third chromosome respectively. They prevent recombination and also carry a visible marker making them easy to identify.

are cycling in the small ventral lateral neurons which explains the rhythmic behaviour observed in these flies (Stanewsky et al., 1998; Helfrich-Forster et al., 2001). In this chapter, I show the results obtained when CRY is overexpressed in the cry^b mutant background. To do so, the *tim-GAL4* driver and the *UAS-cry24B* transgene have been crossed in the cry^b mutant background; details of the crossing schemes are shown in Figure 4.1 and 4.2.

4.2 Results and discussion

4.2.1 Entrainment

Figure 4.3 shows the average locomotor activity pattern of male flies overexpressing CRY in cry^b background under a 12:12 LD regime (*yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b, thereafter named CRY(cry^b)). The relevant controls (*yw*; *tim-GAL4*/+; cry^b/ cry^b and *yw*; *UAS-cry24B*/+; cry^b/ cry^b) are shown for comparison. The two control strains show similar behaviour, namely bimodal activity with peaks centred on the dark/light and light/dark transitions. The morning peak is largely due to the startle response after lights-on rather than to real anticipation of the environmental switch, whereas the evening component of activity is showing normal anticipation. CRY(cry^b) flies are bimodal and they are clearly anticipating the morning activity. Both peaks of activity are centred on lights-on and off, which indicates that the flies entrain normally to the LD cycles. Fourier analysis (table 4.1) also shows that the entrained locomotor activity periods of CRY(cry^b) flies and the controls are very similar and very close to 24h.

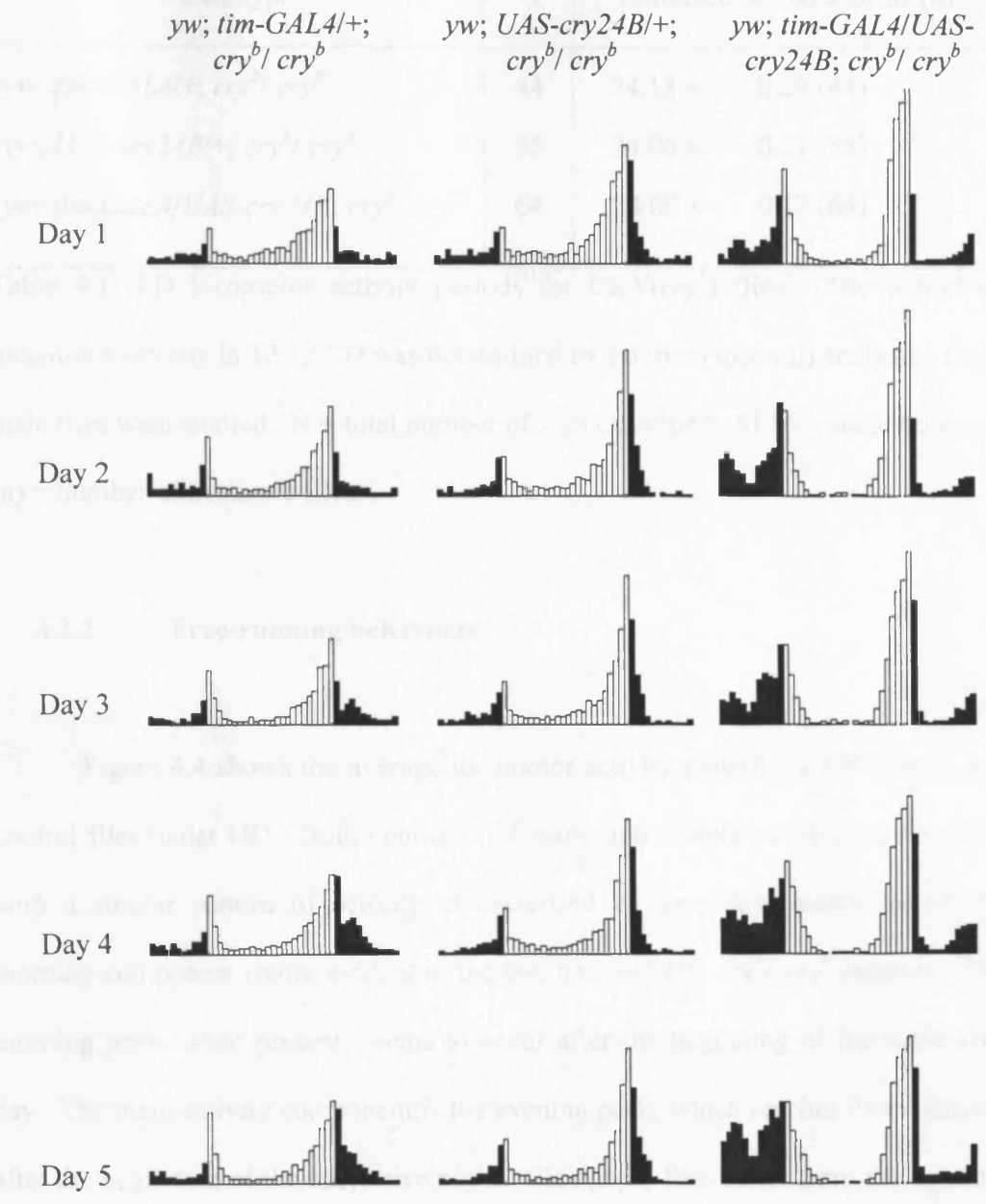


Figure 4.3: LD behaviour of CRY(cry^b) flies. Average locomotor activity pattern for male flies overexpressing CRY in cry^b background (*yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b) and the relevant controls (*yw*; *tim-GAL4/+*; cry^b/ cry^b and *yw*; *UAS-cry24B/+*; cry^b/ cry^b) for 5 days under a 12:12 LD regime. Open bars= light period, filled bars= dark period.

Genotype	N	Entrained period ± SEM (n)
yw; *tim-GAL4/+*; cry^b/ cry^b	44	24.18 ± 0.29 (44)
yw; *UAS-cry24B/+*; cry^b/ cry^b	55	24.06 ± 0.27 (55)
yw; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b	64	24.00 ± 0.17 (64)

Table 4.1: LD locomotor activity periods for CRY(cry^b) flies. The period of locomotor activity in 12:12 LD was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

4.2.2 Free-running behaviour

Figure 4.4 shows the average locomotor activity pattern for CRY(cry^b) and control flies under DD. Both controls (left-hand and middle panels) are rhythmic with a similar pattern of activity characterised by very low amplitude of the morning component (more evident in the *yw*; *tim-GAL4/+*; cry^b/ cry^b control). The morning peak, when present, seems to occur after the beginning of the subjective day. The main activity component is the evening peak, which reaches its maximum after the beginning of the subjective night. CRY(cry^b) flies differ from the controls as they display a robust bimodality with a clear bout of morning activity whose maximum is reached before the subjective day in the first 3 days of the experiments, and just after it for the last two days. The evening bout of activity peaks clearly after the onset of the subjective night. A visual inspection would suggest that it is delayed compared with the controls. Fourier analysis was used to calculate the free-running periods of these flies (table 4.2), showing that controls and CRY(cry^b) flies have the same 24h period ($F_{2,93}$=2.75, ns; appendix 7), hence upregulating CRY in

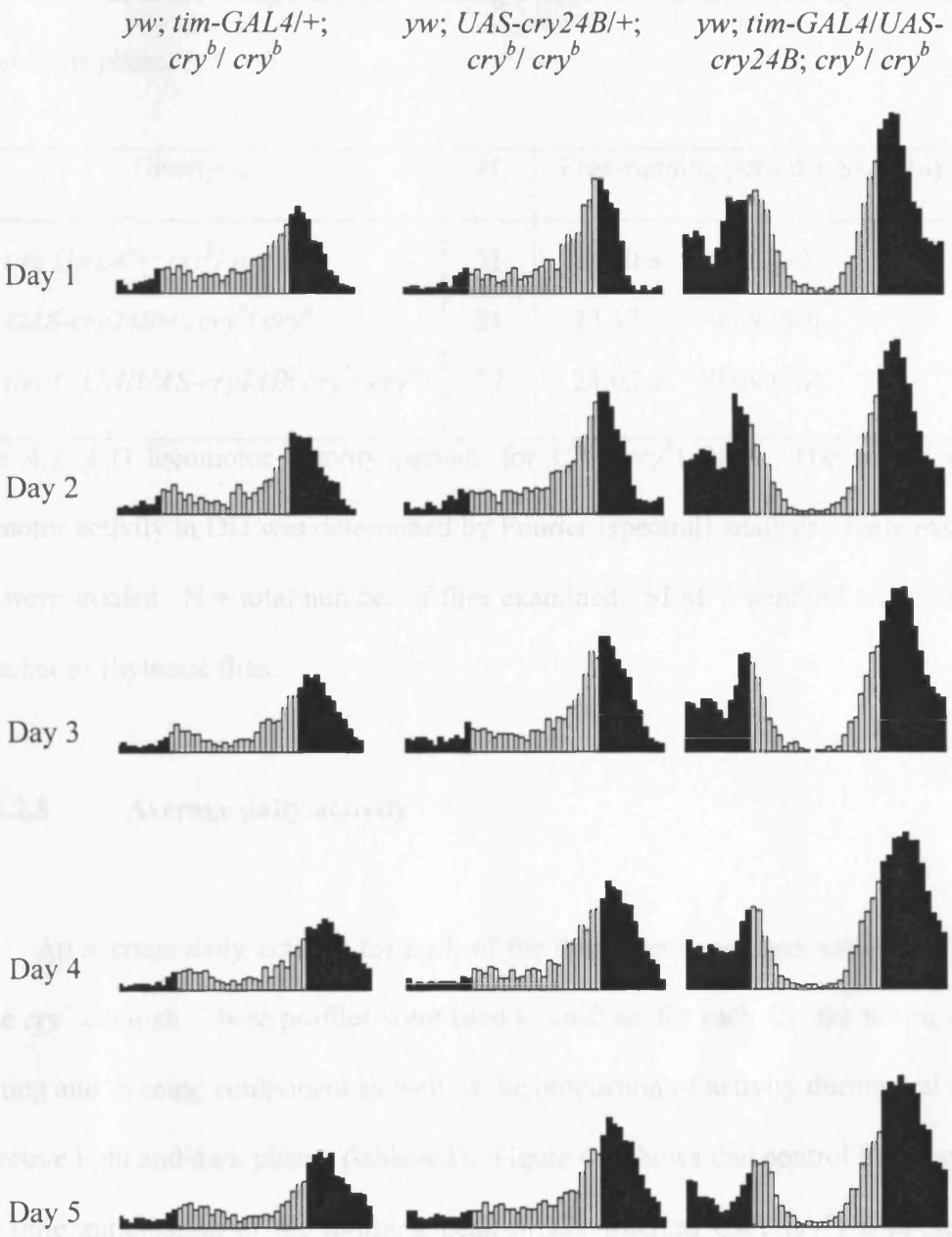


Figure 4.4: DD behaviour of CRY(cry^b) flies. Average locomotor activity pattern for male flies overexpressing CRY in cry^b background (*yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b) and the relevant controls (*yw*; *tim-GAL4/+*; cry^b/ cry^b and *yw*; *UAS-cry24B/+*; cry^b/ cry^b) for 5 days under DD conditions. Grey bars= subjective day period, filled bars= subjective night period.

cry^b mutants does not change the free-running period of locomotor activity but does influence its phase.

Genotype	N	Free-running period ± SEM (n)
yw; *tim-GAL4*/+; cry^b/ cry^b	31	24.30 ± 0.06 (30)
yw; *UAS-cry24B*/+; cry^b/ cry^b	31	24.37 ± 0.09 (30)
yw; *tim-GAL4*/*UAS-cry24B*; cry^b/ cry^b	37	24.03 ± 0.09 (36)

Table 4.2: DD locomotor activity periods for CRY(cry^b) flies. The period of locomotor activity in DD was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

4.2.3 Average daily activity

An average daily activity for each of the three genotypes was calculated as in the cry^+ context. These profiles were used to analyse, for each fly, the timing of morning and evening component as well as the proportion of activity during real or subjective light and dark phases (table 4.3). Figure 4.5 shows that control flies have very little anticipation of the morning peak in LD whereas CRY(cry^b) flies start their morning activity well before lights-on. The timing of the evening peak looks to be advanced in CRY(cry^b) flies compared with controls in which it is closer to the dark to light transition. Another difference is the reduced level of activity observed in CRY(cry^b) flies during the middle of the day. ANOVA reveals that controls are similar except for the timing of MP (appendix 8). Controls have the onset of morning activity at ZT23.07±0.14 (*yw*; *tim-GAL4*/+; cry^b/ cry^b) and ZT22.77±0.17 (*yw*; *UAS-cry24B*/+; cry^b/ cry^b) when CRY(cry^b) flies onset is

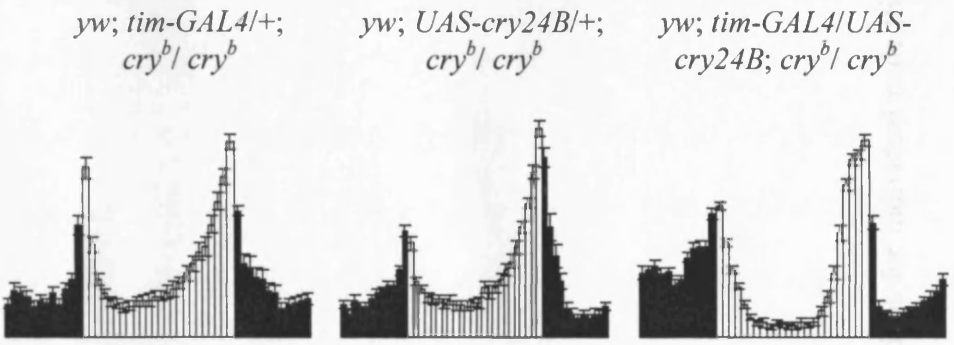


Figure 4.5: LD average daily activity for CRY(cry^b) flies. Average daily activity in 12:12 LD conditions for male flies overexpressing CRY in cry^b background (*yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b) and the relevant controls (*yw*; *tim-GAL4/+*; cry^b/ cry^b and *yw*; *UAS-cry24B/+*; cry^b/ cry^b). Open bars= light period, filled bars= dark period.

Genotype		*yw*; *tim-GAL4*/+; cry^b/ cry^b	*yw*; *UAS-cry24B*/+; cry^b/ cry^b	*yw*; *tim-GAL4*/*UAS-cry24B*; cry^b/ cry^b
Morning activity	Onset	23.07 ± 0.14 (30)	22.77 ± 0.17 (30)	21.76 ± 0.10 (34)
	Peak	0.42 ± 0.06 (30)	0.03 ± 0.07 (30)	0.19 ± 0.06 (34)
	Offset	3.73 ± 0.27 (30)	4.30 ± 0.21 (30)	3.78 ± 0.18 (34)
Evening activity	Onset	8.13 ± 0.31 (30)	7.98 ± 0.21 (30)	8.87 ± 0.12 (34)
	Peak	12.08 ± 0.14 (30)	12.12 ± 0.06 (30)	12.00 ± 0.04 (34)
	Offset	14.67 ± 0.25 (30)	14.48 ± 0.19 (30)	13.51 ± 0.10 (34)
Siesta		4.40 ± 0.34 (30)	3.68 ± 0.21 (30)	5.09 ± 0.25 (34)
Proportion of activity during light phase		0.67 ± 0.03 (30)	0.61 ± 0.03 (30)	0.61 ± 0.02 (34)
Proportion of activity during dark phase		0.33 ± 0.03 (30)	0.39 ± 0.03 (30)	0.39 ± 0.02 (34)

Table 4.3: LD average daily activity for CRY(cry^b) flies. Analysis of average daily activity in 12:12 LD conditions for individual male flies overexpressing CRY in cry^b background as well as the relevant controls.

significantly advanced by one hour ($F_{1,92}$=48.89, P<0.01, appendix 8). There are no differences regarding the offset of morning activity ($F_{1,92}$=0.77, ns, appendix 8). The onset of evening activity is significantly delayed in CRY(cry^b) flies ($F_{1,92}$=9.46, P<0.01, appendix 8). The timing of evening peak is similar (appendix 8). Offset of evening activity is significantly advanced by almost one hour in CRY(cry^b) flies ($F_{1,92}$=22.99, P<0.01, appendix 8). As a result of these observations, the "siesta" time is increased ($F_{1,92}$=10.22, P<0.01, appendix 8). Finally and surprisingly, the distribution of activity is unaffected in CRY(cry^b) flies with 60% of activity during the light phase, similar to the controls (appendix 8). However, this can be explained by the fact that although CRY(cry^b) flies start their morning activity well into the dark phase they also finish their evening activity earlier.

Figure 4.6 shows the average daily activity under DD for CRY(cry^b) flies (*yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b) as well as the controls (*yw*; *tim-GAL4*/+; cry^b/ cry^b and *yw*; *UAS-cry24B*/+; cry^b/ cry^b). The two controls have the morning and evening peaks located on the subjective night/day and day/night transitions respectively. CRY(cry^b) flies show differences from the controls with morning peak located well into the subjective night and evening peak after the beginning of the subjective night. The analysis of individual flies is reported in table 4.4. It reveals that most of the flies used for this experiment have a morning component of activity, there are no differences between controls and CRY(cry^b) flies. ANOVA shows that controls are similar for timing of MP and EP, phase difference and distribution of activity (appendix 9). The morning peak of activity is situated into the subjective day for the controls at CT0.50±0.32 and CT0.41±0.26. CRY(cry^b) flies have their peak about one hour earlier at CT23.49±0.20 ($F_{1,88}$=10.37, P<0.01,

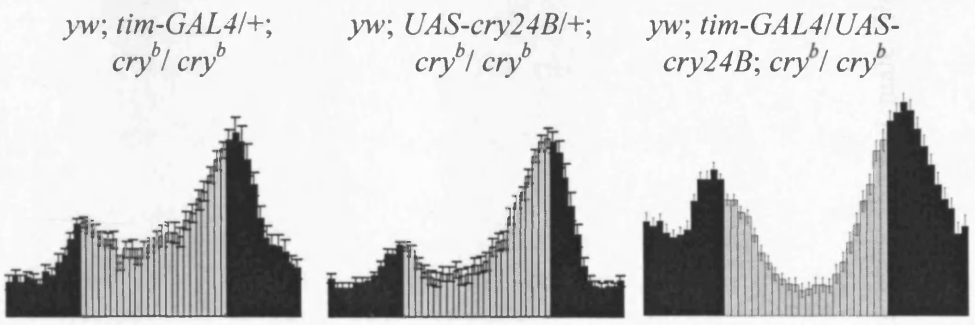


Figure 4.6: DD average daily activity for CRY(cry^b) flies. Average daily activity in DD conditions for male flies overexpressing CRY in cry^b background (*yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b) and the relevant controls (*yw*; *tim-GAL4/+*; cry^b/ cry^b and *yw*; *UAS-cry24B/+*; cry^b/ cry^b). Grey bars= subjective day period, filled bars= subjective night period.

Genotype	*yw*; *tim-GAL4*/+; cry^b/ cry^b	*yw*; *UAS-cry24B*/+; cry^b/ cry^b	*yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b
Number of flies examined	29	29	35
Number of flies where morning peak was present	28 (97%)	27 (93%)	35 (100%)
Morning peak	0.50 ± 0.32	0.41 ± 0.26	23.49 ± 0.20
Evening peak	13.03 ± 0.34	12.05 ± 0.26	13.64 ± 0.24
Phase difference	12.52 ± 0.30	11.87 ± 0.35	14.16 ± 0.24
Proportion of activity during subjective day	0.50 ± 0.04	0.58 ± 0.03	0.31 ± 0.02
Proportion of activity during subjective night	0.50 ± 0.04	0.42 ± 0.03	0.69 ± 0.02

Table 4.4: DD average daily activity for CRY(cry^b) flies. Analysis of average daily activity in DD conditions for individual male flies overexpressing CRY in cry^b background as well as the relevant controls.

appendix 9). The timing of the evening peak is significantly delayed in CRY(cry^b) flies ($F_{1,88}$=9.07, P<0.01, appendix 9). The effects observed on both peaks of activity result in an increased phase difference ($F_{1,88}$=31.25, P<0.01, appendix 9). Advanced morning activity and delayed evening activity also change the distribution of activity with only 30% of total activity occurring during the subjective day period compared to a 50% proportion for controls ($F_{1,88}$=34.50, P<0.01, appendix 9). In conclusion expressing CRY in the cry^b mutant background does not alter the period of locomotor activity of flies (entrained and free-running) but affects the phase of activity. This is characterised by an advance in the morning activity bout both under LD and DD conditions while the effect on the evening component of activity is less pronounced.

4.2.4 Response of CRY(cry^b) flies to constant light exposure

cry^b flies show abnormalities in their light responses (Stanewsky et al., 1998; Emery et al., 2000). For instance, they are rhythmic upon exposure to high level of light (Emery et al., 2000). Controls and CRY(cry^b) flies were analysed under LL conditions for 6 subsequent days, the first one being in normal LD until lights did not go off at ZT12, releasing the flies in LL (Figure 4.7). Both controls are rhythmic in LL, with the evening peak as the main component of activity. The phase of activity changes as soon as the flies enter the LL phase on the evening of the last day in LD. The maximum of evening activity happen well after the beginning of the LL period and this phase is kept on subsequent days in LL. CRY(cry^b) flies are completely different from the controls as they show rhythmicity for one day in LL, then become arrhythmic. This reveals that the upregulation of

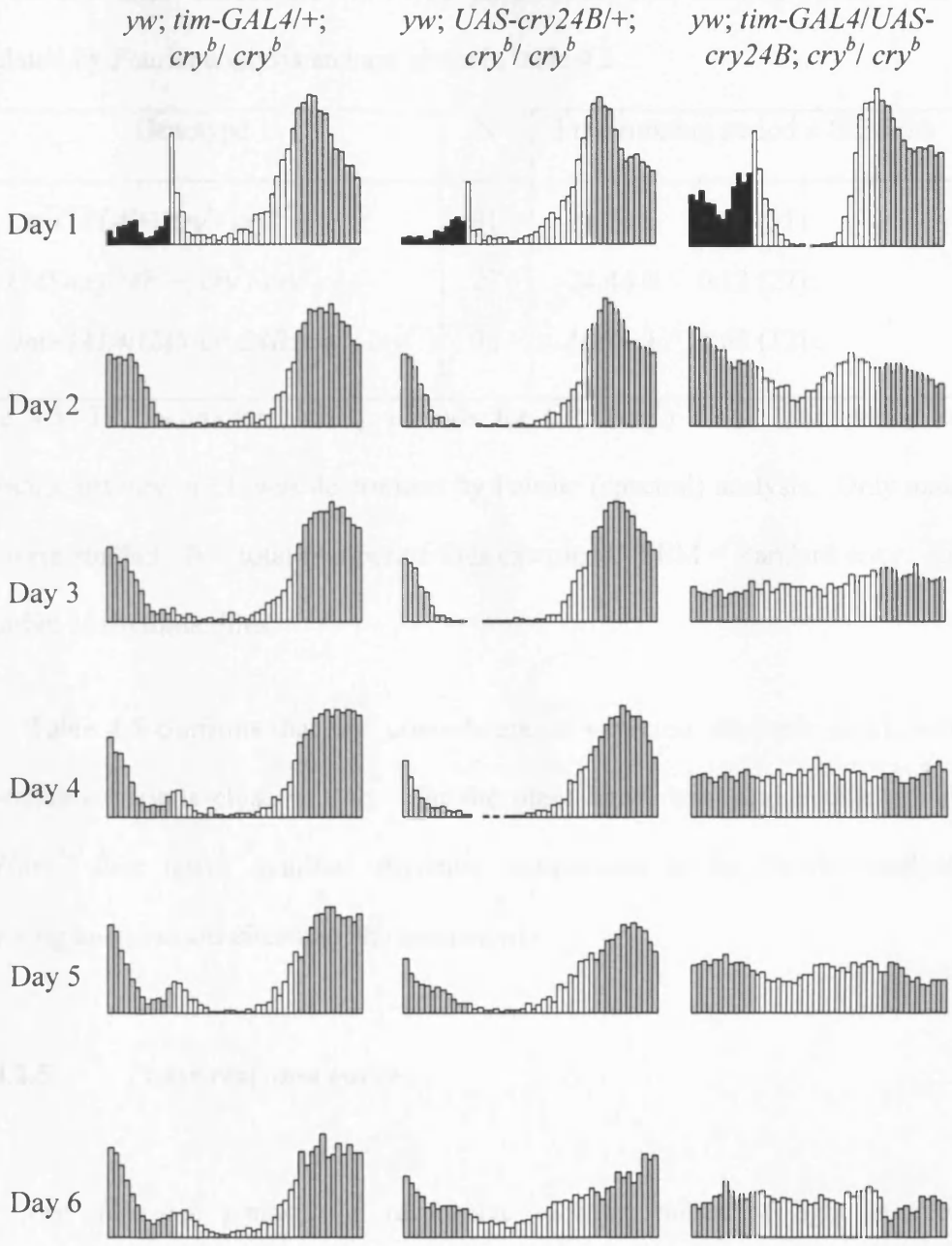


Figure 4.7: LL behaviour of CRY(cry^b) flies. Average locomotor activity pattern for male flies overexpressing CRY in cry^b background (*yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b) and the relevant controls (*yw*; *tim-GAL4*/+; cry^b/ cry^b and *yw*; *UAS-cry24B*/+; cry^b/ cry^b) for 1 day in 12:12 LD followed by 5 days under a LL regime. Open bars= subjective day period, grey bars= subjective night period.

CRY in cry^b flies rescues the wild-type phenotype. Free-running periods were calculated by Fourier analysis and are given in table 4.5.

Genotype	N	Free-running period ± SEM (n)
yw; *tim-GAL4*/+; cry^b/ cry^b	31	24.14 ± 0.05 (31)
yw; *UAS-cry24B*/+; cry^b/ cry^b	27	24.44 ± 0.12 (27)
yw; *tim-GAL4*/*UAS-cry24B*; cry^b/ cry^b	38	18.81 ± 1.67 (12)

Table 4.5: LL locomotor activity periods for CRY(cry^b) flies. The period of locomotor activity in LL was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

Table 4.5 confirms that cry^b controls are, as expected, rhythmic in LL with free-running periods close to 24h. On the other hand, less than a third of all CRY(cry^b) flies tested manifest rhythmic components in the Fourier analysis indicating an increased circadian photosensitivity.

4.2.5 Phase response curve

cry^b flies are almost non responsive to short pulses of light in PRC experiments (Stanewsky et al., 1998). CRY(cry^b) and control flies were subjected to a 5 minutes light pulse at ZT15 and ZT21. These were selected because they give different responses to a light pulse in wild-type as well as being characterised by a differential subcellular localization of the PER and TIM proteins. At ZT15, PER and TIM are mainly cytoplasmic and a phase-delay is observed in a PRC experiment. The situation is opposite at ZT21, with mostly nuclear PER and TIM

and a phase-advance (Young, 1998). Figure 4.8 and table 4.6 illustrate the phase-shifts obtained.

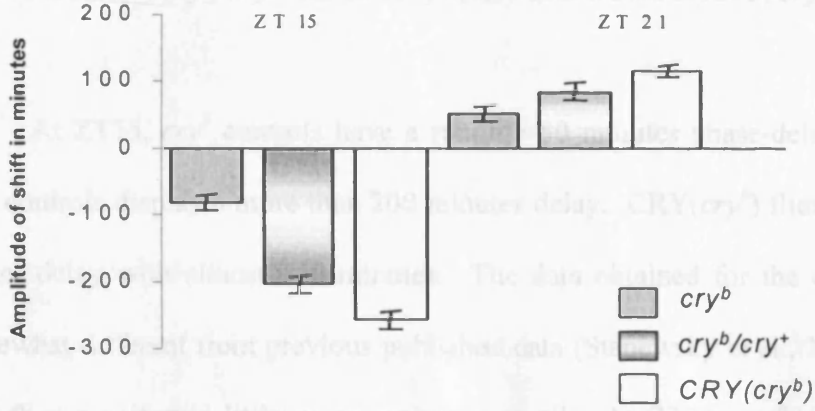


Figure 4.8: PRC of CRY(*cry*^{b}) flies. Phase-shifts for CRY(*cry*^{b}) (*yw*; *tim-GAL4/UAS-cry24B*; *cry*^{b}/ *cry*^{b}) and control flies (*yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b} and *yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*$^+$). Amplitude of shifts obtained in minutes with pulses given at ZT15 and ZT21. Numbers of pulsed flies are given in table 4.6. Numbers of unpulsed control flies were 57 for *yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b}, 73 for *yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*$^+$ and 68 for *yw*; *tim-GAL4/UAS-cry24B*; *cry*^{b}/ *cry*^{b}. Error bars represent standard errors.

Genotype		*yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b}	*yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*$^+$	*yw*; *tim-GAL4/UAS-cry24B*; *cry*^{b}/ *cry*^{b}
Amplitude of shift in minutes at ZT	15 (n)	-81.75 ± 11.91(40)	-205.47±13.27(45)	-257.40 ±13.76(40)
	21 (n)	51.21 ±11.61(35)	84.03 ± 13.09(41)	114.79 ± 8.44 (64)

Table 4.6: PRC of CRY(cry^b) flies. Amplitude of shift obtained in minutes ± SEM with a 5 minutes light pulse given at the indicated ZT. (n) = number of flies pulsed. Numbers of unpulsed control flies were 57 for *yw*; *tim-GAL4*/+; cry^b/cry^b; 73 for *yw*; *tim-GAL4*/+; cry^b/ cry^+ and 68 for *yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b

At ZT15, cry^b controls have a roughly 80 minutes phase-delay, when cry^b/ cry^+ controls display a more than 200 minutes delay. CRY(cry^b) flies have an even bigger delay with almost 260 minutes. The data obtained for the cry^b control is somewhat different from previous published data (Stanewsky et al., 1998) in which cry^b flies manifested little or no response at all. At ZT21, cry^b controls phase-advance by 50 minutes and cry^b/ cry^+ controls by 84 minutes. CRY(cry^b) flies have a phase-advance of more than 110 minutes. Again cry^b flies appear more responsive in this study than in previous work (Stanewsky et al., 1998). Different light intensities used might provide an explanation for these differences between studies. CRY(cry^b) flies are more responsive at both ZTs tested revealing that the wild-type phenotype has been rescued ($F_{1,78}=93.20$, $P<0.01$ at ZT15 and $F_{1,97}=19.80$, $P<0.01$ at ZT21, appendix 10). Interestingly, there seems to be a correlation between levels of CRY and amplitude of response as cry^b/ cry^+ controls are more responsive than cry^b (Although marginally not significant by ANOVA, appendix 10), but not as much as CRY(cry^b) flies which due to the overexpression have more CRY than the other two lines.

4.2.6 Molecular cycling of PER and TIM in CRY(cry^b) flies

Western blots analysis show that PER and TIM do not cycle in head extracts of cry^b mutants (Stanewsky et al., 1998). Moreover high levels of hyperphosphorylated forms of these proteins are found at all times (Stanewsky et al., 1998). PER and TIM molecular oscillations were assayed for CRY(cry^b) and controls in both LD and DD conditions (Figure 4.9). Figure 4.9a shows that in LD there is no oscillation in TIM levels in cry^b controls (*yw*; *tim-GAL4*/+; cry^b/ cry^b), levels are constantly high with hyperphosphorylated forms visible at all time points. CRY(cry^b) flies show cycling of TIM with maximal and minimal levels reached at ZT20 and ZT4-8 respectively. Phosphorylated forms of TIM are seen at night, especially at ZT0. The quantification (Figure 4.9a left-hand panel) reveals that TIM levels in controls are equivalent to the highest levels found in CRY(cry^b) late at night. Similarly, control flies show no cycle for PER in LD with relatively high protein levels. Normal oscillation is rescued in CRY(cry^b) flies with maximum and minimum at ZT20 and ZT8, respectively.

In DD, TIM is again non-cycling in cry^b controls with high levels throughout the circadian cycle and abnormally high levels of hyperphosphorylated forms. CRY(cry^b) flies show oscillation with maximum at CT20 and minimum at CT8. Phosphorylated forms could be seen throughout the cycle but are more abundant during the subjective night. Controls do not show significant cycling for PER in DD whereas CRY(cry^b) flies manifest cycling with maximum at CT0 and minimum at CT4-8. In conclusion, upregulating CRY in tim^+ expressing cells of cry^b mutants restores near wild-type molecular oscillation of PER and TIM in agreement with published observations (Emery et al., 2000).

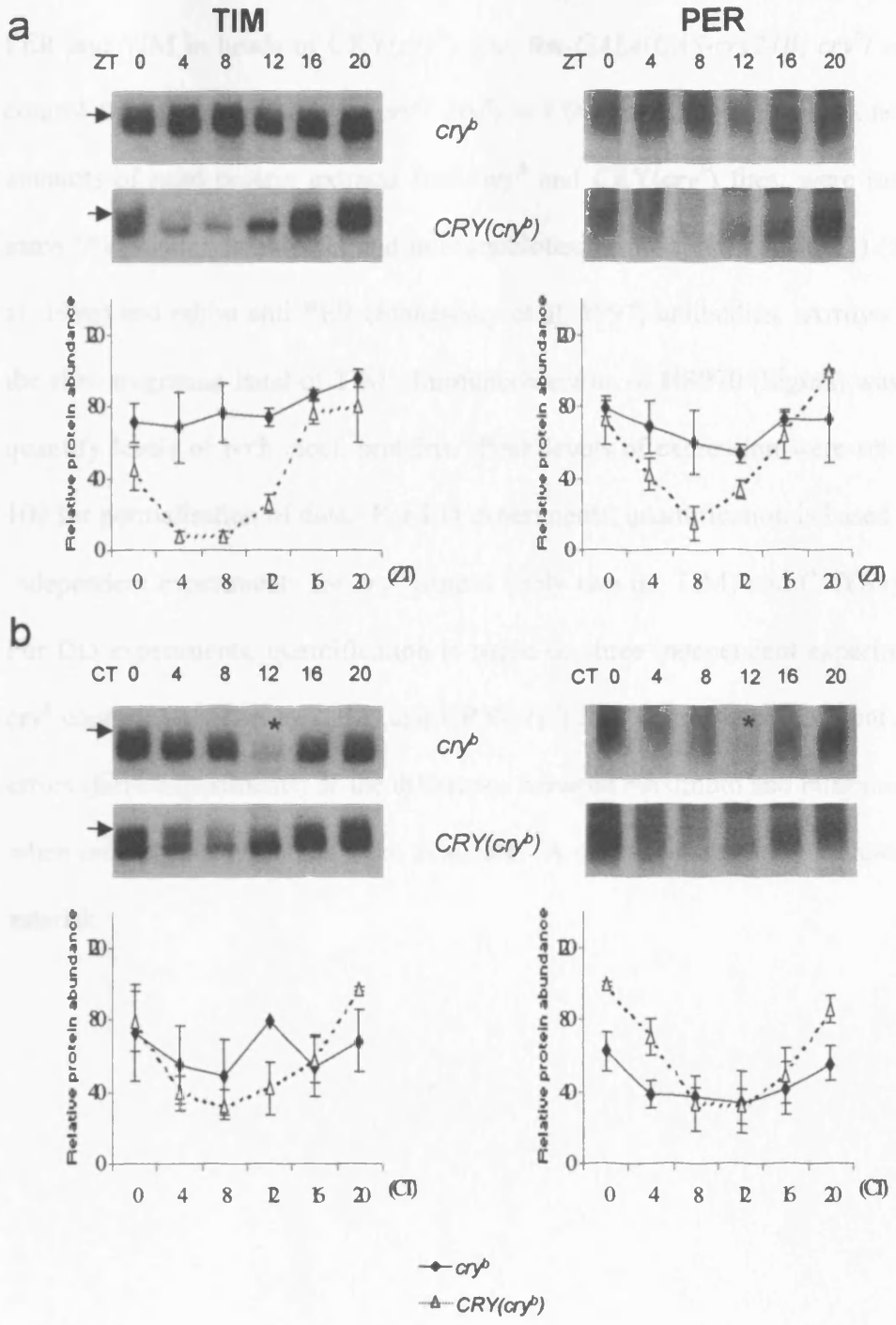

a
TIM
PER
ZT 0 4 8 12 16 20
cry^b
CRY(cry^b)
Relative protein abundance
(ZT)
b
CT 0 4 8 12 16 20
(CT)
cry^b
CRY(cry^b)

Figure 4.9: PER and TIM oscillations in CRY(cry^b) flies. Molecular oscillations of PER and TIM in heads of CRY(cry^b) (*yw*; *tim-GAL4/UAS-cry24B*; cry^b/ cry^b) and control flies (*yw*; *tim-GAL4*/+; cry^b/ cry^b) in LD(a) and DD (b) conditions. Equal amounts of head protein extracts from cry^b and CRY(cry^b) flies, were run on the same 6% polyacrylamide gel and immunoblotted with rat anti-TIM(307) (Myers et al. 1996) and rabbit anti-PER (Stanewsky et al. 1997) antibodies. Arrows indicate the slow migrating band of TIM. Immunodetection of HSP70 (Sigma) was used to quantify levels of both clock proteins. Peak levels of expression were set equal to 100 for normalisation of data. For LD experiments, quantification is based on three independent experiments for cry^b control (only two for TIM) and CRY(cry^b) flies. For DD experiments, quantification is based on three independent experiments for cry^b control (only two for TIM) and CRY(cry^b) flies. Error bars represent standard errors (three experiments) or the difference between maximum and minimum values when only two experiments were available. A degraded sample is indicated by an asterisk.

4.3 Summary

Targeted overexpression of CRY in *tim* expressing cells results in a rescue of all the phenotypes affected by the cry^b mutation. CRY(cry^b) flies are arrhythmic in LL, respond normally to light pulses given at ZT15 and ZT21 and finally show normal molecular oscillations of PER and TIM. The analysis of average daily activity suggests that as CRY(cry^+) flies, CRY(cry^b) have an advanced morning component of activity both in LD and DD conditions. This result gives further support for a possible role of CRY in darkness.

Chapter 5: Overexpression of CRYΔ in a *cry*$^{+}$ background

5.1 Introduction

When 20 amino acids at the C-terminus of CRY are removed to create CRYΔ, the light dependency of CRY interactions in a yeast two-hybrid assay is lost suggesting that CRYΔ behaves as a constitutively active form of the protein as it is able to bind PER and TIM in a light independent manner (Rosato et al., 2001). In this chapter I shall present data regarding the overexpression of CRYΔ in wild-type flies. To do so, several driver lines were used, the previously mentioned *tim-GAL4* line (Emery et al., 1998) which drives expression in all *timeless* expressing cells as well as the lateral neurons specific *Pdf-GAL4* and *gal1118* drivers (Blanchardon et al., 2001; Renn et al., 1999). The hypothesis was that CRYΔ flies should manifest phenotypes normally associated with constant light exposure. CRYΔ flies were assayed for locomotor behaviour as well as molecular cycles of PER and TIM in peripheral and central clocks.

5.2 Results and discussion

5.2.1 Overexpression of CRYΔ in all *timeless* expressing cells

5.2.1.1 Entrainment

Figure 5.1 shows the average locomotor activity pattern for male flies overexpressing CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) as well as the relevant

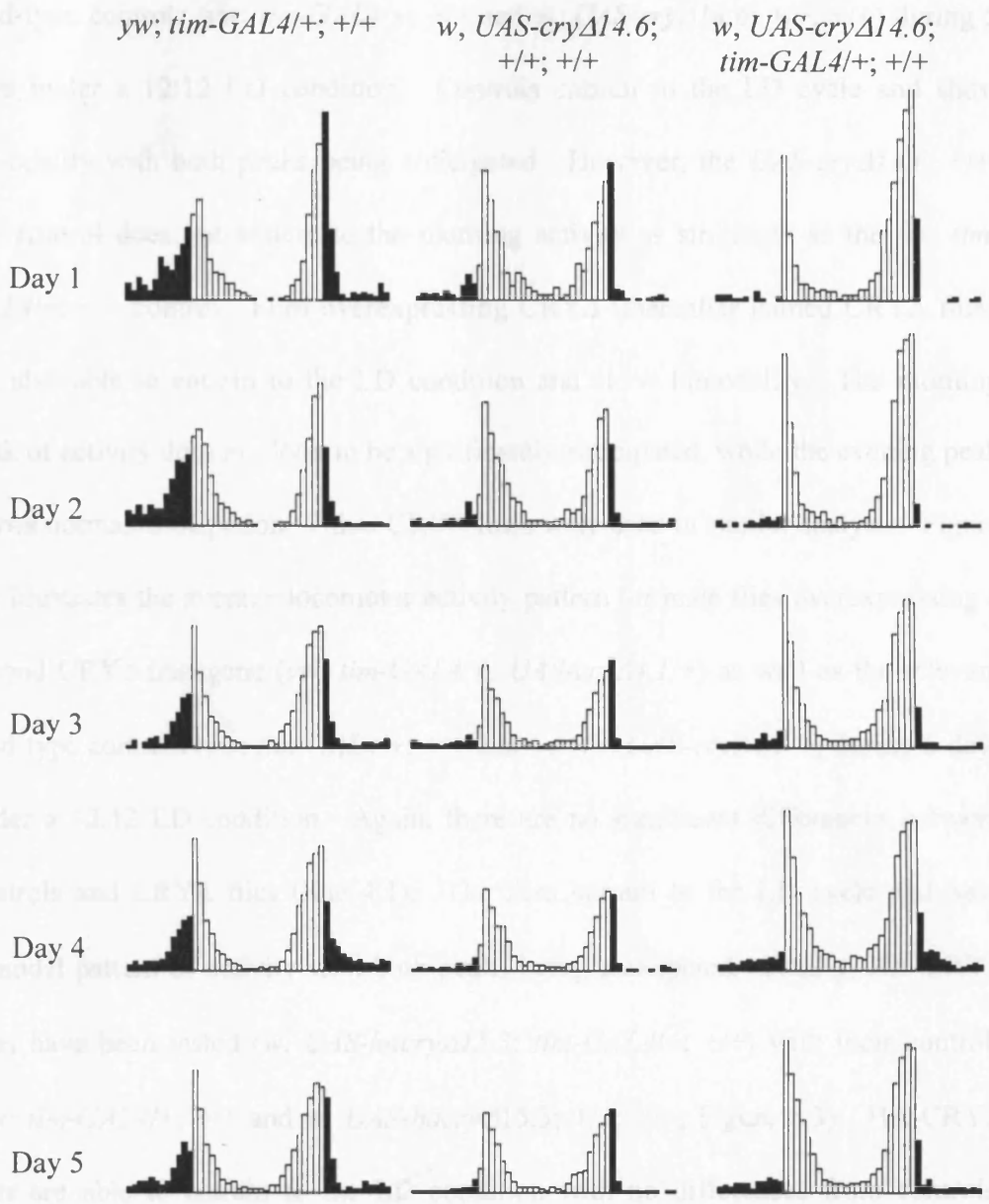


Figure 5.1: LD behaviour of CRYΔ (line 14.6) flies. Average locomotor activity pattern for male flies overexpressing CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *w*, *UAS-cryΔ14.6*; +/+; +/+) during 5 days under a 12:12 LD condition. Open bars= light period, filled bars= dark period.

wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *w*, *UAS-cryΔ14.6*; +/+; +/+) during 5 days under a 12:12 LD condition. Controls entrain to the LD cycle and show bimodality with both peaks being anticipated. However, the *UAS-cryΔ14.6*; +/+; +/+ control does not anticipate the morning activity as strikingly as the *yw*; *tim-GAL4*/+; +/+ control. Flies overexpressing CRYΔ (thereafter named CRYΔ flies) are also able to entrain to the LD condition and show bimodality. The morning peak of activity does not look to be significantly anticipated, while the evening peak shows normal anticipation. Other CRYΔ lines were used in similar analysis. Figure 5.2 illustrates the average locomotor activity pattern for male flies overexpressing a second CRYΔ transgene (*yw*; *tim-GAL4*/+; *UAS-cryΔ4.1*/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *w*; +/+; *UAS-cryΔ4.1*/+) during 5 days under a 12:12 LD condition. Again, there are no significant differences between controls and CRYΔ flies (line 4.1). The flies entrain to the LD cycle and have bimodal pattern of activity with both peaks being anticipated. Finally, HA-CRYΔ flies have been tested (*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) with their controls (*yw*; *tim-GAL4*/+; +/+ and *w*, *UAS-hacryΔ*15.3; +/+; +/+, Figure 5.3). HA-CRYΔ flies are able to entrain to the LD condition with no differences from controls. Fourier analysis was applied to all these genotypes to calculate the entrained locomotor activity periods (table 5.1).

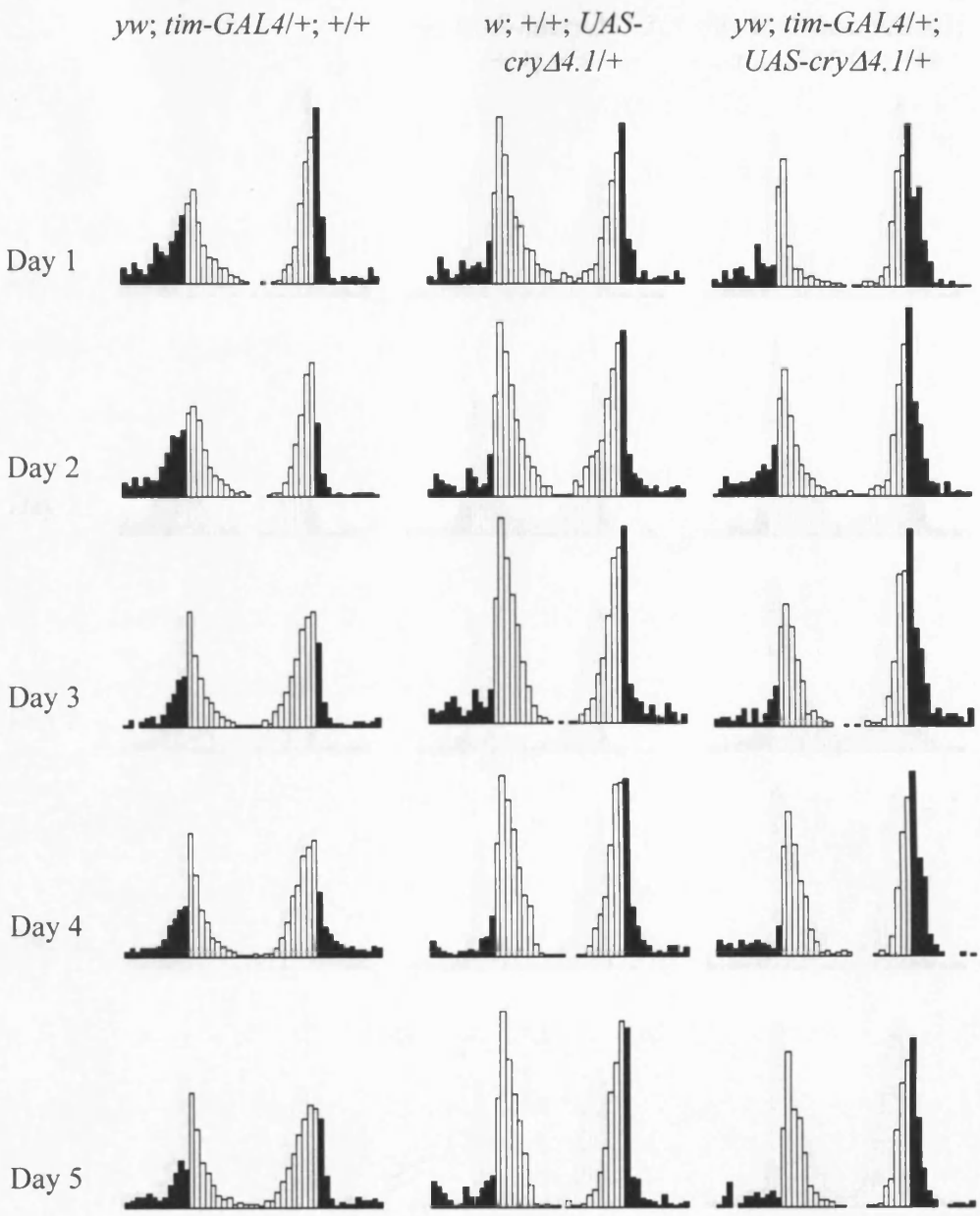


Figure 5.2: LD behaviour of CRYΔ (line 4.1) flies. Average locomotor activity pattern for male flies overexpressing a second CRYΔ transgene (*yw*; *tim-GAL4*/+; *UAS-cryΔ4.1*/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *w*; +/+; *UAS-cryΔ4.1*/+) during 5 days under a 12:12 LD condition. Open bars= light period, filled bars= dark period.

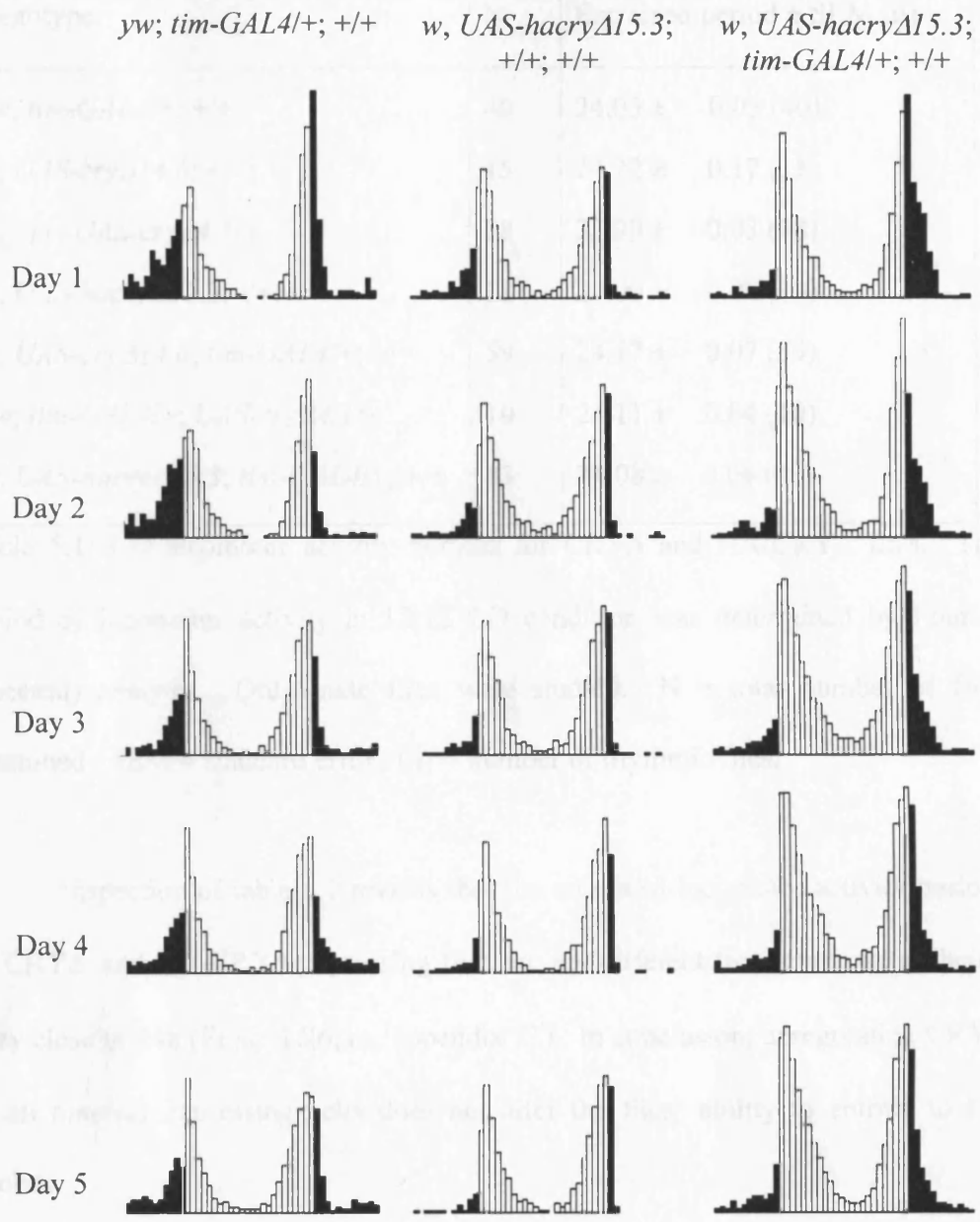


Figure 5.3: LD behaviour of HA-CRYΔ flies. Average locomotor activity pattern for male flies overexpressing HA-CRYΔ (*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *w*, *UAS-hacryΔ15.3*; +/+; +/+, left hand and middle panel respectively) during 5 days under a 12:12 LD condition. Open bars= light period, filled bars= dark period.

Genotype	N	Entrained period ± SEM (n)
yw; *tim-GAL4*/+; +/+	40	24.03 ± 0.03 (40)
w, *UAS-cryΔ14.6*; +/+; +/+	15	24.22 ± 0.17 (15)
w; +/+; *UAS-cryΔ4.1*/+	18	23.99 ± 0.03 (18)
w, *UAS-hacryΔ*15.3;+/+;+/+	18	24.00 ± 0.02 (18)
w, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+	59	24.17 ± 0.07 (59)
yw; *tim-GAL4*/+; *UAS-cryΔ4.1*/+	10	24.11 ± 0.04 (10)
w, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+	43	24.08 ± 0.04 (43)

Table 5.1: LD locomotor activity periods for CRYΔ and HA-CRYΔ flies. The period of locomotor activity in 12:12 LD condition was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

Inspection of table 5.1 reveals that the entrained locomotor activity periods of CRYΔ and HA-CRYΔ expressing flies are not different from controls, all being very close to 24h ($F_{6,196}$=1.26, ns, appendix 11). In conclusion, upregulating CRYΔ in all *timeless* expressing cells does not alter the flies' ability to entrain to LD cycles.

5.2.1.2 Free-running behaviour

Figure 5.4 shows the average locomotor activity pattern of CRYΔ flies (line 14.6) and controls during 5 subsequent days under DD, after an initial period of entrainment of at least 5 days under 12:12 LD cycles. Here again, subjective day period is coloured in grey to allow better visual comparison. Not surprisingly, control flies are rhythmic in DD. The *UAS-cryΔ14.6*; +/+; +/+ control has a very

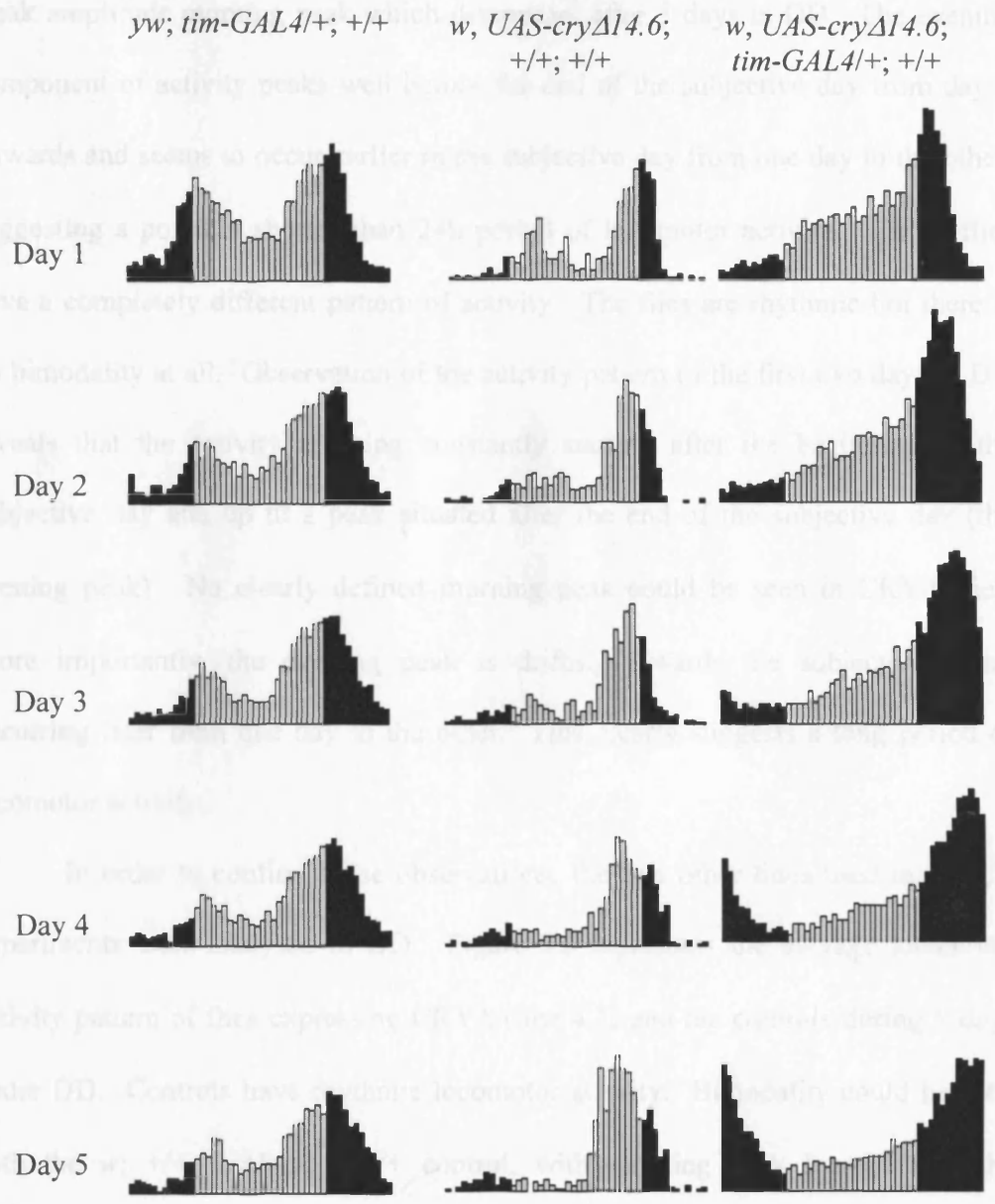


Figure 5.4: DD behaviour of CRYΔ (line 14.6) flies. Average locomotor activity pattern for male flies overexpressing CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *w*, *UAS-cryΔ14.6*; +/+; +/+) during 5 days under DD conditions. Grey bars= subjective day period, filled bars= subjective night period.

weak amplitude morning peak which disappears after 5 days in DD. The evening component of activity peaks well before the end of the subjective day from day 2 onwards and seems to occur earlier in the subjective day from one day to the other, suggesting a possible shorter than 24h period of locomotor activity. CRYΔ flies have a completely different pattern of activity. The flies are rhythmic but there is no bimodality at all. Observation of the activity pattern of the first two days in DD reveals that the activity is rising constantly starting after the beginning of the subjective day and up to a peak situated after the end of the subjective day (the evening peak). No clearly defined morning peak could be seen in CRYΔ flies. More importantly, the evening peak is drifting towards the subjective night, occurring later from one day to the other. This clearly suggests a long period of locomotor activity.

In order to confirm these observations, the two other lines used in the LD experiments were analysed in DD. Figure 5.5 represents the average locomotor activity pattern of flies expressing CRYΔ (line 4.1) and the controls during 5 days under DD. Controls have rhythmic locomotor activity. Bimodality could be seen with the *w*; +/+; *UAS-cryΔ4.1*/+ control, with morning peak located into the subjective day and evening peak occurring well before the beginning of subjective night. Here again, overexpressing CRYΔ results in an apparent lengthening of the locomotor activity period as the evening peak is pushed more into the subjective night from one day to the other. The morning component could be seen on the first day in DD in this CRYΔ line but it then disappears for the remainder of the experiment. Figure 5.6 shows the average locomotor activity pattern of HA-CRYΔ flies (line 15.3) as well as the relevant controls during a similar experiment in DD. The *w*, *UAS-hacryΔ15.3*; +/+; +/+ control shows clear bimodal activity for the first

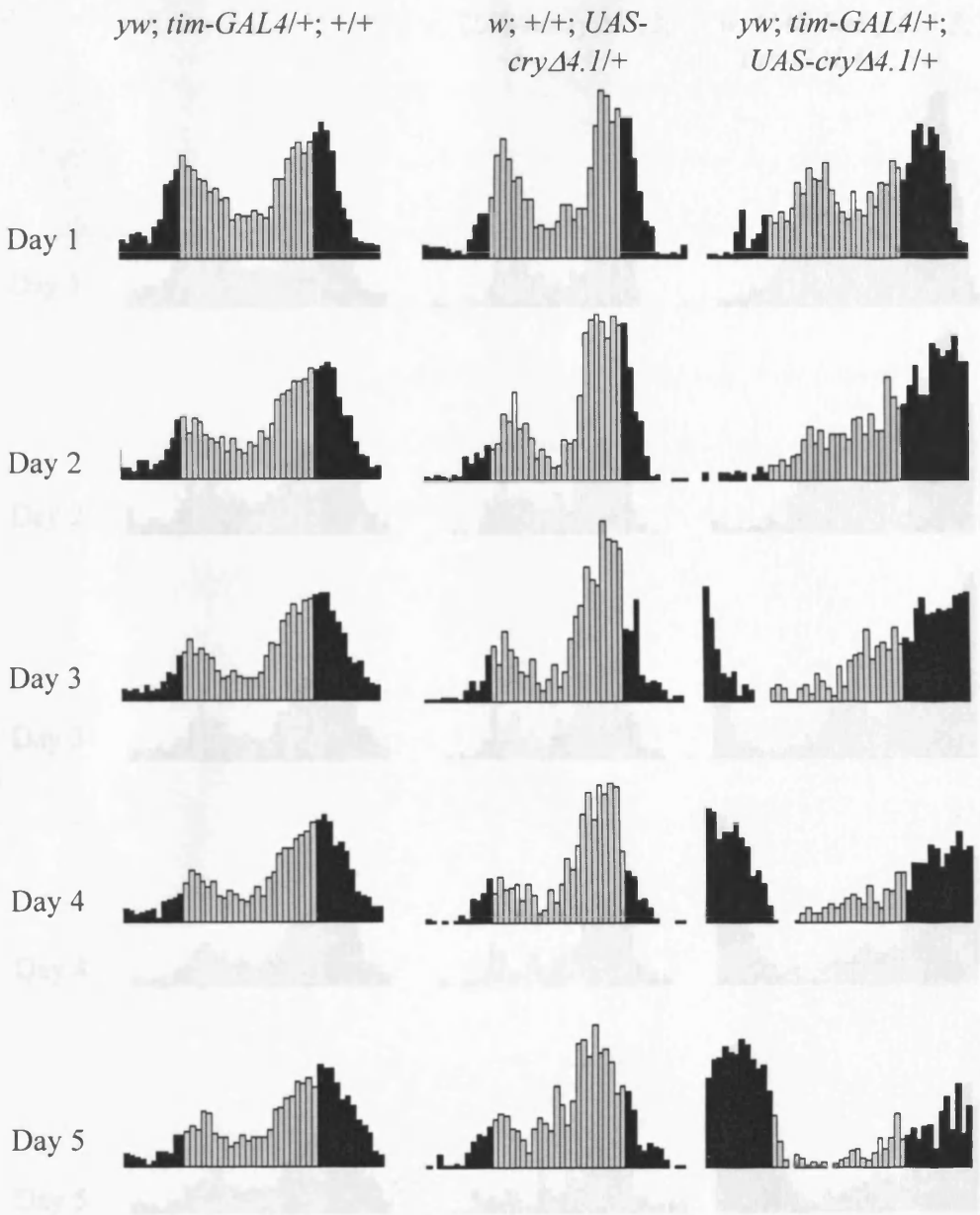


Figure 5.5: DD behaviour of CRYΔ (line 4.1) flies. Average locomotor activity pattern for male flies overexpressing a second CRYΔ transgene (*yw*; *tim-GAL4*/+; *UAS-cryΔ4.1*/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *w*; +/+; *UAS-cryΔ4.1*/+) during 5 days under DD conditions. Grey bars= subjective day period, filled bars= subjective night period.

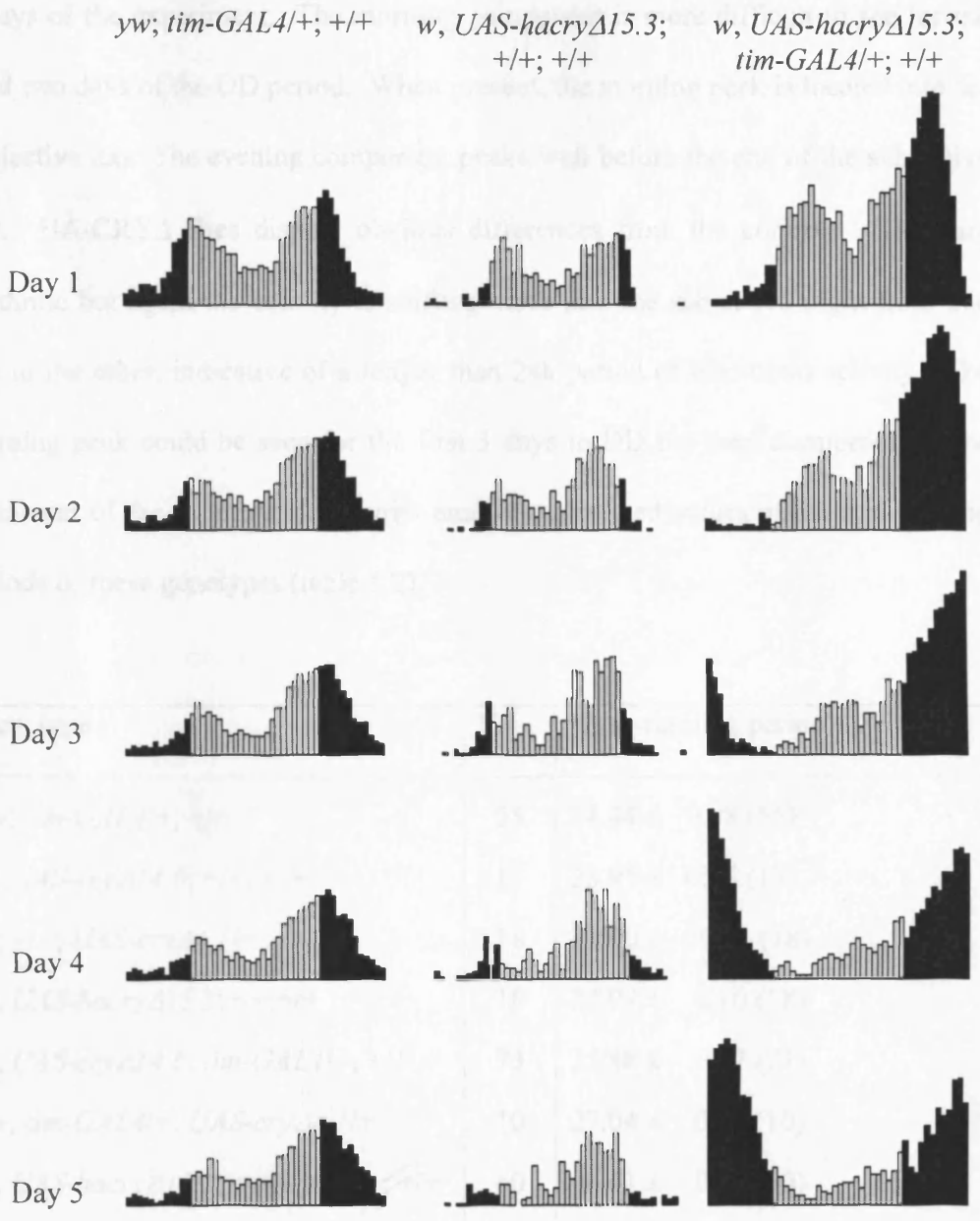


Figure 5.6: DD behaviour of HA-CRYΔ flies. Average locomotor activity pattern for male flies overexpressing HA-CRYΔ (*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *w*, *UAS-hacryΔ15.3*; +/+; +/+) during 5 days under a DD conditions. Grey bars= subjective day period, filled bars= subjective night period.

3 days of the experiment. The morning component is more difficult to see for the final two days of the DD period. When present, the morning peak is located into the subjective day. The evening component peaks well before the end of the subjective day. HA-CRYΔ flies display obvious differences from the controls. They are rhythmic but again the activity is shifting more into the subjective night from one day to the other, indicative of a longer than 24h period of locomotor activity. The morning peak could be seen for the first 3 days in DD but then disappears for the remainder of the experiment. Fourier analysis calculated values of the free-running periods of these genotypes (table 5.2).

Genotype	N	Free-running period ± SEM (n)
yw; *tim-GAL4*/+; +/+	55	24.44 ± 0.08 (55)
w, *UAS-cryΔ14.6*; +/+; +/+	17	23.91 ± 0.17 (17)
w; +/+; *UAS-cryΔ4.1*/+	18	23.90 ± 0.12 (18)
w, *UAS-hacryΔ*15.3;+/+;+/+	18	24.09 ± 0.10 (18)
w, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+	73	25.88 ± 0.07 (73)
yw; *tim-GAL4*/+; *UAS-cryΔ4.1*/+	10	27.04 ± 0.33 (10)
w, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+	60	26.41 ± 0.08 (60)

Table 5.2: DD locomotor activity periods for CRYΔ and HA-CRYΔ flies. The period of locomotor activity in DD was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

Analysis of table 5.2 confirms that CRYΔ flies have long free-running periods of locomotor activity. Depending on the line used, the period is increased

from 1.4h (line 14.6) to 2.6h (line 4.1). The increase is due to the upregulation of CRYΔ as controls have periods close to 24h. Statistical analysis (ANOVA) were carried out and showed that this increase in period length is significant for all lines tested (appendix 11).

5.2.1.3 Average daily activity

In a similar way to what was done for flies overexpressing CRY, individual flies' average daily activity were calculated for CRYΔ flies. These were used for analysis of the timing of morning and evening component of activity as well as to design genotypes average day. Figure 5.7 shows the average daily activity in LD for CRYΔ (line14.6 and 4.1), HA-CRYΔ (line15.3) and controls flies. The results of the individual fly analysis are given in table 5.3. Examination of Figure 5.7 does not reveal substantial differences between CRYΔ and control flies with all genotypes showing bimodal activity with anticipated peaks. Inspection of table 5.3 confirms that in LD, and contrary to CRY flies, CRYΔ flies do not manifest any significant changes in terms of timing of morning and evening components as well as overall distribution of activity (ANOVA, appendix 12).

Figure 5.8 gives the average daily activity in DD for CRYΔ (line 14.6 and 4.1), HA-CRYΔ (line15.3) and controls flies. While controls manifest clear bimodality (less obvious for the *w*, *UAS-hacryΔ15.3*; +/+; +/+ control); CRYΔ flies seem to have an unimodal pattern of activity with the evening peak as the main component. Analysis of the individual flies' data given in table 5.4 reveals that 89-94% of controls display a morning peak whereas only 40-63% of CRYΔ flies show a morning component. This constitutes the only but important difference between

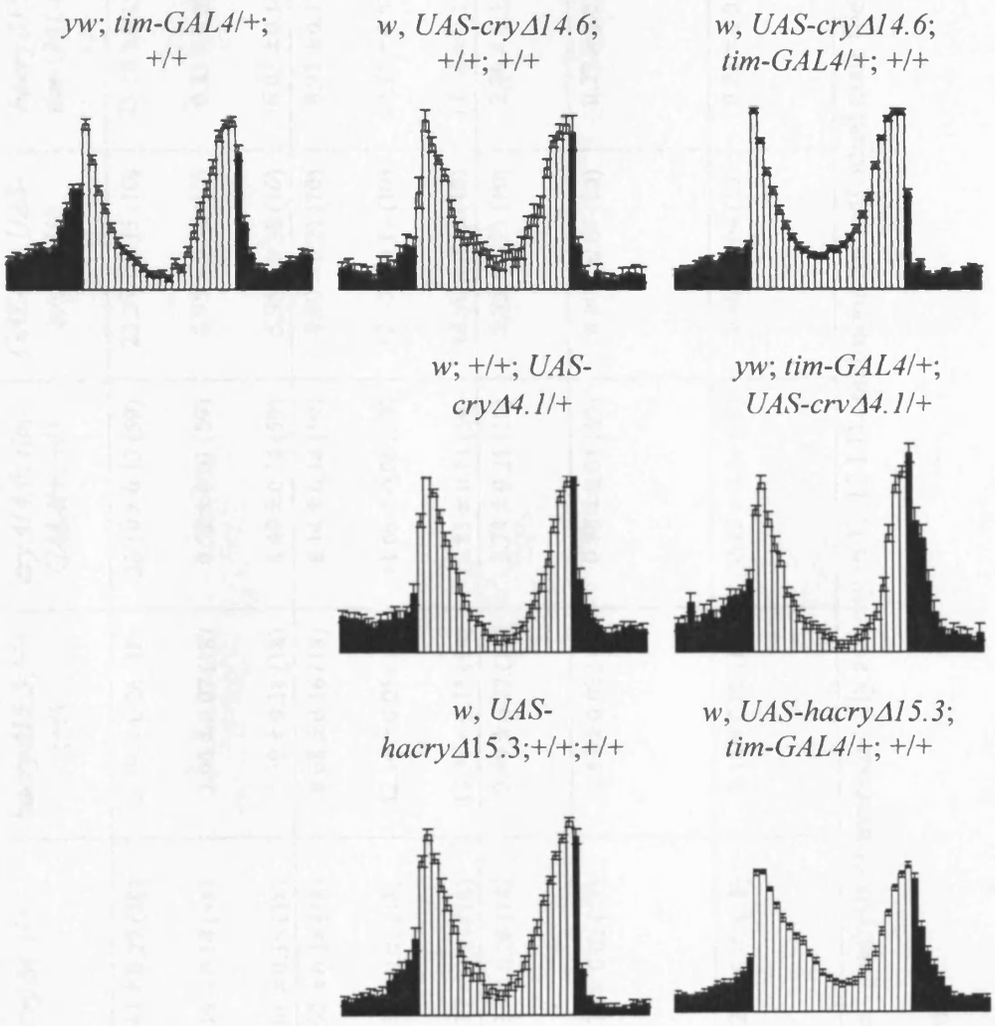


Figure 5.7: LD average daily activity for CRYΔ and HA-CRYΔ flies. Average daily activity in 12:12 LD conditions for CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+ and *yw*; *tim-GAL4*/+; *UAS-cryΔ4.1*/+), HA-CRYΔ (*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) flies and the relevant controls (*yw*; *tim-GAL4*/+; +/+, *w*, *UAS-cryΔ14.6*; +/+; +/+, *w*; +/+; *UAS-cryΔ4.1*/+, and *w*, *UAS-hacryΔ*15.3; +/+;+/+). Open bars= light phase, filled bars= dark phase.

Genotype		*yw*; *tim-GAL4*/+; +/+	*w*, *UAS-cryΔ14.6*; +/+; +/+	*w*; +/+; *UAS-cryΔ4.1*/+	*w*, *UAS-hacryΔ15.3*;+/+ ;+/+	*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+	*yw*; *tim-GAL4*/+; *UAS-cryΔ4.1*/+	*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+
Morning activity	Onset	22.29 ± 0.13 (40)	23.13 ± 0.28 (15)	23.44 ± 0.27 (18)	23.06 ± 0.26 (18)	23.10 ± 0.13 (59)	23.25 ± 0.35 (10)	23.10 ± 0.13 (60)
	Peak	0.60 ± 0.04 (40)	0.83 ± 0.06 (15)	1.14 ± 0.14 (18)	1.06 ± 0.07 (18)	0.52 ± 0.01 (59)	0.95 ± 0.05 (10)	0.83 ± 0.03 (60)
	Offset	5.56 ± 0.17 (40)	5.83 ± 0.27 (15)	5.61 ± 0.19 (18)	5.39 ± 0.18 (18)	4.40 ± 0.14 (59)	5.95 ± 0.30 (10)	6.05 ± 0.14 (60)
Evening activity	Onset	8.42 ± 0.16 (40)	8.63 ± 0.22 (15)	8.92 ± 0.24 (18)	8.08 ± 0.16 (18)	8.14 ± 0.14 (59)	9.80 ± 0.21 (10)	8.91 ± 0.11 (60)
	Peak	11.64 ± 0.12 (40)	11.90 ± 0.25 (15)	12.31 ± 0.06 (18)	12.14 ± 0.05 (18)	11.66 ± 0.06 (59)	12.35 ± 0.11 (10)	12.15 ± 0.07 (60)
	Offset	14.05 ± 0.11 (40)	13.97 ± 0.20 (15)	14.39 ± 0.21 (18)	13.97 ± 0.15 (18)	13.83 ± 0.11 (59)	14.90 ± 0.23 (10)	14.58 ± 0.11 (60)
Siesta		2.86 ± 0.21 (40)	2.80 ± 0.41 (15)	3.31 ± 0.28 (18)	2.69 ± 0.27 (18)	3.74 ± 0.21 (59)	3.85 ± 0.21 (10)	2.86 ± 0.17 (60)
Proportion of activity during light phase		0.69 ± 0.02 (40)	0.81 ± 0.03 (15)	0.74 ± 0.02 (18)	0.82 ± 0.02 (18)	0.88 ± 0.01 (59)	0.60 ± 0.04 (10)	0.77 ± 0.01 (60)
Proportion of activity during dark phase		0.31 ± 0.02 (40)	0.19 ± 0.03 (15)	0.26 ± 0.02 (18)	0.18 ± 0.02 (18)	0.12 ± 0.01 (59)	0.40 ± 0.04 (10)	0.23 ± 0.01 (60)

Table 5.3: LD average daily activity for CRYΔ and HA-CRYΔ flies. Analysis of average daily activity in 12:12 LD conditions for individual male flies overexpressing CRYΔ and HA-CRYΔ as well as the relevant controls.

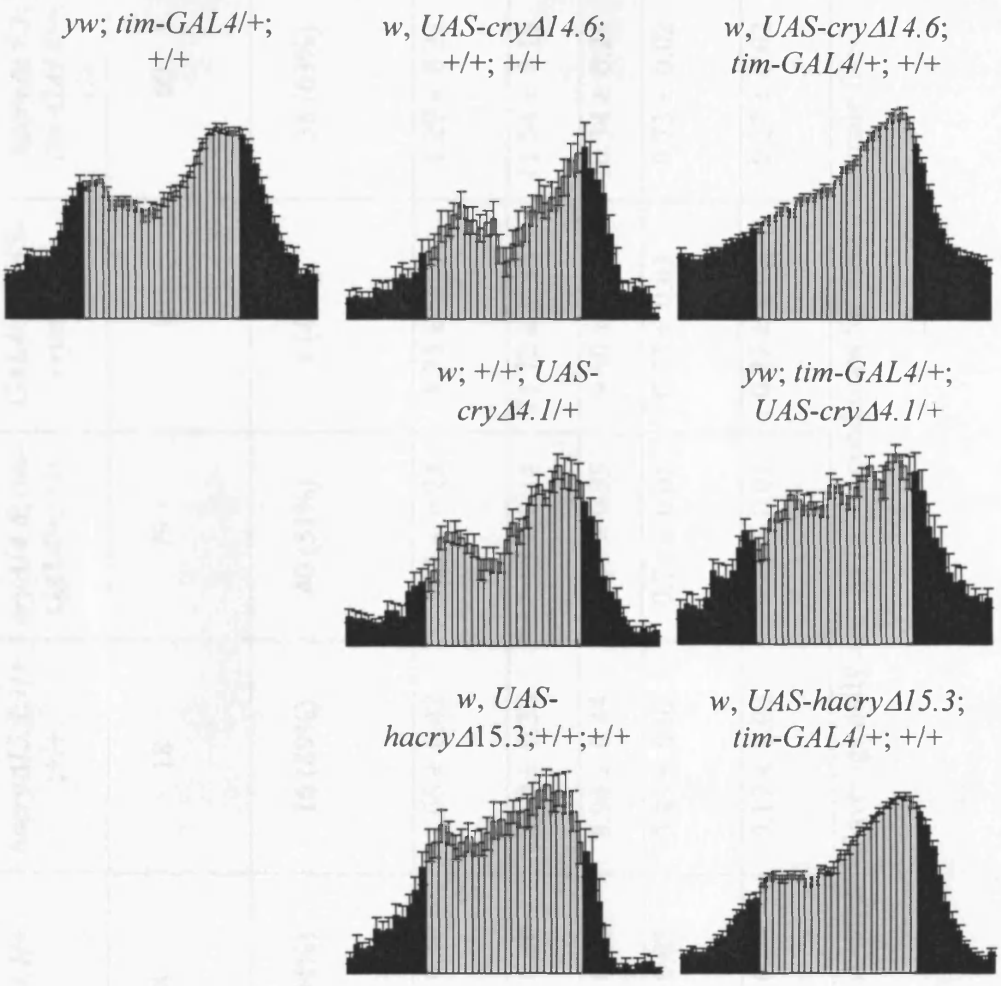


Figure 5.8: DD average daily activity for CRYΔ and HA-CRYΔ flies. Average daily activity in DD conditions for CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+ and *yw*; *tim-GAL4*/+; *UAS-cryΔ4.1*/+), HA-CRYΔ (*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) flies and the relevant controls (*yw*; *tim-GAL4*/+; +/+, *w*, *UAS-cryΔ14.6*; +/+; +/+, *w*; +/+; *UAS-cryΔ4.1*/+, and *w*, *UAS-hacryΔ*15.3; +/+; +/+). Grey bars= subjective day phase, filled bars= subjective night phase.

Genotype	*yw; tim-GAL4/+; +/+*	*w, UAS-cryΔ14.6; +/+; +/+*	*w; +/+; UAS-cryΔ4.1/+*	*w, UAS-hacryΔ15.3;+/+ ;+/+*	*w, UAS-cryΔ14.6; tim-GAL4/+; +/+*	*yw; tim-GAL4/+; UAS-cryΔ4.1/+*	*w, UAS-hacryΔ15.3; tim-GAL4/+; +/+*
Number of flies examined	54	17	18	18	79	10	60
Number of flies where morning peak was present	50 (93%)	16 (94%)	17 (94%)	16 (89%)	40 (51%)	4 (40%)	38 (63%)
Morning peak	0.87 ± 0.20	3.00 ± 0.38	2.21 ± 0.40	1.66 ± 0.42	1.85 ± 0.28	1.75 ± 0.97	1.29 ± 0.22
Evening peak	11.76 ± 0.20	11.22 ± 0.46	10.58 ± 0.40	10.59 ± 0.38	11.18 ± 0.14	11.72 ± 0.43	11.54 ± 0.19
Phase difference	10.89 ± 0.23	8.22 ± 0.43	8.53 ± 0.56	8.94 ± 0.44	9.37 ± 0.35	9.50 ± 1.35	10.34 ± 0.25
Proportion of activity during subjective day	0.65 ± 0.01	0.65 ± 0.06	0.77 ± 0.03	0.83 ± 0.02	0.71 ± 0.01	0.73 ± 0.03	0.73 ± 0.02
Proportion of activity during subjective night	0.35 ± 0.01	0.35 ± 0.06	0.23 ± 0.03	0.17 ± 0.02	0.29 ± 0.01	0.27 ± 0.03	0.27 ± 0.02

Table 5.4: DD average daily activity for CRYΔ and HA-CRYΔ flies. Analysis of average daily activity in DD conditions for individual male flies overexpressing CRYΔ and HA-CRYΔ as well as the relevant controls.

controls and CRYΔ flies as timing of morning (when present) and evening components as well as overall distribution of activity are not affected (ANOVA, appendix 13).

5.2.2 Targetted overexpression of CRYΔ in the lateral neurons

tim-GAL4 drives upregulation of CRYΔ in all known clock cells. Among those, the lateral neurons (LNs) have been identified as the most important cells controlling locomotor behaviour (Dushay et al., 1989; Hardin et al., 1992; Helfrich-Forster, 1998). The ventral LNs (LN_vs) specifically express the neuropeptide PIGMENT DISPERSING FACTOR (PDF) (Renn et al., 1999). A previously described *Pdf-GAL4* line was used to drive the overexpression of CRYΔ in the LN_vs (Renn et al., 1999). For comparison, another line, *gal1118,* was used to drive expression of GAL4 in the LN_vs but also in about 50% of the LN_ds (Blanchardon et al., 2001).

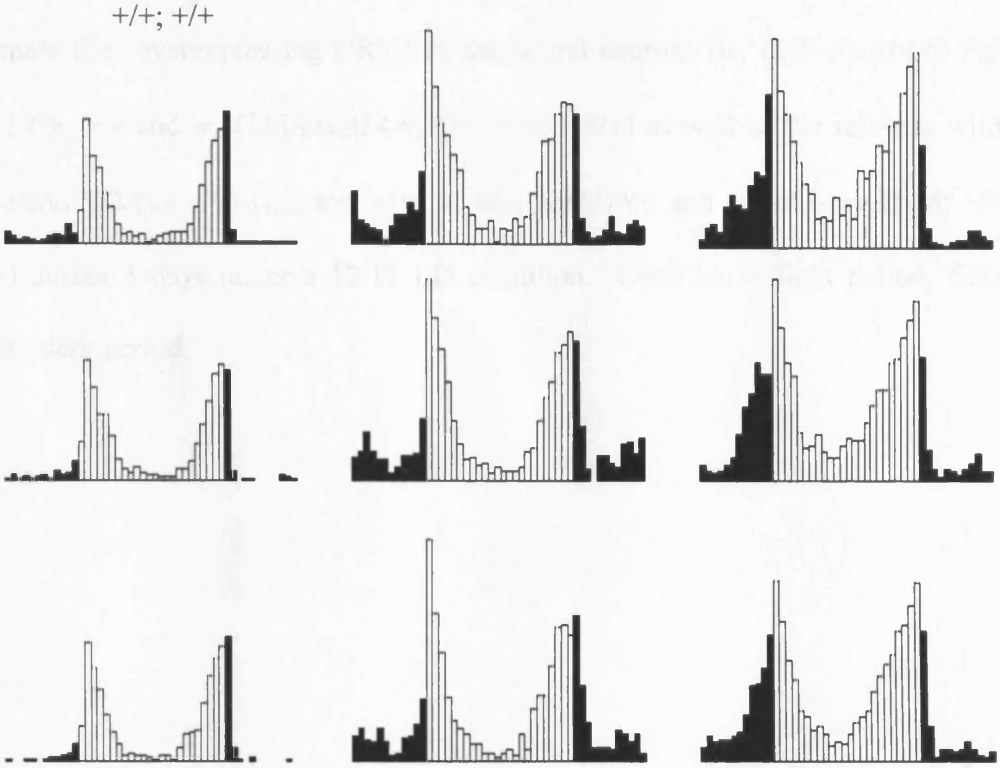
5.2.2.1 Entrainment

Figure 5.9 shows the average locomotor activity pattern of male flies overexpressing CRYΔ in the lateral neurons (*w, UAS-cryΔ14.6*; *Pdf-GAL4*/+, +/+ and *w, UAS-cryΔ14.6*; +/+; *gal1118*/+) as well as the relevant wild-type controls (*yw*; *Pdf-GAL4*/+; +/+; *w*; +/+; *gal1118*/+ and *w*, *UAS-cryΔ14.6*; +/+; +/+) during 3 days under a 12:12 LD condition. Controls show rhythmic bimodal activity in LD with peaks located on the dark/light and light/dark transitions (Figure 5.9 upper panels). Flies overexpressing CRYΔ in the LNs (thereafter called CRYΔ(LNs)) are

w, UAS-cryΔ14.6;
+/+; +/+

yw; *Pdf-GAL4*/+; +/+

w; +/+; *gal1118*/+



w, UAS-cryΔ14.6;
Pdf-GAL4/+

w, UAS-cryΔ14.6;
+/+; *gal1118*/+

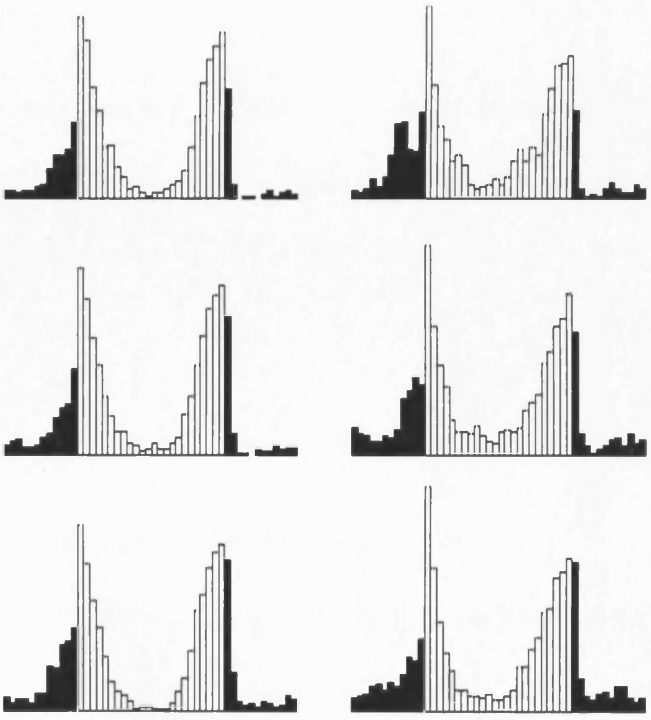


Figure 5.9: LD behaviour of CRYΔ (LNs) flies. Average locomotor activity pattern of male flies overexpressing CRYΔ in the lateral neurons (*w*, *UAS-cryΔ14.6*; *Pdf-GAL4*/+, +/+ and *w, UAS-cryΔ14.6*; +/+; *gal1118*/+) as well as the relevant wild-type controls (*yw*; *Pdf-GAL4*/+; +/+; *w*; +/+; *gal1118*/+ and *w*, *UAS-cryΔ14.6*; +/+; +/+) during 3 days under a 12:12 LD condition. Open bars= light period, filled bars= dark period.

displaying bimodality with no significant differences from the controls indicating that they entrain to the LD cycles. Table 5.5 gives the entrained locomotor activity periods of these flies calculated by Fourier analysis.

Genotype	N	Entrained period ± SEM (n)
w, UAS-cryΔ14.6; +/+; +/+	15	24.22 ± 0.17 (15)
yw; *Pdf-GAL4*/+; +/+	6	24.09 ± 0.08 (6)
w; +/+; *gal1118*/+	19	24.17 ± 0.09 (19)
w, UAS-cryΔ14.6; Pdf-GAL4/+, +/+	20	24.02 ± 0.04 (20)
w, UAS-cryΔ14.6; +/+; *gal1118*/+	22	24.10 ± 0.04 (22)

Table 5.5: LD locomotor activity periods for CRYΔ(LNs) flies. The period of locomotor activity in 12:12 LD condition was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

Analysis of table 5.5 reveals that in LD, there are no differences in period length between CRYΔ(LNs) and control flies further indicating that they entrain to the LD cycles ($F_{4,77}$=0.68, ns, appendix 14). All periods are close to 24h.

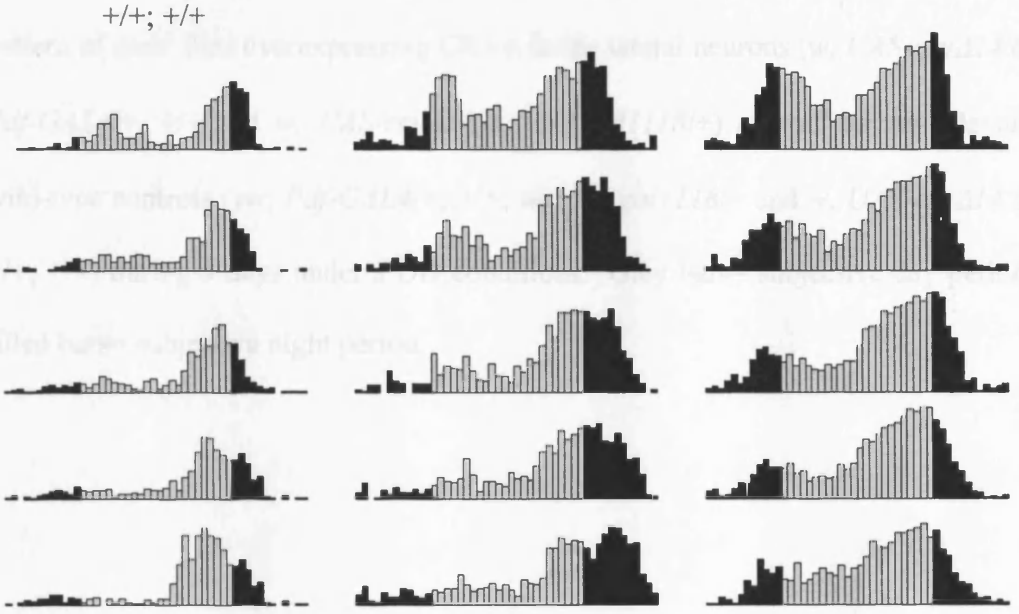
5.2.2.2 Free-running behaviour

Figure 5.10 shows the average locomotor activity pattern of male CRYΔ(LNs) flies as well as the relevant wild-type controls during 5 days under DD conditions after an entrainment period of at least 3 days in a 12:12 LD. Controls are rhythmic in DD. The *UAS-cryΔ14.6*; +/+; +/+ control has a weak morning peak

w, *UAS-cryΔ14.6*;
+/+; +/+

yw; *Pdf-GAL4*/+; +/+

w; +/+; *gal1118*/+



w, *UAS-cryΔ14.6*;
Pdf-GAL4/+

w, *UAS-cryΔ14.6*;
+/+; *gal1118*/+

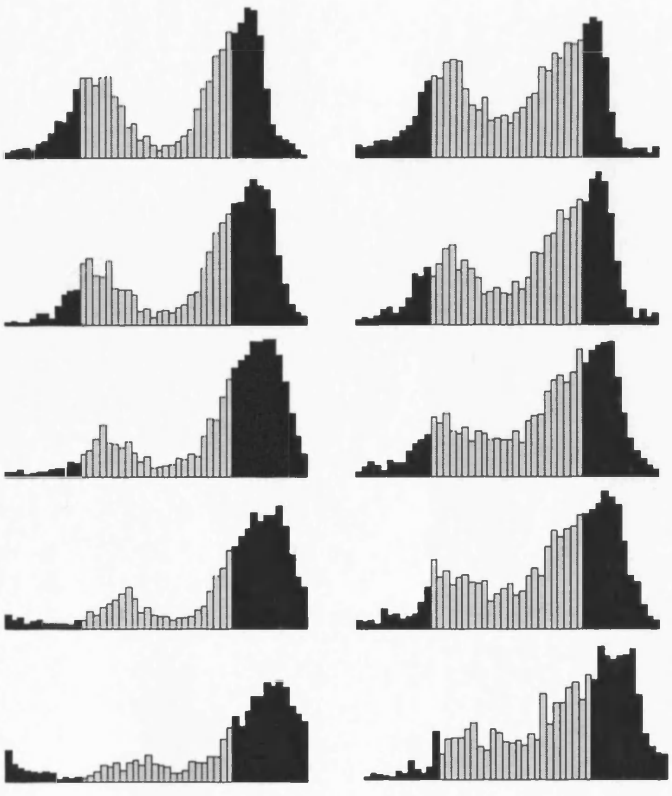


Figure 5.10: DD behaviour of CRYΔ (LNs) flies. Average locomotor activity pattern of male flies overexpressing CRYΔ in the lateral neurons (*w*, *UAS-cryΔ14.6*; *Pdf-GAL4*/+, +/+ and *w, UAS-cryΔ14.6*; +/+; *gal1118*/+) as well as the relevant wild-type controls (*yw*; *Pdf-GAL4*/+; +/+; *w*; +/+; *gal1118*/+ and *w*, *UAS-cryΔ14.6*; +/+; +/+) during 5 days under a DD conditions. Grey bars= subjective day period, filled bars= subjective night period.

located in the subjective day for the first 3 days of the experiment before it disappears. The evening peak occurs well before the end of the subjective day. The *yw*; *Pdf-GAL4*/+; +/+ control shows bimodality throughout the length of the experiment with morning peak located after the beginning of the subjective day and evening peak centred on the subjective day/night transition. Finally, the *w*; +/+; *gal1118*/+ control displays bimodality with morning peak before subjective day and evening peak on the subjective day/night transition. Upregulating CRYΔ with *Pdf-GAL4* as a driver results in the flies being rhythmic and bimodal throughout the 5 days of the experiment. This is in sharp contrast to the result obtained with the *tim-GAL4* driver, when it is difficult to see a morning component on the average locomotor activity pattern. The morning peak is located after the beginning of the subjective day. The evening component peaks in the subjective night, and is drifting more towards the subjective from one day to the other suggesting a long period of locomotor behaviour. The use of *gal1118* confirms these findings. Flies with *gal1118* driven CRYΔ overexpression are rhythmic in DD with clear bimodality. The morning peak is situated in the subjective day and the evening peak is drifting more towards the subjective from one day to the other again suggesting a long period of locomotor activity. Fourier analysis was applied to these flies and the results are given in table 5.6.

Genotype	N	Free-running period ± SEM (n)
w, *UAS-cryΔ14.6*; +/+; +/+	17	23.91 ± 0.17 (17)
yw; *Pdf-GAL4*/+; +/+	13	24.59 ± 0.13 (13)
w; +/+; *gal1118*/+	28	24.26 ± 0.09 (28)
w, *UAS-cryΔ14.6*; *Pdf-GAL4*/+, +/+	50	25.46 ± 0.07 (50)
w, *UAS-cryΔ14.6*; +/+; *gal1118*/+	29	25.02 ± 0.08 (29)

Table 5.6: DD locomotor activity periods for CRYΔ(LNs) flies. The period of locomotor activity in DD was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

As suggested by the average activity pattern, CRYΔ(LNs) flies have significantly longer period of locomotor activity than their controls ($F_{1,68}$=96.02, P<0.01 and $F_{1,61}$=30.90, P<0.01 for *Pdf-GAL4,* $F_{1,44}$=42.95, P<0.01 and $F_{1,55}$=38.35, P<0.01 for *gal1118,* appendix 14). The increase is 0.87h with *Pdf-GAL4* and 0.76h for *gal1118.* This increase is not as large as when CRYΔ was overexpressed more broadly with the *tim-GAL4* driver (1.44h for the same CRYΔ line). This discrepancy could be explained either by different levels of expression or more likely by the differential pattern of expression triggered by the different drivers. In conclusion, CRYΔ(LNs) flies have a longer period of locomotor activity and show bimodality in DD.

5.2.2.3 Average daily activity

Figure 5.11 shows the average daily activity in LD for CRYΔ(LNs) and control flies. No significant differences can be seen between CRYΔ(LNs) flies and controls. The result of the individual flies' analysis are given in table 5.7. Inspection of table 5.7 confirms that in LD, there are no differences between controls and CRYΔ(LNs) flies in terms of timing of morning and evening components as well as overall distribution of locomotor activity. Figure 5.12 shows the average daily activity in DD for CRYΔ(LNs) and control flies. Results of the

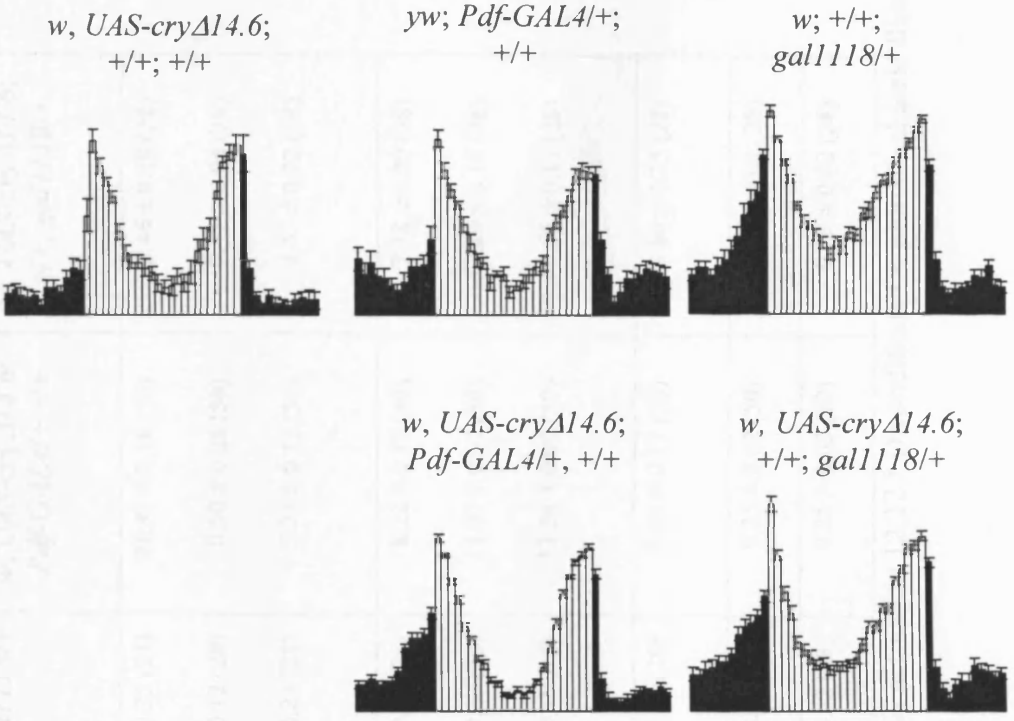


Figure 5.11: LD average daily activity for CRYΔ(LNs) flies. Average daily activity in 12:12 LD conditions for male flies overexpressing CRYΔ in the lateral neurons (*w, UAS-cryΔ14.6*; *Pdf-GAL4*/+, +/+, and *w, UAS-cryΔ14.6*; +/+; *gal1118*/+) as well as the relevant wild-type controls (*yw*; *Pdf-GAL4*/+; +/+; *w*; +/+; *gal1118*/+, and *w, UAS-cryΔ14.6*; +/+; +/+). Open bars= light period, filled bars= dark period.

Genotype		*w, UAS-cryΔ14.6; +/+; +/+*	*yw; Pdf-GAL4/+; +/+*	*w; +/+; gal1118/+*	*w, UAS-cryΔ14.6; Pdf-GAL4/+,+/+*	*w, UAS-cryΔ14.6; +/+; gal1118/+*
Morning activity	Onset	23.13 ± 0.28 (15)	22.92 ± 0.23 (13)	21.73 ± 0.25 (28)	21.93 ± 0.16 (50)	22.46 ± 0.19 (28)
	Peak	0.83 ± 0.06 (15)	0.46 ± 0.04 (13)	0.36 ± 0.12 (28)	0.50 ± 0.08 (50)	0.43 ± 0.06 (28)
	Offset	5.83 ± 0.27 (15)	5.35 ± 0.24 (13)	4.89 ± 0.23 (28)	5.21 ± 0.13 (50)	4.57 ± 0.22 (28)
Evening activity	Onset	8.63 ± 0.22 (15)	8.12 ± 0.22 (13)	7.23 ± 0.17 (28)	8.22 ± 0.12 (50)	7.38 ± 0.20 (28)
	Peak	11.90 ± 0.25 (15)	12.00 ± 0.16 (13)	11.89 ± 0.13 (28)	11.83 ± 0.07 (50)	11.86 ± 0.10 (28)
	Offset	13.97 ± 0.20 (15)	13.96 ± 0.09 (13)	13.75 ± 0.15 (28)	13.54 ± 0.09 (50)	13.55 ± 0.11 (28)
Siesta		2.80 ± 0.41 (15)	2.77 ± 0.35 (13)	2.34 ± 0.26 (28)	3.01 ± 0.17 (50)	2.80 ± 0.25 (28)
Proportion of activity during light phase		0.81 ± 0.03 (15)	0.71 ± 0.03 (13)	0.73 ± 0.02 (28)	0.75 ± 0.02 (50)	0.71 ± 0.02 (28)
Proportion of activity during dark phase		0.19 ± 0.03 (15)	0.29 ± 0.03 (13)	0.27 ± 0.02 (28)	0.25 ± 0.02 (50)	0.29 ± 0.02 (28)

Table 5.7: LD average daily activity for CRYΔ(LNs) flies. Analysis of average daily activity in 12:12 LD conditions for individual male flies overexpressing CRYΔ in the lateral neurons as well as the relevant controls.

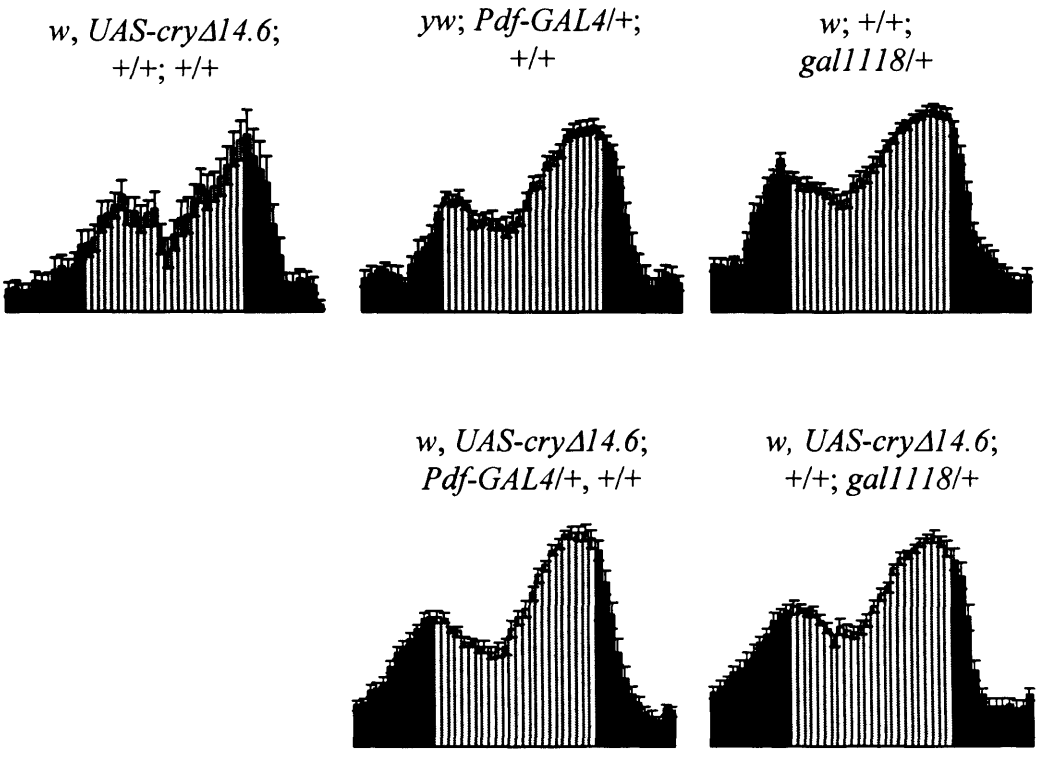


Figure 5.12: DD average daily activity for CRYΔ(LNs) flies. Average daily activity in DD conditions for male flies overexpressing CRYΔ in the lateral neurons (*w, UAS-cryΔ14.6*; *Pdf-GAL4*/+, +/+, and *w, UAS-cryΔ14.6*; +/+; *gal1118*/+) as well as the relevant wild-type controls (*yw*; *Pdf-GAL4*/+; +/+; *w*; +/+; *gal1118*/+, and *w, UAS-cryΔ14.6*; +/+; +/+). Grey bars= subjective day period, filled bars= subjective night period.

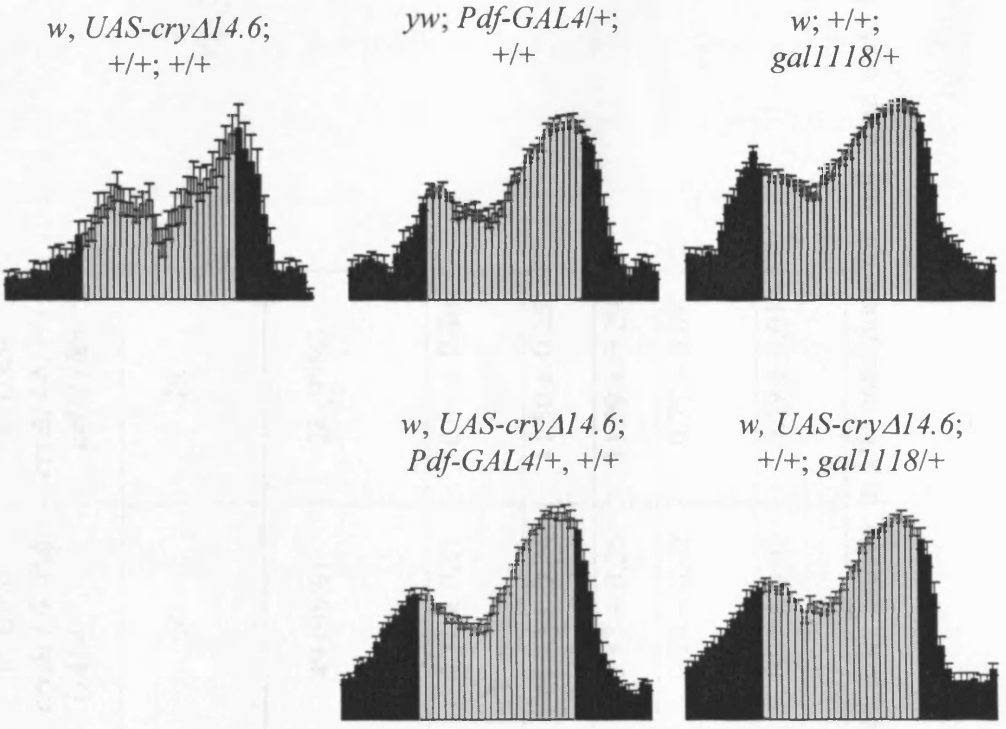


Figure 5.12: DD average daily activity for CRYΔ(LNs) flies. Average daily activity in DD conditions for male flies overexpressing CRYΔ in the lateral neurons (*w*, *UAS-cryΔ14.6*; *Pdf-GAL4*/+, +/+, and *w, UAS-cryΔ14.6*; +/+; *gal1118*/+) as well as the relevant wild-type controls (*yw*; *Pdf-GAL4*/+; +/+; *w*; +/+; *gal1118*/+, and *w*, *UAS-cryΔ14.6*; +/+; +/+). Grey bars= subjective day period, filled bars= subjective night period.

Genotype	*w, UAS-cryΔ14.6*; +/+; +/+	*yw; Pdf-GAL4*/+; +/+	*w*; +/+; *gal1118*/+	*w, UAS-cryΔ14.6; Pdf-GAL4*/+, +/+	*w, UAS-cryΔ14.6*; +/+; *gal1118*/+
Number of flies examined	17	13	28	50	29
Number of flies where morning peak was present	16 (94%)	13 (100%)	25 (89%)	49 (98%)	28 (97%)
Morning peak	3.00 ± 0.38	1.15 ± 0.42	23.64 ± 0.27	0.21 ± 0.21	0.64 ± 0.26
Evening peak	11.22 ± 0.46	11.46 ± 0.27	11.37 ± 0.26	11.03 ± 0.24	11.50 ± 0.25
Phase difference	8.22 ± 0.43	10.31 ± 0.61	11.82 ± 0.42	10.87 ± 0.26	11.09 ± 0.29
Proportion of activity during subjective day	0.65 ± 0.06	0.74 ± 0.03	0.71 ± 0.02	0.70 ± 0.02	0.71 ± 0.01
Proportion of activity during subjective night	0.35 ± 0.06	0.26 ± 0.03	0.29 ± 0.02	0.30 ± 0.02	0.29 ± 0.01

Table 5.8: DD average daily activity for CRYΔ(LNs) flies. Analysis of average daily activity in DD conditions for individual male flies overexpressing CRYΔ in the lateral neurons as well as the relevant controls.

analysis are given in table 5.8. The average daily activity show bimodal pattern of activity for both CRYΔ(LNs) genotypes and do not reveal obvious differences with controls. Inspection of table 5.8 confirms that there are no real differences between controls and CRYΔ(LNs) flies. In conclusion, restricting the overexpression of CRYΔ to the lateral neurons gives a rather different response than a broader upregulation. Namely, almost all CRYΔ(LNs) flies show bimodality in DD whereas CRYΔ overexpressed in all *timeless* expressing cells results in a lower proportion of bimodal flies. This is likely to be caused by the differential pattern of expression induced by the different drivers. Interestingly there are also some similarities between CRYΔ(LNs) and CRYΔ flies, as they all have long periods of locomotor activity. Nevertheless, this increase is not as pronounced in CRYΔ(LNs) flies, suggesting a possible role for non-*Pdf* expressing cells in the control of the locomotor activity periodicity.

5.2.3 CRYΔ and responses to light

5.2.3.1 Response of CRYΔ flies to constant light exposure

5.2.3.1.1 Expression in *timeless* expressing cells

Figure 5.13 illustrates the average locomotor activity pattern of CRYΔ male flies (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) as well as the relevant controls (*yw*; *tim-GAL4*/+; +/+ and *w*, *UAS-cryΔ14.6*; +/+; +/+) during 6 subsequent days, the first one being in normal LD until lights did not go off at ZT 12, releasing the flies in LL. Note that the flies were initially submitted to a period of entrainment of at least 5 days in 12:12 LD cycles. As previously described (chapter 3, Figure 3.7), control flies become arrhythmic upon exposure to constant high level of light exposure. On

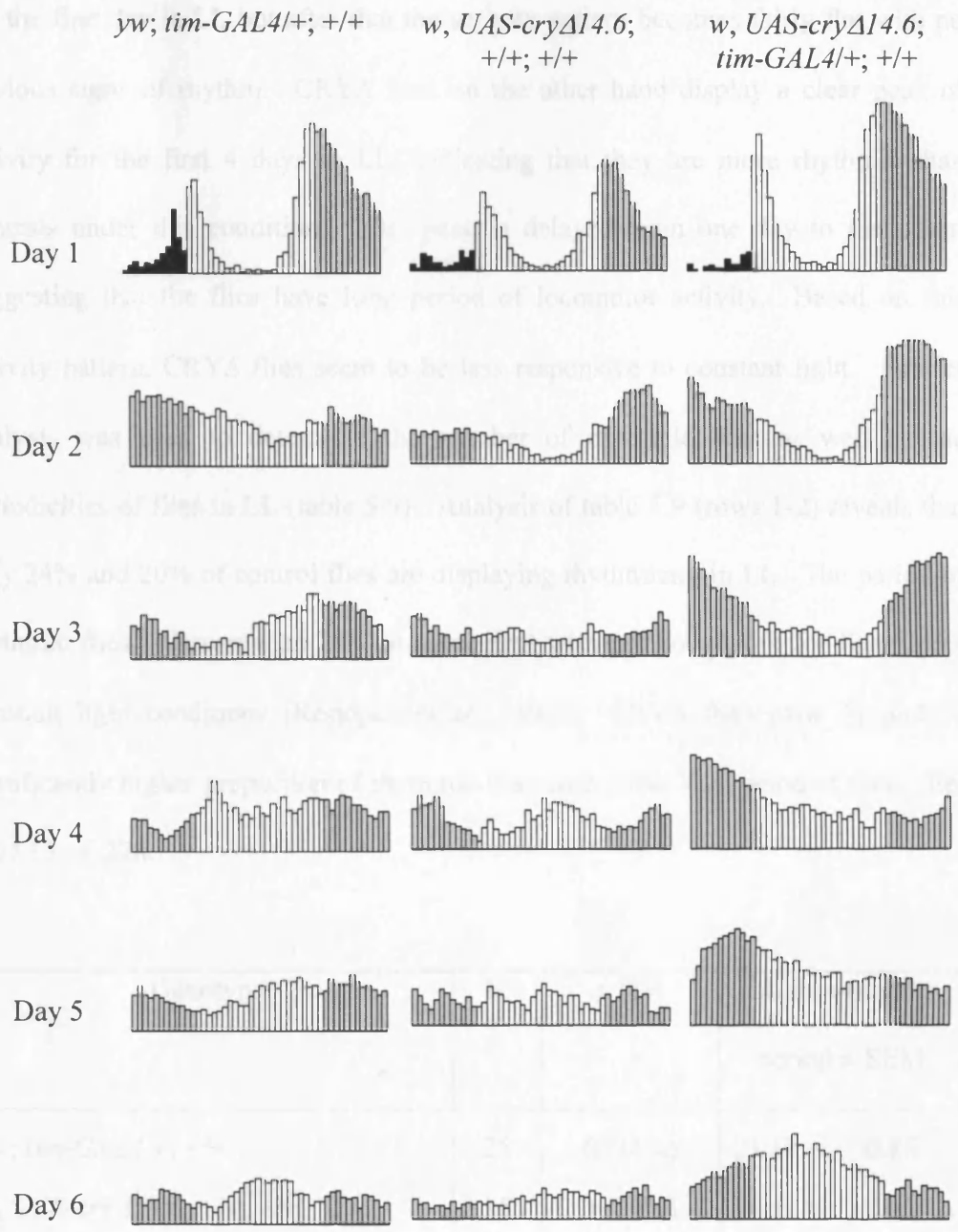


Figure 5.13: LL behaviour of CRYΔ (line 14.6) flies. Average locomotor activity pattern for male flies overexpressing CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+ and *w*, *UAS-cryΔ14.6*; +/+; +/+) during 1 day in 12:12 LD followed by 5 days under LL conditions. Open bars= subjective day period, grey bars= subjective night period.

Figure 5.13, the second control (*w*, *UAS-cryΔ14.6*; +/+; +/+) manifest rhythmicity for the first day in LL but after that the activity pattern becomes fairly flat with no obvious signs of rhythm. CRYΔ flies on the other hand display a clear peak of activity for the first 4 days in LL, indicating that they are more rhythmic than controls under this condition. This peak is delayed from one day to the other, suggesting that the flies have long period of locomotor activity. Based on this activity pattern, CRYΔ flies seem to be less responsive to constant light. Fourier analysis was used to determine the number of rhythmic flies as well as the periodicities of flies in LL (table 5.9). Analysis of table 5.9 (rows 1-2) reveals that only 24% and 20% of control flies are displaying rhythmicity in LL. The period of rhythmic flies is longer than 24h, in agreement with previous work for flies under constant light conditions (Konopka et al., 1989). CRYΔ flies (row 3) show a significantly higher proportion of rhythmic flies with 63%. The period of these flies is 27.13 ±0.27h.

Genotype	N	n (%)	LL free-running period ± SEM
yw; *tim-GAL4*/+; +/+	25	6 (24%)	25.17 ± 0.86
w, *UAS-cryΔ14.6*; +/+; +/+	15	3 (20%)	28.41 ± 1.01
w, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+	35	22 (63%)	27.13 ± 0.27
w, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+	12	9 (75%)	25.49 ± 0.67

Table 5.9: LL locomotor activity periods for CRYΔ and HA-CRYΔ flies. The period of locomotor activity in LL was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. n = number of rhythmic flies.

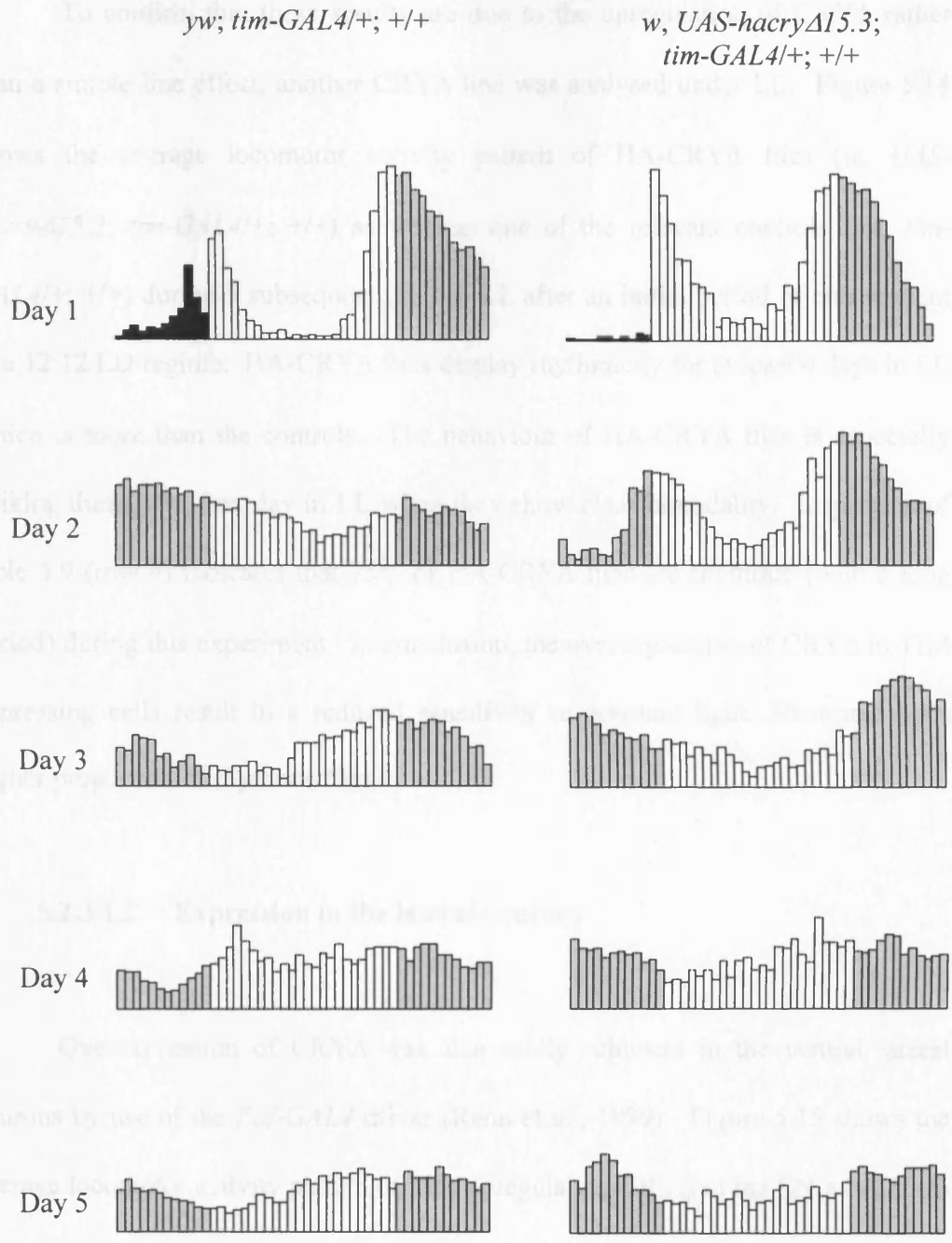


Figure 5.14: LL behaviour of HA-CRYΔ flies. Average locomotor activity pattern for male flies overexpressing HA-CRYΔ (*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) as well as one of the relevant wild-type controls (*yw*; *tim-GAL4*/+; +/+) during 1 day 12:12 LD condition followed by 4 days under a LL regime. Open bars= subjective day period, filled bars= subjective night period.

To confirm that these results are due to the upregulation of CRYΔ rather than a simple line effect, another CRYΔ line was analysed under LL. Figure 5.14 shows the average locomotor activity pattern of HA-CRYΔ flies (*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) as well as one of the relevant controls (*yw*; *tim-GAL4*/+; +/+) during 5 subsequent days in LL after an initial period of entrainment in a 12:12 LD regime. HA-CRYΔ flies display rhythmicity for at least 4 days in LL which is more than the controls. The behaviour of HA-CRYΔ flies is especially striking during the first day in LL when they show clear bimodality. Inspection of table 5.9 (row 4) indicates that 75% of HA-CRYΔ flies are rhythmic (with a long period) during this experiment. In conclusion, the overexpression of CRYΔ in TIM expressing cells result in a reduced sensitivity to constant light, illustrated by a higher proportion of rhythmic flies.

5.2.3.1.2 Expression in the lateral neurons

Overexpression of CRYΔ was also solely achieved in the ventral lateral neurons by use of the *Pdf-GAL4* driver (Renn et al., 1999). Figure 5.15 shows the average locomotor activity pattern of flies upregulating CRYΔ in the LN_vs (*w*, *UAS-cryΔ14.6*; *Pdf-GAL4*/+, +/+) as well as one of the relevant controls (*w*, *UAS-cryΔ14.6*; +/+; +/+) during 5 subsequent days in LL after an initial period of entrainment in a 12:12 LD. The examination of Figure 5.15 reveals that CRYΔ(LN_vs) flies show rhythmicity for the first 2-3 days in LL. Nevertheless, controls themselves display rhythmic activity for the first 1-2 days in LL. Therefore a loss of light sensitivity in CRYΔ(LNvs) can not clearly be concluded from these

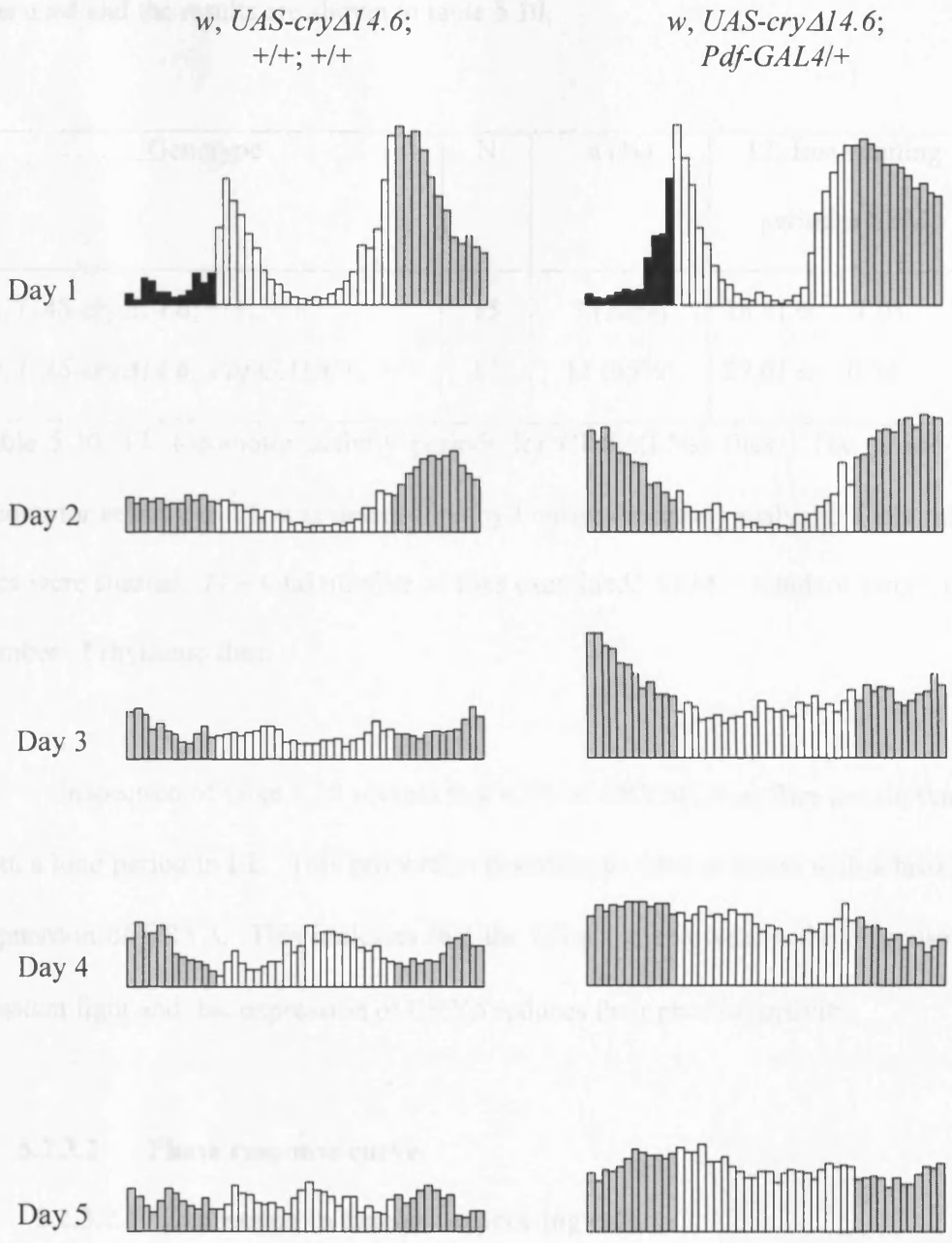


Figure 5.15: LL behaviour of CRYΔ (LNs) flies. Average locomotor activity pattern of male flies overexpressing CRYΔ in the ventral lateral neurons (*w*, *UAS-cryΔ14.6*; *Pdf-GAL4*/+, +/+) as well as one of the relevant wild-type controls (*w*, *UAS-cryΔ14.6*; +/+; +/+) during 1 day under a 12:12 LD condition followed by 4 days in LL. Open bars= subjective day period, filled bars= subjective night period.

data. In order to have a better idea of the number of rhythmic flies, Fourier analysis was used and the results are shown in table 5.10.

Genotype	N	n (%)	LL free-running period ± SEM
w, *UAS-cryΔ14.6*; +/+; +/+	15	3 (20%)	28.41 ± 1.01
w, *UAS-cryΔ14.6; Pdf-GAL4*/+, +/+	17	11 (65%)	27.61 ± 0.54

Table 5.10: LL locomotor activity periods for CRYΔ(LNs) flies. The period of locomotor activity in LL was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. n = number of rhythmic flies.

Inspection of table 5.10 reveals that 65% of CRYΔ(LN_vs) flies are rhythmic with a long period in LL. This proportion is similar to what is found with a broader expression of CRYΔ. This indicates that the LN_vs are involved in the response to constant light and that expression of CRYΔ reduces their photosensitivity.

5.2.3.2 Phase response curve

5.2.3.2.1 Expression in *timeless* expressing cells

Fig. 5.16 shows the PRC for CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) as well as control (*yw*; *tim-GAL4*/+; +/+) flies. The figures used to build the PRC are also reported in table 5.11.

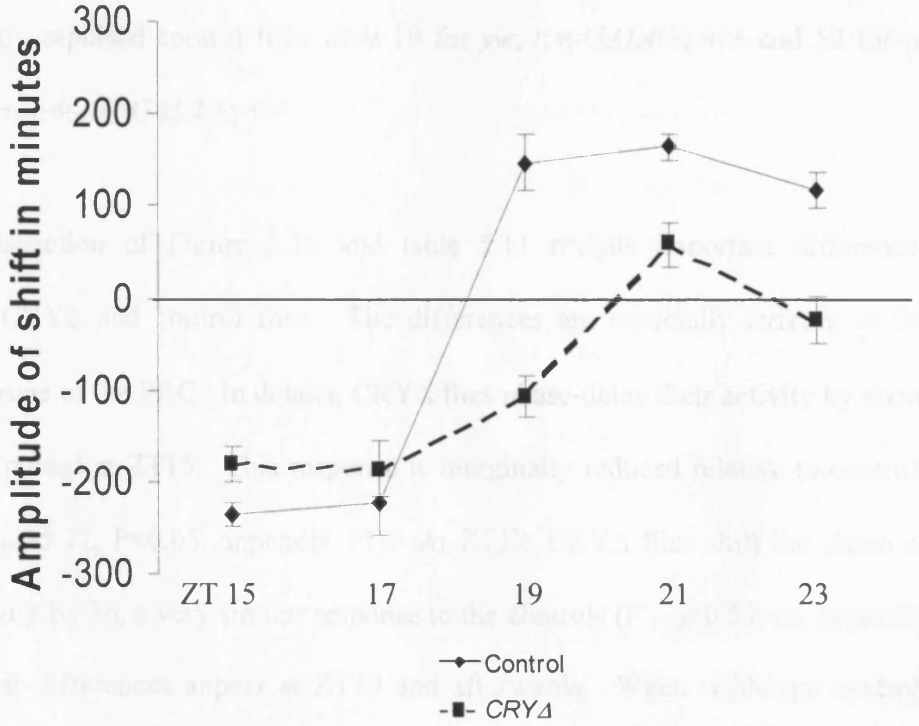


Figure 5.16: PRC of CRYΔ flies. Phase response curve (PRC) for CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) and control (*yw*; *tim-GAL4*/+; +/+) flies. Amplitude of shift obtained in minutes with a 5 minutes light pulse given at the indicated ZT. Numbers of flies pulsed at each time points are indicated in table 5.11. Numbers of unpulsed control flies are 19 for control and 52 for CRYΔ flies. Error bars represent standard errors.

Genotype		*yw*; *tim-GAL4*/+; +/+	*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+
Amplitude of shift in minutes at ZT	15 (n)	-235.71± 12.97 (35)	-181.06 ± 18.81 (35)
	17 (n)	-223.33± 36.09 (9)	-186.77 ± 29.98 (15)
	19 (n)	146.67± 30.60 (9)	-106.48 ± 22.08 (28)
	21 (n)	163.75± 14.33 (24)	58.80 ± 23.98 (23)
	23 (n)	118.24± 19.16 (17)	-24.77 ± 26.33 (15)

Table 5.11: PRC of CRYΔ flies. Amplitude of shift obtained in minutes ± SEM with a 5 minutes light pulse given at the indicated ZT. (n) = number of flies pulsed. Number of unpulsed control flies were 19 for *yw*; *tim-GAL4*/+; +/+ and 52 for *w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+.

Inspection of Figure 5.16 and table 5.11 reveals important differences between CRYΔ and control flies. The differences are especially striking in the advance zone of the PRC. In details, CRYΔ flies phase-delay their activity by about 3h when pulsed at ZT15. This response is marginally reduced relative to controls (1h) ($F_{1,68}$=5.72, P<0.05, appendix 15). At ZT17, CRYΔ flies shift the phase of their activity by 3h, a very similar response to the controls ($F_{1,22}$=0.59, ns, appendix 15). Clear differences appear at ZT19 and afterwards. When wild-type controls display a 2.5h phase advance at ZT19, CRYΔ flies are still showing a phase-delay of more than 1.5h. At ZT21, there is a 1h phase-advance for CRYΔ flies but this is significantly reduced ($F_{1,45}$=14.40, P<0.01, appendix 15) compared to the almost 3h advance obtained with controls. Finally, at ZT23 CRYΔ flies show a 24 minutes phase-delay when controls phase-advance by almost 2h. In conclusion, CRYΔ flies do not respond as well as controls in PRC experiments. The response is especially affected in the advance zone of the PRC (appendix 15).

HA-CRYΔ flies (*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) were also pulsed at ZT19. They phase-delay by more than 5h (-306.43 ±55.18 minutes), confirming the results obtained with CRYΔ flies. The amplitude of the delay is even bigger in HA-CRYΔ flies (300 minutes versus 100 minutes for CRYΔ). This could be the result of a different level of expression of the two transgenes.

5.2.3.2.2 Expression in the lateral neurons

CRYΔ overexpression was specifically targeted in the lateral neurons with use of the *Pdf-GAL4* and *gal1118* driver (Blanchardon et al., 2001; Renn et al., 1999). CRYΔ(LNs) flies were pulsed at ZT 19 and 21. The results are given in Figure 5.17 and table 5.12.

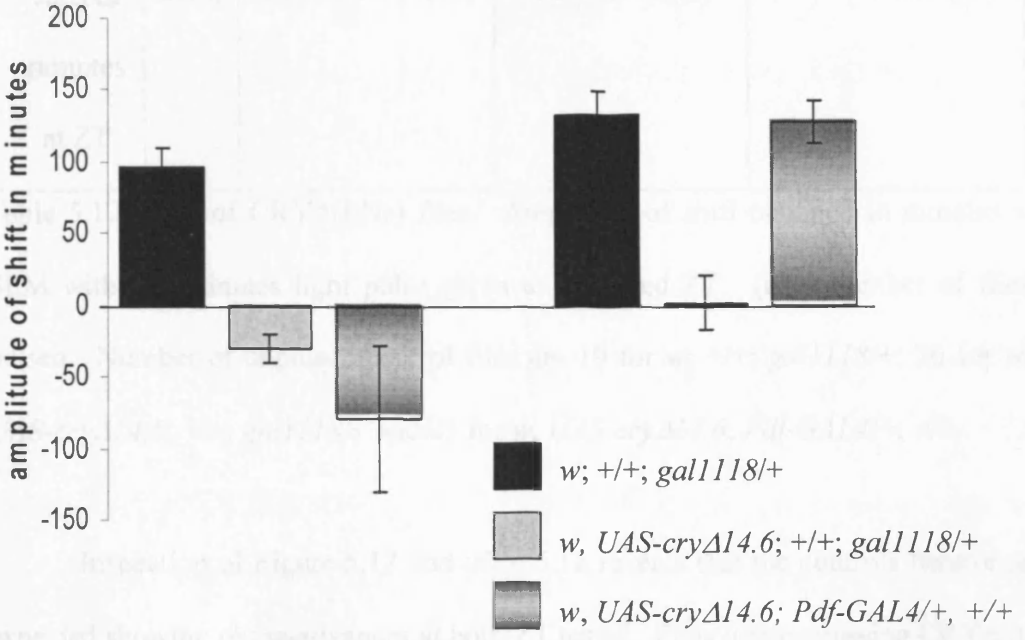


Figure 5.17: PRC of CRYΔ(LNs) flies. Light-pulse phase shift at ZT19 and ZT21. Amplitude of shift obtained in minutes with a 5 minutes light pulse given at the indicated ZT. Only ZT19 and ZT21 were used in this experiment. Note that a pulse given at these time points generates a phase advance in wild-type. Numbers of flies pulsed at each time points are indicated in table 5.12. Numbers of unpulsed control flies are 19 for *w*; +/+; *gal1118*/+; 26 for *w, UAS-cryΔ14.6*; +/+; *gal1118*/+ and 25 for *w*, *UAS-cryΔ14.6*; *Pdf-GAL4*/+; +/+. Error bars represent standard errors.

Genotype		*w*; +/+; *gal1118*/+	*w, UAS-cryΔ14.6*; +/+; *gal1118*/+	*w, UAS-cryΔ14.6; Pdf-GAL4*/+, +/+
Amplitude of shift in minutes at ZT	19(n)	96.6±13.45(10)	-30.1 ± 9.47(15)	-79.2 ±51.23 (9)
	21(n)	133.29±15.74(13)	1.9 ±18.44(15)	127.64 ±14.67 (19)

Table 5.12: PRC of CRYΔ(LNs) flies. Amplitude of shift obtained in minutes ± SEM with a 5 minutes light pulse given at indicated ZT. (n) = number of flies pulsed. Number of unpulsed control flies are 19 for *w*; +/+; *gal1118*/+; 26 for *w, UAS-cryΔ14.6*; +/+; *gal1118*/+ and 25 for *w*, *UAS-cryΔ14.6*; *Pdf-GAL4*/+; +/+

Inspection of Figure 5.17 and table 5.12 reveals that the controls behave as expected showing phase-advances at both ZT tested. Flies overexpressing CRYΔ in all three groups of lateral neurons with the *gal1118* driver show significant differences from the controls ($F_{1,23}=63.06$, $P<0.01$ at ZT19 and $F_{1,26}=28.41$, $P<0.01$ at ZT21, appendix 16) displaying a 30 minutes phase-delay at ZT19 and do not phase-shift at ZT21. This indicates that they are defective in their circadian photosensitivity. However flies in which CRYΔ expression is limited to only the LNvs with use of the *Pdf-GAL4* driver show a rather different response. At ZT19, they phase-delay by more than an hour showing a similar response to CRYΔ(*gal1118*) flies whereas at ZT21 they phase-advance by 2h which is clearly different from CRYΔ(*gal1118*) ($F_{1,22}=1.43$, ns at ZT19 and $F_{1,32}=29.25$, $P<0.01$ at

ZT21, appendix 16). In other words, CRYΔ(LN_vs) flies are affected in their photosensitivity at ZT19 but are responding normally at ZT21 (appendix 16). These results are very interesting because they highlight differences between different subset of clock neurons in the circadian photosensitivity of the clock. However, a full PRC would be needed to have a clearer picture and the control that is missing in this experiment (*yw*; *Pdf-GAL4*/+; +/+) should be added in future analysis. Nevertheless, CRYΔ overexpression in the lateral neurons results in a reduced light sensitivity, indicating that these neurons are involved in circadian photosensitivity.

5.2.4 Molecular oscillations of PER and TIM

Western blots experiments were performed on extracts obtained from CRYΔ flies heads. These were done in LD and DD conditions and PER and TIM molecular cycling was assayed. Figure 5.18 shows representative blots for control (*yw*; *tim-GAL4*/+; +/+), CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) and HA-CRYΔ *(w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) flies as well as quantifications of the level of PER and TIM. The control shows typical oscillations of PER and TIM levels in LD and DD conditions with high levels at the end of the night/subjective night (ZT or CT 16-20) and low levels during the day (ZT or CT4-8). Both proteins also appear to be phosphorylated, with TIM hyperphosphorylated forms being visible late at night in LD conditions and throughout the cycle in DD conditions. PER hyperphosphorylated forms are mainly seen at the end of the night/subjective night and at the beginning of the day/subjective day in LD and DD conditions respectively. CRYΔ and HA-CRYΔ flies show dramatic differences in their pattern of TIM accumulation. Observation of the quantification in Figure 5.18a reveals that

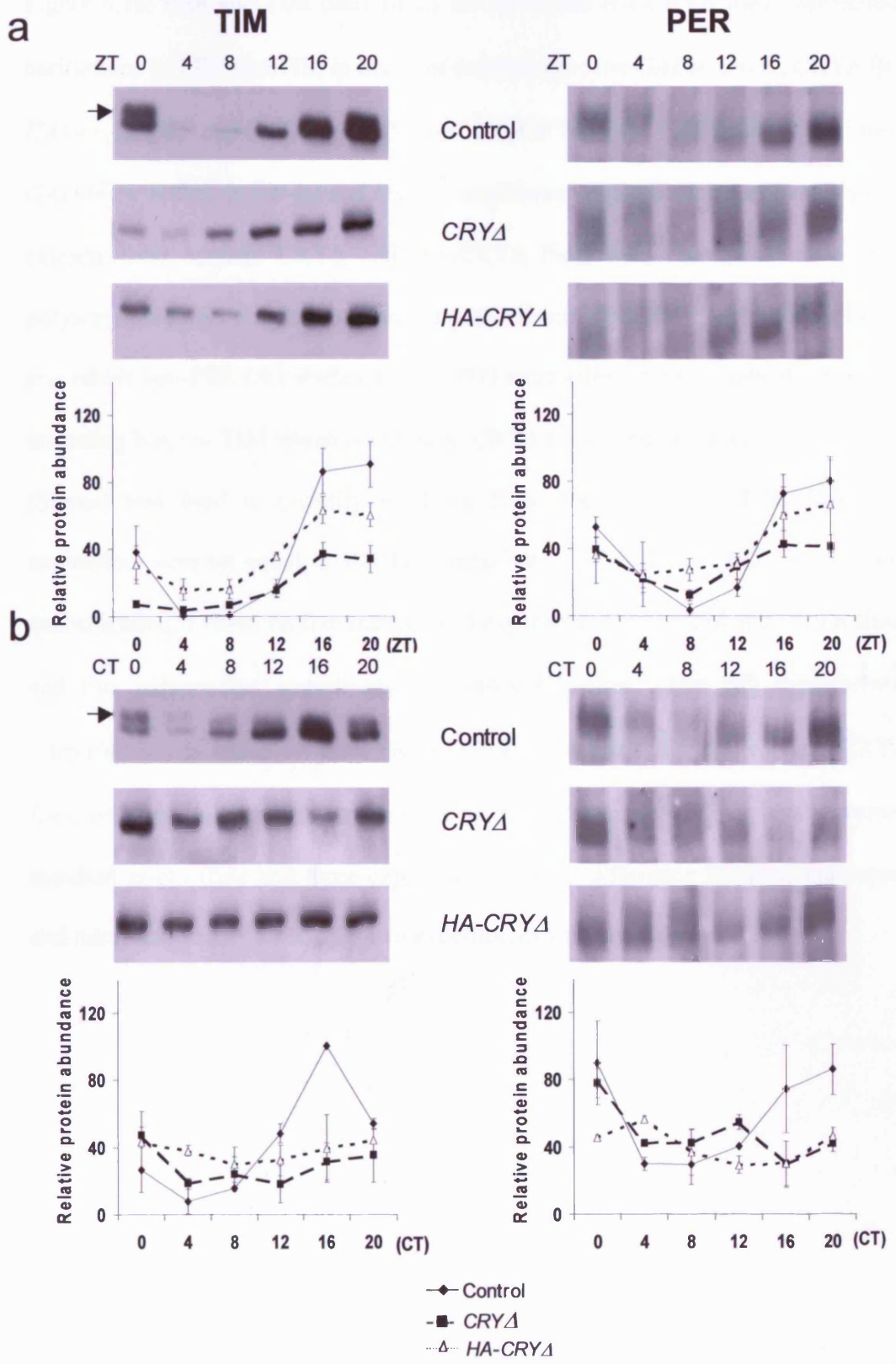

a
TIM
PER
ZT 0 4 8 12 16 20
Control
CRYΔ
HA-CRYΔ
Relative protein abundance
120
80
40
0
0 4 8 12 16 20 (ZT)
b
CT 0 4 8 12 16 20
0 4 8 12 16 20 (CT)
Control
CRYΔ
HA-CRYΔ

Figure 5.18: PER and TIM oscillations in CRYΔ and HA-CRYΔ flies. Molecular oscillations of PER and TIM in heads of controls (*yw*; *tim-GAL4*/+; +/+), CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) and HA-CRYΔ (*w*, *UAS-hacryΔ15.3*; *tim-GAL4*/+; +/+) flies in LD (a) and DD (b) conditions. Equal amounts of head protein extracts from control, CRYΔ and HA-CRYΔ flies, were run on the same 6% polyacrylamide gel and immunoblotted with rat anti-TIM(307) (Myers et al. 1996) and rabbit anti-PER (Stanewsky et al. 1997) antibodies. Arrows indicate the slow migrating band of TIM which is absent in CRYΔ flies. Immunodetection of HSP70 (Sigma) was used to quantify levels of both clock proteins. Peak levels of expression were set equal to 100 for normalisation of data. For LD experiments, quantification is based on five independent experiments for control and CRYΔ flies, and two independent experiments for HA-CRYΔ flies. For DD experiments, quantification is based on three independent experiments for control and CRYΔ flies, and two independent experiments for HA-CRYΔ flies. Error bars represent standard errors (five and three experiments) or the difference between maximum and minimum values when only two experiments were available.

in LD conditions, TIM levels are higher than in controls during the day (ZT 4-12) and lower during the night (ZT16-0). This results in a dampened oscillation of TIM in CRYΔ flies. Moreover, the pattern of TIM oscillation is strikingly different as hyperphosphorylated forms of the protein are not seen at all in CRYΔ flies. TIM appears only as a single band probably accounting for hypophosphorylated forms. In DD conditions, the overall oscillation of TIM is almost abolished in CRYΔ flies with levels being higher than in controls during the subjective day and lower during the subjective night. Again, hyperphosphorylation of TIM is not visible.

PER oscillation is also affected but to a lesser extent. Levels of PER are higher during day/subjective day and lower during night/subjective night in LD and DD conditions respectively resulting in a dampened oscillation. Phosphorylation of PER is visible in CRYΔ flies and it is even possible that phosphorylated PER persist for longer in LD and DD conditions (compare ZT8 or CT8 between controls and CRYΔ flies).

5.2.5 CRYΔ molecular oscillation

The expression of CRYΔ triggers important phenotypes, thus it seemed interesting to check the molecular oscillation of overexpressed CRYΔ. Western blots were performed on HA-CRYΔ flies because of the lack of CRY antibody. Figure 5.19 shows the pattern of accumulation of CRYΔ in LD and DD conditions. As a reference, the blot showing the molecular cycle of HA-CRY (Figure 3.10) is reported. Note that these two blots were not exposed for the same period of time so levels of protein can not be directly compared. It can be seen that levels of CRYΔ are very low and appear to be constant. Even in darkness CRYΔ fails to

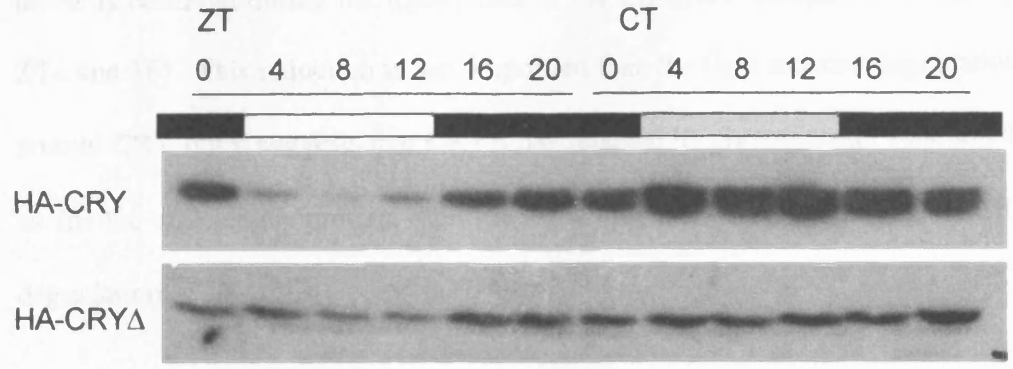


Figure 5.19: HA-CRYΔ molecular oscillations. Molecular oscillation of HA-CRY and HA-CRYΔ in LD (ZT) and DD (CT) conditions. Equal amounts of head protein extracts from HA-CRY and HA-CRYΔ flies, were run on the same 10% polyacrylamide gel and immunoblotted with anti-HA antibody (Sigma). Note that the two blots were not exposed for the same period of time so direct comparison of the overall quantity of protein is not possible (20s for HA-CRY and 5 min for HA-CRYΔ). These experiments were repeated at least 3 times with similar results. Representative blots are shown.

accumulate whereas normal CRY does. However, a slight reduction of CRYΔ levels is observed during the light phase of the LD cycle (compare ZT8 and 12 to ZT4 and 16). This reduction is less important than the light induced degradation of normal CRY but it suggests that CRYΔ has retained its photoreceptor function that, as for the full length protein, starts a signalling pathway culminating in CRYΔ degradation.

5.2.6 Effect of overexpressed CRYΔ on normal CRY

As seen in the previous experiment CRYΔ levels are very low. Because of the lack of a CRY antibody, I could not check directly whether levels of endogenous CRY are affected by CRYΔ overexpression. However, in order to check if overexpression of CRYΔ influences the molecular oscillation of normal CRY that is present in these flies, western blots were performed on transgenics overexpressing both HA-CRY and CRYΔ. Details of the scheme used to cross *UAS-hacry16.1* in *UAS-cryΔ14.6* background are given in Figure 5.20. Representative blots of HA-CRY and CRYΔ,HA-CRY flies collected in LD conditions are shown in Figure 5.21. In HA-CRY flies, the protein is displaying a clear oscillation in its quantity with maximum levels occurring at night (ZT0 and 20). As previously described, the protein is actively degraded upon light exposure so that its levels are very low at the end of the day (ZT8 and 12) (Emery et al., 1998). In flies overexpressing CRYΔ, HA-CRY also shows cycling in its levels; nevertheless the quantification given in Figure 5.21b indicates that HA-CRY levels are higher in CRYΔ flies. This is especially true at ZT4 and 8 when the protein should be degraded upon light exposure suggesting that the light induced

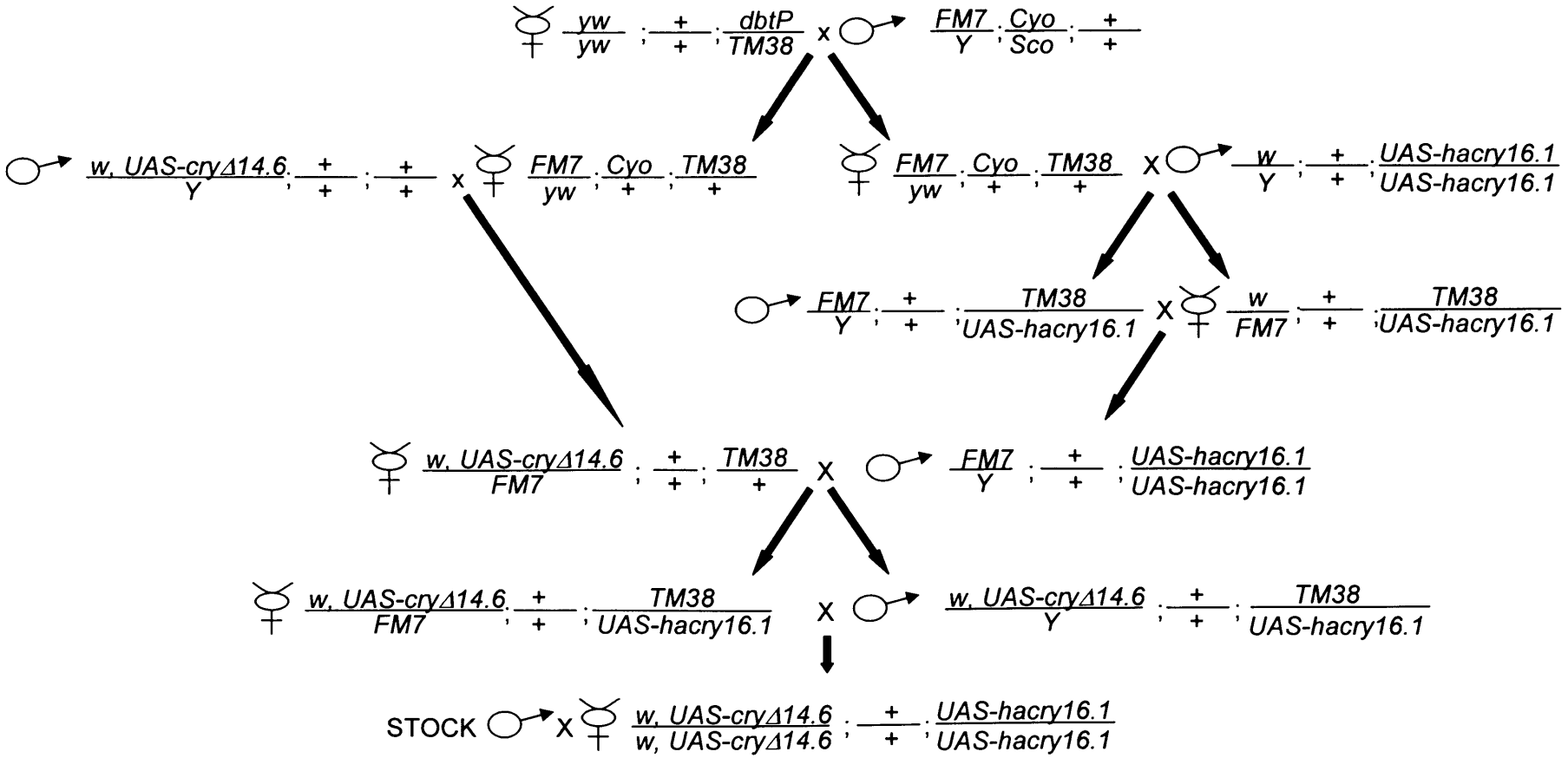


Figure 5.20: scheme used to cross *UAS-hacry16.1* in *UAS-cryΔ14.6* background. FM7, Cyo and TM38 are chromosome balancers for the X, second and third chromosome respectively. They prevent recombination and also carry a visible marker making them easy to identify.

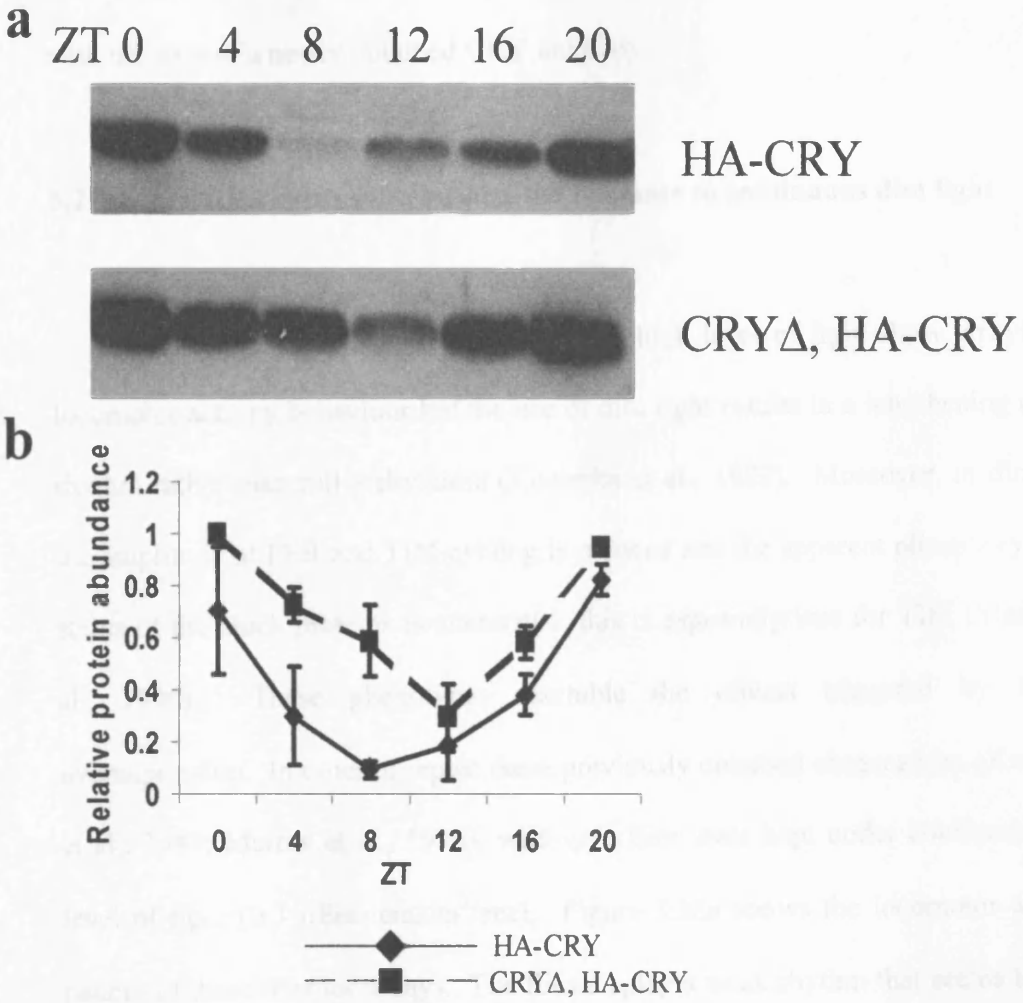


Figure 5.21: HA-CRY molecular oscillations in CRYΔ, HA-CRY flies. **a.** Molecular oscillations of HA-CRY in HA-CRY (*w*; *tim-GAL4*/+; *UAS-hacry16.1*/+) and CRYΔ, HA-CRY (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; *UAS-hacry16.1*/+) flies in 12:12 LD condition. Equal amounts of head protein extracts from HA-CRY and CRYΔ, HA-CRY flies were run on the same 10% polyacrylamide gel and immunoblotted with anti-HA antibody (Sigma). **b.** Quantification of two blots for each genotype using HSP70 (Sigma) as a reference. Error bars represent the difference between maximum and minimum values.

degradation of CRY is affected by the overexpression of CRYΔ. However, to make this absolutely sure levels of endogenous CRY should be checked in future work with the help of a newly obtained CRY antibody.

5.2.7 CRYΔ expression mimics the response to continuous dim light

Wild-type flies kept under continuous high level of light show arrhythmic locomotor activity behaviour but the use of dim light results in a lengthening of the rhythm rather than full arrhythmia (Konopka et al., 1989). Moreover, in dim LL, the amplitude of PER and TIM cycling is reduced and the apparent phosphorylation status of the clock proteins is attenuated, this is especially true for TIM (Marrus et al., 1996). These phenotypes resemble the effects triggered by CRYΔ overexpression. In order to repeat these previously obtained observations (Konopka et al., 1989; Marrus et al., 1996), wild-type flies were kept under continuous low level of light (0.3 μEinsteins/m^2/sec). Figure 5.22a shows the locomotor activity pattern of these flies for 4 days. The flies display a weak rhythm that seems to have a long period. To confirm this, Fourier analysis was used and reveals that 67% of a total of 81 flies are rhythmic under this condition with a long period of activity (28.53±0.37h).

Western blots were also performed on heads of flies subjected to the same dim LL (0.3 μEinsteins/m^2/sec) condition and a representative example is shown in Figure 5.22c. Under this condition TIM levels cycle, in agreement with previous work (Marrus et al., 1996), however hyperphosphorylated forms of the protein that can be seen at ZT0 (just before the beginning of the dim LL phase) are never observed. Therefore, the lengthening of locomotor activity period and the effect on

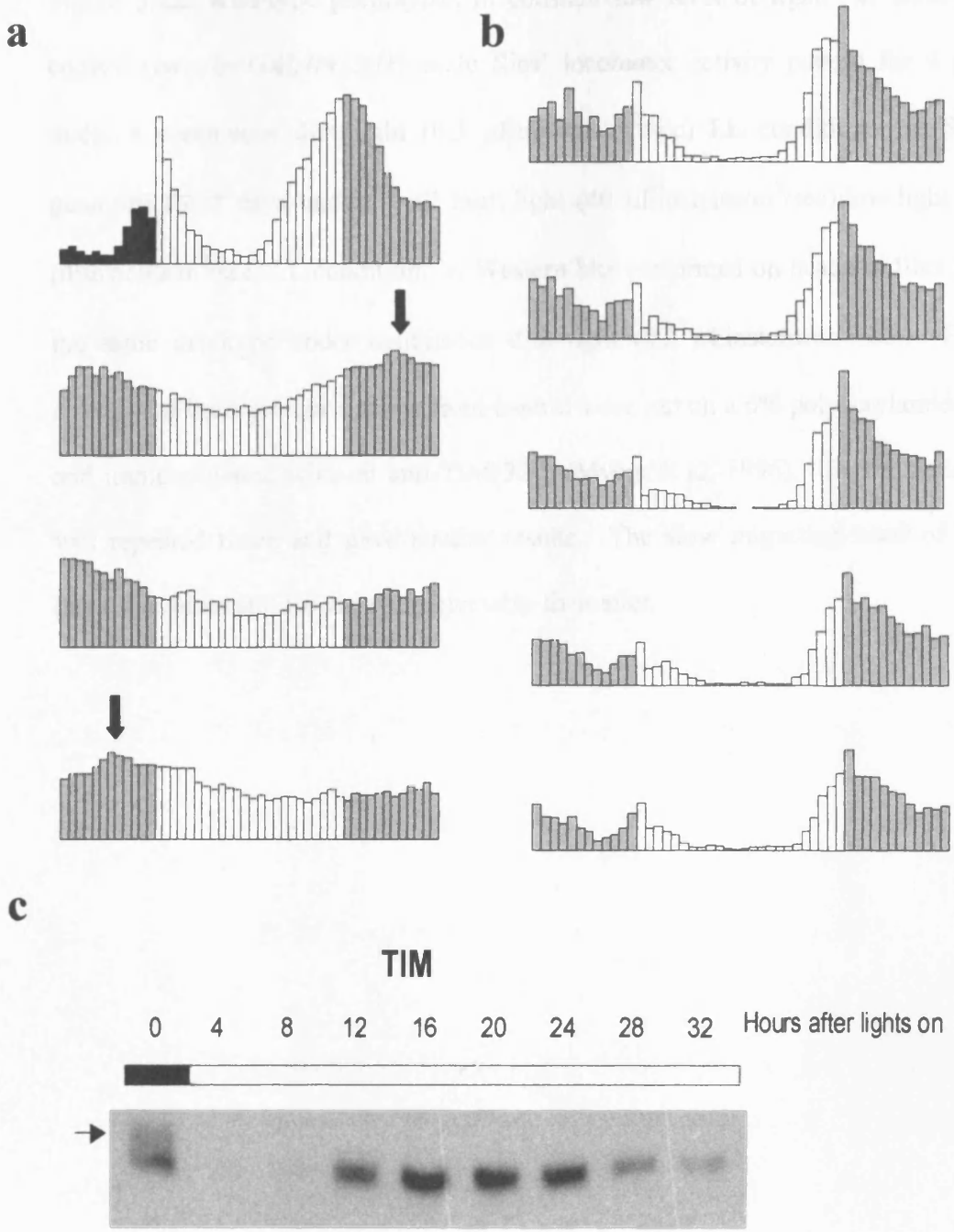

a
b
c
TIM
0
4
8
12
16
20
24
28
32
Hours after lights on

Figure 5.22: Wild-type phenotypes in constant low level of light. **a.** Wild-type control (*yw*; *tim-GAL4*/+; +/+) male flies' locomotor activity pattern for 4 days under a continuous dim light (0.3 μEinsteins/m^2/sec) LL condition. **b.** Same genotype for 5 days under 12:12 high-light (40 μEinsteins/m^2/sec):low-light (0.3 μEinsteins/m^2/sec) LL condition. **c.** Western blot performed on heads of flies from the same genotype under continuous dim light (0.3 μEinsteins/m^2/sec). Equal amounts of head protein extracts from control were run on a 6% polyacrylamide gel and immunoblotted with rat anti-TIM(307) (Myers et al. 1996). This experiment was repeated twice and gave similar results. The slow migrating band of TIM (arrow) evident at ZT 0 is never detectable thereafter.

TIM oscillation observed in dim LL are very similar to phenotypes triggered by CRYΔ overexpression. This and the discovery that CRYΔ is unstable suggests that the CRYΔ protein is constitutively active, giving continuous light information to the pacemaker clock and as a consequence being always degraded. If this is true, it would mean that CRYΔ flies never experience darkness, because a dark phase would be the same as a dim light period. As a result a normal 12:12 LD would in fact be a condition of 12h of bright light followed by 12h of dim light. To test if this condition can entrain the circadian clock, wild-type controls were subjected to a 12:12 high-light (40 μEinsteins/m^2/sec): low light (0.3 μEinsteins/m^2/sec) regime. The activity pattern of these flies is given in Figure 5.22b. It shows that the flies entrain perfectly to such a condition showing rhythmic locomotor activity. Fourier analysis reveals that 100% of 35 flies used in this experiment are rhythmic with a period of 24.15±0.1h.

5.2.8 TIM and PER expression in the lateral neurons of CRYΔ flies

The PRC is thought to reflect changes in the subcellular localization of PER and TIM in clock neurons. Delays occur at times when the clock proteins are mainly cytoplasmic while advances are linked to nuclear localization (Rothenfluh et al., 2000; Young, 1998). Because the PRC is dramatically affected in CRYΔ flies the subcellular localizations of PER and TIM should be affected in clock neurons. To test this, immunohistochemical assays were performed on whole-mount brains collected at ZT19, ZT21 and ZT23. The analysis was focused on the LN_vs because they are easily identified by the cytoplasmic neuropeptide PDF (Renn et al., 1999). The brains were double-labelled with antibodies to cPDH, the crustacean homolog

of PDF (Dircksen et al., 1987; Park et al., 2000) and PER or TIM. Results are presented in Figure 5.23 for PER and 5.24 for TIM. Figure 5.23a shows that in control flies (*yw*; *tim-GAL4*/+; +/+) PER localization is cytoplasmic and nuclear at ZT19 in both types of LN_vs. At ZT21 and ZT23, PER becomes mainly nuclear in the large LN_vs (l-LN_vs) while it is still nuclear and cytoplasmic in the small LN_vs (s-LN_vs) at ZT21 and completely nuclear at ZT23. The nuclear/cytoplasmic (N/C) ratio was calculated for each time point and is indicated by Figure 5.23b. The N/C ratio for the s-LN_vs goes from 1.41 (ZT19) to 2.26 (ZT21) and finally 4.14 (ZT23). For the l-LN_vs it goes from 1.41 (ZT19) to 3.48 (ZT21) and 5.78 (ZT23). This is in agreement with previous work that showed that PER nuclear accumulation occurred earlier in the l-LN_vs (Shafer et al., 2002).

CRYΔ flies (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) on the other hand show a completely different pattern of PER localization. At ZT19 PER is mainly cytoplasmic in both subsets of LN_vs, it then becomes nuclear and cytoplasmic at ZT21 and 23 but never manages to be completely nuclear. The N/C ratio goes from 0.88 (ZT19) to 1.07 (ZT21) and 1.30 (ZT23) for the s-LN_vs and from 0.78 (ZT19) to 0.95 (ZT21) and 1.23 (ZT23) for the l-LN_vs. This indicates that the nuclear accumulation of PER is affected in CRYΔ flies and also suggests that the l-LN_vs are the most affected of the two LN_vs subset. This is because the s-LN_vs N/C ratio at ZT23 is 4.14 for wild-type versus 1.30 for CRYΔ while being reduced from 5.78 to 1.23 in the l-LN_vs. Two-way ANOVA confirms that PER nuclear accumulation is reduced in CRYΔ flies and that the l-LN_vs are more affected (GENOTYPE, $F_{1,244}$= 233.40, $p<<0.01$; CELL TYPE, $F_{1,244}$= 10.20, $p<0.01$; GENOTYPE*CELL TYPE, $F_{1,244}$= 15.48, $p<<0.01$, appendix 17).

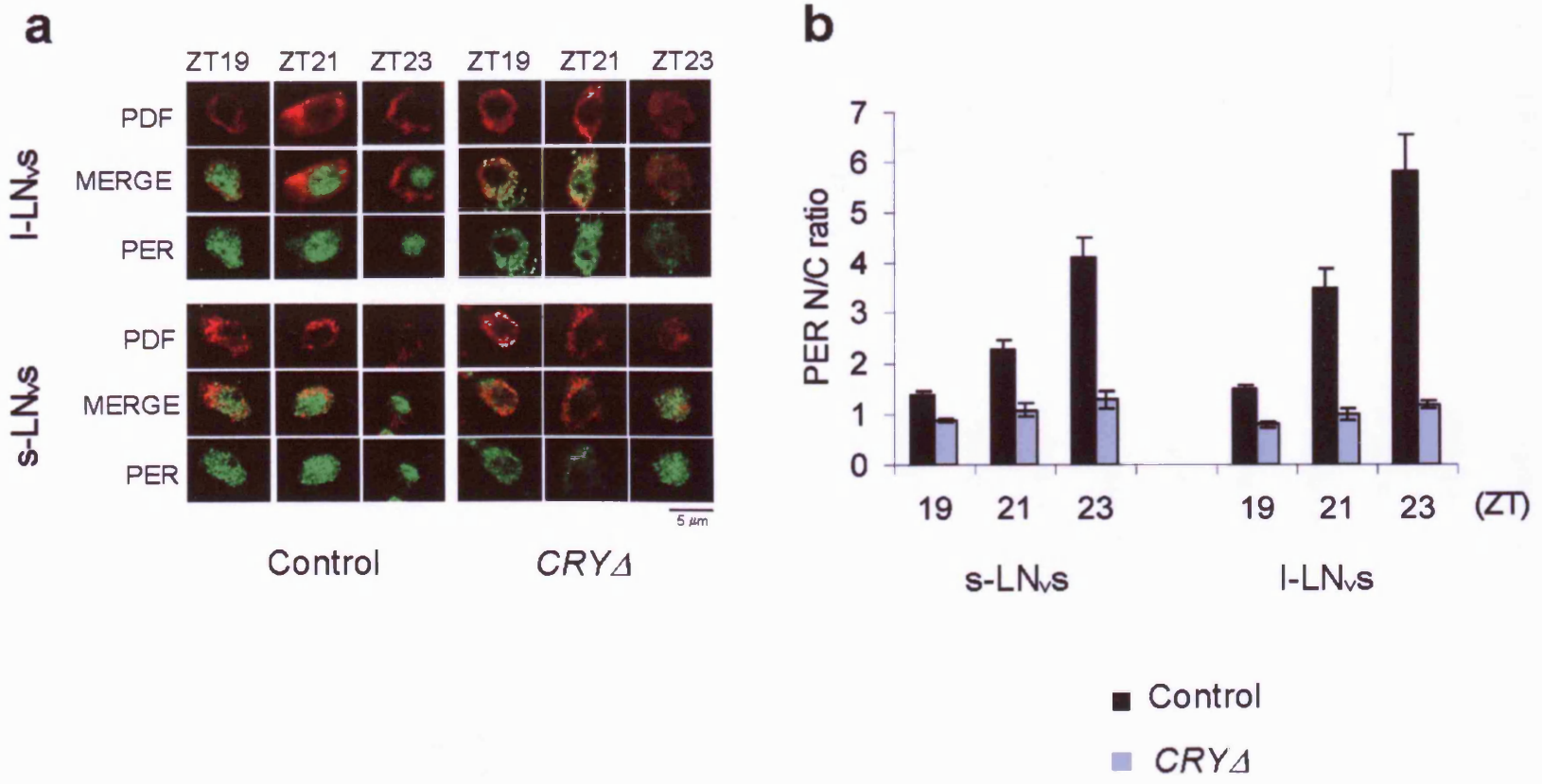
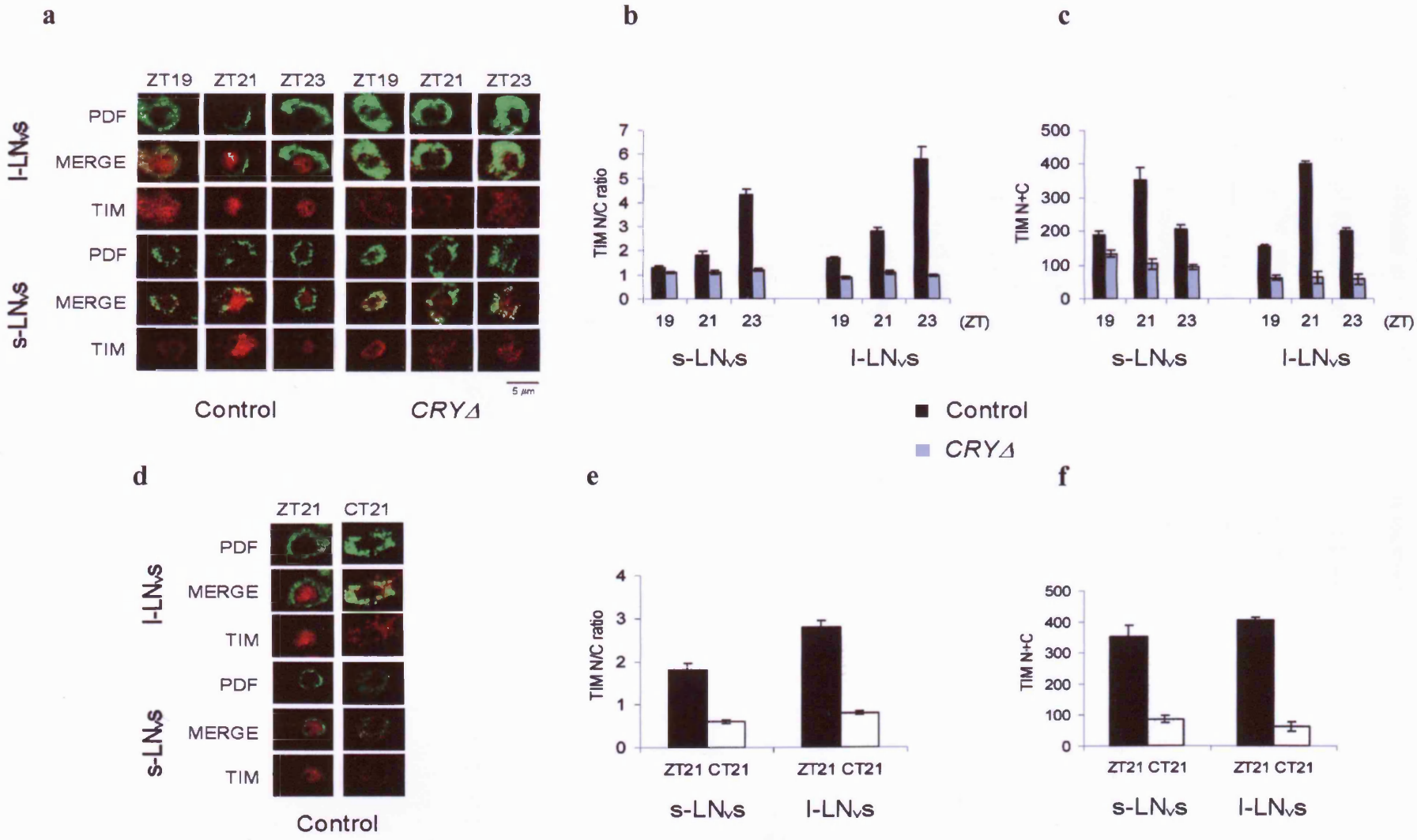

a
ZT19
ZT21
ZT23
ZT19
ZT21
ZT23
l-LNvs
PDF
MERGE
PER
s-LNvs
PDF
MERGE
PER
5 µm
Control
CRYΔ
b
PER N/C ratio
0
1
2
3
4
5
6
7
19
21
23
19
21
23
(ZT)
s-LNvs
l-LNvs
Control
CRYΔ

Figure 5.23: (Dissel et al. 2004) PER immunocytochemistry in CRYΔ flies. **a.** Localization of PER (Stanewsky et al. 1997) (green) and PDF (Park et al. 2000) (red) within the small and large LN_vs of control (*yw*; *tim-GAL4*/+; +/+) and CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) flies at the indicated ZT. PDF is used as a marker of neuronal identity and cytoplasmic localization. **b.** Quantification of the Nuclear/Cytoplasmic (N/C) ratio of PER in the two clusters of LN_vs at the indicated ZT. Quantification data are presented as average±SEM. At least three brains have been analysed for each genotype at every time point, all visible cells were imaged but only those where PDF staining showed a clear distinction between nucleus and cytoplasm were quantified. Due to the high background produced by the anti-PER antibody it was not possible to image a relevant number of cells using the same detector settings, therefore it was not possible to quantify the total level of protein in this experiment.

Figure 5.24a shows that in control flies TIM is becoming mainly nuclear at ZT21. The N/C ratio was calculated and is given in Figure 5.24b. It goes from 1.29 (ZT19) to 1.80 (ZT21) and 4.29 (ZT23) for the s-LN_vs and from 1.67 (ZT19) to 2.82 (ZT21) and 5.77 (ZT23) for the l-LN_vs. Again the l-LN_vs show an advanced nuclear entry of TIM in agreement with published work (Shafer et al., 2002). The overall quantity of TIM has been estimated and the results are illustrated in Figure 5.24c. TIM is at maximum levels at ZT21 for both group of LN_vs. The oscillation is however more pronounced in the l-LN_vs (3 fold amplitude versus 2 fold for the s-LN_vs).

CRYΔ flies again show a strikingly different pattern of TIM accumulation (Figure 5.24a). TIM appears to be at very low levels at the three time points analysed and it is mainly cytoplasmic at ZT19 and then becomes nuclear and cytoplasmic but never gets only nuclear. The N/C ratio (Figure 5.24b) goes from 1.05 (ZT19) to 1.11 (ZT21) and 1.17 (ZT23) for the s-LN_vs and from 0.92 (ZT19) to 1.11 (ZT21) and 1.00 (ZT23) for the l-LN_vs. Therefore, TIM nuclear accumulation is strongly affected in CRYΔ flies and the l-LN_vs seem to be more affected. The overall quantity of TIM is also strongly reduced in both groups of LN_vs (Figure 5.24c) but the reduction is more pronounced in the l-LN_vs. For example compared with control, TIM level is only 30% less in the s-LN_vs of CRYΔ flies at ZT19 while it is reduced by 55% in the l-LN_vs. At later time points, TIM levels are clearly reduced in both groups of LN_vs but there is always more TIM in the s-LN_vs. Two-way ANOVA confirms that TIM nuclear accumulation as well as total levels are reduced by CRYΔ and that the l-LN_vs are more affected (GENOTYPE, $F_{1,292}$= 321.32, $p<<0.01$; CELL TYPE, $F_{1,292}$= 5.98, $p<0.05$; GENOTYPE*CELL TYPE, $F_{1,292}$=8.22,$P<0.01$ for total protein level and


a
ZT19
ZT21
ZT23
ZT19
ZT21
ZT23
l-LNvs
PDF
MERGE
TIM
s-LNvs
PDF
MERGE
TIM
5 µm
Control
CRYΔ
b
TIM N/C ratio
0
1
2
3
4
5
6
7
19
21
23
19
21
23
(ZT)
s-LNvs
l-LNvs
c
TIM N+C
0
100
200
300
400
500
19
21
23
19
21
23
(ZT)
s-LNvs
l-LNvs
Control
CRYΔ
d
ZT21
CT21
l-LNvs
PDF
MERGE
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s-LNvs
PDF
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TIM N/C ratio
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ZT21 CT21
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s-LNvs
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TIM N+C
0
100
200
300
400
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ZT21 CT21
ZT21 CT21
s-LNvs
l-LNvs

Figure 5.24: (Dissel et al. 2004) TIM immunocytochemistry in CRYΔ flies. **a.** Localization of TIM (Yang and Sehgal 2001) (red) and PDF (Dircksen et al. 1987) (green) within the small and large LN_vs of control (*yw*; *tim-GAL4*/+; +/+) and CRYΔ (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; +/+) flies at the indicated ZT. PDF is used as a marker of neuronal identity and cytoplasmic localization. **b.** Quantification of the Nuclear/Cytoplasmic (N/C) ratio of TIM in the two clusters of LN_vs at the indicated ZT. **c.** quantification of total levels (N+C) of TIM in the two clusters of LN_vs at the indicated ZT. **d.** localization of TIM within the small and large LN_vs of control (*yw*; *tim-GAL4*/+; +/+) flies at ZT21 and CT21 (LL). **e.** Quantification of the (N/C) ratio of TIM in the two clusters of LN_vs at ZT21 and CT21 (LL). **f.** quantification of total levels (N+C) of TIM in the two clusters of LN_vs at ZT21 and CT21 (LL). Quantification data are presented as average±SEM. At least five brains have been analysed for each genotype at every time point, all visible cells were imaged but only those where PDF staining showed a clear distinction between nucleus and cytoplasm were quantified.

GENOTYPE, $F_{1,417}$= 399.95, p<<0.01; CELL TYPE, $F_{1,417}$= 20.78, p<<0.01; GENOTYPE*CELL TYPE, $F_{1,417}$= 31.51, p<<0.01 for subcellular distribution) (appendix 17).

Finally, in an attempt to determine whether or not the overexpression of CRYΔ gives a continuous light information to the clock, controls (*yw*; *tim-GAL4*/+; +/+) were used in a comparison between ZT21 and CT21 in LL (Figure 5.24d). It can be seen that constant light strongly affects N/C ratio and overall quantity of TIM in the LN_vs (Figure 5.24d, e and f). TIM remains mainly cytoplasmic with an N/C ratio of 0.61 for the s-LN_vs and 0.78 for the l-LN_vs. The reduction of overall quantity of TIM is 75% for the s-LN_vs and 85% for the l-LN_vs. As a conclusion constant light affects TIM nuclear localization and total quantity in a way consistent with the hypothesis that CRYΔ gives constant light information to the clock.

5.3 Summary

CRYΔ flies show striking phenotypes. They have long period of locomotor activity. The molecular oscillations of PER and TIM in peripheral clocks are strongly affected by CRYΔ both at the overall level and at the phosphorylation status level. This is especially pronounced for TIM. The subcellular localization of PER and TIM and the level of TIM in the lateral neurons are also affected by CRYΔ. The CRYΔ protein itself is very instable and also reduces the light induced degradation of normal CRY expressed in the same flies. Moreover, CRYΔ flies have a reduced light sensitivity in both LL and PRC experiments. Because wild-type flies kept under continuous dim light show similar phenotypes, these results

suggest that CRYΔ is a constitutive form of the protein giving continuous light information to the clock.

Chapter 6: Overexpression of CRYΔ in cry^b background

6.1 Introduction

Overexpression of CRYΔ in cry^+ background results in long period of locomotor activity as well as a reduction of circadian photosensitivity. PER and TIM molecular oscillations are also dramatically affected, especially for TIM as hyperphosphorylated forms of the protein are never seen in CRYΔ flies. The phenotypes associated with CRYΔ overexpression are reminiscent of the phenotypes observed in flies kept under constant low level of light. These discoveries plus the finding that CRYΔ levels are constitutively low suggests that it is a constitutively active form of the protein. To test whether the results obtained by CRYΔ expression are independent of endogenous CRY, CRYΔ was overexpressed in the strong hypomorphic cry^b mutant background (Stanewsky et al., 1998). Figure 6.1 illustrates the scheme used to cross *UAS-cryΔ14.6* in the cry^b mutant background. The scheme to cross *tim-GAL4* in cry^b background was shown in Figure 4.1.

6.2 Results and Discussion

6.2.1 Entrainment

Figure 6.2 shows the average locomotor activity pattern of male flies overexpressing CRYΔ in cry^b mutant background (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/cry^b) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; cry^b/cry^b and *w*, *UAS-cryΔ14.6*; +/+; cry^b/cry^b) during 5 days under a 12:12 LD condition. Both controls entrain to the LD condition, with clear anticipation of the evening peak.

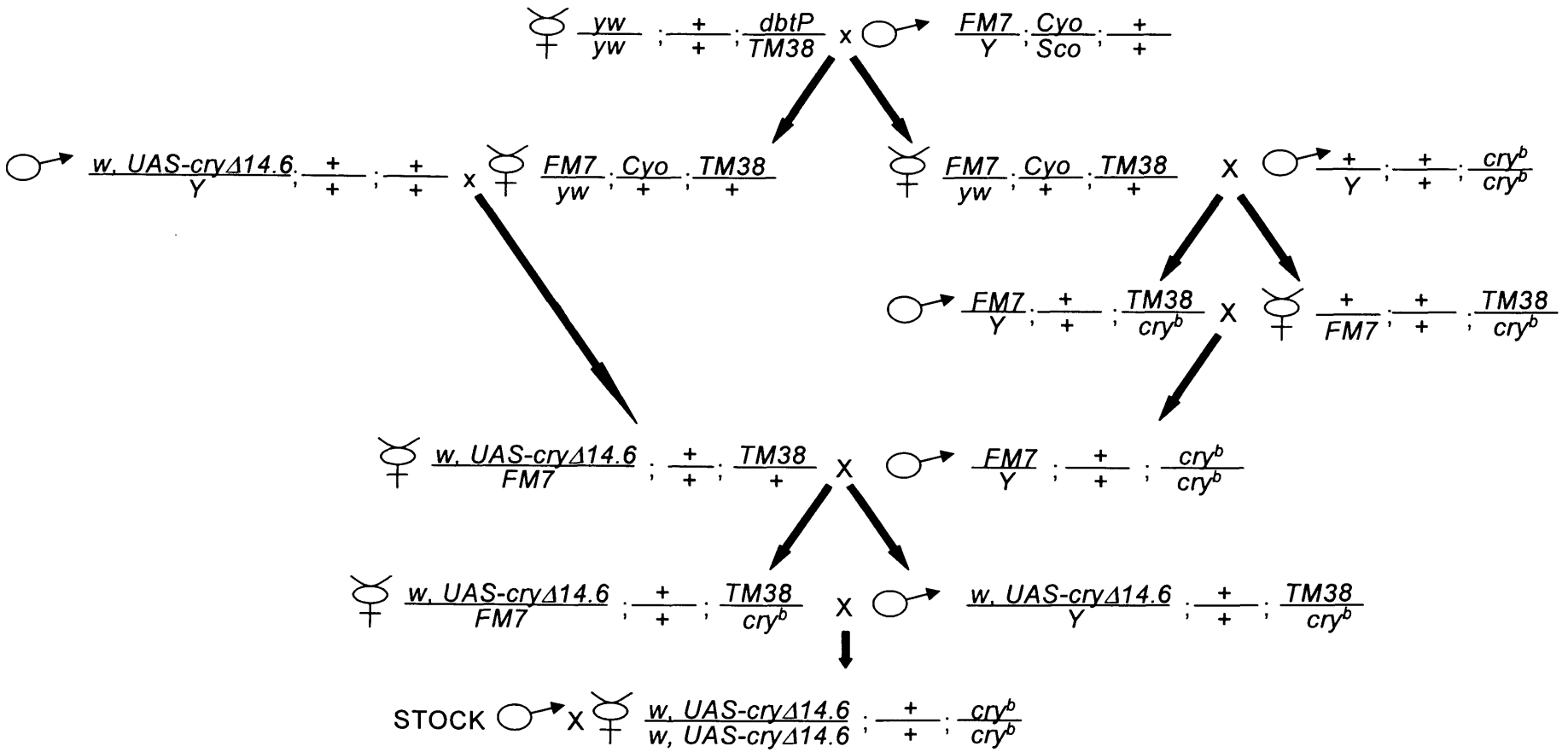


Figure 6.1: Scheme used to cross *UAS-cryΔ14.6* in the *cry*^{b} mutant background. FM7, Cyo and TM38 are chromosome balancers for the X, second and third chromosome respectively. They prevent recombination and also carry a visible marker making them easy to identify.

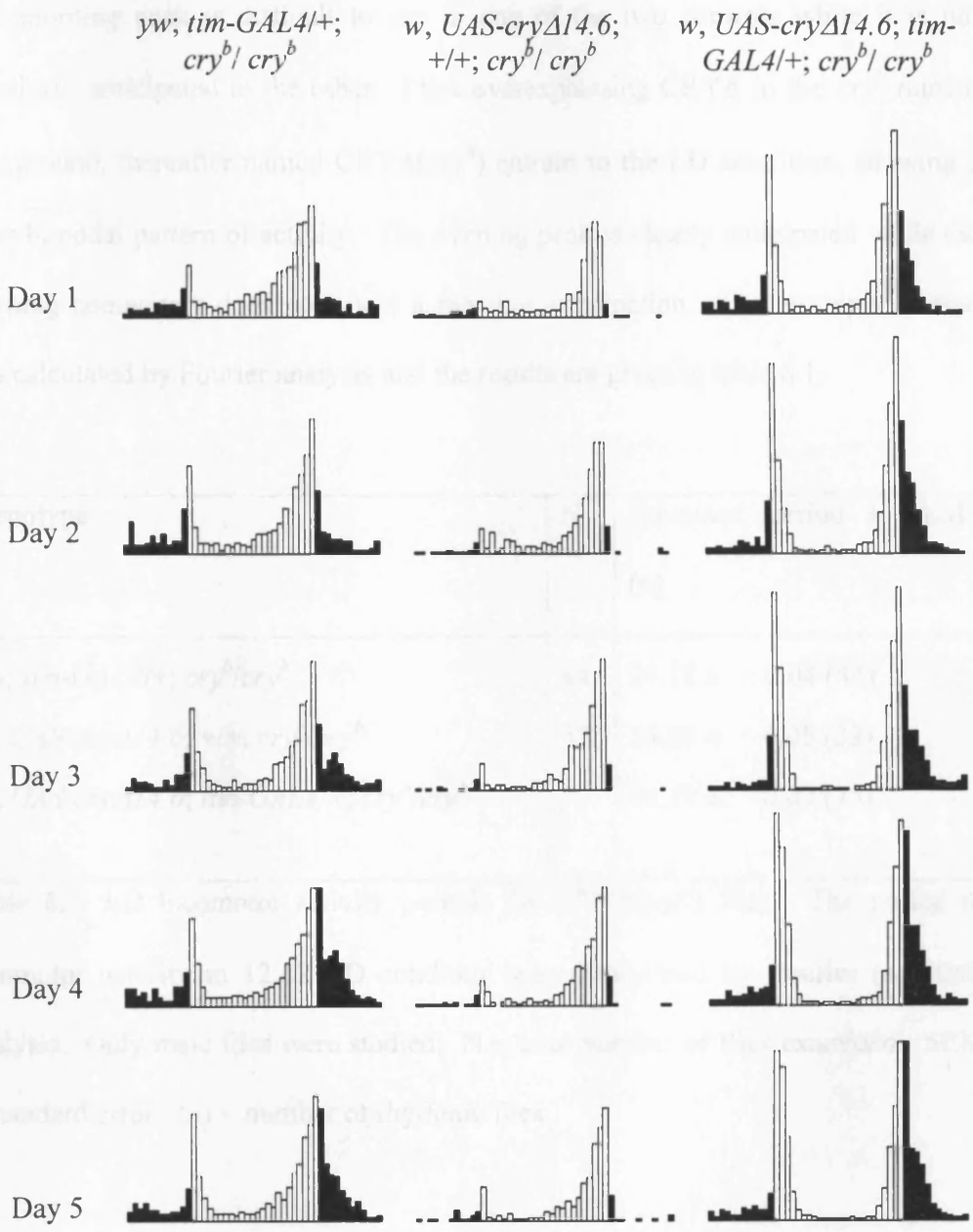


Figure 6.2: LD behaviour of CRYΔ(*cry*^{b}) flies. Average locomotor activity pattern for male flies overexpressing CRYΔ in *cry*^{b} mutant background (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b}) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b} and *w*, *UAS-cryΔ14.6*; +/+; *cry*^{b}/ *cry*^{b}) during 5 days under a 12:12 LD condition. Open bars= day, filled bars= night.

The morning peak is difficult to see in one of the two controls while it is not massively anticipated in the other. Flies overexpressing CRYΔ in the cry^b mutant background, thereafter named CRYΔ(cry^b) entrain to the LD condition, showing a clear bimodal pattern of activity. The evening peak is clearly anticipated while the morning component does not show a massive anticipation. The entrained period was calculated by Fourier analysis and the results are given in table 6.1.

Genotype	N	Entrained period ± SEM (n)
yw; *tim-GAL4*/+; cry^b/cry^b	44	24.18 ± 0.04 (44)
w, *UAS-cryΔ14.6*; +/+; cry^b/cry^b	33	24.27 ± 0.05 (33)
w, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/cry^b	75	24.18 ± 0.02 (75)

Table 6.1: LD locomotor activity periods for CRYΔ(cry^b) flies. The period of locomotor activity in 12:12 LD condition was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

Inspection of table 6.1 confirms that CRYΔ(cry^b) flies entrain to 12:12 LD cycles with period of locomotor activity similar to controls ($F_{2,149}$=1.82, ns, appendix 18).

6.2.2 Free running behaviour

Figure 6.3 shows the average locomotor activity pattern of male CRYΔ(cry^b) flies (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/cry^b) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; cry^b/cry^b and *w*, *UAS-cryΔ14.6*; +/+; cry^b/cry^b) during 5 days under DD conditions. Both controls manifest rhythmic locomotor activity but it is bimodal for only one of the two. CRYΔ(cry^b) flies show a clearly different pattern of locomotor activity. They have a strong evening peak that is moving more and more towards the subjective night indicating a long period of locomotor activity. Fourier analysis confirms that CRYΔ(cry^b) flies have longer period of activity than both controls (table 6.2) ($F_{1,102}$=113.13, P<0.01, appendix 18).

Genotype	N	Free-running period ± SEM (n)
yw; *tim-GAL4*/+; cry^b/cry^b	31	24.39 ± 0.08 (30)
w, *UAS-cryΔ14.6*; +/+; cry^b/cry^b	28	24.24 ± 0.07 (26)
w, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/cry^b	48	25.60 ± 0.11 (48)

Table 6.2: DD locomotor activity periods for CRYΔ(cry^b) flies. The period of locomotor activity in DD conditions was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

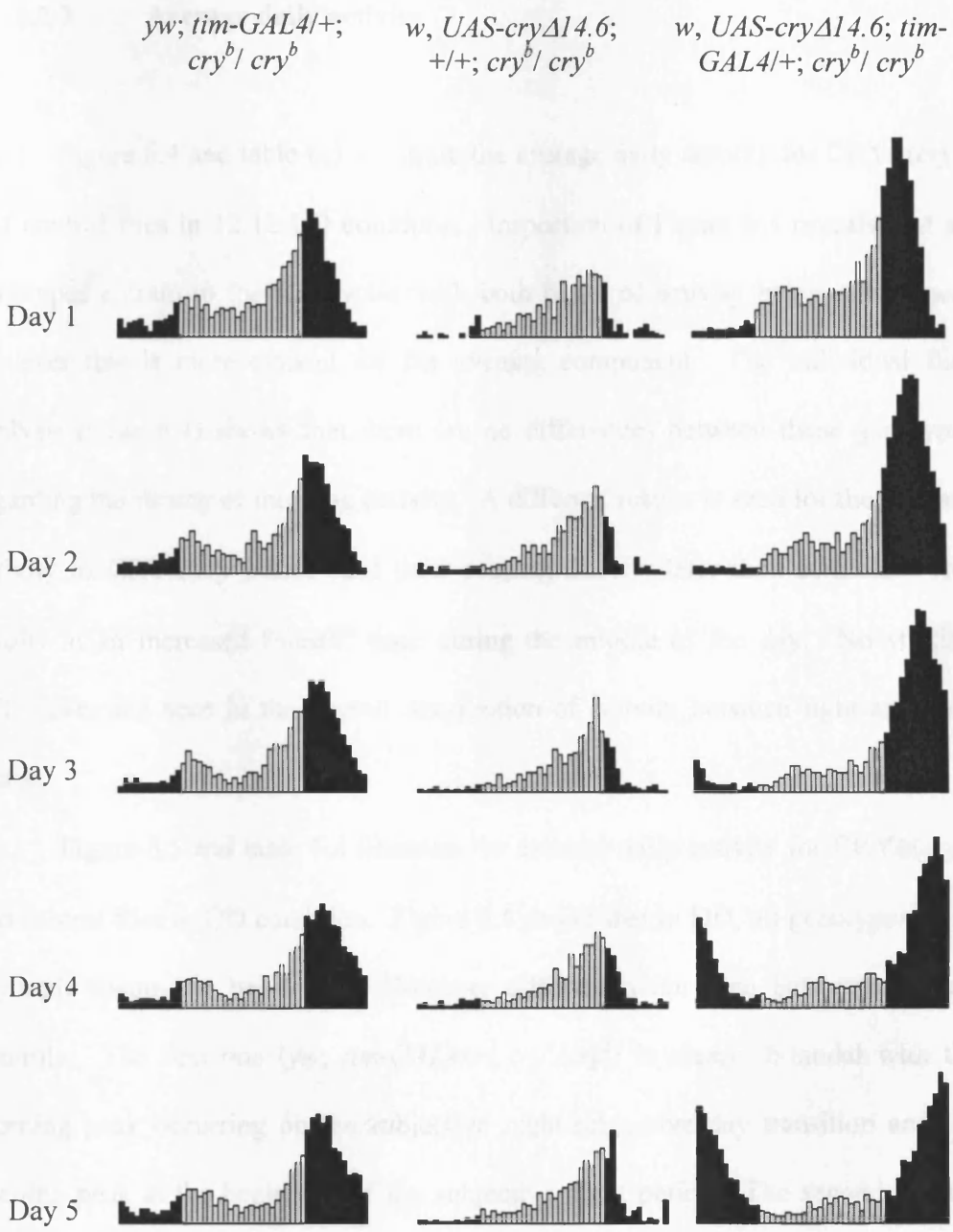


Figure 6.3: DD behaviour of CRYΔ(*cry*^{b}) flies. Average locomotor activity pattern for male flies overexpressing CRYΔ in *cry*^{b} mutant background (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b}) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b} and *w*, *UAS-cryΔ14.6*; +/+; *cry*^{b}/ *cry*^{b}) during 5 days under DD condition. Grey bars= subjective day, filled bars= subjective night.

6.2.3 Average daily activity

Figure 6.4 and table 6.3 illustrate the average daily activity for CRYΔ(cry^b) and control flies in 12:12 LD condition. Inspection of Figure 6.4 reveals that all genotypes entrain to the LD cycles with both peaks of activity being anticipated; however this is more evident for the evening component. The individual flies analysis (table 6.3) shows that there are no differences between these genotypes regarding the timing of morning activity. A different results is seen for the evening activity as CRYΔ(cry^b) flies start their evening activity later than controls. This results in an increased "siesta" time during the middle of the day. No striking differences are seen in the overall distribution of activity between light and dark phase.

Figure 6.5 and table 6.4 illustrate the average daily activity for CRYΔ(cry^b) and control flies in DD condition. Figure 6.5 shows that in DD, all genotypes show rhythmic locomotor behaviour. However differences are seen between the two controls. The first one (*yw*; *tim-GAL4*/+; cry^b/cry^b) is clearly bimodal with the morning peak occurring on the subjective night/subjective day transition and the evening peak at the beginning of the subjective night period. The second control shows a unimodal pattern of locomotor activity peaking well before the end of the subjective day. CRYΔ(cry^b) flies are displaying bimodality, although the morning component has a weak amplitude. The morning peak occurs after the beginning of the subjective day while the evening component peaks on the subjective day/subjective night transition. Inspection of table 6.4 confirms the differences between the two controls observed in Figure 6.5. The first control has morning activity peaking 1h before and evening peak occurring 1h after the second control

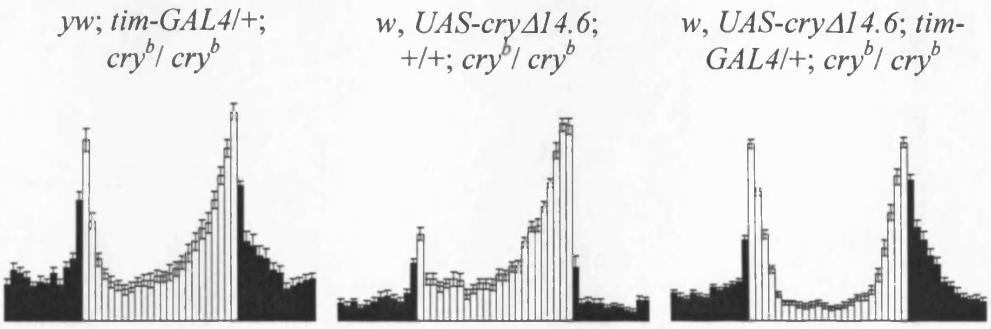


Figure 6.4: LD average daily activity for CRYΔ(*cry*^{b}) flies. Average daily activity in 12:12 LD conditions for male flies overexpressing CRYΔ in *cry*^{b} background (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; *cry*^{b}/*cry*^{b}) and the relevant controls (*yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b} and *w*, *UAS-cryΔ14.6*; +/+; *cry*^{b}/*cry*^{b}). Open bars= light period, filled bars= dark period.

Genotype		*yw*; *tim-GAL4*/+; cry^b/ cry^b	*w*, *UAS-cryΔ14.6*; +/+; cry^b/ cry^b	*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/ cry^b
Morning activity	Onset	23.07 ± 0.14 (30)	23.41 ± 0.17 (23)	23.24 ± 0.12 (41)
	Peak	0.42 ± 0.06 (30)	0.67 ± 0.22 (23)	0.50 ± 0.00 (41)
	Offset	3.73 ± 0.27 (30)	3.89 ± 0.24 (23)	4.06 ± 0.17 (41)
Evening activity	Onset	8.13 ± 0.31 (30)	7.76 ± 0.22 (23)	9.62 ± 0.18 (41)
	Peak	12.08 ± 0.14 (30)	11.67 ± 0.07 (23)	12.24 ± 0.13 (41)
	Offset	14.67 ± 0.25 (30)	13.24 ± 0.19 (23)	15.13 ± 0.18 (41)
Siesta		4.40 ± 0.34 (30)	3.87 ± 0.25 (23)	5.56 ± 0.21 (41)
Proportion of activity during light phase		0.67 ± 0.03 (30)	0.89 ± 0.02 (23)	0.64 ± 0.02 (41)
Proportion of activity during dark phase		0.33 ± 0.03 (30)	0.11 ± 0.02 (23)	0.36 ± 0.02 (41)

Table 6.3: LD average daily activity for CRYΔ(cry^b) flies. Analysis of average daily activity in 12:12 LD conditions for individual male flies overexpressing CRYΔ in cry^b background as well as the relevant controls.

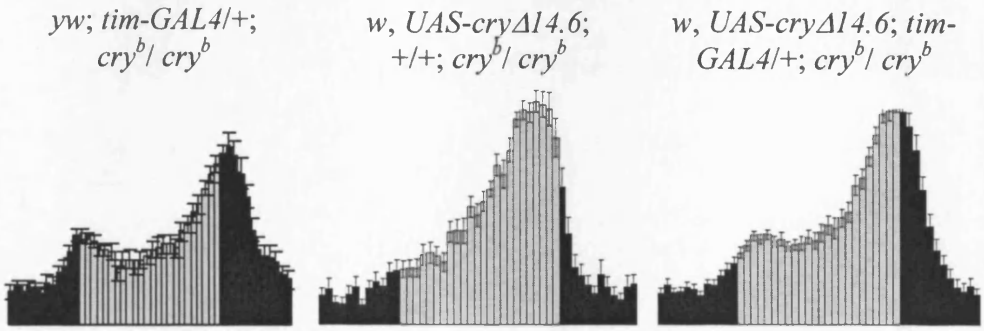


Figure 6.5: DD average daily activity for CRYΔ(*cry*^{b}) flies. Average daily activity in DD conditions for male flies overexpressing CRYΔ in *cry*^{b} background (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; *cry*^{b}/*cry*^{b}) and the relevant controls (*yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b} and *w*, *UAS-cryΔ14.6*; +/+; *cry*^{b}/*cry*^{b}). Grey bars= subjective day period, filled bars= subjective night period.

Genotype	*yw*; *tim-GAL4*/+; cry^b/ cry^b	*w*, *UAS-cryΔ14.6*; +/+; cry^b/ cry^b	*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/ cry^b
Number of flies examined	29	23	41
Number of flies where morning peak was present	28 (97%)	18 (78%)	35 (85%)
Morning peak	0.50 ± 0.32	1.33 ± 0.34	2.13 ± 0.31
Evening peak	13.03 ± 0.34	10.93 ± 0.23	12.01 ± 0.24
Phase difference	12.52 ± 0.30	9.56 ± 0.42	10.10 ± 0.28
Proportion of activity during subjective day	0.50 ± 0.04	0.83 ± 0.02	0.64 ± 0.03
Proportion of activity during subjective night	0.50 ± 0.04	0.17 ± 0.02	0.36 ± 0.03

Table 6.4: DD average daily activity for CRYΔ(cry^b) flies. Analysis of average daily activity in DD conditions for individual male flies overexpressing CRYΔ in cry^b background as well as the relevant controls.

resulting in a 2h phase difference. Another difference is the fact than the second control (*w*, *UAS-cryΔ14.6*; +/+; cry^b/cry^b) shows a reduced proportion of bimodal flies (78% versus 97%). CRYΔ(cry^b) flies are bimodal in a proportion of 85% and the morning activity occurs in the subjective day while evening activity peaks on the subjective day/subjective night transition.

6.2.4 Response of CRYΔ(cry^b) flies to constant light exposure

Figure 6.6 shows the average locomotor activity pattern of male CRYΔ(cry^b) flies as well as controls during 1 day in 12:12 LD condition followed by 7 days under LL condition. The two cry^b controls are rhythmic in LL in agreement with published data (Emery et al., 2000). CRYΔ(cry^b) flies show a different pattern of activity as they are clearly rhythmic for the first 5 days under LL with the evening peak moving towards the right of the activity chart in each subsequent day indicating a long period of locomotor activity. The activity profile becomes flat afterwards indicating no clear rhythmicity and suggesting that some light information reaches the clock in these flies. Fourier analysis was used to calculate the free-running period of these flies and results are shown in table 6.5.

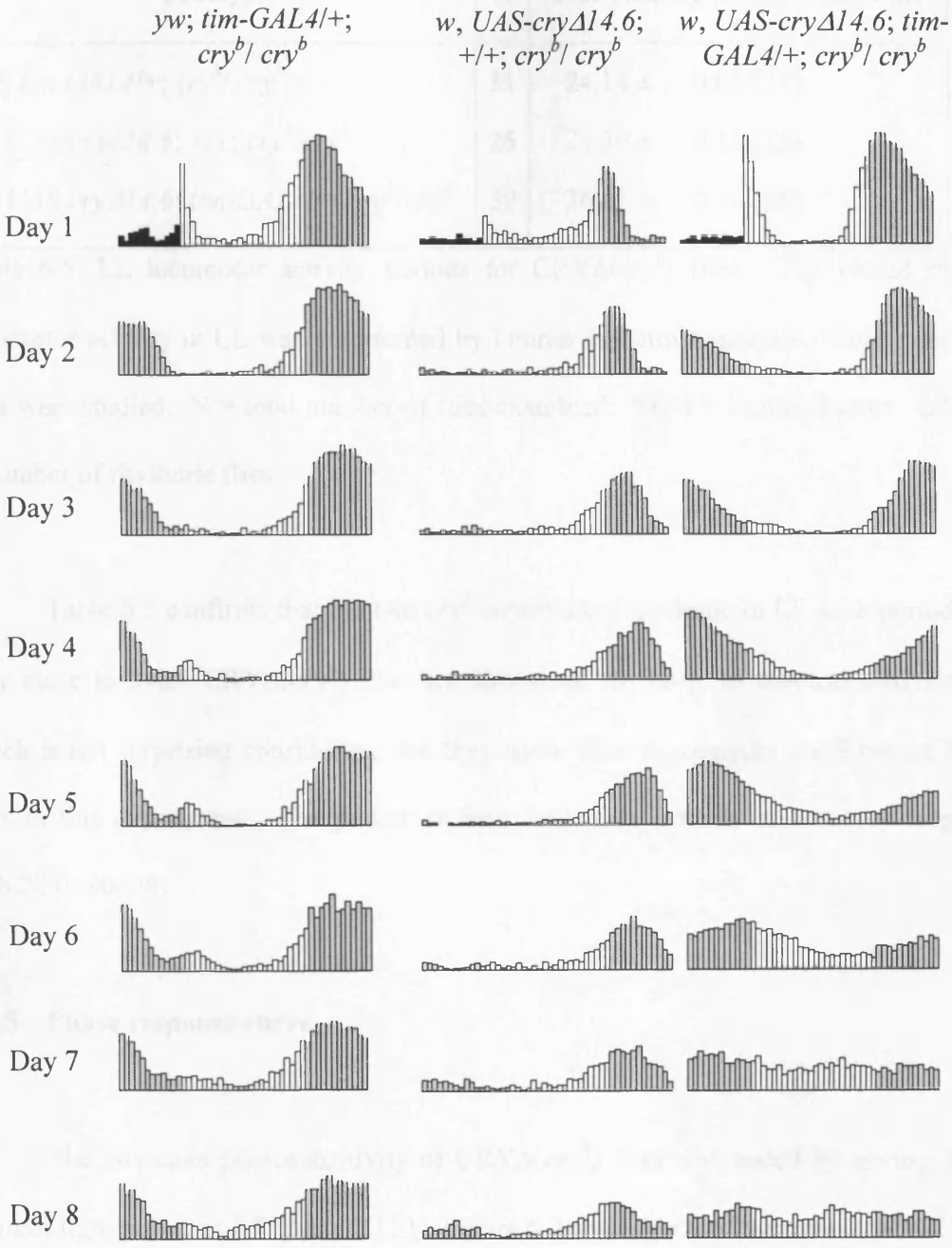


Figure 6.6: LL behaviour of CRYΔ(cry^b) flies. Average locomotor activity pattern for male flies overexpressing CRYΔ in cry^b mutant background (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/ cry^b) as well as the relevant wild-type controls (*yw*; *tim-GAL4*/+; cry^b/ cry^b and *w*, *UAS-cryΔ14.6*; +/+; cry^b/ cry^b) during 1 day in 12:12 LD condition followed by 7 days under LL conditions. Open bars= subjective day, grey bars= subjective night.

Genotype	N	Free-running period ± SEM (n)
yw; *tim-GAL4*/+; cry^b/ cry^b	31	24.14 ± 0.05 (31)
w, *UAS-cryΔ14.6*; +/+; cry^b/cry^b	25	24.30 ± 0.11 (25)
w, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/cry^b	59	26.01 ± 0.19 (58)

Table 6.5: LL locomotor activity periods for CRYΔ(cry^b) flies. The period of locomotor activity in LL was determined by Fourier (spectral) analysis. Only male flies were studied. N = total number of flies examined. SEM = standard error. (n) = number of rhythmic flies.

Table 6.5 confirms that the two cry^b controls are rhythmic in LL with period very close to 24h. CRYΔ(cry^b) flies are almost all rhythmic in spectral analysis which is not surprising considering that they show clear rhythmicity for 5 out of 7 days in this experiment. The period of their locomotor activity is however long with 26.01±0.19h.

6.2.5 Phase response curve

The circadian photosensitivity of CRYΔ(cry^b) flies was tested by giving 5 minutes light pulses at ZT15 and ZT21. Figure 6.7 and table 6.6 illustrates the PRC of CRYΔ(cry^b) and control flies.

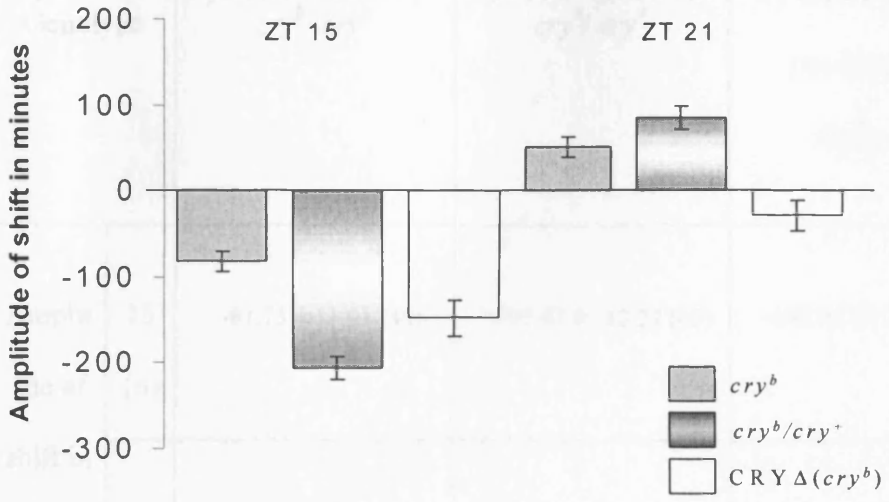


Figure 6.7: PRC of CRYΔ(*cry*^{b}) flies. Light-pulse phase shift at ZT15 and ZT21. A 5 min light pulse was delivered at either ZT15 or ZT21 to the following genotypes: CRYΔ(*cry*^{b}) (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; *cry*^{b}/*cry*^{b}) (31 flies at ZT15, 61 at ZT21), *yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b} (40 flies at ZT15, 35 at ZT21) and *yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*$^+$ (45 flies at ZT15, 41 at ZT21). Number of unpulsed control flies were 57 for *yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*^{b}, 73 for *yw*; *tim-GAL4*/+; *cry*^{b}/ *cry*$^+$ and 64 for *w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; *cry*^{b}/*cry*^{b}. Standard error bars are indicated.

Genotype		*yw*; *tim-GAL4*/+; cry^b/cry^b	*yw*; *tim-GAL4*/+; cry^b/cry^+	*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/cry^b
Amplitude of shift in minutes at ZT	15 (n)	-81.75 ±11.91 (40)	-205.47 ± 13.27 (45)	-147.92 ± 21.54 (31)
	21 (n)	51.21 ± 11.61 (35)	84.03 ± 13.09 (41)	-28.70 ± 17.98 (61)

Table 6.6: PRC of CRYΔ(cry^b) flies. Amplitude of shift obtained in minutes ± SEM with a 5 minutes light pulse given at the indicated ZT. (n) = number of flies pulsed. Numbers of unpulsed control flies were 57 for *yw*; *tim-GAL4*/+; cry^b/cry^b ; 73 for *yw*; *tim-GAL4*/+; cry^b/cry^+ and 64 for *w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/cry^b

Analysis of Figure 6.7 and table 6.6 reveals that at ZT15 CRYΔ(cry^b) flies phase-delay their locomotor activity by 2.5h which is an hour more than cry^b controls but an hour less than cry^b/cry^+ heterozygotes. This suggests that CRYΔ partially rescues full-length CRY activity in cry^b mutants at this time point. However, the situation is different at ZT21 as CRYΔ(cry^b) flies phase-delay by 0.5h instead of showing the usual phase-advance expected at this time point. Analysis by ANOVA reveals that CRYΔ(cry^b) flies respond differently from cry^b controls at both ZTs ($F_{1,69}$=8.08, P<0.01 at ZT15 and $F_{1,94}$=9.94, P<0.01 at ZT21) while they are only marginally different from cry^b/cry^+ heterozygotes at ZT15 ($F_{1,74}$=5.76, P<0.05, appendix 19). In conclusion, the response to short light pulses is partially

functional in the phase-delay zone of the PRC but clearly blunted in the advance zone.

6.2.6 Molecular cycling of PER and TIM in CRYΔ(cry^b) flies

Figure 6.8 shows western blots as well as quantification of PER and TIM levels in CRYΔ(cry^b) and control flies under LD and DD conditions. In agreement with published data (Stanewsky et al., 1998) PER and TIM levels are constitutively high in cry^b controls and are present in highly phosphorylated forms. CRYΔ(cry^b) flies show a different pattern of expression for PER and TIM, principally for TIM. Overall levels and phosphorylation status of the two proteins are reduced, not only compared to cry^b flies but also to wild-type controls (see Figure 5.18). In particular, the slow migrating band of TIM representing higher phosphorylated forms, is not visible in CRYΔ(cry^b) extracts. In CRYΔ(cry^b) flies PER and TIM cycle with a very low amplitude, reaching maximal and minimal levels at the end of the (subjective) night and of the (subjective)day, respectively.

6.3 Summary

Overexpressing CRYΔ in the cry^b mutant background results in a long period of locomotor activity in DD. Moreover, molecular oscillations are rescued, although they do not achieve wild-type characteristics and hyperphosphorylated forms of TIM are completely absent. The functional relevance of CRYΔ is further demonstrated by the fact that CRYΔ(cry^b) flies show physiological responses to light stimuli, as they become arrhythmic under prolonged LL and phase shift to a

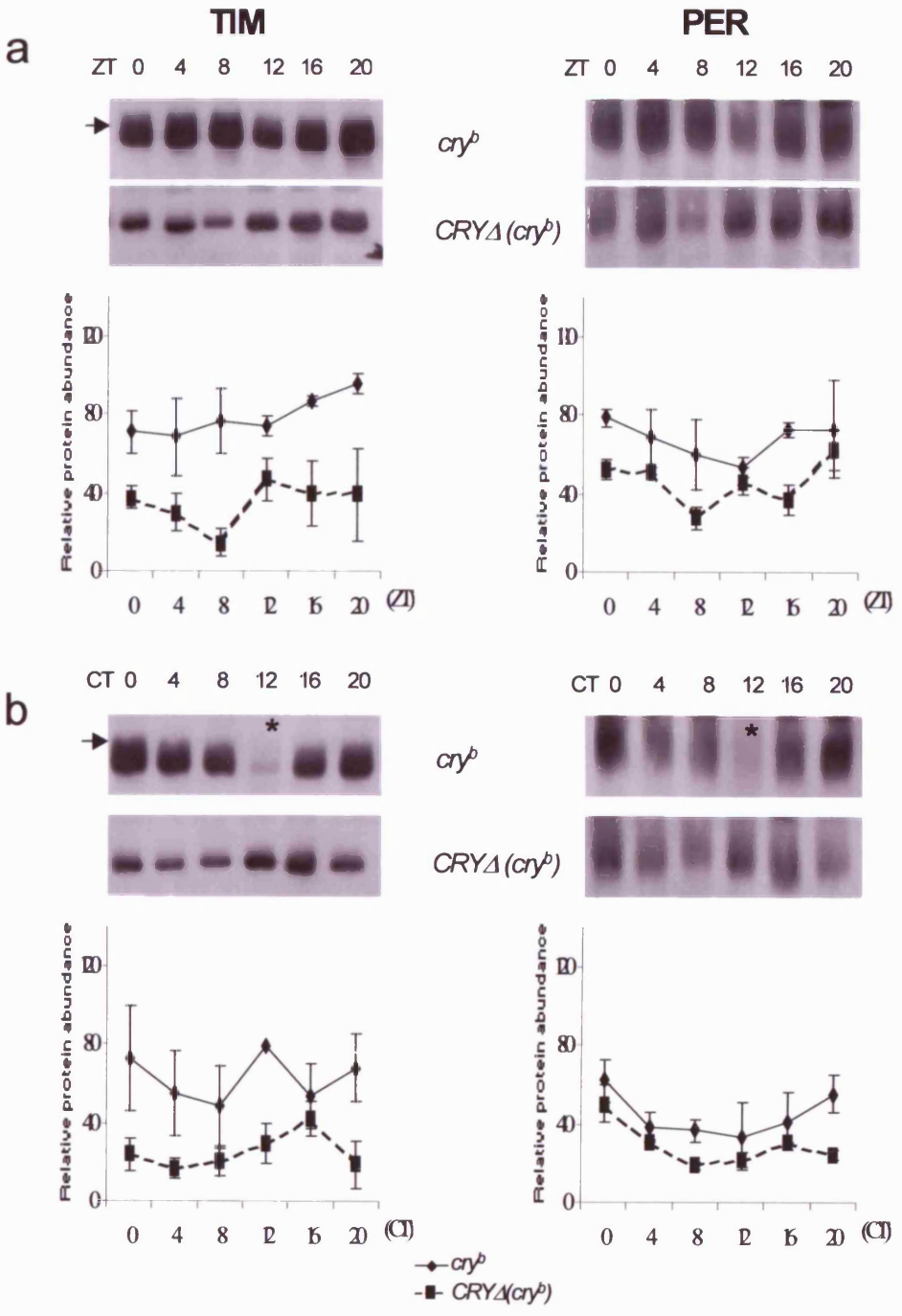

a
TIM
PER
ZT 0 4 8 12 16 20
cry^b
CRYΔ (cry^b)
Relative protein abundance
(ZT)
b
CT 0 4 8 12 16 20
(CT)
cry^b
CRYΔ(cry^b)

Figure 6.8: PER and TIM oscillations in CRYΔ(cry^b) flies. Molecular oscillations of PER and TIM in controls (*yw*; *tim-GAL4*/+; cry^b/ cry^b) and CRYΔ(cry^b) (*w*, *UAS-cryΔ14.6*; *tim-GAL4*/+; cry^b/cry^b) flies in LD (a) and DD (b) conditions. Equal amounts of head protein extracts from cry^b and CRYΔ(cry^b) flies, were run on the same 6% polyacrylamide gel and immunoblotted with rat anti-TIM(307) (Myers et al. 1996) and rabbit anti-PER (Stanewsky et al. 1997) antibodies. Arrows indicate the slow migrating band of TIM which is absent in CRYΔ(cry^b) flies. Immunodetection of HSP70 (Sigma) was used to quantify levels of both clock proteins. Peak levels of expression were set equal to 100 for normalisation of data. For LD experiments, quantification is based on three independent experiments for cry^b control (only two for TIM) and CRYΔ(cry^b) flies. For DD experiments, quantification is based on three independent experiments for cry^b control (only two for TIM) and CRYΔ(cry^b) flies. Error bars represent standard errors (three experiments) or the difference between maximum and minimum values when only two experiments were available. A degraded sample is indicated by an asterisk.

light pulse delivered at ZT15. These results fully confirm the findings presented in the previous chapter and demonstrate that CRYΔ functions independently of endogenous CRY as a constitutively active form of the protein.

Chapter 7: General discussion

CRY is a blue-light sensitive protein involved in circadian photoreception (Emery et al., 1998; Stanewsky et al., 1998) that shows homology with 6-4 photolyases, enzymes that repair UV damaged DNA in a light dependent manner (Carell et al., 2001). The N-terminus of the protein is the region of higher homology whereas the C-terminus represents a specialized, species-specific extension which is not present in photolyases (Emery et al., 1998). In *Drosophila*, overexpression of CRY in clock neurons by means of the GAL4/ *UAS* system (Brand and Perrimon, 1993) leads to enhanced photosensitivity (Emery et al., 1998; Emery et al., 2000b). Conversely, the severe hypomorphic mutation cry^b strongly affects circadian photoresponses (Emery et al., 2000a; Helfrich-Forster et al., 2001; Stanewsky et al., 1998). In peripheral tissues such as the eyes (Stanewsky et al., 1998) the antennae (Krishnan et al., 2001) or the Malpighian tubules (Ivanchenko et al., 2001) cry^b stops the endogenous oscillations, implying a core function for CRY in the clock. Mechanistically, important findings were the discovery that CRY can directly bind to PER and TIM in a light dependent manner in a yeast two-hybrid assay (Ceriani et al., 1999; Rosato et al., 2001) and that, when 20 amino acids from the C-terminus are removed to create CRYΔ, the light dependency of CRY interactions is lost (Rosato et al., 2001). It is believed that CRY acts on the central clock by controlling the light-induced degradation of TIM (Lin et al., 2001). The aim of my thesis was to study the role of CRY and CRYΔ *in vivo*. The work hypothesis was that *in vivo*, CRYΔ but not CRY, would behave as a constitutively active form of the protein triggering behavioural, molecular and cytological

phenotypes consistent with a constitutive light response. To do so, the GAL4/ *UAS* system (Brand and Perrimon, 1993) was used to target the overexpression of CRY or CRYΔ in specific subsets of clock neurons.

7.1 Overexpression of CRY

Overexpressing CRY in all *timeless* competent cells does not affect entrainment to LD cycles nor does it change the free-running period of locomotor activity (Figures 3.1, 3.2, 3.3, 3.4; tables 3.1 and 3.2). Moreover, Western blot analyses of head extracts show that in CRY flies the molecular oscillations of PER and TIM are virtually identical to wild-type controls. As the eyes are the major source of proteins in head extracts, these results suggest that upregulation of CRY does not induce major changes in this peripheral clock (Figure 3.9). The overexpression of a tagged form of the protein, HA-CRY, shows molecular oscillations that are in agreement with published data based on the expression of endogenous CRY (Figure 3.10) (Emery et al., 1998). As previously mentioned, clock neurons targeted overexpression of CRY increases light sensitivity of the circadian clock (Emery et al., 1998). In this thesis, overexpressing CRY in all *timeless* relevant cells also results in enhanced photosensitivity, confirming previous studies. CRY flies seem to become arrhythmic faster and show a reduced proportion of rhythmic flies in constant light (Figure 3.7 and table 3.5). In PRC experiments, CRY flies display phase-advances that have higher amplitude than wild-type controls when pulsed at ZT21 and ZT23 (Figure 3.8 and table 3.6). However, there are no differences in phase-shift amplitudes at earlier time points (ZT15, ZT17 and ZT19). This might imply that the increased photosensitivity of

CRY flies manifests itself more prominently in the advance zone of the PRC. Interestingly, the advance zone of the PRC is correlated with nuclear PER and TIM. It is therefore tempting to suggest that CRY-mediated light responses affect the clock more readily at times when the clock proteins are nuclear.

The use of average daily activity suggests a novel dark role for CRY in the central clock. The pattern of locomotor activity shows that CRY flies have morning and evening peaks of activity pushed towards the night in 12:12 LD cycles (Figure 3.5, table 3.3). As a result, CRY flies show an increased "siesta" during the middle of the day and have a higher proportion of locomotor activity during the night (table 3.3). These results seem to suggest a light-independent role for CRY, at least in the distribution of locomotor activity across the 24h. Indeed, the same phenomenon is observed under DD, namely the subjective morning and evening components are pushed towards the subjective night increasing the phase-difference between the two activity peaks and resulting in a higher proportion of locomotor activity during subjective night (Figure 3.6, table 3.4). The same findings are also confirmed in CRY(cry^b) flies (Figures 4.5 and 4.6, tables 4.3 and 4.4).

It follows that CRY might exert a complex role in central clock cells rather than just being a circadian photoreceptor. It is known that morning and evening activity components are controlled by different subsets of clock neurons, the s-LN_vs and the LN_ds respectively (Grima et al., 2004; Stoleru et al., 2004). As CRY has different effects, namely advancing the morning and delaying the evening activity peaks, it may be suggested that CRY has cell specific functions, speeding up the morning oscillator while slowing down the evening one. Because locomotor behaviour requires the integration of information from different clock neurons

(Peng et al., 2003), it is also possible that CRY influences the communication between the different neurons involved in the control of locomotor activity.

Overexpression of CRY in the cry^b mutant background does not affect entrainment and free-running rhythm in flies (Figures 4.3 and 4.4, tables 4.1 and 4.2). However, and in agreement with published data (Emery et al., 2000b), it rescues the normal arrhythmicity in LL conditions (Figure 4.7, table 4.5) as well as a wild-type response to a light pulse at ZT15 (Figure 4.8, table 4.6). Nevertheless, a closer inspection of tables 3.6 and 4.5 reveals that a pulse given at ZT21 results in substantially higher phase-advances in a cry^+ background. Homozygous cry^+ flies shift by 163 minutes, CRY(cry^+) by 233 minutes, homozygous cry^b by 51 minutes, cry^b/cry^+ heterozygous by 84 minutes and CRY(cry^b) by 115 minutes. Out of these five different genotypes, the one that has the highest amount of CRY shows the biggest phase-advance (CRY(cry^+) 233 minutes). However, the GAL4/*UAS* system overexpresses at constitutively high levels, so it seems reasonable to suggest that CRY(cry^b) have more or at least similar levels of CRY than cry^+ homozygous controls. Surprisingly, cry^+ homozygotes shift more than CRY(cry^b) flies (163 minutes versus 115 minutes). This suggests that the overexpression of CRY in all *timeless* competent cells, does not fully rescue the cry^b mutation, at least regarding the response to a light pulse late at night. One possible explanation is the fact that endogenous CRY is expressed differentially in different types of neurons (Klarsfeld et al., 2004). It is possible that the overexpression of CRY by the *timeless* promoter drives CRY at similar levels in all clock cells disrupting the overall balance based upon differences in the relative levels of expression. Another possibility is that the reduction in late night phase-shift responses is primarily due to high levels of CRY in a group of cells normally expressing this protein at very low levels. The DN2s

are the perfect candidate as they normally express extremely low levels of CRY and cycle with a different phase compared to all other clock neurons, which become particularly evident under prolonged DD (Klarsfeld et al., 2004). Interestingly, overexpression of CRY brings these cells in phase with the other clock neurons (Klarsfeld et al., 2004), suggesting that this phase difference between cells might be accountable for robust phase advances at the level of the organism. Furthermore, the fact that CRY affects the phase-advance portion of the PRC the most, suggests that the most relevant interactions of CRY with PER and TIM likely occur in the nucleus.

Finally, although overall molecular cycles of PER and TIM are rescued in CRY(cry^b) flies (Figure 4.9), inspection of Figures 3.9 and 4.9 suggests that the amplitude of these oscillations is not as robust as in the wild-type control. The same explanations as above might account for this phenomenon.

In conclusion, a previously unknown light-independent role of CRY has been uncovered in this study. Further work should address the contribution of each subset of clock neurons in this new role of CRY. This has recently become possible with the identification of new drivers targetting clock neurons with higher resolution (Grima et al., 2004; Stoleru et al., 2004).

7.2 Overexpression of CRYΔ

The overexpression of CRYΔ leads to dramatic effects, which are evident at the behavioural, molecular and cytological levels. The period of locomotor activity in DD is increased by 1.4 to 2.6h depending on the line and the driver used (Figures 5.4, 5.5, 5.6 and 5.10, tables 5.2 and 5.6). The increase in period length is less

pronounced when CRYΔ overexpression is limited to the lateral neurons, suggesting a role for other clock neurons in controlling the period of locomotor activity. This effect is independent of endogenous CRY as the period of activity is similarly affected by CRYΔ whether it is in cry^+ or in cry^b background (Figure 6.3, table 6.2).

The analysis of average daily activity reveals that when CRYΔ is overexpressed by the *tim-GAL4* driver, the flies have a tendency to lose the morning component of activity in DD conditions (Figure 5.8, table 5.5). Loss of morning activity by CRYΔ requires endogenous CRY as CRYΔ(cry^b) flies show bimodal behaviour (Figure 6.5, table 6.4). Interestingly, bimodality is also maintained in cry^+ background when CRYΔ overexpression is limited to the lateral neurons using either *Pdf-GAL4* or *gal1118* drivers (Figure 5.12, table 5.8). This is surprising given that the LN_vs are supposedly the cells controlling morning activity (Grima et al., 2004; Stoleru et al., 2004) and suggests that other clock neurons might be involved in its control. The *gal1118* driver is expressed in the LN_vs, like *Pdf-GAL4*, but also in some LN_ds and DNs (Blanchardon et al., 2001). It is possible that the additional neurons controlling morning activity together with the s-LN_vs, lay among those DNs and LN_ds that are not expressing the *gal1118* driver.

Molecular oscillations of PER and TIM are dampened in heads extracts of CRYΔ flies (Figure 5.18), showing lower peaks and higher troughs than in wild-type flies. Moreover, the phosphorylation status of TIM is dramatically affected as the slow migrating band, representing the highly phosphorylated forms of the protein is never present. Interestingly, this effect does not require light or endogenous CRY as similar results are found in LD or DD conditions and in both cry^+ and cry^b backgrounds (Figures 5.18 and 6.8).

The subcellular distribution of PER and TIM is also strikingly affected in clock neurons. ICC experiments show that PER and TIM nuclear accumulation is strongly delayed and reduced in LN_vs and that levels of TIM are also greatly reduced (Figures 5.23 and 5.24). The large LN_vs (l- LN_vs) are the most affected by CRYΔ overexpression as they show the greatest reduction in overall levels of TIM and in nuclear accumulation of both proteins, measured as Nuclear to Cytoplasmic ratio (N/C). These neurons occupy a special anatomical position as they are a direct target for visual photoreceptors and, through the s-LN_vs, connect to the whole circadian network (Helfrich-Forster, 2003; Helfrich-Forster et al., 2002; Rieger et al., 2003). Furthermore, compared to the s-LN_vs, the l-LN_vs show earlier nuclear accumulation and higher cycling amplitude of PER and TIM in LD (Figures 5.23 and 5.24) (Shafer et al., 2002), although rhythmicity is lost under constant conditions (Peng et al., 2003; Yang and Sehgal, 2001). This suggests that the clock of l-LN_vs is a dampened oscillator very responsive to light entraining cues. Therefore, the l-LN_vs have both molecular and anatomical characteristics that may enable them to transmit light information to the more robust, self-sustained s-LN_vs that mainly respond to an LD entraining regime. In support of this view, cry^b flies, that show normal molecular cycling in the s-LN_vs but not the l-LN_vs, entrain to a LD regime but have blunted responses to short pulses of light or to LL (Emery et al., 2000a; Stanewsky et al., 1998). The finding that CRYΔ overexpression has a greater impact on the l-LN_vs is in agreement with the hypothesis that these cells might be particularly sensitive to environmental signals.

It has been suggested that highly phosphorylated forms of TIM are particularly important for light responses (Rothenfluh et al., 2000). Therefore, the lack of phosphorylated TIM in CRYΔ flies should be reflected in reduced light

sensitivity. Constant light experiments show that CRYΔ flies take longer than wild-type controls to become arrhythmic (Figures 5.13 and 5.14, table 5.9). Targeted expression in LNs also results in reduced LL sensitivity (Figure 5.15, table 5.10). Interestingly, the PRC of CRYΔ flies is somewhat unexpected. The delay zone of the PRC is unaffected (ZT15 and 17) as CRYΔ flies phase-shift their locomotor activity almost like wild-type (Figure 5.15, table 5.10). The advance zone (ZT19 and afterwards) is on the other hand completely blunted in CRYΔ flies, which phase-delay at ZT19 and are virtually blind to a light pulse at later time points. This difference suggest that the effect of CRYΔ is not uniform throughout the circadian cycle. Alternatively, CRYΔ may have the potential to affect the clock constitutively but the presence of the target is a limiting factor. It is very likely that CRYΔ preferentially triggers degradation of phosphorylated TIM and, as TIM phosphorylation increases throughout the night, CRYΔ is likely not to have access to phosphorylated TIM early at night.

The overexpression of CRYΔ limited to the LNs brings about interesting PRC results (Figure 5.17, table 5.12). When CRYΔ is expressed in all three groups of LNs by use of the *gal1118* driver, the light sensitivity is seriously blunted. However, restricting the expression to the LN_vs results in a defective response at ZT19 but a near normal one at ZT21. These results are very interesting because they indicate that different subsets of clock neurons might be responsible for the response to light pulses at different times. The difference between the *gal1118* and *Pdf-Gal4* drivers lies in the fact that *gal1118* express in about 50% of the LN_ds in addition to the LN_vs (Blanchardon et al., 2001). These neurons seem to have an important effect on the control of the response to a light-pulse given at ZT21.

The CRYΔ protein is very unstable, showing almost constant low levels in LD and DD conditions (Figure 5.19). This could be due to a structural instability of the protein or to a functional response assuming that CRYΔ is a constitutively active form of CRY, an hypothesis that fits well with the phenotypes triggered by CRYΔ. Further support is the demonstration that CRYΔ mimics the effects triggered by exposure of wild-type flies to constant low levels of light (Figure 5.22)(Konopka et al., 1989; Marrus et al., 1996), namely lengthening of the locomotor activity period and disappearance of phosphorylated forms of TIM. In addition, CRYΔ overexpression causes a reduction of light-driven degradation of CRY (Figure 5.21) suggesting that CRYΔ competes with CRY for this degradation pathway. Because of continuous degradation and low expression levels, CRYΔ relays continuous dim-light information to the clock rather than a constant high light signal, which should result in complete behavioural arrhythmia.

There are several lines of evidence suggesting that the CRYΔ protein still retains light sensitivity. Firstly CRYΔ levels are mildly reduced upon light exposure (Figure 5.19). Secondly, in the absence of endogenous CRY, CRYΔ(cry^b) flies become arrhythmic in constant light after several days while cry^b mutants maintain strong rhythmicity (Figure 6.6, table 6.5). PRC experiments also suggest that responses to light pulses are mediated by the CRYΔ protein (Figure 6.7, table 6.6). These results indicate that the photolyase-like part of the CRY molecule is able to respond to light and that the C-terminus regulates stability of the protein. Work by another group using a mutant lacking the C-terminus of CRY also identifies the photolyase-like part as the photoresponsive region of the protein (Busza et al., 2004).

In conclusion, this study has provided behavioural, molecular and immunohistochemical evidence suggesting that flies overexpressing CRYΔ are in a physiological status analogous to continuous dim light exposure. It is shown that the C-terminus is essential for stability suggesting that in *Drosophila,* CRY activation and instability are causally linked. However, CRYΔ is further activated and degraded by light. These observations lead to a model regarding the functioning of *Drosophila* CRY (Figure 7.1). A regulatory molecule may bind to the C-terminus of CRY, maintaining CRY in a "dark" state, either directly or by the action of post-translational modifications. In the "dark" state, CRY would be limited in its ability to engage in protein-protein interactions. Light would promote a conformational change resulting in the release of the repression (either by release of the regulatory protein or by reversal of the regulatory modification) and in more robust or novel binding with other proteins such as clock components and (as yet unknown) signalling partners, which also mediate the degradation of CRY. Under this scenario, the removal of the C-terminus would prevent the binding of the regulator, thus allowing relevant protein-protein interactions to take place under darkness, resulting in constant activation/degradation of CRYΔ. However, light detection could still change the conformation of the photolyase-like part of the molecule, promoting stronger interactions.

7.3 Future work

This study has given some very interesting and exciting results regarding the role and mode of action of CRY in the *Drosophila* circadian clock. However there are still many questions that remain unanswered and more work should be done.

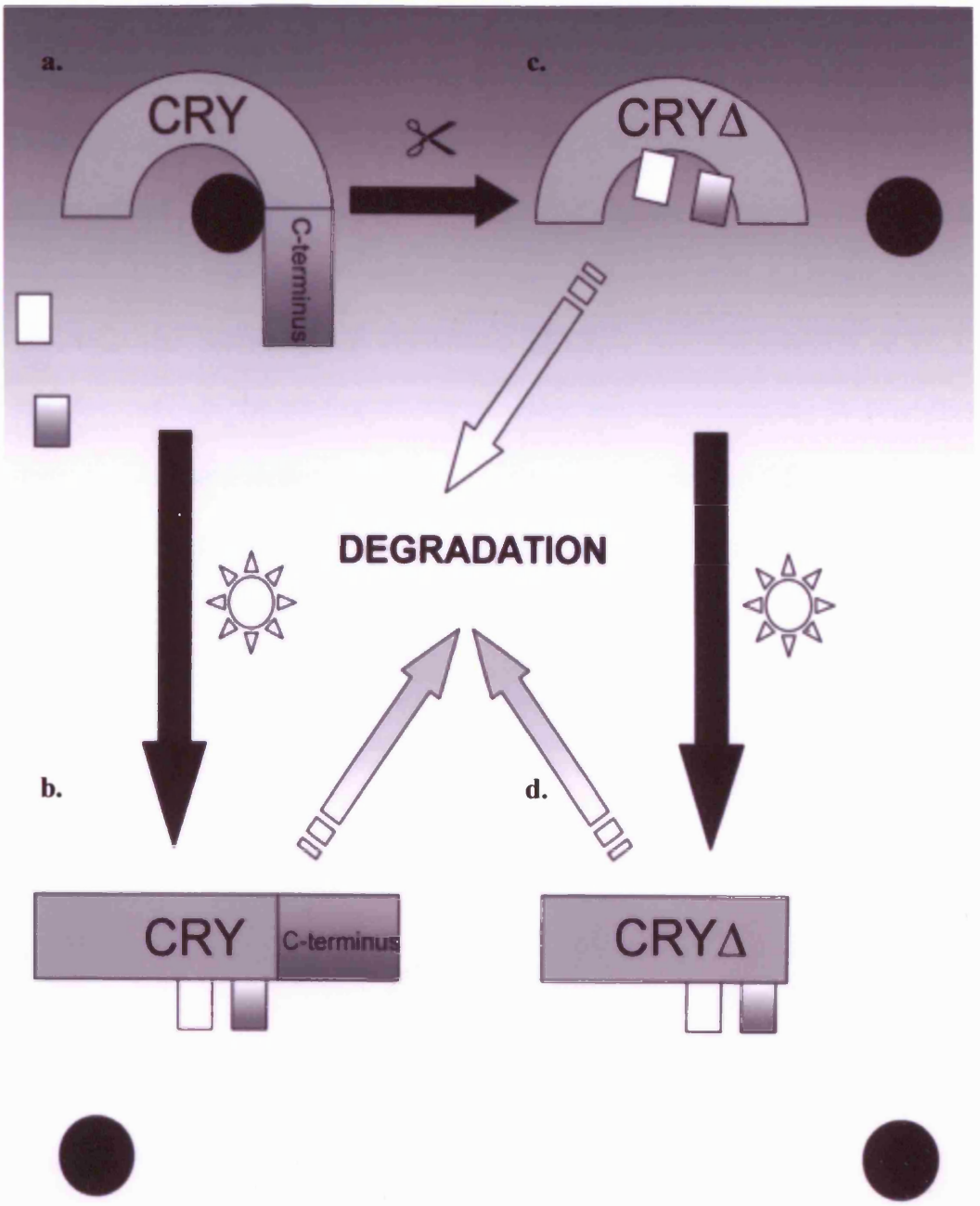

a.
CRY
C-terminus
c.
CRYΔ
DEGRADATION
b.
CRY
C-terminus
d.
CRYΔ

Figure 7.1: (Dissel et al., 2004) Model of CRY function. (**a**) Under darkness a regulatory molecule (black circle) binds the C-terminus, limiting CRY's ability to engage in protein-protein interactions either directly or by action of post-translational modifications. (**b**) Light promotes a conformational change resulting in the release of the repression (either by release of the regulatory protein or by reversal of the regulatory modification) and in binding with signalling partners (squares), also mediating the degradation of CRY. (**c**) The removal of the C-terminus prevents the binding of the regulator, allowing signalling to take place under darkness and consequent constant activation/degradation of CRYΔ. (**d**) Light changes the conformation of CRYΔ, promoting stronger interactions.

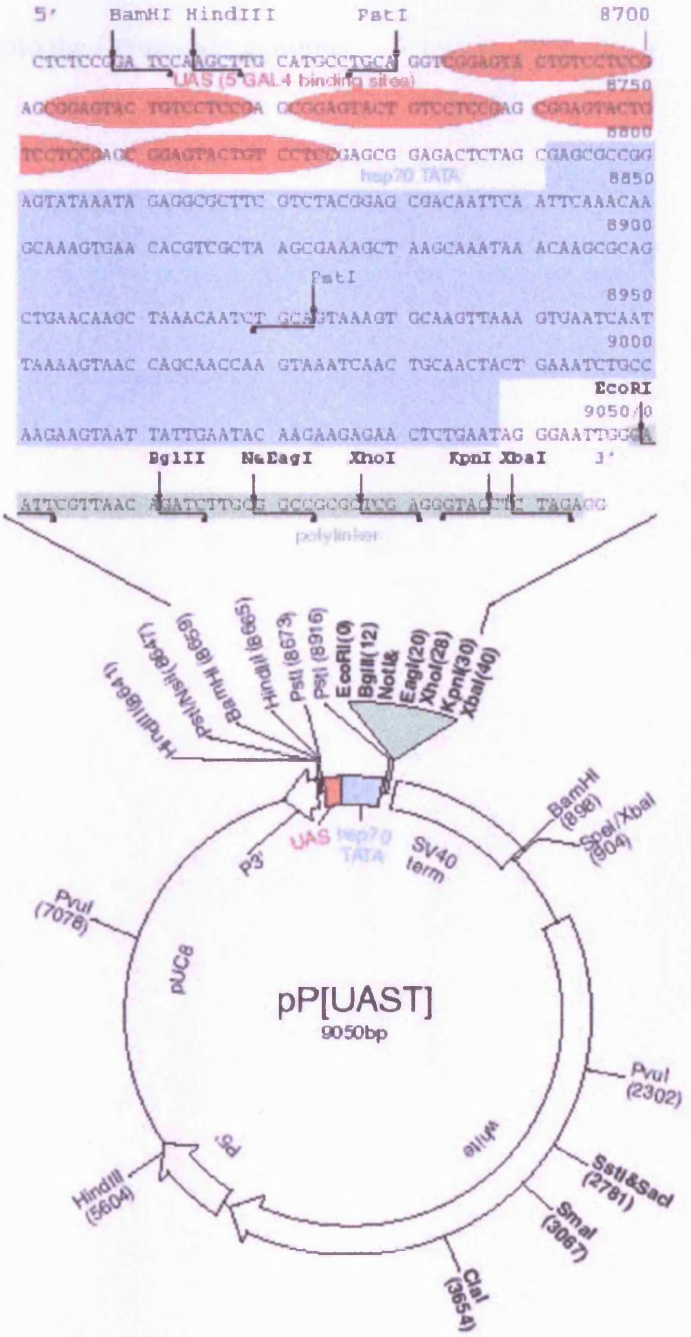
First, in order to overexpress CRY and CRYΔ in cells naturally expressing *cry*, a *cry-GAL4* driver line should be used (Grima *et al.* 2004), and the results should be compared with the effects triggered by the *tim-GAL4* driver. Secondly, the identification of a novel dark function for CRY requires a more thorough investigation. For example, it would be useful to express even more CRY (by use of two copies of the *uas-cry* transgene, for example) or to reduce CRY endogenous levels with dsRNAi techniques. The recent arrival of cell specific driver lines (Grima *et al.* 2004, Stoleru *et al.* 2004) also opens new possibility because it is now possible to target the expression of any transgenes to one cluster of clock neurons only. These new lines should help to further dissect the circadian neural network and to identify the importance of each subset of neurons in the generation of circadian behaviour. These new driver lines may also be used to overexpress CRYΔ in specific neurons. The results obtained in the PRC experiments suggest that different group of neurons might be responsive at different times. The new driver lines will allow to asses the importance of each of the six clusters of clock neurons in response to light pulses.

The CRYΔ protein is very unstable, pointing to the importance of the CRY C-terminus. A major question that should be investigated is what is responsible for the C-terminus controlled stability of CRY? The C-terminus sequence harbours two phosphorylation sites and one PDZ motif. These sites might be related to the control of CRY stability and therefore should be further investigated.

Another line of possible future work is the identification of proteins that interact with CRY and might be involved in circadian rhythmicity. This could be done by coimmunoprecipitation techniques and should help to unravel how CRY signals to the clock and what controls CRY activation and degradation.

APPENDIX 1: the pUAST vector.

PUAST



pUAST (Brand and Perrimon, 1993) consists of five tandemly arrayed optimized GAL4 binding sites (red) followed by the *hsp70* TATA box and transcriptional start

(blue), a polylinker (green) containing unique restriction sites for EcoRI, Bg/II, NotI, Xho, KpnI and XbaI and the SV40 small t intron and polyadenylation site. These features are included in a P-element vector (pCaSpeR3)containing the P element ends (P3' and P5') and the white gene which acts as a marker for successful incorporation into the *Drosophila* genome.

APPENDIX 2: CRY, HA-CRY and control flies have similar period of locomotor activity in 12:12 LD condition.

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.944E-02[a]	4	1.486E-02	.473	.755
Intercept	93946.519	1	93946.519	2992271	.000
GENOTYPE	5.944E-02	4	1.486E-02	.473	.755
Error	6.468	206	3.140E-02		
Total	121745.652	211			
Corrected Total	6.527	210			

a. R Squared = .009 (Adjusted R Squared = -.010)

APPENDIX 3: locomotor activity periods of CRY, HA-CRY and control flies in DD condition.

Line 24B

1. **CRY flies and the *yw*; *tim-GAL4*/+; +/+ control have similar periods**

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.205[a]	1	.205	.800	.372
Intercept	86272.378	1	86272.378	337475.8	.000
GENOTYPE	.205	1	.205	.800	.372
Error	40.647	159	.256		
Total	95842.090	161			
Corrected Total	40.851	160			

a. R Squared = .005 (Adjusted R Squared = -.001)

2. **The *yw*; *UAS-cry24B*/+; +/+ control has different period than CRY flies and the *yw*; *tim-GAL4*/+; +/+ control**

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.498[a]	1	5.498	21.689	.000
Intercept	45067.325	1	45067.325	177788.1	.000
GENOTYPE	5.498	1	5.498	21.689	.000
Error	45.882	181	.253		
Total	108372.189	183			
Corrected Total	51.379	182			

a. R Squared = .107 (Adjusted R Squared = .102)

Line 16.1

1. **Controls have similar periods**

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.339[a]	1	.339	1.153	.286
Intercept	38529.008	1	38529.008	130889.0	.000
GENOTYPE	.339	1	.339	1.153	.286
Error	22.372	76	.294		
Total	46461.767	78			
Corrected Total	22.711	77			

a. R Squared = .015 (Adjusted R Squared = .002)

2. HA-CRY and control flies have different periods

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.321[a]	1	2.321	10.258	.002
Intercept	56806.533	1	56806.533	251035.2	.000
GENOTYPE	2.321	1	2.321	10.258	.002
Error	25.118	111	.226		
Total	66776.139	113			
Corrected Total	27.439	112			

a. R Squared = .085 (Adjusted R Squared = .076)

APPENDIX 4: analysis of average day of CRY, HA-CRY and control flies in 12:12 LD condition.

Line 24B

1. Control flies only differ in timing of evening peak

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	.329[a]	1	.329	.110	.742
	MP	1.277[b]	1	1.277	3.500	.066
	offset mp	11.439[c]	1	11.439	2.881	.095
	ONSET_EP	.319[a]	1	.319	.093	.761
	EP	19.748[d]	1	19.748	11.239	.001
	offset ep	1.356[e]	1	1.356	.806	.373
	SIESTA	15.581[f]	1	15.581	2.338	.132
	ACT_DUR	2.351E-02[g]	1	2.351E-02	2.282	.136
	DARK	2.351E-02[g]	1	2.351E-02	2.282	.136
Intercept	ONSET_MP	4249.103	1	4249.103	1415.278	.000
	MP	10118.439	1	10118.439	27721.750	.000
	offset mp	29193.697	1	29193.697	7352.383	.000
	ONSET_EP	47500.448	1	47500.448	13895.792	.000
	EP	73027.619	1	73027.619	41560.050	.000
	offset ep	91998.517	1	91998.517	54696.893	.000
	SIESTA	2216.936	1	2216.936	332.675	.000
	ACT_DUR	28.653	1	28.653	2780.736	.000
	DARK	4.761	1	4.761	462.051	.000
GENOTYPE	ONSET_MP	.329	1	.329	.110	.742
	MP	1.277	1	1.277	3.500	.066
	offset mp	11.439	1	11.439	2.881	.095
	ONSET_EP	.319	1	.319	.093	.761
	EP	19.748	1	19.748	11.239	.001
	offset ep	1.356	1	1.356	.806	.373
	SIESTA	15.581	1	15.581	2.338	.132
	ACT_DUR	2.351E-02	1	2.351E-02	2.282	.136
	DARK	2.351E-02	1	2.351E-02	2.282	.136
Error	ONSET_MP	180.139	60	3.002		
	MP	21.900	60	.365		
	offset mp	238.239	60	3.971		
	ONSET_EP	205.100	60	3.418		
	EP	105.430	60	1.757		
	offset ep	100.918	60	1.682		
	SIESTA	399.839	60	6.664		
	ACT_DUR	.618	60	1.030E-02		
	DARK	.618	60	1.030E-02		
Total	ONSET_MP	4797.000	62			
	MP	11001.000	62			
	offset mp	32498.000	62			
	ONSET_EP	52000.000	62			
	EP	79115.000	62			
	offset ep	100345.000	62			
	SIESTA	2720.000	62			
	ACT_DUR	31.414	62			
	DARK	6.055	62			
Corrected Total	ONSET_MP	180.468	61			
	MP	23.177	61			
	offset mp	249.677	61			
	ONSET_EP	205.419	61			
	EP	125.177	61			
	offset ep	102.274	61			
	SIESTA	415.419	61			
	ACT_DUR	.642	61			
	DARK	.642	61			

a. R Squared = .002 (Adjusted R Squared = -.015)

b. R Squared = .055 (Adjusted R Squared = .039)

c. R Squared = .046 (Adjusted R Squared = .030)

d. R Squared = .158 (Adjusted R Squared = .144)

e. R Squared = .013 (Adjusted R Squared = -.003)

f. R Squared = .038 (Adjusted R Squared = .021)

g. R Squared = .037 (Adjusted R Squared = .021)

2. CRY flies differ from controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	50.637[a]	1	50.637	12.350	.001
	MP	36.266[b]	1	36.266	32.223	.000
	offset mp	239.579[c]	1	239.579	50.677	.000
	ONSET_EP	129.002[d]	1	129.002	22.949	.000
	EP	1.150[e]	1	1.150	.634	.427
	offset ep	27.688[f]	1	27.688	10.056	.002
	SIESTA	720.185[g]	1	720.185	71.716	.000
	ACT_DUR	1.299[h]	1	1.299	40.826	.000
	DARK	1.299[h]	1	1.299	40.826	.000
Intercept	ONSET_MP	9002.468	1	9002.468	2195.656	.000
	MP	22880.632	1	22880.632	20329.960	.000
	offset mp	64566.621	1	64566.621	13657.359	.000
	ONSET_EP	124609.904	1	124609.904	22167.809	.000
	EP	178911.291	1	178911.291	98640.709	.000
	offset ep	230928.646	1	230928.646	83870.752	.000
	SIESTA	9781.537	1	9781.537	974.044	.000
	ACT_DUR	51.662	1	51.662	1623.521	.000
	DARK	21.461	1	21.461	674.424	.000
GENOTYPE	ONSET_MP	50.637	1	50.637	12.350	.001
	MP	36.266	1	36.266	32.223	.000
	offset mp	239.579	1	239.579	50.677	.000
	ONSET_EP	129.002	1	129.002	22.949	.000
	EP	1.150	1	1.150	.634	.427
	offset ep	27.688	1	27.688	10.056	.002
	SIESTA	720.185	1	720.185	71.716	.000
	ACT_DUR	1.299	1	1.299	40.826	.000
	DARK	1.299	1	1.299	40.826	.000
Error	ONSET_MP	574.018	140	4.100		
	MP	157.565	140	1.125		
	offset mp	661.865	140	4.728		
	ONSET_EP	786.969	140	5.621		
	EP	253.927	140	1.814		
	offset ep	385.474	140	2.753		
	SIESTA	1405.907	140	10.042		
	ACT_DUR	4.455	140	3.182E-02		
	DARK	4.455	140	3.182E-02		
Total	ONSET_MP	9601.000	142			
	MP	23214.000	142			
	offset mp	65513.000	142			
	ONSET_EP	128596.000	142			
	EP	182205.000	142			
	offset ep	235765.000	142			
	SIESTA	12763.000	142			
	ACT_DUR	56.170	142			
	DARK	28.947	142			
Corrected Total	ONSET_MP	624.655	141			
	MP	193.831	141			
	offset mp	901.444	141			
	ONSET_EP	915.972	141			
	EP	255.077	141			
	offset ep	413.162	141			
	SIESTA	2126.092	141			
	ACT_DUR	5.754	141			
	DARK	5.754	141			

a. R Squared = .081 (Adjusted R Squared = .075)

b. R Squared = .187 (Adjusted R Squared = .181)

c. R Squared = .266 (Adjusted R Squared = .261)

d. R Squared = .141 (Adjusted R Squared = .135)

e. R Squared = .005 (Adjusted R Squared = -.003)

f. R Squared = .067 (Adjusted R Squared = .060)

g. R Squared = .339 (Adjusted R Squared = .334)

h. R Squared = .226 (Adjusted R Squared = .220)

Line 16.1

1. Control flies only differ in timing of morning and evening peaks

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	11.914[a]	1	11.914	3.436	.069
	MP	6.547[b]	1	6.547	26.608	.000
	offset mp	6.315[c]	1	6.315	1.132	.292
	ONSET_EP	12.701[d]	1	12.701	2.880	.095
	EP	21.143[e]	1	21.143	12.934	.001
	offset ep	3.525[f]	1	3.525	1.482	.228
	SIESTA	1.104[g]	1	1.104	.107	.745
	ACT_DUR	7.446E-02[h]	1	7.446E-02	5.732	.020
	DARK	7.446E-02[h]	1	7.446E-02	5.732	.020
Intercept	ONSET_MP	4759.470	1	4759.470	1372.616	.000
	MP	10700.642	1	10700.642	43490.733	.000
	offset mp	32131.712	1	32131.712	5759.927	.000
	ONSET_EP	50202.543	1	50202.543	11383.668	.000
	EP	75184.889	1	75184.889	45994.266	.000
	offset ep	92780.922	1	92780.922	39010.919	.000
	SIESTA	2007.581	1	2007.581	193.777	.000
	ACT_DUR	30.768	1	30.768	2368.671	.000
	DARK	4.393	1	4.393	338.183	.000
GENOTYPE	ONSET_MP	11.914	1	11.914	3.436	.069
	MP	6.547	1	6.547	26.608	.000
	offset mp	6.315	1	6.315	1.132	.292
	ONSET_EP	12.701	1	12.701	2.880	.095
	EP	21.143	1	21.143	12.934	.001
	offset ep	3.525	1	3.525	1.482	.228
	SIESTA	1.104	1	1.104	.107	.745
	ACT_DUR	7.446E-02	1	7.446E-02	5.732	.020
	DARK	7.446E-02	1	7.446E-02	5.732	.020
Error	ONSET_MP	211.514	61	3.467		
	MP	15.009	61	.246		
	offset mp	340.288	61	5.578		
	ONSET_EP	269.013	61	4.410		
	EP	99.714	61	1.635		
	offset ep	145.078	61	2.378		
	SIESTA	631.975	61	10.360		
	ACT_DUR	.792	61	1.299E-02		
	DARK	.792	61	1.299E-02		
Total	ONSET_MP	5219.000	63			
	MP	11409.000	63			
	offset mp	34740.000	63			
	ONSET_EP	53963.000	63			
	EP	80478.000	63			
	offset ep	100549.000	63			
	SIESTA	2771.000	63			
	ACT_DUR	33.176	63			
	DARK	5.943	63			
Corrected Total	ONSET_MP	223.429	62			
	MP	21.556	62			
	offset mp	346.603	62			
	ONSET_EP	281.714	62			
	EP	120.857	62			
	offset ep	148.603	62			
	SIESTA	633.079	62			
	ACT_DUR	.867	62			
	DARK	.867	62			

a. R Squared = .053 (Adjusted R Squared = .038)
b. R Squared = .304 (Adjusted R Squared = .292)
c. R Squared = .018 (Adjusted R Squared = .002)
d. R Squared = .045 (Adjusted R Squared = .029)
e. R Squared = .175 (Adjusted R Squared = .161)
f. R Squared = .024 (Adjusted R Squared = .008)
g. R Squared = .002 (Adjusted R Squared = -.015)
h. R Squared = .086 (Adjusted R Squared = .071)

2. HA-CRY flies differ from controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	168.100[a]	1	168.100	44.530	.000
	MP	8.534[b]	1	8.534	10.993	.001
	offset mp	86.885[c]	1	86.885	13.345	.000
	ONSET_EP	37.747[d]	1	37.747	10.281	.002
	EP	3.104[e]	1	3.104	1.844	.178
	offset ep	79.470[f]	1	79.470	25.584	.000
	SIESTA	239.168[g]	1	239.168	20.763	.000
	ACT_DUR	.707[h]	1	.707	40.420	.000
	DARK	.707[h]	1	.707	40.420	.000
Intercept	ONSET_MP	5114.059	1	5114.059	1354.718	.000
	MP	15531.106	1	15531.106	20005.991	.000
	offset mp	45088.027	1	45088.027	6925.486	.000
	ONSET_EP	80128.073	1	80128.073	21824.767	.000
	EP	115992.900	1	115992.900	68906.673	.000
	offset ep	150260.899	1	150260.899	48373.215	.000
	SIESTA	5002.678	1	5002.678	434.299	.000
	ACT_DUR	35.437	1	35.437	2025.633	.000
	DARK	12.489	1	12.489	713.859	.000
GENOTYPE	ONSET_MP	168.100	1	168.100	44.530	.000
	MP	8.534	1	8.534	10.993	.001
	offset mp	86.885	1	86.885	13.345	.000
	ONSET_EP	37.747	1	37.747	10.281	.002
	EP	3.104	1	3.104	1.844	.178
	offset ep	79.470	1	79.470	25.584	.000
	SIESTA	239.168	1	239.168	20.763	.000
	ACT_DUR	.707	1	.707	40.420	.000
	DARK	.707	1	.707	40.420	.000
Error	ONSET_MP	362.400	96	3.775		
	MP	74.527	96	.776		
	offset mp	625.003	96	6.510		
	ONSET_EP	352.457	96	3.671		
	EP	161.600	96	1.683		
	offset ep	298.203	96	3.106		
	SIESTA	1105.822	96	11.519		
	ACT_DUR	1.679	96	1.749E-02		
	DARK	1.679	96	1.749E-02		
Total	ONSET_MP	6691.000	98			
	MP	17222.000	98			
	offset mp	51047.000	98			
	ONSET_EP	86562.000	98			
	EP	126095.000	98			
	offset ep	161852.000	98			
	SIESTA	6133.000	98			
	ACT_DUR	44.151	98			
	DARK	14.199	98			
Corrected Total	ONSET_MP	530.500	97			
	MP	83.061	97			
	offset mp	711.888	97			
	ONSET_EP	390.204	97			
	EP	164.704	97			
	offset ep	377.673	97			
	SIESTA	1344.990	97			
	ACT_DUR	2.387	97			
	DARK	2.387	97			

a. R Squared = .317 (Adjusted R Squared = .310)

b. R Squared = .103 (Adjusted R Squared = .093)

c. R Squared = .122 (Adjusted R Squared = .113)

d. R Squared = .097 (Adjusted R Squared = .087)

e. R Squared = .019 (Adjusted R Squared = .009)

f. R Squared = .210 (Adjusted R Squared = .202)

g. R Squared = .178 (Adjusted R Squared = .169)

h. R Squared = .296 (Adjusted R Squared = .289)

APPENDIX 5: analysis of average day of CRY, HA-CRY and control flies in DD condition.

Line 24B

1. Controls are similar

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	2.982[a]	1	2.982	.432	.513
	EP	6.111E-03[b]	1	6.111E-03	.001	.978
	phase diff	3.258[c]	1	3.258	.355	.553
	ACT_DAY	9.224E-03[d]	1	9.224E-03	.982	.325
	act night	9.224E-03[d]	1	9.224E-03	.982	.325
Intercept	MP	11910.982	1	11910.982	1726.613	.000
	EP	77058.673	1	77058.673	9430.587	.000
	phase diff	28377.758	1	28377.758	3087.642	.000
	ACT_DAY	26.667	1	26.667	2837.864	.000
	act night	7.040	1	7.040	749.222	.000
GENOTYPE	MP	2.982	1	2.982	.432	.513
	EP	6.111E-03	1	6.111E-03	.001	.978
	phase diff	3.258	1	3.258	.355	.553
	ACT_DAY	9.224E-03	1	9.224E-03	.982	.325
	act night	9.224E-03	1	9.224E-03	.982	.325
Error	MP	482.893	70	6.898		
	EP	571.980	70	8.171		
	phase diff	643.353	70	9.191		
	ACT_DAY	.658	70	9.397E-03		
	act night	.658	70	9.397E-03		
Total	MP	14347.000	72			
	EP	91381.000	72			
	phase diff	34360.000	72			
	ACT_DAY	31.633	72			
	act night	9.197	72			
Corrected Total	MP	485.875	71			
	EP	571.986	71			
	phase diff	646.611	71			
	ACT_DAY	.667	71			
	act night	.667	71			

a. R Squared = .006 (Adjusted R Squared = -.008)

b. R Squared = .000 (Adjusted R Squared = -.014)

c. R Squared = .005 (Adjusted R Squared = -.009)

d. R Squared = .014 (Adjusted R Squared = .000)

2. CRY and control flies have different timing of MP, phase difference and distribution of activity but similar timing of EP

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	220.081[a]	1	220.081	36.277	.000
	EP	7.179[b]	1	7.179	.927	.337
	phase diff	306.755[c]	1	306.755	29.153	.000
	ACT_DAY	.531[d]	1	.531	38.705	.000
	act night	.531[d]	1	.531	38.705	.000
Intercept	MP	23176.412	1	23176.412	3820.231	.000
	EP	185169.248	1	185169.248	23922.750	.000
	phase diff	77325.679	1	77325.679	7348.722	.000
	ACT_DAY	51.384	1	51.384	3748.072	.000
	act night	23.746	1	23.746	1732.099	.000
GENOTYPE	MP	220.081	1	220.081	36.277	.000
	EP	7.179	1	7.179	.927	.337
	phase diff	306.755	1	306.755	29.153	.000
	ACT_DAY	.531	1	.531	38.705	.000
	act night	.531	1	.531	38.705	.000
Error	MP	867.546	143	6.067		
	EP	1106.863	143	7.740		
	phase diff	1504.693	143	10.522		
	ACT_DAY	1.960	143	1.371E-02		
	act night	1.960	143	1.371E-02		
Total	MP	24234.000	145			
	EP	186308.000	145			
	phase diff	79208.000	145			
	ACT_DAY	53.806	145			
	act night	26.288	145			
Corrected Total	MP	1087.628	144			
	EP	1114.041	144			
	phase diff	1811.448	144			
	ACT_DAY	2.491	144			
	act night	2.491	144			

a. R Squared = .202 (Adjusted R Squared = .197)

b. R Squared = .006 (Adjusted R Squared = -.001)

c. R Squared = .169 (Adjusted R Squared = .164)

d. R Squared = .213 (Adjusted R Squared = .208)

Line 16.1

1. **HA-CRY differ from the control** *yw*; *tim-GAL4*/+; +/+ **apart from timing of EP**

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	135.832[a]	1	135.832	22.521	.000
	EP	32.243[b]	1	32.243	3.428	.068
	phase diff	300.432[c]	1	300.432	30.153	.000
	ACT_DAY	.212[d]	1	.212	20.077	.000
	act night	.212[d]	1	.212	20.077	.000
Intercept	MP	12776.632	1	12776.632	2118.415	.000
	EP	107595.160	1	107595.160	11439.675	.000
	phase diff	46217.796	1	46217.796	4638.658	.000
	ACT_DAY	29.409	1	29.409	2788.751	.000
	act night	13.336	1	13.336	1264.551	.000
GENOTYPE	MP	135.832	1	135.832	22.521	.000
	EP	32.243	1	32.243	3.428	.068
	phase diff	300.432	1	300.432	30.153	.000
	ACT_DAY	.212	1	.212	20.077	.000
	act night	.212	1	.212	20.077	.000
Error	MP	500.591	83	6.031		
	EP	780.651	83	9.405		
	phase diff	826.980	83	9.964		
	ACT_DAY	.875	83	1.055E-02		
	act night	.875	83	1.055E-02		
Total	MP	14308.000	85			
	EP	111189.000	85			
	phase diff	47483.000	85			
	ACT_DAY	32.358	85			
	act night	14.246	85			
Corrected Total	MP	636.424	84			
	EP	812.894	84			
	phase diff	1127.412	84			
	ACT_DAY	1.087	84			
	act night	1.087	84			

a. R Squared = .213 (Adjusted R Squared = .204)

b. R Squared = .040 (Adjusted R Squared = .028)

c. R Squared = .266 (Adjusted R Squared = .258)

d. R Squared = .195 (Adjusted R Squared = .185)

2. **HA-CRY and** *w*; +/+; *UAS-hacry16.1*/+ **control flies are different**

Line 16.1

1. **HA-CRY differ from the control** *yw*; *tim-GAL4*/+; +/+ **apart from timing of EP**

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	135.832[a]	1	135.832	22.521	.000
	EP	32.243[b]	1	32.243	3.428	.068
	phase diff	300.432[c]	1	300.432	30.153	.000
	ACT_DAY	.212[d]	1	.212	20.077	.000
	act night	.212[d]	1	.212	20.077	.000
Intercept	MP	12776.632	1	12776.632	2118.415	.000
	EP	107595.160	1	107595.160	11439.675	.000
	phase diff	46217.796	1	46217.796	4638.658	.000
	ACT_DAY	29.409	1	29.409	2788.751	.000
	act night	13.336	1	13.336	1264.551	.000
GENOTYPE	MP	135.832	1	135.832	22.521	.000
	EP	32.243	1	32.243	3.428	.068
	phase diff	300.432	1	300.432	30.153	.000
	ACT_DAY	.212	1	.212	20.077	.000
	act night	.212	1	.212	20.077	.000
Error	MP	500.591	83	6.031		
	EP	780.651	83	9.405		
	phase diff	826.980	83	9.964		
	ACT_DAY	.875	83	1.055E-02		
	act night	.875	83	1.055E-02		
Total	MP	14308.000	85			
	EP	111189.000	85			
	phase diff	47483.000	85			
	ACT_DAY	32.358	85			
	act night	14.246	85			
Corrected Total	MP	636.424	84			
	EP	812.894	84			
	phase diff	1127.412	84			
	ACT_DAY	1.087	84			
	act night	1.087	84			

a. R Squared = .213 (Adjusted R Squared = .204)

b. R Squared = .040 (Adjusted R Squared = .028)

c. R Squared = .266 (Adjusted R Squared = .258)

d. R Squared = .195 (Adjusted R Squared = .185)

2. **HA-CRY and** *w*; +/+; *UAS-hacry16.1*/+ **control flies are different**

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	297.420[a]	1	297.420	57.688	.000
	EP	90.591[b]	1	90.591	7.487	.008
	phase diff	716.301[c]	1	716.301	46.069	.000
	ACT_DAY	1.009[d]	1	1.009	55.657	.000
	act night	1.009[d]	1	1.009	55.657	.000
Intercept	MP	9873.490	1	9873.490	1915.071	.000
	EP	68008.065	1	68008.065	5620.973	.000
	phase diff	26055.810	1	26055.810	1675.766	.000
	ACT_DAY	25.247	1	25.247	1392.508	.000
	act night	5.411	1	5.411	298.468	.000
GENOTYPE	MP	297.420	1	297.420	57.688	.000
	EP	90.591	1	90.591	7.487	.008
	phase diff	716.301	1	716.301	46.069	.000
	ACT_DAY	1.009	1	1.009	55.657	.000
	act night	1.009	1	1.009	55.657	.000
Error	MP	283.562	55	5.156		
	EP	665.444	55	12.099		
	phase diff	855.173	55	15.549		
	ACT_DAY	.997	55	1.813E-02		
	act night	.997	55	1.813E-02		
Total	MP	10188.000	57			
	EP	73695.000	57			
	phase diff	31175.000	57			
	ACT_DAY	26.265	57			
	act night	8.894	57			
Corrected Total	MP	580.982	56			
	EP	756.035	56			
	phase diff	1571.474	56			
	ACT_DAY	2.006	56			
	act night	2.006	56			

a. R Squared = .512 (Adjusted R Squared = .503)

b. R Squared = .120 (Adjusted R Squared = .104)

c. R Squared = .456 (Adjusted R Squared = .446)

d. R Squared = .503 (Adjusted R Squared = .494)

APPENDIX 6: analysis of the PRC of CRY and control flies

1. ZT15

Tests of Between-Subjects Effects

Dependent Variable: SHIFT15

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7.018[a]	1	7.018	1.099	.299
Intercept	3036.843	1	3036.843	475.348	.000
GENOTYPE	7.018	1	7.018	1.099	.299
Error	351.377	55	6.389		
Total	3632.500	57			
Corrected Total	358.395	56			

a. R Squared = .020 (Adjusted R Squared = .002)

2. ZT17

Tests of Between-Subjects Effects

Dependent Variable: SHIFT17

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.573E-02[a]	1	3.573E-02	.004	.952
Intercept	1152.865	1	1152.865	121.373	.000
GENOTYPE	3.573E-02	1	3.573E-02	.004	.952
Error	180.472	19	9.499		
Total	1359.263	21			
Corrected Total	180.508	20			

a. R Squared = .000 (Adjusted R Squared = -.052)

3. ZT19

Tests of Between-Subjects Effects

Dependent Variable: SHIFT19

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.681[a]	1	.681	.085	.775
Intercept	465.125	1	465.125	57.939	.000
GENOTYPE	.681	1	.681	.085	.775
Error	128.444	16	8.028		
Total	594.250	18			
Corrected Total	129.125	17			

a. R Squared = .005 (Adjusted R Squared = -.057)

4. ZT21

Tests of Between-Subjects Effects

Dependent Variable: SHIFT21

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	49.185[a]	1	49.185	10.775	.002
Intercept	1614.467	1	1614.467	353.690	.000
GENOTYPE	49.185	1	49.185	10.775	.002
Error	168.892	37	4.565		
Total	1788.750	39			
Corrected Total	218.077	38			

a. R Squared = .226 (Adjusted R Squared = .205)

5. ZT23

Tests of Between-Subjects Effects

Dependent Variable: SHIFT23

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	119.574[a]	1	119.574	20.832	.000
Intercept	1126.604	1	1126.604	196.271	.000
GENOTYPE	119.574	1	119.574	20.832	.000
Error	177.941	31	5.740		
Total	1403.000	33			
Corrected Total	297.515	32			

a. R Squared = .402 (Adjusted R Squared = .383)

APPENDIX 7: Locomotor activity of CRY(cry^b) and control flies in DD

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.494[a]	2	.747	2.750	.069
Intercept	56220.834	1	56220.834	206949.5	.000
GENOTYPE	1.494	2	.747	2.750	.069
Error	25.265	93	.272		
Total	56614.213	96			
Corrected Total	26.759	95			

a. R Squared = .056 (Adjusted R Squared = .036)

APPENDIX 8: Analysis of average day of CRY(*cry*^{b}) and control flies in 12:12LD

1. Controls are similar except for timing of MP

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	5.400[a]	1	5.400	1.922	.171
	MP	8.817[b]	1	8.817	17.027	.000
	offset mp	19.267[c]	1	19.267	2.695	.106
	ONSET_EP	1.350[d]	1	1.350	.162	.689
	EP	6.667E-02[e]	1	6.667E-02	.045	.832
	offset ep	2.017[f]	1	2.017	.344	.560
	SIESTA	30.817[g]	1	30.817	3.251	.077
	ACT_DUR	5.558E-02[h]	1	5.558E-02	2.256	.139
	DARK	5.558E-02[h]	1	5.558E-02	2.256	.139
Intercept	ONSET_MP	5801.667	1	5801.667	2065.241	.000
	MP	9300.150	1	9300.150	17960.334	.000
	offset mp	24080.067	1	24080.067	3368.112	.000
	ONSET_EP	47432.817	1	47432.817	5697.832	.000
	EP	78626.400	1	78626.400	53316.421	.000
	offset ep	101599.350	1	101599.350	17350.365	.000
	SIESTA	3920.417	1	3920.417	413.601	.000
	ACT_DUR	24.399	1	24.399	990.212	.000
	DARK	7.876	1	7.876	319.632	.000
GENOTYPE	ONSET_MP	5.400	1	5.400	1.922	.171
	MP	8.817	1	8.817	17.027	.000
	offset mp	19.267	1	19.267	2.695	.106
	ONSET_EP	1.350	1	1.350	.162	.689
	EP	6.667E-02	1	6.667E-02	.045	.832
	offset ep	2.017	1	2.017	.344	.560
	SIESTA	30.817	1	30.817	3.251	.077
	ACT_DUR	5.558E-02	1	5.558E-02	2.256	.139
	DARK	5.558E-02	1	5.558E-02	2.256	.139
Error	ONSET_MP	162.933	58	2.809		
	MP	30.033	58	.518		
	offset mp	414.667	58	7.149		
	ONSET_EP	482.833	58	8.325		
	EP	85.533	58	1.475		
	offset ep	339.633	58	5.856		
	SIESTA	549.767	58	9.479		
	ACT_DUR	1.429	58	2.464E-02		
	DARK	1.429	58	2.464E-02		
Total	ONSET_MP	5970.000	60			
	MP	9339.000	60			
	offset mp	24514.000	60			
	ONSET_EP	47917.000	60			
	EP	78712.000	60			
	offset ep	101941.000	60			
	SIESTA	4501.000	60			
	ACT_DUR	25.884	60			
	DARK	9.361	60			
Corrected Total	ONSET_MP	168.333	59			
	MP	38.850	59			
	offset mp	433.933	59			
	ONSET_EP	484.183	59			
	EP	85.600	59			
	offset ep	341.650	59			
	SIESTA	580.583	59			
	ACT_DUR	1.485	59			
	DARK	1.485	59			

a. R Squared = .032 (Adjusted R Squared = .015)

b. R Squared = .227 (Adjusted R Squared = .214)

c. R Squared = .044 (Adjusted R Squared = .028)

d. R Squared = .003 (Adjusted R Squared = -.014)

e. R Squared = .001 (Adjusted R Squared = -.016)

f. R Squared = .006 (Adjusted R Squared = -.011)

g. R Squared = .053 (Adjusted R Squared = .037)

h. R Squared = .037 (Adjusted R Squared = .021)

2. CRY(*cry*^{b}) differ from controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	115.196[a]	1	115.196	48.883	.000
	offset mp	4.886[b]	1	4.886	.772	.382
	ONSET_EP	56.859[c]	1	56.859	9.463	.003
	EP	.868[d]	1	.868	.872	.353
	offset ep	97.592[e]	1	97.592	22.985	.000
	SIESTA	95.082[f]	1	95.082	10.225	.002
	ACT_DUR	1.840E-02[g]	1	1.840E-02	.811	.370
	DARK	1.840E-02[g]	1	1.840E-02	.811	.370
Intercept	ONSET_MP	6542.430	1	6542.430	2776.258	.000
	offset mp	34018.929	1	34018.929	5374.647	.000
	ONSET_EP	72633.752	1	72633.752	12088.085	.000
	EP	113129.719	1	113129.719	113623.7	.000
	offset ep	139517.294	1	139517.294	32859.484	.000
	SIESTA	7235.933	1	7235.933	778.126	.000
	ACT_DUR	33.708	1	33.708	1486.342	.000
	DARK	12.329	1	12.329	543.656	.000
GENOTYPE	ONSET_MP	115.196	1	115.196	48.883	.000
	offset mp	4.886	1	4.886	.772	.382
	ONSET_EP	56.859	1	56.859	9.463	.003
	EP	.868	1	.868	.872	.353
	offset ep	97.592	1	97.592	22.985	.000
	SIESTA	95.082	1	95.082	10.225	.002
	ACT_DUR	1.840E-02	1	1.840E-02	.811	.370
	DARK	1.840E-02	1	1.840E-02	.811	.370
Error	ONSET_MP	216.804	92	2.357		
	offset mp	582.316	92	6.330		
	ONSET_EP	552.801	92	6.009		
	EP	91.600	92	.996		
	offset ep	390.621	92	4.246		
	SIESTA	855.525	92	9.299		
	ACT_DUR	2.086	92	2.268E-02		
	DARK	2.086	92	2.268E-02		
Total	ONSET_MP	7946.000	94			
	offset mp	37669.000	94			
	ONSET_EP	78048.000	94			
	EP	122782.000	94			
	offset ep	153782.000	94			
	SIESTA	8297.000	94			
	ACT_DUR	39.078	94			
	DARK	15.172	94			
Corrected Total	ONSET_MP	332.000	93			
	offset mp	587.202	93			
	ONSET_EP	609.660	93			
	EP	92.468	93			
	offset ep	488.213	93			
	SIESTA	950.606	93			
	ACT_DUR	2.105	93			
	DARK	2.105	93			

a. R Squared = .347 (Adjusted R Squared = .340)

b. R Squared = .008 (Adjusted R Squared = -.002)

c. R Squared = .093 (Adjusted R Squared = .083)

d. R Squared = .009 (Adjusted R Squared = -.001)

e. R Squared = .200 (Adjusted R Squared = .191)

f. R Squared = .100 (Adjusted R Squared = .090)

g. R Squared = .009 (Adjusted R Squared = -.002)

APPENDIX 9: Analysis of average day of CRY(cry^b) and control flies in DD

1. Controls are similar

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	.471[a]	1	.471	.050	.824
	EP	30.115[b]	1	30.115	3.157	.081
	phase diff	23.051[c]	1	23.051	1.970	.166
	ACT_DAY	9.031E-02[d]	1	9.031E-02	2.307	.135
	act night	9.031E-02[d]	1	9.031E-02	2.307	.135
Intercept	MP	9160.035	1	9160.035	974.718	.000
	EP	76477.751	1	76477.751	8016.362	.000
	phase diff	32702.396	1	32702.396	2794.854	.000
	ACT_DAY	15.416	1	15.416	393.725	.000
	act night	12.171	1	12.171	310.833	.000
GENOTYPE	MP	.471	1	.471	.050	.824
	EP	30.115	1	30.115	3.157	.081
	phase diff	23.051	1	23.051	1.970	.166
	ACT_DAY	9.031E-02	1	9.031E-02	2.307	.135
	act night	9.031E-02	1	9.031E-02	2.307	.135
Error	MP	498.074	53	9.398		
	EP	505.631	53	9.540		
	phase diff	620.149	53	11.701		
	ACT_DAY	2.075	53	3.915E-02		
	act night	2.075	53	3.915E-02		
Total	MP	9664.000	55			
	EP	77094.000	55			
	phase diff	33388.000	55			
	ACT_DAY	17.544	55			
	act night	14.378	55			
Corrected Total	MP	498.545	54			
	EP	535.745	54			
	phase diff	643.200	54			
	ACT_DAY	2.166	54			
	act night	2.166	54			

a. R Squared = .001 (Adjusted R Squared = -.018)

b. R Squared = .056 (Adjusted R Squared = .038)

c. R Squared = .036 (Adjusted R Squared = .018)

d. R Squared = .042 (Adjusted R Squared = .024)

2. CRY(cry^b) and control flies are different

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	80.305[a]	1	80.305	10.369	.002
	EP	83.567[b]	1	83.567	9.069	.003
	phase diff	327.713[c]	1	327.713	31.253	.000
	ACT_DAY	1.131[d]	1	1.131	34.498	.000
	act night	1.131[d]	1	1.131	34.498	.000
Intercept	MP	12197.639	1	12197.639	1575.005	.000
	EP	125483.567	1	125483.567	13617.848	.000
	phase diff	59435.357	1	59435.357	5668.222	.000
	ACT_DAY	14.652	1	14.652	447.082	.000
	act night	29.397	1	29.397	897.030	.000
GENOTYPE	MP	80.305	1	80.305	10.369	.002
	EP	83.567	1	83.567	9.069	.003
	phase diff	327.713	1	327.713	31.253	.000
	ACT_DAY	1.131	1	1.131	34.498	.000
	act night	1.131	1	1.131	34.498	.000
Error	MP	681.517	88	7.745		
	EP	810.888	88	9.215		
	phase diff	922.743	88	10.486		
	ACT_DAY	2.884	88	3.277E-02		
	act night	2.884	88	3.277E-02		
Total	MP	14060.000	90			
	EP	131387.000	90			
	phase diff	61727.000	90			
	ACT_DAY	21.389	90			
	act night	32.302	90			
Corrected Total	MP	761.822	89			
	EP	894.456	89			
	phase diff	1250.456	89			
	ACT_DAY	4.014	89			
	act night	4.014	89			

a. R Squared = .105 (Adjusted R Squared = .095)

b. R Squared = .093 (Adjusted R Squared = .083)

c. R Squared = .262 (Adjusted R Squared = .254)

d. R Squared = .282 (Adjusted R Squared = .273)

APPENDIX 10: Analysis of the PRC of CRY(cry^b) and control flies

1. No difference between CRY(cry^b) and cry^+ control

at ZT15

Tests of Between-Subjects Effects

Dependent Variable: SHIFT15

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	63.460[a]	1	63.460	7.360	.008
Intercept	5041.072	1	5041.072	584.660	.000
GENOTYPE	63.460	1	63.460	7.360	.008
Error	715.644	83	8.622		
Total	5771.128	85			
Corrected Total	779.105	84			

a. R Squared = .081 (Adjusted R Squared = .070)

at ZT21

Tests of Between-Subjects Effects

Dependent Variable: SHIFT21

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	26.270[a]	1	26.270	4.284	.041
Intercept	1097.585	1	1097.585	179.010	.000
GENOTYPE	26.270	1	26.270	4.284	.041
Error	631.535	103	6.131		
Total	1890.171	105			
Corrected Total	657.805	104			

a. R Squared = .040 (Adjusted R Squared = .031)

2. difference between CRY(cry^b) and cry^b controls

at ZT15

Tests of Between-Subjects Effects

Dependent Variable: SHIFT15

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	685.621[a]	1	685.621	93.204	.000
Intercept	2556.061	1	2556.061	347.475	.000
GENOTYPE	685.621	1	685.621	93.204	.000
Error	573.775	78	7.356		
Total	3815.456	80			
Corrected Total	1259.395	79			

a. R Squared = .544 (Adjusted R Squared = .539)

at ZT21

Tests of Between-Subjects Effects

Dependent Variable: SHIFT21

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	101.606[a]	1	101.606	19.802	.000
Intercept	692.781	1	692.781	135.014	.000
GENOTYPE	101.606	1	101.606	19.802	.000
Error	497.723	97	5.131		
Total	1536.697	99			
Corrected Total	599.329	98			

a. R Squared = .170 (Adjusted R Squared = .161)

APPENDIX 11: Locomotor activity periods of CRYΔ and control flies

1. in 12:12 LD

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.085[a]	6	.181	1.259	.278
Intercept	82884.027	1	82884.027	577115.0	.000
GENOTYPE	1.085	6	.181	1.259	.278
Error	28.149	196	.144		
Total	117877.277	203			
Corrected Total	29.234	202			

a. R Squared = .037 (Adjusted R Squared = .008)

2. In DD

Line 14.6 is different from both controls.

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	65.262[a]	1	65.262	191.384	.000
Intercept	78553.290	1	78553.290	230359.6	.000
GENOTYPE	65.262	1	65.262	191.384	.000
Error	42.625	125	.341		
Total	81154.093	127			
Corrected Total	107.888	126			

a. R Squared = .605 (Adjusted R Squared = .602)

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	53.392[a]	1	53.392	143.025	.000
Intercept	34181.814	1	34181.814	91565.222	.000
GENOTYPE	53.392	1	53.392	143.025	.000
Error	32.851	88	.373		
Total	58639.347	90			
Corrected Total	86.243	89			

a. R Squared = .619 (Adjusted R Squared = .615)

Line 15.3 is different from both controls

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	111.141[a]	1	111.141	326.249	.000
Intercept	73443.197	1	73443.197	215589.3	.000
GENOTYPE	111.141	1	111.141	326.249	.000
Error	38.154	112	.341		
Total	74098.384	114			
Corrected Total	149.295	113			

a. R Squared = .744 (Adjusted R Squared = .742)

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	74.151[a]	1	74.151	238.187	.000
Intercept	35307.658	1	35307.658	113414.7	.000
GENOTYPE	74.151	1	74.151	238.187	.000
Error	23.660	76	.311		
Total	52307.093	78			
Corrected Total	97.811	77			

a. R Squared = .758 (Adjusted R Squared = .755)

Line 4.1 is different from both controls

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	57.639[a]	1	57.639	130.461	.000
Intercept	22352.585	1	22352.585	50593.410	.000
GENOTYPE	57.639	1	57.639	130.461	.000
Error	27.392	62	.442		
Total	39564.237	64			
Corrected Total	85.031	63			

a. R Squared = .678 (Adjusted R Squared = .673)

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	63.464[a]	1	63.464	117.652	.000
Intercept	16682.704	1	16682.704	30927.027	.000
GENOTYPE	63.464	1	63.464	117.652	.000
Error	14.025	26	.539		
Total	17608.503	28			
Corrected Total	77.489	27			

a. R Squared = .819 (Adjusted R Squared = .812)

APPENDIX 12: Analysis of average day for CRYΔ and control flies in LD

Line 14.6

Controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	31.219[a]	1	31.219	9.471	.003
	MP	2.376[b]	1	2.376	8.003	.007
	offset mp	3.201[c]	1	3.201	.690	.410
	ONSET_EP	1.894[d]	1	1.894	.502	.482
	EP	3.007[e]	1	3.007	1.119	.295
	offset ep	.303[f]	1	.303	.145	.705
	SIESTA	.170[g]	1	.170	.021	.886
	ACT_DUR	.157[h]	1	.157	12.714	.001
	DARK	.157[h]	1	.157	12.714	.001
Intercept	ONSET_MP	3872.819	1	3872.819	1174.869	.000
	MP	7874.376	1	7874.376	26525.969	.000
	offset mp	23885.019	1	23885.019	5152.068	.000
	ONSET_EP	36845.967	1	36845.967	9762.554	.000
	EP	55108.970	1	55108.970	20514.665	.000
	offset ep	69876.376	1	69876.376	33505.259	.000
	SIESTA	1399.152	1	1399.152	171.824	.000
	ACT_DUR	24.544	1	24.544	1992.284	.000
	DARK	2.728	1	2.728	221.419	.000
GENOTYPE	ONSET_MP	31.219	1	31.219	9.471	.003
	MP	2.376	1	2.376	8.003	.007
	offset mp	3.201	1	3.201	.690	.410
	ONSET_EP	1.894	1	1.894	.502	.482
	EP	3.007	1	3.007	1.119	.295
	offset ep	.303	1	.303	.145	.705
	SIESTA	.170	1	.170	.021	.886
	ACT_DUR	.157	1	.157	12.714	.001
	DARK	.157	1	.157	12.714	.001
Error	ONSET_MP	174.708	53	3.296		
	MP	15.733	53	.297		
	offset mp	245.708	53	4.636		
	ONSET_EP	200.033	53	3.774		
	EP	142.375	53	2.686		
	offset ep	110.533	53	2.086		
	SIESTA	431.575	53	8.143		
	ACT_DUR	.653	53	1.232E-02		
	DARK	.653	53	1.232E-02		
Total	ONSET_MP	4697.000	55			
	MP	9787.000	55			
	offset mp	30038.000	55			
	ONSET_EP	46341.000	55			
	EP	69140.000	55			
	offset ep	88351.000	55			
	SIESTA	2213.000	55			
	ACT_DUR	29.539	55			
	DARK	5.037	55			
Corrected Total	ONSET_MP	205.927	54			
	MP	18.109	54			
	offset mp	248.909	54			
	ONSET_EP	201.927	54			
	EP	145.382	54			
	offset ep	110.836	54			
	SIESTA	431.745	54			
	ACT_DUR	.810	54			
	DARK	.810	54			

a. R Squared = .152 (Adjusted R Squared = .136)
b. R Squared = .131 (Adjusted R Squared = .115)
c. R Squared = .013 (Adjusted R Squared = -.006)
d. R Squared = .009 (Adjusted R Squared = -.009)
e. R Squared = .021 (Adjusted R Squared = .002)
f. R Squared = .003 (Adjusted R Squared = -.016)
g. R Squared = .000 (Adjusted R Squared = -.018)
h. R Squared = .193 (Adjusted R Squared = .178)

CRYΔ and controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	38.768[a]	1	38.768	10.063	.002
	MP	2.450[b]	1	2.450	13.691	.000
	offset mp	174.523[c]	1	174.523	38.142	.000
	ONSET_EP	13.649[d]	1	13.649	3.283	.073
	EP	.263[e]	1	.263	.157	.693
	offset ep	4.408[f]	1	4.408	1.763	.187
	SIESTA	90.560[g]	1	90.560	9.824	.002
	ACT_DUR	.669[h]	1	.669	71.577	.000
	DARK	.669[h]	1	.669	71.577	.000
Intercept	ONSET_MP	10536.803	1	10536.803	2735.014	.000
	MP	19780.590	1	19780.590	110543.1	.000
	offset mp	55281.891	1	55281.891	12081.860	.000
	ONSET_EP	93246.070	1	93246.070	22430.890	.000
	EP	142443.491	1	142443.491	84741.326	.000
	offset ep	180882.303	1	180882.303	72338.272	.000
	SIESTA	4933.823	1	4933.823	535.217	.000
	ACT_DUR	72.756	1	72.756	7788.861	.000
	DARK	4.583	1	4.583	490.621	.000
GENOTYPE	ONSET_MP	38.768	1	38.768	10.063	.002
	MP	2.450	1	2.450	13.691	.000
	offset mp	174.523	1	174.523	38.142	.000
	ONSET_EP	13.649	1	13.649	3.283	.073
	EP	.263	1	.263	.157	.693
	offset ep	4.408	1	4.408	1.763	.187
	SIESTA	90.560	1	90.560	9.824	.002
	ACT_DUR	.669	1	.669	71.577	.000
	DARK	.669	1	.669	71.577	.000
Error	ONSET_MP	431.487	112	3.853		
	MP	20.041	112	.179		
	offset mp	512.468	112	4.576		
	ONSET_EP	465.588	112	4.157		
	EP	188.263	112	1.681		
	offset ep	280.057	112	2.501		
	SIESTA	1032.457	112	9.218		
	ACT_DUR	1.046	112	9.341E-03		
	DARK	1.046	112	9.341E-03		
Total	ONSET_MP	11065.000	114			
	MP	19812.000	114			
	offset mp	55819.000	114			
	ONSET_EP	93761.000	114			
	EP	142794.000	114			
	offset ep	181327.000	114			
	SIESTA	6110.000	114			
	ACT_DUR	75.052	114			
	DARK	6.181	114			
Corrected Total	ONSET_MP	470.254	113			
	MP	22.491	113			
	offset mp	686.991	113			
	ONSET_EP	479.237	113			
	EP	188.526	113			
	offset ep	284.465	113			
	SIESTA	1123.018	113			
	ACT_DUR	1.715	113			
	DARK	1.715	113			

a. R Squared = .082 (Adjusted R Squared = .074)

b. R Squared = .109 (Adjusted R Squared = .101)

c. R Squared = .254 (Adjusted R Squared = .247)

d. R Squared = .028 (Adjusted R Squared = .020)

e. R Squared = .001 (Adjusted R Squared = -.008)

f. R Squared = .015 (Adjusted R Squared = .007)

g. R Squared = .081 (Adjusted R Squared = .072)

h. R Squared = .390 (Adjusted R Squared = .384)

Line 15.3

Controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	29.292[a]	1	29.292	8.475	.005
	MP	10.305[b]	1	10.305	31.746	.000
	offset mp	1.497[c]	1	1.497	.372	.545
	ONSET_EP	5.797[d]	1	5.797	1.677	.201
	EP	12.483[e]	1	12.483	7.469	.008
	offset ep	.300[f]	1	.300	.164	.687
	SIESTA	1.402[g]	1	1.402	.208	.650
	ACT_DUR	.200[h]	1	.200	18.146	.000
	DARK	.200[h]	1	.200	18.146	.000
Intercept	ONSET_MP	4334.533	1	4334.533	1254.097	.000
	MP	9259.408	1	9259.408	28525.317	.000
	offset mp	26156.669	1	26156.669	6496.070	.000
	ONSET_EP	40356.003	1	40356.003	11673.224	.000
	EP	63556.138	1	63556.138	38030.683	.000
	offset ep	79536.576	1	79536.576	43435.296	.000
	SIESTA	1533.333	1	1533.333	227.009	.000
	ACT_DUR	28.192	1	28.192	2560.304	.000
	DARK	3.017	1	3.017	274.010	.000
GENOTYPE	ONSET_MP	29.292	1	29.292	8.475	.005
	MP	10.305	1	10.305	31.746	.000
	offset mp	1.497	1	1.497	.372	.545
	ONSET_EP	5.797	1	5.797	1.677	.201
	EP	12.483	1	12.483	7.469	.008
	offset ep	.300	1	.300	.164	.687
	SIESTA	1.402	1	1.402	.208	.650
	ACT_DUR	.200	1	.200	18.146	.000
	DARK	.200	1	.200	18.146	.000
Error	ONSET_MP	193.553	56	3.456		
	MP	18.178	56	.325		
	offset mp	225.486	56	4.027		
	ONSET_EP	193.600	56	3.457		
	EP	93.586	56	1.671		
	offset ep	102.544	56	1.831		
	SIESTA	378.253	56	6.755		
	ACT_DUR	.617	56	1.101E-02		
	DARK	.617	56	1.101E-02		
Total	ONSET_MP	4975.000	58			
	MP	10572.000	58			
	offset mp	30955.000	58			
	ONSET_EP	47767.000	58			
	EP	73556.000	58			
	offset ep	93143.000	58			
	SIESTA	2212.000	58			
	ACT_DUR	31.677	58			
	DARK	5.062	58			
Corrected Total	ONSET_MP	222.845	57			
	MP	28.483	57			
	offset mp	226.983	57			
	ONSET_EP	199.397	57			
	EP	106.069	57			
	offset ep	102.845	57			
	SIESTA	379.655	57			
	ACT_DUR	.816	57			
	DARK	.816	57			

a. R Squared = .131 (Adjusted R Squared = .116)

b. R Squared = .362 (Adjusted R Squared = .350)

c. R Squared = .007 (Adjusted R Squared = -.011)

d. R Squared = .029 (Adjusted R Squared = .012)

e. R Squared = .118 (Adjusted R Squared = .102)

f. R Squared = .003 (Adjusted R Squared = -.015)

g. R Squared = .004 (Adjusted R Squared = -.014)

h. R Squared = .245 (Adjusted R Squared = .231)

CRYΔ and controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	38.886[a]	1	38.886	9.629	.002
	MP	.825[b]	1	.825	2.168	.144
	offset mp	34.575[c]	1	34.575	7.767	.006
	ONSET_EP	40.976[d]	1	40.976	12.302	.001
	EP	15.026[e]	1	15.026	9.648	.002
	offset ep	35.573[e]	1	35.573	14.403	.000
	SIESTA	.272[f]	1	.272	.039	.844
	ACT_DUR	3.838E-02[g]	1	3.838E-02	2.808	.096
	DARK	3.838E-02[g]	1	3.838E-02	2.808	.096
Intercept	ONSET_MP	10930.411	1	10930.411	2706.675	.000
	MP	21711.266	1	21711.266	57066.607	.000
	offset mp	65472.202	1	65472.202	14707.647	.000
	ONSET_EP	100770.773	1	100770.773	30253.670	.000
	EP	152401.195	1	152401.195	97850.445	.000
	offset ep	194458.861	1	194458.861	78735.201	.000
	SIESTA	3790.712	1	3790.712	542.976	.000
	ACT_DUR	65.910	1	65.910	4821.858	.000
	DARK	7.523	1	7.523	550.338	.000
GENOTYPE	ONSET_MP	38.886	1	38.886	9.629	.002
	MP	.825	1	.825	2.168	.144
	offset mp	34.575	1	34.575	7.767	.006
	ONSET_EP	40.976	1	40.976	12.302	.001
	EP	15.026	1	15.026	9.648	.002
	offset ep	35.573	1	35.573	14.403	.000
	SIESTA	.272	1	.272	.039	.844
	ACT_DUR	3.838E-02	1	3.838E-02	2.808	.096
	DARK	3.838E-02	1	3.838E-02	2.808	.096
Error	ONSET_MP	468.445	116	4.038		
	MP	44.133	116	.380		
	offset mp	516.383	116	4.452		
	ONSET_EP	386.380	116	3.331		
	EP	180.669	116	1.557		
	offset ep	286.495	116	2.470		
	SIESTA	809.839	116	6.981		
	ACT_DUR	1.586	116	1.367E-02		
	DARK	1.586	116	1.367E-02		
Total	ONSET_MP	11463.000	118			
	MP	21767.000	118			
	offset mp	66093.000	118			
	ONSET_EP	101296.000	118			
	EP	152692.000	118			
	offset ep	194926.000	118			
	SIESTA	4603.000	118			
	ACT_DUR	67.607	118			
	DARK	9.131	118			
Corrected Total	ONSET_MP	507.331	117			
	MP	44.958	117			
	offset mp	550.958	117			
	ONSET_EP	427.356	117			
	EP	195.695	117			
	offset ep	322.068	117			
	SIESTA	810.110	117			
	ACT_DUR	1.624	117			
	DARK	1.624	117			

a. R Squared = .077 (Adjusted R Squared = .069)

b. R Squared = .018 (Adjusted R Squared = .010)

c. R Squared = .063 (Adjusted R Squared = .055)

d. R Squared = .096 (Adjusted R Squared = .088)

e. R Squared = .110 (Adjusted R Squared = .103)

f. R Squared = .000 (Adjusted R Squared = -.008)

g. R Squared = .024 (Adjusted R Squared = .015)

Line 4.1

Controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	66.464[a]	1	66.464	18.841	.000
	MP	14.420[b]	1	14.420	22.424	.000
	offset mp	.117[c]	1	.117	.028	.867
	ONSET_EP	12.003[d]	1	12.003	2.902	.094
	EP	22.161[e]	1	22.161	13.167	.001
	offset ep	5.703[f]	1	5.703	2.443	.124
	SIESTA	9.747[g]	1	9.747	1.413	.240
	ACT_DUR	2.867E-02[h]	1	2.867E-02	2.348	.131
	DARK	2.867E-02[h]	1	2.867E-02	2.348	.131
Intercept	ONSET_MP	4702.878	1	4702.878	1333.118	.000
	MP	9372.765	1	9372.765	14575.358	.000
	offset mp	26665.635	1	26665.635	6450.821	.000
	ONSET_EP	42749.797	1	42749.797	10336.738	.000
	EP	64149.678	1	64149.678	38114.335	.000
	offset ep	81201.289	1	81201.289	34788.719	.000
	SIESTA	1889.127	1	1889.127	273.891	.000
	ACT_DUR	25.321	1	25.321	2073.448	.000
	DARK	4.059	1	4.059	332.379	.000
GENOTYPE	ONSET_MP	66.464	1	66.464	18.841	.000
	MP	14.420	1	14.420	22.424	.000
	offset mp	.117	1	.117	.028	.867
	ONSET_EP	12.003	1	12.003	2.902	.094
	EP	22.161	1	22.161	13.167	.001
	offset ep	5.703	1	5.703	2.443	.124
	SIESTA	9.747	1	9.747	1.413	.240
	ACT_DUR	2.867E-02	1	2.867E-02	2.348	.131
	DARK	2.867E-02	1	2.867E-02	2.348	.131
Error	ONSET_MP	197.553	56	3.528		
	MP	36.011	56	.643		
	offset mp	231.486	56	4.134		
	ONSET_EP	231.600	56	4.136		
	EP	94.253	56	1.683		
	offset ep	130.711	56	2.334		
	SIESTA	386.253	56	6.897		
	ACT_DUR	.684	56	1.221E-02		
	DARK	.684	56	1.221E-02		
Total	ONSET_MP	5273.000	58			
	MP	10675.000	58			
	offset mp	31329.000	58			
	ONSET_EP	49545.000	58			
	EP	73994.000	58			
	offset ep	94382.000	58			
	SIESTA	2484.000	58			
	ACT_DUR	29.538	58			
	DARK	5.761	58			
Corrected Total	ONSET_MP	264.017	57			
	MP	50.431	57			
	offset mp	231.603	57			
	ONSET_EP	243.603	57			
	EP	116.414	57			
	offset ep	136.414	57			
	SIESTA	396.000	57			
	ACT_DUR	.713	57			
	DARK	.713	57			

a. R Squared = .252 (Adjusted R Squared = .238)

b. R Squared = .286 (Adjusted R Squared = .273)

c. R Squared = .001 (Adjusted R Squared = -.017)

d. R Squared = .049 (Adjusted R Squared = .032)

e. R Squared = .190 (Adjusted R Squared = .176)

f. R Squared = .042 (Adjusted R Squared = .025)

g. R Squared = .025 (Adjusted R Squared = .007)

h. R Squared = .040 (Adjusted R Squared = .023)

CRYΔ and controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	ONSET_MP	12.424[a]	1	12.424	2.658	.108
	MP	1.140[b]	1	1.140	1.465	.230
	offset mp	4.732[c]	1	4.732	1.181	.281
	ONSET_EP	50.982[d]	1	50.982	12.941	.001
	EP	8.707[e]	1	8.707	4.768	.033
	offset ep	18.927[f]	1	18.927	8.007	.006
	SIESTA	24.650[g]	1	24.650	3.948	.051
	ACT_DUR	8.711E-02[h]	1	8.711E-02	6.891	.011
	DARK	8.711E-02[h]	1	8.711E-02	6.891	.011
Intercept	ONSET_MP	3341.542	1	3341.542	714.844	.000
	MP	6419.669	1	6419.669	8254.230	.000
	offset mp	18885.732	1	18885.732	4712.446	.000
	ONSET_EP	31483.688	1	31483.688	7991.907	.000
	EP	44696.354	1	44696.354	24478.189	.000
	offset ep	57506.222	1	57506.222	24327.404	.000
	SIESTA	1600.885	1	1600.885	256.390	.000
	ACT_DUR	14.613	1	14.613	1155.965	.000
	DARK	4.074	1	4.074	322.271	.000
GENOTYPE	ONSET_MP	12.424	1	12.424	2.658	.108
	MP	1.140	1	1.140	1.465	.230
	offset mp	4.732	1	4.732	1.181	.281
	ONSET_EP	50.982	1	50.982	12.941	.001
	EP	8.707	1	8.707	4.768	.033
	offset ep	18.927	1	18.927	8.007	.006
	SIESTA	24.650	1	24.650	3.948	.051
	ACT_DUR	8.711E-02	1	8.711E-02	6.891	.011
	DARK	8.711E-02	1	8.711E-02	6.891	.011
Error	ONSET_MP	308.517	66	4.675		
	MP	51.331	66	.778		
	offset mp	264.503	66	4.008		
	ONSET_EP	260.003	66	3.939		
	EP	120.514	66	1.826		
	offset ep	156.014	66	2.364		
	SIESTA	412.100	66	6.244		
	ACT_DUR	.834	66	1.264E-02		
	DARK	.834	66	1.264E-02		
Total	ONSET_MP	6420.000	68			
	MP	12608.000	68			
	offset mp	37074.000	68			
	ONSET_EP	59547.000	68			
	EP	87467.000	68			
	offset ep	111874.000	68			
	SIESTA	3093.000	68			
	ACT_DUR	33.307	68			
	DARK	7.451	68			
Corrected Total	ONSET_MP	320.941	67			
	MP	52.471	67			
	offset mp	269.235	67			
	ONSET_EP	310.985	67			
	EP	129.221	67			
	offset ep	174.941	67			
	SIESTA	436.750	67			
	ACT_DUR	.921	67			
	DARK	.921	67			

a. R Squared = .039 (Adjusted R Squared = .024)

b. R Squared = .022 (Adjusted R Squared = .007)

c. R Squared = .018 (Adjusted R Squared = .003)

d. R Squared = .164 (Adjusted R Squared = .151)

e. R Squared = .067 (Adjusted R Squared = .053)

f. R Squared = .108 (Adjusted R Squared = .095)

g. R Squared = .056 (Adjusted R Squared = .042)

h. R Squared = .095 (Adjusted R Squared = .081)

APPENDIX 13: Analysis of average day for CRYΔ and control flies in DD

Line 14.6

Controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	219.971[a]	1	219.971	26.784	.000
	EP	14.204[b]	1	14.204	1.588	.212
	phase diff	345.967[c]	1	345.967	32.253	.000
	ACT_DAY	2.009E-03[d]	1	2.009E-03	.101	.752
	act night	2.009E-03[d]	1	2.009E-03	.101	.752
Intercept	MP	12211.244	1	12211.244	1486.853	.000
	EP	59321.840	1	59321.840	6632.568	.000
	phase diff	17703.967	1	17703.967	1650.437	.000
	ACT_DAY	19.975	1	19.975	1003.803	.000
	act night	6.219	1	6.219	312.520	.000
GENOTYPE	MP	219.971	1	219.971	26.784	.000
	EP	14.204	1	14.204	1.588	.212
	phase diff	345.967	1	345.967	32.253	.000
	ACT_DAY	2.009E-03	1	2.009E-03	.101	.752
	act night	2.009E-03	1	2.009E-03	.101	.752
Error	MP	525.620	64	8.213		
	EP	572.418	64	8.944		
	phase diff	686.518	64	10.727		
	ACT_DAY	1.274	64	1.990E-02		
	act night	1.274	64	1.990E-02		
Total	MP	15149.000	66			
	EP	82631.000	66			
	phase diff	28728.000	66			
	ACT_DAY	28.748	66			
	act night	9.585	66			
Corrected Total	MP	745.591	65			
	EP	586.621	65			
	phase diff	1032.485	65			
	ACT_DAY	1.276	65			
	act night	1.276	65			

a. R Squared = .295 (Adjusted R Squared = .284)

b. R Squared = .024 (Adjusted R Squared = .009)

c. R Squared = .335 (Adjusted R Squared = .325)

d. R Squared = .002 (Adjusted R Squared = -.014)

CRYΔ and controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	21.415[a]	1	21.415	1.808	.182
	EP	37.838[b]	1	37.838	5.306	.023
	phase diff	116.184[c]	1	116.184	7.613	.007
	ACT_DAY	.390[d]	1	.390	26.537	.000
	act night	.390[d]	1	.390	26.537	.000
Intercept	MP	23127.075	1	23127.075	1952.300	.000
	EP	119549.045	1	119549.045	16765.324	.000
	phase diff	37513.014	1	37513.014	2457.917	.000
	ACT_DAY	49.898	1	49.898	3397.252	.000
	act night	8.510	1	8.510	579.403	.000
GENOTYPE	MP	21.415	1	21.415	1.808	.182
	EP	37.838	1	37.838	5.306	.023
	phase diff	116.184	1	116.184	7.613	.007
	ACT_DAY	.390	1	.390	26.537	.000
	act night	.390	1	.390	26.537	.000
Error	MP	1231.991	104	11.846		
	EP	741.596	104	7.131		
	phase diff	1587.260	104	15.262		
	ACT_DAY	1.528	104	1.469E-02		
	act night	1.528	104	1.469E-02		
Total	MP	25495.000	106			
	EP	129094.000	106			
	phase diff	42715.000	106			
	ACT_DAY	52.733	106			
	act night	11.948	106			
Corrected Total	MP	1253.406	105			
	EP	779.434	105			
	phase diff	1703.443	105			
	ACT_DAY	1.917	105			
	act night	1.917	105			

a. R Squared = .017 (Adjusted R Squared = .008)

b. R Squared = .049 (Adjusted R Squared = .039)

c. R Squared = .068 (Adjusted R Squared = .059)

d. R Squared = .203 (Adjusted R Squared = .196)

Line 15.3

Controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	29.973[a]	1	29.973	3.407	.070
	EP	65.946[b]	1	65.946	8.229	.006
	phase diff	184.837[c]	1	184.837	17.186	.000
	ACT_DAY	.402[d]	1	.402	46.581	.000
	act night	.402[d]	1	.402	46.581	.000
Intercept	MP	10230.882	1	10230.882	1162.894	.000
	EP	57220.855	1	57220.855	7139.812	.000
	phase diff	19060.837	1	19060.837	1772.251	.000
	ACT_DAY	26.504	1	26.504	3071.035	.000
	act night	3.294	1	3.294	381.668	.000
GENOTYPE	MP	29.973	1	29.973	3.407	.070
	EP	65.946	1	65.946	8.229	.006
	phase diff	184.837	1	184.837	17.186	.000
	ACT_DAY	.402	1	.402	46.581	.000
	act night	.402	1	.402	46.581	.000
Error	MP	563.058	64	8.798		
	EP	512.918	64	8.014		
	phase diff	688.330	64	10.755		
	ACT_DAY	.552	64	8.630E-03		
	act night	.552	64	8.630E-03		
Total	MP	13754.000	66			
	EP	81219.000	66			
	phase diff	29519.000	66			
	ACT_DAY	32.600	66			
	act night	7.197	66			
Corrected Total	MP	593.030	65			
	EP	578.864	65			
	phase diff	873.167	65			
	ACT_DAY	.954	65			
	act night	.954	65			

a. R Squared = .051 (Adjusted R Squared = .036)

b. R Squared = .114 (Adjusted R Squared = .100)

c. R Squared = .212 (Adjusted R Squared = .199)

d. R Squared = .421 (Adjusted R Squared = .412)

CRYΔ and controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	5.053[a]	1	5.053	.588	.445
	EP	2.297[b]	1	2.297	.276	.601
	phase diff	.536[c]	1	.536	.045	.833
	ACT_DAY	6.819E-02[d]	1	6.819E-02	5.040	.027
	act night	6.819E-02[d]	1	6.819E-02	5.040	.027
Intercept	MP	19863.822	1	19863.822	2312.136	.000
	EP	118901.528	1	118901.528	14264.289	.000
	phase diff	41567.844	1	41567.844	3465.750	.000
	ACT_DAY	49.871	1	49.871	3686.220	.000
	act night	7.615	1	7.615	562.851	.000
GENOTYPE	MP	5.053	1	5.053	.588	.445
	EP	2.297	1	2.297	.276	.601
	phase diff	.536	1	.536	.045	.833
	ACT_DAY	6.819E-02	1	6.819E-02	5.040	.027
	act night	6.819E-02	1	6.819E-02	5.040	.027
Error	MP	876.293	102	8.591		
	EP	850.232	102	8.336		
	phase diff	1223.377	102	11.994		
	ACT_DAY	1.380	102	1.353E-02		
	act night	1.380	102	1.353E-02		
Total	MP	22114.000	104			
	EP	128743.000	104			
	phase diff	46127.000	104			
	ACT_DAY	54.152	104			
	act night	10.082	104			
Corrected Total	MP	881.346	103			
	EP	852.529	103			
	phase diff	1223.913	103			
	ACT_DAY	1.448	103			
	act night	1.448	103			

a. R Squared = .006 (Adjusted R Squared = -.004)

b. R Squared = .003 (Adjusted R Squared = -.007)

c. R Squared = .000 (Adjusted R Squared = -.009)

d. R Squared = .047 (Adjusted R Squared = .038)

Line 4.1

Controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	90.561[a]	1	90.561	10.368	.002
	EP	53.285[b]	1	53.285	6.335	.014
	phase diff	282.777[c]	1	282.777	21.687	.000
	ACT_DAY	.189[d]	1	.189	19.122	.000
	act night	.189[d]	1	.189	19.122	.000
Intercept	MP	11533.725	1	11533.725	1320.490	.000
	EP	60384.270	1	60384.270	7179.198	.000
	phase diff	19137.106	1	19137.106	1467.706	.000
	ACT_DAY	25.529	1	25.529	2586.785	.000
	act night	4.289	1	4.289	434.593	.000
GENOTYPE	MP	90.561	1	90.561	10.368	.002
	EP	53.285	1	53.285	6.335	.014
	phase diff	282.777	1	282.777	21.687	.000
	ACT_DAY	.189	1	.189	19.122	.000
	act night	.189	1	.189	19.122	.000
Error	MP	567.738	65	8.734		
	EP	546.715	65	8.411		
	phase diff	847.521	65	13.039		
	ACT_DAY	.641	65	9.869E-03		
	act night	.641	65	9.869E-03		
Total	MP	14586.000	67			
	EP	82675.000	67			
	phase diff	29513.000	67			
	ACT_DAY	31.742	67			
	act night	7.724	67			
Corrected Total	MP	658.299	66			
	EP	600.000	66			
	phase diff	1130.299	66			
	ACT_DAY	.830	66			
	act night	.830	66			

a. R Squared = .138 (Adjusted R Squared = .124)

b. R Squared = .089 (Adjusted R Squared = .075)

c. R Squared = .250 (Adjusted R Squared = .239)

d. R Squared = .227 (Adjusted R Squared = .215)

CRYΔ and controls

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	MP	4.420[a]	1	4.420	.434	.512
	EP	.944[b]	1	.944	.102	.750
	phase diff	9.448[c]	1	9.448	.535	.467
	ACT_DAY	1.657E-03[d]	1	1.657E-03	.131	.718
	act night	3.886E-02[e]	1	3.886E-02	3.064	.084
Intercept	MP	3378.617	1	3378.617	331.473	.000
	EP	18232.493	1	18232.493	1974.948	.000
	phase diff	5913.899	1	5913.899	334.942	.000
	ACT_DAY	7.183	1	7.183	569.505	.000
	act night	1.101	1	1.101	86.802	.000
GENOTYPE	MP	4.420	1	4.420	.434	.512
	EP	.944	1	.944	.102	.750
	phase diff	9.448	1	9.448	.535	.467
	ACT_DAY	1.657E-03	1	1.657E-03	.131	.718
	act night	3.886E-02	1	3.886E-02	3.064	.084
Error	MP	703.299	69	10.193		
	EP	637.000	69	9.232		
	phase diff	1218.299	69	17.657		
	ACT_DAY	.870	69	1.261E-02		
	act night	.875	69	1.268E-02		
Total	MP	15592.000	71			
	EP	87473.000	71			
	phase diff	31045.000	71			
	ACT_DAY	33.743	71			
	act night	7.961	71			
Corrected Total	MP	707.718	70			
	EP	637.944	70			
	phase diff	1227.746	70			
	ACT_DAY	.872	70			
	act night	.914	70			

a. R Squared = .006 (Adjusted R Squared = -.008)

b. R Squared = .001 (Adjusted R Squared = -.013)

c. R Squared = .008 (Adjusted R Squared = -.007)

d. R Squared = .002 (Adjusted R Squared = -.013)

e. R Squared = .043 (Adjusted R Squared = .029)

APPENDIX 14: Locomotor activity periods for CRYΔ(LNs) and control flies

1. In LD

Controls and CRYΔ(LNs) are similar

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.398[a]	4	9.960E-02	.679	.609
Intercept	38137.187	1	38137.187	259834.0	.000
GENOTYPE	.398	4	9.960E-02	.679	.609
Error	11.302	77	.147		
Total	47715.754	82			
Corrected Total	11.700	81			

a. R Squared = .034 (Adjusted R Squared = -.016)

2. in DD

CRYΔ(*Pdf-GAL4*) are different from both controls

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	30.393[a]	1	30.393	96.021	.000
Intercept	30920.932	1	30920.932	97687.607	.000
GENOTYPE	30.393	1	30.393	96.021	.000
Error	20.574	65	.317		
Total	42146.257	67			
Corrected Total	50.968	66			

a. R Squared = .596 (Adjusted R Squared = .590)

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7.753[a]	1	7.753	30.903	.000
Intercept	25845.200	1	25845.200	103018.4	.000
GENOTYPE	7.753	1	7.753	30.903	.000
Error	15.304	61	.251		
Total	40283.479	63			
Corrected Total	23.057	62			

a. R Squared = .336 (Adjusted R Squared = .325)

CRYΔ(*gal1118*) are different from both controls

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	13.183[a]	1	13.183	42.954	.000
Intercept	25659.243	1	25659.243	83607.888	.000
GENOTYPE	13.183	1	13.183	42.954	.000
Error	13.504	44	.307		
Total	27886.191	46			
Corrected Total	26.686	45			

a. R Squared = .494 (Adjusted R Squared = .482)

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8.306[a]	1	8.306	38.350	.000
Intercept	34589.736	1	34589.736	159705.3	.000
GENOTYPE	8.306	1	8.306	38.350	.000
Error	11.912	55	.217		
Total	34639.419	57			
Corrected Total	20.218	56			

a. R Squared = .411 (Adjusted R Squared = .400)

APPENDIX 15: Analysis of PRC of CRYΔ and control flies

ZT15

Tests of Between-Subjects Effects

Dependent Variable: SHIFT15

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	58.094[a]	1	58.094	5.724	.020
Intercept	3377.430	1	3377.430	332.765	.000
GENOTYPE	58.094	1	58.094	5.724	.020
Error	690.171	68	10.150		
Total	4125.695	70			
Corrected Total	748.265	69			

a. R Squared = .078 (Adjusted R Squared = .064)

ZT17

Tests of Between-Subjects Effects

Dependent Variable: SHIFT17

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8.356[a]	1	8.356	.586	.452
Intercept	1051.150	1	1051.150	73.658	.000
GENOTYPE	8.356	1	8.356	.586	.452
Error	313.956	22	14.271		
Total	1394.111	24			
Corrected Total	322.311	23			

a. R Squared = .026 (Adjusted R Squared = -.018)

ZT19

Tests of Between-Subjects Effects

Dependent Variable: SHIFT19

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	484.967[a]	1	484.967	35.047	.000
Intercept	12.219	1	12.219	.883	.354
GENOTYPE	484.967	1	484.967	35.047	.000
Error	484.317	35	13.838		
Total	1052.188	37			
Corrected Total	969.284	36			

a. R Squared = .500 (Adjusted R Squared = .486)

ZT21

Tests of Between-Subjects Effects

Dependent Variable: SHIFT21

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	143.746[a]	1	143.746	14.401	.000
Intercept	646.307	1	646.307	64.749	.000
GENOTYPE	143.746	1	143.746	14.401	.000
Error	449.176	45	9.982		
Total	1252.563	47			
Corrected Total	592.921	46			

a. R Squared = .242 (Adjusted R Squared = .226)

ZT23

Tests of Between-Subjects Effects

Dependent Variable: SHIFT23

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	181.070[a]	1	181.070	19.922	.000
Intercept	77.350	1	77.350	8.510	.007
GENOTYPE	181.070	1	181.070	19.922	.000
Error	272.675	30	9.089		
Total	546.958	32			
Corrected Total	453.744	31			

a. R Squared = .399 (Adjusted R Squared = .379)

APPENDIX 16: Analysis of PRC of CRYΔ(LNs) and control flies

CRYΔ(*gal1118*) and control

ZT19

Tests of Between-Subjects Effects

Dependent Variable: SHIFT19

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	107.019[a]	1	107.019	63.060	.000
Intercept	29.482	1	29.482	17.372	.000
GENOTYPE	107.019	1	107.019	63.060	.000
Error	39.033	23	1.697		
Total	157.817	25			
Corrected Total	146.053	24			

a. R Squared = .733 (Adjusted R Squared = .721)

ZT21

Tests of Between-Subjects Effects

Dependent Variable: SHIFT21

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	133.590[a]	1	133.590	28.410	.000
Intercept	141.429	1	141.429	30.077	.000
GENOTYPE	133.590	1	133.590	28.410	.000
Error	122.256	26	4.702		
Total	378.949	28			
Corrected Total	255.846	27			

a. R Squared = .522 (Adjusted R Squared = .504)

CRYΔ(*gal1118*) and CRYΔ(*Pdf-GAL4*)

ZT19

Tests of Between-Subjects Effects

Dependent Variable: SHIFT19

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	15.068[a]	1	15.068	1.435	.244
Intercept	74.666	1	74.666	7.113	.014
GENOTYPE	15.068	1	15.068	1.435	.244
Error	230.933	22	10.497		
Total	308.760	24			
Corrected Total	246.001	23			

a. R Squared = .061 (Adjusted R Squared = .019)

ZT21

Tests of Between-Subjects Effects

Dependent Variable: SHIFT21

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	147.260[a]	1	147.260	29.247	.000
Intercept	156.295	1	156.295	31.041	.000
GENOTYPE	147.260	1	147.260	29.247	.000
Error	161.123	32	5.035		
Total	505.136	34			
Corrected Total	308.383	33			

a. R Squared = .478 (Adjusted R Squared = .461)

CRYΔ(*Pdf-GAL4*) and control

ZT19

Tests of Between-Subjects Effects

Dependent Variable: SHIFT19

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	162.661[a]	1	162.661	12.123	.003
Intercept	1.593	1	1.593	.119	.735
GENOTYPE	162.661	1	162.661	12.123	.003
Error	228.100	17	13.418		
Total	394.510	19			
Corrected Total	390.761	18			

a. R Squared = .416 (Adjusted R Squared = .382)

ZT21

Tests of Between-Subjects Effects

Dependent Variable: SHIFT21

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.274[a]	1	.274	.066	.799
Intercept	583.939	1	583.939	140.468	.000
GENOTYPE	.274	1	.274	.066	.799
Error	124.713	30	4.157		
Total	725.298	32			
Corrected Total	124.986	31			

a. R Squared = .002 (Adjusted R Squared = -.031)

APPENDIX 17: PER and TIM nuclear accumulation and TIM total levels are reduced in CRYΔ flies.

1. PER N/C ratio

Tests of Between-Subjects Effects
Dependent Variable: value

Source	Type III Su	df	Mean Squa	F	Sig.
Corrected Model	594.6235	11	54.05668	49.00333	1.625E-55
Intercept	1049.881	1	1049.881	951.7353	3.52362E-86
GENOTYPE	257.4637	1	257.4637	233.3953	1.99429E-37
CELL	11.25096	1	11.25096	10.1992	0.001589897
TIME	169.2144	2	84.6072	76.69791	1.43323E-26
GENOTYPE * CELL	17.0707	1	17.0707	15.47489	0.000109036
GENOTYPE * TIME	103.796	2	51.89801	47.04646	5.23791E-18
CELL * TIME	7.83989	2	3.919945	3.553499	0.030115023
GENOTYPE * CELL	7.701199	2	3.850599	3.490636	0.032012395
Error	269.1619	244	1.103123		
Total	2041.513	256			
Corrected Total	863.7854	255			

a R Squared = .688 (Adjusted R Squared = .674)

2. TIM N/C ratio

Tests of Between-Subjects Effects
Dependent Variable: V4

Source	Type III Su	df	Mean Squa	F	Sig.
Corrected Model	869.1952	11	79.01775	88.00502	2.2343E-101
Intercept	1630.782	1	1630.782	1816.262	4.7939E-154
V1	359.1021	1	359.1021	399.9454	7.10802E-63
V2	18.65628	1	18.65628	20.7782	6.78502E-06
V3	247.5396	2	123.7698	137.847	1.11037E-46
V1 * V2	28.29298	1	28.29298	31.51096	3.6239E-08
V1 * V3	232.5299	2	116.2649	129.4886	1.8094E-44
V2 * V3	4.884892	2	2.442446	2.720244	0.067027565
V1 * V2 * V3	5.270143	2	2.635071	2.934778	0.054241179
Error	374.415	417	0.897878		
Total	3023.515	429			
Corrected Total	1243.61	428			

a R Squared = .699 (Adjusted R Squared = .691)

3. TIM total levels

Tests of Between-Subjects Effects
Dependent Variable: value

Source	Type III Su	df	Mean Squa	F	Sig.
Corrected Model	2878463	11	261678.4	43.61744	3.21454E-55
Intercept	7994090	1	7994090	1332.482	7.8183E-111
GENOTYPE	1927722	1	1927722	321.3192	5.64387E-49
CELL	35880.88	1	35880.88	5.980744	0.015053398
TIME	505804.2	2	252902.1	42.15456	8.24826E-17
GENOTYPE * CELL	49290.19	1	49290.19	8.215853	0.004454544
GENOTYPE * TIME	582927	2	291463.5	48.58211	6.11636E-19
CELL * TIME	39522.71	2	19761.35	3.293888	0.038492902
GENOTYPE * CELL	11301.02	2	5650.509	0.941846	0.391088739
Error	1751825	292	5999.4		
Total	13231305	304			
Corrected Total	4630288	303			

a R Squared = .622 (Adjusted R Squared = .607)

APPENDIX 18: Locomotor activity of CRYΔ(cry^b) and control flies

1. In LD

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.226[a]	2	.113	1.824	.165
Intercept	79488.494	1	79488.494	1284803	.000
GENOTYPE	.226	2	.113	1.824	.165
Error	9.218	149	6.187E-02		
Total	89024.304	152			
Corrected Total	9.444	151			

a. R Squared = .024 (Adjusted R Squared = .011)

2. In DD

No differences between both controls

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.302[a]	1	.302	1.765	.190
Intercept	32943.004	1	32943.004	192376.0	.000
GENOTYPE	.302	1	.302	1.765	.190
Error	9.247	54	.171		
Total	33135.822	56			
Corrected Total	9.549	55			

a. R Squared = .032 (Adjusted R Squared = .014)

CRYΔ(cry^b) different from control flies

Tests of Between-Subjects Effects

Dependent Variable: PERIOD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	41.965[a]	1	41.965	113.127	.000
Intercept	64402.176	1	64402.176	173611.3	.000
GENOTYPE	41.965	1	41.965	113.127	.000
Error	37.838	102	.371		
Total	64611.151	104			
Corrected Total	79.803	103			

a. R Squared = .526 (Adjusted R Squared = .521)

APPENDIX 19: Analysis of PRC of CRYΔ(*cry*^{b}) and control flies

1. Between CRYΔ(*cry*^{b}) and *cry*$^+$ controls

ZT15

Tests of Between-Subjects Effects

Dependent Variable: SHIFT15

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	67.541[a]	1	67.541	5.763	.019
Intercept	2546.929	1	2546.929	217.337	.000
GENOTYPE	67.541	1	67.541	5.763	.019
Error	867.193	74	11.719		
Total	3731.669	76			
Corrected Total	934.734	75			

a. R Squared = .072 (Adjusted R Squared = .060)

ZT21

Tests of Between-Subjects Effects

Dependent Variable: SHIFT21

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	346.194[a]	1	346.194	21.283	.000
Intercept	83.413	1	83.413	5.128	.026
GENOTYPE	346.194	1	346.194	21.283	.000
Error	1626.655	100	16.267		
Total	2004.134	102			
Corrected Total	1972.849	101			

a. R Squared = .175 (Adjusted R Squared = .167)

2. Between CRYΔ(*cry*^{b}) and *cry*^{b} controls

ZT15

Tests of Between-Subjects Effects

Dependent Variable: SHIFT15

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	84.964[a]	1	84.964	8.083	.006
Intercept	1023.592	1	1023.592	97.374	.000
GENOTYPE	84.964	1	84.964	8.083	.006
Error	725.323	69	10.512		
Total	1775.997	71			
Corrected Total	810.287	70			

a. R Squared = .105 (Adjusted R Squared = .092)

ZT21

Tests of Between-Subjects Effects

Dependent Variable: SHIFT21

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	157.796[a]	1	157.796	9.936	.002
Intercept	12.529	1	12.529	.789	.377
GENOTYPE	157.796	1	157.796	9.936	.002
Error	1492.843	94	15.881		
Total	1650.660	96			
Corrected Total	1650.640	95			

a. R Squared = .096 (Adjusted R Squared = .086)

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