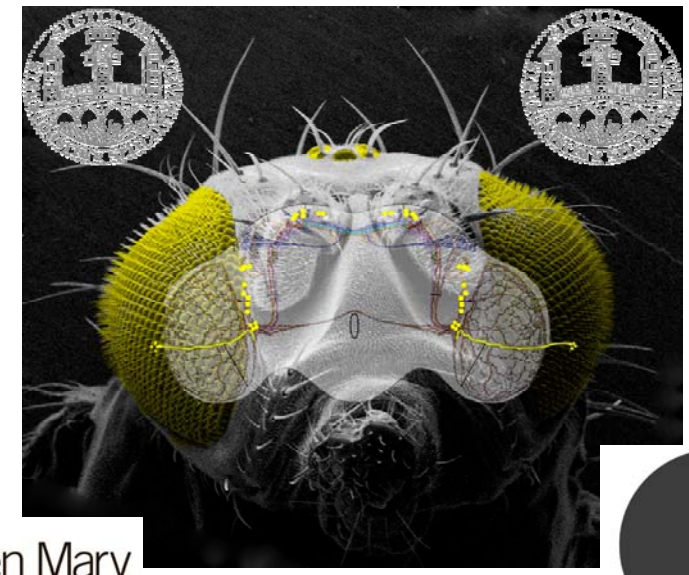
New Insights into Circadian Photoreception and the Molecular Regulation of the Resetting of *Drosophila's* Circadian Clock



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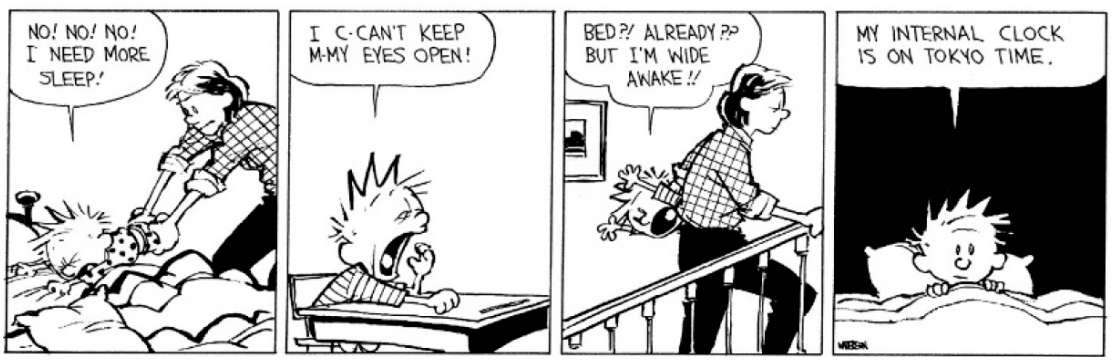
Light-dependent interactions between Cryptochrome and Jetlag regulate circadian clock resetting in *Drosophila*.
(Under Review in Current Biology)



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1. Introduction

"The early bird catcheth the worm."

1.1 General Introduction and History

Evolution has shaped and fine-tuned all living beings to their current existence. Adaptations to different environments like the sea, the woods, or the mountains yielded in wings or fins etc. and thus allowing creatures to conquer new territories and to find their vacant niche. One parameter of our environment is so obvious that it is often neglected or taken for granted - this parameter is the daily changing of day and night. The turning of the earth around its own axis once every 24 hours (23hrs 56min) causes a daily rhythmically change of light and temperature. Virtually all living beings on this planet are exposed to this light and temperature change and hence it is not surprising that they adapted to this 24 hours rhythm and found in that way a new vacant niche – not in terms of space, but in terms of time. There are for example nocturnal, diurnal or crepuscular (i.e. mainly active in dusk or dawn) active animals – all living in the same habitat, but the different activity times allows them to live happily together. Crepuscular insects for instance are only active when it is not too hot and dry on the one hand and when it is not too cold on the other hand. One could argue now that this just reflects a direct response to the changing temperature or light, but when this organism is isolated from all environmental cues, like light, food or temperature it still keeps the same times of activity with a period of close to but not exactly 24 hrs. One speaks in this case of a circadian period (Latin: circa=about and dies=day), i.e. an approximately 24 hrs cycle that is endogenously generated by an organism (Halberg F., 1959). Two questions arise here. First of all, why does an animal need such an endogenous circadian timer? And secondly, how does this circadian clock that sets the pace and time of our activity work? Purely exogenous responses to the environment might be not fast enough to occupy a vacant niche or simply for survival. Otherwise when the time of the opening of this vacant niche is known and anticipated, the organism is at its maximum fitness, at the right time. To give an example, the animal that awakes shortly after sunrise as a response to this changing light environment still needs some time until it reaches its maximum fitness. The animal that gets up 30 minutes before sunrise is at its maximum when the sun finally arises and thus has a big selective advantage. Carl Johnson verified this nicely

in competition experiments with bacteria (Johnson et al., 1998). In those competition experiments he demonstrated that fitness is enhanced when the circadian period resonates with the period of the environmental cycle. In other words, when he competed mutant strains with a period of 22 hours with wild-type bacteria (25 hrs period) he revealed that the mutants outgrow the wild-type under a 22 hours cycle, while the wild-type was victorious in a 25 hours cycle (Johnson et al., 1998). Thus he clearly proved the selective advantage of a circadian clock.

The scientific field of the Chronobiology (Chronos = time) tries to find an answer to the questions, why does an animal need such an endogenous circadian timer and how this circadian clock tells the animal when to sleep and when to be active. And so does the present thesis. The first person who reported circadian behavior of a living being was the French astronomer Jean Jacques Ortous de Mairan in 1729 (deMairan, 1729). He studied the leaf movement of a heliotrope *Mimosa pudica*. This plant opens its leaves during the day and closes the leaves at night. De Mairan's astonishing observation was placing the plant into the dark does not abolish the opening and closing of the leaves at the right time. Thereby he showed the existence of the persistence of this circadian rhythm in the absence of environmental clues. Many more interesting observations should follow, not only in plants but in animals as well. The 20th century brought the genes and genetics to the Chronobiology. Erwin Bünning demonstrated by crossing plants with different periods that the endogenous activity period was genetically inherited (Bünning, 1935). In the following years many other investigators documented the properties of the circadian clock and revealed their generality in organisms ranging from single-celled algae to humans. Colin Pittendrigh for example published in the 1950s a series of papers showing that the fruit fly *Drosophila* emerged from its pupa (eclosion) in a circadian way (Pittendrigh, 1960). After the seismic shift break through

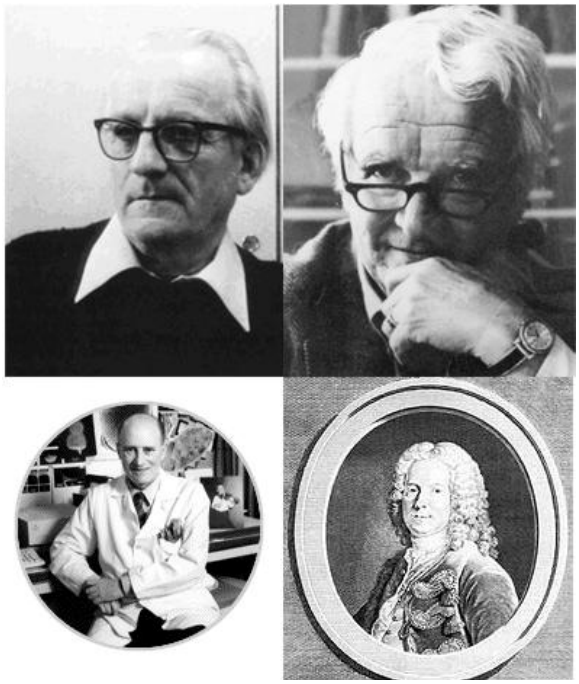


Figure 1-1 Chronobiologists

From upper left clockwise: Erwin Bünning, Colin Pittendrigh, Seymour Benzer, Ortous de Mairan

of Watson and Crick in 1953 (Watson and Crick, 1953) the way was cleared for the discovery of the first genes controlling the circadian rhythm of living beings.

The late Seymore Benzer (1921-2007) and his student Ron Konopka were working with the crepuscular fruit fly *Drosophila melanogaster* in the beginning of the 1970s. After chemical mutagenesis they screened in those flies for animals with abnormal circadian behavior. They could isolate several different fly strains showing abnormal endogenous eclosion period. One strain had a longer period (29 hrs), another strain had a shorter period (19 hrs) and a third strain did not show rhythmic eclosion at all (Figure 1-2). The milestone discovery though was that all three fly strains had a mutation in the same gene locus, located on the *X*-chromosome. This locus was called *period* and the mutants $period^{Long}$, $period^{Short}$ or $period^{01}$ and the first so called 'clock gene' was revealed (Konopka and Benzer, 1971). A few years later other researcher could uncover the exact location of *period* (Bargiello et al., 1984; Jackson et al., 1986; Reddy et al., 1986; Reddy et al., 1984) and found the exact sequence of this gene (Citri et al., 1987). Many more genes should follow this discovery, not only in the fruit fly but in cyanobacteria (Kondo et al., 1993), fungi (McClung et al., 1989) or mammals (Vitaterna et al., 1994) as well. Today our knowledge of the molecular basis of the circadian clock is dramatically increased. In many organisms sophisticated interactions and pathways of the circadian clock have been discovered, but still there is a long way to go until the whole mechanism of circadian clock function is fully understood.

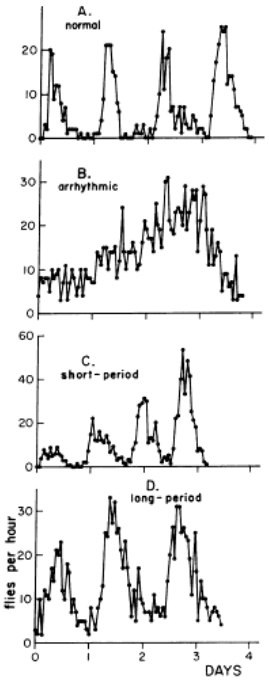


Figure 1-2 Original *period* mutants (PNAS, 1971)
The figures shows eclosion behavior of (A) wild-type, (B) per^{01}, (C) per^{S} and (D) per^{L} animals.

1.2 The Basics and Characteristics of Circadian Clocks

The basic circadian clock simply consists of three different parts. The input component is the part that adapts the core circadian clock to its environment. The endogenous oscillator is the core clock and the third part is the output (Figure 1-3).
Various input factors affecting the fly circadian oscillator are known. The most important factor is light, but other inputs like the temperature (Pittendrigh et al., 1958),

humidity (Halket, 1931), feeding (Stephan et al., 1979) or social interaction (Levine et al., 2002) act as environmental cue, as so called Zeitgeber (Zeit=time, geber= presenter).

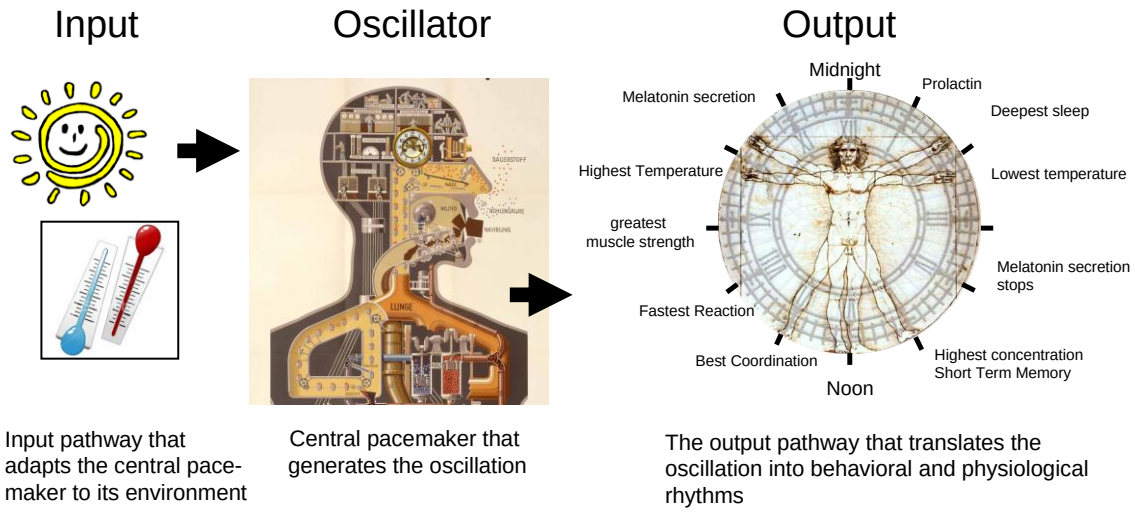


Figure 1-3 A Model of the Circadian System

A schematic illustration of the circadian system. The Input adapts the oscillator via environmental cues like temperature or light to the immediate vicinity. In the Output the organisms rhythmic behavior, hormone control, physical abilities or body temperature are regulated by the circadian oscillator

The oscillator converts and processes the input information to generate oscillation on a molecular basis. Without input from the environment the oscillator generates its own endogenous approximately 24 hrs rhythm. In the output many biological processes are controlled by the central oscillator, to name a few examples: complex behavior like locomotion, rhythmic regulation of the vision, the hearing and smelling or of metabolism functions. Circadian rhythms are defined by well-established criteria that were postulated mainly by Bünning, Aschoff and Pittendrigh. A first criterion is the persistence of the circadian rhythm in the absence of external cues and Zeitgeber – so even in the dark with constant temperature the period should still be approximately 24 hrs. The second criterion is the temperature compensation. The rate of most biochemical processes changes twice to threefold with each 10°C change. In other words, the circadian clock would tell the wrong time, when the temperature rises by 5°C, a not desirable condition. This led to a sophisticated temperature compensation mechanism that is as yet poorly understood (Bruce and Pittendrigh, 1956). The third criterion is that endogenous rhythms of approximately 24 hrs can be adapted or entrained by certain environmental cues, like light-dark cycles (LD cycles) or temperature cycles (see above).

1.3 The Circadian Clock of *Drosophila melanogaster*

A model organism is a species that is extensively studied to understand particular biological phenomena, with the expectation that discoveries made in the model organism will provide insight into the workings of other organisms. In particular, model organisms are widely used to explore potential causes and treatments for human diseases when human experimentation would be unfeasible or unethical. Since the fruit fly made the jump from nature to laboratory in the beginning of last century it brought us many interesting and helpful new findings. Especially now where the full genome of the fly is sequenced (Adams et al., 2000) and the community is so wide spread and big, *Drosophila* has established its firm place in science. No wonder that the circadian clock of *Drosophila* is best investigated of all organisms. Like presumably all circadian clocks *Drosophilas* clock mechanism is based on a negative feedback loop. The basic helix-loop-helix PER-ARNT-SIM transcription factors Clock (Clk) and Cycle (Cyc) can form a heterodimer (Allada et al., 1998; Rutila et al., 1998). This dimer activates the transcription of *period* (*per*) (Konopka and Benzer, 1971) and *timeless* (*tim*) in the nucleus (Konopka and Benzer, 1971; Sehgal et al., 1994) by binding to so called E-Box elements, that can be found in the promotor region of *tim* and *per*. Per and Tim proteins are translated in the cytoplasm and accumulate in the dark, because of the light sensitivity of Tim and the fact that Tim stabilizes Per. The two proteins accumulate in the cytoplasm and enter the nucleus alone or as a heterodimer (Curtin et al., 1995; Shafer et al., 2002). Inside the cell nucleus Per prevents the Clk/Cyc dimer from binding to the DNA and thus inhibits the transcription of *tim* and *per* (Lee et al., 1999; Yu et al., 2006). This

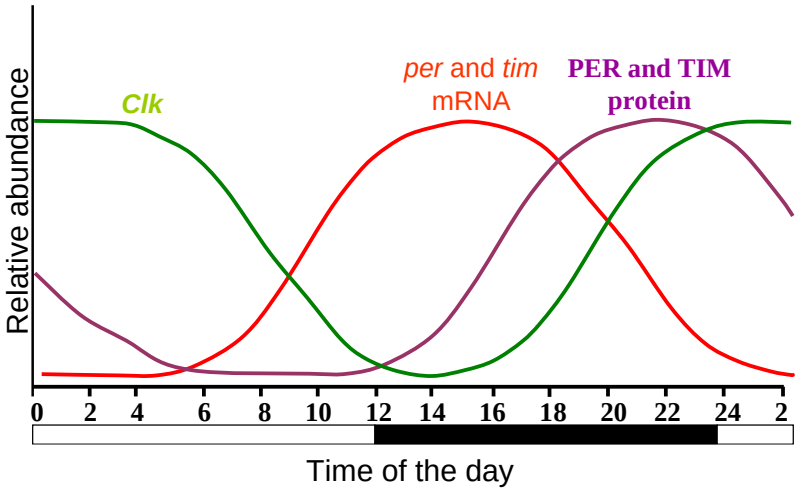


Figure 1-4 The cycling of *per/tim* and Per/Tim

The negative feedback loop generates rhythmic *per/tim* and Per/Tim expression. The mRNA peaks in the beginning of the night, followed by a peak in the protein level 6 hours later. This delay is caused by phosphorylation of Per and Tim and along with this a delayed entry into the nucleus. The Clock protein cycles in the opposite phase.

negative feedback loops hence generates oscillation in the *per/tim* mRNA and protein level. Figure 1-4 shows that the *per* and *tim* mRNA peaks during the early evening, while Per and Tim accumulation and entry into the nucleus peaks in the late night. The reason for the dramatic increase in the night derives first of all from the light sensitivity of Tim. The delayed entry into the nucleus at night is due to phosphorylation induced degradation of Per by Double-Time (Dbt), a homolog of the mammalian casein kinase Iε (Kloss et al., 1998; Price et al., 1998). The phosphorylated Period is subsequently ubiquitinated by the F-Box protein Slimb (Slmb) and degraded in the proteasome (Grima et al., 2002; Ko et al., 2002). On the other hand phosphorylation of Per by the Casein Kinase 2 (CK2) (Lin et al., 2002) and Tim by Shaggy (Sgg) the homolog of the mammalian glykogen synthase kinase 3

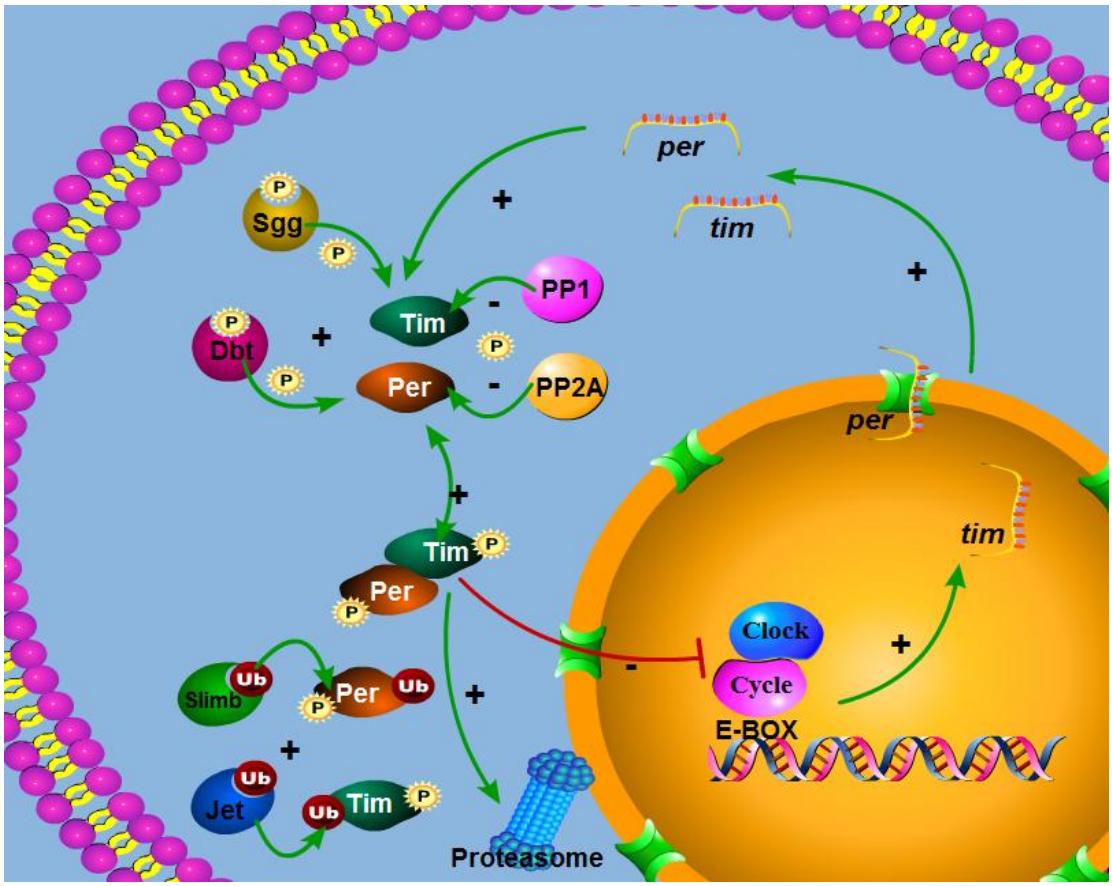


Figure 1-5 The clock mechanism in *Drosophila*

The diagram shows an overview of the important molecular mechanisms in the pacemaker cells. For the sake of clarity some of the described proteins were not included in the drawing. The activation of *tim* and *per* transcription and subsequent translation causes the dimerization of the two proteins, the reentry into the nucleus and here they inhibit their own transcription and thus form the negative feedback loop.

promotes the nuclear entry (Martinek et al., 2001). This whole process of phosphorylation is counterbalanced by phosphatases like the protein phosphatase 2 A (PP2A) that dephosphorylates Per, leading to a stabilization and enhanced nuclear

entry of this protein (Sathyanarayanan et al., 2004). Tim is dephosphorylated by the protein phosphatase 1 (PP1), leading to stabilization of this protein but nuclear entry seems not influenced here (Fang et al., 2007). This model is visualized in Figure 1-5.

Another interlocked feedback loop plays an important role in the regulation of Clk and perhaps of the blue light photoreceptor Cryptochrome (Cry) (Emery et al., 1998; Stanewsky et al., 1998). The Clk/Cyc heterodimer activates the transcription of the *Par-domain protein 1ε (Pdp1*) and *vrille (vri)* (Blau and Young, 1999; Cyran et al., 2003; Glossop et al., 2003). The two basic leucine zipper (bZip) proteins Vri and Pdp1 regulate *Clk* expression by competing for binding to so called V/P-boxes – that can be found in the *Clk* promotor. Vrille represses the transcription of *Clk* and Pdp1 activates *Clk* transcription. This regulation causes *Clk* mRNA levels to cycle in an opposite phase to *period* or *timeless*. This model is visualized in Figure 1-6.

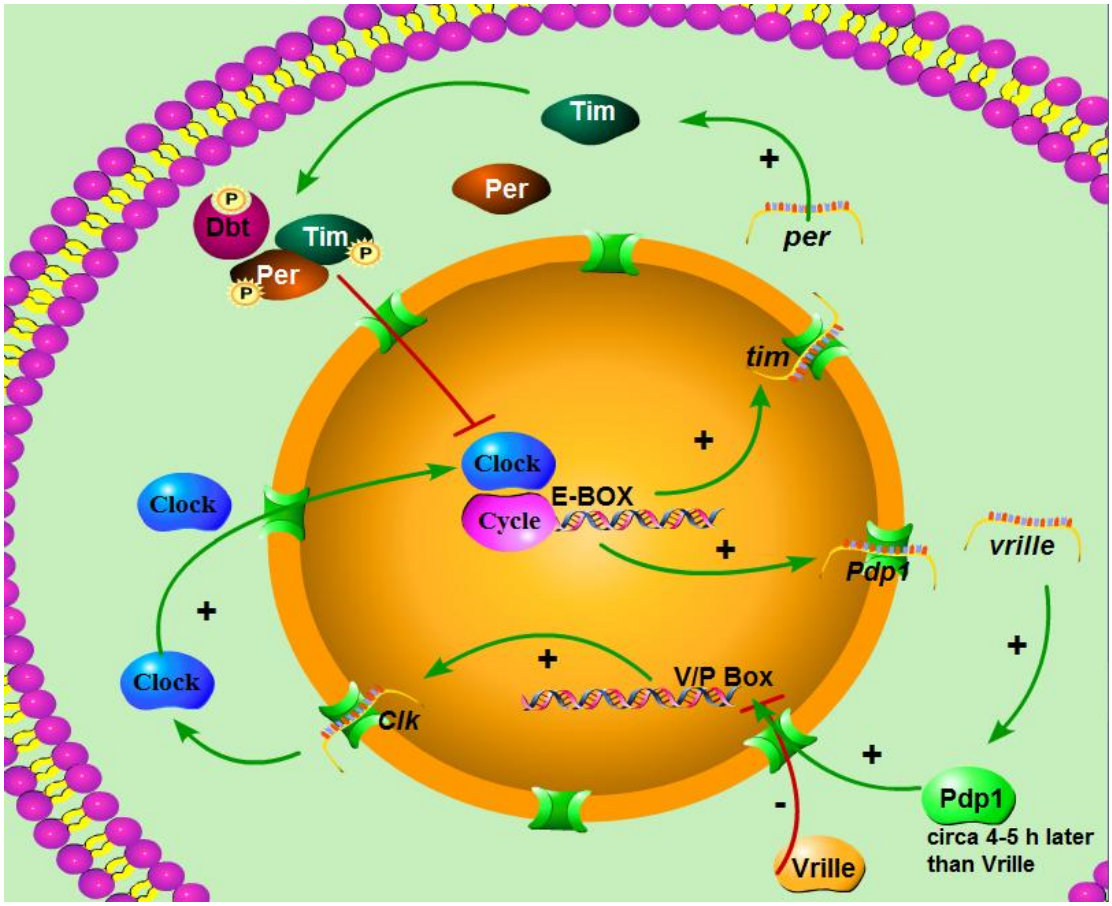


Figure 1-6 The second interlocked feedback loop in the circadian clock of *Drosophila*

A second feedback loop regulates the expression of *Clock* and thus of course of all the clock proteins that have E-Boxes in their promoter region and are activated by Clock. With this model the opposite phase of mRNA expression of Clock compared to Period and Timeless can be explained.

1.4 Input into *Drosophila's* Clock

There are many environmental inputs into the circadian clock, but temperature and especially light are the most potent ones.

Changes in ambient light accompany in most circumstances changes in temperature in nature. But for *Drosophila* temperature changes seem to have only a minor influence compared to ambient light changes (Wheeler et al., 1993). It was shown under laboratory conditions that when the fly was exposed to light and temperature changes at the same time, light is the most potent environmental cue and the effects of the temperature changes are almost negligible. On the other hand temperature has an influence on the fly. If an animal is kept in the laboratory under constant lighting conditions, like constant dark (DD) condition, temperature changes as low as 3°C are sufficient to synchronize the locomotor activity of the flies (Wheeler et al., 1993). Once more it should be stressed, that the circadian clock itself is temperature compensated. The clock can adapt to temperature cycles, but the clock speed does not change twice to threefold when a fly is at lower constant temperature like 20°C or higher constant temperature like 26°C.

Drosophila exhibits a mid-day siesta and is inactive during the hotter part of the day. This adaptation is controlled by an alternative splicing event in an intron within the 3' UTR of *period* (Majercak et al., 1999). At low temperature *per* splicing levels are increased. This leads to an advanced processing of *period* RNA and to an earlier accumulation of Per. Via this way the fly can adapt to seasonal changes (Collins et al., 2004; Majercak et al., 2004; Majercak et al., 1999). The *norpA* gene encodes a Phospholipase–C which is involved in this temperature dependent splicing event. Furthermore *norpA* has a more general function in temperature entrainment, as *NorpA* mutants fail to entrain properly to temperature cycles in constant light (Glaser and Stanewsky, 2005; Glaser, 2006). Another gene, *nocte* influences the temperature entrainment of the fly as well, because *nocte* mutant flies can no longer entrain to temperature cycles (Glaser and Stanewsky, 2005). Finally the circadian blue-light photoreceptor Cryptochrome is involved in the temperature pathway though the proper function for the temperature adaptation is still very vague (Kaushik et al., 2007).

Contrary to the circadian temperature reception more is known about circadian photoreception.

1. 5 Circadian Photoreception in *Drosophila*

The strongest resetting factor for *Drosophila's* circadian clock is light.

The fruitfly has three different photoreceptive organs, containing different Rhodopsins. Those organs are the complex eyes, the ocelli and the Hofbauer-Buchner-Äuglein (HB-eyelet) (Figure 1-7).

Furthermore there are two or more photopigments not restricted to those photoreceptive organs. Cryptochrome, a blue-light sensitive pigment and an as yet unknown photopigment (Helfrich-Forster et al., 2001; Veleri et al., 2003).

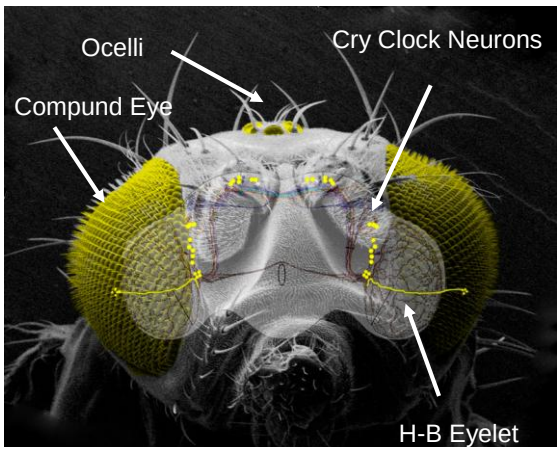


Figure 1- 7
The circadian light input into *Drosophila*
This image shows stained in yellow the light input organs of the adult fly and the Cryptochrome containing cells in the brain.

The response to daily or seasonal changes in light is mainly caused by the degradation of the light sensitive Tim protein. If the fly is briefly exposed to light at night its onset of activity on the next day will be advanced or delayed, depending on the timing of the given lightpulse. If a fly is in constant light conditions, it becomes arrhythmic presumably as a consequence to the lack of Tim (Konopka et al., 1989).

1. 6 Cryptochrome

Cryptochromes are blue-light and ultraviolet light (UV-A) photoreceptors. They share a great similarity with bacterial photolyases. Those photolyases repair UV light (UV-B) induced DNA-damage using blue-light to remove pyrimidin dimers from the DNA (Sancar, 2003). But Cryptochromes lack the DNA repair capacity of the photolyase (Cashmore, 2003). Then again they play an important role in regulating the circadian clock (animals) or in growth and development of plants (Lin and Shalitin, 2003). Crys were first discovered in plants, in the rockcress *Arabidopsis* (Ahmad and Cashmore, 1993), but soon they were found in other plants and in animals like *Drosophila* and humans. The animal Cryptochromes fall into two broad groups. Type1 Crys, like the *Drosophila* Cryptochrome, are circadian photoreceptors. Type2 Cryptochromes, like

the human Cry1 and Cry2 are known for their function in the core clock.

Crys have two domains, a core region with high similarity to the photolyases, and carboxy-terminal 'tails' that vary considerably in length (Figure 1-8). The core domain has conserved binding sites for two cofactors, a flavin-adenin-dinucleotide (FAD) and a second chromophore, the pterin methenyltetrahydrofolate (MTHF). In photolyasas, the second chromophore harvests the light – the resulting excitation energy is transferred to the FAD (catalytic chromophore) (Cashmore et al., 1999). However Cry's photocycle is still under debate.

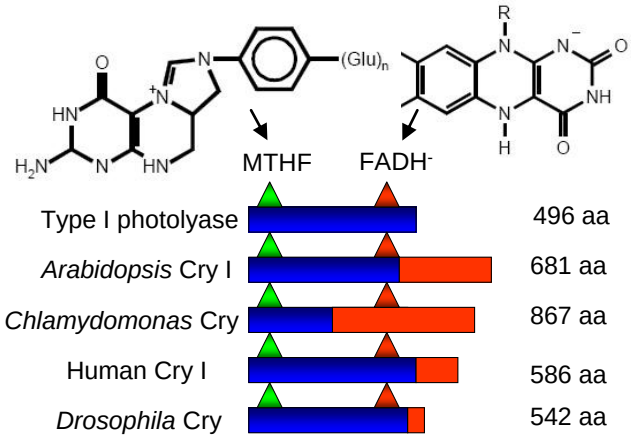


Figure 1- 8 Photolyase region and C-terminus of Cry
The photolyase region (blue) is highly conserved in different species – while the C-terminus (red) differs greatly. The MTHF binding site is painted in green, the FAD binding site in red.

Drosophila has one Cry that acts as a blue-light photoreceptor contributing to photic entrainment of the circadian clocks that control behavior (Stanewsky et al., 1998). Cry may, however, play a different, light-independent role in the so-called 'peripheral' clocks, such as those in the fly antennae or eyes (Collins et al., 2006; Krishnan et al., 2001).

1.7 The Molecular Pathway of *Drosophila's* Circadian Photoreception

In *Drosophila* Cry is activated by light. The model predicts that in darkness a repressor molecule binds to the C-terminus of Cry and prevents the protein from interacting with other proteins (Rosato et al., 2001). A candidate for this repressor molecule is presumably the glycogen synthase kinase 3 Shaggy (Stoleru et al., 2007). Upon illumination Cry changes its confirmation, thus releasing the repressor. Now it can bind to Tim and/or Per. The consequence of this physical interaction is the ubiquitination of Tim mediated by the F-Box protein Jetlag (Koh et al., 2006). The labeling with Ubiquitin marks Tim for the degradation in the proteasome. But not only Timeless is degraded in light conditions, Cryptochrome itself is light-sensitive

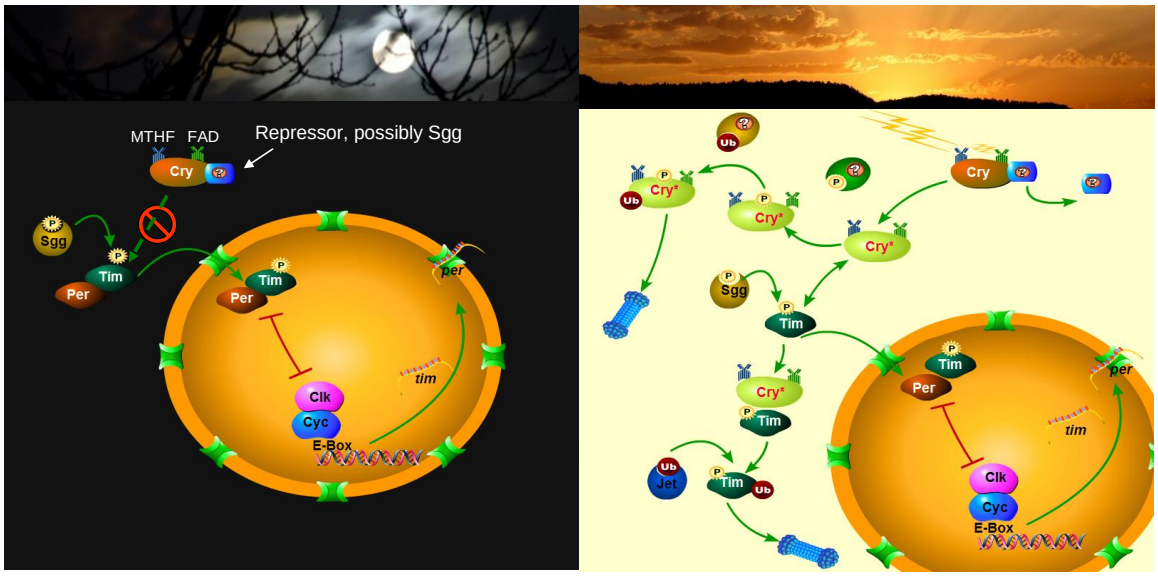


Figure 1- 9 The molecular pathway of *Drosophila's* circadian photoreception
In the darkness (left side) Tim is protected against Cry mediated degradation – most probably caused by Sgg binding to Crys C-terminus. Upon illumination (right side) the repressor is released and Tim degraded in the proteasome. Thus the way is cleared for the next circadian cycle.

and degraded in the proteasome too (Lin et al., 2001; Naidoo et al., 1999; Sathyanarayanan et al., 2008). For a model see Figure 1-9. The degradation of Timeless and the subsequent degradation of Per results in a reset of the circadian clock. When, however, the animals are exposed to light for a prolonged time, the constant degradation of Timeless leads to a loss of a functional circadian clock and to arrhythmic behavior (Konopka et al., 1989). Animals carrying a mutation in the Cryptochrome photoreceptor, like the cry^b (Stanewsky et al., 1998) mutation or cry^m (Busza et al., 2004) alleles, are blind in a circadian sense towards constant light conditions and stay thus rhythmic in LL (Emery et al., 2000). Of course one should keep in mind that Timeless can be degraded in the total absence of Cry as well. Here another, alternative mechanism exists that normally leads to a degradation of Tim in the darkness (e.g. in DD) or fulfills the same task in the absence of functional Cry (Stanewsky et al., 1998).

1.8 Anatomy of *Drosophila's* Circadian System

Drosophilas circadian core clock is located in the brain of the fly. From the approximately 100.000 neurons of the fly's CNS only a tiny subset of about 150 brain neurons control the clock. Those so called 'pacemaker neurons' are defined by the presence of Per or Tim (Taghert and Shafer, 2006). Furthermore those proteins can be

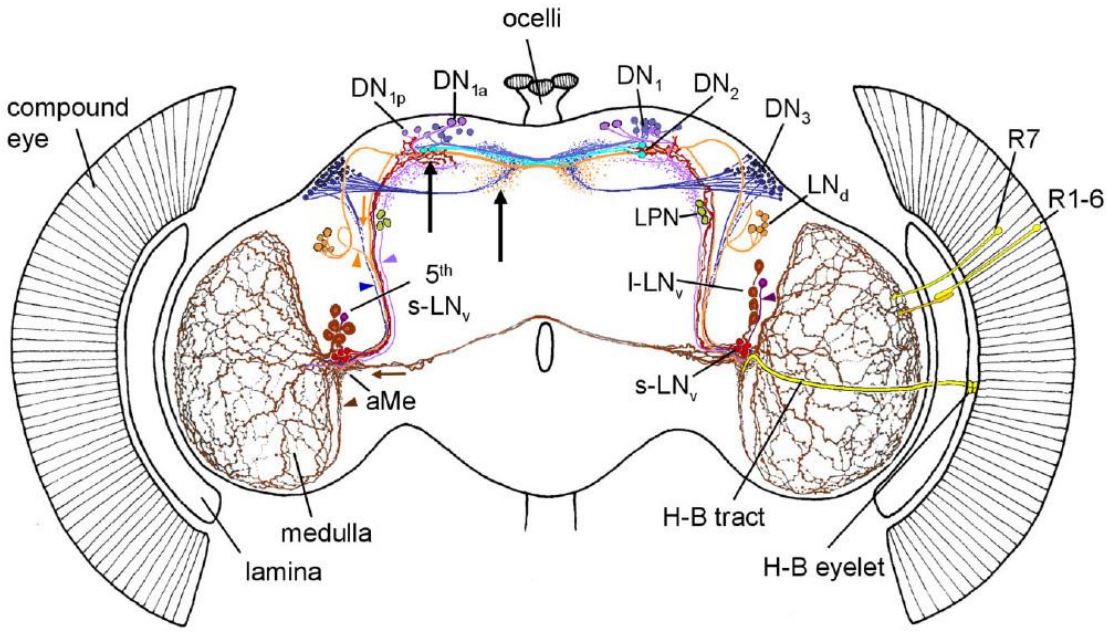


Figure 1-10 Overview of the clock cells in the adult *Drosophila* brain
The picture, showing all the circadian clock neurons plus different light-input routes is taken from C. Helfrich-Förster (Helfrich-Forster et al., 2007a)

found in the photoreceptor cells and in a large number of glia cells in the optic lobes (Zerr et al., 1990). The clock neurons have been divided according to their position in the brain into two major groups – the lateral neurons (LNs) and the dorsal neurons (DNs). Those LNs and DNs are furthermore divided into subgroups. An overview can be seen in Figure 1-10. The LNs are divided into the large ventrolateral neurons (l-LN_vs), the small ventrolateral neurons (s-LN_vs) and the dorsolateral neurons (LN_ds). The DNs are divided into DN_1s, DN_2s and DN_3s see (Helfrich-Forster et al., 2007a) for a detailed overview of the topic.

LNs

There are about 15-18 lateral neurons in each brain hemisphere. Four s-LN_vs and about five l-LN_vs can be detected in the brain. A fifth s-LN_v was just recently discovered. The normal LN_vs express the neuropeptide pigment-dispersing factor (PDF), the 5^{th} s-LN_v is lacking PDF (Kaneko et al., 1997). PDF-immunoreactive l-LN_vs invade the distal medulla and connect the accessory medulla of both brain hemispheres via the posterior optic tract. The function of the l-LN_vs is not yet known, but it was shown, that in constant darkness (DD) the molecular oscillations as well as rhythmic changes of membrane electrical activity stop (Cao and Nitabach, 2008; Veleri et al., 2003). The s-LN_vs on the other hand seem to play the major role in the maintenance of the circadian rhythm in DD and have a special role in regulating the

morning activity of the crepuscular animal. All s-LN_vs show aborizations to the dorsal protocerebrum (Helfrich-Forster et al., 2007a). The 6 LN_ds do not express PDF, and also project to the dorsal protocerebrum. The LN_ds and the special 5^{th} s-LN_v play an important pacemaker role and are thought to regulate the fly's activity in the evening (see below and (Grima et al., 2004; Rieger et al., 2006; Stoleru et al., 2004)).

DNs

About 80 clock neurons can be found in the dorsal brain region and none of them expresses the PDF peptide. The heterogeneous group of about 17 cells close to the center of the two brain hemispheres is named the DN_1s. Recently this group of neurons was divided into to subgroups, the $DN1_{anterior}$ and the $DN1_{posterior}$ (Shafer et al., 2006). The two DN_2 cells are located at the end of the s-LN_vs terminals and are situated directly above the calices of the mushroom bodies. The DN_3 cell group consists of more than 30 cells and is thus the largest clock neuron group.

The most ill defined group of cells is the lateral posterior neurons, LPNs. Those cells are specially implicated in temperature entrainment (Glaser, 2006; Shafer et al., 2006; Yoshii et al., 2005) and always number three to four cells (Shafer et al., 2006). Even though they were discovered a longer time ago still not too much is know about those cells (Kaneko and Hall, 2000; Kaneko et al., 1997).

1.9 Evening and Morning Oscillators

Back in 1976 Pittendrigh and Daan proposed a dual oscillator model based on observations in mammals. Certain light conditions caused desynchronization of the

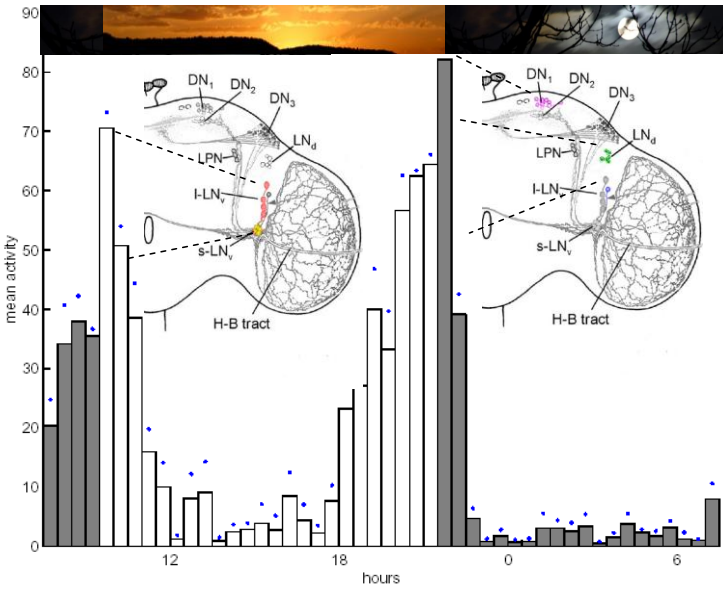


Figure 1-11 The morning and evening cells
Morning and evening activity of a fly are controlled by different subsets of clock neurons. The colored clock neurons are the main oscillator cells for the morning or evening, respectively. The diagram shows the average activity of several flies (n=8), whereby the empty bars represent the activity in the day, the dark colored bars the activity at night. The height of each bar, is positively correlated with the activity level.

internal pacemaker system. They proposed that the period of the morning oscillator is shortened by light, whereas the period of the evening oscillator is lengthened (Pittendrigh, 1976). Two independent studies tried to proof this theory, both using similar genetic mosaic techniques in *Drosophila*. They could reveal that the morning peak in the locomotor behavior is driven by the PDF-positive LN_vs (or now M cells). The evening behavior of the fly is driven by different clock groups, the LN_ds, the 5^{th} s-LN_v and a subset of DN_1 neurons (now called the E cells) (Grima et al., 2004; Rieger et al., 2006; Stoleru et al., 2004). An overview can be seen in Figure 1-11. It should be mentioned that this section is simplified. For example it is now known that the M cells control some aspects of the evening activity as well (Rieger, 2007). On the other hand data suggest that the DN cells contribute not only to the E peak, but also to the M peak (Helfrich-Forster et al., 2007b; Stoleru et al., 2004; Veleri et al., 2003) . The strict separation of E and M cells is hence not accurate.

1. 10 Neurochemistry of the *Drosophila* Circadian Clock

The presence of a large number of neuropeptides is reported in the brain of *Drosophila*. At least 35 different neuropetide precursor genes have been characterized in the fruit fly (Nassel and Homberg, 2006). The widespread clusters of pacemaker neurons suggest that they communicate via peptides. Until today several different neuropeptide transmitters have been identified as candidate signaling molecules in the *Drosophila* circadian clock.

Glutamate is expressed in some DN_1s and DN_3s (Hamasaka et al., 2007) and the neuropeptide Y family peptide NPF is found in three neurons in the LN_d cluster (Lee et al., 2006).

The 18 aa long pigment dispersing factor (PDF) is expressed in the LN_vs (Helfrich-Forster, 1995) and is derived from a 100 aa precursor (Renn et al., 1999). Flies carrying a null mutation in the *Pdf* gene show severe defects in their circadian locomotor activity (Renn et al., 1999). They can still adapt to light changes, but the typical diurnal profile is changed. The morning activity peak is absent and the evening activity peak occurs about 1.5 hrs earlier. Furthermore most of the animals loose their rhythmicity after three days in constant darkness. Although the mRNA and protein

are not rhythmically expressed, the accumulation of the peptide in the terminals of the small LN_vs follows a circadian rhythm (Park et al., 2000).

The receptor of the PDF peptide (PDFR or Han) was recently identified by several studies (Hyun et al., 2005; Lear et al., 2005b; Mertens et al., 2005). The expression pattern of this receptor was unclear until recently. In a novel study, Shafer et al. used a new technique to define the PDF receptive cells (Shafer et al., 2008). They could reveal that the receptive cells are widespread among clock neurons, with the only exception being the l-LN_vs. Other transmitters like acetylcholine, histamine, serotonin and GABA are also involved in the circadian system (Hamasaka and Nassel, 2006; Wegener et al., 2004; Yuan et al., 2005).

1.11 Electrical Activity in Clock Neurons

Synaptic inputs are transduced through transient membrane currents, and downstream outputs are driven by action potentials (APs). Electrical activity has been shown to be important for the function of the clock neurons in generating rhythmic behavior (Nitabach et al., 2002; Nitabach et al., 2005). Recent observation demonstrated that l-LN_v resting membrane potential (RMP), spontaneous AP firing rate, and membrane resistance are cyclically regulated as a function of time of day in 12 hrs light/dark conditions (LD) (Cao and Nitabach, 2008). In contrast, circadian defective per^{01} null mutant l-LN_v membrane excitability is nearly constant in LD. Furthermore electrical silencing of the pigment dispersing factor expressing ventral lateral pacemaker subset of fly clock neurons via expression of an inward-rectifier K^+ channel (Kir2.1) severely disrupts freerunning rhythms of locomotor activity (Nitabach et al., 2002). Those animals become arrhythmic in constant darkness conditions. Under light/dark cycles the locomotor activity is impaired as well. Here the evening activity peak is shifted towards the middle of the day – phenocopying Pdf^{01} mutant animals (Wu et al., 2008). Additionally electrical silencing in the LN_vs abolishes the free-running molecular rhythms in those cells but not the molecular rhythms in LN_d, DN_1, and DN_2 subsets of clock neurons (Wu et al., 2008). As yet the endogenously expressed ion channels and membrane proteins that influence electrical membrane properties of the circadian clock neurons are unknown. Several ion channels have been linked to the *Drosophila* circadian clock, for example the Narrow Abdomen (Na) channel (Lear et al., 2005a), Slowpoke (Slo) (Fernandez et al., 2007) or Shaw (Hodge and Stanewsky, 2008).

1.12 Aim of this Work

When I began to work on circadian rhythms back in 2004 many important facts concerning the circadian photoreception in general and especially in *Drosophila* were already revealed. The main circadian photoreceptor was known (Emery et al., 1998; Stanewsky et al., 1998), the light sensitivity of the Timeless protein and it's function in resetting the circadian clock was already revealed (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). Moreover the impact of light with different photoperiods, different intensities, and the impact of constant light conditions on *Drosophila's* circadian clock was uncovered (e.g. (Emery et al., 2000; Konopka et al., 1989)). But still there was much more in the darkness than known and understood. For example, what is the exact way of the light into the different clock neurons? Can one separate the different neurons into a light and dark oscillator? Who are the other photoreceptors next to Cryptochrome?

Two genetically different fly strains were found in our laboratory that showed an abnormal behavior under constant light conditions. They still behaved rhythmic, though the light should lead to a massive degradation of Tim and thus to a breakdown of the circadian clock. This fact suggested a severe defect in the circadian photoreception of these animals and so it was tempting to further investigate the genetic origin of this malfunction.

In one variant the RNAi mediated knockdown of the gene *lethal (2) 05510* in clock neurons resulted in rhythmic behaviour under LL conditions, however with a period longer than 24 hrs (Peschel, 2004). The other variant, from here on called *Veela*, exhibited an even more drastic phenotype in constant light conditions, namely an almost wild-type period length in LL, resembling the cry^b phenotype (Veleri, 2005).

The investigation of these two strains was expected to lead to new insights into the circadian photoreception of *Drosophila* and perhaps to contribute to a broader knowledge of circadian photoreception and regulation in other living beings, including mammals like the model organisms mouse and rat or us humans.

In a side project of my Ph.D. work, another circadian clock related gene was investigated. A mutation in the Ig transmembrane receptor family protein Roundabout (Robo) causes a shortened circadian period in constant dark (DD) conditions (Berni et al., 2008). Here I was involved in determining the role of *robo* in the circadian clock.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals, enzymes and consumables

If not mentioned otherwise all the consumables were ordered from Roth, Karlsruhe; Merck, Darmstadt; Sigma, St. Louis USA, Fisher, Loughborough ,UK; Eppendorf, Cambridge, UK. Enzymes came from: NEB, Schwalbach; Roche, Basel; Promega, Mannheim; Invitrogen, Karlsruhe; Qiagen, Hilden.

2.1.2 Fly, Bacteria and Yeast Strains

Drosophila melanogaster:

The flies were reared at 25°C or 18°C and 65% humidity in glass or plastic vials with fly food and dried yeast. The ambient regime was set to 12 hours of light and 12 hours of darkness (12:12 LD). The standard fly food was prepared as follows:

1 liter of water, agar 10 g, sucrose 15 g, glucose 33 g, yeast 35g, maize meal 15 g, wheat germ 10g , treacle 30 g, soy flour 1 spoon full, nipagin 10ml and proprionic acid 5ml.

Strain	Description	Literature
Controls		
Canton S		(Konopka et al., 1989)
Df(1) y w		(Lindsley and Zimm, 1992)
general		
y w ;Bl/CyO	balancer 2nd chromosome	(Lindsley and Zimm, 1992)
y w ;H/TM3	balancer 3rd chromosome	(Lindsley and Zimm, 1992)
robo		
robo^{hy} ;8.0 luc 2		this work, R.Stanewsky
8.0 luc 2		(Veleri et al., 2003)
tim-luc-9; robo^{hy}		this work, R.Stanewsky
tim-luc-9		(Stanewsky et al., 1998)
plo: 868; robo^{hy}		this work, R.Stanewsky

Strain	Description	Literature
plo: 868		(Stanewsky et al., 1998)
y w ; robo^{hy}; BG-luc		this work, R.Stanewsky
y w ; BG-luc		(Stanewsky et al., 1997)
quasimodo		
Df(3R) Exel 6144	3R:1328532;1438438	(Parks et al., 2004)
CCAP-Gal4		(Park et al., 2003)
cyc^{01} and cyc^{02}		(Rutila et al., 1998)
UAS-AnfGFP		(Rao et al., 2001)
UAS-Pdf		(Täuber, 2000)
Pdf^{01}		(Renn et al., 1999)
Ep2586		(Rorth, 1996)
P(PZ)l(2)05510/CyO		(Stempfl, 2002)
UASgfp	as in (Helfrich-Forster et al., 2007)	Bloomington Stock Center
actinGal4	described in (Botella et al., 2004)	Bloomington Stock Center
y w; cry^{b}		(Stanewsky et al., 1998)
elavGal4		(Luo et al., 1994)
gmrGal4		(Moses and Rubin, 1991)
pdfGal4		(Park et al., 2000)
timgal4(16),(27),(67),(62)		(Kaneko and Hall, 2000)
Clk^{Jrk}		(Allada et al., 1998)
y per01 w		(Konopka and Benzer, 1971)
1-17/CyO		(Stempfl, 2002)
Veela		
most of the investigated animals are cited in (Peschel et al., 2006) and (Peschel et al., 2008)		
CryGal4(16)		(Emery et al., 2000)
UAS-Sgg		(Martinek et al., 2001)
Sgg$_{RNAi}$	Transformant ID:7005	(Dietzl et al., 2007)
Jet$_{RNAi}$	Transformant ID: 45618	(Dietzl et al., 2007)
timGal4 (62)		(Kaneko and Hall, 2000)

Bacteria:

XL1 Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI$_q$ZΔM15* Tn*10* (Tet$_r$)]. (Stratagene)

Yeast:

EGY48 : *MATα trp1, his3, ura3,* lexAops-LEU2 (Rosato et al., 2001)

The yeast was incubated at 30°C in the light. For experiments in darkness the yeast was wrapped with two layers of aluminum foil.

Cells:

S2 cells:

The S2 cell line was derived from a primary culture of late stage (20-24 hours old) *Drosophila melanogaster* embryos (Schneider, 1972).

The cells were grown in Insect Xpress medium (Cambrex) with 10% fetal bovine serum (Sigma-Aldrich) at 25°C. The cells were kept in 25cm² cell culture flasks (Corning) in 10ml of medium, cells were split every 5 days.

2.1.3 Oligonucleotides and Vectors

Oligonucleotides:

The oligonucleotides were obtained from Invitrogen or Sigma-Aldrich. They had no extra modifications and were desalted.

Name	Sequence	Purpose
quasimodo		
PDF EcorI	GAA TTC GTT CAT TCG CAA GTC TCC TG	S2 cell experiments
PDF XbaI	TCT AGA TTG TTG TAC CAG ATT TCA AGT CG	S2 cell experiments
Cg31547 RNAi EcoRI	GAA TTC AAA CGC CGT GCG ACA GTC	Transformation adult fly
Cg31547 RNAi XhoI As	CTC GAG TGG GAA CGG AAA GCA AGT TG	Transformation adult fly
Cg31547 RNAi XbaI	TCT AGA CAG TTT ATT TTA GGG GGT C	Transformation adult fly
Cg31547 RNAi XhoI Sense	CTC GAG GGC TGG TGC AGG CCC TG	Transformation adult fly
Qsm EcoRI	GAA TTC ATG CTG CTC TCA ATG CAA ATG	Y2H
Qsm XhoI	CTC GAG CTA CAT CAG CCG CCA ACT C	Y2H
Cg31547 EcoRI	GAA TTC ATG GCC GAT CGC TTC CAG	Y2H
Cg31547 XhoI	TCT AGA CTA GGA GTA GAG CGT CAG CAC G	Y2H
microRNA XhoI	CTC GAG CCT AAC GGA TTG CGG ATT T	Transformation adult fly
microRNA EcoRI	GAA TTC AAT TTT CTG CTG CGG ATG	Transformation adult fly
Veela		
Jetlag sense	TGG GAT AGA AGT CGT TCA AGT	detection of *jet*^{c}
Jetlag antisense	TGC CGA TGG CTA ACA GAT	detection of *jet*^{c}
Jet seq 1 sense	AAT CTG CAT GAA CGG GTC G	Sequencing *jetlag*
Jet seq 1 antisense	CAC TGT TTG CGG CTA CGG	Sequencing *jetlag*

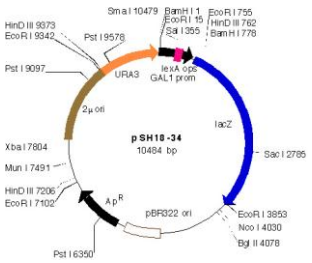
Jet seq 2 sense	CAG CTC CTT GCA CTC CAC	Sequencing *jetlag*
Jet seq 2 antisense	GAA ACC ACG GCG TAA GAT	Sequencing *jetlag*
Jet RNAi EcorI	GAA TTC TGC CGA TGG CTA ACA GAT	Transformation adult fly
Jet RNAi XhoI	CTC GAG CGG ACA ACG TCG AAT GAG	Transformation adult fly
Jet RNAi XbaI	TCT AGA TGC CGA TGG CTA ACA GAT	Transformation adult fly
Jet new EcoRI	GAA TTC ATG TGC ACT CTG CAC CCC	Y2H
Jet new XhoI	CTC GAG CTA GTA AAC AAG GAA ATC GCT G	Y2H
Jet-His XbaI	TCT AGA CGG TAA ACA AGG AAA TCG CTG	S2 cell experiments
Cry His EcoRI	GAA TTC ATG GCC ACG CGA GGG G	S2 cell experiments
Cry His XbaI	TCT AGA AAC CAC CAC GTC GGC CAG	S2 cell experiments

Vectors:

Yeast-2-Hybrid

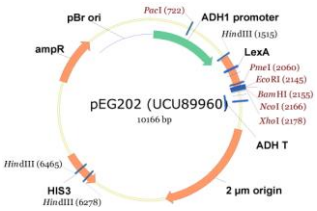
pSH18-34 (Invitrogen/Clontech):

LacZ reporter plasmid, containing eight operators for LexA (LexAop) binding inserted into the unique XhoI site located in the minimal GAL1 promoter (GAL1pro; 0.28 on map).



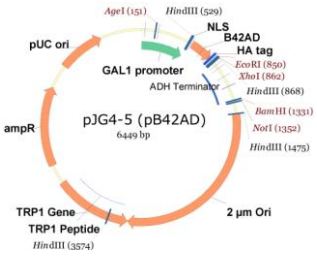
pEG202 (Invitrogen/Clontech):

Contains an ADH promoter that expresses LexA followed by polylinker.



pJG4-5 (Invitrogen/Clontech):

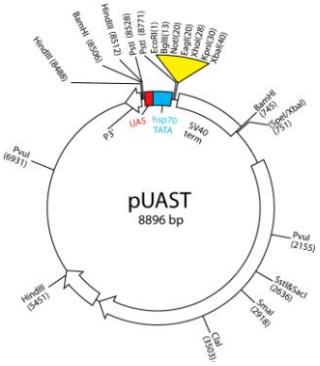
Contains a GAL1 promoter that expresses nuclear localization domain, transcriptional activation domain, HA epitope tag, cloning sites.



Germ line transformation

pUAST (Brand and Perrimon, 1993):

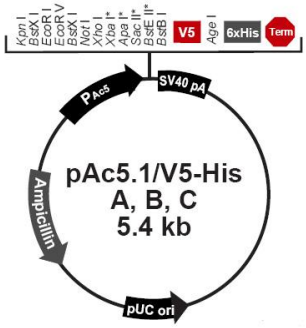
pUAST consists of five tandemly arrayed optimized GAL4 binding sites (red) followed by the hsp70 TATA box and transcriptional start (blue) and a polylinker (yellow).



S2 cells

pAC 5.1 (Invitrogen):

The pAc contains a Drosophila actin 5C (Ac5) promoter for high-level, constitutive expression of the gene of interest in S2 cells



2.1.4 Solutions and Buffers

If not obtained commercially the buffers were prepared as follows:

Name	Inhalt
PCR:	
-10XBuffer	200mM Tris-HCL(pH 8,4), 500mM KCL
-TAE	0,04 M Tris-acetate, 0,001 M EDTA
Gen. DNA:	
-Squishing Buffer	10 mM Tris–Cl (pH 8,2), 1 mM EDTA, 25 mM NaCl und 10 mg/ml proteinase K
Ligation and transformation:	
- Rapid Ligation Buffer	60mM Tris-HCl (pH 7,8) ; 20mM MgCl2 ; 20mM DTT; 2mM ATP; 10% Polyethylene Glycol
- LB Medium	1% Tryptone, 0,5% yeast extract, 1% NaCl
- LB plates	1% Tryptone, 0,5% yeast extract, 1% NaCl, 1,5%Agar
- STET Buffer	10mM Tris-Cl (pH 8,0); 0,1 M NaCl; 1mM EDTA (pH 8,0); 5% TritonX-100

Western Blot:	
- extraction buffer (stock)	HEPES 20mM (pH 7,5) ; KCl 100mM ; 5% Glycerol ;EDTA 10mM;0,1% Triton X-100; ß-Glycerophosphat 20mM; Na_3VO_4 0,1mM pH 10-12
- extraction buffer (fresh)	extraction buffer (stock); 0,5 mM PMSF (in Isopropanol); 20µg/ml Aprotinin; 1mM DTT; 5µg/ml Leupeptin; 5µg/ml Pepstatin (in DMSO)
- extraction buffer CoIP	20mM Hepes(pH 7.5); 100mM KCl; 5% Glycerol; 0,05% NP40, 1mM DTT, protease inhibitor mix
- Electro Phoresis (Elpho)buffer	1,5% Tris, 7,2% Glycin, 0,5% SDS
- sziklai-darlington diamond buffer (Sdbb)	25mM Tris Base; 150mM Glycin; 10% Methanol
- PonceauS	0,5% Ponceau S; 1% Eisessig
- blocking solution	1% BSA Fraction V (Roth) in 50 ml TBST
- TBS(T)	10mM Tris-Cl; 150mM NaCl (pH 7,5) ; (0,005 Tween 20 10%)
- loading buffer	0,3125 M Tris base; 10% SDS; 50% Glycerol; 25% β-Mercaptoethanol; 0,01 % Bromphenolblue
- resolving gel (4.5%)	14,0 ml H_2O sterile; 2 ml 1M Tris pH 6,8; 200 µl 10% SDS; 2,96 ml PAA 30%; 40 µl TEMED; 250 µl 10% APS; 0,6 ml BIS 2%
- stacking gel (e.g. 6%)	12,2 ml H_2O steril; 6,5 ml 1,5M Tris pH 8,8; 250 µl 10% SDS; 4,9 ml PAA 30%; 1,0 ml BIS 2% 12,5 µl TEMED; 125 µl 10% APS
Yeast	
YPD media	10g Yeast extract, 20 g peptone, 20 g Agar, 3ml NaOH, 100ml 20% Glucose (for 1 liter)
selection media (WO)	6,7 g Yeast Nitrogen Base (w/o aa), 20 g Agar (omit for liquid), proper aa, 100ml 20% Glucose (for 1 liter)
10x BU Salts buffer	70g Na_2Hpo_4-$7H_2O$, 30 g NaH_2PO_4, pH 7.0 (for 1 liter)
X-Gal media	6,7 g Yeast Nitrogen Base (w/o aa), 20g Agar, proper aa, 100ml 20% Galactose + 10% Raffinose, 100ml 10x BU salts, 4ml 20mg/ml X-Gal (for 1 liter)
ICC	
PBS 10x	NaH2PO4.H2O 2.83 g, Na2HPO4.2H2O 13.74 g, NaCl 90.00 g. Made up to 1 l with deionized water
PBT (0.3%)	Triton-X100 3 ml, PBS 10X 100 ml. Made up to 1 l with deionized water to get 1X PBT.

Mikroinjection:	
- injection buffer	5mM KCL; 0,1mM Na-Phosphatbuffer (pH 6,8)
- egg laying plates	1,75% Agar; 25% apple juice; 2,5% Sugar; 1% Nipagin (in Ethanol)

2.2 Methods

Genomic DNA

Genomic DNA was obtained by Single Fly DNA preparation (Gloor et al., 1993).

RNA

Isolation of RNA from tissues and cells has been carried out using the TRIZOL or TRIFAST reagent following the protocol as described (Peschel, 2004).

cDNA

RNA was transcribed using the SuperscriptII Kit (Invitrogen), Quantitect Rev RT Kit (Qiagen) or the Taqman Reverse Transcription Kit (Applied Biosystems) following the protocol.

PCR

Was carried out as described (Peschel, 2004). The polymerase was originally isolated from *Thermus aquaticus* (Taq) and has no proof reading capabilities.

qPCR

Quantification of the cDNA was carried out with a Roche Lightcycler as described (Peschel, 2004) or with a Chromo4 Detector (Bio-Rad) using the Power SYBR Green PCR reagent (Applied Biosystems) following the manufacturer's protocol.

Sequencing

PCR fragments (150-1000 bp) were separated using gel electrophoresis, extracted and purified with the miniEluteGel Extraction Kit (Qiagen). The DNA was quantified and

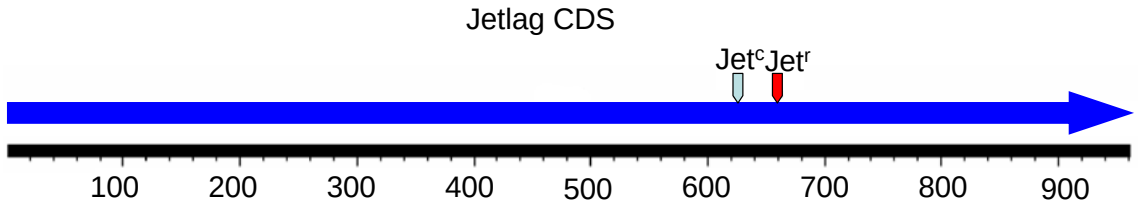
100ng DNA/ 500bp used in the sequencing reaction. For the sequence reaction the BigDye Terminator 1.1 Kit (Applied Biosystems) was used. After precipitation (Sodium acetate/ Ethanol) the DNA was resuspended in HiDi-formamide and detected by GENEART AG (Regensburg) or MWG Biotech (Martinsried).

Cloning

Bacteria were reared and transformed as described (Ausubel, 1996). For small scale plasmid isolation, alkaline lysis minipreps were prepared according to Holmes (Holmes and Quigley, 1981) or using a Qiaprep miniprep Kit (Qiagen). For large scale isolation a Qiaprep midiprep Kit (Qiagen) was used.
Ligation of DNA fragments were carried out with NEB's T4 Ligase and protocol or with the pGem T Easy Vector System (Promega).

In some cases PCR products were used for the cloning. Here a DNA fragment was created using PCR and unique primers carrying restriction sites. These products were purified with the help of gel electrophoresis and the miniEluteGel Extraction Kit (Qiagen) and then subcloned into pGem T Easy vector. All PCR fragments were sequenced to avoid cloning of wrong or mutated fragments.

Jetlag:



Name	Length (nt) Transcript	Length (nt) CDS	Length (aa)	Predicted MW (kD)
Jetlag-PA	1424bp	960bp	319	36,1 kDa

pEG-Jet, pEG-Jet^c, pJG-Jet, pJG-Jet^c

Those vectors were used for Y2H experiments.
The full length *jetlag* or *jetlag^c* coding sequence (960bp) was amplified using *y w* or *Veela* cDNA and the Jet Y2H primer. After subcloning into pGem T Easy the plasmid was EcoRI/XhoI digested and finally ligated into the pEG202 or pJG4-5 vector.

pAc-Jet, pAc-Jet^c, pAc-Jet-His, pAc-Jet^c-His

Those vectors were used for S2 cell transformation.

Further on the *jetlag* and *jetlag^c* EcoRI/XhoI fragments were subcloned into the pUAST vector, respectively, and afterwards EcoRI/XbaI digested. The new fragments were then cloned into the pAc5.1 vector.

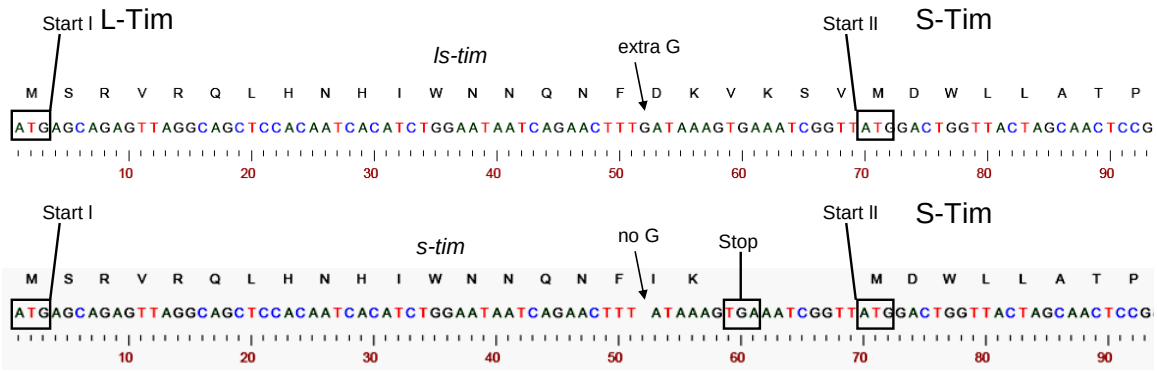
For the cloning of *pAc-Jet-His* and *pAc-Jet^c-His* vector similar cloning steps were applied, only using hereby the Jet-His primer. With the help of this primer the endogenous *jet* stop codon was eliminated, resulting in a Jet-V5-His and Jet^c-V5-His fusion protein, respectively.

pUAST-Jet-RNAi

This vector was used for stable transformation of adult *Drosophilas*.

Genomic DNA and cDNA from *y w* animals was used to generate the two inverted RNAi fragments by PCR and JetRNAi primers. The general cloning steps were accomplished as described (Peschel, 2004).

Timeless:



Name	Length (nt) Transcript	Length (nt) CDS	Length (aa)	Predicted MW (kD)
Timeless-PD	5035 bp	4266 bp	1421	159.2 kDa

pJG-s-Tim, pJG-ls-Tim, pAc-s-Tim, pAc-ls-Tim

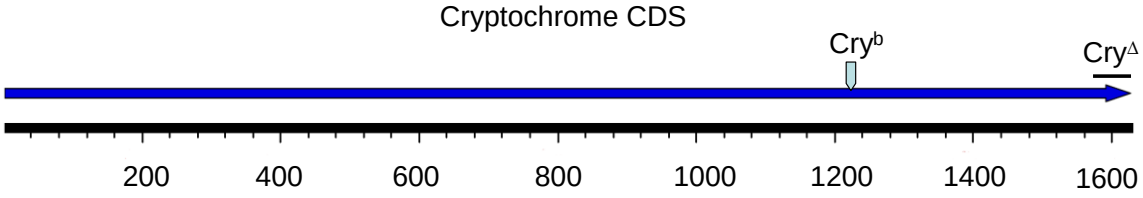
The *pJG* vectors were used for Y2H experiments, while the *pAc* vectors were used for S2 cell transformation.

The pEG-s-Tim and pEG-ls-Tim vectors were a gift from E. Rosato and are described elsewhere (Sandrelli et al., 2007). The vectors were **partially** EcoRI/XhoI digested and the 4.3kb sized EcoRI/XhoI full length Timeless fragments were subcloned into

the pUAST plasmid and finally ligated after an EcoRI/XbaI digest into the pAc5.1 vector.

Cryptochrome:

Name	Length (nt) Transcript	Length (nt) CDS	Length (aa)	Predicted MW (kD)
Cry-PA	1859 bp	1629 bp	542	62,5 kDa



pEG-Cry, pEG-Cry$^{\Delta}$*, pEG-Cry*^{b}

Those vectors were used for Y2H experiments.

The pEG-Cry, pEG-Cry$^{\Delta}$ and pEG-Cry^{b} vectors were a gift from E. Rosato and were described elsewhere (Rosato et al., 2001).

pAc-Cry, pAc-Cry$^{\Delta}$*, pAc-Cry*^{b}*, pAc-Cry-His*

Those vectors were used for S2 cell transformation.

pEG-Cry, pEG-Cry$^{\Delta}$ and pEG-Cry^{b} were EcoRI/XhoI digested and the obtained cry fragments subcloned into the pUAST vector and finally ligated after an EcoRI/XbaI digest into the pAc5.1 vector.

For the generation of the pAc-Cry-His plasmid a PCR based strategy was performed, using the Cry-His primer. After subcloning into pGem T Easy the fragment was obtained by EcoRI/XbaI digestion. This fragment was finally introduced into the pAc5.1 vector.

The Cry-His XbaI primer eliminates the endogenous stop codon thus creating the pAc-Cry-V5-His vector.

*pAc-Luc-Cry, pAc-Luc-Cry*Δ

Those vectors were used for S2 cell transformation.

The pAc-Luc-Cry, pAc-Luc-CryΔ were a gift from R. v. Gelder and are described elsewhere (VanVickle-Chavez and Van Gelder, 2007).

pUAST-Luc-Cry

This vector was used for stable transformation of adult *Drosophilas*. The pAc-Luc-Cry plasmid was EcoRI/XbaI digested and the resulting fragment cloned into pUAST.

Shaggy:

Name	Length (nt) Transcript	Length (nt) CDS	Length (aa)	Predicted MW (kD)
Shaggy-PE	3070 bp	1545 bp	514	53,9 kDa

The pAc-Sgg-V5-His vector was a gift from M.Rosbash and is described elsewhere (Stoleru et al., 2007). This vector was used for transformation of *Drosophila* S2 cells.

Quasimodo:

Name	Length (nt) Transcript	Length (nt) CDS	Length (aa)	Predicted MW (kD)
l(2)05510-PA	1812 bp	1245 bp	414	45,6 kDa

pUAST-qsm, pAc-qsm, pEG-qsm, pJG-Qsm

pUAST-qsm was used for stable transformation of adult *Drosophilas*. *pAc-qsm* was used for transformation of *Drosophila* S2 cells. *pEG* and *pJG-Qsm* were used for Y2H experiments.

The full length coding sequence of the *qsm* gene was PCR generated with the *qsm* Y2H primer. After pGem T Easy subcloning the EcoRI/XhoI fragment was inserted into the pEG202, pJG4-5 and pUAST vector. For the generation of the pAc-qsm vector the pUAST-qsm was EcoRI/XbaI digested and the resulting fragment cloned into the pAc5.1 vector.

Cg31547:

Name	Length (nt) Transcript	Length (nt) CDS	Length (aa)	Predicted MW (kD)
CG31547-PB	3891 bp	3207bp	1068	117.9 kDa

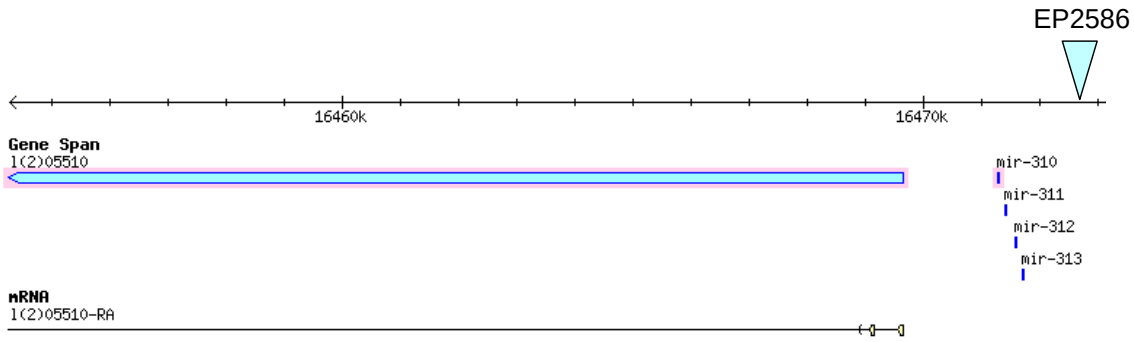
pUAST-Cg31547-RNAi, pUAST-Cg31547, pAc-Cg31547

We used the *pUAST* vectors for for stable transformation of adult *Drosophilas*. The *pAc* vector was used for transformation of S2 cells.

The pOT2 vector containing the GH09711 clone (DGRC Gold Collection) was EcoRI/XhoI digested and the resulting fragment cloned into the pUAST. The GH09711 was not sequenced, because its sequence can be found online (Flybase). This vector was used as template to generate a PCR fragment with the Cg31547 Y2H primer. The PCR fragment was subcloned into pGem T Easy, EcoRI/XhoI digested and cloned into pJG4-5 and pEG202 vector. pUAST-Cg31547 was digested to obtain the EcoRI/XbaI Cg31547

fragment and subsequently ligated into pAc5.1 vector.The Cg31547-RNAi vector was created with a PCR based strategy, using *y w* gDNA and cDNA and the Cg31547 RNAi Primer. The same strategy as in (Peschel, 2004) was followed to create the pUAST-CG31547-RNAi construct.

miRNA:



This figure displays an overview of the insertion site of the microRNAs. The gene span shows the location of the *l(2)05510* gene. Under the gene span one can see the mRNA. Preceeding the *l(2)05510* gene is the microRNA cluster mir-310 to mir-313. *EP2586* is a *P*-element insertion. For more details see Figure 3-5.

The microRNA cluster plasmid, containing the miRNAs 310-313 was created using a PCR based strategy, *y w* gDNA and the miRNA primers. After subcloning into pGEM T Easy an EcoRI/XbaI microRNA fragment was created and cloned into the pUAST vector. We used the *pUAST* vectors for for stable transformation of adult *Drosophilas*.

PDF:

pAc-Pdf

This vector was used for transformation of *Drosophila* S2 cells.

The proPDF DNA was obtained using a PCR based strategy – *y w* gDNA and a PDF primer were used. After subcloning into pGEM T Easy a EcoRI/XbaI PDF fragment was created and cloned into the pAc5.1 vector.

Packard:

Adult flies

Adult flies carrying the *luciferase* gene under circadian promoter control were fed with luciferin containing food, the resulting bioluminescence measured with a Perkin Elmer TopCount NXT. This assay was performed as in (Stanewsky et al., 1997). For a very detailed protocol see (Glaser, 2006).

Cell culture

A 96 well plate (Packard OptiPlate) was filled with 10000 cells/well, whereas only each second well was used. After 24 hours transfection was performed with jetPEI Cationic polymer transfection reagent (PolyPlus Transfection), following the manufacturer's protocol. See also Materials and Method part in Peschel et al., 2008.

Vessel	Amount of DNA (µg)	Volume of NaCl(µl)	Volume of jetPEI(µl)	Volume of jetPEI solution
96-well	0,125	10	0,5	10

After transfection 1% Bioluciferine (=Luciferin (Biosynth, Staad, CH) was added and the 96 well plate sealed with a thin transparent polyethylene sheet. The cells were kept for another 24 hours wrapped in aluminum foil (to avoid early degradation of the Luciferin) and then inserted into the Topcount machine. The detection in the machine was performed at 25°C whereas one plate was measured for 6 minutes in complete darkness. After each measurement the 96 well plate was exposed for another 8 minutes to the surrounding light regime (i.e. Light on or Light Off, depending on the experiment). This cycle was repeated a few hundred times over several days.

Behavior

The locomotor behavior of adult flies was investigated as described (Peschel, 2004).

Antibodies

Antibody	host	marker	dilutionWB	dilution tissue	origin
1st Antibody					
Timeless	rat		1:10000	1:1000	Kaneko et al.,1997
Timeless	rat		1:2000	1:500	Young
Timeless	rat		1:2000	1:500	Sidote et al.,1998
Period	rabbit		1:10000	1:1000	Stanewsky et al.1997a
Cryptochrome	rabbit		1:10000		Yoshii et al.,2008
Cryptochrome	guinea-pig		1:10000		Stanewsky
Cryptochrome	guinea-pig		1:10000		Kyriacou
Quasimodo	rat		1:1000	1:100-1:1000	Stempfl 2002
PDH	rabbit			1:2000	Dirksen et al., 1987
preproPDF	mouse			1:100	Veleri et al., 2003
PDF (*Drosophila*)	rat			1:500	Park et al., 2000
His	mouse		1:5000		Invitrogen
Beta-Gal	mouse			1:200	Promega
2nd Antibody					
rat	goat	HRP	1:25000		Pierce
rat	goat	AlexaFluor 594		1:200	Mol Probes
rabbit	goat	HRP	1:100000		Pierce
rabbit	goat	AlexaFluor 488		1:200	Mol Probes
mouse	goat	HRP	1:2000		Amersham
mouse	goat	AlexaFluor 647		1:200	Mol Probes

Westernblot and Initial S2 Cell Transfection Experiments

The Western blot experiments with proteins from adult *Drosophilas* were performed as reported (Peschel, 2004). For the detection of smaller proteins (less than 70kDa) (QSM, CRY, SGG-His, JET-His) a 10% stacking gel was used, for larger proteins like PER or TIM 6% stacking gels were used.

Western blot from S2 cell proteins were performed as follows:

S2 cells were grown in Insect Xpress medium (Cambrex) with 10% fetal bovine serum at 25°C. For Western blots 100 000 cells, for CoIP assays 2 million cells, and for bioluminescence measurements 15 000 cells were transfected with jetPEI

according to the manufacturer's instruction. The cells were harvested after 48 hours. For proteasome inhibitor experiments MG-132 (50µM) was added. For the bioluminescence measurement 2mM of luciferin was added. For Western blots, cells were transfected with 250 ng to 333 ng of plasmid (from each plasmid, so for example 333 ng from pAc-*cry* and 333 ng from pAc-*sgg*), for CoIP experiments up to 1 µg was used, and for the luciferase experiments cells were transfected with 25 to 40 ng of plasmid. The overall amount of transfected DNA was always the same. Unrelated DNA was used to co-transfect the cells if needed to ensure an equal amount of DNA.

S2 cell protein extraction was performed as follows: Cells were harvested by centrifugation and resuspended in protein-extraction buffer. Loading buffer was added and the cells were boiled before loading the gel. Adult *Drosophila* heads were separated from the bodies, counted and homogenized in protein-extraction buffer. After 5' centrifugation at 4°C supernatant was transferred into a new cup, Loading buffer was added and the protein boiled before loading the gel. Gels were run overnight at 70 V and blotted using a semi-dry blotting apparatus. For a full sized gel (9 cm x 14 cm) we applied 400mA for 90 min. The blot was incubated in blocking solution for 2 hrs. First antibody was added and the blot incubated o/n shaking at 4° C. Blots were rinsed 3x and washed 3x 5' in TBST. Secondary antibody was added and the blot was incubated for two hrs at RT. After repeating the rinsing and washing steps from above the proteins were detected using chemiluminescence substrates for HRP (Pierce).

CoIP

Per sample 25µl of sepharose beads (Protein G Sepharose Fast Flow Beads, Amersham) were used. They were spun down at 2k rpm for 1 min, the supernatant discarded and 1 ml of the CoIP extraction buffer added. After another centrifugation step 1 ml CoIP extraction buffer and 1µl of the antibody per sample was added. The beads were incubated for at least 1 hour at 4°C, rotating. The beads were then spun down and resolved in 40µl fresh extraction buffer per sample. 50µl of this solution was added to the extracted protein. Proteins from S2 cells grown in a six well plate (2ml per well) were extracted as follows. The cells were harvested spinning them down at 4.5k rpm for 4 minutes. After discarding the supernatant 300µl of CoIP extraction buffer was added.

The cells were homogenized and spun down at 5k rpm for 5 minutes. After this step 30 µl of the supernatant were separated. 8µl of the sample loading buffer was added to those 30µl and the sample boiled at 95°C for 4 minutes and frozen at -80°C. The remaining supernatant was added to the 50µl of beads (prepared s. above) and the CoIP rotated overnight at 4°C.

The next day the CoIP samples were spun down 2 minutes at 2k rpm. From the supernatant 30µl were taken, provided with 8µl sample loading buffer, boiled at 95°C and frozen at -80°C. The remaining supernatant was discarded and the beads washed three times with 750µl extraction buffer. After washing, the beads were resuspended in 30µl extraction buffer and boiled with 8µl sample buffer.

Immunocytochemistry (ICC)

Initially flies were entrained for three days to a 12:12 LD cycle at 25°C. To harvest the flies they were anaesthetized with CO_2 and put into the fixing solution. For darkness time points, the fly vials were wrapped with aluminum foil. After two hours of fixation at room temperature the samples were quickly washed three times with PBS. The brains were dissected and cleared from trachea with the help of fine forceps. After another 15 min fixation step the fixing solution was discarded and the brains were washed another 3 times with PBS. The fixed brains were then blocked for 60 min with normal donkey serum (2% NDS in 0.1% PBT). After blocking, primary antibodies were added (dilution see above) and incubated at 4°C for 48 hrs. Brains were washed with 0.3% PBT (5x 15 min) and secondary antibodies, diluted to 1:200 (diluted in 0.3% PBT), were added and the mixture was incubated at 4°C overnight. Finally the brains were mounted in Vectashield on a slide, after another washing step with 0.3% PBT (5X 15 min).

In addition Period antibodies were preabsorbed before dilution against per^{01} embryos; Stanewsky et al., 1997b.

***P*-element Transformation**

Microinjection and *P*-element transformation were performed as described in (Peschel, 2004) with the exception of the pUAST-Luc-Cry plasmid. This plasmid was injected by the Bestgene Company (www.bestgene.com) into *y w* flies.

Yeast-Two-Hybrid

A single yeast colony was inoculated in 6 ml YPD (or the proper selection media). After growing overnight at 30°C (slowly shaking) the cells were harvested. Yeast cells were centrifuged at 6krpm for 2 minutes, the supernatant removed and the cells resuspended in 100 µl of 0.1M LiAc. After 5 minutes of incubation at 30°C the cells were recentrifuged, the supernatant removed and the following ingredients added in the specified order:

240µl (50%) PEG 3350
36µl 1M LiAc
5µl 10mg/ml Salmon Sperm DNA (Invitrogen), boiled at 95°C for 5 min before use.
1.5µg of each plasmid
65µl dH_20

The cells were vortexed for a longer time (about one minute) until fully resuspended. After 30 min of incubation at 30°C they were heat shocked at 42°C for 20 min.
After another centrifugation step at 6krpm (3 min) the cells were resuspended in 200µl of water and very carefully plated onto the proper selective agar plate.
After 40-60 hours of growth at 30°C the resulting transformant colonies were picked and restreacked onto a new selective plate. The cells were amplified for another 24 hours at 30°C and then transferred to the X-Gal plate. After another 12 hours of incubation the interaction should be clearly visible as blue precipitate.

3. Quasimodo

3.1 Background

As a new way for isolating rhythmically expressed genes in *Drosophila* Thomas Stempfl introduced a modification of the classical enhancer trap method. He generated flies carrying a *P*-element construct containing the gene encoding firefly *luciferase* under the control of a weak but constitutively active promoter (Stempfl et al., 2002). In an attempt to isolate circadianly regulated enhancers and genes he then screened for flies expressing this construct rhythmically. Among the 1200 lines about 5 % turned out to be rhythmically expressing ones (Stempfl et al., 2002).

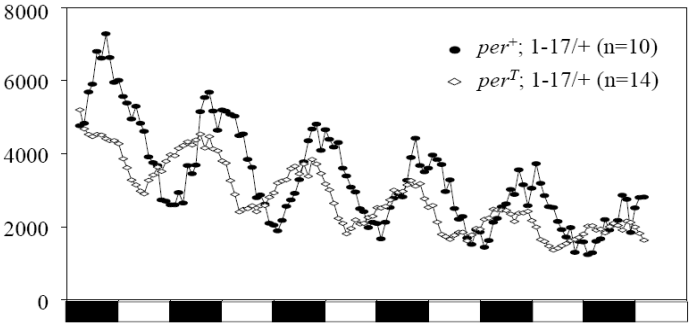


Figure 3-1
Bioluminescence Rhythm in 1-17
The enhancer trap line 1-17, with a *P*- element insertion in the first intron of *Cg13432* clearly showed rhythmic bioluminescence. The experiment was performed in Light/Dark cycles in wild-type *per* and per^T background.

One of the enhancer trap lines that showed rhythmic expression was 1-17 (Figure 3-1). Here the *P*-element insertion was revealed to be located in the first intron of *lethal (2) 05510* or *Cg13432* (Figure 3-5). The bioluminescence rhythm in 1-17 is abolished in clock mutants like per^{01} animals (data not shown), or the period is changed in clock mutants like per^T (Figure 3-1). *P*-element insertions in this gene are connected with developmental phenotypes for example a malformed thorax – *Cg13432* deletions or null mutants are homozygous lethal. The morphological phenotype of the homozygous semi-lethal (only a few escapers survive until adulthood) 1-17 animals – a hunchback - was the reason why this gene was renamed to *quasimodo* (*qsm*) (Stempfl, 2002). Furthermore Stempfl discovered with the help of RNase protection assays that the mRNA of *qsm* shows a circadian fluctuation. The *qsm* mRNA peaks by the end of the night, while it has it's trough by the end of the circadian day. Those results where later verified (Figure 3-2) by qPCR (Peschel, 2004). The involvement in the development of the fly did not allow behavioral studies with certain hypomorphic homozygous alleles or with animal lacking *qsm* completely. To bypass this problem a *P*-element carrying fly strain was generated. In those flies a *qsm* RNAi construct

under the control of an UAS promoter was brought under the control of a circadian clock neuron specific Gal4 driver line.

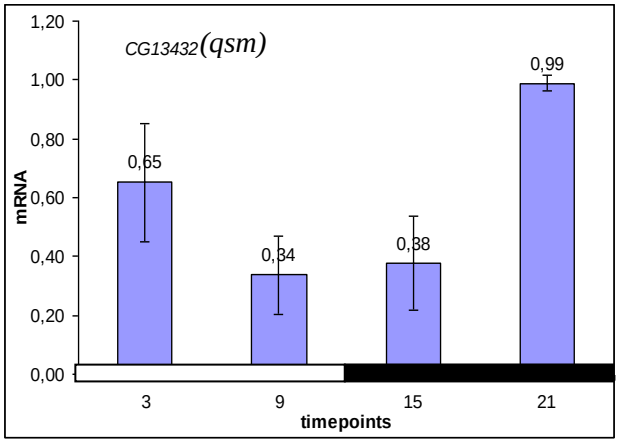


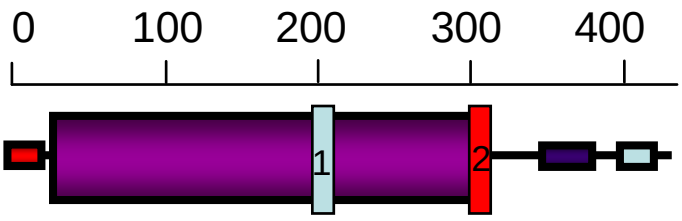
Figure 3-2
Rhythmic expression of *qsm* mRNA
qPCR results from *y w* animals demonstrate a rise in mRNA at ZT21. The peak level was set to one; the other values were calculated relatively to the maximum. Pictured are the mean values of five individual experiments.

First preliminary behavior experiments linked Qsm to circadian photoreception, because RNAi mediated down-regulation of *qsm* caused abnormal rhythmic behavior in LL (Peschel, 2004). This prompted further behavioral experiments, localization of Qsm, and determination of *quasimodo's* influence on the clock proteins Per and Tim in order to reveal *qsm's* function in the circadian clock.

3.2 Introduction

3.2.1 Quasimodo is a Zona Pellucida Protein

The *quasimodo* gene is located on the right arm of the second chromosome. The 1812 bp long mRNA encodes a 414 aa sized protein with a predicted molecular weight of 45.1 kDa. Its molecular function and the biological processes in which it is involved are not known. *In silicio* analysis reveal several predicted domains (Figure 3-3).



Name	Begin	End
Signal peptide	1	23
Peptide 1 (AB)	191	205
Peptide 2 (AB)	302	317
ZP domain	30	300
coiled coil	334	363
low complexity	384	402

Figure 3-3 Quasimodo protein domains
The cartoon shows an overview of Qsm. With almost 300 aa the Zp domain (purple) represents the vast majority of the protein. The two peptides that were used for the antibody production are numbered one and two.

Signal peptide: MLLSMQMWRSLWLAALFCGLAQA. The first 23 aa of Qsm are a signal for the transport of the protein to the membrane. The peptide is cleaved afterwards and is not present in the mature protein.

ZP Domain:

The ZP domain is a common domain in eukaryotic proteins. The abbreviation ZP stands for **z**ona **p**ellucida, the extracellular coat that surrounds all mammalian eggs. This coat is only composed of proteins that contain the ZP domain (ZP1-3). The ZP domain consists of about 260 amino acids with eight conserved cysteine residues and is often glycosylated. Normally all ZP domain proteins share an N-terminal signal peptide that targets them to the secretory pathway through cotranslational import (that means proteins are put into organelles or membranes **during** the actual process of translation) into the endoplasmic reticulum (ER). The functions of ZP domain proteins vary substantially. For example ZP proteins can function as a structural component of eggs, as mechanotransducers or sperm receptors (Jovine et al., 2005).

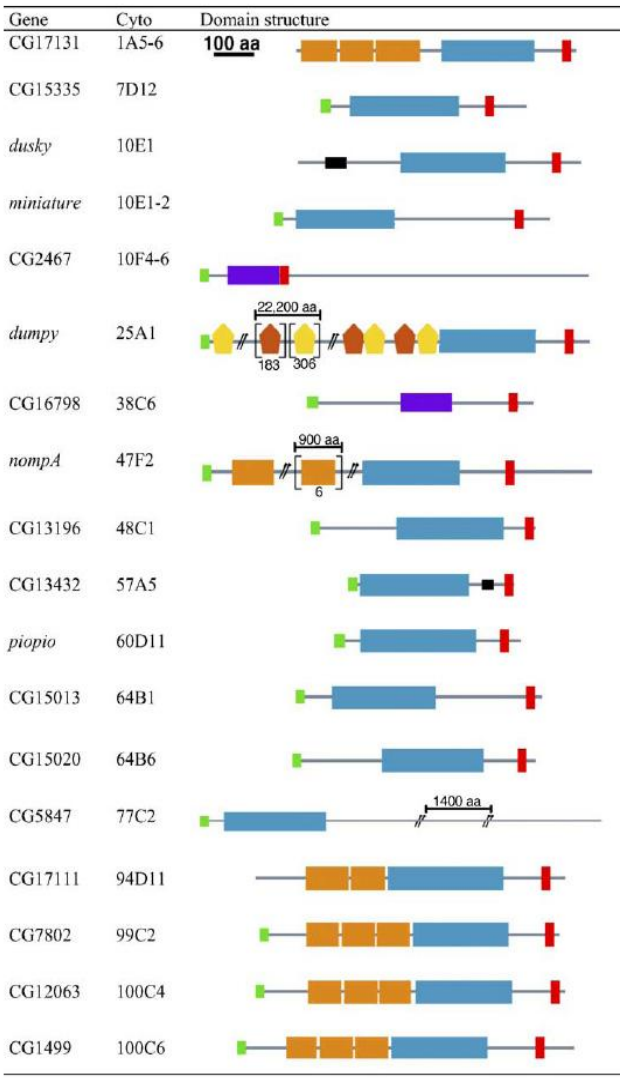


Figure 3-4 Overview of the Drosophila ZP proteins
Picture taken from (Jazwinska and Affolter, 2004)

The different domains are as follows:

signal peptide; PAN domain; EGF domain; DPY module; coiled coil region
transmembrane domain; ZP domain ZP-like domain

Precursors of functional ZP domain proteins generally share a stretch of hydrophobic amino acids following the ZP that either constitutes a single-spanning transmembrane helix or directs attachment of a glycosyl phosphatidylinositol-anchor (GPI-anchor) to nascent polypeptides in the ER. These

features localize the proteins to secretory vesicles that transport them to the plasma membrane of the cell. In *Drosophila* about 20 different ZP domain proteins are known, whereas often the ZP domain is only one of several recognizable motifs found in those proteins (Figure 3-4).

Coiled Coil:

A coiled coil structure is a type of secondary structure composed of two or more alpha helices which entwine to form a cable structure. Many coiled coil type proteins are involved in important biological functions such as the regulation of gene expression e.g. transcription factors (Burkhard et al., 2001). *In silicio* analysis of Qsm revealed that the coiled coil part of Qsm shows remote homologies with the Prickle protein. The functional role of *prickle* lies in the development where they possibly regulate positive neurite formation during brain development (Okuda et al., 2007).

In general one can say not too much about the function of Qsm based on its sequence information. It is an extracellular membrane protein that is linked via a GPI – anchor to the membrane. The whole protein consists of almost only the large ZP domain – no other important domain is visible. The functions of ZP domain proteins vary dramatically. So one can only speculate if Qsm acts as a receptor, mechanotransducer or as a structural component. Despite the different functions of ZP domain proteins it is likely that the domain plays a similar role in all the proteins. From the about 20 ZP domain proteins of *Drosophila* only a few are molecular characterized: *dusky*, *miniature*, *dumpy*, *nompA* and *piopio*.

The *miniature* and *dusky* genes are expressed in cuticle-secreting epithelia and are involved in generating the morphology of adult wing cells. For both *miniature* and *dusky* mutants, wings have a normal number of correctly patterned epidermal cells, but the size of individual cells is significantly reduced. Consequently, wings of mutant flies are smaller than wild-type wings (Roch et al., 2003). It was believed for a long time that *dusky* is involved in the circadian clock. A *dusky* mutation from an ethylmethanesulfonate (EMS) screen, called *Andante*, showed an abnormally long circadian period (Newby et al., 1991). But in fact an additional mutation in *Andante* caused the circadian phenotype. The additional second mutation was found in the *ckIIβ* gene. *dusky* mutations have no effect on rhythmicity (Akten et al., 2003).

nompA is specifically expressed in type I sense organs of the peripheral nervous system by the support cells that ensheath the neuronal sensory process. The structure and location of NompA suggest that it forms part of a mechanical linkage required to transmit mechanical stimuli to the transduction apparatus (Chung et al., 2001).

piopio and *dumpy*. Dumpy is a membrane-anchored fiber (~1µm in length) that is present within the cuticle and provides a strong anchor for the underlying tissue, thereby allowing it to maintain mechanical tension at sites under stress. It is proposed that Dumpy and Piopio interact with each other, possibly through their ZP domains, to form filaments that provide a structural network in the lumenal space of the trachea (Bokel et al., 2005).

3.2.2 The *quasimodo* gene locus

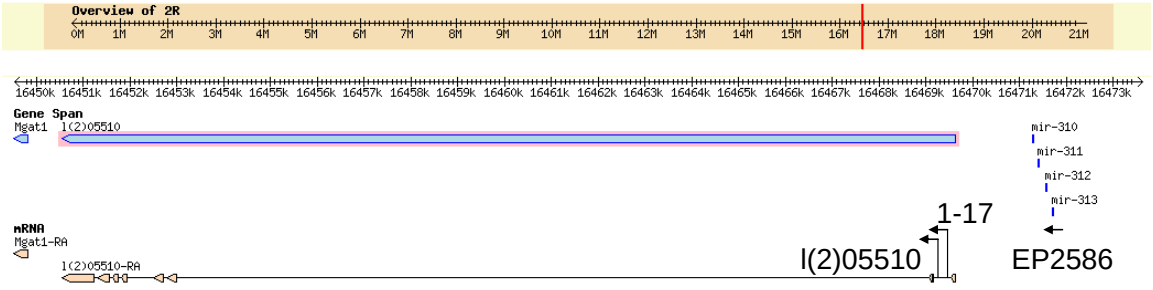


Figure 3-5 The *quasimodo* gene – an overview
The picture shows that *qsm* is located on the right arm of the second chromosome on position 2R:16,450,540...16,469,633 [-]. Under the gene span one can see the mRNA. The next gene after *qsm* is *Mgat1*. The gene preceding *qsm* is the miRNA cluster 310-313. The arrows indicate *P*-element insertions and orientations. The luc-sniffer construct 1-17 is inserted in the first intron. The lacZ line *l(2)05510* can be found almost at the same place as 1-17 in the first intron. The UAS over-expression line from the Rorth collection *EP2586* (Rorth, 1996) is inserted shortly before the start of the *miRNA-313*.

qsm is loacated on the second chromosome (2R), 57A5-57A6. In it's promotor region a microRNA cluster can be found, consisting of *mir-310* to *mir-313* (Figure 3-5). Furthermore this area seems to be a hotspot for *P*-element insertions. More than 20 different *P*-element insertions are located shortly before the microRNA cluster.

3.2.3 MicroRNA Cluster 310-313 and a General Insight into miRNAs

MicroRNAs (miRNAs) are single stranded, 21–23 nucleotide RNAs that are able to repress specific target genes. They do this by base-pairing to target mRNAs, and then either accelerating degradation of the mRNA or inhibiting its translation. miRNA genes are initially transcribed by RNA polymerase II as long primary transcripts (pri-miRNAs) that require subsequent processing to yield a functional mature miRNA.

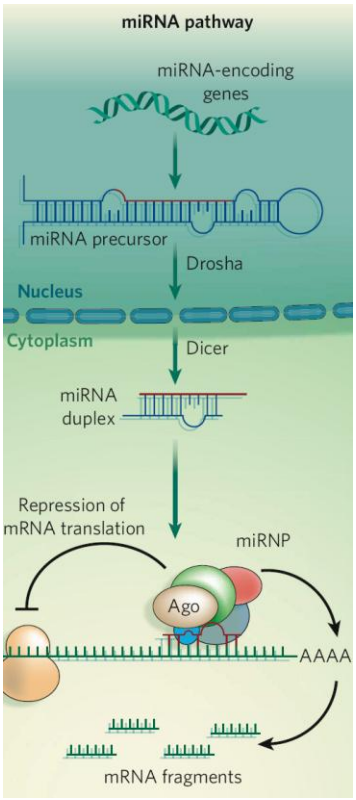


Figure 3-6 miRNA processing
The picture shows the processing of the miRNAs. For detailed description see text. Picture from (Grosshans and Filipowicz, 2008)

Some miRNA genes, like the miRNAs 310-313, are clustered in the genome with an arrangement implying transcription as a multi-cistronic primary transcript. The processing generally occurs in two steps, and is catalyzed by the enzymes Drosha (in the nucleus) and Dicer (in the cytoplasm). One strand of the resulting miRNA duplex, resembling a siRNA, then incorporates into a RNA induced silencing complex (RISC) -like miRNA–ribonucleoprotein (miRNP) complex. The main components of RISC and miRNPs are proteins of the Argonaute (Ago) family. An overview of this process can be seen in Figure 3-6 (Grosshans and Filipowicz, 2008; Jaubert et al., 2007; Seitz and Zamore, 2006; Wu and Belasco, 2008). Because miRNA function requires such a remarkably small amount of complementarity to a target RNA, each miRNA species regulates hundreds of distinct mRNA sequences. The microRNA 310 for example has 462 predicted targets in the genome (http://microrna.sanger.ac.uk).

3.3 Quasimodo Results

3.3.1 Rhythmic Expression of *quasimodo*

An important feature of many circadian genes, conserved across a wide span of evolutionary divergence, is the cyclic expression of their mRNAs. Therefore rhythmically expression of mRNA is a first hint that a gene is involved in the circadian clock. New technologies like qPCR and microarrays have facilitated this analysis enormously. In the preceding diploma thesis and in the Ph. D. thesis by T. Stempfl the rhythmic expression of *qsm* was revealed. The temporal expression pattern of *qsm* resembles *cry/Clk* expression and thus seems to be a target of *vri* repression. There are a number of mRNAs that are regulated similarly to *cry* and *Clk* based on microarray studies (Ceriani et al., 2002; Claridge-Chang et al., 2001; Lin et al., 2002; McDonald and Rosbash, 2001; Ueda et al., 2002). To reveal if *qsm* is under Vri and Clk control, too, we investigated the mRNA level in different clock mutants. The disappearance of rhythmic expression in those mutants demonstrates that *qsm* is under clock control and does not only react to for example the different light regime (Wijnen et al., 2006). The up regulation on the one hand in Clk^{Jrk} and the down regulation on the other hand in per^{01} animals show the *vrille/Clk* regulation of *qsm* (Figure 3-7). Many rhythmically expressed genes are only under clock control, but have no influence on the core clock itself. One speaks here of so called output genes. To answer the question if *qsm* is an output gene or if it feeds back onto the central clock we investigated the expression of Per and Tim in the brain and investigated behavior of animals, where *qsm* is either down regulated by RNAi or up regulated by an over-expression construct.

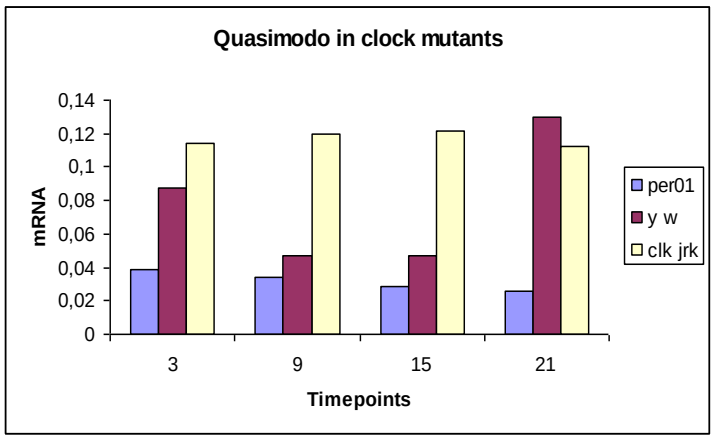


Figure 3-7 *quasimodo* mRNA measured by qPCR
RNA was isolated from control *(y w)*, *per* or *Clk* mutant flies. The time points 3 and 9 are in the light, 15 and 21 in darkness. The rhythmic expression of *qsm* disappears in the clock mutants.

3.3.2 Behavior Analysis

Preliminary data from the preceding diploma thesis demonstrated that under constant light (LL) conditions RNAi mediated knockdown of *qsm* changed the animals circadian behavior. In those animals the mRNA level is decreased, for example in *qsmRNAi/tim*Gal4 flies *qsm* mRNA is knocked down to 30 % (Peschel, 2004). Wild-type flies become arrhythmic in LL, but *qsmRNAi/tim*Gal4 animals still show rhythmic behavior with a circa 27 hour period. To investigate this in more detail animals carrying an UAS-*qsmRNAi* construct were crossed to different driver lines (Table 3-1). Rhythmic behavior was revealed in some of the RNAi lines, depending on the strength and the position of Gal4 expression. The ubiquitous driver *actin*-Gal4 is lethal, when crossed against *qsmRNAi* (Peschel, 2004). To facilitate further investigations a stable recombinant *qsmRNAi*/*tim*Gal4 line was created. The driver line *tim*Gal4 (16) and *tim*Gal4 (67) where used for this purpose. Both of them showed a similar expression pattern, when crossed against a UAS-*gfp*

Genotype	% Rhythmic	n	Period (h)
timGal4 (16)	0	6	
timGal4 (27)	0	6	
pdfGal4	0	6	
gmrGal4	12,5	8	
elavGal4	14,3	7	
QsmRNAi	0	8	
QsmRNAi/timGal4 (16)	85,4	7	28,4
QsmRNAi/timGal4 (27)	62,5	8	27,3
QsmRNAi/gmrGal4	12,5	8	
QsmRNAi/elavGal4	85,4	7	28,6
QsmRNAi/pdfGal4	12,5	8	
yw; cry^{b}	87	8	24,7

Table 3-1 Behavior in LL conditions
QsmRNAi was driven in different neurons with the help of several driver lines.

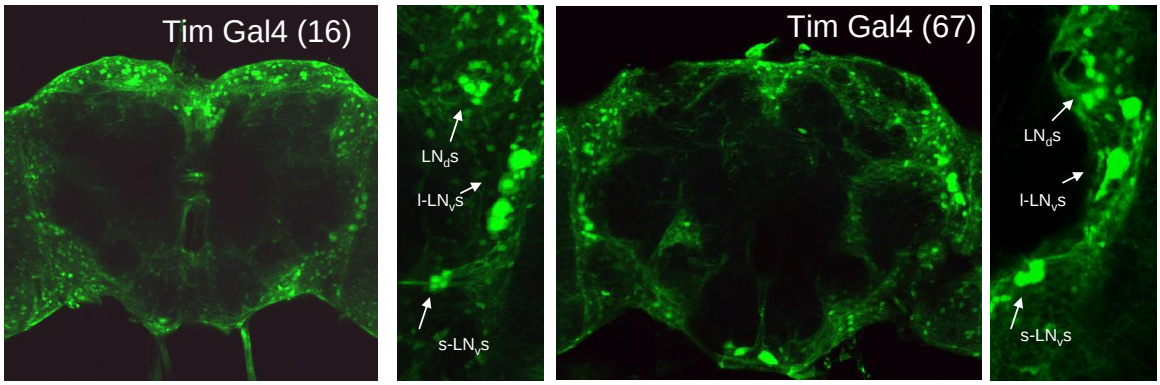


Figure 3-8 *tim*-Gal4 (16) and *tim*-Gal4 (67)
The two investigated circadian driver lines were crossed against UAS-*gfp*. We investigated the GFP expression in wholemount brains.

line. The *tim*Gal4 (16) driver seems to be stronger expressed in the dorsal region, while the *tim*Gal4 (67) line produced a stronger signal in the LN_vs (Figure 3-8). Both driver lines are described in (Kaneko and Hall, 2000) or in (Glossop et al., 2003) and show a low degree of ectopic expression. The recombinant fly lines are further on called q*smRNAi(16)* or Q16 and *qsmRNAi(67)* or Q67 respectively.

A *P*-element insertion, *EP2586*, in the promoter region of *qsm* (Figure 3-5) could be used to over-express the gene following its insertion site. The next gene is *Cg13432*. However it should be mentioned that the insertion occurred immediately upstream of the miRNA cluster 310-313 (Figure 3-5 and 3.3.8). When this line was crossed against several different driver lines the behavior in LL conditions was clearly different from wild-type behavior, but rhythmic behavior was not as obvious as in the *qsmRNAi* lines (Figure 3-9). The behavior could be rather described as complex, complicating the assignment to clear rhythmic behavior or not. Once more we created

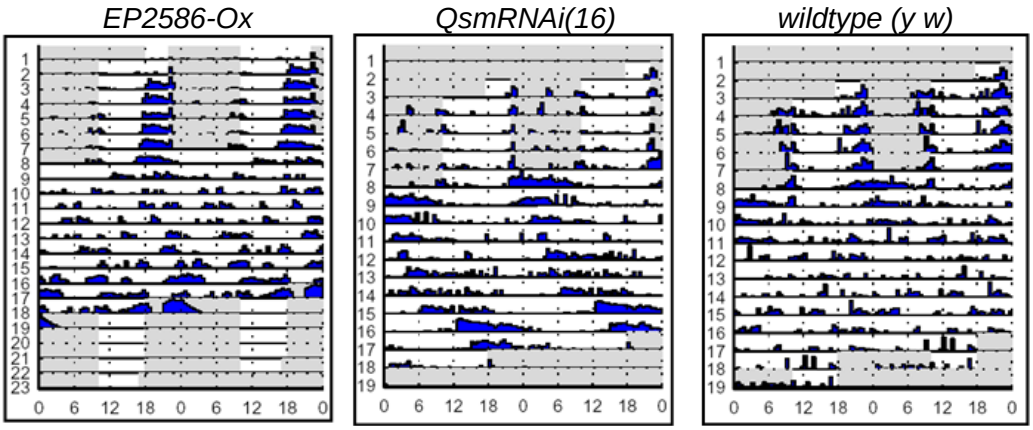


Figure 3-9 *qsmRNAi(16)*, Ep2586-Ox and wild-type animals in constant light conditions
After 3-5 days in 12:12 LD cycles the animals were exposed to constant light. Actograms show the behavior of single animals. While the *y w* and *Ep2586-Ox* animals quickly loose their rhythmicity, q*smRNAi(16)* flies stay rhythmic under LL conditions – but with an elongated period of about 27 hours.

a recombinant line, where *Ep2586* was driven by a *timeless*-Gal4 (27) driver. This line is further on called *Ep2586-Ox*. Under Light/Dark (LD) conditions on the other hand the over expression line did show a severe aberration from wild-type behavior. The evening activity peak was clearly shifted towards the day (Figure 3-10).

This shift in the evening activity and the rhythmic behavior in LL conditions raised the question if the over-expression of *EP2586* or the RNAi mediated knockdown of *qsm* has an influence on the core circadian clock or if its function is located in the output. So first of all we measured Period expression in control flies (*y w*) in comparison to q*smRNAi(16)* and *Ep2586-Ox* animals in LD conditions.

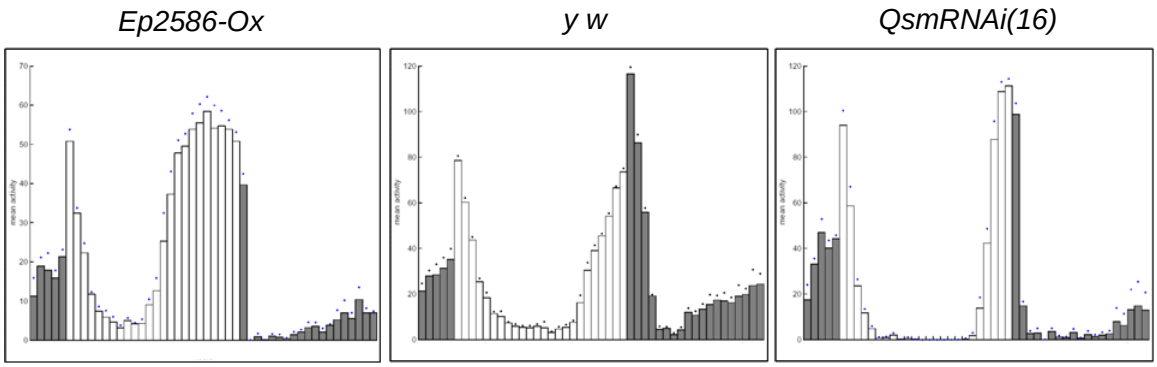


Figure 3-10 Daily average of *qsmRNAi(16)* and *Ep2586-Ox*
Fly activity was measured in 12:12 LD cycle experiments. Per genotype the average of 10 flies is displayed. The dark shaded bars indicate night time the empty bars indicate daytime. The higher the bars are, the higher is the activity of the flies. One can clearly see, that the evening activity of the *EP2586-Ox* animals starts by several hours earlier compared to the wild-type or the *qsmRNAi(16)* animals.

3.3.3 Period and Timeless Expression in the Adult Brain

Figure 3-11 indicates that the degradation of Period in *Ep2586-Ox*, *qsmRNAi(16)* lines is similar like wild-type degradation. The overall amount of Per is slightly higher in the RNAi strain and slightly lower in the over-expression flies. In addition to the lower protein level, Period trough is reached earlier in *Ep2586-Ox* compared to wild-type.

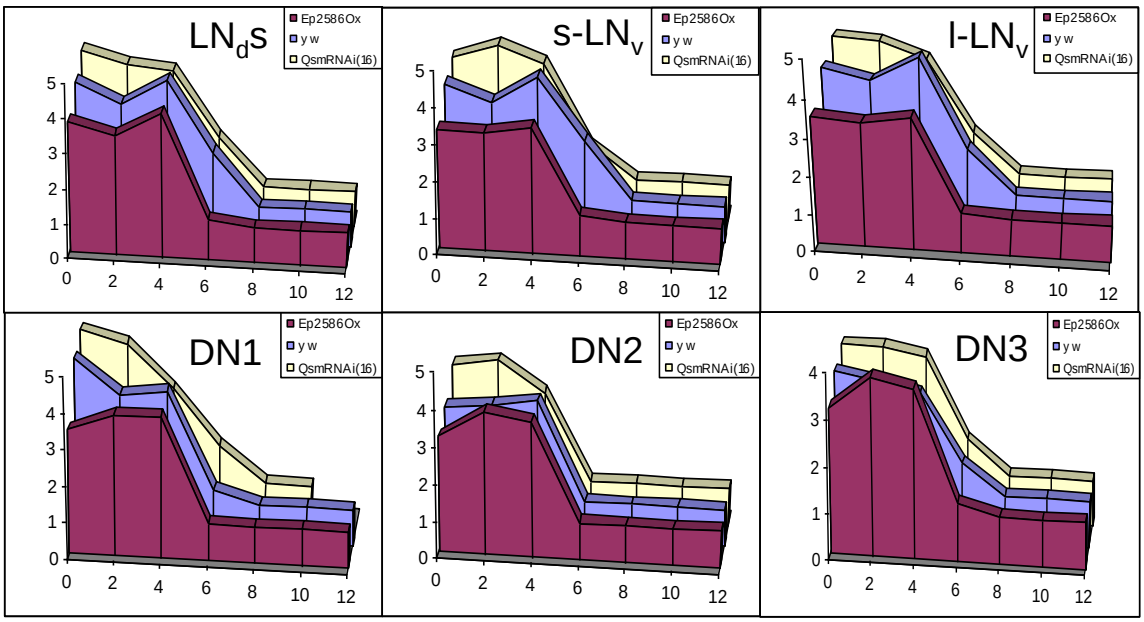


Figure 3-11 Period expression in wholemount brains from *EP2586-Ox*, *qsmRNAi(16)* and *y w*
On the y-axis the overall intensity of the several neuronal groups are displayed. The x-axis shows the different hours – from ZT0 to ZT12. Per genotype and time point about 5 brains (=10 hemispheres) were investigated. Compared to the wild-type the Period protein degrades in *Ep2586-Ox* about 1-2 hours earlier – at least in the lateral neurons. Here we could detect a general lower Period level.

The Timeless protein is degraded very fast under light conditions. In all three investigated fly strains hardly any Tim is visible after 2 hours in the light, as expected (data not shown). Rather unexpected was the result that in the RNAi line Timeless protein was still visible after 4 hours in the light, but only in cells without Period expression. Those extra cells (marked with numbers 1-6) were not linked to the circadian core clock until today (Figure 3-12). But they were all in close vicinity to other clock neurons like the l-LN$_v$s (2), the s-LN$_v$s (3) or the DN$_3$ (1). At later time points, for example at ZT12, Timeless in those cells disappears (data not shown). To see Tim protein at its peak level we investigated *qsmRNAi(16)* brains at ZT23.

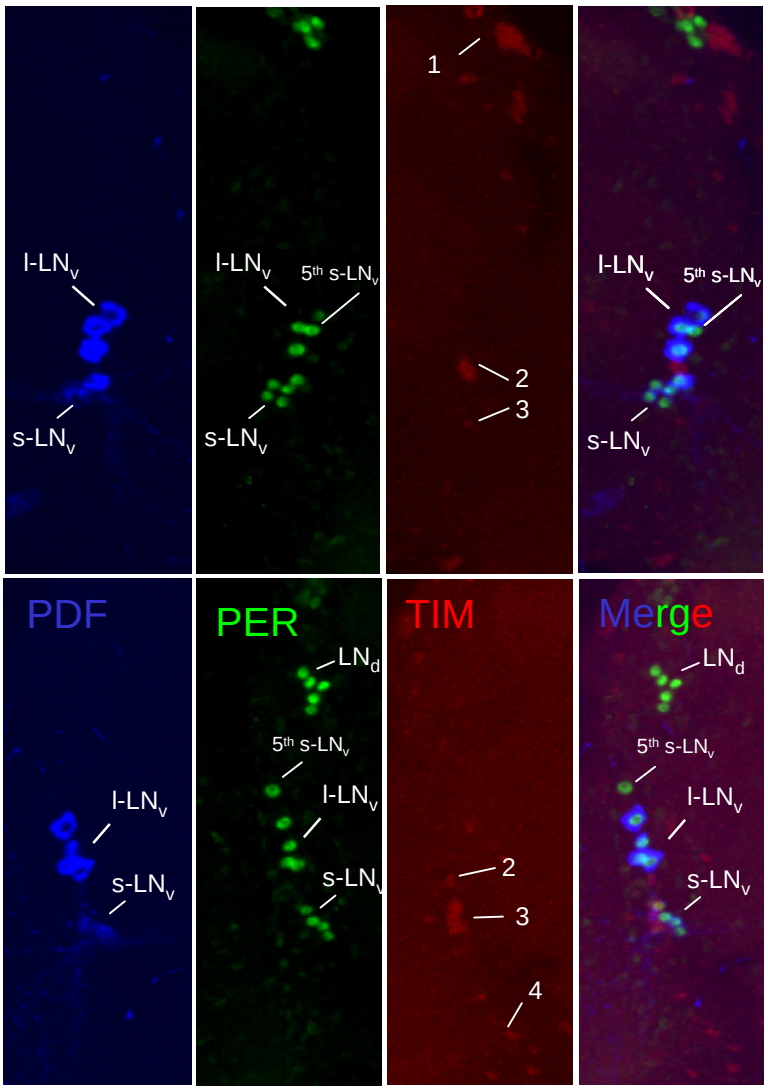


Figure 3-12 Additional Timeless positive neurons
Displayed are two different brains of *qsmRNAi(16)* animals. PDF, Per and Tim are co-immunostained at ZT4. Still a lot of Period protein is not degraded as a response to the light. Tim on the other hand is almost totally degraded. It vanished in all the circadian neuronal groups, like the DNs or the LNs. Only some cells, that are in close proximity to the Per expressing cells show Timeless staining. At ZT4 we could detect extra cells near the DN$_3$s (1), the l-LN$_v$s (2) and the s-LN$_v$s (3). Additionally we could see cells that are more ventrally located (4).

Here another cell cluster could be seen close to the DN_3s (1) and DN_1s (6) (Figure 3-13). In Figure 3-14 (A and C) one additional cell could be seen close to the l-LN_vs (2), a cell cluster could be seen near the lateral protocerebrum, at the base of the optic lobes (4), and some scattered cells near the LN_ds (5). All those cells are never stained in *y w* controls (Figure 3-14 B) or in tim^{01} animals (data not shown), indicating that the observed staining in *qsmRNAi(16)* is 'real'. Consistent with the wholemount stainings we could detect an increase in Tim on Western blots from head extracts as well (Peschel, 2004).

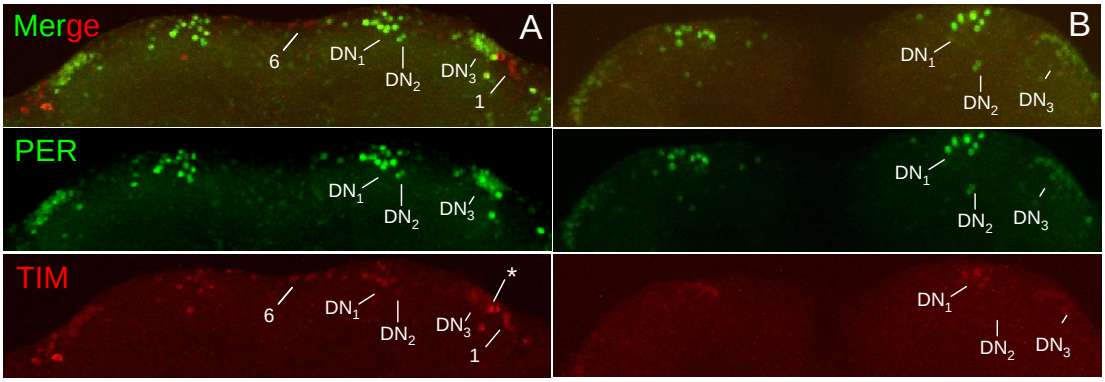


Figure 3-13 Additional Tim positive cells in the dorsal neurons of the brain
Wholemount brains were investigated at ZT23. Here the tissue was co-immunostained with Tim and Per. (A) *qsmRNAi(16)* animals display additional Tim expressing cells. Those cells can be found in close proximity to the DN_3 (1) and DN_1 (6) neurons. Marked with an asterisk is one cell that can be detected in tim^{01} animals as well and thus seems to be unspecific (per. com. S.Veleri). (B) in *y w* animals Tim can be found only co-expressed with Period in the dorsal neurons.

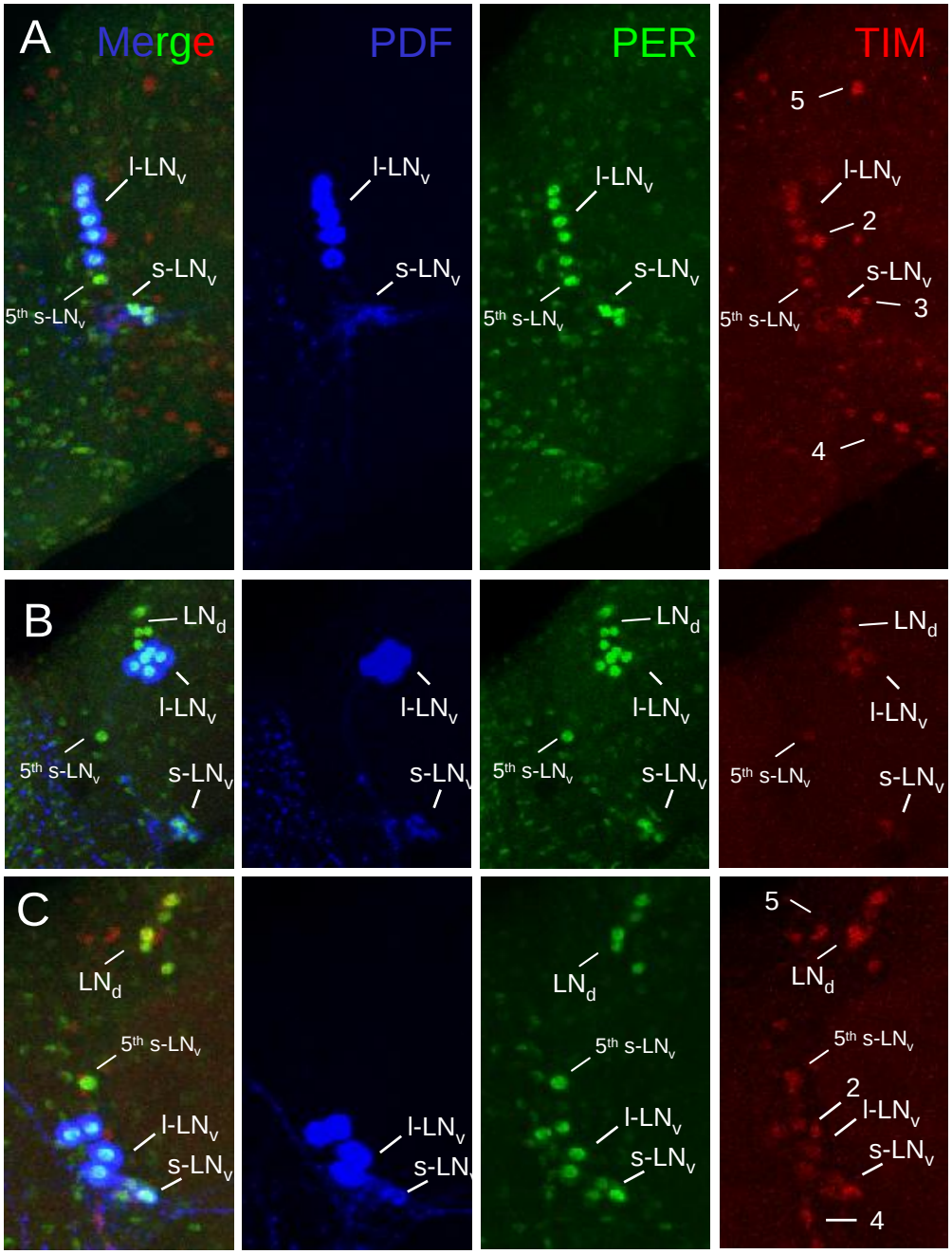


Figure 3-14 Timeless Staining of *qsmRNAi(16)* compared to *y w*
To see the maximum staining of the Tim in the *qsmRNAi(16)* animals we sacrificed the animals at ZT 23. (A) and (C) display different *qsmRNAi(16)* flies, (B) displays the *y w* wild-type control. 6-10 hemispheres were investigated per genotype. In (B) we can see intense staining of the Timeless protein in the LN_ds, l-LN_vs and all the s-LN_vs. Here we can always detect Per and Tim in the same cells. In (A) and (C) we could reveal many additional Tim positive cells in addition to the strongly stained circadian neurons. Again cells close to the l-LN_vs (2), s-LN_vs (3) and the LN_ds (5) are stained by the anti-Tim antibody.

3.3.4 *qsmRNAi(16)* in Constant Light Conditions

Normally Tim is degraded as a response to light. It is a well known fact, that in flies lacking the bluelight photoreceptor Cryptochrome Tim is not degraded to such an extend (Stanewsky et al., 1998). Down regulation of Qsm in clock cells causes rhythmicity in LL – with a 27-28 hours period. Therefore we investigated the clock neurons in those animals in LL to determine which cells are important for the rhythmic behavior in the light and to which level Tim is degraded. Rhythmic Period expression was detected in *qsmRNAi(16)* in the LN_ds and s-LN_vs, while the expression in l-LN_vs was not significantly rhythmic (Figure 3-15). Period peaks in the beginning of the subjective night. The DNs were not investigated. A representative staining series can be seen in Figure 3-16.

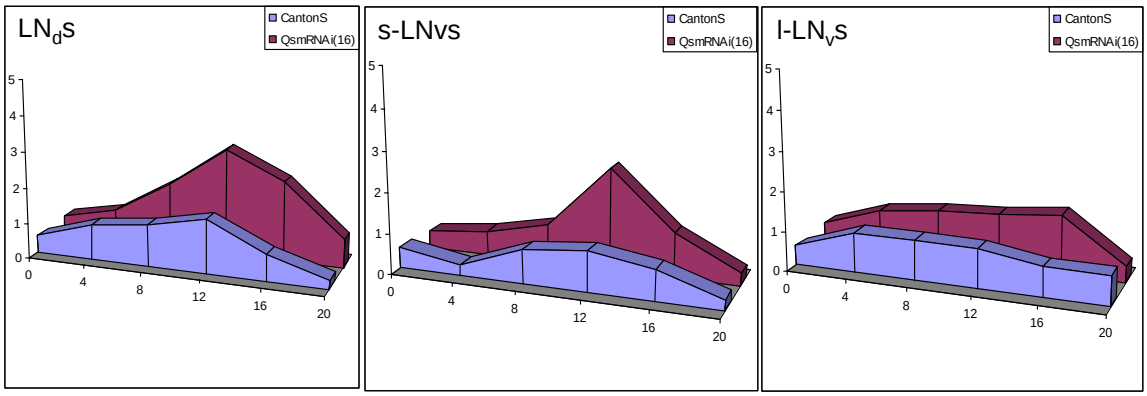


Figure 3-15 Period expression in constant light condition in *CantonS* and *qsmRNAi(16)* flies
The x-axis indicated the circadian time CT0 – CT20, while the y-axis displays the staining intensity. 3-5 brains (6-10 hemispheres) were investigated per time point.

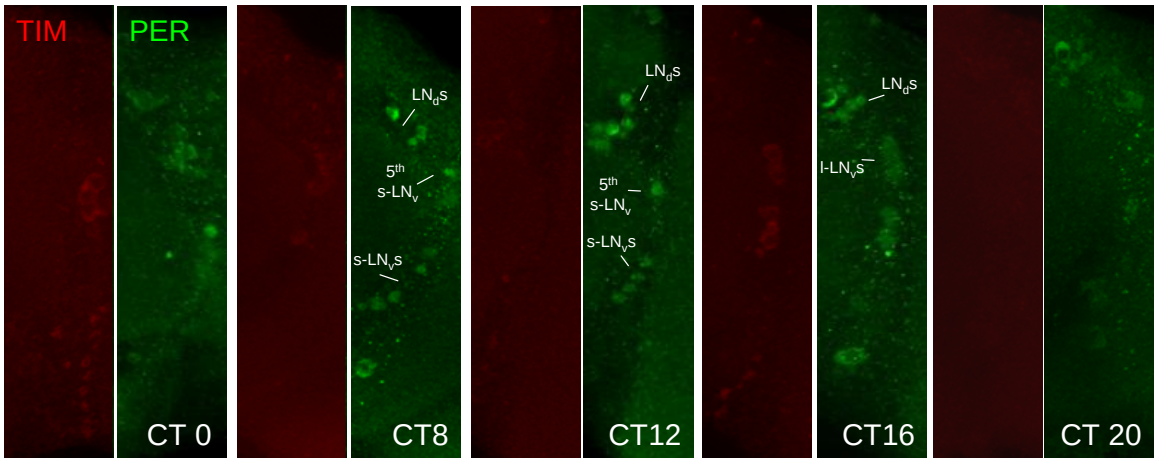


Figure 3-16 Examples of the Period stainings in *qsmRNAi(16)* clock neurons
Timeless and Period co-stainings of *qsmRNAi(16)* brains. Again we could detect Tim positive cells that were surprisingly not Per positive.

As a conclusion one can say that the down-regulation of *qsm* causes abnormal rhythmic behavior in LL conditions. A reason for this phenotype can be found in the rhythmic Period expression in the s-LN_vs and LN_ds. Additionally we could observe a stabilization of the light sensitive protein Timeless in neurons that are in close vicinity to the well known circadian clock neurons, like the LNs or DNs. Whether Tim stabilization in those cells has an influence on the circadian clock is still not known and under investigation. When *qsm* down-regulation is causing the LL rhythmicity one would expect to see endogenous Qsm protein in the clock neurons.

3.3.5 Localization of Quasimodo

Preliminary data from RNA *in situ* stainings indicate that *qsm* is expressed in the adult brain, in cells close to clock neurons or in clock neurons (Peschel, 2004). In order to confirm this data and to investigate where the Qsm protein is expressed in the adult brain two different methods were approached. In the first approach a polyclonal antibody against two different parts of Qsm was used, in a second approach a *P*-element (that contained a β-Galactosidase reporter) in the *qsm* locus (Figure 3-5) was used to detect the Qsm positive cells.

3.3.5.1 Qsm Antibody

In his Ph. D. work Thomas Stempfl generated a polyclonal Qsm antibody (Stempfl, 2002). For the generation of this antibody the two following Qsm peptides were chosen (see Figure 3-3):

1) : **H_2N -** SQD GQK FTR DLT VK **C - $CONH_2$**

2) : **H_2N -** QVG FGR RKR EIS SAN **C - $CONH_2$**

Localization of Qsm

In the first immunostainings of adult *Drosophila* brains with this antibody we used anti-PDF co-immunolabeling to differentiate between the different clock neurons. Here we could see a very intense staining of the l-LN_vs and the s-LN_vs (data not shown). But this set of experiments could not be analyzed properly as the Qsm antibody or the secondary anti-rat antibody unspecifically recognized the PDF labeled cells. Immunostainings with Qsm antibody alone revealed very weak staining of the

cytoplasm or membrane of cells that are in close vicinity to the clock neurons or of the clock neurons. Only occasionally (The experiment was repeated three times. Two times there was intense staining at ZT16, one time this was not the case) at certain time points, like ZT16, a very intense staining could be detected (Figure 3-17). But here the Qsm protein seems to be localized either in the cell nucleus, in the extracellular matrix or in different smaller cells (Figure 3-17). Three different regions were observed, where the Qsm positive cells are near clock neurons. A dorsal cluster of mostly three cells (qDNs), a ventral cluster (qLN$_v$s) close to the s-LN$_v$s and l-LN$_v$s and a second ventral cluster (qLN$_v$s 2) that is even further ventral than the first lateral cluster. The lateral clusters were much more heterogeneous in number and position than the DNs. The qLN$_v$s cells consists of about 10 to 15 cells, the qLNv 2 cluster of about 5 cells. In addition two more clusters, not in close vicinity to clock neurons can be observed in the brain (1 and 2). The largest accumulation of Qsm positive cells is located dorsal-posterior-medial (1). A cluster of 5-8 cells can be found ventral-

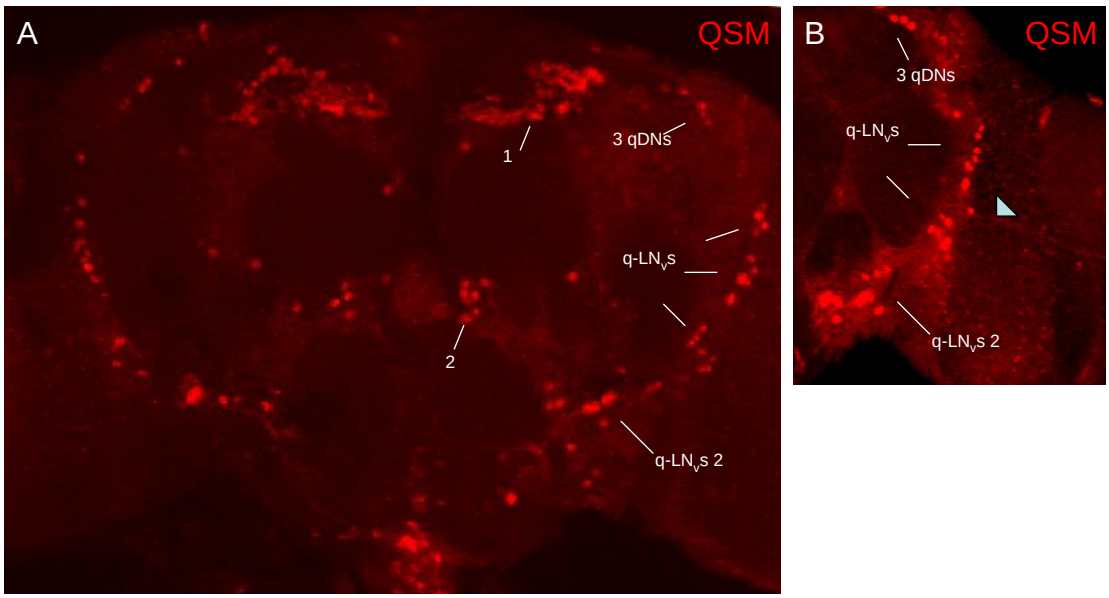


Figure 3-17 *Drosophila* wholemount staining with an anti-Qsm antibody
Adult Drosophila brains were treated with anti-Qsm antibody. The flies were sacrificed at ZT16. (A) displays an overview of one brain. The stainings were not consistent – not always (see above) could we reveal this distinct staining pattern, even if the dilutions/settings and genotype of the investigated animals were the same. In (B) we can see a close up of one of a brain hemisphere. Here we could reveal a projection (marked with an arrow) from cells with a similar position in the brain, like the s-LN$_v$s to the optic lobe. Based on its location, this connection resembles the connection from the extraretinal eyelet to the s-LN$_v$s.

posterior-medial (2) (Figure 3-17). Neuronal projections could not be detected, except for a connection from the q-LN$_v$s to the optic lobe (arrow) (Figure 3-17 B). Based on its location, this connection resembles the connection from the extraretinal eyelet, the so called HB-eyelet to the s-LN$_v$s (Hofbauer and Buchner, 1989).

Temporal Expression of Qsm

The mRNA of *qsm* is rhythmically expressed and under the control of the circadian clock. This suggests that the Qsm protein might be expressed in a rhythmic manner as well. We immunostained adult *Drosophila* brains of flies that were kept for three consecutive days in a 12:12 hour L/D regime in order to investigate rhythmic protein expression. Besides the Qsm antibody an anti-Per antibody was used to distinguish between clock and non-clock cells. In the beginning of the day (ZT0) Qsm is almost exclusively found in the cytoplasm or at the membran. Only in some q-LN_v 2 cells

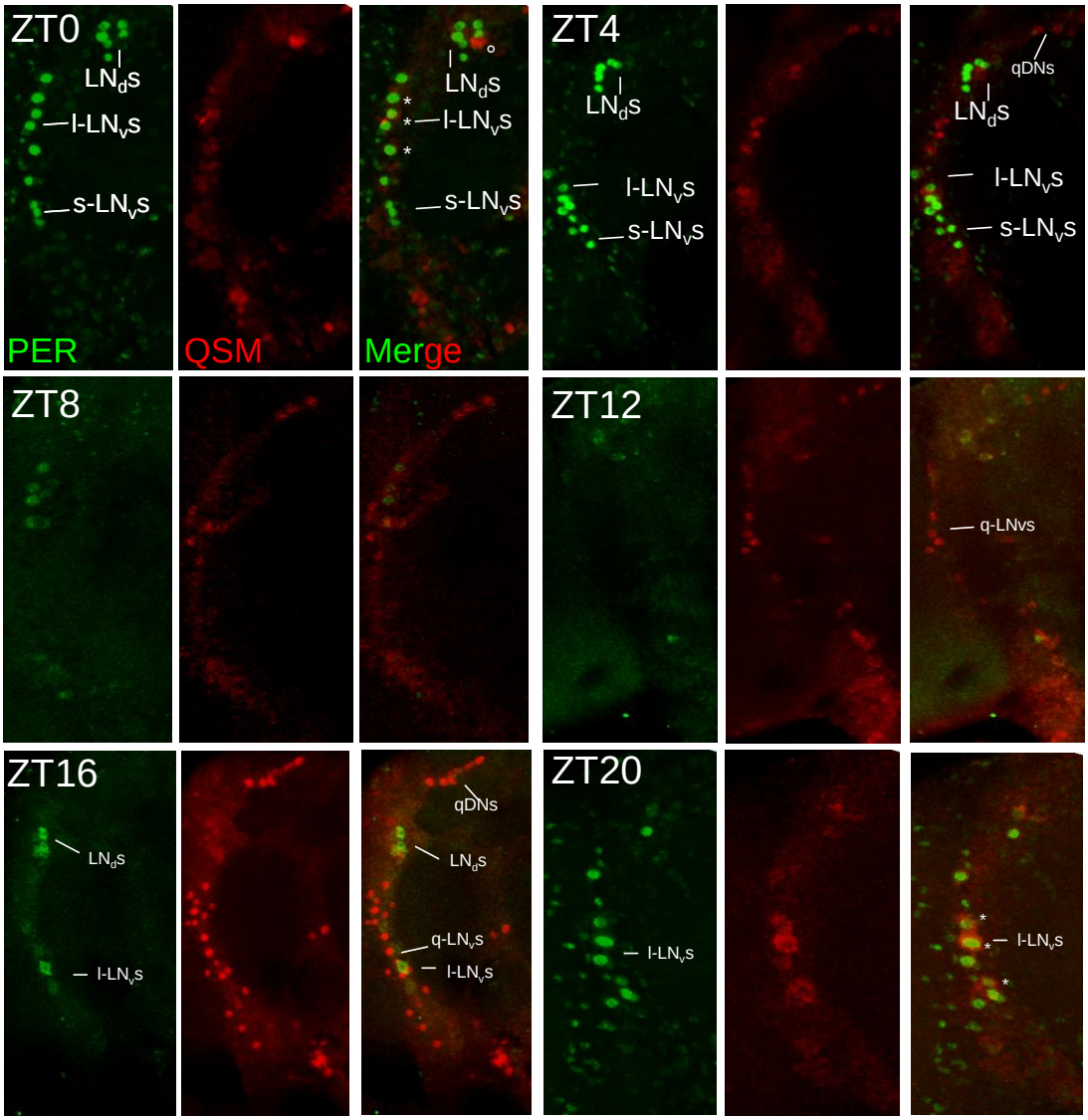


Figure 3-18 Staining series of *y w* animals in LD cycles with anti-Qsm and anti-Per
The lack of a marker protein that is throughout expressed in the clock neurons complicates the mapping of the Qsm positive cells to the clock neurons. The same is true for the assignment if the protein is nuclear or not.

Qsm might be nuclear. The q-LN_vs all show cytoplasmic signals and in two or three cells Qsm might colocalize with Per in the l-LN_vs (see asterisks). In close vicinity to the LN_ds one prominent stained Qsm positive cell can be observed (see circle). At ZT4 we see a similar picture, but the overall anti-Qsm staining is weaker. The 3 qDNs are still prominently stained. At ZT8 a trough level of overall staining can be observed. ZT12 represents the trough level of Period, but Qsm starts to accumulate again. Qsm signals in the q-LN_vs are located now in the nucleus or in smaller cells in close vicinity to the l-LN_vs. At ZT16 the peak level of nuclear (or extracellular)
Qsm accumulation is reached. All q-LN_vs are now nuclear (or extracellular) and stained very brightly. The same intensity can be observed for the 3 qDNs. Only the cells in close vicinity to the LN_ds are not stained at all or display a weak staining of the cytoplasm. AT ZT20 all q-LN_vs are cytoplasmatic again, but the overall staining is stronger than at ZT 0. Again in approximately two q-LN_vs Qsm colocalizes with Period (see asterisks). Those cells are most probably l-LN_vs (Figure 3-18).

3.3.5.2 Reporter Gene Expression in *P (PZ)l(2)05510*^{05510} Animals

Work with the Qsm antibody was problematic and very difficult because the obtained staining patterns were not always reproducible (see above and discussion). Another approach to detect Qsm positive cells was the use of a *P*-element insertion line, the fly strain *P(PZ)l(2)05510*^{05510} (Spradling et al., 1999). Here a *P*-element with the *lacZ* gene was inserted in the first intron of *qsm* (Figure 3-5). Theoretically this *lacZ* gene should be expressed under the control of the *qsm* promoter and thus reveal Qsm expression (Bier et al., 1989). So we investigated adult *Drosophila* brains of flies that were kept for three consecutive days in a 12:12 hour Light/Dark regime. The β-Galactosidase protein is stable for several hours in the adult fly (Helfand and Naprta, 1996; Stanewsky et al., 1997); nevertheless we investigated a time point with high *qsm* mRNA level. Besides the anti-β-Galactosidase antibody an anti-Per antibody was used to distinguish between clock and non-clock cells.
The overview in Figure 3-19 (A) shows a similar expression pattern as the pattern of the Qsm antibody in Figure 3-17. The largest accumulation of Qsm positive cells is located dorsal-posterior-medial (1). Weaker staining can be detected in cells that resemble from their position the qDNs, q-LN_vs 1 and 2. One cell might be in close

vicinity to the LN_ds (see circle). Investigation of the DN_3 region revealed that the β-Gal protein is co-expressed in several DN_3 neurons and in some neurons in close vicinity to the DN_3 cluster (B). A closer look to the l-LN_vs and s-LN_vs revealed, that β-Gal is coexpressed in the l-LN_vs and s-LN_vs and is expressed in cells nearby. Here a PDF antibody was used to discriminate between the large and small LN_vs (C). A close up on one brain hemisphere (D) verified the staining from the overview (A).

The assignement of the Qsm positive cells is still very vague. The bad quality of the anti-Qsm antibody and the therewith connected inconsistence of the staining in one experiment aggravate the interpretation of the results. The similar staining patterns of the *lacZ* experiment make it likely that Qsm is expressed in the s-LN_vs, l-LN_vs, partly

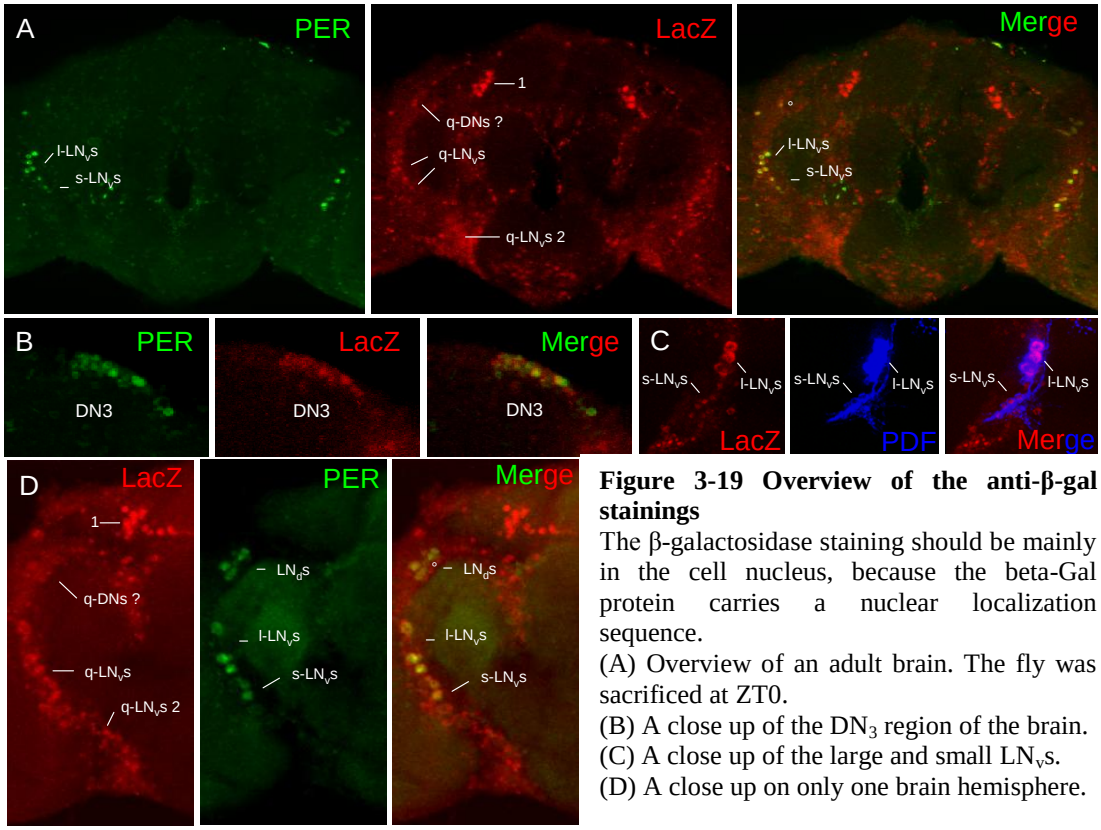


Figure 3-19 Overview of the anti-β-gal stainings
The β-galactosidase staining should be mainly in the cell nucleus, because the beta-Gal protein carries a nuclear localization sequence.
(A) Overview of an adult brain. The fly was sacrificed at ZT0.
(B) A close up of the DN_3 region of the brain.
(C) A close up of the large and small LN_vs.
(D) A close up on only one brain hemisphere.

in the DN_3s and in many other cells in close vicinity to the clock neurons. Qsm is expressed in the cytoplasm or membrane of the cells. Interestingly Qsm can be found at some timepoints of the day, for example at ZT16, in either very small cells or in the nucleus of the cells where it was cytoplasmically expressed at ZT0.

3.3.6 *per*^{01} and Quasimodo

3.3.6.1 Behavior

Animals with a mutated or lacking *period* gene loose their circadian rhythm under light/dark cycles and constant conditions (Konopka and Benzer, 1971). A subtle small peak in activity before ‘‘lights on’’ is described for *per*^{01} animals, indicating some remaining clock function (Helfrich-Förster, 2001). But when the *per*^{01} mutation is combined with a mutation of the blue-light photoreceptor *cryptochrome* a residual rhythmicity was restored (Collins et al., 2005). *per*^{01}*;cry*^{b} animals show a clear anticipation of the lights-off in LD environment. The RNAi mediated knock down of *qsm* in clock cells shows a similar phenotype like the *cry*^{b} mutation. Hence we wanted to investigate the combined effect of *qsm* and *per*^{01} mutations.

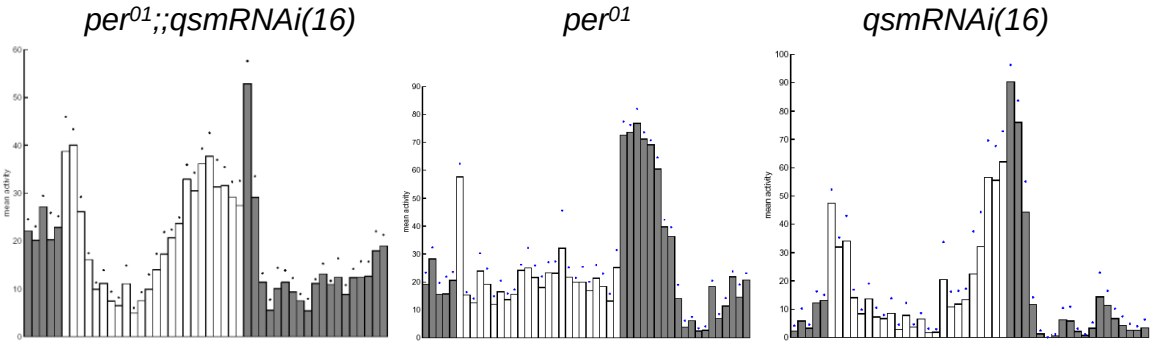


Figure 3-20 Daily average of *qsmRNAi(16)* animals in a *per*^{01} background
The flies were kept in 12:12 LD cycles. An average of 8 flies is displayed per genotype. *per*^{01} flies have lost the ability to anticipate the change of the environmental light conditions. *per*^{01}*;;qsmRNAi(16)* animals on the other hand are still able to anticipate ‘lights-off’. But here, compared to the control animals, a clear phase advance of the activity peak is visible.

In Light/Dark cycles (12:12) *per*^{01} animals only reacted to the light off or light on change with elevated activity, but did not exhibit a circadian rhythm (Figure 3-20). *per*^{01}*;;qsmRNAi(16)* flies on the other hand showed a clear anticipation of the lights-off transition (Figure 3-20). This phenotype obviously resembles that of *per*^{01}*;;cry*^{b} animals (Collins et al., 2005). A similar anticipation of ‘lights-off’ could be revealed, when instead of L/D cycles the flies were kept in constant light and temperature cycles 25:18 °C (data lost). But is this anticipation really circadian entrainment caused by an oscillator or is it generated by a so called ‘hourglass effect’ (Collins et al., 2005; Pregueiro et al., 2005)? In the hourglass mechanism the activity peak always occurs

after a set time, for example always nine hours after lights-on. So we repeated the experiment but this time changed the Light/Dark ratio from 12:12 to 18:6. Once more the *per*^{01} mutants showed only a reaction to the changed light setting (Figure 3-21). The control animals exhibited a shifted evening activity peak – the evening activity

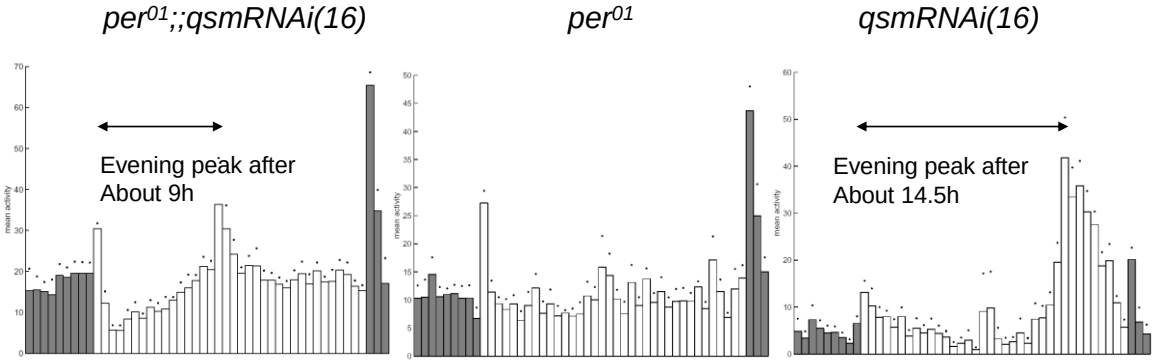


Figure 3-21 Daily average of *qsmRNAi(16)* animals in a *per*^{01} background in a different photoperiod
Flies were kept in 18:6 Light/Dark cycles. An average of 8 flies is displayed per genotype.
While the control flies exhibit a shift of about 3 hours in their evening activity, the *per*^{01} display no evening activity at all. *per*^{01}*;;qsmRNAi(16)* animals show no or only a very subtle change in their evening activity time.

peak is in LD 12:12 after about 12 hours, while in LD 18:6 the maximum evening activity can be detected after about 15 hours. The *per*^{01}*;;qsmRNAi(16)* animals though displayed no or only a very subtle shift in their evening activity – here the maximum activity can be found after nine hours (Figure 3-21).

This observation is evidence that we are looking at an "hourglass effect'. Another proof should come from an additional experiment. A circadian rhythm should be endogenous and should function in the absence of environmental cues. So we investigated the *per*^{01}*;;qsmRNAi(16)* animals under constant light or darkness and temperature condition. As expected the flies lost their rhythmicity under constant

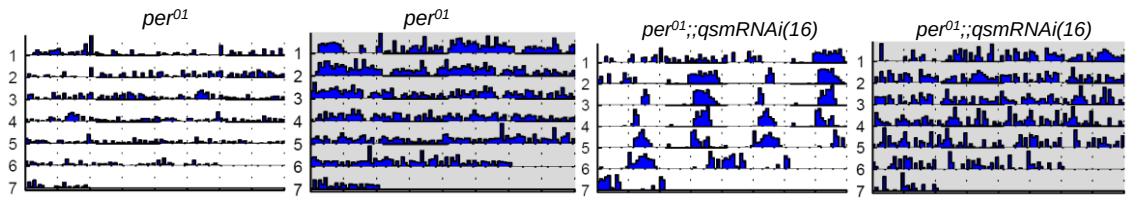


Figure 3-22 Flies with *per*^{01} mutations in constant conditions (LL or DD)
Flies were kept for several days in 12:12 LD conditions and were than released in constant conditions. The animals on the left side of the graph were kept in LL conditions; the flies on the right were kept in DD. The genotype is indicated above the picture.

darkness conditions (Figure 3-22). Also as expected constant light conditions rendered the *per*^{01} animals arrhythmic. The *per*^{01}*;;qsmRNAi(16)* flies also loose their rhythmicity after transfer into LL conditions, but after one to three days they started to exhibit a rhythmic locomotor activity again (Figure 3-22). The period of the rhythm was not 24 hours, but looked rather ultradian (Table 3-2).

Genotype	n	Rhythmic	Autocorrelation (with SEM)	Mesa (hrs with SEM)
per^{01}	16	0	-	-
per^{01}*;;qsmRNAi(16)*	10	60 %	19.4 ± 1.769746	11.7125 ± 0.974588

Table 3-2 *per*^{01} and *qsmRNAi(16)* in LL
While the *per*^{01} animals were all arrhythmic in LL some *per*^{01}*;;qsmRNAi(16)* animals showed rhythmicity. As one can see from the via autocorrelation or Mesa calculated period the assignment of the period is difficult because of the ultradian rhythm.

3.3.6.2 Tim Amount in *per*^{01}

Next we tried to answer the question, what is causing this rhythmicity and/or evening anticipation in *per*^{01}*;;qsmRNAi(16).* First we investigated if the Period lacking animals still showed rhythmic protein expression. A Western blot from whole head extracts revealed that the Timeless protein in *per*^{01} animals still showed light dependent degradation (Figure 3-23) but no sustained rhythmicity in constant conditions (Zeng et al., 1996). Less protein is detected during day time, when the light is on. At night Tim is not degraded and starts to accumulate again. A similar pattern can be observed in *per*^{01}*;;qsmRNAi(16)* . Only here the trough Tim level is reached at ZT9, while in *per*^{01} flies the trough lies at ZT3. All in all slightly more Tim is visible in *per*^{01}*;;qsmRNAi(16)* animals. Keeping in mind, that only four time points were

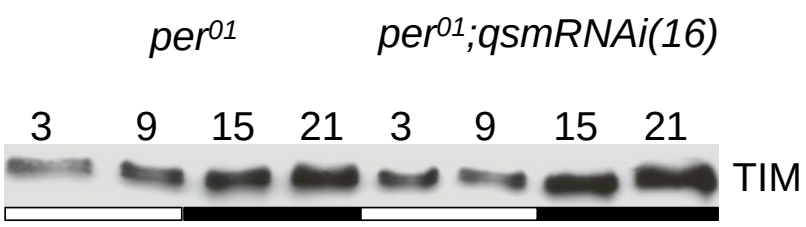


Figure 3-23 Western blot of animals with *per*^{01} mutations in Light/Dark cycles
20 heads per lane were used to investigate the Tim protein amount. The animals were sacrificed at the indicated Zeitgeber times (3,9,15,21). The *per*^{01} mutants on the left show increasing Tim protein level in the dark timepoints (15, 21). The *per*^{01}*;;qsmRNAi(16)* animals on the right blot half display the same phenomenon, but here we could detect more Tim protein at ZT 3 compared to ZT9.

investigated and only a single experiment was performed those results should be handled with care (Figure 3-23).

Next we investigated the Tim level in the circadian clock neurons in *per*^{01} or

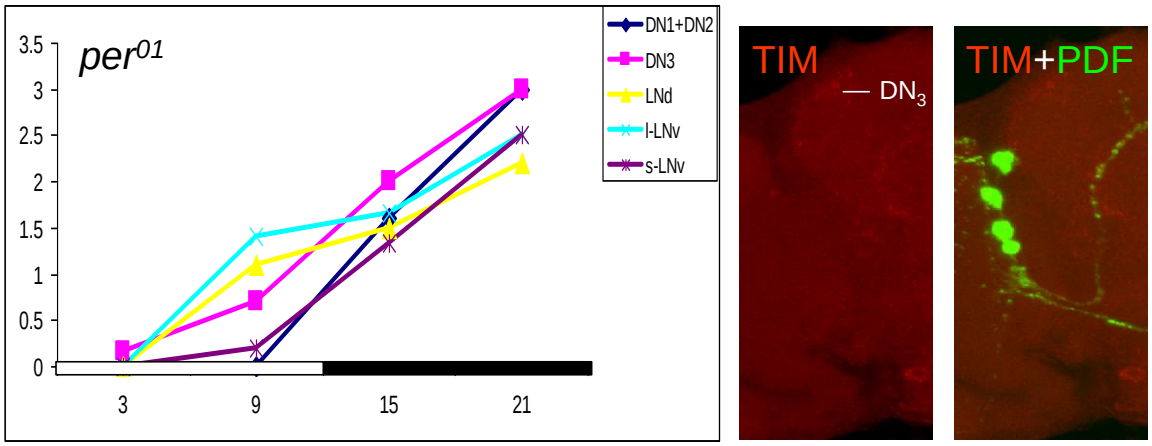


Figure 3-24 Timeless stainings in *per*^{01} wholemounts
Displayed to the left side is a quantification of Tim staining in the different clock neurons in *per*^{01} animals. The y-axis shows the intensity, while the x-axis displays the different ZTs. Only three brains (6 hemispheres) per timepoint were investigated. On the right side is an example of a *per*^{01} brain from ZT3. The only visible staining is in the dorsal neurons.

per^{01}*;;qsmRNAi(16)* flies. Adult animals were kept in 12:12 LD cycles for three days. Wholemount stainings of those animals were then investigated at ZT 3, 9, 15, 21. PDF was used to identify the LN_vs. A first very obvious result was that Tim can be found almost exclusively in the cytoplasm. The results obtained from the Western blot experiments correlated well with those obtained by ICC. *per*^{01} animals show the trough Tim level at ZT3. Here hardly any Tim is visible. Tim levels then increase by the end of the day. The rise of Tim levels continues after "light off", so that it reaches its peak by the end of the night. *per*^{01}*;;qsmRNAi(16)* brains on the other hand still show many Tim containing cells by the beginning of the day. The trough level can be seen at ZT9. In the night Tim accumulates, but in the LN_ds a decreased Tim level can be found already at ZT21. Furthermore at ZT3 Tim can be detected in additional cells, whereas it is uncertain if those cells normally express Period in wild-type animals (Figure 3-25). Those cells most likely correlate with the additional Tim positive cells under LD conditions in *qsmRNAi(16)*. *per*^{01}*;;qsmRNAi(16)* animals behave in a rhythmic manner in LL. But not every *per*^{01}*;;qsmRNAi(16)* fly exhibits this abnormal behavior and not with the same constant period (Table 3-2). For this reason it is very difficult to investigate this in the clock neurons. Hence we investigated this effect only by Western blot, because here we could investigate an average of several flies.

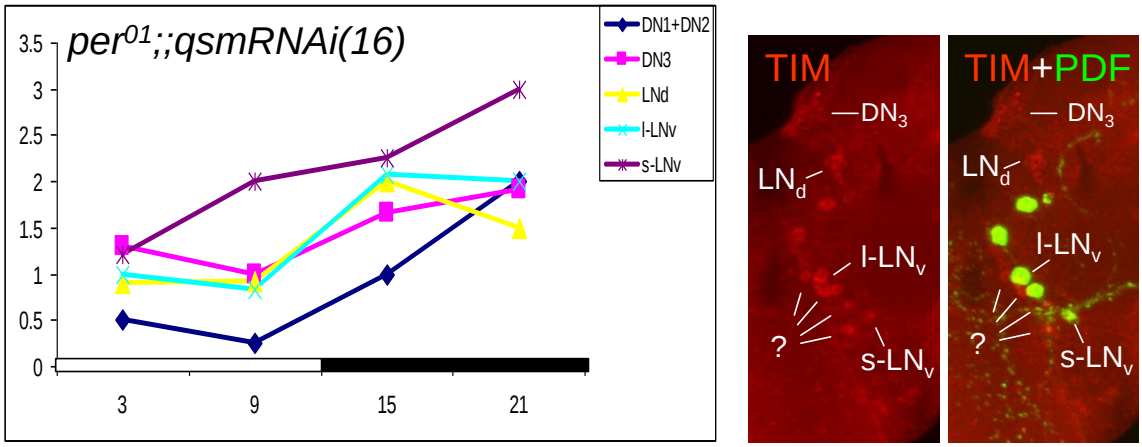


Figure 3-25 Timeless stainings in *per*^{01}*;;qsmRNAi(16)* wholemounts
Displayed to the left side is the quantification of Tim staining in the different clock neurons in *per*^{01}*;;qsmRNAi(16)* animals. The y-axis shows the intensity, while the x-axis displays the different ZTs. Only three brains (6 hemispheres) per timepoint were investigated. On the right side is an example of a *per*^{01}*;;qsmRNAi(16)* brain from ZT3. Clearly one can see Tim staining in the cytoplasm of the LN_ds and in the LN_vs. In addition many more cells in close proximity to the clock neurons are positive for Timeless staining.

Animals were collected at ZT9 and ZT21 or CT9 and CT21 (first day in LL) and the Tim amount and phosphorylation level investigated. Phosphorylated Tim can be observed on the Western blot by a slower migrating form (Martinek et al., 2001). Whilst the *y w* and *qsmRNAi(16)* showed a very clear increase in Tim protein and phosphorylation when ZT9 and ZT21 were compared, the *per*^{01} or *per*^{01}*;;qsmRNAi(16)* mutants showed only a slight increase in total amount of Tim and no change in terms of phosphorylation. In constant light on the other hand there is hardly any Tim protein visible in *y w* animals at CT9 and CT22. *qsmRNAi(16)* flies have more Tim protein at CT9 compared to CT22. The two *period* mutated fly strains both still exhibit more Tim protein, but again no cycling in the protein or phosphorylation level is detectable. In total the protein level is diminished in LL conditions compared to LD (Figure 3-26).

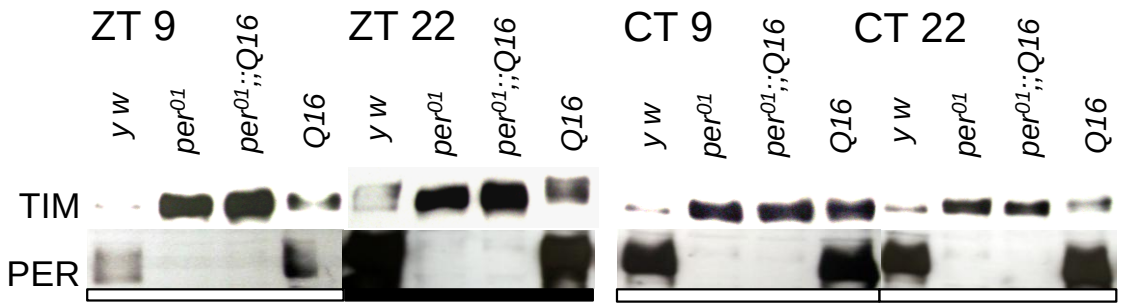


Figure 3-26 Western blot of *per*^{01} mutants in constant light
20 fly heads were investigated per lane. The left half of the blot shows different genotypes in LD cycles, while the right side of the blot exhibits animals from constant light conditions. The ZTs and CTs are as indicated. The upper rows display the Tim staining, while the second row shows the period staining as a control.

3.3.7 PDF and Quasimodo

In a Light/Dark cycle behavior experiment over-expression of the *Ep2586* insertion caused a shift in the evening activity (Figure 3-10). A similar shift can be observed in other clock mutants, for example in animals that lack the pigment-dispersing factor PDF (Pdf^{01}). At the same time Pdf^{01} mutants loose the anticipation of the "lights on" and about 80% of the flies become arrhythmic after the first few days in constant darkness conditions (Renn et al., 1999). When we compared the evening activity of the *EP2586-Ox* animals to transheterozygous *Ep2586-Ox;* Pdf^{01}/+ we could see an enhancement of the advanced evening activity phenotype in those animals (Figure 3-27), while Pdf^{01}/+ flies behaved like wild-type. On the other hand, when we over-expressed PDF in *Ep2586-Ox/UAS-Pdf* animals we could see a slightly diminished phase advance of the evening activity peak.

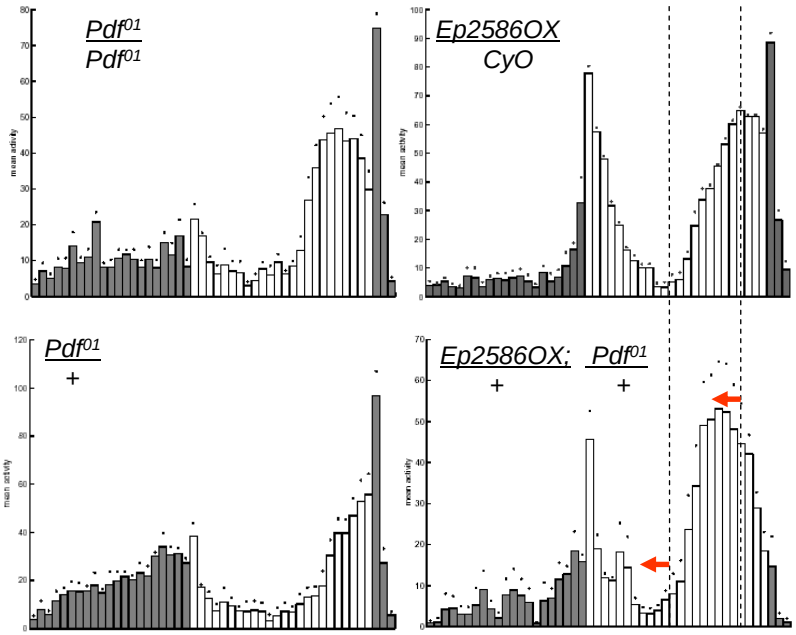


Figure 3-27 Daily average activity plots of *EP2586-Ox*
Illustrated are the daily averages of the behavior of eight flies over a period of five days. Dark shaded bars indicate night time, while empty bars indicate the day time. The dashed lines and the red arrows display the shift in the evening activity of the *EP2586-Ox* animals. In contrary to the Pdf^{01} flies anticipation of the morning is still visible in the *Ep2586/+;*Pdf^{01}/+ animals.

When we over-expressed PDF in the clock neurons with a *tim*-Gal4 driver we could detect no effect (Figure 3-28) and confirmed thus experiments where the *Pdf* gene from the grasshopper *Romalea microptera* was over-expressed in the clock neurons

(Helfrich-Forster et al., 2000). This potential genetic interaction between *qsm* and *Pdf* induced us to investigate the PDF levels in *Ep2586-Ox* animals. PDF is expressed in

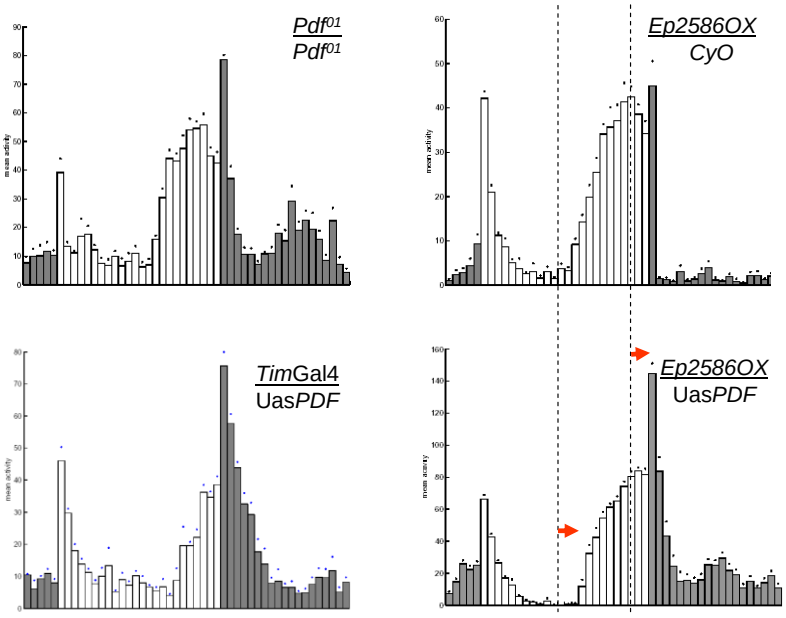


Figure 3-28 Daily Average activity plots of *EP2586-Ox*
Illustrated are the daily averages of the behavior of eight flies over a period of five days. Dark shaded bars indicate night time, while empty bars indicate the day time. The dashed lines and the red arrows display the shift in the evening activity of the *EP2586-Ox* animals. Coexpression of PDF in a *EP2586-Ox* background caused a diminished shift in the evening activity, while over-expression of PDF alone did not change the flies behavior.

the small and in the large LN_vs whereas the s-LN_vs project into the dorsal part of the brain and the l-LN_vs send projections into the optic lobe and across the brain midline.

We compared control (*y w*), *qsmRNAi(16)* and *Ep2586-Ox* brains from flies that were sacrificed at ZT 0 and 9, after entrainment in a 12:12 LD cycle for three days. The two time-points were chosen, because the peak and trough levels of PDF accumulation in the dorsal arborizations are in the beginning of the day and the end, respectively (Park et al., 2000). An anti-PDH antibody was used to detect the PDF peptide. We co-stained the brains with an anti-Tim antibody. In *y w* and *qsmRNAi(16)* animals we can see a very intense staining at ZT0 in the l-LN_vs and s-LN_vs and in their arborizations. An overall higher staining intensity can be detected when *qsm* is down regulated. In contrast the *Ep2586-Ox* animals display very weak PDF staining. The cell bodies of the l-LN_vs are stained, but much weaker than in the wild-type. The s-LN_vs are hardly stained at all. The projections from those cells are barely visible, only the end of the arborizations from the s-LN_vs and some stainings in the optic lobe are visible (Figure 3-29). At ZT9 we can see a decrease in PDF for all the investigated animals, compared to ZT0. In *qsmRNAi(16)* brains we could detect a very high level of PDF, especially the s-LN_vs and their projections contained significantly more PDF compared to the wild-type. The *Ep2586-Ox* line on the other hand almost lacked PDF at all – only in the l-LN_vs we could detect traces of this peptide (Figure 3-29).

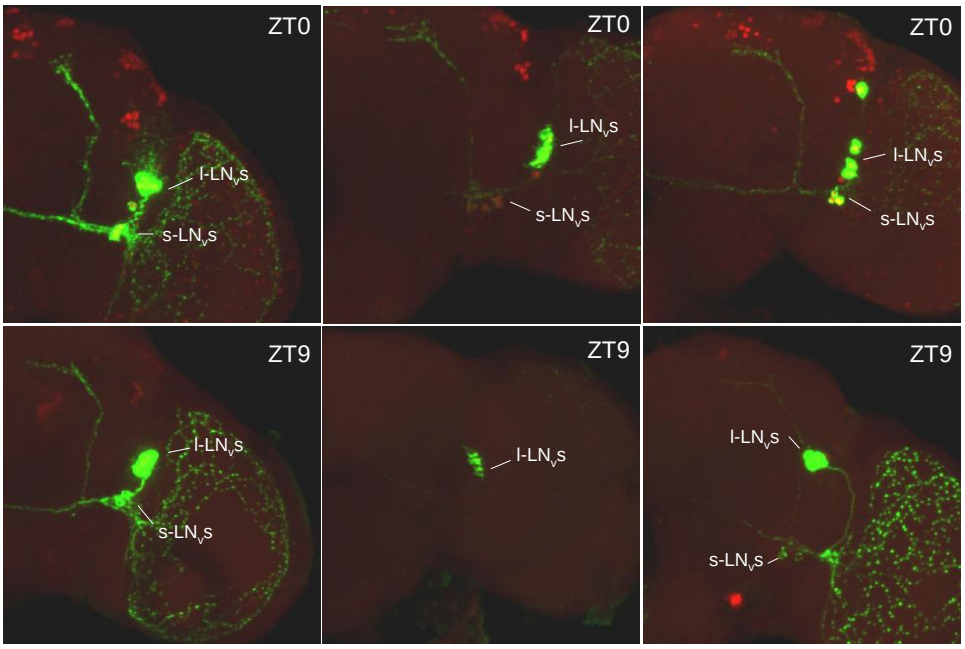


Figure 3-29 PDF level in *Ep2586-Ox*, *qsmRNAi(16)* and *y w* animals at ZT 0 and ZT9
PDF is labeled in green, Tim is labeled in red. As expected and in accordance with others (Park et al., 2000) we could see a reduction of PDF at ZT9, especially in the s-LN$_v$ arborizations, in all three investigated genotypes.

This decrease in PDF can have several causes. First of all we investigated if the *Pdf* expression is up or down regulated. So we isolated mRNA from animals sacrificed at ZT9. As controls we investigated *y w* animals. The same experiment was repeated with animals that over-express PDF. A qPCR revealed, that all three investigated genotypes, *Ep2586-Ox*, *qsmRNAi(16)* and *y w* showed a very similar level of *Pdf* RNA expression. The same result can be seen, when *Pdf* is over-expressed in the three genotypes (Figure 3-30).

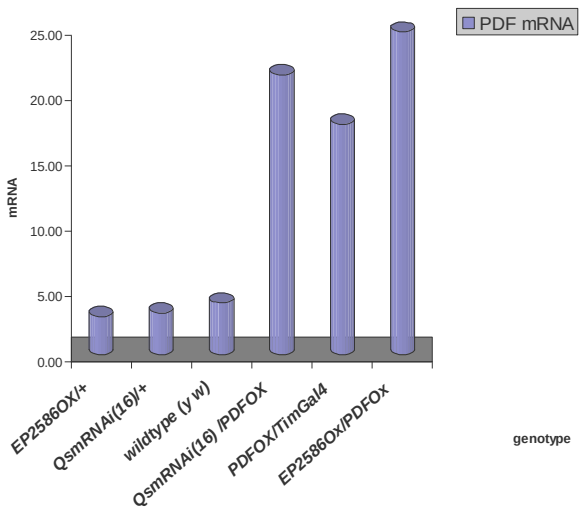


Figure 3-30 *Pdf* RNA expression levels in adult fly heads
The y-axis indicates the relative amount of mRNA expression determined by qPCR. On the x-axis the different genotypes are displayed. Tubulin was used as a housekeeping gene.

Pdf encodes a 102-amino-acid precursor (prepro-PDF). The first 16 amino acids act as a signal peptide. The next 63 amino acids (residues 17-79) form the PDF-associated peptide (PAP). This is followed by the mature 18-amino-acid PDF (residues 83-100). A predicted cleavage site, RKR, is located between the PAP and PDF (Figure 3-31).

Figure 3-31 An overview of the 102-amino-acid PDF precursor
The final functional PDF peptide is amidated at the C-terminus (Rao and Riehm, 1988). Marked with a red asterisk is the position of the Pdf^{01} mutation - a C to A exchange resulting in a Stop Codon.

Another possibility for the reduced PDF levels in *Ep2586-OX* can be a failure in the processing of the mature 18aa PDF peptide. To rule this possibility out we examined brains from flies collected at ZT2. We co-immunostained *Ep2586-Ox* and *qsmRNAi(16)* with an antibody against i) the PDH hormone from *Uca pugilator (Dircksen 1987)*, ii) against the *Drosophila* mature PDF peptide (Park et al., 2000), and iii) the PDF precursor, using the monoclonal antibody nb33 (Veleri et al., 2003). The *Ep2586-Ox* brains showed always a very weak staining, regardless of the used antibody, while on the other hand the *qsmRNAi(16)* animals always exhibited a very strong expression of PDF. We could not find a big difference between the amount of precursor peptide and the mature PDF in both genotypes and thus concluded that the maturation of PDF is not impaired in *Ep2586-Ox* (Figure 3-32).

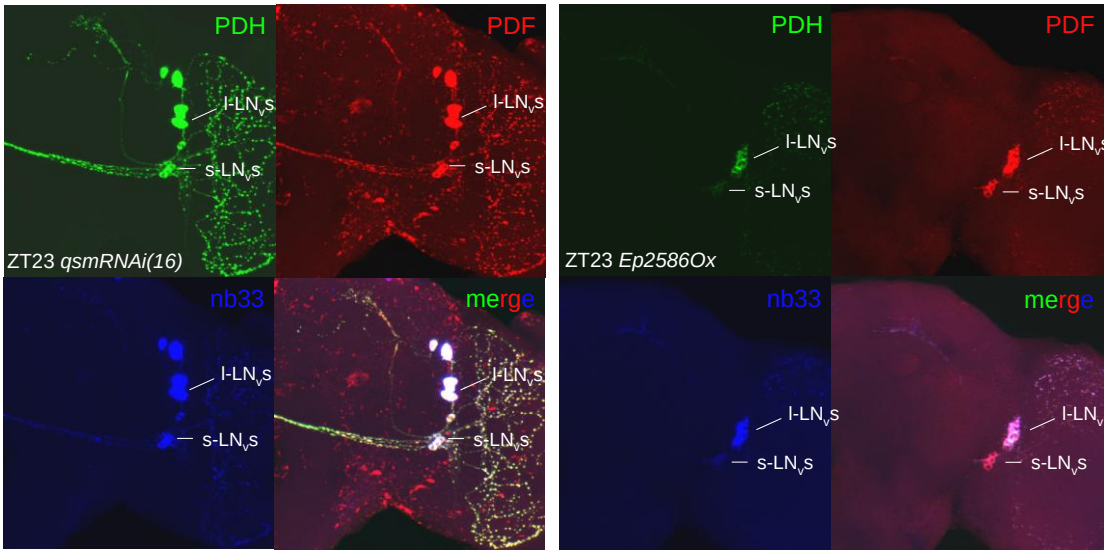


Figure 3-32 Compare of anti-PHD, anti-PDF and nb33 antibodies
The *qsmRNAi(16)* animals on the left side are compared to the *Ep2586-Ox* flies. Only the overall staining intensity differs, but not the levels of mature PDF compared to the PDF precursor peptide.

A different reason why we detected less PDF when we over-express the EP2586 *P*-element insertion can be the release of the peptide. It is generally assumed that PDF is released from the s-LN$_v$s into the dorsal parts of the brain and from the l-LN$_v$s into the optic lobes and across the brain midline. The expression of the peptide in the clock neurons is not under the control of the circadian clock, but the release of PDF into the dorsal parts of the brain is rhythmical (Park et al., 2000). The peak level of PDF can be found in the beginning of the day (ZT1), the trough level can be seen by the end of the day (ZT10). In clock mutants like *per*^{01} or *tim*^{01} this rhythmic PDF release disappears (Park et al., 2000). To test if the release of PDF is malfunctioning in *EP2586-OX* flies we expressed another peptide, a mammalian neuropeptide-GFP fusion gene, the pre-pro-atrial-natriuretic factor–green fluorescent protein [ANFGFP] (Rao et al., 2001) in the clock neurons. Previous work has shown that a transgene consisting Anf-GFP is processed, localized, and released, as would be an endogenous neuropeptide when it is expressed in the nervous system of *Drosophila* using the GAL4/UAS expression system (Husain and Ewer, 2004). Flies were dissected at ZT1. The Anf-GFP was examined by analyzing the GFP signal. In addition we used anti-PDF and nb33 antibodies to visualize the PDF peptide. The *EP2586-Ox* animals did show a weak staining intensity for the Anf-GFP, PDF and the PDF precursor, especially in the s-LN$_v$s and the arborizations. The *qsmRNAi(16)* animals displayed strong staining in all the cell bodies itself and in the arborizations (Figure 3-33).

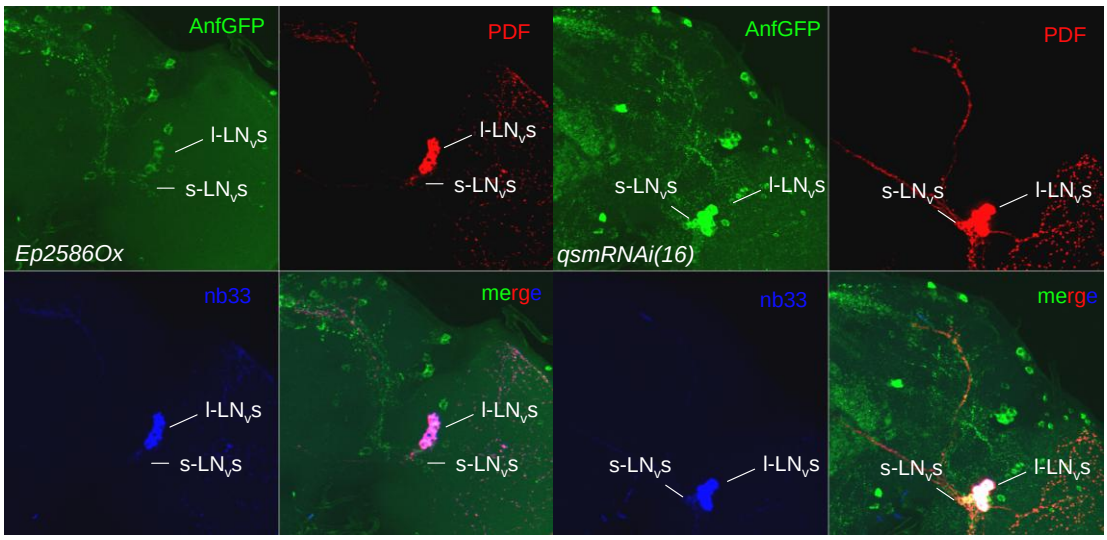


Figure 3-33 Expression of ANF-Gfp in *Ep2586-Ox* and *qsmRNAi(16)*
It was shown, that the Anf-Gfp protein behaves like a normal peptide and thus it should be released in the same way as PDF or other peptides (Husain and Ewer, 2004).

Next we wanted to investigate how the over-expression of PDF influences the *Ep2586-Ox* phenotype. For this reason we immunostained brains from *Ep2586-Ox*, *qsmRNAi(16)* and *timGal4(27)* animals, that were crossed against a *UAS-pdf* *P*-element insertion line.

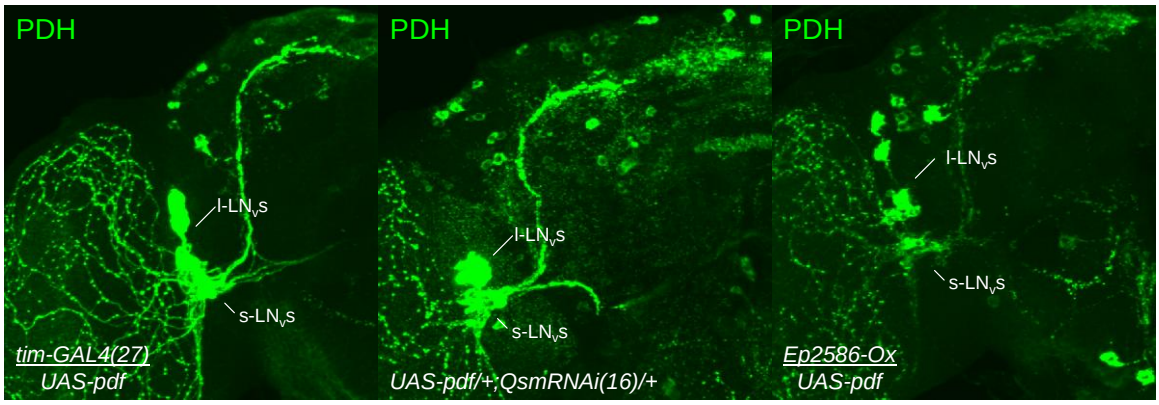


Figure 3-34 PDF is over-expressed in the clock neurons in wild-type, *Ep2586-Ox* and *qsmRNAi(16)* While the arborizations of the s-LN$_v$s are very prominently stained in the control and the RNAi line - not too much protein can be detected in the *EP2586* line.

As a result we could see that the molecular phenotype of the *Ep2586* over-expression is slightly diminished. In the l-LN$_v$s, s-LN$_v$s and in the arborization we could detect much more PDF, compared to the *Ep2586-Ox* alone (Figure 3-34 and 3-29). But when the driver line or the *qsmRNAi(16)* line with the UAS-*pdf* construct are compared to *Ep2586-Ox/Uas-pdf* a drastic reduction of PDF can be clearly seen. Especially the arborizations to the dorsal part of the brains are only weakly stained. Again most of the PDF peptide is here located in the terminals of the arborizations.

Another overall feature of the *EP2586-Ox* cell anatomy was a different shape of the cells. Closer investigation of the cell bodies of the clock neurons revealed, that *Ep2586-Ox* animals displayed an abnormally shrinked shape of the cell (Figure 3-35).

To test if this shape is only an abnormal PDF distribution or if indeed the whole clock cell has a different shape we coexpressed GFP in the clock neurons of *Ep2586-Ox* flies.

When we compared the GFP distribution in *Ep2586-Ox* and *qsmRNAi(16)* l-LN$_v$s we could see, that the cytoplasm volume of *qsmRNAi(16)* is very large. Otherwise the cytoplasm volume in *Ep2586-Ox* cells is shrinked and decreased (Figure 3-35). This shrinked cytoplasm volume might fit to the later postulated connection to a Sodium:potassium:chloride cotransporter Cg31547 (see below).

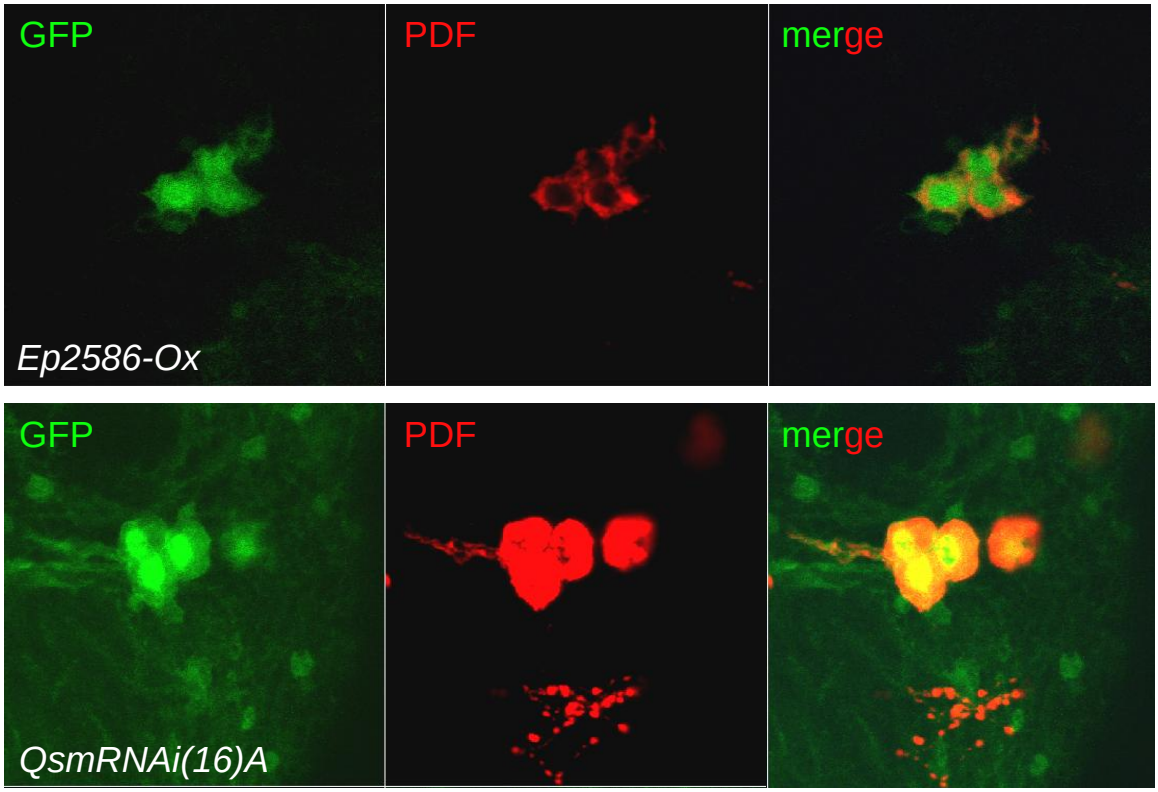


Figure 3-35 The shape of *qsmRNAi(16)* and *Ep2586-Ox* cells
While the nucleus of both genotype exhibits more or less the same size, there is a big difference in the size of the cytoplasm

3.3.8 miRNA Involvement in Circadian Rhythm

T. Stempfl reported in his Ph.D. thesis, that the mRNA level of *qsm* is increased when the *Ep2586* construct is driven by *elav*-Gal4. When we measured the mRNA level of *tim*-Gal4 driven *Ep2586* in qPCR experiments we could only detect a very subtle increase of mRNA (Figure 3-36).

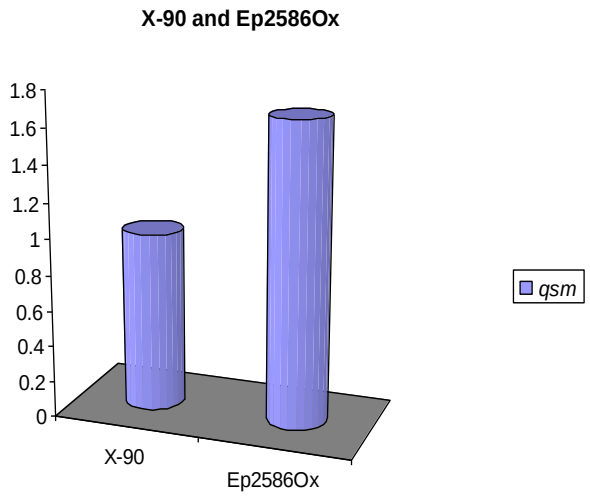


Figure 3-36
Over-expression of *qsm*
The mRNA level in Ep2586-Ox animals was compared to wild-type animals, strain X-90. qPCR of a single experiment did reveal no or only very subtle increase in the Ep2586-Ox animals.

Furthermore we could still see the clock dependent reduction of *qsm* mRNA in the middle of the night in *Ep2586-Ox* animals, although the gene should be over-expressed all the time (Figure 3-37).

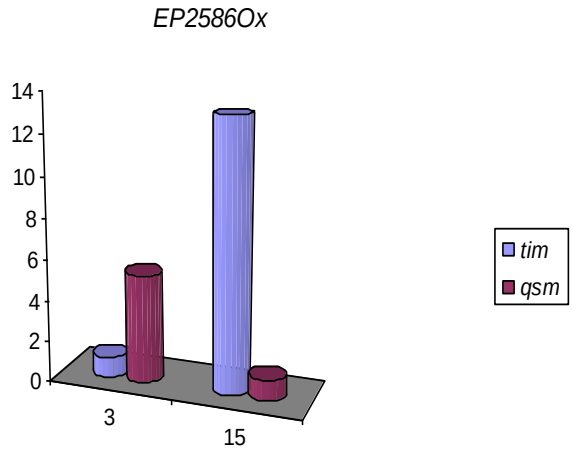


Figure 3-37
Cycling mRNA in *Ep2586-Ox*
mRNA level from animals sacrificed at ZT3 and ZT15 were compared (a single qPCR). Although the *qsm* gene should be over-expressed we still can detect a circadian reduction in the mRNA at ZT15. We compared as a control the *timeless* mRNA level at ZT3 and ZT15.

When we investigated the Qsm protein level of flies that over-express *Ep2586* we could not see any increase of the protein (data not shown). The same phenomenon was reported by Stempfl (Stempfl, 2002). In his thesis he offered several possible reasons why the animals over-expressing *Ep2586* did not show an increased level of Qsm on Western blot. One possibility was the bad quality of the Qsm antibody. The best way to test if the antibody really could detect Qsm on the Western blot or not would be to investigate *qsm* null animals. Because of the lethality of homozygous *qsm* mutants this option was not available.

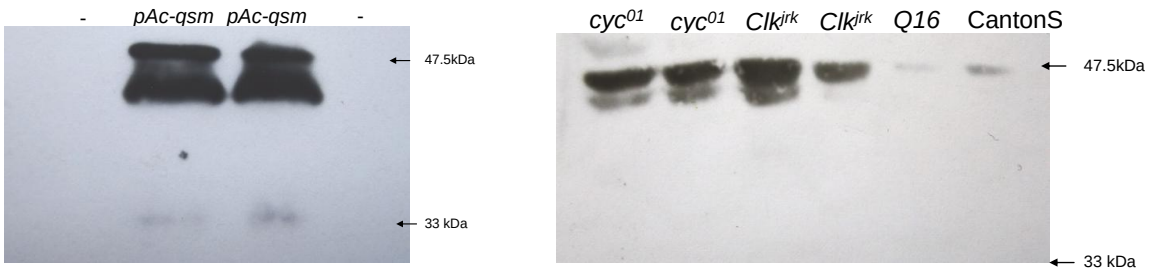


Figure 3-38 Examination of an anti-Qsm antibody on Western blots
(On the left) Protein extracts from S2 cells were investigated by a Western blot. The lanes marked with (-) were loaded with normal S2 cells (lane 1,4), while the lanes marked with *pAc-qsm* were loaded with *pAc-qsm* transformed S2 cells (lane 2,3). A strong band was detected at about 45 kDa a very weak band was detected at 35kDa in lane 2 and three. In lane 1 and 4 we could not detect any staining.
(On the right) Protein extracts from 50 fly heads were investigated. The genotypes are indicated on top of the blot. Here we could detect a band of about 45kDa. This band was elevated in *cyc* or *Clk* mutants, while it was diminished in *qsmRNAi(16)* animals. This blot was accomplished with the help of G.Szabo

For this reason we investigated Qsm levels in *Drosophila* S2 cells. Here *qsm* is not expressed endogenously (data not shown). We transfected cells with a *pAc-qsm* vector. On the Western blot we could detect a prominent staining at 45kDa only in the cells that were transfected with the *pAc-qsm* vector (Figure 3-38). The size of the unprocessed Qsm should be 45 kDa, while the cleaved part of Qsm should be about 31 kDa. Another approach to test if the antibody really detects Qsm on the Western blot or not was to investigate mutants like cyc^{01} or Clk^{Jrk}, where Qsm should be upregulated, when it is regulated in a similar way as *cry*. The protein was indeed upregulated in those animals, in *qsmRNAi(16)* on the other hand the staining was less intense, compared to *CantonS* wild-type control (Figure 3-38).

The qPCR and Western Blot results caused us to wonder, whether *qsm* is really over-expressed in *EP2586* transgenes. Closer investigation of the insertion site of *Ep2586* revealed that the *P*-element is inserted only about 80 bp before the start of the miRNA cluster 310-313. MicroRNAs are transcribed by the RNA polymerase II as long primary transcripts and contain a polyA tail just like a ‘normal’ gene (see above). So we were wondering if only the miRNA cluster is over-expressed in the *Ep2586* transgenes and not *qsm*. This induced us to create further transgenic flies that either carried the *miRNA* gene or the *qsm* gene under the control of an UAS promoter. Next we used two different driver lines, i.e. *Pdf*-Gal4 and *tim*-Gal4, to express both constructs in different clock neurons.

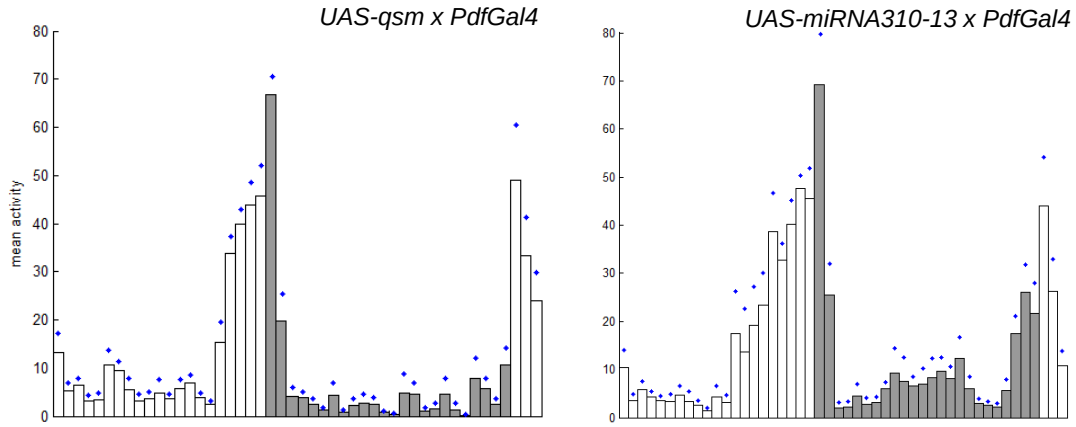


Figure 3-39 *Pdf*-Gal4 driven over-expression of *qsm* or *miRNA 310-13*
We over-expressed either *qsm* or *miRNA310-13* in the PDF neurons. The daily average plot of eight flies over a time of five days does not show any abnormal behavior in 12:12 Light/Dark cycles.

When we used the *pdf*-Gal4 driver line no abnormal locomotor activity in a 12:12 LD cycle was detected, similar to *Ep2586/pdf-Gal4* animals (Figure 3-39 and data not shown). When we crossed the two UAS lines to the *tim*-Gal4*(67)* driver we could detect abnormal behavior only in the UAS-*miRNA 310-13;;tim*-Gal4*(67)* line, whereas UAS-*qsm;tim*-Gal4*(67)* behaved wild-type-like (Figure 3-40). UAS-*miRNA 310-13;;timgal4(67)* animals showed in a 12:12 LD experiment an advanced evening activity, similar like Pdf^{01} or *EP2586-Ox* flies. The miRNA over-expressing

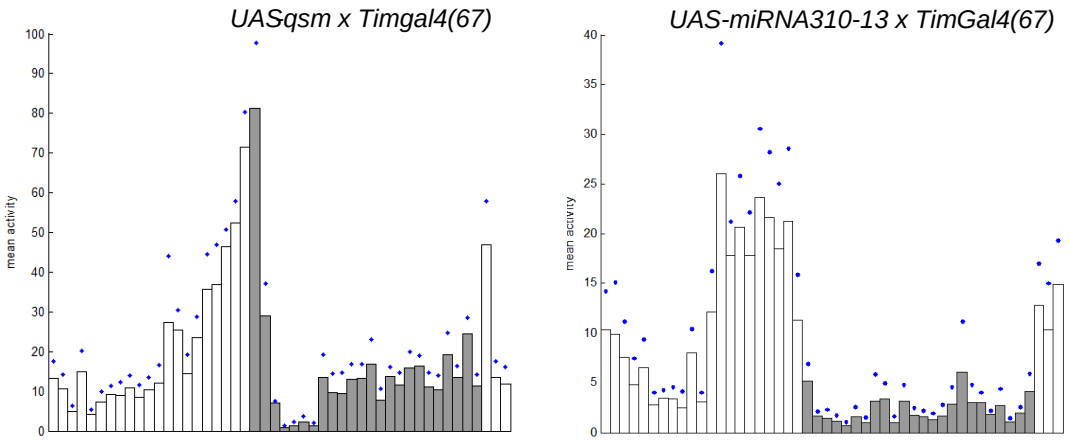


Figure 3-40 Over-expression of *qsm* and *miRNA310-13* in *timeless* expressing cells
We over-expressed either *qsm* or *miRNA310-13* in all *timeless* expressing cells. In the daily average of eight flies over a period of five days we could not detect abnormal behavior in 12:12 Light/Dark cycles after over-expression of *qsm*. Over-expression of the *miRNA310-13* cluster on the other hand induced a striking advance in the evening activity

animals displayed a very low activity level. Furthermore they died after about 4-5 days. This hindered us to perform extended locomotor behavior studies.

Next we wanted to know if in the *UAS-qsm; tim-Gal4* animals *qsm* really was over-expressed. When we isolated mRNA from those animals and performed qPCR

experiments we could see a striking increase in mRNA by the factor of 20 (data lost) compared to wild-type animals.

Detection of Qsm by Western blots confirmed the mRNA data from the qPCR experiments.

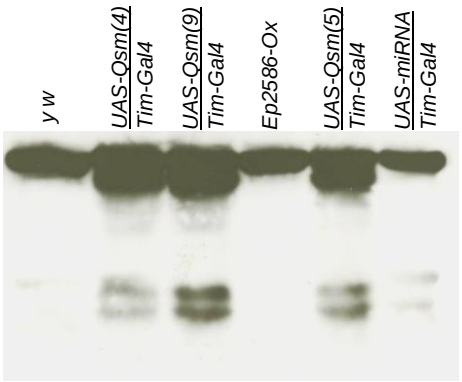


Figure 3-41
Over-expression of Qsm (Western blots)
Indicated on the top of the Western blot are the different genotypes. 25 heads per genotype were used. Three different *UAS-Qsm* insertion lines (number 4, 5 and 9) were investigated. They all carry the same *P*-element, but the construct is inserted at different positions in the genome. As a control *y w* and *Ep2586-Ox* animals were examined. The *UAS-qsm* animals display an increase in Qsm level. Here intensities of the upper band (45 kDa) and especially the faster migrating band (33 kDa) are strongly increased.

Only when we used UAS-*qsm* for expressing of Qsm we could detect a strong increase of the protein on the Western blot. In *Ep2586-Ox* this was not the case (Figure 3-41).

3.3.8.1 Function and Interaction

Still not knowing anything about Quasimodos function we found another crucial hint from a different source. When the over-expression of *Ep2586* was not restricted to the circadian clock neurons only, but was expressed in a much broader region with the pan-neuronal driver line *elav*-Gal4, we could detect severe defects in the morphology of the adult flies. Those animals displayed "juvenile" features of newly eclosed adults. The wings are still unexpanded and the cuticle shows deficits in the tanning (Figure 3-42). The same phenotype is described for animals that have a mutated *bursicon* or *bursicon receptor* gene (Baker and Truman, 2002; Dewey et al., 2004), have a disturbed Bursicon release (Hodge et al., 2005; Luan et al., 2006) from the CCAP cells (Crustacean cardioactive peptide-expressing neurons) or have ablated CCAP cells (Park et al., 2003). When we repeated this experiment and restricted the *Ep2586* expression to the CCAP cells with a *ccap*-Gal4 driver line we obtained the same juvenile phenotype as with the *elav* driver line. When we crossed the UAS-*qsm* and UAS-*miRNA* transgenes to a *ccap*-Gal4 driver line, we could only detect the juvenile phenotype with the miRNA, but never with the Quasimodo over-expression line

(Figure 3-42). This result was recently confirmed by others (Zhao et al., 2008). The Bursicon release is connected to channel or channel-like proteins that control ion transport in the cell. In summary missexpression of channel proteins and miRNAs in CCAP cells causes juvenile phenotypes that were connected to a malfunction in the

Figure 3-42 Juvenile phenotype of flies over-expressing *Ep2586* in all neurons
Bursicon is released within 20 minutes after eclosion. If this peptide is not released, the animals can not unfold their wings and the tanning of the cuticle is abnormal - as exemplified in this figure. The very same phenotype was revealed using the *ccap*-Gal4 driver line and also by expressing the miRNA cluster in the same pattern.

Bursicon release. Expression of NaChBac, EKO, Kir2.1 or Shaw channels all result in a more or less strong juvenile phenotype (Hodge et al., 2005; Luan et al., 2006). After the discovery that the miRNAs and not the *qsm* gene are causing the phenotype of the behavior in LD we investigated if some of the miRNA targets are channel proteins or proteins involved in the ion transport. We used the miRBase from Sanger to find possible targets of the miRNAs 310-313 (Griffiths-Jones et al., 2008). Three channel genes showed a high target probability of the miRNA cluster, *porin*, *Cg8323* and *Cg31547* (Table 3-3).

Cg8323 and Porin are described as transporter proteins and voltage-dependent anion channels, respectively, that are located in the mitochondrial membrane. Cg31547 is a Na-K-Cl-Cotransporter that is located in

313	312	311	310
porin	CG15096	CG3822	CG31547
CG31547	CG8062	CG31547	CG3822
CG4607	CG8323	CG1688	Nhe2
CG8323	CG15555	CG8323	CG1688
porin	porin	CG15555	CG8323
CG17274	CG13893	porin	CG17664
CG6070	CG7342	CG12194	CG17664
CG33528	CG13189	CG7342	blw
Nhe2	CG7385	CG17274	porin

Table 3-3 Predicted miRNA targets
The miRBase homepage from Sanger (http://microrna.sanger.ac.uk/) predicts for the indicated miRNA 310 to 313 about 300 to 400 different targets. The targets with the highest likelihood are displayed in the table, whereas the higher the probability, the further up the protein is in the table. The three genes marked in red, yellow and green encode for proteins that are involved in ion transport.

the cell membrane. Furthermore this protein is, as per a Y2H interaction assay interacting with Qsm (Giot et al., 2003). So we decided to first investigate this transporter protein.

3.3.9 Cg31547

Cg31547 is described as a membrane protein with sodium:potassium:chloride symporter activity. Sodium:potassium:chloride cotransporters (NKCC) mediate the coupled movement of Na, K, and Cl ions across the plasma membrane of most animal cells, and play a vital role in the regulation of ionic balance and cell volume (Xu et al., 1994). Because classical mutants were not available for this gene we investigated RNAi mediated down regulation in clock neurons and otherwise deletions of this gene. When we expressed an UAS-*Cg31547*$_{RNAi}$ construct under the control of a *tim*-Gal4

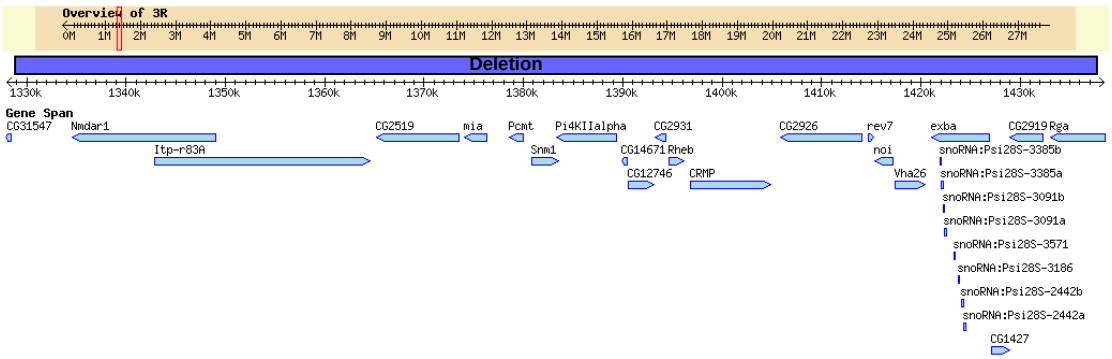


Figure 3-43 The region of the exilixis deletion Df(3R)Exel6144
Starting about 80 bp before the *cg31547* gene the deletion disrupts about 20 genes. None of the genes are in direct connection to the circadian clock. One gene is related to channel proteins. The *Nmdar1* gene encodes a protein with extracellular-glutamate-gated ion channel activity. The actual size of the deletion is indicated on top. The affected genes are shown below.

or *Pdf*-Gal4 driver we could not detect abnormal behavior in 12:12 Light/Dark cycles and under constant conditions, like LL or DD (data not shown). The strength of the RNAi mediated knock down was not yet determined by qPCR. When we knocked down *qsm* and *cg31547* at the same time though we could detect less rhythmicity in LL condition, compared to the *QsmRNAi(16)/TM3* alone. Since we did not determine genetical background effects, e.g. the investigation of the *timeless* and *jetlag* alleles (Peschel et al., 2006) on the second chromosome we have no proof that the diminished rhythmicity is caused by the Cg31547RNAi. The w^{1118}*; Df(3R)Exel6144, P(XP-U)Exel6144/TM6B* deletes the region 3R:1328532..1438438 (83A6;83B6)

(Parks et al., 2004). *Cg31547* is not deleted in this aberration, but its whole promoter region is missing (Figure 3-43).

This deletion is homozygous lethal. When we crossed the deletion with *qsmRNAi(16)* or *EP2586-Ox* we were able to detect an elongation in the period length under DD conditions (Table 3-4). So we could show a genetic interaction between, either Qsm or the Ep2586 line and Cg31547 or one of the deleted genes in *Df(3R)Exel6144*.

Genotype	Period±SEM (h)
Wild-type	23,9±0,1
qsmRNAi(16)	24,3±0,3
Df(3R)Exel6144/+	24,6±0,1
qsmRNAi(16)/ Df(3R)Exel6144	25,9±0,2
Ep2586-Ox	24,4±0,2
Ep2586-Ox/+; *Df(3R)Exel6144/+*	25,4±0,4

Table 3-4 Genetic interaction of Qsm, Ep2586 and the exilixis deletion Df(3R)Exel6144
Animals were kept for several days in LD conditions and released into constant darkness. The investigated period in DD is an average calculated from 10 different animals. Only when the *Df(3R)Exel6144* deletion was combined with the *qsmRNAi(16)* or Ep2586-Ox allele we could detect an elongation of the period.

On the other hand we wanted to investigate the effect of over-expressing Cg31547. For this reason we over-expressed a UAS-*Cg31547* construct in circadian clock cells. First preliminary data did not reveal abnormality when we over-expressed this transgene with a *tim*-Gal4 driver. We did not yet investigate, whether Cg31547 is really over-expressed in those animals and if the construct is functional.

3.4 Discussion

Even though the Qsm protein kept two Ph.D. students busy for the last eight years we are still only at the very beginning of understanding how *qsm* actually influence the circadian clock and - more generally speaking - we still have no proper information about its function in the cell. This recalcitrancy is caused by its diverse phenotypes. The involvement of the miRNA cluster 310-313 does not simplify matters, but makes the whole situation even more complex.

3.4.1 Rhythmic Expression

The rhythmic expression of *qsm* and the fact that this expression vanishes in clock mutants clearly demonstrates that *qsm* is regulated by the circadian clock. The up regulation in C*lk*^{Jrk} and down regulation in *per*^{01} animals suggests a regulation by *vrille*. This is affirmed by anti-Qsm Western blots in *clock* or *cycle* mutants. Here the Qsm protein level is clearly elevated (Figure 3-38). Vri is a transcriptional repressor that inhibits the expression of *Clk*. Vri also represses the rhythmically expression of *cry*, which suggests control of the expression of other output factors as well (Glossop et al., 2003). The fact that *qsm* is down regulated in *per*^{01} animals implies that in the *per*^{01}*;qsmRNAi(16)* flies not only the *period* dependent core clock is destroyed, but also that the low RNAi mediated knocked-down *qsm* levels are further reduced.

3.4.2 Behavior Analysis

RNAi mediated knock-down of *qsm* causes rhythmic behavior in constant light conditions. Using different driver lines we could narrow down the crucial area where *qsm* must be expressed for wild-type like behavior in LL. The eyes and the large and small LN_vs seem to play only a minor role. Keeping in mind, that *qsm* is expressed in (at least partly) the LN_vs, this is an unexpected result. One explanation for this discrepancy can be lower strength of the *Pdf* promoter that results in less RNAi mediated knockdown of *qsm* in the LN_vs (compared to *tim*-Gal4). Investigation with a stronger promoter line like the *Mz520-Gal4* or *gal1118* line might answer this question (Grima et al., 2004). Another explanation can be that *quasimodo* is indeed

expressed in the LN_vs – but the neurons that are important for the behavior in (constant) light are located in the dorsal region. Several recent publications report a major role for these more dorsally located neurons in mediating rhythmic behavior in LL. The over-expression of PER or that of the SHAGGY (SGG) kinase in the PDF-negative clock neurons induced rhythmic behavior in LL (Murad et al., 2007; Stoleru et al., 2007). The rhythmicity was associated with the cycling of PER subcellular localization in some of the DNs, whereas the PDF-expressing cells were molecularly arrhythmic. Furthermore results of Picot et al. indicate that whereas the PDF-expressing morning cells autonomously drive rhythmic behavior in constant darkness, the non PDF-expressing evening cells play this role in constant light, if CRY signaling is abolished or reduced (Picot et al., 2007). Restricting the RNAi mediated knockdown of *qsm* to the dorsal cells only with a specific driver line, e.g. *tim*-Gal4/*cry*-Gal80, might answer this question.

Not only the RNAi mediated knock-down of *qsm*, but as well the over-expression of the *Ep2586 P*-element changes the behavior of the animals. A normal wild-type fly displays a strong bimodal locomotor activity under Light/Dark cycles. The activity starts a few hours before the transition from light to dark or dark to light, respectively. After the transition from dark to light (or vice versa) the locomotor activity drops to a minimum level in the middle of the day (the so called siesta) and in the night time as well. In wild-type animals the evening activity decreases only after dark. In several mutant animals the evening activity peak is shifted towards the day. We could observe a similar advance in the *Ep2586-Ox* animals. Those flies exhibit a shift in their evening activity by several hours; their evening activity starts to drop even before the change from light to dark (Figure 3-10). The same behavior can be observed in Pdf^{01}, han^{5304}, *norpA*, *disco* or per^{Short} mutant animals (Hardin et al., 1992; Hyun et al., 2005; Konopka and Benzer, 1971; Mealey-Ferrara et al., 2003; Renn et al., 1999). While the shift in the activity in per^{S} mutants comes along with a temporal shift in the period expression - in the clock neurons Per^{S} protein levels decline about 4 hr prematurely (Marrus et al., 1996) – the other mutants display a lack of *Pdf* or *Pdf* reception. The *Ep2586-Ox* animals exhibit a small shift in their Per expression level of about 1-2 hrs (Figure 3-11). The possibility of a reduction in the PDF level will be discussed below in the text. *qsmRNAi(16)* animals behave abnormal under constant light conditions, but normal under 12:12 Light/Dark cycles. However we could also reveal a striking difference between the wild-type and the *qsmRNAi(16)* animals at the

molecular level. Normally Tim is degraded very fast as a response to light (Naidoo et al., 1999). In *qsmRNAi(16)* animals we can find a strong reduction of Tim levels in the Period positive (Per (+)) neurons, like the LNs or DNs – similar as in wild-type flies. But in contrast to wild-type flies we could reveal additional, Per negative neurons, where Tim is not degraded very quickly. Here our anti-Tim antibody could detect a strong Tim signal even after several hours in the light. This effect is even more enhanced at ZT 23 and it disappears almost completely at ZT 12. The Tim (+) Per (-) cells can be found in close proximity to the Per (+) cells. Of course the question remains if those cells that seem to solely express the Timeless protein and not the important clock factor Period are important for rhythmic behavior in the adult flies under Light/Dark or constant light conditions. This will be discussed in more detail below.

Under LL conditions we could still detect a very robust rhythm in locomotor activity in the *qsmRNAi(16)* animals. To reveal the molecular basis of this rhythm we investigated the Period and Timeless levels in the circadian clock neurons. Unfortunately we abstained from investigating the dorsal neurons – which is particularly with regard to recent publications a big neglect (Murad et al., 2007; Picot et al., 2007; Stoleru et al., 2007). The investigation of the LNs revealed rhythmicity in Per expression in the s-LN_vs and LN_ds under LL conditions. Once more Timeless staining was revealed even in LL conditions in Per (-) neurons (Figure 3-16). In cry^b brains dissected on the third day in LL, the PDF-positive s-LN_vs and some PDF-negative LNs showed PER cycling, whereas the l-LN_vs and three subsets of DNs did not (Picot et al., 2007). We observed the same rhythmic cells in *qsmRNAi(16)* animals. But Picot et al. clearly could show that the cycling in the s-LN_vs is not necessary for the rhythmic behavior in LL. They concluded that only the lateral neurons of the evening oscillator are sufficient and important for the LL behavior. On the other hand two other groups report that flies over-expressing clock proteins like Period or Shaggy exhibit rhythmic behavior in LL. The only cells that still show rhythmically expression under LL conditions of clock proteins like Pdp1 are some DN_1 cells. The mutual basis of all this results is the importance of the dorsal neurons for the locomotor behavior in LL or perhaps under photoperiods with a longer then 12 hrs light part. With its rhythmic Per expression in the LN_ds in LL *qsmRNAi(16)* fulfills these criteria. Closer investigation of the neglected DN_1s should yield in more important information.

The circadian phenotype caused by the RNAi mediated knock-down of *qsm* in Tim (+) cells with a *tim*-Gal4 driver implies that endogenous *qsm* expression should occur in those cells. Our preliminary result from RNA *in situ* stainings with probes against *qsm* revealed that the expression is indeed in cells close to the LNs, DNs or in the circadian clock cells (Peschel, 2004).

3.4.3 Localization of Qsm

Our first approach to investigate the expression pattern of Qsm was not analyzable. The double staining with the anti-PDF antibody resulted in unspecific binding of the secondary anti-Rat antibody to anti-PDF antibodies. This unspecific binding was not only observed when we investigated Qsm, but as well -only weaker- when the anti-Tim antibody was used.

The usage of the anti-Qsm antibody alone or together with anti-Per revealed a very inconsistent picture. But it is very likely, that the protein is expressed in the large and small LN_vs (Figure 3-18, ZT20), in cells close to these circadian clusters (e.g. Figure 3-18, ZT0), in cells close to the LN_ds (e.g. Figure 3-18, ZT0) and in cells without a circadian connection (Figure 3-17, 1+2). Most of the time Qsm was detected in the cytoplasm or in the cell membrane (Figure 3-18). Taking into account, that from the *in silicio* analysis Qsm is a protein that is linked to the membrane via a GPI anchor the staining pattern was expected. Only at some time points, for example at ZT16 in Figure 3-17 or 3-18, we can see a staining pattern that looks different from the cytoplasmatic/membrane-bound staining. Here the Qsm protein is either located in smaller cells, that are in close proximity of the normal *qsm* cells, or it is now in the nucleus or in a specialized compartment/organelle within the cell. To give an example – the special processing of GPI anchored proteins necessitate a localization of the protein in the endoplasmic reticulum. A third alternative can be that Qsm accumulates – like in Figure 3-18, ZT16 – in small clusters surrounding a l-LN_v cell. This can be interpreted as patches in the membrane of those cells, like in lipid rafts (Simons and Ikonen, 1997). Although only double stainings with markers that clearly distinguish membrane and/or nucleus would allow us to make a clear statement about Qsms' localization in the cell, we can speculate about its place in the cell. On the one hand the signal peptides at the terminus of Qsm clearly mark this protein for the membrane. A nuclear localization signal cannot be found in its amino acid sequence. This makes

it very unlikely that Qsm translocates into the nucleus. But on the other hand we can detect an internal cleavage site in the Qsm sequence. A tetrabasic motive, Arg-Arg-Lys-Arg, is often recognized and cleaved by endoproteases (Rholam et al., 1986). Hence there is a possibility, that Qsm is cleaved at this tetrabasic motive and that the resulting peptide (aa 24 – 306) can now translocate from the membrane to other parts of the cell or the extracellular space and thus producing a different expression pattern.

The *qsm* RNA is rhythmically expressed reaching peak levels at the end of the night/beginning of the day and trough levels by the end of the day and the beginning of the night. We expected to see a similar temporal fluctuation in the protein level. The cytoplasmic/ membrane-bound staining intensity of Qsm indeed seems to peak by the end of the night in a 12:12 LD cycle. But the much stronger nuclear/extracellular expression at ZT 16 suggests the most prominent Qsm expression in the middle of the night. This discrepancy cannot be easily explained. First of all to make a real statement this experiment must be repeated and the exact peak revealed. Additionally the help of membrane-bound or nuclear GFP proteins might answer the question if Qsm is located in the membrane/nucleus or extracellular. Over-expression of Qsm with UAS-*qsm* animals (see below) might provide additional information as well.

A second approach to reveal the exact position of Qsm (+) cells was the usage of a *P*-element insertion line, where the *lacZ* gene was inserted in the first intron of *qsm.* Theoretically the expression of *lacZ* should represent the pattern of *qsm* expression, because both genes should be under the control of the same promoter. Three disadvantages are connected with this approach. First of all, it is not mandatory, that the β-Gal protein (encoded by *lacZ*) exactly reports the expression of Qsm. Important promoter sequences could be located further upstream of the *P*-element insertion or the enhancer could change the expression pattern of Qsm compared to β-Gal. Second, the β-Gal protein is rather stable, resulting in an absence of rhythmic expression of this protein (Stanewsky et al., 1997). This makes it impossible to gain information about the temporal expression of Qsm. And third, the β-Gal protein carries a nuclear localization signal. Hence it can be found almost exclusively in the nucleus and thus provides us with no information about Qsm localization in the cell.

Taken this into account we can nevertheless draw some conclusions from the expression of β-Gal in the fly strain *P(PZ)l(2)05510*^{05510}. Again we could detect β-Gal in the small and large LN_vs. Once more cells in close vicinity to the LNs could be revealed (Figure 3-19, A, C, D). In the dorsal part of the brain we only investigated

the DN_3 region, not the DN1 and DN2 cells. In some of the DN_3 cells Period and β-Gal are co-expressed (Figure 3-19, B).

Under LD and LL conditions additional Tim (+) and Per (-) cells could be detected in *qsmRNAi(16)* animals. Those cells were always in close proximity to the published clock neurons like the LNs or DNs (see Figure 3-12, 3-13, 3-14). Interestingly we could detect Qsm in similar located cells like the Tim (+) Per (-) cells (see Figure 3-17 and 3-18). The q-LN_vs 1 and 2 for example or the additional cells near the DN_3 cell cluster might be the same as the additional Tim cells. The best way to give a clear answer whether *qsm* is expressed in Tim (+) Per (-) cells in *qsmRNAi(16)* animals would be to perform a Qsm/Tim double staining in those animals. The fact that the anti-Qsm and anti-Tim antibody were raised in the same animal (in rat) complicates further investigations of the co-expression of Qsm and Tim in those cells. But if Qsm can be detected in Tim (+) Per (-) cells RNAi mediated knockdown of Qsm in those cells would be the explanation for the additional Tim and perhaps for the observed LL rhythmicity.

3.4.4 *per^{01}* and *quasimodo*

Almost 40 years ago the *period^{01}* phenotype was described for the very first time. *Period* is at the very core of the circadian clock – without a functional *period* gene a fly looses its rhythmicity (Konopka and Benzer, 1971). Our findings that *per^{01};;qsmRNAi(16)* animals still exhibit some rhythmicity caused us to reconsider this hallmark of circadian clocks. Clearly those animals anticipate the lights-off in the evening (Figure 3-20). Even though an experiment using the different photoperiods suggests the involvement of an hourglass mechanism (Figure 3-21), the result that the rhythmicity endures even under constant light conditions argues for a real endogenous rhythm (Figure 3-22). Recent observations demonstrate that in *Drosophila* not only one oscillator contributes to the circadian behavior of the fly, but that different oscillators act together to control this rhythm. In 2004 two different groups reported independently about the existence of a morning and evening oscillator in the flies brain (Grima et al., 2004; Stoleru et al., 2004). Distinct cell groups produce different locomotor behavior (see introduction). A current paper enhances this theory and discusses the possibility of a Dark and Light oscillator. One oscillator that regulates activity in constant darkness or the dark part of one day - the other oscillator

controlling activity in constant light or the light part of the day (Picot et al., 2007). Our current findings suggest that under illumination (LL or the Light part of LD) Period protein is not mandatory for circadian rhythms (Figure 3-22). Without illumination though the animal looses its rhythmicity (in DD or in the Dark part of LD) (Figure 3-22). If we now combine our findings (Chapter 3.3.6) with the Dark and Light oscillator paradigm (Picot et al., 2007) we suggest a modificated model:

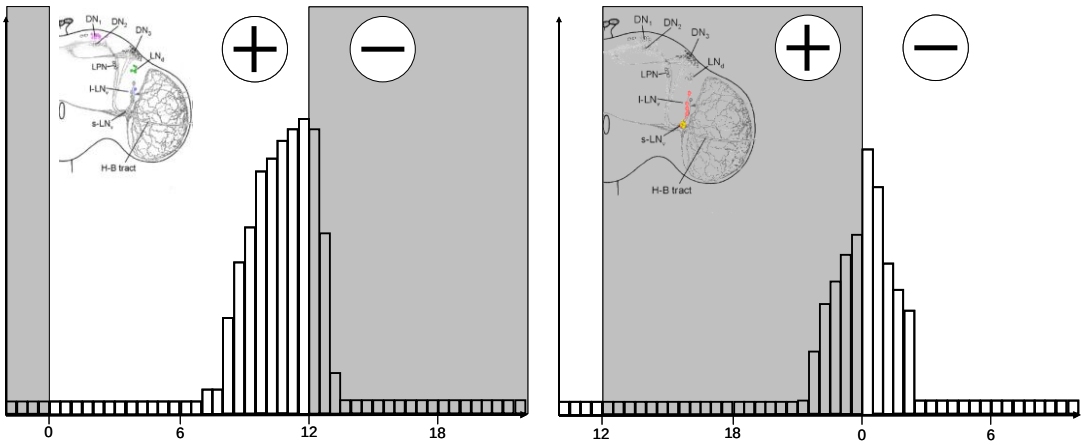


Figure 3-44 The Light and Dark oscillator
The neurons of the so called evening cells contribute to the activity in the light. In those cells light has an activatory role – darkness inhibits the activity (left side). The cells of the so called morning oscillator contribute to the activity in the darkness. Here illumination has an inhibitory effect on the activity (right side).

In a flies' brain two individual oscillators coexist. One 'Dark oscillator' that is located in the small and large LN_vs, generates the activity peak in the morning (Figure 3-44 right). In the night time (or in constant darkness) those cells induce activity in the fly. After illumination/lights-on the activity ceases (Figure 3-44 right).

Contrary the 'Light oscillator' in the LN_ds, the 5th s-LN_v, and the DNs is important for the activity in the evening or in LL. When the darkness starts again, the activity stops (Figure 3-44 left). In the Light oscillator the Period protein seems to be not compulsory. The question remains, why *per*^{01} animals behave arrhythmic in LL conditions and do not exhibit evening anticipation – while *per*^{01}*;;qsmRNAi(16)* animals do behave rhythmic in LL and exhibit evening anticipation? The answer can be found in the connection of the Dark oscillator and the Light oscillator. The dominant Dark oscillator determines the period of the entire system by providing a daily signal to the Light oscillator and is therefore a true cellular Zeitgeber (Stoleru et al., 2005). In wild-type flies the resetting signal from the Dark oscillator may be required to adapt the Light oscillator to different photoperiods. In *per*^{01} animals on the

other hand the information from the Dark to the Light oscillator renders the flies arrhythmic. In *per^{01};;qsmRNAi(16)* animals this signal (from the Dark to the Light oscillator) seems to be impaired. As a consequence to this the evening activity can not be adapted to different photoperiods (Figure 3-21) and the Dark oscillator can not render the animals arrhythmic in LL conditions thus allowing the Light oscillator to free run (Figure 3-22).

Another important clock protein, Tim is intimately involved in the circadian light response. Therefore we investigated if Tim protein alone can drive the behavior in the Light oscillator in *per^{01}* mutant flies. Under LD conditions we still could detect light dependent degradation of Tim – as well in *per^{01}* as in *per^{01};;qsmRNAi(16)* flies. But we were not able to see a significant difference in the phosphorylation. Along with this observation we were not able to detect Tim in the nucleus in wholemount stainings, which implies that Tim phosphorylation – that can be determined by Western blot in form of slower migrating bands (Martinek et al., 2001)- is important for the nuclear localization of Tim, but is not necessary for the light dependent degradation of the protein. This raises the question, if a mainly cytoplasmatic Tim contributes to the evening and/or constant light activity of the *per^{01};qsmRNAi(16)* animals. On the other hand Collins et al. were able to demonstrate the necessity of Tim for at least the evening activity in *per^{01}* flies; *per^{01};cry^{b}* mutants display evening anticipation, while *per^{01};tim^{01};cry^{b}* animals do not (Collins et al., 2005). When we compared the Tim level in adult brains from *per^{01}* and *per^{01};;qsmRNAi(16)* animals we could see some minor differences in the degradation and spacial and temporal localization of Tim. Under LD conditions we could detect a decrease of Tim at ZT9 – this result was revealed by Western Blot and by staining of the brains. Furthermore additionally Tim cells could be observed that might be the previously described Tim (+) Per (-) cells

The fact that only four different time points were investigated does not allow a clear answer about Timeless in *per^{01}* animals. In particular we can not rule out that Tim is localized to the nucleus only during a very narrow time window. Additionally other clock proteins like Pdp1 should be investigated to find out if we still can see a circadian rhythm on a molecular basis in those animals.

3.4.5 PDF and *quasimodo*

The similarity of the *Ep2586-Ox* animals to the Pdf^{01} phenotype caused us to investigate the genetic interaction of *Ep2586* and *Pdf*. If *Ep2586-Ox* animals are impaired in their PDF function we would expect to see a rescue of the advanced evening activity, when we add more PDF to the system and to see an aggravation if we lower the overall amount of PDF. Both predictions proved true - we could see a clear shift in the evening activity in those animals (Figure 3-27 and 28). Contrary to the *Pdf* null mutant, in the *EP2586-Ox/+;* Pdf^{01}/+ double mutant the morning anticipation was not impaired, just like in wild-type or Pdf^{01}/+ animals (Figure 3-27). Regrettably we can not make any conclusion about the behavior of *EP2586-Ox/+;* Pdf^{01}/+ animals in DD (data lost). But again it is very likely, that the postulated Dark oscillator (morning activity and behavior in DD) is not affected by *Ep2586-Ox* and only the signaling from the Dark to the Light oscillator is impaired.

A closer look at the PDF level in *Ep2586-Ox* flies revealed, that indeed the overall amount of this peptide is very low (Figure 3-29). Especially in the s-LN_vs cells and its' arborizations hardly any PDF staining could be detected. Next we investigated why *Ep2586-Ox* animals showed this drastic decrease in PDF. Because the mRNA expression level was normal (Figure 3-30) and the maturation and cleavage of the prepro-PDF to the PDF was not impaired (Figure 3-32) we investigated if the release of the PDF peptide was affected. By using a GFP coupled ANF peptide or by over-expressing PDF we could reveal, that the release of peptides in general is impaired (Figure 3-34 and 3-35). Knowing that the release of PDF is altered in *Ep2586-Ox* raises the question, how the peptide release in the circadian clock neurons is controlled. An important clue comes from Nitabach et al. (Nitabach et al., 2002). They report that the electrically activity of the pacemaker cells control the circadian locomotor behavior by modulating synaptic release of PDF and other neurotransmitters. Furthermore they have shown that electrical silencing of adult *Drosophila* circadian pacemaker neurons through targeted expression of either an open rectifier or inward rectifier K^+-channel stops the free-running oscillations of the circadian molecular clock. Interestingly, electrical silencing of LN_vs phenocopies the Pdf^{01}- null mutant at both behavioral and molecular levels (Renn et al., 1999) except for the complete abolition of free-running cellular oscillation in the LN_vs themselves (Wu et al., 2008). This raised the suspicion that *Ep2586-Ox* alters the electrically

activity in the pacemaker neurons. We were wondering if the over-expression of this ZP domain protein changes the electrically activity in the cell.

3.4.6 The *EP2586* Insertion

An UAS over expression line from the Rorth collection *EP2586* (Rorth, 1996) is inserted shortly before the start of *qsm*. The same is true for the *Ep2356* or *Ep2587* line. All three lines show the same shifted evening activity peak when they are over expressed with the help of a *tim*-Gal4 driver line (Figure 3-10 and (Stempfl, 2002)). The investigation of the over-expression of *qsm* in *Ep2586-Ox* animals revealed that *qsm* mRNA is – if at all – elevated to only a minor extend (Figure 3-36). Since the Gal4 protein is very stable (Allada et al., 2003), the expression of *gal4* under *timeless* control should yield in a massive Gal4 accumulation in the clock neurons and thus to a constituitive upregulation of *qsm*. Hence the *qsm* levels at trough and peak points in *Ep2586-Ox* animals (Figure 3-37) are in contrast with this massive Gal4 expression. Moreover when we investigated the Qsm protein on Western blots in *Ep2586-Ox* we were not able to reveal an upregulation of this gene (data not shown and (Stempfl, 2002). The failure to see an upregulation of Qsm in *Ep2586-Ox* on Western Blots questioned the ability of the anti-Qsm antibody to detect Qsm. But we could clearly demonstrate that our antibody can detect Qsm on Western blot – in S2 cells after expression of *qsm* under the control of an *actin* promoter (Figure 3-38 left) or in different adult *Drosophila* mutants and wild-type flies (Figure 3-38 right).

All those discrepancies caused us to doubt if Qsm is really over-expressed in the *Ep2586-Ox* strain and thus causing the investigated phenotype of an advanced evening activity peak.

In the beginning of this decade the freshly discovered miRNAs became subject of many publications (Zamore and Haley, 2005). New algorithms allowed to find new miRNAs in the *Drosophila* genome and databases were created dealing with the predicted targets of those miRNAs (Griffiths-Jones, 2006; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008). A closer investigation of the insertion locus of *Ep2586*, *Ep2587* and *Ep2356* revealed a miRNA cluster composed of miRNA 310-313 immediately downstream of the insertion site (Figure 3-5). Even though we were not able to investigate the amount of the miRNAs in *Ep2586* it is very likely, that the miRNA gene following the *Ep2586* is over-expressed instead of *qsm*. To give a clear answer to this question we generated transgenic flies that either were over-expressing

the cDNA of *qsm* or the three *miRNAs* under UAS control. When we now over-expressed *qsm* or the *miRNAs* with different clock related Gal4 driver lines we could reveal that only the miRNAs are causing the shift in the evening activity and not the *qsm* gene (Figure 3-39 and 3-40). When we now repeated Western blots with the anti-Qsm antibody to investigate the over-expression of *qsm* we could see a striking up regulation in three fly strains over-expressing the construct under *tim*-Gal4 control (Figure 3-41). An additional hint that in *Ep2586-Ox* animals the miRNA cluster 310-313 is over-expressed was revealed, when we compared over-expression of *Ep2586*, *qsm* or *miRNA310-313* under the control of a *ccap*-Gal4 driver. Only in the *Ep2586* and *miRNA310-313* over-expressing animals we could detect a severe 'juvenile' phenotype (Figure 3-41). This phenotype can be detected, when the release of the peptide Bursicon is disturbed. In a recent publication our results were confirmed (Zhao et al., 2008). When Zhao et al. over-expressed the *EP(2)2587* and *EP(3)3354* lines with a *ccap*-Gal4 driver they revealed the same eclosion phenotype as we did (Zhao et al., 2008). Both EP lines appear to drive the expression of micro-RNAs that all belong to the same family. The *Ep2587* line (same as *Ep2586*) is described above (Table 3-3), the *Ep(3)3354* line expresses the miRNAs *miR-92a*, *miR-92b*. Both clusters effect similar genes and belong to the same family (Table 3-2 and 3-4). Furthermore over-expression of *EP(2)2587* and *EP(3)3354* failed to affect the morphology of the CCAP/bursicon neurons. This caused them to ask whether the secretion of bursicon was affected. They predicted, that over-expression of the miRNAs could have altered the electrical activity (or synaptic efficacy) of neurons within the CCAP/Bursicon cell population that may provide synaptic input to the subset of neurons with neuroendocrine projections (Zhao et al., 2008). The fact that the over-expression of channel proteins like Shaw or Kir2.1 caused the same phenotype focused our research on channel or channel like proteins that are direct targets of both miRNA clusters. A very

92a	92b
CG8028	CG3077
cv	CG33169
CG10947	CG8028
CG33279	cv
CG3077	CG31547
CG33169	Msp-300
nmdyn-D7	CG33279
Grip71	CG10947
CG31547	CG5009
epsin-like	CG13186

Table 3-5 predicted miRNA targets
The miRBase homepage from Sanger (http://microrna.sanger.ac.uk/) predicts for the indicated miRNA 92a and 92b about 400 to 500 different targets. The targets with the highest likelihood are displayed in the table, whereas the higher the probability, the further up the protein is in the table. As in the miRNA cluster 310-313 the co-transporter CG31547 is target of the miRNA 92 cluster.

interesting candidate seems to be the Na-K-Cl co-transporter Cg31547 (Table 3-3 and 3-4).

First preliminary experiments with this gene did not allow us to make a clear assertion if *Cg31547* is the target gene of the miRNAs that causes this circadian phenotype. A deficiency that deletes most of the promoter region of Cg31547 produces an elongation of the circadian period in DD when co-expressed with *Ep2586-Ox* or *qsmRNAi(16)*. But it should be mentioned that in this deletion also the *nmdar1* gene is affected which encodes for a glutamate receptor. Glutamate was already linked to circadian rhythms (Hamasaka et al., 2007). The interaction of this deletion with Qsm might be unexpected. On the other hand an easy explanation for this genetic interaction of *qsm* with *Cg31547* might be that both proteins directly interact. In an Y2H assay it was shown, that Qsm interacts with several different proteins. The highest probability of interaction could be detected for *Cg31547* (Giot et al., 2003).

3.5 Outlook

The primarily revealed different phenotypes of *qsm* complicated our investigation of this gene profoundly. Only in the very end of my work we were able to divide the different phenotypes. We could assign the rhythmic behavior in constant light to the *qsm* gene, while the shift in the evening activity is not caused by the over-expression of *qsm*, but the increased expression of the miRNA cluster 310-313 causes this drastic phenotype. Of course we took one big step forward with this new result – but as well a step back. Whilst the investigation of Qsm is widely advanced, we are now again only at the very beginning of understanding the miRNAs function within the clock. There are hundreds of predicted target genes of the miRNA cluster 310-313, so first of all we have to reveal which of the miRNAs target genes is causing the observed abnormal locomotor activity. With the Na-Ka-Cl co-transporter *Cg31547* we already have a good candidate. But further validations should clarify its involvement. Furthermore it must be validated if this protein really interacts with Qsm. Only after revealing the target gene of the miRNAs we can design new experiments – depending on the function of this miRNA target gene.

For Qsm on the other hand we must clarify if the DNs display rhythmic accumulation of clock proteins or not in LL conditions in *qsmRNAi(16)* flies. Furthermore the presence of Qsm protein in this dorsal group should be investigated. Over-expression

of Qsm in neuronal cells in combination with cellular compartment markers should reveal its cellular localization.

The interesting observation that the knock-down of *qsm* is resulting in rhythmic behavior in animals lacking the per^{01} offers many possibilities and raises a lot of interesting questions. Which neuronal group is important for this *period* lacking clock? Is the Timeless protein alone sufficient to drive the rhythmic behavior in constant light? Is the separation in a Dark and a Light oscillator warrantable? Which other clock proteins are involved in generating this rhythm?

4. *Veela* and Jetlag

4.1 Background

Adaption to the environment leads in general to a higher survival rate and a greater fitness (Darwin, 1859). The fruit fly *Drosophila melanogaster* is equipped with a sophisticated molecular mechanism (called the circadian clock) that allows the animal to anticipate the daily changes of daylight and darkness to adapt to its environment (for a detailed description of this mechanism see general Introduction). The degradation of Tim and the attendant degradation of Per results in a molecular reset of the circadian clock. In animals exposed to light for a prolonged time, the constant degradation of Timeless leads to a loss of a functional circadian clock and to arrhythmic behavior (Emery et al., 2000a; Konopka et al., 1989). Animals carrying a mutation in the Cryptochrome (Cry) photoreceptor, for example the cry^{b} (Stanewsky et al., 1998) or cry^{m} (Busza et al., 2004) allele, behave rhythmic in constant light conditions (LL) (Busza et al., 2004; Emery et al., 2000a).

In his Ph. D. thesis Shobi Veleri discovered that $ninaB^{P360d}$ mutants also stay rhythmic in LL– just like the *cry* mutant animals (Veleri, 2005). *ninaB* or *neither inactivation nor afterpotential B* is a β, β-carotene-15, 15'-monooxygenase, which converts β-carotene to all-trans-retinal. The mutant $ninaB^{P360d}$ therefore lacks functional Rhodopsins (von Lintig et al., 2001; von Lintig et al., 2005). Thus Veleri assumed that the $ninaB^{P360d}$ mutation caused the rhythmic behavior of these flies in LL due to the total lack of functional Rhodopsins (Veleri, 2005). Closer investigation revealed that while the $ninaB^{P360d}$ mutation is located on the third chromosome, the mutation that is causing the circadian phenotype maps to chromosome two. After outcrossing the $ninaB^{P360d}$ bearing third chromosome he renamed the LL-rhythmic mutant fly strain *Veela* (Rowling, 2000). Consequently we had to meiotically map the new genetic variant on chromosome *2* in order to identify the gene that is affected in this mutant. The investigation of this *Veela* mutation was a major project of my Ph.D. thesis.

4.2 *Veela* defines a molecular link between Cry and Tim

The first part of this chapter deals with mapping of the *Veela* mutation.

This investigation yielded in a surprising and unexpected finding. Not one, but two different factors are causing the abnormal behavior in the mutant. Each factor alone impairs the circadian photoreception only to a minor extend. Hence only the combination of both leads to the observed phenotype of rhythmic behavior under constant light conditions. The results of these investigations are presented in the following publication from the year 2006:

N. Peschel, S. Veleri, and R. Stanewsky
Veela defines a molecular link between Cryptochrome and Timeless in the light-input pathway to Drosophila's circadian clock
PNAS, November 14, 2006; 103(46): 17313 - 17318.

Veela defines a molecular link between Cryptochrome and Timeless in the light-input pathway to *Drosophila*'s circadian clock

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Organisms use the daily cycles of light and darkness to synchronize their internal circadian clocks with the environment. Because they optimize physiological processes and behavior, properly synchronized circadian clocks are thought to be important for the overall fitness. In *Drosophila melanogaster*, the circadian clock is synchronized with the natural environment by light-dependent degradation of the clock protein Timeless, mediated by the blue-light photoreceptor Cryptochrome (Cry). Here we report identification of a genetic variant, *Veela*, which severely disrupts this process, because these genetically altered flies maintain behavioral and molecular rhythmicity under constant-light conditions that usually stop the clock. We show that the *Veela* strain carries a natural *timeless* allele (*ls-tim*), which encodes a less-light-sensitive form of Timeless in combination with a mutant variant of the F-box protein Jetlag. However, neither the *ls-tim* nor the *jetlag* genetic variant alone is sufficient to disrupt light input into the central pacemaker. We show a strong interaction between *Veela* and *cryptochrome* genetic variants, demonstrating that the Jetlag, Timeless, and Cry proteins function in the same pathway. *Veela* also reveals a function for the two natural variants of *timeless*, which differ in their sensitivity to light. In combination with the complex array of retinal and extraretinal photoreceptors known to signal light to the pacemaker, this previously undescribed molecular component of photic sensitivity mediated by the two Timeless proteins reveals that an unexpectedly rich complexity underlies modulation of this process.

F-box | polymorphism | photoreception

Most organisms live throughout the year in light/dark (LD) cycles. This natural fluctuation represents a crucial stimulus to adjust the internal circadian clocks to operate in synchrony with the environment (1). Exposure to constant light dramatically affects biological rhythms and molecules comprising the circadian clock in many organisms. In *Drosophila*, the chronic presence of light (constant light, called LL) usually results in behavioral arrhythmicity and a breakdown of molecular oscillations in the circadian clock (2–4). Although the experimental LL situation is artificial, mutations that abolish this LL effect define essential components of light-signaling pathways that synchronize the central pacemaker to the external world. So far, this has been shown for the Cryptochrome (Cry) mutations cry^b and cry^m (5–7). Crys are related to photolyases, blue-light photoreceptors that use harvested light energy to repair UV-damaged DNA (8). In animals and plants, Cry proteins have been shown to function in the circadian system as photoreceptors, clock factors, or both (8, 9).

Opsin-mediated retinal, extraretinal, and Cry-independent photoreception contributes to light synchronization of the circadian clock in *Drosophila* (5, 10, 11). However, the main entrainment pathway is believed to involve light-dependent Cry and Timeless (Tim) and perhaps Period (Per) interactions within the behavioral pacemaker neurons of the fly brain (7, 12, 13). Upon light activation, Cry is thought to undergo a conformational change that allows it to bind to Tim in a way that irreversibly targets this clock protein for degradation by the proteasome (7, 14, 15). This light-induced degradation of Tim is crucial for molecular and behavioral clock resetting. If Tim is degraded prematurely by light pulses given at the end of the night, as a consequence the molecular feedback loops comprising the circadian clock and regulating rhythmic locomotor behavior are phase-advanced. Vice versa, the clock reacts with phase delays in case Tim is degraded by light exposure in the early night (reviewed, for example, in ref. 16).

By characterizing the genetic variant *Veela*, which, like *cry* mutants, behaves abnormally rhythmically in constant light, we identified a factor involved in the Cry-dependent light-input pathway of *Drosophila*. *Veela* genetically interacts with cry^b and shows decreased light sensitivity of Tim degradation. We demonstrate that these effects are caused by the simultaneous presence of a natural (less light-sensitive) form of Tim and a mutation in the F-box protein Jetlag (Jet). Importantly, the same *jetlag* (*jet*) mutant in combination with another natural and common variant of Tim behaves like wild type (WT). Therefore, previous findings attributing observed light-input defects solely to mutations in the *jet* gene (17) need to be revised.

Results

Isolation and Initial Mapping of *Veela*. During behavioral analysis of potential light-synchronization mutants in *D. melanogaster*, we identified a strain that exhibits robust rhythmicity in LL (Fig. 1 and Table 1). The variant mapped to chromosome 2 and, because of its elusive nature (see *Supporting Text*, which is published as supporting information on the PNAS web site), was named *Veela* (18). The mutant showed a semidominant effect: ≈30% of *Veela*/+ flies exhibited weak rhythmicity in LL (Table 1). Behavior in constant darkness was not abnormal (Table 2, which is published as supporting information on the PNAS web site). Mapping by meiotic recombination placed *Veela* between the *aristaless* (*al*) and *dumpy* (*dp*) markers, close to the clock gene *timeless* (refs. 19 and 20; see also *Materials and Methods*).

***Veela* Genetically Interacts with cry^b and Stabilizes Tim in the Light.** Photic responsiveness of Tim is mediated by its light-induced interaction with Cry, resulting in rapid degradation of Tim, thus representing a crucial mechanism by which *Drosophila*'s clock synchronizes to LD cycles (5, 7, 21). Because a mutation in the

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The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: LD, light/dark; LL, constant light; Cry, Cryptochrome; Per, Period; Tim, Timeless; LN, lateral neurons; DN, dorsal neurons; l-LNv, large LN; LRR, leucine-rich repeat; ZT, Zeitgeber time.

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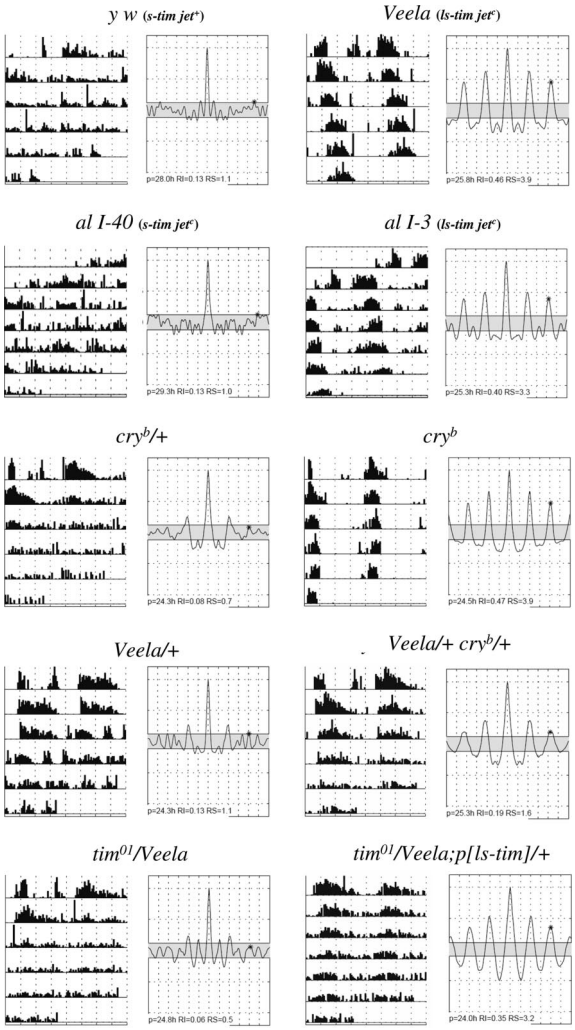

Fig. 1. Locomotor activity of individual flies during constant light. Genotypes are indicated on top of each actogram accompanied by its corresponding autocorrelogram. Actograms show the raw activity of each fly, in which the height of each bar indicates the amount of locomotion during a 30-min interval. Autocorrelograms show period and rhythmicity index values of each fly as an objective way to determine rhythmicity (see *Materials and Methods*). The upper four flies also have their polymorphisms in regard to the *tim* and *jet* variants indicated (see text). Flies to the left are arrhythmic; individuals to the right are rhythmic. *al I-40* and *al I-3* each designate recombinants resulting from meiotic crossing over between two second chromosomes in females heterozygous for *Veela* and the marker combination *al dp b pr*. Both the *I-40* and the *I-3* recombinants carry the *al* marker and the jet^c variant but differ with respect to *tim*. Note that *al I-40* flies behaved arrhythmically in LL (like the controls), although they carry jet^c.

cryptochrome gene (cry^b) also results in anomalous rhythmicity in LL (ref. 6; see also Fig. 1 and Table 1), we reasoned that *Veela* may interfere with the Tim–Cry interaction, perhaps, given our mapping results, because of a mutation in *tim* itself. To examine this possibility, we analyzed the behavior of flies that carried one copy of cry^b in combination with one copy of *Veela*. Strikingly, these double heterozygotes exhibited robust rhythmicity in LL (Fig. 1 and Table 1). Because this phenotype is never observed in heterozygous $cry^b/+$ flies and is much stronger compared with that of *Veela*/+ flies, our results indicate a strong genetic interaction between *Veela* and cry^b (Fig. 1 and Table 1).

These findings suggested that *Veela* impairs light inputs to the clock because of interference with the usual Tim–Cry interaction. If true, light-induced degradation of Tim should be affected in *Veela* flies, and we therefore analyzed Tim abundance during the light portion of the LD cycle [during Zeitgeber times (ZT)0–12; Fig. 2], during which Tim levels are normally very low (5). As expected, Tim abundance in head protein extracts was increased in *Veela* flies (compared with controls monitored between ZT1 and ZT9), whereas we did not see any significant differences during the night (ZT13; Fig. 2). Next, we analyzed Tim levels in *Veela*/+ $cry^b/+$ flies, which also showed robust behavioral rhythms in LL (Fig. 1 and Table 1). Here, too, the doubly heterozygous flies showed increased Tim levels at ZT2 and ZT9 compared with both kinds of singly heterozygous controls (Fig. 2). Importantly, Tim signals in the *Veela*/+ $cry^b/+$ extracts were stronger compared with those of homozygous *Veela* flies and more similar to those of homozygous cry^b flies. Thus, at the molecular level as well as behaviorally, *Veela*

Table 1. Locomotor activity rhythms in constant light

Genotype	N_T	n_{Rhy}	Period	RI	% Rhy
y w	70	2	22.3	0.30	3
wild-type Athens	8	0	—	—	0
cry^b/cry^b	22	21	24.3 ± 0.1	0.33 ± 0.03	96
$cry^b/+$	23	1	27.5	0.30	4
Veela/*Veela*	75	64	25.7 ± 0.1	0.33 ± 0.02	85
Veela/+	35	11	24.4 ± 0.4	0.18 ± 0.01	31
Veela/+ $cry^b/+$	26	20	25.9 ± 0.6	0.24 ± 0.02	77
al I-3/*al I-3* (*ls-tim* jet^c)	10	9	26.2 ± 0.6	0.33 ± 0.04	90
dpp 20/*dpp 20* (*ls-tim* jet^c)	8	6	25.9 ± 0.3	0.20 ± 0.04	75
dp b pr I-28/*dp b pr I-28* (*ls-tim* jet^n)	13	1	25.8	0.27	7
dpp ed 23/*dpp ed 23* (*s-tim* jet^c)	8	0	—	—	0
al I-40/*al I-40* (*s-tim* jet^c)	12	2	24.2	0.16	16
al I-40/*al I-40*	40	2	23.2	0.25	5
$tim^{01}/+$	24	0	AR	—	0
Veela/tim^{01}	42	13	25.9 ± 1.1	0.21 ± 0.02	22
tim^{01}/tim^{01}; *P[ls-tim]*/*P[ls-tim]*	14	1	25	0.20	7
Veela/tim^{01}; *P[ls-tim]*/+	46	29	24.6 ± 0.2	0.25 ± 0.02	63

N_T, total number of flies tested; n_{Rhy}, number of individuals behaving rhythmically in LL. Period, free-running cycle durations (hr ± SEM), determined by autocorrelation; RI, Rhythmicity Index, indicating the significance level associated with a given period (see *Materials and Methods*); % Rhy, percentage of rhythmic flies of a given genotype. Male flies of the indicated genotypes were analyzed. As controls, flies with the *X*-chromosomal body- and eye-color markers *y* and *w* were used (*Materials and Methods*). The *y w* strain used here carries the *s-tim* allele (Fig. 4). The wild-type Athens strain carries the *ls-tim* allele. Genotypes containing a digit in conjunction with a recessive marker designation (e.g., *al I-40*) specify recombinants obtained from crossing homozygous *Veela* flies to multimarker chromosomes (*Materials and Methods*). The *P[ls-tim]* transgene is inserted on chromosome 3 and contains the *ls-tim* version of *timeless* including *tim* promoter sequences (25).

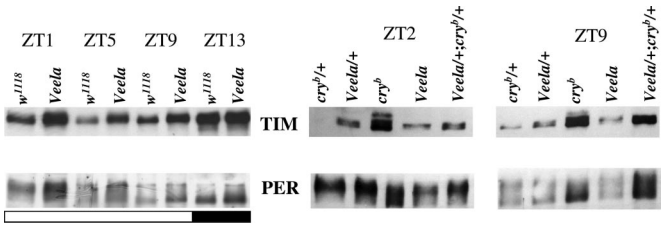

Fig. 2. *Veela* stabilizes Tim during the light portion of LD cycles. Western blots of head extracts from flies collected during LD cycles. Genotypes and time points of collection (ZT) are indicated above the blots. (*Upper*) Anti-Tim. (*Lower*) Anti-Per. Below the left blots, white and black bars indicate when the lights were on and off, respectively. *Veela* and cry^b have similar effects on stabilization of Tim as on Per. Heterodimerization of both proteins has been proposed to stabilize Per (16) and is probably the reason for increased levels of Per in *Veela*, cry^b and *Veela*/+ $cry^b/+$ flies.

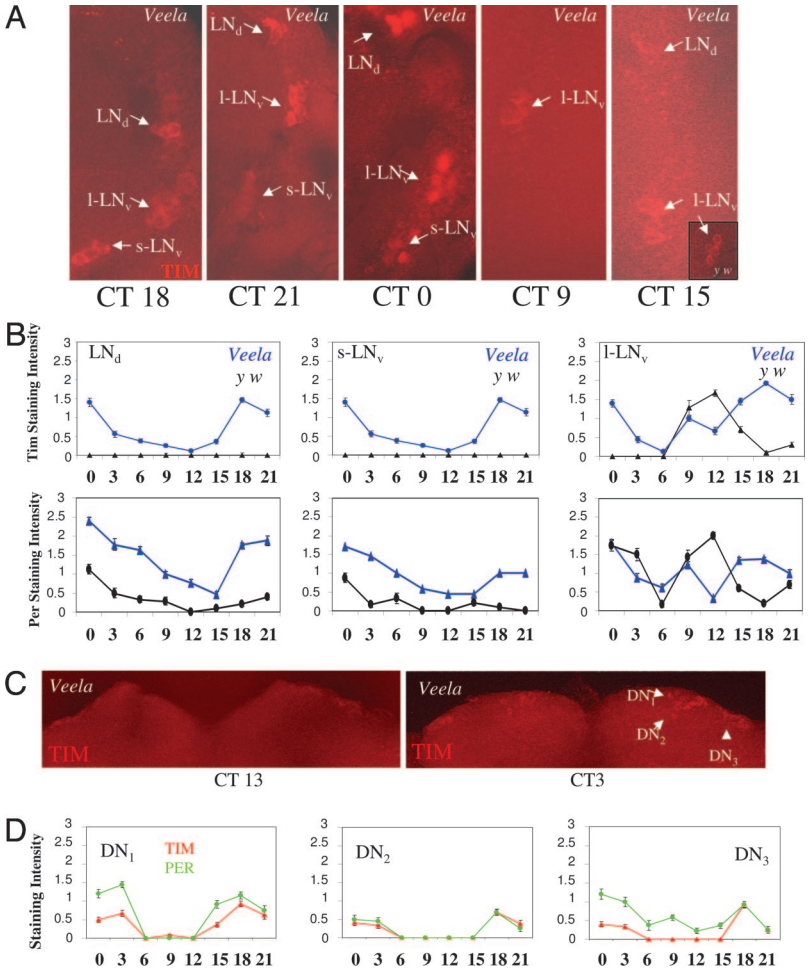

Fig. 3. Rhythmic Tim and Per expression in constant light in clock neurons of *Veela* flies. Control and *Veela* flies were synchronized to 12-h:12-h LD cycles and subsequently released into LL. After 2 days, males were killed at the indicated circadian times (CTs; below the images or *x* axis), and whole-mounted brains were stained with anti-Tim and -Per. (*A*) Rhythmic Tim immunoreactivity during LL in all LN of *Veela* flies and in the cytoplasm of the l-LNvs of *y w* controls (see *Inset*, CT15). (*B*) Quantifications of anti-Tim (*A*) and anti-PER stainings, including *y w* control flies. Note that controls show rhythmic Tim and Per accumulation in the l-LNv. Error bars indicate SEM. (*C*) Rhythmic Tim accumulation in the DNs of *Veela* flies during the second day of constant light. (*D*) Quantification of Per and Tim immunoreactivity in DNs of *Veela* flies during LL. No staining was observed in *y w* control flies under these conditions. Between 12 and 20 (*y w*) or 16 and 25 (*Veela*) brain halves for each time point were analyzed. Error bars indicate SEM.

and Cry interact robustly (Figs. 1 and 2). We have no explanation for why Tim signals in the heterozygous *Veela*/+ flies were stronger compared with those in homozygous *Veela* flies. Similar effects of *Veela* and of the combination of *Veela* with cry^b were observed for the Per protein (Fig. 2 *Lower*), which is thought to be stabilized by heterodimerization with Tim (16).

Tim and Per Are Rhythmically Expressed in the Pacemaker Neurons and Glia Cells of *Veela* Flies in Constant Light. Rhythmic locomotor activity is driven by clock gene expression within certain neurons of the fly brain (22). Based on their location within the lateral and dorsal brain, respectively, these neurons are historically divided into lateral neurons (LNs) and dorsal neurons (DNs): three groups of both LNs [small (s-LNvs), large (l-LNvs), and dorsal LNs] and DNs (cell groups 1–3; ref. 22). Because *Veela* individuals behave rhythmically in LL, we asked which subset of the clock neurons would drive this behavior or whether all neurons would be affected equally by this genetic variant. To answer this question, we stained whole-mounted brains of *Veela* adults during the second day in LL with anti-Tim and -Per. We observed robust rhythmic expression of Tim and Per in all clock-neuronal cell types, indicating that *Veela* disrupts light inputs into all six groups (Fig. 3). In all of these LNs and DNs, peak expression of both Tim and Per were observed at the end of the subjective night (a term for the second half of L in an LL "cycle") through the beginning of the subjective day, demonstrating synchronized expression of clock genes among these cells. As expected, in the LNs of control flies that are behaviorally arrhythmic after 2 days in LL (Fig. 1), no rhythmic expression or accumulation of the two clock proteins was observed, indicating light-induced Cry- and $Veela^+$-mediated constitutive degradation of Tim (Fig. 3*B*). A noticeable exception in the controls involved the l-LNv cells, which showed peaks of Tim and Per signals at the end of the subjective day and an additional Per peak early in the subjective day (Fig. 3*B*). Interestingly, Tim and Per signals in the l-LNs of *y w* controls flies were always cytoplasmic, perhaps explaining why this coordinated clock protein expression in the l-LNvs is not able to drive rhythmic behavior (see Fig. 3*A Inset* and *B*; see also Fig. 1).

Rhythmic and prominent Tim and especially Per expression in LL was also observed in glia cells of the medulla optic lobe in *Veela* flies (Fig. 6, which is published as supporting information on the PNAS web site). cry^b mutant flies also behave rhythmically in LL, and consequently rhythmic accumulation of Per and Tim occurred in the LNs of this mutant in LL (Fig. 7, which is published as supporting information on the PNAS web site). We did not observe rhythmic or significant Tim accumulation within glia cells of cry^b flies, although Per levels did cycle (Fig. 6).

***Veela* Flies Express a Less-Light-Sensitive Form of Tim and Carry a Mutation in the *jet* Gene.** Because *Veela* mapped to the same genetic interval as *tim* and genetically interacts with *cry* (Figs. 1 and 2, Table 1, and *Materials and Methods*), we sequenced the ORF of *tim* in the *Veela* strain but did not find any changes compared with several published WT *tim* sequences (data not shown). We did notice, though, that the *tim* form (*ls-tim*) in the *Veela* strain encodes both the "long" and "short" forms of Tim, in which the longer form contains 23 additional amino acids at its N terminus by use of alternative translation-start codons (ref. 23; Fig. 4). This *ls-tim*

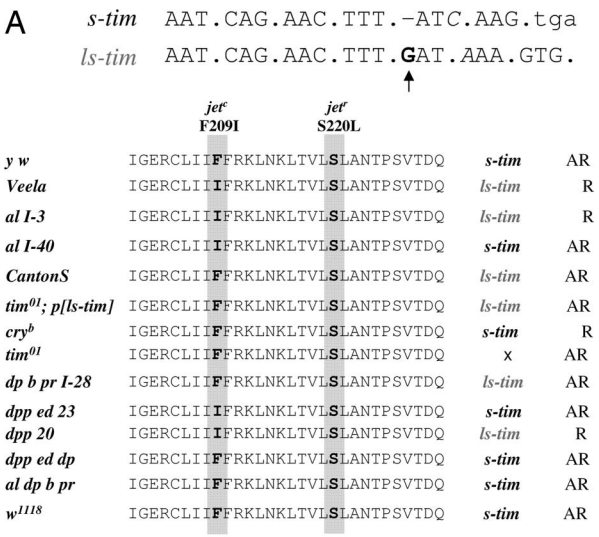

Fig. 4. *tim* and *jet* polymorphisms in Veela and other fly strains. (*A*) (*Upper*) Nucleotide sequence of the two *tim* polymorphisms. A deletion of the G nucleotide at position 294 of the *tim* cDNA (19) results in the generation of a stop codon immediately 5′ of the translational start of *s-tim* (23). Note the additional polymorphism three nucleotides downstream of the G deletion (indicated in italics). Fly lines carrying the single base-pair G deletion produce only the short (more light-sensitive) form of Tim. (*Lower*) Amino acid residues of the fourth and fifth LRR domains of the Jet protein. Highlighted in gray and bold are positions 209 and 220, respectively, which carry the common (F209I) or rare (S220L) polymorphisms (17). Genotypes expressing the respective forms are indicated to the left; their associated *tim* polymorphism along with the LL-behavioral phenotype is indicated to the right (R, rhythmic; AR, arrhythmic in LL). Meiotic recombinants are listed with their second chromosomal markers and a numerical indicator (e.g., *al I-3*). (*B*) Jet polymorphisms in WT fly strains. Displayed are the amino acid residues of the fourth and fifth LRR domains of the Jet protein from different WT fly strains. The fly strains' origin is shown on the left along with the nature of the *tim* allele, if determined (see *Materials and Methods* for details). Natural polymorphisms occurring at positions 161 and 167 are highlighted in gray and bold (Cys to Val and Leu to Ile, respectively). None of the WT strains carried the jet^c or jet^r variant.

variant is common to many strains of *D. melanogaster*, as is exclusive production of the short form (*s-tim*) for other naturally occurring WTs (ref. 23; F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta,

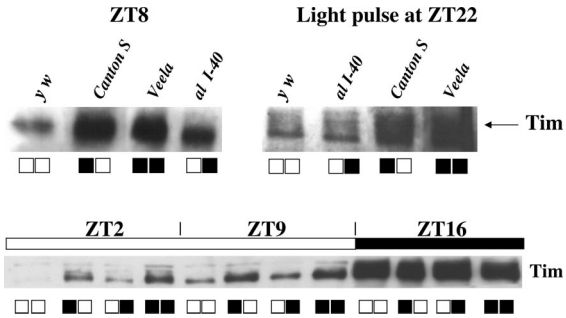

Fig. 5. *ls-tim* flies express a less-light-sensitive form of Tim, which is independent of *jet*. Flies of the indicated genotypes (*Upper*) were raised in 12-h:12-h LD cycles and either collected at the indicated ZT or subjected to a 2-min light pulse at ZT22 and collected at ZT23. Below each lane, the respective allele of *tim* (left square) and *jet* (right square) is indicated for each genotype. Open squares indicate *s-tim* (*Left*) and jet^+ (*Right*), black squares indicate *ls-tim* and jet^c. (*Lower*) Four genotypes, from left to right: *Italy (s-tim)*; *Canton S*; *al I-40*; *b I-27*), all flies had WT eye color. Note that the presence of *ls-tim* leads to a drastic increase in Tim levels, irrespective of *jet* or eye color.

P. Cisotto, *et al.*, unpublished results). We performed Western blot analysis to see whether there are any differences in regard to Tim expression between the *ls-tim* and *s-tim* variants. Tim protein levels in *ls-tim* flies collected during the day were substantially higher compared with *s-tim* (Fig. 5). This was also the case when flies with WT eye color were compared (Fig. 5 *Lower*). Therefore, missing pigments in the white-eyed *y w* flies are not the cause of increased light exposure and degradation of Tim. The same was true when both genotypes where exposed to a light pulse late at night (Fig. 5). Because Tim levels were similar in both genetic variants during the night portion of flies kept in LD cycles (Fig. 5 *Lower*), we conclude that the *ls-tim* allele produces a less-light-sensitive form of Tim. In a recent study, we showed that this difference is caused by a reduced affinity of "long-Tim" to CRY (F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results). However, because flies of the *ls-tim* type do become arrhythmic in LL (Figs. 1 and 4 and Table 1; ref. 2), there must be an additional light-input defect in *Veela* that further stabilizes Tim.

During our mapping experiments, we learned that a genetic variant with a similar LL phenotype to that of *Veela* is located close to our mutant but mapped to the right of *dp* instead (17). Koh *et al.* (17) mapped the phenotype to a gene (*CG8873*, now called *jetlag*) that encodes an F-box protein with leucine-rich repeats (LRR). Although *Veela* mapped to the other side (leftward) of *dp*, we decided to sequence the *jetlag* (*jet*) gene in *Veela* mutant flies because of the similar phenotype of the two mutant strains. Koh *et al.* (17) found four different variants of *jet* in various fly stocks that exist in unknown frequencies in various fly strains of *D. melanogaster*. The variants that correlated with the LL-rhythmic behavioral phenotype were reported to involve a phenylalanine-to-isoleucine substitution in one LRR domain or a serine-to-leucine replacement in the neighboring LRR (ref. 17; Fig. 4). Because they found the former substitution in six of seven behaviorally mutant stocks, the Ile variant was named the *common* (*c*) mutation, and the Leu isoform was named the *rare* (*r*) mutation. The WT variants would apparently encode Phe and Ser at these positions within Jet; however, the strain frequency for these alleles of *jet* was not determined (17). Our sequence analysis of this region in *Veela* revealed that its *jet* variant belongs to the *c* variant type. No other coding changes were found in the *jet* gene of *Veela* flies compared with the published WT sequence, suggesting that the LL-rhythmic phenotype of *Veela* is solely caused by this particular jet^c variant.

jet^c Flies Behave Normally in Constant Light When They also Carry the *s-tim* Allele. Our meiotic mapping placed *Veela* to the left of *dp* (and *jet*). Crucially, two of our genetic recombinants (generated with two

different marker chromosomes) carried the *c* form of the *jet* gene but did not show any phenotype in the LL assay; their behavior was indistinguishable from WT controls (Figs. 1 and 4, Table 1, and *Materials and Methods*). These behavioral-genetic results unequivocally demonstrate that the *c* variant alone is not sufficient to block light input into the circadian clock. Instead, careful inspection of all our genetic recombinants ($n = 26$) revealed that the presence of both the *ls-tim* form and the jet^c variant was correlated one to one with abnormally rhythmic behavior in LL ($n = 11$; Fig. 4 and see *Materials and Methods*). Because our two mapping stocks carry the *s-tim* form (Fig. 4 and see *Materials and Methods*), this explains why we were not able to map *Veela* to the *jet* locus *per se*; the relevant meiotic crossovers would have linked (and did link, in two cases) *s-tim* with jet^c, and this combination is phenotypically normal (Figs. 1 and 4, Table 1, and *Materials and Methods*).

In theory, it would be possible that another factor instead of *ls-tim* is responsible for the light-response defect in combination with jet^c. To firmly establish that the presence of the less-light-sensitive form of Tim is required, we compared the phenotypes of heterozygous jet^c flies, expressing either one copy of *ls-tim* and one copy of *s-tim* (*Veela*/+) or two copies of *ls-tim*, whereby one is carried on a transgene ($Veela/tim^{01}$;*P[ls-tim]*; ref. 24, Fig. 1, and Table 1). Strikingly, the presence of two copies of *ls-tim* resulted in doubling the proportion of LL-rhythmic individuals (31–63%; Table 1), further demonstrating that both the form of Tim associated with reduced light sensitivity (Fig. 5; F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results) and jet^c must be present to block light input into the circadian clock.

jet^c and jet^r Variants Are Most Likely Not Natural Polymorphisms. We investigated the possibility that the *jet* variants described here and in a previous study (17) represent a natural polymorphism as described for the *s-tim* and *ls-tim* alleles (ref. 23; F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results). To this end, we sequenced genomic DNA from 15 WT stocks available from the stock centers as well as from flies collected at five locations in central Europe (F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results; Fig. 4). No strains carrying the jet^c or jet^r variants were identified, but in one case (WT Athens), a base-pair change leading to a single amino change (Cys to Val) at position 161 was identified (Fig. 4). Moreover, several strains harbored a conservative Leu to Ile change at position 167. We analyzed the locomotor behavior of these variants in LL conditions, but their behavior was indistinguishable from other WT controls (Table 1 and data not shown). Although we cannot rule out that the substitutions have subtle consequences for the light sensitivity of the circadian clock, which are not detected in our LL assay, our results indicate they do not grossly alter the function of the Jet protein. Given that we did not identify any WT flies carrying either the jet^c or jet^r alleles, we conclude that both mutations occurred independently and spontaneously in laboratory stocks or were coinduced along with other mutations by chemical mutagenesis. Nevertheless, because we found two new variants in the region harboring the jet^c and jet^r polymorphisms, we cannot rule out that, in other natural strains, additional base-pair changes have occurred in different parts of the *jet* gene, which might more drastically affect the Jet protein and ultimately the light sensitivity of Tim.

Discussion

We identified a genetic variant, *Veela*, that is abnormally rhythmic in constant light, similarly as is shown for mutations affecting the blue-light photoreceptor Cry (6, 7, 10). *Veela*'s phenotype is due to the simultaneous presence of the *ls-tim* allele (encoding a less-sensitive form of Tim) and the jet^c variant encoding a mutant form of the F-box protein Jet (17). We show that *Veela* genetically and molecularly interacts with cry^b, indicating that Tim, Jet, and Cry function in the same circadian light-synchronization pathway. Our findings show that additional factors are necessary to elicit the phenotypes previously associated with *jet* variants (17). In particular, we show that only when jet^c is linked to the *ls-tim* allele, which encodes a less-light-sensitive form of Tim, can abnormal behavioral rhythmicity in LL be observed. The importance of the Jet protein *per se* in the light-entrainment process remains unclear, also when considering certain aspects of the original *jet* study in conjunction with the findings presented here. All control flies used by Koh *et al.* (17) came from a *y w* genetic background (see *Supporting Text*), which we show here carries the *s-tim* allele (Fig. 4). Contrarily, all jet^c or jet^r mutant flies carried the *ls-tim* allele (necessarily; otherwise, they would have behaved like WT). It follows that behavioral and molecular differences between control and mutant flies reported by Koh *et al.* (17) in fact reflect the combined effects of *ls-tim* (vs. *s-tim*) and jet^c (vs. jet^+). In conjunction with our Western blot data showing an increased *jet*-independent stability of the larger Tim form compared with the smaller one (Fig. 5), it seems that the effects on Tim degradation previously attributed to *jet* variants are mainly a reflection of the different features of the two Tim proteins. This may also explain why Koh *et al.* (17) saw only very subtle effects of their mutant Jet proteins on Tim degradation *in vitro*.

Nevertheless, it is clear that *jet* influences the light-input pathway of the circadian clock; WT flies behave arrhythmically in LL, even though they carry *ls-tim*. Moreover, *Veela* strongly interacts with Cry, a crucial protein for circadian light input in flies. Importantly, our findings reveal that, with the current knowledge, an *in vivo* function for *jet*'s F-box protein can be demonstrated only when the available *jet* variants are combined with *ls-tim*. To ultimately resolve the specific function of the Jet protein in the light-input pathway, loss-of-function *jet* mutants (25) or specific RNAi transgenics need to be generated and analyzed chronobiologically (26).

Characterization of *Veela* also led to the assignment of a biological function for the two natural *tim* variants that were identified many years ago (23). We show that Tim encoded by the *ls-tim* allele is more stable after light exposure, and that this increased stability has behavioral consequences when flies are exposed to constant light; if the *ls-tim* allele is linked to jet^c, these flies behave abnormally rhythmically in LL. If jet^c is linked to *s-tim*, the flies behave like WT and become arrhythmic in LL. Therefore, the less-light-sensitive Tim form encoded by *ls-tim* is necessary and sufficient to block light input into the circadian clock of jet^c flies. In nature, the natural polymorphism at the *tim* (and perhaps *jet*) locus might be used to fine-tune the light sensitivity of *Drosophila*'s circadian clock on a purely molecular level (F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results). In conjunction with various anatomical light-input routes that are known to send light to *Drosophila*'s circadian pacemaker (10, 27), our findings reveal a glimpse of the potential complexity of this process. The frequent and random occurrence of *tim* and *jet* variants in currently used laboratory strains also speaks to a more cautious strain selection and genotyping in all studies concerning light-input pathways to the circadian clock.

Materials and Methods

Fly Strains. Stocks of *D. melanogaster* and chromosomal markers were as described (5, 20, 28). *y Df (1)w* (*y w*) flies have yellow body color and white eyes and were initially used as control flies. Because *y w* carries the *s-tim* allele, the WT strain *Canton S* (carrying *ls-tim*, like *Veela* flies) was also used as control during this study. The *Veela* variant was isolated from a stock containing the $ninaB^{360d}$ mutation (29) on chromosome 3 (which was replaced by a WT third chromosome in all *Veela* flies analyzed here). The *ls-tim*-encoding transgene contains the full-length *tim* cDNA (generated by PCR from WT *Canton S* flies) and 6 kb of genomic 5′-flanking material (24). The various WT strains that were used to identify potential natural polymorphisms in the *jet* gene were obtained from the Bloomington stock center. The four WT strains from Moscow and two locations in northern Italy are isofemale lines, which were

generated from individuals collected in 1997 and 2004, respectively. From each of the three locations, a *s-tim* and *ls-tim* line was generated (F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results). The two WT variants from Germany stem from individuals collected in 2006 in Regensburg and near Munich.

Behavioral Analysis. Locomotor rhythms of individual male flies were recorded as described (5). Flies were kept for at least 3 days in 12 h/12 h LD cycles before being transferred to either constant light (300–400 lux LL) or to constant-dark conditions, in which they remained for at least 5 days. Rhythmicity was determined by using autocorrelation and Matlab software as described (30). Flies with period values in the circadian range and with a rhythmicity index value >0.15 were considered rhythmic (see ref. 30).

Chromosomal Mapping of *Veela*. Rhythmic or arrhythmic behavior in LL conditions (indicating the presence or absence of *Veela*, respectively) was used to map the position of *Veela* on chromosome 2. Initially, homozygous *Veela* flies were crossed to the marker stock *al* (map position 0.4) *dp* (13.0) *b* (48.5) *pr* (54.5), all on the left arm of chromosome 2 (20). A total of 22 recombinants was obtained, and all *b pr* ($n = 7$), or single marker *b* ($n = 1$) and *pr* ($n = 1$) recombinants exhibited the *Veela* phenotype. All *al dp* ($n = 4$) *dp b pr* ($n = 4$) *al dp b* ($n = 3$) recombinants were WT, suggesting that *Veela* maps close to *dp*. The two *al* recombinants were WT (I-40) and *Veela* (I-3), respectively (Fig. 1 and Table 1), placing *Veela* between *al* and *dp*, the region that includes the *tim* locus (8.0; ref. 19). Sequence analysis revealed that *Veela* carries the *ls-tim* variant but no other changes in its ORF, which do not occur in other WT or laboratory stocks (Fig. 4 and data not shown). Because other stocks carrying *ls-tim* do not show the *Veela* phenotype, we continued our mapping experiments with a *dpp* (4.0) *ed* (11.0) *dp* (13.0) marker stock in an attempt to separate *Veela* from *tim*. This was not accomplished; of the four total recombinants obtained from *Veela*/*dpp ed dp* females, the one *dpp* recombinant showed the mutant phenotype (Table 1), so that *Veela* must map to the right of *dpp*. All *dpp ed* ($n = 1$) and *ed dp* ($n = 2$) flies were WT (Table 1 and data not shown), placing *Veela* between *dpp* and *dp*, again the region containing *tim*.

Sequence analysis of *jet* in *Veela* flies and several recombinants revealed a correlation between the jet^c variant, *ls-tim*, and the mutant phenotype in all cases ($n = 11$; Fig. 4). Because our two mapping stocks express the *s-tim* variant along with jet^+, our recombinants separated *ls-tim* from jet^c resulting in LL-arrhythmic flies (Figs. 1 and 4; Table 1). Correct meiotic mapping of jet^c would be possible only with both the marker stock and the jet^c strain expressing *ls-tim*.

Western Blot Analysis. Flies of the indicated genotypes were first kept in LD cycles for at least 3 days and collected on dry ice during the indicated ZT in LD. For the light-pulse experiment in Fig. 5, flies were raised identically but exposed to a 2-min light pulse (300–400 lux) at ZT22, allowed to recover for 1 h, and collected on dry ice at ZT23. Preparations of head extracts and protein blots were performed by using anti-PER and -TIM as well as dilutions of these antibodies, as described (5), except that for the blots shown in Fig. 5, a different anti-TIM antibody was applied (31).

Immunohistochemistry. Flies were raised under the same conditions as described above and collected at the indicated circadian times (CTs) during the second day in LL (CTs refer to hours corresponding to ZT in the preceding LD cycles). Whole-mounted brains were prepared and incubated with anti-TIM and -PER, as described (32). Preparations were viewed by using Leica TCS NT (Leica, Deerfield, IL) and Zeiss Meta 510 (Zeiss, Oberkochen, Germany) confocal microscopes. Quantification of stainings was performed (observer blind with regard to genotype) by calculating a staining index, which reflects the number of immunoreactive cells and the staining intensity (on an arbitrary scale from 0 to 4), as described (32).

DNA Sequencing. The *tim* gene of *Veela* flies was sequenced by using genomic DNA and reverse-transcribed RNA fragments, generated by PCR using the methods and oligonucleotides described to sequence the tim^{blind} mutant allele (33). To distinguish between *s-tim* and *ls-tim* in the various fly stocks, the following oligonucleotides were applied to amplify genomic DNA by PCR: 5′-GTGGTTGCGTAATGCCCTGG-3′ (sense) and 5′-GCACCGTCAGATTGACGA-3′ (antisense). Sequencing of *jet* genomic DNA was performed by using oligonucleotides 5′-TGGGATAGAAGTCGTTCAAGT-3 (sense) and 5′-TGCCGATGGCTAACAGAT-3′ (antisense) to determine the variants at the common and rare sites within two LRR-encoding domains. The remaining *jet* DNA sequence was determined by using genomic DNA from *Veela* flies and standard sequencing protocols.

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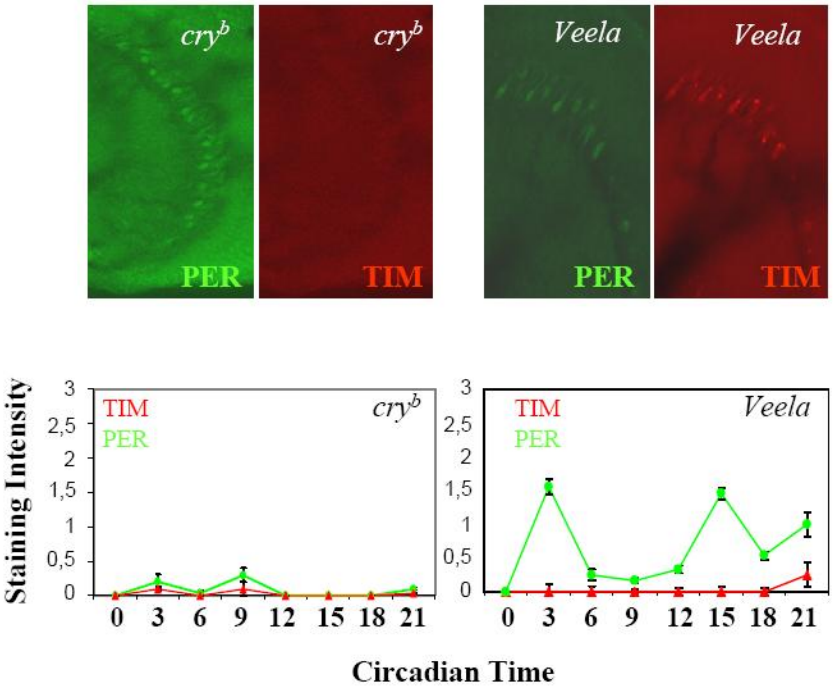

Fig. 6. Rhythmic Timeless and Period accumulation in constant light in glia cells of *Veela* and *cry*^{b} flies. Flies were raised under the same LL conditions as described in the legend to Fig. 3. Both *Veela* and *cry*^{b} flies showed striking expression of clock proteins in optic lobe glia cells. Images show Period and Timeless immunoreactivity of glia cells in the optic lobe of *Veela* and *cry*^{b} flies in LL at circadian time (CT)21. Period signals were detected in both variants, but Timeless immunoreactivity was visible only in *Veela* flies and restricted to CT21. No glia staining was observed in *y w* control flies under these conditions. (*Lower*) Quantifications of Period and Timeless immunoreactivity in glia cells during the second day in LL. Between 16 and 31 brain halves were analyzed for each time point in case of *cry*^{b}. For *y w* controls and *Veela*, the analyzed number of brains is specified in the legend to Fig. 3. Error bars indicate SEM.

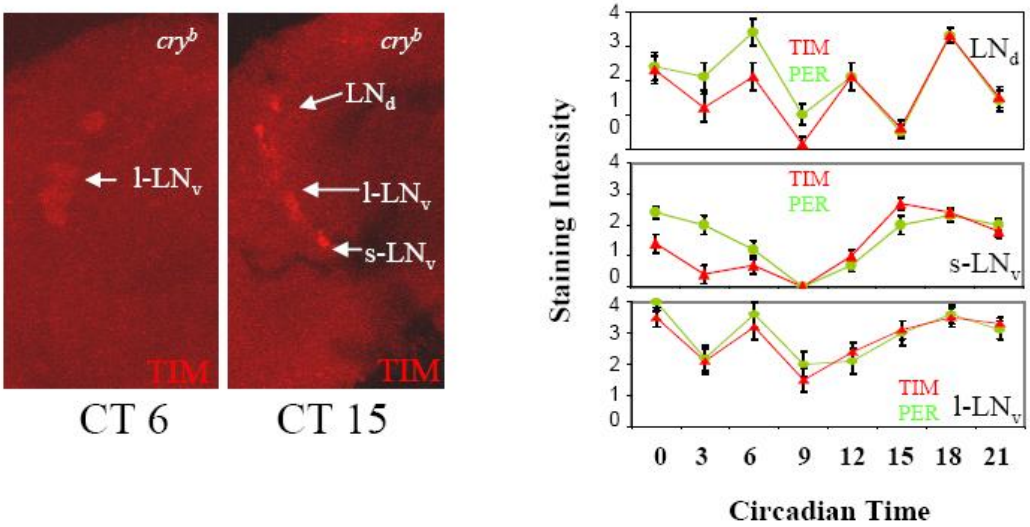

Fig. 7. Rhythmic Timeless and Period immunoreactivity in the brain of *cry*^{b} flies. Images show rhythmic anti-Tim immunoreactivity in LNs of *cry*^{b} flies during the second day in LL (see ref. 1). (*Right*) Quantifications of anti-Tim and -Per signals in *cry*^{b} brains in the three different groups of LNs are shown. For comparison to *y w* control and *Veela* flies, see Fig. 3 *A* and *B*. Between 16 and 31 *cry*^{b} brain halves were analyzed for each time point. Error bars indicate SEM

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Table S1: Locomotor activity rhythms in constant darkness (DD)

Genotype	N_T	n_{Rhy}	Period	RI	% Rhy
y w	19	17	23.8 ± 0.1	0.39 ± 0.02	94
cry^b/cry^b	12	10	23.8 ± 0.1	0.34 ± 0.02	83
$cry^b/+$	12	11	23.9 ± 0.1	0.34 ± 0.02	92
Veela/Veela	20	20	24.3 ± 0.1	0.35 ± 0.02	100
Veela/+	23	22	23.8 ± 0.1	0.44 ± 0.02	96
Veela/+ $cry^b/+$	14	14	23.8 ± 0.1	0.35 ± 0.01	100
tim^{01}/tim^{01}	20	3	26.0 ± 1.5	0.24 ± 0.01	15
$tim^{01}/+$	22	20	23.9 ± 0.1	0.39 ± 0.02	91
$Veela/tim^{01}$	27	27	24.4 ± 0.1	0.36 ± 0.02	100
$*tim^{01}/tim^{01};P[ls\text{-}tim]/+$	16	10	24.5 ± 0.1	n.d.	63

N_T, total number of flies tested; **n_{Rhy}**, number of individuals behaving rhythmically in DD. **Period**, free-running cycle durations (hr ± SEM), determined by autocorrelation; **RI**, Rhythmicity Index, indicating the significance-level associated with a given period (see Materials and Methods). **% Rhy**, percentage of rhythmic flies of a given genotype. Male flies of the indicated genotypes were analyzed. As controls, flies with the *X*-chromosomal body- and eye-color markers *y* and *w* were used (Materials and Methods). *Data taken from (Rutila, 1998).

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Supporting Text

Isolation of *Veela*.

During behavioral analysis of opsin-synthesis mutants in *Drosophila melanogaster*, we identified a strain carrying a mutation in the *ninaB* gene on chromosome 3 (1) that exhibits robust rhythmicity during the chronic presence of light (LL; Fig. 1; Table 1). The *ninaB* gene encodes an enzyme with b,b-carotene-15,15'-dioxygenase activity, and the mutant allele we used ($ninaB^{360d}$) is defective in vitamin A synthesis and therefore should not contain any functional opsins (1). Although we first speculated that the LL rhythmicity observed in *ninaB* mutant flies was due to the complete absence of functional opsins, we demonstrated that this phenotype was not caused by the *ninaB* mutation but instead segregated with chromosome 2. When we replaced the $ninaB^{360d}$ bearing 3rd chromosome with a wild-type chromosome but kept the flies homozygous for the second chromosome originally present in the $ninaB^{360d}$ stock, the flies were still rhythmic in LL. Because of the variant's elusive nature, it was named *Veela* (2). All flies used in the current study carry the outcrossed $ninaB^{+}$, so that any potential influence from opsin depletion can be ruled out.

Discussion

Presence of *s-tim* or *ls-tim* alleles in control and *jet* mutant flies described by Koh *et al.* (3).

As control flies, Koh *et al.* (3) used *y w* flies that were allowed to meiotically recombine with jet^{c} or jet^{r} flies for several generations. Mutant and control stocks were then established from individuals after determining the (mutant or wild-type) sequence at the *jet* locus (3). Unfortunately, the *y w* flies carry the *s-tim* allele (Fig. 4) and are therefore not optimal as controls. The meiotic map distance between *tim* (8.0) and *jet* [13.9: map position of *jet*'s neighboring gene *sgs1* (4)] is »6 cM; in other words, only 6% of all meiotic recombinants will result from a crossover between *tim* and *jet*. In agreement with this estimate, in only 2 of our 26 recombinants a crossover between *tim* and *jet* had occurred (Fig. 4; *Materials and Methods*). Therefore, the mutant flies described in Koh *et al.* (3) still carry *ls-tim* linked with the respective *jet* allele (in fact, they have to, because otherwise they would behave like wild type). Conversely, most of their control flies will still carry *s-tim* linked to jet^{+}, even after

several generations of meiotic crossovers between *y w* and *jet* mutant flies, and the established control stocks are most likely *s-tim*.

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4.3 *jetlag* regulates circadian clock resetting in *Drosophila*

The second part of this chapter deals with the newly discovered F-Box protein Jetlag (Jet).

In general F-Box proteins are part of an E3 ligase. The E3 ligase is a complex of three different protein groups – the so called SCF (S-phase kinase-associated protein 1 (Skp1) – Cullin 1 (Cul1) – F-Box). While Cul1 interacts with Skp1 and an E2 complex, Skp1 interacts with the F-box protein. The E2 complex carries the Ubiquitin and transferes this small marker protein to the substrate. The substrate on the other hand is specifically recognized by the F-Box protein (Figure 4-1).

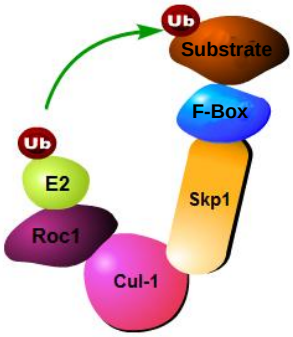

Figure 4-1 The Ubiquitin Ligase complex
The F-Box protein specifically recognizes the substrate. Often F-Box proteins bind only to posttranslational modified proteins, for example only to phosphorylated proteins. Skp1 and Cul1 connect the F-Box protein via the Rice outermost cell-specific gene1 (Roc1) to the E2 complex. From here the Ubiquitin is transferred to the substrate. In general more than one Ubiquitin is translocated to the substrate, thus generating small chains of Ubiquitin connected to the substrate. This substrate is then degraded in the proteasome.

In addition to the N-terminal F-box, other protein domains are typically found at the C-terminus of F-box proteins. Namely the WD40 repeats (WD40) or **l**eucine **r**ich **r**epeats (LRR). Those C-terminal domains are important for the substrate recognition (Ho et al., 2008).

Clock protein degradation in the proteasome is a common feature of regulation in circadian rhythms. In *Arabidopsis thaliana*, establishment of the circadian period relies on targeted degradation of TIMING OF CAB EXPRESSION 1 (TOC1) by the 26S proteasome. ZEITLUPE (ZTL) is the F-box protein that associates with the SCF E3 ubiquitin ligase that is responsible for marking TOC1 for turnover (Mas et al., 2003; Somers et al., 2000).

In mammals the F-box protein FBXL3, a component of the SCF E3 ubiquitin ligase complex, interacts specifically with the CRYPTOCHROME proteins. In FBXL3 mutant mice, expression of the PERIOD proteins PER1 and PER2 is reduced; however, the CRY proteins CRY1 and CRY2 are unchanged. The loss of FBXL3 function leads to a stabilization of the CRY proteins, which in turn leads to

transcriptional repression of the *per* and *cry* genes (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007).

In *Drosophila* the *slimb* (slmb) gene -a member of the F-box/WD40 protein family of the ubiquitin ligase SCF complex that targets phosphorylated proteins for degradation -is an essential component of the circadian clock. Slimb interacts preferentially with phosphorylated Per and stimulates its degradation. In constant darkness, highly phosphorylated forms of the Per and Tim proteins are constitutively present in *slimb* mutants, indicating that their cyclic degradation is impaired (Grima et al., 2002; Ko et al., 2002).

Because levels of Per and Tim oscillate in *slmb* mutants maintained in L/D conditions, light- and clock controlled degradation of Per and Tim do not rely on the same mechanisms and the same proteins (Grima et al., 2002).

Recently it was shown that under illumination Jet binds and ubiquitinates the crucial clock protein Tim and thus targets Tim for the degradation in the proteasome in *Drosophila* S2 cells (Koh et al., 2006). Jetlag contains not only an N-terminal F-box, but also a C-terminal LRR domain. Mutations of this LRR domain, exemplified by the jet^c or jet^r alleles, lead to a reduced ubiquitination of Tim (Koh et al., 2006).

In flies that carry a less-light-sensitive *tim* allele – the so called *ls-tim* allele – the simultaneous presence of the jet^c allele causes behavioral rhythmicity in LL conditions. Animals carrying a more light-sensitive allele of *tim* – the so called *s-tim* allele – in combination with jet^c loose their rhythmicity under constant light conditions and behave like wild type flies. The same is true for animals with a wild-type *jet* allele combined with *ls-tim* (Peschel et al., 2006). The likely reason for why s-Tim is more light sensitive compared to ls-Tim is the weaker binding ability of s-Tim to the blue-light photoreceptor Cryptochrome (Sandrelli et al., 2007).

The interaction of Timeless and Jetlag in S2 cells (Koh et al., 2006) and the different light sensitivity of s-Tim and ls-Tim caused us to investigate the interaction of s-Tim, ls-Tim and Jet. To our great surprise we could not detect an interaction of Tim and Jet in Y2H assays, but instead an interaction between Jet and Cry. A summary of our experiments resulting in a more complex model of the Tim/Jet/Cry interaction and molecular clock resetting by light can be found in the following manuscript:

Light-dependent interactions between Cryptochrome and Jetlag regulate circadian clock resetting in *Drosophila.*

Nicolai Peschel and Ralf Stanewsky (Under review in *Current Biology*)

Light-dependent interactions between Cryptochrome and Jetlag regulate circadian clock resetting in *Drosophila*

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Running head: Cry:Jet interactions regulate clock resetting by light

Summary

Circadian clocks regulate daily fluctuations of many physiological and behavioral aspects in life. They are synchronized with the environment via light- or temperature-cycles [1]. Natural fluctuations of the day length (photoperiod) and temperature necessitate a daily reset of the circadian clock on the molecular level. In *Drosophila* the blue-light photoreceptor Cryptochrome (Cry) mediates a rapid light-dependent degradation of the clock protein Timeless (Tim) via the F-Box protein Jetlag (Jet) and the proteasome, which initiates the resetting of the molecular clock [2, 3]. Cry is degraded in the light as well. While the degradation of Tim is well characterized [4-8] the mechanism for light-dependent degradation of Cry is mostly unknown. Until now it was believed that these two degradation pathways are distinct [4, 9]. Here we reveal that Jetlag also interacts with Cry in a light-dependent manner. After illumination Jetlag induces massive degradation of Cry which can be prevented *in vitro* and *in vivo* by adding Tim as an antagonist. We show that Jetlag causes light-dependant and sequential degradation of Tim *and* Cry and thus reveal an intimate connection between the light-dependent degradation of these two proteins in the proteasome. Our results demonstrate that the competing interaction of Jet with Tim and Cry regulates the daily molecular clock resetting.

Results and Discussion

It was shown recently that the F-Box protein Jetlag (Jet) is closely involved in the resetting mechanism of the circadian clock [2, 3]. Jet is a putative component of a Skp1/Cullin/F-Box (SCF) E3 ubiquitin ligase complex that light-dependently associates with Tim in an embryonic *Drosophila* cell line (S2) in the presence of Cry. This interaction promotes the ubiquitination and degradation of Tim in cultured cells [2]. In nature two *Drosophila* allelic variants of *timeless* can be found, one allele produces a 23 amino-acid N-terminally shortened and more light-sensitive form of Tim (*s-tim*), the other allele encodes both forms (*ls-tim*) [10, 11]. Molecularly, S-Tim's enhanced light-sensitivity is correlated with (and likely due to) an enhanced binding to the circadian blue-light photoreceptor Cry ([10] Figure 1A).

Light-dependent interaction of the F-box protein Jetlag with Cryptochrome in yeast

The hypomorphic *jet*^{c} mutation carries a single amino acid change in the leucine-rich repeat (LRR) region of Jet, which causes flies to be rhythmic in constant light (LL), but only when the flies express the less-light-sensitive L-Tim protein, encoded by the *ls-tim* allele, as is the case in *Veela* flies [3]. The 'LL-rhythmic' *Veela* phenotype resembles that of *cry* mutants [12, 13]. Also similar to *cry*^{b} mutants, homozygous mutant *Veela* flies accumulate abnormally high levels of Tim protein during the light [3, 14]. Strikingly both phenotypes are also observed in transheterozugous *Veela/+; cry*^{b}*/+* flies [3]. This strong genetic interaction between *tim, jet,* and *cry* and prompted us to investigate a potential physical association between Jetlag and Cry proteins in the yeast-two-hybrid system (Y2H). In addition, the two different Timeless isoforms were also tested for interaction with Jetlag or Cryptochrome. In agreement with an earlier study a clear light-dependent interaction between both Tim proteins and Cry was observed, whereby S-Tim interacted stronger with Cry compared to L-Tim (Figure 1A) [10]. Surprisingly we also observed a striking light-dependent interaction between Cry and Jet (Figure 1B), whereas no interaction between Tim and Jet could be revealed in yeast (Figure 1A). Given that Tim and Jet do interact in S2 cells co-transfected with *cry* [2] and our finding that Jet interacts with Cry, an explanation for the lack of Tim:Jet binding could be that Cry is essential for this interaction.

When we tested the interaction between the mutant Jetlag protein (Jet^{c}) and Cryptochrome we revealed that the interaction is significantly weaker compared with the wild-type protein (Figure 1B). Keeping in mind, that the LRR is the binding region for the F-box proteins' substrate [15], this weaker association was expected. Additionally we challenged Jet and Jet^{c} with different Cry mutations. In Cry$^{\Delta}$ the last 20 residues from the Cry C-terminus are missing resulting in strong and light-independent interactions of Cry$^{\Delta}$ with Tim [16]. Similarly, in our Y2H assay we could also reveal a strong light-independent interaction between Jet or Jet^{c} and CryΔ (Figure 1B). The mutant Cry^{b} protein does not interact with Jet or Jet^{c} (Figure 1B), correlating with its inability to bind to Tim in yeast [16].

The Y2H results just described may supply an explanation for the strong genetic interaction between *cry* and *jet* that we had observed in the transheterozygous *Veela/+; cry^{b}/+* flies described above [3]. Although the LL-rhythmicity and increased Tim levels in the transheterozygous flies could also be explained by additive of the single mutations, we think this is rather unlikely given the only mildly dominant (*Veela*) or recessive (*cry^{b}*) character of the genetic variants in question [3, 14]. The strong biochemical and genetic interaction between *cry* and *jet* rather suggests that the Jet:Cry interaction is important *in vivo* and perhaps required for efficient light-induced Tim turnover. Given that we were unable to detect a direct interaction between Jet and Tim in yeast (Figure 1A), this implies that the Jet:Cry complex binds to Tim (via Cry acting as a 'bridge') thereby inducing Tim degradation. Alternatively, binding of Cry to Tim could modify Tim in a way that it now can bind Jet to induce degradation.

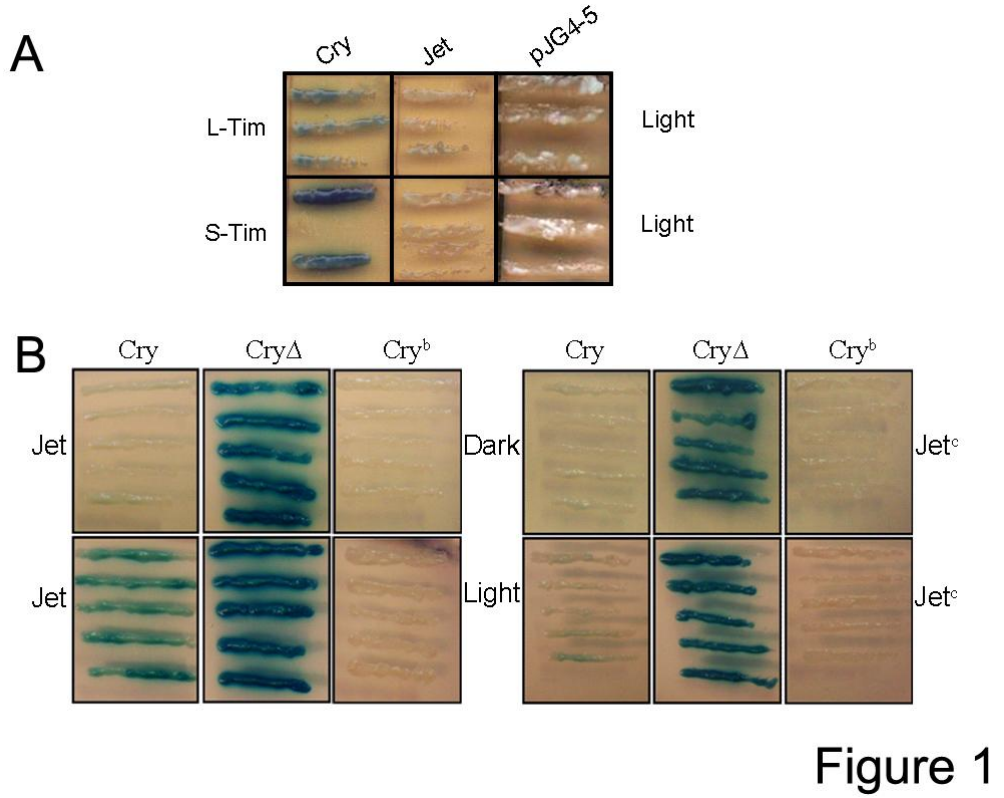

Figure 1: Light dependent Interaction of Cryptochrome and Jetlag in Y2H
(A) Yeast cells were grown in constant light at 30°C. The two different isoforms of Tim interact differentially with Cryptochrome. Binding strength is positively correlated with intensity of the blue staining [10]. No interaction was observed when we challenged the two Tim-isoforms with Jetlag. (B) Yeast was grown in constant light (Light) or under the same conditions, but wrapped in two layers of aluminum foil (Dark).

Jetlag binds to Cryptochrome and Timeless in Drosophila cells

Both scenarios could explain why a simultaneous reduction of Cry and Jet function result in less-efficient Tim degradation. To distinguish between these two possibilities and to find out if the Jet:Tim interaction indeed requires the simultaneous presence of Cry, we performed CoIP experiments in an embryonic *Drosophila* cell line (S2). A full length Jetlag protein fused to a HIS-tag (Jet-H), as well as untagged versions of Cry and Tim proteins, were over-expressed in the embryonic *Drosophila* S2 cell line and immunoprecipitated with anti-HIS antibody. Cells were grown in darkness and exposed to light for 15 min before performing the assay. As expected from the Y2H results, Cry also interacted with Jet-H in S2 cells (Figure 2A). Surprisingly and contrary to the Y2H results, Tim also interacts in S2 cells with Jet-H, without the addition of Cry (Figure 2A). Why could we (and others) detect Tim:Jet interactions in S2 cells, but not in yeast? The reason for this could either be that some crucial phosphorylation step necessary for the detection of Tim by Jet is not performed in yeast cells but does occur in *Drosophila* cells. Alternatively the low endogenous Cry levels in these cells (Figures 2A, B and S5) [8, 17] could promote the Tim:Jet interaction.

When we simultaneously expressed Tim and Cry in the presence of Jet-His, we could detect only minimal amounts of Tim protein in the input or CoIP fractions (Figure 2A, lanes 5, 6). We speculated that the low Tim levels were caused because we reconstituted a fully functional light-sensitive clock-resetting protein complex, resulting in efficient Tim degradation after light-exposure. To test this, we conducted the CoIP experiments also in the presence of the proteasomal inhibitor MG-132 which led to an overall stabilization of the proteins and a clear demonstration of Tim:Jet interactions in S2 cells (Figure 2B, lanes 2-6). The interaction of Tim with Jet is increased in the presence of Cry, supporting the idea that a Jet:Cry complex promotes binding to, and degradation of Tim (Figure 2B, compare lanes 4 and 6). Given the low endogenous Cry levels present in S2 cells we can not unambiguously say if Jet is able to bind Tim without Cry. We did try to carry out the CoIP experiments with cells that were kept in LL for the entire time after transfection to further reduce the endogenous Cry levels. But even though

proteasomal inhibitors were added to the cells (as in Figure 2B), Tim did not accumulate to significant amounts that would have allowed performing CoIP experiments (data not shown). This result implies that residual Cry proteins act as potent photoreceptors in S2 cells, and that perhaps a Cry knock-out cell line needs to be generated to ultimately answer this question.

Nevertheless, our CoIP data does not support a 'bridging' role for Cry in mediating Tim:Jet interactions. In cells transfected with *tim* and expressing only low endogenous levels of Cry, we did observe a Jet:Tim interaction, but not a Jet:Cry interaction (Figure 2 A, B, lanes 3 and 4). Even though the input levels of endogenous Cry and transfected Tim are very low, one would expect to precipitate equal amounts of both proteins bound to Jet, if Cry would indeed form a bridge between Tim and Jet. This was not observed, and we repeatedly precipitated only Tim, indicating the existence of Tim:Jet complexes that are free of Cry. In any case, our data clearly demonstrate interactions between Jet and Cry *in-vitro* and we next wanted to determine if they also play a role in circadian light-entrainment *in vivo*.

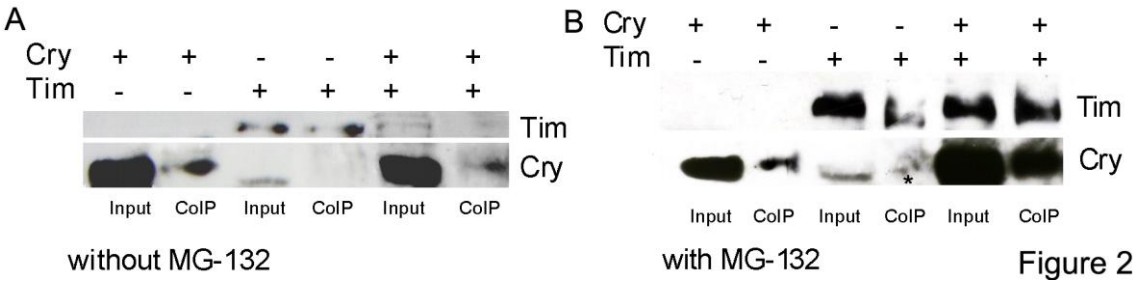

Figure 2: Interaction of Cry and Jetlag in Drosophila S2 cells
pAc-*Jet-His* transfected S2 cells were used for immunoprecipitation with anti-His antibody. The input was compared to the CoIP. Cells were transfected with plasmids as indicated, whereas + is transfected with the vector, - is not transfected. The CoIPs were repeated 3 times with similar results. Before harvesting, the cells were exposed to light for 15 minutes in the absence (A) or presence (B) of MG-132. This proteasomal inhibitor was added under red light 2 hr before cells were exposed to light. The asterisk marks unspecific background staining. The input is loaded on lanes 1, 3, 5, the CoIP on lanes 2, 4, 6. Cry and Tim did could not be precipitated by anti-His antibodies in the absence of Jet-His (Experimental Procedures).

Jetlag promotes Cryptochrome degradation in flies

To determine if Jet plays a role in the light-induced turnover of Cry we measured the level of Cry in adult heads isolated from different *jet* mutant backgrounds (Figure 3A). If the Jet:Cry interaction is biologically relevant, we would—for example—expect to see an effect on the amount of Cry degradation in flies with reduced *jet* function. Indeed, flies with the jet^{c} mutation exhibited mildly increased Cry levels after 2 and 11 hours in light (Figure 3A). Interestingly *s-tim* animals seem to accumulate slightly higher levels of Cry compared to *ls-tim* flies, both in jet^{+} and jet^{c} genetic backgrounds. Cry associates stronger with S-Tim compared to L-Tim (Figure 1A) [10], and in flies this probably leads to a more efficient light-dependent degradation of S-Tim by Jet [3]. This could suggest that a stronger Cry:Tim interaction results in diminished Cryptochrome degradation—in other words Tim could be preferentially degraded at the expense of Cry. Since the differences in Cry degradation caused by jet^{c} were subtle, we wished to confirm this effect by creating a more severe reduction of Jet function. For this, we applied the stronger jet^{r} allele in combination with a deficiency of the *jet* locus [2]. Combining jet^{r} with jet^{c} or the deficiency both lead to substantially increased Cry levels compared to controls and homozygous jet^{c} mutants (Figure 3B). This unequivocally demonstrates that *jet* influences Cry stability *in vivo*. We also noticed that the absence or presence of eye-pigments influences the amount of Cry degradation after light exposure, perhaps because the pigments 'protect' Cry from the light (Supplementary Figure S1). However, all *jet* mutants consistently reduced Cry degradation, independent of eye color, demonstrating involvement of Jet in the Cry degradation process. To further investigate the effect of Jet and the different isoforms of Tim on Cry degradation, and to assure that we only change the factors under investigation, we next analyzed Cry degradation in S2 cells.

Figure 3

Figure 3: jetlag mutants stabilize Cryptochrome in vivo
(A) Flies where kept in 12hr: 12hr LD cycles and were sacrificed at ZT 2 or ZT 11. The protein levels in fly heads were determined by Western blot. Flies with the *jet*^{c} mutation showed a decrease in Cry degradation. All flies had *white* eyes. (B) Flies where kept in 12hr: 12hr LD cycles and were sacrificed at ZT1. *jet*^{r} mutant or deficient flies exhibited a decrease in Cry degradation. All flies are *white*$^{+}$ and carry the *s-tim* allele, except for the Deficiency, which carries *ls-tim*.

Cryptochrome degradation is enhanced by Jetlag and suppressed by Timeless

We and others already showed that *cry* is expressed in S2 cells ([8, 17] and data not shown). However the endogenous Cry protein is unstable in S2 cells and hardly detectable (Figure 2). After isolating RNA from S2 cells we were able to show that *jetlag* is also expressed in these cells, but *tim* RNA could not be detected (Supplementary Figure S2A, and data not shown). Endogenous *jet* expression may explain the previous observation of Tim ubiquitination in S2 cells without co-transfection of Jet [7, 8]. However, without the addition of Cry and Jet, extended light treatment is required for degradation of Tim in this cell-culture system after [7]. This suggests that the amount of Jet (and) or Cry is limiting for triggering Tim degradation. To address this question, we first transformed S2 cells with S-Tim or L-Tim, Cry and Jet, all expressed under the control of a constitutive promoter (Supplementary Figure S2B). Cells transfected with Cry and Tim showed little degradation of Tim, regardless of the Tim form present. In contrast, co-transfection of *jet* led to massive Tim degradation [2], suggesting that the endogenous Jet amount is not sufficient to degrade Tim in an efficient manner. We also observed a slight reduction of Tim degradation after co-transfection of *jet*^{c} and the long isoform of Tim (Supplementary Figure S2B) confirming previous results obtained in adult flies [3].

After establishing conditions that allow recapitulating light-induced degradation of TIM in cell culture, we were now able to examine Cryptochrome levels after illumination. We found that addition of Jet significantly increases the degradation of Cry. Transformation of larger amounts of *jet* plasmid DNA is correlated with increased degradation of Cry (Figure 4A). This effect is indeed caused by extra *jet* and not by less efficient uptake of *cry* plasmid, because transformation with equal amounts of unrelated plasmid DNA did not result in reduced Cry levels (Experimental Procedures and data not shown). When we used the jet^{c} mutation, Cry degradation in the light was reduced but still visible (Figure 4B), confirming the results obtained in adult flies (Figure 3A, B). Both effects are possibly caused by the poorer ability of Jet^{c} to physically interact with Cry (Figure 1B).

To analyze any potential influence of Tim on the degradation of Cry, we transfected cells with Cry alone or in combination with Tim. When the cells were harvested after 10 or 120 minutes of light exposure a dramatic 'protection' of Cry by Tim was observed (Figure 4C). This is in agreement with the idea that Cry and Tim compete for the limiting amounts of endogenous Jet. Indeed, co-transfection of Jet restored the light-induced degradation of CRY, at least after a 2 hr of light-exposure (Figure 4C). To further proof that both proteins are a target of Jet and subsequent proteasomal degradation, the proteasome inhibitor MG-132 was added to cells transfected with Cry and Jet. As previously shown for Tim [2, 7] light- and Jet-dependent degradation of Cry was largely prevented after adding the drug, strongly suggesting that Tim and Cry are degraded via the same pathway (Figure 4D). We do observe a minor Jet-dependent reduction of Cry levels, which seems independent of light and the proteasome (Figure 4D, compare lanes 1 and 3), indicating that at least in S2 cells, Cry may also be degraded via a different, light-independent pathway.

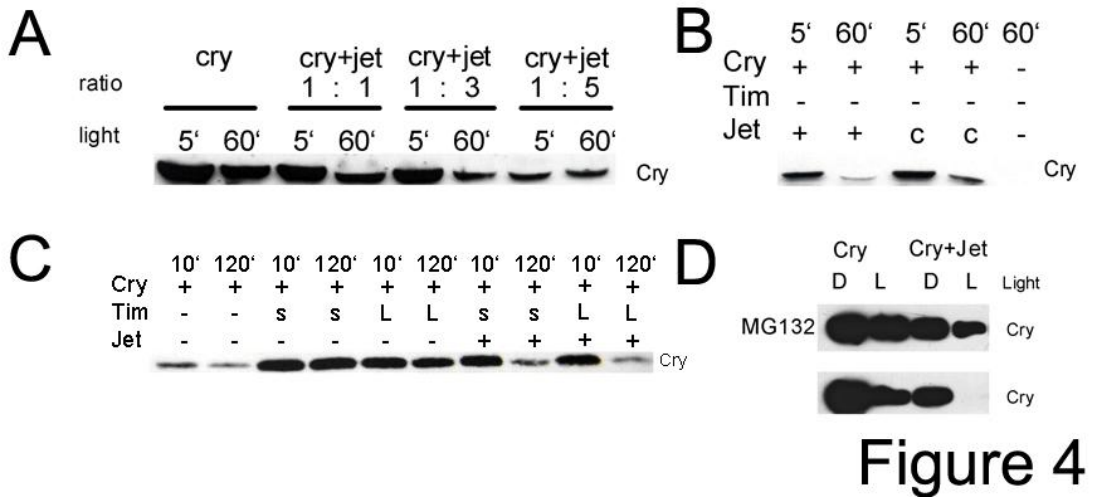

Figure 4

Figure 4: Jetlag promotes Cry degradation in S2 cells
To investigate the degradation of Cry in S2 cells pAc-Cry was coexpressed with different Tim isoforms (pAc-s-Tim or pAc-l-Tim) and with Jet (pAc-Jet) and Jet^{c} (pAc-Jet^{c}), respectively. Protein levels were determined by Western blot; and anti-Cry antibodies were used for the detection [18]. Cells were exposed to light for 5`or 60`before harvesting (A, B). (A) Cry alone (lane 1 and 2) or Cry and Jet were co-expressed in S2 cells. (B) pAc-Cry was co-expressed together with pAc-Jet or pAc-Jet^{c} in S2 cells. (C) pAc-Cry was co-expressed together with different combination of pAc-Jet, pAc-s-Tim, pAc-l-Tim, or both. Cells were harvested 10 and 120 min after "light-on". (D) Cry or Cry and Jet were expressed and cells were harvested in the dark or after 60 min. in the light. The addition of MG-132 strongly decreased the light-dependent degradation of Cryptochrome.

Cryptochrome and Timeless compete for Jetlag in a real-time luciferase Cry-degradation assay

To test the hypothesis that Cry and Tim compete for Jet, we developed an assay allowing us to examine the light-induced degradation of Cry with a higher temporal resolution and in a more quantifiable manner. For this, we applied a constitutively expressed firefly-*luciferase* cDNA fused to full length Cry (Luc-dCry) or to a C-terminal truncated version of Cry (Luc-dCryΔ528) [19]. In S2 cells, the fusion protein Luc-dCry is degraded in a similar way as Cry alone, while the truncated Luc-dCryΔ528 is expressed at a very low level (Figure 5A) [19]. After transient transfection with a plasmid carrying the *luc-dCry* gene under the control of a constitutive *actin* promoter, luminescence was measured in an automated luminescence counter (see Experimental Procedures). After illumination the Luc-dCry protein is degraded swiftly. Importantly, after returning the cells to darkness, Luc-dCry levels recover, demonstrating that the

system nicely reflects the light-dependent degradation and darkness-dependent accumulation of Cry (Figure 5B). When Jet is added to the cells the fusion protein is degraded even faster—an effect not observed when Jet^c is added (Figure 5C). We do observe lower Luc-dCry levels in the dark portion of the day when Jet is present (similar as in Western blots, see Figure 4D), again indicating that there maybe be an additional role for Jet in light-independent degradation of Cry. Co-transfecting *luc-dCry* with *timeless* results in a striking stabilization of Cry in S2 cells (Figure 5B, C) confirming the Western blot results obtained with S2 cells (Figure 4C). The magnitude of this effect depends both on the isoform and on the total amount of Tim (Supplementary Figure S3). S-Tim inhibits Luc-dCry degradation stronger compared to L-Tim indicating again that the stronger S-Tim:Cry interaction stabilizes Cry (cf. Figure 3). Adding Jetlag and Tim at the same time leads to decreased Cry degeneration, compared to transfection with Jet alone (Supplementary Figure S3), but Cry is less 'protected' as if Tim is added alone. Overall, these luciferase results nicely confirm the S2 cell and adult Western blot results, and demonstrate that Jet promotes Cry degradation, which is counteracted by Tim, and especially S-Tim.

Next we wanted to know if this stabilization effect can also be observed in adult flies. Thus we adapted our luciferase assay to the living animal and expressed the Luc-dCry protein in UAS-*luc-dCry* transgenes under the control of a *tim*-Gal4 driver. Endogenous *cry* RNA is rhythmically expressed and this expression is controlled by the circadian clock [20]. Since we used the Gal4/UAS system to express *luc-dcry* in all clock cells, we predict the resulting mRNA to be expressed at constitutive high levels. Nevertheless, we were able to observe robust Luc-dCry oscillations, which are very likely due to light-dependent degradation in our transgenic flies, because a sharp decrease of luciferase signals coincides exactly with 'lights-on' in every cycle (Figure 5D). This result is in agreement with light, but not clock-regulated oscillation of the Cry protein in flies [20]. Over-expression of Tim with a UAS-*tim* transgene led to significantly elevated levels of Luc-dCry during the light phase (Figure 5D), which is quite remarkable given that these flies contain the endogenous wild-type versions of *jet*. Since both transgenic genotypes contained the identical and single copy of the *UAS-luc-dCry* transgene, this difference in the level of Luc-dCry must be due to the over-expression of

Tim. Therefore, the increased daytime Cry levels in our transgenic flies are most likely caused by a stabilization of Cry by Tim, similar as observed in S2 cells. Also similar as in S2 cells, although the Luc-dCry protein is stabilized by Tim, it can still be degraded by light as long as Jet is present (compare Figure 5D with cells in Supplemental Figure S3, where *luc-dcry*, *tim* and *jet* are co-expressed). Interestingly, closer inspection of the of luc-dCry expression in flies reveals that Cry levels in the UAS-*tim* flies already recover during the light phase (Figure 5D), indicating that Tim mainly protects Cry when light is present. A Western-Blot from flies with the same UAS-*tim* transgene under the control of a *tim*-Gal4 driver also reveals a dramatic increase in the levels of Cry and confirms our luciferase results (Supplementary Figure S4). Taken together the Western blot and real-time luminescence data show that Jet supports the light-dependent degradation of Cry *in vitro* and *in vivo*, and that Tim interferes with this process, presumably by competition.

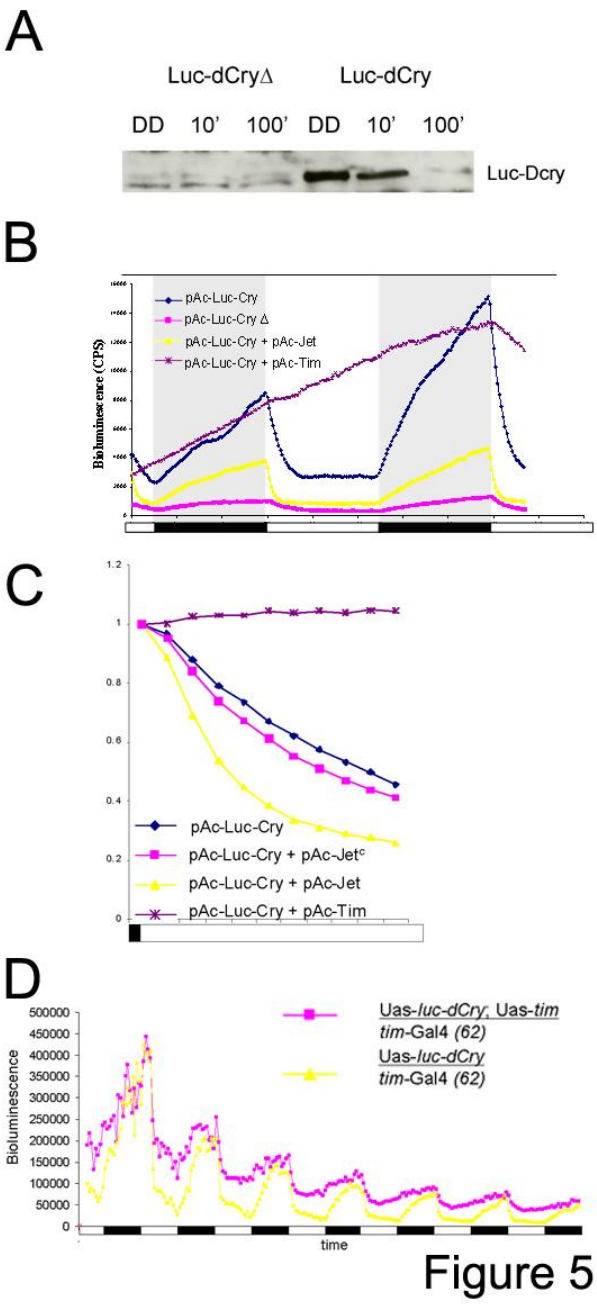

Figure 5

Figure 5: Real-time luciferase assay to determine Cryptochrome degradation

(A) *pAc-Luc-dCry* or *pAc-Luc-dCry*Δ was expressed in S2 cells. Cry levels of transfected cells were determined by Western blot. The fusion protein was light sensitive and degraded after light exposure (lanes 4,5,6). The mutated Luc-dCryΔ was always expressed at a very low level. (B) *pAc-Luc-Cry* or *pAc-Luc-Cry*Δ were expressed in S2 cells or co-expressed with S-Tim or Jet. After addition of luciferin the degradation of the Luc-Cry protein was measured by determining luciferase activity in a Packard Topcount machine. The cells were kept in 12:12 Light/Dark cycles. (C) The *pAc-luc-dCry* plasmid was expressed in S2 cells and the degradation rate after illumination was investigated in the presence of Jet, Jet^c or S-Tim. (D) Adult *Drosophila* animals were investigated in a Packard Top count machine. Luc-dCry was expressed in all clock cells with a *tim*-Gal4 driver. When we expressed Tim at the same time in those cells, the degradation of Cry during the light phase was strongly decreased.

Temporal profile of Timeless and Cryptochrome degradation in fly heads

The fact that Tim stabilizes Cry can most easily be explained if Tim is the preferred target for Jet. If true, one would predict that in flies, light-induced degradation of both Tim and Cry occurs in sequential order; Tim being degraded ahead of Cry. To see if this is the case we simultaneously measured Tim and Cry levels in head extracts of wild-type flies during the first 10 hrs of light in a 12 hr: 12 hr light: dark cycle. Although levels of both proteins start to decrease after the lights are turned on, and trough levels are reached at the same time (ZT4) Tim degradation appears to occur more rapid in the early day (compare ZT0 and ZT2 for Cry and Tim in Figure 6A). This result may suggest that Tim is preferentially degraded after initial light-exposure. Interestingly, a similar result was reported when Cry and Tim degradation kinetics was observed in adult clock neurons. Here too, Tim levels declined very quickly after 'lights on' (ZT1), whereas Cry protein levels where still high at this time and reached their trough later in the day [18].

Conclusions and Model

In *Drosophila* the clock factor Timeless is degraded after illumination resulting in a daily reset and adaptation of the circadian clock to its environment. Here we have demonstrated that the blue-light photoreceptor Cryptochrome directly interacts with the F-Box protein Jetlag in a light-dependent manner. This interaction leads to the degradation of Cry by the proteasome and we unequivocally show that *jet* regulates Cry turnover *in vitro* and *in vivo*. This is an important and surprising observation, given that so far it was assumed that Cry and Tim are degraded via different pathways [4, 9]. In agreement with previous studies we find that Tim also associates with Jet [2], but our results suggest that a posttranslational modification of Tim, induced by its binding to Cry, is a prerequisite for the Jet:Tim association. Cry is dramatically stabilized in the presence of Tim, which can be explained by blocked binding sites for Jet due to the Cry:Tim interaction, or by an increased binding affinity of Jet towards light-activated Tim compared to Cry. Based on our results we now propose a more complex model for light resetting, acknowledging the fact that degradation of Tim and Cry are tightly interwoven (Figure 6B): Light induces a conformational change in Cry, allowing it to

bind to Tim and Jet. Binding of Cry to Jet leads to degradation of Cry in the proteasome. Binding of Cry to Tim leads either to the Tim-Jet interaction, whereas Cry acts as an adaptor (Figure 6B model 1) or to a modification of Tim, most probably a tyrosine phosphorylation [7]. This modification triggers the binding of Jet to Tim, leading to the ubiquitination and subsequent degradation in the proteasome (Figure 6B

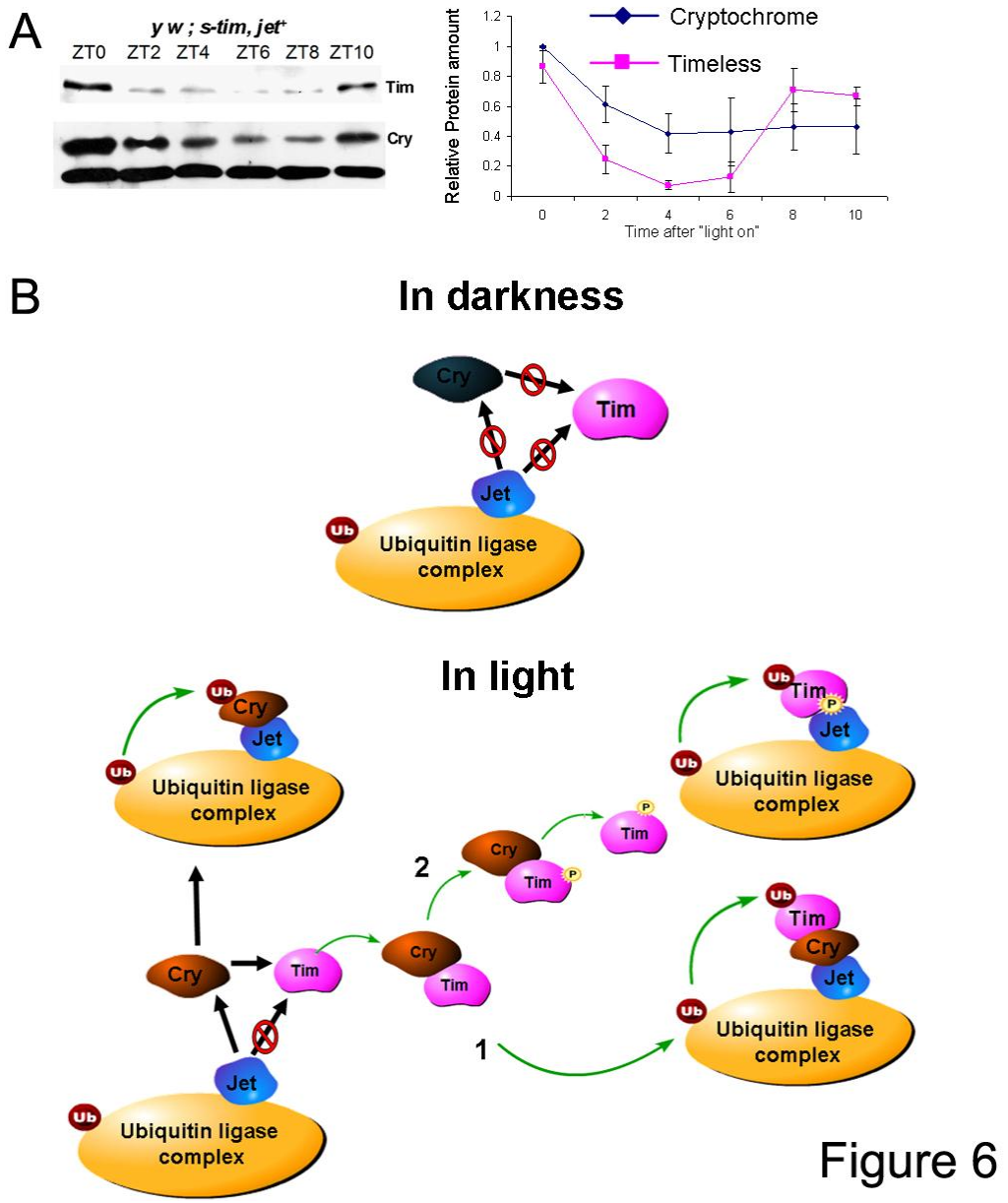

Figure 6

Figure 6: Potential sequential degradation of Tim and Cry and new model for molecular light resetting
(A) *y w* flies with either *s-tim* or *ls-tim* alleles were kept in a 12:12 LD cycle for three days. On the forth day we investigated the Cry and Tim degradation during different ZT times. Left panel shows a representative blot; right panel the the quantification of 4 independent experiments. Error bars indicate SEM. (B) Model of the light-dependent resetting of the circadian clock. The upper part displays the interaction of Jet, Tim and Cry in the darkness, the lower parts shows the interaction in the light. See text for details.

model 2). We favor the latter model, based on our finding that Tim but not Cry can be found in a complex with Jetlag in cells that express endogenous Cry (Figure 2). What could be the advantage of such an interdependent binding and degradation of light-regulated clock proteins? Our results suggest that Tim and Cry may be degraded in a sequential order (Figure 6B). As long as Jet triggers the degradation of Tim, Cry would be spared, either because Cry is still bound to Tim (and thus inaccessible for Jet), or because Jet's affinity to light-activated Tim is much higher than to Cry. After Tim levels have decreased to a critical amount, Cry is no longer needed and is now the prime target of Jet.

Our study reveals a crucial role for the F-Box protein Jetlag in the light-dependent degradation of the blue-light photoreceptor Cry. The same protein is used by the fly to target two important light-input molecules (Cry and Tim) for degradation, presumably in a sequential and logic order. In mammals, the F-box protein Fbxl3 has recently been shown to interact with the Cry1 and Cry2 proteins and to target them for degradation [21, 22]. In contrast to the fly, the mammalian Cry proteins are intimately tight with clock function and consequently mutations in the Fbxl3 gene cause period alterations of the free-running clock [22, 23]. Nevertheless, it also has been shown that mammalian Cry proteins undergo photoconversion of the flavin cofactor, similar as in plants and flies, suggesting that they do fulfill light-dependent functions after all (Hoang et al., 2008). In any case, certain aspects of the Cry degradation mechanism seem to be conserved between flies and mammals, even though it may have lost its light-dependency in the latter.

Experimental Procedures:

***Yeast-Two-Hybrid assay*:** The Cry, Cry^b, Cry^Δ , l-Tim and s-Tim vectors are described elsewhere [10]. Jetlag and $Jetlag^c$ vectors were generated using a PCR-based strategy. The appropriate restriction sites were introduced (Eco RI, Xho I). Before cloning into pEG202 or pJG4-5 vectors the Jetlag full-length fragments were sequenced. The Yeast-Two-Hybrid experiments were performed as in [16]. Each yeast patch descends from an individual transformation. All interactions were repeated at least twice with different yeast transformations.

***Flystrains*:** *jet^c ls-tim , jet^c s-tim, ls-tim* [3] and *cry^b [14]* were described previously**.** The *UAS-Tim* transgenes are described in [24]. *tim-Gal4* driver lines are described in [25]. *tim-Gal4(62)* was applied in Figure 5D and in Supplementary Figure S4; *tim-Gal4(27)* in Supplementary Figures S4 and S5. For the generation of UAS-*Luc-dCry* animals we excised the *luc-dCry* gene from the pAc-*Luc-dCry* vector and ligated it into a pUAST vector [26]. The pUAST-*Luc-dCry* vector was injected into *y w* animals by BestGene Inc.

***Western blots*:** Fly head Western blots have been carried out as in [3]. In each case equal numbers of fly heads were loaded per genotype (determined by counting). S2 cell protein extraction was performed as follows: Equal numbers of cells (determined by counting, see below) were harvested by centrifugation and resuspended in protein-extraction buffer [3]. Loading buffer was added and the cells were boiled before loading the gel (Resolving gel was 10%). Gels were blotted on Nitrocellulose using a semi-dry blotting apparatus and equal transfer of proteins was controlled by PonceauS staining. All Western blots were repeated 2-4 times under identical conditions with similar results. Cryptochrome antibody was raised in rabbit [18] and used 1:10000. Tim antibody was raised in rat and used as in [3]. Contrary to other reports [4, 8, 16], our Cry antibody detects a Cry band at about 50 kDa suggesting that Cry does not run with its predicted molecular weight of 60 kDa on SDS-Page gels (Supplementary Figure S5).

***S2 cell experiments*:** S2 cells were grown in Insect Xpress medium (Cambrex) with 10% fetal bovine serum at 25°C. For Western blots 100 000 cells, for CoIP assays 2 million cells, and for bioluminescence measurements 15 000 cells were transfected with

jetPEI according to the manufacturer's instruction. The cells were harvested after 48 hours. For proteasome inhibitor experiments MG-132 (50µM) was added. For the bioluminescence measurement 2mM of luciferin was added. For Western blots, cells were transfected with 250 ng of plasmid, for CoIP experiments up to 1 µg was used, and for the luciferase experiments cells were transfected with 25 to 40 ng of plasmid. The overall amount of transfected DNA was always the same. Unrelated plasmid DNA was used to co-transfect the cells when needed to ensure an equal amount of total transfected DNA. All plasmids described were cloned via Eco RI and Xba I into the pAc 5.1 vector (Invitrogen). The full length cDNAs of *jetlag*, *jetlag*^{c}, *l-tim*, *s-tim* and *cry* were introduced into the Eco RI and Xba I sites of the pAc 5.1 vector. His-tagged Jetlag was generated by eliminating the stop codon of *jetlag* and introducing the cDNA into the pAc 5.1 VH5/His vector. The pAc-*luc-dCry* and pAc-*luc-dCry*Δ were a generous gift from S. VanVickle-Chavez and R. Van Gelder.

***Co-immunoprecipitation (CoIP)*:** The experiment was performed as in [4], except that the Sepharose beads were coated with anti-His antibody for precipitation of Jet-His and the proteins bound to Jet (Invitrogen). In order to test if Cry or Tim are able to bind to the anti-His coated beads unspecifically, the CoIP experiments were also performed in the absence of Jet-His. In no case, binding of Cry or Tim was observed in the absence of Jet-His (data not shown).

***Luciferase assay*:** Luciferase levels of the transfected cells and adult flies were measured as described in [27]. The cells were kept in 12 hr: 12 hr LD cycles at 25°C and bioluminescence was measured once every hr.

Acknowledgements

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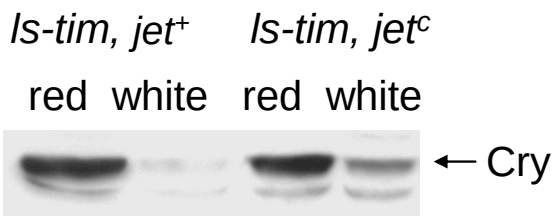
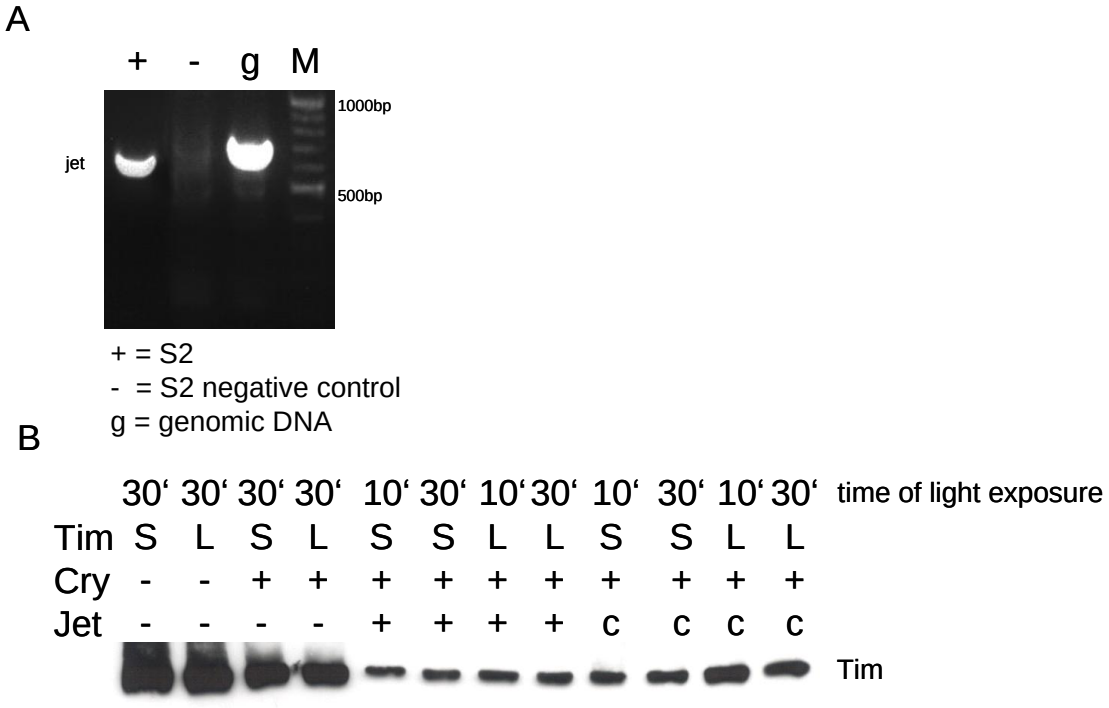
Supplemental Data

Figure S1: Influence of the eye color on Cryptochrome stability in the light



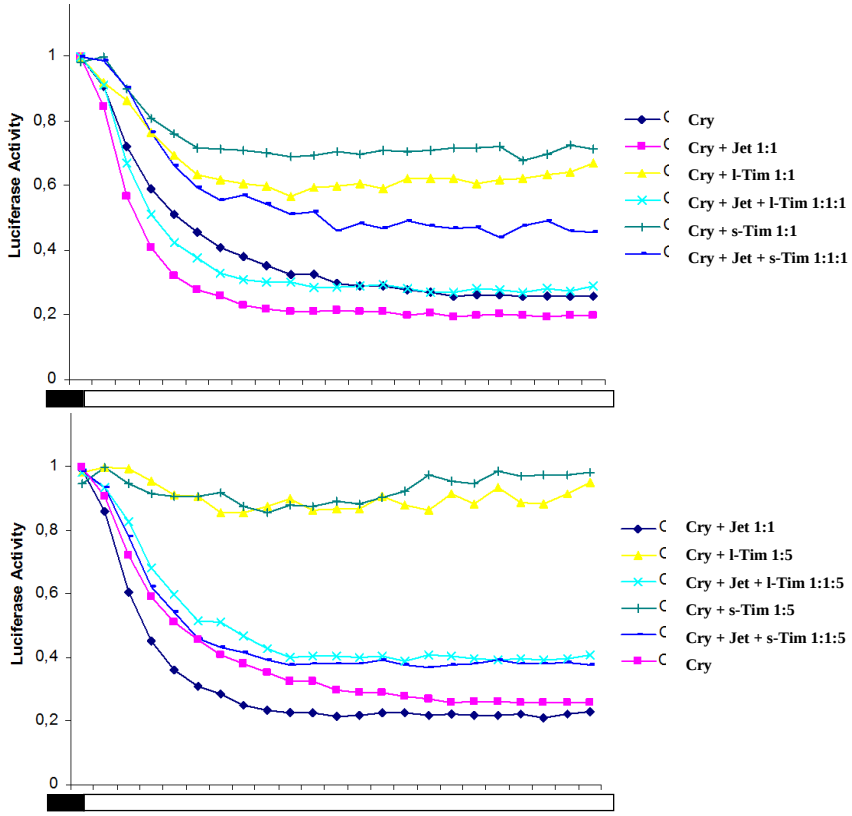
Protein head extracts from red-or white-eyed animals were investigated on Western blots. Lane 1 and 2 exhibit red- and white-eyed flies with a normal *jet* allele, while lane 3 and 4 represent animals with jet^c mutations. There is a clear difference visible between red- and white- eyed flies, whereas jet^c animals, red- or white-eyed, always show less degradation of Cry, compared to jet^+.

Figure S2: Expression of Jetlag in S2 cell culture



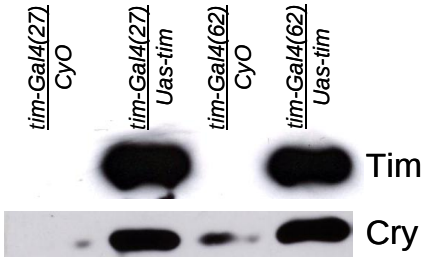
(A) Endogenous *jet* can be detected in S2 cells. A band corresponding to *jet* was detected by PCR, following the reverse transcription of S2 cell RNA (+). This PCR product was not seen in the negative control (-), a slightly bigger band was seen, when genomic fly DNA (still carrying a *jet* intron) was used as a template. (B) Timeless was expressed in S2 cells from pAc-*s-tim* or pAc-*l-tim* vectors (corresponding to the different Tim isoforms), or co-expressed with pAc-*cry* and/or wild-type *jet* (from pAc-*jet*) or the jet^c (from pAc-jet^c). Tim protein levels were assessed by Western blot with anti-Tim antibody. The cells were exposed to light for 10 min or 30 min before being harvested. In the first two lanes S-Tim (S) or L-Tim (L) was expressed alone. In lane 3 and 4 Cry was co-expressed, leading to a slight decrease of Tim. In the next four lanes Jet and Cry were co-expressed, inducing strong Tim degradation. The last four lanes show slightly increased Tim levels, resulting from Jet^c and Cry co-expression, more clearly visible after co-expression of L-Tim (last 2 lanes).

Figure S3: Degradation of Luc-dCry



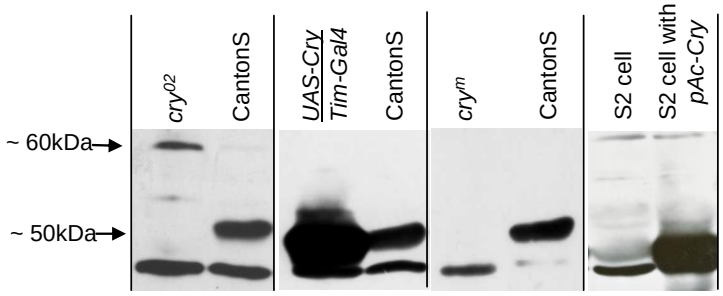
The pAc-*luc-dCry* plasmid was expressed in S2 cells and the degradation rate after illumination was investigated in the presence of Jet, Jet^{c} and/or S-Tim and L-Tim. Cells were kept in 12 hr: 12hr LD cycles and bioluminescence was measured 4 times per hr. The maximum Luc-dCry level for each combination was set to 1. The ratio of transfected Cry, Jet and/or Tim encoding plasmid DNA is indicated in the legend.

Figure S4: Overexpression of Timeless increases Cry stability in adult flies



In transgenic animals a UAS-*tim P*-element was driven with different *timeless-Gal4* drivers (Experimental Procedures). Flies were kept in a 12 hr: 12hr LD cycle and sacrificed at ZT6. In the control, Tim and Cry are strongly degraded. When Tim is over expressed, Cryptochrome levels are increased as well.

Figure S5: Specificity test of the Cryptochrome antibody



Our anti-Cry antibody [1] detects a 50 kDa band in wild-type flies (*Canton S*), which is absent in *cry* loss-of-function mutant *cry02* [2]. A strong signal of the same size is obtained when we use *tim-Gal4* and UAS-*cry* constructs to over-express Cry in all clock cells of the adult fly. In *cryb* (data not shown) and *crym* [3] mutant animals the intensity of 50 kDa band is strongly reduced or the band is absent, respectively. In S2 cells we can detect a very weak band at 50 kDa. When we express pAc-*cry* in those cells we can also detect a very prominent band running at 50 kDa. Taken together, these results demonstrate that our antibody specifically detects Cry.

Supplementary references

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4.3.1 Introduction Shaggy

The Glycogen-Synthase-Kinase 3 GSK3 ortholog Shaggy (Sgg) has an important function in the *Drosophila* circadian clock (Martinek et al., 2001). Increased *sgg* function results in period shortening- whereas decreased function causes substantial period lengthening of the molecular oscillator and behavioral rhythms. Furthermore, mammalian GSK3-β can phosphorylate *Drosophila* Tim *in vitro.* Over-expression of *sgg* in adult flies increased the amount of hyperphosphorylated Tim forms on Western blots. This is accompanied by an advanced nuclear entry of Tim and subsequently also of Period. A decrease of *sgg* produces almost no hyperphosphorylated Tim (Martinek et al., 2001). Thus the postulated function of Sgg in the *Drosophila* circadian clock is the phosphorylation of Tim thereby controlling it's nuclear entry and light sensitivity, because the hyperphosphorylated Tim is the preferred target of light-dependent degradation (Martinek et al., 2001).

Recently another interesting function of Shaggy was revealed (Stoleru et al., 2007). These investigators could reveal a striking stabilization of Cryptochrome caused by Shaggy. CoIP assays demonstrated a direct interaction of Cry and Sgg *in vitro* and *in vivo*– whereas the predicted interaction between Sgg and Tim could not be detected, a difficulty also encountered by others (Stoleru et al., 2007). Furthermore these authors could demonstrate that over-expression of Sgg in the DNs causes LL rhythmicity. A question that is totally untouched and not addressed in this publication is why the over-expression of a kinase that is thought to phosphorylate the Tim protein to generate a more light-sensitive form of Tim, causes *stabilization* of Tim and Cry resulting in LL rhythmicity?

To obtain answers to this interesting question we broadened our investigations about the interaction of Cryptochrome, Timeless and Jetlag and introduced yet another player – Shaggy.

4.3.2 Shaggy Results

A very dramatic effect of Sgg on the accumulation of Cry can be observed in S2 cells. Without Sgg, Cry accumulates only to very low levels – even in darkness. The expression of Shaggy stabilizes Cry so profoundly that it can be detected even under

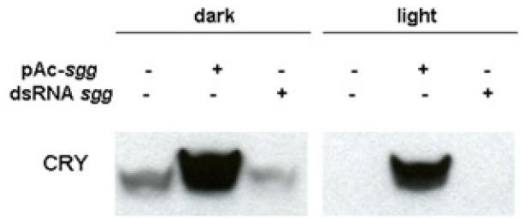

Figure 4-2 Sgg stabilizes Cry in S2 cells
This picture is taken from Stoleru at al. (2007).
The transfected plasmids are indicated at the top. The cells on the left were kept in darkness, the cells on the right under illumination. The anti-Cry antibody used for this Western blot is described in Emery et al. (1998).

illumination (Figure 4-2). We first wanted to investigate, if Sgg mediated stabilization of Cry could be reversed by co-expression of Jetlag in *Drosophila* S2 cells (Figure 4-3).

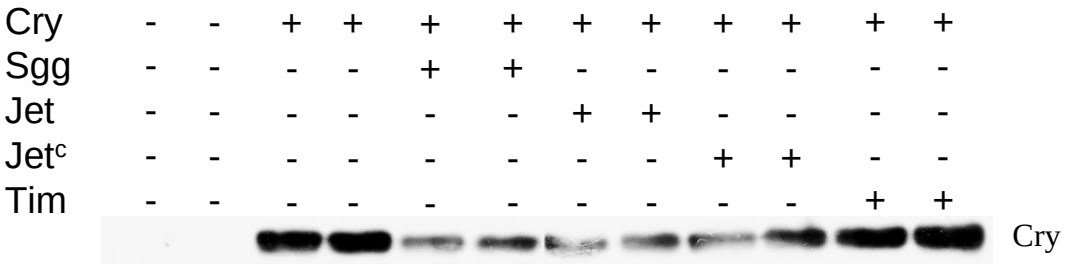

Figure 4-3 Degradation of Cry in the presence of Shaggy
The transfected plasmids are indicated at the top and left, while (+) is transfected, (-) indicates no transfection. The cells were kept in darkness. The antibody used to detect Cry Protein is described in (Yoshii et al., 2008).

Surprisingly we were not able to reveal the stabilization effect of Sgg on Cry. To verify that the pAc-*Sgg* plasmid (a gift from Pipat Nawathean) was the correct one, we partially sequenced the plasmid and affirmed thereby that it was the proper plasmid (data not shown). Several repetitions of similar experiments yielded comparable results (data not shown). Every time we co-transfected Sgg we could never discover an increase in Cry level, but a decrease.

Next we wanted to be sure, that *Sgg* is really expressed in our S2 cell culture system. In the pAc-*sgg* plasmid *sgg* is directly fused to a V5 and His tag (Figure 4-4).

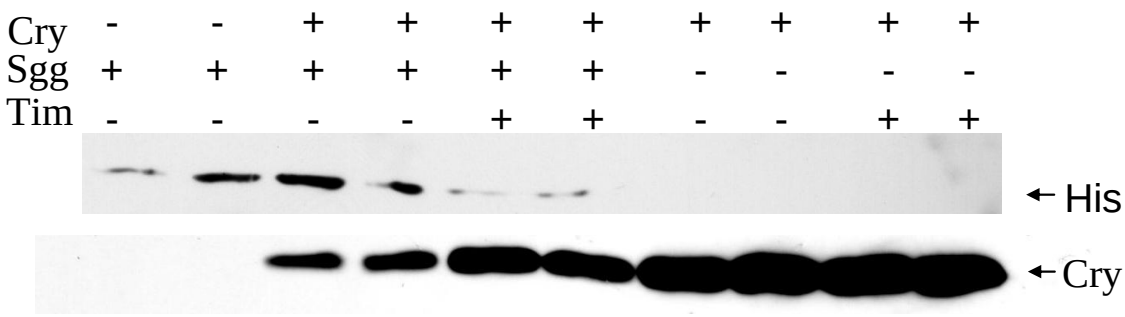

Figure 4-4 Shaggy-His in S2 cells
The transfected plasmids are indicated at the top. The cells were kept in darkness. The antibody used to detect Cry Protein is described in (Yoshii et al., 2008). The antibody used to detect the Shaggy-His protein was anti-His (Invitrogen).

The detection of the His-tagged Sgg demonstrates that the protein is expressed. The reason for the different amount of Sgg protein (esp. in lane 4 and 5) is not known. Different transformation efficiency might be a reason. This was only a single experiment and should be repeated to reveal the cause for the different amount of Sgg-His on the blot.

Stoleru et al. revealed stabilization of Cry in adult flies overexpressing Shaggy under

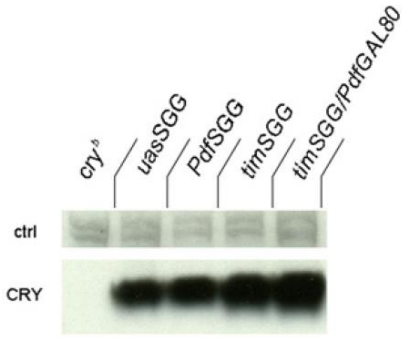

Figure 4-5 Shaggy mediated Stabilization of Cry
This picture is taken from Stoleru at al. (2007).
The investigated genotypes are indicated at the top. Animals were sacrificed in the dark (DD CT22). The upper bands are unspecific and act as a loading control. The anti Cry antibody is described in Emery et al. (1998). The experiment was repeated under illumination and produced similar results (data not shown).

a *timeless*-Gal4 promotor (Figure 4-5). The stabilization was even pronounced when the expression of Shaggy was restricted to the PDF-negative neurons (Stoleru et al., 2007). To investigate if our particular S2 cell culture system is responsible for the absence of Cry stabilization we examined adult flies that either over-expressed *shaggy* or where *shaggy* was knocked down by RNAi (Figures 4-6 and 4-7). Again we were not able to detect a clear effect of Shaggy on Cry – like a stronger degradation when Sgg was knocked down by RNAi.

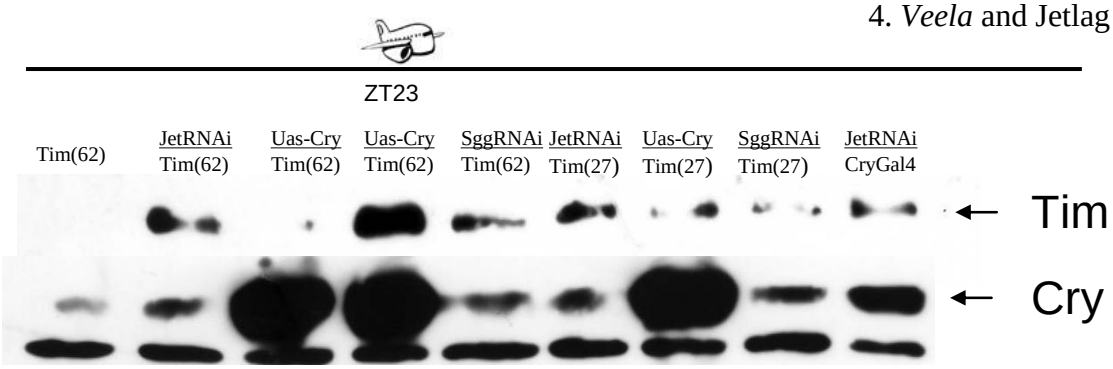

Figure 4-6 Down-regulation of Shaggy in adult flies
The genotype of the animals are as indiacted on top. The upper row marks the Timeless protein, while the lower row indicates Cry. All flies were sacrificed at ZT1 – except for lane four. In this lane the flies were sacrificed in the dark at ZT23. Tim(62) and Tim(27) are different *timeless*Gal4 transgenic alleles.

When we repeated this experiment in the dark at ZT23 we were not able to see a stabilization effect of Shaggy on Cry (Figure 4-7). Again we wanted to be sure that the investigated fly strains really over-expressed the Shaggy protein. For this reason we performed a behavior experiment in DD conditions. Here the transgenic flies expected to over-express *sgg* in all clock cells exhibited the previously described shorter period of about 21 hours (Martinek et al., 2001). The Sgg_{RNAi} animals behaved similar to wild-type animals (data not shown). We did not investigate the mRNA level of those animals and thus can not be sure if Shaggy is indeed knocked down by RNAi.

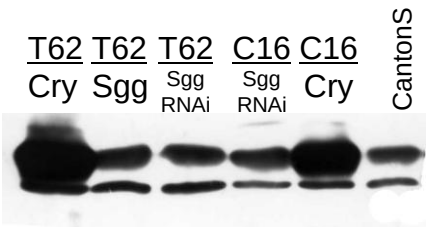

Figure 4-7 Over-expression and down-regulation of Shaggy in adult flies (dark)
All flies (20 heads per lane) were sacrificed for the Western blot investigation at ZT 23. The genotypes are indicated on top. The upper band shows the Cry protein, while the lower band is unspecific band. Abbreviations: T62= *tim*-Gal4 (62), C16 = *cry*-Gal4 (16), Cry = UAS-*cry*, Sgg= UAS-*sgg*, Sgg_{RNAi} = UAS-Sgg_{RNAi}.

When we detected Cry in our Western Blot assays we could see a prominent Cry band at 50kDa size (Peschel et al., 2008). Stoleru et al. detected a protein on the Western Blots with a larger size of about 55-60kDa (personal communication D.Stoleru) with an antibody from Emery et al. (1998). So we were wondering if the different antibodies might detect different modified forms of Cry, like phosphorylated or ubiquitinated Cry.

Because Shaggy is a kinase we wanted to investigate if perhaps Cryptochrome is phosphorylated by this protein. For this reason we co-transfected S2 cells with Cryptochrome and/or Shaggy and investigated the Cry level from those cells in light, darkness and after phosphatase treatment (Figure 4-8).

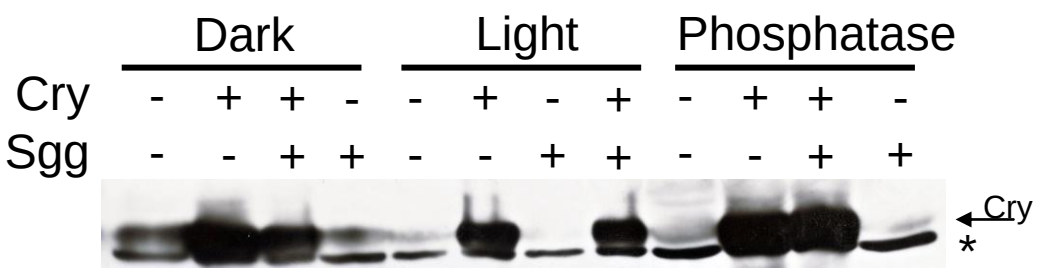

Figure 4-8 Phosphatase treatment of Sgg and Cry expressing cells.
The presence or absence of transfected plasmids is indicated with (+) and (-), respectively. Cells were either sacrificed in darkness, in light, or they were treated with a λ-phosphatase for 1 hour (after being in dark). The asterisk marks a non specific band. Cry antibody is described in (Yoshii et al., 2008).

Again we could not detect a stabilization effect when we coexpressed Shaggy. When pAc-*sgg* was used for the transfection we could detect a decrease in Cry protein. This was notable diminished when we treated the cells with a λ-phosphatase (especially in the dark sample, although here the non specific band seems to be weaker). We could not detect any signs of phosphorylated Cry with our antibody.

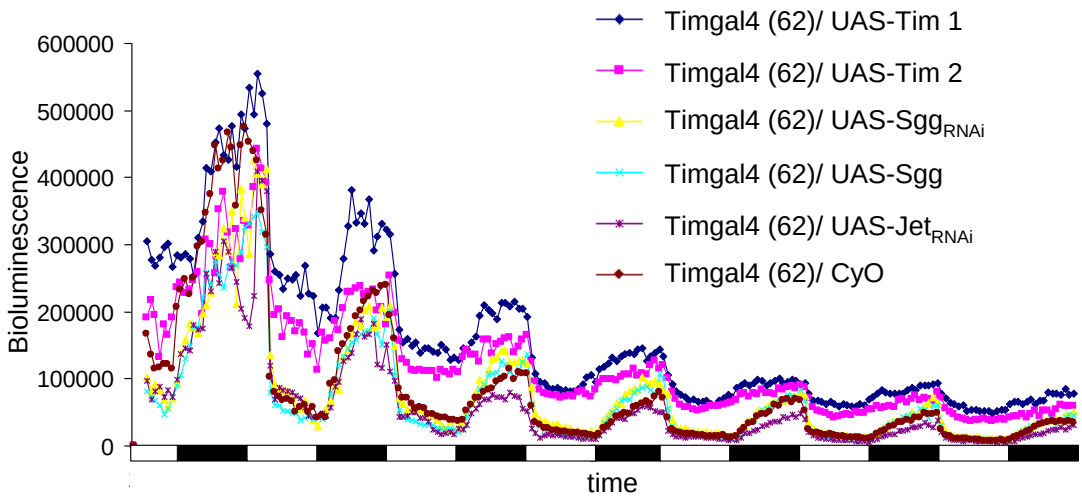

Figure 4-9 Bioluminescence of adult *Drosophila* animals carrying a Luc-dCry reporter.
Adult *Drosophila* transgenic flies were measured in a Packard Topcount machine. The genotype of the flies is displayed on the right. All animals carried furthermore one chromosomal copy of a UAS-Luc-dCry insertion. The x-axis indicates the time, the black and white bars at the bottom indicate the daily change of light and darkness (Light/Dark ratio 12:12 hr). The y-axis indicates the bioluminescence level (in Counts per second CPS) and thus the luciferase amount. Per genotype 8 animals were investigated.

Finally we wanted to investigate the potential and in our hands so far irreproducible stabilization effect of *sgg* on Cry in our luciferase assay. For this reason we over-expressed Shaggy in the presence of Luc-dCry in adult flies. Additionally we investigated animals where we attempted to knock down Shaggy or Jetlag by RNAi (Figure 4-9). Once more we could not detect a strong stabilization effect on Cry after

shaggy over-expression. When we coexpressed Jet_{RNAi} in the presence of *Luc-dCry* we could see a slightly decreased bioluminescence signal, compared to the control. But it should be mentioned that here (Jet_{RNAi}) female flies were investigated and all other animals were male (see discussion). Next we tested single tissues, (i.e.wings) of flies from the various genotypes. In contrary to the whole animal this time we detected a clear upregulation of the bioluminescene in wings were *shaggy* was co-expressed with Luc-dCry (Figure 4-10, light-blue track).

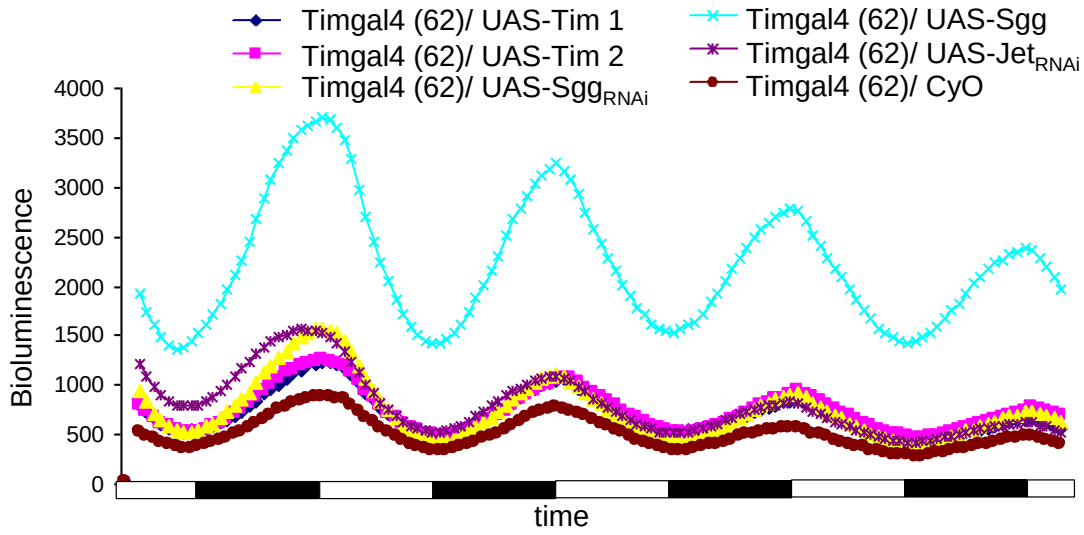

Figure 4-10 Bioluminescence of adult *Drosophila* wings kept in semi culture conditions. Adult *Drosophila* transgenics were dissected and the wings separated and measured in a Packard Topcount machine. The genotype of the investigated fly wings is displayed on the top. All animals carried in addition a UAS-Luc-dCry insertion. The black and white bars marked at the bottom indicate the daily change of light and darkness (Light/Dark ratio 12:12 hr).Per genotype 6 animals were investigsted. The y-axis indicates the bioluminescence level (in CPS) and thus the luciferase amount. The x-axis indicates the time, the black and white bars at the bottom indicate the daily change of light and darkness (Light/Dark ratio 12:12 hr).

4.3.3 Discussion Shaggy

The current model of the molecular regulation of circadian photoreception in *Drosophila* suggests that in darkness a yet unknown factor binds to the C-terminus of Cry. This factor acts as an inhibitor and hinders Cry from binding to other proteins like Jet or Tim. In the light this factor is released and now the blue-light photoreceptor is able to directly interact with Tim and/or Jet (Dissel et al., 2004; Rosato et al., 2001; Stoleru et al., 2007). After the discovery that Sgg stabilizes Cry - from Stoleru et al. in 2007 - we predicted that this unknown factor is Shaggy. Shaggy is expressed only at a

low level in S2 cells. Consequently this would imply that the Cry level in those cells is very low because only the Sgg-Cry complex protects Cry from degradation– in darkness and in light. If no inhibitor is bound to the C-terminus of Cry it is free to bind other proteins like Jet and thus becomes targeted for degradation in the proteasome. When additional Sgg is expressed in S2 cells, only the amount of Sgg would then limit the amount of Cry. We thought that this could be an explanation for the stabilization-effect seen by Stoleru et al. (Stoleru et al., 2007). Another effect observed when *shaggy* is over-expressed is the stabilization of Timeless (Stoleru et al., 2007). A fact that is hard to explain, considering that a prime function of Cry is thought to be the light-dependent Tim degradation (Stanewsky et al., 1998). Over-expression of Cry in adult flies leads to more light sensitive flies (Emery et al., 1998). The absence of the blue-light photoreceptor leads to a dramatic stabilization of Tim- especially the hyperphosphorylated form (Stanewsky et al., 1998) - and thus causes rhythmicity under constant light conditions (Emery et al., 2000a). Why should the stabilization of Cry via Sgg over-expression induce the same rhythmicity in LL? If the stabilization effect is real, an explanation for this result could be the fact that Jetlag induces the degradation of Timeless **and** Cryptochrome. Stabilization of Cry by Sgg and the accompanying increase of Cry could compete with Tim for Jetlag interaction. So the high Cry level would lead to an increase of Jet:Cry complexes and thus inhibit Jet binding to Tim and thereby inhibiting Tim degradation.

In fact it was shown that Tim over-expression causes a massive stabilization of Cry (Peschel et al., 2008). Will an increase of Cry induce a stabilization of Tim as well? We could reveal by Western blots that this is not the case (Figure 4-6). Furthermore under illumination Tim is the preferred target of Jetlag (Peschel et al., 2008). Of course one should keep in mind that these observations were seen under LD conditions. Constant light could change the degradation rate of Cry and Tim. Another explanation for the Shaggy mediated stabilization of Cry and Tim could be, that the binding of Shaggy to Cry hinders Cry-Jet interaction (leading to Cry stabilization) and the Cry-Tim interaction (leading to Tim stabilization). Stoleru et al. demonstrated that the Cry stabilization could be detected under illumination as well (Stoleru et al., 2007 and Figure 4-2). If this is the case this would imply that even under illumination the proposed Sgg-Cry complex does not seize to exist and that Shaggy hinders Cry interaction with other proteins even in the light. The normally in wild-type flies

observed fast light-dependent degradation of Cry and the interaction of Cry with other proteins like Tim makes this very unlikely.

Our failure to repeat the Cry stabilization by Sgg in S2 cells (Figure 4-3, 4-4,) and in adult animals (Figure 4-7) questions now the model that Sgg stabilizes Cry. Our Western blot results even argue for a decrease of Cry in the presence of Shaggy - at least for the observed 50 kDa Cryptochrome protein (Figure 4-3, 4-4). If this observation is true one could explain the observed stabilization of Tim and the rhythmic behavior in LL after *sgg* over-expression easily with a diminished Cry level. The Shaggy kinase may interact with Cryptochrome and phosphorylate Cry. This phosphorylated Cryptochrome can now interact with an F-Box protein like Jetlag and is subsequently degraded in the proteasome. Tim on the other hand is not degraded because either Cry is absent (it is degraded already) or because the Shaggy-phosophorylated-Cry is the preferred Jet target. A decrease of Cry levels would also easily explain the rhythmic behavior of Sgg over-expressing flies in LL.

Interestingly mCRY2 is phosphorylated at Ser557 in the mouse SCN and liver, in which the Ser557-phosphorylated form accumulates. The priming phosphorylation of mCRY2 at Ser557 allows subsequent phosphorylation at Ser553 by glycogen synthase kinase-3 (GSK-3), resulting in proteasomal degradation of mCRY2 (Harada et al., 2005; Kurabayashi et al., 2006). Recent observations predict a phosphorylation of dCry by the same kinases and phosphatases affecting Tim and Per (C. Hansen, personal communication). The question remains, why Stoleru et al. observed a drastic stabilization of Cry on Western blots after Shaggy overexpression (Figure 4-2)? Possibilities to explain the discrepancy between our and their results could be the usage of different S2 cells or the usage of different antibodies.

Different S2 cells, like S2 and S2R+ cells display minor differences. The over-expression of Wingless (Wg) induces Armadillo (Arm) stabilization and accumulation in the adult animal and in S2R+ cells, but not in normal S2 cells. The reason is the absence of the Wg receptor, Dfrizzled-2 in S2 cells (Yanagawa et al., 1998). So even minor differences between our and the by Stoleru et al. investigated cells might yield in different results.

Another explanation could be, if the antibody utilized by Stoleru et al. (Emery et al., 1998; Stoleru et al., 2007) preferably recognizes phosphorylated Cry, one could explain the described overall weak quality of the antibody (Emery et al., 2000b) (because normally the phosphorylated form of Cry is not the main form) and one

could explain the observed discrepancy in size (50kDa versus 55-60kDa). It would also explain why they could detect a stabilization or upregulation of Cry on their blots, because they detect the strongly phosphorylated form of Cry! When Sgg phosphorylates Cry the resulting phosophorylated form of Cry, which is the one that is light activated and targeted for degradation can not be revealed by us, because our antibody detects 'naked' Cry, which does not exist anymore after phosphorylation/light activation.

To avoid the difficulties with the different antibodies and cells we wanted to investigate the degradation of Cry in adult flies that express a Luciferase protein fused to Cryptochrome. This protein reports light-dependent degradation of Cry via a reduction of Luciferase levels reflected by decreased bioluminescence emission (Peschel et al., 2008). A first unexpected result was the slightly lower level of Luc-dCry in the Jet_{RNAi} transgenic flies. To explain this result we should mention that it was never tested if the *jet* mRNA is really down-regulated in *jet-RNAi* flies. Furthermore one should mention that the investigated animals were (in contrast to all other investigated flies) female and thus might exhibit minor differences in protein levels.

A second unexpected observation was seen when we over-expressed Shaggy in the presence of Luc-dCry. In adult flies the over-expression seems to have only a minor (if at all) effect, affirming our Western Blot results. In S2 cells expressing Luc-dCry and Shaggy we could even detect a decrease in bioluminescence and Cry level (data not shown). But when we investigated single tissues (i.e., wings) from adult flies the over-expression of Shaggy caused an up regulation of Luc-dCry – especially in the darkness (Figure 4-10). If this result is caused by Shaggy and not by some genetic background effects, again an explanation that fits to the overall model is not easy. (But it would finally confirm the results from Stoleru et al. (2007)).

An explanation for the discrepancy in the bioluminescence level between adult whole animals and wings (from the very same animals) could be that in wings and whole animals the composition of proteins is different. Some proteins encountered in for example the head might not be expressed in wing cells and thus result in different Cry level. Another important fact is that the *cryptochrome* gene seems to have a different function in the central brain as a circadian photoreceptor and in the peripheral tissue as a repressor molecule that is closer involved in the core control of the circadian clock.

4.3.4 Outlook Shaggy

An interesting way to explain the discrepancies between our own observations – Sgg does not increase Cryptochrome level and even decreases Cry amount – and the observation by Stoleru et al. (2007) that Sgg stabilizes Cry, would be the use of different antibodies. One antibody (Stoleru et al., 2007) that preferably recognizes phosphorylated Cry would report an Cryptochrome increase after Shaggy over-expression, because Sgg might phosphorylate Cry and thus transform most of the protein into the by this antibody preferably recognized form of Cry. Another antibody (Peschel et al., 2008, Yoshii et al., 2008) that does not recognize the phosphorylated form of cry, but the normal form would not detect this increase of Cryptochrome. And, assuming that the phosphorylated form of Cry is the most light-sensitive and thus instable form, even detect a strong decrease of overall Cry level. Because phosphorylated forms of proteins migrate in a slower form on Western blots one even can explain in this way the discrepancy between the observed sizes of Cry on Western blots (50kDa versus 55-60kDa). Additionally one can easily explain with this model why an over-expression of Sgg produces LL-rhythmic animals.
So the best way to test this model would be to compare the two different antibodies (Emery et al., 1998 and Yoshii et al., 2008). If those two antibodies really show the postulated different Cry level and protein size on Western Blots one can proof our theory and additionally show that Cry is phosphorylated by Shaggy. Further experiments with phosphatase treatments should even result in more interesting findings.

5. The *roundabout* Gene's Function in Circadian Rhythm

5.1 Introduction

The *roundabout* (*robo)* gene in *Drosophila* was first discovered in a mutant screen for genes that control axonal pathfinding in the CNS midline. In *robo* mutants too many axons cross and recross the midline (Kidd et al., 1998). Robo appears to function as a repulsive axon guidance receptor on growth cones that responds to a midline ligand. The protein is expressed at the growth cone filopodia and responds to the repulsive diffusible ligand SLIT emanating from the midline (Kidd et al., 1999). Furthermore the lack of ROBO function during puparium formation leads to defects in the position of sensory terminals as well as the malformation of the commissures connecting the two olfactory lobes in *Drosophila* (Jhaveri et al., 2004). In a recent publication Berni et al. reported the discovery of a hypomorphic *robo* allele $robo^{hypomorph}$ ($robo^{hy}$) that alters the locomotor behavior under constant conditions (DD) (Berni et al., 2008). This mutant expresses about half the normal amount of Robo protein. Under constant darkness conditions homozygous mutant $robo^{hy}$ animals display a locomotor period of about 23.4 hr. This shortening of the period was never observed in heterozygous mutants. When a UAS-*robo* animal was used to express a higher level of *robo* in the $robo^{hy}$ animals, the animals displayed a more wild-type-like period of 23.65 hrs. The eclosion period on the other hand is not shortened in $robo^{hy}$. Despite the expected role in circuit assembly, the PDF network displayed only subtle defects in the axon trajectory. This observation caused Berni et al. to suggest a potential acute role of ROBO in the circadian rhythm of the adult fly. The authors also demonstrated a genetic interaction of *robo* with *Pdf*, the only synchronizing factor so far described in the circadian network of flies. This underscores that ROBO could modulate the signaling downstream of the small LN_vs. Furthermore the authors were able to correlate the period shortening defect in locomotor behavior with an earlier entry of Per into the nucleus (Berni et al., 2008).

My contribution to this project was to investigate the expression levels of the clock gene products encoded by the *per* and *tim* genes in $robo^{hy}$ mutant adult flies and tissues using the real-time *luciferase* (*luc)* reporter gene system (Brandes et al., 1996). The various reporter genes we applied differed in their spatial expression pattern due to various

amounts of regulatory and coding sequences present in each construct that were used to drive *luciferase* expression. The following *per*- and *tim-luc* reporter genes were applied:

BG-*luc*

The BG-*luc* construct carries the *period* promoter region, and 2/3rds of the Period coding region fused to the *luciferase* gene (Figure 5-1) (Stanewsky et al., 1997b).

plo

In this construct the *luciferase* gene is controlled only by the *period* promoter and thus represents *period* mRNA transcription (Figure 5-1) (Brandes et al., 1996, Stanewsky et al., 1997b).

8.0-*luc*

Here a part of the first *period* intron is followed by almost the full length *period* ORF. No upstream promoter sequence controls here the expression (Figure 5-1) (Veleri et al., 2003). It should be mentioned that this construct is not expressed in all the clock cells, but only in a subset –i.e. in the dorsal neurons in the brain (Veleri et al., 2003).

tim-luc

luciferase is under the control of the *timeless* promoter and thus represents the *timeless* mRNA expression (Figure 5-1) (Stanewsky et al., 1998).

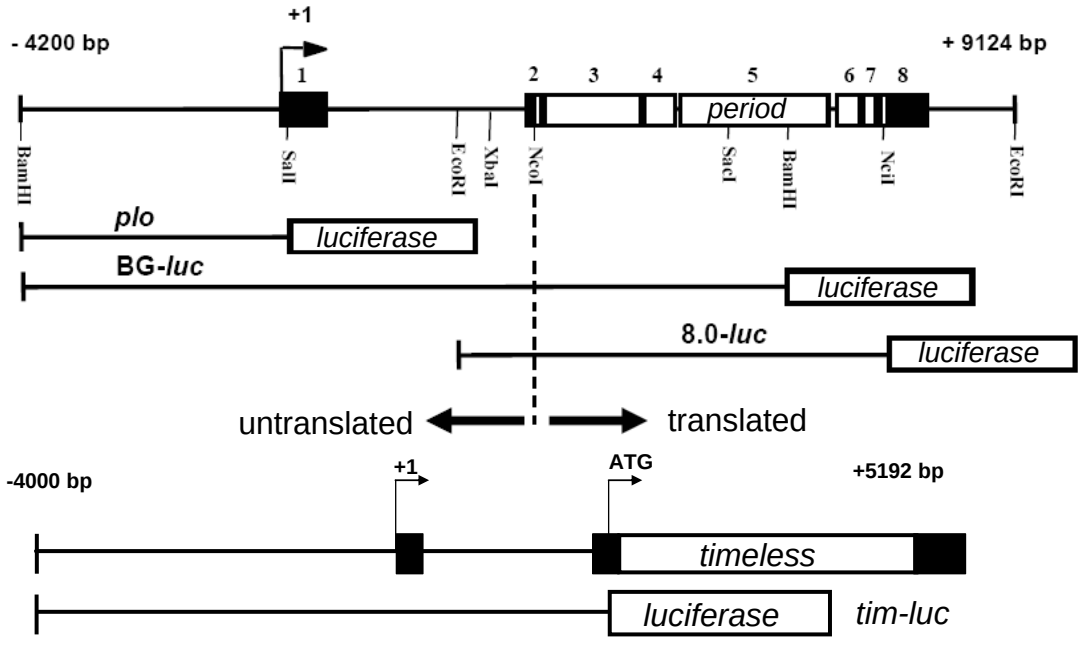

Figure 5-1 Overview of different luciferase contructs
This picture was adapted from (Stanewsky et al., 2002). Luciferase cDNA is controlled by different circadian regulatory elements. Those elements are important for expression of the *timeless* and *period* genes. BG-*luc* and 8.0-*luc* constructs encode for fusion proteins (between Period and Luciferase).

5.2 Results

To investigate the effect of the *robo*^{hy} mutation on *period* and *timeless* or Per expression we compared different circadian reporter in wild-type or *robo*^{hy} background.

5.2.1 Adult Flies

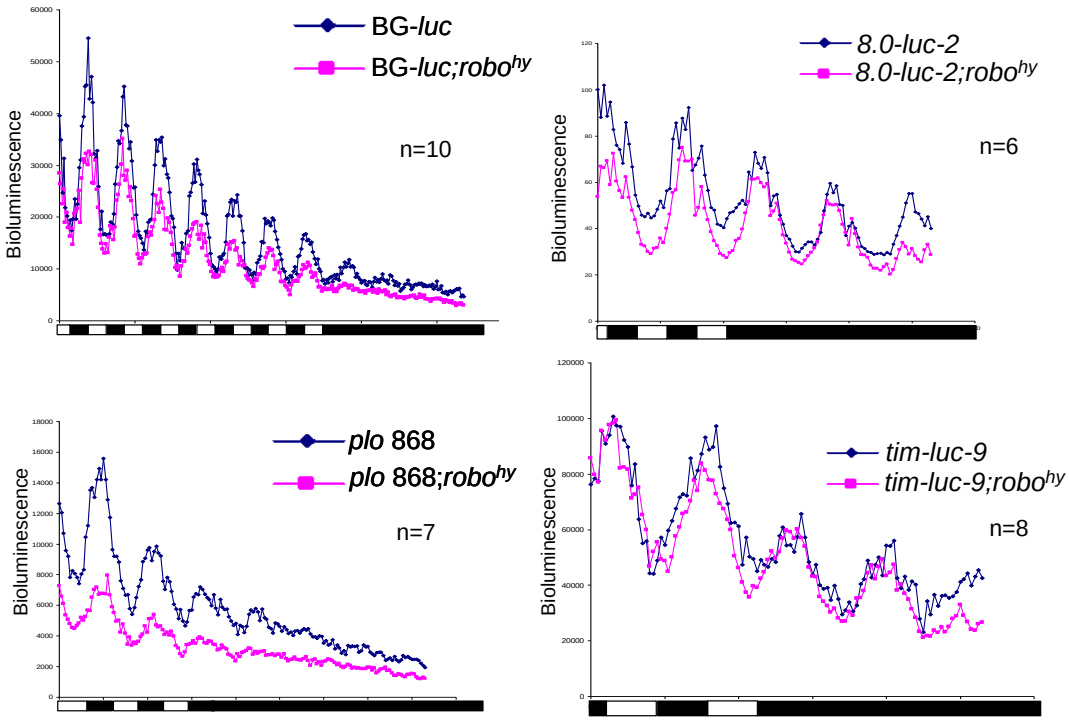

Figure 5-2 Adult animals with control and *robo*^{hy} genetic backgrounds.
Different reporter constructs were investigated in wild-type animals or in *robo*^{hy} background. The y-axis indicates the bioluminescence level (in counts per second, or CPS) and thus the luciferase amount. The x-axis indicates the time. Animals were first kept for several days in L/D conditions and then released into constant darkness. The black and white bars indicate light on or off. The different reporter constructs are described in Figure 5-1.

We first investigated the influence of *robo* on Per in adult flies. Western Blot results revealed a down regulation of Per in *robo*^{hy} animals (data not shown, personal communication J. Berni). We could detect the same in the luciferase assay. Not only the protein, but the mRNA is down regulated as well. In the 8.0-luc-2 animals the reduction in Per is only very mild or not present. In those animals we only look at the Per expression in the dorsal neurons of the fly. This reduction can not be seen, when we used

tim-luc as a reporter. The down regulation seems to be independent of the ambient light regime and can be detected in L/D or in constant darkness.

5.2.2 Semi Cultures

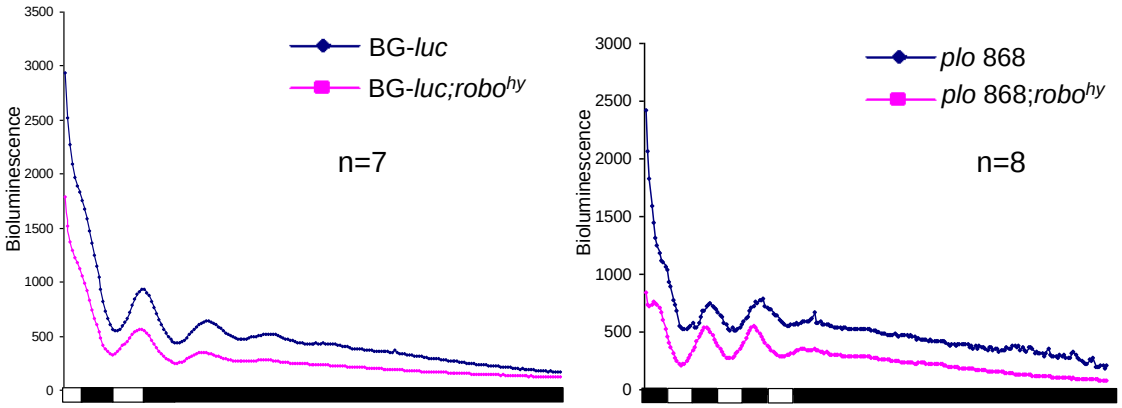

Figure 5-3 Bioluminescence in body parts of *robo^{hy}* animals.
Different reporter constructs were investigated in body parts of wild-type and *robo^{hy}* mutant animals. The y-axis indicates the bioluminescence level (in CPS) and thus the luciferase amount. The x-axis indicates the time. Animals were first kept for several days in L/D conditions and then released into constant darkness. The different reporter constructs are described in figure 5-1. The black and white bars indicate light on or off.

The experiment was repeated with separated body parts, for example isolated wings. Here we could reveal the same down regulation as was seen in the whole animals.

5.3 Discussion

Animals with a hypomorphic allele of the *roundabout* gene, *robo^{hy}*, exhibit a shorter locomotor activity period than wild-type under constant darkness. Preliminary results support the theory that PER entry into the nucleus in the LN_vs is affected in those animals, i.e. it was detected sooner in *robo^{hy}* than in *w^{1118}* flies (Berni et al., 2008). Western blot experiments revealed that the Period protein is down regulated in *robo$^{h\,y}$* head extracts (per. communication J.Berni). The results from the bioluminescence assays revealed a similar, overall lower amount of Period protein expression (Figure 5-2). This effect can

not only be seen in constant darkness conditions, but under LD cycles as well. Otherwise animals with an 8.0-*luc* construct display only a very mild (if at all) down-regulation of this protein. The BG-*luc* reporter is expressed in many tissues of the adult fly, while the 8.0-*luc* is expressed only in some neurons in the brain (Stanewsky et al., 1997; Veleri et al., 2003). From this result we assume that the down-regulation of Per happens only in the periphery and not in the brain – or at least not in the DNs. The 'promoter only' *plo* construct represents *period* mRNA transcription in the fly (Stanewsky et al., 1997). Here we revealed down regulation of *period* in $robo^{hy}$ background. This overall lower mRNA level that can be observed in the $robo^{hy}$ background can of course directly result in an overall lower Period protein level. *timeless* expression on the other hand is not down regulated or even seems to be slightly upregulated (Figure 5-2 and data not shown). It was assumed that *robo* is mainly expressed and active in the brain (Berni et al., 2008). Therefore we investigated if the overall down regulation of Per and *per* is only caused by a signal from the brain to the periphery that is disturbed in the $robo^{hy}$ mutants or if the mechanism of *robo* in the circadian rhythm is more widespread all over the body. For this reason we analyzed *per* expression in isolated body parts of the adult fly. The isolated wings do not have the connection to the brain anymore and therefore are lacking the potential signal that is send from the brain to the periphery. But the isolated tissues also show an overall down regulation of Period expression (Figure 5-3). Based on our results we conclude that the function of *robo* in the adult fly is more widespread than expected and that no signal from the brain is necessary to cause the down regulation of Per in $robo^{hy}$ animals. More experiments with RNAi-mediated down-regulation, or over-expression of *robo* in specific tissues will hopefully lead to a clearer picture. Stainings of different body parts with anti-Robo antibodies should reveal if *robo* is indeed widely expressed in the adult fly.

6. Supplementary Information

6.1 Summary

6.1.1 Summary English

Circadian rhythms are so widespread under the inhabitants of the earth that it is no wonder that circadian clocks are focus of investigation for a very long time. The many genetic and molecular possibilities of the model organism *Drosophila melanogaster* make the fruit fly the perfect organism for the investigation of the circadian clock on the molecular level. For this reason the current thesis deals with the investigation of the circadian clock of *Drosophila* with the focus on the circadian photoreception.

In the first part of this work we investigated the gene *lethal (2) 05510* or *quasimodo (qsm)*. Our investigations revealed, that the *qsm* mRNA is rhythmically expressed under the control of the circadian clock. When this gene is down-regulated via RNAi technique in the crucial clock neurons of adult flies those animals still behave rhythmic under constant illumination. Constant light normally renders flies arrhythmic, because of a light dependent degradation of the important clock protein Timeless (TIM). Furthermore some neuronal groups still exhibited a rhythmic fluctuation of the Period (PER) protein under constant illumination – this was never detected in wild-type animals. We could show that QSM is expressed –at least partly- in clock neurons and in neurons in close proximity to those neurons. Whereas the localization and intensity of the protein changes with the time, surprisingly the troughs and peaks do not correlate with the mRNA troughs and peaks.

Another interesting observation was the restored rhythmicity of per^{01}animals when *qsm* was down regulated in constant light conditions in per^{01} background. Until now it was believed, that *period* is mandatory for circadian rhythm. The fact that per^{01}*;;qsmRNAi(16)* animals are rhythmic under constant light condition or anticipate the evening under LD conditions brought us to rethink. What really renders those animals rhythmic in LL is still subject of investigation. We discussed in this context a separation of the circadian clock into several oscillators – one important for the control of the circadian clock in the darkness, one important for the control of the circadian clock in the light. In the “Light-clock” PER does not play the important role it holds in the “Dark-clock”.

Furthermore we could reveal that the over-expression of the *EP2586* insertion – a *P*-element insertion close to the *qsm* start - does not approach *quasimodo* but the miRNA cluster 310-313. This cluster is located in the promoter region of *qsm*. When those miRNAs are overexpressed in the clock neurons we could detect a different behavior under Light/Dark cycles. In those animals the release of the circadian neuropeptide PDF seems to be impaired. Because similar phenotypes are reported for animals where ion channels are over-expressed or down regulated we focused our search for the targets of the miRNAs to channel and channel like proteins. A good candidate for a target channel protein is Cg31547. First preliminary results still do not allow us to make a clear assertion.

The second part of this work deals with a mutant fly that behaves rhythmic under LL conditions as well. We could show that in this mutant fly – called *Veela* - not one but two different factors are causing the abnormal behavior: *Veela* animals carry a mutation in the F-Box protein Jetlag (JET). *jet* is important for the light dependent degradation of TIM. Furthermore *Veela* animals carry a less light sensitive natural allelic variant of the Timeless protein – encoded by the so called *ls-tim* gene. Each factor alone impairs the circadian photoreception only to a minor extend. Hence only the combination of both leads to the observed phenotype under constant light conditions.

We could reveal that the Jetlag protein has an additional function in the circadian clock. Jetlag also interacts with Cry in yeast and in *Drosophila* S2 cells in a light-dependent manner. After illumination Jetlag induces massive degradation of Cry which can be prevented *in vitro* and *in vivo* by adding Tim as an antagonist. We show that Jetlag causes light-dependant and sequential degradation of Tim and Cry. With this sophisticated mechanism the degradation of TIM and CRY and thus the resetting of the circadian clock in the light is regulated.

Another protein that is intimately involved in the degradation of TIM and CRY is Shaggy. Recent observations demonstrate that Shaggy stabilizes Cryptochrome in the light. Our preliminary results could not affirm a stabilization effect of Shaggy on Cry. Here we discussed the possibility that the function of Shaggy is not located in the stabilization of Cry by binding to its C-terminus and thus acting as a repressor. We rather speculated that Shaggy phosphorylates Cry and facilitates thus the degradation of this protein.

In a third part we investigated the effect of the *roundabout* gene (*robo*) on the circadian rhythm. Here we could reveal, that the PER decreasing effect of Robo in the adult fly is widespread through the whole body of the fly and that the function of robo is not restricted to the brain.

6.1.2 Zusammenfassung

Circadiane Rhythmen sind unter den Bewohnern dieses Planeten so weit verbreitet, dass es nicht verwunderlich ist, dass die circadianen Uhren schon seit sehr langer Zeit im Fokus von vielen Untersuchungen stehen. Die vielen genetischen und molekularen Möglichkeiten des Model-Organismus *Drosophila melanogaster* machen die Fruchtfliege zu einem perfekten Organismus für die Untersuchungen der circadianen Uhr auf molekularer Ebene. Darum handelt diese Doktorarbeit von der Untersuchung der inneren Uhr von *Drosophila* mit dem Schwerpunkt auf der circadianen Photorezeption.

Im ersten Teil der Arbeit wurde das Gen *lethal (2) 05510* oder - auch *quasimodo (qsm)* genannt - untersucht. Diese Untersuchungen konnten zeigen, dass die *qsm* mRNA rhythmisch unter der Kontrolle der circadianen Uhr exprimiert wird. Wenn diese Gen mit Hilfe der RNAi Technik in den wichtigen Uhrenneuronen herab reguliert wird, verhalten sich diese Tiere immer noch rhythmisch unter dauerhafter Beleuchtung. Dieses konstante Licht bewirkt normalerweise wegen des lichtabhängigen Abbaus des wichtigen Uhrenproteins Timeless (Tim) arrhythmisches Verhalten. Weiterhin zeigen manche neuronale Gruppen immer noch rhythmische Fluktuation des Period (PER) Proteins im Licht – dieses konnte in wildtypischen Tieren nie beobachtet werden. QSM Protein konnte – zumindest teilweise – in den Uhrenneuronen und in Neuronen die sich in unmittelbarer räumlicher Nähe dazu befanden nachgewiesen werden. Während sich der Expressionsort und -intensität des Proteins mit der Zeit veränderten, waren die Hoch- und Tiefpunkte der Protein Expression überraschenderweise nicht identisch mit den mRNA Maximas und Minimas. Eine weitere wichtige Beobachtung war, dass das rhythmische Lokomotorverhalten in per^{01} mutanten Tieren zum Teil wieder hergestellt werden konnte, wenn hier gleichzeitig *qsm* im Dauerlicht herab reguliert wurde. Bis jetzt wurde angenommen, dass *period* unabdingbar für die circadiane Rhythmik ist. Die Tatsache, dass per^{01}*;;qsmRNAi(16)* Tiere rhythmisch im Dauerlicht sind oder aber den Abend in einem Licht/Dunkel Experiment antizipieren brachte uns dazu das

Period Protein in einem neuen Licht zu sehen. In diesem Zusammenhang wurde eine Aufteilung der circadianen Uhr in mehrere Oszillatoren postuliert– wobei einer wichtig für die Kontrolle der circadianen Uhr im Dunkeln ist und ein Oszillator wichtig für die Kontrolle der circadianen Uhr im Licht ist. In der „Licht-Uhr“ spielt das Period Protein nicht die wichtige Rolle die es in der „Dunkel-Uhr“ inne hat.

Weiterhin konnte gezeigt werden, dass die Überexpression der Insertion *Ep2586* – eine *P*-Element Insertion in der Nähe des *qsm* Startes – keine direkte Überexpression von *qsm* bewirkt, sondern eine Überexpression des microRNA Clusters 310-313. Dieses Cluster befindet sich in der Promotorregion von *qsm*. Wenn die microRNAs in den Uhrenneuronen über exprimiert wurden konnte unter Licht/Dunkel Bedingungen ein vom Wildtyp abweichendes Lokomotor Verhalten feststellen werden. Bei diesen Tieren scheint die Abgabe des Neuropeptids PDF gestört zu sein. Da für Tiere bei denen Ionenkanäle vermehrt oder verringert exprimiert wurden ähnliche Phänotypen festgestellt werden konnten, wurde die Suche nach den Zielgenen der microRNAs auf Kanal und Kanal-ähnliche Proteine eingeengt. Ein guter Kandidat für ein solches Ziel Kanal Protein ist das Gen *cg31547*. Erste vorläufige Ergebnisse erlauben aber bis lange keine klare Aussage dazu.

Der zweite Teil dieser Arbeit handelt von mutanten Fliegen, die sich im Dauerlicht (LL) auch rhythmisch verhalten. Es konnte gezeigt werden, dass in diesen mutanten Fliegen – *Veela* genannt – nicht ein sondern zwei unterschiedliche Faktoren dieses anormale Verhalten hervorrufen. *Veela* Tiere weisen eine Mutation im F-Box Protein Jetlag (Jet) auf. *jet* spielt eine wichtige Rolle für die lichtabhängige Degradierung von TIM. Weiterhin tragen *Veela* Tiere eine weniger lichtsensitive allelische Variante des Timeless Proteins. Diese wird durch das so genannte *ls-tim* Gen kodiert. Jeder Faktor für sich genommen schränkt die circadiane Lichtrezeption nur zu einem bestimmten Grad ein. Deshalb bewirkt nur die Kombination beider Faktoren den beobachteten Phänotyp unter konstanten Licht-Verhältnissen. Es konnte auch gezeigt werden, dass Jetlag eine weitere Funktion in der circadiane Rhythmik aufweist. Jetlag interagiert auch mit Cryptochrome in Hefe und in *Drosophila* S2 Zellen in lichtabhängier Weise. Nach Lichtgabe bewirkt Jetlag starken Abbau von Cryptochrome, welcher *in vitro* und *in vivo* durch Zugabe von TIM als ein Antagonist vermindert werden kann. Durch diesen ausgeklügelten Mechanismus wird der Abbau von Tim und Cry reguliert und dadurch der Reset der circadianen Uhr im Licht reguliert.

Ein weiteres Protein welches eng mit der lichtabhängigen Degradierung von Tim und Cry verbunden ist, ist Shaggy (Sgg). Neuere Untersuchungen zeigten, dass Shaggy das Cryptochrome Protein im Licht vor dem Abbau schützt. Vorläufige Ergebnisse dieser Arbeit konnten den stabilisierenden Effekt von Shaggy auf Cryptochrome nicht bestätigen. In diesem Zusammenhang wurde nun eine Möglichkeit diskutiert, dass die Funktion von Shaggy nicht in der Stabilisierung von Cry durch beispielsweise eine Anlagerung von Shaggy an den C-Terminus von Cry zu suchen ist. Viel eher wurde spekuliert, dass Shaggy Cry phosphoryliert und dadurch die Degradierung des Proteins erleichtert.

In einem letzten Teil wurde der Effekt des *roundabout* Genes (*robo*) auf die circadiane Rhythmik hin untersucht. Hierbei konnte gezeigt werden, dass der Effekt den Robo auf das Period Protein aufweist (eine verminderte Expression von Per) sich nicht auf den Kopf der Fliege beschränkt, sondern im ganzen Fliegenkörper zu sehen ist. Dadurch konnte gezeigt werden, dass die Funktion von *robo* nicht auf das Gehirn der Fliege beschränkt ist.

6.2 Appendices

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Citations

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INGRATITUDE

Declaration

Hiermit erkläre ich, diese Dissertation selbstständig angefertigt und dabei keine anderen Hilfsmittel als die angegebenen benutzt zu haben. Alle aus der Literatur entnommenen Daten und Abbildungen sind als solche gekennzeichnet. Die hier vorliegende Dissertation wurde weder vollständig noch teilweise einer anderen Fakultät vorgelegt.

Regensburg, den

Nicolai Peschel