A putative role for TANGO in the circadian clock of *Drosophila melanogaster*

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Abstract

A putative role for TANGO in the circadian clock of *Drosophila melanogaster*

Luiz Guilherme Bauzer

Living organisms can anticipate predictable environmental cycles using self-sustained pacemakers found in the brain and in a variety of tissues. This internal clock generates rhythms that can persist even in the absence of environmental time cues. For the circadian clock to be functional it must have the ability to entrain, i.e. to respond to environmental cues such as the 24 h light-dark cycle. The clock mechanism involves negative feedback loops associated with daily oscillations of several genes expression at both the RNA and protein levels. The mammalian *Aryl Hydrocarbon Receptor Nuclear Translocator* (*ARNT*) homologous gene *tango* (*tgo*) is essential for normal *Drosophila melanogaster* development. TANGO (TGO) is a transcription factor, which belongs to the PAS (Per-Arnt-Sim) superfamily and, in the yeast-two- hybrid system, physically interacts with the circadian proteins CLOCK (CLK) and CRYPTOCHROME (CRY), suggesting a possible role for *tgo* in the circadian clock. Miss-expression of *tgo* does not interfere with the 24h period of locomotor activity but causes abnormal behavioural responses to light. However, the generalised reduction of TGO promotes an internal desynchronization between the morning and evening clock oscillators, suggesting that TGO might be involved in neuronal crosstalk. Moreover, miss-expression of *tgo* seems to promote an advance in the phase of expression of the TIMELESS protein, a key regulator of circadian light-responses in *Drosophila*. Finally, TGO was shown to be involved in sleep regulation, as revealed by a reduction in the amount of sleep in flies overexpressing or down-regulating this protein. In conclusion, this study indicates that TGO does not control the inner molecular cogs of the clock but strongly suggests a potential function for TGO in the input and/or output mechanisms and also a role in the sleep regulation process.

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Chapter 1: Introduction

1.1 Biological rhythms

Ever since life first appeared on Earth organisms have been living in time structured environments. Astronomical generated rhythms have strong effects on organisms living in a range of habitats as diverse as lands and oceans. Only deep soil/oceanic forms of life can escape from environmental rhythms such as daily cycle of light and darkness, phases of the moon, tides and seasons of the year, which are imposed by the Earth movement around the Sun and the rotation around its tilted axis.

A common feature of astronomical generated rhythms is their precision and regularity. Organisms cannot evolve adaptations to rare and unpredictable events but they can easily adapt to predictable and rapid cycles in the environment. Repetitive external conditions are a rich source of information that creates opportunities to be exploited. It is extremely advantageous to the organisms to be able to predict changes before they occur. An organism that is physiologically prepared before the external changes occur (i.e. sun rise, drop in temperature, food availability, predator avoidance, etc) has a bigger chance to leave progeny, while the others can go extinct. Therefore it is not surprisingly to observe that high eukaryotes (and some prokaryotes) can anticipate predictable cyclic events, an extraordinary adaptation to the challenges posed by the environment. Biological rhythms have evolved to match a quite impressive range of periodicity. Those matching the 24 hours period of the Earth's rotation around its axis are called circadian rhythms (in Latin *Circa* = about, *Diem* = day). Endogenous oscillations evolved to match less than 24-hours periodicity, such as those from tidal (~12.4 h) cycles, are denoted ultradian rhythms. Finally, cycling biological phenomena possessing a period longer than 24 hours, such as the lunar (~29.4 days) or annual (~a year)

cycles, are defined as infradian rhythms. Nothing is known about the molecular architecture of ultradian and infradian clocks, hence they will not be discussed here any further

1.2 Biological clocks

The competitive advantage of possessing an endogenous anticipatory system promoted the evolution of biological clocks (Pittendrigh, 1965; 1967; 1993), which are inherited timing devices controlling rhythms of many physiological and behavioural functions. One of the most conspicuous characteristics of biological clocks is the self-sustaining oscillations. They are found in a variety of tissues driving rhythms that persist in the absence of environmental time cues generating natural free-running periods (τ) close to, but generally different from that of the solar day.

For the clock to be functional and to accurately measure the time, temperature compensation is also required. Living organisms more than double the rate of biochemical processes with every 10°C rise in temperature (Saunders *et al.*, 2002). This fact would make time measurement an impossible task without the temperature compensation properties of the clock. During evolution this challenge has been met. However, the detailed manner in which temperature compensation is achieved in biological clocks remains obscure (Huang *et al.*, 1995; Kurosawa and Iwasa, 2005; Peixoto *et al.*, 1998; Sawyer *et al.*, 1997).

Another property of the biological clock is the ability to be entrained, i.e. to respond to environmental cues in a process by which extrinsic stimuli change or reset its phase. Through entrainment the clock synchronise to environmental variables so that its period becomes equal to the period of the entrainment agent, the *Zeitgeber* (from the German for time giver), and adopts a fixed phase relationship with it. Multiple independent oscillators can be found throughout the body and they are controlled by different inputs. As a consequence, rhythms

will also entrain to non-photic *Zeitgebers* including cycles of temperature, food availability, social interactions and some physiological or pharmacological agents (Ben-Sholomo and Kyriacou, 2002; Castillo *et al.*, 2004; Duffy *et al.*, 1996; Gardani *et al.*, 2005; Joy and Turek, 1992; Levine *et al.*, 2002; Mistlberger and Skene, 2004; Rensing and Ruoff, 2002; Stokkan *et al.*, 2001; Turek *et al.*, 1995; Yoshii *et al.*, 2005).

A master clock governs peripheral tissues rhythms in several organisms. The anatomical location of the central circadian pacemaker is known for most animals whereas plants appear to lack central pacemakers. The current conceptual view of the molecular clock is based on the hypotheses that rhythmicity is generated by a negative feedback loop operating through the self-regulated expression of clock genes and their protein products using transcriptional and translational checkpoints (Allada, 2003; Allada *et al.*, 2001; Blau, 2003; Stanewsky, 2003; Young and Kay, 2001). The known molecular feedback loops in different species are strongly related to the light input pathways. This fact leads to the assumption that the clock evolved from light transduction mechanisms (Crosthwaite *et al.*, 1997).

Biological clocks control a wide variety of behaviour and physiological activities in living organisms. In insects, daily rhythms of locomotor activity, feeding, mating, olfaction, emergence from the pupal case and seasonal control of alternate development pathways, are all processes under clock control amongst several others (Saunders *et al.*, 2000). The biological clock also controls rhythms in vector-borne disease transmission that affects millions of people all over the world. Due to its importance, several species of insect vectors have had their activity rhythms described but very little is know concerning the molecular components of the circadian clock in this group of organisms (Meireles-Filho *et al.*, 2006). Leaf movements, photosynthesis and flowering time are amongst the common manifestations of the biological clock in plants. Disruption of clock function in mammals results in abnormalities in many physiological functions, resulting in increases in risks of

cardiovascular and gastrointestinal diseases, sleep abnormalities and cancer (Hastings *et al.*, 2003; Fu and Li, 2003). In humans, the most frequent effect experienced by the clock disruption is jet lag, characterized by several physiological disturbances after travelling across different time zones. Also, human sleep disorders such as familial advanced sleep phase syndrome (FASPS) and delayed sleep phase syndrome are linked to defective clock functions (Toh *et al.*, 2001; Archer *et al.*, 2003). Finally, recent works (Gorbacheva *et al.*, 2005; Green, 2005) have shown that sensitivity to chemotherapeutic agents depends on the specific molecular state of the circadian clock. All different fields mentioned above underlies the importance of understanding how biological clocks work.

1.3 The *Drosophila* circadian clock

1.3.1 Oscillator

Drosophila melanogaster is an outstanding model organism for studying many areas of biology, including circadian rhythms (the history of which was chronicled by Weiner, 1999). Several genes involved in interconnected transcriptional-translational feedback loops that constitute the inner part of the clock has been characterized (reviewed in Chang, 2006; Hall, 1995; 1996; Hardin, 2005; Helfrich-Foster, 2005, Rosato *et al.*, 2006; Rosbash *et al.*, 1996; Sehgal *et al.*, 1996; Stanewsky, 2002; Zordan *et al.*, 2003). Interestingly, the principle of the molecular feedback loop is used also in circadian oscillators of fungi, plants, and vertebrates, with the molecular components being remarkably conserved between fruit flies and mammals (reviewed in Allada *et al.*, 2001; Dunlap, 1999; Young and Kay, 2001). The mechanism of the molecular clock is conceptually quite simple; oscillation of the mRNA level is the first step toward sustainable molecular rhythms. Post-transcriptional and post-translational controls accomplish the circadian clock by affecting protein stability (Price *et al.*,

1998; Sathyanarayanan *et al.*, 2004; Suri *et al.*, 1998) or controlling subcellular localization (Cyran *et al.*, 2005; Martinek *et al.*, 2001; Saez and Young, 1996).

The first clock component to be characterized was the *period* (*per*) locus, identified by chemical mutagenesis analysis of the fly X chromosome (Konopka & Benzer, 1971). The initial character used for the screen was emergence from the pupal case of fly populations. *per* was also characterized as a fundamental regulator of the daily rhythm of locomotor activity (Konopka & Benzer, 1971). The successful identification of *per* spawned subsequent genetic and biochemical screens to identify additional circadian clock components. Mutants for the autosomal locus *timeless* (*tim*) were found to show molecular rhythms similar to *per* mutants, exhibiting characteristics indicative of a true clock component (Sehgal *et al.*, 1994). Positional cloning was used to isolate *tim* (Sehgal *et al.*, 1995) which was cloned (Myers *et al.*, 1995) and whose product was identified as an interaction partner of PERIOD (PER) during a yeast two-hybrid screening (Gekakis *et al.*, 1995). PER protein belongs to the PAS domain super family (see section 1.4 of this chapter), a group of proteins involved in the detection of, and adaptation to, environmental changes (Gu *et al.*, 2000; Huang *et al.*, 1993; Lindebro *et al.*, 1995; Pellequer *et al.*, 1998; 1999; Taylor and Zhulin, 1999). PAS are interaction domains through which PER interacts with TIMELESS (TIM) (Sehgal *et al.*, 1994). So far those are the only motifs known to be conserved among widely diverse clock proteins (Taylor and Zhulin, 1999).

The *Drosophila* circadian oscillator is composed of two intracellular feedback loops, the first involving *per/tim* genes and the second based upon the cycling of expression of the *Clk* gene (Glossop *et al.*, 1999; Hardin *et al.*, 1990). Figure 1.1 shows a generalized molecular circadian clock model with the principal components of the *per/tim* feed-back loop. Under exposure to cycles of 12 h light and 12 h dark (12:12 LD), both *per* and *tim* genes are rhythmically expressed and their mRNAs reach maximum levels at the beginning of the night.

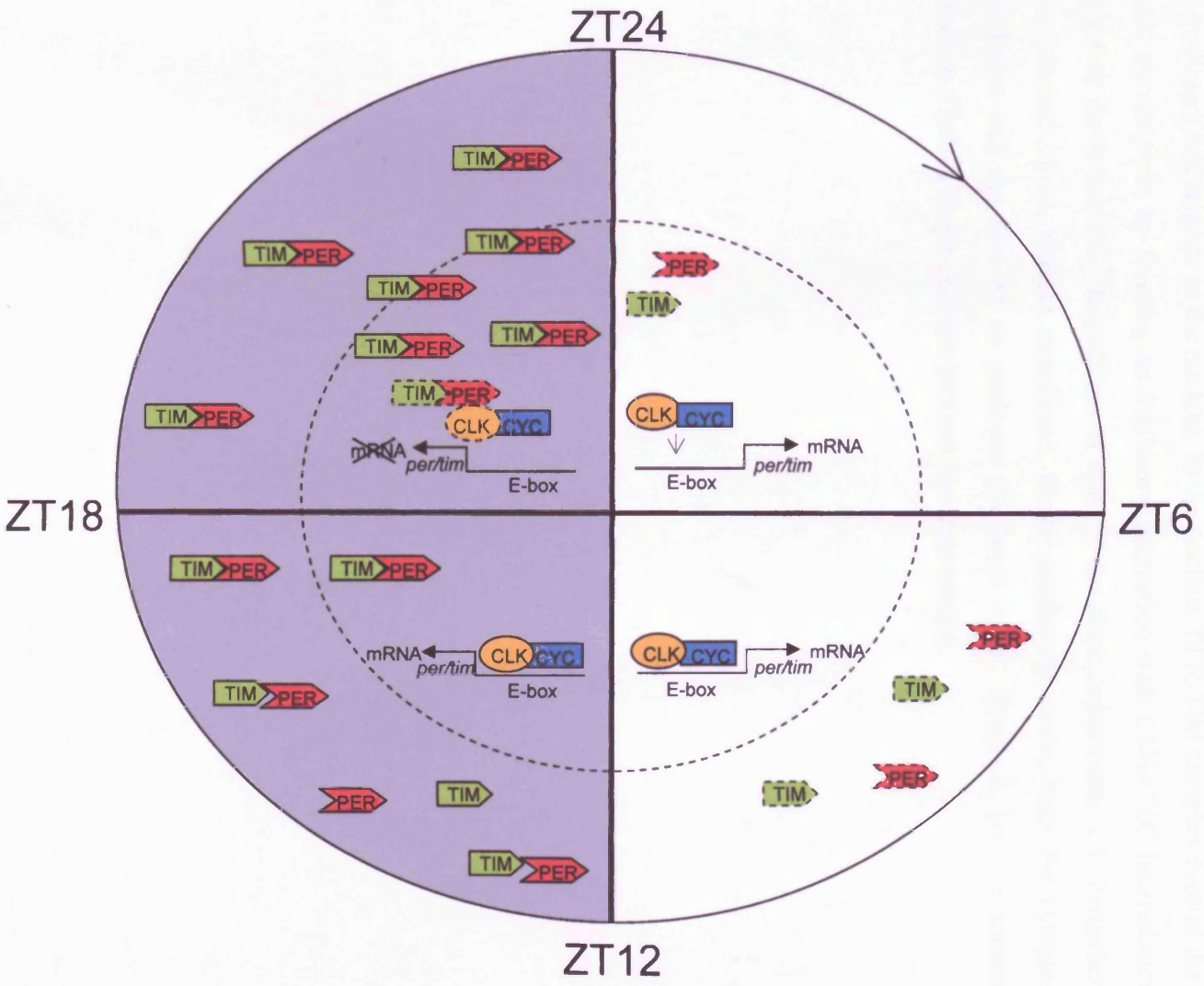

ZT24
ZT18
ZT6
ZT12
TIM
PER
CLK
CYC
mRNA
per/tim
E-box

Figure 1.1

A generalized molecular circadian clock model showing the principal components of the *Per/Tim* feed-back loop. Gray and white semi-circumferences represent the scotophase and photophase in a 12: 12 h light-dark cycle, respectively. TIM and PER start to accumulate at the beginning of the night, as indicated by the clock section between the points ZT12 and ZT18. At the second half of the night, PER and TIM reach their maximum level and form heterodimers that migrate to the nucleus. In the nucleus, PER-TIM complex control their own genes transcription by forming an inhibitory interaction with CLK-CYC heterodimers and triggering the proteasome degradation of most of the clock components. ZT, *Zeitgeber* time. Inner dashed circle, nuclear membrane. Outer continuous circle, both the cytoplasmatic membrane and the face of an analogue 24 hours clock. Black X, *per/tim* transcription cessation. Dashed shapes, protein proteasomal degradation.

PER and TIM proteins also cycle showing a peak of expression with a 4 to 6h lag, towards the end of the night. During the late day/early night, the concentration of PER and TIM in the cytoplasm is high enough to favour formation of a PER-TIM complex. The formation of this dimer is delayed or accelerated by the mammalian CASEIN KINASE 1 ε (CK1ε) homolog DOUBLETIME (DBT) (Kloss *et al.*, 1998; Price *et al.*, 1998) and PROTEIN PHOSPHATASE 2A (PP2A) (Sathyanarayanan *et al.*, 2004), that shortens or lengths circadian period by differential phosphorylation of PER. The premature degradation of PER in the cytoplasm requires the ubiquitin ligase product SUPERNUMERY LIMBS (SLIMB) (Grima *et al.*, 2002; Ko *et al.*, 2002) and it ceases only with the build up of TIM, itself phosphorylated by the product of the GLUCOSE SYNTHASE KINASE 3 (GSK3) homolog SHAGGY (SGG) (Martinek *et al.*, 2001). TIM phosphorylation by SGG promotes nuclear localization of PER-TIM heterodimers, which migrates to the nucleus in the middle of the night as a result of their mutual stabilisation and cooperation (Ashmore *et al.*, 2003). Molecular details of this process are still under debate (Meyer *et al.*, 2006). PER and TIM proteins lack conventional DNA-binding sequences and therefore, in the nucleus, they regulate their own transcription by blocking the action of CLOCK (CLK) (Allada *et al.*, 1998; Darlington *et al.*, 1998; King *et al.*, 1997) and CYCLE (CYC) (Rutila *et al.*, 1998), two basic-helix-loop-helix (bHLH)/PAS transcription factors. As a heterodimer with CYC, CLK binds regulatory elements, called E-boxes, which are known to be binding sites for bHLH transcription factors and are present in the promoter region of several rhythmically expressed genes within and downstream of the circadian clock (Darlington *et al.*, 1998; Hao *et al.*, 1997; Lee *et al.*, 1999). Once in the nucleus, PER is eventually degraded by DBT phosphorylation, a process that reactivates transcription in the *per/tim* feedback loop where non-repressed CLK heterodimerize with CYC (via PAS) binding to CACGTG E-boxes in the *per* and *tim* promoter regions (Lee *et al.*, 1999). Whilst CYC is constitutively expressed (Glossop *et al.*, 1999), it was believed for a long time that CLK abundance cycled with a peak during late

night/early morning and a trough during the late day/early night (Lee *et al.*, 1998). However, it was recently shown that constitutive levels of nuclear CLK regulate rhythmic transcription in circadian oscillator cells (Houl *et al.*, 2006).

PER and TIM are necessary for rhythmic transcription of *Clk* (Bae *et al.*, 1998) and, not surprisingly, the *per/tim* loop is coupled to a second one via CLK. Figure 1.2 shows a generalized molecular circadian clock model with the principal components of the *Clk* feedback loop. Under 12:12 LD cycles, CLK/CYC binds E-boxes driving high level expression of *vrille* (vri) and *Par domain protein 1* (*Pdp1*) mRNA during the day and the early night (Blau and Young, 1999; Cyran *et al.*, 2003; Glossop *et al.*, 2003). PDP1 has been hypothesized to be the direct activator of *Clk* transcription, while VRI seems to be the repressor (Cyran *et al.*, 2003). In this model, VRI accumulates in phase with its mRNA and binds the VRI/PDP1 box (V/P box) regulatory elements in the promoter region of *Clk*, thus inhibiting its transcription (Cyran *et al.*, 2003; Glossop *et al.*, 2003). PDP1 accumulates to high levels during the mid to late evening reactivating *Clk* transcription through antagonistic binding to V/P boxes (Cyran *et al.*, 2003).

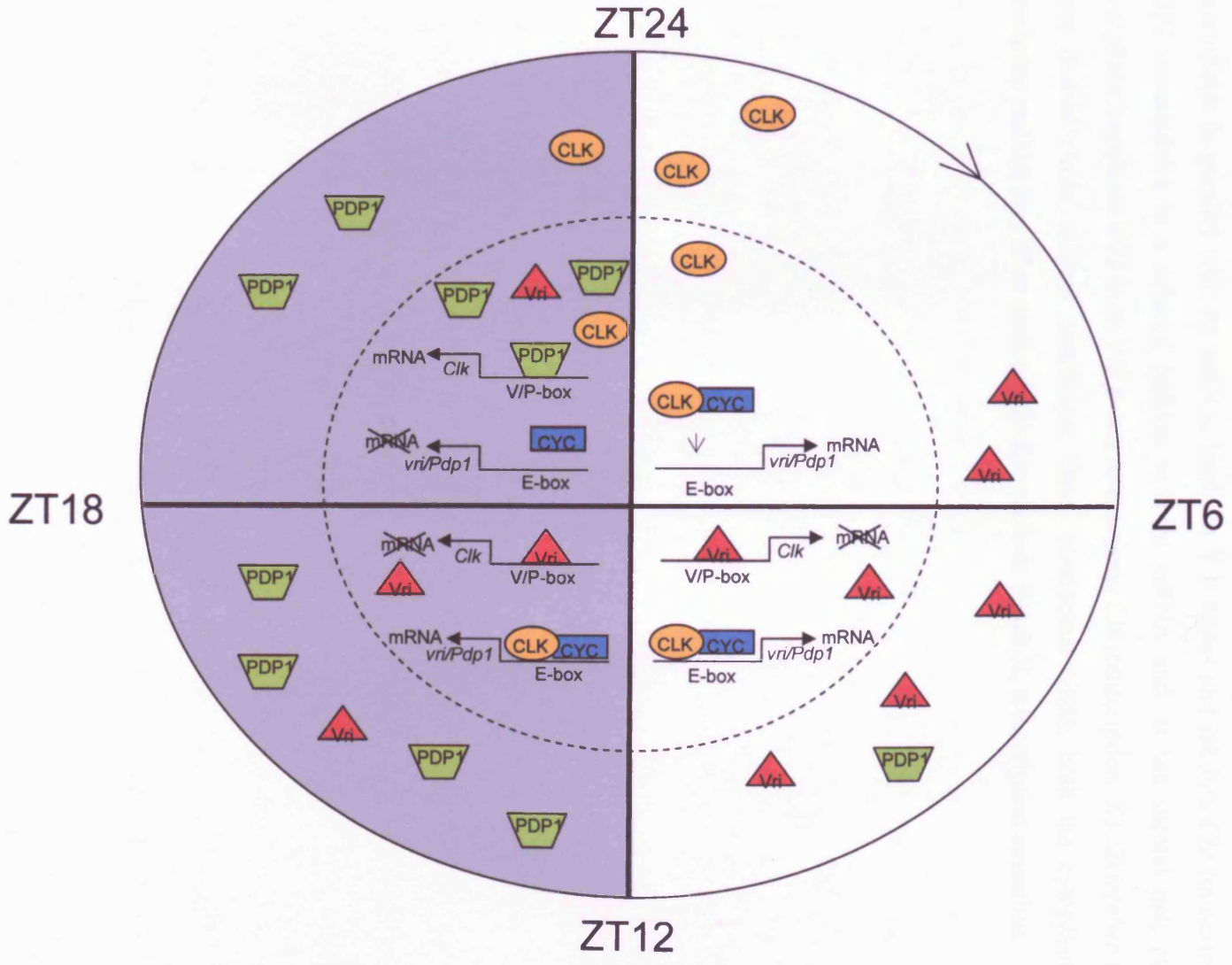

ZT24
ZT18
ZT6
ZT12
CLK
PDP1
Vri
mRNA
Clk
V/P-box
CYC
vri/Pdp1
E-box

Figure 1.2

A generalized molecular circadian clock model showing the principal components of the *Clk* feed-back loop. Gray and white semi-circumferences represent the scotophase and photophase in a 12: 12 h light-dark cycle, respectively. CLK-CYC heterodimers bind to E-boxes and activate *vri* and *Pdp1* transcription at the first half of the photophase. VRI protein accumulates in parallel with its mRNA, binds to V/P boxes and inhibits *Clk* transcription. PDP1 accumulates in a delayed fashion with its mRNA and, at the second half of the scotophase, supplants VRI from V/P boxes to derepress *Clk* transcription. ZT, *Zeitgeber* time. Inner dashed circle, nuclear membrane. Outer continuous circle, both the cytoplasmatic membrane and the face of an analogue 24 hours clock. Black X, transcription cessation.

1.3.2 Neuro-architecture

The use of antibodies raised against clock proteins and the expression of reporter genes under clock promoters have shown the wide distribution of clock cells all over the fly body and at all stages of development, from embryo to adult (Plautz *et al.*, 1997; Sheeba *et al.*, 2001). Studies elucidating the pattern of expression of clock genes are most abundant for *per* (Ewer *et al.*, 1992; Frisch *et al.*, 1994; Kaneko *et al.*, 1997; Kaneko and Hall, 2000; Liu *et al.*, 1988; 1992; Saez and Young, 1988; Siwicki *et al.*, 1988; Stanewsky *et al.*, 1997; Zerr *et al.*, 1990) and to a lesser extent for *tim* (Hunter-Ensor *et al.*, 1996; Kaneko *et al.*, 1997; Kaneko and Hall, 2000; Myers *et al.*, 1996; Yang *et al.*, 1998). Only a few studies have investigated the expression of *vri* (Blau and Young, 1999), *dbt*, *Clk* and *cyc* (Houl *et al.*, 2006; Kloss *et al.*, 1998; So *et al.*, 2000).

In general, these studies have revealed that the expression of clock genes is quite widespread, being found in a variety of neuronal and non-neuronal tissues in the fly head and body (Liu *et al.*, 1988; Plautz *et al.*, 1997; Siwicki *et al.*, 1988). In the adult head, PER is localised in photoreceptors cells of the compound eyes, the ocelli and the extraretinal eyelet Hofbauer-Buchner organ (Helfrich-Forster *et al.*, 2002). The last is derived from the Bolwig's organ, a larval photosensory system (Malpel *et al.*, 2002). Many glia cells also express PER and, finally, strong expression is found in six clusters of neurons that due to their anatomical position have been called the Lateral Neurons (LNs, three clusters) and the Dorsal Neurons (DNs, three clusters) (Siwicki *et al.*, 1998; Zerr *et al.*, 1990). Outside the head PER is expressed in the gut, salivary glands, gonads, fat body, malphigian tubules and epidermis (Saunders *et al.*, 2002), suggesting the existence of peripheral clocks that contributes to the physiology of the organism (Giebultowicz, 1999; 2000). With the help of an *in vivo* luciferase assay, PER oscillation was demonstrated even in living individual flies under free-running conditions (Brandes *et al.*, 1996; Plautz *et al.*, 1997; Stanewsky *et al.*, 1997).

Clock genes expression in specific neurons in the lateral and dorsal brain generates cell-autonomous molecular circadian oscillations. These clusters are connected to each other and contribute differentially to the control of behavioural rhythmicity (Helfrich-Forster, 2003). Figure 1.3 shows the anatomical distribution of cells expressing clock genes in the *Drosophila* brain. The adult brain expression patterns of *per* and *tim* are virtually identical. Three cells in the lateral posterior brain, the Lateral Posterior Neurons (LPNs), were previously thought to be immunoreactive with antisera against TIM but not to contain noticeable amounts of PER (Kaneko and Hall, 2000). However, it was recently shown that a canonical PER/TIM oscillation does occur in the LPNs under a 12:12 LD cycle, bringing them to a pacemaker cell category (Shafer *et al.*, 2006). Like *per* and *tim*, *vri* is expressed in the photoreceptor cells of the compound eyes (Blau and Young, 1999; Kloss *et al.*, 1998; So *et al.*, 2000) and appears to be present in all adult LNs (Blau and Young, 1999). A broad distribution of *dbt*, *Clk* and *cyc* throughout the cortical regions of the brain and optic lobes was detected by *in situ* hybridizations (Kloss *et al.*, 1998; So *et al.*, 2000).

In the fly brain the lateral neurons (LNs) are of particular interest. Of the *per*-expressing cells, only some LNs are present at an early developmental stage (Helfrich-Forster, 1997; Kaneko *et al.*, 1997) and only these show a persistent cycling in the amount of PER throughout metamorphosis (Kaneko *et al.*, 1997). Three clusters of LNs can be distinguished in the anterior brain: the dorsal LNs (LN_ds) and the small and large ventral LNs (*s*-LN_vs and *l*-LN_vs, respectivelly) (Ewer *et al.*, 1992; Frisch *et al.*, 1994, Helfrich-Forster, 2003). Robust rhythmicity could be observed in flies expressing *per* only in the LNs, as revealed by studies involving transgenic flies (Frisch *et al.*, 1994). In contrast, genetic mosaic studies showed that *per* expression in cells other than the LNs was not sufficient to provoke robust activity rhythms (Ewer *et al.*, 1992). Anatomical studies using *disconnected* (*disco*) mutants (Dushay *et al.*, 1989; Hardin *et al.* 1992; Zerr *et al.* 1990) support the idea that LN_vs are pacemaker neurons. In most *disco* mutant flies, the LN_vs are completely absent (Helfrich-

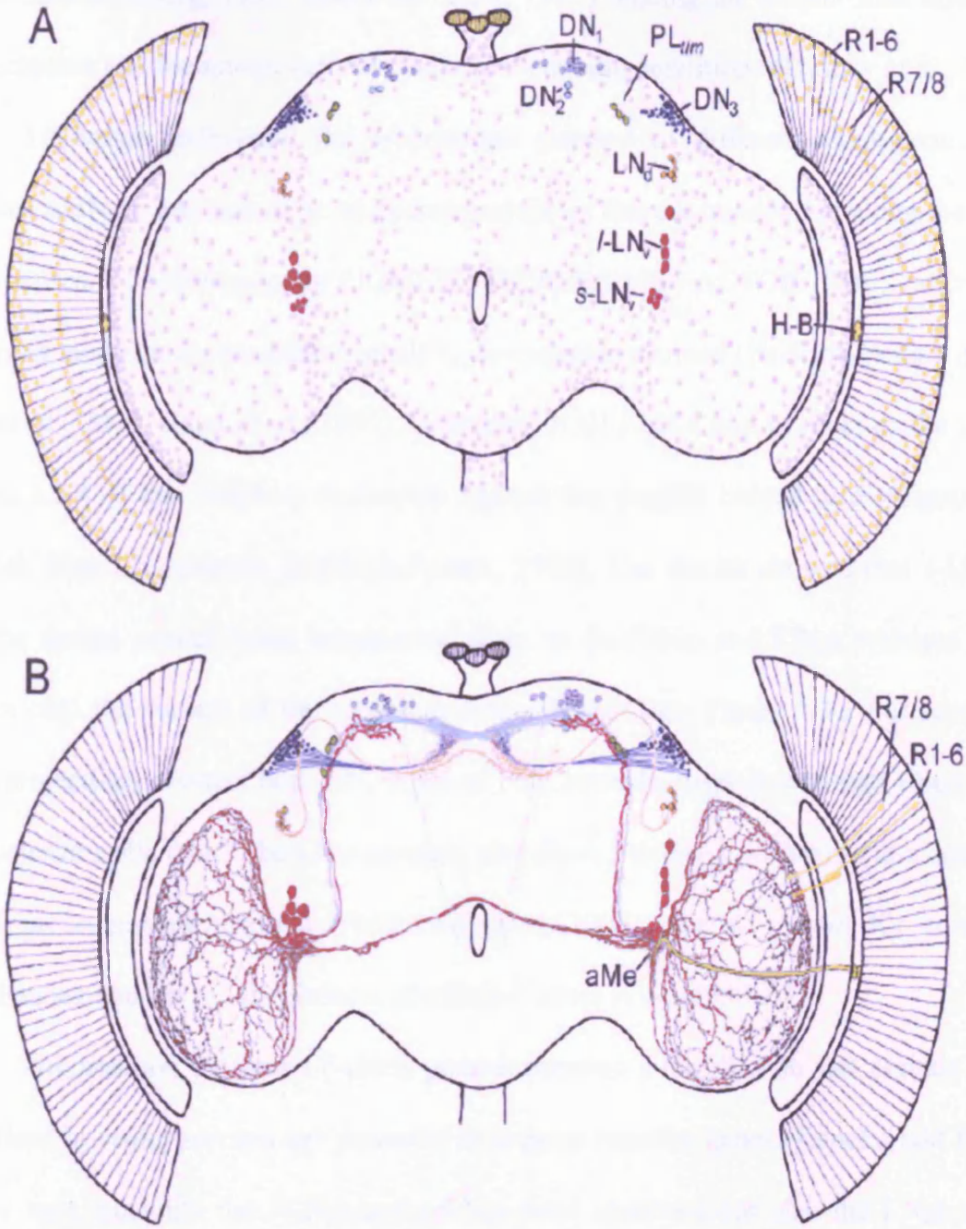


Figure 1.3

Cells expressing clock genes in the *Drosophila* brain. A: The two clusters of LN_vs and the LN_ds are shown in red and orange, respectively. The DNs are shown in blue, glial cells in pink and photoreceptor nuclei in yellow. The three cells labelled in green were previously thought to express TIM but no PER (Pl_{tim}). B: Projections from the *per*/*tim*-expressing neurons. aMe: accessory medulla. (Helfrich-Forster, 2003).

Forster and Homberg, 1993; Helfrich-Forster, 1997) rending the mutant flies arrhythmic for both eclosion and locomotor activity under free-running conditions (Dushay *et al.*, 1989).

To better understand the arborisation patterns of different clock-gene expressing neurons, analysis was also done on neurotransmitters that are transported along the neurites to the synapses. The neuropeptide PIGMENT-DISPERSING FACTOR (PDF), which probably servers as mediator for circadian signals to downstream neurons (Helfrich-Forster *et al.*, 2000; Park *et al.*, 2000, Renn *et al.*, 1999), is present in all *l*-LN_vs and in most of the *s*-LN_vs (see section 1.3.4 of this chapter). Antiserum against this peptide helped to distinguish the fiber network from LN_v clusters (Helfrich-Forster, 1997). The results showed that *s*-LN_vs project into the dorsal central brain terminating close to the DN_1s and DN_2s whereas the *l*-LN_vs project onto the surface of the second optical neuropil (see Figure 1.3). Furthermore, the *l*-LN_vs projections connect both LN_v types of both brain hemispheres trough fibers running in the posterior optic tract. The LN_vs neurons also show a dense arborisation in a small neuropil called the accessory medulla (Helfrich-Forster, 2003), that is known for containing the circadian pacemaker in other insects (Helfrich-Forster *et al.*, 1998).

Arborisation patterns of clock gene-expressing cells that do not contain PDF were visualised by using *per* and *tim* promoter to express reporter genes (Kaneko and Hall, 2000). In this way, not only the *l*-LN_vs and *s*-LN_vs were observed but also the LN_ds and all DN clusters (DN_1s, DN_2s and DN_3s). Generally, all these *per*/*tim*-expressing neurons were shown to send main projections into the dorsal protocerebrum where their terminals largely overlap (see Figure 1.3). This brain area has connections to several other sites and also houses much of the neurosecretory system, leading to the conclusion that circadian signals may be transferred electrically or/and via humoral pathways to effectors organs (Kaneko and Hall, 2000).

1.3.3 Input

The input pathway is traditionally perceived as the route by which entraining signals from the environment reach the clock, adjusting both period and phase to match the external conditions. Daily environmental cycles of light, temperature, food, and social interactions are all capable of entraining circadian oscillators (Hardin, 2005). Light is the major input to the clock and *D. melanogaster* possesses multiple photoreceptive pathways (Helfrich-Forster *et al.*, 2001). Genetic analysis has revealed the contribution of external photoreceptors, such as the compound eyes and ocelli, and of non-canonical photoreceptors, such as the Hofbauer-Buchner eyelet and cells containing the blue light sensitive protein CRYPTOCHROME (CRY) and other unknown photopigments (Helfrich-Forster *et al.*, 2001; 2003). All these pathways have been shown to contribute to light dependent entrainment of behavioural rhythms in *Drosophila*, although the presence of eyes is not strictly necessary (Wheeler *et al.*, 1993; Yang *et al.*, 1998). The *cryptochrome* gene (*cry*) is expressed in head and body (Egan *et al.*, 1999; Ishikawa *et al.*, 1999; Okano *et al.*, 1999; Selby and Sancar, 1999) including the small ventral neurons (*s*-LN_vs) (Emery *et al.*, 2000; Helfrich-Forster, 1998). CRY is a protein of 542 amino acids (Emery *et al.*, 1998) that shows homology to photolyases, enzymes that directly repair DNA lesions caused by UV-light in several organisms (Carell *et al.*, 2001). Both CRY and photolyases are highly conserved and bind to two chromophores, a pterin or deazaflavin and Flavin Adenin Dinucleotide (FAD). Although photolyases can bind DNA, CRY cannot, despite the fact that the putative DNA binding domains are well conserved between CRY and photolyases (Emery *et al.*, 1998). CRY proteins differ from photolyases for an extension of the C-terminus, which is likely to be important for their function (Cashmore *et al.*, 1999; Partch and Sancar, 2005). Unlike the photosensitive part of the protein which is highly conserved, the C-terminus is extremely diverged, even between paralogs within a species (Emery *et al.*, 1998). *Drosophila* is an exception in having only one type of CRY.

Other insects usually possess two types of CRY (Zhu *et al.*, 2005), where one works like a repressor and the other like a photopigment, giving good evidence that not all CRYs conserved the ability to react to light. Mammals are peculiar as both the CRYs they express seem to be involved in the repression function, although circumstantial evidence suggested a limited photosensitive role in a few tissues (Partch and Sancar, 2005). Fruit flies were for a time located at the other extreme as its CRY was known mainly for its photosensitive function. However, it was recently shown that even in *Drosophila* CRY had also acquired a repression function, working as an essential clock component in *Drosophila* peripheral tissues such as antennae (Krishnan *et al.*, 2001), malpighian tubules (Ivanchenko *et al.*, 2001) and eyes (Collins *et al.*, 2006).

Once activated by light, CRY interacts with TIM triggering the rapid degradation of both TIM and CRY. In *Drosophila*, TIM protein is degraded within 30 to 90 minutes of light exposure (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996). The degradation of TIM (which negatively affects PER stability) feeds back to the core of the clock and is interpreted as a resetting stimulus (Ceriani *et al.*, 1999; Ivanchenko *et al.*, 2001; Lin *et al.*, 2001; Stanewsky *et al.*, 1998). While TIM is highly sensitive to this input in circadian tissues, it is not light sensitive in the ovary (Rush *et al.*, 2006). Recently it was demonstrated that the circadian photoreceptor CRY is not expressed in ovarian tissues. Remarkably, ectopic CRY expression in the ovary was sufficient to cause degradation of TIM after exposure to light. In addition, PER levels were reduced in response to light when CRY was present, as observed in circadian cells. Hence, CRY was shown to be a missing key component for the light input pathway in the ovary (Rush *et al.*, 2006).

A missense mutation within the flavin-binding region of *Drosophila cry* (cry^b) interferes with light entrainment of locomotor activity (Stanewsky *et al.*, 1998). Although being behaviourally rhythmic, flies carrying this mutation are unable to reset their clocks in response to short light pulses. Interestingly, flies carrying cry^b mutation remain behaviourally

rhythmic under intense constant light (LL) (Emery *et al.*, 2000); a condition that renders wild-type flies arrhythmic (Emery *et al.*, 2000; Konopka *et al.*, 1989). Previous reports demonstrated that CRY can interact with TIM and PER (Ceriani *et al.*, 1999; Lin *et al.*, 2001; Rosato *et al.*, 2001). However, no interaction was detected between CRY and PER without TIM (Busza *et al.*, 2004).

cry RNA levels oscillate peaking at the beginning of the morning and showing low levels in the middle of the night (Emery *et al.*, 1998). Canonical clock mutants abolish rhythmic expressing of *cry*, indicating that its transcription is controlled by the circadian clock (Emery *et al.*, 1998). Interestingly, the clock does not regulate CRY protein levels as it continuously accumulates throughout the night being degraded only upon light exposure *via* the proteasome pathway (Emery *et al.*, 1998; Lin *et al.*, 2001). Although being expressed in most larval and adult neuronal groups expressing the PER protein, CRY is not expressed in the specific set of larval Dorsal Neurons DN_2s (Klarsfeld *et al.*, 2004) in which PER cycles in antiphase to all other known cells (Kaneko *et al.*, 1997). Forced expression of CRY in larval DN_2 promotes a PER oscillation in normal phase, indicating that their unique antiphase rhythm is related with their lack of *cry* expression (Klarsfeld *et al.*, 2004). In adults, CRY is expressed in the two ventral Lateral Neurons (LN_vs) clusters, in the dorsal lateral neurons (LN_ds) and most of the dorsal neurons DN_1. CRY is weakly expressed in the two DN_2 dorsal neurons and in one quarter of the DN_3 dorsal neurons (Klarsfeld *et al.*, 2004).

Light is not the only modality that can entrain the clock. There are non-photic entrainment signals, such as temperature and pheromonal social cues, which obviously require different circuits than those conveying information from the eyes or other photoreceptive structures. It has long been known that temperature cycles can entrain circadian clocks (Chang, 2006; Wheeler *et al.*, 1993). It was recently shown that a mutation that disrupts the phototransduction cascade ($norpA^{p41}$) and a novel allele of the yet unidentified *nocte* gene impair circadian thermal entrainment (Glaser and Stanewsky, 2005).

Social entrainment requires the antennal involvement as the signals sent to synchronize group-housed flies are olfactory in nature. Yet, it is possible that tactile and/or auditory cues contribute to social entrainment in addition to olfactory signals (Levine *et al.*, 2002).

1.3.4 Output

Throughout output pathways the clock regulates the specific physiological processes that govern the two best studied rhythms in *Drosophila*, adult locomotion and pupal eclosion. While adult activity rhythms appear to be controlled by the *s*-LN_vs and LN_ds, the eclosion is suggested to be controlled only by the *s*-LN_vs (Helfrich-Forster, 2003).

Animal circadian clocks employ neuropeptides as signalling molecules. They operate within the brain master clock to synchronise the oscillations of individual clock neurons and also to transfer signals to downstream neurons. In *Drosophila*, the first clock neurotransmitter to be localized and to have its gene cloned was the Pigment-Dispersing Factor (PDF)(Helfrich-Forster, 1995; Park and Hall, 1998). The LNs release the neuropeptide PDF that is required for the rhythm of locomotor activity in adult fly (Renn *et al.*, 1999). Recent evidences indicate that PDF is rhythmically secreted into the dorsal protocerebrum, requiring the normal function of the clock proteins PER, TIM, CLK, CYC and VRI (Blau and Young, 1999; Park *et al.*, 2000). Interestingly, constitutive ectopic PDF expression in cells that project into the dorsal protocerebrum disturbs activity and the overexpression of *Pdf* gene disrupts flies rhythmic emergence from their puparia (Helfrich-Foster *et al.*, 2000; Park *et al.*, 2000). Recently, three different works opened the way to understand signalling pathways within and outside the circadian clock by identifying the PDF receptor that has long remained a mystery. A systematic screen of receptors candidates for sensitivity to PDF was performed by employing a transient functional expression of receptor cDNAs in mammalian HEK293

cells (Mertens *et al.*, 2005). Measures using a promiscuous G protein subunit indicated sensitivity of CG13758 to PDF. This G protein-coupled receptor (GPCR) named PDF RECEPTOR (PDFR) elevated cAMP levels when expressed in HEK293 cells. Also, *Pdfr* mutants displayed increased circadian arrhythmicity. Finally, immunocytochemistry expression pattern of the receptor revealed its presence in a subset of the clock neurons as well as in the protocerebrum, local that have been described to harbour the relay areas between the fly master clock and rhythmic behaviour (Mertens *et al.*, 2005). The search for genes involved in temperature sensing (Hyun *et al.*, 2005) and for ion channel mutants that could disrupt the circadian clock (Lear *et al.*, 2005) also leaded to the identification of CG13758 as a PDF receptor. The P insertion mutant *han* preferred different temperatures during the day and night and reduced the transcription level of CG13758, promoting arrhythmic behaviour in constant darkness (Hyun *et al.*, 2005). In the mutant *groom-of-PDF* (*gop*), a spontaneous retrotransposon disrupted a coding exon of CG13758 being accompanied by phase-advance oscillations of the core clock protein PER (Lear *et al.*, 2005).

Wild-type *D. melanogaster* exposed to a generic day of 12 hours of daylight followed by 12 hours of darkness (12 :12h LD) exhibits two distinct bouts of locomotor activity that are controlled by the circadian clock, as inferred from the loss of anticipatory activity of both light transitions in *per*-null mutants (Hall, 2003). The contribution of the different clock neurons to the rest-activity pattern has recently gained new information (Grima *et al.*, 2004; Stoleru *et al.*, 2004). Target expression of PER was used to restore the clock function of specific subset of lateral neurons in *per*-null mutant flies. It was shown that PER expression restricted to the LN_vs only restores the morning activity whereas expression of PER in both the LN_vs and LN_ds also restores the evening activity. Also, the same study showed that the morning oscillator is sufficient to drive the circadian system as the LN_vs alone could generate 24h activity rhythms (Grima *et al.*, 2004). Similar results were shown by using the proapoptotic gene *hid* to induce cell-specific ablation in both the LN_vs and LN_ds (Stoleru *et*

al., 2004). A more recently discovered output pathway gene is *takeout* (*to*), whose expression occurs in the cardia, crop and antennae (Sarov-Blat *et al.*, 2000) and may provide temporal information for feeding and antennal rhythms (Krishnan *et al.*, 1999).

At least in part the pupal eclosion rhythms have a distinct output pathway. This was evidenced by the requirement of *lark* (Newby and Jackson, 1993), whose gene product LARK expression is eliminated in *per*-null flies. LARK is a RNA-binding protein which cycles in abundance in some neurons of the brain and ventral ganglionic mass, co-localizing with a neuropeptide CCAP (Crustacean Cardioative Peptide) (Gammie and Truman, 1999) that controls ecdysis behaviour. The fact that LARK reaches its highest level during the day in wild-type flies suggests that it acts as a repressor of eclosion (Saunders *et al.*, 2002).

1.4 PAS domain superfamily

The PAS domain is a signature of proteins that play direct or indirect roles in the detection and adaptation to environmental changes. This large, multifunctional interaction domain monitors a wild variety of biological processes such as neurogenesis, tubule formation, carcinogen metabolism, response to hypoxia, response to changes in light, biological rhythms and the overall energy level of a cell (reviewed in Gu *et al.*, 2000; Taylor and Zhulin, 1999).

PAS domains have been identified in proteins from both prokaryotes and eukaryotes including kinases, chemoreceptors, photoreceptors, circadian clock proteins, voltage activated ions channels, cyclic nucleotide phosphodiester and transcription factors regulating responses to hypoxia, xenobiotics, embryological development and biological rhythms (Taylor and Zhulin, 1999). PAS domains are not confined to specific phylogenetic groups, however not all species have PAS domains (Taylor and Zhulin, 1999). Analysis of completely sequenced

bacterial and archaeal genomes revealed that some species contain no recognizable PAS domains whereas others have abundant PAS domains (Ponting and Aravind, 1997; Zhulin *et al.*, 1997). The *Drosophila* circadian clock is one of the most studied PAS-dependent signalling pathways found in higher eukaryotes and is represented by the gene products of *per*, *Clk* and *cyc*. A role for other clock PAS domains proteins in the clock input is without experimental support. TIM is the only clock protein demonstrated to respond rapidly to light and it does not have a PAS domain (Suri *et al.*, 1998; Yang *et al.*, 1998).

PAS is an acronym formed from the family's founding member's names: the *Drosophila* PER clock protein (Huang *et al.*, 1993; Lindebro *et al.*, 1995; Pellequer *et al.*, 1998; 1999), the vertebrate ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR (ARNT) (Hankinson, 1995) and the *Drosophila* SINGLE-MINDED protein (SIM) (Nambu *et al.*, 1991). The consensus PAS domains emerged from sequences comparisons after SIM and ARNT were cloned (Crews *et al.*, 1988). The ARNT protein forms heterodimers with two other PAS proteins: the ARYL HYDROCARBON RECEPTOR (AHR), involved in the response of xenobiotics (Hankinson, 1995); and the mammalian HYPOXIA-INDUCIBLE FACTOR 1 (HIF1), involved in hypoxia response mechanisms (Wang *et al.*, 1995). In *Drosophila*, SIM acts as a master regulator of CNS midline development, being required for activation of midline gene expression (Nambu *et al.*, 1990, 1991) and repression of lateral neuroectodermal expression (Chang *et al.*, 1993; Mellerick and Nirenberg, 1995; Xiao *et al.*, 1996). The PAS domain is best described as a region of homology to these three founding members. It typically encompasses 250-300 amino acids and contains two 50-residue conserved sequences termed PAS-A and PAS-B repeats (Crews *et al.*, 1988; Hoffman *et al.*, 1991; Jackson *et al.*, 1986; Nambu *et al.*, 1991). Recent studies suggested that PAS domains comprise a region of approximately 100 to 120 amino acids (Taylor and Zhulin, 1999). In higher eukaryotes PAS domain functions as a surface for both homotypic interactions with other PAS proteins and heterotypic interactions with cellular

chaperones such as the 90 kDa heat shock protein (Hsp 90) (Denis *et al.*, 1988; Perdew, 1988). It is typical to find PAS domains acting in pairs as eukaryotic transcriptional activators. Finally, in the case of AHR, the PAS domain can also bind small molecules ligands surfaces such as the environmental contaminant dioxin (Burbach *et al.*, 1992; Dolwick *et al.*, 1993; Poland *et al.*, 1994).

Most of the PAS proteins studied in mammals and *Drosophila* also contain basic-helix-loop-helix (bHLH) motifs immediately N-terminal to theirs PAS domains. Amongst all the *Drosophila* circadian clock PAS domain protein representatives only PER does not have this motif. The HLH domains participate in homotypic dimerization between two bHLH-PAS proteins. They also physically interact within the major groove of target regulatory elements found in DNA by positioning the basic regions (Murre *et al.*, 1994), which implicates them as DNA-binding transcription factors.

The specificity of transcriptional factors in activating target genes is determined by their PAS domains. Chimeras constructed from the TRACHEALESS (TRH) and SINGLE-MINDED (SIM) proteins from *Drosophila* showed that replacement of the TRH PAS domain by the analogous region of SIM produced a chimera with a functional specificity of a SIM protein in gene activation (Zelzer *et al.*, 1997). In the condition of signal transduction molecules, the carboxyl termini of some bHLH-PAS proteins have been found to function as transcriptional activation or repression domains (Franks and Crews, 1994; Jain *et al.*, 1994; Li *et al.*, 1994; Yamaguchi and Kuo, 1995). However, despite the relative conservation of both the bHLH and the PAS domains, most PAS proteins show little sequence homology in their C-terminal sequences.

1.5 TANGO and the *Drosophila* circadian clock

In *Drosophila*, *tango* (*tgo*) is orthologous to mammalian *Arnt* (Sonnenfeld *et al.*, 1997), whose product functions as a heterodimer with the ARYL HYDROCARBON RECEPTOR (AHR) to metabolize dioxins (Brown *et al.*, 2005; Hoffman *et al.*, 1991). Sequence analysis of the complete gene and corresponding embryonic cDNA clones indicate that *tgo* is highly related to mammalian *Arnt*, both in sequence and structure (Sonnenfeld *et al.*, 1997). Figure 1.4 shows both the structural motifs and amino acid sequences from the highly related protein products of *tgo* and *Arnt*. The bHLH domains of human ARNT and *Drosophila* TGO are 92% identical whereas the PAS domains are 53% identical (Sonnenfeld *et al.*, 1997). Although both ARNT and TGO C-terminal regions have glutamine-rich sequences, this very important region is generally unrelated in primary sequence between these proteins. ARNT has been shown to contain strong C-terminal transactivation domain (Jain *et al.*, 1994; Li *et al.*, 1994; Whitelaw *et al.*, 1994; Yamaguchi and Kuo, 1995) a fact that is still to be shown in TGO. Only some regions in the C-terminal end of TGO were suggestive of transactivation (Mitchell and Tjian, 1989). A feature of *Drosophila* TGO is the presence of a histidine-proline rich region, referred as PRD repeat, of unknown function in the C-terminus and not found in other ARNT proteins (Sonnenfeld *et al.*, 1997). Recent work has shown that an intact sequence of PRD repeats is essential for TGO to form heterodimer with TRH to trigger a proper activation of the gene *breathless* (*btl*) (Ohshiro and Saigo, 1997) in early tracheal cells (Sonnenfeld *et al.*, 2005). Sequence analysis of *tgo* genomic and cDNA clones revealed a 2.9 kb gene possessing a single intron of 142 bp within the 5`- untranslated region (Sonnenfeld *et al.*, 1997). The simplicity of the exon-intron structure of the *tgo* gene is in contrast to the mammalian *Arnt* gene that is much larger and structurally complex (Maltepe *et al.*, 1997).

A

B

```
                                        N-terminus
Tango                                                                    MDEANIQD     8
H Arnt   MAATTANPEMTSDVPSLGPAIASGNSGPGIQGGGAIVQRAIKRRPGLDFDDDGEGNSKFLRCD•DQMSN•    70

                 Basic          Helix 1          Loop          Helix 2
Tango    KERFA SRENHCEIERRRR NKMTAYITELSDMVP TCSALARKPD KLTILRMAVAHMKAL RGTGNTS    73
H Arnt   ••••- A••••S••••••• ••••••••••••••• •••••••••• •••••••••S•••S• •••••••   134

             Spacer 1                                PasA
Tango    SDGTYKPSFLTDQEL KHLILEAADGFLFVVSCDSGRVIYVSDSVTPVLNYTQSDWYGTSLYEHIHPDDR   142
H Arnt   T••S•••••••••••• ••••••••••••I•••ET•••V••••••••••QP••E•F•ST••DQV••••V   203
                                F-------------------PR-----------------H--D

                                        Spacer 2
Tango    EKIREQLS TQESQNAGRILDLKSGTVKKEGHQSSMRLSMGARRGFICRMRVGNVN--PESMV SGHLNR   208
H Arnt   D•L••••• •S•NALT•••••••T•••••••Q•••••MC••S••S••••••C•SSSVD•E•VN RLSFV•   271

                                        PasB
Tango    LKQRNSLGPSRDG-TNYAVVHCTGYIKNWPPTDMFPNMHMERDVDDMSSHCCLVAIGRLQVTSTAA-NDM   276
H Arnt   NRC••G••SVKE•EPHFV•••••••••A•••AGV--SLPDDDPEAGQG•KF••••••••••••SPNCT••   339

Tango    SGSNNQSEPITRHAMDGKFTFVDQRVLNILGYTPTELLGKICYDFFHPEDQSHMKESFDQVLKQKGQMFS   346
H Arnt   •NVCQPT•••S••NIEGI•••••H•CVATV••Q•Q•••••NIVE•C•••••QLLRD••Q••V•L•••VL•   409
               F---------------- PR -----------------H--D

Tango    LLYRARAKNSEYVWLRTQAYAFLNPYTDEVEYIVCTNSSGKTMHGAPLDAAAA HTPEQVQQQQQQQQEQ   415
H Arnt   VMF•F•S•NQ•WL•M••SSFT•Q•••S••I•••I•••TNV•NSSQE•RPTLSN TIQRPQLGPTANLPLE   478

Tango    HVYVQAAPGVDYARRELTPVGSATNDGMYQTHMLAMQAPTPQQQQQQQQRPGSAQTTPVGYTYDTTHSPY   485
H Arnt   MGSGQLAPRQQQQQTELDMVPGRDGLASYNHSQVVQPVTTTGPEHSKPLEKSDGLFAQDRDPRFSEIYHN   548

                                        tgo1  X
Tango    SAGGPSPLAKIPKSGTSPTPVAPNSWAALRPQQQQQQQQPVTEGYQYQQTSPARSPSGPTYTQLSAGNGN   555
H Arnt   INADQSKGISSSTVPATQQLFSQGNTFPPTPRPAENFRNSGLAPPVTIVQPSASAGQMLAQISRHSNPTQ   618

                                        PRD repeat
Tango    RQQAQPGAYQAGPPPPPNAPGMWDWQQAGGHPHPPHPTAHPHHPHAHPGGPAGAGQPQGQEFSDMLQMLD   625
H Arnt   GATPTWTPTTRSGFSAQQVATQATAKTRTSQFGVGSFQTPSSFSSMSLPGAPTASPGAAAYPSLTNRGSN   688

Tango    HTPTTFEDLNINMFSTPFE                                                     644
H Arnt   FAPETGQTAGQFQTRTAEGVGVWPQWQGQQPHHRSSSSEQHVQQPPAQQPGQPEVFQEMLSMLGDQSNSY   758

H Arnt   NNEEFPDLTMFPPFSE                                                        774
```

Figure 1.4

Comparison of the highly related *Drosophila* TGO and mammalian ARNT proteins. A: Sequence organization of TGO and ARNT derived from cDNA clone sequences. The N terminus is to the left. Colour schemes represent important protein motifs. Red, bHLH domains. Blue and lighter blue, PasA domain and its Pas repeat (PR), respectively. Green and lighter green, PasB domain and its Pas repeat (PR), respectively. Purple, glutamine (Q)-rich stretches. Cyan, histidine-proline-rich PRD-repeat only present in TGO. B: The protein sequences of TGO and ARNT are compared. Amino acid numbering is shown on the right. Dots indicate sequence identity and deletion, respectively. Blank spaces divide different sequence regions. The position of the premature translation stop (X) corresponding to tgo^{1} EMS mutation is shown above the TGO protein sequence (Adapted from Sonnenfeld *et al.*, 1997).

The product of *tgo* forms heterodimers with the products of *single-minded* (*sim*), *trachealess* (*trh*), *spineless* (*ss*) and *dysfusion* (*dys*) during embryogenesis (Crews *et al.*, 1988; Emmons *et al.*, 1999; Isaac and Andrew, 1996; Jiang and Crews, 2003; Ohshiro and Saigo, 1997; Thomas *et al.*, 1988; Wilk *et al.*, 1996; Sonnenfeld *et al.*, 1997; Zelzer *et al.*, 1997). Analysis *in vivo* revealed a TGO requirement for its partner-mediated processes (Emmons *et al.*, 1999; Ohshiro and Saigo, 1997; Sonnenfeld *et al.*, 1997; Zelzer *et al.*, 1997). Indeed, both cell culture analysis and *in vivo* studies showed that a DNA enhancer element acts as a binding site for both SIM-TGO and TRH-TGO heterodimeres controlling CNS midline and tracheal transcription. TGO is known to require a dimerization partner for nuclear translocation as evidenced by the lack for recognizable nuclear localization sequence (Sonnelfeld *et al.*, 1997). Mutations in the *tgo* gene revealed both CNS midline and tracheal defects (Sonnenfeld *et al.*, 1997).

Another *Drosophila* PAS domain that has been identified is the human HIF1 homolog SIMILAR (SIMA)(Nambu *et al.*, 1996), that also contains a bHLH domain and is inducible by hypoxia (Bacon *et al.*, 1998) controlling its response (Lavista-Llanos *et al.*, 2002). SIMA functions as a sensor indicating that hypoxia signalling pathway is conserved between flies and human. Given the role that hypoxia has as a developmental signal in the vascular tubes formation (Semenza *et al.*, 1998), it is possible that trachealogenesis can also respond directly to low oxygen levels and that THR and TGO are the sensors in that pathway (Wilk *et al.*, 1996; Isaac and Andrew, 1996).

It is reasonable to assume that a protein in possession of a good PAS dimerization domain could play a role in more than one PAS signalling system. Amongst all the clock proteins in possession of bHLH-PAS domains it was shown that *Drosophila* TGO, CYC and their human homolog's, ARNT and BMAL1, belong to a distinct family of bHLH-PAS proteins (Jiang and Crews, 2003). Figure 1.5 shows a phylogram generated using representatives of all families of bHLH-PAS proteins compared and aligned by using the

bHLH domain protein sequences. The evolutionary conservation of the TGO/ARNT/CYC/BMAL1 sub-family suggests a functional relationship amongst them. Indeed, during a yeast-two-hybrid screening (Gekakis *et al.*, 1998), it was shown that mammalian CLK interacts with ARNT. The high homology between TGO and ARNT proteins suggests that the former might interact with components of the clock machinery as well. Using a yeast-two-hybrid assay it was shown that TGO physically interacts not only with *Drosophila* CLK but also with CRY (E. Rosato, personal communication).

Another piece of evidence suggesting a role for *tgo* in the clock comes from a study comparing the effects of Clk^{Jrk} and cyc^{0} mutations on the immunostaining of PDF (Park *et al.*, 2000). Expression of *pdf* in the arrhythmic Clk^{Jrk} mutant was found to be strikingly abnormal showing no detectable levels of both *pdf* mRNA and PDF in larval LN cells and in the *s*-LN_vs of adults. Interestingly, some developmental defects were induced by Clk^{Jrk} evidencing the pleiotropic character of this gene. Projections from *l*-LN_vs were shown to be abnormal in approximately 50% of the mutant brains. Analysis of *Pdf* expression in cyc^{0} mutants indicated similar but less severe effects than those caused by Clk^{Jrk}. Most of the larval LN cells and adults *s*-LN_vs expression of both mRNA and PDF were weakly detected in cyc^{0} mutants in contrast with the elimination of such signal in Clk^{Jrk} flies. About 25% of the adult cyc^{0} brains exhibited an abnormal dorsal projection. The milder effects of cyc^{0} on neuron cells development and *pdf* expression suggest the existence of another PAS-containing transcription factor that, in the absence of CYC, could partner with CLK providing a biologically relevant function. Also, it was suggested that the stronger effects of Clk^{Jrk} on *pdf* expression could have been due to this mutant protein ability to form inactive complexes not only with CYC but also with a putative PAS factor (Park *et al.*, 2000).

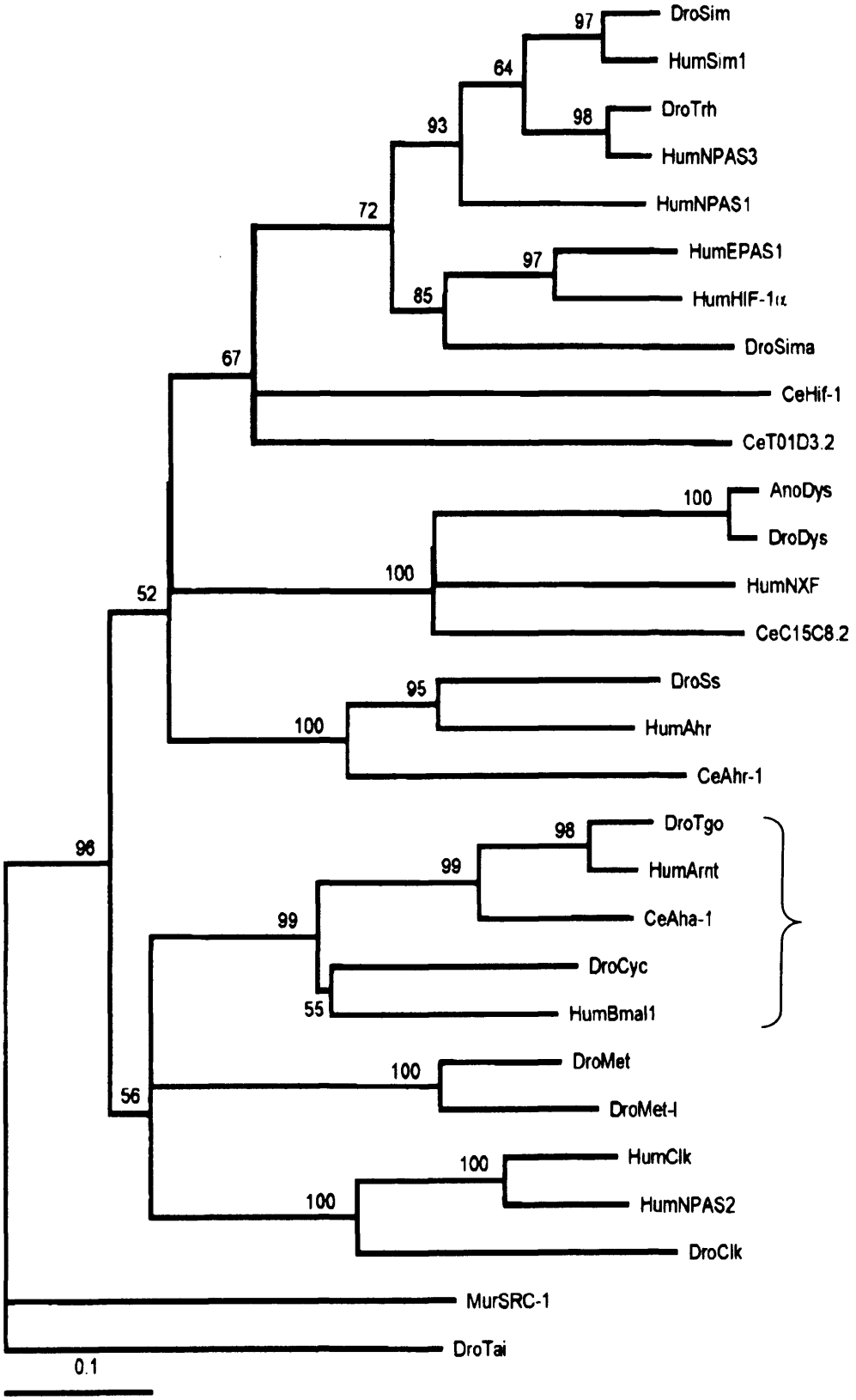

DroSim
HumSim1
DroTrh
HumNPAS3
HumNPAS1
HumEPAS1
HumHIF-1α
DroSima
CeHif-1
CeT01D3.2
AnoDys
DroDys
HumNXF
CeC15C8.2
DroSs
HumAhr
CeAhr-1
DroTgo
HumArnt
CeAha-1
DroCyc
HumBmal1
DroMet
DroMet-l
HumClk
HumNPAS2
DroClk
MurSRC-1
DroTai
0.1
97
64
98
93
72
97
85
67
100
100
52
95
100
98
99
99
55
96
100
56
100
100

Figure 1.5

The phylogram demonstrates that dTGO, hARNT, dCYC and hBmal1 represent a distinct and evolutionary conserved subfamily of bHLH-PAS proteins (bracket). The scale represents the fraction of no identical amino acids residues along each branch. Numbers along each branch are the bootstrap confidence limits with 1,000 repetitions. The species designation precedes each protein acronym (Ano, *A.gambie*; Ce, *C. elegans*; Dro, *Drosophila*; Mur, murine; Hum, human) (Adapted from Jiang and Crews, 2003).

1.6 Objectives

The main purpose of this project is to investigate a possible role for *tgo* in both the circadian clock and in the mechanisms that control rest/activity in *Drosophila melanogaster*. To better understand a putative regulation of *tgo* in any aspect of the behaviour biology, I sought to search and characterize behavioural and biochemical changes in lines over-expressing and down-regulating TGO protein in contrast to normal control flies.

Chapter 2: Material and methods

2.1 General techniques

2.1.1 Fly stocks

All experiments were performed with *D. melanogaster* adult flies reared on standard sucrose-yeast medium (46.3g/l of sucrose, 46.3g/l of dry yeast, 7.1g/l of agar and 2g/l of the anti-mycotic nipagin). All stocks were kept either at 25°C or 18°C, in temperature-controlled rooms, under an artificial rectangular cycles of 12 hours light and 12 hours dark (LD 12:12). The strains used are described as follows:

***w*; *act*-GAL4**

Carries a P-element on chromosome 2 in which a promoter from *actin* gene drives the ubiquitous expression of the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) protein GAL4. Obtained from Bloomington Stock Centre.

***Clk*^{jrk}**

Strain carrying a mutation in the fly gene *clock*, located on the left arm of the third chromosome (3L). An amino acid replacement at position 776 results in premature stop codon that truncates the protein, deleting most of the C- terminal activation domain but not the DNA binding one. Obtained as a gift from M. Rosbash.

cyc^0

Strain carrying a mutation in the fly gene *cycle*, located on the left arm of the third chromosome (3L). A nonsense mutation at amino acid 159 promotes a premature stop codon deleting the entire PAS B domain. Obtained as a gift from M. Rosbash.

***yw; tim*-GAL4**

Carries a P-element on chromosome 2 in which a promoter from the clock gene *timeless* drives the expression of the yeast *S. cerevisiae* protein GAL4. Obtained as a gift from P. Emery.

***w; tubP*-Gal80^{TS}**

Carries a P-element on chromosome 3 in which a *tubulin* promoter drives the ubiquitous expression of the yeast *S. cerevisiae* protein GAL80, which inhibits GAL4 action. This particular allele is temperature sensitive, i.e. it is inactive at temperatures above 29°C. Obtained from Bloomington Stock Centre.

w; CyO/Sco; MKRS/TM6

Chromosome balancer line carrying useful visible markers and used in crosses schemes. Obtained from Bloomington Stock Centre.

yw; tubP*-GAL4/*TM3(Sb)

Carries a P-element on the first chromosome 3 in which a *tubulinP* promoter drives the ubiquitous expression of the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) protein GAL4. The other chromosome 3 is a balancer carrying a visible marker (stubble bristle). Obtained from Bloomington Stock Centre.

***tim*^{01}**

Strain in which a P-element promoted an internal deletion at the *timeless* gene on the second chromosome. Obtained as a gift from M. Young.

***tgo*^{1}**

A hypomorph mutant for the *tango* gene located on the third chromosome that is lethal in homozygosis. It has a termination codon at amino acid 532 that deletes a proline-rich region (the PRD repeat) and a glutamine-rich region from the protein. Obtained as a gift from S. Crews.

***tgo*^{5}**

A tango mutant phenotype that is close to a null allele. No molecular data is available for this mutation. Obtained as a gift from S. Crews.

UAS *tango*

The yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) UAS promoter drives the expression of *tango* cDNA. The strains carry a P element inserted on the chromosome X (line 52.1) or chromosome 3 (line 25.1). Referred to as UAS *tgo* throughout. Produced in the laboratory.

UAS *tango* intron *tango*

The yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) UAS promoter drives the expression of *tango* cDNA cloned as 2 fragments in opposite orientation separated by an intron. The transcription of this construct results in the production of double stranded RNA which mediates degradation of endogenous *tgo* mRNA by RNA interference. The strains carry a P element inserted on the chromosomes 2 (line 3 and 1y). Referred to as UAS *tint* throughout. Produced in the laboratory.

w^{1118}

Carries a null mutant for the *white* gene thus producing white coloured eyes. This is the standard strain used for the production of transgenic flies and is generally used as a wild-type control in the different experiments. Obtained from Bloomington Stock Centre.

2.1.2 Crosses

Males and virgin females were anesthetised with dioxide carbon (CO_2) and scored under a stereo microscope. The desired genotypes were brought together in a vial for breeding

and kept at 25°C in temperature-controlled rooms under an artificial rectangular cycles of 12 hours light and 12 hours dark (LD 12:12). Parents were removed from vial as soon as pupae were detected to avoid mixing with F1 generation. The generation of specific strains will be discussed in the relevant chapters.

2.1.3 Statistical analysis

All statistical tests applied in this study were performed using the computer software SPSS (11.0.1) for Windows. In order to know how well the distribution of sample data conformed to normal distribution, a test known as the Kolmogorov-Smirnov goodness of fit was applied. If the populations from which the samples were drawn were normally distributed with equal variances, the statistical significance of results was evaluated via Analysis of Variance (ANOVA). In case where the null hypotheses (hypothesis of no difference) were rejected, the Bonferroni *Post Hoc* analysis was performed in order to check in a single test which genotypes differed from the others. When the assumptions underlying ANOVA were not met, that is, when the populations from which the samples were drawn were not normally distributed with equal variances, two alternatives methods were applied. Initially, a data transformation was performed by using log10 or Square Root. If the samples persisted being not normally distributed, the non-parametric alternative to ANOVA, the Kruskal-Wallis test, was used to test the hypothesis of equal location parameters. In the cases where Kruskal-Wallis test rejected the null hypothesis, the pair wise Mann-Whitney test was applied in order to detect which genotypes contributed to the detected differences.

2.2 Specific techniques

2.2.1 Locomotor activity experiments

Locomotor activity was measured under controlled light dark (LD) cycles and constant temperature using the *Drosophila* activity monitoring system Drosophix (Pixel srl, Padova, Italy). One male fly was individually housed in a small cylindrical glass tube (0.5 cm diameter, 8 cm length) filled approximately with 2cm deep sugar/agar medium in one end and closed at the other end with cotton. Each tube was loaded in a channel into the activity monitor. Each channel was equipped with an infrared light emitter and detector. Whenever a moving fly interrupted the infrared light beam this was counted as one event. The number of counts over a specified period of time (bin, usually 30 min.) were recorded and stored by a computer. Flies that died during the course of the experiment were not included in any subsequent analysis. At the end of the experiment, the activity data were transferred to an Excel spreadsheet for analysis.

Calculation of τ

The period (τ) of locomotor activity of each fly was calculated using Autocorrelation (available in the SPSS/PC+ Version 2.0 software package) and high resolution Spectral Analysis (Kyriacou *et al.*, 1989; Roberts *et al.*, 1987) employing the CLEAN algorithm. Autocorrelation analysis examines the correlation between the sequential 30 minutes activity bins, corresponding to whole activity record of a fly, as a function of time, namely how bin 1 correlates with bin 2 (lag 1), bin 2 with 3 (lag 2), etc. For instance, if a fly has a period of 24 hours, the highest correlation coefficients were produced by bin 1 and bin 49, bin 2 and bin 50 etc. Significant rhythmicity in an autocorrelogram was one where the correlation coefficient

(r) itself showed cycling, and the peak was equal or greater than the 95% confident limits. Correlograms were produced which displays the correlation coefficients plotted against each 30 min time lag, up until the 116th lag (or 58 hours). The highest peak above the 95% confidence limit, which is in the range of 15-40 hours, is taken to be the free running period (τ) of the fly.

The spectral analysis uses the CLEAN algorithm (Kyriacou and Hall, 1989; Roberts *et al.*, 1987) and gives a more accurate estimate of the period and can be used to confirm or reject the biological significance of the autocorrelation. The spectral analysis breaks down a signal to its sine and cosine waves, and the frequencies that provided the closest matches to the data are displayed as a spectrogram. Monte-Carlo simulations were used to generate approximate 95% and 99% confidence limits by randomizing the data for each fly 100 times and performing spectral analysis on these data. The highest peak shown above the 99% confidence limit is taken as the period of the fly. The locomotor activity profiles of *D. melanogaster* are bimodal, thus eventually the highest peak was approximately 12 hours. In this case the ~ 12 h peaks were doubled before being recorded as the period of the fly. Autocorrelograms and spectograms were judged to be rhythmic or arrhythmic on the basis of a blind assessment of their correlograms. If a record gave a significant period with autocorrelation, but not spectral analysis, or vice versa, then the record was judged arrhythmic. Because the autocorrelation periods were recorded as a multiple of 30 minutes, the spectral analysis period was used for further statistical analysis.

Evaluation of ϕ

Another important circadian parameter is the state of the oscillation within a period, ie., the phase (ϕ).When calculating the phase, it is important to state which overt reference point has been used, e.g. onset of activity, peak of activity, drop of 50% of the peak value, etc.

Finally, the phase reference point is compared with a fixed environmental reference such as lights on, lights off, light pulse, etc.

Phase shift analysis and phase-response curve (PRC)

Experimental flies were split in two groups and placed in different incubators that were set to the same light/dark cycle and temperature conditions. Initially both groups were entrained to a LD 12:12 cycle for at least 3 days. In one incubator, flies were subjected to light perturbation lasting 5 minutes either at the beginning (ZT13; ZT15) or at the end (ZT21) of the last scotophase. Locomotor activity of *D. melanogaster* was then allowed to free run in DD for the subsequent five days. The recorded locomotor activity data was transferred to an Excel spreadsheet and each group had its activity profile analysed. Flies showing phase shift ($\Delta\Phi$) due to the single light pulses exposure were denominated pulsed flies. The second group made the unpulsed control. Attainment of a new phase by the overt rhythm might not be instantaneous and the ultimate steady state might be reached via a series of transient cycles. Because of that the phase shift was measured by looking only at the third evening peak after the pulse. A reference point was determined by taking the bin number relative to an activity drop of 50% of the peak value. This was repeated for each individual fly in the pulsed and unpulsed groups. The bin numbers of the unpulsed flies were averaged and the individual bin numbers of the pulsed flies subtracted from the unpulsed average. Finally, these subtracted values were averaged to give the phase shift amplitude for a given genotype subjected to light perturbations at a particular ZT. A positive value corresponded to an advance phase shift ($+\Delta\Phi$) while a negative value indicated a delay phase shift ($-\Delta\Phi$).

2.2.2 Behavioural analysis of sleep

Flies are considered to be in a sleep-like state during periods of quiescence that last five minutes or longer (Hendricks *et al.*, 2000a; Shaw *et al.*, 2000), hence the monitoring of locomotor activity in 1 min bins offered a reliable measure of sleep. Periods of waking and quiescence can be quantified using the Drosophila Activity Monitoring System (Trikinetics), that was validated for sleep measurement by direct visual observation and high resolution videography (Hendricks *et al.*, 2000a; Shaw *et al.*, 2000).

Three days old flies maintained in LD 12:12, at 25°C, were loaded in activity tubes during the light phase and were let to rest or sleep deprived during the dark phase until the beginning of the following light cycle. To induce sleep deprivation, activity tubes were positioned, as a group of about 10, inside a 50ml polypropylene vial. This was closed with a lid but the opening of lateral windows assured proper ventilation. The vials were attached on a vertical mechanical wheel whose continuous rotation (6 rotations per minute) caused the activity tubes, and the flies within, to drop rhythmically to the bottom of the tube preventing any rest. Controls not sleep-deprived were put in a tray in the same room where the sleep deprivation was taking place. Immediately after lights on, both sleep deprived flies and controls were loaded in the activity monitors and recorded for the next 24 hours under LD 12:12. The recorded locomotor activity data was transferred to an Excel spreadsheet and each group had its amount of sleep computed.

2.2.3 Western blot

Protein extraction

Adult flies were sampled at specific time points under an artificial rectangular cycles of 12 hours light and 12 hours dark (LD 12:12). Flies were anesthetised with CO_2, collected into a 1.5 ml micro centrifuge tube and immediately frozen in liquid nitrogen. Heads were disconnected from the body by vigorous shaking and collected with a sieve on dry ice. Ice cold lysis buffer (0.1M KCl, 20mM HEPES pH7.5, 5% glycerol, 0.1% Triton X-100, 10mM EDTA, 1mM DTT, 0.5 mM phenylmethylsulfonylfluoride, 10 μgml^{-1} aprotinin, 5 μgml^{-1} leupeptin, 5 μgml^{-1} pepstatin) was added as twice the estimated volume of heads. Using a plastic micropestle the heads were homogenised for about 30 seconds, always keeping the sample on ice. Debris was removed by spinning the sample for 5 minutes at maximum speed at 4°C and transferring the supernatant to a new tube on ice. The protein extracts were equalised before loading using the Bradford reagent. Briefly, 800µl dH2O and 200µl Bradford reagent were mixed in a cuvet before adding 1µl of head protein extract. After 5 minutes incubation at room temperature the optical density (OD) at 595 nm was measured for each sample. Lysis buffer was used to dilute the most concentrated protein samples so to achieve equal OD_{595} values.

Protein separation by electrophoresis

Proteins extracts were separated in a 6% (TIM) or 10% (TGO) SDS-polyacrylamide gel. The 6% resolving gel was made of 4ml Tris 2M pH8.8, 200µl 10% SDS, 4ml 30% acrylamide, 160µl ammonium persulphate and 24µl TEMED for a final volume of 20ml. The 10% resolving gel was made of 3.7ml Tris 2M pH8.8, 200µl 10% SDS, 6.6ml 30% acrylamide, 80µl 25% ammonium persulphate and 12µl TEMED for a final volume of 20ml.

The resolving gel ingredients were mixed and immediately poured between the gel plates. Just after the gel was set, the stacking gel ingredients (1ml Tris 1M pH6.8, 1.5ml 30% acrylamide, 100µl 10% SDS, 20µl 25% ammonium persulphate and 10µl TEMED for a final volume of 10ml) were mixed and poured between the plates on the top of the resolving gel. Finally, 3x protein loading buffer (188mM Tris pH6.8, 6% (v/v) SDS, 30% (v/v) glycerol, 15% (v/v) β–mercaptoethanol, 0.03% (w/v) bromophenol blue) was added to each sample that were then boiled, spun down and loaded. The Broad Range Prestained SDS-PAGE Standards, BioRad, was used as protein weight marker. The gel was run at 10mA until the dye entered the resolving phase of the gel. The current was increased up to 30mA until the samples reached the bottom. The gel running buffer consisted of 2.5mM Tris, 0.25M Glycine, 0.1% (v/v) SDS.

Protein electro-blotting

Immediately after the samples have run to the bottom of the gel, they were electro-blotted onto a nitrocellulose membrane (PROTRAN, Schleicher and Schuell) at 400mA for 3 hours. The system was kept on ice to avoid overheating. The blotting buffer consisted of 40mM Tris, 40mM glycine, 0.0375% (v/v) SDS, 20% MetOH for a final volume of 5 l.

Incubation with antibodies

Antibodies were used according to the following final dilutions:
Rat anti-TIM (gift from M. Young 1: 1000)
Rabbit anti-TIM (produced by Neosystem, 1:1000)
Goat anti Rat HRP conjugated (Sigma, 1: 8000)
Mouse anti-HSP70 (Sigma, 1: 50000)
Goat anti-Rabbit HRP conjugated (Sigma, 1: 6000)
Rabbit anti-Mouse HRP conjugated (Sigma1: 6000)

The nitrocellulose membranes carrying the proteins were blocked during at least one hour at room temperature in TBST (10mM Tris-Cl pH7.5, 150mM NaCl and 0.05% w/v Tween-20) containing 5% (w/v) milk powder. They were then incubated with the primary antibody diluted in TBST plus 5% milk for 2-3 hours at 4°C with agitation. The antibody solution was then poured off and the membranes were washed tree times in TBST for 5 minutes. The membranes were incubated for 1 hour with a horseradish peroxidase-conjugated secondary antibody in TBST plus 5% milk. A further three 5 minutes washes were then carried out in TBST prior to the incubation with the signal detection reagents.

Signal generation and detection

The luminescent signal was created by incubating the membranes in a solution made of 0.1M Tris-Cl (pH 8.5), 6.25 µM luminol, 6.38 µM p-coumaric acid and 2.7 mM H_2O_2 for 1 minute. This signal was then detected on an auto-radiograph film (Fuji). The developed film was digitalised with a scanner and the corresponding image was analysed using the Scion Image for Windows software (beta 4.0.2 version). The amount of protein corresponding to each band was calculated as the average pixel intensity minus the background (i.e., the average pixel intensity of an area of equal size next to the band to quantify) times the area of the signal corresponding to the band.

Acetone powder purification

To reduce cross reactivity of the rabbit anti-TIM antibody, a 1:10 dilution of the serum was pre-incubated overnight with acetone powder from tim^0 flies. Heads were powdered in liquid nitrogen with mortar and pestle. The powder was homogenised in saline (0.9% NaCl). After 5 minutes incubation in ice, 8 ml of fresh acetone (-20 °C) per 2 ml of cell suspension was added. The samples were mixed, incubated at 0°C for 30 minutes and spun down. After a second treatment with fresh acetone, the pellet was transferred to a clean piece of filter paper,

spread and allowed to air drying at room temperature. The acetone powder was added to a final concentration of 1%, incubated for 30 min at 4°C with rabbit anti-TIM antibody and spun down. The supernatant was used as a source of anti-bodies for the assay.

2.2.4 Anti-body staining

Expression of TGO in the *Drosophila* brain was determined by immunostaining with TGO anti-body (Sonnenfeld *et al.*, 1997). Brains were dissected in PBS and fixed in 4% PFA for 2 hours at room temperature. Before being transferred to methanol (MetOH), the samples were left over night at -20°C. Next day, the brains were washed for 5 minutes in 75% MetOH in PBSTr (PBS plus 0.5% Triton-X). Two subsequent 5 min washes were performed with 50% and 25% MetOH in PBSTr, respectively, and finally three 5 min washes with PBSTr. Samples were blocked in 10% sheep serum in PBSTr for 2 hours at room temperature. The brains were incubated with anti-TGO anti-body (1: 50) diluted in 10% sheep serum in PBSTr and left over-night at 4°C. At the following day, eight 15 min washes in PBTr were performed before the samples were incubated over-night at 4°C with anti-mouse horseradish peroxidase-conjugated secondary anti-body (1:6000) in 10% sheep serum diluted in PBTr. The brains were again washed eight times for 15 min. and pre-incubated for 20 min in 1% diaminobenzidine (DAB) in PBS before the addition of 1, 5 µl of 6% H_2O_2. The reaction was monitored under a dissecting microscope until it reached a satisfactory level of staining. The reaction was stopped by washing three times for 5 min with PBS and then the brains were fixed for 20 min in 4% PFA. The fixative was removed with three 5 min washes in PBS and the samples were preserved in 80% glycerol at 4°C. Preparations were observed with a Nikon Optiphot-2 microscope, using Plan 10x (16/0.17, 0.30 DIC) and Plan 20x (16/0.17, 0.50 DIC) objectives. Pictures were taken with a Nikon Coolpix 4500 digital camera.

Chapter 3: TANGO expression in *Drosophila* head

3.1 Introduction

The embryonic expression of TGO has been extensively examined by immuno-staining with monoclonal anti-body (Emmons *et al.*, 1999; Sonnenfeld *et al.*, 1997; Ward *et al.*, 1998). TGO was found to be broadly distributed throughout embryogenesis (Ohshiro and Saigo, 1997; Sonnenfeld *et al.*, 1997), although enhanced levels were observed in the developing trachea and CNS. The protein distribution, determined by using TGO anti-body, has been shown to be similar to its mRNA distribution (Sonnenfeld *et al.*, 1997). The transcripts of *tgo* were shown to be found either in the cell nucleus or in the cytoplasm, depending on cell type and time of development (Sonnenfeld *et al.*, 1997). Later studies confirmed that nuclear localization of TGO is developmentally regulated, being localized to the cytoplasm in most cells except the CNS midline, salivary duct, tracheal, antennal segment and peripheral nervous system cells, where it accumulates in the nuclei (Emmons *et al.*, 1999; Ward *et al.*, 1998). TGO nuclear localization correlates with bHLH-PAS proteins functioning as transcription factors and corresponds to the cells in which SINGLE-MINDED (SIM), TRACHEALESS (TRH) or SPINELESS (SS), dimerization partners of TGO, are present. This fact provided *in vivo* evidence that SIM, TRH and SS form transcriptional competent hetorodimers with TGO (Emmons *et al.*, 1999; Ohshiro and Saigo, 1997; Sonnenfeld *et al.*, 1997; Ward *et al.*, 1998).

Interestingly, TGO immuno-staining analysis showed that only in those cells in which it is part of a heterodimeric complex is the protein concentrated in the embryonic nuclei (Ward *et al.*, 1998). The situation is different in mammals since ARNT has a nuclear localization sequence, absent in TGO, which can localize the protein into the nuclei in the absence of any known bHLH-PAS counterpart (Eguchi *et al.*, 1997; Pollenz *et al.*, 1994).

One important implication of the *Drosophila* studies is that TGO does not form transcriptional active homodimers during embryogenesis (Ward *et al.*, 1998). Moreover, biochemical studies have not been carried out to determine whether *Drosophila* TGO can homodimerize or not. This is in opposite situation to mammalian ARNT that can homodimerize *in vitro* and in cell culture, binding DNA and activating transcription (Sogawa *et al.*, 1995, Sonnenfeld *et al.*, 1997; Swanson *et al.*, 1995).

TGO expression in *Drosophila* adult brain is not as well characterized as in embryos. *In situ* hybridization (ISH) staining for *tgo* in wild-type *Drosophila* (Codd, 2004) revealed a very wide spread pattern of distribution, resembling that seen for *per* (Kloss *et al.*, 1998). Moreover, staining was observed in both the retinas, suggesting that *tgo* is expressed within the photoreceptors. Nothing is known about the circadian profile of the protein in adult head. Recent genome-wide analysis of gene expression in *D. melanogaster* using microarrays (Claridge-Chang *et al.*, 2001; McDonald and Rosbash, 2001; Ueda *et al.*, 2002; Wijnen *et al.*, 2006) have revealed a large number of oscillating genes. The results indicated that *tgo* was not included in the group of cycling genes. However, since screening was based on robust and persistent gene expression, cycling genes giving rise to less persistent, damping oscillations may well have been overlooked. For a better characterization of a role for TGO in the circadian clock, it is necessary to determine the specific localization of *tgo* transcripts within the central pacemakers and also within the photoreceptors structures and whether the gene product cycles or not in adult heads. In this chapter I shall present data on TGO expression in *Drosophila* adults as indicated by utilization of anti-TGO monoclonal antibody for immuno-staining of the brain and for Western blot experiments on heads.

3.2 Material and methods

3.2.1 Immuno-staining

Drosophila strain used was w^{1118}. Before being immobilized with CO_2, flies were kept under a 12:12 LD cycle at 25°C. Whole brains were extracted from both male and female adults. Antibody staining and imaging processing were carried out according to Chapter 2.

3.2.2 Western blot

Drosophila strain used was w^{1118}. Flies subjected to a 12: 12 h LD conditions at 25°C were collected every two hours during a 24h day course. Proteins were extracted from both male and female adult heads. Refer to Chapter 2 for the detailed description of Western blot experiments.

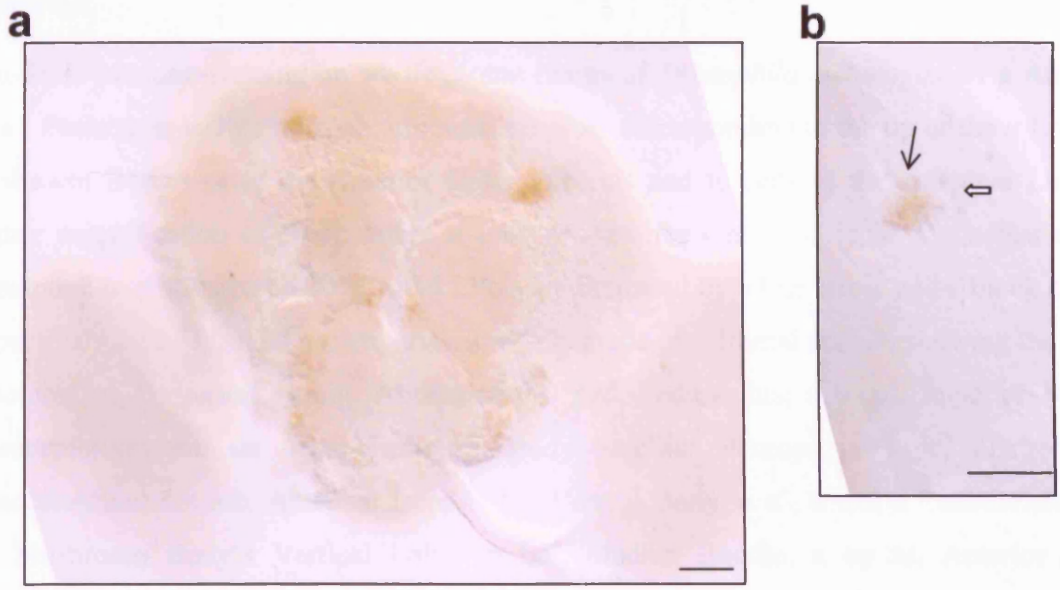
3.3 Results

3.3.1 Immuno-staining

Figures 3.1 and 3.2 show diaminobenzidine (DAB) staining for TGO on whole mount *Drosophila* brains. The reaction was performed with a monoclonal antibody (Sonnenfeld *et al.*, 1997) available from the Hybridoma bank at the University of Iowa. When compared with the schematic provided (Fig. 3.1c), the anterior brain (Fig. 3.1a) shows that anti-TGO immunoreactivity is putatively present in the following brain structures; tip of the α lobe of the Mushroom Bodies or Anterior Optic Tubercle, Antennal Lobe and Sub-esophageal ganglion. The specificity of the signal is supported by the nuclear localisation of the staining (see Fig 3.1b), which is expected only in cells expressing functional partners of TGO. The posterior side (Fig 3.2a) of the brain also shows widespread distribution of immunoreactivity in the Protocerebrum, in the Tritocerebrum and in the Sub-esophageal ganglion. No staining was detected in the Optic Lobes and in the retina (Fig 3.2b), which is at odd with previous results from *in situ* hybridisation.

3.3.2 Western blot

Figure 3.3 shows a Western blot analysis of TGO in heads of *D. melanogaster* normally expressing the protein. Adult flies were shown to be able to express high levels of TGO. Moreover, it was shown that TGO does not cycle, as no progressive difference in levels of expression was observed between the time points analysed. The small level detected at ZT 2 seems to be sample error loading as indicated by the low level of HSP70 at the same collection point.


a
b

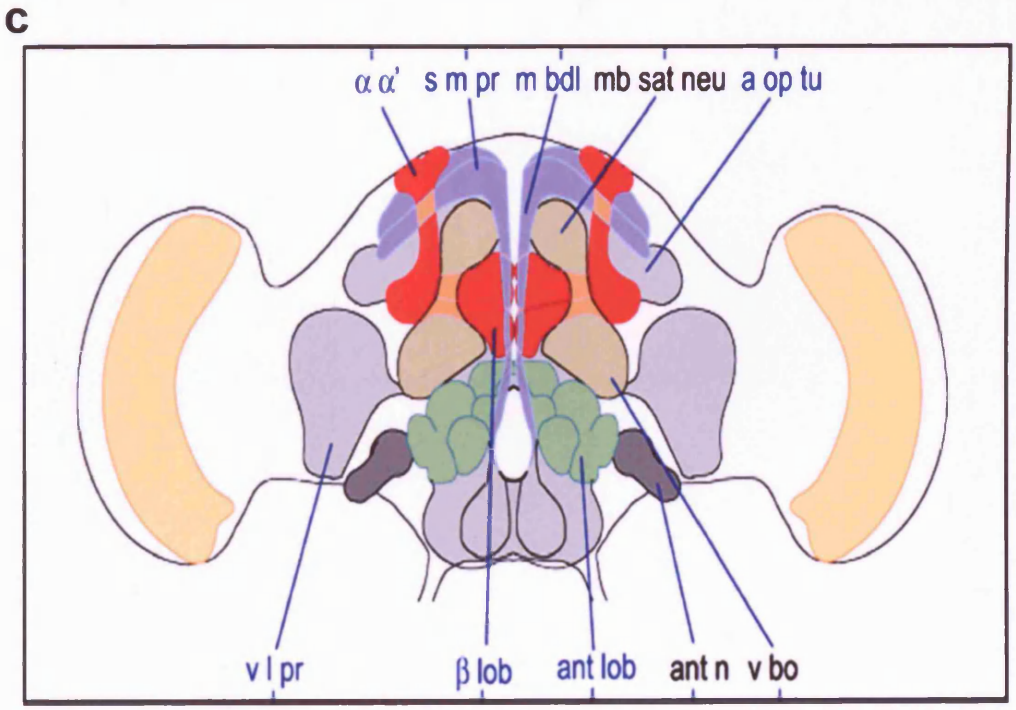

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Figure 3.1

Anti-TGO immuno-staining on whole mount brains of *Drosophila melanogaster*. **a** Anterior view. Staining is visible in a pair structure, possibly corresponding to the tip of the α Lobe of Mushroom Bodies or to the Anterior Optic Tubercle and to cells of the Antennal Lobe. **b** Higher magnification of the putative α lobe or Anterior Optic Tubercle. Cells that might correspond to clock neurons DN2s and LPNs are indicated by a line arrow and a block arrow, respectively (see Fig. 1.3 for comparison). **c** Schematic of a frontal section showing the main structures of *Drosophila* brain. Abbreviations: ped, Pedunculus; s m pr, Superior Medial Protocerebrum; mb sat neu, Mushroom Body Satellite Neuropil; v l pr, Ventrolateral Protocerebrum; ant lob, Antennal Lobe; v bo, Ventral Body, α α`, α and α ` subdivisions of the Mushroom Body`s Vertical Lobe; m bdl, Median Bundle; a op tu, Anterior Optic Tubercle; β lob, β subdivision of the Mushroom Body`s Medial Lobe; ant n, Antennal Nerve (see http://flybrain.neurobio.arizona.edu/Flybrain/html/atlas/schematic/frontschem1.html). Scale bars correspond to 50 μm.

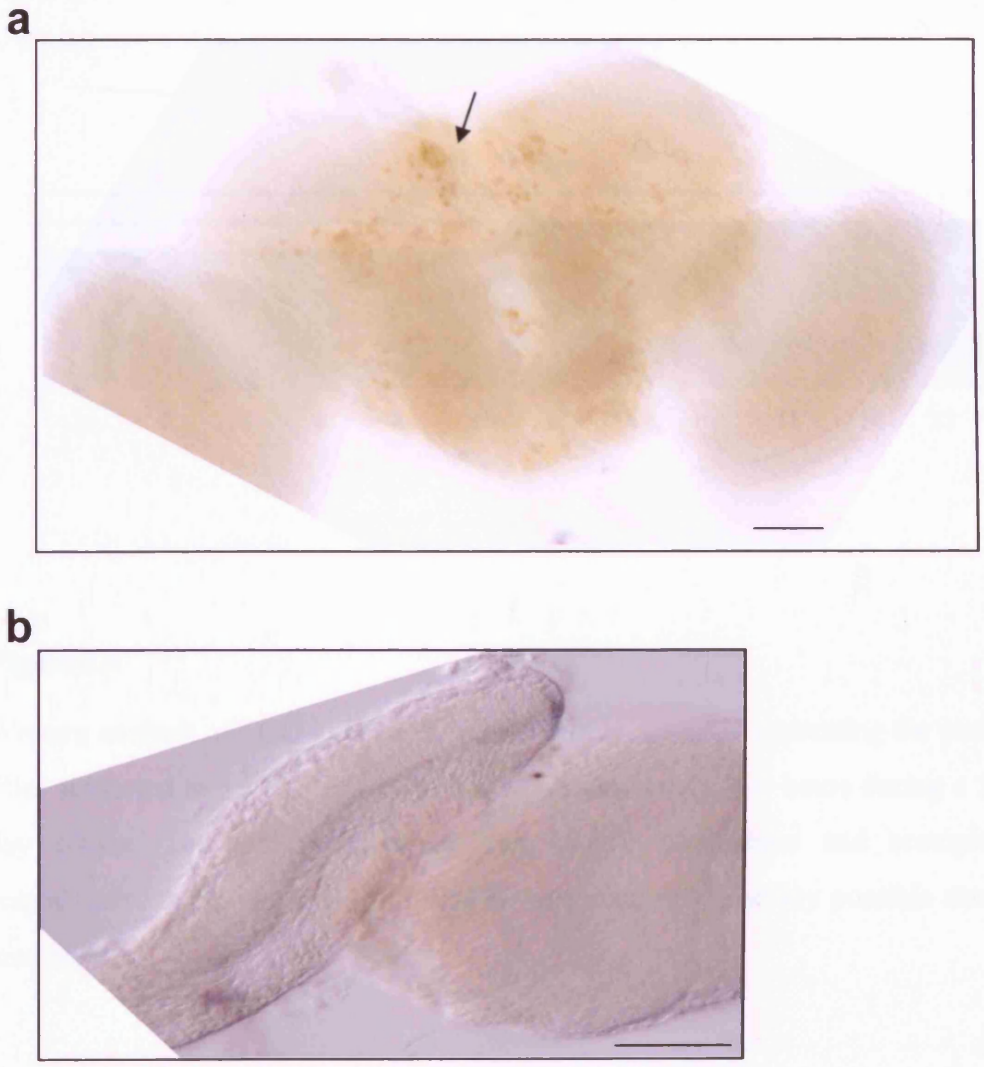


Figure 3.2

Anti-TGO immuno-staining on whole mount brains of *Drosophila melanogaster*. **a** Posterior view of the brain showing widespread distribution of the immuno-signal. Line arrow shows possible DN1s (see Fig. 1.3 for comparison). **b** Retina and Optic Lobe show no immuno-staining. Scale bars correspond to 50 mm.

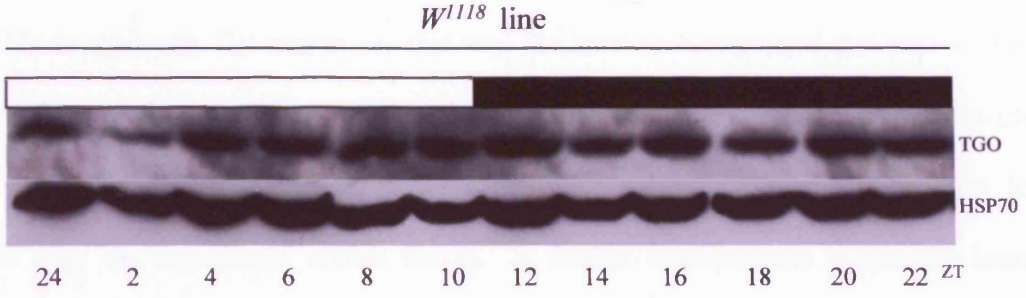


Figure 3.3

Western analysis of TGO in adult *D. melanogaster* normally expressing the protein. Flies subjected to a 12:12 LD cycle were collected every two hours during a 24 h day course. The white and black bars denote photophase and scotophase, respectively. Immunodetection of HSP70 was done to check any possible sample loading error.

3.4 Discussion

Several attempts to obtain staining using a standard protocol, without the addition of methanol, failed. This suggests that the available anti-TGO antibody might recognise an epitope hidden in a hydrophobic pocket of the protein, which becomes exposed after denaturation. Moreover, the pictures presented are from a single experiment that turned out impossible to replicate. The reason for that was the level of background that was always too high to allow a clear detection of the signal. This was likely caused by the secondary antibody, as the original batch of anti-mouse HRP secondary antibody was no longer available after the experiment shown above. A further consideration is that the immuno-signal was only observed after an enzymatic amplification provided by the DAB reaction. The use of a fluorescent-conjugated secondary antibody never resulted in staining. These hurdles prevented double-staining experiments to compare the expression of TGO with markers for the circadian clock (for instance PER and TIM) or for the Kenyon cells (for example, EYELESS). Nevertheless, the pattern of expression suggests that TGO is not present in the Lateral Neurons, but could be present in some Dorsal Neurons, like the DN2s (Figure 3.1b) and a subset of the DN1s and DN3s (Figure 3.2a). Further attempts must be made to map the expression of TGO in conjunction with clock markers, possibly using a combination of ISH with anti-body staining and also employing a fluorescent-tyramide method (http://probes.invitrogen.com/media/publications/222.pdf), as to benefit from the confocal microscopy. It will also be important to re-examine *tgo* ISH to resolve the issue of the expression in photoreceptor cells, clarifying whether *tgo* mRNA is indeed found in the retinas and whether or not they express TGO. To overcome artefacts that might affect ISH and immuno-staining, these methods could be combined to PCR and western blotting using dissected eyes. Finally, Western blot experiment revealed that *tgo* product is highly expressed in *Drosophila* head and also that it does not cycle at 12: 12 LD condition.

Chapter 4: Overexpression of TANGO in *Drosophila melanogaster*

4.1 Introduction

The TGO protein belongs to a broad class of proteins characterized by the possession of PAS domains that are used as a signal sensor in many signalling pathways (Gu *et al.*, 2000; Oshiro and Saigo, 1997; Sonnenfeld *et al.*, 1997; Taylor and Zhulin, 1999). The circadian clock is one of a number of PAS-dependent signalling pathways which, in higher eukaryotes, have the function to tune the organism to the environment (Allada, 2003; Allada *et al.*, 2001; Blau, 2003; Chang, 2006; Dunlap, 1999; Hall, 1995; 1996; Hardin and Siwick, 1995; Hardin, 2005; Helfrich-Foster, 2005; Rosato *et al.*, 2006; Rosbash *et al.*, 1996; Sehgal *et al.*, 1996; Stanewsky, 2002; Stanewsky, 2003; Young and Kay, 2001; Zordan et al., 2003). It is reasonable to assume that a protein PAS dimerization domain could play a role in more than one PAS signalling system. During a yeast-two-hybrid screening (Gekakis *et al.*, 1998), it was shown that mammalian CLOCK (CLK) interacted with Aromatic Hydrocarbon Receptor Nuclear Translocator (ARNT), a member of the PAS superfamily (Gu *et al.*, 2000). In *Drosophila*, *tango* (*tgo*), the ortholog of mammalian *Arnt*, codes for a protein involved in several aspects of development (Sonnenfeld *et al.*, 1997). Moreover, the homology between these two proteins suggests that TGO, like ARNT, might interact with components of the clock machinery. Indeed, using a yeast-two-hybrid assay our group has shown that TGO physically interacts not only with *Drosophila* CLK, but also with dCRY (E. Rosato, personal communication). A simple way to investigate a possible function of *tgo* in the circadian clock is by manipulating its expression in clock cells without altering its expression in other tissues. In this chapter I shall present data regarding the overexpression of TGO in clock cells and its effects on the regulation of *Drosophila* circadian clock.

4.2 Material and methods

The expression of TGO was manipulated by using the GAL4/UAS binary system (Brand and Perrimon, 1993; Brand *et al.*, 1994; Phelps and Brand, 1998). Transgenic *Drosophila* lines expressing the yeast transcriptional activator GAL4 (driver lines) fused to the *timeless* (*tim*) promoter were used to drive gene expression in clock neurons. Any gene of interest can then be driven in the clock neurons by creating transgenic flies (reporter strain) carrying the relevant cDNA downstream of several copies of the Upstream Activation Sequence (UAS), the binding site of GAL4. As *Drosophila* have no endogenous GAL4, the gene of interest is only expressed when the driver and the reporter lines are crossed, bringing the GAL4 and UAS elements together. The GAL4 system is temperature sensitive, showing little activity at 15°C, while driving strong expression at 29°C (Brand *et al.*, 1994; Kramer and Staveley, 2003).

In order to obtain TGO overexpression, UAS-*tgo* constructs were created and, following microinjection, two transgenic fly lines were recovered; line 52.1 carrying an X chromosome insertion, and line 25.1 carrying a chromosome 3 insertion. The desired genotype was obtained by bringing together the *yw*; *tim*-GAL4 driver line with the UAS-*tgo* reporter lines. In order to minimize variables throughout this study, all crosses were performed using virgin female drivers (*yw*; *tim*-GAL4) against male reporters (UAS-*tgo*). Only male F1 flies were used for the locomotor activity analysis. Both male and females F1 flies were used for western blot experiments. Exceptionally, the crosses involving the line 52.1 were performed in the opposite direction i.e., using male drivers (*yw*; *tim*-GAL4) against virgin female reporters (UAS-*tgo52.1*) due to the fact that in this line the construct is located at the X chromosome. In this way male F1 would still carry the UAS-*tgo* construct to successfully overexpress the protein. Refer to Chapter 2 for the detailed description of the crosses, locomotor activity and western blot experiments.

4.3 Results and discussion

4.3.1 Assessment of TGO overexpression via protein quantification

Important features as phosphorylation of proteins and quantification of their level of expression are based upon biochemical analysis on head extracts. It has to be taken into consideration that head extracts mostly reflect the peripheral clock located in the eyes whereas behaviour depends upon clusters of neurons in the brain. An important point considered before conducting experiments with the aforementioned transgenic lines was to assess their efficiency in overexpressing TGO.

Figure 4.1 shows a western blot experiment made with lines overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/ + and UAS-*tgo52.1*; *tim*-GAL4/+; +/+) and their common control (*w*; *tim*-GAL4/ +; +/ +). Flies were raised at 25°C and collected in the middle of the photophase (ZT6). Equal amounts of whole head protein extracts were run on a 10% polyacrylamide gel and blotted onto nitrocellulose membrane. TGO was detected with a mouse anti-TANGO antibody (Developmental Studies Hybridoma Bank at the University of Iowa, 1:1000) and a HRP-conjugated rabbit anti-mouse (Sigma, 1: 6000). Both the transgenic lines analysed were shown to be able to overexpress TGO. After confirming that these "tools" were working properly; a number of phenotypes began to be investigated in order to assess whether or not *tgo* up regulation would affect the circadian clock of *D. melanogaster*.

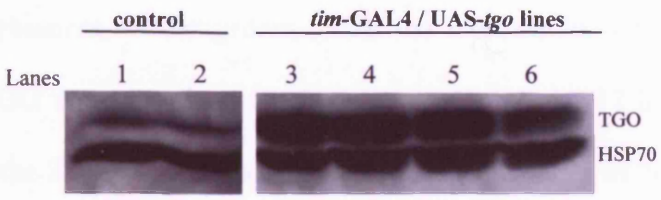


Figure 4.1

Western blot analysis of TGO in control and overexpressing flies. Samples were loaded as duplicates from different extractions. Immunodetection of HSP70 was done to check any possible sample loading error. Lanes 1 and 2, control flies (*w*; *tim*-GAL4/+; +/+). Lanes 3 and 4, overexpressing flies carrying the UAS-*tgo25.1* construct (w; *tim*-GAL4/+; UAS-*tgo25.1*/+). Lanes 5 and 6, overexpressing flies carrying the UAS-*tgo52.1* construct (UAS-*tgo52.1*; *tim*-GAL4/+; + /+). The whole film obtained in this experiment is shown in appendix 1.

4.3.2 Entrainment by light cycles

Analysis of the behavioural rhythms can be made in order to investigate if the overexpression of TGO can affect entrainment. Endogenous oscillators (the 'biological clock') are expected to adjust their period (τ) such as to match that of cycling parameters in the external environment, or *Zeitgebers* (from the German for time giver). Initially, flies overexpressing TGO were entrained for 5 days to a 12 h light: 12 h dark cycle (LD 12:12) which represents the *Zeitgeber*. The activity, recorded as a series of 30 min bins, for the flies of each genotype, was averaged and double plotted. Figure 4.2 shows the average locomotor activity pattern of male flies overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/+) as well as the relevant controls (*w*; *tim*-GAL4/+; +/+ and *w*; + /+; UAS-*tgo25.1*/+). The experiments were conducted at 25°C.

A perfect coupling between the self sustained oscillator and the *Zeitgeber* was observed for all flies examined. Thus, TGO overexpression did not affect entrainment under the conditions shown above; in fact the overexpressing flies showed the same bimodal pattern as the controls with peaks of activity centred on the dark/light and light/dark transitions (Klarsfeld *et al.*, 2003, Rosato and Kyriacou, 2006). Table 4-1 shows that the period of locomotor activity (τ) is the same for all genotypes regardless the amount of TGO protein produced. This was statistically confirmed by analysis of variance (ANOVA, $F_{2, 175} = 0.245$, $P > 0.05$, see appendix 2).

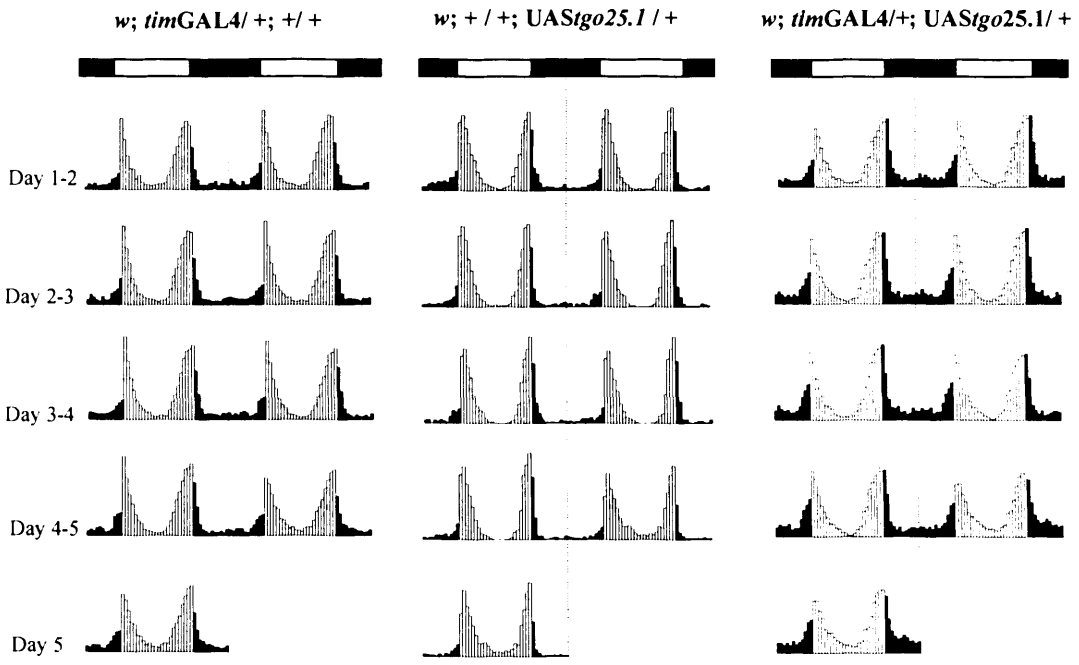


Figure 4.2

Double plot of locomotor activity profiles (average amount of activity – Y axis – as a function of time – X axis) for *D. melanogaster* overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/+) and relevant controls (*w*; *tim*-GAL4/ +; +/+ and *w*; + / +; UAS-*tgo25.1*/+). Shown are 5 days of activity under a LD 12:12 regime. Open bars, photophase. Shaded bars, scotophase.

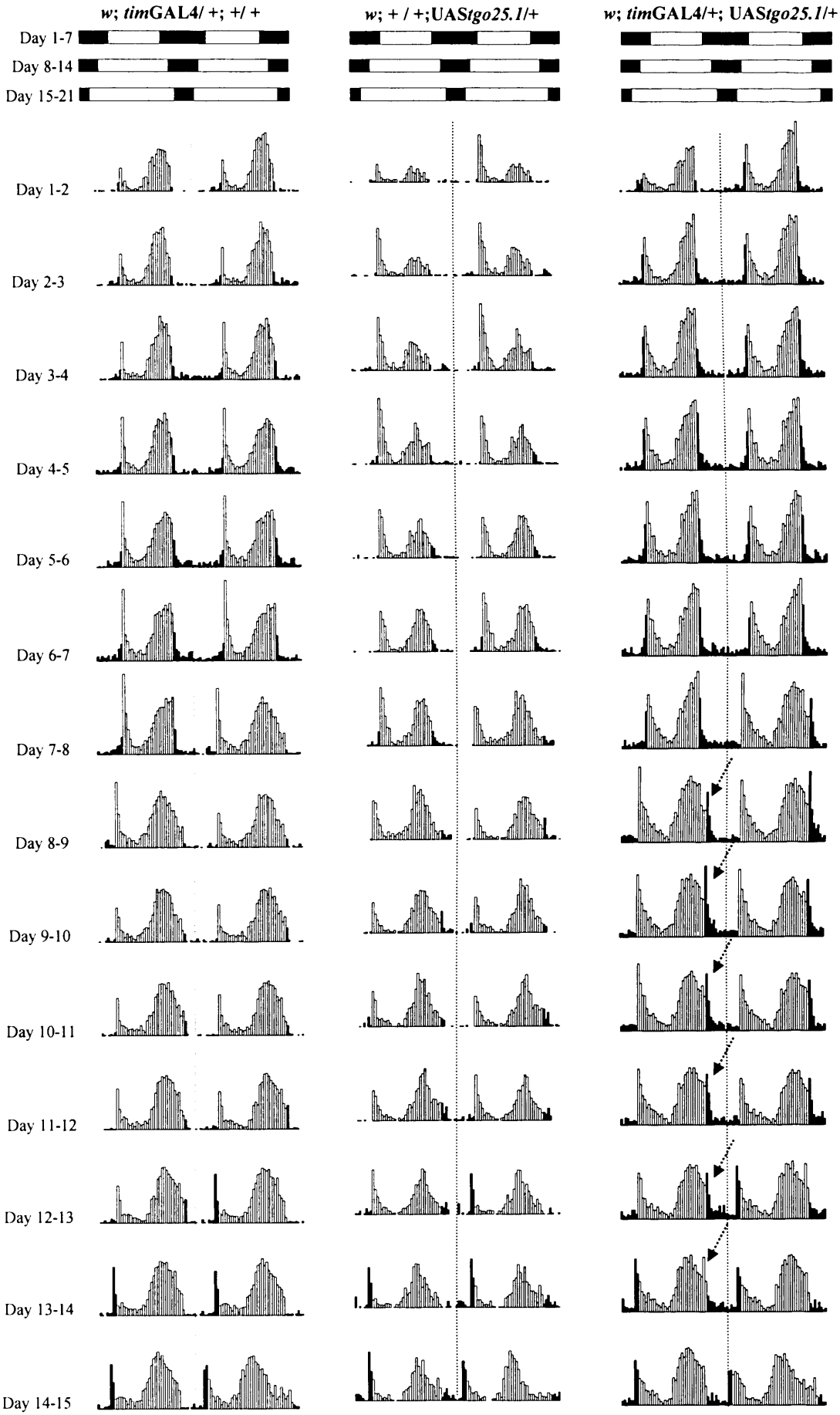
Table 4-1

Entrained locomotor activity rhythms for flies overexpressing TGO and controls. The entrained period was determined by autocorrelation and Fourier analysis and the results compared. The period length given by spectral analysis is presented. Average and standard error of the mean (SEM) are shown. N= number of flies examined.

Genotype	Entrained period ± SEM	N
w; *tim*-GAL4/ +; +/ +	24.07 ± 0.01	68
w; + / +; UAS-*tgo25.1* / +	24.05 ± 0.02	41
w; *tim*-GAL4/+; UAS-*tgo25.1* / +	24.06 ± 0.02	69

Apart from synchronizing the clock, light can also influence activity rhythms by direct modulation. In diurnal animals light often promotes activity, whereas it inhibits activity in nocturnal animals (Rieger *et al.*, 2003). When such effects of light are present, they often conceal the activity controlled by the circadian clock. Therefore they are called the "masking effect" of light (Mrosovsky, 1999; Mrosovsky *et al.*, 1999). Further investigation on entrainment was performed by changing the length of the photophase and scotophase so that the peak of activity would not coincide with the times of light transitions in a 12: 12h LD cycle. The reason for this is that the "masking effect" could be obscuring the results. To do so, flies were allocated in an incubator initially set to12:12 LD and after one week the incubator had its photophase increased by 4 hours (16:8 LD). Finally, after the second week the photophase was once again increased by 4 hours (20:4 LD). The temperature was always kept constant at 20°C. The transitions between the light and the dark phases were instantaneous (abrupt transition).

The recorded locomotor activity data was transferred to an Excel spreadsheet for analysis. The bin corresponding to the highest level of activity was chosen as the representative point for the activity peak for each genotype. The two initial days after LD conditions changes were considered as transition and therefore they were not computed. The measurements were done only on the third day after the start of each new LD cycle. The average time of the evening peak of activity was calculated for each genotype in every LD condition and the values were compared. Figure 4.3 shows the average locomotor activity patterns for flies overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/+) and controls (*w*; *tim*-GAL4/+; +/+ and *w*; +/+; UAS-*tgo25.1*/+) subject to weekly increases of the photoperiod. Table 4-2 summarizes the number of flies and the evening peak av erage time for each genotype and each photoperiod analysed. ANOVA analysis revealed that the line overexpressing TGO was significantly different from both the controls at LD 12:12 ($F_{2, 50}$ = 8.7, $P < 0.05$, appendix 3), at LD 16: 8 ($F_{2, 45}$ = 8.57, $P < 0.05$, appendix 3) and at LD 20: 4 ($F_{2, 37}$ = 4.68, $P < 0.05$, appendix 3). However, Bonferroni *Post Hoc* comparisons revealed that *tim*-GAL4/UAS-*tgo25.1* and UAS-*tgo25.1*/+ flies were not different at LD 20:4. This could have been due either to the reduced number of flies that survived the third week of the experiment or to a lack of effect in such conditions.


w; timGAL4/ +; +/ +
w; + / +;UASstgo25.1/+
w; timGAL4/+; UASstgo25.1/+
Day 1-7
Day 8-14
Day 15-21
Day 1-2
Day 2-3
Day 3-4
Day 4-5
Day 5-6
Day 6-7
Day 7-8
Day 8-9
Day 9-10
Day 10-11
Day 11-12
Day 12-13
Day 13-14
Day 14-15

Continuation

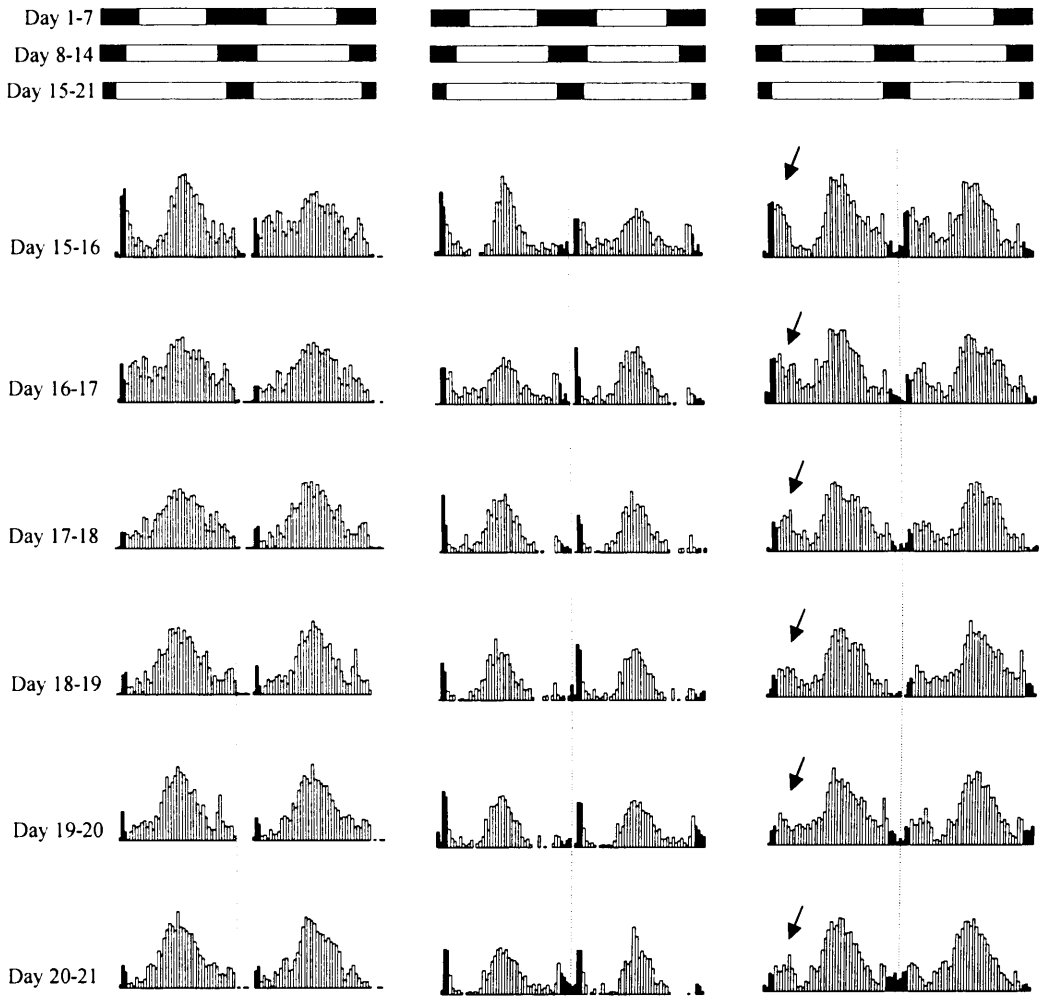


Figure 4.3

Double plot of locomotor activity profiles (average amount of activity – Y axis – as a function of time – X axis) for *D. melanogaster* overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/+) and relevant controls (*w*; *tim*-GAL4/ +; +/ + and *w*; + / +; UAS-*tgo25.1*/+). Dashed arrows indicate a startle response for lights off in the experimental line. Filled arrows indicate a morning peak persisting only in the flies overexpressing TGO. Shown are 7 days of activity under LD 12:12, followed by 7 days under LD 16: 8 and finally 7 days under LD 20: 4. Open bars, photophase. Shaded bars, scotophase.

Table 4-2

Evening peak of locomotor activity for flies overexpressing TGO and controls. The time of peak was calculated for each fly individually. Average and standard error of the mean (SEM) are shown. N= number of flies examined.

Genotype	Evening Peak Time ± SEM (ZT)					
	12:12 LD	**N**	**16: 8 LD**	**N**	**20: 4 LD**	**N**
w; *tim*-GAL4/+; +/+	9.18 (±0.25)	17	9.0 (±0.29)	15	5.2 (±0.45)	15
w; +/+; UAS-*tgo25.1*/+	9.0 (±0.38)	15	9.27 (±0.24)	13	6.0 (±0.42)	9
w; *tim*-GAL4/+; UAS-*tgo25.1*/+	10.52 (±0.26)	21	10.04 (±0.25)	20	7.19 (±0.47)	16

Figure 4.3 also shows two additional interesting features caused by TGO overexpression. Dashed arrows indicate a evening startle response that only occurs during the second week of experiment in the line overexpressing the protein, when the flies were exposed to 16: 8 LD. Black arrows indicate the maintenance of the morning peak in the third week of the experiment (20: 4 LD). Under an extreme long photoperiod, control flies were shown to loose the morning component of the activity. Only the experimental line showed the typical bimodal pattern of locomotor activity observed in the previous weeks of LD cycles.

Despite all the differences observed in the locomotor activity profile, flies overexpressing TGO showed essentially the same 24-h rhythm of locomotor activity under entrainment conditions as summarized in Table 4-3. A slightly longer but still significant period was only observed for the control line w; +/+; UAS-*tgo25.1*/+ and only at 12:12 LD (Kruskal-Wallis, X^2= 17.89, df = 2, P <0.05, appendix 4). For the two subsequent LD cycles (16: 8 LD and 20: 4 LD) the period was essentially the same for all the genotypes analyzed (ANOVA, $F_{4, 77}$ = 1.74, P > 0.05 and $F_{4, 72}$ = 1.76, P > 0.05, respectively, appendix 4).

Table 4-3

Entrained locomotor activity rhythms for flies overexpressing TGO and controls. The entrained period was determined by autocorrelation and Fourier analysis and the results compared. The period length given by spectral analysis is presented. Average and standard error of the mean (SEM) are shown .N= number of flies examined

Genotype	Entrained period ± SEM					
	12:12 LD	**N**	**16: 8 LD**	**N**	**20: 4 LD**	**N**
w; *tim*-GAL4/+; +/+	24.06 (±0.05)	34	24.19 (±0.04)	15	24.02 (±0.07)	15
w; +/+; UAS-*tgo25.1*/+	24.47 (±0.09)	33	24.18 (±0.09)	13	23.94 (±0.18)	9
w; *tim*GAL4/+; UAS*tgo25.1*/+	24.01 (±0.04)	44	24.18 (±0.05)	18	23.97 (±0.07)	18

These results suggest that TGO overexpression might affect light input mechanisms of the clock. Thus, it is possible to speculate that is the photophase of the circadian cycle to be mainly affected by the overexpression of TGO, as evidenced by the fact that behavioural effects become clearer the longer the photophase is. Interestingly, this is the part of the day we know the least about circadian terms. During the day both TIM and PER are largely absent from the cellular scene and CLK, although present at high levels, is inhibited by VRI (Allada *et al.*, 1998; Darlington *et al.*, 1998; Glossop *et al.*, 1999; Houl *et al.*, 2006; King *et al.*, 1997). Nevertheless, this is when the circadian behaviour of flies unfolds. Clearly, many other components are involved suggesting that some might be dependent upon TGO.

4.3.3 Free-running behaviour

Rhythms of activity of *D. melanogaster* overexpressing TGO were analyzed in continuous darkness (DD) and constant temperature. In this way, the endogenous periodicity (τ) of each fly could be computed without the interference of external variables. The same flies analyzed under entrainment of 12:12 h LD conditions at 25°C (Section 4.3.2 of this chapter) were subjected to free-running conditions for five subsequent days. Figure 4.4 shows the average locomotor activity pattern of flies overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/+) as well as the relevant controls (*w*; *tim*-GAL4/+; +/+ and w; +/+; UAS-*tgo25.1*/+). No difference was detected in the bimodal locomotor activity profiles for all genotypes analysed. In all cases the amplitude of the morning component decreased with time leading to a unimodal activity pattern. This in agreement with previous work comparing rhythms of locomotor activity in *D. melanogaster* wild-type strains (Canton S, Berlin, and Oregon R) that underlined the multioscillatory nature of the *Drosophila* circadian system (Helfrich-Forster, 2000b). Table 4-4 shows that all genotypes had a similar circadian period (ANOVA, $F_{2, 319} = 2.05$, $P > 0.05$, appendix 5).

Table 4-4

Free running locomotor activity rhythms for flies overexpressing TGO and controls. The endogenous period was determined by autocorrelation and Fourier analysis and the results compared. The period length given by spectral analysis is presented. Average and standard error of the mean (SEM) are shown. N= number of flies examined.

Genotype	τDD ± SEM	N
w; *tim*-GAL4/+; +/+	24.72 ± 0.06	104
w; +/ +; UAS-*tgo25.1*/+	24.56 ± 0.07	98
w; *tim*-GAL4/+; UAS-*tgo25.1*/+	24.71 ± 0.06	120

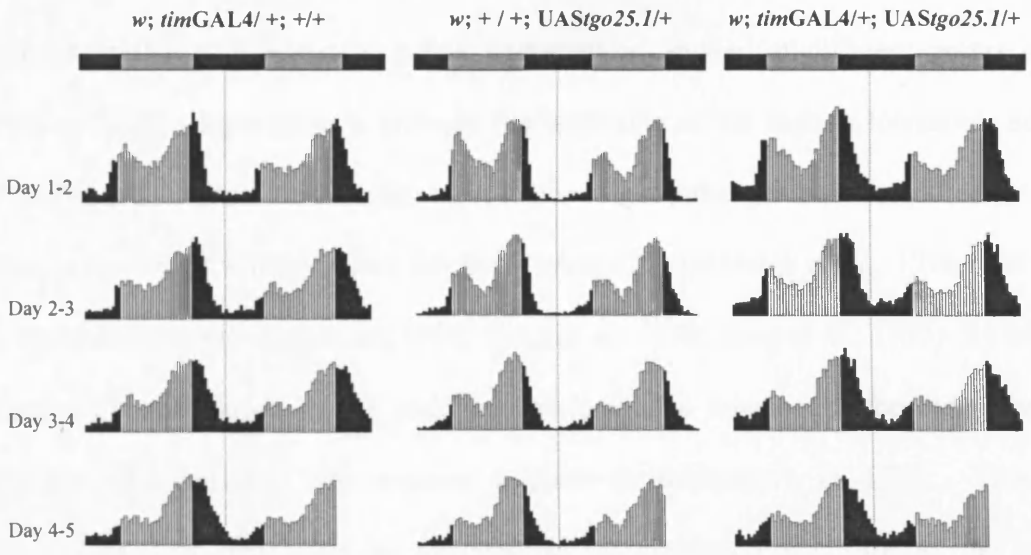


Figure 4.4

Double plot of locomotor activity profiles (average amount of activity – Y axis – as a function of time – X axis) for *D. melanogaster* overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/+) and relevant controls (*w*; *tim*-GAL4/ +; +/+ and *w*; + / +; UAS-*tgo25.1*/+). Shown are 5 days of activity under constant darkness (DD). Gray bars, subjective day. Black bars, subjective night.

4.3.4 Phase shift analysis and phase-response curve (PRC)

Classic experiments of phase-response curve (PRC) test the ability of the endogenous oscillator to adjust to single perturbations applied at different points of the circadian cycle. If a light pulse is given at the beginning of the night a locomotor activity phase delay ($-\Delta\Phi$) is promoted. On the other hand, a light pulse given at the end of the night promotes a locomotor activity phase advance ($+\Delta\Phi$) (Hunter-Ensor *et al.*, 1996; Lee *et al.* 1996; Myers *et al.*, 1996; Suri *et al.*, 1998; Yang *et al.*, 1998; Zeng *et al.*, 1996). By plotting the phase-shift experienced by the endogenous clock as a function of the time when the perturbation was delivered, we produce a phase-response-curve or PRC. This non-parametrical (i.e. in opposition to parametrical or prolonged exposure to the cycling entraining cue) assay for studying entrainment was first developed by Colin Pittendrigh (1965, 1966) and applied to *D. pseudoobscura* eclosion rhythms. Generally, differences in the phase shift after a light pulse might be due to either a difference in photoresponse mechanisms or a difference in the phase of the endogenous clock. However, by using the anchored-PRC protocol, where the light pulse is given during the scotophase of the last LD, the endogenous clock will possess a defined phase so that differences in shift will reflect a difference in the photosensitivity. In order to check if TGO overexpression alters the clock photosensitivity, flies up-regulating the protein and the relevant controls were subject to a 5 minutes pulse at ZT 13, ZT 15 and ZT 21 and the phase shift was calculated (see Figure 4.5 and Table 4-5).

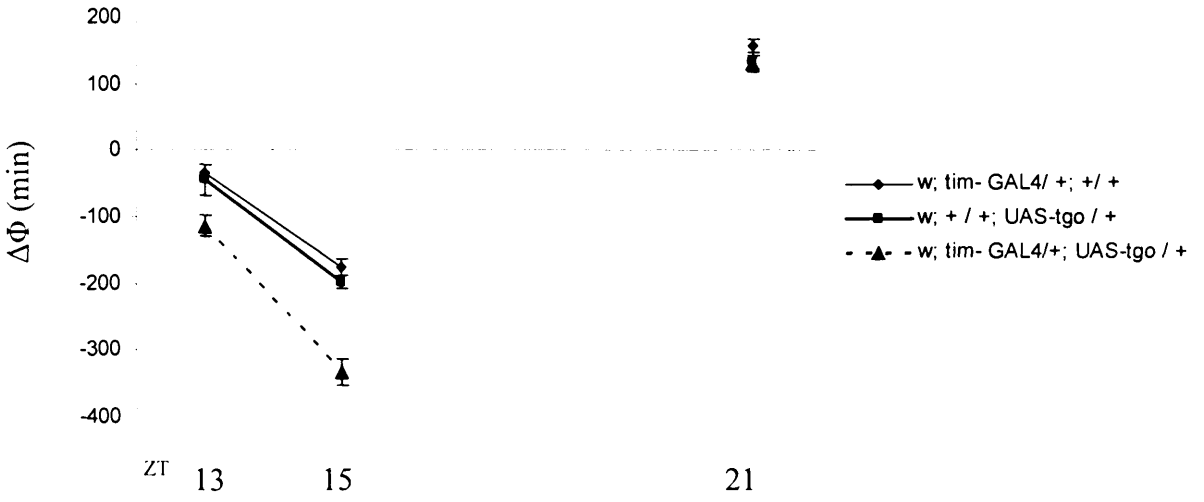


Figure 4.5

Phase Response for *D. melanogaster* overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/+) and relevant controls (*w*; *tim*-GAL4/+; +/+ and w; +/+; UAS-*tgo25.1*/+). Number of flies used to calculate the phase shift magnitudes are indicated in Table 4-5. Average and standard error of the mean (SEM) are shown.

Table 4-5

Phase shift (minutes) after a 5 min light pulse at ZT13, 15 and 21 for flies overexpressing TGO and controls. $N_{P;\,U}$ = number of pulsed and unpulsed flies, respectively.

Genotype	ΔΦ in minutes ± SEM					
	ZT 13	$N_{P;\,U}$	ZT 15	$N_{P;\,U}$	ZT 21	$N_{P;\,U}$
w; *tim*-GAL4/+; +/+	-34.47 (±14.37)	19; 14	-176.32 (±11.42)	37; 23	158.60 (±10.27)	38; 37
w; +/+; UAS-*tgo25.1*/+	-44.50 (±23.97)	23; 16	-199.08 (±10.04)	43; 53	136.27 (±11.43)	64; 53
w; *tim*-GAL4/+; UAS-*tgo25.1*/+	-113.62 (±15.21)	27; 16	-333.4 (±18.68)	30; 33	131.60 (±12.75)	48; 49

Two time domains can be defined in the PRC shown above: (i) a phase delay domain in the early subjective night, when light pulses reset the fly clock to late afternoon; (ii) a phase advance domain, late at night, where light pulses advance flies into the early morning. Each of

these domains has a molecular correlate: the delay domain corresponds to the time of PER and TIM cytoplasm accumulation, while during the advance domain PER and TIM are in the nucleus (Rutila *et al.*, 1998). Examination of Figure 4.5 reveals that the amplitude of the delay domain in the TGO overexpression PRC is roughly double of that seen with control flies. ANOVA analysis points out that the difference found is highly significant at ZT 13 ($F_{2, 66}$ = 5.64, $P < 0.05$, appendix 6) and ZT15 ($F_{2, 107}$ = 37.96, $P < 0.05$, appendix 6). Subsequent Bonferroni *Post Hoc* tests indicated that *tim*GAL4/UAS*tgo25.1* flies were different form both the controls. Interestingly, at ZT 21, the amplitude of the advance domain in the TGO overexpression PRC showed no difference when compared with the controls (ANOVA, $F_{2, 147}$ = 1.28, $P > 0.05$, appendix 6). It suggests that TGO overexpression no longer interfere with PER and TIM molecular dynamics when they are located in the nucleus. In conclusion, flies overexpressing TGO seem to behave similarly to the control in the phase advance domain but when the pulse is given at early night (ZT13 and ZT15) they show a more pronounced response in the phase delay domain, revealing an increased sensitivity to light in the early night.

4.3.5 Overexpression of TANGO in a Clk^{jrk} and cyc^{0} background

Good evidence suggesting a possible role for *tgo* in the circadian clock of *D. melanogaster* comes from a study comparing the effects of Clk^{jrk} and cyc^{0} mutations on the immunostaining of PDF, a neuropeptide involved in clock output in insects (Park *et al.*, 2000). Clk^{jrk} showed more severe effects on both the expression of PDF and the development of the lateral neurons (the main clock cells in the brain) than cyc^{0}, perhaps indicating the existence of another PAS-containing transcription factor that can associate with CLK providing a biological relevant function. If this hypothesis is true, one could expect a rescue in

locomotor activity rhythms of the arrhythmic Clk^{jrk} and cyc^{0} mutant flies overexpressing TGO. Two hypothetical situations could be imagined: (i) in Clk^{jrk} mutants, the CLK protein possesses an amino acid replacement at position 776 resulting in premature stop codon that truncates the protein, deleting most of the C- terminal activation domain but not the DNA binding one. Since TGO was shown to bind CLK in yeast-two-hybrid assays (E. Rosato, personal communication), a putative interaction with truncated CLK would allow TGO C-terminal activation domain to trigger the transcription activation on *per* and *tim* making the flies rhythmic, (ii) cyc^{0} mutant flies overexpressing TGO may also have its rhythm rescued if one assumes that TGO would bind CLK to replace the CYC PAS deficient protein produced by cyc^{0} mutation. Figures 4.6 and 4.7 show the crossing schemes used to obtain *D. melanogaster* carrying *tim*-GAL4 in a Clk^{jrk} and cyc^{0} mutant background, respectively. Figures 4.8 and 4.9 show the crossing schemes used to obtain *D. melanogaster* carrying UAS-*tgo52.1* in a Clk^{jrk} and cyc^{0} mutant background, respectively. By bringing the newly obtained lines together TGO protein was overexpressed in arrhythmic mutant flies. Figure 4.10 shows the average locomotor activity pattern of flies overexpressing TGO in a Clk^{jrk} background (UAS-*tgo52.1*; *tim*-GAL4/+; Clk^{jrk}/Clk^{jrk}) as well as in relevant controls (*w*; *tim*-GAL4/+; Clk^{jrk} /+ - UAS-*tgo52.1*; +/+; Clk^{jrk} /+ - UAS-*tgo52.1*; +/+; Clk^{jrk} / Clk^{jrk} - +; *tim*-GAL4/+; Clk^{jrk}/Clk^{jrk}). TGO overexpression was shown not to be able to rescue rhythmicity in the Clk^{jrk} mutant flies. The endogenous period was determined by autocorrelation and Fourier analysis and the results compared (see Table 4-6). In agreement with the locomotor activity profile, 100% of the flies carrying Clk^{jrk} in homozygosis were arrhythmic, regardless of the amount of TGO present. Control flies carrying only one copy of Clk^{jrk} were all rhythmic.

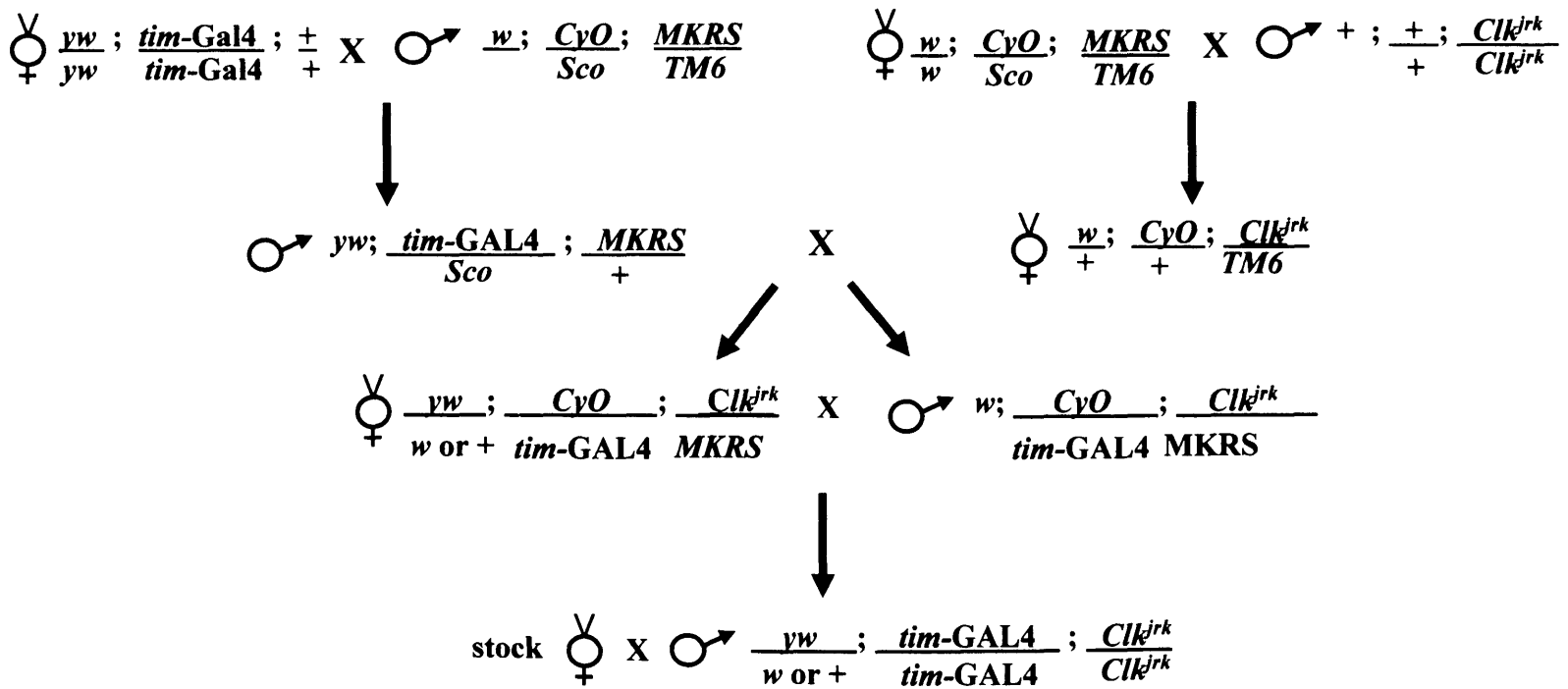


Figure 4.6

Scheme used to move *tim*-GAL4 into the *Clk* ^{jrk} background. *CyO* and *MKRS/TM6* are chromosome balancers used to prevent recombination in the second and third chromosomes, respectively. All balancers also carried a visible marker for identification.

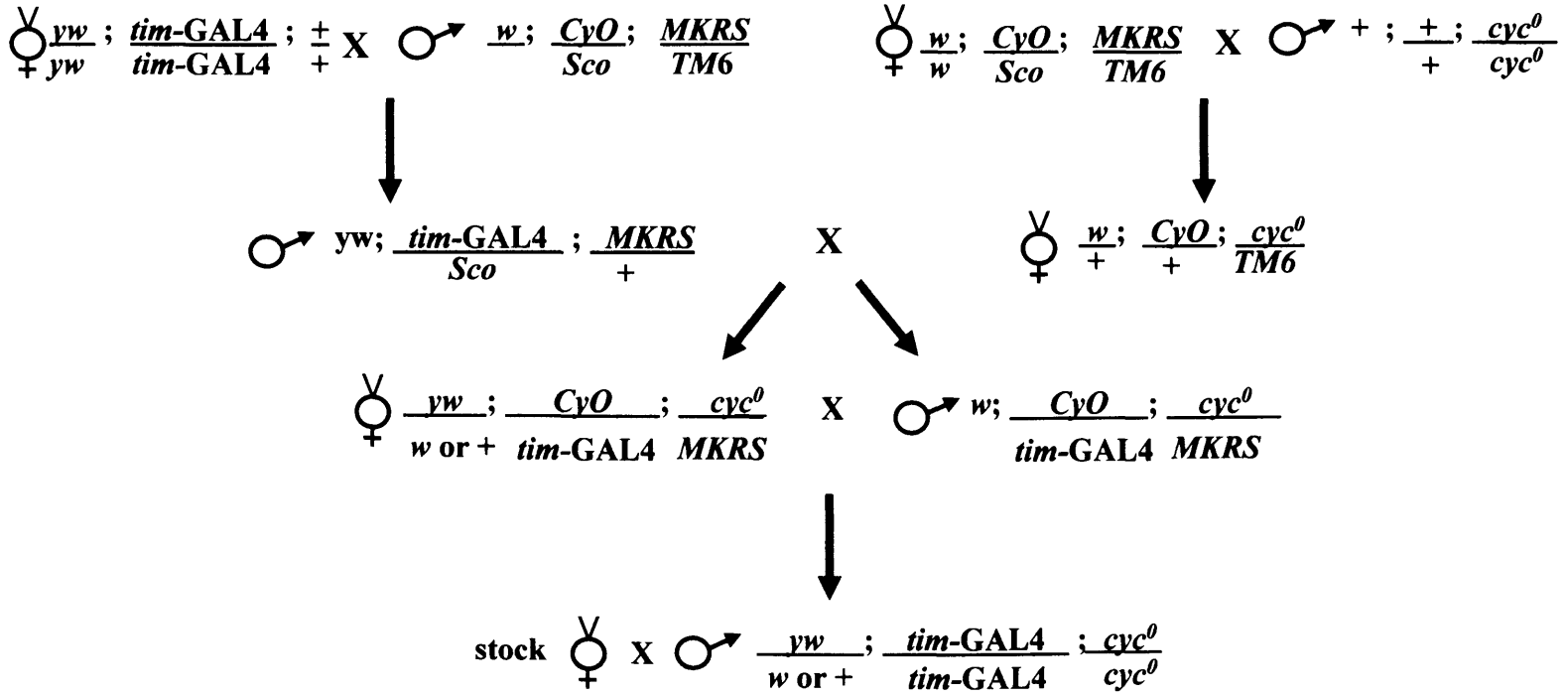


Figure 4.7

Scheme used to move *tim*-GAL4 into the cyc^0 background. *CyO* and *MKRS/TM6* are chromosome balancers used to prevent recombination in the second and third chromosomes, respectively. All balancers also carried a visible marker for identification.

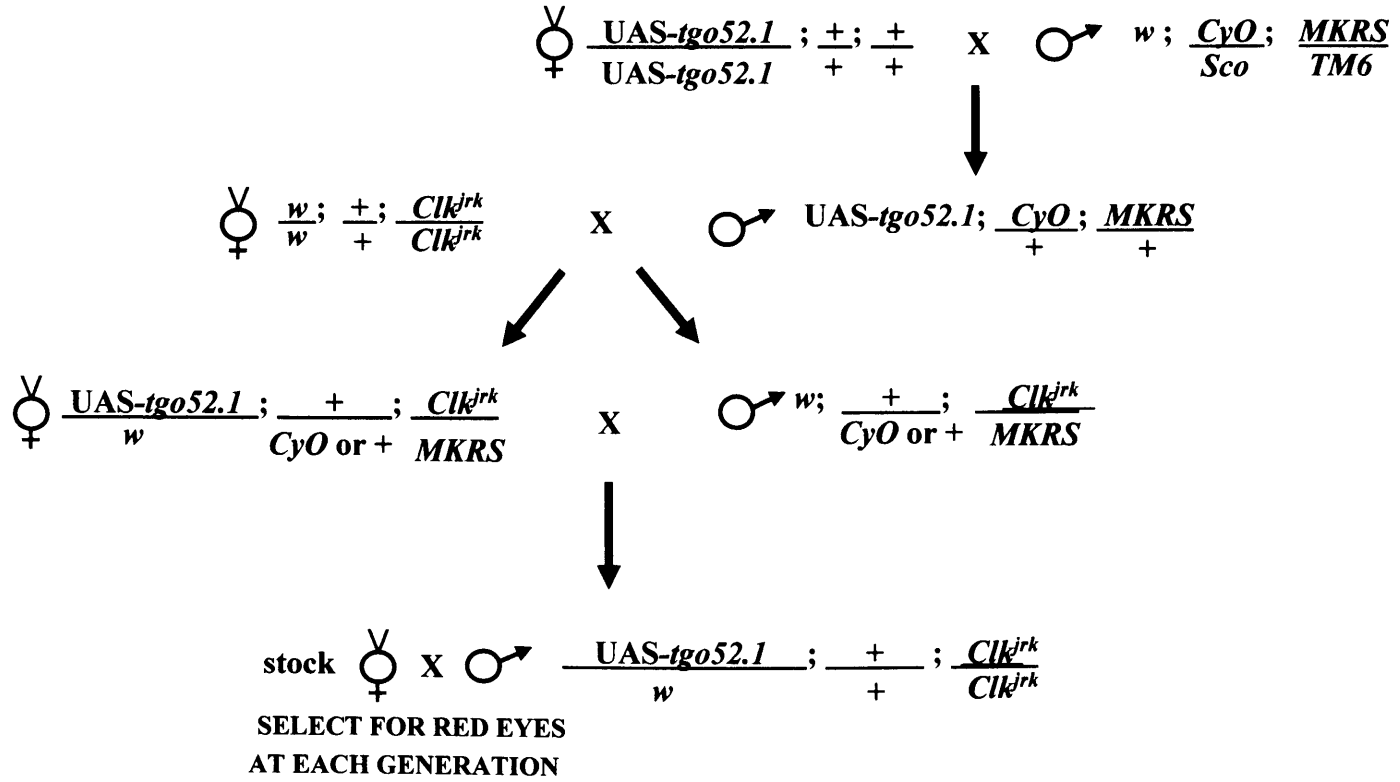


Figure 4.8

Scheme used to move *UAS-tgo52.1* into a *Clk^jrk* background. *CyO* and *MKRS/TM6* are chromosome balancers used to prevent recombination in the second and third chromosomes, respectively. All balancers also carried a visible marker for identification.

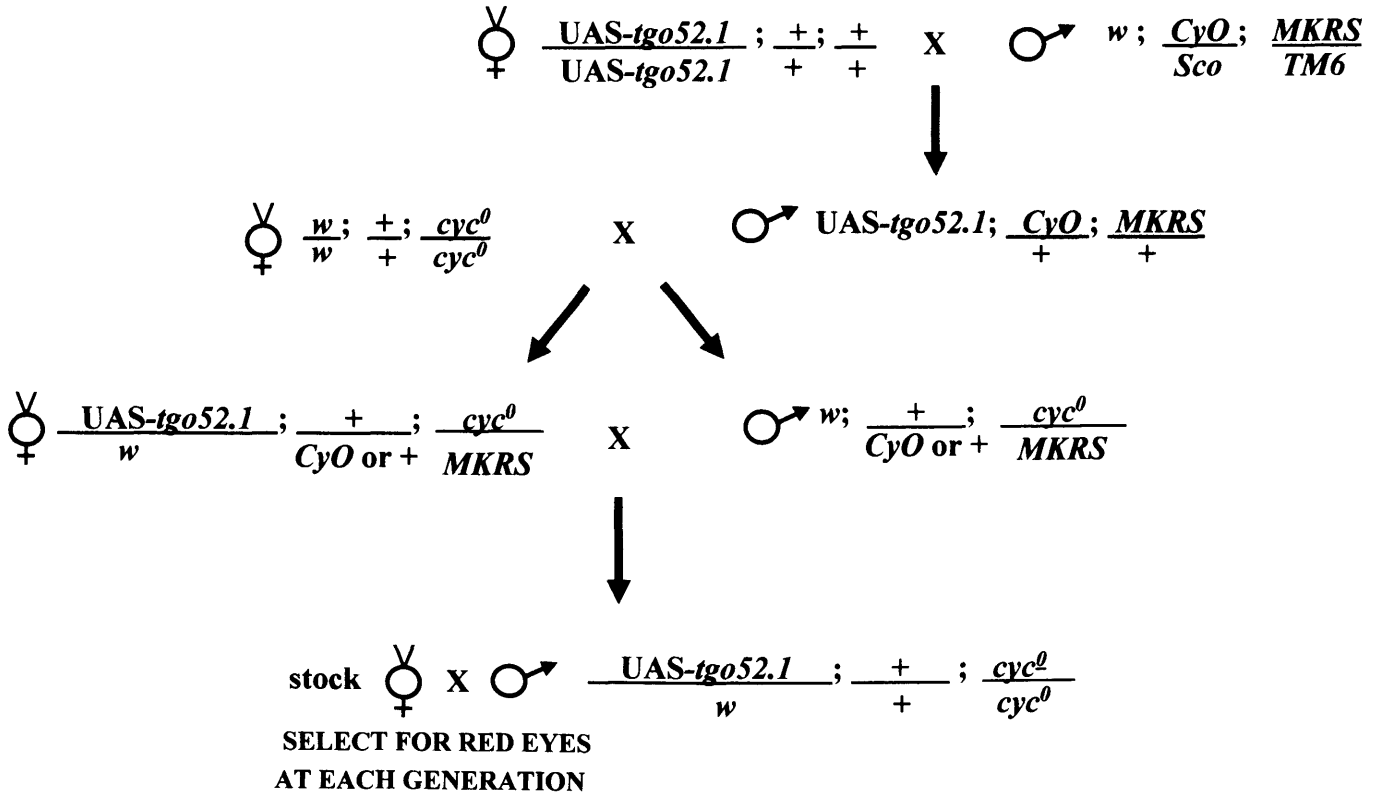


Figure 4.9

Scheme used to move UAS-*tgo52.1*into a *cyc*^{0} background. *CyO* and *MKRS/TM6* are chromosome balancers used to prevent recombination in the second and third chromosomes, respectively. All balancers also carried a visible marker for identification

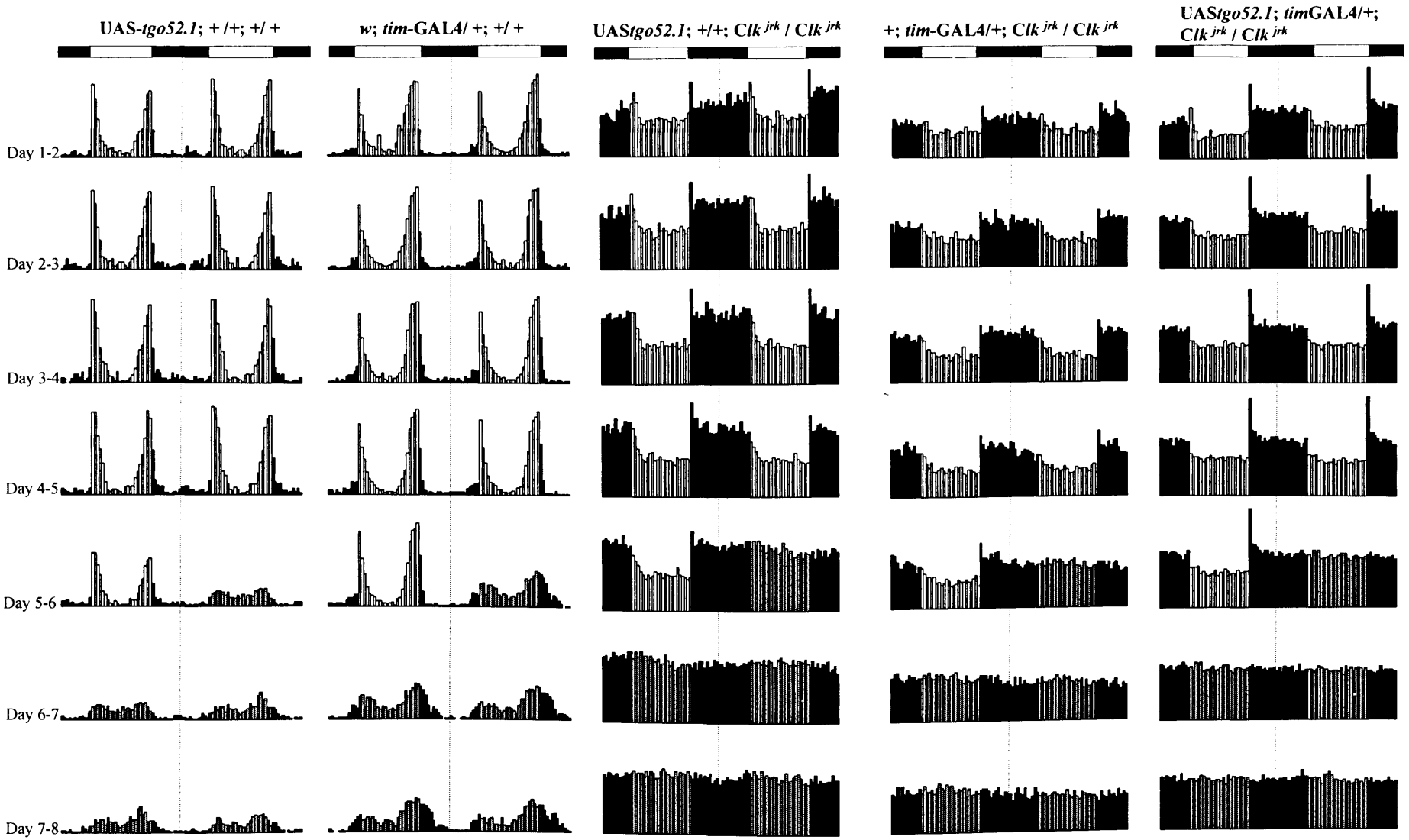

UAS-tgo52.1; + /+; +/ +
w; tim-GAL4/ +; +/ +
UAStgo52.1; +/+; Clk jrk / Clk jrk
+; tim-GAL4/+; Clk jrk / Clk jrk
UAStgo52.1; timGAL4/+; Clk jrk / Clk jrk
Day 1-2
Day 2-3
Day 3-4
Day 4-5
Day 5-6
Day 6-7
Day 7-8

Figure 4.10

Double plot of locomotor activity profiles (average amount of activity – Y axis – as a function of time – X axis) for *D. melanogaster* overexpressing TGO in Clk^{jrk} background (UAS-*tgo52.1*; *tim*-GAL4/+; Clk^{jrk}/Clk^{jrk}) and relevant controls (*w*; *tim*-GAL4/+; Clk^{jrk}/+, UAS-*tgo52.1*; +/+; Clk^{jrk}/+ , UAS-*tgo52.1*; +/+; Clk^{jrk} /Clk^{jrk}, +; *tim*-GAL4/+; Clk^{jrk}/Clk^{jrk}). Shown are 5 days in LD 12:12 h followed by 3 days in constant darkness (DD). In LD white and black bars represent photophase and scotophase, respectively. In DD gray and dark bars represent subjective day and the subjective night, respectively.

Table 4-6

Free running locomotor activity rhythms for flies overexpressing TGO in a Clk^{jrk} background and controls. The endogenous period was determined by autocorrelation and Fourier analysis and the results compared. The period length given by spectral analysis is presented. N= number of flies examined.

Genotype	τDD ± SEM	N
w; *tim*-GAL4/ +; Clk^{jrk} / +	24.36 ± 0.09	27
UAS-*tgo*; + /+; Clk^{jrk} / +	24.18 ± 0.15	22
UAS-*tgo*; +/+; Clk^{jrk} / Clk^{jrk}	Arrhythmic	39
+; *tim*-GAL4/+; Clk^{jrk} / Clk^{jrk}	Arrhythmic	40
UAS-*tgo*; *tim*-GAL4/+; Clk^{jrk} / Clk^{jrk}	Arrhythmic	62

Moreover, Figure 4.11 shows that TGO was not able to rescue rhythmicity also in cyc^0 flies. Indeed, autocorrelation and Fourier analysis revealed that 100% of the flies carrying cyc^0 in homozygosis were arrhythmic regardless the amount of TGO produced (see Table 4-7). Control flies carrying only one copy of cyc^0 were all rhythmic although with a longer period as previously reported (Rutila *et al.*, 1998).

Table 4-7

Free running locomotor activity rhythms for flies over-expressing TGO in a cyc^0 background. The endogenous period was determined by autocorrelation and Fourier analysis and the results compared. The period length given by spectral analysis is presented. N= number of flies examined.

Genotype	τDD ± SEM	N
w; *tim*-GAL4/+; cyc^0/+	25.27 ± 0.08	40
UAS-*tgo*; +/+; cyc^0/+	24.68 ± 0.05	49
UAS-*tgo*; +/+; cyc^0/cyc^0	Arrhythmic	24
+; *tim*-GAL4/+; cyc^0/cyc^0	Arrhythmic	19
UAS-*tgo*; *tim*-GAL4/+; cyc^0/cyc^0	Arrhythmic	49

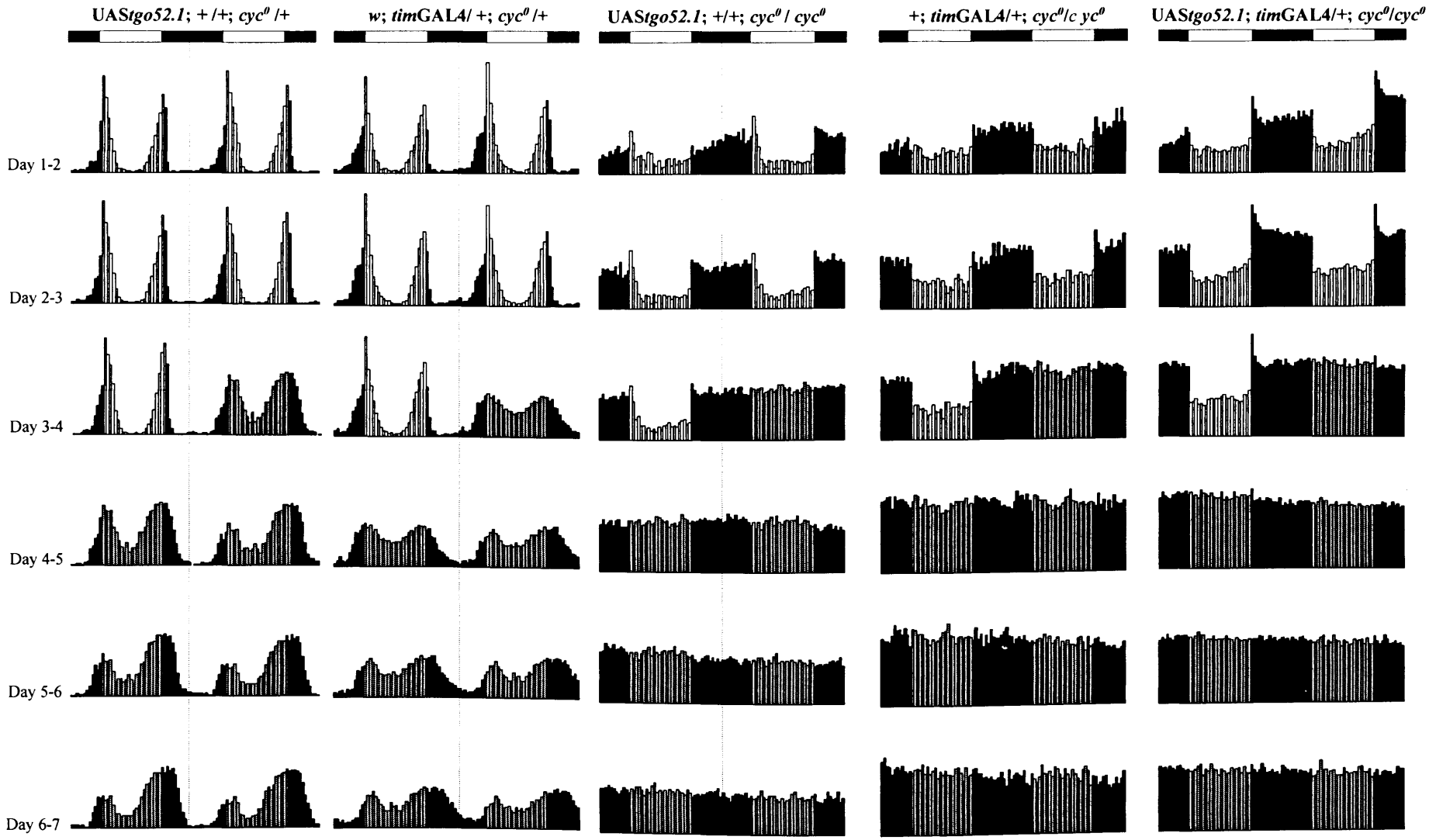

UAS*tgo52.1*; + /+; *cyc*^{0} /+
w; *tim*GAL4/ +; *cyc*^{0} /+
UAS*tgo52.1*; +/+; *cyc*^{0} / *cyc*^{0}
+; *tim*GAL4/+; *cyc*^{0}/*c yc*^{0}
UAS*tgo52.1*; *tim*GAL4/+; *cyc*^{0}/*cyc*^{0}
Day 1-2
Day 2-3
Day 3-4
Day 4-5
Day 5-6
Day 6-7

Figure 4.11

Average distribution of locomotor activity profiles (average amount of activity – Y axis – as a function of time – X axis) for *D. melanogaster* overexpressing TGO in a cyc^0 background (UAS-*tgo*; *tim*-GAL4/+; cyc^0 / cyc^0) and relevant controls (*w*; *tim*-GAL4/ +; cyc^0/ + , UAS-*tgo*; + /+; cyc^0/ + , UAS-*tgo*; +/+; cyc^0/ cyc^0, +; *tim*-GAL4/+; cyc^0/ cyc^0). Shown are 3 days in LD 12:12 h followed by 4 days in constant darkness (DD). In LD white and black bars represent photophase and scotophase, respectively. In DD gray and dark bars represent subjective day and the subjective night, respectively.

4.3.6 Molecular cycling of TIM

The effects of TGO overexpression on the peripheral clock were analyzed by western blot experiments on protein extracts from the head, where the eyes constitute the largest part of the total amount of tissue. Molecular cycles of TIM are observed in 12:12h LD conditions in wild-type fly (Edery *et al.*, 1994; Hunter-Ensor *et al.*, 1996). The oscillation is such that TIM levels peak at the end of the night (ZT20) and show a through during the day. The low levels found during the day just reflects TIM light sensibility properties that causes the protein to be degraded minutes after lights on and to accumulate again around lights off (ZT12). In order to analyse if TGO up-regulation could interfere on TIM cycling dynamics, flies subjected to a 12:12 h LD conditions at 25°C were collected every two hours during a 24 h day course. Equal amounts of head protein extracts were run in a 6% polyacrylamide gel. Blots were probed with rat anti-TIM antibodies (1: 1000) and a HRP-conjugated goat anti-rat (Sigma, 1: 8000). Figure 4.12 shows the average molecular oscillation of TIM in flies overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/+) compared to the control (*w*; *tim*-GAL4/+; +/+) obtained from six independent immunoblot experiments. The experimental line showed a small advance in the timing of TIM rise and fall. However, ANOVA analysis revealed that the difference found was not statistically significant ($F_{13, 58} = 0.421$, $P > 0.05$, appendix 7).

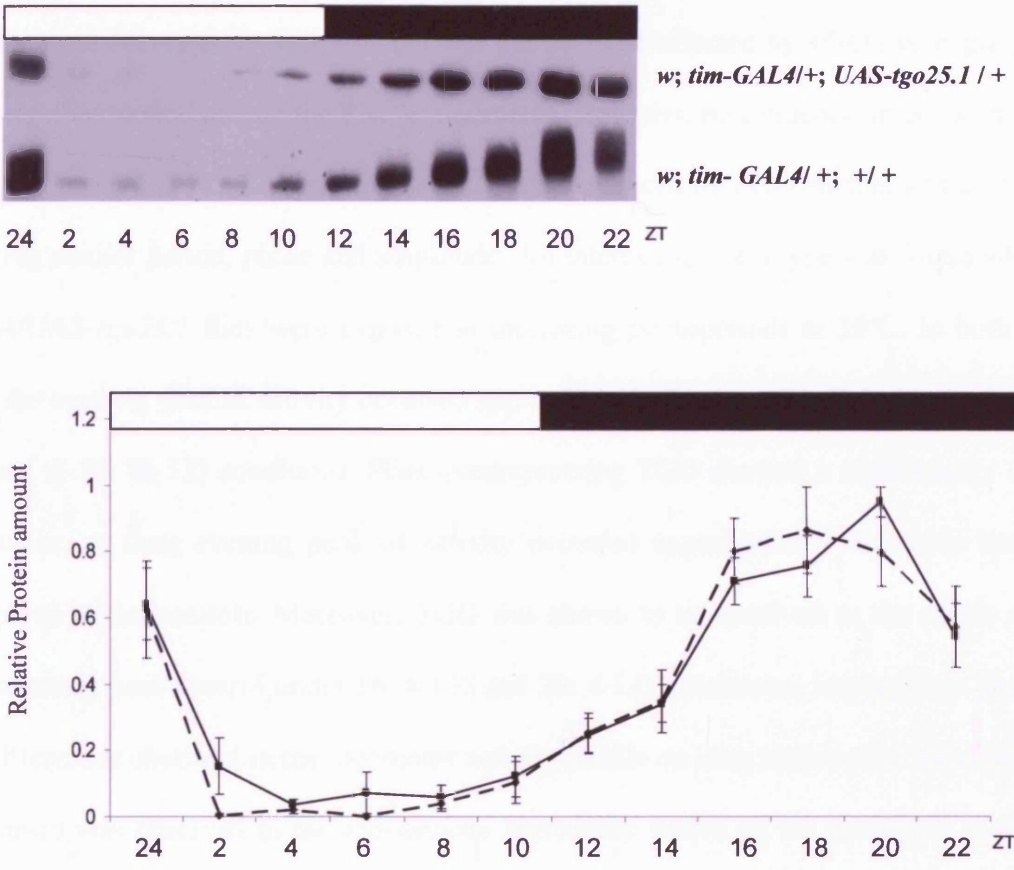


Figure 4.12

Western blot analysis of TIM in flies overexpressing TGO and control. A representative blot is shown on the top for each genotype. The white and black bars denote photophase and scotophase, respectively. On the bottom, average ± SEM of TIM oscillation from six independent experiments. For each gel the strongest band was set equal to 100, the others were normalized accordingly. Continuous line: *w*; *tim*-GAL4/+; +/+. Dashed line: *w*; *tim*-GAL4/+; UAS-*tgo25.1* / +.

4.4 Summary

The overexpression of TGO in clock cells by using the GAL4/UAS binary system was obtained successfully. Entrainment was shown to be affected by TGO overexpression on the long photoperiod and on the PRC experiments. However, no influence on entrainment was observed under LD 12: 12 conditions at 25°C, as evidenced by experimental and control flies showing similar period, phase and amplitude. An interesting phenotype was found when *tim*-GAL4/UAS-*tgo25.1* flies were exposed to increasing photoperiods at 20°C. In both control lines the evening peak of activity occurred approximately 9 hours after lights on under 12:12h LD and at 16: 8h LD conditions. Flies overexpressing TGO showed a significantly different behaviour, as their evening peak of activity occurred approximately one hour later when compared to the controls. Moreover, TGO was shown to be involved in the startle response and morning peak control under 16: 8 LD and 20: 4 LD conditions, respectively. Despite all the differences observed in the locomotor activity profile on long photoperiod experiments, no difference was observed in the endogenous periodicity within all the genotypes analyzed. It indicates that TGO overexpression does not interfere with the core mechanisms of the circadian clock. Indeed, all the genotypes analysed under constant darkness conditions at 25°C showed the same endogenous period (τ), regardless the amount of TGO protein produced. This suggests that the effect described under long photoperiod is limited to the phase of the clock but does not influence its periodicity. Phase shift experiments on flies overexpressing TGO revealed striking differences in the delay zone of the PRC after 5 minutes light pulse was given at ZT13 and at ZT15. While no difference was detected in the phase advance domain, flies up-regulating the protein showed a more pronounced response in the phase delay domain. Despite the molecular similarity between *tgo* and *cyc* genes and the fact that TGO protein was shown to bind CLK in yeast-two-hybrid essays, no circadian rhythm rescue was observed in lines overexpressing TGO in both Clk^{jrk} and cyc^{0} backgrounds.

Finally, Western blot analysis was performed on head extracts of flies overexpressing TGO. Statistical analysis revealed that the small phase advance observed in the molecular cycling of TIM was not significant.

Chapter 5: Down-regulation of TANGO in *Drosophila melanogaster*

5.1 Introduction

Just before the current circadian clock negative feed back oscillator models were defined, it had already been proposed that bHLH-PAS proteins such as CLK and ARNT might interact with other PAS proteins to control the periodicity of biological rhythms (Huang *et al.*, 1993; Lindebro *et al.*, 1995; King *et al.*, 1997). Null mutations and gene silencing are normally applied to partially or completely reduce levels of proteins in a chosen system to be analysed. By using null mutants of the *Clk* and *cyc* genes it was shown that their respective PAS products were indeed involved in the core mechanisms of the circadian clock (Allada *et al.*, 1998; Darlington *et al.*, 1998; King *et al.*, 1997, Rutila *et al*, 1998).

Reverse genetic techniques were also applied here as an approach to discover a putative function of *tgo* in the circadian clock. After the protein reduction process, a possible phenotype that could be derived from the gene of interest was investigated. TGO protein is found broadly distributed throughout embryogenesis, with enhanced levels in certain cell types including the trachea and CNS (Sonnenfeld *et al.*, 1997). Due to its importance for developmental processes, *tgo* null mutations are not practical for investigations into the molecular basis of the circadian clock as they are lethal when homozygous (Emmons *et al.*, 1999; Sonnenfeld *et al.*, 1997). Thus, three complementary approaches were applied to successfully reduce the expression of TGO in adult flies without triggering its lethality effects; (1) specific protein reduction limited to clock cells throughout all the developmental stages and adult phase, (2) Total protein reduction in the whole organism only during the adult phase, (3) 50% reduction of the protein in the whole organism throughout all the developmental stages and adult phase.

In this chapter I shall present data regarding the down-regulation of TGO and its effects on the regulation of *Drosophila* circadian clock.

5.2 Material and methods

Double stranded RNA (dsRNA) can trigger the silencing of a cognate gene as a result of a complex mechanism known as RNA interference (RNAi, Kennerdell & Carthew, 2000). In *Drosophila*, a way to produce dsRNA is to clone two cDNA fragments in opposite orientation in a vector used for fly transformation. In order to overcome the difficulty of cloning palindromic DNA molecules in a plasmid, a spacer sequence can be allocated between the 2 inverted cDNA fragments (Piccin *et al.*, 2001). If the spacer corresponds to the sequence of an intron, the pairing of the dsRNA could be facilitated by the splicing mechanism (Smith *et al.*, 2000). Coupling this strategy with the GAL4/UAS binary system (Brand and Perrimon, 1993; Brand *et al.*, 1994; Phelps and Brand, 1998) the expressing of dsRNA and consequent gene silencing can be restricted to specific tissues, cell types and/or time of development.

In order to promote TGO down-regulation, a construct called UAS-*tango-intron-tango*, or UAS-*tint*, was created by cloning a sequence of *tgo* cDNA as two fragments in opposite orientation separated by the second intron of the *single-minded* (*sim*) gene (Ozkaya, 2001). Following microinjection, two transgenic fly lines were recovered; line 3 and line 1y, both carrying a chromosome 2 insertion.

Reduction of TGO protein only in clock cells was achieved by bringing together the *yw; tim*-GAL4 driver (see Chapter 4) line and the dsRNA producing reporter UAS-*tint* lines. In order to minimize variables throughout this study, all crosses were performed using virgin female drivers (*yw; tim*-GAL4) against male reporters (UAS-*tint*).

Generalised TGO reduction was performed by combining UAS-*tint* lines with the ubiquitous driver *actin*-GAL4. Since *tgo* is an essential gene for development, it's completely down-regulation could only occur after the flies reached the adult phase. To do so, the conventional UAS/GAL4 binary system was regulated by a temperature-sensitive *S. cerevisiae* GAL80 molecule, which was driven by an ubiquitous *tubulineP* promoter (*tubP-*

GAL80^{TS}) to repress GAL4 transcriptional activity at permissive temperatures (Mcguire *et al.*, 2004). Virgin females carrying both the *act*-GAL4 and *tub*GAL80^{TS} constructs (*w*; *act*-GAL4/*Cyo*; *tubP*-GAL80^{TS}/*tub*-GAL80^{TS}) were crossed to males carrying the UAS-*tint* reporter constructs. F1 flies were maintained at 25°C throughout their developmental stage so that GAL80^{TS} could inhibit GAL4 activity. Consequently the RNAi system remained inactive not triggering TGO down-regulation. As soon as the flies hatched, they were transferred to 29°C so that GAL80 ^{TS} was inactivated. Thus GAL4 activity was no longer blocked finally making TGO down-regulation process to take place globally in adult flies.

Finally, in order to get 50% TGO reduction the null mutations tgo^1 and tgo^5 were used in heterozygosis. Male w^{1118} were mated to *w*; +/+; tgo^1(or tgo^5)/ TM6 virgin females flies, where TM6 is a balancer carrying a visual marker. The F1 generation was screened and selected against the TM6 balancer. Refer to Chapter 2 for the detailed description of the crosses, locomotor activity and western blot experiments.

5.3 Results and discussion

5.3.1 Assessment of RNAi performance via TGO quantification and crossing experiments

Before conducting experiments with the aforementioned down-regulating lines, flies were assessed regarding their efficiency in reducing TGO expression. Initially, the UAS-*tint* lines were used in crosses performed to check the viability of adult flies when down-regulation of TGO was driven by a strong and ubiquitously expressed driver. Virgin female flies carrying the *tubP*-GAL4 construct balanced with *TM3(Sb)* on chromosome 3 (*yw*, +/+; *tubP*-GAL4/*TM3(Sb)*) were crossed to UAS-*tint3* and UAS-*tint1y* males and left at 25°C under a 12:12h LD cycle. *Sb* is a common maker producing a stubble bristle phenotype. The

F1 generation was analysed and a completely deviation from the 50% expected distribution amongst stubble and normal bristles was observed (see Table 5-1). The lack of normal stubble phenotype indicated that all flies down-regulating TGO by bring together both the *tubp*-GAL4 and UAS-*tint* constructs were not able to survive the early developmental stages.

Table 5-1

Crosses performed to check the UAS-*tint* constructs functionality. N_{F1}, number of flies analysed in the F1 generation. N_{sb}, number of flies showing a stubble bristle phenotype. N_{nb}, number of flies showing a normal bristle phenotype.

Crosses (♂ x ♀)	N_{F1}	N_{sb}		N_{nb}	
		Expected	**Observed**	**Expected**	**Observed**
w, UAS-*tint3*/UAS-*tint3*, +/+ x *yw*, +/+ ; *tubP*-GAL4/*TM3(Sb)*	68	34	68	34	0
w, UAS-*tint1y*/UAS-*tint1y*, +/+ x *yw*, +/+ ; *tubP*-GAL4/*TM3(Sb)*	71	35.5	71	35.5	0

After checking that the constructs where working properly, western blot experiments were performed on whole heads and compared. Molecular observations should not be extrapolated to pacemaker's neurons as the head mainly comprises peripheral clocks. Figure 5.1 shows a western blot experiment made with UAS/GAL4 lines down-regulating TGO (*w; tim*-GAL4/UAS-*tint3; +/+* and w; *tim*-GAL4/UAS-*tint1y; +/+*), *tgo* mutants lines (*w; +/ +; tgo*^{1}*/+* and *w; +/ +; tgo*^{5}*/+*) and a control line (*w; tim*-GAL4/+; *+/+)*.

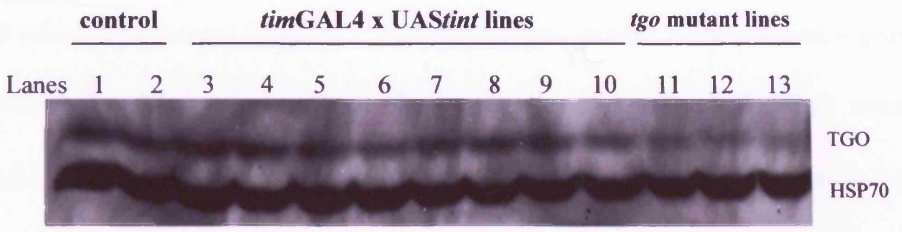


Figure 5.1

Western blot analysis of TGO in control and down-regulating flies. Samples were loaded as duplicates from different extractions. Immunodetection of HSP70 was done to check any possible sample loading error. Lanes 1 and 2, control flies (*w*; *tim*-GAL4/+; +/+). Lanes 3 and 4, down-regulating flies carrying the UAS-*tint3* construct (w; *tim*-GAL4/ UAS-*tint3*; +/+). Lanes 5 and 6, down-regulating flies carrying the UAS-*tint1y* construct (w; *tim*-GAL4/ UAS-*tint1y*; +/+). Lanes 7 to 10, down-regulating flies carrying two other UAS-*tint* constructs that were available (UAS-*tint1R1* and UAS-*tint51.1*). Lanes 11 and 12, flies carrying only one copy of the tgo^{1}mutant. Lane 13, flies carrying only one copy of the tgo^{5} mutant. The whole film obtained in this experiment is shown in appendix 1.

Flies were raised at 25°C and collected in the middle of the photophase (ZT6). Equal amounts of whole head protein extracts were run on a 10% polyacrylamide gel and blotted onto nitrocellulose membrane. TGO was detected with a mouse anti-TANGO antibody (Developmental Studies Hybridoma Bank at the University of Iowa, 1:1000) and a HRP-conjugated rabbit anti-mouse (Sigma, 1: 6000). The *tgo* mutant lines showed a greater level of protein reduction when compared to the controls. Reduction of TGO using the *tim*-GAL4/UAS-*tint* system was not as evident. Extra western blots experiments were performed for these two experimental lines. The graphs on Figure 5.2 represent the average relative amount of TGO for all experimental lines above mentioned compared to their controls. As it can be depicted from the graph shown, TGO was reduced by approximately 10% and 30% in the UAS-*tint3* and UAS-*tint1y* strains, respectively. The mutant lines showed a 60% reduction when compared to the control. Considering the error limit range of the technique this reduction is within the range that cover the 50% reduction expected on heterozygous lines.

Finally, a complete reduction of TGO seems to be achieved when the activation of the UAS/GAL4 system was temporally controlled by the *tub*-GAL80^{TS} construct at 29 °C (see Figure 5.3). Lines up-regulating TGO were used as a positive control indicating that the system was working. The figure also shows levels of constitutively expressed HSP70 protein in order to indicate any possible sample loading error. The graph presented indicates that TGO was severely reduced in those flies after three days of exposure at 29 °C. However, the HP70 shows that the down-regulating lines have been loaded less, about half the amount of the controls. If one assumes that the bands in the controls are so weak that they wouldn't be visible if reduced to half of its intensity, so the amount of TGO reduction is still to be confirmed when using this approach.

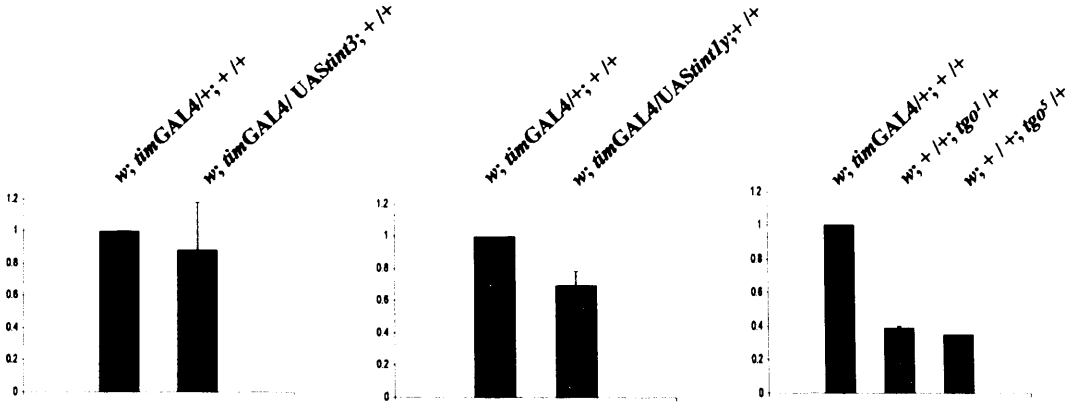


Figure 5.2

The graphs show the relative amount of TGO for each genotype analyzed. Peak levels of expression were set equal to 100 for the control lines in order to normalize the data. For *tim*-GAL4/UAS-*tint3* flies, quantification was based on 9 sample loadings obtained from 3 independent experiments. For *tim*-GAL4/UAS-*tint1y* flies, quantification was based on 3 sample loadings obtained from 2 independent experiments. The tgo^1 mutant strain had its quantification based on 2 samples obtained from a single experiment. Only one sample from a single experiment was used for tgo^5 mutant strain quantification. Errors bars represent either standard errors or deviation from the mean.

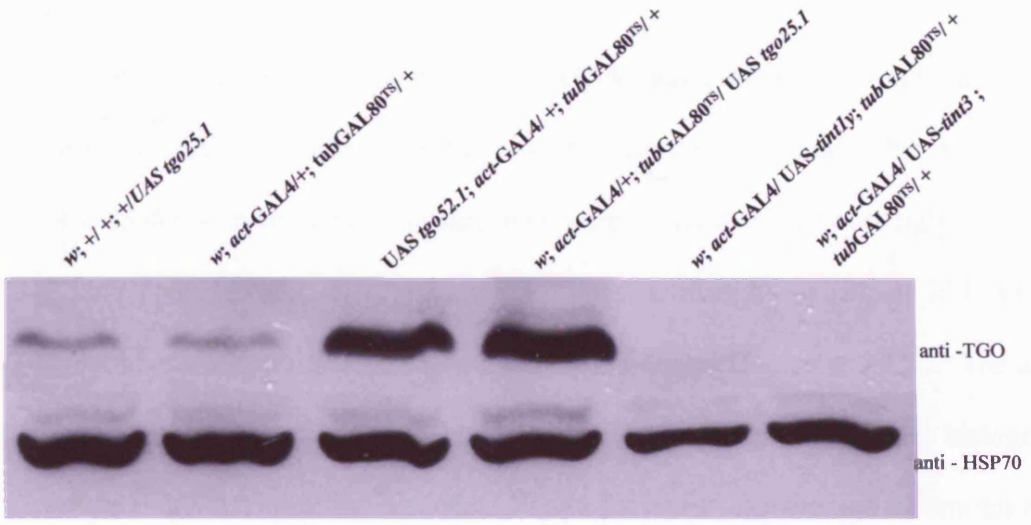


Figure 5.3

TGO quantification in adult flies down-regulating the protein ubiquitously. Western blot was performed with equal amounts of adult whole head protein extracts. Samples were initially imunoblotted with mouse anti-TGO. Lately mouse anti HSP70 was used to standardize. Peak levels of expression were set equal to 100 in order to normalize the data. *w*; +/+; +/UAS-*tgo25.1* and *w*; *act*-GAL4/+; *tub*-GAL80^{TS}/+ were the controls. UAS-*tgo52.1*; *act*-GAL4/+; *tub*-GAL80^{TS}/+ and *w*; *act*-GAL4/+; *tub*-GAL80^{TS}/UAS-*tgo25.1*; up-regulating lines. *w*; *act*-GAL4/UAS-*tint*; *tub*-GAL80^{TS}/+ (strains 3 and 1y); down-regulating lines.

5.3.2 Entrainment by light cycles

A putative role for TGO in the central clock was evaluated throughout behaviour analysis of lines with reduced levels of the protein. Flies down-regulating the protein by the *tim*-GAL4/UAS-*tint* system and *tgo* mutant lines were exposed to 24 hours light: dark cycles with a 12 hour photoperiod (LD 12:12) during 5 days in order to investigate if TGO down-regulation could affect entrainment. The experiments were conducted at 25 °C. The average activity level for each bin was used to build up an average locomotor activity histogram for each genotype under this specific condition. Figure 5.4 shows the average locomotor activity pattern of flies down-regulating TGO by the UAS/GAL4 system (*w*; *tim*-GAL4/ UAS-*tint*3; +/ +), together with *tgo* mutants flies (*w*; +/ +; tgo^1/ + and *w*; +/ +; tgo^5/ +) and two controls (*w*; *tim*-GAL4/ +; +/ + , *w*; UAS-*tint3* /+; +/ +). The results show that these flies were able to entrain with a period (τ) of about 24 h (see Table 5-2). Kuskal-wallis test revealed that the difference found amongst the groups analyzed was not significant (X^2= 11.38, df = 6, P > 0.05, appendix 8). As it happened with up-regulating flies, reduced levels of TGO still allows the fly to have its locomotor activity entrained, showing the same bimodal fashion as the controls, i.e. with peaks centred on the dark/light and light/dark transitions (Klarsfeld *et al.*, 2003, Rosato and Kyriacou, 2006).

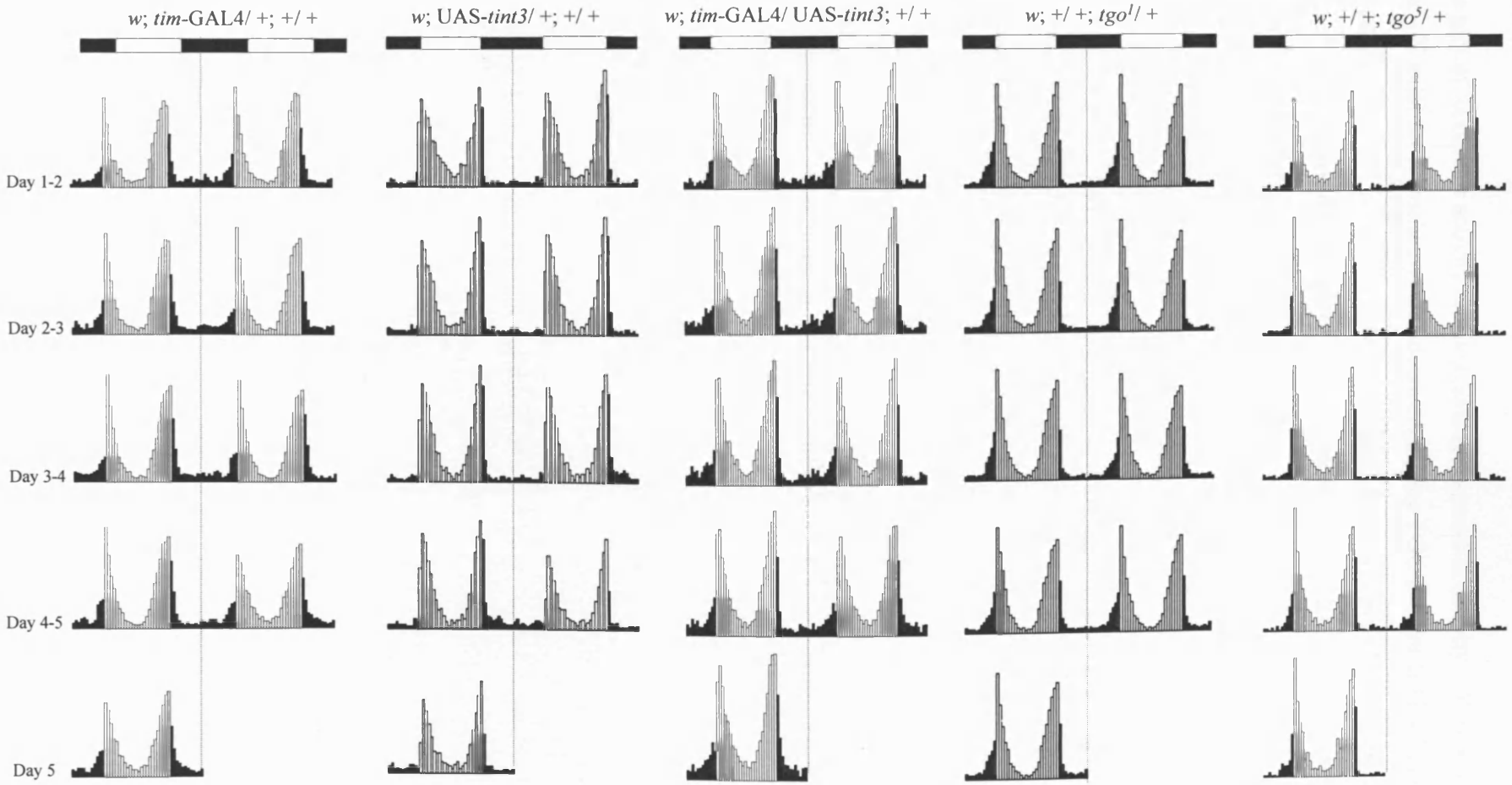

w; tim-GAL4/ +; +/ +
w; UAS-tint3/ +; +/ +
w; tim-GAL4/ UAS-tint3; +/ +
w; +/ +; tgo1/ +
w; +/ +; tgo5/ +
Day 1-2
Day 2-3
Day 3-4
Day 4-5
Day 5

Figure 5.4

Double plot of locomotor activity profiles (average amount of activity – Y axis – as a function of time – X axis) for *D. melanogaster* down-regulating TGO (*w*; *tim*-GAL4/UAS-*tint3*; +/+ , *w*; +/ +; tgo^1/ + , *w*; +/ +; tgo^5/ +) and two controls lines (*w*; *tim*-GAL4/ +; +/+ and *w*; UAS-*tint3*/ +; +/ +). Shown are 5 days of activity under a 12:12 LD cycle regime. Open bars, photophase. Shaded bars, scotophase.

Table 5-2

Entrained locomotor activity rhythms for flies down-regulating TGO and controls. The entrained period was determined by autocorrelation and Fourier analysis and the results compared. The period length given by spectral analysis is presented. Average and standard error of the mean (SEM) are shown. N= number of flies examined.

Genotype	Entrained period ± SEM	N
w; tim-GAL4/+; +/+	24.07 ± 0.01	68
w; UAS-*tint3*/+; +/+	23.99 ± 0.03	42
*w; tim*GAL4/ UAS-*tint3;* +/+	24.09 ± 0.02	89
w; +/+; tgo^1/+	24.08 ± 0.04	61
w; +/+; tgo^5/+	24.16 ± 0.05	34

In order to evaluate the effects of a full TGO down-regulation on entrainment, experiments were conducted at 29 °C using the line carrying both the *act*-GAL4 and *tub*-GAL80^{TS} constructs. Figure 5.5 shows the average locomotor activity pattern of male flies ubiquitously down-regulating TGO (*w*; *act*-GAL4/UAS-*tint3*; *tub*-GAL80^{TS}/+ and *w*; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80^{TS}/+) as well as the relevant controls (*w*; UAS-*tint3*/+; +/+ - *w*; UAS-*tint1y*/+; +/+ - *w*; *act*-GAL4/ +; *tub*-GAL80^{TS}/+). The files were exposed to a 12 h light 12 h darkness cycle (LD 12:12) for 5 days. Table 5.3 shows the period calculated for each genotype. No significant difference in period was detected among the genotypes analyzed (Kruskal-Wallis X^2= 5.82, df = 4, $p > 0.05$, appendix 9). However, an interesting feature could be observed in the activity profiles of both lines with reduced levels of TGO. Visual inspection of Fig 5.5 revealed a shoulder in the evening peak where flies down-regulating TGO show a secondary peak of activity in the night. This might reflect a tendency for a longer period in those flies that are interrupted by the startle response caused by the lights off in such LD conditions.

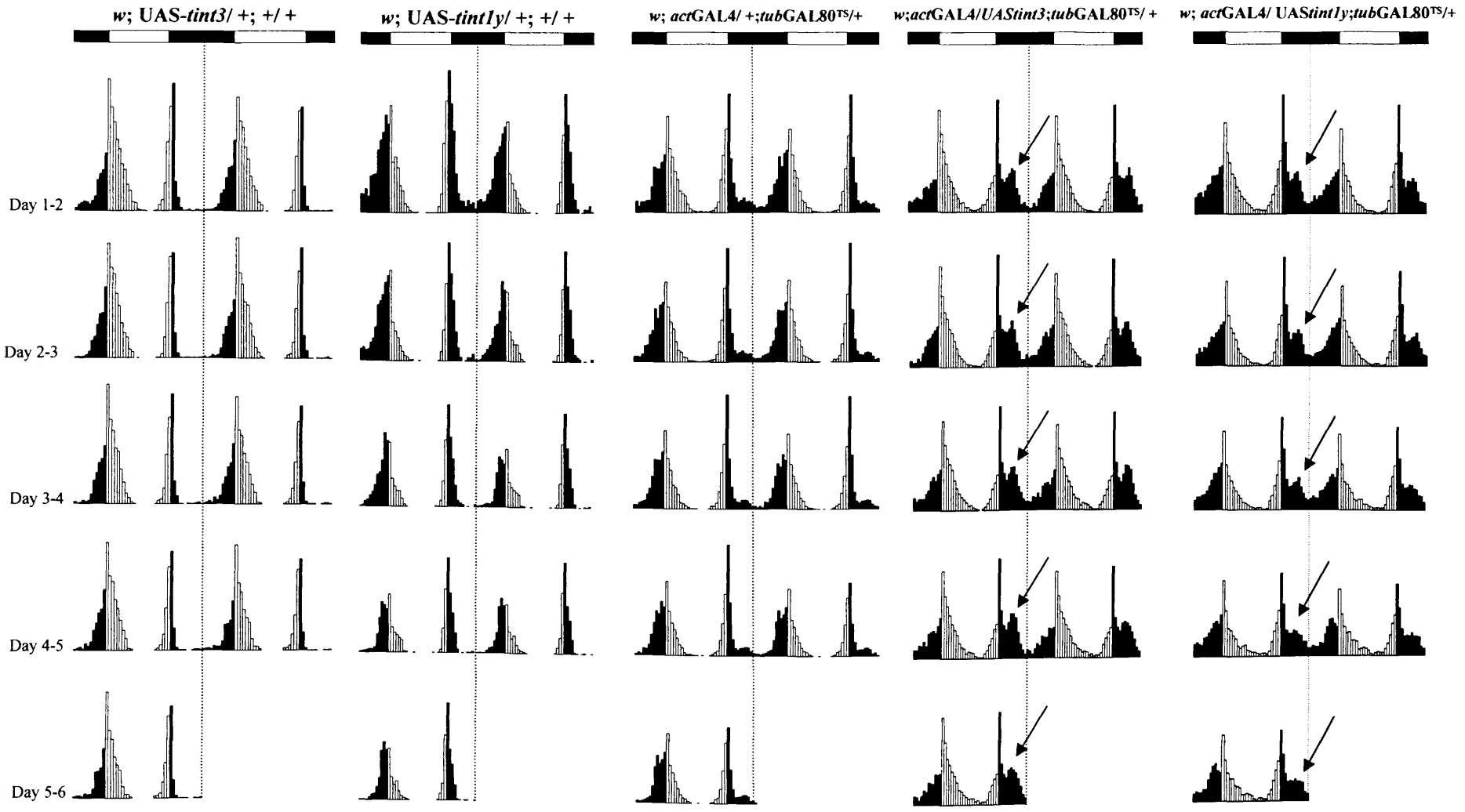

w; UAS-tint3/ +; +/ +
w; UAS-tint1y/ +; +/ +
w; actGAL4/ +;tubGAL80TS/+
w;actGAL4/UAStint3;tubGAL80TS/ +
w; actGAL4/ UAStint1y;tubGAL80TS/+
Day 1-2
Day 2-3
Day 3-4
Day 4-5
Day 5-6

Figure 5.5

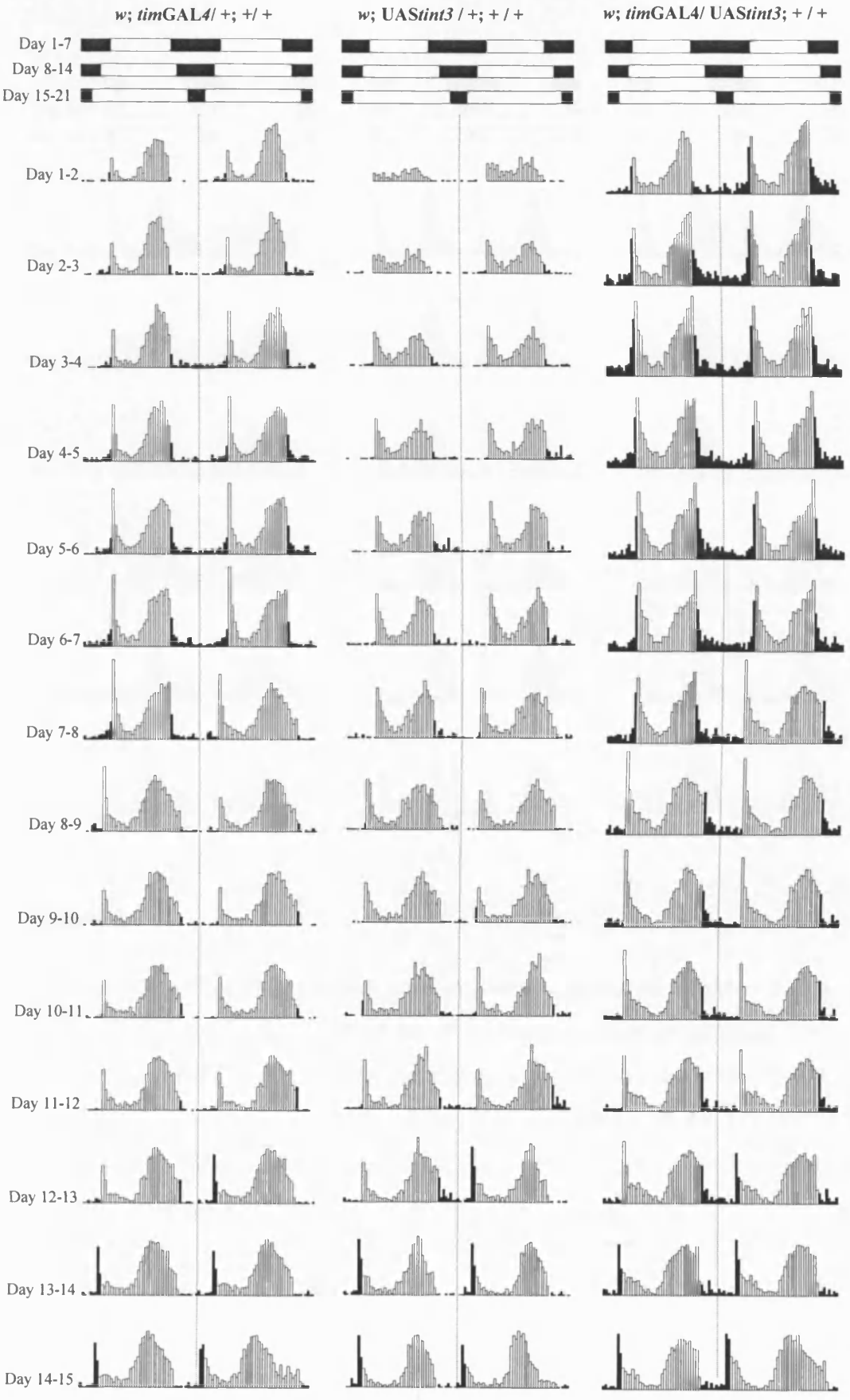
Double plot of *D. melanogaster* locomotor activity profiles (average amount of activity – Y axis – as a function of time – X axis) for a ubiquitous TGO down-regulation (*w*; *act*-GAL4/ UAS-*tint3*; *tub*-GAL80^{TS}/+ and *w*; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80^{TS}/+) and three control lines (*w*; *act*-GAL4/+; *tub*-GAL80^{TS}/+ - *w*; UAS-*tint3*/ +; +/ + - *w*; UAS-*tint1y*/ +; +/+). Arrows indicate a shoulder in the evening peak occurring only in flies down-regulating TGO. Shown are 5 days of activity under a 12:12 LD cycle regime. Open bars, photophase. Shaded bars, scotophase.

Table 5-3

Entrained locomotor activity rhythms for flies down-regulating TGO and controls. The entrained period was determined by autocorrelation and Fourier anal ysis and the results compared. The period length given by spectral analysis is presented. Average and standard error of the mean (SEM) are shown. N= number of flies examined.

Genotype	Entrained period ± SEM	N
w; UAS-*tint3*/+; +/+	23.93 ± 0.04	47
w; UAS-*tint1y*/+; +/+	24.00 ± 0.04	46
w; *act*-GAL4/+; *tub*-GAL80^{TS}/+	23.98 ± 0.03	55
w; *act*-GAL4/UAS-*tint3*; *tub*-GAL80^{TS}/+	24.05 ± 0.04	57
w; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80^{TS}/+	24.12 ± 0.05	60

Finally, male flies were subject to a change in photoperiod that was gradually lengthened to test how reduced expression of TGO could affect masking (see Chapter 4 for details). Control of light permitted the exposure of flies to any desired photoperiod, for example to 16 h light and 8 h darkness (LD 16: 8). The transitions between the light and the dark phases were instantaneous (abrupt transition). Experiments were conducted as detailed in Chapter 2. Figure 5.6 shows the average locomotor activity pattern of male flies down-regulating TGO (*w*; *tim*-GAL4/UAS-*tint3*; +/+) as well as the relevant controls (*w*; *tim*-GAL4/+; +/+ and *w*; UAS-*tint3*/+; +/+) during 7 days under LD 12: 12, followed by 7 days under LD 16:8 and finally 7 days under LD 20: 4. The experiments were conducted at 20 °C. The locomotor activity profiles showed that different genotypes had the peak of activity occurring at distinct times. Table 5.4 summarizes the number of flies analysed and the average time of peak occurrence relative to lights on (ZT 0) for each of the photoperiod length analysed. ANOVA analysis revealed a significant difference among the genotypes at 12:12 LD ($F_{2, 54}$= 28.99, $p < 0.05$, appendix 10), 16: 8 LD ($F_{2, 51}$ = 18.33, $p < 0.05$, appendix 10) and 20: 4 LD ($F_{2, 47}$ = 14.17, $p < 0.05$, appendix 10). Subsequent Bonferroni *Post Hoc* comparison


w; timGAL4/ +; +/ +
w; UASstint3 / +; + / +
w; timGAL4/ UASstint3; + / +
Day 1-7
Day 8-14
Day 15-21
Day 1-2
Day 2-3
Day 3-4
Day 4-5
Day 5-6
Day 6-7
Day 7-8
Day 8-9
Day 9-10
Day 10-11
Day 11-12
Day 12-13
Day 13-14
Day 14-15

Continuation

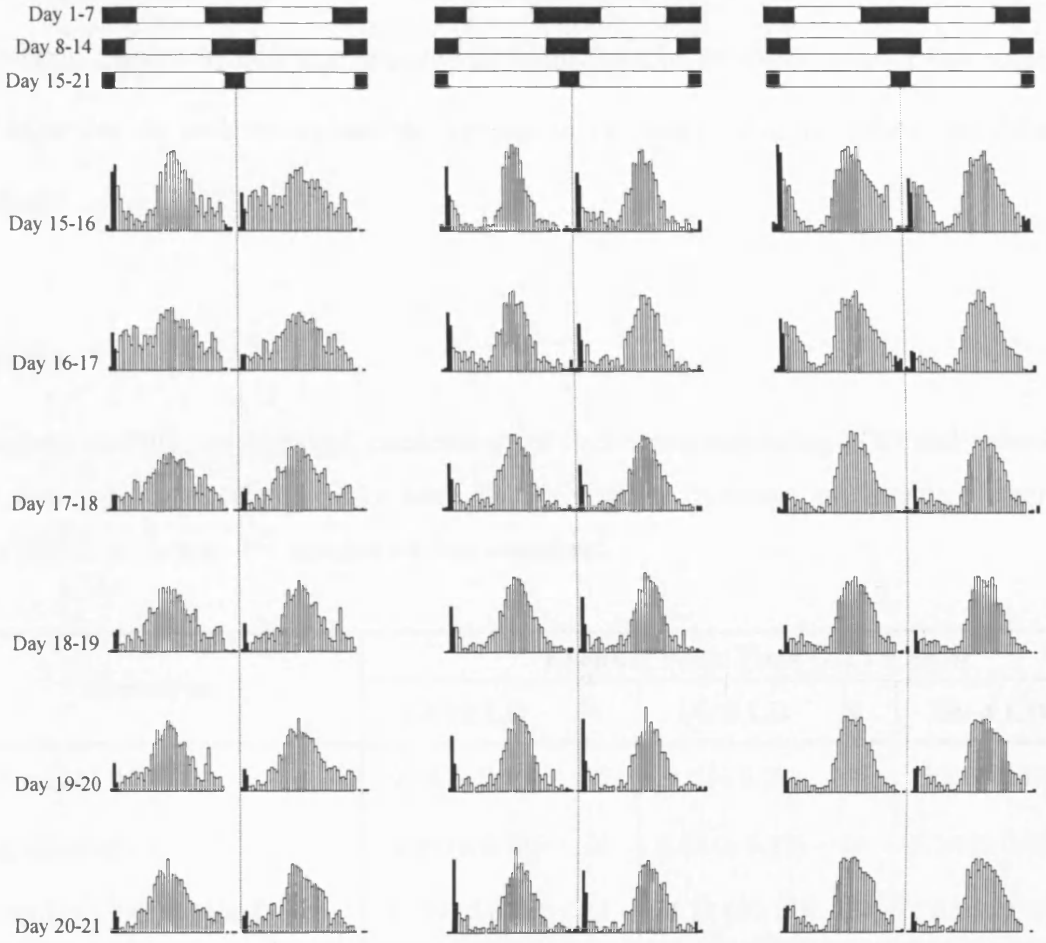


Figure 5.6

Double plot of locomotor activity profiles (average amount of activity – Y axis – as a function of time – X axis) for *D. melanogaster* down-regulating TGO (*w*; *tim*-GAL4/ UAS-*tint3*; + / +) and relevant controls (*w*; *tim*-GAL4/ +; +/ + and *w*; +/ +; UAS-*tint3*/ +). Shown are 7 days of activity under LD 12:12, followed by 7 days under LD 16: 8 and finally 7 days under LD 20: 4. Open bars, photophase. Shaded bars, scotophase.

showed that *tim*-GAL4/UAS-*tint3* flies were significantly different from both the controls at 12: 12 LD and 16: 8 LD ($p < 0.5$). During the last week (20: 4 LD) the experimental line no longer showed a difference to one of the controls (*w; UAS-tint3/+; +/+*). This could be due to the reduced number of flies that survived the third week of the experiment or this could be an indication that at such conditions the protein in no longer able to induce the differential behaviour.

Table 5-4

Locomotor activity evening peak occurrence for flies down-regulating TGO and controls. The peak time point was calculated for each fly individually. Average and standard error of the mean (SEM) is shown. N= number of flies examined.

Genotype	Evening Peak Time (ZT) ± SEM					
	12:12 LD	N	16: 8 LD	N	20: 4 LD	N
w; tim-GAL4/+; +/+	9.18 (± 0.25)	17	9.0 (± 0.29)	15	5.2 (± 0.45)	15
w; UAS-*tint3* /+; +/+	8.69 (± 0.36)	16	9.43 (± 0.37)	16	7.14 (± 0.45)	16
w; tim-GAL4/UAS-*tint3; +/+*	11.39 (± 0.24)	24	11.22 (±0.19)	23	8.08 (± 0.29)	19

Despite the differences observed in the locomotor activity profile of flies down-regulating TGO, no difference in the periodicity was observed within all the genotypes analyzed. The number of flies used in the experiment and the calculated period are summarized in Table 5-5. Exceptionally, a significant difference in period was observed under 12:12 LD conditions (using Kruskal-Wallis, $X^2= 17.58$, df = 2, $p << 0.05$, appendix 11). However, pairwise comparisons using Mann-Whitney test revealed that flies down-regulating TGO were different from just one of the controls ($p < 0.05$ against *w*; UAS-*tint3*/+; +/+ control and $P > 0.05$ against *w*; *tim*-GAL4/+; +/+ control). This indicates that the difference

observed was more due to a line effect rather than an effect of TGO down-regulation. For the two other subsequent LD cycles (16: 8 LD and 20: 4 LD) the period was essentially the same for all the genotypes analyzed (ANOVA, $F_{4, 77}$ =1.74, P > 0.05 and $F_{4, 72}$ =1.76, P > 0.05, appendix 11), including the flies up-regulating TGO (see Chapter 4).

Table 5-5

Period of locomotor activity for flies down-regulating TGO and controls. The entrained period was determined by autocorrelation and Fourier analysis and the results compared. The period length given by spectral analysis is presented. Average and standard error of the mean (SEM) are shown. N= number of flies examined

Genotype	Entrained period ± SEM					
	12:12 LD	N	16: 8 LD	N	20: 4 LD	N
w; tim-GAL4/+; +/+	24.06 (±0.05)	34	24.19 (±0.04)	15	24.02 (±0.07)	15
w; UAS-*tint3* /+; +/+	24.38 (±0.08)	29	24.31 (±0.06)	15	24.07 (±0.07)	16
w; tim-GAL4/UAS-*tint3;* +/+	24.11 (±0.04)	50	24.1 (±0.04)	22	23.81 (±0.06)	19

The results suggested that *tgo* might be involved in the control of the clock input mechanism, mainly the response for light. The mechanism by which TGO might be controlling the different responses for long days is still unclear.

5.3.3 Free-running behaviour

Rhythms of activity of *D. melanogaster* down-regulating TGO were analyzed for 5 days under DD conditions. The same flies analyzed under entrainment of 12:12 h LD conditions at 25°C (Section 5.3.2 of this Chapter) were subject to free-running conditions for five subsequent days. The average activity level for each bin was then used to build up an

average locomotor activity histogram for each genotype under this specific condition. Figure 5.7 shows the average locomotor activity pattern of male flies down- regulating TGO (*w*; *tim*-GAL4/ UAS-*tint*3; +/+ - *w*; +/+; tgo^1/+ - *w*; +/+; tgo^5/+) as well as the relevant controls (*w*; *tim*-GAL4/+; +/+ and *w*; UAS-*tint3*/+; +/+). Table 5-6 shows the endogenous period (τ) computed for lines reducing TGO. Although ANOVA revealed a difference amongst the groups analysed ($F_{6, 600} = 14.55$, $p < 0.05$, appendix 12) the periods were within a "wild- type" range.

Table 5-6

Free running locomotor activity rhythms for flies down-regulating TGO and controls. The endogenous period was determined by autocorrelation and Fourier analysis and the results compared. The period length given by spectral analysis is presented. Average and standard error of the mean (SEM) are shown. N= number of flies examined.

Genotype	τ_{DD} ± SEM	N
w; tim-GAL4/ +; +/ +	24.72 ± 0.06	104
w; UAS-*tint3* / +; +/ +	24.07 ± 0.08	73
w; tim-GAL4/UAS-*tint3;* +/ +	24.56 ± 0.06	117
w; +/ +; tgo^1/ +	24.20 ± 0.07	60
w; +/ +; tgo^5/ +	24.14 ± 0.08	35

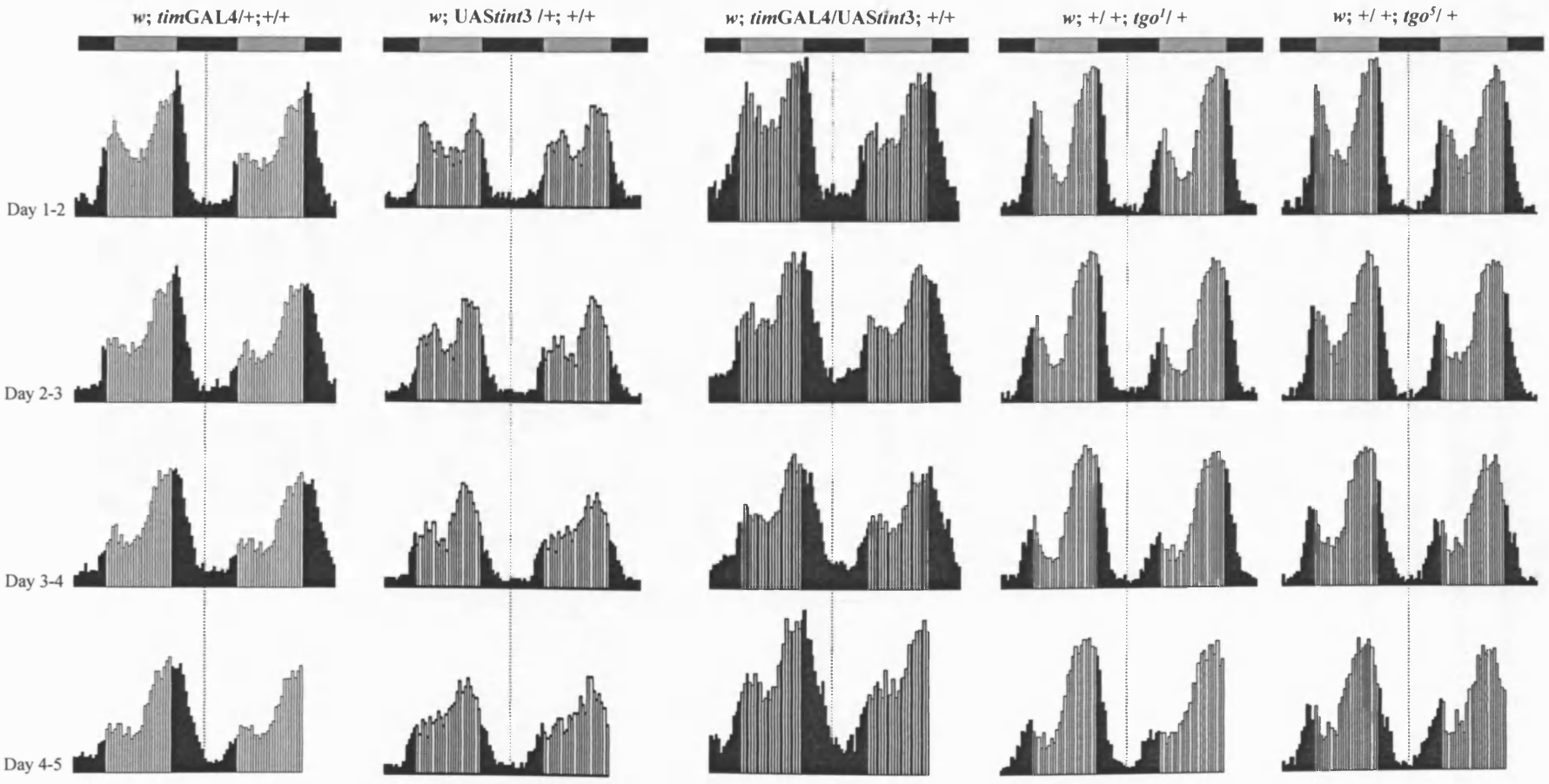

w; timGAL4/+;+/+
w; UAStint3 /+; +/+
w; timGAL4/UAStint3; +/+
w; +/ +; tgo1/ +
w; +/ +; tgo5/ +
Day 1-2
Day 2-3
Day 3-4
Day 4-5

Figure 5.7

Double plot of locomotor activity profiles (average amount of activity – Y axis – as a function of time – X axis) for *D. melanogaster* down-regulating TGO (*w*; *tim*-GAL4/UAS-*tint3*; +/ + , *w*; +/ +; tgo^{1}/ + and *w*; +/ +; tgo^{5}/ +) and relevant controls (*w*; *tim*-GAL4/ +; +/ + and *w*; UAS-*tint3*/ +; +/ +). Shown are 5 days of activity under constant darkness (DD). Gray bars, subjective day. Black bars, subjective night.

In order to evaluate if the endogenous period (τ) is affected by full TGO down-regulation, experiments were conducted at 29°C using the line carrying both the *act*-GAL4 and *tub*-GAL80^{TS} constructs. Figure 5.8 shows the average locomotor activity pattern of male flies down-regulating TGO (*w*; *act*-GAL4/UAS-*tint3*; *tub*-GAL80^{TS}/+ and *w*; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80^{TS}/+) as well as the relevant controls (*w*; UAS-*tint3*/+; +/+ - *w*; UAS-*tint1y*/ +; +/+ - *w*; *act*-GAL4/+; *tub*-GAL80^{TS}/+). Table 5-7 shows the period calculated for each genotype. A highly significant difference in period was detected among the genotypes analyzed (Kruskal-Wallis X^2= 76.89, df = 4, P < 0.05, appendix 13). Mann-Whitney pair wise comparisons revealed that both lines down-regulating TGO were highly different from the controls (P<0.05 for both).

Table 5-7

Free running locomotor activity rhythms for flies down-regulating TGO and controls. The entrained period was determined by autocorrelation and Fourier analysis and the results compared. The period length given by the most prominent spectral components is presented. Average and standard error of the mean (SEM) are shown. N= number of flies examined.

Genotype	τ_{DD} ± SEM	N
w; UAS-*tint3*/+; +/+	23.65 ± (0.09)	47
w; UAS-*tint1y*/+; +/+	24.02 ± (0.06)	46
w; *act*-GAL4/+; *tub*-GAL80^{TS}/+	24.51 ± (0.16)	55
w; *act*-GAL4/UAS-*tint3*; *tub*-GAL80^{TS}/+	26.33 ± (0.27)	21
w; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80^{TS}/+	25.59 ± (0.28)	22

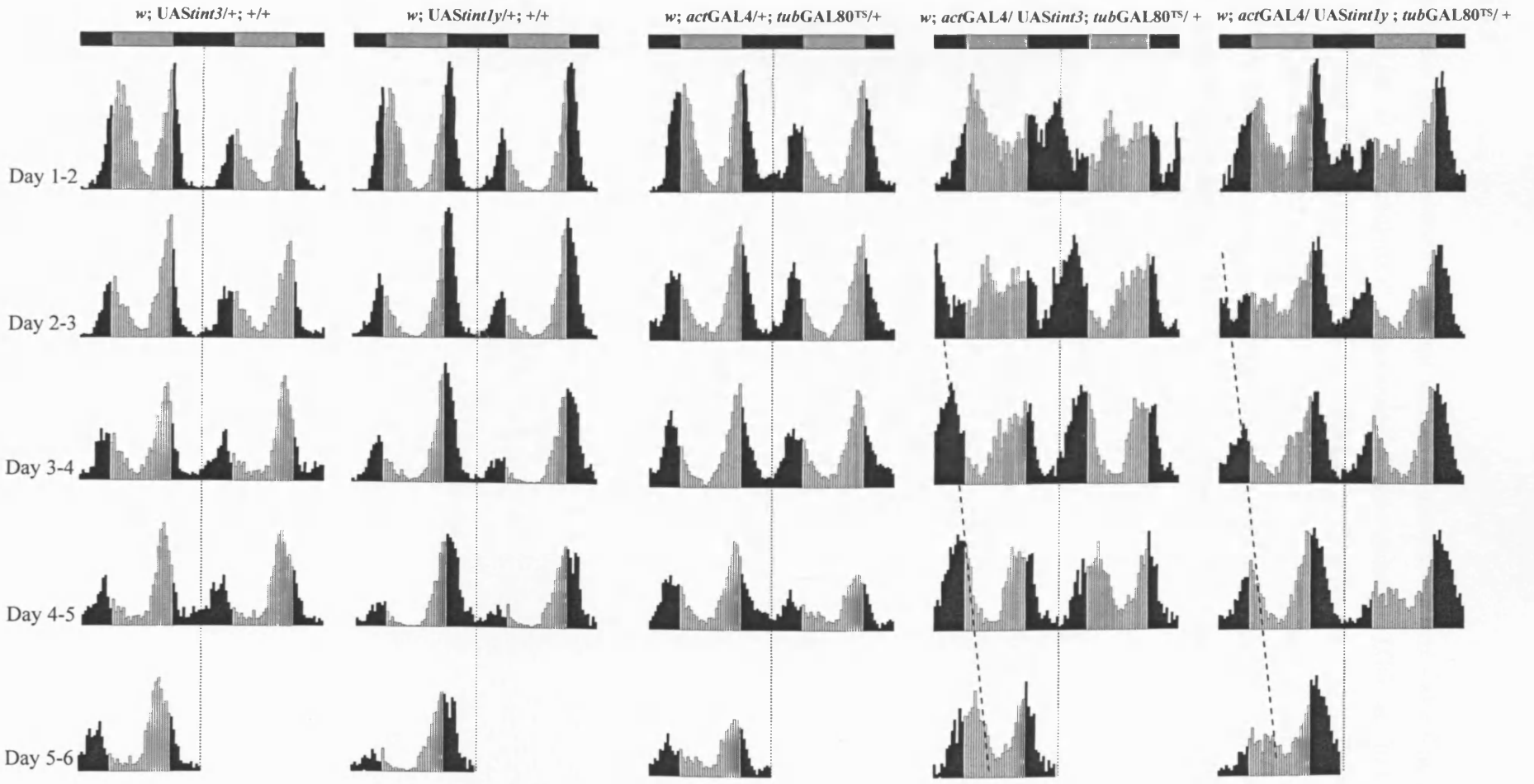

w; UASstint3/+; +/+
w; UASstint1y/+; +/+
w; actGAL4/+; tubGAL80TS/+
w; actGAL4/ UASstint3; tubGAL80TS/ +
w; actGAL4/ UASstint1y ; tubGAL80TS/ +
Day 1-2
Day 2-3
Day 3-4
Day 4-5
Day 5-6

Figure 5.8

Double plot of locomotor activity (average amount of activity – Y axis – as a function of time – X axis) for *D. melanogaster* ubiquitously down-regulating TGO at 30°C (*w*; *act*-GAL4/UAS-*tint3*; *tub*-GAL80^{TS}/+ and *w*; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80 ^{TS}/+) as well as the relevant controls (*w*; UAS-*tint3*/+; +/+, *w*; UAS-*tint1y*/+; +/+ and *w*; *act*-GAL4/+; *tub*-GAL80 ^{TS}/+). Tilted dashed line indicates the morning peak long period. Shown are 5 days of activity under constant darkness (DD). Gray bars, subjective day. Black bars, subjective night.

The results indicated that the effects observed when TGO is ubiquitously reduced is not directly related to the clock cells but to an unknown area in the brain devoted to the control of the activity. Interesting to note that while the evening peak did not move the morning peak did. This shows that the change in period of the morning peak was unable to reset the evening peak, indicating a function disruption in the neural circuit and in the oscillator-coupling mechanism that normally ensure a proper relationship between the timing of morning and evening locomotor activity (Stoleru *et al.*, 2005).

Another interesting observation is that the lack of TGO protein might interfere with the longevity of the flies. This suggestion is based on the fact that only half of the total flies analysed during the LD cycle of the experiment were able to survive the 10 days covering both the LD and DD cycles (see Table 5-7).

5.3.4 Phase shift analysis and phase-response curve (PRC)

Classic experiments of phase-response curve (see Chapter 4) were performed for lines down-regulating TGO. In order to investigate if *tgo* could be acting on the photosensitivity of the circadian clock, flies down-regulating the protein (*w*; *tim*-GAL4/UAS-*tint*3; +/ + ; *w*; +/ +; tgo^{1}/+ and *w*; +/+; tgo^{5}/+) as well as the relevant controls (*w*; *tim*-GAL4/+; +/+ and *w*; UAS-*tint3*/+; +/+) were subjected to 5 minutes light pulses at ZT 13, ZT15 and ZT21 and the phase shift was calculated (see Figure 5.9 and Table 5-8)

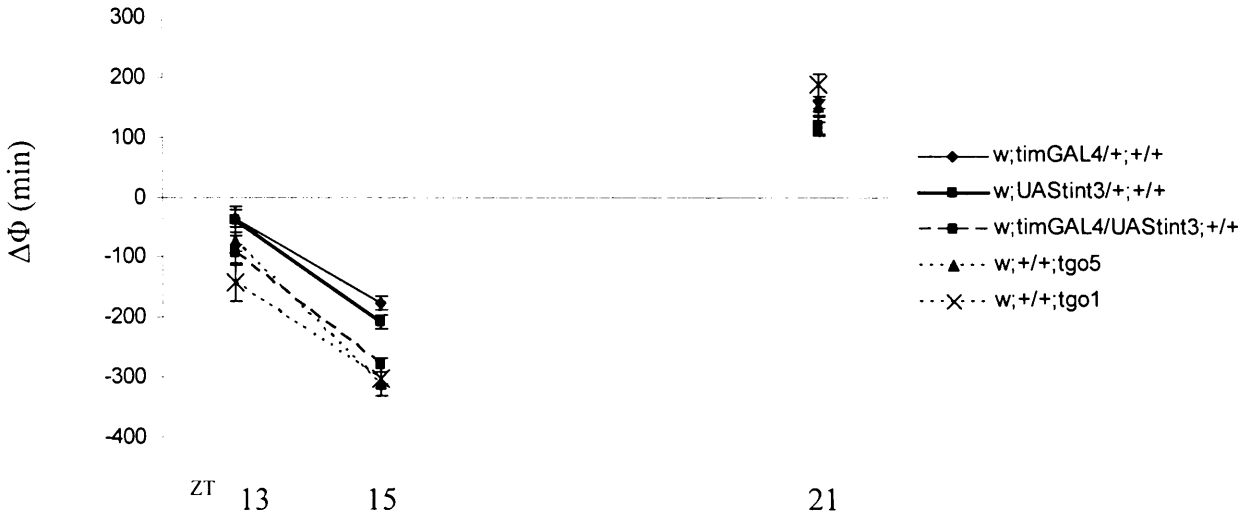


Figure 5.9

Phase Response for *D. melanogaster* down-regulating TGO (*w*; *tim*-GAL4/ UAS-*tint*3; +/+ - *w*; +/+; *tgo*^{1}/+ - *w*; +/+; *tgo*^{5}/+) and the relevant controls (*w*; *tim*-GAL4/+; +/+ and *w*; +/+; UAS-*tint3*/+). Number of flies used to calculate the phase shift magnitudes are indicated in Table 5-8. Average and standard error of the mean (SEM) are shown.

Table 5-8

Phase shift (minutes) after a 5 min light pulse at ZT13, 15 and 21 for flies down-regulating TGO and controls. $N_{P;\,U}$= number of pulsed and unpulsed flies, respectively.

Genotype	ΔΦ in minutes ± SEM					
	ZT 13	$N_{P;\,U}$	ZT 15	$N_{P;\,U}$	ZT 21	$N_{P;\,U}$
w; tim-GAL4/+; +/+	-34.47 (±14.37)	19; 14	176.32 (±11.42)	37; 23	158.60 (±10.27)	38; 37
w; UAS-*tint3*/+; +/+	-39.86 (±24.65)	14; 5	-207.84 (±10.1)	39;39	119.18 (±14.44)	58;39
w; tim-GAL4/UAS-*tint3*; +/+	-94.41 (±15.56)	27; 18	-279.08 (±11.99)	35; 32	114.45 (±11.30)	39; 41
w; +/+; *tgo*^{1}/+	-143.00 (±29.05)	15; 11	-301.58 (±9.55)	19; 13	186.82 (±18.26)	22; 22
w; +/+; *tgo*^{5}/ +	-74.35 (±16.46)	18; 18	-311.79 (±20.01)	28; 27	149.81 (±12.65)	32; 28

From Table 5-8 it can be seen that the mutant line *w*; +/ +; tgo^1/ + behaves as an outlier for two time points, ZT 13 and ZT 21. In the line analysed, tgo^1 occurs on the same chromosome as *ebony*. This mutation, used as a phenotypic marker reveals pleiotropic behavioural defects (Hodgetts, 1972; Hodgetts and Konopka, 1973; Newby and Jackson, 1991; Kyriacou *et al.*, 1978) such as reduced phototaxis and optomotor response (Heisenberg, 1972) and, in homozygosis, arrhythmic locomotor activity (Newby and Jackson, 1991). Thus, this line was removed from the statistical analysis for the purpose of simplification. Examination of Figure 5.9 reveals that at ZT15 the amplitude of the delay domain in the TGO reducing lines PRC was roughly the double of that seen for the control flies. This difference was highly significant (ANOVA, $F_{6, 224} = 22.04$, $p < 0.01$, appendix 14). However, ANOVA analysis points out that the difference found was not significant at ZT 13 ($F_{3, 74} = 2.89$, $p > 0.05$, appendix 14). Interestingly, as it occurred with the up-regulating lines analysed in Chapter 4, at ZT 21 the amplitude of the advance domain in the TGO down-regulation PRC showed no difference when compared with the controls (ANOVA, $F_{5, 273} = 1.55$, $p > 0.05$, appendix 14), suggesting that the reduction of TGO no longer interfere with PER and TIM molecular dynamics when they are located in the nucleus. In conclusion, flies reducing levels of TGO seem to behave similarly to the control in the phase advance domain and early night (ZT13). At ZT15, however, they show a more pronounced response revealing an increased sensitivity to light in the early night.

5.3.5 Molecular cycling of TIM

The effects of TGO down-regulation on the peripheral clock were analyzed by western blot experiments on protein extracts from the head, where the eyes constitute the largest part of the total amount of tissue. In order to analyse if TGO down-regulation could interfere on

TIM cycling dynamics, flies subjected to a 12:12 h LD condition at 25°C were collected every two hours during a 24 h day course. Equal amounts of head protein extracts were run in a 6% polyacrylamide gel. Blots were probed with rat anti-TIM antibodies (1: 1000) a HRP-conjugated goat anti-rat (Sigma, 1: 8000). Figure 5.10 shows the average molecular oscillation of TIM in flies down-regulating TGO (w; *tim*-GAL4/UAS-*tint3*; +/+) compared to the control (w; *tim*-GAL4/+; +/+) obtained from seven independent immunoblot experiments. The experimental line revealed that TIM level reaches half its maximum amount at ZT14 while in the control it only happens at ZT 16. Another interesting feature observed in Figure 5.10 is that whilst in the control flies the protein peak happened at ZT 20, in flies down-regulating TGO the peak occurred 2 hours before (ZT18). Despite all the differences observed, ANOVA analysis revealed that the changes in TIM expression pattern were not statistically significant ($F_{13, 65} = 1.15$, $P > 0.05$, appendix 15).

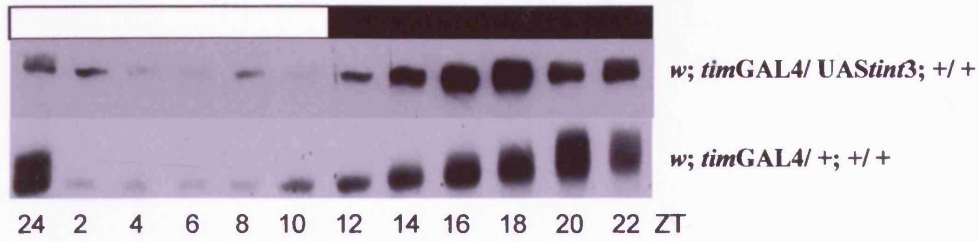
5.4 Summary

The down-regulation of TGO in clock cells by using the GAL4/UAS binary system was obtained successfully. When driven by the *tim*-GAL4 construct, reduction was approximately 10% and 30% in the UAS-*tint3* and UAS-*tint1y* strains, respectively. The *act*-GAL4 driver seemed to induce a complete TGO reduction in the flies. Because only one gel was obtained, the exactly amount of protein that was down-regulated has still to be confirmed. As expected, flies carrying one copy of the *tgo* mutant gene (tgo^5 or tgo^1) showed approximately 50% reduction of the protein.

The *w*; *tim*-GAL4/UAS-*tint*3; +/+ genotype showed no difference for entrainment under a 12:12 LD cycle and also for the free-running period. However, distinct behaviours were observed when compared to the controls in the long photoperiod and light pulse experiments. In the first, the experimental line showed a strong evening peak delay under

12:12 LD and 16: 8 LD conditions. In the second, flies down-regulating the protein showed a more pronounced response in the phase delay when a 5 minutes light pulse was given at ZT15. Finally, the *w*; *tim*-GAL4/UAS-*tint*3; +/+ showed a different profile for TIM molecular oscillation during a 24 h day course. Although not being statistically significant, the early build up of the protein suggested some molecular influence.

The ubiquitous TGO reduction induced by the *actin*-GAL4 driver (*w*; *act*-GAL4/UAS-*tint3*; *tub*-GAL80^{TS}/+ and *w*; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80^{TS}/+) showed no difference in the endogenous period at 12: 12 LD. However, a secondary peak of activity in the night reflected a tendency for a longer period in those flies, which was interrupted by the startle response caused by the lights-off in such LD conditions. Indeed, the free running period for *act*GAL4/UAS*tint* flies were shown to be longer when compared to the controls. Interestingly, the morning and evening peaks of activity were differentially affected during DD and LD, respectively. This suggests that a deficiency in crosstalk between clock neurons might be induced by TGO down-regulation. Finally, the *tgo* mutant genotypes were shown to share almost the same distinct phenotypes observed for the *tim*-GAL4/UAS-*tint3* flies in the entrainment, free running and phase shift experiments here conducted.



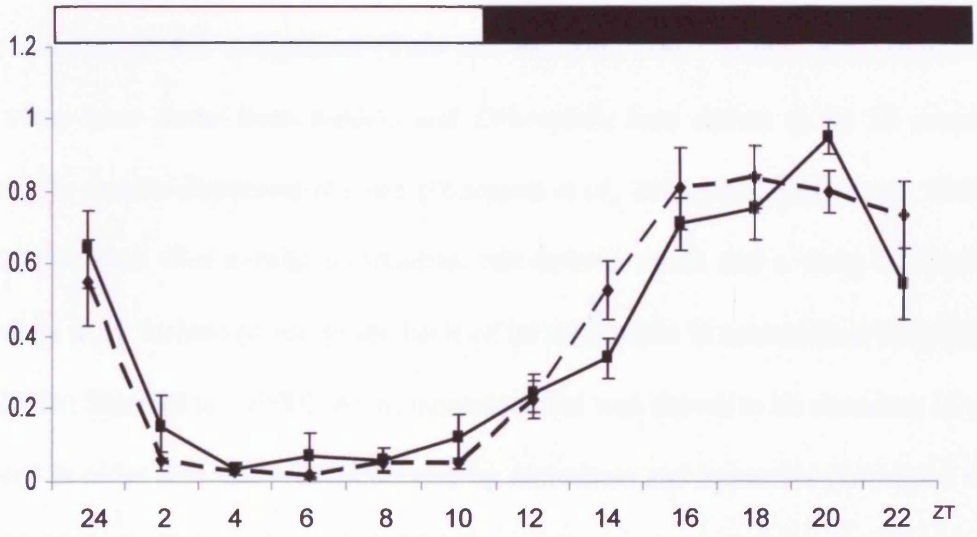


Figure 5.10

Western blot analysis of TIMELESS in flies down-regulating TGO and control. A representative blot is shown on the top for each genotype. The white and black bars denote photophase and scotophase, respectively. On the bottom, average ± SEM of TIM oscillation from seven independent experiments. For each gel the strongest band was set equal to 100, the others were normalized accordingly. Continuous line: *w*; *tim*-GAL4/ +; +/ +. Dashed line: *w*; *tim*-GAL4/ UAS-*tint3*; + / +.

Chapter 6: Behavioural analysis of sleep

6.1 Introduction

Sleep is an essential biological process whose function remains unknown (Campbell and Tobler, 1984; Greenspan *et al.*, 2001; Rechtschaffen, 1989; Tobler, 2000). Abundant evidence exists that several sleep disorders and many aspects of the normal sleep have a strong genetic component (Tafti and Franken, 2002; Toth, 2001). Important insights into sleep have come from models and *Drosophila* was shown to be an excellent model system for genetic dissection of sleep (Greespan *et al.*, 2001; Hendricks *et al.*, 2000a; Shaw *et al.*, 2000). Fruit flies exhibit a circadian rest-activity cycle and a sleep-like state has been described in *D. melanogaster* on the basis of its similarities to mammalian sleep (Hendricks *et al.*, 2000a; Shaw *et al.*, 2000). As in mammals rest was shown to be abundant in young flies, reduced in older flies and was modulated by stimulants and hypnotics (Greespan *et al.*, 2001; Hendricks *et al.*, 2000a; Shaw *et al.*, 2000).

Sleep in *Drosophila* is defined by universal criteria that include prolonged periods of quiescence, reduced responsiveness to external stimuli, increased arousal threshold, rapid reversibility and homeostatic regulatory mechanism (Campbell and Tobler, 1984; Hendricks *et al.*, 2000b; Tobler, 2000). During periods of quiescence that last five minutes or longer flies are unresponsive to mild external stimuli but can be quickly aroused with stronger stimulation (Greenspan *et al.*, 2001). This fact made to be universally accepted that inactivity period spanning five minutes is the minimum amount of time the fly should be resting to be considered in a sleep like state (Greespan *et al.*, 2001, Shaw and Franken, 2003). Confirming this definition, an independent group has succeeded in recording field potentials from *Drosophila* and has found reliable electrophysiological changes between periods of sleep and

waking (Nitz *et al.*, 2002). Recently two groups have identified the *Drosophila* adult mushroom bodies, a neural structure central to memory and learning, as the site promoting sleep regulation (Joiner *et al.*, 2006; Pitman *et al.*, 2006).

Sleep is clearly a circadian rhythm and it is regulated by both a circadian and a homeostatic process (Borbely, 1982, 1998; Daan *et al.*, 1984). The first gives time context to most physiological processes ensuring proper entrainment between internal rhythms and the external alterations in photoperiod. Thus, the distribution of sleep over the 24h day is strongly determined by the circadian process. The second tracks sleep need, which accumulates in the absence of sleep and decreases in its presence (Shaw and Franken, 2003). The homeostatic regulation is characteristically independent to the circadian clock (Greespan *et al.*, 2001). In mammals the independence of these two systems has been demonstrated by evaluating sleep following lesion in the suprachiasmatic nucleus (SCN), the site of the principal circadian pacemaker. Following such lesions, sleep deprived animals exhibited an increase in sleep duration and intensity indicating that homeostatic mechanisms are not dependent upon an intact circadian system (Tobler, 2000). The same independence in this relationship was demonstrated in flies, but without ablating the structure responsible for generation of circadian rhythms. The clock was evaluated by analysing mutants of the *period* locus (per^0), which indicated the presence of both homeostatic response and rest in the arrhythmic flies (Greenspan *et al.*, 2001; Hendricks *et al.*, 2000a). A study in both per^0 and *timeless* (tim^0) mutants indicated that the role of the two genes in rest regulation might be different (Hendricks *et al.*, 2000a). In contrast to what was found for per^0 flies, tim^0 flies showed a lack of rest rebound after 3-6h sleep deprivation suggesting for the *tim* gene a function beyond its role in the circadian clock.

The example above gives us a glimpse on the inherent pleiotropic nature of genes, especially with respect to behaviour (Greenspan, 2001). One pathway that has been shown to have an influence in sleep drive and whose members show a remarkable amount of pleiotropy

is the one that comprises the circadian clock (Shaw and Franken, 2003), whose genes have been implicated in courtship, feeding, addiction, and learning and memory (Andretic *et al.*, 1999; Kyriacou and Hall, 1982; Sarov-Blat *et al.*, 2000). Other clock genes were then evaluated to better understand the interaction between homeostatic and circadian processes. A study comparing homeostasis in the canonical loss-of-function mutants *Clock* (Clk^{jrk}), *cycle* (cyc^{01}), *period* (per^{01}) and *timeless* (tim^{01}) revealed that these genes influenced homeostasis to varying degrees (Shaw and Franken, 2003). While wild-type flies with an intact circadian clock reclaimed approximately 40-50% of their lost sleep, three mutants (Clk^{jrk}, $per^{01}$and tim^{01}) reclaimed 100% of their lost sleep. cyc^{01} flies appeared to be much more sensitive to the effects of sleep loss, exhibiting sleep rebounds six times larger than wild-type flies. More importantly, when the deprivation was extended past 10h, cyc^{01}flies started to die, suggesting that the increase in recovery sleep after shorter deprivations reflected an acceleration of the deleterious effects of awaking rather than an impairment of the recovery process. Interestingly, in this study tim^{0} flies (previously shown to lack rest rebound after 3-6h sleep deprivation) also reclaimed 100% of lost sleep after 7, 9 and 12h of sleep deprivation. More recently it was shown that this abnormal response in cyc^{01}flies was sexually dimorphic, being reduced or absent in males and exaggerated in females (Hendricks *et al.*, 2003).

It is worth note that flies have not been the only genetic organism in which investigations into sleep have been influenced by chronobiology. In mouse the *Clock* mutation was shown to affect NREM (non rapid-eyes movement) type of sleep by decreasing its time, delta power and consolidation (Naylor *et al.*, 2000). Opposite sleep changes were observed in mice lacking both the *Cryptochromes* (*Cry1,* $2^{-/-}$), consistent with *Clock* and *Cry* being positive and negative regulators of the murine circadian clock, respectively (Wisor *et al.*, 2002).

Several environmental changes can cause sleep deprivation and there is strong evidence that PAS proteins are involved in their adaptive responses to overcome dysfunctions

that sleep loss and stress might bring (Shaw *et al,* 2002). Two facts characterize the bHLH-PAS protein TGO as a strong candidate for controlling key components in the sleep regulation process. The first is its interaction in yeast-two hybrid with two proteins involved in sleep homeostasis, CLK and CRY (Shaw & Franken, 2003; Cirelli, 2003; Wisor *et al.,* 2002). The second is TGO molecular similarity to CYC, the clock gene that so far have shown the strongest influence in sleep homeostasis process.

6.2 Material and methods

The effects of TGO overexpression on sleep regulation and recovery was analysed in F1 male flies from two different experimental lines (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/ + and UAS-*tgo52.1*; *tim*-GAL4/+; +/ +) and their respective controls (*w*; *tim*-GAL4/ +; +/ + - *w*; + / +; UAS-*tgo25.1*/ + - UAS-*tgo52.1*; +/+; +/ +).

The effects of TGO down-regulation on sleep regulation and recovery was analysed in F1 male flies from two different experimental lines (*w*; *tim*-GAL4/ UAS-*tint*3; +/ + and *w*; *tim*-GAL4/ UAS-*tint*1y; +/ +) and their respective controls (*w*; *tim*-GAL4/ +; +/ + - *w*; UAS-*tint*3 /+; +/ + - *w*; UAS-*tint1y*/ +; +/ +).

Refer to Chapter 2 for the experiments conduction details.

6.3 Results

Generally, all genotypes showed the sleep-wake distribution typical of wild-type males of *D. melanogaster*, with high levels of sleep in both the scotophase and middle of the photophase (siesta). Nevertheless, as in wild-type flies, the light-to-dark and dark-to-light transitions were accompanied by a pronounced decrease in sleep time for all lines analysed. Figure 6.1 shows the daily time course (1 hour interval) of the amount of sleep in two lines

overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo 25.1*/ + and UAS-*tgo52.1*; *tim*-GAL4/+; +/ +) and their respective controls (*w*; *tim*-GAL4/ +; +/+ - *w*; + / +; UAS-*tgo25.1*/+ - UAS-*tgo52.1*; +/+; +/+). As it can be depicted from the graph, the overexpression of TGO promoted a strong reduction in the amount of sleep, mainly between ZT7 - ZT9 and ZT16 - ZT19. Both the experimental lines showed an amount of sleep varying from 65 to 72% of the total time (see Table 6.1). Analysis by ANOVA shows a significant difference among the lines ($F_{4, 380} = 24.44$, $P << 0.05$, appendix 16). Bonferroni *Post Hoc* comparisons reveal that one of the lines overexpressing TGO (*w*; *tim*-GAL4/+; UAS-*tgo25.1*/ +) was particularly affected.

An interesting phenotype was also found when analysing lines with low levels of TGO. Figure 6.2 shows the daily time course of the amount of sleep in two lines down-regulating the protein (*w*; *tim*-GAL4/ UAS-*tint*3; +/ + and *w*; *tim*-GAL4/ UAS-*tint*1y; +/ +) and their respective controls (*w*; *tim*-GAL4/ +; +/ + - *w*; UAS-*tint3* /+; +/ + - *w*; UAS-*tint1y*/ +; +/ +). The graph analysis reveals a high reduction in the amount of sleep in the experimental flies when compared to the controls. Both lines down- regulating TGO showed an amount of sleep varying from 66.2 to 67.5 % of the total time, a reduction of at least 3% when compared to the controls (see Table 6.1). Statistical analysis revealed a significant difference amongst the genotypes (ANOVA $F_{4, 359} = 21.01$, $P < 0.01$, appendix 17). Subsequent Bonferroni *Post Hoc* comparisons showed that although certain level of difference was detected within the control group, the experimental lines were significantly different from all the controls. Interestingly, sleep reduction happened essentially during the photophase, between ZT4 and ZT6, where control flies spent up to 90% of their time at a sleep state. Roughly, the test animals slept for 80% of their time during the same period.

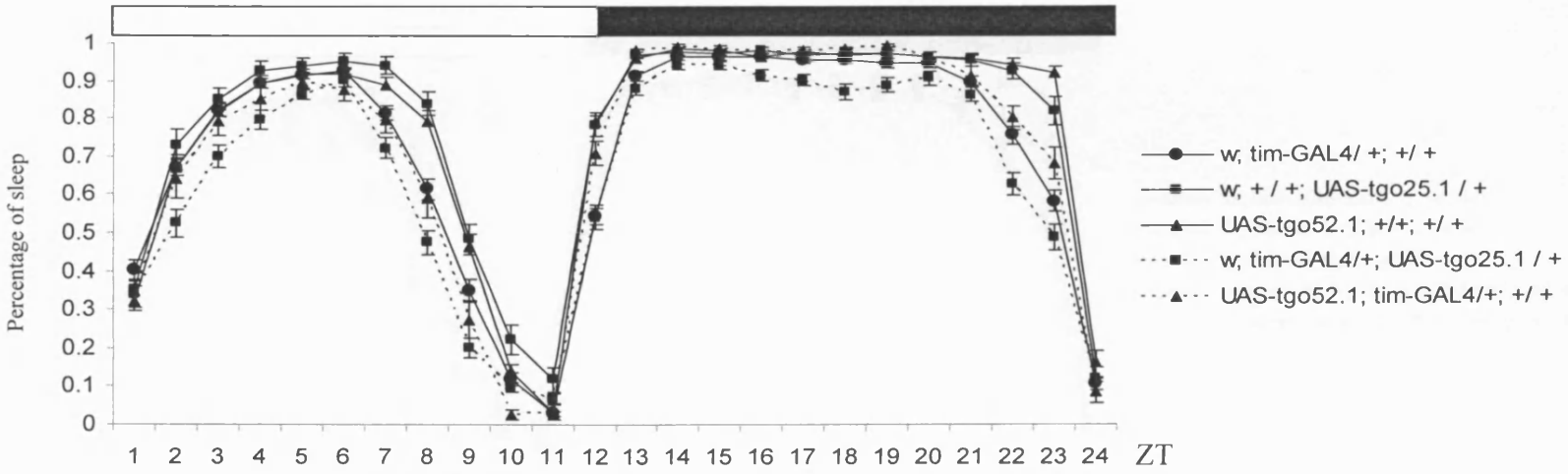


Figure 6.1

Daily time course (1 hour interval) of the amount of sleep in two lines overexpressing TGO and their respective controls. The bar on the top represents the photopahse (white) and the scotophase (black) of a 12: 12 h LD cycle. The y axis shows the percentage of sleep during each one hour computation. The x axis indicates the ZT time point from which the sleep computation begun for each hour. The Standard Error of the Mean (SEM) is given for all time points presented.

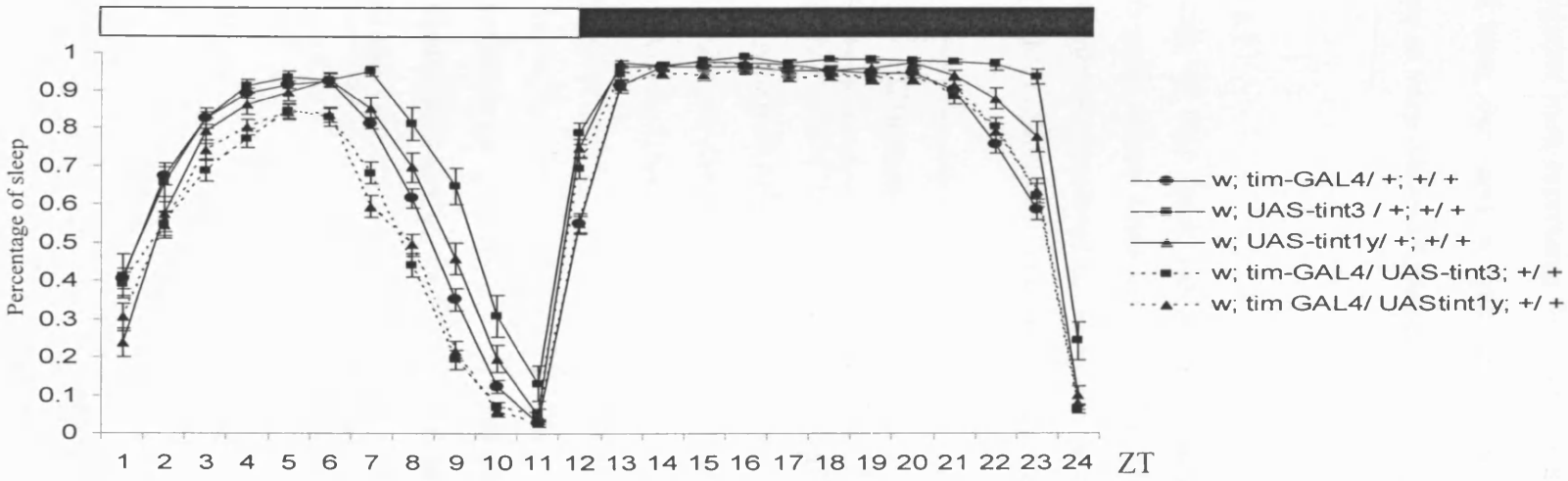


Figure 6.2

Daily time course (1 hour interval) of the amount of sleep in two lines down regulating TGO and their respective controls. The bar on the top represents the photopahse (white) and the scotophase (black) of a 12: 12 h LD cycle. The y axis shows the percentage of sleep during each one hour computation. The x axis indicates the ZT time point from which the sleep computation begun for each hour. The Standard Error of the Mean (SEM) is given for all time points presented.

Finally, experiments performed to check sleep homeostasis control in *D. melanogaster* miss-expressing TGO were not conclusive. This was due to the fact that two control lines, one used in both up and down regulation experiments, did not show any evidence of sleep rebound following 12 h sleep deprivation (see Table 6.1).

Table 6.1

Percentage of time spent asleep in *D. melanogaster* miss-expressing TGO and relevant controls under normal conditions and after 12 hours of sleep deprivation. Mean sleep amount was obtained as percentage of the total time of a 24-h day (12:12 LD). Standard error of the mean (SEM) is shown. N= number of flies examined.

Genotype	Sleep (%) ± SEM			
	Baseline	N	Deprived	N
UAS-*tgo52.1*;+/+; +/ +	76.77 (±1.70)	73	81.45 (±1.98)	64
w;+/ +; UAS-*tgo25.1* /+	78.15 (±2.37)	50	83.22 (±2.14)	45
UAS-*tgo52.1*; *tim*-GAL4/+; +/+	71.64 (±2.52)	39	69.31 (±2.48)	37
w; *tim*-GAL4/+; UAS-*tgo25.1*/+	64.90 (± 2.17)	89	68.58 (±2.23)	86
w; *tim*-GAL4/+; +/+	71.10 (±1.54)	134	71.90 (±1.93)	124
w; UAS-*tint1y*/ +; +/+	73.89 (±2.30)	48	82.83 (±2.19)	49
w; UAS-*tint*3 /+; +/ +	80.24 (±2.40)	34	82.70 (±1.92)	36
w; *tim*-GAL4/UAS-*tint*1y; +/+	66.26 (±1.96)	83	62.23 (±2.77)	75
w; *tim*-GAL4/UAS-*tint*3; +/ +	67.57 (±1.99)	65	69.79 (±2.32)	59

6.4 Summary

This study has examined a possible role for TGO in sleep regulation. The results obtained seem to confirm this hypothesis as both TGO up and down-regulation promote a significant reduction, of at least 3 %, in the daily requirement of sleep. The results provide evidence that TGO might have a different pathway of controlling sleep when compared to the canonical circadian clock mutants per^{0}, tim^{0}, Clk^{jrk}, and cyc^{01}. That is because despite of the arrhythmic phenotype and the striking differences on sleep rebound, all these mutants showed the same amount of rest as wild-type flies when not sleep deprived (Greenspan *et al.*, 2001; Hendricks *et al.*, 2000a; Shaw and Franken, 2003). Conversely, miss-regulation of TGO is responsible for a short sleep phenotype. This is similar to the effect of *shaker* mutants, where a point mutation in the conserved domain of the gene promotes a sleep reduction of one-third of the wild-type amount (Cirelli *et al.*, 2005).

Finally, this study failed to characterize sleep homeostasis in the genotypes analysed as the lack of sleep rebound could not be correlated to TGO up or down- regulation. The main reason for this was lack of sleep rebound in two control strains, suggesting that either strain position effects or technical artefacts might have influenced these data.

Chapter 7: General discussion

7.1 Overview

The *Drosophila tango* (*tgo*) gene is a member of the bHLH-PAS family which is characterized by the presence of the basic helix-loop-helix (bHLH) and Per-Arnt-Sim (PAS) domains (Crews, 1998; Ohshiro and Saigo, 1997; Sonnenfeld *et al.*, 1997, Sonnenfeld *et al.*, 2005). *tgo* is orthologous to the vertebrate *Aryl hydrocarbon receptor nuclear translocator* (*Arnt*) whose product functions as a heterodimer with the Aryl hydrocarbon Receptor (AhR) to metabolize dioxins (Hoffman *et al.*, 1991). A functional analysis of a role for *tgo* during CNS midline and tracheal development in *Drosophila* embryos has been previously reported (Ohshiro and Saigo, 1997; Sonnenfeld *et al.*, 1997; Zelzer *et al.*, 1997). Interaction studies indicate that TGO and mammalian ARNT are partners for a number of phylogenetic diverse bHLH-PAS proteins (Crews *et al.*, 1988; Emmons *et al.*, 1999; Isaac and Andrew, 1996; Jiang and Crews, 2003; Ohshiro and Saigo, 1997; Thomas *et al.*, 1988; Wilk *et al.*, 1996; Sonnenfeld *et al.*, 1997; Zelzer *et al.*, 1997). These observations indicate TGO/ARNT as a broadly expressed dimerization platform for bHLH-PAS proteins. Hence, they constitute evolutionary conserved transcriptional regulators that are found in most multicellular organisms such as mammals, *Drosophila* and fish (Pollenz *et al.*, 1996).

Daily rhythms of physiology and behaviour are generated by endogenous circadian oscillators which are controlled by several clock genes (reviewed in Chang, 2006; Dunlap, 1999; Hall, 1995; 1996; Hardin, 2005; Hardin and Siwick, 1995; Helfrich-Foster, 2005; Rosato *et al.*, 2006; Rosbash *et al.*, 1996; Seghal *et al.*, 1996; Stanewsky, 2002; Zordan *et al.*, 2003). The current *Drosophila* circadian clock model suggests that *Clock* and *cycle* bHLH encoded products dimerize by means of their PAS domain to activate *period* (*per*) and

timeless (*tim*) transcription. After PER and TIM proteins accumulate in the cytoplasm and form heterodimers, they translocate to the nucleus and negatively regulate their own genes by interfering with CLK: CYC function. This negative feed-back loop is regulated by post-transcriptional endogenous systems, such as protein phosphorialation, that modulates the stability of certain clock components according to a specific time of the day (Edery, 1999). Light acts as an exogenous modulator by destabilization of TIM protein throughout the eyes and extra ocular pathways promoting its degradation (Stanewsky *et al.*, 1998; Suri *et al.*, 1998; Yang *et al.*, 1998).

A plethora of data offers the tantalizing hypothesis that TGO might act as a component of the circadian clock. For instance, in a yeast-two-hybrid system both TGO and its mammalian homologue (ARNT) interact with CLK (Gekakis *et al.*, 1998). Additionally, it was shown that TGO weakly interacts with CRY (E. Rosato, personal communication). Preliminary in *situ* hybridization results showed that TGO and CRY are co-expressed in a large portion of the *Drosophila* head which might include the pacemaker cells (Codd, 2003). Moreover, TGO expression in the adult brain of *Drosophila* was determined by immunostaining with TGO anti-body (Chapter 3). The results suggest that TGO has a wide pattern of expression and that it might be present also in a sub-set of clock cells in the dorsal brain, although not in the Lateral Neurons.

Finally, TGO was shown to have a strong molecular analogy to CYC (Jiang and Crews, 2003) which suggests that they might have overlapping or related functions. Indeed, TGO could be one of the PAS transcription factor able to substitute CYC during the development of the *s*-LN_vs, providing a possible explanation for the milder effect of cyc^0 compared to the Clk^{jrk} mutation in a developmental phenotype (Park *et al.*, 2000).

During the work carried out for this thesis, the role of TGO on the *Drosophila* circadian clock and in the mechanisms that control rest/activity was evaluated by means of the overexpressing and down-regulation of the protein in the flies. The results obtained support a

function for TGO in the input and/or the output of the clock and in the mechanisms regulating sleep.

7.2 Overexpression of TANGO in *Drosophila melanogaster*

The overexpression of TGO induced by *tim*-GAL4 driver was shown not to interfere with the clock mechanisms that govern the rhythmicity period of locomotor activity. This conclusion was reached after the observation that experimental flies showed no behavioural difference in period, phase and amplitude in both 12: 12h LD and DD conditions when compared to controls (Table 4-1 and Table 4-4, respectively).

However, TGO overexpression elicits a higher response to a light pulse delivered at ZT13 and ZT15 (Table 4-5). Interestingly, those represent the time when TIM, a photosensitive molecule (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996) is being built up. According to the current model of clock function, the behavioural delay after a light pulse is delivered early in the night represents the time required for TIM levels to go back to normal, after light-mediated degradation of the protein (Hunter-Ensor *et al.*, 1996; Lee *et al.* 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996; Suri *et al.*, 1998; Yang *et al.*, 1998).

An experiment where flies were entrained to a long photoperiod also revealed a strong influence of TGO overexpression in the pathways related to light responses. Interestingly, under a 16: 8 LD cycle and at a constant temperature of 20°C, the experimental flies showed a burst of activity after lights-off. This was similar to the pa radoxical masking, an effect sometimes observed in mutants that lack any of several photoreceptors, including the compound eyes (Rieger *et al.*, 2003).

An additional effect on entrainment became apparent under extremely long days (20:4 LD) as flies overexpressing TGO showed a pronounced morning peak, that was absent or severely reduced in control flies (Figure 4.3). In wild-type flies, the evening activity bout is

always the most prominent peak which persists under constant conditions, whereas the morning activity is much reduced under such conditions and may even disappear (Wheeler *et al.*, 1993; Helfrich-Forster, 2000). Considering that this extreme photoperiod is quite close to a regime of constant light, it is not surprising to see the morning peak disappearing in control flies. Thus, it is possible that the morning peak persistence in the experimental flies was related to the overexpression of TGO in clock cells.

No rhythmicity rescue was observed when TGO was overexpressed in both Clk^{jrk} and cyc^{0} mutants background (Figure 4.10 and 4.11, respectively). This result was expected for Clk^{jrk} which is not a null mutant. The semi-dominance of this mutation is a reflection of the residual activity of CLK^{JRK} (Allada *et al.*, 1998) and therefore a high affinity heterodimerization between CYC: CLK^{JRK} could have occurred. Alternatively, one might suggest that TGO could bind to the truncated CLK^{jrk} protein but could not replace CLK activation domain. Since TGO was shown to bind CLK in a yeast-two-hybrid assay (E. Rosato, personal communication) and a putative PAS containing transcription factor was suggested to bind CLK in cyc^{0} mutant flies (Park *et al.*, 2000), rescue of rhythmicity was more likely to be seen when TGO was overexpressed in a cyc^{0} background. However, the results suggested that TGO was not able to substitute for CYC as no rhythmicity rescue was observed. It is also possible to assume that whenever TGO: CLK heterodimers are formed in the pacemaker cells they would only affect TGO-dependent genes, such as those carrying the CNS midline element (CME) and previously shown to be the target sequence of SIM:TGO and TRH:TGO (Oshiro and Saigo, 1997; Sonnenfeld *et al.*, 1997; Zelzer *et al.*, 1997).

All the assumptions above can also be applied when interpreting the function of TGO within the peripheral clock cells, as western blot analysis showed that the molecular cycling of TIM was not significantly affected by TGO overexpression. However, one must take into consideration that the low resolution given by western blot analysis might have caused small differences impossible to be detected. Alternatively, it is also possible that posttranslational

regulation of TIM could have masked changes in *tim* mRNA expression. These should be further investigated *via* quantitative PCR.

7.3 Down-regulation of TANGO in *Drosophila melanogaster*

The core of the circadian clock was shown not to be affected by TGO down-regulation when induced by the *tim*-GAL4 driver. The locomotor activity period, phase and amplitude were the same between experimental and control lines in both 12: 12h LD and DD conditions (Tables 5-2 and 5-6, respectively). It is well known that during embryogenesis *tgo* function is required in processes such as CNS midline cell differentiation and tracheal tubule and antennal development (Emmons *et al.*, 1999; Ohshiro *et al.*, 1997; Sonnenlfeld *et al.*, 1997; Zelzer *et al.*, 1997). However, it is not known if TGO is involved in the development of central pacemaker cells. The normal functioning of the clock cells where TGO was down-regulated presented a good indication of their integrity. This result strongly suggests that TGO is not controlling any developmental aspect in the central clock. Moreover, immunohystochemistry has shown that TGO is not found in important central clock cells such as the Lateral Neurons (Figure 3.1a).

Interestingly, the down-regulation of TGO was shown to affect light responses. When exposed to long photoperiods at 20°C, *tim*-GAL4/UAS-*tint3* male flies showed a later occurrence of the evening activity offset (Figure 5.6). The photoperiod duration and ambient temperature have strong effects on the time of evening activity. Over a wild range of photoperiods, the evening peak occurs around the light-to-dark transition (Majercak *et al.*, 1999; Qiu and Hardin, 1996; Rieger *et al.*, 2003; Shafer *et al.*, 2004). However, ambient temperature regulates the evening activity by modulating the proportion that occurs during either daytime or night time hours. As temperature increases, the evening peak becomes

progressively more nocturnal (Majercak *et al.*, 1999), an adaptive response to minimize the risks of desiccation during the hot midday. Interestingly, the results indicate that the low temperature will bring the evening peak from the light-to-dark transition hours to the middle of the photophase, regardless of its duration. However, when TGO is down-regulated the evening peak occurs during the lights transitions as if the flies were not exposed to a low temperature.

Moreover, *tim*-GAL4/UAS-*tint3* male flies showed a more pronounced phase shift after a light pulse was delivered at the beginning of the night (Table 5-8). It is interesting to note that the waveform of TIM levels in flies down-regulating TGO, as revealed by western blot experiments, shows a tendency for an early accumulation of the protein in the peripheral clock (Figure 5.10). The results indicate that at ZT13 and ZT15 a higher level of TIM is present in *tim*-GAL4/UAS-*tint3* flies when compared to the controls. Hence, more TIM will be degraded in the experimental flies that consequently will require more time to recover their normal amount levels. How a change is the peripheral clock would affect the phase shift is currently unknown.

Entrainment and light pulse experiments were also conducted in flies carrying the *tgo* mutants in heterozygosis. The results were basically the same as those obtained when TGO was down-regulated by the *tim*-GAL4 drivers. This confirms that a partial reduction of TGO is enough to affect the mechanisms involved in the photosensitivity of the circadian clock.

The most striking and unexpected finding of the current study was observed when TGO was ubiquitously down-regulated by using the strong *act*-GAL4 driver. The results suggest that the strong reduction of TGO all over the organism promotes an internal desynchronization between the morning and evening oscillators. A long standing model assumes that the clock consists of two groups of synchronised oscillators, one governing the morning and the other governing the evening activity of the animal (Pittendrigh and Daan, 1974). Internal desynchronization into two free-running components was recently described

for *cry*^{b} mutants under constant light conditions, where behavioural rhythm dissociation was related to a ventral/dorsal brain PER expression dissociation (Yoshii *et al.*, 2004). In contrast to previous observations (Grima *et al.*, 2004; Stoleru *et al.*, 2004), it was recently suggested that the *s*-LN$_{v}$ cells control not only the morning but also the evening activity bout whereas the dLN would govern only the evening oscillator. Moreover, these neural groups were shown to respond differently to light and to be completely desynchronized from one another by constant light in flies missing the compound eyes, where the evening component showed a longer period and the morning component showed a short period (Rieger *et al.*, 2006). Here, a complex pattern of activity was observed in *act*-GAL4/UAS-*tint3* and *act*-GAL4/UAS-*tint1y* male flies. It was recently shown that the morning oscillator can determine the clock pace of other neuron groups including the evening cells (Stoleru *et al.*, 2005). However, flies with very low levels of TGO exposed to a constant darkness (DD) conditions showed a static evening peak whereas the morning peak was shown to move with a longer period (Figure 5.8). The results indicate that, without TGO, the intrinsic clock program maintained by evening cells is no longer able to receive a resetting signal from the morning cells. Interestingly, under 12:12h LD, these same flies show a morning peak that is synchronised with the lights-on signal but with a double bout of activity in the evening peak, one corresponding to the lights-off signal and the other occurring later, during the dark phase (Figure 5.5). Taken together, these results suggest that, under LD conditions, the morning cells can be entrained by the LD signal. However, the evening cells could entrain only partially, suggesting that an entraining signal from the morning cells is also normally required. Under DD conditions, the morning cells showed a longer rhythm but were unable to pass on this information to the evening cells, which continued to cycle at the normal pace. The data suggested that TGO is an essential key regulatory molecule that keeps the crosstalk between the morning and evening oscillators. There are two possibilities that could explain the results observed; either TGO acts on relay stations that connect the morning and evening

oscillators, or TGO affects the morning cells (*s*-LN_vs), through some of the DNs where it seems to be expressed (Figure 3.1). Additional immunohistochemistry experiments are required to clear this issue.

7.4 Behavioral analysis of sleep

The genetic tools available allowed the investigation of TGO up and down-regulation on the sleep regulation processes. Under a 12: 12h LD conditions, experimental flies showed a small, but significant, reduction in sleep amount when compared to the controls (Table 6-1). Mushroom bodies are known to be a critical region for promoting and regulating sleep in *Drosophila* (Joiner *et al.*, 2006; Pitman *et al.*, 2006). Because *tim* is not expressed in the mushroom bodies (Kaneko and Hall, 2000), it is likely that *tim*-GAL4 has not driven TGO miss-expression in those cells. This fact strongly suggests that *tim* expressing neurons, which directly or indirectly interact with the mushrooms bodies, might be involved in the control of sleep processes. An interaction between sleep and circadian processes has been described in *Drosophila*, zebrafish, and mouse (Franken *et al.*, 2000; Hendricks *et al.*, 2001; Naylor *et al.*, 2000; Shaw *et al.*, 2002; Zhdanova *et al.*, 2001). Sleep may have evolved from the circadian cycle of rest and activity; however it may have become advantageous to uncouple sleep need from circadian control (Shaw, 2003). Given the pleiotropic nature of clock genes, it is predicted that some of the clock components will play a clock-independent role in sleep processes. In this thesis, TGO has been shown to be involved in the light regulation of the circadian clock and in the cellular crosstalk among circadian neurons (See Chapter 4 and 5) and therefore its involvement in sleep regulation might not be surprising.

A word of caution has to be given based on traditional studies whose results show that environmental stimuli can have a profound effect on sleep (reviewed in Shaw and Franken,

2003). A given mutation may produce subtle changes in several environmental signalling pathways such as temperature, light or auditory stimuli that could result in sleep disruptions. Consequently, the involvement of this mutation on sleep regulation may or may not be direct. This fact is particularly more important when analysing PAS proteins, naturally involved in environmental signalling pathways. Finally, even if the particular mutation plays a more causal role, some problems of interpretation will persist. As an example, the TGO miss-expression flies could be awake either because they do not need to sleep or just because they can not sleep.

It is currently unexplained why both down- and up-regulation of TGO generates the same phenotypic effects on light response processes and on reduction of sleep. One possible explanation lies in the nature of TGO as a heterodimeric transcription factors that requires physical interaction with a specific bHLH partner to be able to move inside the nucleus and activate transcription (Crews *et al.*, 1988; Emmons *et al.*, 1999 Ward *et al.*, 1998). Perhaps, at very high levels, TGO is able to homodimerise in the same way as its mammalian homologue ARNT does (Sogawa *et al.*, 1995; Sonnenefeld *et al.*, 1997; Swanson *et al.*, 1995). However, unlike its mammalian counterpart, TGO would not be functionally active. Alternatively, the possibility exists that TGO, either as a homo or a heterodimer, could drive the overexpression of downstream genes setting in motion a negative feedback resulting in the down-regulation of its own interacting partners. Under both scenarios overexpression of TGO would ultimately result in down-regulation of its function.

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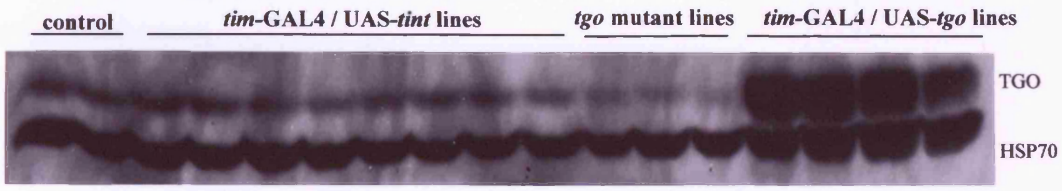
Appendix 1: Analysis of TGO in adult flies miss-expressing the protein and control



Appendix 2: Entrained locomotor activity rhythms in *D. melanogaster* overexpressing TGO and controls under a 12:12 LD cycle

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: period

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.009[a]	2	.004	.245	.783
Intercept	97209.131	1	97209.131	5446741	.000
genotype	.009	2	.004	.245	.783
Error	3.123	175	.018		
Total	103042.488	178			
Corrected Total	3.132	177			

a. R Squared = .003 (Adjusted R Squared = -.009)

Appendix 3: Evening peak of locomotor activity in *D. melanogaster* overexpressing TGO and controls under 12:12 LD, 16: 8 LD and 20: 4 LD cycles

1) 12: 12 LD

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: peak

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	104.712[a]	2	52.356	8.702	.001
Intercept	21074.713	1	21074.713	3502.706	.000
genotype	104.712	2	52.356	8.702	.001
Error	300.835	50	6.017		
Total	22291.000	53			
Corrected Total	405.547	52			

a. R Squared = .258 (Adjusted R Squared = .229)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: peak

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	3.00	-.3529	.86893	1.000	-2.5054	1.7996
	4.00	-3.0476*	.82923	.002	-5.1018	-.9935
3.00	1.00	.3529	.86893	1.000	-1.7996	2.5054
	4.00	-2.6947*	.80027	.004	-4.6771	-.7122
4.00	1.00	3.0476*	.82923	.002	.9935	5.1018
	3.00	2.6947*	.80027	.004	.7122	4.6771

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 1: *w*; +/+; UAS-*tgo25.1*/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 4: *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

2) 16: 8 LD

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: peak

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	77.882[a]	2	38.941	8.572	.001
Intercept	18806.420	1	18806.420	4139.733	.000
genotype	77.882	2	38.941	8.572	.001
Error	204.431	45	4.543		
Total	20087.000	48			
Corrected Total	282.313	47			

a. R Squared = .276 (Adjusted R Squared = .244)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: peak

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	3	.5385	.80766	1.000	-1.4700	2.5469
	4	-2.2615*	.75934	.014	-4.1499	-.3732
3	1	-.5385	.80766	1.000	-2.5469	1.4700
	4	-2.8000*	.72801	.001	-4.6104	-.9896
4	1	2.2615*	.75934	.014	.3732	4.1499
	3	2.8000*	.72801	.001	.9896	4.6104

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 1: *w*; +/+; UAS-*tgo25.1*/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 4: *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

3) 20: 4 LD

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: peak

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	123.750[a]	2	61.875	4.678	.015
Intercept	6584.257	1	6584.257	497.839	.000
genotype	123.750	2	61.875	4.678	.015
Error	489.350	37	13.226		
Total	7742.000	40			
Corrected Total	613.100	39			

a. R Squared = .202 (Adjusted R Squared = .159)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: peak

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	3.00	1.6000	1.53337	.911	-2.2453	5.4453
	4.00	-2.3750	1.51530	.377	-6.1750	1.4250
3.00	1.00	-1.6000	1.53337	.911	-5.4453	2.2453
	4.00	-3.9750*	1.30703	.013	-7.2527	-.6973
4.00	1.00	2.3750	1.51530	.377	-1.4250	6.1750
	3.00	3.9750*	1.30703	.013	.6973	7.2527

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 1: *w*; +/+; UAS-*tgo25.1*/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 4: *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

Appendix 4: Entrained locomotor activity rhythms in *D. melanogaster* overexpressing TGO and controls under 12:12 LD, 16: 8 LD and 20: 4 LD cycles

1) 12:12 LD

- Kruskal-Wallis test

Ranks

	genotype	N	Mean Rank
period	25.1 x w	33	74.74
	TG4 x W	34	50.99
	TG4 x 25.1	44	45.82
	Total	111	

Test Statistics[a,b]

	period
Chi-Square	17.891
df	2
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: genotype

- Mann-Whitney pair wise comparison test

a) *w*; +/+; UAS-*tgo25.1*/+ x *w*; *tim*-GAL4/+; +/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	25.1 x w	33	41.56	1371.50
	TG4 x W	34	26.66	906.50
	Total	67		

Test Statistics[a]

	period
Mann-Whitney U	311.500
Wilcoxon W	906.500
Z	-3.231
Asymp. Sig. (2-tailed)	.001

a. Grouping Variable: genotype

b) *w*; +/+; UAS-*tgo25.1*/+ x *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	25.1 x w	33	50.18	1656.00
	TG4 x 25.1	44	30.61	1347.00
	Total	77		

Test Statistics[a]

	period
Mann-Whitney U	357.000
Wilcoxon W	1347.000
Z	-3.911
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: genotype

c) *w*; *tim*-GAL4/+; +/+ x *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	TG4 x W	34	41.82	1422.00
	TG4 x 25.1	44	37.70	1659.00
	Total	78		

Test Statistics[a]

	period
Mann-Whitney U	669.000
Wilcoxon W	1659.000
Z	-.863
Asymp. Sig. (2-tailed)	.388

a. Grouping Variable: genotype

2) 16: 8 LD

1. One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: period

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.354[a]	4	.088	1.744	.149
Intercept	46297.980	1	46297.980	912774.8	.000
genotype	.354	4	.088	1.744	.149
Error	3.906	77	.051		
Total	47959.487	82			
Corrected Total	4.260	81			

a. R Squared = .083 (Adjusted R Squared = .035)

3) 20: 4 LD

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: period

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.706[a]	4	.176	1.710	.157
Intercept	41197.229	1	41197.229	399284.8	.000
genotype	.706	4	.176	1.710	.157
Error	7.429	72	.103		
Total	44206.189	77			
Corrected Total	8.135	76			

a. R Squared = .087 (Adjusted R Squared = .036)

Appendix 5: Free-running locomotor activity rhythms in *D. melanogaster* overexpressing TGO and controls

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: period

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.645[a]	2	.822	2.046	.131
Intercept	194449.163	1	194449.163	483795.6	.000
genotype	1.645	2	.822	2.046	.131
Error	128.214	319	.402		
Total	196057.028	322			
Corrected Total	129.859	321			

a. R Squared = .013 (Adjusted R Squared = .006)

Appendix 6: Phase shift after a 5 min light pulse at ZT13, 15 and 21 in *D. melanogaster* overexpressing TGO and controls

1) ZT 13

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: shift

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	100.239[a]	2	50.119	5.644	.005
Intercept	309.540	1	309.540	34.857	.000
genotype	100.239	2	50.119	5.644	.005
Error	586.107	66	8.880		
Total	1049.105	69			
Corrected Total	686.345	68			

a. R Squared = .146 (Adjusted R Squared = .120)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: shift

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	3.00	.3345	.92385	1.000	-1.9349	2.6040
	4.00	-2.3039*	.84558	.025	-4.3811	-.2267
3.00	1.00	-.3345	.92385	1.000	-2.6040	1.9349
	4.00	-2.6385*	.89235	.013	-4.8306	-.4464
4.00	1.00	2.3039*	.84558	.025	.2267	4.3811
	3.00	2.6385*	.89235	.013	.4464	4.8306

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 1: *w*; +/+; UAS-*tgo25.1*/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 4: *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

2) ZT 15

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: shift

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	520.031[a]	2	260.016	37.958	.000
Intercept	6676.132	1	6676.132	974.605	.000
genotype	520.031	2	260.016	37.958	.000
Error	732.959	107	6.850		
Total	7609.935	110			
Corrected Total	1252.991	109			

a. R Squared = .415 (Adjusted R Squared = .404)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: shift

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	3.00	.7585	.58689	.597	-.6690	2.1859
	4.00	-4.4773*	.62261	.000	-5.9916	-2.9630
3.00	1.00	-.7585	.58689	.597	-2.1859	.6690
	4.00	-5.2358*	.64302	.000	-6.7997	-3.6718
4.00	1.00	4.4773*	.62261	.000	2.9630	5.9916
	3.00	5.2358*	.64302	.000	3.6718	6.7997

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 1: *w*; +/+; UAS-*tgo25.1*/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 4: *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

3) ZT21

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: shift

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	19.334[a]	2	9.667	1.227	.296
Intercept	3219.398	1	3219.398	408.580	.000
genotype	19.334	2	9.667	1.227	.296
Error	1158.285	147	7.879		
Total	4464.664	150			
Corrected Total	1177.619	149			

a. R Squared = .016 (Adjusted R Squared = .003)

Appendix 7: Molecular cycling of TIM in *D. melanogaster* overexpressing TGO and control

- Two-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: TIMquant

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.701[a]	13	.285	5.996	.000
Intercept	25.213	1	25.213	530.919	.000
Genotype	.002	1	.002	.044	.834
ZTcollection	3.581	6	.597	12.568	.000
Genotype * ZTcollectior	.120	6	.020	.421	.862
Error	2.754	58	.047		
Total	33.168	72			
Corrected Total	6.456	71			

a. R Squared = .573 (Adjusted R Squared = .478)

Appendix 8: Entrained locomotor activity rhythms in *D. melanogaster* down-regulating TGO (*tim*-GAL4 driver and *tgo* mutants) and controls under a 12:12 LD cycle

- Kruskal-Wallis test

Ranks

	genotype	N	Mean Rank
period	25.1 x w	41	200.74
	TinT3 x w	42	158.07
	TG4 x w	68	211.57
	TG4 x 25.1	69	201.70
	TG4 x TinT3	89	215.10
	TGO1 x x	61	187.97
	TGO5 x w	34	236.09
	Total	404	

Test Statistics[a,b]

	period
Chi-Square	11.379
df	6
Asymp. Sig.	.077

a. Kruskal Wallis Test

b. Grouping Variable: genotype

Appendix 9: Entrained locomotor activity rhythms in *D. melanogaster* down-regulating TGO (*act*-GAL4 driver) and controls under a 12:12 LD cycle

- Kruskal-Wallis test

Ranks

	genotype	N	Mean Rank
period	1	47	138.21
	2	48	113.20
	3	53	122.60
	4	60	141.73
	5	54	139.30
	Total	262	

Test Statistics[a,b]

	period
Chi-Square	5.818
df	4
Asymp. Sig.	.213

a. Kruskal Wallis Test

b. Grouping Variable: genotype

Appendix 10: Evening peak of locomotor activity in *D. melanogaster* down-regulating TGO and controls under 12:12 LD, 16: 8 LD and 20: 4 LD cycles

1) 12: 12 LD

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: peak

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	343.251[a]	2	171.626	28.999	.000
Intercept	23220.183	1	23220.183	3923.424	.000
genotype	343.251	2	171.626	28.999	.000
Error	319.591	54	5.918		
Total	25674.000	57			
Corrected Total	662.842	56			

a. R Squared = .518 (Adjusted R Squared = .500)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: peak

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2.00	3.00	-.9779	.84737	.761	-3.0717	1.1158
	5.00	-5.4167*	.78517	.000	-7.3567	-3.4766
3.00	2.00	.9779	.84737	.761	-1.1158	3.0717
	5.00	-4.4387*	.77119	.000	-6.3442	-2.5332
5.00	2.00	5.4167*	.78517	.000	3.4766	7.3567
	3.00	4.4387*	.77119	.000	2.5332	6.3442

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 2: *w*; UAS-*tint3*/+; +/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 5: *w*; *tim*-GAL4/UAS-*tint3*; +/+

2) 16: 8 LD

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: peak

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	201.718[a]	2	100.859	18.328	.000
Intercept	22759.904	1	22759.904	4135.921	.000
Genotype	201.718	2	100.859	18.328	.000
Error	280.652	51	5.503		
Total	24888.000	54			
Corrected Total	482.370	53			

a. R Squared = .418 (Adjusted R Squared = .395)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: peak

Bonferroni

(I) Genotype	(J) Genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2.00	3.00	1.2500	.84309	.433	-.8371	3.3371
	5.00	-3.1848*	.76367	.000	-5.0753	-1.2943
3.00	2.00	-1.2500	.84309	.433	-3.3371	.8371
	5.00	-4.4348*	.77854	.000	-6.3621	-2.5075
5.00	2.00	3.1848*	.76367	.000	1.2943	5.0753
	3.00	4.4348*	.77854	.000	2.5075	6.3621

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 2: *w*; UAS-*tint3*/+; +/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 5: *w*; *tim*-GAL4/UAS-*tint3*; +/+

3) 20: 4 LD

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: peak

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	283.156[a]	2	141.578	14.171	.000
Intercept	10586.524	1	10586.524	1059.636	.000
genotype	283.156	2	141.578	14.171	.000
Error	469.564	47	9.991		
Total	11764.000	50			
Corrected Total	752.720	49			

a. R Squared = .376 (Adjusted R Squared = .350)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: peak

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2.00	3.00	3.9125*	1.13599	.004	1.0922	6.7328
	5.00	-1.8454	1.07250	.276	-4.5081	.8173
3.00	2.00	-3.9125*	1.13599	.004	-6.7328	-1.0922
	5.00	-5.7579*	1.09173	.000	-8.4683	-3.0475
5.00	2.00	1.8454	1.07250	.276	-.8173	4.5081
	3.00	5.7579*	1.09173	.000	3.0475	8.4683

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 2: *w*; UAS-*tint3*/+; +/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 5: *w*; *tim*-GAL4/UAS-*tint3*; +/+

Appendix 11: Entrained locomotor activity rhythms in *D. melanogaster* down-regulating TGO and controls under 12:12 LD, 16: 8 LD and 20: 4 LD cycles

1) 12:12 LD

- Kruskal-Wallis test

Ranks

	genotype	N	Mean Rank
period	Tint3 x w	29	77.55
	TG4 x W	34	47.94
	TG4 x TinT3	50	51.24
	Total	113	

Test Statistics[a,b]

	period
Chi-Square	17.584
df	2
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: genotype

- Mann-Whitney pair wise comparison test

a) *w*; UAS-*tint3*/+; +/+ x *w*; *tim*-GAL4/+; +/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	Tint3 x w	29	40.93	1187.00
	TG4 x W	34	24.38	829.00
	Total	63		

Test Statistics[a]

	period
Mann-Whitney U	234.000
Wilcoxon W	829.000
Z	-3.746
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: genotype

b) *w*; UAS-*tint3*/+; +/+ x *w; tim*-GAL4/UAS-*tint3;* +/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	Tint3 x w	29	51.62	1497.00
	TG4 x TinT3	50	33.26	1663.00
	Total	79		

Test Statistics[a]

	period
Mann-Whitney U	388.000
Wilcoxon W	1663.000
Z	-3.610
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: genotype

c) *w*; *tim*-GAL4/+; +/+ x *w; tim*-GAL4/UAS-*tint3;* +/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	TG4 x W	34	41.06	1396.00
	TG4 x TinT3	50	43.48	2174.00
	Total	84		

Test Statistics[a]

	period
Mann-Whitney U	801.000
Wilcoxon W	1396.000
Z	-.492
Asymp. Sig. (2-tailed)	.623

a. Grouping Variable: genotype

2) 16: 8 LD

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: period

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.354[a]	4	.088	1.744	.149
Intercept	46297.980	1	46297.980	912774.8	.000
genotype	.354	4	.088	1.744	.149
Error	3.906	77	.051		
Total	47959.487	82			
Corrected Total	4.260	81			

a. R Squared = .083 (Adjusted R Squared = .035)

3) 20: 4 LD

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: period

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.706[a]	4	.176	1.710	.157
Intercept	41197.229	1	41197.229	399284.8	.000
genotype	.706	4	.176	1.710	.157
Error	7.429	72	.103		
Total	44206.189	77			
Corrected Total	8.135	76			

a. R Squared = .087 (Adjusted R Squared = .036)

Appendix 12: Free running locomotor activity rhythms in *D. melanogaster* down-regulating TGO (*tim*-GAL4 driver and *tgo* mutants) and controls

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: period

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	33.929[a]	6	5.655	14.549	.000
Intercept	305659.154	1	305659.154	786412.1	.000
genotype	33.929	6	5.655	14.549	.000
Error	233.205	600	.389		
Total	364614.965	607			
Corrected Total	267.135	606			

a. R Squared = .127 (Adjusted R Squared = .118)

- Bonferroni *Pos-Hoc* multiple comparisons test

Genotype 1: *w*; +/+; UAS-*tgo25.1*/+

Genotype 2: *w*; UAS-*tint3*/+; +/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 4: *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

Genotype 5: *w; tim*-GAL4/UAS-*tint3; +/+*

Genotype 6: *w; +/ +; tgo^{1}/ +*

Genotype 7: *w; +/ +; tgo^{5}/ +*

Multiple Comparisons

Dependent Variable: period

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.4840*	.09639	.000	.1899	.7781
	3.00	-.1583	.08777	1.000	-.4261	.1095
	4.00	-.1526	.08488	1.000	-.4115	.1064
	5.00	-.0029	.08537	1.000	-.2633	.2576
	6.00	.3535*	.10220	.012	.0417	.6653
	7.00	.4163*	.12276	.016	.0418	.7909
2.00	1.00	-.4840*	.09639	.000	-.7781	-.1899
	3.00	-.6423*	.09519	.000	-.9327	-.3519
	4.00	-.6365*	.09254	.000	-.9189	-.3542
	5.00	-.4868*	.09299	.000	-.7706	-.2031
	6.00	-.1305	.10864	1.000	-.4619	.2010
	7.00	-.0677	.12818	1.000	-.4587	.3234
3.00	1.00	.1583	.08777	1.000	-.1095	.4261
	2.00	.6423*	.09519	.000	.3519	.9327
	4.00	.0057	.08352	1.000	-.2491	.2606
	5.00	.1554	.08402	1.000	-.1009	.4118
	6.00	.5118*	.10107	.000	.2035	.8202
	7.00	.5746*	.12183	.000	.2029	.9464
4.00	1.00	.1526	.08488	1.000	-.1064	.4115
	2.00	.6365*	.09254	.000	.3542	.9189
	3.00	-.0057	.08352	1.000	-.2606	.2491
	5.00	.1497	.08100	1.000	-.0974	.3968
	6.00	.5061*	.09857	.000	.2053	.8068
	7.00	.5689*	.11977	.000	.2035	.9343
5.00	1.00	.0029	.08537	1.000	-.2576	.2633
	2.00	.4868*	.09299	.000	.2031	.7706
	3.00	-.1554	.08402	1.000	-.4118	.1009
	4.00	-.1497	.08100	1.000	-.3968	.0974
	6.00	.3564*	.09899	.007	.0543	.6584
	7.00	.4192*	.12011	.011	.0527	.7857
6.00	1.00	-.3535*	.10220	.012	-.6653	-.0417
	2.00	.1305	.10864	1.000	-.2010	.4619
	3.00	-.5118*	.10107	.000	-.8202	-.2035
	4.00	-.5061*	.09857	.000	-.8068	-.2053
	5.00	-.3564*	.09899	.007	-.6584	-.0543
	7.00	.0628	.13260	1.000	-.3418	.4674
7.00	1.00	-.4163*	.12276	.016	-.7909	-.0418
	2.00	.0677	.12818	1.000	-.3234	.4587
	3.00	-.5746*	.12183	.000	-.9464	-.2029
	4.00	-.5689*	.11977	.000	-.9343	-.2035
	5.00	-.4192*	.12011	.011	-.7857	-.0527
	6.00	-.0628	.13260	1.000	-.4674	.3418

Based on observed means.

*. The mean difference is significant at the .05 level.

Appendix 13: Free running locomotor activity rhythms in *D. melanogaster* down-regulating TGO (*act*-GAL4 driver) and controls

- Kruskal-Wallis test

Ranks

	group	N	Mean Rank
period	1	44	77.65
	2	46	52.92
	3	50	92.38
	4	22	144.07
	5	21	152.21
	Total	183	

Test Statistics[a,b]

	period
Chi-Square	76.895
df	4
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: group

- Mann-Whitney pair wise comparison test

a) *w*; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80^{TS}/+ x *w*; UAS-*tint1y*/+; +/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	w; UAS-tint1y/ +; +/ +	44	24.07	1059.00
	w; act- GAL4/ UAS-tint1y ; tubGAL80TS/ +	22	52.36	1152.00
	Total	66		

Test Statistics[a]

	period
Mann-Whitney U	69.000
Wilcoxon W	1059.000
Z	-5.668
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: genotype

b) *w*; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80^{TS}/+ x *w*; *act*-GAL4/+; *tub*-GAL80^{TS}/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	w; act-GAL4/ UAS-tint1y ; tubGAL80TS/ +	22	51.39	1130.50
	w; act-GAL4/ +; tubGAL80TS/ +	50	29.95	1497.50
	Total	72		

Test Statistics[a]

	period
Mann-Whitney U	222.500
Wilcoxon W	1497.500
Z	-4.012
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: genotype

c) *w*; UAS-*tint1y*/+; +/+ x *w*; *act*-GAL4/+; *tub*-GAL80^{TS}/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	w; act-GAL4/ +; tubGAL80TS/ +	50	51.05	2552.50
	w; UAS-tint1y/ +; +/ +	44	43.47	1912.50
	Total	94		

Test Statistics[a]

	period
Mann-Whitney U	922.500
Wilcoxon W	1912.500
Z	-1.348
Asymp. Sig. (2-tailed)	.178

a. Grouping Variable: genotype

d) *w*; *act*-GAL4/UAS-*tint3*; *tub*-GAL80^{TS}/+ x *w*; UAS-*tint3*/+; +/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	w; act-GAL4/ UAS-tint3 ; tubGAL80TS/ +	21	55.05	1156.00
	w; UAS-tint3/ +; +/ +	46	24.39	1122.00
	Total	67		

Test Statistics[a]

	period
Mann-Whitney U	41.000
Wilcoxon W	1122.000
Z	-5.986
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: genotype

e) *w*; *act*-GAL4/UAS-*tint3*; *tub*-GAL80^{TS}/+ x *w*; *act*-GAL4/+; *tub*-GAL80^{TS}/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	w; act-GAL4/ UAS-tint3 ; tubGAL80TS/ +	21	53.10	1115.00
	w; act-GAL4/ +; tubGAL80TS/ +	50	28.82	1441.00
	Total	71		

Test Statistics[a]

	period
Mann-Whitney U	166.000
Wilcoxon W	1441.000
Z	-4.530
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: genotype

f) *w*; UAS-*tint3*/+; +/+ x *w*; *act*-GAL4/+; *tub*-GAL80^{TS}/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	w; UAS-tint3/ +; +/ +	46	37.02	1703.00
	w; act- GAL4/ +; tubGAL80TS/ +	50	59.06	2953.00
	Total	96		

Test Statistics[a]

	period
Mann-Whitney U	622.000
Wilcoxon W	1703.000
Z	-3.881
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: genotype

g) *w*; *act*-GAL4/UAS-*tint1y*; *tub*-GAL80^{TS}/+ x *w*; *act*-GAL4/UAS-*tint3*; *tub*-GAL80^{TS}/+

Ranks

	genotype	N	Mean Rank	Sum of Ranks
period	w; act- GAL4/ UAS-tint1y ; tubGAL80TS/ +	22	18.95	417.00
	w; act- GAL4/ UAS-tint3 ; tubGAL80TS/ +	21	25.19	529.00
	Total	43		

Test Statistics[a]

	period
Mann-Whitney U	164.000
Wilcoxon W	417.000
Z	-1.635
Asymp. Sig. (2-tailed)	.102

a. Grouping Variable: genotype

Appendix 14: Phase shift after a 5 min light pulse at ZT13, 15 and 21 in *D. melanogaster* down-regulating TGO and controls

1) ZT 13

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: shift

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	57.197[a]	3	19.066	2.899	.041
Intercept	303.041	1	303.041	46.079	.000
genotype	57.197	3	19.066	2.899	.041
Error	486.664	74	6.577		
Total	914.407	78			
Corrected Total	543.861	77			

a. R Squared = .105 (Adjusted R Squared = .069)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: shift

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2.00	3.00	.1796	.90326	1.000	-2.2692	2.6284
	5.00	-1.8185	.84459	.207	-4.1082	.4712
	7.00	-1.1496	.91385	1.000	-3.6271	1.3278
3.00	2.00	-.1796	.90326	1.000	-2.6284	2.2692
	5.00	-1.9981	.76792	.067	-4.0800	.0838
	7.00	-1.3293	.84350	.716	-3.6160	.9575
5.00	2.00	1.8185	.84459	.207	-.4712	4.1082
	3.00	1.9981	.76792	.067	-.0838	4.0800
	7.00	.6688	.78035	1.000	-1.4467	2.7844
7.00	2.00	1.1496	.91385	1.000	-1.3278	3.6271
	3.00	1.3293	.84350	.716	-.9575	3.6160
	5.00	-.6688	.78035	1.000	-2.7844	1.4467

Based on observed means.

Genotype 2: *w;* UAS-*tint3*/+; +/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 5: *w; tim*-GAL4/UAS-*tint3;* +/+

Genotype 7: *w;* +/+; tgo^5/ +

2) ZT 15

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: shift

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	863.222[a]	6	143.870	22.038	.000
Intercept	16078.311	1	16078.311	2462.910	.000
genotype	863.222	6	143.870	22.038	.000
Error	1462.311	224	6.528		
Total	18184.644	231			
Corrected Total	2325.534	230			

a. R Squared = .371 (Adjusted R Squared = .354)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: shift

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.2919	.56498	1.000	-2.0282	1.4444
	3.00	.7585	.57294	1.000	-1.0022	2.5192
	4.00	-4.4773*	.60780	.000	-6.3451	-2.6094
	5.00	-2.6668*	.58167	.000	-4.4543	-.8793
	6.00	-3.4166*	.70385	.000	-5.5796	-1.2536
	7.00	-3.7570*	.62046	.000	-5.6637	-1.8503
2.00	1.00	.2919	.56498	1.000	-1.4444	2.0282
	3.00	1.0504	.58637	1.000	-.7516	2.8524
	4.00	-4.1854*	.62048	.000	-6.0922	-2.2786
	5.00	-2.3749*	.59490	.002	-4.2031	-.5467
	6.00	-3.1247*	.71483	.000	-5.3214	-.9279
	7.00	-3.4651*	.63288	.000	-5.4100	-1.5202
3.00	1.00	-.7585	.57294	1.000	-2.5192	1.0022
	2.00	-1.0504	.58637	1.000	-2.8524	.7516
	4.00	-5.2358*	.62773	.000	-7.1649	-3.3067
	5.00	-3.4253*	.60246	.000	-5.2767	-1.5739
	6.00	-4.1751*	.72113	.000	-6.3912	-1.9589
	7.00	-4.5155*	.63999	.000	-6.4822	-2.5487
4.00	1.00	4.4773*	.60780	.000	2.6094	6.3451
	2.00	4.1854*	.62048	.000	2.2786	6.0922
	3.00	5.2358*	.62773	.000	3.3067	7.1649
	5.00	1.8105	.63571	.101	-.1431	3.7641
	6.00	1.0607	.74913	1.000	-1.2415	3.3629
	7.00	.7203	.67138	1.000	-1.3430	2.7835
5.00	1.00	2.6668*	.58167	.000	.8793	4.4543
	2.00	2.3749*	.59490	.002	.5467	4.2031
	3.00	3.4253*	.60246	.000	1.5739	5.2767
	4.00	-1.8105	.63571	.101	-3.7641	.1431
	6.00	-.7498	.72808	1.000	-2.9873	1.4877
	7.00	-1.0902	.64782	1.000	-3.0810	.9006
6.00	1.00	3.4166*	.70385	.000	1.2536	5.5796
	2.00	3.1247*	.71483	.000	.9279	5.3214
	3.00	4.1751*	.72113	.000	1.9589	6.3912
	4.00	-1.0607	.74913	1.000	-3.3629	1.2415
	5.00	.7498	.72808	1.000	-1.4877	2.9873
	7.00	-.3404	.75943	1.000	-2.6742	1.9934
7.00	1.00	3.7570*	.62046	.000	1.8503	5.6637
	2.00	3.4651*	.63288	.000	1.5202	5.4100
	3.00	4.5155*	.63999	.000	2.5487	6.4822
	4.00	-.7203	.67138	1.000	-2.7835	1.3430
	5.00	1.0902	.64782	1.000	-.9006	3.0810
	6.00	.3404	.75943	1.000	-1.9934	2.6742

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 1: *w*; +/+; UAS-*tgo25.1*/+

Genotype 2: *w;* UAS-*tint3*/+; +/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 4: *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

Genotype 5: *w; tim*-GAL4/UAS-*tint3; +/+*

Genotype 6: *w; +/+; tgo*^{1}*/+*

Genotype7: *w; +/+; tgo*^{5}*/ +*

3) ZT21

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: shift

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	65.755[a]	5	13.151	1.553	.174
Intercept	5323.775	1	5323.775	628.727	.000
genotype	65.755	5	13.151	1.553	.174
Error	2311.639	273	8.468		
Total	7899.002	279			
Corrected Total	2377.394	278			

a. R Squared = .028 (Adjusted R Squared = .010)

Appendix 15: Molecular cycling of TIM in *D. melanogaster* down-regulating TGO and control

- Two-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: TIMquant

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.916[a]	13	.301	7.094	.000
Intercept	29.058	1	29.058	684.368	.000
Genotype	.034	1	.034	.805	.373
ZTcollection	3.612	6	.602	14.178	.000
Genotype * ZTcollection	.294	6	.049	1.153	.342
Error	2.760	65	.042		
Total	37.105	79			
Corrected Total	6.676	78			

a. R Squared = .587 (Adjusted R Squared = .504)

Appendix 16: Time spent asleep during a 24 h period in *D. melanogaster* overexpressing TGO and controls

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: sleep

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.813[a]	4	.203	24.440	.000
Intercept	168.442	1	168.442	20265.282	.000
genotype	.813	4	.203	24.440	.000
Error	3.158	380	.008		
Total	201.965	385			
Corrected Total	3.971	384			

a. R Squared = .205 (Adjusted R Squared = .196)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: sleep

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	3.00	.07046*	.015108	.000	.02780	.11312
	4.00	.13254*	.016113	.000	.08704	.17803
	6.00	.01378	.016736	1.000	-.03347	.06104
	8.00	.06513*	.019477	.009	.01013	.12012
3.00	1.00	-.07046*	.015108	.000	-.11312	-.02780
	4.00	.06208*	.012467	.000	.02688	.09728
	6.00	-.05668*	.013262	.000	-.09413	-.01923
	8.00	-.00533	.016588	1.000	-.05217	.04151
4.00	1.00	-.13254*	.016113	.000	-.17803	-.08704
	3.00	-.06208*	.012467	.000	-.09728	-.02688
	6.00	-.11876*	.014396	.000	-.15940	-.07811
	8.00	-.06741*	.017508	.001	-.11684	-.01798
6.00	1.00	-.01378	.016736	1.000	-.06104	.03347
	3.00	.05668*	.013262	.000	.01923	.09413
	4.00	.11876*	.014396	.000	.07811	.15940
	8.00	.05135*	.018083	.048	.00029	.10241
8.00	1.00	-.06513*	.019477	.009	-.12012	-.01013
	3.00	.00533	.016588	1.000	-.04151	.05217
	4.00	.06741*	.017508	.001	.01798	.11684
	6.00	-.05135*	.018083	.048	-.10241	-.00029

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 1: *w*; +/+; UAS-*tgo25.1*/+

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 4: *w*; *tim*-GAL4/+; UAS-*tgo25.1*/+

Genotype 6: UAS-*tgo52.1*;+/+; +/ +

Genotype 8: UAS-*tgo52.1; tim*-GAL4/+; +/ +

Appendix 17: Time spent asleep during a 24 h period in *D. melanogaster* down-regulating TGO and controls

- One-way ANOVA test

Tests of Between-Subjects Effects

Dependent Variable: sleep

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.584[a]	4	.146	21.010	.000
Intercept	151.337	1	151.337	21789.926	.000
genotype	.584	4	.146	21.010	.000
Error	2.493	359	.007		
Total	184.379	364			
Corrected Total	3.077	363			

a. R Squared = .190 (Adjusted R Squared = .181)

- Bonferroni *Pos-Hoc* multiple comparisons test

Multiple Comparisons

Dependent Variable: sleep

Bonferroni

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2.00	3.00	.0914*	.01600	.000	.0462	.1366
	5.00	.1267*	.01764	.000	.0769	.1765
	7.00	.0647*	.01868	.006	.0120	.1175
	9.00	.1398*	.01697	.000	.0919	.1878
3.00	2.00	-.0914*	.01600	.000	-.1366	-.0462
	5.00	.0353	.01260	.054	-.0003	.0709
	7.00	-.0267	.01402	.579	-.0663	.0129
	9.00	.0484*	.01164	.000	.0156	.0813
5.00	2.00	-.1267*	.01764	.000	-.1765	-.0769
	3.00	-.0353	.01260	.054	-.0709	.0003
	7.00	-.0620*	.01586	.001	-.1068	-.0172
	9.00	.0131	.01380	1.000	-.0258	.0521
7.00	2.00	-.0647*	.01868	.006	-.1175	-.0120
	3.00	.0267	.01402	.579	-.0129	.0663
	5.00	.0620*	.01586	.001	.0172	.1068
	9.00	.0751*	.01511	.000	.0324	.1178
9.00	2.00	-.1398*	.01697	.000	-.1878	-.0919
	3.00	-.0484*	.01164	.000	-.0813	-.0156
	5.00	-.0131	.01380	1.000	-.0521	.0258
	7.00	-.0751*	.01511	.000	-.1178	-.0324

Based on observed means.

*. The mean difference is significant at the .05 level.

Genotype 2: *w;* UAS-*tint*3 /+; +/ +

Genotype 3: *w*; *tim*-GAL4/+; +/+

Genotype 5: *w; tim*-GAL4/UAS-*tint*3; +/ +

Genotype 7: *w;* UAS-*tint1y*/ +; +/ +

Genotype 9: *w; tim*-GAL4/UAS-*tint*1y; +/ +