Unravelling the function of the novel gene*, day,* in *Drosophila melanogaster*.

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In zoos, as in nature, the best times to visit are sunrise and sunset. That is when most animals come to life. They stir and leave their shelter and tiptoe to the water's edge. They show their raiment's. They sing their songs. They turn to each other and perform their rites. The reward for the watching eye and the listening ear is great. I spent more hours than I can count a quiet witness to the highly mannered, manifold expressions of life that grace our planet. It is something so bright, loud, weird and delicate as to stupefy the senses. Yann Martel.

Abstract

Circadian rhythms in biological outputs are seen in numerous phyla that live in cyclical environments. Regulation of these behaviours has been examined in the fruit fly *Drosophila melanogaster*, with many of its genes sharing mammalian homologs. One component of the molecular clockwork of particular interest is the light activated *cryptochrome* (*cry*) gene; *cry* has been shown to be important for a number of light driven behavioural outputs, including but not limited to: entrainment, response to brief light pulses, sensitivity to magnetic stimulus and negative geotaxis. *A yeast two hybrid screen (hybrigenics)* revealed *cry* associating with a novel gene primarily in darkness; the gene CG15803 displayed a light dependent binding to CRY and was re-named DAY (Dark Active in Yeast). The goal of this research was to characterise DAY and examine the consequences of DAY-CRY binding on behavioural rhythmicity, as well as further examine the influence of *cry's* C-terminus in regulating its function. This was accomplished using unique transgenic constructs aimed at: removing *day*, cloning tagged versions of *day* cDNA for protein work, creating *day* promoter lines which give information on its cellular location and examining behaviour of *cry* C-terminus mutants. Using this methodology uncovered a number of phenotypes for flies lacking *day* and hints at a possible role for *day* in regulating *cry* in one particular clock cell. The mutation of the C-terminus of *cry's* revealed some residues important for regulation of *cry*.

Acknowledgements

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Abbreviations

CDS: Coding Sequence

Co-IP: Co-ImmunoPrecipitation

CT: Carboxyl Terminus

DAM: Drosophila Activity Monitor

DAY: Dark Active in Yeast

DD: Dark Dark

DN: Dorsal Neurons

DSB: Double Strand Break

HR: Homologous Recombination

LD: Light Dark

LL: Light Light

LN: Lateral Neurons

PDF: Pigment Dispersing Factor

PI: Pars Intercerebralis

TIC: Time In Copula

Tim-GAL4: TG4

UAS: Upstream Activating Sequence

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1 Introduction

1.1 Clocks

The rotation of the earth has profound consequences on the behaviour of the creatures that reside on it. Timing behavioural events to maximise optimal conditions is vital for success, in a biological context, success equates to survival and reproduction. The decorum of natural selection dictates that organisms that can sense and adapt to cyclical and seasonal conditions will out-compete their rivals obtaining an evolutionary advantage. To this end numerous forms of life have invested genetic capital in carrying a biological clock, from cyanobacteria to humans. There are various forms of rhythmic behaviour ranging from ultradian (events occurring inside of 24 hours), infradian (longer than 24 hours) and circadian (close to 24 hours). To be termed 'circadian' the property in question must comply with the following:

- Receptive to environmental conditions and stimuli, termed *Zeitgebers*.
- Persistence of rhythmicity in conditions once the stimulus is permanently removed.
- Temperature compensated, whereby a deviation in temperature does not influence the timing of the occurrence.

The discovery that the complex control of behaviour could be exerted at the level of nucleotides came in the 1970s, when mutation experiments on the fruit fly *Drosophila melanogaster* created mutants that displayed: short, long and arrhythmic eclosion from pupae (Konopka & Benzer, 1971). *Drosophila* had been installed as a laboratory tool since the 1900s; when CW Woodworth introduced fruit flies into a laboratory

setting. The use of *Drosophila* as a model was utilised to answer questions on inheritance and mutation with TH Morgan awarded the Nobel Prize in 1933 (inheritance) and HJ Muller 1946 (mutation) (noble prize.org).

The importance of circadian behaviour has been demonstrated in a number of studies that have demonstrated a reduction in fitness in flies that behave arrhythmically. Males in particular were found to release less sperm and were less successfully in reproducing (Beaver *et al.*, 2002), furthermore, in models of neuro-degeneration and stress, flies without a functional clock deteriorate faster (Krishnan *et al.*, 2009; Krishnan *et al.*, 2012). Circadian research in *Drosophila* has tangible benefits to human well-being with the human *period* gene identified due to its homology with the sequenced *Drosophila* counter-part. Studies have shown circadian rhythms to be compromised in metabolic disorders (Sookoian *et al.*, 2008), cancer (Kuo *et al.*, 2009) and mental illness (Pallier *et al.*, 2007). Therefore understanding the genetics of circadian behaviour may be beneficial for understanding physical and mental wellbeing in humans; *Drosophila* is an excellent model for molecular studies with its ease of genetic manipulation and short generation time.

One property of a clock is its ability to respond to stimuli and there are a number of known inputs into the circadian clock chiefly: light and temperature. These inputs converge on the oscillator which accepts stimuli and processes the information causing a change in a particular output. As stated previously the first recorded output was eclosion, when flies emerge from their pupal casing, due to the infrequent occurrence of this event most laboratories measure the locomotor activity of flies instead.

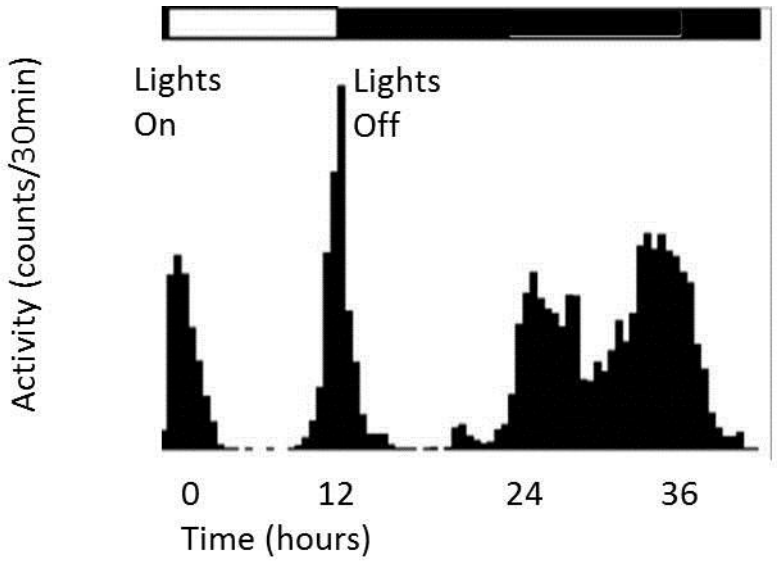


Figure 1-1: Average activity profile under LD (light dark) and DD (dark dark) conditions.

Wild type *D. melanogaster* flies, vertical black bars indicate activity.

White horizontal bar = lights on, horizontal black bars lights = off.

The figure above gives a typical plot of activity for one day of LD followed by 24 hours of DD conditions. The main points of interest are the peaks at the lights on and off transitions in LD, prior to the peaks there is a gradual build in activity levels termed anticipation. The following day of DD has notable differences in profile, the peaks are attenuated and the activity is more broadly distributed across the first 12 hours of D. Also of note is the cessation of activity after the morning and evening peak, periods of prolonged inactivity have been demonstrated to correspond to sleep and will be discussed in more detail later.

The discovery of the clock gene *period* gave invaluable information on one component of the genetic oscillator that generates rhythmic behaviour, the following sections detail the rest of the clock components discovered thus far and the mechanisms that underlie circadian outputs.

1.2 Negative feedback loop

To generate 24 hour periodicity requires the presence of cyclical transcription and post-transcriptional/translational control of genes and their products, a genetic model detailing the components and mechanisms necessary for circadian behaviour has been devised. Referred to as the feedback loop it contains both positive and negative limbs (Glossop *et al.*, 1999). The negative feedback limb begins with the transcription factors CLOCK (CLK) and CYCLE (CYC); these bind through PAS (PER, ARNT, SIM) and basic helix-loop helix (bHLH) domains. As heterodimers CLK and CYC bind circadian E-box promoter sites, promoting the expression of clock controlled genes (CCGs) (Darlington *et al.*, 1998; Rutila *et al.*, 1998; Allada *et al.*, 1998). Two important genes transcribed in this fashion are *period* (*per*) and *timeless* (*tim*), once translated, the proteins accumulate in the cytoplasm and after a delay of 6 hours move into the nucleus, repressing their transcription (Hardin *et al.*, 1992; Edery *et al.*, 1994). This negative feedback and its timing is controlled and also requires the presence of other proteins to assist in halting CLK-CYC transcription. The cessation of transcription is a two-step process whereby PER binds the CLK on the DNA halting transcription, this abolishes the CLK binding to DNA and allows CLK to be hyper-phosphorylated (Menet *et al.*, 2010). It is thought that a PER-CLK complex mediates

the acquisition of kinase enzymes such as DOUBLETIME (DBT); as PER mutants that lack the DBT binding domain are ineffective in terminating CLK transcription (Kim *et al.*, 2007). The role of TIM in the negative feedback loop is indicated by tim^{01} flies (*tim* null allele), as these flies are behaviourally arrhythmic. Another consequence of tim^{01} was reduced levels of PER, indicating that TIM may help stabilise PER, allowing cytoplasmic accumulation of the proteins (Sehgal *et al.*, 1994; Price *et al.*, 1998; Saez & Young, 1996; Vosshall *et al.*, 1994). The association between TIM and PER has consequences on the 24 hour period, evidenced by the tim^{UL} mutant. In this mutant enhanced binding of TIM to PER increased the period length in DD to 33 hours; negative feedback was diminished and *tim*/*per* expression was prolonged (Rothenfluh *et al.*, 2000).

An additional layer of control over the period length is exerted via the entry of TIM and PER into the nucleus. The per^{L} mutation (28 hour period) discovered in the seminal work of Konopka and Benzer (1971), was shown to increase the time taken for PER to enter the nucleus (Konopka & Benzer, 1971; Meyer *et al.*, 2006). Originally it was thought that TIM PER nuclear translocation could only occur with the formation of a heterodimer, as PER cannot enter the nucleus in a tim^{01} background and vice-versa (Saez & Young, 1996; Vosshall *et al.*, 1994; Myers *et al.*, 1996). However, evidence that TIM could enter nuclei without PER and the accumulation of PER in clock cell nuclei prior to TIM entry cast doubt on this notion (Ashmore *et al.*, 2003; Shafer *et al.*, 2002). Furthermore PER was detectable in nuclei in a tim^{01} background when coupled with DBT mutants, these reduced RNA levels (dbt^{P}) or diminished kinase activity (dbt^{AR}) (Cyran *et al.*, 2005). Thus these studies show that TIM PER binding was dispensable for

nuclear entry of both proteins. Although more recently a *tim* mutant was discovered, which fails to enter the nucleus rendering flies arrhythmic in DD; PER could no longer be detected in the nucleus in this mutant (Hara *et al.*, 2011). The mutation (tim^{PL}) is caused by a single base change which is thought to alter secondary structure preventing phosphorylation by an unknown kinase, but still allowing TIM to bind PER (Hara *et al.*, 2011). The importance of TIM in regulating the nuclear entry of PER had also been shown by (Saez *et al.*, 2011), whereby mutating the nuclear localisation signal of TIM dramatically reduced the quantity of PER in the nucleus, despite the proteins being able to dimerise. From these experiments a model has been proposed, in wildtype flies, TIM binds PER in the cytoplasm with excess TIM moving in and out of the nucleus contributing to an unknown role but not inhibiting CLK-CYC (Ashmore *et al.*, 2003). In the late evening when high levels of PER are obtained TIM binding assists in removing or negating inhibitory phosphorylation of PER, that have been administered by DBT, PER can then enter the nucleus. TIM and PER can both do this separately but they may also move together, repressing CLK-CYC (Meyer *et al.*, 2006; Hara *et al.*, 2011). Therefore the importance of TIM and PER on controlling period length is evident; genes or mutations which can influence the stability, binding affinity or nuclear entry of TIM and PER are also important in regulating the clock (Hara *et al.*, 2011).

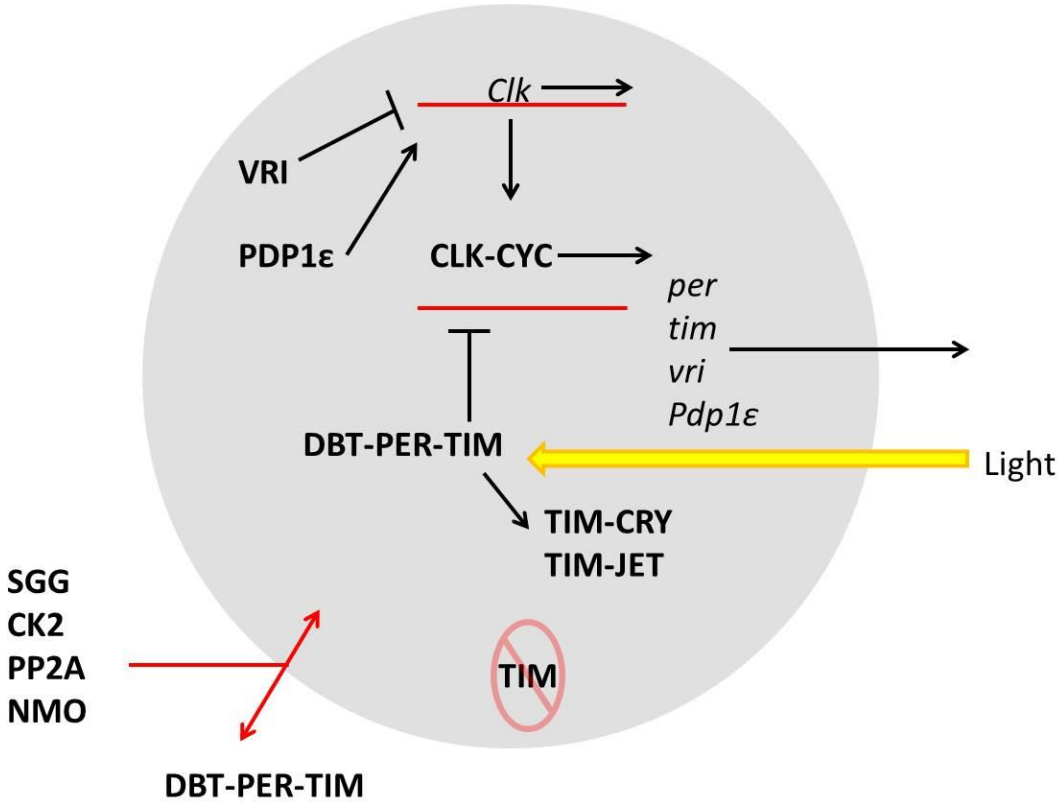


Figure 1-2: Overview of clock controlled feedback loops.

The key features are CLK-CYC nuclear (black circle) transcription of genes which are translated in the cytoplasm. The DBT-PER-TIM complex moves into the nucleus with the aid of the kinase shown with the double ended red arrow. CLK-CYC transcription is repressed; light destroys this inhibition as TIM is degraded by a CRY mediated process which also involves the JET protein, discussed later. The VRI and PDP1ε act directly on the *Clk* promoter inhibiting and activating respectively.

1.3 Kinase and phosphatases of the circadian clock

The phosphorylation status of TIM and PER plays a key role in dictating period, genes responsible for post-translational modifications of TIM and PER have been explored in some detail; they allow fine tuning of CLK-CYC repression by modulating PER and TIM. The influence of DBT on the clock has been analysed through a number

of mutants, DBT^S (short) and DBT^L (long), which decrease and increase the period respectively (Price *et al.*, 1998). The mechanism through which DBT appears to exert the vast majority of its clock influence is in regulating the stability of PER and its transport into the nucleus (Price *et al.*, 1998; Kloss *et al.*, 1998). DBT degrades PER in the cytoplasm preventing its accumulation, the hyperphosphorylated version of PER is then a target for the SLIMB protein. SLIMB is an F-box/WD40-repeat protein that acts as an ubiquitin ligase, marking PER for degradation in the proteasome pathway (Ko *et al.*, 2002; Grima *et al.*, 2002). An important residue PER for this degradation pathway is serine (S47) as phosphorylation of this residue by DBT creates a site for SLIMB to bind, prompting degradation of PER (Chiu *et al.*, 2008). Therefore post translational modifications by DBT have been shown to influence the levels of PER and its ability to halt CLK-CYC transcription, indicating the importance of phosphorylation as a mechanism of regulation in the negative feedback limb (Kim *et al.*, 2007; Price *et al.*, 1998; Kloss *et al.*, 1998; Ko *et al.*, 2002; Grima *et al.*, 2002).

Aside from DBT there are other well documented post-translational enzymes that are important for regulating 24 hour periodicity, such as: SHAGGY (SGG), CASEIN KINASE II (CK2), PROTEIN PHOSPHATASE 2A (PP2A), and NEMO.

SGG is a serine-threonine kinase which was discovered in a behavioural screen and was found to shorten the period when overexpressed (Martinek *et al.*, 2001). The mechanism of this period shortening was correlated to earlier PER TIM nuclear accumulation and phosphorylation of TIM *in-vivo (Martinek et al., 2001)*.

Mutation of the CK2 protein has been shown to increase period lengths and IHC analysis showed expression of CK2 in the lateral clock neurons (LNs) (Lin *et al.*,

2002; Akten *et al.*, 2003), these neurons will be discussed in further detail later in this chapter. The *in-vitro* phosphorylation of TIM and PER by CK2 was shown, with additional analysis the period increase was correlated to a delay in PER entry into the nucleus of larval clock neurons (Lin *et al.*, 2002). *In-vivo* phosphorylation of PER was confirmed later (Lin *et al.*, 2005); with TIM also shown to be a phosphorylation target of CK2 with accumulation and entry affected in CK2 mutants (Meissner *et al.*, 2008).

The NEMO (NMO) kinase has been shown to be involved in phosphorylating the serine 596 of PER *in-vivo*, further phosphorylation's of PER were uncovered *in-vitro* but these sites are not characterised fully yet (Chiu *et al.*, 2008). Aside from PER, NMO was found in complexes with TIM and CLOCK; overexpression of NMO increased the period by 1.5 hours (Yu *et al.*, 2011). This overexpression delayed nuclear accumulation of PER and TIM but also decreased the levels of CLK, presumably the net result of these actions is greater PER TIM nuclear repression which increases the period (Yu *et al.*, 2011).

In order for phosphorylation to be an effective layer of control, it needs to be removable. The PROTEIN PHOSPHATASE 2A (PP2A) enzyme is a heterotrimeric protein with a conserved catalytic unit, a structural subunit and a regulatory sub-unit (Sathyanarayanan *et al.*, 2004). Two regulatory subunits, *twins* and *widerborst*, were found to shorten and lengthen period respectively, both sub-units are regulated in a circadian fashion but in different phases, thus this may account for their converse actions (Sathyanarayanan *et al.*, 2004). PP2A is thought to modulate PER, increasing its stability and regulating its nuclear transport, much like the circadian kinases mentioned thus far (Sathyanarayanan *et al.*, 2004).

1.4 Positive limb of *Clk* control

The negative feedback limb described above details the repression of CLK-CYC transcription by the PER-TIM complex; however, these are not the only genes that control the activity of CLK. One such gene, called *vrille* (*vri*), was identified using differentially display methodology (Blau & Young, 1999). RNA was extracted from heads, examining the cDNA that was differentially expressed at ZT14 and ZT20 in wildtype (WT), cycling of *vri* was abolished in a per^{01} (*per* null allele) background, suggesting that *vri* was a CCG (Blau & Young, 1999). It was identified in the LNs and heterozygotes for the *vri* null allele shortened the period of locomotor activity by 0.4-0.8 hours (Blau & Young, 1999). VRI is a basic leucine zipper (bZIP) transcription factor and was found to bind the promoter of *Clk* at V/P sites and inhibit transcription; this negative repressive action results in low C*lk* levels when VRI is at its peak (Glossop *et al.*, 2003; Cyran *et al.*, 2003). However, a confusing result was that the peak levels of C*lk* occurred during the late night when PER-TIM repression was at its maximum, this implicated a transcription factor for positive regulating C*lk* transcription (Allada *et al.*, 1998). Work in mammals had already revealed such regulatory proteins and the positive element of this second loop was correlated to the action of the PAR DOMAIN PROTEIN 1ε (PDP1ε), much like VRI it is transcribed through CLK-CYC activation (Cyran *et al.*, 2003). The isoform PDP1ε accumulates slower than VRI by 3-6 hours and competitively binds the same portion of the C*lk* promoter, activating transcription of *Clk (Cyran et al., 2003)*. The dual loop control of *Clk* levels is an indication of its importance in controlling molecular rhythmicity

1.5 Regulation is something chosen

The molecular oscillator composed primarily of two interlocked feedback loops was expanded with the discovery of the *clockwork orange* (*cwo*) gene (Glossop *et al.*, 1999; Matsumoto *et al.*, 2007; Kadener *et al.*, 2007; Lim *et al.*, 2007). CWO was located in all clock neurons, a robust finding, obtained using two independent reporter lines and antibodies against CWO (Matsumoto *et al.*, 2007; Kadener *et al.*, 2007; Shafer *et al.*, 2006). Partial knockdown of *cwo* was achieved using lines carrying transposon insertions in the first intron. In these lines, in DD, there was a significant decrease in rhythmicity compared to controls, while flies that were rhythmic displayed longer periods (Kadener *et al.*, 2007). CWO functions as a bHLH transcription factor and is expressed in cyclical fashion via CLK-CYC transcription; the *cwo* promoter carries a number of E-box sites (Matsumoto *et al.*, 2007). These sites are not only important for CLK binding but also CWO can bind its own promoter inhibiting transcription; demonstrated in *cwo* null flies that have very high levels of CWO (Richier *et al.*, 2008). It should be noted that *in-vitro* cell work showed that CWO could also act as a repressor of clock gene transcription through e-box sites, but there is also conflicting evidence of CWO activating expression of *per*, *tim*, *vri* and *pdp1ε* *in-vivo* and amplifying their expression (Matsumoto *et al.*, 2007; Richier *et al.*, 2008). The timing of transcriptional repression vs activation is thought to be distinct and that the levels of fluctuating clock genes may guide to CWO to either promote or reduce transcription of target genes (Richier *et al.*, 2008).

The main molecular components of the circadian clock discussed above are believed to control the central oscillator in constant conditions, however, these conditions are unlikely to be experienced in the wild, a more pertinent question is to ask how these components sense changes in the environment and respond.

1.6 Light entrainment and *cryptochrome*

Light is the most heavily studied *zeitgeber* in the field of circadian rhythms, with standard LD conditions capable of entraining most flies, even those with clock gene mutations. The discovery that light exposure reduced levels of TIM abundance was the first indication of how light may manifest change in the timing of molecular cycling (Myers *et al.*, 1996). After exposing flies to light there was a marked decrease in TIM levels, when assayed after a recovery period in darkness the levels of TIM were found to be increased (Myers *et al.*, 1996). Light causes TIM to be tagged with ubiquitin and is then degraded by the proteasome (Naidoo *et al.*, 1999). The influence of light on locomotor activity takes a number of forms, constant light renders flies arrhythmic, constant dim light gives rhythmic flies a longer period and pulses of light are capable of shifting the peak of activity depending on when they are administered (Emery *et al.*, 2000a; Pittendrigh, 1960). A light pulse administered in the early evening produces a delay while a pulse administered in the late night causes an advance in the peak of activity. Delays are thought to be the consequence of TIM degradation, with the time taken for TIM levels to re-build after a light pulse determining the delay length. An advance occurs later when *tim* RNA levels are not sufficient in quantity to effectively replenish TIM.

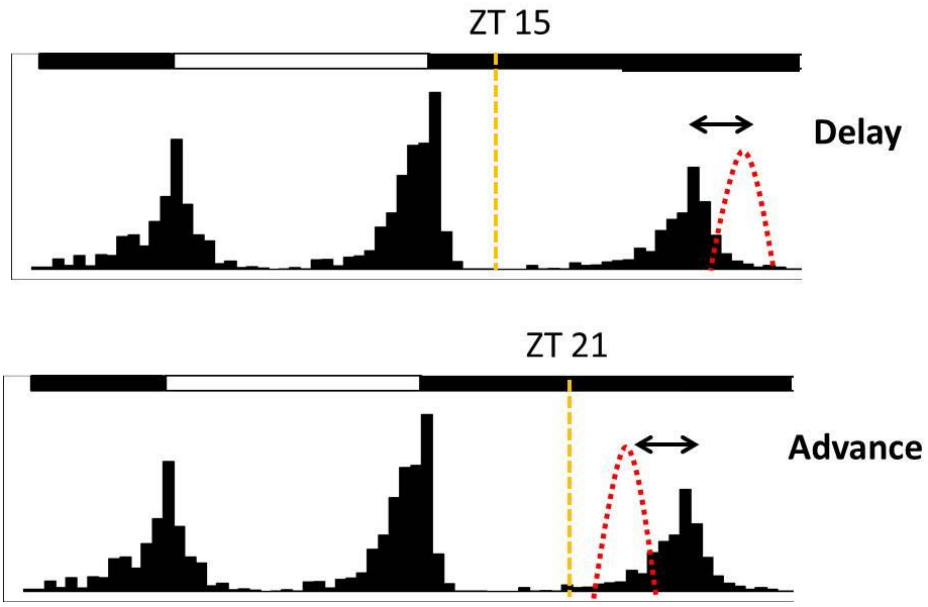


Figure 1-3: Phase shift diagram.

Vertical black bars indicate level of activity, yellow dashed bars show light being administered and red dashed lines highlight where the new peak of activity would occur. Horizontal bars: white= lights on, black lights off.

The revelation that light could be recognised by the product of the *cryptochrome* (*cry*) gene helped to unravel the mechanisms behind light entrainment. *Drosophila* CRY is ancestrally similar to DNA (6-4) photolyases, which recognise blue light and repair DNA damage. CRY no longer repairs DNA damage, structurally it has a conserved N-terminus that binds the co-factors pterin and flavin, as well as a C-terminus that is species-specific (Cashmore *et al.*, 1999). Analysis of *cry*'s function was achieved when the mutant cry^{b} was uncovered (Emery *et al.*, 1998; Stanewsky *et al.*, 1998), they found that the mutation caused cessation of the cycling of PER and TIM in DD and LD, however, the locomotor activity was rhythmic in DD with a wild type period and also

demonstrated an ability to re-entrain to a shift in the light regime (Stanewsky *et al.*, 1998). The re-entrainment of cry^{b} flies to differing light regimes indicated that *cry* was not entirely responsible for light entrainment, when coupled with the $norpA^{P24}$ mutation (which causes the compound eyes and ocelli to be un-responsive to light) the double mutant took much longer to re-entrain to shifts in the light regime but was still capable of doing so (Stanewsky *et al.*, 1998). This was compounded further when $norpA^{P24}$ was introduced into a *cry* null back ground (Dolezelova *et al.*, 2007), however, 'true' circadian blindness was thought to be achieved by combining cry^{b} with $glass^{60j}$ mutation (Helfrich-Forster *et al.*, 2001). GLASS is a transcription factor involved in the formation of the eye, with *glass* mutants lacking ocelli, retinal photoreceptor cells and the Hofbauer Buchner (H-B) eyelet, which are cells that underneath the retina projecting to the clock LN_vs cells (discussed later) (Helfrich-Forster *et al.*, 2001). Combining both *cry* and *glass* mutations abolished entrainment, with single mutants showing an ability to re-entrain.

Another novel effect on behaviour controlled by *cry* was the maintenance of rhythmic locomotor behaviour in constant light, as wild-type flies cannot display rhythmic locomotor activity in this condition (Emery *et al.*, 2000a). The ability to sense light and shift locomotor behaviour is something that requires *cry* but not exclusively as other light sensing pathways are involved. The molecular mechanisms behind light responses are discussed below.

1.7 Molecular mechanism of light sensing

It had been shown that TIM was light sensitive and that CRY has a role in light entrainment (Myers *et al.*, 1996; Emery *et al.*, 1998; Stanewsky *et al.*, 1998). CRY binds to TIM in a light dependent fashion promoting the degradation of TIM (Naidoo *et al.*, 1999; Busza *et al.*, 2004). TIM was shown to be more sensitive to brief illumination than CRY which required continuous light in order to be broken down (Busza *et al.*, 2004). The light dependence of TIM-CRY binding is conferred by the C-terminus (CT) as deletion of 20 amino acids (CRYΔ) destroyed this regulation allowing CRYΔ to bind in both L and D conditions (Rosato *et al.*, 2001; Dissel *et al.*, 2004).

There is thought to be a PER-TIM-CRY complex with *in-vitro* work demonstrating that CRY can even bind PER in the absence of TIM (Rosato *et al.*, 2001). The CT of *cry* has been dissected *in-silico* and reveals a number of binding motifs and potential phosphorylation targets that may allow it to bind different protein/complexes, discussed in detail chapter 9 (Hemsley *et al.*, 2007). Other genes that help *cry* perform its signalling duties will now be discussed.

LL rhythmicity was a critical phenotype in finding the gene *jetlag* (*jet*), flies carrying the jet^{c} or jet^{r} mutations could no longer respond to constant LL (Koh *et al.*, 2006). JET was found to facilitate the degradation of TIM, and is an ubiquitin ligase that marks TIM for proteasome trafficking. In order for JET to function it requires CRY to bind TIM *in-vitro* (Koh *et al.*, 2006). The interaction between CRY-TIM and JET was dissected further by Peschel *et al* (2006 and 2009); in this work they took advantage of a natural *tim* allele that produces long and short isoforms of *tim*, ls-TIM, and a short allele, s-TIM (Rosato *et al.*, 1997). *Tim* isoforms were shown to have different affinity

towards CRY with LS-TIM binding weakly when compared with S-TIM, and these binding preferences were thought to manifest themselves as varying sensitivity to light; S-TIM was more light sensitive than LS-TIM (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007). It was demonstrated that the JET^{c} mutant that is normally rhythmic in LL and can be restored to wild-type by introducing the S-TIM allele (more light sensitive) in place of LS-TIM (Peschel *et al.*, 2006; Peschel *et al.*, 2009). JET was also shown to degrade CRY in the same manner as TIM, but TIM degrades faster than CRY (Peschel *et al.*, 2009).

Other components of light sensing pathways have also been picked up including the membrane bound *quasimodo* gene (*qsm*) and the chromatin remodelling enzyme KISMET (KIS) (Dubruille *et al.*, 2009; Chen *et al.*, 2011). Although the control of KIS over light perception is not fully understood, the Inhibition of the enzyme using RNA interference (RNAi) gave rhythmicity in LL but did not reduce mRNA levels of *tim*, *cry*, *jet* or *sgg* (Dubruille *et al.*, 2009). Highlighting that there may be regulation of this behaviour beyond the genes already described. Another gene found to be important for regulating rhythmicity in LL was the membrane bound QUASIMODO. Down regulating *qsm* using RNAi gave an increase in the percentage of rhythmicity in LL and an increase in the levels of TIM (Chen *et al.*, 2011). This was shown to be independent of *cry*, with *qsm* only showing partial cell overlap with *cry* (Chen *et al.*, 2011).

In summary both CRY and JET are important in controlling LL rhythmicity by regulating the degradation of TIM, however, neither protein is directly responsible for the regulation of the LD or DD activity profiles (Stanewsky *et al.*, 1998; Koh *et al.*, 2006). A caveat to this is CRYΔ, which can influence the clock, increasing the period

due to an interaction with TIM in darkness (Dissel *et al.*, 2004). The recent discovery of genes such as *kis* and *qsm* reveal components that can transmit light information and influence LL behaviour, with *qsm* function independent of *cry (Dubruille et al., 2009; Chen et al., 2011)*. Identifying new components will help to elucidate the role of light on the locomotor activity with respect to entrainment, phase shifts and LL behaviour.

In order to understand how *cry* performs the tasks attributed to it and the role of *cry* in clock homeostasis, the neurons regulating behavioural rhythmicity must be analysed.

1.8 Clock neurons

Having analysed the functions of the many genes involved in the molecular maintenance of 24 hours, it is worth considering the neurons that transmit the cycling of the cellular oscillator into neuronal communication which results in rhythmic behaviour (Dunlap, 1999).

Approximately 150 neurons have been identified and termed 'clock' cells, colloquially speaking, these neurons should have cycling levels of PER and TIM and modification of these cells should influence the distribution or phase of activity in LD or DD. The cells are divided, based upon their location, with two primary groups the dorsal neurons (DNs) and lateral neurons (LNs) reviewed in (Helfrich-Forster, 2003; Kaneko & Hall, 2000). These neurons had been shown to immunoreactive to the PER antibody and have been shown to display cycling levels of PER that were altered in clock mutants (Siwicki *et al.*, 1988; Zerr *et al.*, 1990). The LN's are segregated into the

small (s-LN_vs), large (l-LN_vs), the fifth s-LN_vs (non-PDF expressing), dorsal (LN_ds) and Lateral posterior neurons (LPNs); the DN's are termed DN1s, DN2s and DN3s (Shafer *et al.*, 2006; Helfrich-Forster, 2003; Kaneko & Hall, 2000; Kaneko *et al.*, 1997). Each group vary in cellular abundance and expression of particular clock genes, furthermore specific roles have been attributed to each cell group in the control of circadian behaviour, which will now be discussed.

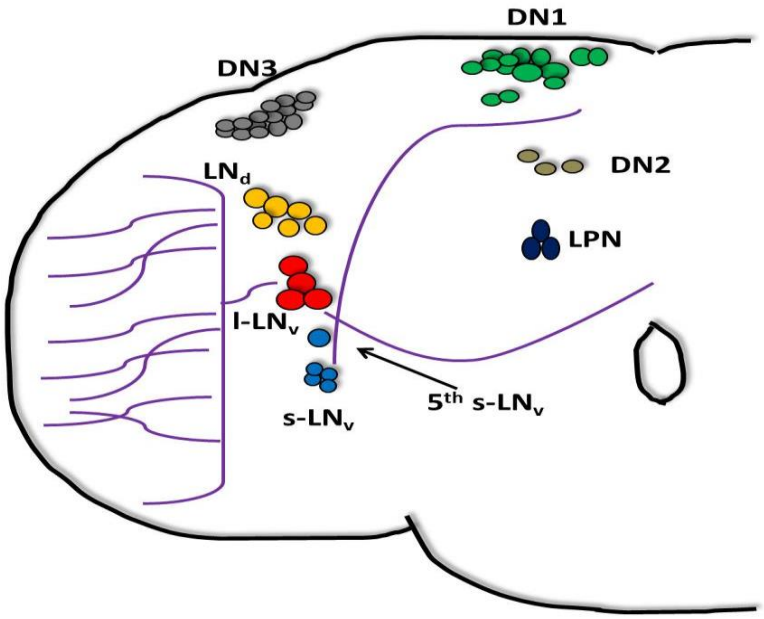


Figure 1-4: Neuroanatomy of *Drosophila* clock neurons.

Purple lines represent PDF neuropeptide projections emanating from the s-LN_vs and l-LN_vs.

1.9 PDF cells and the LNs

Expression of the *Pigment dispersion factor* (*Pdf*) gene is limited to the s-LN_vs and l-LN_vs, although one s-LN_v does not produce *Pdf* (Renn *et al.*, 1999). This neuro-peptide was shown to be crucial for rhythmicity as *Pdf* null (P*df*^{01}) flies became rapidly arrhythmic after 1-3 days in DD, those that maintained rhythmicity had short periods, in LD, with the evening peak advanced by one hour and finally there was a loss of morning anticipation (Renn *et al.*, 1999). In the same work the cells expressing PDF were abolished using lethal neuro-toxins and a similar result was seen with a reduction in the level of locomotor rhythmicity and a shorter period (Renn *et al.*, 1999). Immunostaining of PER in P*df*^{01} flies demonstrated that the cycling was desynchronised between the s-LN_vs neurons and in the anatomically distinct LN_ds PER was entering the nucleus in an advanced phase (Lin *et al.*, 2004). With the PDF projections showing termini in areas adjacent to clock cells, a model was conceived whereby PDF is required to keep synchrony between these cell groups (Lin *et al.*, 2004).

Further understanding of the role of PDF came through analysis of the PDF receptor (PDFR), mutations in this gene showed similar behaviour to *Pdf*^{01} flies, early evening peak in LD, reduced rhythmicity in DD and no morning anticipation (Hyun *et al.*, 2005; Mertens *et al.*, 2005). Full rescue of this phenotype was only achieved using a transgene of ~70 kb and gave the complete picture of PDFR expression (Im & Taghert, 2010). Clock cell expression of PDFR was seen in 6 of the DN1s, the 5th s-LN_vs and three of the LN_ds, as well as weaker expression in two of the l-LN_vs, four s-LN_vs and four DN 2+3 cells (Im & Taghert, 2010). Aside from clock cell expression, PDFR cells in close proximity to the PDF projections were observed but whose functions are

mainly un-characterised (Im & Taghert, 2010). The staining of PDFR in the DN1s is of particular interest, as rescue of the morning anticipation and DD rhythmicity phenotypes of PDFR mutants was achieved using a very specific cell type driver that express in 16-20 DN1 neurons (Zhang *et al.*, 2010). They demonstrated that the molecular clock of these DN1 cells was sensitive to PDF re-setting and highlight *in-vivo* neuronal connectivity between PDF positive and negative cell types (Zhang *et al.*, 2010).

The role of PDF in circadian rhythms has been studied extensively; this has led to sub-division of the clock neurons in relation to controlling locomotor activity. However, PDF signalling is only part of this picture, analysing the influence of the remaining neurons has been attempted in order to obtain a complete picture of neural control of locomotor activity.

1.10 Clock cell communication

The generation of diurnal behaviour is thought to be the consequence of a dual oscillator controlling the twin peaks of activity (Pittendrigh & Daan, 1976). The control of activity bouts in *Drosophila* has been dissected by driving expression of *per* in different combinations of clock cells and examining the effect on LD and DD behaviour. This research proposed that the PDF expressing cells controlled morning activity, while the remaining clock cells were important for evening activity but not integral to controlling rhythms in constant darkness (Grima *et al.*, 2004; Stoleru *et al.*, 2004).

The control of the LNs over DD rhythmicity has also been demonstrated in a number of studies. Recently the gene *unfulfilled* (*unf*) has been documented; this gene

is only expressed in the s-LN_vs clock cells, with loss of function mutations rendering flies completely arrhythmic in DD (Beuchle *et al.*, 2012). Interestingly for this phenotype to occur *unf* must be compromised during development; knockdown of *unf* in adult tissue led to increased periods but no loss of rhythmicity (Beuchle *et al.*, 2012). Furthermore the *disconnected* (*disco*) mutant lacks neuronal connections between the eyes and the optic lobe, resulting in the elimination of many of the LN cells and leaving many flies arrhythmic. Although arrhythmicity was not observed in flies that carried one s-LN_vs or l-LN_vs cell, with their appropriate projections intact (Helfrich-Forster, 1998).

These data seems to support the notion that the LNs are primarily important for maintaining DD behaviour as specific manipulation of these cell groups only have dramatic consequences for behaviour; this has led to many referring to the small and large LNs as the 'master' oscillator. It should be remembered that DD conditions are rarely experienced in the wild, and even low intensities of light are shown to influence the clock (Bachleitner *et al.*, 2007). Therefore the influence of light on clock cells shall be discussed.

1.10.1 Clock cells in light

Electrical activity of clock cells is vital to their function; studies have shown the influence of altered membrane properties on circadian rhythmicity (Nitabach *et al.*, 2002). In 2006 the electrophysiology of the l-LN_vs was documented showing these cells to be sensitive to light; artificially hyperpolarising the l-LN_vs abolished this behaviour (Park & Griffith, 2006). Furthermore the firing of the l-LN_vs was found to be regulated

in both a circadian and light based manner (Sheeba *et al.*, 2008b). This light response was found to be attenuated in cry^b mutants and removed in *cry* null flies, furthermore firing ceased when wave-lengths of light outside of CRY's action spectra were used to stimulate the l-LN_vs (>500nm) (Fogle *et al.*, 2011). This firing was found to occur without optic inputs, and cells normally un-responsive to light became light sensitive upon *cry* induction (Fogle *et al.*, 2011).

As stated previously the rhythmicity of flies in constant light is low, a novel finding was that overexpressing *per* in clock neurons could restore rhythmicity (Emery *et al.*, 2000a; Murad *et al.*, 2007). They found that the most important cells in *per* overexpression for LL rhythmicity were the DN1 neurons, moreover the DN1 did not require *Pdf* in order to control this behaviour (Murad *et al.*, 2007). The effect of bright light is to create arrhythmic behaviour, however, in constant dim light rhythmicity is maintained. In per^{01} flies this behaviour is abolished and it was shown that *per* expression in just 4 neurons could rescue dim light rhythmicity; these neurons were the three *cry* positive LN_ds and the fifth s-LN_vs (non-*Pdf* cell) (Rieger *et al.*, 2009). The bi-modal pattern of activity was retained, with the authors of this work suggesting that the group of 4 cells is split with two cells controlling the morning activity and the other two cells controlling the evening (Rieger *et al.*, 2009). This suggests that the small and large LNs are dispensable for morning activity and rhythmicity if assayed in different lighting conditions (Rieger *et al.*, 2009).

It is not just continuous light that can influence behaviour and light pulses in the early evening or late night can causes the peak of activity to delay or advance (Pittendrigh, 1960). The proposed molecular mechanism involves TIM degradation

through CRY mediated interactions, discussed previously (Emery *et al.*, 1998; Stanewsky *et al.*, 1998). A heterogeneous effect of light pulse administration was seen at the neuronal level as a light pulse administered at ZT 15 (delay phase) caused no significant reduction of TIM in the small and large LNs, while TIM is absent from the LN_ds and DNs after this light pulse (Tang *et al.*, 2010). A pulse delivered at ZT 21 reduces TIM in half of the LNs as well as the other clock neurons; this study suggests that at the molecular level, clock cells have differing sensitivity to light, dependent on the time of its administration (Tang *et al.*, 2010).

The results of the studies described above highlight the varying impact of light on different clock cell types; with certain cell groups controlling behaviour in particular conditions. The visual input into clock cells is varied with the optic nerves innervating the LN_vs, *cry* expression in all clock cells bar the DN2-3s and some of the LN_ds/DN1s and *quasimodo* expression in 14-17 DN3s, the PDF minus s-LN_v, 2 DN2s and 1 LN_d (Helfrich-Forster *et al.*, 2001; Chen *et al.*, 2011; Yoshii *et al.*, 2008). The existence of multiple sensing pathways demonstrates lights importance on locomotor behaviour and how there are intricate differences in how cells respond to light linked to the light sensing inputs they receive at the molecular or neuronal level.

1.11 Circadian regulation of additional outputs: sleep and courting

1.11.1 Sleep

The precise function of sleep is still a matter of debate; although its importance is evident as this behaviour is seen in a diverse range of creatures and when restricted the effect is detrimental to the organisms' well-being. Akin to circadian rhythms, sleep

is a complex behaviour that can be studied at the genetic level using *Drosophila.* There are a number of criteria that must be fulfilled to be termed as sleep behaviour reviewed in (Greenspan *et al.*, 2001):

- Increased arousal threshold
- Quiescence
- Homeostatic rebound after prolonged wakefulness

There was significant overlap in the features of sleep between human and *Drosophila* with pharmacological stimulants increasing wakefulness in a dose dependent manner; older flies displaying fragmented sleep and resting flies unable to react to stimuli sufficiently (Greenspan *et al.*, 2001; Hendricks *et al.*, 2000; Shaw *et al.*, 2000; Hendricks *et al.*, 2003a).

The distribution of activity in *Drosophila* is controlled by the circadian oscillator; therefore it would seem likely that clock cells would control sleep. Measuring the ability of sleep deprived flies to undertake homeostatic re-bound, demonstrated that per^{01} responded in a wildtype fashion (Shaw *et al.*, 2000). The same study showed that C*lk* and *cyc* were influential in the regulation of sleep, with mutations in both genes raising average activity length; furthermore *cyc* mutation was shown to prevent any homeostatic re-bound in LD and DD (Hendricks *et al.*, 2003b). Efforts to identify genes important for sleep or regulated by sleep themselves have revealed a vast number of candidates, (reviewed in Sehgal & Mignot, 2011; Crocker & Sehgal, 2010). Some examples of sleep genes include: *Shaker* a potassium channel (Cirelli *et al.*, 2005), epidermal growth factor receptor (EGFR) (Foltenyi *et al.*, 2007)

and arylalkylamine N-acetyltransferase (DAT), an enzyme involved in the catabolism of monoamines (Shaw *et al.*, 2000). These three genes indicate a role of neuronal transmission and signalling, the identity of neurons that regulate sleep behaviour will now be discussed.

1.11.2 Sleep at the level of the neuron

The identification of clock genes that also affect sleep made clock neurons obvious candidates for the regulation of this complex behaviour. The neurotransmitter GABA has been shown to initiate sleep behaviour (Agosto *et al.*, 2008); receptors for GABA were shown to be present on the LN_vs neurons (Parisky *et al.*, 2008). Down-regulating the receptor for GABA in the LN_vs led to increased wakefulness and conversely over-expressing the receptor led to increased sleep, this marked out these cells as wake promoting under the control of GABA-ergic neurons (Parisky *et al.*, 2008). Further behavioural analysis of the LN_vs expressing excitatory or inhibitory membrane elements revealed that exciting these neurons caused increased nocturnal activity (Sheeba *et al.*, 2008a). Attempts were made to dissect the influence of the s-LN_vs from the l-LN_vs on this behaviour, by using a driver specific for the l-LN_vs, the sleep phenotype was maintained (Sheeba *et al.*, 2008a). Although there was expression in a number of other peptidergic cells that were also found to influence sleep. To ensure it was the l-LN_vs causing this sleep phenotype ablation of only the s-LN_vs was accomplished using a toxic Huntingtin fragment (Q128-Htt), the s-LN_vs were more susceptible to this neurotoxin as they develop first (Sheeba *et al.*, 2010). The flies

lacking s-LN_vs neurons displayed increased nocturnal activity, suggesting that the l-LN_vs can act as arousal neurons independently of the s-LN_vs (Sheeba *et al.*, 2010).

Two other major neuronal sleep centres are the neurons of the mushroom bodies (MB) and the pars intercerebralis (PI) (Foltenyi *et al.*, 2007; Joiner *et al.*, 2006). The regulation of sleep within the MB clusters is heterogeneous, as manipulations of particular cells produced increased sleep whilst the others drove longer activity bouts (Joiner *et al.*, 2006). This disparity in function can be attributed to the innervation of particular cell subsets by cells expressing neurotransmitters with conflicting roles in sleep regulation. Dopamine has been shown to promote arousal whilst serotonin evokes sleep; the receptors of each neurotransmitter are anatomically distinct (Andretic *et al.*, 2005; Yuan *et al.*, 2006). The dopamine arousal pathway is particularly interesting as arousal can be sub-divided into: startle response and normal wakefulness, arousal was examined by blasting the flies with air repeatedly for brief durations; flies with deficient dopamine receptors did not respond like wildtype flies (Lebestky *et al.*, 2009).

Recently details have emerged which links dopamine signalling with *cry* and the l-LN_vs (Kumar *et al.*, 2012). It was shown that the C*lk* mutant Clk^{jrk} displayed elevated levels of nocturnal activity; normal sleep levels can be restored by placing the C*lk* mutation into a *cry* null background. The cells responsible for this behaviour were the l-LN_vs, which contained higher levels of *cry* in a C*lk* mutant background. Furthermore, the levels of tyrosine hydroxylase (TH) enzyme involved in synthesising dopamine were also found to be elevated in C*lk* mutants. Blocking the output of dopamine neurons in a Clk^{jrk} background suppressed nocturnal activity levels. Increasing the

levels of *cry* in the l-LN_vs in a background where dopamine levels are normal did not lead to increased night time arousal; the reason for this could be that both *cry* and dopamine must be more abundant to produce nocturnal activity. Thus the l-LNvs and dopaminergic neurons may converge on the same target to produce nocturnal activity or modulate each other (Kumar *et al.*, 2012).

1.11.3 Courtship

Another complex behaviour that shares cells and genes in common with the circadian clock is courting/copulation behaviour. Courting is a regulated process with a number of sensory inputs. Male courtship behaviour consists of: orienting (male starts following the female), tapping (male touches the female with fore-leg), singing (vibrating wing), licking (male licks female genitals) and attempted copulation and if the female is receptive then copulation (Hall, 1994).

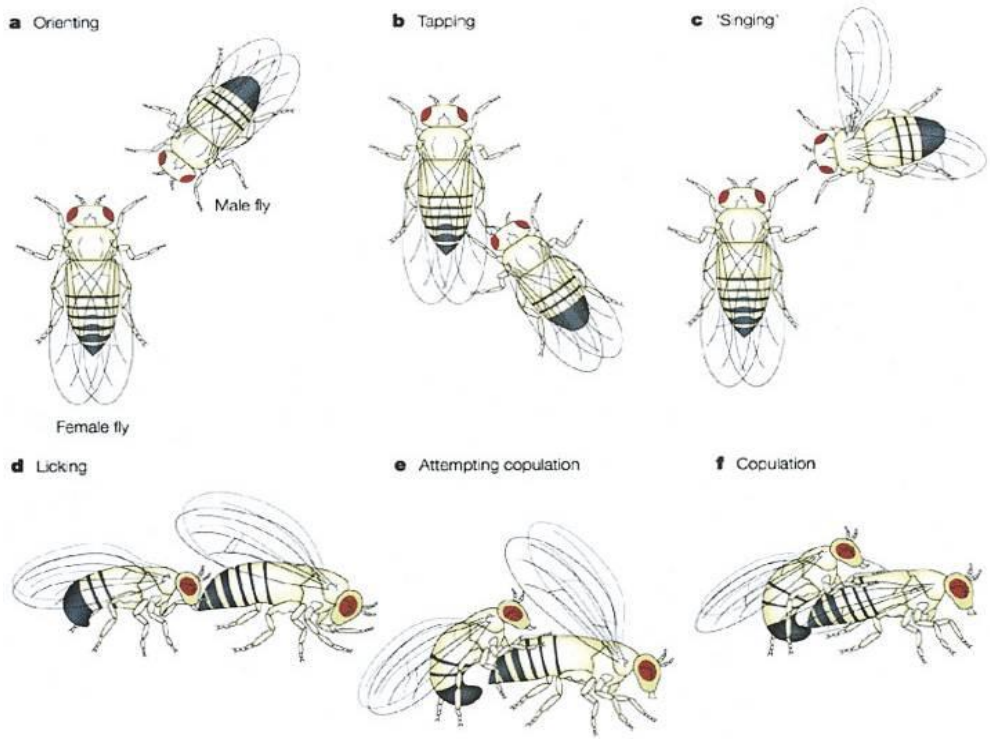


Figure 1-5: Acts in *Drosophila* courtship.

The six stages of Drosophila courtship whereby the male recognises the female (orientating), the male then begins to make contact with the female (tapping), wing extrusion is then performed by the male (singing), additional contact is then made by the male with its proboscis (licking) and then the male will attempt copulation these attempts may then lead the female to accept the male (copulation). Figure taken from (Emmons & Lipton, 2003)

One of the key genes in development of courtship behaviours and sex determination is the transcription factor *fruitless* (*fru*) (Ryner *et al.*, 1996; Ito *et al.*, 1996). f*ru* is 180 kb in size with 18 different isoforms and 4 promoters, splicing of *fru* is integral to sex determination with a male and female transcript generated (FRU^M, male and FRU^F, female). In females' the gene sex-lethal gene (*Sxl*) produces a full length transcript of the transformer (*tra*) mRNA, this is not present in males; non-functional *tra* mRNA is made in males. TRA controls the splicing of *fru* and mutations

that ablate the function of *tra* or the *tra-2* gene cause females to produce the FRU^M isoform, displaying male courtship behaviour. The effect of FRU^F expression in males is similar as it removes the ability to court females and moreover mutation of the *fru* gene in males led to an increase in homosexual behaviour with males courting each other forming chains (Ryner *et al.*, 1996; Ito *et al.*, 1996). Part of the mechanism of FRU action is controlled epigenetically, in males FRU binds the transcription co-factor BON (BON), once bound chromatin modifying enzymes, Heterochromatin protein 1a (HP1a) or Histone deacetylase (HDAC1) are recruited that regulate gene transcription (Ito *et al.*, 2012). With the authors concluding that HDAC1 was responsible for modifications that cause masculinisation of neurons, while HP1a counter act these changes (Ito *et al.*, 2012).

Links establishing a role for the clock in the regulation of courting behaviour have been established, the timing of mating activity was abolished in the per^{01} clock mutant (Sakai & Ishida, 2001). Different mating rhythms were observed between *D.pseudoobscura* and *D.melanogaster*, when rescue of a per^{01} phenotype in *melanogaster* was achieved using the *pseudoobscura per* gene, the same mating profile as *pseudoobscura* was witnessed (Tauber *et al.*, 2003). The length of copulation or time in copula (TIC) was also controlled by clock genes; per^{01} mutation had an effect on TIC as it lengthened the copulation duration significantly as did tim^{01}. This appeared to suggest a role for the clock in regulating this behaviour, except that C*lk* and *cyc* mutants were not significantly different from controls (Beaver & Giebultowicz, 2004). Moreover rescuing per^{01} in the central brain did not recover the TIC phenotype

confirming that the central oscillator is not directly involved (Beaver & Giebultowicz, 2004).

A big step in linking courtship behaviour to clock regulation was accomplished when NEUROPEPTIDE F (NPF) was uncovered (Brown *et al.*, 1999). NPF is involved in feeding, metabolism and ethanol sensitivity, reviewed in (Nassel & Wegener, 2011), but was also shown to be expressed in a sexually dimorphic pattern with males carrying 26 NPF cells and females 20 (Lee *et al.*, 2006). Identification of NPF expression in clock cells was accomplished originally highlighting three LN_ds, although recently this has expanded to include 1-3 l-LN_vs and the fifth non-PDF expressing s-LN_vs (Lee *et al.*, 2006; Hermann *et al.*, 2012). Expressing *tra* in NPF cells abolished courtship behaviour in males; this result coupled with data revealing that limiting the firing of *tim* cells gave significantly less mating in both males and females emphasising the link between courting behaviour and circadian rhythmicity revealing shared components (Lee *et al.*, 2006; Kadener *et al.*, 2006).

1.12 Additional *cry* phenotypes

At this stage we have discussed the circadian clock and the molecular regulation that controls it, as well as looking at the neuronal control of the clock on locomotor activity. One gene in particular, *cry,* has been shown to be important regulation of some of these behaviours, although the complete picture of how *cry* performs these tasks is not clear. The next section details additional phenotypes controlled by *cry* and examining the possible mechanistic control exerted by *cry*, followed by a description of a novel putative CRY interacting protein discovered in our laboratory.

There appears to be a functional role for *cry* in the entrainment to heat pulses, 25°C to 37°C, as these pulses appear to be capable of causing phase delays but not advances in wild type flies (Kaushik *et al.*, 2007). In cry^b flies there was no shift in behaviour when a heat pulse was applied, the same for a light pulse, this behaviour was moderately rescued by only expressing *cry* in the s-LN_vS and l-LN_vS (Emery *et al.*, 1998; Stanewsky *et al.*, 1998; Kaushik *et al.*, 2007). This response to heat seems to be caused by the interaction between PER and CRY; per^L mutants are normally hypersensitive to temperature pulses but in a cry^b background these responses are diminished (Kaushik *et al.*, 2007). Recently the action of a heat pulse was shown to be equivalent of a pulse of dim light and the same neurons (*cry* expressing LN_ds and DNs) showed in-phase TIM degradation (Tang *et al.*, 2010).

A study by Gegear et al (2008) demonstrated that *cry* was essential in naïve and trained responses to magnetic fields; whereby light filtered in the *cry* activating blue region (<420 nm) abolished wildtype responses to magnetic fields (Gegear *et al.*, 2008). Reduced response to magnetic stimuli was also shown in cry^b/cry^0 backgrounds and flies overexpressing *cry* were shown to be hypersensitive to magnetic stimulation (Yoshii *et al.*, 2009). It would be logical to assume that seasonal/circadian rhythms and magnetic field sensing were complementary with many migration patterns occurring at a particular time of year, however, magneto-sensitivity appears to occur regardless of a functional clock (Gegear *et al.*, 2008). Although the wavelength of the magnetic field appears to effect the period length, with a significant number of flies showing a period increase at 300µT if exposed to blue light (Yoshii *et al.*, 2009). Therefore, *cry* is

involved in light dependent magneto-sensing regardless of a functional clock, but the mechanism of action is not fully understood.

The discovery that the rhythmic expression of transgenic luciferase transcripts was abolished in a cry^b background, yet locomotor activity was akin to wild-type was perplexing to the field (Stanewsky *et al.*, 1998). As most of the light from a luciferase assay stems from peripheral tissue i.e. non-clock cells, levels of cycling TIM and PER was examined in the eye, showing arrhythmic expression of these proteins (Stanewsky *et al.*, 1998; Stanewsky *et al.*, 1997). This result and *in-vitro* cell work by Collins et al (2006), suggested that in peripheral tissues CRY can influence the transcriptional cascade mediated by CLK-CYC, repressing it much like the mammalian CRY (Collins *et al.*, 2006; van der Horst *et al.*, 1999). This notion that *cry* can function differently between clock cells and other cell groups opened up the possibility that *cry* function is not cell type ubiquitous. Furthermore it has been shown that the CRY levels between the sub-sets of clock cells are not homogenous, CRY is low in the s-LN_vs compared to LN_ds under LD conditions but was elevated in DD (Yoshii *et al.*, 2008). An additional discovery was that the cycling of CRY was of high amplitude in the LN_ds but not in the s-LN_vs, moreover the kinetics of CRY degradation was shown to be different between the small and large LN_vs (Yoshii *et al.*, 2008). This hints at the possibility that CRY is regulated differently within a clock cell context. The actions of CRY appear to extend beyond TIM degradation in regulating clock behaviours. Tang (2010) recently found that TIM was not degraded in the s-LN_vs during a delay light pulse, however, down regulating *cry* with RNAi in the s-LN_vs did influence the delay length (Tang *et al.*, 2010).

A non-TIM mediated response to light could be the result of *cry* influencing the membrane properties (Fogle *et al.*, 2011).

The revelation that CRY is necessary for the firing of the l-LN_vs neurons upon exposure to light, unveils a new non-TIM degradation pathway through which *cry* can influence locomotor activity (Fogle *et al.*, 2011). CRY mediated neuronal firing was shown to be important for night time arousal and the same study also found that the response to mechanical stress was abolished in cry^0 flies but remained in cry^b (Kumar et al., 2012). This suggests that proteins that interact with CRY to produce a mechanical stress response can still interact with CRY^b.

Therefore proteins that interact with CRY may help to explain some of the complexity related to how *cry* performs many of the tasks attributed to it. Clock proteins such as TIM, PER and JET have well documented interactions with CRY in clock related phenomena, however, the varying CRY kinetics in different clock cells (Yoshii *et al.*, 2008), non-TIM mediated firing of cells (Fogle *et al.*, 2011), tissue specific CLK-CYC transcription repression (Stanewsky *et al.*, 1998; Collins *et al.*, 2006) and clock independent sensing of magnetic stimuli (Gegear *et al.*, 2008) all suggest that other proteins may interact with CRY to assist in the regulation of particular phenotypes.

1.13 *CG15803* and aims of project

For the purposes of identifying proteins that interact a yeast-two hybrid screen was undertaken (Hybrigenics); using CRY as bait the screen revealed a protein interacting with CRY in a light dependent manner.

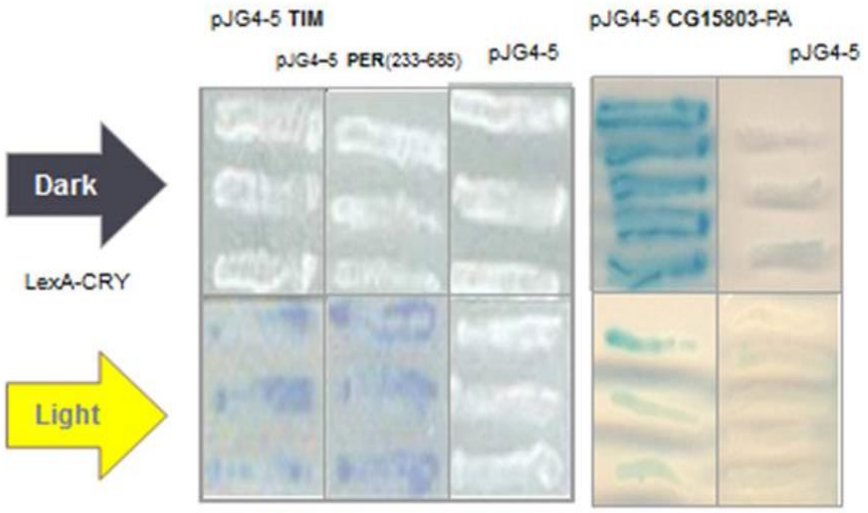


Figure 1-6: Yeast-two hybrid of CRY interacting proteins, columns ordered left to right.

Column 1: TIM challenged with CRY in Darkness (top column) and light (bottom column). White staining indicates a lack of binding, blue binding has occurred.

Column 2: PER challenged with CRY in Darkness (top column) and light (bottom column). White staining indicates a lack of binding, blue binding has occurred.

Column 3: Vector control challenged with CRY in Darkness (top column) and light (bottom column). White staining indicates a lack of binding, blue binding has occurred.

Column 4: CG15803 challenged with CRY in Darkness (top column) and light (bottom column). White staining indicates a lack of binding, blue binding has occurred.

Column 5: Vector control challenged with CRY in Darkness (top column) and light (bottom column). White staining indicates a lack of binding, blue binding has occurred.

The figure above shows a novel protein that interacts with CRY primarily in darkness (column 4), therefore the CG15803 gene was re-named *dark active in yeast* (*day*).

Work in our laboratory has shown the cDNA of this transcript to cycle in LD conditions and *in-situ* hybridisation showed staining in potential clock cell loci in the brain.

Structural analysis data reveals DAY carries 4 PDZ domains, which are protein interaction motifs. Proteins carrying more than one PDZ are associated with a scaffold function where proteins typically located at the membrane are organised to ensure efficient signalling processes or to polarise cells; reviewed in (Sierralta & Mendoza, 2004). One example of a characterised PDZ gene is *discs large* (*dlg*) which is known to be involved development in epithelial cell polarity and is located both pre and post synaptically in the larval neuromuscular junction determining the location of the potassium channel *Shaker* (Tejedor *et al.*, 1997). Therefore based on the motif analysis alone we can examine signal related function and possible developmental roles for *day*. RNA analysis of developing flies shows expression of *day* rising after 14 hours in embryonic development and continuing expression in adult tissue, this could indicate a role for *day* in development (Graveley *et al.*, 2011).

A yeast-two hybrid experiment with CRYΔII, carrying a large deletion (50 aa) of the C-terminus (CT) of CRY, abolished binding of CRY to DAY, marking the CT of CRY as a potential interaction domain. The regulation of the light responsive binding of CRY to PER and TIM has already been shown to be controlled by the CT of CRY (Rosato *et al.*, 2001). Challenging DAY with CRYΔ caused constitutive binding in both light and dark akin to PER and TIM (Dissel *et al.*, 2004). Moreover a number of motifs and potential phosphorylation sites on CRY's CT have been identified *in-silico.* Particular CT residues were mutated and their *in-vitro* binding assays with known clock proteins were performed (Hemsley *et al.*, 2007). However, *in-vivo* studies with these CRY mutants

has not been attempted; to address the impact of substituting residues, locomotor activity analysis has been conducted on lines overexpressing these CRY mutations in clock cells (detailed in chapter 9). Characterising these mutants may then allow predictions to be made about their association with DAY helping to understand the relationship between DAY and CRY more clearly.

Therefore having identified a potential CRY interactor, DAY, a selection of genetic tools are required to examine if DAY and CRY possess a functional relationship *in-vivo*. These tools can potential be used in:

- Establishing the coding sequence (CDS) and intron boundaries of *day*
- Protein work with overexpressed DAY, trying to identify CRY binding
- Knockout of the *day* gene
- Examination of the function of *day* in knock-out flies
- Characterising the cellular expression of *day-GAL4* constructs
- Analysing the function of the cells expressing *day-GAL4*
- Behavioural analysis of *cry* CT mutants

With this data it will be possible to understand if there is functional relationship between DAY and CRY and the consequences of this binding.

2 Materials and Methods

The following protocols cover the conventional methodology as specialised protocols will be addressed in their respective chapters.

2.1 DNA extraction single fly

DNA was extracted from single flies for polymerase chain reaction (PCR) using the following protocol. The fly is anaesthetised and placed into a 1.5 ml tube where it is homogenised in 50-100 µl of squishing buffer. Squishing buffer is composed of:

- 10 mM Tris-HCl pH 8.2
- 1 mM EDTA
- 25 mM NaCl
- 200 µg/ml Proteinase k

This solution is then incubated at 37°C for 30 min, after which it is then heated to 95°C for 2 min. 1-2 µl was used for subsequent amplification (Gloor *et al.*, 1993).

2.2 DNA extraction from multiple flies

In order to extract DNA from groups of flies the E.Z.N.A® insect DNA kit (omega, USA) was employed. Fifty flies were collected and then frozen in liquid Nitrogen, using

a pestle and mortar the frozen flies were ground into a powder. The resulting powder was then transferred into a clean tube, after which the protocol used is as stated in the kit. The average yield for 50 flies was ~400 ng/µl.

2.3 PCR

When PCR was performed it was either for 'cloning' or 'diagnostic' purposes. Cloning PCRs were performed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs Inc, USA), this proof reading Taq was used to minimise the number of errors that are inherent in the PCR procedure. All cloning was performed using DNA derived from the fly strain w^{1118}. Diagnostic PCRs were performed using standard Taq polymerase (Kappa Biosystems, USA). The concentration of reagents and a standard temperature profile are listed below.

Reagent	Volume (µl)
5x Buffer	4 µl
10 mM dNTPs	0.4 µl
Primer For 10 mM	2 µl
Primer Rev 10 mM	2 µl
Template DNA	2 µl
Phusion Polymerase	0.2 µl
H2O	To 20 µl

Table 0-1: Standard 20 µl PCR mix

Temperature (°C)	Number of cycles	Time
95	1	2 min
95		20 secs
See primer table for specific annealing Temps	30	30 secs

72		Dependent on product size 30 secs per 1 kb Phusion 1 min per 1 kb Kappa
72	1	10 mins
10	1	store

Table 0-2: Standard thermocycler program

PCR reactions were carried out on a DYAD™ DNA Engine (MJResearch, USA) or a G-Storm GS4 thermal cycler (G-Storm, UK). Gradient programs were utilised to determine optimum annealing temperature of all the primers used in this work.

2.4 Agarose gel electrophoresis

Agarose gel solutions ranging from 0.8-1.5% were made using 0.5% TBE (109 g/L Tris, 55 g/L Boric Acid, 9.3 g/L EDTA in water). Concentrations of gels and the voltage/running times were tailored to the product size, with a standard voltage range of 80-120 volts lasting 45-100 min. To determine the endpoint of the gel loading dye was added to all samples and ladders (0.25% Bromophenol blue, 0.25% Xylene cyanol). Visualisation was achieved using the Gene Genius apparatus (Syngene, UK), with 2 µl of ethidium bromide dissolved in a 50-100 ml gel. The primary ladders chosen were: Hyperladder IV, Lambda and Phix 174 (Bioline, UK).

2.4.1 Gel extraction procedure

DNA was recovered using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA). The only deviation in protocol was an additional spin cycle of 2 mins at

14,000 rpm after the last wash to ensure excess alcohol was removed. This was crucial for successful ligations.

2.5 Bacterial transformation

The XL1-MRF' blue *E. coli* cells (Stratagene, UK) were used for plasmid transformation using the following protocol:

- 40 µl of cells were defrosted.
- 2 µl of plasmid DNA (5 ng/µl) added to cells.
- The DNA/cell mixture was added to an ice cold electroporation cuvette and shocked at 1.5 kV, 1000 Ω, and 25 µF using a Biorad Gene Pulser (Biorad, Germany).
- 1 ml of Luria Bertani (LB) broth was added to the mixture which was then shaken for 1 hour at 37°C.
- 100 µl of a dilution series ranging from 1/1000 to 1 was applied to LB agar (LBA) plates with the appropriate antibiotic for selection purposes.
- Plates were grown at 37°C overnight, from which, single colonies were chosen for growth in liquid culture and 'mini-prep' analysis

Transformation experiments that involved a ligation procedure were run with a positive control (plasmid allowed to self-ligate) and a negative control (DNA insert only with no plasmid).

2.6 Plasmid retrieval (mini-prep)

To obtain the required concentration/purity of plasmid DNA the E.Z.N.A® HP Plasmid Mini Kit I (Omega, USA) was utilised. There were no modifications made to the protocol provided.

2.7 Restriction digest

Restriction digests were performed using NEB enzymes and appropriate buffers (1-4). Reactions typically consisted of 1x buffer, 1x Bovine Serum Albumin (BSA), no more than 5% (in vol) of restriction enzyme and DNA/H_2O to volume. The reaction size/concentration of DNA was correlated to task. Smaller concentrations of DNA and volumes (typically 20 µl) were used for determining ligation success. Greater concentrations of DNA and 50 µl volumes were utilised for further cloning steps. Digestions were incubated for 1-2 hours as longer times run the risk of un-specific cutting and so were avoided.

2.8 Ligation

Ligation procedures were undertaken using the New England Biolabs inc T4 ligase, a typical reaction comprised of:

- 10x ligase buffer 1.5 µl
- ~ 5-30 ng/µl DNA template 5 µl

〉 Ligase 1 µl 400 units

〉 H_2O 7.5 µl

Reaction conditions were 4°C, overnight for blunt-end ligations and room temperature, 2 hours for sticky-ends joining. Ligation reactions were halted with, 1.5 µl of 3M NaAc and 50 µl of Isopropanol. The ligated DNA was then precipitated by centrifugation (20 min at 13,000 rpm on an Eppendorf microfuge), washed twice with 80% ethanol and re-suspended in 5 µl of H_2O.

2.9 Shrimp alkaline phosphatase

The efficiency of ligation steps was improved using an Antarctic shrimp phosphatase (New England biolabs inc, USA), preventing self- ligation of vectors. Reactions were assembled as follows:

〉 10 µl DNA ~10-50 ng/µl

〉 1 µl SAP (5 units)

〉 3 µl restriction enzyme buffer (1-4)

〉 16 µl H_20

Once reactions were complete they were incubated for 1 h at 37°C; heat inactivation (65 °C for 30 min) preceded further steps.

2.10 Sequencing

Cloned DNA fragments were either sent for Sanger sequencing analysis at the Protein and Nucleic Acid Chemistry (PNACL, University of Leicester) or sequencing reactions were carried out in the laboratory. This was performed adding

- 1 µl Big Dye Ready Reaction Mix v3.1 (vhbio, UK)
- 3.5 µl 5x Big Dye Terminator Buffer
- 10 µl DNA (20-30 ng/kb)
- 3.2 µl primer (1 µm)
- H_20 to 20 µl

The mixture was incubated on a thermocycler using the profile:

96°C 10 sec
50°C 5 sec
60°C 4 min
} 30 cycles

10°C store

Unbound dyes were eliminated using Performa DTR Gel Filtration Cartridges (vhbio, UK) that were spun at 820 g for 1 min. The sequencing reactions were then run on a capillary electrophoresis apparatus (Applied Biosystems 3730) by PNACL.

2.11 Sequencing Vector pBluescript

The pBluescript® II KS (Stratagene, UK) vector was selected as a vessel for cloning. It possess a divergent multiple cloning site (MCS), suitable antibiotic detection system, Lac Z blue/white selection and M13 sequencing primer sites. Appendix I contains a map of this plasmid.

2.12 p-UAST vector

All of the overexpression flies that have been utilised have been created in the p-UAST vector (*Drosophila* Genomics Resource Center, USA). It is a P-element vector that carries: mini-white gene for tracking, 5x UAS elements that are recognised by GAL4 and appropriate cloning motifs/ selection system. Vector map in appendix I.

2.13 RNA extraction

The extraction of RNA was performed using sixty heads from w^{1118} flies. The heads were homogenised with 200 µl of trizol (Invitrogen) in a 1.5 ml microfuge tube, after which 1 ml of trizol was added to the solution and left for 5 min at room temperature. The sample was spun at 14,000 rpm for 10 min at 4°C, with the supernatant dispensed into a fresh tube and the remaining detritus discarded. 200 µl of chloroform was added to the supernatant and then inverted fifty times, after which the sample was left to settle for 3 min. The mixture was then spun for 15 min at 4°C, the supernatant removed and kept before 500 µl of Isopropanol was added and then left for 10 min at room temperature. This was then spun for 10 min at 4°C, the

supernatant was discarded and the remaining pellet was washed with 1 ml of 75% ethanol. The ethanol was decanted and the sample was spun at 8000 rpm for 5 min at 4°C. Residual ethanol was removed and the pellet was re-suspended in μl of ultra-pure water.

2.14 DNase treatment/cDNA generation

Before cDNA synthesis, the extracted RNAs were subjected to DNase treatment (Ambion, UK). The reaction consisted of:

- 1 μg Nucleic acids
- 1 μl DNase buffer
- 1 μl DNase
- 3 μl H_2O

This was left at Room temperature for 15 mins and then the DNAse was deactivated using 1 μl EDTA and 10 mins at 65°C. cDNA was generated using ImProm-II Reverse Transcriptase (Promega, USA) .The reagents:

- 3 μl DNase treated RNA
- 1 μl oligo DT primers
- 1 μl H_2O

were mixed together and then incubated at 70°C for 5 mins before resting on ice.

Then the remaining components were added:

- 4 µl 5x buffer
- 2.4 µl $MgCl_2$
- 1 µl DNTPs
- 1 µl RNAsein
- 1 µl Reverse Transcriptase
- 6.6 µl ddH_20

The mixture was then placed into a thermocycler using the following program:

25°C 5 mins

42°C 60 mins

70°C 15 mins

1 µl of the above mixture containing cDNA was used for each 20 µl PCR reaction; any remaining volume was stored at -80°C.

2.15 Immunohistochemistry

Whole flies were collected and fixed, in a solution containing 4% paraformaldehyde (PFA), 1% triton and 0.1% DMSO, for 2 hours at room temperature or overnight at 4°C. Flies were washed in phosphate buffer solution (PBS) with 0.1% triton five times and dissected in 1x PBS. Post dissection the brains were washed five

times in a 1% PBST (PBS with 1% triton added). A 10% goat serum in 0.5% PBST was applied for 2 hours at room temperature or at 4°C overnight as a blocking solution.

Primary solutions consisted of 1x PBS with 10% Goat serum and 0.1% NA Azide with above antibody diluted appropriately. Secondary solutions made with 1x PBS and 10% Goat serum and respective dilution of appropriate antibody. Secondary staining took 2.5-3 hours in dark conditions or for rabbit biotin 1 week at 4°C. Brains mounted onto glass slides in 80% glycerol and 3% Propyl gallate. Visualisation was achieved using a laser scanning confocal microscope (Olympus FV1000) and images created using the FV-10 ASW 2.0 viewer program.

2.16 Western blotting

2.16.1 Protein extraction

Flies were collected in 50 ml polyproylene tubes and immediately frozen in liquid Nitrogen. To separate heads from bodies they were vortexed for about 1 min for three times and cooled in liquid nitrogen between vortexing attempts. Fly heads were retained on a metallic sieve and then moved into a metal plate, the procedure was performed on a bed of dry ice. Heads were then collected into fresh 1.5 ml microfuge tubes and homogenised with 1.5x extraction buffer (0.1% triton, 10 mM EDTA, 1 mM DTT, 0.05 mM PMSF, 10 µg/ml Aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin in HEMG: 1 M KCL, 0.1 M HEPES pH 7.5, glycerol 5%). The extract was spun for 5 min at 13,000 rpm and the supernatant was moved to a new tube. To quantify the extracted proteins, 1 µl of protein sample was mixed with 800 µl of water and 200 µl of Bradford reagent (Sigma-Aldrich, USA) into a cuvette were the mixture was allowed to settle for

5 mins at RT. Then the optical density (OD) was measured at 595 nm with a spectrophotometer (Eppendorf, Germany). The OD values were used to equalise the protein content of each sample before loading.

2.16.2 Resolving and stacking gels

The following solutions were mixed to prepare a SDS acrylamide gel to run on a Hoefer (Germany) large tank system

Resolving gel (10% 40 ml)	Stacking gel (7% 15 ml)
〉 H_20 19 ml	H_20 11.1 ml
〉 2 M Tris pH 8.8 7.4 ml	1 M Tris pH 6.8 1.5 ml
〉 SDS 20% 200 µl	SDS 20% 75 µl
〉 APS 25% 160 µl	APS 25% 30 µl
〉 TEMED 24 µl	TEMED 20 µl
〉 Acrylamide 30% 13.3 ml	Acrylamide 30% 2.25 ml

10 µl of loading buffer (188 mM Tris HCl pH 6.8, 6% v/v SDS, 30% glycerol, 15% v/v βMecaptoethanol, 0.03% Bromophenol Blue) was added to 20 µl of all samples. These were then heated at 95°C for 5 mins and spun at 13,000 rpm for 30 secs, before being loaded into the wells of the gel along with an appropriate protein ladder (pre-stained, wide range New England Biolabs inc, USA).

Gels were run on Tricine-Glycine buffer (Tris base 30.2 g, Glycine 188 g, 950 ml H_20, 0.1% fresh SDS), at 10 mA overnight.

2.16.3 Blotting

After electrophoresis the proteins were transferred on to a nitrocellulose membrane (Shleicher and schuell, Germany) using a custom made (Biomedical mechanical workshop at the University of Leicester) electroblotting apparatus and transfer buffer (Tris base 5.28 g, glycine 2.93 g, 20% SDS 1.88 ml, Methanol 200 ml, H_2O to 1 L). The run lasted for 2.5-3 hours on ice at 400 mA.

2.16.4 Staining and detection

The membrane was blocked for 1 hour in 100 ml of TBST (10 mM Tris HCL pH 7.5, 150 mM NaCl, and 0.05% w/v Tween 20) with 5% dry milk powder. The primary antibody (anti-HA mouse, Sigma, 1:10,000) diluted in a 5% milk TBST solution was then applied, for 2 hours with agitation. The primary was then removed and the residual unbound anti-body was washed off with three 20 min washes of TBST. The membrane was the incubated with the secondary antibody (HRP conjugated anti-mouse, Sigma, 1:6000) in milk TBST buffer for 1 hour, followed by further washes (20 min TBST performed thrice).

Detection of proteins was performed by chemoluminescence using an in house made solution (0.1M Tris HCl pH 6.8, 6.25 µM Luminol, 6.38 µM Coumaric acid, 2.7 mM H_2O_2) which was applied for ~1 min followed by a 1 min exposure to a X-ray film.

2.17 Fly stocks

Experiments were performed with D*. melanogaster* adults*,* which have been kept and crossed at either 25°C or 18°C in light dark (LD) 12:12 conditions. All stocks were grown on maize food (7 L H_20, 360 g maize meal, 396.5 g glucose, 250 g brewer's yeast, 62.5 g agar, 94 ml nipagin and 15 ml propionic acid). The following genotypes were used extensively; however, transgenic lines created in our laboratory will be referred to in their respective chapters.

Genotype	Description
w^{1118}	A fly strain which no longer carries the *white* gene and as a consequence no eye pigment. This line is used for transgenic injections and crossed to generate controls in activity experiments.
CS/+	Canton-S (CS) is a laboratory wild type strain; CS has been back crossed 6 times to the w^{1118} strain above (CS/+).
***w*;CyO/Sco;TM6b/MKRES**	This line was used for balancing constructs on the second and third chromosomes.
FM7a	Used for balancing on the X chromosome

Table 0-3: Common stocks

2.18 Locomotor activity, set up

Rhythmic locomotor activity was assessed using the *Drosophila* Activity Monitoring (DAM) system (TriKinetics inc, USA). Monitors are equipped with an

infrared (IR) emitter-detector system for each of their 32 channels, and through a computer connection, allow continuous recording of each IR beam break with 1 min resolution. 10cm glass tubes were filled with 2 cm of sugar food (110 g sucrose, 5 g agar, 110 g yeast, 2.4 L H_20) and capped with a cotton bung at the other end ensuring no escape of flies. Each tube was then loaded into a channel and the monitors were housed in a temperature and light controlled incubator (LMS, UK).

2.18.1 Activity analysis

The number of beam breaks was recorded every minute using the DAM system 2.1.3 software. Raw data was extracted in 30 minute bins for each channel to generate actograms, using Microsoft Excel macros (macros generated at the University of Leicester, Green E., Kyriacou CP, un-published data), data was plotted and analysed.

The individual activity data of a fly collected under free-running conditions will contain information on the flies' endogenous period. To analyse this data autocorrelation and CLEAN spectral analysis are employed. CLEAN is an algorithm originally designed by radioastronomers and the significance of the rhythmic components is assessed via a MonteCarlo approach. The data is randomly re-arranged 100 times and the CLEAN algorithm is re-run. This data is visualised on a graph with period values on the x axis with the amplitude of the oscillation on the y axis. The 95% and 99% confidence limits are computed and peaks above the 99% limit were selected as the period value, in some instances multiple peaks are generated and the flies were termed complex rhythmic. If no individual period component crosses the 99% threshold then the autocorrelation was analysed to determine if the fly is rhythmic.

Autocorrelation analysis allows resolution of rhythmic behaviour in individual flies by calculating a correlation coefficient between data points (Rosato & Kyriacou, 2006).

3 Analysis of *day* locus

3.1 Introduction

To re-iterate briefly we have discovered a gene, *day*, whose protein product displays a light dependent interaction with CRY in our yeast system. In order to understand the consequences of this binding we first sought to examine the *day* locus. The *day* locus has been updated a number of times in the *Drosophila* database Flybase since it has become known to our laboratory; therefore PCRs were done on DNA/cDNA to confirm the absence and presence of essential features of the gene. These included PCRs along the CDS of *day* and additional PCRs into the neighbouring introns to confirm that the start and end of transcription are correct. Furthermore a cDNA clone has been documented on Flybase containing a stretch of regulatory 3' day DNA, marked in green in the figure below, thus primers were also constructed to confirm the existence of this cDNA.

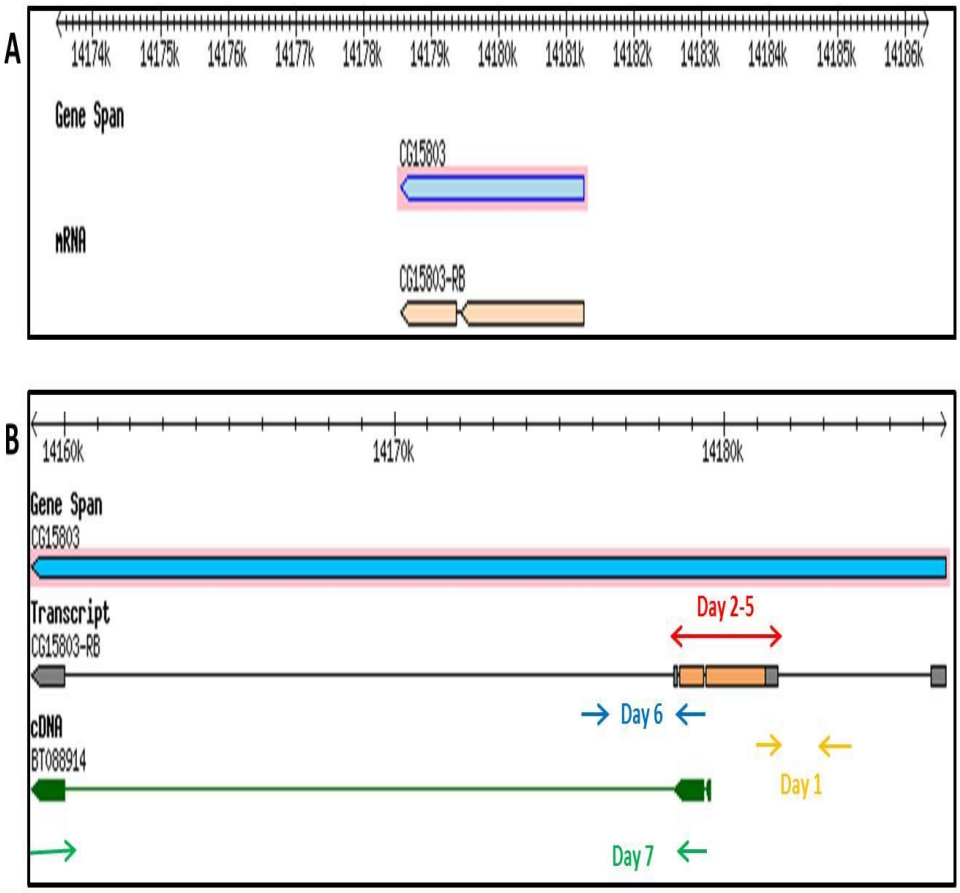


Figure 3-1: Overview of *day* locus and primer sites.

A: *day* (*CG15803*) locus as shown in Flybase in 2009, B: Updated description (2011) of the *day* locus with 5' and 3' regulatory elements added; arrows represent primer binding sites. Images obtained using 'GBrowse' from the *Drosophila* web-site Flybase.org.

3.2 Materials and methods

Overlapping primers were designed, amplifying the CDS and upstream/downstream of juxtaposing intron sequence.

Primer name	Sequence	Annealing Temp (°C)	Band size
Day 1F	GTTAGGGCCTTGATCACCAC	68	1000 bp
Day 1R	TCAGCAGCATTATCCGAGTG		
Day 2F	ACCACGTACGTTTCCGTTTC	55	900 bp
Day 2R	AGGTGGAGGTCATCGATCTG		
Day 3F	GCTTTCACAGGTGCCTCATT	55	1000 bp
Day 3R	AGAACCGGCACATTGAGACT		
Day 4F	TCAAGGCTGATCCCTAGACC	50	1000 bp
Day 4R	TGGAGCTGCACAAGAATGTC		
Day 5F	CTCCAGTTCCTGTTCCGTTTT	55	1000 bp
Day 5R	AATGAGGCACCTGTGAAAGC		
Day 6F	CATGAAAAGCCTGTGGTCAA	55	900 bp
Day 6R	ACATAGTGGCAGCCCCTTC		
Day 7F	AATGTCCGCGAATTCTTGAG	55	900 bp
Day 7R	ACATAGTGGCAGCCCCTTC		
Cry SDF	ATTTCGTTGCGCACACATAC	55	900 bp
Cry SDR	AAACGCATCCGATTGTAACC		

Table 3-1: *day* primers

Primer pairs 2-5 amplify the entire CDS of *day*. The Day 1 primers begin at the start of the *day* coding sequence (CDS) and span 1000 bp upstream; these primers are intended to ensure the presence of an intron before the CDS and thus should fail to amplify the cDNA sample. The Day 6 primers concern the 3' end of the gene and begin at the very end of the CDS extending into the neighbouring intron by 1000 bp, these

primers should not function with the cDNA template. An additional regulatory sequence at the 3' end of the *day* locus was reported more recently. Day 7 primers should be capable of amplifying it but only in the cDNA sample as the intron separating the day stop codons and sequence is in excess of 10 kb. The final primer set is designed to amplify the first intron of the *cry* gene; these were used to identify genomic contamination in the cDNA template.

3.3 Results

The primers set 2-5 were designed to cover the entire CDS and were successful in amplifying both cDNA and genomic templates. The amplifications carried out with Primers sets 1 and 6 confirmed the intron/exon boundaries upstream and downstream of the coding region as they do not amplify the cDNA. The amplification with primer set 7 confirmed the presence of a non-coding exon starting about 10 kb downstream from the stop codon. The set of primers designed for the intron of *cry* confirmed the validity of these results as no genomic contamination was present in the cDNA template.

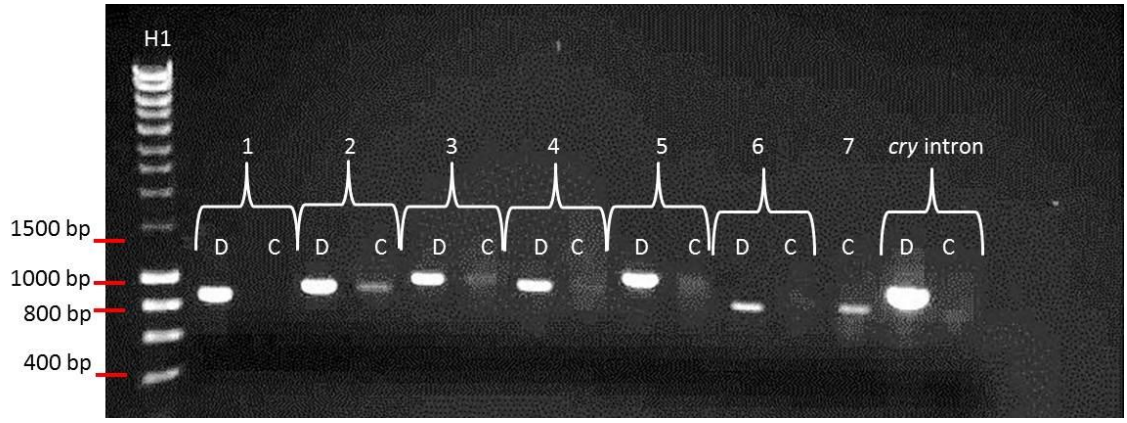


Figure 3-2: PCR of *day* CDS and flanking sequences.

H1 Hyperladder, C= cDNA template, D=Genomic template.

1. Day 1 primers: the amplification band is seen in D but not in C.

2. Day 2 primers: the amplification bands are seen in both the D and C lanes.

3. Day 3 primer: the amplification bands are seen in both the D and C lanes.

4. Day 4 primer: the amplification bands are seen in both the D and C lanes.

5. Day 5 primer: the amplification bands are seen in both the D and C lanes.

6. Day 6 primer: the amplification band is seen in D but not in C.

7. Day 7 primer: Only C sample should amplify and does so.

Cry SD primer: only amplification in D sample.

3.4 Discussion

The PCR analyses described above started at a time when the annotation of the *Drosophila* genome was still quite imprecise. To understand the function of *day* I wanted (1) to design a strategy of gene knock-out (KO) by homologous recombination, (2) to use existing UAS-day lines for the overexpression of DAY, (3) to produce *day-GAL4* lines to aid in the identification of the cells expressing *day* and to allow their subsequent manipulation. All of the above required us to ascertain the correct intron/exon structure of the gene and to locate where the putative promoter and enhancers sequences, may lie. This PCR analysis was completed at an early stage of the project and the results obtained were used to plan the work outlined above. More recently the database has been updated using high-throughput sequencing data. These have revealed the presence of an additional non-coding exon approximately 5 kb before the start codon, which has pushed the putative promoter region further upstream. The coding region remains unaffected; therefore the strategies adopted for the production of *day* KO and for generating *UAS-day* lines remained valid. However, there has been an effect on the design of the *day-GAL4* lines, which will be discussed in chapter 7. It would have been possible to confirm the existence of this regulatory sequence using the same approach shown above, however, because of the high level of confidence associated with these novel techniques and the limited information this would give on the research questions posed it was not performed.

4 Generation of transgenic tools I: HA-STREP(II)*day*

4.1 Introduction

Confirming the interaction of DAY and CRY proteins *in-vivo* will be a critical step in establishing a functional relationship between these two proteins. In particular determining the light dependence of this binding, as mentioned previously there is a lot of data concerning the role of *cry* in light driven behaviours, but a dark function for *cry* has only recently been shown experimentally (Kumar *et al.*, 2012). Could *day* be a modulator of *cry* during these dark periods? Before commencing this work there was no antibody available for DAY that functions on a western blot and the CRY antibody is not sensitive enough to the lower levels of CRY present in a light sample. To ascertain the likelihood of DAY and CRY binding *in-vivo* requires a co-IP protocol with tagged UAS-*day* and *cry.* The fly lines UAS-HA*day* and UAS-MYC*cry* were already available in our laboratory. MYC is a short peptide sequence of 10 amino acids taken from the *c-myc* gene, while the HA tag is derived from a glycoprotein from the influenza virus envelope. Although in possession of a tagged version of *day* (HA) an additional tag was selected to create a double-tagged version of UAS-*day* more suitable for co-IP experiments. The STREP(II) tag reliably binds to the StrepTactin ligand. Its small size (8 amino acids), high levels of affinity to its ligand and capability of being eluted at physiological pHs make it an ideal tag (Skerra & Schmidt, 1999). Moreover a double tagged *day* construct would allow pull-down experiments to be carried out with each tag individually, then mass spectrometry analysis would allow you to compare the proteins binding to DAY in each sample, allowing the identification of overlapping proteins interacting with DAY between tagged samples.

The work described below details the cloning and insertion of the STREP (II) tag into the UAS-HAday construct, shows western blotting analysis of lines expressing UAS-HA-STREP(II) *day* and details a co-IP experiment undertaken with said genotype.

4.2 Materials and methods

Only standard methods and reagents were used for this work, referred to in the general methods section (Chapter 2). All transgenic lines created in this work are listed in appendix 2.

4.3 Results

A construct carrying the cDNA of *day*, directly fused at its 5' end, with the sequence for the hemagglutinin (HA) tag, was already available in the laboratory as a pBluescript(II) plasmid (PBS). The strategy was then to add, in frame, the sequence encoding the STREP(II) tag between the HA coding fragment and *day* cDNA. Two complementary oligonucleotides were designed, shown below, exploiting the presence of an *EcoRI* site at the boundary between *HA* and *day* sequences.

Primer name	Sequence	Annealing Temperature (°C)
*EcoRI*f	5'-AATTCTGGTCCCACCCCCAGTTCGAGAAGG	
EcoRIr	5'- AATTCCTTCTCGAACTGGGGGTGGGACCAG	79°C

Table 4-1: STREP(II) primers

The primers were brought together and annealed at 79°C; this resulted in the generation of a STREP(II) sequence flanked by *EcoRI* sites.

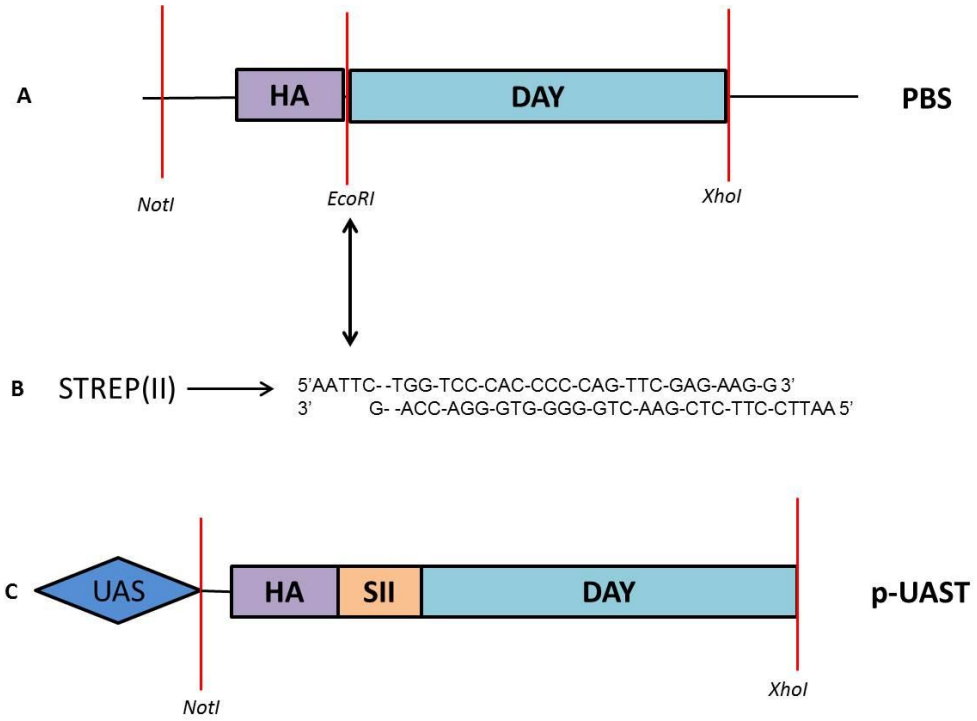


Figure 4-1: Cloning strategy for generating double tagged *day*.

A: original single HA tagged day cDNA in PBS, B: STREP(II) annealed oligos with EcoRI ends, C: completed construct with STREP (II) in p-UAST. SII= STREP(II).

The STREP(II) sequence was generated through the annealing of complementary oligos, it was then ligated into the PBS vector containing HA-*day*, via the *EcoRI* site. The STREP(II) sequence could potentially ligate in either orientation, to ensure the insertion was orientated correctly, sequencing was undertaken. A clone with STREP(II) correctly inserted was sub-cloned into p-UAST using *NotI* and *XhoI,* commercial injection of the plasmid was undertaken at the Department of Genetics, University of Cambridge using conventional injection techniques (Rubin & Spradling, 1982).

To test the levels of protein expression Western blotting was carried out on head samples from five UAS-HA-STREP(II)-*day* lines. These fly lines were crossed to the *tim*-GAL4 driver (TG4) which overexpresses tagged *day* in the clock cells and the progeny were raised in 12:12 LD conditions. Heads were taken and proteins extracted as stated in chapter 2.15, fly lines are listed in appendix 2.

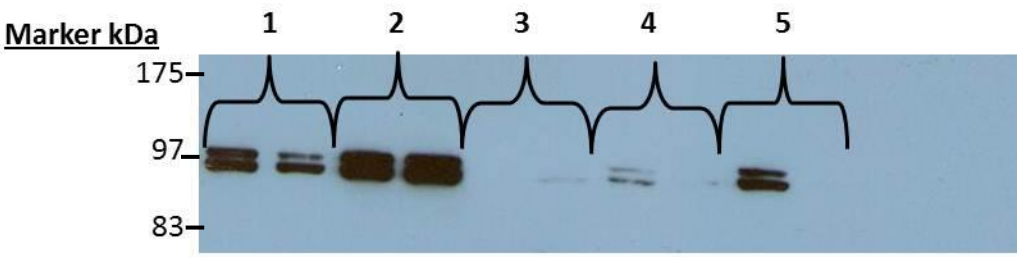


Figure 4-2: HA-STREPII-DAY protein expression in clock cells from different transgenic lines.

Each number represents to a cross between a HA-STREP-day line (genotypes listed below) and *tim*-GAL4 (TG4). For each cross the F1 were sampled at ZT12 (left) and ZT24 (right). 1: DSIIF1, 2: DSII S3, 3:DSII T5,4: DSII F2 and 5: DSII S65.

2 out of the 5 tested lines successfully overexpressed DAY to an appropriate visible level (samples 1 +2) and the size of the band is correct. It is apparent that there are two bands present in the blot, this double band could be a consequence of intron splicing. Within the coding sequence of DAY residues an intron of 57 bp, although the resolution of the gel does not allow for the difference in size of the doublet to be separated, it could be that the difference in size of the two bands may be caused by intron splicing. Sampling the overexpression at two time points does not appear to give a consistent difference in the abundance of DAY, suggesting that DAY overexpression is not effected by either light or dark conditions. From this experiment a line DSIIS3 (lane 2) was selected for further analyses.

4.4 DAY-CRY co-IP assay

The experiment described below was a collaboration with a PostDoc in the laboratory, Dr Celia Hansen, whom directly performed the immunoprecipitation. Flies carrying *tim-GAL4 (TG4)*, UAS-HA-STREP(II)-*day* and UAS-MYC-*cry* were entrained in LD at 25°C. These flies co-expressed HA-STREP(II)DAY and MYC-CRY in all clock cells. As a control, TG4 UAS-MYC*cry* was crossed to w^{1118}, hence no UAS-*day* expression. The 'Dark sample' (D) flies were collected at ZT23.5, whereas the 'Light sample' (L) consisted of flies maintained for 30 min under bright white light starting from ZT23.5. Light and dark samples were frozen in liquid Nitrogen and protein extraction was performed from heads as described in Materials and Methods (Chapter 2). 400 μl of protein extract was subjected to immunoprecipitation using sepharose beads coated with strepTactin (GE Healthcare Bio-sciences AB, USA), which bind to STREP(II). Detection was achieved using anti-MYC (1:8000, Sigma) and anti-HA (1:10000, Sigma) antibodies.

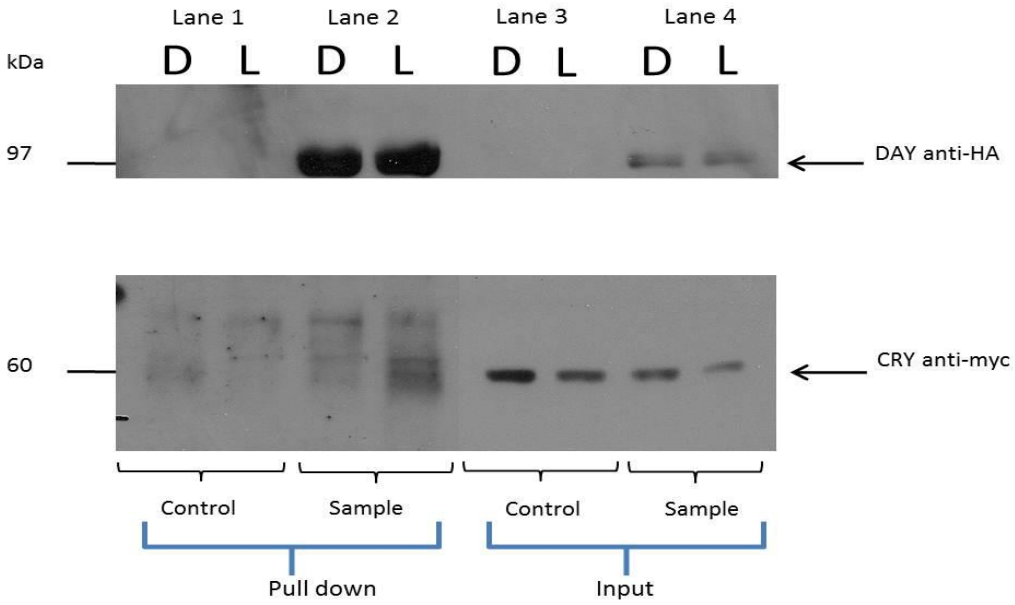


Figure 4-3: DAY and CRY co-immunoprecipitate in clock cells.

Lane 1: Pull down on genotype *w*;TG4/+;UAS-MYC*cry*/+.

Lane 2: Pull down on *w*;TG4/UAS-HA-STEP(II)*day*; UAS-MYC*cry*/+.

Lane 3: Input protein sample (unchallenged with co-IP beads) from control flies, genotype same as lane 1.

Lane 4: Input protein sample from lines co-expressing tagged *cry* and *day*, same genotype as lane 2.

In lane 1 the control sample has been challenged with strepTactin beads, as this genotype does not carry any target protein, any bands in this lane serve as an indicator as to the level of unbound proteins present. The sample ran in lane 2 co-expresses tagged DAY and CRY HA staining reveals strong bands in both D and L conditions, confirming that HA-STREP(II)DAY binds the strepTactin beads. The MYC antibody indicates the presence of CRY that has either bound HA-STREP(II)DAY or has bound un-specifically. Comparison of lane 1 with 2 reveals a relatively intense band in the L sample of lane 2 at 60 kDa, which is the expected size of CRY. The input sample of the control genotype (lane 3) demonstrates that HA antibody is not un-specifically binding

to proteins the same size as HA-STREP(II)*day* and that the MYC epitope is recognised by its appropriate antibody; while the input sample in lane 4 confirms that the HA antibody is working effectively.

Therefore this experiment asserts that tags and antibodies are binding their respective substrates correctly, as well suggesting that CRY has bound DAY in light conditions, when over-expressed *in-vivo*.

4.5 Discussion

The binding of DAY to CRY in a heterologous system is important but does not definitively mean that the two proteins interact in *Drosophila in-vivo.* To address this concern a tagged UAS-*day* construct was modified to incorporate the STREP(II) tag. Overexpression of DAY and CRY is needed due to the limitations with antibodies raised against both proteins, furthermore a singly tagged copy of HA-day is still unsuitable as the antibodies for the HA protein are approximately the same size as CRY. Finally a dual tagged DAY will be useful for pull-downs performed in parallel; comparing the mass spectrometry analysis of the individual pull-downs may reveal common interactor proteins. This is important as previous work in our laboratory with CRY revealed a long list of interacting proteins, having a large set of proteins raises the concern that some will be false positives, if a dual tagged pull-down is employed it may give the data more credibility.

Tagging UAS-day with STREP(II) was accomplished and western blots with lines injected with this construct reveal DAY is expressed correctly, whilst it also appears

that there is no reliable difference in levels of UAS-*day* in light or dark. The lack of difference in light and dark samples was not an un-expected result as western blots undertaken in a similar manner by other colleagues yielded the same result (data not shown). From this western experiment the line with the highest level of UAS-HA-STREP(II)DAY was chosen for co-IP analysis. The co-IP, performed by Dr C.Hansen, was successful showing CRY is present in a pull-down with DAY. This binding appeared to be more abundant in light, with the dark sample showing only marginally more protein than the control. If this result is correct then it contradicts the yeast data, primarily a dark interaction between DAY and CRY, indicating that either the yeast system is not accurately reproducing *in-vivo* conditions or that the co-IP favours light activated CRY in some fashion. CRY co-IPs are known to be challenging, the reasons why are not clear. Potential hypotheses may be that either the conditions of co-IP are too severe for CRY to maintain its interactions/ stability, or that the number CRY interactors is too great and that isolating single protein-protein complexes is difficult. Future experiments could utilise a cross linking procedure that could help overcome the difficulties experienced with CRY co-IP (Juschke & Knoblich, 2008).

5 Generation of transgenic tools II: *day* knockout

5.1 Introduction

Drosophila gene targeting was first carried out successfully in 2000 by Rong and Golic; they successfully rescued a mutant *yellow* strain with its wild type allele (Rong & Golic, 2000). In this work, ends-in targeting was performed whereby the transgenic donor undergoes homologous recombination (HR) and is incorporated into the target area of the genome, causing a duplication that can be removed later in a process called reduction. This technique is suitable for generating mutant alleles of genes and null alleles (Rong & Golic, 2001). The procedure requires introduction of a transgenic donor (carrying homologous sequences with the target) via P element mediated transformation and excision of the donor from the genome using both FLP-FRT site specific recombination and restriction by *I-SceI* endonuclease. The presence of a linear double strand DNA, carrying regions of homology with a desired genomic target allows donor and target to engage in homologous recombination, albeit at a low frequency.

A variation of the system was employed for this research, termed ends-out targeting (Rong & Golic, 2001; Gong & Golic, 2003a). Ends-out HR employs the same enzymes and sequences as ends-in; however, the configuration of HR is altered so that successful ends-out targeting creates a null allele with-out the need for a reduction step.

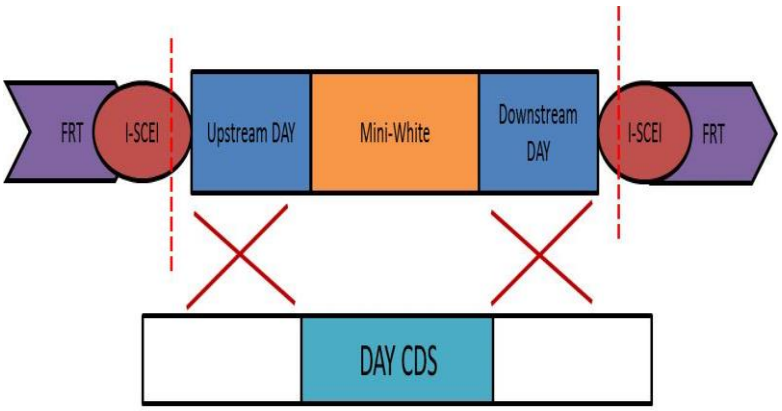


Figure 5-1: Ends-out targeting overview.

Dashed red lines indicate DSB with solid red lines representing HR.

5.2 Methods

In order to complete ends-out HR, the pW25 vector was obtained from the '*Drosophila* Genomics Resource Center' (www.dgrc.cgb.indiana.edu).

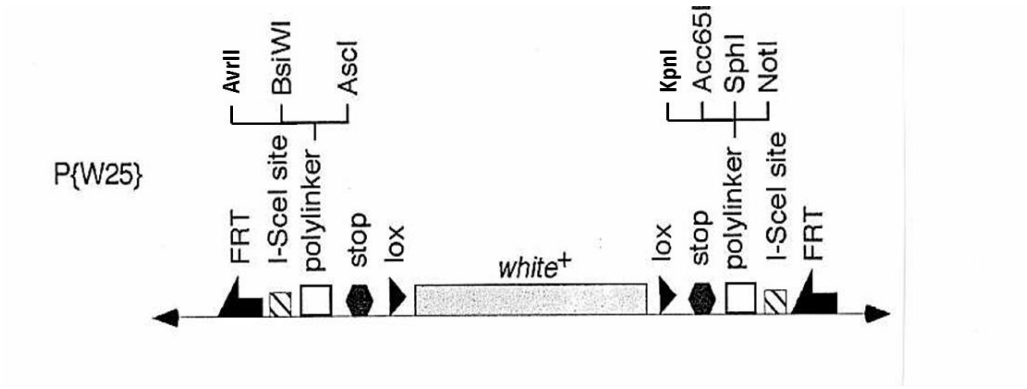


Figure 5-2: Vector map of pW25.

***White*$^{+}$ refers to the mini-*white* gene.**

The upstream *day* sequence from the start codon into the adjacent intron spanning 3 kb was cloned using Phusion® Taq (NEB), into the *AscI* and *AvrII* restriction sites located in the pW25 plasmid. A sequence of equal length was cloned from the stop codon into its neighbouring intron (downstream sequence) and transferred into the *NotI* and *KpnI* sites of pW25; using the primers listed below. Successful homologous recombination has been performed with anything from 2 kb to 9 kb of flanking sequence, but longer sequences had greater success (Bi & Rong, 2003).

Primer name	Sequence	Annealing temperature
Dayf-*AscI*	5'-[phos]GCAGGCGCGCCATCGTTGATCAGATCGATGA	55
Dayr-*AvrII*	5'- [phos]GCACCTAGGTGAAGCACACTCGCAAGTACA	
Daydownf-*NotI*	5'-[phos]GCAGCGGCCGCCTGCCAGTTGTTCTTATGAGC	55
Daydownr-*KpnI*	5'- [phos]GCAGGTACCGCGATTCGACCAGCACATAG	

Table 5-1: Homologous recombination primers.

Cloned regions were sequenced and the plasmid showing the highest percentage of sequence identity (as determined by BLAST) was selected for transformation into flies.

The latter was performed commercially (Fly Facility, France) using standard methodology (Rubin & Spradling, 1982).

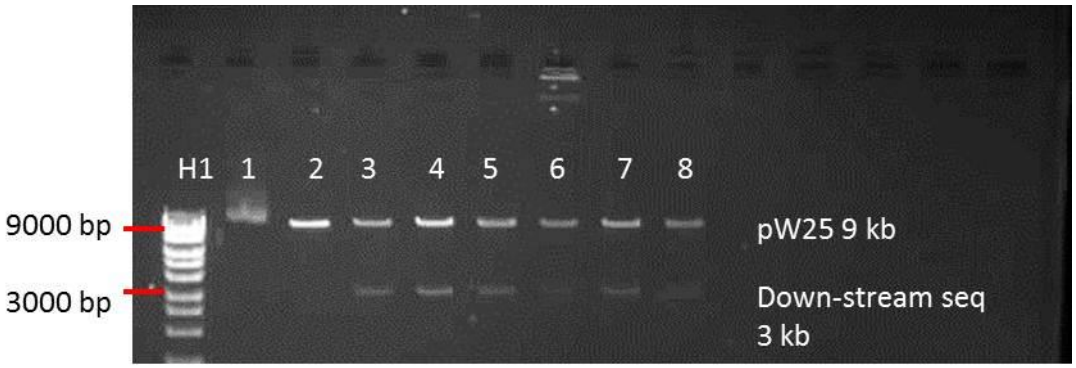


Figure 5-3: Digests of pW25.

An agarose gel of pW25 after *NotI* and *KpnI* digestions. H1 Hyper-ladder, Lane 1 uncut plasmid, Lane2 linearised pW25 only, Lanes 3-8 clones carrying down-stream day sequence (3 kb).

5.2.1 Crossing scheme/flies

The injected fly lines were balanced and one line (*w*;FRP52, here after referred to as the donor) on the second chromosome was chosen. The chromosomal location of the donor is significant, as potential knock-outs are identified by tracking the movement of the donor (by virtue of the *mini-white*$^{+}$ marker) from its original chromosomal location to that of the target; thus only donors on the X or second chromosome were suitable. HR was performed using the following fly lines (obtained from Dr Steve Goodwin, Glasgow, now Oxford University).

Name	Genotype	Description
M690	*y w*/Y,*HS-hid*; *HS-I-SCEI*, *HS-FLP*/CyO; +	Carries three genes under heat shock control, *I-SCE-I*, *FLP* and *hid*. FLP and SCEI are enzymes that excise the construct and introduce double strand breaks, respectively. The *hid* gene, inserted onto the Y chromosome, encodes for a pro-apoptotic protein, hence developing males die after heat shock. Must be grown at 18°C
M699	*y w*; *eyFLP5*;+	Carries the *FLP* gene under an eye specific promoter, on the second chromosome.
M701	*y w* eyFLP2; +; Ly/TM3	Carries the *FLP* gene under an eye specific promoter on the second chromosome. The third chromosomes carry the marker gene Ly and the balancer TM3.
M700	*y w* eyFLP2; Pin/ CyO;+	Carries the *FLP* gene under an eye specific promoter on the X chromosome. The second chromosomes carry the marker gene Pin and the balancer CyO

Table 5-2: Fly strains used in homologous recombination.

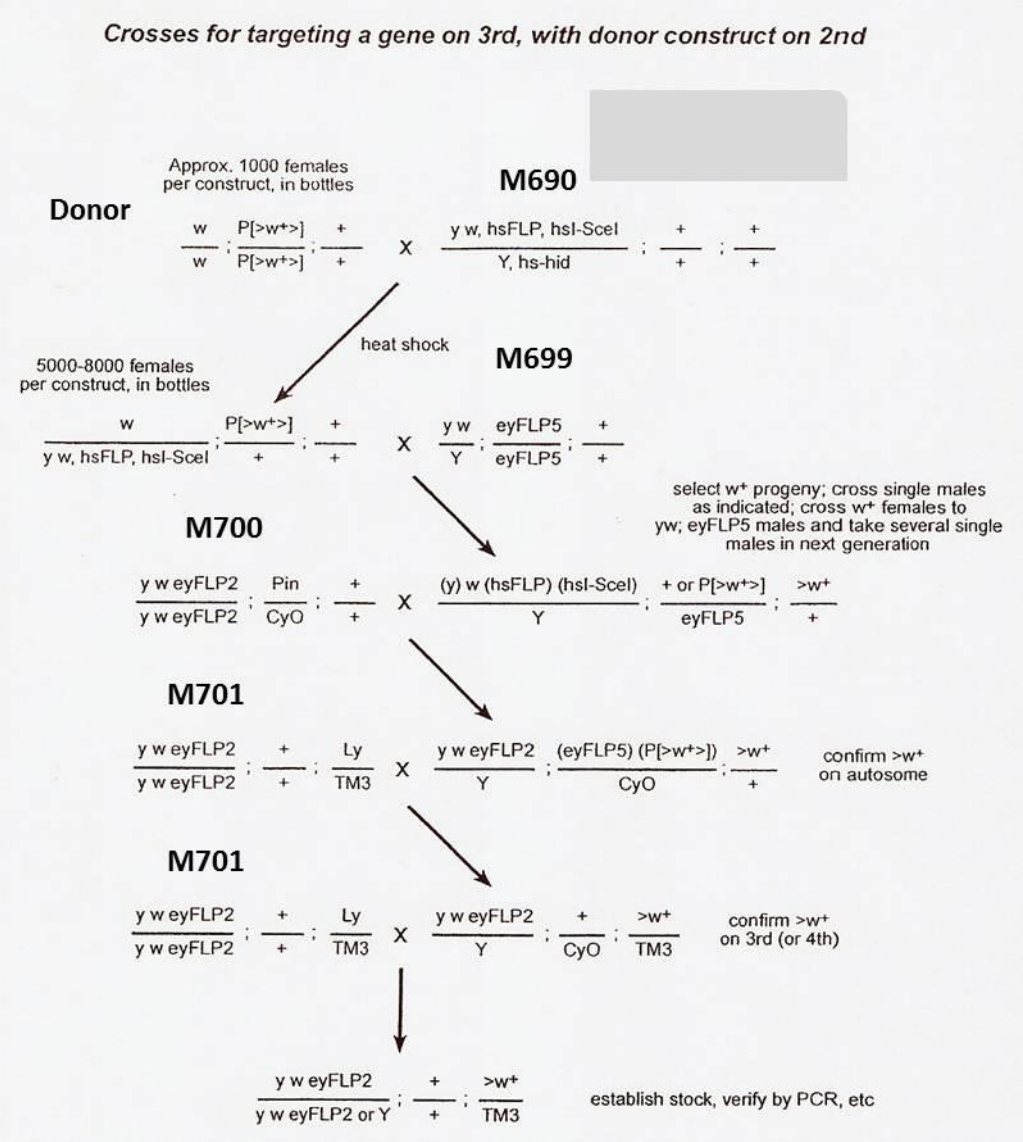


Figure 5-4: Homologous recombination crossing scheme.

Details of crossing scheme:

<u>Cross 1</u>

〉 1000 donor virgin females placed in 200 vials with M690 males.

〉 Heat shock applied on day 4 for 1.5 hours at 38°C, with an additional 1 hour heat shock at day 5 at 38°C.

Cross 2

〉 5000 mosaic eyed virgins collected into 1000 vials with M699. The mosaicism is a consequence of the donor construct being excised and indicates successful FRT recombination. The induced FLP enzyme creates a recombination event between the FRT sites leaving one FRT site in the genome and allowing the donor to be excised from the genome as circular product, the other FRT site is lost in the recombination event.

Cross 3

〉 Males with solid red eye colour selected and crossed to M700 virgins.

〉 Females with solid red eyes crossed to M699 and the male offspring crossed to M700.

〉 When red eyed flies were found the vial was marked so that different targeting events could be isolated. With successful targeting events solid red eye colour is desired as successful HR events remove one of the FRT sites, therefore any flies with mosaic eyes still carry two FRT and do not represent a successful targeting event. To ensure efficacy of this screening procedure the M700 line, as well as the M701 line, carries the FLP gene driven in the eye. Technical note:

it is very common to find a large % of flies with no eye colour due to complete excision of the donor which is not then re-integrated successfully.

Cross 4

- Red eyed males balanced on the second chromosome with CyO are selected and crossed to M701.

Cross 5

- Red eyed males balanced on the second and balanced on the third with TM3 are chosen and crossed to M701.
- The progeny of this cross reveal which chromosome the *white*$^{+}$ gene segregates with and thus indicates if a targeting event has occurred.
- If such an event has occurred then the flies balanced on the third are crossed and kept as a stock.

PCRs of the *day* locus were performed using the standard conditions stated in chapter 2, with DNA extractions carried out from single or multiple flies. The fly strain Df(3R)Exel6178 was also used in PCR analysis, and it carries a genomic deletion spanning 90F4-91A5 (*day* locus 91A3), Bloomington stock number 7657.

5.3 Results, PCR analysis

On completion of the aforementioned crossing scheme, 6 fly lines (F, M, G, U, O, P) representing potential targeting events (movement of *white*$^{+}$ from chromosome 2 to 3) were isolated. In order to establish if a knock-out had arisen, overlapping PCRs of the entire coding sequence had to be executed. The primer sets used for the analysis were Day2, Day3, Day4 and Day5, which have been described in Chapter 3.

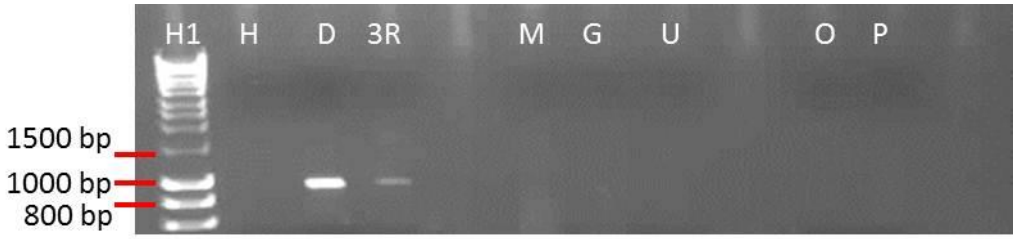


Figure 5-5: *Day* 2 PCR of potential HR targeting events.

DNA extracted from single flies, Lanes: H1 Hyper-ladder, H H_20, D positive control for *day* gene, 3R deficiency line carrying a large deletion spanning the *day* locus, M-G-U-O-P lanes represent individual fly lines that may carry a deletion of *day*.

The gel shown above gives the following information: no bands in the negative control, a correctly sized band in the positive control, a band half as intense in the *day* deficient 3R line compared to the positive control (2 copies of *day*) and no bands present in the M,G,U,O,P lines.

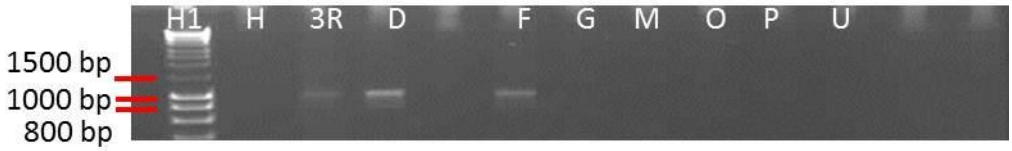


Figure 5-6: *Day* 3 PCR of potential HR targeting events.

DNA extracted from single flies, Lanes: H1 Hyper-ladder, H H_20, D positive control for *day* gene, 3R deficiency line carrying a large deletion spanning the *day* locus, F, M, G, U, O, P lanes represent individual fly lines that may carry a deletion of *day*.

The Day 3 PCR is arranged in the same manner as before with the same findings, no band in negative control, positive bands in 3R and D genotypes (again less intense staining in 3R than D sample), but with an additional line 'F' that was also suspected to be carrying a *day* deletion. From this PCR it is evident that the F genotype carries this particular region of the *day* CDS while the G, M, O, P, U samples are negative.

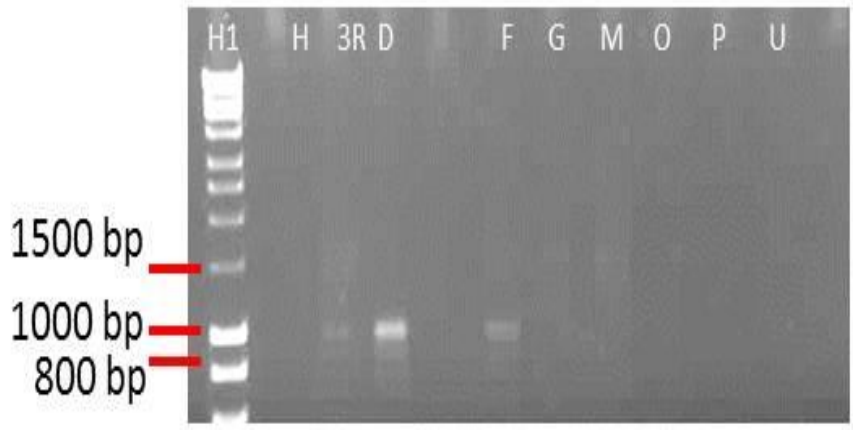


Figure 5-7: Day 4 PCR of potential HR targeting events.

DNA extracted from single flies, Lanes: H1 Hyper-ladder, H H_20, D positive control for *day* gene, 3R deficiency line carrying a large deletion spanning the *day* locus, F, M, G, U, O, P lanes represent individual fly lines that may carry a deletion of *day*.

Again this gel has the same essential features as the two previous with no bands present in the G, M, O, P, U lines.

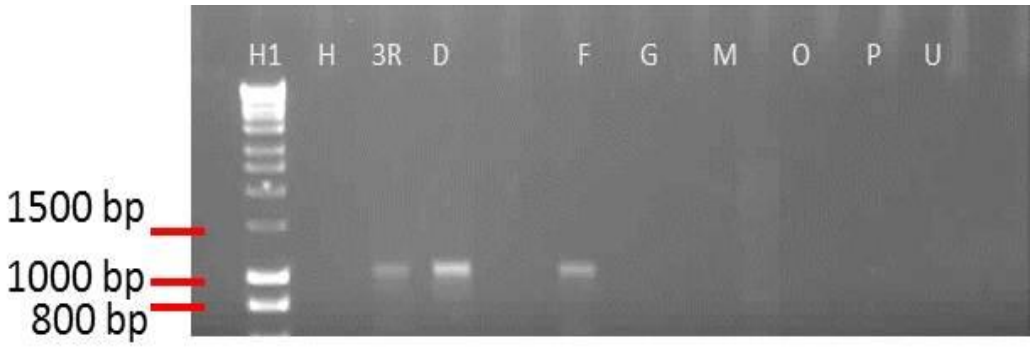


Figure 5-8: Day 5 PCR of potential HR targeting events.

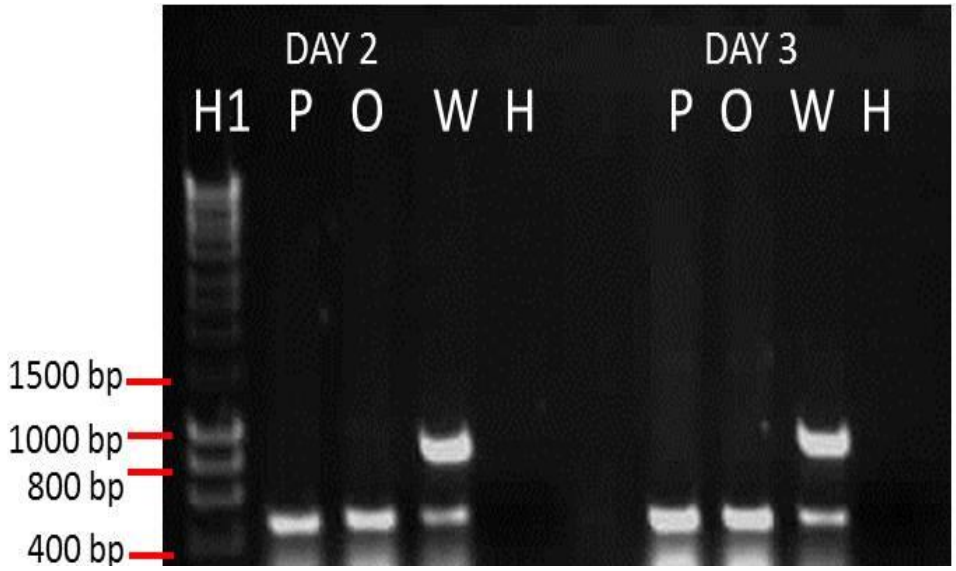
DNA extracted from single flies, Lanes: H1 Hyper-ladder, H H_20, D positive control for *day* gene, 3R deficiency line carrying a large deletion spanning the *day* locus, F, M, G, U, O, P lanes represent individual fly lines that may carry a deletion of *day*.

This final PCR has the same findings as the previous 3 showing no bands in the G, M, O, P, U samples but is positive in the 3R, D and F genotypes, therefore it appears that G-U are knockouts of the *day* gene.

The *day* CDs spans 3 kb, with the 4 primer sets used amplifying 900 bp-1000 bp, therefore the overlapping nature of this primer set gives good coverage of the *day* locus and it is apparent that no CDS PCRs work on the G, M, O, P, U genotypes and are null alleles of *day*. These lines were analysed further while the F line was discarded

due to the presence of most of the *day* CDS. The *day* null flies were backcrossed to flies of the genotype w^{1118} six times; this has been shown to be crucial in negating the influence of background on potential phenotypes (O'Keefe *et al.*, 2007).

Upon completion of the backcrossing protocol, DNA was obtained from a preparation of 50 flies for each of the *day* null lines. The extraction procedure (chapter 2.2) yielded a significant quantity of DNA that was amplified to re-confirm the absence of the *day* gene in the P and O lines, the w^{1118} genotype was also subjected to the same extraction procedure as a control for the technique.



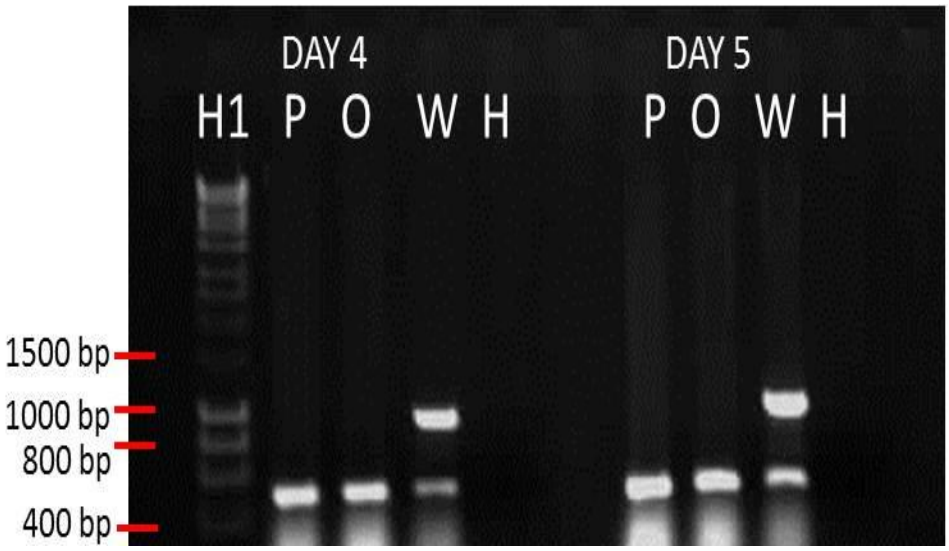


Figure 5-9: CDS PCRs of backcrossed *day* null lines P +O.

PCR primer sets are listed above their respective gels. Lanes: H1 Hyper-ladder, P and O back crossed *day* zero lines, W w^{1118} and H H_20. Band in w^{1118} lane serves as positive control while confirmation of the DNA quality was accomplished by performing a PCR for *tim* in the reaction mixture (500bp) band.

The absence of *day* in either of the P or O lanes signifies that backcrossing has not re-introduced *day* via recombination. By taking 50 flies we can be confident that this is a fair representation of the population; a *tim* control was run to ensure that the PCR was carried out successfully. Therefore, the HR protocol has been carried out successfully, with 5 lines generated from a cross of 5000 (0.1% success rate). Backcrossing should standardise the background, all *day* null experiments performed were carried out using the *lines* P and O, now termed day^{OO} and day^{0P} or *day* zero P/O (due to formatting constraints on GraphPad prism 5).

5.4 Discussion

In order to establish a functional relationship between *cry* and *day*, as well as determine any non-*cry* related function of the *day* gene, ends-out homologous recombination was selected to remove *day* (Rong & Golic, 2000; Rong & Golic, 2001). This technique has been employed successfully in this instance to create a null of the *day* CDS. This approach allows targeted gene disruption, which is more definitive than traditional approaches such as RNAi or mutants carrying P-element insertions in locations proximal or within the gene of interest. The HR approach has an inherent set of problems, with the most obvious being the low success % (taken as an average, as individual loci vary greatly in success rates) requiring the maintenance and crossing of thousands of flies, as well as each donor site having variable probabilities with regards to successful HR (Rong & Golic, 2001; Gong & Golic, 2003b). Even if successful targeting is obtained this does not always lead to the generation of a null allele as it is possible to have legitimate recombination at one end of the gene and a non-homologous exchange at the other, whereby the donor construct is incorporated leaving the gene intact, to some extent (Gong & Golic, 2004). More efficient gene specific mutagenesis has been accomplished using a ϕC31 phage integrase, whereby recombination sites attP are recognised by their partner attB which flanks a modifying sequence (Groth *et al.*, 2004). The attP is stably incorporated into the genome using the standard P-element system, attP embryos are injected with the integrase and an attB plasmid containing a modifying sequence is inserted between the attP sites accurately and readily (Groth *et al.*, 2004). There are a number of variants utilising these tools: SIRT (site-specific integrase mediated repeated targeting), IMAGO (Integrase–mediated approach for gene knock-out) and genomic engineering reviewed

in (Huang *et al.*, 2009). Essentially these approaches all require that the attP site be inserted within your gene of interest and use either ends in/out to accomplish this, therefore unless a line has been generated previously then there is no direct advantage in using an integrase approach versus the approach used in this work. With the exception that once an attP is inserted then additional modifications can be made readily giving a greater degree of options to the user (Huang *et al.*, 2009). The solution to readily modifying specific genes in a non-laborious manner may be through the zinc finger nuclease (ZFN) approach: this technique requires the user to take advantage of the specific binding sites recognised by dimerised zinc finger proteins and then introduce a non-specific DSB. The DNA repair machinery of the cell will then fix this cleavage using non-homologous end joining (NHEJ) or the HR machinery (Beumer *et al.*, 2008). If NHEJ is carried out deletion mutations are achieved, however if the RNA of the ZFN is co-injected with a circular plasmid containing a homologous sequence, then it is possible to insert mutations into the gene, modifying it (Beumer *et al.*, 2008). The ZFN approach appears to be efficient with a 7% success rate at the *ry* locus reported, much greater than HR (Rong & Golic, 2001; Beumer *et al.*, 2008). Designing target sequences using the zincfinger tools web-site (www.zincfingertools.org) allows the researcher a degree of confidence in designing the ZFN, with simple cloning steps to create ZFNs. Testing the ZFN requires protein expression work or you may precede to make ZFN RNA and attempt to inject (Carroll *et al.*, 2006).

Although these approaches were available to us, we chose the HR method and managed to obtain a null with this technique. There were other options for exploring the function of the *day* gene, as RNAi is available from the Vienna *Drosophila* RNAi

centre and a *Minos* element was inserted into the first *day* intron approximately one and half years into my PhD (Metaxakis *et al.*, 2005); more recently modified *Minos* elements have been inserted within the *day* locus (Venken *et al.*, 2011). These elements will be discussed in detail later when analysing the location and possible function of cells expressing *day*. It may have been challenging to employ these elements for functional analysis of *day* without a clearer indication of possible *day* phenotype(s). Indeed *day* RNAi experiments using Vienna RNAi lines and ones generated within our laboratory gave stochastic results when clock related phenotypes were studied, experiments performed by a colleague (data not shown). It was therefore integral to the analysis of *day* function that a knockout was obtained; the work detailed above indicates how this task was accomplished and confirms the absence of *day* using overlapping primers of the *day* locus. Additional confirmation could be accomplished using a Southern blotting approach, but due to time constraints and the robust PCR approach used it was not attempted. Backcrossing is an essential step in eliminating background mutations and permits comparisons to be made between *day* null flies and control genotypes (O'Keefe *et al.*, 2007).

6 Physiology and behavioural examination of *day*^{0} mutants.

6.1 Introduction

The data presented in this chapter examines the impact of removing the *day* gene on: chill-coma, lifespan and locomotor activity rhythms under light/dark (LD), constant darkness (DD) and constant light (LL) conditions. As well as analysing the behavioural consequences of increased levels of *day* expression.

A potential role for DAY in chill-coma was uncovered through a large screening that was carried out on the *Drosophila* Genetic Reference Panel (DGRP) fly lines. These represent 192 inbred lines derived from flies caught in the wild in Releigh, North Carolina. These flies have been fully sequenced and are a resource, available to the whole *Drosophila* community, for whole genome association mapping of quantitative trait loci (Mackay *et al.*, 2012).

A single nucleotide polymorphism (SNP) at position 14175232, within the fourth intron of *day*, was associated with a different response to chill-coma. The assay works by subjecting flies to prolonged exposure to cold temperatures thus inhibiting the firing of synapses, which results in full anaesthesia. This state can be reversed by increasing the ambient temperature, which allows the flies to regain their physical composure, (reviewed in Macmillan & Sinclair, 2011). The length of the recovery time can be recorded and comparisons made. This procedure was applied to *day*^{0} flies to validate the relevance of DAY for this phenomenon.

Analysis of the amino acid residues of DAY reveals 4 PDZ motifs. Proteins of this nature are known to be involved in development (Bilder, 2001); RNA expression data

from Flybase (Flybase.org) shows *day* expression peaking at 20 hours into the embryonic development (Graveley *et al.*, 2011). Therefore to determine if removing *day* has any adverse effect on the overall health of the fly, lifespan analysis was performed.

From the data presented thus far it is unclear if the binding of CRY to DAY occurs in light, as suggested by the co-IP (chapter 4), or darkness indicated by the yeast binding data (chapter 1). By analysing the locomotor activity of flies maintained in constant light or darkness, it may be possible to observe behavioural differences in either setting. This may give information on whether *day* is required in the clock under these particular conditions.

Finally, ascertaining the influence of *day* through overexpression studies using broad cell type drivers was accomplished. These experiments examined the role of *day* in regulating the period of locomotor activity measured in DD.

6.2 Methods

6.2.1 Chill coma

Chill coma experiments were undertaken by collecting flies on the first day of their eclosion in groups of 10 (males and females separated). After 3-5 days of LD at 25°C, the flies were changed into empty clear plastic vials and placed on ice for 3 hours. The flies were analysed in a paired fashion, with chilling occurring between ZT 4-6. After the time had elapsed the vials were emptied into clear plastic petri dishes and rapidly each fly was placed onto their back. The time for each fly to awake and

transfer from their backs to feet was recorded. Data was analysed using the GraphPad Prism 5 program and one way ANOVA was used to test for significance, with Bonferroni post-hoc analysis to compare individual genotypes or if only two genotypes were analysed Student's un-paired t-test was performed.

6.2.2 Longevity

To calculate the average lifespan newly eclosed males were collected and sorted into groups of 10. To prevent ill health, flies were pushed into fresh vials twice a week, using a mild amount of CO_2. The number of flies left alive was recorded on an almost daily basis. Collected data was used to assign the average life-span of each genotype, statistical significance of data was analysed using the Mantel-Cox test on GraphPad prism 5, longevity analyses.

6.2.3 Fly stocks

The locomotor and physiological assays performed in this work used the following genotypes.

Genotype	Description
w;;day^{0P} and w;;day^{0O}	Two independent *day*-null lines. Source: this work
w;;actin5C-GAL4	Driver line expressing GAL4 ubiquitously, under the *actin5C* promoter s, Source: Bloomington Stock centre
w;UAS-HAday5m;	UAS line for the expression of Ha tagged DAY Source: previous work in the laboratory
w,Elav-GAL4;;	Driver line expressing GAL4 in all neurons. Source: Bloomington Stock centre

Figure 6-1: Fly stocks utilised.

6.2.4 Sleep analysis

Sleep analysis is performed after running standard locomotor assays detailed in chapter 2.18.Flies were aged 3-4 days and kept in groups of 10-20 prior to be loaded into individual activity tubes using standard activity monitoring techniques described in Materials and Methods. Data was extracted in 5 minute bins from 8am onwards, at this time lights on occurs and data extraction must start at 8am or the macro fails to analyse the remaining bins correctly. To account for adaptation differences/stress responses to their new environment data was analysed after 1 full day of LD conditions, typically on the third day, using the Be-fly! macro (Green E., Kyriacou CP) un-published data. 5 minute bins containing no activity were recorded as a sleep episode. Sleep data was tested for significance using GraphPad Prism 5 program using one way ANOVA unless stated otherwise.

6.3 Results

6.3.1 Chill coma

Males of the *day*^{0} genotype were collected and individual chill coma recovery times were recorded and compared to controls of the same background. d*ay*^{0P} was paired with CS/+ (genotype described in Materials and Methods) while *day*^{0O} was tested with the *w*^{1118} genotype, the pairing of *day*^{0} genotypes with controls reduces the impact of variables such as increased room temperature. Any circadian effect was controlled for by performing the experiments within ZT 4-6. The recovery times of individual assays were combined for each genotype with the data analysed using a one

way ANOVA and Bonferroni post-hoc, revealing a significant increase in the recovery time in both *day*^{0} lines compared to controls, statistics shown in figure 6-2.

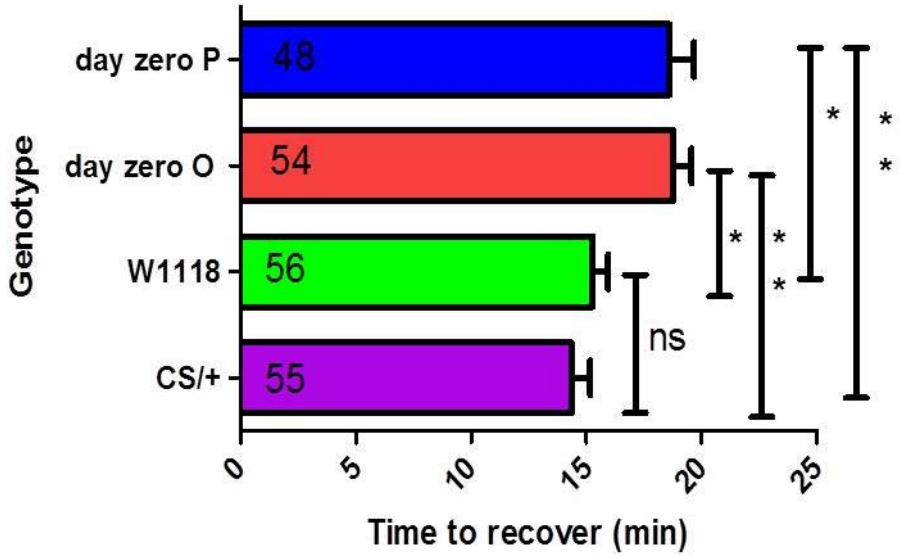


Figure 6-2: Mean recovery times from chill coma for *day*^{0} males.

Red and blue bars represent *day*^{0}, green and purple the controls, numbers within columns indicate N. ANOVA,$F_{3,209}$=7.350 P <<0.001, Bonferroni post hoc *day*^{0P} vs CS/+ P=0.01, *day*^{0P} vs *w*^{1118} P=0.05, *day*^{0O} vs CS/+ P=0.01, *day*^{0O} vs *w*^{1118} P=0.05.

Therefore there was a significant increase in the recovery time of *day*^{0} male flies. To assess the impact of gender, the same experiment was repeated with female flies, using only the *day*^{0P} line.

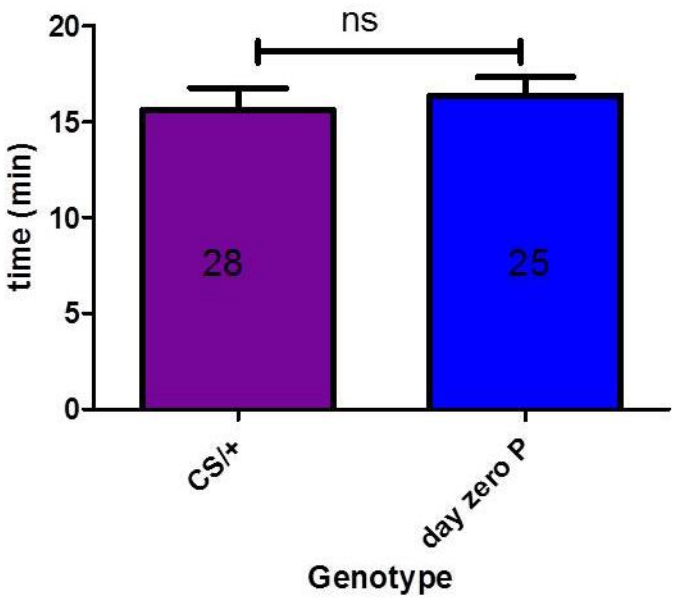


Figure 6-3: Female *day*^{0P} chill coma mean recovery times.

Control purple, blue *day*^{0P}, N number within column.

One line of *day*^{0} female flies showed no significant difference in recovery time from the control. Data was analysed using an un-paired Student's t test P=0.6328. There is a clear gender specific difference in the response to chill coma with *day*^{0} males taking significantly longer to recover from this cold stress.

6.3.2 Longevity

The results from the longevity study are shown as the survival percentage against time (days) below. To re-iterate briefly 100 flies per genotype (ten vials of ten) were collected within 1 week for all genotypes tested and survival data was recorded

for all the genotypes each day, brief CO_2 exposure was required to transfer flies to fresh vials.

Genotype	Median survival (days)	N
w;;*day*^{0P}	51	100
w;;*day*^{0O}	53	100
w^{1118}	48	100
CS/+	36.5	100

Table 6-1: Longevity analysis (median survival times) of *day*^{0} flies versus controls.

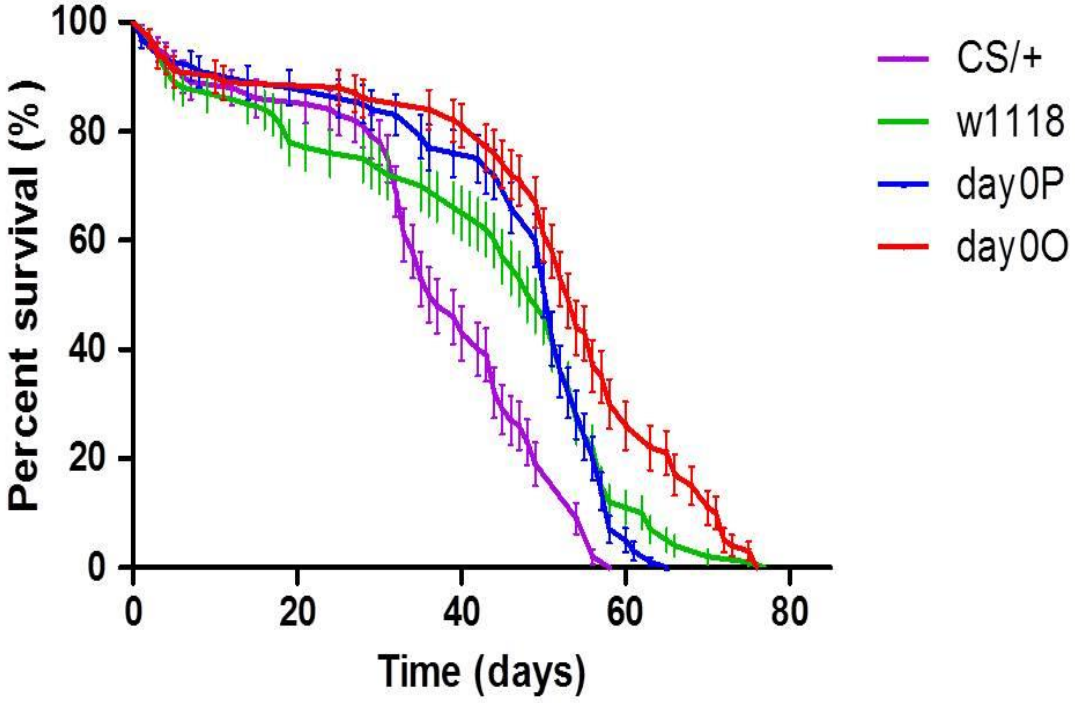


Figure 6-4: Male longevity data plotting survival percentage versus time.

From the table and graph presented above it is clear that there are differences in survival, with CS/+ flies perishing earlier than the w^{1118} genotype and the day^{0} lines. The median survival lengths of day^{0} lines versus the w^{1118} genotype were slightly longer, however, viewing the data as a survival percentage versus time gives a similar curve. Mantel cox curve comparisons gave significant differences between day^{0O} vs day^{0P} (P=<0.0001), significant difference with w^{1118} vs day^{0O} (P=0.0008), but there was no significant difference between w^{1118} vs day^{0P} (P= 0.68). All curve comparisons with CS/+ were significantly different (day^{0P} P = <0.0001, w^{1118} P = <0.0001 and day^{0O} P = 0.008). The longevity data presented appears to suggest the following: CS/+ genotype has a reduced life-span, while the day^{0} flies seem to flank the w^{1118} genotype with

day^{0P} having a reduced life span, whilst day^{0O} takes longer for the percentage of flies to reduce but the experiment terminates after a similar number of days to w^{1118}. Taken together with the median it would appear that this experiment doesn't reveal a consistent trend and that day^0 flies appear to possess a similar length of lifespan to w^{1118}. At this stage it is unclear why CS/+ would have a significantly reduced life span.

6.3.3 Locomotor behaviour of day^0

On obtaining day^0 flies that were backcrossed to w^{1118} their locomotor behaviour was subsequently analysed, flies were kept in LD and then transferred into DD conditions at 25°C.

Genotype	Period ± SEM	N	Arrhythmic (%)
w;;day^{0P}	24.06 ± 0.09	34	15 %
w;;day^{0O}	24.24 ± 0.13	24	38%
w^{1118}	23.64 ± 0.08	24	17%
CS/+	24.15± 0.16	11	9%

Table 6-2: Locomotor period analysis of day^0 flies in DD at 25°C.

Analysing the period data shown above using a one way ANOVA with a Bonferroni post hoc test gave significant differences between the day^0 lines and w^{1118}

but not CS/+ (ANOVA $F_{3,89}$=5.235 P=0.0023; Bonferroni post-hoc *day*^{0P} vs *w*^{1118} P=0.05, *day*^{0O} vs *w*^{1118} P=0.01, CS/+ vs *day*^{0P} = ns, CS/+ vs *day*^{0O} = ns). The CS/+ genotype is not significantly different from either *w*^{1118} or the *day*^{0} lines and gives a high level of lethality in the activity analysis, which is consistent with what is seen in the longevity analysis; measuring the locomotor activity of a greater number of flies will give a more robust number. This data seems to indicate that *day* has some involvement in controlling the period of locomotor activity when *day*^{0} flies are compared solely to the *w*^{1118} genotype, however, the CS/+ genotype is not significantly different. It is not apparent what might be causing these subtle increases in *day*^{0} flies as background should be equivalent between all 4 genotypes. It seems unlikely that *day* has an effect on the period as despite the differences with *w*^{1118}, the period is of *day*^{0} flies is effectively 24 hours for both mutants. Another feature of this DD analysis is the level of arrhythmicity with the *day*^{0O} showing a much higher percentage of arrhythmic behaviour, it is unlikely to be a consequence of *day* loss as the *day*^{0P} has levels of arrhythmia similar to controls.

6.3.4 Does *day* have any effect on LL behaviour?

The ability of *cry* to convert photic information into arrhythmic locomotor behaviour is well documented, therefore LL experiments were undertaken to determine what influence *day* could have on this behaviour.

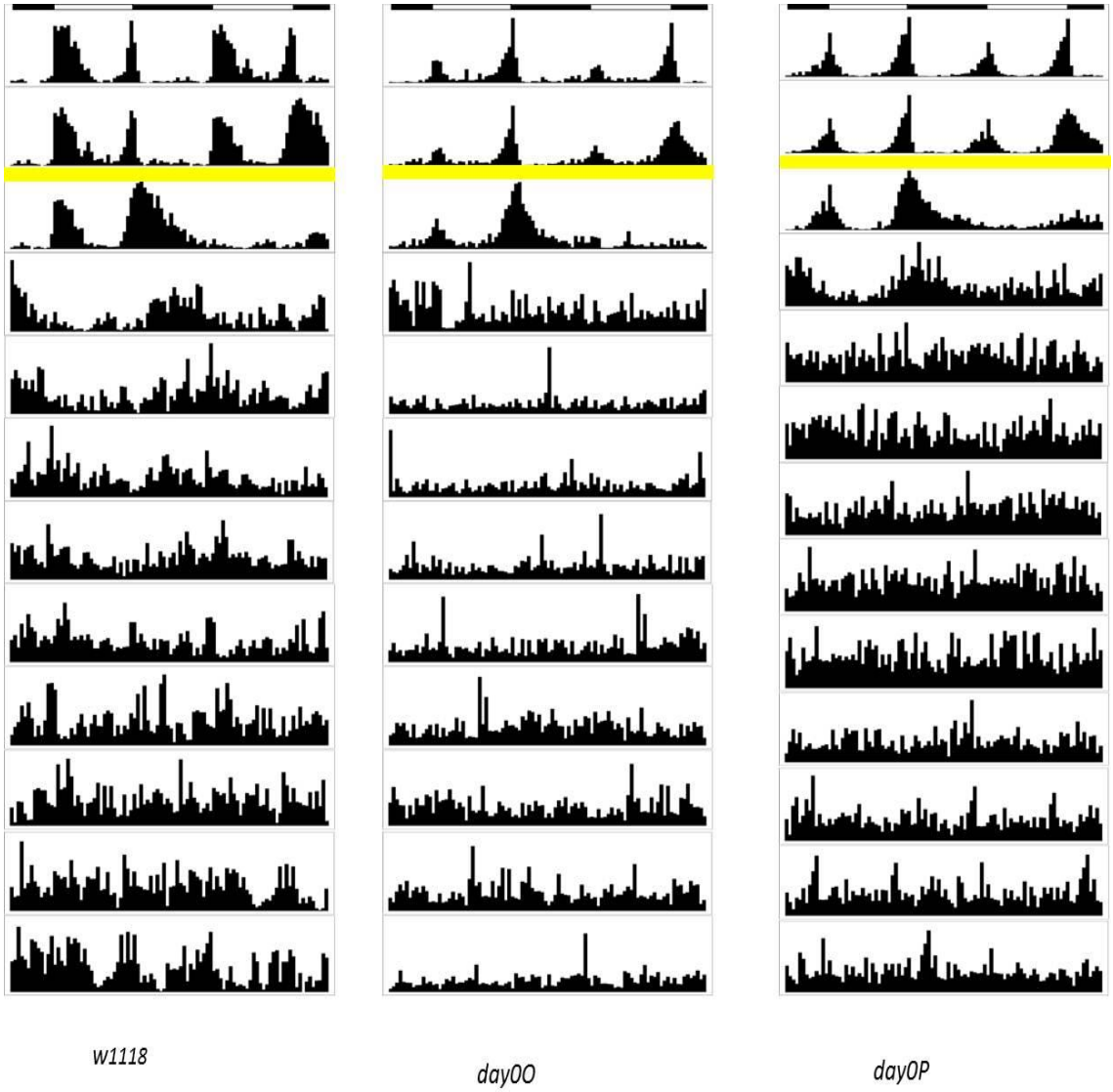


Figure 6-5: LL locomotor behaviour of *day*^{0} flies and *w*^{1118} control at 25°C.

Mean LD activity for 4 days, horizontal bars indicate light conditions (black off, white on) yellow bar represents the transition into LL conditions.

Genotype	N	Arrhythmic (%)	Complex rhythms
w;;*day*^{0P}	20	94%	1
w;;*day*^{0O}	20	100%	0
w^{1118}	19	94%	1

Table 6-3: Breakdown of LL behaviour.

Table displays data on arrhythmicity, experiment lethality and complex rhythms.

The activity profile of these two *day*^{0} lines show standard arrhythmic behaviour, thus the removal of *day* has no influence on the severe LL phenotype.

6.3.5 LD activity of *day*^{0} flies at 25°C

At this point constant conditions have been examined; the data was compiled from a large group of LD experiments with the third day of an LD cycle analysed as described in Materials and Methods.

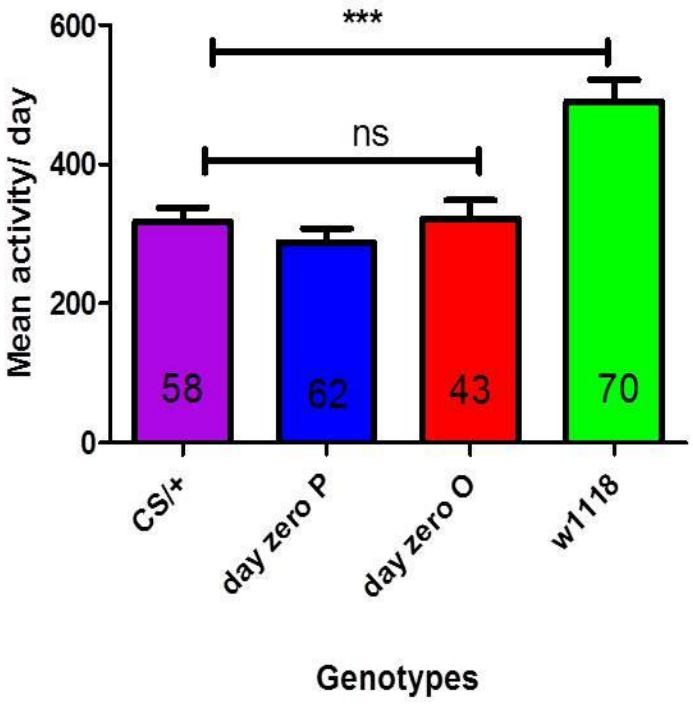


Figure 6-6: Mean total levels of activity during a 24 hour period for *day*^{0} flies.

The locomotor activity mean for an entire day was calculated and showed that flies of the *w*^{1118} genotype were significantly more active than the CS/+ control and the *day*^{0} lines ($F_{3,229}$ = 14.15, P = <0.0001; Bonferroni post hoc: CS/+ vs *w*^{1118} P = 0.001, *day*^{0P} vs *w*^{1118} P=0.001, *day*^{0O} vs *w*^{1118} P = 0.001, CS/+ vs *day*^{0P} = ns, CS/+ vs *day*^{0O} = ns). Although there was a trend for *day*^{0} flies to display less activity, although when tested this was not significant compared to CS/+ in Bonferroni post-hoc analysis.

6.3.6 Sleep analysis of *day*^{0}

The level of activity of *day*^{0} flies appears normal, but the distribution of activity may be altered giving rise to sleep phenotypes in either the light or dark period of an LD cycle. The pooled data shown above was examined for sleep phenotypes.

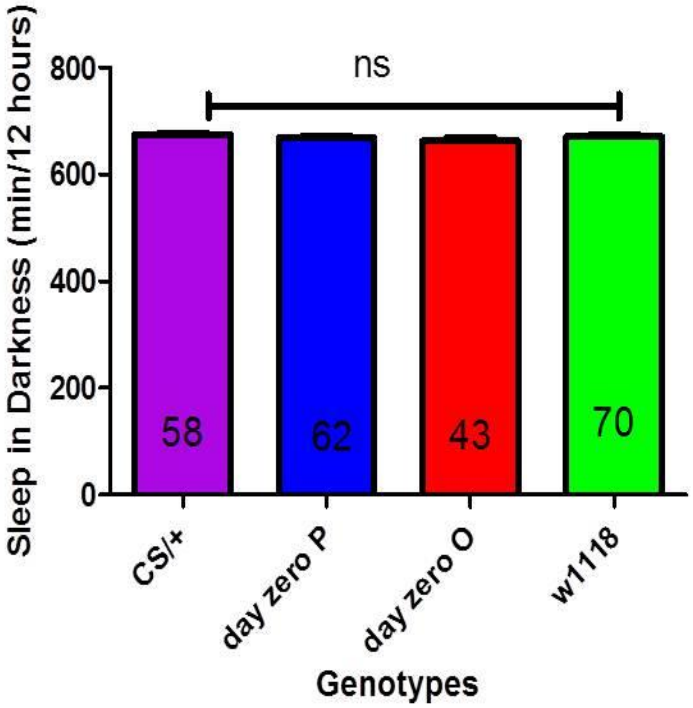


Figure 6-7: Sleep in darkness during LD cycle of *day*^{0} flies and controls at 25°C.

The total sleep in darkness shows no differences between any of the lines tested ($F_{3, 229}$ = 1.049, P= 0.37). Sleep in light was also tested giving the following results.

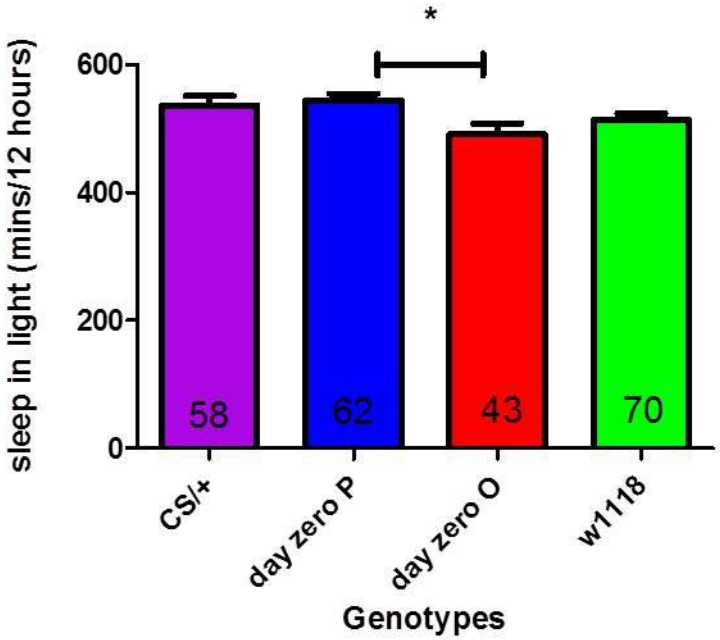


Figure 6-8: Sleep during light phase of LD cycle for *day*^{0} flies and controls at 25°C. One way ANOVA $F_{3,229}$ = 3.253, P =0.0225; Bonferroni post hoc *day*^{0P} vs *day*^{0O} P =0.05, *day*^{0P} vs CS/+ P = ns, *day*^{0P} vs *w*^{1118} P = ns, *day*^{0O} vs *w*^{1118} P = ns, *day*^{0O} vs CS/+ P= ns.

The sleep in light is less uniform than darkness and a significant difference between the *day*^{0} lines is observed, showing ~50mins difference. This result is unexpected and could be the consequence of a smaller number for the *day*^{0O} genotype.

6.3.7 Does over-expressing DAY have any influence on the clock?

The absence of *day* has no effect on the 24 hour period, but it was unclear what effect increased levels of *day* would have. Therefore *day* was expressed using ubiquitous cell type drivers: *actin*-GAL4 (all cells) and *elav*-GAL4 (all neurons).

Genotype	Period ± SEM	N	Rhythmic (%)
***w*;;*actin*-GAL4/+**	23.70 ± 0.05	19	95 %
***w*;UASHA*day*5M/+;*actin*-GAL4/+**	23.65 ± 0.06	9	90%
***w*;UASHA*day*5M/+**	23.98 ± 0.05	18	100%
***elav*-GAL4;+;+**	23.63 ± 0.05	17	100%
***elav*-GAL4;UASHA*day*5M/+**	23.52 ± 0.07	19	100%

Table 6-4: *day* overexpression analysis.

Averaged period values obtained in DD 25°C with SEM, red font indicates over-expression of *day* ($F_{5,94}$ = 11.14, P =<0.0001; Bonferroni post hoc: actin-GAL4/+ vs *w*;UASHAday5M/+;actin-GAL4/+ P = ns, *w*;UASHAday5M/+ vs *w*;UASHAday5M/+;actin-GAL4/+ P = 0.05, elav-GAL4;+;+ vs elav-GAL4;UASHAday5M/+ P = ns, *w*;UASHAday5m/+ vs elav-GAL4;HAday5M/+ P = 0.001).

Over-expression of *day* has no influence on the period, as neither line was significantly different from both the GAL4 and UAS controls, see Table 6-4.

6.4 Discussion

Removing *day* has a significant impact on the flies' response to chill-coma; chill coma is a relatively simple assay whereby a fly is brought into an unconscious state by applying cold temperatures (Ashburner, 1989; Jean David *et al.*, 1998). Understanding the underlying mechanisms for this condition may reveal what function *day* may have in this process. Many of the mechanisms highlighted thus far have concerned: signal transmission (Rodgers *et al.*, 2010), ion disruption/ion channels (Zachariassen *et al.*, 2004; Loughney *et al.*, 1989) and the membrane environment (Hazel, 1995). These

mechanisms are distinct but closely related, coupled with the *day* PDZ structural data and the known function of PDZ proteins as membrane scaffolds, it is conceivable that *day* may play a part in signal transmission. This is not the case for day^0 females who respond in a wild type manner, this suggests differences in *day* expression between males and females. At present it is unclear at this stage what mechanistic role *day* has on chill coma.

The life span of adult day^0 flies was examined to determine if *day* had functional consequences for this particular phenotype. Perhaps surprisingly the most significant difference was seen in the CS/+ control that displayed a reduced lifespan. Day^0 showed a similar median length of life as the w^{1118} control group, although there was a significant difference between day^{0O} and w^{1118} in the survival curve analysis, these differences were not seen when day^{0P} was compared to w^{1118}. These differences could be a result of simple biological variation, or the numerous food changes incurred, perhaps to achieve better resolution with this assay more flies need to be tested. However, as the day^0 mutants are not both significantly different from the control then it is most probable that *day* has no consequences in adult lifespan. One reason why there may be little consequence for *day* loss in lifespan analysis could be due to the presence of proteins that are sufficient to compensate, as there also other well documented PDZ proteins including *Patj* and *discs large 1.* It is also important to remember that only adult life span has been examined, which does not rule out a developmental role for *day*; examining the number of larvae or pupae that survive versus controls , this may reveal a role in the success rate or perhaps even the timing of these behaviours. If a gene is important for lifespan you might expect to it

conserved throughout species. Searches for orthologs reveal a limited number of organisms express the d*ay* gene and the only non-*Drosophila* species is the ant. It could be possible that the *day* is a more recent evolutionary addition with a specific behavioural and niche function in insects.

The final question asked in this chapter was: is *day* involved in the circadian clock or involved in clock outputs? The effect of *day* absence was examined in LD, DD and LL conditions. The DD data suggested that the period of locomotor activity was increased with respect to w^{1118} but not CS/+. Although this data is not completely definitive it does suggest no involvement for *day* in the maintenance of 24 hour rhythmicity in constant darkness. Evidence to support this conclusion, could be, that over expressing *day* within a broad number of cells (including clock cells), also doesn't give any significant period differences. Perhaps this is unsurprising as cry^{0} flies also have a wild-type period (Dolezelova *et al.*, 2007), it could be that a DAY-CRY interaction, if it occurs *in-vivo* it is not involved in the regulation of period. One output controlled by *cry* is the regulation of locomotor activity in LL, with flies lacking *cry* becoming rhythmic in LL conditions (Emery *et al.*, 2000a). Analysis of day^{0} lines in this condition gave a high degree of arrhythmicity in both mutant lines and the control, suggesting that the light perception pathways are unaffected in day^{0} flies.

Finally the locomotor activity in LD was characterised showing that: day^{0} mutants had comparable levels of daily activity to the CS/+ control but not $w^{1118,}$ which displayed significantly more activity. The reason for elevated activity in the w^{1118} genotype could be a consequence of lacking the *white* gene, as a lack of eye pigmentation may make them more sensitive to the 12 hours of light; elevating their

activity accordingly. Sleep in darkness was uniform across genotypes highlighting no direct role for *day* in sleep regulation. The only significant difference in light sleep was between the *day* null lines, the reason for this difference is not clear, however as *day*^{OO} lines showed an increase in their mean activity and although it was not significant it may underlie the differences seen with sleep in light. The data collected seem to suggest that the activity levels and distribution of activity of *day*^{0} flies are not significantly different from both controls; this idea fits with the period results suggesting that *day* is not directly involved in controlling the locomotor activity of circadian rhythms.

7 Construction and analysis of day-GAL4 lines

7.1 Introduction

The yeast protein GAL4 has become an extremely valuable tool in *Drosophila* genetics as it binds specifically to UAS and promotes transcription (Fischer *et al.*, 1988). Further elucidation of *day* function can be achieved by generating GAL4 lines driven by upstream *day* sequences. Generating these lines would provide information on cellular location of *day*; work undertaken in our laboratory has attempted to address this question using *in-situ* hybridisation, but the resulting staining was either too broad or of insufficient intensity to be of use. Moreover, a *day-GAL4* line would also give functional data on the role of cells expressing GAL4. To obtain *day-GAL4* lines lengths of upstream sequence were chosen including: 1 kb, 1.5 kb, 3 kb, 4.5 kb and 6 kb. The decision to create a 6 kb line of upstream sequence was taken after an update to the gene span was made on Flybase (www.Flybase.org). This update identified a potential 5' regulatory element that could be important, it should also be noted that regulatory elements at the 3' end were also added. 6 kb upstream sequence incorporates this 5' region (shown in grey in the transcript figure below) and continues several bases into the first exon.

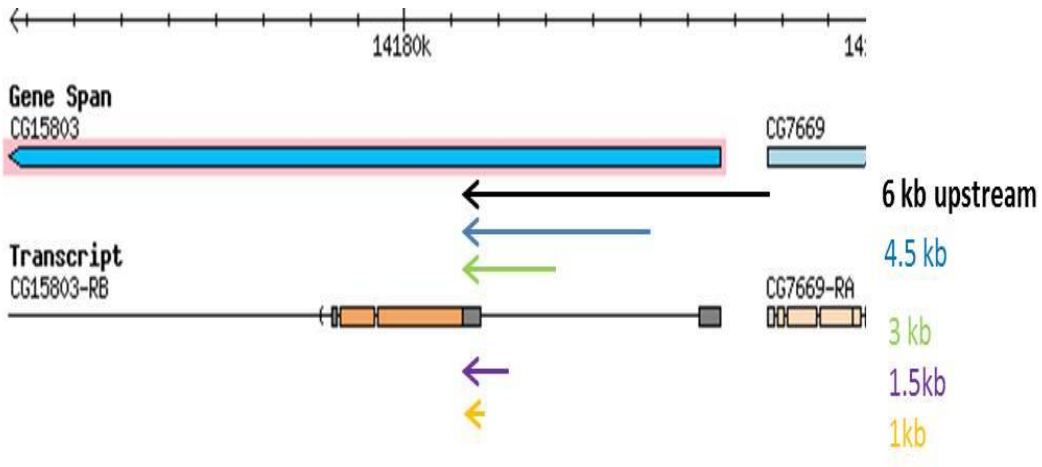


Figure 7-1: Overview of upstream sequences of *day* used in GAL4 experiment. Image taken from 'GBrowse', Flybase.org.

By constructing various lengths of upstream sequence it will be possible to dissect and identify regulatory regions. Construction of *day*-GAL4 lines has a number of potential uses including:

- Rescue of day^0 (null) phenotypes.
- Identifying cells that express *day*.
- Modifying the clock (negative feedback loop)/neuronal output of these cells to determine function.

Establishing the function and location of potential *day* expressing cells will assist in detecting potential phenotypes that may be altered in day^0 flies. The methodology required in attempting to address these questions is listed below.

7.2 Methods

7.2.1 Cloning protocol

In order to generate cellular reporters of *day* expression the pPTGAL vector was selected (Sharma *et al.*, 2002). All of the lengths of upstream sequence were cloned in the *Not I* and *KpnI* sites. The primers used can be located beneath this passage; the 6 kb sequence was cloned as three separate fragments that were subsequently ligated together to ensure high sequence fidelity.

Primer name	Sequence	Annealing temperature (°C)	Band size (kb)
jh upgal4-for-kpn	[phos]GGTACCCCATCGTTGATCAGATCGATGA		
jh upgal4 rev not 1kb	[phos]GCGGCCGCAACATGACACAGTAACTTCAA	62	1 kb
jh upgal4 rev-not 1.5kb	[phos]GCGGCCGCCGAGGCCCATATCATGGCATT	72.7	1.5 kb
jh upgal4 rev not 3kb	[phos]GCGGCCGCTGAAGCACACTCGCAAGTACA	67.9	3 kb
jh upgal4 rev not 4.5kb	[phos]GCGGCCGCGCTGCAGCAATTTGGCTAAGA	67.9	4.5 kb
JH G4f1Fwd NOT	[phos]GCGGCCGCAGCTTGAGTAGTTGCTATTTG	55	2.2 kb
JH G4F1Rev NCO	[phos]CTATCACAACTCTTCGCAATT		

JH G4f2 fwd NCO	[phos]CAGTCAATGCGGCTAGACCAA	60	1.8 kb
JH G4f2 Rev Bsm	[phos]GGATCGGGCCATTGTTGGCCG		
JH G4f3 Fwd Bsm	[phos]GACTCAGAACAAGCGGCGTTA	59	2.6 kb
jh G4f3 Rev KPN	[phos]GGTACCCATCGTTGATGAGATCG		

Table 7-1: *day* upstream primers.

The cloning was carried out with Phusion® Taq (NEB) digestion and sequencing analysis ensured high degrees of sequence similarity. All sequences were selected for the greatest degree of homology available, according to BLAST search, and sent for injection commercially (Department of Genetics, Cambridge).

7.2.2 Antibodies for immunofluorescence

IHC was performed using the following dilutions of primary and secondary antibodies. All solutions were made in 0.5% PBS buffer with 10% goat serum, see material and methods for details.

Name	Dilution	Animal	Provider	Duration
Anti-GFP	1:1000	Rabbit	Invitrogen	3 days-1 week

Anti-GFP	1:500	Mouse (monoclonal)	Abcam	1 week
Anti-CRY	1:500	Rabbit	Custom made(Neosystems now polypetides, France)	1 week (Flies kept in DD for 3+ days)
Anti-DAY777	1:500	Rabbit	Custom made (Eurogentec, Belgium)	1 week

Figure 7-2: Primary antibodies.

Name	Dilution	Animal	Provider	Duration
Anti-Rabbit cy3	1:200	Goat	Jackson	2.5-3 hours
Anti-Mouse cy2	1:200	Goat	Jackson	2.5-3 hours

STREP cy5	1:600	Goat	Jackson	2.5-3 hours
Anti-Rabbit Biotin	1:300	Goat	Jackson	1 week

Figure 7-3: Secondary antibodies used.

To achieve visualisation of cells using the CRY and DAY777 antibody, they were applied for 1 week, removed, stained with rabbit-biotin for another week and then STREP cy5 was used. CRY antibody staining was carried out after raising the flies in DD to promote CRY levels, while DAY777 flies were kept in LD.

7.2.3 Fly stocks

Fly lines used in this chapter are described below.

Genotype, Individual lines are reported in parenthesis	**Description**
***w;*UAS-*CLK*Δ1; and *w;*UAS-CYCΔ103;**	These constructs are dominant negative forms of the genes clock and cycle, they allow binding of their respective partners, but lack DNA binding domains. The result is that cyclical transcription of genes is halted in the cells expressing these constructs (Tanoue *et al.*, 2004)
***w;NaChBac* (4);**	Bacterial Sodium channel that is activated by depolarisation and is expected to increase membrane excitability, increasing firing of the neurons expressing it (Nitabach *et al.*, 2006).

day-GAL4 (F1, F4, 62T) [6]	3 lines of the 6 kb upstream sequence placed in front of GAL4
***day-GAL4* (S3,T3) [4.5]**	2 lines of 4.5 kb sequence placed in front of GAL4
***day-GAL4* (S2,T3) [3]**	2 lines of 3 kb sequence placed in front of GAL4
***w*;;UAS-*actin*GFP5C**	UAS line expressing *actin* fused with GFP, source: Bloomington

Figure 7-4: Description of genotypes utilised.

7.3 Results

7.3.1 Cloning of upstream sequences

Confirmation of successful cloning is shown below with 4 of the 5 upstream sequences below (6 kb data not shown).

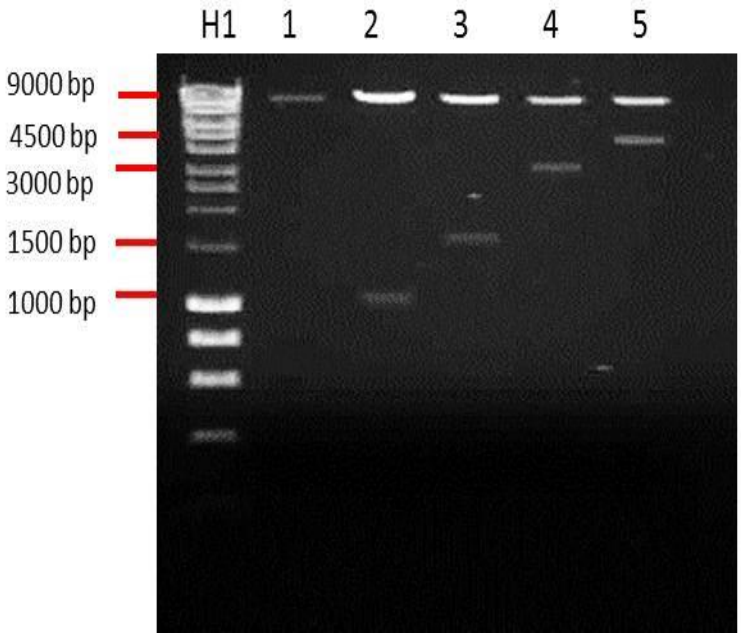


Figure 7-5: *Not I* and *Kpn I* digestion of pPTGAL vector (9 kb) lengths of upstream sequence.

Lanes: H1- Hyper ladder, 1) pPTGAL only linearised, 2) 1 kb of upstream *day* sequence, 3) 1.5 kb upstream sequence, 4) 3 kb upstream, 5) 4.5kb upstream.

7.3.2 Sleep analysis of *day-GAL4* [6] lines in *LD* conditions

To test whether *day* positive cells are involved in sleep or in controlling rhythms of locomotor activity, *day-GAL4* [6] and *UAS-CLKΔ* flies were crossed, to express a dominant negative form of CLK (Tanoue *et al.*, 2004). *CLK*Δ is a partially defective protein that lacks some of the DNA-binding region but maintain the protein-dimerisation domain. Hence *CLK*Δ can sequester CYC in a dimer that is not competent for transcription resulting in clock de-regulation. For sleep analysis locomotor activity data was recorded under LD conditions at 25°C.

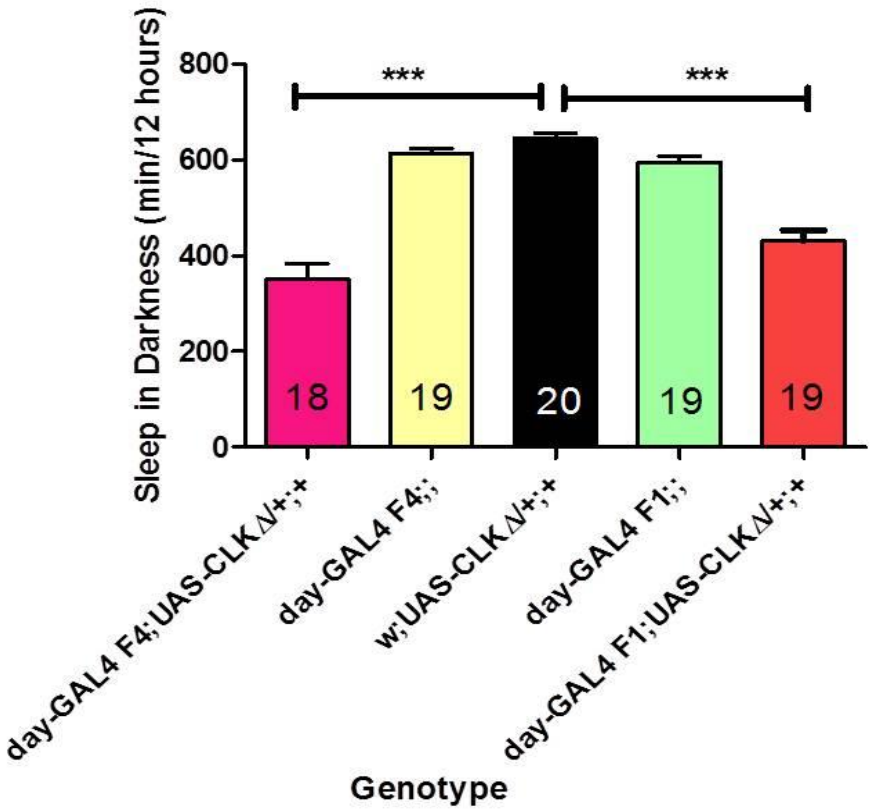


Figure 7-6: *CLK*Δ affect sleep levels when expressed in *day*-positive cells.

Sleep levels were measured during the second day in LD at 25°C, during 12 hours of darkness. *day-GAL4* F4 [6] ,*CLK*Δ is shown in pink, *day-GAL4* F1 [6] ,*CLK*Δ is shown in red, *day-GAL4* F4 [6];; control in yellow, *day-GAL4* F1 [6];; control in green, and UAS-*CLK*Δ/+ in black. N value for each genotype inside their respective column. One way ANOVA $F_{4,89}$ = 44.84, P = <0.0001; Bonferroni post hoc: *day-GAL4* F4 [6];*CLK*Δ/+ vs *day-GAL4* F4 [6];; P = 0.001, *day-GAL4* F4 [6];*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+ P = 0.001, *day-GAL4* F1 [6];UAS-*CLK*Δ vs *day-GAL4* F1 [6];; P = 0.001 and *day-GAL4* F1 [6];UAS-*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+ P = 0.001.

Figure 7-6 shows the sleep data for the progeny of two lines of *day-GAL4* [6] crossed to UAS-*CLK*Δ, the data is taken from the second day of LD where the average range of sleep in the controls is 595 min to 644 min, with the mutants sleeping on average 350-431 min. The profile of locomotor activity shows that mutants expressing

*CLK*Δ were displaying more activity during the dark period of a LD cycle and thus were sleeping less, see figure 7-6 for statistics.

To ensure that the phenotype we are witnessing is a sleep phenotype and not hyper-activity, the mean waking activity was analysed. This variable excludes inactive data and measures the mean activity in a 5 minute bin for each genotype, see Figure 7-7. Doing this analysis showed no differences between flies overexpressing *CLK*Δ and controls, suggesting that the former are not simply hyperactive ($F_{4,85}$ = 3.275, P = 0.0151; Bonferroni post hoc: *day-GAL4* F4 [6];*CLK*Δ/+ vs *day-GAL4* F4 [6];; P = ns, *day-GAL4* F4 [6];*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+ P = ns, *day-GAL4* F1 [6];UAS-*CLK*Δ/+ vs *day-GAL4* F1 [6];; P = ns and *day-GAL4* F1 [6];UAS-*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+ P = ns).

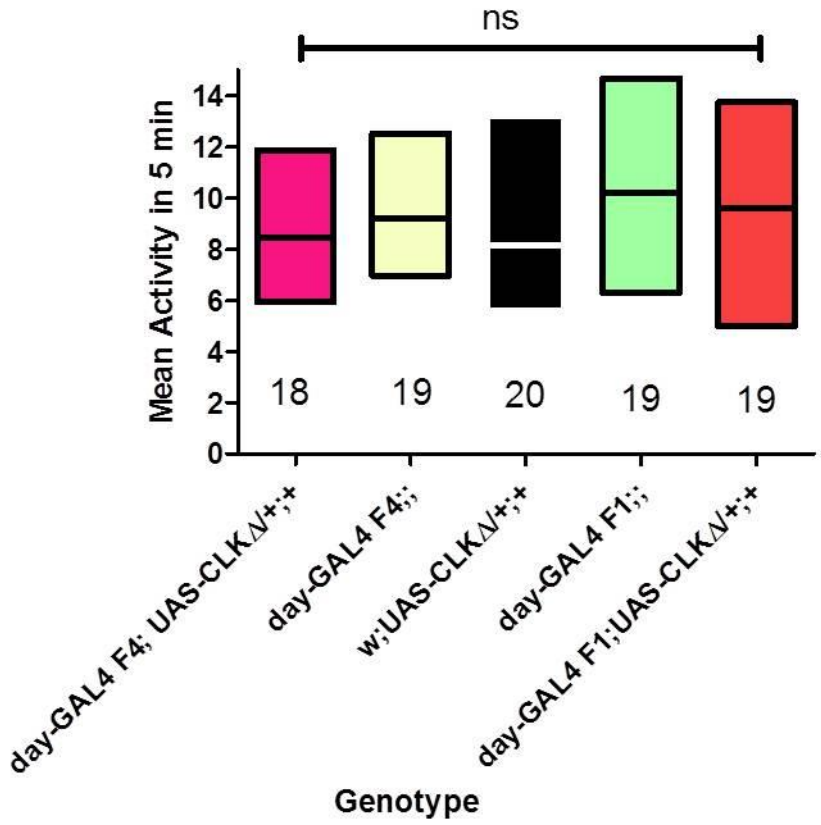


Figure 7-7: The mean waking activity of *day-GAL4* [6], *CLK*Δ flies.

Average activity count/ total number of activity bins, of *day-GAL4*, UAS-*CLK*Δ. Line through column represents the mean flanked by the highest and lowest values. No significant differences seen in the mutant strain versus controls. One way ANOVA $F_{4,85}$ = 3.275, P = 0.0151; Bonferroni post hoc: *day-GAL4* F4 [6];*CLK*Δ/+ vs *day-GAL4* F4 [6];; P = ns, *day-GAL4* F4 [6];*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+ P = ns, *day-GAL4* F1 [6];UAS-*CLK*Δ/+ vs *day-GAL4* F1 [6];; P = ns and *day-GAL4* F1 [6];UAS-*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+ P = ns.

Finally to establish if there was any activity phenotype during light conditions sleep in light was examined with progeny of *day-GAL4* lines expressing UAS-*CLK*Δ. This analysis gave no significant differences between the mutant line and both control, see figure 7-8. Mutants expressing UAS-*CLK*Δ do not display significant increases of activity during

the entire 24 hours; only during dark period do they increase their activity, thereby sleeping less.

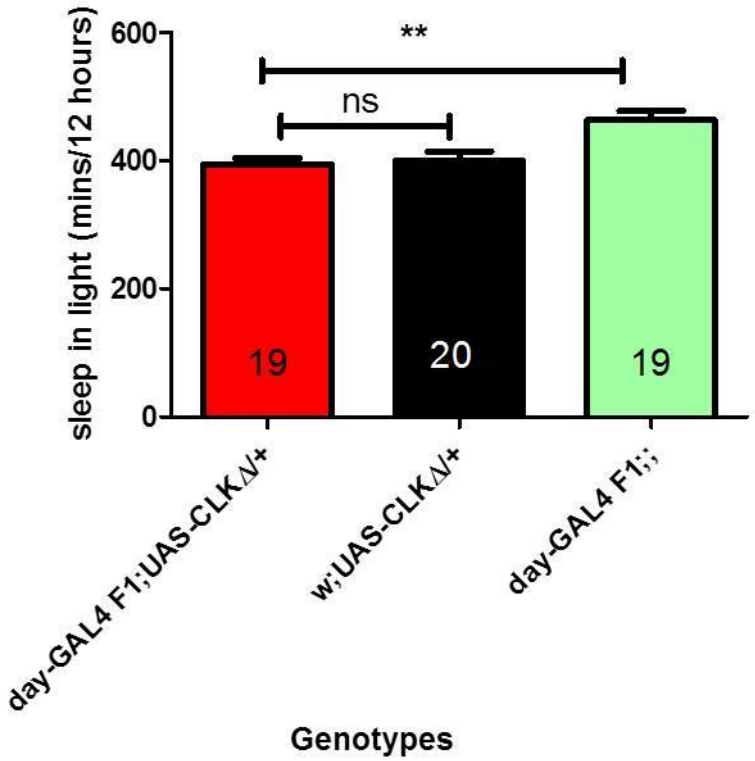


Figure 7-8: Sleep in light during LD 25°C conditions for *day-GAL4* F1 [6], UAS-*CLK*Δ.

Showing the mutant line only varying significantly from one control, red column vs green. One way ANOVA $F_{2,57}$ = 8.707, P= 0.0005: Bonferroni post hoc: *day-GAL4* F1 [6];UAS*CLK*Δ/+ vs *w*;UAS-*CL*KΔ/+ P = ns, *day-GAL4* F1 [6];UAS*CLK*Δ/+ vs *day-GAL4* F1 [6];; P = 0.01.

7.3.3 DD conditions

To rule out the influence of light on this *day-GAL4*,*CLK*Δ sleep in darkness phenotype, the first day of DD data was analysed. In this protocol flies were exposed to 12 hours of darkness then the sleep contained in the remaining 12 hours of darkness was analysed, see figure 7-9.

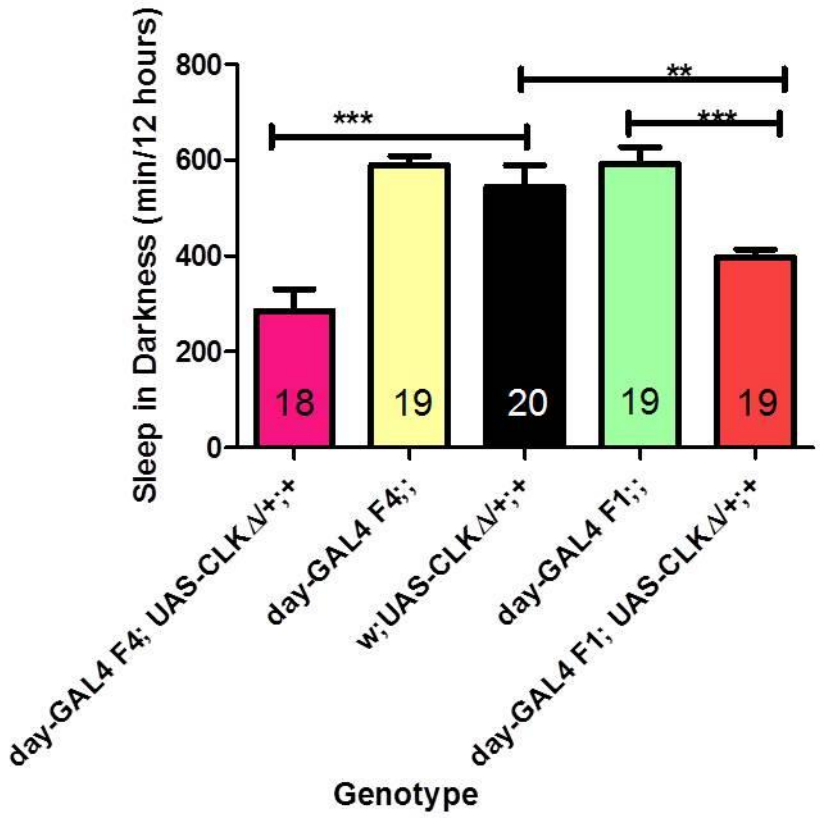


Figure 7-9: day-GAL4 [6] expressing UAS-*CLK*Δ sleep in darkness recorded.

Sleep in darkness after a prior 12 hour dark phase. ANOVA ($F_{4,89}$ = 15.20, P = <0.0001; Bonferroni: *day-GAL4* F1 [6];UAS-*CLK*Δ/+ vs *day-GAL4* F1 [6];; P = 0.001, *day-GAL4* F1 [6];UAS-*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+ P = 0.01, *day-GAL4* F4 [6];UAS-*CLK*Δ/+ vs *day-GAL4* F4 [6];; P = 0.001, *day-GAL4* F4 [6];UAS-*CLK*Δ/+ vs w;UAS-*CLK*Δ/+ P = 0.001)

The result is identical to the LD data, with lines expressing UAS-*CLK*Δ showing reduced levels of sleep in DD, ruling out a light effect on this particular sleep phenotype. Furthermore direct comparisons of sleep quantity for each of the two *day-GAL4* [6] lines crossed to UAS-*CLK*Δ during LD and DD, using a Student's unpaired t-test to analyse significance (*day-GAL4* F1 [6];UAS-*CLK*Δ/+ LD vs *day-GAL4* F1 [6];UAS-*CLK*Δ/+

DD P = 0.2108 and *day-GAL4* F4 [6];UAS-*CLK*Δ/+ LD vs *day-GAL4* F4 [6];UAS-*CLK*Δ/+ DD P = 0.2542).

7.3.4 Period of locomotor activity analysis of *day-GAL4* [6] lines

The decrease in sleep demonstrates an ability of the *day*-GAL4 [6] driver to influence the activity/rest balance; changes in periodicity were also examined.

Genotype	Period ± SEM	N	Rhythmic (%)
***day-GAL4* F4 [6];;**	24.65 ± 0.21	19	100%
***day-GAL4* F4 [6];UAS-*CLK*Δ/+**	24.36 ± 0.10	9	90%
***w*;UAS-*CLK*Δ/+;+**	23.68 ± 0.06	39	100%
***day*-GAL4 F1 [6];UAS-*CLK*Δ/+**	24.08 ± 0.13	14	93%
***day*-GAL4 F1 [6];;**	23.83 ± 0.05	39	100%

Table 7-2: Mean period of flies expressing UAS-*CLK*Δ with *day-GAL4* [6].

Measured in DD at 25°C, mutants in red font, and controls in black. One way ANOVA $F_{6,153}$ = 12.69, P = <0.0001; Bonferroni post hoc: *day-GAL4* F4 [6];; vs *day-GAL4* F4 [6];UAS-*CLK*Δ/+ P = ns, *w*;UAS-*CLK*Δ/+ vs *day-GAL4* F4 [6];UAS-*CLK*Δ/+ P = 0.001, *day-GAL4* F1 [6];; vs *day-GAL4* F1 [6];UAS-*CLK*Δ/+ P = ns, *w*;UAS-*CLK*Δ/+ vs *day-GAL4* F1 [6];UAS-*CLK*Δ/+ P = 0.01).

The period values observed in the mutant lines do vary significantly but not from both controls, see table 7-2.

The expression of *CLK*Δ should disrupt the clock negative feedback loop in the cells that it is expressed in; with *day*-GAL4 [6] expressing *CLK*Δ there are no changes in

the overall periodicity, but the distribution of sleep is altered in a light independent manner, suggesting a role for these cells in sleep/wake regulation.

7.3.5 Activity using CYCΔ

A UAS-*CYC*Δ construct exists that is equivalent to *CLK*Δ and using the same locomotor assay it should be possible to examine differences between the two. Expression of *CYC*Δ gave the same sleep phenotype with a significant reduction in the amount of sleep in darkness; when a comparison is made with *CLK* the reduction in the amount of sleep in darkness is not significantly different between *CLK*Δ and *CYC*Δ.

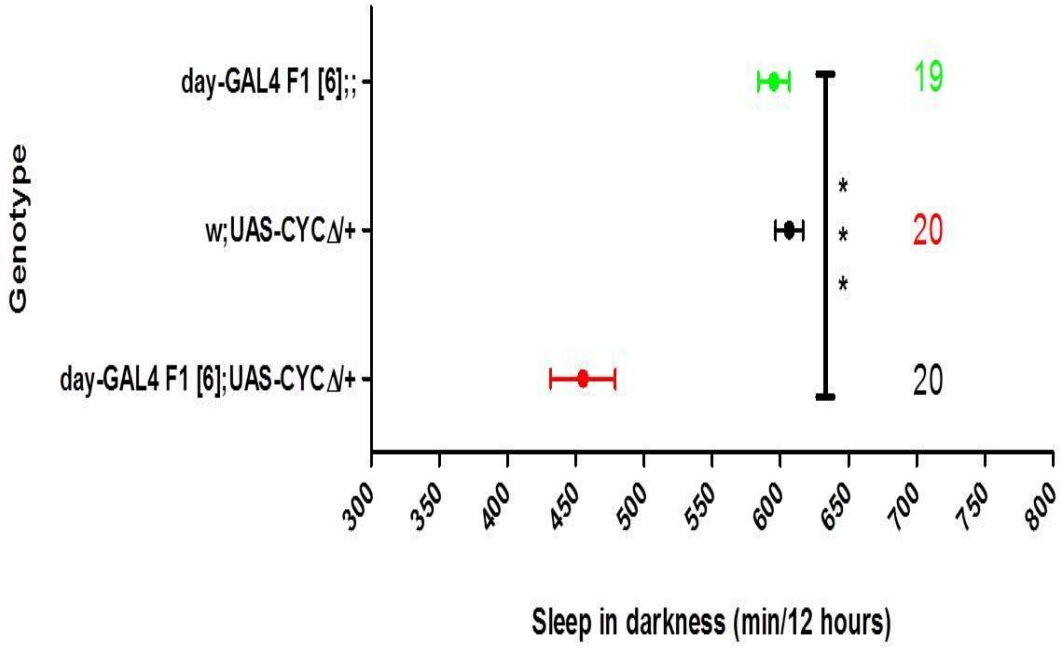


Figure 7-10 Sleep in darkness profile of *day*-GAL4 [6] driving UAS-CYCΔ.

Sleep recorded in LD 25°C; mutant in red, UAS control black and GAL4 control green. One way ANOVA $F_{2,56}$ = 26.71, P = <0.0001; Bonferroni post hoc: *day-GAL4* F1

[6];UAS-*CLK*Δ/+ vs *day-GAL4* F1 [6];; P =0.001, *day-GAL4* F1 [6];UAS-*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+ P =0.001.

Therefore the UAS-*CYCΔ* behaves in much the same manner as it *CLK*Δ counterpart in reducing the levels sleep in darkness, see figure 7-10.

7.3.6 Increasing neuronal output with *day-GAL4* [6]

The experiments driving modified CLK and CYC in potential *day* expressing cells indicate a role for these molecules and cells in sleep regulation, however, it would be useful to understand how these cells regulate such behaviour. In order to address this question neuronal output can be examined by decreasing membrane potential. Expression of the sodium channel *NaChBac* is thought to increase neuron excitability by allowing membranes to depolarise faster (Nitabach *et al.*, 2006). Locomotor activity was recorded for flies expressing the *NaChBac* construct driven using one of the lines of *day-GAL4* [6] used in the previous experiments.

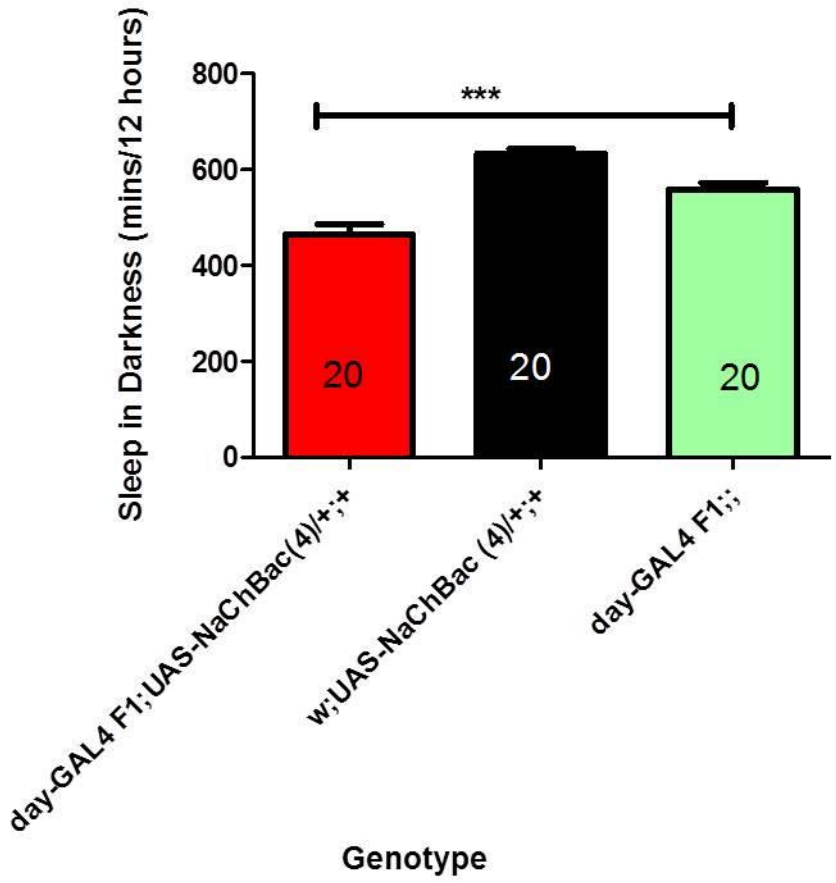


Figure 7-11: Sleep in darkness analysis of *day*-GAL4 [6] crossed to *NaChBac*.

Sleep recorded in LD at 25°C, mutant shown in red, black UAS control and green GAL4 control. One way ANOVA $F_{2,57}$ = 28.58, P= <0.0001; Bonferroni post hoc: *day-GAL4* F1 [6];; vs *day-GAL4* F1 [6];UAS-*NaChBac*/+ (4) P =0.001, *day-GAL4* F1 [6] vs w;UAS-*NaChBac*/+ P = 0.001.

A significant reduction in the total amount of sleep in darkness was observed when *NaChBac* was expressed; this result is akin to the *CLK*Δ overexpression seen with *day*-GAL4 [6] described previously, see figure 7-11.

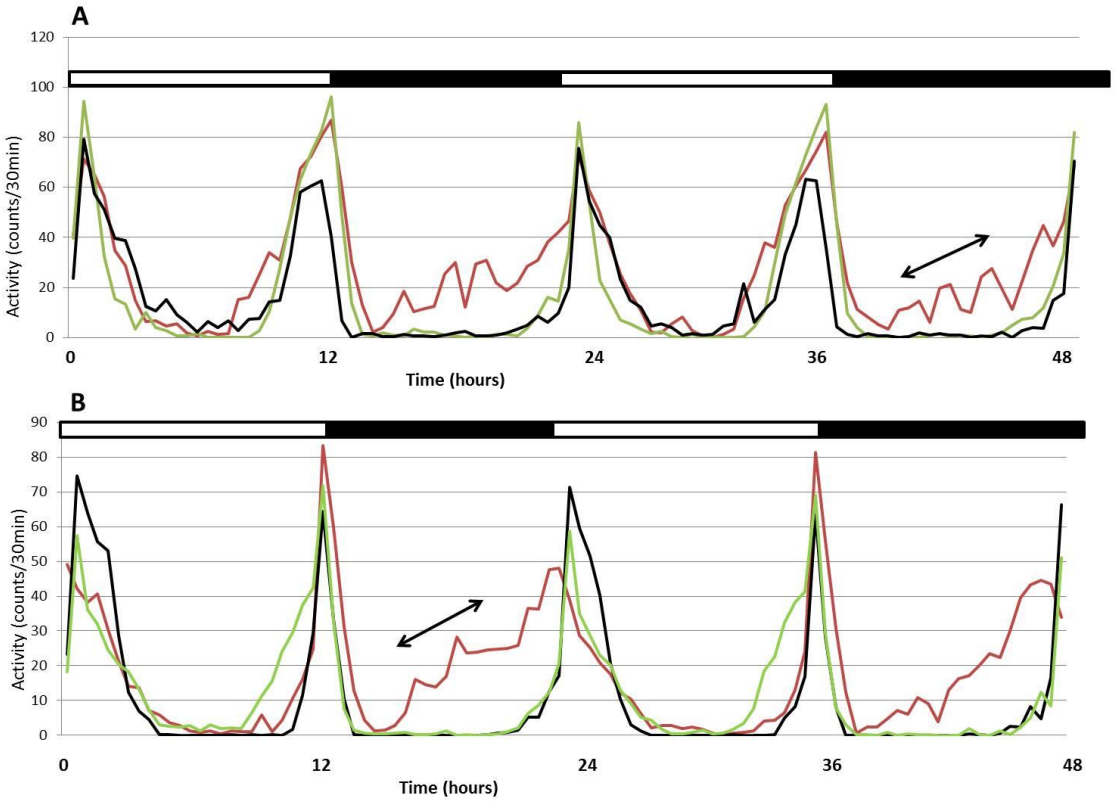


Figure 7-12: Average activity distribution of *day*-GAL4 [6] expressing *NaChBac* and UAS-*CLK*Δ in LD conditions at 25°C.

A: *day*-GAL4 F1 [6] crossed to UAS-*CLK*Δ, yellow and black horizontal bars indicate lights on and off respectively. Mutant lines in red, UAS control black and GAL4 control green, B: *day*-GAL4 F1 [6] crossed to *NaChBac*, labelled in exact same manner as graph A. Black arrows highlight the differing activity profile of night time disruption. N= 20 for all genotypes.

The profile of the two mutant genotypes is very similar in that both increase activity respective to their controls after lights off. Both *CLK*Δ and *NaChBac* cause night time arousal, however, the arrow in graph A highlights how the *CLK*Δ mutants display a bout of activity followed by rest and then a return to activity; this activity gradually increases and peaks at lights on. *NaChBac* expression causes a gradual increase in

activity which follows a linear-like progression and then peaks before lights on (black arrow graph B). The morning peak in flies carrying *day-GAL4* [6] and *NaChBac* flies is advanced by 1 bin length (30 mins) this would hint at some level of circadian control. To examine any differences in circadian clock phenotypes, the period of locomotor activity was examined.

Genotype	Period ± SEM	N
***day*-GAL4 F1 [6];UAS-*NaChBac* (4)/+**	23.60 ± 0.15	19
***w*;UAS-*NaChBac* (4)/+**	24.12 ± 0.14	16
***day-GAL4* F1 [6];;**	23.83 ± 0.05	39

Table 7-3: **Period analysis of *day-GAL4* [6] expressing *NaChBac*.**

Flies tested in DD conditions at 25°C. One way ANOVA $F_{8,186}$ = 10.99, P = <0.0001; Bonferroni post hoc: *day-GAL4* F1 [6];; vs *day-GAL4* F1 [6];UAS-*NaChBac* (4)/+ P = ns, *w*;UAS-*NaChBac* (4)/+ vs *day-GAL4* F1 [6];UAS-*NaChBac* (4)/+ P= 0.001.

Again akin to *CLK*Δ overexpression no significant change in the period is observed from both controls when expressing *NaChBac* with the *day-GAL4* driver [6], see table 7-3.

7.3.7 Other lengths of GAL4

As stated in chapter 3 "Analysis of *day* locus" the upstream region of *day* has revealed an additional regulatory sequence. Downstream of this regulatory element 4

shorter lengths (1, 1.5, 3 and 4.5 kb) have been cloned into the pPTGAL vector, as described above. It may be possible to dissect the upstream *day* region by using varying lengths of sequence and employing the same types of activity experiment. These may reveal behavioural differences that could be attributed to the presence or absence of transcriptional enhancer/repressor elements. Therefore the same locomotor studies using UAS-*CLK*Δ were attempted for two of the lengths.

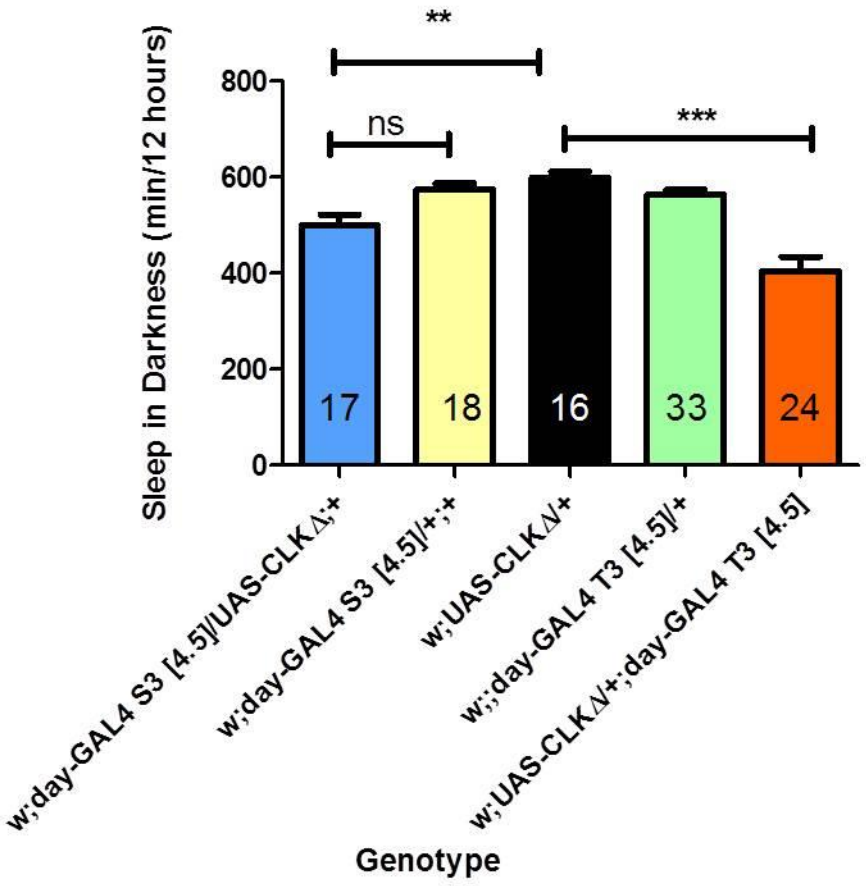


Figure 7-13: Locomotor activity analysis examining sleep in darkness of *day-GAL4* [4.5] driving UAS-*CLK*Δ, in LD at 25°C, mutant.

Mutant lines are shown in blue (S3) and orange (T3) with the UAS control in black and the GAL4 controls in yellow and green. One way ANOVA $F_{4,103}$ = F 17.07, P= <0.0001; Bonferroni post hoc: w;*day-GAL4* S3 [4.5]/UAS-*CLK*Δ/+ vs *w*;*day-GAL4* S3 [4.5]/+; P = ns, *w*;*day-GAL4* S3 [4.5]/UAS-*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+ P = 0.01, *w*;UAS-

***CLK*Δ/+;*day-GAL4* T3 [4.5]/+ vs *w*;;*day-GAL4* T3 [4.5]/+ P = 0.001, *w*;UAS-*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+;*day-GAL4* T3 [4.5]/+ P = 0.001.**

Of the two lines tested, one (T3), is significantly different from both controls showing a reduction in sleep in darkness, while the S3 line displays a similar trend but is not significant from both controls, see figure 7-13.

The same experiment was carried out using *day-GAL4* [3] lines with UAS-*CLK*Δ, analysing sleep in LD conditions.

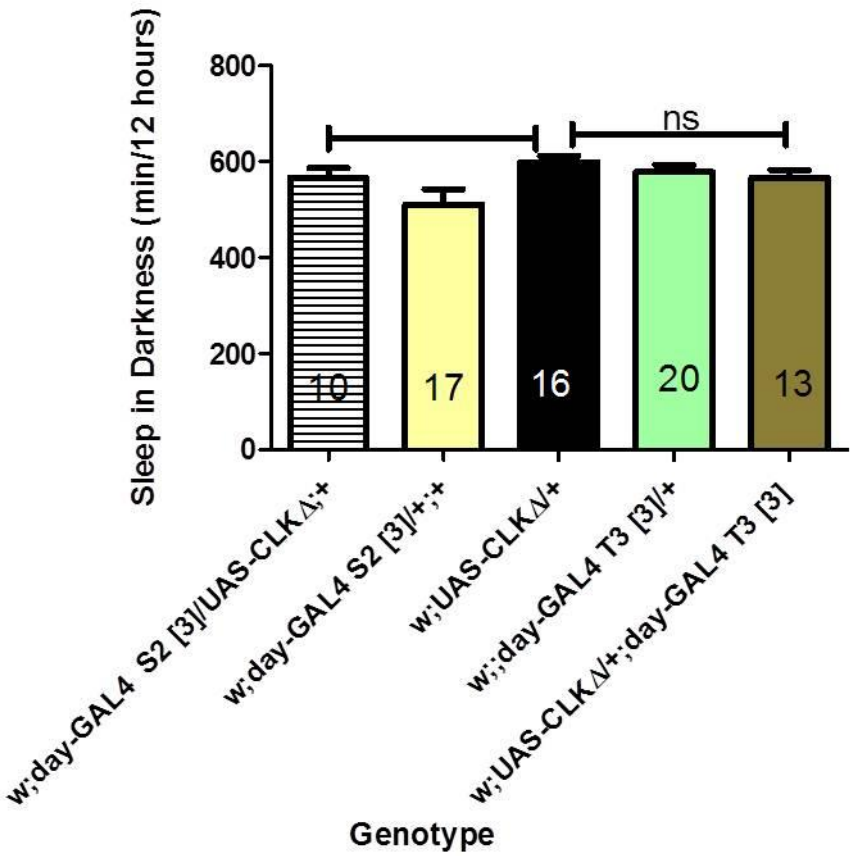


Figure 7-14: Sleep in darkness of 3 kb day-GAL4 enhancer driving UAS-*CLK*Δ, LD 25°C.

Mutants marked with horizontal black bars (S2) and gold (T3), black UAS control and yellow and green the GAL4 controls. One way ANOVA $F_{4,71}$ =2.688, P = 0.0380;

Bonferroni post hoc: *w*;*day-GAL4* S2 [3]/UAS-*CLK*Δ; vs *w*;*day-GAL4* S2 [3]/+;+ P = ns, *w*;UAS-*CLK*Δ/+ vs *w*;*day-GAL4* S2 [3]/UAS-*CLK*Δ/+ P = ns, *w*;;*day-GAL4* T3 [3]/+ vs *w*;UAS-*CLK*Δ/+;*day-GAL4* T3/+ [3] P= ns, *w*;UAS-*CLK*Δ/+ vs *w*;UAS-*CLK*Δ/+;*day-GAL4* T3 [3]/+ P = ns.

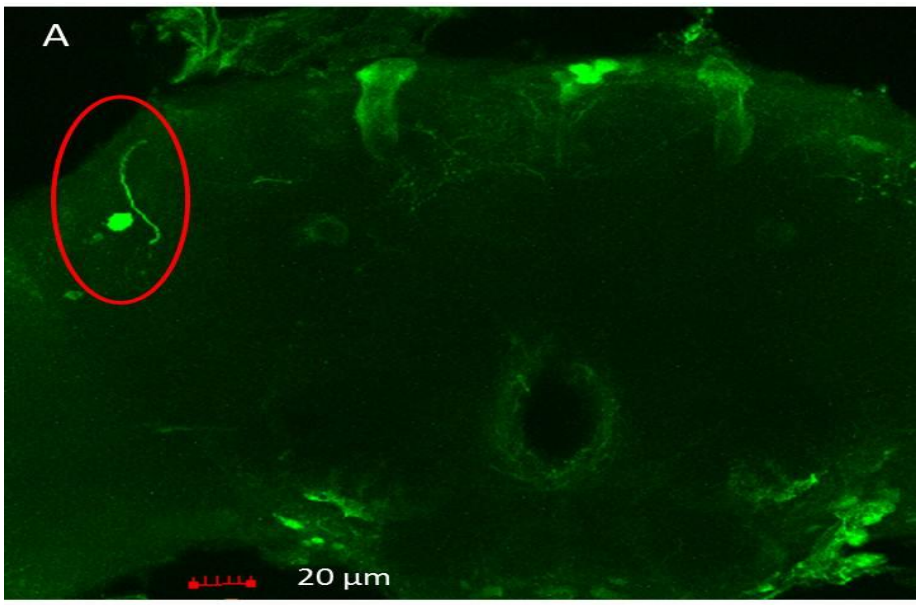
Of the two lines tested neither showed a significant reduction in sleep, see figure 7-14. Therefore by shortening the length of upstream *day* sequence we have lost the sleep phenotype seen with the two previous longer lengths (6 kb and 4.5 kb).

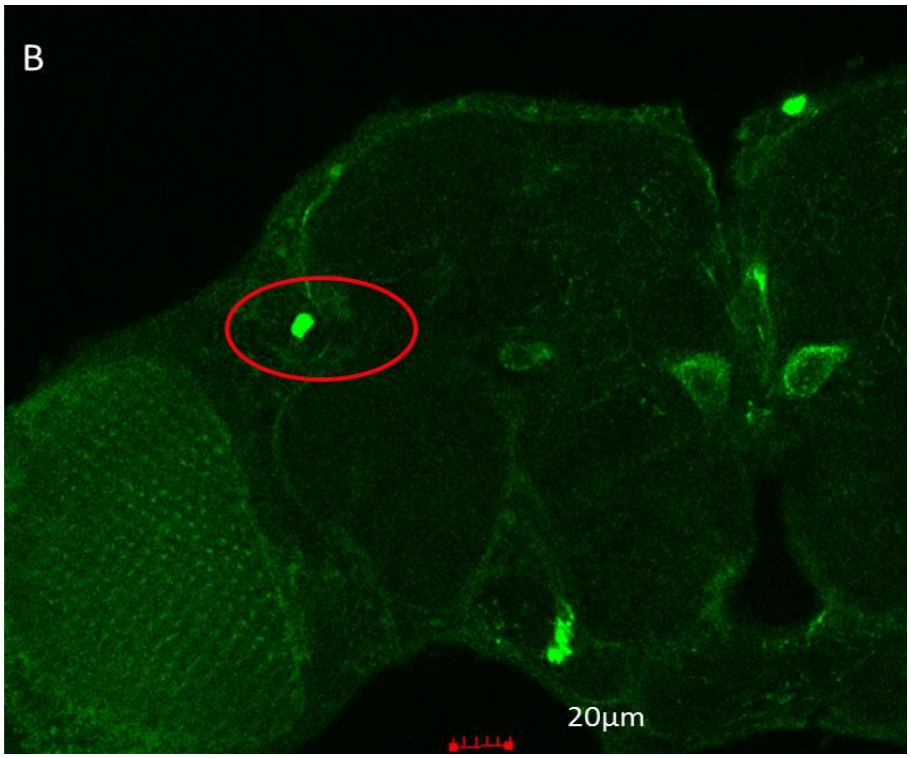
7.4 Which cells express *day*?

Identifying cellular expression of *day* would significantly contribute to addressing the function of the gene as it may be possible to identify specific cell bodies with known involvement in regulatory processes. To address this question the *day-GAL4* [6] lines were examined, as were independent *Minos* insertions that have recently been identified (Venken *et al.*, 2011).

7.4.1 IHC of *day*-GAL4 [6]

Coupling the activity data seen thus far with data revealing the location of cells expressing *day*, could help in addressing the function of *day*. To this end IHC was carried out using *day*-GAL4 [6] crossed to UAS-GFP.


A
20 µm


B
20µm

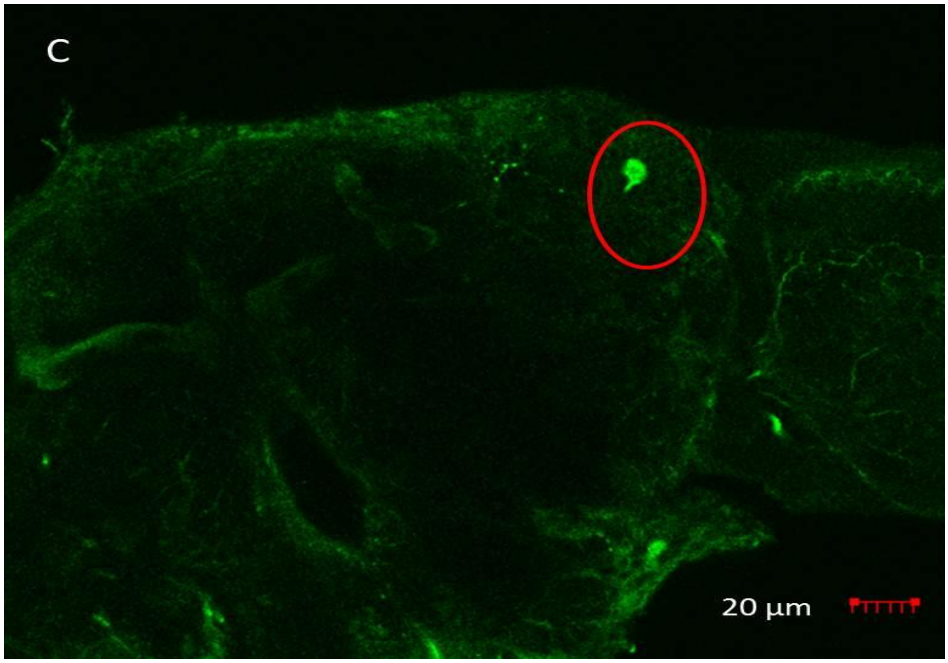


Figure 7-15: **IHC analysis of three lines of *day-GAL4* [6] expressing UAS-GFP.**

Anterior brain stacks at x 40 magnification. Flies raised in standard LD conditions and fixed at ZT 4. Staining with anti-GFP (Rabbit) and anti-rabbit cy3. A: *day-GAL4* F1 [6], B: *day*-GAL4 62-T [6] and C: *day*-GAL4 6F [6]. Red circle highlights a cell common to all three and thought to be a clock neuron.

These independent insertions, allow identification of common cell staining patterns and cells giving a possible readout of endogenous *day* expression. All three images focus on the anterior side of the brain, showing signal coming from a cell with a projection looping into the dorsal hemisphere of the brain (circled in red). The location of this cell is similar to one of the LN_d neurons (diagrammatic depiction of this cell is in chapter 1.8). Further experiments have been undertaken to confirm this and shall be discussed later in this chapter. Images A and C clearly have the mushroom bodies (MB) present with the α α' and β' lobes visible, furthermore image C shows antennal nerve staining. Not all the cells shown in these images have been described due to the broad staining patterns generated by these lines, some of this staining is likely to be line

specific and dependent on the enhancer sequences surrounding the *day-GAL4* [6] P-element. Finally it is partially evident that within the red circle in image A lie two cells, this dual cell staining was verified using different lines (data not shown).

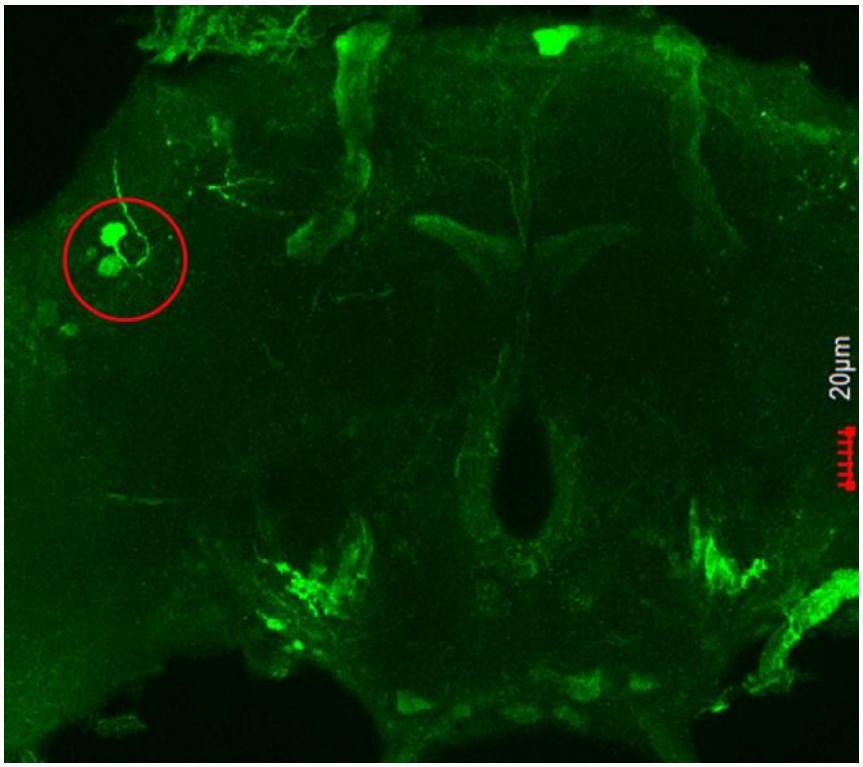


Figure 7-16: **IHC of *day-GAL4* F1 [6] expressing UAS-GFP.**

Anterior brain stacks at x 40 magnification. Flies raised in standard LD conditions and fixed at ZT 4. Staining with anti-GFP (Rabbit) and anti-rabbit cy3. Red circle highlights two cells that show similarities with LN_ds.

This double cell staining is not present in all of the images taken, but has now been verified in two different lines suggesting that there may be two LN_d neurons expressing *day-GAL4* [6]. The respective UAS and GAL4 controls for this experiment are shown below.

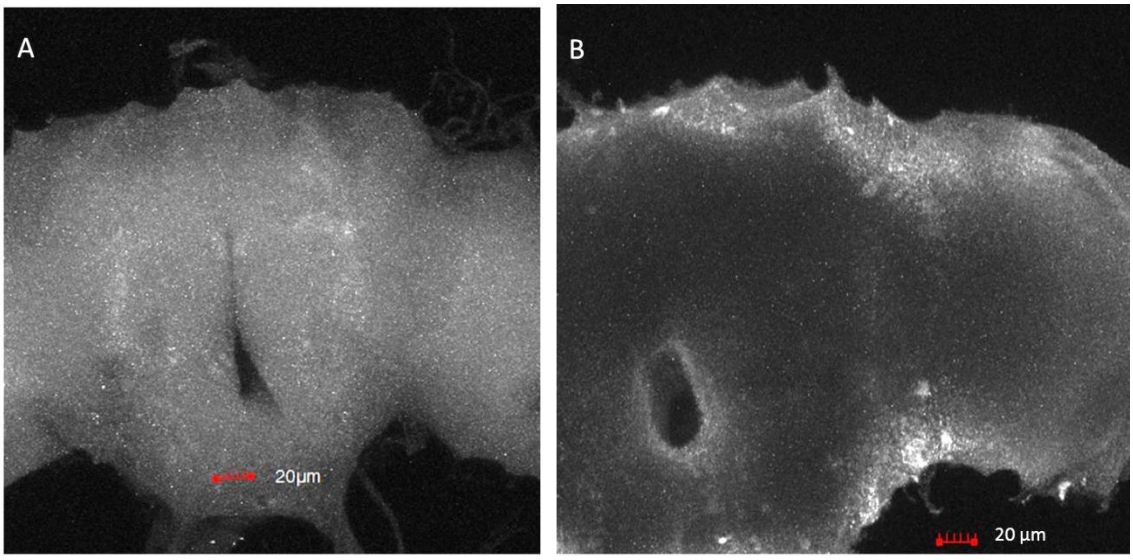


Figure 7-17: **Controls of *day-GAL4* F1 [6] IHC experiment**.

A: w;UAS-GFP crossed to w^{1118}, B: *day*-GAL4 F1 [6] crossed to w^{1118}. Controls were stained with the same antibodies and were raised in the same conditions as the figures shown above.

The controls for this experiment show no specific cell body staining in the regions visualised in the *day-GAL4* [6], UAS-GFP IHC, therefore the antibodies are specific and the staining we have seen with *day-GAL4* [6] is credible. Having analysed *day-GAL4* [6] in males it could be interesting to repeat this experiment using females to test for similarities in expression.

7.4.2 Female brain *day*-GAL4 [6] staining

Females of the *day*-GAL4 [6]; UAS-GFP genotype were analysed, in an attempt to analyse sex specific differences in the upstream sequence. We have some indication

that they may be gender differences based on the results of the chill coma experiment, where females behaved as wild type.

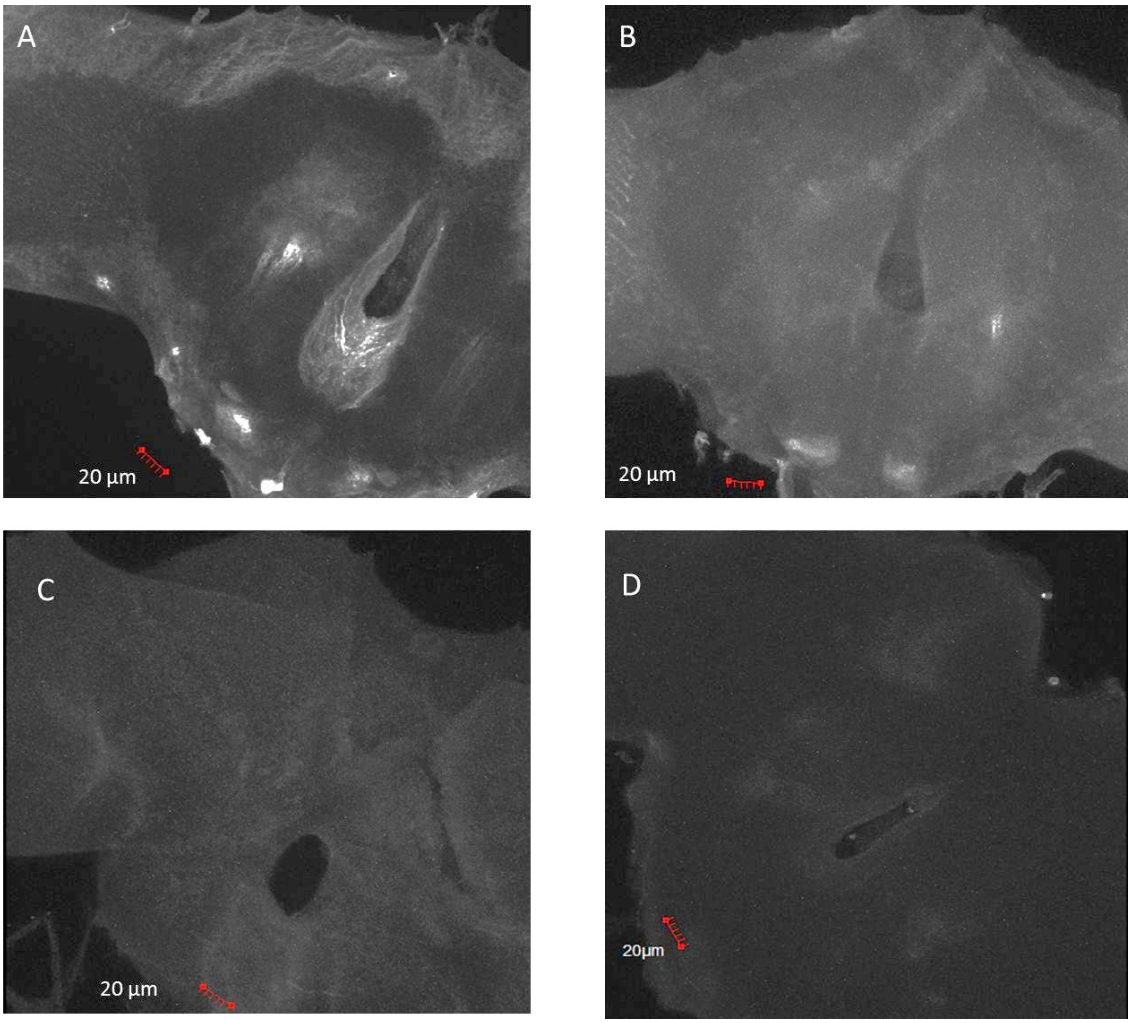


Figure 7-18: IHC female *day-GAL4* [6] UAS-GFP.

A+B *day*-GAL4 F1 [6] UAS-GFP, C: UAS-GFP/+ and D: *day*-GAL4 F1 [6]. Stained with anti-GFP rabbit, anti-rabbit cy3 and were raised in LD conditions at 25°C and sampled at ZT 4. Images taken with 40x magnification and are stacked images (posterior and anterior).

There are no clear cell bodies present in these stacks (figure 7-18); there are patterns in the suboesophageal ganglia (SOG) (image A). While image B shows what could be

antennal nerve staining, however, overall there appears to be a vast reduction in cell fluorescence, this could be a problem with the line as only one has been used or it could simply highlight sexual dimorphic expression of GAL4; the controls do not show specific cell groups.

7.4.3 Minos Mediated Integration Cassette (MiMIC) lines

There are inherent faults in determining expression patterns completely on staining generated using upstream sequences. It is possible for that within the 6 kb sequence are enhancers for other genes which would be indistinguishable from the gene of interest, furthermore the three lines of male *day-GAL4*,UASGFP highlight how the positioning of the P-element may also added additional cellular staining. Therefore another method is required to verify the staining seen previously. In an attempt to address this concern, we have performed IHC with two transgenic MiMIC insertion lines (Venken,K.J. 2011). These lines use the *Minos* transposable element; this flanks an enhanced GFP gene (EGFP) and *att*P sites that allow modification with an integrase enzyme. The figure below indicates the presence of two of these insertions in the 5' and 3' ends of the *day* locus.

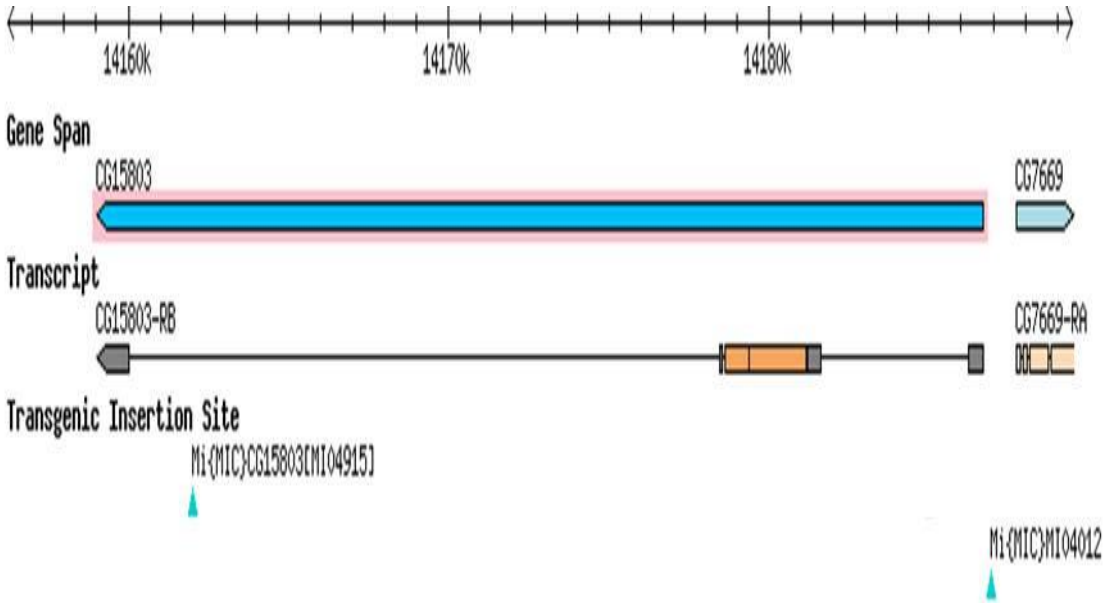


Figure 7-19: Flybase GBrowse image revealing position of transgenic MiMIC insertions.

Turquoise triangles indicate the position of MiMICs in the gene span of *day*.

The EGFP contained within the MiMIC construct is promoter-less and therefore it should act as an enhancer trap, whereby *day* expressing cells may express the EGFP. An IHC experiment was undertaken on fly lines carrying either insert and then correlating this expression with *day*-GAL4 [6] may give a more reliable indication of cells that express *day*. The multiple sites present in the MiMIC construct are shown below, followed by IHC analysis of the MiMIC lines.

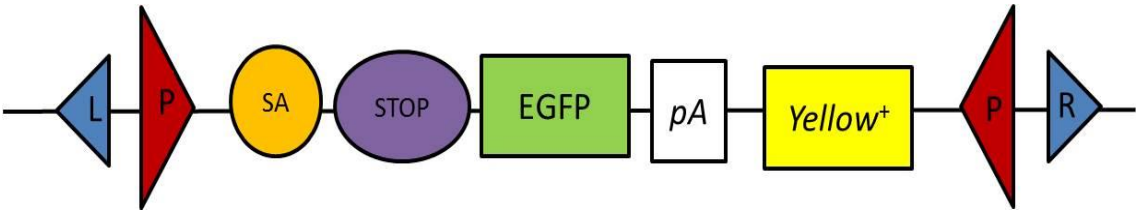


Figure 7-20: Diagram depicting the sites present in the MiMIC construct.

L + R are the two *Minos* inverted repeats, P = inverted *attP* ϕC31 integrase sites, SA= splice acceptor, Stop= stop codon sequence, EGFP= *enhanced green fluorescent protein*, pA= polyadenylation signal, *yellow*$^{+}$= marker.

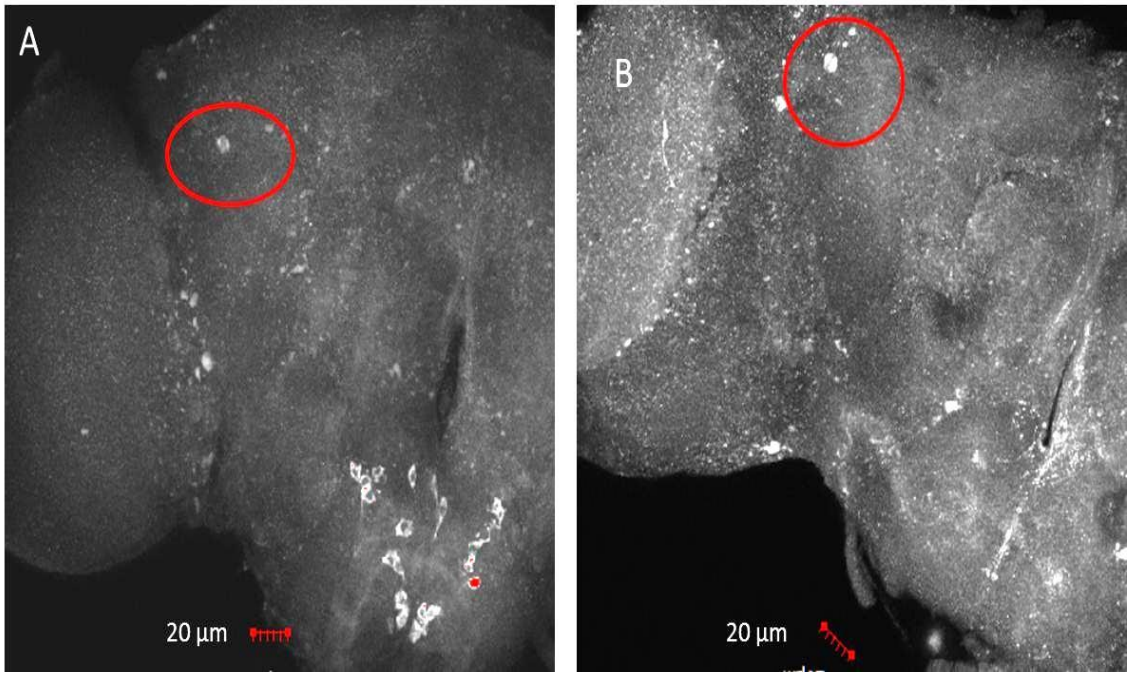


Figure 7-21: IHC of MiMIC lines.

A: MiMIC line 36939 5' insertion, B: MiMIC line 37870, 3' insert. Flies raised in LD and sampled at ZT4-6. EGFP stained with anti-GFP rabbit, anti-rabbit cy3, images taken at 40x magnification, single sections are shown. Red circle highlights cell of interest.

Using the MiMIC lines for IHC reveals a similar pattern of expression between the two lines (figure 7-21), which are located at opposite ends of the *day* locus. Both lines highlight one discrete cell body, but it is unknown if this cell is the same between the two genotypes. Unlike the *day-GAL4* [6]; *UAS-GFP* images shown earlier, expression of EGFP is not as abundant in both intensity of staining and number of cells highlighted. This difference in intensity of staining is to be expected as the *day-GAL4* [6] lines are overexpressing, whilst the EGFP expression from the MiMIC inserts should represent the natural pattern of *day* expression, which will be expressed at lower levels.

Additional images were taken showing similar patterns of EGFP expression in the posterior side of the brain, figure 7-22.

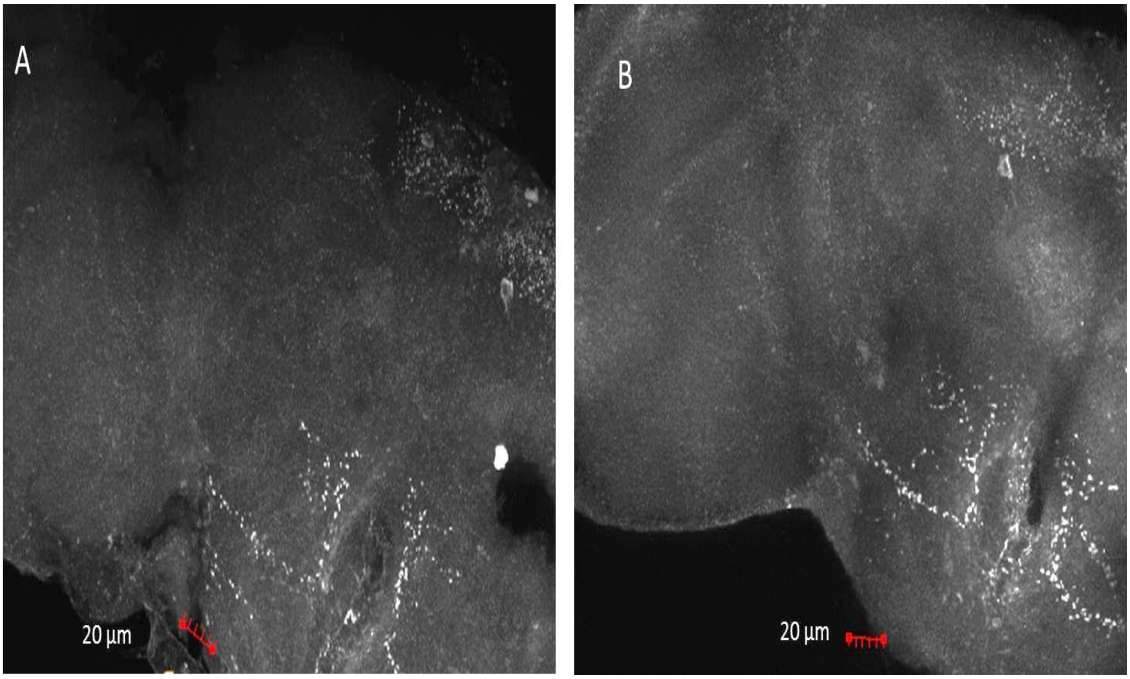


Figure 7-22: IHC of MiMIC lines; stack of posterior images.

A: MiMIC line 36939 5' insertion, B: MiMIC line 37870, 3' insert. Flies raised in LD and sampled at ZT4-6. EGFP stained with anti-GFP rabbit, anti-rabbit cy3, images taken at 40x magnification.

Again these images show a similar pattern of EGFP staining; in particular the SOG is highlighted, located laterally, as well as similar staining on the dorsal side of the brain. These data seem to be suggesting that both constructs are expressed in the same types of cell. Finally posterior images were taken with the *day-GAL4*.UAS-GFP genotype to allow for comparisons to the MiMIC lines.

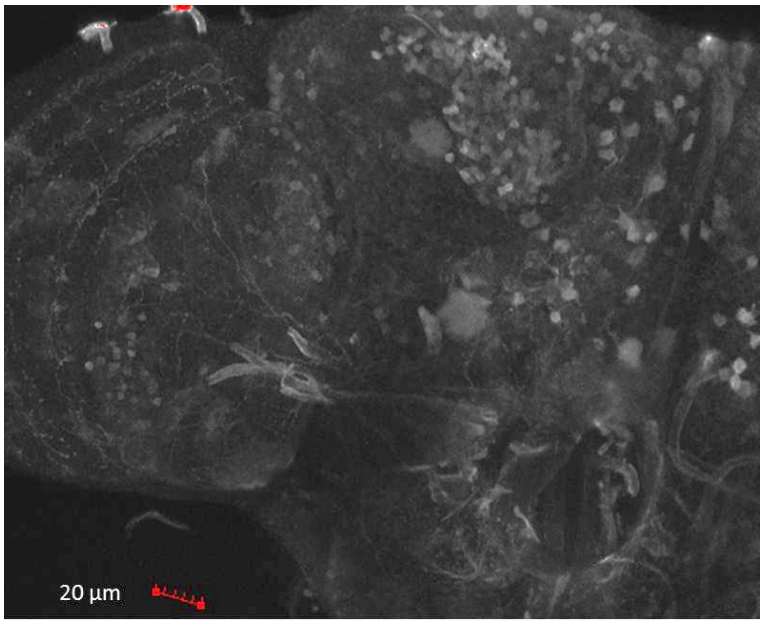


Figure 7-23: IHC of *day-GAL4* F1 [6];UAS-GFP lines posterior stacked images.

Flies raised in LD and sampled at ZT4-6. EGFP stained with anti-GFP rabbit, anti-rabbit cy3, images taken at 40x magnification.

Using the *day-GAL4* [6], UAS-GFP line we see similar dorsal staining as was seen with the MiMIC lines, the pattern is somewhat broader in the *day-GAL4* genotype and this may be a consequence of greater overexpression. The SOG staining in the *day-GAL4* genotype is not akin to what was observed using the MiMIC lines. Therefore by comparing the expression patterns of *day-GAL4* to MiMIC lines we do see some similarities (dorsal posterior staining and possible LN_d overlap), as well as differences (varying SOG patterns and no MB staining in the MiMIC lines).

7.4.4 Does *day-GAL4* overlap with CRY?

So far we have examined where *day* may be expressed, but as *day* was brought to our attention through its light dependent binding of CRY, it is important to establish if there is any overlap in cells expressing *cry* and *day*. The IHC data shown previously led us to conclude that there might be staining in the LN_d neuron(s). To answer this question IHC was undertaken using *day-GAL4* [6], UAS-GFP with co-staining using the CRY antibody.

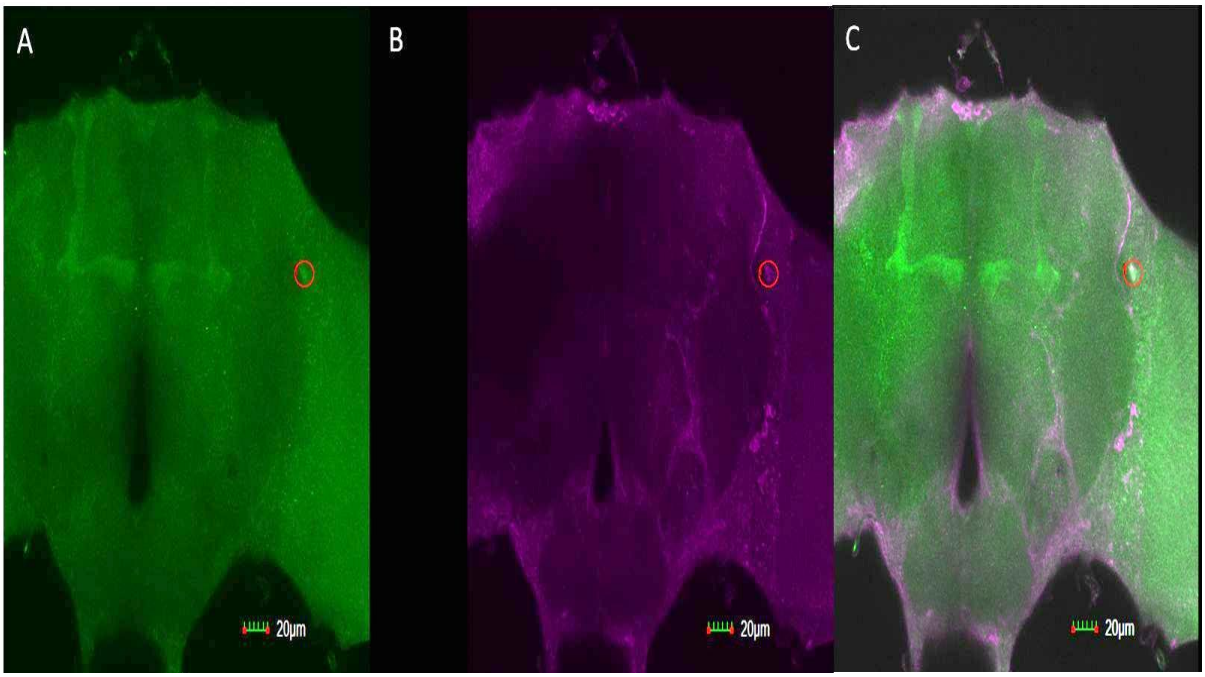


Figure 7-24: IHC of *day-GAL4* F1 [6]; UAS-GFP with CRY antibody.

A: GFP staining, B: CRY antibody staining, C: Overlap of image A and B. Images presented are stacks of the anterior stained with GFP (anti-GFP mouse, anti-mouse cy2) and CRY antibody (anti-CRY rabbit, anti-rabbit biotin and STREP cy5). Red circle highlights common cell staining. Flies were raised in DD to raise the levels of CRY.

Images of 5 slices were stacked in the LN_d region, GFP staining was not as abundant in this experiment and this is likely to be a consequence of a less sensitive GFP antibody (mouse instead of rabbit). In the previous IHC of *day-GAL4* F1 [6] (rabbit antibody), there was sometimes two cells that may have been LN_ds, however only one is seen in this experiment. Image A shows GFP staining and some features such as the MBs are still visible, as well as a cell highlighted by the red circle. The purple stain in image B is of the CRY antibody and within the red circle are three LN_d cell bodies, this is consistent with published reports showing three *cry* positive LNds (Yoshii *et al.*, 2008).

To visualise this staining more clearly zoomed images (single slice not stack) were created (figure 7-25).

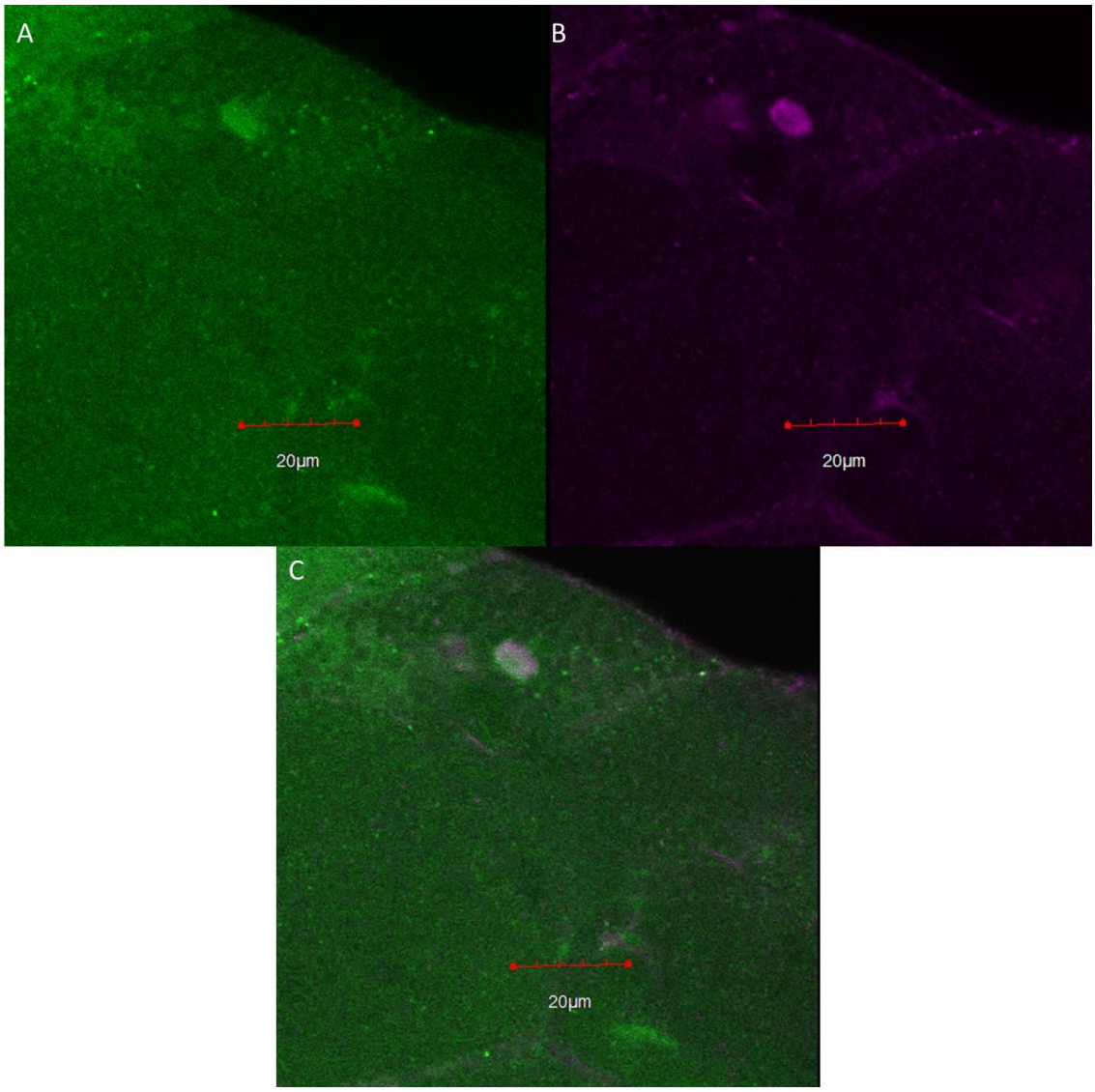


Figure 7-25: Zoomed in *day-GAL4* [6], UAS-GFP co-stained with CRY.

A: GFP staining, B: CRY staining and C: the overlap of images A and B. Stained in the same way as the previous figure. Single slice image.

From this figure it is clear that there is overlap between the green and purple signals, thus day-GAL4 [6] expresses in a cell that overlaps with the CRY antibody. The position

of the cell indicates that it is likely to be an LN_d cell, of which there are three CRY positive LN_ds.

To confirm this GAL4 and UAS controls were examined in parallel for this experiment and are shown below.

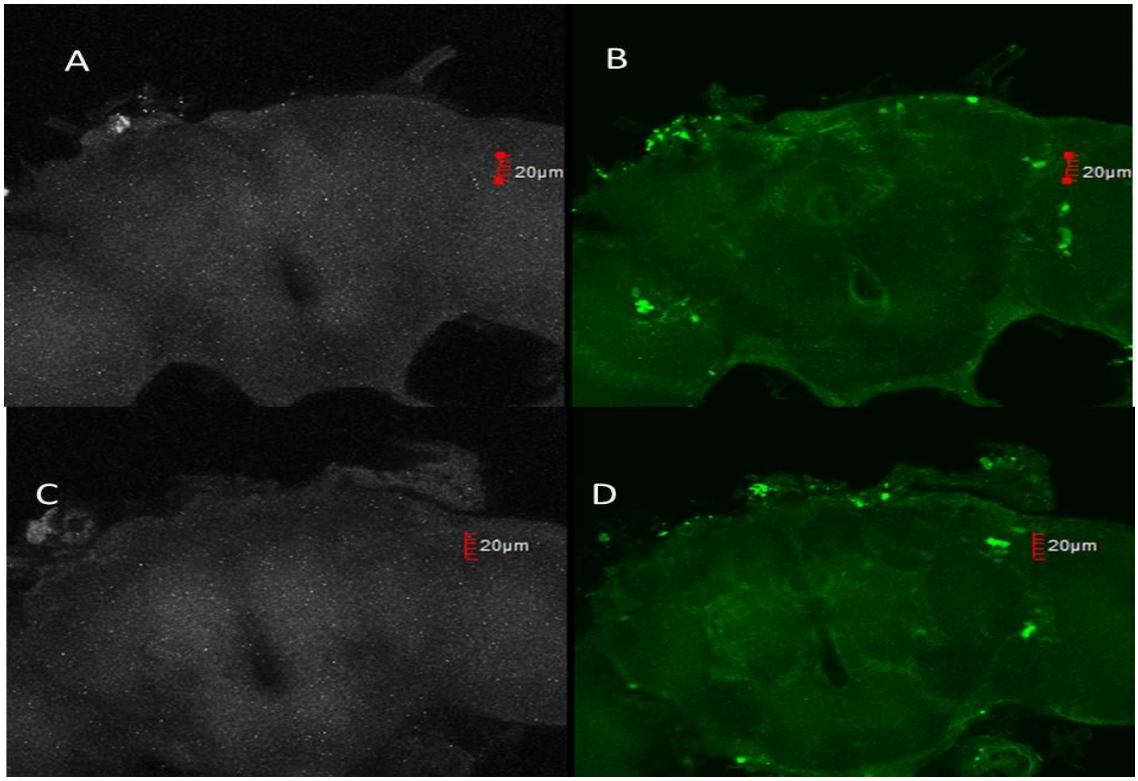


Figure 7-26: Control staining of *day-GAL4* [6], UAS-GFP experiment.

A: *w*;UAS-GFP/+ stained with GFP antibody, B: *w*;UAS-GFP/+ stained with CRY antibody. C: *day*-GAL4 F1 [6];; GFP stain, D: *day*-GAL4 F1 [6];; CRY staining.

No GFP signal is present in either of the controls (*w*;UAS-GFP/+ and *day-GAL4* F1 [6];;) and the CRY antibody works effectively in both control genotypes, showing some of the l-LN_vs cells in sample B and D.

7.4.5 DAY antibody

An additional way to verify the staining of *day-GAL4* [6] and confirm the presence of DAY expressing cells more specifically would be to use an antibody specific for DAY. This antibody was generated by Eurogentec (Belgium) and used a short peptide sequence of DAY; the data below show IHC experiments using this antibody. The IHC experiments carried out with the DAY antibody in this sub-chapter were performed by Dr C. Hansen.

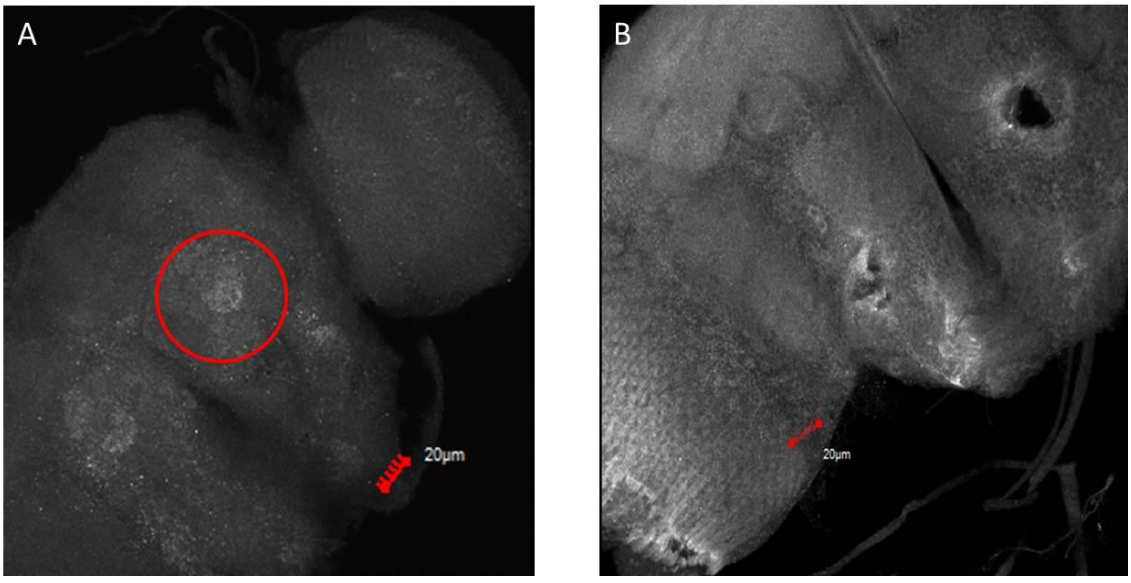


Figure 7-27: DAY 777 antibody staining.

A:CS/+ male flies raised in LD and stained with DAY777 for 1 week followed by anti-rabbit biotin amplification for an additional week and then stained with STREP-cy5, B: *day^{0P}* male stained in the same conditions. Images taken at 40X magnification and an anterior stack is shown. Red circle highlights staining.

The staining produced by the DAY antibody is very diffuse and single images do not show cell body staining (figure 7-27). Taking a stack of the brain does show staining in the antennal lobe neuropil. This staining was consistently seen in a number of individuals and therefore to verify it is accurate the same experiment was performed in *day^{0}* lines. The staining shown in the *day^{0O}* line shows different staining to the CS/+ control shown previously, there is a very low level of staining in the *day^{0O}* line but the antennal lobe structures are not seen as clearly as before. This indicates that although the DAY antibody does not appear to stain much it does appear to stain selectively.

The staining seen with the DAY antibody appears to be marking the antennal glomeruli structures, to confirm this with *day-GAL4* [6], the UAS-actinGFP line was used. In this genotype the proteins ACTIN and GFP are fused together, thus this line is more suited for marking projections and neuron terminals. This cross gave a marked difference in the pattern of GFP expression, shown below figure 7-29.

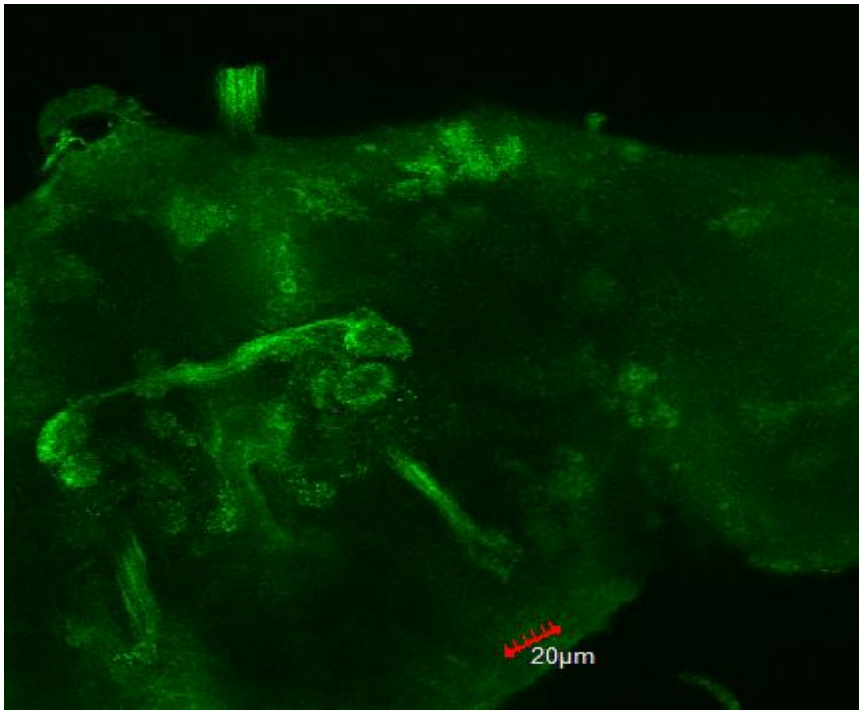


Figure 7-28: IHC of male *day-GAL4* F1 [6]; UAS-actinGFP flies.

Experiments performed with flies raised in LD, using anti-GFP rabbit antibody stained with anti-rabbit cy2. Anterior stack shown.

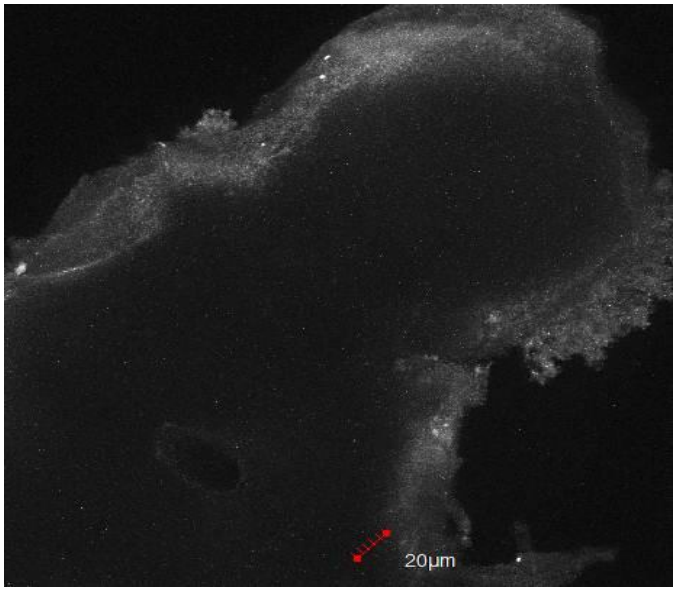


Figure 7-29: Control staining of UAS-*actinGFP*/+.

UAS-*actinGFP* crossed to w^{1118}. Sample was stained with the sample dilution of antibody and for the same length of time as in the previous figure. GAL4 control for this antibody was shown in:

Figure 7-17: **Controls of *day-GAL4* F1 [6] IHC** experiment.

The overexpression of ACTIN-GFP has given clearer depiction of the antennal lobe glomeruli which was not seen as clearly using regular GFP, however, it is unclear from these pictures if the staining of the DAY antibody is overlapping with the staining shown in the ACTIN-GFP image. Again there is a structure resembling the antennal nerve projecting located ventrally to the lobe staining suggesting the presence of *day-GAL4* [6] expression in the antenna.

7.4.6 *Day-GAL4* [6] and the antennae

To examine if *day-GAL4* is expressed in the antennae, *day-GAL4* [6] flies were crossed to UAS-GFP and the resulting progeny examined. The antennae of flies raised in LD were removed and mounted on glass slides and visualised within 1 hour of their removal.

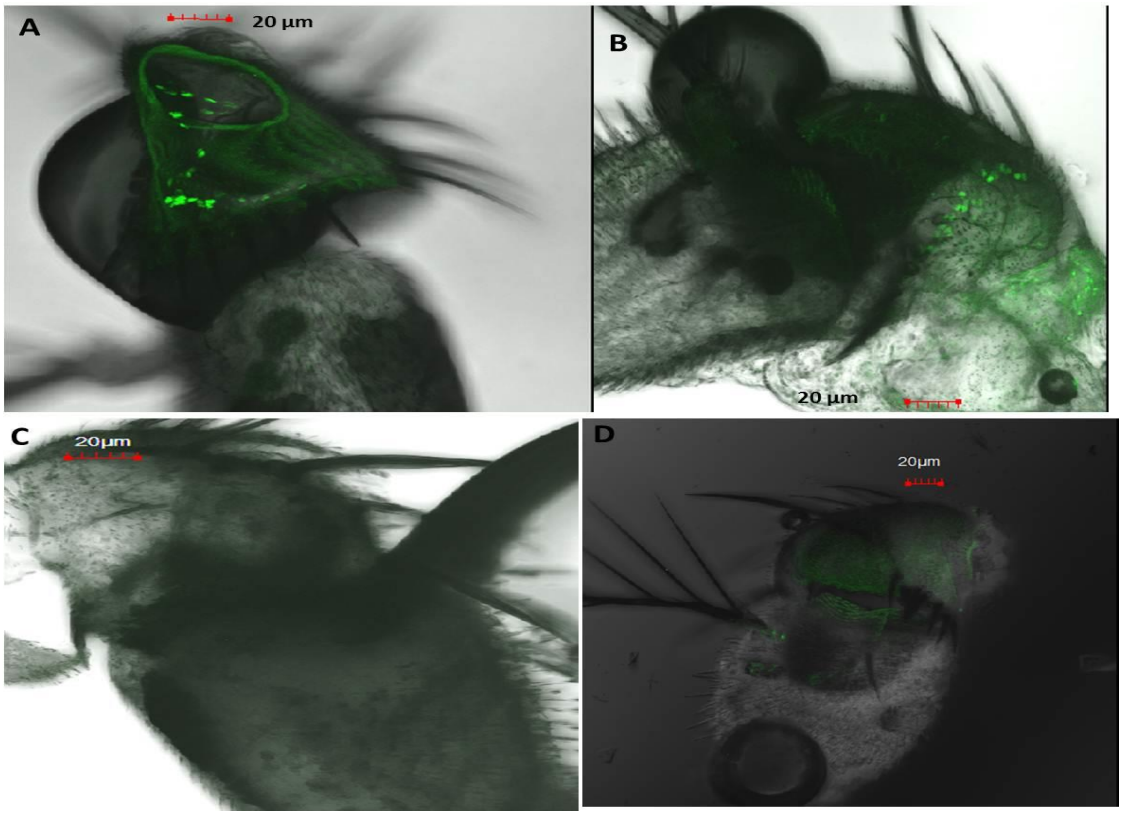


Figure 7-30: *day-GAL4* [6], UAS-GFP staining in the antennae of male flies.

A+B: *day-GAL4* F1 [6], UAS-GFP, C: *w*;UAS-GFP/+, D: *day-GAL4* F1 [6];;. No antibody was used to visualise GFP, images taken with confocal microscope at 40X magnification.

There is cell staining in the Johnston organ shown in images A + B, while the controls do not show clear cell like patterns, instead they have a hazy signal which is

background. CRY is also known to be expressed in this organ and the same experiment was repeated for *cry*-GAL4(13), however, the GAL4 control was not tested.

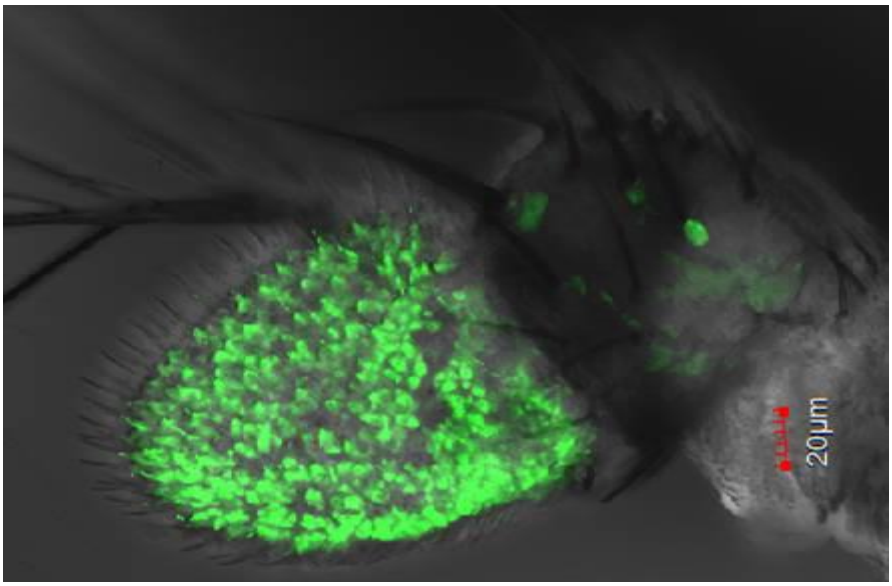


Figure 7-31: Cry-GAL4(13), UAS-GFP antennae.

Experiments were performed in a homologous manner to the *day-GAL4* [6] experiment.

The staining with *cry*-GAL4(13) shows marked differences to *day-GAL4* [6], with the vast majority of GFP from *cry*-GAL4 (13) expressed in the third segment of the antennae.

7.5 Discussion

The aim of the work presented in this chapter was to create and characterise lengths of upstream *day* sequence and examine what this tells us about the function of *day*, by examining the location of cells expressing GAL4 and then manipulating these

cell groups and examining the consequences. Additional MiMIC lines and DAY antibody staining (antibody work carried out by Dr C.Hansen) is also shown in attempt to verify staining seen in the GAL4 lines.

The first parameter examined was the locomotor activity of flies expressing the dominant negative *CLK*Δ, using the longest stretch of *day* upstream sequence caused a sleep disruption phenotype whereby the sleep in darkness, but not light, was reduced significantly. This suggests that the *day-GAL4* [6] driver is expressed in cells responsible for either sleep promotion or arousal. When the firing of these cells is increased with *NaChBac* the same arousal phenotype is seen. This seems to suggest that these are arousal cells rather than sleep promoting cells that are expressing the GAL4. Observing the sleep profile of these cells showed different arousal patterns with *day-GAL4* [6], *NaChBac* flies increasing their activity in a more linear fashion than flies expressing *CLK*Δ, which display more erratic bouts of activity. This hints at a difference in how this arousal is being generated, it could be possible that a functional clock in these cell is required to maintain a firing rhythm, when disrupted with *CLK*Δ these neurons might fire out of phase promoting bouts of arousal that aren't sustained. In contrast *NaChBac* expression may be causing continuous firing giving rise to increased nocturnal activity as the night progresses. What is behind this linear increase in activity? Perhaps these neurons expressing *NaChBac* become more important for the regulation of night time activity as time passes and impose themselves raising activity, or this activity rise could be due to other waking promoting cells 'chiming in' and causing the premature peak of activity, for example clock cells promoting the anticipation of the morning peak. Of course it could even be a combination of both

these neural mechanisms. The fact that an increase of activity is not seen during the light phase suggests that these cells are not responsible for total activity distribution. By combining this activity data with the IHC carried out with *day-GAL4* [6] it may be possible to identify potential cellular regulators of this arousal phenotype.

The *day-GAL4* [6] activity data is extremely similar to behavioural data presented in (Joiner *et al.*, 2006), where the fly line, *c309*-GAL4, behaved in a similar fashion and was found to be express GAL4 in the α, β and γ neurons of the MBs. The *day-GAL4* [6] lines showed expression of GFP in the α and β, but not the γ structures. Furthermore the study design is not identical as Joiner *et al* brought about changes by overexpressing *protein kinase A* (PKA). Therefore It is not definitive that the MBs are responsible for *day-GAL4* [6] arousal phenotype as the MBS have heterologous effects on sleep regulation and our cellular staining is not identical (Joiner *et al.*, 2006). Moreover the result with *day-GAL4* [6], *NaChBac* is interesting as not only is the sleep in darkness affected but also the morning peak was advanced by 30min. This could suggest that circadian clock neurons are being manipulated, the PDF neurons are known to be involved in gating of the morning peak and have been implicated in arousal (Renn *et al.*, 1999; Sheeba *et al.*, 2008a). Again analysing *day-GAL4* [6], UAS-GFP IHC data does not show obvious cell bodies in the accessory medulla (aMe) where the PDF expressing cells are located. The difficulty with analysing the data from the *day-GAL4* [6],UAS-GFP IHC is that a large number of cells/neuropil are present; to identify specific clock neurons requires double staining with clock protein antibodies. This was attempted using the CRY antibody, uncovering one *cry* positive LN_d cell but unfortunately the GFP antibody staining was not as effective in this experiment and

thus this may be why no other clock neurons have been identified. Work with this driver could be repeated with either a TIM or PER antibody (mouse based antibody that would allow GFP rabbit to be re-used) that would highlight all clock cells and identify clock cells overlapping with *day-GAL4* [6] expression, possibly helping to explain this arousal phenotype. The absence of any other circadian phenotypes such as period changes when expressing the *CLK*Δ and *NaChBac* constructs, suggests that any potential overlapping clock neurons are unimportant for controlling the period in the conditions measured. The PDF cells are not the only clock cells implicated in arousal; work on the potassium channel *Shaw* showed night time arousal phenotypes, when it was overexpressed in clock cells. Overexpressing Shaw is thought to reduce the spontaneous firing of action potentials and when this was restricted to only the dorsal clock neurons this arousal phenotype continued (Hodge & Stanewsky, 2008). It is difficult to understand at this stage which GAL4 expressing cells are causing the arousal phenotype observed using *day-GAL4* [6]. A possible way to identify these arousal cells could be to use the smaller lengths of *day-GAL4*. Activity data suggested that two of the 3 kb *day-GAL4* lines did not give this arousal phenotype. If an IHC experiments were performed on these fly lines it could allow for comparisons to be made the cell groups expressing the differing lengths of GAL4, this may highlight the cells responsible for arousal.

Although this arousal phenotype is interesting it does not give much direct data on what DAY does and its relationship with CRY. The IHC analysis on *day-GAL4* [6] lines was supposed to give information on *day* expression, however, taking the IHC data without comparing it to another independent method of identifying *day* expressing

cells would be dangerous. The cloned upstream *day* sequences may carry enhancers for other genes as well as GAL4 expression from neighbouring genes located around the P element insertion of the GAL4 line. Verifying GAL4 staining and ensuring that it is representative of endogenous gene expression is important and difficult. The 5.5 kb promoter sequence of cry-GAL4 was found to be expressed in all of the LN neurons, however, more recent analysis with a more sensitive CRY antibody showed similar expression but only half of the LN_ds were stained; although this antibody did stain cells in a cry^{out} background (Yoshii *et al.*, 2008; Emery *et al.*, 2000b).

Fortunately two MiMIC insertions were reported in distinct regions of the *day* locus that could give more data on the location of *day* expressing cells (Venken *et al.*, 2011). The two insertions displayed very similar patterns of EGFP expression which is encouraging as there is ~ 24 kb between the insertions, suggesting that these lines are reliable reporters for *day* expression. Although there is no confirmation if the cells expressed in the MiMIC lines overlap with each other or if the cell expressed in both MiMIC lines is a LN_d cell, this would be of particular interest, as *day-GAL4* [6] expressing in two cells in a similar location and using the CRY antibody one was confirmed as an LN_d. The other potential LN_d expressed by *day-GAL4* [6] was not seen in the CRY antibody overlap (possibly due to the ineffectiveness of the antibody), so determining the identity of this cell would also be very interesting as there are three *cry* positive LN_ds (Yoshii *et al.*, 2008). Could *day-GAL4* be expressed in two of them? The LN_ds have been characterised and aside from a heterologous expression of CRY they also have differing projections patterns (Yoshii *et al.*, 2008) .

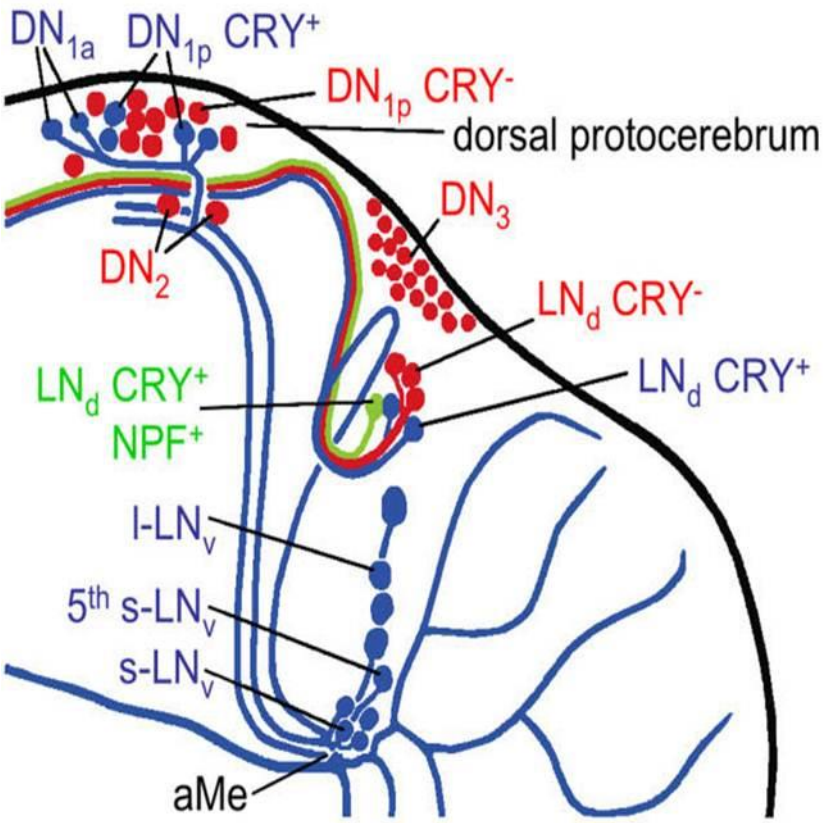


Figure 7-32: Diagrammatic depiction of the clock cells and their projections. Taken from Yoshii *et al.*, (2008).

Using the 7-33 figure above it was possible to try to identify which LN_d expressed *day-GAL4* [6] due to the presence of CRY and its projection pattern, as *day-GAL4* [6] never sends projections to the aMe, projecting only to the dorsal protocerebrum. The sexual dimorphic expression of *day-GAL4* [6], whereby females have different *day-GAL4* [6] expression, is particularly interesting especially as one of the LN_d is positive for NPF which is also known to be expressed differently between males and females (Yoshii *et al.*, 2008; Lee *et al.*, 2006). The finding that *day-GAL4* [6] is not expressed at the same intensity or within the same cells, between males and females could also be tested

using the MiMIC lines. Whilst performing an IHC with the NPF antibody and *day-GAL4* [6] as well as the MiMIC lines, with males, would in confirm which LN_d GAL4 is expressed and confirm that MiMIC staining is present in an LN_d cell.

The DAY antibody is another tool that could identify cells, however, unfortunately the DAY antibody staining is particularly faint and only highlights the antennal lobes, but this staining is specific as day^{00} did not show the same pattern. When *day-GAL4* [6] expresses a GFP (ACTIN-GFP) that labels axons and termini a similar pattern of expression is seen, indicating possible overlap between GAL4 and antibody. Due to the presence of antennal nerve structures the antennae was examined in *day-GAL4*,UAS-GFP flies. These flies gave cell body staining in the Johnston organ which is known to control olfaction, gravity sensing and hearing, (reviewed in Eberl, 1999; Leinwand & Chalasani, 2011; Beckingham *et al.*, 2005). Of these geotaxis is particularly interesting as *cry* is involved in this process and is also expressed in the antennae, although there does not appear to be clear overlap between the two staining patterns (Toma *et al.*, 2002). Moreover *cry* has also been shown to be important for olfaction rhythmicity, where the antenna shows circadian variation in detecting stimuli (Tanoue *et al.*, 2004). Therefore the results of antibody staining combined with *day-GAL4* [6] analysis have identified potential phenotypes to investigate validating our approach.

The IHC results may have helped to confirm a function for *day* by highlighting sexually dimorphic staining and confirming the presence of *day-GAL4* [6] within a clock cell. These results have been built on in the following chapters where geotaxis, courtship and more locomotor activity analysis was carried out with the aim of

establishing a role for *day*. The recent discovery of the MiMIC lines is also a powerful tool for undertaking molecular experimentation on *day,* as a whole host of genes can be readily transferred into the site through recombination with attP and attB sites (Groth *et al.*, 2004; Baer & Bode, 2001). One possible strategy may be to insert GAL4 at these loci. These smaller subsets of cells, seen in the MiMIC IHC, can be made to overexpress *NaChBac* or *CLK*Δ, which may yield more specific data on the possible function of *day* expressing cells.

8 Behavioural and courtship examination

8.1 Introduction

This chapter attempts to address the possible functional consequences of DAY-CRY binding, as well as identify phenotypes produced by absence of the *day* gene. In order to address these concerns the following experiments have been undertaken.

- Geotaxis assay
- Courting of male and female day^0 flies.
- Further locomotor experiments with day-GAL4 [6].
- Constant dim light analysis.
- Locomotor response to light pulses.

Armed with data from these experiments, it should be possible to shed light on the function of *day*.

8.2 Methods

8.2.1 Geotaxis

Geotaxic assays were performed by collecting newly eclosed male in groups of 10 and raising them in LD for 4 days. After 4 days flies are tapped without anaesthesia into a double ended vial (vials fixed together with tape) which has been marked 8cm from the bottom. Flies were allowed to acclimatise to their surroundings for 1 minute

before the vials are tapped down firmly, leaving all the flies at the bottom of the tube. The number of flies that cross the 8cm mark after 10 seconds is recorded. This was done three times for each genotype with a 1 minute recovery period after tapping and a number of biological replicates were used. Experiments were carried out in red light to avoid a phototaxic response. Data was collected as a percentage of flies that climbed and comparisons were made using a Student's t test.

8.2.2 Courting

To implement a single choice courting assay virgin males and females were collected upon eclosion and housed in individual vials for 4 days at 25°C LD 12:12. Virgin females of the required genotype were selected first and introduced into a Perspex box (5mm height, 10mm diameter), followed by a male, without anaesthesia. The following parameters were recorded:

- Time taken to initiate courting behaviour.
- Time female accepts male.
- Copulation end.

If copulation had not occurred within a 20 minute time period, then the couple were discarded. All experiments were carried out at 25°C between ZT 0- ZT 3 hours, with all genotypes backcrossed six times to the w^{1118} background.

8.2.3 Dim light activity

Dim light locomotor activity experiments were carried in the same fashion as outlined in Materials and Methods, however, there were the following modifications to the method: 2 tubes containing LED blue lights was placed into the incubator one as a source of dim light, the other was set at a higher intensity to entrain the flies (>>0.400 μV/cm^2). Only 1 shelf in the incubator (top) was used so that the distance between the light source(s) and the activity monitors was constant. The LED tubes were attached to a power pack and plugged into a timer which produced 12:12 bright blue light for 2 days followed by only constant dim blue light of ~0.400 μV/cm^2, this intensity was used as it had previously been shown in our laboratory to be dim enough to allow wild type flies to maintain rhythmicity (G.Fedele, un-published data). All light recordings were made with a photometer and experiments were carried out at 20°C. After 2 days of LD using the brighter LEDs, constant dim light was set for 6 days, data was analysed using the activity analysis protocol detailed previously.

8.2.4 Phase shift experiments

Flies (aged 3-7 days) were maintained under LD 12:12 for 3 days at 25°C. On the final night of LD a 20 min pulse of light was administered at either ZT 15 or ZT 21, after which they were maintained in DD for 1 week. Control flies (same genotype) were subjected to the same conditions bar the light pulse. On completion of the experiment data was compiled in 30 min bins and the third day of DD was selected for phase analysis. Focussing on the evening bout of activity, the reference point for phase was chosen (per individual fly), as the bin whose activity was equal or lower than 50% of

the peak. The average for all flies from the same genotype and conditions was considered the average phase. By comparing individual pulsed flies with the average phase of control flies it was possible to determine the average phase shift and its variance.

8.2.5 Fly genotypes

Below are the main fly stocks used predominately in this chapter.

Genotype	Description
***w*;;UAS-*day*3m**	Line expressing cDNA of *day* backcrossed to *white*
w*;;*cry-GAL80	The yeast gene GAL80 prevents GAL4 from UAS activation of transcription. GAL80 expression is dictated by upstream promoter sequences, in this instance the *cry* promoter (Stoleru *et al.*, 2004).
cry^{02}	A null allele of the *cry* gene, generated through homologous recombination (Dolezelova *et al.*, 2007)
***w*;UAS-*cry*24B**	*cry* overexpression line, created with the cDNA of *cry* cloned into p-UAST vector.
***day-GAL4* F1 [6];;**	One line of the 6 kb upstream sequence of *day* used predominately throughout this chapter.

Table 8-1: Fly stocks utilised.

In some instances these lines are in either a cry^0 or day^0 background, achieved through crossing flies using balanced stocks referred to in Materials and Methods.

8.3 Results

8.3.1 Geotaxis assay

The presence of *day-GAL4* [6] staining in the antennae led us to question if there was a role for *day* in geotaxis. Geotaxis assays were performed on day^{0P} flies and the CS/+ genotype in darkness, however, there was no significant difference seen between the two groups (Student's t test. P = 0.14). With day^{0P} showing 78% of flies climbing and CS/+ was 85%, N =30 for both genotypes. Thus *day* seems to have no regulation over this behaviour.

8.3.2 Courtship analysis of day^{0} males and females

Based on the findings of chapter 7 it would appear that there is evidence of cellular overlap of *day* and *cry* in the LN_ds. The fact that one of the LN_d cells is known to be sexually dimorphic (Lee *et al.*, 2006) and that no staining is present in female *day-GAL4* [6] brains may hint at a possible role for *day* in courtship behaviour.

The latency of male flies to display courtship behaviour once introduced to virgin females, and the length of the copulation episode (TIC) was measured for day^{0} and control flies. The latency for males once introduced to begin courting behaviour (time until mating) was not significantly different from CS/+ and day^{0P} flies, un-paired Student's t-test (P = 0.4363).

Males lacking *day* exhibited a significant reduction in TIC compared to all of the tested genotypes, see figure 8-1. However, the day^{0} loci carry the $white^{+}$ gene driven by the HSP70 promoter, which is ubiquitously expressed. w$hite^{+}$ is known to influence courting behaviour (Zhang & Odenwald, 1995), the UAS-*day*3m line also carries the

white^{+} gene and serves as a control having been backcrossed to w^{1118} six times. Therefore to rule out any influence of *white*$^{+}$ on this behaviour, the TIC of UAS-*day*3m males was recorded in the exact same manner as day^{0} flies. The UAS-*day*3m line showed a significant increase in TIC compared to all of the lines tested, see figure 8-1, suggesting that *white*$^{+}$ could increases TIC, contrasting with what we see with day^{0}. The controls did not vary significantly from each other, despite CS/+ carrying the *white* gene expressed under its endogenous promoter. It could be conceived that the effect seen with UAS-*day*3m is either a positional effect of the P element or a result of UAS leakage and that potentially overexpressing *day* could result in extended TIC times.

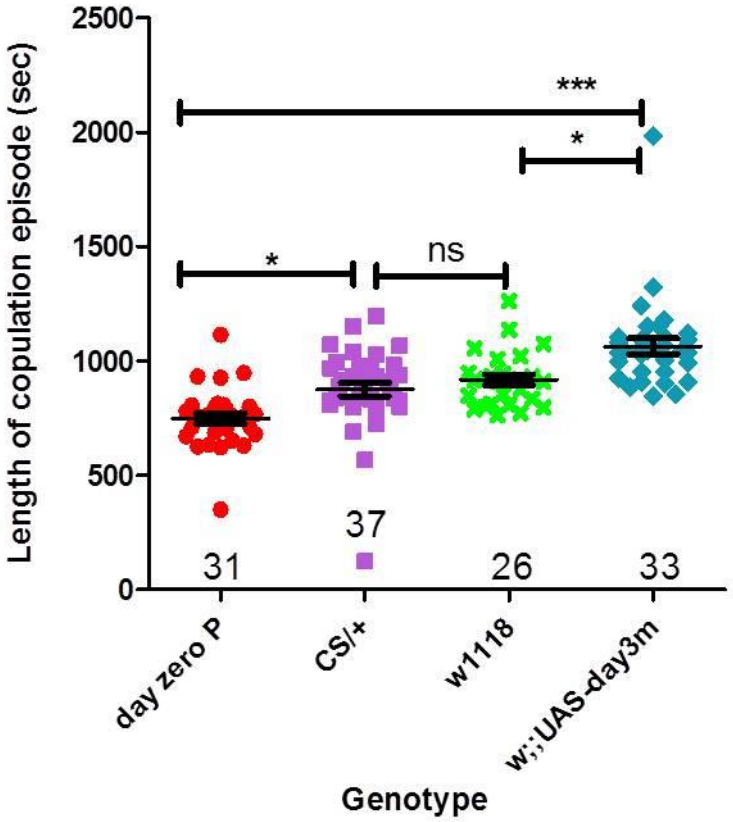


Figure 8-1: Plot of mean TIC with SEM of males with wild-type females.

Males are paired with wild type CS/+ females, after being raised in LD conditions after 4 days, black bars highlight the mean of the length of a copulation episode. N value shown below genotype. One way ANOVA ($F_{3,123}$=20.74, P= <0.0001: Bonferroni post hoc: *day*^{0P} vs CS/+ P = 0.05, *day*^{0P} vs *w*^{1118} P= 0.001, *day*^{0P} vs *w*;;UAS-day3m P = 0.001, *w*;;UAS-DAY3m vs *w*^{1118} P = 0.01, *w*;;UAS-DAY3m vs CS/+ P = 0.001, CS/+ vs *w*^{1118} P = ns).

To examine if expressing *day* leads to increased TIC length or if there is a position effect in UAS-*day*3m the following data was obtained from a colleague (Dr C Hansen).

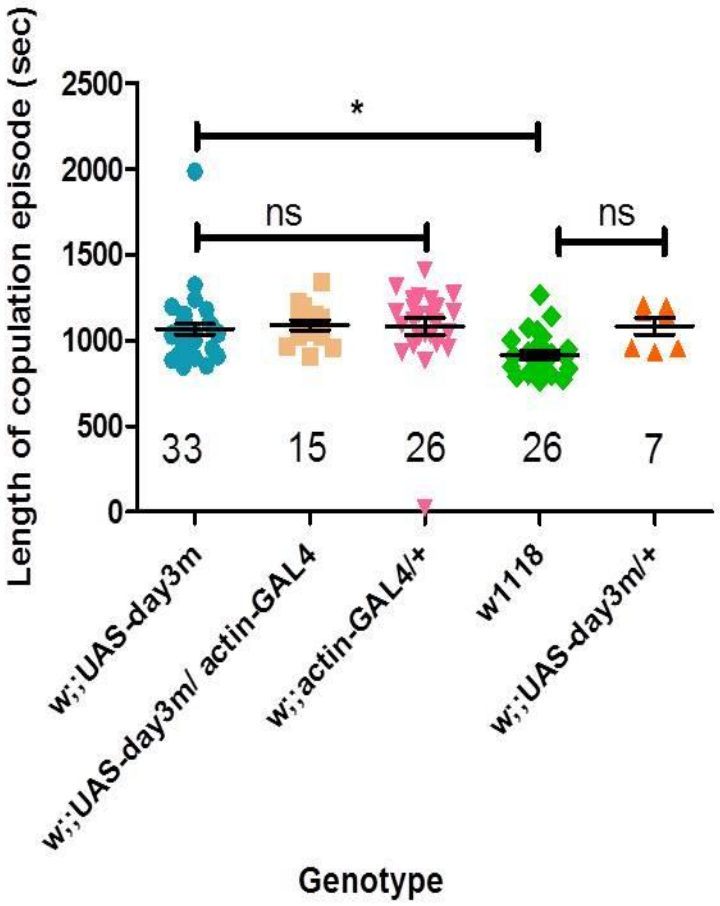


Figure 8-2: TIC experiments performed with UAS-DAY3m crossed to *w*;;actin-GAL4 and their controls.

Experiments were undertaken in the same fashion as the experiment described above. Data for the *w*;;UAS-day3m line and w^{1118} have been recorded by myself, whilst the expression of *w*;;UAS-day3m was performed by Dr C.Hansen. Black bars indicate the mean and SEM, N values shown below genotype. One way ANOVA ($F_{4,104}$ = 3.809, P = 0.0062; Bonferroni post hoc: *w*;;UAS-day3m vs *w*;;UAS-day3m/actin-GAL4 P = ns, *w*;;UAS-day3m vs *w*;;UAS-day3m/+ P= ns, *w*;;UAS-day3m vs *w*;;actin-GAL4/+ P = ns, w^{1118} vs *w*;;UAS-day3m/actin-GAL4/+ P = 0.05, w^{1118} vs *w*;;UAS-day3m/+ P = ns, w^{1118} vs actin-GAL4/+ P = 0.05).

Figure 8-2 shows what occurs when *day* is over-expressed using the *actin*-GAL4 driver (ubiquitous expression), all lines used were in a w^{1118} background. Increasing *day*

levels with *actin*-GAL4 does not increase the TIC significantly compared to its controls, while these lines are significantly increased for TIC when compared to w^{1118}, except for *w*;;UAS-day3m/+, see figure 8-2. This data highlights how overexpression of *day* has no effect on copulation length; however, three of these $white^{+}$ lines are significantly longer for TIC compared to w^{1118} suggesting that the $white^{+}$ may be causing increased TIC.

8.3.3 Female day^{0} courtship behaviour

Females of the day^{0} phenotype were also subjected to courtship analysis. The main parameter analysed was the willingness of virgin females to accept their partner (time between the start of courtship to the beginning of copulation).

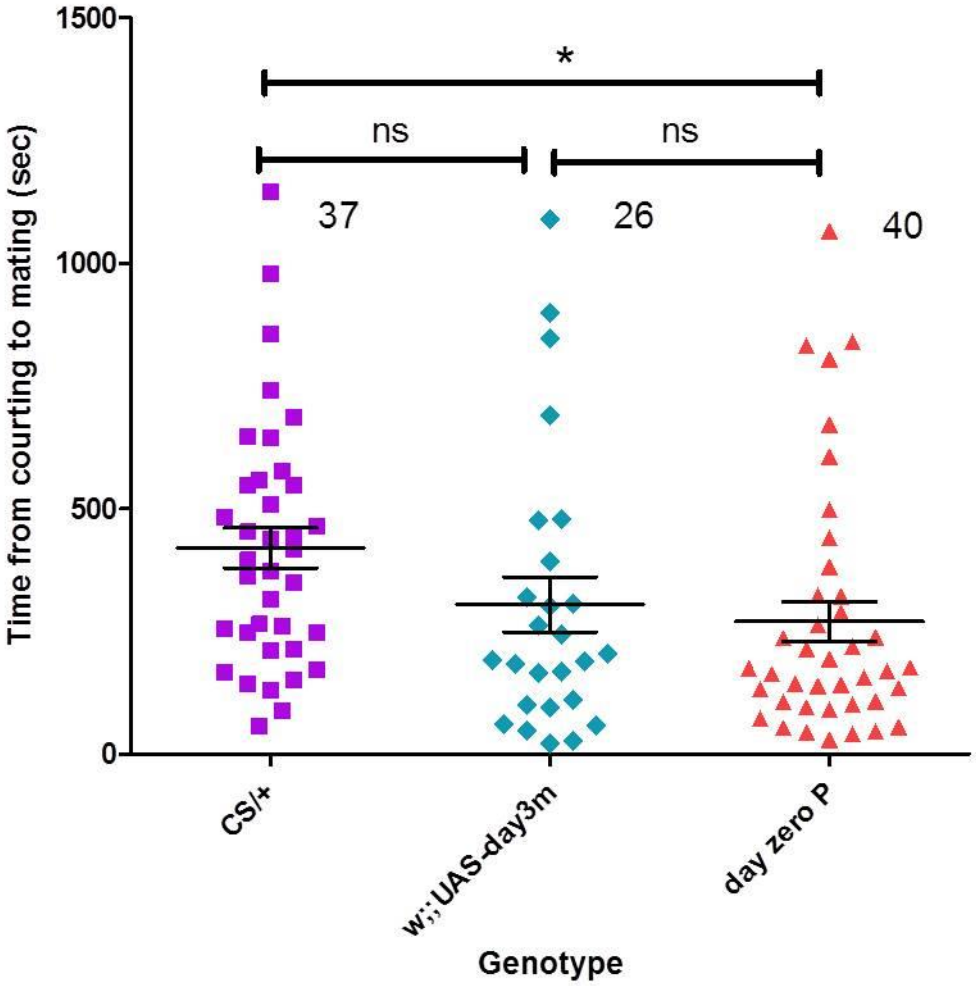


Figure 8-3: Receptivity of females to courting from CS/+ males.

Receptivity measured by recording the time measured from the beginning of courting to the acceptance of the male by the female, mean of acceptance time shown with SEM as black bars. Numbers to the right of genotype data represent the N value. One way ANOVA $F_{2,100}$ = 3.349, P= 0.0391; Bonferroni post hoc: CS/+ vs day^{0P} P= 0.05, CS/+ vs *w*;;UAS-day3m P = ns, *w*;;UAS-day3m vs *day*^{0P} P = ns.

Significant differences were originally seen between CS/+ and *day*^{0} females, with *day*^{0P} females having a shorter courting episode before accepting the male. However, using the control line UAS-*day*3m gave no significant difference from *day*^{0P} and from CS/+.

8.3.4 Final activity experiments

The behavioural influence of cells expressing *day* and *cry* can be examined using *cry*-GAL80. GAL80 prevents GAL4 activating UAS sites; therefore using *cry-GAL80* (Stoleru *et al.*, 2004) will prevent *cry* expressing cells from expressing UAS elements (Ma & Ptashne, 1987). Repeating the same overexpression experiments from chapter 7, overexpressing *CLK*Δ and *NaChBac*, but in combination with *cry-GAL80* could give an indication of what day^{+} cry^{-} cell(s) may be responsible for.

Firstly *CLK*Δ was overexpressed using *day-GAL4* [6], *cry-GAL80* and the profile of LD locomotor activity was recorded. Originally expressing *CLK*Δ with *day-GAL4* [6] resulted in a reduction in the sleep in darkness, therefore by introducing a *cry-GAL80* element to this cross do we still see the same phenotype? The *day-GAL4* [6],UAS-*CLK*Δ,*cry-GAL80* appears to show the same amount of sleep in darkness as *day-GAL4* [6],UAS-*CLK*Δ, shown below.

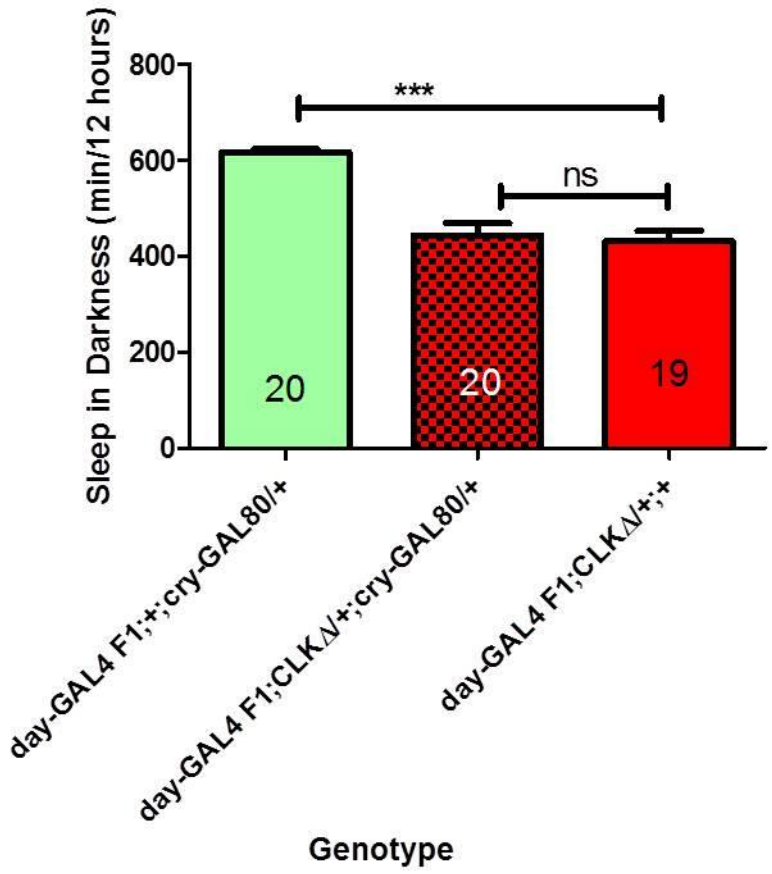


Figure 8-4: The mean of sleep in darkness of genotypes expressing UAS-*CLK*Δ with day-GAL4 /*cry-GAL80* or without *cry-GAL80*.

Red checked column represents *day-GAL4* F1 [6], UAS-*CLK*Δ, *cry-GAL80*, green column is the GAL4, *cry-GAL80* control and solid red column is day-GAL4 F1 [6] UAS-*CLK*Δ. Performed in LD conditions at 25°C, numbers within columns represent N values. One way ANOVA $F_{2,56}$ = 28.21, P= <0.0001; Bonferroni post hoc: *day-GAL4* F1 [6];UAS-*CLK*Δ/+;*cry-GAL80*/+ vs *day-GAL4* F1 [6];+;*cry-GAL80*/+ P = 0.01, *day-GAL4* F1 [6];UAS-*CLK*Δ/+;*cry-GAL80*/+ vs *day-GAL4* F1 [6];UAS-*CLK*Δ;+ P = ns)

At present one of the controls has not been run (*w*;UAS-*CLK*Δ/+;cry-GAL80/+) due to difficulties rearing the stock, however, expressing UAS-*CLK*Δ with *day*-GAL4 cry-GAL80 gave a significant difference from the GAL4 control but not from *day*-GAL4,UAS-*CLK*Δ, see figure 8-4. This suggests that the *cry-GAL80* transgene has no significant influence

on controlling the sleep disruption caused by UAS-*CLK*Δ, but awaits confirmation due to the absence of 1 control.

To completely rule out the influence of *cry*, performing the same experiment in a cry^{02} background would also be informative. As *cry-GAL80* only prevents cells from expressing the UAS element, by removing *cry* you can examine if it is necessary for this arousal/sleep disruption phenotype making a distinction between the cells that express *cry* and the protein.

Expressing UAS-*CLK*Δ in a cry^{02} background gives an unclear finding as some night episodes appear to be severely disrupted whilst others are not, see figure 8-5. There was a high degree of lethality giving a small N and high standard error. Repeating this experiment would be informative, viewing the profile (shown below) that the activity in darkness is erratic with some dark episodes showing greater levels of activity in the mutant lines, but this does not appear to be as consistent as *day-GAL4* [6], UAS-*CLK*Δ. Analysing each of the dark episodes may give varying degrees of significance; this result could indicate that *cry* is involved in regulating this behaviour but it may need to be repeated to improve its robustness.

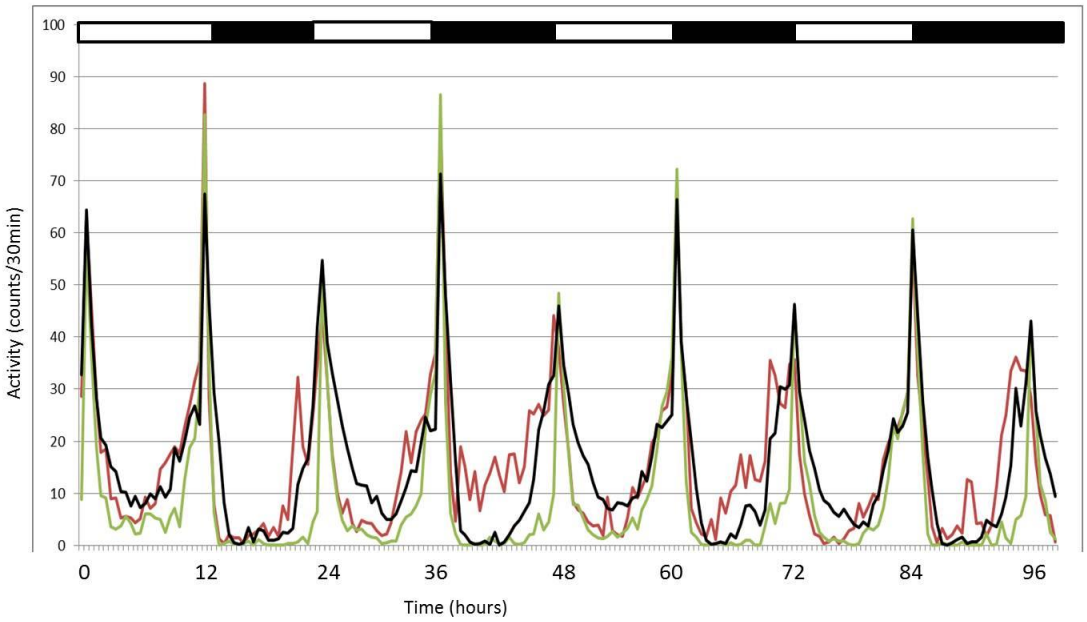


Figure 8-5: LD profile of locomotor activity of *day-GAL4* [6], UAS-*CLK* in a *cry*^{02} background.

Red line is *day-GAL4* [6] F1, UAS-*CLK cry*^{02}, N= 11, green is *day-GAL4* F1 [6] *cry*^{02} control, N=20 while black is UAS-*CLK*Δ *cry*^{02} control, N= 19. Yellow and black bars indicate lights on and off respectively.

Originally *day*-GAL4, *NaChBac* showed a reduction in night time sleep and a peak prior to lights on, *day*-GAL4, *NaChBac* was analysed with *cry-GAL80* to see if this trend continued.

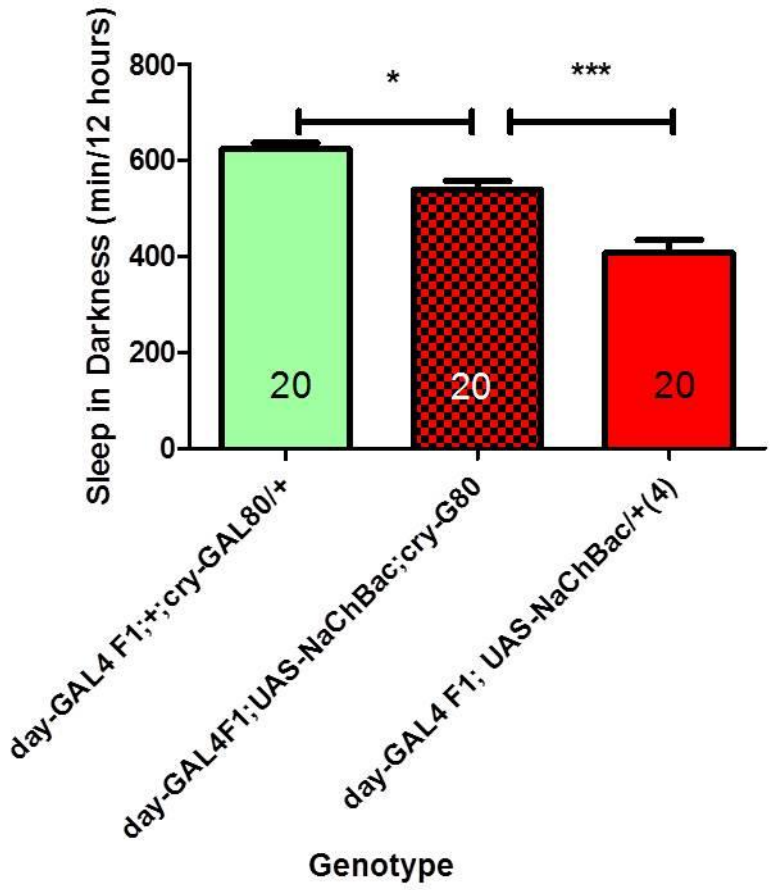


Figure 8-6: Mean sleep in darkness of *day-GAL4* F1 [6] flies expressing *NaChBac* with *cry-GAL80*.

Red and black checked column is the *day-GAL4* F1 [6] driver expressing *NaChBac* with *cry-GAL80*, solid red column is overexpression of *NaChBac* only with *day-GAL4* F1 [6] and the GAL4 control is shown in green. Performed in LD conditions at 25°C, N values shown within columns. One way ANOVA ($F_{2,57}$ = 27.45, P = <0.0001).

Again the UAS control is missing (*w*;UAS-*CLKΔ*/+;*cry-GAL80*/+); comparison of *day*-GAL4 *NaChBac cry*-GAL80 to the GAL4 control shows a significant decrease in sleep ($F_{2,57}$ = 27.45, P = <0.0001), although this reduction is not as severe as previously seen

in when expressing *NaChBac* without *cry*-GAL80. The profile was also examined to check for the presence of an early morning peak in activity.

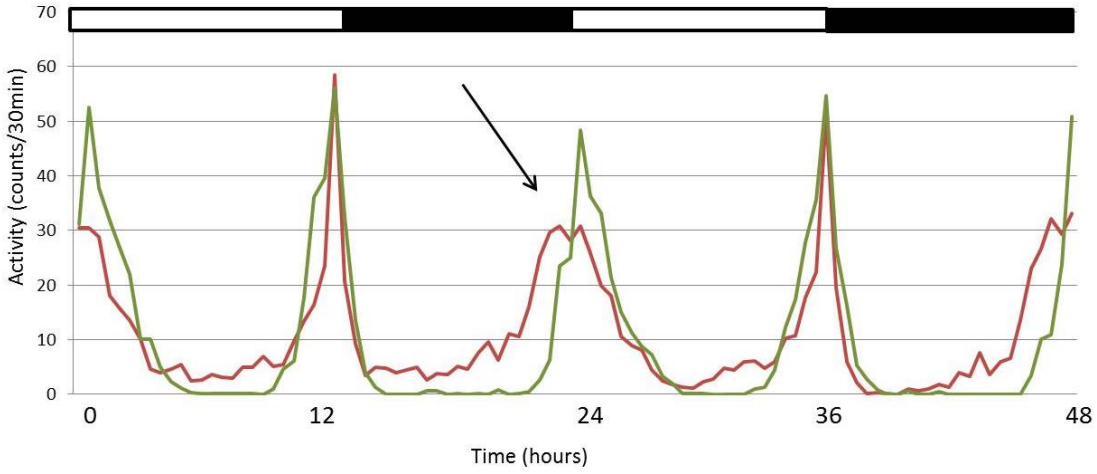


Figure 8-7: LD profile of *day*-GAL4 F1 [6] *NaChBac* at 25°C.

Red line: *day*-GAL4 F1 [6];UAS-*NaChBac* (4)/+;*cry*-GAL80/+, N=20, Green: *day*-GAL4 F1 [6];+;*cry*-GAL80/+, N=20.Yellow bar lights on, black lights off. Black arrow indicates the presence of the morning peak.

The profile above shows the mutant in red and the GAL4 control in green. The black arrow indicates a peak prior to lights on in the mutant line. This behaviour is similar to what is seen without using *cry-GAL80* in *day*-GAL4 F1 [6], UAS-*NaChBac*, the two are compared below.

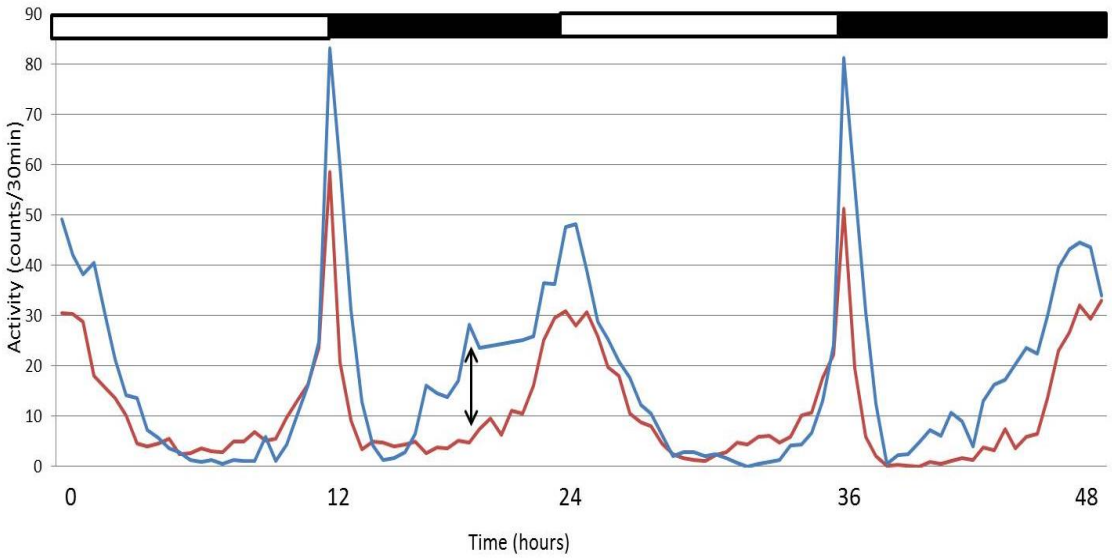


Figure 8-8: Profile differences between *day-GAL4* F1 [6] expressing *NaChBac* with *cry-GAL80* compared to the same mutants lacking *cry-GAL80*.

Red line: *day*-GAL4 F1 [6]; UAS-*NaChBac* (4)/+;*cry-GAL80*/+, N=20 blue: *day*-GAL4 F1 [6]; UAS-*NaChBac* (4)/+ N=20. Performed in LD conditions at 25°C. Black arrow highlights differences in activity between the two mutants.

The peak in morning activity is the same between the two lines, but there are differences in the levels of activity between the two lines in darkness highlighted by the black arrow, these were shown to be significant (Figure 8-6; Bonferroni post hoc: day-GAL4 F1 [6]; UAS-NaChBac (4)/+ vs day-GAL4 F1;UAS-*NaChBac* (4)/+; *cry*-GAL80/+ P = 0.001).

8.3.5 Overexpression of *cry* with *day*-GAL4

The manipulations performed with UAS-*CLK*Δ and *NaChBac* are useful in determining what potential function(s) the cells expressing *day*-GAL4 [6] have, yet

they fail to directly address the possible functional relationship of DAY-CRY binding. To address this point overexpression of *cry* (*w*;UAS-*cry*24B;+) was undertaken using *day*-GAL4 [6] in *day* positive and negative backgrounds. Knowing that *day-GAL4* [6] and *cry* expression overlap; overexpressing *cry* in day^{+} and day^{0} could reveal differences.

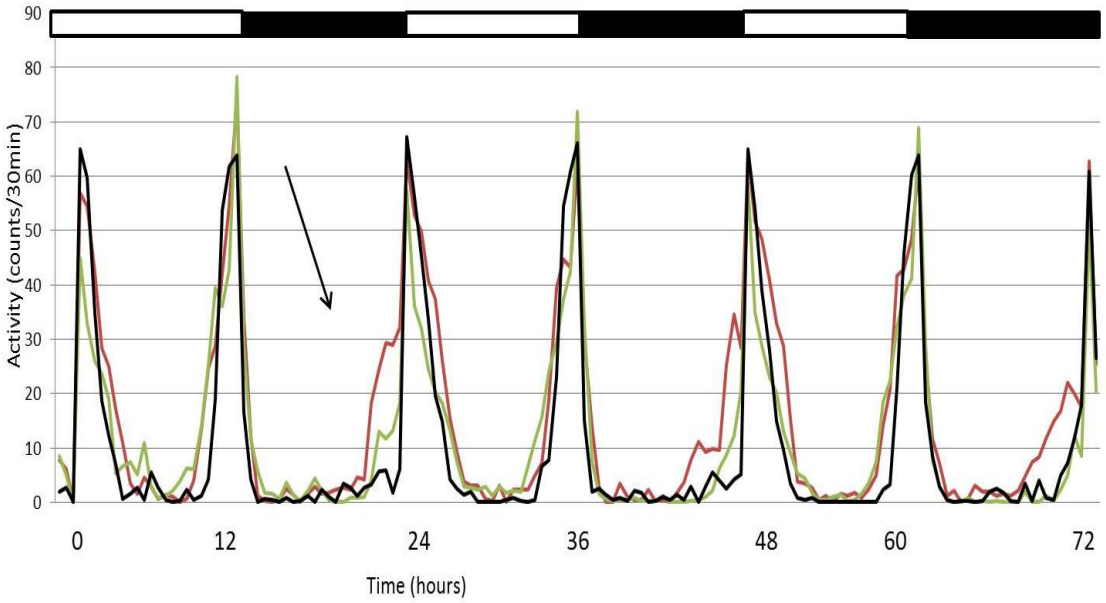


Figure 8-9: LD profile of flies overexpressing cry with *day-GAL4* [6] cells and controls.

Red line= *day-GAL4* F1 [6], UAS-*cry24B* N=20, black = UAS-*cry24B* N=20, green = *day-GAL4* F1 [6] N=20. Horizontal white and black bars indicate lights on and off respectively, performed at 25°C.

The black arrow in figure 8-9 highlights the only difference in the three profiles; the mutants show increased anticipation of the morning peak. This phenotype is similar to *NaChBac* overexpression, although not as severe. This rise in activity shown by overexpressing *cry* is more likely to anticipation rather than sleep disruption, as sleep

analysis shows that *cry* overexpression is not significantly different from both controls when the complete sleep in darkness of 12 hours is tested ($F_{2,59}$ = 4.273, P = 0.0187; Bonferroni post hoc: *day-GAL4* F1 [6];UAS-cry24B/+ vs *day-GAL4* F1 [6];; P = ns, *day-GAL4* F1 [6];UAS-cry24B/+ vs *w*;UAS-cry24B/+ P = 0.05). The consequence of overexpressing *cry* with *day-GAL4* [6] appears to be causing a more subtle night time effect than either *CLK*Δ or *NaChBac* and there seems to be no change in the light profile of overexpressing *cry*.

The same experiment performed above was repeated but this time in a *day*^{0} background.

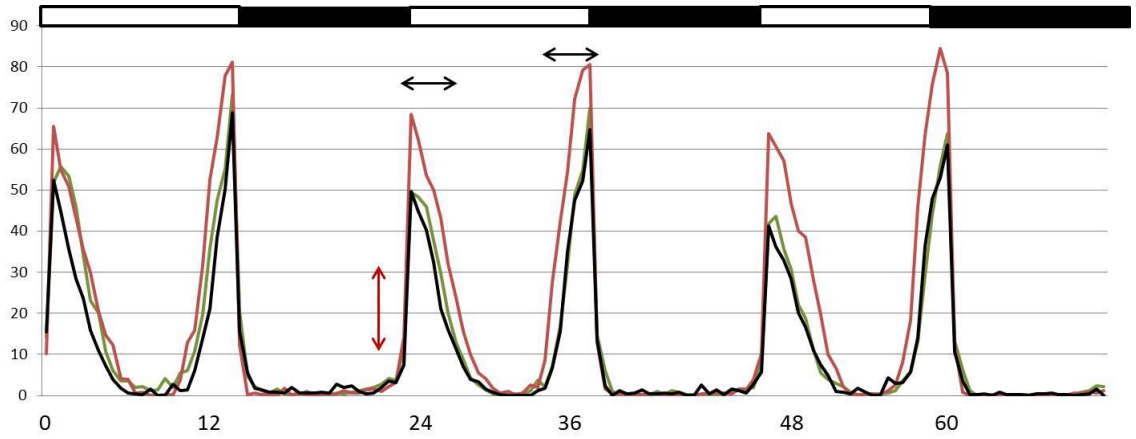


Figure 8-10: LD profile of flies overexpressing *cry* in *day-GAL4* F1 [6] cells in a *day*^{0P} background and their controls.

Red line *day-GAL4* F1 [6], UAS-*cry24B day*^{0P} N=40, black = UAS-*cry24B day*^{0P} N= 36, green = *day-GAL4* F1 [6] *day*^{0P} N=40. Horizontal white lights on and black bars indicate lights off , performed at 25°C.

There are a number of features to this LD profile (figure 8-10), firstly the anticipatory behaviour seen before when overexpressing *cry* is no longer present (red arrow), and secondly the activity of the morning and evening peak is increased in both levels and duration (black arrows), this appears to become more severe as the experiment continues. This was tested by averaging the individual activity of each fly for the first 3 hours of the morning on day 1 and day 3. These were then tested for significance to determine if the activity within the first 3 hours of the morning varied significantly. On the first day of LD there was no difference between the mutant line and control, although it was close to significance ($F_{2,111}$ = 2.393, P= 0.0960). This was not the case on day 3 as the lines overexpressing *cry* in a day^{0} background showed significantly greater amounts of activity in the morning ($F_{2,111}$ = 9.776, P= 0.0001; Bonferroni post hoc: *day-GAL4* F1 [6];UAS-*cry24B*/+;day^{0P} vs *day-GAL4* F1 [6];;day^{0P} P = 0.01, *day-GAL4* F1 [6];UAS-*cry24B*/+;day^{0P} vs *w*;UAS-*cry24B*/+;day^{0P} P = 0.001). Measuring the last three hours of the light episode (ZT9-12) of day 3 also showed significant differences in the mean of activity between the mutant lines and controls ($F_{2,111}$ = 20.50, P= <0.0001), but the activity on day 1 was also significantly elevated in the late day ($F_{2,111}$ = 8.976, P= 0.0002). The DD data of this experiment has not been analysed as the *day-GAL4* F1 [6] line crossed to *w;;P* showed a period increase suggesting that this line has recently been contaminated in some way as in all other activity experiments this GAL4 line gave wild type periods as shown in chapter 7. The LD data has been analysed as the *day-GAL4* F1 control is behaving the same as the UAS-control, however, repeating this experiment with a fresh *day-GAL4* [6] line is essential, the *day-GAL4* F1 [6], UAS-*cry24B* experiments were clean and it is only the day^{0} *cry* overexpressing experiments that are affected.

8.3.6 Dim light

So far all experiments have been carried out in standard light conditions, which when applied constantly, causes arrhythmicity. However, using lower intensities of light renders flies rhythmic but with an increased period. Dim light experiments were carried out on *day*^{0} flies at 20°C instead of the standard 25°C used so far. Flies live longer at 20°C and the dim light protocol used was originally obtained from a colleague (G.Fedele), who studied dim light in this fashion and who required long running activity experiments.

Dim blue light	**N**		**DD**	**N**
Genotype	**Rhythmicity (%)**		**Genotype**	**Rhythmicity (%)**	
CS/+	**70%**	**20**	**CS/+**	**68%**	**20**
***day*^{00}**	**17%**	**20**	***day*^{00}**	**74%**	**20**
***w*^{1118}**	**5%**	**20**	*w*^{1118}	**59%**	**20**

Table 8-2: Table displaying the % of rhythmic flies in DD and dim light conditions at 20°C.

Immersing *day*^{0} flies in constant blue dim light gave an increased level arrhythmicity when compared to CS/+ control genotype. This was tested using a Fisher's two tailed exact test and gave significance (P=0.0298). Only one line of *day*^{0} has been tested thus far and repeating this experiment with both lines may lend more weight to the findings of this experiment. This data suggests that *day*^{0} flies are more sensitive to light than controls. The *w*^{1118} genotype is also more sensitive to light when compared to CS/+ (P=0.0260). This is likely to be a consequence of lacking eye pigment, as all lines are in the same background.

8.3.7 Light Pulse

Following on from the dim light experiment, this could suggest an increased sensitivity of light. To test this hypothesis light pulse experiments were carried out at ZT 15 and 21, using white light for 20mins at 25°C.

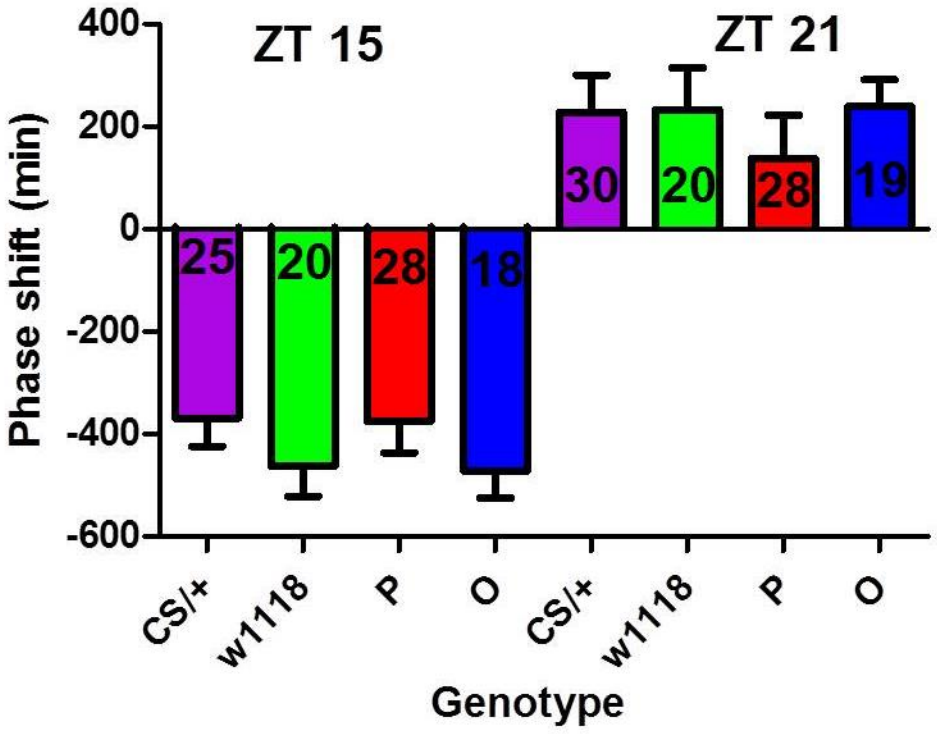


Figure 8-11: Light pulse analysis of *day*^{0} lines versus controls.

Size of the shift achieved shown in minutes. Timing of pulse is stated above the columns, numbers inside columns represent sample N.

The delays and advances seen are not significant different from the controls for both the lines of *day*^{0} and between *day*^{0} genotypes, (delay: $F_{3,87}$ = 0.8456, P = 0.4726, advance: $F_{3,94}$ = 0.4078, P =0.7477) . This data seems to suggest that *day* is not involved in controlling the advance or delay behaviour of the circadian regulated phase shifting response.

8.4 Discussion

This chapter has sought to build on the results of the previous chapters and establish a role for *day* in a behavioural output. One finding was the presence of *day-GAL4* staining in the antennae; knowing that *cry* is expressed there and that cry^{b} fly's display flies abnormal climbing behaviour, we sought to examine if day^{0} behaved differently (Toma *et al.*, 2002). There was no significant difference with day^{0} flies but there may be a reason for this, as the method used by Toma *et al* was a geotaxis maze where flies make a series of up and down decisions. This difference in approach could be addressed to confirm that *day* really is not involved in geotaxis, although other studies have used the tapping based experiment design to analyse the antennae in geotaxis (Sun *et al.*, 2009), although in this study they used larger tubes and recorded in 15 cm in 15 secs instead of the 8cm in 10 secs used in this study.

The differences between males and females seen with *day-GAL4* [6] expression and the LN_d data hinted at a role for *day* in a gender specific output. Courtship is one such process and single choice courting experiments were undertaken with day^{0} flies. This gave significant results with male day^{0} flies displaying shorter TIC times than controls, however, day^{0} lines carry $white^{+}$ as a marker used for tracking. Unfortunately $white^{+}$ has consequences on courting behaviour as ectopically expressed $white^{+}$ caused a change in courting behaviour in male flies, who displayed homosexual behaviour (Zhang & Odenwald, 1995). This experiment did not look specifically at TIC, to try to address the role of $white^{+}$ we used UAS-*day3m* that carries two copies of $white^{+}$, this line did not give the same phenotype as day^{0} and show extended TIC compared to w^{1118}. The possibility that we are observing an effect of the P element insertion in the

UAS-*day3m* line seems unlikely as the *actin*-GAL4/+ genotype has the same TIC length, with both lines significantly increased compared to w^{1118}. If $white^{+}$ is involved in TIC regulation then it would be possible to remove it from the day^{0} locus using the CRE protein, as this gene is flanked by *lox* sites; re-analysing the courting of day^{0} flies would then separate the possible influences of day^{0} and $white^{+}$. Genes that are also responsible for TIC length include: *coi*, *fic*, *Ken and Barbie*, *fru*, *per* and *tim* (Beaver & Giebultowicz, 2004; Yamamoto *et al.*, 1997; Lee *et al.*, 2001). Although despite finding a number of TIC mutants the cells responsible for TIC regulation and genetic pathways are unclear; *coi*, *fic* and *ken and barbie* all reduce TIC similar to *day* reviewed in (Yamamoto *et al.*, 1997), whilst mutants for *fru*, *per* and *tim* extend it (Beaver & Giebultowicz, 2004; Lee *et al.*, 2001). The *per* and *tim* finding is particularly interesting as CRY is known to bind these proteins and could also bind DAY, however one important finding of this study was that central expression of PER could not rescue the TIC phenotype (Beaver & Giebultowicz, 2004). If there is a *day-cry-per-tim* pathway important for regulating TIC it may residue in cells outside of the brain. The *fru* and *fic* genes are important for development; furthermore overexpressing *cry* has been shown to accelerate the development cycle when flies are maintained in LL conditions versus DD, possibly highlighting a role for *cry* in development (Vieira *et al.*, 2012; Ryner *et al.*, 1996; Ito *et al.*, 1996; Grant *et al.*, 1992). Therefore examining *day* expression in tissues outside the brain during development may give information on TIC regulation. However, before experiments of this nature are attempted it would be wise to knock out the $white^{+}$ gene from *day* to ensure that it is directly involved in this process.

The female receptivity was also examined but gave a confusing picture as *day*^{0} flies were significantly more receptive than CS/+ but not the w;;UAS-*day3m* line. This could suggest that the *day*^{0} female effect is merely a consequence of *white*$^{+}$ expression; additional transgenic lines need to be tested to rule out a position effect caused by the P element insertion or again removing the *white*$^{+}$ gene from females and re-testing courting behaviour would be advisable.

Following on from the activity/IHC data shown previously (chapter 7) whereby expression of the *CLK*Δ and *NaChBac* constructs caused sleep disruption and a *cry* expressing LN_d overlapped with *day-GAL4* [6] expression, to dissect the influence of this cell on sleep disruption, the *cry-GAL80* construct was used (Stoleru *et al.*, 2004). When expressing *CLK*Δ no significant difference was seen between *day-GAL4* [6] lines with or without *cry-GAL80*, suggesting that the impact of *cry* expressing cells is limited with respect to this sleep phenotype we are observing. The notion that *cry* is not responsible for this phenotype was re-tested in a different manner by performing the cross in a *cry*^{02} background. The data from this experiment suggests that *cry* may be important as the sleep disruption was erratic, with some night episodes displaying greater sleep disruption than others. Although this result is not completely conclusive in establishing a role for *CLK*Δ mediated sleep disruption using *day-GAL4* [6], there is some similarities with the results of (Kumar *et al.*, 2012). They found that in the C*lk*^{jrk} background flies displayed high levels of nocturnal activity, which were reduced when *cry* was removed with the *cry*^{b} mutation. The results of our work and theirs are similar in that both highlight a *cry* mediated arousal process when C*lk* driven processes are altered negatively.

The results using *NaChBac* overexpression with *day-GAL4* [6],*cry-GAL80* is interesting as they show a slightly different result from the *CLK*Δ,*cry-GAL80* analysis. The expression of *NaChBac* with *cry-GAL80* gave significant reduction of sleep in darkness from the one control line tested; however, this sleep reduction was not as significant as lines without *cry-GAL80*, suggesting that *cry* expressing cells are important for the amplitude of this sleep disruption. This result is different from the *CLK*Δ,*cry-GAL80* experiment described above and may highlight a different mechanism of action between the processes controlling sleep disturbance when overexpressing *CLK*Δ vs *NaChBac*. Another phenotype caused by *NaChBac* expression was an advanced morning peak. This is still present in flies expressing *NaChBac,cry-GAL80*, hinting that *cry* expressing cells are not responsible for this behaviour.

Finally locomotor behaviour analysis of flies overexpressing *cry* with *day-GAL4* [6] were performed in a *day* positive and null background. It should be remembered that at this stage the *day-GAL4* [6] F1 line had been contaminated giving a longer period. As the GAL4 control behaved identically to the UAS only the LD data has been analysed. This data was particularly interesting as two phenotypes were observed, firstly that expressing *cry* with *day-GAL4* [6] caused anticipation of the morning peak; thus far all manipulations using *day-GAL4* [6] appear to have consequences during the dark phase of LD. This is not evidence for a direct functional role for *cry* in this behaviour as *day-GAL4* [6] will express *cry* ectopically in other cells; expressing *cry* in cells that were normally un-responsive to light caused them to become light sensitive (Fogle *et al.*, 2011). This experiment highlights how *cry* expression may cause effects in cells that do not normally express *cry*. However, what is particularly interesting about this

experiment is that when repeated in *day*^{0} this anticipation disappears, but the morning and evening activity peaks are elevated significantly, although for the morning peak this was not apparent on the first day of LD. This data links *cry* and *day* as it seems that *day* is necessary for the presence of *day-GAL4* [6] mediated anticipatory behaviour, as well as preventing high amplitude morning and evening activity during the L portion of an LD cycle. The fact that both the morning and evening peak is affected is interesting as the clock cells have been sub-divided into morning (LNvs) and evening (remaining clock cells) (Grima *et al.*, 2004; Stoleru *et al.*, 2004). Thus it is conceivable that *day-GAL4* [6] is expressing in two distinct clock cell groups or that this one cell can control both peaks. If the LD behavioural data is coupled the binding data discussed previously (in yeast DAY binds CRY in darkness and the co-IP DAY binds CRY in light), then it could be proposed that CRY and DAY bind in both light and dark conditions. Functionally the consequence of this binding in dark may facilitate a rise in anticipatory behaviour, whilst in light, DAY prevents excessive activity at the morning and evening peaks, suggesting that DAY could be acting as a repressor of locomotor activity in light conditions and as an activator in darkness. Evidence that supports this notion is seen in the constant dim light experiment, where one line of *day*^{0} flies displayed greater arrhythmicity, although it should be remembered that these two experiments were carried out at two different temperatures (25°C overexpressing *cry* and 20°C for dim light). It seems likely that CRY could be causing this dim light arrhythmicity, as it has an established role in light mediated behaviour (Emery *et al.*, 2000a; Stanewsky *et al.*, 1998; Emery *et al.*, 2000b).Theoretically there may be two ways of explaining this behaviour in *day*^{0} flies: 1) that DAY acts a repressor of CRY in light conditions and by removing *day,* CRY

mediated changes in neuron firing causes the arrhythmicity in dim light. 2) that *day*^{0} flies have elevated levels of CRY and consequently this causes the arrhythmicity; possibly through increased neuron firing, in the LN_d where DAY and CRY overlap (discussed later). This explanation assumes that the increased arrhythmicity seen in dim light conditions is caused by CRY. This needs to be shown and could be accomplished by using *cry* RNAi in a *day*^{0} background, then analysing the locomotor behaviour in dim light conditions, if these flies displayed a higher % of rhythmicity then it would suggest that CRY is responsible for this arrhythmicity. If this was accomplished then a test of the two hypotheses proposed previously could be evaluated by performing an IHC on *day*^{0} flies using the CRY antibody, comparing this to appropriate controls and looking for elevated levels of CRY during the morning and evening transition periods or in dim light. The only caveat with this approach is that the CRY antibody may not be sensitive enough in L conditions.

The arrhythmicity in dim light phenotype observed is interesting as (Rieger *et al.*, 2009) showed that the LN_ds are very important for behaviour in dim light and rescued the *per*^{0} phenotype by expressing *per* in only the LN_ds and fifth small s-LN_vs; this activity analysis was carried out at 20°C which is the same temperature as our dim light experiment. Therefore knowing that *day-GAL4* [6] expresses in one *cry* expressing LN_d and that the LN_ds are important for dim light behaviour (Rieger *et al.*, 2009), it would be a reasonable to assume that *day* is required in this particular sub-set of clock cells. Proving that the *day*^{0} cells responsible are the LN_ds could be achieved by rescue experiments for the dim light phenotype, using the Mai179-GAL4 PDF-GAl80 driver

combination overexpressing *day* in the LN_ds and fifth s-LN_vs (Grima *et al.*, 2004; Stoleru *et al.*, 2004).

The final experiment carried out was to analyse the ability of day^0 to respond to either a delay or advance light pulse. The data indicates no significant difference in either type of pulse in day^0 flies, which could be viewed as contradictory to the theory that day^0 flies are more sensitive to light. However, there are a number of possible explanations why day^0 flies may be more sensitive to light but don't show any differences in response to a light pulse. Firstly the light intensity used may be too strong, using a lower intensity of light may be a more effective way to elucidate differences in light sensitivity. This has already been shown in work where *cry* was overexpressed (Emery *et al.*, 2000a; Emery *et al.*, 1998; Emery *et al.*, 2000b), showing increased sensitivity to advance and delay pulses; depending on which clock cells were manipulated (Emery *et al.*, 1998; Emery *et al.*, 2000b). Also the impact of temperature may be important as dim light experiments were conducted at 20°C whilst light pulses were at 25°C. Again *cry* has been shown to be important for entrainment to temperature pulses (37°C) (Kaushik *et al.*, 2007) and cry^0 flies are thought to be less rhythmic at 20°C (Dolezelova *et al.*, 2007). However I have not directly examined the influence of temperature on day^0. Repeating these light pulse experiments with these factors considered may yield interesting data.

9 *Cry* mutants

9.1 Intro

Drosophila cry is a functionally divergent signalling protein involved in, but not limited to: light perception (Emery *et al.*, 1998; Stanewsky *et al.*, 1998), temperature entrainment (Kaushik *et al.*, 2007), magneto-sensitivity (Gegear *et al.*, 2008; Yoshii *et al.*, 2009) and sleep/wake states (Kumar *et al.*, 2012). In order to partake in all of these pathways it is likely that CRY interacts with different complements of protein complexes in cells that may control one or more of the pathways described above. Identifying proteins that bind CRY *in-vivo* has proved difficult; therefore the question was re-addressed: which amino acid residues are important for CRY's function? The C-terminus (CT) of *cry* has been shown to be much more variable across species than the N terminus which is highly conserved (Cashmore *et al.*, 1999).

Prior work in our laboratory highlighted the importance of the CT for the binding of TIM and PER in a yeast two hybrid system, whereby the deletion of 50 CT amino acids (dCRYΔII) abolished the binding of CRY to TIM and PER (Hemsley *et al.*, 2007). The same yeast two hybrid system also revealed the role of the CT in regulating the light response of CRY, as the CRYΔ construct, which is truncated by 20 amino acids, is constitutively active and capable of binding TIM and PER in darkness and light (Dissel *et al.*, 2004). Flies expressing CRYΔ displayed longer periods in the presence and absence of functional *cry* (*cry*^{b}) and CRYΔ was able to reduce levels of TIM and PER *in-vivo* (Dissel *et al.*, 2004). Following on from this work the CT was dissected *in-silico* to identify important structural domains that permit *cry* to carry out specific functions,

this work identified PDZ and TRAF2 ligand motifs. Three single point mutations in the CT and a chimeric GFP *cry*-CT construct was constructed, in an attempt to understand the regulation of *cry* in more detail. The mutations are as described below:

- S (526) which is a hypothetical phosphorylation site and part of the TRAF 2 ligand motif. Conversion of Serine (526) to D mimics a constitutive phosphorylation, displaying increased interaction with TIM and PER in yeast (Hemsley *et al.*, 2007).

- V (531) part of the PDZ domain and an aliphatic residue, mutated to K, introducing a positively charged residue generating electrostatic repulsion. Yeast data indicates still capable of binding TIM and PER (Hemsley un-published data).

- E (530) another residue located in the PDZ domain, modified to Proline causing in-stability of the predicted α helix. This mutation led to no interaction with TIM and PER in light or dark, in yeast (Hemsley *et al.*, 2007).

- The chimeric *cry* construct encodes the CT amino acids from 491 to 542, upstream of the CT are the entire GFP gene and the N-terminus is tagged with the STREP(II) tag.

The three residue mutants detailed above give a possible opportunity to examine the differences in CRY's ability to bind TIM/PER in at least two settings: enhanced (S.D) and abolished (E.P), provided that the yeast data is accurate *in-vivo*. The V.K mutation may also display diminished binding to TIM and PER but obtaining this data would require a

quantitative yeast two hybrid experiment. The GFP *cry* construct is an interesting tool for attempting to address what direct influence the CT of CRY has on many of the behaviours it exhibits.

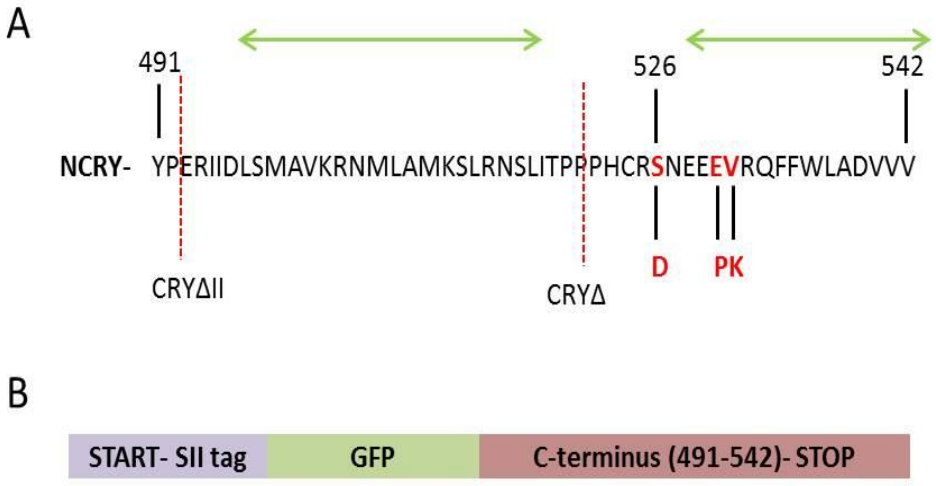


Figure 9-1: Illustrated CRY mutants.

A: Depicts the amino acid composition of CRY's C-terminus, starting at 491 and depicting the location of substitution mutants (in red). Amino acids to the right of dashed lines are absent in the CRYΔII and CRYΔ mutants, with green arrows indicating secondary structure helixes. B: Diagrammatic representation of the chimeric GFP *cry* C-terminus mutant, SII=STREP(II).

9.2 Methods

9.2.1 Cloning of *cry* mutants

The design and generation of the S.D, V.K and E.P mutants was performed as stated in Hemsley 2007. Essential codon changes were manufactured using specific-

site mutation PCR and these sequences were cloned into the p-UAST expression vector and injected (Hemsley *et al.*, 2007).

In order to create a chimeric STREP(II) GFP *cry*-CT construct, primers were designed to amplify GFP and *cry*.

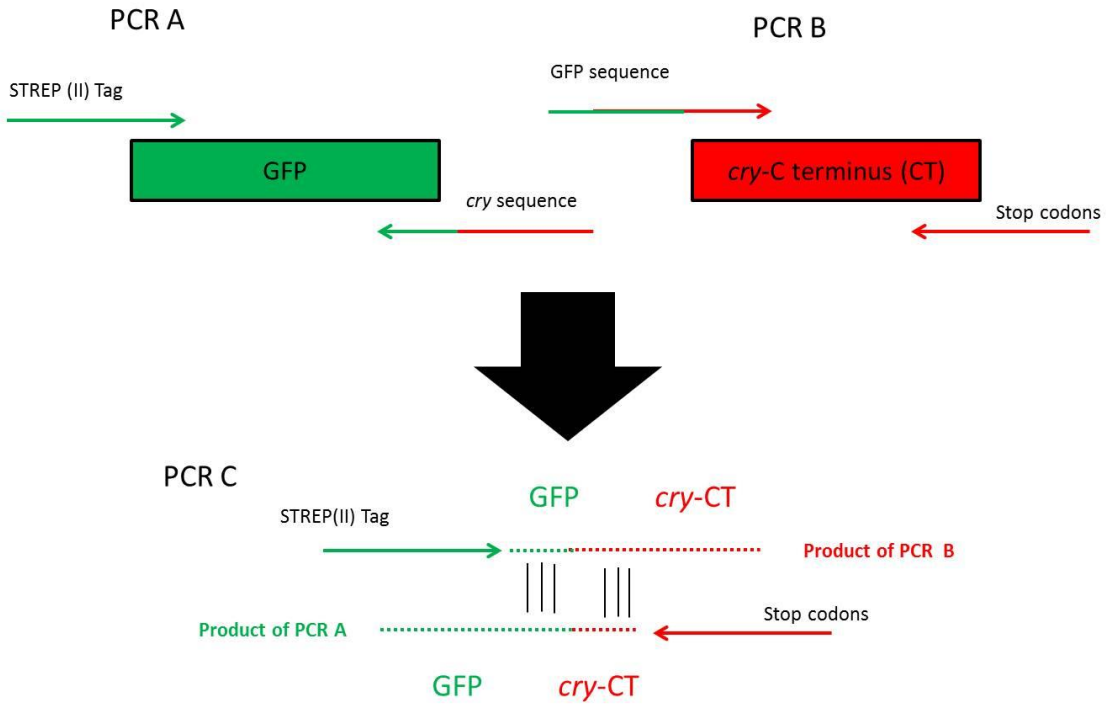


Figure 9-2: Diagrammatic representation STREP(II) GFP*cry*-CT mutant PCR.

PCR A: The GFP gene was amplified from a plasmid held in our laboratory; the forward primer contains a start codon and the STREP(II) tag, the reverse primer possesses the relevant GFP sequence and an additional stretch of bases complementary to *cry* CT.

PCR B: The forward primer carries a stretch of complementary GFP bases and the start of the CT of *cry*, the reverse primer completes the *cry* sequences and has stop codons to terminate translation.

PCR C: The products of PCRs A+B (after gel extraction) are added together with the forward primer of PCR A and the reverse of PCR B.

In order to carry out this experiment the following primers were designed:

Primer Name	Sequence	Annealing Temp (°C)
	PCR A	
JH GFP For EcoRI	[phos]GAATTCCAACGGATGTGGTCCCACCCCCAGTTCGAGAAGATGGTGAGCAAGGGCGAGGAG	55
JH GFP Rev 492	ATCAATGATCCGCTCCGGATACTTGTACAGCTCGTCCATGCC	
	PCR B	
JHCRY For GFP	ATGGACGAGCTGTACAAGTAATATCCGGAGCGGATCATTGAT	50
JH CRY Rev Xho	[phos]CTCGAGCTATCAAACCACCACGTCGGCCAG	

Table 9-1: Primers for GFP*cry*-CT mutant

PCR C was undertaken at 55°C using the methodology stated previously.

9.2.2 Fly stocks used

The lines used were primarily clock drivers to ascertain the influence of *cry* on the 24 hour periodicity.

Genotype	Description
***w*;*tim*-GAL4;+ (TG4)**	Driver expressing GAL4 in all types of clock cells.
***w*;PDF-GAL4 ;+**	Driver for small and large lateral neurons.
w*;TG4;*cry-GAL80	Cell type driver, expressing in the DNs
***w*;*cry*39-GAL4;*cry*^{02}**	*cry* driver in *cry*^{02} background.
***UAS-cry*S.D (8F(X), 2M, 4M)**	Serine to Aspartic acid *cry* substitution. Mimics phosphorylation.
UAS-*cry*V.K (s61, s62, fmt2)	Valine to Lysine substitution. Electrostatic repulsion.
UAS-*cry*E.P (s61, s62, s63)	Glutamatic acid to Proline substation. Destroys secondary structure.
UAS-GFPcry-CT (1F,12T, 10S, 23S 6T, 9T)	A tagged GFP *cry*-CT fusion.
***w*;UAS-*cry*24B;+**	Line expressing HA- wild type *cry*

Table 9-2: Fly genotypes used for investigating *cry* CT function.

The TG4, PDF and transgenic *cry* mutant lines were also crossed into a *cry*^{02} background using standard balancing protocols.

9.2.3 *Tim* Genotyping

It has been shown that there are natural variants of the *tim* allele that do not effect period, one producing a long *tim* isoform (L-TIM) 1421 amino acids as well as a short *tim* allele (S-TIM) 1398 amino acids, combined (LS-TIM), whilst the other variant only produces the S-TIM isoform (Rosato,E. 1997; Sandrelli *et al.*, 2007). The L-TIM isoform was shown to have a diminished binding of *cry* in a yeast system but TIM binding to PER was unaffected. It is thought that this diminished binding manifests

itself as a reduced response to brief light pulses in the L-*tim* genotype versus S-*tim*; LS-*tim* flies displayed an intermediary behaviour so were less sensitive than S-*tim* but more L-*tim* (Tauber *et al.*, 2007). *In-vivo* LL experiments using transgenic L and S *tim* in natural populations showed no significant difference in their ability to transmit constant light to arrhythmicity. To determine the presence of LS or S tim, genotyping was performed as in Tauber 2007 using the primers listed below.

Primer Name	Sequence	Annealing Temp (°C)
LSTIM F	TGGAATAATCAGAACTTTGA	55
STIM F	TGGAATAATCAGAACTTTAT	
LSTIM R	AGATTCCACAAGATCGTGTT	
TIM CON F	CATTCATTCCAAGCAGTATC	55
TIM CON R	TATTCATGAACTTGTGAATC	

Table 9-3: Primers for LS and S *tim*

9.3 Results

9.3.1 Behavioural analysis of un-crossed homozygote lines

The endogenous periods of three of the *cry* mutant lines (S.D, V.K and E.P) were tested to ensure that they did not carry clock mutations, by examining locomotor activity in darkness. All three lines had period values close to 24 hours, ranging from 23.9-24.3; this is within the range seen for many of the control lines that I have tested throughout this work.

9.3.2 Period analysis of *cry* mutants

The following data looks at the effects of overexpression of each of the *cry* mutants using different clock cell GAL4 drivers the composition of cells manipulated is shown below:

- TG4 (*tim*-GAL4): all tim expressing cells, all clock cells
- PDF: the LN_vs, excluding the fifth s-LN_vs
- TG4;*cry-GAL80*: DN2 and DN3 neurons and a subset of DN1s
- *Cry*: the LN_vs, LN_ds, and a small number of DN1s

To establish a role for the *cry* mutants in behavioural rhythmicity, overexpression experiments using the TG4 driver were performed (Table 9-4). Overexpressing wild type *cry* has no effect on the period, however, the S.D, V.K and E.P lines all gave significant increases (3 lines examined for S.D and V.K, 4 lines tested for E.P with only 1 line not showing significance, E.Pfmt1). The GFPcry-CT mutant showed very slight period increases in 3 of the lines (1f, 10s and 12T), however, 3 lines did not show period increases (23S, T6 and 9T). The UAS-GFPcry-CT 1F line that did show period increases when overexpressed with TG4 was used in the remaining behavioural experiments.

These experiments were repeated in a cry^{02} background to observe if removing *cry* has any impact on this period phenotype seen in the mutant lines (Table 9-5). Again wild type *cry* overexpression does not significantly increase the period, this is

also true of the S.D mutants as the cry^{02} background has nullified their period lengthening effects seen in the previous experiment. The V.K and E.P mutations continue to give period increases, whilst the UAS-GFPcry-CT 1F mutant no longer shows a period increase. Thus there appears to be a *cry* dependent effect in the period lengthening abilities of S.D and GFPcry-CT mutants, while V.K and E.P are unaffected by the removal of endogenous *cry*.

The PDF neurons also express *cry*, any cell type specific effects of *cry* mutant expression can be analysed using the PDF driver. A subset of the lines used in the TG4 experiments were analysed (1 line per genotype), in cry^{+} and cry^{02} backgrounds. All of the mutants and wild type *cry* showed a significant increase in period when overexpressed with the PDF driver. Removing *cry* halted the period increases seen when overexpressing wild type *cry*, however, the S.D, V.K and E.P mutants continued to increase the period when overexpressed in the cry^{02} background. The GFP-cry-CT line was not analysed in cry^{02}. There appears to be a trend in the data where the S.D line has period ~ equal in cry^{+} and cry^{02}, in V.K the period increase is moderately increased (< 1 hour) in the cry^{02} background and E.P;cry^{02} line shows an increase of 1 hour in the cry^{02} background compared to its cry^{+} counterpart. This data hints that the possible severity of the mutant has influences on the length of period increase seen when *cry* is removed in the PDF cells.

When we overexpress with PDF-GAL4 we are targeting cells that normally express *cry* and with TG4 we express *cry* in these same cells as well as other *cry* expressing cells and also ectopically in a sub-set of clock cells. By combining TG4 with *cry-GAL80* we can target expression to cells that have no endogenous expression of

cry. TG4;*cry-GAL80* overexpression was performed on the mutant *cry* lines giving no change in period for all the mutant lines (E.P was significantly different from both controls, however it was longer than one and shorter than the other, suggesting that those biological differences are not meaningful). Wild type *cry* was not tested; the lack of change seen in the period of locomotor activity suggests that the period increases seen previously may not be manifested in cells lacking endogenous *cry*.

Finally we can use cry39-GAL4 to express the mutants in cells that only express *cry*, with the exception of 3 extra LNds (Yoshii *et al.*, 2008; Emery *et al.*, 2000b). *Cry* overexpression was only carried out in a cry^{02} background; wild type *cry* showed no significant change as did the V.K mutant, while S.D, E.P and GFPcry-CT displayed longer periods. In this experiment we have targeted *cry* cells and have seen increased periods in three of the four mutants, the V.K result is somewhat surprising as this mutant has influenced behaviour in all driver combinations/*cry* backgrounds studied thus far. Using a cry-GAL4 line in a cry^{+} background could also be done to examine differences between cry^{+} and cry^{02} for this specific driver. The data discussed can be found below.

Genotype	Period ± SEM	N	Statistical significance from controls	Affected neurons
S.D				
UAS-*cry*SD8F(X);;	23.9 ± 0.2	12	not tested	NA
UAS-*cry*SD2M/CyO	24.3 ± 0.2	12	not tested	NA
TG4				
UAS-cryS.D8F(X);TG4/+	25.88 ± 0.10	35	a	All clock cells
UAS-cryS.D8F(X);;	23.72 ± 0.06	15		Control
w;UAS-cryS.D4M/TG4;+	24.87 ± 0.14	11	b	All clock cells
w;UAS-cryS.D4M/+	23.38 ± 0.08	20		Control
w;UAS-cryS.D2M/TG4	24.80 ± 0.15	10	c	All clock cells
w;UAS-cryS.D2m/+	23.37 ± 0.05	4		control
y w;TG4/+	23.39 ± 0.05	62		control
PDF				
w;UAS-cryS.D4m/PDF;+	25.22 ± 0.10	17	d	PDF cells
w;PDF-GAL4/+	23.77 ± 0.07	38		control
w*;TG4;*cry-GAL80				
UAS-cryS.D8F(X);TG4/+;*cry-GAL80*	23.42 ± 0.06	15	e	PDF^- CRY^- cells
w;TG4/+;*cry-GAL80*/+	23.37 ± 0.06	17		control
V.K				
w;UAS-cryV.Ks61/CyO;	23.9 ± 0.09	17	not tested	NA
w;UAS-cryV.Ks62;	24.3 ± 0.06	17	not tested	NA
w;UAS-cryV.Ks63;	24.1 ± 0.08	13	not tested	NA
TG4				
w;UAS-cryV.Ks61/TG4	25.85 ± 0.11	18	f	All clock cells
w;UAS-cryV.Ks61/+	23.51 ± 0.05	32		control

w;UAS-cryV.Ks62/TG4	24.96 ± 0.11	31	g	All clock cells
w;UAS-cryV.Ks62/+	23.92 ± 0.09	39		control
UAS-cryV.Kfmt2;TG4/+	24.47 ± 0.08	28	h	All clock cells
UAS-cryV.Kfmt2;+	24.12 ± 0.07	30		control
PDF				
w;UAS-cryV.Ks61/PDF-GAL4	25.11 ± 0.08	28	i	PDF
w*;TG4;*cry-GAL80				
w;UAS-cryV.Ks61/TG4;*cry-GAL80*/+	23.56 ± 0.10	7	j	PDF^- CRY^- cells
E.P				
w;UAS-cryE.Ps61/CyO;	24.14 ± 0.10	15	not tested	NA
w;UAS-cryE.Ps63;	24.2 ± 0.17	8	not tested	NA
TG4				
w;UAS-cryE.Ps61/TG4	26.08 ± 0.14	16	k	All clock cells
w;UAS-cryE.Ps61/+	24.02 ± 0.04	30		control
w;UAS-cryE.Ps62/TG4	25.49 ± 0.12	29	l	All clock cells
w;UAS-cryE.Ps62/+	23.87 ± 0.07	32		control
w;UAS-cryE.Ps63/TG4	25.15 ± 0.13	32	m	All clock cells
w;UAS-cryE.Ps63/+	23.76 ± 0.08	34		control
UAS-cryE.Pfmt1;TG4/+	24.12 ± 0.15	17	n	
UAS-cryE.Pfmt1;;	23.75 ± 0.05	15		
PDF				
w;UAS-cryE.Ps61/PDF-GAL4	25.88 ± 0.17	16	o	PDF
w*;TG4;*cry-GAL80				
w;UAS-cryE.Ps61/TG4;*cry-GAL80*/+	23.61 ± 0.07	14	p	PDF^- CRY^- cells
GFPcry-CT				
TG4				
UAS-GFPcry-CT 1F; TG4/+	24.68 ± 0.10	10	q	All clock cells

UAS-GFPcry-CT 1F;+	23.93 ± 0.05	33		control
w;TG4/+;UAS-GFPcry-CT 12T/+	24.23 ± 0.07	20	r	All clock cells
w;+;UAS-GFPcry-CT 12T	23.48 ± 0.04	20		control
w;UAS-GFPcry-CT10S/TG4	24.2 ± 0.15	11	s	All clock cells
w;UAS-GFPcry-CT10S/+	23.61 ± 0.06	19		
w;UAS-GFPcry-CT23S/TG4	24.00 ± 0.01	19	t	All clock cells
w;UAS-GFPcry-CT23S/+	23.32 ± 0.11	15		control
w;TG4/+;UAS-GFPcry-CT T6/+	24.03 ± 0.09	19	u	All clock cells
w;;UAS-GFPcry-CT T6/+	23.69 ± 0.05	19		control
w;TG4/+;UAS-GFPcry-CT 9T/+	24.1 ± 0.09	19	v	All clock cells
w;;UAS-GFPcry-CT 9T/+	23.71 ± 0.10	19		control
PDF				
UAS-GFPcry-CT 1F;PDF-GAL4/+	24.77 ± 0.06	20	w	PDF
w;TG4;*cry-GAL80*				
UAS-GFPcry-CT 1F;TG4/+;*cry-GAL80*/+	23.87 ± 0.04	20	x	PDF^- CRY^- cells
Wild type *cry* (UAS-cry24B)				
TG4				
w;UAS-cry24B/TG4	23.94 ± 0.04	33	y	All clock cells
w;UAS-cry24B/+	23.77 ± 0.08	28		control
PDF				
w;UAS-cry24B/PDF-GAL4	24.79 ± 0.15	33	z	PDF

Table 9-4: Period analysis of overexpressing *cry* mutants and their controls.

All experiments carried out at 25°C for >1 week, after 2-3 days of LD entrainment. The genotype period + SEM and N value are all reported. Statistical analysis: one way ANOVA and Bonferroni post hoc to confirm statistical significance of overexpression line from both controls. These data are reported below:

a ($F_{2,108}$ = 232.3, P = <0.0001; Bonferroni post hoc: UAS-cryS.D8F(X);+ vs UAS-cryS.D8F(X);TG4/+ P = 0.001, *w*;TG4/+ vs UAS-cryS.D8F(X);TG4/+ P = 0.001),

b ($F_{2,89}$ = 50.11 P = <0.0001; Bonferroni post hoc: *w*;UAS-cryS.D4m/+ vs *w*;UAS-cryS.D4m/TG4 P = 0.001, *w*;TG4/+ vs *w*;UAS-crySD.4m/TG4 P = 0.001),

c ($F_{2,72}$ = 26.11, P= < 0.0001; Bonferroni post hoc: *w*;UAS-cryS.D2m/+ vs *w*;UAS-cryS.D2m/TG4 P = 0.001, *w*;TG4/+ vs *w*;UAS-cryS.D2m/+ P = 0.001),

d ($F_{2,72}$ = 98.82, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryS.D4m/+ vs *w*;UAS-cryS.D4m/PDF P = 0.001, *w*;PDF/+ vs *w*;UAS-cryS.D4m/PDF P =0.001),

e ($F_{2,45}$ = 8.075, P = 0.0010; Bonferroni post hoc: UAS-cryS.D8F(X);TG4/+;*cry-GAL80*/+ vs *w*;TG4/+;*cry-GAL80*/+ P = ns, UAS-cryS.D8F(X);TG4/+;*cry-GAL80*/+ vs UAS-cryS.D8F(X);; P = 0.01),

f ($F_{2,108}$ = 221.9, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryV.Ks61/+ vs *w*;UAS-cryV.Ks61/TG4 P = 0.001, *w*;TG4/+ vs *w*;UAS-cryV.Ks61/TG4 P = 0.001),

g ($F_{2,128}$ = 47.97, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryV.Ks62/+ vs *w*;UAS-cryV.Ks62/TG4 P = 0.001, *w*;TG4/+ vs *w*;UAS-cryV.Ks62/TG4 P = 0.001),

h ($F_{2,116}$ = 16.13, P = <0.0001; Bonferroni post hoc: UAS-cryV.Kfmt2;; vs UAS-cryV.Kfmt2;TG4/+ P =0.01, *w*;TG4/+ vs UAS-cryV.Kfmt2;TG4/+ P = 0.001),

i ($F_{2,95}$ = 129.1, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryV.Ks61/+ vs *w*;UAS-cryV.Ks61/PDF P = 0.001, *w*;PDF/+ vs *w*;UAS-cryV.Ks61/PDF P =0.001),

j ($F_{2,53}$ = 1.399, P = 0.2558; Bonferroni post hoc: *w*;UAS-cryV.Ks61/+ vs *w*;UAS-cryV.Ks61/TG4;*cry-GAL80*/+ P = ns, *w*;TG4/+;*cry-GAL80*/+ vs w;UAS-cryV.Ks61/TG4;*cry-GAL80*/+ P = ns),

k ($F_{2,104}$ = 197.6, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryE.Ps61/+ vs *w*;UAS-cryE.Ps61/TG4 P = 0.001, *w*;TG4/+ vs *w*;UAS-cryE.Ps61/TG4 P = 0.001),

l ($F_{2,119}$ = 119.2, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryE.Ps62/+ vs *w*;UAS-cryE.Ps62/TG4 P 0.001, *w*;TG4/+ vs *w*;UAS-cryE.Ps62/TG4 P = 0.001),

m ($F_{2,124}$ = 66.74, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryE.Ps63/+ vs *w*;UAS-cryE.Ps63/TG4 P = 0.001, *w*;TG4/+ vs *w*;UAS-cryE.Ps63/TG4 P=0.001),

n ($F_{2,90}$ = 2.951, P = 0.0574; Bonferroni post hoc: UAS-cryE.Pfmt1;; vs UAS-cryE.Pfmt1;TG4/+ P = 0.05, *w*;TG4/+ vs UAS-cryE.Pfmt1;TG4/+ P = ns),

o ($F_{2,81}$ = 131.6, P =<0.0001; Bonferroni post hoc: *w*;UAS-cryE.Ps61/+ vs *w*;UAS-cryE.Ps61/PDF P = 0.001, *w*;PDF/+ vs *w*;UAS-cryE.Ps61/PDF P =0.001),

p ($F_{2,58}$ = 37.21, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryE.Ps61/+ vs *w*;UAScryE.Ps61/TG4;*cry-Gal80*/+ P 0.001, w;TG4/+;*cry-GAL80*/+ vs *w*;UAScryE.Ps61/TG4;*cry-Gal80*/+ P =0.05),

q ($F_{2,101}$ = 17.95, P = <0.0001; Bonferroni post hoc: UAS-GFPcry-CT 1F;; vs UAS-GFPcry-CT 1F;TG4/+ P =0.001, *w*;TG4/+ vs UAS-GFPcry-CT 1F;TG4/+ P =0.001),

r ($F_{2,97}$ = 23.23, P = <0.0001; Bonferroni post hoc: *w*;;UAS-GFPcry12T/+ vs *w*;TG4/+;UAS-GFPcryCT12T P = 0.001, *w*;TG4/+ vs *w*;TG4/+;UAS-GFPcry-CT12T/+ P = 0.01),

s ($F_{2,88}$ = 10.26, P = <0.0001; Bonferroni post hoc: *w*;UAS-GFPcry-CT10S/+ vs *w*;UAS-GFPcry-CT10S/TG4 P = 0.001, *w*;TG4/+ vs *w*;UAS-GFPcryCT10S/TG4 P = 0.05),

t ($F_{2,92}$ = 15.29, P = <0.0001; Bonferroni post hoc: *w*;UAS-GFPcry-CT23S/+ vs *w*;UAS-GFPcry-CT23S/TG4 P = 0.001, *w*;TG4/+ vs *w*;GFPcry-CT23S/TG4 P = ns),

u ($F_{2,97}$ = 4.739, P = 0.0109; Bonferroni post hoc: *w*;;UAS-GFPcry-CT T6/+ vs w;TG4/+;UAS-GFPcry-CT T6/+ P = 0.01, *w*;TG4/+ vs *w*;TG4/+;UAS-GFPcry-CT T6/+ P = ns),

v ($F_{2,96}$ = 4.425, P = 0.0145; Bonferroni post hoc: *w*;;UAS-GFPcry-CT9T/+ vs *w*;TG4/+;UAS-cry-CT9T/+ P = 0.01, *w*;TG4/+ vs *w*;TG4/+;UAS-cryCT-9T/+ P = ns),

w ($F_{2,88}$ = 49.14, P = <0.0001; Bonferroni post hoc: UAS-GFPcry-CT 1F;; vs UAS-GFPcryCT1F;PDF/+ P = 0.001, *w*;PDF/+ vs UAS-GFPcry-CT 1F;PDF/+ P = 0.001),

x ($F_{2,67}$= 21.37, P= <0.0001; Bonferroni post hoc: UAS-GFPcry-CT 1F;TG4/+;*cry-GAL80*/+ vs UAS-GFPcry-CT 1F;+, P = ns, *w*;TG4/+;*cry-GAL80*/+ vs UAS-GFPcry-CT 1F;TG4/+;*cry-GAL80*/+ P = 0.001),

y ($F_{2,119}$ = 2.177, P = 0.1179; Bonferroni post hoc: *w*;UAS-cry24B/+ vs *w*;TG4/UAS-cry24B P = ns, *w*;TG4/+ vs *w*;UAS-cry24B/TG4 P = ns),

z ($F_{2,80}$ = 28.30, P =<0.0001; Bonferroni post hoc: *w*;UAS-cry24B/+ vs *w*;UAS-cry24B/PDF P = 0.001, *w*;PDF/+ vs *w*;UAS-cry24B/PDF P = 0.001).

Genotype	Period ± SEM	N	Statistical significance from controls	Affected neurons
S.D				
TG4;*cry*^{02}				
UAS-cryS.D8F(X);TG4/+;*cry*^{02}	24.09 ± 0.12	13	a	All clock cells
UAS-cryS.D8F(X);+;*cry*^{02}	23.40 ± 0.11	7		Control
w;UAS-cryS.D4M/TG4;*cry*^{02}	24.57 ± 0.09	18	b	All clock cells
w;UAS-cryS.D4M/+;*cry*^{02}	23.59 ± 0.11	18		Control
y w;TG4/+;*cry*^{02}	24.37 ± 0.12	13		Control
PDF;*cry*^{02}				
w;UAS-cryS.D4m/PDF;*cry*^{02}	25.26 ± 0.18	18	c	PDF
w;PDF-GAL4/+;*cry*^{02}	23.59 ± 0.11	18		Control
Cry;*cry*^{02}				
w;cry39-GAL4/UAS-cryS.D4m;*cry*^{02}	25.06 ± 0.16	16	d	CRY^{+}
w;cry39-GAL4/+;*cry*^{02}	24.54 ± 0.13	16		Control
V.K				
TG4;*cry*^{02}				
w;UAS-cryV.Ks61/TG4;*cry*^{02}	25.89 ± 0.25	16	e	All clock cells
w;UAS-cryV.Ks61/+;*cry*^{02}	23.75 ± 0.08	16		Control
PDF;*cry*^{02}				
w;UAS-cryV.Ks61/PDF-GAL4;*cry*^{02}	26.32 ± 0.17	17	f	PDF
w;cry39-GAL4;*cry*^{02}				Control
Cry;*cry*^{02}				
w;cry39-GAL4/UAS-cryV.Ks61;*cry*^{02}	24.53 ± 0.09	17	g	CRY^{+}
E.P				
TG4;*cry*^{02}				

w;UAS-cryE.Ps63/TG4;*cry*^{02}	24.92 ± 0.13	19	h	All clock cells
w;UAS-cryE.Ps63/+;*cry*^{02}	23.76 ± 0.07	18		Control
PDF				
w;UAS-cryE.Ps61/PDF-GAL4;*cry*^{02}	26.81 ± 0.17	6	i	PDF
w;UAS-cryE.Ps61/+;*cry*^{02}	23.63 ± 0.08	15		Control
Cry;*cry*^{02}				
w;cry39-GAL4/UAS-cryE.Ps61;*cry*^{02}	25.20 ± 0.08	18	j	CRY^{+}
GFPcry-CT				
TG4				
UAS-GFPcry-CT 1F; TG4/+;*cry*^{02}	24.69 ± 0.16	19	k	All clock cells
UAS-GFPcry-CT 1F;+;*cry*^{02}	23.16 ± 0.09	18		Control
Cry;*cry*^{02}				
UAS-GFPcry-CT 1F; cry-39-GAL4/+;*cry*^{02}	25.01 ± 0.13	17	l	CRY^{+}
Wild type *cry* (UAS-cry24B)				
TG4				
w;UAS-cry24B/TG4;*cry*^{02}	23.99 ± 0.14	17	m	All clock cells
w;UAS-cry24B/+;*cry*^{02}	23.49 ± 0.14	16		Control
PDF				
w;UAS-cry24B/PDF-GAL4;*cry*^{02}	24.01 ± 0.14	13	n	PDF
cry-GAL4;*cry*^{02}				
w;UAS-cry24B/cry39;*cry*^{02}	24.65 ± 0.11	15	o	CRY^{+}

Table 9-5: Period analysis of overexpression of cry mutants in a *cry*^{02} background.

Data collected and analysed in the same manner shown in the previous table.

a ($F_{2,28}$ = 14.80, P = <0.0001; Bonferroni post hoc: UAS-CryS.D8F(X);TG4/+;*cry*^{02} vs *w*;TG4/+;*cry*^{02} P = ns, UAS-cryS.D8F(X);; vs UAS-cryS.D8F(X);TG4/+ P = 0.01),

b ($F_{2,48}$ = 23.67, P =<0.0001; Bonferroni post hoc: *w*;UAS-cryS.D4m/TG4;*cry*^{02} vs *w*;TG4/+;*cry*^{02} P = ns, *w*;UAS-cryS.D4m/+;*cry*^{02} vs *w*;UAS-cryS.D4m/TG4;*cry*^{02} P = 0.001),

c ($F_{2,46}$ = 36.25, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryS.D4m/+;*cry*^{02} vs *w*;UAS-cryS.D4m/PDF;*cry*^{02} P = 0.001, *w*;PDF/+;*cry*^{02} vs *w*;UAS-cryS.D4m/PDF;*cry*^{02} P = 0.001),

d ($F_{2,47}$ = 29.07, P = <0.0001; Bonferroni post hoc: *w*;UAS-crySD4m/+;*cry*^{02} vs *w*;UAS-cryS.D4m/cry39;*cry*^{02} P = 0.001, *w*;cry39/+;*cry*^{02} vs *w*;UAS-cryS.D4m/cry39;*cry*^{02} P = 0.05),

e ($F_{2,61}$ = 56.41, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryV.Ks61/+;*cry*^{02} vs *w*;UAS-cryV.Ks61/TG4;*cry*^{02} P = 0.001, *w*;TG4/+;*cry*^{02} vs *w*;UAS-cryV.Ks61/TG4;*cry*^{02} P = 0.001),

f ($F_{2,62}$ = 120.6, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryV.Ks61/+;*cry*^{02} vs *w*;UAS-cryV.Ks61/PDF;*cry*^{02} P = 0.001, *w*;PDF/+;*cry*^{02} vs *w*;UAS-cryV.Ks61/PDF;*cry*^{02} P =0.001),

g ($F_{2,46}$ = 22.32, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryV.Ks61/+;*cry*^{02} vs *w*;UAS-cryV.Ks61/cry39-GAL4;*cry*^{02} P = 0.001, *w*;cry39-GAL4/UAS-cryV.Ks61;*cry*^{02} vs *w*;cry39-GAL4/+;*cry*^{02} P = ns),

h ($F_{2,47}$ = 29.17, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryE.Ps63/+;*cry*^{02} vs *w*;UAS-cryE.Ps63/TG4;*cry*^{02} P = 0.001, *w*;TG4/+;*cry*^{02} vs *w*;UAS-cryE.Ps63/TG4;*cry*^{02} P = 0.01),

i ($F_{2,31}$ = 156.1, P =<0.0001; Bonferroni post hoc: *w*;UAS-cryE.Ps61/+;*cry*^{02} vs *w*;UAS-cryE.Ps61/PDF;*cry*^{02} P = 0.001, *w*;PDF/+;*cry*^{02} vs *w*;UAS-cryE.Ps61/PDF;*cry*^{02} P = 0.001),

j ($F_{2,42}$ = 73.27, P = <0.0001; Bonferroni post hoc: *w*;UAS-cryE.Ps61/+;*cry*^{02} vs *w*;UAS-cryE.Ps61/cry39;*cry*^{02} P = 0.001, *w*;cry39/+;*cry*^{02} vs *w*;cry39/UAS-cryE.Ps61;*cry*^{02} P = 0.001),

k ($F_{2,47}$ = 17.37, P = <0.0001; Bonferroni post hoc: UAS-GFPcry-CT 1F;;*cry*^{02} vs UAS-GFPcry-CT 1F;TG4/+;*cry*^{02} P = 0.001, UAS-GFPcry-CT 1F; TG4/+;*cry*^{02} vs *w*;TG4/+;*cry*^{02} P = ns),

l ($F_{2,48}$ = 34.01, P = <0.0001; Bonferroni post hoc: UAS-GFPcry-CT 1F;;*cry*^{02} vs UAS-GFPcry-CT 1F;cry39/+;*cry*^{02} P = 0.001, *w*;cry39/+;*cry*^{02} vs UAS-GFPcry-CT 1F; cry39/+;*cry*^{02} P = 0.05),

m ($F_{2,43}$ = 9.842, P = 0.0003; Bonferroni post hoc: *w*;TG4/+;*cry*^{02} vs *w*;UAS-cry24B/TG4;*cry*^{02} P = ns, *w*;UAS-cry24B/+;*cry*^{02} vs *w*;UAS-cry24B/TG4;*cry*^{02} P = 0.05),

n ($F_{2,39}$ = 12.84, P = <0.0001; Bonferroni post hoc: *w*;PDF/UAS-cry24B;*cry*^{02} vs *w*;PDF/+;*cry*^{02}, P = ns, *w*;UAS-cry24B/+;*cry*^{02} vs *w*;UAS-cry24B/PDF;*cry*^{02} P = 0.05),

o ($F_{2,44}$ = 22.41, P = <0.0001; Bonferroni post hoc: *w*;UAS-cry24B/+;*cry*^{02} vs *w*;UAS-cry24B/cry39;*cry*^{02} P = 0.001, *w*;cry39-GAL4/+;*cry*^{02} vs *w*;cry39-GAL4/UAS-cry24B;*cry*^{02} P = ns).

9.3.3 Tim genotyping of *cry* mutants

The *tim* LS/S backgrounds of the *cry* mutant lines were examined and the data has been summarised in the table below; while the agarose gel analysis can be seen in appendix 3.

Genotype	tim
***w*;cry24B/TG4;*cry*^{02}**	**S-tim only**
***w*;cryS.D4m/TG4;*cry*^{02}**	**S-tim only**
***w*;cryV.Ks62/TG4;*cry*^{02}**	**LS and S**
***w*;cryE.Ps61/TG4;*cry*^{02}**	**LS and S**
UAS-GFPcry-CT 1F;;*cry*^{02}	**LS and S**
***w*;TG4;*cry*^{02}**	**S-tim only**

Table 9-6: Table showing the *tim* composition in each of the *cry* mutant lines.
10 flies were analysed per genotype.

The genotyping reveals a heterogeneous composition of LS and S-*tim* between the mutant genotypes, the *cry*^{02} genotype has been published as S-*tim* only (Dolezelova *et al.*, 2007) and therefore it seems likely that the V.K, E.P and GFPcry-CT mutants have been injected into a *w*^{1118} strain carrying LS-*tim*. While the TG4, S.D and 24B lines, which were injected several years prior to these mutants, were made in an S-*tim* background.

9.3.4 Constant light phenotype

One phenotype observed when is *cry* is removed is the maintenance of rhythmicity in constant light, the ability of each mutant to rescue this phenotype was tested using TG4; the table below summarises these findings.

Genotype	Rhythmic (%)	N	Complex rhythms (%)
w^{1118}	6%	19	6%
cry^{02}	100%	17	76%
w;TG4/+;cry^{02}	100%	20	60%
Wild type *cry*			
w;UAS-*cry*24B/TG4;cry^{02}	55%	20	30%
w;UAS-cry24B/+;cry^{02}	94%	18	33%
S.D			
w;UAS-*cry*S.D4m/TG4;cry^{02}	20%	20	5%
w;UAS-cryS.D4m/+;cry^{02}	90%	18	67%
UAS-*cry*S.D8f(x); TG4/+;cry^{02}	45%	20	0%
UAS-cryS.D8F(X);+;cry^{02}	100%	20	60%

w;UAS-*cry*S.D2m/TG4;*cry*^{02}	25%	20	10%
w;UAS-cryS.D2m/+;*cry*^{02}	100%	19	58%
V.K			
w;TG4/UAS-*cry*V.Ks61;cry^{02}	92%	37	19%
w;UAS-cryV.Ks61/+;*cry*^{02}	100%	29	52%
w;TG4/UAS-*cry*V.Ks62;*cry*^{02}	100%	19	32%
w;UAS-cryV.Ks62/+;*cry*^{02}	95%	19	63%
UAS-*cry*V.Kfmt2;TG4/+;*cry*^{02}	65%	20	20%
UAS-cryV.Kfmt2;+;*cry*^{02}	100%	18	67%
E.P			
w;TG4/UAS-*cry*E.Ps61;*cry*^{02}	100%	20	40%
w;UAS-cryE.Ps61/+;*cry*^{02}	95%	19	50%
w;TG4/UAS-*cry*E.Ps62;*cry*^{02}	100%	19	5%
w;UAS-cryE.Ps62/+;*cry*^{02}	100%	20	55%
GFPcry-CT			
UAS-GFP*cry*-CT 1F;TG4/+;*cry*^{02}	75%	20	40%
UAS-GFP*cry*-CT 1F;+;cry^{02}	90%	20	65%

Table 9-7: Rhythmicity in LL of *cry* mutants driven by TG4.

Flies were kept in 25°C LL conditions for >1 week after 2-3 days of LD entrainment, % of rhythmicity is shown as is the % of complex rhythmicity.

This data gives an indication of the ability of the mutant to rescue the arrhythmicity normally seen in LL; the TG4 driver was used as it is expressed in all clock cells. Wild type *cry* overexpression showed a significantly reduction in the level of rhythmicity compared to its controls (Fisher's, 2 tailed test: *w*;UAS-cry24B/+;cry^{02} vs *w*;UAS-cry24B/TG4;cry^{02}, P = <0.0012 and *w*;TG4/+;cry^{02} vs *w*;UAS-cry24B/TG4;cry^{02}, P = <0.0089). The three S.D lines were also tested in an identical fashion, giving the same level of significance from both controls: cryS.D2m P=<0.0001, S.D4m P=<0.0001 and cryS.D8F(X) P = 0.0001. Therefore the S.D mutation has shown successful rescue. Two of the V.K mutants did not show significant differences in rhythmicity, V.Ks61 P= 0.2497 and V.Ks62 P = 1.00. Although one line of V.K was significant from the controls: UAS-cryV.Kfmt2;;cry^{02} vs UAS-cryV.Kfmt2;TG4/+;cry^{02}, P = 0.0087 and *w*;TG4/+;cry^{02} vs UAS-cryV.Kfmt2;TG4/+;cry^{02}, P = 0.0083. The E.P mutant lines were not tested for significance as they are identical to controls, whilst the GFPcry-CT mutant were also not significant (P = 0.4). Thus the S.D line is capable of rescuing the LL phenotype while the V.K (2 out of 3 lines), E.P and GFPcry-CT mutants are not capable of doing so. The S.D lines give an even lower level of rhythmicity than wild type, but comparing the most severe S.D mutant (4M) with wild type *cry* overexpression was not significant (P = 0.344). It is also interesting to note that in the mutant lines that were predominately rhythmic (V.K, E.P and GFPcry-CT) the percentage of complex rhythmicity was consistently lower when the mutant *cry* was expressed versus controls. Complex rhythmicity is assigned to individual flies when they show two significant period values and are confirmed as rhythmic in the autocorrelation analysis.

9.4 Discussion

The C-terminus (CT) of *cry* has been shown to be functionally important for facilitating the binding of the clock proteins PER and TIM (Rosato *et al.*, 2001). Furthermore a number of potential protein binding sites were identified on the CT (Hemsley *et al.*, 2007), as well as confirming that a large deletion of the CT (CRYΔII) abolished the interaction between DAY and CRY in yeast (Hemsley, unpublished data). Therefore by studying mutations in the CT we may be able to identify residues important for CRY function and can use these mutants to help characterise the relationship between DAY and CRY more clearly. To briefly re-iterate the point mutations are: S.D, mimics phosphorylation, V.K, electrostatic repulsion and E.P, introduces a turn damaging secondary structure.

Overall there was a heterogeneous effect on locomotor behaviour when overexpressing the *cry* mutant lines. With TG4, wild type *cry* has no effect on the period in either cry^{+} or cry^{02} backgrounds, while the S.D mutation increased the period in cry^{+} and had no effect in cry^{02}. The inability of the S.D mutant to affect the period in the cry^{02} background suggests that an interaction between the mutant and wild type is required. This interaction could be physical, with S.D forming dimers with wild type and influencing the period. An alternate hypothesis would be an indirect mechanism of action where *cry* is required in the signalling process that creates these elevated period values. Both V.K and E.P lengthen the period independently of *cry*, while *GFPcry-CT* gave modest but significant increases in half the lines tested, this could reflect expression level differences. Analysing the level of overexpression achieved for

each mutant would ensure that the expression achieved is comparable between mutants.

Overexpressing with PDF-GAL4 was undertaken to examine cell type specific effects of mutant expression. The S.D mutant caused increased periods but this time in both $cry^{+ \text{ and } 02}$ backgrounds. If *cry* S.D needed to form dimers to produce period lengthening effects then the increased period seen with PDF;cry^{02} should not be possible; suggesting that *cry* S.D does not need to physically associate with *cry* to influence the period. Interestingly overexpressing wild type *cry* gave an increased period in *cry* dependent fashion, showing significant period increases in a cry^{+} environment. The *cry* dependence of this result is interesting and indicates how other cells must express *cry* to produce period changes. Furthermore it shows that *cry* can influence the clock in DD in a cell type specific manner. How it accomplishes this is uncertain but it is unlikely to be through an interaction with PER and TIM due to the light dependence of this reaction. Data in our laboratory suggests that when you overexpress *NaChBac* with PDF-GAL4 a period increase is also seen (S.Dissel, unpublished data). Perhaps *cry* overexpression causes increased firing in the PDF cells increasing the period; if the cells downstream of this firing do not possess *cry* then the consequences of this firing are circumvented. The V.K and E.P mutants again affect the period in both *cry* backgrounds, although larger values were seen in the cry^{02} background versus cry^{+} suggesting that they are more potent when *cry* is absent. Although this is far from conclusive as the V.K line was less than 1 hour different and the E.P line has a small N value (6), repeating these experiments may reveal that overexpressing these mutants in cry^{02} is more potent than cry^{+}. Finally the *GFPcry-CT*

line chosen showed a significant increase in period, indicating that the *cry* CT can influence locomotor behaviour. Repeating this experiment with a control for GFP ensuring it has no effect by itself, as well as overexpressing *GFPcry-CT* with PDF-GAL4 in a cry^{02} context would be useful. Furthermore the *GFPcry-CT* lines that did not show significant differences when overexpressed with TG4 could be repeated using the PDF driver, as the PDF cells appear to be sensitive to *cry* manipulations.

One important finding was that overexpression of all the mutants using TG4 *cry-GAL80*, gave no effect on behaviour (wild type not tested). This suggests that the cells manipulated with this driver are not affected or that they have no influence over the period in DD. This second theory can be dismissed as manipulations using *NaChBac* overexpression in these cells alters the period (Dissel, unpublished data). Therefore it could be that the cells lacking endogenous *cry* are deficient in proteins/process's that have caused the increased periods seen thus far.

The mechanisms through which these behavioural changes may be occurring is possibly bespoke for each mutation, however, one possible common feature of these mutants may be dominant negative behaviour, whereby each mutant can sequester proteins blocking them from acting as normal. This interaction is likely to be with clock proteins due to the behaviour observed. Another hypothesis is that this period increase is the result of altered neuronal output, perhaps these mutations are not regulated effectively at the level of the membrane causing alteration in the polarisation of the neurons that they express in. The PDF cells appear to be the most sensitive to manipulations with *cry* made in DD as even wild type *cry* altered the period; this is unsurprising as the PDF cells are already known to show control of DD

rhythms more so than other clock cells (Renn *et al.*, 1999; Helfrich-Forster, 1998). Re-attempting these studies with a driver for TG4/PDF-GAL80 would be informative; if no changes in period were seen with this driver it would confirm that the PDF cells are important for the phenotypes we have been witnessing. This would re-highlight the importance of this cell type and hint at a possible pathway specific to these cells which is sensitive to *cry* manipulation; or alternatively that the overexpression of the *cry* mutants is having off target effects that manifest themselves as period changes in PDF cells. The DD analysis of *cry* mutants has revealed some interesting findings, but as *cry* is known for being a light activated protein it may be more pertinent in addressing the function of these mutations, to discuss the LL data (Emery *et al.*, 1998; Stanewsky *et al.*, 1998).

The LL behaviour allows for an assessment of the *cry* mutants that is more binary in nature (rhythmic or arrhythmic) and thus is easier to assess. These experiments reveal that:

- Wild type *cry* and S.D rescue the cry^{02} phenotype
- V.K, E.P and GFPcry-CT fail to rescue

If we couple this result with the yeast data published (Hemsley *et al.*, 2007), where the S.D mutant displayed an increased ability to bind TIM and this interaction was abolished in the E.P mutant, then it would seem that the V.K and *GFPcry-CT* mutant also lack the ability to bind TIM, or that they can bind TIM but this does not manifest itself as behavioural arrhythmicity. Confirmation of TIM binding could be attempted in yeast two-hybrids for V.K and *GFPcry-CT*. One potential caveat is the presence of the

LS-*tim* allele, which the V.K, E.P and GFPcry-CT lines all carry; however, the LS-tim allele was shown to have no effect on the LL phenotype (Tauber *et al.*, 2007) and the driver line was homozygous for the S-*tim* allele, so overexpressed mutant lines are heterozygous in *tim* composition. However, it would be interesting to follow up this work by examining the ability of each mutant to respond to a light pulse. If this work was to be attempted, then it would be cleaner to place each mutant in either a homozygous S-*tim* or LS-*tim* background. Moreover the gene *jet* has been implicated in LL rhythmicity and although the *jet* background of the cry^{02} has been published as free from *jet* mutations, the *cry* mutant lines have not been examined (Dolezelova *et al.*, 2007). This would be an important step in verifying that the V.K, E.P and GFPcry-CT do not carry *jet* mutations that would confound the LL analysis: this is unlikely in the wild type and S.D lines as they show arrhythmicity.

Each of the mutants was designed for specific purposes which will now be discussed individually. The S.D was designed to test the importance of a serine phosphorylation. It appears mutating this residue has no impact on the ability of *cry* to sense light, but the LL data did show a trend with a greater percentage of arrhythmic flies with *cry* S.D, although not significantly different from wild type. If the yeast data is correct then the S.D mutant may bind TIM more effectively, this could be tested by light pulse experiments with lower light levels or shorter duration times, coupled with experiments analysing TIM levels run in parallel conditions; this may reveal a difference between wild type and *cry* S.D in light sensitivity/TIM degradation dynamics. Although the LL data is comparable between wild type and S.D is similar, the DD data is not, as S.D influenced the period in more driver lines than wild type,

whether this reflects an increased ability of S.D to partake in a specific *cry* mediated dark signalling pathway or if this is a merely an off target clock effect is not clear at this stage.

The V.K mutant adds a positively charged residue into the PDZ domain of *cry*, in doing so we see that V.K cannot rescue the behavioural arrhythmicity normally seen in LL conditions. Thus we may have identified a residue important for light regulation; when this residue is removed in the CRYΔ mutant (as well as 19 others) *cry* is capable of constitutive binding of PER and TIM. Therefore it seems unlikely that the V residue is normally important for direct binding of PER and TIM as it is absent in the CRYΔ mutant. It is conceivable that this site controls interactions with other proteins but it is unclear at this what these proteins may be. The E.P mutation gives the same LL phenotype as the V.K mutant, furthermore E.P mutations have been shown to abolish binding of TIM and PER; this is likely due to an alteration in secondary structure. Again the E.P motif is absent in CRYΔ and thus it is possible that disrupting this sites has downstream consequences on *cry's* ability to bind TIM and PER effectively preventing arrhythmicity in LL. Analysing the levels of TIM in LL for both mutants would confirm that TIM is not being degraded like in wild type flies. The stability of E.P and V.K could also be addressed in LL, perhaps these mutants are more susceptible to light induced degradation? Again the DD data is similar for both mutants with overexpression causing period increases (exception: cry39,V.Ks61), it seems probable due to the similarity in LL data that they could be causing the period increases seen in DD through the same mechanisms.

Finally the CT of *cry* was tested to ask if it could influence behaviour without the N-terminus. The DD data suggests that it may, but is driver dependent with TG4 showing modest increases and PDF and *cry39* showing larger ones. The LL behaviour was not rescued, but there was a non-significant trend in the data suggesting that overexpressing the mutant made the flies more arrhythmic. One potential use of this mutant is as a potential tool to analyse proteins that may interact with CRY. In chapter 4 the technical challenges of co-IP with CRY were highlighted, by expressing only the CT of CRY it may be possible to confirm interaction of this mutant with known CRY binding proteins, if this was successful then maybe GFPcry-CT could be used as a tool to identify more proteins that are capable of binding the CT of CRY that have not been characterised. If successful we could also use GFPcry-CT to confirm an interaction with DAY.

10 Discussion

DAY locus and protein work

The identification of a novel protein interacting with CRY in a heterologous system was the bedrock for the research undertaken to characterise and define this relationship functionally. Trying to decipher a role for *day* specifically in *cry* related processes is challenging due to the broad range phenotypes that are altered after *cry* is manipulated (Emery *et al.*, 2000a; Emery *et al.*, 1998; Stanewsky *et al.*, 1998; Dolezelova *et al.*, 2007; Fogle *et al.*, 2011; Collins *et al.*, 2006). The primary goal of this research was to identify and examine the role of *day* with particular emphasis on behaviour and *cry* associated phenotypes. In order to address such concerns a number of unique transgenic constructs were designed and tested, while examinations at both the RNA and DNA level were undertaken to explore key features of this gene.

Examining the *day* gene was an important task, although the establishment that the CDS sequence of *day* was expressed in adult *Drosophila* heads was work that had been done in our laboratory previously analysing cDNA, it was important to revisit this finding due to a number of updates made at the *day* locus. In doing so the *day* CDS was again amplified successfully from cDNA, as was an additional 3' non-coding exon previously identified on 'Flybase'; its presence is confirmed in our analysis. The intron boundaries of *day* were also examined to ensure that the CDS was not longer than had already been reported, this data confirmed that the sequences analysed were not present in the CDS, however, a 5' non-coding exon was added to *day* suggesting that this approach or experiment design is not completely effective. Establishing the

features of the *day* locus was crucial for designing a number of the transgenic lines including UAS-*day and* UAS-STREP (II)*day* (CDS), the *day-GAL4* lines and the HR donor construct. For the UAS-*day* and HR flies these updates to the *day* locus do not appear to have any impact. For the *day-GAL4* this may not be the case as the largest GAL4 line made prior to the 5' update was 4.5 kb, this upstream sequence did not contain this 5' element, so a larger 6 kb sequence was cloned to incorporate this element. Although direct IHC comparisons have not been made between the two lengths it is probable that there will be different staining patterns between the two and this re-enforces the importance of working on a locus that is completely defined.

Analysis at the protein level with double tagged UAS-day constructs demonstrated that the STREP(II) tag had been successfully incorporated upstream of the *day* CDS, furthermore when analysed through western blotting with the TG4 line driving expression, it was shown that there was a DAY doublet formed where the second intron is spliced in or out; this result is confirmed by additional western blots performed previously. Exon splicing is known to be important for giving genes alternate functions and efficiencies, one such study in rats demonstrated that there was a difference between splice forms in assembling potassium channels resulting in varying electrophysiological responses (Godreau *et al.*, 2003). Although this was an exon splicing difference, intron retention has been shown to be important for determining tissue specific expression of the *erect wing* gene (Koushika *et al.*, 1999). The western blot analysis was performed on male and female heads; knowing that there is a sexual dimorphic expression differences using *day-GAL4* and that splicing is crucial in the sex determination pathway, which has consequences on courtship

behaviour, it may be interesting to ask if there are gender specific differences in the splicing of the *day* intron; sex determination splicing reviewed in (Black, 2003).

A co-IP experiment aimed at trying to identify CRY and DAY interaction was undertaken using the STREP(II)day transgenic lines; the double tagged construct was important as the DAY antibody generated does not function in western blots and the HA antibody appears at a similar band size to CRY. The data from the co-IP suggest that DAY and CRY can bind *in-vivo*. Experience in our laboratory suggests that co-IP experiments with CRY are challenging for reasons that are unclear although possibly related to oxidative state of CRY in light versus darkness and the number of transient interactions it may participate in. An attempt was made to address these problems as a unique construct, UAS-GFPcry-CT, was cloned encoding the CT of *cry* with upstream GFP and STREP(II) that can be used to pull down and detect this construct. The behavioural data available for this line suggest that this mutant may influence the clock. Performing yeast two-hybrid experiments with this mutant and CRY interactors such as PER, TIM and DAY would confirm that the CT of this mutant is capable of binding, then co-IP experiments with this line could be carried out which could reveal a number of unknown CRY binding proteins.

DAY function and expression

To address the function of *day* expression the gene was knocked out using a HR ends out approach. Doing so gave five lines of day^{0} flies, however, this technique, although effective in this instance, will most likely be superseded by the ZFN technique

described earlier (Beumer *et al.*, 2008). Confirmation of a successful knockout was performed using multiple PCRs; additional confirmation is revealed using the DAY antibody which no longer stains the antennal lobes in the day^0 lines unlike controls. Analysis of the locomotor behaviour of day^0 lines showed it was not significantly altered in LD and DD conditions at 25°C. When day^0 flies were kept in constant dim light at 20°C arrhythmicity was observed, thus forming a link between *day* and the circadian clock. The experiments such as overexpression of *day* which showed no change in behaviour could be re-addressed in these dim light conditions, perhaps revealing more interesting data on *day* function. Furthermore if day^0 is causing arrhythmicity then seems likely that it must be expressed in clock cells, the LN_ds seem probable targets as *day-GAL4* is expressed in one CRY expressing cell. The LN_ds are heterogeneous for three neuropeptides: neuropeptide F (NPF), short neuropeptide F (sNPF) and ion transport peptide (ITP) (Johard *et al.*, 2009). Again having identified sexual dimorphic expression of *day-GAL4*, a chill coma phenotype only in male flies and courting differences in day^0 male flies we postulate that this LN_d is likely to express NPF. There are three NPF positive LN_ds, but only one is cry^+; there is also NPF expression in the LN_vs (Lee *et al.*, 2006; Hermann *et al.*, 2012).

The locomotor behaviour of flies when NPF cells were manipulated showed a decreased evening peak and a normal period when the LN_ds expressing NPF are ablated (Hermann *et al.*, 2012). A direct comparison between this data and the results of *day-GAL4* [6] show some similarities, when the NPF expressing LN_ds were removed no change in period was observed, although not an identical manipulation we halted the clock in *day-GAL4* [6], UAS-*CLK*Δ flies and did not observe differences (Hermann *et*

al., 2012). Furthermore they found differences in the evening peak which we see in Figure 7-12 graph B, although not tested for significance, *day-GAL4* [6], *NaChBac* expression gave more activity at the evening peak. One difference between the activity profiles is that the *day-GAL4* [6] manipulations also gave differences in the morning peak (advanced), the LN_vs have been attributed to the control of the morning peak and it is conceivable that *day-GAL4* [6] expresses in the LN_vs cells (Grima *et al.*, 2004; Stoleru *et al.*, 2004). Thus these comparisons between NPF cells and *day-GAL4* [6] ones reveal some similarities, furthermore if we factor in the mating behaviour of day^0 flies where the timing of copulation length is affected we could conceive of a model where in day^0 flies NPF expression is altered. However, it would be important to verify the staining seen with *day-GAL4* [6] is really representative of *day* expression. The DAY antibody cannot accomplish this task as it produces images with insufficient staining intensity, furthermore it seems likely, due to the staining patterns/PDZ data, that DAY is not located in cell bodies and more at the termini of neurons; making overlapping staining more challenging. Promising data from the MiMIC lines showing very similar staining patterns between two independent insertions and some overlapping features with *day-GAL4* [6], indicating that we have adequate tools to verify the *day-GAL4* [6] expression data. Once a confirmed NPF/DAY/CRY cell is established then the courting behaviour and dim light phenotypes could potentially be followed up with rescue experiments for each phenotype.

Establishing a functional relationship between DAY and CRY was attempted by using the *day-Gal4* [6] driver to overexpress *cry* with or without *day*. This data revealed that without *day, cry* overexpression caused significantly increased morning

and evening peaks, there was also a loss of morning anticipation seen previously when expressing CRY in *day*$^{+}$. Thus the distribution of activity in the light and dark phase has been altered; these differences could be related to binding properties of DAY-CRY in either condition. A model portraying how this interaction may work is shown below.

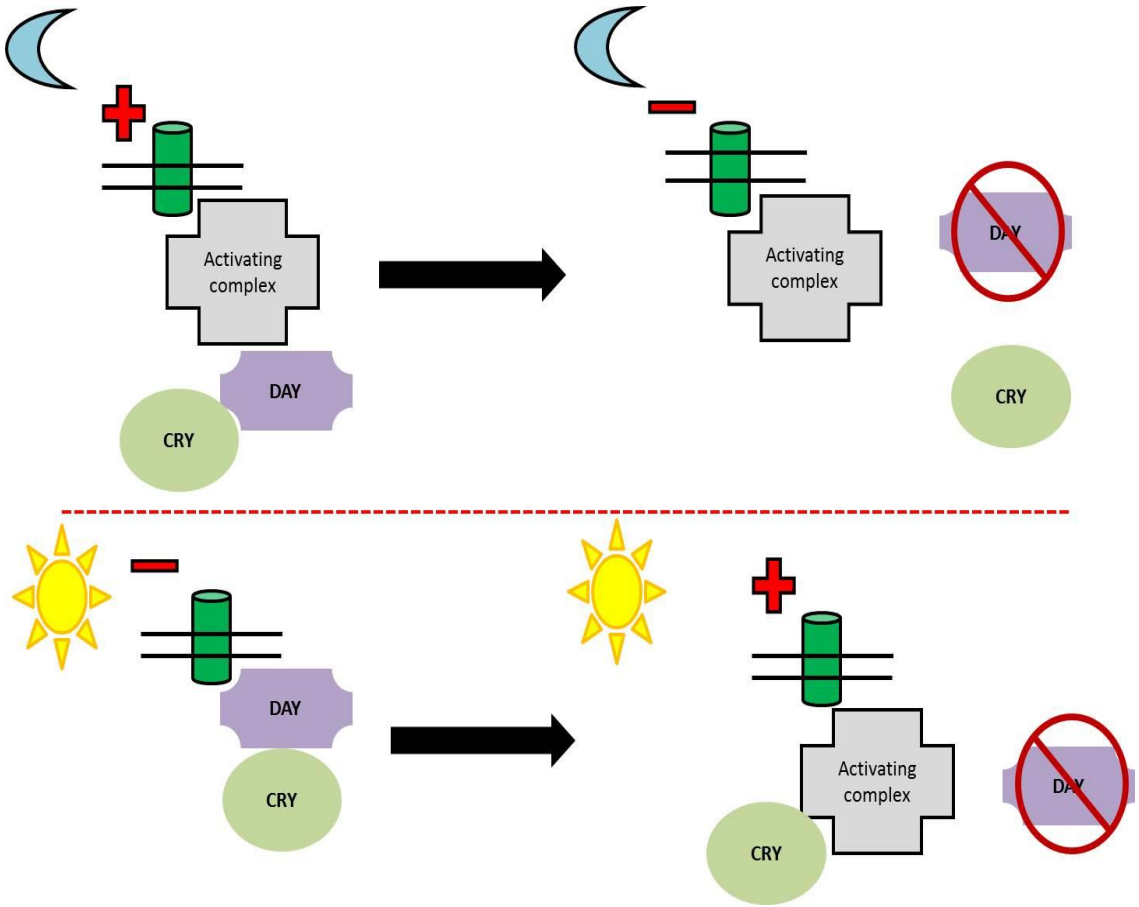


Figure 10-1: DAY CRY model.

Model depicting the possible light regulated mechanism of DAY CRY binding. Top half (above red line) show dark interaction where DAY and CRY binding promoting signalling, to the right in *day*^{0} flies this activation does not occur/inefficient. Bottom half light interaction, DAY binds CRY acting as a repressor, in *day*^{0} this repression does not occur and there is increased firing. Green column represent signalling channel, + and – denote activity at the membrane.

If *day* is responsible for regulating either of these responses (increased LD peaks and night time arousal) then why do day^{0} flies not show either of these phenotypes? The answer could be that the levels of endogenous *cry* do not reach sufficient levels or *cry* is not activated effectively, perhaps because these cells are more sensitive to dim light. Again *cry* will be expressed ectopically so there may be network interactions to take into account.

Cry mutants

Establishing a functional relationship between DAY and CRY would be important as we could then dissect differences using the *cry* mutants that we have characterised in this work. Each of the single base pair mutants could be tested for their ability to rescue the phenotypes shown when expressing *cry* using *day-GAL4* [6], determining the importance of the residue for these behaviours. The behavioural data collected showed that these single base pair mutations had consequences on their ability to sense light as well as producing behavioural differences in DD, dependent on what neuronal cell type they were expressed in and the *cry* context. Further studies could continue to examine these light responses and correlate differences with either molecular abnormalities or neuronal firing.

Therefore this work has attempted to identify and characterise the relationship between DAY and CRY, a lot has been accomplished in trying to achieve this goal, particularly in the generation of genetic tools. The results have also identified three different *day* phenotypes and it seems likely that at least one concerns CRY (dim light).

In that respect this work has been successful, however, the mechanisms concerning how *day* is involved in all these phenotypes still requires additional work. However, I am confident that with the tools accumulated and the leads gathered it would not be difficult to establish the importance of *day* in these behaviours.

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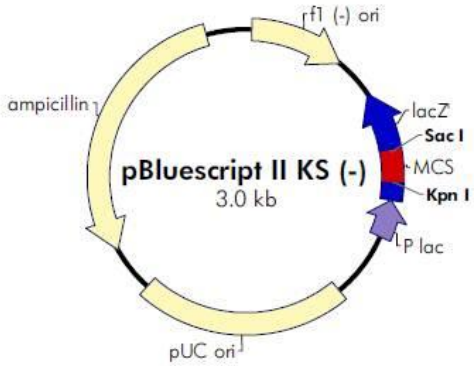
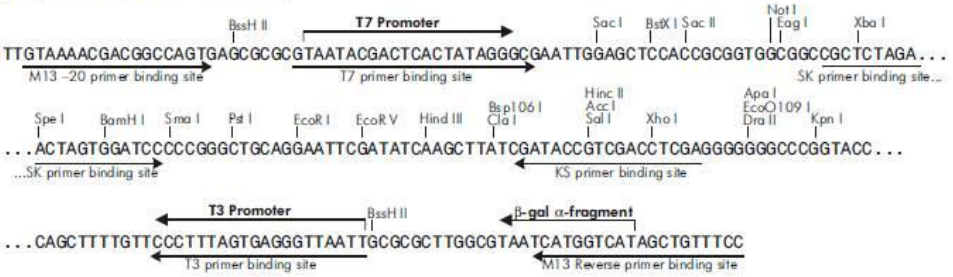
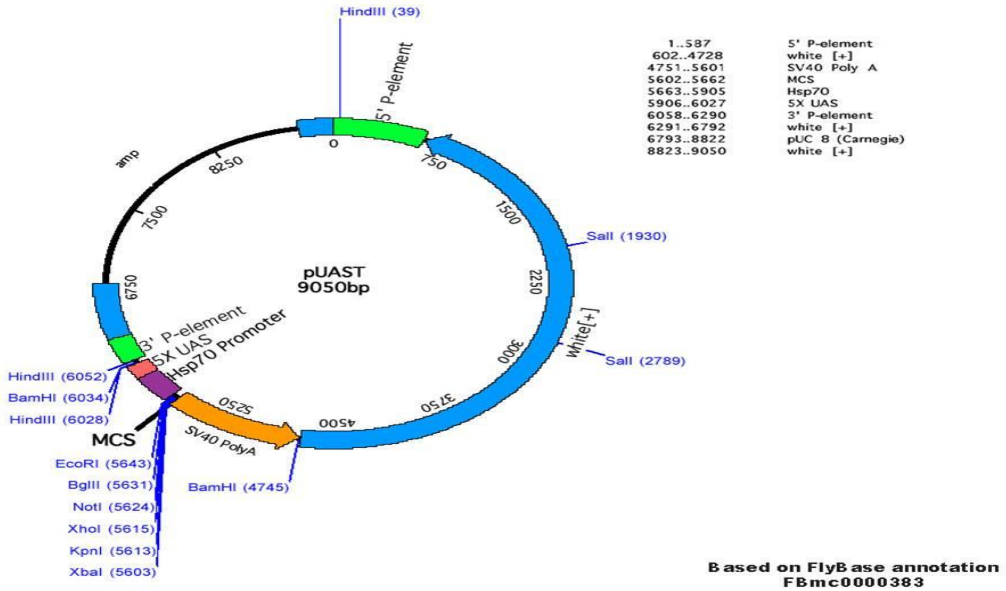
Appendix 1

f1 (–) origin 21–327
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



pBluescript II KS (+/–) Multiple Cloning Site Region (sequence shown 598–826)





Vector maps of PBS and p-UAST.

Appendix 2

List of transgenic stocks

Tagged HA-STREP(II)-DAY (DSII) lines genotype names

DSII F1, DSII F2, DSII F3- X chromosome

DSII S1, DSII S2, DSII S3, DSII s64, DSII S65- Second chromosome

DSII T2, DSII T4, DSII T5- Third chromosome

DAY GAL4 lines

1 kb

GAL4 1 kb F1, GAL4 1 kb F2- X chromosome, GAL4 1 kb S1, GAL4 1 kb S2, GAL4 S3- Second chromosome, GAL4 1 kb T1, GAL4 1 kb T2- Third chromosome.

1.5 kb

GAL4 1.5 kb F1, GAL4 1.5 kb F3- X chromosome, GAL4 1.5 kb S1, GAL4 1.5 kb S2, GAL4 1.5 kb S3- Second chromosome, GAL4 1.5 kb T1, GAL4 1.5 kb T2, GAL4 1.5 kb T3.

3 kb

GAL4 3kb F1, GAL4 3kb F2- X chromosome, GAL4 3 kb S1, GAL$ 3 kb S2, GAL4 3 kb S3- Second chromosome, GAL4 3 kb T2, GAL4 3 kb T3- Third chromosome.

4.5 kb

GAL4 4.5 kb F1, GAL4 4.5 kb F2, GAL4 4.5 kb F3- X chromosome, GAL4 4.5kb S1, GAL4 4.5 kb S2, GAL4 4.5 kb S3- Second chromosome, GAL4 4.5 kb T1, GAL4 4.5 kb T2, GAL4 4.5 kb T3 – Third chromosome.

6 kb

GAL4 6 kb F1, GAL4 6 kb F4, GAL4 6 kb F6- X chromosome, GAL4 6 kb S2, GAL4 6 kb S5- Second chromosome, GAL4 3T, GAL4 62T- Third chromosome.

Homologous recombination lines

FRP52 1, FRP52 2, FRP52 3, FRP52 4, FRP52 6.

FRP52/CyO-Second chromosome, line used for HR.

CRY mutants

S.D

crySD8f(x) - X chromosome, crySD2M, crySD4M, crySD6M, crySD9M- Second chromosome

V.K

cryV.K F1, cryV.k F2, cryV.K F3- X chromosome, cryV.K S1, cryV.K S2, cryV.K S3- Second chromosome, cryV.K T1, cryV.K T3-Third chromosome.

E.P

cryE.P S1, cryE.P S2, cryE.P S3- Second chromosome, cryE.P T1, cryE.P T3- Third chromosome.

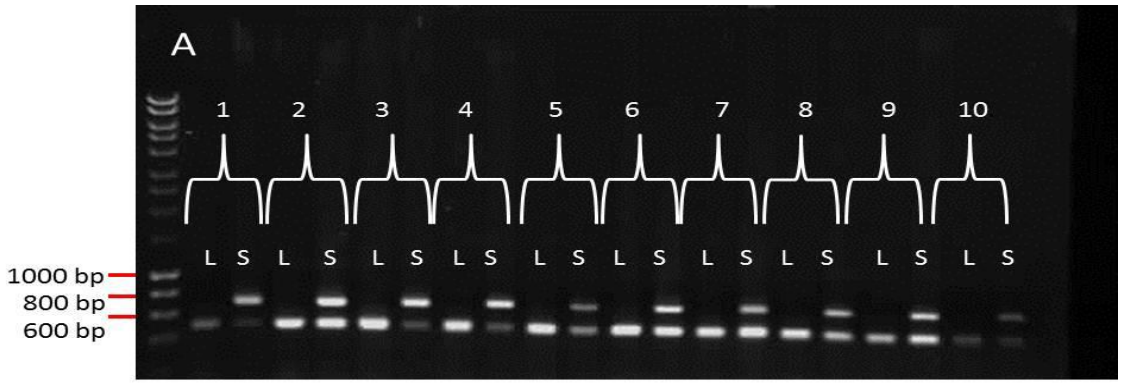
GFPcry-CT

GFPCRYCT 1F, GFPcry-CT 3F- X chromosome, GFPcry-CT 10S, GFPcry-CT 23S- Second chromosome, GFPcry-CT 6T, GFPcry-CT 7T, GFPcry-CT 9T, GFPcry-CT 12T- Third chromosome.

day zero lines

w;;G, *w*;;M, *W*;;O, *W*;;P, *w*;;U, Third chromosome.

Appendix 3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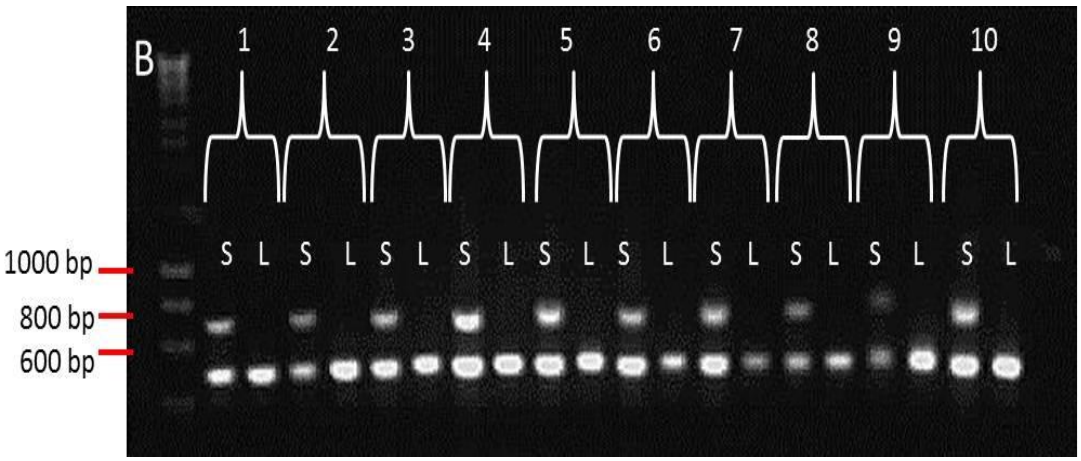
800 bp
600 bp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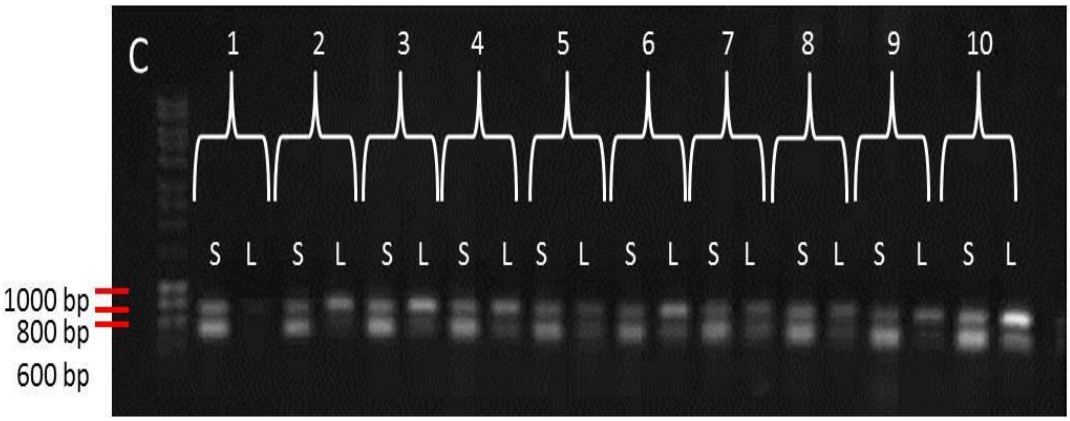
800 bp
600 bp


C
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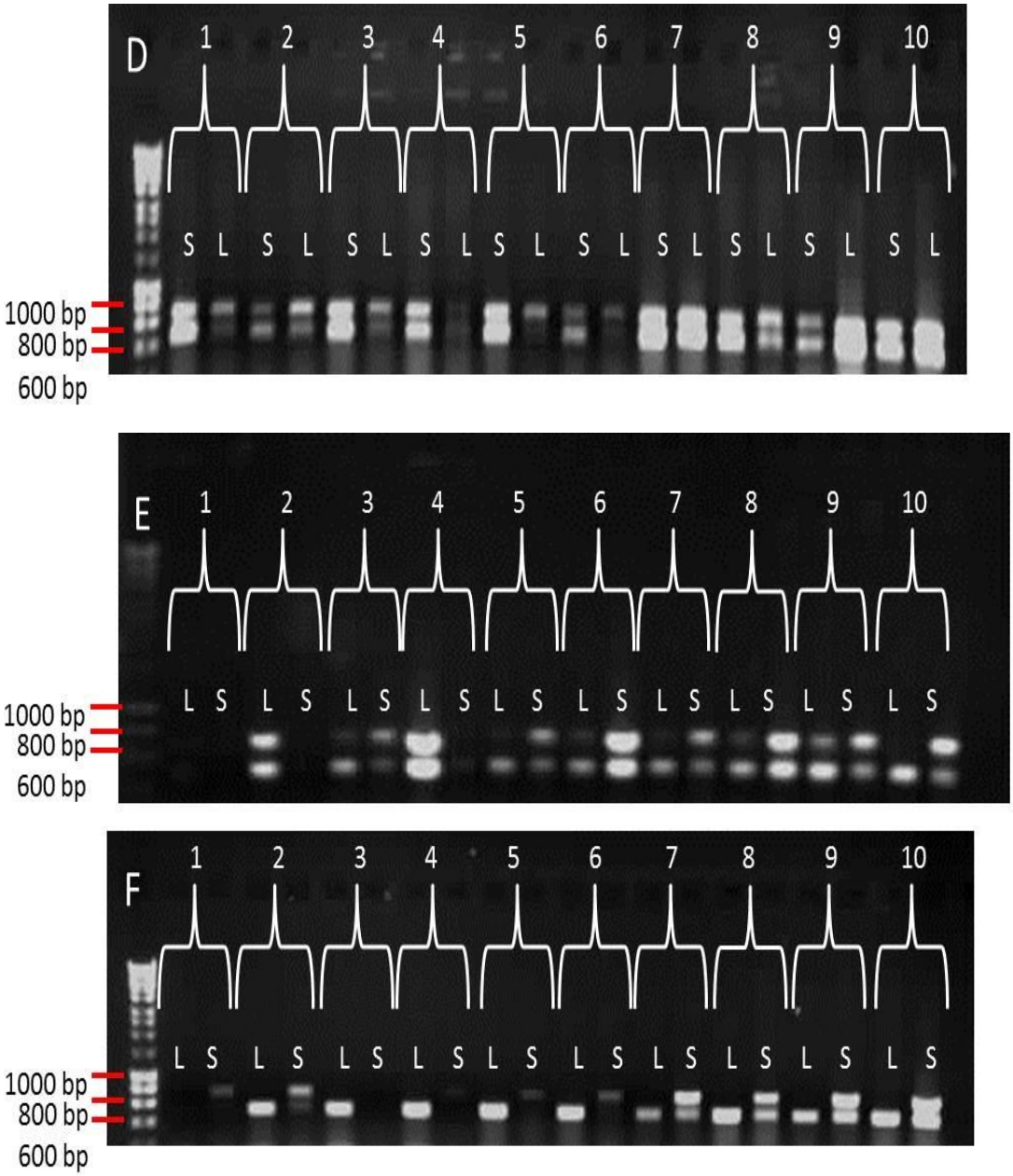
1000 bp
800 bp
600 bp



Genotyping of LS *tim* and S-*tim* alleles.

Marker is hyper ladder, ten flies used for each genotype numbered 1-10, S = S-*tim*, L = LS-*tim*. A: *w*;crySD4M/TG4;*cry*^{02} genotype= S-*tim*, B: *w*;cry24B/TG4;*cry*^{02} = S-*tim*, C: *w*;cryV.Ks62/TG4;*cry*^{02} = LS-*tim* and S-*tim*, D: *w*;cryE.Ps61/TG4;*cry*^{02} = LS and S-*tim*, E: UAS-GFPcry-CT 1F;;*cry*^{02} = LS and S-*tim*, F: *w*;TG4;*cry*^{02} = S-*tim*.