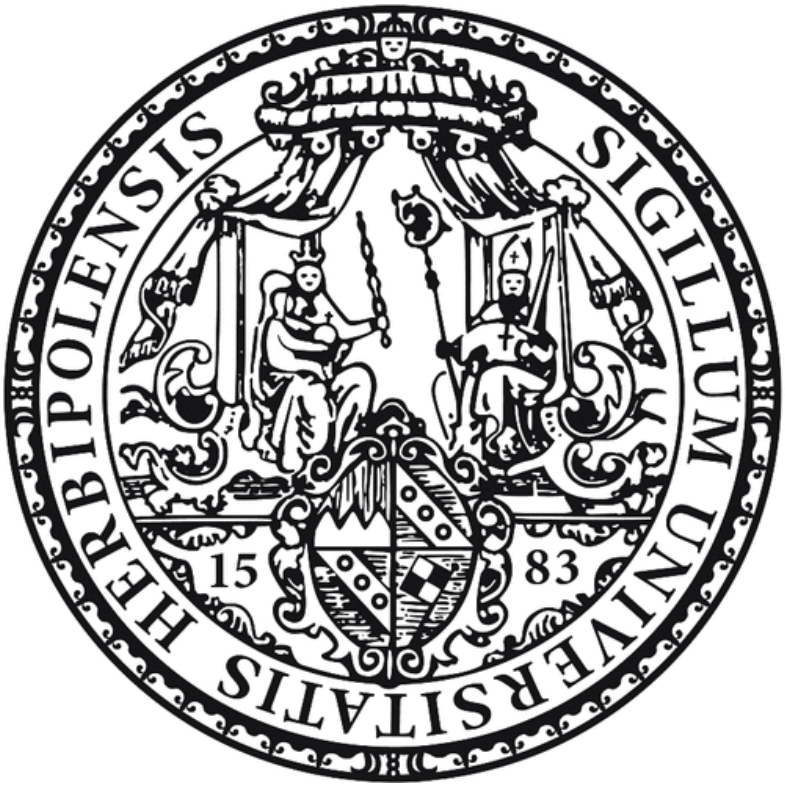
The Impact of Thermogenetic Depolarizations of Specific Clock Neurons on *Drosophila melanogaster's* circadian clock

Der Einfluss thermogenetischer Depolarisationen spezifischer Uhrneurone auf *Drosophila melanogasters* circadiane Uhr



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submitted by

Saskia Eck

from Haßfurt

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Submitted on**: ……..**

Office stamp

Members of the Thesis Committee (Promotionskomitee):

Chairperson**: Prof. Dr. Thomas Dandekar**

Primary Supervisor**: Prof. Dr. Charlotte Förster**

Supervisor (Second): **Prof. Dr. Christian Wegener**

Supervisor (Third): **Prof. Dr. André Klarsfeld**

Supervisor (Fourth): **Dr. Dirk Rieger**

Date of Public Defense: ……

Date of Receipt of Certificates: ……..

"Die Zeit ist die wichtigste Zutat im Rezept des Lebens"

Charles Darwin (1809-1882)

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Zusammenfassung

Die Rotation der Erde um ihre eigene Achse hat periodisch verändernde Umweltbedingungen, wie beispielsweise Veränderungen in den Lichtverhältnissen und der Temperatur, zur Folge. Um das Verhalten, die Physiologie und den Metabolismus eines Organismus an stets wiederkehrende Veränderungen anzupassen, haben sich endogene/circadiane Uhren entwickelt, die es dem Organismus erlauben diese Umweltbedingungen zu antizipieren. In der Chronobiologie, einem wissenschaftlichen Fachbereich, der sich mit der Untersuchung der zugrunde liegenden Mechanismen der Inneren Uhr befasst, dient die Taufliege *Drosophila melanogaster* als nützlicher Modellorganismus. Die Innere Uhr der Taufliege ist anatomisch eher einfach organisiert, weist trotz alledem jedoch Homologien zum Säugersystem auf. Auch im Rahmen dieser Doktorarbeit diente die Taufliege daher dazu grundlegende Netzwerkeigenschaften der circadianen Uhr zu untersuchen.

Die Innere Uhr von *Drosophila melanogaster* besteht aus ungefähr 150 Uhrneuronen, die sich im zentralen Nervensystem der Fliege befinden. Diese Uhrneurone können, bezüglich ihrer anatomischen Position im Gehirn in die Gruppe der dorsalen Neurone (DN1, DN2, DN3), sowie in die der lateralen Neurone untergliedert werden (LPN, LN_d, s-LN_v, l-LN_v). Funktionell werden diese Uhrneuronengruppen als Morgen- und Abendoszillatoren (M- und E-Oszillatoren) klassifiziert, da sie für unterschiedliche Verhaltensanteile in der Laufaktivität der Fliege unter Licht-Dunkel-Verhältnissen (LD) verantwortlich sind. Die s-LN_v stellen dabei die Morgenoszillatoren (M-Oszillatoren) dar und werden als Hauptschrittmacher betrachtet, da sie die Geschwindigkeit der Uhr unter konstanten Bedingungen (Dauerdunkel; DD) bestimmen. Die Gruppe der Abendoszillatoren (E-Oszillatoren) besteht aus den LN_d, einigen DN1 und der fünften s-LN_v (5^{th} s-LN_v) und ist für die richtige Terminierung der Abendaktivität in LD zuständig. All diese Uhrneurone sind funktionell nicht unabhängig voneinander, sondern bilden komplexe neuronale Verschaltungen untereinander aus, die durch einen hohen Grad an Plastizität bezüglich ihrer Reaktion auf unterschiedliche Umweltparameter (Zeitgeber), wie Licht oder Temperatur, gekennzeichnet sind.

Obwohl bereits vieles hinsichtlich der Funktion und der Bedeutung einiger Gruppen von Uhrneuronen bekannt ist, ist das genaue Zusammenspiel unter ihnen immer noch recht unklar. Um die Mechanismen, die in den Kommunikationsprozessen

zwischen verschiedenen Uhrneuronen involviert sind, zu untersuchen, machten wir Gebrauch von *dTrpA1,* einem thermosensitiven Kationenkanal, der es durch die Applizierung von Temperaturpulsen (TP) über 29°C ermöglicht, Neuronen in der intakten und sich frei bewegenden Fliege zeitlich begrenzt und zellspezifisch zu depolarisieren. Mithilfe verschiedener Uhr-spezifischer *GAL4*-Treiberlinien und der Verabreichung von TP zu verschiedenen Zeitpunkten des circadianen Zyklus in DD, war es uns möglich Rückschlüsse auf die Eigenschaften der Inneren Uhr anhand von Phasen-Verschiebungsexperimenten zu ziehen. Die hervorgerufenen Phasenverschiebungen im Laufverhalten, die durch die Aktivierung spezieller Uhrneuronen hervorgerufen wurden, wurden dabei als Phasen Responz Kurve (engl. *phase response curve*; PRC) dargestellt.

Die Depolarisierung aller Uhrneurone verschob die Phase der Aktivität am stärksten, insbesondere in der Phasen-Verzögerungszone der PRC. Wurden ausschließlich die M-Oszillatoren zusammen mit den l-LN_v (PDF^+ Neurone: s-LN_v & l-LN_v) depolarisiert, wurden ebenso Phasenverschiebungen nach vorne, wie auch nach hinten hervorgerufen, jedoch reichten die Verschiebungen nach vorne deutlich in den subjektiven Tag hinein. Daraus schlussfolgerten wir, dass Licht inhibitorischen Einfluss in diesem Bereich der PRC haben muss, da typische Licht-PRCs nicht derart ausgeprägte Vorverschiebungen aufweisen. Aufgrund des vollständigen Lichtausschlusses in den PRC-Versuchen dieser Doktorarbeit fehlt jedoch dieser Licht-vermittelte inhibitorische Einfluss zu den PDF^+ Neuronen und führt daher zur zeitlich stark ausgeprägten Phasen-Vorverschiebungszone. Diese Ergebnisse lassen daher vermuten, dass ein inhibitorisch wirkender Licht-vermittelter Eingang zu den PDF^+ Neuronen von den photorezeptiven Organen (Hofbauer-Buchner Äuglein, Photorezeptoren der Komplexaugen, Ocellen) oder von anderen Uhrneuronen existieren muss, der die Phasen-Vorverschiebungen während des subjektiven Tages unterdrückt.

Um Kenntnis über den molekularen Status der Uhr in der Verzögerungs- und Phasen-Vorverschiebungszone zu erlangen, wurden Färbungen gegen das Protein Period (PER), eines der zentralen Bestandteile der Inneren Uhr und gegen das Neuropeptid Pigment Dispersing Factor (PDF) angefertigt. Der zeitliche Verlauf im Auf- und Abbau des PER Proteins spiegelte die Phasenverschiebungen im Verhalten der Experimentalfliegen wider, wohingegen die Kontrollen weitestgehend unauffällig blieben. Zudem waren nur diejenigen Neurone von einer unmittelbaren Verschiebung

der PER Protein Oszillation betroffen, die depolarisiert wurden, was auf einen schnellen Zell-autonomen Prozess schließen lässt.

Die molekulare Verknüpfung, die zwischen der Depolarisation der Uhrneuronen und der Verschiebung der molekularen Uhr-Oszillation fungiert, ist immer noch unbekannt. Diesem Thema wurde nachgegangen, indem CREB (engl. *cAMP responsive element binding protein*) in den großen ventrolateralen Neuronen (l-LN_v) quantifiziert wurde, da diese Neuronen unerwarteterweise und am wirksamsten auf die artifizielle Depolarisation mit einer starken PER-Akkumulation reagiert haben. In vorherigen Arbeiten wurde bereits angenommen, dass CREB in die circadiane Rhythmik involviert sei, indem es an Regulationssequenzen des *period* Gens bindet (Belvin et al., 1999) und somit dessen Transkription aktiviert. Wir konnten zeigen, dass die Menge an CREB Protein in den l-LN_v circadian reguliert wird, da diese am Ende der subjektiven Nacht im Vergleich zum Ende des subjektiven Tages deutlich erhöht ist. Dieser Effekt konnte durch die artifizielle Depolarisation, aber unabhängig von deren Zeitpunkt, weiter verstärkt werden. Zudem deuten die Ergebnisse darauf hin, dass die Akkumulation des CREB Proteins mit dem Zeitpunkt des Anstiegs des PER Proteins in den l-LN_v koinzidiert. Das lässt die Vermutung zu, dass CREB als molekulare Verbindung zwischen dem elektrischen neuronalen Status und der molekularen Uhr dienen kann.

Zusammenfassend lässt sich sagen, dass die zeitlich begrenzte Depolarisation mithilfe von *dTrpA1* signifikante Phasenverschiebungen im Verhalten wie auch auf der Proteinebene hervorrufen kann. Eine artifizielle Depolarisation zu Beginn der subjektiven Nacht verursacht Phasenverschiebungen nach hinten, wohingegen eine Depolarisation zum Ende der subjektiven Nacht Phasenverschiebungen nach vorne zur Folge hat. Die Aktivierung aller Uhrneurone brachte eine PRC hervor, die weitestgehend einer Licht-PRC gleicht. Die Depolarisierung der PDF^+ Zellen hingegen ergab eine PRC, die sich insbesondere bezüglich der ausgeprägten Phasen-Vorverschiebungszone von einer Licht-vermittelten PRC unterscheidet. Die Innere Uhr scheint somit die Fähigkeit zu besitzen, exzitatorische und inhibitorische Eingänge in komplexer Art und Weise zu verarbeiten. Obwohl der in dieser Doktorarbeit gewählte experimentelle Ansatz hochgradig artifiziell ist, war es uns gerade durch den Ausschluss von Licht möglich, neue Schlussfolgerungen bezüglich der Kommunikation innerhalb des Netzwerks und dessen Lichtinformations-Eingänge zu ziehen.

Summary

The rotation of the earth around its own axis determines periodically changing environmental conditions, like alterations in light and temperature. For the purpose of adapting all organisms' behavior, physiology and metabolism to recurring changes, endogenous clocks have evolved, which allow the organisms to anticipate environmental changes. In chronobiology, the scientific field dealing with the investigation of the underlying mechanisms of the endogenous clock, the fruit fly *Drosophila melanogaster* serves as a beneficial model organism. The fruit fly's circadian clock exhibits a rather simple anatomical organization, but nevertheless constitutes homologies to the mammalian system. Thus also in this PhD-thesis the fruit fly was used to decipher general features of the circadian clock's interneuronal communication.

Drosophila melanogaster's circadian clock consists of about 150 clock neurons, which are located in the central nervous system of the fly. These clock neurons can be subdivided regarding to their anatomical position in the brain into the dorsal neurons (DN1s, DN2s, DN3s), as well as into the lateral neurons (LPNs, LN_ds, s-LN_vs, l-LN_vs). Functionally these clock neuron clusters can be classified as Morning- and Evening oscillators (M- and E- oscillators), driving different parts of the fly's locomotor activity in light-dark conditions (LD). The Morning-oscillators are represented by the s-LN_vs and are known to be the main pacemakers, driving the pace of the clock in constant conditions (constant darkness; DD). The group of Evening-oscillators consists of the LN_ds, the DN1s and the 5^{th} s-LN_v and is important for the proper timing of the evening activity in LD. All of these clock neurons are not functionally independent, but form complex neuronal connections, which are highly plastic in their response to different environmental stimuli (Zeitgebers), like light or temperature.

Even though a lot is known about the function and the importance of some clock neuron clusters, the exact interplay between the neurons is not fully known yet. To investigate the mechanisms, which are involved in communication processes among different clock neurons, we depolarized specific clock cells in a temporally and cell-type restricted manner using *dTrpA1*, a thermosensitive cation channel, which allows the depolarization of neurons by application of temperature pulses (TP) above 29°C to the intact and freely moving fly. Using different clock specific *GAL4*-driver lines and applying TPs at different time points within the circadian cycle in DD enabled us with

the help of phase shift experiments to draw conclusions on the properties of the endogenous clock. The obtained phase shifts in locomotor behavior elicited by specific clock neuronal activation were plotted as phase response curves (PRCs).

The depolarization of all clock neurons shifted the phase of activity the strongest, especially in the delay zone of the PRC. The exclusive depolarization of the M oscillators together with the l-LN_vs (PDF^+ neurons: s-LN_vs & l-LN_vs) caused shifts in the delay and in the advance zone as well, however the advances were severely enhanced in their temporal occurrence ranging into the subjective day. We concluded that light might have inhibitory effects on the PDF^+ cells in that particular part of the PRC, as typical light PRCs do not exhibit that kind of distinctive advances. By completely excluding light in the PRC-experiments of this PhD-thesis, this photic inhibitory input to the PDF^+ neurons is missing, probably causing the broadened advance zone. These findings suggest the existence of an inhibitory light-input pathway to the PDF^+ cells from the photoreceptive organs (Hofbauer-Buchner eyelet, photoreceptor cells of compound eyes, ocelli) or from other clock neurons, which might inhibit phase advances during the subjective day.

To get an impression of the molecular state of the clock in the delay and advance zone, staining experiments against Period (PER), one of the most important core clock components, and against the neuropeptide Pigment Dispersing Factor (PDF) were performed. The cycling of PER levels mirrored the behavioral phase shifts in experimental flies, whereas the controls were widely unaffected. As just those neurons, which had been depolarized, exhibited immediate shifted PER oscillations, this effect has to be rapidly regulated in a cell-autonomous manner.

However, the molecular link between clock neuron depolarization and shifts in the molecular clock's cycling is still missing. This issue was addressed by CREB (*cAMP responsive element binding protein*) quantification in the large ventrolateral neurons (l-LN_vs), as these neurons responded unexpectedly and strongest to the artificial depolarization exhibiting a huge increase in PER levels. It had been previously suggested that CREB is involved in circadian rhythms by binding to regulatory sequences of the *period* gene (Belvin et al., 1999), thus activating its transcription. We were able to show, that CREB levels in the l-LN_vs are under circadian regulation, as they exhibit higher CREB levels at the end of the subjective night relative to the end of the subjective day. That effect was further reinforced by artificial depolarization, independently of the time point of depolarization. Furthermore the

data indicate that rises in CREB levels are coinciding with the time point of increases of PER levels in the l-LN$_v$s, suggesting CREB being the molecular link between the neuronal electrical state and the molecular clock.

Taking together, the results indicate that a temporal depolarization using *dTrpA1* is able to significantly phase shift the clock on the behavioral and protein level. An artificial depolarization at the beginning of the subjective night caused phase delays, whereas a depolarization at the end of the subjective night resulted in advances. The activation of all clock neurons caused a PRC that roughly resembled a light-PRC. However, the depolarization of the PDF$^+$ neurons led to a PRC exhibiting a shape that did not resemble that of a light-mediated PRC, indicating the complex processing ability of excitatory and inhibitory input by the circadian clock. Even though this experimental approach is highly artificial, just the exclusion of light-inputs enabled us to draw novel conclusions on the network communication and its light input pathways.

1 Introduction

1.1 Circadian clocks - Basics & Characteristics

Evolution is the change in heritable traits of biological populations over successive generations, which enables organisms in a long-term manner to adapt to their environment by natural selection, biased mutations, genetic drift or gene flow (Hall & Hallgrímsson, 2008). To increase an organism's fitness in comparison to its competitors it is advantageous to be prepared to changes in the environment, which can reduce fitness, if happening unexpectedly. The rotation of the earth around its own axis takes about 23 hours 56 minutes, defines one day and hence provides daily periodic changes. Every single turning of the earth causes predictable recurring changes in light and temperature conditions with higher temperatures and light intensities during the day and lower temperatures and light intensities during the night. Thus it is not surprising that an adaption to these daily, rhythmic changes evolved to obtain fitness advantages. These adaptations already took place on lower evolutionary levels of biological organization and development like in bacteria (e.g. cyanobacteria *Synechococcus*, Johnson et al., 1996; Kondo et al., 1997; Ishiura et al., 1998), algae (e.g. *Chlamydomonas reinhardtii*; Bruce, 1970, 1972; Bruce & Bruce 1978; Mergenhagen, 1984; Kondo et al., 1991; Johnson et al., 1992) and plants (e.g. *Mimosa pudica*; de Mairan, 1729; Hill, 1757; Duhamel duMonceau 1759; Zinn, 1759). Endogenous time-keeping systems are called *endogenous* or *circadian* clocks/oscillators, which enable the organisms to adjust their physiological, biochemical and behavioral responses to daily environmental changes. Since circadian clocks are not exactly ticking with a 24 hour-period, Halberg defined 1959 the so-called *endogenous* period as *circadian* period (Latin *circa: approximately*, *dies: day*), which gets apparent under constant conditions (constant darkness; DD).
1986 Johnson and Hastings came up with a simplified model of the organization of these circadian oscillators dividing the circadian clock into 3 main components (Fig. 1): the *input* to the clock, the *core clock* and the clock *output*. As *input* environmental parameters or so-called *Zeitgebers* (German *Zeit*: *time*, *-geber*: *presenter*; Aschoff 1960) such as light intensity (e.g. Emery et al., 1998), temperature (Pittendrigh et al., 1958), feeding (Stephan et al., 1979), social interaction (Levine et al., 2002) or humidity (Halket, 1931) provide information for the organism regarding the time of day to properly synchronize or *entrain* the endogenous, molecular system

to the external cycle. Light functions as the main Zeitgeber, apart from temperature (Pittendrigh et al., 1958). Since both of them together are the most reliable environmental cues during the whole course of the year, the animal can adjust its clock appropriately and thus is able to predict e.g. the rise of the sun in a confident manner (*anticipation* of e.g. behavior). The *core clock* processes input parameters and complex input combinations (e.g. light intensity with temperature) to generate molecular oscillations, that in turn cause rhythmically occurring *outputs* like behavior (e.g. locomotor activity on the level of a single animal or e.g. eclosion behavior on the *Drosophila* population level), changes in the physiological state (e.g. metabolic functions) or other clock-controlled processes.

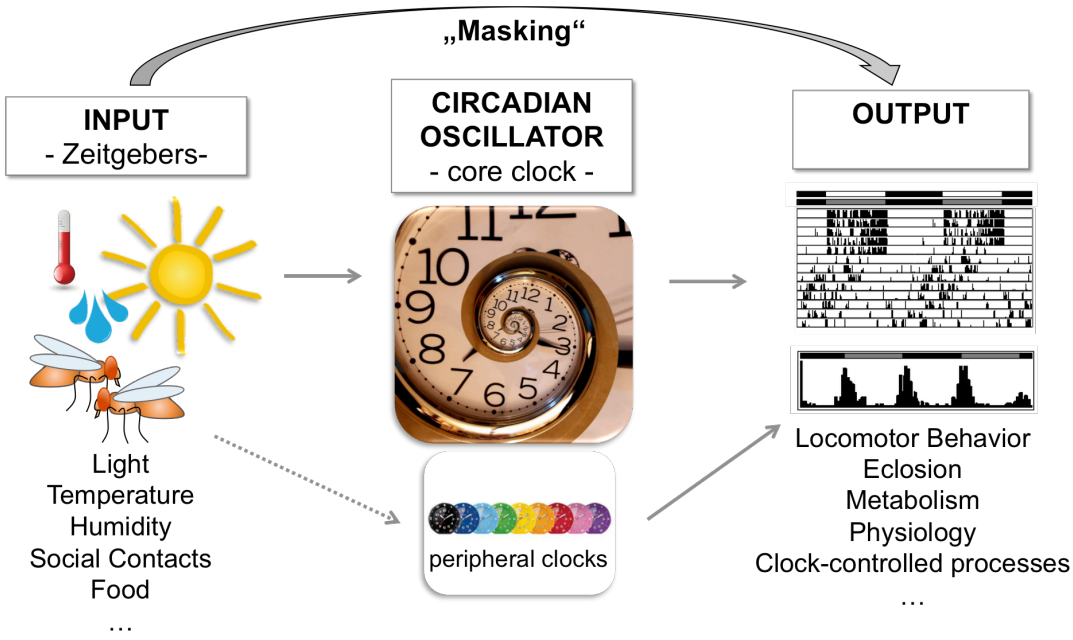

Figure 1 Simplified model of the circadian clock system
The core circadian oscillator receives input by Zeitgebers (e.g. light, temperature, humidity, social contacts or food availability). The circadian clock is synchronized to these Zeitgebers and mediates either indirectly via peripheral clocks in different tissues (in *Drosophila* e.g. in the Malphigian tubules, the eyes or the antennae (Plautz et al., 1997; Giebultowicz, 2001)) or directly oscillating outputs (e.g. rhythms in behavior, metabolism or physiology). Environmental cues can also bypass the circadian clock and evoke fast, non clock-controlled output responses known as masking effects (e.g. reaction to lights on without any anticipation) (adapted from Golombek & Rosenstein, 2010).

To distinguish between responses which are just spontaneous reactions to changes in e.g. light conditions, which are actually simply bypassing the clock (*masking;* Mrosovsky, 1999) and real clock-controlled outputs, Bünning (Bünning, 1935), Aschoff (Aschoff, 1960) and Pittendrigh (Pittendrigh, 1954, 1960, 1966, 1967, 1981; Pittendrigh et al., 1958; Pittendrigh & Minis, 1964), the most important early

"Chronobiologists" (Greek *chronos*: *time*) defined three fundamental properties, which are approved as hallmarks for actual circadian rhythms: First of all, circadian rhythms have to be generated by a self-sustained oscillator, whose oscillations continue or *free-run* even without any input by *Zeitgebers* with an endogenous *circadian* period of about 24 hours. Secondly, the circadian clock can be synchronized or *entrained* by Zeitgebers, like light or temperature among others (Pittendrigh et al., 1958). The last criterion is the circadian clock's property of being temperature compensated, meaning that even at higher temperatures the circadian clock keeps its pace and its periodicity stable over a wide physiological range (Pittendrigh 1954; Bruce & Pittendrigh, 1956).

Even though the general features of the circadian clock are highly conserved and were already nicely described by behavioral experiments of for example Jean Jacque Ortous de Mairan in the 18th century (deMarain, 1729), the underlying clock mechanisms remained highly elusive for a long time. To investigate the underlying mechanisms of the circadian clock, which was by then a "black box", *Drosophila melanogaster* turned out to be a perfect model organism in the lab of chronobiologists. Since *Drosophila* is highly genetically accessible, has a short generation time with a high reproduction rate and a relative simple neuronal composition in comparison to mammals, the use of the fruit fly opened a wide range of possibilities. As in the early 1970s *Drosophila* genetics came into play, Seymour Benzer and Ron Konopka performed chemical mutagenesis screens ending up with several *Drosophila* clock mutants. One of these clock mutants exhibited a longer period (Per^{L}), one had a shorter period (Per^{S}) and another lost its rhythmicity completely in constant darkness (DD) (per^{01}). It turned out that all three fly strains had a mutation in the same gene, *period (per)*, which was the first clock gene to be investigated (Konopka & Benzer, 1971). In the following years the field of chronobiology celebrated groundbreaking scientific outcomes and a lot of core clock components were identified (reviewed in Helfrich-Förster, 2014). Other clock genes like *timeless* (Sehgal et al., 1994), *clock* (Allada et al., 1998), *cycle* (Rutila et al., 1998) and their interactors *double-time* (Price et al., 1998), *vrille* (Blau et al., 1999), *shaggy* (Martinek et al., 2001), *casein kinase2* (Lin et al., 2002), *slimb* (Ko et al., 2002; Akten et al., 2003), *pdp1* (Cyran et al., 2003) and *pp2a* (Sathyanarayanan et al., 2004) were discovered.

1.2 The circadian clock of *Drosophila melanogaster*

By identifying the core clock component *period* in the 1970s Konopka and Benzer opened up the field of molecular and cellular based chronobiology in *Drosophila melanogaster*. Apart from the investigation of other clock genes in the 1990s, these results allowed in follow-up experiments to pin-point the anatomical position of the circadian clock in the fruit fly to a small neuropil next to the optic lobes in the brain of the fly, to the accessory medulla (aMe) (Konopka et al., 1983; Helfrich, 1986; Dushay et al., 1989). Especially the generation of clock protein specific antibodies allowed the morphological description and analysis in a way that we are today lucky to know most of the anatomical features of the circadian clock network (reviewed in Helfrich-Förster, 2002; Helfrich-Förster et al., 2007).

1.2.1 The clock neuron network

The circadian clock of *Drosophila melanogaster* consists of about 150 clock neurons, positioned in the central nervous system of the fly. All so-called *pacemaker* neurons have to be *per definitionem* immunoreactive against the core clock proteins TIM and PER (Taghert & Shafer, 2006) and can be subdivided according to their anatomical position in the brain, into the dorsal neurons (DNs) and the lateral neurons (LNs). The dorsal neurons can further be classified into the cluster of the DN1 neurons, which consists of two anterior DN1 cells ($DN1_a$), which have been shown to be already present in larvae (Klarsfeld et al., 2004) and of about fifteen posterior DN1 neurons ($DN1_p$) (Shafer et al., 2006). Furthermore two DN2 neurons and about 30-40 DN3 cells can be found per brain hemisphere. The cluster of the DN1 neurons has been functionally implicated to be among others downstream targets of the Pigment Dispersing Factor positive (PDF^+) neurons, the s-LN_vs (Grima et al., 2004; Stoleru et al., 2004; Shafer et al., 2008; Cusumano et al., 2009; Zhang et al., 2010) whereas the DN2 neurons have been shown to be involved in temperature entrainment of the circadian clock (Miyasako et al., 2007; Picot et al., 2009). The function of the last and biggest DN group, the DN3s, is unfortunately unknown so far.

The second major group of clock neurons is that of the lateral neurons (LNs). They can be subdivided into three lateral posterior neurons (LPNs), six PDF negative (PDF^-) dorsolateral neurons (LN_ds), four PDF^+ large ventrolateral neurons (l-LN_vs), as well as into four PDF^+ small ventrolateral neurons (s-LN_vs) per brain hemisphere (Helfrich-Förster, 1995, Helfrich-Förster et al., 2007). Next to the l- and s-LN_vs one

single PDF$^-$ small ventrolateral neuron (5th s-LN$_v$) is located (Kaneko et al., 1997). In contrast to that of the DNs, the role of most of the LNs in the circadian clock network is well understood. It was shown that the s-LN$_v$s are the main pacemakers in constant darkness (DD) and that they are representing the Morning oscillators (M cells) together with some of the DN1 neurons. The M cells mainly set the phase of the morning activity peak in light-dark conditions (LD) (Helfrich-Förster, 1998; Renn et al., 1999; Park et al., 2000; Blanchardon et al., 2001; Nitabach et al., 2002; Peng et al., 2003; Lin et al., 2004; Stoleru et al., 2004, 2005). Three out of six PDF$^-$ LN$_d$s (CRY$^+$ LN$_d$s), some DN1s and the 5th PDF$^-$ s-LN$_v$ are defined as evening oscillators (E cells), setting the fly's evening activity (E peak) in LD, are implemented in temperature entrainment of the clock and are furthermore thought to be the main pacemakers under LL-conditions (Grima et al., 2004; Rieger et al., 2006; Stoleru et al., 2004; 2007; Yoshii et al., 2010; reviewed in Hermann-Luibl & Helfrich-Förster, 2015). Since the l-LN$_v$s among others express Cryptochrome (CRY), a blue light receptor (Emery et al., 1998; Stanewsky et al., 1998; Yoshii et al., 2008), and additionally provide dense connections across the medulla, the third optic neuropil of the fly (Helfrich-Förster et al., 2007), these cells are assumed to be coincidence detectors for light (Shang et al., 2008). Indeed there is experimental evidence that these neurons are important for arousal, as they show increased electrical excitability upon illumination and thus probably deliver light information from the photoreceptive organs (compound eyes, H-B eyelet, ocelli) to the clock network (Sheeba et al., 2008; Fogle et al., 2011). Solely the function of the LPN group is not really clear yet, but they might be involved in temperature entrainment (Kaneko et al., 1997; Kaneko & Hall, 2000; Yoshii et al., 2005; Glaser PhD-thesis, 2006; Shafer et al., 2006; Miyasasko et al., 2007; Lee et al., 2013).

The immunoreactivity of the projections of the clock neurons against PDF as well as the usage of ectopically expressed fluorescent markers, like the green-fluorescent protein (GFP), allowed the determination of the arborization patterns of the various clock neurons to unravel possible network interactions. It turned out that there are two main projection targets of the clock neurons in the central nervous system of the fly: the accessory medulla (aMe), which is known to be the principal pacemaker center of insects (Page, 1985; Helfrich-Förster et al., 2007) and the superior brain, where neurosecretory and hormone secreting neurons (*pars intercerebralis, pars lateralis*) are located (Helfrich-Förster et al., 2007). PDF-staining experiments

exhibited the l-LN$_v$s sending PDF$^+$ fibers into the ventral elongation of the ipsilateral aMe, having dense arborizations across the ipsilateral and contralateral medulla, thus connecting both hemispheres via the *posterior optic commissure (POC)*. The PDF$^+$ s-LN$_v$s and the 5th PDF$^-$ s-LN$_v$ innervate the aMe and send projections to the *superior protocerebrum*, connecting the aMe with the DNs (Helfrich-Förster et al., 2007). The group of the LN$_d$s is more heterogeneous in its arborization pattern. Three LN$_d$s innervate the *superior protocerebrum* and send projections to the ipsilateral and contralateral aMe, whereas the remaining ones project to the contralateral side of the brain (Helfrich-Förster et al., 2007). The arborization pattern of the LPNs is not known so far, but there seems to be an overlap with the projections of other neurons (Helfrich-Förster et al., 2007). The dorsal neurons commonly innervate the *superior protocerebrum*. Just some DN1s (DN1$_a$s and two DN1$_p$s) and DN3s send projections to the ipsilateral aMe (Shafer et al., 2006; Helfrich-Förster et al., 2007). The remaining DN1 cells may also run to the aMe, others may run to the contralateral brain, others may do both (Helfrich-Förster et al., 2007).

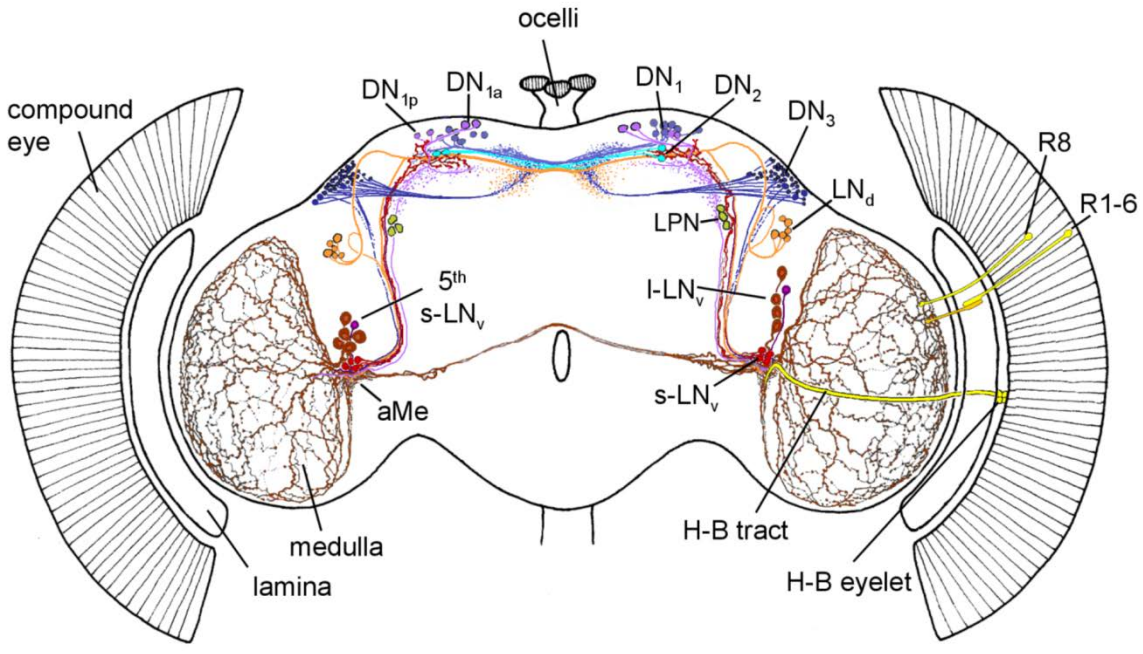

Figure 2 Schematic overview of the circadian clock network of *Drosophila melanogaster*
The clock neurons can be divided into two main groups of neurons: the dorsal neurons (DNs) and the lateral neurons (LNs). The DNs consist of the DN1$_{a/p}$s, DN2s and DN3s. The LNs are composed of the LPNs, LN$_d$s, l-LN$_v$s, s-LN$_v$s and the 5th s-LN$_v$. The arborizations of the different clock neuron subclusters are shown in corresponding colors to the color of their cell bodies (from Helfrich-Förster et al., 2007).

1.2.2 The molecular clock mechanism

The core molecular clock of *Drosophila melanogaster* consists of two negative interlocked transcriptional and translational feedback loops, which are present in each particular clock neuron (Hardin et al., 2011). The clock causes a ~24 hour rhythm in *per* and *tim* mRNA as well as PER and TIM protein accumulation, which also persists in DD, demonstrating the free-running ability of the clock. Due to complex phosphorylation, dephosphorylation and translocation processes the protein cycling of PER and TIM are 6-8 hours delayed compared to the peaking of mRNA levels (Fig. 3) (Kloss et al., 1998; Price et al., 1998; Martinek et al., 2001; Lin et al., 2002; Sathyanarayanan et al., 2004; Brown et al., 2012).

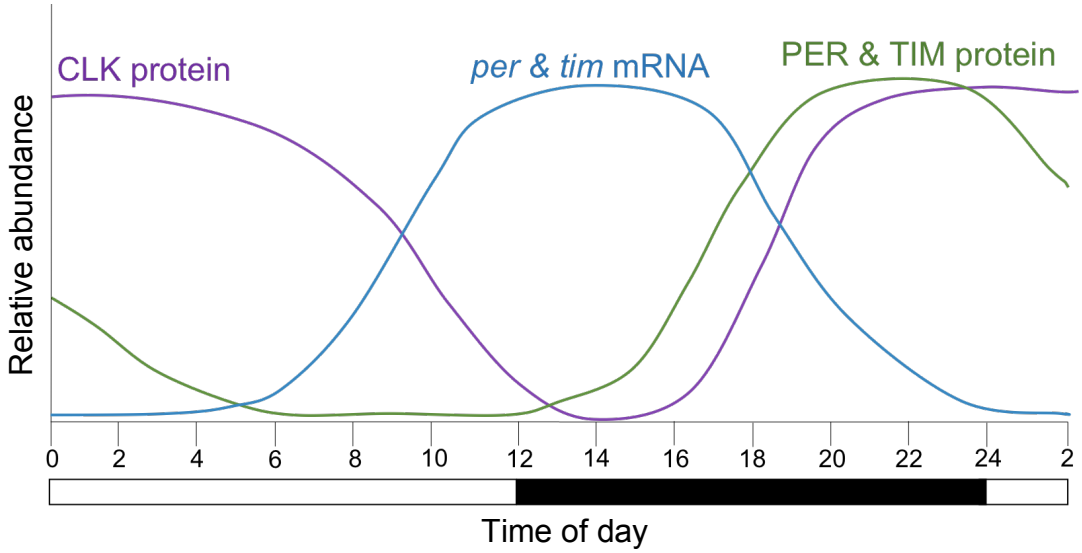

Figure 3 Oscillations of *per* & *tim* mRNA and PER, TIM & CLK protein levels during one circadian cycle

The core molecular negative feedback-loop generates a cycling of *per* and *tim* mRNA levels and PER and TIM protein levels. The mRNA levels peak in the beginning of the night, whereas the proteins peak 6-8 hours after the mRNAs reaching the highest levels around the end of the night. The second feedback loop causes the CLK protein cycling in antiphase to *per* and *tim* mRNA levels (adapted from Peschel PhD-thesis, 2008; reviewed in e.g. Peschel & Helfrich-Förster, 2011).

Key components of the core feedback loop are the transcription factors CLOCK (CLK) and CYCLE (CYC) that bind as heterodimers (CLK-CYC) to the E-boxes of *period* and *timeless* gene promoter regions (Sehgal et al., 1994), thus enhancing *per* and *tim* transcription, apart from regulating the transcription of other clock-controlled genes (*ccg*) (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). After the transcription and translocation of *per* and *tim* mRNA to the cytoplasm, both mRNAs are translated (Fig. 4). Once PER and TIM have accumulated in the cytoplasm, both proteins form a heterodimer (TIM-PER) (Price et al., 1998). Without TIM bound to PER, PER is susceptible to phosphorylation by Double-time (DBT), is subsequently

ubiquitinated by the F-Box protein Slimb (SLMB) and degraded by the proteasome (Grima et al., 2002; Ko et al., 2002). Just in the heterodimerized form PER and TIM (Curtin et al., 1995; Shafer et al., 2002) are able to enter the nucleus, mediated by a nuclear localization signal of TIM (Saez et al., 2011) and several phosphorylation signals, such as the phosphorylation of TIM by Shaggy (SGG) (Martinek et al., 2001) or of PER by Casein Kinase 2 (CK2) (Lin et al., 2002). After being translocated to the nucleus TIM-PER inhibit their transcription by PER binding to CLK (Martinek et al., 2001; Lin et al., 2002; Akten et al., 2003). This in turn causes the phosphorylation of CLK and CLK-CYC is released from the E-boxes of *tim* and *per* genes. Without CLK-CYC bound to the E-boxes, the transcription of *per* and *tim* stops. Less TIM and PER can accumulate in the cytoplasm that can inhibit CLK-CYC to bind to the E-boxes of *per* and *tim.* More *per* and *tim* expression can take place again, resulting in more PER and TIM protein, what in turn defines the beginning of a new cycle (Hardin, 2011).

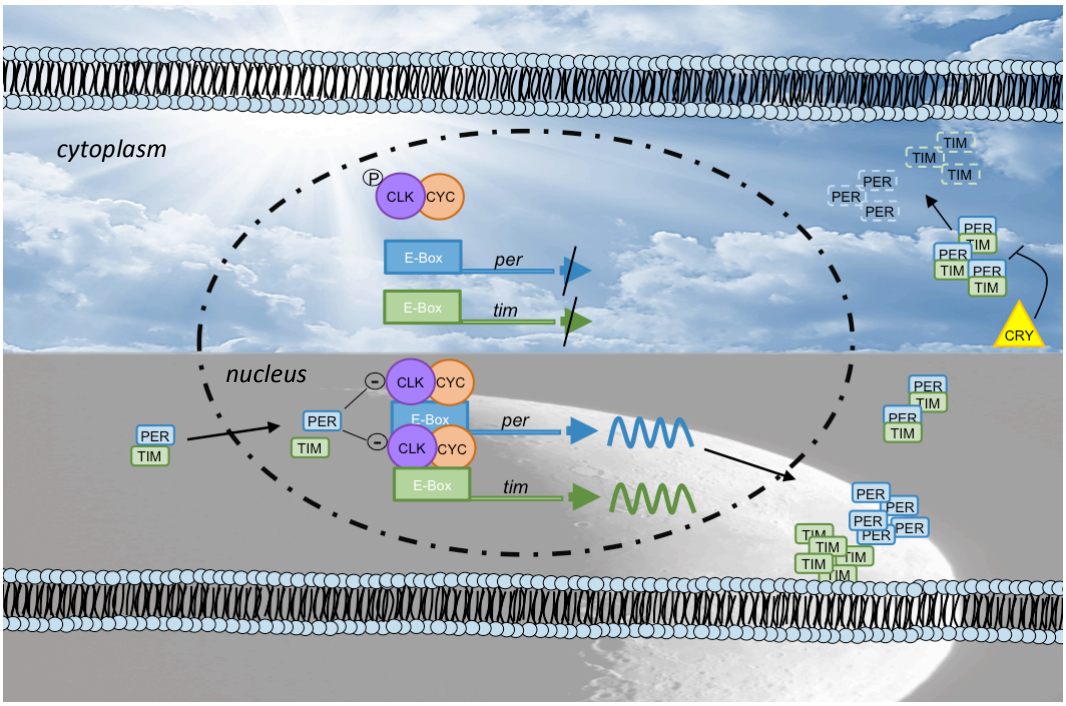

Figure 4 Simplified core clock mechanism of *Drosophila melanogaster*
The transcription factors CLK-CYC bind to the E-boxes of *per* and *tim* genes to activate *per* and *tim* transcription during the dark period. TIM and PER protein accumulate in the cytoplasm and form heterodimers to repress their own transcription by inhibiting CLK binding to the E-boxes of *per* and *tim*. During the light phase CRY mediates the light-dependent phosphorylation and degradation of TIM. In the absence of TIM PER is unstable and is degraded, too. The inhibition of CLK cannot take place and subsequently the cycle is reset (adapted from Paranjpe & Sharma, 2005; Blau et al., 2007; Peschel & Helfrich-Förster, 2011).

The complexity of the circadian mechanism is further increased, as CRY as a blue light-receptor is mediating the light-dependent degradation of TIM in a cell-autonomous manner (Emery et al., 1998; Stanewsky et al., 1998; Emery et al., 2000; Krishnan et al., 2001; Lin et al., 2001). CRY is expressed in many clock neurons (Fig. 7), thus enabling the circadian clock to be well synchronized to environmental changes in light-dark conditions (LD) (Emery et al., 1998; Stanewsky et al., 1998; Yoshii et al., 2008) (Fig. 4).

The second feedback loop also includes CLK and CYC as main components. CLK-CYC heterodimers activate the transcription of *par-domain protein 1ε (Pdp1)* and *vrille (vri)* (Blau & Young, 1999; Cyran et al., 2003; Glossop et al., 2003). VRI and PDP1 both compete for binding to the V/P-box in the promoter region of *clk* with VRI repressing *clk* transcription and PDP1 activating it, but with a delay of about 4-5 hours relative to VRI's repression. These mechanisms cause a cycling in *clk* mRNA levels, which is in antiphase to the cycling of *per* and *tim* mRNA levels (Fig. 3 & Fig. 5).

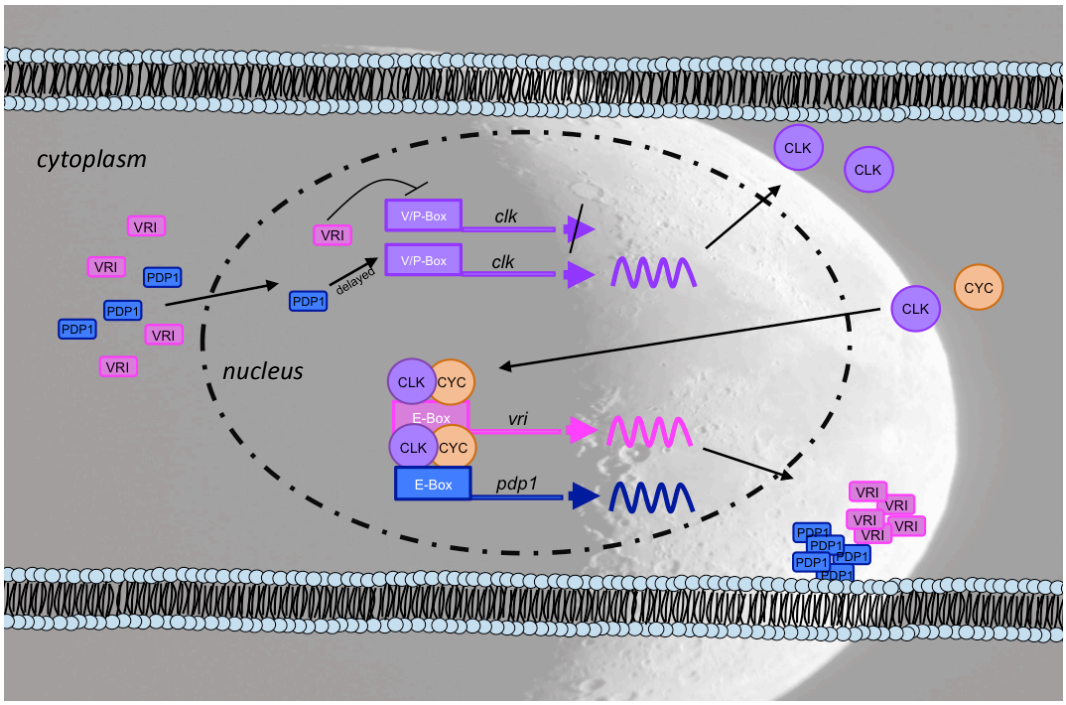

Figure 5 Second interlocked feedback loop of *Drosophila melanogaster*
The second feedback loop consists of CLK-CYC dimers, which bind to the E-boxes of *vri* and *pdp1* genes, thus activating their transcription during the night. VRI and PDP1 influence in turn the transcription of *clk*: VRI is inhibiting the transcription and PDP1 is activating the transcription of *clk*, but with a delay of 4-5 hours relative to VRI's repression. That causes the cycling of CLK, which is in antiphase to the cycling of PER and TIM (Fig. 3) (adapted from Paranjpe & Sharma, 2005; Blau et al., 2007; Peschel & Helfrich-Förster, 2011).

1.2.3 The dual oscillator model

Drosophila melanogaster as a crepuscular animal exhibits a bimodal activity pattern in locomotor behavior rhythms under light-dark conditions (LD), displaying a pronounced morning activity peak (M peak) around dawn and an evening activity peak (E peak) around dusk (Fig. 6, middle). The fruit fly's highly reliable and characteristic bimodal activity pattern has been investigated extensively during the last years to proof the so-called *dual oscillator model*. Originally investigating mammals, Pittendrigh and Daan have postulated 1976 the existence of a dual oscillator mechanism, which is generating the two distinctive peaks occurring at lights -on and -off. They proposed the existence of a morning oscillator (M oscillator), whose period is shortened by light, whereas the evening oscillator's period (E oscillator) is lengthened, respectively (Pittendrigh & Daan, 1976). During the last years with this regard, efforts in *Drosophila's* chronobiology have been very successful showing that the principle of the dual oscillator model holds true also for the fruit fly's circadian clock. The M oscillator has been shown to be represented by the PDF^+ s-LN_vs, which time locomotor activity to the morning. Furthermore these cells were shown to constitute the main pacemaker of the clock, as they are important for free-running locomotor behavior in DD (Fig. 6). M cells speed up their molecular pace upon light exposure to advance the phase of activity. The E oscillator covers more than one distinct cluster of clock neurons, hence including some of the DN1 neurons, three CRY^+ LN_ds and the 5^{th} s-LN_v cell per brain hemisphere (Grima et al., 2004; Stoleru et al., 2004; Rieger et al., 2006; Picot et al., 2007) (Fig. 6). These E cells are thought to slow down the speed of the molecular clock in response to light to properly delay the evening peak.

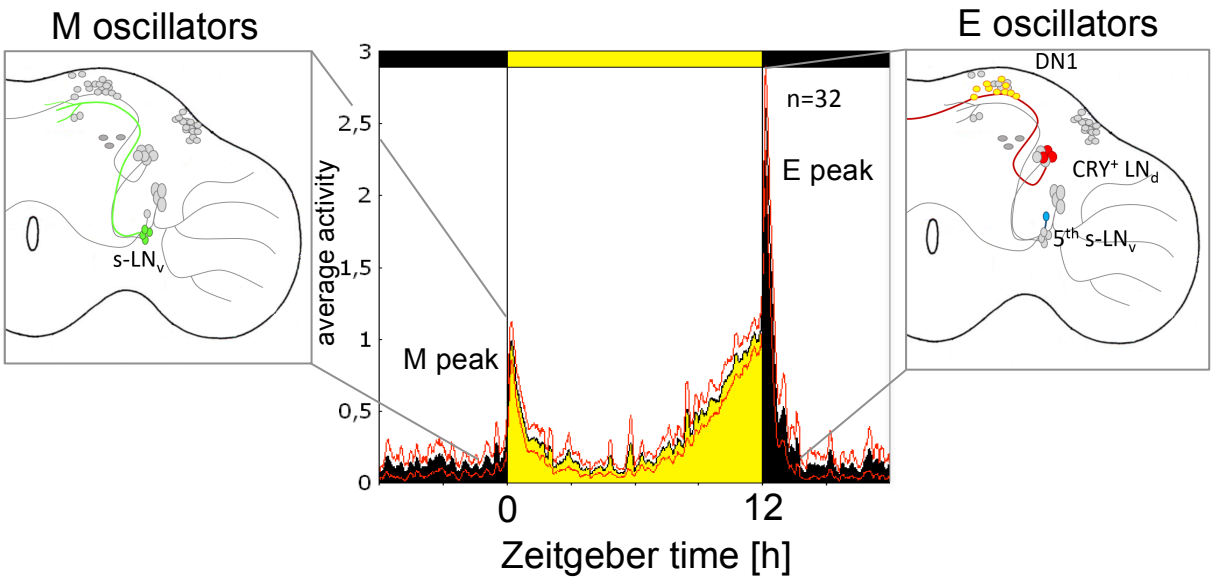

Figure 6 Morning and Evening oscillators
Drosophila exhibits two main activity peaks of locomotor behavior in light-dark conditions, one starting before lights-on, which is referred to as M peak and one before lights-off, referred to as E peak of locomotor activity. This characteristic activity pattern relies on proper working M oscillators, namely the s-LN_vs and E oscillators, represented by some DN1s, three CRY^+ LN_d and the 5^{th} PDF^- s-LN_v. The diagram shows the average activity (mean±SEM) of wild type *Ala* flies (n=32) in LD 12:12 (indicated by black and yellow colors) at 20°C.

During the last years it got more and more evident that indeed the principal of the dual oscillator model fits quite well for *Drosophila*, however it appears to be oversimplified. Different clock neurons contribute to varying degrees to locomotor activity rhythms, which is highly dependent on the environmental conditions the fly experiences (Rieger et al., 2009; Zhang et al., 2010; Yoshii et al., 2012). Along that line it was shown under different temperature conditions (Dubruille & Emery, 2008; Sehadova et al., 2009; Gentile et al., 2013) and day lengths (Rieger et al., 2006; Stoleru et al., 2007) that in long photoperiods the two peaks of activity move further apart from each other. However in shorter photoperiods the peaks were closer together, as the flies followed dawn and dusk (Vanin et al., 2012; Menegazzi et al., 2013). This ability to properly shift the main portion of activity to a certain time of the day has evolutionary relevance, since this procedure has to occur daily during the course of the year from e.g. wintertime (short photoperiods: shorter days, longer nights) to summertime (longer photoperiods: longer days, shorter nights) and vice versa. Allowing the fly to advance the morning peak and to delay the evening peak in longer photoperiods, the M oscillators have to accelerate their molecular clock, whereas the E oscillators have to decelerate. That feature of both oscillators fits to the predictions of the Pittendrigh and Daan dual oscillator model (1976), stating that

the M oscillator shortens its period in light, whereas the E-oscillator lengthens its period upon increasing light. This differential adjustment of the pace is thought to be mainly mediated by the neuropeptide Pigment Dispersing Factor (PDF; 18 amino acids) (Helfrich-Förster, 1995; Wu et al., 2008; Wülbeck et al., 2008; Picot et al., 2009; Yoshii et al., 2009; Shafer & Yao, 2014). The basal and central role of PDF in mediating phase relationships in the circadian clock network in insects was demonstrated by an experiment, in which PDF was injected into brains of cockroaches. There, PDF was able to reset the phase of free-running behavioral rhythms efficiently (Petri & Stengl, 1997). Also in *Drosophila* PDF is thought to be the most important neuropeptide to mediate neuronal synchronization after clock neuron activation (Nitabach et al., 2006; Wu et al., 2008; Yoshii et al., 2009; Shafer & Yao, 2014) and as such serves as an output signal to downstream neurons and arousal centers to elicit activity rhythms (Cusumano et al., 2009; Lear et al., 2009; Shafer et al., 2009; Im et al., 2011; Seluzicki et al., 2014). By binding to the PDF receptor (PDFR), PDF predominantly decelerates E cells and accelerates M cells (Hyun et al., 2005; Mertens et al., 2005; Yoshii et al., 2009; Im & Taghert, 2010; Im et al., 2011) and plays an essential role in mediating synchronization of M and E oscillator with each other, since the loss of PDF alters molecular oscillations in M and E cells differently. Thus, it has been shown that the loss of PDF signaling desynchronizes the daily rhythms of PER accumulation and subcellular localization in M cells, but produces an advanced phase and reduced amplitude of PER oscillations in E cells (Lin et al., 2004; Lear et al., 2005). Furthermore PDF mutant flies (pdf^{01}) show severe defects in locomotor activity regarding their M and E peaks of activity (Renn et al., 1999). Although the flies were still able to entrain to LD conditions, the M peak was completely missing and the E peak was advanced for about 2 hrs in comparison to wild type flies. This indicates the importance of PDF signaling on M cell activity and on the timing of E cell activity. A possible explanation for the dissimilar responses of M and E cells to the same signal has been published by Duvall and Taghert in 2013: Even though the PDF-signal to M and E cells itself as well as the PDF-receptor (PDFR) expressed in M and E cells (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005; Shafer et al., 2008; Im & Taghert, 2010) are similar, M and E cells have been shown to express different types of adenylyl cyclases (ACs), which as part of the downstream signaling pathway of PDF may mediate an acceleration in M cells, but a deceleration of pace in E cells (Duvall & Taghert, 2013).

1.2.4 The influence of light & temperature on the clock

Zeitgebers like light and temperature are main input factors to entrain the circadian clock to the environment. Light is known to be the strongest Zeitgeber and its input to the clock is mainly mediated cell-autonomously by the blue light receptor Cryptochrome (dCRY1) (Emery et al., 1998; 2000; Stanewsky et al., 1998), whose absorption spectrum lies in the UV- (λ≈ 360 nm) and in the blue range (λ≈ 430-460 nm) of light (Ozturk et al., 2011). Functionally it has been shown that a conformational change of the C-terminus of CRY is necessary for proper signaling and light-dependent cell-autonomous TIM degradation (Ozturk et al., 2011), apart from a non cell-autonomous TIM degradation pathway, which might be critical for circadian behavioral photoresponses as well (Lamba et al., 2014). dCRY is known to be expressed in about six DN1 neurons, three LN_ds and in all of the LN_vs in the circadian clock (Fig. 7), but also in the cytoplasm of the photoreceptor cells of the compound eyes (Yoshii et al., 2008). There, CRY enhances the sensitivity of the compound eyes to light in the rhabdomeric microvilli of the photoreceptor cells (Schlichting PhD-thesis, 2015).

Although CRY is fundamental for rapid entrainment of the clock to photic stimuli, entraining the clock to light input does not necessarily require CRY (Kistenpfennig et al., 2012). The visual system of the fly, consisting of the photoreceptive organs (compound eyes, ocelli and the Hofbauer-Buchner eyelet (H-B eyelet; Hofbauer & Buchner, 1989)) contributes apart from dCRY to mediate light information to the circadian clock network and enables the fly to employ the entire spectrum of light (H-B eyelet: 480 nm; photoreceptors of compound eyes: 350-650 nm) to accurately time the circadian phase of the clock to the natural environment (Fig. 2; Hofbauer & Buchner, 1989; Helfrich-Förster; 2002). The compound eyes are known to be important for providing color, shape and motion vision input (Menne & Spatz, 1977; Yamaguchi et al., 2010), whereas the ocelli have been shown to be involved in perceiving small changes in light intensity (Goodman, 1970; Hu et al., 1978). The H-B eyelet, which projects to the aMe, has been shown to be directly involved in circadian entrainment (Hofbauer & Buchner, 1989; Yasuyama & Meinertzhagen, 1999; Helfrich-Förster et al., 2002; Rieger et al., 2003; Schlichting PhD-thesis, 2015).

Even though the transmission mechanism of light information between the photoreceptor cells (PRs) of the compound eyes and the H-B eyelet to the circadian clock of insects is not fully understood, there is some evidence that the PRs might

mediate light information via histamine to the clock, since they express histidine decarboxylase (Hdc), a protein necessary for histamine biosynthesis (Hardie et al., 1987; Burg et al., 1993). The targets of this signaling might be the l-LN_vs since they are immunoreactive to the inhibitory histamine receptor HisCl, whereas the s-LN_vs are HisCl negative (Hong et al., 2006). Functionally this putative histamine signaling from the eyes to the clock might be responsible for phase delaying effects on the evening activity in LD conditions (Rieger et al., 2006).

The H-B eyelet is immunoreactive against histamine and choline acetyl transferase (ChAT), an enzyme responsible for the synthesis of the neurotransmitter acetylcholine (ACh) (Pollack & Hofbauer, 1991; Yasuyama & Salvaterra, 1999), suggesting an inhibitory signaling pathway via histamine and an excitatory input pathway via ACh from the H-B eyelet to the clock (Schlichting PhD-thesis, 2015). ACh in insects is known to supply fast synaptic excitatory transmission via nicotinic acetylcholine receptors (nAChRs) (reviewed in Dupuis et al., 2012), whereas histamine is reported to have inhibitory effects on downstream neurons via histamine-gated chloride channels (Hardie, 1989; Pantazis et al., 2008). A possible intersection of the photic input to the clock at the stage of the LNs via neurotransmitters has been demonstrated, as the PDF-positive LNs have been shown to be downstream targets of the precursor of the H-B eyelet by experiments performed in *Drosophila* larvae. ACh released from the H-B eyelet's larval precursor, the Bolwig organ (Bolwig 1946; Malpel et al., 2002) was shown to serve as excitatory input factor onto the PDF-positive LNs, as the LNs responded with increased Ca^{2+} levels, suggesting this signal to represent the photic input to the clock in larvae (Wegener et al., 2004; Keene et al., 2011; Yao et al., 2012). Furthermore, it was demonstrated that ACh signaling elicits PDF release from in the LN_vs, which in turn resets the DN1s (Klarsfeld et al., 2011), indicating that the PDF^+ neurons are in charge receiving light information and mediating light-dependent clock responses among the clock neurons. Also in the adult fly excitatory pathways via acetylcholine might mediate light-input like in larvae, as both adult large and small LN_vs are responsive to cholinergic agonists and as both terminate very close to the accessory medulla (Helfrich-Förster et al., 2002, 2007; Malpel et al., 2002; McCarthy et al., 2011; Lelito & Shafer, 2012; Yoshii et al., 2015), suggesting a cross talk among the clock neurons (Yuan et al., 2011; Yao et al., 2012).

Along that line, Schlichting and colleagues recently showed by cAMP imaging also in adults that the H-B eyelet specifically excites the s-LN$_v$s, but not the l-LN$_v$s. The depolarization of the s-LN$_v$s was additionally shown to phase advance the clock in free-running conditions (Schlichting PhD-thesis, 2015). However the link between the neuronal depolarization (excitatory input e.g. via the H-B eyelet) and hyperpolarization (inhibitory input via the compound eyes/H-B eyelet) to the molecular clock remains mainly elusive.

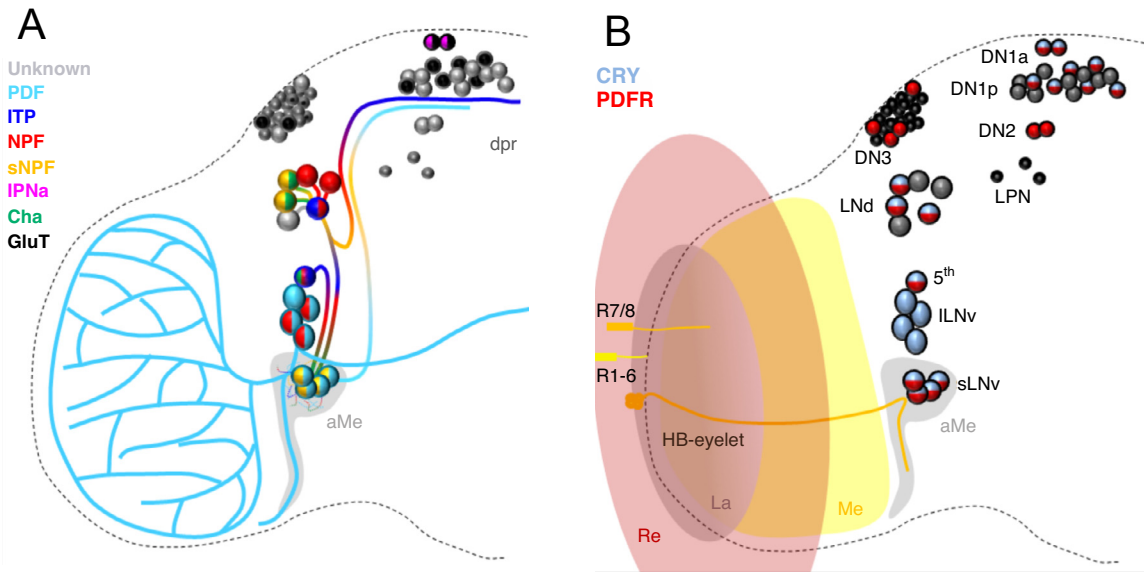

Figure 7 Scheme of the expression pattern of (A) neuropeptides, fast neurotransmitters, (B) Cryptochrome and PDFR in the circadian clock of *Drosophila melanogaster*
(A) The peptide expression in the clock network shows the heterogeneity of the clock neurons even within one cluster. PDF is important for proper clock cell-synchronization across the network and is expressed in 4 s-LN$_v$s and 4 l-LN$_v$s per hemisphere. The neuropeptide constitution of some DN1s, the DN3, one LN$_d$ and the LPNs is still unknown. **(B)** The PDF receptor (PDFR) is expressed in all s-LN$_v$s, three LN$_d$s and some DNs. CRY as important light mediating component is expressed in all LN$_v$s, three LN$_d$s and in about six DN1s. (aMe: accessory medulla; dpr: dorsal/superior protocerebrum (from Hermann-Luibl & Helfrich-Förster, 2015))

Besides the photic entrainment and the proper adjustment to LD-cycles, *Drosophila* is also able to synchronize the clock to time cues such as those provided by daily temperature cycles (T-cycles; Pittendrigh et al., 1958; Yoshii et al., 2005; Glaser PhD-thesis, 2006; Shafer et al., 2006; Goda et al., 2014). The clock of some insects, lizards and vertebrates including that of *Drosophila* is highly sensitive to temperature changes of 1-3 degrees Celsius (Underwood & Calaban, 1987; Francis & Coleman; 1988; Wheeler et al., 1993; Lahiri et al., 2005). In *Drosophila* these tiny temperature changes can be sensed either by certain clock neurons themselves e.g. the LPNs (Yoshii et al., 2005, 2009; Busza et al., 2007; Miyasako et al., 2007; Picot et al., 2009; Lee et al., 2013) or by the aristae and/or chordotonal organs of the antennae

(Sayeed & Benzer, 1996; Sehadova et al., 2009; Gallio et al., 2011). However, to entrain sufficiently to T-cycles is no general feature of all clock neurons, as the l-LN$_v$s are not able to entrain to changes in temperature (Miyasako et al., 2007; Yoshii et al., 2009). Also the prior concept that CRY$^-$ clock neurons are preferably entrained by temperature and CRY$^+$ neurons by light might be oversimplified. More likely is that both CRY$^+$ and CRY$^-$ neurons synergistically play a role in temperature entrainment, since it has been shown that the CRY$^+$ clock cells are sufficient for proper entrainment of the clock to T-cycles, even though also the CRY$^-$ can perform that task (Yoshii et al., 2010).

Apart from that an ionotropic glutamate receptor (IR25a) was shown to be important to entrain to low-amplitude T-cycles (Chen et al., 2015). Moreover the TRP family of ion channels constitutes further thermoreceptors in the fly (Montell, 2011), among which only two have been implicated in temperature entrainment (Das et al., 2016). Those are *pyrexia* (Wolfgang et al., 2013) and *Drosophila* TRPA1 (*dTrpA1*; Transient Receptor Potential Cation Channel A1). The latter one was demonstrated to be involved in temperature entrainment in 2013 by Lee and colleagues, where they were able to show that *dTrpA1* is expressed in a few brain cells, the subesophageal ganglion and eight cells in the thoracic ganglion (Hamada et al., 2008; Shih & Chiang, 2011). Amongst those the anterior cell (AC) neurons appear to be the main internal thermosensors, also being in charge of integrating temperature information from peripheral sensors (Tang et al., 2013). *dTrpA1* was shown to contribute to a normal activity pattern during temperature entrainment, apart its other functions in avoidance of noxious tastants (Kang et al., 2010; Kim et al., 2010), insect repellents (Kim et al., 2010), excessively bright light (Xiang et al., 2010) and uncomfortably warm und slightly suboptimal temperatures (Viswanath et al., 2003; Rosenzweig et al., 2005; Kwon et al., 2010; Neely et al., 2011; Shen et al., 2011; Kang et al., 2012; Zhong et al., 2012). However, it was demonstrated that *dTRPA1* does not appear to be a *direct* temperature sensor in the pacemaker neurons (except potentially in the LPNs), but might act indirectly on the clock, since the mutation of *trpA1* influenced the temperature-dependent cycling of PER expression in some pacemaker neurons. Roessingh et al. (2015) recently demonstrated that *dTrpA1* is not required for temperature synchronization in a broad temperature range (16°C: 25°C and 20°C: 29°C T-cycles), but instead is necessary to repress activity during the warm fraction of the day. In conflict to that, Green et al. (2015) showed that *dTrpA1* is

required for the additional afternoon activity peak, occurring at high temperatures (35°C) in seminatural conditions. But as *dTrpA1* expression is not required in clock or AC neurons to obtain that phenotype, the neurons in which *dTrpA1* has to be expressed are not known up to now. The data, which are available regarding the necessity and the function of *dTrpA1* in thermosensation and temperature synchronization of the clock appear quite inconsistent so far. However, all data support the notion that there might be no direct interaction or expression of *dTrpA1* with/within the clock, thus favoring *dTrpA1* playing a rather minor role in entraining the clock to T-cycles.

Besides specialized organs and particular neurons being in charge of sensing temperature cues, the core molecular clock itself is highly susceptible to perceive changes in temperature as well (Wheeler et al., 1993; Glaser & Stanewsky, 2005; Boothroyd et al., 2007; Busza et al., 2007; Currie et al., 2009; Sehadova et al., 2009). On the transcriptional level Boothroyd and colleagues (2007) were able to show that 3'-terminal introns of *per* and *tim* genes were spliced in a thermally sensitive manner, thus allowing the fly to adapt to changes in temperature by regulating the amount of available PER and TIM protein already on the transcriptional level. At low temperatures *per* splicing is increased (Majercak et al., 1999), thus leading to an earlier accumulation of *per* mRNA and protein, in turn causing an advance in molecular clock cycling. NorpA, a Phospholipase C (PLC), has been shown to be involved in that process (Glaser & Stanewsky, 2005; Glaser PhD-thesis, 2006).

Apart from entraining the clock, temperature is also able to shift the phase of the circadian clock when applied in short-term pulses. Thus it has been shown in wild type flies that both PER and TIM proteins are rapidly downregulated after short temperature pulses (TPs) resulting in phase delays, if the TP is administered during the early night (Sidote et al., 1998). However TPs in the late night did not cause phase advances, as it would have been the case for administering light pulses (LP) at this time.

All these experiments indicate the strong dependence upon the Zeitgeber type, intensity and duration as well as upon the clock's sensitivity to entrain to environmental cues. The ability of the clock to adjust and to entrain to Zeitgebers enables the investigator to understand the underlying mechanisms, like stimuli perception and procession, besides the determination of the valence of certain stimuli to the clock.

1.3 Phase Response Curves (PRC) as read-out of the phase shifting ability of the clock

“Understanding entrainment is equal understanding the circadian system” is one statement in “The Art of Entrainment” by Roenneberg et al. (2003), which perfectly illustrates the importance of decoding major features of entrainment to decode the mechanism of the circadian clock. Entrainment is most efficiently done by light, the main Zeitgeber of the clock, which is influencing the clock in several ways. To unravel the underlying mechanisms of light affecting the clock, two remarkable models by Aschoff and Pittendrigh tried to explain the effects of light on the clock already in the 1960s. Aschoff postulated in his model *parametric* (or continuous, tonic) effects of light on the clock’s *period* (τ), whereas Pittendrigh assumed *non-parametric* (or discrete, phasic) effects on the clock’s *phase* (Daan, 1977; Roenneberg, et al., 2003). The so-called Aschoff’s rule of parametric light effects in general assumed that the period length (τ) of the circadian oscillator of night-active (nocturnal) animals should be lengthened by increasing light intensity or photoperiods, as well as by constant light (LL), whereas a continuously shortened period should be exhibited by day-active (diurnal) animals in these conditions (Pittendrigh, 1960). In other words, τ is a function of light intensity in constant light conditions with a shortening of τ with increasing light intensity accompanied with an increase of activity levels at the same time (Aschoff, 1960). Although that assumption worked out well for a lot of animal species, it did not fit to all diurnal organisms, as e.g. *Drosophila* as crepuscular to diurnal animal gets immediately arrhythmic in LL (Konopka et al., 1989). In 1966 Colin Pittendrigh proposed another model, known as the *non-parametric* model of light. This model claims that light has an effect on the *phase* Φ of the circadian clock, thus enabling the clock to compensate for the deviation of τ (endogenous period of the oscillator) and T (external period of the Zeitgeber) in a daily manner ($\tau - T = \Delta\Phi$). If this model holds true, light administered at certain times of the day should influence the clock’s phase differently. To investigate this issue, classical light-phase response curve (PRC) experiments were performed, which actually do demonstrate and do proof the non-parametric impact of light on the clock.

PRCs are in general suitable tools to investigate the sensitivity of the clock to various stimuli. The classical PRC experiments, which are referred to above, allowed for instance the investigation of the impact of light upon the phase in locomotor activity

by application of light pulses (LP) at different times of the subjective day and night in constant darkness (DD). The magnitude of these changes in phase is plotted in a PRC with advances (positive values) or delays (negative values) on the ordinate and the circadian time of the stimulus (CT) plotted on the abscissa. *Per definitionem* the circadian time (CT) in DD corresponds to the Zeitgeber-time (ZT) in LD multiplied by 24/τ to obtain “circadian” hours (Johnson, 1990). Furthermore the circadian time 0 is defined as the beginning of the subjective day. Therefore a PRC is a plot of phase-shifts as a function of circadian phase of a stimulus, like pulses of light, temperature or pulses of drugs or chemicals. Chronobiologists basically distinguish two different types of PRCs: type 1 PRCs display rather small phase-shifts, usually not exceeding 6 hours with continuous transitions between delays and advances. Type 0 PRCs exhibit larger phase shifts with discontinuity, hence breakpoints of transitions between delay and advance phase-shifts (Winfree, 1970; Johnson, 1990). The terms “type 1” and “type 0” refer to the slope of the curve, when the shifts are plotted as a phase transition curve (PTC; plot of “new phase” vs. “old phase”) (Johnson, 1990). A type 1 PRC has an average slope of 1 (45° angle), when plotted as a PTC, whereas a type 0 PRC has an average slope of 0 (0° angle) (Johnson, 1990).

How difficult it is to actually understand entrainment to understand the clock, like the quote in the beginning of this chapter says, becomes even more apparent, considering that not all organisms have the similar ability to phase shift their clock ($\Delta\Phi=\tau-T$) (Johnson, 1990). There are species-specific as well as individual differences in the amplitude and shape that an animal’s PRC exhibits. Parameters like stimuli strength, intensity or duration as well as the nature of the stimulus *per se* can influence a PRC in amplitude and shape (Pittendrigh, 1981; Johnson, 1990). Thus the same organism can exhibit both types of phase resetting depending on the strength of the stimulus (Saunders, 1978), e.g. in light dose (*Culex*: 7.5min vs. 120min light pulses) or drug dosage (*Gonyaulax*: anisomycin 0.1 vs. 0.3 µM), hence converting type 1 to type 0 resetting upon increasing light intensity (Johnson, 1990).

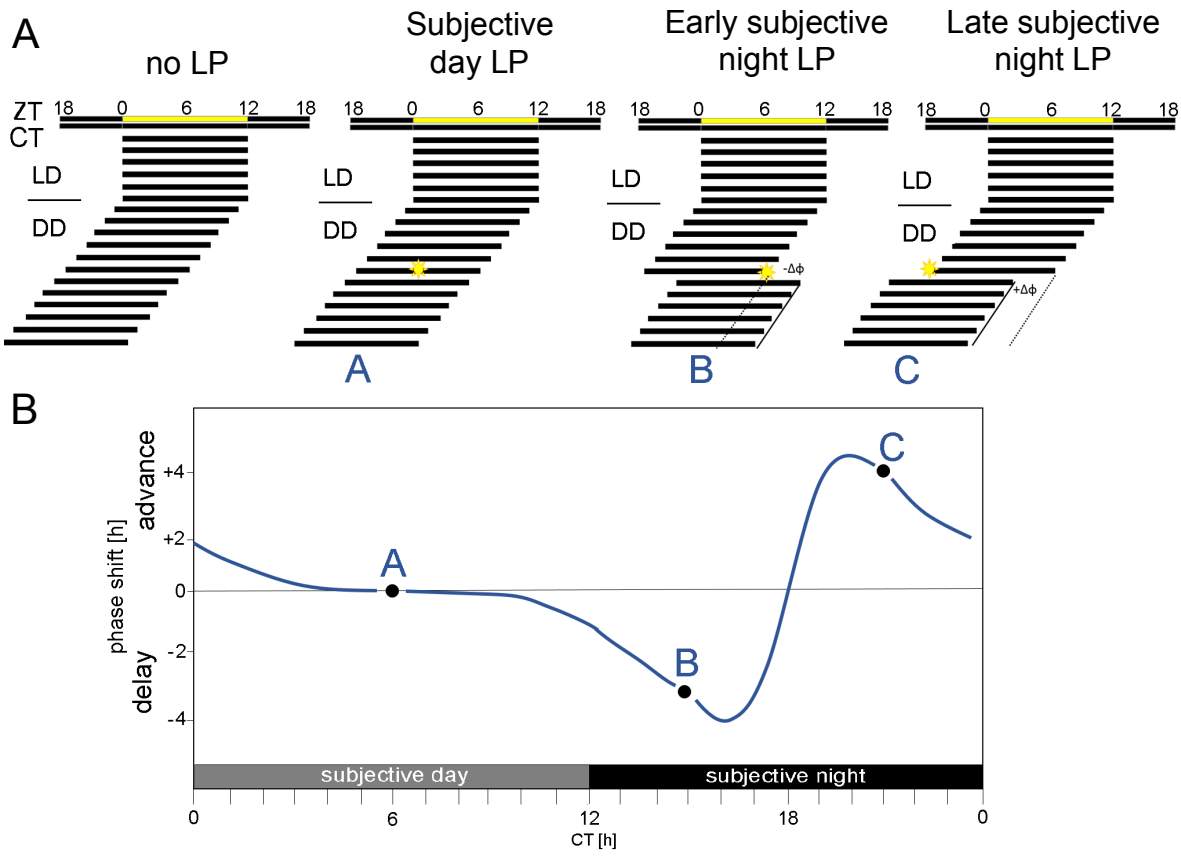

Figure 8 Scheme of a type 1 photic phase response curve
(A) Simplified actograms of a diurnal animal. The locomotor activity of the animal is indicated by the black bars. The black/yellow and black/black bars above the actograms indicate the light regime the animal is experiencing. First the animal is entrained to LD 12:12, followed by constant darkness (DD). The symbol of the sun represents the light pulse (LP), which is applied to the animal at different times of the subjective day (CT0-CT12) or night (CT12-CT24/0) in DD, after the animal was already in free-run (τ<24 hrs). **(B)** The response of the animal to the LP is plotted as an idealized PRC type 1, with delays (negative values) and advances (positive values), depending on the time the LP is administered. During the subjective day (CT0-CT12) nearly no or just little phase shifts occur. This part of the PRC is called the “dead zone” of the PRC (A). LPs applied during the end of the subjective day and the early subjective night, are delaying the clock (B), whereas LPs applied during the late subjective night advance the clock (C) (adapted from Golombek & Rosenstein, 2010).

By studying the behavioral output of the clock one can also see huge differences in the clock’s ability to respond to the same stimulus, which is highly dependent upon the time of day the stimulus is given. Representative light-pulse PRCs of circadian oscillators show typically delays in the early subjective night (CT12-CT15) and pronounced advances in the late subjective night (CT19-CT23), with little or no phase shifts during the subjective day (CT0-CT12) (Fig. 8) (e.g. Suri et al., 1998; Kistenpfennig et al., 2012). The portion of the PRC during the subjective day, where no phase shifts occur, is called the “dead zone” of the PRC (Fig. 8) (Johnson, 1990).

But what are the underlying molecular mechanisms that determine the shifting ability of the clock? During the subjective day (CT0-CT12) LPs do not shift the clock, since TIM levels are anyway relatively low (Fig. 3 & Fig. 8A). LPs applied at the beginning of the subjective night (CT12-CT15) cause modest to strong phase delays, as the TIM protein, which has accumulated in the cytoplasm is degraded and remains at lower levels much longer than it would have been without a LP. But *tim* mRNA levels are high at this point of the circadian cycle (Fig. 3), thus a re-accumulation of TIM protein is carried out rapidly. However the heterodimerization of PER and TIM is delayed as well as the nuclear entry of both proteins, resulting in a phase delaying effect on the clock. Earlier LP-induced TIM-degradation and hence phase advances occur, if LPs are applied during the end of the subjective night (CT18-CT23). At this particular time of the circadian cycle *tim* mRNA levels are low anyway (Fig. 3) and TIM/PER levels are highest. Hence LPs cause an earlier light-dependent TIM degradation and thus preponed *tim* and *per* transcription, advancing the phase of the clock. For the clock the LP at CT18-CT23 mimics an early, advanced dawn, whereas the LP at CT12-CT15 mimics a late, delayed dusk (Johnson, 1990; Golombek & Rosenstein, 2010; Hardin, 2011).

1.4 CREB (cAMP responsive element binding protein) - a putative link between the neuronal electrical state of clock neurons and the molecular clock

CREB (=cAMP responsive element binding protein) was identified 1987 as a transcription factor binding to CRE-sequences (=cAMP responsive element; Montminy & Bilezikjian, 1987) of particular genes, which had been shown to be involved in adaptive behavioral responses to various external stimuli, such as stress (Borsook et al., 1994; Tan et al., 1996), growth factor stimulation (reviewed by Segal & Greenberg, 1996), learning and memory (Bourtchuladze et al., 1994, Yin et al., 1994, 1995; Silva et al., 1998) and circadian rhythms (Ginty et al., 1993; Stehle et al., 1993; Foulkes et al., 1996). Along that line, the cAMP signal transduction pathway has been reported to be involved in light mediated resetting of the circadian clock in a variety of species, like in the sea slug *Aplysia californica* (Eskin & Takahashi, 1983), the fungus *Neurospora crassa* (Techel et al., 1990), as well as in mammals (Prosser & Gillette, 1989, 1991). In mammals it was demonstrated that light entrainment of the circadian clock requires CREB activity (Ding et al., 1997) and that a lengthening of

the circadian period can be obtained by CREB overexpression in the core circadian clock in the brain of the animal, the suprachiasmatic nucleus (SCN). Moreover CREB is well known to be part of the molecular response of the cells to increased electrical activity in the SCN in mammals, where it is phosphorylated and induces *mPer1* transcription after light input (Ginty et al., 1993; Obrietan et al., 1999). In homology to the mammalian CREB and its functions, in *Drosophila* two *creb* genes, *dCREB1* (or dCREBA) and *dCREB2* (most similar to the mammalian CREB/CREM) have been identified (Abel et al., 1992; Smolik et al., 1992; Usui et al., 1993; Yin et al., 1995). *Drosophila per* has been shown to exhibit three dCREB2 binding sites at position -3210, -2990 and -1335 (Belvin et al., 1999), which potentially allow CREB to directly interact with the molecular clock. The binding of dCREB to CRE-sequences in the *per* gene is assumed to promote *per* transcription like in mammals, as Mizrak et al. (2012) demonstrated that the overexpression of CREB in the fly's main pacemaker neurons, the LN_vs, is able to lengthen the free-running period of locomotor activity. In contrast, dCREB2 loss-of-function mutations diminished the oscillation of *per* transcripts and caused a shortening of the circadian locomotor rhythm to an average of 22.8 hours (Belvin et al., 1999). Moreover it was shown that the mutation of *dunce*, which encodes a cAMP-specific phosphodiesterase, caused increased phase delays after LP-application (Levine et al., 1994). All these pieces of experimental evidence indicate that CREB might link the clock neurons' electrical, environmental and network input to circadian gene expression and as such might gate neuronal responsiveness to membrane activity as a function of time, serving as an internal Zeitgeber (Mizrak et al., 2012).

1.5 Aim of the present PhD-Thesis

Rather than simply transmitting the molecular state of one clock neuron to another, the upcoming opinion is that neural activity reinforces molecular clock oscillation (Mizrak et al., 2012). This view is supported by experiments, in which electrically activating (depolarizing) or silencing (hyperpolarizing) of the LN_vs in *Drosophila* was shown to cause changes in clock protein cycling (Nitabach et al., 2002, 2006; Wu et al., 2008; Mizrak et al., 2012). Since it is neither certainly known how neuronal activation of particular clock neurons is processed within the clock network to cause phase shifts in behavior, nor how an artificial depolarization affects the clock on the molecular level, the question rises what the molecular mechanisms downstream of membrane depolarization might be. To address this, the present PhD-thesis was composed of three major aims:

The first aim of this thesis was to determine the contribution of different clock neuron clusters to the phase shifting ability at different time points of the circadian cycle using different clock specific *GAL4* lines. Furthermore it was to find out whether a temporal depolarization of specific clock neurons can generally mimic light input during the whole circadian cycle, like it was recently claimed by Guo et al. (2014). For this purpose phase response curve experiments were performed using the thermosensitive cation channel *dTrpA1* to depolarize clock neurons in a temporally and spatially restricted manner.

The second major topic delved into the question, what the consequences of artificial clock neuron depolarization on the molecular level might be. Given that changes in electrical activity caused by depolarization alters the output of the clock (behavior), the molecular state of the clock should be influenced, too. To address this issue immunocytochemical experiments were performed and quantitatively analyzed. Changes in PER cycling, as representative of the core clock should shed light on the molecular state after TP application and clock neuron depolarization.

The third aim of this work was to find out, what pathway and putative candidates might be in charge to serve as link between the alterations of the molecular state of the clock and cation influx, derived from clock neuron depolarization. The cAMP responsive element binding protein (CREB) seemed to be a quite feasible candidate to mediate information transfer between the electrical state of clock neurons and the transcriptional/translational program of the clock, as Mizrak and colleagues showed (2012) that dCREB2 levels are regulated by neuronal activity in the LN_vs and as

normal levels of dCREB2 were required for 24-hour rhythms. Along that line the last part of the thesis addressed the putative role of dCREB as mediator of neuronal excitation and the molecular clock by quantification of dCREB protein levels in the l-LN_vs.

2 Material & Methods

2.1 Material

2.1.1 Fly Strains

All fly strains that were used in this thesis are summed up in Table 1 and were kept for long-term maintenance and for development at 18°C ± 0.2°C with 60-65% of relative humidity. All flies were reared on standard cornmeal/agar medium (0.8% agar, 2.2% sugar-beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour, and 0.3% hydroxybenzoic acid) in a LD-cycle of 12:12.

Table 1 Fly strains used in the present thesis
stock collection: fly lines of the Department of Neurobiology & Genetics Würzburg;
BL: Bloomington *Drosophila* Stock Center, Indiana University, USA

Genotype	Source	Reference/Comments
Wild type/Balancer		
y w	stock collection	wild type
w^{1118}	stock collection	wild type
w; CyO/Sco; TM6B/MKRS	BL # 3703	double balancer
GAL4-lines		
y w; PdfGAL4; +	J.C. Hall	Renn et al., 1999
w; clk856GAL4; +	O.T. Shafer	Gummadova et al., 2009
w; mai179GAL4/CyO; +	F.Rouyer	Grima et al., 2004
w; clk4.1MGAL4; +	P. Emery	Zhang et al., 2010
y w; R6GAL4/y+CyO; +	stock collection	Helfrich-Förster et al., 2007
UAS-lines		
w; P{UAS-TrpA1(B).K}attP16	stock collection	Rosenzweig et al., 2005
w; P{UAS-TrpA1(B).K}attP2/ TM6B, Tb^{1}	BL #26264	P. Garrity
GAL80-line		
y w; PdfGAL80$_{96A}$	M. Rosbash	Stoleru et al., 2004
fluorescence-protein expressing fly line		
clk856epac	recombination by C. Luibl	Gummadova et al., 2009; Nikolaev et al., 2004

To obtain experimental lines virgins of *w*; *clk856GAL4*, *y w*; *PdfGAL4, w; mai179GAL4, y w; R6GAL4 or w; clk4.1MGAL4* were crossed to *w; UASdTrpA1* males. Furthermore as controls w^{1118} or *y w* virgin females were crossed to *UASdTrpA1* males and *clk856GAL4*, *PdfGAL4, mai179GAL4, R6GAL4* and *clk4.1MGAL4* virgins were crossed to w^{1118} males to obtain heterozygous control flies for behavioral experiments as well as for immunocytochemistry. To restrict *dTrpA1*

expression in *clk856/TrpA1* flies to the PDF$^-$ neurons another set of experiments was performed. *w*; *clk856GAL4;TM6B/MKRS* flies were combined with *w*; *CyO/Sco; UASTrpA1/TM6B,Tb* to obtain a genetically stable *w; clk856GAL4/CyO;UASTrpA1/TM6B* line. This fly line was then crossed to *y w; PdfGAL80$_{96A}$/CyO* for each experiment. Furthermore *w; UASdTrpA1/TM6B,Tb, w; clk856GAL4/CyO* and *w; PdfGAL80/ CyO* were crossed to *w^{1118}* to get heterozygous control flies. For all of the experiments only male flies of an age of 3 to 6 days were used.

2.1.2 Antibodies

Table 2 shows the list of all antibodies used in this thesis, including the used dilution and further information regarding their immunogen, donor animal, reference and source. For storage antibody stocks were kept at -20°C in 50% Glycerol. The working solution of the antibody was kept at 4°C in the fridge with 0.02% NaN_3, diluted in 0.5% PBT (recipe in "6 *Appendix"* chapter "6.1.2 *Additional Material"*) to the given concentration below.

Table 2 Antibodies used in this thesis
DSHB: Developmental Studies Hybridoma Bank at the University of Iowa, USA; Invitrogen/ThermoFisher Scientific Inc. Waltham MA, USA

Primary Antibody	Immunogen	Donor Animal	Dilution	Reference/Source
anti-PER	Baculovirus expressed full length *Drosophila* PER protein	rabbit, polyclonal	1: 2.000	Stanewsky et al., 1997/R. Stanewsky
anti-PDFc7	amidated *Drosophila* PDF peptide (NSELINSLLSLP KNMNDANH2)	mouse, monoclonal	1: 2.000	DSHB, J. Blau
anti-VRI	Histidin fused VRI (coding region) expressed in Sf9 insect cells	guinea pig, polyclonal	1: 3.000	Glossop et al., 2003 / P.Hardin
anti-CREB	gST-full length fusion protein	rabbit, monoclonal	1: 1.000	ThermoFisher scientific
Secondary Antibody	Immunogen	Dilution		Source
Alexa Fluor 488	goat anti-rabbit	1: 200		Molecular Probes (Invitrogen)
Alexa Fluor 555	goat anti-guinea pig	1: 200		Molecular Probes (Invitrogen)
Alexa Fluor 635	goat anti-mouse	1: 200		Molecular Probes (Invitrogen)
Alexa Fluor 647	goat anti-mouse	1: 200		Molecular Probes (Invitrogen)

2.2 Methods

2.2.1 The *GAL4/UAS* System

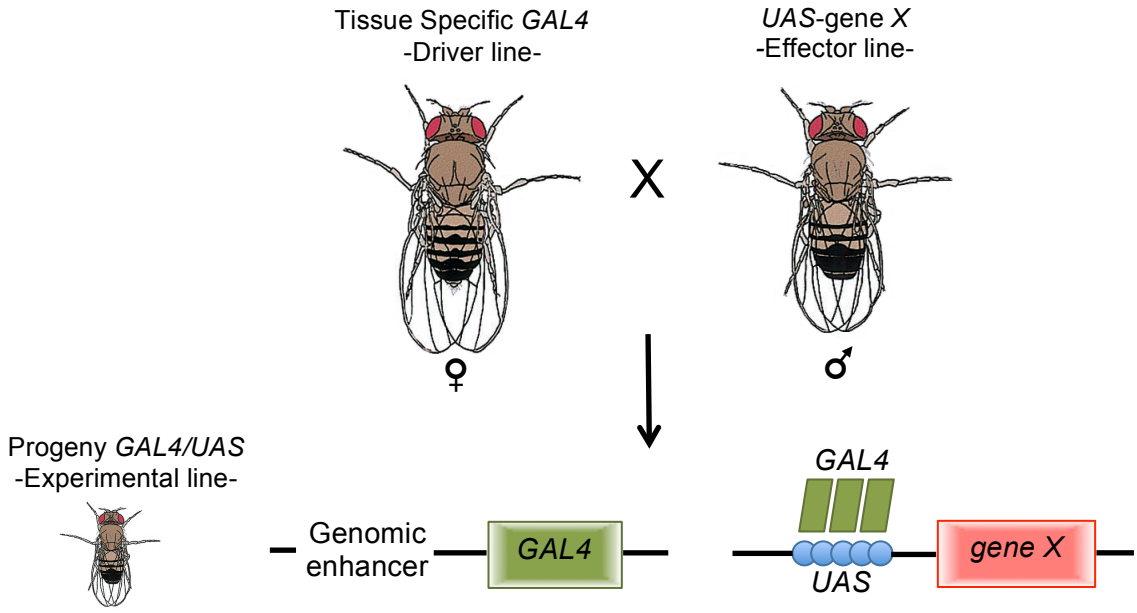

Figure 9 Schematic model of the binary *GAL4/UAS* system in *Drosophila melanogaster*
The *GAL4/UAS* system allows temporally and spatially restricted expression of genes of interest (gene *X*) in a tissue and cell-type specific manner (modified from St. Johnston, 2002 & from Duffy, 2002).

The binary *GAL4/UAS* system makes use of the yeast transcriptional activator *GAL4*, which allows tissue or cell-type specific and restricted gene expression of distinct genes (gene *X*) in *Drosophila melanogaster in vivo*. A huge collection of fly lines is available that express *GAL4* under the control of nearby genomic enhancers of genes of interest, offering a big variety of cell-type and tissue-specific driver lines (Brand & Perrimon, 1993), thus allowing to address almost any anatomical structure in the fly. The principle of this system includes two transgenic parental fly lines. One parental line serves as driver line, whereas the second parental line represents the effector line. The driver line expresses the tissue or cell-type specific *GAL4* construct, while the effector line contains the upstream-activating sequence (*UAS)* in a gene of interest *X,* which serves as the target sequence of the *GAL4* transcriptional activator. The progeny of the driver and effector line contain both, the *UAS* and the *GAL4* construct in their genome (Fig. 9). By binding of the tissue- or cell-type specific *GAL4* to the *UAS* of gene *X,* the transcription of gene *X* can exclusively take place in the cells and tissues covered by the *GAL4*, but not in any other cells or tissues

(Fischer et al., 1988). That enables the investigator to perform cell-type specific manipulations, for example by expression of *UASdTrpA1* in the nervous system neuronal activation, of *UASGFP* tissue- and cell-specific visualization via fluorescence and of *UASKir* tissue- and cell-specific silencing of cells (St. Johnston, 2002).

Another fine-tuning tool of the *Drosophila GAL4/UAS* system was introduced 1999 by Lee and Luo. They cloned *GAL80*, another transcriptional regulator of the yeast into the fly, thus enabling an even more spatially restricted expression pattern of the *UAS*-controlled gene. By binding to *GAL4, GAL80* represses the expression of *GAL4* exclusively in cells, where *GAL80* is present, hence preventing the expression of the gene of interest in *GAL4* expressing cells or tissues. Apart from permanently active *GAL80s,* also temperature sensitive *GAL80* constructs (*GAL80^{ts}*) are available that are active at lower (~18°C) and inactive at elevated (~32°C) temperatures. That enables the investigator to further restrict the expression of *GAL80* in a temporal manner to e.g. a specific developmental period by simply increasing or decreasing the temperature at a certain time. Whenever the temperature is increased, *GAL80* releases *GAL4*, which can bind to *UAS*, thus enabling the expression of the gene of interest in a specific subset of cells at a particular time *in vivo*.

Besides the possibility of choosing the appropriate temperature to inactivate thermosensitive *GAL80* constructs (*GAL80^{ts}*) in a temporal manner on purpose, *GAL4* constructs (and putatively also *PdfGAL80$_{96A}$*) are known to exhibit a certain temperature dependence, however to a much lower extent. The *Drosophila GAL4* has its minimal activity at 16°C, while 29°C provides a balance between maximal *GAL4* activity and marginal negative effects on fertility and viability at high temperatures (reviewed in Duffy, 2002).

2.2.2 The Thermogenetic Tool *dTrpA1*

The field of thermogenetics allows the modulation of neuronal activity in response to changes in ambient temperature using genetically encoded molecular sensitizers (Bernstein et al., 2012), which permit precise control of neuronal electrical activity in a temporal and spatial restricted manner via the *GAL4/UAS* system. The robustness and technical simplicity of *dTrpA1*-mediated neuronal activation with relatively limited investment in equipment and materials made this thermogenetic tool favored to investigate neuronal circuit properties by studying behavior (Bernstein et al., 2012).

These thermosensitive cation channels or “ThermoTRPs”, to whose family *dTrpA1* (Hamada et al., 2008) belongs, dramatically change their conductance by simply increasing temperature (Fig. 10) (Jordt et al., 2003; Dhaka et al., 2006). Besides, these “ThermoTRPs” exhibit an excellent thermal sensitivity and a highly robust activity, thus also relatively weak promoters can be used and TPs can be delivered to the freely moving and behaving fly by ambient warming (Hamada et al., 2008). That is advantageous as it is simple and non-invasive, hence predestinated for behavioral long-term recordings. Furthermore *dTrpA1* drives neuronal activation starting at ~25°C (Hamada et al., 2008; Pulver et al., 2009), which is in the fly’s preferred temperature range of ~24-27°C (Sayeed et al., 1996; Hamada et al., 2008; Bernstein et al., 2012), albeit it has been shown that in experimental approaches higher temperatures (25-31°C or higher) are necessary to elicit behavioral changes (Parisky et al., 2008; Krashes et al., 2009; Carrillo et al., 2010; von Philipsborn et al., 2011; Kohatsu et al., 2011).

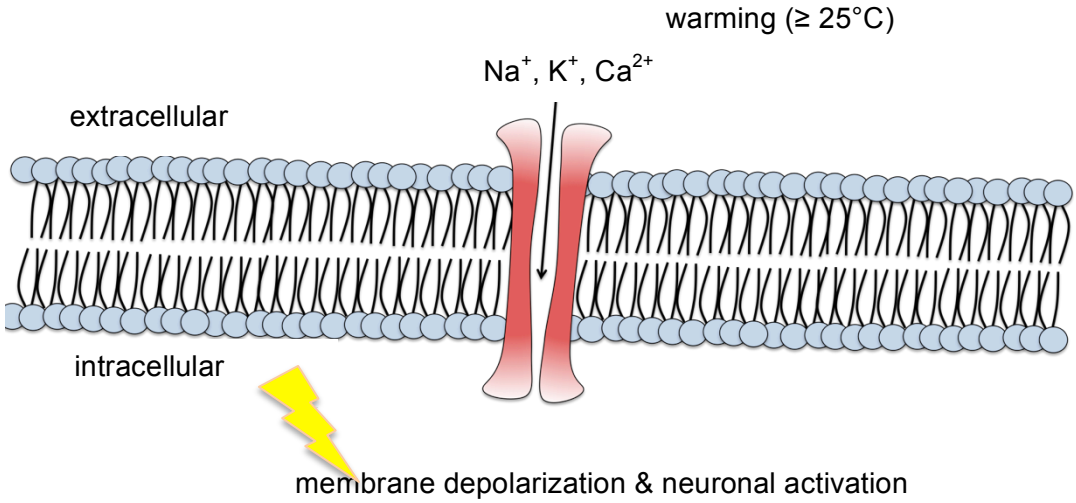

Figure 10 Schematic model of *dTrpA1* activation in *Drosophila melanogaster*
By increasing ambient temperature above 25°C, thermosensitive cation channels open in *dTrpA1*-expressing neurons and increase their conductance for Na^+, K^+ and Ca^{2+}, hence causing a membrane depolarization and activation of these specific neurons.

In the experimental approaches performed in this thesis, temperatures of 32°C were used to reliably activate *dTrpA1* in specific clock neuron clusters using the *GAL4/UAS* system.

2.2.3 Behavior

2.2.3.1 Recording the Locomotor Activity of Flies

Locomotor activity of individual male flies was recorded photo-electrically in 1-min intervals using the commercially available *Drosophila* Activity Monitoring (DAM) System (Trikinetics, Inc., Waltham, MA; USA), counting the infrared light-beam crossings of each fly per interval during the whole recording time. For each monitor 32 flies were transferred into recording tubes (65 mm x 5 mm), which were filled to one third with sugar-agar medium (2% agar, 4% sucrose) and were closed by an air-permeable polyurethane foam plug (Fig. 11A & 11B).

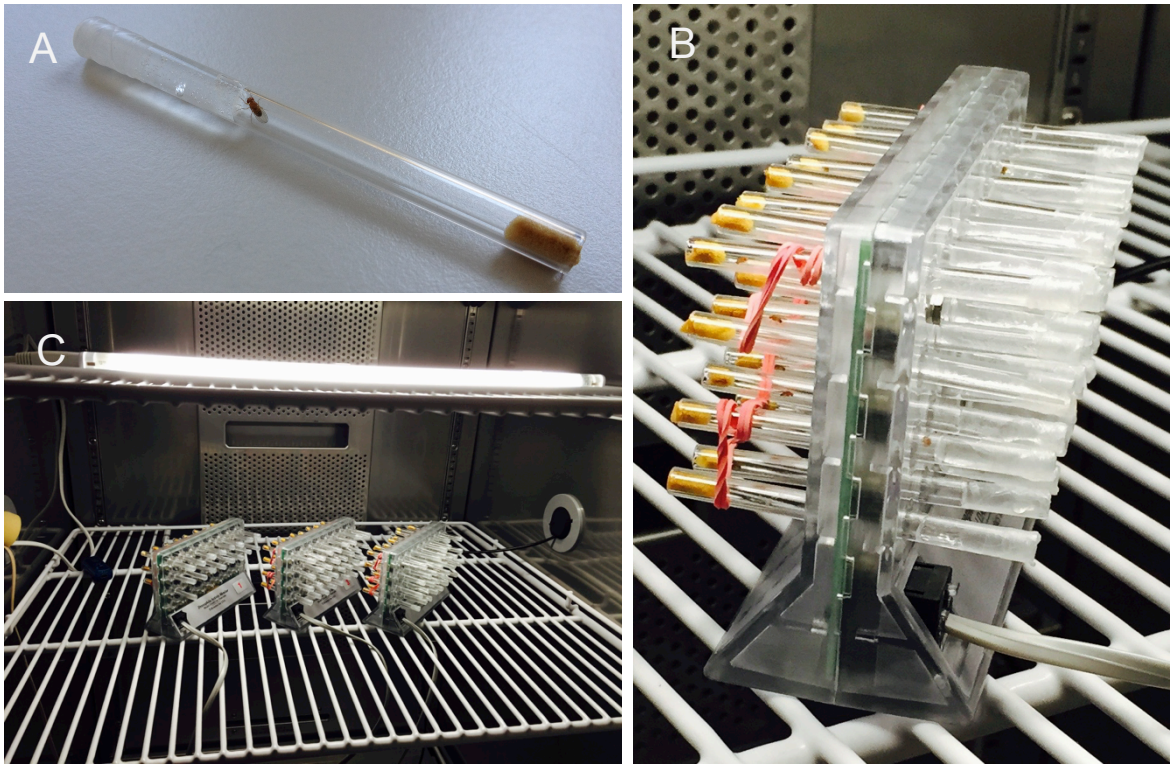

Figure 11 The *Drosophila* Activity Monitoring (DAM) System
(A) Flies were individually put in glass tubes with sugar-agar supply. **(B)** 32 individual male flies were placed into one monitor. By walking around, the fly interrupts the infrared light beam. The number of interruptions per minute is recorded for each single fly individually by a computer. **(C)** The activity recordings were performed in a temperature-controllable and cooling incubator with a light source, adjusted to about 500 lux.

The activity monitors were placed into a cooling incubator (MIR-553; Sanyo Electric Co., Ltd., Osaka, Japan) to perform the entrainment to LD 12:12 at 20°C ± 0.2°C for 5 days. Subsequently a TP of three hours of 32°C ± 0.2°C on the second day in constant darkness (DD2) was applied at different circadian times (CTs) of the subjective day (Fig. 11C). Illumination, derived from a white light source, was set to ~ 500 lux (Voltcraft MS-1300 digital luxmeter 0.1-50.000 lux). Experimental

genotypes as well as their respective controls were always recorded at the same time and in the same incubator to exclude artifacts and side effects.
The given CT in the plots indicates the time point one hour after the beginning of the three hour-TP, as it took one hour to reach 32°C inside the recording tubes, where the flies were. The temperature gradient was determined inside the tubes using the digital thermometer PeakTech® 5110 (with bead probe P TF-50).
To account for slightly different endogenous free-running periods of individual flies in DD the actual time point of TP application was determined for each single fly separately by hand.
CT 0 was defined as the beginning of the subjective day and CT 12 as the beginning of the subjective night. Thus, CT 1 to CT 24/0 is the duration of one endogenous cycle (endogenous period, τ). The CT of the TP was calculated by multiplying the real hour by 24 h/τ for each fly (Johnson, 1990). Correspondingly, the phase shifts were indicated as circadian hours (actual hours were multiplied by 24 h/τ).

2.2.3.2 Data Analysis

The raw data of individual flies were recorded as text files by the DAMSystem Software and were displayed as actograms using ActogramJ ((v0.9; Schmid et al., 2011) a plugin of Fiji; v2.0.0-rc-30/1.49s (Schindelin et al., 2012)). For quantification of the behavioral responses to TPs the phase of activity before and after the pulse was determined by the offset of the evening peak, as this peak was more reliable in constant conditions in comparison to the morning activity peak. The difference between the offset of the evening activity before and after the TP was measured by extrapolation of both phases by means of the Fiji tool „*Activity on- and offset*“. The phase shift of activity, which occurs when flies were released from LD in DD without any manipulation, was averaged and subtracted from the mean shift value for each genotype. Mean phase shifts (±SEM) were calculated out of the individual phase shifts of each single fly and genotype that experienced the TP at the same time.
Since the endogenous periods of the different fly lines were not significantly different and close to 24 hours, the mean phase shifts (±SEM) of all individual flies of each genotype were plotted against the time of TP application to enable the statistical comparison of the magnitude of the phase shift between different genotypes.
The calculation of endogenous periods (free-running rhythm τ) in DD of different genotypes was done by χ^2-periodogram analysis ($p<0.05$).

For better overview and comparison of the phase shifting data of different genotypes, advance and delay zone phase shifts were pooled and plotted as boxplots, providing apart from the median value also information about the distribution of the data. The advance zone data set includes data obtained from all flies of a genotype temperature-pulsed from CT21-CT24. The delay zone data set comprises data of all flies of a genotype temperature-pulsed from CT12-CT15.

2.2.3.3 Statistics

Phase shifts of temperature-pulsed experimental flies and respective controls were tested for a significant difference in magnitude of shifting by a Wilcoxon signed-rank test with holm correction, done in R (R 3.0.2 GUI 1.62 Snow Leopard build (6558), S. Urbanek, H.J. Bibiko, S.M. Iacus, © R Foundation for Statistical Computing, 2012). Values were considered to be significantly different at *p<0.05, highly significant at **p<0.01 and most significant at ***p<0.001. Significance levels are indicated by asterisks within all graphical charts.

2.2.4 Immunocytochemistry

2.2.4.1 Entrainment & Staining protocol

Only 3- to 6-day old males were used and entrained for at least 5 days to LD 12:12 at 500 lux (Voltcraft MS-1.300 digital luxmeter 0.1-50.000 lux) at 20°C and collected at the referred circadian times (CTs) in DD. Corresponding to the strongest effects in the behavioral phase response curve experiments TPs were applied in the delay zone (CT12-CT15) or the advance zone (CT24-CT3), respectively. Experimental flies and controls were fixed in freshly thawed 4% paraformaldehyde (PFA) in phosphate buffer with 0.1%TritonX-100 (PBT 0.1%, pH 7.4) at room temperature in the dark on an Eppendorf tube-inverter. After three hours of fixation, the flies were washed twice with phosphate buffer for 15 minutes each, followed by one washing step with phosphate buffer with 0.5% TritonX-100 (PBT 0.5%) for 15 minutes. The brains were dissected in PBT 0.5% and blocked in 5% normal goat serum (NGS) in PBT 0.5% at 4°C overnight.

PERIOD staining protocol

For PER-staining experiments the primary antibodies rabbit anti-PER (1:2.000) and mouse anti-PDFc7 (1:2.000) were incubated for 72 hours at 4°C. The brains were rinsed five times 10 minutes each with PBT 0.5%. The secondary fluorescence-

conjugated antibodies solutions including Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) (1:200) and Alexa Fluor® 635 Goat Anti-Mouse IgG (H+L) (1:200) were incubated for three hours at room temperature. Afterwards the brains were rinsed five times for 10 minutes in PBT 0.5%, once in PBT 0.1% and subsequently embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). All brains of one experiment were processed in exactly the same way during the whole staining protocol to enable comparison among the different genotypes.

CREB staining protocol

For performing the CREB-staining experiments the monoclonal primary rabbit anti-CREB antibody (1:1.000), mouse anti-PDFc7 (1:2.000) and the polyclonal guinea pig antiserum against the clock protein VRI (1:3.000) were used to counterstain and identify clock neurons. The brains were incubated for 72 hours and were washed five times à 10 minutes in PBT 0.5%. The secondary fluorescence-conjugated antibodies Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L), Alexa Fluor® 555 Goat Anti-Guinea Pig IgG (H+L) and Alexa Fluor® 647 Goat Anti-Mouse IgG (H+L) were diluted 1:200 in PBT 0.5% with 5% NGS and incubated for three hours. Afterwards the brains were rinsed five times for 10 minutes in PBT 0.5%, once in PBT 0.1% and subsequently embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). All brains of one experiment were processed in exactly the same way during the whole staining protocol to allow comparison among the different genotypes.

2.2.4.2 Microscopy & Image Analysis

PERIOD Image Acquisition & Quantification

For PER quantification brains were analyzed by laser confocal microscopy using a Leica TCS SPE confocal microscope (Leica microsystems, Wetzlar, Germany). Single confocal stacks of 2 µm thickness were taken with 400 Hz using the 488 nm laser to visualize PER (Alexa Fluor®488) and the 635 nm laser to visualize PDF (Alexa Fluor®635). All brains were processed similarly and the same settings were kept for all of the scans, time points and genotypes. PER staining intensity was calculated in grayscale units, which ranged between 0 (black; no staining) and 255 (white; saturated staining) using the intensity-measuring tool in Fiji in 8-10 hemispheres per time point and genotype. Mean PER values of different cell groups were calculated in a square-shaped area of 3x3 pixels in the brightest focal plane. The background staining was measured similarly. After background correction mean

PER intensity values (±SEM) were plotted against the time point of collection for each neuronal subgroup and genotype.

CREB Image Acquisition & Quantification

For CREB quantification confocal images were obtained using a Leica SP8 confocal microscope (Leica microsystems, Wetzlar, Germany). For CREB intensity quantification all samples were processed in the same way and all laser settings were kept unchanged for the whole experiment. Confocal stacks of 2 µm thickness were taken with 400 Hz. For CREB visualization the 488 nm (Alexa Fluor®488), for PDF the 647 nm (Alexa Fluor®647) and for VRI the 555 nm (Alexa Fluor®555) lasers were used. CREB staining intensities of Z-stacks were quantified in grayscale units, which ranged between 0 (black; no staining) and 255 (white; saturated staining) in Fiji using the „*Freehand selections"*-tool. Regions of interest (ROIs) were defined by revolving the PDF^+ large ventral lateral neurons (l-LN_vs) using the PDF-immunoreactivity signal. The mean intensity (±SEM) of the ROIs was averaged, background corrected and plotted against the time point of collection.

2.2.4.3 Statistics

The mean values of PER- and CREB-staining intensity in the different clock cell clusters of different time points, treatments and genotypes were statistically compared using the Wilcoxon rank-sum test in R with holm correction (R 3.0.2 GUI 1.62 Snow Leopard build (6558), S. Urbanek, H.J. Bibiko, S.M. Iacus, © R Foundation for Statistical Computing, 2012).

2.2.5 Whole brain-culturing

2.2.5.1 Petri Dish Preparation

One day in advance of brain dissection and culturing, sterile glass bottom petri dishes (35 mm in diameter) were prepared with black adhesive tape, exhibiting a hole in the middle, where the brains were placed in the day after. To sterilize the petri dishes properly, the dishes were incubated in 70% EtOH overnight in a sterile cell culture bench. Just shortly before the brains were dissected, the prepared petri dishes were treated with UV-irradiation for at least 30 minutes under the laminar flow of a sterile cell culture bench.

2.2.5.2 Sterile Dissection & Explant Treatment

In general, cell culture specific good laboratory practice was strictly adhered to. In advance of dissection the working space for dissection was prepared and sterilized. Three dissection dishes were prepared: one containing 96% EtOH for sterilizing the flies and two filled with sterile filtered Ca^{2+}-free Ringers solution for dissection and collection of dissected brains. The latter one was covered and placed on ice. Three to four day old adult flies were anesthetized on ice and shortly sterilized in 96% EtOH before they were dissected. The dissection time was kept as short as possible. Dissected brains were then transferred to the collection dish on ice.

2.2.5.3 Embedding & Monitoring of the Viability of the brains

After dissection, a single droplet (about 50 µl) of 0.5% agarose (in Ca^{2+}-free Ringers) was pipetted into the hole of the adhesive tape, which was placed on the glass bottom of the dish and sterilized in advance (see chapter "2.2.5.1 *Petri Dish Preparation"* in "2 *Material & Methods*"). The brains were quickly placed on the agarose-droplet with the antennal lobes up and were softly dipped into the agarose. 2 ml insect cell culture medium was added. From day 2 *in vitro* onwards half of the medium was carefully refreshed every second day. The viability of the brains was monitored by visualizing *epac* fluorescence using a fluorescence or confocal scanning microscope. The explants were considered to be alive whenever all clock neuron clusters displayed strong fluorescence at low laser intensities with normal features of cell labeling. Recombined *clk856epac* flies (recombination done by C. Luibl) were predestinated for the establishment of the method as *epac* is stably and strongly expressed in this fly line, thus no fly crossings were required. Furthermore the *clk856*-promoter, controlling the expression of the *epac* sensor, drives expression in all of the clock neurons (Gummadova et al., 2009; Nikolaev et al., 2004), enabling the investigator to check the viability of the brains via the maintenance of the fluorescence in all clock neurons. And last, the classical *epac* can easily be excited by using excitation wavelengths around 420 nm (Salonikidis et al., 2008).

3 Results

3.1 Investigation of clock network properties by means of phase shift experiments

3.1.1 The impact of temperature pulses in *Drosophila melanogaster's* light PRC´s advance and delay zone

To investigate the influence of TPs and timed neuronal activation via *dTrpA1*, control flies and several clock neuron specific *GAL4* lines were used. As latter ones include different clock cell clusters (Fig. 12; for anti-GFP & anti-PER staining see chapter "6.1.1 *Supplementary Data*" in "6 *Appendix"*), it was possible to exclusively address various subsets. To those flies, TPs were applied at certain times of the circadian cycle in DD.

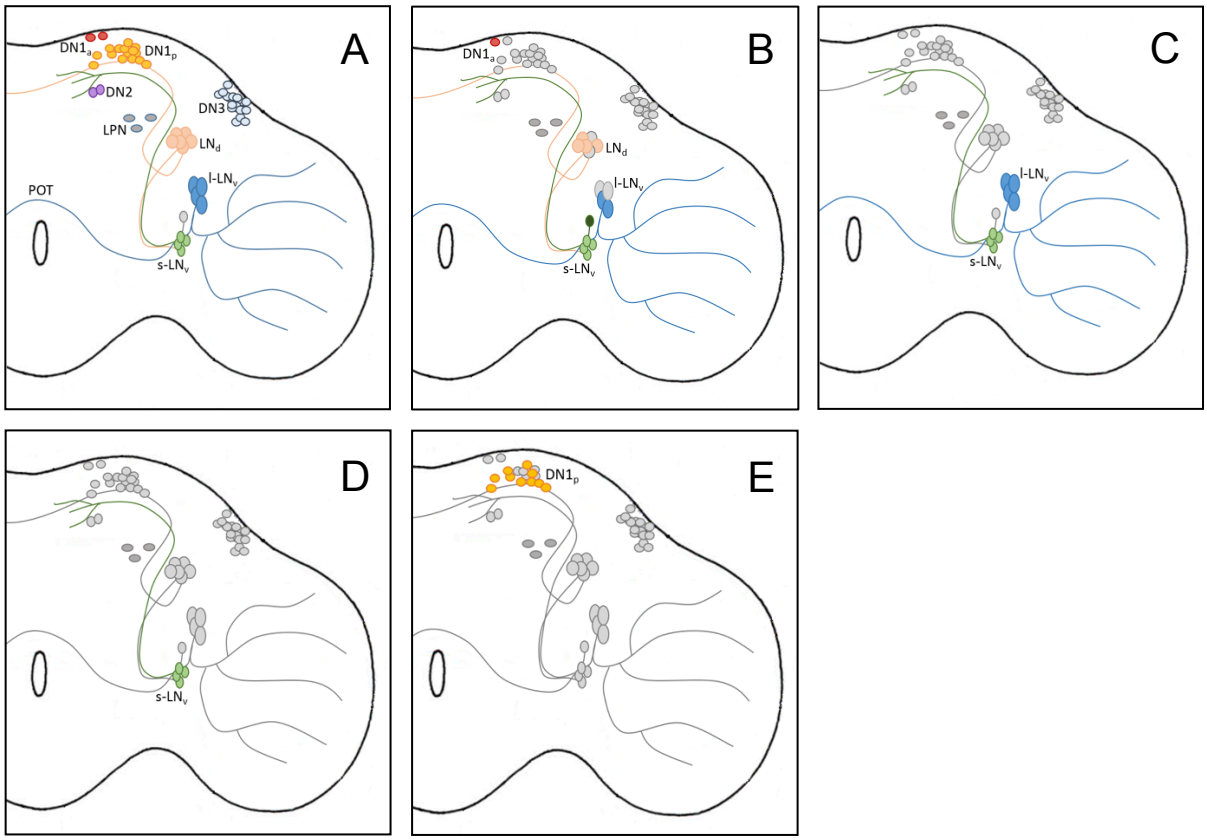

Figure 12 Schematic overview of the expression pattern of the different *GAL4* lines
(A) *clk856GAL4* expresses in all clock neurons, including the DN1s, DN2s, DN3s, LPNs, LN_ds, s-LN_vs and l-LN_vs (Gummadova et al., 2009). **(B)** *mai179GAL4* expresses in one $DN1_a$ neuron, three-four LN_ds (including three CRY^+ LN_ds), all s-LN_vs and some l-LN_vs per brain hemisphere (Grima et al., 2004). **(C)** *PdfGAL4* expresses in four s-LN_vs and the l-LN_vs, but not in the 5th s-LN_v (Renn et al., 1999). **(D)** *R6GAL4* expresses in four PDF^+ s-LN_vs per brain hemisphere (Helfrich-Förster et al., 2007). **(E)** *clk4.1MGAL4* expresses in 8-10 $DN1_p$ neurons per hemisphere (Zhang et al., 2010). (For anti-GFP and anti-PER staining of driver lines see chapter "6.1.1 *Supplementary Data"* in "6 *Appendix").*

It is known from light-PRC experiments that the clock is particularly sensitive to stimuli during the early night around CT 15 (referred to as *delay* zone) and the late night around CT 21 (referred to as *advance* zone) (reviewed in Golombek & Rosenstein, 2010). Furthermore Guo et al. (2014) reported recently that TPs administered at CT 15 and CT 21 to *Pdf/TrpA1* flies cause effects that mimic behavioral phase shifts upon standard LPs applied at these time points of the circadian cycle. Thus TPs were applied to experimental flies (*clk856/TrpA1, mai179/TrpA1, Pdf/TrpA1, R6/TrpA1 & clk4.1M/TrpA1*) and respective controls around CT 15 and CT 21. Representative double plotted actograms of flies temperature pulsed in the delay zone are shown in Fig. 13.

Flies, which express *dTrpA1* in all of the clock neurons (*clk856/TrpA1*) showed a strong delay when the TP was applied from CT12-CT15 in comparison to respective controls. Controls just experienced the TP, but did not express *dTrpA1* and thus were not depolarized (Fig. 13A, column 1 "*Experimental line*" in comparison to controls Fig. 13A second & third column "*GAL4/+", "+/UAS"). mai179/TrpA1* flies, which express *dTrpA1* in most of the lateral neurons and in a few dorsal neurons did not significantly delay the phase of locomotor activity in the delay zone (Fig. 13B). Depolarizing exclusively the PDF^+ s-LN_v neurons (*R6/TrpA1*) (Fig. 13D) or some of the $DN1_p$s (*clk4.1M/TrpA1*) (Fig. 13E) did also not significantly alter the phase of activity compared to just temperature-pulsed controls. The depolarization of the PDF^+ s-LN_vs and l-LN_vs together, however, caused weak delays (Fig. 13C & Fig. 19).

Thus just the neuronal activation of all clock neurons was able to strongly shift the phase of locomotor behavior in the delay zone. However, the data indicates that the TP *per se* has an impact on the clock as well, since controls, which did not experience a depolarization, did shift their phase in response to temperature (Fig. 13 second & third column), even though those phase shifts were much weaker.

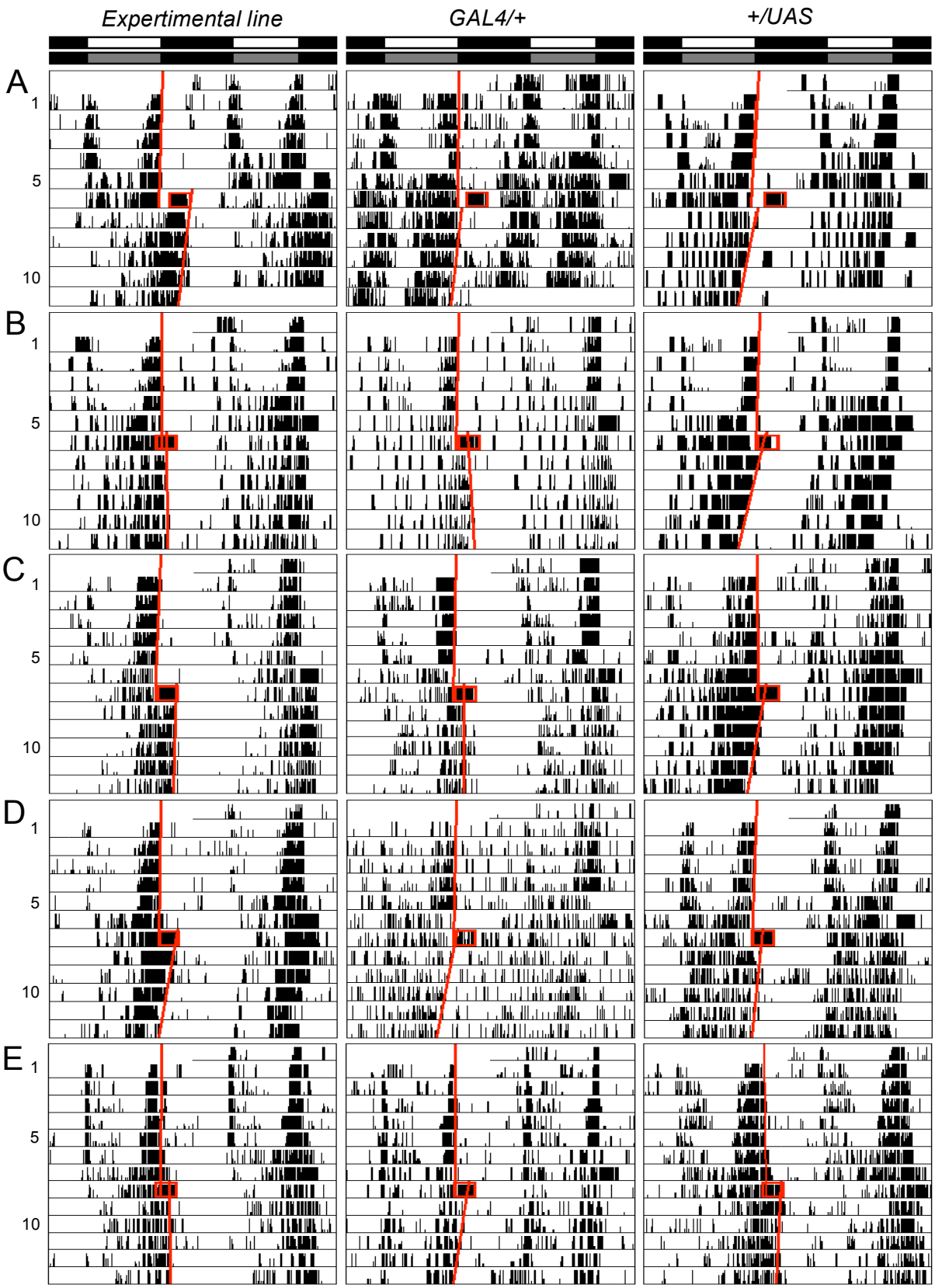

Figure 13 Representative double plotted *delay* zone actograms of (A) *clk856/TrpA1*, (B) *mai179/TrpA1,* (C) *Pdf/TrpA1,* (D) *R6/TrpA1, (E) clk4.1M/TrpA1* and respective controls
(A) TPs in the delay zone (CT12-CT15) strongly shifted the phase of locomotor activity in *clk856/TrpA1* (first column), **(C)** but just modestly in *Pdf/TrpA1* flies compared to controls (second & third column of **A/C**). **(B, D, E)** The depolarization of other subsets of clock neurons did not elicit significant delays. First column: *experimental lines*; second/third column: *GAL4- & UAS-controls*. Black/white bars indicate LD 12:12, followed by DD (black/gray bars). Red boxes indicate the TP on day two in DD. Red lines show the extrapolation of the offset of activity before and after the TP.

In the advance zone (CT21-CT24) the depolarization of all clock neurons (*clk856/TrpA1*) or of M cells, some l-LN_vs and some E cells (*mai179/TrpA1*) modestly shifted the phase of locomotor behavior in comparison to controls (Fig. 14A & Fig. 14B). Remarkably, the exclusive depolarization of PDF^+ s- and l-LN_v neurons in *Pdf/TrpA1* or of PDF^+ s-LN_vs in *R6/TrpA1* flies caused highly significant phase advances (Fig. 14C & Fig. 14D). However, the activation of some DN1s, representing some of the dorsally located downstream targets of the PDF^+ neurons, did not affect the phase of locomotor activity, as there was no significant difference between *clk4.1M/TrpA1* and respective controls (Fig. 14E).

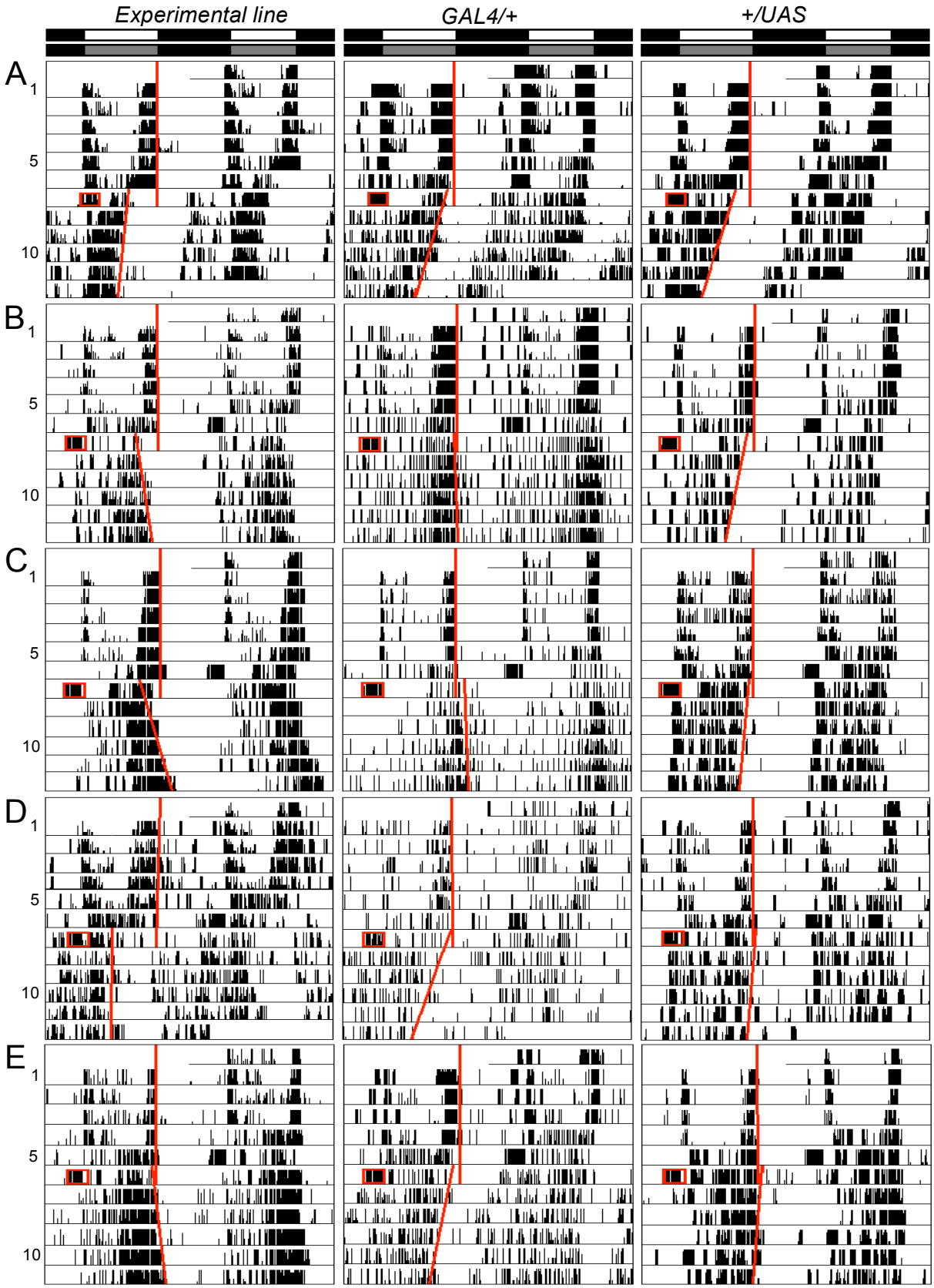

Figure 14 Representative double plotted *advance* zone actograms of (A) *clk856/TrpA1*, (B) *mai179/TrpA1,* (C) *Pdf/TrpA1,* (D) *R6/TrpA1, (E) clk4.1M/TrpA1* and respective controls
TPs in the advance zone (CT21-24) shifted the phase of locomotor activity in *clk856/TrpA1* **(A),** *mai179/TrpA1* **(B)**, *Pdf/TrpA1* **(C)** and *R6/TprA1* flies **(D)** (first column of **A-D**), but not of *clk4.1M/TrpA1* flies **(E)** compared to controls (second & third column of each row).
First column: *experimental lines*; second/third column: *GAL4- & UAS-controls*. Black/white bars indicate LD 12:12, followed by DD (black/gray bars). Red boxes indicate the TP on day two in DD. Red lines show the extrapolation of the offset of activity before and after the TP.

Pooled phase shift data for delay (CT12-CT15) and advance zone depolarizations (CT21-CT24) are summarized in Fig. 15. TPs applied in the delay zone cause strong phase delays, when all clock neurons are addressed (*clk856/TrpA1*), while the activation of certain subsets of clock neurons does not sufficiently shift the activity in that particular time window (Fig. 15A).

When the TP was applied in the advance zone (CT21-CT24) the depolarization of all clock neurons (*clk856/TrpA1*) and the activation of the M cells, some l-LN$_v$s and some of the E cells (*mai179/TrpA1*) phase advanced the clock modestly. The depolarization of the PDF$^+$ neurons (*Pdf/TrpA1)* or of M cells (*R6/TrpA1)* in the advance zone elicited the strongest responses. However, the depolarization of the dorsally located downstream targets of the PDF$^+$ neurons, of the DN1s, did not significantly alter the phase of locomotor activity in comparison to respective controls (*clk4.1M/TrpA1*) (see chapter "6.1.1 *Supplementary Data"* in "6 *Appendix").*

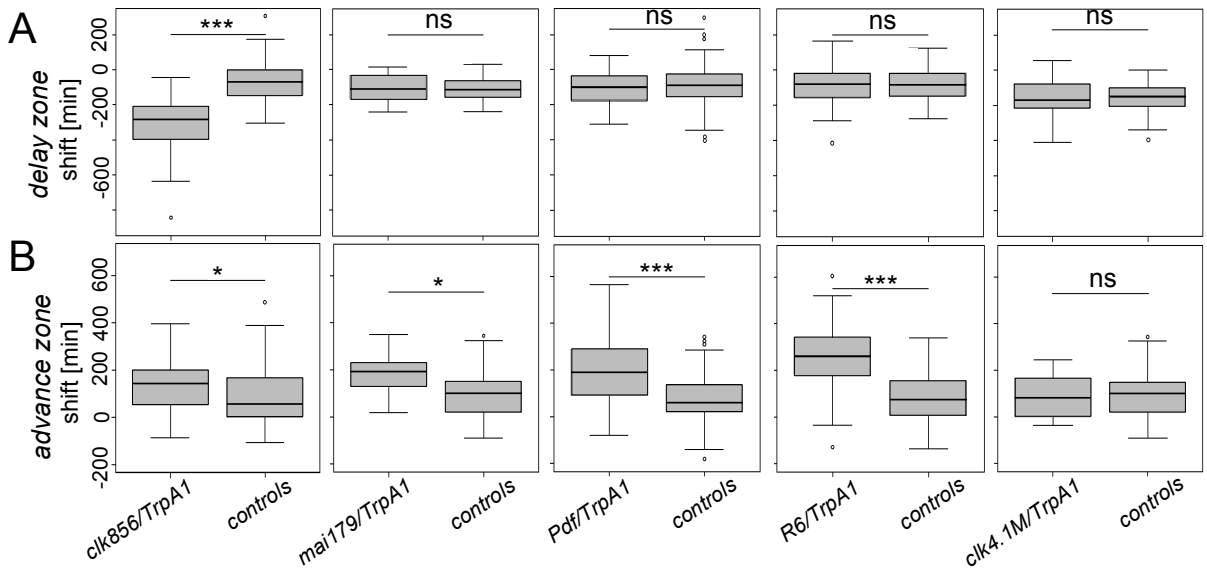

Figure 15 Phase shifting ability of *clk856/TrpA1*, *mai179/TrpA1*, *Pdf/TrpA1, R6/TrpA1* and *clk4.1M/TrpA1* flies with respective controls after TPs in (A) the delay zone (CT12-CT15) and (B) the advance zone (CT21-CT24)

(A) TPs administered in the delay zone (CT12-CT15) mainly shifted the phase of *clk856/TrpA1* flies, but not of any other genotype. **(B)** TPs applied in the advance zone (CT21-CT24) strongly shifted the phase of locomotor activity in *Pdf/TrpA1* and *R6/TrpA1* flies. Weaker but significant advances were also exhibited by *clk856/TrpA1* and *mai179/TrpA1* flies relative to controls. *clk4.1M/TrpA1* flies showed no difference from controls neither in the delay nor in the advance zone. Boxplots show the median, the first and third quartile of the dataset. Whiskers indicate maximum/minimum and open circles outliers of the data set. Asterisks indicate significant differences (*p<0.05; **p<0.01; ***p<0.001; ns = no significant difference; Wilcoxon rank sum test). "*controls"* include pooled phase shift data of respective *GAL4/+* and *+/UAS* controls.

The phase shifts in locomotor behavior of control flies, which do not express *dTrpA1* and are thus not neuronally activated, suggest that the clock is highly susceptible to changes in ambient temperature, underlining the importance of temperature as a potent Zeitgeber. Temperature changes *per se* can sufficiently contribute to the phase adjustment of the fly's circadian clock, what might play a rather minor role in LD, since light is the most powerful Zeitgeber, but might get apparent in DD.

The artificial depolarization of different clock neuron clusters provided insights into the complex phase adjustment ability of the clock. By depolarizing exclusively the PDF^+ neurons or just the M cells the clock severely advances its phase, whereas an ubiquitous clock neuron depolarization enables the clock to mainly delay its phase, even though there are modest advances as well. This indicates that the degree and direction of phase shifting is highly dependent upon the type of clock neuron cluster(s), which is/are activated as well as upon the time of the circadian cycle the TPs are applied.

3.1.2 Investigation of depolarization-dependent phase shifting of the clock during one entire circadian cycle

The results of advance and delay zone phase shift-experiments (see "3 *Results*", chapter "*3.1.1 The impact of temperature pulses in Drosophila melanogaster's light PRC´s advance and delay zone"*) already gave an impression of the most important clock neuron clusters being involved in phase shifting during the different time windows. However, it remained unclear whether the data of TP-phase shifting of the missing time points would mimic the data one would obtain by photic stimuli phase shifting, as it was assumed by Guo et al. (2014). To address this possibility, TPs were administered during the whole circadian cycle in DD to obtain an entire phase response curve. We focused on *clk856/TrpA1*, thus on the depolarization of all clock neurons, and *Pdf/TrpA1*, hence on the depolarization of the PDF^+ LN_vs, as these two genotypes gave us strong phenotypes in prior experiments and as they cover the most important clock neuron groups (Fig. 13, Fig. 14, Fig. 15).

The whole time course of *clk856/TrpA1* flies' phase-shifting pattern is shown in Fig. 16A. The depolarization of all clock neurons mainly causes phase delays in the beginning of the subjective night. TPs at the end of the subjective night until the

beginning of the subjective day cause modest phase advances. Both controls (*GAL4/+, +/UAS*) show temperature dependent alterations in phase (Fig. 16B & 16C), even though their shifts exhibited much lower amplitudes than those of flies that experienced neuronal activation (*clk856/TrpA1*; Fig. 16A). That effect gets apparent in the PRC (Fig. 17), in which mean phase shift values (±SEM) of *clk856/TrpA1* and both controls are plotted against the circadian time (CT).

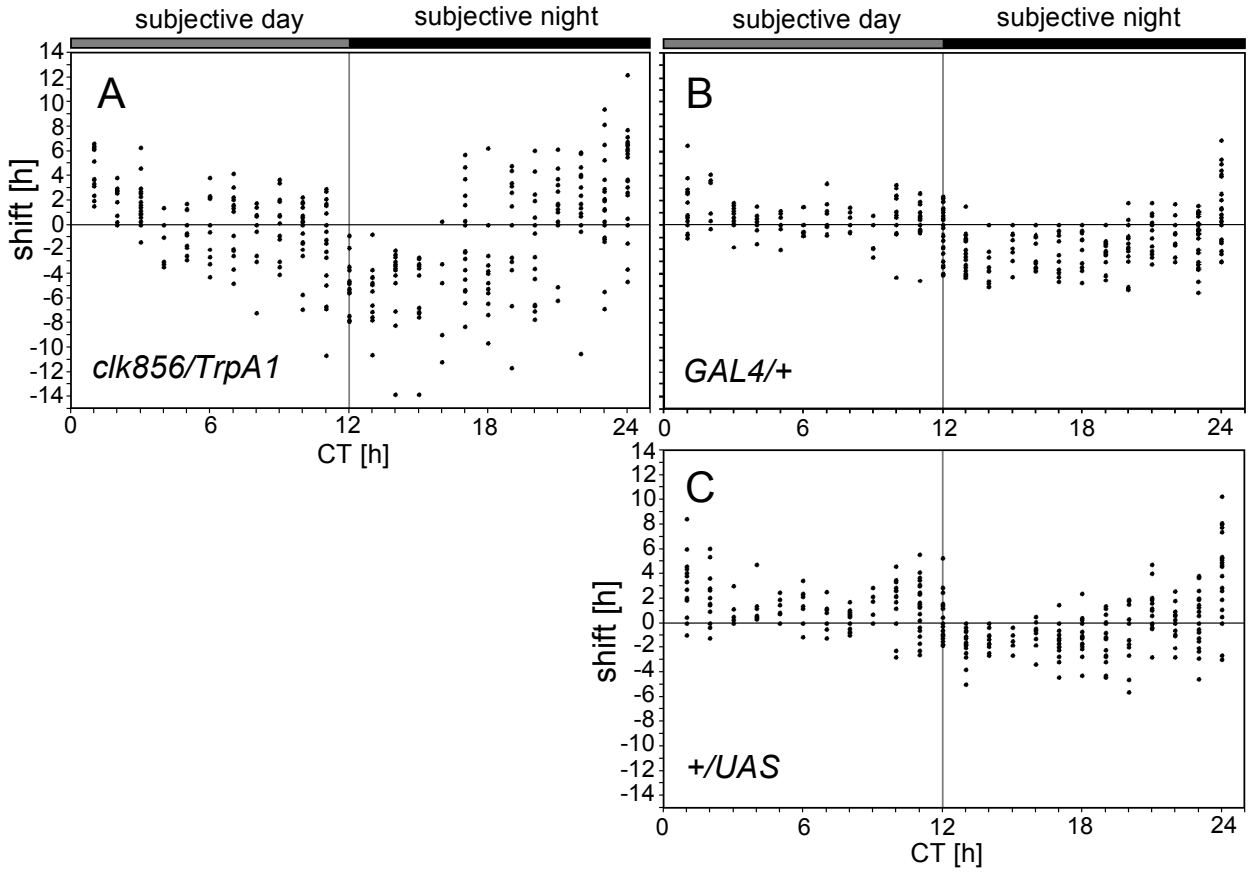

Figure 16 Phase response curves of *clk856/TrpA1* (A) and respective controls (B, C)
(A) TPs administered in the delay zone (CT12-CT15) caused strong delays in *clk856/TrpA1* flies. However these flies exhibited phase advances upon depolarization as well. **(B, C)** TPs applied to both controls, which solely experienced the TP, also shifted the phase of locomotor activity, however, to a much lower extent than *clk856/TrpA1* flies that additionally experienced a depolarization. Each single dot indicates one fly. Gray and black bars indicate subjective day and subjective night in DD. Advances are shown as positive values, whereas delays are shown as negative values. The abscissa indicates the time point, at which the TP reached 32°C in circadian time (CT).

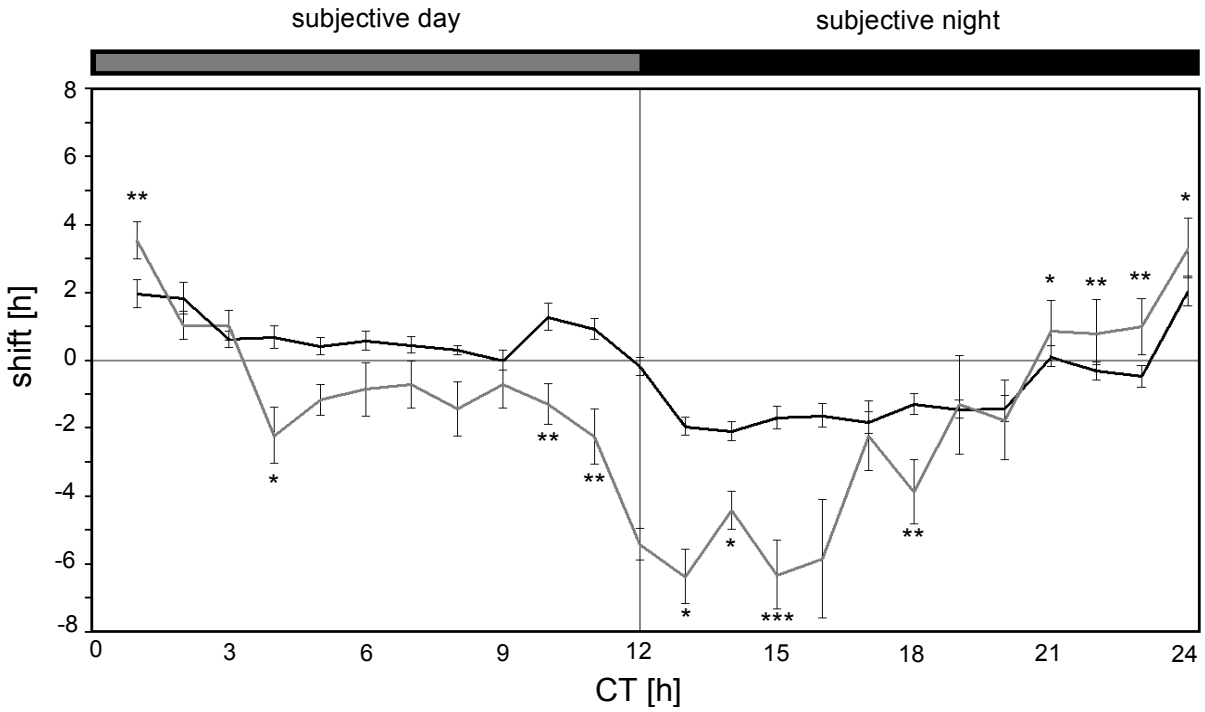

Figure 17 Phase response curves of *clk856/TrpA1* and the mean of respective controls
TPs administered in the delay zone (CT12-CT15) cause strong delays in *clk856/TrpA1* flies (gray line). However, TPs in the advance zone elicited phase advances upon depolarization as well. Both controls (black line), which solely experienced the TP, also shifted the phase of locomotor activity, however with a much lower amplitude than depolarized *clk856/TrpA1* flies. Mean (±SEM), asterisks indicate significant differences (*p<0.05, **p<0.01, ***p<0.001; Wilcoxon rank sum test).

The simultaneous depolarization of all clock neurons is able to shift the phase of locomotor activity in both directions, thus allowing the clock to phase advance and to phase delay. The ability to delay in comparison to advance the phase, is slightly asymmetric with bigger delays than advances, indicating that the simultaneous depolarization of all clock neurons might primarily decelerate the speed of the molecular clock.

Control flies shifted the phase of activity in response to TPs, however to a much lower extent. The shape of the PRC of the controls resembles a flat light-PRC, as there is a pronounced dead zone during the subjective day, as well as an advance and a delay zone during the particular fractions of the subjective night. Considering that the amplitude of a PRC represents the impact of a Zeitgeber on the clock, the rather low amplitude of the temperature-mediated PRC of controls indicates a rather weak influence of temperature as a Zeitgeber. Furthermore, as we obtained phase delays and advances in the circadian cycle, where these would have been expected for a typical light-PRC, the depolarization-mediated PRC resembles the shape of a type 1 light-PRC.

To further gain insight into the circadian clock's phase shifting properties, the same experiment was performed using *Pdf/TrpA1* flies, as the PDF$^+$ neurons were demonstrated to be important for phase shifts (Guo et al., 2014) and as the s-LN$_v$s are known to be the main pacemakers of the clock in DD. The activation of the PDF$^+$ neurons caused highly significant phase advances and modest phase delays. Remarkably, advance zone TPs do not solely affect the height of the phase shift amplitude, but also cause alterations in the shape of the PRC, as the PRC exhibits a very pronounced advance zone. The advance zone covers about one half of the subjective day, holding the strongest shifts around CT 3 (Fig. 18 & Fig. 19).

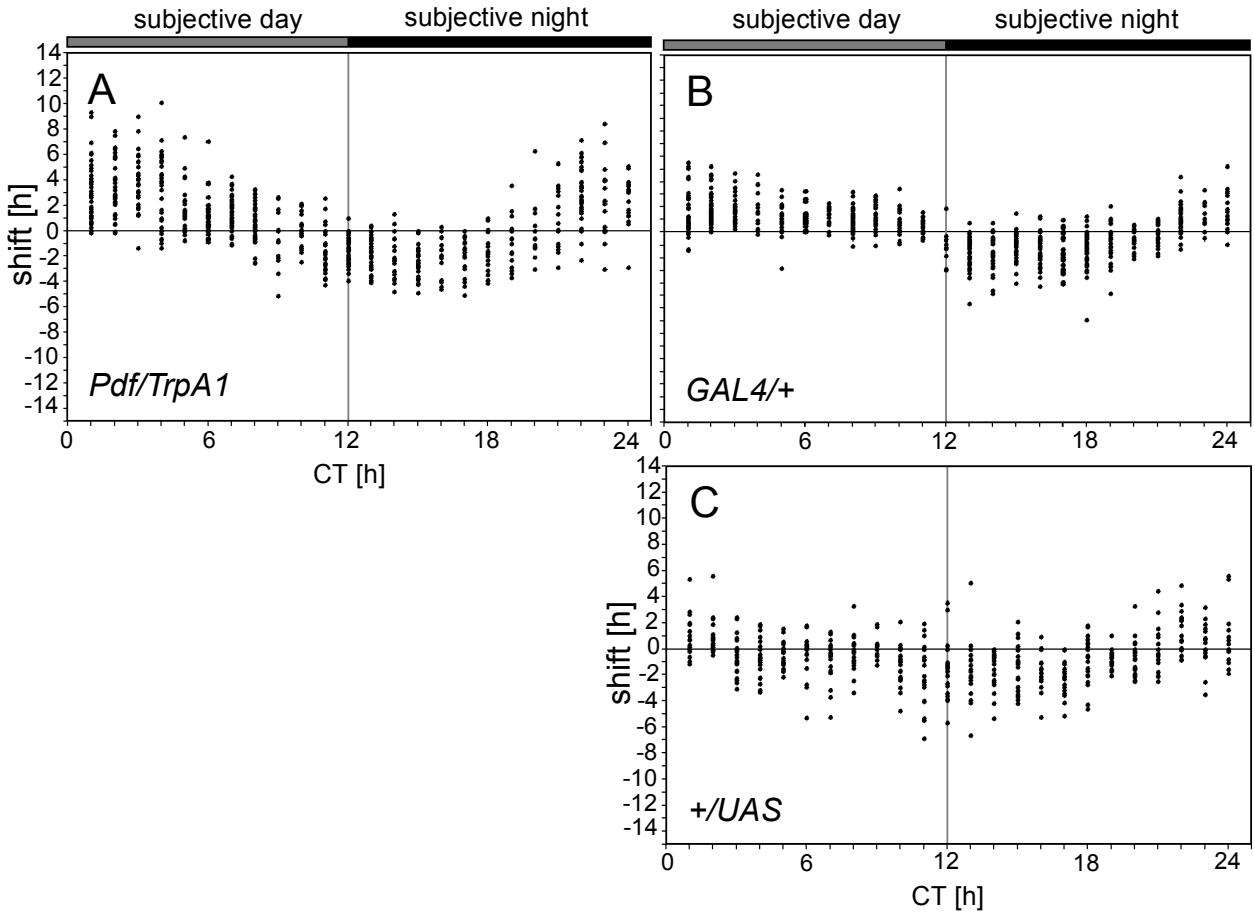

Figure 18 Phase response curves of *Pdf/TrpA1* (A) and respective controls (B, C)
(A) TPs administered in the advance zone (CT21-CT24) caused strong advances in *Pdf/TrpA1* flies. However those flies exhibited phase delays upon depolarization as well. **(B, C)** TPs applied to both controls, which solely experienced the TP, also shifted the phase of locomotor activity, however with a lower amplitude than depolarized *Pdf/TrpA1* flies. Each single dot indicates one fly. Advances are shown as positive values, whereas delays are plotted as negative values. The abscissa indicates the time point, at which the TP reached 32°C in circadian time (CT).

As the advance zone drastically reaches into the subjective day, the second half of the subjective day shows a narrowed dead zone fraction from CT7-CT10 in *Pdf/TrpA1* flies. This effect is specifically due to the depolarization of the PDF^+ LN_vs, since both controls do not exhibit such a pronounced advance zone, but display a rather flat PRC, which is quite comparable to controls in *clk856/TrpA1* experiments (Fig. 16, Fig. 17, Fig. 18, Fig. 19).

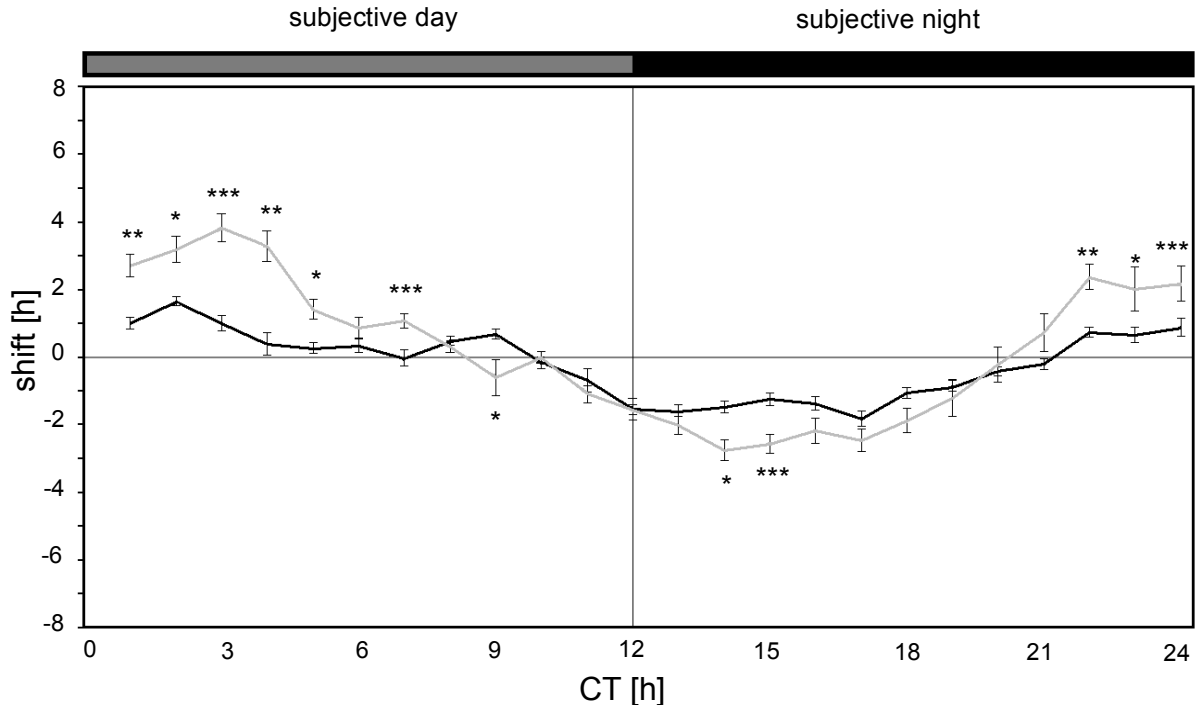

Figure 19 Phase response curves of *Pdf/TrpA1* and the mean of respective controls
TPs administered in the advance zone (CT21-CT24) caused severe and highly significant advances in *Pdf/TrpA1* flies (gray line). However, these flies exhibited modest phase delays upon depolarization as well. TPs applied to both controls (black line), which solely experienced the TP, also shifted the phase of locomotor activity, however to a much lower extent than depolarized *Pdf/TrpA1* flies. Mean (±SEM), asterisks indicate significant differences (*p<0.05, **p<0.01, ***p<0.001; Wilcoxon rank sum test).

The comparison of the PRCs of *clk856/TrpA1, Pdf/TrpA1* and controls provide three conspicuities: First, there seems to be a temporal dependence in the sensitivity of the clock's phase shifting ability upon TPs *per se* (Fig. 20, black line "*controls"*). TPs in DD are able to delay the clock at the beginning of the subjective night ("delay zone") and to advance it at the end of the subjective night ("advance zone") (Fig. 20). TPs during the course of the subjective day, however, did not affect the phase of locomotor behavior. The temperature-mediated shifting pattern is comparable to that

of a moderate light-PRC in amplitude and transition phases. Second, there seems to be a dependence upon the clock neuron cluster, which is depolarized: Upon “ubiquitous” clock neuronal activation (*clk856/TrpA1*) the clock predominantly delays its phase (Fig. 20, dark gray line) in the expected delay zone time-window. TPs applied in the advance zone shifts the clock just modestly in these flies. Upon PDF$^+$ specific clock neuronal activation though, the clock is mainly able to advance its phase (*Pdf/TrpA1, R6/TrpA1*), exhibiting a broadened advance zone, which ranges into the subjective day (*Pdf/TrpA1*). That suggests a highly differential sensitivity of certain clock neurons upon timed depolarization probably also including the differential sensitivity of their downstream targets. Third, the transitions from advances to delays and vice versa vary among the PRCs: While the controls show gently inclining transitions with a distinct dead zone during the subjective day, the depolarization of all clock neurons (*clk856/TrpA1*) causes strong, steep transitions and high PRC amplitudes, especially in the delay zone. The depolarization of the PDF$^+$ neurons (*Pdf/TrpA1*) cause more modest, shallow transitions from advances to delays and vice versa (Fig. 20).

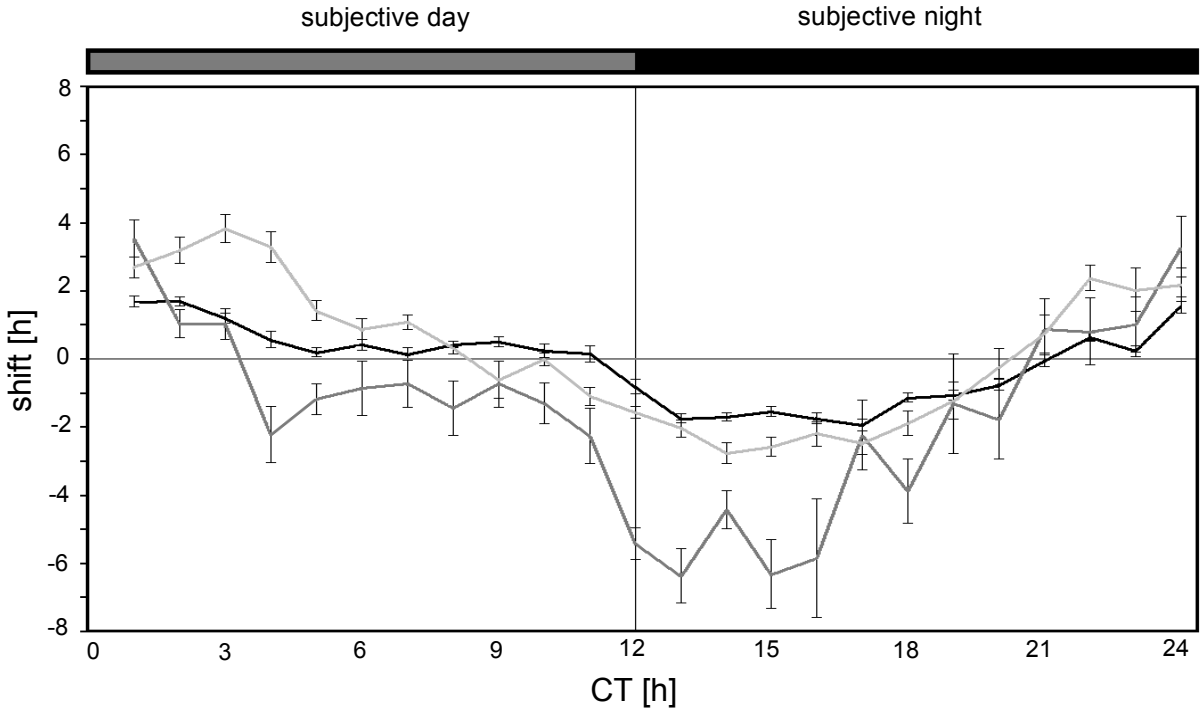

Figure 20 Comparison of the PRCs of *clk856/TrpA1* & *Pdf/TrpA1* and the mean of respective controls

TPs administered in the advance zone (CT21-CT24) cause enhanced advances in *Pdf/TrpA1* flies (light gray line), reaching up to one half into the subjective day. TPs applied in the delay zone (CT12-CT15) cause severe phase delays, when all clock neurons were depolarized (*clk856/TrpA1*; dark gray line). Controls (black line), which did solely experience the TP, shifted the phase of locomotor activity, however to a much lower extent. Mean (±SEM); Gray and black bars indicate subjective day and subjective night.

3.1.3 Excluding the PDF$^+$ neurons from ubiquitous clock neuron depolarization causes diminished phase delays and phase advances

To decipher the influence of the PDF$^+$ neurons in *clk856/TrpA1* flies in delay zone and advance zone phase shifting, *PdfGAL80* experiments were performed. Driving *PdfGAL80* in *clk856/TrpA1* excluded the PDF$^+$ neurons from depolarization via *dTrpA1*. TPs were applied in the advance and delay zone, respectively. Additional data of the phase shifting ability of experimental lines and controls are shown in "6 *Appendix",* chapter "6.1.1 *Supplementary data".*

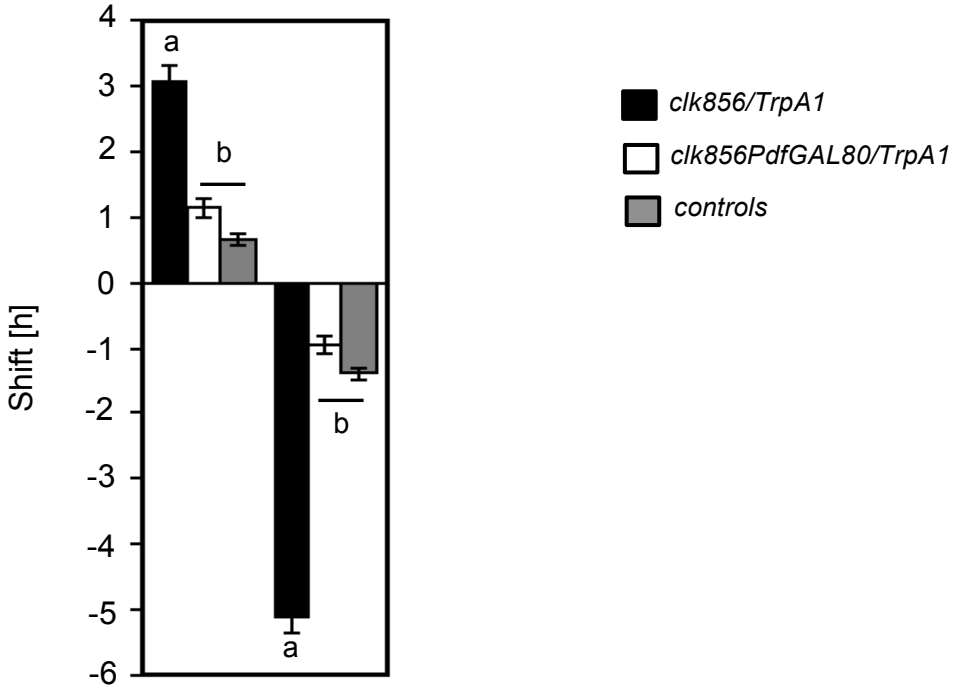

Figure 21 Shifting ability of *clk856/TrpA1* flies with and without PDF$^+$ neuron depolarization in the delay and advance zone

TPs at the end of the subjective night (advance zone) caused significantly higher phase advances of *clk856/TrpA1* flies (black bar) than of *clk856PdfGAL80/TrpA1* flies (white bar) and controls (gray bar). The same was true when TPs were applied at the beginning of the subjective night (delay zone). (Mean±SEM; advances were plotted as positive values, delays were plotted as negative values; Wilcoxon rank sum test with holm correction; different letters indicate significant differences, $p<0.05$).

Phase shifts of *clk856/TrpA1* and *clk856PdfGAL80/TrpA1* flies elicited by *TrpA1* activation in the advance (CT21-CT24) and delay zone (CT12-CT15) are shown in Fig. 21. *clk856/TrpA1* flies (Fig. 21; black bar) that experience the depolarization of all clock neurons, exhibit stronger phase shifts than *clk856PdfGAL80/TrpA1* flies that lack the depolarization of the PDF$^+$ neurons (Fig. 21; white bar) independently of the time point of TP application. Thus, flies that lack the depolarization of the PDF$^+$ neurons are neither able to phase advance nor to phase delay their activity properly,

suggesting that the PDF$^+$ neurons mediate the phase advancing ability of the clock when TPs are applied in the advance zone at the end of the subjective night. However, the neuronal activity of the PDF$^+$ neurons appears also necessary for phase delaying the clock, as flies that lack the depolarization of the PDF$^+$ neurons are not able to phase delay more than their respective controls (gray bar). This indicates a key role of the PDF$^+$ neurons for both phase delays and advances. Nevertheless, the depolarization of the PDF$^+$ neurons appears not to be sufficient to cause strong phase delays, as *Pdf/TrpA1* flies exhibit weaker phase delays compared to *clk856/TrpA1* flies after *TrpA1* activation (Fig. 15 & Fig. 20).

3.1.4 Temperature pulses applied in the delay zone shift PER cycling in M and E cells in *clk856/TrpA1* flies, mirroring the phase shifts in behavior

Ubiquitous clock neuron depolarization (*clk856/TrpA1*) generates prominent phase delaying locomotor behavior as observable output of the clock. But is the shift in behavior also accompanied by a shift of the molecular oscillations in clock protein levels, as a representative of the state of the core clock? To address this question, PER staining experiments were performed, which were in correspondence to the "delay zone" behavioral experiments in *clk856/TrpA1*. A TP was applied from hour 36-39 on the second day in DD (Fig. 22B, red box), PER levels were determined every two hours for three consecutive days in DD and were statistically compared to respective controls.

Flies, which did not experience a TP, but had the same genotype as experimental flies exhibited a continuance of the PER cycling in amplitude and periodicity over the whole time course in DD (Fig. 22A). PER levels in the LNs (except in the l-LN$_v$s and LPNs) as well as in the DN1s (Fig. 22A, green and dark gray curve) showed stably persisting and high amplitude oscillations. The cycling in the LPNs seemed to persist as well in DD, however with a much lower amplitude (Fig. 22 A, orange curve). In the l-LN$_v$s (Fig. 22A, red curve) and the DN2s (Fig. 22A, light blue curve) PER cycling was dampened, indicating that in DD, without any artificial depolarization, the network is not able to sufficiently maintain the oscillation of PER in all clock neuron clusters.

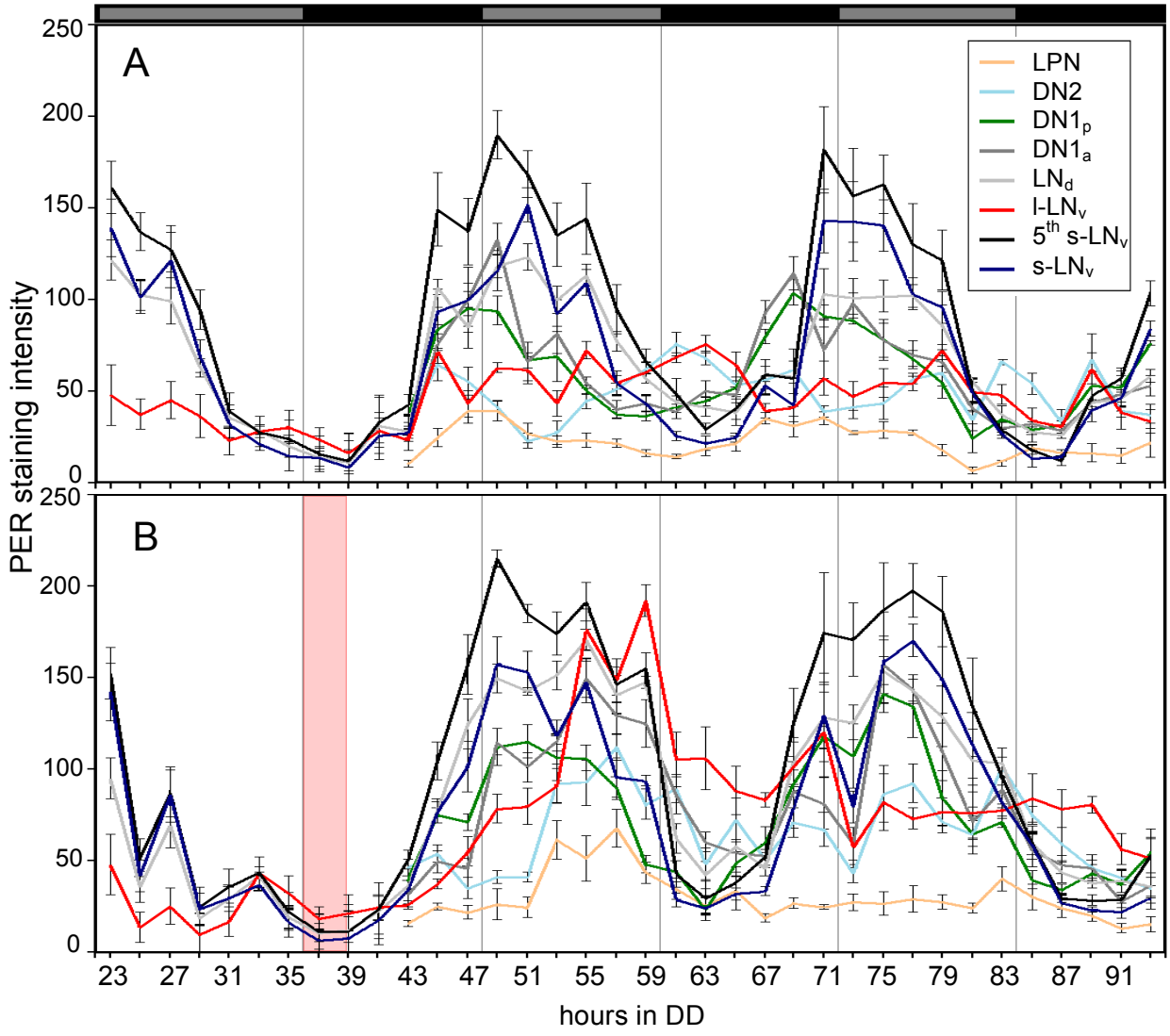

Figure 22 PER protein cycling on three consecutive days in DD in (A) non-temperature pulsed and (B) temperature pulsed *clk856/TrpA1* flies

PER cycling in non-temperature pulsed **(A)** and temperature pulsed *clk856/TrpA1* flies **(B)** continued in DD. The cycling in most of the clock neuron clusters kept the same amplitude during the whole time course**. (A)** In non-temperature pulsed flies solely the PER oscillations of the l-LN$_v$s (red curve), the LPNs (orange curve) and the DN2s (light blue curve) dampened. **(B)** In temperature pulsed *clk856/TrpA1* flies PER started to cycle at least once after application of the TP in the l-LN$_v$s (red curve), LPNs (orange curve) and DN2s (light blue curve). The red box indicates the TP from hour 36-39 in DD. (Mean grayscale value (±SEM); Gray and black bars indicate subjective day and subjective night, respectively.)

After application of the TP from hour 36-39 in DD (Fig. 22B, red box) PER cycling was altered immediately in all investigated clock neuron groups: The clock neurons exhibited a delayed PER peaking time in comparison to unpulsed control flies. The group of the l-LN$_v$s (Fig. 22B, red curve), the LPNs (Fig. 22B, orange curve) and the DN2 (Fig. 22B, light blue curve) responded once by displaying one peak of PER accumulation in the first cycle after the neuronal manipulation of the whole network.

Particularly the l-LN$_v$s increased PER levels rapidly, but this effect was restricted to the first cycle after depolarization, since PER levels declined afterwards and did not show another round of accumulation (Fig. 22B, red curve). Notably, those three clock neuron clusters that exhibited permanently low PER levels if not depolarized (Fig. 22A), showed a delay in PER accumulation in comparison to all other clock cell groups after activation (Fig. 22B).

The cell cluster-wise comparison illustrates the differences in PER protein levels between treated *clk856/TrpA1* flies *(clk856/TrpA1 with TP;* Fig. 23, red curves*)* and both controls (Fig. 23; gray & black curves) even better, as it reveals the pronounced delay in PER oscillations in the s-LN$_v$s, the 5th s-LN$_v$s, the LN$_d$s and the DN1s, which persisted at least for two consecutive cycles in DD and occurred immediately after TP application. Neuronal clock clusters, whose PER levels normally do not oscillate under constant conditions, namely the l-LN$_v$s, the LPNs and the DN2s, remarkably showed at least one cycle of PER oscillations after the TP (Fig. 23). In particular the l-LN$_v$s increased PER protein levels strongly once, but afterwards PER cycling dampened out again. Increases of PER levels in the LPNs and DN2s are more moderate in comparison to the l-LN$_v$s and seem to occur with a slight delay compared to PER levels of other clock neuron clusters.

In conclusion, the prominent delay in PER cycling does correspond to the direction and the amount of the phase delay the flies exhibited in the behavioral experiments in this specific time window. This indicates that the molecular clock itself is indeed mirroring the phase shifting effect in behavior and might constitute the underlying process controlling the phase shift in behavior.

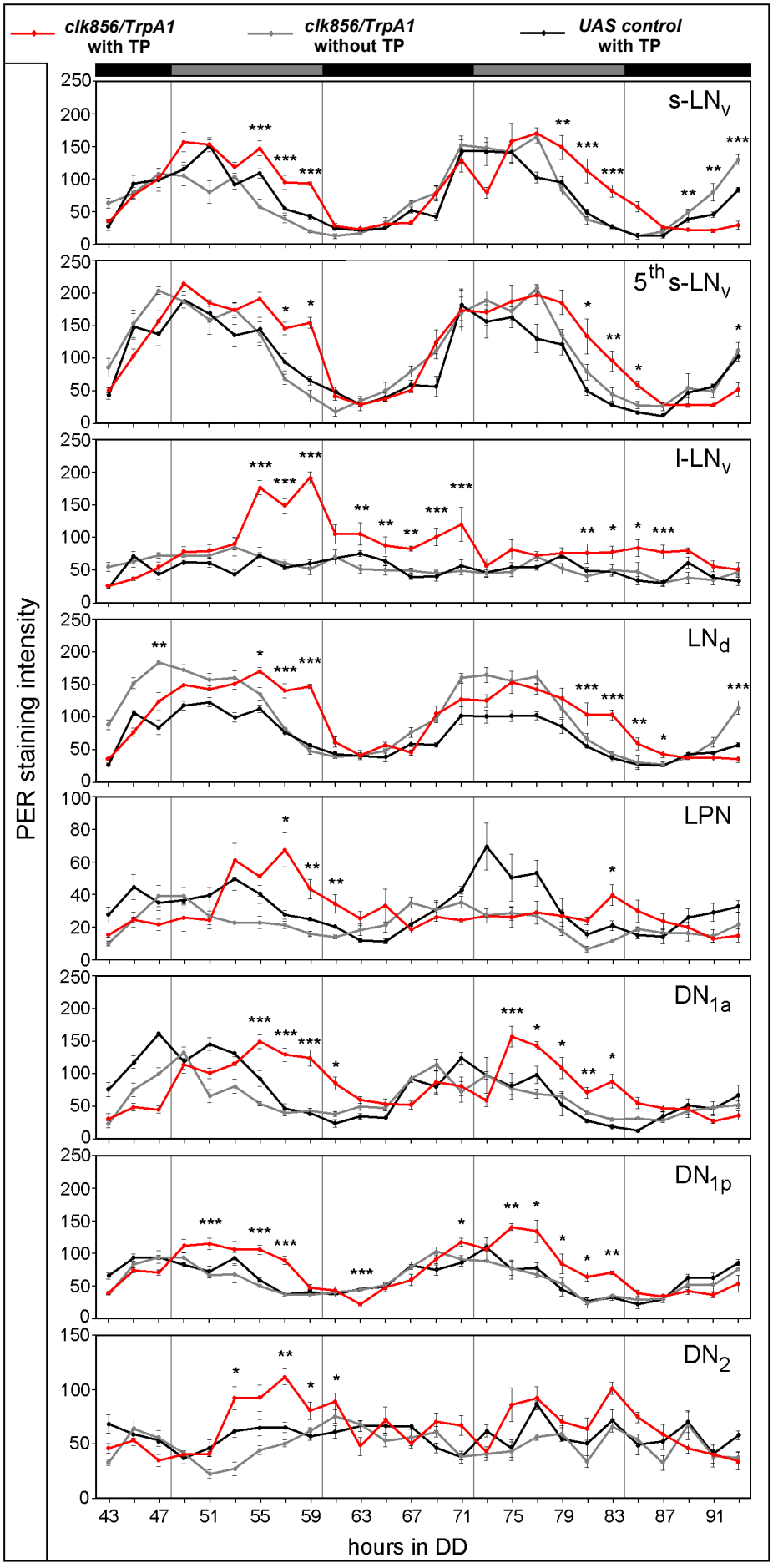

Figure 23 Comparison of PER protein cycling in DD in delay zone temperature pulsed *clk856/TrpA1* flies and controls

PER cycling in *clk856/TrpA1* with TP (red curves) is delayed relative to PER cycling of controls (gray & black curves). In *clk856/TrpA1* with TP the l-LNvs show a strong increase in PER levels. The LPNs and DN2s exhibit one cycle of PER accumulation, while the controls did not.
(Gray and black bars indicate subjective day and night, respectively. Mean(±SEM) in grayscale units; *p<0.05; **p<0.01; ***p<0.001; Wilcoxon rank sum test with holm correction).

3.1.5 Temperature pulses applied in the advance and delay zone shift PER cycling in PDF$^+$ neurons in *Pdf/TrpA1* flies

Immunohistochemical experiments of flies with ubiquitous clock neuron depolarization (*clk856/TrpA1*) provided insight into the molecular state of the clock, which indeed mirrored the behavioral output of the clock. The same experiment was performed for *Pdf/TrpA1* flies in that particular time window, in which most prominent changes in PER levels occurred in *clk856/TrpA1* flies, from hour 55 to 61 in DD, after a TP was applied in the delay zone.

The exclusive depolarization of the PDF$^+$ neurons via *dTrpA1* did indeed change PER levels, but just exclusively in those clock neurons, which had been depolarized, thus in the s-LN$_v$s and l-LN$_v$s. PER levels in all other clock neuron clusters, which had been investigated, remained unaffected (Fig. 24).

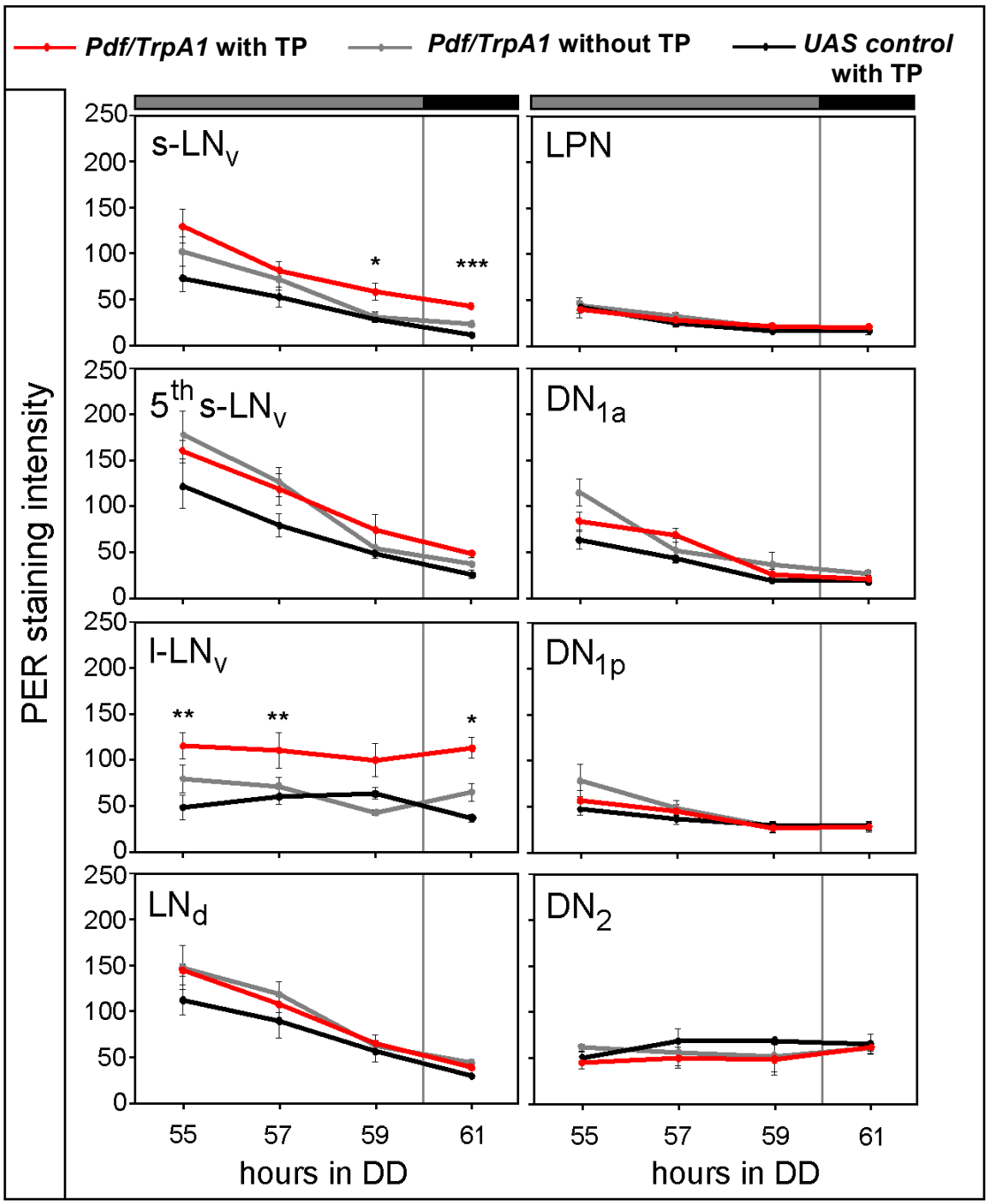

Figure 24 Comparison of PER protein cycling in DD in delay zone temperature pulsed *Pdf/TrpA1* flies and controls

PER levels in *Pdf/TrpA1* flies with TP (red line) are significantly higher in the s-LN$_v$s and l-LN$_v$s in comparison to controls (gray & black lines), but are unaffected in other clock neuron clusters.
(Gray and black bars indicate subjective day and night, respectively. Mean(±SEM) in grayscale units; *p<0.05; **p<0.01; ***p<0.001; Wilcoxon rank sum test with holm correction).

As most prominent phase shifts occurred in the advance zone in *Pdf/TrpA1* flies, immunohistochemical experiments were performed in correspondence to the behavioral advance zone experiments. TPs applied to *Pdf/TrpA1* flies during the advance zone resulted in immediately preponed PER accumulation (Fig. 25), but also mainly in those clock neurons, which had been depolarized via *dTrpA1*. Hence just in the PDF^+ s-LN_vs and l-LN_vs increases in PER levels occurred earlier, whereas PER levels in other clock neuron groups seemed widely unaffected. That was consistent with the immunohistochemical delay zone results of *Pdf/TrpA1* flies.

However, a lagged communication from the PDF^+ neurons to the DN1s after the depolarization of the PDF^+ neurons cannot be excluded, as the DN1 neurons are known to be downstream targets of the LN_vs (amongst others) and as there is one significant earlier time point of PER peaking in the DN1s. However, a clear and significant effect in other clock neurons than the PDF^+ neurons is lacking.

Overall, this implements that in the delay zone the exclusive depolarization of the PDF^+ neurons caused slight changes in PER levels mirroring the modest effects in behavior. The number of neurons, which shifted PER cycling, may account for the rather modest delays in behavior compared to *clk856/TrpA1*, in which nearly all clock neurons exhibited delayed PER levels, resulting in huge phase delays also in behavior. TPs applied in the advance zone, however, are potent to strongly advance PER accumulation in the PDF^+ LN_vs and possibly also in other clock neurons (DN1s) as well as to remarkably shift locomotor behavior in comparison to controls. Even though the rapid shifts of the molecular clock were mainly restricted to the manipulated neurons, the PDF^+ neurons are highly potent to advance the clock.

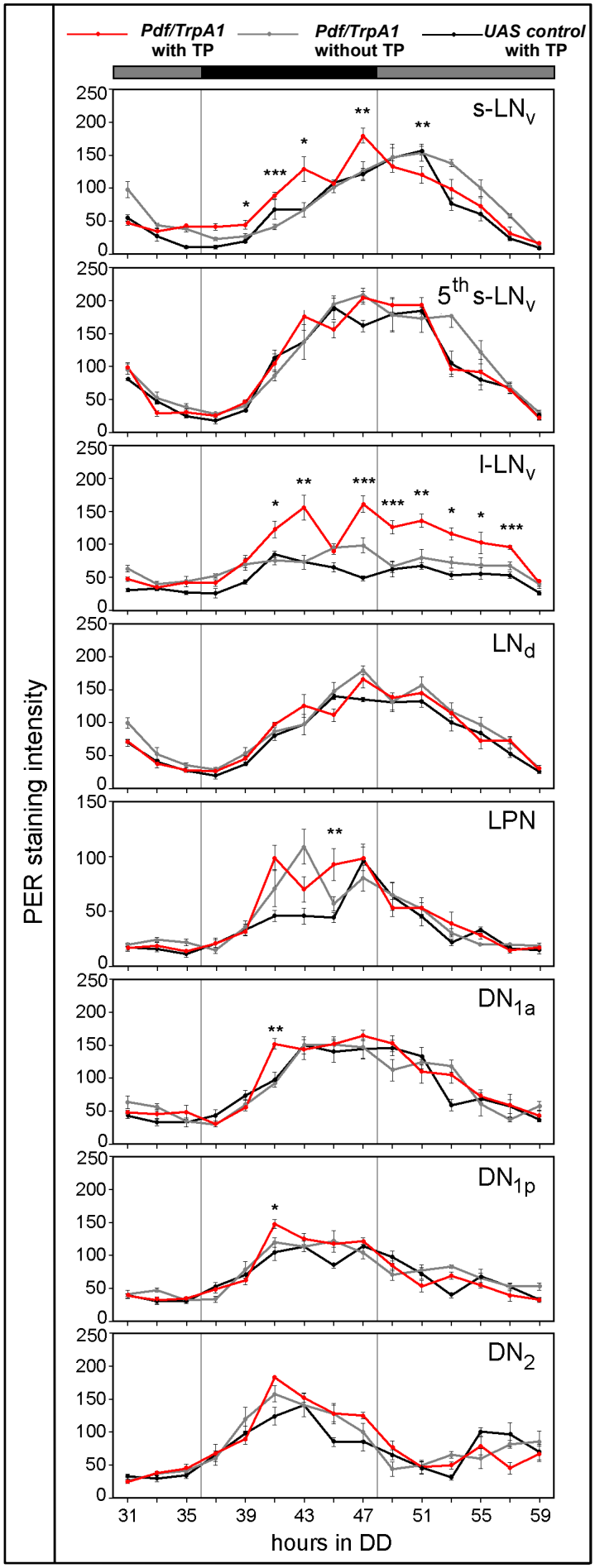

Figure 25
Comparison of PER protein cycling in DD in advance zone temperature pulsed *Pdf/TrpA1* flies and controls

PER cycling in *Pdf/TrpA1* flies with TP (red curve) is significantly advanced in PDF$^+$ neurons compared to PER oscillations in controls (gray & black curves).
The l-LN$_v$s exhibited a strong increase in PER levels in experimental flies.
(Gray and black bars indicate subjective day and night, respectively. Mean±SEM in grayscale units; *p<0.05; **p<0.01; ***p<0.001; Wilcoxon rank sum test with holm correction).

3.1.6 Temperature pulses applied in the delay zone do not significantly shift PER cycling in *mai179/TrpA1* flies

The depolarization of all clock neurons (*clk856/TrpA1*) strongly delayed and modestly advanced locomotor activity. The pronounced phase delaying effect was accompanied by severe delays in molecular PER cycling in all clock neurons. The prominent phase advancing effect in *Pdf/TrpA1* flies' behavior was also paralleled by phase advances in PER accumulation, however just in clock neurons, which were addressed by depolarization. *mai179/TrpA1* flies just show significant advances, whereas TPs in the delay zone did not cause significant phase delays. Why is this happening in these flies? Are some neurons delaying their molecular clock while others are advancing? Thus is there a counteracting effect that somehow prevents the flies from phase shifting in behavior? For that reason these flies were investigated in another immunocytochemical experiment: *mai179/TrpA1* flies were depolarized in the delay zone and sampled in that time window, in which the depolarization of all clock neurons (*clk856/TrpA1*) exhibited the strongest effects (hour 55-61 in DD). The depolarization of most of the LN_vs and some DNs in the delay zone using *mai179/TrpA1* did not shift PER cycling sufficiently. Just at hour 55 in DD (apart from some significant peaks of PER at hour 59 (Fig. 26)) PER levels were increased in some clock neuron clusters. Thus not just the manipulated clock neurons exhibited altered PER levels after TP application in *mai179/TrpA1* flies. However, there was neither a coherent pattern of clock neurons exhibiting higher PER levels which may resemble the *mai179GAL4*-driver line expression pattern, nor was there a clear delay of PER accumulation (Fig. 26). If there was an effect at all, *mai179/TrpA1* flies exhibited rather an advance of PER accumulation than a delay, indicating a potential counteracting and disturbed PER cycling pattern within the clock network, which might be the reason for preventing phase delays in behavior.

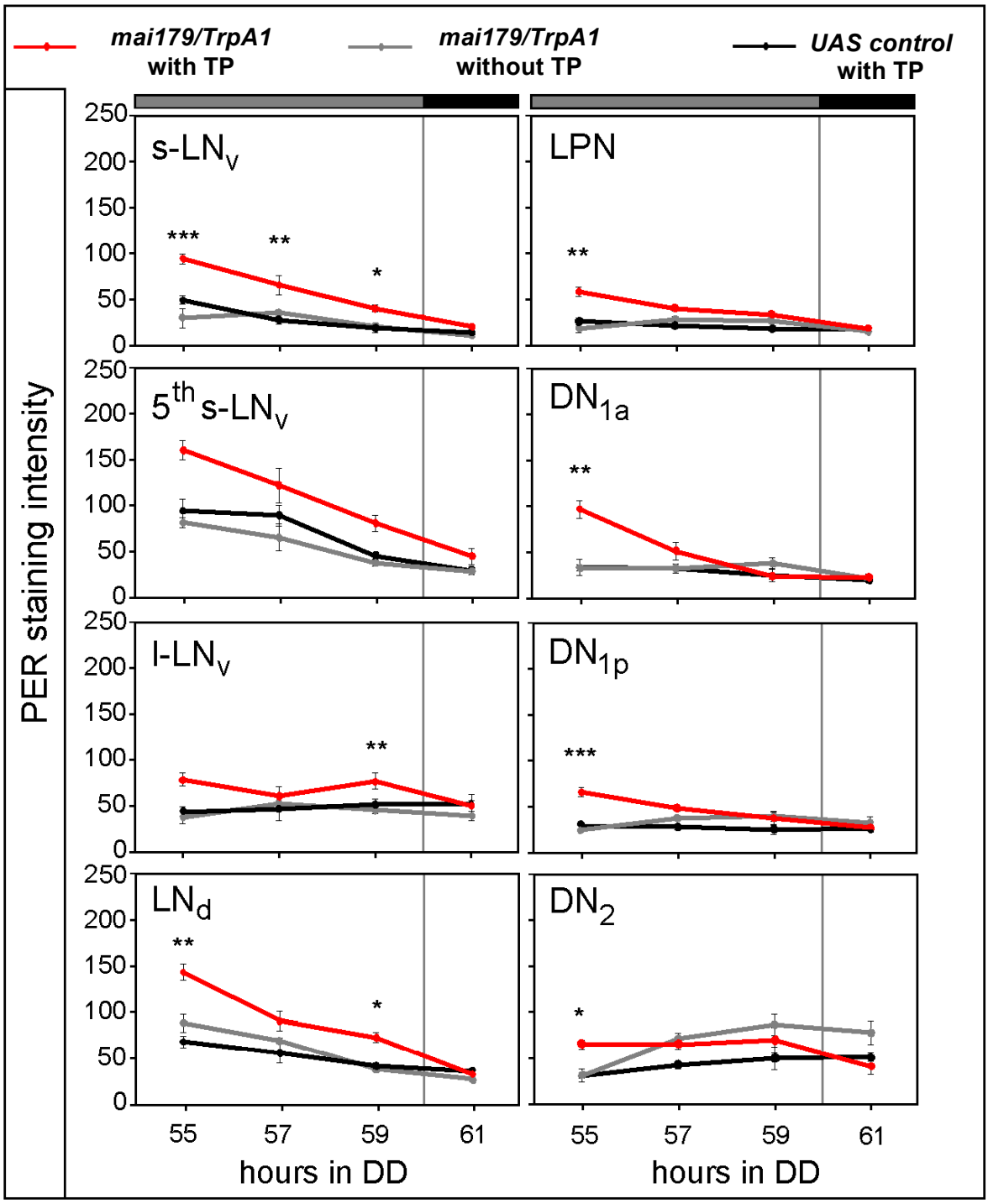

Figure 26 Comparison of PER protein cycling in DD in delay zone temperature pulsed *mai179/TrpA1* flies and controls

PER levels in *mai179/TrpA1* flies with TP (red line) are significantly higher in the s-LN$_v$s, LN$_d$s, LPNs and DNs in comparison to controls. (Gray and black bars indicate subjective day and night, respectively. Mean±SEM in grayscale units; *p<0.05; **p<0.01; ***p<0.001; Wilcoxon rank sum test with holm correction).

3.1.7 Depolarization-mediated rises in PER levels coincide with rises in CREB levels in the large ventrolateral neurons

The PER-immunocytochemical approach demonstrated that the observed immediate alterations in PER oscillations in Pdf/TrpA1 and clk856/TrpA1 flies are mainly restricted to the respective manipulated neurons. That might indicate that the extraordinary strong and fast response of especially the l-LN_vs in the advance zone (*Pdf/TrpA1*) as well as in the delay zone (*Pdf/TrpA1*, *clk856/TrpA1*) may occur due to the cell-autonomous depolarization via *dTrpA1*. Second messengers as cAMP and Ca^{2+} are known to be responsible for intracellular responses to changes in membrane potential in neurons (Purves et al., Neuroscience, 2nd edition, Sunderland (MA): Sinauer Associates, 2001). Also within the clock particularly cAMP has recently been shown to be involved in interneuronal communication via PDF as well as in PER protein stabilization (Li et al., 2014). Furthermore Ca^{2+} might be the most prominent cation (apart from sodium and potassium), entering the neuron after *TrpA1*-activation (Latorre et al., 2009). To address whether cAMP and/or Ca^{2+} are specifically involved in the mechanism of mediating the signal of membrane-depolarization after *dTrpA1* activation to the molecular clock, CREB (cAMP responsive element binding protein) staining experiments were performed right after TP application in *clk856/TrpA1, Pdf/TrpA1* flies and respective controls (Fig. 27 & Fig. 28). The sampling was set to the time point when PER accumulated in the l-LN_vs, thus 9 hours after the beginning of the TP (Fig. 28A). Since CREB is expressed in a variety of cells in the nervous system, like in glia cells, clock neurons and non-clock cells (Tanenhaus et al., 2012), CREB quantification was restricted to the l-LN_vs, as those cells exhibited the strongest effects in PER accumulation.

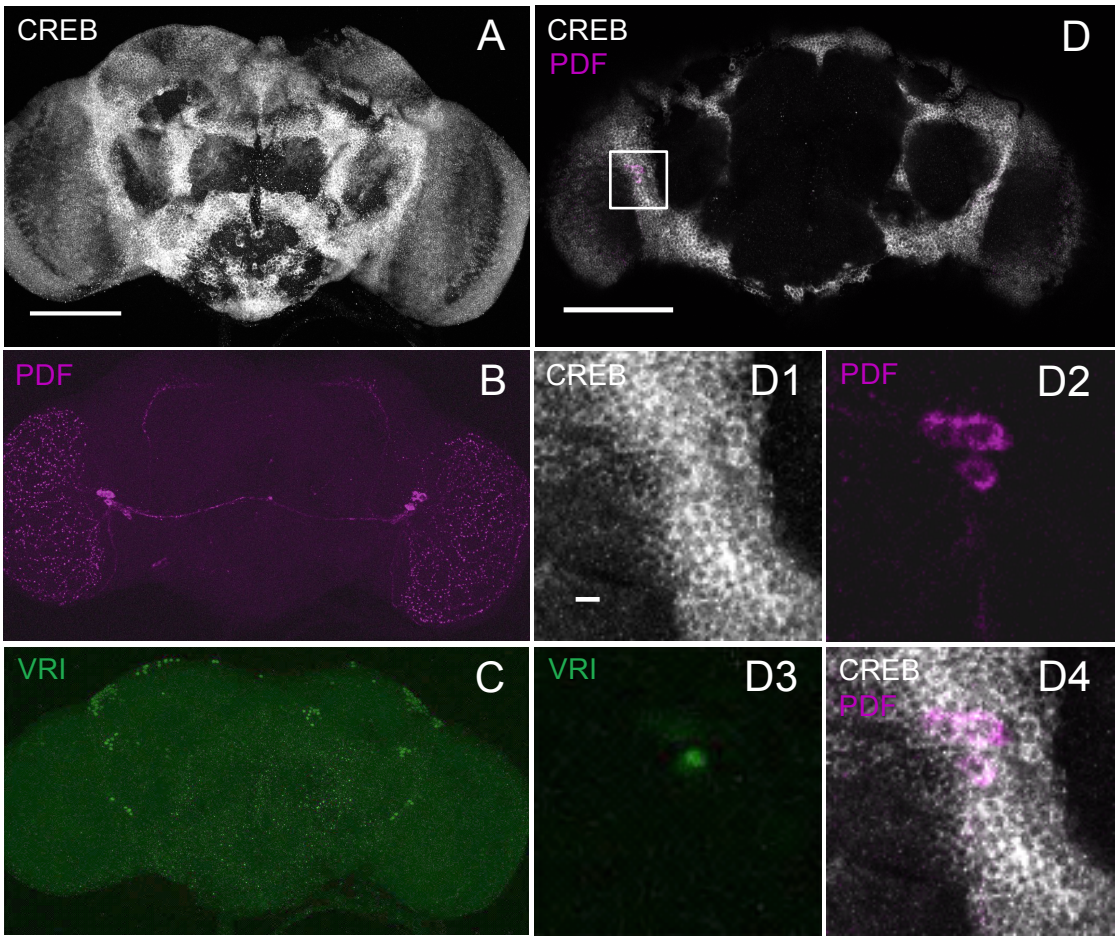

Figure 27 Representative CREB staining pattern after TP application
Male adult flies treated with a TP showed strong CREB staining (gray) in most of the cells of the central nervous system. By means of the counterstaining against **(B)** PDF (magenta) and **(C)** VRI (green) l-LN$_v$s were identified and CREB levels were quantified. **(A)** CREB is expressed in a large number of cells in the brain. **(D)** At that focal plane where the cell bodies of the l-LN$_v$s were largest, l-LN$_v$s were defined as regions of interest (ROI) using Fiji and CREB levels were measured in grayscale units **(D4).** (**D1-D4)** show the magnification of the white box in **(D).** For further information regarding quantification please see chapter "2.2.4.2 *Microscopy & Image Analysis" in "*2 *Material & Methods"*. Scale bar in **(A)** 100 µm (applies to **(B)-(D))** and in **(D1)** 1 µm (applies to (**D2)-(D4)**).
All images show Z projections for the purpose of illustration.

The quantification of CREB remarkably revealed that CREB overall levels in the l-LN$_v$s were as double as high at the end of the subjective night ("delay zone TP") relative to the end of the subjective day ("advance zone TP"). That effect was irrespective of the genotype or treatment (Fig. 28B), suggesting a general circadian cycling of CREB in the l-LN$_v$s. Interestingly, both experimental lines showed significantly higher CREB levels after TP application compared to respective controls in the delay as well as in the advance zone. These results indicate a coincidence of rises in CREB and PER levels, furthermore suggesting a putative link between cell-activation and the molecular clock mediated by CREB and the cAMP/PKA-pathway and Ca^{2+} (Fig. 28).

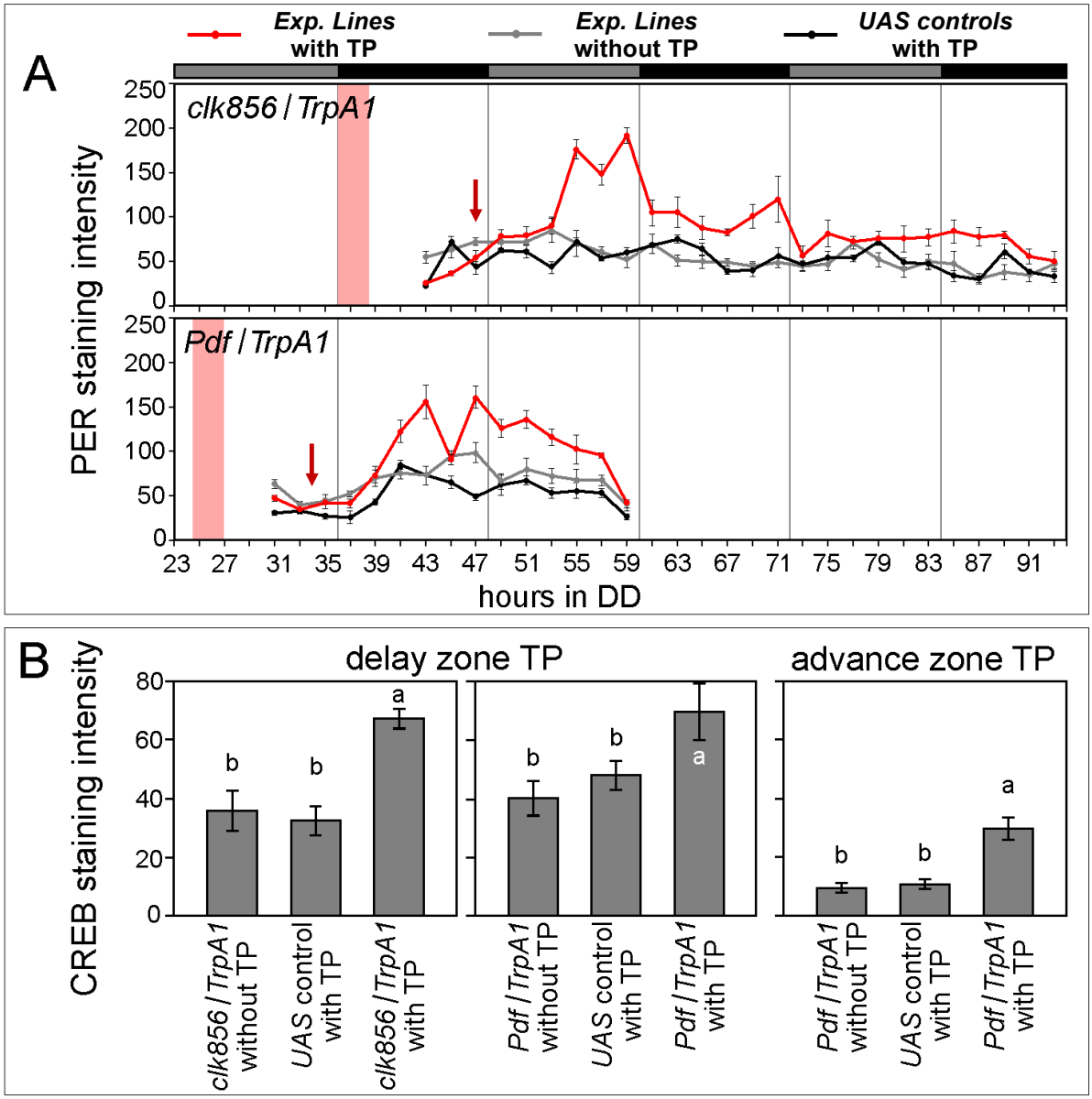

Figure 28 CREB accumulation coincides with rises of PER levels in the l-LN$_v$s after *dTrpA1* activation

(A) Both experimental lines (red curves) exhibited strongly increased PER levels after *dTrpA1* activation relative to respective controls (gray & black curves). Red arrows indicate the beginning of the rise in PER levels as well as the time point of CREB-staining quantification.

(B) The quantification of CREB levels in the l-LN$_v$s after depolarization via *dTrpA1* in the delay (*clk856/TrpA1, Pdf/TrpA1* and controls) or the advance zone (*Pdf/TrpA1* and controls) revealed significantly higher amounts of CREB in the l-LN$_v$s in both experimental lines compared to their controls. Rises in CREB coincide with rises in PER levels in the delay and in the advance zone. (Gray and black bars indicate subjective day and night, respectively. Mean±SEM in grayscale units; different letters indicate significant differences. Wilcoxon rank sum test with holm correction, $p<0.01$, ≥20 hemispheres per time point and genotype.)

3.2 Establishment of the whole-brain *ex vivo* culturing technique for long-term luciferase experiments

For the purpose of long-term luciferase-assays on explanted brains the whole-brain culturing method was adapted from Ayaz et al. (2008) and was established in our laboratory. Fig. 29 shows some representative brains after more than 2 weeks in culture. The shape of the brains looked unaltered compared to freshly dissected brains (for comparison see e.g. staining in chapter "6.1.1 *Supplementary Data"* in "6 *Appendix"*), suggesting rather marginal degeneration processes and/or bacterial contamination, even though such processes cannot be generally excluded and fully evaluated with the help of those images. However the strong fluorescence signal in most of the clock neurons indicate that the brains seemed to be quite healthy, functional and alive, as just living cells are able to produce such a signal. Not just the LNs exhibited strong fluorescence (Fig. 29A & Fig. 29B), but also the dorsal neurons (Fig. 29C), indicating that also neurons, which are at the surface of the brain and are thus more prone to degenerative processes, were still alive.

The successful establishment of the whole-brain culturing method in our department allows follow up experiments, in which long term observations of clock neuronal communication *ex vivo*, thus without any input to the clock (via e.g. the eyes), can be performed. Since luciferase fused to various clock promoters (Stanewsky et al., 1997, 1998, 2002; Veleri et al., 2003; Glaser & Stanewsky, 2005), highly sensitive charge-coupled device (CCD) cameras and microscopic equipment are available, long-term experiments observing the luminescence as read-out of the clock are possible. Luminescence is indeed a proper indicator of the clock's status, thus a manipulation of some clock neurons may alter the output und this can be monitored by altered luminescence.

Since the pure communication among the clock neurons, without interfering input from other parts of the body (like e.g. the photoreceptive organs) could not be investigated in an intact fly and as immunocytochemical approaches can just provide information that represents rather a snapshot of the molecular clock at the particular time of sampling, the whole brain-culturing method allows the investigation of the interaction of particular clock neuron clusters over days and opens a high variety of potential experiments.

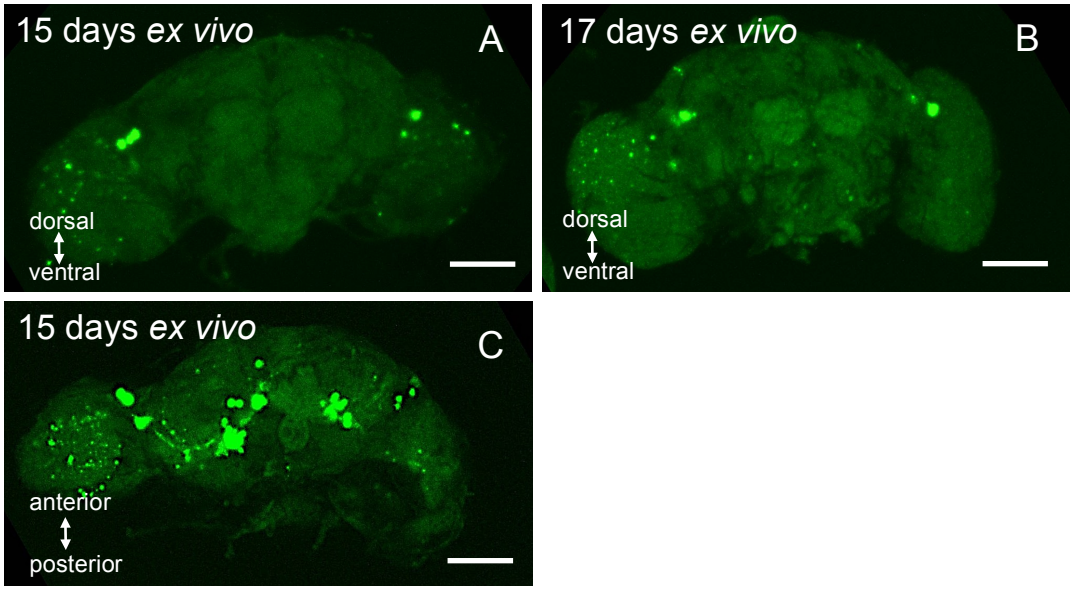

Figure 29 *clk856epac* brain explants after more than 14 days *ex vivo*
The brains appear quite healthy as indicated by the strong fluorescence signal of the clock neurons in culture. **(A, B)** Both the LNs and **(C)** the DNs exhibit strong fluorescence. For the culturing protocol please see chapter "2.2.5 *Whole brain-culturing*" in "2 *Material & Methods"*.
Scale bar 100 µm. **(A)-(C)** are Z projection images for the purpose of illustration.

4 Discussion

The neuronal electrical state of clock neurons is coupled to 24-hour rhythms in gene expression, resulting in clock-controlled circadian output (Mizrak et al., 2012). Since the strong relationship between electrical activity and transcription defines the functional state of clock neurons (Colwell, 2011; Mizrak et al., 2012), an emerging view is that neural activity supports molecular clock oscillations rather than just mediating clock neuron communication (Mizrak et al., 2012), thus ensuring the maintenance of robust rhythms in LD and in particular in constant conditions (Nitabach et al., 2002). Several publications were able to provide experimental evidence that a hyperexcitation of the LN_vs is potent to cause severe changes and phase shifts in clock protein levels in certain clock neurons (Nitabach et al., 2002, 2006; Wu et al., 2008; Depetris-Chauvin et al., 2011), facilitating the mediation of stimuli like e.g. light information to the network (Sheeba et al., 2008), besides the cell-autonomous CRY-dependent light-input pathway. However, the exclusive and pure response of the clock to neuronal depolarization and the consecutive communication among the clock neurons after such a depolarization remains uncertain.

4.1 Clock network properties – Behavior & Phase Response Curves

Guo et al. (2014) claimed recently that an artificial depolarization using *dTrpA1* expressed in PDF^+ neurons is able to mimic light input, since a depolarization at CT 21 advanced and at CT 15 delayed the clock to an extent, which was comparable to shifts of standard light-PRCs. By performing phase shift experiments with *clk856/TrpA1* and *Pdf/TrpA1* flies, covering the whole time course of one day in DD, I was able to demonstrate that clock neuronal depolarization is not unconditionally mimicking photic input, emphasizing this view to be much oversimplified.

Phase shifts after depolarization of all clock neurons

The depolarization of all clock neurons (*clk856/TrpA1*) yielded an asymmetric PRC with pronounced delays of about 6 hours in maximum, but with weaker advances of at most 4 hours. Amplitudes of light-PRCs are highly dose-dependent (Johnson, 1990) and indeed can also exhibit asymmetric proportions of delays and advances (e.g. Kistenpfennig et al., 2012). Considering that the main features of a light-PRC

might also apply to a depolarization-mediated PRC, high PRC-amplitudes after neuronal activation via *dTrpA1* may account for huge effects on the molecular clock, which might be comparable to a high intensity LP in a light-PRC. Although the approach of timed depolarization via *dTrpA1* is highly artificial, the PRC of *clk856/TrpA1* flies exhibited phase delays and advances at those parts where it would have been expected for a standard light-PRC. However, the depolarization-mediated PRC exhibited slight delays during the course of the subjective day.

How to explain these slight delays plausibly?

The *dead zone* of a PRC might represent a buffered state of the clock, as normally neither advances nor delays take place during that particular time. Nevertheless, M cells display high levels of electrical activity during the subjective day (Cao & Nitabach, 2008; Sheeba et al., 2008), a feature that might exert no or just weak influence on the phase relationship of activity, as no phase shifts occur at that portion of the light-PRC. Since in the behavioral experiments using *clk856/TrpA1* not just M cells (PDF^+ s-LN_vs), but also PDF^+ l-LN_vs, E cells, LPNs and DNs were depolarized, the additional depolarization of E cells or of other clock neurons might unbalance the buffered system of the clock during the subjective day, determining weak phase delays. E cells currently regain more attention with regard of being potent to exert influence on the whole network and being in charge of (firing dependent) phase-resetting and adjustment, among those particularly the LN_ds (Guo et al., 2014; Roberts et al., 2015, Yoshii et al., 2015). Along that line, E cells were proven to respond with a much stronger TIM degradation than the M cells did after a similar artificial depolarization. Furthermore, it has been shown recently by Yoshii and colleagues (2015) that rescuing *cry* in the 5^{th} s-LN_v and the LN_ds consistently caused an improvement in the speed of re-entrainment to shifted LD conditions. Another study demonstrated, using an *ex vivo* approach that M and E cells in brain explants recover differently in speed after LP application at ZT 21. Even though a LP was applied in the advance zone, the LN_ds appeared to recover synchrony faster than all other clock neuron groups (Roberts et al., 2015). All these pieces of evidence underline the importance of the E cells and especially of the LN_ds for clock network readjustment and synchronization not just in the delay zone (Guo et al., 2014; Yoshii et al., 2015), but also in the advance zone of a PRC (Roberts et al., 2015). These clock neurons are the first cells to resynchronize to shifted conditions after LP

application as well as after artificial depolarization, putatively being able to dictate to the rest of the network how to re-adapt properly.

During the early night *(delay zone)* the depolarization of all clock neurons caused extraordinary strong phase delays. As in the early night M cells are usually exhibiting low electrical activity at least in LD conditions (Cao & Nitabach, 2008), M cells are now artificially hyperexcited by *dTrpA1*, apart from the activation of the E cells and other clock neurons. The simultaneous excitation of the PDF^+ LN_vs, 4 LN_ds and 5^{th} s-LN_v using *DvPdfGAL4* (Bahn et al., 2009) in this particular time window was already demonstrated to be responsible for phase delays of about 3.5 hours (Guo et al., 2014). The exclusive depolarization of the PDF^+ neurons, however, appeared also to be sufficient to evoke phase delays, though to a lower extent (around 2.5 hours of delay) (Guo et al., 2014), indicating a supportive function of the PDF^+ neurons to the E cells in phase delaying the clock. Since Guo and colleagues used a driver line, which does not include the DNs and the LPNs as the *clk856GAL4*-driver line does, the delay they observed after clock neuron depolarization was not as strong as the shifts in the present work (about 6 hours). Those disparities in the expression pattern of both *GAL4* lines might determine the differences in the behavioral phase shifting ability, as at least one of these two clusters of clock neurons is of importance for phase resetting. Some of the DN1s are reported to be part of the E cell cluster (Rieger et al., 2006) and E cells have been shown to respond to PDF signaling with a deceleration of their molecular pace (Wu et al., 2008; Yoshii et al., 2009), what makes them predestinated for phase delaying. Moreover, the DN1s were demonstrated to be the most light-sensitive neurons in the early night and are known to have the capacity to serve as regulators of phase and/or amplitude of the M and E peak in LD, indicating a role of these cells in the process of phase delaying (Tang et al., 2010) as well as in proper phase adjustment of the clock (Zhang et al., 2010). Furthermore, the $DN1_p$s have been shown to directly contact a subset of neurons of the neuroendocrine *pars intercerebralis*, whose neurons have been implicated to integrate light and temperature inputs together with circadian signals (Cavanaugh et al., 2014), potentially influencing activity rhythms (Zhang et al., 2010). The depolarization of the “incomplete” E cell cluster (just four LN_ds and the 5^{th} s-LN_v) by Guo and colleagues using the *DvPdfGAL4*-driver line, though, may not represent the complete subset of E clock neurons, which may normally mediate phase delays, as some of them are not included in the *GAL4* line and are hence

lacking the temporal depolarization via *dTrpA1*. That might result in a much weaker delay than obtained by ubiquitous clock neuron activation (*clk856/TrpA1*).

But what might be the molecular mechanism downstream of the depolarization in the delay zone? In the early night PER/TIM proteins are at very low levels, but *per* and *tim* mRNA levels are highest. After artificial depolarization via *dTrpA1,* protein kinase A (PKA) might be activated, which stabilizes PER. PER abundance might be rapidly increased (Li et al., 2014). In the presence of highly accumulated PER, SUPERNUMERARY LIMBS (SLMB) an E3 ubiquitin ligase, which controls the stability of phosphorylated PER and TIM, preferentially interacts with phosphorylated TIM, favoring its degradation (Grima et al., 2012). But as there is much PER and TIM due to the prior stabilization of PER, the degeneration of both proteins takes longer, the cycle is slowed down, thus enabling the clock to delay its phase. Alternatively, another ubiquitin ligase CULLIN-3 (CUL-3) might be involved in that process, as CUL-3 was implicated in particular to be involved in promoting delay zone phase shifts and to participate in altering TIM-levels (Grima et al., 2012; Guo et al., 2014). But a prerequisite for CUL-3 interaction with the core clock component TIM are low levels of PER. In the early night PER levels are low and TIM is predominantly cytoplasmic. This enables CUL-3 to physically interact with low-phosphorylated TIM, forming a complex with TIM and thereby allowing its accumulation. To slow down the pace of the clock, thus delaying it, TIM has to receive accumulation-mediating signals longer than it would have received without neuronal activation. Albeit such a particular mechanism might be possible, the experimental evidence still has to be delivered.

The *M cells might mainly control the advance zone portion of the PRC*, as those neurons are known to mediate phase advances. However, the advance zone does not exhibit such a pronounced amplitude as it is the case for the delay zone, suggesting that the E cells, which are primarily able to delay, are in favor to control the whole network in *clk856/TrpA1* flies after ubiquitous depolarization.

Phase shifts after the depolarization of the PDF $^{+}$ neurons

The PRC of *Pdf/TrpA1* flies showed a more shallow transition between delays and advances with a distinct, but narrowed *dead zone* during the subjective day. The shifts reached at most around 5 hours of advances and about 2 hours of delays. The delay zone was located like it would have been for a light-PRC around CT 13 to

CT 17, but there was a remarkable temporal enhancement of the advance zone, which ranged from CT 21 to CT 6/7. Among the PDF^+ neurons, which have been addressed by depolarization in this experiment are the l-LN_vs. These neurons have been shown to have a higher resting membrane potential, frequency of spontaneous action potential firing rate and burst firing pattern during the subjective day relative to the subjective night (Cao & Nitabach, 2008; Sheeba et al., 2008). The electrical activity of the l-LN_vs during the day in turn has been shown to be directly linked to light-arousal, which appears to be unique among the neurons of the circadian network, since the s-LN_vs as well as all other clock neuron groups were insufficient to do so (Shang et al., 2008). The second PDF^+ clock neuron group being addressed in *Pdf/TrpA1* is the cluster of the s-LN_vs. The s-LN_vs' resting membrane potential is most depolarized around lights-on (Cao & Nitabach, 2008), indicating in general a clock-controlled membrane excitability of these clock neurons (Mizrak et al., 2012). Accounting for these circadian regulated electrical properties, an additional artificial depolarization at the beginning of the subjective day (*advance zone*) without the counteracting performance of depolarized E cells (like it has been the case in *clk856/TrpA1* flies) may further increase and even temporally expand the advancing ability into the subjective day, as the threshold to generate action potentials is achieved much easier. A fundamental role in advancing the phase of behavior for the l-LN_vs has already been reported (Shang et al., 2008). The l-LN_vs were able to phase advance the activity in response to LPs administered at ZT 21. This response was highly dependent on the number of l-LN_vs being present: The less l-LN_vs were present, the weaker the advances were, supporting the view that the l-LN_vs mediate light information and serve as intersect of light-input from the photoreceptive organs to the circadian clock network. This effect was restricted to the advance zone, since no significant differences could be detected among controls and the experimental flies, which were lacking the l-LN_v neurons, at ZT 15.

Even though the l-LN_vs are implicated in light-dependent advance phase shifts, it is to point out that the depolarization-PRC of the PDF^+ neurons is definitely not mirroring a light-PRC. A typical light-PRC exhibits a pronounced dead zone, which is strongly narrowed in the depolarization-mediated PRC of *Pdf/TrpA1* flies. What can account for these differences?

Light has direct impact on the l-LN_vs' neuronal excitability mediated via CRY (Sheeba et al., 2008) and is perceived additionally by the photoreceptive organs of the fly (the

compound eyes, the ocelli and the H-B eyelet). Among these the photoreceptors of the compound eyes and the H-B eyelet have been shown to be histaminergic (Pollack & Hofbauer, 1991; Yasuyama & Salvaterra, 1999), what makes them predestinated to deliver light-dependent inhibitory input to the clock by coupling to histamine-gated chloride channels (HisCl) in downstream neurons (Hardie, 1989; Pantazis et al., 2008) such as the l-LN$_v$s (Hong et al., 2006). Whenever there is light, it is perceived by the photoreceptive organs, which then might release histamine (Helfrich-Förster, 2002). At the same time the l-LN$_v$s increase their firing rate and membrane potential in response to the similar photic stimulus mediated by CRY (Shang et al., 2008; Sheeba et al., 2008; Fogle et al., 2011). After its release, histamine might bind to HisCl on the l-LN$_v$s, causing an electrical inhibition or at least a partial compensation for the depolarization, which occurs due to cell-autonomous light-perception by CRY. The inhibitory input might prevent l-LN$_v$ firing, determining the dead zone of a light-PRC. That implies that the l-LN$_v$s might serve as coincidence detector or computational unit that processes depolarization signals generated by the l-LN$_v$s cell-autonomously due to light-perception via CRY and the inhibitory inputs from the photoreceptors of the compound eyes or the H-B eyelet via HisCl. Along that line, Kistenpfennig et al. postulated (2012) that, as CRY-less flies' light-sensitivity has to be mediated by the photoreceptive organs to obtain phase shifts in light-PRC experiments, certain photoreceptors may transmit photic information to the clock only in the morning and the evening, thus in the advance and delay zone. Hence they suggested that different photoreceptive organs might be responsible for certain parts of the PRC (Kistenpfennig et al., 2012), being supportive of the idea that there might be a possible mechanism of inhibition restricted to dawn.

Experimental evidence, which reinforces the notion of light-mediated inhibition in the subjective morning was delivered by patch clamp recordings of l-LN$_v$s after histamine application to explanted brains. These neurons responded with a severely dampened spiking frequency, thus electrical inhibition, which was highly dependent on the dose and duration of histamine application to the brains (Buhl E., personal communication; Schlichting PhD-thesis, 2015). Along that line, histamine was described to have delaying effects on the clock's output, as histamine deficient flies (*hdc*^{JK910} = histidine decarboxylase; loss of function mutant; (Burg et al., 1993)) were unable to properly delay their E peak (Schlichting PhD-thesis, 2015). That bears functional relevance, as it has been shown that photic input signals from the eyes to the clock efficiently

decelerate molecular oscillations (Rieger et al., 2006). The l-LN$_v$s are moreover morphologically ideally positioned to comply with that task, as they are exhibiting extensive arborizations within the optic lobes and between both hemispheres (Helfrich-Förster et al., 2007). Hence, this data supports the notion of the existence of inhibitory photic input from the photoreceptive organs to the clock via histamine, which intersects most probably at the l-LN$_v$s to the clock network.

But apart from the processes taking place in LD conditions, such as light-mediated inhibition, *dTrpA1*-PRC experiments were performed in DD, thus lacking any photic input. Instead, the l-LN$_v$s, which exhibit higher intrinsical electrical activity during the early day anyway (Sheeba et al., 2008), were further depolarized by *dTrpA1*. As light-input was described to increase membrane potential in a CRY dependent manner (Fogle et al., 2011), the artificial depolarization might mimic CRY-mediated cell-autonomous excitatory light input on the cellular level of the l-LN$_v$s. However, the inhibitory photic input via histamine to the l-LN$_v$s, which is released from the photoreceptor cells of the compound eyes and/or the H-B eyelets is missing, as the experiments were performed in DD. Hence no inhibition of the l-LN$_v$s occurs and the depolarization of the l-LN$_v$s does not have to compensate for the inhibition at that time. Instead, the neurons are further excited due to artificial depolarization, exceeding the thresholds for action potential generation, likely causing the observable phase shifts and the enhanced advance zone from CT 1 to CT 6/7. That notion fits quite well, if one considers that on the molecular level short-term temporal electrical silencing keeps PER levels unaltered in the LN$_v$s (Depetris-Chauvin et al., 2011), but that permanent silencing changes PER levels (Nitabach et al., 2002, 2006). This suggests that in LD conditions indeed short-term temporal electrical silencing/inhibition might occur at that particular time of the circadian cycle. That may happen without any interruption of the molecular cycling of the clock, thus leaving PER levels unchanged. Hence unaltered PER levels may resemble the PER oscillation status in that part of the PRC, resulting in the distinctive “buffered” behavior of flies and in the shape of the dead zone in a standard light-PRC.

Even though the l-LN$_v$s appear to be essential neurons in mediating the alterations in phase in *Pdf/TrpA1* flies, the role of the s-LN$_v$s, which have been addressed in *Pdf/TrpA1* flies as well and the signaling pathway from the large to the small LN$_v$s had to be investigated. This is of particular interest since the s-LN$_v$s as M cells and main pacemakers are known to be strongly coupled to the rest of the network via

PDF signaling and glycine as a classical fast neurotransmitter (Yasuyama & Meinertzhagen, 2010; Gorostiza et al., 2014; Frenkel et al., 2016 in revision), which enables them best to control and to reset the whole network.

In contrast to the quite conspicuous advance zone phase shifts, the *delay zone* shift is more modest in *Pdf/TrpA1* flies. The l-LN$_v$s, which are probably generating the huge effects in the PRC's advance zone (apart from the s-LN$_v$s), have been reported to be unable to mediate delay zone phase shifts in response to light (Shang et al., 2008). Nevertheless, both small and large LN$_v$s were reported to be sufficient for phase delaying the clock (Guo et al., 2014). To figure out whether just the s-LN$_v$s might be responsible for the ability of phase shifting in *Pdf/TrpA1* flies, another set of experiments (*R6/TrpA1*) was performed, which allowed the exclusive depolarization of the s-LN$_v$s. Depolarizing the s-LN$_v$s produced proof of the importance of the s-LN$_v$ neurons for phase advancing the clock, as *R6/TrpA1* flies exhibited strong advances, which were comparable to *Pdf/TrpA1* shifts, when depolarized in the advance zone. The shifting ability of *R6/TrpA1* flies, however, was restricted to the advance zone, as there was no significant difference relative to controls in the delay zone. This data supports the notion that both s- and l-LN$_v$s might be necessary for delay zone phase shifts as it was shown that l-LN$_v$s alone were not able to phase delay (Shang et al., 2008) and as just *Pdf/TrpA1*, but not *R6/TrpA1* flies exhibited delays. Furthermore, the huge advances obtained by *R6/TrpA1,* however, indicate that apart from the l-LN$_v$s also the s-LN$_v$s might be a target of inhibition by a pathway comparable to the inhibitory photic input of histamine to the l-LN$_v$s. Histamine as a direct inhibitory signal to the s-LN$_v$s appears unlikely, as the s-LN$_v$s are HisCl negative (Hong et al., 2006). However, other inhibitory neurotransmitters like γ-aminobutyric acid (GABA) can comply with that task in the s-LN$_v$s, since GABA$_B$ receptors have been identified on the s-LN$_v$s (Dahdal et al., 2010). Along that line, it was demonstrated that the co-application of GABA with the cholinergic receptor agonist carbamoylcholine is able to significantly reduce the excitatory Ca^{2+} responses to carbamoylcholine in the LN$_v$s and decreases cAMP levels, indicating also an important role of GABA on the phase and/or period of the molecular clock in the s-LN$_v$s (Lelito & Shafer, 2012).

Phase shifts after the depolarization of the PDF$^-$ neurons

To further decipher the role of the PDF$^+$ neurons in *clk856/TrpA1* flies, phase shifting experiments using *PdfGAL80* in *clk856/TrpA1* were performed to exclude PDF$^+$

neurons from depolarization via *dTrpA1*. Thereby I focused on the delay and advance zone, respectively. Excluding the PDF$^+$ neurons, thus the s-LN$_v$s (M cells) and the l-LN$_v$s from depolarization in the advance zone caused significantly weaker phase advances (about 1 hr; Fig. 21, gray bar) compared to *clk856/TrpA1* (about 3 hrs; Fig. 21, black bar).

Considering the M cells mainly being able to advance the phase of activity in response to PDF and assuming the l-LN$_v$s especially being important for phase advancing behavior (Shang et al., 2008), the diminished advancing ability of *clk856PdfGAL80/TrpA1* flies meets the expectations. The M cells' key role for phase advances is also further proven by the exclusive depolarization of the s-LN$_v$s (*R6/TrpA1*) or both the s- and l-LN$_v$s (*Pdf/TrpA1)* in the present work. These experiments demonstrate that the neuronal activation of the s-LN$_v$s is sufficient to evoke strong phase advances and indicate along with the *GAL80* experiments that the M cells (and the l-LN$_v$s) might also be responsible for the phase advances in *clk856/TrpA1* flies.

TPs applied in the delay zone elicited significant phase delays in *clk856/TrpA1* flies (about 5 hrs), but not in *clk856PdfGAL80/TrpA1* flies (about 1 hr). Latter ones were not significantly different from controls (Fig. 21, white bar), indicating that the simultaneous depolarization of PDF$^+$ neurons and E cells is necessary to cause strong phase delays (*clk856/TrpA1*). The exclusive depolarization of E cells (with others, as the DN3s, LPNs, DN2s), though, appears not to be sufficient to elicit phase delays (*clk856PdfGAL80/TrpA1)*, but requires at least the activation of both clusters of PDF$^+$ neurons (s-LN$_v$s, l-LN$_v$s) (*Pdf/TrpA1*), since the depolarization of the s-LN$_v$s just advanced, but did not delay the clock (*R6/TrpA1*). The activation of both PDF$^+$ neuron groups, however, was able to cause modest delays (*Pdf/TrpA1*). These results are consistent with the data of Guo et al. (2014), as they reported that the simultaneous depolarization of M and E cells (PDF$^+$ LN$_v$s, 4 LN$_d$s, 5th s-LN$_v$ using *DvPdfGAL4* (Bahn et al., 2009)) causes strong delays, but the exclusive depolarization of most of the E cells (four LN$_d$s and the 5th s-LN$_v$) was not able to shift the phase of activity in the early night (about 0.5-1 hour).

Although the *PDFGAL80-* results measured up to our expectations and shifts of *clk856PdfGAL80/TrpA1* flies were not significantly different from controls but from *clk856/TrpA1* flies, one has to keep in mind that eventually due to the properties of the *PdfGAL80$_{96A}$* construct the inhibition of the *GAL4* expression might have been

interrupted for three hours, as the TP of 32°C was applied for this fraction of time. Comparable to the general temperature dependence of *GAL4*, *PdfGAL80$_{96A}$* might be temperature dependent as well, even though, Stoleru and colleagues, who created this fly line in 2004, did not comment on that possibility. Experiments in our department, however, indicate that the long-term maintenance at higher temperatures (< 30°C) of experimental flies, carrying the *PdfGAL80$_{96A}$* construct may impair the ability of *PdfGAL80$_{96A}$* to prevent *GAL4* expression (C. Luibl, P. Menegazzi, personal communication). Even though this effect might just apply to experiments that necessitate permanent high temperatures, this possibility cannot be excluded.

Phase shifts after the depolarization of the DN1s

As the essential role of the PDF$^+$ neurons in phase shifting has been shown in prior experiments, it was to find out, whether some of the dorsally located downstream target neurons of the PDF$^+$ neurons can at least partly mimic *Pdf/TrpA1* or *R6/TrpA1* effects after artificial activation. Even though the DN1$_p$s are known to be sufficient to generate the M peak in LD (Zhang et al., 2010; Seluzicki et al., 2014), the depolarization of a subset of those clock neurons (8-10 DN1$_p$s) using *clk4.1M/TrpA1* did not significantly shift the phase of locomotor activity neither in the delay nor in the advance zone of the PRC. This outcome was not necessarily expected, as it has been shown that signaling from the DN1s via glutamate is indeed able to alter the pace of the clock by influencing the LN$_v$s: Glutamate released from the DN1 and perceived by the LN$_v$s in LD and DD (Hamasaka et al., 2007) is able to play two distinct roles in the *Drosophila* circadian circuit depending on the receptor that receives the signal in the LN$_v$s. Glutamate can promote LN$_v$s synchrony when received by the metabotropic glutamate receptor mGluRA, which mediates long-term regulatory processes of the clock (Collins et al., 2012, 2014) and modulates the rhythmic adult behavior pattern (Hamasaka et al., 2007). Another receptor, the ionotropic GluCl receptor was shown to be responsible for rapid behavioral responses to light as well as for circadian regulation of light-avoidance behavior in larvae (Collins et al., 2012). Although glutamate release from the DN1s may represent a potential pathway that reinforces rhythmicity (Hamasaka et al., 2007), its ability might be restricted, as it is of common notion that the s-LN$_v$s are the main pacemakers in DD. Since the s-LN$_v$ neurons were not depolarized via *dTrpA1* in

clk4.1M/TrpA1 flies, the DN1s may not be able to sufficiently counteract the molecular clock's status of the master pacemaker. Furthermore, the cluster of DNs exhibits a high degree of heterogeneity, as just half of them express for instance PDFR and CRY, suggesting also great differences in their network properties and in their susceptibility to artificial depolarization as well as in their output. Along that line, the DNs are described to require PDF signaling from the LN_vs to exhibit a synchronous molecular cycling in DD among each other (Yoshii et al., 2009), further underpinning their diversity. The activation of a subset (just 8-10 $DN1_p$s) of this in any case very diverse cell group, hence may have different effects on particular neurons of this cluster, which may be counteracting and preventing distinct signaling to other clock neuron groups to generate major alterations in clock output.

Phase shifts after the depolarization of M and E oscillator neurons

Next, it was to test whether an artificial activation of M cells, a small number of l-LN_vs and some E cells (Grima et al., 2004), using the *mai179GAL4*-driver line would resemble rather an ubiquitous or a PDF^+ cell restricted neuronal activation. The artificial depolarization did not significantly delay, but did significantly advance the phase of activity in comparison to controls, indicating that the depolarization of the M cells (s-LN_vs) predominate the network, thus causing phase advances. The synergistical depolarization of some E cells (some DNs, three LN_ds, the 5^{th} s-LN_v per hemisphere) together with the M cells and some l-LN_vs, however, appears not to be sufficient to cause phase delays, as there was no significant difference between *mai179/TrpA1* flies and respective controls. How to explain that result? *Pdf/TrpA1* flies exhibited modest delays, but *R6/TrpA1* flies did not. The only difference between both genotypes is the lack of depolarization of the l-LN_vs in *R6/TrpA1* flies, indicating that the l-LN_vs might be necessary (together with the s-LN_vs) to evoke phase delays. As the *mai179GAL4*-driver line drives expression just in a small number of l-LN_vs, the depolarization of only a few l-LN_vs might not be sufficient to phase delay the clock. That appears plausible, since such a quantity-dependent effect was already described for the l-LN_vs in light-mediated advancing behavior (Shang et al., 2008).

Alternatively one can challenge the dual oscillator model by raising a (less likely and daring) hypothesis: The three LN_ds and the 5^{th} s-LN_v, which are included in the *mai179GAL4*-driver line are CRY^+ neurons. In general, it was suggested by Yoshii et

al. (2010) that the presence of CRY might be an indicator for preferential entrainment to light, while it has been shown that the CRY^- LN_ds are preferentially entrainable to changes in temperature (Yoshii et al., 2005; Busza et al., 2007; Miyasako et al., 2007; Tomioka et al., 2008). The CRY^+ E cells actually share these preferential entrainment properties to light relative to temperature with the LN_vs, thus also with the M cells. In LD conditions CRY's function in E neurons is to phase advance, hence to keep E activity during daytime by mediating TIM degradation followed by a decline in PER at the beginning of the day, hence determining E activity onset (Menegazzi et al., 2013). Further experiments have shown that CRY overexpression in E cells is able to advance E activity in LD and is also sufficiently able to counteract the PDFR-mediated signaling in these cells (Zhang et al., 2009; Seluzicki et al., 2014). PDF is known to accelerate the speed of the clock in $PDFR^+$ M cells, whereas it slows down the clock in $PDFR^+$ E cells (Yoshii et al., 2009; Duvall & Taghert, 2012, 2013). Thus, the deceleration effects in the E cells in response to PDF might be prevented by (over)expression of CRY. Indeed CRY^+ LN_ds and 5^{th} s-LN_v have been reported to escape the decelerating PDF signal, instead behaving and responding like M cells and exhibiting a lengthened period in DD (Yoshii et al., 2009). Another publication was able to show that the membrane depolarization of CRY^+ neurons is probably potent to mimic light input, but independently of CRY-TIM interactions in a cell-autonomous manner (Fogle et al., 2011). Effects like these have been described for the l-LN_vs, which among other clock neurons express CRY (Yoshii et al., 2008). They responded to moderately bright light by elevation of spontaneous firing frequency up to 200%. This electrophysiological light response was highly dependent on CRY and on the conductance of membrane potassium channels. The light-activation of CRY appears to cause an increase of neuronal membrane potential, hence the depolarization of clock neurons by increasing the firing rate via potassium channel modulation (Fogle et al., 2011), meaning that CRY activation causes the depolarization of the cell. That in turn may activate downstream signaling pathways in the neuron, which influence the pace of the clock, thus accelerating the clock in a way probably working like the mechanism, which causes the phase advances of the E peak in LD via CRY. These effects might exclusively apply to the l-LN_vs, as they are the light-mediating and arousal-clock neurons, however, the underlying mechanism could theoretically occur in other CRY^+ neurons as well, albeit experimental evidence is missing. In *mai179/TrpA1* flies three CRY^+ LN_ds and the

CRY^+ 5th s-LN_v per hemisphere as representatives of the E cells were depolarized, additionally to the M cells and to a small number of l-LN_vs (Grima et al., 2004). Even though they are E neurons according to the original classification of the dual oscillator model, they might behave differently in that particular approach. It is of common notion that after PDF-neuron depolarization PDF is most probably released for phase adjustment of certain clusters of the clock network, thus decelerating E cells (Yoshii et al., 2009; Guo et al., 2014). But as CRY has been shown to be able to antagonize PDFR signaling (Zhang et al., 2009), meaning in the case of the CRY^+ E cells preventing the deceleration of the clock, it might be possible that these delaying effects on the E cells are attenuated or abolished. The strong artificial depolarization and thus activation of the CRY^+ E cells may resemble a molecular situation like it would be for CRY overexpression, which has been shown to be potent to advance the phase of the E peak, at least in LD conditions.

But since the network of the circadian clock is obviously a system of extraordinary high complexity (e.g. Yao & Shafer, 2014) and the dual oscillator model of accelerating one and decelerating the other part of the clock (Pittendrigh & Daan, 1976) is oversimplified, the illustrations done so far remain as hypotheses, which have to be elucidated by further experiments.

The depolarization of different subsets of clock neurons reveals the plasticity of the system

The first subset of experiments makes me summarize that the simultaneous depolarization or neuronal firing of neither the M cells (*Pdf/TrpA1, R6/TrpA1),* nor of some of the CRY^+ DN1s (*clk4.1M/TrpA1*) nor of the M and some of the E cells (*mai179/TrpA1*) together mimic a typical light-PRC. However, the simultaneous depolarization of all clock neurons (*clk856/TrpA1*) resembles the shape of a type 1 light-PRC the most.

The effect of firing dependent phase shifting appears to be highly dependent upon the time window the TP was applied as well as on the clock neuron type being addressed by depolarization. Whereas depolarization-dependent effects in the delay zone seem to be additive, thus enhancing phase shifting when all clock neurons were activated, this might not apply to the dead zone or advance zone of a PRC. Delays could be obtained by activation of the PDF^+ neurons (*Pdf/TrpA1*) as well as by ubiquitous clock neuron (*clk856/TrpA1*) depolarization, supporting the view that light-

PRC-like delays seem to require both the depolarization of the PDF^+ neurons and of some E neurons. In the dead and advance zone, processing mechanisms of E and M cell electrical activity might be regulated in more complex manner with regard to the resulting clock output. The advancing phase shifts can be achieved by M cell depolarization, even to a much higher extent than in light-PRCs with regards to the temporal range of the advance zone. The inhibitory input putatively derived from the photoreceptors or other neurons (clock neurons/interneurons) to the clock in the advance zone might thus indeed restrict the temporal occurrence during the day. These indications bring me to the conclusion that certainly the advancing ability of the clock might be regulated in a more complicated way, including not just electrical activation, but also receiving inhibitory photoreceptive input that enables adaptation. This is supported by the assumption of Guo et al. (2014), postulating that under normal conditions delays were more simply firing-dependent than advances, whereas the latter ones may require a more complicated combination of light and firing.

The system gains further complexity if one considers other putatively involved factors, which have been just shortly if at all discussed in the hypotheses so far: Apart from PDF, which is undoubtedly the most important peptide orchestrating the synchronization of the network, also other neuropeptides have been shown to be expressed in certain clock neuron clusters. Some LN_ds do not just express the PDFR and CRY, but also Neuropeptide F (NPF), apart from the 5^{th} s-LN_v and some l-LN_vs (Hermann et al., 2012), which has been reported to be relevant for circadian rhythmicity in general (Lee et al., 2006; Hermann et al., 2012; He et al., 2013). The NPF receptor (NPFR) might be expressed in some DN1s and some LN_ds, indicating that NPF might act as another intra-network communication signal among the clock neurons. Small neuropeptide F (sNPF) exhibits also a restricted expression pattern with regard to the clock network, as it is expressed in the s-LN_vs and two CRY^+ LN_ds (Johard et al., 2009). sNPF is mainly thought to control sleep and to mediate circadian output of the clock, as it causes the hyperpolarization of motor centers (Vecsey et al., 2013). Another peptide, Ion transport peptide (ITP), is expressed in just one LN_d, which is NPF^+, CRY^+ and $PDFR^+$ and in the 5^{th} s-LN_v, which also expresses CRY and PDFR (Johard et al., 2009). ITP in the circadian clock network is mainly setting the timing of the E peak and is slightly shortening the free-running period. Thus ITP appears to counteract PDF, which controls the M peak and

lengthens the period in DD (Hermann-Luibl et al., 2014). However the neurons, which express the receptor of ITP are not known so far. Apart from the neuropeptides, also classical neurotransmitters as glycine (Frenkel et al., 2016 in revision), GABA (Dahdal et al., 2010), acetylcholine (Johard et al., 2009), histamine (Pantazis et al., 2008; Schlichting PhD-thesis, 2015) and glutamate (Hamasaka et al., 2007; Collins et al., 2012, 2014) affect circadian organization of activity. Furthermore, direct electrical contacts via gap junctions among cells are known to mediate fast information transfer. Gap junctions have been long disregarded in chronobiology, but as there is a huge variety of neuronal contact in e.g. the superior protocerebrum or the accessory medulla (aMe) between the DN1s and the s-LN_vs or the LN_ds and the s-LN_vs (Beckwith & Ceriani, 2015), which do additionally experience pruning and outgrowing (e.g. of the dorsal terminals of s-LN_vs) in a circadian regulated manner (Gorostiza et al., 2014), the investigation of gap junctions being involved in clock neuron communication is overdued and might allow a more detailed understanding of the circadian network.

Although there are pieces of evidence for excitatory and inhibitory light-input pathways to the clock, the exact mechanism how light-information is transduced to and mediated among the clock neurons remains elusive. The data of the present thesis proofs further evidence for the involvement of a putative inhibitory input from the photoreceptive organs to the clock. The mention of all putatively involved peptides, neurotransmitters and gap junctions should point out how little we actually know about the orchestration of the whole network, how complicated it is regulated and how highly plastic the fly‘s circadian clock is (reviewed in Muraro et al., 2013). To deal with complex environmentally fixed and predictable oscillations as well as with stochastically and variable events, requires both the ability to respond to changes in relevant cues and the ability to prevent responses to uninformative input (Beckwith & Ceriani, 2015). For this reason the hierarchy of clock neurons in the clock network seems to be highly plastic and flexible to be best prepared for a changing environment.

The impact of short-term temperature pulses on the clock’s behavioral output

Another issue that necessarily has to be discussed is the PRC of heterozygous controls, which did not express *dTrpA1* and are thus not depolarized, but did just experience the TP *per se*. The PRCs of these controls nicely illustrate the influence

of temporally restricted increases of ambient temperatures, thus of TPs from 20°C to 32°C (ΔT 12°C). TPs in the early night cause modest delays of about 2 hours. Late night to early day TPs result in moderate phase advances of around 2 hours. Thus, the shape of the temperature-PRC is quite comparable to that of a low intensity light-PRC, as it exhibits rather low amplitude. PRC-amplitudes are highly stimuli-dependent (Johnson, 1990). Light, which is the most important Zeitgeber, would *per se* yield higher PRC-amplitudes than secondary entrainment cues like e.g. temperature (Pittendrigh, 1960). Although, not only the type of stimulus is valuable, but also stimuli duration and intensity do both substantially influence the general shape of a PRC (Johnson, 1990). In the last years several publications reported of quite high ambient temperatures, which appeared to be required to properly phase shift the circadian clock of *Drosophila* (Edery et al., 1994; Sidote et al., 1998; Kaushik et al., 2007). In wild type *CantonS* flies TPs of 37°C for 30 minutes have been shown to evoke just early night delays, but no late night advances (Sidote et al., 1998). TPs below 37°C, such as TPs of 30°/34°C, did cause no or just little behavioral phase shifts (Kaushik et al., 2007), indicating that shifting the clock by modest increases of temperature appears to require longer application times lasting several hours (Zimmerman et al., 1968; Chandrasherkan, 1974). Temperatures of 32°C for 3 hours, as the phase shifts of the controls in this thesis indicate, could be sufficient though to phase shift the activity in the delay as well as in the advance zone. Thus, lower temperatures than 37°C, but longer TP durations are able to enhance the shifting ability in *both* directions, into the advance as well as into the delay zone. Although the flies used as controls in the present thesis are no wild type flies, they should not comprise any genetic differences to the wild type, which might improve or impair the shifting ability of the clock, thus considering them to display a wild type-like phase shifting pattern and PRC.

But why do controls of this thesis in contrast to flies of Sidote et al. (1998) exhibit both advances and delays? First, one has to keep in mind that the shape of a PRC might slightly differ due to stimulus strength and duration. Sidote and colleagues (1998) tested flies, which experienced a TP for 30 minutes of 37°C, thus they applied shorter lasting but higher TPs than used in this thesis. Furthermore, by just applying TPs at CT15 and CT21, at time points, which are surely known to cause the greatest phase shifts in a standard light-PRC (e.g. Pittendrigh & Minis, 1964; Pittendrigh, 1967), but not necessarily in a temperature-PRC, one can miss (as it turned out to be

true for *Pdf/TrpA1* flies in the *dTrpA1* experiments by Guo et al. (2014)) crucial time points. Along that line, also controls of this thesis exhibit a phase delay of about 2 hours at CT 15, but nearly no or just little phase advance at CT 21, which is quite consistent with their phase shifting data at CT 15 and CT 21. Nonetheless, the control flies of this work indeed exhibit an advance, since the advance zone begins later around CT 22 and ranges to around CT 3, which they would have missed. Furthermore, reading their “Material & Methods” section indicates that their way of locomotor data acquisition was somehow unfortunate, since they had to remove the flies from the TriKinetic monitors to apply the TP in a water bath. To eliminate startle responses evoked by the handling, they simply excluded the consecutive days after temperature treatment from the calculation, however, that procedure may possibly have caused some side effects or artifacts, since locomotor behavior is a quite sensitive read out. Moreover, the fact that TPs applied in the delay zone (CT 15) caused strong phase delays in behavior, which paralleled long-term delays in PER and TIM cycles in abundance and phosphorylation for at least 2 days, whereas TPs in the advance zone caused nearly no phase advances in behavior, but significant differences in PER and TIM protein levels, suggests that Sidote and colleagues (1998) may have overlooked behavioral phase advances. That might probably have happened because of choosing the wrong time point for testing the flies, due to their experimental protocol, the execution of their phase shift experiments and/or data analysis.

But what might be the underlying mechanism causing phase shifts, when TPs were applied in the advance or delay zone? Since Sidote and colleagues (1998) were able to show that the decline and accumulation of PER and TIM as well as the mRNA cycling pattern of both were consistent with the effects in behavior in magnitude and direction, like it is for light, the question rose whether the same mechanisms are in charge to mediate phase shifts after light as well as after TP application. If true, the mechanisms after TP application might be in analogy with the LP mechanisms: Delays might result from the delayed decline in the levels of *per* and *tim* transcripts, following a TP at CT 15. This might be caused by the retarded nuclear entry of the PER-TIM complex, like it was described for light-PRCs (Lee et al., 1996). The delayed entry of PER-TIM is most probably due to a longer lasting degradation of TIM in the cytoplasm. In contrast, the advancing effect can be explained by premature disappearance of nuclear TIM in the late night, which is accompanied by

the earlier hyperphosphorylation and degradation of PER. Both TIM and PER require the presence of each other to enter the nucleus (Vosshall et al., 1994; Myers et al., 1996; Saez & Young, 1996), suggesting that the temporary absence of either clock protein in the cytoplasm will advance the entire cycle.

But what kind of mechanism is able to mediate temperature information to the clock, thus facilitating longer lasting or premature TIM degradation, like CRY does in the case of light? Temperature-sensitive splicing events of *tim* and *per* genes have already been described several years ago (Majercak et al., 1999, 2004; Boothroyd et al., 2007), which affect PER and TIM abundance and are thus able to influence the pace of the clock. Sidote et al. (1998) were able to detect shifts in *tim* and *per* mRNA, when TPs were applied at CT 15. This is in accordance to the finding that the 3'–terminal intron of *per* is spliced to a much lower extent at elevated temperatures, which then cause phase delays, since there is a delayed protein accumulation (Majercak et al., 1999, 2004; Boothroyd et al., 2007). However, Sidote and colleagues (1998) did not find any differences in mRNA levels in the advance zone, suggesting that the transcription level may not be affected at CT 21. In that case a posttranscriptional mechanism, which is regulated in a thermally sensitive manner, might affect core clock protein levels, as PER and TIM levels were strongly shifted. This posttranscriptional mechanism, however, might be restricted to the advance zone of the PRC.

The impact of short-term temperature pulses on Drosophila melanogaster

All mentioned explanations for the effects in behavior underline the high capability of the organism and the clock to sense and process temperature information as a potent Zeitgeber. The effect of TPs on the clock, which can distinctively phase shift circadian locomotor activity, was mainly discussed in the thesis part right above. However, temperature *per se* can effectively bypass the clock, evoking so-called masking effects and physiological processes of the organism can be affected that purely arise from elevated temperatures. That brings me to the big CAVEAT of the thermogenetic approach:

Raised temperatures *per se* can cause effects deriving from heat shock response mechanisms of the organism. Elevated temperatures such as 37°C used by Sidote et al. (1998) are highly potent to elicit a full-blown heat shock response in *Drosophila melanogaster* (Velazquez et al., 1983; Lindquist; 1986). More modest temperatures,

however, like e.g. 29°C and below just cause slight, if at all heat shock protein induction in *Drosophila* cells (Velazquez et al., 1983). The usage of thermogenetic tools like *dTrpA1*, whose kinetics allow the channels to open just above 29°C in a physiological context (like in a freely moving fly; Bernstein et al., 2012), may provoke slight heat shock responses and epiphenomena unrelated to the circadian resetting mechanism, as thermally denaturated proteins are prime targets for proteolysis by the ubiquitin-proteasome system (Sidote et al., 1998; Hershko & Ciechanover, 1992; Jentsch, 1992). On account for this, pure temperature-induced shifts have to be subtracted from the depolarization-mediated phase shift effects. Just after the consideration of these “background”-phase shifts, the evaluation of the phase shift data can be conducted in circadian respects. Therefore all experiments of experimental lines and controls have necessarily been performed in parallel to minimize side effects and artifacts. Despite that disadvantage, thermogenetic tools have been proven to be best suited to manipulate neurons in a temporally restricted manner in flies, which additionally enable the investigator to easily address specific neurons by means of the *GAL4/UAS*-system. Optogenetics, which are currently rushing into the field of neurobiology, may offer further prospects for experiments. But also the optogenetic approach comprises some weaknesses for chronobiologists, since most of the channelrhodopsins exhibit excitation spectra, which broadly overlap with the excitation range of CRY or even the rhodopsins of the compound eyes (Salcedo et al., 1999; Stavenga & Arikawa, 2008; Ozturk et al., 2011; Dawydow et al., 2014). However, there are now optogenetic fly lines available, whose excitation spectra are 45 nm red shifted relative to previous channelrhodopsins, which allow the activation of neurons using longer wavelengths (Klapoetke et al., 2014). Nevertheless, since light is the most important Zeitgeber, which is even more potent than temperature, optogenetics do not preserve from performing the experiments with caution, albeit side effects like high temperature artifacts (e.g. heat shock responses) can be excluded (reviewed in Bernstein et al., 2012).

4.2 Clock network properties – PER Protein Cycling & CREB

Persistence of PER cycling in constant conditions

One of the hallmarks of the circadian clock is the free-running ability even in absence of any Zeitgebers. This free-running ability can be observed in the behavior of single housed flies, when kept in constant temperature and constant light conditions, e.g. at

20°C and in constant darkness (DD). Flies maintained under these conditions and recorded in *Drosophila* activity monitors (DAM) show rhythmic locomotor activity with a period of about 24 hours, depending on the particular genotype. Locomotor behavior as such, as most evident clock output, is assumed to widely mirror the state of the molecular clock with PER being a core clock representative. PER degradation and accumulation kinetics, apart from TIM protein kinetics, are most influential in determining the state of the molecular clock. Thus, the pattern of PER protein cycling is thought to parallel the behavioral output of the clock even after a manipulation. Monitoring PER protein cycling of flies with free-running, thus unaltered clocks, demonstrates that most of the clock neuron groups keep their pace of cycling in high synchrony to other clusters. The amplitude of PER accumulation in each cluster is maintained at least from the second day in DD on without any dampening effects, except of three clock neuron groups.

Among those clock neuron groups, which are exhibiting a dampening of PER oscillation in DD are the l-LN$_v$s. These neurons were demonstrated to be important for arousal and for mediating light-input to the clock (Shang et al., 2008). Without any photic input, however, they might lose their trigger and thus their group-internal synchrony. In constant darkness single l-LN$_v$s have been described to get out of molecular synchrony from the second cycle in constant conditions on (Shafer et al., 2002). On the first day of DD PER was reported to remain mainly in the nucleus of the l-LN$_v$s, whereas TIM gets predominantly cytoplasmic. On the second day in DD PER and TIM were almost undetectable (Shafer et al., 2002), indicating that the separation of PER and TIM in different cellular compartments cause the degradation of both proteins, since they need to dimerize to prevent protein phosphorylation and subsequently degradation. The second clock neuron cluster that lack prominent PER cycling in constant conditions was the group of the DN2 neurons. These neurons are thought to be responsible for the temperature input to the clock (Picot et al., 2009; Yoshii et al., 2010). But as there are no temperature cues in constant conditions (DD, constant 20°C), the DN2 might lose their trigger for maintaining PER cycling synchrony like the l-LN$_v$s do without light-input. The third group is that of the LPNs. The LPNs are also thought to be involved in temperature entrainment, since other functions are not known so far (Miyasako et al., 2007; Yoshii et al., 2010). As such the LPNs, maybe due to the same reasons as for the DN2s, might lose their internal synchrony, causing the dampening of the PER oscillation.

So far it was not surely known whether there are inhibitory mechanisms actively causing the dampening of PER cycling in the l-LN$_v$s, DN2s and LPNs in DD. This data, along with the data of a prior publication (Shafer et al., 2002), support the notion that the molecular cycling in individual clock neurons of each of those three clusters rather gets out of synchrony than being inhibited by another mechanism. Thus the dampening effect of PER oscillation rather derives from the way of data analysis, as PER levels are quantified and averaged for all cells of a brain and genotype of an experiment.

The impact of short-term temperature pulses on the molecular clock

The comparison of temperature pulsed and non-temperature pulsed controls allows the evaluation of the impact of TPs on the molecular clock *per se* without the involvement of depolarization. TPs seem not to affect PER protein kinetics significantly, since the PER cycling of both controls was not different from each other. Sidote and colleagues (1998) investigated the influence of TPs (37°C) on the clock of wild type flies and demonstrated that TPs at all times in a daily cycle elicited dramatic and rapid decreases in PER and TIM protein levels (Sidote et al., 1998), even in the absence of a functional clock in *per*^{01} and *tim*^{01} mutants. However, those effects were restricted to delays, as just little if any advance zone shifts of PER and TIM cycling in abundance and phosphorylation occurred. In the advance zone PER and TIM accumulation profiles of temperature pulsed flies and untreated controls were undistinguishable from each other in their hands, even though TPs administered in the late night were accompanied by transient and rapid increases in the speed of PER-TIM cycling in experimental flies (Sidote et al., 1998). They observed first a temperature-induced degradation of PER and TIM, followed by an extremely quick PER and TIM (re)accumulation, which was in turn followed by another round of degradation. Since mRNA levels of *per* and *tim* were widely unaltered in comparison to their untreated controls, they suggested that the mechanism(s) taking place in the late night, has/have to be posttranslationally regulated, but are highly potent to advance PER and TIM protein cycling. TPs in the early night caused strong phase delays, which exhibited protein degradation kinetics like after a LP.

The PER cycling data of all controls in this thesis (temperature pulsed controls vs. non-temperature pulsed controls) did not show these effects after TP application, neither in the advance nor in the delay zone, indicating that the shifts of PER and

TIM accumulation they observed mainly arose or were at least reinforced by temperature dependent degradation processes such as heat shock responses. Such heat shock responses have been shown to be already elicited by elevated temperatures above 29°C (Velazquez et al., 1983; Lindquist, 1986), suggesting that heat pulses of 37°C definitely elicit these responses, thus causing premature protein degradation via the proteasomal pathway, in turn allowing an earlier transcription of *per* and *tim* mRNA. If the effect on PER and TIM degradation might be due to heat shock responses, that kind of protein degradation would be unspecific and not restricted to clock proteins. That might be the case, since Sidote and colleagues (1998) mentioned that the effects were independent of a functional clock, as the effects could also be observed in per^{01} and tim^{01} clock mutants. Using more modest temperatures, but longer application times like in the experiments in the present work, may have prevented such severe side effects, since there was no significant difference between the PER cycling of temperature pulsed and non-temperature pulsed controls.

The impact of clock neuron depolarization on the molecular clock

Apart from the effects of pure temperature-related degradation processes on PER levels, which were apparently rather neglectable in the experiments of this thesis, the effects of clock neuron depolarization on PER levels were investigated in comparison to both controls. Ubiquitous clock neuron depolarization (*clk856/TrpA1*) in the early night caused significant delays in PER accumulation in all investigated clock neuron groups in comparison to controls, especially in the s-LN_vs, the 5^{th} s-LN_v and the LN_ds, but also in the DN1s. The strongest response, however, was exhibited by the l-LN_vs. Whereas the l-LN_vs of both controls lack any PER oscillation in DD, the l-LN_vs of temperature pulsed *clk856/TrpA1* flies showed a strong and fast increase in PER protein levels. The peaking of PER accumulation in the l-LN_vs occurred simultaneously to the prolongation of PER accumulation in all other clock neurons, suggesting the involvement of a fast and synchronous signal, which promotes PER accumulation at that particular time. Remarkably, also the DN2s and LPNs responded at least once with a slight rise in PER levels, but the cycling dampened rapidly.

In *Pdf/TrpA1* flies mostly those neurons exhibited a rapid shift of PER accumulation, which experienced the depolarization via *dTrpA1*, namely the s- and the l-LN_vs. PER

levels in all other clock neuron clusters were not convincingly shifted during the course of the first circadian cycle, suggesting the (fast) mechanism to be cell-autonomously regulated.

The PER staining of *mai179/TrpA1* partly reinforces the notion of a fast cell-autonomous depolarization-mediated shift in PER levels, since temperature pulsed *mai179/TrpA1* flies showed mainly (but not exclusively) increased PER levels in those cell clusters, which had been addressed due to *GAL4* expression, hence the s-, l-LN_vs, the 5^{th} s-LN_v, the LN_ds and some DNs. On the other hand, the somehow disturbed pattern of clock neurons that responded with an alteration in PER levels may account for the inability of these flies to generate a common clock output to e.g. phase delay locomotor behavior. But the message in *mai179/TrpA1* flies is not as clear, since the PER oscillation pattern is hard to evaluate on the basis of just four time points. Unfortunately, the staining experiments were not performed in the advance zone, where significant phase advances occurred in experimental flies relative to controls.

As all clock neurons responded concurrently and immediately after artificial depolarization in *clk856/TrpA1* flies, a fast cellular signal seems to exist that mediates the burst and the prolongation in PER protein accumulation. This cellular signal appears to be independent of PDF and might have occurred instantaneously in all clock neuron clusters cell-autonomously, since all and just depolarized neurons were affected at the same time (indicated by *Pdf/TrpA1* flies).

However, a slower and putatively PDF-mediated effect on PER levels in the downstream targets of the PDF^+ neurons cannot be excluded, as just one cycle of PER quantification was performed in *Pdf/TrpA1* flies. Thus it is not sure whether PDF would have influenced the phase of PER oscillations in $PDFR^+$ neurons in the second cycle, thus causing lagged effects in the PDF downstream neurons' molecular clock. That result is quite noteworthy, as it was assumed that PDF^+ neuronal firing might mimic light-input to the clock (Guo et al., 2014) and as such was thought to cause a rapid release of PDF to adjust the phase in downstream cells.

As the PER cycling data suggest a fast cell-autonomous mechanism downstream of the membrane depolarization of the clock neurons, the assumption of a direct connection to the clock's molecular transcriptional and translational feedback loop

had to be confirmed by experimental evidence. For that purpose the l-LN$_v$s were chosen to be object for further investigation, as these neurons constitute a special cluster of clock neurons functioning as a link between the light-input pathways and the clock (Shang et al., 2008). As such, they were demonstrated to alter their resting membrane potential in response to light in milliseconds followed by an increase in action potential firing within seconds. These effects were shown to occur in a CRY dependent manner and even in absence of any photoreceptive input, indicating a cell-autonomous mechanism in these cells (Sheeba et al., 2008; Fogle et al., 2011). Thus the l-LN$_v$s are predestinated to investigate the downstream mechanism of depolarization, as they appear to rapidly respond with increased electrical activity to light in a cell-autonomous manner, an electrical state that might have been mimicked by artificial depolarization. The burst of PER accumulation and thus the reset of the molecular state might resemble downstream effects that may occur after cell-autonomous light-perception in these neurons.

The *Drosophila* cAMP responsive element binding protein (dCREB) appeared to be a plausible candidate to link the clock's neuronal electrical state and the molecular cycling, since it was shown to be involved in circadian rhythms and phase resetting as well as in influencing *per* transcription (Belvin et al., 1999; Mizrak et al., 2012). Thus, in order to decipher the underlying mechanism, CREB staining experiments were performed to investigate whether the beginning of PER accumulation may coincide with increases in CREB levels in the l-LN$_v$s of depolarized *clk856/TrpA1* and *Pdf/TrpA1* flies. Although it is not surely known whether the antibody used in this thesis recognizes the activated or inactivated form of CREB or both, the quantification of CREB levels in the l-LN$_v$s revealed significant differences in depolarized *clk856/TrpA1* and *Pdf/TrpA1* flies relative to respective controls, which coincided with rises in PER levels. That might suggest a specific binding of the antibody to the activated form of CREB. Data of Fropf et al. (2014) support that assumption, as they performed luciferase experiments, which showed higher dCREB2 transcriptional activity in clock neurons at the end of the subjective night relative to the beginning of the subjective night, a pattern that is in accordance to the quantified CREB levels in the l-LN$_v$s.

CREB quantification reveals that CREB levels in the l-LN$_v$s were significantly higher in experimental flies compared to controls, independently of the time point the depolarization occurred. However, there was a time of day specific difference in

overall CREB levels, since levels were reduced to about 50% in the delay zone compared to the advance zone, indicating that the critical and sufficient level of CREB expression might be time of day dependent.

But what might happen to PER on the molecular level that in the end causes phase shifts in behavior? CREB is activated by several second messengers like cAMP or Ca^{2+} (Mayr & Montminy, 2001). These signals are thought to regulate the phosphorylation of Ser 230 of CREB in *Drosophila* (or Ser 133 in mammals), which is located in the kinase-inducible domain (KID) (Chrivia et al., 1993; Arias et al., 1994). Among a variety of kinases, which are potentially able to phosphorylate CREB, the cAMP-dependent protein kinase (PKA) is most likely to phosphorylate and activate CREB (Shaywitz & Greenberg, 1999). PKA itself is activated by increases in cAMP levels. As increasing cAMP and Ca^{2+} levels are known to be a measure of neuronal activity, both second messengers might be upregulated by artificial membrane depolarization via *dTrpA1*. This suggests that after the artificial depolarization of the clock neurons cAMP and Ca^{2+} are increased and PKA is activated, which in turn can phosphorylate and thus activate CREB. Activated CREB can bind to CRE sequences of the *per* gene, hence increasing *per* transcription. The effects of enhanced *per* transcription might be, however, regulated in a circadian way, since CREB levels in the clock neurons are low at the end of the subjective day (Belvin et al., 1999; Fropf et al., 2014). This causes a weak activation of *per* transcription due to just low levels of CREB at that particular time.

After an artificial depolarization, though, levels of activated CREB increase, hence leading to an enhanced *per* transcription. *per* and *tim* mRNA levels reach their highest point of expression at the beginning of the subjective night and PER protein levels are low at that time. Huge increases of activated CREB due to the depolarization might cause higher rates as well as longer lasting *per* transcription, what might be responsible for delayed PER protein accumulation.

At the end of the subjective night overall CREB as well as PER/TIM protein levels are higher. Further increases of activated CREB might not lead to increases in *per* transcription due to high PER levels. These high PER levels might counteract *per* transcription efficiently. But if the artificial depolarization occurs at the end of the subjective day just before PER/TIM levels are highest, CREB might support premature *per* transcription, because PER levels are not high enough to prevent transcription sufficiently. That may cause an advance of *per* transcription and PER

accumulation. Thus, the inhibition of their own transcription by PER/TIM, which would occur due to high protein levels, is counteracted and prevented by premature CREB-mediated *per* transcription, leading to earlier increases in *per* mRNA and PER protein, which can accumulate and dimerize with TIM much earlier. Thus the preponed accumulation of PER results in earlier degradation, since the degradation machinery both phosphorylates and ubiquitinates and finally degrades PER earlier. Premature high PER levels probably result in a stabilization of TIM, which enables the whole molecular clock to adjust its phase, which is in accordance with the observations in behavior.

All this data together support the notion that indeed CREB might be upregulated upon neuronal activation and might serve as a link and molecular gate for phase shifting, allowing shifts to occur during the advance or delay zone, but inhibiting the process during e.g. the dead zone of the subjective day or in the middle of the night. Even though CREB's involvement in that process still has to be considered as a hypothesis and the underlying mechanism remains unclear, that kind of regulation might enable the system to be fine-tuned in the fly as it is in mammals (Ding et al., 1997; Tischkau et al., 2000, 2003).

4.3 Conclusion

The present PhD-thesis intended to decipher the communication within the circadian clock network of *Drosophila melanogaster* by temporally and spatially restricted clock neuron activation using the thermogenetic tool *dTrpA1*.

The depolarization of all clock neurons evoked the strongest phase shifts in behavior, especially in the delay zone. The shape and temporal range of the advance and delay zone were comparable to a high intensity light-PRC (e.g. Kistenpfennig et al., 2012), indicating that the simultaneous activation of all clock neurons might resemble the electrical situation of the clock neurons after a LP the most. Depolarizing exclusively the PDF^+ neurons caused modest delays, but highly temporally extended advances. The advance zone covered one half of the subjective day, underlining the major role of the PDF^+ neurons in phase advancing the clock. Follow-up experiments allowed to determine the M cells (PDF^+ s-LN_vs) being responsible and sufficient to evoke strong phase advances (*R6/TrpA1*), but being unable to evoke phase delays. These results were further confirmed by excluding the PDF^+ neurons from

depolarization in *clk856/TrpA1* flies. *clk856PdfGAL80/TrpA1* flies exhibited significantly weaker phase shifts in both the advance and delay zone, indicating that the depolarization of the PDF^+ neurons is not just necessary for phase *advances*, but is also required for phase *delays*.

On the molecular level elevated ambient temperatures *per se* did not significantly affect the clock. Artificial clock neuron depolarization, however, caused a shifted molecular PER cycling that mirrored the behavioral effects with respect to the direction as well as with regards to the extent of the phase shifts. These fast and immediate effects, though, were specifically restricted to the clock neurons being addressed by depolarization, indicating a rapid cell-autonomous mechanism. The putative release of PDF after artificial PDF^+ cell depolarization might not occur immediately, but most probably with a temporal delay.

As fast changes in PER levels appeared not to be mediated via PDF, but cell-autonomously, CREB was shown be a plausible candidate connecting the electrical neuronal state with the molecular clock. CREB in the l-LN_vs is regulated in a circadian manner and is upregulated upon neuronal activation, coinciding with rises in PER levels.

The hypothetical mechanism, which might lead to phase shifts in behavior is summed up in Fig. 30: Whenever a TP of 32°C is applied and temperature exceeds 29°C, *dTrpA1* is activated. Flies, which express *dTrpA1* in certain clock neurons, experience the activation of this particular subset of cells. *dTrpA1* channels open and cations are able to enter the neurons, causing an increase of membrane potential, thus depolarizing the cell. Exclusively in these neurons, Ca^{2+} as a measure of neuronal activity might be directly and cAMP might be indirectly increased. Both cAMP and Ca^{2+} might activate a variety of kinases, amongst others cAMP-dependent protein kinase A (PKA), which can phosphorylate and thus activate CREB. Activated CREB might in turn affect the molecular clock by binding to cAMP responsive element (CRE)-sequences upstream of the *per* gene. CREB exerts its influence on *per* transcription, thus affecting the pace of the molecular clock. Hence, CREB can constitute the putative link between membrane potential/neuronal activity and the core molecular clock. As such CREB might act as molecular gate that impose the time of day from the electrical state of the clock neurons to the transcriptional program.

This kind of approach and experimental design, with its total elimination of light-input and the resulting differences in behavioral outcomes in comparison to light-PRCs, enabled us to gain insights into the clock's neuronal properties. The data revealed the l-LN_vs and/or s-LN_vs being direct or indirect targets of light-mediated inhibitory input to the clock. Furthermore it was demonstrated that the artificial depolarization of the PDF^+ neurons did not immediately affect downstream neurons, but downstream neurons might rather be directed with a temporal delay. Therefore we concluded that the rapid cellular response after clock neuron depolarization has to be regulated cell-autonomously. That mechanism might include CREB as a potential molecular link between neuronal activation and the molecular clock.

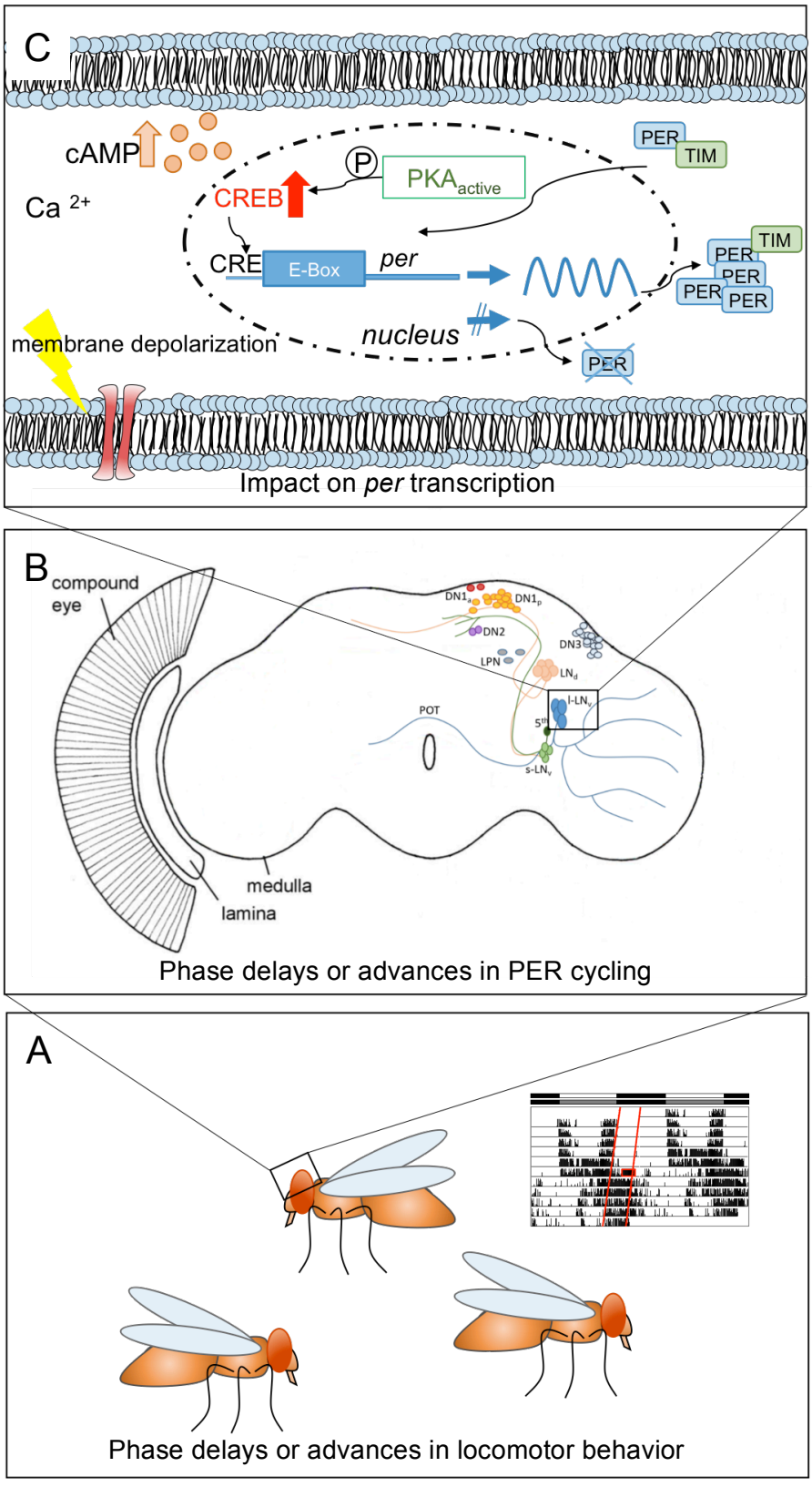

Figure 30 Hypothetic model of the mechanism taking place after artificial clock neuron depolarization

(A) TPs were applied to experimental flies in the delay or advance zone, which responded with phase shifts in behavior. Just those clock neurons, which have been depolarized, shifted their phase of PER cycling significantly, suggesting a cell-autonomous mechanism. **(B)** *clk856/TrpA1* and *Pdf/TrpA1* flies' l-LN$_v$s exhibit strong increases in PER levels after depolarization. **(C)** The beginning of PER accumulation coincided with increases in CREB levels, indicating CREB being involved in mediating information of the clock neurons' excitatory state and the core molecular clock.

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6 Appendix

6.1 Supplementary

6.1.1 Supplementary Data

Table 3 Locomotor activity rhythms in constant darkness (DD)
n_{tested}: total number of tested flies; n_{rhythm}: number of rhythmic flies; period *τ*: free-running period in DD without TP; % rhythm: percentage of statistically rhythmic flies (X^2-periodogram analysis; $p<0.05$)

Genotype	n_{tested}	n_{rhythm}	*period τ [h] (±SEM)*	*Power (±SEM)*	*% rhythm*
clk856 Experiments					
w^{1118}	32	24	23.93±0.06	46.90±2.30	75
UASTrpA1	16	12	23.29±0.11	29.95±1.47	75
clk856GAL4	16	11	23.62±0.09	34.10±2.22	69
clk856/TrpA1	32	28	24.02±0.06	37.17±1.62	88
clk856GAL4/+	32	24	23.51±0.06	40.88±1.75	75
+/UASTrpA1	32	29	23.84±0.06	46.62±2.07	91
Pdf Experiments					
Pdf/TrpA1	32	30	24.15±0.13	34.01±0.84	94
PdfGAL4/+	32	28	24.37±0.08	35.94±1.24	88
+/UASTrpA1	32	25	23.23±0.07	35.16±2.04	78
clk4.1M Experiments					
clk4.1M/TrpA1	64	61	23.57±0.05	44.96±1.33	95
clk4.1MGAL4/+	32	26	23.80±0.06	38.22±1.16	81
+/UASTrpA1	32	29	23.66±0.09	41.07±2.26	91
mai179 Experiments					
mai179/TrpA1	64	49	23.98±0.04	31.92±3.05	77
mai179GAL4/+	55	39	24.18±0.04	28.37±2.18	71
+/UASTrpA1	64	52	23.65±0.05	26.77±2.53	81
R6 Experiments					
R6/TrpA1	17	15	23.31±0.15	11.48±1.11	88
R6GAL4/+	28	5	23.72±0.10	8.91±0.29	18
+/UASTrpA1	29	29	22.82±0.82	11.97±0.67	100
PdfGAL80 Experiments					
clk856/TrpA1	64	58	23.41±0.04	26.21±1.00	91
clk856GAL4/+	56	48	23.72±0.03	33.81±1.49	86
+/UASTrpA1	64	50	23.40±0.03	29.29±1.23	78
clk856PdfGAL80/TrpA1	64	55	23.22±0.03	36.84±1.59	86
PdfGAL80/TrpA1	32	23	23.22±0.07	31.64±2.17	72
clk856GAL4/PdfGAL80	64	51	23.24±0.03	38.40±1.88	80

mai179/TrpA1 phase shift experiments

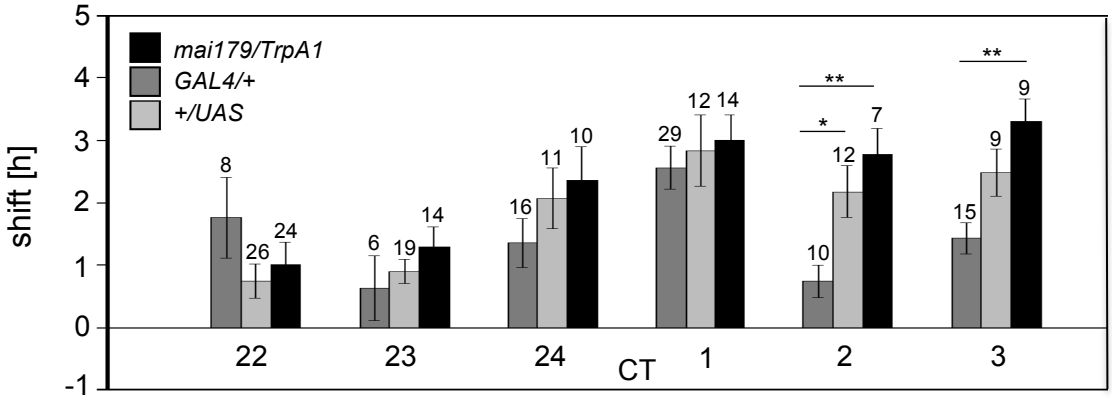

Figure 31 *mai179/TrpA1* and controls with TP in the advance zone
dTrpA1 expression in M and E neurons advanced the phase of activity. The numbers indicate the number of flies per genotype and time point. (Mean±SEM; wilcoxon rank sum test with holm correction; *p<0.05; **p<0.01; ***p<0.001.)

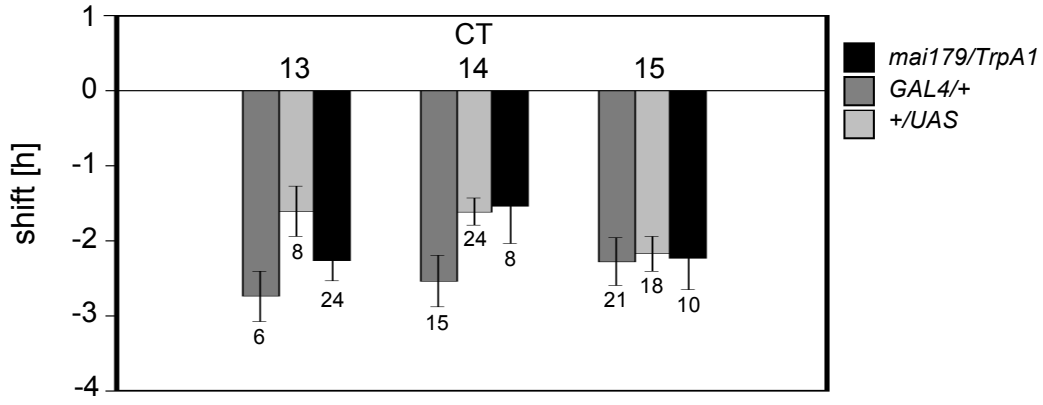

Figure 32 *mai179/TrpA1* and controls with TP in the delay zone
dTrpA1 expression in M and E neurons did not significantly affect the ability to phase shift the clock in the delay zone. The numbers indicate the number of flies per genotype and time point. (Mean±SEM; wilcoxon rank sum test with holm correction.)

R6/TrpA1 phase shift experiments

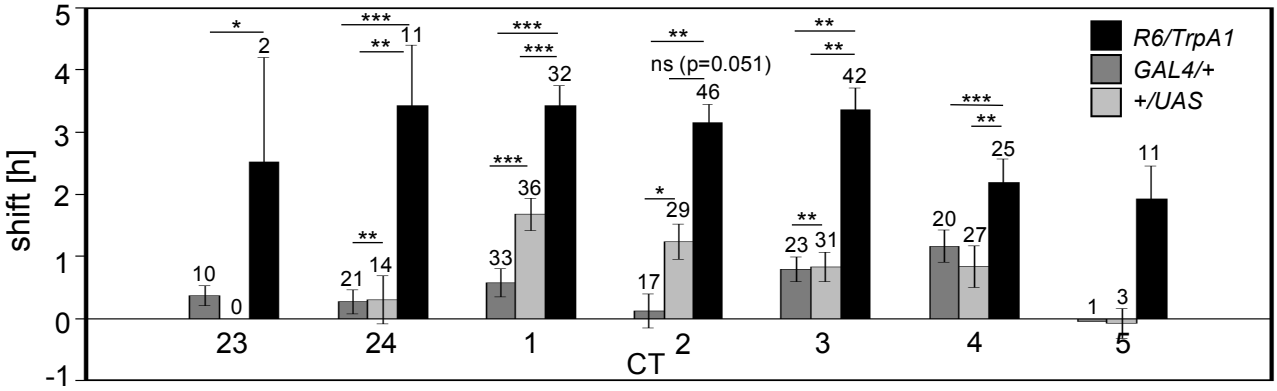

Figure 33 *R6/TrpA1* and controls with TP in the advance zone
dTrpA1 expression in the PDF$^+$ s-LN$_v$ neurons did severely enhance the ability to phase advance the clock after TP application in the advance zone. The numbers indicate the number of flies per genotype and time point. (Mean±SEM; wilcoxon rank sum test with holm correction; *p<0.05; **p<0.01; ***p<0.001; ns:no significant difference.)

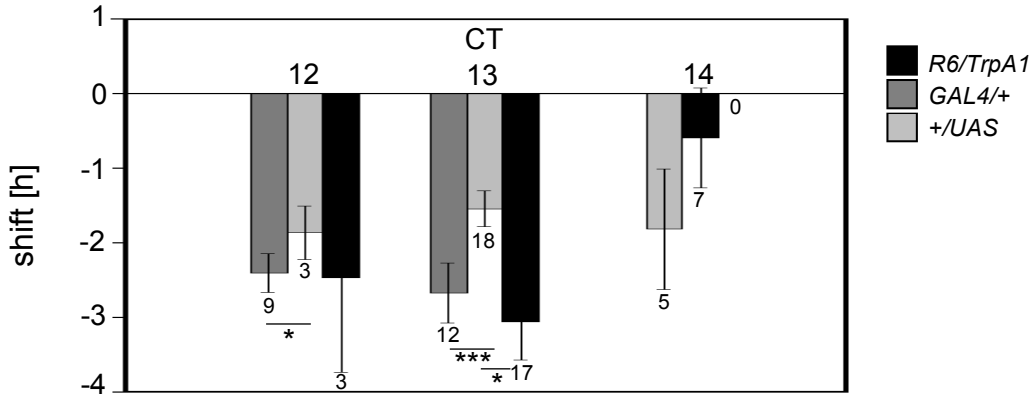

Figure 34 *R6/TrpA1* and controls with TP in the delay zone
dTrpA1 expression in the PDF$^+$ s-LN$_v$ neurons did not significantly affect the ability to phase shift the clock after TP application in the delay zone. The numbers indicate the number of flies per genotype and time point. (Mean±SEM; wilcoxon rank sum test with holm correction; *p<0.05; **p<0.01; ***p<0.001.)

clk4.1M/TrpA1 phase shift experiments

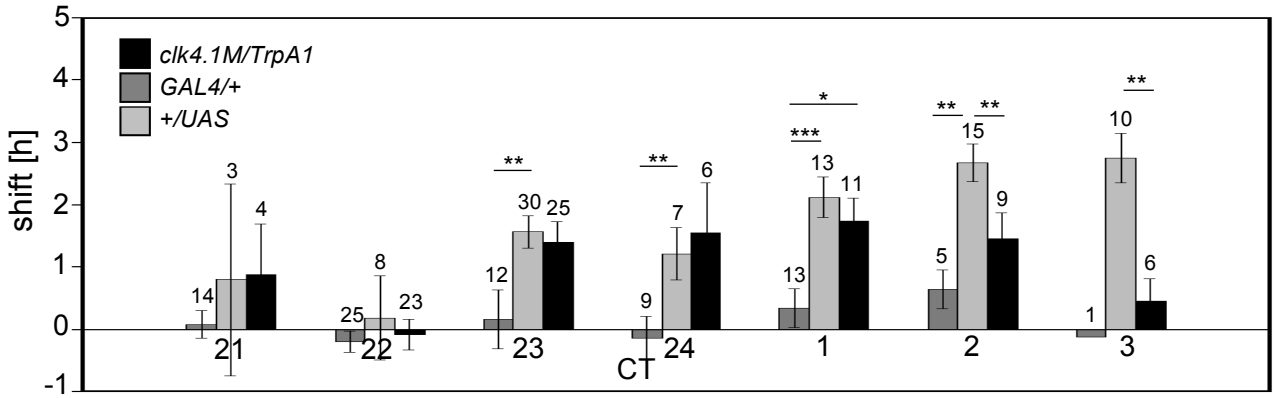

Figure 35 *clk4.1M/TrpA1* and controls with TP in the advance zone
dTrpA1 expression in 8-10 $DN1_p$ neurons did not significantly affect the ability to phase shift the clock in the advance zone. The numbers indicate the number of flies per genotype and time point. (Mean±SEM; wilcoxon rank sum test with holm correction; *p<0.05; **p<0.01; ***p<0.001.)

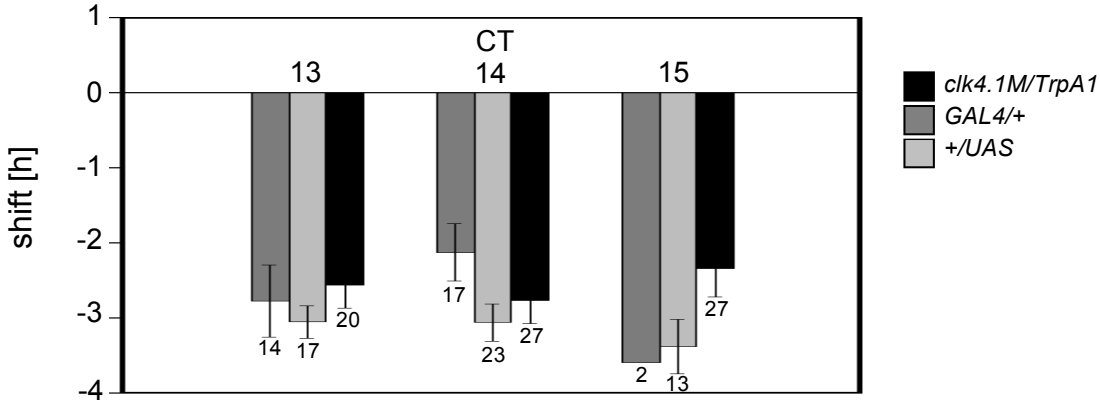

Figure 36 *clk4.1M/TrpA1* and controls with TP in the delay zone
dTrpA1 expression in 8-10 $DN1_p$ neurons did not significantly affect the ability to phase shift the clock in the delay zone. The numbers indicate the number of flies per genotype and time point. (Mean±SEM; wilcoxon rank sum test with holm correction.)

PdfGAL80 phase shift experiments

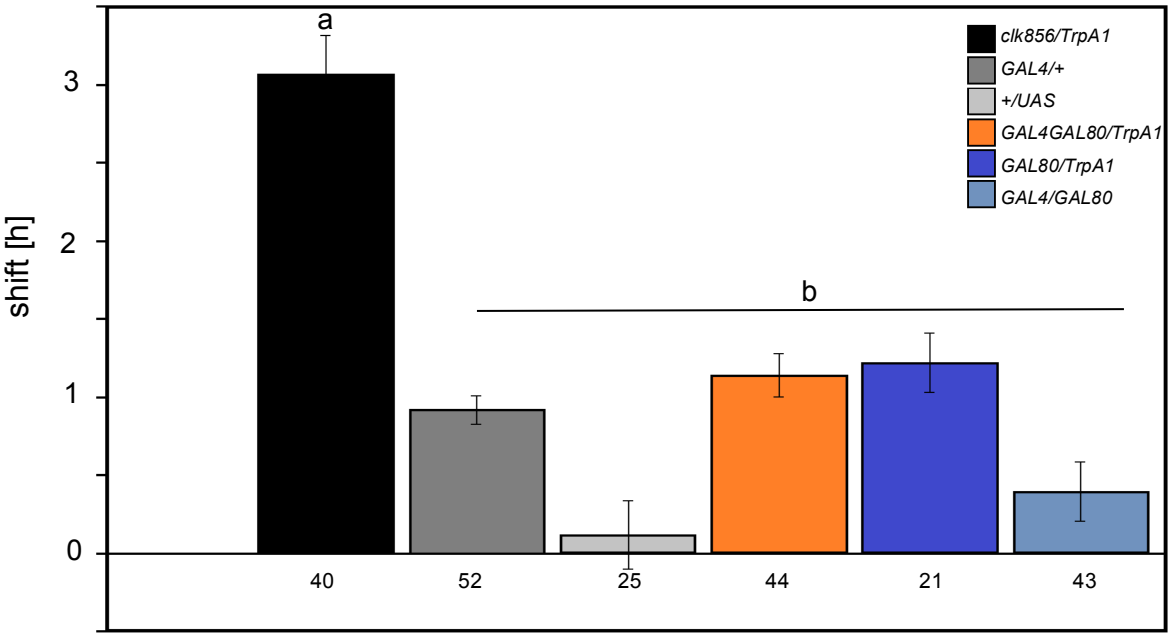

Figure 37 *PdfGAL80* experiment advance zone (CT22 - CT01)
Expressing *PdfGAL80* excluded the PDF$^+$ neurons from depolarization in *clk856/TrpA1* flies. After activation of the remaining neurons by TP application in the advance zone, *clk856PdfGAL80/TrpA1* flies showed weaker phase advances than *clk856/TrpA1*, which still include the PDF$^+$ neurons. That indicates an important role of the PDF$^+$ neurons in advancing the phase of activity. Numbers indicate the number of flies per genotype. (Mean±SEM; wilcoxon rank sum test with holm correction; different letters indicate significant differences p<0.05).

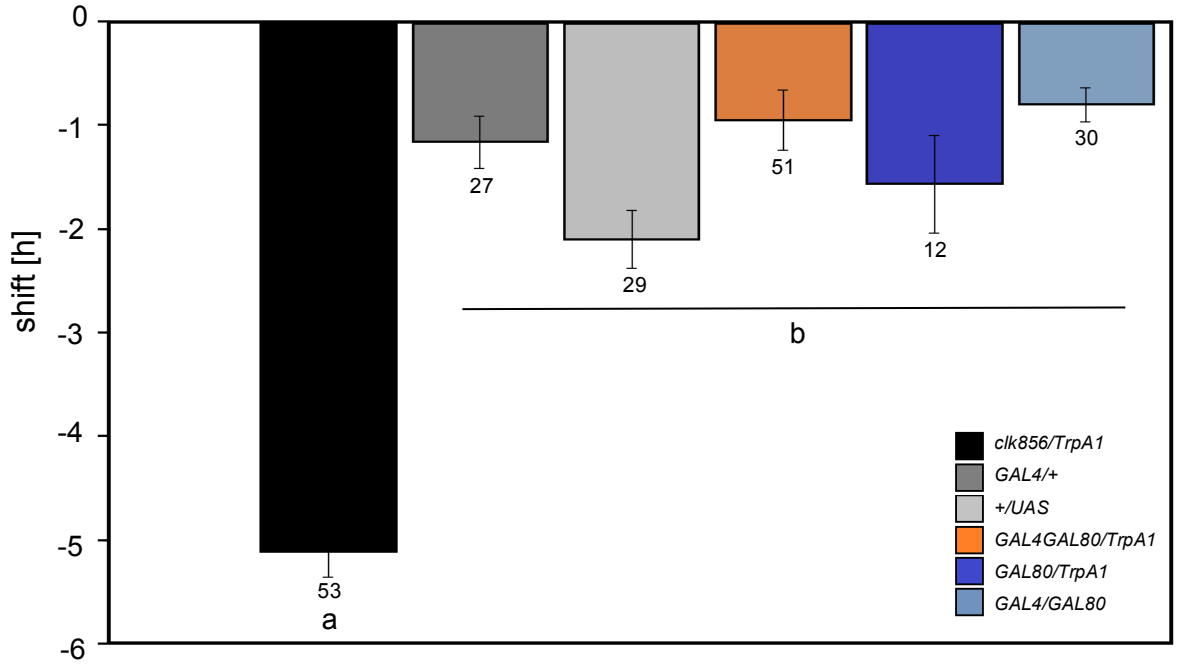

Figure 38 *PdfGAL80* experiment delay zone (CT12 - CT15)
Expressing *PdfGAL80* excluded the PDF$^+$ neurons from depolarization in *clk856/TrpA1* flies. After activation of the remaining neurons by TP application in the delay zone, *clk856PdfGAL80/TrpA1* flies showed weaker phase delays than *clk856/TrpA1*, which still include the PDF$^+$ neurons. That indicates that the PDF$^+$ neurons might also be important for proper phase delays. (Mean±SEM; wilcoxon rank sum test with holm correction; different letters indicate significant differences p<0.05).

GFP & PER staining in the brain of clock neuron specific driver-lines

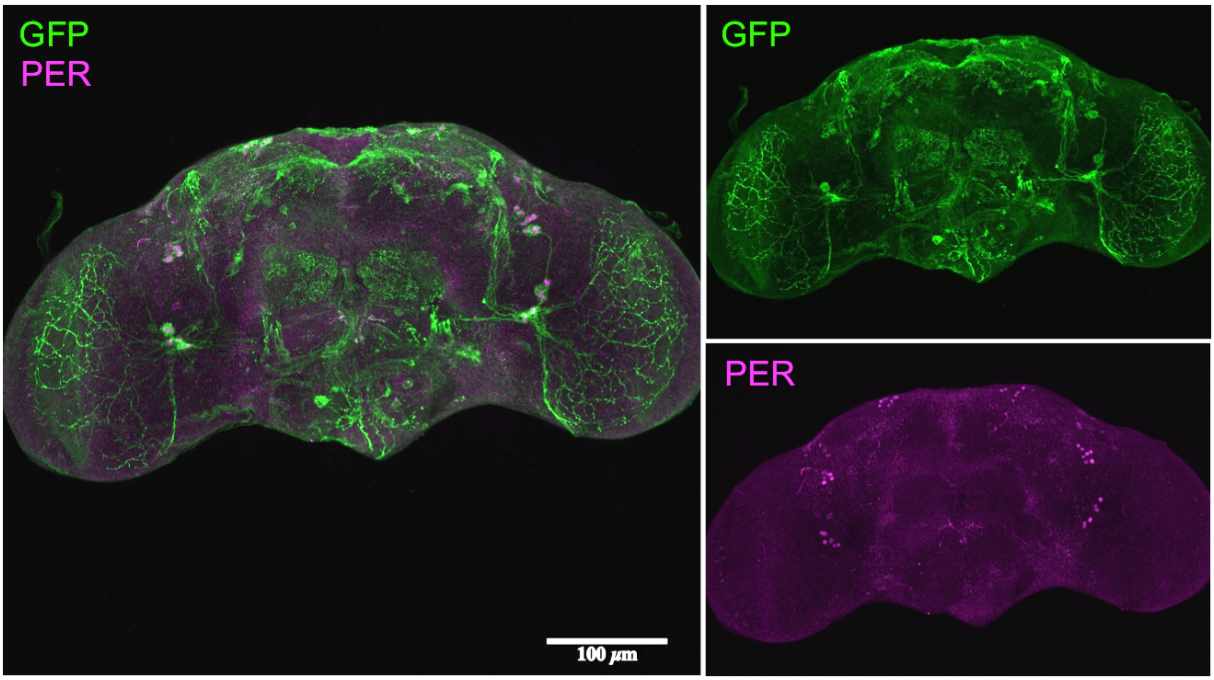

Figure 39 Expression pattern of *clk856/10xGFP* with antibody staining against PER (magenta) & GFP (green) (Gummadova et al., 2009)
clk856GAL4 expresses in the DN1s, DN2s, DN3s, the LPNs, the LN_ds and the LN_vs. Apart of the expression in the clock neurons, there are also non-clock GFP^+ neurons. The brains were temperature-treated like the TP-pulsed flies in the experiments to obtain a representative expression of the *GAL4*. Scale bar 100 µm.

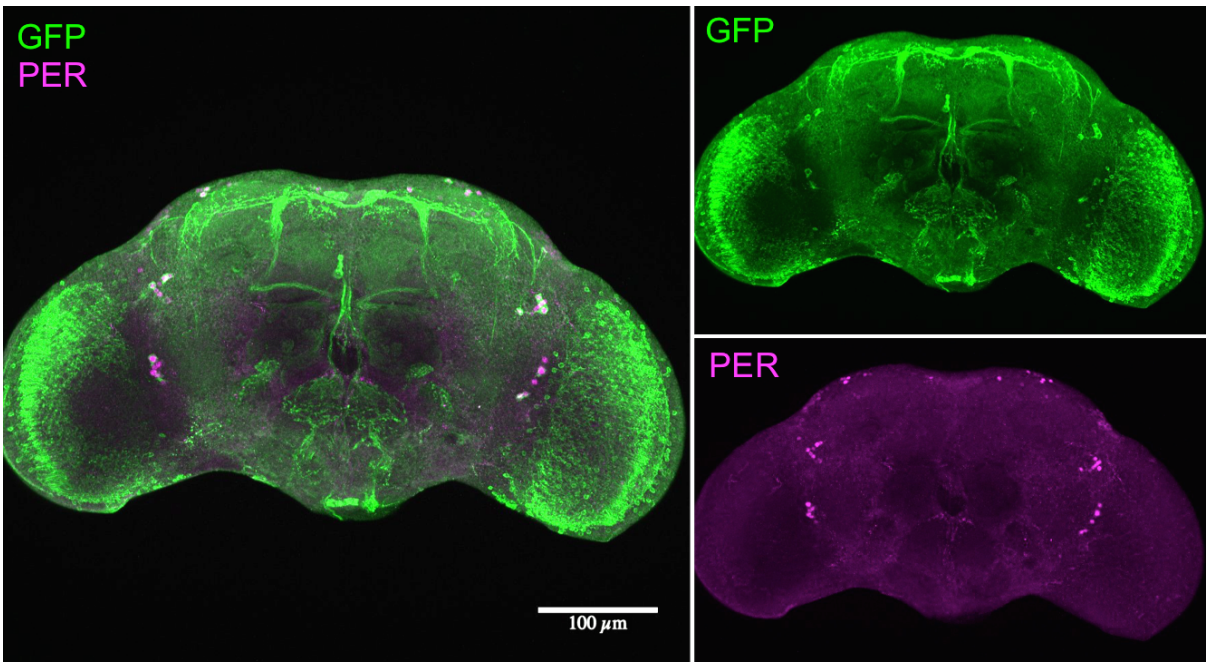

Figure 40 Expression pattern of *mai179/10xGFP* with antibody staining against PER (magenta) & GFP (green) (Grima et al., 2004)
mai179GAL4 expresses in 3-4 LN_ds, in all s-LN_vs (including the 5^{th} s-LN_v) and some l-LN_vs. Apart from the expression in the clock neurons, there are also non-clock GFP^+ cells in the optic lobes and the mushroom bodies. The brains were temperature-treated like the TP-pulsed flies in the experiments to obtain a representative expression of the *GAL4*. Scale bar 100 µm.

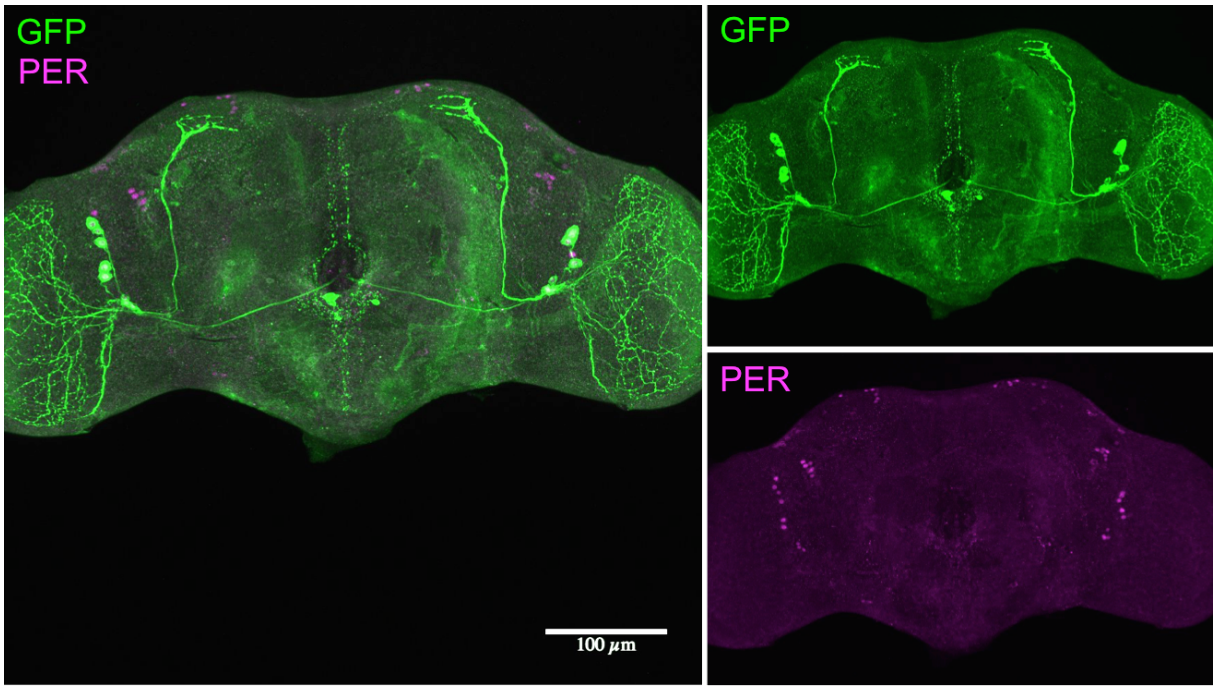

Figure 41 Expression pattern of *Pdf/10xGFP* with antibody staining against PER (magenta) & GFP (green) (Renn et al., 1999)
PdfGAL4 expresses in the PDF$^+$ s-LN$_v$s and the l-LN$_v$s. The brains were temperature-treated like the TP-pulsed flies in the experiments to obtain a representative expression of the *GAL4*.
Scale bar 100 µm.

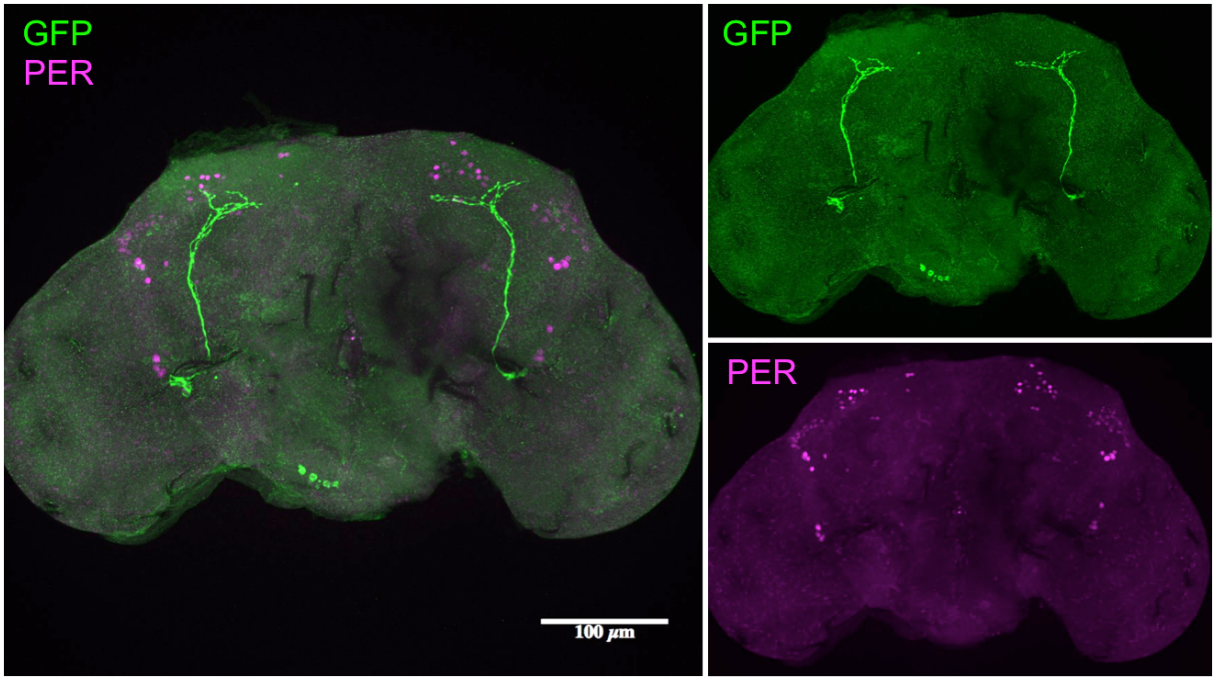

Figure 42 Expression pattern of *R6/10xGFP* with antibody staining against PER (magenta) & GFP (green) (Helfrich-Förster et al., 2007)
R6GAL4 expresses in the PDF$^+$ s-LN$_v$s. The brains were temperature-treated like the TP-pulsed flies in the experiments to obtain a representative expression of the *GAL4*. Scale bar 100 µm.

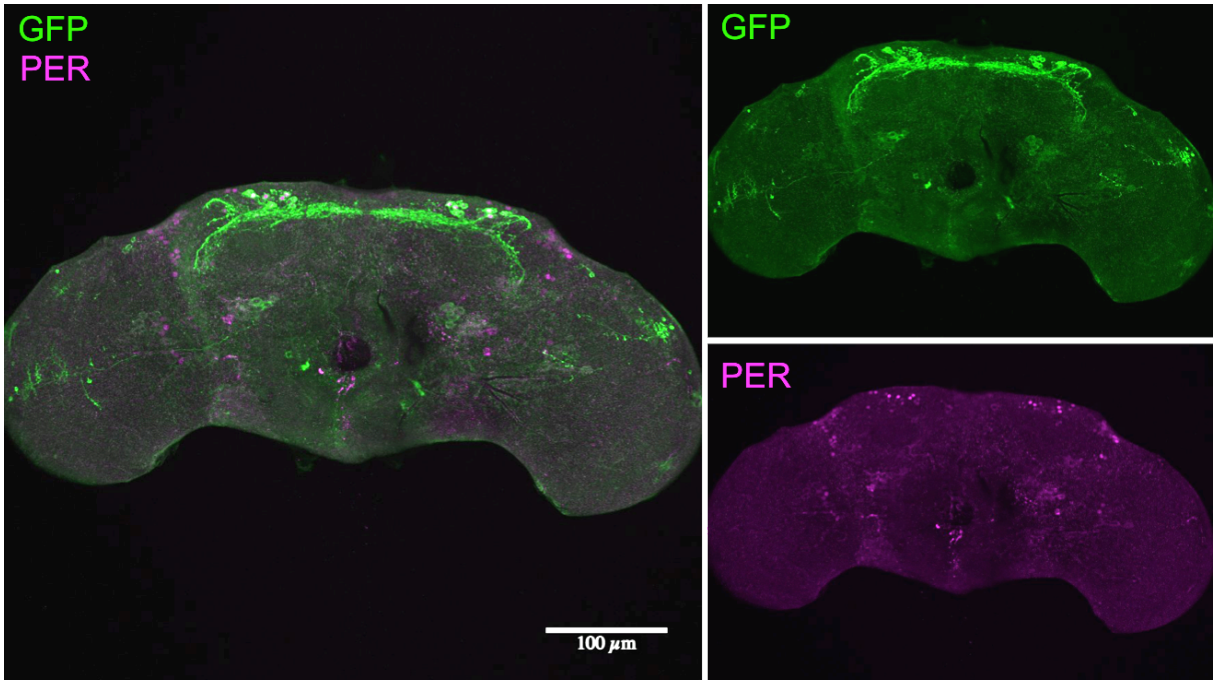

Figure 43 Expression pattern of *clk4.1M/10xGFP* with antibody staining against PER (magenta) & GFP (green) (Zhang et al., 2010)

clk4.1MGAL4 expresses in the 8-10 $DN1_p$s. The brains were temperature-treated like the TP-pulsed flies in the experiments to obtain a representative expression of the *GAL4*. Scale bar 100 µm.

GFP, Rh1 & Rh6 staining in the eye of clk856/TrpA1 flies and controls

To exclude the possibility that the strong effects in behavior and on PER protein levels are due to unspecific expression in the eye in the *clk856GAL*-driver line, GFP was expressed via the *GAL4/UAS* system and immunocytochemical experiments were performed against GFP, against Rh1 and Rh6, to colocalize the inner and outer rhabdomeres in *clk856/10xGFP* and controls.
There was no specific staining in the eye of *clk856/10xGFP* flies (Fig. 44 A1 & A2). Fig. 44 A2 shows the Z projection of all layers of the GFP staining of Fig. 44 A1.

In both the *GAL4* and *UAS* controls (Fig. 45 & Fig. 46) there was nearly the same amount of unspecific binding of the antibody like in the experimental line (Fig. 44), indicating that there is no ectopic expression of *GAL4* in the eye in *clk856GAL4* flies, which may have influenced the outcome of the experiments.

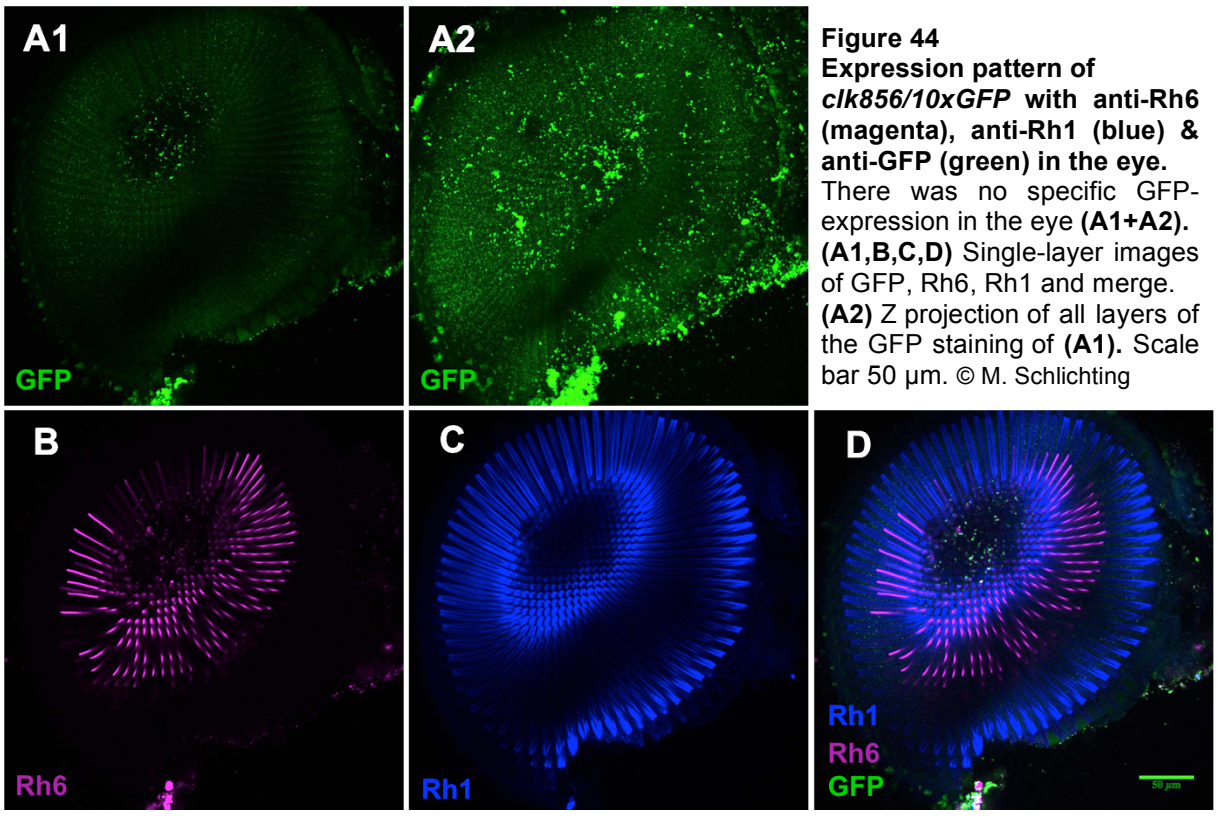

Figure 44
Expression pattern of *clk856/10xGFP* with anti-Rh6 (magenta), anti-Rh1 (blue) & anti-GFP (green) in the eye. There was no specific GFP-expression in the eye **(A1+A2).** **(A1,B,C,D)** Single-layer images of GFP, Rh6, Rh1 and merge. **(A2)** Z projection of all layers of the GFP staining of **(A1).** Scale bar 50 µm. © M. Schlichting

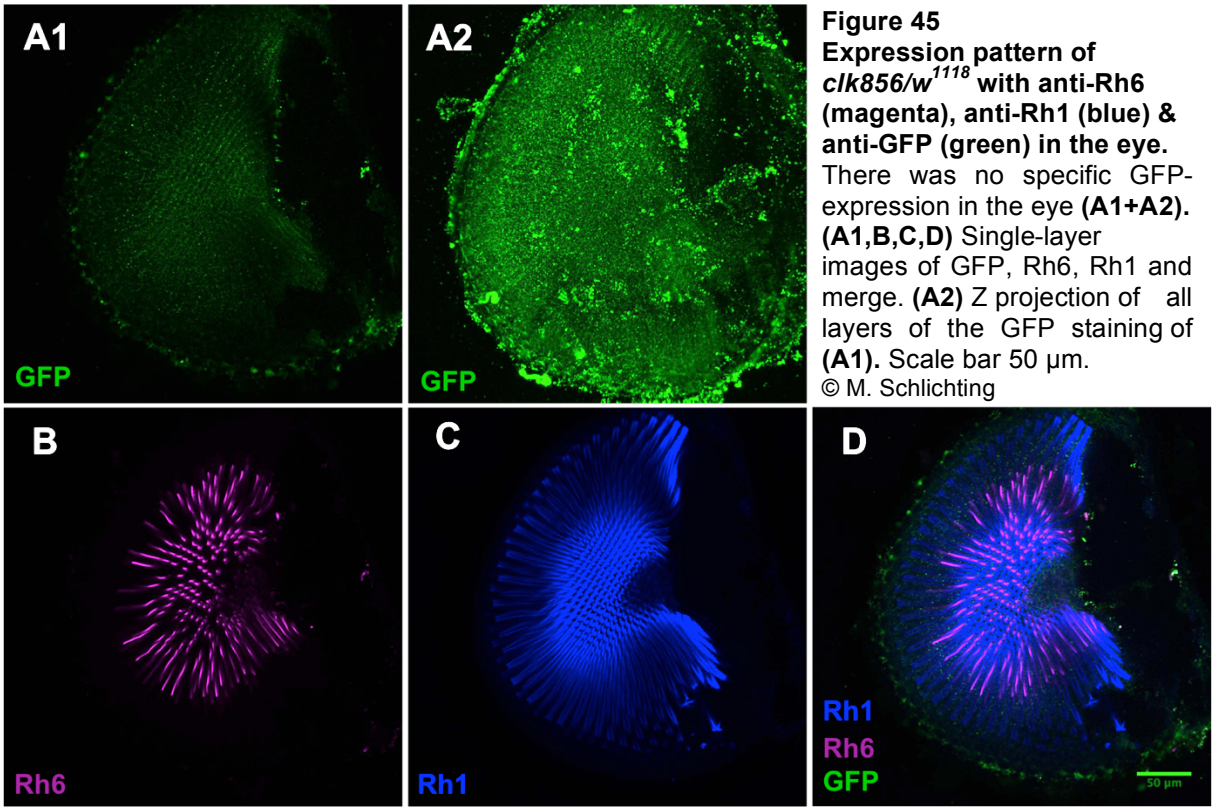

Figure 45
Expression pattern of *clk856/w^{1118}* with anti-Rh6 (magenta), anti-Rh1 (blue) & anti-GFP (green) in the eye. There was no specific GFP-expression in the eye **(A1+A2). (A1,B,C,D)** Single-layer images of GFP, Rh6, Rh1 and merge. **(A2)** Z projection of all layers of the GFP staining of **(A1).** Scale bar 50 µm.
© M. Schlichting

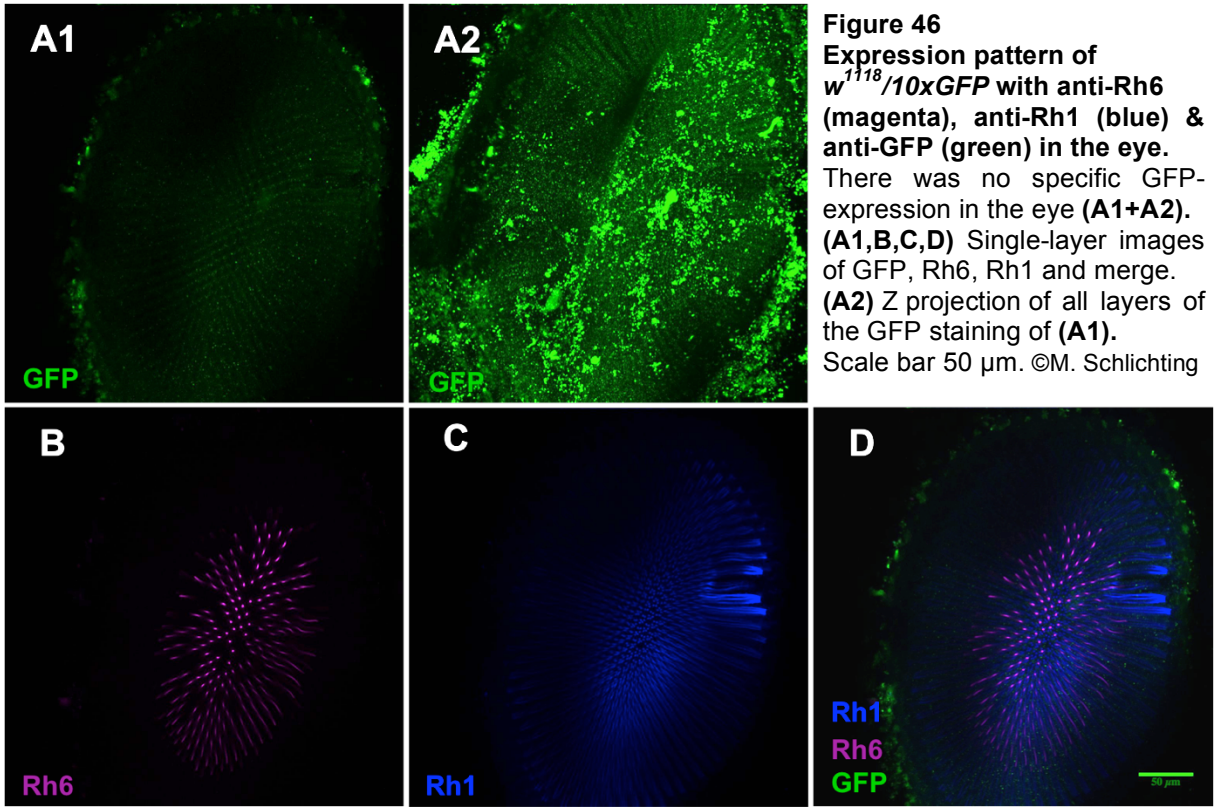

Figure 46
Expression pattern of *w^{1118}/10xGFP* with anti-Rh6 (magenta), anti-Rh1 (blue) & anti-GFP (green) in the eye. There was no specific GFP-expression in the eye **(A1+A2). (A1,B,C,D)** Single-layer images of GFP, Rh6, Rh1 and merge. **(A2)** Z projection of all layers of the GFP staining of **(A1).** Scale bar 50 µm. ©M. Schlichting

6.1.2 Additional Material

Table 4 Buffers, Media & Additional Substances

Buffer/Media	*Incredients/Source*
Standard *Drosophila* rearing medium	0.8% agar, 2.2% sugar-beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour, 0.3% hydroxybenzoic acid, H_2O
Locomotor Behavior	
TriKinetics medium	2.0% agar-agar (danish; Carl Roth), 4.0% sucrose (AppliChem), H_2O
Immunocytochemistry	
phosphate buffered saline (PBS)	1x, (SIGMA-Aldrich (10x)), pH 7.4
phosphate buffered saline (PBS), TritonX-100 0.1% (PBT 0.1%)	1x, (SIGMA-Aldrich (10x)), 0.1% TritonX-100 (Carl Roth), pH 7.4
phosphate buffered saline (PBS), TritonX-100 0.5% (PBT 0.5%)	1x, (SIGMA-Aldrich (10x)), 0.5% TritonX-100 (Carl Roth), pH 7.4
Sodium-Azide (NaN_3)	0.02% in 1x PBS (2% stock solution, SIGMA-Aldrich)
Paraformaldehyde (PFA 4%)	4% PFA in 0.1M PBT (0.1% TrX-100)
Blocking Solution	5.0% Normal Goat Serum (NGS) in PBT 0.5% (SIGMA-Aldrich)
Fixogum Rubber Cement	Marabu GmbH & Co. KG (Tamm, Germany)
Whole brain-culturing	
Ca^{2+}-free Ringers solution	4 g NaCl (SIGMA-Aldrich), 0.37 g KCl (Carl Roth), 1.9 g $MgCl_2$ (AppliChem), 0.84 g $NaHCO_3$ (SIGMA-Aldrich), 41.1 g Sucrose (AppliChem), 1.2 g HEPES (Carl Roth), add 1 l sterile ddH_2O
0.5% Agarose in Ca^{2+}-free Ringers solution	0.1 g agarose (Ultra Pure Agarose GIBCO BRL; Life Technologies; Scotland), 20 ml Ca^{2+}-free Ringers solution, solved in microwave, sterile filtrated
Culture medium	80% Schneider's Insect Medium (SIGMA- Aldrich), 20% fetal bovine serum (PAA), after sterile filtration: 1% Penicillin-Streptomycin (SIGMA-Aldrich) solved in microwave, sterile filtrated

6.1.3 Abbreviations

#	number
Ach	acetylcholine
aMe	accessory medulla
BL	Bloomington *Drosophila* Stock Center
°C	degree Celsius
Ca^{2+}	calcium
cAMP	cyclic adenosyl-monophosphate
CCD	charge-coupled device
ccg	clock-controlled genes
clk	*clock* gene
CLK	Clock protein
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
cry	*cryptochrome* gene
CRY	Cryptochrome gene product
CT	circadian time [τ]
cyc	*cycle* gene
CYC	Cycle protein
DAM	*Drosophila* activity monitoring
Δ	delta/ difference
DD	constant darkness
DNs	dorsal neurons
dTrpA1	*Drosophila* TrpA1
DSHB	Developmental Studies Hybridoma Bank
e.g.	for example (lat. “exempli gratia”)
et al.	and others (lat. “et alii”)
E	evening
Fig.	figure
GABA	γ-aminobutyric acid
GAL4	transcriptional activator of yeast
GFP	Green Fluorescent protein
Hz	Hertz
hr/s	hour/s
K^+	potassium
l	liter
LD	light-dark
LD-cycle	light-dark cycle
LL	constant light
l-LN_vs	large ventrolateral neurons
LN_d	dorsolateral neurons
LP	light pulse
LPN	lateral posterior neurons
mRNA	messenger RNA
M	morning
µm	micrometer
ml	milliliter
Me	Medulla
min	minute
mm	millimeter
n	sample size
nm	nanometer
Na^+	sodium
NaN_3	sodium-azide
-	negative
NGS	normal goat serum
λ	wavelength [nm]
LNs	lateral neurons
PBS	Phosphate buffered saline
PDF	Pigment dispersing factor
PDFR	PDF receptor
pdp1	*par domain protein 1* gene
PDP1	Par domain protein 1
per	*period* gene
PER	Period protein
PFA	paraformaldehyde
Φ	phase of rhythm
PKA	Protein kinase A
+	positive
PR	photoreceptor cell
PRC	phase response curve
PTC	phase transition curve
RNA	Ribonucleic acid
ROI	region of interest
RT	room temperature
SCN	suprachiasmatic nucleus
SD	standard deviation
SEM	standard error of the mean
s-LN_vs	small ventrolateral neurons
τ	free-running rhythm (endogenous period)
T	period in ZT (period of Zeitgeber)
T-cycle	temperature cycle
tim	*timeless* gene
TIM	Timeless protein
TP	temperature pulse
TrpA1	transient receptor potential cation channel A1
TrX-100	TritonX-100
UAS	upstream activating sequence
vri	*vrille* gene
VRI	Vrille protein
ZT	zeitgeber time [T]

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Curriculum Vitae

Affidavit

I hereby confirm that my thesis entitled *„The impact of thermogenetic depolarizations of specific clock neurons on Drosophila melanogaster's circadian clock*" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 12.05.2016

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation *„Der Einfluss thermogenetischer Depolarisationen spezifischer Uhrneurone auf Drosophila melanogasters circadiane Uhr"* eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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