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

PD Dr. Ralf Stanewsky

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Die Arbeit wurde angeleitet von PD. Dr. Ralf Stanewsky

Prüfungsausschuß:

Vorsitzender: Prof. Dr. Reinhard Sterner
Erster Gutachter: PD. Dr. Ralf Stanewsky
Zweiter Gutachter: Prof. Dr. Charlotte Förster
Dritter Gutachter: Prof. Dr. Günter Hauska
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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.

![Diagram of the circadian clock](image)

**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 Zeusgeber 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 Zeusgeber for the circadian clock. Other Zeusgebers like temperature (Pittendrigh et al., 1958), humidity (Halke t, 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 photoisomerization 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 effector enzyme, phosphoinositide--specific phospholipase C (PLCβ), encoded by the norpA gene in Drosophila. It hydrolyses the minor membrane phospholipid phosphotidylinositol 4,5--bisphosphate (PIP2) to produce soluble inositol 1,4,5--triphosphate (InsP3) 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 Ca2+ levels, by releasing Ca2+ from InsP3--sensitive stores and also by activating Ca2+ 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 Ca2+ permeable (Hardie, 1991) and mediated by at least two channels encoded by the trp (transient
receptor potential) gene and a homologue with approximately 40% sequence identity, \textit{trpl} (\textit{trp--like}) (Hardie and Minke, 1992; Niermeyer \textit{et al.}, 1996; Philips \textit{et al.}, 1992).

### 2.2.2 Cryptochromes

Cryptochromes (CRY) were first discovered in \textit{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 \textit{Arabidopsis}, CRY1 and CRY2 mRNA levels oscillate with a circadian rhythm (Harmer \textit{et al.}, 2000). At protein level CRY1 expression is not affected by light, whereas CRY2 expression is negatively regulated by blue light (Ahmad \textit{et al.}, 1998a; Lin \textit{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 \textit{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 \textit{et al.}, 1996). CRY1 inhibits the hypocotyl elongation in blue light (Lin \textit{et al.}, 1998), and it plays a role in light--dependent anthocyanin accumulation (Ahmad \textit{et al.}, 1995). In circadian clock function the \textit{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, cryb 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), DN1s, DN2s and DN3s; and three groups of lateral neurons (LNs), large--lateral neurons ventral (l--LNvs), small--lateral neurons ventral (s--LNvs), and lateral neurons dorsal (LNds). The DN1s are present in the dorsal brain and the LNs are present in the lateral brain. The DN1s 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 LNvs reaching up to the medulla (Me). The DN1s also send axonal projections towards the esophagus (Es). The DN2s 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 DN2s send axonal projections locally within the dorsal brain. The DN3s
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_{3}s 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 perikarya 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 \textit{period (per)} and the \textit{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_{1}s, DN_{2}s and DN_{3}s. 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_{1}s are located in the most dorsal cortex of the brain and are composed of 8--17 cells. The DN_{2}s 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_{3}s are located in the lateral superior protocerebrum and consists of ~40 neurons with small somata. These LNs and DNs rhythmically express \textit{PERIOD (PER)} and \textit{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 \textit{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 I--LNv sends 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--LNv sends projections into the dorso--medial brain and their axons terminate dorso--frontally of the mushroom--body calyces (Fig. 2). The LNv sends 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--LNv and LNv. Some of the DN1s project their axons towards the LNv and to the esophagus (Hall, 2005). The DN3s either send axons towards the mid--dorsal brain (Kaneko and Hall, 2000) or towards the LNv (Fig. 2; Veleri et al., 2003).

3.1.3 Functions of the brain clock neurons
Currently, it is believed that the s--LNv 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--LNv together with the I--LNv 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--LNv and I--LNv 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--LNv 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 LN₃s 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--LN₃s and l--LN₃s, either by cell ablation or by mutation (Pdf⁰¹), had a much milder effect on the locomotor activity than the knock--out of s--LN₃,s, l--LN₃,s and LN₄,s by the disco² mutation, which lacks all LNs but retains the DN₃s (Zerr et al., 1990; Hardin et al., 1992; Veleri et al., 2003). The Pdf⁰¹ flies for example displayed a rhythm with short period for at least one week in DD, which may be generated from the LN₄,s (Helfrich--Förster, 2002). Recently, it was shown that the LN₃,s contribute to the E peak (Grima et al., 2004; Stoleru et al., 2004).

It was shown that the LN₃,s 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 LN₃,s 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 DN₃s, although their projections have been studied intensely (Kaneko and Hall, 2000; Helfrich--Förster, 2003). A role for DN₁,s in light mediated locomotor rhythms has been implicated by Klarsfeld et al. (2004). Recently, the DN₂,s and a subset of the DN₁,s were shown to contribute to the E peak (Stoleru et al., 2004). The above mentioned studies did not look at specifically on the DN₃,s’ role in circadian functioning; therefore their function is largely unknown (see more about DN₃s in Chapter 4). Besides the LNs and DN₃s, 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
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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 (period01) 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\(^{01}\) ClkJrk 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 ClkJrk 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 overexpression 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--LNvs) by the molecular clock mechanism described above. In fact, the s--LNvs were shown to maintain self--sustained molecular oscillations in DD for multiple days, but in contrast in I--LNvs 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,s, 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 DN1s and in the l--LN,s, and anti--phase oscillations in the DN2s; 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*<sup>b</sup>, results in constant levels of TIM in the eye photoreceptors and in the LNs, except the s--LN<sub>s</sub>s and the LN<sub>s</sub>s (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*<sup>b</sup> flies can LD synchronize behavioral rhythms and molecular synchronization of TIM.
in the s--LN, 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 cryb 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 cryb 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 cryb 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 cryb flies (Ivanchenko et al., 2001). This findings suggest that CRY is also a photoreceptor in the peripheral clocks. On contrary in LD in adult cryb flies the TIM oscillations continued in the s--LN, and in DN (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 Bmal1--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 extrahepatic tissues like eye) knockout (rbp\(^{-/}\)) mice exhibit either normal photic entrainment or become diurnal. In contrast, (vitamin A--repleted) rbp\(^{+/}\) cry\(^{1/-}\) cry\(^{2/-}\) mice have reduced light sensitivity, while (vitamin A--depleted) rbp\(^{+/}\) cry\(^{1/-}\) cry\(^{2/-}\) mice, that are additionally on a vitamin A--depleted diet, presumably lack functional opsin 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 opsin 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 cryb 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 cryb 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 feedback 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,s 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*<sup>1</sup>;*glass*<sup>60j</sup>. *sine oculis* (*so*) 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*<sup>60j</sup> 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<sub>s</sub> (Helfrich--Förster et al., 2001, Blanchardon et al., 2001). However, *so*<sup>1</sup>*;gl*<sup>60j</sup> 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<sup>P41</sup>;cry<sup>b</sup> 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<sup>P41</sup>;cry<sup>b</sup> 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<sup>P41</sup>;cry<sup>b</sup> flies the molecular synchronisation of PER and TIM expression in the s--LN<sub>v</sub>s and in the DN<sub>1</sub>s 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<sup>P41</sup>;cry<sup>b</sup> flies was eliminated by combining <i>glf</i><sup>60j</sup> with cry<sup>b</sup>, thus the <i>glf</i><sup>60j</sup> cry<sup>b</sup> double mutant flies were absolutely circadian blind (Helfrich--Förster et al., 2001). What additional photoreceptive structures were affected by the <i>glf</i><sup>60j</sup> mutation? As mentioned above, the H--B eyelet and the DN<sub>1</sub>s are additionally removed in <i>glf</i><sup>60j</sup> flies. Therefore, these structures <i>per se</i> pose as the possible candidates to mediate light signaling into the circadian clock in the norpA<sup>P41</sup>;cry<sup>b</sup> flies.

The H--B eyelet qualifies as one of the relevant candidates for mediating residual light signals into the s--LN<sub>v</sub>s for 2 additional reasons: 1) It has direct neural projections towards the s--LN<sub>v</sub>s (Fig. 2) (Hofbauer and Buchner, 1989; Yasuyama and Meinertzhagen, 1999; Malpel et al., 2002), which retained molecular
synchronization of PER and TIM in \textit{norpA}^{P41};\textit{cryb}^b flies (Helfrich--Förster \textit{et al.}, 2001). The H--B eyelet sends direct neural projections towards the s--LN\_s implicating a role for the former in the circadian function. 2) The H--B eyelet expresses the \textit{Rh 5} and \textit{Rh 6} genes (Yasuyama and Meinertzhagen, 1999; Malpel \textit{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\_s (Fig. 2) like the H--B eyelet (Kaneko and Hall, 2000). Possibly, the DN\_1\_s can entrain the s--LN\_s through this anatomical pathway (Helfrich--Förster \textit{et al.}, 2001). It is inferred that the DN\_1\_s play a role in LD entrainment because \textit{disconnected} mutant flies, which lack the LN\_s but not the DN\_1\_s, are able to entrain to LD (Zerr \textit{et al.}, 1990; Hardin \textit{et al.}, 1992; Helfrich--Förster, 1998, Blanchardon \textit{et al.}, 2001). Like the s--LN\_s, the DN\_1\_s also retained synchronized molecular cycling in \textit{norpA}^{P41};\textit{cryb}^b flies (Helfrich--Förster \textit{et al.}, 2001).

Yet another promising candidate is a novel putative rhodopsin gene hereon called \textit{rhodopsin 7} (\textit{Rh 7}) (Adams \textit{et al.}, 2000). If \textit{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 \textit{Drosophila} rhodopsin \textit{Rh 5}. \textit{Rh 7} also shows a 12.5\% homology with the retinal photoisomerase from the Japanese squid. The predicted protein of \textit{Rh 7} encodes for a G protein coupled photoreceptor like other \textit{Drosophila} rhodopsins (FlyBase).

Although the H--B eyelet, the DNs and \textit{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 \textit{norpA}^{P41};\textit{cryb}^b flies. A novel mutant, \textit{Veela}, is drastically disabled to perceive/transmit the constant light signal into the circadian clock similar to \textit{cryb}^b phenotype. Therefore, possibly \textit{Veela} could define such an unorthodox photoreceptor/photopigment. Furthermore, it also possible that a functional \textit{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<sup>P44</sup>;cry<sup>b</sup> 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 <i>D. melanogaster</i>, 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 <i>D. melanogaster</i>.
CHAPTER 2

GENERAL MATERIALS AND METHODS

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Chapter 2
General Materials and Methods

1 Materials

1.1 Fly stocks

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

The \( \text{norpA}^{P41} \) used here was in \( w \) background, hereon called only \( \text{norpA}^{P41} \).

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

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

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

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\textsubscript{2}O

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\textsubscript{2}O
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$_2$PO$_4$.H$_2$O 2.83 g
Na$_2$HPO$_4$.2H$_2$O 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

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<td><em>Expand High Fidelity PCR System</em></td>
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<tr>
<td>Restriction enzymes, T4--Ligase, Calf</td>
<td>Invitrogen</td>
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<td>Qiagen</td>
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<tr>
<td><em>QIAquick</em> Gel Extraction Kit, <em>QIAGEN</em>®*</td>
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<td><em>Plasmid Midi Kit</em></td>
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<tr>
<td>BigDye®Terminator v1.1 Cycle Sequencing kit</td>
<td>AB Applied Biosystems</td>
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<tr>
<td>dNTP set</td>
<td>Roth</td>
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<tr>
<td>PeqGOLD Trifast</td>
<td>(PeqLab)</td>
</tr>
<tr>
<td>All other chemicals, salts and solutions</td>
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</tr>
<tr>
<td></td>
<td>Biozym, Difco, Eurobio, Fluka,</td>
</tr>
<tr>
<td></td>
<td>Merck, Roth.</td>
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<tr>
<td>Paraformaldehyde</td>
<td>Electron Microscopy Sciences</td>
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<tr>
<td>Luciferin</td>
<td>Biosynth</td>
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<tr>
<td>H₂O</td>
<td>Cleaned and deionized with the</td>
</tr>
<tr>
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<td>System Milli Q Plus, Millipore</td>
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</table>

### 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.
Chapter 2 General Materials and Methods

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

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, $\tau$--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 downstream of UAS (Brand and Perrimon, 1993; Fig. 1).

![GAL4/UAS-System](image)

**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) downstream 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 DN1s and LN2s and in the fifth s-LNv cell by using cry promoter to drive GAL4 and blocking its action in the LNv.s 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 CO2 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 infrared beam for recording as an event (e.g., a hash--density of 25 means that the fly should cross 25 times the infrared 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 \( \chi^2 \) periodogram and autocorrelation analyses (see for example Table 1 and Table 2 in Chapter 6).

2.4.2 \( \chi^2 \) 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 \( \chi^2 \) 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 CO₂ 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 per₀₁ 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?

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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\textsuperscript{P41};cry\textsuperscript{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\textsubscript{v}s, the DN\textsubscript{1}s, (and to a certain extent also in the LN\textsubscript{d}s) 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\textsuperscript{60j} cry\textsuperscript{b} flies. They are disabled in CRY mediated signaling, like in norpA\textsuperscript{P41};cry\textsuperscript{b} flies. The gl\textsuperscript{60j} cry\textsuperscript{b} flies further miss the compound eyes and the ocelli, which are already non--functional in norpA\textsuperscript{P41};cry\textsuperscript{b} flies. But compared to norpA\textsuperscript{P41};cry\textsuperscript{b} flies the gl\textsuperscript{60j} cry\textsuperscript{b} flies additionally miss the Hofbauer--Buchner (H--B) eyelet and the DN\textsubscript{1}s (Helfrich--Förster et al., 2001). [The norpA\textsuperscript{P41} mutation does not remove any photoreceptor structures but only results in phototransduction block whereas gl\textsuperscript{60j} removes photoreceptor structures like compound eyes, ocelli as well as the H--B eyelet and the DN\textsubscript{1}s (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\textsubscript{v}s (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
Chapter 3 The H--B eyelet a circadian photoreceptor

hosting the elusive photoreceptor, responsible for the residual light re--entrainment retained in the norpA<sup>P41</sup>;cry<sup>b</sup> flies.

![Image](image.png)

**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^ kid flies (which lack the compound eyes and ocelli, but retain the H--B eyelet, the DN1s, 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^ kid;gt^ G/j flies showed orthodromic phase shifting (by advancing 6 h the activity rhythm in the same direction of LD shift) but so^ kid 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^ kid;gt^ G/j 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_r s. The Tetanus--Toxin Light Chain (TtxLC) blocks chemical synaptic transmission by cleaving the neuronally expressed synaptobrevin protein (Sweeney 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_r s 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,s, 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).
Figure 2 Genetic cross between Rh 5--GAL4 female (virgin) and UAS--txx male flies to bring together the GAL4 and UAS elements. The Rh 5--GAL4 and UAS--txx 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 $\chi^2$ periodogram analyses (see Table 2).
Chapter 3 The H--B eyelet a circadian photoreceptor

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-LNv's in norpA<sup>P41</sup>;cry<sup>b</sup> flies. norpA<sup>P41</sup>;cry<sup>b</sup> flies can synchronize to a new LD cycle, but need many days to do so (Stanewsky <em>et al</em>., 1998; Emery <em>et al</em>., 2000b; Helfrich--Förster <em>et al</em>., 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 <em>et al</em>., 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 <em>et al</em>., 2002). For expressing tetanus toxin in the H--B eyelet the GAL4/UAS system was applied (Brand and Perrimon, 1993). The Rh5 promoter is active in the H--B eyelet (Malpel <em>et al</em>., 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<sup>P41</sup>;cry<sup>b</sup> flies (hereon called H--B<sup>TD</sup>:norpA<sup>P41</sup>;cry<sup>b</sup>) 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--BTD: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--BTD:norpA^{P41};cry^{b} flies was noticed in 50
lux green light: (Veleri, S. and Helfrich--Fürster, 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--BTD: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 (yw) 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--BTD: 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 yw 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 yw 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<sup>P41</sup>:cry<sup>b</sup> 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 $\tau > 24$ 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 norpAP41; cryb 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 norpAP41; cryb 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: norpAP41; cryb 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 norpAP41 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; cryb flies took 2.9 ± 0.1 days (Fig. 5B and Table 1; Helfrich–Förster et al., 2001) and the non–transgenic norpAP41; cryb flies needed 4.5 ± 0.5 days (Fig. 5B and Table 1). The transgenic norpAP41; UAS–ttx; cryb needed 3.3 ± 0.3 days and norpAP41; Rh 5–GAL4/Cyo; cryb 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 norpAP41; cryb flies they needed on average 3.9 ± 0.2 days for re–entrainment (Table 1). The H–B TD: norpAP41; cryb 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: norpAP41; cryb flies are assumed to be affected in the photic signaling to the LNv,s and the PDF expressing LNv,s 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<sup>b</sup> 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<sup>TD</sup>:norpA<sup>P41</sup>;cry<sup>b</sup> flies were in a norpA<sup>P41</sup>;cry<sup>b</sup> 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<sup>P41</sup>/Y;cry<sup>b</sup> flies on behavioral re--entrainment in 65 lux

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total no. of flies tested (N)</th>
<th>% of flies re--entrained to 6 h phase delay (n)</th>
<th>No. of days needed for re--entrainment ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>y w/Y</td>
<td>39</td>
<td>100 (39)</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>yw/Y;cry&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12</td>
<td>100 (12)</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>norpA&lt;sup&gt;P41&lt;/sup&gt;/Y;cry&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21</td>
<td>48 (10)</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>norpA&lt;sup&gt;P41&lt;/sup&gt;/Y;UAS--ttx;cry&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40</td>
<td>56 (23)</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>norpA&lt;sup&gt;P41&lt;/sup&gt;/Y;Rh 5--GAL4/CyO;cry&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36</td>
<td>36 (13)</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Transgenic and non--transgenic combined:norpA&lt;sup&gt;P41&lt;/sup&gt;/Y;cry&lt;sup&gt;b&lt;/sup&gt;</td>
<td>97</td>
<td>47 (46)</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>norpA&lt;sup&gt;P41&lt;/sup&gt;/Y; Rh 5--GAL4/UAS--ttx;cry&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58</td>
<td>0 (0)</td>
<td>--</td>
</tr>
</tbody>
</table>
### Table 2  Effect of tetanus toxin expression in the H–B eyelet on behavioral rhythms of norpA<sup>P41</sup>/Y;<i>cry</i><sup>b</sup> flies in 65 lux

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LD</th>
<th>Shifted LD regime</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N  (n)</td>
<td>Period ± SEM (h)</td>
<td>% Rhy.</td>
</tr>
<tr>
<td>y w/Y</td>
<td>39 (38)</td>
<td>24.2 ± 0.04</td>
<td>97</td>
</tr>
<tr>
<td>yw/Y;&lt;i&gt;cry&lt;/i&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12 (12)</td>
<td>24.4 ± 0.06</td>
<td>100</td>
</tr>
<tr>
<td>norpA&lt;sup&gt;P41&lt;/sup&gt;/Y;&lt;i&gt;cry&lt;/i&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23 (10)</td>
<td>24.5 ± 0.08</td>
<td>43</td>
</tr>
<tr>
<td>norpA&lt;sup&gt;P41&lt;/sup&gt;/Y; UAS--ttx;&lt;i&gt;cry&lt;/i&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41 (37)</td>
<td>24.2 ± 0.1</td>
<td>90</td>
</tr>
<tr>
<td>norpA&lt;sup&gt;P41&lt;/sup&gt;/Y; Rh 5--GAL4/Cyo;&lt;i&gt;cry&lt;/i&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42 (31)</td>
<td>24.3 ± 0.1</td>
<td>74</td>
</tr>
<tr>
<td>Transgenic and non-transgenic combined:norpA&lt;sup&gt;P41&lt;/sup&gt;/Y;&lt;i&gt;cry&lt;/i&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>106 (78)</td>
<td>24.3 ± 0.03</td>
<td>74</td>
</tr>
<tr>
<td>norpA&lt;sup&gt;P41&lt;/sup&gt;/Y;Rh 5--GAL4/UAS--ttx;&lt;i&gt;cry&lt;/i&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61 (50)</td>
<td>24.1 ± 0.04</td>
<td>82</td>
</tr>
</tbody>
</table>

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 resynchronization (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 *yw* flies showed typical bimodal activity in initial LD, on 3 LS and Lst LS, in shifted new LD regime; in comparison *cry* flies needed more time to resynchronize.

On the day 3 of the new LD regime, the *yw* and *cry* flies had re–synchronized their evening peaks while the *norpA*;*cry* 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*T:*norpA;*cry* 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*T:*norpA;*cry* flies as opposed to the slow re–entrainment observed in *norpA*;*cry* flies (Fig. 4D and Table 2). This is also indicated by the shorter free run period values exhibited by the H–
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\[ \text{B}^{\text{TD}}:\text{norpA}^{\text{P41}};\text{cry}^{b} \text{ flies (Fig. 4D) compared to the transgenic norpA}^{\text{P41}};\text{cry}^{b} \text{ flies (Fig. 4B, C and Table 2). On the day 5 of shifted LD, all the genotypes showed a similar behavior as they had in the day 3 of shifted LD (Fig. 4 and Table 2) indicating that the H--B}^{\text{TD}}:\text{norpA}^{\text{P41}};\text{cry}^{b} \text{ flies could not resynchronize, even after prolonged exposure to the new LD regime.} \]

**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\textsuperscript{TD}:norpA\textsuperscript{P41};cry\textsuperscript{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\textsuperscript{TD}:norpA\textsuperscript{P41};cry\textsuperscript{b} flies to re--entain 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\textsuperscript{P41};UAS--ttx;cry\textsuperscript{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\textsuperscript{P41};UAS--ttx;cry\textsuperscript{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\textsubscript{s} and PER and TIM in the DN\textsubscript{2}s and DN\textsubscript{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\textsubscript{s} and DN\textsubscript{1}s. This discrepancy may owe to the difference in entrainment conditions existed in both experiments. Furthermore, the presence of the w\textsuperscript{+} transgene along with GAL4 might have influenced our result.

In the norpA\textsuperscript{P41};UAS--ttx;cry\textsuperscript{b} flies there was minimum cycling amplitude in the DN\textsubscript{3}s, followed by the DN\textsubscript{2}s (Fig. 9; cf. Helfrich--Förster et al., 2001) and DN\textsubscript{1}s. In lateral neurons, the s--LN\textsubscript{s} and l--LN\textsubscript{s} (only for PER; the l--LN\textsubscript{s} result contrasts Helfrich--Förster et al., 2001) showed high amplitude of cycling but the LN\textsubscript{s}s
showed relatively weaker amplitude oscillations (cf. Helfrich--Förster et al., 2001). Helfrich--Förster et al. (2001) showed almost no PER cycling in the I--LNₜₛ.

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_{s}s 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_{3}s) and small lateral ventral neurons (s--LN_{s}s). Panels F and H show lateral neurons: small and large ventral lateral neurons (s+l--LN_{s}s) 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<sup>P41</sup>;UAS--ttx;cry<sup>B</sup> 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 \( \text{norpA}^{P41};\text{UAS–ttx;cry}^b \) flies (Fig. 6B–column 1 row 1; Fig. 8, Fig. 9).

4.2.1 Synchronization of \textsc{timeless} expression to a 6 h phase delayed LD of 65 lux white light in the H–B\textsuperscript{TD}::\text{norpA}^{P41};\text{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 \( \text{norpA}^{P41};\text{UAS–ttx;cry}^b \) control flies (Fig. 10 upper panels and Fig. 11). This was expected for the DN\textsubscript{2}s, DN\textsubscript{3}s and l–LN\textsubscript{v}s, because TIM expression in these cells was not synchronized in the initial LD cycle (Fig. 9B). But the DN\textsubscript{1}s, s–LN\textsubscript{v}s and LN\textsubscript{2}s 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\textsuperscript{TD}::\text{norpA}^{P41};\text{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 \( \text{norpA}^{P41};\text{cry}^b \) 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_{s}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_{s}s (B), and the LN_{s}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 DN1,2s (E), the s--LN,s and the l--LN,s (F) were strongly stained in comparison to the ZT 22 (G). The LN,s 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\textsuperscript{P41};\textit{Rh 5--GAL4/UAS--ttx;\textit{cry}^b} flies TIM expression shows desynchronization only in the LN\textsubscript{s}s. In the s--LN\textsubscript{s}s and l--LN\textsubscript{s}s 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\textsuperscript{TD}:norpA\textsuperscript{P41};\textit{cry}^b flies indicating that they had kept their original entrained activity phase (Fig. 6B, column 3).

TIM expression in the H--B\textsuperscript{TD}:norpA\textsuperscript{P41};\textit{cry}^b flies kept synchronization with the initial LD regime for 5 days, except in the LN\textsubscript{s}s (Fig. 11; Fig. 12 lower panels; Fig. 13; and Fig. 14 lower panels). The l--LN\textsubscript{s}s 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\textsuperscript{P41};\textit{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\textsuperscript{P41};UAS--ttx;\textit{cry}^b (TTX) control and lower panels show norpA\textsuperscript{P41}; \textit{Rh 5--GAL4/UAS--ttx;\textit{cry}^b} (Rh5/TTX) brains. In the TTX at ZT 10 (A) and ZT 22 (B) the DN\textsubscript{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 DN1+2s, s--LNvs and LNds displayed synchronized TIM expression in line with initial LD cycle in norpA<sup>P41</sup>;Rh 5--GAL4/UAS--ttx;cryb compared to controls. (A) The DN<sub>1+2+3</sub>s showed synchronized TIM expression in y w flies. In norpA<sup>P41</sup>;UAS--ttx;cryb flies the DN<sub>1+2</sub>s showed desynchronized TIM expression. On contrary in norpA<sup>P41</sup>;Rh 5--GAL4/UAS--ttx;cryb flies the TIM expression was synchronized to the initial LD cycles, therefore, more TIM staining at ZT 10, indicates that norpA<sup>P41</sup>;Rh 5--GAL4/UAS--ttx;cryb 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<sup>P41</sup>;UAS--ttx;cryb flies the LNs show desynchronized TIM expression. In norpA<sup>P41</sup>;Rh 5--GAL4/UAS--ttx;cryb flies TIM expression shows desynchronization only in the l--LNvs. In the s--LNvs and LNds 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–LNₜs and LNₜs remained synchronized with the initial LD regime in flies expressed tetanus toxin in the H–B eyelet in a norpA⁣⁺⁴¹;cryᵇ background. Upper panels show norpA⁣⁺⁴¹;UAS–txx;cryᵇ (TTX) control and lower panels show the norpA⁣⁺⁴¹;Rh 5–GAL4/UAS–txx;cryᵇ (Rh5/TTX) brains. In the TTX at ZT 10 (A) the I–LNₜs
were more strongly stained compared to ZT 22 (C), whereas the s--LN,s and LN,s stained strongly at both time points (A, B, C, D). In Rh5/TTX flies the s--LN,s and LN,s stained more strongly at ZT 10 (E, F) compared to ZT 22 (G), whereas the l--LN,s 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,s (Yang and Sehgal, 2001; Shafer et al., 2002; Veleri et al., 2003). The TIM expression was desynchronized in the LN,s on the day 3 of shifted LD regime; however, on the day 5 they showed synchronization with initial LD, similar to the s--LN,s. Possibly the LN,s are entrained by the s--LN,s 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--BTD: 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--BTD: 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 yw control flies. In the norpA^{P41};UAS--tx;cry^{b} flies the synchronization was less pronounced in some subsets of clock neurons compared to the yw flies (Fig. 15 upper panels and Fig. 16). In initial LD cycles, the DN_{2+3}s and the LN,d,s showed less synchronization (Fig. 9A). Thus, the s--LN,s and l--LN,s mainly showed a decrement in
synchronization compared to the initial LD regime. In the H–B TD: norpA^{P41}; cry^{b} flies the DN_{1}s and I–LN_{s} 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_{1}s and I–LN_{s}. 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
DN1s (D) and I–LNvs (E) were strongly stained. Weak staining was also seen in the LNds (E) at ZT 10 whereas the s–LNvs and LNvs showed stronger staining at ZT 22 (G and H).

Figure 16 Quantification of anti–PER staining in dorsal (DN1–3s) and lateral neurons (LNs) on the day 3 of the shifted new LD regime. (A) The DN1s show perturbed PER synchronization in norpA

\( ^{P41} \); Rh 5–GAL4/UAS–ttx;cryb compared to the controls. (B) The I-

-LNvs also show perturbed PER synchronization in norpA

\( ^{P41} \); Rh 5–GAL4/UAS–ttx;cryb compared to the controls.

s–LNvs and LNvs were synchronized as in control flies (Fig. 15 lower panels and Fig. 16). The DN2s and DN3s are also not synchronized in the controls in the initial LD regime. This indicates that in the DN1s and I–LNvs PER synchronization is affected on day 3 in the H–B

\( ^{TD} \); norpA

\( ^{P41} \); cryb 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 \textit{norp}A^{P41}; UAS--\textit{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--BTD: \textit{norp}A^{P41}; cry^b flies, like in the day 3, the DNs were still not synchronized with the new LD but the s--LN_s, I--LN_s and l--LN_s were synchronized (Fig. 17 lower panels and Fig. 18) indicating that the l--LN_s receive 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--BTD: \textit{norp}A^{P41}; cry^b flies compared to the \textit{norp}A^{P41}; cry^b flies. At ZT 22 (corresponding to ZT 4 in initial LD) PER expression was more compared to ZT 10

\textbf{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 \textit{norp}A^{P41}; cry^b double mutants lost synchronization of PER expression to the new LD regime in the DN_1s. Upper panels show \textit{norp}A^{P41}; UAS--\textit{ttx}; cry^b (TTX) control and lower panels show the \textit{norp}A^{P41}; Rh 5--GAL4/UAS--\textit{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).
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(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

![Graph A](image)

![Graph B](image)

**Figure 18** Quantification of anti--PER staining in dorsal neurons (DN 1--3s) and lateral neurons (LN) on the day 5 of shifted LD cycle. The DN 1s show non--synchronized PER expression in norpA<sup>P41</sup>;Rh 5--GAL4/UAS--ttx;cry<sup>b</sup> flies compared to control flies (A); whereas synchronization of PER expression in the l--LN<sub>s</sub> 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 CRYB to PER might be allowing PER to
resynchronize to new LD regime in the H--B TD: norpA\textsuperscript{P41};cry\textsuperscript{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\textsuperscript{P41};cry\textsuperscript{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\textsubscript{s}, DN\textsubscript{1}s and LN\textsubscript{0}s (Helfrich--Förster
et al., 2001), which suggested the existence of an additional extra--retinal
photoreceptor. g\textsuperscript{60j} cry\textsuperscript{b} flies lacked the residual behavioral and molecular
synchronization mentioned above. The norpA\textsuperscript{P41};cry\textsuperscript{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 g\textsuperscript{60j} flies are morphologically
defective in the compound eyes, ocelli and in addition the H--B eyelet and the
DN\textsubscript{1}s are depleted. Therefore, g\textsuperscript{60j} cry\textsuperscript{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\textsubscript{s} (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\textsubscript{s} 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\textsubscript{s}. 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 LNₜₛ are tetanus toxin sensitive

The Tetanus Toxin Light Chain (TtxLC) 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--LNₜₛ to the DNₜₛ 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--LNₜₛ as well as the behavioral resynchronization in dim light. Therefore, the synaptic connections from the H--B eyelet to the s--LNₜₛ 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 *norpA⁴¹, cry⁷* flies could re--entrain to a new LD regime but not the *gl⁶⁰, cry⁷* flies, which lacked the H--B eyelet and DN₁ₛ along with the compound eyes and ocelli.
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(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 Meinertz-Hagen, 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_s 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 retrospect, 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\(_{i}\)s (Hofbauer and Buchner, 1989; Yasuyama and Meinertzhagen, 1999). The s--LN\(_{i}\)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\(_{i}\)s (Fig. 14 lower panels; and Fig. 13B).
Besides in the s–LNvs TIM expression remained synchronized to the initial LD regime in the l–LNvs and the DN1s in the H--B TD:norPA41;cryb flies (Fig. 10 lower panels; Fig. 11B; Fig. 12; and Fig. 14), both on the day 3 and 5 (except for the l–LNvs) of the new shifted LD regime. By day 5 the l–LNvs might be nonsynchronized in the absence of any entraining signals from the s–LNvs, as they behave in DD (Veleri et al., 2003; Yang and Sehgal, 2001). It was surprising to see that TIM was affected in the DN1s in the H--B TD:norPA41;cryb flies because no connection between the H--B eyelet and the DN1s was shown yet. Then how could the H--B eyelet influence the TIM expression in the DN1s? Possibly, in an indirect route from the H--B eyelet via the s--LNvs to the DN1s. The DN1s project axons towards the s--LNvs and the s--LNvs 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 DN1s could affect the behavioral rhythm. Interestingly the s--LNvs and DN1s were the only neurons that retained synchronized molecular cycling in LD of 100 or 1000 lux in the norPA41;cryb 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--LNvs and the DN1s.

A role for the LNvs 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 LNvs might also have contributed to the free running behavior seen in the H--B TD:norPA41;cryb flies because the LNvs showed similar synchronization as in the s--LNvs (Fig. 13B).

Surprisingly, in the H--B TD:norPA41;cryb flies PER expression was in synchronization with the new shifted external LD in LNvs (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 CRYB 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, s and in the DN, s (Fig. 13) of the H-B\textsuperscript{TD}:\textit{norpA}\textsuperscript{p41};\textit{cry}\textsuperscript{b} flies. TIM is regarded as one of the crucial clock components in the \textit{Drosophila} circadian clock (Stanewsky, 2002), and it responds immediately to light signals, indicating that TIM mediates photic resetting of the circadian clock in \textit{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\textsuperscript{TD}:\textit{norpA}\textsuperscript{p41};\textit{cry}\textsuperscript{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 \textit{norpA} encoded PLC?

The \textit{norpA}\textsuperscript{p41};\textit{cry}\textsuperscript{b} flies are capable of entraining to LD cycles. They have the H-B eyelet intact, which expresses \textit{Rh 5} and \textit{Rh 6} (Yasuyama and Meinertzhagen, 1999; Malpel et al., 2002). It is believed that \textit{Rh 5} in R 8 cells utilizes the PLC (Montell, 1999). Is it possible for the H-B eyelet to use this \textit{Rh 5} (may be \textit{Rh 6} also) to signal the photic information bypassing \textit{norpA} encoded PLC?

Possibly, extra-retinal rhodopsins utilize an alternative signaling route. However, \textit{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 (Gärtnner, 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 outside the compound eyes might be using a different transduction machinery which may not be requiring the PLC encoded by the norpA gene. Therefore, in norpAP41;cryP 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 CRyB could be present in norpAP41;cryb 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 LNvs 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--BTD:norpAP41;cryb 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 norpAP41;cryb 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

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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--LNv,s) 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 (DN3,s and DN2,s) and in the s--LNv,s 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--LNv,s as a molecular pacemaker oscillator (Veleri *et al*., 2003).
2 Materials

2.1 Flies

disco² (Steller et al., 1987).

Pdf⁰¹ (Renn et al., 1999)

Pdf⁰¹ used in this study was in a y w background, hereon called Pdf⁰¹.

per⁰¹ 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™-96, Packard) with fly food (100 µl solution of 1% agar, 5% sucrose and 15 mM luciferin [Biosynth], dissolved in dH₂O water). After the food was solidified, flies anaesthetized by CO₂ 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’ ($\tau$), ‘relative amplitude error’ (rel--amp), and the average CT peak time of expression (phase). Rhythmic flies (\% rhythmic) had $\tau$ 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--LNv,s or the l--LNv,s or in the LNd,s 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\textsubscript{s} and it enabled to study these neurons (Grima \textit{et al.}, 2004). In another

![Diagram](image)

**Figure 1** Exon/Intron structure of the \textit{period} locus and transgenic fragments of the \textit{period} gene with or without reporter fusion genes. Upper most panel: structure of the 13.2 kb genomic DNA fragment containing the \textit{per} gene (Veleri \textit{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 \textit{per} constructs with or without reporter genes (\textit{lacZ} or \textit{luciferase}) is shown. In our study we have used the \textit{luciferase} reporter gene fused to a promoterless 8.0 kb \textit{per} gene fragment (bottom construct).

‘subtracting’ approach Stoleru \textit{et al.} (2004) showed how to utilize GAL\textit{80} (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 \textit{per\textsuperscript{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 \textit{et al.}, 2004; Stoleru \textit{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 DN1s, DN2s, DN3s (and rarely in the LN4s) 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-LNVs) maintain sustained expression of PER oscillations after 5 days in DD. In addition, we showed that the DN2s and DN3s 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 *g^{60} 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 *norpAP^{41};cry^b* flies. Furthermore, we show a putative *Rh 7* gene expresses in the dorsal brain very close to the DN1+2s (see in Chapter 5). Possibly there exist neuronal connections between the DN1+2s and the cells expressing *Rh 7*. Therefore, *Rh 7* could be the missing photopigment responsible for light entrainment of the DNs in *norpAP^{41};cry^b* double mutant flies. This novel putative photoreceptor may be functioning in the high intensity light in *norpAP^{41};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.
A Self-Sustaining, Light-Entrainable Circadian Oscillator in the Drosophila Brain

Shobi Veleri,1 Christian Brandes,2 Charlotte Helfrich-Förster,1 Jeffrey C. Hall,2 and Ralf Stanewsky1,*

1Universität Regensburg
Institut für Zoologie
Lehrstuhl für Entwicklungsbiologie
93040 Regensburg
Germany
2Department of Biology
Brandeis University
Waltham, Massachusetts 02454

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 damping 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 damping 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 damping 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...
7.2. per luc:9 construct. Frisch et al. [10] had shown that expression of the period-luciferase fusion gene, which lacks 5'-flanking regulatory 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, or 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 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 period 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 per01 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...
behavioral rhythms in per\textsuperscript{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\textsuperscript{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 rhythm to per\textsuperscript{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\textsuperscript{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\textsuperscript{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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Percent Rhythmic</th>
<th>τ (hr)</th>
<th>rel-amp</th>
<th>Phase (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XLG-luc:1</td>
<td>51</td>
<td>43.1</td>
<td>22.7 ± 0.4</td>
<td>0.50 ± 0.02</td>
<td>23.6 ± 1.0</td>
</tr>
<tr>
<td>discoXLG-luc:1</td>
<td>44</td>
<td>75.0</td>
<td>23.2 ± 0.1</td>
<td>0.53 ± 0.02</td>
<td>23.1 ± 0.5</td>
</tr>
<tr>
<td>perδXLG-luc:1</td>
<td>47</td>
<td>21.3</td>
<td>21.5 ± 0.4</td>
<td>0.61 ± 0.03</td>
<td>2.6 ± 1.7</td>
</tr>
<tr>
<td>XLG-luc:2</td>
<td>43</td>
<td>93.0</td>
<td>22.6 ± 0.1</td>
<td>0.48 ± 0.02</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>discoXLG-luc:2</td>
<td>33</td>
<td>84.8</td>
<td>23.0 ± 0.1</td>
<td>0.45 ± 0.02</td>
<td>22.9 ± 0.5</td>
</tr>
<tr>
<td>8.0-luc:9</td>
<td>110</td>
<td>85.5</td>
<td>23.1 ± 0.1</td>
<td>0.41 ± 0.01</td>
<td>23.4 ± 0.3</td>
</tr>
<tr>
<td>disco8.0-luc:9</td>
<td>407</td>
<td>74.7</td>
<td>23.5 ± 0.1</td>
<td>0.38 ± 0.01</td>
<td>23.3 ± 0.3</td>
</tr>
<tr>
<td>perδ8.0-luc:9</td>
<td>69</td>
<td>24.6</td>
<td>24.6 ± 0.5</td>
<td>0.59 ± 0.02</td>
<td>23.5 ± 1.2</td>
</tr>
<tr>
<td>8.0-luc:9;gl60j</td>
<td>81</td>
<td>91.4</td>
<td>22.2 ± 0.1</td>
<td>0.43 ± 0.01</td>
<td>23.1 ± 0.2</td>
</tr>
<tr>
<td>8.0-luc:9;cryb</td>
<td>56</td>
<td>51.8</td>
<td>22.5 ± 0.2</td>
<td>0.49 ± 0.02</td>
<td>1.0 ± 1.6</td>
</tr>
<tr>
<td>8.0-luc:9;gl60j cryb</td>
<td>48</td>
<td>43.8</td>
<td>22.7 ± 0.3</td>
<td>0.54 ± 0.02</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>8.0-luc:11</td>
<td>92</td>
<td>37.0</td>
<td>24.3 ± 0.2</td>
<td>0.56 ± 0.02</td>
<td>23.6 ± 0.7</td>
</tr>
<tr>
<td>perδ8.0-luc:11</td>
<td>33</td>
<td>6.1</td>
<td>25.3 ± 0.5</td>
<td>0.57 ± 0.11</td>
<td>21.0 ± 0.7</td>
</tr>
</tbody>
</table>

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 perδ;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 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\textsuperscript{2}; 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-LN\textsubscript{v}, t-LN\textsubscript{v}, and LN\textsubscript{d}) except DN2 cells, which were not stained in this particular brain. Note that only the two LN\textsubscript{v} groups coexpress XLG-luc and PDF.

(C–K) per\textsuperscript{2}; 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 LN\textsubscript{d} (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-LN\textsubscript{v}, LN\textsubscript{d}, and DN2, cells as well as within the DN3 cluster, but not in the t-LN\textsubscript{v} and DN1 cells (Figures 5 and 6, Table S2; cf. [12, 13]). This indicates that PER expression cycles in the s-LN\textsubscript{v} and LN\textsubscript{d} 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 “anti-phase” 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 DN 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\textsuperscript{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\textsuperscript{60j} flies carrying a tim-gal4 and UAS-gfp transgene [20].
Whole-mounted brains were inspected for GFP expression, and robust signals were detected in all known clock neurons (Figure S1). In the glass60 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 glass60 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;glass60 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 glass60 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;cryb 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 glass60 was combined with...
we demonstrated that a true circadian oscillator is lost under 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 cycling 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 neuromodulator 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 cycling 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 cycling—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\(^{8.0-luc}9\) 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 LNv or PER expression therein. Interestingly, all genetic variants in which the LNv 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\(^{8.0}\) mutant, indicating that the deleted photoreceptors and neurons are not required for light input into the DN3 cell group. Applying the cry\(^{+}\) 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\(^{8.0}\) cry\(^{+}\) 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 (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\(^{8.0}\) cry\(^{+}\) 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 LN 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\(^{8.0}\) and tim\(^{+}\) 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 per-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\(^+\) mutant is described in [21], and per\(^{+}\) and glass\(^{8.0}\) 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\(^{8.0}\) cry\(^{+}\) 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 transgensics, 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 22 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 [14]. Generally, flies were entrained for one day in 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 rabbit anti-TIM [17] or rabbit anti-PER [14] at 1:1000 dilution. The anti-PDF stainings 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. Numbers of stained neurons were determined with a high-magnification lens (further zoomed digitally 3×).

Acknowledgments

We thank Alois Hofbauer for comments on the manuscript and the nb33 antibody, Michael Rosbash for TIM antibodies, Christine Winter for germline transformation of the 8.0-luc construct, and Johannes Landskron for mapping the Drosophila nervous system. Neuron 12, 555–570. 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). 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.

Behavior

Locomotor activity of adult males was monitored automatically and analyzed as described [14]. Generally, 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 rabbit anti-TIM [17] or rabbit anti-PER [14] at 1:1000 dilution. The anti-PDF stainings 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. Numbers of stained neurons were determined with a high-magnification lens (further zoomed digitally 3×).

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References


Supplemental Data

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

Shobi Veleri, Christian Brandes, Charlotte Helfrich-Förster, Jeffrey C. Hall, and Ralf Stanewsky

**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/NcoI *per* subclone, which extended from +5449 to +6658 (+1 = transcription start) and in which the NcoI site was changed to an XhoI site via the Klenow fill-in reaction at the NcoI 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 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 the 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 CG19093, 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 χ² periodogram analysis (α = 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* 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* 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**


Whole-mounted brains from “photoreceptor normal” or glass60j 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). glass60j 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 glass60j flies, yet the number of projections from the dorsal brain toward the LNV cluster seems not to be significantly reduced in comparison with glass+/H11001 (arrows).

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Percent Rhythmic</th>
<th>( \tau ) (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>y w</td>
<td>29</td>
<td>90</td>
<td>23.8 ± 0.1</td>
</tr>
<tr>
<td>per1;</td>
<td></td>
<td></td>
<td>33.4 ± 5.4</td>
</tr>
<tr>
<td>per1; XLG-luc:1</td>
<td>64</td>
<td>47</td>
<td>23.2 ± 0.1</td>
</tr>
<tr>
<td>per1; XLG-luc:2</td>
<td>49</td>
<td>53</td>
<td>24.0 ± 0.1</td>
</tr>
<tr>
<td>per1; 8.0-luc:9</td>
<td>48</td>
<td>2</td>
<td>28.6</td>
</tr>
<tr>
<td>disco; 8.0-luc:9</td>
<td>35</td>
<td>9</td>
<td>25.7 ± 1.4</td>
</tr>
</tbody>
</table>

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 \( \chi^2 \) periodogram; only flies showing periods in combination with a “power” value ≥10 and a time bin “width” ≥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

<table>
<thead>
<tr>
<th>Genotype (time point)</th>
<th>DN1</th>
<th>DN2</th>
<th>DN3</th>
<th>s-LNv</th>
<th>l-LNv</th>
<th>LNd</th>
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<tr>
<td><strong>yw (ZT23)</strong></td>
<td>9.7 ± 0.6</td>
<td>0.8 ± 0.2</td>
<td>9.7 ± 0.7</td>
<td>3.2 ± 0.3</td>
<td>3.4 ± 0.3</td>
<td>5.3 ± 0.5</td>
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<tr>
<td></td>
<td>(29/30)</td>
<td>(12/30)</td>
<td>(29/30)</td>
<td>(28/30)</td>
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<td>(27/30)</td>
</tr>
<tr>
<td><strong>per</strong>;XLG-luc:1 (ZT23)</td>
<td>3.8 ± 0.5</td>
<td>0.9 ± 0.3</td>
<td>2.4 ± 0.7</td>
<td>1.4 ± 0.5</td>
<td>3.4 ± 0.5</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>(14/16)</td>
<td>(8/16)</td>
<td>(8/16)</td>
<td>(7/16)</td>
<td>(14/16)</td>
<td>(14/16)</td>
</tr>
<tr>
<td>8.0-luc:9 (ZT23)</td>
<td>7.8 ± 0.9</td>
<td>1.9 ± 0.1</td>
<td>9.6 ± 1.1</td>
<td>4.5 ± 0.5</td>
<td>5.9 ± 0.6</td>
<td>2.7 ± 0.6</td>
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<tr>
<td></td>
<td>(14/14)</td>
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<td>(14/14)</td>
<td>(15/15)</td>
<td>(15/15)</td>
<td>(9/19)</td>
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<tr>
<td>8.0-luc:9 (ZT11)</td>
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<td>0</td>
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<td>(0/14)</td>
<td>(0/14)</td>
<td>(0/14)</td>
<td>(0/14)</td>
</tr>
<tr>
<td><strong>per</strong>;8.0-luc:9 (ZT23)</td>
<td>3.4 ± 1.0</td>
<td>0.6 ± 0.3</td>
<td>5.1 ± 0.5</td>
<td>0.3 ± 0.3</td>
<td>0.64 ± 0.5</td>
<td>1.8 ± 0.8</td>
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<tr>
<td></td>
<td>(9/14)</td>
<td>(4/14)</td>
<td>(14/14)</td>
<td>(1/14)</td>
<td>(2/14)</td>
<td>(5/14)</td>
</tr>
<tr>
<td><strong>yw (CT 23)</strong></td>
<td>4.2 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>4.2 ± 0.5</td>
<td>2.6 ± 0.2</td>
<td>2.3 ± 0.3</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
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<td>(32/44)</td>
<td>(37/44)</td>
<td>(30/44)</td>
<td>(33/44)</td>
</tr>
<tr>
<td><strong>yw (CT11)</strong></td>
<td>3.1 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>2.1 ± 0.3</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>(28/33)</td>
<td>(18/33)</td>
<td>(4/33)</td>
<td>(2/33)</td>
<td>(20/33)</td>
<td>(12/33)</td>
</tr>
<tr>
<td><strong>disco</strong> (CT21)</td>
<td>3.6 ± 0.7</td>
<td>0.2 ± 0.2</td>
<td>3.2 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>4.0^*</td>
</tr>
<tr>
<td></td>
<td>(13/18)</td>
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<td>(12/18)</td>
<td>(0/18)</td>
<td>(0/18)</td>
<td>(1/18)</td>
</tr>
<tr>
<td><strong>disco</strong> (CT23)</td>
<td>4.5 ± 0.7</td>
<td>0.1</td>
<td>5.3 ± 0.8</td>
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<tr>
<td></td>
<td>(16/20)</td>
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<td>(0/20)</td>
</tr>
<tr>
<td><strong>disco</strong> (CT9)</td>
<td>6.0 ± 0.7</td>
<td>1.6 ± 0.2</td>
<td>0.3 ± 0.2</td>
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<tr>
<td></td>
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<td>(17/21)</td>
<td>(4/21)</td>
<td>(0/21)</td>
<td>(0/21)</td>
<td>(0/21)</td>
</tr>
<tr>
<td><strong>disco</strong> (CT11)</td>
<td>5.1 ± 0.6</td>
<td>1.6 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(19/21)</td>
<td>(17/21)</td>
<td>(3/21)</td>
<td>(0/21)</td>
<td>(0/21)</td>
<td>(0/21)</td>
</tr>
</tbody>
</table>

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$_v$s and all the l--LN$_v$s express PDF (Helfrich--Förster, 1995; Kaneko and Hall, 2000; Stoleru et al., 2004); and the s--LN$_v$s axonal terminals show rhythmic accumulation of PDF (Helfrich--Förster, 2003). The DNs and LN$_d$s do not express PDF (Helfrich-Förster, 2003). However, a direct PDF--mediated signaling from the s--LN$_v$s 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$_v$s (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$_v$s 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$_v$s and in LN$_d$s of Pdf$^{01}$ mutant flies in DD...
Figure 2 Bioluminescence rhythm records from 8.0–luc:9 transgenic flies in y w, disco2 and Pdf01 genetic backgrounds. 8.0–luc:9 transgenic flies in the above genetic backgrounds showed a non-dampening bioluminescence (disco2 had an improved performance than the y w flies). In the Pdf01 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,s and LN ds 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 ds
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 Pdf01 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–LNvs, LN4s, DN2s and DN3s but not in the l–LNvs and DN1s (Veleri et al., 2003). PER cycling in the s–LNvs 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 DN2s PER expression was cycling in anti-phase with the s–LNvs and DN3s (Veleri et al., 2003). Similarly, the larval DN2s 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 DN2s 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 DN2s is a default state of it in the absence of entraining LD cycles. It is possible that the larval DN2s 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 DN2s (Klarsfeld et al., 2004). Artificial expression of cry in the larval DN2s resulted in the phase reversal of PER expression rhythm in synchrony with the s–LNvs and DN3s. It is suggested that the DN2s 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 DN2s resume their default state (Veleri et al., 2003).
In contrast to the constant levels of PER expression in the l--LNv,s and DN1,s, in DD (Veleri et al., 2003), *tim* mRNA expression was reported to cycle still on the day 8 of DD in the l--LNv,s and DN1,s, 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--LNv,s “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*\(^D1\) and *disco*\(^2\) backgrounds they showed only 3% and 29% rhythmicity, respectively (Table 1). In *per*\(^+\) and *per*\(^D1\) 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$_1$s (see in

![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.](image)

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$^{D1}$ 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$_1$s and DN$_3$s, and in 1--2 LN$_d$s, the 8.0--luc:9 transgene expressed similarly in the DN$_1$s and DN$_3$s and also in DN$_2$s (4 hemispheres) and in LN$_d$s (5 hemispheres) (Table 3). The transgene expressed in the l--LN$_v$s of only one brain hemisphere out of nine tested flies and none in the s--LN$_v$s (Table 3). The absence of 8.0--luc:2 transgene's expression in the s--LN$_v$s correlated with the inability to rescue the behavioral rhythm in DD, in a per$^{D1}$ background (Table 2). The expression of 8.0--luc:2 transgene in the DNs and
LN_δs only correlated with anticipation of the light to dark transition in behavioral rhythms of these transgenic flies in LD in a \( \text{per}^{01} \) background (Table 2) (cf., Grima et al., 2004; Stoleru et al., 2005).

### 4.2.2 Transgenic lines 8.0--\text{luc}:4 and 8.0--\text{luc}:11

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

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**Figure 3B, C** Bioluminescence rhythm records from different 8.0--\text{luc} transgenic fly lines in \( y\ w \) and \( \text{disco}^2 \) genetic backgrounds in DD. (B) 8.0--\text{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\textsuperscript{2} genetic background the rhythm dampened off even quicker. (C) 8.0--luc:11 transgenic flies both in y w and disco\textsuperscript{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.
	ransgenic flies indicating a broader expression pattern. In the per\textsuperscript{4} 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\textsuperscript{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\textsuperscript{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\textsubscript{3}s showed expression (Table 3). Like in the 8.0--luc:2 transgenic line, lack of transgene expression in the s--LN\textsubscript{v}s 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\textsubscript{3}s only correlated with the presence of an E peak and absence of a M peak in behavioral rhythms, in LD in a per\textsuperscript{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\textsubscript{3}s (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\textsubscript{1}s and DN\textsubscript{3}s (Table 3). The DN\textsubscript{1}s contain a dampened molecular oscillator (Veleri et al., 2003). Therefore,
expression of the transgene simultaneously in the DN1s and DN3s 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 DN1s than in the DN3s (Table 3). The transgene also expressed in few of the I--LNvs, another dampened molecular oscillator; and in the LN$s$ (Table 3). Therefore, this transgenic line showed dampened molecular oscillation too. In 8.0--luc:11 flies, the transgene expressed almost equally in the DN1s and in the DN3s, and very little in the I--LNvs 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 DN1s and in the DN3s 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 DN3s 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$s$.

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).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N (n)</th>
<th>% rhythmic</th>
<th>Bioluminescence expression level (CPS)</th>
<th>period ± SEM (h)</th>
<th>Phase ± SEM (h)</th>
<th>Rel-amp ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0–luc:2/TM3</td>
<td>65 (56)</td>
<td>85</td>
<td>52.4 ± 2.0</td>
<td>23.3 ± 0.1</td>
<td>23.5 ± 0.3</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>per^D1/Y;8.0–luc:2/TM3</td>
<td>38 (1)</td>
<td>3</td>
<td>63.8</td>
<td>25.9</td>
<td>6.1</td>
<td>0.51</td>
</tr>
<tr>
<td>disco2/Y;8.0–uc:2/+</td>
<td>41 (12)</td>
<td>29</td>
<td>36.5 ± 1.2</td>
<td>22.8 ± 0.5</td>
<td>24.0 ± 1.6</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>8.0–luc:4</td>
<td>67 (35)</td>
<td>52</td>
<td>4345 ± 131</td>
<td>23.9 ± 0.1</td>
<td>1.6 ± 0.4</td>
<td>0.55 ± 0.02</td>
</tr>
<tr>
<td>per^D1/Y;8.0–luc:4</td>
<td>52 (0)</td>
<td>0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>disco2/Y;8.0–uc:4/+</td>
<td>121 (18)</td>
<td>15</td>
<td>4223 ± 187</td>
<td>24.4 ± 0.4</td>
<td>22.9 ± 1.0</td>
<td>0.61 ± 0.02</td>
</tr>
<tr>
<td>8.0–luc:9</td>
<td>110 (94)</td>
<td>85</td>
<td>29.4 ± 1.8</td>
<td>23.1 ± 0.1</td>
<td>23.4 ± 0.3</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>per^D1/Y;8.0–luc:9/+</td>
<td>69 (17)</td>
<td>25</td>
<td>96.9 ± 1.9</td>
<td>24.6 ± 0.5</td>
<td>23.5 ± 1.2</td>
<td>0.59 ± 0.02</td>
</tr>
<tr>
<td>disco2/Y;8.0–uc:9/+</td>
<td>407 (304)</td>
<td>75</td>
<td>52.9 ± 3.5</td>
<td>23.5 ± 0.1</td>
<td>23.3 ± 0.3</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>8.0–luc:9;Pdf01</td>
<td>28 (24)</td>
<td>86</td>
<td>23.4 ± 0.6</td>
<td>22.7 ± 0.1</td>
<td>23.3 ± 0.4</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>8.0–luc:11/CyO</td>
<td>92 (34)</td>
<td>37</td>
<td>702 ± 32</td>
<td>24.3 ± 0.2</td>
<td>23.6 ± 0.7</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td>per^D1/Y;8.0–uc:11/CyO</td>
<td>33 (2)</td>
<td>6</td>
<td>233</td>
<td>25.3</td>
<td>21.0</td>
<td>0.57</td>
</tr>
<tr>
<td>disco2/Y;8.0–luc:11/+</td>
<td>40 (0)</td>
<td>0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
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 per01 background, mostly all the transgenic flies were arrhythmic. In disco2 and Pdf01 backgrounds, 8.0--luc:9 transgenic flies showed robust rhythmicity (in fact, in disco2 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 ± 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LD</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Genotype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>y w/Y</em></td>
<td>37</td>
<td>34</td>
</tr>
<tr>
<td><em>per^{01}/Y</em></td>
<td>34</td>
<td>21</td>
</tr>
<tr>
<td><em>disco^2/Y</em></td>
<td>34</td>
<td>26</td>
</tr>
<tr>
<td>8.0--*luc:2/TM3</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>*per^{01}/Y;8.0--luc:2/TM3</td>
<td>37</td>
<td>28</td>
</tr>
<tr>
<td>*per^{01}/Y;8.0--luc:2/+</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>8.0--<em>luc:4</em></td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>8.0--*luc:4/CyO</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>*per^{01}/Y;8.0--luc:4</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>*per^{01}/Y;8.0--luc:4/CyO</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>8.0--<em>luc:9</em></td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>8.0--*luc:9/CyO</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>*per^{01}/Y;8.0--luc:9</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>n</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td>per&lt;sup&gt;01&lt;/sup&gt;/Y;8.0--luc:9/CyO</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>disco&lt;sup&gt;2&lt;/sup&gt;/Y;8.0--luc:9</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>disco&lt;sup&gt;2&lt;/sup&gt;/Y;8.0--luc:9/+</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>8.0--luc:11/CyO</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>per&lt;sup&gt;01&lt;/sup&gt;/Y;8.0--luc:11/CyO</td>
<td>43</td>
<td>42</td>
</tr>
<tr>
<td>per&lt;sup&gt;01&lt;/sup&gt;/Y;8.0--luc:11/+</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>disco&lt;sup&gt;2&lt;/sup&gt;/Y;8.0--luc:11/+</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

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 $\chi^2$ 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 (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<sup>01</sup> background, in the LN<sub>s</sub> correlated with the inability to show the M, and the expression of per gene in the DNs or the LN<sub>d</sub>s 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DN₁s</th>
<th>DN₂s</th>
<th>DN₃s</th>
<th>s--LNₙs</th>
<th>l--LNₙs</th>
<th>LNₙs</th>
</tr>
</thead>
<tbody>
<tr>
<td>y w/Y</td>
<td>9.67 ± 0.63*</td>
<td>0.80 ± 0.18</td>
<td>9.73 ± 0.69</td>
<td>3.2 ± 0.25</td>
<td>3.37 ± 0.33</td>
<td>5.3 ± 0.48</td>
</tr>
<tr>
<td>ZT 23 N = (15/15)</td>
<td>(29/30)§</td>
<td>(12/30)</td>
<td>(29/30)</td>
<td>(28/30)</td>
<td>(28/30)</td>
<td>(27/30)</td>
</tr>
<tr>
<td>*per⁰¹;XLG--luc:1--1</td>
<td>3.75 ± 0.45</td>
<td>0.94 ± 0.25</td>
<td>2.38 ± 0.65</td>
<td>1.44 ± 0.45</td>
<td>3.38 ± 0.46</td>
<td>4.38 ± 0.52</td>
</tr>
<tr>
<td>ZT 23 N = (8/8)</td>
<td>(14/16)</td>
<td>(8/16)</td>
<td>(8/16)</td>
<td>(7/16)</td>
<td>(14/16)</td>
<td>(14/16)</td>
</tr>
<tr>
<td>*per⁰¹;8.0--luc:2/TM6B</td>
<td>2.89 ± 0.49</td>
<td>0</td>
<td>4.44 ± 0.29</td>
<td>0</td>
<td>0.22</td>
<td>1.72 ± 0.44</td>
</tr>
<tr>
<td>ZT 23 N = (9/9)</td>
<td>(13/18)</td>
<td>(0/18)</td>
<td>(18/18)</td>
<td>(0/18)</td>
<td>(1/18)</td>
<td>(9/18)</td>
</tr>
<tr>
<td>*per⁰¹;8.0--luc:4</td>
<td>6.5 ± 0.65</td>
<td>0.123 ± 0.13</td>
<td>5.69 ± 0.87</td>
<td>0</td>
<td>0.94 ± 0.43</td>
<td>3.75 ± 0.53</td>
</tr>
<tr>
<td>ZT 23 N = (8/8)</td>
<td>(15/16)</td>
<td>(1/16)</td>
<td>(13/16)</td>
<td>(0/16)</td>
<td>(4/16)</td>
<td>(13/16)</td>
</tr>
<tr>
<td>*per⁰¹;8.0--luc:9D</td>
<td>3.36 ± 0.97</td>
<td>0.57 ± 0.25</td>
<td>5.07 ± 0.46</td>
<td>0.29 ± 0.29</td>
<td>0.64 ± 0.46</td>
<td>1.79 ± 0.76</td>
</tr>
<tr>
<td>ZT 23 N = (7/21)</td>
<td>(9/14)</td>
<td>(4/14)</td>
<td>(14/14)</td>
<td>(1/14)</td>
<td>(2/14)</td>
<td>(5/14)</td>
</tr>
<tr>
<td>*per⁰¹;8.0--luc:11/CyO</td>
<td>5.13 ± 0.46</td>
<td>0</td>
<td>4.69 ± 0.66</td>
<td>0</td>
<td>0.5</td>
<td>4.38 ± 0.57</td>
</tr>
<tr>
<td>ZT 23 N = (8/8)</td>
<td>(15/16)</td>
<td>(0/16)</td>
<td>(14/16)</td>
<td>(0/16)</td>
<td>(2/16)</td>
<td>(14/16)</td>
</tr>
</tbody>
</table>

* 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 per01 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-LNvs 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 LNv's); 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--LNv's. 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 LNv's 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\(_c\)s, which could be correlated with the partial behavioral
rescue in DD in a $per^{D1}$ 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 $per^{D1}$ 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 $per^{D1}$ 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N (n)</th>
<th>percent rhythmic</th>
<th>Bioluminescence expression level (CPS)</th>
<th>(\tau \pm \text{SEM}) (h)</th>
<th>phase (\pm \text{SEM}) (h)</th>
<th>rel--amp (\pm \text{SEM})</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0--luc:2--1</td>
<td>6 (5)</td>
<td>83</td>
<td>49.9 (\pm 8.5)</td>
<td>23.9 (\pm 0.3)</td>
<td>5.8 (\pm 4.3)</td>
<td>0.50 (\pm 0.05)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--1</td>
<td>6 (3)</td>
<td>50</td>
<td>21.7 (\pm 0.8)</td>
<td>25.9 (\pm 1.6)</td>
<td>1.7 (\pm 1.9)</td>
<td>0.62 (\pm 0.03)</td>
</tr>
<tr>
<td>8.0--luc:2--2</td>
<td>6 (5)</td>
<td>83</td>
<td>253 (\pm 10)</td>
<td>25.9 (\pm 3.1)</td>
<td>23.1 (\pm 5.3)</td>
<td>0.54 (\pm 0.03)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--2</td>
<td>6 (1)</td>
<td>17</td>
<td>239</td>
<td>23.2</td>
<td>9.7</td>
<td>0.52</td>
</tr>
<tr>
<td>8.0--luc:2--3</td>
<td>6 (2)</td>
<td>33</td>
<td>185 (\pm 8)</td>
<td>23.3</td>
<td>3.8</td>
<td>0.38</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--3</td>
<td>3 (1)</td>
<td>33</td>
<td>92</td>
<td>21.6</td>
<td>14.7</td>
<td>0.65</td>
</tr>
<tr>
<td>8.0--luc:2--6</td>
<td>5 (5)</td>
<td>100</td>
<td>26 (\pm 2)</td>
<td>23.5 (\pm 0.2)</td>
<td>12.6 (\pm 1.4)</td>
<td>0.47 (\pm 0.05)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--6</td>
<td>6 (2)</td>
<td>33</td>
<td>25</td>
<td>22.6</td>
<td>3.2</td>
<td>0.61</td>
</tr>
<tr>
<td>8.0--luc:2--15</td>
<td>6 (6)</td>
<td>100</td>
<td>21.3 (\pm 1.0)</td>
<td>23.4 (\pm 0.2)</td>
<td>6.9 (\pm 3.5)</td>
<td>0.51 (\pm 0.03)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--15</td>
<td>4 (3)</td>
<td>75</td>
<td>20.9 (\pm 0.8)</td>
<td>22.8 (\pm 0.4)</td>
<td>2.8 (\pm 1.7)</td>
<td>0.58 (\pm 0.06)</td>
</tr>
<tr>
<td>8.0--luc:2--19</td>
<td>4 (4)</td>
<td>50</td>
<td>24.3 (\pm 0.7)</td>
<td>23.9 (\pm 0.4)</td>
<td>9.8 (\pm 1.7)</td>
<td>0.56 (\pm 0.13)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--19</td>
<td>6 (5)</td>
<td>83</td>
<td>29.9 (\pm 0.9)</td>
<td>23.0 (\pm 0.1)</td>
<td>0.3 (\pm 0.8)</td>
<td>0.56 (\pm 0.04)</td>
</tr>
<tr>
<td>8.0--luc:2--20</td>
<td>5 (5)</td>
<td>100</td>
<td>24.0 (\pm 0.7)</td>
<td>22.8 (\pm 0.2)</td>
<td>17.8 (\pm 4.2)</td>
<td>0.46 (\pm 0.02)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--20</td>
<td>4 (1)</td>
<td>25</td>
<td>23.9</td>
<td>22.4</td>
<td>2.2</td>
<td>0.58</td>
</tr>
<tr>
<td>8.0--luc:2--21</td>
<td>5 (5)</td>
<td>100</td>
<td>21.4 (\pm 0.7)</td>
<td>22.9 (\pm 0.3)</td>
<td>22.8 (\pm 4.9)</td>
<td>0.44 (\pm 0.05)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--21</td>
<td>6 (3)</td>
<td>50</td>
<td>29.8 (\pm 1.1)</td>
<td>22.5 (\pm 0.8)</td>
<td>3.1 (\pm 2.1)</td>
<td>0.62 (\pm 0.02)</td>
</tr>
<tr>
<td>8.0--luc:2--22</td>
<td>4 (3)</td>
<td>75</td>
<td>24.1 (\pm 0.8)</td>
<td>23.4 (\pm 0.2)</td>
<td>10.7 (\pm 0.4)</td>
<td>0.38 (\pm 0.05)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--22</td>
<td>6 (2)</td>
<td>36</td>
<td>32.2</td>
<td>23.6</td>
<td>22.5</td>
<td>0.50</td>
</tr>
<tr>
<td>8.0--luc:2--25</td>
<td>6 (4)</td>
<td>67</td>
<td>21.2 (\pm 0.7)</td>
<td>23.3 (\pm 0.2)</td>
<td>4.8 (\pm 5.4)</td>
<td>0.55 (\pm 0.02)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--25</td>
<td>2 (0)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>8.0--luc:2--33</td>
<td>6 (3)</td>
<td>50</td>
<td>24.5 (\pm 0.9)</td>
<td>23.8 (\pm 0.7)</td>
<td>22.7 (\pm 2.3)</td>
<td>0.50 (\pm 0.09)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--33</td>
<td>6 (2)</td>
<td>36</td>
<td>22.3</td>
<td>23.7</td>
<td>22.5</td>
<td>0.40</td>
</tr>
<tr>
<td>8.0--luc:2--34</td>
<td>3 (1)</td>
<td>33</td>
<td>32.5</td>
<td>23.1</td>
<td>1.3</td>
<td>0.37</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--34</td>
<td>6 (1)</td>
<td>17</td>
<td>28.7</td>
<td>23.8</td>
<td>21.9</td>
<td>0.61</td>
</tr>
<tr>
<td>8.0--luc:2--36</td>
<td>4 (4)</td>
<td>100</td>
<td>31 (\pm 1)</td>
<td>22.9 (\pm 0.2)</td>
<td>0.5 (\pm 0.6)</td>
<td>0.56 (\pm 0.03)</td>
</tr>
<tr>
<td>disco(^2)/Y:8.0--luc:2--36</td>
<td>5 (1)</td>
<td>20</td>
<td>19</td>
<td>24.3</td>
<td>19.7</td>
<td>0.55</td>
</tr>
</tbody>
</table>

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^{P1} background. Experimental details and analysis are as described for Table 2.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LD</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>E</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--1</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--2</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--3</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--6</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--15</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--19</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--20</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--21</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--22</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--25</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--33</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--34</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>per^{P1}/Y;8.0--luc:2--36</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

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 \textit{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 \textit{per} transgenes studied in the past.

The \textit{per} genomic locus spans the 3B1--2 region of the X chromosome in \textit{Drosophila} (Smith and Konopka, 1981). Molecular cloning of DNA sequences including those of \textit{per} allowed to generate germ--line transformants for \textit{per}, and certain sub--segments of \textit{per} DNA could rescue locomotor rhythms in \textit{per}^{01} flies (Zehring et al., 1984). Among the different sub--segments of \textit{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 \textit{per}^{01} flies in DD with a period of longer than 24 h
Chapter 4 A light--entrainable circadian oscillator in the dorsal brain

(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 LNvs. Similar 8.0 kb sub--segments of per rescued behavior of per01 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, LNvs, LNds 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 LNVs; 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 per01 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 LNds and LNvs without the
Table 6 Promoterless per DNA sub–segments’ spatial expression and behavioral rescue.

<table>
<thead>
<tr>
<th>per sub–segment (in kb)</th>
<th>Expression pattern in per&lt;sup&gt;α1&lt;/sup&gt; in LD</th>
<th>Behavioral rescue in DD in per&lt;sup&gt;α1&lt;/sup&gt;</th>
<th>τ ± SEM in DD in per&lt;sup&gt;α1&lt;/sup&gt; (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2:2 7.2:9</td>
<td>LN&lt;sub&gt;s&lt;/sub&gt;&lt;sup&gt;•&lt;/sup&gt;, LN&lt;sub&gt;s&lt;/sub&gt;&lt;sup&gt;•&lt;/sup&gt; DNs, LN&lt;sub&gt;s&lt;/sub&gt;&lt;sup&gt;•&lt;/sup&gt; s, photoreceptors, gut</td>
<td>Yes</td>
<td>26.4 ± 0.1 26.0 ± 0.1</td>
<td>Frisch et al., 1994 Frisch et al., 1994</td>
</tr>
<tr>
<td>7.2:2 7.2:9</td>
<td>Not analyzed</td>
<td>Yes</td>
<td>26.0 ± 0.1 25.9 ± 0.1</td>
<td>Frisch et al., 1994 Frisch et al., 1994</td>
</tr>
<tr>
<td>8.0:2(b) 8.0:3(b)</td>
<td>Not analyzed</td>
<td>No</td>
<td>--</td>
<td>Zehring et al., 1984 Zehring et al., 1984 Zehring et al., 1984</td>
</tr>
<tr>
<td>8.0:4(b) 8.0:11(b)</td>
<td>Not analyzed</td>
<td>Yes*</td>
<td>25.5 ± 1.5 25.0</td>
<td>Zehring et al., 1984 Zehring et al., 1984 Zehring et al., 1984</td>
</tr>
<tr>
<td>8.0:14(b) 8.0:24(a)</td>
<td>Not analyzed</td>
<td>Yes*</td>
<td>24.9 ± 0.3 24.9</td>
<td>Zehring et al., 1984 Zehring et al., 1984 Zehring et al., 1984</td>
</tr>
<tr>
<td>8.0:4</td>
<td>No signal detected</td>
<td>Yes*</td>
<td>24.4 ± 0.5 24.4</td>
<td>Zerr et al., 1990</td>
</tr>
<tr>
<td>8.0--2a 8.0--10a 8.0--12a 8.0--24a 8.0--44 8.0--47a 8.0--54a 8.0--79a 8.0--80a 8.0--AV</td>
<td>Not analyzed</td>
<td>Yes</td>
<td>24.1 ± 0.1 25.4 ± 0.9 24.6 ± 0.3 24.2 ± 0.2 25.5 ± 1.0 27.9 ± 1.7 27.0 ± 1.5 29.5 ± 2.3 25.4 ± 0.3 26.0 ± 0.5</td>
<td>Liu et al., 1991 Liu et al., 1991 Liu et al., 1991 Liu et al., 1991 Liu et al., 1991 Liu et al., 1991 Liu et al., 1991 Liu et al., 1991 Liu et al., 1991 Liu et al., 1991</td>
</tr>
<tr>
<td>8.0:6</td>
<td>Wide in all brain with varying intensity DN&lt;sub&gt;s&lt;/sub&gt;, LN&lt;sub&gt;s&lt;/sub&gt;, photoreceptor, lamina, in body (RNA blot)</td>
<td>Not analyzed</td>
<td>--</td>
<td>Cooper et al., 1999 Cooper et al., 1999 Cooper et al., 1999 Cooper et al., 1999 Cooper et al., 1999 Cooper et al., 1999 Cooper et al., 1999 Cooper et al., 1999 Cooper et al., 1999</td>
</tr>
<tr>
<td>8.0--lacZ (originally named 8.0--β-gal)</td>
<td></td>
<td></td>
<td></td>
<td>Liu et al., 1991</td>
</tr>
<tr>
<td>8.0--lac:2 8.0--lac:2--20&lt;sup&gt;<strong>&lt;/sup&gt; 8.0--lac:2--22&lt;sup&gt;</strong>&lt;/sup&gt; 8.0--lac:4&lt;sup&gt;###&lt;/sup&gt; 8.0--lac:9 8.0--lac:11&lt;sup&gt;#&lt;/sup&gt;</td>
<td>DN&lt;sub&gt;s&lt;/sub&gt;,LN&lt;sub&gt;s&lt;/sub&gt;,l--LN&lt;sub&gt;s&lt;/sub&gt;&lt;sup&gt;•&lt;/sup&gt;§ No signal detected DN&lt;sub&gt;s&lt;/sub&gt;,LN&lt;sub&gt;s&lt;/sub&gt;,l--LN&lt;sub&gt;s&lt;/sub&gt;&lt;sup&gt;•&lt;/sup&gt;§ No signal detected DN&lt;sub&gt;s&lt;/sub&gt;,LN&lt;sub&gt;s&lt;/sub&gt;,l--LN&lt;sub&gt;s&lt;/sub&gt;&lt;sup&gt;•&lt;/sup&gt;§ DN&lt;sub&gt;s&lt;/sub&gt;,LN&lt;sub&gt;s&lt;/sub&gt;,l--LN&lt;sub&gt;s&lt;/sub&gt;&lt;sup&gt;•&lt;/sup&gt;§ DN&lt;sub&gt;s&lt;/sub&gt;,LN&lt;sub&gt;s&lt;/sub&gt;,l--LN&lt;sub&gt;s&lt;/sub&gt;&lt;sup&gt;•&lt;/sup&gt;§</td>
<td>Yes* Yes* Yes* Yes* Yes*</td>
<td>23.5 (1/14) 24.4 ± 0.1 23.8 (2/8) 26.2 ± 0.8 28.6 (1/10) 23.7 ± 0.5</td>
<td>Veleri et al., 2003</td>
</tr>
</tbody>
</table>

# , ### Level of expression in the eye photoreceptors.
* Weakly rhythmic, see Table 2 for details on 8.0--lac. ** 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 LNds 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, 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, 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 \(\text{per}^{01}\) background indicates that the \(8.0--\text{luc}:9\) might not be expressed in the s--\(\text{LN}_v\)s. However, the discrepancy between the expressions pattern of \(8.0--\text{lacZ}\) and \(8.0--\text{luc}:9\) remains wide open. Nevertheless, the partial behavioral rescues observed in other \(8.0--\text{luc}\) lines (Table 2, 5) indicate that the \(8.0--\text{luc}\) could also be expressed in the \(\text{LN}_v\)s. It is possible that \(8.0\) \(\text{kb}\) has a sequence information to target into the s--\(\text{LN}_v\)s that may be absent in the \(8.0--\text{luc}\). Possibly, \(\text{lac}--\text{Z}\) 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 \(8.0\) \(\text{kb}\) transgenic flies? Possibly temporal features of the \(\text{per}\) 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 \(\sim\)24 h. Therefore, it is also possible that the posttranslational control of \(\text{PER}\) could bring about a temporal difference (e.g., Sathyanarayanan \textit{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 \(\text{PER}\) could possibly shorten or lengthen the free running period, respectively (Smith and Konopka, 1981). Changes in the phosphorylation state of \(\text{PER}\) caused by DBT affects the turnover of the former and therefore the free running period length of \textit{Drosophila} (Price \textit{et al.}, 1998). Therefore, possibly a different half--life of \(\text{PER}--\text{LUC}\) could affect the free running period length. It was shown that in the absence of PDF the cytoplasmic retention of \(\text{PER}\) is reduced, and hence these flies showed a shorter period (Lin \textit{et al.}, 2004). The firefly luciferase has a SKL tripeptide in its C--terminal, which localizes it to the peroxisomes (Keller \textit{et al.}, 1987). Whereas \(\text{PER}\) has both a nuclear and cytoplasmic localization signal (Yildiz \textit{et al.}, 2005). The presence of multi--location target sequences in the \(\text{PER}--\text{LUC}\) fusion protein may cause some kind of ‘conflict’ and hence a delay to shuttle \(\text{PER}\) 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 norpAP41;cryb and gi60j cryb 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 norpAP41;cryb 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--LNvs 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

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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

\[ \text{mutant flies.} \]

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

\[ \text{mutant flies the} \]

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

\[ \text{mutant flies} \]

(Stanewsky et al., 1998). In the double mutant, norpA

\[ \text{;cry} \]

mutant flies both rhodopsin and cryptochrome mediated phototransduction into the circadian clock is blocked. However, the norpA

\[ \text{;cry} \]

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Type</th>
<th>Literature and source</th>
</tr>
</thead>
<tbody>
<tr>
<td>recA1 endA1 gyrA96 thi--1</td>
<td>E. coli --</td>
<td>Stratagene</td>
</tr>
<tr>
<td>hsdR17 supE44 relA1 lac</td>
<td>XL 1-- Blue Electro competent Cells</td>
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</tr>
<tr>
<td>[F’ proAB lacIqZ.M15 Tn10 (Tetr)]</td>
<td></td>
<td></td>
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</tbody>
</table>

#### 2.3 Vectors

<table>
<thead>
<tr>
<th>Name</th>
<th>Literature and source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM--T Easy I cloning kit</td>
<td>Promega</td>
</tr>
<tr>
<td>pTGAL4</td>
<td>Sharma et al., 2002</td>
</tr>
</tbody>
</table>

#### 2.4 DNA Oligonucleotides

Primers were designed to create a desired restriction site, and were obtained from Invitrogen.
Chapter 5 Rh 7--a novel rhodopsin–like gene in Drosophila

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Remarks</th>
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<tr>
<td>Rh 7 III (BamH I)</td>
<td>5’ acatggatcccccagacatcgcgtagccat 3’</td>
<td>at the 5’ end a BamH I site created</td>
</tr>
<tr>
<td>Rh7 R3 (EcoR I)</td>
<td>5’ tacagaattccgccttttagctgcaagactc 3’</td>
<td>at the 5’ end a EcoR I site created</td>
</tr>
</tbody>
</table>

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)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gDNA</td>
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</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>2.0</td>
</tr>
<tr>
<td>Forward primer--Rh 7 III (BamH I) (7.5 µM)</td>
<td>2.0</td>
</tr>
<tr>
<td>Reverse primer--Rh 7 R3 (EcoR I) (7.5 µM)</td>
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</tr>
<tr>
<td>Reaction Buffer 10X</td>
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<tr>
<td>Sterile dH₂O</td>
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Enzyme Mixture (10 µl)

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</tr>
<tr>
<td>Expand High fidelity Taq Polymerase</td>
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</tr>
<tr>
<td>Sterile dH₂O</td>
<td>7.8</td>
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</table>

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

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<th>Annealing temp.</th>
<th>Polymerization temp.</th>
<th>No. of cycles</th>
</tr>
</thead>
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<td>95°C</td>
<td>53°C</td>
<td>68°C</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4 min</td>
<td>0 min</td>
<td>0 min</td>
<td>1X</td>
</tr>
<tr>
<td>II</td>
<td>50 sec</td>
<td>1 min</td>
<td>4 min</td>
<td>18X</td>
</tr>
<tr>
<td>III</td>
<td>50 sec</td>
<td>1 min</td>
<td>6 min</td>
<td>15X</td>
</tr>
<tr>
<td>IV</td>
<td>50 sec</td>
<td>1 min</td>
<td>12 min</td>
<td>1X</td>
</tr>
</tbody>
</table>

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 $\text{Rh 7--P}$ PCR product was generated using the oligonucleotides $\text{Rh 7 III}$ and $\text{Rh 7 R3}$, and it was cloned into the pGEM--T Easy. By EcoRI digestion the $\text{Rh 7--P}$ 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 EcoRI site in the multiple cloning site (MCS) in pTGAL. The clockwise 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 pTGA4, 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 (BamHI); Rh 7 R3 (EcoRI)] (Fig 1A). This PCR product was used for cloning and subcloning as mentioned (see Appendix 1 for the complete sequences cloned).

To ensure that the Rh 7 promoter was cloned into pTGA4 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 EcoRI.

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: \textit{Rh 7:9--1/UAS--\(\tau\)--GFP} and \textit{Rh 7:2B/UAS--\(\tau\)--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](image)

**Figure 4** GFP reporter gene expression in the \textit{Drosophila} brains under control of the \textit{Rh 7} promoter using the GAL4/UAS system. \textit{Rh 7:9--1/UAS--\(\tau\)--GFP} and \textit{Rh 7:2B/UAS--\(\tau\)--GFP} flies expressed \(\tau\)--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 \textit{Rh 7:2B}, some cells close to lateral clock neurons (LNss)--called lateral cells (LCs) and close to dorsal clock neurons (DNss)--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--\(\tau\)--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 (\(Rh\,7:1\text{A}/TM3\)) did not show any detectable GFP signals. In \(Rh\,7:2\text{B}/UAS--\(\tau\)--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--\(\tau\)--GFP flies without the \(Rh\,7--GAL4\) construct (UAS--\(\tau\)--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 \textit{et al.}, 1998; Emery \textit{et al.}, 2000b; Helfrich--Förster \textit{et al.}, 2001). Rhodopsin mediated phototransduction in the compound eyes and the ocelli is non--functional in \(norpA^{P41}\) flies (Pearn \textit{et al.}, 1996; Helfrich--Förster \textit{et al.}, 2001). Cryptochrome function is completely or nearly abolished in the \(cry^{b}\) mutant flies (Stanewsky \textit{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 \textit{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 (Devreotes, 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 phototransduction 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 phototransducton 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 DN$s$ 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 DN$s$ but also in cells close to the LN$s$. In the present study, at least one transgenic fly line showed GFP expression in the LCs, near the LN$_{0}$s and s--LN$_{v}$s; 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 LN$s$. 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 LN$s$ 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 DN1+2s 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?

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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.
Chapter 6 Veela involved in the circadian input

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^{P_{360d}} (von Lintig et al., 2001), hereon called ninaB^{P_{360d}}.
w; ninaB^{P_{315}} (von Lintig et al., 2001), hereon called ninaB^{P_{315}}.
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).

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'GAACTGTGCAGATGACTGG3'
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'GCACTTCTTTCTGGCTGCTAC3'
TIM--10578 5'GCAGTCGTGGAGTCTGCTGTC3'

2.2.2 antisense direction
TIM--568Rev 5'GCACCGTCAGATTGACGA3'
TIM--680rev 5'GCTTCTTGGCTGTGTACAG3'
TIM--1150rev 5'CTGATACTCCTGACCCTTGC3'
TIM--1773rev 5'CATCATTGCTGCTGCTGG3'
TIM--2277rev 5'TCACCACCAAGTGCTTC3'
TIM--2379rev 5'CCTCAGATCGGACATTTTCGC3'
TIM--2609rev 5'GTCCTCAAACAGAATGCC3'
TIM--2748rev 5'TCGCACAGTTCATAGTCC3'
TIM--2900rev 5'AGTGTGTCATATCCCTCC3'
TIM--3500rev 5'CGAGTCGTCATACATTGTCTCC3'
TIM--3748rev 5'ATCGAGATACACCGACGG3'
TIM--4444rev 5'TCGAACGAGGACTTCATG3'
TIM--4695rev 5'TCGGATATCGGATCGGACGTG3'
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.
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 \( \text{Veela} \) 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 \( \text{Veela} \) 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 \( \chi^2 \) 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 dH2O (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 timblind (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 ninaBP360d flies showed rhythmic behavior in LL similar to the cry\(^b\) flies (Fig. 2). The ninaB gene encodes an enzyme \(\beta,\beta'\)--carotene--15,15'--dioxygenase, which cleaves the \(\beta--\)carotene into two molecules of retinal (von Lintig et al., 2001). The ninaB\(^{P360d}\) flies can not split the \(\beta--\)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 \textit{ninaB}^{P360d} flies. Thereafter, it was named \textit{Veela} (owing to its disguising nature!).

The \textit{Veela} mutant flies showed normal behavior in LD cycles like \textit{yw} control flies (Fig. 2, Table 1 and Table 2). But in LL \textit{Veela} mutant flies showed robust rhythmic behavior (like \textit{cry}^b flies), while \textit{yw} flies exhibited arrhythmic behavior (Fig. 2 and Table 1). Interestingly, there was a difference in period--length between the \textit{Veela} mutant flies and \textit{cry}^b flies in LL; i.e. 25.7 ± 0.12 h for \textit{Veela} mutant flies compared with 24.3 ± 0.1 h for \textit{cry}^b flies. However, in DD, both the \textit{Veela} mutant and \textit{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 \textit{Veela} mutant fly was clearly different in LL (25.7 ± 0.12 h) and DD (24.3 ± 0.11 h). In summary, the \textit{Veela} mutant flies were rhythmic in LL like the \textit{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 \textit{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 \textit{et al.}, 1989).

4.2 \textit{cry} interacts with \textit{Veela}

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

When both the \textit{Veela} and \textit{cry}^b mutations were combined by genetic crossings, transheterozygous (\textit{Veela}/+;\textit{cry}^b/+) flies showed rhythmic behavior in LL like homozygous (hz) \textit{Veela} flies (Fig. 3, Fig. 2 and Table 1). The period of \textit{Veela}/+;\textit{cry}^b/+ flies was 25.9 ± 0.6 h, not significantly different from \textit{Veela} (hz) mutant flies (25.7 ± 0.12 h). The heterozygous (ht) \textit{Veela}/+ flies showed only weak rhythmic behavior in LL (Fig. 3 and Table 1) compared to \textit{Veela} (hz) flies. Their period was significantly shorter compared to \textit{Veela} (hz) flies and the percentage of flies showing rhythmic behavior was reduced by 2.7 fold in \textit{Veela} (ht) (Table 1).
Figure 2 Actograms and periodograms show that Veela mutant flies stay rhythmic in constant light similar to cry<sup>b</sup> 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)
Chapter 6 Veela involved in the circadian input

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 aristless (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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>n</th>
<th>period ± SEM (h)</th>
<th>RI ± SEM</th>
<th>% Rhy.</th>
<th>N</th>
<th>n</th>
<th>period ± SEM (h)</th>
<th>RI ± SEM</th>
<th>% Rhy.</th>
</tr>
</thead>
<tbody>
<tr>
<td>y w /Y</td>
<td>74</td>
<td>73</td>
<td>23.9 ± 0.03</td>
<td>0.35 ± 0.01</td>
<td>99</td>
<td>70</td>
<td>2</td>
<td>22.3</td>
<td>0.30</td>
<td>3</td>
</tr>
<tr>
<td>y w /Y; cry²</td>
<td>22</td>
<td>21</td>
<td>24.3 ± 0.10</td>
<td>0.36 ± 0.03</td>
<td>96</td>
<td>22</td>
<td>21</td>
<td>24.3 ± 0.10</td>
<td>0.33 ± 0.03</td>
<td>96</td>
</tr>
<tr>
<td>y w /Y; cry²/+</td>
<td>24</td>
<td>22</td>
<td>24.1 ± 0.04</td>
<td>0.41 ± 0.01</td>
<td>92</td>
<td>23</td>
<td>1</td>
<td>27.5</td>
<td>0.30</td>
<td>4</td>
</tr>
<tr>
<td>y w /Y; Veela</td>
<td>75</td>
<td>74</td>
<td>24.0 ± 0.03</td>
<td>0.37 ± 0.01</td>
<td>99</td>
<td>75</td>
<td>64</td>
<td>25.7 ± 0.12</td>
<td>0.33 ± 0.02</td>
<td>85</td>
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<tr>
<td>y w /Y; Veela/+</td>
<td>35</td>
<td>35</td>
<td>24.1 ± 0.04</td>
<td>0.45 ± 0.02</td>
<td>100</td>
<td>35</td>
<td>11</td>
<td>24.4 ± 0.40</td>
<td>0.18 ± 0.01</td>
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<tr>
<td>y w /Y; Veela/+; cry²/+</td>
<td>26</td>
<td>26</td>
<td>24.1 ± 0.04</td>
<td>0.45 ± 0.01</td>
<td>100</td>
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<td>25.9 ± 0.60</td>
<td>0.24 ± 0.02</td>
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<td>+/Y; Df (3R) cry/TM6B</td>
<td>21</td>
<td>21</td>
<td>23.9 ± 0.10</td>
<td>0.34 ± 0.02</td>
<td>100</td>
<td>21</td>
<td>8</td>
<td>22.4 ± 0.40</td>
<td>0.25 ± 0.03</td>
<td>38</td>
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<tr>
<td>+/Y; Df (3R) cry/+</td>
<td>12</td>
<td>8</td>
<td>24.1 ± 0.13</td>
<td>0.32 ± 0.03</td>
<td>67</td>
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<tr>
<td>y w /Y; Veela/+; Df (3R) cry/+</td>
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<td>24</td>
<td>24.1 ± 0.02</td>
<td>0.4 ± 0.01</td>
<td>100</td>
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<td>19</td>
<td>24.9 ± 0.10</td>
<td>0.30 ± 0.02</td>
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<tr>
<td>y w /Y; tim²⁺</td>
<td>40</td>
<td>40</td>
<td>23.9 ± 0.05</td>
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<td>24.2 ± 0.05</td>
<td>0.45 ± 0.02</td>
<td>100</td>
<td>24</td>
<td>0</td>
<td>AR</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>y w /Y; Veela/tim²⁺</td>
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<td>55</td>
<td>24.1 ± 0.02</td>
<td>0.43 ± 0.01</td>
<td>100</td>
<td>42</td>
<td>13</td>
<td>25.9 ± 1.10</td>
<td>0.21 ± 0.02</td>
<td>22</td>
</tr>
<tr>
<td>+/Y; Df (2L) tim²⁰/CyO</td>
<td>22</td>
<td>20</td>
<td>24.0 ± 0.04</td>
<td>0.36 ± 0.03</td>
<td>91</td>
<td>21</td>
<td>6</td>
<td>23.6 ± 1.10</td>
<td>0.19 ± 0.02</td>
<td>29</td>
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<tr>
<td>y w /Y; Df (2L) tim²⁰/Veela</td>
<td>29</td>
<td>28</td>
<td>24.0 ± 0.03</td>
<td>0.36 ± 0.02</td>
<td>97</td>
<td>27</td>
<td>5</td>
<td>23.7 ± 0.81</td>
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<td>+/Y; Df (2L) tim²⁰+/tim²⁰</td>
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<td>24.0 ± 0.03</td>
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<td>93</td>
<td>27</td>
<td>0</td>
<td>AR</td>
<td>-</td>
<td>0</td>
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<td>w/Y: tim²⁺; P(w⁺ tim⁺)</td>
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<td>24.0 ± 0.10</td>
<td>0.35 ± 0.02</td>
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<td>14</td>
<td>1</td>
<td>25</td>
<td>0.20</td>
<td>7</td>
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<tr>
<td>y w /Y; Veela/tim²⁺; P(w⁺ tim⁺)/+</td>
<td>46</td>
<td>46</td>
<td>23.9 ± 0.02</td>
<td>0.41 ± 0.01</td>
<td>100</td>
<td>46</td>
<td>29</td>
<td>24.6 ± 0.18</td>
<td>0.25 ± 0.02</td>
<td>63</td>
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</table>

‘N’ is total number of flies tested and ‘n’ is number of flies showed rhythmicity in LL. ‘RI’ is Rhythm Index.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>LD</th>
<th>DD</th>
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<td></td>
<td>N</td>
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<tr>
<td>y w /Y</td>
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<td>19</td>
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<td>y w /Y; cryβ</td>
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<td>23</td>
</tr>
<tr>
<td>y w /Y; Veela/+; cryβ/+</td>
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<td>15</td>
</tr>
<tr>
<td>+/Y; Df(3R) cry/ TM6B</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>y w /Y; Veela/+; Df(3R) cry/+</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>y w /Y; tim01</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>y w /Y; tim01/+</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>y w /Y; Veela/tim01</td>
<td>27</td>
<td>27</td>
</tr>
</tbody>
</table>

'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.

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Marker(s)</th>
<th>Rhy. in LL?</th>
<th>Period $\pm$ SEM (h)</th>
<th>N(n)</th>
<th>% Rhy.</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>I--3</td>
<td>al</td>
<td>Yes</td>
<td>26.3 ± 0.22</td>
<td>8 (8)</td>
<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>I--7</td>
<td>b pr</td>
<td>Yes</td>
<td>25.2 ± 0.31</td>
<td>3 (3)</td>
<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>I--11</td>
<td>pr</td>
<td>Yes</td>
<td>25.6 ± 0.18</td>
<td>7 (4)</td>
<td>57</td>
<td>Veela</td>
</tr>
<tr>
<td>I--12</td>
<td>b pr</td>
<td>Yes</td>
<td>24.7 ± 0.50</td>
<td>6 (4)</td>
<td>67</td>
<td>Veela</td>
</tr>
<tr>
<td>I--18</td>
<td>b pr</td>
<td>Yes</td>
<td>26.2 ± 0.22</td>
<td>9 (4)</td>
<td>40</td>
<td>Veela</td>
</tr>
<tr>
<td>I--27</td>
<td>b</td>
<td>Yes</td>
<td>25.6 ± 0.22</td>
<td>8 (8)</td>
<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>I--32</td>
<td>b pr</td>
<td>Yes</td>
<td>25.4 ± 0.27</td>
<td>7 (7)</td>
<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>I--42</td>
<td>b pr</td>
<td>Yes</td>
<td>26.0 ± 0.32</td>
<td>6 (6)</td>
<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>I--45</td>
<td>b pr</td>
<td>Yes</td>
<td>25.8 ± 0.24</td>
<td>7 (6)</td>
<td>86</td>
<td>Veela</td>
</tr>
<tr>
<td>I--49</td>
<td>b pr</td>
<td>Yes</td>
<td>26.1 ± 0.21</td>
<td>6 (5)</td>
<td>83</td>
<td>Veela</td>
</tr>
<tr>
<td>II--1</td>
<td>b cn</td>
<td>Yes</td>
<td>24.9 ± 0.23</td>
<td>6 (3)</td>
<td>50</td>
<td>Veela</td>
</tr>
<tr>
<td>II--11</td>
<td>cn</td>
<td>Yes</td>
<td>25.8 ± 0.16</td>
<td>6 (5)</td>
<td>83</td>
<td>Veela</td>
</tr>
<tr>
<td>II--15</td>
<td>bw</td>
<td>Yes</td>
<td>26.6 ± 0.23</td>
<td>5 (3)</td>
<td>60</td>
<td>Veela</td>
</tr>
<tr>
<td>II--23</td>
<td>cn</td>
<td>Yes</td>
<td>25.5 ± 0.15</td>
<td>6 (3)</td>
<td>50</td>
<td>Veela</td>
</tr>
<tr>
<td>II--25</td>
<td>b cn</td>
<td>Yes</td>
<td>25.3 ± 0.27</td>
<td>5 (3)</td>
<td>60</td>
<td>Veela</td>
</tr>
<tr>
<td>II--26</td>
<td>bw (w)</td>
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<td>25.3 ± 0.60</td>
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<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>II--31</td>
<td>bw</td>
<td>Yes</td>
<td>25.6 ± 0.30</td>
<td>6 (6)</td>
<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>II--37</td>
<td>bw</td>
<td>Yes</td>
<td>25.5 ± 0.18</td>
<td>6 (6)</td>
<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>II--45</td>
<td>bw</td>
<td>Yes</td>
<td>26.1 ± 0.24</td>
<td>6 (6)</td>
<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>II--53</td>
<td>bw (w)</td>
<td>Yes</td>
<td>25.4 ± 0.42</td>
<td>4 (4)</td>
<td>100</td>
<td>Veela</td>
</tr>
<tr>
<td>II--55</td>
<td>b cn</td>
<td>Yes</td>
<td>25.2 ± 1.70</td>
<td>5 (2)</td>
<td>40</td>
<td>Veela</td>
</tr>
<tr>
<td>II--56</td>
<td>b cn (w)</td>
<td>Yes</td>
<td>24.7 ± 0.38</td>
<td>5 (4)</td>
<td>80</td>
<td>Veela</td>
</tr>
<tr>
<td>II--58</td>
<td>b cn</td>
<td>Yes</td>
<td>25.0 ± 0.37</td>
<td>4 (3)</td>
<td>75</td>
<td>Veela</td>
</tr>
</tbody>
</table>

Note: I--5 to II--58 represent different fly lines tested for recombination with Veela in LL conditions. The table includes markers tested, their recombination status, period with standard error of the mean, number of flies tested, percentage of rhythmic flies, and corresponding remarks.
Table 4 List of silent mutations found in the *Veela* TIM protein.

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

* Indicates that the *Veela* sequence was identical to the TIM--PD but there was a nucleotide change in the control flies (*ninaBP315*).
Figure 3 Actograms, periodograms and daily averages indicate that Veela and cry interact. Heterozygous cry<sup>b</sup> 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 tim01 or Df(2L)tim02/+ compliment for Veela? Could a tim transgene (in tim01 background) rescue the wild type LL behavior in Veela flies? Therefore, we combined the Veela and tim01 mutations (heterozygously) and tested their behavior in LL. The Veela/tim01 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)tim02 flies also showed similar behavior (Table 1). From the above results it seems that Veela is complimented by tim01 or Df(2L)tim02/+ . However, it was difficult to discern the contribution of tim01 or Df(2L)tim02/+ 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 tim01 flies show arrhythmic behavior in LL. Combining heterozygous Veela mutation with a heterozygous tim01 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 tim01;P(w+ tim+) (Rutila et al., 1998) generated Veela mutant flies carrying the timeless transgene (Veela/tim01;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/tim01;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/tim01;P(w+ tim+)/+ transgenic flies showed rhythmic behavior in LL (Table 1). The rhythmic Veela/tim01;P(w+ tim+)/+ transgenic flies showed a period of 24.6 ± 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/tim01 (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 \(\textit{ninaB}^{P315}\) flies were used as controls. This was done because the \textit{Veela} mutant was recovered from the original \(\textit{ninaB}^{P360d}\) fly stock, and both \(\textit{ninaB}^{P360d}\) and \(\textit{ninaB}^{P315}\) fly stocks were generated in the same mutagenesis screen performed at Purdue University (von Lintig \textit{et al.}, 2001; Schneuwly, S., personal communication).

Figure 6 (continues in next page)

**Figure 6** Actograms and autocorrelograms indicate that the \(\textit{tim}\) transgene did not rescue \textit{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) \textit{Veela} mutants show
robust rhythmic behavior in LL (autocorrelogram--asterisk above the cut--off lines). (C) The \textit{tim}^{01} flies exhibit arrhythmic behavior.

Figure 6 (D) The \textit{tim} transgene control flies show arrhythmic behavior in LL. (E) \textit{Veela} flies carrying a \textit{tim} transgene (\textit{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 \textit{tim} gene in the \textit{Veela} mutant fly revealed that it has 3 nucleotide changes that result in amino acid substitutions (Fig. 7) compared to the control flies (\textit{ninaB}^{P315}).

1) The control \textit{ninaB}^{P315} flies had a guanine (G) deletion at position 52 (the positions are with reference to \textit{tim}--\textit{PD} cDNA; CG 3234--\textit{PD}, FlyBase) but the \textit{Veela} mutant flies did not (Fig. 7A). To know whether this G deletion was specific for the \textit{Veela} mutant flies, further sequencing for this region of \textit{tim} was performed on Canton--S, Oregon--R and \textit{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 \textit{Veela} mutant flies but the \textit{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 ninaBP315 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 ninaBP315 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, timblind (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 timblind and for BG--luc are kindly provided by Peschel, N. and Wülbeck, C., respectively); and timblind flies did not show rhythmic behavior in LL (Wülbeck, C., personal communication), this transition can not be responsible for the Veela phenotype.
Chapter 6 Veela involved in the circadian input

**A** G--deletion results in TIM--short form

<table>
<thead>
<tr>
<th></th>
<th>TIM-PD-1421</th>
<th>Oregon-R</th>
<th>Veela</th>
<th>timblind</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCCGTCGCGCGGAGCAGCATTCCTGAGACGCTGTCATGACGCTG</td>
<td>TCCGTCGCGCGGAGCAGCATTCCTGAGACGCTG</td>
<td>TCCGTCGCGCGGAGCAGCATTCCTGAGACGCTG</td>
<td>TCCGTCGCGCGGAGCAGCATTCCTGAGACGCTG</td>
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</tr>
<tr>
<td>stop codon</td>
<td>G</td>
<td>A</td>
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<td>G</td>
<td>G</td>
</tr>
<tr>
<td>alternate</td>
<td>stop codon</td>
<td>start codon</td>
<td>alternate</td>
<td>stop codon</td>
<td>start codon</td>
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</tbody>
</table>

**B** Glycine to serine at amino acid 449

<table>
<thead>
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<th>TIM-PD-1421</th>
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<th>Veela</th>
<th>timblind</th>
<th>Consensus</th>
</tr>
</thead>
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<td></td>
</tr>
</tbody>
</table>

**C** Isoleucine to methionine at amino acid 1284

<table>
<thead>
<tr>
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<th>TIM-PD-1421</th>
<th>Oregon-R</th>
<th>Veela</th>
<th>Canton-S</th>
<th>ninaBP315</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
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<td>TCCGTCGCGCGGAGCAGCATTCCTGAGACGCTGTCATGACGCTG</td>
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<td>TCCGTCGCGCGGAGCAGCATTCCTGAGACGCTG</td>
<td>TCCGTCGCGCGGAGCAGCATTCCTGAGACGCTG</td>
<td>TCCGTCGCGCGGAGCAGCATTCCTGAGACGCTG</td>
<td>TCCGTCGCGCGGAGCAGCATTCCTGAGACGCTG</td>
</tr>
</tbody>
</table>

**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*<sup>P315</sup> *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*<sup>blind</sup> 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*<sup>P315</sup> 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{b} mutation abolishes PER and TIM cycling in total fly head extracts in LD (Stanewsky et al., 1998), however, norpA\textsuperscript{P41};cry\textsuperscript{b} flies maintained PER and TIM cycling in the s-LN\textsubscript{s} and DN\textsubscript{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\textsuperscript{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\textsubscript{r}s, l--LN\textsubscript{r}s and LN\textsubscript{d}s) 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\textsuperscript{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\textsubscript{r}s 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\textsuperscript{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\textsuperscript{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--LNₙₛ and l--LNₙₛ indicates small and large --later ventral neurons, respectively. LNₙₛ are dorsal lateral neurons.

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--LNₙₛ (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 vs 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 vs (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* flies also showed synchronized cycling of PER and TIM in the pacemaker neurons. The synchronized cycling of PER and TIM in the s--LN vs 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 vs (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 vs, which was never reported earlier.
Figure 11

- **y w/Y**
  - Glia cells
- **y w/Y; veela**
  - s--LNₜ,s
- **y w/Y; cryb**
  - s--LNₜ,s
- **y w/Y; Veela**
  - Glia cells
- **y w/Y; cryb**
  - s--LNₜ,s
  - LNₜ,s
  - I--LNₜ,s
  - LNds
**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 3\textsuperscript{rd} 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\textsuperscript{p*} flies but not in *yw* 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 cryb 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 cryb 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). cryb 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 cryb 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 cryb flies is immune to the damaging effects of LL. At 2000 lux LL, cryb 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 cryb 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 \textit{ninaB}^{P315} and \textit{yw} flies (Fig. 7A). Oregon--R, Canton--S and the \textit{Veela} mutant flies had a G in position 52 (Fig. 7A), as reported in the data base for \textit{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 \textit{Veela} phenotype? If the longer TIM form is responsible for the \textit{Veela} phenotype all the \textit{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 \textit{tim} gene from Canton--S, Oregon--R, and \textit{yw}. 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 \textit{yw} flies (Fig. 6A), which encodes the shorter TIM form. Therefore, the G at position 52 is not responsible for the \textit{Veela} phenotype.

The second nucleotide change found in the \textit{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 \textit{timblind} mutant flies (Fig. 7B ; Wülbeck et al., 2004), and they show rhythmic behavior in LL (Wülbeck, C., personal communication). \textit{BG--luc} flies (Stanewsky et al., 2002) also have the same nucleotide change. Therefore, this amino acid change is not responsible for the \textit{Veela} phenotype.

A third nucleotide change in \textit{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 \textit{ninaB}^{P315} and \textit{yw} 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 \textit{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 \textit{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 cryb 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,s was delayed in Veela compared with cryb 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).

yw flies showed conspicuous cytoplasmic staining in the l--LN,s 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,s (Fig. 9 lower panels, and Fig. 11). Similarly, Veela and cryb flies showed cytoplasmic staining in the l--LN,s at CT 9 and CT 6 respectively (Fig. 8B and Fig. 10 lower left half panels). The cytoplasmic staining pattern of the l--LN,s also followed a phase relationship correlating with the period length of Veela and cryb flies in LL (Fig. 2 and Table 1). However, the implication of cytoplasmic staining in the l--LN,s in LL is yet to be understood. Further, it would be interesting to know why and how yw 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.,
Veela and cry\(^b\) flies showed synchronized PER and TIM expression in the glia cells in LL but y\(\text{w}\) 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\(_s\)s. 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\(_s\)s and therefore they exhibit arrhythmic behavior. Moreover, to our surprise, there was some cytoplasmic staining for both PER and TIM in the l--LN\(_s\)s 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\(_s\)s and s--LN\(_s\)s. 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\textsuperscript{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\textendash{}LN\textsubscript{s}, 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_{1:s}). The H--B eyelet projects axons toward the s--LN_{v}s pacemaker neurons. Both in the s--LN_{v}s and in the DN_{1:s} 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 photoreceptor 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.


## Appendix 1

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N (n)</th>
<th>percent rhythmic</th>
<th>Bioluminescence expression level (CPS)</th>
<th>period ± SEM (h)</th>
<th>phase ± SEM (h)</th>
<th>rel--amp ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0--luc:2--1</td>
<td>6 (5)</td>
<td>83</td>
<td>49.9 ± 8.5</td>
<td>23.9 ± 0.3</td>
<td>5.8 ± 4.3</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>disco2/Y;8.0--luc:2--1</td>
<td>6 (3)</td>
<td>50</td>
<td>21.7 ± 0.8</td>
<td>25.9 ± 1.6</td>
<td>1.7 ± 5.9</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>8.0--luc:2--15</td>
<td>6 (6)</td>
<td>100</td>
<td>21.3 ± 1.0</td>
<td>23.4 ± 0.2</td>
<td>6.9 ± 3.5</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>disco2/Y;8.0--luc:2--15</td>
<td>4 (3)</td>
<td>75</td>
<td>20.9 ± 0.8</td>
<td>22.8 ± 0.4</td>
<td>2.8 ± 1.7</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td>8.0--luc:2--19</td>
<td>4 (4)</td>
<td>50</td>
<td>24.3 ± 0.7</td>
<td>23.9 ± 0.4</td>
<td>9.8 ± 1.7</td>
<td>0.56 ± 0.13</td>
</tr>
<tr>
<td>disco2/Y;8.0--luc:2--19</td>
<td>6 (5)</td>
<td>83</td>
<td>29.9 ± 0.9</td>
<td>23.0 ± 0.1</td>
<td>0.3 ± 0.8</td>
<td>0.56 ± 0.04</td>
</tr>
<tr>
<td>8.0--luc:2--20</td>
<td>5 (5)</td>
<td>100</td>
<td>24.0 ± 0.7</td>
<td>22.8 ± 0.2</td>
<td>17.8 ± 4.2</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>disco2/Y;8.0--luc:2--20</td>
<td>4 (1)</td>
<td>25</td>
<td>23.9</td>
<td>22.4</td>
<td>2.2</td>
<td>0.58</td>
</tr>
<tr>
<td>8.0--luc:2--21</td>
<td>5 (5)</td>
<td>100</td>
<td>21.4 ± 0.7</td>
<td>22.9 ± 0.3</td>
<td>22.8 ± 4.9</td>
<td>0.44 ± 0.05</td>
</tr>
<tr>
<td>disco2/Y;8.0--luc:2--21</td>
<td>6 (3)</td>
<td>50</td>
<td>29.8 ± 1.1</td>
<td>22.5 ± 0.8</td>
<td>3.1 ± 2.1</td>
<td>0.62 ± 0.02</td>
</tr>
<tr>
<td>8.0--luc:2--22</td>
<td>4 (3)</td>
<td>75</td>
<td>24.1 ± 0.8</td>
<td>23.4 ± 0.2</td>
<td>10.7 ± 0.4</td>
<td>0.38 ± 0.05</td>
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<td>36</td>
<td>32.2</td>
<td>23.6</td>
<td>22.5</td>
<td>0.50</td>
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<td>67</td>
<td>21.2 ± 0.7</td>
<td>23.3 ± 0.2</td>
<td>4.8 ± 5.4</td>
<td>0.55 ± 0.02</td>
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<tr>
<td>disco2/Y;8.0--luc:2--25</td>
<td>2 (0)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>8.0--luc:2--33</td>
<td>6 (3)</td>
<td>50</td>
<td>24.5 ± 0.9</td>
<td>23.8 ± 0.7</td>
<td>22.7 ± 2.3</td>
<td>0.50 ± 0.09</td>
</tr>
<tr>
<td>disco2/Y;8.0--luc:2--33</td>
<td>6 (2)</td>
<td>36</td>
<td>22.3</td>
<td>23.7</td>
<td>22.5</td>
<td>0.40</td>
</tr>
<tr>
<td>8.0--luc:2--34</td>
<td>3 (1)</td>
<td>33</td>
<td>32.5</td>
<td>23.1</td>
<td>1.3</td>
<td>0.37</td>
</tr>
<tr>
<td>disco2/Y;8.0--luc:2--34</td>
<td>6 (1)</td>
<td>17</td>
<td>28.7</td>
<td>23.8</td>
<td>21.9</td>
<td>0.61</td>
</tr>
</tbody>
</table>

‘N’ is the number of flies tested and ‘n’ is number of rhythmic flies.
## Appendix 2

### Table 5 Supplement

Transgenic fly lines, mobilized from \textit{8.0-luc:2}, screened for locomotor behavior in LD and DD.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LD</th>
<th></th>
<th></th>
<th>DD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n</td>
<td>% rhy.</td>
<td>Anticipation of light–dark transitions</td>
<td>period ± SEM (h)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>E</td>
<td></td>
<td>M</td>
<td>E</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--4</td>
<td>8</td>
<td>5</td>
<td>63</td>
<td>- +</td>
<td>24.2 ± 0.07</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--5</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>- +</td>
<td>24.1 ± 0.06</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--7</td>
<td>8</td>
<td>5</td>
<td>63</td>
<td>- +</td>
<td>24.1 ± 0.04</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--9</td>
<td>8</td>
<td>5</td>
<td>63</td>
<td>- +</td>
<td>24.0 ± 0.08</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--10</td>
<td>8</td>
<td>3</td>
<td>38</td>
<td>- +</td>
<td>23.9 ± 0.3</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--11</td>
<td>8</td>
<td>7</td>
<td>88</td>
<td>- +</td>
<td>24.0 ± 0.4</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--12</td>
<td>7</td>
<td>5</td>
<td>71</td>
<td>- +</td>
<td>24.2 ± 0.10</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--13</td>
<td>8</td>
<td>6</td>
<td>75</td>
<td>- +</td>
<td>24.4 ± 0.17</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--16</td>
<td>8</td>
<td>7</td>
<td>88</td>
<td>- +</td>
<td>24.2 ± 0.36</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--17</td>
<td>7</td>
<td>5</td>
<td>71</td>
<td>- +</td>
<td>24.1 ± 0.13</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--18</td>
<td>8</td>
<td>7</td>
<td>88</td>
<td>- +</td>
<td>24.3 ± 0.10</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--23</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>- +</td>
<td>24.2 ± 0.06</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--26</td>
<td>7</td>
<td>5</td>
<td>71</td>
<td>- +</td>
<td>24.2 ± 0.10</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--27</td>
<td>8</td>
<td>7</td>
<td>88</td>
<td>- +</td>
<td>24.3 ± 0.10</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--28</td>
<td>8</td>
<td>6</td>
<td>75</td>
<td>- +</td>
<td>24.4 ± 0.11</td>
</tr>
<tr>
<td>\textit{per}^{P} /Y;8.0--luc:2--29</td>
<td>8</td>
<td>7</td>
<td>88</td>
<td>- +</td>
<td>24.3 ± 0.11</td>
</tr>
<tr>
<td>Lineage</td>
<td>N</td>
<td>n</td>
<td>Period (h) ± Error</td>
<td>Blood (h)</td>
<td>Dead</td>
</tr>
<tr>
<td>---------------</td>
<td>----</td>
<td>----</td>
<td>-------------------</td>
<td>-----------</td>
<td>------</td>
</tr>
<tr>
<td>per01/Y;8.0--luc:2--30</td>
<td>8</td>
<td>6</td>
<td>75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>per01/Y;8.0--luc:2--35</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>per01/Y;8.0--luc:2--37</td>
<td>8</td>
<td>6</td>
<td>75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>per01/Y;8.0--luc:2--38</td>
<td>8</td>
<td>7</td>
<td>88</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>per01/Y;8.0--luc:2--41</td>
<td>3</td>
<td>2</td>
<td>33</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>per01/Y;8.0--luc:2--42</td>
<td>10</td>
<td>7</td>
<td>70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>per01/Y;8.0--luc:2--43</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>per01/Y;8.0--luc:2--44</td>
<td>8</td>
<td>6</td>
<td>75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>per01/Y;8.0--luc:2--45</td>
<td>8</td>
<td>5</td>
<td>63</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

‘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

tccccccgacatcgatgcatggcattttccatcgaatcccgcaaacgaatatgtattcactacattttaaa
actggcaacagattaacgcaacgcattacatattggggtcgggaacaaacgcggatgtgcctgctttaaaccg
cgggattctaatcatgatagcattgtttaaaccgcaacacagcatcttagctcaatttccatcgaatcccg
ttatattttagccgtatatttatttttttctcactacattttctccatcgaatcccgcaaacgaatatgtattcactacattttaaa
actggcaaacagattaacgcaacgcattacatattggggtcgggaacaaacgcggatgtgcctgctttaaaccg
actggcaaacagattaacgcaacgcattacatattggggtcgggaacaaacgcggatgtgcctgctttaaaccg
actggcaaacagattaacgcaacgcattacatattggggtcgggaacaaacgcggatgtgcctgctttaaaccg
actggcaaacagattaacgcaacgcattacatattggggtcgggaacaaacgcggatgtgcctgctttaaaccg
The underlines indicate the oligonucleotide region chosen for designing the oligonucleotide for PCR reaction.
Appendix--2

Homology matrix (DNAMAN 4.0)

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

The homology matrix shows the percentage of amino acid sequences conserved between Rh 7 and other rhodopsins (*D. melanogaster*) 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.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh1-NCBP</td>
<td><img src="" alt="Alignment" /></td>
</tr>
<tr>
<td>Rh2</td>
<td><img src="" alt="Alignment" /></td>
</tr>
<tr>
<td>Rh5</td>
<td><img src="" alt="Alignment" /></td>
</tr>
<tr>
<td>Rh4</td>
<td><img src="" alt="Alignment" /></td>
</tr>
<tr>
<td>Rh3</td>
<td><img src="" alt="Alignment" /></td>
</tr>
<tr>
<td>Rh6</td>
<td><img src="" alt="Alignment" /></td>
</tr>
<tr>
<td>OPN4</td>
<td><img src="" alt="Alignment" /></td>
</tr>
<tr>
<td>Retinochromes</td>
<td><img src="" alt="Alignment" /></td>
</tr>
</tbody>
</table>

continues...
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
Acknowledgements

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I thank Charlotte Helfrich--Förster for the innumerable helps, discussions and for the privilege given me to use her abundant reference collection. I’m indebted to her for the introduction and guidance given for me on confocal microscopy. I acknowledge her for kindly sharing the unpublished data. Furthermore for me, she was a source of inspiration as an excellent and artful chrono--neuroanatamist.

I also thank Georg Kuhn for his expert advices on confocal microscopy.

I thank Alois Hofbauer for extending many ‘instant answers’ on my queries to suffice my fragmentary anatomical knowledge.

I thank Stephan Schneuwly for providing me the access to use all facilities in the ‘Lehrstuhl’ without any constraints. Also, for maintaining a stimulating intellectual milieu. I appreciate all his creative work group members, who assured a warm and pleasant atmosphere, which always eased my ‘Fly Pushing’. I also appreciate all, for interlacing me into the fabric of ‘Lehrstuhl--Gemeinnütziger Verein’, and making my stay in Regensburg a wonderful experience.

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