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Belle and Aurora A are novel components of the circadian machinery in *Drosophila melanogaster*

Direttore della Scuola : Prof. Giuseppe Zanotti
Coordinatore d'indirizzo: Prof. Paolo Bonaldo
Supervisore : Prof. Rodolfo Costa

Dottorando : Elena Carbognin

Abstract

The circadian clock is a self-sustained system that synchronizes the physiology and behaviour of most organisms with the light-dark (LD) cycles. In humans, several pathological conditions, including cancer, are related to the deregulation of the circadian clock. *Drosophila melanogaster* is one of the most important models for the study of the circadian mechanism. The proteins Timeless (Tim) and Period (Per) are two main components of the master clock, which is located in the brain. The synchronization of the circadian clock to environmental changes is mostly achieved by blue light-dependent degradation of Tim, via the photoreceptor Cryptochrome (Cry). Cry mediates the daily resetting of the clock by light, binding to Tim and targeting it for degradation via proteasome. The oscillator is able to keep circadian time even in the absence of external stimuli and consists of a network of genes that function in transcriptional/translational feedback loops. Among the post-translational modification that regulate clock proteins amount, localization and activity, phosphorylation is likely the most important one.

We have initiated a study in order to identify new molecular partners for Cry, both in light and dark.

1) The search for new partners of Cry by co-IP and mass spectrometry led to the identification of Belle, an ATP-dependent RNA helicase. Belle is expressed in the glial cells in fly adult brain, where also Cry is expressed. Moreover, the expression of this RNA-helicase oscillates in light-dark cycles (LD) and constant dark conditions (DD) in wild-type flies. Moreover, flies mutant for this gene do not show the typical rhythmic activity profile and show an impairment in Per expression in a subset of circadian neurons. Thereafter the downregulation of the gene causes reduced photophobicity in 3rd instar larvae and significant defects in the locomotor activity, suggesting an impairment of the circadian clock. Our data suggest that Belle is a novel "clock" protein involved in the circadian machinery of *Drosophila*, where it could act in the post-transcriptional control of circadian components.

2) It has been shown that the C-terminus of Cry contains putative targets for phosphorylation that play a role in the modulation of protein activity. Cry activation by light could be due to a conformational change or a release of a repressor. Phosphorylation could play an important role in the light dependent activation of the protein. In fact, AtCry1 and AtCry2 in plants (*Arabidopsis thaliana*) and mCry2 in mammals are phosphorylated after light exposure. *in vitro* experiments, performed in our lab, showed that *Drosophila* Cry is a putative target of the Serine-Threonine kinase Aurora A. Moreover, *in silico* analyses revealed that Cry C-terminus seems to contain consensus motifs which could be a target for this kinase. In particular, two Serine residues belonging to the SLIT and SNEE kinase consensus motifs in the C-terminal region result specifically phosphorylated. We have conducted a study in order to verify the *in vivo* role for Aurora A in Cry regulation. Analysis of single and double transgenic mutants for the residues target of phosphorylation seem to exclude a role for this sites in mediating Cry degradation after light exposure. However, experiments performed in flies over-expressing Aurora A show modifications in the circadian control of their rhythmic behaviour and display an increased degradation of Cry after light exposure. Taken together, these data suggest a putative implication of Aurora A in the regulation of Cry activity

Riassunto

La maggior parte degli organismi viventi sincronizza le proprie attività fisiologiche e comportamentali con l'alternanza di cicli luce-buio, che si ripetono con un periodo di circa 24 ore per effetto della rotazione terrestre. Ciò è permesso grazie ad un orologio endogeno, detto circadiano (dal latino *circa diem*): esso rappresenta l'esempio più significativo del controllo temporale dei processi biologici. Nei mammiferi controlla l'alternanza di sonno e veglia, la secrezione di ormoni e le fluttuazioni della temperatura corporea; nelle piante la fioritura e le attività foto sintetiche; nei cianobatteri i processi di fissazione dell'azoto e del metabolismo di alcuni amminoacidi; nei funghi la regolazione dei fenomeni di sporulazione; nel moscerino della frutta, *Drosophila melanogaster*, l'attività locomotoria e la schiusa pupale.
L'orologio circadiano è capace di percepire alterazioni di variabili ambientali e può sincronizzarsi in risposta a queste ultime, tuttavia è anche un sistema in grado di "autosostenersi" essendo attivo anche in assenza di stimoli esterni.
In *Drosophila*, evento fondamentale per la sincronizzazione dell'orologio in risposta alle variazioni ambientali è la degradazione in presenza di luce della proteina Timeless (Tim), questa è mediata dalla sua interazione con il fotorecettore della luce blu Cryptochrome (Cry). In seguito a fotoattivazione, Cry interagisce con Tim che viene fosforilata, ubiquitinata e degradata dal proteasoma. Successivamente, anche Cry viene degradata via proteasoma dopo esposizione alla luce ma il meccanismo che regola questo processo rimane ancora largamente sconosciuto.
Il lavoro qui presentato si divide in 2 parti, la prima è volta allo studio di un interattore molecolare di Cry e della sua caratterizzazione come componente dell'orologio circadiano, la seconda è volta all'analisi del ruolo di una chinasi nella regolazione e nella degradazione di Cry dopo esposizione alla luce.

Parte 1
Uno studio condotto nel nostro laboratorio volto alla ricerca di nuovi partner molecolari di Cry ha portato all'identificazione di Belle, una RNA elicasi ATP-dipendente. Questa proteina è espressa nelle cellule gliali del cervello di *Drosophila*, dove anche la proteina Cry è espressa, avvalorando l'ipotesi che vi sia un'interazione fisica tra le due. Esperimenti di Real-time PCR condotti su estratti di teste di adulti *wild-type* hanno evidenziato che l'espressione di questa RNA elicasi oscilla nelle 24 ore sia in alternanza di luce-buio (LD) sia in assenza di stimolo luminoso (DD). Inoltre, individui mutanti per il gene *belle*, mostrano un profilo locomotorio aritmico e difetti nell'espressione della proteina orologio Per in alcuni neuroni fondamentali per la generazione della ritmicità. Oltre a ciò, la caratterizzazione di linee in cui l'espressione del gene *belle* è ridotta ha evidenziato sia una riduzione della ritmicità circadiana negli adulti, sia un deficit a livello di risposta fotofobica dopo esposizione alla luce nelle larve terzo stadio, suggerendo che Belle sia richiesto per il normale funzionamento dell'orologio molecolare.
Nel complesso, i risultati ottenuti avvalorano l'ipotesi che la proteina Belle non sia solo un interattore del fotorecettore Cry ma anche un nuovo componente dell'orologio centrale.

Parte 2

È stato osservato che l'estremità C-terminale di Cry ha un ruolo predominante nel regolare le risposte alla luce in *Drosophila.* Infatti la rimozione di questo dominio sembra eliminare la necessità di luce per l'interazione con le proteine orologio Tim e Per. Mediante saggi *in vitro* condotti nel nostro laboratorio*,* è stato osservato che Cry viene fosforilata da Aurora A, una serin treonin chinasi coinvolta nel ciclo cellulare. Mediante spettrometria di massa sono stati individuati due siti di fosforilazione che corrispondono alla Serina 529, nella sequenza consenso SLIT, e della Serina 540, nella sequenza consenso SNEE, entrambi localizzati nella regione C-terminale.

Allo scopo di verificare l'effettivo ruolo della chinasi Aurora A nella regolazione dell'attività di Cry, è stata condotta un'analisi su linee linee transgeniche di *Drosophila* in cui i due residui sono stati mutati singolarmente o contemporaneamente. In queste linee è stata valutata la sensibilità alla luce in confronto alla linea selvatica. I risultati ottenuti non hanno evidenziato una sostanziale differenza a livello di sensibilità alla luce e ciò può essere dovuto al fatto che la variante mutata di Cry è espressa a livelli molto bassi se confrontata con la forma selvatica. Tuttavia, esperimenti condotti su linee che over-esprimono la chinasi Aurora A hanno evidenziato sia un incremento nella degradazione di Cry dopo esposizione alla luce continua, sia un deficit nella risposta circadiana a "pulse" di luce somministrati durante la notte soggettiva. Questi risultati suggeriscono che Aurora A potrebbe avere un ruolo nell'orologio circadiano ed in particolare nella regolazione dell'attività di Cry in seguito ad esposizione alla luce.

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INTRODUCTION

General introduction

An organism could optimize its behaviour and physiology by responding to daily and seasonal changes of the environment. In fact, an effect of the earth rotation is the variation of light, temperature, humidity and other physical parameters that occur with a period of about 24 hours. Yet virtually all organisms from Cyanobacteria to humans have evolved an internal circadian clock (from the latin words "circa dies") that allows them to anticipate daily environmental changes and alter their behaviour and physiology accordingly.
Biological rhythms originated probably in the earliest cells to protect DNA replication from damages caused by ultraviolet radiations during the daytime. Henceforth they evolved to increase the fitness of the organisms in their natural habitat (Beaver et al., 2002). For examples many flowers open and close in certain times of day and honeybees plan their visits to plants according to these cycles, improving the pollination (Sweeney, 1987).
Many daily rhythms are also present in humans. Among them the sleep/wake cycle is the most evident but circadian clocks in mammals have been shown to control also body temperature, hormonal release, rates of drug detossification, bone growth, liver regeneration and cell divisions (Gorbacheva et al., 2005; Fu at al., 2005).

The beginning of the circadian research

The existence of an endogenous clock was first reported by French geophysicist Jean-Jacques de Mairan in 1729 (de Mairan, 1729). Having observed a daily cycle of leaf opening and closing, he asked what would happen to this rhythm in the absence of environmental cues by moving the plant to his dark wine cellar. He found that leaves continued to show daily cycles of opening and closing even in constant darkness (DD), indicating the existence of an internal clock.
Many of the pioneering experiments on circadian rhythms were performed in Drosophila. For example, Colin Pittendrigh (Pittendrigh, 1954) demonstrated that the clock that drives rhythms in eclosion (hatching of adults from their pupal cage) runs with a 24 hors period, free-runs with a circadian rhythm in DD, can be reset by light delivered during darkness and was independent from temperature when the changes occurred in a range of about 10°C (temperature compensation).
It was the identification of the first clock gene mutants by Konopka and Benzer (Konopka and Benzer ,1971) in Drosophila that opened the door which ultimately led to a detailed molecular understanding of how intracellular clocks tic and how they are reset by light.

Main features of circadian rhythms

Circadian clocks have three fundamental properties:

- They persist under constant environmental conditions (such as constant temperature, darkness or light) with a period of about 24 hours, called *free running* period
- They can be entrained (or synchronized) by environmental stimuli called *Zeitgebers* (time givers), such as temperature, food, social interactions and light
- They are temperature compensated meaning that they proceed with almost the same free running period in the range of physiological temperatures (for most of the biological processes the reaction rate doubles when the temperature increases by 10°C).

In a schematic view, circadian clocks are characterised by three main components:

- An input pathway, which represents all the ways to perceive environmental stimuli and relay them to the central oscillator;
- The central pacemaker, an internal mechanism that generates the rhythmicity. It is composed of molecular elements which are expressed rhythmically with a period of 24 hours;
- An output pathway, which represents all the ways to influence and drive circadian phenotypes, such as behaviour or metabolism.

Circadian clocks are present in virtually all the cells of all the organisms. In eukaryotes, such as *Drosophila* and mammals, they are distributes in all tissues. However we can distinguish between pacemaker cells (in the brain) and peripheral clocks, expressed in other tissues of the body. The biological functions of many of the peripheral clocks is still unknown, but it is reported that they control the rhythmicity of specific tissues. Insects and mammals peripheral clocks are different: while in *Drosophila* they can perceive light stimuli and respond in a brain independent way, in mammals they are not permeable to the environmental stimuli, meaning that signals from the brain are the input cues of the peripheral clocks (Stanewsky, 2003).

Zeitgeber time and Circadian time

Studying the circadian clock is important to discriminate between Zeitgeber time (ZT) and Circadian time (CT). The main difference is the presence or the absence of external environmental cues able to entrain the core of the clock. In fact, ZT is based on the presence of a stimulus which synchronize the endogenous clock to the environmental changes. CT instead is not characterized by the presence of an external stimulus but it refers to constant light or temperature conditions. This condition is also called *free running*.
In experimental conditions of ZT, individuals are for example maintained in 12:12 light-dark (LD) cycles (12 hours of light and 12 hours of darkness) at constant temperature. In this case the Zeitgeber is the light. In a LD 12:12 cycle, ZT0 correspond to the light switch on, while ZT12 correspond to the light switch off. It is also possible to entrain organisms to temperature cycles: in this case, the light is kept constant while the temperature oscillates during the 24 hours.

In experimental conditions of CT, individuals experience constant regimes of light (or dark) and temperature. The canonical free-running conditions are constant darkness and constant temperature.

1. The input pathways

Different input pathways relay the circadian pacemaker to the external environment and synchronize the clock according to these changes.

1.1 Light entrainment

The capability of the clock to synchronize its rhythms with the environment represents an important feature for all the organisms. In a general sense the sensory task of photoentrainment is complex, but can be considered fundamentally the same for all organisms. Therefore, we should predict that the photo-entrainment pathway will show convergent features in highly divergent organisms.
The stable daily change in the environment provides the most reliable indicator of the time of the day. As a result, most organisms use changes in the quality and quantity of light around dawn and dusk as their primary Zeitgeber to effect what has come known as "photoentrainment" (Roenneberg and Foster 1997). Many studies on light effects on organisms pointed out that blue-light responses are conserved in all organisms, from cyanobacteria to plants and animals. Among them bacteria photoriactivation, plants phototropism and photomorphogenesis, cyanobacteria, insect and mammals circadian entrainment are the most relevant.

Cryptochromes

Cryptochromes are receptors for blue and ultraviolet light that share sequence similarity to DNA photolyases, DNA-repair enzymes that use blue light to repair UV induced DNA damage by removing pyrimidine dimers from DNA (Sancar 2003). Cryptochromes in general do not posses photolyase activity.
According to their sequence similarities, cryptochromes can be clustered into three subfamilies: plant cryptochromes, animal cryptochromes and cryptochrome-DASH proteins (CRY-DASH).
The first cryptochrome gene to be identified was *Arabidopsis* CRY1 (Ahmad and Cashmore 1993), and cryptochromes were soon found by homology in other plant species and in animals. Soon after the cloning of the first 6-4 photolyase from *Drosophila* (Todo et al., 1993; Todo et al., 1996), a related sequence was discovered in the human EST databases, that encodes human cryptochrome 1 (hCry1) (Todo et al.,1996; Hsu et al., 1996). Cryptochromes

have been found in various animal lineages, including insects, fish amphibians and mammals. Animal cryptochromes act as components of the circadian clock that controls daily behavioural and physiological rhythms and photoreceptors that mediate entrainment of the circadian clock to light (Cashmore 2003).
As mentioned before cryptochromes show structural similarity to DNA photolyases despite the fact that they do not possess photolyase activity. Most chryptochromes (but not CRY-DASH) are composed of two domains, an amino-terminal photolyase-related region (PHR) and a carboxy terminal domain (Todo 1999). The PHR region binds two cofactors: one is flavin adenine dinucleotide (FAD) and the other is the cromophore 5,10-methenyltetrahydrofolate (pterin or MTHF) (Lin et al., 1995; Malhotra et al., 1995) The carboxy-terminal domain of cryptochromes is generally less conserved than the PHR region. In fact it is longer in plant than in animal, and CRY-DASH lacks this domain.

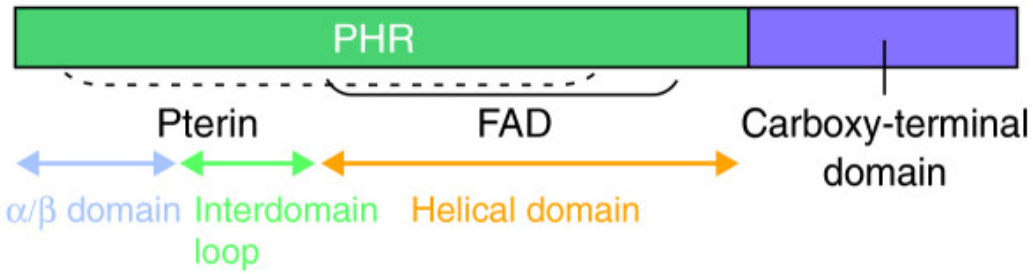


Figure 1: A schematic representation of a typical photolyase/cryptochrome protein. The parts of the PHR region bound by Pterin and FAD are indicated by brackets. From Lin and Todo, 2005.

Cryptochromes are ubiquitously expressed in the organs and tissues of all organisms, and they are generally nuclear proteins that regulate gene expression. The best studies animal cryptochromes are the *Drosophila* Cry and the animal mCry1/hCry1 and mCry2/hCry2 (Emery et al., 1998; Kume et al., 1999), and the Arabidopsis AtCry1 and AtCry2.
So far Cryptochromes signal transduction pathways are not fully understood, however it has been suggested that they are regulated by mechanisms such as redox state, phosphorylation and protein-protein interaction.
It is reported that Cryptochrome activation, such as photolyases, is due to an intramolecular electron transport in three Tryptophan residues (Trp triad) (Froy et al., 2002; Partch and Sancar, 2005; Zeugner et al., 2005). The conversion to Alanine of two Tryptophan residues in the "Trp triad" causes the loss of the light-dependent activity of dCRY (Froy et al., 2002; Partch and Sancar, 2005).
Phosphorylation has an important role in the regulation of the activity of Plant and animal cryptochromes. For example, the C-terminal region of both *Arabidopsis* Cryptochromes undergo to a light-dependent phosphorylation. In fact AtCRY1 is phosphorylated by the kinase activity of the photoreceptor Phytochrome A (PHYA) when exposed to red light (Ahmad et al., 1998) and AtCRY2 is phosphorylated when exposed to blue light (Shalitin et al., 2002). It was also demonstrated that AtCRY1 and human CRY1 (hCRY1) bind ATP and show auto-phosphorylation activity (Bouly et al., 2003; Partch and Sancar, 2005). Finally the

C-terminal phosphorylation of mammalian CRY2 (mCRY2) is a foundamental event for its regulation and oscillation (Harada et al., 2005).
Recent studies indicated that one photon absorption by Cryptochromes causes changes in protein complexes composition. For example, AtCRY1 form homodymers after light exposure (Sang et al., 2005). Moreover the general model of the signal transduction mechanism of Cryptochromes and photolyases suggests that flavin photon absorption is optimal if the protein binds a substrate in the dark (Sancar, 2003; Partch and Sancar, 2005).

Cryptochromes in plants

Plants life is strongly influenced by light. For this reason plants evolved a chromophores system which allows an optimal light perception (Dunlap, 1999).
Light perception is achieved by Cryptochromes and 4 types of phytochromes: PhyA, PhyB, PhyD, PhyE (Devlin and Kay, 2001). All of these molecules are able to perceive light within a specific light intensity and wavelength.
The first Cryptochrome isolated in plants was in *Arabidopsys thaliana* with the isolation of a light insensitive mutant (Ahmad and Cashmore, 1993). In *Arabidopsis* in fact, plants grown in dark show a reduced epicotyl compared to plants kept in light and this response is modulated by blue, red and infrared light. The mutant was named Atcry1 and the protein was affiliated to the flavoprotein family even if the photolyasic activity was lost (Lin and Todo, 2005). A second gene was then found (Atcry2), homolog to photolyases and a third gene (Atcry3) affiliated to the CRY-DASH superfamily, with transcription repressor activity (Brudler et al., 2003).
Genetical studies demonstrated that Cryptochromes interact with phytochromes in the regulation of the morphogenic development during flowering and photosynchronization of the circadian clock. This suggests that Arabidopsis Cryptochromes are not directly involved in the core of the clock but in the light signal transduction and in the input pathway (Salomè and McClung, 2004).
It appears that Cryptochromes control developmental changes in plants via changes of gene expression in response to light. CRY1 and CRY2 together are responsible for blue-light dependent changes in gene expression of up to 10-20% of the *Arabidopsis* genome (Ma et al., 2001). There are two mechanisms by which they may affect nuclear gene-expression changes in response to light: first, a cryptochrome molecule may interact with proteins associated with the transcriptional machinery to affect transcription directly; second, the plant cryptochrome may interact with proteins exerting other cellular functions to regulate the stability, modification and cellular trafficking of the transcriptional regulators.

Mammalian cryptochromes

Mammalian cryptochromes are predominantly nuclear proteins but they can also be found in the cytosol. The two genes *mcry1* and *mcry2* are expressed in almost all tissues. Mcry2 is strongly expressed in the retina and transcription of mcry1 is particularly present in the hypothalamus suprachiasmatic nuclei (SCN) where it oscillates (Miyamoto and Sancar, 1998).

Mammalian cryptochromes perform both light-dependent and light-independent functions in the regulation of the circadian clock. KO mice lacking one or both cryptochrome genes show a an impairment in the ability to induce expression of clock genes or the protoonco-gene c-fos in response to light (Thresher et al., 1998; Selbi et al., 2000). Moreover the pupils of mutant mice lacking both cry1 and cry2 have reduced reflex responses to light (van Gelder et al., 2003).

These observations indicate that the Cry proteins play an essential and light–independent function in the mammalian central circadian oscillator, but they are not the only photoreceptors mediating light control of the clock. In fact it has been shown that the mouse *cry* mutant retains its ability to mediate light input unless the function of the visual pigments is also disrupted at the same time (Selby et al., 2000).

Drosophila Cryptochrome

Drosophila Cry is a predominant nuclear protein that mediates regulation of the circadian clock by light (Emery et al., 1998; Stanewsky et al., 1998), although it can also be found in the cytosol (Ceriani et al., 1999). There is only one *cryptochrome* gene (d*cry)* in the fly genome, located on the third chromosome. This gene is rhythmically expressed, with a maximum at the beginning of the day (ZT1-6). This oscillation is completely abolished in clock mutants (Emery et al., 1998). dCRY protein levels oscillate only in LD cycles while in constant light the protein is rapidly degraded and in constant dark it accumulates continuously (Emery et al., 1998). These data suggest a double regulation for *cryptochrome*: a clock controlled gene expression and a post-transcriptional and post-translational control on the protein exerted by light.

d*cry* encodes a protein of 541 aminoacids with a high homology to plants and mammals cryptochromes.

The first *cry* mutant, cry^{baby} (cry^{b}), was identified by chemical mutagenesis in a screen for clock genes expression mutants in peripheral tissues (Stanewsky et al., 1998). The mutant phenotype was the disruption of the *per* and *tim* cycling under light-dark conditions (LD) in head extracts (mostly eye contribution). Since the molecular oscillations were normal when cry^{b} were entrained to temperature cycles, CRY was proposed to be required for the light entrainment of the cellular oscillators of the eye clock (Stanewsky et al., 1998). A missense mutation in the flavin-binding site rendered thus the CRY^{B} protein insensitive to light. Indeed

in cry^b mutants the behavioural rhythms were normal in both LD cycles and constant darkness (DD) (Stanewsky et al.,1998).
In agreement with unaffected molecular cycling in their clock neurons, the behavioural rhythms of cry^b mutants could be entrained by light-dark cycles, although they took more days than wild-type flies to adjust their activity to the new LD regimes when submitted to a shift in the LD schedule (Stanewsky et al., 1998; Emery et al., 2000). This suggest that cry^b flies were partially impaired for circadian photoreception.

In wild-type flies, short light pulses can reset the clock. A useful description of the phase-dependent response of the clock to light is the Phase Response Curve (PRC). Flies released under free-running conditions (ie: DD conditions) show different responses to light pulses given at different times of the subjective day or night, with the responses reflecting the status of the molecular clock at that precise time. Light pulses given during the subjective day do not affect the clock and this part of the PRC is thus called "dead zone". Brief light pulses given in the early subjective night phase delay the activity of the flies, as if the light phase of the day was prolonged. In contrast, light pulses given in the late subjective night phase advance the activity of the flies, as if the day was starting earlier. In *cry*^{b} mutants these responses to light stimuli were lost, their molecular clock was thus insensitive to short light pulses (Stanewsky at al., 1998; Emery et al., 2000).

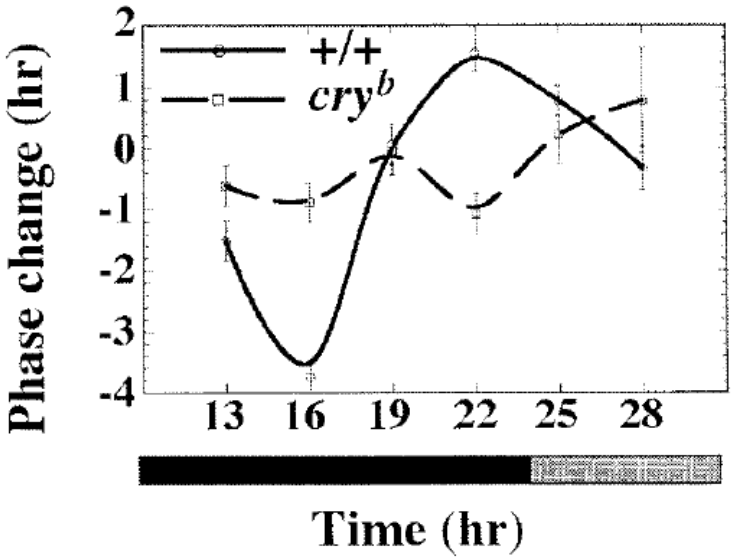


Figure 2: Phase Response Curve (PRC). In wild type flies (+/+) light pulses in early subjective night and in late subjective night phase delay and advance respectively the activity of the flies. *cry*^{b} mutants do not respond to short light pulses. Black bar: subjective night; grey bar: subjective day. From Stanewsky et al., 1998).

Wild-type flies become soon arrhythmic in LL, because of the constitutive activation of CRY by blue-light resulting in the absence of TIM accumulation. An important and unique characterized phenotype of the *cry*^{b} mutants was the persistence of a strong rhythmicity under constant light conditions (LL) (Emery et el., 2000).

Altogether, these results suggested that CRY was involved in light perception by the behavioural clock, although CRY-independent pathways exist for the entrainment by LD cycles through the visual system. In contrast, CRY appears to be the only circadian photoreceptor in the peripheral clocks.
Finally, cry^b mutants were also shown to display behavioural and molecular light-independent defects in peripheral clocks such as the antennae and Malpighian Tubules (Stanewsky et al., 1998).
It has been also reported that *Drosophila* Cry doesn't function only as a light responsive protein but also as a transcriptional repressor required for the correct specification of clock cells and for the oscillation of peripheral circadian clocks (Collins et al., 2006).

Recently, a study showed that Cry is also necessary for light-dependent magnetosensitive responses in *Drosophila melanogaster* (Yoshii et al., 2009 ; Gegear et al., 2008). Wild-type flies showed significant naive and trained responses to a magnetic field while Cry-deficient cry^0 and cry^b flies do not display this phenotype. (Gegear et al., 2008). The present data establish the circadian clock of *Drosophila* as a model system for CRY-dependent magnetic sensitivity. Furthermore, given that CRY is expressed in multiple tissues of *Drosophila*, including those potentially implicated in fly orientation, future studies may yield insights that could be applicable to the magnetic compass of migratory birds and even to potential magnetic field effects in humans

The circadian photoreception in *Drosophila melanogster*

Drosophila perceives the light inputs through three different pathways: by retinal photoreceptors (compound eyes and ocelli), by extraretinal photoreceptors (the Hofbauer-Buchner (HB) eyelet) and by the CHRYPTOCHROME (CRY) inner photoreceptor. The elimination of all of them resulted in a blind circadian system (Helfrich-Foster et al., 2001).

The compound eyes (the retinal photoreceptors of *Drosophila*) are constituted od about 800 ommatidia each. An ommatidium is an optical unit that contains 6 "outer photoreceptors" (R1-6) and 2 "inner photoreceptors" (R7-8) cells, able to catch photons. The R1-6 photoreceptors are involved in motion detection and image formation and project into the lamina. They mostly express rhodopsin 1 (Rh1), which is the opsin with the broader absorption spectrum. The inner photoreceptors are involved in color vision and project in the medulla. They express one of four different rhodopsins (Rh3, Rh4, Rh5 or Rh6). R7 contains the Rh3 or Rh4 (ultraviolet absorbing visual pigment) while R8 contains Rh5 or Rh6 (blue and green absorbing pigment respectively) with about 30% of y-type (Rh3 in R7, Rh5 in R8) ommatidia and 70% of p-type (Rh4 in R7, Rh6 in R8) ones (Montell et al., 1987; Zuker et al., 1987; Papatsenko et al., 1997; Pichaud et al., 1999; Wang and Montell, 2007).
When rhodopsin absorbs light, the isomerisation of the chromophore from the 11- *cis* to all-*trans* retinal, leads to the formation of an active metarhodopsine, which in turn activates G-

protein (Gq). A subsequent cascade of events involving Phospholipase Cβ (PLC), allows the opening of the Transient Receptor Potential (TRP) and the Transient Receptor Potential-Like (TRPL) cation channels (Montell and Rubin, 1989). This leads to the depolarization of the photoreceptor membrane and the consequent release of neurotransmitters.
Responses of the *Drosophila* behavioural clock to light have been shown to occur for wavelengths between 400 and 600 nm (Suri et al., 1998; Helfrich-Forster et al., 2002). Recently it was reported that the double mutants for the Rh1-encoding gene (neither inactivation nor afterpotential E, ninaE flies) (O'Tousa et al., 1985) and Rh6-encoding gene as well as mutants for PLC (no receptor potential A, norpA flies) (Bloomquist et al., 1988) were unable to entrain under red light-dark cycles (RD), suggesting that the compound eyes indeed might be involved in the phototransduction of red light (Hanai et al., 2008).
Another structure is located in the vertex of the fly's head: the three ocelli. They consist of about 70-90 photoreceptor neurons, which contain a thin layer of pigment cells expressing the Rh2 opsins, a violet light absorbing pigments (Pollock and Benzer, 1988). Their role in the circadian photoreception has not been determined.

The HB eyelet is an extra-retinal circadian photoreceptor located between the retina and the lamina in the fly's optic lobe (Hofbauer and Buchner, 1989). This particular light-percepting organ derives from the larval visual system or Bolwig Organ, which is composed of twelve photoreceptors: eight green sensitive (expressing Rh6) and four blue-sensitive (expressing Rh5) ones (Sprecher er al., 2007). During metamorphosis, all the Rh6-expressing cells die and the four Rh5-expressing larval photoreceptors switch from the blue to the green chromophore absorbing pigment, thus becoming the four photoreceptor of the adult H-B eyelet, expressing Rh6 (Sprecher and Desplan, 2008).
The four adult photoreceptors project directly to the aMe, where their axons contact the PDF-expressing LNvs (small ventral lateral neurons) (Hofbauer and Buchner, 1989; Yasuyama and Meinertzhagen, 1999; Helfrich-Forster et al., 2002, Malpel et al., 2002). Recently it has been shown that the synaptic transmission between the HB-eyelet and the LNvs is important to synchronize the small LNvs clock neurons (Veleri et al., 2007). The analysis of the action spectra of the H-B eyelet suggested thai it is sensitive to wavelengths of approximately 480nm, but it alone cannot provide strong entrainment to the circadian clock (Helfrich-Forster et al., 2002).

1.2 Temperature entrainment

The first real proof that temperature was an effective Zeitgeber for the circadian clock came late in the 1960s with the recordings of the synchronized circadian eclosion rhythms of *Drosophila pseudobscura* under DD conditions and temperature cycles (Zimmerman, 1969). Subsequently, the locomotor activity of *Drosophila melanogaster* was also found to be entrained under thermoperiodic conditions even when temperature changed only as little as 3 °C (Wheeler et al., 1993). Surprisingly, this rhythmicity persists when flies are placed in constant light, condition that normally render flies arrhythmic (Yoshii et al., 2002; Glaser and

Stanewsky, 2005). A chemical mutagenesis screen identified *nocte* (*no circadian temperature entrainment*) as a mutant for circadian temperature entrainment. *nocte* flies synchronized normally in LD cycles and were rhythmic in DD conditions, whereas they abolished temperature entrainment (Glaser and Stanewsky, 2005; Sehadova et al., 2009).
Another gene involved in temperature synchronization is *norpA (no receptor potential A)*. the protein encodes PLC, which is part of the phototransduction pathway in photoreceptors. The PLC had also a non –photic role in regulating the *per* gene alternative splicing, relaying the external temperature information to the molecular clock (Collins et al., 2004; Majercak et al., 2004).
The blue light photoreceptor CRY was also found to mediate circadian temperature responses in *Drosophila*. A heat-phase response curve was generated revealing that heat pulses could delay but not advance the clock phase and these responses were lost in the absence of CRY (Kaushik et al., 2007).
These results point out the importance of temperature for the entrainment of the clock. Daily light changes are followed by temperature changes and together they finely regulate the molecular clock and behaviour. Short cold days promote the efficient splicing of an intron in the *per* gene as well as light induction of *tim* transcription, which led to a rapid accumulation of the PER and TIM proteins and thus an earlier clock phase. Long warm days result in a less efficient splicing of *per* transcript and no induced *tim* transcription. The slow accumulation of PER/TIM complexes induces a later clock phase (Cheng et al., 1998; Majercak et al., 1999; Majercak et al., 2004). Recent studies also reveal regulatory mechanisms based on alternative splicing also for the *tim*'s gene transcript, where cold temperature promote the transcription of truncated TIM protein (TIM^{COLD}) that could regulate the period length of the behavioural clock (Boothroyd et al, 2007).

2:the central clock of *Drosophila melanogaster*

The first negative feedback loop

The first negative feedback loop is based on the *per*, *tim*, *clk* and *cyc* genes and their products. CLK and CYC are transcriptional factors that contain the basic-helix-loop-helix (bHLH) DNA-binding domain and PER-ARNT-SIMS (PAS) protein-protein interaction domains (Huang et al., 1993; Allada et al., 1998; Rutila et al., 1998). From midday to early night, CLK/CYC heterodimers recognize specific binding sites for the bHLH domains (CACGTG), called E-boxes, in *per* and *tim* genes' promoters, to activate their transcription (Darlington et al., 1998). *per* e *tim* mRNA start to accumulate with a peak early in the evening (Hao et al., 1997; McDonald et al., 2001; Wang et al., 2001). However, the peak of PER and TIM proteins does not occur until late in the night, about 4-6 h after *per* and *tim* mRNA peaks. This temporal delay is due to posttranslational mechanisms, such as protein phosphorylation and ubiquitination that promote their degradation.
In the cytoplasm, PER and TIM form heteodimers (Lee et al., 1996; Zeng et al., 1996; Meyer et al., 2006) and move into the nucleus where they repress the positive transcriptional activity

of CLK/CYC, by inducing CLOCK protein phosphorylation and the removal of the complex from the DNA (Yu et al., 2006). This results in the decrease of *per* and *tim* mRNA transcription. TIM first and then PER are degraded, which allows the CLK/CYC activators to start the cycle anew. Recent studies have demonstrated that PER and TIM enter into the nucleus independently (Shafer et al., 2002; Meyer et al., 2006), but *in vivo* and *in vitro* experiments indicated that TIM alone is not sufficient to repress the CLK/CYC activity, while nuclear PER can negatively regulate *per* and *tim* transcription (Rothenfluh et al., 2000; Chang and Reppert, 2003). A new bHLH transcription factor, CLOCKWORK ORANGE (CWO) has been recently isolated, whose loss or downregulation induces a lengthening of the behavioural period (Kadener et al., 2007; Lim et al, 2007; Matsumoto et al., 2007; Richier et al., 2008). CWO is required for robust *per* and *tim* RNA cycling but its exact function in the transcriptional loop remains unclear.

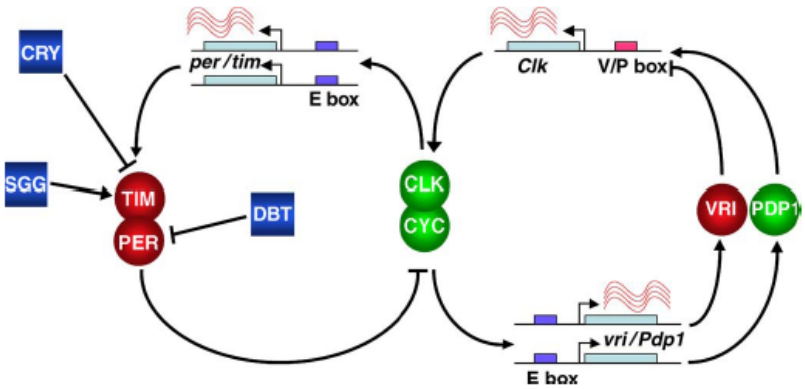


Figure 3: Interlocked feedback loops in Drosophila melanogaster. From Collins and Blau, 2007.

The second interlocked feedback loop

An additional regulation on the activity of the CLK/CYC complex occurs through the regulation of the *clock* gene transcription. In the second feedback loop, CLK/CYC heterodimers bind the E-boxes of the *vrille* (*vri*) and *PAR domain protein 1 e* (*Pdp1e*) genes. VRI and PDP1e are two basic leucine zipper (bZip) transcription factors of the *clock* gene, identifies as a repressor and an activator, respectively. As a consequence of the transcriptional regulation of their genes, VRI and PDP1e proteins accumulate at different times: first VRI inhibits the transcription of the *clock* gene; then only 3-6 h later, PDP1e starts to accumulate, thus promoting the expression of the *clock* mRNA (Cyran, 2003). The abundance of the clock mRNA thus shows circadian cycling, with a peak that is in antiphase with the one of the per and tim mRNA and in phase with the maximum amount of PER and TIM proteins (Allada et al., 1998; Bae et al., 1998; Darlington et al., 1998; Rutila et al., 1998). Recently the role of PDP1e in the second feedback loop was questioned. Benito et al., suggested that PDP1e has a role in regulating the behavioural output of the circadian oscillator and not in the core clock itself (Benito et al, 2007).

2.2 post-transcriptional and post-translational mechanisms

One important feature of circadian oscillators is their ability to generate self-sustained 24h rhythmicity. As described above, they are based on interlocked feedback loops in which clock proteins negatively regulate their own gene transcription. Therefore, a delay between gene expression cycling and protein cycling is required to maintain the oscillator dynamic.
Different molecular mechanisms have evolved to avoid the steady state and adjust the period and the amplitude of the rhythms according to the external environment. Rhythmic clock gene expression is important to generate rhythmic protein accumulation, but several lines of evidence suggest that cycling protein level do not depend only on cycling mRNA levels (Cheng et al., 1998; Yang and Sehgal, 2001; Grima et al., 2004; Harms et al., 2004). Indeed, posttranscriptional and mostly posttranslational mechanisms have evolved to generate proteins' cycling and adjust the proper pace of the clock.

Posttranscriptional modifications

In *Drosophila melanogaster*, the *per* gene is regulated by alternative splicing. Two different *per* mRNA were found to be involved in the seasonal adaptation of the flies: in warm days the inefficiency of *per* splicing and the subsequent slow *per* mRNA accumulation led the flies to prefer a nocturnal activity (delayed evening activity), thus avoiding the hot midday sun. In contrast, cold temperature promoted splicing and faster PER accumulation, inducing an advance of the evening activity. How splicing efficiency regulates the accumulation of PER protein remains unclear since the encoded proteins are the same (Collins et al., 2004; Majercak et al., 2004).

Posttranslational modifications

Phosphorylation, dephosphorylation and ubiquitination are fundamental mechanisms to control activity and cycling of the circadian transcription factors.

Delay in the accumulation of PER and TIM proteins
The time lag of about 4-6 h between the *per* and *tim* mRNA peaks and the accumulation of the PER and TIM proteins in the cytoplasm, results from the phosphorylation of PER by Casein Kinase I epsilon homologue DOUBLE-TIME (DBT) (Kloss et al., 1998; Price et al., 1998). Casein Kinase II (CKII) also affects PER phosphorylation and seems to require TIM to do so (Lin et al., 2002; Akten et al., 2003; Lin et al., 2005; Meissner et al., 2008; Smith et al., 2008). A model describing the delay between transcription of *per* and *tim* genes and accumulation of the encoded proteins, predicts that the new synthesized PER monomers are rapidly phosphorylated by DBT and then degraded until TIM protein reaches a threshold concentration that allows the formation of the PER/TIM complexes (Kloss et al., 1998). The

TIM binding likely prevents DBT-dependent phosphorylation of PER (Kloss et al., 2001). TIM stability is controlled by CKII, which regulates its abundance (Meissner et al., 2008)., whereas the Glycogen Synthase Kinase 3b ortholog SHAGGY (SGG) controls TIM nuclear lacalization (Martinek et al., 2001).
PER and TIM proteins can be also dephosphorylated and thus stabilized by the protein phosphatase 2A (PP2A) and by the protein phosphatase 1A (PP1A) respectively (Sathyanarayanan et al., 2004; Fang et al., 2007). Once phosphorylated in the cytoplasm, PER (and probably TIM) is recognized by Supranumerary Limbs (SLMB), a F-box/WD40 repeat E3 ubiquitin ligase protein, that promotes its degradation via the proteasome pathway (Grima et al., 2002; Ko et al., 2002; Chiu et al., 2008).

Nuclear translocation and turnover regulation of the PER/TIM complex
As described above, DBT, CKII and SGG kinases act in the cytoplasm to phosphorylate PER and TIM. In doing so they control also the translocation of the PER and TIM proteins into the nucleus. Phosphorylation of PER and TIM also regulate the timing of their nuclear entry. Once in the nucleus PER, phosphorylated by DBT, represses the activity of the CLK/CYC complex and is then degraded (preceded by TIM) via the proteasome pathway (Cyran et al., 2005; Kivimae et al., 2008).
An accurate balance between kinases and phosphatases thus appears to regulate the nuclear translocation and turnover of PER and TIM.

CLOCK regulation
The *clk* mRNA also cycles in a circadian manner (Bae et al, 1998; Darlington et al., 1998). In addition, a rhythm in the phoshorylation state of the CLK protein was revealed recently (Kim and Edery, 2006; Yu et al., 2006). The kinase DBT and the protein phosphatase PP2A play an important role in the regulation of the phosphorylation of the CLK protein in the nucleus. The balance between the hyper- and hypo- phosphorylated states appears to regulate the total levels of CLK and the transcriptional activity of the CLK/CYC complex (Kim and Edery, 2006). In the evening, the hypo- phosphorylate CLK is associated with DNA and is temporally correlated with the increase of *per* and *tim* transcripts, thus suggesting that it is the active form of the protein. In contrast, the hyper- phosphorylated for of CLK is unbound to DNA and is temporally correlated with decreasing *per* and *tim* mRNA levels. It has been proposed that the phosphorylation of PER by DBT induces the phosphorylation of CLK and its unbinding from DNA (Yu et al., 2006).

Light-dependent TIM degradation

Post-translational mechanisms, such as phosphorylation and ubiquitination, are also involved in setting the phase of the molecular clock according to the external light-dark cycles. TIM is the key clock protein that connects the molecular clock to the external light inputs. In the presence of light, CRY undergoes a conformational change that induces its binding to TIM and sends the latter to proteasomal degradation pathway. CRY transmits light information to

the clock through light-dependent interaction with the core clock protein TIM (Ceriani et al., 1999) and possibly PER (Rosato et al., 2001). The CRY-TIM interaction involves another protein called JETLAG (Koh et al., 2006) which is also involved in the light dependent degradation of CRY (Peschel et al., 2009). It has been demonstrated that this enteraction is mediated by the carboxy-terminus of CRY protein. This has been demonstrated using CRYΔ which laks the C-terminal end (approximately last 20 amino acids). CRYΔ is constitutively active and binds PER and TIM in light and dark (Dissel et al., 2004). This C-terminal region of CRY is extremely diverse between species while the N-terminal region is more conserved.

2.3 Anatomical organisation of the central clock

The circadian clock of *Drosophila melanogaster* that controls locomotor activity rhythms is located in the brain and relies of about 70 clock neurons for each brain hemisphere. These clock neurons are bilaterally clustered in two main groups: the Dorsal Neurons (DN) that lay in the dorsal protocerebrum and the Lateral neurons (LN) that lay between the optic lobe and the central brain (Zerr et al., 1990; Ewer et al., 1992; Frisch et al., 1994). According to their location, morphology or properties, the neurons of these two clusters can be further sub-divided respectively in three different groups: the Dorsal Neurons in DN1s, DN2s and DN3s; the Lateral Neurons in small Ventral Lateral neurons (s-LNvs and the 5th s-LNv), the large ventral Lateral Neurons (l-LNvs) and dorsal Lateral neurons (LNds). Recently, an additional group of lateral neurons was found in the posterior brain, called the lateral Posterior Neurons (LPN) (Kaneko and Hall, 2000; Yoshii et al., 2005; Shafer et al., 2006; Helfrich-Forster et al., 2007).

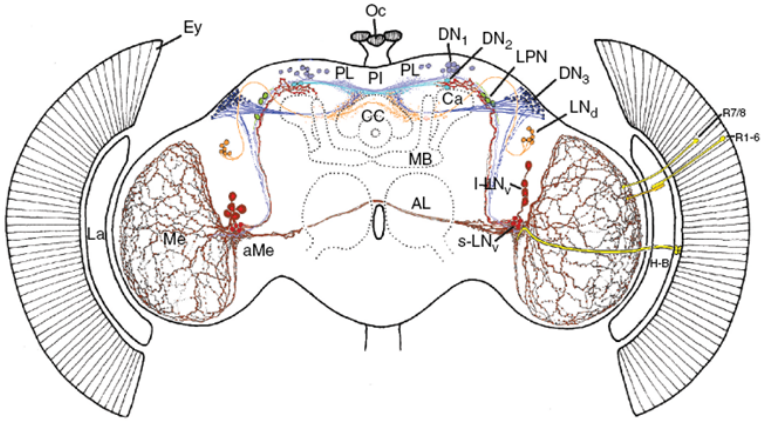


Figure 4: Schematic representation of Drosophila brain. From Helfrich-Forster et al., 2007)

Clock neurons and rhythmic behaviour

Small ventral Lateral Neurons (s-LNvs and the 5th s-Nvs)

Four s-LNvs are located between the optic lobe and the central brain and express the neuropeptide Pigment Dispersing Factor (Helfrich-Forster, 1995; Helfrich-Forster, 1997; Kaneko et al., 1997; Shafer et al., 2006). They remain ipsilaterally and project toward the dorsal protocerebrum, near the DN2s. They arborize in the central accessory medulla (aMe).
The 5th s-LNv is located a bit more dorsally, near the l-LNvs and it is the only ventral lateral neuron that does not express PDF (Helfrich-Forster, 1997; Kaneko et al., 1997; Shafer et al., 2006). Ir projects toward the aMe where it arborizes in the central part of the aMe and sends projections to the dorsal part of the brain (Helfrich-Forster et al., 2007).

Large ventral Lateral Neurons (l-LNvs)

Four to six PDF-expressing l-LNvs lay dorsally to the s-LNvs (Helfrich-Forster, 1995; Helfrich-Forster, 1997; Kaneko et al., 1997; Shafer et al., 2006). They send a projection towards the aMe, where it makes a ventral elongation (v aMe) and then arborizes in the Medulla. These neurons also send projections to the controlateral hemisphere, through the Posterior Optic Tract (POT). This projection arborizes in the controlateral Medulla (Helfrich-Forster et al., 2007).

Dorsal Neurons (DNs)

The dorsal neurons can be divided in three different clusters.
In the adult brain there are about 14-16 DN1s neurons per hemisphere that project toward the dorsal protocerebrum, with the projections running to the controlateral side of the brain and forming the dorso-anterior commissure. (Kaneko and Hall, 2000; Helfrich-Forster, 2003). Some of them also send fibres down to the aMe, close to the dorsal projections of the PDF-expressing s-LNvs. Two of the DN1s are located in the anterior dorsal brain, separated from the others. These two cells are called $DN1_{anterior}$ (DN1a) and conversely to the other DN1s, do not express the GLASS transcription factor. These neurons express the neuropeptide IPN-amide (Hamasaka et al., 2007). Similarly, two other DN1s located in the posterior brain were named DN1posterior (DN1p). both DN1a and DN1p project ventrally to the ipsilateral accessory Medulla (Kaneko and Hall, 2000; Klarsfeld et al., 2004; Shafer et al, 2006; Helfrich-Forster et al., 2007).
The two DN2s cells lie close to the terminals of the s-LNvs. Their projections were identified by the analysis of *glass* mutants, in which the GLASS-expressing DN1s were removed. This study revealed that the DN2 also participate to the dorso-anterior commissure, sending their projections to the controlateral side of the brain.
The last group of dorsal neurons, the DN3s, are located in the lateral superior protocerebrum. This cluster of cells consists of about 40 clock neurons per hemisphere tha extend their projections below the DN1 commissure in the dorsal part of the brain (Kaneko and Hall, 2000; Shafer et al., 2006; Helfrich-Forster et al., 2007).

Lateral Posterior Neurons

Three neurons located in the posterior part of the lateral brain (LPN) were first identified as only TIM-expressing cells. Other studies then demonstrated that these are PER and TIM expressing neurons, therefore they are now considered as clock neurons that appear to be

involved in temperature entrainment (Kaneko and Hall, 2000; Yoshii et al., 2005; Shafer et al., 2006). Their projections' pattern remains unknown.

Besides the clock neurons, the drosophila brain also has PER-expressing glial cells (Zerr et al., 1990; Helfrich-Forster, 1995). These cells are localized close to the sLNvs, LNds, DN1s and DN3s. A recent study highlighted the important contribution of these cells in modulating the rhythmic output that is driven by the clock neurons (Suh and Jackson, 2007).

3: The output pathway

The identification of the neurotransmitters that modulate the circadian behaviour of *Drosophila melanogaster* is required to understand the outputs of the clock and the functioning of the neuronal circadian network. Glutamate and a few neuropeptides like PDF, neuropeptide F (NPF) and the neuropeptide precursor-like protein 1 (NPLP1) were found to be expressed in some particular clusters of clock cells. Only the role of the PDF neuropeptide is starting to be characterized.

Glutamate

Vescicular glutamate transporters (DvGluT) were recently found to be strongly expressed in the DN1a and some DN1s and DN3s cells, while the metabotropic glutamate receptor (dmGluRA) was detected in the small LNvs (Hamasaka et al., 2007). Behavioural locomotor analysis of flies in which DMGluRA expression was severely affected in the LNvs, modified their activity pattern in LD (increase of dark activity) and DD conditions (lengthened free running period). It is thus possible that the DN1s influence the activity output of the brain oscillator via glutamate release (Hamasaka et al., 2007).

Neuropeptide precursor-like protein 1 (NPLP1)

The neuropeptide NPLP1 was moderately detected in many neurons of the CNS and strongly expressed in the DN1s (Shafer et al., 2006). No receptor or any potential role have yet been identified.

Neuropeptide F (NPF)

The NPF, structurally and functionally related to the mammalian neuropeptide Y (NPY), is expressed in some endocrine cells and in the male Drosophila brain (Lee et al., 2006). Among the clock neurons, it is expressed in three LNds: one CRY-positive LNd and two CRY-negative LNds (Yoshii et al., 2008). The role of the neuropeptide in the circadian circuit is not clear. The sex-controlled expression of te neuropeptide suggested that it could be involved in the regulation of the sexually dimorphic profile of the rest-activity behaviour in flies (Lee et al., 2006).

Pigment Dispersing Factor (PDF)

The PDF neuropeptide was found to be expressed in the small and large LNvs exclusively, but not in the 5th s-LNv (Helfrich.Forster, 2005; Park and Hall, 1998). The analysis of the behavioural activity of the pdf^{0} flies (null allele) revealed that PDF is a key component of the circadian clock output. Under LD conditions, flies depleted for PDF were still entrained by light-dark cycle but they did not anticipate the lights-ON transitions and the evening activity bout was phase-advanced by at least 2 hours. In DD conditions, flies were largely arrhythmic or displayed very weak free-running rhythms with a short period of about 22 h (Renn et al., 1999).

The *pdf* gene was suggested to be positively regulated by the CLK and CYC transcriptional factors indirectly (Park et al., 2000). Although no *pdf* mRNA oscillations were detected in the LNvs neurons, a PDF rhythm was observed in the dorsal axonal terminals of the small LNvs (Park et al., 2000). In early mornins, higher degree of arborizations and axonal processes of the s-LNvs projections were presented in the dorsal protocerebrum, whereas during the night, only few branches could be clearly distinguished. These rhythms persisted in LD and DD conditions and were abolished in *per*^{0} and *tim*^{0} mutants, demonstrating the role of the circadian clock in controlling the synaptic plasticity of these clock neurons (Park et al., 2000 ; Fernandez et al., 2008).

State of the art

Drosophila melanogaster Cryptochrome (dCRY) is the blue light photoreceptor involved in the photic input pathway to the circadian clock. It mediates the resetting of the circadian molecular oscillation by light, a foundamental step in circadian rhythmicity, by binding to Timeless (dTIM) and targeting it to degradation via proteasome (Ceriani et al., 1999 ; Naidoo et al., 1999 ; Lin et al., 2001). The activation of dCRY by light could require a conformational change or the release of a putative repressor (Rosato et al., 2001). In fact, we previously hypothesized that the activation of dCRY by light is mediated by specific "regulators" that bind the C-terminus of the protein, which has been proven to regulate the light-dependence of dCRY activity. This hypothesis was supported by the observation that the C-terminus of dCRY is an hotspot for molecular interactions: in fact, by *in silico* analysis and experimental validation we could identify, in this small region, several protein-protein interaction motifs, such as PDZ and TRAF2, known to be present in proteins that participate in several processes, as DNA binding, signal transduction, protein–protein interaction and targeting proteins for proteasome-mediated degradation (Hemsley et al., 2007).
In both plants and animals, phosphorylation plays an essential role in regulating cryptochrome activity. In *Arabidopsis thaliana*, both AtCRY and AtCRY2 are phosphorylated and activated upon a light stimulus and in AtCRY2 this modification occurs specifically at the C-terminus (Shalitin et al., 2002, 2003) In mammals, the activity of mCRY1 and mCRY2 is regulated by CKIE and MAPK respectively mediated phosphorylation and moreover the phosphorylation of mCRY2 occurs at the C-terminus and in a circadian manner (Eide et al., 2002; Sanada et al., 2004; Harada et al., 2005).

A study was initiated in our laboratory in order to:
1) identify possible partners of dCRY under light/dark conditions
2) investigate about a possible role of phosphorylation as post-translational modification that regulates dCRYactivity

1) Identification of dCRY partners
In order to find new molecular interactors of dCRY and better understand dCRY regulation mechanism by light, an immunoprecipitation approach was used. In particular head protein extracts from transgenic flies expressing HACRY in *tim* positive cells were subjected to a Co-Immunoprecipitation assay by using an anti-HA antibody, the immunocomplexes separated by SDS-PAGE and gel bands corresponding to the putative interactors were analyzed by mass spectrometry. This approach was followed for individuals maintained both in the dark and light conditions and results are reported in figure 1.

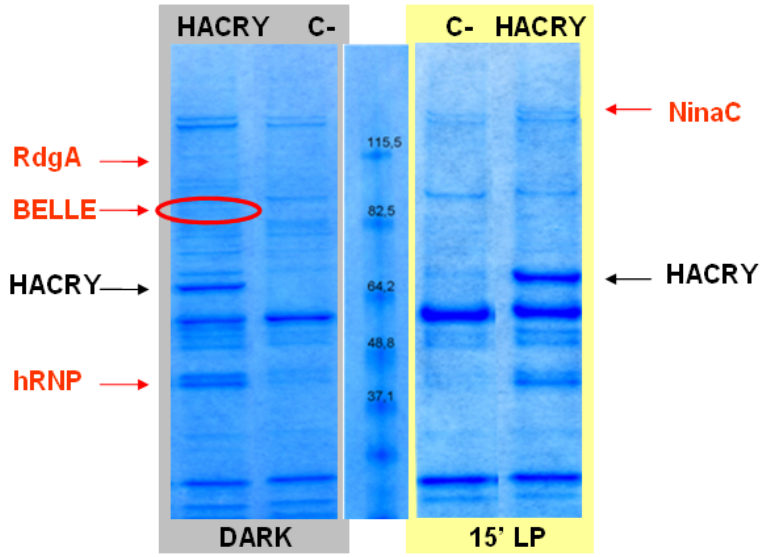


Figure 1: HACRY immunoprecipitation in the dark and after 15' of light exposure. Putative HACRY interactors are highlighted in red.

This strategy led to the identification of 3 interactors in the dark, corresponding to the proteins Belle, Retinal degeneration A (RdgA) and heterogeneous RiboNucleoProtein (hRNP) and one interactor in the light, corresponding to the protein Neither Inactivation Nor Activation C (NINAC).

The protein Belle (an RNA helicase) was chosen for further analyses because it is reported that another RNA helicase, FRH (Frequency-interacting RNA Helicase), forms a complex with the protein FRQ (Frequency), an essential component of the circadian oscillator in *Neurospora crassa* (Cheng et al., 2005). The two proteins share both the RNA helicase ATP-dependent domain and the DEAD BOX domain, and this is very conserved for proteins involved in RNA molecule metabolism. In *Neurospora*, the FRH/FRQ complex is predominantly express in the cytoplasm and is a fundamental element for the transcriptional/translational feedback loop. Moreover FRH was also reported to form a complex with White Collar-1 (WC1) and White Collar-2 (WC2) proteins (Hunt et al., 2010; Guo et al., 2010; Shi et al., 2010; Cheng et al.,2005). In particular, WC1 is the blue light photoreceptor in *Neurospora* (Froehlich et al., 2002).

RNA helicases are highly conserved enzymes that use ATP to bind or remodel RNA or ribonucleoprotein complexes (RNPs) (Figure 2) (Tanner and Linder, 2001). They are found in all kingdoms of life (Anantharaman et al., 2002).

Based on sequence and comparative structural and functional analyses, all helicases are classified into six superfamilies (SFs) and all eukaryotic RNA helicases belong to SFs 1 and 2. Eucaryotic RNA helicases display a conserved helicase core surrounded by C- and N-terminal domains (Zhang and Grosse, 2004; Cui et al., 2008). C- and N-terminal domains are thought to be crucial for the cellular specificity of helicases, as they facilitates the interactions with other proteins or recruitment of the proteins to specific complexes(Lattmann et al., 2010; Karginov et al., 2005).

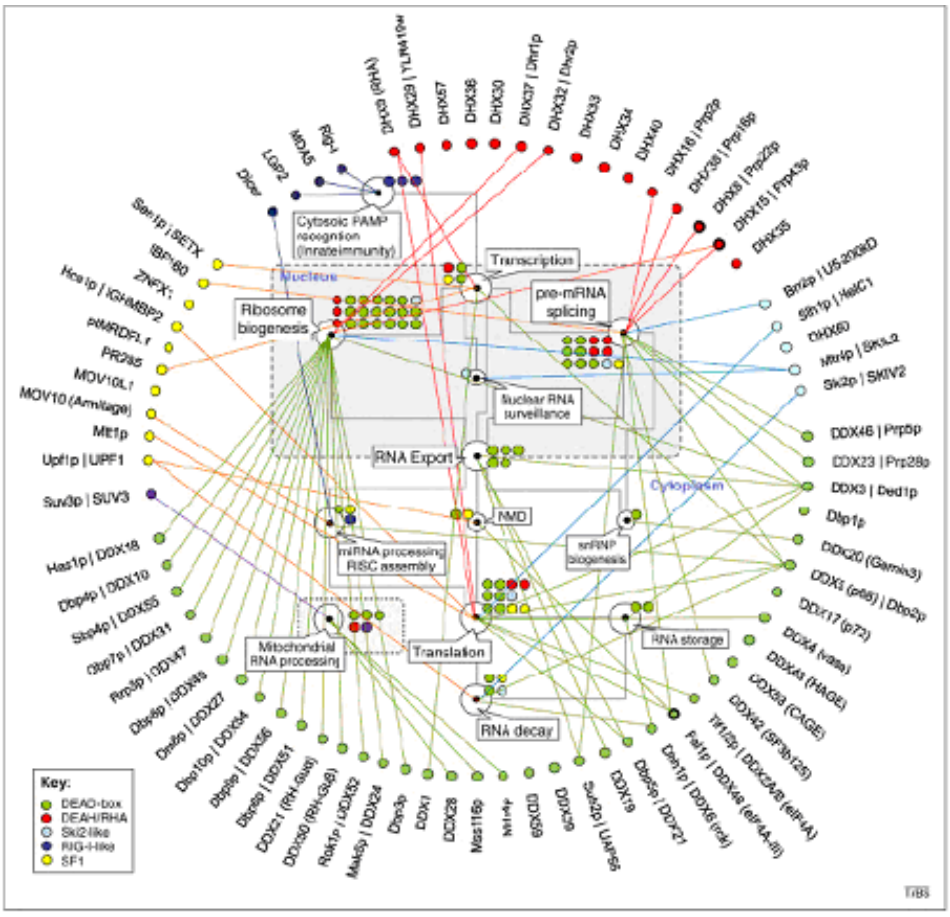


Figure 2: cellular roles of eukaryotic RNA helicases.

Belle is classified as a DEAD-box RNA helicase. DEAD-box proteins are defined through the conservation of multiple different motifs including the D–E–A–D (Asp–Glu–Ala–Asp) motif for which they were named (Linder et al., 1989). Several members of this family have been demonstrated to function as ATP-dependent RNA helicases, capable of unwinding double-stranded RNA (Tanner and Linder, 2001) and to have a role as RNPases, acting to remodel RNA–protein interactions (Fairman et al., 2004; Jankowsky et al., 2001; Linder et al., 2001). Moreover they have been linked to every stage of RNA processing including splicing, ribosome biogenesis, nuclear export, degradation, translation and specific regulatory events. For one highly conserved subfamily, which includes members in yeast (Ded1p), Xenopus (An3), mice (PL10), and humans (DDX3), data suggest both a general cellular requirement as well as a developmental regulatory function.

Drosophila protein Belle, was assigned to the Ded1p subfamily of DEAD-box proteins. It is broadly expressed and is essential for viability and organismal growth. In fact, strong mutations in Belle cause lethality by larval growth arrest. Like its mammalian homologues, Belle is required for male fertility. In addition, Belle is also required for female fertility in *Drosophila* and localizes in nuage and at the oocyte posterior (Johnstone et al., 2005). Moreover, Belle is reported to be a factor that coordinate splicing and translational regulation (Worringer et al., 2009).

Another study-based on a screen to find genes required for RNAi in *Drosophila* S2 cells, Belle was identified to be a component of the RISC machinery and to interact with the protein Argonaute 2 (Ulvila et al., 2006).
The aim of this study was the characterization of the protein Belle as Cryptochrome interactor and a putative new clock component in *Drosophila*.

2) dCRY phosphorylation upon light activation
In order to characterize dCRY regulation by light, a second study was initiated to understand whether dCRY is phosphorylated upon light activation.
The search for phosphorylated forms of dCRY was performed by mono- and bidimensional electrophoresis separation of enriched samples of dCRY protein and subsequent mass spectrometry analysis. Although this strategy has led to unsuccessful results, *in vitro* kinase assays were performed. HACRY was immunoprecipitated from head extracts of flies overexpressing the fusion under control of *timeless* promoter. An *in vitro* assay was performed on HACRY using human Aurora A. Samples were subjected to two-dimensional (2D) electrophoresis and results showed that the presence of human Aurora A increases the phosphorylation of HACRY by 3 folds (Figure 3). This clearly indicates that HACRY is a substrate for Aurora A *in vitro*.

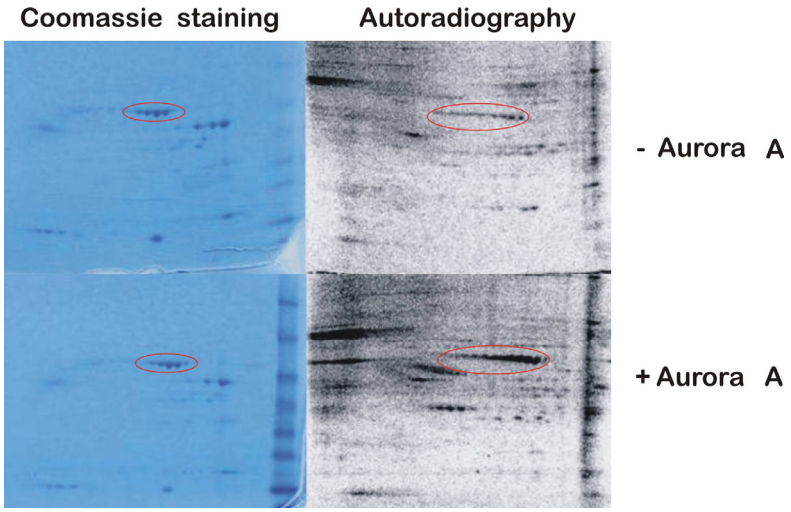


Figure 3. HACRY is substrate for Aurora A *in vitro*

Aurora-A was discovered in a screen for Drosophila mutations affecting the poles of the mitotic spindle function (Glover et al., 1995). The Aurora kinases are importantly involved in cell cycle and they localize to centrosomes, spindle poles, spindle from prophase to metaphase but predominantly to the spindle during telophase (Bischoff et al., 1998). In the centrosomes they play a role in bipolar spindle assembly and maturation of duplicated centrosomes (Portier et al., 2007). Aurora kinases are involved in multiple functions of mitosis. In particular, Aurora A is involved in mitotic entry, separation of centriole pairs, accurate bipolar spindle assembly, and alignment of metaphase chromosomes and completion of cytokinesis (Marumoto et al, 2003).

Aberrant expression of Aurora kinases may disturb checkpoint functions particularly in mitosis and this may lead to genetic instability and trigger the development of tumors. In many tissues, Aurora A kinase over-expression leads to genetic instability (aneuploidy), which may cause cancer. Aurora A gene was first named as BTAK (Breast Tumor Activated Kinase), because its mRNA is found to over-expressed in breast tumors and plays a critical role in breast tumor cells transformation (Sen et al., 1997). In the recent years, Aurora A kinases have gained much attention since they were identified as *bona fide* oncogenes (Katayama and Sen, 2010)

A mass spectometry analysis was performed in order to identify those HACRY residues specifically phosphorylated by Aurora A. HACRY spots obtained from 2D gel were subjected to digestion with LysC, Trypsin and Chymotrypsin. The tryptic peptide mixtures were analyzed by mass spectrometry (NanoLC-MSMS) and the generated spectra were characterized with MASCOT algorithm, that uses mass spectrometry data to identify proteins and determine phosphorylation status of target peptides from primary sequence databases (www.matrixscience.com). 65% of HACRY sequence was covered by this analysis (in red in Figure 4).

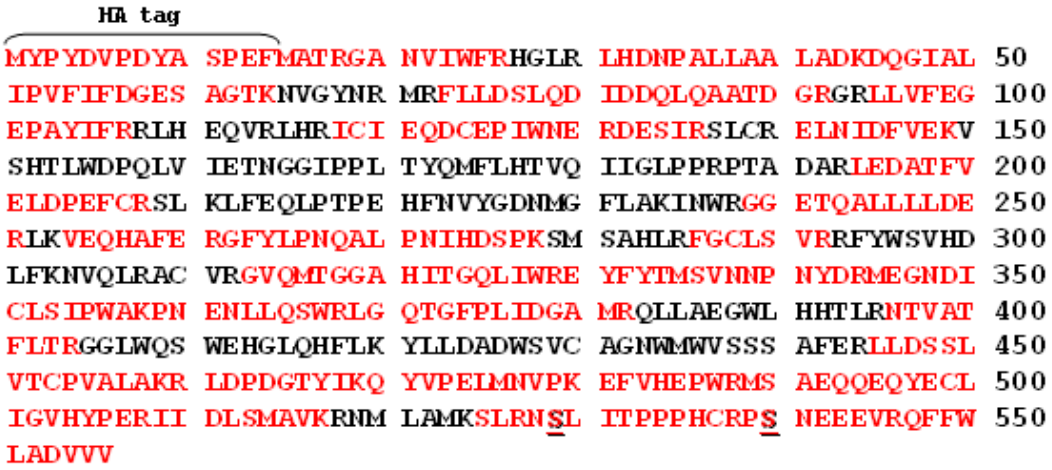


Figure 4. HACRY sequence covered by NanoLC-MSMS analysis

Mass spectrometry analysis indicated that Aurora A phosphorylates the HACRY C-terminus at the level of Serine 529, in the SLIT kinase target consensus, and Serine 540, in the SNEE kinase target consensus. The analysis revealed that Serine 529 is a preferential target for Aurora A, as the phosphorylation occurs with a ten fold higher stoichiometry compared to that of Serine 540. These data were confirmed by *in vitro* kinase assays performed on synthetic peptides with conserved phosphorylation consensus and peptides where Serine 529 or Serine 540 were replaced by Alanine. Results clearly showed that mutating specifically these residues the phosphorylation was abolished.
For all these reasons, the aim of this study was to investigate the role of Aurora A kinase in dCRY activity regulation.

MATERIALS AND METHODS

5.1 Fly Stocks and Maintenance

Flies were raised on a standard yeast–glucose–agar medium (Roberts and Standen 1998) and were maintained at 18° C, 70% relative humidity, on a 12h light: 12h dark cycle.

5.2 Fly strains

***w*^{1118}** : this fly strain was used as control strain (*wild-type*). It is characterized by:

- white (w, 1-1.5), recessive allele caused by a deletion in the white locus which determines the "white eyes" phenotype; the gene is implicated in the prosuction and distribution of homocrome pigments (brown) and pteridine pigment (red) present in the compound eyes and ocelli of the adult fly

UAS*belle*RNAi: this line was used to induce (by crossing with a Gal4 driver) specific silencing of the *belle* gene. A 325bp RNAds recognizes and binds to *belle* coding region at position 1660, causing specific *belle* mRNA degradation. This line is available on Vienna Drosophila RNAi Center (VDRC) (#6299).

UAS*aurora*GFP (#0466, #0467): this transgenic lines were generated as described in Berdnik and Knoblich, 2002. The Aurora A coding region was cloned into a vector containing a b-globin leader and one copy of GFP.S65T (M. Schaefer and J.A.K., unpublished data). The GFP.Aurora fusion was cloned into pUAST expression vector and transgenic flies were generated following standard procedures.

w;;UAS-HA*cry* 16.1: transgenic line which express the fusion protein HACRY after activation by the UAS-GAL4 system. It is characterized by:

- white (w; 1-1.5)
- UAS-HAcry 16.1: the yeast upstream activation sequence (UAS) is the binding site for the GAL4 protein and to induce the transcription of the construct. The UAS sequence controls the expression of dcry full-lenght cDNA fused at the n-terminal with the HA (hemoagglutinin) coding sequence (Dissel et al., 2004).

yw;*tim*Gal4: this strain was used to activate the tissue specific transcription of the analyzes genes by the UAS-GAL4 system. It is characterized by:

- white
- yellow (y, 1-0.0): it controls the pigmentation pattern of the cuticule in adults and the buccal cuticule pigmentation in the larvae. Mutants for this locus appear yellow-brown coloured.
- timGal4: the Gal4 sequence is controlled by the promoter of the circadian gene timeless, whose transcription oscillates during the 24 hours with a peak of maximum expression at the beginning of the night. This driver express in all

tissues where are expressed both elements of the central clock (lateral neurons that drive the circadian rhythmicity- Emery et al., 1998) and elements of the pheripheral clock (Plautz et al, 1997).

***pdf*Gal4**: this strain was used to activate the tissue specific transcription of the analyzed genes by the UAS-GAL4 system. It is characterized by:

- pdfGal4: the Gal4 sequence is controlled by the promoter of the pdf gene. This driver express in the small and large ventral lateral neurons (LNvs) (Kaneko and Hall, 2000).

***cry*gal4**: it is characterized by:

- cryGal4: the Gal4 sequence is controlled by the promoter og the cry gene. This driver express in the small and few large LNvs and 3 LNds CRY positive (Picot et al., 2007).

yw; P{GAL4−*ninaE*.GMR}12; P{UAS−InR.A1325D}3 *(*GMR-Gal4): glass enhancer driving GAL4 in the eye disc, provides strong expression in all cells behind the morphogenetic furrow. This line is available on Bloomington Stock Center Indiana University (#8440).

w; P{GAL4-*ninaE*.GMR}12 (ninaEGal4): glass enhancer driving Gal4 in the eye disc. Provides strong epression in all cells behind the morphogenetic furrow. This line is available on Bloomington Stock Center Indiana University (#1104)

***tim*^{0}**: homozygous line which displays an arrhythmic phenotype. This line is characterizes by a deletion of 64bp in the exon7 in position4937-5000 of the timeless gene, leading to the formation of a protein that terminate 329aa downstream the mutation (Myers et al., 1995).

***per*^{0}**: loss of function allele. Homozygous line which displays an arrhythmic phenotype. This line is characterized by a point mutation (position X:2,583,791..2,583,791) of the period gene, leadind to a stop codon within the per locus.

***cry*^{OUT}**: This line is characterized by a deletion in the cry gene. To generate this deletion, the *P*-element line *P(XP)cry*^{d10630} was crossed to a transposase producing Δ*2–3* strain (Robertson et al., 1988). *P(XP) cry*^{d10630} is inserted 57 base pairs upstream of the transcription start site of the *cry* gene and also carries the *mini-white* gene as selectable marker (Yoshii et al., 2008).

***cry*^{0}**: this line is characterized by a knock out of the entire cry gene. The construct for HR was designed such that the entire coding sequence of the cry allele would be replaced by mini-white (Dolezelova et al., 2007).

***yw*;P{EPgy2}*bel*^{EY08943}**: transgenic line obtained by a P-element insertion (P{EPgy2}bel^{EY08943}) in the first exon of the belle gene regulatory region (Bellen et al., 2004), on the third chromosome. This line is available on Bloomington stock centre Indiana University (#19945). It is characterized by:

- white

- yellow
- bel ^{EY08943}: allele associated to the P-element insertion. This allele determines the phenotype (vital and fertile).

P{PZ}*bel*$^{cap-1}$ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}: transgenic line obtained by a P-element insertion (P{PZ}*bel*$^{cap-1}$) in the first exon of bel gene regulatory region (Bellen et al., 2004), on the third chromosome. This line is available on Bloomington stock center Indiana University (#11778). It is characterized by:

- ry^{506}: recessive null allele caused by a deletion in the rosy locus, which determines the phenotype (red-brown eye) induced by the accumulation of the xanthine and ipoxanthine substrates for the deleted gene XHD (xanthine dehydrogenase) in adult eye.

yw;P{EPgy2}*aur*^{EY03490}: transgenic line obtained by a P-element insertion (P{EPgy2}aur^{EY03490}) on the third chromosome (location: 3R:7,788,892..7,788,892). The insertion is at nucleotide position +33 relative to the aur transcription start site (Shilova et al, 2006). This line is viable and fertile and available on Bloomington stock center Indiana University (#15904).

***aur*$^{87Ac-3}$/MKRS**: transgenic line obtained by EMS chemical mutation (Glover et al., 1995). It presents two non synonymous mutations:

- position 488: Glicine- Aspartate
- position 785: Alanine- Valine

This line is vital and presents mitotic cell cycle and neuroanatomical defects. It is available on Bloomington stock center Indiana University (#6188).

5.3 Immunocytochemistry and quantification of staining intensity

Flies were synchronized by light-dark cycles (LD 500 lux:0 lux) at least for 3 days, then transferred to constant darkness (DD) depending on the experiment. Whole flies were quickly fixed in buffered 4% paraformaldehyde for 2 hour at room temperature. Dissected brains were fixed in 4% paraformaldehyde in PBS (10mM Na_2HPO_4, 10mM NaH_2PO_4, 140mM NaCl pH 7.4) for 1 hour and washed in PBST 1X (PBS + 1% Triton) (4 x 10 min). The brains were blocked in 1% BSA at room temperature for 2 hours and subsequently incubated in primary antibodies overnight at 4°C. Primary antibody was washed in PBST 1X (PBS + 1% Triton) (4 x 10min) and brains were incubated with secondary antibody for 3 hours at room temperature. Secondary antibody was washed in PBS 1X (4 x 10min) and brains were mounted using Vectashield (Vector Laboratories Inc.).
Fluorescence signals were analyzed with a confocal microscope (Vico).
Fluorescence intensity was quantified from digital images with the ImageJ software (NIH). We applied the formula: $I = 100(S - B)/B$, which gives the fluorescence percentage above

background (where S is fluorescence intensity and B is average intensity of the region adjacent to the positive cell).

anti-PER	primary	rabbit	1:1:1250
anti-PDF	primary	mouse	1:300
anti-GFP	primary	mouse	1:1000
anti-rabbit Alexa 488	secondary	goat	1:250
anti-mouse Alexa 569	secondary	goat	1:250

5.4 Protein extraction and western Blot.

Flies of the indicated genotypes were first kept in LD cycles for at least 3 days and collected on dry ice every three hours during the indicated ZT in LD or at the third day of DD depending on the experiment. Proteins extracts were made from 30-40 heads for each time point. Frozen heads were homogenized in 40µl of ice-cold extraction buffer (20mM Hepes pH7.5, 100mM KCl, 5mM DTT, 5% glycerol, 5mM EDTA, 0,5% Triton X-100) with the following adds: Apoprotinin: 1µl; PepstatinA: 50µl; Leupeptin: 2µl; DTT:1µl. Samples were centrifuged for 15 minutes at 4°C and supernatant collected. Proteins were quantified with the Bradford method and loading buffer and DTT were added to samples proportionally to the volumes. Samples were boiled at 95° for 10 min before loading the gel (Resolving gel was 4-12% (Invitrogen)). Gels were blotted on Nitrocellulose using a blotting apparatus (Invitrogen) (1h at 30V) and equal transfer of proteins was controlled by Ponceau staining. All western blots were repeate at least 3 times under identical conditions with similar results. PVDF membrane was then incubated for 1h in 5% milk blocking solution and subsequentely incubated in primary antibody overnight at 4°C. The membrane was washed in TBST solution (0.01M Tris-HCl pH7.5, 0.14M NaCl, 0.05% Tween-20, 0.05% Triton) (3 x 10min) and incubated with secondary antibody for 2 hours at room temperature. Membrane was washed in TBST solution (3 x 10min) and incubated with revelation solution (2.25mM Luminol, 0.2mM cumaric acid, 0.1M Tris-Hcl pH8.5, 0.01% H_2O_2). proteins were them revealed with the chemiluminescent system.

anti-Belle	primary	rabbit	1:2000
anti-HA	primary	mouse	1:2000
anti-GFP	primary	mouse	1:1000
anti-Hsp70	primary	mouse	1:10000
anti-Aurora	primary	rabbit	1:1000
anti-rabbit	secondary	goat	1:10000
anti-mouse	secondary	goat	1:10000

5.5 Protein extraction and immunoprecipitation.

Flies of the indicating genotypes were first kept in LD cycles for at least 3 days and collected in dry ice at ZT24 (lights on) and after 15' of light exposure. Protein extracts were made from 200 heads for each time point. Frozen heads were homogenized in 800µl of extraction buffer (20mM Hepes, 100mM KCl, 2,5 mM EDTA, 5% glycerol, 0.5% Triton, 1 tablet protease inhibitors, 0.5M vanadate, 0.1M DTT). Samples were incubated for 2h at 4°C, centrifuged for 10min at 4°C and proteins quantified (OD 595) from the supernatant using the Bradford method.
Immunoprecipitation was then performed using an anti-HA affinity matrix (Roche Applied Science) following manifacturer's instructions. 50 to 100 ml of anti-HA affinity matrix was incubated with samples at 4°C for 30 min and pellet matrix was centrifuged for 5-10 seconds. The matrix was subsequently washed three times with 1ml of cold extraction buffer and then resuspended in next wash solution. 1 to 2 matrix volumes of LDS were addes to final washed matrix pellet and boil for 5 minutes at 90°C. The supernatant was then loaded on 4-12% precast gel (Invitrogen) for SDS PAGE electrophoresis.

5.6 RNA extraction and real-time PCR

RNA was extracted using the reagent Trizol (Invitrogen) as indicated in manifacturer data sheet instructions. 50 frozen heads were homogenized in 1ml of Trizol and incubated for 5 min at room temperature. 0.2ml of Cloroform were added to the sample followed by 15min of centrifugation at 13000rpm. Liquid phase was transferred in 0.5ml of Isopropanol and centrifugated as before. The pellet was washed in 70% ethanol and diluted in 10µl of DEPC water.
1:50 dilution of the RNA solution was quantified with a spettrofotometer. 260/280 absorbance was measured to evaluate purification efficiency . RNA concentration was then calculated with the formula: RNAµg/µl=A260 x eluition factor x 40/1000.
For retrotranscription 1µg of RNAwas addeto to 1µl of dNTPs (10µM) and 1µl oligo dT (10µM) and incubated for 5min at 70°. Subsequentely, 4ml of reaction buffer 5x (Invitrogen), 2ml DTT (0.1M), 1ml RNAse OUT (100 u/ml) and 1ml SuperscriptII (100 u/ml) (Invitrogen) were added to the sample and incubated at 42° for 1h and 75° for 15 min.
The primers used for the realtime PCR were designed with the Primer 3 Software available at (http://www.basic.nwu.edu/biotools/Primer3.html). Real-time PCR was performed on a Rotor Gene 3000 (Corbett Research) by using the following primers: 5'-AACCAAAATGGCACAGGTCT-3' and 5'-GCGTTGTTATTGCCACCAC-3' for Belle mRNA and 5'-ATCGGTTACGGATCGAACAA-3' and 5'-GACAATCTCCTTGCGCTTCT-3' for the housekeeping rp49 mRNA.
SYBR Green (Applied Biosystems, Foster City, CA) fluorescent marker was used to generate semiquantitative data. PCR premixes containing all reagents except for target cDNAs were prepared and aliquoted by a Robotic Liquid Handling System (CAS-1200, Corbett Robotics)

into PCR tubes (Corbett Research). The PCR reaction was carried out following the program: 95_ for 30 sec, 60_ for 30 sec, 72_ for 45 sec, for 40 cycles, followed by a final 10 min at 72_. The amplification efficiencies (E ¼ 10_1/slope) for each sample were calculated on the basis of the results of three amplification reactions, each with a different quantity of the template (6, 18, or 54 ng of total reverse transcribed RNA), according to Pfaffl (2001). Each reaction was performed in duplicate. The amplification efficiency, obtained for each sample, was used to estimate the relative level of expression between the control sample and the others samples (for time courses the control samples was ZT0): $R=Etarget^{\Delta Ct\ target}/Ereference^{\Delta Ct\ reference}$ where Etarget is the amplification efficiency of Belle mRNA; Ereference is the amplification efficiency of rp49 mRNA; ΔCt target and ΔCt reference are the difference between cycle threshold (Ct) of control and samples for Belle mRNA and rp59 mRNA respectively.

5.7 Locomotor activity analysis

Locomotor rhythms of individual male flies were recorded with Drosophila activity monitors (TriKinetics, Waltham, MA). Flies were kept for at least 3 days in 12 h_12 h LD cycles before being transferred to either constant light (LL) or to constant-dark conditions (DD), in which they remained for at least 5 days depending on the experiment. Data were recorded every 5 minutes over the 24 hours, analyzed with the Phyton software(ref) and plotted in the Actograms. Rhythmicity in constant conditions (either LL or DD) was determined by using the CLEAN autocorrelation algorithm (from Dr. J. Lehar, MIT, Cambridge, Mass., USA), which uses sinusoidal functions to find a rhythmicity (Figure1).

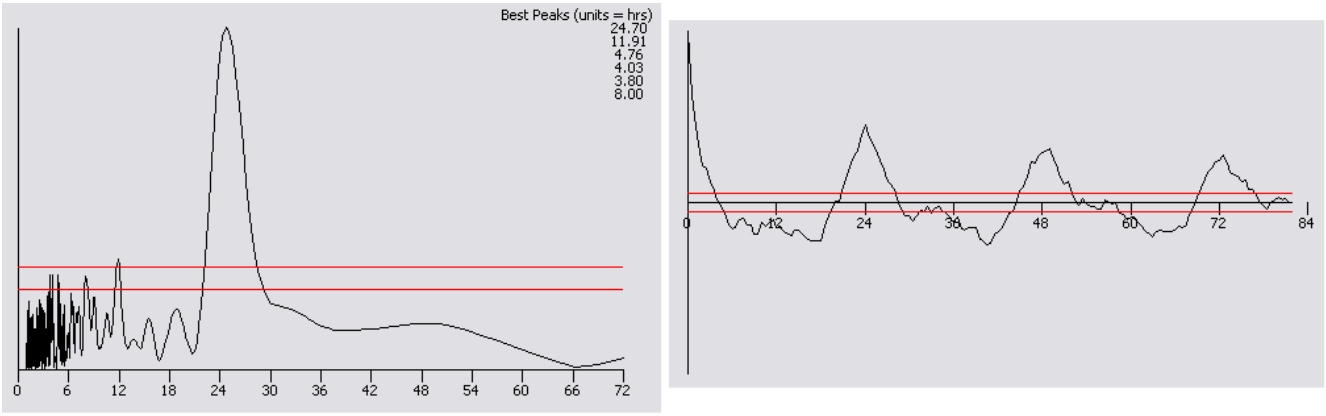


Figure 1:Representation of a graph obtained from the CLEAN analysis

In addition, it uses a range of significativity of 95% and 99% to estimate significantly rhythmic data (red lined). The period is defined by the highest peak over the the significativity range (left).

5.8 Photophobicity test

Larval photophobic behaviour was tested as described in Mazzoni et al., 2005, with minor modifications. A 10cm Petri dish with 15 ml of 1.5% Bactoagar was used, and half of the lid was covered with black electric tape. Early third instar larvae were removed from food and washed with PBS for 30 seconds. 15 to 20 larvae were distributed along the junction between light and dark, and plates were illuminated from above using a cool light lamp. After 10 minutes at room temperature, the number of larvae in each half was counted. Plates with larvae borrowing into agar were discarded. Larvae crawling on the walls of Petri dishes were not included. For this experiment, w^{1118} larvae were used as positive control, per^{0} and tim^{0} larvae were used as negative controls.

5.9 Egg-to-adult viability

200 fertilized eggs derived from crosses UASbelRNAi/timGAL4 and UASbelRNAi/w^{1118} were collected on standard yeast–glucose–agar medium in a petri dish. The fertilized eggs were incubated at 23° and the number of individuals for each crossed reaching third instar larva, pupa, or adult, and the relative percentages, were calculated.

5.10 Plasmids construction and transgenics generation

Plasmid pJG4-5*cry* (Rosato et al., 2001), which carries an in-frame HA tag, was used as template to amplify a *HAcry*. The primers used were: 5NotHACRY: 5'-GCGGCCGCATGTACCCTTATGATGTGCCAGATTATGCC-3', that adds a NotI site (underlined) and 3ScaCRY: 5'-GGTGTAGAAGTACTCTCGCCAGATC-3', located 1002 bp from the *HAcry* start codon, downstream to an internal ScaI site. The amplified fragment was cloned into the PCR® 2.1-TOPO® vector using the TOPO TA Cloning® Kit (Invitrogen, CA, USA) and sequenced. The 5' region of *HAcry* was extracted with NotI and ScaI. CRY_S(515)A, CRY_S(515)D, CRY_S(526)A and CRY_S(515)A/S(526)A were produced by mutagenic PCR. A 766 bp fragment corresponding to the 3' of the gene was amplified by a common forward primer, 5ScaCRY 5'-TCAAGAATGTCCAGTTGCGCGCC-3, located upstream an internal ScaI site, and specific reverse primers adding an XhoI site (underlined) and carrying the desired nucleotide substitutions generating the mutations, indicated in bold italics: CRY_S515A 5'-CTCGAGTCAAACCACCACGTCGGCCAGCCAGAAGAACTGACGCACTTCCTCCTCGTTGGATGGTCGGCAATGCGGCGGCGGGGTGATCAGCG***C***ATTTCGGGAG-3';
CRY_S515D 5'-CTCGAGTCAAACCACCACGTCGGCCAGCCAGAAGAACTGACGCACTTCCTCCTCGTTGGATGGTCGGCAATGCGGCGGCGGGGTGATCAG***ATC***ATTTCGGGAG-3';
CRY_S526A 5'

CTCGAGTCAAACCACCACGTCGGCCAGCCAGAAGAACTGACGCACTTCCTCCTCG TTGG***C***TGGTCG-3'; CRY_S(515)A/S(526)A 5'-CTCGAGTCAAACCACCACGTCGGCCAGCCAGAAGAACTGAC GCACTTCCTCCTCGTTGG***C***TGGTCGGCAATGCGGCGGCGGGGTGATCAGCG**C**ATT TCGGGAG. The amplified fragments were cloned and sequenced. The mutagenized 3'moieties of *cry* were extracted with ScaI and XhoI. The four mutated forms of HA*cry* were obtained by 3 fragments ligations in pUASt linearized by NotI and XhoI. The constructs were microinjected by standard techniques at BestGene Inc. (U.S.A.) and several transgenic lines were obtained. Briefly, the constructs with the w^+ marker were injected into w^{1118} embryos. Nine independent transformant lines were established for each construct. *FM7* (X chromosome), *CyO* (chromosome 2) and *TM3(sb)* (chromosome 3) balancers were used to balance these lines. Lines carrying the different transgenes were chosen on the basis of their homozygous viability. These new alleles of *HACRY* were then expressed in circadian neurons using the timGal driver for molecular experiments. These new alleles were also expressed in flies with a cry^0 background by sequential crosses for behavioural experiments (Figure 2)

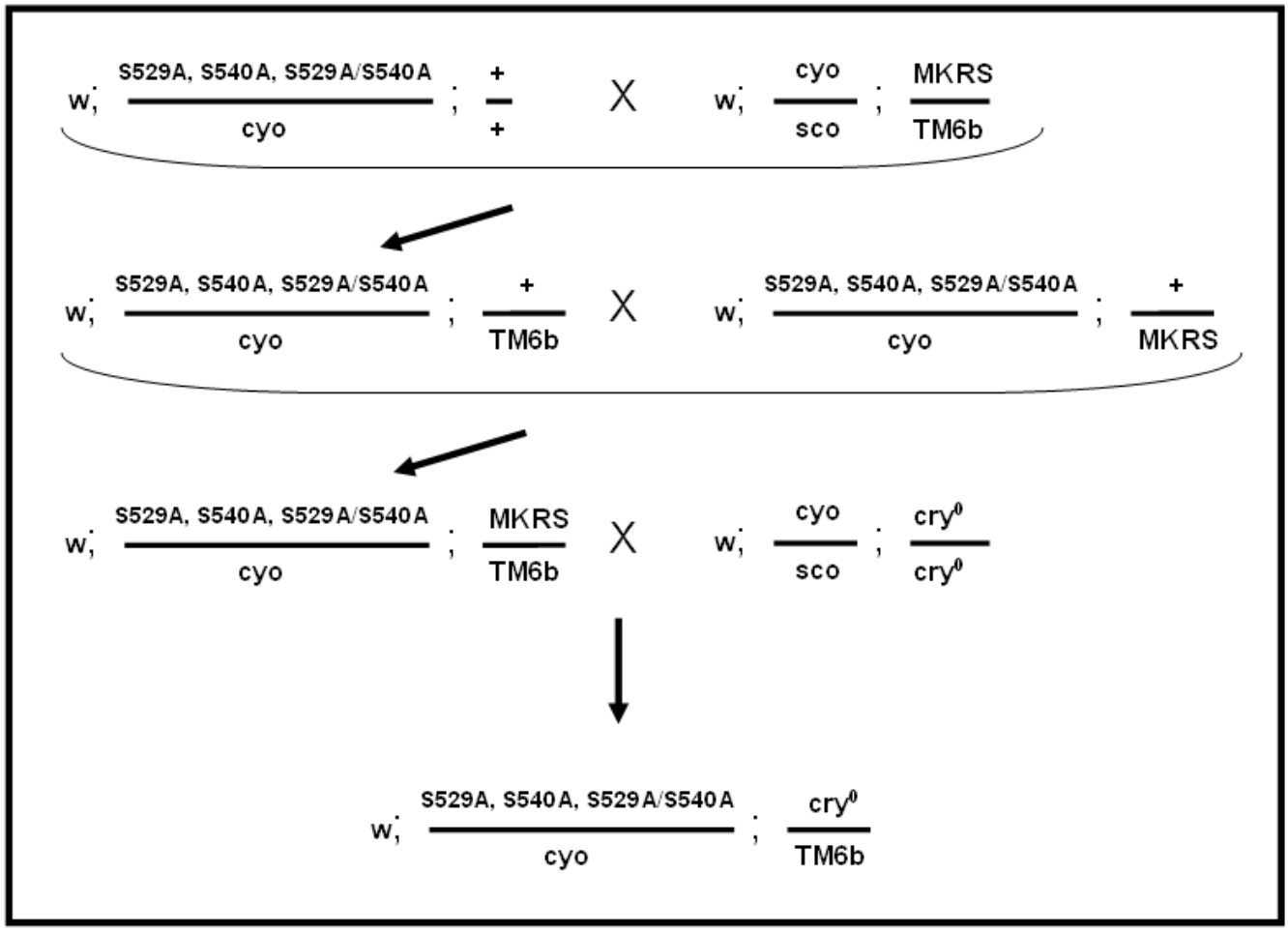


Figure2: schematic representation of sequential crosses performed to obtain flies lacking the endogenous *cry* (on the third) and carrying the mutated form of HACRY on the second chromosome.

5.11 Statistical Analysis

If not otherwise specified, statistical analyses were performed employing the ANOVA F test followed when appropriate by Fisher LSD *post hoc* test with the Statistica 5.0 package (Statsoft Inc.). To determine whether *belle* expression levels in heads were significantly rhythmic, cross correlations of the mean qPCR data to a sine wave of 24 h were computed, using the software Circlewave (Hut RA).

RESULTS I

6.1 The RNA helicase Belle is a new partner of Cryptochrome

Recently, a study was initiated in our laboratory in order to identify new molecular partners for Cry, both in light and dark. Co-IP and mass spectrometry analyses led to the identification of Belle, an ATP-dependent RNA helicase, which was found to interact with Cry in the dark.
In order to confirm the data obtained by MS, co-immunoprecipitation and western-blot were performed using transgenic flies expressing a tagged version of Cry (HACRY) under the control of the driver *tim*GAL4. In these flies HACRY is expressed at the same level as Cry and oscillates during the 24 hours (Dissel et al., 2004). Individuals were collected at ZT24 and after a 15 minutes of light pulse given at the same time-point. Head protein extracts were subjected to immunoprecipitation with an anti-HA affinity matrix and the HACRY immunocomplex subjected to western-blot and probed with an anti-Belle antibody, that should give a signal at around 80 KDa (Johnstone et al., 2005). The same blot was hybridized also with an anti-HA antibody, in order to assess the specificity of the interaction.

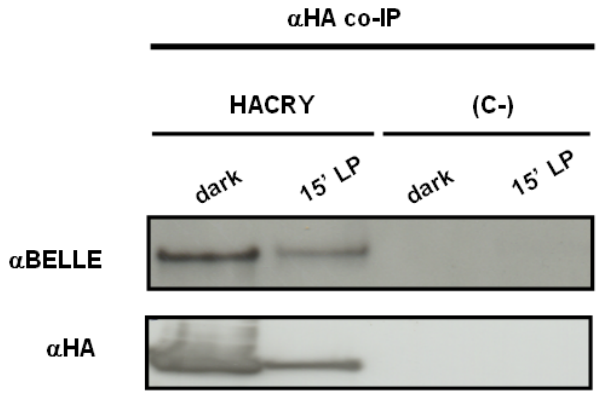


Figure 6.1: Co-immunoprecipitation and western-blot showing the interaction between Cry and Belle in dark and after light exposure in flies overexpressing HACRY (*yw*;*tim*-GAL4/+; UAS-*HAcry*/+). *tim*-GAL4 flies were used as control (C-). Heads were collected at ZT24 and after a 15 min light pulse given at the same time-point.

As shown in Fig. 6.1, the anti-Belle antibody recognizes a protein of the expected molecular weight specifically in the samples where HACRY is present, and not in the controls, demonstrating an interaction between the two proteins either in the dark and after 15 minutes of light exposure.

Belle is expressed in *Drosophila* brain

Clock proteins resides in the brain (70 clock neurons for each hemisphere) and in particular in two main clusters of neurons: the lateral neurons and the dorsal neurons. The molecular oscillation of clock genes activity in these neurons controls and defines the locomotor activity rhythm.

It is known the Belle is expressed in germ cells throughout all developmental stages and in larval neurons (Johnstone et al., 2005, Rolls et al., 2007), but no information were available regarding its expression in the adult fly head. In order to characterize Belle expression in the fly brain, an immunocytochemistry experiment was performed. For this purpose, a *belle*-GFP line was used (FlyTRAP stock center) which allows the detection of Belle protein by using an αGFP antibody. Flies were maintained in standard light-dark conditions, brains were dissected and GFP expression was detected with the specific αGFP antibody.

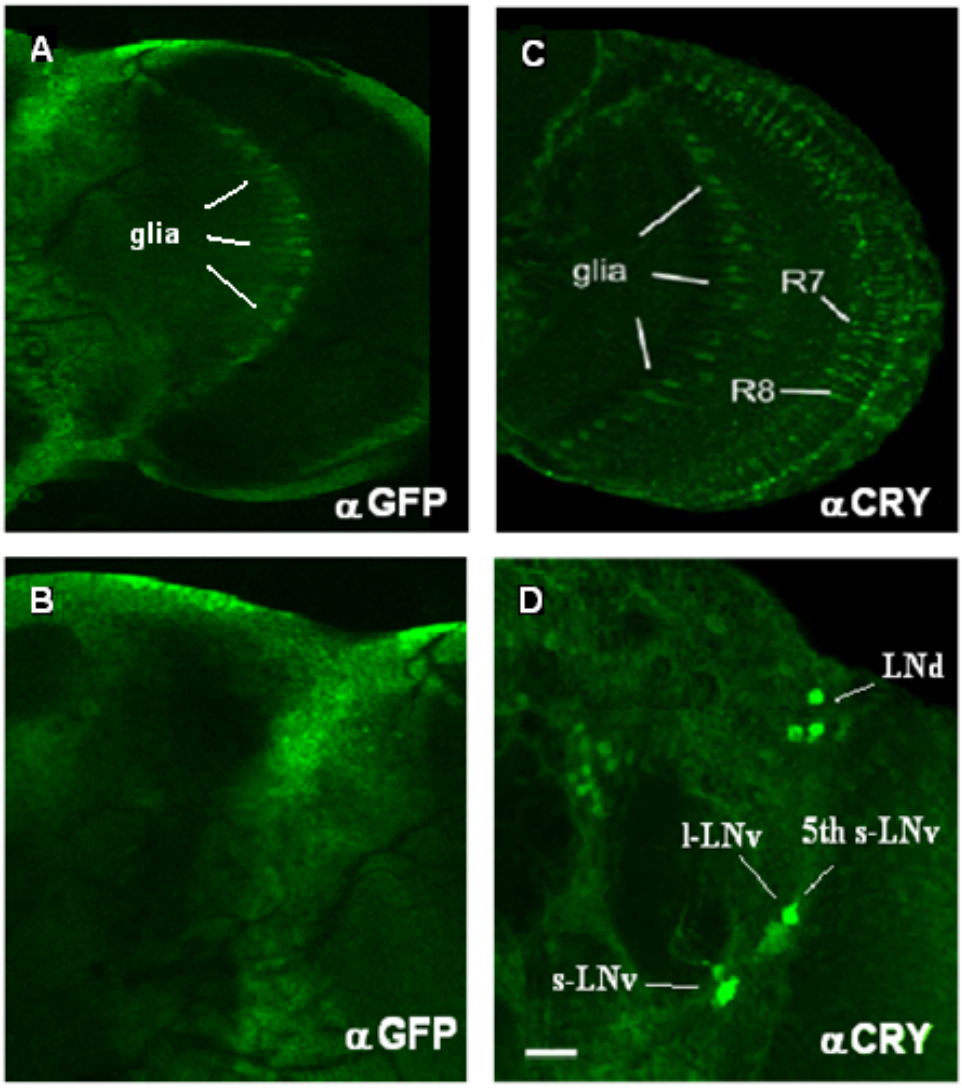


Figure 6.6: Anti GFP labelling in 3 days old *belle*-GFP flies (B and D). A: anti-Cry labeling in the medulla of the right optic lobe. Labelling is found in the terminals of photoreceptor cells R7 and R8 of the right compound eye and in glia cells in the second optic chiasma (A) (Yoshii et al., 2008). C:anti-Cry labelling in clock neurons (Yoshii et al., 2008).

As shown in figure 6.6 (A), Belle is expressed in the glial cells in the optic lobe. It is reported that also Cry is expressed in the same brain area (B) (Yoshii et al., 2008) and this result could support the idea of a physical interaction between the two proteins. However no Belle expression was found in the circadian neurons and in the photoreceptors (C), where Cry is highly expressed (D) (Yoshii et al., 2008). Due to a technical problem with the antibody, it

wasn't possible to verify directly the co-localization of the endogenous proteins, but the same expression pattern supports the idea of a functional interaction of the two proteins.

6.2 Analysis of *belle* expression

It is known that mRNA and protein levels of key clock components oscillate in the brain with a periodicity of about 24 hours. In particular, *per* and *tim* mRNA levels show a maximum of expression in the early evening followed by a 4-6 hours delayed peak in the respective proteins. This temporal delay is due to posttranslational mechanisms, such as protein phosphorylation and ubiquitination that promote their degradation (Cheng et al., 1998; Yang and Sehgal, 2001; Grima et al., 2004; Harms et al., 2004).
In order to reveal a possible control of the circadian clock in the expression of *belle,* mRNA and protein levels were analysed during the 24 hours. To do so wild-type flies w^{1118} were entrained for at least 3 days after hatching in 12:12 light:dark regimes and collected every three hours during the 24 hours. For oscillation analysis in constant dark conditions (DD) flies were subjected to standard entrainment for 2 days, moved to DD conditions and collected on the third day of DD every three hours during the 24 hours.

For each time point, proteins were extracted from heads and Belle was detected by western blot with the specific anti-Belle antibody. The αHsp70 (Heat Shock Protein 70) antibody was used as loading control.

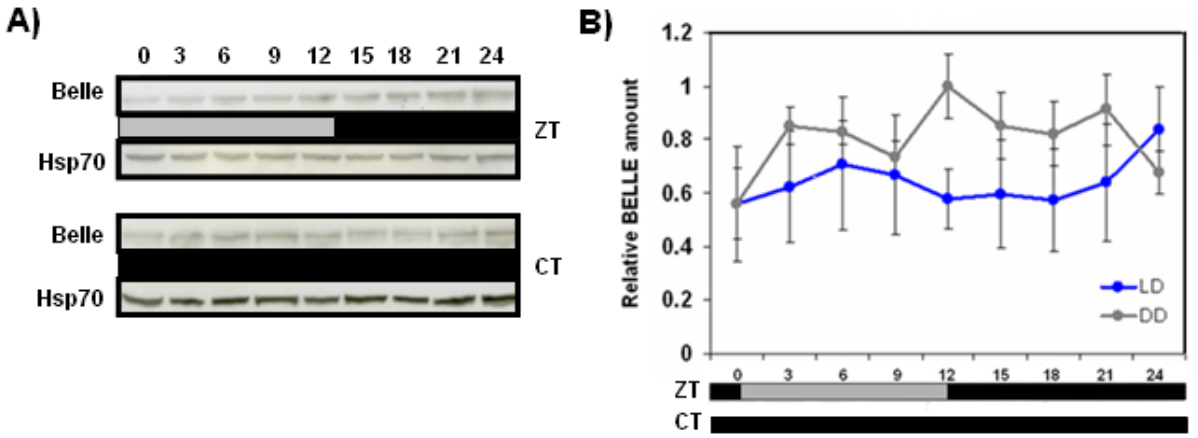


Figure 6.2: Representative western blot on heads extracts from w1118 in LD and DD conditions . (B) Quantification of Belle levels. Relative abundance of Belle was defined as a ratio with HSP70. ZT: Zeitgeber Time, CT:Circadian Time.

Figure 6.2 shows the result obtained from the immunodetection. In A, a representative western blot performed both in LD and DD is shown; in B the graph corresponding to the quantification of three independent replicates is reported. No statistically significant protein oscillation was detected neither in LD conditions nor in DD. However Belle expression seems to be slightly higher in the light and lower in the dark.

The analysis of mRNA expression was performed by Real-time PCR on total RNA extracted from flies heads maintained in light-dark and constant darkness regimes. In fact, one of the most important features of the circadian clock is the capability to maintain the endogenous rhythm in absence of stimuli from the environment (Allada and Chung, 2010)
Wild-type flies w^{1118} were synchronized by 12:12 LD cycles and collected every three hours during the 24 hours, either in LD regime or at the third day after being transferred to constant darkness (DD). *belle* signal was quantified on the reference gene rp49, which expression is constant during the 24 hours.

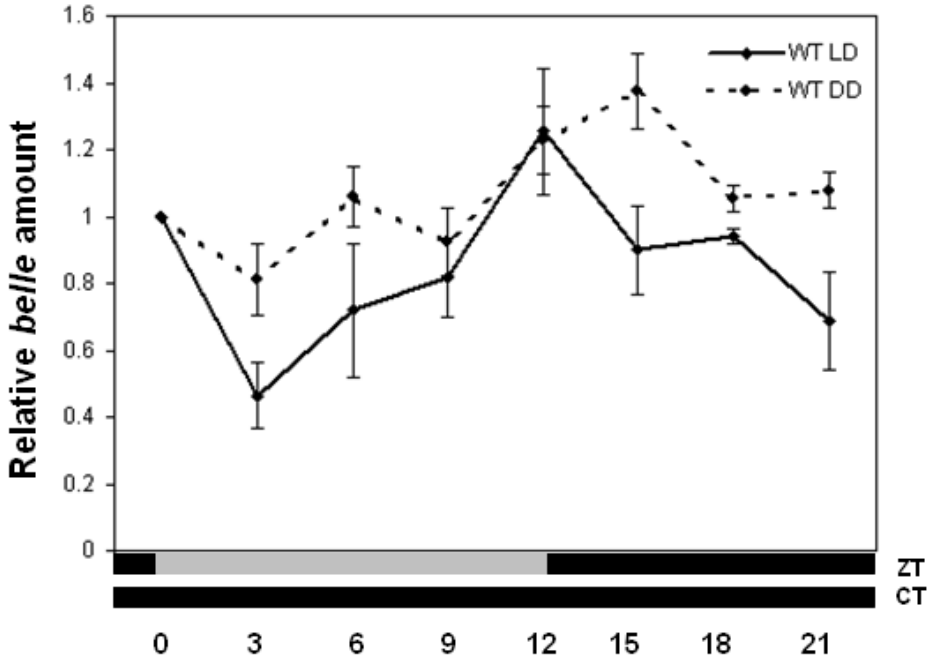


Figure 6.3: Quantification of *belle* mRNA expression in LD and DD conditions in the wild-type. RT-PCR on heads extracts from w1118. *belle* signal was quantified on the reference gene rp49. *belle* fluctuations in LD are statistically significant (ANOVA analysis, circlewave analysis).

belle expression appears to oscillate both in LD and in DD conditions. In particular, in light-dark cycles it shows a peak around ZT12 while in constant darkness the peak is slightly delayed and is present 3 hours later, at ZT15. These results are in line with a previous microarray study showing that *belle* expression oscillates during the 24 hours, with maximum in the dark phase of the cycle (ZT17- Claridge-Chang et al, 2001) These result suggest that this RNA helicase might be a component of the circadian clock machinery.
In order to verify whether the expression of *belle* is controlled by the central clock, we analyzed *belle* mRNA levels in flies mutants for the two main clock components: *period (per)* and *timeless (tim),* both in LD and DD conditions. Flies were collected as described previously and results are plotted in figures 6.4 and 6.5.

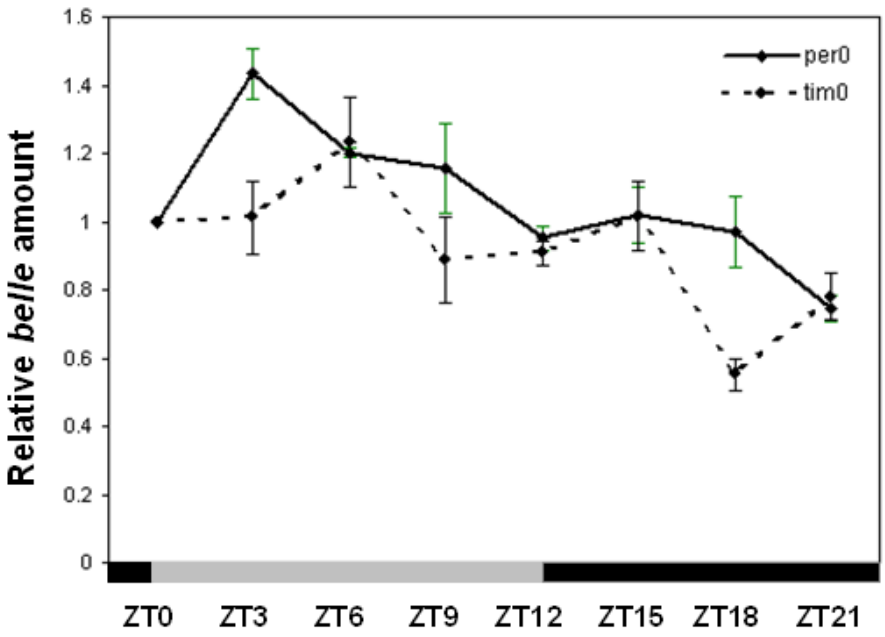


Figure 6.4: belle mRNA expression in LD conditions in *per*^{0} and *tim*^{0} mutants. RT-PCR on heads extracts from *per*0 and *tim*0. *belle* fluctuations are statistically significant (ANOVA analysis, circlewave analysis).

In light-dark cycles in *per*^{0} mutants *belle* mRNA peaks in the early morning while in *tim*^{0} mutants it reaches a maximum 3 hours later. Thus an oscillation of the gene is still present but shifted of 12 hours compared to the *wild-type*. These data suggest an involvement of these two main clock component in the regulation of *belle* expression.
We then analyzed the expression of *belle* in the clock mutants in constant darkness conditions and results are reported in figure 6.4.

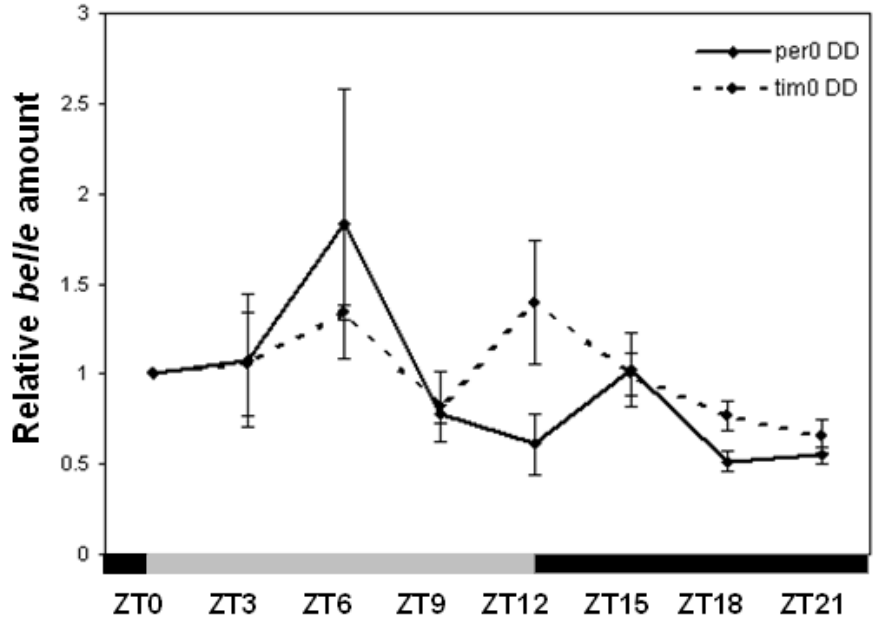


Figure 6.5: belle mRNA expression in DD conditions in *per*^{0} and *tim*^{0} mutants. RT-PCR on heads extracts from *per*^{0} and *tim*^{0}

In both mutants the oscillation of the gene seems to be abolished when flies are kept in constant darkness. These results could suggest a role of the light in the regulation of *belle* gene expression.

6.3 Post-transcriptional gene silencing of *belle* via RNA interference

To test whether *belle* is required as a functional component of the clock, the effect of *belle* downregulation on the fly circadian behavior was studied. For this purpose an RNAi line (#6299 VDRC Vienna) was used. This line has an UAS-RNAi transgene located on the third chromosome and, when crossed to a GAL4 line, expresses a 324 nucleotides double strand RNA which promotes the degradation of *belle* mRNA, *via* RNAinterference.

*bel*RNAi/*tim*Gal4

In a first experiment, aimed at the characterization of the effects of *belle* downregulation in circadian neurons, the *bel*RNAi line was crossed with the *tim*GAL4 driver This cross showed to be lethal at the larval stage, as none of the resulting larvae were able to undergo into metamorphosis. For this reason a vitality test was performed. UAS*bel*RNAi line was crossed in parallel with *tim*GAL4 flies and with w^{1118} as control. Every stage (embryos, larvae, pupae, adults) was followed and individuals were counted. As in the UAS-*bel*RNAi line the transgene is in heterozygosity with a balancer chromosome with MKRS marker, it is expected to have 50% of resulting individuals with the integrated transgene (grey) and 50% with the MKRS marker (black).

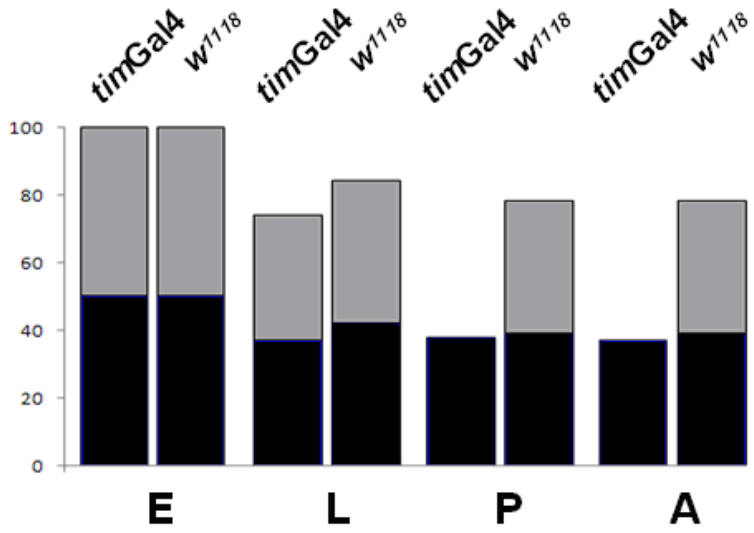


Figure 6.6: Effects of *belle* downregulation on egg-to-adult viability calculated as percentages of third instar larvae (L), pupae (P) and adults (A) relative to the number of embryos (E).

Flies heterozygous *bel*RNAi/TM3Sb where crossed to *tim*Gal4 or w^{1118}. For both crosses grey and black bars represent *bel*RNAi and TM3Sb individuals respectively.

As shown in figure 6.6, the control cross with w^{1118} line gave the expected ratio at all the developmental stages analysed whereas the cross with *tim*GAL4 gave only pupae and adults with the MKRS marker, suggesting that expression of the interference in *tim* cells was lethal at larval stage.

To verify that the effect on larval development in *bel*RNAi lines was specifically due to the downregulation of *belle*, a real time PCR was performed in *wild-type* and downregulated 3rd instar larvae. In particular, larvae were collected at the end of the day, when *belle* expression reaches its maximum level, and quantitative PCR was performed. Results in figure 6.7 clearly show a strong reduction of *belle* expression in *bel*RNAi larvae compared to wild-type, suggesting that the inability of this larvae to undergo metamorphosis could be due to *belle* downregulation.

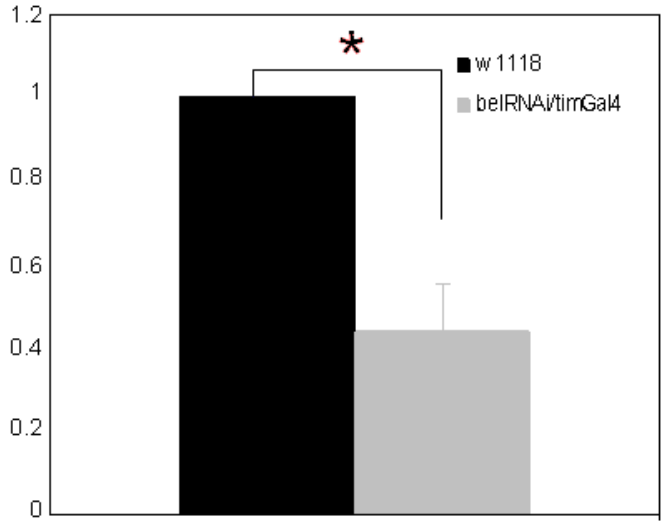


Figure 6.7: Average amount (3 replicates) of *belle* mRNA in w^{1118} fly heads (black) and *bel*RNAi/*tim*Gal4 fly heads (grey).

Photophobic response of *belle* RNAi larvae

Due to the lethality at larval stages of *belle* downregulated individuals, it was not possible to perform the canonical circadian locomotor activity analyses. However, a circadian test for larvae is known, which is the characterization of the circadian photophobic response (Mazzoni et al., 2005). It is reported that w^{1118} larvae kept in LD cycle, shifted to DD and tested for light avoidance (photophobicity) show a circadian modulation of this behaviour, with a peak response toward the end of the night and lowest responses toward the end of the

day and this . Moreover this phenotype seems to be controlled by the clock, as it is impaired in *per*^{0} mutants (Mazzoni et al., 2005).
The photophobic response relies on circadian neurons that not only receive light information for entrainment of their clock, but they also translate this information to produce a rapid photophobic response. Larvae in which circadian neurons where either ablated or electrically silenced were as defective in light avoidance as larvae lacking all photoreceptor cells (Mazzoni et al., 2005). This indicates that circadian neurons in Drosophila larvae are an essential part of a neuronal circuit that leads to a rapid behavioural response such as light avoidance (Mazzoni et al., 2005).
The photophobic response was measured as in Mazzoni et al., (2005), 3rd instar larvae were placed in a petri dish half covered with black tape and after 10 minutes larvae found in the "dark side" were considered photophobic while larvae found in the "illuminated side" were considered not photophobic or blind. In order to characterize photophobic response in *belle* knockdown lines, UAS*bel*RNAi line was crossed to *tim*GAL4 driver; w^{1118} line was used as positive control while per^{0} and tim^{0} lines as negative control and the photophobic response was measured in the middle of the light phase (ZT6). As reported in Mazzoni et al., (2005), this is higher in this moment of the day and the difference in the response between positive and negative controls is more pronounced.

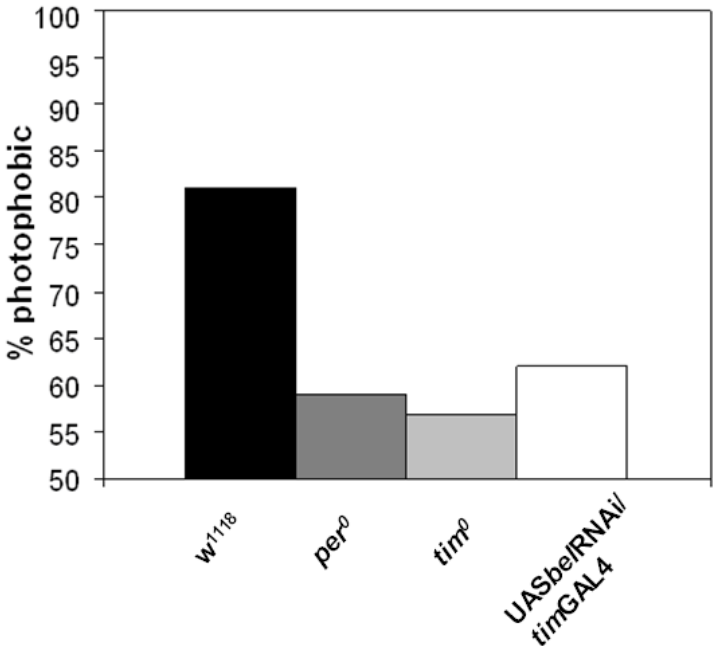


Figure 6.8: Photophobic response of w^{1118} (black), per^{0} (dark grey), tim^{0} (light grey) and UAS*bel*RNAi/*tim*GAL4 (white) individuals. Bars represent average percentage of photophobicity on 10 replicates.

As shown in figure 6.8, larvae of the strain w^{1118} prefer darkness to light (about 80%) in an immediate light/dark boundary passing test and show a significant reduction in motility in the dark; tim^{0} and per^{0} larvae distribute randomly in the petri dish, confirming that ablation of circadian cells results in defective light avoidance. These results are in accordance to those

obtained by Mazzoni et al., (2005).UAS*bel*RNAi/*tim*GAL4 larvae show a behaviour similar to that of clock mutants, with only 60% of are photophobic individuals, suggesting an impairment in light perception and a role of *belle* in the photophobic response.

Other-GAL4 drivers

In order to further characterize the role of *belle* in the circadian clock, the knockdown was performed only in a precise subset of neurons. In particular *gmr*GAL4, *ninaE*GAL4 drivers, that express in the photoreceptors, *cry*Gal4, that express in some circadian neurons and in photoreceptor cells (Emery et al., 2000) and *pdf*GAL4 driver, that express in 4 small and one large lateral neurons (Kaneko and Hall, 2000) were used. The specific cells where the different drivers are active are shown in Table1.
First of all, the expression of the different GAL4 drivers was verified, by crossing the driver to a reporter UAS-GFP line and detecting the endogenous GFP expression in the progeny.

DRIVERS	EXPRESSION
pdfGAL4	4sLNvs, 1LNvs
cryGAL4	DN1, 3/6 LNds, 5th s-LNvs, 1LNVs
gmrGAL4	Photoreceptors, glial cells, HB tract
ninaEGAL4	Photoreceptors, glial cells, HB tract

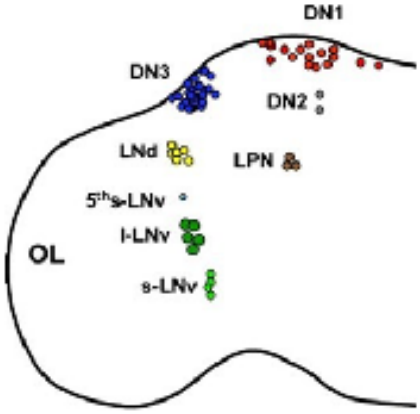


Table 1:drivers expression in the brain. Schematic representation of circadian neurons in one brain hemisphere (right).

Panels in figure 7.9 show, in green, GFP expression of one brain hemisphere.

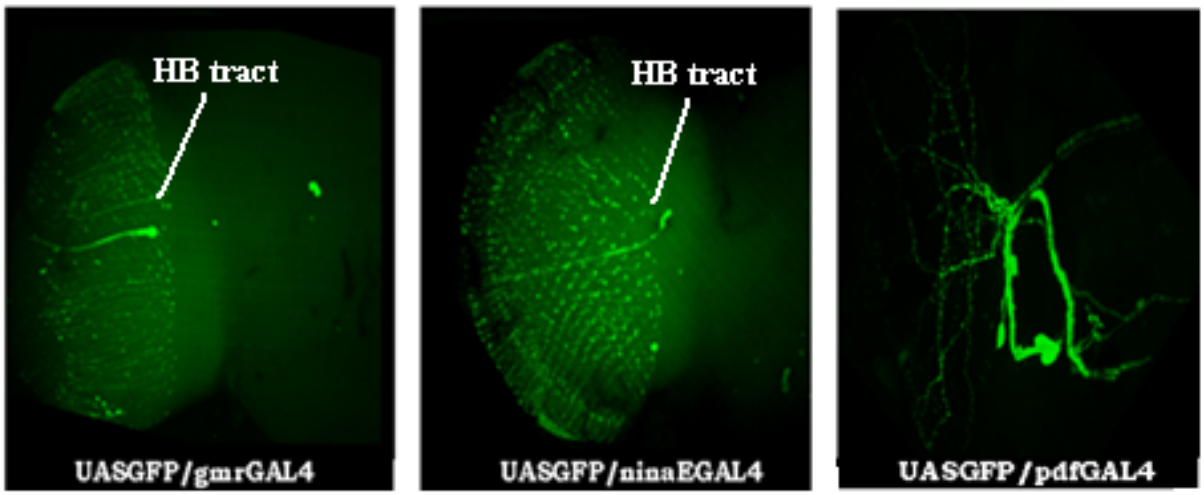


Figure 6.9: endogenous Gfp expression in brains by using 3 different drivers. Images were acquired with a confocal microscope (Vico).

Results indicate that GFP is expressed in the reported brain areas, confirming the specificity of the drivers used. In fact with *gmr*GAL4 and *ninaE*Gal4, GFP expression was found in the photoreceptor cells in the optic lobe and in the HB tract. Using the *pdf*Gal4 driver, GFP expression is present in the circadian neurons and on the projections.

Circadian behaviour of *belle* down-regulated lines

The genetic, molecular and anatomical dissection of the circadian clock in *Drosophila* and other higher organisms relies on the quantification of rhythmic phenotypes. The method currently in use is the analysis of fly locomotor activity rhythms, which is the preferred readout for measuring the rhythmicity under a variety of conditions. This phenotype provides a relatively simple, reliable and robust output for the circadian clock. Most of the mutational screens performed to date in the search for genes involved in circadian rhythmicity were based on monitoring Drosophila mutants for alterations in the circadian pattern of locomotor activity.

Flies locomotor activity presents a bimodal profile where flies anticipate lights-on in the morning and lights-off in the evening (Allada and Chung, 2010).

Circadian locomotor activity was analyzed in flies where the expression of *belle* was down-regulated with each of the drivers described previously. Each driver was also crossed with the w^{1118} strain and the progeny used as a control. In figure 6.10 a representative double plot for the locomotor activity of a single fly is reported.

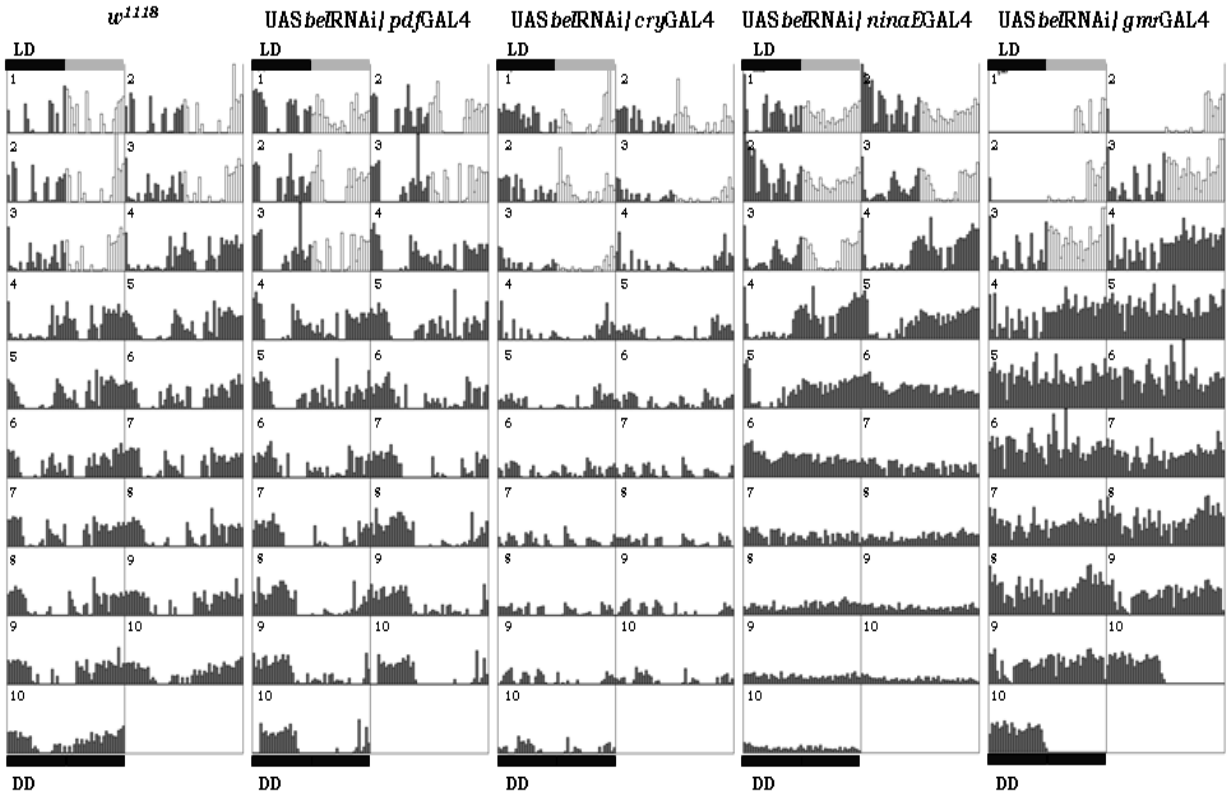


Figure 6.10: Representative locomotor activity doubleplot of a single fly recorded for 3 days in LD conditions and 7 days in DD conditions.

w^{1118} flies display normal locomotor activity in LD conditions with a morning peak and an evening peak corresponding to lights on and lights off respectively. In DD they show a rhythmic unimodal phenotype. UAS*bel*RNAi/*pdf*Gal4 flies show the canonical bimodal profile in LD and rhythmicity in DD, with a slightly longer period. *belle* knock down flies in Cry positive, NinaE positive and Gmr positive cells showed, in most of the cases, an arrhythm profile in constant darkness conditions.
Percentage of rhythmicity and mean period are reported in table 2.

GENOTYPE	% RHYTHMIC FLIES	AVERAGE τ (± SEM)
w^{1118}	96 (24/25)	24.65 ± 0.08
UAS*bel*RNAi/*pdf*Gal4	85.71 (48/56)	24.94 ± 0.1
*pdf*Gal4/w^{1118}	96.29 (26/27)	24.54 ± 0.09
UAS*bel*RNAi/*cry*Gal4	65.38 (17/36)	24.89 ± 0.07
*cry*Gal4/w^{1118}	95.83 (23/24)	24.67 ± 0.07
UAS*bel*RNAi/*gmr*Gal4	52.63 (20/38)	23.99 ± 0.23
*gmr*Gal4/w^{1118}	82.75 (24/25)	24 ± 0.12
*bel*RNAi/$nina^{E}$Gal4	68 (17/25)	23 ± 0.15
$nina^{E}$Gal4/w^{1118}	80 (13/15)	24.32 ± 0.12
*bel*RNAi/w^{1118}	80 (12/15)	24.32 ± 0.14

Table 2: Locomotor activity analysis of flies expressing belRNAi in different clusters of neurons.

In particular, the downregulation of *belle* in all photoreceptors (with *gmr*GAL4 and *ninaE*GAl4 drivers) causes higher percentage of arrhythmicity in constant conditions, Also the downregulation in Cry positive cells (which comprises also some photoreceptor cells) causes an impairment in the circadian locomotor activity, but the effect is reduced compared to that caused by the downregulation in all the photoreceptors. Moreover, when we drive the RNAi of *belle* in Pdf positive neurons, no effects in terms of arrhythmicity are observed. This result confirms the specificity of the observed phenotype, as our previous experiments showed that *belle* is not expressed in these neurons. Taken together the result of these experiments suggest that the expression of *belle* is important for the correct functioning of the circadian machinery.

6.4 Characterization of *belle* mutant lines

In order to further investigate on the role of *belle* in the circadian machinery, we decided to perform circadian locomotor activity analyses on two insertional mutant lines for *belle*

(*#11778* and *#19945* from flybase), that have a P-element insertion in the regulatory region of the gene (Bellen et al., 2004).

Analysis of *belle* expression

First of all we decided to ascertain whether the P-element insertion alters the circadian oscillation of *belle* expression, and for this purpose 3 days old flies were kept in standard 12:12 light-dark cycles, collected every three hours during the 24 hours and gene expression was analyzed by Real-time PCR.

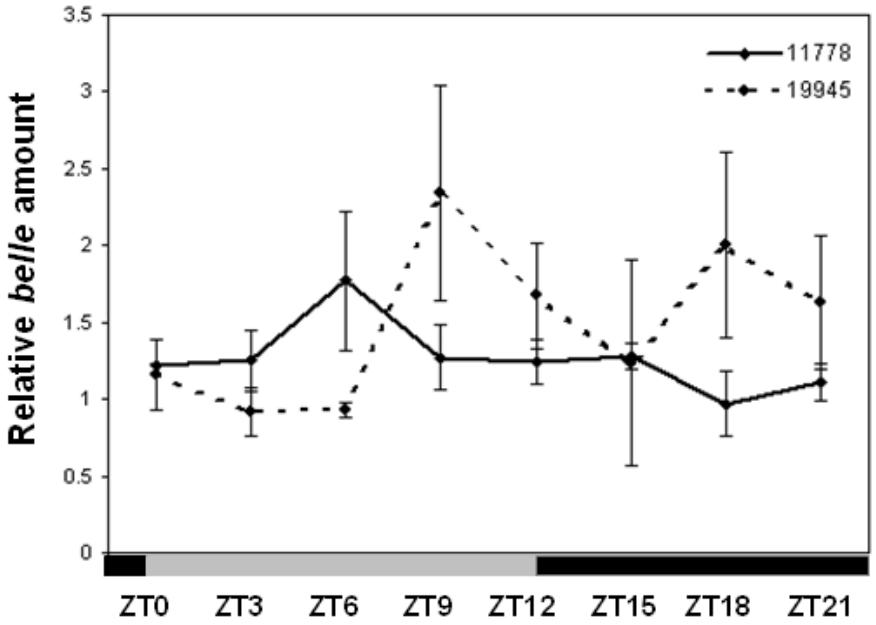


Figure 6.11: belle mRNA expression in LD conditions in #11778 and #19945 P-element mutants. RT-PCR on heads extracts from *#11778* and *#19945* P-element mutants.

Results reported in Figure 6.11 show the average amount of *belle* expression during the 24 hours. For both mutants the expression level of the gene is not downregulated compared to wild-type (wild-type expression is referred to 1) but the oscillation during the 24 hours is lost, indicating an impairment of the circadian oscillation of *belle*.light-dark cycles. This result indicates that in the mutants *belle* expression is impaired.

Analysis of the circadian locomotor activity

Locomotor activity analysis of the two p-element mutants was performed. Flies were entrained to 3 days of light dark cycles al constant temperature (23°C) followed by 5 days of constant darkness, to detect if the endogenous clock is able to maintain a cycle in the absence of light.

In figure 6.7 a representative double plot for the locomotor activity of a single fly is reported. During the first 3 days of entrainment wild-type flies (left) present the canonical bimodal profile with two peaks of activity corresponding to light-dark transitions and the 'siesta' between them. *#11778* mutant flies (centre) present the bimodal profile during the first day of recording, but already at day 3 the morning peak is lost and the activity is shifted in the middle of the day. #19945 mutant flies (right) have the same behaviour as *#11778* and furthermore they also show the loss of the morning anticipation.
In constant darkness the mutant flies loose the rhythmicity already at the first day of constant conditions, while the rhythmic profile of wild-type is maintained throughout the experiment.

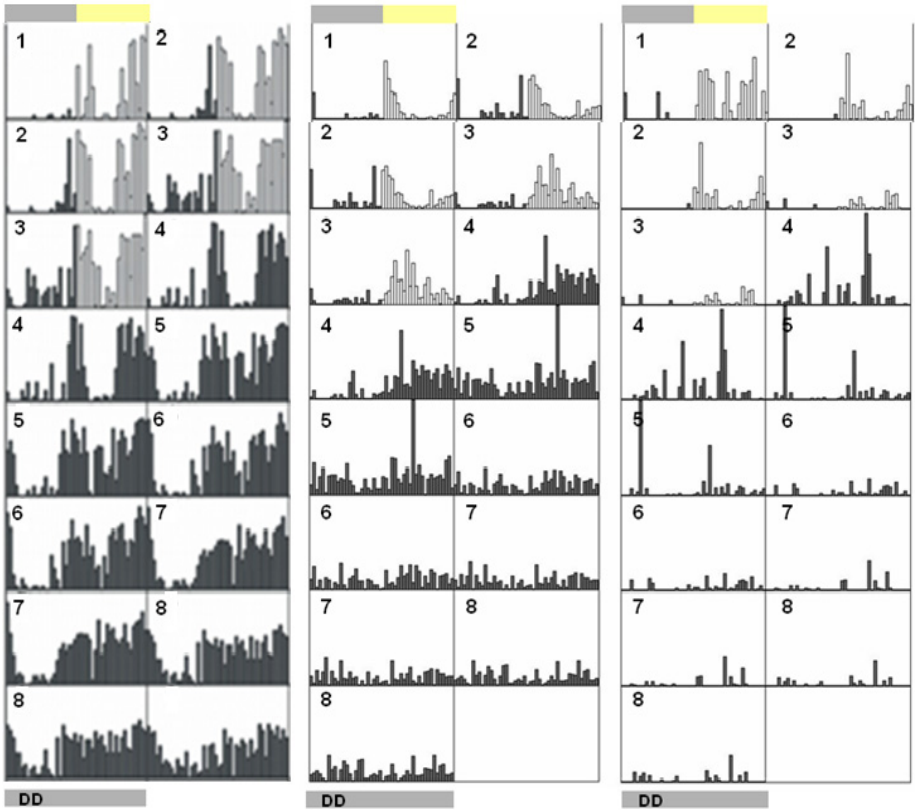


Figure 6.12: Representative locomotor activity doubleplot of a single fly recorded for 3 days in LD conditions and 5 days in DD conditions.

Results reported in figure 6.12 where also quantified and reported in table 3. For each strain the percentage of rhythmicity, the period and the percentage of morning onset where calculated. It is important to highlight that the two mutant lines present an impaired percentage of rhythmicity in constant darkness and an impaired percentage of morning onset calculated on the third day of entrainment. No defects where monitored concerning the period, which is comparable to the *wild-type*.

GENOTYPE	% RHYTHMIC FLIES	AVERAGE t (± SEM)	% MO
w1118	100 (25/25)	24.76 ± 0.057	100 (25/25)
#11778	57 (29/47)	24.66 ± 0.19	76 (36/47)
#19945	24 (7/29)	24.14 ± 0.25	69 (20/29)

Table 3: Locomotor activity analysis of P-element mutant flies

The doubleplot analysis shows also an evidence of a reduced amount of activity of the mutants compared to the wild-type. The average activity on three days of light-dark has been calculated (figure 6.13) and is clearly visible that these mutant lines display not only defects in the canonical circadian phenotype but also in the amount of movements during the 24 hours.

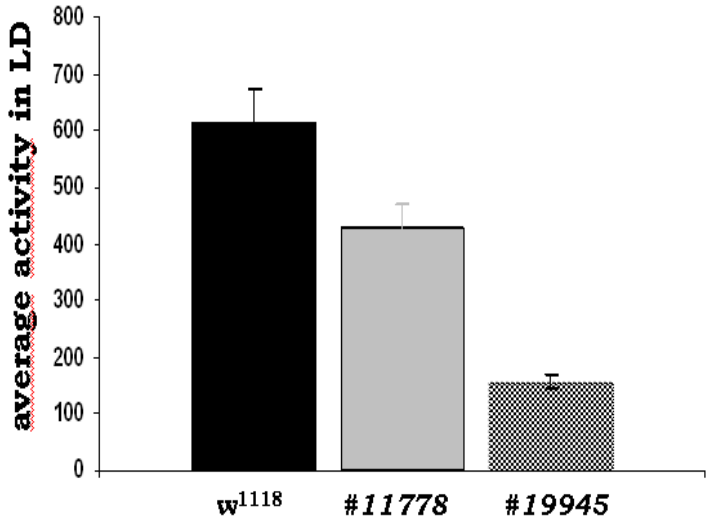


Figure 6.13: quantification of average activity of wild-type and mutants of 3 days of LD.

Analysis of Per protein oscillation

The circadian clock of *Drosophila melanogaster* that controls locomotor activity rhythms is located in the brain and relies of about 70 clock neurons for each brain hemisphere. According to their location, morphology or properties, the neurons of these two clusters can be further sub-divided respectively in three different groups: the Dorsal Neurons in DN1s, DN2s and DN3s; the Lateral Neurons in small Ventral Lateral neurons (s-LNvs and the 5th s-LNv), the large ventral Lateral Neurons (l-LNvs) and dorsal Lateral neurons (LNds). In the different clusters of neurons

Period and Timeless expression oscillates during the 24 hours, with a maximum around ZT0 and a minimum around ZT12.

We have hypothesized that the impairment of the circadian locomotor activity in *belle #11778 and #19945* mutants (57% and 24% respectively of rhythmicity in constant conditions) could be due to an impaired circadian expression of these key clock proteins. In particular we focused on Period, either because it is a main component of the central clock and also because an antibody for this protein is available in our laboratory.
In order to test this hypothesis, 3 days old flies were monitored for locomotor activity for 2 days in light-dark cycles and 5 days in constant darkness. Data were analyzed and arrhythmic flies and flies arrhythmic lacking morning anticipation (MA) were collected separately every 4 hours during the 24 hours. Brains were subsequently dissected and αPer antibody was used to detect Per expression in the neurons. αPdf antibody was utilized for the detection of the different clusters of neurons.

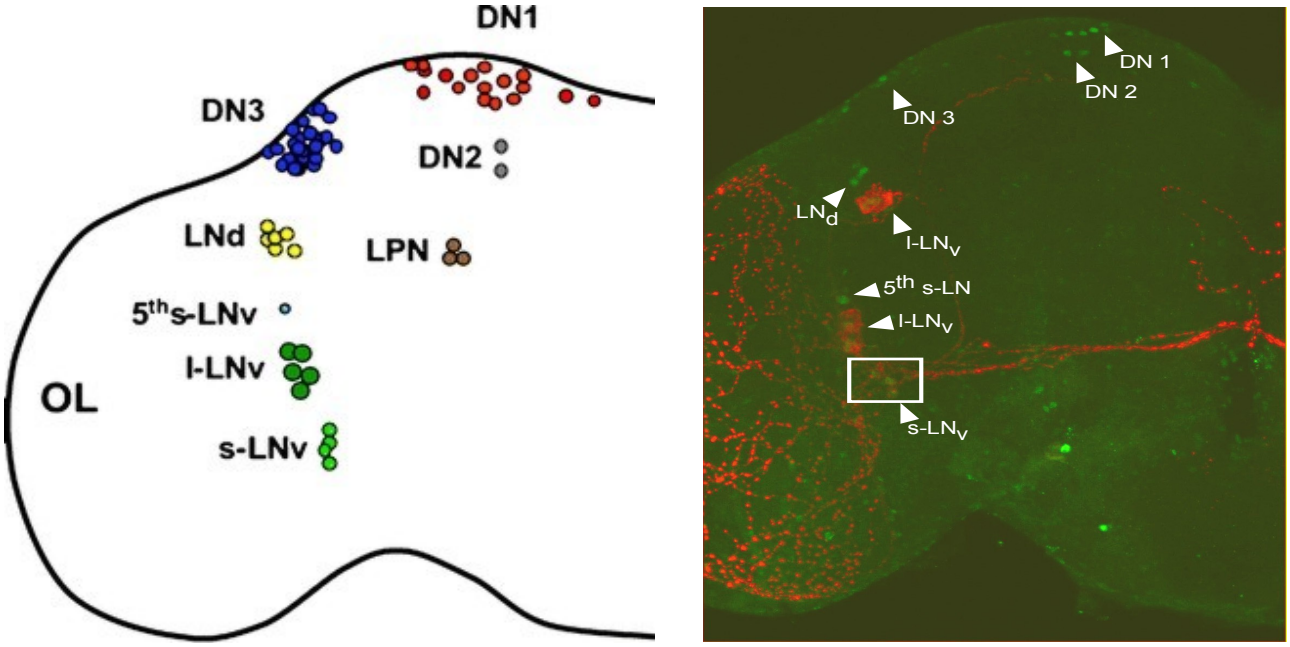


Figure 6.14: representative picture of circadian neurons in one brain hemisphere (left) and example of Per-Pdf double staining in one brain hemisphere (right).

In figure 6.14 it is reported an example of Per staining in one hemisphere in a wild-type fly. Per protein is easily visible in all groups of neurons while Pdf expression is present in the ventral neurons (but not the 5th s-LNv) and in the l-LNvs.

#11778

The signal in all groups of neurons was quantified and plotted and results are reported in figure 6.15.

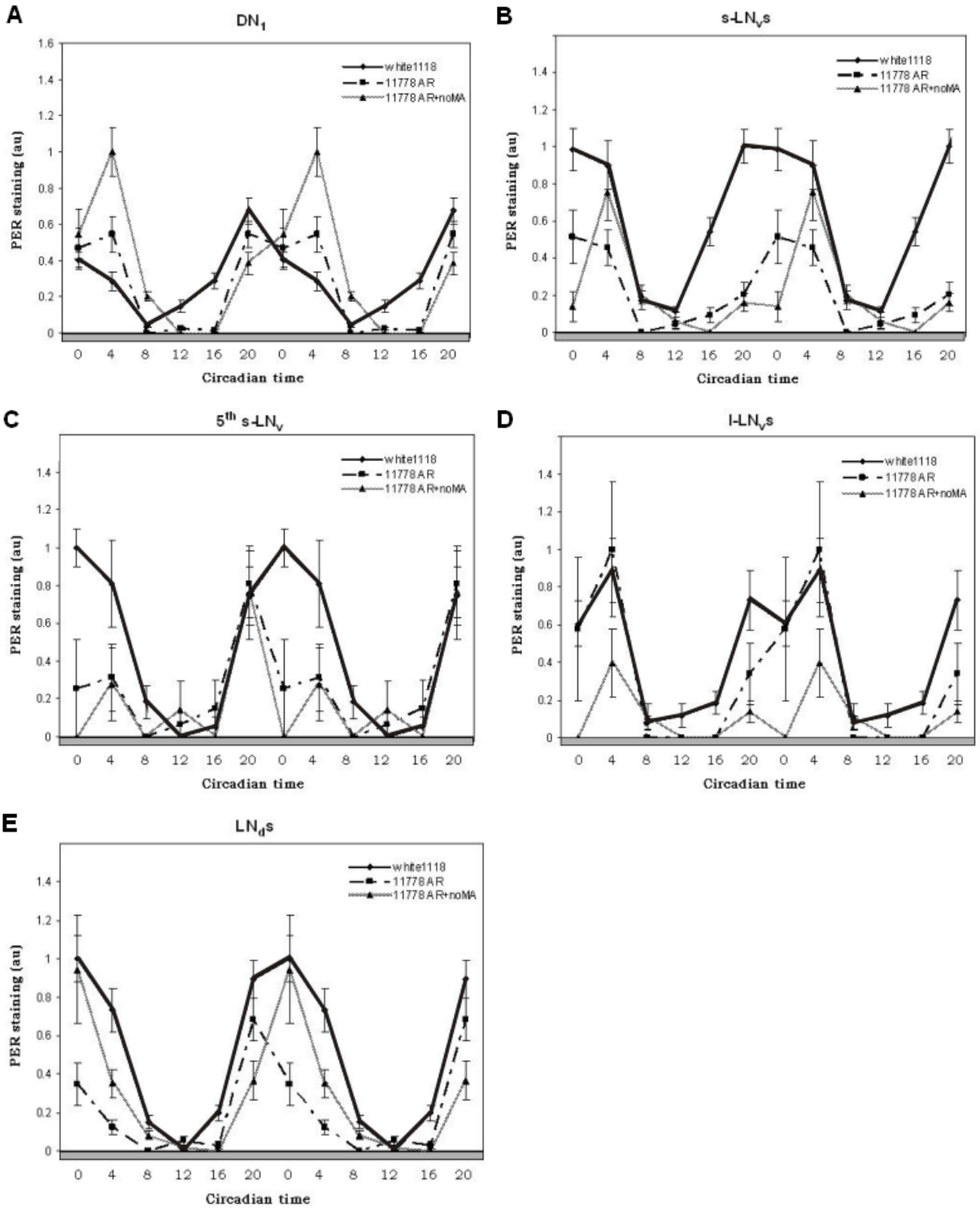
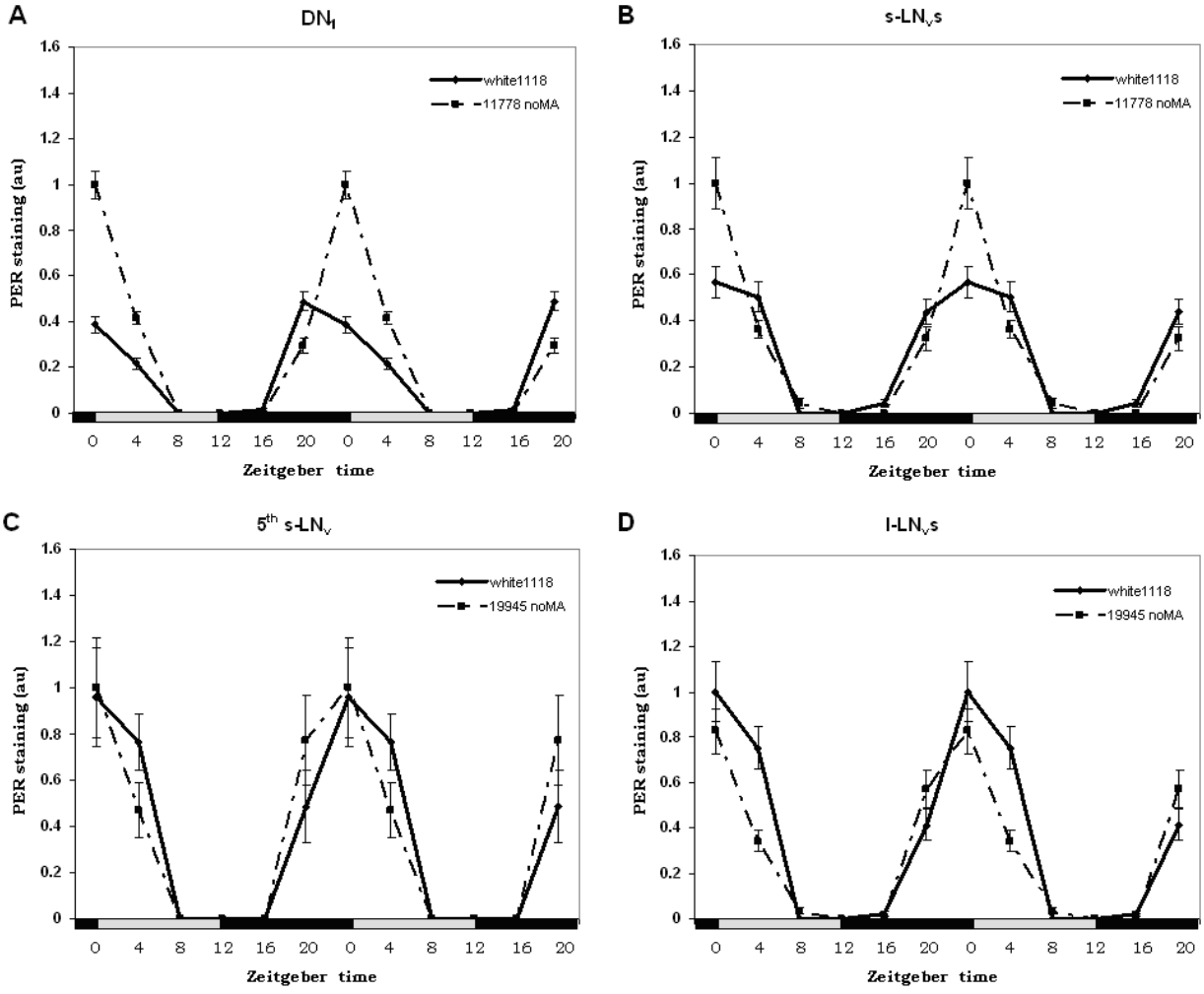


Figure 6.15: Doubleplot of Per expression in all groups of neurons in wild-type brains (w^{1118}), *#11778* arrhythmic (AR) flies and *#11778* arrhythmic flies lacking morning anticipation (MA) at day 3 in LD. Fluorescence intensity was quantified from digital images with ImageJ software. Fluorescence percentage (corrected for the background) was obtained by: I=100x(S- B)/B. ANOVA statistical analysis was used.

As expected, in the wild-type, Per expression reaches a maximum peak at ZT0 and a minimum al ZT12. On contrary, *#11778* mutant line show defects in some clusters of neurons. In particular, in DN1s (A) and in the sLNvs (B) it displays a delay in the phase of

about 8 hours while in the 5thLNv the phase is advanced. Moreover in almost all groups of neurons the total amount of Per expression seems to be reduced compared to *wild-type*.

In the locomotor activity experiment, *#11778* mutant lines showed also a reduced presence of the morning anticipation at the third day of LD. In order to verify if this effect could be due to an impaired Per expression in the neurons, 3 days old flies where monitored for three days in light-dark cycles, data were analyzed and only flies lacking the morning anticipation were collected as previously described. Brains were then dissected, stained as before and signal was quantified. Results are reported in figure 6.16.



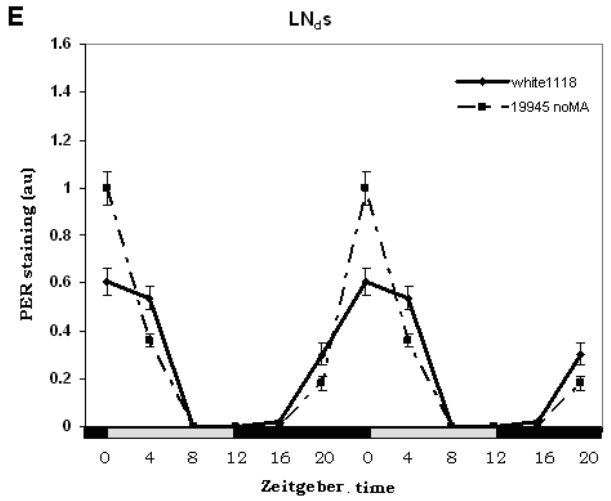
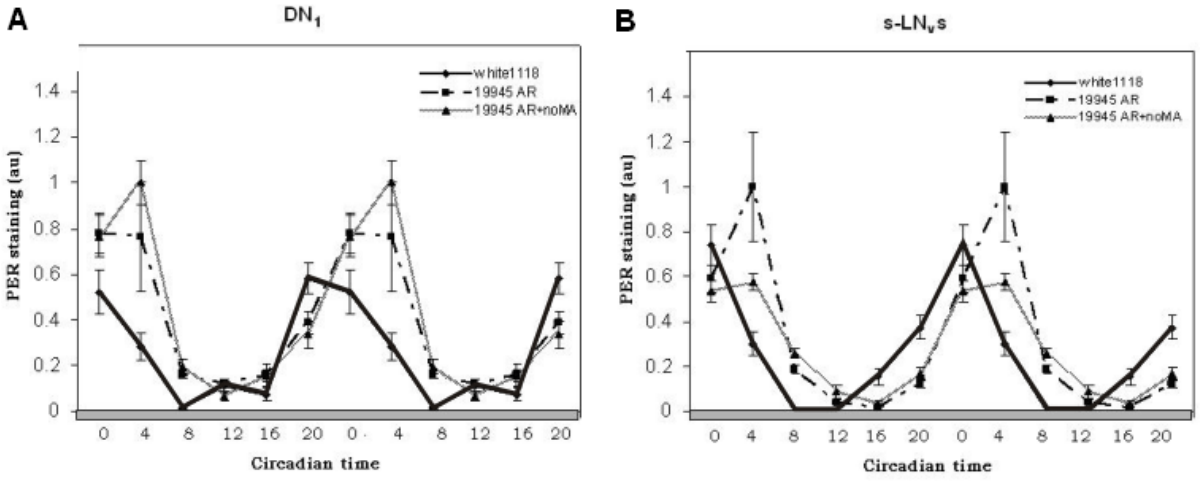


Figure 6.16: Doubleplot of Per expression in all groups of neurons in wild-type brains (w^{1118}), and *#11778* arrhythmic flies lacking morning anticipation (MA) at day 3 in LD. Fluorescence intensity was quantified from digital images with ImageJ software. Fluorescence percentage (corrected for the background) was obtained by: I=100x(S- B)/B. ANOVA statistical analysis was used.

Results reported in figure 6.18 show differences of Per expression in the Dorsal neurons (A), small Lateral neurons (B) and Lateral Dorsal neurons (E). In all these groups of neurons, Per expression is higher in the mutants compared to wild-type at the moment when the light switch on (ZT0), suggesting an higher accumulation of the protein during the dark phase. This results indicate that probably this impairment in Per expression could be responsible for the lack of the morning anticipation in this mutant line.

#19945

From the locomotor activity analysis, *#19945* mutant line appeared to be the most affected. In fact it displays 76% of arrhythmicity and a strong decrease in the presence of the morning anticipation. For these reasons the same analysis was also performed with this mutant line and results are reported in figure 6.17.



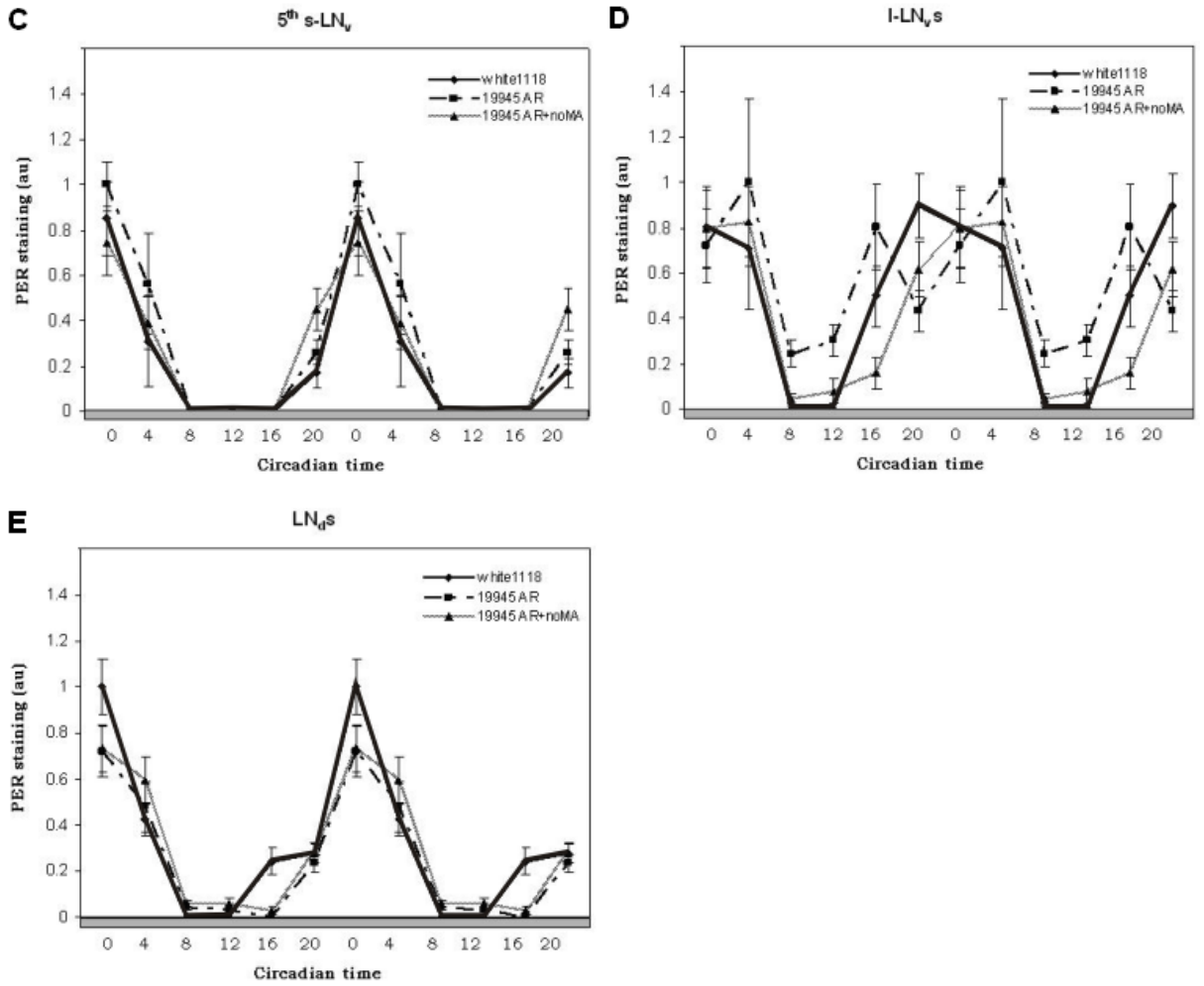


Figure 6.17: doubleplot of Per expression in DD in all groups of neurons in wild-type brains (w^{1118}), *#19945* arrhythmic (AR) flies and *#19945* arrhythmic flies lacking morning anticipation (MA) at day 3 in LD. Fluorescence intensity was quantified from digital images with ImageJ software. Fluorescence percentage (corrected for the background) was obtained by: I=100x(S- B)/B. ANOVA statistical analysis was used.

In *#19945* flies Per expression (for both arrhythmic flies and arrhythmic without morning anticipation) is comparable to *wild-type* in all group of neurons except s-LNvs and DN1. In fact, in these two groups Per expression is considerable higher and, more important, the peak is shifted. In particular in the sLNvs there is a delay in the peak of expression of about 8 hours while in DN1s only arrhythmic flies present a delay of about 4 hours. This results suggest that the impairment in the locomotor activity of this mutant line could be due to an impairment in the expression of Per protein.

In the locomotor activity experiment also *#19945* mutant lines showed also a reduce presence of the morning anticipation at the third day of LD. For this reason, as previously described, flies lacking the morning anticipation were collected and Per staining in the brains was analyzed.

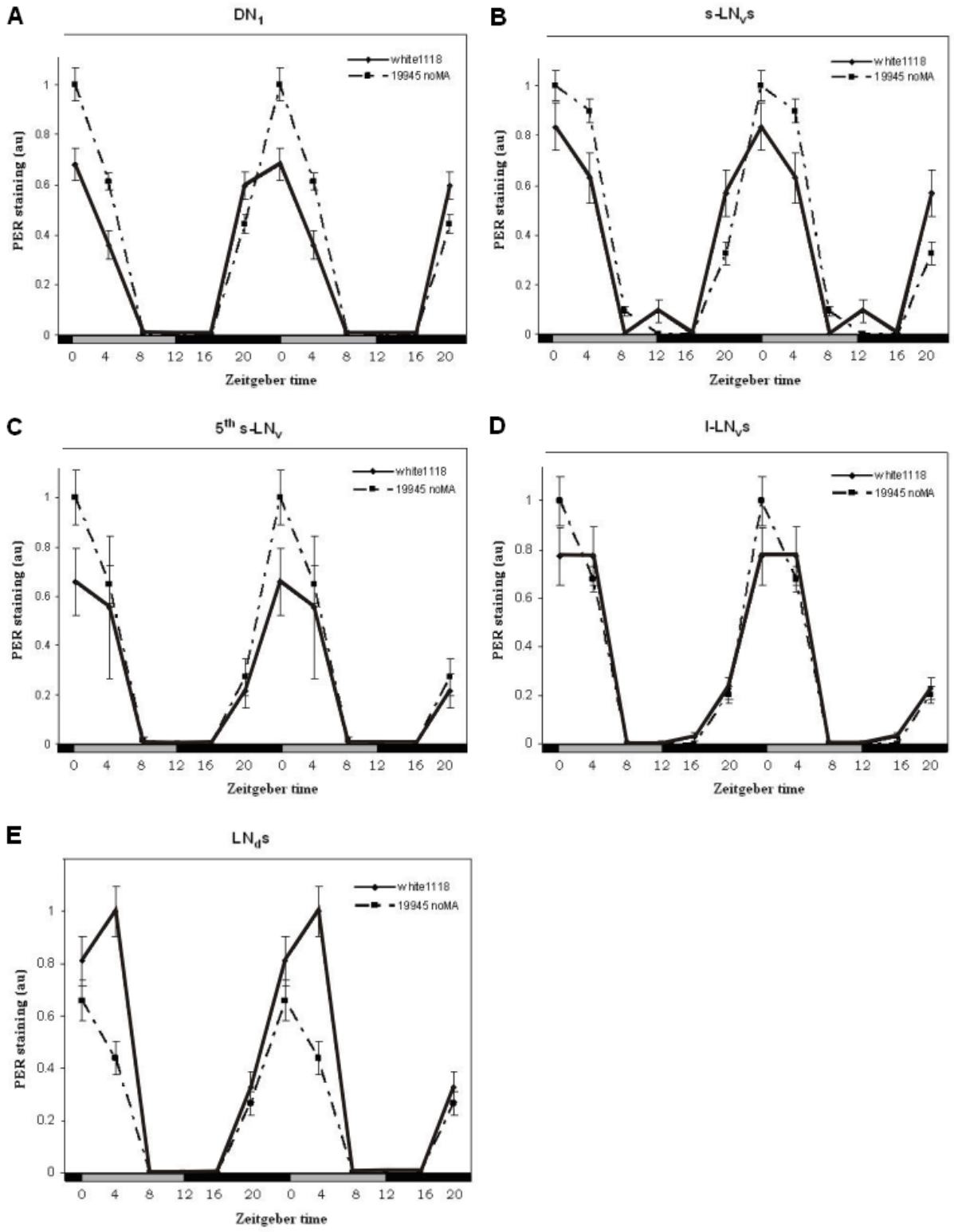


Figure 6.18: Doubleplot of Per expression in all groups of neurons in wild-type brains (w^{1118}), and *#19945* arrhythmic flies lacking morning anticipation (MA) at day 3 in LD. Fluorescence intensity was quantified from digital images with ImageJ software. Fluorescence percentage (corrected for the background) was obtained by: I=100x(S- B)/B. ANOVA statistical analysis was used.

Results reported in figure 6.18 show differences of Per expression in the dorsal neurons (A) and Lateral Dorsal neurons (E). In the Dorsal Neurons, Per expression is higher in the mutants compared to wild-type when the light switch on (ZT0) while in the Lateral Dorsal neurons Per

expression is lower that the wild-type 4 hours after lights on, suggesting an higher instability and degradation of the protein. This results indicates that probably this impairment in Per expression could be responsible for the lack of the morning anticipation in this mutant line.

DISCUSSION I

The synchronization of the circadian clock with the environmental changes is achieved by light dependent degradation of the clock protein TIMELESS (Tim) via the blue light photoreceptor CRYPTOCHROME (Cry) (Stanewsky et al., 1998). Flies carrying mutations in the *cryptochrome* gene as cry^b and cry^m, show defects in their synchronization to light (Stanewsky et al., 1998; Busza et al., 2004). On the other hand, overexpression of *cry* enhances the light responsiveness of the clock (Ishikawa et al., 1999; Emery et al., 2000). In the presence of light, Cry interacts with Tim which is subsequently phosphorylated and degraded (Naidoo et al., 1999; Koh et al., 2006) and this event leads to the "resetting" of the clock. Cry is also degraded in the light but whereas the degradation of Tim is well characterized (Busza et al., 2004 Lin et al., 2001; Peschel et al., 2006; 2009), the mechanism involved in the regulation and light-dependent degradation of Cry is mostly unknown. Moreover, it has been reported that *Drosophila* Cry doesn't function only as a light responsive protein but also as a transcriptional repressor required for the correct specification of clock cells and for the oscillation of peripheral circadian clocks (Collins et al., 2006).

A study was initiated in our laboratory, in order to find new molecular partners of Cry, both in light and dark conditions. This led to the identification of new genes putatively involved in the circadian clock machinery of *Drosophila melanogaster* both in its fine-tuning and synchronization to external cues. Isolating new proteins that interact with Cryptochrome will help to understand how the diverse activities of this protein are regulated, and will lead to the identification of clock components that might have tissue-specific roles.
A co-immunoprecipitation approach, followed by mass spectrometry analysis, led to the identification of the protein Belle. Belle is an RNA helicase previously described to have a fundamental role in the sRNA mechanism. It was chosen as candidate for further investigation as it was reported that, in *Neurospora crassa*, an RNA helicase named FRH (Frequency interacting RNA Helicase) has a crucial role in the circadian clock, as it interacts with Frequency (FRQ) and the WHITE-COLLAR complex (WCC) to mediate negative feedback control (Shi et al., 2009; Hunt et al., 2010).
First of all, the interaction between Cry and Belle was verified by immunoprecipitation of head extracts from flies overexpressing a tagged version of the cryptochrome, HACRY. Results showed that the interaction between the two proteins occurs in the dark and it is maintained after light exposure. This data was supported by the results of experiments aimed at the immunolocalization of Belle in the *Drosophila* adult brains. By using a Belle-GFP tagged strain, we found that Belle is expressed in the glial cells in the optic lobe, were also Cry is present (Yoshii et al., 2008). The co-localization of the two proteins in certain brain areas, corroborates the idea of a functional interaction between Cry and Belle.

Key circadian clock components oscillate in 12:12 light:dark conditions with a period of about 24 hours. To test if Belle may be a circadian component, its expression was temporally characterized. Western Blot analysis on heads extracts didn't show statistically significant results, although Belle expression seems to be high during the light hours and decrease during the dark time. It is important to underline that whole head extracts comprise also structures of the eyes not visualized in the ICC experiments, that probably express Belle but not in a

circadian manner. To test whether Belle levels oscillate in the 24 hours, protein expression should be specifically monitored in the glial cells by using the BelleGFP line and searching for circadian variations of GFP levels.

belle gene expression during the 24 hours was evaluated by Real Time PCR on total RNA extract from heads. We observed a circadian oscillation of belle mRNA, with levels lower during the day and higher during the night, and a peak of expression at lights off. Moreover, in absence of light stimuli belle mRNA continues to oscillate, an important feature of clock genes, whose rhythmic expression is maintained also without a light input. These results suggest an involvement of the circadian machinery in the regulation of *belle* expression. Moreover, interestingly *belle* expression pattern mirrors CRY expression profile in LD, which is low during the day and higher during the night (Stanewsky et al., 1998).

In order to further investigate if *belle* is regulated by the clock, *belle* expression pattern was analyzed in heads of flies mutant for 2 main clock components, *period* and *timeless* (per^0 and tim^0 mutants), both in light and dark. In these mutants, *belle* oscillates in LD, but with a phase delayed compared to wild-type, suggesting that these genes contribute to regulate *belle expression*. In absence of light the oscillation is completely abolished, suggesting that also light has an effect on the regulation of this gene. For this reason it is possible to hypothesize the presence of two regulatory mechanisms: an intrinsic one, mediated by clock components, and an extrinsic one, light dependent.
It is worthy to notice that the oscillation of Per and Tim proteins occur with the same phase as that of *belle* mRNA, and this is in agreement with the hypothesis that Per and Tim can promote *belle* circadian transcription. This hypothesis is supported by the fact that in absence of Per and/or Tim, *belle* expression is deregulated.

In order to verify if Belle is functionally required for clock functioning, the effect of *belle* downregulation on the fly circadian behaviour was studied, by using a *belle* RNAi line and specifically driving the knock down in desired cells.
belle knock down in *tim* expressing cells (comprising all circadian neurons) resulted to be lethal at the larval stage, as the belle mutant (Johnstone et al., 2005), and this could be due to the fact that *tim* is expressed in the embryo, where Belle is necessary for vitality (Johnstone et al., 2005). However it was possible to characterize the photophobic behaviour of larvae (Mazzoni et al., 2005). We observed that the knock down of *belle* causes a reduction in the photophobicity of larvae compared to the wild type, the same as per^0 and tim^0 clock mutants. The clock is necessary to mediate the photophobicity behaviour and these results clearly demonstrate the role of Belle in light avoidance.

Due to the lethality of *belle* knock down in all circadian cells, different drivers were used to direct *belle* RNAi only in some precise adult brain neurons, without alter the expression in other organism districts important for vitality. In particular *belle* down regulation was driven in the optic lobe (gmrGAL4 and ninaEGAL4), where also *belle* is expressed, in a subset of

circadian neurons and some photoreceptor cells (cryGAL4) and in a restricted portion of circadian cells, specifically 4 sLNvs and 1LNv (pdfGal4).
The progeny obtained from these crosses was vital and we therefore analyzed these flies for circadian locomotor activity in dark conditions.
We observed that the downregulation of belle in those cells where the two proteins
Results showed an impaired rhythmicity in *gmr*- and *ninaE*- knock down flies. In particular, in these individuals the percentage of rhythmic flies is strongly reduced compared to the wild-type. Also the downregulation in Cry positive cells (which comprises also some photoreceptor cells) causes an impairment in the circadian locomotor activity, albeit less pronounced. The specificity of this phenotype is confirmed by the fact that when we drive the knock-down in pdf positive cells, where *belle* is not expressed, no effects on the rhythmicity are observed.
Our results suggest that Belle and Cry, although expressed together in some brain cells and forming stable complexes, do not act in the same pathway in the circadian photoreception of adult flies. In fact, it is well established that, in contrast to *belle*-RNAi flies, Cry mutants are perfectly rhythmic in constant conditions (Stanewsky et al., 1998; Dolezelova et al., 2007). However we can still reasonably hypothesize a role for Belle in the generation of circadian rhythmicity.
In order to further understand the role of Belle in the circadian mechanism, *belle* P-element mutant lines were used. In these lines *belle* expression is comparable to wild type in terms of mRNA level, but the oscillation in the 24 hours is abolished. The same lines were also tested for circadian locomotor activity in LD and DD conditions and they displayed an alteration in the phenotype both in light-dark cycles, with a lack of the morning peak, and in constant dark conditions, with a strong impairment in the percentage of rhythmicity. These data suggest that the oscillation of *belle* mRNA has an important role in the generation of the rhythmic phenotype, as the deregulation of the gene is *per se* sufficient to alter a phenotype.

Based on the hypothesis that the impairment of the circadian locomotor activity in these mutants could be due to an altered circadian expression of some key clock components, we decided to characterize, by immunocitochemistry, the oscillation of Period in the different clusters of neurons known to be responsible for the generation of the rhythmic behaviour. In particular we analyzed separately flies showing either an alteration of the rhythm in entrainment conditions (i.e. absence of morning anticipation), and/or absence of rhythmicity in free running. We revealed that, in all cases, the expression of Per is altered in the mutants compared to the wild-type, in two clusters of neurons, the sLNvs and the DN1s. In particular, in these cells we observe a difference in the expression levels of the protein when the flies are in the entrainment phase, and, more notably a difference in the phase of its expression when the flies are in free-running.
This strongly suggests that the deregulation of the *belle* gene is sufficient to alter the circadian rhythm, at both molecular and behavioural levels, and supports the idea that Belle likely has a role as a component of the central clock.

The point that remains to be investigated is the following: Belle together with Cry is expressed in the glial cells in the optic lobe and Tim and Per are expressed in the circadian neurons. How could these two groups of neurons communicate?
It is reported that glial cells are associated with the clock neurons (Helfrich-Forster, 1995), contain a clock independent from that of the lateral neurons (Zerr et al., 1990), and could play a role in the normal functioning of the circadian clock (Ewer et al., 1992). A recent study shows indeed that glia cells are required for normal circadian locomotor rhythms (Suh and Jackson, 2007).
Moreover, studies have shown that subtypes of mammalian glia (astrocytes) can regulate the excitability of neurons through the regulated release of ‘‘gliotransmitters’’ (glutamate, ATP, cytokines. Adenosine and growth factors), and it has become clear that there are reciprocal neuron-glia signalling systems that regulate neuronal excitability (Fields and Burnstock, 2006; Haydon, 2001; Haydon and Carmignoto, 2006).
Therefore, it is possible to hypothesize a communication between Belle and clock proteins through signal molecules release.

The aim of this work was to identify novel molecular mechanisms that regulates Cry activity. Belle physically interacts with Cry both in light and dark but is seems to have also a role *per se* in the molecular mechanism of the central clock. As reported, Cry interacts with Tim and promotes its degradation but it seems also to interact with Belle. Moreover *belle* transcription is regulated by Per Tim and the light stimulus.
Belle is also requested to stabilize Per (as shown by ICC experiments) and probably Tim. So far, we still don’t know what is the exact mechanism underlying this regulation but we hypothesize that the RNA binding protein activity of Belle could stabilize *per* mRNA. Moreover the mutual activation of Per/Tim and *belle* and the fact that both of these proteins are inhibited by light could suggest that they are a part of a feedback mechanism. In fact in the dark they stabilize each other.
Finally our study suggests that Belle, together with CRY, might be involved in the molecular clock of glial cells as well as in their synchronization to LD cycles; albeit this has to be proved in further studies.

RESULTS II

7.1 Role of the Kinase Aurora A in Cry regulation

Phosphorylation is one of the most common post-translational regulation systems in circadian clocks, and modulates both rhythmic expression of molecular elements and response to light (Mehra A. et al., 2009)

Previous experiments of immunoprecipitation and in vitro phosphorylation carried out in our laboratory showed that HACRY is specifically recognized and phosphorylated by the Serine Threonine kinase Aurora A. Moreover, mass spectometry analysis showed that Aurora A phosphorylates HACRY at the C-terminus, and in particular two Serine residues, respectively S529 and S540, belonging the SLIT and SNEE consensus motifs. This result was also validated in vitro with synthetic peptides, showing that the substitution of the Serine 529 with Alanine causes the complete abolishment of the kinase activity. Taken together these results indicate that these two residues are specific substrates of Aurora A kinase.

In order to verify the role of Aurora A kinase in HACRY phosphorylation and regulation, a study was started and two parallel approaches were used:

- Study of *Drosophila* lines overexpressing Aurora A kinase
- Study of *Drosophila* mutant lines with mutations at the level of the two Serines, putative targets of phosphorylation

7.2 HACRY-Aurora interaction

In order to detect an interaction between HACRY and Aurora A *in vivo*, a co-immunoprecipitation assay was performed. The UAS-HACRY line was crossed with the driver timGAL4 to induce an overexpression of HACRY in *tim* positive cells. Individuals obtained were collected in the dark (ZT24) and after 15 minutes of light exposure. Protein extracts were prepared from fly heads and an HA matrix was used to pull down HACRY. The protein Aurora A was detected by western blot with a specific antibody (from Berdnik and Knoblich, 2002).

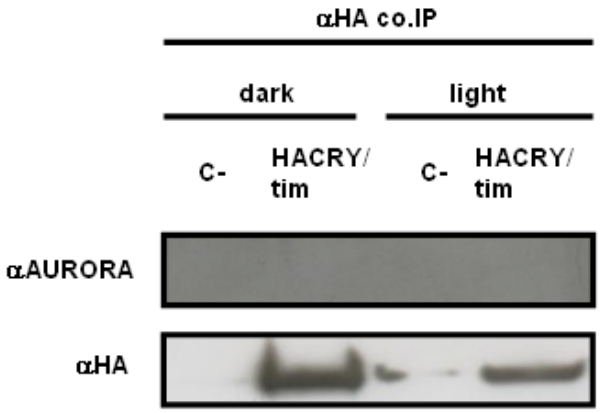


Figure 7.1: Co-immunoprecipitation and western blot for the detection of the interaction between HACRY and Aurora in dark and light in three days old flies over expressing HACRY (*tim*GAL4/+; UAS-HACRY/+) in the circadian neurons. *tim*GAL4 flies were used as control. Heads were collected at ZT24 and after a 15 min light pulse given at the same time point.

As shown in Fig. 7.1, no signal corresponding to Aurora A was observed, while the presence of HACRY in the immunoprecipitate is revealed by the anti-HA antibody. Thus, no interaction was detected between the two proteins, neither in the dark nor in the light.

In order to clarify if the previous result was due to a technical problem, the opposite approach was followed, by using a transgenic line (*#0466* from Berdnik and Knoblich, 2002) overexpressing, always with the UAS-GAL4 system, an Aurora-GFP chimeric protein in *tim* positive cells.

Individuals were then collected, as before, in the dark and after 15 minutes of light exposure, for Aurora A over-expressing strain and only in the dark for control flies. Protein extracts were prepared from fly heads, a GFP matrix (Vector laboratories) was used to pull down Aurora-GFP and the protein Cry was detected by western blot with a specific αCry antibody (Alpha Diagnostic Int.).

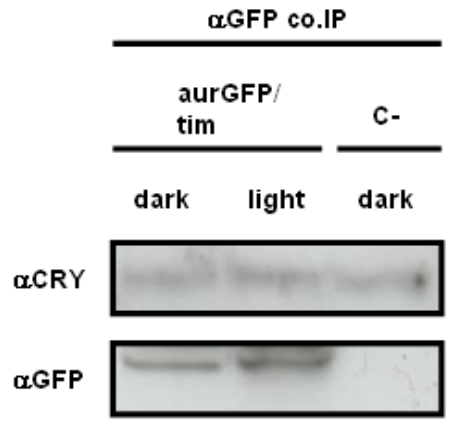


Figure 7.2: co-immunoprecipitation and western blot for the detection of the interaction between HACRY and Aurora in dark and light in three days old flies overexpressing *aurora*-GFP (*tim*GAL4/+; UAS-*aurora*GFP) in the circadian neurons. *tim*GAL4 flies were used as control. Heads were collected at ZT24 and after a 15 min light pulse. αGFP matrix was used (Vector laboratories).

As shown in Fig. 7.2, a signal corresponding to Cry protein was detected, but since it is present also in the negative control it was considered as aspecific signal. In conclusion, also with this approach, no interaction was detected between Aurora and Cry, neither in the dark nor in the light.

7.3 Characterization of Aurora A overexpressing lines and *aurora a* mutants

In order to underline a role of Aurora A kinase in the circadian molecular mechanism in *Drosophila*, several flies lines where used for molecular and behavioural experiments. In particular: a) a line obtained by EMS mutagenesis (*#6188*-Bloomington Stock Centre) which is vital only in heterozygosis, b) a line with a p-element inserted in the 5'UTR (*#15904* Bloomington Stock Centre), and 2 UAS*aurora* lines (*#0466* and *#0467*) (Berdnik and Knoblich, 2002) that, when crossed with the *tim*Gal4 driver, induce the overexpression of Aurora A in the *tim* positive circadian neurons.

Analysis of locomotor activity in constant dark

The analysis of the locomotor activity in absence of external stimuli is a standard protocol to evaluate the functionality of the circadian clock.

For this reason, the above described lines were first entrained for 3 days in 12:12 light:dark cycles at constant temperature and then monitored for 7 days at constant darkness conditions. For each genotype the percentage of rhythmicity and the average period (τ) were quantified and results are reported in table 1.

GENOTYPE	% RHYTHMIC FLIES	AVERAGE t (± SEM)
P element *aur*-mutant (#15904)	100 (12/12)	23.89 ± 0.27
EMS-*aur*-mutant(#6188)	100 (31/31)	23.61 ± 0.14
UASaur/timGAL4 (#0466)	100 (24/24)	24.7 ± 0.2
UASaur/timGAL4 (#0467)	100 (29/29)	24.77 ± 0.11
*tim*Gal4/w^{1118}	100 (20/20)	24.46 ± 0.11

Table 1: Locomotor activity analysis of *aurora* mutants and Aurora A over expressing flies in constant dark (DD) conditions.

All lines show a normal rhythmicity in absence of light stimuli and the average period is comparable to control. This indicates that an endogenous clock is still present and functional in the strains analyzed.

Analysis of locomotor activity in constant light

Another fundamental feature of circadian clocks is the circadian photoreception. Drosophila perceives the light inputs through three different pathways: by retinal photoreceptors (compound eyes and ocelli), by extraretinal photoreceptors (the Hofbauer-Buchner (HB) eyelet) and by the Cryptochrome (Cry) inner photoreceptor.

Light input resets the clock, in fact wild-type flies become soon arrhythmic in LL, because of the constitutive activation of Cry by light, resulting in the absence of Tim accumulation. Flies mutants for one or more component of the photoreception system show defects in the circadian behaviour in constant light conditions. An important and unique characterized phenotype of the cry^b and cry^0 mutants was the persistence of a strong rhythmicity under constant light conditions (LL) (Stanewsky et al., 1998; Dolezelova et al., 2008).

For these reason, in order to verify a possible role of Aurora A kinase in the circadian photoreception, the same lines used previously were monitored in constant and saturating light intensity (about 800 lux) conditions. Results were quantified as described above and are reported in tab 2.

GENOTYPE	% ARRHYTHMIC FLIES	% RHYTHMIC FLIES	AVERAGE t (± SEM)
P element *aur*-mutant *#15904*	90.5 (19/21)	9.5 (2/21)	23.89 ± 0.27
EMS-*aur*-mutant/+ *#6188*	80.4 (41/51)	19.6 (10/51)	23.61 ± 0.14
tim-aur (1) *#0466*	62 (24/39)	38 (15/39)	25.7 ± 0.2
tim-aur (2) *#0467*	50 (18/36)	50 (18/36)	24.77 ± 0.11
*tim*Gal4/w^{1118}	91 (22/24)	9 (2/24)	24.46 ± 0.11

Table 2: Locomotor activity analysis of *aurora* mutants and Aurora A overexpressing flies in constant light (LL) conditions. t*im*Gal4 line crossed with w^{1118} was used as positive control.

In this case, p-element insertion line *#15904* and EMS mutant line *#6188* show a low percentage of rhythmicity, comparable to the control strain. Aurora A overexpressing lines (*#0466* and *#0467*), instead, show a high percentage of rhythmicity in constant light (38% and 50%), indicating an impairment of these individuals in the light perception or in the resetting of the clock after light exposure. This data suggest a possible role of Aurora A kinase in the circadian photoreception mechanism.

Analysis of the Phase Response Curve

In wild-type flies, short light pulses can reset the clock at the molecular level. A useful description of the phase-dependent response of the clock to light is the Phase Response Curve (PRC). Flies released under free-running conditions (ie: DD conditions) show different responses to light pulses given at different times of the subjective day or night, with the responses reflecting the status of the clock at that precise time. Light pulses given during the subjective day do not affect the clock and this part of the PRC is thus called "dead zone". Short light pulses given in the early subjective night phase delay the activity of the flies, as if the light phase of the day was prolonged. In contrast, light pulses given in the late subjective night phase advance the activity of the flies, as if the day was starting earlier. These effects are due to Tim degradation: in the early night Tim levels are low (Tim starts to accumulate when the light switch off) and a short pulse of light causes its degradation and a delay in the subsequent accumulation; on the other hand, a short pulse late in the night, when Tim levels are high, causes a rapid degradation and for this reason an advance in the phase of the clock. In *cry*^{b} mutants these responses to light stimuli were lost, their molecular clock was thus insensitive to short light pulses (Stanewsky at al., 1998; Emery et al., 2000).

UAS*aur*/*tim*Gal4 lines and UAS*aur*/*w*^{1118} were entrained for three days in standard light:dark conditions at constant temperature. During the 3rd night a short pulse of light (20 minutes) was given (in independent experiments) at different moments during the night: ZT15, ZT17, ZT19, ZT21, ZT23. Flies were then kept in constant darkness for 7 days and anticipations or delays were quantified on the 3rd day in constant darkness conditions.

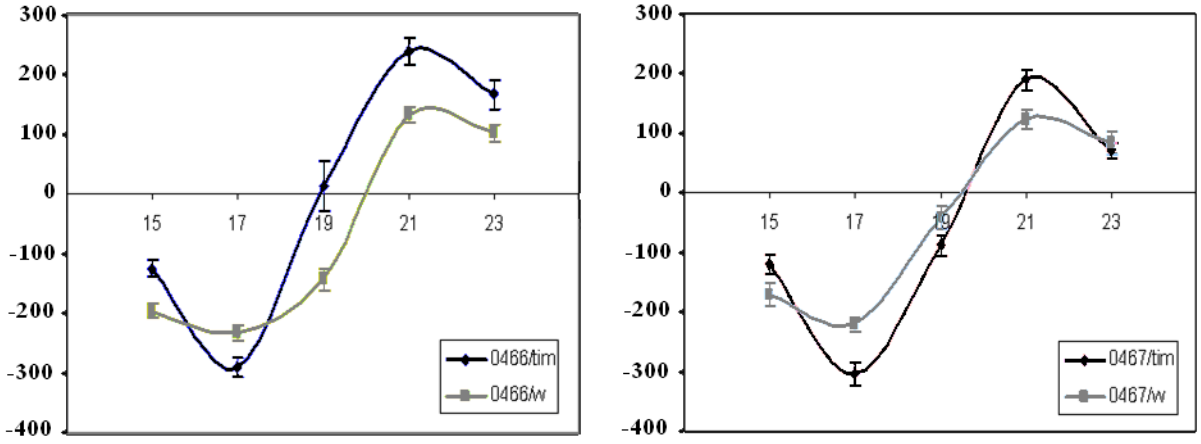


Figure 7.3: Phase Response Curve (PRC) graph of 2 Aurora A over expressing lines and respective controls. Phase advances are represented as positive values, phase delays are represented as negative values. Advance or delays (represented in minutes) are calculated as difference in the phase between flies subjected to light pulses and flies of the same phenotype maintained in constant dark (DD).

Results shown in figure 7.3 indicate delay (negative values) and anticipation (positive values) in the phase of both Aurora A overexpressing lines (*#0466* and *#0467*) together with respective controls. Both Aurora A overexpressing lines and controls display the maximum delay response at ZT17 and the maximum anticipation response at ZT21, but in Aurora A

overexpressing lines the light pulse elicits higher effect. Indeed, both at ZT17 and ZT21, overexpressing lines show an higher response compared to respective controls, indicating an higher sensitivity to light stimulus. This results indicate again a possible role for the kinase Aurora A in light perception.

Study of dCRY stability

cry gene is rhythmically expressed, with a maximum at the beginning of the day (ZT1-6). Cry protein levels oscillate only in LD cycles while in constant light the protein is rapidly degraded and in constant dark it accumulates constantly (Emery et al., 1998).

In order to verify if there is an effect of Aurora A on Cry light-dependent degradation, the stability of the photoreceptor was analyzed in flies over-expressing Aurora A in circadian neurons. In particular, flies were kept for 3 days in constant darkness, to promote the accumulation of Cry, and then collected in the dark (CT24) and after 15 minutes, 30 minutes and 1 hour of light exposure. Head protein extracts were subjected to western-blot and the endogenous Cry detected with an αCry antibody (Alpha Diagnostic).

In figure 7.4, the result obtained for line *#0466* is reported.

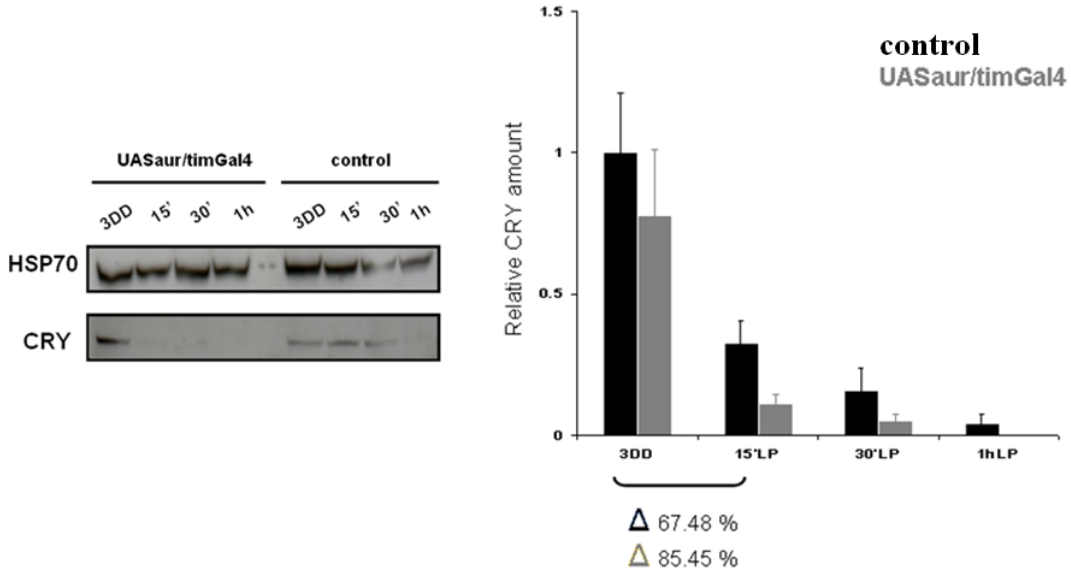


Figure 7.4: Analysis of Cry degradation in Aurora A over expressing line *#0466*. Representative western blot of Cry and HSp70 signal in UAS*aur*/*tim*Gal4 and control flies (left). Average of 3 replicates of relative Cry amount (right).

Cry expression in the Aurora A overexpressing line *#0466* is comparable to control in the dark. However, after progressive light exposure intervals, Cry degradation is more prominent

in these line compared to control, and this effect is already evident after 15 minutes of light exposure. This result suggests that the enhanced kinase activity results in a more rapid degradation of Cry, and indicating that Aurora A kinase could have a role in Cry degradation.

In Aurora A overexpressing line *#0467,* however, the stability of Cry seems not to be affected. In fact, in figure 7.5 is reported a western blot showing that there are no difference either in Cry accumulation in the dark and degradation after light exposure, in the two kind of lines.

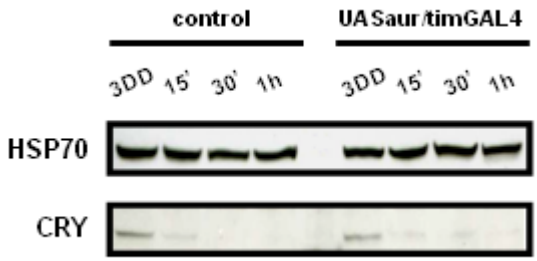


Figure 7.5: Analysis of Cry degradation in *A*urora *A* over expressing line *#0467*. Representative western blot of CRY and HSp70 signal in UAS*aur*/*tim*Gal4 and control flies.

7.4 Analysis of HACRY variants

Previous mass spectrometry experiments showed that Aurora A phosphorylates HACRY at the C-terminus and in particular 2 Serine residues (S529, S540) belonging to the SLIT and SNEE consensus motifs. In figure 7.6 sequences covered by the analysis are reported (red) and the two Serines targets of phosphorylation are underlined.

tag HA

MYPYDVPDYA SPEFMATRGA NVIWFRHGLR LHDNPALLAA LADKDQGIAL 50
IPVFIFDGES AGTKNVGYNR MRFLLDSLQD IDDQLQAATD GRGRLLVFEG 100
EPAYIFRRLH EQVRLHRICI EQDCEPIWNE RDESIRSLCR ELNIDFVEKV 150
SHTLWDPQLV IETNGGIPPL TYQMFLHTVQ IIGLPPRPTA DARLEDATFV 200
ELDPEFCRSL KLFEQLPTPE HFNVYGDNMG FLAKINWRGG ETQALLLLDE 250
RLKVEQHAFE RGFYLPNQAL PNIHDSPKSM SAHLRFGCLS VRRFYWSVHD 300
LFKNVQLRAC VRGVQMTGGA HITGQLIWRE YFYTMSVNNP NYDRMEGNDI 350
CLSIPWAKPN ENLLQSWRLG QTGFPLIDGA MRQLLAEGWL HHTLRNTVAT 400
FLTRGGLWQS WEHGLQHFLK YLLDADWSVC AGNWMWVSSS AFERLLDSSL 450
VTCPVALAKR LDPDGTYIKQ YVPELMNVPK EFVHEPWRMS AEQQEQYECL 500
IGVHYPERII DLSMAVKRNM LAMKSLRNSL ITPPPHCRPS NEEEVRQFFW 550
LADVVV

Figure 7.6 HACRY amminoacidic sequence. In red is represented the sequence covered by mass spectrometry analysis. Sites of Aurora phosphorylation are underlined.

In order to test *in vivo* the specificity of this residues in Cry regulation and/or stability, 4 different transgenic mutant lines were generated:

Single mutants:

1 UAS-HACRY S529A: substitution of Serine 529 with Alanine, a non-phosphorylable aminoacid
2 UAS-HACRY S529D: substitution of Serine 529 with Aspartate, an amino-acid that mimics a constitutive phosphorylated state
3 UAS-HACRY S540A: substitution of Serine 540 with Alanine, a non-phosphorylable aminoacid.

Double mutant:

4 UAS-HACRY S529A S540A: substitution of both Serine 529 and 540 with Alanine.

All these lines are vital and fertile and the presence of the specific mutations was verified by gene sequencing.

7.4.1 Single mutants

Single mutant lines were used for both molecular and behavioural experiments. In this experiment we analyzed 4 lines in which the transgene has been placed in a *cry* null background, in order to characterize only the effect of the mutated form of Cry.

Analysis of HACRY stability

In order to analyze whether the specific mutation alters HACRY stability after light exposure, western blot experiments were performed and signal was detected with the specific αHA antibody. In particular, UAS-HACRY mutants (SLIT and SNEE mutants) were crossed to the *tim*GAL4 driver and the progeny was entrained for three days in standard light-dark conditions. Individuals were then collected at ZT24 and after 30', 1h and 3h of light exposure. As control the progeny resulting from UAS-HACRY crossed with *timGAL4* was used.

Results obtained are shown in figure 7.7.

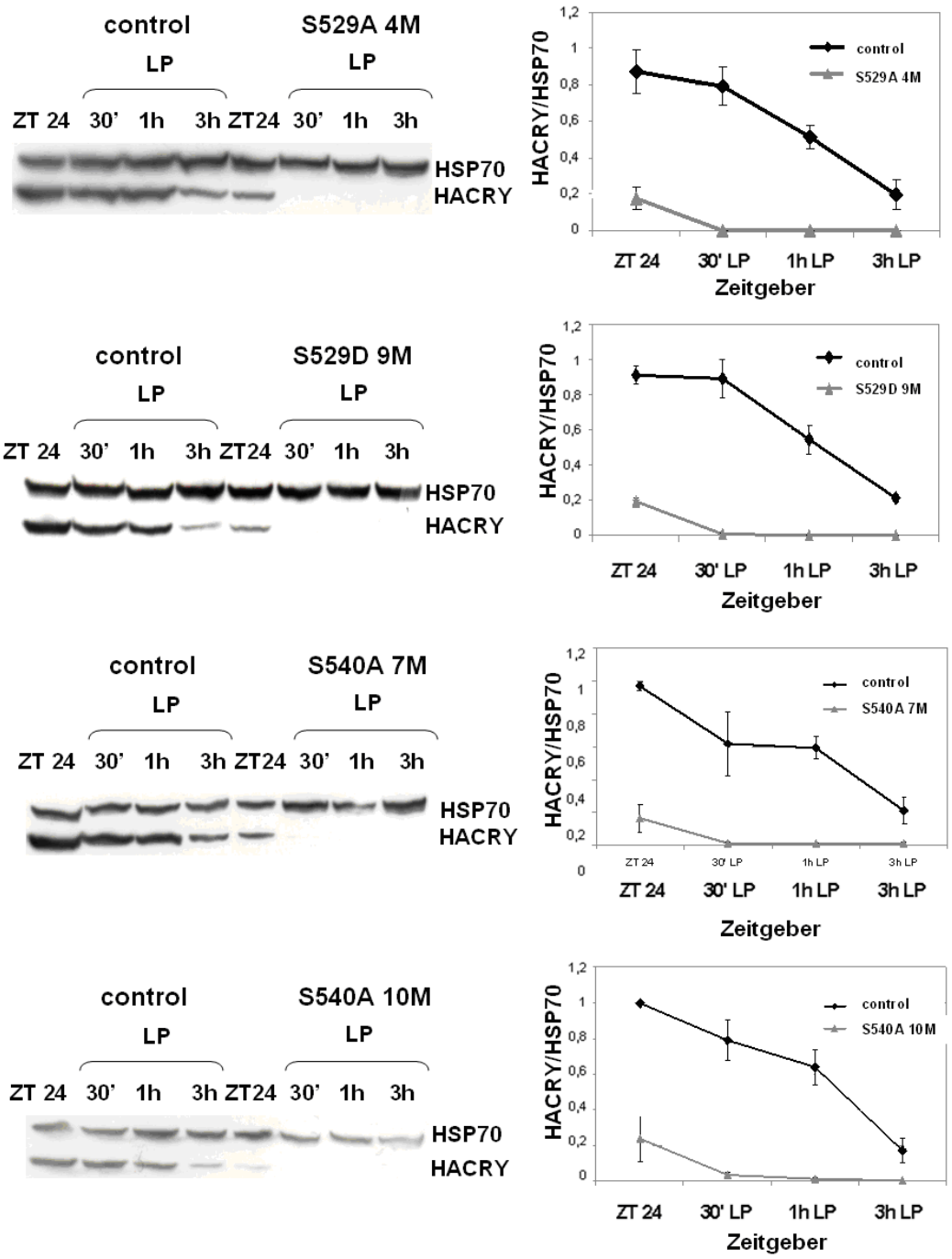


Figure 7.7: HACRY degradation analysis in single mutants and control flies. HSP70 and HACRY signals detected by western blot (left) and average HACRY expression (3 replicates) (right).

Western blot analyses reveal that all the lines express the transgene. However, in control line (left) HACRY expression is high in the dark (ZT24) and it is progressively degraded after light exposure. On contrary, transgenic lines show a reduced expression already at ZT24 compared to control, and the protein is then completely degraded after 30 min of light exposure. Graphs on the right show the quantification obtained by normalization of HACRY signal on HSP70 signal, an housekeeping protein, and results reported are the average of three independent experiments. Graphs show that for all transgenic lines (grey) HACRY signal is

lower that control line signal (black). However light seems to have the same effect in all mutant lines, promoting a rapid degradation of the protein. These results indicate that no differences, in terms of HACRY stability, can be observed when Serines residues are mutated.

These results highlighted the fact that HACRY amount, in the mutants, is reduced compared to control lines, already in the dark before light exposure. This effect could either be due to a reduced transgene expression in the mutagenized lines or to a higher instability of HACRY protein in the dark. In order to discriminate between these two hypothesis, an RNA levels quantification was performed by Real-time PCR. For this purpose, only two transgenic lines, one with the mutation with Alanine (S540A 10M) and one with Aspartate (S529S), were characterized as examples. Flies were collected at ZT 24 when the expression of *hacry* is at the maximum level and RNA was extracted from heads. *hacry* signal was quantified on the reference gene *rp49*, whose expression is constant during the 24 hours. Results obtained are reported in figure 7.8.

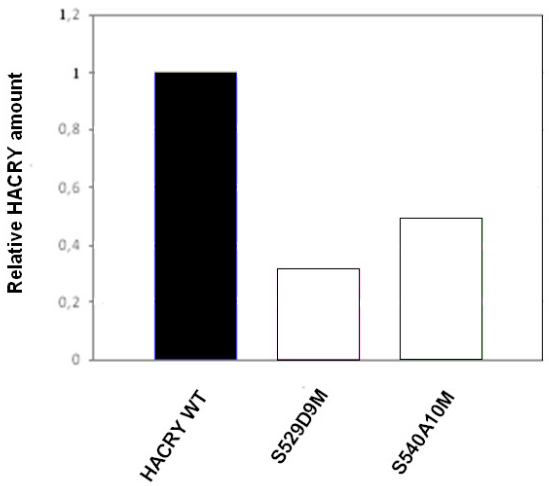


Figure 7.8: Analysis of transgene expression on heads from control line (HACRY WT) and 2 single mutant line (S529D 9M and S549A 10M).

Hacry mutated amount is reduced in these 2 lines compared to the wild-type form, suggesting that reduced western blot signal was due to a reduced transgene expression in the mutagenized lines. This result could be explained by a positional effect of the insertion of the transgene.

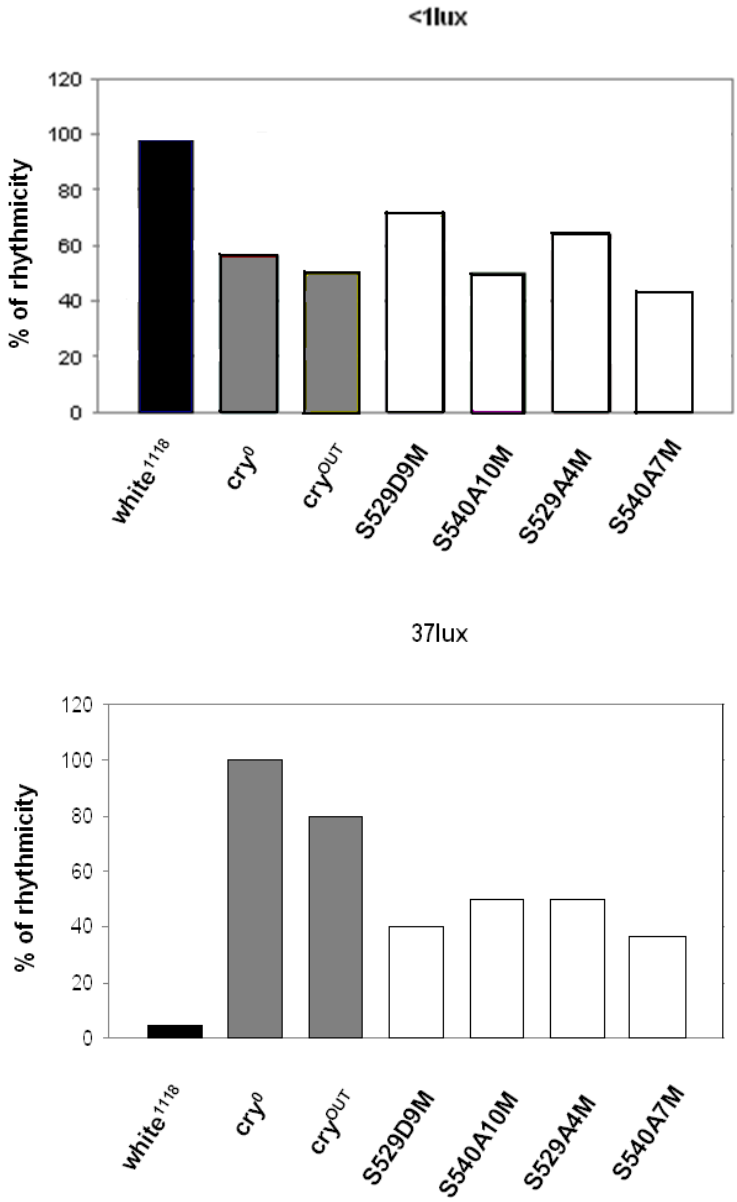
Circadian behaviour in constant light

Single mutant lines were also used in behavioural experiments, in order to characterize the response of this lines to light and eventually to detect defects in light perception.

For this reason, UAS-HACRYmut/*tim*GAL4 individuals were kept in standard 12:12 light dark cycles and then moved to constant light. In particular flies were subjected to 3 different

light intensities: <1lux, 37lux and 400lux. Wild-type flies in constant light conditions show an arrhythmic locomotor profile, due to the light sensitivity of proteins responsible for the clock resetting (Rosato et al., 2006).

In this experiments w^{1118} strain was used as positive control and cry^{0} and cry^{OUT} mutants as negative controls. Results are shown in figure 7.9.



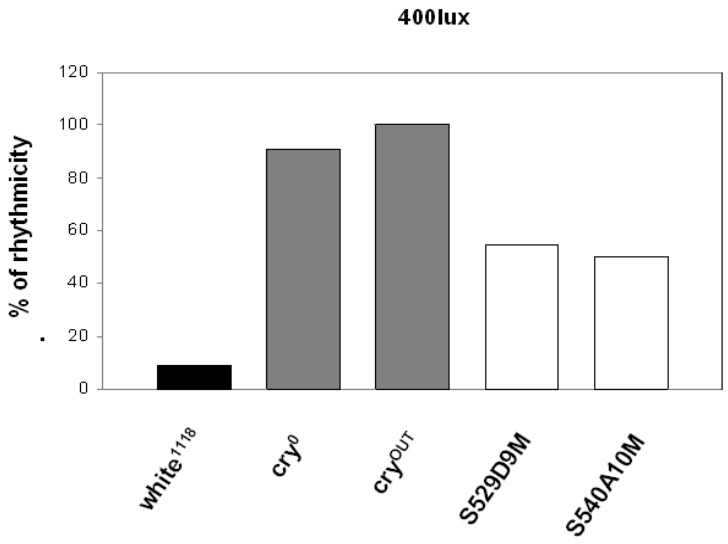


Figure 7.9: representation of percentage of rhythmicity at 3 different light intensities (<1, 37, 400 lux) in positive control (w^{1118}), negative controls (cry^0, cry^{OUT}) and single mutants (in white).

At low light intensity (dim light) wild-type flies and *hacry* mutants are rhythmic, as non saturating light is not efficient in clock resetting and is capable to entrain the clock (Dolezelova et al., 2007). However, at 37lux and 400lux wild type flies become almost completely arrhythmic, while cry^0 and cry^{OUT} mutants have an high rhythmicity, confirming that no functional Cry photoreceptor is present in these lines.

HACRYmutant lines show a percentage of rhythmicity which is higher than the wild-type but lower than *cry* null mutants and this result could be due to the lower HACRY expression in these lines compared to controls. Moreover, it is important to note that transgenic lines where Serine is replaced by Alanine show a constant percentage of rhytmicity when subjected to different light intensities, while the line where Serine is replaced byAspartate has an higher percentage of rhythmic at <1lux light which decreases with the increasing light intensity. This result suggest that the mimicking of a constitutive phosphorylation of cryptochrome impairs the light photoreception at low light intensities, but not at higher, saturating, light.

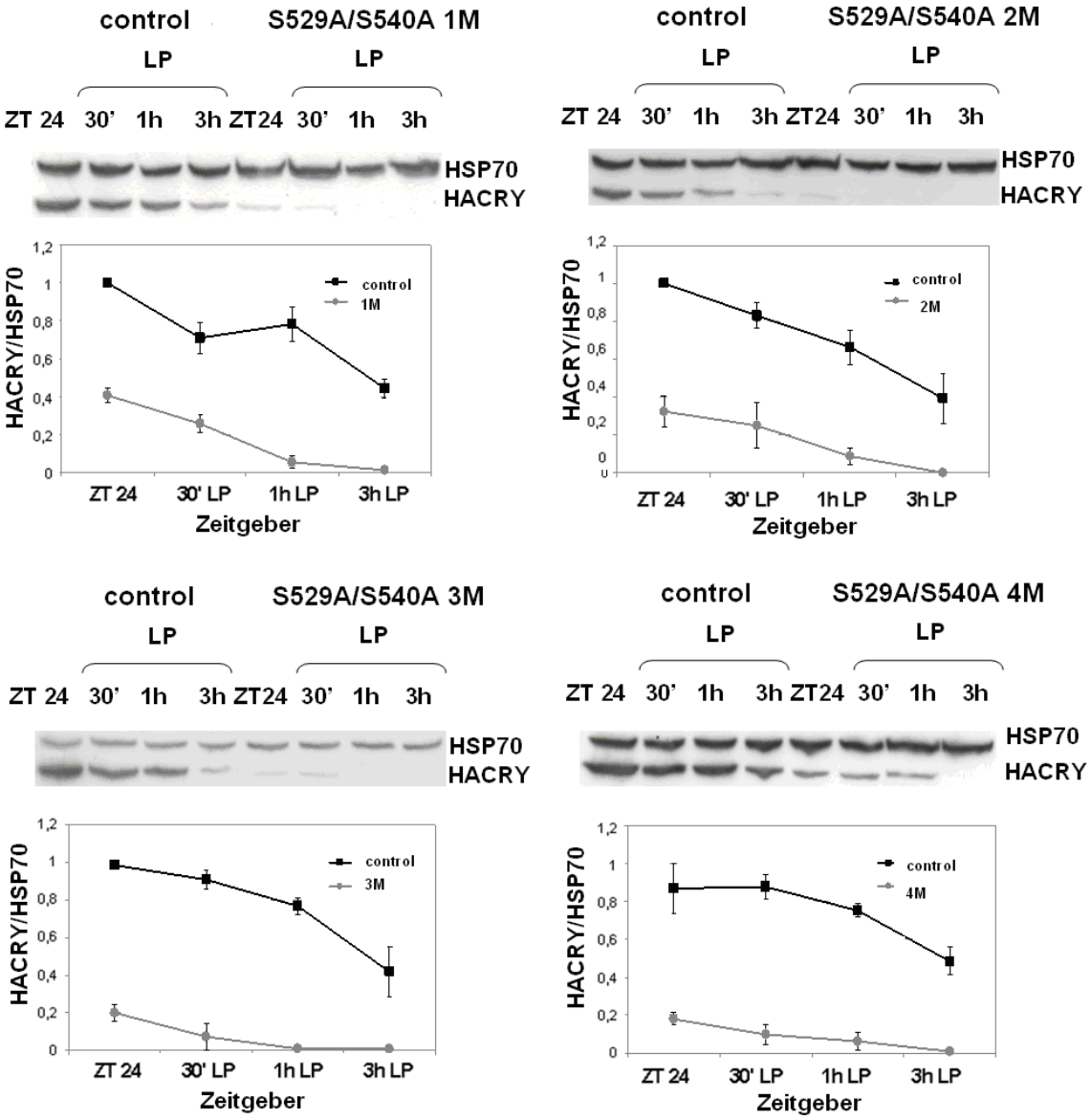
7.4.2 Double mutants

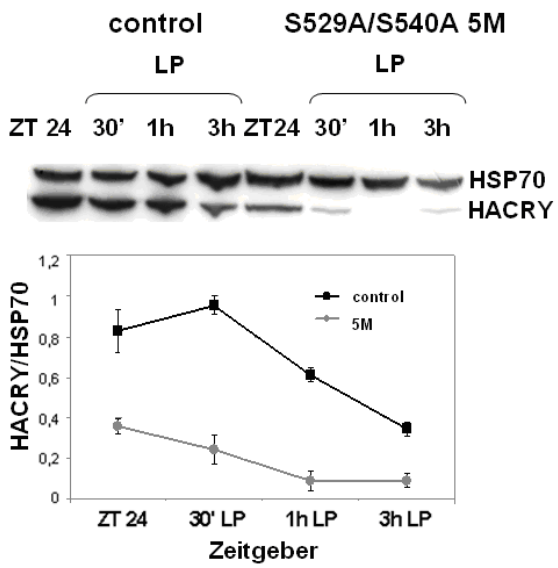
Analysis of HACRY stability

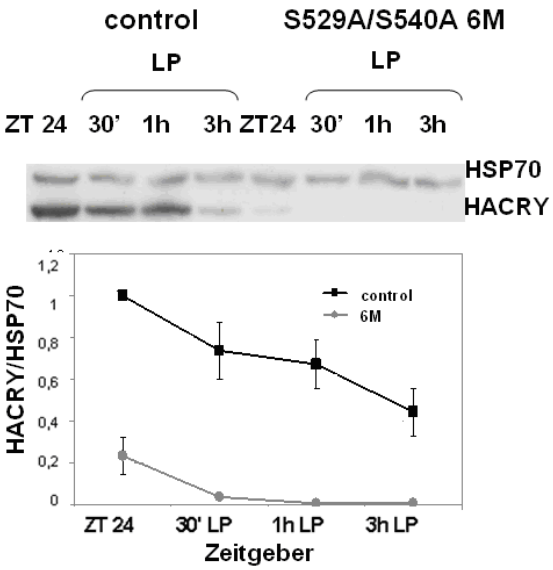
The same approach was used for double mutant lines. In these strains, HACRY carries substitutions of both Serine 529 and Serine 540 with Alanine, in order to abolish phosphorylation at the same time in both sites.

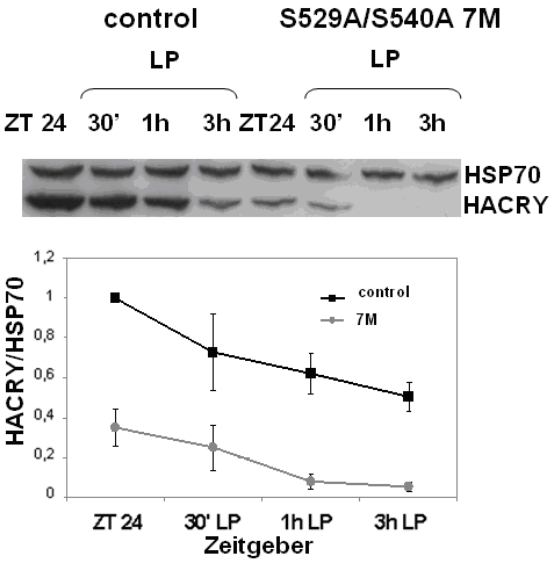
For this construct 10 independent lines were obtained and each of them was analyzed by western blot as described before. These lines carry the transgene in the second or in the third

chromosome and are in a *cry*+ background. Results of ibridization and signal quantification are reported in figure 7.10.




control
S529A/S540A 5M
LP
LP
ZT 24 30' 1h 3h ZT24 30' 1h 3h
HSP70
HACRY
HACRY/HSP70
control
5M
ZT 24
30' LP
1h LP
3h LP
Zeitgeber


control
S529A/S540A 6M
LP
LP
ZT 24 30' 1h 3h ZT24 30' 1h 3h
HSP70
HACRY
HACRY/HSP70
control
6M
ZT 24
30' LP
1h LP
3h LP
Zeitgeber


control
S529A/S540A 7M
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ZT 24 30' 1h 3h ZT24 30' 1h 3h
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HACRY
HACRY/HSP70
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7M
ZT 24
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1h LP
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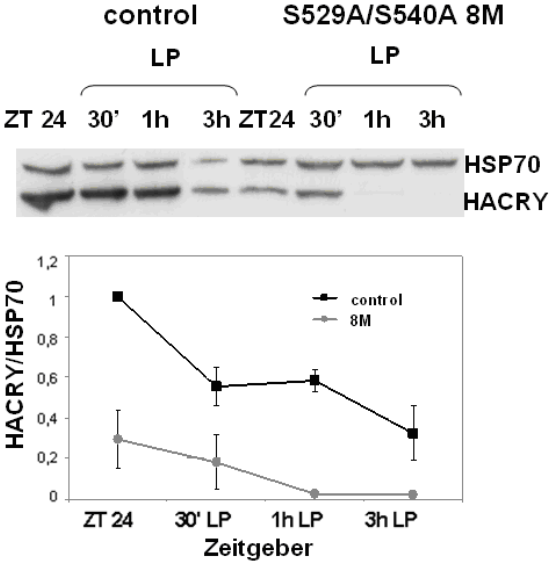
Zeitgeber


control
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ZT 24 30' 1h 3h ZT24 30' 1h 3h
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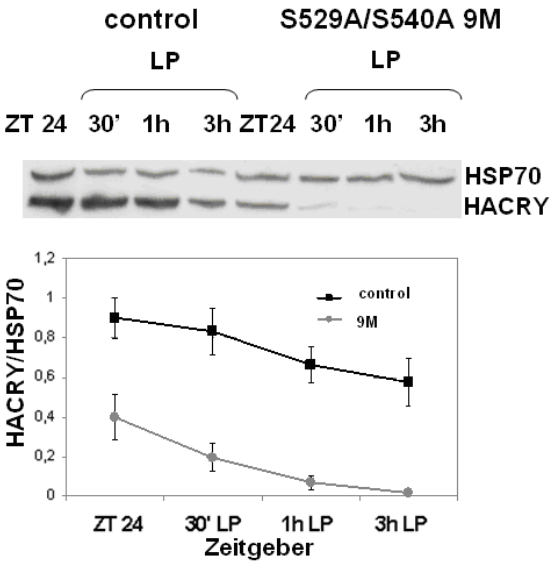
1h LP
3h LP
Zeitgeber


control
S529A/S540A 9M
LP
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ZT 24 30' 1h 3h ZT24 30' 1h 3h
HSP70
HACRY
HACRY/HSP70
control
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ZT 24
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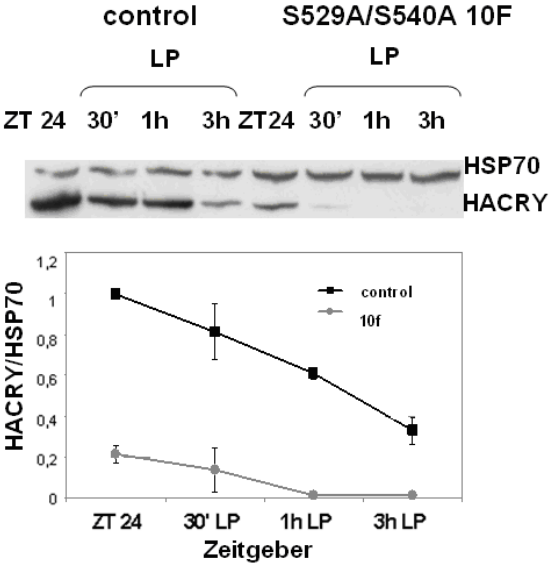
Zeitgeber


control
S529A/S540A 10F
LP
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ZT 24 30' 1h 3h ZT24 30' 1h 3h
HSP70
HACRY
HACRY/HSP70
control
10f
ZT 24
30' LP
1h LP
3h LP
Zeitgeber

Figure 7.10: HACRY stability analysis in double mutant and control flies. HSP70 and HACRY signals detected by western blot (top) and average HACRY expression (3 replicates) (bottom).

Results obtained show that HACRY expression in all these lines is strongly reduced compared to the control and signal is no longer detectable after 1 hour of light exposure. Moreover the kinetic of degradation of transgenic lines (between ZT24 and 30'LP) is comparable to that of the control. Taken together these results show that inhibition of phosphorylation at both target sites results in an higher instability of HACRY after light exposure suggesting that Cry phosphorylation in these sites does not represent a signal for the light-dependent degradation of Cry.

However we might hypothesize that phosphorylation play a role in the regulation of Cry activity.

Circadian behaviour in constant light

Double mutant lines were also tested for rhythmicity in constant light conditions in order to verify if the inhibition of both sites of phosphorylation could result in light perception defects. To perform this experiment, 4 out of 10 transgenic lines with the insertion in the second chromosome were placed in a cry^0 background, in order to monitor only the effect of the mutated form of HACRY without the contribution of the endogenous Cry. These individuals were entrained for 3 days in LD cycles and that shifted for 10 days to constant saturating light (400lux). Results were analyzed and plotted.

Figure 7.11 show results of 2 transgenic lines (HACRY S529A S540A 6M and 7M) as example.

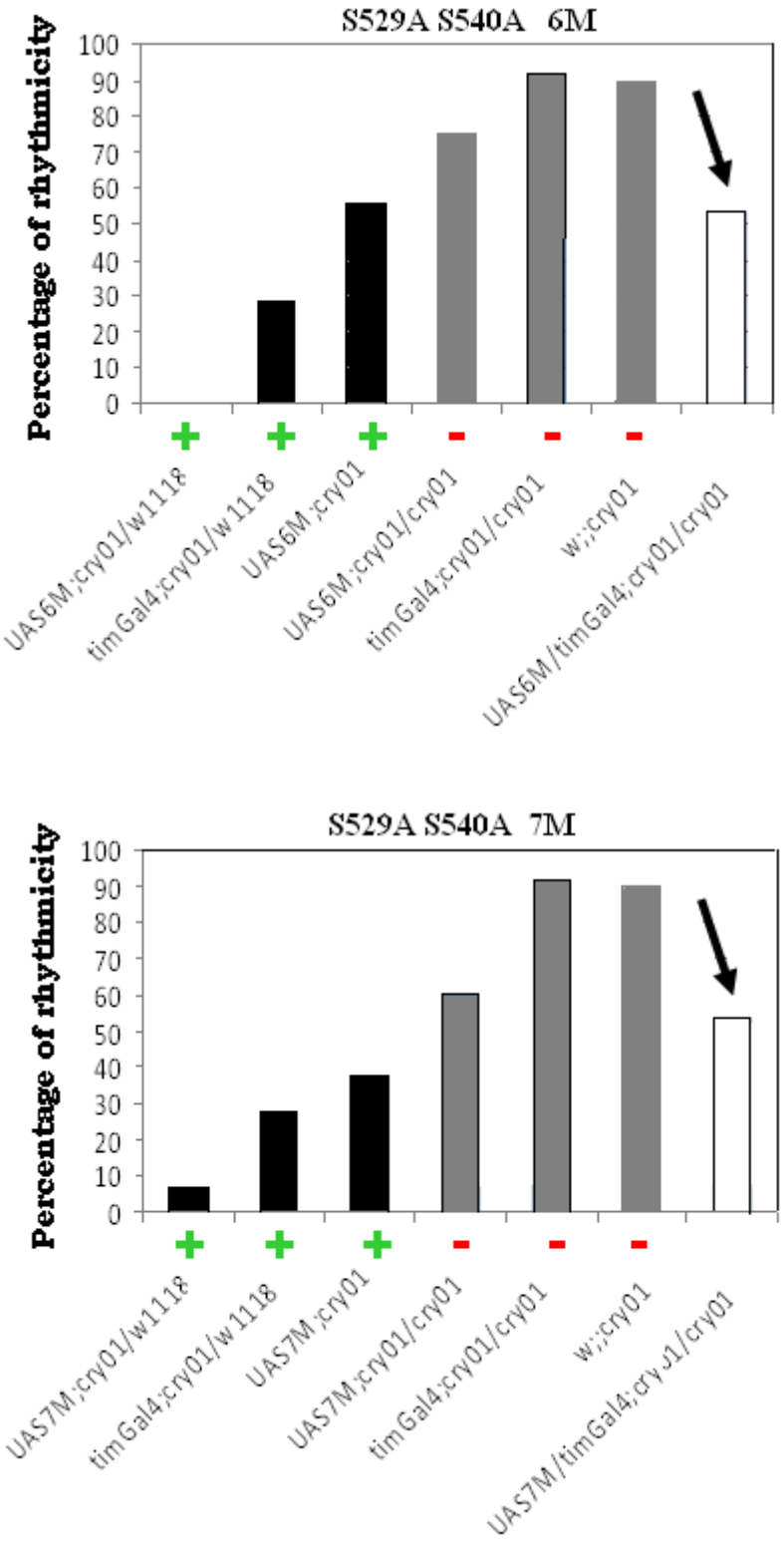

Figure 7.11: Percentage of rhythmicity at saturating light intensities (<1, 37, 400 lux) in positive control (+), negative controls (-) and 2 double mutant lines as examples (arrows).

Figure 7.11 shows percentage of rhythmicity calculated for 3 positive controls (black), which are lines in a wild-type background, 3 negative controls (grey,) which are lines in a *cry*^{0} background, and the double mutant (white). Positive controls are capable to see light and for

this reason are expected to be highly arrhythmic in constant light. On contrary, negative controls are expected to be highly rhythmic. Both transgenic lines show a reduce percentage of rhythmicity compared to cry^0 lines but higher compared to wild-type strains. This results could be due to a reduced capability of the mutated form of HACRY to perceive light suggesting an impairment in light photoreception. However, the reduced expression of the transgene could also influence the behavioural effect of this lines.

DISCUSSION II

Phosphorylation is a post-translational regulation mechanism very common in circadian clocks (Mehra A. *et al*., 2009). In *Drosophila*, Cryptochrome mediates a rapid light-dependent degradation of the clock protein Timeless, which initiates the resetting of the molecular clock. Cry is also degraded in the light but whereas the degradation of Tim is well characterized, the mechanism for light-dependent degradation of Cry is mostly unknown. For this reason, a study was initiated in our laboratory in order to investigate on the putative role of phosphorylation in Cry activity regulation. *In vitro* phosphorylation assays performed on heads extracts over-expressing HACRY in Tim positive cells, indicated the Serine-Threonine kinase Aurora A as an enzyme that promotes HACRY phosphorylation. Moreover, mass spectrometry analysis indicated that Aurora A phosphorylates the HACRY C-terminus at the level of Serine 529, in the SLIT kinase target consensus, and Serine 540, in the SNEE kinase target consensus. In addition, *in vitro* kinase assays performed with syntetic peptides mutated in these specific residues resulted in the abolishment of phosphorylation, confirming the previous analysis.

The present study is focused on the characterization of the role of Aurora A kinase in Cry activity regulation.

First of all, we attempted to reveal the existence of a physical interaction between Cry and Aurora A *in vivo* by immunoprecipitation with an anti-HA affinity matrix on head extracts over-expressing HACRY, and western-blot with an anti-Aurora A antibody (Berdnik and Knoblich, 2002) This approach didn't reveal any physical interaction between the two proteins. The opposite strategy was then used: an immunoprecipitation assay was performed with an anti-GFP matrix on head extracts over-expressing Aurora A fused to GFP, followed by a western-blot with an anti-Cry antibody. Unfortunately also in this case the interaction between Cry and Aurora A was not detectable. A possible explanation for this result could be that the interaction was tested only at ZT24 (when CRY is at maximum levels) and after 15 minutes of lights exposure. It is possible that Cry and Aurora A interact at another ZT which would be more difficult to be revealed due to lower levels of Cryptochrome. Moreover the kinetics of the interaction could be very specific and fast, and for this reason very difficult to visualize with standard techniques.

In order to verify if Aurora A has a role in Cry regulation, two mutant lines and two *aurora* over expressing lines were characterized. In particular: a line obtained by EMS mutagenesis, a line with a P-element inserted in the 5'UTR and 2 UAS*aurora* lines (Berdnik and Knoblich, 2002) that, when crossed with the *tim*Gal4 driver, induce the over-expression of Aurora A in the *tim* positive circadian neurons. Behavioural experiments, performed in constant conditions (both constant light and constant dark), showed that the over-expression of Aurora A results in an impairment of the locomotor behaviour in constant light. In particular, wild-type flies loose completely the rhythmicity of their locomotor activity when exposed to constant light, due to the absence of accumulation of Tim and Cry (Konopka and Benzer, 1971). In contrast, both Aurora A over-expressed lines showed an high percentage of rhythmicity in constant light, suggesting a role of this protein in the light perception mechanism, probably related to

Cry deregulation. In fact, it is reported that *cry* mutants (cry^0 and cry^b) in the same conditions of constant light are rhythmic, as the light perception pathway is disrupted (Stanewsky et al., 1998; Dolezelova et al., 2007).

These results prompted us to investigate about the effects of Aurora A over-expression at the molecular level and, in particular, Cry degradation dynamic was analyzed in these aurora A over-expressing lines. In particular flies were kept for three days in constant dark, in order to allow endogenous Cry accumulation and collected at the end of the subjective night (CT24) and after light exposures of increasing length (15', 30', 1h). Results showed that in one of the tested Aurora A over-expressing lines the degradation of Cry after light exposure occurs with a faster kinetics compared to control line, as the protein is completely degraded already after 15 minutes. This data suggests a possible role of Aurora A in the mechanism of light-dependent Cry degradation, and the effect on the circadian locomotor behaviour could be explained by an effect of the kinase on Cry stability.

In order to further investigate on the effect of aurora A over-expression and its relation to light input pathway, another behavioural test was performed. It is known that short light pulses can reset the clock (due to Tim degradation) and pulses given at the beginning or at the end of the subjective night can delay or anticipate respectively the phase of the clock. This peculiar circadian phenotype is called phase response curve (PRC). Flies displaying impairment in the light input pathway show an impairment in the PRC (Stanewsky et al., 1998). Aurora A over-expressing flies were then subjected to short light pulses at different Zeitgebers during the dark phase in the third day of LD (from ZT13 every 2 hours) and the effects, delay or anticipation of the phase, calculated after three days in constant darkness. Both lines showed an increase of the response, either in terms of delay or anticipation. The increased phase delay observed early in the night can be explained by the fact that in this Aurora over-expressing lines Tim levels are lower than in control flies, and the same mechanism could also be hypothesized for phase advance. Reduced Tim levels could be explained by a more efficient Cry-mediated degradation. In fact, light activates Cry that subsequently promotes a stronger degradation of Tim. This implies either that Cry is present at higher levels, or, rather, that the cryptochrome is more efficient. We lean towards this second hypothesis, also because previous results obtained from Cry dynamic degradation analyses in these lines showed that, already after 15 minutes of light, Cry levels are strongly reduced compared to the control. This observation, then, supports the idea that in these flies Cry protein is more efficient in mediating Tim degradation.

However, it is necessary to underline that short light pulses (about 15 minutes) and continuous light have different effects on clock resetting. In fact Cry defective cry^b flies don't respond to light pulses in a standard PRC analysis, but are still able to resynchronize the clock after a phase shift of 4 hours if the light is given in standard LD 12:12 cycles (Stanewsky et al., 1998). Another experiment that could be performed with these flies is the analysis of clock resynchronization after phase-shift.

The second part of the project was focused on the analysis of Cry degradation after light exposure in lines over-expressing an HACRY fusion where Ser529 and Ser540, putative sites of Aurora A phosphorylation, were mutagenizad. In particular, single mutants were generated, where S529 was mutagenized either towards Alanine (S529A) or towards Aspartic (S529D), and S540 was replaced by Alanine (S540A), and double mutants with both residues mutagenized towards Alanine (S529A,S540A).

Western blot analyses, that reveal specifically the HACRY fusion, showed that the substitution of the residues target for Aurora A phosphorylation doesn't influence the temporal degradation of Cryptochrome after light exposure, indicating that the phosphorylation at these specific sites is not responsible for Cry degradation. It is important to underline that *hacry* expression in these transgenic lines was strongly reduced compared to control, already in the dark. For this reason this couldn't be the best molecular model to analyze the degradation dynamic. However it could be interesting to investigate on Cry dependent Tim degradation in these lines, as Aurora A mediated phosphorylation at those specific sites could mediate the regulation of the cryptochrome.

HACRY single and double mutants were also tested for circadian locomotor activity in constant light conditions. Wild-type flies are arrhythmic in constant light, phenotype due to the continuous degradation of clock proteins, while *cry* mutants (cry^b and cry^0) show a rhythmic phenotype. HACRY single and double mutants show in this condition a reduced percentage of rhythmicity compared to cry^0 lines, albeit higher compared to wild-type. This phenotype could indicate an impairment in the light perception, probably due to a deregulation of the photoreceptor. However, the effect of mutation in HACRY on light perception could be even higher than those observed, due to the fact that, as already mentioned, these transgenes are expressed at a level lower than the control.

Taken together the results obtained indicate that Aurora A kinase have a role in Cry activity regulation, in particular regarding the light perception. It remains to clarify if the putative sites of phosphorylation in the C-terminus of Cry are responsible for the activity regulation. For this reason, a preliminary experiment was done using the strategy of the yeast two hybrid system, that allows to detect the *in vitro* interaction between two proteins. It is already reported that the interaction of dCRY with dPER and dTIM in a yeast two-hybrid assay is a reliable readout of its functioning (Hemsley et al., 2007). This experiment showed that these residues are important for Cry activity, in terms of interaction with Per, as the mutated forms of Cryptochrome bind Per with a lower affinity, compared to wild-type. This preliminary result is a first evidence of the role of these residues in the light-dependent regulation of Cry activity, mediated by the C-terminus of the protein.

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