Analysis of the light--entrainment pathways for the circadian clock of *Drosophila melanogaster*

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER NATURWISSENSCHAFTLICHEN FAKULTÄT III - BIOLOGIE UND VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

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“Om Asato maa sad--gamaya; tamaso maa jyotir--gamaya”

(*Brihadaranyaka Upanishads* 1.3.28; 800--400 B.C.)

(O’ Lord lead me from the unreal to the real; lead me from the darkness to light)

"With each dawn and each dusk, we connect with the world in its time"

Dedicated to...

Professor Dr. V. K. Sasidharan (late)…

Who imparted on me the cardinal virtues in Life and in Science.
Who helped to mould my thoughts for a realistic world.

Ms. A. C. Pushpalatha…

Who instilled inside me, in the early years of my learning,
the thought to pursue a scientific carrier.

My beloved parents and sisters…

They could only watch in amazement what I'm doing and where I'm going on…
Their presence in my life keeps the journey going and elating…
and their constant thoughts, prayers and blessings are the impetus for my spirit…
without that I wouldn't have done anything…

unter Anleitung von

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CHAPTER 1

CIRCADIAN PHOTORECEPTION IN *DROSOPHILA MELANOGASTER*

Chapter 1

Circadian photoreception in *Drosophila melanogaster*

1 Introduction

Light rays emanating from the sun are essential for maintaining life on earth. Since earth rotates on its own axis, the level of light signals fluctuates rhythmically with a period of ~24 h on the earth's surface. Hence organisms experience day--night changes. To adapt to this changing environmental settings organisms have evolved a circadian clock, which synchronizes their physiology and behavior to the environment. Moreover, the circadian clock allows the organism to anticipate a regular temporal change in the environment. The broad distribution of circadian clocks in prokaryotic and eukaryotic organisms may be due to the adaptive advantage it attributes on the organisms; it was shown that the presence of a functional circadian clock improves the reproductive fitness of both the cyanobacteria (Ouyang *et al.*, 1998) and fruit flies (Beaver *et al.*, 2002).

In a simplified view, the circadian clock consists of three basic components, an input, an oscillator and an output (Fig. 1). The input pathways feed temporal information about the environment to the oscillator and it modulates the output functions, i.e. the organism's physiology and behavior.

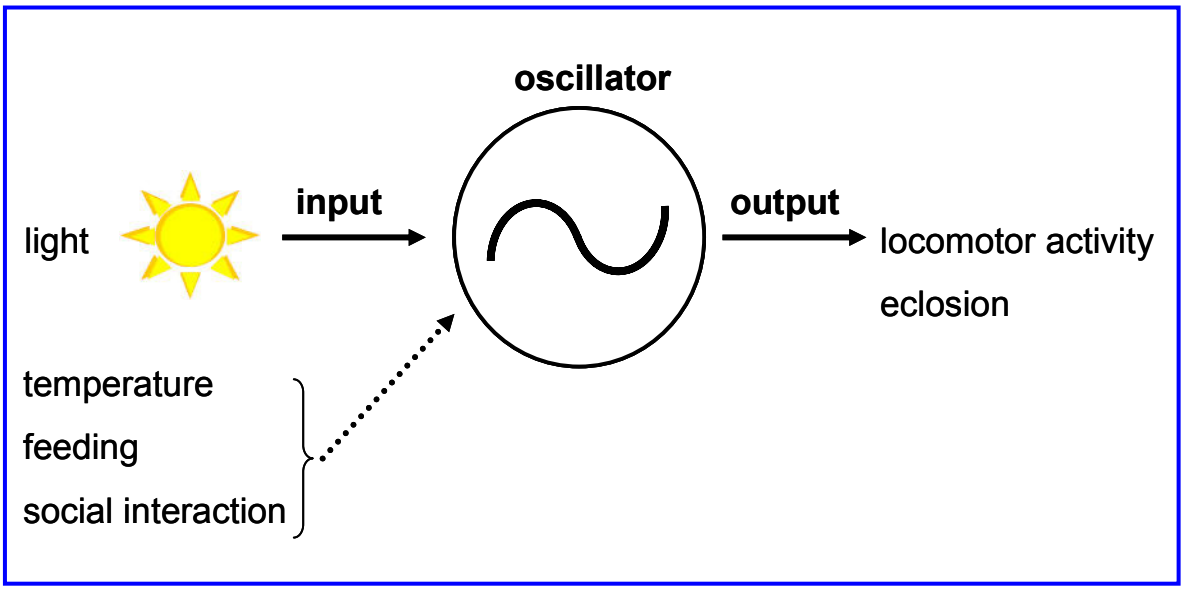

Figure 1 Basic model of the circadian clock. It has three components, the input, the oscillator and the output. The input constitutes various temporal cues like light,

temperature, time of feeding, social cues etc., transmitting from the environment into the circadian clock *via* different signal receptors, like photoreceptors. The oscillator is core of the circadian clock, which functions based on a molecular feed--back loop mechanism. Every clock cell has an oscillator. It receives the input and modifies/generates the outputs. The output comprises the general physiology and behavior of the organism, like locomotor activity and eclosion rhythms.

The light--dark cycles (LD) in nature are one of the most important and potent *Zeitgeber* or temporal cues available to organisms for synchronizing the circadian clock to the environment (Pittendrigh *et al.*, 1958; Helfrich--Förster, 2002). But light is not the only *Zeitgeber* for the circadian clock. Other *Zeitgebers* like temperature (Pittendrigh *et al.*, 1958), humidity (Halket, 1931); feeding (Stephan *et al.*, 1979) and social interactions (Levine *et al.,* 2002a) can also entrain the circadian clock. The circadian clock of *Drosophila melanogaster* (*D. melanogaster*) is synchronised by light signals perceived through the photoreceptors. In *D. melanogaster,* so far seven photopigment molecules have been reported, i.e. six rhodopsins (Montell, 1999) and one cryptochrome (Emery *et al.*, 1998; Stanewsky *et al.*, 1998).

The circadian rhythm is endogenous in nature and can free run in constant darkness (DD) with a period close to 24 h. It is thought to be generated by the circadian oscillator based on a molecular feed--back loop knitted by the clock genes and their products (Hardin *et al*., 1990; Dunlap, 1990). There are two major classes of circadian oscillators: the self--sustaining and dampening oscillators (or slave--oscillators). In the self--sustaining oscillators the molecular oscillations sustain in DD (Yang and Sehgal, 2001; Shafer *et al*., 2002; Veleri *et al*., 2003), and are more central in nature, i.e. present in the central nervous system (CNS). In the dampening oscillators the molecular oscillations dampen quickly in DD (Plautz *et al*., 1997a), and most of them are present in the peripheral tissues (Plautz *et al*., 1997a); some are also present in the CNS (Yang and Sehgal, 2001; Shafer *et al*., 2002; Veleri *et al*., 2003). Both of these oscillators rhythmically express the clock genes *period* and *timeless* in LD (Zerr *et al*., 1990; Hunter--Ensor *et al*., 1996).

The output from the circadian oscillators is mainly the physiological or behavioral activity of the organism. In *Drosophila*, the best studied circadian outputs are the locomotor activity rhythms and eclosion, hatching of a fly from the pupal case (Pittendrigh *et al*., 1967; Konopka *et al*., 1971). The locomotor activity rhythm can be observed in an individual organism whereas the eclosion rhythm has to be observed always on a population basis. Both of these rhythms require the clock genes *period* and *timeless* (Konopka *et al*., 1971; Sehgal *et al*., 1994).

2 Photoreceptors and photopigments in *Drosophila*

2.1 Photoreceptors

In *Drosophila* two externally visible photoreceptor structures are present, a pair of compound eyes and the ocelli (Fig. 2). *Drosophila* also has a putative photoreceptor structure called the Hofbauer--Buchner (H--B) eyelet (Hofbauer and Buchner, 1989) (Fig. 2), its (putative) precursor in larvae is known as the Bolwig Organ (BO) (Yasuyama and Meinertzhagen, 1999; Helfrich—Förster *et al.*, 2002). These photoreceptors express the retinal based photopigment rhodopsin. Recently a new class of flavin based photoreceptor was discovered called cryptochrome, CRY (Emery *et al*., 1998; Stanewsky *et al*., 1998). It is expressed in the circadian rhythm controlling cells (Egan *et al*., 1999; Emery *et al*., 2000b; Klarsfeld *et al*., 2004).

2.2 Photopigments

2.2.1 Rhodopsins

The rhodopsins are retinal based photopigments where retinal serves as the chromophore (von Lintig *et al*., 2001). In *D. melanogaster* there are 6 different rhodopsin (Rh) molecules (Montell, 1999). The maximum sensitivity for different rhodopsins varies (as written in parenthesis): Rh 1 (478 nm) and Rh 5 (437 nm) absorb in the blue range, Rh 2 (420 nm) absorbs in the violet range, Rh 3 (345 nm) and Rh 4 (375 nm) absorb in the UV range, and Rh 6 (508 nm) absorbs in the green range (Salcedo *et al*., 1999). Most of them express in the photoreceptor cells (R 1--R8) of the compound eyes. Rh 1 expresses in R 1--6, Rh 3 and Rh 4 express in non--overlapping subsets of R 7 cells, and Rh 5 and Rh 6 express in mutually exclusive subsets of R 8 cells, whereas Rh 2 expresses in the ocelli (Salcedo *et al*., 1999; Montell, 1999). *Rh* 5 and *Rh 6* genes both express in the

BO, but never together in the same cells as could be seen by reporter gene expression in BO fibers contacting the pacemaker cells (Malpel *et al*., 2002). In the H--B eyelet cells Rh 6 protein is expressed (Malpel *et al*., 2002; Yasuyama and Meinertzhagen, 1999) and a weak expression of the *Rh 5* gene was detected in the H--B eyelet cells with a *Rh 5*--GAL4 transgene but an antibody against Rh 5 could not detect any signal from the H--B eyelet (Malpel *et al*., 2002).

2.2.1.1 Rhodopsin initiated visual transduction cascade

Rhodopsins are G protein coupled receptors (GPCR), and the rhodopsin based phototransduction involves factors encoded by many genes (Ranganathan *et al*., 1995; Montell, 1999; Hardie and Raghu, 2001). Therefore, eliminating or disabling any of these gene products is expected to stop the phototransduction cascade. Rhodopsin is activated by absorption of light by the covalently bound chromophore 11--*cis* 3--hydroxy retinal (Vogt and Kirschfeld, 1984). The resulting photo isomerization to all--*trans* retinal triggers the conversion of rhodopsin to the active metarhodopsin state, which catalyses the activation of a heterotrimeric G protein. This involves exchange of GTP for GDP and subsequent dissociation of the Gα subunit, which remains active until the bound GTP is hydrolyzed. The activated G protein binds to and activates the effecter enzyme, phosphoinositide--specific phospholipase C (PLCβ), encoded by the *norpA* gene in *Drosophila.* It hydrolyses the minor membrane phospholipid phosphotidylinositol 4,5--bisphosphate (PIP_2) to produce soluble inositol 1,4,5--triphosphate ($InsP_3$) and diacylglycerol (Hardie, 2001). It results, by a yet unknown mechanism, in the activation of cation--permeable channels and membrane depolarization (Hardie, 2001). $norpA^{P41}$ is a null mutation for phospholipase C, and hence $norpA^{P41}$ flies can not perform the above catalytic reaction (Pearn *et al*., 1996). Therefore, in $norpA^{P41}$ flies the rhodopsin based phototransduction *via* PLCβ is not functional. The phosphoinositide cascade is of central importance in controlling cellular Ca^{2+} levels, by releasing Ca^{2+} from $InsP_3$--sensitive stores and also by activating Ca^{2+} influx through specific channels in the plasma membrane. The central role of PLC in invertebrate photoreceptors is not disputed, but how activation of PLC is linked to opening of the light sensitive channels remains unresolved (Hardie, 2001). In *D. melanogaster* the light sensitive conductance is highly Ca^{2+} permeable (Hardie, 1991) and mediated by at least two channels encoded by the *trp* (*t*ransient

*r*eceptor *p*otential) gene and a homologue with approximately 40% sequence identity, *trpl* (*trp*--*l*ike) (Hardie and Minke, 1992; Niermeyer *et al*., 1996; Philips *et al*., 1992).

2.2.2 Cryptochromes

Cryptochromes (CRY) were first discovered in *Arabidopsis*. CRYs are flavin containing blue light photoreceptors related to bacterial photolyases (Ahmad and Cashmore, 1993). The photolyases are flavoproteins, which repair the DNA damage caused by UV--B irradiation (Sancar, 2003). Subjecting DNA molecules to UV--B exposure result in pyrimidine dimer formation, generally a thymidine dimer, which may lead to mutation if not repaired. Photolyase absorbs blue or UV--A light and as a result an electron from the photolyase bound flavoprotein is transferred to the damaged pyrimidine dimer. The incoming electron causes an isomerization reaction in the pyrimidine dimer, and subsequently cleaves it and yields the monomer. Finally an electron is returned to the photolyase. In this respect, photolyases are photoreceptors able to mediate blue light--dependent redox reactions (Cashmore, 2003).

Cryptochromes are found in algae, ferns, plants and animals (Lin, 2002). In *Arabidopsis*, *CRY1* and *CRY2* mRNA levels oscillate with a circadian rhythm (Harmer *et al*., 2000). At protein level CRY1 expression is not affected by light, whereas CRY2 expression is negatively regulated by blue light (Ahmad *et al*., 1998a; Lin *et al*., 1998). CRY2 expression is correlated with its functions in de--etiolation and photoperiodic flowering. The de--etiolation function of CRY2 is largely limited to relatively low light (Lin *et al*., 1998). In plants grown in short--day photoperiods the CRY2 protein level is lower during the day but higher in the night, this cycling level of CRY2 was not apparent in long--day photoperiod. This differential expression of CRY2 depending on photoperiods may provide a mechanism for the plant to sense differences in the day length, on which the flowering mechanisms rely, which is accomplished together with the phytochromes. CRY1 promote floral initiation in various light conditions (Mozley and Thomas, 1995; Bagnall *et al*., 1996). CRY1 inhibits the hypocotyl elongation in blue light (Lin *et al*., 1998), and it plays a role in light--dependent anthocyanin accumulation (Ahmad *et al*., 1995). In circadian clock function the *cry1* mutant

shows longer period length than the wild type plants, both in low and high intensities of blue light whereas *cry1* mutant shows a slight change in period length only in low intensity blue light (Somers *et al.*, 1998). However, both play a role in the circadian clock because double mutants have a much longer period length than either the *cry1* or *cry2* monogenic mutants (Devlin and Kay, 2000). *cry1* is well known to be the major blue light receptor regulating the light induced expression of flavonoid biosynthesis gene such as *CHS* [chalcone synthase] (Kubasek *et al*., 1992). *cry1* and *cry2* are involved in light regulation of the chloroplast transcription (Thum *et al*., 2001).

In the monarch butterfly a role for cryptochrome in the light entrainment of eclosion rhythm has been implicated (Froy *et al*., 2003). On the embryonic chicken iris exposure to light causes its constriction. It is a non--visual photo--response. Knockdown expression of cryptochrome decreased iris photosensitivity. But knockdown expression of melanopsin expression did not decrease iris photosensitivity more than the controls. Therefore, a role for cryptochrome in non--visual photoreception in chick iris has been suggested (Tu *et al*., 2004).

In *D. melanogaster* CRY is encoded by the *cry* gene (Emery *et al*., 1998; Stanewsky *et al*., 1998). It is expressed in most of the clock neurons (Egan *et al*., 1999; Emery *et al*., 2000b; Klarsfeld *et al*., 2004). CRY is a crucial component of the circadian input pathway used for the photic entrainment of *Drosophila* circadian clock (e.g., Emery *et al*., 2000a; Stanewsky *et al*., 1998). This was shown by an apparent null mutation, cry^b blocks CRY mediated circadian photoreception and it affects the photic entrainment of molecular oscillations while the temperature entrainment is retained (Stanewsky *et al*., 1998). (See section 4.5 below for a more detailed discussion on CRY in the circadian clock function).

2.2.2.1 Cryptochrome initiated signal transduction cascade

The elaborate molecular mechanism of CRY signaling is not known (Lin, 2002). However, results from recent studies indicate that CRY interact with other proteins (Ceriani *et al*., 1999; Busza *et al*., 2004), suggesting that absorption of a photon may trigger a change of protein--protein interactions. The interaction with CRY

may result in altered sub cellular localization of light--signaling protein, or changes in ion homeostasis, gene expression, or other cellular activities.

Cryptochromes have two chromophores: a folate based (5, 10--methenyl tetrahydrofolate, MTHF) and a flavin based (Flavin Adenine Dinucleotide, FAD). In photolyase, the mechanism of light absorption and DNA repair is as follows: First photolyase binds to the DNA lesion; a photon absorbed by MTHF chromophore excites it. The resulting excitation energy is transferred to the catalytic chromophore FAD. A single electron is then transferred from FAD to the cyclobutane ring of pyrimidine dimer to generate two pyrimidines. An electron is transferred back from remaining pyrimidine radical to FAD thus restoring the redox status of the cofactor (Sancar, 1994). Assuming a similar mechanism in cryptochrome, an electron transfer may happen between flavin of cryptochrome and a signaling molecule in its vicinity. Alternatively, an electron transfer could occur between flavin and the protein moiety of the cryptochrome, resulting in conformational change within the photoreceptor. Either of these changes could lead to biochemical modifications such as phosphorylation of the cryptochrome, and alternation of protein--protein interaction between the cryptochrome and signaling proteins interacting to it. Light--dependent phosphorylation of recombinant CRY1 protein by phytochrome A protein was shown *in vitro* (Ahmad *et al*., 1998b).

The interaction of CRY with other proteins varies between *Drosophila* and *Arabidopsis*. In *Drosophila*, the C--terminal of CRY interacts with its partner, whereas in *Arabidopsis* the N--terminal of CRY interacts with its partners. Moreover, in *Drosophila* CRY mediated light dependent--degradation of its interacting proteins occurs in the presence of light whereas in *Arabidopsis* degradation of its interacting partners occurs in the darkness (Cashmore 2003 and refs. therein).

3 Circadian oscillators

3.1.1 Clock cells in the brain--the central clocks

Cells expressing clock genes are called clock cells. Molecular--genetic studies revealed the presence of numerous putative clock cells throughout *Drosophila*'s

body (Hall, 1995), but most of the studies were focused on brain clock neurons (Zerr *et al*., 1990; Ewer *et al*., 1992; Frisch *et al*., 1994; Helfrich--Förster, 1995; Kaneko and Hall, 2000), because already Konopka *et al*. (1983) had located the pacemaker center controlling rhythmic behavior in the brain. The clock neurons in the brain are regarded as the central clock cells and are classified into two main groups based on their anatomical positions. Laterally positioned groups of neurons called lateral neurons (LNs) and dorsally positioned groups of neurons called dorsal neurons (DNs)--(Fig. 2; Kaneko, 1998).

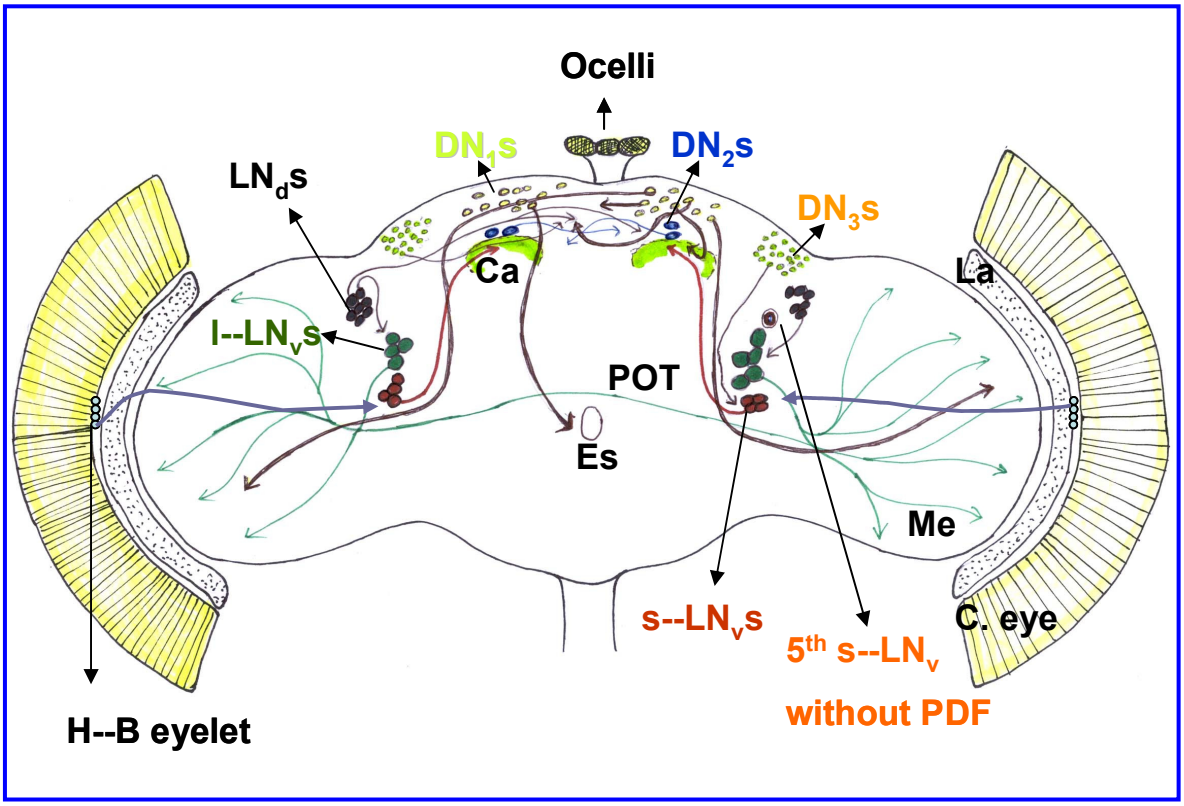

Figure 2 Schematic view of *Drosophila* adult brain showing the clock neurons and their axonal projections along with the rhodopsin photoreceptors. There are three groups of dorsal neurons (DNs), DN_1s, DN_2s and DN_3s; and three groups of lateral neurons (LNs), large--lateral neurons ventral (l--LN_vs), small--lateral neurons ventral (s--LN_vs), and lateral neurons dorsal (LN_ds). The DNs are present in the dorsal brain and the LNs are present in the lateral brain. The DN_1s are in the most dorsal cortex and composed of 8--17 cells (Kaneko, 1998). They send axonal projections, locally within the dorsal brain, and towards the LN_vs reaching up to the medulla (Me). The DN_1s also send axonal projections towards the esophagus (Es). The DN_2s are, present in the roof of the calyx (Ca) of the mushroom body, typically consist of two cells mostly positioned in a horizontally or vertically line to each other. The DN_2s send axonal projections locally within the dorsal brain. The DN_3s

are located in the lateral superior protocerebrum and consist of approximately 40 neurons with small somata compared to other DN subgroups. The axonal projections from the DN_3s course towards the mid dorsal brain and also towards the LN_vs. The l--LN_vs are present in the lateral brain and composed of 4--6 cells. Their perikariya are relatively larger than the DNs. The l--LN_vs have profuse axonal projections in the optic lobe. Their axonal projections also course to the contra--lateral optic lobe *via* the posterior optic tract (POT). The s--LN_vs constitute 4--5 cells relatively smaller than the l--LN_vs. Axonal projections from the s--LN_vs course into the dorso--medial brain close to the calyx. The LN_ds comprises 5--6 cells and located in the dorso--lateral brain. Their axons project into the mid--dorsal brain and also towards the LN_vs. All the DNs and the LNs express the *period* (*per*) and the *timeless* (*tim*) genes. The LN_vs express a clock output factor, called pigment dispersing factor (PDF, shown in red letters); however, there is an exception to this, the fifth s--LN_v (5^{th} s--LN_v, orange letters) that does not express PDF. The compound eyes (C. eye) are the main external photoreceptors perceiving the light signals for the circadian clock. The ocelli, another external photoreceptor, are present in the mid dorsal apex of the head case. Further, an extra--retinal photoreceptor called Hofbauer--Buchner (H--B) eyelet, found in the posterior border of the compound eye, between retina and lamina (La). They are about 4 cells. Their axons project to the vicinity of LN_vs.

The LNs are subdivided into three groups based on their relative position in the lateral brain: small lateral neuron ventral (s--LN_vs), large lateral neuron ventral (l--LN_vs) and lateral neuron dorsal (LN_ds). The DNs are also subdivided into three groups based on their relative position in the dorsal brain: DN_1s, DN_2s and DN_3s. All these cells are placed symmetrically in both brain hemispheres. The s--LN_vs consist of 4--5 cells, the l--LN_vs of 4--6 cells and the LN_ds of 5--6 neurons. The DN_1s are located in the most dorsal cortex of the brain and are composed of 8--17 cells. The DN_2s are situated at the roof of the calyces of the mushroom body and are typically of two cells, positioned either in a vertical or horizontal line to each other (Fig. 2). The DN_3s are located in the lateral superior protocerebrum and consists of ~40 neurons with small somata. These LNs and DNs rhythmically express PERIOD (PER) and TIMELESS (TIM) in LD (Kaneko, 1998; Hunter--Ensor *et al*., 1996). Four of the s--LN_vs and the entire l--LN_vs express the pigment dispersing factor (PDF), a clock output factor (Renn *et al*., 1999), but the fifth s--LN_v cell, the LN_ds and the DNs do not express PDF (Helfrich--Förster, 1995; Kaneko *et al*, 1997; Stoleru *et al*., 2004; Kaneko and Hall, 2000). Since *Pdf*--null

mutants exhibit severe defects in behavioral rhythms (Renn *et al*., 1999), PDF is considered as an important circadian mediator. It was suggested that PDF could serve as a circadian neuromodulator of the activity of neurons in the superior protocerebrum and in the medulla (Helfrich--Förster *et al*., 1995).

3.1.2 Networking of the brain clock neurons

Present knowledge about the clock neuronal networking mainly stemmed out of anti--PDF immunostaining studies in *Drosophila* brain (Helfrich--Förster, 1995; Helfrich--Förster, 2003). It was further extended by the studies using transgenic--driven neurite markers (Renn *et al*., 1999; Kaneko and Hall, 2000; Park *et al*., 2000; Veleri *et al*., 2003; Stoleru *et al*., 2004). The l--LN_vs send axons across the brain midline (*via* the posterior optic track) to the vicinity of contra--lateral LNs, and these axons arborize on the surface of the medulla (Fig. 2). The s--LN_vs send projections into the dorso--medial brain and their axons terminate dorso--frontally of the mushroom--body calyces (Fig. 2). The LN_ds send axons mainly to the dorsal brain (Fig. 2; Kaneko and Hall, 2000). Most of the DNs project their axons to the vicinity of axon terminals from the s--LN_vs and LN_ds. Some of the DN_1s project their axons towards the LN_vs and to the esophagus (Hall, 2005). The DN_3s either send axons towards the mid--dorsal brain (Kaneko and Hall, 2000) or towards the LN_vs (Fig. 2; Veleri *et al*., 2003).

3.1.3 Functions of the brain clock neurons

Currently, it is believed that the s--LN_vs host the central circadian pacemaker because PER expression continued to cycle in DD for one or two or five or 9 days (Yang and Sehgal, 2001; Shafer *et al*., 2002; Veleri *et al*., 2003; Helfrich--Förster, 2003; Lin *et al*., 2004; Veleri and Wülbeck, 2004). Besides controlling the circadian output the s--LN_vs together with the l--LN_vs may couple single pacemaker cell groups and thus accomplish the synchronization among all the clock cells (Helfrich--Förster, 1998; Peng *et al*., 2003; Lin *et al*., 2004). Recently, the PDF expressing s--LN_vs and l--LN_vs were shown to control the morning activity peak (Grima *et al*., 2004; Stoleru *et al*., 2004). These cells are believed to be responsible for the anticipation of dark to light transition, displayed by flies as the morning activity peak, hereon called M peak. The PDF less fifth s--LN_v contributes to the anticipation of light to dark transition, displayed by flies as the evening activity peak, hereon called E peak (Stoleru *et al*., 2004).

A role for the LN_ds in locomotor activity can be inferred from the studies of Blanchardon *et al*. (2001) and Renn *et al*. (1999). A functional knock--out of the s--LN_vs and l--LN_vs, either by cell ablation or by mutation (*Pdf*^{01}), had a much milder effect on the locomotor activity than the knock--out of s--LN_vs, l--LN_vs and LN_ds by the *disco*^{2} mutation, which lacks all LNs but retains the DNs (Zerr *et al*., 1990; Hardin *et al*., 1992; Veleri *et al*., 2003). The *Pdf*^{01} flies for example displayed a rhythm with short period for at least one week in DD, which may be generated from the LN_ds (Helfrich--Förster, 2002). Recently, it was shown that the LN_ds contribute to the E peak (Grima *et al*., 2004; Stoleru *et al*., 2004).

It was shown that the LN_vs alone could maintain the activity rhythms in DD, hence argued that the morning oscillator is sufficient to maintain the free running rhythms (Grima *et al*., 2004). However, in free running conditions only the evening activity peak remains and the morning activity peak is weak or even disappears (Wheeler *et al*., 1993; Helfrich--Förster, 2000). Therefore, it is conceivable that at least some of the LN_vs might be contributing to the E peak, too.

Compared to the LNs where a role has been postulated for the behavioral oscillators, very little is known about the function of the DNs, although their projections have been studied intensely (Kaneko and Hall, 2000; Helfrich--Förster, 2003). A role for DN_1s in light mediated locomotor rhythms has been implicated by Klarsfeld *et al*. (2004). Recently, the DN_2s and a subset of the DN_1s were shown to contribute to the E peak (Stoleru *et al*., 2004). The above mentioned studies did not look at specifically on the DN_3s' role in circadian functioning; therefore their function is largely unknown (see more about DNs in Chapter 4). Besides the LNs and DNs, numerous glia cells in the brain also rhythmically express PER (Zerr *et al*., 1990; Ewer *et al*., 1992; Kaneko, 1998) and TIM (Hunter--Ensor *et al*., 1996; Kaneko and Hall, 2000). They may be also contributing to the behavioral rhythms (Ewer *et al*., 1992).

3.2 Other clock gene expressing cells--the peripheral clocks

In addition to the clock cells in the brain there are many clock cells all over the *Drosophila'*s body. The analysis of a transgenic reporter fusion gene revealed that

per is rhythmically expressed and in synchrony with the LD in peripheral tissues like antennae, proboscis, eyes, cells in the thoracic ganglion, gut, Malpighian tubules, and testes (Liu *et al*., 1988; Giebultowicz *et al*., 2000), except in ovary, where *per* is constitutively expressed (Hardin, 1994). Therefore it was suggested that many of these peripheral tissues contain an intrinsic circadian oscillator (Liu *et al*., 1988).

4 Clock work mechanisms

Circadian clocks function is based on a molecular--feed back mechanism. All the clock cells have the basic components of the clock work mechanism. However, there are subtle variations in the way the central and peripheral clocks accomplish the clock work mechanism. Below I shall explain the basic components required for, and the principle behind the clock work mechanism.

4.1 Basic components and principle behind the molecular clock work in the *Drosophila* circadian clock

The isolation of a rhythm mutant (*period*^{01}) in *D. melanogaster* allowed first molecular studies of the circadian clock (Konopka and Benzer, 1971). This discovery encouraged many researchers to take up additional genetic and molecular approaches targeted to identify additional clock genes. As a result, many more clock genes like, *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*), *double--time* (*dbt*), *vrille (vri), shaggy* (*sgg*) and *Par domain protein 1* (*Pdp 1*) (Sehgal *et al*., 1994; Allada *et al*., 1998; Rutila *et al*., 1998; Price *et al*., 1998; Blau and Young, 1999; Martinek *et al*., 2001; Cyran *et al*., 2003) were added to the repertoire of the clock gene family. They are the basic components required for the clock work mechanism in *Drosophila*.

The basic principle behind the clock work mechanism is an interconnected positive--negative feed back loops (Fig. 3). In the positive limb of the first loop the clock genes *per* and *tim,* and in the second loop *Pdp 1* and *vri*, are activated transcriptionally by the heterodimer CLOCK (CLK): CYCLE (CYC) (Fig. 3). Whereas, in the negative limb, the products of *per* and *tim*, which form also a heterodimer--PER:TIM moves from the cytoplasm to the nucleus and binds to CLK:CYC. The formed PER:TIM--CLK:CYC complex is not anymore able to activate *per* and *tim*, thus the PER:TIM complex stops its own production. In the

second loop binding of PER:TIM to CLK:CYC also stops the further activation of *vri and Pdp 1,* which ultimately enhances the PDP 1 mediated transcription of *Clk* (Stanewsky, 2003).

4.2 Molecular mechanisms of the *Drosophila* circadian clock

4.2.1 CLK and CYC act as transcriptional activators

CLK and CYC are transcription factors containing a PAS (PER--ARNT--SIM) dimerization domain and a basic helix--loop--helix (bHLH) domain involved in DNA binding. If one of them is mutated, flies lose their eclosion and locomotor rhythms (Allada *et al*., 1998; Rutila *et al*., 1998). Therefore, they are crucial clock components. Moreover, *Clk* and *cyc* mutants negatively affect the expression levels of other clock genes like *per*, *tim* and *vri* (Allada *et al*., 1998; Rutila *et al*., 1998; Blau and Young, 1999), thus CLK and CYC positively influence transcription of other genes. Indeed, the CLK:CYC heterodimer binds to the E--box sequences--a stretch of six consensus nucleotides recognized by bHLH transcription factors (Kyriacou and Rosato, 2000)--in the promoters of *per* and *tim* and activates their transcription (Darlington *et al*., 1998; Lee *et al*., 1999).

4.2.2 Regulation of transcriptional activators by PER, TIM, DBT and SGG

The PER protein is a founding member of the PAS proteins, but lacks the bHLH DNA--binding domain. PER forms a dimer with TIM (Gekakis *et al*., 1995). TIM is not a PAS protein but it contains three ARMADILLO--like dimerization domains and two of them are meant to act as PER binding sites (Kyriacou and Hastings, 2001; Saez and Young, 1996). The PER:TIM heterodimer moves into the nucleus and binds to the CLK:CYC heterodimer, which stops *per* and *tim* transcription by interfering with the functioning of CLK:CYC on the *per* and *tim* promoters (Fig. 3; Lee *et al*., 1998, 1999; Bae *et al*., 2000). However, there is evidence that PER can function on its own, as it alone could move into the nucleus (Shafer *et al*., 2002)..

The PER protein is phosphorylated by the DOUBLE--TIME (DBT) kinase in the cytoplasm (Kloss *et al*., 1998, Price *et al*., 1998), which renders PER unstable until it dimerizes with TIM. The SHAGGY (SGG) phosphorylates TIM (Fig. 3), and thus SGG regulates the nuclear entry of TIM (Martinek *et al*., 2001). The nuclear entry of PER is similarly regulated by DBT (Bao *et al*., 2001).

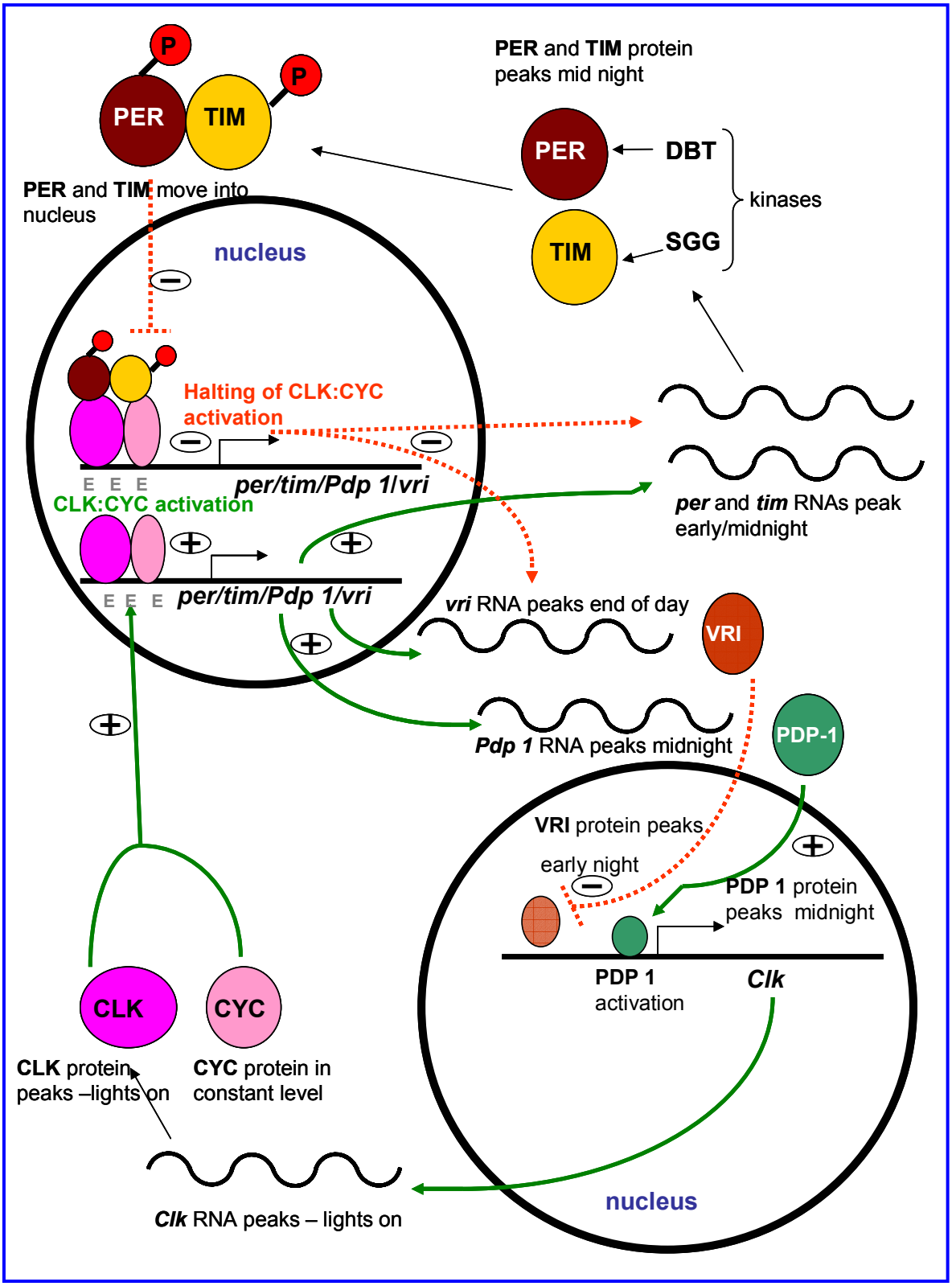

Figure 3 Schematic view of the circadian clock work mechanism in *Drosophila*. It is based on the interconnected feed--back loops. In the first loop, the CLOCK:CYCLE (CLK:CYC) dimer complex binds to the E--boxes (represented by multiple E) in the *per* and *tim* promoter, thus positively control their transcription. In early/midnight, *per* and *tim* RNA levels peak in the cytoplasm. During the late night PERIOD (PER) and TIMELESS (TIM)

accumulate in the cytoplasm, subsequently they are phosphorylated by DOUBLE--TIME (DBT) and SHAGGY (SGG), respectively. The phosphorylated PER and TIM (with a P attached in the picture) form the heterodimer PER:TIM. In the late night, PER:TIM shuttles into the nucleus and binds to CLK:CYC, which closes the loop. The binding of PER:TIM to CLK:CYC stops the transcriptional activation of *per* and *tim,* thus achieves a negative feed--back on their own production levels.

In the second loop, again CLK:CYC positively activates the transcription of *vrille* (*vri*) and *Par domain protein 1* (*Pdp 1*). Due to their slight temporal difference in transcription *vri* RNA peaks in the end of day but *Pdp 1* RNA peaks in the midnight. Correspondingly, VRILLE (VRI) peaks in the early night and PAR DOMAIN PROTEIN 1 (PDP 1) peaks in the midnight. When CLK levels are high during the late day, by then VRI level rises and depresses further transcription of the *Clk* gene by binding to its promoter. In contrast, PDP 1 activates the *Clk* gene transcription in the midnight, since PDP 1 peaks 3--4 h later in the night than VRI. Therefore, in the midnight the *Clk* gene transcription proceeds and by early in the day the *Clk* RNA peaks in the cytoplasm. During the daytime, VRI and PDP 1 levels are low in the cytoplasm, and at that time the CLK level is high in the cytoplasm, leading to heterodimer formation with CYC (CLK:CYC). This heterodimer moves into the nucleus and binds to *vri* and *Pdp 1* resulting once again in their transcriptional activation. However, binding of PER:TIM to CLK:CYC in the nucleus, stops the transcriptional activation of *vri* and *Pdp 1* similar to *per* and *tim*. Thus, the CLK interconnects both the feed--back loops. (For more clarity, the events in the nucleus were split for each feed--back loop).

By midnight, PER, TIM and DBT enter the nucleus perhaps as a complex (Curtin *et al*., 1995; Kloss *et al*., 2001). The phosphorylation of PER and TIM continues in the nucleus by the respective kinases, DBT and SGG (Edery *et al*., 1994; Zeng *et al*., 1996; Price *et al*., 1998; Martinek *et al*., 2001). Thus, it was implicated that SGG might also be accompanying the PER, TIM and DBT complex. This complex likely binds to the CLK:CYC dimer and stops the transcription of *per* and *tim*.

To reinitiate *per* and *tim* transcription by CLK:CYC the repression by PER:TIM on CLK:CYC must be relieved. Therefore, the PER:TIM heterodimer must be either detached from CLK:CYC or degraded. Upon light exposure, TIM is degraded and eases the DBT mediated phosphorylation of PER, which is subsequently degraded. In the PER:TIM complex PER is stable, therefore it is suggested that

TIM also inhibits nuclear function of DBT (Rothenfluh *et al*., 2000). Therefore, ultimately, TIM dissociation (and degradation) from the PER:TIM repressor complex defines the period length of a molecular cycle. SGG phosphorylates TIM, which promotes the latter's transport from the cytoplasm to the nucleus (Martinek *et al*., 2001). Similarly, SGG mediated phosphorylation of TIM in the nucleus might trigger the latter's dissociation from the PER:TIM complex. The dissociation of phosphorylated TIM from PER makes it vulnerable for rapid degradation by light signals (Martinek *et al*., 2001) or even in the absence of light signals; because it was shown that TIM levels fall before lights are 'on' in LD cycle (Zeng *et al*., 1996). However, PER alone can act as potent repressor in the complete absence of TIM (Rothenfluh *et al*., 2000). That is why the *per* and *tim* transcription remains depressed even after TIM is degraded by light in LD cycles (Zeng *et al*., 1996; So and Rosbash, 1997).

4.2.3 CLK, VRI and PDP 1 enhances *per* and *tim* transcription

CLK is one of the transcriptional activators for *per* and *tim*. The *Clk* gene shows rhythmic expression of both its RNA and protein like *per* and *tim*, but the phase of both oscillations is opposite to that of *per* and *tim* RNA. This opposite phase suggested the existence of a second feed--back loop. This additional loop, with *Clk* in the centre, possibly amplifies *per* and *tim* transcriptional rhythms by increasing the amplitude. PER and TIM positively affect the *Clk* RNA and protein levels as per^{01} and tim^{01} mutants have low levels of both *Clk* RNA and protein (Bae *et al*., 1998; Lee *et al*., 1998). On the other hand, per^{01} Clk^{Jrk} and per^{01} cyc^{01} double mutants showed high levels of *Clk,* suggesting that CLK and CYC repress the *Clk* expression but *per* and *tim* derepress the negative action of CLK and CYC on *Clk* expression (Glossop *et al*., 1999).

The bZIP transcription factors, VRILLE (VRI) and PAR domain protein 1 (PDP 1) are expressed with a similar phase as *per* and *tim*, indicating that both are regulated by the same mechanism as *per* and *tim* (Fig. 3; Blau and Young, 1999; Cyran *et al*., 2003). This suggests that the CLK:CYC dimer that effects *per* and *tim* expression may control *vri* and *Pdp 1* expression too. Indeed, the *vri* and *Pdp 1* RNA levels are low in Clk^{Jrk} and cyc^{01} mutants (Blau and Young, 1999; Cyran *et al*., 2003). Moreover CLK activates *vri* expression *in vitro* in an E--box dependent

manner (Blau and Young, 1999). Similar E--boxes are also present in the *Pdp 1* promoter (Cyran *et al*., 2003).

The expression of the transcription factor *vri* is positively activated by CLK:CYC, and during early night the VRI protein enters the nucleus and inhibits the transcription of *Clk.* In contrast, PDP 1 activates *Clk* expression during the late night (Cyran *et al*., 2003). Thus VRI and PDP 1 have an opposite effect on *Clk.* However, the expression of these proteins is temporally displaced by 3--6 h. PDP 1 is produced maximally around midnight and VRI during early night, therefore the PDP 1 eventually causes a rise in production of CLK in the daytime.

vri levels are intermediate in per^{01} and tim^{01} mutants (Blau and Young, 1999), probably due to the relaxed transcription of *vri* by CLK:CYC, resulting in building up of VRI, which would explain the low levels of *Clk* RNA in per^{01} and tim^{01} mutants (Bae *et al*., 1998; Lee *et al*., 1998). An overexperssion of *vri* results in a reduction of *per* and *tim* RNA levels (Blau and Young, 1999), possibly by reducing CLK, which in turn down regulates *per* and *tim* expression. The two described feed--back loops are interconnected *via* CLK. CLK acts as a pivot as follows: binding of PER:TIM to CLK:CYC depresses the activation of *per* and *tim* and simultaneously releases the CLK:CYC--dependent repression of *Clk* transcription mediated by VRI (Fig. 3).

4.3 Molecular clocks can be driven by light

One of the basic tenets of the circadian clock is that it can free run in the absence of *Zeitgebers*. Indeed, the behavioral rhythms are sustained for lifelong in DD (Helfrich, 1986; Dowse, *et al*., 1987). The oscillations triggering this behavior are believed to be generated in the pacemaker neurons (s--LN_vs) by the molecular clock mechanism described above. In fact, the s--LN_vs were shown to maintain self--sustained molecular oscillations in DD for multiple days, but in contrast in l--LN_vs the molecular oscillations dampened quickly (Veleri *et al*., 2003; Shafer *et al*., 2002; Yang and Sehgal, 2001; Lin *et al*., 2004). Also in most of the peripheral circadian oscillators, like sensory bristles in leg and wings, or in Malpighian tubules the molecular oscillations dampen quickly in the absence of *Zeitgebers* (Plautz *et al*., 1997a; Giebultowicz *et al*., 2000). However, reinstating the LD cycles could

boost the dampening molecular oscillations back to normal in the l--LN_vs, and in peripheral oscillators (Plautz *et al*., 1997a; Giebultowicz *et al*., 2000). This shows that light signals function as a potent amplifier of the molecular oscillations. Two more observations give a clue that light might be resetting the molecular oscillation. In DD wild type flies show desynchronized molecular oscillations in the DN_1s and in the l--LN_vs, and anti--phase oscillations in the DN_2s; and the locomotor activity rhythms free run in DD. But in LD the molecular oscillations in all the clock neurons are synchronized, and the locomotor activity rhythms are also synchronized, to the external LD cycles. How are this light resetting of the circadian clock achieved? How is the light signals integrated into the molecular clock work mechanism to reset it on a daily basis?

First, let us look which of the clock components are directly affected by light signals. TIM is a crucial clock component required for both eclosion and locomotor rhythms (Sehgal *et al*., 1994). It is rapidly degraded by light signals in LD or by the light pulses in DD (Hunter--Ensor *et al*., 1996; Lee *et al*., 1996; Myers *et al*., 1996; Zeng *et al*., 1996). It dimerizes with PER and it is believed that thus PER is stabilized (Zeng *et al*., 1996). The dependence of PER stability on TIM and the latter's light labile nature explains how light resets the molecular oscillations (Fig. 4). The light signals decompose the nuclear TIM, independently whether it has already formed a dimer with PER or not. The decomposition of TIM results in enhanced phosphorylation of PER and its subsequent degradation (Rothenfluh *et al*, 2000). The degradation of PER:TIM releases the repression it exerted on CLK:CYC, which mediates the transcription of *per* and *tim*.

In the early night, a light pulse degrading TIM can be compensated by more TIM production due to high levels of *tim* RNA at this time of day. Therefore, the molecular clock takes an extended time to finish the cycle after a light pulse in the early night, hence it exhibits a phase delay. In the late night, the light pulse degrades TIM but by that time there is not much *tim* RNA to compensate for the premature degradation of TIM. Thus the clock attains the completion of the cycle at an earlier point and begins the next cycle. Therefore a light pulse given at later night results phase advance of the circadian clock. Exploiting this mechanistic

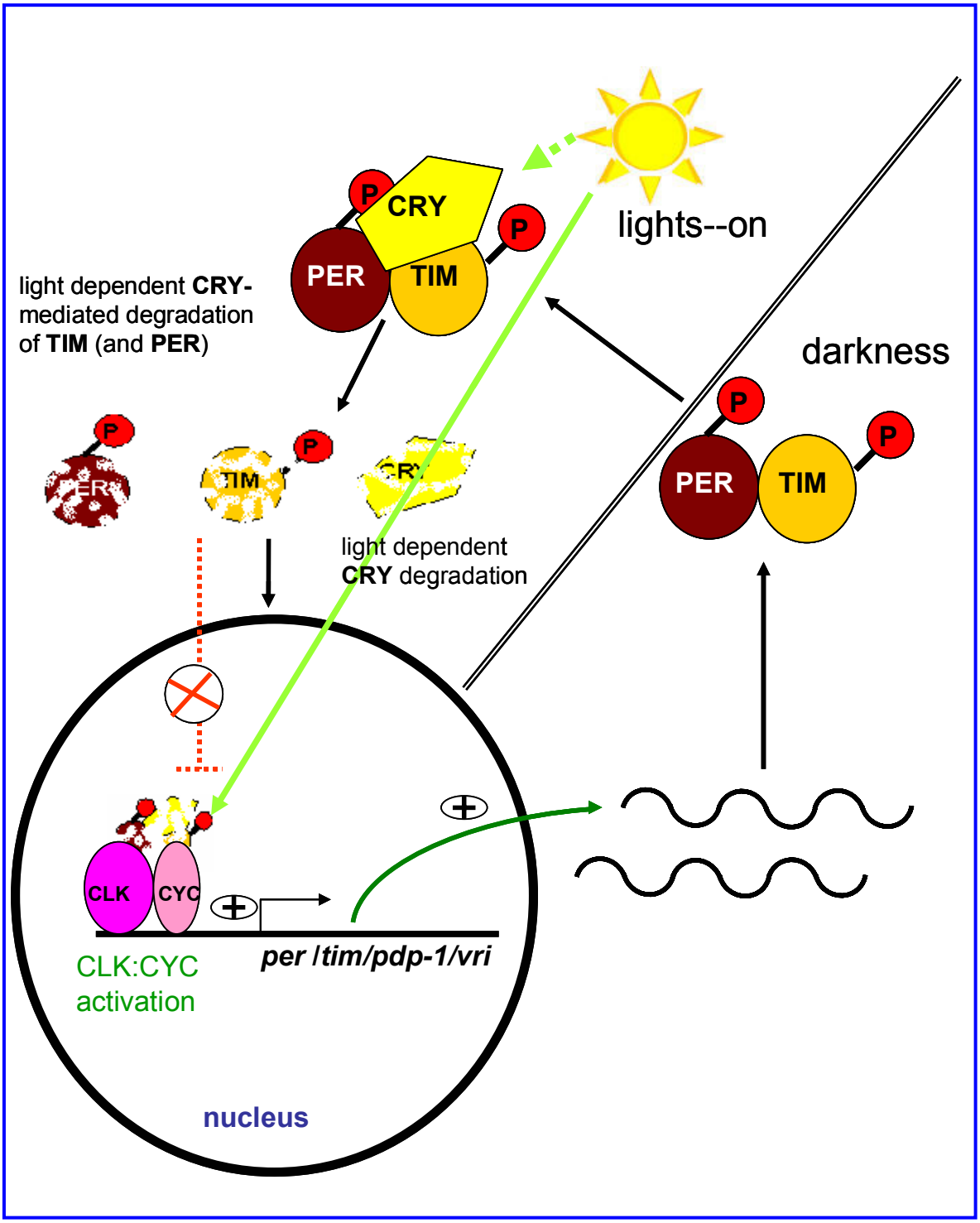

Figure 4 Light resetting of the molecular circadian clock in *Drosophila*. The feed--back loop works as explained in Fig. 3. Phosphorylated PER is unstable both in the light and dark. In the dark, however, PER forms a complex with TIM. This complex is stable in the dark. May be cryptochrome (CRY) is also part of this complex. Upon light exposure in the daytime, CRY mediates light signaling to TIM, and light activated TIM is degraded in the proteasome. Once the TIM is degraded, phosphorylated PER in the nucleus is also decomposed subsequently. CRY may be also degraded by the light signals. Thus, light signals degrade the PER:TIM complex. Its binding to CLK:CYC depressed the activation

of *per, tim, vri* and *Pdp 1*. The degradation of PER:TIM complex relieves the depression of CLK:CYC mediated transcriptional activation. Thus, light signals effect the resetting of the circadian clock work mechanism through a CRY mediated mechanism. (Presently, there is evidence for the existence of an additional CRY--independent mechanism for photic resetting of the circadian clock).

quality of the clock, phase shift experiments were done to understand the light resetting mechanism of the circadian clock (Yang *et al*., 1998; Suri *et al*., 1999). Phase shift experiments corroborated the notion that TIM mediates light resetting of the circadian clock. However, how the light signals reach TIM remained to be resolved.

4.4 Which photopigment(s) integrates light signals into the molecular circadian clock?

Now I shall elaborate the possible photopigments, which could potentially transmit the light signals to TIM. Obviously, compound eyes are the major photoreceptor organs in *Drosophila*, which express the majority of rhodopsins (see in 2.2.1). The compound eyes and other extra ocular photoreceptors are involved in the LD synchronization of both the molecular and behavioral rhythms (Stanewsky *et al*., 1998; Helfrich--Förster *et al*., 2001; Rieger *et al*., 2003). However, *Rh 1* in the compound eyes and the phototransduction cascade down stream from it furnished by *trp* and *trpl* are not required for circadian entrainment of behavior and molecular entrainment of TIM in the LNs (Yang *et al*., 1998). Circadian entrainment is also possible in complete eyeless flies (Rieger *et al*., 2003). This indicates that, additionally, an extra--ocular, non--opsin photopigment might be signaling into the circadian clock. The discovery of a flavin based photopigment, CRY substantiated for such an extra--ocular photopigment/photoreceptor (Emery *et al*., 1998, 2000b; Stanewsky *et al*., 1998). An apparent null mutation, cry^b, results in constant levels of TIM in the eye photoreceptors and in the LNs, except the s--LN_vs and the LN_ds (Emery *et al*., 1998; Stanewsky *et al*., 1998; Helfrich--Förster *et al*., 2001). Hence CRY is regarded as the main circadian photoreceptor for light--induced TIM degradation (Fig. 4). Moreover, a direct interaction between CRY and TIM has been demonstrated *in vitro* (Ceriani *et al*., 1999) and *in vivo* (Busza *et al*., 2004), which supports the view that CRY mediates light signaling to TIM. However, cry^b flies can LD synchronize behavioral rhythms and molecular synchronization of TIM

in the s--LN_vs, indicating the existence of an additional (perhaps it might be a opsin--based one) light--dependent degradation mechanism for TIM, which is CRY--independent. To conclude, it is very likely that TIM receives light signals *via* CRY--dependent and--independent routes to reset the circadian clock.

4.5 Functions of cryptochrome

4.5.1 CRY plays different roles in the central and peripheral circadian oscillators in *Drosophila*

In cry^b flies luciferase reported *per* rhythm was found to be lost, which mainly originated from the compound eyes (Stanewsky *et al*., 1998). The olfactory response sensitivity rhythm and rhythmic clock gene expression were disrupted in cry^b flies in DD (Krishnan *et al*., 2001), suggesting that CRY is more than a photoreceptor at least in this tissue, and that it occupies a place in the clock work mechanism. *cry* is required for maintaining circadian oscillations in the peripheral tissues.

In LD, TIM expression was constitutively high in the photoreceptor cells of cry^b flies (Stanewsky *et al*., 1998) but reinstating CRY expression in the photoreceptor cells restored the synchronized TIM oscillations (Emery *et al*., 2000b). The TIM oscillations in Malpighian tubules did not respond to light pulses in cry^b flies (Ivanchenko *et al*., 2001). This findings suggest that CRY is also a photoreceptor in the peripheral clocks. On contrary in LD in adult cry^b flies the TIM oscillations continued in the s--LN_vs and in DN_1s (Helfrich--Förster *et al*., 2001). This means that CRY plays different roles in the central and peripheral circadian oscillators. In the central oscillators, CRY is not essential for light entrainment of TIM because its role may be substituted by other yet unknown circadian photoreceptor(s) (CRY--independent mechanism). Instead, in the peripheral oscillators, CRY is required for light--mediated degradation of TIM (CRY--dependent mechanism). A direct interaction of CRY with TIM in light, *in vitro* (Ceriani *et al*., 1999) and *in vivo* (Busza *et al*., 2004) supports this belief. CRY may also interact with PER in a light--dependent manner (Rosato *et al*., 2001).

The phosphorylated forms of both PER and TIM form a stable heterodimer. However, interaction of CRY with TIM (may be with PER, too) results in TIM

degradation in a light--dependent manner (Fig. 4; Naidoo *et al*., 1999). Possibly, the CRY interaction activates a tyrosine kinase mediated phosphorylation, followed by ubiquitination of TIM resulting in its decomposition in the proteasome (Lin *et al*., 2001; Grima *et al*., 2002). CRY is also degraded by the proteasome in a light--dependent manner, but light induced ubiquitination of TIM precedes the CRY degradation (Lin *et al*., 2001). CRY degradation requires electron transfer, fulfilled by a CRY cofactor, FAD.

In conclusion, the light mediated CRY interaction with TIM, and the subsequent degradation of the latter may be responsible for high amplitude molecular cycling of the clock gene products in LD. This may be true mostly for the peripheral oscillators because the central oscillators can maintain clock gene cycling independent of CRY.

4.5.2 CRY is a core clock component in the mammalian clock

There are two CRY encoding genes in mammals, i.e. *mCry1* and *mCry2.* Mice which are double knockouts for *mCry1* and *mCry2* show complete loss of circadian rhythmicity (van der Horst *et al*., 1999), suggesting that *mCrys* are part of the central circadian clock mechanism. These genes are rhythmically expressed in the suprachiasmatic nuclei (SCN--the central circadian pacemaker in mammals), with a similar phase like the mammalian *period* genes (*mPers*) but in the opposite phase to *Bmal 1*--the *cyc* equivalent in mammals (Miyamoto and Sancar, 1998; Kume *et al*., 1999). Inhibition of BMAL 1:CLK mediated transcription results in high levels of mCRY1 and mCRY2 proteins in the SCN. Consistent with a negative role of mCRYs in the feed--back loop, *mCry* RNA levels are severely reduced and non--cycling in the SCN of *Clk* mutant mice (Kume *et al*., 1999). This indicates that mCRYs play a negative feed--back role like TIM in *Drosophila* (Stanewsky, 2003). The mCRYs are able to block the BMAL 1:CLK mediated transcription *in vitro*, independent of mPER, thus indicating a direct interplay between mCRYs and the BMAL 1:CLK (Kume *et al*., 1999; Shearman *et al*., 2000). Therefore, CRY is a crucial clock component in the mammalian circadian clock. It is suggested that CRY has substituted for *Drosophila* TIM in the mammalian circadian clock (Stanewsky, 2003).

In the peripheral clock (skeletal muscles) of *Clk* mutant mice the *mCry1* and *mCry2* RNA expression rhythms were dampened and phase advanced, and totally abolished, respectively (Kume *et al*., 1999). Moreover, the presence of the canonical E--box in the *mCry1* promoter suggested that CLK directly mediate the *mCry1* transcription by binding to the E--box (Kume *et al*., 1999). In *mCry* double knockout mice the mPER1 and mPER2 were predominantly seen in the cytoplasmic compartment, suggesting mCRYs are required for the nuclear transport of mPERs (Lee *et al*., 2001). Taken together, these data indicate that *mCry1* and *mCry2* are part of the clock work mechanism in the peripheral clocks, too.

4.5.3 Is CRY a photoreceptor for the mammalian circadian clock?

In mammals, eyes are required for circadian photoreception because enucleation eliminates circadian responses to light, but the rods and cones are not necessary (Forster and Helfrich--Förster, 2001). The pupillary constriction following light exposure to eyes does not require rods and cones. But the maximum sensitivity for this response was around 479 nm implicating an opsin--type photopigment is involved (Lucas *et al*., 2001a). Melanopsin was suggested as the responsible photopigment because it is expressed in the inner retina (Provencio *et al*., 2000). It is also found in a subset of retinal ganglion cells, which are part of the retinal hypothalamic tract connecting the eye with the SCN (Hannibal *et al*., 2002; Hatter *et al*., 2002). These subsets of ganglion cells are intrinsically light sensitive and their photic--response correlates with the photic entrainment (Berson *et al*., 2002; Hatter *et al*., 2002). Recently, it has been demonstrated that human melanopsin can function as a photopigment, but in a way typical for invertebrate opsins (Melyan *et al*., 2005; Panda *et al*., 2005; Qiu *et al*., 2005). In fact melanopsin shows highest sequence similarity to invertebrate opsins (Provencio *et al*., 2000).

The mCRYs are also potential candidates for the photic entrainment of the circadian clock because they are expressed in the ganglion cell layer and inner nuclear layer of retina (Miyamoto and Sancar, 1998). Another line of evidence comes from the observation that *mCry* double knockout mice are able to entrain to the LD, while in DD they displayed arrhythmic behavior (van der Horst *et al*., 1999). This LD behavior was argued as masking response to light (van der Horst

et al., 1999; Mrosovsky, 2001). However, this masking response was eliminated in the *mCry* double knockout mice in a *rodless* background, while *rodless* mice alone were able to entrain to the LD (Selby *et al*., 2000). The above finding indicated that both rods and mCRYs are involved in the masking behavior. Therefore, rods and mCRYs could contribute to the photic signaling. However, the spectral evidence suggested that the photic entrainment in rodless and coneless mice may not be mediated by CRY (Berson *et al*., 2002) despite the fact that determining the precise action spectrum of CRY chromophore remains difficult (Cashmore, 2003) because flavin exists in three different redox states (Lin *et al*., 1995). More recently, it was shown that vitamin A--depleted, retinol binding protein (required for transporting the retinol from liver to extraheptic tissues like eye) knockout ($rbp^{-/-}$) mice exhibit either normal photic entrainment or become diurnal. In contrast, (vitamin A--repleted) $rbp^{-/-}$ $cry1^{-/-}$ $cry2^{-/-}$ mice have reduced light sensitivity, while (vitamin A--depleted) $rbp^{-/-}$ $cry1^{-/-}$ $cry2^{-/-}$ mice, that are additionally on a vitamin A--depleted diet, presumably lack functional opsins and cryptochromes were affected in most of their behavioral and molecular responses to light (Thompson *et al*., 2004). Therefore, the authors have suggested that both cryptochromes and opsins regulate non--visual photic responses. In conclusion, mCRYs may be part of the multiple photopigments responsible for the light detection for photic entrainment of the SCN clock in mammals (Stanewsky, 2003; Forster and Helfrich--Förster, 2001).

4.6 The circadian clock mechanism crashes in constant light

Aschoff (1979) found that constant light (LL) shortens the intrinsic circadian period of diurnal mammals and lengthens that of nocturnal mammals. However, in arthropods LL generally lengthens the free running circadian period. High intensity LL results in extreme effects on the animal behavior and ultimately they behave arrhythmic. In intense LL *D. melanogaster* also exhibits arrhythmic behavior (Konopka *et al*., 1989). Nevertheless, cry^b mutants show rhythmic activity in LL similar to that in DD (Emery *et al*., 2000a). per^s flies have a short free running period in DD (Konopka *et al*., 1989). per^s; cry^b flies displayed a short period in LL, reinforcing the free running nature of the period shown by cry^b flies in LL (Emery *et al*., 2000a). Therefore, it was suggested that the cry^b flies are severely disabled to perceive and/or transmit constant light signals into the circadian clock (Emery *et*

al., 2000a). However, in LL of 100 lux the cry^b flies have a slightly longer period than in DD (Helfrich--Förster *et al*., 2001), which indicates that minute levels of light input reaches the circadian clock. In cry^b flies the response to short light pulses is poor (Stanewsky *et al*., 1998), again indicating a problem in light signaling transduction to the circadian clock. Based on the above findings CRY is regarded as major circadian photoreceptor/photopigment mediating the photic entrainment in *D. melanogaster* (Emery *et al*., 2000a; Emery *et al*., 1998; Stanewsky *et al*., 1998).

Possibly, in LL a CRY (or an unknown factor) mediated light--dependent mechanism continuously degrades TIM as described above (Naidoo *et al*., 1999; Ceriani *et al*., 1999; Busza *et al*., 2004). PER may also be affected by CRY in a light--dependent manner (Rosato *et al*., 2001). Therefore, LL ultimately results in degradation of TIM; subsequently to that of PER, and finally the molecular feed--back to CLK:CYC is eliminated. As a result the molecular clock work mechanism crashes in LL.

Our observations (see in Chapter 6 section 4.7) suggest that the molecular oscillations are lost in the s--LN_vs in LL in *Drosophila*. This could cause the arrhythmic behavior in LL. However, quite recently it has been demonstrated that in LL, the pacemaker cells in the SCN retain molecular oscillations but with desynchronized phases among each other (Ohta *et al*., 2005). Perhaps the molecular mechanisms in the pacemaker neurons of *Drosophila* and mammals react differently to constant light signals. As mentioned earlier, CRY behaves differently in *Drosophila* and mammalian central clocks. Therefore, it is conceivable that there may be different mechanisms to deal with LL in the *Drosophila* and mammalian circadian clocks.

5 A novel photopigment mediates light entrainment of the circadian clock

5.1 Multiple photopigments involved in light entrainment of the circadian clock

The circadian oscillators must be entrained to the 24 h day--night changes in the environment to serve as reliable clocks. The light--dark cycles in the environment are the most important *Zeitgebers* for synchronization of the circadian clock. For this purpose the light signals are perceived by the photopigments of the photoreceptors and transferred into the circadian clock. In *Drosophila*, eclosion and activity rhythms are very sensitive to light signals (Helfrich--Förster and Engelmann, 2002). LD cycles of light intensity less than 1 lux is enough to entrain both the eclosion and activity rhythms (Winfree, 1970). Spectral sensitivity of the circadian clock was tested by studying the phase delaying and phase advancing effects of monochromatic light. This has allowed unraveling the photopigments involved in the circadian photoreception. Light pulses of 15 min (0.1 lux) are enough to phase shift the eclosion rhythms, and the blue light is the most effective wavelength (Winfree, 1970). Light pulses in the wavelength--range of 420--480 nm were maximally effective for phase advancing and phase delaying but above 540 nm the phase shifting was ineffective (Frank and Zimmermann, 1969). In less intense light, phase advancing was ten times more sensitive to 442 nm compared to phase delaying (Chandrashekaran and Engelmann, 1973). Whereas, at higher light intensities (perhaps saturating levels) this difference disappeared, suggesting the involvement of two different photoreceptor pigments or different primary processes being responsible for light absorption during phase advance and phase delay. Phase delaying of the eclosion rhythm also showed that blue light of 473, 435 and 375 nm is of most effective (Klemm and Ninnemann, 1976). The maxima of the action spectrum for the eclosion rhythm indicated that a flavoprotein and not a carotenoid (rhodopsin) serves as the photopigment for phase delay (Klemm and Ninnemann 1976), which was further supported by the fact that the flies grown on a carotene free food were not affected by the photosensitivity of their eclosion rhythm while the visual photoreception was reduced three times (Zimmermann and Goldsmith, 1971). Moreover, it has been shown that the compound eyes are not required for synchronizing the eclosion rhythms (Engelmann and Honegger, 1966). Besides the eclosion rhythms, the locomotor rhythms were also

synchronized to LD in the different blind mutants (Helfrich and Engelmann, 1983; Dushay *et al*., 1989; Wheeler *et al*., 1993). Therefore, the compound eyes are not necessary for the circadian photoreception. Even though, the compound eyes do contribute to the circadian photoreception in adult *Drosophila* as shown in the following studies. Action spectra for the activity rhythms varied among wild type and carotenoid deprived, eyeless or otherwise blind flies (Blaschke *et al*., 1996; Ohata *et al*., 1998). The activity rhythm showed sensitivity near red (500 nm) but the eclosion rhythm did not (Blaschke *et al*., 1996; Ohata *et al*., 1998). The spectral response curve showed a maximum of sensitivity at 500 nm with some red sensitivity for the activity rhythm (Suri *et al*., 1998), while the eclosion rhythm is insensitive to red light (Helfrich--Förster, 1997). These findings suggested that rhodopsin contributes mainly for light entrainment of the locomotor rhythms (Helfrich--Förster and Engelmann, 2002).

All the known rhodopsin expressing photoreceptors could be removed by the double mutant *sine oculis*^{1}*;glass*^{60j}. *sine oculis* (*so*^{1}) flies lack the compound eyes and ocelli because a homeobox gene required for the visual system development is affected in this mutant (Cheyette *et al*., 1994). Mutations in the *glass* gene, which encodes a transcription factor necessary for the general development of photoreceptor cells (Moses *et al*., 1989), impair development of all known external and internal visual structures. Flies carrying the loss of function allele *gl*^{60j} lack all ommatidial photoreceptors and the ocelli, as well as the primary and secondary pigment cells in the compound eyes (Lindsley and Zimm, 1992; Kunes *et al*., 1993). Recently it was found that they also lack the H--B eyelet and the DN_1s (Helfrich--Förster *et al*., 2001, Blanchardon *et al*., 2001). However, *so*^{1}*;gl*^{60j} flies are still capable of LD synchronization and phase--shifting the circadian locomotor rhythms (Hall, 1998a; Helfrich--Förster *et al*., 2001). The above result indicates that a non--rhodopsin photopigment plays a role in circadian photoreception.

The discovery of a flavin based CRY in *Drosophila* (Emery *et al*., 1998; Stanewsky *et al*., 1998), and CRY's absorption maximum at 420 nm (Selby and Sancar, 1999) matches that of a non--rhodopsin photopigment suggested that it as a best candidate for the non--rhodopsin photic entrainment of the circadian clock. Expression of CRY in the pacemaker neurons supported this view (Egan *et al*.,

1999; Emery *et al*., 2000a). Indeed, finally CRY's role in circadian photoreception was demonstrated in *Drosophila* (Emery *et al*., 1998; Stanewsky *et al*., 1998). In conclusion, rhodopsins and CRY are involved in the photic entrainment of the circadian clock.

5.2 Why to look for a novel circadian photoreceptor in *Drosophila*?

As outlined above rhodopsins and cryptochrome concurrently furnish the task of circadian photoreception in *D. melanogaster* (Helfrich--Förster, 2002). In $norpA^{P41};cry^{b}$ double mutant flies both rhodopsin and cryptochrome mediated phototransduction is blocked into the circadian clock (Stanewsky *et al*., 1998; Helfrich--Förster *et al*., 2001). However, this double mutant flies are still able to entrain to LD, although it takes them much longer time to synchronize their behavior to shifted LD (i.e. an experimentally--induced jet--lag situation (Stanewsky *et al*., 1998; Emery *et al*., 2000b)). Therefore, in $norpA^{P41};cry^{b}$ double mutant flies the circadian clock is not absolutely blind. This indicates that the circadian clock receives light input through additional, extra--retinal photoreceptors/photopigments independent of *norpA* and *cry*. Moreover, in $norpA^{P41};cry^{b}$ flies the molecular synchronisation of PER and TIM expression in the s--LN_vs and in the DN_1s by light is still possible (Helfrich--Förster *et al*., 2001), again pointing to an alternate pathway(s) for the light--dependent TIM degradation in addition to the known CRY--dependent TIM degradation mechanism.

5.3 Where to look for a novel circadian photoreceptor in *Drosophila*?

The residual behavioral and molecular synchronization retained in the $norpA^{P41};cry^{b}$ flies was eliminated by combining gl^{60j} with cry^{b}, thus the gl^{60j} cry^{b} double mutant flies were absolutely circadian blind (Helfrich--Förster *et al.*, 2001). What additional photoreceptive structures were affected by the gl^{60j} mutation? As mentioned above, the H--B eyelet and the DN_1s are additionally removed in gl^{60j} flies. Therefore, these structures *per se* pose as the possible candidates to mediate light signaling into the circadian clock in the $norpA^{P41};cry^{b}$ flies.

The H--B eyelet qualifies as one of the relevant candidates for mediating residual light signals into the s--LN_vs for 2 additional reasons: 1) It has direct neural projections towards the s--LN_vs (Fig. 2) (Hofbauer and Buchner, 1989; Yasuyama and Meinertzhagen, 1999; Malpel *et al*., 2002), which retained molecular

synchronization of PER and TIM in *norpA^{P41};cry^{b}* flies (Helfrich--Förster *et al*., 2001). The H--B eyelet sends direct neural projections towards the s--LN$_v$s implicating a role for the former in the circadian function. 2) The H--B eyelet expresses the *Rh 5* and *Rh 6* genes (Yasuyama and Meinertzhagen, 1999; Malpel *et al*., 2002). Since it expresses these photopigments, which absorb in blue and green lights and are involved in the circadian photoreception (Frank and Zimmermann, 1969), the H--B eyelet might be playing a role in the circadian photoreception.

Similarly, the DN$_1$s possibly host an additional extra--retinal photoreceptor/photopigment. The DN$_1$s also send axonal projections toward the s--LN$_v$s (Fig. 2) like the H--B eyelet (Kaneko and Hall, 2000). Possibly, the DN$_1$s can entrain the s--LN$_v$s through this anatomical pathway (Helfrich--Förster *et al*., 2001). It is inferred that the DN$_1$s play a role in LD entrainment because *disconnected* mutant flies, which lack the LN$_v$s but not the DN$_1$s, are able to entrain to LD (Zerr *et al*., 1990; Hardin *et al*., 1992; Helfrich--Förster, 1998, Blanchardon *et al*., 2001). Like the s--LN$_v$s, the DN$_1$s also retained synchronized molecular cycling in *norpA^{P41};cry^{b}* flies (Helfrich--Förster *et al*., 2001).

Yet another promising candidate is a novel putative rhodopsin gene hereon called *rhodopsin 7* (*Rh 7)* (Adams *et al*., 2000). If *Rh 7* indeed turns out to encode a functional opsin, it may represent a novel circadian photopigment. Its predicted amino acid sequence shows a maximum 30.7% homology to *Drosophila* rhodopsin *Rh 5. Rh 7* also shows a 12.5% homology with the retinal photoisomerase from the Japanese squid. The predicted protein of *Rh 7* encodes for a G protein coupled photoreceptor like other *Drosophila* rhodopsins (FlyBase).

Although the H--B eyelet, the DNs and *Rh 7* pose as potential candidates for an elusive circadian photoreceptor/photopigment, it is equally possible that a totally unorthodox molecule effects the residual circadian entrainment in the *norpA^{P41};cryb* flies. A novel mutant, *Veela,* is drastically disabled to perceive/transmit the constant light signal into the circadian clock similar to *cry^{b}* phenotype. Therefore, possibly *Veela* could define such an unorthodox photoreceptor/photopigment. Furthermore, it also possible that a functional *Veela*

molecule may be participating either upstream or down stream of CRY in mediating the light signals to TIM in the circadian photoreception pathway. It is equally possible that any of the proposed candidates alone or any combinations of them may be mediating the residual light entrainment in the *norpA^{P41};cry^{b}* flies. Finally, the possibility always remains open for a totally novel molecule to be discovered, which may function as a novel circadian photopigment/photoreceptor.

In search for a novel circadian photoreceptor in *D. melanogaster*, all four above possibilities were systematically investigated by applying behavioral, neurogenetic, immuno--histochemical and molecular biological approaches. In the following chapters I shall elaborate on my efforts to identify and localize such an elusive novel circadian photoreceptor in *D. melanogaster*.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Chapter 2

General Materials and Methods

1 Materials

1.1 Fly stocks

norpA^{P41} (Pearn *et al.*, 1996).

The *norpA*^{P41} used here was in *w* background, hereon called only *norpA*^{P41}.

norpA^{P41}*;cry*^{b} (Stanewsky *et al*., 1998).

Df(1) y w (Lindsley and Zimm, 1992), hereon termed *y w.*

y w;cry^{b} (Stanewsky *et al*., 1998), hereon called *cry*^{b}*.*

Other fly stocks used are mentioned in the relevant chapters.

1.2 Fly foods

1.2.1 Yeast--Corn--Agar *Drosophila*--medium

Potable tap water	1 l
Agar	8 g
Rüben Sirup	22 g
Malt extract	80 g
Beer Yeast	18 g
Soya meal	10 g
Corn meal	80 g
Nipagin (mold inhibitor)	3 g

1.2.2 Sugar--Agar medium (Food for locomotor behavioral experiments)

Sucrose 4 g

Bactoagar 2 g

Made up to 100 ml with dH_2O

1.2.3 Luciferin fortified fly food for bioluminescence assay

Sucrose 5 g

Bactoagar 1 g

15 mM luciferin

Made up to 100 ml with dH_2O

1.3 Antibodies for immuno--histochemistry

1.3.1 Primary and secondary Antibodies

The following primary antibodies were used for immuno--histochemical studies.

Polyclonal rabbit anti--PER (Stanewsky *et al*., 1997b).

Polyclonal rat anti--TIM (Kaneko *et al*., 1997).

The application of the following secondary antibodies enabled simultaneous visualization of anti--PER and anti--TIM staining by fluorescence microscopy.

Anti--rabbit: AlexaFluor 488, green (Molecular Probes)

Anti--rat: AlexaFluor 594, red (Molecular Probes)

1.3.2 Other reagents for immuno--histochemistry

Normal Donkey Serum (Jackson ImmunoResearch Inc.)

Vectashield (Vector Laboratories Inc.)

1.4 Solutions for histological procedures

1.4.1 PBS 10X

$NaH_2PO_4.H_2O$ 2.83 g

$Na_2HPO_4.2H_2O$ 13.74 g

NaCl 90.00 g

Made up to 1 l with deionized water

1:10 dilution in deionized water for 1X working solution.

1.4.2 PBT (0.3%)

Triton--X100 3 ml

PBS 10X 100 ml

Made up to 1 l with deionized water to get 1X PBT.

1.5 Chemicals, reagents, kits and enzymes

Item	Source of supply
Expand High Fidelity PCR System	Roche
Restriction enzymes, T4--Ligase, Calf Intestine Alkaline Phosphatase (CIAP), 1 kb DNA ladder	Invitrogen
Oligonucleotides, RT--SuperScript III--Kit	Invitrogen
QIAquick PCR Purification Kit , QIAquick® Gel Extraction Kit, QIAGEN® Plasmid Midi Kit	Qiagen
BigDye®Terminator v1.1 Cycle Sequencing kit	AB Applied Biosystems
dNTP set	Roth
PeqGOLD Trifast	(PeqLab)
All other chemicals, salts and solutions	Amersham Pharmacia, BioRad, Biozym, Difco, Eurobio, Fluka , Merck, Roth.
Paraformaldehyde	Electron Microscopy Sciences
Luciferin	Biosynth
H_2O	Cleaned and deionized with the System Milli Q Plus, Millipore

1.6 Instruments

1.6.1 Forceps

For micro dissection of the *Drosophila* brain, fine quality forceps were used (Forceps No. 5; Dumont™, Switzerland).

1.6.2 Microscopes

The following microscopes were used for microscopic examinations and/or dissections.

Leitz Wetzlar compound light microscope

Leica TCS NT Confocal Microscope

Zeiss stemi 2000 compound light microscope

Zeiss Meta 510 Confocal Microscope

1.6.3 PCR machines

Whatman Biometra® TGardient (Bimetra)

Stratagene® -Robocycler Gradient 40

1.6.4 Electroporator

Stratagene Electroporator 1000

1.6.5 Scintillation Counter

TopCount Multiplate Scintillation Counter (Packard)

1.7 Data management tools and web resources

Standard Softwares from Microsoft® and Adobe Photoshop 7, CorelDRAW11 were employed.

DNASTAR V 5.03 (DNASTAR Inc., USA)

DNAMAN 4.0 (Lynnon Biosoft, Quebec, Canada)

Chromas 1.45 (Conor McCarty, School of Health Science, Griffith University, Australia)

LSM 5 Image Browser V 3.2.0.115 (Carl Zeiss GmbH, Germany)

MultAlin v 5.4.1, online free software at http://www.toulouse.inra.fr/multalin.html (Corpet, 1988)

National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/

Flybase http://flybase.bio.indiana.edu

Blast http://www.ncbi.nlm.nih.gov/BLAST/

2 Methods

2.1 Maintaining the flies

The flies were maintained on Yeast--Corn--Agar *Drosophila*--medium. The flies were housed in glass tubes in a controlled environment (25°C or 18°C, and at 65% relative air humidity; the lighting for the room was set to regular cycles of 12 h: 12 h light--dark, 7:30 a.m. light 'on' and 7:30 p.m. light 'off').

2.2 Genetic crosses

The genetic crosses performed for particular purpose are depicted as pictures in the respective chapters.

2.3 GAL4/UAS system

The GAL4/UAS system was used to express a gene of interest (e.g. tetanus toxin, τ--GFP) in a targeted cell or tissue under the control of a gene promoter (e.g. *Rh 7 or Rh 5* promoter or *Pdf* promoter), which expresses in the desired target cell or tissue (Brand and Perrimon, 1993). This system works based on the principle that a promoter of interest drives expression of the GAL4 sequences, which then goes and binds to the yeast Upstream Activator Sequences (UAS) and activates the transcription of a gene of interest fused down stream of UAS (Brand and Perrimon, 1993; Fig. 1).

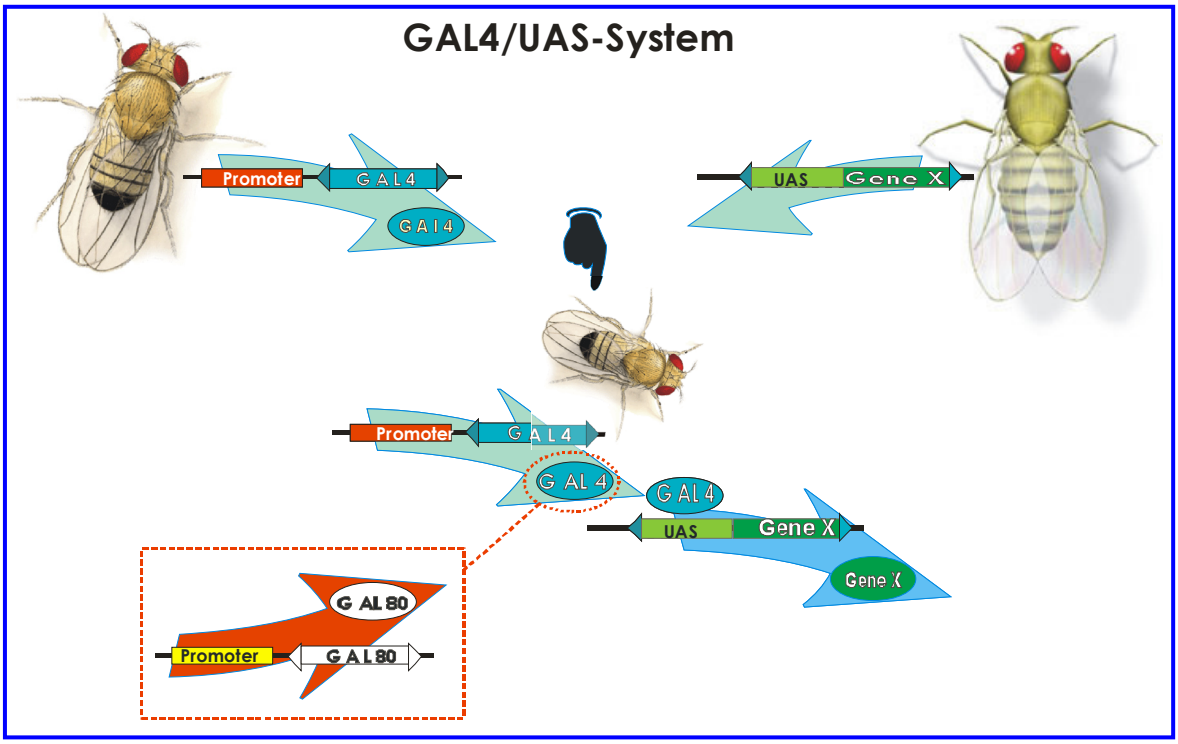

Figure 1 Principle of the GAL4/UAS system. A first transgenic fly carries a target cell or tissue specific gene promoter in front of the GAL4 sequences and a second transgenic fly carries a gene of interest (Gene X) down stream of Upstream Activating Sequences (UAS). A cross between these two transgenic flies produced progenies, which have both GAL4 and UAS elements. In the cells where the promoter is active, it drives the expression of GAL4, which binds to the UAS sequences and activates expression of the gene of interest. In the figure top left and right flies are male and female virgin, respectively. Expression of GAL4 and GAL80 elements together in the same cells blocks

GAL4 action on UAS element, which results in halting of UAS mediated expression of a gene of interest.

The action of GAL4 elements could be blocked by simultaneously expressing GAL80 elements in the same cells (Fig. 1). GAL80 binds to GAL4 and blocks the latter's action on UAS elements. This enables for targeting the clock neurons in a subtracting strategy as used by Stoleru *et al*. (2004). They restricted expression of an apoptotic gene in the DN_1s and LN_ds and in the fifth s--LN_v cell by using *cry* promoter to drive GAL4 and blocking its action in the LN_vs by expressing GAL80 under the control of *Pdf* promoter.

2.4 Behavioral locomotor activity testing and analyses

The locomotor activity rhythms were monitored as described in Hamblen *et al*. (1986) and Hamblen--Coyle *et al*. (1992). Three to four days old male flies were briefly anaesthetized by CO_2 and each fly was loaded into a glass tube (65 mm length, 5 mm diameter). The glass tubes were filled 2--2.5 cm with food (sugar--agar medium). The "food--end" of the tube was sealed by paraffin and the other end was plugged by cotton, after the fly was loaded. Tubes were placed into a *Drosophila* Activity Monitor (32 tubes/monitor), which was then placed in an incubator (illuminated by ~300--400 lux white light but for testing flies, which were expressing tetanus toxin in the H--B eyelet, 65 lux white light was used ;25°C).

The entrainment conditions employed for specific purposes are described in the respective chapters. Generally, flies were first entrained for 5--8 days in 12 h: 12 h LD, followed by 5--8 days of DD/LL. For light shift experiments, following the initial LD, the lights came 'on' 6 h later (light shift, LS) compared to the initial LD. Finally, flies were released into constant darkness (DD) for 5 days.

Locomotor activity was monitored and data acquisition was done automatically by the DAM system (Sulzman, 1986; Hamblen *et al*., 1986). The collected data were transferred from the Apple computer to a P C. Data were analyzed according to Hamblen *et al*. (1986) using *The Brandeis Rhythm Package Analyses software*, http://hawk.bcm.tmc.edu or with the help of MATLAB R 12 (Levine *et al*., 2002b).

2.4.1 Actogram

Actogram represents the raw activity data obtained from individual fly over the experimental time. It is plotted as an event record. The strength of the event record in the actogram can be manipulated by using the function called hash density, which defines the number of times a fly needed to cross the infra red beam for recording as an event (e.g., a hash--density of 25 means that the fly should cross 25 times the infra red beam to register as one event). The locomotor activity for individual flies for each day was 'double plotted', so that the subsequent days were plotted side by side (see for example Fig. 4 in Chapter 3). It helps to visualize better the activity rhythm. The locomotor activity data were collected every 30 min (cf. Hamblen--Coyle *et al*., 1992).

Actograms for the entire duration of the experiment were generated for each fly and were used to visually inspect whether a fly behaved rhythmic or arrhythmic in LD, DD and LL. The strength of rhythmicity for each fly was separately estimated for LD, LL and DD employing χ^2 periodogram and autocorrelation analyses (see for example Table 1 and Table 2 in Chapter 6).

2.4.2 χ^2 periodogram

This paradigm detects any periodic recurrence of activity in the given data. It uses the Fast Fourier Transformation (FFT), which decomposes the data signal into sinusoidal waves and determines the period of the rhythm as well as its robustness (Klarsfeld *et al*., 2003).The range of period to be detected can be specified by the user (like 15 h to 30 h). The program presents the output both in form of a spectral graphics and as numerical data. The significance of a period detected by the periodogram can be estimated by the combination of two criteria. Period estimates with 1) 'Power' values (height of the χ^2 periodogram peak above the 5% significance line, in arbitrary units) greater or equal to 10 in conjunction with 2) 'Width' values (number of period values in 0.5 h increments above that line) greater or equal to 2 were considered to be rhythmic (Kaneko *et al*., 2000). Periodogram analysis was performed on data sets extending from 4--6 days (Sokolove and Bushnell, 1978).

2.4.3 Autocorrelation

This function is based on the relationship between the values of a signal and the same signal some time later, and gives information about the periodicity in the signal (Levine *et al*., 2002b). The autocorrelation is an analytical tool used for evaluating the rhythmic component in a data set (Levine *et al*., 2002b). A rhythmic component above the cut--off line (see in Chapter 6 Fig. 4, the shaded area in the autocorrelogram) with a rhythm index (RI) of > 0.17 indicated a significant rhythm.

2.5 Histological and immuno--histochemistry protocols

2.5.1 Initial LD entrainment

Flies were generally entrained to 12 h: 12 h LD cycles initially (in required light intensities and the temperature was maintained at 25°C) for at least 3 days. Following the LD, as per requirement light shift (LS) or constant darkness (DD) or constant light (LL) were employed for another 5--days. For staining flies were selected at defined circadian time in LD or LL or DD.

2.5.2 General protocol for fixing, dissecting, staining and mounting

Flies were collected and anaesthetized by CO_2 at defined time--points before fixing. For dissection the proboscis was pulled out with forceps, subsequently the head case was opened (all this was done under a Zeiss stemi 2000 compound light microscope), quickly rinsed in 1X PBS (phosphate buffered saline) and the flies were immediately placed in an Eppendorf tube (2 ml) containing 4% paraformaldehyde, pH 7.4 (prepared in 0.1% PBT--PBS with 0.1% Triton X--100) for fixing. Fixing was performed for 5 min at room temperature (RT), followed by 5 min on ice. Subsequently, the fixing solution (fix) with flies was mixed on a shaker for 20 min at RT. After that, the fix was discarded and the flies were rinsed with 1X PBS. Brains were dissected out, cleared from trachea with the help of fine forceps under a Leitz Wetzlar compound light microscope. Specimens were placed back into fix and mixed on a shaker for another 15 min at RT, subsequently fix was discarded and brains were rinsed in 1X PBS for 5 min (on ice). Brains were then incubated in 0.1% PBT for 15 min and rinsed in 1X PBS for 5 min, followed by blocking in normal donkey serum (NDS--2% in 0.3% PBT) for 30 min. After washing in 0.3% PBT, primary antibodies were added at a dilution of 1:1000 (diluted in 0.3% PBT), for both rabbit anti--PER serum (preabsorbed before dilution against per^{01} embryos; Stanewsky *et al*., 1997b) and rat anti--TIM, and

incubated at 4°C for 48 h. Brains were washed in 0.3% PBT (5X 15 min) and secondary antibodies, diluted to 1:200 (diluted in 0.3% PBT), were added (anti--rabbit: AlexaFluor 488 green; anti--rat: AlexaFluor 594 red) and the mixture was incubated at 4°C overnight. Excess secondary antibodies were washed off by 0.3% PBT (5X 15 min). Finally, brains were washed with PBS and placed in 0.1% PBT before being mounted in Vectashield.

Other methods used for this study are described in the relevant chapters.

CHAPTER 3

THE H--B EYELET--A CIRCADIAN PHOTORECEPTOR FOR PHASE SETTING IN TWILIGHT?

Chapter 3

The H--B eyelet--a circadian photoreceptor for phase setting in twilight?

1 Introduction

The circadian clock in *Drosophila melanogaster* is synchronized by the environmental LD cycles. Light signals captured by rhodopsins and cryptochrome essentially assure the synchronization of the circadian clock (Helfrich--Förster, 2002; Hall, 2000). With the help of $norpA^{P41};cry^{b}$ double mutation it is possible to simultaneously block light signals from both compound eyes and CRY to enter the circadian clock. This affects synchronization drastically, but does not make the circadian clock absolutely blind (Helfrich--Förster *et al*., 2001; Stanewsky *et al*., 1998; Emery *et al*., 1998, 2000b). This suggests existence of an additional extra--retinal photoreceptor. At the molecular level, synchronization of PER and TIM oscillations in the s--LN_vs, the DN_1s, (and to a certain extent also in the LN_ds) is still possible in the double mutants, pointing to alternate pathways for light--dependent TIM degradation in addition to the known CRY--dependent mechanism (Helfrich--Förster *et al*., 2001). The above mentioned behavioral and molecular synchronization was absent in gl^{60j} cry^{b} flies. They are disabled in CRY mediated signaling, like in $norpA^{P41};cry^{b}$ flies. The gl^{60j} cry^{b} flies further miss the compound eyes and the ocelli, which are already non--functional in $norpA^{P41};cry^{b}$ flies. But compared to $norpA^{P41};cry^{b}$ flies the gl^{60j} cry^{b} flies additionally miss the Hofbauer--Buchner (H--B) eyelet and the DN_1s (Helfrich--Förster *et al*., 2001). [The $norpA^{P41}$ mutation does not remove any photoreceptor structures but only results in phototransduction block whereas gl^{60j} removes photoreceptor structures like compound eyes, ocelli as well as the H--B eyelet and the DN_1s (see more in Chapter 1 section 5.1)]. The H--B eyelet is a group of 4 cells, with characteristic features of photoreceptor, found near the posterior border of the adult eye between the retina and lamina (Fig. 1; Hofbauer and Buchner, 1989; Yasuyama and Meinertzhagen, 1999). It sends axons towards the LN_vs (Fig. 1; Hofbauer and Buchner, 1989; Yasuyama and Meinertzhagen, 1999; Malpel *et al*., 2002; Helfrich--Förster *et al*., 2002). Therefore, the H--B eyelet stands as a potential candidate

hosting the elusive photoreceptor, responsible for the residual light re--entrainment retained in the *norpA*^{P41};*cry*^{b} flies.

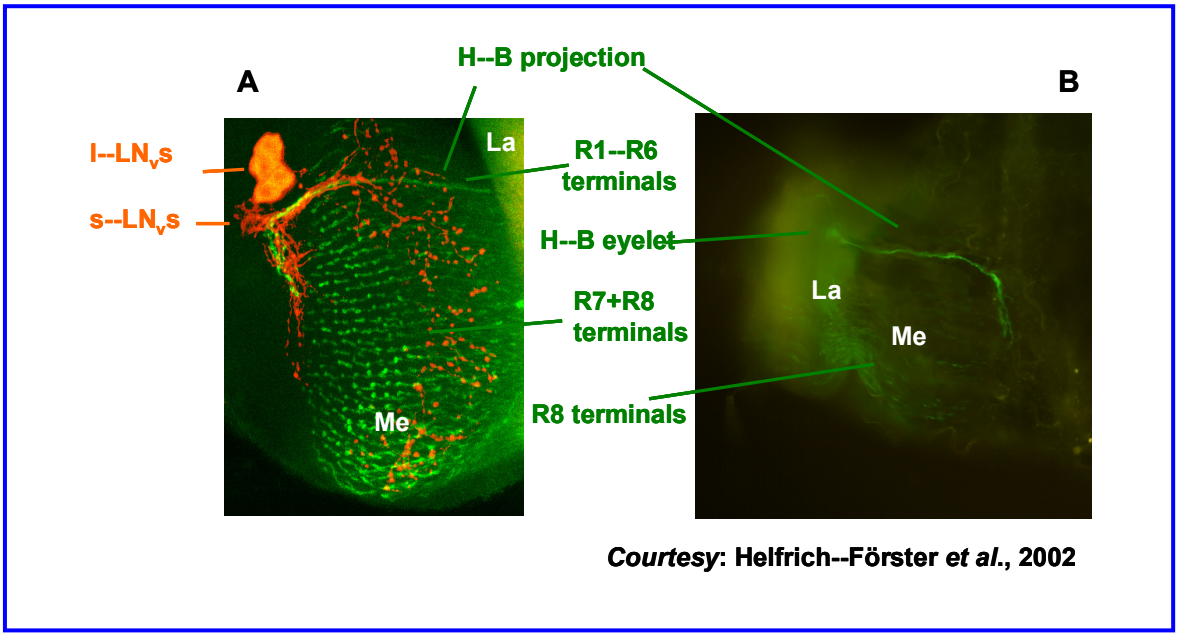

Figure 1 The H--B eyelet projection terminating close to the lateral neurons. (A) The green staining represents the photoreceptors (*gmr*--driven expression of GFP) and red staining represents lateral neurons and their optic lobe arborizations (anti--PDF). (B) *Rh 6* driven GFP expression in the H--B eyelet and its projection towards the lateral neurons position. R1--R8 are the photoreceptor cells. R8 cells are *Rh 6* positive. *La* and *Me* mean lamina and medulla, respectively.

The Bolwig organ (BO) is the precursor of the adult H--B eyelet (Malpel *et al*., 2002; Helfrich--Förster *et al*., 2002). It functions as a larval visual system in *Drosophila* and consists of a pair of 12 cells. Disappearance of BO is followed by the appearance of the H--B eyelet. Both of them express *Rh 5*, *Rh 6* and *norpA* encoded PLC (Malpel *et al*., 2002). The BO projects fibres towards the larval LNs, like the H--B eyelet does in the adult (Malpel *et al*., 2002; Helfrich--Förster *et al*., 2002). BO mediates light entrainment of the circadian clock in third instar larva (Busto *et al*., 1999). Quite recently, it has shown that the pacemaker neurons receive light input from the BO, and it plays a role in resetting the circadian clock by sending signals to the pacemaker neurons. BO is a sensory system without a molecular clock. It transmits rapid photophobic signal to the pacemaker neurons (Mazzoni *et al*., 2005). This means that the BO can transmit photic signal to the pacemaker neurons to affect the behavior. Therefore, possibly the H--B eyelet also

could function similar way to affect the pacemaker neurons. Further, it has been shown that the Bolwig's nerve is required for the development or the maintenance of the dendritic arborizations of the larval LNs (Malpel *et al*., 2002).

A role for the H--B eyelet was implicated in synchronization to extreme photoperiods, i.e. it seems to impart a kind of "after--effects" on the internal clock following entrainment in long day photoperiods (Rieger *et al*., 2003). In 20:4 LD, so^{1} flies (which lack the compound eyes and ocelli, but retain the H--B eyelet, the DN_1s, and cryptochrome) mostly free ran with a significantly longer period (24.9 h). In DD, they showed free running behavior with a longer period, indicative of the last photoperiod they experienced. In 20:4 LD, flies lack the compound eyes, ocelli and defective in the H--B eyelet were unable to light entrain, which was more affected when cry^{b} mutation was combined to the former mutants. However, the H--B eyelet alone is a less effective circadian photoreceptor compared to the compound eyes and cryptochrome (Helfrich--Förster *et al*., 2002; Rieger *et al*., 2003). In a 6 h LD phase advance $so^{1};gl^{60j}$ flies showed orthodromic phase shifting (by advancing 6 h the activity rhythm in the same direction of LD shift) but so^{1} flies showed anti--dromic phase shift (by delaying 6 h the activity rhythm in the opposite direction of LD shift), i.e. similar to a phase delay. This anti--dromic phase shift occurred mainly at 480 nm, the putative sensitivity maximum of the H--B eyelet photopigment. Whereas in a minority of $so^{1};gl^{60j}$ flies anti--dromic phase shift occurred at 420 nm. Therefore, a role for the H--B eyelet was proposed in phase delays but this behavior is explicit only in absence of the compound eyes (Helfrich--Förster *et al*., 2002).

The hypothetical role for the H--B eyelet in circadian synchronization of $norpA^{P41};cry^{b}$ flies was tested by blocking the putative synaptic transmission from it to the s--LN_vs. The Tetanus--Toxin Light Chain (TxTxLC) blocks chemical synaptic transmission by cleaving the neuronally expressed synaptobrevin protein (Sweeny *et al*., 1995; Martin *et al*., 2002). Kaneko *et al*. (2000) have shown that tetanus toxin expressed in the clock cells under control of *per*--GAL4 or *tim*--GAL4 caused abnormal behavioral rhythms. However, expression of tetanus toxin under the control of *Pdf*--GAL4 promoter showed reduced behavioral abnormalities suggesting that the synaptic transmission from the s--LN_vs to the dorsal

protocerebrum may not be blocked by tetanus toxin. For targeted expression of tetanus toxin in the H--B eyelet the GAL4/UAS binary system was adopted (see more in Chapter 2 Methods, section 2.3; Brand and Perrimon, 1993). The *Rh 5* promoter drives the tetanus toxin expression in the H--B eyelet (Mealey--Ferrara *et al*., 2003) because *Rh 5* gene is active there (Malpel *et al*., 2002). After blocking the neural transmission from the H--B eyelet to the s--LN_vs, we analyzed the locomotor behavioral rhythms during LD and jet--lag conditions. We further examined the PER and TIM expression in the clock neurons of these flies.

Here I shall demonstrate that indeed the H--B eyelet plays a role in the circadian photoreception, probably as a low intensity light sensor required for resetting the circadian clock after a phase delay. In dim light blocking the neural signals from the H--B eyelet resulted in non--synchronized free running behavior and non--synchronized molecular cycling of TIM in the pacemaker neurons of *Drosophila*.

2 Materials

2.1 Flies

norpA^{P41};Rh5--GAL4*/CyO;cry^{b}* (Mealey--Ferrara *et al*., 2003).
*norpA^{P41};*UAS--*ttx;cry^{b}* (Mealey--Ferrara *et al*., 2003).

3 Methods

3.1 GAL4/UAS system

The GAL4/UAS system was used to express the tetanus toxin under the control of *Rh 5* promoter using the *norpA^{P41};Rh 5*--GAL4*/CyO;cry^{b}* and *norpA^{P41};*UAS--*ttx;cry^{b}* transgenics (Mealey--Ferrara *et al*., 2003; Brand and Perrimon, 1993). See in Chapter 2 Methods, section 2.3 for more about the GAL4/UAS system.

3.2 Genetic crosses

To bring the GAL4 and UAS elements together, the following cross was performed (Fig. 2).

P:

$$\frac{w\ norpA^{P41}}{w\ norpA^{P41}}\ ;\ \frac{Rh5-\mathrm{GAL4}}{CyO}\ ;\ \frac{cry^{b}}{cry^{b}}\quad\times\quad\frac{w\ norpA^{P41}}{Y}\ ;\ \frac{\mathrm{UAS}-ttx}{\mathrm{UAS}-ttx}\ ;\ \frac{cry^{b}}{cry^{b}}$$

F1:

$$\frac{w\ norpA^{P41}}{Y}\ ;\ \frac{Rh5-\mathrm{GAL4}}{\mathrm{UAS}-ttx}\ ;\ \frac{cry^{b}}{cry^{b}}$$

Figure 2 Genetic cross between *Rh 5*--GAL4 female (virgin) and UAS--*ttx* male flies to bring together the GAL4 and UAS elements. The *Rh 5*--GAL4 and UAS--*ttx* transgenic flies were balanced with *CyO*. In the F1 generation flies without *CyO* balancer were selected for further studies. Four to five days aged male flies from the F1 generation were selected for preparing whole mounted brains.

3.3 Behavioral testing and analyses

The locomotor activity rhythms were monitored as described in Hamblen *et al*. (1986) and Hamblen--Coyle *et al*. (1992). For more details see in Chapter 2 Methods, section 2.4. The behavioral testing incubator was illuminated by 65 lux white light and maintained at 25°C. The flies were first entrained for 5--8 days in 12 h: 12 h LD, followed by 5 days of LD where the lights came ‘on’ 6 h later (light shift, LS) compared to the initial LD. Finally, flies were released into constant darkness (DD) for 5 days, see Fig. 3.

Actograms for the entire duration of the experiment were generated for each fly and were used to visually inspect (see below the protocol for a blind qualitative analysis) whether a fly qualified for re--entrainment to a 6 h phase delay or not. The resynchronization of the morning and/or evening peak with the new LD regime was regarded a sign of re--entrainment. Rhythm strength for each fly, separately for LD, LS and DD, was estimated by employing χ^2 periodogram analyses (see Table 2).

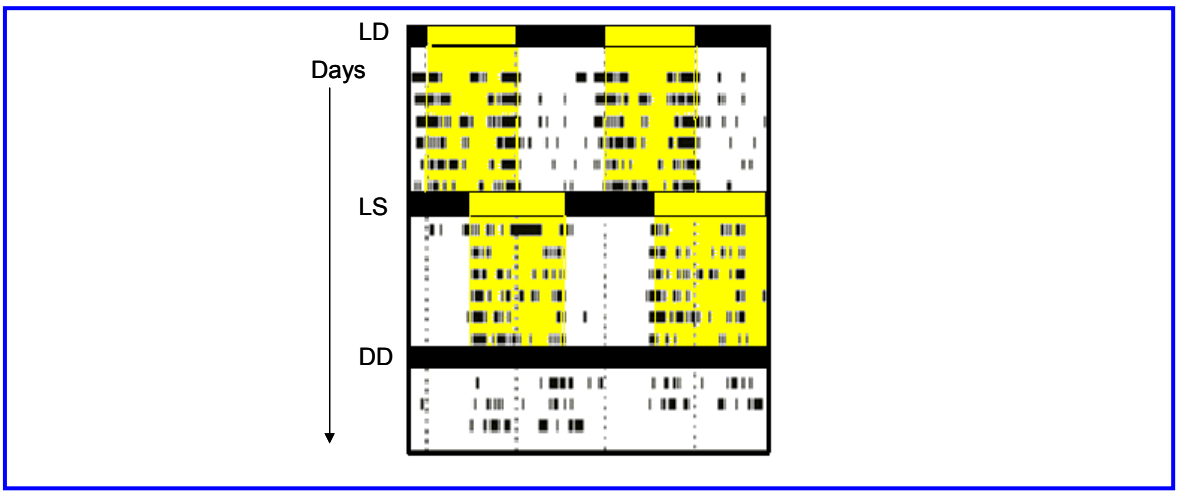

Figure 3 Schematic view of an actogram showing 6 h phase delayed behavioral light shift experiment where the original 12 h: 12 h LD cycle was shifted by 6 h after 6 day in the initial LD regime. During the first 5--6 days the fly was subjected to a 12 h: 12 h light--dark (LD) cycles. The yellow shade indicates when the lights were 'on' (65 lux) and a white field indicates lights 'off'. The locomotor activity of the fly shows a typical bimodal pattern, i.e. more activity (black vertical stripes) around light 'on' and light 'off'. After the initial LD regime, light was switched on 6 h later (LS) compared to the previous day and flies were tested in the newly phased 12 h:12 h LD cycle. After 6 days flies were released into constant darkness (DD). The horizontal bars--either alternate black and yellow or full black--indicate light 'off' and 'on', or continuous darkness.

3.4 Behavioral re--entrainment: Blind Test protocol

The following procedure was employed for inspecting whether a fly re--entrained or not, and if the fly re--entrained, how many days needed to re--entrain to the 6 h phase delayed LD of 65 lux.

1) Three individual persons were given the actograms for 'blind' inspection (there was no information on the actograms about the genotype). They were asked to inspect and judge whether a fly re--entrained to the 6 h phase delayed LD regime or not. The flies were first classified either as re--entrained or not re--entrained (see Table 1). For each individual fly that re--entrained, the inspector assigned a 'value', representing the number of days a fly needed to re--entrain to the 6 h phase delayed regime.
2) If two persons out of three judged that a fly had re--entrained, it was classified as re--entrained.

3) An average value (number of days needed to re--entrain) for each fly was obtained by averaging the values assigned by three (or 2) individual persons.
4) Individuals with the same genotypes from 4 different experiments were pooled after the step 3.
5) An average value for each genotype was calculated from the pooled data and tabulated in Table 1.

3.5 Immuno--histochemistry

Flies were entrained to 12 h: 12 h LD cycles (65 lux, 25°C) for at least 3 days; for light shift (LS) the LD entrained flies were subjected to a 6 h phase delayed LD with respect to the original LD cycle (see Fig. 2). Flies for staining were entrained in the same incubator, where the behavioral experiments were done. For further details on dissection and immuno--histochemistry see in Chapter 2 Methods, section 2.5.

3.6 Quantification of the neuronal staining

The number of clock neurons that stained for anti--PER and/or anti--TIM in individual brain halves was determined by manual counting using a confocal microscope. The average number of stained neurons for each neuronal group was determined for each time point. Then the numbers were normalized for individual subsets of clock neurons. The average number of neurons stained for each subset in the *y w* control flies at the peak time point (ZT 22) was set as 100% for the respective subset. From this value, for the corresponding subset of neurons in other genotypes, a value was calculated. Normally, the *y w* control flies had the highest score compared to the rest of the genotypes.

As a measure of light--synchronized expression of PER and TIM we determined an “amplitude of clock protein cycling”: The difference between the average number of neurons stained at ZT 22 (peak time point) and ZT 10 (trough time point) was calculated and plotted in Figures 9, 11,13,16 and 18 as “PER or TIM amplitude of cycling”. A strong positive value (Fig. 9) indicated that PER and/or TIM were synchronized to the new phase delayed LD regime and a small positive value or negative value (Fig. 11A, Fig. 13) indicated no synchronization to the new

LD regime. A small positive value means that PER and/or TIM expression were almost equal (i.e. desynchronized) at the two time--points investigated, therefore subtraction of ZT 10 value from ZT 22 resulted in an integer close to zero (i.e. close to the x--axis in histograms; Figures 9, 11,13,16 and 18). A negative value means that PER and/or TIM were expressed more at ZT 10 compared to ZT 22. Further, it indicates that the neurons retained the synchronization from their initial LD regime and were not resynchronized to the new shifted LD regime.

4 Results

4.1 Re--entrainment of locomotor rhythms in the H--B eyelet defective flies to a 6 h phase delayed LD (65 lux)

To test whether the H--B eyelet plays a role in circadian entrainment, we tried to block the putative synaptic transmission from the H--B eyelet to the s--LN_vs in *norpA*^{P41}*;cry*^{b} flies. *norpA*^{P41}*;cry*^{b} flies can synchronize to a new LD cycle, but need many days to do so (Stanewsky *et al*., 1998; Emery *et al*., 2000b; Helfrich--Förster *et al*., 2001). Thus simultaneous blocking of light signals from both the compound eyes and CRY affected synchronization drastically, but did not make the circadian clock absolutely blind. One possibility for this residual re--entrainment ability is that the circadian clock is getting light input from a putative circadian photoreceptor, the H--B eyelet because it projects axons towards the pacemaker neurons (Hofbauer and Buchner, 1989; Yasuyama and Meinertzhagen, 1999; Malpel *et al*., 2002). In an attempt to make the H--B eyelet non--functional, tetanus toxin was expressed in the H--B eyelet cells since this substance blocks synaptic transmission (Martin *et al*., 2002). For expressing tetanus toxin in the H--B eyelet the GAL4/UAS system was applied (Brand and Perrimon, 1993). The *Rh 5* promoter is active in the H--B eyelet (Malpel *et al*., 2002), therefore it was used to drive expression of tetanus toxin in the H--B eyelet by the GAL4/UAS system (see Fig. 2). The putative H--B eyelet transmission defective *norpA*^{P41}*;cry*^{b} flies (hereon called H--B^{TD}:*norpA*^{P41}*;cry*^{b}) were then tested for their capability to re--entrain to a 6 h phase delayed LD of 65 lux. In addition to its activity in the H--B eyelet the *Rh5*--GAL4 driver used here was earlier shown to express in 30% of R8 PRs (Pichaud and Desplan, 2001).

The *norpA^{P41};cry^{b}* flies were shown to take a very long time (5--10 days) to re--entrain into a new LD regime of 1000 lux (Emery *et al*., 2000b), but in a new LD regime of 16 lux blue light, *norpA^{P41};cry^{b}* flies showed all range of behaviors, i.e. 40% thoroughly re--entrained and 40% did not, 13% started free run in initial LD itself and 7% were arrhythmic (Stanewsky *et al*., 1998). In the present study, the H--B^{TD}:*norpA^{P41};cry^{b}* flies showed no difference in re--entrainment behavior compared to the *norpA^{P41};cry^{b}* flies in bright light of ~400 lux (i.e., thorough, but slow re-entrainment). An effect for the H--B^{TD}:*norpA^{P41};cry^{b}* flies was noticed in 50 lux green light: (Veleri, S. and Helfrich--Förster, C., unpublished observation) where they showed a defect in re--entrainment (data not shown). This raised the question whether the specific wavelength and/or low light intensity caused the observed defect in the H--B^{TD}:*norpA^{P41};cry^{b}* flies. Therefore, a similar experiment was repeated in 65 lux white light, and it was found that indeed the observed defect was due to low intensity light but not the wave length. Therefore, to assess the photic re--entrainment of a given fly, the time needed to re--entrain to a 6 h phase delayed LD cycle of 65 lux white light was determined. Flies were first exposed to 5--8 days of LD, followed by 5 days of a 6 h phase delayed LD, and finally released into 5 days of DD (Fig. 3 and Fig. 4).

In the initial LD regime, the different genotypes showed varying percentages of entrainment (Table 2), which were comparable to an earlier report (Stanewsky *et al*., 1998). In our experiments, 97% of the wild type (*y w*) and 100% *cry^{b}* flies showed entrainment compared to a 93% and 84%, respectively, reported earlier (Stanewsky *et al*., 1998). In the initial LD regime, the H--B^{TD}: *norpA^{P41};cry^{b}* flies were able to synchronize to the dim LD (Fig. 6B--column 3, first row). With regard to re--entrained or not (judged by 'blind' visual inspection) all the tested *y w* and *cry^{b}* flies re--entrained (100%) to the 6 h phase delay (Fig. 5A and Table 1). The transgenic and non--transgenic *norpA^{P41};cry^{b}* flies showed significantly lesser re--entrainment than the *y w* and *cry^{b}* flies (Fig. 4B, C; Fig. 5A and Table 1). 48% of the non--transgenic *norpA^{P41};cry^{b}* flies showed re--entrainment (Table 1). 36% and 56% of the transgenic *norpA^{P41};Rh 5*--GAL4*/CyO;cry^{b}* and *norpA^{P41};*UAS--*ttx;cry^{b}* flies showed re--entrainment in 6 h phase delay, respectively (Fig. 5A and Table 1). When data for transgenic and non--transgenic *norpA^{P41};cry^{b}* flies were pooled

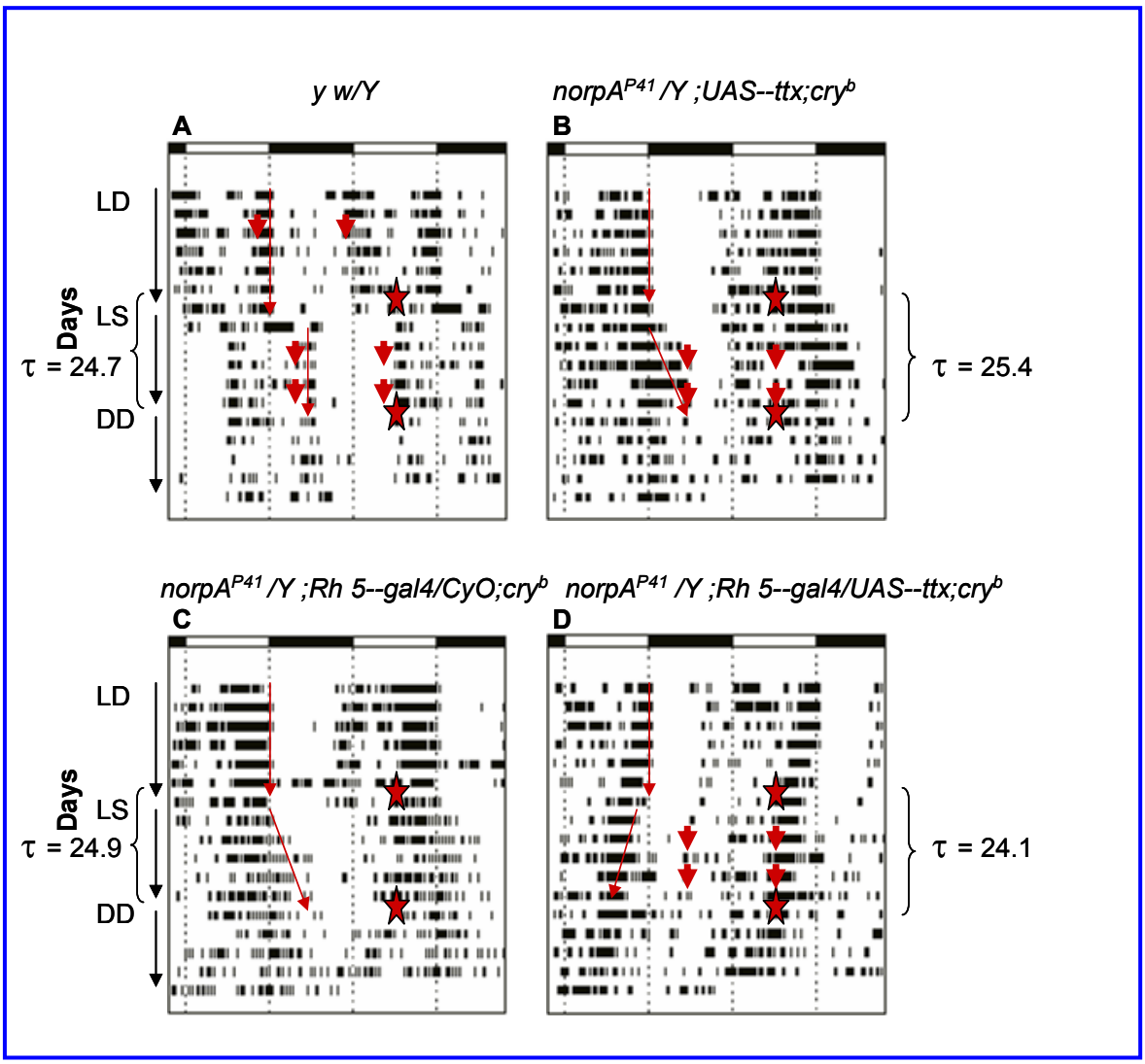

Figure 4 Actograms show that tetanus toxin expressed in the H--B eyelet in *norpA^{P41};cry^{b}* double mutant flies affect their capability to re--entrain to a 6 h phase delayed LD cycle with 65 lux white light. The flies were first entrained to 12 h: 12 h LD cycles (65 lux) for eight days. On the day 8 the LD cycle was delayed by 6 h (LS). After five days in the new 12 h: 12 h LD regime flies were released into DD. The upper and lower red stars in each actogram indicate time of the LS and DD, respectively. The thin red lines indicate progress of the evening peak during (re)--entrainment; quick re--entrainment is represented by a vertical line; a gradual re--entrainment is represented by a sloppy line (slanting towards right, indicating a τ >24 h); and no re--entrainment is indicated by a sloppy line (slanting towards left, indicating a $\tau \leq 24$ h). The red bold arrows indicate the time points at which flies were sacrificed to perform anti--PERIOD and anti--TIMELESS staining (all genotypes were sampled at the same time points). The upper (only in A), middle and lower rows of red bold arrows (in A and correspondingly in B, D) indicate third day in the initial LD, third day and fifth day in shifted LD, respectively (first and second arrows in each row indicate ZT 10 and ZT 22).

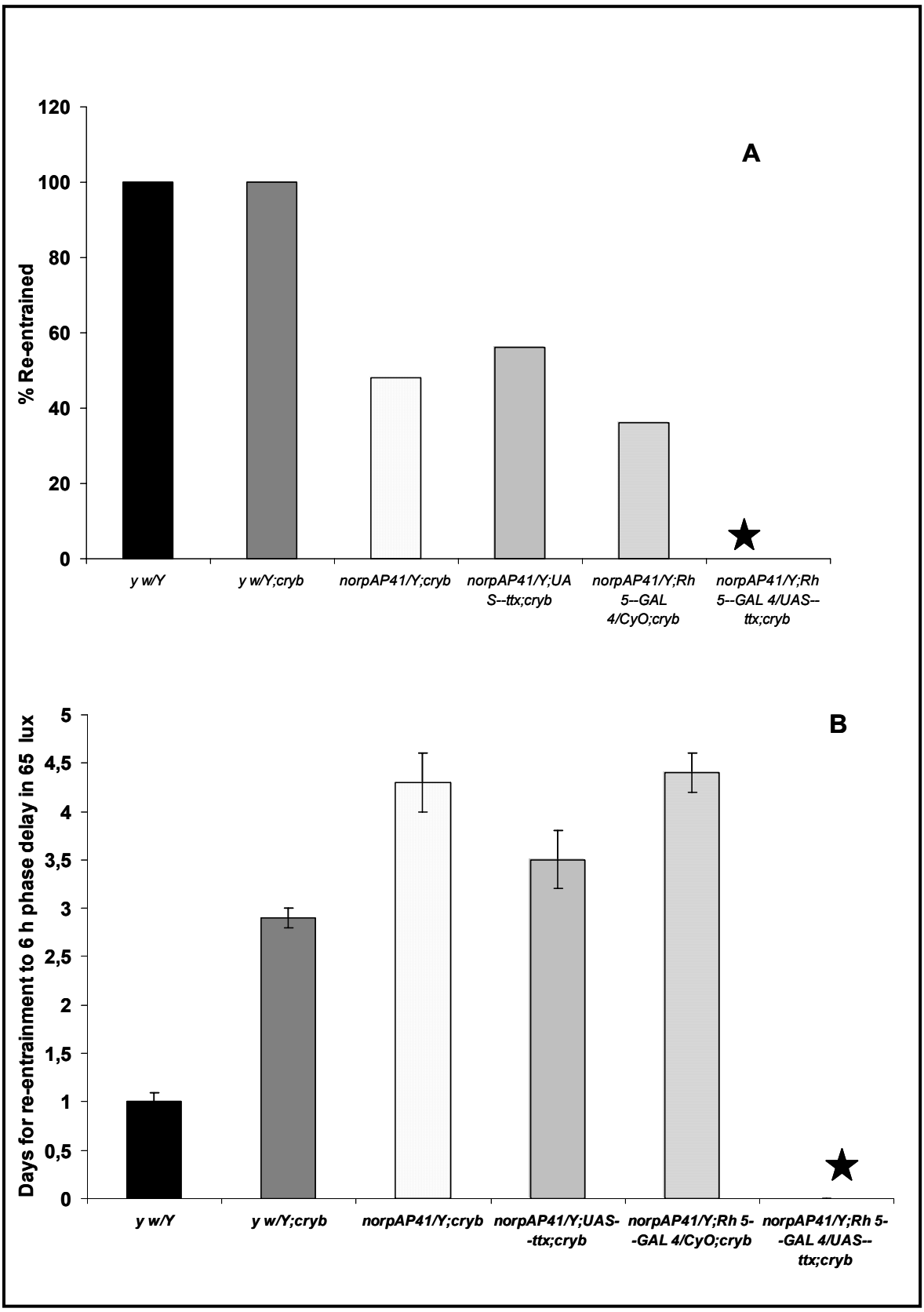

Figure 5 Quantification of locomotor activity behavior of *D. melanogaster* showing percentage of the flies re--entrained and the number of days needed for re--entrainment to a 6 h phase delayed LD cycle (65 lux white light). (A) Percentage of flies re--retrained: *y w*

flies showed 100 % re--entrainment whereas expression of tetanus toxin in a *norpA^{P41}; cry^{b}* background resulted in 100 % non re--entrainment (star). (B) Shows the number of days needed for re--entrainment in 6 h phase delay: *y w* flies needed less than a day for re--entrainment, in contrast tetanus toxin expressing *norpA^{P41}; cry^{b}* flies failed to re--entrain even after the 5th day (indicated by a star). (See in Methods, section3.4 for how the ability and time needed for re--entrainment was determined).

and analyzed, 47% were re--entrained (Table 1). In contrast to all the control flies mentioned above, none of the H--B^{TD}: *norpA^{P41};cry^{b}* flies showed re--entrainment in a 6 h phase delayed LD (Fig. 4D; Fig. 5A and Table 1). It seemed that they rather exhibited a free running behavior because 88% of them displayed a short period of 24.2 ± 0.1 h in the shifted LD regime compared to the control flies (Fig. 4D, Table 2). When the *norpA^{P41}* flies needed more time to re--entrain to a shifted LD, they show a period of >24 h, quickly re--entrained flies show a period close to 24 h and in free running conditions the period was <24 h (Helfrich--Förster *et al*., 2001).

With respect to the number of days needed for re--entrainment (judged by 'blind' visual inspection) all genotypes differed (Fig. 5B). The *y w* flies re--entrained very quickly (1 ± 0.1 day) to the 6 h phase delay (Fig. 4A; Fig. 5B and Table 1; cf. Emery *et al*., 2000b; cf. Helfrich--Förster *et al*., 2001). The *y w;cry^{b}* flies took 2.9 ± 0.1 days (Fig. 5B and Table 1; Helfrich--Förster *et al*., 2001) and the non--transgenic *norpA^{P41};cry^{b}* flies needed 4.5 ± 0.5 days (Fig. 5B and Table 1). The transgenic *norpA^{P41};*UAS--*ttx;cry^{b}* needed 3.3 ± 0.3 days and *norpA^{P41};Rh 5*--GAL4*/CyO;cry^{b}* flies needed 4.2 ± 0.3 days to re--entrain (Fig. 4B, C; Fig. 5B and Table 1). When data were pooled for transgenic and non--transgenic *norpA^{P41};cry^{b}* flies they needed on average 3.9 ± 0.2 days for re--entrainment (Table 1). The H--B^{TD}:*norpA^{P41};cry^{b}* flies completely failed to re--entrain (Fig. 4D; Fig. 5B and Table 1) and consequently the time needed for re--entrainment could not be calculated.

Since the H--B^{TD}:*norpA^{P41};cry^{b}* flies are assumed to be affected in the photic signaling to the LN_vs and the PDF expressing LN_vs contribute to the M peak (Stoleru *et al*., 2004), we decided to analyze a possible effect of the H--B eyelet specifically on M (and/or E) peak. We inspected the daily average activity plots

separately on the day 3 and last day of the 6 h phase delayed LD regime. In the initial LD regime the *y w* flies showed the typical bimodal activity pattern in LD (Fig. 6A; cf. Helfrich--Förster *et al*., 2001). The *cry*^{b} flies showed a weaker M peak than the *y w* flies (Fig. 6A; cf. Helfrich--Förster *et al*., 2001). All genotypes, including the H--B^{TD}:*norpA*^{P41}*;cry*^{b} flies were in a *norpA*^{P41}*;cry*^{b} genetic background, showed a higher activity pattern and their evening activity peak was more spread than the *y w* flies (Fig. 6A and Fig. 6B).

Table 1 Effect of tetanus toxin expression in the H--B eyelet of *norpA*^{P41}*/Y;cry*^{b} flies on behavioral re--entrainment in 65 lux

Genotype	**Total no. of flies tested (N)**	**% of flies re--entrained to 6 h phase delay (n)**	**No. of days needed for re--entrainment ± SEM**
y w/Y	39	100 (39)	1 ± 0.1
yw/Y;cry^{b}	12	100 (12)	2.9 ± 0.1
norpA^{P41}*/Y;cry*^{b}	21	48 (10)	4.5 ± 0.5
norpA^{P41}*/Y;*UAS--*ttx;cry*^{b}	40	56 (23)	3.3 ± 0.3
norpA^{P41}*/Y;Rh 5--*GAL4*/CyO;cry*^{b}	36	36 (13)	4.2 ± 0.3
Transgenic and non--transgenic combined:norpA^{P41}*/Y;cry*^{b}	97	47 (46)	3.9 ± 0.2
norpA^{P41}*/Y; Rh 5--*GAL4*/*UAS--*ttx;cry*^{b}	58	0 (0)	--

Table 2 Effect of tetanus toxin expression in the H--B eyelet on behavioral rhythms of *norpA^{P41}/Y;cry^{b}* flies in 65 lux

Genotype	**LD**			**Shifted LD regime**			**DD**		
	N (n)	**Period ± SEM (h)**	**% Rhy.**	**N (n)**	**Period ± SEM (h)**	**% Rhy.**	**N (n)**	**Period ± SEM (h)**	**% Rhy.**
y w/Y	39 (38)	24.2 ± 0.04	97	39 (22)	24.8 ± 0.12	56	37 (22)	24 0 ± 0.1	60
yw/Y;cry^{b}	12 (12)	24.4 ± 0.06	100	12 (9)	25.3 ± 0.14	75	11(6)	25 0 ± 0.7	55
norpA^{P41}/Y;cry^{b}	23 (10)	24.5 ± 0.08	43	22 (9)	24.9 ± 0.2	41	22 (1)	23.5	4.5
norpA^{P41}/Y; UAS--*ttx;cry^{b}*	41 (37)	24.2 ± 0.1	90	41 (36)	24.9 ± 0.1	88	40(7)	23.5 ± 0.2	18
norpA^{P41}/Y; Rh 5-- GAL4*/CyO;cry^{b}*	42 (31)	24.3 ± 0.1	74	41 (29)	24.7 ± 0.1	71	37 (4)	23.7 ± 0.7	11
Transgenic and non-transgenic combined:norpA^{P41}/Y;cry^{b}	106 (78)	24.3 ± 0.03	74	104(74)	24.9 ± 0.1	71	99 (12)	23.6 ± 0.2	12
norpA^{P41}/Y;Rh 5-- GAL4/UAS--*ttx;cry^{b}*	61 (50)	24.1 ± 0.04	82	60 (53)	24.2 ± 0.1	88	56 (20)	23.7 ± 0.1	36

N is the number of flies tested and 'n' is the number of flies showed rhythmic behavior

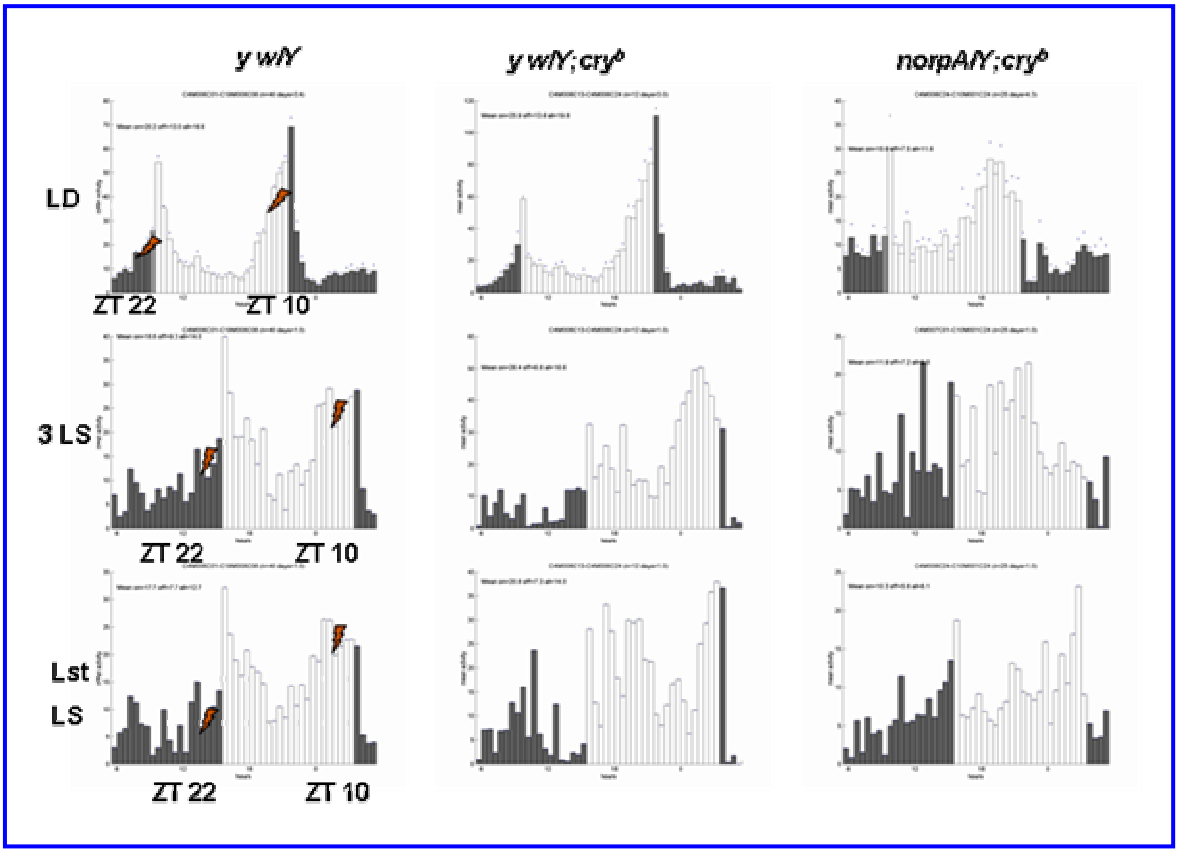

Figure 6A Daily average plots for *D. melanogaster* in the initial LD regime during re--synchronization (or not) to the shifted LD cycle. The upper row represents the initial LD cycle (3--5 days), the middle and the lower rows represent the day 3 (3 LS) and the day last (Lst LS) in shifted LD, respectively. The different columns represent the indicated genotype. The red flash symbols (left ZT 22, right ZT 10) on each plot indicate time when the flies were sacrificed for immuno--histochemistry. The *y w* flies showed typical bimodal activity in initial LD, on 3 LS and Lst LS, in shifted new LD regime; in comparison cry^b flies needed more time to resynchronize.

On the day 3 of the new LD regime, the *y w* and cry^b flies had re--synchronized their evening peaks while the $norpA^{P41};cry^b$ control flies failed to do so: their activity offsets remained almost in synchrony with the initial LD regime (Fig. 6A and Fig. 6B; cf. Stanewsky *et al*., 1998). This indicated that these flies were unable to re--entrain to the 6 h phase delayed LD (cf. Stanewsky *et al*., 1998). In the H--B^{TD}:$norpA^{P41};cry^b$ flies this problem was more aggravated (Fig. 6B column 3). The activity offset was shifted more towards the left. This may be an indication of the free run of the H--B^{TD}:$norpA^{P41};cry^b$ flies as opposed to the slow re--entrainment observed in $norpA^{P41};cry^b$ flies (Fig. 4D and Table 2). This is also indicated by the shorter free run period values exhibited by the H--

B^{TD}:*norpA^{P41};cry^{b}* flies (Fig. 4D) compared to the transgenic *norpA^{P41};cry^{b}* flies (Fig. 4B, C and Table 2). On the day 5 of shifted LD, all the genotypes showed a similar behavior as they had in the day 3 of shifted LD (Fig. 4 and Table 2) indicating that the H--B^{TD}:*norpA^{P41};cry^{b}* flies could not resynchronize, even after prolonged exposure to the new LD regime.

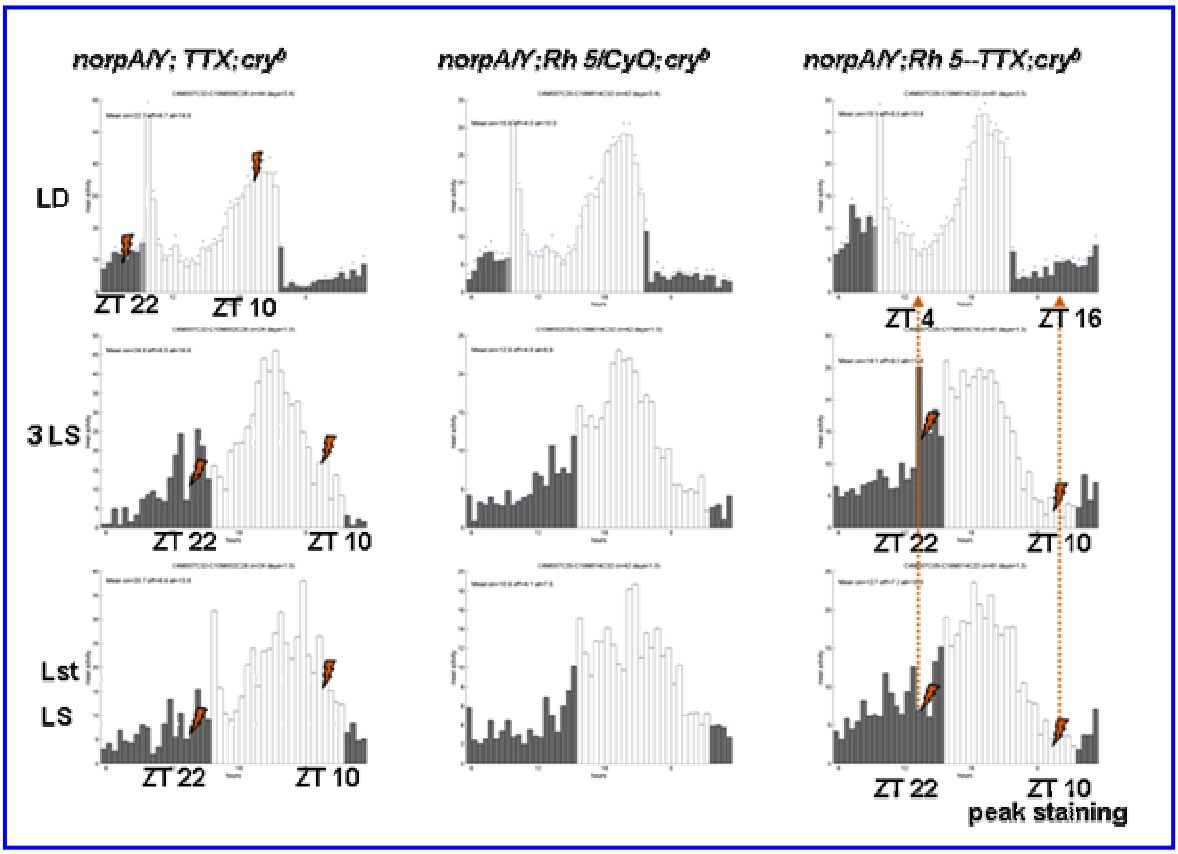

Figure 6B The *norpA^{P41};cry^{b}* flies displayed synchronized behavior in the initial LD and in the shifted LD regime they needed more time to resynchronize compared to *cry^{b}* flies (6A column 3). The transgenic *norpA^{P41};cry^{b}* flies also showed a bimodal entrainment in the initial LD but in the shifted LD cycle their activity peak showed an offset from the initial LD (column 1, 2). The H--B^{TD}:*norpA^{P41};cry^{b}* flies were synchronized with the initial LD regime but not resynchronized with shifted LD regime (column 3). Instead of resynchronization to the new LD regime they kept the original phase. The red dotted arrows in indicate the corresponding ZT in the initial LD regime.

In summary, blocking the synaptic signal from the H--B eyelet in the *norpA^{P41};cry^{b}* flies resulted in the total inability to resynchronize to a 6 h phase delayed LD

regime of 65 lux. Instead of adjusting to the new LD regime the H--B^{TD}:*norpA^{P41};cry^{b}* flies showed free running behavior.

4.2 Synchronization of TIMELESS and PERIOD expression to LD of 65 lux white light

Failure of the H--B^{TD}:*norpA^{P41};cry^{b}* flies to re--entrain to a shifted (dim) LD cycle prompted us to investigate whether PER and TIM expression was affected in the clock neurons. First, flies were entrained to a 12 h: 12 h LD of 65 lux and the PER and TIM levels were measured at 2 time--points at which clock protein expression is high and low, respectively (Kaneko, 1998; Helfrich--Förster *et al*., 2001; Yang and Sehgal, 2001). This was done to assure that the clock proteins were normally synchronized before the flies were phase shifted by 6 h. Amplitude of clock protein cycling is a measure of light--synchronized expression of PER and TIM: the difference between the average number of neurons stained at ZT 22 (peak time point) and ZT 10 (trough time point) was calculated (see details in Methods 3.6). The PER and TIM expression levels were tested in clock neurons of the *y w* and *norpA^{P41};*UAS--*ttx;cry^{b}* control flies. In *y w*, PER and TIM expression was synchronized in all groups of clock neurons, i.e. the peak level of clock protein expression occurred at ZT 22 and trough level of clock protein expression was observed at ZT 10 (Fig. 7 and Fig. 9). In the *norpA^{P41};*UAS--*ttx;cry^{b}* flies overall PER and TIM cycling was synchronized with the initial LD regime, in the subsets of clock neurons (except TIM in the l--LN$_v$s and PER and TIM in the DN$_2$s and DN$_3$s), but the amplitude of oscillation was reduced, in turn indicative of desynchronization in these neurons (Fig. 8 and Fig. 9). On contrary, Helfrich--Förster *et al*. (2001) reported that in LD PER showed robust cycling only in the s--LN$_v$s and DN$_1$s. This discrepancy may owe to the difference in entrainment conditions existed in both experiments. Furthermore, the presence of the *w^{+}* transgene along with GAL4 might have influenced our result.

In the *norpA^{P41};*UAS--*ttx;cry^{b}* flies there was minimum cycling amplitude in the DN$_3$s, followed by the DN$_2$s (Fig. 9; cf. Helfrich--Förster *et al*., 2001) and DN$_1$s. In lateral neurons, the s--LN$_v$s and l--LN$_v$s (only for PER; the l--LN$_v$s result contrasts Helfrich--Förster *et al*., 2001) showed high amplitude of cycling but the LN$_d$s

showed relatively weaker amplitude oscillations (cf. Helfrich--Förster *et al*., 2001). Helfrich--Förster *et al*. (2001) showed almost no PER cycling in the l--LN$_v$s.

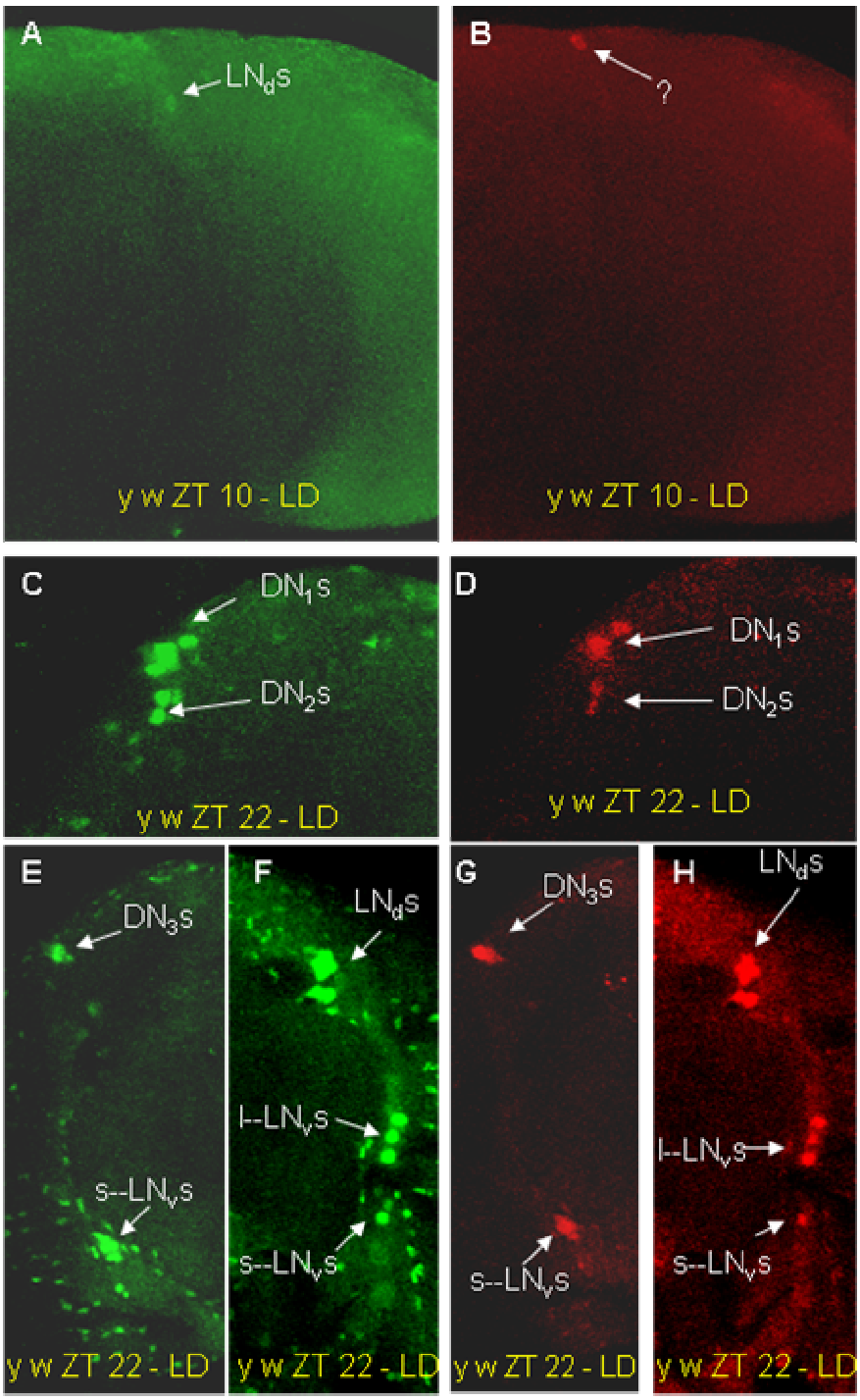

Figure 7 Whole mounted brains of *y w* flies stained for anti--PERIOD (PER--green) and anti--TIMELESS (TIM--red) in LD in 65 lux. Uppermost panels show the trough time point

(ZT 10) for both clock proteins PER and TIM. At ZT 10 no conspicuous staining for PER (A) and TIM (B) was visible (except for the single LN_ds seen in this particular picture)--the question mark in (B) indicates an unspecific cell stained against anti--TIM. The middle and lower panels show the various clock neurons stained against anti--PER and anti--TIM at the peak time point (ZT 22). Panels C and D show the dorsal neurons 1 and 2 (DN_1+$_2$s). Panels E and G show dorsal neurons 3 (DN_3s) and small lateral ventral neurons (s--LN_vs). Panels F and H show lateral neurons: small and large ventral lateral neurons (s+l--LN_vs) and lateral neurons dorsal (LN_ds).

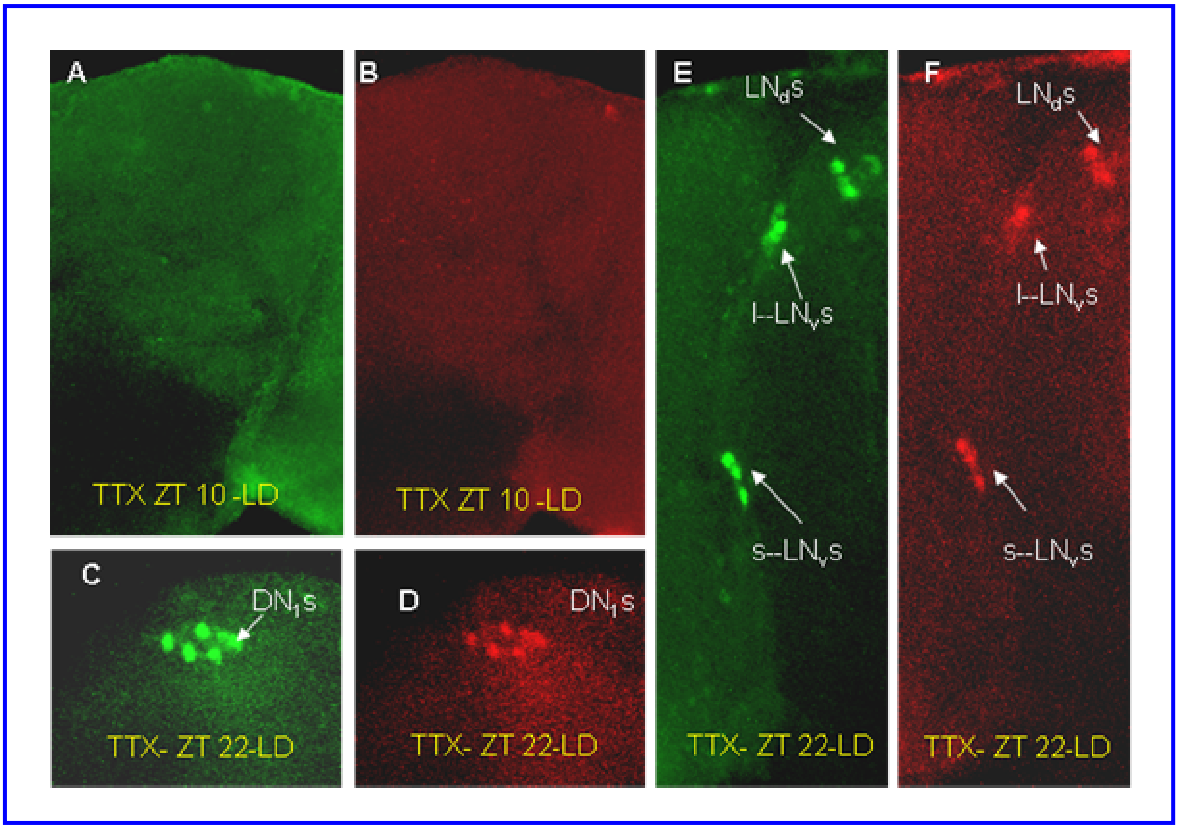

Figure 8 *norpA*^{P41};UAS--*ttx;cry*^{b} (TTX) *D. melanogaster* brains (whole mounted) stained for anti--PER (green) and anti--TIM (red) in LD in 65 lux. Uppermost panels show the trough time point (ZT 10) for both the clock proteins PER and TIM. At ZT 10 no conspicuous staining for PER (A) and TIM (B) was visible. Middle and lower panels show various clock neurons (as indicated) stained at the peak time point (ZT 22) against the anti--PER (C and E) and anti--TIM (D and F).

In LD, the peak level of PER and TIM staining in the clock neurons of *y w* flies was negatively correlated with their locomotor activity pattern (cf. Helfrich--Förster *et al*., 2001). Similar results were obtained in the present study (Fig. 6A--column1

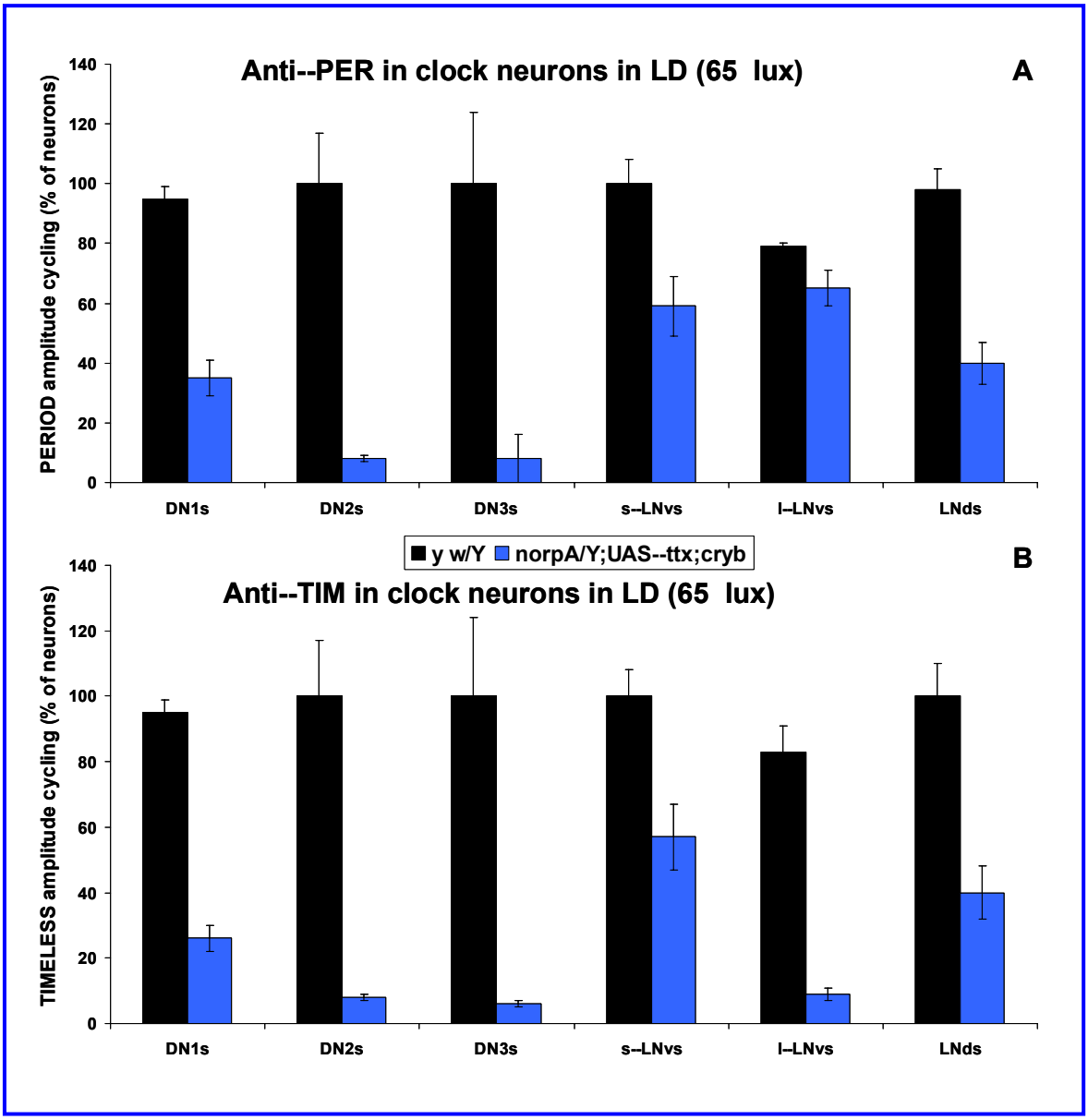

Figure 9 Quantification of the neuronal staining in *y w* and *norpA*^{P41};UAS--*ttx;cry*^{b} flies for PER (A) and TIM (B). As a measure of light--synchronized expression of PER and TIM "amplitude of clock protein cycling": the difference between the average number of neurons stained at ZT 22 (peak time point) and ZT 10 (trough time point) was calculated. A strong positive value indicated that PER and/or TIM expression was synchronized to the LD regime and a small positive or negative value indicated no synchronization to the LD regime. A small positive value means that PER and/or TIM were expressed almost equally (i.e. desynchronized) at the two time--points investigated, therefore subtraction of ZT 10 value from ZT 22 resulted in an integer close to zero. A negative value means that PER and/or TIM were expressed more at ZT 10 compared to ZT 22. Further, it indicates that the neurons are not synchronized to LD regime.

row 1 and Fig 7). At a time of peak activity, i.e. around ZT 10--12 (Fig. 6A: time of animal sacrifice for immuno--histochemistry is indicated by the flash arrows in the figure), there was no or minimum staining in the clock cells (Fig. 7, Fig. 9), and at a time of less activity, i.e. around ZT 22--23, there was high intensity staining. This was also true for the *norpA^{P41};*UAS--*ttx;cry^{b}* flies (Fig. 6B--column 1 row 1; Fig. 8, Fig. 9).

4.2.1 Synchronization of TIMELESS expression to a 6 h phase delayed LD of 65 lux white light in the H--B^{TD}:*norpA^{P41};cry^{b}* flies

The TIM expression level was investigated on the day 3 and day 5 in a 6 h phase delayed experiment (65 lux). On the day 3, TIM expression was synchronized to the new LD regime in all subsets of clock neurons in the *y w* flies (Fig. 11). But all the subsets of clock neurons were not synchronized very well in *norpA^{P41};*UAS--*ttx;cry^{b}* control flies (Fig. 10 upper panels and Fig. 11). This was expected for the DN_2s, DN_3s and l--LN_vs, because TIM expression in these cells was not synchronized in the initial LD cycle (Fig. 9B). But the DN_1s, s--LN_vs and LN_ds are less synchronized in the new LD shifted flies, which might explain why they need more time to resynchronize behaviorally. Interestingly, in the H--B^{TD}:*norpA^{P41};cry^{b}* flies none of the subsets of clock neurons were synchronized to the new LD regime (Fig. 10 lower panels, and Fig. 11). In fact, there was reversed phase for the molecular cycling. This is because molecular cycling remained synchronized to the initial LD regime. Therefore, in the shifted new LD regime, at the trough time point (ZT10) there was more staining, which corresponds to the ZT 16 of the initial LD (Fig. 6B, column 3 first row), a circadian time when PER and TIM start accumulate in the cytoplasm (Shafer *et al*., 2002). At the peak time point (ZT 22) in the shifted new LD regime there was less staining, which corresponds to the ZT 4 of the initial LD (Fig. 6B, column 3 first row), a circadian time when less PER and no TIM were visible (Shafer *et al*., 2002). Due to this reversed phase of peak staining in the new LD regime, our calculation for the cycling amplitude gave a negative value (Fig. 11). This correlated with reversed activity pattern in these flies (Fig. 6B, column 3 row 2) compared to the control flies. The activity pattern was shifted to left, compared to the control flies indicating that these flies did not resynchronize, not even slowly as *norpA^{P41};cry^{b}* flies do (Fig. 4B, C, D). There was

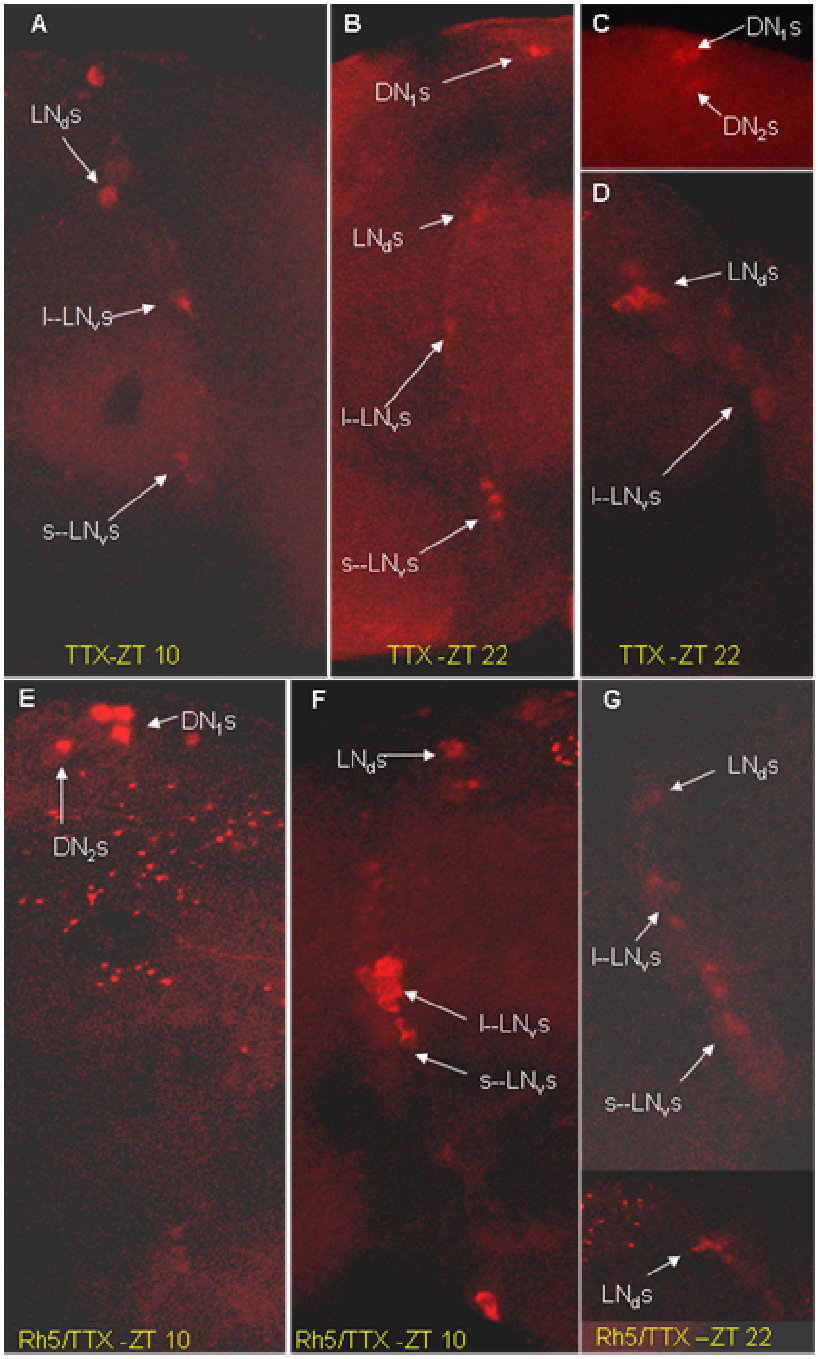

Figure 10 Whole mounted brains stained for anti--TIM on day 3 of shifted LD cycle. Expression of tetanus toxin in the H--B eyelet in a *norpA*^{P41};*cry*^{b} double mutant resulted in failure to resynchronize TIM expression in DNs and LN$_v$s. Upper panels show *norpA*^{P41};UAS--*ttx;cry*^{b} (TTX) control and lower panels show *norpA*^{P41}; *Rh 5*--GAL4/UAS--*ttx;cry*^{b} (Rh5/TTX) brains. In TTX at ZT 22 the DN$_{1+2}$s (C), the s--LN$_v$s (B), and the LN$_d$s (D) were strongly stained, whereas at ZT 10 all these cell groups were weakly stained

(A). In contrast, in Rh5/TTX at ZT 10 the DN_{1+2}s (E), the s--LN_vs and the l--LN_vs (F) were strongly stained in comparison to the ZT 22 (G). The LN_ds were stained similarly at both time points (F, G).

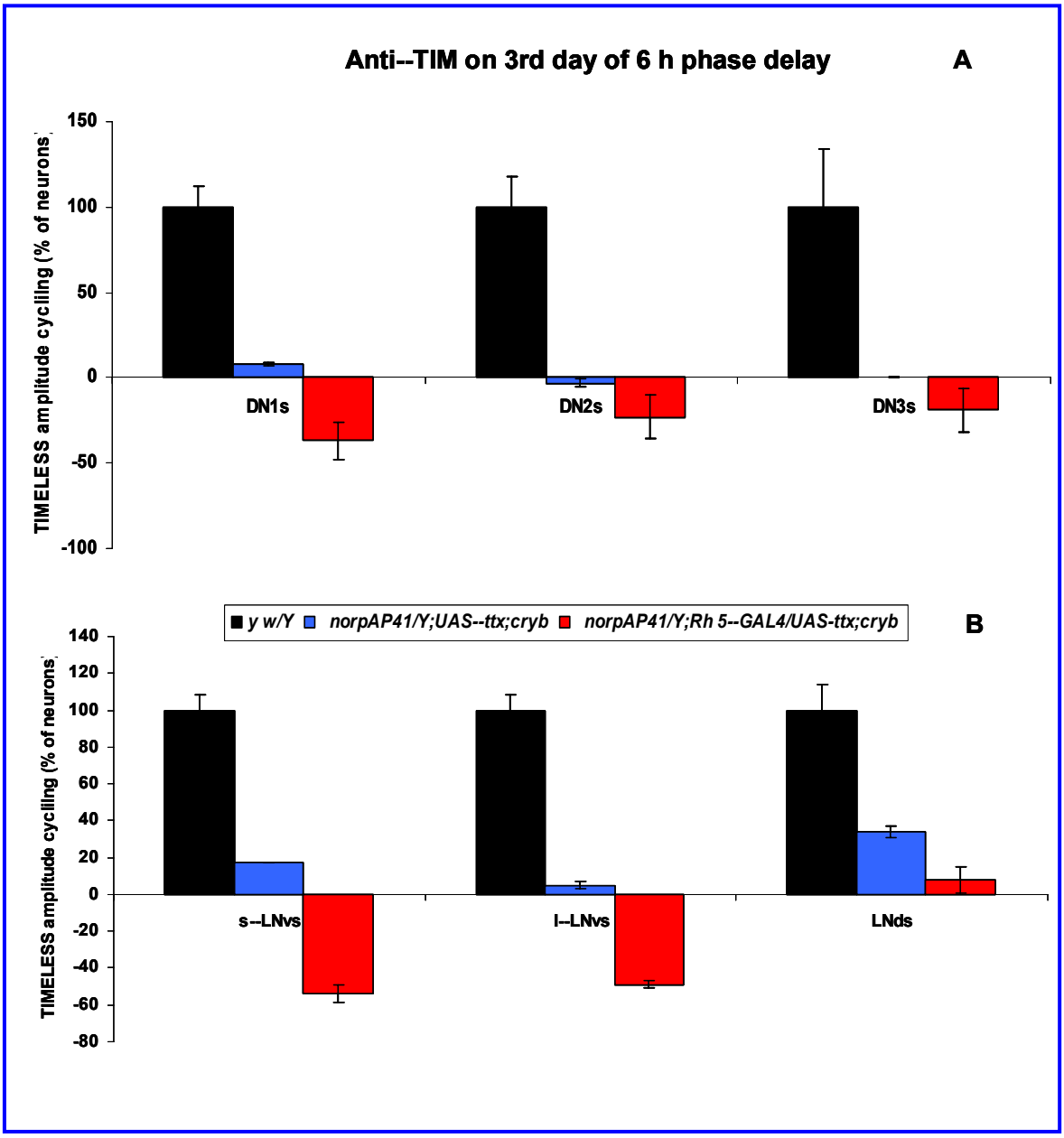

Figure 11 Quantification of anti--TIM staining in the DNs and LNs on day 3 in shifted LD cycle. (A) The TIM staining in DNs is synchronized in *y w* flies but it is desynchronized in *norpA*^{P41};UAS--*ttx;cry*^{b} flies. On contrary the TIM staining is higher at ZT 10 in *norpA*^{P41};*Rh 5*--GAL4/UAS--*ttx;cry*^{b} flies, therefore they show a negative value. This indicates that *norpA*^{P41};*Rh 5*--GAL4/UAS--*ttx;cry*^{b} flies were synchronized to the initial LD cycles. The initial synchronization is reflected in the new LD regime. (B) Similarly, in *y w* flies all the LNs display synchronized TIM expression and in *norpA*^{P41};UAS--*ttx;cry*^{b} flies

the LNs show desynchronized TIM expression. In *norpA^{P41};Rh 5*--GAL4/UAS--*ttx;cry^{b}* flies TIM expression shows desynchronization only in the LN_ds. In the s--LN_vs and l--LN_vs the TIM expression shows synchronization to the initial LD regime.

minimum activity at ZT 10 (and maximum staining) and *vice versa* at ZT 22 (Fig. 6B, column 1 row 2). Especially the drastic activity cut off after the lights 'off' of the original light regime was preserved in the H--B^{TD}:*norpA^{P41};cry^{b}* flies indicating that they had kept their original entrained activity phase (Fig. 6B, column 3).

TIM expression in the H--B^{TD}:*norpA^{P41};cry^{b}* flies kept synchronization with the initial LD regime for 5 days, except in the LN_ds (Fig. 11; Fig. 12 lower panels; Fig. 13; and Fig. 14 lower panels). The l--LN_vs though retained synchronization of TIM expression to the initial LD regime until day 3 in the new shifted LD, but later on

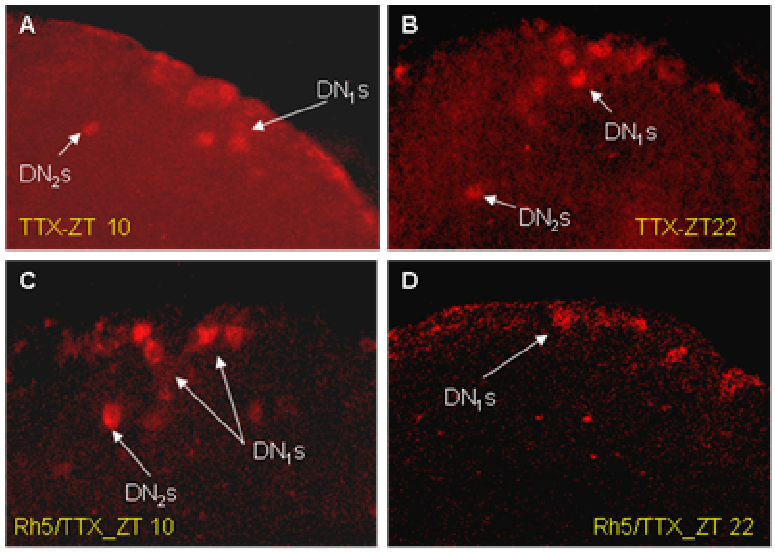

Figure 12 Whole mounted brains stained for anti--TIM on day 5 in the shifted LD regime. Expression of tetanus toxin in the H--B eyelet in a *norpA^{P41};cry^{b}* double mutant background results in inability to synchronize the TIM expression to the new shifted LD cycles in DNs. Upper panels show *norpA^{P41};*UAS--*ttx;cry^{b}* (TTX) control and lower panels show *norpA^{P41}; Rh 5*--GAL4/UAS--*ttx;cry^{b}* (Rh5/TTX) brains. In the TTX at ZT 10 (A) and ZT 22 (B) the DN_{1+2}s were stained equally, whereas in Rh5/TTX more neurons were stained at ZT 10 (C, D).

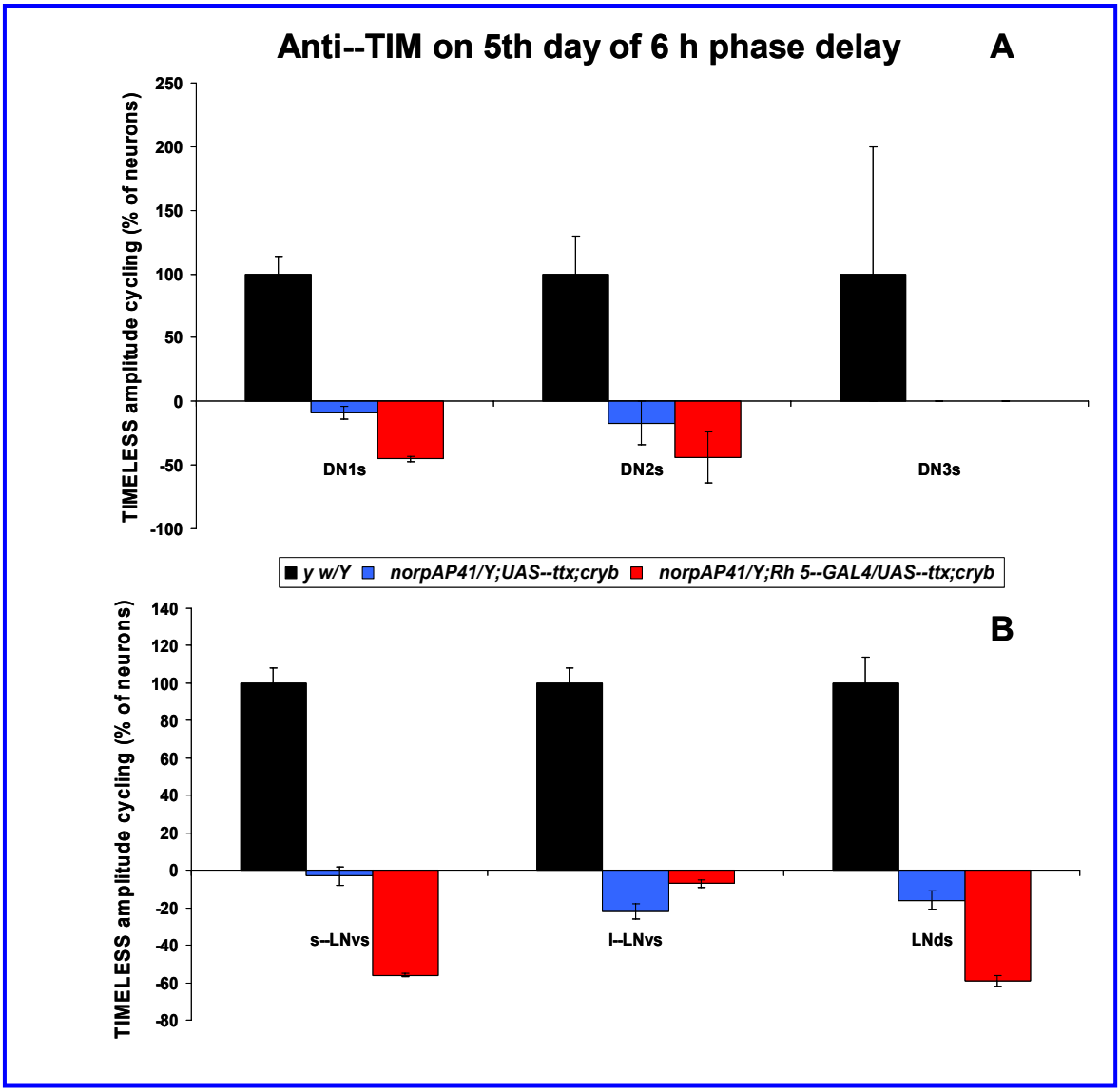

Figure 13 Quantification of anti--TIM staining in DNs and LNs on day 5 in the shifted LD regime. The DN_{1+2}s, s--LN_vs and LN_ds displayed synchronized TIM expression in line with initial LD cycle in *norpA^{P41};Rh 5*--GAL4/UAS--*ttx;cry^b* compared to controls. (A) The DN_{1+2+3}s showed synchronized TIM expression in *y w* flies. In *norpA^{P41}*;UAS--*ttx;cry^b* flies the DN_{1+2}s showed desynchronized TIM expression. On contrary in *norpA^{P41};Rh 5*--GAL4/UAS;*ttx;cry^b* flies the TIM expression was synchronized to the initial LD cycles, therefore, more TIM staining at ZT 10, indicates that *norpA^{P41};Rh 5*--GAL4/UAS--*ttx;cry^b* flies did not entrain to the new shifted LD cycles. (B) Similarly, in *y w* flies all the LNs display synchronized TIM expression and in *norpA^{P41}*;UAS--*ttx;cry^b* flies the LNs show desynchronized TIM expression. In *norpA^{P41};Rh 5*--GAL4/UAS--*ttx;cry^b* flies TIM expression shows desynchronization only in the l--LN_vs. In the s--LN_vs and LN_ds the TIM expression shows synchronization to the initial LD regime.

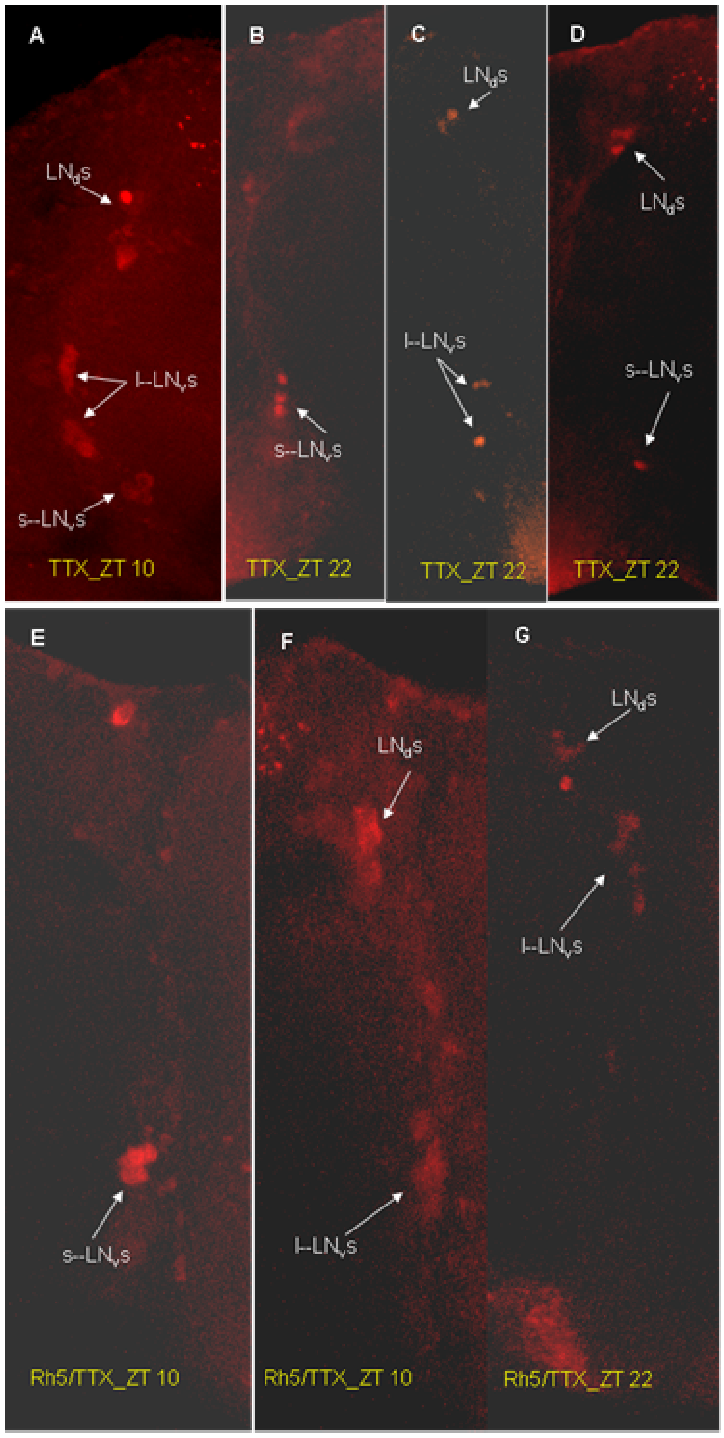

Figure 14 Whole mounted brains stained for anti--TIM on day 5 in the shifted LD regime. The TIM expression in the s--LN_vs and LN_ds remained synchronized with the initial LD regime in flies expressed tetanus toxin in the H--B eyelet in a *norpA*^{P41};*cry*^{b} background. Upper panels show *norpA*^{P41};UAS--*ttx;cry*^{b} (TTX) control and lower panels show the *norpA*^{P41};*Rh 5*--GAL4/UAS--*ttx;cry*^{b} (Rh5/TTX) brains. In the TTX at ZT 10 (A) the l--LN_vs

were more strongly stained compared to ZT 22 (C), whereas the s--LN_vs and LN_ds stained strongly at both time points (A, B, C, D). In Rh5/TTX flies the s--LN_vs and LN_ds stained more strongly at ZT 10 (E, F) compared to ZT 22 (G), whereas the l--LN_vs were equally stained at both time points, indicating their synchronization to the initial LD cycle is lost.

(by day 5) they had lost this synchronization. This may be because they lack any direct light input and behave like they were released into DD. It was reported that in DD rhythmic oscillations of PER and TIM are lost in the l--LN_vs (Yang and Sehgal, 2001; Shafer *et al*., 2002; Veleri *et al*., 2003). The TIM expression was desynchronized in the LN_ds on the day 3 of shifted LD regime; however, on the day 5 they showed synchronization with initial LD, similar to the s--LN_vs. Possibly the LN_ds are entrained by the s--LN_vs later on (Stoleru *et al*., 2004). As was the case on the day 3 of the new LD cycle, on the day 5 there was minimal activity at ZT 10 and more at ZT 22 (in the shifted LD cycle), which corresponds to the ZT 16 and ZT 4 of the initial LD cycle, respectively (Fig. 6B, column 3 row 3). This was correlated with a staining maximum at ZT 10 and minimal TIM signals at ZT 22 (Fig. 13 and Fig. 14 lower panels). Taken together, these results indicate that the TIM expression retained its phase from the initial LD regime and it was not resynchronized with the new shifted LD regime. This non--synchronization of TIM expression correlates with the non--synchronized behavioral activity pattern in the new shifted LD. Therefore, TIM expression seems to be the dominant signal for regulating the behavioral activity in LD cycles.

4.2.2 Synchronization of PERIOD expression to a 6 h phase delayed LD of 65 lux white light in the H--B^{TD}:*norpA^{P41};cry^{b}* flies

The PER expression level was also investigated on the day 3 and on the day 5 in a 6 h phase delayed LD of 65 lux in the H--B^{TD}:*norpA^{P41};cry^{b}* and control flies. On the day 3, the PER expression was well synchronized to the new 6 h phase delayed LD in all the subsets of clock neurons in *y w* control flies. In the *norpA^{P41};*UAS--*ttx;cry^{b}* flies the synchronization was less pronounced in some subsets of clock neurons compared to the *y w* flies (Fig. 15 upper panels and Fig. 16). In initial LD cycles, the DN_{2+3}s and the LN_ds showed less synchronization (Fig. 9A). Thus, the s--LN_vs and l--LN_vs mainly showed a decrement in

synchronization compared to the initial LD regime. In the H--B^{TD}:*norpA^{P41};cry^{b}* flies the DN_1s and l--LN_vs were not synchronized to the new shifted LD regime but the

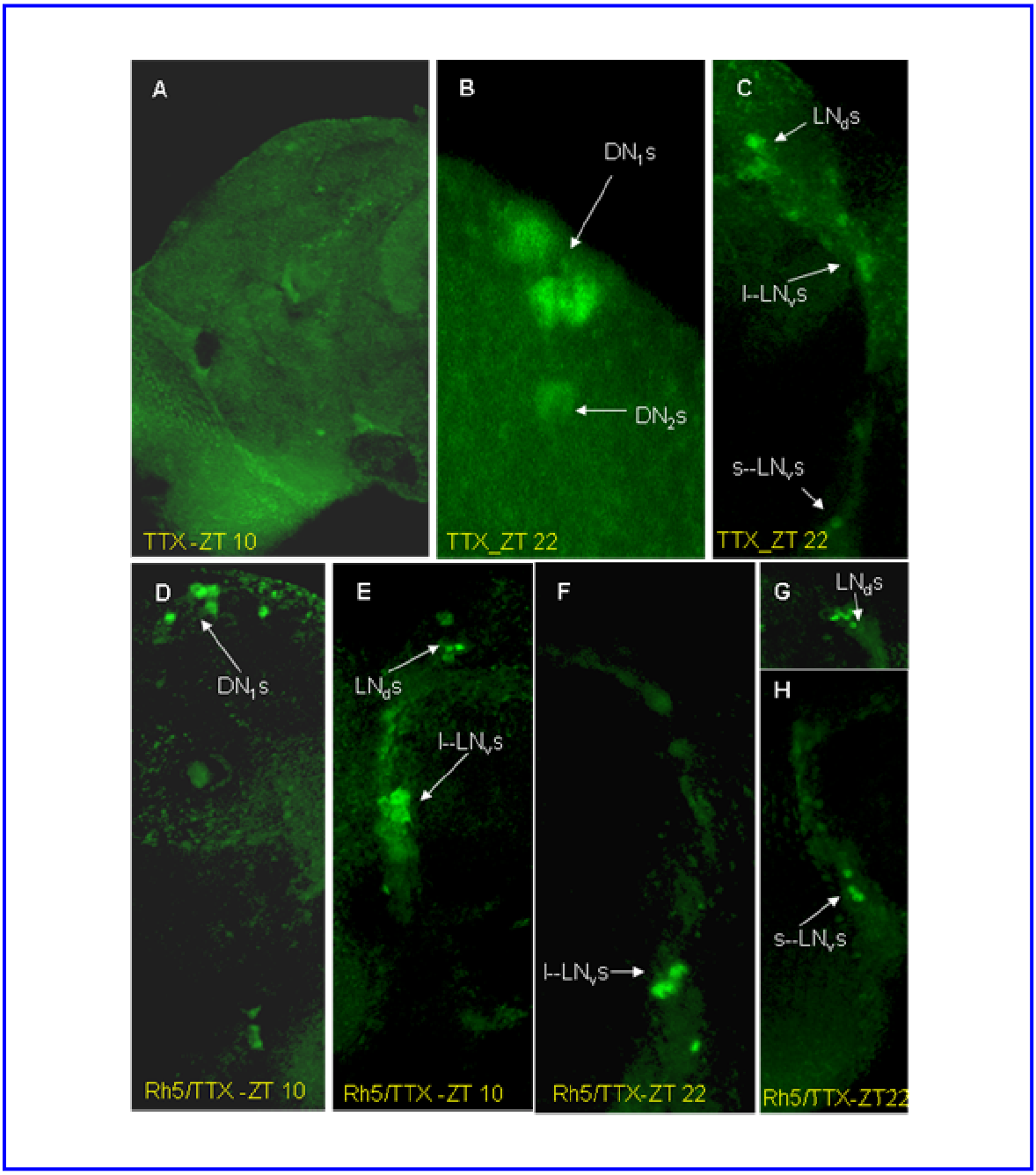

Figure 15 Whole mounted *D. melanogaster* brains stained for anti--PER on the day 3 of the shifted LD cycle. Expression of tetanus toxin in the H--B eyelet in a *norpA^{P41};cry^{b}* double mutant perturbed the synchronized PER expression in the DN_1s and l--LN_vs. Upper panels show the *norpA^{P41};*UAS--*ttx;cry^{b}* (TTX) control and lower panels show the *norpA^{P41};Rh 5*--GAL4/UAS--*ttx;cry^{b}* (Rh5/TTX) brains. At ZT 10 there was no staining in TTX (A) compared to strong staining at ZT 22 (B, C); whereas in Rh5/TTX at ZT 10 the

DN_1s (D) and l--LN_vs (E) were strongly stained. Weak staining was also seen in the LN_ds (E) at ZT 10 whereas the s--LN_vs and LN_ds showed stronger staining at ZT 22 (G and H).

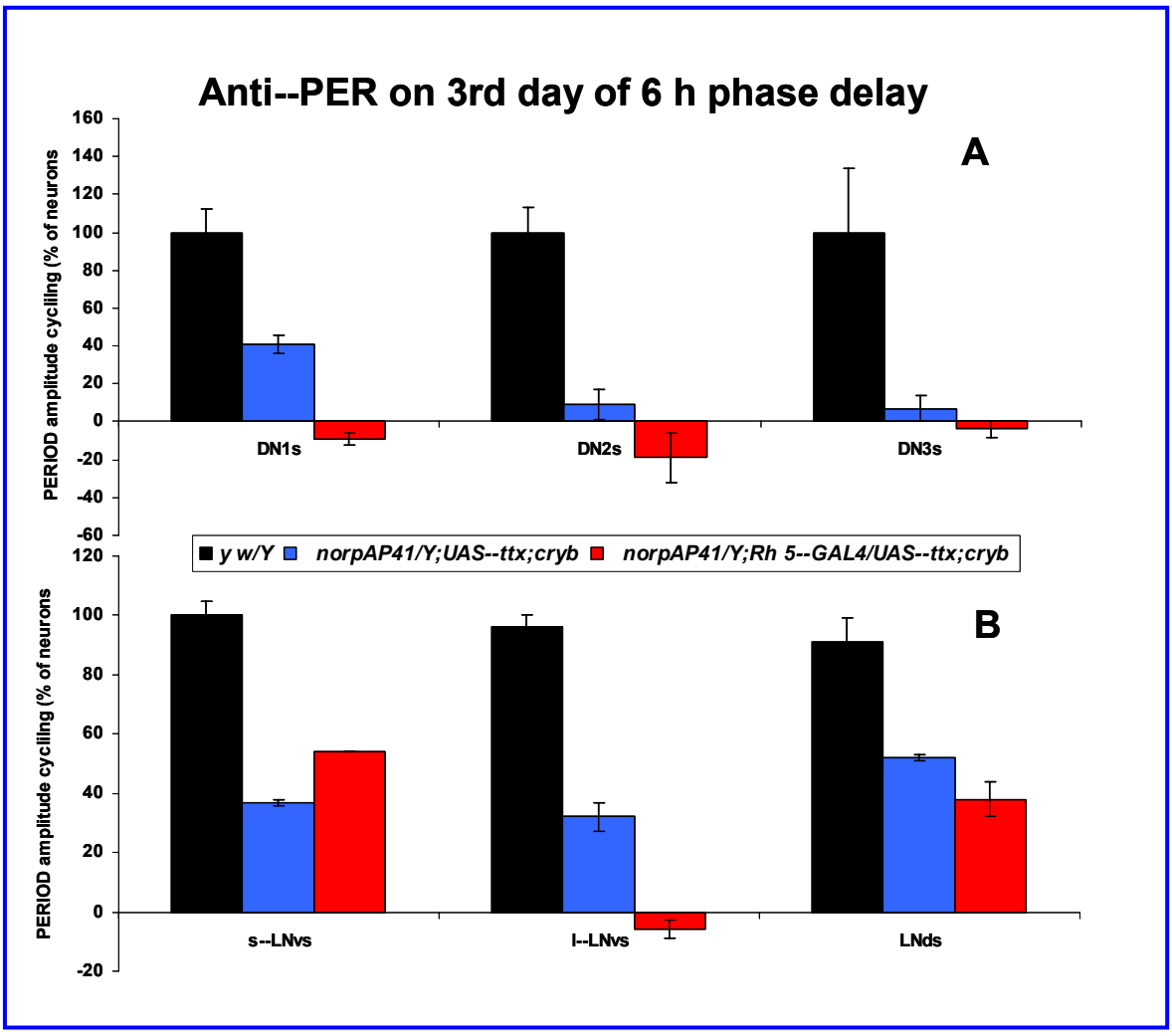

Figure 16 Quantification of anti--PER staining in dorsal (DN_{1-3}s) and lateral neurons (LNs) on the day 3 of the shifted new LD regime. (A) The DN_1s show perturbed PER synchronization in *norpA^{P41};Rh 5*--GAL4/UAS--*ttx;cry^b* compared to the controls. (B) The l--LN_vs also show perturbed PER synchronization in *norpA^{P41};Rh 5*--GAL4/UAS --*ttx;cry^b* compared to the controls.

s--LN_vs and LN_ds were synchronized as in control flies (Fig. 15 lower panels and Fig. 16). The DN_2s and DN_3s are also not synchronized in the controls in the initial LD regime. This indicates that in the DN_1s and l--LN_vs PER synchronization is affected on day 3 in the H--B^{TD}:*norpA^{P41};cry^b* flies.

On day 5 of the 6 h phase delayed new LD, again the *y w* flies showed a synchronized expression of PER in all the subsets of clock neurons (Fig. 18). In

the *norpA*^{P41}; UAS--*ttx;cry*^{b} flies, except for the DN_2s and DN_3s, all the subsets of clock neurons were synchronized to the new LD regime (i.e. they behaved similar to the flies stained in the original LD) (Fig. 9A; Fig. 17 upper panels; and Fig. 18). In the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies, like in the day 3, the DNs were still not synchronized with the new LD but the s--LN_vs, l--LN_vs and l--LN_ds were synchronized (Fig. 17 lower panels and Fig. 18) indicating that the l--LN_vs receive

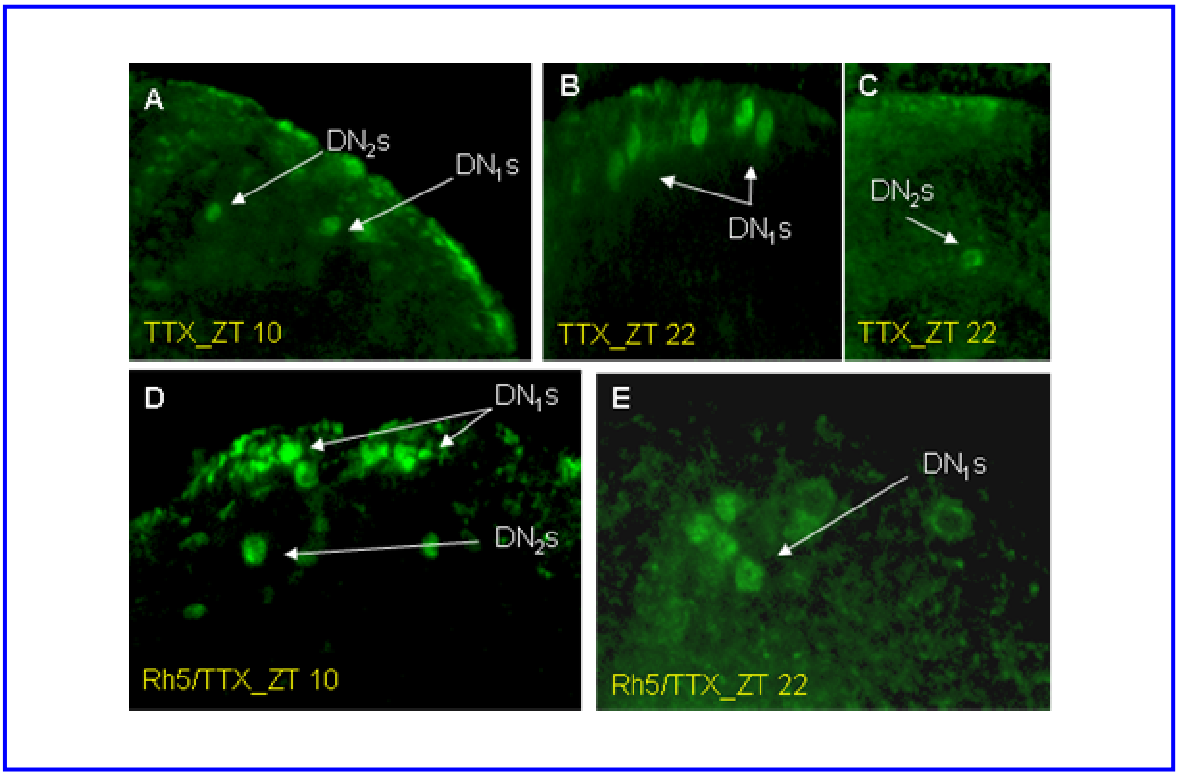

Figure 17 Whole mounted brain stained for anti--PER on the day 5 of the shifted LD regime. Tetanus toxin expression in the H--B eyelet in *norpA*^{P41};*cry*^{b} double mutants lost synchronization of PER expression to the new LD regime in the DN_1s. Upper panels show *norpA*^{P41};UAS--*ttx;cry*^{b} (TTX) control and lower panels show the *norpA*^{P41};*Rh 5*--GAL4/UAS--*ttx;cry*^{b} (Rh5/TTX) brains. In TTX, the DNs were predominantly stained at the peak time point (ZT 22, B, C) compared to ZT 10 (A). In contrast in Rh5/TTX, many DNs were stained strongly at ZT 10 (D) and at ZT 22 (E).

entraining signals from the other lateral clock neuronal groups. In summary, synchronization of PER expression was affected only in the DN_1s by day 5 in the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies compared to the *norpA*^{P41};*cry*^{b} flies. At ZT 22 (corresponding to ZT 4 in initial LD) PER expression was more compared to ZT 10

(ZT 16 initial LD) which did not very well correlate to the reciprocal relationship between staining and locomotor activity pattern, as seen for TIM (Fig. 6B) . However, how PER expression was synchronized to the shifted LD regime in LNs needs to be resolved because TIM could not resynchronize in the same flies. Therefore, a direct light signaling via TIM to PER in the shifted LD regime is most

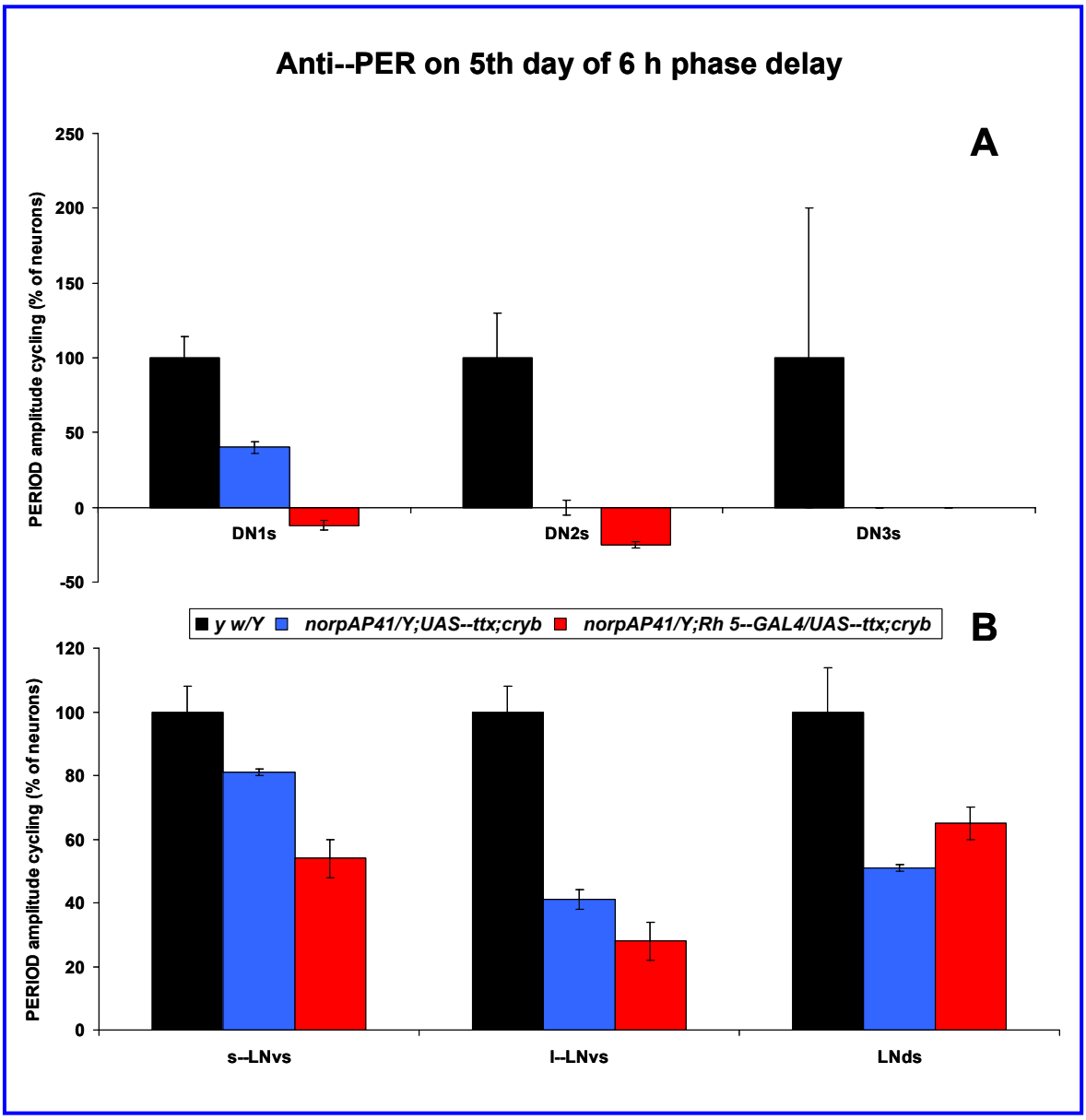

Figure 18 Quantification of anti--PER staining in dorsal neurons ($DN_{1--3}s$) and lateral neurons (LN) on the day 5 of shifted LD cycle. The DN_1s show non--synchronized PER expression in *norpA*^{P41}*;Rh 5*--GAL4/UAS--*ttx;cry*^{b} flies compared to control flies (A); whereas synchronization of PER expression in the l--LN_vs was restored as opposed to the day 3 (B).

unlikely. PER is shown to directly interact with CRY *in vitro* (Rosato *et al*., 2001), therefore, a leaky signaling from CRY^{B} to PER might be allowing PER to resynchronize to new LD regime in the H--B^{TD}:$norpA^{P41}$;cry^{b} flies. These results suggest that TIM is the driving force for the circadian clock in LD.

5 Discussion

In *D. melanogaster* synchronization of the circadian clock to the environmental LD is accomplished by light signals concurrently received through rhodopsins and CRY (Helfrich--Förster, 2002; Hall, 2000), which are blocked in $norpA^{P41};cry^{b}$ flies (Stanewsky *et al*., 1998; Emery *et al*., 2000b; Helfrich--Förster *et al*., 2001). However, the double mutants still could do behavioral resynchronization in response to shifted LD cycles though they needed prolonged time (Stanewsky *et al*., 1998; Emery *et al*., 2000). Moreover, they maintained the molecular synchronization of TIM and PER in the s--LN_vs, DN_1s and LN_ds (Helfrich--Förster *et al*., 2001), which suggested the existence of an additional extra--retinal photoreceptor. gl^{60j} cry^{b} flies lacked the residual behavioral and molecular synchronization mentioned above. The $norpA^{P41};cry^{b}$ flies look morphologically normal but are functionally disabled in for both rhodopsin and CRY photoreception (see more in Chapter 1, section 2.2). In contrast, the gl^{60j} flies are morphologically defective in the compound eyes, ocelli and in addition the H--B eyelet and the DN_1s are depleted. Therefore, gl^{60j} cry^{b} flies are also functionally disabled in rhodopsin (perhaps except any putative rhodopsin present in the brain) and CRY signaling (Helfrich--Förster *et al*., 2001; Blanchardon *et al*., 2001). The H--B eyelet sends neuronal projections to the proximity of LN_vs (Hofbauer and Buchner 1989; Yasuyama and Meinertzhagen, 1999; Malpel *et al*., 2002; Helfrich--Förster *et al*., 2002). Therefore, a role for the H--B eyelet was implicated for mediating some aspects of light entrainment (Helfrich--Förster *et al*., 2001; Helfrich--Förster *et al*., 2002). Very recently, it was shown that the larval precursor of the H--B eyelet, the BO, signals photic information to the s--LN_vs and thus mediates photophobic behavior in larva (Mazzoni *et al*., 2005).

The H--B eyelet's role in circadian photoreception was tested by blocking the putative synaptic transmission from it to the LN_vs. Flies simultaneously non--functional for the H--B eyelet, rhodopsins and CRY were tested for their ability to

resynchronize behavioral and molecular rhythms to a shifted LD cycles (i.e. equivalent to the jet lag experience) in dim light (65 lux).

5.1 Synaptic connections from the H--B eyelet to the LN_vs are tetanus toxin sensitive

The Tetanus Toxin Light Chain (TxTxLC) blocks chemical synaptic transmission by cleaving the neuronally expressed synaptobrevin protein (Sweeny *et al*., 1995; Martin *et al*., 2002). Tetanus toxin expressed in the clock cells under the control of *per*--GAL4 or *tim*--GAL4 showed substantially decreased behavioral rhythms in DD and in LD cycles. Locomotor activity level during night increased and the morning activity peak was reduced (Kaneko *et al*., 2000). However, expression of tetanus toxin under the control of *Pdf*--GAL4 promoter showed only minor behavioral abnormalities suggesting that the synaptic transmission from the s--LN_vs to the DNs in the dorsal protocerebrum may not be affected by the tetanus toxin expression. Tetanus toxin expression in the H--B eyelet affected the molecular resynchronization in the s--LN_vs as well as the behavioral resynchronization in dim light. Therefore, the synaptic connections from the H--B eyelet to the s--LN_vs are tetanus toxin sensitive.

5.2 Behavioral resynchronization of the circadian clock in low intensity light requires the H--B eyelet

It was proposed that the H--B eyelet plays a role in behavioral resynchronization of the circadian clock, especially in phase delay (Helfrich--Förster *et al*., 2002; Rieger *et al*., 2003). To maintain a synchronized behavior with the changing environment, the organisms need to reset their circadian clock on a daily basis, which means that in a first step the molecular cycling needs to be adjusted. In nature synchronization of circadian rhythms is most efficiently accomplished by twilight (Helfrich--Förster *et al*., 2001; Panda *et al*., 2003). During twilight the light intensity is very low. Therefore, a photoreceptor that senses very low intensity light is possibly involved in resetting of the circadian clock. The H--B eyelet's role in circadian photoreception is relevant in this context because: 1) The $norpA^{P41};cry^{b}$ flies could re--entrain to a new LD regime but not the gl^{60j} cry^{b} flies, which lacked the H--B eyelet and DN_1s along with the compound eyes and ocelli

(Helfrich--Förster *et al*., 2001; Blanchardon *et al*., 2001). 2) The H--B eyelet sends direct neural connection towards the pacemaker neurons (Hofbauer and Buchner, 1989; Yasuyama and Meinertzhagen, 1999; Helfrich--Förster *et al*., 2002; Malpel *et al*., 2002). 3) A role for the H--B eyelet in resynchronization to a changed photoperiod was implicated (Rieger *et al*., 2003) and a role for it was proposed in phase delays (Helfrich--Förster *et al*., 2002). 4) Blocking the putative synaptic transmission from the H--B eyelet to the LN_vs in a $norpA^{P41};cry^{b}$ genetic background affected the resynchronization in dim green light (Veleri, S. and Helfrich--Förster, C., unpublished observations). 5) It expresses *Rh 5* and *Rh 6* (Malpel *et al*., 2002) with an absorption maximum for blue and green light, respectively (Montell, 1999) which could effect light entrainment of the circadian clock. 6) In high intensity light the H--B^{TD}: $norpA^{P41};cry^{b}$ flies showed normal re--entrainment (Veleri, S., unpublished observation). Therefore, a putative role for the H--B eyelet in sensing in low intensity light was investigated.

In natural condition the light intensity is very low at dawn and dusk, by which the circadian clocks are normally synchronized in nature (Helfrich--Förster, *et al*., 2001; Panda *et al*., 2003). 65 lux light is within the limits of light intensity present at dawn (Gasio *et al*., 2003). In 65 lux white light, the H--B^{TD}:$norpA^{P41};cry^{b}$ flies were unable to do behavioral resynchronization to a 6 h phase delayed LD cycle (Fig. 4D, Fig. 5) as was earlier seen in green dim light (data not shown). Similar results were reported by Mealey--Ferrara *et al*. (2003), where the authors showed that expressing tetanus toxin in the H--B eyelet in $norpA^{P41};cry^{b}$ flies reduced its capability for re--entrainment. At 25 lux H--B^{TD}:$norpA^{P41};cry^{b}$ flies entrained to an initial 12 h: 12 h LD but showed difficulty to re--entrain to a new LD of longer photo period (13 h: 13 h LD). In our experiments, the effect seen at 65 lux light intensity was not reproducible at higher light intensities of 300--400 lux (data not shown). Since the effect is observed only in dim light we suggest that the H--B eyelet is specialized to sense low intensity light, and indeed it plays a role in the circadian photoreception. In retrospection, this finding corroborated a proposed role for the H--B eyelet in mediating phase delays (Helfrich--Förster *et al*., 2002).

Instead of resynchronization to the new shifted LD cycles the H--B^{TD}:$norpA^{P41};cry^{b}$ flies exhibited free running behavior during the shifted regime, whereas the

norpA^{P41};*cry*^{b} flies slowly adjusted their behavior to the new LD regime (Fig. 4B, C; Fig. 5 and Table 2). This indicated that at low intensity light blocking neural signals from the H--B eyelet precluded residual light input reaching the circadian clock in the *norpA*^{P41};*cry*^{b} flies. Therefore, a role for the H--B eyelet as a circadian photoreceptor in low intensity light has been demonstrated here. But how can one explain the normal resynchronization of the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies to higher intensities of light? One possible explanation is that yet another extra--retinal photoreceptor might be functioning in high intensity light, which signals to the circadian clock to attain resynchronization. Perhaps the dorsal neurons (DNs) contain such an additional extra--retinal photoreceptor (see more in Chapter 4 about DNs). The alternate explanation may be that *cry*^{b} is not absolutely devoid of functional cryptochrome and a leaky signaling may still be possible at higher light intensities (as discussed in Mealey--Ferrara *et al*., 2003) or tetanus did not blocked 100% neural transmission to the pacemaker neurons in our experiments. It is also possible that *norpA* is not completely interrupting the signal transduction cascade.

5.3 Light signaling from the H--B eyelet affected resynchronization of the molecular clock in the pacemaker neurons

The finding that the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies displayed a distinct behavioral impairment in resynchronization to a 6 h phase delayed LD of 65 lux prompted us to test whether the behavioral defect exhibited by these flies had any detectable molecular basis. The blocking of neural signals emanating from the H--B eyelet resulted in the inability of these flies to resynchronize the TIM expression to the new LD regime in the pacemaker neurons both on day 3 and 5 of shifted LD. The TIM expression therefore stayed synchronized to the initial LD regime (Fig. 10 lower panels; Fig. 11B; Fig. 14 lower panels; and Fig. 13). This is indeed conceivable because the H--B eyelet possibly has direct connections with the s--LN$_v$s (Hofbauer and Buchner, 1989; Yasuyama and Meinertzhagen, 1999). The s--LN$_v$s are the central behavioral circadian pacemaker neurons in *Drosophila* (Helfrich--Förster, 1998; Kaneko, 1998). The free running behavioral rhythm observed in 6 h phase delayed LD of 65 lux in the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies could be therefore correlated with free running molecular oscillation in the s--LN$_v$s (Fig. 14 lower panels; and Fig. 13B).

Besides in the s--LN_vs TIM expression remained synchronized to the initial LD regime in the l--LN_vs and the DN_1s in the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies (Fig. 10 lower panels; Fig. 11B; Fig. 12; and Fig. 14), both on the day 3 and 5 (except for the l--LN_vs) of the new shifted LD regime. By day 5 the l--LN_vs might be non-synchronized in the absence of any entraining signals from the s--LN_vs, as they behave in DD (Veleri *et al*., 2003; Yang and Sehgal, 2001). It was surprising to see that TIM was affected in the DN_1s in the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies because no connection between the H--B eyelet and the DN_1s was shown yet. Then how could the H--B eyelet influence the TIM expression in the DN_1s? Possibly, in an indirect route from the H--B eyelet via the s--LN_vs to the DN_1s. The DN_1s project axons towards the s--LN_vs and the s--LN_vs send axons towards the dorsal protocerebrum (see in Chapter 1 Fig. 2; Kaneko and Hall, 2000). Hence, it is possible that the desynchronized but reverse phased TIM cycling in the DN_1s could affect the behavioral rhythm. Interestingly the s--LN_vs and DN_1s were the only neurons that retained synchronized molecular cycling in LD of 100 or 1000 lux in the *norpA*^{P41};*cry*^{b} flies (Helfrich--Förster *et al*., 2001), and in the current study they turned out to be the most drastically affected by the expression of tetanus toxin in the H--B eyelet. Thus, the previous and novel results combined indicate that the H--B eyelet synchronizes the s--LN_vs and the DN_1s.

A role for the LN_ds in the behavioral circadian rhythm was inferred (Renn *et al*., 1999; Blanchardon *et al*., 2001; Grima *et al*., 2004; Stoleru *et al*., 2004). The synchronized TIM expression in tune with the initial LD regime in the LN_ds might also have contributed to the free running behavior seen in the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies because the LN_ds showed similar synchronization as in the s--LN_vs (Fig. 13B).

Surprisingly, in the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies PER expression was in synchronization with the new shifted external LD in LNs (Fig. 15 lower panels; Fig. 16B; and Fig. 18B) but the behavioral rhythm was not resynchronized to the new LD regime (Fig. 6B column 3 row 2, --3). This indicates that the TIM expression level may override the PER expression levels to control the LD behavior. However, the mechanism of PER's synchronization with the shifted LD regime remains to be resolved. It is possible that residual CRY^B function transduces the

light signals directly to PER as suggested by Rosato *et al*. (2001). It is interesting to note that the TIM expression largely remained synchronized to the initial LD regime in the s--LN_vs and in the DN_1s (Fig. 13) of the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies. TIM is regarded as one of the crucial clock components in the *Drosophila* circadian clock (Stanewsky, 2002), and it responds immediately to light signals, indicating that TIM mediates photic resetting of the circadian clock in *Drosophila* (e.g. Hunter--Ensor *et al*., 1996). Therefore, blocking a light signal from the H--B eyelet might have resulted in non--synchronization of TIM expression to the new external LD and it retained the initial synchronization profile. TIM levels correlated with the behavioral activity pattern displayed by the H--B^{TD}:*norpA*^{P41};*cry*^{b} flies. When the flies were more active at ZT 22, they showed minimum staining for TIM, and at ZT 10 when the activity was low TIM staining in pacemaker cells was high. Such a negative correlation between locomotor activity and staining was already reported (Helfrich--Förster *et al*., 2001; Yoshii et al., 2004).

5.4 Extra--retinal rhodopsins may not be using the *norpA* encoded PLC?

The *norpA* ^{P41}*;cry*^{b} flies are capable of entraining to LD cycles. They have the H-B eyelet intact, which expresses *Rh 5* and *Rh 6* (Yasuyama and Meinertzhagen, 1999; Malpel *et al*., 2002). It is believed that *Rh 5* in R 8 cells utilizes the PLC (Montell, 1999). Is it possible for the H--B eyelet to use this *Rh 5* (may be *Rh 6* also) to signal the photic information bypassing *norpA* encoded PLC?

Possibly, extra--retinal rhodopsins utilize an alternative signaling route. However, *norpA* is expressed in the H--B eyelet (Malpel *et al*., 2002). May be the H--B eyelet hosts redundant signaling pathways. The following evidence from the literature is presented to support such a possibility.

Rhodopsins are photoreceptors falling in the category of seven transmembrane helixes (7 TH) receptors (Gärtner, 2000). In animal visual opsin, the phototransduction is mediated through a G protein present in the cytoplasm (Franke *et al*., 1992). In contrast, the bacterial sensory opsin accomplishes signaling directly via transducer complex proteins present in the plasma membrane (Gordeliy *et al*., 2002). This means that the signal transduction mechanism from rhodopsin could be varying according to the context, as

suggested by Panda *et al*. (2005) for melanopsin mediated light signaling in the mammalian brain.

Another variation in the 7 TH is exemplified by the retinal G protein coupled receptor (RGR). It is a photopigment that binds retinal as a chromophore like the other rhodopsins. It is expressed in the retinal epithelium (RPE), a monolayer of cells under the retina, in the mammalian eye (Jiang *et al*., 1993; Spudich *et al*., 2000). RGR is believed to have an entirely different function than the rhodopsins. RGR has a retinal photoisomerase function in the RPE. Nevertheless, the presence of the ERY motif that binds G protein in RGR suggests a likely function in signal transduction (Spudich *et al*., 2000; Lamb and Pugh, 2004). However, it lacks the HEK motif that is believed to be needed for G protein activation present in the invertebrate opsin (Gärtner, 2000).

Alternatively, for signaling from 7 TH a G protein coupling may not be necessary always. They can signal via other protein molecules either in the plasma membrane or in the cytoplasm. During the past few years a growing body of evidence suggests that not all 7 TH receptors use G protein coupled signal transduction mechanisms (Hall *et al*., 1999). For example, in *Drosophila* arrestin binds directly to the rhodopsin to inactivate the activated rhodopsin (Ebrey and Koutalos, 2001), it also functions as the adapter molecule to couple GPCRs to the activation of SRC--like kinases; (Schmidt--Ruppin strain of avian sarcoma virus (ASV) subgroup C: *src* gene has a phosphorylation function in ASV) and to facilitate the formation of multimolecular complexes (McDonald *et al*., 2000). (See in Chapter 5, section 5.1 for a detailed discussion about the G protein independent signaling mechanisms in 7 TH).

Based on the alternatives for signal transduction mechanism in the animals and in the bacteria from rhodopsins, and the varying functions of similar 7 TH receptors, like rhodopsin and RGR, it could be speculated that *Rh 5* and *Rh 6* expressed out side the compound eyes might be using a different transduction machinery which may not be requiring the PLC encoded by the *norpA* gene. Therefore, in $norpA^{P41};cry^{b}$ flies *Rh 5* and *Rh 6* in the H--B eyelet might be functioning to transduce light signals to the circadian clock. The expression of *Rh 5* driven

tetanus toxin expression in the H--B eyelet affected this signaling. Downstream of *norpA* the genes *trp* and *trpl* function in the phototransduction cascade (see in Chapter 1, section 2.2.1). It has shown that *trp* and *trpl* are not required for photic entrainment of the circadian clock (Yang *et al*., 1998). Therefore, it is possible to speculate that the photic entrainment of circadian clock bypasses the classical visual transduction cascade.

Yet another possibility was suggested by Mealey--Ferrara *et al*. (2003); a leaky signaling from CRY^{B} could be present in $norpA^{P41};cry^{b}$ flies. If *Rh 5* utilizes the proposed alternative machinery without a PLC, putative signaling from R 8 cells also might be affected by *Rh 5* driven tetanus toxin expression, since it expresses *Rh 5*. Maybe *Rh 5* in the H--B eyelet and R 8 cells utilize different signaling routes dependent on the spatial context. However, expression of *norpA* in the H-B eyelet questions this. If it is not utilized why is it expressed in the H--B eyelet? Perhaps, a redundancy of signaling mechanism exists in the H--B eyelet. Maybe for entrainment there exists one *norpA*--independent signaling route. Since BO functions to signal rapid photophobic behavior (Mazzoni *et al*., 2005), possibly the H--B eyelet also could perform this function, and for this it may utilize another *norpA*--dependent signaling route. Further experiments are necessary to answer these questions.

6 Conclusion

The results presented so far demonstrate that the H--B eyelet plays a role in the behavioral and molecular re--synchronization of the circadian clock in dim light conditions. Therefore, function of the H--B eyelet could be to sense the twilight signals and fine tune the internal circadian clock. Synaptic connections from the H--B eyelet to the LN_vs are tetanus toxin sensitive. Blocking synaptic signaling from the H--B eyelet to the pacemaker neurons, along with the PLC and CRY mediated signaling, abolished the re--synchronization of TIM cycling in pacemaker neurons and resulted in free running locomotor behavioral rhythms during the shifted LD regime. The TIM expression in the pacemaker neurons was negatively correlated with the behavioral activity pattern in the $H\text{--}B^{TD}$:$norpA^{P41};cry^{b}$ flies. These results offer strong evidence for the H--B eyelet to function as a circadian

photoreceptor, independent of PLC encoded by *norpA*. Future research is necessary to decipher the nature of the responsible photopigment(s). *Rh 5* and *Rh 6* are likely candidates but it is questionable if they function independently of the *norpA* encoded PLC because *norpA* is expressed in the H--B eyelet. It is likely that rhodopsins outside the classical photoreceptor cells in the compound eyes might utilize novel signaling mechanisms. Further research is needed to clarify such possibilities. Finally, our results indicate that yet another photoreceptor functions in high intensity light in the $norpA^{P41};cry^{b}$ flies. Possibly it is *Rh 5* and *Rh 6* present in R 8 cells or a novel photoreceptor yet to be identified.

CHAPTER 4

THE DORSAL BRAIN OF *DROSOPHILA MELANOGASTER* HOSTS A SELF--SUSTAINED, LIGHT ENTRAINABLE CIRCADIAN OSCILLATOR

Chapter 4

The dorsal brain of *Drosophila melanogaster* hosts a self--sustained, light entrainable circadian oscillator.

1 Introduction

Most of the organisms show a regular pattern in their physiology and behavior in tune with the environmental LD cycles. Most of the physiological functions and the behavioral activities repeat approximately every 24 h hence they are called circadian rhythms. True circadian rhythms free run in constant darkness (DD) and are therefore considered as endogenous in origin. They are entrainable to the environmental day--night changes. It is believed that the cycling level of clock gene expression is the basis of the endogenous rhythm (see more in Chapter 1, section 4.2). In *Drosophila* the locomotor activity rhythm sustains for weeks in DD (e.g., Helfrich, 1986; Dowse *et al*., 1987). However, a molecular basis for the sustained behavioral rhythm was not proved beyond skepticism because the molecular cycling dampens quickly in DD (e.g., Stanewsky *et al*., 1997a). All attempts to demonstrate the sustained molecular rhythm in a living fly or tissue in DD failed (Stanewsky *et al*., 1997a; Plautz *et al*., 1997a; Giebultowicz *et al*., 2000). Nevertheless, in a subset of clock neurons (s--LN_vs) sustained clock gene cycling for a limited time in DD was reported (Blanchardon *et al*., 2001; Klarsfeld *et al*., 2004; Shafer *et al*., 2002; Yang and Sehgal, 2001; Lin *et al*., 2004). In the present study, we could demonstrate that molecular oscillation sustains for extended times in a subset of clock neurons in DD (Veleri *et al*., 2003). We have demonstrated that *per* oscillation in DD *in vivo* sustains for five days in the dorsal neurons (DN_3s and DN_2s) and in the s--LN_vs pacemaker neurons (Veleri *et al*., 2003). This was attained by employing a real--time luciferase reporter system (Stanewsky *et al*., 1997a), adopted for restricted spatial expression. In conjunction with immunostainings this reporter system has identified a novel molecular circadian oscillator in the dorsal brain of *D. melanogaster* and established the s--LN_vs as a molecular pacemaker oscillator (Veleri *et al*., 2003).

2 Materials

2.1 Flies

disco^{2} (Steller *et al*., 1987).

Pdf^{01} (Renn *et al*., 1999)

Pdf^{01} used in this study was in a *y w* background, hereon called *Pdf*^{01}.

per^{01} used in this study was in a *y w* background (Konopka and Benzer, 1971)

3 Methods

3.1 Assaying bioluminescence from living transgenic fly

Time--based automated bioluminescence assays of luciferase activity in individually monitored *luc*--containing flies were performed as described by Stanewsky *et al.* (1997a). In short, 2--4 days aged flies were collected, which were reared under a regular 12 h: 12 h LD cycles, before being placed in an assay plate. The plates were prepared by filling each well of a white 96--well microtiter plate (Optiplate^{TM}-96, Packard) with fly food (100 μl solution of 1% agar, 5% sucrose and 15 mM luciferin [Biosynth], dissolved in dH_2O water). After the food was solidified, flies anaesthetized by CO_2 were placed in the well and covered with a transparent dome (lids of PCR cups; there were two pin holes for aeration), which allowed to restrain the flies in the respective wells (see more in Stanewsky *et al*, 1997a). Plates were sealed by a thin transparent polyethylene sheet. The sheet also was pierced twice over each well for aeration. The flies were pre--fed on this food for approximately 30 min before an experiment began. The fly--containing plates were then placed in a TopCount Multiplate Scintillation Counter (Packard), that was programmed to count bioluminescence automatically once per hour per fly. The room temperature was maintained at 25 °C, and the room was humidified by an automatic mechanical device.

To record the very low counts of bioluminescence (e.g., from *8.0--luc:9* flies), the flies were first entrained by 12 h: 12 h LD, followed by constant darkness (DD) for 5--7 days, which allowed to reduce the noise in the detected signal due to the

presence of background light in the room in LD cycles. This helped to identify the self--sustained molecular oscillator in the DNs of these transgenic flies.
The data were plotted (uncorrected as to when, during a given hour, a fly's plate was read) as counts per second (CPS) and then processed analytically for period and phase. The analysis was done as described by Plautz *et al.* (1997b) and Stanewsky *et al.* (1997a). Briefly, data were analyzed with FFT--NLLS to determine 'period' (τ), 'relative amplitude error' (rel--amp), and the average CT peak time of expression (phase). Rhythmic flies (% rhythmic) had τ values of 24 $\pm$ 5 h and rel--amp errors < 0.7 (see details in the experimental procedures in Veleri *et al*., 2003).

3.2 Behavioral analysis and immuno--histochemistry

Behavioral analysis and immuno--chemistry was done as described by Veleri *et al.* (2003), and for more details see also in Chapter 2 Methods, sections 2.4, 2.5.

4 Creating "mosaic" transgenic flies expressing *per* in the subsets of clock neurons

A 7.2 kb of promoterless *per* gene was shown to express specifically in LNs (Frisch *et al*., 1994). Therefore, we have generated a similar transgene, *8.0--luc* (Fig. 1). It has 800 bp more first intron material at 5' region than the *7.2* kb fragment. *8.0--luc* missed the sequences that code for last 10 amino acids at the C--terminal of PER and *per*'s 3' untranslated region (UTR). Moreover, it was fused to the luciferase cDNA serving as reporter gene (Stanewsky *et al*., 1997a). The luciferase reporter system allowed us to monitor the rhythmic *per* expression as reported by luciferase in real--time in the individual flies *in vivo*.

We hoped that we might get transgenic flies expressing the *8.0--luc* transgene in a spatially restricted manner such as in the s--LN_vs or the l--LN_vs or in the LN_ds or may be in a combination of these subsets. Furthermore, we also mobilized one of the *8.0--luc* lines to create even more lines with a potentially interesting spatial and temporal expression pattern. In a similar spatially restricted targeted expression approach, enhancer trap element *Mz520--GAL4* was shown to express in subsets

of the LN_vs and it enabled to study these neurons (Grima *et al*., 2004). In another

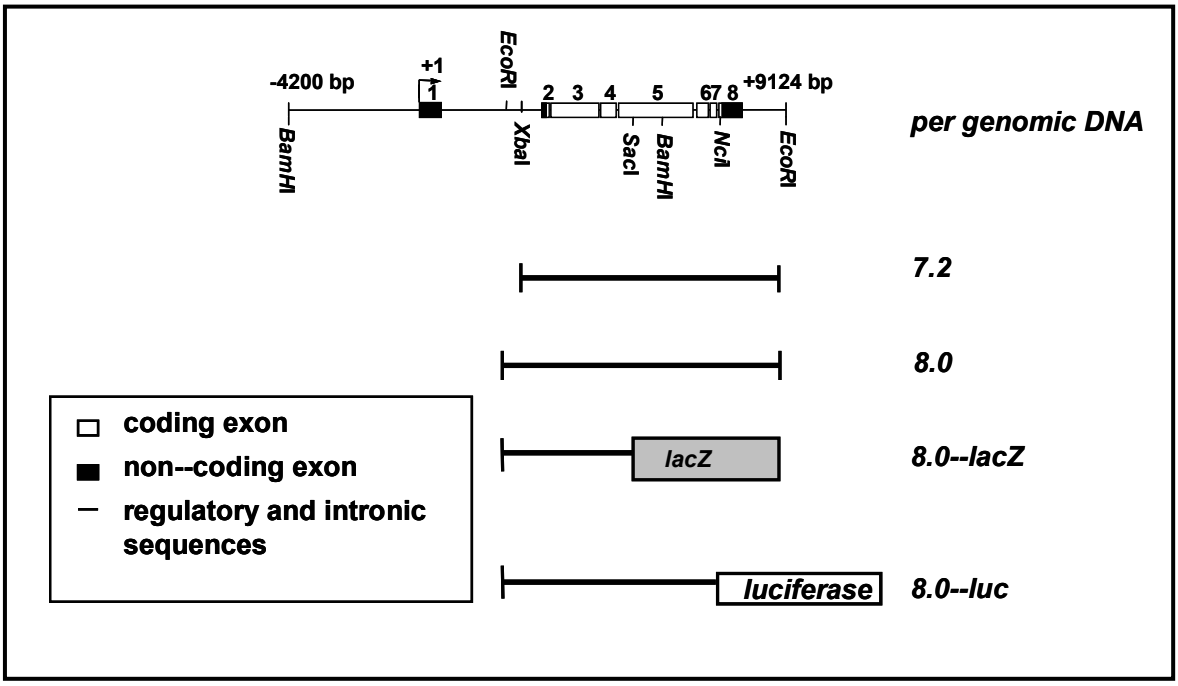

Figure 1 Exon/Intron structure of the *period* locus and transgenic fragments of the *period* gene with or without reporter fusion genes. Upper most panel: structure of the 13.2 kb genomic DNA fragment containing the *per* gene (Veleri *et al*., 2003). Restriction enzyme positions indicate the 5' and 3' ends of the constructs shown below; +1 indicates the transcription start point. Lower panels: the extent of various promoterless *per* constructs with or without reporter genes (*lacZ* or *luciferase*) is shown. In our study we have used the *luciferase* reporter gene fused to a promoterless 8.0 kb *per* gene fragment (bottom construct).

'subtracting' approach Stoleru *et al*. (2004) showed how to utilize *GAL80* (see in Chapter 2 Fig. 1 and section 2.3) to study the clock neurons of interest. In our approach, it would be possible to screen large numbers of flies for expressing the transgene in potential clock cells by looking at the bioluminescence rhythms in DD. Further, the ability of the subset of clock cells expressing the transgene to rescue behavior could be tested in a per^{01} background, in DD. This would give an idea about the transgene expressing clock cell's circadian function, and thus further extend the understanding on the clock cells as demonstrated by recent studies (Grima *et al*., 2004; Stoleru *et al*., 2004).

4.1 A DN specific mosaic transgenic line: *8.0--luc:9*

We have generated transgenic flies in which expression of *per* gene is restricted to certain subsets of clock neurons (see Materials and Method in Veleri *et al*., 2003). One of our mosaic transgenic flies (*8.0--luc:9*) showed robust rhythmic expression in DD, with little or no dampening of the luciferase--reported rhythmicity. The restricted expression of *period--luciferase* transgene (*8.0--luc:9*) in the DN_1s, DN_2s, DN_3s (and rarely in the LN_ds) permitted us to monitor reporter gene activity specifically from these cells in real--time. We also showed by anti--PER staining on the whole mounted brains that the previously described pacemaker neurons (s--LN_vs) maintain sustained expression of PER oscillations after 5 days in DD. In addition, we showed that the DN_2s and DN_3s maintained self--sustained expression of PER oscillation, after 5 days in DD, suggesting that these cells are responsible for the sustained luciferase rhythms (Veleri *et al*., 2003).

4.1.1 DN oscillator is light entrainable

The self--sustained DN oscillator is light entrainable, and it could synchronize the behavioral rhythms to LD (see Fig. 3 in Veleri *et al*., 2003). The light input into the DN oscillator involves *cryptochrome* (Veleri *et al*., 2003). However, blocking CRY and rhodopsin mediated signaling (by applying *norpA; cryb* and gl^{60j} cry^{b} double mutants) did not make the DN oscillator non--synchronizable to LD, indicating the existence of a novel putative photoreceptor synchronizing the DNs. Such a novel photoreceptor may be functioning within or outside the DNs. This finding is interesting because it could support a *norpA* and *cry* independent light signaling mechanism into the circadian clock discussed in the previous chapter (Chapter 3), as well as yet another novel putative photoreceptor functioning in high intensity light in the $norpA^{P41};cry^{b}$ flies. Furthermore, we show a putative *Rh 7* gene expresses in the dorsal brain very close to the DN_{1+2}s (see in Chapter 5). Possibly there exist neuronal connections between the DN_{1+2}s and the cells expressing *Rh 7*. Therefore, *Rh 7* could be the missing photopigment responsible for light entrainment of the DNs in $norpA^{P41};cry^{b}$ double mutant flies. This novel putative photoreceptor may be functioning in the high intensity light in $norpA^{P41};cry^{b}$ flies. The following article (Veleri *et al*., 2003) is presented for details on our finding about the novel DN oscillator, a light entrainable circadian oscillator.

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A Self-Sustaining, Light-Entrainable Circadian Oscillator in the *Drosophila* Brain

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Summary

Background: The circadian clock of *Drosophila* is able to drive behavioral rhythms for many weeks in continuous darkness (DD). The endogenous rhythm generator is thought to be generated by interlocked molecular feedback loops involving circadian transcriptional and posttranscriptional regulation of several clock genes, including *period*. However, all attempts to demonstrate sustained rhythms of clock gene expression in DD have failed, making it difficult to link the molecular clock models with the circadian behavioral rhythms. Here we restricted expression of a novel *period-luciferase* transgene to certain clock neurons in the Drosophila brain, permitting us to monitor reporter gene activity in these cells in real-time.
Results: We show that only a subset of the previously described pacemaker neurons is able to sustain PERIOD protein oscillations after 5 days in constant darkness. In addition, we identified a sustained and autonomous molecular oscillator in a group of clock neurons in the dorsal brain with heretofore unknown function. We found that these "dorsal neurons" (DNs) can synchronize behavioral rhythms and that light input into these cells involves the blue-light photoreceptor *cryptochrome*.
Conclusions: Our results suggest that the DNs play a prominent role in controlling locomotor behavior when flies are exposed to natural light-dark cycles. Analysis of similar "stable mosaic" transgenes should help to reveal the function of the other clock neuronal clusters within the fly brain.

Introduction

It is generally believed that cyclic expression of clock genes is the driving force for behavioral and physiological rhythms in organisms possessing a circadian clock. In *Drosophila* a set of clock genes, organized in regulatory feedback loops, is involved in the generation of the molecular circadian clock. The *period* (*per*) and *timeless* (*tim*) genes are transcribed in a rhythmic fashion and are activated by a heterodimer consisting of the two basic-helix-loop-helix-PAS transcription factors CLOCK and CYCLE (CLK/CYC). PER and TIM proteins subsequently repress their own expression by inhibiting CLK/CYC function in the nucleus and thereby closing the negative feedback loop (reviewed in [1]).

In flies the most prominent biological rhythm controlled by the circadian clock is the rest-activity cycle. Under free-running conditions of constant darkness and constant temperature (DD), this rhythm persists for at least five weeks (e.g., [2]). In stark contrast to these robust behavioral rhythms, studies of clock-gene expression under such free-running conditions revealed a rapid dampening of molecular oscillations within 2–4 days [1]. It is therefore not proven if cycling gene products are required for generating behavioral rhythmicity. Several arguments have been put forward in order to explain the observed discrepancies. (1) Molecular rhythms are usually measured after extraction of mRNA or protein from many individuals (typically 30–50) at a given time of day. Since the internal free-running periods vary slightly between the different animals, this will lead to an overall amplitude dampening the longer the flies are kept in DD (e.g., [3, 4]). (2) Many tissues within one fly contain circadian clocks (e.g., [5, 6]). Without entrainment cues they could internally desynchronize, resulting in damped molecular rhythms when all such tissues are monitored simultaneously; the same could also apply for the clock cells *within* a given tissue. (3) There is a qualitative difference between "pacemaker oscillators" (e.g., those driving robust behavioral rhythms) and "peripheral oscillators" (e.g., the fly's eyes, in which clock gene expression has been analyzed for the majority of chronomolecular studies); only bona-fide pacemakers are able to maintain molecular oscillations in DD. Establishment of *luciferase* as a real-time reporter gene helped to rule out the first possibility; recordings from individual transgenic *per-luc* flies also showed rapid dampening in DD [7]. Together with the finding that isolated *Drosophila* body parts and organs contain circadian clocks (e.g., [5, 6, 8, 9]), this made the second possibility seem likely. But the fact that transcriptional rhythms in individually cultured body parts and organs also rapidly dampen in DD favors the third argument [5, 6, 9]. Therefore, true circadian molecular oscillations could be a unique feature of a set of brain neurons known to control behavioral rhythmicity (e.g., [10, 11]).

Little is known about the features of molecular oscillations in these brain neurons and the function of the various neuronal groups. Among these neurons are the behavioral pacemaker neurons called "lateral neurons" (or LNs), located bilaterally between the optic lobes and the central brain [1]. Clock gene expression within a ventrally located LN group (LNv) has been demonstrated to be sufficient for driving behavioral rhythms in DD [10, 11]. The LNv group consists of five small cells (s-LNv) and four large ones (l-LNv), which project to the dorsal brain and to the optic lobes, respectively [1]. Interestingly, in the l-LNv's PER and TIM, protein oscillations immediately stop under constant conditions, whereas they continue for at least 2 days in DD within the s-LNv's

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[12, 13]. This points to different roles in circadian clock function for the two LNv neuronal groups. The LNv's comprise a minority of clock gene-expressing neurons. There is also a more dorsally located group of LNs (LNd), consisting of approximately six cells on each side of the brain, as well as three bilateral groups of clock neurons in the dorsal brain (approximately 15 DN1s, 2 DN2s, and 40 DN3s [1], Figure S1). All these neurons send projections to the same dorsal brain area to which the s-LNv's project, but not much is known with regard to the function of these cells or whether they contain sustained molecular oscillators [1].

The current study aimed to dissect the biological function of the different clock neurons in the CNS and to ask whether molecular oscillations within at least some of these neurons are the driving force for behavioral rhythms under constant conditions. For this, we generated a transgenic type that is a stable *period*-expression "mosaic": a novel *per-luciferase* construct that generates a fusion protein in only a subset of the clock neurons. Luciferase activity robustly oscillates in DD and, surprisingly, these oscillations do not depend on the LNv pacemaker neurons but instead are generated in the DN3. Although we found that these DN3′s are unable to drive behavioral rhythms in DD, PER expression in these dorsal cells—and probably in two other groups of DNs—mediates synchronized locomotion under LD conditions. We furthermore showed that the phasing of this rhythmicity depends on cryptochrome acting as a light-input factor.

Results

Generation of a Promoterless *period-luciferase* Gene to Create Stable *period* Mosaics

In an attempt to dissect the function of the various clock-neuronal clusters and to demonstrate nondampening molecular oscillations in free-running environmental conditions, our approach was to generate stable mosaics in which expression of the *per* clock gene is restricted to certain subsets of pacemaker neurons in the brain. Based on previous observations, we generated a *period-luciferase* fusion gene, which lacks 5′-flanking *per* sequences, and created transgenic lines carrying this construct. Frisch et al. [10] had shown that expression of a *per* transgene (called *7.2*) lacking these regulatory sequences either does not occur, for certain genomic insertion sites, or is restricted to subsets of the known PER-expressing cells, including the LNs. Compared with the original *7.2* kb *per* transgene, the new one contains an additional 0.8 kb of the first intron and was named *8.0-luc* (Figure 1).

Non-Dampening *period* Expression Independent of the Behavioral Pacemaker Neurons

Out of 11 independently isolated *8.0-luc* lines, three showed luminescence expression significantly above background (Figure 2, Table 1, and data not shown). One of these lines (*8.0-luc:9*) was robustly rhythmic in DD, with little or no dampening of the luciferase-reported rhythmicity (Figure 2). Robust free-running molecular cycling was also indicated by the high percentage of

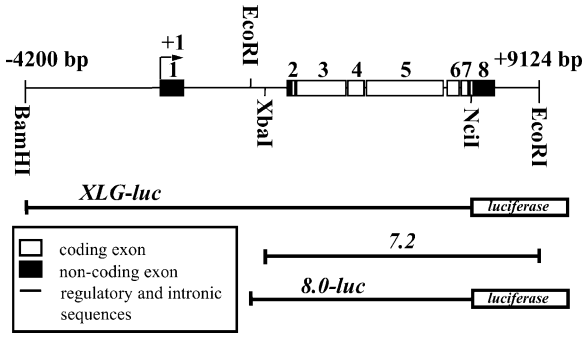

Figure 1. Exon/Intron Structure of the *period* Locus and *period-luciferase* Fusion Genes

Upper panel: structure of a 13.2 kb genomic DNA fragment containing the *per* gene is shown. Restriction enzyme positions indicate the 5′ and 3′ ends of the constructs shown below; +1 indicates the transcription start. Lower panel: the extent of genomic *per* sequences of the *per-luc* transgenes used in this study as well as that of the original *7.2* construct [10]. Note that both *XLG-luc* and *8.0-luc* contain the entire *per* ORF except for 10 amino acids at the C-terminal end (cf. *XLG-lacZ* [14]).

rhythmically expressing flies, which was determined after numerical analysis of the raw data (robust rhythms are associated with low relative-amplitude errors, as described in the Experimental Procedures and documented by the "rel-amp" values in Table 1). In contrast, a control transgenic type, containing the 5′-flanking regulatory region of *per* in addition to almost all of its coding sequences (*XLG-luc*, Figure 1), showed rapid dampening of molecular oscillations in DD (Figure 2, Table 1). Most likely *XLG-luc* is expressed in all known *per*-expressing cells, based on our own observations (Figures 4A and 4B; data not shown) as well as on the description of the spatial expression pattern mediated by an *XLG-lacZ* transgene, which contains the same *per* sequences fused to a different reporter [14]. Probably as a consequence of this widespread expression-pattern—including tissues and cells that run out of phase with each other or dampen out completely—*XLG-luc* flies exhibit rapid dampening in DD even as individuals (Figure 2). The remaining *8.0-luc* lines (*4* and *11*) exhibited higher levels of reporter activity compared with *8.0-luc:9*; this was correlated with rapid dampening of luciferase expression in DD (Table 1 and data not shown), probably as a result of more widespread transgene expression in the two strains compared with *8.0-luc:9*.

Sustained molecular rhythmicity of *8.0-luc:9* expression depends largely on the presence of endogenous PER: Overall rhythmicity was reduced and associated with high rel-amp errors when this transgenic type was tested in a per^{01} genetic background (Table 1). This could be due to a negative impact of the luciferase part on PER function in the PER-LUC fusion protein, or to a lack of additional transcriptional regulation of the endogenous *per* gene (see below).

Because *8.0-luc:9* flies exhibited the lowest overall luminescence levels, we suspected that expression in this line might be restricted to certain clock neurons in the brain. Thus, these *8.0-luc* flies, in parallel with the *XLG-luc* type, were tested for their ability to restore

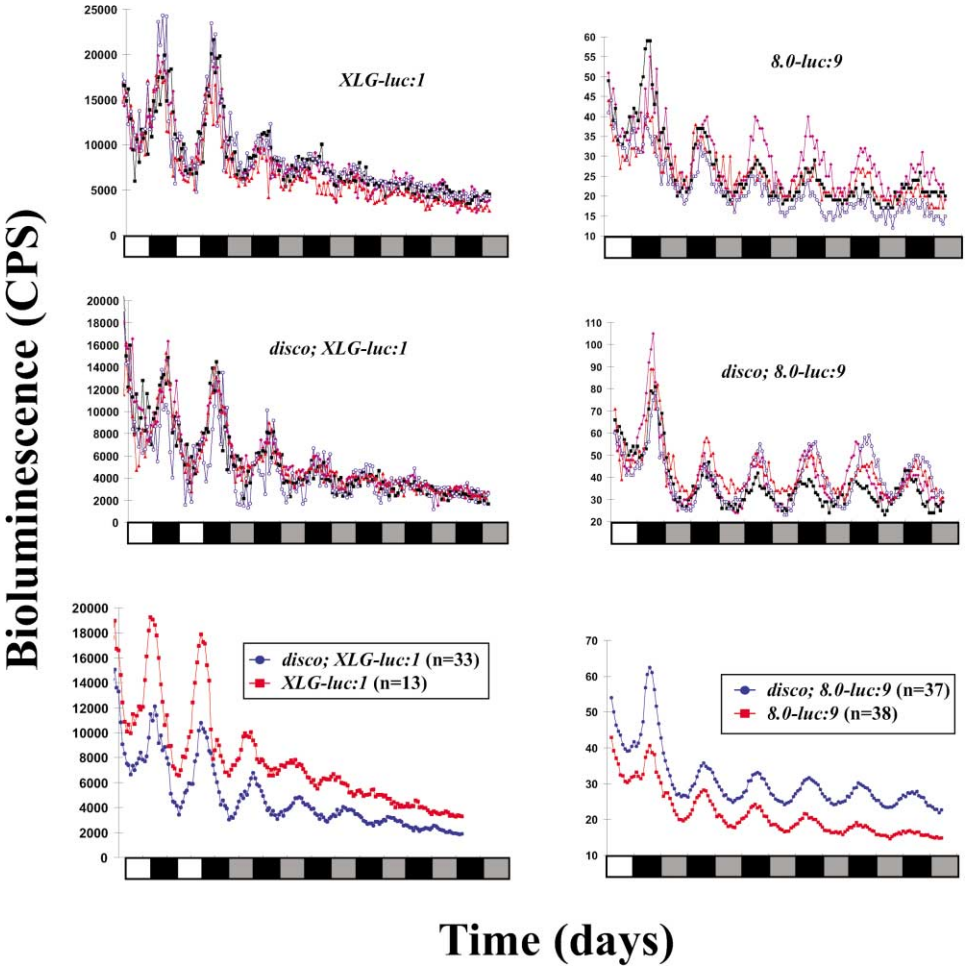

Figure 2. Bioluminescence Rhythms Recorded from *XLG-luc* and *8.0-luc:9* Transgenics in Wild-Type and *disco* Genetic Backgrounds

Left panel: recordings of 4 *XLG-luc* individuals that were determined to be "rhythmic" after FFT-NLLS analysis (see Experimental Procedures) in a *disco*$^{+}$ (top) compared with a *disco* (middle) genetic background. Note the rapid dampening after transfer to DD; this dampening also becomes apparent after recordings from many rhythmic individuals of each genotype are averaged (bottom). Right panel: recordings of four rhythmic *8.0-luc:9* individuals in *disco*$^{+}$ (top) and *disco* (middle) backgrounds. Note that the *8.0-luc:9* records show no (or almost no) dampening. The weak dampening observed in the average plots (bottom) is a consequence of the different individual free-running periods. Bioluminescence was measured in counts per second (CPS). The black and white bars under each panel indicate when lights were off (12 hr) and on (12 hr), respectively. Gray bars indicate subjective daytime in DD (12 hr).

behavioral rhythms in *per*^{01} mutant flies. *XLG-luc* restored robust behavioral rhythms in about 50% of the flies tested (Table S1 in the Supplemental Data), indicating that the luciferase part of the fusion protein does not have a severe impact on PER's biological activity. Moreover, under LD conditions *per*^{01}*;XLG-luc* flies showed synchronized behavioral rhythms similar to those of wild-type controls (Figure 3, cf. [15]). In contrast, the *8.0-luc:9* transgene was unable to restore behavioral rhythmicity to *per*^{01} flies in DD (Table S1). Nevertheless, *8.0-luc:9* individuals did show synchronized behavior in LD cycles (Figure 3), especially the characteristic anticipation of the lights-off transition in the evening (cf. [15]). This behavior is indicative of an at least partially functioning clock and clearly differs from that of *per*^{01} animals, which simply react to the LD transition (Figure 3, cf. [15]). Therefore, it seems as if the *8.0-luc:9* transgene would be expressed at least in a subset of clock neurons within the brain.

To find out whether *8.0-luc:9* is expressed in the LN cells, we crossed the transgene into a *disco* genetic background, *disco* being a mutation that largely eliminates LN brain cells ([4, 11]; Figure 5; Table S2). We expected *8.0-luc*-driven luminescence to disappear if the construct were solely expressed in the LNs. But neither the (low) level of reporter signal nor the robust rhythmic expression was affected by *disco* (Figure 2), indicating that *8.0-luc:9* is not expressed in the LNs. Thus, the oscillator responsible for the sustained molecular rhythmicity is independent of these brain neurons.

8.0-luc:9 Is Expressed in All Dorsal Neuronal Clusters

Because DNs 1–3 are present in *disco* flies [4, 16], we suspected *8.0-luc:9* to be expressed within these cells. Indeed, anti-PER immunostainings on whole-mounted brains of *per*^{01}*;8.0-luc:9* flies showed that PER signals were largely restricted to cells located in the region of

Table 1. Bioluminescence Rhythms of *XLG-luc* and *8.0-luc* Transgenic Lines in Various Genetic Backgrounds

Genotype	n	Percent Rhythmic	τ (hr)	rel-amp	Phase (hr)
XLG-luc:1	51	43.1	22.7 ± 0.4	0.50 ± 0.02	23.6 ± 1.0
disco;XLG-luc:1	44	75.0	23.2 ± 0.1	0.53 ± 0.02	23.1 ± 0.5
per^{01};XLG-luc:1	47	21.3	21.5 ± 0.4	0.61 ± 0.03	2.6 ± 1.7
XLG-luc:2	43	93.0	22.6 ± 0.1	0.48 ± 0.02	0.7 ± 0.5
disco;XLG-luc:2	33	84.8	23.0 ± 0.1	0.45 ± 0.02	22.9 ± 0.5
per^{01};XLG-luc:2	40	0			
8.0-luc:9	110	85.5	23.1 ± 0.1	0.41 ± 0.01	23.4 ± 0.3
disco;8.0-luc:9	407	74.7	23.5 ± 0.1	0.38 ± 0.01	23.3 ± 0.3
per^{01};8.0-luc:9	69	24.6	24.6 ± 0.5	0.59 ± 0.02	23.5 ± 1.2
8.0-luc:9;gl^{60j}	81	91.4	22.2 ± 0.1	0.43 ± 0.01	23.1 ± 0.2
8.0-luc:9;cry^{b}	56	51.8	22.5 ± 0.2	0.49 ± 0.02	1.0 ± 1.6
8.0-luc:9;gl^{60j} cry^{b}	48	43.8	22.7 ± 0.3	0.54 ± 0.02	3.0 ± 1.3
8.0-luc:4	67	52.2	23.9 ± 0.1	0.55 ± 0.02	1.6 ± 0.4
per^{01};8.0-luc:4	52	0	-	-	-
8.0-luc:11	92	37.0	24.3 ± 0.2	0.56 ± 0.02	23.6 ± 0.7
per^{01};8.0-luc:11	33	6.1	25.3 ± 0.5	0.57 ± 0.11	21.0 ± 0.7

Flies were recorded for 5–7 days in constant darkness (DD) at 25°C. Data were analyzed with FFT-NLLS software to determine "period" (τ), "relative amplitude error" (rel-amp), and the average CT peak time of expression (Phase). Rhythmic flies (% rhythmic) had τ values of 24 ± 5 hr and rel-amp errors <0.7 (as described in the Experimental Procedures). Prior to the experiment, flies were entrained to 12 hr:12 hr LD cycles at 25°C for at least 3 days.

the DNs (Figures 4C, 4F, and 4I; Table S2). Moreover, this immunoreactivity (PER-IR) was only observed at ZT23 (ZT = Zeitgeber Time; specifies time in a 12 hr:12 hr LD cycle with respect to lights on [ZT0] or lights off [ZT12]) and not at ZT11 (a classic feature of temporally varying PER-IR, e.g., [4]), indicating that DN expression is the source of the robust luminescence rhythms emanating from *8.0-luc:9* flies (Table S2). Additionally, double-labeling experiments with anti-PER and anti-TIM confirmed that *8.0-luc:9* is indeed expressed in the DN1, DN2, and DN3 neuronal clusters (Figures 4C–4K). We further analyzed PER's spatial expression by staining tissue sections of whole *per^{01};8.0-luc:9* flies. Consistent with the low luminescence levels emanating from whole intact animals, we did not observe any PER staining significantly above background outside the central brain (data not shown); this is in agreement with results obtained from the *7.2 per* transgenic type [10].

Only Certain Groups of Clock Neurons Contain a Circadian Oscillator

Because *8.0-luc:9* is expressed in all three DN clusters, we sought to determine whether all or only a subset of the DNs are responsible for the observed molecular rhythmicity in DD. We stained control and *disco* flies with anti-PER antibodies at CT11 (CT = circadian time, which specifies the time in DD when lights would have been on [subjective day] or off [subjective night] with

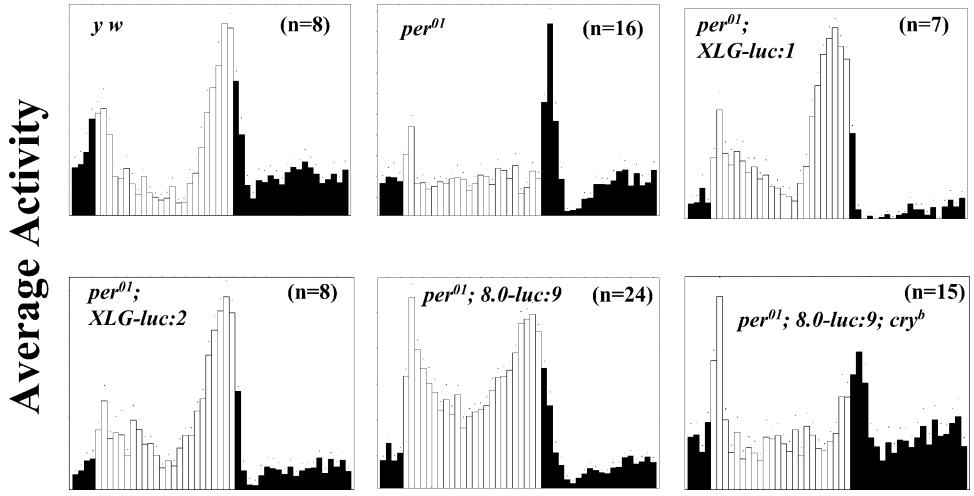

Figure 3. Average Rest-Activity Pattern under Light-Dark Conditions

Male flies of the indicated genotype were analyzed for 5–7 days under 12 hr:12 hr LD cycles at 25°C. White bars indicate activity levels when the lights were on; black bars indicate activity levels when they were off. SEMs are indicated by the dots above each column. Note that all genotypes, except *per^{01}*, increase their locomotion before lights off.

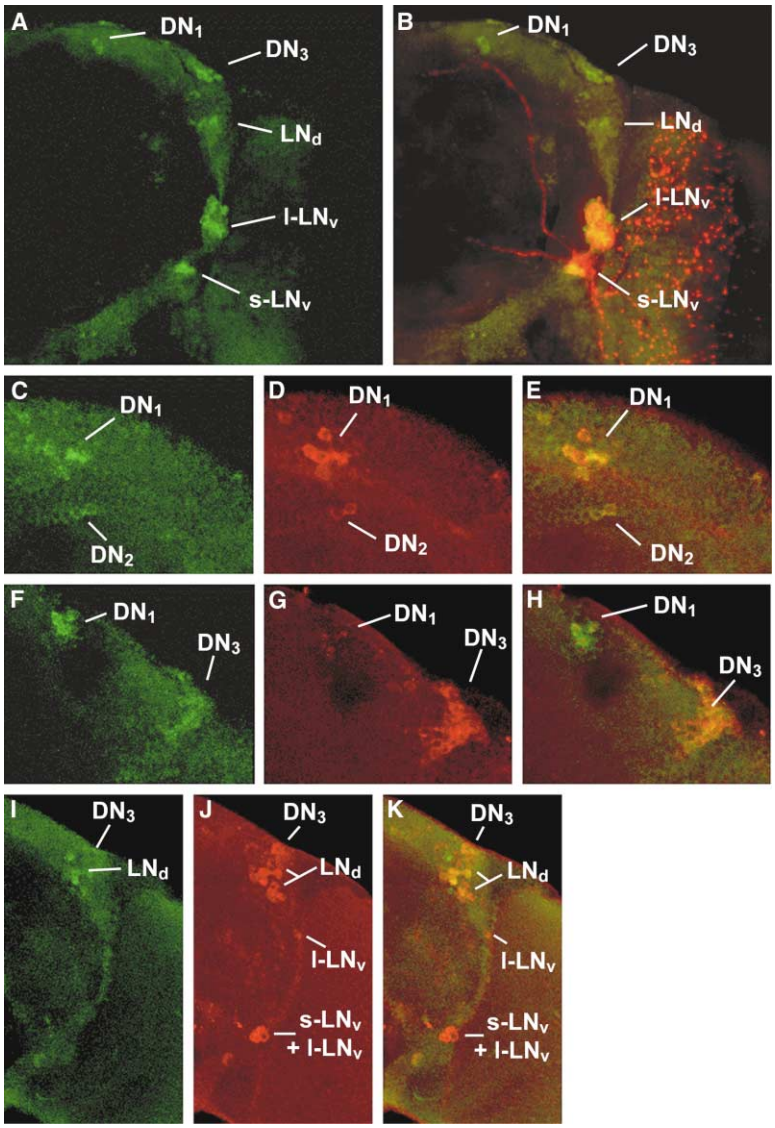

Figure 4. Spatial Expression Pattern of PER-LUC Fusion Proteins in Brains of *XLG-luc:1* and *8.0-luc:9* Transgenics

(A and B) *per*^{01}*; XLG-luc:1* flies stained with anti-PER (green) and anti-PDF (red) at ZT23. The *XLG-luc* transgene is expressed in all different groups of clock neurons (DN1, DN3, s-LNv, l-LNv, and LNd) except DN2 cells, which were not stained in this particular brain. Note that only the two LNv groups coexpress *XLG-luc* and PDF.

(C–K) *per*^{01}*; 8.0-luc:9* brains double-labeled (at ZT23) with anti-PER (green) to reveal transgene expression and anti-TIM (red) to track endogenous TIM expression. (C, F, and I) confocal image showing anti-PER signals or anti-TIM only (D, G, and J) or both (E, H, and K). (C, D, and E) Posterior optical sections showing labeling in the DN1 and DN2; (F, G, and H) posterior optical sections of another brain showing the DN1 and DN3; (I, J, and K) anterior sections showing the LNs and a subset of the DN3. Note that TIM signals are visible in all LNs and DN1-3, but PER immunoreactivity is largely restricted to DN1-3, with the exception of occasional staining in the LNd ([I–K]; Table S2).

respect to the previous LD cycle) and CT23 (also at CT9 and CT21 for the *disco* specimens) after keeping them for 5 days in DD (Figure 5). In the controls there was a significant difference in PER staining intensity between the two time points only in s-LNv, LNd, and DN2, cells as well as within the DN3 cluster, but not in the l-LNv and DN1 cells (Figures 5 and 6, Table S2; cf. [12, 13]). This indicates that PER expression cycles in the s-LNv and LNd cells, which would be consistent with the behavior-controlling pacemaker function inferred for these subsets of the lateral neurons [10].

PER levels in the DN2 cells were high during the subjective day and low during subjective night, similar to the antiphase cycling described for larval precursors of DN2s [17]. In *disco* individuals, although they are behaviorally arrhythmic in DD (e.g., [18]), significant differences in PER-IR were observed at CT9 and CT11 versus CT21 and CT23 in the DN3, and clear "antiphase" differences in the DN2 were discerned (Figures 5 and 6; Table S2). These results indicate that the DN3s are responsible for the robust circadian luminescence rhythms observed in *8.0-luc:9* flies and confirm that this oscillator operates independently of the LNs. Because there are only two DN2 cells in each brain hemisphere, the antiphase-cycling observed in these neurons is not able to overcome the DN3-mediated rhythms that comprise the majority of *8.0-luc*-expressing DN cells (approximately 80 DN3 cells total [1]).

Light Input into the Newly Identified Circadian Oscillator

A final set of experiments took advantage of our ability to monitor *8.0-luc:9* expression "on-line." We asked which photoreceptors are responsible for synchronizing PER expression within the DN brain cells. Thus, we analyzed *8.0-luc:9* expression in the background of the *glass*^{60j} mutation; it removes all external photoreceptors, a photoreceptive structure known as the H-B eyelet, and DN1 brain cells [19]. To specify the degree of DN1 loss and to better resolve the projection pattern of the DN3 cells, we labeled all LN and DN groups in wild-type and *glass*^{60j} flies carrying a *tim-gal4* and *UAS-gfp* transgene [20].

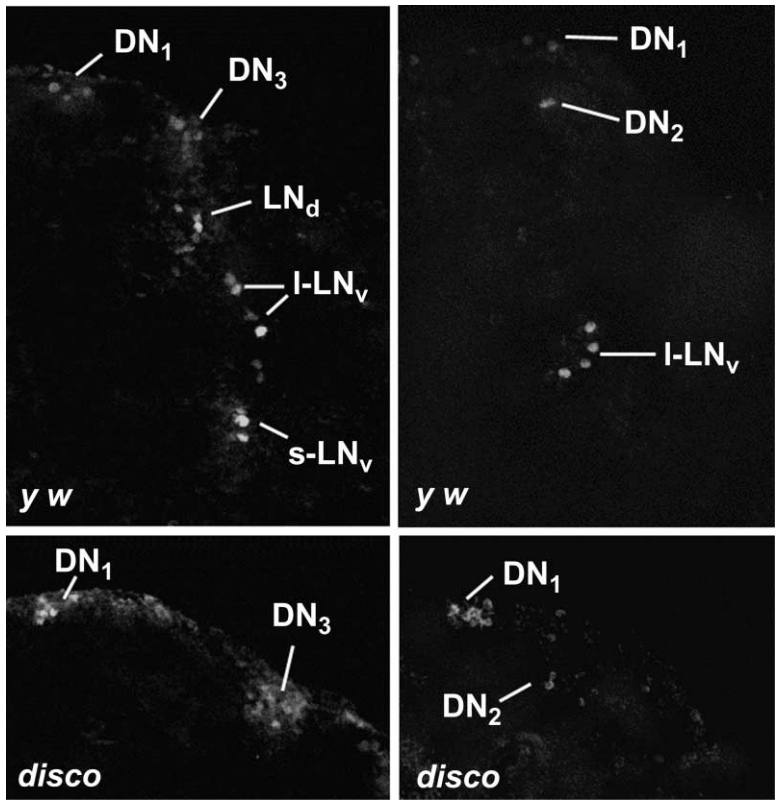

Figure 5. PER Expression under Free-Running Conditions

Anti-PER staining in *y w* (non-transgenic control) and *disco;8.0-luc:9* brains, on the fifth day of DD. Left and right panels show staining of the indicated genotype at CT23 and CT11, respectively. Note that in *y w*, s-LNv and DN3 cells are predominantly stained during subjective night; these results are similar to the signals observed within DN3 cells in *disco* brains. In both genotypes, the DN2s are mainly stained during subjective day, indicating antiphase oscillations in this neuronal type.

Whole-mounted brains were inspected for GFP expression, and robust signals were detected in all known clock neurons (Figure S1). In the *glass*^{60j} mutants, only two of the DN1 cells are left, whereas numbers and locations of DN2 and DN3 cells seemed unaffected. DN1 perikarya have been shown to send axons toward the location of LNv cells [20]; we now show that certain projections from the DN3 cells terminate near the LNv cells as well. Interestingly, the density of projections from the dorsal brain toward the LNv region seemed not to be reduced in *glass*^{60j} flies compared with the wild-type, indicating that the DN3 cells contribute significantly to the observed arborizations contacting the LNv cells (arrows in Figure S1).

8.0-luc:9;glass^{60j} flies were exposed to at least three 12 hr:12 hr LD cycles before being monitored for luciferase expression in DD. Such reporter oscillations were clearly synchronized among different animals, which shows that the structures and cell types removed by *glass*^{60j} are not necessary for the light input into the DN3 (Figure 7; Table 1).

Next we determined whether the blue-light photoreceptor encoded by the *cryptochrome* (*cry*) gene plays a role in this process. Although *8.0-luc:9;cry*^{b} flies, which lack functional CRY protein [21], showed synchronized oscillations, the phase of peak expression was delayed by several hours as compared with the *cry*$^{+}$ situation (Figure 7; Table 1). Even after *glass*^{60j} was combined with

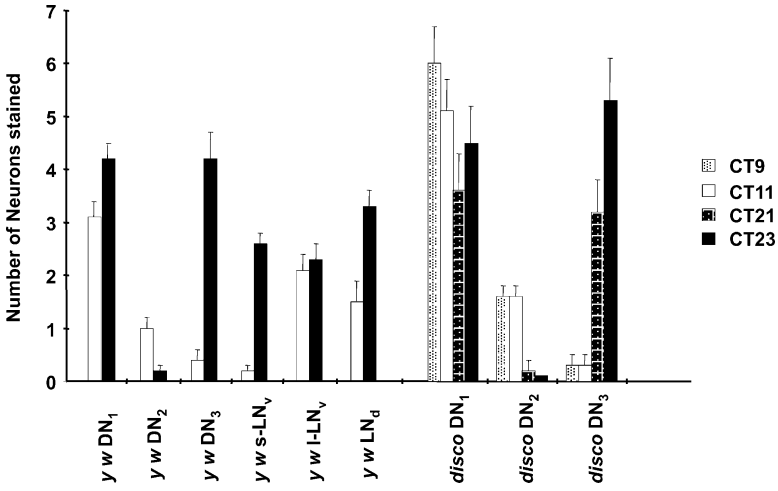

Figure 6. Quantification of PER Expression after 5 Days in DD

y w (*disco*$^{+}$) and *disco* flies were entrained to 12 hr:12 hr LD cycles for 3 days, then released into 5 days of DD, after which they were collected at the indicated CT. Whole-mounted brains were stained with anti-PER. Brain hemispheres were analyzed separately, and the average numbers of stained cells along with the SEM were plotted for each neuronal group.

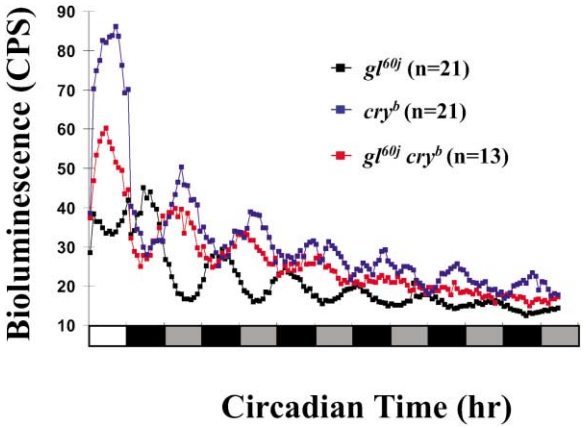

Figure 7. Bioluminescence Rhythms Recorded from *8.0-luc:9* Transgenics in Different Photoreceptor-Defective Mutant Backgrounds

8.0-luc:9 transgenics carrying the indicated photoreceptor mutation were analyzed in DD after initially being entrained to at least three cycles of 12 hr:12 hr LD at 25°C. All genotypes show clearly synchronized oscillations. Note that the two genotypes involving cry^b demonstrate a phase of peak expression that is delayed with respect to the $glass^{60j}$ case or others that included the cry^+ allele (Figure 2, Table 1). Black, white, and gray bars are as in Figure 2.

cry^b, *8.0-luc:9* oscillations could still be synchronized (Figure 7; Table 1). These findings indicate that CRY contributes to synchronization of the DN3s but also suggest that another input route contributes to light entrainment of these dorsal-brain cells.

cry^b had little influence on the free-running properties of the DN3 oscillator; rhythmic expression was robust (Figure 7), although the proportion of rhythmic individuals was reduced as compared with the proportion for transgenic individuals carrying the normal cry^+ allele (Table 1). This result argues that *cry* plays only a minor role as a clock factor in neurons that control locomotor behavior.

Finally, we attempted to correlate synchronization of molecular rhythmicity with behavioral entrainment. Rest/activity cycles of doubly mutant per^{01};*8.0-luc:9*;cry^b flies could be entrained to a 12 hr:12 hr LD cycle, but with a less pronounced anticipation of the lights-off transition than for the cry^+ case (Figure 3). Moreover, the per^{01};*8.0-luc:9*;cry^b flies displayed an "evening peak" of locomotor activity that was delayed as compared with the per^+ control, which correlates with observations of the molecular oscillations.

Discussion

Free-Running Circadian Oscillators in the Fly Brain

Using a novel promoterless *period-luciferase* transgene, we demonstrated that a true circadian oscillator is located in the dorsal brain of the fly. Given the discrepancy between long-lived circadian locomotor rhythms in constant conditions (DD) on one hand and the rapid dampening of molecular oscillations on the other, we asked if in at least certain brain neurons clock gene cyclings would continue in DD. We show that the s-LNv cells display substantial PER oscillations in DD, indicating that they contain a sustained molecular oscillator able to drive behavioral rhythms. In contrast, oscillations in the LNd cells occurred with a reduced amplitude, suggesting that this cell type drives the weak behavioral rhythmicity observed in flies lacking all LNv cells or mutant for the LNv-specific neuropeptide PDF [22].

Surprisingly, we found that in addition to the two LN groups, dorsal-brain clock neurons with so-far-unknown function—the DN3 cells—contain a circadian oscillator. This oscillator is independent of the LNs as demonstrated by the robust *8.0-luc:9* reporter gene rhythms in a *disco* mutant background as well as by the sustained PER oscillations in the DN3s of *disco* flies after they were kept for 5 days in constant darkness. These results are even more astonishing in light of *disco*'s behavioral arrhythmicity in DD ([18]; Table S1), which is probably caused by the elimination of all three LN groups. In the present study only one of the *disco* individuals was immunoreactive for PER in any of the three LN clusters, implying almost complete elimination of these cell types in the stock we used (Table S2).

The DN2 cells seem to be special, owing to the fact that larval precursors of the cells show PER and TIM cycling with an opposite phase (in both LD and DD conditions) from other clock neurons in these developing animals [17]. In adults, clock protein cycling in the DN2 cells is synchronized with that in the other neurons under LD conditions and for the first 2 days in DD ([16, 19]; Table S2). Here we show that after 5 days in DD, PER expression within these cells cycles, again with a phase *opposite* to that of the s-LNv and DN3 cells (Figure 6). A likely explanation is that opposite-phase cycling is the default state of the DN2 oscillator and that only light input can synchronize it with the other clock cells. If this is true, DN2 in larvae would lack any photic input and only acquire it during or after metamorphosis.

But why do the DN2 and DN3 clusters contain a circadian oscillator even though they do not use it to drive free-running behavior? The simplest explanation would be that all neuronal clock cells—and perhaps even non-neuronal ones—contain such a true oscillator but that coupling among the DN2 and DN3 cells (plus the s-LNv and LNd cells) is especially tight.

Alternatively, the nonrhythmic l-LNv and DN1 cells, as well as peripheral tissues in which clock gene cyclings occur, could contain damped oscillators. These would be unable to maintain oscillations for prolonged times in DD, as suggested by previous reports in which transcriptional rhythms of various cultured body parts rapidly dampened in DD [5, 6, 9]. But it is still possible that the clock proteins robustly oscillate in all or a subset of these tissues. To determine to what extent all clock-gene expressing cells contain a circadian oscillator, one would need to monitor clock protein cyclings—perhaps by applying our *XLG-luc* transgenic flies—at the level of single cells in the various tissues.

Chronobiological Functions of the Dorsal Neurons

Although the DN3 brain cells contain a circadian oscillator, these neurons are not able to drive behavioral rhythms in DD (Table S1). This result is not surprising

in light of the fact that *disco* mutant flies, lacking LNv and LNd cells but containing all DN groups, behave rhythmically in DD only for a maximum of 2 days before turning completely arrhythmic [11, 16, 18]. Nevertheless, *disco* mutants are able to synchronize their behavior to LD cycles, similar to what we observed for per^{01};*8.0-luc:9* flies (e.g., [11, 15, 16, 18]; Figure 3). Similar effects on LD and DD behavior were obtained with flies in which the LNv cells had been ablated by expression of cell-death genes specifically in this cell group [16, 22]. Therefore, it seems clear that the DNs are able to drive synchronized behavior under LD conditions in the absence of LNs or PER expression therein. Interestingly, all genetic variants in which the LNs are either missing (e.g., in *disco*) or lack the LNv-specific neuropeptide PDF, show shortened (<24 hr) free-running behavioral rhythms (for a limited time these individuals stay rhythmic in DD, e.g., [11, 22]). Similarly, free-running periods for *8.0-luc:9*-reported molecular rhythms are about 1–2 hr shorter compared with those of *8.0-luc:4* and *8.0-luc:11* (Table 1), indicating that the DN3 oscillator has a <24 hr endogenous period. Therefore, in wild-type flies this dorsal-neuronal oscillator could function as a modulator of period length because it is principally determined by the s-LNv and LNd clusters.

Photoreceptors Involved in the Synchronization of the Dorsal Neurons

The DN1 and DN3 cells also send projections toward the s-LNv cell bodies (Figure S1; [20]). Given the ability of these dorsal neurons to mediate synchronized behavior, it seems possible that they contribute to light entrainment of these cells or receive light signals via these processes. Molecular rhythms of *8.0-luc:9* expression could still be synchronized in the $glass^{60j}$ mutant, indicating that the deleted photoreceptors and neurons are not required for light input into the DN3 cell group. Applying the cry^{b} mutant revealed that synchronization of the DN3 is still possible, but molecular cycles as well as locomotor peaks occurred with a substantial phase delay (Figures 3 and 7; Table 1). This shows that cryptochrome (CRY) is involved in the light synchronization of the DN3, consistent with the observation that the *cry* gene is transcribed in these cells [23]. In the $glass^{60j}$ cry^{b} double mutant, molecular oscillations could still be synchronized (Figure 7). Compared with the single mutants, the rhythms dampened more rapidly in DD, indicating that the doubly mutant individuals either have more variable period values and/or that internal clock function is somehow impaired more drastically by simultaneous removal of *cry* and *glass* function (Table 1).

Although behavioral rhythms of this double mutant could not be entrained by LD cycles, they showed decreased locomotion immediately after lights on and an increase after lights off, especially at high light intensities (which has been attributed to "masking" effects of light [19]). Flies in the current study were kept in similar bright-light conditions (500-700 lux) before their luminescence rhythms were analyzed, and it is therefore possible that the DN3s mediate the masking response in doubly mutant $glass^{60j}$ cry^{b} flies. In any event, some light-sensing abilities are retained by the DN3 cells, which are able to synchronize molecular rhythms in the absence of external photoreceptors, H-B eyelet, DN1, and CRY; but the photopigment mediating these responses is not known. In this regard, the existence of an "extra" such input route to the fly's brain clock (in addition to the routes that use external eyes, H-B cells, and CRY's blue-light reception) was inferred from results reported by Malpel et al. [24].

Conclusions

We were able to analyze biological rhythms (synchronization of behavior) and the underlying molecular oscillations (*per-luc* reporter gene rhythms in the DN cells) in parallel in the intact animal. This allowed us to elucidate a chronobiological role for heretofore poorly characterized dorsal-brain neurons—the clock-gene-expressing DN3 cells. These neurons contain a true circadian oscillator, which functions independently from the behavioral pacemaker neurons (within LN cells) and contributes to synchronized locomotor rhythms under light-dark conditions. Our study directs attention toward analyzing the specific functions of the different clock neuron clusters within the fly brain. Although the importance of the s-LNv cells in controlling sustained behavioral rhythms is well established (e.g., [11, 22]), specific ablation of the 2 LNv groups, or lack of the LNv-specific neuropeptide PDF, does not lead to complete arrhythmicity in free-running conditions [22]. Moreover, expression of a neurotoxin in all (LN plus DN) groups of clock neurons led to behavioral phenotypes similar to those of flies carrying per^{01} and tim^{01} loss-of-function mutations [25]; also, *disco* flies—lacking the LNv and LNd neuronal clusters—still show synchronized behavior under LD cycles (e.g., [11, 15, 16, 18]). Taken together, these findings point to a contribution of the LNd and DN neuronal clusters in regulating behavioral rhythms. The current molecular, anatomical, and behavioral results—obtained by our "stable mosaic" strategy—point to the meaning of these separate neural substrates. Future generation of similar mosaics, in which clock genes would be expressed in other subsets of clock neurons, should help to decipher further chronobiological functions of various neuronal groups in the *Drosophila* brain.

Experimental Procedures

Drosophila Strains and Generation of *period-luciferase* Constructs

All strains used carried an X chromosome marked with *y w* (*y Df(1)w;* [26]), resulting in white eye and yellow body color. The cry^{b} mutant is described in [21], and per^{01} and $glass^{60j}$ are described in [26]. The *disco* allele used in the current study was $disco^{2}$, which is molecularly identical to $disco^{1}$ [27], the mutant used in two of the cited studies [11, 16]. The $glass^{60j}$ cry^{b} double mutant was generated by Helfrich-Förster et al. [19]; the *tim-gal4* and *UAS-gfp* flies are described in [20]. For generation of the *XLG-luc* and *8.0-luc* transgenics, see the Supplemental Data.

Analysis of Bioluminescence Rhythms

Luciferase expression of individual flies carrying the *XLG-luc* or *8.0-luc* transgenes was measured as described in [28]. Prior to each experiment, flies were entrained for at least 3 days to a 12 hr:12 hr LD cycle at 25°C and kept in the same regime for the first 1–2 days of the experiment. Subsequently, lights were turned off for good, and flies were monitored for 5–6 days in constant darkness (DD).

Raw data were plotted and analyzed with Import and Analysis software [29]. For details, see the Supplemental Data.

Behavior

Locomotor activity of adult males was monitored automatically and analyzed as described (e.g., [14]). Generally, flies were entrained for one day in 12 hr:12 hr LD at 25°C and then assayed for locomotor activity for the next 5 days in the same LD regime; this was followed by 7 days in constant darkness (DD). For generation of behavioral plots and data analysis, see the Supplemental Data.

Immunohistochemistry

Prior to collection at ZT11 and ZT23, male flies were entrained for at least 3 days under 12 hr:12 hr LD conditions (with a light intensity of approximately 1000 lux). (For DD experiments, flies were kept in constant darkness after 3 days entrainment in LD and were collected during the fifth day of DD). Whole-mounted brains were stained with polyclonal rat anti-TIM [17] or rabbit anti-PER [14] at 1:1000 dilution. The anti-PDF stainings shown were done with the monoclonal antibody *nb33* diluted 1:100 (Figure 4) or with an antibody against crab PDH (Figure S1) as described in [30]. The respective fluorescent secondary antibodies (AlexaFluor [Molecular Probes, OR]) were diluted 1:200. For details regarding the staining protocols and *nb33*, see the Supplemental Data. Brains were stored at 4°C until confocal observations were made with a Leica TCS NT microscope. Numbers of stained neurons were determined with a high-magnification 63× lens (further zoomed digitally 3×).

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A Self-Sustaining, Light-Entrainable Circadian Oscillator in the *Drosophila* Brain

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Supplemental Experimental Procedures

Generation of *XLG-luc* and *8.0-luc* Transgenics

We generated *XLG-luc* as follows: we generated a 1.2 kb BamHI/NciI *per* subclone, which extended from +5449 to +6658 (+1 = transcription start) and in which the NciI site was changed to an XhoI site via the Klenow fill-in reaction at the NciI and XhoI sites of pBluescript II KS(+). Modified XhoI and BamHI restriction sites were used for cloning this fragment in frame to the 5′ end of firefly luciferase cDNA contained in a pCaSpeR 4 transformation vector with a SV40 polyadenylation site [S1]. Finally, a 9.8 kb BamHI *per* fragment (−4200 to +5449) was cloned in frame in front of the *per-luc* fusion part. The final *XLG-luc* construct contains an approximately 10.8 kb *per* fragment including the 5′-flanking regulatory region as well as the entire coding region (except those encoding the last ten C-terminal amino acids) fused to the *luciferase* cDNA (Figure 1). To generate *8.0-luc* instead of the 9.8 kb BamHI *per* fragment, we cloned a 4.1 kb EcoRI/BamHI *per* fragment (+1369 to +5449) in frame to the *per-luc* fusion part. As a result, the final *8.0-luc* construct lacks the 5′-flanking regulatory region, the first non-coding exon, and approximately 50% of *per*'s first intron (Figure 1). We generated two independent *XLG-luc* and 11 independent *8.0-luc* lines by transforming *y w* embryos with a given construct, all of which carried the mini-*white*$^{+}$ gene as a marker (as in [S1]). In only three out of the 11 *8.0-luc* lines was *luciferase* expression detectable (see the main text and Table 1). Analysis of DNA sequences flanking the *8.0-luc:9* insertion revealed that it inserted into the first intron of *CG18093*, a gene encoding a tyrosine/serine/threonine phosphatase. There are no reports connecting this phosphatase with the circadian system or rhythmic gene expression [S2].

Analysis of Bioluminescence Rhythms

Data were analyzed for period, amplitude, and phase by a Fourier transform-non-linear least-squares (FFT-NLLS) multicomponent cosine analysis [S3]. In the current study, all records that had periods in the range of 24 ± 5 hr and relative amplitude errors (rel-amp) <0.7 were considered to be rhythmic. The rel-amp is obtained by dividing the 95% confidence interval of the amplitude estimate by the amplitude estimate (ratio of amplitude error to most probable amplitude). This value ranges from 0 to 1, where 0 indicates a rhythm with infinite precision and 1 indicates a rhythm that is not statistically significant. Rel-amps <0.7 indicate that the bioluminescence rhythm is due to rhythmic gene expression (see [S1] for how this cut-off was determined).

Plotting and Analysis of Behavioral Data

We generated the daily average histograms for the LD part of the experiment shown in Figure 3 by first superposing locomotor activity from a single male and then superposing the daily activity of all flies from the same genotype. Locomotor periods in DD were analytically determined by χ^2 periodogram analysis ($\alpha = 0.05$). The program also indicates the strength of the behavioral rhythm (cf. [S4]) by computing "power" values (roughly the height of the periodogram peak) and the number of 0.5 hr bins crossing the significance line ("width"). Only flies showing periods in combination with powers ≥10 and width ≥2 were considered to be significantly rhythmic and had their period values listed in Table S1 (averages for all rhythmic flies from a given genotype).

Immunohistochemistry

Flies were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS [pH 7.4]) at 4°C for 4 hr (2 hr at 20°C for anti-PDF immunohistochemistry shown in Figure 4). Fixed flies were washed 4 × 5 min in PBS (on ice). Brains were dissected in PBS and washed first in 0.1% Triton X-100 in PBS (0.1% PBT) (3 × 5 min) and then in 1% PBT for 10 min. After the washes, brains were blocked in 2% donkey serum in 0.3% PBT for 30 min and rinsed in 0.3% PBT for 5 min. Primary antibodies were added at a dilution of 1:1000, for both rabbit anti-PER serum (preabsorbed against *per*^{01} embryos) and rat anti-TIM, and incubated at 4°C for two nights. For staining the PDF cells shown in Figure 4, the mouse monoclonal antibody *nb33* was used at a dilution of 1:100. *nb33* specifically labels PDF-expressing cells, and no signals are detected in *Pdf*^{01} mutant flies (A. Hofbauer, personal communication). The extra primary antibody was washed off by 0.3% PBT (5 × 15 min). Secondary goat antibodies, diluted to 1:200, were added (anti-rabbit: AlexaFluor 488 green; anti-rat: AlexaFluor 594 red; or anti-mouse: AlexaFluor 568 red [Molecular Probes, OR]), and the mixture was incubated at 4°C overnight. Excess secondary antibodies were washed off by 0.3% PBT (5 × 15 min). Finally, brains were rinsed with PBS and placed in 0.1% PBT before being mounted in Vectashield (Vector Laboratories, CA).

Supplemental References

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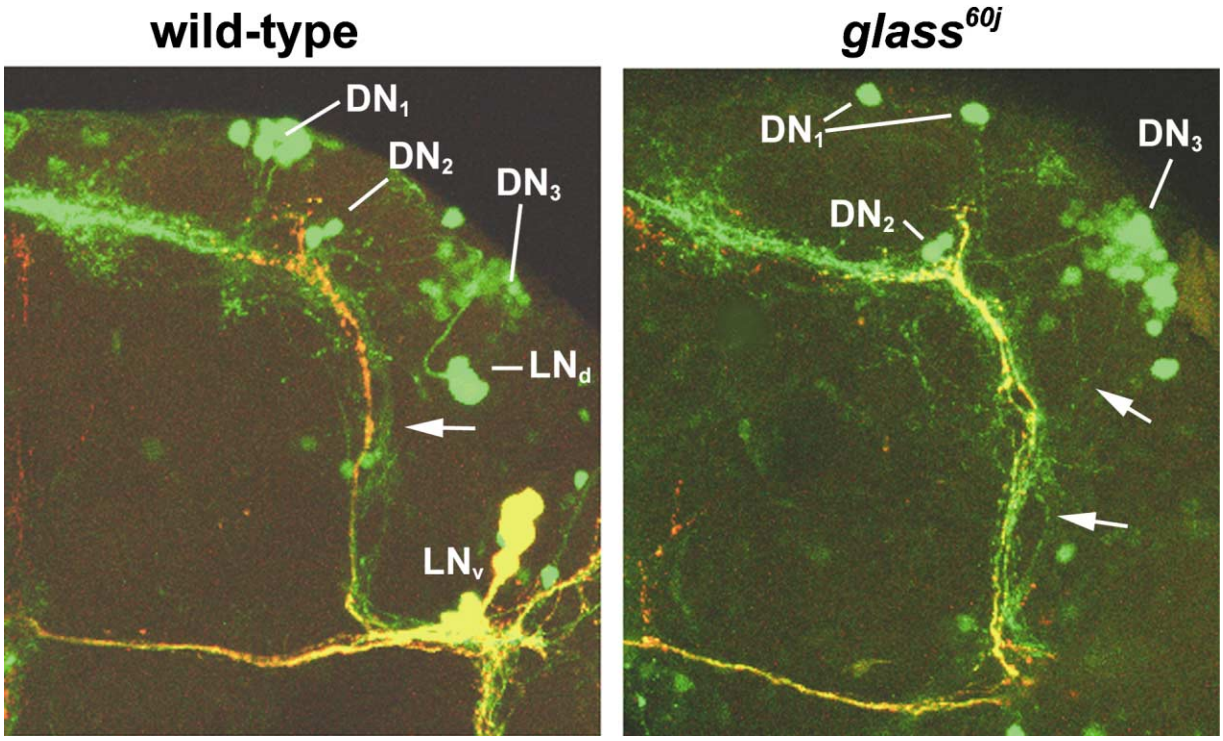

Figure S1. *tim*-Driven Reporter Gene Expression in Clock Neurons of *glass*$^{+}$ and *glass*^{60j} Flies

Whole-mounted brains from "photoreceptor normal" or *glass*^{60j} flies carrying the *tim-gal4* and *UAS-gfp* transgenes were immunostained with an antibody against PDF (red). *tim* expression was visualized by GFP fluorescence (green). Note that only the LNv cells express both PDF and *tim* (indicated in yellow). *glass*^{60j} flies have normal LNv and LNd cells, but they were excluded from the optical sections shown so that the LN and DN axonal projections would be visible. Note that only two DN1 cells are present in *glass*^{60j} flies, yet the number of projections from the dorsal brain toward the LNv cluster seems not to be significantly reduced in comparison with *glass*$^{+}$ (arrows).

Table S1. Free-Running Behavioral Rhythms of *XLG-luc* and *8.0-luc:9* Transgenic Lines in Various Genetic Backgrounds

Genotype	n	Percent Rhythmic	τ (hr)
y w	29	90	23.8 ± 0.1
per^{01}	41	5	33.4 ± 5.4
per^{01}; *XLG-luc:1*	64	47	23.2 ± 0.1
per^{01}; *XLG-luc:2*	49	53	24.0 ± 0.1
per^{01}; *8.0-luc:9*	48	2	28.6
disco; 8.0-luc:9	35	9	25.7 ± 1.4

Flies were entrained to 12 hr:12 hr LD cycles for 5 days and then released into DD, where their behavior was monitored for an additional 5–7 days. Period values were determined by χ^2 periodogram; only flies showing periods in combination with a "power" value $\geq$10 and a time bin "width" $\geq$2 were considered to be rhythmic (cf. [S4]). The different transgenic types were also tested in clock-normal genetic backgrounds (i.e., *per*$^{+}$ and *disco*$^{+}$) like the *y w* controls, such flies showed normal locomotor activity, ruling out any negative effects of transgene expression on clock function (data not shown).

Table S2. PER Immunoreactivity in Control and *8.0-luc:9* Transgenics under Entrainment and Free-Running Conditions

	Average Number of Neurons Stained ± SEM (Hemispheres Stained/Hemispheres Analyzed)					
Genotype (time point)	DN1	DN2	DN3	s-LNv	l-LNv	LNd
y w (ZT23)	9.7 ± 0.6 (29/30)	0.8 ± 0.2 (12/30)	9.7 ± 0.7 (29/30)	3.2 ± 0.3 (28/30)	3.4 ± 0.3 (28/30)	5.3 ± 0.5 (27/30)
per^{01};XLG-luc:1 (ZT23)	3.8 ± 0.5 (14/16)	0.9 ± 0.3 (8/16)	2.4 ± 0.7 (8/16)	1.4 ± 0.5 (7/16)	3.4 ± 0.5 (14/16)	4.4 ± 0.5 (14/16)
8.0-luc:9 (ZT23)	7.8 ± 0.9 (1414)	1.9 ± 0.1 (14/15)	9.6 ± 1.1 (14/14)	4.5 ± 0.5 (15/15)	5.9 ± 0.6 (15/15)	2.7 ± 0.6 (9/15)
8.0-luc:9 (ZT11)	0 (0/14)	0 (0/14)	0 (0/14)	0 (0/14)	0 (0/14)	0 (0/14)
per^{01};8.0-luc:9 (ZT23)	3.4 ± 1.0 (9/14)	0.6 ± 0.3 (4/14)	5.1 ± 0.5 (14/14)	0.3 ± 0.3 (1/14)	0.64 ± 0.5 (2/14)	1.8 ± 0.8 (5/14)
per^{01};8.0-luc:9 (ZT11)	0 (0/16)	0 (0/16)	0 (0/16)	0 (0/16)	0 (0/16)	0 (0/16)
y w (CT 23)	4.2 ± 0.3 (40/44)	0.2 ± 0.1 (4/44)	4.2 ± 0.5 (32/44)	2.6 ± 0.2 (37/44)	2.3 ± 0.3 (30/44)	3.3 ± 0.3 (33/44)
y w (CT11)	3.1 ± 0.3 (28/33)	1.0 ± 0.2 (18/33)	0.4 ± 0.2 (4/33)	0.2 ± 0.1 (2/33)	2.1 ± 0.3 (20/33)	1.5 ± 0.4 (12/33)
disco (CT21)	3.6 ± 0.7 (13/18)	0.2 ± 0.2 (2/18)	3.2 ± 0.6 (12/18)	0 (0/18)	0 (0/18)	4.0* (1/18)
disco (CT23)	4.5 ± 0.7 (16/20)	0.1 (1/20)	5.3 ± 0.8 (17/20)	0 (0/20)	0 (0/20)	0 (0/20)
disco (CT9)	6.0 ± 0.7 (19/21)	1.6 ± 0.2 (17/21)	0.3 ± 0.2 (4/21)	0 (0/21)	0 (0/21)	0 (0/21)
disco (CT11)	5.1 ± (0.6) (19/21)	1.6 ± 0.2 (17/21)	0.3 ± 0.2 (3/21)	0 (0/21)	0 (0/21)	0 (0/21)

Flies were either entrained to 12 hr:12 hr LD cycles for 3 days and collected on the fourth day at the indicated ZT or released into 5 days of DD and collected at the indicated CT. Whole-mounted brains were fixed and stained with anti-PER. Brain hemispheres were analyzed separately, and the average numbers of stained cells (± SEM) were determined for each neuronal group. In parentheses is given the number of brain hemispheres showing at least one stained cell of the respective neuronal group in relation to the total number of brain hemispheres analyzed. The *disco* mutation largely eliminates the LNs, but in one particular brain (*) four LNd cells were stained, and we cannot rule out that in some of the other *disco* brains LNs are present but do not express PER.

4.1.2 PDF is not required for self--sustained molecular oscillations in the DNs

The pigment dispersing factor (PDF) is a neuropeptide, which acts as an output factor in the circadian clock (Helfrich--Förster, 2003). Four out of five s--LN_vs and all the l--LN_vs express PDF (Helfrich--Förster, 1995; Kaneko and Hall, 2000; Stoleru *et al*., 2004); and the s--LN_vs axonal terminals show rhythmic accumulation of PDF (Helfrich--Förster, 2003). The DNs and LN_ds do not express PDF (Helfrich--Förster, 2003). However, a direct PDF--mediated signaling from the s--LN_vs to DNs was suggested (Peng *et al*., 2003). *Pdf*^{01}--null mutant flies lose behavioral and molecular rhythmicity after several days in DD but retain both behavioral and molecular rhythms in LD (Renn *et al*., 1999; Peng *et al*., 2003). Therefore, it was suggested that for robust sustained behavioral and molecular oscillations in DD, PDF is required as an important intercellular communicator molecule (Peng *et al*., 2003).

The *8.0--luc:9* transgene is mainly expressed in the DNs, which showed sustained molecular oscillations even after 5 days in DD (see above, Veleri *et al*., 2003). Therefore, we have investigated the role of PDF in maintaining the sustained molecular rhythms in DNs. Our results show that PDF is not required for maintaining the self--sustained molecular oscillation (luciferase--reported PER expression) emanating from the DNs (Fig. 2). The *8.0--luc:9D;Pdf*^{01} transgenic flies showed sustained rhythmic expression of PER in DD at least until 5 days in DD (Fig. 2). Our results indicate that free running and synchronized molecular rhythms (of PER expression) do not require PDF, as well as the s--LN_vs (Fig. 2). Because the *disco*^{2}*;8.0--luc:9* transgenic flies (which lack the LNs; Helfrich--Förster, 1998) also showed sustained rhythmic expression of PER. In fact, *disco*^{2}*;8.0--luc:9* transgenic flies had an improved performance than the wild type flies. This may be because some kind of control exerted by the LN_vs on DNs may be absent in *disco*^{2} background. Therefore, even in absence of communication between the LNs and DNs, and in absence of external light input, a synchronized molecular oscillation could be maintained. Recently it was shown that the PER expression cycles in the individual s--LN_vs and in LN_ds of *Pdf*^{01} mutant flies in DD

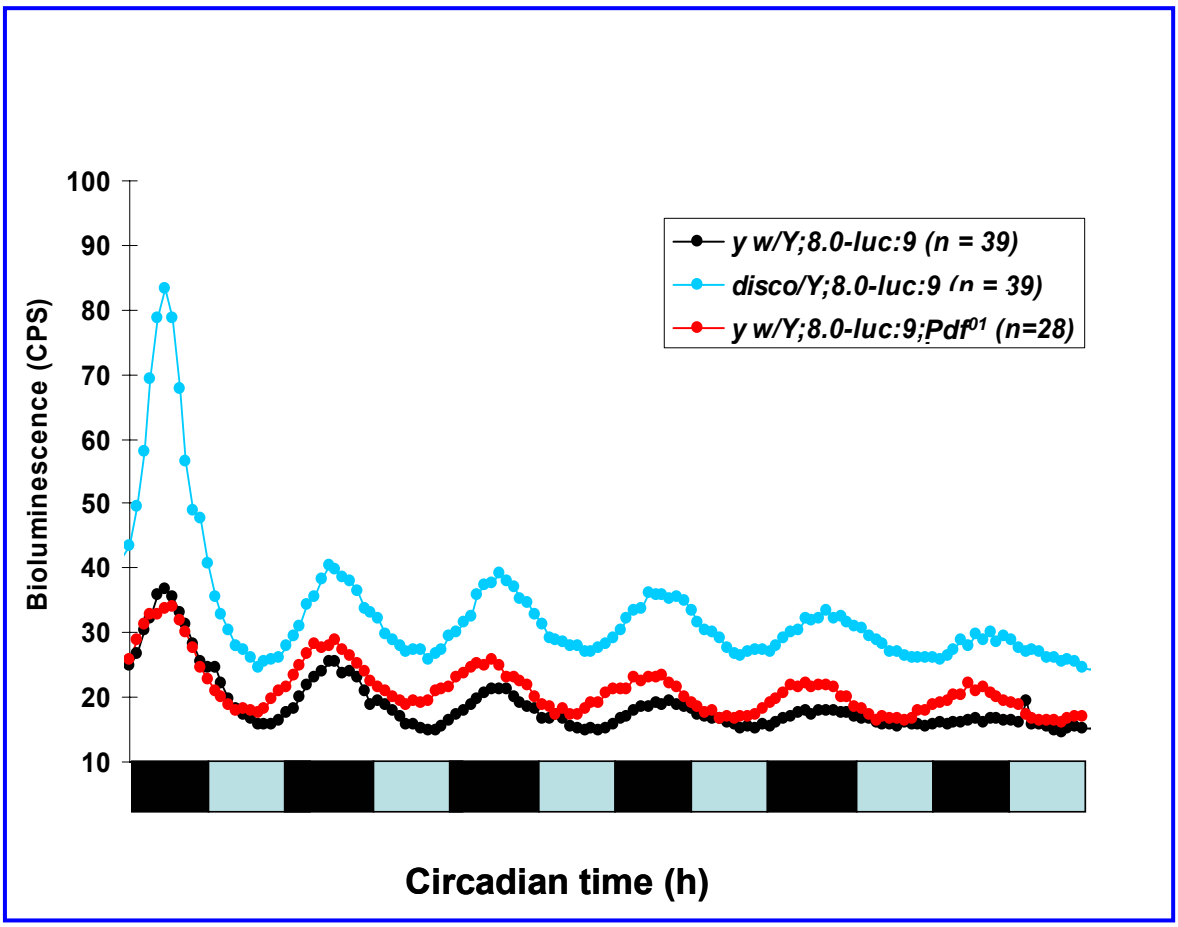

Figure 2 Bioluminescence rhythm records from *8.0--luc:9* transgenic flies in *y w*, *disco*^{2} and *Pdf*^{01} genetic backgrounds. *8.0--luc:9* transgenic flies in the above genetic backgrounds showed a non--dampening bioluminescence (*disco*^{2} had an improved performance than the *y w* flies). In the *Pdf*^{01} genetic background, the record showed a shorter period. The bioluminescence record was averaged for many animals ('n' in the legend), which were statistically rhythmic. In the x--axis, the gray and black bars indicate subjective day and subjective night, respectively, in DD. The y--axis indicates bioluminescence expression levels in counts per second (CPS).

(Lin *et al*., 2004). Above findings show that in DD sustained PER cycling continues, in the DN oscillators (Veleri *et al*., 2003), and in the s--LN$_v$s and LN$_d$s oscillators, in the absence of PDF (Lin *et al*., 2004). However, PDF was shown to be required for *tim* mRNA cycling in DD (Peng *et al*., 2003). Possibly, PDF may be required for transcriptional control of *tim* but not for the translational or posttranslational control of *per*. It is known that the cyclic PER protein expression is under posttranslational control of the protein phosphatase 2A (Sathyanarayanan *et al*., 2004). In the absence of PDF, the nuclear shuttling PER rhythm in the LN$_d$s

was phase advanced, and thus resulted in a short free running behavioral period (Lin *et al*., 2004). In our analysis of PER bioluminescence rhythms in the DNs of Pdf^{01} flies also showed a short period molecular rhythm (Fig. 2 and Table 1). Therefore, PDF may also be needed for the phase setting of nuclear shuttling rhythms of PER in the DNs.

4.1.3 Transcriptional vs. posttranscriptional regulation of molecular oscillations in the clock neurons

Self--sustained molecular oscillations are maintained by a subset of clock neurons in the *Drosophila* brain (Veleri *et al*., 2003). On the fifth day after the transition from LD to DD, anti--PER staining reveals cycling in the s--LN_vs, LN_ds, DN_2s and DN_3s but not in the l--LN_vs and DN_1s (Veleri *et al*., 2003). PER cycling in the s--LN_vs in DD was consistently reported for multiple days ([2 days] Shafer *et al*., 2002; [5 days] Veleri *et al*., 2003; [2 days] Yang and Sehgal, 2001; [3, 6 and 9 days] Lin *et al*., 2004), and thus confirms this neuronal group as a self--sustained molecular oscillator. Interestingly, in the DN_2s PER expression was cycling in anti--phase with the s--LN_vs and DN_3s (Veleri *et al*., 2003). Similarly, the larval DN_2s show anti--phase cyclic expression of both PER and TIM, under LD and DD (Kaneko *et al*., 1997; Klarsfeld *et al*., 2004). In contrast to the larval data PER expression has been shown to cycle in DN_2s of adult flies in synchrony with other clock neurons under LD conditions as well as for the first day in DD (Blanchardon *et al*., 2001). The return of anti--phase cycling after a long time in DD suggests that the anti--phase cycling of the DN_2s is a default state of it in the absence of entraining LD cycles. It is possible that the larval DN_2s do not get any light input and therefore show an anti--phase cycling. Indeed, it was shown that *cry*, a blue light photopigment, is not expressed in the larval DN_2s (Klarsfeld *et al*., 2004). Artificial expression of *cry* in the larval DN_2s resulted in the phase reversal of PER expression rhythm in synchrony with the s--LN_vs and DN_3s. It is suggested that the DN_2s are secluded from the light input until a certain stage of the metamorphosis is reached, and therefore, the PER oscillations remained in the anti--phase default state (Kaneko *et al*., 1997). In extended DD, the adult DN_2s resume their default state (Veleri *et al*., 2003).

In contrast to the constant levels of PER expression in the l--LN_vs and DN_1s, in DD (Veleri *et al*., 2003), *tim* mRNA expression was reported to cycle still on the day 8 of DD in the l--LN_vs and DN_1s, as well as in all other subsets of the clock neurons (Peng *et al*., 2003), regardless to the fact whether they were considered as dampened or self--sustaining oscillators according to the results of our study. These profound differences in the temporal expression pattern of PER protein and *tim* mRNA (also observed by Lin *et al*., 2004) are problems yet to be solved in further studies. Peng and colleagues also claimed that mRNA oscillation of the l--LN_vs "adapt" to constant conditions by becoming rhythmically expressing once again, after being arrhythmic about 2 days into DD, immediately following transfer from LD to DD. This is in stark contrast to the arrhythmic (constitutive) protein expression in these cells in DD (Veleri *et al*., 2003; Yang and Sehgal, 2001; Lin *et al,* 2004). Conceivably there may be differential gene regulation at various levels of gene expression such as transcription and translation (e.g., Cheng and Hardin, 1998; Stanewsky *et al*., 1997b; Stanewsky *et al*., 2002; Sathyanarayanan *et al*., 2004). The meaning of this divergence in regulation of mRNA and protein rhythms in various circadian oscillators is yet to be understood.

4.2 Other *8.0--luc* transgenic lines investigated

Besides the *8.0--luc:9* transgenic flies, we also analyzed 3 other transgenic mosaic fly lines, *8.0--luc:2*, *8.0--luc:4* and *8.0--luc:11*, carrying the same fusion gene but at different locations in the *Drosophila* genome. Further, the *8.0--luc:2* transgene in the flies was mobilized for creating even more lines with a potentially interesting spatial and temporal expression pattern.

4.2.1 Transgenic line *8.0--luc:2*

8.0--luc:2 transgenic flies showed rhythmic bioluminescence in DD for 4--5 days (Fig. 3A), with a similar low expression level of luciferase like the *8.0--luc:9* transgenic line (Table 1). In a per^+ background 85% of the *8.0--luc:2* transgenic flies showed rhythmic luciferase expression in DD, but in per^{01} and $disco^2$ backgrounds they showed only 3% and 29% rhythmicity, respectively (Table 1). In per^+ and per^{01} backgrounds, a high percentage of them showed rhythmic locomotor activity in LD with a characteristic anticipation of the light to dark

transition (E peak), an indication of the functional LN_ds and some DN_1s (see in

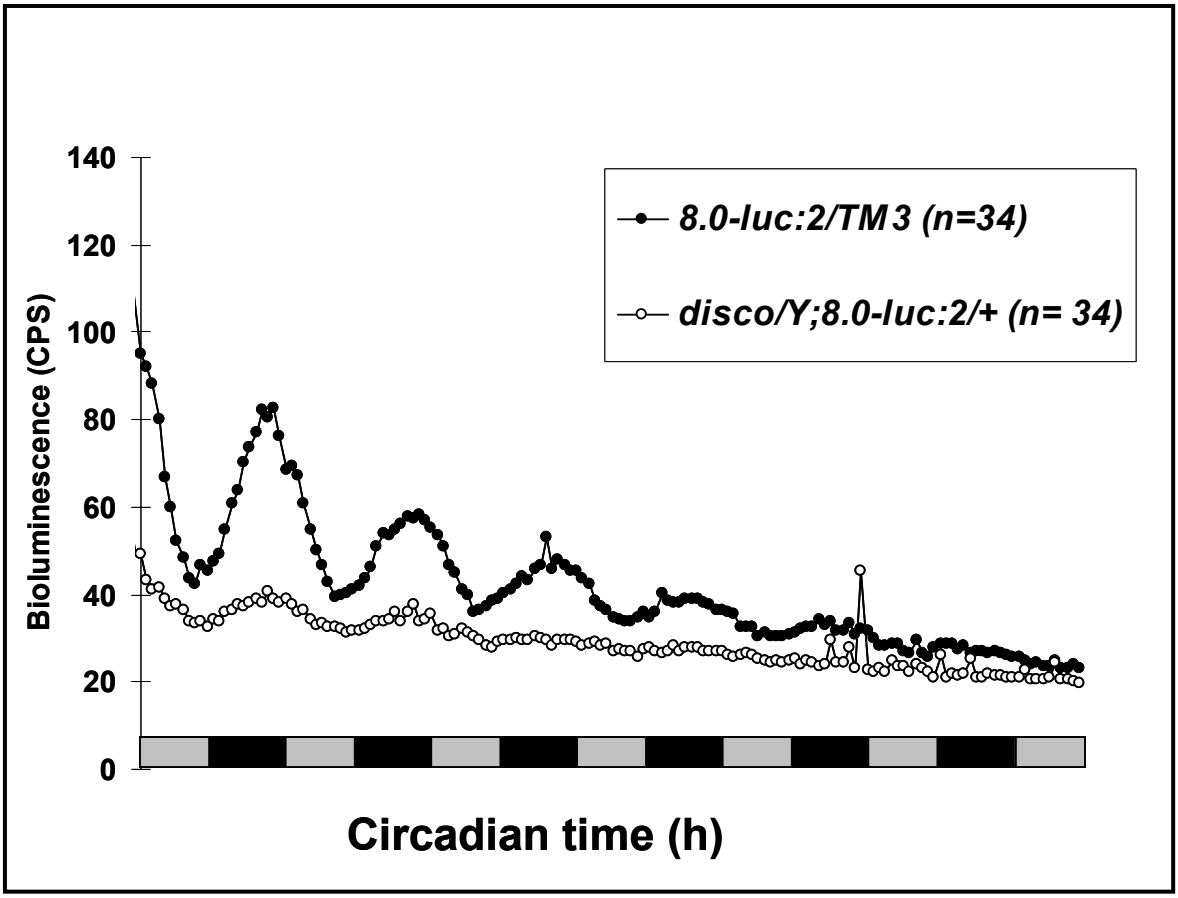

Figure 3A Bioluminescence rhythm records from different *8.0--luc* transgenic fly lines in *y w* and *disco*^{2} genetic backgrounds in DD. (A) *8.0--luc:2* transgenic flies in a *y w* genetic background showed a slow dampening bioluminescence but quickly dampened to arrhythmic in a *disco*^{2} genetic background. The axes are as described in Fig. 2.

Chapter 1, section 3.1.3; Stoleru *et al*., 2004; Grima *et al*., 2004; Veleri *et al*.,2003). But in DD the transgene could rescue behavioral rhythms in a *per*^{01} background in only 7% of the tested flies (1 out of 14 flies) and the *8.0--luc:9* transgene rescued 10% (1 out of 10 flies) in the same conditions (Table 2). The transgene *8.0--luc:2* expressed mainly in the DN_1s and DN_3s, and in 1--2 LN_ds , the *8.0--luc:9* transgene expressed similarly in the DN_1s and DN_3s and also in DN_2s (4 hemispheres) and in LN_ds (5 hemispheres) (Table 3). The transgene expressed in the l--LN_vs of only one brain hemisphere out of nine tested flies and none in the s--LN_vs (Table 3). The absence of *8.0--luc:2* transgene's expression in the s--LN_vs correlated with the inability to rescue the behavioral rhythm in DD, in a *per*^{01} background (Table 2). The expression of *8.0--luc:2* transgene in the DNs and

LN_ds only correlated with anticipation of the light to dark transition in behavioral rhythms of these transgenic flies in LD in a *per*^{01} background (Table 2) (cf., Grima *et al*., 2004; Stoleru *et al*., 2005).

4.2.2 Transgenic lines *8.0--luc:4* and *8.0--luc:11*

The transgenic fly lines *8.0--luc:4* and *8.0--luc:11* showed higher levels of luciferase expression (Fig. 3B, C and Table 1) compared to the *8.0--luc:2*

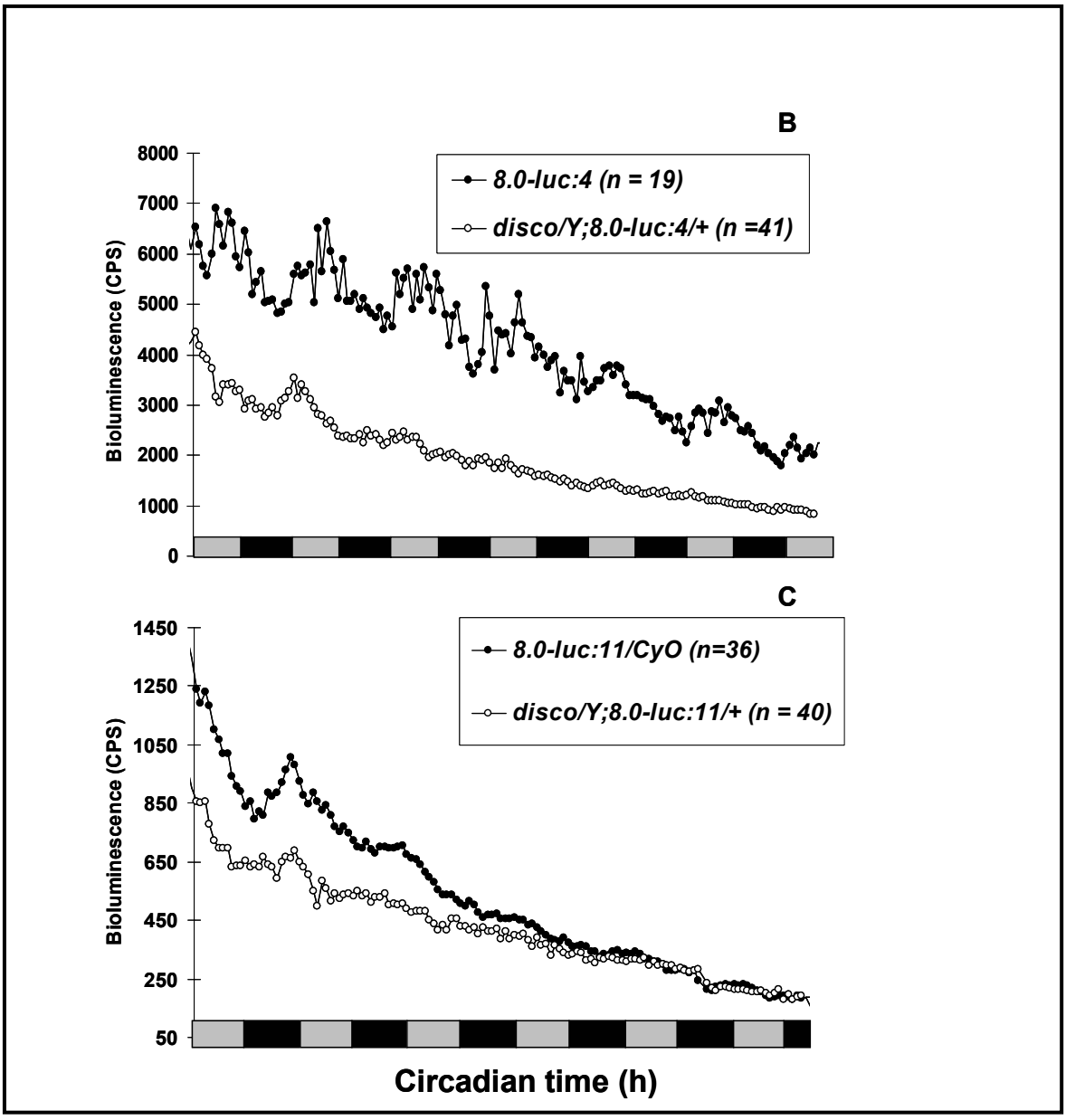

Figure 3B, C Bioluminescence rhythm records from different *8.0--luc* transgenic fly lines in *y w* and *disco*^{2} genetic backgrounds in DD. (B) *8.0--luc:4* transgenic flies in a *y w* genetic background showed a slowly dampening bioluminescence rhythms (with high

level of bioluminescence expression), and in a *disco*^{2} genetic background the rhythm dampened off even quicker. (C) *8.0--luc:11* transgenic flies both in *y w* and *disco*^{2} genetic backgrounds showed a quickly dampening bioluminescence rhythm. (Note that lines *8.0--luc:4* and *8.0--luc:11* showed higher bioluminescence expression levels (B, C), compared to a low expression level in *8.0--luc:2* line (Figure 3A), indicating the expression of the transgenes in many clock cells in addition to the clock neurons in the brain. This may result in quick dampening due to internal desynchronization among different clock gene expressing cells in DD). The axes are as described in Fig. 2.

transgenic flies indicating a broader expression pattern. In the *per*$^{+}$ background *8.0--luc:4* and *8.0--luc:11* transgenic flies showed 52% and 37% rhythmicity, respectively, in terms of luciferase expression in DD (Table 1). In the *disco*^{2} background, *8.0--luc:4* flies showed 15% rhythmic expression but none of the *8.0--luc:11* showed rhythmic expression (Table 1). In a *per*^{01} background, both transgenic fly lines showed synchronized behavior in LD with wild type--like anticipation of light to dark transition, but in DD a very low percentage of them were rhythmic (Table 2). The transgenes *8.0--luc:4* and *8.0--luc:11* were expressed in the clock neurons in a similar pattern as described for *8.0--luc:2* except that a higher number of DNs and LN_ds showed expression (Table 3). Like in the *8.0--luc:2* transgenic line, lack of transgene expression in the s--LN_vs of *8.0--luc:4* and *8.0--luc:11* transgenic flies correlated with the failure to rescue behavioral rhythms in DD (Table 2, Table 3). Similar to *8.0--luc:2* transgenic flies, the expression of the transgene in DNs and LN_ds only correlated with the presence of an E peak and absence of a M peak in behavioral rhythms, in LD in a *per*^{01} background (Table 2).

4.2.3 Spatial expression pattern of the transgene is correlated with dampening or non--dampening bioluminescence expression in DD

In DD, *8.0--luc:9* flies showed robust rhythmic bioluminescence expression (Fig. 2 in Veleri *et al*., 2003) compared to *8.0--luc:2, 8.0--luc:4 and 8.0--luc:11* flies (Fig. 3). This may be because in *8.0--luc:9* flies the transgene was mainly expressed in higher number of the DN_3s (Table 3), which define a self--sustained molecular oscillator (Veleri *et al*., 2003), compared to the other lines. In *8.0--luc:2* flies the transgene almost equally expressed in the DN_1s and DN_3s (Table 3). The DN_1s contain a dampened molecular oscillator (Veleri *et al*., 2003). Therefore,

expression of the transgene simultaneously in the DN_1s and DN_3s might have caused dampened oscillations in this line due to internal desynchronization among different clock neurons (cf. *XLG--luc:1* in Fig. 2 in Veleri *et al*., 2003). Similarly, in *8.0--luc:4* flies the transgene expressed relatively more in the DN_1s than in the DN_3s (Table 3). The transgene also expressed in few of the l--LN_vs, another dampened molecular oscillator; and in the LN_ds (Table 3). Therefore, this transgenic line showed dampened molecular oscillation too. In *8.0--luc:11* flies, the transgene expressed almost equally in the DN_1s and in the DN_3s, and very little in the l--LN_vs compared to in the *8.0--luc:4* flies (Table 3). This reduced expression of the transgene in the *8.0--luc:11* flies is evident in the comparatively lesser bioluminescence count displayed by them compared to the *8.0--luc:4* flies (Fig. 2B, C). The *8.0--luc:11* flies too showed dampening molecular oscillations possibly due to the expression in the DN_1s and in the DN_3s like in the *8.0--luc:2* flies. In the eye photoreceptors of transgenic flies, the *8.0--luc:4* transgene stained strongly, and the *8.0--luc:11* transgene stained weakly whereas the *8.0--luc:2* transgene did not give any signal (data not shown). This relative expression levels of the transgene in the eye photoreceptors of the above mentioned transgenic lines is evident in their bioluminescence record (Fig. 3A, B, C).

In a *disco*^{2} background the *8.0--luc:9* flies showed better rhythmicity than the *8.0--luc:2, 8.0--luc:4 and 8.0--luc:11* flies (Fig. 3; Fig. 2 in Veleri *et al*., 2003). This may be because the transgene in *8.0--luc:9* flies are expressed more in the self--sustained DN_3s than in the other lines (Table 3). The improved rhythmicity in *disco*^{2} background may be because the DNs are relieved from some kind of coupling control exerted by the LN_vs.

In a *per*^{01} background, all these transgenics displayed largely arrhythmic bioluminescence expression in DD (Table 1). Maybe the promoterless *per* gene is disabled in its capacity to function in the feed--back loop (see in Chapter 1) compared to the endogenous *per* gene. For example, for transcriptional activation of *per* by CLK:CYC the promoter E--boxes in *per* is necessary (see Chapter 1, section 4.2 and Fig. 3 therein), which is absent in our *8.0--luc* constructs (Fig.1).

Table 1 Bioluminescence rhythms of various *8.0--luc* transgenic flies in different genetic backgrounds (DD).

Genotype	N (n)	% rhythmic	Bioluminescence expression level (CPS)	period ± SEM (h)	Phase ± SEM (h)	Rel--amp ± SEM
8.0--luc:2/TM3	65 (56)	85	52.4 ± 2.0	23.3 ± 0.1	23.5 ± 0.3	0.39 ± 0.01
per^{01}/Y;8.0--luc:2/TM3	38 (1)	3	63.8	25.9	6.1	0.51
disco^{2}/Y;8.0--uc:2/+	41 (12)	29	36.5 ± 1.2	22.8 ± 0.5	24.0 ± 1.6	0.54 ± 0.03
8.0--luc:4	67 (35)	52	4345 ± 131	23.9 ± 0.1	1.6 ± 0.4	0.55 ± 0.02
per^{01}/Y;8.0--luc:4	52 (0)	0	--	--	--	--
disco^{2}/Y;8.0--uc:4/+	121 (18)	15	4223 ± 187	24.4 ± 0.4	22.9 ± 1.0	0.61 ± 0.02
8.0--luc:9	110 (94)	85	29.4 ± 1.8	23.1 ± 0.1	23.4 ± 0.3	0.41 ± 0.01
per^{01}/Y;8.0--luc:9/+	69 (17)	25	96.9 ± 1.9	24.6 ± 0.5	23.5 ± 1.2	0.59 ± 0.02
disco^{2}/Y;8.0--uc:9/+	407 (304)	75	52.9 ± 3.5	23.5 ± 0.1	23.3 ± 0.3	0.38 ± 0.01
8.0--luc:9;Pdf^{01}	28 (24)	86	23.4 ± 0.6	22.7 ± 0.1	23.3 ± 0.4	0.27 ± 0.02
8.0--luc:11/CyO	92 (34)	37	702 ± 32	24.3 ± 0.2	23.6 ± 0.7	0.56 ± 0.02
per^{01}/Y;8.0--uc:11/CyO	33 (2)	6	233	25.3	21.0	0.57
disco^{2}/Y;8.0--luc:11/+	40 (0)	0	--	--	--	--

Table 1 Summary of bioluminescence rhythms recorded from different *8.0--luc* transgenic flies in various genetic backgrounds in DD. In a per^{+} background the transgenic fly lines showed varying degrees of rhythmic expression of luciferase. In a per^{01} background, mostly all the transgenic flies were arrhythmic. In $disco^{2}$ and Pdf^{01} backgrounds, *8.0--luc:9* transgenic flies showed robust rhythmicity (in fact, in $disco^{2}$ background the rhythmic expression level was improved). Flies were recorded for 5--7 days in DD at 25°C. Data were analyzed with FFT--NLLS software to determine 'period' (τ), 'relative amplitude error' (rel--amp), and the average CT peak time of expression (phase). Rhythmic flies (% rhythmic) had τ values of 24 $\pm$ 5 h and rel--amp errors < 0.7 (see details in the experimental procedures in Veleri *et al*., 2003). Prior to the experiment, flies were entrained to 12 h: 12 h LD cycles at 25°C for at least 3 days. 'N' and 'n' indicate total number of flies tested and number of flies that showed rhythmic bioluminescence in DD. Bioluminescence expression level is represented as counts per second (CPS).

Table 2 Locomotor behavior rhythms of various *8.0--luc* transgenic flies in different genetic background in LD and DD.

Genotype	**LD**						**DD**			
	N	**n**	**% rhythmic**	**Anticipation of dark to light (M) or light to dark (E) transitions**		**period ± SEM (h)**	**N**	**n**	**% rhy.**	**period ± SEM (h)**
				M	**E**					
y w/Y	37	34	92	++	+++	24.0 ± 0.03	35	31	89	23.9 ± 0.07
per^{01}/Y	34	21	62	-	-	23.9 ± 0.05	33	2	6	31.5
disco^{2}/Y	34	26	76	-	+++	24.3 ± 0.1	33	6	18	24.9 ± 0.6
8.0--luc:2/TM3	12	10	83	+	++	23.9 ± 0.1	11	9	82	24.0 ± 0.12
per^{01}/Y;8.0--luc:2/TM3	37	28	76	-	+++	23.9 ± 0.04	37	0	0	--
per^{01}/Y;8.0--luc:2/+	14	14	100	-	+++	24.0 ± 0.05	14	1	7	23.5
8.0--luc:4	13	9	69	+	+++	23.9 ± 0.07	11	6	55	24.6 ± 0.19
8.0--luc:4/CyO	12	12	100	+	++	23.9 ± 0.09	10	9	90	24.5 ± 0.1
per^{01}/Y;8.0--luc:4	27	23	85	-	+++	23.9 ± 0.07	23	3	13	26.2 ± 0.8
per^{01}/Y;8.0--luc:4/CyO	12	8	67	-	+++	23.6 ± 0.13	9	1	11	28.4
8.0--luc:9	12	10	83	+	++	22.4 ± 0.05	11	8	73	24.6 ± 0.08
8.0--luc:9/CyO	11	11	100	+	++	23.9 ± 0.04	11	11	100	24.0 ± 0.07
per^{01}/Y;8.0--luc:9	36	32	89	-	++	24.1 ± 0.04	36	0	0	--

per^{01}/Y;8.0--luc:9/CyO	12	10	83	-	+	23.9 ± 0.06	10	1	10	28.6
$disco^{2}$/Y;8.0--luc:9	11	5	46	-	+++	24.2 ± 0.2	11	1	9	23.5
$disco^{2}$/Y;8.0--luc:9/+	15	12	80	-	+++	24.2 ± 0.1	13	2	15	24.6
8.0--luc:11/CyO	16	16	100	+	+++	24.2 ± 0.03	16	16	100	24.7 ± 0.09
per^{01}/Y;8.0--luc:11/CyO	43	42	98	-	+++	24.1 ± 0.03	43	3	7	25.4 ± 1.1
per^{01}/Y;8.0--luc:11/+	15	15	100	-	+++	23.9 ± 0.07	15	4	27	23.7 ± 0.5
$disco^{2}$/Y;8.0--luc:11/+	10	10	100	-	+++	24.4 ± 0.07	10	1	10	22.9

N is total number flies tested, and 'n' is number rhythmic.

Table 2 Summary of locomotor behavioral rhythms in LD and DD of various *8.0--luc* transgenic flies. Flies were entrained to 12 h: 12 h LD cycles for 5 days and then released into DD for another 5--7 days. Period values were determined by χ^2 periodogram; only flies showing periods in combination with a 'power' value ≥ 10 and a time bin 'width' ≥ 2 were considered to be rhythmic (see in Chapter 2 Methods, section 2.4.2). The circadian clock enables flies to anticipate the changes in the light level. Flies react to the transition of dark to light in the morning, seen as the morning activity peak (M). Similarly, they also react to the light to dark transitions in the evening, seen as evening activity peak (E). The M and E in LD were judged by analyzing the average daily activity for 5 days. A fly with all the subsets of clock neurons expressing *per* display a strong M and E. Lack of *per* gene expression, in a *per^{01}* background, in the LN_vs correlated with the inability to show the M, and the expression of *per* gene in the DNs or the LN_ds correlated with the presence of the E. The anticipation is graded in an arbitrary scale (-, no anticipation; +, minimum anticipation; ++, medium anticipation; +++, good anticipation).

Table 3 Shows expression pattern of the *8.0--luc* transgene in the clock neurons.

Genotype	**DN_1s**	**DN_2s**	**DN_3s**	**s--LN_vs**	**l--LN_vs**	**LN_ds**
y w/Y ZT 23 N = (15/15)	9.67 ± 0.63* (29/30)§	0.80 ± 0.18 (12/30)	9.73 ± 0.69 (29/30)	3.2 ± 0.25 (28/30)	3.37 ± 0.33 (28/30)	5.3 ± 0.48 (27/30)
per^{01};XLG--luc:1--1 ZT 23 N = (8/8)	3.75 ± 0.45 (14/16)	0.94 ± 0.25 (8/16)	2.38 ± 0.65 (8/16)	1.44 ± 0.45 (7/16)	3.38 ± 0.46 (14/16)	4.38 ± 0.52 (14/16)
per^{01};8.0--luc:2/TM6B ZT 23 N = (9/9)	2.89 ± 0.49 (13/18)	0 (0/18)	4.44 ± 0.29 (18/18)	0 (0/18)	0.22 (1/18)	1.72 ± 0.44 (9/18)
per^{01};8.0--luc:4 ZT 23 N = (8/8)	6.5 ± 0.65 (15/16)	0.123 ± 0.13 (1/16)	5.69 ± 0.87 (13/16)	0 (0/16)	0.94 ± 0.43 (4/16)	3.75 ± 0.53 (13/16)
per^{01};8.0--luc:9D ZT 23 N = (7/21)	3.36 ± 0.97 (9/14)	0.57 ± 0.25 (4/14)	5.07 ± 0.46 (14/14)	0.29 ± 0.29 (1/14)	0.64 ± 0.46 (2/14)	1.79 ± 0.76 (5/14)
per^{01};8.0--luc:11/CyO ZT 23 N = (8/8)	5.13 ± 0.46 (15/16)	0 (0/16)	4.69 ± 0.66 (14/16)	0 (0/16)	0.5 (2/16)	4.38 ± 0.57 (14/16)

* Average plus SEM (apply to all entries in the table)

§ (Number of brain hemispheres stained/Number of total brain hemispheres--apply to all entries in the table)

N = Number of brains stained/Total number of brains in experiments.

Table 3 PER immunoreactivity in *y w* control flies and in different *8.0--luc* transgenic fly lines (in a per^{01} background) in LD. Flies were entrained to 12 h: 12 h LD cycles for 3 days and collected on the fourth day at the indicated ZT. Whole mounted brains were fixed and stained with anti--PER. Brain hemispheres were analyzed separately, and the average numbers of stained cells (± SEM) were determined for each neuronal subgroup. In parenthesis the number of brain hemispheres showing at least one stained cell of the respective neuronal subgroup in relation to the total number of the brain hemispheres analyzed is given.

4.2.4 Newly mobilized transgenic lines *8.0--luc:2--20* and *8.0--luc:2--22*

Similar to the "mosaic" transgenic flies discussed above we generated additional "mosaic" transgenic flies, searching for lines which express the transgene in the s--LN_vs only. We were looking for a transgenic fly line, which satisfies the following requirements:1) should be expressing bioluminescence rhythmically in DD; 2) should be arrhythmic in a *disco*^{2} background (due to absence of LN_vs); 3) should be behaviorally rhythmic in DD in a *per*^{01} background. Fulfilling these conditions may be an indication that the transgene expresses exclusively in the s--LN_vs. Therefore, we further genetically mobilized the P element carrying the transgene *8.0--luc:2* by performing the following genetic crosses (Fig. 4).

We had generated 48 new transgenic fly lines and tested these lines to see whether the transgene was expressed rhythmically in DD. 43 lines were showing rhythmic luciferase expression in DD (see in Appendix 1, Table 4 Supplement) but only 13 among them showed partial behavioral rescue in DD in a *per*^{01} background (Table 4 and Table 5). Among these 13 lines, 2 lines (*8.0--luc:2--20* and *8.0--luc:2--22*) were chosen for further investigation because they exhibited very low levels of slow dampening luciferase expression (Fig. 5 and Table 4), and they also showed partial behavioral rescue (Table 5) in DD. Moreover, they showed reduced bioluminescence rhythms in a *disco*^{2} background (Fig. 5 and Table 5), indicating that the transgene expressed in LNs. We presumed that these fly lines might be expressing the transgene in a subset of pacemaker clock neurons, and therefore displayed slow dampened molecular rhythms (Fig. 5), like the *8.0--luc:9* lines. Therefore, anti--PER staining was performed on whole mounted brains of these two transgenic fly lines (in a *per*^{01} background), at known peak and trough time points for PER, i.e. ZT 23 and ZT 11 (Veleri *et al*., 2003). However, no anti--PER signal was detected in these transgenic fly brains (data not shown). This may be due to the extremely low level of transgene expression found in these flies (Fig. 5), compared to *8.0--luc:9* transgenic flies (see Fig. 2 in Veleri *et al*., 2003). Possibly, *8.0--luc:2--20* and *8.0--luc:2--22* transgenic flies may be expressing the transgene in the LN_ds or DNs because they displayed anticipation of the light to dark transition in LD in a *per*^{01} background (Table 5). A quickly dampening rhythmic

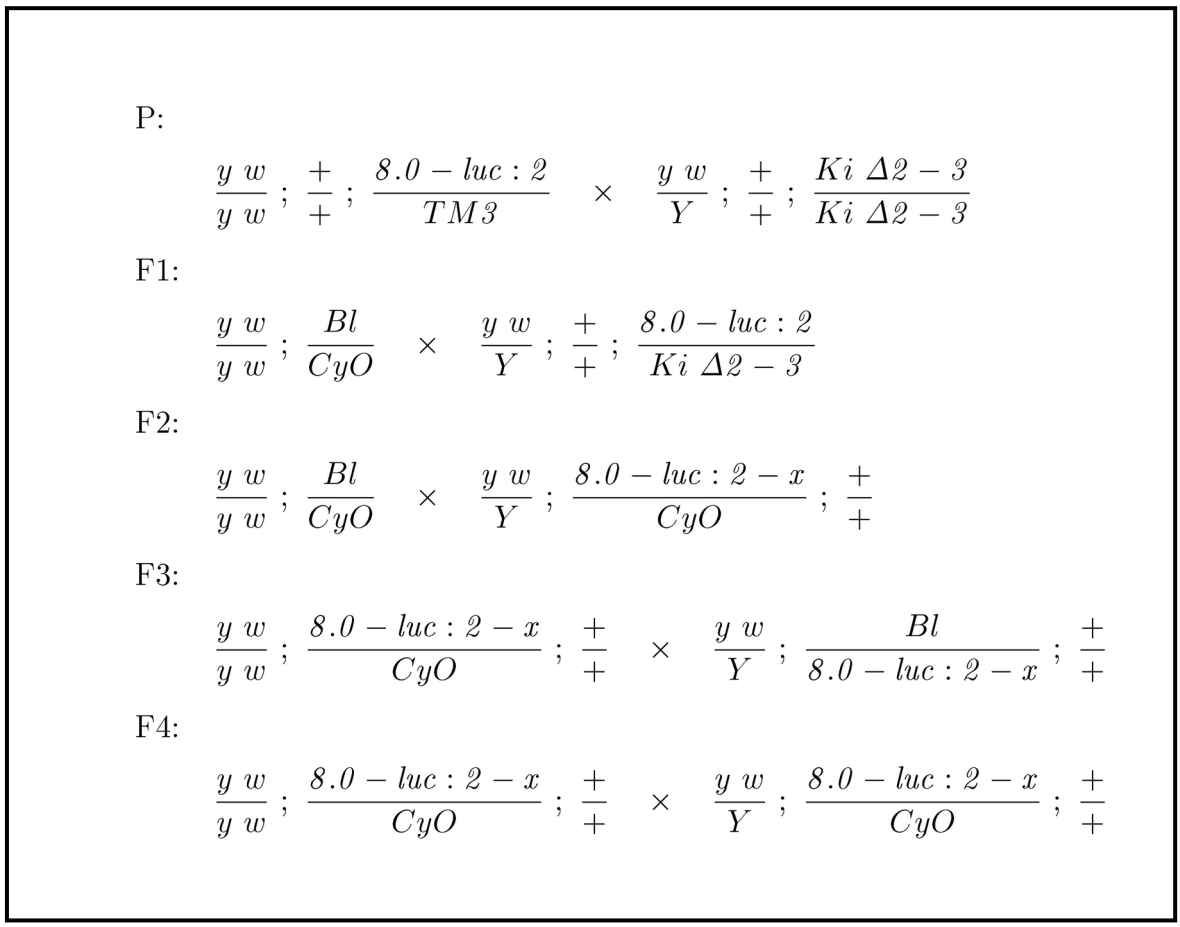

Figure 4 Genetic crosses performed for creating the mosaic flies from *8.0--luc:2* flies. First of all to mobilize the P element carrying transgene (*8.0--luc:2,* balanced with *TM3*--marked by *Stubble, Sb*), a transposase element is brought in by genetic crossing (mass cross) to transgenic flies carrying the transposase (*Ki Δ 2--3*). Out of this cross, to assure the presence of both the transgene and transposase, flies with red eyes, without *Sb* and with *Kinked* (*Ki*) were selected. In F1 cross, single female *y w;Bl/CyO* flies were crossed to single male red mosaic eyed *8.0--luc:2* flies with the marker *Ki*. In F2 cross, single male flies with distinct colored eye (potential P element 'jumps', with out *Ki*) were crossed to white eyed single female *y w;Bl/CyO* flies. In F3 cross, the potential 'jumps' balanced with *CyO* or *Bl* were crossed to each other (mass cross). Out of this cross, only if *w+* females or *w+* and *w* males, hatch, P element is on X chromosome (none of our lines had P on X chromosome). Out of F3 cross, if all the flies carrying *Bl/CyO* markers have a white eye (*w*), the transgene is present on the second chromosome (*8.0--luc:2--20,--22* have the transgene on the second chromosome). To get homozygous transgenic flies F4 cross was performed (mass cross) by crossing each other the balanced (*CyO*) transgenic flies. Flies without *CyO* and with colored eyes were saved for further investigations. For crosses always virgin females were selected.

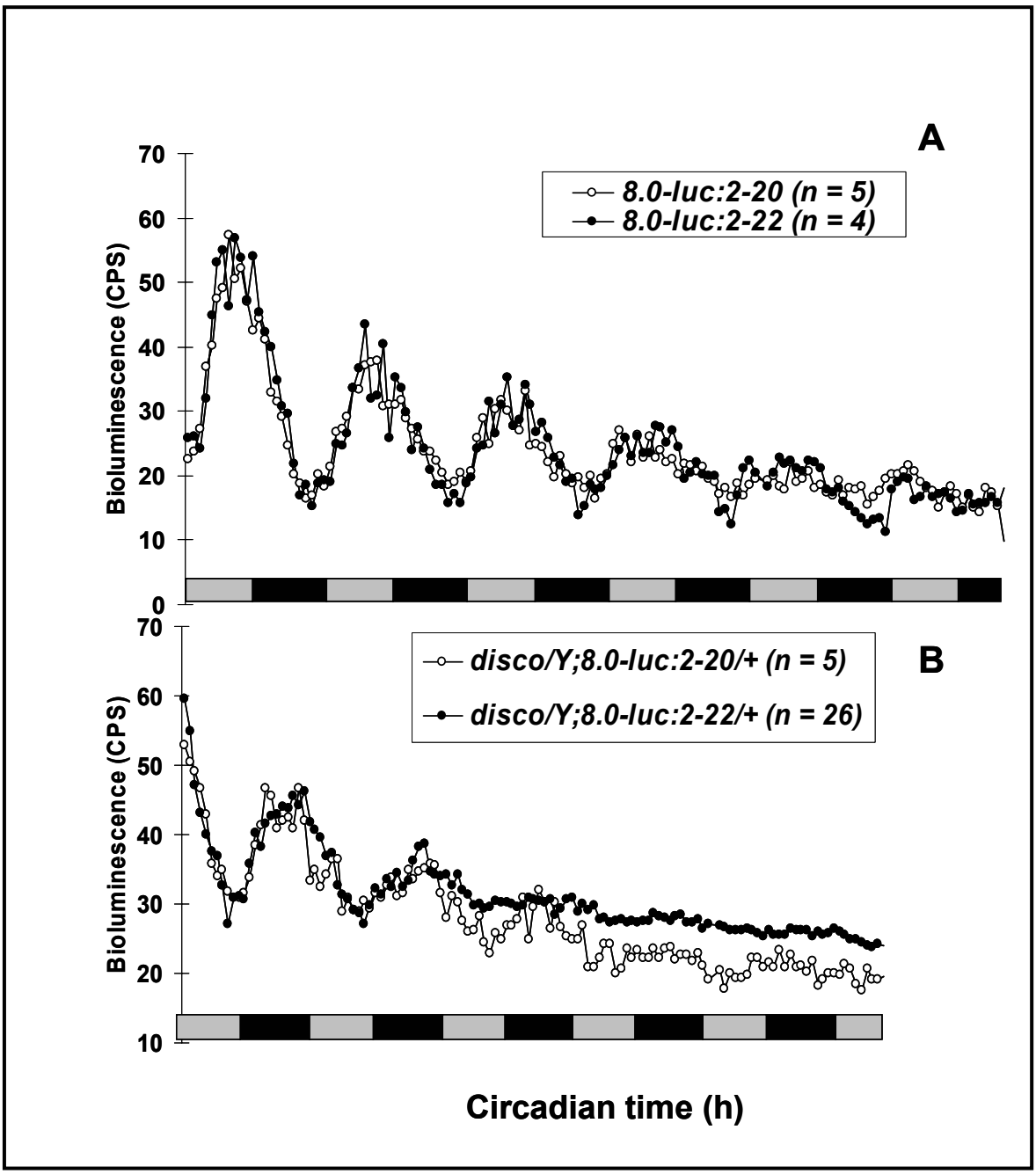

Figure 5 Bioluminescence rhythm records from *8.0--luc:2--20* and *8.0--luc:2--22* transgenic flies lines in *y w* and *disco*^{2} genetic backgrounds in DD. (A) In a *y w* genetic background both *8.0--luc:2--20* and *8.0--luc:2--22* flies showed a slowly dampening bioluminescence rhythm. (B) In a *disco*^{2} genetic background, the bioluminescence rhythm dampened quickly in both lines, pointing to the possible expression of transgene in the LNs. The axes are as described in Fig. 2.

luciferase expression was seen in a *disco*^{2} background for *8.0--luc:2--20* and *8.0--luc:2--22* transgenic flies (Fig. 4B and Table 4), indicating the transgene might also be expressed in the LN_vs, which could be correlated with the partial behavioral

rescue in DD in a *per*^{01} background (Table 5). Such a double--check of behavioral rescue in DD and dampened rhythmic luciferase expression in a *disco*^{2} background were one of our major criteria for selecting interesting lines expected to express the transgene in the pacemaker clock neurons.

In conclusion, the generation of stable "mosaic" *per*--expressing transgenic flies and analyzing their circadian rhythm with a multi--pronged experimental approach enabled us to successfully identify a novel self--sustained molecular oscillator in the DNs, which is entrainable by LD cycles. In this approach, we were at least able to isolate one mosaic line, which expresses the transgene in the DNs. It helped us to focus on the DNs. The possibility of a multi--level check to screen for mosaic lines assures the likelihood to isolate transgenics, which show expression pattern in the different subsets of clock cells. First of all the bioluminescence gives an impression whether it is rhythmic or not. Crossing this level most probably confines to a rhythm relevant cell. Further, depending on the cell group(s) where the transgene expresses, there is a possibility to get dampening or non--dampening bioluminescence expression in DD. In addition, checking the transgene's ability to rescue behavioral rhythms in DD in *per*^{01} flies shows the possible expression pattern of the transgene, which could be finally traced in the cells directly by immuno--staining. This approach may further help to study the nature and function of other clock neuronal groups in the *Drosophila* brain.

4.2.5 Disadvantages and advantages of our mosaic creating strategy

Since our *8.0--luc* transgene lacks the promoter region (Fig. 1), its expression depends mainly on the insertion site in the genome. Therefore, the efficiency of isolating a transgene expressing line is lesser (cf. Frisch *et al*., 1994), compared to the targeted expression subtraction strategy employing *GAL80* (Stoleru *et al.*, 2004; see in Chapter 2 Fig. 1), and the targeted expression assured by a promoter (Grima *et al*., 2004). The expression of promoterless *per* may be more aggravated in a *per*^{01} background due to the reasons discussed earlier.

Table 4 Summary of bioluminescence rhythms recorded from different *8.0--luc* transgenic flies lines (genetically mobilized from *8.0--luc:2*), in *per*$^{+}$ and *disco*^{2} genetic backgrounds. Experimental conditions and analysis are as described for Table 1.

Genotype	N (n)	percent rhythmic	Bioluminescence expression level (CPS)	τ ± SEM (h)	phase ± SEM (h)	rel--amp ± SEM
8.0--luc:2--1	6 (5)	83	49.9 ± 8.5	23.9 ± 0.3	5.8 ± 4.3	0.50 ± 0.05
disco^{2}*/Y;8.0--luc:2--1*	6 (3)	50	21.7 ± 0.8	25.9 ± 1.6	1.7 ± 5.9	0.62 ± 0.03
8.0--luc:2--2	6 (5)	83	253 ± 10	25.9 ± 3.1	23.1 ± 5.3	0.54 ± 0.03
disco^{2}*/Y;8.0--luc:2--2*	6 (1)	17	239	23.2	9.7	0.52
8.0--luc:2--3	6 (2)	33	185 ± 8	23.3	3.8	0.38
disco^{2}*/Y;8.0--luc:2--3*	3 (1)	33	92	21.6	14.7	0.65
8.0--luc:2--6	5 (5)	100	26 ± 2	23.5 ± 0.2	12.6 ± 0.1	0.47 ± 0.05
disco^{2}*/Y;8.0--luc:2--6*	6 (2)	33	25	22.6	3.2	0.61
8.0--luc:2--15	6 (6)	100	21.3 ± 1.0	23.4 ± 0.2	6.9 ± 3.5	0.51 ± 0.03
disco^{2}*/Y;8.0--luc:2--15*	4 (3)	75	20.9 ± 0.8	22.8 ± 0.4	2.8 ± 1.7	0.58 ± 0.06
8.0--luc:2--19	4 (4)	50	24.3 ± 0.7	23.9 ± 0.4	9.8 ± 1.7	0.56 ± 0.13
disco^{2}*/Y;8.0--luc:2--19*	6 (5)	83	29.9 ± 0.9	23.0 ± 0.1	0.3 ± 0.8	0.56 ± 0.04
8.0--luc:2--20	5 (5)	100	24.0 ± 0.7	22.8 ± 0.2	17.8 ± 4.2	0.46 ± 0.02
disco^{2}*/Y;8.0--luc:2--20*	4 (1)	25	23.9	22.4	2.2	0.58
8.0--luc:2--21	5 (5)	100	21.4 ± 0.7	22.9 ± 0.3	22.8 ± 4.9	0.44 ± 0.05
disco^{2}*/Y;8.0--luc:2--21*	6 (3)	50	29.8 ± 1.1	22.5 ± 0.8	3.1 ± 2.1	0.62 ± 0.02
8.0--luc:2--22	4 (3)	75	24 .1± 0.8	23.4 ± 0.2	10.7 ± 0.4	0.38 ± 0.05
disco^{2}*/Y;8.0--luc:2--22*	6 (2)	36	32.2	23.6	22.5	0.50
8.0--luc:2--25	6 (4)	67	21.2 ± 0.7	23.3 ± 0.2	4.8 ± 5.4	0.55 ± 0.02
disco^{2}*/Y;8.0--luc:2--25*	2 (0)	--	--	--	--	--
8.0--luc:2--33	6 (3)	50	24.5 ± 0.9	23.8 ± 0.7	22.7 ± 2.3	0.50 ± 0.09
disco^{2}*/Y;8.0--luc:2--33*	6 (2)	36	22.3	23.7	22.5	0.40
8.0--luc:2--34	3 (1)	33	32.5	23.1	1.3	0.37
disco^{2}*/Y;8.0--luc:2--34*	6 (1)	17	28.7	23.8	21.9	0.61
8.0--luc:2--36	4 (4)	100	31 ± 1	22.9 ± 0.2	0.5 ± 0.6	0.56 ± 0.03
disco^{2}*/Y;8.0--luc:2--36*	5 (1)	20	19	24.3	19.7	0.55

N is total number of flies tested, ‘n’ is number of rhythmic flies.

Table 5 Summary of locomotor behavioral rhythms in LD and DD of various transgenic fly lines genetically mobilized from *8.0--luc:2*. These 13 transgenic lines out of 43 showed rhythmic behavior in DD in a per^{01} background. Experimental details and analysis are as described for Table 2.

Genotype	**LD**						**DD**			
	N	**n**	**% rhy.**	**Anticipation of light--dark transitions**		**period ± SEM (h)**	**N**	**n**	**% rhy.**	**period ± SEM (h)**
				M	**E**					
per^{01}/Y;8.0--luc:2--1	8	6	75	-	++	24.2 ± 0.07	8	2	25	24.1
per^{01}/Y;8.0--luc:2--2	8	8	100	+	+++	24.3 ± 0.05	8	1	13	24.7
per^{01}/Y;8.0--luc:2--3	8	8	100	+	+	24.3 ± 0.06	8	1	13	22.5
per^{01}/Y;8.0--luc:2--6	8	6	75	-	+++	24.2 ± 0.07	8	1	13	24.2
per^{01}/Y;8.0--luc:2--15	7	4	57	-	+	24.1 ± 0.13	5	1	20	24.3
per^{01}/Y;8.0--luc:2--19	8	7	88	-	++	24.2 ± 0.06	8	3	38	24.7 ± 0.4
per^{01}/Y;8.0--luc:2--20	8	7	88	-	++	24.3 ± 0.09	8	3	38	24.4 ± 0.1
per^{01}/Y;8.0--luc:2--21	8	7	88	-	++	24.2 ± 0.08	8	3	38	24.6 ± 0.2
per^{01}/Y;8.0--luc:2--22	8	3	38	-	+++	24.5 ± 0.06	8	2	25	23.8
per^{01}/Y;8.0--luc:2--25	8	8	100	+	+	24.3 ± 0.06	8	4	50	24.4 ± 0.5
per^{01}/Y;8.0--luc:2--33	8	7	88	-	-	24.1 ± 0.1	8	1	13	22.5
per^{01}/Y;8.0--luc:2--34	8	7	88	+	+	24.3 ± 0.1	8	3	38	24.4 ± 0.5
per^{01}/Y;8.0--luc:2--36	8	7	88	-	-	24.1 ± 0.12	8	1	13	24.7

N is total number of flies tested, 'n' is number of rhythmic flies.

Nevertheless, the real--time expression monitoring has crucial multiple advantages compared to traditional molecular biology methods. Our approach allows screening a large number of animals simultaneously for rhythmic expression of the transgene, and thus could save a lot of time. It also allows repeated time measurements of the transgene expression from the individual flies, which is impossible in traditional molecular biology methods (Stempfl *et al*., 2002). The multiple measurements are a crucial criterion to judge the circadian expression of a gene. These possibilities are difficult to attain in the other strategies mentioned earlier. Because *8.0--luc* transgene lacks the promoter, it enabled us to specifically restrict the expression to subsets of clock neurons, and thus helped us to identify a sustained molecular oscillator in the dorsal brain. This may be rather difficult to achieve in a GAL4 mediated approach, where a subset specific promoter would be inevitable.

5 Circadian clock information in the 8.0 kb promoterless *period* DNA

The *8.0--luc* transgenic lines discussed above exhibited varying degree of spatial expression patterns of the transgene in the clock neurons (Table 3). Below I shall briefly discuss the possible reasons for this varying spatial expression. Further, I shall compare the *8.0--luc* transgene's expression pattern and its behavioral rescue to other *per* transgenes studied in the past.

The *per* genomic locus spans the 3B1--2 region of the X chromosome in *Drosophila* (Smith and Konopka, 1981). Molecular cloning of DNA sequences including those of *per* allowed to generate germ--line transformants for *per,* and certain sub--segments of *per* DNA could rescue locomotor rhythms in per^{01} flies (Zehring *et al*., 1984). Among the different sub--segments of *per* genomic locus, the *8.0* kb sub--segment draws special attention (Fig. 1), because it lacks an extensive 5' flanking region (including the promoter, where E--boxes for CLK:CYC binding are located [see Chapter 1, section 4.2 and Fig. 3 therein]; first non--coding exon and major part of the first intron). This sub--segment was able to rescue locomotor rhythms in per^{01} flies in DD with a period of longer than 24 h

(Table 6). Therefore it was concluded that the *8.0* kb sub--segment contained crucial sequences of the *per* locus, coding for the control of *Drosophila*'s biological clock (Zehring *et al*., 1984). However, an anti--PER antibody failed to detect any signal derived from the *8.0* kb sub--segment in the adult head, but the 8.0 constructs rescued locomotor rhythms in 50% of the animals' analyzed (Zerr *et al*., 1990). Therefore, it might be expressed in the LN_vs. Similar *8.0* kb sub--segments of *per* rescued behavior of per^{01} flies in DD, but their expression pattern was not tested (Zehring *et al.*, 1984; Liu *et al.*, 1991; Cooper *et al*., 1994; Table 6). Later on, the spatial expression pattern of the *8.0* kb sub--segment was investigated by using a *period--lacZ* (*8.0--lacZ*: in original paper named as *8.0--βgal*) reporter fusion gene (Fig. 1; Liu *et al*., 1991); this fusion gene had lesser 3' flanking DNA sequences (and it did not encode the full PER) than the original *8.0* kb sub--segment but the same 5' end (Fig. 1). The *8.0-lacZ* was expressed in DNs, LN_vs, LN_ds and in the eye photoreceptors (RNA blot experiments shown that *8.0--lacZ* is also present in the body), though less intensely than a larger reporter construct, which carried full 5' flanking region of *per* including the promoter (Liu *et al*., 1991). It was implicated that the absence of a major part of the first intron restricted the broad tissue specific expression of *per* to certain subsets of clock gene expressing cells, including the pacemaker neurons. The absence of first intron may also inhibit splicing of the small introns 2, 3 and 4 (Fig. 1**;** Liu *et al*., 1991).

Similarly, a *7.2* kb sub--segment, carrying 800 bp less 5' flanking material than the *8.0* kb sub--segment (Fig. 1) also rescued locomotor rhythms in few transgenic lines in DD, again with a longer period. In one *7.2* transgenic fly line, the transgene was expressed exclusively in the LNs; in another line it was additionally expressed in the eye photoreceptors and DNs (Table 6; Frisch *et al*., 1994). The *per--luc* reporter gene (*8.0--luc*) was similar to the *8.0* kb fragment used by Liu *et al*. (1991) except that the *8.0--luc* missed the sequences that code for last 10 amino acids of PER at the 3' flanking end and *per*'s 3' UTR, and that it was fused to the luciferase cDNA (Fig. 1). Transgenics *8.0--luc*:--2,.--4,--9 and --11, could not largely rescue the rhythmic behavior in per^{01} flies in DD (Table 2; Veleri *et al*., 2003). However, 13 of the newly mobilized *8.0--luc:2* lines showed partial rescue in DD (Table 5). The *7.2* kb transgene expressed in the LN_ds and LN_vs without the

Table 6 Promoterless *per* DNA sub--segments' spatial expression and behavioral rescue.

***per* sub--segment (in kb)**	**Expression pattern in *per*^{01} in LD**	**Behavioral rescue in DD in *per*^{01}**	**$\tau \pm$ SEM in DD in *per*^{01} (h)**	**Reference**
7.2:2	LN_ds, LN_vs	Yes	26.4 $\pm$ 0.1	Frisch *et al.*, 1994
7.2:9	DNs, LN_ds, LN_vs, photoreceptors, gut	Yes	26.0 $\pm$ 0.1	Frisch *et al.*, 1994
7.2:2	Not analyzed	Yes	26.0 $\pm$ 0.1	Cooper *et al.*, 1994
7.2:9	Not analyzed	Yes	25.9 $\pm$ 0.1	Cooper *et al.*, 1994
8.0:2(b)	Not analyzed	No	--	Zehring *et al.*, 1984
8.0:3(b)	Not analyzed	Yes	25.5 $\pm$ 1.5	Zehring *et al.*, 1984
8.0:4(b)	Not analyzed	Yes*	25.0	Zehring *et al.*, 1984
8.0:11(b)	Not analyzed	Yes*	ca 28	Zehring *et al.*, 1984
8.0:14(b)	Not analyzed	Yes*	ca 28	Zehring *et al.*, 1984
8.0:24(a)	Not analyzed	Yes	24.9 $\pm$ 0.3	Zehring *et al.*, 1984
8.0:4	No signal detected	Yes	24.4 $\pm$ 0.5	Zerr *et al.*, 1990
8.0--2a	Not Analyzed	Yes	24.1 $\pm$ 0.1	Liu *et al.*, 1991
8.0--10a	Not Analyzed	Yes	25.4 $\pm$ 0.9	Liu *et al.*, 1991
8.0--12a	Not Analyzed	Yes	24.6 $\pm$ 0.3	Liu *et al.*, 1991
8.0--24a	Not Analyzed	Yes	24.2 $\pm$ 0.2	Liu *et al.*, 1991
8.0--44	Not Analyzed	Yes	25.5 $\pm$ 1.0	Liu *et al.*, 1991
8.0--47a	Not Analyzed	Yes	27.9 $\pm$ 1.7	Liu *et al.*, 1991
8.0--54a	Not Analyzed	Yes	27.0 $\pm$ 1.5	Liu *et al.*, 1991
8.0--79a	Not Analyzed	Yes	29.5 $\pm$ 2.3	Liu *et al.*, 1991
8.0--80a	Not Analyzed	Yes	25.4 $\pm$ 0.3	Liu *et al.*, 1991
8.0--AV	Not Analyzed	Yes	26.0 $\pm$ 0.5	Liu *et al.*, 1991
8.0:4	Not Analyzed	Yes	24.4 $\pm$ 0.2	Cooper *et al.*, 1994
8.0:24a	Not Analyzed	Yes	25.0 $\pm$ 0.2	Cooper *et al.*, 1994
8.0:24b	Not Analyzed	Yes	25.0 $\pm$ 0.2	Cooper *et al.*, 1994
8.0--lacZ (*originally named 8.0--β--gal*)	Widely in head with varying intensity; DNs, LNs, photoreceptor, lamina, in body (RNA blot)	Not analyzed	--	Liu *et al.*, 1991
*8.0--luc:2***	DNs,LN_ds,l--LN_vs§	Yes*	23.5 (1/14)	Veleri *et al.*, 2003
*8.0--luc:2--20***	No signal detected	Yes*	24.4 $\pm$ 0.1	
*8.0--luc:2--22***	No signal detected	Yes*	23.8 (2/8)	
8.0--luc:4 ##	DNs,LN_ds,l--LN_vs§	Yes*	26.2 $\pm$ 0.8	
8.0--luc:9	DNs,LN_ds,l--LN_vs§	Yes*	28.6 (1/10)	
8.0--luc:11 #	DNs,LN_ds,l--LN_vs§	Yes*	23.7 $\pm$ 0.5	

, ## Level of expression in the eye photoreceptors.
* Weakly rhythmic, see Table 2 for details on *8.0--luc.* ** See Table 5 for the mobilized lines' free running period. § See Table 3.

promoter, first untranslated exon and first intron. However, in spite of having 800 bp extra 5' flanking sequences in the *8.0--luc* transgene, it largely failed to express in LNs (very few LN_ds expressed, see Table 3) (Veleri *et al*., 2003).

The differences in expression pattern of the *8.0* kb and *8.0--luc* might be due to the spatial information in the 3' UTR. The neighboring transcription factors and/or other unknown factors regulating a neighboring gene might be influencing their expression strength. However, the *8.0--lacZ* and *8.0--luc* expressed in the clock neurons in the head (Table 6; Liu *et al*., 1991; Veleri *et al*., 2003), *7.2* kb sub--segments also could express in the head (Frisch *et al*., 1994). It indicates that these sub--segments still carry some crucial spatial information to target expression to the clock cells. Possibly, the differences among different versions of *8.0* kb sub--segment (with or without a reporter gene) may be due to the presence of a fusion protein, e.g. luciferase in our study. It could also due to the presence/absence of the 3' UTR. Almost all the *8.0* kb sub--segment without a reporter fusion gene rescued rhythmic behavior in DD (Table 6; Zehring *et al*., 1984; Liu *et al*., 1991; Cooper *et al*., 1994). Most of the *8.0* kb sub--segment with *luc* reporter gene could not rescue rhythmic behavior in DD (Table 6 and in Appendix 2, Table 5 Supplement; Veleri *et al*., 2003). But partial behavioral rescue was observed in some transgenic lines (Table 2, 5 and 6). It is not tested yet whether *8.0--lacZ* could rescue rhythmic behavior in DD (Liu *et al*., 1991). The inability of *8.0--luc:9* to rescue behavior in per^{01} flies in DD might be justified by the lack of expression of it in the LN_vs of *8.0--luc:9* transgenic flies (Veleri *et al*., 2003). The expression of *8.0--lacZ* in the LNs but not *8.0--luc:9* remains intriguing because if *8.0--lacZ* has all the spatial information to target expression to the LNs, the *8.0--luc:9* should also carry almost all this information. One possibility is that the *8.0--luc:9* fusion product targets differently than the *8.0--lacZ* fusion product. Alternatively, *8.0--lacZ* could lack some "restrictive regulatory elements". It is possible that the luciferase protein and β--GAL affect the stability and turnover of the fusion product. However, the *8.0--luc:9* transgene expression in the DNs suggests that it is unlikely that the fusion protein affected the spatial expression pattern. A rather remote possibility is that the strength of expression of the transgene *8.0--luc:9* in the LN_vs is extremely weak, which may be beyond the sensitivity of detection methods we employed. But failure to rescue the behavioral

rhythms in DD in a *per*^{01} background indicates that the *8.0--luc:9* might not be expressed in the s--LN_vs. However, the discrepancy between the expressions pattern of *8.0--lacZ* and *8.0--luc:9* remains wide open. Nevertheless, the partial behavioral rescues observed in other *8.0--luc* lines (Table 2, 5) indicate that the *8.0--luc* could also be expressed in the LN_vs. It is possible that *8.0* kb has a sequence information to target into the s--LN_vs that may be absent in the *8.0--luc.* Possibly, *lac--Z* could be more sensitive than anti--PER.

5.1 Influence of the transgene on the free running period

Most of the behaviorally rescued transgenic flies exhibited a longer period length (Table 6). Why does the free running period length differ among the different *8.0 kb* transgenic flies? Possibly temporal features of the *per* gene expression may be different for the promoterless constructs. They lack E--boxes, where the transcriptional activation is effected (see Chapter 1, section 4.2 and Fig. 3 therein). However, very few of the transgenics showed a free running period ~24 h. Therefore, it is also possible that the posttranslational control of PER could bring about a temporal difference (e.g., Sathyanarayanan *et al*., 2004). Moreover, depending on the site of insertion in the genome, the transcriptional elements neighboring the transgene may affect its expression level. An increased or decreased production of PER could possibly shorten or lengthen the free running period, respectively (Smith and Konopka, 1981). Changes in the phosphorylation state of PER caused by DBT affects the turnover of the former and therefore the free running period length of *Drosophila* (Price *et al*., 1998). Therefore, possibly a different half--life of PER--LUC could affect the free running period length. It was shown that in the absence of PDF the cytoplasmic retention of PER is reduced, and hence these flies showed a shorter period (Lin *et al*., 2004). The firefly luciferase has a SKL tripeptide in its C--terminal, which localizes it to the peroxisomes (Keller *et al*., 1987). Whereas PER has both a nuclear and cytoplasmic localization signal (Yildiz *et al*., 2005). The presence of multi--location target sequences in the PER--LUC fusion protein may cause some kind of ‘conflict’ and hence a delay to shuttle PER into the nucleus. Therefore, this may result in a longer free run period. This seems highly unlikely, because all *8.0* transgenic lines with or without a reporter gene produce a longer free run period (Table 6).

6 Conclusion

We have demonstrated that by using a promoterless *per* it is possible to create mosaic flies which express *per* in restricted subsets of clock neurons. This approach helped us to identify a novel molecularly self--sustained circadian oscillator in the DNs, which is entrainable by light--dark cycles. This oscillator entrains to LD even in $norpA^{P41};cry^{b}$ and gl^{60j} cry^{b} flies. Therefore it is possibly entrained by a novel putative photopigment, functioning independent of *norpA* and *cry*. But *cry* is required for proper phasing of PER oscillations in DNs. This indicates the existence of a non--classical light signaling route in *Drosophila*. This novel putative photoreceptor may be involved in signaling high intensity light in the $norpA^{P41};cry^{b}$ flies, as pointed out in the previous chapter. The DN oscillator does not require PDF to sustain its rhythmicity. We also established that the s--LN_vs are a *bona fide* molecularly self--sustained circadian oscillator. *per* expression shows differences at the transcriptional and translational level, depending on the different subsets of clock neurons. The 8.0 kb promoterless *per* carries spatial information to target its expression in the brain clock neurons, therefore it could be used to create mosaic flies expressing *per* in subsets of the clock neurons. This kind of mosaic approach may help to further widen our understanding about the different clock neurons in the brain.

CHAPTER 5

A NOVEL, RHODOPSIN--LIKE GENE, IS EXPRESSED IN THE *DROSOPHILA MELANOGASTER* BRAIN

Chapter 5

A novel, Rhodopsin--like gene, is expressed in the *Drosophila melanogaster* brain.

1 Introduction

The circadian clock is entrained by light signals perceived through the retinal and extra--retinal photoreceptors like rhodopsins and cryptochrome. In *Drosophila* six rhodopsins (Montell, 1999) and a cryptochrome (Stanewsky *et al*., 1998; Emery *et al*., 1998) have been reported. Phototransduction *via* rhodopsin is blocked in the $norpA^{P41}$ mutant flies. The *norpA* gene encodes for a phospholipase C (PLC), which is involved in the phototransduction cascade leading from rhodopsin (Montell, 1999; see Chapter 1, section 2.2.1.1). In $norpA^{P41}$ mutant flies the phototransduction cascade in the compound eyes and ocelli is affected (Pearn *et al*., 1996). *cry* encodes for the flavin based blue light photoreceptor cryptochrome and its function is completely or nearly abolished in cry^{b} mutant flies (Stanewsky *et al*., 1998). In the double mutant, $norpA^{P41};cry^{b}$ flies both rhodopsin and cryptochrome mediated phototransduction into the circadian clock is blocked. However, the $norpA^{P41};cry^{b}$ double mutant flies are still able to synchronizable to light--dark cycles (LD) but it takes them much longer to re--entrain to shifted LD [i.e. equivalent to a jet lag experience] (Stanewsky *et al*., 1998; Emery *et al*., 2000b). This indicates that the circadian clock receives light input through a novel photoreceptor independent of *norpA* and *cry* (Hall, 2000). In the previous chapters I have shown evidence for a new putative photoreceptor in the dorsal brain of *Drosophila* and in the H--B eyelet.

A promising candidate for a novel circadian photopigment is the novel putative rhodopsin gene (*Rh 7*), which was published in the *D. melanogaster* genome project (Adams *et al*., 2000). The gene maps to the third chromosome and its predicted amino acid sequence shows maximum homology to *Drosophila* rhodopsin *Rh 5,* i.e. 30.7% and minimum homology to *Rh 6*, i.e. 25.0% (see Appendix 2). *Rh 7* amino acid sequence also shows a 12.5% homology with the cephalopod retinochrome (retinal photoisomerase) from the Japanese squid

(*Todarodes pacificus*). (DNAMAN 4.0 software was used to see the amino acid sequences homology). The predicted protein of *Rh 7* encodes for a G protein coupled photoreceptor like other *Drosophila* rhodopsins (FlyBase).

In this chapter I describe the spatial expression profile of the putative *Rh 7* gene in the adult fly. Part of its promoter was cloned in front of GAL4 to drive the GFP reporter gene in a GAL4/UAS--GFP reporter system (Brand and Perrimon, 1993; Mollereau *et al*., 2000). Using this system I could demonstrate that the *Rh 7* promoter is active in the *D. melanogaster* brain.

2 Materials

2.1 Fly stocks

w;UAS--τ--GFP (Brand, 1995).

2.2 Microorganisms

Genotype	Type	Literature and source
recA1 endA1 gyrA96 thi--1 hsdR17 supE44 relA1 lac [F´ *proAB lacI*^{q}*Z.M15* Tn*10* (Tet$_{r}$)]	*E. coli* -- XL 1-- Blue Electro competent Cells	Stratagene

2.3 Vectors

Name	Literature and source
pGEM--T Easy I cloning kit	Promega
pTGAL4	Sharma *et al*., 2002

2.4 DNA Oligonucleotides

Primers were designed to create a desired restriction site, and were obtained from Invitrogen.

Name	Sequence	Remarks
Rh 7 III (*BamH* I)	5' acatggatccccgacatcgatagccat 3'	at the 5' end a *BamH* I site created
Rh7 R3 (*EcoR* I)	5' tacagaattccgctttttagctgcaagactc 3'	at the 5' end a *EcoR* I site created

3 Methods

3.1 Molecular biology methods

The general molecular biology procedures were done as detailed in Sambrook and Russel (2001); any change from the standard procedure is mentioned separately. The complete strategy used for cloning a fragment of the *Rh 7* promoter is depicted in the scheme (Fig. 1A, B).

3.2 Genomic DNA preparation

Genomic DNA was prepared using the single--fly DNA preparation protocol for PCR developed by Gloor *et al*. (1993).

3.3 Polymerase Chain Reaction (PCR)

For a final reaction volume of 50 μl:

Reaction Mixture (40 μl)

Component	Volume (μl)
gDNA	1.0
dNTPs (10 mM)	2.0
Forward primer--*Rh 7III* (*BamH* I) (7.5 μM)	2.0
Reverse primer--*Rh 7 R3* (*EcoR* I) (7.5 μM)	2.0
Reaction Buffer 10X	4.0
Sterile dH_2O	29.0

Enzyme Mixture (10 μl)

Component	Volume (μl)
Reaction Buffer 10X	1.0
Expand High fidelity Taq Polymerase	1.2
Sterile dH_2O	7.8

PCR settings: (Stratagene® --Robocycler Gradient 40)

Window	Melting temp.	Annealing temp.	Polymerization temp.	No. of cycles
	95°C	53°C	68°C	
I	4 min	0 min	0 min	1X
II	50 sec	1 min	4 min	18X
III	50 sec	1 min	6 min	15X
IV	50 sec	1 min	12 min	1X

To minimize unspecific PCR--products standardized hot start PCR was employed (Chou *et al*., 1992). For a *hot start* PCR, an Eppendorf tube containing the Reaction Mixture was placed in a Robocycler for 4 min at 95°C for melting the DNA double strand. The Enzyme Mix was directly added into the Reaction Mixture after the 4 min of melting. To optimize the PCR reactions, a pilot PCR reaction was performed by employing a temperature gradient for annealing ranging from 51°C to 58°C. 53°C was found to be optimal.

3.4 Cloning and sub--cloning of the PCR amplified DNA

A 1.4 kb DNA fragment amplified by PCR was separated on a TAE Agarose gel (Fig. 3A) and later extracted and purified using the standard *QIAquick® Gel Extraction Kit* (Qiagen). It was then cloned into the PCR cloning vector pGEM--T Easy (as in Fig. 1A) following the protocol provided (Promega). This clone was used for electro--transforming *E. coli* XL--1--Blue--Cells (Stratagene). The transformant cells were double selected on an ampicillin, X--Gal plate. For small scale qualitative analysis of plasmids, alkaline lysis minipreps were done (Ausubel *et al*., 1996). To obtain larger and cleaner amounts of plasmid DNA from the

positive clone “Midi--preps” were performed using the *QIAGEN® Plasmid Midi Kit* (Qiagen).

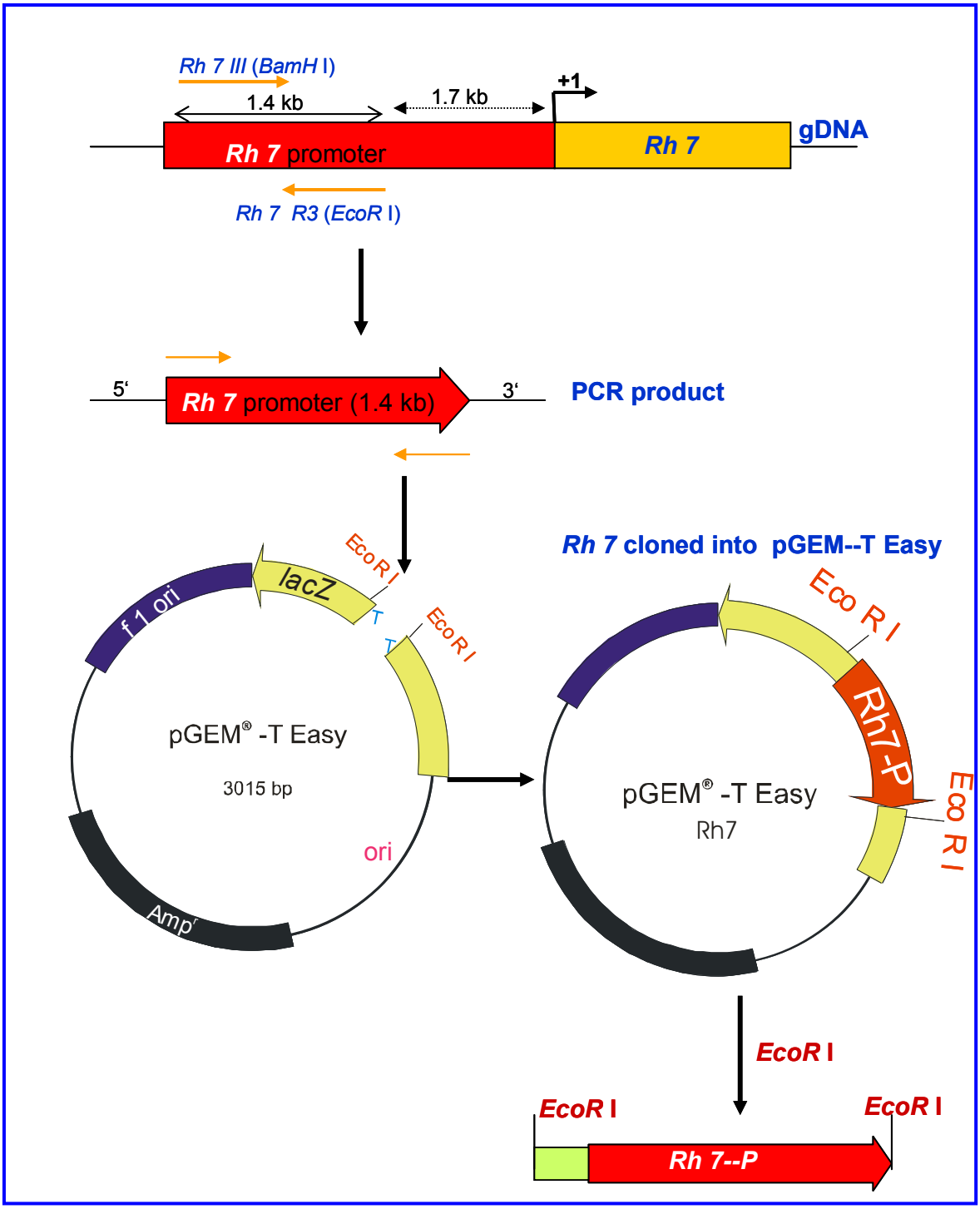

Figure 1A From gDNA a 1.4 kb *Rh 7--P* PCR product was generated using the oligonucleotides *Rh 7 III* and *Rh 7 R3*, and it was cloned into the pGEM--T Easy. By *EcoR* I digestion the *Rh 7--P* retrieved from pGEM--T Easy. The 1.4 kb cloned DNA lacks immediately following it another 1.7 kb promoter fragment.

The *Rh 7 promoter*--fragment was retrieved from the pGEM--T Easy after *EcoR* I restriction enzyme digestion and then sub--cloned into pTGAL4 (as in Fig. 1B).

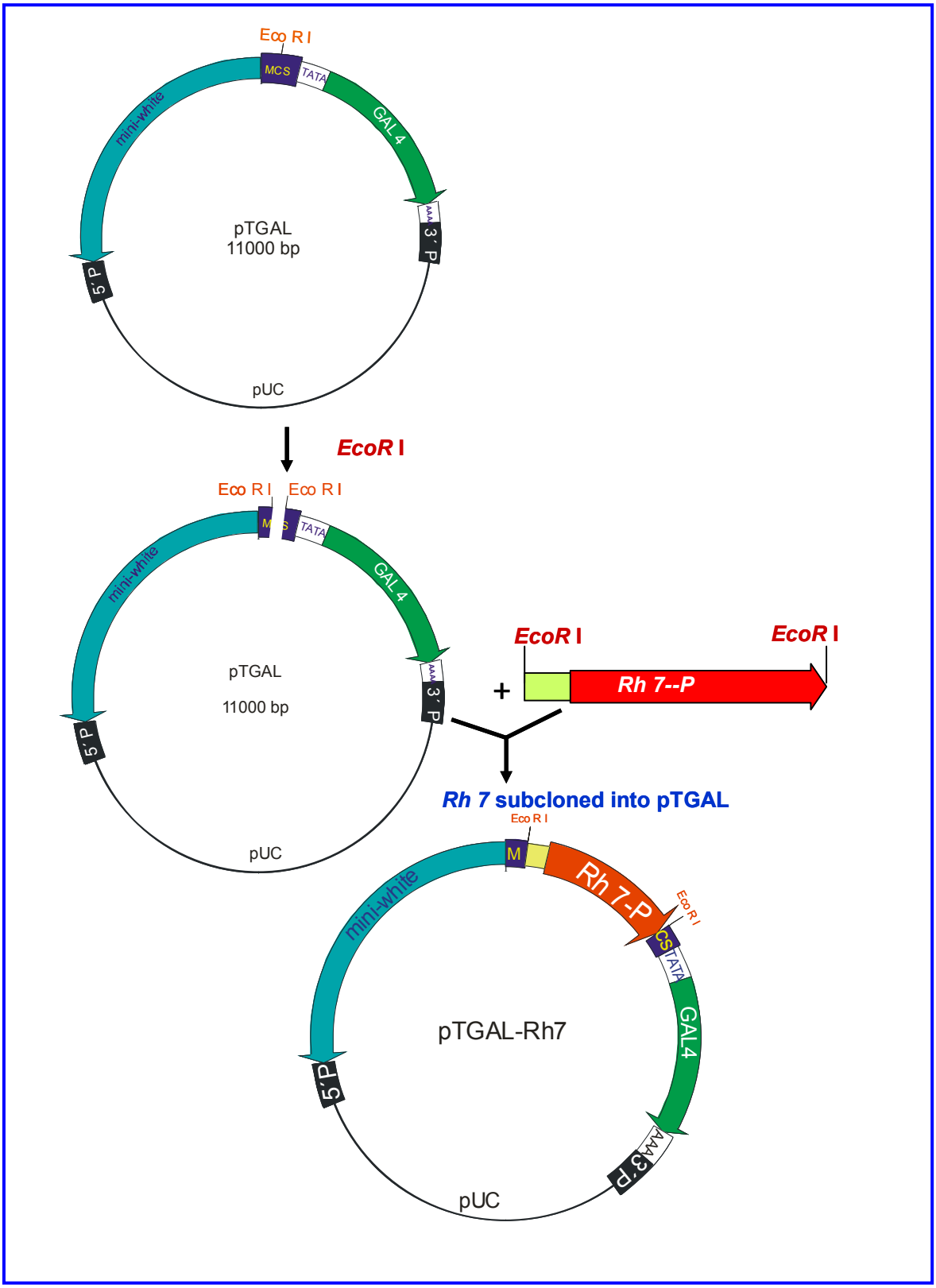

Figure 1B The 1.4 kb *Rh 7--P* DNA fragment was sub cloned from pGEM--T Easy into the pTGAL. *Rh 7--P* DNA fragment is retrieved from pGEM--T Easy by *EcoR* I digestion, and

is cloned into the *EcoR* I site in the multiple cloning site (MCS) in pTGAL. The clock--wise direction of *Rh 7--P* DNA fragment in pTGAL represents the correct orientation. The orientation of cloning was tested by restriction analysis (see more in Fig. 3).

The transformed cells were double selected as mentioned earlier. Subsequently, mini and midi plasmid preparations were performed to generate sufficient amounts of pure plasmid DNA (pTGAL4--*Rh 7*) for micro--injection.

3.5 Generation of germ--line transformants and stable transgenic lines

The pTGAL4--*Rh 7* was used to generate germ--line transformants as described by Peschel (2004). A total of 6 transgenic lines were generated according to Peschel (2004). P element insertions were mapped to a particular chromosome using standard genetic crosses (Greenspan, 1997). The following three lines were used for further investigations.

1) *Rh 7:9--1/CyO*
2) *Rh 7:1A/TM3*
3) *Rh 7:2B/TM3*

3.6 GAL4/UAS system

The GAL4/UAS system was used to express the GFP under the control of the *Rh 7* promoter (Brand and Perrimon, 1993). For details about the GAL4/UAS system, see in Chapter 2 Methods, section 2.3.

3.7 Genetic crosses

To combine the GAL4 and UAS elements the following cross was performed (Fig. 2). Four to five days aged male flies from the F1 generation were selected for preparing whole mount brains (see more in Chapter 2 Methods, section 2.5).

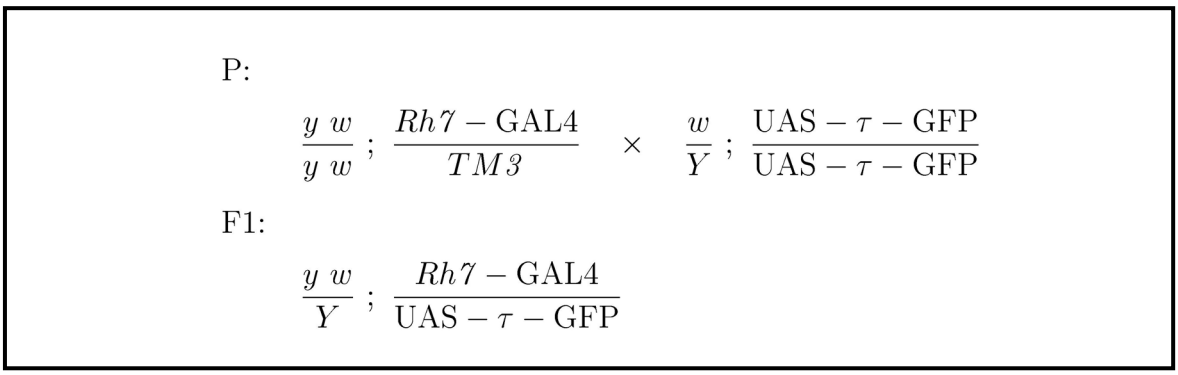

Figure 2 Genetic cross between *Rh 7*--GAL4 female (virgin) and UAS--τ--GFP male flies to bring together the GAL4 and UAS elements. The *Rh 7*--GAL4 flies were balanced with *TM3* (the marker *Stubble, Sb*, enables to track *TM3*). In the F1 generation flies without *TM3* balancer (no *Sb*) were selected for further studies.

3.8 Microscopy

Whole mounted brains prepared from τ--GFP expressing transgenic flies were inspected under Zeiss LSM 510 META confocal microscope. To visualize the green fluorescence, brains were excited with a blue Argon laser (488 nm).

4 Results

4.1 Cloning of the *Rh 7* promoter

To investigate the *Rh 7* expression profile, the GAL4/UAS system was chosen (Brand and Perrimon, 1993). In order to clone the presumptive *Rh 7* promoter in front of the GAL4 sequences in pTGAL4, first DNA sequences, expected to contain the *Rh 7* promoter were PCR amplified from *D. melanogaster* genomic DNA (gDNA) using the *Rh 7* promoter specific oligonucleotides [*Rh 7 III* (*BamH* I); *Rh 7 R3* (*EcoR* I)] (Fig 1A). This PCR product was used for cloning and sub--cloning as mentioned (see Appendix 1 for the complete sequences cloned).

To ensure that the *Rh 7* promoter was cloned into pTGAL4 in the correct orientation a restriction analysis was done. Correct orientation results in a DNA fragment of ~200 bp after *Bgl* II digestion (Fig. 3B). Wrong orientation would produce a larger DNA fragment of 1.2 kb.

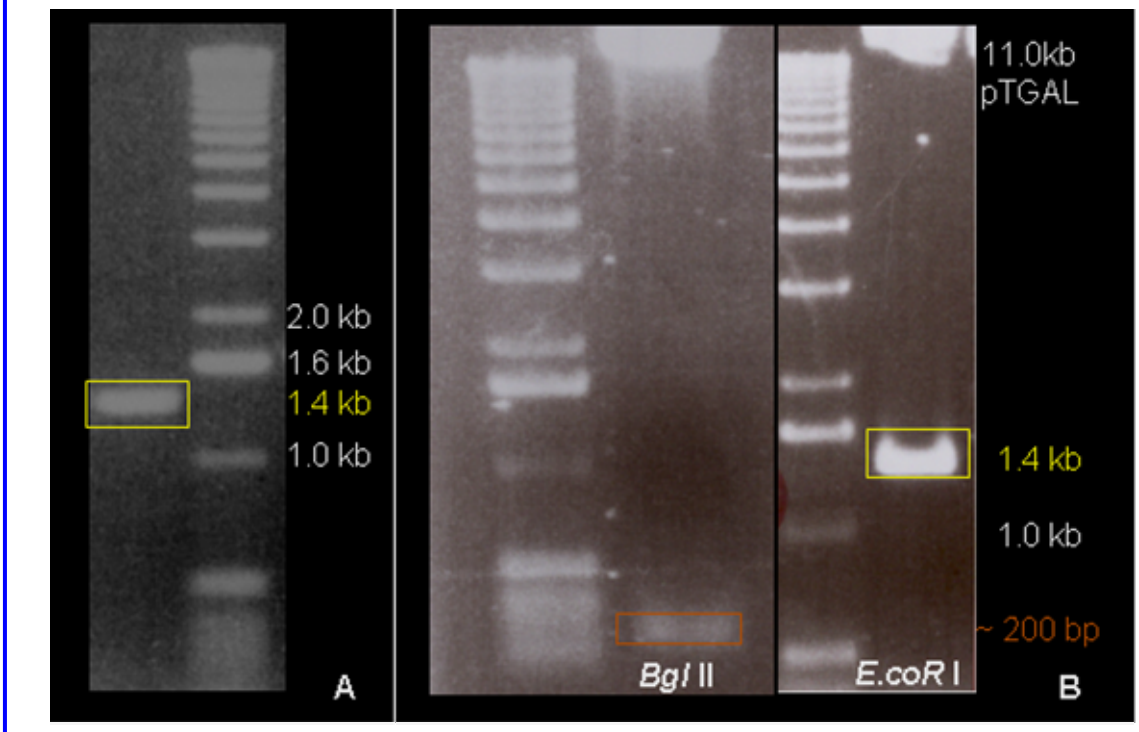

Figure 3 The gel photographs showing different DNA fragments generated for cloning the *Rh 7* promoter into pTGAL4. (A) Left panel depicts a 1.4 kb PCR product (in yellow rectangle) amplified from genomic DNA by the *Rh 7* promoter specific oligonucleotides. Right panel shows a 1 kb DNA ladder used for identifying DNA size. (B) The left panel shows a *Bgl* II digestion of pTGAL4--*Rh 7*. A 200 bp DNA fragment (red rectangle) was expected only if *Rh 7* is inserted in the correct orientation. The right panel shows the 1.4 kb recovered from the pTGAL4--*Rh 7* by *EcoR* I.

4.2 The spatial expression of *Rh 7* promoter mediated GFP in the *Drosophila* brain

y w flies were transformed with the pTGAL4--*Rh 7*, and six stable transgenic lines were established. Out of this six transgenic lines, three lines: *Rh 7:9--1/CyO*, *Rh 7:2B/TM3* and *Rh 7:1A/TM3* were further investigated for the spatial expression pattern of the GFP reporter gene driven by the *Rh 7* promoter. For this, *Rh 7*--GAL4 female transgenics were crossed to a UAS--τ--GFP male transgenics and the F1 male flies (see Fig. 2) were used for preparing whole mounted brains. The *tau* protein linked GFP enables to label the whole extent of cell morphology in

exquisite detail except the cell nucleus, because *tau*--GFP reveals the microtubule network within the cell (Brand, 1995).

Whole mounted brains of transgenic flies were prepared and tested for GFP reporter activity. Among the three tested lines, two lines: *Rh 7:9--1/*UAS--τ--GFP and *Rh 7:2B/*UAS--τ--GFP were expressing GFP in the sub--esophageal neurons, which sent fibers towards the mid--dorsal protocerebrum (Fig. 4A, B), but the third

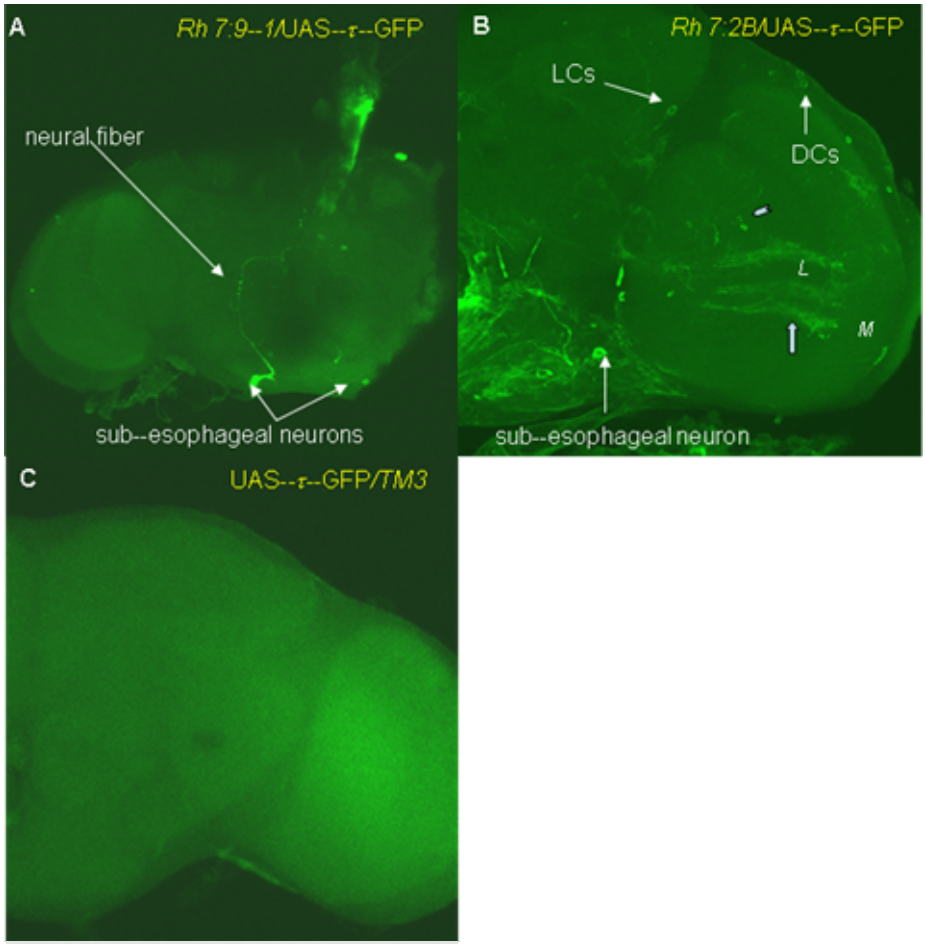

Figure 4 GFP reporter gene expression in the *Drosophila* brains under control of the *Rh 7* promoter using the GAL4/UAS system. *Rh 7:9--1/*UAS--τ--GFP and *Rh 7:2B/*UAS--τ--GFP flies expressed τ--GFP in the brain. Mainly two sub--esophageal neurons expressed GFP (A, B). These sub--esophageal neurons sent fibers towards the mid--dorsal protocerebrum. In the transgenic line *Rh 7:2B*, some cells close to lateral clock neurons (LNs)--called lateral cells (LCs) and close to dorsal clock neurons (DNs)--called dorsal cells (DCs), some cells in the lobula (arrow head), and three bundles of axons in the optic

lobe, running from the medulla to lobula (bold arrow) also showed GFP activity (B). Control flies (UAS--τ--GFP/*TM3*) not carrying the *Rh 7* promoter cloned in front of GAL4 did not show any GFP signals. In control flies the optic lobe and the dorsal brain did not show any trace of GFP expression at all (C). M means medulla and L means lobula.

transgenic fly line (*Rh 7:1A/TM3*) did not show any detectable GFP signals. In *Rh 7:2B/*UAS--τ--GFP some cells in the position of dorsal clock neurons and lateral clock neurons (see in Chapter 1 Fig. 2), here on called the dorsal cells (DCs) and the lateral cells (LCs), respectively, displayed GFP activity (Fig. 4B). We named them based on their relative positions in the brain. In the same flies GFP signals were also visible in few axon--like bundles in the optic lobe (Fig. 4B) however, the exact origin of these bundles was not revealed.

To prove if the GFP signals were indeed driven by the presumptive *Rh 7* promoter sequences, the same UAS--τ--GFP flies without the *Rh 7*--GAL4 construct (UAS--τ--GFP/*TM3*) were inspected for GFP expression. Brains prepared from these control flies showed no detectable GFP signal (Fig. 4C). Therefore, the expression of GFP seen in Fig. 4 (A, B) is exclusively driven by the *Rh 7* promoter.

5 Discussion

Light signals perceived by rhodopsins in the retinal and extra--retinal photoreceptors and, by cryptochrome in the clock neurons entrain the circadian clock in *D. melanogaster*. The involvement of both rhodopsins and cryptochrome in circadian light--entrainment has been unequivocally demonstrated in earlier studies (Stanewsky *et al*., 1998; Emery *et al*., 2000b; Helfrich--Förster *et al*., 2001). Rhodopsin mediated phototransduction in the compound eyes and the ocelli is non--functional in $norpA^{P41}$ flies (Pearn *et al*., 1996; Helfrich--Förster *et al*., 2001). Cryptochrome function is completely or nearly abolished in the cry^{b} mutant flies (Stanewsky *et al*., 1998). However, the $norpA^{P41}$; cry^{b} flies are still capable of accomplishing the circadian entrainment, though it takes a prolonged time compared to wild type or single mutant flies (Helfrich--Förster *et al*., 2001). How is light--entrainment achieved in the $norpA^{P41}$; cry^{b} flies? The involvement of a circadian photoreceptor other than the known rhodopsins and cryptochrome was

postulated (Helfrich--Förster *et al*., 2001; Malpel *et al*., 2002), which allowed speculating that there is a novel phototransduction cascade bypassing the classical PLC mediated visual transduction cascade. Once the existence of a new photoreceptor had been envisioned, the major challenge was to identify it.

The genome sequencing and gene annotation of *D. melanogaster* has predicted and documented many putative new genes (Adams *et al*., 2000). Among them was a putative novel rhodopsin gene that has been designated as rhodopsin 7 (*Rh 7*) with an inscription in FlyBase as CG 5638. It maps cytologically to 68F8--69A1 on the third chromosome (FlyBase). The prediction was based on the amino acid sequence homology to known rhodopsins, i.e. rhodopsins 1 to 6. Comparison of predicted protein (amino acid) sequence of *Rh 7* with the known *Drosophila* rhodopsin proteins revealed that it has a maximum sequence homology of 30.7% to *Rh 5* and a minimum sequence homology of 25.0% with *Rh 6* (Appendix 2). This was a rational value to work on further because similarly predicted and identified genes even had lower sequence homology (e.g., Nagel *et al*., 2002). *Rh 7* amino acid sequence also showed 12.5% homology with retinochrome of squid (*Todarodes pacificus*) (see Appendix 2 for percentage comparison and Appendix 3 for a complete sequence homology among all *Drosophila* rhodopsins and retinochrome). Moreover, *in silico,* it has been predicted that the *Rh 7* protein contains 7 transmembrane helixes [7 TH] (FlyBase). The 7 TH architecture is a characteristic of the rhodopsin family of proteins, and therefore *Rh 7* was placed in the rhodopsin--like G protein coupled receptor (GPCR) superfamily.

5.1 *Rh 7* a GPCR or a retinal photoisomerase?

Below I shall discuss the probability for *Rh 7* to function as a signaling molecule. The predicted amino acid sequence of *Rh 7* encodes for a 7 TH receptor coupled to a G protein (FlyBase). The *Rh 7* sequence has a conserved lysine (K) in the seventh helix like all the known *Drosophila* rhodopsins (Fig. 5A), to which the chromophore retinal binds (Gärtner, 2000). In 7 TH the second and third cytoplasmic loops are believed to interact with the G protein transducin (Franke *et al*., 1992). They are required for activation of transducin. Multiple deletion or

replacement of amino acids in these loops resulted in failure of the transducin activation (Franke *et al*., 1992).

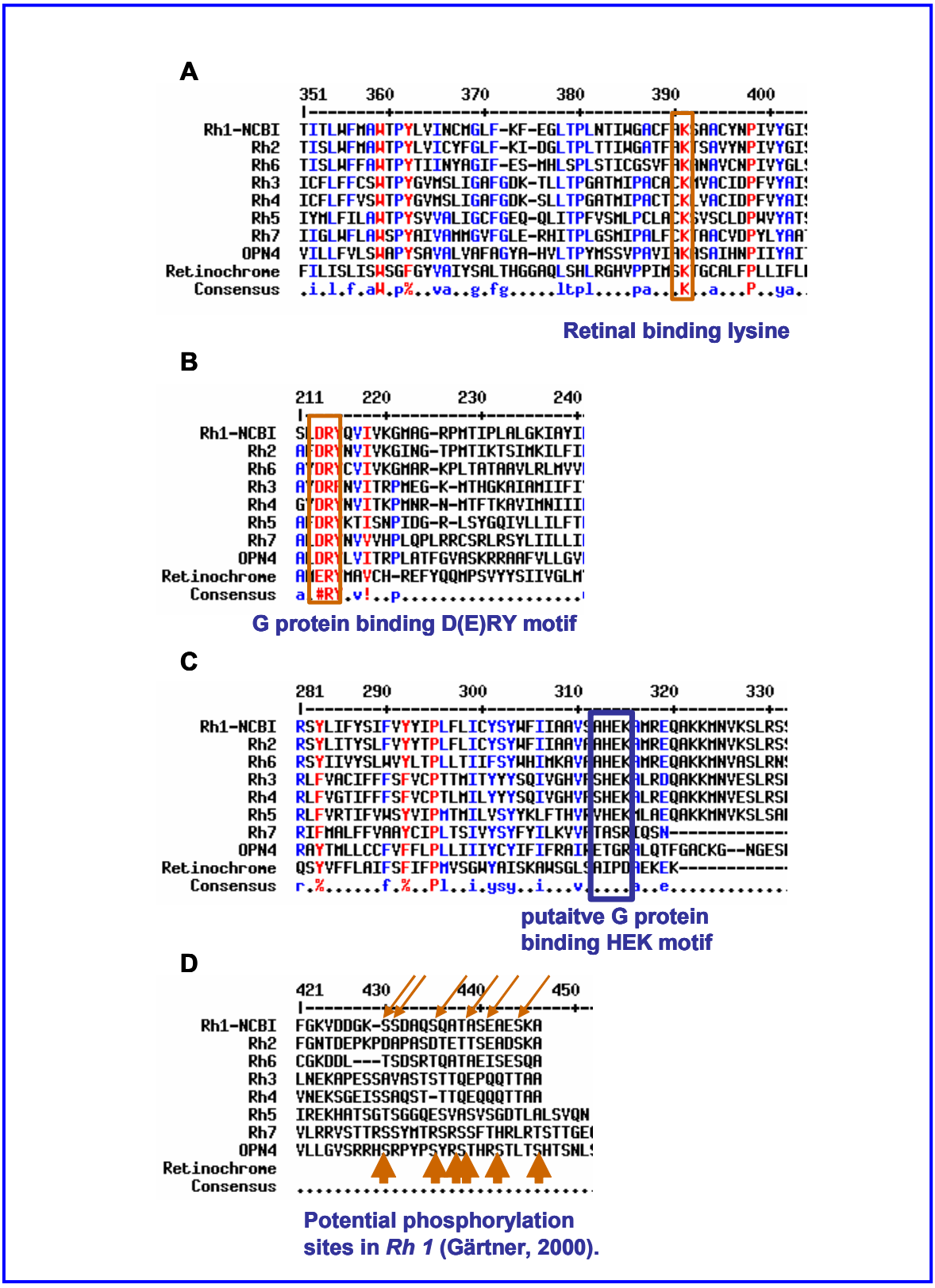

Figure 5 Amino acid sequence comparison between *Rh 7* and rhodopsins (*D. melanogaster*) and retinochrome (*Todarodes pacificus*). (A) The conserved chromophore,

retinal, binding lysine (marked by the box) is present in *Rh 7*. (B) The G protein interacting D(E)RY motif (marked by the box) in the second cytoplasmic loop is conserved in all the sequences, including *Rh 7.* (C) The putative G protein binding HEK motif (marked by the box) is absent in *Rh 7*, melanopsin and retinochrome. (D) The potential phosphorylation sites (marked by red arrows) on serine (S) and threonin (T) are present in *Rh 1*. Similar potential phosphorylation sites (marked by bold red arrow heads) are present in *Rh 7* and melanopsin but retinochrome lacks the corresponding entire C--terminal region.

In beginning of the second cytoplasmic loop presence of the conserved DRY motif is characteristic of all the known *Drosophila* rhodopsins (Fig. 5B; Gärtner, 2000); and in bovine visual pigment there is a corresponding ERY motif (Gärtner, 2000). The first two amino acids, i.e. D(E)R are essential for the activation of the G protein transducin because the double mutant for ER failed to activate transducin (Franke *et al*., 1992). *Rh 7* has this important DRY motif (Fig 5B). Therefore, possibly it can bind and activate transducin.

A second conserved HEK motif at the beginning of the third cytoplasmic loop is characteristic of all the known *Drosophila* rhodopsins (Fig. 5C) but not in vertebrates, which have instead a KEA motif (Gärtner, 2000). HEK is believed to be interacting with transducin (Gärtner, 2000). Since vertebrates lack the HEK motif it is assumed that in invertebrates a different activation process for the G protein might take place (Gärtner, 2000). However, replacement of KEA by TQA essentially left the transducin activation intact (Franke *et al*., 1992). Due to the absence of HEK motif both in *Rh 7* and retinochrome (Fig. 5C), it is thought that *Rh 7* is a retinal photoisomerase like retinochrome. However, it is not unlikely that *Rh 7* can function like the vertebrate opsin without the HEK motif. Moreover, recently it was demonstrated that a photoisomerase like photoreceptor (melanopsin) could to function as a signaling molecule (Panda *et al.*, 2005). Human melanopsin (Opn4) has similar sequences like the *Rh 7.* Melanopsin has lysine for chromophore binding (Fig. 5A), DRY motif for G protein bind and action (Fig. 5B). It also has the potential phosphorylation sites at the C--terminal (Fig. 5D). But it also lacks the HEK motif like in Rh 7 (Fig. 5C). However, it can activate the TRP cation channels involved in the phototransduction signaling (Panda *et al.*, 2005).

Retinochrome is a photopigment present in visual cells of cephalopods and gastropods. It is stored in the myeloid bodies of the inner segment, while rhodopsin is located only in the rhabdomal microvilli of the outer segment. (Hara--Nishimura *et al*., 1990). Retinochrome facilitates the photoregeneration of 11--*cis* retinal from all--*trans* retinal (Pepe and Cugnoli, 1992). In retinochrome the chromophore isomerases from *all--trans* to 11--*cis* retinal by irradiation, whereas in rhodopsin it is *vice versa* (Hara--Nishimura *et al*., 1990). Retinochrome lacks the C--terminal region (Fig. 5D), the potential phosphorylation sites required for arrestin binding and subsequent deactivation of activated rhodopsin (Kuhn and Wilden, 1987), therefore it was suggested that it may have only a catalytic role but no signaling function (Spudich *et al*., 2000). Similar potential phosphorylation sites are present in *Rh 7* (Fig. 5D), indicating that it may have a function in signal transduction.

A retinal photoisomerase is also found in mammalian retinal pigment epithelium (RPE) in the eye (Spudich *et al*., 2000). RPE is a specialized cell monolayer that lies adjacent to the photoreceptors and performs the function of regenerating the 11--*cis* retinal from all--*trans* retinal (Spudich *et al*., 2000). RPE and Müller cells express the retinal G protein coupled receptor (RGR), which is a typical 7 TH protein (Jiang *et al*., 1993). It binds all--*trans* retinal, and has an absorption maximum at 370 and 469 nm. Upon illumination with either UV or visible light, the all--*trans* chromophore bound to RGR is photoisomerized to 11--*cis* retinal. The all--*trans* retinal in the visual rod is transported to the RPE and there it is converted to 11--*cis* retinal by the RGR (Lamb and Pugh, 2004). Since RGR is present in the RPE and not in rod cells and catalyses the all--*trans* to 11--*cis* retinal reaction it is assumed that RGR has only an isomerase function. It is unlikely that the small number of RGR in the eye (about 1/100 that of rhodopsins) can solely regenerate all the 11--*cis* retinal required for the rhodopsins (Lamb and Pugh, 2004). Moreover, RGR contains a G protein binding domain. Therefore, a signal transduction function seems likely for RGR in addition to the 11--*cis* retinal regeneration (Spudich *et al*., 2000; Lamb and Pugh, 2004). Given the above possibilities that retinochrome can function as a GPCR (including the latest finding on melanopsin), it is likely that *Rh 7* also can function as a GPCR.

Alternatively, if *Rh 7* is not a GPCR can it perform a signal transduction function? During the past few years a growing body of evidence suggests that not all signal transduction leading from 7 TH receptor is mediated by G protein activation (Hall *et al*., 1999). For instance, the cAMP receptors of the slime mold *Dictyostelium discoideum* are 7 TH receptors that induce chemotaxis of undifferentiated cells into an aggregated fruiting body. These chemotactic effects are mediated through G protein activation (Devretoes, 1994). However, aggregated *Dictyostelium* cells undergo a number of cAMP receptor--mediated transcriptional changes that are independent of G protein activation, since cells with G protein subunits deleted still exhibit these changes following stimulation by cAMP (Jin *et al*., 1998; Hall *et al*., 1999). The G protein independent signaling mechanism in the above case is, however, unknown. In *Drosophila*, arrestin binds directly to the rhodopsin to inactivate the activated rhodopsin (Ebrey and Koutalos, 2001), it also functions as the adapter molecule to couple GPCRs to the activation of SRC--like kinases; and to facilitate the formation of multimolecular complexes (McDonald *et al*., 2000). In archea, the sensory rhodopsins I and II mediated phototranduction is accomplished by the interaction with the transducer complex embedded within the plasma membrane (Gordeliy *et al*., 2002) in contrast to the visual rhodopsins, which interact with G proteins in the cytoplasm (Franke *et al*., 1992). Therefore, as an alternative to GPCR mediated signaling, it is possible that *Rh 7* can transduce the photic signal through an unknown protein present in the membrane.

Furthermore, our results presented in Chapter 3 and Chapter 4 indicate possible existence of a *norpA* independent light signaling. Moreover, enzymes downstream of *norpA* in the phototransduciton cascade, like *trp* and *trpl* are not essential for photic entrainment of the circadian clock (Yang *et al*., 1998). All these findings strongly point towards a novel light signaling route bypassing the classical visual transduction cascade.

In summary, the data presented above and evidence from the literature strongly support that *Rh 7* can perform a signaling function. The only compelling evidence that suggests *Rh 7* is a photoisomerase like the retinochrome is based on the absence of a HEK motif in both, which is, however, absent in vertebrate opsins. However, melanopsin functions as a light signaling molecule without this motif.

5.2 *Rh 7* expression in the *Drosophila* brain and its implications on photoreception

The GAL4/UAS system has been successfully utilized for studying gene expression profiles using multiple reporter systems (Brand and Perrimon, 1993; Mollereau *et al*., 2000). The GFP reporter was used to study specific promoter controlled reporter expression (e.g. Magness *et al*., 2004). Therefore, the advantages of the GAL4/UAS system in combination with GFP were harnessed to investigate the putative expression of the *Rh 7* gene. A 1.4 kb fragment of the *Rh 7* promoter was successfully cloned in front of GAL4, and transgenic flies were generated. *In silico* analysis revealed that the 1.4 kb fragment contains promoter elements (Mazzoni, E. O. and Desplan, C., personal communication). However, the cloned promoter fragment lacked 1.7 kb remains of the promoter sequences, immediately downstream of the 1.4 kb fragment analyzed (see Fig.1A). Among the three transgenic lines studied only *Rh7:9--1/*UAS--τ--GFP and *Rh 7:2B/*UAS--τ--GFP were expressing GFP in brains (Fig. 4); in the third line expression was under the limit of detection.

The finding that the *Rh 7* promoter is active in sub--esophageal neurons, and in DCs and LCs is in agreement with another study, which showed (using UAS--GFP reporter) that the *Rh 7* promoter is active in similar cells in independently isolated transgenic fly lines carrying the same *Rh 7*--GAL4 construct (Bachleitner, W., personal communication). Moreover, an antibody made against *Rh 7* also stained cells in the same regions in the *Drosophila* brains (Bachleitner, W., personal communication). The GFP expressing sub--esophageal neurons sent neural fibers to the dorsal protocerebrum but their terminal targets were still obscure.

The *Rh 7* promoter was active in the DCs and LCs. These cells were not the same dorsal and lateral clock neurons, which express PER and TIM (see in Chapter 1 Fig. 2; Kaneko, 1998) because a staining with anti--PER in the transgenic flies carrying the *Rh 7* promoter did not show any co--localized GFP signal (Bachleitner, W., personal communication). Nevertheless, expression of the *Rh 7* gene in the DCs makes sense in light of the recent findings that molecular oscillations in the DNs are entrained by photoreceptors other than the known rhodopsins and cryptochrome (see in Chapter 4 about DNs; Veleri *et al*., 2003).

Since the DCs are intermingled with the DN_{1+2} cells (Bachleitner, W., personal communication), possibly the former could interact with the latter. If true, this implicates that the photopigment that synchronizes the DN circadian oscillator is indeed *Rh 7*.

The harboring of a novel photoreceptor in the dorsal brain is implicated by an earlier study, which used the luciferase reporter gene driven by a promoterless *period* transgene (Veleri *et al*., 2003). The localization of *Rh 7* expression in DCs close to the DN_{1+2}s in the present study is promising. Because, both studies arrived at the same assumption, postulating that a novel photoreceptor is present in the dorsal brain (in dorsal cells closely associated with the DN_{1+2}s). These merging lines of evidence from two independent studies strongly support the hypothesis that the DNs are synchronized by the putative *Rh 7* photopigment. In a different perspective, the result of the current study delimited the putative photoreceptor beyond the dorsal brain because the *Rh 7* promoter expressed not only in the cells near the DNs but also in cells close to the LNs. In the present study, at least one transgenic fly line showed GFP expression in the LCs, near the LN_ds and s--LN_vs; independent *Rh 7*--GAL4 lines showed similar results in another study (Bachleitner, W., personal communication). This foretells that the novel putative photoreceptor is expressed not only in cells near the DN_{1+2}s but also in other cells which are near the LNs. Perhaps these cells may be irrelevant for the circadian clock. If *Rh 7* is not a circadian photopigment perhaps it plays a role in the non--circadian photoreception like the photophobic reaction to light by the larva (Mazzoni *et al*., 2005). It is also possible that *Rh 7* plays a role both in the circadian and non--circadian photoreception. Expression of *Rh 7* also in the sub--esophageal neurons may support this view. Mazzoni *et al*. (2005) reported that one fiber from BO reaches beyond the LNs deep into the brain. But its targets were not clear in larva. The significance of the sub--esophageal neuronal expression of *Rh 7* is yet to be understood.

6 Conclusion

The expression pattern of a *Rh 7* promoter driven reporter--gene has been demonstrated in the *Drosophila* brain. The findings may substantiate the existence of a predicted gene, *Rh 7,* in the *D. melanogaster* genome. Given its potential expression in a group of cells located in the same brain area where the DN_{1+2}s are present, it is possible that *Rh 7* contributes to the minute light--responsiveness of the $norpA^{P41};cry^{b}$ flies. It may substitute for the missing putative photopigment that signals in high intensity light in the $norpA^{P41};cry^{b}$ flies. Thus, the demonstration of *Rh 7* promoter activity in the *Drosophila* brain creates room for investigating its possible role in the circadian photoreception and/or non--circadian photoreception. However, expressing the promoter of a putative gene is only the preliminary evidence to support its existence. To confirm that *Rh 7* encodes a real photoreceptor, functional evidence is needed. Expression of *Rh 7* in a heterologous system could enable to test whether it is a photoreceptor or not. If it is a real photopigment, it would show a characteristic absorption maximum for a particular wavelength. It could also be tested if expression of *Rh 7* in the R1--R6 PRs in a *ninaE* mutant background (which lacks *Rh 1* expression in the R1--6 PRs; Zucker *et al*., 1985) enables them to detect light signals? Creating a Rh 7 mutant and testing its capability to light entrain the circadian clock could answer a putative role of it in the circadian photoreception. Once *Rh 7* is affirmed as a real photopigment, it would be pertinent to explore the phototransduction cascade downstream of *Rh 7*. Given the remaining light--responsiveness of $norpA^{P41}; cry^{b}$ flies it is possible that *Rh 7* can function without the *norpA* encoded PLC and cryptochrome. Absence of a conserved motif in *Rh 7* may indicate such a novel mode of phototransduction in *Drosophila*. *Rh 7* may not be utilizing the classical visual transduction cascade. Identification of such a novel route may give unforeseen insights into the whole mechanism of phototransduction.

CHAPTER 6

A NOVEL MUTANT, *VEELA*, INVOLVED IN THE CIRCADIAN LIGHT INPUT PATHWAY OF *DROSOPHILA MELANOGASTER*?

Chapter 6

A novel mutant, *Veela*, involved in the circadian light input pathway of *Drosophila melanogaster*?

1 Introduction

Light is the most salient environmental input into the circadian clock of Drosophila (Hall, 2005). The circadian clock is normally reset by light signals on a daily basis. Light signals are transduced into the circadian clock *via* the rhodopsin mediated phototransduction cascade and also *via* the largely unknown cryptochrome mediated cascade (see more in Chapter 1, section 2.2). In spite of the fact that light resets the circadian clock, an uninterrupted light exposure to wild type flies results in arrhythmic behavior (Konopka *et al.*, 1989). The constant light (LL) condition collapses the clock work mechanism (Price *et al.*, 1995; Marrus et al, 1996). In LL TIM is continuously degraded by the CRY mediated light--dependent mechanism, which ultimately results in interruption of the negative feed back control on the CLK:CYC dimer (see more in Chapter 1, section 4.6). By blocking CRY (a specialized circadian photoreceptor, Stanewsky *et al.*, 1998) mediated light input pathway, the circadian clock becomes immune to the collapsing effects of LL: cry^b mutant flies show rhythmic behavior in LL similar as if they were in DD (Emery *et al.*, 2000a). Therefore, rhythmic behavior of a fly in LL is an indicator of a defect in the light input pathway into the circadian clock. Here, I describe and characterize a novel mutant, *Veela*, that behaves similar to the cryptochrome mutant (cry^b) in LL. The gene defined by *Veela* is likely to be involved in the circadian light input pathway. Possibly, it could perform a novel function in the light transduction cascade into the circadian clock. It may be part of the CRY--signaling mechanism.

2 Materials

2.1 Flies

Canton--S (Konopka *et al.*, 1989).

Oregon--R (Csink and McDonald, 1995).

y w;*Veela* (this study), hereon called *Veela.*

w;*ninaB*^{P360d} (von Lintig *et al.*, 2001), hereon called *ninaB*^{P360d}*.*

w;*ninaB*^{P315} (von Lintig *et al.*, 2001), hereon called *ninaB*^{P315}*.*

tim^{01} (Myers *et al.*, 1995; Sehgal *et al.*, 1994).

Df(3R)DI--BXR/+ (Alton *et al.*, 1989). This deficiency uncovers the region of the *cry* gene (Stanewsky *et al.*, 1998), therefore hereon termed as *Df(3R)cry/+*.

Df(2L)23F3--23F6, which uncovers *tim*, therefore hereon termed *Df(2L)tim*^{02} (Myers *et al.*, 1995).

w/Y;tim^{01}*;P(w*$^{+}$ *tim*$^{+}$*)* (Rutila *et al.*, 1998), hereon called *tim*^{01}*;*P(*w*$^{+}$ *tim*$^{+}$).

al dp b pr (Lindsley and Zimm, 1992).

b cn bw (Lindsley and Zimm, 1992).

Bl (Lindsley and Zimm, 1992).

H (Lindsley and Zimm, 1992).

TM6B/+ (Lindsley and Zimm, 1992).

CyO/+ (Lindsley and Zimm, 1992).

2.2 DNA Oligonucleotides

2.2.1 Sense direction

TIM--222B	5’CCTCAATGTTCGCAGTCGAC3’
TIM--568	5’TCGTCAATCTGACGGTGC3’
TIM--1150	5’GCAAGGGTCAGGAGTATCAG 3’
TIM--1452	5’ACTACCAACCCAACGTCCAG 3’
TIM--1773	5’CAGCAACGACGATGATGG3’
TIM--2379	5’GCGAAATGTCCGATCTGAGG3’
TIM--2475	5’CCAATCACATCCTCCTAC3’
TIM--2748	5’GAACTGTGCGATGACTGG3’
TIM--3345	5’CACTGCATCTGCAAGCAG3’
TIM--3365	5’GCAAGCAGAAGTCCATTCCG3’

TIM--3403	5'GAGCAATCCACTACGATG3'
TIM--3500	5'GGAGACAATGTACGGACTCG3'
TIM--4265	5'CCTGAACATGCTAAACACC3'
TIM--1957	5'GTGGTTGCGTAATGCCCTGG3'
TIM--4636	5' GCACTTCTTCTGGCTGGTCAC3'
TIM--10578	5'GCAGTCGTGGAGTCTGCTGAGTC3'

2.2.2 antisense direction

TIM--568Rev	5'GCACCGTCAGATTGACGA3'
TIM--680rev	5'GCTTCCTTGCTGGTGTACAG3'
TIM--1150rev	5'CTGATACTCCTGACCCTTGC3'
TIM--1773rev	5'CATCATCGTCGTTGCTGG3'
TIM--2277rev	5'TCACCACCAAGTGCATTC3'
TIM--2379rev	5'CCTCAGATCGGACATTTCGC3'
TIM--2609rev	5'GTCCTCCAACAGAATGCC3'
TIM--2748rev	5'TCGCACAGTTCATAGTCC3'
TIM--2900rev	5'AGTGTGTCCATTTCCTCC3'
TIM--3500rev	5'CGAGTCCGTACATTGTCTCC3'
TIM--3748rev	5'ATCGAGATACACCGACGG3'
TIM--4444rev	5'TCGAACGAGGACTTCATG3'
TIM--4695rev	5'TCGGATATCGGATCGGATCG3'
TIM--4938rev	5'GCATAGGCAAACAGTCTG3'

3 Methods

3.1 Genetic crosses

The following crosses (Fig. 1) were performed to map the *Veela* mutation by meiotic recombination of the *Veela* mutants with a multi--marker version of chromosome 2. For crossing, female virgins were always selected. Four to five day old male flies from the F4 generation were selected for phenotypical analysis.

Cross I

P (mass crosses):

$$\frac{+}{+}\ ;\ \frac{al\ dp\ b\ pr}{al\ dp\ b\ pr} \quad \times \quad \frac{y\ w}{Y}\ ;\ \frac{Veela}{Veela}$$

F1 (mass crosses):

$$\frac{y\ w}{+}\ ;\ \frac{Veela}{al\ dp\ b\ pr} \quad \times \quad \frac{y\ w}{Y}\ ;\ \frac{Bl}{CyO}$$

F2 (single males and 3 females):

$$\frac{y\ w}{y\ w}\ ;\ \frac{Bl}{CyO} \quad \times \quad \frac{+}{Y}\ ;\ \frac{[al\ dp\ b\ pr\ Veela]}{CyO}$$

F3 (3-5 males and females):

$$\frac{y\ w}{+}\ ;\ \frac{[al\ dp\ b\ pr\ Veela]}{CyO} \quad \times \quad \frac{y\ w}{Y}\ ;\ \frac{[al\ dp\ b\ pr\ Veela]}{CyO}$$

F4:

$$\frac{y\ w}{Y}\ ;\ \frac{[al\ dp\ b\ pr\ Veela]}{[al\ dp\ b\ pr\ Veela]}$$

Cross II

P:

$$\frac{+}{+}\ ;\ \frac{b\ cn\ bw}{b\ cn\ bw} \quad \times \quad \frac{y\ w}{Y}\ ;\ \frac{Veela}{Veela}$$

F1:

$$\frac{y\ w}{+}\ ;\ \frac{Veela}{b\ cn\ bw} \quad \times \quad \frac{y\ w}{Y}\ ;\ \frac{Bl}{CyO}$$

F2:

$$\frac{y\ w}{y\ w}\ ;\ \frac{Bl}{CyO} \quad \times \quad \frac{+}{Y}\ ;\ \frac{[b\ cn\ bw\ Veela]}{CyO}$$

F3:

$$\frac{y\ w}{+}\ ;\ \frac{[b\ cn\ bw\ Veela]}{CyO} \quad \times \quad \frac{y\ w}{Y}\ ;\ \frac{[b\ cn\ bw\ Veela]}{CyO}$$

F4:

$$\frac{y\ w}{Y}\ ;\ \frac{[b\ cn\ bw\ Veela]}{[b\ cn\ bw\ Veela]}$$

Figure 1 Genetic crosses performed to produce meiotic recombination of *Veela* with chromosome 2 markers. Cross I To produce the *Veela* recombinants with the recessive

markers *aristaless* (*al*), *dumpy* (*dp*), *black* (*b*) and *purple* (*pr*) present on the left arm of chromosome 2. For parental cross (P), female flies (red eyes, w^+) homozygous for the recessive markers *al, dp*, *b* and *pr* were crossed to homozygous *Veela* mutant male flies (white eyes, *w*), in mass. For F1 cross, red eyed female flies were crossed to white eyed y w;*Bl/CyO* male flies. For the F2 cross, 3 white eyed y w;*Bl/CyO* female flies were crossed to red eyed single male fly, without *Bl* marker and with *CyO*. For F3 cross, 3--5 red eyed males, without *Bl* and with *CyO,* were crossed to 3--5 red eyed females without *Bl* and with *CyO.* In F4 generation, flies homozygous for the marker(s) *al*, *dp*, *b* and *pr* were selected for testing their locomotor behavior in LD and LL.

Cross II To produce the *Veela* recombinants with the recessive markers *black* (*b), cinnabar* (*cn*) and *brown* (*bw*) present on the right arm of chromosome 2. All the steps were identical as in cross I except instead of flies with markers *al, dp*, *b* and *pr* in cross I, flies with markers *b, cn, bw* were used in cross II.

3.2 Behavioral testing and analyses

The locomotor activity rhythms were monitored as described in Hamblen *et al.* (1986) and Hamblen--Coyle *et al.* (1992). See in Chapter 2, section 2.4 for more details. The behavioral testing incubator were illuminated by ~300--400 lux white fluorescence light and maintained at 25 °C. The flies were first entrained for 5--8 days in 12 h: 12 h light--dark cycles (LD), followed by 5--8 days in constant light (LL) or constant darkness (DD).

Actograms for the entire duration of the experiment were generated for each fly and were used to visually inspect whether a fly behaved rhythmic or arrhythmic in LD/LL/DD. The strength of rhythmicity for each fly was separately estimated for LD, LL and DD employing χ^2 periodogram analysis (see Table 1 and 2).

3.3 Immuno--histochemistry

Flies were entrained to 12 h: 12 h LD (25°C) for at least 3 days and then subjected to LL (see Fig. 2). Flies were collected at defined time--points before fixing on the second day in LL. See details on dissection and histological protocols in Chapter 2, section 2.5.

3.4 Quantification of the immuno--staining in the clock neurons and glia cells

The clock neurons stained for both anti--PER and anti--TIM were visually inspected under a confocal microscope to assign a staining index for individual brain halves. The staining intensity was graded by a second person (a scale ranging from 0--4). The average number for each neuronal group was estimated, for each time point.

3.5 Genomic DNA preparation

Genomic DNA was prepared using the single--fly DNA preparation protocol for PCR developed by Gloor *et al.* (1993).

3.6 RNA isolation

Flies were entrained for 3 days in 12 h: 12 h LD in an entrainment incubator. Since *tim* RNA is expressed maximally between ZT 12 and 16 (Hunter--Ensor *et al.*, 1996) flies were collected at ZT 15 for total RNA preparation and flash frozen by liquid nitrogen. The heads of flies (~50) were separated from the body by vortexing, and straining through a wire--mesh. Heads were homogenized with 250 μl Trifast, subsequently centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was transferred into 50 μl fresh chloroform and vortex for 15 sec. After 3 min at RT, the mixture was centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was added to 125 μl isopropanol and mixed well, and kept at RT for 10 min. To precipitate RNA, the mixture was centrifuged at 10000 rpm for 10 min at 4°C. The pellet was washed with 800 μl 75% EtOH (DEPC), air--dried and re--suspended in 15 μl dH_2O (DEPC). 1 μl each of RNAsin and DNAse were added to the re--suspended RNA and incubated at 37°C for 20 min followed by 10 min heat inactivation of DNAse at 65°C.

3.7 Reverse transcription PCR (RNA --> cDNA)

The reverse transcription reaction was performed according to Invitrogen standard protocol (RT--SuperScript III--Kit).

3.8 Polymerase Chain Reactions

To cover the entire of length ~4.5 kb target *timeless* cDNA, primer walking was performed. Oligonucleotide primers used were originally generated for sequencing tim^{blind} (Wülbeck *et al.*, 2004), except TIM--1957 (to amplify a DNA fragment

immediately upstream of the transcription start), TIM--4636, TIM--10578 (covering an intron piece between exon 16 and 17) and TIM--568 Rev. Using these primers DNA fragments of up to 800 bp were amplified by PCR from cDNA and/or gDNA templates. The DNA fragments amplified by PCR were separated on a TAE Agarose gel and later extracted and purified using the standard *QIAquick® Gel Extraction Kit* (Qiagen). It was then used as a template for sequencing reactions.

3.9 Sequencing Reaction

The sequencing reactions were done using a fluorescent--dideoxynucleotide based BigDye®Terminator v1.1 Cycle Sequencing kit (AB Applied Biosystems) and an *ABI capillary Sequencer* (GeneArt). The output data from *ABI capillary Sequencer* was read and analyzed by employing Chromas, MultAlin and DNASTAR softwares.

4 Results

4.1 *Veela* mutant flies behave rhythmically in constant light

Wild type flies behave rhythmically in LD and DD. But subjecting the fly to LL results in arrhythmic (AR) behavior (Konopka *et al.*, 1989), caused by continuously decomposing the TIM clock protein (Price *et al.*, 1995; Myers *et al.*, 1996). A mutation in the *cryptochrome* gene, cry^b, blocks the transduction of light signals to TIM (Stanewsky *et al.*, 1998), and thus renders the cry^b flies immune to the AR--inducing effect of LL. As a result the cry^b flies stay rhythmic in LL (Emery *et al.*, 2000a; Helfrich--Förster *et al.*, 2001). Surprisingly, the $ninaB^{P360d}$ flies showed rhythmic behavior in LL similar to the cry^b flies (Fig. 2). The *ninaB* gene encodes an enzyme β,β--carotene--15,15'--dioxygenease, which cleaves the β--carotene into two molecules of retinal (von Lintig *et al.*, 2001). The $ninaB^{P360d}$ flies can not split the β--carotene to produce the retinal chromophore, and therefore they lack functional rhodopsins. Since the $ninaB^{P360d}$ flies have no functional rhodopsins we tried to analyze how they get the light signals into the circadian clock. It was believed that the $ninaB^{P360d}$ caused the rhythmic behavior of these flies in LL due to its total lack of functional rhodopsins. When we tried to link this behavior to the chromosome 3 carrying the $ninaB^{P360d}$ mutation by chromosomal segregation, we

realized that the LL--phenotype actually mapped to chromosome 2 of the *ninaB*^{P360d} flies. Thereafter, it was named *Veela* (owing to its disguising nature!).
The *Veela* mutant flies showed normal behavior in LD cycles like *y w* control flies (Fig. 2, Table 1 and Table 2). But in LL *Veela* mutant flies showed robust rhythmic behavior (like *cry*^{b} flies), while *y w* flies exhibited arrhythmic behavior (Fig. 2 and Table 1). Interestingly, there was a difference in period--length between the *Veela* mutant flies and *cry*^{b} flies in LL; i.e. 25.7 $\pm$ 0.12 h for *Veela* mutant flies compared with 24.3 $\pm$ 0.1 h for *cry*^{b} flies. However, in DD, both the *Veela* mutant and *cry*^{b} flies showed similar period values like the controls, i.e. all showed a free running period of nearly 24 h (Table 2). Thus, the period of *Veela* mutant fly was clearly different in LL (25.7 $\pm$ 0.12 h) and DD (24.3 $\pm$ 0.11 h). In summary, the *Veela* mutant flies were rhythmic in LL like the *cry*^{b} flies but had a longer period. However, in DD both had a similar free running period like the wild type flies. The longer period value in LL for *Veela* mutant flies is an indication that some residual light signals reach the circadian clock otherwise it would have a shorter period like in DD (Konopka *et al.*, 1989).

4.2 *cry* interacts with *Veela*

The above result indicated that *Veela* might encode a molecule which participates in phototransduction to the circadian clock. In the *Drosophila* circadian clock CRY and TIM are believed to be directly involved in the light signaling mechanism, i.e., CRY captures the light signals and relays them to TIM by direct physical interaction (Ceriani *et al.*, 1999; Busza *et al.*, 2004). Therefore, it seems likely that *Veela* is somehow required for the functional CRY:TIM interaction. To test this we studied the potential genetic interaction of *Veela* with *cry* in LL.

When both the *Veela* and *cry*^{b} mutations were combined by genetic crossings, transheterozygous (*Veela/+;cry*^{b}*/+*) flies showed rhythmic behavior in LL like homozygous (hz) *Veela* flies (Fig. 3, Fig. 2 and Table 1). The period of *Veela/+;cry*^{b}*/+* flies was 25.9 $\pm$ 0.6 h, not significantly different from *Veela* (hz) mutant flies (25.7 $\pm$ 0.12 h). The heterozygous (ht) *Veela/+* flies showed only weak rhythmic behavior in LL (Fig. 3 and Table 1) compared to *Veela* (hz) flies. Their period was significantly shorter compared to *Veela* (hz) flies and the percentage of flies showing rhythmic behavior was reduced by 2.7 fold in *Veela* (ht) (Table 1).

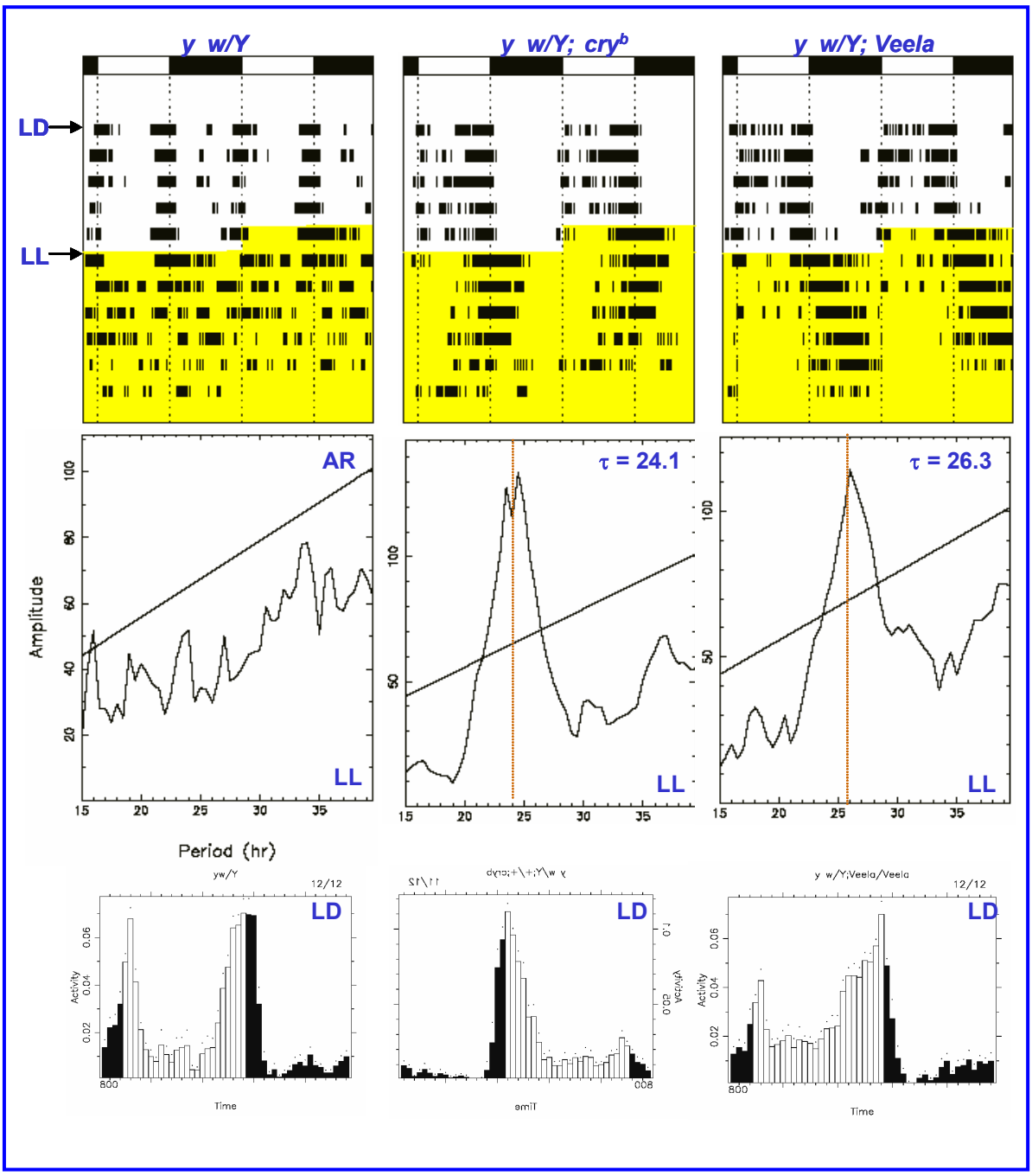

Figure 2 Actograms and periodograms show that *Veela* mutant flies stay rhythmic in constant light similar to *cry*^{b} flies. Flies were first entrained to 12 h: 12 h Light--Dark (LD) cycles for 5 days. Subsequently they were subjected to constant light (LL) for 5 days. The upper, middle and lower panels show actograms, periodograms and daily averages respectively, for the indicated genotypes. Yellow shading in actograms indicates LL. The alternate black and white bars at the top of the actograms indicate light 'off' (ZT 12--24) and light 'on' (ZT 0--12), respectively. The periodograms represent the LL part of the experiment. The peak above the oblique line (5% significance) in each periodogram indicates the free running period (τ) estimate. *y w* flies become arrhythmic in LL, whereas *Veela* and *cry* mutant flies exhibit rhythmic behavior in LL. The red vertical line (dotted)

indicates the free running period value in LL. AR means arrhythmic. The daily average is shown only for the LD part. Black (filled) and white (unfilled) histograms in the daily average represent the activity in the night (ZT 12--24) and day (ZT 0--12), respectively.

Heterozygous cry^b flies failed to show any rhythmic behavior in LL (Fig. 3 and Table 1). A deficiency (heterozygous) covering the *cry* gene in (*Df(3R)cry/*+) showed 33% rhythmicity, however cry^b/+ flies showed only 4% rhythmicity in LL (Table 1) *Veela/+;Df(3R)cry/*+ flies showed similar behavior in LL, except that the period length was slightly different (Table 1). In summary, the *Veela/+;cryb/*+ flies displayed the full effect of the *Veela* (hz) mutation. This genetic interaction indicates that the *Veela* gene product is required for *cry* mediated light signaling to the circadian clock.

4.3 Mapping the *Veela* mutation

The *Veela* mutation segregates with chromosome 2, and to further refine its approximate location, meiotic recombination was applied. *Veela* flies were recombined with standard recombination markers available for the second chromosome (see Materials, 2.1). The recessive markers *aristaless* (*al*), *dumpy* (*dp*), *black* (*b*), and *purple* (*pr*) are present on the left arm of chromosome 2, in the order just written with *al* being at the distal end from the centromere. The recessive markers *cinnabar* (*cn*) and *brown* (*bw*) are present on the right arm of chromosome 2, *cn* being located proximal to the centromere (Lindsley and Zimm, 1992). The *Veela* mutant flies were crossed either with flies carrying *al dp b pr* or *b cn bw* markers (see Fig.1)

Table 1 Locomotor activity rhythms in LD and LL

						LL				
Genotype	**N**	**n**	**period ± SEM (h)**	**RI ± SEM**	**% Rhy.**	**N**	**n**	**period ± SEM (h)**	**RI ± SEM**	**% Rhy.**
y w /Y	74	73	23.9 ± 0.03	0.35 ± 0.01	99	70	2	22.3	0.30	3
y w /Y; cry^b	22	21	24.3 ± 0.10	0.36 ± 0.03	96	22	21	24.3 ± 0.10	0.33 ± 0.03	96
y w /Y; cry^b/+	24	22	24.1 ± 0.04	0.41 ± 0.01	92	23	1	27.5	0.30	4
y w /Y;Veela	75	74	24.0 ± 0.03	0.37 ± 0.01	99	75	64	25.7 ± 0.12	0.33 ± 0.02	85
y w /Y;Veela/+	35	35	24.1 ± 0.04	0.45 ± 0.02	100	35	11	24.4 ± 0.40	0.18 ± 0.01	31
y w /Y;Veela/+; cry^b/+	26	26	24.1 ± 0.04	0.45 ± 0.01	100	26	20	25.9 ± 0.60	0.24 ± 0.02	77
+/Y;Df (3R) cry/TM6B	21	21	23.9 ± 0.10	0.34 ± 0.02	100	21	8	22.4 ± 0.40	0.25 ± 0.03	38
+/Y;Df(3R)cry/+	12	8	24.1 ± 0.13	0.32 ± 0.03	67	12	4	24.2 ± 0.90	0.20 ± 0.03	33
y w/ Y;Veela/+; Df(3R)cry/+	24	24	24.1 ± 0.02	0.4 ± 0.01	100	24	19	24.9 ± 0.10	0.30 ± 0.02	79
y w /Y;tim^{01}	40	40	23.9 ± 0.05	0.35 ± 0.02	100	40	2	23.2	0.25	5
y w /Y;tim^{01}/+	24	24	24.2 ± 0.05	0.45 ± 0.02	100	24	0	AR	-	0
y w /Y;Veela/tim^{01}	55	55	24.1 ± 0.02	0.43 ± 0.01	100	42	13	25.9 ± 1.10	0.21 ± 0.02	22
+/Y;Df(2L) tim^{02}/CyO	22	20	24.0 ± 0.04	0.36 ± 0.03	91	21	6	23.6 ± 1.10	0.19 ± 0.02	29
y w /Y;Df(2L) tim^{02}/Veela	29	28	24.0 ± 0.03	0.36 ± 0.02	97	27	5	23.7 ± 0.81	0.17 ± 0.03	19
+/Y;Df(2L)tim^{02}/ tim^+	27	25	24.0 ± 0.03	0.35 ± 0.02	93	27	0	AR	-	0
w /Y;tim^{01}; P(w^+ tim^+)	10	9	24.0 ± 0.10	0.35 ± 0.02	90	14	1	25	0.20	7
y w /Y; Veela/tim^{01}; P(w^+ tim^+)/+	46	46	23.9 ± 0.02	0.41 ± 0.01	100	46	29	24.6 ± 0.18	0.25 ± 0.02	63

'N' is total number of flies tested and 'n' is number of flies showed rhythmicity in LL. 'RI' is Rhythm Index

Table 2 Locomotor activity rhythms in LD and DD.

	LD					DD				
Genotype	**N**	**n**	**period ± SEM (h)**	**RI ± SEM**	**% Rhy.**	**N**	**n**	**period ± SEM (h)**	**RI ± SEM**	**% Rhy.**
y w /Y	20	19	23.9 ± 0.04	0.34 ± 0.02	95	19	17	23.8 ± 0.07	0.39 ± 0.02	94
y w /Y; cry^b	12	12	23.9 ± 0.05	0.37 ± 0.01	100	12	10	23.8 ± 0.10	0.34 ± 0.02	83
y w /Y; cry^b/+	12	12	24.2 ± 0.29	0.32 ± 0.02	100	12	11	23.9 ± 0.10	0.34 ± 0.02	92
y w /Y;Veela	20	20	24.0 ± 0.05	0.36 ± 0.02	100	20	20	24.3 ± 0.14	0.35 ± 0.02	100
y w /Y;Veela/+	23	23	23.9 ± 0.27	0.43 ± 0.02	100	23	22	23.8 ± 0.10	0.44 ± 0.02	96
y w /Y;Veela/+; cry^b/+	15	15	23.9 ± 0.02	0.41 ± 0.01	100	14	14	23.8 ± 0.07	0.35 ± 0.01	100
+/Y;Df(3R)cry/ TM6B	8	8	23.8 ± 0.02	0.47 ± 0.02	100	8	8	23.8 ± 0.02	0.47 ± 0.02	100
y w/Y;Veela/+; Df(3R)cry/+	8	7	23.9 ± 0.04	0.35 ± 0.04	88	8	8	23.8 ± 0.16	0.41 ± 0.06	100
y w /Y;tim^{01}	22	17	23.9 ± 0.09	0.32 ± 0.01	73	20	3	26.0 ± 1.53	0.24 ± 0.01	15
y w /Y;tim^{01}/+	22	22	23.9 ± 0.05	0.39 ± 0.02	100	22	20	23.9 ± 0.07	0.39 ± 0.02	91
y w /Y;Veela/tim^{01}	27	27	23.9 ± 0.04	0.37 ± 0.01	100	27	27	24.4 ± 0.06	0.36 ± 0.02	100

'N' is the total number of flies tested and 'n' is number of flies showed rhythmicity. 'RI' is Rhythm Index

Table 3 Behavioral analysis of flies tested for recombination with *Veela* in LL.

Line No.	Marker(s)	Rhy. in LL?	Period ± SEM (h)	N(n)	% Rhy.	Remark
I--3	*al*	Yes	26.3 ± 0.22	8 (8)	100	*Veela*
I--7	*b pr*	Yes	25.2 ± 0.31	3 (3)	100	*Veela*
I--11	*pr*	Yes	25.6 ± 0.18	7 (4)	57	*Veela*
I--12	*b pr*	Yes	24.7 ± 0.50	6 (4)	67	*Veela*
I--18	*b pr*	Yes	26.2 ± 0.22	9 (4)	40	*Veela*
I--27	*b*	Yes	25.6 ± 0.22	8 (8)	100	*Veela*
I--32	*b pr*	Yes	25.4 ± 0.27	7 (7)	100	*Veela*
I--42	*b pr*	Yes	26.0 ± 0.32	6 (6)	100	*Veela*
I--45	*b pr*	Yes	25.8 ± 0.24	7 (6)	86	*Veela*
I--49	*b pr*	Yes	26.1 ± 0.21	6 (5)	83	*Veela*
II--1	*b cn*	Yes	24.9 ± 0.23	6 (3)	50	*Veela*
II--11	*cn*	Yes	25.8 ± 0.16	6 (5)	83	*Veela*
II--15	*bw*	Yes	26.6 ± 0.23	5 (3)	60	*Veela*
II--23	*cn*	Yes	25.5 ± 0.15	6 (3)	50	*Veela*
II--25	*b cn*	Yes	25.3 ± 0.27	5 (3)	60	*Veela*
II--26	*bw (w)*	Yes	25.3 ± 0.60	3(3)	100	*Veela*
II--31	*bw*	Yes	25.6 ± 0.30	6 (6)	100	*Veela*
II--37	*bw*	Yes	25.5 ± 0.18	6 (6)	100	*Veela*
II--45	*bw*	Yes	26.1 ± 0.24	6 (6)	100	*Veela*
II--53	*bw (w)*	Yes	25.4 ± 0.42	4 (4)	100	*Veela*
II--55	*b cn*	Yes	25.2 ± 1.70	5 (2)	40	*Veela*
II--56	*b cn (w)*	Yes	24.7 ± 0.38	5 (4)	80	*Veela*
II--58	*b cn*	Yes	25.0 ± 0.37	4 (3)	75	*Veela*
I--5	*al dp*	No	--	8 (0)	0	*Veela*$^+$
I--15	*al dp*	No	--	3 (0)	0	*Veela*$^+$
I--21	*dp b pr*	Yes	25.5	8 (1)	13	*Veela*$^+$
I--23	*dp b pr*	Yes	24.5	7 (1)	14	*Veela*$^+$
I--25	*al dp*	No	--	8 (0)	0	*Veela*$^+$
I--28	*dp b pr*	Yes	25.8	8 (1)	13	*Veela*$^+$
I--31	*al dp b*	Yes	25.0	8 (1)	13	*Veela*$^+$
I--38	*al dp*	No	--	8 (0)	0	*Veela*$^+$
I--40	*al*	No	--	8 (0)	0	*Veela*$^+$
I--41	*al dp b*	No	--	8 (0)	0	*Veela*$^+$
I--43	*al dp*	No	--	8 (0)	0	*Veela*$^+$
I--46	*al dp b*	No	--	8 (0)	0	*Veela*$^+$
I--51	*dp b pr*	No	--	8 (0)	0	*Veela*$^+$
I--52	*al dp*	Yes	25.0	8 (1)	13	*Veela*$^+$
I--55	*al dp*	Yes	--	8 (0)	0	*Veela*$^+$
I--58	*al dp*	Yes	26.5	8 (1)	13	*Veela*$^+$
II--6	*b cn*	No	--	3 (0)	0	*Veela*$^+$
II--9	*b cn*	No	--	6 (0)	0	*Veela*$^+$
II--10	*b cn*	No	--	5 (0)	0	*Veela*$^+$
II--13	*bw*	No	--	5 (0	0	*Veela*$^+$
II--14	*bw*	No	--	5 (0	0	*Veela*$^+$
II--24	*b cn*	No	--	5 (0)	0	*Veela*$^+$
II--27	*b cn*	No	--	5 (0)	0	*Veela*$^+$
II--57	*b cn*	No	--	5 (0)	0	*Veela*$^+$

Table 4 List of silent mutations found in the *Veela* TIM protein.

Sl. No.	nucleotide change	position in TIM--PD
1	CTG → CTT (L)	263
2	CCC → CCA (P)	288
3	CCT → CCA (P)	304
4	TCT → TCC (S)	306
5	AAT → AAC (N)	311
6	GAG → GAA (E)	400
7	CTA → CTT (L)	423
8	TCG → TCA (S)	477
9	GCC → GCA (A)	495
10	CTC → CTG (L)	498
11	TGT → TGC (C)	506
12	GAT → GAC (D)	541
13	CTG → CTA (L)	607
14	ACT → ACA (T)	727
15	CTT → CTC (L)*	740
16	ATT → ATC (I)*	817
17	TTT → TTC (F)*	851
18	GGC → GGT (G)	1330
19	TCG → TCA (S)	1361

* Indicates that the *Veela* sequence was identical to the TIM--PD but there was a nucleotide change in the control flies (*ninaB*^{P315}).

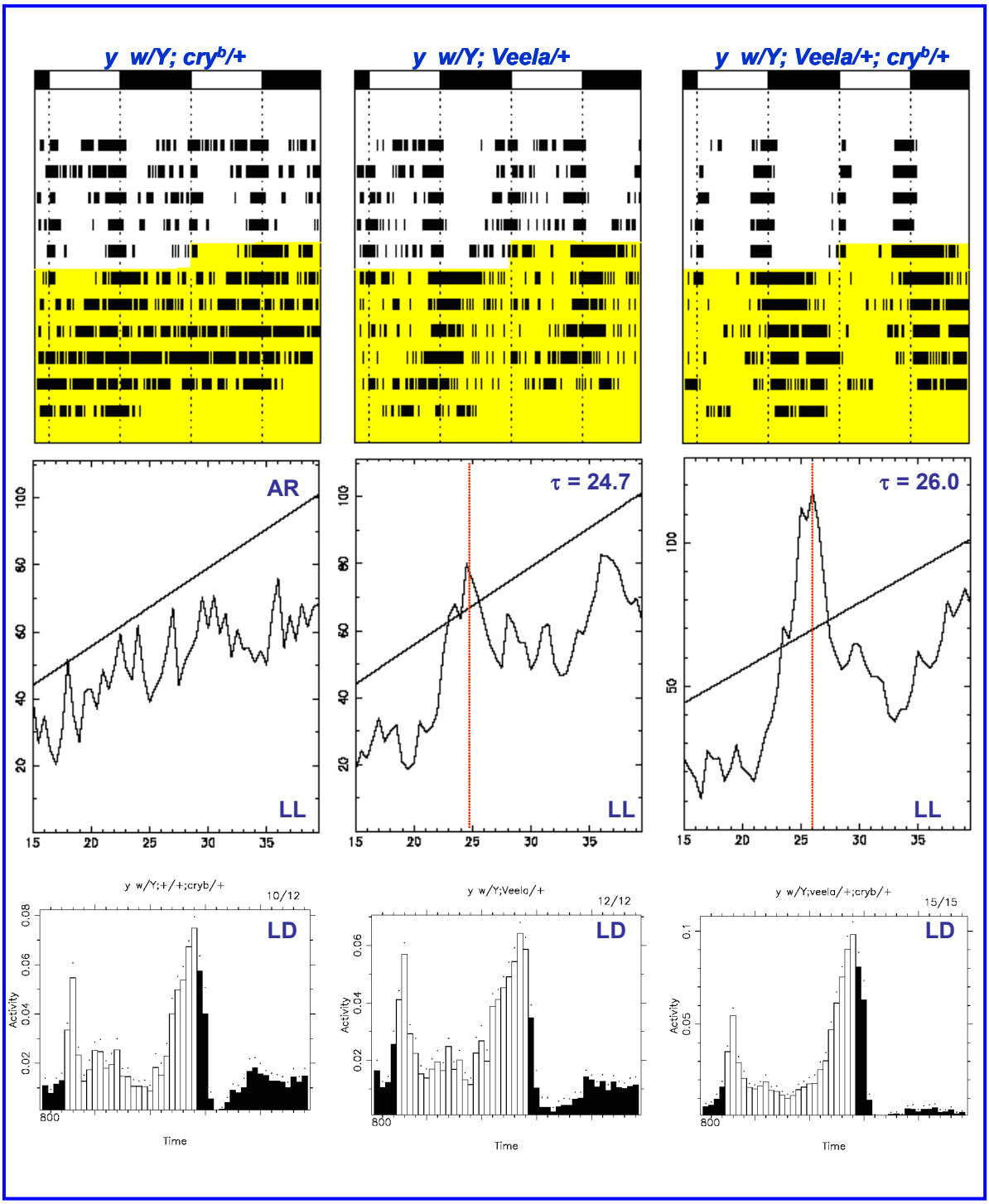

Figure 3 Actograms, periodograms and daily averages indicate that *Veela* and *cry* interact. Heterozygous *cry*^{b} flies display arrhythmic behavior in LL and heterozygous *Veela* mutant flies display a weaker (see Table 1 for RI) rhythm in LL compared to the homozygous ones (Fig. 2). However, combining both the *cry* and *Veela* mutations transheterozygously restored the homozygous *Veela* mutant phenotype. For intra--figure labels see Fig. 2.

The crossings produced fly strains carrying various combinations of markers on chromosome 2 (see Table 3). In order to determine if they also carry the *Veela* mutation, these recombinants were tested for their behavior in LL. A rhythmic fly (with a characteristic long period of *Veela*) indicated that the *Veela* mutation was recombined with a particular marker combination. Conversely, if the fly was arrhythmic in LL a wild type copy of the *Veela* gene was associated with respective marker. The behavioral data of the recombinants indicated that the *Veela* mutation grossly mapped between *al* and *dp* on the left arm of the chromosome 2 (Fig. 4), a region also containing the *tim* gene (Myers *et al.*, 1995).

The recombinant fly line I--3 (*al:Veela*), which had only *al* marker showed the characteristic rhythmic behavior of the *Veela* mutant fly in LL (Fig. 4A and Table 3). This indicated that the *Veela* mutation maps to the right side of *al* (Fig. 4E). A second recombinant fly line I--40 (*al:Veela*) again had the same *al* marker, but behaved arrhythmic (Fig. 4B and Table 3), which indicates that a recombination event occurred after the *Veela* locus (Fig. 4E). It also indicated that the *Veela* mutation maps distal to the *dp*. A third recombinant line I--32 (*b pr: Veela*) and 6 other lines carrying both the *b* and *pr* markers, also showed the same characteristic rhythmic behavior of the *Veela* mutant fly in LL (Fig. 4C and Table 3), which indicated that the *Veela* mutation maps to the left of both *b pr* markers (Fig. 4E). A fourth recombinant fly line I--52 (*al dp: Veela*) and 8 other lines with same markers, displayed arrhythmic behavior in LL like the *y w* flies (Fig. 4D and Table 3). This indicated that the *Veela* mutation does not map to the right side of *dp* (Fig. 4E). Summing up the above assumptions, it can be deduced that the *Veela* mutation maps between the *al* and *dp* markers.

4.4 *Veela* could be a *tim* allele?

Like *timeless* the *Veela* mutation was mapped between the *al* and *dp* markers (Myers *et al.*, 1995). Assuming that *Veela* might be a *tim* allele we asked the following questions: Could tim^{01} or $Df(2L)tim^{02}$/+ compliment for *Veela*? Could a *tim* transgene (in tim^{01} background) rescue the wild type LL behavior in *Veela* flies? Therefore, we combined the *Veela* and tim^{01} mutations (heterozygously) and tested their behavior in LL. The *Veela*/tim^{01} flies showed rhythmic behavior in LL (Fig. 5

and Table 1) like that of *Veela*/+ flies (Fig. 3 and Table 1). The *Veela/Df(2L)tim*^{02} flies also showed similar behavior (Table 1). From the above results it seems that *Veela* is complimented by *tim*^{01} or *Df(2L)tim*^{02}/+. However, it was difficult to discern the contribution of *tim*^{01} or *Df(2L)tim*^{02}/+ because of *Veela*'s semidominant nature. Alternatively, the above results indicate that *Veela* is a new tim allele.

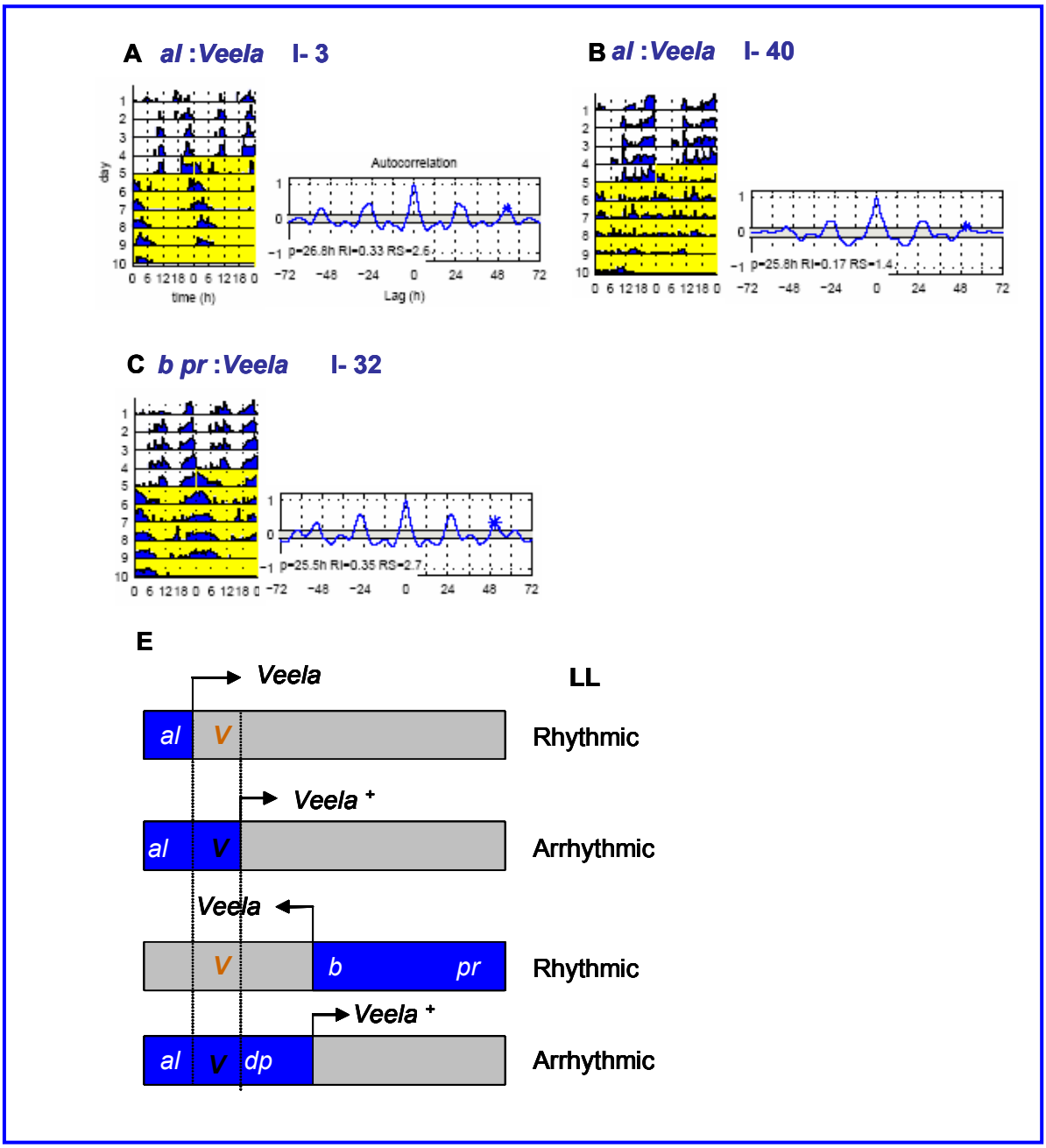

Figure 4 Actograms and autocorrelograms of *Veela* mutants recombined with various chromosomal markers (*al dp b pr*). The actogram and autocorrelogram for an individual fly is displayed side by side. The autocorrelograms represent only the LL part of the experiment. The yellow shade indicates LL. Flies were first entrained to 12 h: 12 h LD cycles for 5 days. Subsequently flies were subjected to LL for 5 days. The recombinant

lines I--3 (*al:Veela*) and I--32 (*b pr:Veela*) displayed rhythmic behavior in LL (A, C), whereas the recombinant lines I--40 (*al:Veela*) and I--52 (*al dp:Veela*) displayed arrhythmic behavior in LL (B, D). The above results indicated that the *Veela* mutation maps between *al* and *dp* on chromosome 2 (E). The '*V*' in red and black indicate mutant (*Veela*) and wild type (*Veela*$^{+}$), respectively. An asterisk in autocorrelogram above the cut--off lines (bordering the shaded area) represents a significant rhythm.

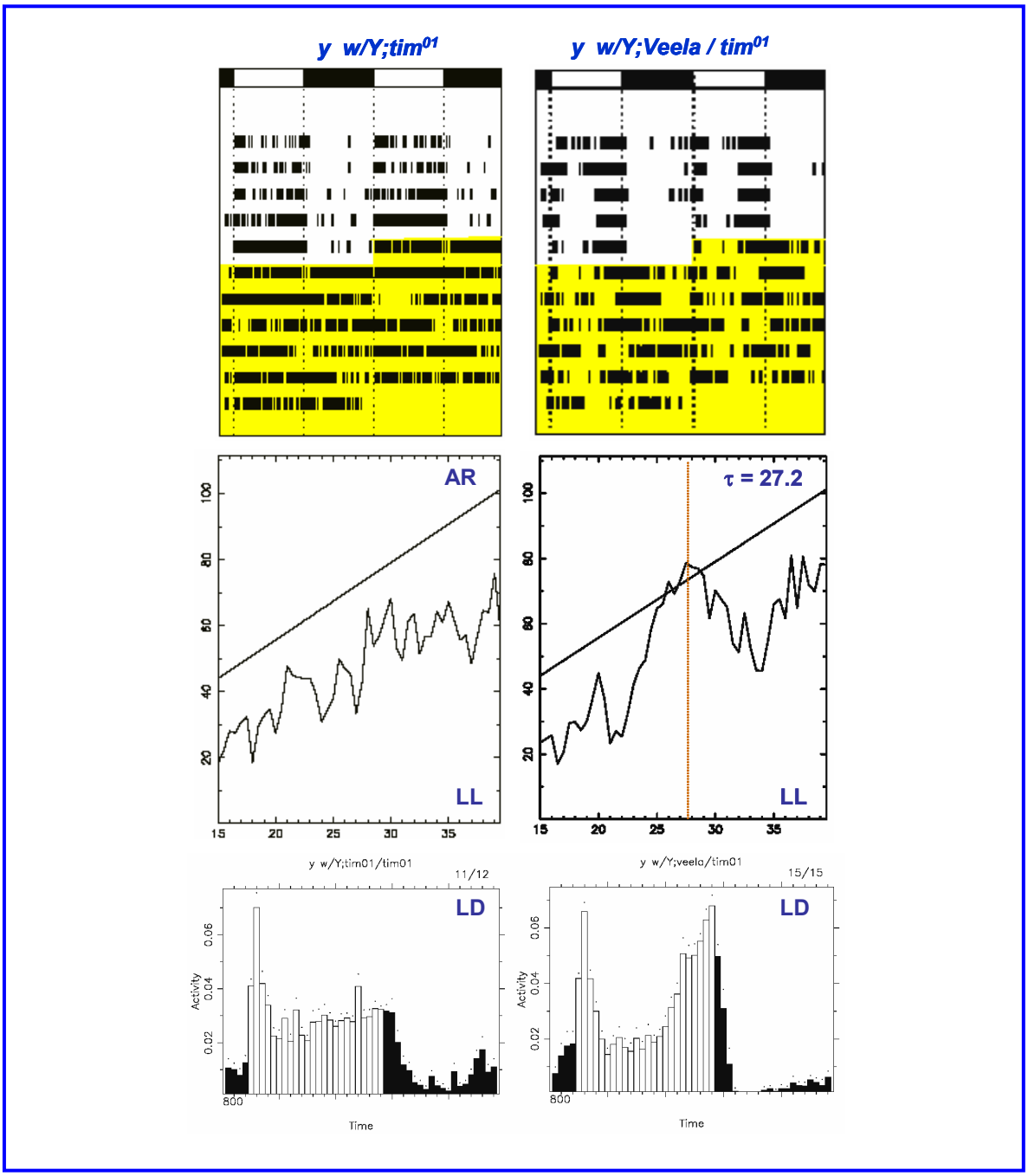

Figure 5 Actograms and periodograms show an ambiguous interaction between *Veela* and *tim*. The *tim*^{01} flies show arrhythmic behavior in LL. Combining heterozygous *Veela* mutation with a heterozygous *tim*^{01} showed a weak characteristic long free running period

of homozygous *Veela* flies with similar rhythm strength as heterozygous *Veela* flies (Table 1).

4.5 *Veela* could be rescued by a *tim* transgene?

Based on the above assumption that *Veela* might be a *timeless* allele, a further attempt was made to rescue the wild type behavior in the *Veela* mutant flies. If *Veela* is a *timeless* allele, a *timeless* transgene should rescue the wild type behavior in the *Veela* mutant flies in LL (i.e. they should become AR in LL). A genetic crossing between *Veela* mutant flies and a transgenic fly carrying the *timeless* transgene in a *timeless* loss of function background $tim^{01};P(w^{+}\ tim^{+})$ (Rutila *et al.*, 1998) generated *Veela* mutant flies carrying the *timeless* transgene ($Veela/tim^{01};P(w^{+}\ tim^{+})/+$). These flies were then monitored in LL to see whether the transgene could rescue the wild type behavior or not.

The behavioral data from the LL experiments using $Veela/tim^{01};P(w^{+}\ tim^{+})/+$ showed that the transgene could not rescue the wild type behavior in the *Veela* mutant flies (Fig. 6E and Table 1). 63% of the tested $Veela/tim^{01};P(w^{+}\ tim^{+})/+$ transgenic flies showed rhythmic behavior in LL (Table 1). The rhythmic $Veela/tim^{01};P(w^{+}\ tim^{+})/+$ transgenic flies showed a period of 24.6 $\pm$ 0.18 h, which was similar to that of *Veela* (ht), except that a higher number of transgenic flies showed rhythmic behavior in LL (Table 1). Since a higher percentage of the transgenics showed rhythmicity in LL than the *Veela* (ht) or $Veela/tim^{01}$ (Table 1) we considered that there is no rescue by the transgene, otherwise more transgenic flies should have shown arrhythmic behavior. Higher number of rhythmic flies could be caused by the eye color (from the w^{+} in the transgene). Maybe the eye pigment is shielding the light signals entering *via* the compound eyes.

4.6 Do *Veela* mutant flies have any nucleotide change in *timeless* to accounting for its phenotype?

Since the *Veela* mutation mapped to the same region where the *tim* gene is present and the complementation experiments were not unambiguously interpretable, it was possible that *Veela* is a *timeless* allele. Therefore, it was decided to sequence the *tim* gene in the *Veela* mutant flies in order to find out whether there existed any real nucleotide change(s) compared to the wild type gene. Along with the *Veela* mutant

strain *ninaB*^{P315} flies were used as controls. This was done because the *Veela* mutant was recovered from the original *ninaB*^{P360d} fly stock, and both *ninaB*^{P360d} *and ninaB*^{P315} fly stocks were generated in the same mutagenesis screen performed at Purdue University (von Lintig *et al.*, 2001; Schneuwly, S., personal communication).

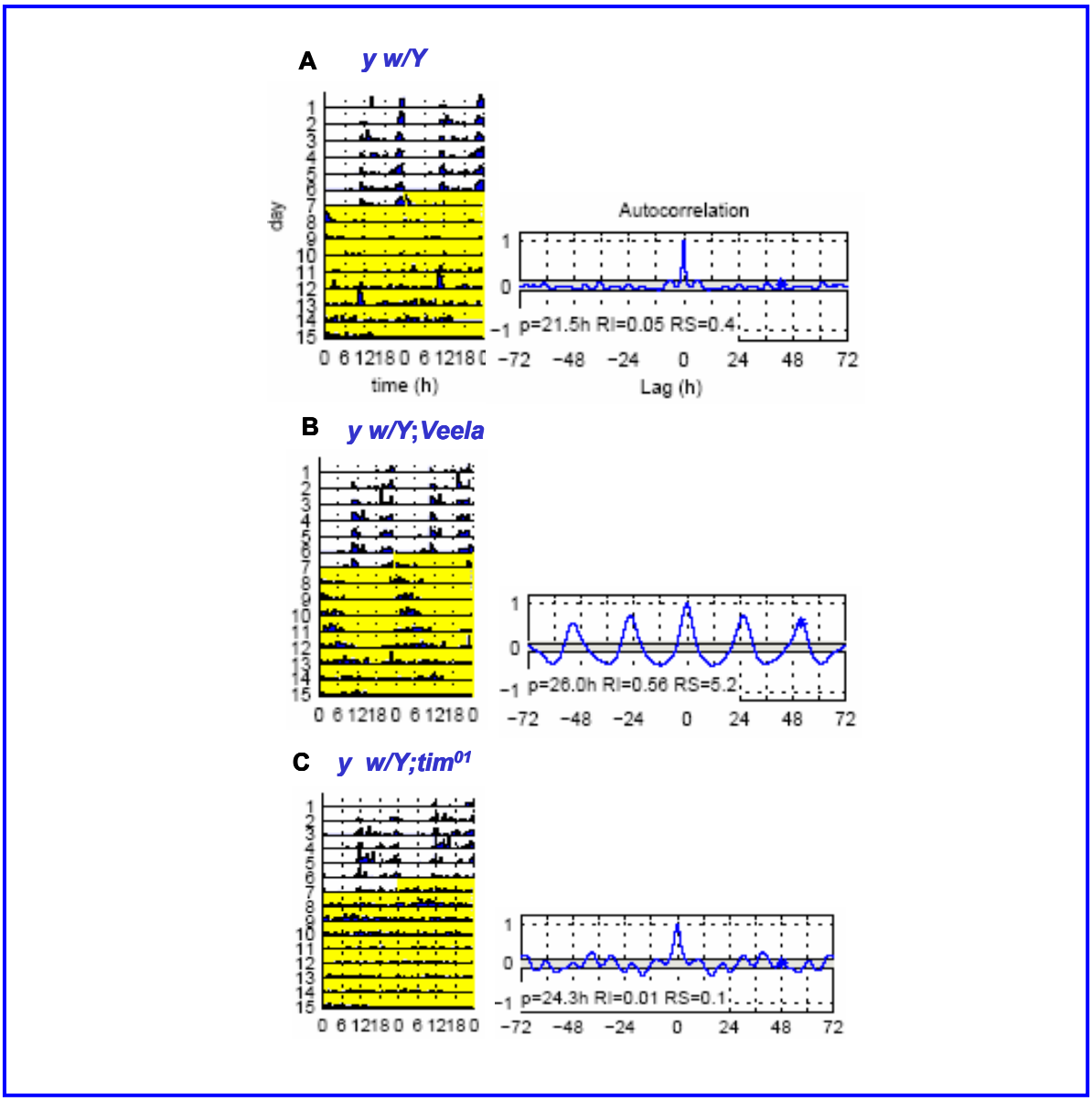

Figure 6 (continues in next page)

Figure 6 Actograms and autocorrelograms indicate that the *tim* transgene did not rescue *Veela* mutation. The flies were first entrained to 12 h: 12 h LD cycles for 6 days. Subsequently flies were subjected to constant light (LL) for 8 days. (A) *y w* flies show arrhythmic behavior in LL, actogram and the autocorrelogram (asterisk within the cut--off line, shaded area in the plot) displayed no significant rhythm. (B) *Veela* mutants show

robust rhythmic behavior in LL (autocorrelogram--asterisk above the cut--off lines). (C) The *tim*^{01} flies exhibit arrhythmic behavior.

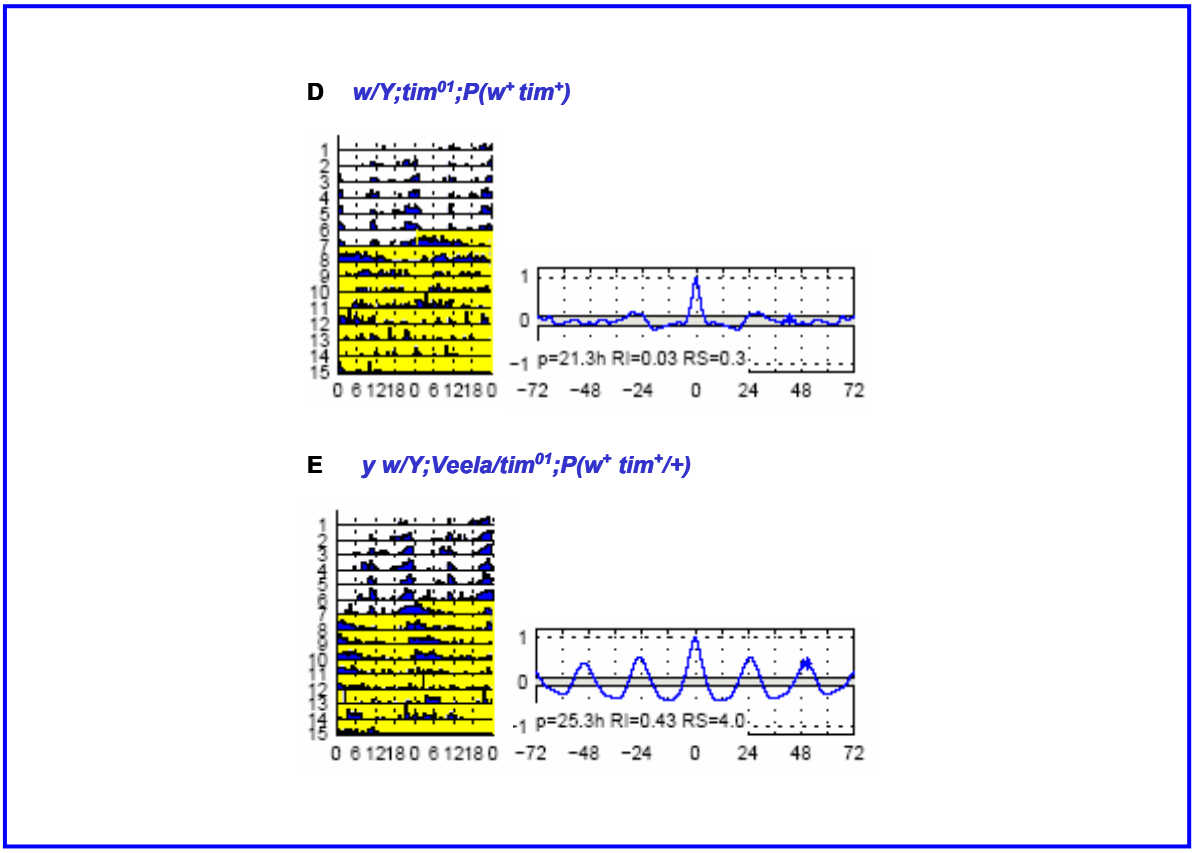

Figure 6 (D) The *tim* transgene control flies show arrhythmic behavior in LL. (E) *Veela* flies carrying a *tim* transgene (*tim*^{01}) could not rescue the wild type behavior (asterisk above the cut--off line indicates a significant period in LL).

Sequencing and analysis of the *tim* gene in the *Veela* mutant fly revealed that it has 3 nucleotide changes that result in amino acid substitutions (Fig. 7) compared to the control flies (*ninaB*^{P315}).

1) The control *ninaB*^{P315} flies had a guanine (G) deletion at position 52 (the positions are with reference to *tim--PD* cDNA; CG 3234--PD, FlyBase) but the *Veela* mutant flies did not (Fig. 7A). To know whether this G deletion was specific for the *Veela* mutant flies, further sequencing for this region of *tim* was performed on Canton--S, Oregon--R and *y w* flies. As shown in Fig. 7A, Canton--S and Oregon--R strains had a G at position 52 (the sequence data for Canton--S and Oregon--R strains are kindly provided by Landskron, J) like the *Veela* mutant flies but the *y w* flies had a G deletion at position 52. It has been reported that flies with

a G deletion at position 52 encode a predicted TIM protein with a 23 amino acid truncation at the N--terminus (Rosato *et al.*, 1997). Following the G deletion, the third triplet codon is a stop codon (TGA), which results in premature halt of the translation. But starting from position 71 there is another ATG, which codes for the alternate start codon, following the premature termination of shorter TIM (Fig. 7A; Rosato *et al.*, 1997). It follows, that *Veela* mutant flies are able to generate the long and short forms of TIM. The *tim* sequence at position 52 in the *Veela* mutant flies was identical to the reported *tim--PD* (FlyBase) and *tim* in Canton--S flies. Canton--S flies are arrhythmic in LL (Konopka *et al.*, 1989). Therefore, it was concluded that the presence of G at position 52 is not responsible for the *Veela* phenotype. Again, at position 55 there was an adenine (A) in *Veela* mutant flies, but a cytosine (C) was found in $ninaB^{P315}$ flies (Fig. 7A). All strains that had a G at position 52 invariably carried an A at position 55, and a G deletion at position 52 always accompanied with a C at position 55 (cf. Rosato *et al.*, 1997) (Fig. 7A). Since the G deletion resulted in a shorter TIM, for which the open reading frame (ORF) had started downstream of position 55, this substitution of A to C has no consequence on the amino acid level. Since the A at position 55 is conserved between the reported *tim--PD* sequence (FlyBase) and *Veela* it can not be responsible for the observed phenotype.

2) A second nucleotide change was found in the *Veela* mutant flies at position 1345 (Fig. 7B). The *Veela* mutant flies had an A whereas the $ninaB^{P315}$ had a G at position 1345, like the reported *tim--PD* sequence (FlyBase). This transition resulted in an amino acid change from glycine to serine in the *Veela* mutant flies at amino acid position 449 of TIM--PD. Since another mutant fly, tim^{blind} (Wülbeck *et al.*, 2004), and *BG--luc* flies (Stanewsky *et al.,* 2002) had an identical A nucleotide at the same position like *Veela* (Fig. 7B; the sequence data for tim^{blind} and for *BG--luc* are kindly provided by Peschel, N. and Wülbeck, C., respectively); and tim^{blind} flies did not show rhythmic behavior in LL (Wülbeck, C., personal communication), this transition can not be responsible for the *Veela* phenotype.

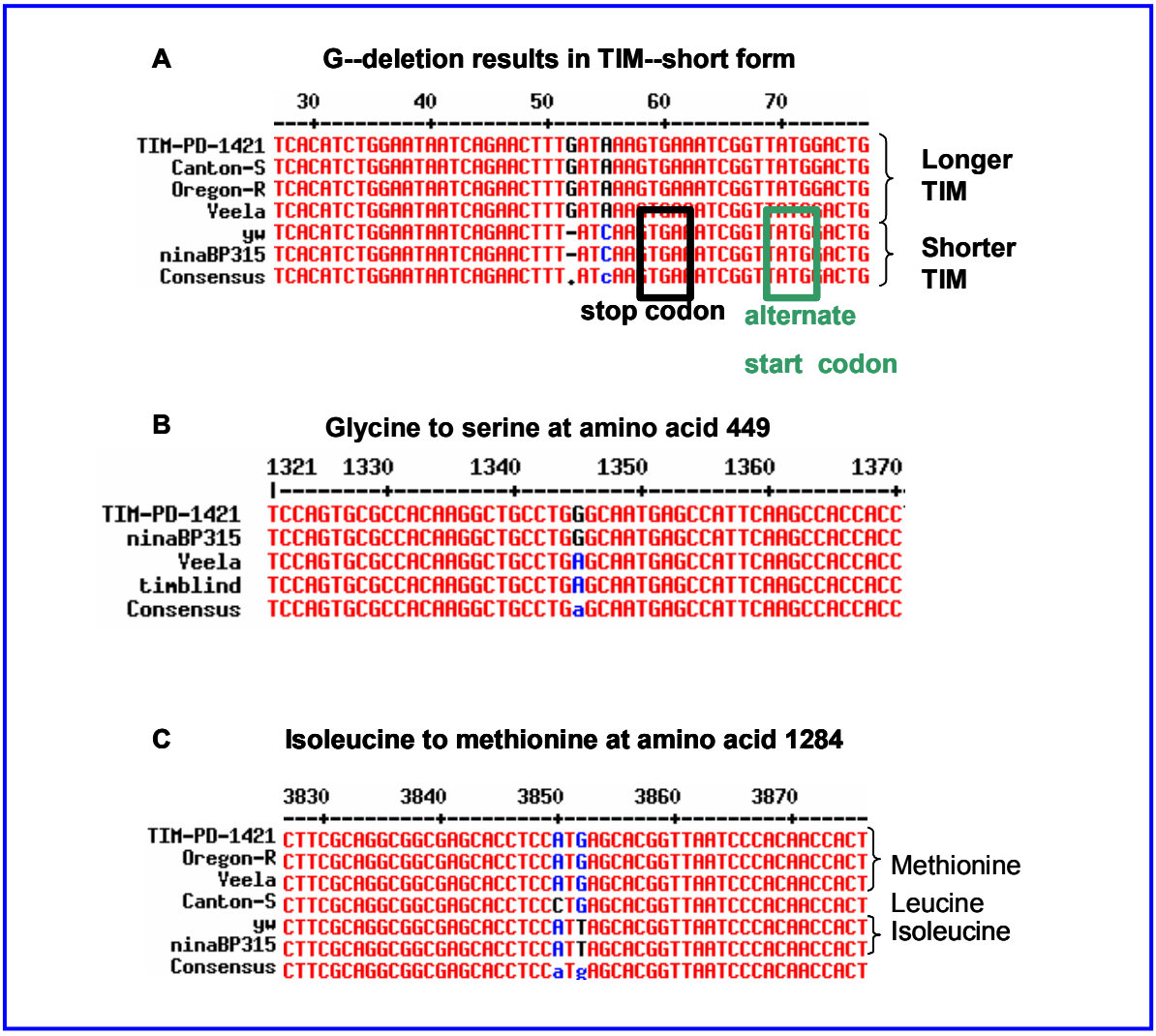

Figure 7 Nucleotide changes revealed by sequencing the *tim* gene in *Veela* mutant flies. (A) At position 52, with reference to the TIM--PD--1421 cDNA, there was a G in *Veela*, resulting in a 23 amino acid elongation of TIM protein at the 5' end compared to TIM encoded by the y w and *ninaB*^{P315} *tim* genes. The G deletion results in a frame shift, thus the third codon after the deletion becomes a stop codon (TGA), indicated by the black box (cf. Rosato *et al.*, 1997), which is followed by a subsequent start codon (indicated by the green box). Canton--S, Oregon--R, and *Veela* flies encode the longer TIM form (TIM--PD) as reported in the database. (B) At position 1345, *tim* in *Veela* flies has an A to G substitution, which results in a glycine to serine change at amino acid 449 of TIM--PD. A similar change is found in *tim*^{blind} and *BG--luc* flies. (C) At position 3852, in *Veela tim* shows a T to G transversion, which results in an isoleucine to methionine change at amino acid 1284 of TIM--PD. *y w* and *ninaB*^{P315} flies also have an isoleucine at position 1284, whereas Oregon--R and the reported database encode a methionine at position 1284. In Canton--S flies *tim* encodes a leucine at this position. The TIM--PD (FBpp0077255) is obtained from *y[1] cn[1] bw[1] sp[1]* strain (FlyBase).

3) A third change was found at position 3852 in the *Veela* mutant flies (Fig. 7C). At position 3852 the *Veela* mutant flies had a G whereas the *ninaB*^{P315} flies had a thymine (T). This transversion from A to T results in an amino acid change from isoleucine to methionine in the *Veela* mutant flies at amino acid position 1284 of TIM--PD. However, further sequencing of Oregon--R strain revealed that it also had a G at position 3852 like the *Veela* mutant flies. Thus Oregon--R and *Veela* mutant flies had a conserved G at position 3852 as reported for *tim--PD* (Fig. 7C). *y w* flies had an isoleucine like the *ninaB*^{P315} flies, and surprisingly the Canton--S had a leucine instead in the same position (Fig. 7C). Taken together these data indicated the existence of a polymorphism at position 3852 of *tim*. Since Oregon--R and *Veela* flies have the identical sequence (Fig. 7C) at this position, it follows that the methionine at position 3852 is not responsible for the *Veela* phenotype. Besides, the three major nucleotide changes detailed above, *Veela* has 16 and *ninaB*^{P315} flies has 3 silent mutations in *tim* (see Table 4).

4.7 In the pacemaker neurons of *Veela* mutant flies synchronized PERIOD and TIMELESS cycling is maintained in LL

The rhythmic behavior of *Veela* mutant flies in LL prompted us to investigate how the clock proteins TIM and PER behaved in the pacemaker neurons under the same condition. The clock protein TIM is highly sensitive to light and it is quickly degraded after light exposure (Hunter--Ensor *et al.*, 1996). Reduction in TIM levels is accompanied by a depletion of PER (Price *et al.*, 1998). In the whole process CRY is believed to transduce light signals to TIM (Ceriani *et al.*, 1999; Busza *et al.*, 2004; Stanewsky *et al.*, 1998).

The clock proteins PER and TIM were investigated in the clock neurons of the *Veela* mutant flies on the second day in LL every three hours throughout the circadian day, ranging from CT 0 to CT 21 (CT means subjective circadian time; from CT 0 to CT 12 is subjective day [i.e. when the light would have been “on” during a 12 h: 12 h LD]. Since the *cry*^{b} flies are also rhythmic in LL, they were also investigated in parallel along with *y w* flies as controls. It is known that the *cry*^{b} mutation abolishes PER and TIM cycling in total fly head extracts in LD (Stanewsky *et al.*, 1998), however, *norpA*^{P41}*;cry*^{b} flies maintained PER and TIM cycling in the s--LN$_v$s and DN$_1$s (Helfrich--Förster *et al.*, 2001; see in Chapter 3, section 4.2).

Possibly, a novel photopigment mediates light signaling, which could function independent of *norpA* and *cry.*

The anti--PER and anti--TIM staining on whole mounted brains of *Veela* mutant flies revealed that both clock proteins were cycling in a synchronized manner in the pacemaker clock neurons that control locomotor behavior (Fig. 8A, B and Fig. 11). cry^b flies also showed a similar circadian profile of PER and TIM cycling in the pacemaker clock neurons (Fig. 10 and Fig. 11). Anti--PER and anti--TIM staining was hardly seen in the *y w* flies on corresponding time points (Fig. 9 and Fig. 11).

In *Veela* mutant flies, PER and TIM started accumulating in the cytoplasm of LNs (s--LN_vs, l--LN_vs and LN_ds) at CT 18 (Fig. 8A upper panels) and showed maximum levels in the cytoplasm at CT 21 (Fig. 8A middle panels). PER and TIM were in the nucleus of LNs at CT 0 (Fig. 8A lower panels). anti--PER and anti--TIM staining were at minimum levels in LNs at CT 12 (Fig. 8B lower panels). In *Veela* mutant flies, the glia cells also stained for both PER and TIM at CT 21 (Fig. 11; Fig. 12 row 2 right half panels), thus PER and TIM showed circadian cycling not only in the pacemaker cells but also in glia cells as in LD and DD conditions (e.g. Zerr *et al.*, 1990; Stanewsky *et al.*, 1997b; Hunter--Ensor *et al.*, 1996; Kaneko and Hall, 2000). Moreover, at CT 3 and CT 15 the glia cells showed only anti--PER staining (Fig. 11; Fig. 12--row 1 right half panels, --row 2 left half panels). In cry^b flies, peak levels of anti--PER and anti--TIM staining were visible in the cytoplasm of the pacemaker cells at CT 15 (Fig. 10 upper left half panels).

At CT 18 anti--PER staining was nuclear in the s--LN_vs but anti--TIM staining remained in the cytoplasm (Fig. 10 upper right half panels), and it remained cytoplasmic. At CT 9, both anti--PER and anti--TIM staining was at trough levels (Fig. 10 lower right half panels and Fig. 11). It is noteworthy that anti--PER and anti--TIM staining in the pacemaker neurons was visible earlier in cry^b flies (CT 15) compared to *Veela* mutant flies (CT 18) (Fig. 8; Fig. 10; and Fig. 11). The advanced clock protein staining in the pacemaker neurons correlated with a shorter period in LL for cry^b flies compared with *Veela* flies (Fig. 2 and Table 1). The glia cells showed strong anti--PER staining at CT 3 and CT 9 (Fig. 11; and Fig. 12 row

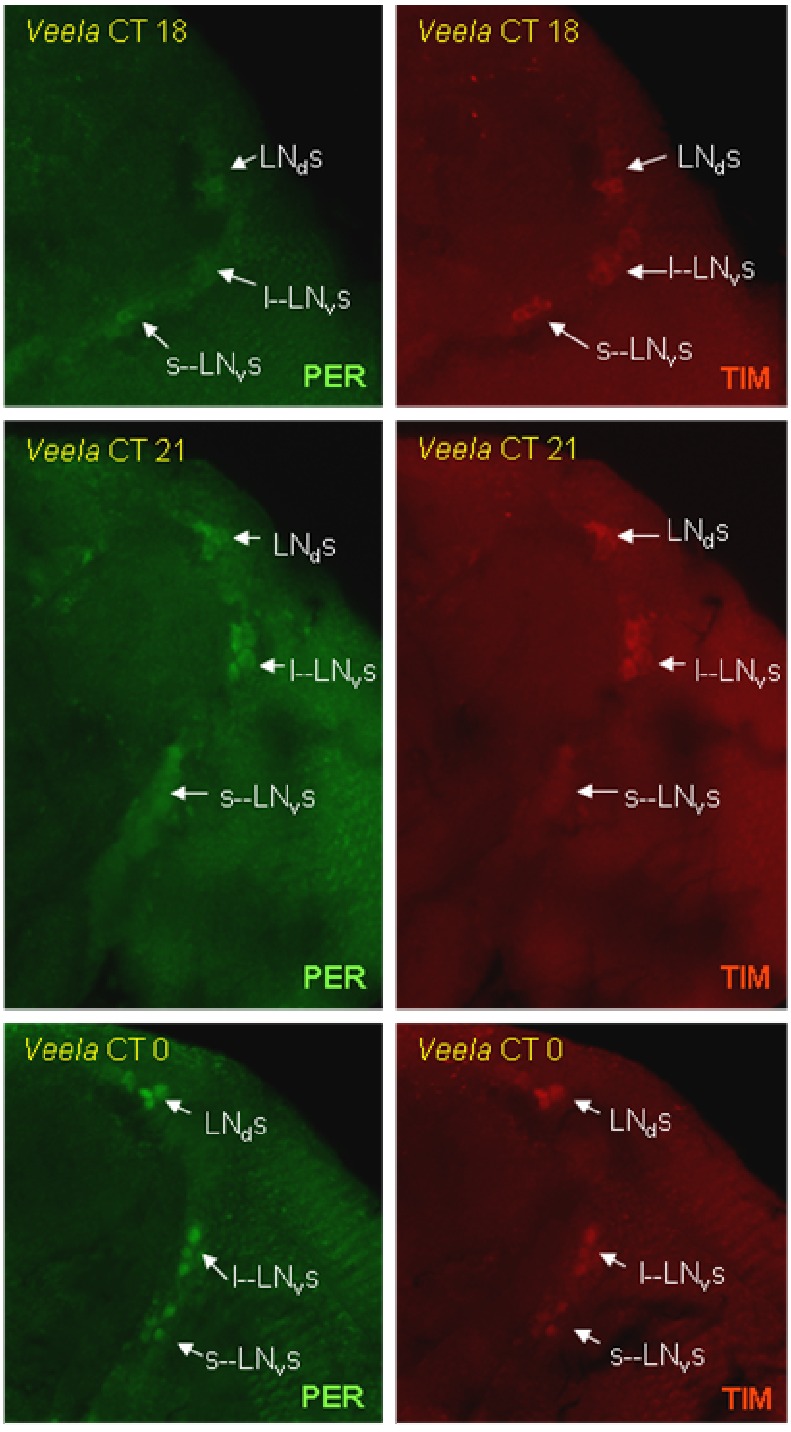

Figure 8A *Veela* mutant flies exhibit coordinated cycling levels of clock proteins in the pacemaker neurons. Whole mounted brains were stained for anti--PERIOD (green) and anti--TIMELESS (red) in *Veela* mutant flies on the second day in LL. At CT 18, PER and TIM were weakly detectable in the cytoplasm in the indicated cell groups (Fig. 8A upper panels), and at CT 21 PER and TIM were more strongly stained in the cytoplasm of the

indicated cell groups (Fig. 8A middle panels). At CT 0, PER and TIM were nuclear in the indicated cell groups (Fig. 8A lower panels).

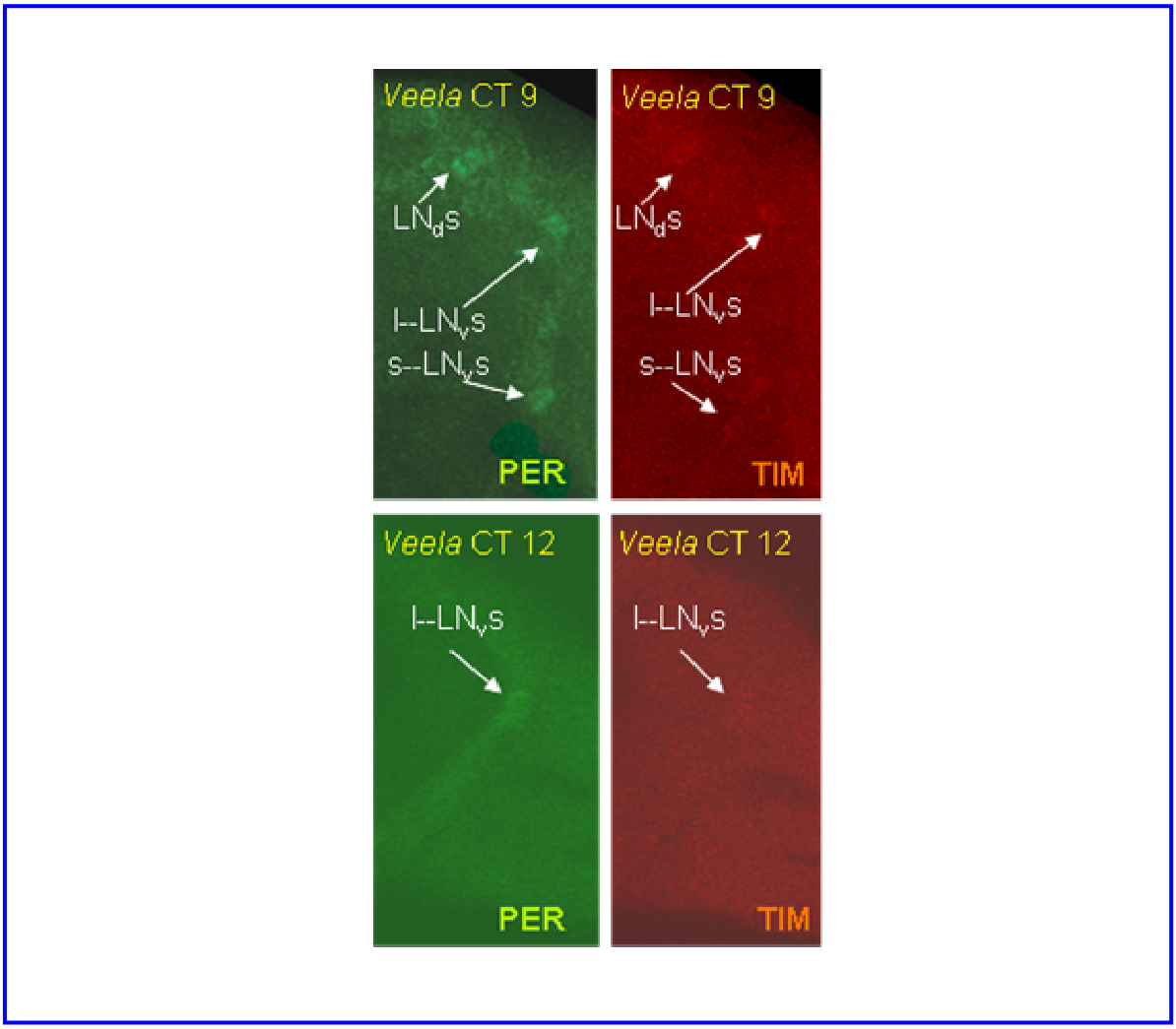

Figure 8B At CT 12, brains were largely negative for both PER and TIM signals (Fig. 8B lower panels) and the same was true at CT 9 except some cytoplasmic staining in the LNs. CT indicates subjective circadian time, where CT 0 to CT 12 indicates the subjective day (where the lights would have been 'on' in a LD cycle) and CT 12 to CT 24 subjective night (where the lights would have been 'off' in a LD cycle). The s--LN_vs and l--LN_vs indicates small and large --later ventral neurons, respectively. LN_ds are dorsal lateral neurons.

3 panels). In *y w* flies, anti--PER and anti--TIM staining was essentially absent in the pacemaker neurons at most of the time points investigated (Fig. 8, and Fig. 10). However, surprisingly at CT 12 there was strong cytoplasmic staining for both anti--PER and anti--TIM in the l--LN_vs (Fig. 9 upper panels; and Fig. 11), and also a weak cytoplasmic staining at CT 9 (Fig. 9 lower right half panels). Moreover, at CT

0 all LNs showed weak cytoplasmic staining for anti--PER (Fig. 9 lower left half panels).

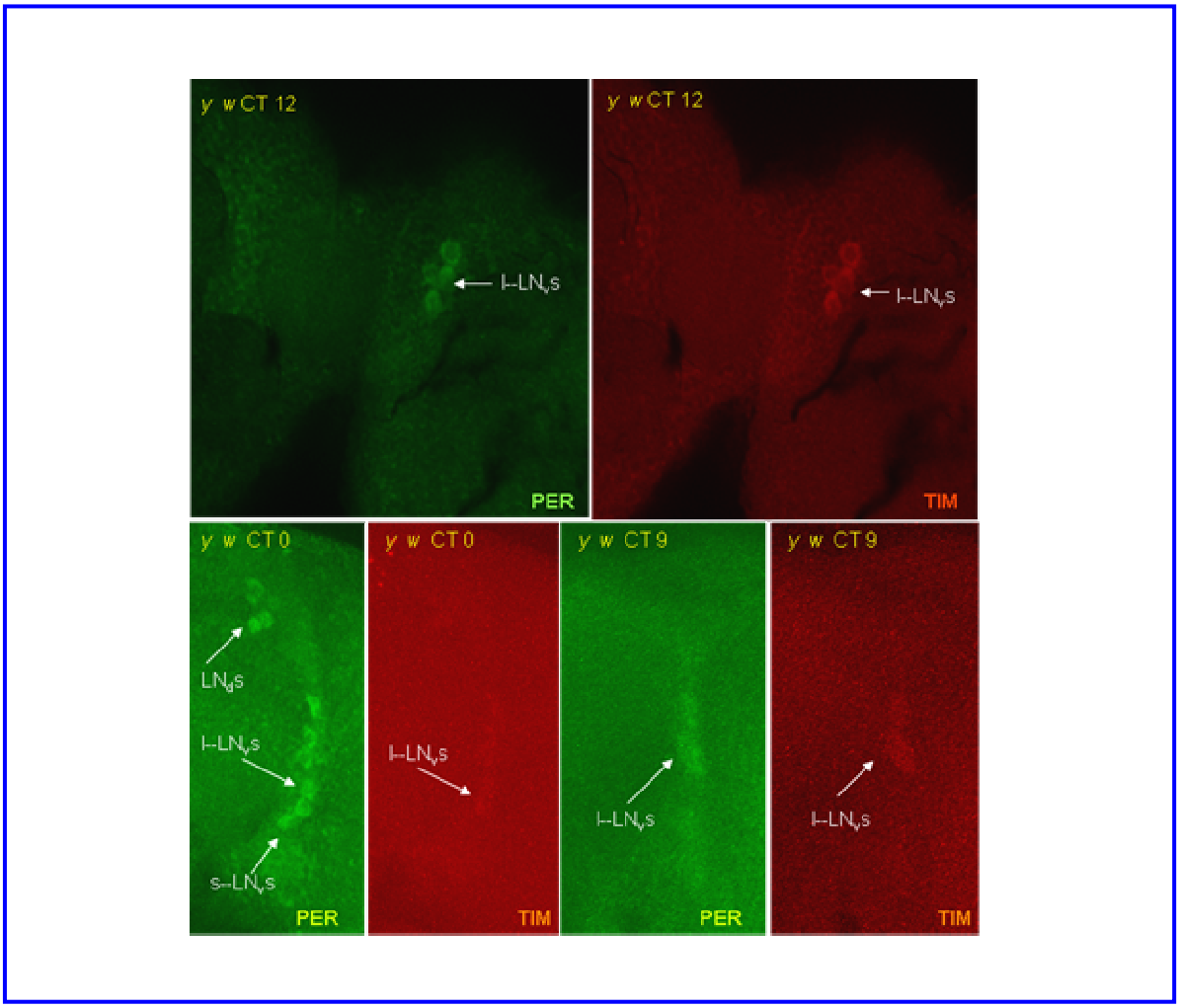

Figure 9 In wild type flies (*y w*) clock proteins expression in LL is restricted to a single time point and neuronal type. Whole mounted brains were stained for anti--PER (green) and anti--TIM (red) in *y w* flies on the second day in LL. At CT 12, flies displayed cytoplasmic staining in the l--LN$_v$s for both anti--PER and anti--TIM (upper panels). At CT 0 there was very weak anti--PER cytoplasmic staining but no anti--TIM in lateral neurons (lower left 2 panels). At CT 9 there is weak cytoplasmic staining only in the l--LN$_v$s (lower right 2 panels).

In conclusion, PER and TIM levels cycle in a circadian fashion in the pacemaker neurons and glia cells of *Veela* mutant flies. Similarly, *cry^b* flies also showed synchronized cycling of PER and TIM in the pacemaker neurons. The synchronized cycling of PER and TIM in the s--LN$_v$s explains the rhythmic behavior of both *Veela*

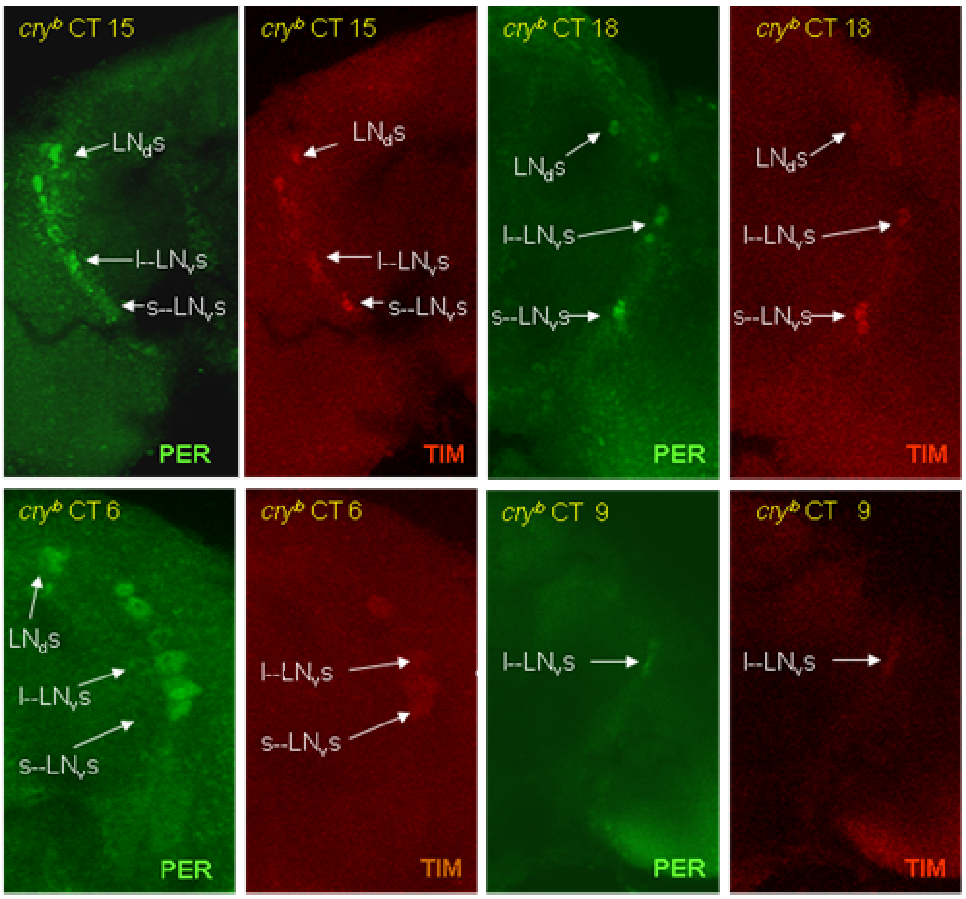

Figure 10 *cry*^{b} flies maintain coordinated cycling levels of clock proteins in pacemaker cells in LL. Whole mounted brains were stained for anti--PER (green) and anti--TIM (red) on the second day in LL. At CT 15 and CT 18 the lateral neurons were strongly staining for both PER and TIM (upper panels), and at CT 9 weak staining for both PER and TIM was seen in the l--LN$_v$s (lower right 2 panels). At CT 6 the LNs show cytoplasmic staining for both PER and TIM (lower left 2 panels).

and *cry*^{b} flies in LL. The absence of a similar molecular cycling in the *y w* flies explains their arrhythmic behavior in LL. Even though the *y w* flies did not show any synchronized cycling of PER and TIM in pacemaker cells in LL, they did show anti--PER and anti--TIM staining at CT 12 in the l--LN$_v$s, which was never reported earlier.

Figure 11

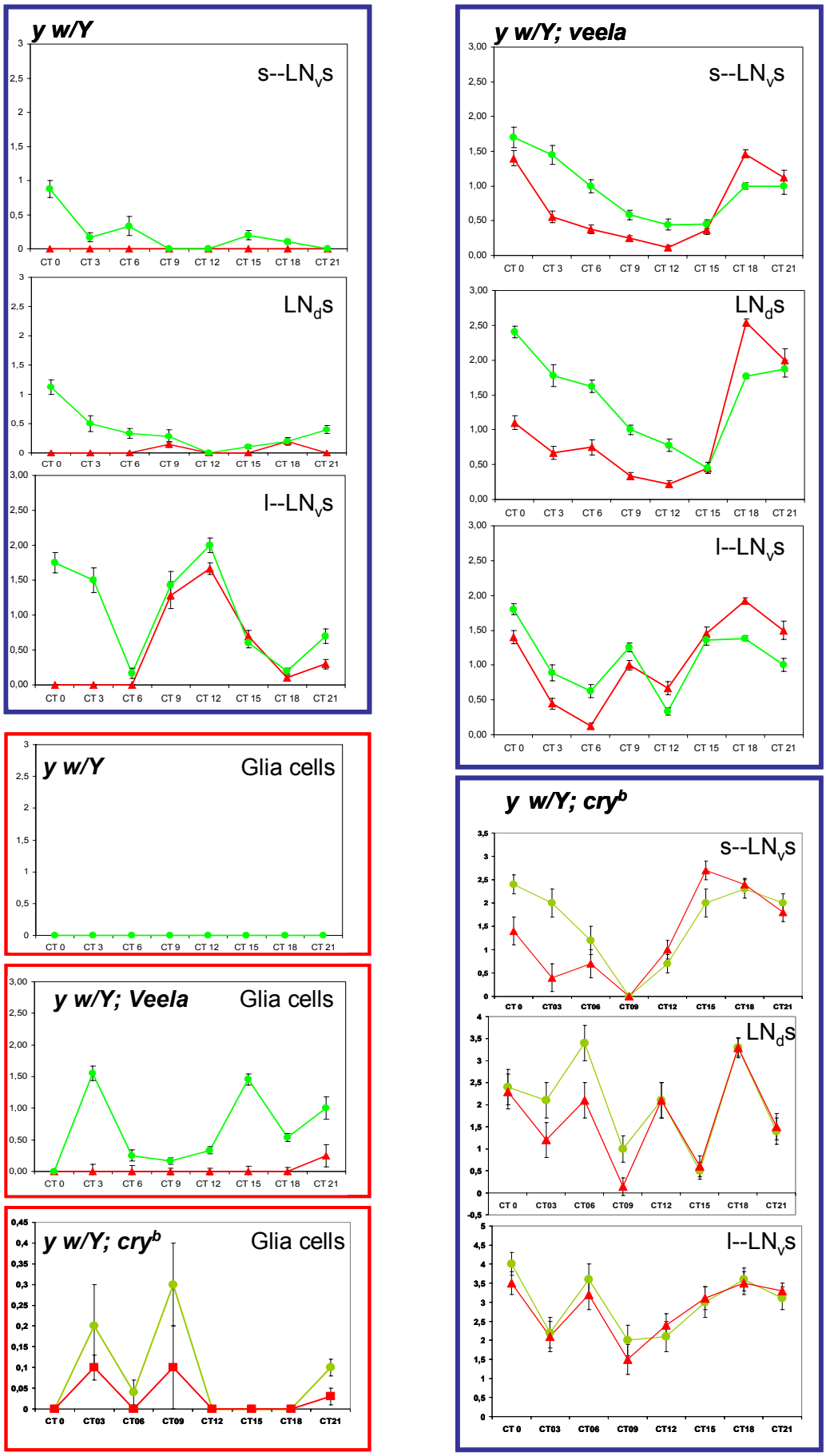
y w/Y
s--LNvs
LNds
l--LNvs
y w/Y Glia cells
y w/Y; Veela Glia cells
y w/Y; crybGlia cells
y w/Y; veela
s--LNvs
LNds
l--LNvs
y w/Y; cryb
s--LNvs
LNds
l--LNvs
CT 0 CT 3 CT 6 CT 9 CT 12 CT 15 CT 18 CT 21
CT 0 CT03 CT06 CT09 CT12 CT15 CT18 CT21

Figure 11 Quantification of anti--PER and anti--TIM staining in lateral neurons and glia cells on the second day in LL. PER and TIM expression is indicated by green and red lines, respectively. The x--axis represents the subjective circadian time (CT) and the y--axis represents the staining index. On the x--axis time starts at CT 0 and the every 3rd until CT 21. On y--axis the staining index is graded by a scale of 0--4.

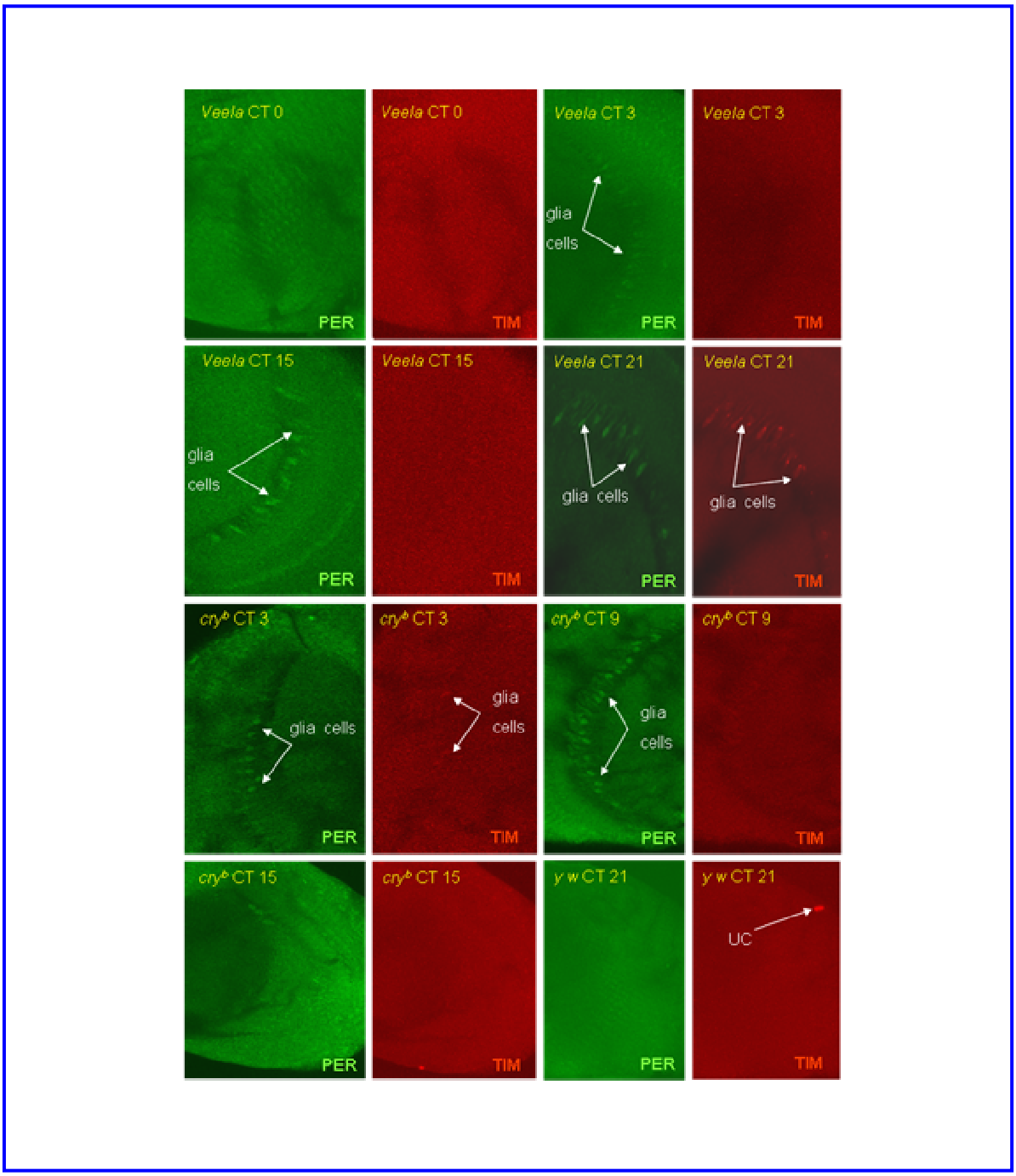

Figure 12 Glia cells show coordinated cycling levels of clock proteins in *Veela* and *cry*^{b} flies but not in *y w* flies. Whole mounted brains were stained for both anti--PER (green) and

anti--TIM (red) on the second day in LL. At C T0 and CT 3 there is no staining in *Veela* flies (first row of panels). The glia cells show strong signals for both PER and TIM in *Veela* flies at CT 21 (2 right panels in row 2). At CT 15 *Veela* flies show only PER staining (2 left panels in row 2). In cry^b flies, there is weak staining at CT 3 and CT 9 for anti--PER (row 3 panels). At CT 15 there is no staining in cry^b flies (two left panels in the row 4). *y w* flies largely showed no staining at any time point (a representative picture from CT 21 is presented) (two right panels in the row 4). UC indicates an unspecific cell stained against anti--TIM antibody.

5 Discussion

In natural conditions, the circadian clock of *Drosophila* is daily reset by LD cycles. Subjecting the fly artificially to continuous high intensity light results in arrhythmic locomotor activity (Konopka *et al.*, 1989; Helfrich--Förster *et al.*, 2001). cry^b flies remain rhythmic in intense constant light (Emery *et al.*, 2000a) because this mutation possibly blocked CRY and light mediated degradation of TIM (Stanewsky *et al.*, 1998; Ceriani *et al.*, 1999; Busza *et al.*, 2004) and thus renders the clock work mechanism immune against the damaging effects of LL. Based on the above results CRY is regarded as a dedicated circadian photoreceptor in *Drosophila* (Emery *et al.*, 2000a). Therefore, rhythmic behavior of a fly in LL indicates an impediment in the light input pathway of the circadian clock.

5.1 Is *Veela* a component of the circadian input pathway?

The novel mutant *Veela* exhibited behavioral rhythmicity in LL similar to the cry^b flies (Fig. 2 and Table 1). This indicated that the *Veela* mutation might be impairing a molecule involved in the light input pathway into the circadian clock of *Drosophila*. Wild type flies show a lengthened period in dim LL, and this period lengthening progressively increased with higher light intensities and finally results in AR (Konopka *et al.*, 1989). The circadian clock in cry^b flies is immune to the damaging effects of LL. At 2000 lux LL, cry^b flies showed a free running behavior with a period close to the free running ca. 24 h period of wild type files in DD, and the authors proclaimed that the cry^b clock can not ‘see’ LL (Emery *et al.*, 2000a). The normal free running period indicates an absolute blockage in light input.

However, at 100 lux LL, adult cry^b flies showed a period lengthening compared with the DD--period (Helfrich--Förster *et al.*, 2001). The period lengthening of cry^b flies in LL indicates that residual light signals seep into the circadian clock. *Veela* mutant flies exhibited a longer period compared to the cry^b flies (Table 1). In DD, the *Veela* mutant flies displayed a free running period similar to wild type flies (Table 2), which implies that the *Veela* mutation does not affect the basic clock work mechanism responsible for the free running behavior in *Drosophila*. Again, similar to cry^b flies *Veela* mutant flies also behaved normally in LD (Fig. 2, Table 1 and Table 2), which implied the mutation specifically blocks the LL effects on the circadian clock similar to cry^b. Finally, the longer and shorter free running periods, in LL and in DD, respectively (Table 1 and Table 2), of *Veela* flies indicate that residual light signals enter their circadian clock, but more substantially as in cry^b flies.

5.2 Does *Veela* interact with *cry* and *tim*?

In cry^b flies rhythmic behavior in LL was visible only when the mutation was homozygous for cry^b; i.e. it is a recessive mutation. In case of *Veela* mutant flies, both homozygous and heterozygous flies exhibited rhythmic behavior in LL, in spite of the fact that the period length was shorter and rhythms were weaker (compare the RI in Table 2) in heterozygous flies (Fig. 2 and Table 1) compared to homozygous flies (Fig. 3 and Table 1). Moreover, homozygous flies showed 85% rhythmicity whereas heterozygous flies showed 31%. To attain full efficiency of the *Veela* mutation a homozygous situation may be necessary. Therefore, *Veela* is a semidominant mutation. On the other hand, combining the *Veela* mutation with cry^b (both mutations in heterozygous situation) resulted in a phenotype similar to the *Veela* mutation in homozygous situation (Fig. 3 and Table 1). From this result it could be inferred that *Veela* interacts with *cry*. Given the similar behavior of both *Veela* and cry^b flies in LL, it can be predicted that both of them function in the same phototransduction pathway(s) into the circadian clock or alternatively they might interact directly (Fig. 3 and Table 1). Since the *Veela* and cry^b mutant flies show similar behavior in LL, it is possible that *Veela* interacts with TIM, as CRY does with TIM (Ceriani *et al.*, 1999; Busza *et al.*, 2004).

Sequencing of the *tim* gene in *Veela* revealed that it is an independent mutation. Therefore, from the behavioral experiments employing *Veela/tim*^{01} flies one could get an idea about the possible interaction between *tim* and *Veela* genes. However, the results we obtained were ambiguous and therefore could not be interpreted. Among the tested flies 22% of *Veela/ tim*^{01} flies showed rhythmic behavior similar to *Veela* /+ flies in LL (Table 1). On the other hand the *Veela* /+; *cry*^{b}/+ flies showed 77% rhythmicity in LL, like the homozygous *Veela* flies (Fig. 3 and Table 1). Therefore, constructing any opinion about the interaction between *Veela* and *cry* was more assimilable than for *Veela* and *tim*.

5.3 Is *Veela* a *timeless* allele?

The *Veela* mutation was meiotically mapped between *al* and *dp* on chromosome 2 (Fig. 4), a region where the *timeless* gene is located (Myers *et al.*, 1995). In the context of a possible interaction between *Veela* and *cry* (Fig. 3) and mapping of the *Veela* mutation close to *tim* we speculated that *Veela* might be a novel *tim* allele. We tried to test this hypothesis in two ways: a) Could a *tim* transgene rescue the wild type behavior in the *Veela* mutant flies in LL? b) Did the *tim* sequence show any significant nucleotide change(s) in the *Veela* mutant flies? Solid positive answers for these two questions were required for accepting the above hypothesis.

Answer to the first question was that the *tim* transgene could not rescue the wild type behavior in the *Veela* mutant flies (Fig. 6E and Table 1). Out of the tested flies, 63% of the flies showed rhythmic behavior in LL indicating a more *Veela* like phenotype in LL (Table 1). But, it was not certain whether the *tim* transgene could rescue the wild type behavior in the *Veela* mutant flies because of its semidominant nature. Since the *tim* transgene failed to compliment the *Veela* phenotype, it is likely that *Veela* is not a *tim* allele. To confirm this assumption we sequenced the *tim* gene region of the *Veela* mutant flies.

Sequencing of the *tim* gene revealed that there were 3 nucleotide changes in the *Veela* mutant flies compared to the control strain, *ninaB*^{P315} (Fig. 7). First of all there was a G at position 52 (with reference to *tim*-- PD cDNA, CG 3234 --PD, FlyBase) in the *Veela* mutant flies, whereas the *ninaB*^{P315} flies had a G deletion at the same position. It has already been reported that some strains of *Drosophila*

have a G deletion at position 52, which results in a TIM protein truncated by 23 amino acids (Rosato *et al.*, 1997). The G deletion results in a premature termination of the first exon because the third triplet codon after the G deletion encoded for a stop codon (TGA) (Fig. 7A). The G deletion was found in the *ninaB*^{P315} and *y w* flies (Fig. 7A). Oregon--R, Canton--S and the *Veela* mutant flies had a G in position 52 (Fig. 7A), as reported in the data base for *tim*--PD (cf. Rosato *et al.*, 1997). TIM--PD is a longer form of TIMELESS with 1421 amino acids and it has a G at position 52 (FlyBase).

Could the longer TIM protein form be responsible for the *Veela* phenotype? If the longer TIM form is responsible for the *Veela* phenotype all the *D. melanogaster* strains with the longer TIM form should be rhythmic in LL. In order to answer this question we also sequenced this specific region of *tim* gene from Canton--S, Oregon--R, and *y w.* Canton--S and Oregon--R flies have the longer TIM form (Fig. 7A), but Canton--S flies show arrhythmic behavior in LL (Konopka *et al.*, 1989), like *y w* flies (Fig. 6A), which encodes the shorter TIM form. Therefore, the G at position 52 is not responsible for the *Veela* phenotype.

The second nucleotide change found in the *Veela* mutant flies is at position 1345. This nucleotide change from G to A results in a glycine to serine amino acid change, at amino acid 449 of TIM--PD (Fig. 7B). An identical nucleotide change is found in the *tim*^{blind} mutant flies (Fig. 7B ; Wülbeck *et al.*, 2004), and they show rhythmic behavior in LL (Wülbeck, C., personal communication). *BG--luc* flies (Stanewsky *et al.*, 2002) also have the same nucleotide change. Therefore, this amino acid change is not responsible for the *Veela* phenotype.

A third nucleotide change in *Veela* was found at position 3852 (Fig. 7C). A transversion from T to G, which results in an amino acid change from isoleucine to methionine, with reference to the sequence from the *ninaB*^{P315} and *y w* flies. But again, at the corresponding position (amino acid at 1284) TIM--PD and Oregon--R also has a methionine pointing to another polymorphism in the *tim* gene (Fig. 7C). Moreover, Canton--S flies encode a leucine instead of isoleucine at this position (Fig. 7C). Therefore, the methionine at position 3852 can not be responsible for the *Veela* phenotype. In conclusion, the critical analyses of the rescue and sequencing

data did not support the notion that *Veela* is a *tim* allele. Hence, this hypothesis was redeemed. However, further fine mapping of *Veela* is presently underway, and has already revealed that *Veela* can be genetically separated from the *tim* gene (Peschel, N., personal communication).

5.4 *Veela* does not disrupt cycling of molecular clock components in the pacemaker cells in LL

In LD, the molecular clock components PER and TIM show synchronized expression profiles in the clock neurons (Kaneko, 1998; Hunter--Ensor *et al.*, 1996). Changing from LD to LL stops the molecular cycling in clock neurons (Zerr *et al.*, 1990). Hence, wild type flies show arrhythmic behavior in LL (Konopka *et al.*, 1989; Helfrich--Förster *et al.*, 2001). *Veela* mutant flies showed robust PER and TIM cycling in the LNs in LL (Fig. 8 and Fig. 11), and as a result they exhibit rhythmic behavior in LL (Fig. 2, Fig. 11 and Table 1). The cry^b flies relatively short period in LL compared to *Veela* (Table 1) may be associated with molecular cycling in the lateral neurons (Fig. 9 and Table 1).The peak phase of molecular cycling in s--LN_vs was delayed in *Veela* compared with cry^b flies (Fig. 11; Fig. 8A; and Fig. 10). A delayed molecular cycling may be responsible for the longer period length of *Veela* flies in LL (Fig.2 and Table 1), and is probably caused by residual light input reaching the circadian clock (Konopka *et al.*, 1989).

y w flies showed conspicuous cytoplasmic staining in the l--LN_vs for both PER and TIM at CT 12 (Fig. 9 upper panels, and Fig. 11). At a previous time point, CT 9, also weak staining was seen in the l--LN_vs (Fig. 9 lower panels, and Fig. 11). Similarly, *Veela* and cry^b flies showed cytoplasmic staining in the l--LN_vs at CT 9 and CT 6 respectively (Fig. 8B and Fig. 10 lower left half panels). The cytoplasmic staining pattern of the l--LN_vs also followed a phase relationship correlating with the period length of *Veela* and cry^b flies in LL (Fig. 2 and Table 1). However, the implication of cytoplasmic staining in the l--LN_vs in LL is yet to be understood. Further, it would be interesting to know why and how *y w* flies exhibit this feature in LL.

PER and TIM show synchronized expression in the glia cells in LD and DD conditions (e.g. Zerr *et al.*, 1990; Stanewsky *et al.*, 1997b; Hunter--Ensor *et al.*,

1996; Kaneko and Hall, 2000). *Veela* and *cry^b* flies showed synchronized PER and TIM expression in the glia cells in LL but *y w* flies did not (Fig.11 and Fig. 12). The glia cells' expression of clock proteins also displayed the characteristic phase difference between *Veela* and *cry^b* flies, and this difference correlated with their long and short period length respectively. It was reported that PER expression in the glia cells was sufficient to manifest a weak behavioral rhythm (Ewer *et al.*, 1992). However, the rhythmic behavior of the *Veela* mutant flies may not be generated in the glia cells because of the robust molecular cycling in the s--LN_vs. But it is possible that the glia cells contribute to the rhythmic behavior observed in *Veela* and *cry^b* flies (Ewer *et al.*, 1992).

The results presented here imply that the *Veela* mutation causes a serious inefficiency to process the constant light input into the circadian clock. Therefore, the *Veela* mutant flies are resistant to the damaging effects of LL. *Veela* flies might be experiencing a subjective DD in LL. However, their longer period length indicates that they receive some light signals. A complete blindness to light signals should result in free running periods of ca. 24 h.

Wild type flies experience a drastic effect in LL: PER and TIM are continuously cleared in LL from the s--LN_vs and therefore they exhibit arrhythmic behavior. Moreover, to our surprise, there was some cytoplasmic staining for both PER and TIM in the l--LN_vs at CT 12 in LL (Fig. 9 upper panels). This is an intriguing observation never reported before. This indicates that the clock work mechanism is different in various subsets of the clock neurons and they react differently to the light signals as exemplified by the difference in the l--LN_vs and s--LN_vs. In the *Veela* mutant flies, glia cells also showed anti--PER and anti--TIM staining in LL at CT 21, and it seems that the glia cells exhibited nuclear staining for both PER and TIM at CT 21 (Fig. 12 row 2 right half panels). This adds to the differences and complexity in the clock work mechanism in different clock cells. Yet another difference is seen between the mammalian and the *Drosophila* circadian clock. It was shown very recently that pacemaker cells in the SCN get desynchronized in LL but rhythmicity within individual neurons does not collapse (Ohta *et al.*, 2005), whereas in *Drosophila* the molecular mechanism crashes. However, in both cases LL induces AR behavior.

6 Conclusion

The behavioral and histological data presented here strongly suggest that *Veela* is involved in the circadian light input pathway, comparable to *cry*. Blocking the light input pathway into the circadian clock both in the *Veela* and *cry* mutant flies spared the damaging effects of constant light on the clock work mechanism. A possible interaction between *Veela* and *cry* is likely, because of their strong genetic interaction observed in transheterozygous animals. Moreover, in agreement with the robust behavioral rhythms in LL, *Veela* and cry^b flies maintained molecular cycling of PER and TIM in the pacemaker neurons. In the *Drosophila* clock rhythmic gene expression crashes in LL in the s--LN_vs, contrasting the desynchronization observed in the mammalian SCN. The mapping of the *Veela* mutation between *al* and *dp* initially suggested it to be a novel *timeless* allele, but the sequencing data did not support this hypothesis. Mapping of the *Veela* mutation to its precise position may unravel its exact nature and role in the circadian light input pathway. Further, it may provide a novel paradigm in circadian photoreception.

Summary

In *Drosophila*, naturally the circadian clock is entrained by environmental light--dark cycles. Photoreceptors like rhodopsins and cryptochrome perceive light signals for light entrainment of the circadian clock. It is known that there is a light--dependent CRY:TIM interaction, leading to the degradation of TIM coupled with molecular re--setting of the clock--gene cyclings. However, functional double mutants (*norpA^{P41};cry^{b}*), blocking simultaneously the known rhodopsin mediated transduction cascade and the largely unknown cryptochrome mediated transduction cascade do not confer the circadian clock absolutely blind to light signals. The double mutants' circadian clock still shows residual light entrainment ability, indicating that a novel photoreceptor entrains the circadian clock. On the other hand, in *gl^{60j} cry^{b}* double mutants the circadian clock is absolutely blind to light signals. The *gl^{60j}* mutation removes morphological structures like the compound eyes and the ocelli (signaling from them is blocked by *norpA^{P41}*); *gl^{60j}* in addition removes the H--B eyelet and a subset of DNs (DN_1s). The H--B eyelet projects axons toward the s--LN_vs pacemaker neurons. Both in the s--LN_vs and in the DN_1s the molecular PER and TIM cyclings can still be synchronized by light in *norpA^{P41};cry^{b}* double mutant flies. Therefore, we have investigated a possible role of the H--B eyelet in residual light entrainment of the circadian clock.

We have shown that the H--B eyelet functions to sense dim white light. Blocking light signaling from it affects the synchronization of molecular TIM cycling in the pacemaker neurons and in a subset of the DNs. However, at higher intensities of light no effect was seen. Therefore, we further investigated a possible role of the DNs, a subset of which also maintained light--synchronized molecular circadian oscillation in *norpA^{P41};cry^{b}* double mutant flies. We have shown that the DN_{2+3}s host a self--sustained molecular oscillator, which is entrainable by light dark cycles. Further, it was demonstrated that a novel *norpA* and *cry* independent photoreceptor in the dorsal brain could entrain the DN oscillator. As a putative candidate, we have investigated the *Rh 7* gene and shown that it is indeed expressed in proximity to the DNs and LNs. Possibly, *Rh 7* bypasses the classical visual *norpA* dependent phototransduction cascade. The data presented in this thesis support the existence of a novel circadian photo*receptor* and--*pigment* and

set the stage for analysis of the signaling pathways involved. Finally, isolation of the novel mutant *Veela* presents a potential missing link in the CRY:TIM signaling and interaction mechanism, which is the central part of the *Drosophila* light--entrainment pathway.

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Appendix 1

Table 4 Supplement. Bioluminescence rhythms of transgenic lines, mobilized from *8.0--luc:2,* in various genetic backgrounds (in DD).

Genotype	N (n)	percent rhythmic	Bioluminescence expression level (CPS)	period ± SEM (h)	phase ± SEM (h)	rel--amp ± SEM
8.0--luc:2--1	6 (5)	83	49.9 ± 8.5	23.9 ± 0.3	5.8 ± 4.3	0.50 ± 0.05
disco^2/Y;8.0--luc:2--1	6 (3)	50	21.7 ± 0.8	25.9 ± 1.6	1.7 ± 5.9	0.62 ± 0.03
8.0--luc:2--15	6 (6)	100	21.3 ± 1.0	23.4 ± 0.2	6.9 ± 3.5	0.51 ± 0.03
disco^2/Y;8.0--luc:2--15	4 (3)	75	20.9 ± 0.8	22.8 ± 0.4	2.8 ± 1.7	0.58 ± 0.06
8.0--luc:2--19	4 (4)	50	24.3 ± 0.7	23.9 ± 0.4	9.8 ± 1.7	0.56 ± 0.13
disco^2/Y;8.0--luc:2--19	6 (5)	83	29.9 ± 0.9	23.0 ± 0.1	0.3 ± 0.8	0.56 ± 0.04
8.0--luc:2--20	5 (5)	100	24.0 ± 0.7	22.8 ± 0.2	17.8 ± 4.2	0.46 ± 0.02
disco^2/Y;8.0--luc:2--20	4 (1)	25	23.9	22.4	2.2	0.58
8.0--luc:2--21	5 (5)	100	21.4 ± 0.7	22.9 ± 0.3	22.8 ± 4.9	0.44 ± 0.05
disco^2/Y;8.0--luc:2--21	6 (3)	50	29.8 ± 1.1	22.5 ± 0.8	3.1 ± 2.1	0.62 ± 0.02
8.0--luc:2--22	4 (3)	75	24 .1± 0.8	23.4 ± 0.2	10.7 ± 0.4	0.38 ± 0.05
disco^2/Y;8.0--luc:2--22	6 (2)	36	32.2	23.6	22.5	0.50
8.0--luc:2--25	6 (4)	67	21.2 ± 0.7	23.3 ± 0.2	4.8 ± 5.4	0.55 ± 0.02
disco^2/Y;8.0--luc:2--25	2 (0)	--	--	--	--	--
8.0--luc:2--33	6 (3)	50	24.5 ± 0.9	23.8 ± 0.7	22.7 ± 2.3	0.50 ± 0.09
disco^2/Y;8.0--luc:2--33	6 (2)	36	22.3	23.7	22.5	0.40
8.0--luc:2--34	3 (1)	33	32.5	23.1	1.3	0.37
disco^2/Y;8.0--luc:2--34	6 (1)	17	28.7	23.8	21.9	0.61

'N' is the number of flies tested and 'n' is number of rhythmic flies.

Appendix 2

Table 5 Supplement. Transgenic fly lines, mobilized from *8.0--luc:2,* screened for locomotor behavior in LD and DD.

Genotype	LD						DD			
	N	n	% rhy.	Anticipation of light--dark transitions		period ± SEM (h)	N	n	% rhy.	period ± SEM (h)
				M	E					
per^{01}/Y;8.0--luc:2--4	8	5	63	-	++	24.2 ± 0.07	8	0	0	--
per^{01}/Y;8.0--luc:2--5	8	8	100	-	+++	24.1 ± 0.06	8	0	0	--
per^{01}/Y;8.0--luc:2--7	8	5	63	+	++	24.1 ± 0.04	8	0	0	--
per^{01}/Y;8.0--luc:2--9	8	5	63	-	+++	24.0 ± 0.08	8	0	0	--
per^{01}/Y;8.0--luc:2--10	8	3	38	+	+++	23.9 ± 0.3	8	0	0	--
per^{01}/Y;8.0--luc:2--11	8	7	88	-	+++	24.0 ± 0.4	8	0	0	--
per^{01}/Y;8.0--luc:2--12	7	5	71	-	++	24.2 ± 0.10	8	0	0	--
per^{01}/Y;8.0--luc:2--13	8	6	75	-	+++	24.4 ± 0.17	8	0	0	--
per^{01}/Y;8.0--luc:2--16	8	7	88	-	+++	24.2 ± 0.36	8	0	0	--
per^{01}/Y;8.0--luc:2--17	7	5	71	-	++	24.1 ± 0.13	8	0	0	--
per^{01}/Y;8.0--luc:2--18	8	7	88	-	++	24.3 ± 0.10	8	0	0	-
per^{01}/Y;8.0--luc:2--23	8	8	100	-	+	24.2 ± 0.06	8	0	0	--
per^{01}/Y;8.0--luc:2--26	7	5	71	-	-	24.2 ± 0.10	8	0	0	--
per^{01}/Y;8.0--luc:2--27	8	7	88	-	-	24.3 ± 0.10	8	0	0	--
per^{01}/Y;8.0--luc:2--28	8	6	75	-	++	24.4 ± 0.11	8	0	0	--
per^{01}/Y;8.0--luc:2--29	8	7	88	-	+++	24.3 ± 0.11	8	0	0	--

per^{01}/Y;8.0--luc:2--30	8	6	75	-	-	23.9 ± 0.15	8	0	0	--
per^{01}/Y;8.0--luc:2--35	6	6	100	-	+	24.3 ± 0.08	8	0	0	--
per^{01}/Y;8.0--luc:2--37	8	6	75	-	-	24.0 ± 0.15	8	0	0	--
per^{01}/Y;8.0--luc:2--38	8	7	88	-	+	23.9 ± 0.14	8	0	0	--
per^{01}/Y;8.0--luc:2--41	3	2	33	-	-	24.1	8	0	0	--
per^{01}/Y;8.0--luc:2--42	10	7	70	-	-	24.3 ± 0.16	8	0	0	--
per^{01}/Y;8.0--luc:2--43	8	8	100	-	-	24.1 ± 0.08	8	0	0	--
per^{01}/Y;8.0--luc:2--44	8	6	75	-	-	23.9 ± 0.09	8	0	0	--
per^{01}/Y;8.0--luc:2--45	8	5	63	-	-	24.1 ± 0.07	8	0	0	--

‘N‘ is the number of flies tested and ‘n’ is number of rhythmic flies.

Appendix--1

The 1.4 kb *Rh 7* promoter region cloned into pTGAL in front of GAL 4

ttcccccgacatcgatagccatggcttttccatcgaatcccgcaaacgaatcaatagttatcactacctttaaa
actggcaaacagattaacgcaacgccattacattaatttggggtcggaaaacaaacggagtgggatatttc
cggttttcaaactgatgataggttgtctaaacgcgcacagatcttagctaattcagtgggcaaaccagcctag
tcaatcagatttaacagccataattgatctgaacaaaggaatgctttatatcttcgatgttaacaatattcatatg
tatattatttgcgtcatttcatttaacttgtctccagtgaaaacttcaaagggaactgatatttttttttttgtttaaaca
attagttatgttatggcaaaataatagggatttaattgcaggcaaaaaagtttaatacaatcaagaattgcaa
attaattaatgcattgcaatgccaagcgaatagatcgacaaaaaataaaacaattaattaaacaactagca
agcagataatccaacagaattttaatacgcacagcgtacggttgccgcttattttctacgcttttttttttggtaact
ttttaggccagttatgactgactcatcccgcaaagttcggctttggcaatggaaatttttcacctgaacttgggc
tgttaagcactttgcattgtcgttcatctgggcaagccgagggttaaaaaaatcaggtaaggcgtgtattaaa
acgacctccgtgatatgtcaagcgagcaaatatgtatgtacatatgtacatacataaatgtcatttttctacaa
gcagctaaaatcagtctaaaatctggaaaacttgcgcatagaacacttacacttatgtatgtcccgtgtttata
atagactcttttgtattccttttttttttgtacaagtcactaattactctgctcggggtttttgttttttttttttttggagcaa
aggggaatcagcagacagagacagcatctgaaatggagaaagaataagtgctttttttctttgctgtgaattg
caagctatgaaaaatatatgtatgtacgtatatttttatttgtgagtactaacagccggcggcgaatgcgtaaat
gtcgtataatttagtgccacaaagcaaaacttttaatatctaaaccggaaagacagccggcaaaagttcgtt
ggtgtgcacatgctctatgtatgagaaaatgtctgcgtctaaaggcgtttttgtttaactccctttttgggggaatgt
cacaaatggtatctgatttttttaagttttaaggggtcattgtcgtaaaagaatatcatatatgtatgaatatttaaa
atgcacttaatcattcgagtcttgcagctaaaaagcgg

The underlines indicate the oligonucleotide region chosen for designing the oligonucleotide for PCR reaction.

Appendix--2

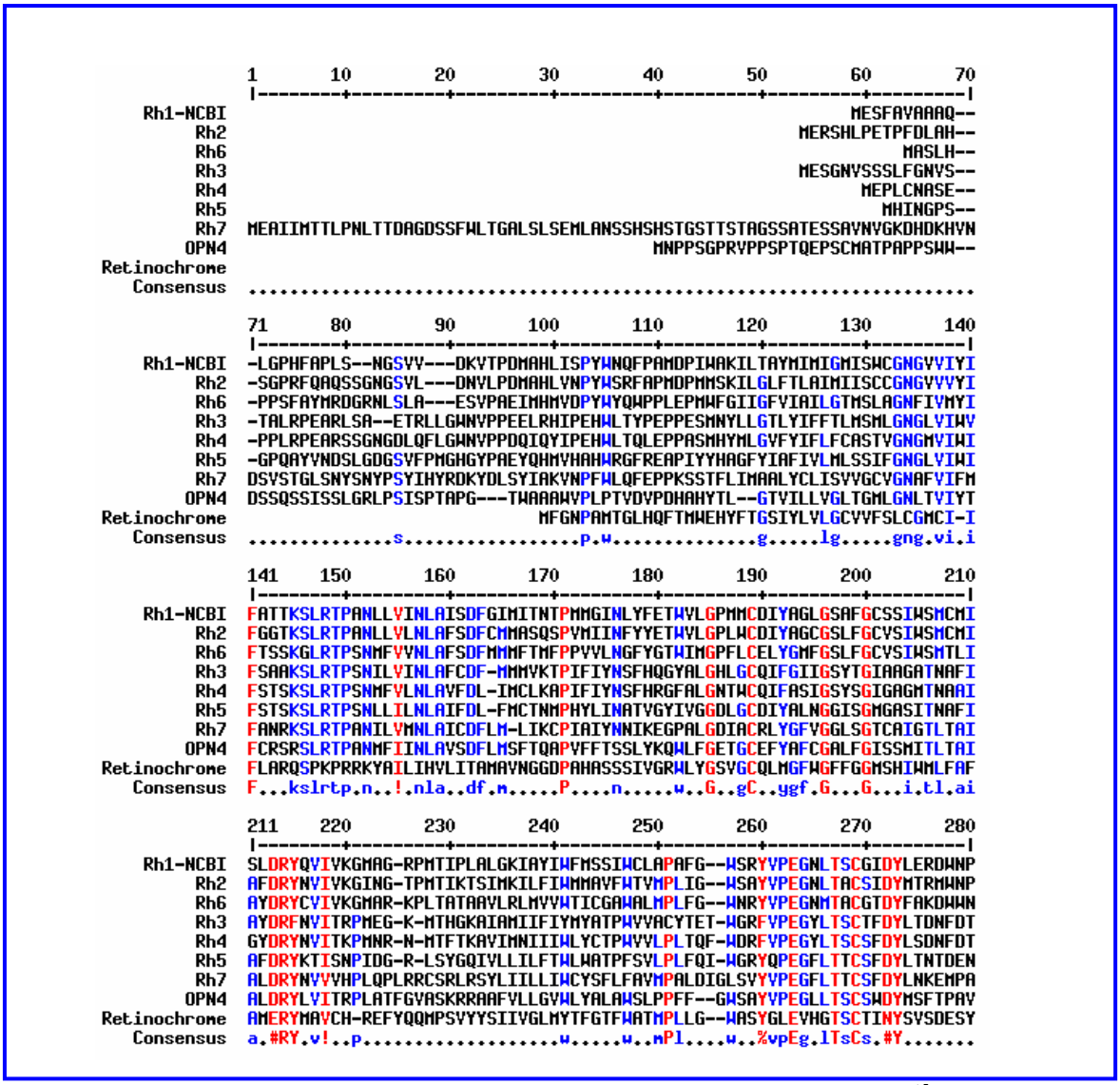
Homology matrix (DNAMAN 4.0)

Retinochrome	100%		-	-	-	-	-	-
Rh 1	23.7%	100%	-	-	-	-	-	-
Rh 2	22.8%	70.3%	100%	-	-	-	-	-
Rh 3	20.0%	39.0%	40.0%	100%	-	-	-	-
Rh 4	19.4%	40.4%	41.4%	75.2%	100%	-	-	-
Rh 5	18.6%	36.7%	35.3%	45.4%	47.7%	100%	-	-
Rh 6	21.7%	53.3%	52.8%	36.4%	36.5%	33.1%	100%	-
Rh 7	12.5%	26.8%	26.2%	29.3%	30.2%	30.7%	25.0%	100%

The homology matrix shows the percentage of amino acid sequences conserved between *Rh 7* and other rhodospins (*D. melanogster*) and retinochrome (*Todarodes pacificus*). Red highlight indicates a maximum sequence homology between *Rh 7* and *Rh 5*. Yellow highlight indicates a minimum sequence homology between *Rh 7* and *Rh 6*. The gray highlight indicates the sequence homology between *Rh 7* and retinochrome. The last (right end) entry in each row indicates the maximum value for sequence homology to the respective rhodopsin or retinochrome in the same row. To compare to a rhodopsin or retinochrome of interest, follow the column where it has 100% value and then look at the row where it crosses with other rhodopsins or retinochrome.

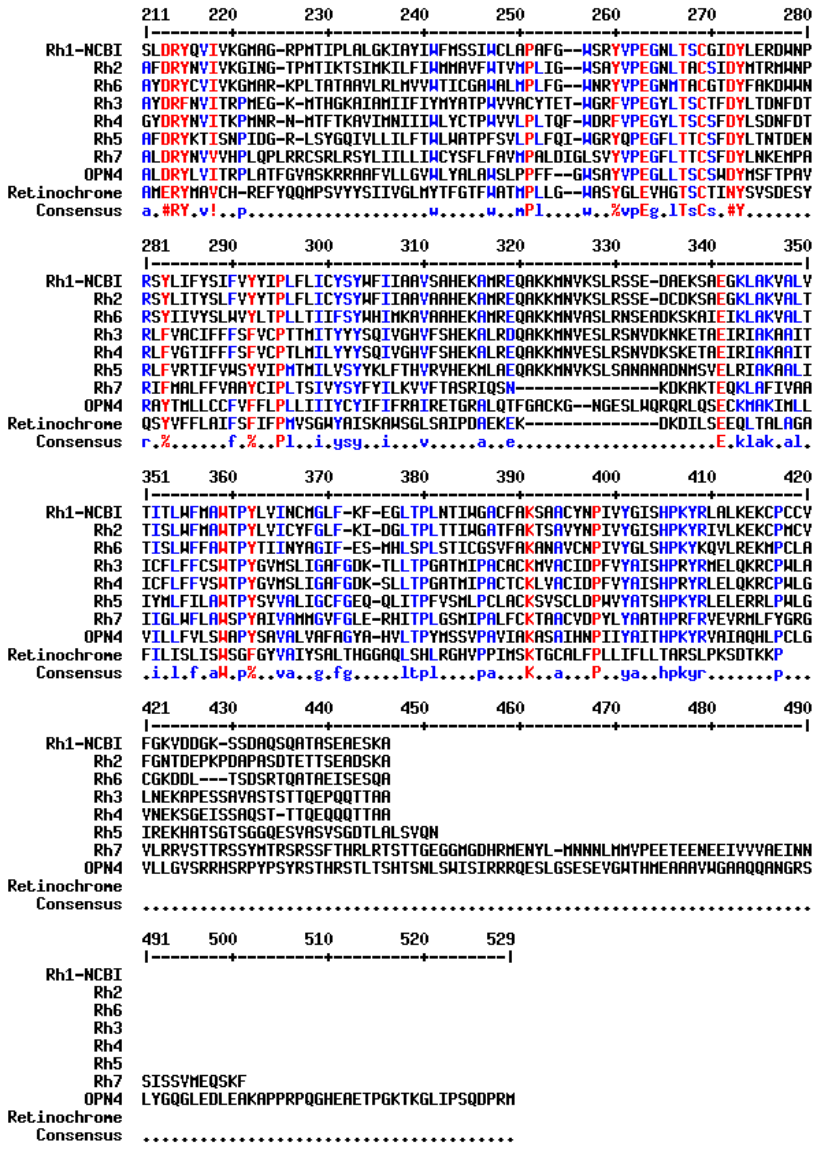
Appendix--3

Comparison of complete protein sequence among the different known *Drosophila* opsins, putative *Rh 7* in *Drosophila melanogaster*, human melanopsin and squid retinochrome.



continues…

Appendix--3


211 220 230 240 250 260 270 280
281 290 300 310 320 330 340 350
351 360 370 380 390 400 410 420
421 430 440 450 460 470 480 490
491 500 510 520 529
Rh1-NCBI
Rh2
Rh6
Rh3
Rh4
Rh5
Rh7
OPN4
Retinochrome
Consensus
Rh 1 -- NCBI -- P06002
Rh 2 -- NCBI -- P08099
Rh 3 -- NCBI -- P04950
Rh 4 -- NCBI -- P08255
Rh 5 -- NCBI -- P091657
Rh 6 -- NCBI -- CAB6821
Rh 7 -- NCBI -- NP524035
Drosophila melanogaster
Retinochrome -- NCBI -- CAA40422
Todarodes pacificus
Opsin 4 (Melanopsin) -- NCBI -- Q9UHM6
Homo sapiens

Declaration

I hereby declare that to the best of my knowledge and belief all the data presented in this Dissertation is absolutely created by me. Any known concept or literature from others used in this Dissertation is duly acknowledged.

This Dissertation or any part of it was not submitted to no other Faculty for the same purpose.

Regensburg,
22 February, 2005.

Shobi Veleri

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