Isolation of new genes involved in temperature synchronization of the circadian clock of

*Drosophila melanogaster*

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of the requirements of the University of London

for the Degree of *Philosophiae Doctor* (Ph.D.)

September 2010
I certify that this thesis, and the research to which it refers, are the product of my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline. I acknowledge the helpful guidance and support of my supervisor, Professor Ralf Stanewsky.

September 2010

Alekos Simoni
Circadian clocks regulate behaviour and physiology of many organisms and keep them in synchrony with the environment. *Drosophila*’s circadian clock is mainly synchronized by natural light-dark cycles and temperature fluctuations, both at molecular and behavioural levels. The mechanisms underlying temperature entrainment are poorly understood, but previous studies have shown that this process can be genetically dissected.

In this work, I isolated several mutants which interfere with the temperature synchronization of *Drosophila*’s circadian clock. Three variants were isolated in a chemical EMS-mutagenesis screen monitoring putative second- and third-chromosomal mutations. The mutants behave normal in light-dark cycles suggesting that they specifically interfere with temperature entrainment. In a different, RNAi-based screen, a Forkhead-domain transcription factor encoding gene was isolated, which shows defective circadian activity of *per* expression and PER accumulation in temperature-entrainment condition, when down-regulated. Finally, a candidate approach led me to identify three genes encoding proteins belonging to the TRP family of ion channels. Mutations in the *pyrexia*, *trpM* and *trpA1* genes show abnormal temperature synchronization of locomotor behaviour, similar to our EMS-candidates.

The isolation and analysis of those mutations are described, as well as a behavioural analysis of the already-known “temperature-mutant” *nocte*. In particular, I discuss the involvement of chordotonal organs as structures required for temperature entrainment of the clock and the role of *nocte* for signalling the temperature information from the periphery to the brain.

The rest-activity pattern is a well-studied circadian output behaviour; the pupal emergence, named eclosion, is another behaviour strictly regulated by the circa-
dian clock. Here we show that genes important for entrainment of adult locomotor behaviour to temperature do not play the same role in regulating the synchronization of eclosion. To gain insight into the synchronization mechanisms of eclosion, I studied how different entrainment conditions affect the phase and free-running period of eclosion.
Ai miei amati genitori,
che anche da lontano
mi siete sempre stati vicini.
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Sara who did not want to play.

Vahid for showing me the other side of the world.

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The Football World Cup, for setting the edges of my Ph.D.
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Abbreviations

♂  male
♀  virgin female
20E 20-hydroxyecdysone peptide
aME Accessory medulla
bHLH basic helix-loop-helix
BO Bolwig’s Organ
CCAP Crustacean cardioactive peptide
ch chordotonal
ck1ε casein kinase 1ε
ck2 casein kinase 2
clk Clock
CNS Central nervous system
cps count per second
cry cryptochrome

cT  Circadian Time
cwo clockwork orange
cyc  cycle
dbt doubletime
DD Constant Darkness
DMAS Drosophila Activity Monitor System
DN1 Dorsal neuron group 1
DN2 Dorsal neuron group 2
DN3 Dorsal neuron group 3
DNA DeoxyRibonucleic Acid
EH Eclosion hormone
EMS Ethyl Methane-sulfonate
ERG Electroretinogram
es external sensory
ETH Ecdysis-trigger hormone
FFT-NLLS Fast Fourier transform non-linear least squares
gsk3β glicogensytase kinase 3β
HB Hofbauer-Buchner
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>HEK</td>
<td>Human Embryonic Kidney cells</td>
</tr>
<tr>
<td>iav</td>
<td>incative</td>
</tr>
<tr>
<td>JO</td>
<td>Johnston’s Organ</td>
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<tr>
<td>l-LN_v</td>
<td>large-ventrolateral neuron</td>
</tr>
<tr>
<td>LD</td>
<td>Light-Dark cycle</td>
</tr>
<tr>
<td>LL</td>
<td>Constant Light</td>
</tr>
<tr>
<td>LN_d</td>
<td>dorsolateral neuron</td>
</tr>
<tr>
<td>LPN</td>
<td>Lateral posterior neuron</td>
</tr>
<tr>
<td>luc</td>
<td>luciferase</td>
</tr>
<tr>
<td>mya</td>
<td>million years ago</td>
</tr>
<tr>
<td>nan</td>
<td>nanchung</td>
</tr>
<tr>
<td>nocte</td>
<td>no circadian temperature entrainment</td>
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<tr>
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<td>no receptor potential A</td>
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<tr>
<td>PAS</td>
<td>Per-Arnt-Sim, or period - aryl hydrocarbon receptor nuclear translocator - single-minded</td>
</tr>
<tr>
<td>PDF</td>
<td>Pigment dispersing factor</td>
</tr>
<tr>
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<td>Par domain protein 1</td>
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<td>per</td>
<td>period</td>
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<td>Prothoracic Gland</td>
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<td>Pars lateralis</td>
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<td>PNS</td>
<td>Peripheral nervous system</td>
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<td>Posterior optic tract</td>
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<td>protein phosphatase 1</td>
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<td>Prothoracicotropic hormone</td>
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<td>Ring Gland</td>
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<td>RNA interference</td>
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<td>s-LN_v</td>
<td>small-ventrolateral neuron</td>
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<td>SCF</td>
<td>Skp1/Cul1/F-box protein complex</td>
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<td>Standard Error of the Mean</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>sgg</td>
<td>shaggy</td>
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<tr>
<td>slimb</td>
<td>supernumerary limbs</td>
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<tr>
<td>TC</td>
<td>Temperature Cycles</td>
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<tr>
<td>tim</td>
<td>timeless</td>
</tr>
<tr>
<td>trp</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>trpl</td>
<td>trp-like</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
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<td>vrrille/Pdpl site</td>
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<tr>
<td>vri</td>
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<tr>
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<td>Vibration-Silence cycles</td>
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<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
<tr>
<td>wtrw</td>
<td>water witch</td>
</tr>
<tr>
<td>ZT</td>
<td>Zeitgeber Time</td>
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</table>

VDRC Vienna *Drosophila* RNAi Center
There once was a scientist doing an experiment on the reaction of fleas. He had trained a flea to jump on command. The scientist would command the flea “Jump Flea!” and the flea would jump. Then the scientist would proceed to pull off one of the fleas legs with a pair of tweezers and write a comment in his notebook.

The scientist did this many times until the flea had only one leg left. The scientist said “Jump Flea!” and the flea made it’s best effort to jump, which the scientist recorded in his notebook.

After he pulled off its last leg, the scientist commanded the flea to jump, and after repeating the command many times without the flea responding he jotted down in his notebook, “After the flea looses all of his legs it becomes completely deaf.”

“For every behavioural observation, there is an equal and opposite observation.”

Seymour Benzer ¹.

¹Quoted in Mealey-Ferrara et al. (2003)
Chapter 1

Introduction

*Time is an illusion. Lunchtime doubly so.*

Douglas Adams

1.1 Chronobiology and circadian clocks

The first report of the existence of circadian clock dates back to the eighteen century, and an observation of a French astronomer, Jean-Jacques d’Ortus de Mairan (1729). The leaves of the heliotrope *Mimosa pudica* turn to the sun and open during the day and close during the night (hence the name, *helios*=sun, *tropos*=movement). De Mairan observed that the leaf movement was not a passive response to the environment, but persisted when the plant was enclosed in a dark cabinet and not exposed to the daily oscillation of light and dark. De Mairan cleverly concluded that there must have been some endogenous mechanism which was telling the plant the time of the day and he proposed the existence of a “circadian clock” (although the term circadian has been coined only in the 1950s by Franz Halberg, as recalled in the publication Halberg et al., 2003).

Two centuries later the origin of this mysterious mechanism has been revealed
Figure 1.1: The three-component structure of circadian clocks. The components of the “input pathway” receive the cycling information from the environment and transmit them to the “central oscillator”. Rhythmic behaviours are then generated via the “output pathway”.

Circadian clocks are endogenous oscillators shaped by evolution to make the organisms able to perceive time and to be synchronized with the cycling environment in order to anticipate reproducible environmental changes.

Three properties characterize circadian clocks. First, they are endogenous and self-sustainable. The clocks generate rhythms with a period of about 24 hours (circa dies) that persist even in absence of any environmental stimulus. Secondly, the clock can be synchronized (entrained) by cycling environmental conditions in order to be in-phase with the environment. Last, the clocks are temperature compensated, which means that the period is almost the same over a wide range of constant physiological temperatures. This last properties is summarized by the formula \( Q_{10} \approx 1 \). \( Q_{10} \) indicates the rate of change of chemical reactions as a consequence of temperature increase of 10°C \(^1\). For most biochemical reactions, the rate is generally between 2 and 3. For biological clocks, the rate is about 1, which means that circadian systems are temperature compensated (Pittendrigh, 1954).

The simplified structure of a circadian system is based on three components

\(^1\)\( Q_{10} \) is calculated as \( Q_{10} = \left( \frac{R_2}{R_1} \right)^{10/(T_2-T_1)} \), where \( R \) is the rate and \( T \) is the temperature (in Celsius).
(Figure 1.1). The central oscillator is capable of generating sustained circa 24-hour cycles of gene expression and protein accumulation. The central oscillator (or pacemaker) can be entrained by rhythmically changing environmental conditions. The second component that constitutes the circadian clock is the input pathway, which includes the structures arranged to perceive the cycling stimuli in the environment and to transmit these information to the central oscillator. The stimuli able to entrain the circadian clock are called Zeitgeber (German Zeit=time and Geber=giver). For Drosophila, the main Zeitgebers are daily light-dark cycles and temperature fluctuations (Pittendrigh et al., 1958; Zimmerman et al., 1968), although other factors can synchronize the circadian clocks, for instance social interactions (Levine et al., 2002b). The generation of rhythmic behaviours and physiological processes is regulated through the third component of the circadian clock — the output pathway. In Drosophila, many complex behaviours are under the control of the circadian clock and the most studied ones are adult emergence (eclosion) and locomotor behaviour (see below).

1.2 The circadian clock of Drosophila melanogaster

1.2.1 The central oscillator

The current understanding of circadian systems has achieved great contributions thanks to studies conducted on Drosophila. After the pioneering works from Konopka and Benzer (1971) and the isolation of the first clock mutants, the molecular basis of circadian clocks has been established. Now, many “clock genes” are known to be required for the generation of 24 hour rhythms and the picture is
The generation of a rhythm of 24 hours is determined by the presence of two interlocked feedback loop of gene expression and repression. The basic helix-loop-helix (bHLH) PAS-domain transcription factors CLOCK (CLK) and CYCLE (CYC) (Allada et al., 1998; Bae et al., 1998; Rutila et al., 1998) form heterodimers and bind the E-box sequences \((CACGTG)\) (Kyriacou and Rosato, 2000) in the promoters of the target genes \textit{period (per)} and \textit{timeless (tim)} (Konopka and Benzer, 1971; Sehgal et al., 1994). PER and TIM proteins accumulate in the cytoplasm, dimerize and then migrate to the nucleus (Saez and Young, 1996; Shafer et al., 2002) where they block the CLK/CYC complex (Lee et al., 1999), inhibiting their own transcription. Some studies have proposed that PER and TIM dissociate before entering the nucleus (Shafer et al., 2002), and that PER homodimerization is important for nuclear entry (Landskron et al., 2009).

The time in which PER and TIM accumulate in the cytoplasm is strictly regulated by post-transcriptional events and ubiquitin-mediated degradation. Phosphorylation of PER is mediated by the kinase DOUBLE TIME (DBT), which is the homologue of the mammalian \textit{casein kinase 1\epsilon} (Kloss et al., 1998; Price et al., 1998). The F-box protein SLIMB in the ubiquitin-proteasome pathway interacts with DBT-phosphorylated PER and mediates its degradation (Ko et al., 2002). The PER/DBT complex can also enter the nucleus regulating the activity of CLK (Kim and Edery, 2006). TIM is phosphorylated by GLYCOGEN SYNTHASE KINASE 3\(\beta\)/SHAGGY (GSK3\(\beta\)/SGG) (Martinek et al., 2001) and CK2 (Meissner et al., 2008). The action of PROTEIN PHOSPHATASE 2A (PP2A) and PROTEIN PHOSPHATASE 1 (PP1) (Sathyanarayanan et al., 2004; Fang et al., 2007) on PER and TIM, respectively, promotes the accumulation and the stability of the protein in the cytoplasm and the subsequent entry in the nucleus. While PER
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**Figure 1.2:** The schematic of the circadian clock of *Drosophila melanogaster*. Two negative feedbacks loops are interlocked with each other. In the first loop, CLK and CYC regulate the expression of *per* and *tim* by binding the E-box regulatory sequences on their promoter region. The phosphorylation activity of SGG and DBT determines the stability of TIM and PER and the time before the proteins enter the nucleus where they eventually inhibit their own transcription. In the second loop, CLK/CYC activate the expression of *vri* and *Pdp1ε*. Once the respective proteins have been translated, VRI and PDP1 repress and activate, respectively, the expression of *Clock*, competing for the same V/P box binding site. CRY mediates the light-dependent degradation of TIM, resetting the clock with the light signal. Figure taken from Collins and Blau (2007).

and TIM repress their own transcription, their concentration gradually decreases. As a result, CLK and CYC are then free to start activating *per* and *tim* again, and the cycle continues. In wild-type (wt) flies, this feedback loop takes circa 24 hour to repeat itself.

In *Drosophila*, a second feedback loop is interlocked with the first. In addition to activating the expression of *per* and *tim*, the CLK/CYC complex activates the transcription of *Par domain protein 1ε (Pdp1ε), vrille (vri)* and *clockwork orange (cwo)* (Cyran et al., 2003; Kadener et al., 2007; Lim et al., 2007). The basic zipper transcription factor PDP1 activates the expression of CLK, whereas VRI represses it, competing for the binding site on its promoter (V/P site). This results in *Clk* mRNA cycling in anti-phase compared to *per* and *tim*: the latter phase peak in
the early night and the former in the morning. CWO belongs to the same feedback loop and acts as a repressor for CLK/CYC-mediated activation of \( vri \) and \( Pdp1 \) and for its own transcription (Kadener et al., 2007; Lim et al., 2007). However, CWO has also been proposed to act also as an activator for CLK targets (Richier et al., 2008).

### 1.2.2 The input of the clock: light and temperature

The two-loop model described above explains the first characteristic of the circadian clocks, such as the ability to generate and self-maintain a circa 24-hour rhythm. The rhythm is \emph{circa} 24 hour, but \emph{not exactly} 24. This means that the clock has to be continuously reset to be constantly in phase with the 24-h environmental cycles. In \emph{Drosophila}, daily temperature oscillations and the light-dark cycle are the strongest known signals perceived by the clock as Zeitgeber. Although light is a more potent signal compared to temperature (Wheeler et al., 1993), the two act together to fine tune and reinforce the fly entrainment in a synergistic way (Boothroyd et al., 2007; Yoshii et al., 2009a).

#### Light entrainment

The fly possess several photoreceptors which act in orchestra to sense the light signal and transmit it to the central pacemaker. The fly’s photoreceptors are the compound eye, the ocelli and the Hofbauer-Buchner eyelet (HB-eyelet) (Helfrich-Förster et al., 2001; Helfrich-Förster, 2002; Veleri et al., 2007). However, the main circadian photoreceptor is the blue-light sensitive protein \textit{CRYPTOCHROME} (CRY) expressed within the clock neurons where it mediates the light-dependent degradation of TIM (Emery et al., 1998, 2000; Stanewsky et al., 1998). In darkness, CRY is kept in an inactive form by an unknown repressor (Rosato et al.,
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When light is on, CRY is activated, and it binds directly to TIM, triggering its degradation via the proteasome (Ceriani et al., 1999; Busza et al., 2004). The light-dependent degradation of TIM is mediated by the F-box protein JET-LAG (Koh et al., 2006; Peschel et al., 2006, 2009), which is part of the SCF E3 ubiquitin ligase complex.

Light-dependent degradation of TIM is a crucial event for resetting the circadian clock. In fact, when TIM is degraded, the PER/TIM complex cannot be formed and PER is a target of phosphorylation events by DBT, which leads to PER degradation (see above). This results in a delay of PER and TIM entry in the nucleus and a resetting point for their own transcription (Dunlap, 1999).

Exposure to constant light (and constant temperature) induces arrhythmic behaviour (Konopka et al., 1989) due to continuous degradation of TIM by CRY. The $cry^b$ mutation, which severely affects CRY function, largely prevents light-dependent TIM degradation resulting in a severe reduction of circadian light responses and in rhythmic behaviour under constant light conditions (Stanewsky et al., 1998; Emery et al., 1998, 2000). These data were recently confirmed by the generation of the null mutant $cry^0$ (Dolezelova et al., 2007). Over-expression of CRY, instead, induces hypersensitivity to light (Emery et al., 1998), confirming the prominent role of CRY in the light entrainment.

CRY mutants are not completely circadian blind, but the circadian light sensitivity is much reduced and light entrainment is slower (Stanewsky et al., 1998; Emery et al., 1998, 2000). Only the removal of all photoreceptors makes the fly circadianly blind and unresponsive to the light stimulus (Helfrich-Förster et al., 2001). However, the input pathways which mediate CRY-independent light entrainment are still unclear.
Temperature entrainment

In addition to LD cycles, temperature cycles (TC) also can entrain the circadian clock. Temperature robustly synchronizes the eclosion rhythm of *Drosophila pseudoobscura* (Zimmerman et al., 1968), the fly’s locomotor behaviour (Wheeler et al., 1993) and the molecular oscillations of clock proteins (Stanewsky et al., 1998).

Temperature cycles (TC) can also entrain the circadian clock in LL, both at behavioural (Tomioka et al., 1998; Yoshii et al., 2002, 2005) and molecular levels (Glaser and Stanewsky, 2005), a situation that usually induces the fly to be arrhythmic (Konopka et al., 1989). It is generally believed that LL stops the clock, since TIM and PER are not cycling under LL and constant temperature. However, TC in LL restore the circadian oscillation of PER and TIM (Glaser and Stanewsky, 2005). Work from Tomioka et al. (1998) and Yoshii et al. (2002) suggest an even “better entrainment” to temperature cycles in LL compared to DD, based on the observation that *per*<sup>s</sup> and *per*<sup>L</sup> mutants fail to synchronize their locomotor activity to temperature in DD whereas they do so in LL. Also wild-type flies entrain to TC faster in LL compared to DD. In addition, Yoshii et al. (2002) showed that that wild-type flies can entrain to a wide range of thermoperiods in LL but only to 12:12 hr warm-cold cycles in DD, indicating that temperature is a stronger Zeitgeber in LL than in DD. The effect of temperature as Zeitgeber on the clock is further confirmed by experiments involving temperature pulses and temperature step-up and step-downs, which can change the phase of free-running behaviour (Sidote et al., 1998; Kaushik et al., 2007; Yoshii et al., 2007). Interestingly, the phase response induced by heat pulses (37°C) is mediated by the photoreceptor CRY (Kaushik et al., 2007), since *cry*<sup>b</sup> exhibits reduced (or almost zero) heat phase responses. The model proposes that heat facilitates the interaction between PER and TIM and facilitate also the interaction of the PER-TIM complex with the
active CRY. CRY:TIM-PER interaction leads to TIM degradation, which advance or delay the clock (Kaushik et al., 2007).

Although CRY seems not to be required for temperature entrainment in a more physiological range (Stanewsky et al., 1998; Busza et al., 2007), it mediates the interaction with PER/TIM in a temperature-dependent manner, similarly to the light-dependent interaction between CRY and TIM/PER (Rosato et al., 2001; Kaushik et al., 2007).

The ability of the circadian clock to synchronize to temperature cycles, with an amplitude as little as 3°C (Wheeler et al., 1993) is of interest if we consider that the clock is temperature compensated (Pittendrigh, 1954; Konopka et al., 1989). The chemical reactions underlying the circadian system are buffered to keep a constant rate at different physiological temperatures. However, the same system is able to interpret temperature oscillations as a Zeitgeber, in a process which is still mainly unknown. The protein PER is probably involved in the temperature compensation of the clock, since per$^s$ and per$L^L$ mutant flies, in addition to having, respectively, a short and long period (Konopka and Benzer, 1971), are not temperature compensated (Konopka et al., 1989). It has also been proposed that the polymorphic repeats of Threonine-Glycine (Thr-Gly) residues in the PER protein mediate the adaptation of various populations of Drosophila melanogaster living in different latitudes to keep the same period length at different temperature (Sawyer et al., 1997).

The peak phase of molecular oscillations and behavioural rhythms under temperature entrainment are a few hours advanced compared to LD entrainment (Glaser and Stanewsky, 2005; Busza et al., 2007; Boothroyd et al., 2007). However, the natural profile of temperature cycles is delayed by several hours compared to the one of light (Boothroyd et al., 2007; Yoshii et al., 2009a) and thus the result-
The phase of rhythms entrained by the respective Zeitgeber in nature is essentially the same. In natural condition, light and temperature act probably synergically to enhance and fine-tune the entrainment (Boothroyd et al., 2007; Yoshii et al., 2009a).

Although the mechanisms by which TC act on the central clock are still unknown, several contributions address the effect of temperature itself on the regulation of per and tim expression. Also, the pattern of the fly’s locomotor activity is modulated by different constant temperatures by a mechanism which involves temperature-dependent alternative splicing of intron 8 in the 3′-untranslated region (UTR) of per (Majercak et al., 1999, 2004). At lower temperature (18°C) and short photoperiods, the spliced version of per mRNA is preferred, which results in an advanced accumulation of the PER protein, correlated with an early activity phase. In warm temperatures (29°C) the unspliced version is favoured correlated with a later activity phase (Majercak et al., 1999). Interestingly, under these two temperature conditions, difference in tim expression are also observed: at 29°C the expression of tim is higher than at 18°C (Majercak et al., 1999). In addition, tim is also expressed in two different transcript according the temperature (Boothroyd et al., 2007). One transcript, named tim\textsuperscript{cold}, is more abundant at 18°C (in LD) while the other (the normal one) is favoured at 25°C. Interestingly, the tim\textsuperscript{cold} transcript generates a truncated TIM protein (Boothroyd et al., 2007) but its physiological role has not yet been identified. The modulation of the PER and TIM phase in different temperature and photoperiods thus contributes to the “seasonal adaptation” of the fly’s behaviour (Collins et al., 2004; Majercak et al., 1999, 2004). In warmer and longer days, the fly’s behaviour is shifted towards dawn and dusk, whereas in cold and short days the fly is more active during the relatively mild hours in the afternoon.
Temperature variations are interpreted by the clock not only for the purpose of seasonal adaptation, but also as a Zeitgeber that entrains the clock. However, the molecular mechanisms by which the temperature entrains the clock are still mainly unknown. To date, only two genes have been proposed as components required for the molecular entrainment of the circadian clock to temperature: \textit{nocte} and \textit{norpA} (Glaser and Stanewsky, 2005, 2007; Sehadova et al., 2009).

\textit{nocte} has been isolated in an EMS chemical mutagenesis screen as a variant with severe defects on synchronization of the circadian clock to temperature cycles (Glaser and Stanewsky, 2005). The \textit{nocte} gene has been cloned and encodes a large Glutamine-rich protein with no evident homology with any known domains or proteins (Sehadova et al., 2009). RNAi-mediated down-regulation of the gene in peripheral tissues, and specifically in the chordotonal (ch) organs, with the \textit{F-gal4} driver (Kim et al., 2003), compromises temperature entrainment, similar to the \textit{nocte} mutants. Analysis of several ch organ mutants revealed these structures to be required for temperature entrainment (Sehadova et al., 2009), although the molecular mechanisms underlying the entrainment remain unclear. Nevertheless, it appears that temperature and light entrainment display clear differences in the way the signal is transmitted to the central clock. Light acts directly on the clock neurons through the photoreceptor action of CRY (see above). The fly’s brain, instead, cannot alone interpret the temperature signal, but it requires peripheral sensory tissues for the entrainment to take place (Sehadova et al., 2009).

Part of this thesis contributed to the publication of the work by Sehadova et al. (2009), and it will be addressed in the chapter 7.2.

The product of the gene \textit{norpA} (\textit{no receptor potential A}) is the enzyme Phospholipase C (PLC). PLC has a prominent role in the visual phototransduction cascade in the fly’s compound eyes, and \textit{norpA} mutants are completely blind
Glaser and Stanewsky (2005) showed that norpA mutant flies exhibit defects in entrainment of the circadian clock to temperature, and the phenotype resembles very much the one of the nocte mutants. Previous studies have also shown the involvement of PLC in the light input pathway of the clock, by combining to norpA and cry mutants (Stanewsky et al., 1998; Emery et al., 1998; Helfrich-Förster et al., 2001). An additional involvement for the norpA gene has been suggested in the regulation of the temperature-dependent alternative splicing of the 3'-UTR region of per (see above). norpA mutants favour the spliced version of the per mRNA in warm temperature and long photoperiods, in a light-independent manner (Collins et al., 2004; Majercak et al., 2004). This temperature-dependent splicing event of per is not required for temperature entrainment (Glaser and Stanewsky, 2005; Currie et al., 2009), since the two transcripts are expressed at equal levels during TC in wild-type and nocte mutant flies (Glaser and Stanewsky, 2007). In contrast, norpA mutants favour the “cold” variant (the spliced transcript) during the temperature cycles (Glaser and Stanewsky, 2007), confirming the involvement of norpA in the temperature regulation of per splicing.

PLC has also been proposed to play a role in the themopreference behaviour mediated by TRP channels (Kwon et al., 2008). Larval norpA mutants lose the ability to distinguish between 18°C and 24°C and the model suggests that the TRPA1 channel — required for thermopreference behaviour (see below) — acts downstream of a temperature dependent signalling cascade mediated by PLC (Kwon et al., 2008). The role played by the TRP channels in temperature sensation will be addressed in more details in Chapter 5 and Section 1.3.
Temperature entrainment in different model organisms

Periodic temperature oscillations with period of around 24 hours, even in the range of 1–2°C can entrain the circadian clock of all poikilothermic organisms, beside *Drosophila*. In homeothermic organism, temperature cycles can also cause entrainment, although with considerable individual difference, and only of they are of rather high amplitude (reviewed by Rensing and Ruoff, 2002).

In *Neurospora*, temperature entrains the circadian clock by changing the level of the FREQUENCY (FRQ) protein. At higher temperature, FRQ oscillates at higher level than in low temperature, whereas the level of *frq* oscillation varies little between high and low temperature (Liu et al., 1998). Temperature steps-up and steps-down increase or decrease, respectively, the level of FRQ, causing phase-shift, similar to those obtained after light pulse. Interestingly, in *Neurospora* temperature cycles are even a stronger Zeitgeber than light. Conidiation occurs mainly during the dark phase of a LD regime, and during the cold-phase in temperature entrainment regime. With conflicting light and temperature cycles (light and cold temperature to dark and high temperature), the conidiation rhythms follow the temperature rather than the light (Liu et al., 1998).

The molecular clock of Zebrafish (*Danio rerio*) can also be synchronized by temperature cycles. 24-hour oscillations of 4°C in DD can entrain the expression of clock genes, and setting the phase of free-running after released to constant conditions (Lahiri et al., 2005). Temperature steps shift the phase of clock genes expression and change their expression level: expression of *per4* and *cry3* are down- and up-regulated following a temperature increase and decrease, respectively, while the opposite effect is observed for *cry2a*. Expression of other genes, like *clock1*, *per2* or *β-actin* is not affected by temperature steps, indicating a gene-specific response (Lahiri et al., 2005). In addition, the protein expression level,
amplitude and phosphorylation of CLOCK, one of the central clock-protein, are temperature dependent, suggesting that posttranscriptional effects are also under temperature control.

The effect of periodic temperature changes is a less powerful Zeitgeber on the circadian clock of endothermic animals respect to ectothermic (poikilothermic) animals. The reason is probably that a homeostatic regulation of the body temperature should render the organism less sensitive to temperature fluctuations (Rensing and Ruoff, 2002). However, even for endothermic animals, temperature cycles can entrain the circadian clock: the locomotor activity of rodents can be synchronized to temperature changes but entrainment to temperature is much slower and less strong than light (Refinetti, 2010). Cultured pineal cells of chicks exhibit rhythmic melatonin production in constant conditions and this rhythm can be synchronized to temperature cycles (Rensing and Ruoff, 2002). Cultured rat fibroblast can entrain clock and clock-controlled genes expression to temperature cycles, suggesting the ability of temperature oscillations to autonomously synchronize cells in vitro (Brown et al., 2002). In an organismic level, it has been shown that temperature cycles can entrain peripheral clock in mice liver without affecting the phase of the central clock in the SCN (Brown et al., 2002). In addition, natural body temperature fluctuations can delay the dampening of cycling gene expression in peripheral oscillators (Brown et al., 2002).

1.2.3 The neuronal architecture of the circadian clock

The central oscillator of D. melanogaster is located in the central nervous system (CNS). Several groups of neurons rhythmically express clock genes, and were named according to their anatomical position and size (Kaneko and Hall, 2000; Shafer et al., 2006; Helfrich-Förster et al., 2007). In the adult Drosophila
brain there are circa 150 clock neurons, which can be divided to six main groups (Helfrich-Förster et al., 2007). Three groups of lateral neurons are located in the centro-lateral region of the brain and are named dorsolateral neurons (LN_d) and small and large ventrolateral neurons (s-LN_v and l-LN_v). The three other groups are located dorsally and are called dorsal neurons group 1, 2 and 3 (DN_1, DN_2 and DN_3; Figure 1.3A). All six groups of neurons are required for generating rhythmicity but the LN_v s and LN_d s seem to be more important since they are necessary and sufficient for maintenance of rhythmic behaviour in the absence of environmental cues (Grima et al., 2004; Shafer et al., 2006). As shown in Figure 1.3A, four s-LN_v s neurons and five l-LN_v s neurons are located in the brain. The s-LN_v s project into the accessory medulla (aME) and to few DN_1 and DN_3 cells. The l-LN_v s project through the posterior optic tract (POT) onto the surface of both medullae. The fifth s-LN_v cell, which does not express the PIGMENT DISPERSING FACTOR (PDF), arborizes in the aMe and runs toward the dorsal brain (Helfrich-Förster et al., 2007). Near the posterior surface of the brain, three to four cells of lateral posterior neurons (LPNs) are located close to the dorsal projections of the s-LN_v s (Shafer et al., 2006). The more dorsally located 5–8 LN_d also project into the dorsal brain. The dorsal region consists of more than 80 neurons and none of them express Pdf. The ~17 DN_1 cells are subdivided in two subclasses, the two DN_1A and the DN_1P (for “anterior” and “posterior”, Shafer et al., 2006). The two DN_2 cells are located in the proximity of the projection from the LN_v s and are believed to play an important role in temperature entrainment (Yoshii et al., 2005; Shafer et al., 2006). The biggest group of dorsal neurons is the DN_3, which is constituted of ~40 cells.

The light input pathways from the R1–R6 and R7/R8 photoreceptor cells of the compound eye terminate in the lamina and in the medulla, respectively, whereas
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the projections from the 4 cells of the Hofbauer-Buchner (HB) eyelet overlap with dendritic terminals of the s-LNsvs in the aME (Helfrich-Förster et al., 2007). The circadian photoreceptor CRY is expressed in all the LNVs (including the 5th PDF-negative), in three of the six LNdv6s, in the DNv1As and six of the DNv1Ps (Yoshii et al., 2009b).

The pathway by which the temperature signals synchronize the central clock is still unclear. Indications suggest that the DNv2 and the LPN neurons are required for temperature entrainment (Yoshii et al., 2005; Glaser and Stanewsky, 2007; Miyasako et al., 2007). \textit{Pdf}01 mutant flies are still able to entrain to temperature cycles (Yoshii et al., 2005) suggesting an important role for the \textit{Pdf}-negative neurons and the non-requirement of this neuropeptide in the process.

The larval brain of \textit{Drosophila melanogaster} is much simpler than the adult one and consists of only three groups of clock neurons: five LNs (four of which express PDF), and two pairs of dorsal neurons (DNv1 and DNv2) (Kaneko et al., 1997; Helfrich-Förster, 2005 and Figure 1.3B). The photoreceptor CRY is expressed only in the PDF-positive LNs and in the DNv1 (Klarsfeld et al., 2004). Interestingly, the DNv2 cells express PER in anti-phase compared to the other groups of neurons (Kaneko et al., 1997; Klarsfeld et al., 2004; Picot et al., 2009) and the phase of PER expression is reversed during metamorphosis (Kaneko et al., 1997). The DNv2 cells seem to be required for temperature entrainment of the larval clock (Picot et al., 2009), whereas the LNs are required for light entrainment through the larval visual system, the Bolwig’s Organ (BO) (Kaneko et al., 1997; Malpel et al., 2002).

The contribution of each clock neuronal group to circadian rhythmicity is still object of debate. In 1976 Pittendrigh and Daan proposed the existence of two independent oscillators which govern the morning and evening activity. Their idea was based on studies of free-running activity in rodents and the observation
of the “splitting” phenomenon. Activity sometimes “splits” in two independent components with different free-running periods under certain light or darkness conditions, suggesting the existence of (at least) two oscillators.

In *Drosophila* several studies support the idea of two oscillators. Mosaic analysis revealed that the morning anticipatory activity under light-dark cycles is driven by PDF-positive LN$_{v}$s (M, morning cells). The cells which contribute to the evening (E) activity are the 5th-LN$_{v}$, the LN$_{d}$ and some DN$_{1}$ and are called E cells (Grima et al., 2004; Stoleru et al., 2005). However, the situation is not completely clear and the original Pittendrigh and Daan’s model may not fit to *Drosophila*.

The s-LN$_{v}$s (M-cells) seem also to contribute to the evening bout of activity, at least in LL. This observation is based on experiments of *cry*$_{b}$ mutants analysed in LL conditions: the free-running evening peak “split” in a long and a short component (Rieger et al., 2006). This split behaviour is observed also in wild-type flies released in low light intensity. Analysis of PER cycling in specific groups of neurons revealed that the short component is driven by the s-LN$_{v}$, which are therefore proposed to be “main cells”, rather than “morning cells” (Rieger et al., 2006). A recent work by Zhang et al. (2010) showed that the DN$_{1}$ neurons, in addition to contribute to the evening bout of activity, control also the morning activity under high intensity light. However, their contribution is under environmental control: at high light intensity and high temperature, the DN$_{1}$s are unable to generate the evening activity, whereas at low temperature, the morning bout of activity is inhibited (Zhang et al., 2010).

Therefore, the regulation of the activity does not depend only on specific groups of neurons, but by the interaction with the environment. Such complexity of organization is probably required to deal with an environment in which multiple
variables can changes, predictably in many cases, but also erratically (Dubruille and Emery, 2008) and the separation between E and M cells is probably a simplification.

1.2.4 The output: locomotor behaviour and eclosion

Many behavioural and physiological processes are under control of the circadian clocks, ranging from sleep (Shaw et al., 2000), to memory (Lyons and Roman, 2009), feeding (Xu et al., 2008), egg-laying (S. Hari Dass and Sharma, 2008), chemosensation (Chatterjee et al., 2010), courtship and mating (Sakai and Ishida, 2001) and immunity (Lee and Edery, 2008). However, the most studied output behaviours are the locomotor behaviour (activity) and the eclosion rhythm, probably because of the automated nature of the Drosophila Activity Monitor System (DAMS).

The first output of the circadian clock that was studied in detail has been the emergence from the pupal case (eclosion). Although the adult emergence occurs only once in the fly’s lifetime, it is considered to be a circadian rhythm. This is because a population of pupae manifests an eclosion rhythm if they are not necessarily synchronous developmentally, but are fully synchronous in their circadian oscillations (Skopik and Pittendrigh, 1967).

Eclosion occurs at the early (and wettest) hours of the day because emerging flies lose water at high rate compared to mature flies and they fail to expand their wings at low humidity (Pittendrigh, 1954) — hence Drosophila got its name, from the Greek drosos, “dew” and philos, “lover”. The event of eclosion is controlled by a cascade of peptide hormones produced in the prothoracic gland (PG) and in the CNS. Ecdysis, the shedding of cuticle at defined stages of development and growth (Nässel, 2000), initiates with a decreasing titer of 20-hydroexdysone (20E,
produced in the PG), which leads to the titer increase of the eclosion hormone (EH) and the ecdysis-trigger hormone (ETH) — produced, respectively, by the neurosecretory EH-cells and crustacean cardioactive petide (CCAP)-cells (Figure 1.3B and reviewed by Helfrich-Förster, 2005). The increase of EH and ETH triggers a rhythmic release of CCAP, which eventually leads to eclosion (Figure 1.4). The regulation of the EH by the PG is under control of the prothoracicotropic hormone (PTTH), which is produced by secretory cells in the pars intercerebralis/lateralis (PI, PL). The circadian clock controls the timing of the eclosion event through three pathways: (i) Projections of the larval LN_v's overlap with the ones of the EH cells (see arrow heads in Figure 1.3B); (ii) DN_2 projections overlap dendritic fibres of the CCAP cells (see arrows); (iii) the third pathway connects the LN_v's to the PTTH cells (via the DN_2) and then to the PG (Siegmund and Korge, 2001; Helfrich-Förster, 2005 and Figure 1.4).

Although the anatomy of the circadian clock neurons is well characterized, little is known about the neurotransmitters implied in the generation of rhythmic behaviour. Only few neurotransmitters are known to be expressed in the clock
neurons. The pigment dispersing factor (PDF) is expressed in the small (except the 5th) and large LNv.s and it is required for maintenance of circadian rhythm under constant conditions and normal locomotor activity under LD (Renn et al., 1999). IPNamid (IPNa) is expressed only in the DN1A (Shafer et al., 2006), and its requirement is still unclear. Recently, other three neuropeptides have been found in a subsets of lateral neurons (Johard et al., 2009). The ion transport peptide (IPT) is expressed in one CRY-positive LNd and in the 5th PDF-negative s-LNv. The long neuropeptide F (NPF) is expressed only in 3 LNd.s of male but not female flies, whereas the small neuropeptide F (sNPF) is found in the four PDF-positive LNv.s and in two NPF-negative LNd.s (Johard et al., 2009), but the functional role of those neuropeptides remains uncertain.

1.3 TRP channels and thermosensation

The ability to perceive environmental stimuli is of fundamental importance for the survival of organisms. For poikilothermic organisms such as *Drosophila*, abrupt changes of temperature can have lethal consequences. Temperature is not only a noxious stimulus, though: perceiving environmental changes (e.g. daily temperature fluctuations) can have selective advantages, for instance it allows synchronization of the circadian clock.

Members of the transient receptor potential (TRP) family of ion channels play important roles in sensory physiology and are primary sensors for both physical (heat, light, mechanical stress) and chemical (pH, pheromones, capsaicin) external stimuli (reviewed by Voets and Nilius, 2003; Montell, 2005).

Sensory organs in *Drosophila* fall in two categories. Type I are multicellular organs, consisting of one to four neurons and specialized support cells. Type I
organs are further classified into two subgroups: external sensory (es) organs and chordotonal (ch) organs (see below). Type II are single, non-ciliated, multidendritic neurons (Kernan, 2007). TRP channels are found in both type I and type II sensory organs (see Table 1.1).

The TRP channels get their name from a Drosophila mutant (trp) that showed a “transient” instead of a “sustained” response to bright light in an electroretinogram (ERG) recording (Figure 1.5 and Montell, 2005). After that, many related channels have been isolated, and have been found in diverse organisms, from C. elegans, to humans (Hardie, 2007). In Drosophila there are 13 members belonging to the TRP family, which are divided into 7 classes based on sequence comparison (Figure 1.6 and Table 1.1). The common structure of TRP channels is based on six transmembrane domains with the pore loop permeable to cations situated between the fifth and the sixth transmembrane segments. Channels belonging to the TRPC, TRPV, TRPA and TRPN class have ankyrin repeats at the N-terminus, and their basic structure is conserved between organisms (Venkatachalam and Montell, 2007).

The TRPC class is primarily involved in the visual system via the phototransduction cascade mediated by the PLC, although it is not completely clear by which mechanisms the channels are opened after activation of PLC (Montell, 2005). Many members of the TRP family are activated through mechanical stim-
CHAPTER 1. INTRODUCTION

Figure 1.6: Dendrogram of the 13 TRP channel proteins in Drosophila and their classification into 7 classes based on sequence comparison. Figure modified from Montell (2005).

ulation (reviewed by Damann et al., 2008). Those include the products of the genes *no mechanoreceptor potential C* (*nompC*, TRPN), *painless* (*pain*, TRPA), *nanchung* (*nan*) and *inactive* (*iav*, TRPV). NOMPC is required for the mechanotransduction current in the sensory bristle and mechanosensory organs, whereas NAN and IAV are required in the fly’s hearing system, mediated by the Johnston’s Organ (JO) (see below). PAIN has been identified as a component required for nociception (Tracey et al., 2003). When wild-type larvae are touched with a heated probe (≈40°C) they vigorously roll sideways to escape the stimulus. *pain* mutants fail to exhibit this behaviour (Tracey et al., 2003). PYREXIA (PYX) and TRPA1 are needed to properly distribute in a thermal gradient, and are specifically required to avoid high temperatures (Lee et al., 2005; Rosenzweig et al., 2005). Recently, a role for TRPA1 in chemical nociception has been indicated: Kang et al. (2010) proposed that the avoiding response to reactive electrophiles (noxious tissue-damaging agents, such as allyl isothiocyanate, *N*-methylmaleimide...
CHAPTER 1. INTRODUCTION

or cinnamaldehyde) is entirely TRPA1-dependent and TrpA1 mutants lack this response. NAN and WATER WITCH (WTRW) have been implicated in hygrosensation and the channels can sense dry and most air, respectively (Liu et al., 2007). IAV, PAIN and PYX seem to be required also for the geotaxis behaviour mediated by the fly’s antennae (Sun et al., 2009). TRP and TRPL, in addition to playing a role in the visual signal transduction cascade, seem to be implied in cold avoidance (Rosenzweig et al., 2008). TRPML has been implicated in lysosome-mediated autophagy by clearance of toxic macromolecules and of apoptotic cells. trpml mutants exhibit impaired autophagy which lead to neurodegenerative processes (Venkatachalam et al., 2008). Recently, the TRPM channels has been proposed to regulate the intake of extracellular magnesium (Mg$^{2+}$) from the hemolymph. High concentration of Mg$^{2+}$ in fly diet increases lethality of trpm mutants and reduces the size of fat bodies and of the whole larva (Hofmann et al., 2010). TRPM is expressed in Malpighian tubules, the fly counterpart of the mammalian kidneys.

In flies, the organ required to sense the temperature is believed to be located in the antennae. Wild-type flies strongly prefer 24°C if are let to distribute in a thermal gradient within the physiological range (Sayeed and Benzer, 1996). Genetic or surgical removal of the third antennal segment inhibits this thermopreference behaviour, and flies distribute randomly all over the temperature gradient (Sayeed and Benzer, 1996). Mutants for pyrexia and TrpA1 show similar phenotypes, and mutant larvae as well as adult flies have defects in thermotaxis behaviour (Lee et al., 2005; Rosenzweig et al., 2005, 2008). Expression analysis of the PYX channels revealed that they are expressed in the third antennal segment (Lee et al., 2005), confirming the requirement of that organ in thermotaxis. However, thermotaxis behaviour (the ability to chose a preferred temperature in a gradient) and temperature sensation (the perception of temperature as hot or cold noxious stim-
<table>
<thead>
<tr>
<th>Class</th>
<th>Gene</th>
<th>Described function</th>
<th>Expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPM</td>
<td>trpM</td>
<td>Mg(^{2+}) homeostasis</td>
<td>Malpighian tubules</td>
<td>Hofmann et al. (2010)</td>
</tr>
<tr>
<td>TRPV</td>
<td>nanchung (nan)</td>
<td>hearing, hygrosensation, geotaxis</td>
<td>ch organs</td>
<td>Kim et al. (2003); Gong et al. (2004); Liu et al. (2007); Sun et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>inactive (iav)</td>
<td>hearing, geotaxis</td>
<td>ch organs</td>
<td>Gong et al. (2004); Sun et al. (2009)</td>
</tr>
<tr>
<td>TRPA</td>
<td>TrpA1</td>
<td>warm avoidance, chemical nociception</td>
<td>central brain neurons</td>
<td>Hamada et al. (2008); Rosenzweig et al. (2005, 2008); Kang et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>painless (pain)</td>
<td>nociception, geotaxis</td>
<td>multidendritic neurons</td>
<td>Tracey et al. (2003); Sun et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>pyrexia (pyx)</td>
<td>high temperature tolerance, geotaxis</td>
<td>ch organs, multidendritic neurons</td>
<td>Lee et al. (2005); Sun et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>water witch (wtrw)</td>
<td>hygrosensation</td>
<td>mechanosensory neurons?</td>
<td>Liu et al. (2007)</td>
</tr>
<tr>
<td>TRPC</td>
<td>trp</td>
<td>vision, cold avoidance</td>
<td>photoreceptor cells</td>
<td>Montell and Caterina (2007); Rosenzweig et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>trpl</td>
<td>vision, cold avoidance</td>
<td>photoreceptor cells</td>
<td>Montell and Caterina (2007); Rosenzweig et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>trp-γ</td>
<td>vision</td>
<td>photoreceptor cells</td>
<td>Xu et al. (2000)</td>
</tr>
<tr>
<td>TRPN</td>
<td>nompC</td>
<td>mechanoreception</td>
<td>ch and es organs</td>
<td>Walker et al. (2000)</td>
</tr>
<tr>
<td>TRPML</td>
<td>trpml</td>
<td>autophagy</td>
<td></td>
<td>Venkatachalam et al. (2008)</td>
</tr>
</tbody>
</table>

Table 1.1: List of the 13 *trp* channel encoding *Drosophila* genes. TRP channels are classified in 7 classes. The functions and expression pattern of TRP channels (if known) are indicated.
The pain mutant larvae do not respond to heat stimulation (Tracey et al., 2003) but mutant adult flies have normal thermotaxis behaviour (Rosenzweig et al., 2008). *pyrexia* and *TrpA1* mutants instead compromise both warm avoidance and thermotaxis (Lee et al., 2005; Rosenzweig et al., 2008).

The diverse spatial distribution of TRP channels throughout the fly body and organs emphasizes the requirement of the TRP channels in the many physiological roles and functions they play. At the same time, the different expression pattern of channels implicated in related processes (e.g. thermosensation) exemplifies the complex nature of the sensory physiology and the requirement of many players to fine tune and regulate temperature sensation.

### 1.4 Chordotonal organs

As mentioned above, type I sensory organs are classified into external sensory (es) organs and chordotonal (ch) organs (Kernan, 2007). Es organs are formed by external mechanosensory bristles, innervated by a single neuron. In contrast, ch organs lack any external part and are attached to the inside of the cuticle. The

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**Figure 1.7**: Schematic of the fly’s head, antenna, and a single scolopidium of the Johnston’s Organ. Numbers 1, 2 and 3 represent the three antennal segments. Modified from Sun et al. (2009)
basic unit of a ch organ is called scolopidium, and each ch organ may be formed by hundreds of scolopidia. Each scolopidium contains a liquid-filled capsule called scolopale, which encloses the sensory cilia of one to three ch neurons (Figure 1.7). Chordotonal organs are found at nearly all exoskeletal joints and between joints within limbs and body segments in insects (Field and Matheson, 1998). The known function of ch organs in limbs is proprioception, but a role in vibration detection and graviception is also possible, though unclear (Kernan, 2007). The most prominent ch organ in flies is the Johnston’s Organ (JO), the fly’s ear (Figure 1.7). It is located in the second antennal segment and it is constituted of almost 480 neurons located in more than 200 scolopidial units specialized in sensing near-field acoustic signals of courtship songs (Eberl and Boekhoff-Falk, 2007). Although the main function of the JO is hearing, it is also required to sense gravity and the JO neurons can be classified into subgroups according to their different role (Kamikouchi et al., 2009).

1.5 Aim of this work

Since the 1970s, with the pioneering research of Seymour Benzer and colleagues — a fascinating account of his work is well depicted by Jonathan Weiner in *Time, Love, Memory* (1999) — the investigation of the fly’s behaviour moved down to the scale of single genes and their interactions.

After the truly groundbreaking work of Konopka and Benzer (1971), in which they isolated the first clock mutant *period*, many other components of the circadian clock of *D. melanogaster* have been isolated using genetic screens (reviewed by Stanewsky, 2003; Hall, 2005). Today, the central mechanisms generating circadian rhythmicity are quite well understood. Nevertheless, much remains to be
clarified. In particular, the mechanisms by which temperature cycles synchronize
the circadian clock are poorly known. At present, only 2 genes have been reported
to be required for temperature entrainment, nocte and norpA. nocte has been
isolated in a EMS mutagenesis screen performed by a former Ph.D. student in
our group, aimed at the isolation of novel components required for temperature
synchronization of the circadian clock (Glaser and Stanewsky, 2005; Glaser, 2006).
Similarly to nocte, norpA also has been reported to be required for temperature
synchronization of the clock. However, the exact role of these two genes is not
known yet and many questions are still to be answered. Which are the other
components that mediate temperature synchronization? Where are the circadian
temperature sensors and what is the molecular nature? How does the nocte gene
play its role in this process? Which structures are necessary to mediate tempera-
ture entrainment?

This work tries to answer some of these open questions. In order to address
these issues, the main aim of my Ph.D. was to identify new players and novel
components that play a role in the circadian temperature entrainment. By iso-
lating new factors and trying to understand their role in the process of circadian
rhythmicity we aimed on providing new insight in the mechanisms of tempera-
ture entrainment. Following the long path of research conducted in Drosophila,
we made use of forward and reverse genetic screens to isolate new components.
The power of the RNAi technique has been combined with a bioluminescence as-
say able to monitor real-time expression of one of the central components of the
circadian clock as read out for our screens.
Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Fly stock

*Drosophila melanogaster* were kept and raised in 25°C or 18°C in plastic vials containing fly food and dry yeast. The ambient was set to 12:12 hr light-dark cycles and 65% relative humidity.

The fly food was prepared as follow:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1 litre</td>
</tr>
<tr>
<td>Agar</td>
<td>10 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>15 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>33g</td>
</tr>
<tr>
<td>Yeast</td>
<td>35g</td>
</tr>
<tr>
<td>Maize meal</td>
<td>15g</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>10g</td>
</tr>
<tr>
<td>Treacle</td>
<td>30g</td>
</tr>
<tr>
<td>Soya flour</td>
<td>1 table spoon</td>
</tr>
<tr>
<td>Nipagin</td>
<td>10 ml</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Fly strain | Reference/source
---|---
Control | Konopka et al. (1989)
Canton S | Lindsley and Zimm (1992)
*Df(1) y w* |
### MATERIALS AND METHODS

#### period-luciferase
- **y w ; BG-luc**
- **y w ; plo ; (LT21)**
- **y w ; XLG-luc (line 1.1)**

#### Balancers
- **y w ; Bl/CyO**
- **y w ; Dr/TM3**

#### GAL4 driver
- **y w ; tim-gal4/CyO ;** (line 27)
- **y w ; tim-gal4 ;** (line 16)
- **y w ; tim-gal4 ;** (line 62)
- **y w ; tim-gal4 ;** (line 67)
- **y w ; cry-gal4BN**
- **y w ; pdf-gal4**
- **elav-gal4 ;**
- **y w ; F-gal4 ;**
- **y w ; F-gal4 (line 33-5)**
- **y w ; nocte-gal4 (line B3) ;**
- **y w ; repo-gal4**

#### UAS lines
- **y w ; UAS-cry ;** (line 24.5)

#### Mutant
- **y per$^{P1}$ w ; ;**
- **y w ; Pdp$^{P1}$**
- **norpa$^{P41}$ ; ;**
- **y w nocte$^1$ ; ;**

RNAi lines were obtained from the Vienna *Drosophila* RNAi Center (VDRC, Dietzl et al., 2007) and the National Institute of Genetics - Fly Stock Center (Japan, [http://www.shigen.nig.ac.jp/fly/nigfly/](http://www.shigen.nig.ac.jp/fly/nigfly/)), as indicated in the text (see Table 5.1).

Mutants for the *trp* channels encoding genes used in this study and relative references are listed in Table 5.1.
CHAPTER 2. MATERIALS AND METHODS

2.2 Methods

2.2.1 EMS mutagenesis

Ethyl Methane-sulfonate (EMS) induces point mutations by ethylation of the $O\text{-}6$ position of guanine (G) and the $O\text{-}4$ position of thymine, which allows mispairing during DNA duplication (Roberts, 1998). EMS mutagenesis has been performed as described by T.A. Grigliatti (Roberts, 1998). Three-day old male flies were collected in group of 30, placed in a plastic vials containing a piece of filter paper soaked with water and let them to starve for 5–6 hours. The flies were then transferred in a second plastic vial containing a filter paper soaked with 200 $\mu$l of EMS-sucrose solution (5% sucrose, 25 mM EMS $^1$ in water coloured by blue food dye). Flies were let to feed EMS-sucrose solution for 12 to 16 hours, then were transferred to a new vial with fresh food where they were allowed to recover for about 24 h prior to mating.

EMS fed males were then crossed to virgin females according the crossing scheme depicted in Figure 3.2.

2.2.2 Bioluminescence assay

per-luc constructs

In vivo real-time monitoring of gene expression has been performed by the use of fusion constructs in which the central clock component period was fused to the sequence of the luciferase gene from the firefly Photinus pyralis. Analysis of per-luc expression in living organisms is possible thanks to the short half-life of the luciferase (Brandes et al., 1996).

$^1$The EMS concentration has been increased to 30 mM in the second half of the mutagenesis screen in order to increase the mutagenesis rate.
In this work we used three different constructs (Figure 2.1). \textit{plo} (for promoter luciferase only) carries only the promoter region of \textit{per} fused to the luciferase gene (Stanewsky et al., 1997b). \textit{BG-luc} in addition to \textit{plo} expresses two-third of the \textit{PER} protein (Stanewsky et al., 1997b) and \textit{XLG-luc} expresses the whole \textit{PER} except the last 10 aa (Veleri et al., 2003). The presence of the 5’-UTR region of \textit{per} allows the expression of the three constructs in the same spatial and temporal distribution of the endogenous \textit{per} gene. Given that \textit{per} is expressed in almost every tissue, \textit{per-luc} expression was monitored in the whole adult fly and in isolated body parts (namely legs) kept in insect culture medium (Plautz et al., 1997).

\section*{Cultures preparation}

For monitoring adult flies, alternated-skipped wells of a 96-well Microplate (Packard OptiPlate, Perkin-Elmer) were filled with 100 µl of luciferin-medium (1% Bacto-
agate, 5% sucrose and 15mM Biosynth luciferin). 2–6 day old per-luc flies were anaesthetized with diethyl ether, placed in the luciferin-filled well and covered with plastic caps (PCR tube lid with air holes) to keep the flies during the measurement in the correct position relative to the Z-axis. The plate was sealed with an adhesive sealing film (TopSeal, Perkin-Elmer), which was pierced to allow flies to breath.

For tissue cultures preparation, the 96-well Microplate was filled with 100 µl of sterile tissue culture medium (85.9% M3-insect culture medium, 12% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin mixture, 1% Biosynth-luciferin, 0.5% Insulin; all reagents except luciferin from Sigma-Aldrich). Dissection of legs form diethyl ether-anaesthetized flies was carried out on 2% Bacto-agar dissection plates using sharp forceps. The 6 legs of individual flies were placed in a single well. The plate was then covered by an adhesive sealing film (TopSeal, Perkin-Elmer).

**Data analysis**

*period-luciferase* expression was monitored in the TopCount automated bioluminescence counter (Perkin-Elmer, TopCount NXT), in 65% relative humidity and in the light and temperature conditions as indicated in each experiments. Temperature cycles conditions were achieved by oscillating the temperature of the room where the TopCount counter was located. This resulted in a gradual ramping of temperature, which lasted for about 2 hours from cold to warm and about 4–6 hours from warm to cold.

Raw data were collected and analysed with the Brass analysis software (Andrew Millar lab) operating in Excel. Data were plotted as bioluminescence readings (counts per second, cps) as a function of time (Zeitgeber Time, h). Only
cultures that showed robust and uniform expression within the approximately 1 week of monitoring were included in data analyses.

Rhythmicity analysis has been performed as described (Plautz et al., 1997; Stanewsky et al., 1997b; Glaser and Stanewsky, 2005) via a Fast-Fourier transform-non-linear least square (FFT-NLLS) multicomponent cosine analysis to determine period, phase, and a metric called relative-amplitude error (rel-amp error).

The statistical significance of each derived rhythmic component is assessed by way of the relative amplitude error, defined as the ratio of, in the numerator, the amplitude error (one-half the difference between the upper minus the lower 95% amplitude confidence limits) to, in the denominator, the most probable derived amplitude magnitude. Theoretically, this metric will range from 0.0 to 1.0; 0.0 indicating a rhythmic component known to infinite precision (i.e. zero error), 1.0 (or greater) indicating a rhythm that is not statistically significant (i.e. error equal to (or exceeding) the most probable amplitude magnitude), and intermediate values indicative of varying degrees of rhythmic determination. In this study, all flies and cultures that had a period value within the range of 24 ± 1.5 hr and rel-amp error < 0.7 were considered rhythmic (as in Plautz et al., 1997; Stanewsky et al., 1997b).

### 2.2.3 Locomotor behaviour

Analysis of locomotor activity was performed using the *Drosophila* Activity Monitor System (Trikinetics Inc., Massachusetts). 2–6 day old flies were placed inside a small glass tube (5 mm diameter × 65 mm length), in which at one side fly food (2% Bacto-agar, 4% sucrose) was placed to sustain the fly over the course of the experiment. The food end of each tube was then sealed with paraffin wax to prevent desiccation and the tube was plugged with cotton. The DAM locomotor
monitor measures the simultaneous individual activity of 32 flies. As a fly walks back and forth from one end of its tube to the other, its passage is detected by an infra-red beam which bisects the tube, and counted. The data are sampled every 30 min.

The DAM monitors were located inside a light- and temperature-controlled incubator where the fly’s activity was monitored for up to three weeks.

The raw data were then collected and analysed using a signal-processing toolbox (Levine et al., 2002a,c) implemented in Matlab (MathWorks), which generated the graphical representation of the activity and analysis of rhythmicity.

**Actogram:** Graphical double-plotted representation of activity versus time. A given row shows two consecutive days of activity; the second such day is re-plotted in the left half of the next row down (thus, consecutive days of locomotion can be viewed both horizontally and vertically); heights of bars within a given actogram row reflect varying amounts of locomotion per half-hour data-collection bin. Shaded areas represent dark phase (or cold phase during temperature cycles conditions) (Figure 2.2A).

**Histogram:** Daily average activity of several individual flies during entrainment conditions. Every bar indicates average activity during 30 min bin. Dots indicate SEM. Grey bars indicate average activity during lights-off (or cold) and white bars during lights-on (or warm) (Figure 2.2B).

**Filtered histogram:** Filtered version of the daily average activity to which a 4-h low-pass Butterworth filter has been applied. In addition, the peaks of activity are automated calculated, and the relative intensity values (normalized to the highest peak) are shown in percentage. Pink and blue areas represent lights-on and lights-off (or warm and cold), respectively (Figure 2.2C).
Autocorrelation: Correlogram has been used to determine periodicity and whether the rhythms were statistically significant. The asterisk above the third peak indicates the point used to assess the Rhythms Index (RI), a measure of rhythm strength. If RI is equal to or greater than the numerical height of the confidence line, then the rhythm is significant (by definition, the height of the peak is $\geq$ the height of the confidence interval used to determine statistical significance). The Rhythmicity Statistic (RS) is obtained from the ratio of the RI value to the 95% confidence line. Thus, RS provides a numerical accounting of significance for an individual specimen or an average signal. When RS is $\geq 1$, the rhythm is statistically significant. In this study we considered the rhythmicity significant when the RS value was $\geq 1.5$ (Figure 2.2D).

Maximum entropy spectral analysis (MESA): Spectral density analysis calculates the period of the rhythm. Asterisks are placed over the highest peak shown. Autocorrelation and MESA provide numerical estimates of periodicity using different statistical approaches, but in this study autocorrelation was used to determine rhythmicity (Figure 2.2E).

X-Y activity plot: Activity data are plotted as counts (in 30 min) vs. time. This representation was mainly used for plotting eclosion activity (Figure 2.2G).

X-Y filtered activity plot: Filtered version of the X-Y activity plot to which a 4-h low-pass Butterworth filter has been applied to “smooth” the activity profile (Figure 2.2H).

Circular phase: An average estimate of peak phase, obtained for each specimen, is plotted as a point on a unit circle. A mean vector, extending from the centre of the unit circle towards the diameter is calculated for each group.
of points; the direction of the vector indicates mean peak phase for the group and the length of the vector represents the variability or dispersion between the points (phase estimates for each specimen). The internal black circumference represents 100% coherence between individuals of the same group. The closer the vector is to the black line, the more coherent the group is. The Watson-Williams-Stevens test returns an F-statistic that is used to evaluate whether the mean (M) phase vectors are significantly different from one another and whether the dispersion (D) within the groups is statistically significant (Figure 2.2F).

Statistical analysis: Daily-average activity of individual flies was imported in the GraphPad PRISM 4 software (GraphPad Software, San Diego California, United States). A Two-way analysis of variance (ANOVA) was performed in order to determine statistical interaction of a given genotype with the wild-type control. A Bonferroni Post-test was then performed to determine the time points that showed significant difference of activity compared to control ($P<0.05$).

Statistical analysis was performed with the aid of GraphPad PRISM 4 software (GraphPad Software, San Diego California, United States).

2.2.4 Uncoordination behaviour

Uncoordination behaviour has been performed as described in Sehadova et al. (2009), see Chapter 7.
CHAPTER 2. MATERIALS AND METHODS

Four to eight bottles of the desired genotype were raised in 12:12 hr LD conditions at 25°C or 18°C or as stated in each specific experiment. Pupae of various ages were then harvested by fluctuation method in a plastic tub filled with 20–22°C water: A given bottle was submerged in the tub and vigorously shaken in order to release the pupae from the bottle walls. With the use of a brush, all the pupae were removed from the bottles. Pupae were collected by fluctuation with a metal...
sieve and positioned on a dry paper towel to dry. Larvae were separated from the pupae, and the former discarded. Once the pupae were dry, they were glued (with standard glue stick) over the surface of the eclosion disc (Trikinetics Inc.) and let dry for 5–10 min. The disc was inverted and placed on top of a glass funnel, whose inside surface had been siliconized by a non-toxic sigmacote (Jersey-Cote, Lab Scientific) to prevent newly hatched flies to stick to the funnel. The funnel plus disc were inserted into an eclosion monitor (Trikinetics Inc.) and placed into a light- and temperature-controlled incubator. The eclosion monitor included a solenoid device that “taps down” on top of the disc 3 times every 15 minutes to help the eclosed flies to pass through the funnel stem, where an infrared detector was automated recording every eclosion event. Eclosion was usually monitored for 6–7 days.

The data were then acquired as eclosion events per 30 min intervals by the Drosophila Activity Monitor System (Trikinetics Inc.) similarly to that used to register locomotor activity. Data were analysed by the signal-processing toolbox (Levine et al., 2002a,c) implemented in Matlab (MathWorks). Data were plotted as number of eclosed flies as a function of time (per 0.5 hr bin, “raw activity”) and also after smoothing them by application of a 4-h low-pass Butterworth filter (“filtered activity”). Rhythmicity analysis has been performed as for locomotor activity (see above and Levine et al., 2002a,c).

2.2.6 Western Blot

For analysis of PER protein oscillation, 20–25 flies per time points were collected in liquid nitrogen and stored at -80°C. Heads were removed by vortex and counted on dry ice. The proteins were extracted in 40 µl of extraction buffer (20 mM HEPES pH 7.5, 100 mM KCl, 5% glycerol, 10 mM EDTA, 0.1% Triton X-100, 20mM β-
glycerophosphate, 100 µM Na$_3$VO$_4$ pH 10–12, 0.5 mM PMSF, 20 µg/ml aprotinin, 1 mM DTT, 5 µg/ml Leupeptin, 5 µg/ml pepstatin) to which protease inhibitor was added. After discarding the cell residues, the SDS loading buffer (0.3M TRIS, 10% SDS, 50% glycerol, 25% β-mercapto-ethanol, 0.01% Bromophenol blue) was added, the sample was boiled for 5 min and load on the 4.5% SDS stacking gel and 6% resolving gel. The protein separation was carried out at 70 V, 400 mA and 5W for 16 hours. The separated proteins were transferred into a nitrocellulose membrane (Protran, Whatman) by a “semi-dry blotter” for 1 h at 25 V, 400 mA and 150 W. Ponceau staining was used to control the proteins transfer. The membrane was then blocked for 2 h in 2% BSA in TBST (8.18 g NaCl, 5 ml 2M Tris-HCl pH 7.5, 0.05% Tween-20 for 1 L water) at room temperature. The membrane was incubated with the primary antibody (Rabbit anti-PER, 1:10000, Stanewsky et al., 1997a) at 4°C overnight and with the HRP secondary antibodies (Goat anti-Rabbit, 1:166000) for 2 h at room temperature. The blot was incubated for 5 min with the Pierce SuperSignal HRP kit and developed on X-ray film.

Quantification of the bands was performed with the ImageJ software, by subtracting the background signal and normalizing to the maximum value.

### 2.2.7 Mechanical stimulation

The effect of mechanical stimulation of the fly’s daily pattern of locomotor behaviour was investigate by mounting a DAMS monitor (Trikinetics Inc.) on top of a loudspeaker (ProSound, Power Amp 1600). A ~5 cm thick polystyrene separator was placed between loudspeaker and DAMS monitor to insulate the monitor from possible temperature oscillations generated by the loudspeaker. The stimulus sequence, which was played continuously for 12 hours, consisted of a 40 Hz tone (duration: 0.5 sec; root mean square (RMS) amplitude: 1 V; acceleration:
CHAPTER 2. MATERIALS AND METHODS

Figure 2.3: Recording of the vibration stimulus sequence applied to flies. 0.5 sec of 40 Hz tone (RMS amplitude of 1 V and acceleration of 63 m/s$^2$) was followed by 0.5 sec of 200 Hz (RMS amplitude 2.5 V and acceleration of 158 m/s$^2$) and 0.5 sec of silence. The sequence was repeated for 12 hour followed by 12 hours of silence (background noise).

63 m/s$^2$) followed by a 200 Hz tone (0.5 sec, 2.5 V and 158 m/s$^2$) and 0.5 sec of silence (Figure 2.3). The 12-hour stimulus was followed by 12 hours without any stimulation (“silence”). The resulting vibration of the fly vials were measured with an accelerometer (Brüel & Kjær, Charge Amplifier type 2635) that was coupled to the behaviour monitor. “Silence” is defined as background noise in the room, with an intensity of the order of a ten thousandth lower compared to the stimulus applied.

The vibration stimulus was sampled for 3 sec every 30 min together with the temperature. The temperature was measured with a thermosensor located inside one of the behaviour tubes where flies were placed. Data acquired using the Spike2 software coupled with the Power 1401 mkII (CED, Science Products).

Experiments were conducted in darkness at 21°C.

For the antennae ablated experiments, flies were anaesthetized under CO$_2$ and the antennae were manually ablated using sharp forceps. All the three antennal segments were removed. Wild-type flies with and without antennae were monitored at the same time.
Chapter 3

Results

Mutagenesis Screen

3.1 EMS mutagenesis screen

To isolate novel genes which play a role in temperature synchronization of the circadian clock of *Drosophila melanogaster*, we performed a screen of chemically-induced mutants. Ethyl Methane-sulfonate (EMS) induces point mutations by ethylation of the $O$-6 position of guanine (G) and the $O$-4 position of thymine, which allows mispairing during DNA duplication (Roberts, 1998). The concentration of 25 to 30 mM EMS used for the mutagenesis (see Materials and Methods) is expected to generate 50–80% lethal recessive mutations on each autosome (Roberts, 1998). An average of 47.3% of lines generated following our EMS treatment were recessive lethal (see Table 3.1), suggesting an effective ratio of inducing mutations in essential genes along the two autosomes. Although, we noticed a large variability between different treatments (from 15 to 85% lethality). If 47.3% of lines generated were recessive lethal, we can estimate that a similar number of
CHAPTER 3. MUTAGENESIS SCREEN

<table>
<thead>
<tr>
<th></th>
<th>Total (n)</th>
<th>Homozygous viable (n)</th>
<th>Homozygous lethal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lines generated</td>
<td>3044</td>
<td>1637</td>
<td>47.3</td>
</tr>
<tr>
<td>Chromosome 2</td>
<td>1738</td>
<td>962</td>
<td>45.7</td>
</tr>
<tr>
<td>Chromosome 3</td>
<td>1306</td>
<td>675</td>
<td>51.1</td>
</tr>
</tbody>
</table>

TABLE 3.1: Summary of the EMS mutagenesis screen. The total number of mutant lines generated is indicated. All the homozygous viable lines have been tested in temperature entraining conditions.

non-essential genes (and hypomorphic — non lethal — alleles of essential genes) have been induced.

We generated 3044 EMS mutant lines, 1738 for chromosome 2 and 1306 for chromosome 3. A total of 1637 lines were homozygous viable and have been tested in an automated bioluminescence assay monitoring real-time expression of period-luciferase (per-luc) in living flies, as previously described (Plautz et al., 1997; Stanewsky et al., 1997b; Glaser and Stanewsky, 2005). The flies were raised in a 12:12 hr light-dark (LD) cycles and then analysed in constant light (LL) and 12:12 hr 25°C:16°C temperature cycles (TC).

Initially, for the first quarter of the screen, the temperature cycles were applied in-phase compared to the previous LD entrainment (warm-phase corresponded to the light-phase and the cold-phase corresponded to the dark-phase). In the subsequent part of the screen the phase of the TC was opposite compared to the LD entrainment (cold-phase corresponded to the previous light-phase and the warm-phase corresponded to the dark-phase). The temperature entrainment regime was changed, because given the low rate of mutants isolated during the first part of the screen, we decided for a more stringent paradigm to discriminate between flies able to re-synchronize to the new temperature entrainment and those who were not (potential temperature entrainment mutants), rather than keeping the previous LD-cycle phase.

We utilized three different per-luc transgenic types (Figure 3.1), each of them
Figure 3.1: Structure of the *period* locus and *period-luciferase* transgenes used in this study. The structure of the 13.2 kb genomic DNA fragment containing the *per* gene is shown in the upper part (from Stanewsky et al., 1997a). White bars: coding exon. Black bars: non-coding exon. Line: regulatory and intronic sequences. Note that the *plo* construct contains only the promoter region of *per* fused with the *luciferase* cDNA sequence (modified from Stanewsky et al., 1997a).

containing genomic DNA of the *period* gene, fused with the coding sequence of the firefly *luciferase* gene (Brandes et al., 1996). One line, called *plo* (promoter *luciferase* only) carries only the promoter region of *period* directly fused with the *luciferase* cDNA. The *plo* construct is inserted on chromosome 2 and it was used in the EMS mutagenesis screen for testing putative mutants on chromosome 3. Another line (called *BG-luc*) carries the promoter of *per*, the N-terminal two-third of the coding sequence of the PER protein and the *luc* gene (Stanewsky et al., 1997b). This line carries the *BG-luc* transgene on the chromosome 3 and therefore was used as background for isolating putative mutations on chromosome 2. The third line used, *XLG-luc*, contains the same *per* and *luc* sequence as *plo*, but in addition carries the DNA encoding the entire PER protein, with the exception of the last 10 amino acids (Veleri et al., 2003). For practical reasons this line was used as read-out during the RNAi screen (see chapter 4).

Given the different nature of the two *per-luc* lines utilized in the mutagenesis
CHAPTER 3. MUTAGENESIS SCREEN

A. Generation of putative mutations on chromosome 2

P: ♀♀yw w ; BlCyO; + × ♂yw Y; BG−luc / BG−luc (EMS-fed males)

F1: ♀yw w ; BlCyO; + × ♂yw ; +; BG−luc+ / BG−luc+ (single male cross)

F2: ♀♀yw w ; DrTM3 × ♂yw ; +; DrCyO; + BG−luc+ / +

F3: ♂yw Y; + × ♂yw Y; +; Dr/Dr → bioluminescence assay

B. Generation of putative mutations on chromosome 3

P: ♀♀yw w ; DrTM3 × ♂yw plo; + (EMS-fed males)

F1: ♀yw w ; DrTM3 × ♂yw ; plo+; Dr/Dr × + (single male cross)

F2: ♀♀yw w ; plo+; DrTM3 × ♂yw ; plo+; DrTM3

F3: ♂yw Y; plo+ × ♂yw Y; plo+; DrTM3 → bioluminescence assay

Figure 3.2: Crossing scheme applied to screen for novel mutation affecting temperature entraining of the circadian clock on (A) chromosome 2 and (B) chromosome 3. Stars indicate chromosomes with potential EMS-induced mutations. Bl/CyO and Dr/TM3 are balancer chromosome for autosome 2 and 3, respectively (Lindsley and Zimm, 1992). ♀: virgin female flies, ♂: male flies.

screen, we wanted to trace both the effect of mutations on the transcriptional and post-translational level of period, by exposing plo and BG-luc to mutagenesis, respectively. plo, in fact, is lacking the translated region of PER, thus the bioluminescence readings correlates with the expression of per mRNA. The BG-luc line, instead, expresses also PER protein, therefore it gives indications of the post-transcriptional regulation of PER-LUC protein.

Figure 3.2 shows the crossing scheme we applied to generate autosomal variants specific for chromosome 2 and chromosome 3. As visible from the crossing scheme, the line analysed in our bioluminescence assay carried only one specific autosome with homozygous EMS-induced mutations. However, also the other au-
to some could have carried EMS-induced mutations. Let’s consider for instance the crossing scheme regarding isolation of mutants for chromosome 2 (Figure 3.2A). The lines we tested in our bioluminescence assay were $w^{+} / w^{+} ; +^{*} / +^{*} ; B G^{-} l u c^{*} / + B G^{-} l u c^{*}$, which potentially load mutations on chromosome 3. Anyway, almost all of the lines we tested carried only one EMS-treated chromosome 3 (with orange eyes — indicating the presence of the BG-luc transgene) and the homologue was non-treated ($B G^{-} l u c^{*} / +$). This because for practical reason we selected in the F2 only one parent with orange eyes. This reduced the possibility to isolate mutants on the third chromosome. In addition, eventual mutations on chromosome 3 were not balanced and would have been lost by recombination. Therefore the eventual mutant phenotype would not be seen in the following generations and the line not taken into further investigations. Analogue approach applied for chromosome 2 (Figure 3.2B).

### 3.2 Isolation of mutant lines

It has been shown that temperature cycles can also synchronize per-luc expression in isolated organs and body parts, such as legs, wings, abdomen and head (Glaser and Stanewsky, 2005). Interestingly, isolated body parts, particularly legs, exhibit a less erratic and “smoother” bioluminescence expression of per-luc compared to that of the intact fly, in which luminescence rhythms are relatively noisy (e.g. Glaser and Stanewsky, 2005). The small variability between isolated legs of different individuals of the same genotype, does not require the need to test a large number of flies of the same genotype to distinguish a potential mutant from a wild-type. This can be important in a big screen for mutants, since it reduces the costs of the screen, in terms of time and money.
CHAPTER 3. MUTAGENESIS SCREEN

Recordings of isolated legs during LL and 25:16°C TC

A)

B)

C)

Figure 3.3: Average bioluminescence recordings from legs of EMS mutant and control flies (BG-luc) during LL and 12:12 hr 25:16°C TC. The phase of the previous LD entrainment was opposite compared to the TC applied here and this explains why control flies take up to 2 days to synchronize to the TC. Red/blue bars at bottom indicate warm-phase (25°C) and cold-phase (16°C), respectively. Error bars indicate SEM. Number of individuals is indicated.
For those reasons, we monitored the expression of per-luc in isolated legs, maintained in luciferin-enriched insect tissue-culture media and subjected to constant light and 12:12 hr 25°C:16°C temperature cycle conditions (see Materials and Methods for details). We isolated 3 lines labelled 2T-30, 2T-38 and 2P-42 that exhibited mutant phenotype, i.e. reduced rhythmicity during LL and TC conditions (Figure 3.3). In all the three lines, per-luc expression in isolated legs is drastically reduced compared to untreated control (BG-luc) and other EMS-treated lines (see Figure 3.3 and Table 3.2). There is drastic reduction of per-luc level of expression, together with a lower amplitude of oscillation. For line 2P-42, the remaining per-luc rhythm exhibits an earlier phase (see below). The two lines 2T were isolated in a batch originating from a single EMS treatment, which produced 82% of homozygous lethal mutant flies, suggesting a very effective mutagenesis. The origin of the two lines from the same treatment includes also the possibility that the two lines are clones, i.e. they carry the same mutation (see below). The treatment which generated the line 2P-42, instead, produced 46% homozygous lethal mutant lines.

For unknown reasons, the BG-luc control did not exhibit a consistent rhythmicity between the different independent experiments. Therefore, we compared the EMS-treated lines among themselves, since almost all showed a clear and robust rhythmicity in LL and TC conditions, such as the line 2X-8 exemplified in Table 3.2, which is an EMS-treated line that shows a wild-type phenotype. We considered a line to be mutant in those conditions if it exhibited a mutant phenotype in at least three independent experiments. The three lines isolated have been the only ones to fulfil this criteria and therefore selected for further characterization.

The mutagenesis screen has been performed analysing per-luc expression in dissected legs. We then monitored bioluminescence expression in the whole fly.
### FFT-NLLS analysis of bioluminescence oscillation of EMS mutants and control (BG-luc).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rhythmic (%)</th>
<th>n</th>
<th>τ (hr) ± SEM</th>
<th>Rel-Amp ± SEM</th>
<th>Phase ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LL and temperature cycle entrainment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated legs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2T-30</td>
<td>26.7</td>
<td>15</td>
<td>24.7 ± 0.27</td>
<td>0.20 ± 1.13</td>
<td>20.2 ± 1.13</td>
</tr>
<tr>
<td>2T-38</td>
<td>37.9</td>
<td>29</td>
<td>25.1 ± 0.31</td>
<td>0.30 ± 1.13</td>
<td>20.6 ± 0.43</td>
</tr>
<tr>
<td>2P-42</td>
<td>47.2</td>
<td>36</td>
<td>25.1 ± 0.17</td>
<td>0.28 ± 0.68</td>
<td>21.8 ± 0.40</td>
</tr>
<tr>
<td>BG-luc</td>
<td>58.3</td>
<td>12</td>
<td>25.3 ± 0.37</td>
<td>0.19 ± 0.01</td>
<td>17.5 ± 1.02</td>
</tr>
<tr>
<td>2X-8</td>
<td>100</td>
<td>4</td>
<td>24.9 ± 0.10</td>
<td>0.23 ± 0.05</td>
<td>23.2 ± 0.34</td>
</tr>
<tr>
<td>Whole fly</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2T-30</td>
<td>26.9</td>
<td>26</td>
<td>24.5 ± 0.67</td>
<td>0.61 ± 0.02</td>
<td>18.6 ± 1.21</td>
</tr>
<tr>
<td>2T-38</td>
<td>25.0</td>
<td>8</td>
<td>23.9 ± n.c.</td>
<td>0.53 ± n.c.</td>
<td>20.7 ± n.c.</td>
</tr>
<tr>
<td>2P-42</td>
<td>22.2</td>
<td>9</td>
<td>23.1 ± n.c.</td>
<td>0.54 ± n.c.</td>
<td>20.0 ± n.c.</td>
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<tr>
<td>BG-luc</td>
<td>66.7</td>
<td>21</td>
<td>23.7 ± 0.73</td>
<td>0.54 ± 0.03</td>
<td>18.2 ± 0.97</td>
</tr>
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<td><strong>LD entrainment and constant temperature (25°C)</strong></td>
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<td></td>
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</tr>
<tr>
<td>Isolated legs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2T-30</td>
<td>100</td>
<td>12</td>
<td>23.9 ± 0.07</td>
<td>0.25 ± 0.04</td>
<td>23.4 ± 0.17</td>
</tr>
<tr>
<td>2T-38</td>
<td>100</td>
<td>8</td>
<td>24.0 ± 0.21</td>
<td>0.30 ± 0.04</td>
<td>23.1 ± 0.31</td>
</tr>
<tr>
<td>2P-42</td>
<td>93.3</td>
<td>15</td>
<td>23.9 ± 0.06</td>
<td>0.19 ± 0.02</td>
<td>23.2 ± 0.10</td>
</tr>
<tr>
<td>BG-luc</td>
<td>100</td>
<td>15</td>
<td>23.8 ± 0.05</td>
<td>0.24 ± 0.03</td>
<td>23.1 ± 0.13</td>
</tr>
<tr>
<td><strong>DD and temperature cycle entrainment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Isolated legs</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2T-30</td>
<td>100</td>
<td>23</td>
<td>24.3 ± 0.06</td>
<td>0.12 ± 0.01</td>
<td>19.6 ± 0.23</td>
</tr>
<tr>
<td>2T-38</td>
<td>100</td>
<td>22</td>
<td>24.2 ± 0.04</td>
<td>0.13 ± 0.01</td>
<td>18.7 ± 0.27</td>
</tr>
<tr>
<td>2P-42</td>
<td>95.6</td>
<td>23</td>
<td>24.2 ± 0.06</td>
<td>0.14 ± 0.01</td>
<td>19.9 ± 0.29</td>
</tr>
<tr>
<td>BG-luc</td>
<td>100</td>
<td>24</td>
<td>24.3 ± 0.07</td>
<td>0.16 ± 0.01</td>
<td>19.7 ± 0.30</td>
</tr>
<tr>
<td>Whole fly</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2T-30</td>
<td>54.5</td>
<td>22</td>
<td>24.1 ± 0.39</td>
<td>0.55 ± 0.03</td>
<td>19.2 ± 0.89</td>
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<td>2T-38</td>
<td>78.5</td>
<td>14</td>
<td>24.5 ± 0.17</td>
<td>0.46 ± 0.03</td>
<td>20.0 ± 0.91</td>
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<tr>
<td>2P-42</td>
<td>62.5</td>
<td>24</td>
<td>24.4 ± 0.21</td>
<td>0.59 ± 0.04</td>
<td>17.8 ± 0.98</td>
</tr>
<tr>
<td>BG-luc</td>
<td>63.2</td>
<td>19</td>
<td>25.0 ± 0.41</td>
<td>0.51 ± 0.04</td>
<td>19.5 ± 1.12</td>
</tr>
</tbody>
</table>

Table 3.2: FFT-NLLS analysis of bioluminescence oscillation of EMS mutants and control (BG-luc). Whole adult flies or isolated legs were entrained in LL and 12:12 hr 25:16°C temperature cycles, in 12:12 hr LD and constant temperature (25°C) or in DD and 12:12 hr 25:16°C TC, as indicated. EMS mutant flies are weakly rhythmic compared to non-treated control (BG-luc) and a treated non-mutant line (2X-8). See text for more details. FFT-NLLS analysis was applied to calculates “period” (τ), “relative amplitude error” (Rel-Amp) and “phase” only of rhythmic flies. Flies were considered rhythmic if rel-amp error was ≤0.7 and the period valued was in the range of 24±1.5 hr. SEM indicates Standard Error of the Mean.
(Figure 3.4 and Table 3.2). Line 2T-30 and 2T-38 show strongly reduced rhythmicity compared to control (Figure 3.4A–B). Analysis of plots of individual-fly recordings of 2T-30 and 2T-38 (Figure 3.5) reveals that per-luc expression of the different individuals is weakly (if at all) rhythmic and out of phase each other, explaining the overall flat bioluminescence reading when the average is plotted. Line 2P-42 (Figure 3.4C), instead, seems to cycle with an opposite phase compared to control, although with a minor amplitude. Noticeably, we observed a steep increase of LUC activity just after temperature rising and a fast decrease after temperature dropping, suggesting more a temperature response rather than synchronization to TC with an opposite phase. This is also suggested by inspection of recording from individual flies (Figure 3.5) which also shows that not all the flies exhibit the same phase. This phenotype is reminiscent of per-luc expression in isolated brains, which do not entrain to temperature: if a brain of a wild-type per-luc fly is dissected from the body, it cannot synchronize per expression to TC, but rather reacts to temperature step-up with an steep increase of per-luc expression (Sehadova et al., 2009).

Isolated legs of the three EMS mutant lines synchronize normally to LD cycles, in terms of period, phase and expression level compared to control, as shown in Figure 3.6, with a peak of per expression towards the end of the night (ZT 23, see Table 3.2), indicating a specific defect on temperature entrainment.

We then investigated whether the mutant phenotype was manifested also during DD and TC. Figures 3.3 and 3.8 show bioluminescence expression of BG-luc and the three EMS mutant lines, in DD and temperature cycles. Interestingly, isolated legs of mutants could entrain to TC with a comparable period, phase and amplitude to the control (Figure 3.3 and Table 3.2), but with a subtle reduced per expression level (and no differences between the different mutated lines). Note
Average recordings of adult flies during LL and 25:16°C TC

A)

B)

C)

**Figure 3.4:** Average bioluminescence recordings of whole adult flies of control (BG-luc) and EMS mutants during LL and 12:12 hr 25:16°C TC. Conditions and coloured bars are as described in Figure 3.3. Grey error bars indicate SEM.
Individual recordings of adult flies during LL and 25:16°C TC

**A)**

**B)**

**C)**

**D)**

**Figure 3.5:** Individual bioluminescence recordings of whole adult flies of control (BG-luc) and EMS mutants during LL and 12:12 hr 25:16°C TC. Average recordings and conditions are shown in Figure 3.4.
Bioluminescence recordings of isolated legs during LD and 25°C

**Figure 3.6:** Bioluminescence expression of EMS mutant lines and controls in LD conditions. A) Control flies (BG-luc) entrain normally to LD cycles (at constant 25°C) with a peak of *per* expression towards the end of the night (ZT23). B–D) EMS mutant lines show no significant difference of *per-luc* expression during light-dark cycles compared to controls. White bars at the bottom of the plot indicate light-on. Black bars indicate light-off.

that during temperature entrainment (LL or DD) the peak of *per-luc* expression occurs 2–3 hours earlier than during LD entrainment. This observation differs slightly compared to previous reports, for instance Glaser et al (2005). The divergence can be related to different technical experimental conditions in which the flies were monitored (10:14 hr 25:18°C vs 12:12 hr 25:16°C). However, my data correlates also with an advance evening peak of locomotor activity during TC entrainment compared to LD (see below and Discussion). An explanation could be a difference in light and temperature cycles as they occur in natural environmental
conditions: light arises earlier in the morning than temperature, and the latter increases more gradually during the day and peaks later than light, generating a gap between temperature and light profile (Boothroyd et al., 2007; Currie et al., 2009; Yoshii et al., 2009a and Discussion).

PER-LUC luminescence in the whole fly revealed distinct phenotypes among the 3 mutant lines during DD and TC. Average per expression in line 2T-30 is flat (Figure 3.8). FFT-NLLS analysis shows that only half of the flies are weakly rhythmic (Table 3.2), and inspection of individual recordings (Figure 3.9) shows that the remaining rhythmic ones are out of phase with each other during temperature entrainment. This explains the overall flat per-luc reading when the average of all flies is plotted.

Line 2T-38 synchronizes to temperature cycles in DD, even if the level of per-luc expression is reduced (similarly as for isolated legs of the same genotype). Although not all the flies are strongly in phase with each other as visible from recordings of individual flies (Figure 3.9) and large standard error in the FFT-NLLS analysis, they appear to be in phase with the BG-luc control (Figure 3.8 and 3.9).

Adult 2P-42 flies exhibit the same phenotype in LL and DD and temperature cycles: flies seem synchronized with an opposite phase compared to control (Figure 3.8). Also, inspection of individual recordings (Figure 3.9) shows that most of the flies exhibit the same phase. However, the steep increase of LUC activity just after the temperature goes up, and the steep decrease after temperature goes down suggests more temperature reaction (masking effect) rather than entrainment.

The fact that isolated body parts during DD and temperature cycles entrain, while the whole fly does not, is very interesting. In fact, this is similar to the phenotype of other already described “temperature mutants”, e.g. nocte1 or norpAP41,
Figure 3.7: Average bioluminescence recordings of isolated legs of control (BG-luc) and EMS mutants during DD and 12:12 hr 25:16°C TC. Conditions and coloured bars are as described in Figure 3.3.
Recordings of adult flies during DD and 25:16°C TC

**A)**

![Graph A]

**B)**

![Graph B]

**C)**

![Graph C]

**Figure 3.8:** Average bioluminescence recordings of whole adult flies of control (BG-luc) and EMS mutants during DD and 12:12 hr 25:16°C TC. Conditions and coloured bars are as described in Figure 3.3.
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Individual recordings of adult flies during DD and 25:16°C TC

Figure 3.9: Individual bioluminescence recordings of whole adult flies of control (BG-luc) and EMS mutants during DD and 12:12 hr 25:16°C TC. Mean recordings and conditions are plotted in Figure 3.8.

in which per-luc expression in isolated body parts synchronise to TC, but it does not when the whole animal is considered (Gentile C., Simoni A., Stanewsky R., in preparation and see Discussion).

Because the line 2T-30 and 2T-38 were isolated from a batch of lines generated by the same EMS treatment, we performed a complementation test to rule out the possibility that they affected the same gene or that the different phenotypes observed were due to the generation of allelic variants of the same gene. This case is rare but indeed possible. In the EMS screen which led to the isolation of the first clock gene, three different mutants were isolated with different phenotypes. All mapped to the very same locus period (Konopka and Benzer, 1971).

We crossed the 3 mutants to each other and then tested the progeny for their
CHAPTER 3. MUTAGENESIS SCREEN

Complementation test:

Recordings of isolated legs during LL and 25:16°C TC

A)

B)

C)

Figure 3.10: Complementation test of EMS mutants. Recording of PER-LUC in isolated legs from lines (A) 2T-30/2T-38, (B) 2T-30/2P-42 and (C) 2P-42/2T-38 during LL and 12:12 hr 16:25°C temperature cycles. All the mutants complemented each other, indicating that each EMS mutant line affects a different gene. Red and blue bars indicate warm and cold, respectively. Note that the phase of TC is opposite to that of the previous LD (in which the flies have been raised, not shown). Legs take up to two days to synchronize to temperature.
ability to entrain \textit{per-luc} expression to TC in constant light. Figure 3.10 shows that all the mutants complement and the wild-type phenotype is restored. This suggests that the 3 EMS lines affect different genes and this may explain the variety of phenotypes observed in different light/temperature conditions between the lines.

3.3 Behavioural analysis of the EMS mutants

3.3.1 Entrainment in constant light and temperature cycles

In the previous section we described that our three candidate temperature-entrainment mutants affect synchronization of \textit{per-luc} expression to temperature cycles during constant light. Next, we addressed whether the observed molecular phenotype was also reflected at the behavioural level.

The rest-activity pattern of the fly is one of the best studied behavioural outputs of the circadian clock. The locomotor behaviour of a wild-type male under light-dark cycles displays a bimodal pattern of activity (as shown in Figure 3.11B, left column). The fly is very active in the morning, anticipating the transition from dark to light, then displays an afternoon \textit{siesta} of very low activity before becoming again very active in the evening, again anticipating the transition from light to dark.

During constant light and temperature cycles (Figure 3.11C) locomotor activity is unimodal: the morning peak of activity disappears, flies are mainly active towards the end of the warm phase, anticipating the transition from warm to cold. In the three mutant lines we isolated the morning anticipation during LD is only weakly present (if at all, see Figure 3.11B). The reason for this can be the differ-
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A  

Average actogram

Canton S (9)  $2T-30;+$ (15)  $2T-38;+$ (15)  $2P-42;+$ (16)

B  

Light-Dark cycle (25°C)

C  

Constant Light and 25:16°C temperature cycle

Figure 3.11: Rest-activity pattern of EMS mutant lines and control during LD cycles and LL & TC. A) Double-plot average actogram of wild-type (Canton S), $2T-30$, $2T-38$ and $2P-42$. Flies were first exposed to 12:12 hr LD cycle at constant temperature for 7 days and then to constant light (LL) and 12:12 hr 25:16°C temperature cycles (arrows) for the following 7 days, in which the phase of temperature was opposite compared to the previous LD (warm corresponding to dark phase and cold corresponding to the previous light phase). B) Average histograms showing daily average activity of flies during the LD entrainment days. C) Daily average activity of flies during the subsequent entrainment to LL and TC. Shaded areas in the background correspond to the dark-phase (during LD entrainment) or the cold-phase (during TC entrainment). Dots above bars in the histograms represent SEM. Number of individuals analysed is indicated in brackets.

ent genetic background of the EMS-treated lines. BG-luc flies lack the morning anticipatory activity (see Figure 5 in Glaser and Stanewsky, 2005).

The mutant flies have a less pronounced siesta but show a robust anticipation
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Figure 3.12: Daily average activity of controls (red line) and EMS mutants (green, blue and black, as indicated) during the warm-phase only of LL and TC of the histograms depicted in Figure 3.11C. Activity of the different genotypes is overlapped to see the effect of the mutation pattern and on the activity level. Two-way Anova was performed to determine overall statistical interaction between Canton S and EMS mutants for the range ZT 0–12 (left) and ZT 6–12 (right). Coloured bars underneath represent the time points in which each mutant shows significant difference compared to Canton S control (Bonferroni posttest \( P < 0.05 \)). Error bars indicate SEM.

Unlike Canton S, all three mutant lines show a sharp increase of activity after the lights-off transition in the evening. In contrast, the mutants behaviour looks drastically different during temperature entrainment (Figure 3.11C and 3.12). Analysis of behaviour was performed first by inspection of actograms and histograms of daily average activity (Figure 3.11) and then by determining statistical interactions between activity of each mutant lines with the wild-type control (Figure 3.12). Anova was performed on the warm-phase only of temperature entrainment. We considered both the whole warm phase (ZT 0–12, left plot) and the second half of the warm phase (ZT 6–12, right plot), independently. In this way, we could monitor both the effects of the mutations on the overall activity (included the startle response induced by the steep increase of temperature) and the effect specifically on the peak of activity which anticipates the transition from warm to cold.

Unlike Canton S, all three mutant lines show a sharp increase of activity after
CHAPTER 3. MUTAGENESIS SCREEN

A

Canton S (12) 2T-30;+ (11) 2T-38;+ (11) 2P-42;+ (11)

B

C Daily average activity at 29:25°C Temperature cycles

D Daily average activity at 25:20°C Temperature cycles

E Daily average activity at 20:16°C Temperature cycles
temperature rise and they remain active for the whole warm phase (Figure 3.12). Line 2T-30 does not show any anticipation of the transition warm-cold, with highly significant interaction with control ($F_{(11,252)}=3.90$, $P<0.0001$, Two-way Anova).

Interestingly, when we consider only the second half of the warm phase (ZT6–12), mutant 2T-38 and 2P-42 do not differ from control (interaction not significant, Anova) in term of activity level and pattern. However, the three mutants interact with Canton S (in the range ZT 0–12), indicating an overall effect on the activity pattern.

The temperature cycles applied in this study to elicit entrainment were 25:16°C. We wondered if mutant flies were responding in a different way at different temperature intervals. This was also done in the light of the poor, but visible, ability of 2T-38 ad 2P-42 to entrain to 25:16°C. It is known that a temperature cycle as little as 2–3°C is enough to synchronize behaviour in wild-type flies (Wheeler et al., 1993). We monitored the behaviour of flies at three different temperature intervals (in LL) to try to understand if distinct mutant lines were able to synchronize specifically to a certain temperature range, but fail to entrain when a wide temperature range was applied. Figure 3.13 shows the behaviour of the EMS mutant

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**Figure 3.13 (preceding page):** Behaviour activity of EMS mutant lines and control in LL and different temperature intervals.

A) Average actogram of wild-type control (Canton S), 2T-30, 2T-38 and 2P-42 putative mutant lines. Flies were first entrained in LD cycles at 25°C (not shown) followed by 5 days of LL and 12:12 hr – 29:25°C temperature cycles which was 6 hr delayed compared to the previous LD (warm phase delayed compared to the previous light phase). This was followed by 6 days of LL and 12:12 hr – 25:20°C temperature cycles again delayed by 6 hr compared to the previous TC. Finally, the flies were exposed to 6 days of LL and 12:12 hr – 20:16°C temperature cycles, delayed 6 hr compared to the previous TC. B) Activity peak phase of control and mutant flies. Conditions are the same as in (A). C–E) Daily activity plot of control and mutant lines during LL and 29:25°C (C), 25:20°C (D) and 20:16°C (E). Grey shadows (A, B) and grey bars (C–E) represent cold-phase (in any different temperature intervals) while white areas/bars indicate warm phase. Error bars in (B) indicate SEM. Number of individuals is indicated in brackets.
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Daily average activity at 29:25°C TC

![Graph showing daily average activity at 29:25°C TC with statistical analysis for 2T-30, 2T-38, and 2P-42.]

- 2T-30: $F_{(23,432)} = 4.60, P < 0.0001$
- 2T-38: $F_{(23,480)} = 1.71, P < 0.05$
- 2P-42: $F_{(23,432)} = 1.65, P < 0.05$

Daily average activity at 25:20°C TC

![Graph showing daily average activity at 25:20°C TC with statistical analysis for 2T-30, 2T-38, and 2P-42.]

- 2T-30: $F_{(11,216)} = 2.26, P < 0.05$
- 2T-38: ns
- 2P-42: ns

Daily average activity at 20:16°C TC

![Graph showing daily average activity at 20:16°C TC with statistical analysis for 2T-30, 2T-38, and 2P-42.]

- 2T-30: $F_{(23,432)} = 4.96, P < 0.0001$
- 2T-38: $F_{(23,480)} = 1.87, P < 0.0001$
- 2P-42: $F_{(23,432)} = 4.41, P < 0.0001$

- 2T-30: ns
- 2T-38: ns
- 2P-42: ns

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lines and controls at constant light and 29:25°C, 25:20°C and 20:16°C temperature intervals. As before, activity was plotted in form of actograms and histograms of the individual genotypes (Figure 3.13) and by overlapping the activity of the all genotypes for each condition in single plots, where statistical analysis was performed (Figure 3.14). Wild-type flies strongly entrain to all three temperature intervals. Note also the effect of “seasonal adaptation” of the clock (Majercak et al., 1999; Collins et al., 2004; Stoleru et al., 2007): The phase of evening activity is moving towards the centre of the day (i.e. warm phase in a temperature entrainment regime) during a cold interval (20:16°C), the equivalent of an autumn day. In a warm range (29:25°C), like a summer day, the fly’s activity is shifted more to the end of the day (see Figure 3.13B, first column). The advantage of this behaviour pattern could be to avoid the hottest part of the day during summer, preventing excessive dehydration, while in colder seasons activity mainly occurs during mildest hours before dusk.

The overall pattern of activity of the three mutants is significant different form Canton S control in any temperature intervals (Figure 3.14). All the mutants, in contrast to Canton S, exhibit a strong peak of activity after the transition from cold to warm (morning) and a less pronounced anticipation of the transition from warm to cold (evening), indicating defective entrainment to temperature. However, when a Two-way Anova is restricted to the second half of the warm phase (ZT 6–12, i.e. the evening anticipation peak), we found statistical difference from

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**Figure 3.14 (preceding page):** Daily average activity of controls (red line) and EMS mutants (green, blue and black, as indicated) during the warm-phase only of LL and different temperature intervals of the histograms depicted in Figure 3.13C–E. Two-way Anova was performed to determine statistical interaction between Canton S (CS) control and each EMS mutant lines in the range ZT 0–12 (left) and ZT 6–12 (right). Coloured bars underneath indicate the time points in which each mutant shows significant difference compared to Canton S (Bonferroni posttest $P < 0.05$).

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control only for the mutant line 2T-30 during LL and 29:25° C TC (F(11,216)=2.26, \( P<0.05 \), Figure 3.14). Therefore, the lines 2T-38 and 2P-42, although exhibiting a stronger reaction peak in response to the temperature increase, are able to entrain to the different temperature intervals, in terms of exhibiting a clear evening anticipation peak of activity comparable to control (Two-way Anova interaction not significant). In addition, from overlapped daily activity plots in Figure 3.14 it is visible that at 29:25°C the activity level of the mutants is higher compared to control, whereas at 20:16°C the activity level is lower (with statistical significance, Anova, data not shown), suggesting again that the passive response to temperature is more pronounced in the mutant than in control flies.

We thus observed a clear difference of activity at different temperature intervals in LL. The clearest effect is observed for 2T-30 at 29:25°C TC. Interestingly, all the lines which exhibit a mutant phenotype at 25:16°C entrain (weakly) at 25:20°C and 20:16°C (which are “part” of 25:16°C). This indicates, surprisingly, that mutants fail to synchronize to higher temperature cycles than to lower ones.

### 3.3.2 Entrainment in constant darkness and temperature cycles

It is known that temperature cycles can entrain locomotor activity in constant darkness (Wheeler et al., 1993). We first entrained flies to LD cycles and then applied 12:12 hr 25:16°C TC in constant darkness in phase with the previous LD. Subsequently, a new temperature regime was applied, which was 6 hour delayed compared to the previous and then flies were released to DD and 25°C where free run activity was monitored. In addition to plot the activity in form of average actograms and daily activity histograms (Figure 3.15), the behaviour analysis software calculates also the average peak phase of activity, which have been reported.
in Table 3.3. Then, Two-way Anova was performed to determine statistical interaction between the mutants and control. During the first temperature regime, Canton S flies move the evening activity earlier during the warm phase, compared to the previous LD, stabilizing to ZT7.8 (Figure 3.15B and Table 3.3 “pre-shift”). A minor peak of activity occurs just after temperature-up (ZT0.3), probably induced by the steep increase of temperature. A similar “temperature response” was observed in the same conditions also in per\textsuperscript{\alpha1} flies (Tomoka et al., 1998), suggesting that it is clock-independent. After a 6-hr temperature shift, the main peak of activity in wild-type flies stabilizes at ZT3.6, much earlier compared to LL (compare Figures 3.15 to 3.11). The activity take 3–4 days to reach a stable phase, indicating that in DD flies synchronize their behaviour to TC more slowly compared to LL (in which they take 2–3 days). A burst of activity still occurs just after the temperature increase (Figure 3.15C), but it is not considered by the phase analysis, which apply a 4-hr low-pass filter to the data (Table 3.3). When flies are released in DD (and 25\textdegree C) the phase of free-running activity follows the previous temperature entrainment, indicating that the flies are fully synchronized (Figure 3.15D and Table 3.3).

The mutant line 2T-30 is unable to synchronize its behaviour to TC in DD (Figure 3.15). As soon as the flies are subjected to TC, their activity is characterized by a strong reaction to temperature just after the cold to warm transition (ZT0.8) and an overall low activity during the warm phase. Although the “reaction” peak of mutants and control is comparable, both before and after the temperature shift (Figure 3.16), in case of the mutants a second “entrainment” peak exhibited by control flies does not follow. Before the temperature shift, the analysis of variance revealed not significant difference compared to control in term of statistical interaction when the second half of the warm phase only is consid-
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A  Average actogram

Canton S (16)  2T-30;+ (16)  2T-38;+ (16)  2P-42;+ (16)

B  DD and 25:16°C TC

Canton S (16)  2T-30;+ (16)  2T-38;+ (16)  2P-42;+ (16)

C  DD and 25:16°C TC after 6 hr temperature shift

D  DD and 25°C (Day 1-3)
ered, but a clear difference of activity level is visible (Figure 3.14). After the
temperature shift (Figure 3.15C) the “temperature mutant” phenotype is even
clearer (and highly significant). Subsequent free-running activity of 2T-30 flies in
DD is mainly arrhythmic (Figure 3.15D, Table 3.4 and see below).

2T-38 exhibits a main peak of activity just after the cold to warm transition,
before and after the shift (Figure 3.15 and Table 3.3), suggesting a reaction to
temperature, rather than synchronization. The peak of entrained activity which
characterize Canton S flies, is barely visible, but not significant (Figure 3.16).
Mutant flies subsequently released to constant conditions free-run with a phase at
CT0.8 (Figure 3.15D and Table 3.4), 3 hours earlier than Canton S, and a second
peak of free-running activity occurs in the same position as in controls.

Activity of line 2P-42 is comparable to controls, in terms of activity pattern
and number of days required to reach a stable phase (Figure 3.15 and Table 3.3).
Before the temperature shift, the activity is synchronized to temperature cycles,
it peaks two hour later than control (ZT9.8 vs 7.8) and although the activity level
of the “entrainment” peak is lower than Canton S it is not statistically different

**Figure 3.15 (preceding page):** Rest-activity pattern of EMS mutant lines and controls
during DD and TC. A) Double-plot average actogram of wild-type (Canton S), 2T-30,
2T-38 and 2P-42. Flies were first entrained to 12:12 hr LD cycle at constant temperature
(25°C) for 4 days and then exposed to DD and 12:12 hr 25:16°C TC (arrows) for the
following 4 days in which the phase of temperature was the same as the previous LD
(warm corresponding to previous light phase and cold corresponding to the dark phase).
Subsequently, a new temperature regime was applied 6 hr delayed compared to the
previous TC, followed by release to DD and 25°C (arrowheads), where free-running
activity was analysed. Note that the evening activity during TC is advanced compared
to LD. B) Histogram showing daily average activity of flies during the first DD and
TC entrainment (4 days). C) Daily average activity of flies during the subsequent
entrainment to DD and TC (13 days). D) Average free-running activity during the first 3
days of DD and 25°C. Shaded areas in the background correspond to the dark/cold phase
and white areas to light/warm phase. In (D) light- and dark-grey shadings represent
subjective day (warm) and night (cold), respectively. Dots above bars in the histograms
represent SEM. Number of individuals analysed is indicated in brackets.
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Daily activity at DD and 25:16°C TC

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3_16a}
\caption{Daily average activity of controls (red line) and EMS mutants (green, blue and black, as indicated) during the warm-phase only of DD and TC histograms depicted in Figure 3.15B–C. Two-way Anova was performed to determine statistical interaction between Canton S (CS) control and each EMS mutant lines in the range ZT 0–12 (left) and ZT 2–12 or ZT 6–12 (right), as indicated. Coloured bars underneath represent the time points in which the respective mutants show significant difference compared to Canton S (Bonferroni posttest $P < 0.05$).}
\end{figure}

Daily activity after 6 hour temperature shift

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3_16b}
\caption{Daily average activity of controls (red line) and EMS mutants (green, blue and black, as indicated) during the warm-phase only of DD and TC histograms depicted in Figure 3.15B–C. Two-way Anova was performed to determine statistical interaction between Canton S (CS) control and each EMS mutant lines in the range ZT 0–12 (left) and ZT 2–12 or ZT 6–12 (right), as indicated. Coloured bars underneath represent the time points in which the respective mutants show significant difference compared to Canton S (Bonferroni posttest $P < 0.05$).}
\end{figure}
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Peak phase (ZT)\(^a\)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LD</th>
<th>DD + TC pre-shift</th>
<th>DD + TC post-shift</th>
<th>DD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canton S</td>
<td>12.3</td>
<td>7.8(^b) 0.3 (77)(^c)</td>
<td>3.6 – 3.8</td>
<td>2T-30</td>
</tr>
<tr>
<td>2T-30</td>
<td>11.8</td>
<td>0.8 10.3 (82)</td>
<td>1.3 6.8 (72) 1.3</td>
<td></td>
</tr>
<tr>
<td>2T-38</td>
<td>12.3</td>
<td>0.3 10.8 (54)</td>
<td>0.8 5.8 (51) 0.8</td>
<td></td>
</tr>
<tr>
<td>2P-42</td>
<td>11.8</td>
<td>9.8 0.8 (89)</td>
<td>0.8 4.8 (84) 4.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: Peak phase analysis of EMS mutants and controls during DD and TC entrainment. Peak phase (in hr) are calculated from 4-hr lowpass filtered versions of the histograms depicted in Figure 3.15. During LD entrainment, only the evening peak is considered, which occurs at the transition between light and dark (around ZT12). During temperature entrainment, before and after the temperature shift (pre- and post-shift, respectively), two main activity peaks are present and the respective values are reported. The phase of the major peak (relative amplitude of 100%) is displayed in the first column. The phase of a minor, secondary peak, is displayed in the second column (and relative peak amplitude is shown in brackets). Note that Canton S flies display only one activity peak after the temperature shift.

\(^a\) Phase relative to the entrainment regime: 0 = lights-on or temperature-up. 12 = lights-off or temperature-down. For DD conditions, CT values relative to the previous temperature regime are considered. Phase calculated only for rhythmic flies.

\(^b\) Major peak. Relative peak intensity 100%

\(^c\) Minor peak. Relative peak intensity is indicated in brackets (%)

(Figure 3.16). After the temperature shift (Figure 3.15C) the peak of activity is even lower than control and occurs 1.2 hr later, determining a statistically significant difference from Canton S (Figure 3.16). However, analysis of actogram and histogram reveals that a synchronized activity peak is indeed visible, and free-running activity phase after temperature entrainment follows the previous TC (Table 3.3 and Figure 3.15D), suggesting that 2P-42 flies are synchronized to temperature in DD.

### 3.3.3 Free-run rhythmicity of EMS mutants

Wild-type flies strongly entrain to LD, as well as to TC. After any entrainment condition, be it light or temperature, the rhythm persists in constant conditions. We proceed by monitoring the free-running activity of the EMS mutants compared
Table 3.4: Free-running locomotor activity of EMS mutant and wild-type control. Flies were entrained in light or temperature conditions (as indicated) and then released in DD and constant temperature (25°C). Free-running period (τ) of rhythmic flies is calculated and autocorrelation values are shown. RI (Rhythmicity Index) and RS (Rhythmicity Statistic) determines the statistical significance and strength of the rhythm. RI and RS > 0.1 and 1.0 respectively, indicate statistical significance (based on autocorrelation). See Materials & Methods for details. SEM: Standard Error of the Mean.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>% rhythmic</th>
<th>τ (h) ± SEM</th>
<th>RI ± SEM</th>
<th>RS ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>After LD entrainment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canton S</td>
<td>42</td>
<td>97.6%</td>
<td>24.4 ± 0.1</td>
<td>0.38 ± 0.02</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>2T-30</td>
<td>59</td>
<td>42.4%</td>
<td>24.0 ± 0.8</td>
<td>0.26 ± 0.02</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>2T-38</td>
<td>47</td>
<td>51.1%</td>
<td>24.8 ± 0.2</td>
<td>0.29 ± 0.02</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>2P-42</td>
<td>37</td>
<td>81.1%</td>
<td>24.3 ± 0.2</td>
<td>0.28 ± 0.02</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>After LL + 25:16°C temperature entrainment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canton S</td>
<td>23</td>
<td>82.6%</td>
<td>24.7 ± 0.2</td>
<td>0.25 ± 0.01</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>2T-30</td>
<td>22</td>
<td>36.4%</td>
<td>24.0 ± 0.3</td>
<td>0.24 ± 0.02</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>2T-38</td>
<td>15</td>
<td>66.7%</td>
<td>24.1 ± 0.5</td>
<td>0.16 ± 0.02</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>2P-42</td>
<td>16</td>
<td>43.8%</td>
<td>23.7 ± 0.8</td>
<td>0.21 ± 0.03</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>After DD + 25:16°C temperature entrainment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canton S</td>
<td>36</td>
<td>91.7%</td>
<td>24.2 ± 0.21</td>
<td>0.29 ± 0.02</td>
<td>2.30 ± 0.18</td>
</tr>
<tr>
<td>2T-30</td>
<td>31</td>
<td>16.1%</td>
<td>24.0 ± 1.19</td>
<td>0.21 ± 0.03</td>
<td>1.62 ± 0.23</td>
</tr>
<tr>
<td>2T-38</td>
<td>33</td>
<td>69.7%</td>
<td>24.4 ± 0.24</td>
<td>0.28 ± 0.02</td>
<td>2.27 ± 0.18</td>
</tr>
<tr>
<td>2P-42</td>
<td>40</td>
<td>87.5%</td>
<td>23.7 ± 0.20</td>
<td>0.27 ± 0.02</td>
<td>2.20 ± 0.15</td>
</tr>
</tbody>
</table>

to wild-type control, after light and temperature entrainment. The phase of the clock can be reset by the LL to DD transition and we did not address this possibility for our EMS mutants. However, we calculated free-running rhythmicity after LD, LL and TC and DD and TC conditions.

Line 2T-30 exhibits a very low rhythmicity in DD after any entrainment conditions. After LD cycles, 42% of flies are still rhythmic; after LL and TC, only 36% shows rhythmicity and the percentage drop to 16% after DD and TC (Table 3.4).

50% of 2T-38 mutant flies are not rhythmic after LD entrainment, while more than 65% free-run after temperature entrainment (both in LL or DD). In contrast to 2T-30 this allows determination of the phase of the free-running activity peak.
after TC. Although the flies do not look synchronized during temperature cycles, the free-running phase follows that of the previous warm phase, suggesting that the clock is actually entrained and it free-runs in phase with the previous temperature cycle (see Figure 3.15A and Table 3.3).

Mutant line 2P-42 is weakly rhythmic (44%) after LL and TC, while it free-runs after LD and DD & TC (more than 80% of flies are rhythmic). The free-running phase is synchronized to the previous regime, and the rest-activity pattern during TC resembles that of wild-type (Figure 3.15), indicating that 2P-42 can entrain to temperature only in DD and not in LL.

Free-running period is slightly longer than 24 hours for all the genotypes (after any entrainment conditions), with no significant difference among control and mutants. The only exception is 2P-42 after LL and TC, which free-runs with a 23.7 hr period. Note, though, a low rhythmicity and a large SEM (Table 3.4).

Circadian clocks are temperature compensated. This means that free-running period is almost invariant over a wide range of physiological temperatures and the “temperature factor” ($Q_{10}$) is about 1 (see Introduction and Pittendrigh, 1954). The mechanism underlying temperature compensation is not known. However, mutations in the period gene compromise temperature compensation: per$^{L}$, for instance, in addition to exhibiting a very long free-running period (27–30 hr) also has impaired temperature compensation (Konopka et al., 1989 and Figure 3.17).

We investigated whether the EMS mutants exhibited a different free-running behaviour at different constant temperatures. We first entrained mutant and control flies to LD and then released them in DD, at three different temperatures: 18°C, 25°C and 29°C. All EMS-mutants tested show similar period length compared to the wild-type control (Figure 3.17) and they are all temperature compensated. Line 2T-30 has a slightly longer period at 29°C but this is probably reflecting
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Figure 3.17: Average free-running period length of controls and EMS mutants at different constant temperatures. EMS mutant and wild-type (Canton S) flies are temperature compensated. per<sup>L</sup> in addition to exhibiting a long free-running period also lacks temperature compensation (27.2 hr at 18°C, 28.4 hr at 25°C and 29.5 hr at 29°C). White columns indicate free-running period of rhythmic flies at 18°C, grey columns at 25°C and black columns indicate period at 29°C. Error bars indicate SEM. Number of individuals tested and of rhythmic flies are shown in the table underneath the graph.

3.4 2T-30 is a locomotor output mutant

Given that line 2T-30 is largely arrhythmic in DD after any entrainment condition (Table 3.4 and Figure 3.18B), we wondered if this mutation affects the central clock mechanism.

Together with locomotor activity, a different behavioural output of the clock
Chapter 3. Mutagenesis Screen

Figure 3.18: Locomotor activity of 2T-30 and controls. Average actogram (n=16), single-fly actogram, autocorrelation, MESA and χ²-periodogram of (A) Canton S and (B) the 2T-30 mutant. Flies were entrained in 5 LD cycles and then released to DD (25°C). Autocorrelation, MESA and periodogram are calculated during the DD part of the experiment only. Data of one representative fly are shown.

is the eclosion rhythm, i.e. the adults emergence from the pupal case. Eclosion exemplifies a population rhythm that is equally affected by clock mutants (perL,
CHAPTER 3. MUTAGENESIS SCREEN

A  Canton S (n=1736)

B  2T-30 (n=1586)

Figure 3.19: Eclosion profile of (A) Canton S and (B) 2T-30 during 3 days of a LD cycle, followed by 4 days in DD (20°C). The ordinates of the two upper plots depict number of emerging adults per 30 min bin. The total number of eclosed flies is indicated next to the genotype. Left-hand panel (raw activity) displays the actual number of eclosed flies, and the right-hand panel displays 4-hr low-pass filtered version of the left plot (filtered activity). The lower plots show analysis of eclosion rhythms performed in the same way as that for locomotor activity (see Figure 3.18).
per* and per01) as locomotor rhythms (Konopka and Benzer, 1971). Figures 3.18 and 3.19 shows a comparison of locomotor and eclosion behaviour of 2T-30 and wild-type control in a LD to DD experiment. As described above, locomotor behaviour of adult 2T-30 flies is largely arrhythmic in DD. The left-hand panel (in 3.18B) shows an average actogram in which the typical locomotor activity during LD to DD is visible; the activity becomes arrhythmic as soon as the mutant flies are released to DD. The right-hand panel shows an actogram of a single representative fly (compare to wild-type control in A). We then investigated free-running adult-emergence profiles of 2T-30 in the same condition. In a LD cycle, wild-type flies mainly eclose in the morning, before the dark-to-light transition (Figure 3.19). This rhythm persists in constant conditions, with a period of circa 24 hours (Pittendrigh, 1954). Both Canton S and 2T-30 exhibited eclosion rhythmicity (Figure 3.19A–B), and interestingly, 2T-30 exhibited a even “sharper” emergence peak than Canton S (probably due to different genetic backgrounds. See Chapter 6 for more details about eclosion). The eclosion rhythm of 2T-30 is strong and persists for several days in DD, with a period of 23.8 hours. The eclosion rhythm is generated by the central clock located in the brain and in the prothoracic gland (PG), and particularly the LN neurons and the PG are required to maintain rhythmicity in DD (Blanchardon et al., 2001; Myers et al., 2003 and Introduction). The 2T-30 mutation also does not alter rest-activity pattern in LD. Taken together, these data indicate that the mutation, which characterizes line 2T-30, does not affect central oscillator genes. The gene mutated reveals a new component of the locomotor output pathway, in addition to being defective in temperature entrainment.
3.5 Uncoordination phenotype

A previous genetic screen led to the isolation of a temperature mutant, named \textit{nocte}, affecting temperature entrainment (Glaser and Stanewsky, 2005, 2007). It has been recently shown by our group that chordotonal (ch) organs are involved in synchronization of the circadian clock of \textit{Drosophila melanogaster} to temperature cycles (Sehadova et al., 2009). In this paper we reported that \textit{nocte} mutants exhibit an “uncoordinated” phenotype when exposed at 37°C for 90 minutes (for more details see Chapter 7 and Cook et al., 2008), likely caused by fluid loss from ch organs due to structural defects.

We questioned whether the \textit{2T-30} mutants exhibit a similar phenotype. Figure 3.20 shows that \textit{2T-30} flies lose the ability to walk properly if exposed to 37°C for 90 minutes and exhibit an “uncoordinated” phenotype. After increasing environmental humidity in parallel to temperature (37°C) — by introducing a filter paper soaked with water in the chamber where the flies were monitored — we were able to rescue completely their uncoordinated phenotype. Given that the same phenotype is observed in \textit{nocte} (Sehadova et al., 2009) and \textit{spam} mutants (Cook et al., 2008), and that both genes are affecting the structural function of the chordotonal organs, it is possible that \textit{2T-30} also has an effect at the structural level of the ch organs. Further studies investigating morphological and structural alterations of ch organs will shed light on a possible ch role of this mutant to the temperature signalling pathway.
Figure 3.20: Uncoordination phenotype of EMS mutant 2T-30 and control (Canton S). Flies were raised at 25°C and then transferred to 37°C where they were monitored every 15 minutes for their ability to walk. The percentage of normal walking flies compared to the uncoordinated ones is shown. A) Mutant flies exposed to heat and low relative humidity became quickly uncoordinated. B) The uncoordination phenotype can be completely rescued by exposing the flies to high temperature with high humidity. At least 3 independent experiments (of 10 flies per genotype each) have been performed. Error bars indicate SEM. In (B), Canton S and 2T-30 data overlap.
3.6 Mapping of mutant line 2T-30

3.6.1 Complementation test

The EMS mutagenesis screen led to the isolation of three mutants with impaired per-luc synchronization to temperature cycles and defects on entrainment of the locomotor activity to TC. To reveal which genes are affected and elucidate the molecular basis of temperature entrainment it is crucial to map the mutations.

Given that the chemical-induced mutant line 2T-30 exhibited the strongest phenotype among the three lines isolated in term of aperiodic per-luc expression, arrhythmicity of locomotor behaviour in constant conditions and inability to entrain rest-activity pattern to TC, we decided to initiate our mapping experiments with this mutation.

The mutagenesis and crossing scheme applied have been performed in order to isolate putative mutations linked to chromosome 2 and 3 (see Figure 3.2). According to this scheme, 2T-30 is located on chromosome 2. We first investigated whether the mutation complemented the other 2nd-chromosome mutants, known to affect circadian clock. tim01 and vrille1 are central clock mutants (Sehgal et al., 1994; Blau and Young, 1999) and were our first candidates.

Homozygous 2T-30 flies were crossed with homozygous tim01 and vrille1 flies. The heterozygous progeny for 2T-30 and tim (or vri) mutations were tested for their ability to entrain to TC. If 2T-30 affected the same gene as, for instance, tim01, the progeny would not possess a normal copy of the gene and therefore would exhibit the mutant phenotype as the parental lines. In contrast, if the gene affected in line 2T-30 is different from tim (or vri), complementation would occur resulting in a wild-type phenotype. We also tested a range of 2nd chromosome transient receptor potential (trp) channel mutants, because they are candidates
for mediating temperature entrainment (see Chapter 5).

Figure 3.21A–H shows complementation test between 2T-30 with two clock genes (tim and vri), a TRP channel mutant (trpM) which exhibits temperature entrainment defects (see Section 5 for more details) and three more TRP channel mutants which do not show temperature entrainment defects (painless, nompC and double mutant trp trp\(^l\)). The wild-type phenotype of the transheterozygous tim\(^{01}\)/2T-30 was only partially restored in this condition (LL and TC). We then repeated the complementation test and assayed the progeny 2T-30/tim\(^{01}\) in a LD to DD experiment (Figure 3.21I–K), since tim\(^{01}\) and most of the 2T-30 mutants are arrhythmic in DD (Sehgal et al., 1994 and Table 3.4). All the tested flies showed strong rhythmicity in DD conditions, indicating that the two genes complemented each other and therefore 2T-30 does not affect timeless.

Genetic complementation analysis of 4 trp genes suggests that 2T-30 does not affect trpM, painless, trp trp\(^l\) nor nompC (Figure 3.21E–H). Although 2T-30/pain\(^3\) is not completely normal, complementation to the the allele pain\(^1\) restores completely the wild-type phenotype. Both the pain alleles are induced by P-element insertion in the start region of the pain gene and they are both null mutation (Tracey et al., 2003).

The attempt to map the mutation by meiotic recombination with second multiple-marked chromosome which carry recessive mutations associated with morphological markers in known positions, failed. At the moment of writing a finer localization of the mutation is being carried out by deficiency mapping covering all the left arm of chromosome 2.
CHAPTER 3. MUTAGENESIS SCREEN

Figure 3.21: Complementation test between 2T-30 and 2nd-chromosome mutants during LL and 12:12 hr − 25:16°C TC. Rest-activity pattern of (A) Canton S, wild-type control, (B) 2T-30 mutant control, (C) 2T-30 over tim01, (D) 2T-30 over vri1, (E) 2T-30 over trpM, (F) 2T-30 over painless3, (G) 2T-30 over painless1 and (H) 2T-30 over trp trpl. White bars represent average activity during warm (25°C) and grey bars activity during cold phase (16°C). Number of individual is indicated in brackets. (I, J) Average actogram of y w; tim01/tim01 (n = 12) and y w; tim01/2T-30 (n = 16), respectively, for 4 days in LD followed by 7 days in DD (25°C). Note that DD rhythmicity is rescued in the heterozygous flies (100%, τ = 24.1±0.05, RI=0.51±0.02, RS=4.8±0.20). (K) DD analysis of a representative y w; tim01/2T-30 fly.

3.7 Summary

• Screen of 1637 EMS-induced mutant lines analysing per-luc expression in isolated legs during LL and 12:12 hr 25:16°C TC.

• Isolation of three lines, 2T-30, 2T-38 and 2P-42, which have impaired per-luc expression specifically during TC (both in LL and DD) and normal in LD conditions.

• Locomotor activity of the three mutant lines is affected specifically during
25:16°C TC and normal during LD conditions. In particular, they all exhibit a sharp peak of activity after the increase of temperature and a less pronounced peak of activity in the second half of the warm phase. Statistical analysis at different and smaller temperature intervals revealed that all mutants exhibit overall defects of entrainment to temperature, but only mutant line $2T-30$ fail to synchronize at 29:25°C TC when considering the evening anticipation peak only. In DD and TC, $2T-30$ mutants do not synchronize their locomotor activity to temperature and they are mainly arrhythmic in constant conditions. $2T-38$ flies exhibit mainly a reaction peak to temperature and the entrainment peak is barely visible (and not significant). Line $2P-42$, instead synchronize to TC in DD but not in the same extent as controls.

- Locomotor activity of $2T-30$ mutants is mainly arrhythmic in constant conditions, while adult emergence is normal, suggesting that the mutation affects the locomotor output pathway in addition to the temperature entrainment one.

- The mutant line $2T-30$ exhibit an “uncoordinated” phenotype when exposed to high temperature and low humidity, suggesting involvement of the chordotonal organs for the gene affected.

- $2T-30$ complement with tim, vri, trpM, pain and double mutant trp trpl in LL and TC, suggesting that the mutation does not hit any of those second chromosome genes.
Chapter 4

Role of the forkhead domain 3F gene in the circadian clock

4.1 Screen of 148 RNAi lines for defects in the temperature synchronization of the circadian clock

In parallel to the chemical mutagenesis screen, we screened a library of RNAi lines generated by the National Institute of Genetics Fly Stock Center (Japan) and generously provided by François Rouyer’s group (CNRS, Paris, France).

We combined the UAS-GAL4 system (Brand and Perrimon, 1993) with the bioluminescence assay monitoring real-time per-luc expression in living flies. Driven by GAL4, the RNAi flies produce double-stranded RNA which induces knockdown of the specific target gene in vivo. We used a tim-gal4 (line 27) driver line in order to down-regulate the expression of targeted genes in all clock cells (tim-expressing cells, see Kaneko and Hall, 2000).
In Appendix A are listed the 148 lines we screened, which covered 96 different genes (for most of the genes, 2 independent insertion lines were available). Figure 4.1 shows the crossing scheme we applied to generate flies in which we could assay the RNAi lines for their ability to entrain XLG-luc expression to temperature cycles in constant light. For reasons described in the previous chapter, we monitored per-luc expression in isolated legs, kept in an insect-tissue culture media. The XLG-luc transgene encodes for the full PERIOD protein (except for the last 10 aa) fused to LUCIFERASE (see Figure 3.1). This construct can partially rescue rhythmicity and restore per spatial distribution in a per^{01} background (Veleri et al., 2003).

Among the 148 lines screened we isolated one which failed to synchronize per expression to temperature in isolated legs. tim-gal4 down-regulation of 12632-R2 led to misexpression of per-luc during LL and TC and during LD cycle (Figure 4.2). The line 12632-R2 affects the gene CG12632 (Flybase annotation symbol) which encodes the FORKHEAD DOMAIN 3F transcription factor, or FD3F (see below for more details on the gene). In both LD and TC conditions, XLG-luc expression is drastically reduced in isolated legs of tim-driven RNAi flies, in terms of cycling amplitude and overall expression level. The two upper graphs of Figure 4.2 show the bioluminescence readings of control and fd3F-RNAi-R2, respectively. In TC

**Screen of UAS-RNAi library driven by tim-gal4**

$$\text{P: } \varnothing \varnothing \text{ UAS-RNAi } \times \varnothing \varnothing \frac{y w}{y} \text{; tim-gal4; } \frac{XLG-luc}{CyO}\text{; TM3}$$

$$\text{F}_{1}: \varnothing \varnothing \frac{+}{y} ; \text{ tim-gal4; } \frac{XLG-luc}{UAS-RNAi} \text{, or } \frac{+}{y} ; \text{ tim-gal4; } \frac{XLG-luc}{UAS-RNAi} \rightarrow \text{ to test}$$

**Figure 4.1:** An UAS-RNAi library was crossed to tim-gal4 (line 27), in order to knockdown specific genes in all the clock cells. Driver line carries also a per-luc transgene (XLG-luc, line 1:1). CyO and TM3 are balancer chromosome for autosome 2 and 3, respectively. ♀: virgin female flies, ♂: male flies.
Average reading from isolated legs

A Light-Dark (25°C)

B fd3F-RNAi

C Normalized

D LL and 12:12 hr 25:16°C

E fd3F-RNAi

F Normalized

Figure 4.2: XLG-luc bioluminescence expression from isolated legs of fd3F-RNAi-R2 and control flies in light and temperature entrainment. A–B) LUC readings of controls and flies in which fd3F is down-regulated by the tim-gal4 driver (line 27), respectively, during LD cycles (25°C). D–E) per-luc expression during LL and 12:12 hr 25:16°C temperature cycles. In both conditions, the level of per-luc expression and the cycling amplitude is drastically reduced in the RNAi line compared the control (which carries the same XLG-luc). (C, F) To better visualize the effective down-regulation of the period-luciferase gene caused by the RNAi line, we plotted the same graphs as above normalizing the bioluminescence reading to the average value for each genotype. Control XLG-luc: black dashed line. fd3F-RNAi-R2: red solid line. Number of individuals tested is indicated in each graph with the genotype. In LD graphs, white/black bars at the bottom indicate light and dark phase, respectively. In TC graphs, red/blue bars indicate warm and cold phase, respectively. cps: counts per second. ZT: Zeitgeber Time (ZT0 = lights-on or temperature-up. ZT12 = lights-off or temperature-down).
conditions, the expression levels are between 1500 and 3000 counts per second (cps) for the control and between 400–800 cps for the RNAi line. During LD conditions, the difference is similar, if not even higher: 2000–6000 cps for control versus 400–1000 cps of the RNAi. Given the very low level of LUCIFERASE activity in the RNAi flies compared to controls (which carry the same XLG-luc transgene), we normalized the expression values to the average values for each genotype (Figure 4.2C,F). This allows a better visualization of the effects of fd3F down-regulation not only on the overall per-luc expression levels, but also on the amplitude of per-luc cycling. The amplitude of fd3F-RNAi is 2-fold reduced in LD and 1.5-fold in TC conditions, compared to control. Note also that the phase of per-luc expression during LL and TC is 2–3 hours advanced compared to LD conditions, as we already observed in the previous chapter (Section 3.2).

Analysis of per-luc expression of tim-gal4;fd3F-RNAi-R2 in the whole intact fly was not performed because tim-gal4:27-driven fd3F-RNAi expression induces adult lethality (flies die 2–3 days after hatching, see below).

A second RNAi line targeting the same gene is available, 12632R-1 (Table A.1). When driven by tim-gal4, it induces adult lethality in the same way as the line 12632R-2. Analysis of per-luc expression in legs in LL and 12:12 hr 25:16°C TC gives comparable results, in term of reduction of per-luc expression level and 2-fold decrease of cycling amplitude (Figure 4.3). This suggests specificity of the phenotype of the line 12632R-2, rather than insertional effects due to the presence of the RNAi transgene. Given the reproducibility of the phenotype between the 2 lines, the line 12632R-1 was not investigated further.

Hereafter, all the results of this section refer to the line 12632R-2, which we will call fd3F-RNAi.
Figure 4.3: XLG-luc bioluminescence expression of fd3F-RNAi-R1 and controls in LL and 12:12 hr 25:16°C temperature entrainment. per-luc expression level is drastically reduced and the cycling amplitude is 2-fold decreased. For description of plots, see Figure 4.2
4.2 Behavioural analysis of \textit{fd3f}-RNAi

<table>
<thead>
<tr>
<th>Driver</th>
<th>Lethality (stage)</th>
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<tbody>
<tr>
<td>\textit{tim-gal4:27}</td>
<td>Adult day 2–3</td>
</tr>
<tr>
<td>\textit{tim-gal4:16}</td>
<td>Adult day 4–5</td>
</tr>
<tr>
<td>\textit{tim-gal4:67}</td>
<td>Adult day 2–3</td>
</tr>
<tr>
<td>\textit{nocte-gal4}</td>
<td>Pupae</td>
</tr>
<tr>
<td>\textit{repo-gal4}</td>
<td>Pupae</td>
</tr>
<tr>
<td>\textit{elav-gal4}</td>
<td>n.l.</td>
</tr>
<tr>
<td>\textit{cryBN-gal4}</td>
<td>n.l.</td>
</tr>
<tr>
<td>\textit{Pdf-gal4}</td>
<td>n.l.</td>
</tr>
<tr>
<td>\textit{F-gal4}</td>
<td>n.l.</td>
</tr>
</tbody>
</table>

Table 4.1: Different lethality effects on \textit{fd3F}-RNAi caused by several driver lines. n.l.: no lethality

When the \textit{fd3F} gene is down regulated with the \textit{tim-gal4:27} driver, flies do not survive the third day of adult stage, suggesting severe defects induced by the RNAi. Light-microscopy inspection did not reveal any gross morphological abnormalities. We tested the effect of several \textit{tim-gal4} lines, which only differ for the insertional position on the chromosomes but not for spatial distribution of \textit{timeless} expression (Kaneko and Hall, 2000). As stated in Table 4.1, there is a slight difference between the onset of lethality induced by line 16 (4–5 days) and lines 27 and 67 (2–3 days). Kaneko and Hall (2000) showed that the \textit{tim-gal4} lines applied in this study have the same spatial expression both in larvae and in the adult. However, there could be some slight spatially differences not detected by Kaneko and Hall and also differences in the expression level (caused by insertion site).

Since \textit{tim-gal4}-induced lethality, locomotor activity of \textit{tim-gal4}/\textit{fd3F}-RNAi flies is difficult to be analysed and we could monitor only 4 days of activity in LD conditions. Although it looks as if the morning peak, which anticipates the transition from dark to light, is missing (or not very pronounced), the LD activity looks normal and comparable to control (Figure 4.4F).

Expressing \textit{fd3F}-RNAi with other drivers, which express in different tissues than \textit{tim-gal4}, does not induce adult lethality and therefore the rest-activity pat-
12:12 hr Light-Dark cycles (25°C)

<table>
<thead>
<tr>
<th></th>
<th>Line</th>
<th>Duration</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Canton S (10)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>elav-gal4 (16)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>cryBN-gal4 (12)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>pdf-gal4 (18)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>F-gal4 (11)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>tim-gal4 (12)</td>
<td></td>
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LL and 12:12 hr 25:16°C Temperature cycles

<table>
<thead>
<tr>
<th></th>
<th>Line</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Canton S (10)</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>elav-gal4 (15)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>cryBN-gal4 (12)</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>pdf-gal4 (16)</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>F-gal4 (10)</td>
<td></td>
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</tbody>
</table>

**Figure 4.4:** Rest-activity patterns of *fd3F*-RNAi driven by different GAL4 driver lines, as indicated. Flies were first synchronized to LD cycles for 5–7 days at 25°C (A–F), then subjected for 7 days to constant light and 12:12 hr 25:16°C temperature cycles which was in opposite phase compared the previous LD (G–K). F) *tim-gal4* -driven silencing of *fd3F* induces lethality. However, when line 16 is used, flies survive until day 4–5 of adult stage, so it is possible to monitor rest-activity pattern up to 4–5 days. Only activity during LD entrainment has been analysed and it is normal. Number of individuals tested is indicated in brackets.
terns could be monitored (Figure 4.4 and Table 4.1). When we used the neuronal-specific driver \textit{elav-gal4}, which express GAL4 in the CNS and PNS (Luo et al., 1994), \textit{fd3F}-RNAi flies can synchronize both to LD and LL and TC with no difference compared to control (4.4B,G).

Interestingly, a driver widely expressed like \textit{elav-gal4} does not kill the flies, while \textit{tim-gal4} does (Table 4.1). This suggests either that FD3F executes essential functions in non-neuronal \textit{tim}-expressing cells or that \textit{elav-gal4} is not as strongly expressed as \textit{tim-gal4}. Moreover, when we silenced \textit{fd3F} with the \textit{nocte-gal4} driver, which is broadly expressed in the fly, including neurons and glia cells in CNS (Sehadova et al., 2009), \textit{fd3F} flies do not hatch (flies die at the pupal stage). Given that the glia-specific driver \textit{repo-gal4} (Sepp et al., 2001) also induces \textit{fd3F}-RNAi pupal lethality, we could speculate an essential role for FD3F in glia cells.

We then investigated locomotor behaviour of \textit{fd3F}-RNAi when down-regulated by drivers specific to subgroups of clock neurons. CRY is expressed in small- and large-LN\textsubscript{v}s, 3 LN\textsubscript{d}s and some DN\textsubscript{1} neurons (Yoshii et al., 2009b). If \textit{fd3F} down-regulation is restricted to \textit{cry} expressing cells using a \textit{cry-gal4} line (Emery et al., 2000), flies entrain normally to LD cycles (Figure 4.4C) and to temperature cycles: the evening peak of activity is advanced and flies are more active during the cold phase compared to controls (Figure 4.4I). The mean activity during the cold phase (per 30 minutes interval) is 20.3 for \textit{cry-gal4/}fd3F-RNAi versus 9.0 for the wt control. The overall pattern of activity, in particular if we consider the anticipation from warm to cold, is normal. We observed similar results when we crossed \textit{pdf-gal4} to UAS-fd3F-RNAi. In this case, \textit{fd3F} is silenced in the PDF-positive LN\textsubscript{v}s (Park et al., 2000). Locomotor activity in LD cycles is again normal but in TC flies do not exhibit a clear and pronounced evening anticipation of the transition to 16°C and flies are more active during the cold phase (mean activity
A more severe temperature entrainment phenotype was seen when we silenced \textit{fd3F} with \textit{F-gal4} (Figure 4.4K), a chordotonal (ch) organ driver line (Kim et al., 2003; Sehadova et al., 2009). LD entrainment is not affected (Figure 4.4E) but, interestingly, temperature entrainment in LL is compromised (Figure 4.4K). There is no evening anticipatory peak but a gradual increase of activity towards the middle of the day (as in \textit{Pdf-gal4}) but not a clear reduction afterwards. The overall level of activity in the cold phase is comparable to the controls (mean activity 12.6) but flies exhibit mainly a reaction to temperature changes, typical phenotype of “temperature mutants” (Glaser and Stanewsky, 2005; Sehadova et al., 2009). Although these are preliminary results, and analysis of \textit{F-gal4}-driven \textit{fd3F}-RNAi need to be addressed more in details and in different conditions, this is very intriguing. Our group already reported the importance of ch organs for temperature entrainment (see further sections and Sehadova et al, 2009). This data suggest a good potential candidate (a forkhead transcription factor) for TC-dependent transcription regulation in ch organs (see below and Discussion).

However, it is important to mention that \textit{F-gal4} is not expressed exclusively in neurons of ch organs as reported by Kim et al. (2003), but also in a number of putative chemoreceptive and mechanoreceptive external sensory organs located in the legs, labial and maxillary pulpus, wings, haltere and antennae. It is also expressed in the retina and neurons of the central brain, most prominently within the antennal lobes (Sehadova et al., 2009). I mention this because the function of FD3F could be executed in different structures than — or not only in — ch organs.

Finally we examined whether the down-regulation of the forkhead transcription factor has any effects on the free-running locomotor activity in constant conditions.
CHAPTER 4. ROLE OF FD3F

Table 4.2: Free-running locomotor rhythmicity of control (non-driven RNAi line) and fd3F-RNAi driven by cry-gal4 and Pdf-gal4. Flies were entrained in LD (for at least 3 days) then released to DD and constant temperature (25°C). The free-running period ($\tau$) of rhythmic flies is calculated and autocorrelation values are shown. RI: Rhythmicity Index. RS: Rhythmicity Statistic. SEM: Standard Error of the Mean.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>% rhythmic</th>
<th>$\tau$ (h) ± SEM</th>
<th>RI ± SEM</th>
<th>RS ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>+:fd3F-RNAi</td>
<td>11</td>
<td>90.1%</td>
<td>24.1 ± 0.2</td>
<td>0.45 ± 0.02</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>cry-gal4/fd3F-RNAi</td>
<td>18</td>
<td>94.4%</td>
<td>24.5 ± 0.1</td>
<td>0.45 ± 0.02</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Pdf-gal4;fd3F/+</td>
<td>18</td>
<td>100%</td>
<td>24.4 ± 0.1</td>
<td>0.47 ± 0.03</td>
<td>4.9 ± 0.3</td>
</tr>
</tbody>
</table>

When we silenced FD3F with cry-gal4 or Pdf-gal4 and monitored activity in constant conditions, flies free-run with a strong rhythm and normal period (Table 4.2). This suggests that FD3F does not execute essential clock function in Pdf- and cry-cells. Since Pdf-expressing ventro-lateral neurons (LNv,s) are necessary to generate DD free-run activity (Frisch et al., 1994; Blanchardon et al., 2001), we can speculate that FD3F function is not required to generate free-running activity driven by the Pdf cells.

4.3 FD3F affects PERIOD accumulation in LL and TC

We showed that tim-gal4-driven down-regulation of fd3F compromised period-luciferase expression, both during LD and during LL and temperature cycles. We wondered whether this is reflected at the protein level, i.e. if temporal PER accumulation is normal or not, as suggested by the abnormal per-luc expression. For this, we quantified PERIOD protein levels isolated from head-extracts of individuals that have been previously entrained in LD or LL and TC, at 4 hr intervals.

We showed that tim-gal4:16 driven knocked-down of fd3F induces adult lethality at day 4–5, thus we entrained flies to different environmental conditions from
the time of eclosion, in order to have enough days of entrainment before the flies died.

During LD entrainment, PER protein oscillates with a peak at ZT21 and trough at ZT9, both in control and in \textit{tim-gal4/fd3F-RNAi} flies (Figure 4.5). In the RNAi flies, we observed a slight extension of the PER trough (until ZT13), even though with a prominent error, indicating non-continuity between independent experiments. These data are conflicting with the previously described \textit{per-luc}
oscillation results, which indicated defects on per expression also in LD conditions. However, per-luc expression has been monitored in isolated legs, while the PER protein has been quantified from head-extracts.

During LL and TC entrainment, PER protein in wild-type heads oscillates with a smaller amplitude compared to LD conditions (Figure 4.5B–C), and the protein peaks 2–3 hours earlier (compare also Glaser and Stanewsky, 2005). This fits well with the advanced peak of per-luc expression and the earlier evening peak of locomotor activity in LL and TC compared to LD entrainment, described in the previous chapter (Section 3.2). PER in fd3F-RNAi flies, is highly accumulated and it seems to weakly oscillate with opposite phase compared the control, but with no statistical significance ($p > 0.05$, 1-way Anova). It is also difficult to distinguish different PER bands corresponding to the different forms of the phosphorylated protein (compare control at ZT1, for instance, and Edery et al., 1994), suggesting defects in PER phosphorylation and subsequent degradation during the warm phase of TC.

### 4.4 fd3F alters the phase of eclosion

RNAi-mediated down-regulation of fd3F in all clock cells (via tim-gal4) induces early lethality and therefore behavioural analysis of the knocked-down flies is restricted to a few days only. Therefore, we opted to investigate another circadian rhythmicity to complement the limited locomotor rest-activity pattern analysis.

We investigated the eclosion activity of fd3F-RNAi flies driven by tim-gal4 during LL and TC conditions. Pupae of Canton S wild-type cultures synchronize their eclosion rhythm to TC in 2 days and the peak of emergence occurs towards the end of the cold phase (Figure 4.6A–B). We silenced fd3F with 3 different
CHAPTER 4. ROLE OF FD3F

Constant light and 25:16°C Temperature cycles

A  Canton S (1332)

B  y w (1753)

C  tim-gal4:16/fd3F-RNAi (772)

D  tim-gal4:67/fd3F-RNAi (1889)

FIGURE 4.6
E  *tim-gal4:27:fd3F-RNAi* (1205)

F  *tim-gal4/fd3F-RNAi* (3753)

G
tim-gal4 driver lines (16, 67 and 27, see M&M), and then monitored the eclosion activity during LL and TC entrainment. The eclosion occurs rhythmically, synchronized with TC, but with a different phase compared the control. The flies eclose mainly during the warm phase (25°C) and no distinct eclosion peak is visible (Figure 4.6C–E). We did not observe any difference of eclosion activity when different tim-gal4 lines were used to silence fd3F. Therefore, we could combine the results of the 3 independent experiments in a single plot in which the phenotype was even more prominent (Figure 4.6F). Control cultures move their eclosion phase from the previous “LD-entrained” to the “TC-entrained” in the first 2 days, and they stabilize their phase with the temperature cycles from day 3. In contrast, fd3F-RNAi cultures always (and immediately) hatch in the warm-phase. Although this might be interpreted as lack of entrainment, analysis of the clock mutant per01 suggests that fd3F-RNAi exhibits a difference of eclosion phase rather than lack of synchronization (see chapter 6, Figure 6.2 and Discussion).

**Figure 4.6 (preceding page):** Eclosion profiles of control and fd3F-RNAi cultures in LL and TC. A–F) The left columns show double-plot actograms of flies emerged during LL and 12:12 hr 25:16°C TC, which was in opposite phase compared to the previous LD entrainment. Only the last dark cycle of the previous LD entrainment is indicated (in A–D). The two right-hand columns depict, respectively, the total number of flies emerged as a function of time (“raw activity”, middle plot) and 4-hr low-pass filtered versions of the middle plots (“filtered activity”, right-hand plot, see M&M for more details). The total number of flies emerged is indicated in brackets next to the genotype. Shaded areas refer to temperature conditions (grey shading: 16°C; white shading: 25°C). A–B) Canton S and y w control flies synchronize eclosion to TC in 2 days and, once synchronized, exhibit peak of emergence during the last part of the cold phase (ZT22–23). C–E) fd3F-RNAi driven by tim-gal4 eclose with a different phase, emerging mainly at 25°C, regardless of the tim-gal4 line used (16, 67 and 27, respectively). We thus combined the 3 independent experiments (F) to better visualize this phenomenon. G) Quantification of number of flies eclosed during the warm phase (white bar) compared the cold phase (grey bar). There is a statistically significant preference (F(1,2)=26.88, P<0.05, two-way ANOVA) in fd3F-RNAi to emerge during the warm phase compared to control flies. Plot generated from average of 3 independent experiments. Error bars indicate SEM.
To support the idea that *tim-gal4*/*fd3F*-RNAi has a different eclosion phase, we calculated the number of flies eclosed during the warm phase, compared to the ones emerged in the cold. Figure 4.6G shows a striking difference between the number of *fd3F*-RNAi flies which eclose during the warm phase (66 ± 5.6%) compared the control (32 ± 5.8%). The analysis of variance between the RNAi line and control reveals statistical significance \( F(1,2) = 26.88, P < 0.05, \text{ Two-way Anova} \).

The eclosion profile of *fd3F*-RNAi, induced by *tim-gal4* (line 67) has been monitored in free-running conditions (Figure 4.7). After LD entrainment, control flies (Canton S and *y w*, in our case) emerge rhythmically in DD with a strong period of circa 24 hours (24.5 and 23.8 hours, respectively). *fd3F*-RNAi flies also eclose rhythmically in constant conditions, with a period of 24.3 hours, although the rhythm is not as strong as in the control flies: the profile is “noisier” and the peak less “sharp” compared to control, which is also indicated by the lower RS values.

The eclosion peak of wt cultures is centred to the transition between the subjective dark- to light-phase, following the pattern of eclosion during LD cycles, where the majority of flies emerge at dawn (Figure 4.7 and Qiu and Hardin, 1996). Interestingly, *fd3F* knocked-down flies eclose mainly during the whole part corresponding the subjective day, and the peak is “broader” and centred at the middle of the subjective light-phase. This correlates with the eclosion activity during TC, which is phase-shifted (towards the warm phase) compared to the controls (Figure 4.7 and 4.6).

It has been shown previously that mutants can affect the phase of eclosion. Mutants for the RNA-binding protein LARK, for instance, exhibit an early-eclosion phenotype: *lark* mutant eclose several hours earlier than control both during LD
Eclosion of control and \textit{fd3F}-RNAi cultures in free-running conditions

\textbf{A} \textit{yw} (2016)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.7a}
\caption{Flies were entrained in LD and then released to DD (at 20°C). Only the last cycle of LD is included in the plot. The 3 left-hand columns are as described in Figure 4.6. The most right-hand plot depicts the autocorrelation values calculated for the DD part only. A) \textit{yw} and (B) Canton S flies exhibit free-running rhythm with a period of 23.8 and 24.5 hr, respectively. C) \textit{tim-gal4/fd3F}-RNAi (line 67) eclosed rhythmically with a period of 24.3 hr. Number of flies eclosed is indicated in brackets. D) Eclosion activity of the cultures depicted in (A–C) to which a 5-point moving average has been applied. \textit{fd3F}-RNAi flies eclose with a later phase compared to controls (arrows). Light- and dark-grey areas indicate subjective days and nights, respectively.}
\end{figure}

\textbf{B} Canton S (1147)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.7b}
\end{figure}

\textbf{C} \textit{tim-gal4/fd3F}-RNAi (1100)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.7c}
\end{figure}

\textbf{D}
and TC entrainment (Newby and Jackson, 1993). Accumulation of the clock proteins PER and TIM in lark mutant is normal as well as locomotor activity, suggesting that LARK acts specifically on the “eclosion output pathway” of the clock (McNeil et al., 1998; Schroeder et al., 2003). Our data, however, indicate abnormal PER-LUC expression and PER accumulation in fd3F-RNAi adults, together with the delayed-phase of eclosion and abnormal behaviour of adults during TC (when driven by F-gal4). The transcription factor nature of the gene, suggests more a role on clock gene expression, rather than on the output pathway (see Discussion).

4.5 Role and function of forkhead transcription factors

In the RNAi screen, monitoring real-time per-luc expression in tissue cultures kept in LL and TC, we isolated the line CG12632 as showing impaired period synchronization. The line affects the gene forkhead domain 3F (or fd3F) which encodes a forkhead domain containing protein. What are forkhead proteins?

Forkhead domains, known also as “winged helix”, are a family of DNA-binding domains highly conserved between different eukaryotic transcription factors, from yeast to humans (Lai et al., 1993). They were first discovered in Drosophila as proteins required for the proper development of the terminal structures of the anterio- and posterio-gut. forkhead mutants fail to develop correctly and the embryo shows a “spiked head” — hence the name (Weigel et al., 1989). While the detailed temporal and spatial expression of forkhead domain proteins differ, they are expressed in many, if not all, tissues (Lai et al., 1993). The mammalian counterparts of Drosophila’s forkhead proteins are Hepatocyte Nuclear Factor-3 (HNF-
3), named because they are required to activate specifically expression of genes in the rat and mouse liver (Clark et al., 1993). Interestingly, mammalian HNF-3 transcription factors are under the control of the circadian clock and their expression is regulated by clock-controlled gene (CCG) promoters (Bozek et al., 2009). HNF-3 mRNA expression is reduced by the Clock mutation and up-regulated in Cry-deficient mice (Oishi et al., 2003).

In Drosophila there are 17 forkhead domain protein encoding genes. Only 6 of them are characterized and studied; Those include forkhead (fkh), sloppy paired 1 (slp1), sloppy paired 2 (slp2), crocodile (croc), jumeaux (jumu) and biniou (bin) (reviewed by Kaufmann and Knochel, 1996; Lee and Frasch, 2004). Phylogenetic analysis with each other and with mouse orthologues allows classification of 13 members (out of 17) to 10 subgroups (Figure 4.8). The classification considers sequence comparison with forkhead domain in mouse and rat rooted with the forkhead domain sequence from the yeast protein Fhl1p as an outgroup (Lee and Frasch, 2004). The remaining 4 genes cannot be grouped within any subclass that are known in chordates, and are named according their cytological position (Lee and Frasch, 2004). One of these 4 is fd3F which maps to position 3F on chromosome X (3F2), and it does not have a mouse homologue.

fd3F mRNA expression is broadly distributed during early embryonic stages (until stage 12, see Figure 4.9, taken from Lee and Frasch, 2004). Interestingly, after stage 12, fd3F expression is restricted to a subgroup of cells which, based upon their position and arrangements, corresponds to chordotonal sensory organs and their precursor (Lee and Frasch, 2004). These observations are in good agreement with our findings, which showed that F-gal (ch organ-specific) down-regulation of fd3F exhibits defects of synchronization of locomotor behaviour to temperature cycles (Figure 4.4K).
CHAPTER 4. ROLE OF F3Df

Figure 4.8: Phylogenetic tree of *Drosophila* and mouse forkhead domains. The tree is constructed from sequence alignment of all *Drosophila* forkhead domains with each other and with mouse orthologs, using the forkhead domain sequence from the yeast protein Fhl1p as an outgroup. *Drosophila* proteins are shown in black boxes. CG numbers refer to Computed Gene Products as predicted by the Berkeley Drosophila Genome Project (BDGP). The bar denotes 10% divergence. Nodes with a bootstrap value of < 50% are unmarked, those at 50–75% are marked +, 77-95% ++, and 95-100% ++++. This figure is taken from Lee and Frasch (2004).
Given that ch organs are required for clock entrainment to TC (Sehadova et al., 2009), we can speculate that $fd3F$ plays a role in this mechanism and in these structures. The transcription factor nature of the protein suggests a possible regulation of important target genes required for temperature entrainment ($nocte$?), which, so far, are mainly unknown.

It has been reported that a gene related to the class of forkhead domain transcription factors is involved in circadian rhythm. *Circadianly Regulated Gene 1* ($Crg-1$) is circadianly expressed with the same phase as *per* and *tim* and its spatial distribution overlaps with that of *per* — at least in *Drosophila* heads (Rouyer et al., 1997). Interestingly, genomic sequence analysis of different *Drosophila* species showed that $Crg-1$ is a chimeric gene originating from a genomic duplication and fusion of the $fd3F$ and *Tousled-like kinase* ($tlk$) genes (Hogan and Bettencourt, 2009 and Figure 4.10). $fd3F$ is present in all the *Drosophila* species according to this study, while $Crg-1$ is absent in evolutionary related species like *D. simulans* and *D. yakuba* and appears only in *D. melanogaster* (which originated $\sim$2.3 million years ago) (Hogan and Bettencourt, 2009). This explains why $Crg-1$ exhibits high sequence similarity with $fd3F$. However, even if $Crg-1$ probably acquired the novel function as circadian regulator only after the duplication event (Hogan and Bettencourt, 2009), we can not exclude a possible circadian regulation for $fd3F$ as
Analysis of protein interaction libraries based on genome-wide yeast 2-hybrid data, reveals putative interactions of FD3F with 2 proteins: NINAE and X11L. We considered three independent Drosophila interaction databases, BioGrid (Stark et al., 2006), IntAct (Hermjakob et al., 2004) and Mint (Zanzoni et al., 2002): the three of them indicated identical putative interactions.

The neither inactivation nor afterpotential E (ninaE) gene encodes the major visual pigment protein (Rhodopsin 1) contained in Drosophila photoreceptor cells.
R1–R6 (O’Tousa et al., 1985). *ninaE* mutants lack the rhodopsin protein Rh1 and although the light-sensitivity of the mutant is reduced in circadian context (Stanewsky et al., 1998), it does not affect the light-dependent degradation of TIM (Yang et al., 1998).

The *Drosophila* X11L is homologue to the mammalian X11L proteins, which interact with the cytoplasmic domain of the amyloid precursor protein (APP) and act as adapter proteins during the regulation of neural function (Hase et al., 2002). X11L plays an important role in the preservation and/or degeneration of neuronal functions (Hase et al., 2002). In *Drosophila*, the expression of the X11L is neural tissue-specific, and its overexpression results in destruction of the eye morphology probably due to enhanced developmental apoptosis (Hase et al., 2002; Vishnu et al., 2006).

Based on these observations, it is difficult to speculate a role for FD3F, in particular, it is not known whether *fd3F* is co-expressed with *ninaE* and/or *x11l*. Furthermore, the interactions are suggested by databases which are based on the yeast system and thus the interactions may not occur *in vivo*.

### 4.6 Summary

- *tim-gal4*-driven knock-down of *fd3F* reduces the expression level and cycling amplitude of *per-luc* both during LD and TC conditions.

- PER protein during TC is not cycling and is highly expressed throughout the day, while it is normal in LD conditions.

- Adult *tim-gal4* down-regulated flies do not survive more than 4–5 days after hatching and display a normal locomotor activity in LD.
• *F-gal4*-driven down-regulation compromises the ability of the flies to synchronize their rest-activity pattern to TC.

• *fd3F* embryonic expression is restricted to ch organs suggesting an active role for the transcription factor in development of the ch organs, supporting the observation of locomotor synchronization defects of the RNAi line when driven by *F-gal4*.

• Eclosion activity is rhythmic in DD conditions, and therefore the central oscillator is not affected. However, *tim-gal4*-driven *fd3F*-RNAi flies eclose mainly during the warm phase in temperature entrainment conditions with implications to the regulation of eclosion phase.
Chapter 5

Involvement of TRP channels in temperature synchronization of the circadian clock

5.1 Background

In the previous chapters, we described two screens we performed in order to isolate novel components playing a role in the entrainment of the circadian clock of Drosophila melanogaster. The screens were based on random chemical mutagenesis and screen of RNAi library, respectively. These two approaches, although different, share very similar principles, i.e. “forward genetics”. This means that we started inducing mutants (or making use of available RNAi libraries) and screen for a phenotype — in our case, defects of entrainment of the circadian clock to TC. The mutation responsible for the mutant phenotype is then identified (step unnecessary in the case of the RNAi screen) and further analyses are conducted to determine the role of the gene affected and the function being studied.
The work described in this chapter made use of a “reverse genetics” approach. Instead of searching for the genetic basis of a particular phenotype (defect of temperature entrainment), we used a candidate gene approach. For this, we utilized the RNAi technique (and the availability of genomic RNAi libraries), and specific mutants to knock-out (or knock-down, in case of RNAi) genes which we thought might be possible candidates for playing a role in the temperature entrainment mechanism.

The transient receptor potential (TRP) family of cation channels are ubiquitously involved in sensory physiology. They are highly conserved between organisms, from nematodes to human and they respond to light, temperature, touch, pain, sound, humidity and mechanical stress (reviewed by Clapham, 2003; Montell, 2005; Minke and Parnas, 2006; Hardie, 2007).

In *Drosophila* there are 13 *trp* channel encoding genes, belonging to 7 different subgroups, or classes (Table 5.1). Most of them have been implied to function in several physiological sensory responses (Table 5.1).

Although the mechanisms underlying the temperature synchronization of the circadian clock is only poorly understood, it is reasonable to imagine the requirement of temperature sensors, able to “sense” and transmit the temperature information from the external, environmental world, to the internal, physiological environment (and thereafter, to the central clock). Given the distribution and variety of TRP channels in sensory physiology, they were our first candidates as putative components for the temperature input pathway to the circadian clock.
<table>
<thead>
<tr>
<th>Class</th>
<th>Gene</th>
<th>Mutant alleles</th>
<th>RNAi</th>
<th>Described function</th>
<th>References</th>
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<tr>
<td>TRPM</td>
<td>trpM</td>
<td>trpM&lt;sup&gt;EY01618&lt;/sup&gt;, trpm&lt;sup&gt;1&lt;/sup&gt;, trpm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2+2*</td>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt; homeostasis</td>
<td>Hofmann et al. (2010)</td>
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<td>TRPV</td>
<td>nanchung</td>
<td>nan&lt;sup&gt;q5&lt;/sup&gt; †, nan&lt;sup&gt;36a&lt;/sup&gt; †</td>
<td>2</td>
<td>hearing, hygrosensation, geotaxis</td>
<td>Kim et al. (2003); Gong et al. (2004); Liu et al. (2007); Sun et al. (2009)</td>
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<td>Hofmann et al. (2010); Sun et al. (2009)</td>
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<td>Hamada et al. (2008); Rosenzweig et al. (2005, 2008); Kang et al. (2010)</td>
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<td>nociception, geotaxis</td>
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<td>high temperature tolerance, geotaxis</td>
<td>Lee et al. (2005); Sun et al. (2009)</td>
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<td>Montell and Caterina (2007); Rosenzweig et al. (2008)</td>
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<td>autophagy</td>
<td>Venkatachalam et al. (2008)</td>
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Table 5.1: List of the 13 trp channel encoding Drosophila genes. TRP channels are classified in 7 subgroups or classes. The function of TRP channels (if known) is indicated. Several mutants targeting trp genes are available and only the one marked with daggers (†) have been assayed in this study. RNAi lines used in this Thesis were provided by the VDRC, except the ones marked with an asterisk which were provided by the NIG Fly Stock Center (and the number of insertion lines indicated). “n.a.”: not available.
5.2 Analysis of RNAi targeting trp genes

Our “reverse genetic” approach involved the use of the RNAi technique of available lines (see Table 5.1) combined with the bioluminescence assay, monitoring real-time expression of per-luc transgenes in living flies. We silenced trp channel genes in all clock cells, crossing RNAi lines with a tim-gal4 driver (line 27, Kaneko and Hall, 2000). Flies were monitored in our automated bioluminescence assay for their ability to synchronize per-luc (XLG-luc) in LL and 12:12 hr 25:16°C TC. Initially, we restricted our analysis to monitoring per-luc expression in isolated legs. Among the 23 RNAi lines available (covering 11 different genes) we did not observe any major defects of per-luc expression during LL and TC (data not shown).

We wondered if this was a false negative determined by the nature of the RNAi technique with the combination of the tim-gal4 driver line. RNAi does not induce null mutation of targeted genes and the expression pattern of many TRP channels is not known. It is possible that a more comprehensive driver might be more useful. We extended our investigation to several mutants available against trp channel encoding genes (Table 5.1). To our knowledge, there is not so far any study which connects the TRP channels family to the circadian clock, with the exception of trp and trpl in the phototransduction cascade required for the Drosophila visual system (Niemeyer et al., 1996; Yang et al., 1998).

5.3 Behavioural analysis of trp mutants

5.3.1 Behaviour analysis in constant light and TC

We analysed 12 mutant lines, targeting 7 trp encoding genes, for their ability to entrain locomotor activity to TC. Flies were entrained in LD first and then released
in LL and TC, which were in opposite phases compared the previous LD cycles (Figure 5.1). One line exhibited entraining defects in LD cycles. $nan^{36a}$ does not synchronize to LD but rather reacts to lights-on and lights-off: High peaks of activity arise just after the change of conditions, with a lack of typical anticipatory patterns both in the morning and in the evening (Figure 5.1B). Interestingly, during LL and TC the activity looks more “normal” (Figure 5.1O), although the evening peak of activity arises later and persists more during the cold phase, compared to control (Figure 5.1N). A second allele affecting the same $nanchung$ gene ($nan^{dy5}$) also lacks the anticipatory activity from dark to light, whereas the evening peak of activity is normal in both LD and LL and TC (Figure 5.1C, P). $nan$ has been identified as a TRP channel required for hearing, and is expressed in larval and adult chordotonal organs (Kim et al., 2003). $nan^{36a}$ and $nan^{dy5}$ were generated by $P$-element imprecise excision and lack the first intron, and the first four introns, respectively (Kim et al., 2003). The two mutants alleles have been reported to be sedentary and mildly “uncoordinated” (Kim et al., 2003), and this might explain the inability of $nan^{36a}$ to properly synchronize to LD cycles. However, this is not the case for the allele $nan^{dy5}$, which is normal.

Another line exhibits minor entrainment defects both during LD and TC conditions. The double mutant $trp^{302};trp^{P343}$ affects the 2 related TRP channels $trp$ and $trpl$ genes, which are components required for the visual phototransduction cascade (Niemeyer et al., 1996). During LD conditions, $trp^{302};trp^{P343}$ flies anticipate the light-dark transitions but their behaviour lacks a pronounced siesta and flies are instead very active during the whole day (Figure 5.1M). Also during LL and TC the activity is not normal, the flies are more active during the warm phase and the peak of activity that usually anticipates the transition from warm to cold is (if present at all) advanced (Figure 5.1Z).
12:12 hr Light-Dark cycles (T=25°C)

A  Canton S (10)

D  trpM (10)

G  TrpA ins (16)

J  pyx²/pyx³ (16)

M  trpD02;trpP343 (27)

B  nan³⁶a (10)

E  pain³ (9)

H  pyx² (16)

I  pyx³ (16)

K  trpP²⁴³ (7)

L  trpD02 (11)
CHAPTER 5. TRP CHANNELS

LL and 12:12 hr 25:16°C Temperature Cycles

N  Canton S (9)  O  nan^{36a} (10)  P  nan^{dy5} (11)

Q  trpM (10)  R  pain^{3} (9)  S  pain^{2451} (8)

T  TrpA^{ins} (10)  U  pyz^{2} (15)  V  pyz^{3} (8)

W  pyz^{2}/pyz^{3} (12)  X  trp^{F343} (6)  Y  trp^{102} (9)

Z  trp^{102};trp^{F343} (8)
The *trpl;trp* double mutant is visually blind but the circadian clock can still be reset by light (through non-visual light signalling, i.e. CRY) but the light-induced TIM degradation is attenuated (Yang et al., 1998). It has been reported that the *trpl^{302};trp^{P343}* double mutants have impaired cold avoidance behaviour, when mutant larvae are let to choose between room-temperature and cold-temperature (15°C) in a thermal gradient (Rosenzweig et al., 2008).

Among the lines tested, we found 4 mutant lines (affecting 3 genes) which showed abnormal locomotor activity specifically during TC conditions. Mutants for *pyrexia* (*pyx*), *trpM* and TrpA1 have normal LD behaviour but fail to synchronize to TC: no anticipatory activity for the transition from warm to cold and a pronounced startle response after temperature step-up (*TrpA1*) or step-down (*pyx* and *trpM*). For *pyrexia*, two different alleles show very similar results, together with the transheterozygous line *pyx^{2}/pyx^{3}* , indicating that the phenotype is specific to the *pyrexia* gene.

The *pyx* mutants have been isolated in a screen aimed to isolate thermopreference mutants (Lee et al., 2005). The channel is made of two isoforms, PYX-PA and PYX-PB, resulting from alternative transcripts. *pyx^{2}* is characterized by a *P*-element inserted 538 bp upstream from the first translation codon which greatly decreases the expression of the PYX-PB transcript and increases that of the PYX-PA. *pyx^{3}*, which has been induced by *P*-element hopping, is a null allele, and it

**Figure 5.1** (preceding page): Daily average profiles of TRP channel mutants during LD cycles and LL and TC. Flies were first synchronized in 12:12 hr LD cycles at 25°C (A–M) and then subjected to LL and 12:12 hr 25:16°C TC (N–Z) which were in opposite phase compared to the previous LD. All the genotypes included in the figure have been tested at least twice, with reproducible results. *trpM* (D, Q), *TrpA1* (G, T) and *pyx* (H–J, T–W) lines display mutant phenotype specifically in LL and TC (and not in LD conditions). See text for more details. Grey shading represents activity during dark-phase in LD or cold-phase in TC. Number of individuals is indicated in brackets next to the genotype.
CHAPTER 5. TRP CHANNELS

does not express any of the two PYX transcripts (Lee et al., 2005). *pyx* mutant flies do not distribute normally when they are subjected to a temperature gradient, they are less responsive to heat stress and they paralyse faster than wild-type if exposed to noxious warm temperature (40°C) (Lee et al., 2005). It has also been shown by Lee et al. (2005) that PYX channels are gated by temperature, at least when expressed in *X. leavis* oocytes or HEK cells. *In vivo* studies of PYX distribution revealed that it is widely expressed in larval central and peripheral nervous system (Lee et al., 2005). In the adult, it is expressed in both multidendritic (type II) and nonmultidendritic (type I) sensory neurons innervating bristles, in the maxillary palps, proboscis, and antennae (Lee et al., 2005). A more recent paper (Sun et al., 2009), studying the function of different TRP channels in geotaxis within the chordotonal neurons of Johnston’s organ (which is the specialized organ for hearing, located in the antennae, see Introduction), revealed the expression of *pyx* in the cap cells of the scolopidium.

The *trpM* mutant is caused by a *P*-element insertion (Flybase symbol *P-{EPgy2}CG34123EY01618*) in the coding region of the *CG34123* gene, or *trpM*. TRPM has been recently described as required for magnesium (Mg\(^{2+}\)) intake in the Malpighian tubules (Hofmann et al., 2010). Given that two mutants alleles generated by Hofmann et al. (2010) are pupal lethal, the allele we used (named *trpM*) is presumably hypomorphic. In our assay, *trpM* exhibits a very similar phenotype to the *pyx* mutants. It belongs to the TRPM class and *trpM* mutant flies are normal in a thermopreference assay (Rosenzweig et al., 2005). The mammalian ortholog to *trpM* is TRPM3, which is activated by cell swelling (reviewed by Kraft and Harteneck, 2005). The other six members of the mammalian TRPM class are associated with taste transduction, sensation of cool temperatures and Mg\(^{2+}\) absorption in the intestine (Hofmann et al., 2010).
TrpA1 has been already described as a TRP channel required for warm avoidance in Drosophila larvae (Rosenzweig et al., 2005, 2008) and adults (Hamada et al., 2008). \textit{TrpA1}\textsuperscript{ins} null mutant has been generated via site-directed insertional disruption which change its reading frame resulting in a truncated protein (Hamada et al., 2008). \textit{TrpA1} is expressed in three groups of cells in the adult brain, the anterior cells (AC), the ventral cells (VC) and the lateral cells (LC) neurons, but only the AC neurons (which projects to the antennal lobe) seem to be necessary and sufficient to restore normal thermopreference in \textit{TrpA1} mutants and to act as “internal” thermosensors (Hamada et al., 2008) in addition to the “external” one, located in the third antennal segment (Sayeed and Benzer, 1996). Recently, TRPA1 has been proposed to be required for chemical nociception and \textit{TrpA1} mutants fail to respond to reactive electrophiles, i.e. allyl isothiocyanate, $N$-methylmaleimide or cinnamaldehyde (Kang et al., 2010).

### 5.3.2 Behaviour analysis at different temperature intervals

The different TRP channels can be activated and respond to warm and cold in a wide range of temperatures (Montell and Caterina, 2007). To address whether certain genes or mechanisms operate in restricted temperature intervals, and to identify the specific role that each isolated \textit{trp} mutant plays in the process of temperature entrainment of the circadian clock, we investigate the locomotor behaviour at different and smaller temperature intervals than the one utilized previously. We first entrained the flies to 12:12 hr LD cycles and then monitored their activity in LL and three different TC: 29:25°C, 25:20°C and 20:16°C.

Figure 5.2 shows the locomotor activity of the four TRP channel mutants and controls in LL and different temperature intervals. Flies were first entrained in LD (25°C) then transferred to LL and 12:12 hr 29:25°C TC for 5 days. Subsequently,
Figure 5.2
CHAPTER 5. TRP CHANNELS

I  $pyx^2$ (19)  M  $pyx^3$ (20)

J  LL and 12:12 hr 29:25°C TC  N

K  LL and 12:12 hr 25:20°C TC  O

L  LL and 12:12 hr 20:16°C TC  P

FIGURE 5.2
Figure 5.2: Locomotor activity of TRP channel mutants and controls in LL and different temperature intervals. The plot on top depicts average actograms. The lower plots depict daily average activity (left) and a low-pass filtered version of the same plot (right) from which the average peak phase (in ZT) has been extrapolated (and depicted in Table 5.2), in LL and 12:12 hr 29:25°C, 25:20°C and 20:16°C TC, respectively (as indicated). Flies have been entrained in LD first and then subjected to LL and the different temperature intervals, each of those were 6-hr phase delayed compared to the previous conditions (see shaded areas in the actogram). LD part is not shown. The experiment has been performed twice. In the second repetition, the TC intervals have been applied in the reverse order, i.e. from 20:16°C to 29:25°C. The results were similar, excluding that ageing effects caused the failure of entrainment during the cold interval (data not shown). Shaded areas (grey in the actograms and daily average or blue in the peak-phase plots) represent activity during the cold-phase. Number of individuals tested is indicated in brackets.
Activity at 29:25°C TC

Activity at 25:20°C TC

Activity at 20:16°C TC

Figure 5.3: Daily average activity of controls (red line) and TRP mutants (green, blue, grey and black, as indicated) during the warm-phase only in LL and different temperature intervals of the histograms depicted in Figure 5.2B–D, F–H, J–L, N–P, R–T. Two-way Anova was performed to determine statistical interaction between Canton S (CS) control and each TRP mutant lines in the range ZT 0–12. Coloured bars underneath represent the time points in which the respective mutants show significant difference compared to Canton S control (Bonferroni posttest $P < 0.05$).

The temperature regime was then changed to 25:20°C and delayed by 6 hr compared the previous regime. After 6 days, the temperature cycles regime was shifted again by other 6 hr (delay) and changed to 20:16°C. Activity was first analysed by monitoring both actograms and histograms (Figure 5.2). Subsequently, by overlapping the daily activity of the mutants and control in the same plot and performing a Two-way Anova to determine statistical interactions of each mutant

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with the controls in the warm phase only (Figure 5.3). In addition, the phase of
the activity peak and amplitude of the rhythm were calculated (Table 5.2).

Canton S flies entrain their locomotor activity to any of the three temperature
intervals. As described in the previous chapter (Section 3.3.1) Canton S flies nicely
display the effect of “seasonal adaptation” of behaviour to different temperature
intervals (Figure 5.2A–D, Figure 5.3 and Table 5.2).

TrpA1 INS flies do not entrain at 20:16°C TC and 25:20°C TC and very weakly, if
at all, at 29:25°C, but not in a way comparable to controls (Figure 5.2E–H). Two-
way Anova finds highly significant interaction with Canton S at any temperature
intervals (Figure 5.3) and the pattern of activity during the warm phase is weakly
cycling (amplitude 2.8) only at 29:25°C, whereas it is not rhythmic at 25:20°C and
at 20:16°C TC (Table 5.2). To support this observation, note that the main peak
of activity occurs just after temperature changes (phase values in Table 5.2).

Analysis of actograms and histograms of pyx2 flies revealed that they synchro-
nize their rest-activity pattern to temperature cycles in the warm range (29:25°C),
very weakly in the mid range (25:20°C) and not in the cool (20:16°C). Two-
way Anova suggests significant difference from control at any temperature intervals
and this can be explained by the different phase of activity: the peak of evening
activity during 29:25°C occurs 2.5 hours earlier than in controls and 1.5 hours
later during 25:20°C TC (Table 5.2). In the cold range, pyx2 activity is flat (am-
plitude not significant) and flies do not exhibit any peak of activity in terms of
anticipation of the warm-cold transition (Figure 5.2L Figure 5.3 and Table 5.2).

The behaviour of pyx3 mutants resembles very much that of pyx2: entrainment
in 29:25°C and 25:20°C intervals, but not at 20:16°C TC. The phase of activity
in the two higher temperature intervals occurs two hours earlier than control,
resulting in an significant difference of the overall activity compared to control
when Anova is performed (Figure 5.3). This is supported also by the overall higher level of activity of the mutants compared to Canton S in all the three temperature intervals. Activity in the cold range (20:16°C) is flat (amplitude not significant), and flies are not synchronized. The PYX channel is required for warm avoidance and the mutants lack the ability to react to noxious warm (Lee et al., 2005), so one would expect inability to entrain in a warm temperature interval. Yet our results reveal the opposite effect on temperature entrainment: both pyrexia mutant alleles we assayed entrain to 29:25°C TC but not at all at 20:16°C.

trpM mutants entrain normally to LL and 25:20°C TC, in a way comparable to control (Figure 5.2 and Figure 5.3). Similarly to pyx\(^2\) and pyx\(^3\), the shape of the daily average activity during 29:25°C TC is similar to that of wild-type control, but the phase is 2 hours earlier and the activity level higher, resulting in an overall statistically significant interaction with control. trpM mutants fail to synchronize their activity to the cold interval 20:16°C: the activity pattern is flat (amplitude not significant, Table 5.2) and it exhibits only a sharp increase when temperature drops to 16°C (Figure 5.2 and Figure 5.3).

Looking at the actograms in Figure 5.2, one could argue that the mutants inability to synchronize their behaviour in the cold interval 20:16°C could be consequence of ageing effects, since the cold temperature interval occurs at the end of the experiment. To address this question, the same experiment was performed with opposite temperature regimes, i.e. starting with the cool range 20:16°C and increasing up to 29:25°C. The results were comparable and the mutant lines showed the same activity patterns, regardless of the order in which the temperature intervals had been applied (data not shown).
### LL and Temperature cycles

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### DD and Temperature cycles

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**Table 5.2:** Summary of peak phase and amplitude of locomotor activity in LL or DD and different temperature intervals. The phase values (in ZT) are calculated from 4-hr low-pass filtered versions of daily average histograms depicted in Figure 5.1, 5.2, 5.4 and 5.6. When more than one peak of activity was observed, only the main one is considered (100% relative intensity). Colour backgrounds summarize the behaviour of flies which were entrained (green), not entrained (red) or weakly entrained (hypomorphic behaviour, yellow) in the different conditions. Amplitude was calculated by dividing the the maximum and minimum activity level of daily average activity. One-way Anova was used to calculate whether the activity oscillation between peak and trough was significant ($P<0.05$. ns: $P>0.05$).

#### 5.3.3 Behaviour analysis in constant darkness and TC

All the behavioural analysis shown so far has been performed in constant light (and TC). It is known that at least some TRP channels are involved in the phototransduction cascade, for instance, TRP and TRPL (Niemeyer et al., 1996). Both are not directly activated by light, but through a phospholipase C (PLC)-mediated signalling pathway (reviewed by Katz and Minke 2009). We therefore wondered if the constant presence of light could have some direct effects on the channels that may alter behaviour, in spite of the putative role on the temperature synchronization of the clock. We thus monitored the ability of the *trp* mutants to entrain to temperature cycles in DD.
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A Canton S (8) B trpM (12) C TrpA1ins (16)

D pyx2 (14) E pyx3 (16) F norpAP41 (12)
The locomotor activity pattern of wild-type flies in DD and TC resembles in shape the activity pattern during LL and TC, exhibiting a unimodal pattern (and not a bimodal one as in LD entrainment) but with a different phase. The peak of activity does not occur towards the end of the warm phase (around ZT10), but at beginning of the thermo-phase, soon after the raise of temperature (around ZT3, Figure 5.4A). During DD and TC conditions, temperature-entrainment mutant flies, such as *norpA*P41 (Glaser and Stanewsky, 2005), exhibit a so called “startle response”, which occurs just after the temperature increases (ZT0–1) (see Figure 5.4F). In LL and TC conditions, the circadianly-regulated peak (at around ZT10) is far apart from the startle response at ZT0 so it is “easier” to discriminate mutant flies from wild-type. In DD and TC, the situation is different, since the circadianly-regulated peak partially overlaps the startle response peak at the beginning of the day (warm-phase). For this reasons, it can be misleading to judge the behaviour of a fly only on the position of the activity peak of histograms (during TC conditions). However, careful inspection of the activity pattern, gives in many cases the possibility to discriminate whether the activity is reaction to temperature or (in addition) circadianly regulated. One way to test if the locomotor activity is synchronized to temperature is to monitor the transients from one condition to another and to check free-running phase when the flies are released to constant conditions.

Figure 5.4 shows actograms, histograms and peak phase analysis of *trp* mu-
Figure 5.5: Daily average activity of controls (red line), TRP mutants (green, blue, grey and black) and norpA (light blue), as indicated, during the warm-phase only in DD and 25:16°C TC of the histograms depicted in Figure 5.4. Two-way Anova was performed to determine statistical interaction between Canton S (CS) control and each mutant lines in the range ZT 0–12. Coloured bars underneath represent the time points in which the respective mutants show significant difference compared to control (Bonferroni posttest \( P < 0.05 \)). Error bars indicate SEM.

tants and controls during (25°C) to DD and 12:12 hr 25:16°C TC (after initial LD entrainment) and subsequent release to constant conditions. The activity peak of wild-type flies occurs 3 hours after the transition from cold to warm and it takes 2–3 transition cycles to stabilize. When wild-type flies are then released to constant conditions, the initial phase of free-running activity follows the phase observed during entrainment.

trpM flies exhibit a very similar behaviour to Canton S controls, although their peak phase during TC occurs one hour earlier (ZT2.3 versus ZT3.3 of control, see Table 5.2), resulting in significant interaction with control in an Anova test (Figure 5.5). trpM behaviour exhibits transients before reaching a stable phase and it is distinct from a pure temperature-reaction activity, which mainly occurs...
just after the cold-warm transition (compare with norpA\textsuperscript{P41}, Figure 5.6F). Also, free-running activity after TC follows the behaviour peak observed at the end of entrainment (Figure 5.4B), suggesting the trpM flies entrain their activity to 25:16°C TC. This result indicates a striking difference of behaviour during TC between LL and DD for the trpM mutant flies.

\textit{TrpA1}\textsuperscript{ins} activity is very similar to a “temperature mutant”: a prominent behavioural activity peak just after temperature increase from the first day of the new regime and no clear transients are observed (Figure 5.4C). Two-way Anova revealed highly significant interaction with Canton S, and the main peak of activity occurs 1.5 hours earlier then control. Also free-running activity after TC is not clearly synchronized with the behaviour during TC (Figure 5.4C). These results are consistent with the inability of \textit{TrpA1}\textsuperscript{ins} mutants to synchronize their behaviour to LL and TC (Figure 5.1).

Similar to trpM, pyx\textsuperscript{2} flies are able to entrain to TC during DD (Figure 5.4D). This is particularly evident if considering the free-running activity after TC entrainment (see below and Figure 5.4D). pyx\textsuperscript{3} shows a similar synchronization to temperature than pyx\textsuperscript{2}: the activity peak during TC follows the temperature and the free-run activity after TC conditions is dampened but in phase with the previous activity. For both mutants, Two-way Anova revealed significant difference form control (Figure 5.5), probably because they exhibit an higher response to temperature increase, an overall higher activity level and an earlier phase of activity peak (1 and 1.5 hours, respectively, Table 5.2).

Interestingly, pyx and trpM mutants do not entrain locomotor behaviour to 25:16°C TC in LL but they do in DD. We had also observed different behaviour among the mutants assayed in LL when smaller temperature intervals were applied, so we investigated if this was the case also in constant darkness.
Figure 5.6 and Figure 5.7 show rest-activity patterns of TRP mutants and controls in DD and different temperature intervals.

Canton S flies entrain to any of the three temperature intervals we applied. The activity shows clear transients when flies are subjected to the different TC and they persist longer than in LL. Interestingly, the seasonal adaptation of the behaviour is also less pronounced compared to LL and TC condition (figure 5.2A–D). Those observations are in agreement with previous results of “stronger” entrainment in LL compared to DD (see Tomioka et al., 1998; Yoshii et al., 2002; Glaser and Stanewsky, 2005 and Discussion).

Analysis of variance between $TrpA1^{ins}$ flies and Canton S exhibit statistical interaction at any temperature intervals (Figure 5.7). By inspection of the actograms and histograms (Figure 5.6), $TrpA1$ flies do not exhibit the same activity pattern as controls. In particular, we did not observe any transients and the activity peak of $TrpA1^{ins}$ flies occurs just after the temperature raise.

$trpM$ mutants entrained to 25:20°C and 20:16°C temperature interval and weakly at 29:25°C TC. Interestingly, $trpM$ entrain to the cold 20:16°C TC (a condition to which it does not entrain in LL, compare Figure 5.2T). Anova analysis restricted to the warm-phase only shows interaction with Canton S at 29:25°C, probably as a consequence of the lower activity level and the slightly earlier activity phase (0.5 hour). However, the pattern of activity (Figure 5.7) and the amplitude of oscillation (Table 5.2) is comparable to control suggesting $trpM$ flies are weakly entrained (but not in the same extent as controls).

$pyx^2$ flies exhibit a different activity profiles compared to control in the 29:25°C and 25:20°C intervals, while it is comparable to control ($P>0.05$, Two-way Anova) in the cold interval (Figure 5.7). Interestingly, in the 29:25°C and 25:20°C ranges, the activity peak is shifted towards the end of the warm phase (ZT10), the activity
A Canton S (12)  

B DD and 12:12 hr 29:25°C TC  

C DD and 12:12 hr 25:20°C TC  

D DD and 12:12 hr 20:16°C TC  

E TrpA1 (14)  

F  

G  

H  

**Figure 5.6**
Figure 5.6: A–T) Locomotor activity of TRP channel mutants and control in DD and different temperature intervals. The plots have been described in Figure 5.2. Flies were entrained in 12:12 hr LD cycle (not included in the graphs) and then subjected to DD and different TC (29:25°C, 25:20°C and 20:16°C respectively, as indicated) which were 6-hr phase delayed and advanced, respectively, compared to the previous entrainment regime (see shading). Note that in DD and TC, the main peak of activity seats at the beginning of the warm phase. See text for more details. Average phase values of the main activity peak (100% relative intensity) are summarized in Table 5.2.
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Activity at 29:25°C TC

Activity at 25:20°C TC

Activity at 20:16°C TC

Figure 5.7: Daily average activity of controls (red line) and TRP mutants (green, blue, grey and black, as indicated) during the warm-phase only in DD and different temperature intervals of the histograms depicted in Figure 5.6B–D, F–H, J–L, N–P, R–T. Two-way Anova was performed to determine statistical interaction between Canton S (CS) control and each TRP mutant lines in the range ZT 0–12. Coloured bars underneath represent the time points in which the respective mutant shows significant difference compared to Canton S (Bonferroni posttest $P < 0.05$). Error bars indicate SEM.
profile is relatively noisy, the cycling amplitude is lower than control as well as
the overall activity level. A similar behaviour is observed for pyx^3 in the same
ranges. Instead, the behaviour of the pyx^3 allele at 20:16°C seems more a reaction
to the temperature increase, rather than synchronization (Figures 5.6 and 5.7). In
fact, the activity is more concentrated during the warm-phase (and the level higher
than control), and we did not observe transients between the different temperature
intervals.

Among the TRP channel mutants we analysed, TrpA1^{ins} exhibits the most
severe phenotype of defective temperature entrainment. In addition, from my
observations it emerges a very complex picture in which it is challenging how
to classify mutations affecting trp genes that also affect aspects of temperature
entrainment of the circadian clock. It appears that it is crucial to consider the
conditions in which the flies are assayed, in terms of light or darkness and interval
of temperature cycles in order to judge how a TRP channel affects temperature
entrainment. Some mutant lines can exhibit the phenotype in certain conditions,
but not in other, and *vice versa*. These observations could also help to understand
the functions that a specific channel plays. For instance, the ability to entrain
only in one specific temperature range, could mean the non-requirement of that
particular channel in that specific range. In other words, the different behaviour of
trp mutants could help to generate a “temperature map” where different channels
respond to different temperature cycles, thereby allowing the fly to sense, react,
and ultimately entrain to different environmental conditions.
5.4 *per-luc* expression in *trp* mutants

I next investigate if *trp* mutants affect directly the expression of the *period* gene and its product when assayed in temperature cycles conditions. I first assayed *per-luc* expression in RNAi lines against *TrpA1, trpM* and *pyx* driven by *tim-gal4*, both in isolated legs and in the whole adult fly. Four independent RNAi lines are available against *trpM*, and one each against *TrpA1* and *pyx* (see Table 5.1).

Flies were first entrained to LD (25°C) and then subjected to LL and 12:12 hr 25:16°C TC in opposite phase compared the previous LD regime. Note that wild-type adult flies take up to 2 days to entrain to the new temperature regime, while isolated legs are fully synchronized to temperature after 1 day of transition (Figure 5.8). Overall *per-luc* (XLG-*luc*) expression appeared normal in isolated legs during LL and TC in all the RNAi lines we assayed (Figure 5.8, left column), as already discussed in Section 5.2. Expression levels in legs of *tim-gal4*-driven *TrpA1*-RNAi and *pyx*-RNAi were slightly lower than in controls, but similar in terms of rhythmicity, amplitude and phase. Interestingly, when we monitored *per-luc* expression in the whole adult fly, we observed that PER-LUC in *trpM*-RNAi (line R4) is not cycling at all, while it is normal in *pyx*-RNAi and *TrpA1*-RNAi. We tested 4 different RNAi lines against *trpM* and only one (line R4) shows the phenotype (reproducible among different experiments). This can be interpreted in 2 different ways. The first explanation is that the other 3 RNAi lines are not efficient or they do not knock-down the *trpM* gene enough in order to manifest the phenotype. Secondly, it could be that the phenotype induced by *trpM*-RNAi (line R4) is not due to silencing the *trpM* gene, but due to insertional effects of the RNAi line itself, and this would be supported by the results obtained with the XLG-*luc* transgenic mutant line (see below). However, the observation that *per-luc* expression synchronizes to temperature in legs but not in the context
Average recordings of TRP-RNAi lines in LL and TC

<table>
<thead>
<tr>
<th>Isolated legs</th>
<th>Whole adult fly</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> <em>trpM</em>-RNAi</td>
<td><img src="image1" alt="" /></td>
</tr>
<tr>
<td><strong>B</strong> <em>TrpA1</em>-RNAi</td>
<td><img src="image2" alt="" /></td>
</tr>
<tr>
<td><strong>C</strong> <em>pyx</em>-RNAi</td>
<td><img src="image3" alt="" /></td>
</tr>
</tbody>
</table>

**Figure 5.8:** Flies were first entrained in LD and then subjected to LL and 12:12 hr 25:16°C TC which was in opposite phase compared to the previous LD regime. The last day of LD is included in the plots (except in (B) left column, where only LL and TC part is included). Black line represents bioluminescence readings from control flies. Blue line represents reading from *tim-gal4*-driven RNAi lines. White/black bars indicate light and dark phase. Red/blue bars indicate 25°C and 16°C phase, respectively. Error bars (grey) indicate SEM. Number of individuals tested (n) is indicated.
of the whole animal is reminiscent of the phenotype of the known “temperature mutants” nocte and norpA\textsuperscript{P41} (Glaser and Stanewsky, 2005; Sehadova et al., 2009 and C. Gentile, A. Simoni, R. Stanewsky, in preparation). It is interesting to note that this phenotype appears in several different mutants, as for example in the EMS mutants (in DD and TC, described in the Section 3), nocte, norpA and now \textit{trpM}. At the moment, the explanation for this phenomenon remains unknown, but further investigation will hopefully help to understand the underlying mechanism.

The RNAi technique is an extremely powerful genetic tool, however it is not free from drawbacks. RNAi knock-down may be not sufficient to induce a phenotype (in particular when dealing with potential signal transduction events that can be amplified) or RNAi may be driven in the wrong cells (see Discussion). To solve those problems, we also generated transgenic flies carrying the XLG-\textit{luc} transgene in the \textit{TrpA1\textsuperscript{ins}}, \textit{trpM} and \textit{pyx} mutant backgrounds.

In order to compare the results obtained with the RNAi lines with results of chromosomal mutation in the same genes we monitored TRP channel mutants for their ability to synchronize \textit{per-luc} expression in LL and 12:12 hr 25:16°C TC. \textit{trpM};XLG-\textit{luc} flies entrain PER-LUC expression to temperature. Expression in isolated legs is equivalent to wild-type controls, and in adult flies the \textit{per-luc} expression reaches a stable and synchronized phase to TC one day earlier than controls (Figure 5.9). \textit{trpM} mutants exhibit a small (and bigger than control) rise in PER-LUC expression directly after the temperature increase (particularly evident at ZT12 of day 3 of TC, Figure 5.9A, right). Two-way Anova performed in the range ZT 48–144 (i.e. for four days after the moment \textit{per-luc} expression is synchronized to TC) revealed no statistical interaction between the genotypes, indicating no significant difference between \textit{trpM} XLG-\textit{luc} and control both in isolated legs and in the whole adult flies.
Average recordings of TRP mutant lines in LL and TC

**A** \textit{trpM}

Isolated legs

\begin{itemize}
  \item Black line represents bioluminescence recordings from control flies (XLG-\textit{luc}).
  \item Blue line represents XLG-\textit{luc} in TRP channel mutant backgrounds, as indicated.
\end{itemize}

Whole adult fly

\begin{itemize}
  \item Interaction not significant
\end{itemize}

\textbf{B} \textit{TrpA1}

Isolated legs

Interaction not significant

Whole adult fly

\begin{itemize}
  \item Interaction not significant
  \item \(F_{(95,1746)} = 1.54, P < 0.001\)
\end{itemize}

**FIGURE 5.9:** Average bioluminescence recordings of (A) TRPM and (B) TRPA1 mutant lines in LL and TC. Flies were first entrained to LD and then subjected to LL and 12:12 hr 25:16°C TC which was in opposite phase compared the previous LD regime. The last day of LD is included in the plots. Black line represents bioluminescence recordings from control flies (XLG-\textit{luc}). Blue line represents XLG-\textit{luc} in TRP channel mutant backgrounds, as indicated. Number of individual tested is indicated next to the genotype. White/black bars indicate light and dark phase. Red/blue bars indicate 25°C and 16°C phase, respectively. Error bars (grey) indicate SEM. Two-way Anova was performed in the range ZT 48–144 to determine statistical interaction of each mutant with control (results reported under each plot).
The $TrpA1^{ins}$ XLG-luc flies entrain to TC, but not in the same extent compared to control. *per-luc* expression in isolated legs is strongly reduced in terms of amplitude only during LL and TC and not in LD (Figure 5.9B). Analysis of variance revealed interaction not significant but an highly significant genotype effect ($F_{(1,1746)} = 66.4$, $P < 0.0001$), as a results of the lower amplitude of cycling, but not an overall effect on the rhythmic *per-luc* expression and phase (Figure 5.9). PER-LUC expression in the whole adult flies of $TrpA1$ mutant is lower compared to control in terms of cycling amplitude. It is instead comparable with the wild-type control in terms of overall bioluminescence levels, suggesting defects in synchronization rather than generally repressing PER expression. Two-way Anova performed in the range ZT 48–144 shows highly significant interaction between the mutant and control ($F_{(95,1746)}=1.54$, $P < 0.001$). Interestingly, we noticed also a 2-hr phase delay of *per-luc* expression in the $TrpA1$ mutant compared to wild-type control during LD conditions. This is noticeable only in adult flies, but not in isolated legs (Figure 5.9B).

The defects in period expression during TC for $TrpA1^{ins}$ mutant correlates well with the observation that this mutant shows the most severe “temperature phenotype” at the behavioural level in many temperature conditions we assayed, suggesting an important role for this TRP channel in the temperature entrainment pathway. We also generated transgenic lines carrying XLG-luc in $pyx^2$ and $pyx^3$ mutant backgrounds. At the moment it is not possible to present any results from those lines. For time reasons the experiments have not been completed and further investigation will determine whether *per-luc* expression in pyrexia mutants is affected. Additional experiments monitoring *per-luc* expression in the $TrpA1$, $pyx$ and $trpM$ mutant backgrounds are also needed under the conditions in which they exhibited a phenotype in our behaviour assays.
5.5 Summary

- Analysis of trp channel encoding genes in LL and 25:16°C TC revealed that pyrexia, TrpA1 and trpM exhibit impaired synchronization of locomotor activity specifically to TC and normal activity in LD conditions.

- tim-gal4-driven knock-down of TRP-RNAi lines did not give any results.

- Behavioural analysis at different temperature intervals in LL shows that pyx and trpM have entrainment defects specifically at cold interval (20:16°C) and normal entrainment at 25:20°C and 29:25°C TC. TrpA1 mutants entrain weakly (if at all) at 29:25°C TC and do not at 25:20°C and 20:16°C.

- pyx and trpM synchronize their behaviour in DD to 25:16°C TC and even to small temperature intervals (29:25°C, 25:20°C and 20:16°C). Similarly, pyx3 entrain at 29:25°C and 25:20°C but not in the range 20:16°C. trpA1 flies weakly entrain to all temperature intervals but not in the same extent as control (statistical interaction highly significant).

- tim-gal4-driven RNAi lines against pyx and trpA1 do not induce any effect on per-luc expression in LL and TC. On the opposite, per-luc expression in tim-gal4-induced trpM-RNAi is not rhythmically cycling in LL and TC when the whole adult flies are analysed and normal in isolated legs.

- XLG-luc expression in trpM mutant background is comparable to control. TrpA1 mutant flies exhibit a down-regulation of XLG-luc expression both in isolated legs and in the context of the whole fly (in terms of cycling amplitude).
Chapter 6

Circadian regulation of eclosion

6.1 Eclosion profile of “temperature mutants”

In the previous chapters, we discussed the isolation of new components which play a role in the temperature synchronization of the circadian clock. We examined the isolation of three mutants from an EMS mutagenesis screen, of three trp channel encoding genes (pyx, trpM and trpA1) and of one forkhead transcription factor encoding gene (fd3F) which show defects in temperature entrainment. The isolation of the mutant lines was based on the analysis of adult locomotor activity or monitoring real-time expression of period-luciferase transgenes in temperature entrainment regimes. In the past, similar approaches led to the isolation of two other “temperature mutants”, nocte and norpA (Glaser and Stanewsky, 2005, 2007; Sehadova et al., 2009).

In my Ph.D. I also investigated whether the novel “temperature mutants” exhibited defects in the synchronization of the circadian clock that regulates the emergence of adult flies from the pupal stage.

Eclosion rhythms can be entrained, not only by light-dark cycles, but also by
CHAPTER 6. CIRCADIAN REGULATION OF ECOSSION

Constant light and 25:16°C Temperature cycles

A Canton S (1332, 3)

B y w (1753, 2)

C 2T-30 (1882, 2)

D 2T-38 (1447, 2)

Figure 6.1
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Figure 6.1
CHAPTER 6. CIRCADIAN REGULATION OF ECLOSION

I. nocte\textsuperscript{P} (1303, 1)

Figure 6.1: Eclosion profile for control and “temperature mutant” cultures during LL and TC conditions. The left-hand column shows double-plotted actograms of flies that emerged during a LL and 12:12 hr 25:16°C TC, which was in opposite phase compared to the previous LD entrainment. Only the last dark cycle of the previous LD entrainment is indicated (except in C–E, where the full last LD cycle is included). The two right-hand columns depict, respectively, the total number of flies emerged as a function of time (“raw activity”) and 4-hr low-pass filtered versions of the left plots (“filtered activity”, see M&M and Levine et al., 2002a for more details). The total number of hatched flies and the number of experiment repetitions are indicated in brackets with the genotypes. Shaded areas refer to temperature conditions (grey shading: 16°C; white shading: 25°C). A–B) Canton S and y w control cultures synchronize eclosion to TC in 2 days and exhibit peak of emergence during the last part of the cold phase (ZT22–23). C–J) All the “temperature mutant” lines we tested show similar synchronization to TC compared to the control. Note the presence of a secondary peak, notably in day 3 and 4 of temperature entrainment, just after the transition cold-warm, which is probably a gating response induced by steep rise of temperature (see text). Repetitions showed reproducible results.

temperature cycles (Zimmerman et al., 1968). However, not many studies focus on the regulation of eclosion entrainment during temperature cycles, particularly in constant light conditions. In order to correlate the eclosion results with our locomotor behaviour data, I investigated eclosion in LL under temperature cycles.
Cultures for the desired genotype were raised in 12:12 hr LD cycles (at 20°C) and monitored for eclosion after exposing the cultures to LL and 12:12 hr 25:16°C TC which was in the opposite phase compared to the previous LD. The eclosion profile under TC conditions, after establishing a stable phase, exhibits a clear 24 hour rhythm, and flies emerge in a 6–8 hour window which occurs shortly before the cold to warm transition. The timing of eclosion is controlled by the circadian clock, which imposes a “gate” that opens only during certain time of the day (Qiu and Hardin, 1996).

Wild-type control cultures (Canton S and y w) synchronize their eclosion rhythm to the TC after 2 transient days (Figure 6.1A–B), stabilizing the peak of emergence at the end of the cold phase after day 3. A second eclosion peak occurs just after the temperature rise (particularly noticeable in day 3 and 4 of temperature entrainment). This second peak occurs probably in response to the steep increase of temperature, inducing the pupae developmentally mature enough, to hatch. This second temperature-induced eclosion peak has been mentioned previously in the same conditions (Newby and Jackson, 1993).

We monitored the eclosion activity of 8 different mutant lines: 2T-30, 2T-38, 2P-42, pyx2, pyx3, trpM, nocteP and norpAp41 (fd3F was analysed in the chapter 4). All of them exhibited a mutant phenotype (to different extents) when adult locomotor activity was monitored during temperature entrainment regime (see chapters 3, 5, Sehadova et al., 2009 and Glaser and Stanewsky, 2005, respectively). Surprisingly, all lines eclose with a normal pattern, synchronizing emergence after 2 days of TC and stabilizing the eclosion peak at the end of the cold phase (Figure 6.1). The only exception might be line 2T-30 (Figure 6.1C), which displays a “noisier” pattern of eclosion, pupae take one day longer to be synchronized with temperature and the gating window is wider then wild-type control (up to 12
CHAPTER 6. CIRCADIAN REGULATION OF ECLOSION

hours). 2T-30 cultures exhibit also a more pronounced rise in eclosion directly after temperature rise (see day 4 and 5 of TC in Figure 6.1C). Although the pattern appears nosier, 2T-30 cultures can entrain to TC (see also Figure 6.2B).

The only mutant line we observed having defects on entrainment of eclosion to TC has been described in the previous chapter (4.4). When the transcription factor fd3F is down-regulated in all the clock cells (via a tim-gal4 driver line), the phase of eclosion is shifted towards the warm phase.

To determine eclosion we utilize an automated monitor which counts the number of flies that hatch as a function of time (see M&M for details). For this reason, we could test only true breeding (not balanced) cultures, since we could not select the desired genotype from the eclosed progeny. Thus, we could not test genotypes like nocte1, in which heterozygous females do not express the phenotype. Here I report the eclosion data from nocteP, a hypomorphic nocte allele (Sehadova et al., 2009).

It was surprising that all genotypes we assayed exhibited a clear entrainment of eclosion — lines which had manifested a distinct mutant phenotype at the behavioural and molecular level in the adult. Our data suggest that entrainment of the clock that regulates eclosion involves different components and/or pathways compared to the adult clock.

To challenge further the ability to synchronize to TC even more than in the previous regime, I subjected 2T-30 and control cultures to a temperature shift (in LL) and monitored the ability to re-synchronize the eclosion clock.

Cultures were raised in LD conditions and the pupae were transferred to LL and 12:12 hr 25:16°C TC. After 3 days, the temperature regime was advanced for 6 hours and eclosion was monitored. Canton S (control) and 2T-30 (mutant) cultures shift the peak of eclosion according to the temperature cycles, exhibiting
two days of transients and resynchronizing the emergence peak to the end of the cold phase (Figure 6.2). Clock mutant \( \text{per}^{01} \) cultures, on the contrary, are unable to synchronize to temperature cycles: they do not eclose with a 24-hr period but instead manifest bursts of emergence after any temperature transitions (Figure

A  Canton S (1286, 1)

B  \( 2T-30 \) (1598, 1)

C  \( \text{per}^{01} \) (1076, 1)

**Figure 6.2:** Re-synchronization of ecolision rhythms after 6-hr advanced temperature shift in LL. For description of the plots see Figure 6.1. Shift occurred at day 4, as shown by the shaded area in the actograms. A) Canton S and (B) EMS mutant \( 2T-30 \) cultures re-synchronize ecolision emergence to temperature cycles in 2 days. C) Ecolision of \( \text{per}^{01} \) cultures is not synchronized to TC: flies emerge mainly after all temperature changes most likely as a reaction to temperature increase and decrease.
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Figure 6.3: per<sup>01</sup> cultures eclose with a 12-hour period. Flies emerge mainly after all temperature changes most likely as a reaction to temperature increase and decrease.

6.2C), therefore exhibiting a 12-hr period (Figure 6.3).

6.2 Effects of TC on the eclosion period

Given that adult “temperature mutants” do not exhibit eclosion phenotypes during LL and 25:16°C TC, I investigated in more detail the regulation of the circadian clock to TC, by monitoring the eclosion rhythm after temperature entrainment in constant conditions.

I raised cultures of Canton S and 2T-30 in LD and exposed them to LL and 12:12 hr 25:16°C TC (which was 6 hr delayed compared to the previous LD) for 3 days at the early pupal stage, and then released them in DD and constant temperature to monitor the eclosion rhythm. As shown in Figure 6.4A,B, the two different cultures synchronize eclosion to temperature and, when released in DD, they display a clear rhythm, which persists for several days, and is in phase with the previous temperature regime. Very surprisingly, I observed that flies emerge with a short (and reproducible) period of 20–21 hour. Canton S exhibit a 20 hour period and 2T-30 a 21 hour period (Figure 6.4). Given that the short period of
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eclosion is stable (i.e. persists for several days) and is a common feature between cultures of different genetic background, we assume that it is not affected by the mutant tested.

Recent work from the Rouyer group proposed a model for temperature entrainment of the larval brain (Picot et al., 2009). Monitoring PER accumulation

A \hspace{1cm} \text{Canton S (1499, 3)}

\begin{center}
\begin{tabular}{ccc}
\textbf{Actogram} & \textbf{Raw Activity} & \textbf{Filtered Activity} \\
\includegraphics[width=0.3\textwidth]{actogram_canton_s.png} & \includegraphics[width=0.3\textwidth]{raw_activity_canton_s.png} & \includegraphics[width=0.3\textwidth]{filtered_activity_canton_s.png} \\
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{ccc}
\textbf{Autocorrelation} & \\
\includegraphics[width=0.3\textwidth]{autocorrelation_canton_s.png} & \\
\end{tabular}
\end{center}

B \hspace{1cm} \text{2T-30 (1156, 1)}

\begin{center}
\begin{tabular}{ccc}
\textbf{Actogram} & \textbf{Raw Activity} & \textbf{Filtered Activity} \\
\includegraphics[width=0.3\textwidth]{actogram_2t30.png} & \includegraphics[width=0.3\textwidth]{raw_activity_2t30.png} & \includegraphics[width=0.3\textwidth]{filtered_activity_2t30.png} \\
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{ccc}
\textbf{Autocorrelation} & \\
\includegraphics[width=0.3\textwidth]{autocorrelation_2t30.png} & \\
\end{tabular}
\end{center}

C \hspace{1cm} \text{Pdf01 (2397, 2)}

\begin{center}
\begin{tabular}{ccc}
\textbf{Actogram} & \textbf{Raw Activity} & \textbf{Filtered Activity} \\
\includegraphics[width=0.3\textwidth]{actogram_pdf01.png} & \includegraphics[width=0.3\textwidth]{raw_activity_pdf01.png} & \includegraphics[width=0.3\textwidth]{filtered_activity_pdf01.png} \\
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{ccc}
\textbf{Autocorrelation} & \\
\includegraphics[width=0.3\textwidth]{autocorrelation_pdf01.png} & \\
\end{tabular}
\end{center}

Figure 6.4: Free-running eclosion rhythms after 3 days of LL and TC entrainment. Cultures of Canton S, 2T-30 and Pdf01 flies were raised in LD (20°C) until early pupae, which were then collected and transferred to the eclosion discs. Those were subjected to 3 full 25:16°C temperature cycles in LL which were 6-hr delayed compared to the previous LD and then release in constant darkness (at 20°C). Only 2 of the 3 days of LL and TC entrainment are included in the plots. The three left-hand plots have been described in Figure 6.1. The most right-hand plot depicts autocorrelation which gives period values, calculated for the DD part only. Grey shaded areas represent 16°C (during LL and TC) or darkness (20°C). White areas represent 25°C (during LL and TC). “p”, period (hr). “RI”, Rhythmicity Index. “RS”, Rhythmicity Statistic (RS 1.5 indicates rhythmicity, see M&M for details). Number of total emergents and experiment repetition is indicated in brackets with the genotype.
in the different groups of larval clock neurons, they described that the DN$_2$s can directly be entrained by TC and synchronize PER expression in the LNs through a PDF-independent (unknown) pathway. They proposed that the CRY-negative DN$_2$s are required to entrain the larval central clock to temperature cycles (Picot et al., 2009).

To address whether the short period was driven by the DN$_2$s and if \textit{Pdf} was required in the synchronization of the eclosion to temperature cycles, we monitored \textit{Pdf}$^{01}$ mutant cultures. Surprisingly, \textit{Pdf}$^{01}$ cultures eclosed rhythmically with a short period of 22 hours, which persists for several days, comparable to wild-type (Figure 6.4C).

Short eclosion period of wild-type cultures has never been reported before. Moreover, Myers et al. (2003) reported that \textit{Pdf}$^{01}$ mutants exhibit aperiodic eclosion after LD entrainment. We wondered if the short eclosion period, and rhythmicity of \textit{Pdf}$^{01}$ cultures, were determined by the different conditions we used. Therefore, we investigated the free-running period of the same genotypes after entrainment to LD. Figure 6.5 shows free-running rhythms of Canton S, \textit{y w}, \textit{2T-30} and \textit{Pdf}$^{01}$ cultures. They all entrain to light-dark cycles and flies eclose with a circa 24 hour free-running rhythm, which persists for several days. \textit{Pdf}$^{01}$ cultures are strongly rhythmic for 3 days (with a 23.3 hour period) and then the rhythmicity gradually dampens, resulting in aperiodic eclosion after day 5 (Figure 6.5D). Interestingly, my observations are in disagreement with data published by the Sehgal group, which showed that eclosion of \textit{Pdf}$^{01}$ mutants (and flies lacking pdf-expressing cells) is arrhythmic in DD (Myers et al., 2003). Thus, we showed that (a) 3 days of entrainment in LL and TC during the pupal stage shortens the period of eclosion of cultures which exhibit a normal 24 period after LD entrainment and (b) that \textit{Pdf}$^{01}$ eclose rhythmically and comparable to control after LL.
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A  Canton S (1071, 2)

B  y w (2016, 1)

D  2T-30 (1847, 1)

D  Pdfo1 (2672, 3)

Figure 6.5: Free-running eclosion rhythms after LD entrainment. Cultures of Canton S, y w, 2T-30 and Pdfo1 flies were raised in LD (20°C) and then released into constant conditions (DD and 20°C) where the eclosion activity was monitored. Plots have been described in Figure 6.1. Total numbers of emerged flies and experiment repetitions are indicated in brackets next to the genotypes. All the cultures eclose rhythmically with a circa 24-hr period. Pdfo1 rhythm persists strongly for 3 days (with a 23.3 hr period) and then gradually dampens. White/black bars at top indicate subjective day and night, respectively.

and TC and that rhythmicity persist for 3–4 days in DD after LD entrainment.

Since we did not observe any gross differences in the eclosion pattern between 2T-30 and Canton S cultures (in constant conditions), we proceeded by monitoring the emergence profiles of Canton S (control) and Pdfo1 (mutant) to address the
CHAPTER 6. CIRCADIAN REGULATION OF ECLOSION

question on the origin of the short period observed after LL and TC entrainment.

This short period phenotype was obtained after exposing pupal cultures glued to “eclosion discs” to TC for 3 days. Since we assume that the free-running period is clock controlled, we wondered if we could observe the same short free-running period if TC entrainment was restricted exclusively to the larval stages, in order to discriminate whether the short period was generated by the larval or the pupal clock.

Thus, we restricted temperature cycle entrainment (in LL) from day 2 of development until the first pupa appeared in the cultures, and then transferred to constant darkness (at 20°C). The free-running eclosion rhythm was then monitored (Figure 6.6). Canton S flies eclosed rhythmically with a 22.8 hour period. The eclosion profile is “noisier” and the peak less sharp than in the previous conditions, probably because the cultures had been in DD for up to five days before eclosion was monitored, and therefore individuals within the population being out of phase each other. It is also interesting to note that the phase during free-run of eclosion compared to that after temperature entrainment is reversed: The peak of emergence is centred to the second half of the corresponding warm phase (CT10–12) versus ZT22–6 during temperature entrainment. This nicely correlates with the reported data that larval-only TC entrainment reverses the phase of adult locomotor activity (Picot et al., 2009 and see below). When Picot et al. restricted TC entrainment to larva only stages (in DD) and then monitored free-running adult locomotor activity, they observed that the phase of activity was opposite compared to activity of flies subjected to larval only LD entrainment (Picot et al., 2009).

\textit{Pdf}^{01} cultures exhibit aperiodic eclosion in the same conditions (Figure 6.6B). The \textit{Pdf}^{01} rhythm does not persist for more than 5 days in DD (after LD entrain-
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A  Canton S (1306)

Figure 6.6: Free-running eclosion rhythms after LL and TC entrainment restricted to larval stages. Cultures were subjected to LL and 12:12 hr 25:16°C TC only during the three larval stages (from day 2 of development until the first pupa appeared in the culture), then were transferred to DD (20°C) where the eclosion rhythm was monitored. A) Canton S rhythms persist in DD for several days with a period of 22.8 hour. B) Pdf01 flies exhibit aperiodic eclosion. Red/blue bars at top indicate subjective warm- and cold-phase, respectively (relative to larval-only TC entrainment). Number of flies eclosed is indicated in brackets next to the genotype. Experiment in this conditions was performed once.

ment, Figure 6.5) and, if TC entrainment is restricted to larvae only, cultures are kept in DD for 5 days before eclosion is being monitored. Taken together, these two observations could explain why the Pdf mutant is arrhythmic in the current experiment. The Canton S free-running period of 22.8 hour lies in between the short period (20 hr) observed after 3 days of TC entrainment restricted to the pupal stage and the “normal” 24 hr period after LD entrainment.

Next, we asked which eclosion period cultures would exhibit when exposed to TC throughout development. We entrained Canton S and Pdf01 cultures to LL and 25:16°C from late embryo to pupae and then released them in DD. Canton S and Pdf01 cultures emerged with a short period of 22.5 and 21.8 hours, respectively
(Figure 6.7), similar to the results obtained when TC entrainment is applied to larvae or pupae only. This observation suggests that the timing at which the TC is applied during development does not determine the period of free-running eclosion, but are rather the conditions (LL and TC) themselves.

Figure 6.9 summarizes the experiments described above and illustrates free-running period of Canton S and \textit{Pdf}^{01} cultures after LL and TC entrainment applied at different times during development. It appears that there is no clear correlation between the developmental stage at which TC is applied and the resulting free-running eclosion period in DD. TC restricted to larval stage only, pupae only or during all developmental stages result in a short period of 22.5, 21 and 22.5 hours, respectively, for Canton S cultures. Similarly, temperature entrainment restricted to pupae or during all developmental stages induces a period of 21.5 and 21.8 hours, respectively, for \textit{Pdf}^{01} mutants, whereas eclosion is arrhythmic in larva-only temperature entrainment.

To address whether the short free-running period of eclosion after TC during the pupal stage was induced by the continuous exposure to light we also monitored free-running activity after entraining cultures to DD and TC. Canton S and \textit{Pdf}^{01} cultures were entrained in DD and 12:12 hr 25:16°C TC throughout development before being transferred to the eclosion disc (see Figure 6.9) and then released to constant conditions (at 20°C). As depicted in Figure 6.8, Canton S eclosion is rhythmic, but the free-running period is not stable and seems to change over the days. From day 1 to day 3 (of DD) flies eclose with a short, 22.5 hr period (Figure 6.8A,C). During the subsequent days, the eclosion activity exhibits a 24 hr rhythm, and the rhythmicity dampens gradually over the days. \textit{Pdf}^{01} cultures also exhibit rhythmic eclosion, with a stable short period of 20.8 hr (Figure 6.8B). The rhythm is strong for the first 3 days (with a 20.5 hr period, Figure 6.8D) and
then gradually dampens and becomes very “noisy”.

The short period exhibited by Canton S flies is quite surprising, since previous reports have shown a strong and stable eclosion rhythm with a 24 hr period after DD and temperature cycle entrainment (Zimmerman et al., 1968). However, Zimmerman and Pittendrigh’s observation were based on experiments conducted on *D. pseudoobscura* and not on *D. melanogaster*.

For the time being, it is difficult to speculate which components of the circadian clock contribute to generate the short period of free-running eclosion after entrainment to TC. It is also largely unclear which contribution each group of neurons plays in regulating locomotor behaviour in the adult fly during temperature entrainment. Even more unclear (and less studied) is which different neuronal group drives eclosion rhythms. Nevertheless, the short eclosion period after TC

![Figure 6.7](image)

**Figure 6.7:** Free-running eclosion rhythms after 9 days of LL and TC entrainment. Cultures of Canton S and *Pd*01 flies were raised in LL and 25:16°C TC since day 2 of development then were transferred to DD and 20°C where free-running periods were calculated. The last cycle in LL and 25:16°C is included in the plots (white/grey shading in day 0). For description of plots, see Figure 6.1. Both Canton S (A) and *Pd*01 (B) flies eclose with a short rhythm of 22.5 and 21.8 hours, respectively. Numbers of flies eclosed and experiment repetitions are indicated in brackets next to the genotype.
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A Canton S (1269)

B Pdf01 (3306)

C Canton S

D Pdf01
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entrainment, the pdf-independence of the rhythms, and indications from published work (Picot et al., 2009) suggested an important role for the DN2 neurons in the temperature entrainment of eclosion.

We can speculate a role for the larval DN2s in setting the period of eclosion. We observed that TC applied to larval stages only reverse the eclosion phase and this is not the case for pupal-only TC entrainment. It has been reported that the larval DN2s have an opposite phase of PER expression (compared to the LNs) in LD (Kaneko et al., 1997; Picot et al., 2009) and that they reverse their PER-expression phase during metamorphosis (Kaneko et al., 1997). Larval LNs are required to generate eclosion rhythms (Blanchardon et al., 2001) — they are the only clock-gene expressing neurons during pupation (Kaneko et al., 1997; Helfrich-Förster et al., 2007) — and we propose here that the eclosion phase is determined by the temperature sensitive DN2s.

6.3 Summary

- Components required for entrainment of the adult locomotor activity to TC do not affect the ability of cultures to synchronize their eclosion rhythms to temperature. This suggests the involvement of different structures and

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**Figure 6.8 (preceding page):** Free-running eclosion rhythms after DD and TC entrainment. A–B) Cultures of Canton S and Pdfl01 were entrained in DD and 12:12 hr 25:16°C since day 2 of development until pupae were loaded on the “eclosion disc”. Then, cultures were released in DD (and 20°C), where free-running emergence was monitored. The last day of TC is included in the plots (white/grey areas indicate 25°C and 16°C, respectively). C–D) Analysis of rhythmicity considering only the first 3 days after TC entrainment. A) Canton S flies eclose rhythmically with a period of 24 hours. The rhythmicity is very noisy and dampens quickly. The free-running period changes from 22.5 the first 3 days (C) to 24 hours during subsequent days. B) Pdfl01 cultures eclose with a stable rhythm of 20.8 hr (20.5 hr in the first 3 days, D). After 3–4 days, the eclosion profile of both genotypes becomes “noisier” and the peak less “sharp”. Number of flies eclosed is indicated in brackets with the genotype. Experiment performed once.
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#### Figure 6.9: Summary of free-running eclosion periods after different light and temperature entrainment conditions.

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Top 2 rows depict a visual representation of the *Drosophila* life cycle and a rough estimation of the days it takes to go through the different stages (during TC). See text for more details of the precise number of days in which the cultures have been exposed to the different Zeitgebers. Arrow indicates the moment when the cultures were transferred from bottles to the “eclosion discs” and when data collection started (grey background days: duration of data collection). Cultures were entrained in different entrainment conditions, as indicated, and the period calculated after release to DD. LD is 12:12 hr light-dark and 20°C. TC is 12:12 hr 25:16°C. DD refers to constant darkness and 20°C. The period of eclosion (τ, in hr) is calculated for the last 4 or 5 days of DD. Images of the *Drosophila* life cycle are taken from the cover of *Science*, Vol. 297, Issue 5590 (2002) “n.r.” indicates not rhythmic.
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pathways for temperature entrainment of eclosion clock compared to the adult clock.

- \textit{pdf}^{01} mutants exhibit rhythmic eclosion in DD, after light and temperature entrainment.

- TC applied at different times during development modulate the free-running period of eclosion, in a largely PDF-independent manner. Pupal TC entrainment drastically shortens free-running eclosion period of wild-type cultures, and similarly of \textit{pdf}^{01} mutants.

- Larval-only temperature entrainment reverse the phase of eclosion compared to light-dark entrainment.

- We propose that the free-running phase and period of eclosion after TC are determined by the DN\textsubscript{2}s
Chapter 7

nocte and peripheral sensory tissues

7.1 Background

In a previous EMS mutagenesis screen, aimed on the isolation of novel factors involved in the temperature entrainment of the circadian clock, a new genes has been isolated. *no circadian temperature entrainment* (*nocte*) shows impaired *per-luc* synchronization as well as abolished entrainment of the locomotor behaviour to temperature cycles (Glaser and Stanewsky, 2005). Later, the mutation has been mapped on the X chromosome and the gene identified (Glaser, 2006).

In this chapter, the following work is discussed, which led to the publication of the article “Temperature entrainment of *Drosophilas* circadian clock involves the gene *nocte* and signaling from peripheral sensory tissues to the brain” in the journal *Neuron* (Sehadova et al., 2009).
7.2 My contribution to the paper

The contribution I had for the publication of the paper was the behavioural analysis of the two nocte alleles and of chordotonal organ mutants in LL and TC (Figure 4). In particular, I analysed the behaviour of nocte\textsuperscript{i}, nocte\textsuperscript{P}, eys\textsuperscript{395}, eys\textsuperscript{734}, spam\textsuperscript{1}, tilB\textsuperscript{1} and smetana (the transheterozygous smet/Df(smet) and double mutant nocte\textsuperscript{P};smet/Df(smet)). All those mutants lines showed defects of temperature entrainment in LL and 25:16°C TC. Similarly, analysis of the several nocte-RNAi lines driven by F-gal4 have been tested in LL and TC, and quantification of behaviour is depicted in Figure S5. We observed that lines from both types of RNAi constructs can results in either normal or mutant phenotype, indicating that positional effects of the insertion sites influence the efficiency of the RNAi effect.

Next, I showed that both nocte alleles exhibit an uncoordinated phenotype, similar to spam mutants, after prolonged exposure to high temperature (37°C). The uncoordination phenotype is rescued when flies are exposed to to the same high temperature at >90% humidity (Figure 7).

I also showed that stopping the clock in the ch organs neurons by over-expressing a dominant negative form of cycle (cyc-\textDelta, Tanoue et al., 2004), under the F-gal4 promoter, does not prevent the flies to entrain to TC in LL (Figure 8). This suggests that temperature entrainment does not require a functional clock in ch organ to take place. Finally, I demonstrated that removal of either the 3\textsuperscript{rd} antennal segment, or the whole antenna results in “entrained” behaviour (Figure S5). This demonstrates that ch organs located in the antennae are not required for temperature entrainment
Temperature Entrainment of Drosophila’s Circadian Clock Involves the Gene nocte and Signaling from Peripheral Sensory Tissues to the Brain

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SUMMARY

Circadian clocks are synchronized by the natural day/night and temperature cycles. Our previous work demonstrated that synchronization by temperature is a tissue autonomous process, similar to synchronization by light. We show here that this is indeed the case, with the important exception of the brain. Using luciferase imaging we demonstrate that brain clock neurons depend on signals from peripheral tissues in order to be synchronized by temperature. Reducing the function of the gene nocte in chordotonal organs changes their structure and function and dramatically interferes with temperature synchronization of behavioral activity. Other mutants known to affect the function of these sensory organs also interfere with temperature synchronization, demonstrating the importance of nocte in this process and identifying the chordotonal organs as relevant sensory structures. Our work reveals surprising and important mechanistic differences between light- and temperature-synchronization and advances our understanding of how clock resetting is accomplished in nature.

INTRODUCTION

Circadian clocks regulate many biological processes so that they occur at beneficial times for the organism. Although these clocks are self-sustained and continue to run under constant conditions, they are synchronized with the environment by so called “Zeitgebers” (Dunlap et al., 2004). Two prominent Zeitgebers are the natural light-dark and temperature cycles that are able to synchronize the circadian clock of Drosophila and other organisms (see Boothroyd and Young, 2008; Dubrulle and Emery, 2008; Glaser and Stanewsky, 2007 for recent reviews). Although our knowledge regarding light entrainment of both fly and mammalian clocks is quite advanced, relatively little is known about temperature synchronization. Light is generally considered to be the more powerful Zeitgeber, but a temperature cycle with only 2°C–3°C amplitude robustly synchronizes Drosophila behavioral rhythms (Wheeler et al., 1993). In mammals, chick, and zebrafish, similar low-amplitude temperature rhythms (equivalent to body-temperature rhythms) are able to synchronize clock gene expression in the suprachiasmatic nucleus (SCN) and peripheral clock cells (Barrett and Takahashi, 1995; Brown et al., 2002; Herzog and Huckfeldt, 2003; Kornmann et al., 2007; Lahiri et al., 2005; Prolo et al., 2005), exemplifying the potential strength of this Zeitgeber. Moreover, as shown for Drosophila (Glaser and Stanewsky, 2005), temperature synchronization of clock gene expression in these organisms occurs in tissue- or cell-autonomous manners, indicating that similar mechanisms are involved in ectothermic and endothermic animals.

Drosophila’s daily locomotor rhythmicity profile is bimodal, exhibiting major activity peaks in the morning and evening (e.g., Wheeler et al., 1993). This bimodality is regulated by several groups of clock neurons in the fly brain (see Sheeba et al., 2008 for a recent review). Recent work has revealed that a group of ventrally located neurons controls mainly the morning activity peak of fly behavior (M-cells), whereas more dorsally located cells regulate evening activity (E-cells) (Sheeba et al., 2008). These neurons control locomotor rhythms, and cyclically express several clock genes and proteins in synchrony with light-dark or temperature cycles (e.g., Yoshi et al., 2005; Zerr et al., 1990).

While clock neurons are mainly cell autonomously synchronized by light via Cry, it is not known how temperature signals reach the brain clock. It is formally possible that temperature sensitive neurons express a circadian temperature receptor that is able to synchronize the molecular clock within the pacemaker neurons (Hamada et al., 2008). Alternatively, temperature could be sensed by other neurons in the brain or by sensory structures in other parts of the fly, which then signal to the clock neurons. Two mutations that interfere with temperature entrainment, both molecularly and behaviorally, have been identified and could therefore shed light on the temperature entrainment mechanism (Glaser and Stanewsky, 2005). Mutants in the norpA...
gene, which encodes for the enzyme phospholipase C, are not able to synchronize to temperature cycles (Glaser and Stanewsky, 2005), indicating that a G protein-coupled signal transduction cascade might be involved. The mutated gene of the other temperature-entrainment-deficient variant (nocte) was not known until now.

Here we demonstrate that isolated Drosophila brains are not able to synchronize to temperature cycles. Since they do synchronize to light-dark cycles, these findings indicate that the brain requires temperature input from the periphery. We further reveal the molecular identity of the nocte gene, which encodes a large glutamine-rich protein with unknown function. Downregulation of nocte in peripheral tissues, including neurons of specific sensory structures (chordotonal [ch] organs), thoroughly disrupts temperature entrainment of behavioral rhythms. Similarly, other mutants known to affect the structure and function of ch organs also interfere with temperature entrainment, and mutant nocte alleles exhibit structural as well as physiological defects of sensory organ function. Moreover we show that a functional clock within these sensory structures is not required for behavioral temperature entrainment to occur, indicating that temperature information must be interpreted in a temporal fashion by downstream clock neurons in the thoracic central nervous system (CNS), or by the brain pacemaker neurons themselves. Our findings demonstrate the existence of a periphery-to-brain signaling pathway, identify the responsible sensory structures, and uncover fundamental differences between the light- and temperature-entrainment pathways of the fly circadian clock.

RESULTS

Tissue-Autonomous Synchronization to Temperature Cycles Is Restricted to Peripheral Organs

The Drosophila circadian clock can easily be entrained by temperature cycles (or steps), both in constant darkness (DD) and constant light (LL) (Busza et al., 2007; Glaser and Stanewsky, 2005; Matsumoto et al., 1998; Stanewsky et al., 1998; Wheeler et al., 1993; Yoshii et al., 2002, 2005, 2007). We previously showed that molecular synchronization can occur on a tissue-autonomous level. Isolated body parts of flies expressing two different period-luciferase (per-luc) constructs showed entrained bioluminescence oscillations when kept in LL and temperature cycles (Glaser and Stanewsky, 2005). When we performed these experiments, we noticed that isolated brains showed a 12 hr phase-advanced bioluminescence peak compared to all other isolated tissues (Figure 1A and Glaser and Stanewsky, 2005). This phase advance was not observed in LD cycles at constant temperature (Figure 1A), perhaps because the increase in Per-Luc expression occurs in clock neurons within the brain or in ectopic locations, we imaged brain bioluminescence signals using a highly sensitive imaging system (Figure 2). Brains were kept in cell culture medium in LL and temperature cycles and imaged at a time corresponding to the peak of luc-reported expression (ZT8, Figures 1A and 1B).

In a control experiment, brains were kept in LD cycles and constant temperature and imaged at ZT0, a time of high bioluminescence levels in brains and other tissues) kept in LD (Figure 1A). In the LD control brains, luminescence signals could be detected in regions corresponding to various groups of clock neurons, presumably large and small Lateral Neurons ventral (LNvs), the dorsal Lateral Neurons (LNds), and two groups of the Dorsal Neurons (DNs) (Figure 2A, upper panel). Expression was also found in the ocelli and in the retina (Figure 2A, upper panel), previously shown to express per (Hall, 2003).

Surprisingly, brain expression in temperature cycles was not confined to cells that usually express clock genes. In a clock-normal genetic background, bioluminescence signals were restricted to the dorsal brain (Figure 2A). Compared to the LD expression pattern, the dorsal expression domain appeared broader, indicating that in addition to the DN located in this region, other cells now express the XLG-luc construct. Moreover, signals were clearly absent from brain regions where the lateral clock neurons are usually located. This cannot be explained by the constant presence of light (LL) (cf. Zerr et al., 1990), because Per is expressed in these clock neurons under LL and temperature cycling conditions in the intact animal (Yoshii et al., 2005). Strikingly, an almost identical expression pattern was observed in the tim01 genetic background, further indicating the non-clock-related nature of luc-reported expression (Figure 2A). Similarly, in a CikXk mutant background, BG-luc expression occurred in a central brain region corresponding to the calyces of the mushroom bodies not known to contain any clock-gene-expressing cells (Figure 2A). Note that both XLG-luc and BG-luc transgenes are expressed in clock neurons encoding 2/3 or the entire Per protein fused to Luc, respectively; both expressed under control of a 4.2-kb DNA fragment from the per promoter (Stanewsky et al., 1997; Veleri et al., 2003). Both the norpA and nocte mutations, previously shown to abolish molecular and behavioral synchronization by temperature (Glaser and Stanewsky, 2005), did not prevent the increase in brain per-luc expression after the temperature rise (Figure 1B). Next, we analyzed per-luc oscillations in the clock mutant backgrounds of tim01 and CikXk (Allada et al., 1998; Sehgal et al., 1994). Both mutations were previously shown to disrupt temperature entrainment at behavioral and molecular levels (Glaser and Stanewsky, 2005, 2007; Yoshii et al., 2002, 2005, 2007). Although temperature-induced oscillations were strongly suppressed in most clock mutant tissues analyzed, the brains again showed sharp increases of expression immediately after the change to the warm temperature (Figure 1B).
during LD cycles (Stanewsky et al., 1997; Veleri et al., 2003; Figure 2A), indicating that the dramatic difference of spatial signal distribution between the two transgenic types observed in isolated brains during temperature cycling conditions depends more on the transgenic insertion site than on regulatory sequences contained within the transgene. Importantly, in neither case does the spatial expression pattern significantly overlap with that of clock-gene-expressing cells under LD conditions.

**Figure 1. Cultured Brains Synchronize to Light-Dark, but Not to Temperature, Cycles**

Isolated body parts or whole flies were either kept in light-dark cycles (LD) at 25°C or in constant light and 25°C:16°C temperature cycles (LL & C~) as indicated in the figure.

(A) (Left) Phase comparison of bioluminescence peaks obtained from XLG-luc transgenic flies, in which the entire period (per) gene is fused to the luciferase (luc) cDNA. (Right) XLG-luc brains kept in LL & C~ (top) or LD (bottom). Error bars indicate SEM. See Experimental Procedures for details about phase determination.

(B) Averaged bioluminescence recordings of different body parts from XLG-luc and BG-luc (containing a transgene encoding for two-thirds of the Per protein fused to luc) flies in different mutant backgrounds.

(C) Bioluminescence recordings from the 8.0-luc:9 transgenic type. This promoter-less per-luc construct encodes the entire Per protein and is predominantly expressed in dorsal clock neurons. White/gray and black bars indicate light/warm or dark/cold phase of the LD or temperature cycle, respectively.
conditions, pointing to a complete lack of spatial regulation of per under these conditions.

The ectopic Per-Luc expression observed in cultured brains—along with the lack of such signals from clock neurons—raised the possibility that spatial clock gene expression is generally altered in LL and temperature cycles compared to LD cycles at constant temperature. To test this, we imaged tissues known to synchronize under these conditions as well as in LD cycles (Glaser and Stanewsky, 2005; Figure 1A). Both legs and wings showed very similar spatial Per-Luc bioluminescence expression patterns under the two entrainment regimes, showing that peripheral clock tissues can be synchronized by light and temperature (Figure 2B).

In agreement with an earlier study describing per-gal4-driven GFP expression (Plautz et al., 1997), we also observe Per-Luc expression in potential mechanosensory and chemosensory cells along the wing margin and veins. In addition to what has been reported, we detected strong Per-Luc signals originating from the base of the wing, from the joints of the various leg segments (Figure 2B), and from segments of the haltere (inset in Figure 2B).

The 8.0-luc:9 line contains a promoterless per-luc fusion gene, which is expressed within a subpopulation of the DN

and occasionally in some LNd cells, but not in peripheral clock cells (Veleri et al., 2003). When 8.0-luc:9 adults were tested in LL and temperature cycles, bioluminescence peaks (presumably reflecting Per expression in brain clock neurons only) occurred late in the cold phase, similar as for the other Per-Luc transgenics tested (Figure 1C). When isolated brains were analyzed, we again observed a 12 hr phase shift, indicated by increased bioluminescence levels immediately following the temperature step up (Figure 1C).

Our results show that the brain has to be in the context of the intact fly in order for clock-neuronal gene expression to be synchronized by temperature cycles, and implies that in whole flies temperature entrainment involves signaling from peripheral tissues to the brain. As we will show below, this involves the gene nocte, a locus previously identified to play a role in temperature entrainment (Glaser and Stanewsky, 2005).

**The nocte Gene Encodes a Large Glutamine-Rich Protein**

In order to learn more about the function of nocte in temperature entrainment, we cloned the gene. Using meiotic mapping involving visible marker mutations and single nucleotide polymorphisms (SNPs), nocte was mapped to the 9A2–9D3 interval on the X chromosome (Experimental Procedures). Fine mapping using deficiencies placed nocte within the 9C1–9D2 interval containing 12 genes, flanked by the proximal breakpoint of Df(1)uc52,fly252 (which removes 8E3-5;9C1; Tweedie et al., 2009) and the distal breakpoint of Df(1)ED7010 (removing 9D3;9D4; Ryder et al., 2004) (Experimental Procedures). A chromosomal duplication covering this region (Dp(1;2)w75D 9A2;10C2) rescued the...
molecular temperature entrainment defects of the original nocte mutant (Figure 3A), which confirmed the results of our mapping experiments. Next, we analyzed available mutations for the 12 candidate genes (Tweedie et al., 2009). One $P$ element insertion line exhibited a temperature entrainment phenotype comparable to that observed in the original nocte mutant (Figures 3A, 4B, and 4C): wild-type flies anticipate the transition to the cold phase by an increase of their activity levels, which usually peak in control, but not in nocte1 (n=8) and Dp(1;2) (n=8). The EMS-induced nocte1 mutant was rescued by the Dp(1;2)v+75d (Dp(1;2)) covering the X chromosomal region 9A2–10C2.

(A) Bioluminescence recordings of BG-luc (left) and XLG-luc (right) adults in wild-type and nocte mutant flies. The Dp(1;2)v+75d(Dp(1;2)) covers the X chromosomal region 9A2–10C2 and rescues the phenotype of the EMS-induced nocte1 mutant.

(B) Nocte gene structure, mRNA transcripts (gray: noncoding regions, blue: coding regions), location of the two mutant alleles, and the target regions of two nocte RNAi constructs (brown bars). In addition to the two transcript types reported in flybase (CG17255-RA and RB), we identified two additional transcripts by RT-PCR (RC and RD). All transcripts encode the same predicted protein and differ only in regard to the 5'-UTR (see also Figure S2).

(C) The predicted Drosophila Nocte protein has weak homology to the mammalian GRP-1 protein (red) and to the BAT2 domain of MHCIII genes (orange). nocte1 results in a truncated protein as indicated. In addition to other Drosophilidae, potential Nocte homologs were found in the mosquitoes Aedes aegypti and Anopheles gambiae (Diptera), in the wasp Nasonia vitripennis (Hymenoptera), and in the beetle Tribolium castaneum (Coleoptera) (see Experimental Procedures for details).

Figure 3. The nocte Gene Encodes a Large Glutamine-Rich Protein

(A) Bioluminescence recordings of BG-luc (left) and XLG-luc (right) adults in wild-type and nocte mutant flies. The Dp(1;2)v+75d(Dp(1;2)) covers the X chromosomal region 9A2–10C2 and rescues the phenotype of the EMS-induced nocte1 mutant.

(B) nocte gene structure, mRNA transcripts (gray: noncoding regions, blue: coding regions), location of the two mutant alleles, and the target regions of two nocte RNAi constructs (brown bars). In addition to the two transcript types reported in flybase (CG17255-RA and RB), we identified two additional transcripts by RT-PCR (RC and RD). All transcripts encode the same predicted protein and differ only in regard to the 5'-UTR (see also Figure S2).

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several hours before the actual transition (Busza et al., 2007; Glaser and Stanewsky, 2005), while flies carrying the chemically induced nocte allele do not show this anticipation, and simply react to temperature changes (Figure 4B) (Glaser and Stanewsky, 2005). Similarly, 80% of the flies carrying the insertion P[iacW]CG17255007164, or females heterozygous for the insertion and the original nocte allele, do not synchronize properly to temperature cycles, though they do entrain to LD (Figures 4C, 4G, and S1 available online). The insertion associated with this line is located in the first intron of the gene CG17255 (Tweedie et al., 2009) (Figures 3B and S2) and results in the generation of abnormally spliced CG17255 transcripts (Figure S2). We sequenced the open reading frame (ORF) of this gene in the original nocte mutant and in the background strain used to induce the original mutation (Experimental Procedures) and found that this mutant contains a single base pair change at nucleotide 5119 of the CG17255 cDNA. This alteration introduces a premature stop codon (CAG → TAG) at position 1707 of the predicted protein, which normally is 2309 amino acids long (Figure 3C). Both mutations interfere with molecular and behavioral temperature entrainment, fail to complement each other, and affect the same transcription unit (Figures 3A, B, 4B, 4C, and S1). We therefore conclude that disruption of CG17255 causes the observed phenotypes and named this gene nocte. The original ethyl methane sulfonate (EMS)-induced allele will from now on be referred to as nocte1; the P element insertion as nocte2. The nocte2 mutation maps to a portion of the gene’s ORF that encodes one of several poly-glutamine stretches of Nocte. Apart from this feature and several poly-alanine stretches, Nocte has no apparent homologies to any other protein in the databases, except for a small region of similarity to the mammalian BAT2 domain at its N terminus (the overall similarity to the 70 N-terminal residues is weak, but it includes 11 identical amino acids). The BAT2 protein is encoded by a gene belonging to the MHCIII class genes, but its function is unknown (Banerji et al., 1990). Nocte does not contain any cysteine residues, suggesting that it is an intracellular protein. Although Nocte has no apparent DNA binding domain, the presence of poly-Q and poly-A stretches also suggests that Nocte may function as transcription cofactor (Riley and Orr, 2006), which is further supported by a stretch of 268 amino acids showing weak homology to the mammalian Glutamine Rich Protein 1 (GRP-1) (Figure 3C; Cox et al., 1996). Comparison with available genome sequences revealed that nocte is distributed among insects (i.e., not only Drosophila; Figure 3C), but no obvious vertebrate homolog was identified (Experimental Procedures).

**Downregulation of nocte in Peripheral Cells Interferes with Temperature Synchronization**

Based on our finding that isolated peripheral tissues robustly synchronize to temperature cycles, but isolated brains do not (Figures 1 and 2), we wondered if nocte function in peripheral tissues may be required for temperature entrainment of the whole animal. For this, we generated two nocte UAS-RNAi transgenes (1 and 2) (Figure 3B, Experimental Procedures), and combined them (separately) with several gal4-containing transgenes that drive expression in various regions of the peripheral nervous system (PNS). Both UAS-nocteRNAi lines result in substantial downregulation of nocte mRNA in third-instar larvae, when crossed to tim-gal4 or nocte-gal4 (see below) driver lines, reducing mRNA levels to 20%–35% of peak levels (Figure S3). Synchronization to temperature cycles was analyzed by monitoring locomotor activity first in a 12 hr: 12 hr LD cycle at constant 25°C, followed by exposure to an out-of-phase 12 hr: 12 hr temperature cycle in LL for 1 week (previous light phase corresponded to the cryophase [16°C] and previous dark phase to the warm temperature [25°C]). This was followed by another such cycle, in which the onset of the warm phase was delayed by 6 hr compared to the initial temperature cycling regime (Figure 4). In this regime wild-type flies require 2–3 days to synchronize to the first temperature cycle and about the same number of days to resynchronize to the shifted temperature cycle. These “transients” are especially obvious when the activity peak phase plot (next to the actograms) is inspected (see Experimental Procedures and Figure S4 for how these plots were generated and how the ability to synchronize was determined and classified). In the daily average plots below the actograms, wild-type entrained behavior is characterized by a robust and defined activity peak in the second half of the warm phase, reflecting an anticipation of the transition to cold temperature (Figure 4A; Glaser and Stanewsky, 2005). Strikingly, the F-gal4 transgenic (two independent insertion lines; Experimental Procedures), which is predominantly expressed in the neurons of the ch organs (Kim et al., 2003; Figures 5J–5R), produced a severe temperature entrainment defect (Figures 4D and 4G) when used to drive nocte RNAi-1 or 2. In the mutant plots the characteristic transients are missing or reduced and the animals largely react to the new temperature cycle. Apart from the reaction to the temperature change, the mutants remain rather constitutively active during the warm phase, not exhibiting the distinct anticipatory peak in the second half of the warm phase. Interestingly, ~25% of the nocte” mutant animals showed normal entrainment to temperature cycles, and another 25%

Figure 4. Downregulation of nocte in Peripheral Sensory Structures and Ch Organ Mutants Interferes with Behavioral Synchronization to Temperature Cycles

(A–F) Average actogram (left panels), activity peak phase (right panels), and daily activity plots (lower three panels) of flies that were entrained to 12 hr: 12 hr LD cycles for 3 days (LD) followed by 6 days of LL and 12 hr: 12 hr temperature cycles (25°C/16°C; LL + TC preshift) in which the warm and cold phase were in antiphase to the previous LD cycle. Subsequently, the onset of the warm phase was delayed by 6 hr compared to the initial regime (LL + TC postsift). Number of individual flies tested is indicated in (G). Note that all flies, except Canton S controls, show abnormal entrainment to temperature cycles but normal synchronization to LD. White bars or areas indicate light or warm phase and gray bars (areas) indicate dark or cold phase in LD and temperature entrainment conditions, respectively. Dots above the daily average bars indicate SEM. (G) Summary and quantification of behavior in temperature cycles for all genotypes tested. For nocte-RNAi lines 2:1b and 1:3 are shown (Figure S3); for tim-gal4 line, 16 was used. For classification criteria and methods see Experimental Procedures and Figure S4.

Neuron
Peripheral Tissues Synchronize Brain Clock

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S

nocte

T

nocte

period

Relative RNA concentration

Ext. body parts

Relative RNA concentration

- Head  - Brain  - Testis  - Leg  - Wing  - Ovary

- Head  - Brain  - Testis  - Leg  - Wing  - Ovary

S T

0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0

0 4 8 1 2 1 6 2 0

ZT

0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0

0 4 8 1 2 1 6 2 0

ZT

0 0.3 0.5 0.8 1.0 1.3 4 1 6

ZT 4  ZT 16

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were weakly entrained (Figure 4G). This indicates that the nocteP allele is a hypomorph, which is not surprising given that normally spliced nocte mRNAs can be detected in nocteF flies (Figure S2).

As in nocte mutants, entrainment to LD cycles was not affected in F-gal4/nocte-RNAi flies, indicating a specific defect in temperature synchronization (Figures 4B–4D). Some nocte RNAi insertion lines (from both types) resulted in only mild temperature entrainment defects or even wild-type behavior (Figures SSA and SSD), indicating that positional effects of the transgene insertion site influence expression levels and RNAi efficiency (although levels of downregulation mediated by such “weak” RNAi lines were not assessed molecularly). These latter results, along with another control in which F-gal4 was used to drive deaf1-RNAi (deaf1 encodes a DNA binding protein unrelated to circadian clocks and ch organ function) (Veraksa et al., 2002; Figure 4G), also demonstrate that the temperature entrainment defects are not elicited by the F-gal4 driver line alone.

F-gal4 Is Expressed in Ch and External Sense Organs

Adult ch organs are located at the joints between limb segments and are internally attached to the cuticle. They function as stretch receptors and the ch organs in adult legs and wings have been implicated in proprioception, whereas the one in antennae mediates hearing (Kernan, 2007). Neurons in larval body wall ch organs exhibit temperature-dependent calcium changes (Liu et al., 2003), but no connection between adult ch organs and temperature reception has been reported so far.

Careful analyses of UAS-mdCD8gfp and UAS-rfp expression driven by the F-gal4 transgene revealed that in addition to neurons of the ch organs (Figures 5Q and SR), F-gal4 is also expressed in a number of putative chemoreceptive and mechanoreceptive cells (external sense [es] organs) located in the labial and maxillary palpus (Figure 5J), first antennal segment (Figure 5K), wing (Figure 5L), haltere (Figure 5N), and leg (Figures 5O–5Q). Within the wing, F-gal4-positive cells were detected in the wing base, the ventral wing margin, and all wing veins, particularly in the regions close to the wing base. In the haltere and leg, F-gal4-positive cells are located in the cortex of every segment; marker signals were especially abundant in the capitelium of the haltere, the distal part of the femur, and the proximal part of the tibia. We also detected limited F-gal4 expression in the brain (Figures S6 and S8).

Mutations Affecting the Ch Organs Show Deficits in Temperature Entrainment

To confirm the potential role of ch and es organs in temperature entrainment, we analyzed mutants affecting the eyes shut (eys) a.k.a. spacemaker (spam) gene that encodes a proteoglycan expressed in the interrhabdomeral space within the eye as well as within the luminal space of ch and es organs (Husain et al., 2006; Zelhof et al., 2006; Figure 6G). eys mutants lack the interrhabdomeral space, and as a consequence rhabdomeres are in close contact, leading to visual impairment, but no mechanosensory defects have been described (Husain et al., 2006). We tested three eys/spam alleles: eys734 and eys955 behave as loss-of-function mutants with respect to the rhabdomere phenotype, although Eys protein was detected in both mutants (Husain et al., 2006). The spam1 allele is protein null and exhibits the same rhabdomere phenotype as the other two eys alleles (Zelhof et al., 2006). All three alleles showed wild-type behavior under LD conditions (Figure 4E and data not shown). Strikingly, in temperature cycles, 80% to 100% of the eys/spam mutant flies were mainly active during the warm phase, did not show the typical transients after transfer to a new temperature regime, and did not anticipate the temperature decrease, whereas the spam1 protein null allele showed the most severe phenotype (Figures 4E and 4G). The temperature synchronization defects observed for all three eys/spam alleles again indicate that ch and/or es organs are required for synchronization to temperature cycles and suggest that this function can be separated from their role in mechanoreception. Importantly, reducing eys/spam function in the ch and es organs using F-gal4/eys-RNAI also resulted in severely impaired behavioral synchronization to temperature in a manner similar to that as observed with nocte-RNAI (Figure 4G).

To specifically address the role of ch organs in temperature synchronization, we applied mutations of genes that are known to play a role in mechanoreception mediated by these organs and also retained normal es organ function. touch insensitive larvaeB (tilB) mutants have normal bristle receptor potentials but lack adult ch organ function, at least that of Johnston’s organ (Eberl et al., 2000; Kernan, 2007). tilB encodes a protein conserved in ciliated eukaryotes that is required for cilial structure and function (Kavle, 2007; Tweedie et al., 2009). In tilB-mutant spermatids the axonemal structures of the cilia are disrupted, indicating that ciliary motility is impaired—the likely
cause of sterility associated with this mutation (Caldwell et al., 2003). Although no such structural defect can be observed in adult ch organs at the light microscopy level, the same defect may underlie the deafness observed in tilB mutants (Kernan, 2007). smetana (smet) was isolated in a genetic screen for auditory mutants, and like tilB\textsuperscript{1}, causes male sterility and deafness, indicating a structural defect of the axoneme, but the mutated gene is not known (Caldwell et al., 2003). Strikingly, most tilB\textsuperscript{1} (90\%) and smet/Df(smet) (65\%) mutants show no or only weak synchronization to temperature cycles (Figures 4F and 4G). In order to determine potential genetic interactions between nocte and ch-organ-specific mutants, we generated a nocte\textsuperscript{1}; smet/ Df(smet) double mutant. Interestingly, the double mutant flies exhibited a more severe temperature entrainment phenotype compared to the single mutants, indicating an additive effect and the involvement of both genes in the same process (Figure 4G). These results strongly implicate ch organs and the axonemal cytoskeleton surrounding the ch organ cilia as crucial components of the temperature input pathway.

**nocte Is Expressed in Many Tissues, Including Ch and Es Organs**

To determine if nocte is indeed expressed in the adult ch and es organs, we generated a nocte-gal4 transgene by cloning an \(\sim\)2 kb genomic DNA fragment upstream of the nocte transcription start into a gal4 transformation vector (Experimental Procedures). Crossing several independently isolated nocte-gal4 insertion lines to a reporter strain containing UAS-mCD8gfp and UAS-rfp transgenes revealed identical widespread, but not ubiquitous, activity of the nocte promoter fragment (Figures 5A–5I, S6, and S8). Positive tissues included those that can be synchronized by temperature cycles in isolation, but they also included the brain (Figures 5A–5I, S6, and S8). To validate the spatial expression pattern of nocte, we performed real-time PCR experiments on RNA isolated from different body parts, which confirmed the broad expression pattern observed with nocte-promoter-driven reporter expression (Figure 5S). Our expression data are also in good agreement with those reported in FlyAtlas for CG17255 (Chintapalli et al., 2007). Quantitative RNA expression analysis revealed that nocte is neither circadianly nor temperature-dependently regulated in the tissues analyzed (Figure 5T, and data not shown).

Importantly, when we compared reporter signals in F-gal4 and nocte-gal4 flies, the ch and es organs of the adult legs, wings, haltere, and antennae were found to express nocte (Figures 5A–5I; S6, and S8). Quantitative RNA expression analysis revealed that nocte is neither circadianly nor temperature-dependently regulated in the tissues analyzed (Figure 5T, and data not shown).

**Johnston’s Organ Is Not Required for Temperature Entrainment**

Because the antennal ch organ (Johnston’s organ, located in the second antennal segment) of Drosophila is a highly specialized organ mediating hearing, we speculated that it is not required for temperature synchronization. On the other hand, a previous report (Sayeed and Benzer, 1996) revealed a receptor for temperature preference behavior to be located in the third antennal segment. We had already shown that the antennae...
are not required for temperature-cycle-induced molecular clock gene oscillations (Glaser and Stanewsky, 2005). Here we tested flies, in which either the third antennal segments or the whole antenna (including Johnston’s organ) were mechanically ablated, for their ability to synchronize behaviorally to LD and temperature cycles (Figures S5B and S5C). During both environmental cycles, behavior of the manipulated flies was very similar to that of wild-type, demonstrating that the antennae are not required for temperature entrainment. Although we cannot rule out a contribution of Johnston’s organ, it seems clear that one or several of the other adult ch organs (Figures 5C–5I and 5L–5R) are sufficient for clock synchronization by temperature.

**nocte Mutants Exhibit Visible Ch Organ Defects**

Based on the involvement of nocte and ch organs in temperature entrainment, it was possible that nocte mutants affected ch organ structure. First we analyzed RFP expression in F-gal4/

**nocte**-RNAi/riP and nocte1; F-gal4/riP flies and could not detect any gross structural abnormalities in the adult ch organ (Figure S7 and data not shown). In order to reveal potential alterations in the fine structure of ch organs, we crossed a gfp-nompA reporter gene into the genetic background of nocte1 and nocte2. gfp-nompA encodes a GFP-NompA fusion protein, expressed under the control of the endogenous nompA promoter and recapitulating the spatial pattern of nompA expression (Chung et al., 2001). NompA is a transmembrane protein containing extracellular ZP domains, and is specifically expressed in the dendritic cap of ch and es organs (Figures 6A–6G; Chung et al., 2001). Inspection of GFP-NompA expression in femur ch organs revealed that between 60% (noc-te1) and 100% (noc-te1) of the noc-te mutant flies tested contain dendritic caps that appear to have physical gaps, or spatially suppressed GFP-NompA expression (Figures 6D–6F and Table S1 available online). Since NompA is critical for transmission of mechanical stimuli from sensory structures to the sensory neuron (Chung et al., 2001), the structural defect, or the NompA expression phenotype we observed, indicates that ch organ function is also impaired in nocte mutants.

**nocte Mutants Exhibit a Temperature-Dependent “Uncoordinated” Phenotype**

The above results suggest that a structural defect in ch organs is responsible for the temperature synchronization defects observed in nocte, tilB, and smet mutants. Interestingly, one allele of the eys/spam locus (spam1) also effects ch and es organ structure, but in a temperature- or humidity-dependent manner (Cook et al., 2008). It was shown that the Eys/Spam protein within the scolopale of ch organs conserves the shape and function of this structure after water loss induced by exposure to excessive heat or osmotic shock. In spam1 mutants this structural conservation is lost and the scolopales undergo dramatic cellular deformation, leading to flies that exhibit an irreversible uncoordinated locomotor phenotype after prolonged exposure to 37°C (Cook et al., 2008). Given that both eys/spam (including spam1) and nocte mutants fail to entrain to temperature cycles (Figures 4B, 4C, 4E, and 4G), we wondered if nocte mutants also exhibit the same temperature-dependent uncoordinated phenotype. For this, we exposed control and nocte mutant flies to 37°C for 90 min and counted the number of flies that fell over during this time in 15 min intervals (Experimental Procedures; Cook et al., 2008). Both nocte alleles showed an uncoordinated phenotype, with more flies falling over the longer they were exposed to the high temperature (Figure 7). This phenotype was again more pronounced in nocte1 compared to nocte2, further suggesting that the latter allele is a weak hypomorph. Interestingly, and as in the case of spam1 mutants, uncoordination was largely prevented when flies were exposed to the same high temperature at 90% humidity (Figure 7). This indicates that water loss from the scolopale also results in gross structural defects and cellular deformations of noc-te ch organs, which may also explain why these organs fail to mediate temperature synchronization when mutated.

**Temperature Entrainment Does Not Require a Functional Clock in the Ch Organs**

We wanted to determine if a clock is required in the peripheral tissues expressing F-gal4. For this, we expressed a dominant-negative form of the cycle gene (cycD) in either all clock-gene expressing cells or the F-gal4 pattern only. The UAS-cycD line causes arrhythmcity under DD and constant temperature.
conditions (Tanoue et al., 2004). As expected from the results obtained with the *tim* and *Clk* mutants (Figures 1B and 2A), UAS-*cyc*-Δ expression driven in all clock cells using the *tim-gal4* driver resulted in abnormal temperature entrainment (Figure 8A) (Yoshii et al., 2002, 2005). In contrast, when UAS-*cyc*-Δ was restricted to the *F-gal4*-expressing cells, temperature entrainment appeared normal (Figure 8B). This result is in good agreement with our failure to detect clock gene expression in ch organs (Figure S7), and also explains why *tim-gal4/UAS-noc-teRNAi* flies entrain normally to temperature cycles (Figure 4G).

**DISCUSSION**

**Temperature Entrainment of the Brain Clock Requires Signals from the Periphery**

We show here unequivocally that isolated brains are not able to synchronize their circadian clock to temperature cycles, whereas they do entrain to LD. Tissue-autonomous synchronization to LD cycles is very likely mediated by the blue light photoreceptor *Cry*, which is expressed within a large subset of lateral and dorsal clock neurons (Benito et al., 2008; Yoshii et al., 2008). Likewise, synchronization of peripheral clock cells to light-dark and temperature cycles is tissue autonomous (Figure 1A). In contrast, brains depend on signals from the periphery for temperature entrainment to occur, indicating different temperature entrainment mechanisms for peripheral clock cells and central brain clock neurons. A possible reason for this may be that clock neurons need to be “protected” from imminent influences of temperature changes, which can occur very sporadically in nature. In fact, work from the Emery lab has shown that, even within the brain, a certain subset of light-responsive clock neurons that mainly controls the behavioral morning activity (M-cells) seems to repress temperature responsiveness of a different group of clock neurons (Busza et al., 2007).
Molecularly, this block could be mediated by Cry expression in the clock neurons, because it has been shown that the cry\textsuperscript{B} mutation enhances the amplitude of temperature-entrained clock gene expression (Glaser and Stanewsky, 2007). This suggests that some neurons are more responsive to a certain Zeitgeber than others and vice versa. For example, the Cry-negative neurons may be more important as temperature sensors as it was shown for the DN2 and Lateral Posterior Neurons (LPNs) located in the dorsal and lateral brain (Miyasako et al., 2007). These two neuronal groups do not (so far) belong to the M- and E-cell groups, and they can mediate aspects of temperature-entrained behavior in the absence of the M- and E-cells (Busza et al., 2007). In larvae the Cry-negative DN2 also play a prominent role under temperature entrainment conditions, where they seem to determine the phase of the other clock neurons in the larval brain (Picot et al., 2009).

It is conceivable that such a division of sensitivity to environmental signals, including complex protection from temperature signals in certain neurons, is required for stable synchronization to natural light-dark and temperature cycles. Since temperature cycles are a less reliable Zeitgeber compared to light-dark cycles, it would make sense that a peripheral temperature input is received only by a subset of clock neurons. These Cry-negative neurons are usually entrained by the light-responsive Cry-positive neurons, but under certain environmental conditions they could turn into the dominant neurons—now synchronizing the light-responsive neurons and activity rhythms to temperature cycles.

Perceived temperature in these neurons occurs non-cell-autonomously (i.e., via the pericapsule) perhaps ensures that their input can more easily be controlled (i.e., shut off) by the clock-neuronal network.

**Ch Organs as Circadian Temperature Receptors**

We applied a set of PNS-gal\textsuperscript{4} driver lines to home in on the tissues responsible for circadianly relevant temperature reception. This strategy was based on three observations: (1) PNS cells have been reported to express per, although a function for this expression is not known (Plautz et al., 1997); (2) isolated tissues containing these PNS cells are able to synchronize per expression to temperature cycles (Figures 1, 2, and Glaser and Stanewsky, 2005); and (3) nocte expression is also found within PNS cells of these tissues (Figure 5).

We found that F-gal\textsuperscript{4}, previously reported to be expressed specifically in ch organs, leads to disruption of temperature synchronization when crossed to nocte-RNAi. Although this immediately suggested that ch organs are crucial for this form of entrainment, careful inspection of the F-gal\textsuperscript{4} expression pattern revealed that this driver is also active in es organs (Figure 5) and in a few neurons in the brain (Figures S6 and S8). Although nocte is also expressed in the brain, the spatial expression pattern of both genes appears to be distinct (Figures S6 and S8). Nevertheless, we cannot rule out that nocte and F-gal\textsuperscript{4} are coexpressed in a few brain neurons and that F-gal\textsuperscript{4}-mediated downregulation in brain neurons contributes to the observed temperature entrainment phenotype in F-gal\textsuperscript{4}/nocte-RNAi flies. In fact, nocte’s broad expression pattern in the brain does include the LNVs, and we could show that one LNv is also positive for F-gal\textsuperscript{4} (Figure S8). But several observations strongly suggest that it is the prominent expression of F-gal\textsuperscript{4} and nocte in ch organs that is mediating temperature entrainment. First, isolated brains do not synchronize to temperature cycles, indicating that nocte expression in the brain is not sufficient to mediate entrainment. Second, we found that mutations known to affect ch organ structure and function (tibl, eys, spam) also interfere with temperature entrainment. Third, expression or function in the brain for any of these genes has not been described, and even if they do act in the brain it seems very unlikely that they are all expressed in the same putative “temperature entrainment cells.” Fourth, if clock function is compromised in the one F-gal\textsuperscript{4}-positive LNv via expression of the dominant-negative form of cyc, behavioral synchronization to temperature cycles is not affected (Figure 8B).

The tibl and smet mutants applied here are known to specifically affect ch function and leave es organ function intact (Caldwell et al., 2003; Eberl et al., 2000). Similarly, the eys\textsuperscript{734/395} alleles retain normal mechanosensory function, but are thought to exhibit a molecular defect of the sensory dendrite of ch organs (Husain et al., 2006). All these mutations interfere with entrainment to temperature, but not to light-dark, cycles. Together with the prominent expression of F-gal\textsuperscript{4} and nocte in ch organs, these findings strongly implicate ch organs as mediators of temperature entrainment, at least within the temperature interval applied in this study (25°C-16°C).

**How May Ch Organs Perceive Temperature?**

Ch organs can function as stretch receptors and have been implicated in mediating proprioception, gravireception, and vibration detection (Kernan, 2007). In contrast to external sensory cells, adult ch organs do not contain external bristles, and are attached to the inside of the cuticle. They consist of one to several hundred sensory units (scopulae), and each of them contains a liquid-filled capsule (scolopale) that harbors the sensory endings of one to three neurons (Figure 6; Kernan, 2007). Interestingly, the Eys/Spam protein can be detected at the border between the ch neuron cell body and the lumen of the scolopale, and close to a characteristic dilation of the ch cilia (Cook et al., 2008; Husain et al., 2006). spams mutants exhibit a massive cellular deformation of the scolopale after exposure to 37°C (Cook et al., 2008). This deformation can be prevented by exposing the mutants to >90% humidity during the high temperature period. This suggests that the cellular deformation is caused by water loss from the hemolymph, which leads to water loss from the scolopale and subsequent neuronal deformation (Cook et al., 2008). eys/spams mutants show normal mechanoreceptor responses at room temperature, indicating that the presence of Eys/Spam protects the scolopale from excessive heat, probably by preventing water loss (Cook et al., 2008; Husain et al., 2006). nocte mutants exhibit the same temperature- and humidity-dependent uncoordinated phenotype as spams mutants, indicating a similar cellular deformation induced by excessive heat (Figure 7). Given that mutants of both genes also fail to synchronize to temperature cycles, we suspect that both phenotypes are related. As we show here, both nocte alleles lead to a structural defect in the dendritic cap of the ch organ (or misexpression of the dendritic cap protein NomP). It is conceivable that this defect also leads to excessive water loss at high temperatures, which would explain nocte’s
uncoordinated phenotype. For temperature entrainment to work properly it seems therefore absolutely crucial that the scolopale is protected from effects of extreme temperatures.

On the other hand, the ch organs must be able to sense subtle changes of temperature alterations in the fly’s physiological range in order to function as circadian temperature sensors. In larvae, both ch organs and es neurons in the body wall react by increasing $[\text{Ca}^{2+}]$ after raising or lowering the temperature (Liu et al., 2003), suggesting that they are also capable of detecting temperature changes in the adult. Considering that two other temperature entrainment mutants, tbl and smet, very likely affect the axonemal cytoskeleton structure of the ch cilia, we believe that perhaps dynamic properties of the ch cilia underlie temperature entrainment. The cilia in stimulated femoral ch organs of grasshoppers show active bending (i.e., not a passive reaction to the mechanical stimulus) close to the region where the cilia enters the scolopale (Moran et al., 1977). This ciliar bending presumably activates the ch neuron, which propagates the signal to the thoracic CNS. Interestingly, the same study showed that the femoral ch organ behaves tonically—in other words, it keeps firing at the same rate as long as the mechanical stimulation doesn’t change (Moran et al., 1977). This inability to adapt to an environmental stimulus is exactly what would be required for a circadian temperature receptor, because it is necessary that it tracks subtle changes in temperature over time. Our current hypothesis therefore postulates the scolopale as an active unit for circadian temperature reception. Eys/Spam and Nocte are required to protect the unit from water loss at different temperatures, rendering the cilia able to react to subtle changes in temperature by actively changing its shape (perhaps by bending). The degree of ciliar bending then determines the firing frequency of the ch neuron, which is tightly coupled to the ambient temperature.

The Role of the nocte Gene Product in Temperature Entrainment
Both nocte alleles show similar phenotypes in regard to temperature entrainment, dendritic cap, and uncoordination phenotypes, although nocte$^d$ always exhibits more severe defects than nocte$^c$. This suggests that nocte$^d$ is a hypomorphic allele, a suggestion also supported by the observation that it is able to generate normally spliced transcripts in addition to aberrant ones (Figure S2). We also have evidence that nocte$^f$ is not a null allele, because (1) we can detect a truncated protein of the predicted size on western blots probed with an anti-Nocte serum, and (2) driving nocte-RNAi with broadly expressed gal4 driver lines (e.g., nocte-gal4, tim-gal4) leads to adult lethality (C.G., H.S., A.S., A.G., and R.S., unpublished data).

Downregulation of nocte using F-gal4 results in a severe temperature entrainment defect, confirming that this transcription unit is involved in the process. Because F-gal4 is expressed within the neurons and cilia of ch organs, this behavioral defect indicates that nocte is also expressed in ch organ neurons (Kim et al., 2003). Based on the potential structural defect observed in nocte mutants, the Nocte protein may be required for the proper connection between the scolopale and the dendritic cap or proper expression and distribution of temperature-entrainment-relevant gene products along the cilia (Figure 6). This would also explain the structural defect or nompA misexpression phenotype caused by both nocte alleles (Figures 6D–6F and Table S1), which presumably underlies the observed temperature entrainment phenotype.

Requirement of a Functional Clock in Ch Organs
Our findings indicate that a functional clock within peripheral sensory structures important for temperature entrainment is not required (Figures 8 and S8). We therefore propose a model in which ch organ neurons, which do not possess a functional clock, send temperature information to peripheral clock neurons in the thoracic CNS, or directly to the more temperature-sensitive clock neurons within the brain (see above). A similar pathway has recently been described for sex peptide (SP) signaling, in which specific SP-receptor-expressing neurons located within the female reproductive tract signal to the CNS (Häsemeyer et al., 2009).

For daily temperature entrainment to work, temperature signals need to be interpreted by clock neurons in a time-dependent (i.e., circadian) manner in order to result in coordinated clock protein cycling and synchronized behavior controlled by these neurons. Neuronal brain clocks totally depend on these signals to become entrained by temperature, since they cannot synchronize in culture (Figures 1 and 2). Because isolated brains cell-autonomously synchronize to light (Figure 1), our findings reveal a fundamental difference between these two entrainment pathways.

EXPERIMENTAL PROCEDURES

Flies
For a detailed description of fly stocks used and generated for this study see Supplemental Experimental Procedures.

Bioluminescence Recordings and Imaging
Bioluminescence rhythms emanating from whole flies or individual body parts were recorded essentially as described (Glaser and Stanewsky, 2005) using an automated bioluminescence counter (Topcount, Perkin-Elmer). For more details, also regarding the generation of bioluminescence images shown in Figure 2 using the LuminoviewLV200 imaging system (Olympus, Tokyo, Japan), please refer to the Supplemental Experimental Procedures.

Mapping of nocte
By using visible markers, SNPs, chromosomal deficiencies, and insertions, nocte was mapped to the CG7725 transcription unit at position 9C6–9D1 on the X chromosome. For details see Supplemental Experimental Procedures.

Generation of noc te Constructs
Nocte RNAi constructs were designed using fusions between nocte genomic and cDNA as described previously (Kalidas and Smith, 2002). For details see Supplemental Experimental Procedures.

Behavioral Analysis
Locomotor activity rhythms were recorded automatically using the Drosophila Activity Monitoring (DAM) system (Trikinetics, Waltham, MA) as previously described (Glaser and Stanewsky, 2005). Flies were initially synchronized and recorded in LD (12 hr: 12 hr) at a constant temperature of 25°C for 3–5 days and then released to LL and temperature cycles (12 hr: 12 hr, 25°C:16°C) in opposite phase to that of the initial LD cycle (i.e., cryophase corresponded to light, and warm phase to dark portion of the LD cycle). For the experiments shown in Figure 4, the temperature cycle was shifted after 6 days, so that the warm phase occurred in a 6 hr delay compared to the initial
Peripheral Tissues Synchronize Brain Clock

temperature cycle, as indicated by the shading in the locomotor-activity plots (called actograms). For further details, please refer to the Supplemental Experimental Procedures.

Uncoordinated Assay and Quantification
The assay was performed as previously described (Cook et al., 2008). Flies were raised at 25°C and 10 young males were placed in a Petri dish and transferred for 90 min to a 37°C incubator where they were observed every 15 min. Relative humidity in the incubator was between 20%–30%. High humidity was obtained by adding a filter paper soaked in water to the Petri dish where the flies were monitored. The percentage of normal walking flies was determined by counting the number of flies walking at a certain time point and comparing it to the number of flies unable to walk or completely uncoordinated (i.e., lying on their backs or sides). High and low humidity observations were performed at the same time.

Fluorescence Microscopy and Immunohistochemistry
See Supplemental Experimental Procedures.

RNA Exactions and qPCR
Adult Drosophila (y w) were entrained for 3 days both under LD 25°C and LL 25°C:16°C (12 hr: 12 hr) conditions, and then collected at Zeitgeber Time (ZT) 0, 4, 12, 16, and 20. Collection and dissection of 10 individuals started 30 min before and finished 30 min after each ZT. Wings, legs, and heads were immediately transferred to dry ice, brains, ovaries, and testis to RNA later solution (Ambion), and stored at −80°C until RNA extraction. Two different UAS-nocte-RNAi transgenics (nocte-RNAi:1:3 and nocte-RNAi:1:3) were crossed with tim-gal4:6;7 and noc-te-gal4: M3. Crosses were kept at 18°C for 20 days and then transferred to 25°C. After four days at 25°C, five third instar larvae were collected from each cross and from two of the parental lines (nocte-RNAi:1:3 and tim-gal4:6;7) and processed for quantitative PCR using a Reverse Transcription Reagents Kit (Applied Biosystems) (see Supplemental Experimental Procedures).

nocte Transcripts in nocte Mutants
Two samples of two adult female flies were collected in parallel from Canton S and nocte strains, and RNA extraction was performed as described above for samples used in qPCR and eluted in a final volume of 200 μl of H2O. Reverse transcription reactions were performed with High Capacity RNA-to-CDNA Master Mix (Applied Biosystems) according to the manufacturer’s instructions. PCR products from both strains were generated with nocte-specific primers and sequenced as described in the Supplemental Experimental Procedures.

Comparative Sequence Analysis to Identify nocte Homologs
Nocte protein sequence from Drosophila melanogaster was initially blasted (blastn tool) against both the whole-genome shotgun reads on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the available genomic sequences of insects on the Vector Base website (http://www.vectorbase.org/Tools/BLAST/). Several insect species were identified as potentially containing nocte homologs. In order to obtain a more specific blast result, the genomic regions with the best hits were downloaded and blasted (blastx tool) against the GeneBank protein sequences of Drosophila melanogaster in Flybase website (http://flybase.org/blast/). The most reliable candidates are presented in Figure 3C.

SUPPLEMENTAL DATA
Supplemental data for this article include eight Supplemental Figures, one Supplemental Table, and Supplemental Experimental Procedures and can be found at http://www.cell.com/neuron/supplemental/S0896-6273(09)00638-2.

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Temperature Entrainment of Drosophila’s Circadian Clock Involves the Gene nocte and Signaling from Peripheral Sensory Tissues to the Brain

Figure S1: Impaired temperature synchronization in nocte\textsuperscript{1}/nocte\textsuperscript{p} females.

Group actograms, peak phase of activity (A, B upper left and right panels, respectively) and daily average activity (A, B middle and bottom panels) of flies that were entrained to 12 hr: 12 hr LD cycles for 3 days followed by two weeks of LL and 12 hr: 12 hr temperature cycles (25:16°C) in which the warm- and cold-phase were in anti-phase to the previous LD cycle (i.e., cold-phase corresponds to the light-phase) are shown. A) nocte\textsuperscript{1}/FM6 females were crossed to nocte\textsuperscript{p} males and locomotor rhythms of heterozygous nocte\textsuperscript{1}/nocte\textsuperscript{p} F1 females were analyzed. B) nocte\textsuperscript{p}/FM6 females from the same cross served as controls. Note that control females exhibit a steep and gradual activity increase after a trough in the early morning, whereas the mutant females show high activity throughout almost the whole warm phase. Higher activity of females vs. males during the day is also typical for flies entrained to LD cycles (Helfrich-Förster, 2000). White and black areas or bars in all behavioral plots indicate light or warm and dark or cold phase, respectively. Error bars and dots in peak phase and daily average plots indicate SEM’s. C) Quantification of ability to synchronize behavioral rhythms to temperature cycles (see Experimental Procedures for details).
**Figure S2:** nocte transcripts in the nocte\(^b\) mutant.

A) Transcripts from two independent RNA extractions (from two adult female flies each) from Canton S (i, ii) and nocte\(^b\) flies (iii, iv), obtained by PCR with primers that amplify a ca. 1.2 kb wild type nocte fragment (varies according to the different transcripts), starting from the 5’-UTR region, and spanning the insertion site of the \(P\)-element in nocte\(^b\), and the start codon. B) Schematic representation of the nocte wild type transcripts cloned and sequenced from samples in (A). C) Abnormally spliced transcripts cloned and sequenced from nocte\(^b\). The upper transcript contains all necessary sequences to encode a normal Nocte protein. The middle transcript encodes a predicted fusion protein between proteins encoded by \(P\)-element sequences and 59 out-of-frame amino acids encoded by nocte. The bottom example encodes short (20 amino acid) peptides initiated at an ATG upstream of the predicted nocte ATG, which are terminated at an out-of-frame stop codon in the nocte coding region.

**Figure S3:** Efficiency of nocte downregulation by RNAi.

Relative amounts of nocte RNA in larvae expressing two different \(UAS\)-nocteRNAi constructs (lines 2:1b and 1:3) under the control of two different drivers (nocte-gal4:M3 and tim-gal4:67). nocte mRNA levels in two of the parental lines (\(UAS\)-nocteRNAi:1:3 and tim-gal4:67) were used as control. For all samples expression was normalized to rp49. For both drivers, Analysis of Variance (ANOVA) indicates that the difference between larvae expressing nocte RNAi and the control is highly significant (\(P<0.005\)). Error bars indicate SEM.
**Figure S4:** Examples and classification of flies with normal or impaired abilities to synchronize to temperature cycles.

Locomotor activity plots of individual flies during the same LD and temperature entrainment regime shown in Figure 4. To classify each fly as ‘entrained’, ‘weakly entrained’, or ‘not entrained’ we generated an individual actogram (left column), and an average activity plot for each day of the experiment (middle and right columns, showing the activity during one day in the life of that particular fly, respectively). Flies were classified as ‘entrained’ if they exhibited a clear and sharp activity peak in the warm phase (arrows in the upper panel), or ‘weakly entrained’, if that peak appeared broader and the behavior during the warm phase was more erratic (arrows in the middle panel). For easier identification of the entrained peak, especially in more noisy records, a three-point-moving-average filter was applied to the raw data (dots connected by red line). ‘Not entrained’ flies did not show a behavioral peak during the warm phase but stayed active constitutively or showed erratic behavior. All flies did show a response to the temperature changes by increasing or decreasing their activity abruptly after the temperature changes (arrow heads) (see Experimental Procedures for more details). The flies shown here were from the *Canton S* (upper panel) and *eys* strains (middle and lower panels).

**Figure S5:** Normal temperature-entrained behavior of ‘weak’ UAS-nocteRNAi lines, and flies with impaired antennae in temperature cycles.

**A-C** Group actograms and daily average activity of flies that were entrained to 12 hr: 12 hr LD cycles for 5-7 days followed by one week of LL and 12 hr: 12 hr temperature cycles (25:16°C) in which the warm- and cold-phase were in anti-phase to the previous
LD cycle (i.e., cold-phase corresponds to the light-phase). **A)** The *nocte-RNAi* line shown here belongs to type 2 (Figure 3B) (line 2-1a) and was crossed to the *F-gal4* insertion on chromosome 2. **B, C)** Ablation of either the 3rd antennal segment or the whole antenna resulted in ‘entrained’ behavior demonstrating that the ch organ in the antenna is not required for temperature synchronization. Dots in daily average plots indicate SEM’s. **D)** Quantification of behavior in temperature cycles of all *nocte-RNAi* lines after crossing to the *F-gal4* line located on chromosome 3. Note that lines from both types of *nocte-RNAi* can result in normal or mutant behavior, indicating that positional effects of the insertion site influence the efficiency of the RNAi effect. Similarly, the *F-gal4* line located on chromosome 3 (*F-gal4-33-5*) produced stronger phenotypes compared to the chromosome 2 insertion line (compare *nocte-RNAi-2-1a* in panel A with panel D).

**Figure S6:** Overview of *F-gal4* and *nocte-gal4* driven reporter gene expression in the brain. **(A, B)** Expression of *UAS-gfp* driven by *nocte-gal4* in the brain. GFP was detected in many neurons and glia cells within the central brain, subesophageal ganglion (SOG), and in the optic lobes (OL). A cluster of neurons in the ventro lateral brain (arrows) corresponds to the ventral lateral clock neurons (LNvs) (see Figure S8). Dorsally from the LNvs is a group of neurons (arrowheads) from which one cell projects dorsally into the midbrain (open arrowheads). This S-shape axonal projection is typical for a subset of the dorsal lateral clock neurons (LNd) suggesting that this neuron represents an LNd (Figure S8). About 20 large neurons are clustered in the pars intercerebralis (PI). In the SOG, neurons are marked in the lateral and ventral regions. Some of them (open arrows)
are located close to the intake of the connectives to the spinal nerve cord. Extensive GFP signals were found in all compartments of the mushroom bodies including α, β, and γ lobes (α, β, γ), pedunculus (P), and calyx (Ca). (C, D) Brain expression of UAS-gfp driven by F-gal4. The dorsal brain harbours a group of about 3 small and 1 large neurons (arrowheads) with typical S-shape axonal projection (open arrowhead) running first to the dorsal and then to the central brain where they ramify. Varicose fibres then split in a thin nerve bundle which extents into the contralateral brain hemisphere. In the dorsal brain close to the posterior surface, a cluster of about 5 small cells (asterisks) contains GFP. Five bilateral neurons (daggers) are clustered in the anterior deutocerebrum. The most prominent signal emanates from neuronal arborisations within the antennal lobes and antennal nerves (arrows) probably originating from neurons of the antennal chordotonal organs. These arborisations form two thin nerve bundles. One interconnects the arborisation in the contralateral brain hemispheres. The second one runs dorsomedially to the brain midline where it merges with axonal projections arising from a bilateral cluster of about 4 large neurons located in the ventral area of the SOG (open arrows) and then runs parallel to the contralateral bundle into the pars intercerebralis where it vanishes. This nerve fascicle divides into two small bilateral ramifications in the wedge between the proto- and deutocerebrum. A small neuron (double arrowheads) projecting to the central brain as well as to the medulla is located in the ventral optic tract. Signals in the retina (R) can represent an autoflorescence, since in the control experiment (UAS-gfp flies) a signal was detected in this area. Scale bar = 100 μm.
**Figure S7:** Reduction of nocte expression does not grossly interfere with development of chordotonal organs, which express F-gal4 but not tim-gal4. (A-G) UAS-rfp and nocteRNAi:1:3 driven by F-gal4 in external body parts. Widespread distribution of RFP was detected in nuclei of the putative chemo- and mechanoreceptors cells in the mouthpart (A), antennae (A, B), wing (C, D), haltere (E) and leg (F, G). Positive signals were also found in neurons of the chordotonal organs (arrows) in all external tissues tested. Down-regulation of nocte expression did not cause any significant changes in distribution of RFP, suggesting that nocte is not essential for development of F-gal4-expressing sensory organs (compare with Figure 5I-P). (H-N) UAS-mCD8gfp and UAS-rfp driven by tim-gal4:62. GFP and RFP signals were detected in mechano- and chemoreceptor cells in the mouthpart (H), antennae (H, I), wing (J, K), haltere (L), and leg (M, N). No signals were detected in the chordotonal organs (arrows). This was also the case for the other tim-gal4 lines used in this study. labp = labial palpus; mxp = maxillary palpus; ant = antenna. Scale bar (A, C, F, H, J, M) = 100 μm, (B, D, E, G, I, K, L, N) = 10 μm.

**Figure S8:** nocte-gal4 and F-gal4 expression in canonical clock neurons in the brain. Upper panel: Period expression in nocte-gal4/UAS-rfp flies was determined by anti-Per immunostaining (green) to identify dorsally located clock neurons co-expressing nocte-gal4 (red). At least 4 DN1 and 1 LNd co-express Period and nocte-gal4 (arrowheads in merged image). Middle panel: Anti-PDH signals (red) in nocte-gal4/UAS-gfp brains reveal that nocte-gal4 is expressed in all ventrally located PDH-expressing clock neurons (LNvs). Lower panel: All clock neuronal groups were identified by anti-Per
**Figure S1**
Figure S2
Figure S3
Figure S5
Figure S8
Table S1: Quantification of dendritic cap defects or NompA misexpression in nocte mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>control (FM6)</th>
<th>nocte&lt;sup&gt;l&lt;/sup&gt;</th>
<th>nocte&lt;sup&gt;p&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of individual flies checked</td>
<td>10</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>number of flies showing defects (%)</td>
<td>1 (10)</td>
<td>4 (100)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>average number of caps visualized per femur/average number of defective caps (%)</td>
<td>30/1 (3)</td>
<td>20/7 (35&lt;sup&gt;1&lt;/sup&gt;)</td>
<td>22/6 (27)</td>
</tr>
</tbody>
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Males homozygous for the gfp-nompA reporter construct were crossed to nocte<sup>l</sup>/FM6 and nocte<sup>p</sup>/nocte<sup>p</sup> females. In the F1, 4-6 legs of each male individual from the indicated genotype (each carrying one copy of the gfp-nompA reporter) were dissected and mounted on a glass slide in a glycerol:water (2:1) solution. Freshly mounted legs were then directly inspected by confocal microscopy and one femur was scored for the presence and frequency of the dendritic cap phenotype per fly.<sup>1</sup> Note that although every nocte<sup>l</sup> fly exhibited the phenotype, the frequency of defective dendritic caps within one femur varied substantially between individuals: it ranged from 15% to 70% between individual flies.
**Experimental Procedures**

**Flies:** As control flies either wild type *Canton S* or *y Df(1)w* (*y w*) were used (Lindsley and Zimm, 1992). *XLG-luc; 8.0-luc.9* (Veleri et al., 2003), and *BG-luc* (Stanewsky et al., 1997) transgenics have been described previously as have the *Clk^brk* (Allada et al., 1998), *tim^01* (Sehgal et al., 1994) rhythm mutants and the *norpA^P41* null mutation (Lindsley and Zimm, 1992). The original *nocte^l* allele was isolated after chemical mutagenesis as described (Glaser and Stanewsky, 2005). The *P{XP}CG17255^d07154* insertion in the first intron of *nocte* (*nocte^p* ) was obtained from the Exelixis collection at Harvard University, Boston, MA (USA) (Thibault et al., 2004). A duplication covering the chromosomal interval 9A2-10C2 *[Dp(1;2)v^+75d]* was obtained from the Bloomington stock center as were the *X*-chromosomal deficiencies and multi marker chromosome bearing stocks used in the mapping tests. Two different insertion lines of *F-gal4* were applied in this study, one is inserted on chromosome 2 (*F-gal4*), one on chromosome 3 (*F-gal4-33-5*) (Kim et al., 2003). The *UAS-cycΔ:24* line was described previously (Tanoue et al., 2004) as well as *eys^395*, *eys^734*, and *spam^l* mutants (Husain et al., 2006; Zelhof et al., 2006), and the *tilB^l* and *smetana* mutants (Caldwell et al., 2003). The reporter strain expressing a *gfp-nompA* fusion protein under control of *nompA* promoter sequences was generated in the laboratory of M. Kernan (Chung et al., 2001). The *eys* and *deaf-RNAi* stocks were obtained from the National Institute of Genetics Fly Stock Center (Japan). A transgenic type carrying *tim-gal4* (lines 16, 27, 62, 67) (Kaneko and Hall, 2000) was used to drive *UAS-cycΔ:24*, to visualize *tim* expression in peripheral tissues (whereby *tim-gal4* drove expression of *UAS-mCD8gfp* and *UAS-rfp*), and to drive *UAS-nocte-RNAi* to determine
the efficiency of *nocte* mRNA reduction or potential effects on temperature-entrained behavior. Transgenic flies carrying either of the two *nocte-RNAi*, and the *nocte-gal4* constructs (for details see below) were generated in a *y w* genetic background by BestGene Inc. Ten independent lines were generated for each construct. Six type-1 and six type-2 *nocte-RNAi* constructs were tested, out of which four (type-1) and two (type-2) cause temperature entrainment phenotypes after crossing to *F-gal4* (Figure S5).

Experiments shown in Results were performed with lines *UAS-nocte-RNAi-1:3* and *UAS-nocte-RNAi-2:1b* (except the one shown in Figure S5A, where *UAS-nocte-RNAi-2:1a* was used). For *nocte-gal4* three independent insertion lines (F10, M3, M6) were crossed to *UAS-gfp* (nuclear), *UAS-mCD8gfp*, and *UAS-rfp* (all reporter lines were obtained from the Bloomington stock center) and found to exhibit identical expression patterns in brain and peripheral clock tissues. The *nocte-gal4:M3* line was applied in the experiments shown here. To visualize nuclear and membrane expression at the same time, a true-breeding stock containing *UAS-rfp* and *UAS-mCD8gfp* was generated by standard crossings.

**Bioluminescence recordings and imaging:** Flies or tissues were kept either in LL and temperature cycling conditions (12 hr: 12 hr, 16°C: 25°C), or in LD (12 hr: 12 hr at 25°C) for the entire duration of the experiment. Raw data was plotted and analyzed (including determining the peak phase of expression plotted in Figure 1A) using Brass software (Locke et al., 2005) as described (Glaser and Stanewsky, 2005). For the images shown in Figure 2, tissues were dissected under M3 Insect Medium (Sigma) and kept for three days in culture medium (M3 Insect Medium with fetal Bovine serum, Penicillin-Streptomycin,
Insulin and Luciferin [200 µM, Biosynth]) in light and temperature regimes as stated in
Results text. The entrained tissues were imaged in the LuminoviewLV200 (Olympus,
Tokyo, Japan) at a time corresponding to peak expression of PER in the Topcount
luminometer (at ZT18 for brains and external tissues in LD cycles and at ZT11 and ZT18
for brains and external tissues, respectively, in LL and temperature cycles). Brains were
exposed for 25 min and external tissues for 15 min at a gain of 255. Bioluminescence
images were analyzed and merged with bright-field images using CellM imaging software
for life science microscopy (Olympus).

Mapping of nocte: 118 meiotic recombinants between the nocte and multi marker
chromosome y w cv v f car were established. Testing these recombinants for molecular
(using bioluminescence measurements, see above) and behavioral (see below)
temperature-entrainment phenotypes placed nocte between cv (cytological map position
5A13) and v (9F11). Further mapping using Single Nucleotide Polymorphisms (SNP’s),
which distinguish the nocte and marker chromosomes, placed nocte between CG15245
(9A2) and CG2124 (9D3). The accuracy of this mapping was confirmed by covering
phenotypic effects of the nocte mutation with Dp(1;2)v^{+75d} (9A2-10C2) (Figure 3A). For
further mapping, chromosomal deficiencies with molecularly defined breakpoints were
applied (Ryder et al., 2004). From five deficiencies tested [Df(1)ED6991, Df(1)ED7005,
Df(1)ED7010, Df(1)ED429, and Df(1)ED7042] only Df(1)ED7005 uncovered effects of
the nocte mutation (both in bioluminescence and behavioral assays), placing nocte in the
interval between 9B3 and 9D3. This region is bordered by the genes CG15309 and
CG15296, an interval containing 35 genes (Tweedie et al., 2009). Another deficiency
[Df(1)c52,flw^{52}, which removes 8E3-5;9C1] complements \textit{nocte} but not \textit{flw}, thereby further reducing the interval to the 12 genes between \textit{flw} (9B14-C1) and \textit{CG15296} (9D3) (Tweedie et al., 2009). No obvious candidates could be identified among the 12 remaining genes so that we initiated sequence determination of all candidates along with analysis of available mutations and \textit{P}-element insertions. This approach resulted in the identification of a \textit{P}-element insertion (\textit{P}\{XP\}CG17255\textsuperscript{d07154}) in the gene \textit{CG17255}, which exhibits very similar temperature entrainment phenotypes compared to the original \textit{nocte} mutation (Figures 3A and 4C).

\textbf{Generation of \textit{nocte} constructs:} For RNAi construct 1 genomic DNA containing \textit{nocte} exon 2, intron 2, exon 3 and intron 3 was amplified by PCR and cloned into pUAST via EcoRI and NotI. An inverted cDNA fragment encoding exons two and three was subsequently cloned as a NotI-XhoI fragment into the same vector to create a fusion between the genomic DNA and the reversed cDNA. For \textit{nocte} RNAi construct 2 the 3' end of exon 4, intron 4, exon 5 and intron 5 were chosen and cloned into pUAST as a BglII-NotI fragment. An inverted cDNA fragment encoding the 3' end of exon 4 and exon 5 was fused to this genomic DNA fragment by cloning it subsequently into the NotI and XbaI sites of the same vector. Primer sequences for \textit{nocte} RNAi constructs: RNAi construct 1 genomic forward: GCGGACGAAATTCACAAACCCACCAGAAAGACTGAAACA; RNAi construct 1 genomic reverse: CCGGCACGCAGCGCCGCTAAATGGAAAAATTCACCCAGTTAAGG; RNAi construct 1 cDNA forward:
CCGGCGACGCGGCCGCTTCGACTGAGACTAAGAGATTGT; RNAi construct 1 cDNA reverse: GCATCCGCTCGAGATGAGTACACTGGGGGGAAG; RNAi construct 2 genomic forward: GGCAGATCTAAAATGAATCTTTATGCGGCTCCAC; RNAi construct 2 genomic reverse: CCGGCGACGCGGCCGCTGAAGAGGAAAGAAGGATGAATTAGG; RNAi construct 2 cDNA forward: CCGGCGACGCGGCCGCGCCGTAGTTCTTGTGA; RNAi construct 2 cDNA reverse: GCGGTCTAGAATGAATCTTTATGCGGCTCCAC.

The *nocte* promoter region contained 3.646 kb of DNA upstream of the predicted start codon and was amplified from genomic DNA using primers that introduce XbaI and SpeI sites. The resulting fragment was then cloned into pPTGAL4. PCR primers to amplify the *nocte* promoter region were:

- **Forward:** GGCTCTAGAGGAGTCAGATTCAGATTCCGGC
- **Reverse:** TGGCGCACTAGTGCTGCCAATAGATCCTCATTAG

**Behavioral analysis:** Daily average histograms and actograms were plotted using the fly toolbox and MATLAB software (Levine et al., 2002). Quantification of the ability to resynchronize to temperature cycles was performed by first generating an actogram and daily histograms for individual flies. All individuals from each genotype were then scrutinized for their activity peak at each day of the experiment. To facilitate detection of peak activity a three-point moving average filter was applied to the data (red-dotted line in Figure S4) (Garnett, 1997). Wild-type flies typically show two activity peaks in the entrainment regime applied here: one peak immediately after the temperature rise, which we interpret as a ‘response’ to the environmental change, rather than entrainment (Glaser...
and Stanewsky, 2005; Yoshii et al., 2005). In addition a major activity peak occurs in the second half of the warm phase. This peak is not observed in clock or temperature-entrainment mutants and is therefore referred to as the ‘entrained’ peak. If present, this entrained peak was determined (by eye) and assigned a numerical value for each day and each individual and tracked throughout the experiment (Phase plots in Figure 4). These animals were classified as ‘entrained’ (Figure 4) and examples are shown in Figure S4. Most mutant animals did not show a clear entrained peak but were either constitutively active or inactive during the warm phase. Since they did show a response to the temperature increase, this ‘response’ peak was tracked and plotted in Figure 4. These individuals were classified as ‘not entrained’ (Figure 4) and examples are shown in Figure S4. In addition, some individuals displayed an intermediate behavior. They did show characteristics of an entrained peak, which was usually less pronounced and noisier compared to wild type flies and controls. Also, these flies often displayed a more drastic response to the warm transition compared to controls. These individuals were classified as ‘weakly entrained’ (Figure 4) and typical examples are shown in Figure S4.

**Fluorescence Microscopy and Immunohistochemistry:** To observe GFP and/or RFP signals tissues of transgenic flies were dissected in *Drosophila* Ringer solution and fixed in 4% paraformaldehyde overnight at 4 ºC. Samples were then washed 3 times for 15 min in 0.1 M sodium phosphate buffer (pH 7.4) then 3 times for 10 min in phosphate-buffer saline (PBS) supplemented with 1% Triton X-100 (PBS-T) at room temperature. Following 5 min rinse in distilled water, samples were mounted in Vectashield medium (Vector laboratories, Burlingame, Calif.) and examined under a LSM-510 META
confocal microscope (Zeiss, Germany). In control assessments, the original UAS-
mCD8gfp, UAS-gfp, and UAS-rfp lines were used to verify the absence of Gal4-
indepedent signals (data not shown). For immunostaining with monoclonal 22C10 antibodies, samples were blocked for 2 hr in 5% normal goat serum in PBS-T after the fixation and washing steps. This was followed by incubation with the primary antibody (dilution 1:100, Developmental Studies Hybridoma Bank) in blocking solution over night (4°C) and washing steps described above. Samples were then incubated with Phalloidin-
TRITC (1:1000, Sigma) and secondary Alexa 647 goat anti-mouse antibodies (Molecular Probes). Samples were then washed with PBS-T (3 x 20 min) and rinsed in water (5 min) before being mounted and inspected as described above. Control and nocte mutant flies expressing the gfp-nompA construct were obtained after crossing homozygous gfp-nompA males (inserted on chromosome 3) to nocte1/FM6 and nocte2/FM6 females. Legs from 2-
3 day old nocte1 mutant and FM6 control males generated by this cross (both carrying one copy of the gfp-nompA construct) were dissected in Ringer solution and cut at the proximal end of the femur to allow antibody penetration. For quantification of the ch organ structural defect observed in nocte mutants see legend to Table S1. For anti-Per and anti-PDH immunostainings (Figure S8) fixed and washed preparations were subsequently blocked with 10% normal goat serum in PBS-T for 2 h at room temperature and incubated for 2 days with anti-PER at 4°C (Veleri et al., 2003) or with affinity purified anti-crab-PDH (Hodge and Stanewsky, 2008) (both diluted 1:1000 in PBS-T). After washing, samples were incubated with AlexaFluor 488 (green) or AlexaFluor 594 (red), respectively (both diluted 1:300 in PBS-T).
RNA extraction, Reverse Transcription reaction, and qPCR: Total RNA was extracted with 0.5 ml of TRI Reagent (Ambion) followed by purification with Lithium Chloride solution (Ambion) according to the manufacturers’ instructions. The volume of H$_2$O used for the final elution of the RNA depended on the nature of the sample, according to the potential cDNA synthesis inhibition detected for each one, which was determined as follows: standard curves of RNA dilutions from each sample were performed for every pair of primers to be used in the quantitative PCR. For 10 flies the volume of H$_2$O used to elute the extracted RNA from each sample (volume in which no inhibition of cDNA synthesis was observed for any pair of primers) was: heads 100 μl, brains, legs, and wings 40 μl, ovaries and testis 200 μl, and 5 3rd instar larvae in 800 μl. cDNA synthesis was performed with Reverse Transcription Reagents Kit (Applied Biosystems) in 10 μl reactions according to the manufacturer’s instructions. For quantitative PCR, first, the 10 μl cDNA from each sample was diluted 10 times, and then qPCR reactions were prepared as follows: 7.5 μl Power SYBR Green PCR Master Mix (Applied Biosystems), 4.0 μl of cDNA, each primer at 0.5 μM final concentration and H$_2$O to a 15 μl final volume reaction. The primers used in the qPCRs were as follows (always in 5’ -> 3’ orientation): rp49 sense CGATATGCTAAGCTGTCACA, rp49 antisense CGCTTGTTCGATCCGTAACC, period sense CAACAAAGTCGGTGTCAGAC, period antisense GTCTTGACCAGGCATGCTCTG, noce sense AAGACTACGGCCGCGTG, noce antisense CCAAGGGTCGTCATGCT (note that the pair of primers used for noce targets a region that does not overlap with any of the two noce-RNAi constructs used). Reactions were performed in a Chromo4 Detector (Bio-Rad) under the following temperature conditions: hot start at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for
30 seconds, and 10 seconds at a reading temperature (reading of the signal). The reading temperature for a pair of primers is generally ~3-5°C lower than the melting temperature of the amplicon they generate, and higher than that of the potential primer dimer. The reading temperature for the pair of primers used and listed above were 77°C for rp49 and period, and 80°C for timeless and nocte. For each sample, three replicas of the reaction were run in parallel, and the average of their Ct (threshold cycle) values (excluding outliers) was considered for quantification. The relative quantification was determined using the comparative C\textsubscript{T} method, also known as the ΔΔC\textsubscript{T} method, or the 2^{-ΔΔC}_{T} method (Livak and Schmittgen, 2001; Pfaffl, 2001), using rp49 as control. The Applied-Biosystems User Bulletin #2: Relative quantification of gene expression (1997, updated on 10/2001, available on line) was also used as support guide for the calculations, all performed in Microsoft Excel software. For the statistical analysis, analysis of variance (ANOVA) and LSD Post-Hoc test (\(P<0.05\)) were performed when possible (for details see legend of figures).

**nocte transcripts in nocte\textsuperscript{p} mutants:** PCR was conducted with 2.0 \(\mu\)l of cDNA, 12.5 \(\mu\)l of LongAmp Taq 2x Master Mix (New England Biolabs), a pair of primers for nocte gene to a final concentration of 0.5 \(\mu\)M each (sense: 5’- GCGGCAAACCTTTATGTTGGA -3’; antisense: 5’- CGTGTCCCTGTGGTAATTGC -3’), and \(\text{H}_2\text{O}\) to a final volume reaction of 25 \(\mu\)l. The pair of primers used amplifies a ca. 1.2-kb wild type nocte fragment (varied slightly according to the different transcripts; see Figure 3B), starting from the 5’-UTR region, and spanning the insertion site of the \(P\)-element in nocte\textsuperscript{p} and the start codon of the wild-type transcripts. For each strain, the two independent PCR reactions were pooled and
purified with MicroSpin S-400 HR Columns (GE Healthcare Life Sciences), cloned in pGEM-T Easy Vector (Promega) according to the manufacturer’s instructions, and used to transform XL1 Blue cells. On average 25 clones from each transformation were sequenced.

Supplemental References:


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7.3 Mechanical stimulation of ch organs synchronizes fly’s locomotor activity

Recent findings suggest that chordotonal organs are required for the temperature-dependent entrainment of circadian clocks (Sehadova et al., 2009). Insects use ch organs to convert sound and other mechanical stimuli into action potentials that then are propagated to the central nervous system (Eberl, 1999; Albert et al., 2007). As ch organs are mechanosensory organs, I wondered whether the mechanical stimulation of ch organs alone could be sufficient for circadian clock entrainment. Specifically, I asked if a vibratory stimulus that was designed to excite ch organs could act as Zeitgeber and therefore entrain the circadian clock. In collaboration with Dr Jörg T. Albert (Ear Institute, University College London, UK), we set-up a mechanical stimulation apparatus which allowed us to measure the circadian patterns of locomotor activity of flies subjected to 12:12 hr “vibration:silence” (VS) cycles (see M&M for more details). Briefly, flies were subjected for 12 hours to a continuous 2-component stimulus of 200 Hz and 40 Hz vibrations followed by 12 hours of “silence” (i.e. background noise with an intensity of the order of a ten-thousandth lower compared to the stimulus applied). The two frequency have been chosen in order to stimulate both the Johnston’s Organ (200Hz) and the other, non-hearing, ch organs in the fly’s body (40Hz).

Flies were first entrained to 12:12 hr LD cycles and then transferred to DD and the VS was applied “in phase” with the previous LD (“vibration” corresponding to the previous day and “silence” corresponding to the previous night). After 5 days, the VS regime was delayed by 6 hours compared to the previous one and kept like that for 7 days. Next, another 6 hour VS shift (delay) was applied, so that the VS stimulation resulted in opposite phase compared to the initial LD
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Figure 7.1: Average locomotor activity of wild-type and antennae-ablated flies (subjected to VS cycles) and controls (not subjected to VS cycles). A, B) Wt (n=15) and flies with bilateral ablation of all antennal segments (n=16) were first entrained in LD cycles (2 days). Then, flies were transferred to constant darkness and 12:12 hr “vibration-silence” (VS) cycles were applied in phase with the previous LD (arrows). After 5 days, the VS cycles were shifted of 6 hr (delayed) and kept for 7 days. Next, a subsequent 6-hr delayed shift was applied so to have the VS cycles in opposite phase compared to the initial LD regime. After that, flies were release in constant condition (arrow heads) and the phase was calculated (see Figure 7.5). C, D) Wt (n=6) and flies with bilateral ablation of the three antenna segments (n=14) were entrained to LD cycles and then released in DD served as controls. Free-running period are given in Table 7.1. White and grey shades represent “vibration” and “silence”, respectively (or light and dark, for the first 2 days).
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Table 7.1: Free-running period of wild-type and antennae ablated flies subjected to vibration-silence (VS) stimulation and control (not subjected to VS). Period (\(\tau\)) and rhythmicity statistic (RS) are calculated only for rhythmic flies. Total number of flies tested is indicated (n).

<table>
<thead>
<tr>
<th>Subjected to VS</th>
<th>Not subjected to VS</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Rhythmic</td>
</tr>
<tr>
<td>Wild-type</td>
<td>28</td>
</tr>
<tr>
<td>Ant ablated</td>
<td>26</td>
</tr>
</tbody>
</table>

Analysis of average actograms and daily activity revealed that wild-type Canton S flies subjected to the VS stimulation exhibit different rest-activity patterns compared to free-running controls (Figure 7.2A–D). They display a sharp increase of locomotor activity after the transition from “silence” to “vibration”, plus a major peak of activity during the vibration phase. The main peak of activity of flies subjected to the mechanical vibration occurs at the end of the vibration in the first VS cycles, then moves to the mid of the vibration part during the second VS cycles and after the second shift, it arises soon before the transition from “silence” to “vibration” (Figure 7.2B–D). The increase of activity occurring after vibration-on is reminiscent of the startle response induced by light and temperature. Wild-type flies not subjected to the VS cycles free-run with a period of 24 hours (Figure 7.1 and Table 7.1) and the phase of the main peak of activity follows the previous LD entrainment (Figure 7.2E–H). The differences on the activity phase and pattern induced by the exposure to the mechanical stimulation is conspicuous, and it does not seem to be a reaction (masking) response to the vibration stimulus (see below).

The fly’s hearing organ is located in the second antennal segment (Caldwell and Eberl, 2002). It is composed of an array of specialized scolopidial cells, named
Figure 7.2: Daily average activity of wild-type flies subjected to 12:12 hr VS cycles and control. See Figure 7.3 legend for details.
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Antennae ablated

LD cycles

A

First VS cycles (in-phase)

B

F

Second VS cycles (6 hr delayed)

C

G

Third VS cycles (6 hr delayed)

D

H
Johnston’s organ (JO). Beside the auditory JO, the fly possesses a series of further ch organs which are mainly located in the animals’ legs and thought to serve proprioceptive or vibratory functions. In order to assess the role of the fly’s antennal ch organ for the observed behavioural changes, we removed the flies’ antennae prior to subjecting them to the VS regime. As shown in Figure 7.3A–D, the pattern of activity of antennae-ablated flies is comparable to that of wt flies subjected to the same VS condition (Figure 7.2A–D). In contrast, the activity of antennae-ablated flies not subjected to VS is comparable to that of wild-type (with antennae) not subjected to VS (Figures 7.2E–H and 7.3E–H, respectively). This suggests that the pattern of locomotor activity during VS conditions is not mediated by JO only, and therefore it is not perceived by the flies as an acoustic signal only.

However, we observed two characteristics of the behaviour of antennae ablated flies. The increase of activity just after “vibration-on” is reduced compared to wild-type flies, suggesting that this “response” is mediated by the fly’s ear (JO) rather than by the non-antennal ch organs. Secondly, antennae ablated flies are more active during the “silence” phase compared to wild-type flies. This phenomenon is more evident after the VS shift (Figure 7.3C,D). To support this observation, we quantified the relative activity of flies during the “silence” phase of flies subjected to the VS conditions, compared to the control flies kept in DD (free-running conditions). As shown in Figure 7.4, 65% of the total activity of antennae ablated flies...
flies occurs during the “silence” phase, compared to the 45% of wild-type flies with antennae, in a reproducible way between independent experiments ($F_{(1,5)}=45.4$, $P < 0.01$, Two-way Anova). Free-running control flies do not show differences of relative activity in the corresponding part of the day, indicating that the shift of activity is not caused by the ablation of antennae per se, but that the effect induced by the lack of antennae depends on the vibration stimulation. The reason for this intriguing phenomenon is still unclear.

To investigate whether the locomotor activity pattern was synchronized to the VS stimulus or the vibration was only masking the locomotor activity inducing a passive response, we analysed the phase of free-running activity after releasing the flies to constant conditions, compared to the phase of flies which have not been exposed to the mechanical stimulation. From the actograms depicted in Figure
7.1 it is already clear that there is a difference in phase between flies exposed to VS (upper part) and controls (lower plots). Nevertheless, we estimated the free-running phase of individual flies and determined the mean phase and intensity in order to quantify the visual impression of the actograms. The circular phase analysis (see Levine et al., 2002a,c and M&M for details) is depicted in Figure 7.5. Every dot in the plot represents the mean free-running peak phase time of single flies plotted in a circular graph with the mean phase of the group depicted by a vector starting from the center and pointing towards the time (expressed in Circadian Time, CT0 = vibration-on, CT12 = vibration-off, or equivalent time for the control). The phase of wild-type and antennae ablated flies (blue stars) is 2.0 and 1.4 hours, respectively. The phase difference compared to the respective controls, which have not been subjected to the vibration (red circle), is 9.8 and 8.5 hours, respectively (Figure 7.5). The mean phase difference between flies subjected to VS and control is strongly significant (M = 100 means p < 0.001).

Interestingly, the dispersion of phase values (given by the strength of the vector in Figure 7.5) tends to be higher (and the vector smaller) for flies subjected to VS (blue asterisks) compared to control (red circles), both for wt and antennae ablated flies.

Therefore, the VS conditions change both the locomotor activity patterns during the stimulation, and during the subsequent free-run. Taken together, these findings indicate that flies can be entrained by the “vibration-silence” cycles and they do not exhibit a passive (masking) response only. The ablation of the antennae does not prevent the flies from synchronizing to the VS stimulation, and thus the antennae are not necessary for this behaviour, as they are not required for the fly to entrain to TC. However, flies lacking antennae exhibit a different behaviour, and the difference can be measured and quantified. The vibration stimulus is per-
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A Wild-type

B Antennae ablated

Figure 7.5: Circular phase analysis of activity peaks during free-run after VS regime of wt and antennae ablated flies compared to control. The plots represent the mean peak phase time values over the DD days of each individual flies after VS treatment (blue asterisks) and non-treated controls (red circles). The mean phase is represented as a vector with the mean direction indicating the time and the magnitude of the vector indicating the variability (dispersion) within the group. The internal black circumference represents 100% coherence between individuals of the same group. The closer the vector is to the black line, the more coherent the group is. The phase is calculated from the origin (0 h) and within the range $-\pi \leq \text{phase} \leq \pi$. The phase difference compared to control is 9.8 hours for wild-type (A) and 8.5 hours for flies with antennae ablated (B). M values $\geq 95$ indicate statistical significance of the mean phase difference between the two groups (Rayleigh’s test). D values indicate dispersion within the group. In my data, the mean phase is statistically significant ($p < 0.001$), whereas the distribution of phase of the two groups cannot be statistically distinguished ($p > 0.5$). Number of individual is given in brackets, next to the phase values of each group.

Vibratory stimulus are perceived by fly’s mechanosensory organs, most likely of both type I and type II (i.e. external sensory plus chordotonal organs and multidendritic neurons, respectively, see Introduction). The fly’s system is partially redundant, and this explain why removal of antennae does not abolish the response to the vibration stimulus. Therefore, antennae are not necessary for this behaviour, but they contribute to it.
inantly by ch organs. My preliminary work of entrainment of the circadian clock by mechanical stimulation gives additional support to the idea of ch organs being required for the entrainment of the clock. In this respect, we could speculate a model were temperature is perceived by ch organs in a similar way than ch organs perceive vibration. The mechanism underlying this process is still unknown and awaits an explanation.

However, much is still to be done. Analysis of clock mutants and ch organ mutants will reveal if there is a link between the circadian clock and the ch organs which mediate entrainment. Vibration-pulse experiments together with the analysis of locomotor behaviour in different VS regimes (“vibro-period”) and application of different stimuli (in terms of frequency, duration and intensity) will provide additional details to the vibration stimulus as novel Zeitgeber.

7.4 Summary

- Isolated *Drosophila* brains are not able to synchronize to TC but need information from the periphery.

- Down-regulation of *nocte* in peripheral sensory (chordotonal) organs mimic the effect of the mutant.

- Mutant for ch organs exhibit defects of temperature entrainment similar to the *nocte* mutants.

- *nocte* is required for signalling the temperature information form the periphery to the brain in order to synchronize the central clock.

- *nocte* exhibits an “uncoordinated” phenotype when expose to 37°C for 90 min, in a similar way to *spam* mutants.
• A functional clock is not required in ch organs to execute temperature entrainment neither are required the ch organs in the antennae.

• Flies can entrain their behaviour to 12:12 hr vibration-silence (VS) cycles that stimulates mechanosensory organs. VS phase the locomotor activity during the stimulation and the subsequent free-running activity.

• The ablation of the antennae does not prevent the flies from synchronizing to the VS regime, suggesting that antennae are not required for entrainment.
Chapter 8

Discussion

8.1 The power of genetic screens: Isolation of novel components for the entrainment of the circadian clock

To address the question of how the circadian clock of *Drosophila melanogaster* can be synchronized to TC, we proceeded by searching for novel components which affect the ability of the fly to entrain. In this work, we made use of several different and complementary approaches all aimed on the isolation of new mutants and genes.

The first approach we used to isolate novel genetic variants was an EMS chemical mutagenesis. EMS mutagenesis reveals itself as an extremely powerful tool of investigation in *Drosophila*. Most of the components of the circadian clock in *Drosophila* have been isolated with EMS variants. In the 1970s the isolation of the *period* mutants by Konopka and Benzer (1971) opened the door to the genetic dissection of complex behaviours, such as those controlled by the circadian clock.
Only two decades after, the EMS-mutagenesis approach gave further important contributions. In 1998 four more components of the circadian clock were isolated: \textit{doubletime} \citep{Kloss1998, Price1998}, \textit{Clock} \citep{Allada1998, Bae1998}, \textit{cycle} \citep{Rutila1998} and \textit{cryptochrome} \citep{Stanewsky1998} all from EMS mutagenesis screens. The following year, \textit{vrille} was discovered as component required for circadian rhythmicity \citep{Blau1999}. More recently, the “temperature mutant” \textit{nocte} has been isolated by our group in an EMS screen \citep{Glaser2005}, indicating that the temperature entrainment pathway can also be genetically dissected.

My work follows the previous investigations of EMS-induced variants which affect circadian rhythmicity. In this work we screened 1637 chemical-induced mutant lines and the screen has been successful with the isolation of 3 novel variants which show defects in the synchronization of the circadian clock to temperature cycles (Section 3). The 3 mutants were named \textit{2T-30}, \textit{2T-38} and \textit{2P-42}. They all map to chromosome 2 and have been isolated in a bioluminescence assay monitoring real-time expression of \textit{period-luciferase} in isolated legs in a temperature entrainment regime. The two mutants \textit{2T-30} and \textit{2T-38} were isolated from a batch of lines originated in a single EMS treatment, which led the possibility that the two variants affected the same gene and they were potential clones. Complementation tests revealed that the two mutants complement each other (Figure 3.10), excluding the possibility that they map to the same gene.

EMS mutagenesis is unpredictable: it introduces random mutations, it is unbiased and can lead to unexpected results. The extensive use of random mutagenesis screens in the laboratories is supported by power of the fly genetics. The combination of a well-designed experimental read-out, and the feasibility of genetic manipulation, makes \textit{Drosophila} a perfect tool for screening of variants showing
a desired phenotype among a population of random chemical-induced mutants. The downside of the approach is that you need to map the mutation by meiotic recombination, a process which is usually very complex and time consuming (as in my case).

To get around this problem, a complementary approach used a library of RNAi lines targeting specific genes. In *Drosophila* two main libraries are available to the public domain and they cover over 90% of the predicted protein-coding genes (http://www.shigen.nig.ac.jp/fly/nigfly and Dietzl et al., 2007). In this work, we performed two RNAi screens, one targeting 148 randomly-chosen RNAi lines, provided by François Rouyer (and originating from the NIG-FLY Stock Centre, Japan) and a second targeting specifically TRP channels encoding genes (RNAi lines provided by the Vienna Drosophila RNAi Center, VDRC). From the former, we isolated one line which showed impaired synchronization of *per-luc* expression in isolated legs, both during LD cycles and TC conditions. The RNAi line targets the forkhead transcription factor *fd3F*, whose function is still unknown. The latter, TRP-RNAi candidate approach screen, did not give any positive results (see below).

In the post-genomic era, more and more studies make use of RNAi libraries as efficient tools to silence gene expression where mutant alleles are not available (recently reviewed by Boutros and Ahringer, 2008 and Belles, 2010). The RNA interference combined with the UAS-GAL4 system (Brand and Perrimon, 1993) adds the possibility to silence target genes in specific tissues or specific developmental stages, by the use of appropriate *promoter-gal4* driver lines. This allows performing knock-down studies of genes, where mutants might otherwise be lethal, by restricting the effect to specific tissues or cells. There are also some disadvantages of the RNAi technique. The phenotype associated with a certain RNAi line
can be related to positional insertion of the RNAi construct itself. Also, RNAi is often inefficient, resulting in knock-down rather than loss-of-function of the targeted genes, often preventing the desired phenotype from arising. The UAS/GAL4 system can give a false negative result in situation where the expression pattern of the targeted gene is not known. Those last two disadvantages, could be the reasons why we did not obtain any phenotype from the \textit{trp}-targeting RNAi screen approach.

As a complementary approach, we also analysed 12 mutant lines affecting 7 — out of the 13 — \textit{trp} genes. Four mutants (affecting three genes), exhibited defects of entrainment of the circadian clock specifically to temperature cycles. Mutants of the \textit{pyrexia}, \textit{trpA1} and \textit{trpM} genes fail to synchronize locomotor activity to TC, but they are normal under LD conditions. Although the respective RNAi lines did not produce a similar phenotype, the analysis of mutants supported our hypothesis that TRP channels may be involved in the temperature entrainment of the circadian clock.

Therefore, a non complete silencing of TRP channel encoding genes could have been the reason for the absence of phenotypes. Many TRP channels are formed by different subunits and some form heteromultimeric complexes, as reported by the Montell group for TRP and TRPL (Xu et al., 1997). If the double-stranded RNA targets only one subunit, this still might allow the non-silenced subunits to form a functional channel. The efficiency of the interference mechanism can be enhanced by overexpression of components of the RNAi machinery, specifically \textit{Dicer2} (Dietzl et al., 2007). The efficiency increase is particularly evident for the RNAi lines generated by the VDRC compared to the ones from the NIG-FLY Stock Center (this work and François Rouyer, personal communication). In this work, we did not make use of additional \textit{Dicer} to trigger the interference, mainly because of
the difficulty in generating flies carrying simultaneously *UAS-Dicer, tim-gal4* and *per-luc*, together with the RNAi construct. The presence of overexpressed DICER is also associated with an increase of false positive results due to off-target effects (Dietzl et al., 2007). When DICER is overexpressed, the process that produce small RNAs from double-stranded RNAs (dsRNAs) is enhanced and it raises the probability of mismatches and gaps in pairing of the small RNA to the targeted genes (Dietzl et al., 2007).

Another reason for the lack of *trp*-RNAi effect could be related to the specific driver line. We restricted our analysis on clock cells-driven RNAi, using a *tim-gal4* driver to knock-down TRP channels. This was based on our initial hypothesis that cells required for temperature entrainment of the circadian clock must indeed possess a clock. Our recent findings (Sehadova et al., 2009) suggest that this is not necessarily the case, and structures required for the temperature entrainment of the clock, namely the chordotonal organs, may not possess a clock. Therefore, the absence of the phenotype arising from *tim-gal4*-driven RNAi can be a results *in se*: this may speak towards the hypothesis that TRP channels are not required in clock cells, but their putative function as thermoreceptors, or at least as components required in the temperature entrainment pathway, is not executed in clock-possessing cells, as suggested by the temperature entrainment defects of “true” TRP channel mutants.

This work shows that genetic screens are a powerful and effective way to investigate and dissect complex biological processes, such as the entrainment of the circadian clock. EMS chemical-mutagenesis continues to be a good resource for the isolation of novel variants, even considering the drawback of the complicated and time consuming mapping process. The specific candidate approach can combine the huge availability of RNAi lines and genetic aberrations (mutants, *P*-element
insertions, deficiencies, etc.) also providing a complementary tool for the isolation of novel components required for specific mechanism.

8.2 \textit{per-luc} expression in novel mutants reveals differences between light and temperature entrainment

The three EMS mutant lines exhibit a drastic reduction of \textit{per-luc} expression in isolated legs, in term of overall expression levels and cycling amplitude specifically during TC conditions, while they exhibit normal \textit{per-luc} expression in LD cycles (Figure 3.3 and 3.6). \textit{per-luc} expression in the whole adult fly during TC entrainment is compromised too (Figure 3.4): 2T-30 and 2T-38 exhibit non-cycling \textit{per} expression, indicating either malfunction at the central pacemaker resulting in complete arrhythmic \textit{per} expression at the whole fly level, or dyssynchrony among central and peripheral clocks, generating an overall flat \textit{per} expression due to different oscillating phases. Adult \textit{per-luc} expression in the line 2P-42 is rhythmic and exhibits an opposite phase compared to controls, both in LL (Figure 3.4) and in DD and TC (Figure 3.8). This can be explained in two ways. (a) The mutation alters the endogenous phase of \textit{per} expression and makes it cycling in opposite phase or (b) the bioluminescence expression is not circadianly regulated but instead it exhibits a mere reaction to temperature increase and decrease. Given that \textit{per-luc} expression in DD and TC in isolated legs is normal, this suggests that peripheral oscillators are entrained to temperature (at least in DD) and that the overall \textit{per} expression is out of phase between central and peripheral oscillators. Interestingly, a similar phenomenon occurs in \textit{nocte} and \textit{norpA} temperature.
entrainment mutant flies (Gentile C., Simoni A. and Stanewsky R., in preparation) in which per-luc expression entrain to TC in isolated legs but not in the whole fly. From my data it emerges also that a trp mutant exhibits the same phenotype: per-luc expression in tim-gal4 driven trpM-RNAi is rhythmic in isolated legs, but aperiodic in the adult fly (Figure 5.8) during LL and TC. A similar phenomenon is observed in trpA1ins XLG-luc flies: per-luc expression in TC in isolated legs is only reduced in terms of amplitude, whereas in the whole fly it is barely cycling (Figure 5.9).

At the moment we still do not have an explanation for this intriguing phenomenon, but it appears a common feature of different mutants affecting the entrainment of period expression to temperature cycles. This may reflect a clear difference between the light versus the temperature entrainment pathway. The light entrainment signal acts directly on the central pacemaker, through the light-dependent action of CRY in the clock neurons (Stanewsky et al., 1998; Emery et al., 1998, 2000). As recently reported by our group (Sehadova et al., 2009), it emerges that peripheral structures are instead required to entrain the fly to TC and that the temperature signal is transmitted from the periphery to the brain. The ability of some mutant tissues (legs), to entrain, while the whole mutant fly cannot, may indicate a “hierarchy of entrainment”, in which the mutant disrupts part of the pathway required for the entrainment of the whole organisms, while specific isolated components can still be synchronized to temperature.

Interestingly, we observed that per-luc expression of trpM mutants is not affected in LL and TC, while tim-gal4-driven RNAi down-regulation of trpM abolishes rhythmic per-luc expression in the adult fly (but not in isolated legs, Figure 5.9 and 5.8). This seems to suggest that the RNAi induces a stronger phenotype than the mutant, which is generated by a P-element inserted to the coding region.
of the gene. However, at the behavioural level, the situation is opposite: trpM-RNAi does not induce any effect while the P-element does. Recently, TRPM has been indicated as required for the intake of Mg$^{2+}$ from the hemolymph to the Malpighian tubules (Hofmann et al., 2010). In this work, the authors generated two mutant alleles of the trpM gene, named trpm$^1$ and trpm$^2$. trpm$^1$ was generated by imprecise excision of the P-element P[EY01618] that removed three exons (C9–C11). trpm$^2$ was generated by insertion of the $w^+$ gene in place of the exons C2–C4 by ends-out homologous recombination (Hofmann et al., 2010). Both the alleles generated are pupal lethal (Hofmann et al., 2010). This strongly suggests that the mutant we used in our study (the P-element inserted in the 3’ splice site of exon C11) is a hypomorphic allele and therefore may explain why the mutant line does not induce mutant phenotype in certain assays (bioluminescence) whereas it does in others (behaviour).

As mentioned in Chapter 3.2, for unknown reasons, the BG-luc control did not display consistent rhythmicity between experiments. The 3 mutants have been originated from EMS-fed BG-luc flies, therefore they should share the same genetic background. However, as depicted in the crossing scheme in Figure 3.2, the lines we assayed (F$_3$ generation) have been genetically rearranged (via recombination) on chromosome 1 and 3 with the balancer line $y$ w; $\frac{B_{1/3}}{C_{1/3}}$; $\frac{H}{TM3}$ (or $y$ w; $\frac{+}{+}$; $\frac{H}{TM3}$, in case of potential EMS-induced mutants on chromosome 3). Therefore, although the mutant lines have been originated from BG-luc, they do not share the same genetic background (independently of the additional EMS-induced mutations). Although this does not explain why BG-luc flies did not exhibit consistent rhythmicity, it may explain why the majority of EMS-fed lines — all minus the 3 isolated as mutants — showed clear rhythmicity and could therefore be used as internal controls for the screen (see for instance line 2X-8 in Table 3.2).
8.3 Behaviour analysis of the novel temperature mutants

8.3.1 Locomotor behaviour in LL and TC

Behavioural analysis of the seven novel mutants shows interesting differences between the lines. Initially, locomotor behaviour has been analysed for the ability to re-entrain to LL and TC after a 12 hour shift compared to the previous LD entrainment (except for *tim-gal*-driven *fd3F*-RNAi, see below). Wild-type control flies resynchronize their rest-activity pattern to LL and TC after 2 transient days (Figure 3.11). The mutants exhibit an abnormal behaviour pattern during LL and TC. They show a drastic increase of activity (unlike Canton S) immediately after temperature goes up (*2T-30, 2T-38, 2P-42, trpA1\textsuperscript{ins}* or temperature goes down (*trpM* and *pyx*) and the peak of activity that usually anticipate the transition from warm to cold, is absent (*2T-30* and *trpA1\textsuperscript{ins}* or only barely visible (*2T-38, 2P-42, pyx* and *trpM*). The activity pattern during LD conditions is normal except for the 3 EMS mutants and *trpM*, which are lacking the morning peak that normally anticipates the transition from dark to light. This could be related to the genetic background, at least for the EMS mutants, since BG-*luc* flies also lack the anticipatory activity before the light goes on, see Figure 5A in Glaser and Stanewsky (2005).

These data show that the isolated mutants exhibit clear defects in adjusting their locomotor behaviour during LL and temperature cycles.
8.3.2 Temperature entrainment differences between LL and DD

The analysis of novel mutants involved in temperature entrainment had initially been performed in constant light and temperature cycles. The reasons why constant light was chosen versus constant darkness were based on the work from Tomioka et al. (1998) and Yoshii et al. (2002), in which they showed that temperature cycles are a stronger Zeitgeber in LL compared to DD, in terms of the ability to entrain either wild-type flies to different thermoperiods, or to synchronize long and short period variants to TC. A possible explanation could be the residual (dominant) effect of the previous LD entrainment during DD and TC. Temperature, being a weaker Zeitgeber than light (Pittendrigh et al., 1958; Wheeler et al., 1993), may not be strong enough to conflict the free-running rhythm set by the light-dark cycle.

However, the use of constant light (rather than DD) during temperature entrainment prevents the possibility of monitoring free-running rhythms after releasing the flies to constant conditions (in DD and constant temperature). In order to determine proper entrainment (i.e. the peak of activity aligned with that observed during entrainment) and to investigate any direct effects of light itself on the ability to synchronize to TC, we also compared to the locomotor activity of the novel “temperature mutants” in DD and 12:12 hr 25:16°C TC conditions.

Wild-type locomotor activity in DD and TC exhibits a similar pattern compared to the one in constant light, exhibiting a single unimodal peak of activity (versus the bimodal pattern of activity in LD). However, the phase of activity is advanced, and flies are mainly active at the beginning of the warm day (displaying the main activity peak at ZT3.8, Table 3.3). Previous work studying temperature entrainment in DD has shown that the activity pattern is bimodal, and the main
peak of activity occurs at the end of the warm phase (Tomioka et al., 1998; Yoshii et al., 2002; Busza et al., 2007). These results were based on experiments performed in 30:25°C and 29:20°C temperature cycles, respectively, and the different temperature intervals may explain the different patterns of activity in DD. A recent work from Yoshii et al. (2009a) using conditions comparable to mine (26:16°C) also showed a single peak of activity during the first half of the thermophase. In addition, different constant temperatures affect the shape of free-running activity (Majercak et al., 1999), and a higher temperature (29°C) induces the activity to persist with a bimodal pattern for several days in DD, whereas cold temperature (18°C) induce an unimodal pattern of free-run activity. This may be the case also when high-interval (29:20°C or 30:25°C) temperature cycles are applied, explaining the unimodal activity of my observation at the “low” temperature interval of 25:16°C.

In many cases, it was difficult to judge whether the locomotor activity during DD and TC was entrained, or if the flies were only reacting to the temperature changes, given that the main activity peak partially overlaps with the startle response induced by the increase of temperature. In these situations we need to consider both the activity during the transient days (if present) and the free-running activity once the flies have been released to constant conditions. Considering these difficulties, a picture emerges in which some mutants are able to entrain to DD and 25:16°C TC (2T-38, 2P-42, trpM, pyx² and pyx³, Figure 3.15 and 5.4) in term of (a) exhibiting clear transients until resynchronization from LD to TC conditions (comparable to controls) and (b) synchronized free-running activity in constant conditions, following the previous TC. Other lines (2T-30 and TrpA₁mnw) exhibit a mutant phenotype: no transient days but a burst of activity in response to the temperature increase at the first day of TC, and free-running activity is not
synchronized to the previous TC regime (Figure 3.15 and 5.4).

8.3.3 *Entrainment to different temperature intervals*

The temperature intervals chosen for this study were based on an agreement between several research groups collaborating in an European Integrated Project focused on the entrainment of the *Drosophila* circadian clock (EUCLOCK). The 25:16°C temperature cycles correspond to average recordings taken in natural conditions at the end of September to the beginning of October (to which correspond 12:12 hr LD cycles), in northern Italy (Yoshii et al., 2009a).

Our idea was to reveal whether entrainment of the mutants at some specific temperature ranges could give us hint about the function of the genes affected in our novel mutants, thus we investigated locomotor activity of flies exposed to different temperature intervals of 29:25°C, 25:20°C and 20:16°C.

The fly’s behaviour adapts to the seasonal variations of photoperiod and temperature, concentrating the activity to the milder part of the day in short and cold days (spring and fall) and shifting the activity towards dawn and dusk in long and warm summer days, perhaps to avoid dessication (Majercak et al., 1999; Collins et al., 2004; Stoleru et al., 2007). We observed this phenomenon after monitoring locomotor activity of wild-type flies in different temperature intervals. Interestingly, the effect of temperature is again more pronounced in LL than in DD (Figures 5.2 and 5.6, respectively), presumably for the reasons described above of “better entrainment” in LL versus DD (Tomioka et al., 1998; Yoshii et al., 2002; Glaser and Stanewsky, 2007). The effect of the seasonal adaptation of the behaviour to different temperature intervals is less pronounced in all novel mutants compared to wild-type flies both in LL and in DD (Figures 3.13, 5.2 and 5.6).

Analysis of behaviour at different temperature intervals showed that some mu-
tiant lines can synchronize their locomotor activity in certain ranges, but not in others. Interestingly, line 2P-42 and 2T-38, for instance, entrain to 25:20°C and 20:16°C, but not in the 25:16°C regime. This indicates that the mutant lines, surprisingly, fail to synchronize to larger temperature intervals (25:16°C), while they do at small intervals (25:20°C), suggesting some kind of “entrainment repression” induced by certain temperature ranges. This is also suggested by results obtained with the trp mutants (summarized in Table 5.2). pyx and trpM mutants fail to synchronize their locomotor activity to 20:16°C TC but they entrain to 25:20°C and 29:25°C TC, suggesting indeed that the cold temperature range somehow “represses” entrainment in these TRP channel mutants, whereas the high temperature interval effects more the EMS mutants. TrpA1ins exhibit the most severe defects in temperature entrainment compared to the other mutant lines we assayed. Locomotor activity in DD and different temperature intervals confirms the idea that some genes are important for certain temperature ranges (29:25°C), whereas other genes are important in different intervals (e.g. trpM and pyx mutants in 20:16°C), although the effect in DD is less pronounced than in LL. This is even more surprising given that pyx and trpA1 have been isolated as mutants failing to avoid a warm noxious stimulus (Lee et al., 2005; Rosenzweig et al., 2005). This suggests that thermal preference and temperature entrainment, although they may be mediated by the same players — TRP channels — indeed involve different mechanisms (see below). The existence of several thermal TRP channels might be explained by the requirement of the same thermal sensor to play different roles at opposite sides of temperature spectrum. For instance, PYX and TRPA1 may be required either to respond to a warm noxious stimulus (above 40°C) and also to respond to cold TC (below 20°C) in terms of circadian entrainment. This idea is supported by experiments conducted in larvae by the Montell
group, where they propose that TRPA1, in addition to act as thermosensor for noxious temperature, could also function indirectly for temperature sensation in the comfortable range (Kwon et al., 2008).

Although it seems that TRP channels are involved in the entrainment of the clock to temperature cycles, it is still unknown how the temperature stimulus is processed in a circadian manner. The temperature stimulus as a noxious response is mediated in a very small time-scale, in the order of seconds, or milliseconds. This makes sense in the way that the fly must respond immediately to the stimulus to avoid life threatening situations. For a stimulus to be circadianly significant must act in the order of hours and must sense not an absolute temperature value but temperature oscillations (independently of the absolute values). The circadian clock is much less sensitive to temperature than to light, perhaps because it must resist dramatic short-term perturbations of temperature, due to irregular environmental conditions, as proposed by Currie et al. (2009).

In this context, TRP channels mediate entrainment not directly responding to temperature, but rather function through second messenger pathway, or signal amplification mechanism, maybe mediated by a tonic stimulus response of the channels, rather than phasic (transient) stimulus. Recently, it has been shown that TRPA1 controls thermotaxis behaviour acting downstream of a PLC-dependent signalling cascade (Kwon et al., 2008), similar to the PLC-coupled pathway resulting in TRP and TRPL opening in the visual system (Niemeyer et al., 1996). It is already known that PLC is also involved in temperature entrainment (Glaser and Stanewsky, 2005; Sehadova et al., 2009), although its role in the process is still unclear. It is possible that norpA mediates a signal cascade associated with TRP channel opening in the temperature entrainment pathway. However, this also implies that the receptor is still elusive. If we compare to the visual transduction
Further studies, and a deeper investigation of the available mutants — also combining TRP mutants with \textit{norpA}, for instance — will help to better understand the role of these proteins in the temperature entrainment of the clock. At the moment of writing, a member of our group is proceeding on mapping the mutations and the isolation of the genes affected by the EMS mutants will hopefully clarify some aspect of the entrainment mechanism. The attempt to map the 2\textit{T-30} mutant by meiotic recombination suggests that the mutation lays on the left arm of chromosome 2.

Also, more analysis in different light and temperature conditions, monitoring the fly’s activity in different temperature intervals, are needed. In particular, analyses of the free-running phase of activity after TC at different intervals, in order to discriminate proper synchronization from temperature-driven effect, are necessary. My preliminary work in this direction, discussed in this thesis, shows the benefits of the approach to dissect in more detail the ability of specific mutants to synchronize the locomotor activity in different conditions. It will also be extremely important to monitor real-time \textit{per-luc} expression (or clock protein accumulation and expression) during, and after, temperature entrainment at different intervals, to correlate — and complement — my behavioural results.

8.3.4 \textbf{2\textit{T-30} is a locomotor output mutant}

Analysis of free-running locomotor behaviour of the isolated mutants revealed that \textit{2\textit{T-30}} flies are largely arrhythmic in constant conditions (Table 3.4). To address whether the mutants affect the central clock mechanism, we investigated the eclosion rhythm. Cultures of \textit{2\textit{T-30}} exhibit a strong rhythm with a period of 23.8 hours when they are released to DD after LD entrainment (Figure 3.19), indi-
cating that the central pacemaker is not compromised. Given that the rhythmic locomotor behaviour is strongly jeopardized, this suggests that the 2T-30 mutant affects a gene related to the locomotor output, in addition to the temperature input pathway.

8.4 Regulation of the eclosion circadian clock

8.4.1 Adult “temperature mutants” do not affect eclosion

In the present thesis we have investigated whether the novel temperature mutants we isolated, in addition to the 2 known temperature mutants, nocte1 and norpAP41, compromise the ability of the circadian clock to synchronize their eclosion activity to temperature cycles, as much as they do for the adult locomotor activity.

The timing of adult emergence is controlled by the circadian clock and can be synchronized by temperature cycles (Zimmerman et al., 1968; Newby and Jackson, 1993). Wild-type cultures raised in LD, resynchronize the eclosion activity to LL and TC after 2 transient days. The eclosion window, or gate, after they establish a stable phase, occurs at the end of the cold phase, just before the transition to warm (Figure 6.1).

The phase of eclosion rhythm during TC occurs few hours earlier than during LD cycles, in which flies eclose in a broad peak mainly during the first half of the light phase (Qiu and Hardin, 1996). This correlates well with per-luc recordings and PER accumulation in LL and TC conditions (Section 3.2 and 4.3), in which we showed that during TC, per expression and PER accumulation is 2–3 hours advanced compared to LD conditions. The advance eclosion activity in TC, compared to LD, fits well also with an earlier phase of locomotor activity in TC compared to LD.
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We observed also a second peak of eclosion occurring immediately after the temperature rise, likely induced by the steep increase of temperature, promoting the flies developmentally mature enough that missed the eclosion gate, to hatch (also shown by Newby and Jackson, 1993). During LD conditions, the light-on to light-off transition is the moment in which the clock measures the developmental state in order to trigger the eclosion in the next available eclosion gate (Qiu and Hardin, 1996). It is not known at which point during the day this state is determined during temperature entrainment.

The ability to re-entrain to a 6hr temperature shift (Figure 6.2) confirms that TC are indeed a strong Zeitgeber perceived by the clock. However, there is a clear difference between light-dark entrainment and temperature entrainment. The eclosion profile of per01 cultures suggests that temperature per se induces a clock-independent eclosion event occurring after change of conditions. During temperature cycles, per01 cultures exhibit a 12-hr eclosion rhythm, and flies emerge after any temperature transitions, suggesting clock-independent temperature-driven eclosion events (Figure 6.2 and 6.3). During LD conditions, per01 eclosion is arrhythmic and the flies emerge throughout the light and dark phase (Konopka and Benzer, 1971; Qiu and Hardin, 1996). Unlike for the locomotor behaviour of per01 mutant flies, which shows masking effects, the eclosion rhythm cannot be completely light-driven (Qiu and Hardin, 1996), but it can exhibit a light-induced (light-on) response if a light pulse is applied only after the normal gate is opened (McNabb and Truman, 2008). The ability of temperature changes to drive the eclosion in per01 flies, suggests that the second eclosion peak occurring after temperature step-up, in wild-type cultures, is temperature driven (masking), and not clock-controlled.

In the current study we analysed the eclosion activity of seven novel (2T-30,
2T-38, 2P-42, pyx², pyx³, trpM and fd3F) and 2 already described (nocte¹ and norpA²) “temperature mutants” in LL and TC conditions. To our surprise, all the cultures analysed exhibit a normal eclosion activity in TC conditions, synchronizing the emergence to TC after 2 transient days, in a way comparable to wild-type cultures. The only exception is fd3F, which exhibits an altered phase of the eclosion rhythm, and will be therefore discussed separately (see below). To our knowledge, this is the first time that adult “temperature mutants” have been assayed in eclosion. My data suggest that the genes which affect temperature entrainment in the adult do not play the same function in the synchronization of the eclosion-regulating clock to TC.

8.4.2 Role of the DN² neurons in the regulation of eclosion

The neuronal architecture which eventually triggers the eclosion event in a circadian manner is not fully understood. Even less clear are the differences between light-dark and temperature entrainment. However, recent work from Picot et al. (2009) shed some light on the role of the DN² neurons in the larval brain in temperature entrainment. The authors propose that the light-blind CRY-negative DN²s (Klarsfeld et al., 2004) are necessary (and sufficient) to entrain the larval brain to TC. In LD, the PDF-positive LNs synchronize PER oscillations both through the presence of CRY and through the visual system and they synchronize PER expression in the DN²s via the neuropeptide PDF. During TC, the DN²s are directly entrained by temperature and they synchronize the LNs through a PDF-independent pathway. Previous studies in LD have shown that PER cycles in the DN²s with an opposite phase compared to all other groups of larval neurons (Kaneko et al., 1997; Klarsfeld et al., 2004) and that forced expression of CRY can reverse their internal phase (Klarsfeld et al., 2004), in a PDF-independent manner.
My data showed that temperature entrainment applied during development can modulate both the free-running eclosion period and phase. A single light-dark cycle applied as early as at the first larval stage can phase the circadian clock and drive a rhythmic locomotor behaviour (Sehgal et al., 1992; Kaneko et al., 2000; Malpel et al., 2004). Larval-only TC entrainment generates an adult locomotor activity which is in opposite phase compared to larval-only LD entrainment (Picot et al., 2009). My data complement these studies, showing that eclosion activity also exhibits an opposite phase after larval-only TC entrainment (Figure 6.6) compared to LD entrainment. Although the LNs and the prothoracic gland (PG) are required to generate eclosion rhythms (Kaneko et al., 1997; Blanchardon et al., 2001; Myers et al., 2003), my data showed that PDF is not required to generate rhythmic eclosion events both after LD or TC entrainment. We therefore propose that temperature entrainment is mediated by the DN2s — in agreement with Picot et al.’s results (2009) — and that it is PDF independent. Previous studies have shown that the removal of the larval visual system can change the phase of adult locomotor activity (Malpel et al., 2004). The authors speculated that the DN2s are responsible to drive the opposite phase of adult activity, given their opposite phase of PER cycling. My results strongly support this idea, suggesting that temperature entrainment could supplement the lack of the information through the visual system and phase the clock to gate a rhythmic eclosion. However, in this respect it is not clear how the DN2s can be synchronized. Mealey-Ferrara et al. (2003) proposed the existence of 3 pathways for the entrainment of the eclosion circadian clock. The 1st pathway involves the larval visual system (opsin- and norpA-mediated); the 2nd requires CRY as photoreceptor and a 3rd, mysterious pathway, suggested by the light-entrainability of the eclosion clock in mutants.
lacking the first 2 pathways (Mealey-Ferrara et al., 2003). This mysterious 3rd pathway could mediate the entrainment of the eclosion through the DN_2s, in a situation when the light-pathway is not effective, such as in TC entrainment, consistent with my results.

Very interestingly, we observed that the free-running eclosion period of wild-type cultures entrained in LL and TC is 21 hours (Figure 6.4, 6.7 and 6.9). This is the case for temperature entrainment restricted to both larval-only and pupal-only stages, as well as for TC applied during all developmental time. Based on the hypothesis that during temperature entrainment the DN_2s are the neuron that set phase and period of eclosion, we propose that the free-running period of the DN_2s is shorter than 24 hours. This is strongly supported by the same short eclosion period exhibited by Pdf^{01} mutants and from observation in adult flies: Pdf^{01} mutants are mainly arrhythmic in DD, but the remaining rhythmic flies exhibit a short, 21-hour periodicity (Renn et al., 1999).

The phase of PER oscillations in the adult DN_2s is the same in all clock neurons during LD conditions (Kaneko et al., 1997; Blanchardon et al., 2001). Thus, the adult DN_2s — which derive from the larval DN_2s — change their internal phase of PER cycling during metamorphosis (Kaneko et al., 1997). This is likely due to the synchronization with the LN_v_s, which are the only group of neurons which express PER throughout the metamorphosis (Kaneko et al., 1997; Helfrich-Förster et al., 2007) and are also responsible for the developmental time-memory (Kaneko et al., 2000).

In the future, a more detailed analysis of the developmental time at which temperature entrainment can elicit a short free-running period of eclosion is needed. Also, analysis of eclosion period and phase of culture which overexpress CRY in specific groups of larval clock neurons will be performed, together with the modu-
lation of the internal speed of the clock in specific cells, in order to address which are the component that drive the short free-running period.

8.4.3 \textit{fd3F} alters the phase of eclosion

Among the seven novel “temperature mutants” isolated in this work, one exhibited an eclosion phenotype in LL and temperature entrainment. We showed that \textit{tim-gal4}-driven down-regulation of the transcription factor FD3F resulted in an abnormal phase of eclosion activity: flies emerged preferentially during the warm phase and without any defined peak (Figure 4.6). The pattern of emergence is unequivocally different from any other cultures we assayed, including the clock mutant \textit{per}^{01} (Figure 6.2). The rhythmic free-running eclosion activity excludes the involvement of \textit{fd3F} in the central clock mechanism, but rather suggests a role in the regulation of eclosion gating.

Further investigations are necessary to clarify the effective role of the FD3F transcription factor in this context. Many indications suggests that forkhead transcription factors are expressed during development (see Section 4.5). Lethality in different developmental stages of the \textit{fd3F-RN}Ai driven by \textit{tim-gal4}, \textit{repo-gal4} and \textit{nocte-gal4} suggests that FD3F is indeed required development of the fly. The spatial and temporal expression profile of \textit{fd3F} will help to understand which roles it plays. Published data and this work suggest a direct involvement for FD3F in the chordotonal organs (Lee and Frasch, 2004 and below). So far there are no known connections between the chordotonal organs and the regulation of eclosion. Our data showed that ch organs are required for the adult fly to entrain to temperature (see Sehadova et al., 2009 and below) and \textit{fd3F} could be one component that mediates the process.


8.5 Chordotonal organs and temperature entrainment

Accumulating evidence indicates the involvement of chordotonal (ch) organs in the synchronization of the circadian clock of *Drosophila* to temperature cycles. The gene *nocte* plays a crucial role in the temperature entrainment. EMS-induced *nocte* mutant flies fail to synchronize *per-luc* expression and locomotor activity specifically to temperature cycles as they behave normally in LD conditions (Glaser and Stanewsky, 2005, 2007; Sehadova et al., 2009). Down-regulation of *nocte* with the *F-gal4* driver reproduces the phenotype of the mutant. *F-gal4* (originated by fusion of the *nanchung* promoter to *gal4* sequences) was previously reported to be expressed specifically in ch organs (Kim et al., 2003) but its expression was found also in some external sensory organs and few neurons in the brain (Sehadova et al., 2009). Analysis of ch organs mutants, such as *tilB*, *smetana* and *eyes shut*, confirmed the role of ch organs in temperature entrainment (Sehadova et al., 2009). My work contributed to these findings including new and unpublished data, which support the idea that ch organs are required for temperature entrainment.

The transcription factor FD3F seems to be expressed exclusively in ch organs, at least in the embryo (Lee and Frasch, 2004). We do not have any data concerning the spatial expression profile in the adult. However, *F-gal4*-driven down-regulation of *fd3F* disrupts temperature synchronization of locomotor activity (Figure 4.4) in a similar way as *nocte-RNAi*. This clearly indicates a role for *fd3F* in the adult ch organs too.

Rouyer et al. (1997) described that the circadian regulated gene 1 (*crg-1*) is rhythmically expressed in phase with the *per* gene and expressed in the same pat-
tern in fly heads. Recently, it turned out that *crg-1* is a chimeric gene, originated by a duplication event that occurred only in *Drosophila melanogaster* (and not in evolutionary related species) which led to the fusion of the 5’-region of *fd3F* and the 3’-UTR region of *Toulouse-like kinase* (*Tlk*) (Hogan and Bettencourt, 2009 and Figure 4.10). Although the *crg-1* cDNA has been detected by PCR (Hogan and Bettencourt, 2009), CRG-1 protein has never been detected (F. Rouyer, personal communication).

It is possible that the circadian regulation of *crg-1* has been gained after the duplication event, occurred probably less than 2.3 mya (Hogan and Bettencourt, 2009). However, it is also possible that the *fd3F* gene is circadianly regulated too, because the regulation region is shared between the two genes. The *fd3F*-RNAi construct does not target *crg-1*, suggesting that the phenotypes we observed in temperature entrainment are specific to FD3F and not to CRG-1. Given the transcription factor nature of the gene, it is very tempting to look for the possible circadian regulation of FD3F itself, and for possible regulation of clock genes, such as *period* and *timeless* by *fd3F*. Although the presence of a functional clock in chordotonal organ neurons (as defined by *F-gal4*) is not required for temperature entrainment (Sehadova et al., 2009), this does not exclude the possibility that *fd3F* is circadianly regulated. In fact, the assumption of ch organs non-clock requirement for temperature entrainment is based on experiments in which the clock was stopped with the *F-gal4* driver, which is expressed in the ch organs neurons (Kim et al., 2003; Sehadova et al., 2009). Neuronal-specific (*elav-gal4*-driven) knock-out of *fd3F* does not give any effects in temperature entrainment (Figure 4.4), suggesting that FD3F could play a role in non-neuronal cells in the chordotonal organs. This is also indicated by pupal lethality induced by *repo-gal4* and *nocte-gal4*, but not by *elav-gal4* driven *fd3F* knock down. *nocte* expression
in the scolopidium is broader than \textit{F-gal4} (Sehadova et al., 2009) although at the moment it is not clear exactly in which additional cells \textit{nocte} is expressed. This could suggest a role for FD3F in glia cells.\ PER is expressed in glia cells (Zerr et al., 1990; Ewer et al., 1992; Kaneko and Hall, 2000) and analysis of \textit{per}^{+/per^{01}} mosaics showed that rhythmic behaviour can be observed when PER expression is restricted to glia cells (Ewer et al., 1992). Moreover, a role for the glia cells in circadian rhythms has been proposed (Suh and Jackson, 2007). The glia cells surrounding the ch neurons might be a good location for the presence of a peripheral clock, where FD3F could play its role as a transcription factor. Alternatively, the \textit{F-gal4} is perhaps a “stronger” driver than \textit{elav-gal4}, explaining the non-effect induced with \textit{elav-gal4}. More works need to be done to identify which structures require FD3F and at what level the transcription factor executes its role.\ \textit{per, tim} and \textit{nocte} will be tested as possible targets and, in parallel, \textit{fd3F} expression will be tested in clock and \textit{nocte} mutants.

The structure of the chordotonal organs is determined by the expression of many different genes and proteins which allow the organ to develop and execute the many functions played in \textit{Drosophila}. The implications of ch organs go beyond that of stretch receptor function. In the limb joints ch organs contribute to nociception, whereas the biggest ch organ located in the antennae (named Johnston’s organ, JO) mediates hearing (reviewed by Kernan, 2007) and geotaxis (Sun et al., 2009). Several TRP channels belonging to the 3 superfamilies are expressed in ch organs, implicated in transducing touch, sounds and mechanical pain: \textit{nompC} (TRPN), \textit{nanchung, incative} (TRPV), and \textit{painless} (TRPA). It has recently been shown that \textit{pyrexia} (TRPA) is involved in regulation of negative geotaxis mediated by the JO and that it is expressed in the cap (or attachment) cells which connect the scolopidium to the cuticle (Sun et al., 2009).\ \textit{pyx} mutants do not affect
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Figure 8.1: Drawing of the elements of a scolpidium and the putative location of the genes expressed. Two neurons (n) are present which culminate in a basal body (bb). From the bb, the axoneme of the long cilium (c, red) is assembled, including the ciliary dilation (cd, yellow). The dendritic cap (dc, green) connects the scolopale to the cuticle. The location of the genes expressed in the scolpidium is indicated with colours. **Spam/eye shut** is expressed in the scolopale space (ss, blue) and in the basal region surrounding the basal body. **nompA** is expressed in the dendritic cap and **pyx** in the dendritic cells that surround it (green). Many genes are expressed in the ch neurons and in the cilium, including **nompB**, **nompC**, **smetana** (**sme**) and the TRP channels **nan**, **pain** and **iav**. **Beethoven** (**btv**) is also probably expressed in the cilium. Modified from Caldwell and Eberl (2002).
hearing (Sun et al., 2009), whereas it mediates warm avoidance (Lee et al., 2005)
and temperature entrainment (this work). The fly’s antennae are also required for
thermotaxis behaviour, but this function is executed by the third antennal segment
(Sayeed and Benzer, 1996) whereas the JO is located in the second segment. The
fly antennae seem not to be required for synchronizing the circadian clock to TC,
since its physical removal does not prevent the fly to entrain to temperature cycles
(Glaser and Stanewsky, 2005; Sehadova et al., 2009). However, the expression
of TRP channels in the ch organs which have been implicated in temperature
entrainment is unlikely to be a coincidence. Interestingly, as shown in the Figure
8.1, PYX expression is detected in the proximity of the dendritic cap (the apical
part of the scolopale), which is the structure that manifests a morphological defect
in *nocte* mutants (Sehadova et al., 2009). Moreover, PYX is not expressed in the
chordotonal neurons, in contrast with the other ch TRP channels (Sun et al.,
2009), suggesting again that the non-neuronal cells of the ch organs may indeed
play a role in the temperature entrainment process. Although *nocte* probably plays
its role in the ch organ neurons — based on the observation that *F-gal4* driven
*nocte* knock down induces the phenotype —, it may also effect the development
of non-neuronal structures, which surround the ch neuron.

We also identify TRPA1 as component required for temperature entrainment.
Both PYX and TRPA1 are required for thermodosis and warm-avoidance (Lee
et al., 2005; Rosenzweig et al., 2005, 2008; Hamada et al., 2008). Interestingly,
cool and warm avoidance are distinct mechanisms, and involve different TRP
channels: cold avoidance is mediated by TRP and TRPL, and warm avoidance by
TRPA1, PYX and PAIN, indicating that *Drosophila* use different TRPs to respond
to different discrete ranges of temperature (Rosenzweig et al., 2008). However, in
contrast to PYX and TRPA1, PAIN is not required for thermodosis (Rosenzweig
et al., 2008) nor temperature entrainment (this work). *atonal* (*ato*) is a proneural gene required for the specific formation of the ch organs (Jarman et al., 1995). *ato* mutants lack all larval and adult ch organs (Jarman et al., 1995) but exhibit normal thermotaxis behaviour (Rosenzweig et al., 2005). Given that *TrpA1* is not expressed in ch organs (Rosenzweig et al., 2005) this suggest that ch organs are not required for thermotaxis.

Taken together, these data suggest that (a) thermotaxis, temperature avoidance and ch organs-mediated temperature entrainment are distinct mechanisms, (b) some TRP channels might have different functions mediating some of these mechanisms, but not others, and (c) *Drosophila* use thermosensory reception structures and overlapping players to execute different and distinct sets of behavioural responses.

Each scolopidium in the ch organs contains an internal liquid-filled capsule-like structure called scolopale (see Introduction and Figure 8.1). The EYESHUT (SPAM) protein is located at the border between the ch neurons and the scolopale (Figure 8.1). Interestingly, *eys* mutants exhibit a massive deformation of the scolopale due to loss of water by evaporation in flies that are exposed to high temperature (37°C) for one hour (Cook et al., 2008). This results in an “uncoordination” phenotype, which prevents the flies for walking properly, and eventually they fall over (Cook et al., 2008). Increasing the environmental humidity, avoiding the water to escape the scolopale, can rescue this phenotype. The same “uncoordination” phenotype was observed for *nocte* (Sehadova et al., 2009) and the 2T-30 mutants (Figure 3.20). Since all the three mutants also exhibit similar defects in entraining their locomotor activity to temperature cycles, this suggests they fulfil related functions in maintaining ch organ activity.
Finally, based on the observation that ch organs seem required to mediate temperature entrainment, we tried to synchronize fly locomotor behaviour by direct stimulation of the ch organs with a mechanical stimulus. Our preliminary data showed that cycles between 12 hr of “vibration” stimulus (2-component of frequencies of 40 and 200 Hz) and 12 hr of “silence” (in constant darkness), can synchronize the locomotor behaviour (Figure 7.1, 7.2 and 7.3). Phase analysis of free-running activity after vibration entrainment showed that physical removal of the antennae does not prevent the clock to synchronize to the stimulus. This correlates with previous observations of non-requirement of the antennae for temperature entrainment of the clock (see above), and suggests that the stimulus applied is perceived by the the fly ch organs as a whole and not exclusively as an acoustic response (mediated by the antennae only).

Analysis of clock and temperature mutants combined with molecular studies of clock genes are needed to confirm that the circadian clock is indeed synchronized by the mechanical stimulation. However, these preliminary data strongly suggest the role played by the ch organs in mediating the temperature entrainment of the circadian clock of *D. melanogaster* and provide a new fascinating tool for studying the temperature entrainment pathways.
Chapter 9

Conclusions

In this thesis I showed that genetic screens are still a powerful approach to isolate new components involved in complex mechanisms, such as the temperature entrainment of the circadian clock. By a forward genetic approach I isolated three novel EMS variants that have impaired synchronization of per-luc expression and locomotor activity to temperature cycles. In a screen of RNAi lines I identified one forkhead domain transcription factor (fd3F) that, when down-regulated, reduces the expression level and cycling amplitude of per-luc in isolated legs both during LD and TC conditions. tim-gal4-driven knock-down of fd3F alters the phase of eclosion and induce lethality in early adults. In a candidate approach screen of mutant lines for trp channels encoding genes I identified three TRP channels that are required for entrainment of the circadian clock in LL and TC. Mutants for pyrexia, trpM and trpA1 affect entrainment of locomotor activity to temperature cycles and analysis of behaviour at different temperature intervals suggested that pyx and trpM are required for synchronization to cool temperatures, whereas TrpA1 is required in any temperature intervals we applied.

With this work I provided additional evidence that temperature entrainment
in *Drosophila* is mediated by the periphery, which autonomously receives temperature signal from the environment and transmit it to the central clock in the brain. In particular, in support of our recent publication (Sehadova et al., 2009), my data indicate that chordotonal organs mediate circadian temperature entrainment. *nocte* expression in the ch neurons and specific ch organ genes are required for temperature entrainment (Sehadova et al., 2009). *fd3F* is also expressed in ch organs (Lee and Frasch, 2004) and I showed that ch neuron down-regulation of *fd3F* affects entrainment of locomotor activity to temperature. In addition, PYX channels is also expressed in ch organs and the 2T-30 mutant exhibits an “uncoordination” behaviour if exposed to high environmental temperature stress probably due to structural defects of ch organs, in a similar way to *nocte* and *spam* (Sehadova et al., 2009). Mechanical stimulation of mechanosensory organs using a vibratory stimulus can entrain fly’s locomotor activity, in a similar was as temperature does, providing additional evidence of the importance of ch organs in the temperature entrainment pathway.

Finally, I showed that temperature entrainment of the clock that controls eclosion timing requires different components and structures compared to the adult clock. In this Thesis I showed that temperature entrainment applied during development induce short, 21 hour, free-running eclosion rhythms, which are *Pdf*-independent. In addition, larval-only temperature entrainment reverses the phase of eclosion compared to larval-only LD entrainment. We proposed that the DN2 group of clock neurons determine the phase of eclosion during temperature entrainment conditions and have an endogenous short free-running period, which is manifested after temperature entrainment conditions.
Appendix A

RNAi lines

List of the 148 RNAi lines generated by the National Institute of Genetics Fly Stock Center (Japan) screened for defects of synchronization of XLG-\textit{luc} to temperature cycles in LL. The lines have been crossed to \( y^{w} w^{y}, \text{tim}^{g} \text{al4:27}^{CygO}, \text{XLG-luc1-1}^{TM3} \). All the lines listed below have been assayed in our bioluminescence assay in constant light and 12:12 hr 25:16°C temperature cycles.
APPENDIX A. RNAI LINES

<table>
<thead>
<tr>
<th>List of RNAi lines screened</th>
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<td>1401R-1 3412R-1 5087R-2 7288R-2 9144R-2 10952R-2 12345R-1 14884R-3</td>
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<td>1401R-2 3412R-3 5279R-2 7399R-1 9497R-2 11324R-2 12345R-2 15010R-2</td>
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<tr>
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<td>3249R-3 5076R-2 7288R-1 9144R-1 10952R-1 12227R-2 14884R-1</td>
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**Table A.1:** List of RNAi lines from National Institute of Genetics Fly stock center (Japan) screened for abnormalities in entrainment to temperature cycles when driven by *tim-gal4*. Every line is labelled according the “CG annotation symbol” of the gene (from Flybase) followed by insertion number (R). A total of 148 lines, covering 96 specific genes have been screened (for most of them 2 independent insertion lines were available).
Appendix B

DEG/ENaC Channels

List of RNAi targeting degenerin/epithelial sodium channels (DEG/ENaC) genes which have been tested for defect on synchronization of the circadian clock to temperature cycles. The RNAi lines were provided by the Vienna Drosophila RNAi Centre (Vienna) and have been crossed to $\frac{y^w}{y^w}; \frac{tim-gal4:27}{CyO}; \frac{XLG-luc:1-1}{TM3}$. All the lines listed below have been assayed in our bioluminescence assay in constant light and 12:12 hr 25:16°C temperature cycles. All of them showed normal phenotype, in term of entraining per-luc expression to temperature cycles.
### Table B.1: List of RNAi targeting DEG/ENaC channel genes which were tested for defects on temperature entrainment of the circadian clock. The “line name” refers to the Flybase annotation symbol followed by insertion number (according to the VDRC).

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<td>ripped pocket</td>
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<tr>
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APPENDIX B. DEG/ENAC CHANNELS
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Light and temperature control the contribution of specific DN1 neurons to Dro-

compensation of the circadian oscillation in drosophila pseudoobscura and its