Jihočeská univerzita v Českých Budějovicích
Přírodovědecká fakulta

Perception of periodical temperature cycles regulating the circadian clock in larvae of *Drosophila melanogaster*

Master thesis

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České Budějovice 2023

Opelka, J., 2023: Perception of periodical temperature cycles regulating the circadian clock in larvae of *Drosophila melanogaster*. Mgr. Thesis, in English. – 53 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Abstract:

The circadian rhythms control many biological processes in almost all living organisms, from the single cells to mammals. The evolution of this system allows organisms to respond to and predict changes in the external environment. After light, the most important cue for synchronizations of the circadian clock is the change in temperature during the day and night. Synchronization by thermocycles has been extensively studied in adults of *Drosophila melanogaster*, but little is known about the ontogenetic development of sensory organs for thermocycle perception. In this work, the distribution and function of Ionotropic Receptor 25a (IR25a) was investigated in *D. melanogaster* larvae. This receptor was shown to play an important role in the entrainment to the low temperature cycles in adults. The study revealed similarities in the molecular mechanism of the low temperature cycles perception in adults and larvae of *D. melanogaster*, however this signaling pathway differs in several aspects.

Prohlášení:

Prohlašuji, že jsem autorem této kvalifikační práce a že jsem ji vypracoval pouze s použitím pramenů a literatury uvedených v seznamu použitých zdrojů.

V Českých Budějovicích dne 13. dubna 2023 …….

Bc. Jakub Opelka

Poděkování:

Chtěl bych poděkovat všem členům Střediska mikroskopie a histologie Entomologického ústavu Biologického centra Akademie věd v Českých Budějovicích. Jmenovitě především své školitelce Mgr. Haně Sehadové Ph.D. za její cenný čas, odborné vedení a trpělivost do posledních minut Také bych chtěl poděkovat Prof. Ivo Šumanovi Ph.D. za cenné lekce angličtiny. Dále bych rád poděkoval lidem, kteří mi usnadnili práci s pro mě novými metodami, především Mgr. Markétě Vrchotové, Mgr. Václavu Brabci, Ing., Michalovi Šerýmu. Ph.D. a Mgr. Šárce Podlahové. V neposlední řadě děkuji své přítelkyni a celé mé rodině za neutuchající podporu a trpělivost.

Obsah

1 Introduction

1.1 Biological rhythms

Biological rhythms are a fundamental aspect of homeostasis: "everything is rhythmic unless proven otherwise." These rhythms are endogenous, that is, they are intrinsically determined and persist without external influences. However, the timing and level of functioning of these rhythms are ultimately determined by the interaction of environmental stimuli with internal environmental factors. Life is constantly influenced by external stimuli, many of which exhibit cyclic patterns. Daily and seasonal patterns of light, food availability, and temperature are predictable, and animals, including humans, have the ability to predict these cycles of environmental events through cyclic changes in their internal conditions. These rhythmic patterns of prediction have clear benefits and contribute to survival (Pittendrigh 1993). Biological rhythms can be classified by their period, which refers to the interval between two starting points. Circadian rhythms occur approximately every 24 hours and control rhythmic fluctuations in body temperature and levels of certain hormones. Ultradian rhythms have periods shorter than 24 hours and include respiration and heart rate, while infradian rhythms have periods longer than 24 hours and include the menstrual cycle. Circaanual rhythms have periods of up to one year, e.g., seasonal rhythms such as diapause. Rhythms also have amplitude, which is the difference between maximum and minimum values and the average value. Internal oscillators are adjusted by external factors, also known as "zeitgebers," or "time-givers", such as light-dark cycles and temperature cycles that promote synchronization of the circadian clock or the various biotic cycles in organisms ranging from bacteria to animals. These factors can also promote a change in clock phase in either direction depending on the time, which in turn can alter the timing of daily behaviors such as rest-activity cycles, mating, egg hatching, and feeding. In addition, there are other cyclic processes that can interact with each other, such as tidal and circalunar rhythms. The frequencies of human biological rhythms cover almost every time period, and understanding these rhythms is important to medicine because many drugs exhibit rhythmic variations in pharmacokinetics and pharmacodynamics. In addition, the development of many diseases of civilization and aging are influenced by imbalanced biological rhythms.

1.1.1 Circadian rhythms

Circadian rhythms are biological processes that occur approximately every 24 hours and are present in most living organisms, including humans. The mechanisms underlying circadian rhythms differ among organisms, but they share a number of core clock genes, highlighting the evolutionary conservation of the core clock mechanism across species (Dunlap, 1999; Bell-Pedersen et al., 2005). These rhythms influence various physiological functions such as hormone production, metabolism, and sleep-wake cycles. Disturbances in circadian rhythms, such as those that occur during shift work or jet lag, can have negative effects on health, such as sleep disturbances, metabolic disorders, and increased risk of cancer. Self-sustaining rhythms are controlled by internal clocks. These clocks must be synchronized with the external world on a daily basis. A characteristic feature of circadian rhythms is their ability to function in the absence of external stimuli, although these stimuli play an important role in synchronizing or entraining these rhythms (Pittendrigh and Daan, 1976). The entrainment, adjustment, or resetting the circadian clock by an environmental stimulus called a "zeitgeber" refers to important environmental factors that can reset the clock, such as light, temperature change, timing of food availability, and exercise. However, the most important of these factors is light exposure (Dunlap et al., 2004). Subjective day is the internal physiological state corresponding to the time when an organism was regularly exposed to daylight, while subjective night refers to the internal physiological state corresponding to the time when an organism was regularly exposed to darkness. Even under constant conditions, due to the cyclic oscillation of the circadian clock, the organism follows a 24-hour cycle in which subjective day and night alternate (Figure 1).

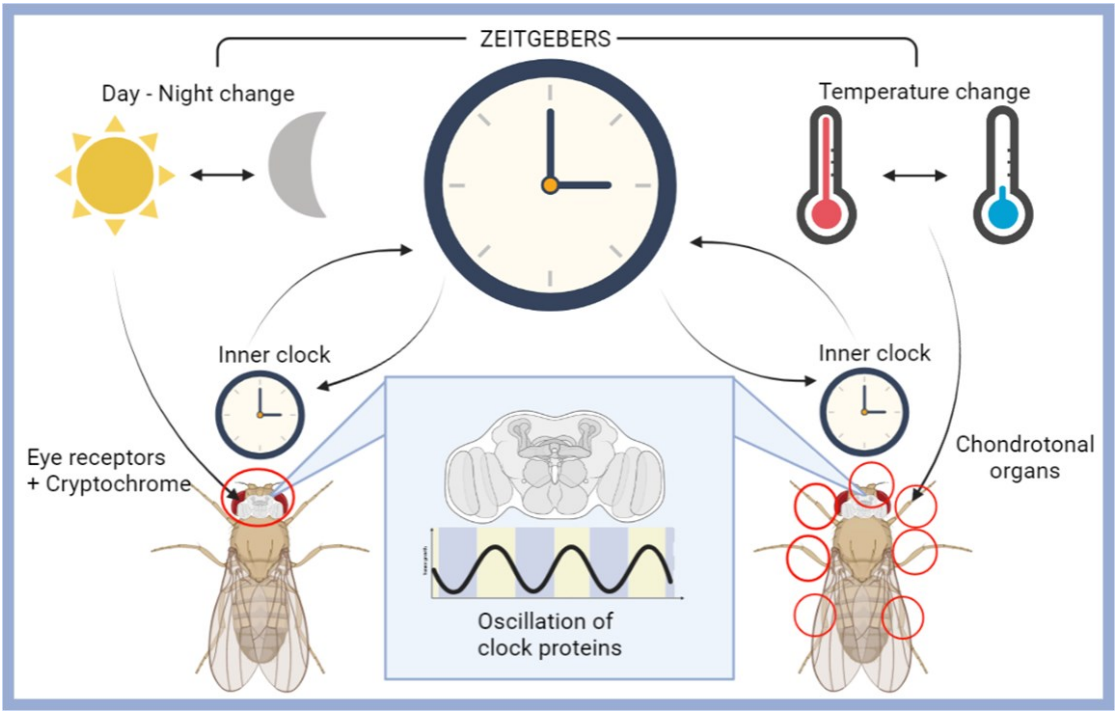

Figure 1: Simplified scheme of circadian synchronization to environmental zeitgebers in *Drosophila melanogaster*. The main zeitgebers for synchronizing the circadian clock are the day/night cycle and the hot/cold cycle. Drosophila has several organs (marked by red circles) responsible for the perception of these inputs. It synchronizes its internal clock based on these inputs.

1.2 Circadian clock

The circadian clock is a localizable functional unit capable of generating endogenous, self-sustaining oscillations and controlling other rhythms in the body. In the past, the most widely accepted model of the mechanism of the circadian clock, the fundamental circadian oscillator, was based on single cells. However, neuronal interactions between several different groups of pacemaker neurons and brain parts appear to play a critical role in generating rhythmic behavior. Remarkably, these interactions may even be required to maintain correct molecular oscillations within interacting pacemaker cells, even in the absence of environmental stimuli that mark the passage of time. The endogenous period of the internal clock controls circadian rhythms in a constant environment with an internal period of approximately 24 hours. The relatively low sensitivity of the master oscillator to the temperature of the external environment is called temperature compensation, which means that temperature changes in the environment do not affect the clock itself. On the other hand, the main oscillator can integrate information about temperature changes through its temperature-sensitive organs or cells, but this influence has not been fully explored. There is little difference between poikilotherms and homoiotherms in the use of temperature

synchronization. In homoiotherms, the central oscillator uses reduced body temperature during sleep to reset the peripheral slave clock, whereas in poikilotherms this synchronization occurs at the behavioral level. In *Drosophila*, this behavior is referred to as TRP activity, which is controlled by the master oscillator but simultaneously synchronized by temperature cycles. Not all environmental synchronizers are zeitgebers; some of the factors may have a masking effect that is a direct response to environmental factors, which is often seen in cases where the synchronizer is too weak to regulate rhythm in behavior but strong enough to affect activity when it is present.

In the past, attention has focused on the distinct properties of different individual clock cells comprising a core set of clock genes that regulate large networks of gene transcription through both direct transcriptional/translational activation/repression and recruitment of proteins that alter chromatin state. However, recent studies suggest that the circadian neuronal circuit may consist of multiple autonomous components. The full extent of the mechanisms by which gene regulation and oscillations of clock proteins in cells maintain clock pacemakers that integrate and control behavior is still under investigation. In more complex organisms, there are more complex clocks with a specific hierarchy. The main pacemaker ("master oscillator") is capable of self-sustaining oscillations and synchronous output, even when isolated from surrounding structures. The lower level of control consists of semi-autonomous oscillators whose individual cells are capable of oscillations but cannot produce a synchronous output signal without the synchronizing influence of the pacemaker. The slave oscillators are capable of oscillating only when there is a regular or at least intermittent signal from the main oscillator. The main oscillator is located in the brain of an animal and many semi-autonomous and slave oscillators are scattered in the periphery of the body. Clock gene production is an intrinsic property of all clock cells in various tissues; however, these peripheral oscillators are less complex and under the control of the main oscillator. (Yoo et al., 2004). Similarly, the insect brain has a main oscillator in the accessory medulla (aMe), which lies between the central brain and the optic lobes (Tomioka et al., 2010). The result of whole-organism synchronization is behavioral changes that correspond to light and temperature cycles and anticipate energy needs with organism activities (such as feeding and sleeping), which helps the various tissue and organ systems coordinate and optimize their performance (Bass and Takahashi, 2010).

1.2.1 Circadian clock network in *Drosophila melanogaster*

D. melanogaster, commonly known as the fruit fly, is a valuable model organism because it can be easily and inexpensively bred under laboratory conditions and has a short life cycle. It has advanced genetic tools that can be used to study individual clock neurons separately, as well as methods based on visualization of neuronal activity that illuminate communication in the circadian clock network. In studying the circadian clock in Drosophila, measurements of locomotor activity, which is closely linked to circadian regulation, can also be used. During a 12-hour daylight cycle followed by a 12-hour dark cycle (LD), wild-type D. melanogaster flies exhibit two distinct phases of locomotor activity. These occur at dawn and dusk, as in many crepuscular insect species. The activity peaks begin before the light is turned on and off, suggesting that the flies anticipate these intermittent changes at LD conditions. Both morning and evening anticipation require an endogenous circadian oscillator. Many arrhythmic mutants cannot synchronize their activity with the environment, resulting in their activities being restricted to different times. However, they also respond to environmental changes immediately after lights are turned on and off.

Recent studies suggest that the oscillations of the important clock genes *period* (*per*), *timeless* (*tim*), *cycle* (*cyc*), and *clock* (*Clk*) (Forster et al., 2007b) in individual clock cells are as important as the mutual integration of the individual oscillators. Moreover, their individual phases together form a harmonic and time-dependent sequence of outputs that synchronize the whole organism. Clock cells are divided into functional rhythmic centers that cooperate or compete to regulate the various rhythmic behaviors that govern the fly's daily activity pattern. (Muraro et. Al., 2013) Two groups of circadian neurons, known as M (morning) and E (evening) cells, are involved in the control of morning and evening activity peaks in Drosophila and operate separately, although they communicate with each other via neuropeptides to generate two domains of locomotor rhythm. (Stoleru et al., 2004; Grima et al., 2004). In contrast, the following studies suggest that the classification between E- and M-cells is too strict because E-cells can control both E- and M-oscillations under more natural environmental conditions. (Yoshii et al., 2010; Bywalez et al., 2012; Menegazzi et al., 2012) The remaining groups of circadian neurons exhibit rhythmic but unsynchronized activity that depends on master oscillators that contribute to network products at multiple phasic times. (Liang et al., 2019; Muraro et al., 2013).

The *Drosophila* brain consists of about 150 clock neurons divided into several oscillator subgroups that cooperate in complex rhythmicity. The subgroups are named according to their anatomical distribution in the dorsal and lateral protocerebrum and their relative size (Luibl et al., 2015). The lateral neurons in the fruit fly brain are divided into four generally recognized subgroups: the large ventrolateral neurons (l-LNV) and the small ventrolateral neurons (s-LNV), the 5th small ventrolateral neuron (5th small LNV), the dorsolateral neurons (LNd), and the lateral posterior neurons (LPN). Immunocytochemical studies using anti- TIM and anti- PER antibodies show that these proteins are rhythmically expressed in all LNVs, but in some of them with different phase. Several studies have shown that the s-LNVs located near aMe are the central pacemakers (Tomioka et al., 2010). These results are supported by immunohistochemical studies of PER oscillations in the s-LNVs, which are more pronounced than in the l-LNVs, and oscillations in the s-LNVs act as the main oscillator for circadian rhythms in constant darkness. In addition, the s-LNVs play a critical role in regulating morning locomotor activity under LD conditions and in communicating with the E oscillators through the neuropeptide pigment-dispersing factor (PDF). (Ewer et al., 1992; Siwicki et al., 1988; Zerr et al., 1990; Frisch et al., 1994). The l-LNVs likely have the main function in gating and relaying light inputs to the s-LNVs, which promote wakefulness, as they largely inervate the medulla and have high expression of the light-sensitive protein cryptochrome (CRY) (Förster, 2014). Oscillatory activity follows the s-LNV in the morning (Shang et al., 2008). The fifth s-LNV is a PER and TIM positive neuron that does not express PDF and is located between the clusters of LNV cells. Both groups are associated with the evening peak of locomotor activity (E cell group) (Grima et al., 2004; Stoleru et al., 2004), and their neuronal activity anticipates dusk. (Liang et al., 2016; Peng et al., 2003; Lin et al., 2004). Although LPNs of the last lateral group have long been uncharacterized, recent studies (Reinhard et al., 2022) have identified neuropeptides and connections between other clock neurons but have said little about their possible function. LPNs express the neuropeptides allatostatin A (AstA), allatostatin C (AstC), and diuretic hormone 31 (DH31) in double-peak oscillations, suggesting that they are involved in the control of sleep and feeding in a type of temperature cycle (Chen et al., 2016; Ni et al., 2019; Miyasako et al., 2007; Yoshii et al., 2005).

The dorsal neurons are divided into three subgroups of dorsally positioned clock neurons, including the following groups DN1, DN2, and DN3. DN1 is not a very uniform cluster, whose parts have different characteristics and are therefore divided into DN1a and

DN1p cells. Some DN1s neurons oscillate together with DN2 neurons in counterphase to s-LNVs and maintain their period even under constant light conditions (LL) in the presence of temperature cycling (TC). In addition, DN1 cells integrate multiple inputs, one of which is the synchronization of M and E cells and the other comes from anterior cells (AC) and taste projection neurons TPN-II. By integrating these signals, DN1 contribute to the regulation of locomotor activity and sleep (Zhang et al., 2010; Liang et al., 2017; Guo et al., 2016). DN2 cells are located near the terminals of the small LNVs, but their function is not yet DN2 Because the somata of DN2 cells are located near the PDF-positive terminals of the s-LNVs, they are synchronized by the PDF neuropeptide in LD. However, under DD or LL conditions, they can synchronize at TC and regulate TPR activity. DN3 neurons are a heterogeneous group in which some cells are synchronized by TC and others by LD. Experiments with pdf0 mutants have shown that DN3 contribute to the synchronization of locomotor activity, especially to the evening peak. This differential timing allows the network to generate a sequence of neuronal outputs that regulate behavioral rhythms during the day and night (Miyasako et al., 2007).

The clock network of the larval brain is less complex than that of the adult and consists of four s- LN, the 5th LNS, and two pairs of dorsal neurons, DN1 and DN2. The LNs are located near the larval optic neuropile. They all survive to the adult stage (Kaneko and Hall, 2000; Shafer et al., 2006). In addition, eclosion hormone (EH) and crustacean cardioactive peptide (CCAP) are involved in the regulation of respiration and metamorphosis and are subject to circadian regulation. CRY is expressed only by PDF LNs and DN1s clock neurons. (Klarsfeld et al., 2004). Larval light- and temperature-sensitive organs are much simpler. The only known organ that provides light input through the visual system is the Bolwig organ (BO), which has only 12 cells and expresses only two rodopsins RH5 or RH6 that project to the PDF-expressing LNs (Kaneko et al., 2000; Malpel et al., 2004). Rhodopsins consist of the protein subunit opsin and a vitamin A-derived chromophore. Although they are primarily photoreceptors, they also have the ability to sense temperature, which is described in more

detail in the chapter on temperature synchronisation. Together with CRY, they are the only light inputs available to the larval clock (Figure 2).

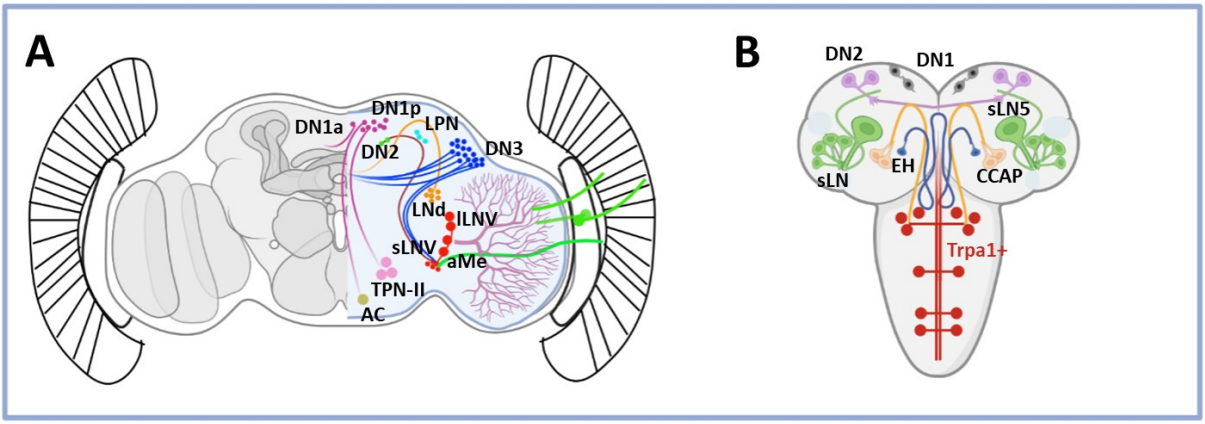

Figure 2: Scheme of distribution of groups of individual clock oscillators and their interrelationships and connections in the brains of adult (**A)** and larva (**B)**. Synchronization of the organism occurs by communication of individual groups of oscillators s-LNV + some DN1 (M-oscillator) and LNd + DN1 + 5th-LNV (E-oscillators). AC and TPN-II are the cells involved in temperature and taste input to the clock cells. Eclosion hormone (EH), and crustacean cardioactive peptide (CCAP) is involved in the regulation of respiration and metamorphosis.

Clock cell groups were identified based on the expression of clock genes that oscillate daily in all clock cells. A particular group of clock proteins initiates and controls changes in their own equilibrium state and in the levels of their mRNAs. These changes result in rhythmic waves of gene and protein expression that together form a repetitive translational-transcriptional loop. The first gene whose expression was detected in the context of the cellular clock was per, such that the translated protein PER also inhibits transcription of the mRNA of per, further highlighting the role of PER in this feedback loop as a transcriptional repressor. These results support the view that there is a molecular cell clock (Konopka and Benzer, 1971; Sehgal et al., 1994; Rutila et al., 1996). Other core clock proteins have been discovered, namely TIM, CYC, CLK, and together with PAR domain protein 1 (PDP1), clockworkorange (CWO), vrille (VRI), unfulfilled (UNF), and ecdysone-induced protein 75 (E75) form a total of three transcriptional loops, of which the *per/tim* loop is the most important. There are certainly other proteins, transcription factors, and kinases involved that can assist in temperature compensation and accelerate or alter the amplitude of the core clock proteins. This is usually done by stabilizing protein complexes, phosphorylating/dephosphorylating or preventing their entry into the nucleus. The best known modifiers are kinases such as double time (DBT), casein kinase 2 (CK2) and GSK-3ss aka Shaggy (SGG). The first feedback loop begins with the CLK/CYC dimer, which activates transcription of the *per* and *tim* genes by

binding to their E-boxes. The mRNA of *per* and *tim* migrate to the cytoplasm where translation into the proteins PER and TIM begins. When the organism is exposed to light, the TIM/CRY dimer is normally formed, and TIM is continuously degraded until evening. Similarly, the PER protein is destabilized and degraded to slow its accumulation. In the evening, TIM and PER form a dimer stabilized by DBT that can migrate into the nucleus and bind to CLK/CYC. This leads to the repression of *per* and *tim* mRNA transcription. At dusk, CRY is reactivated and can initiate degradation of the TIM protein, leading to restoration of transcription of *per* and *tim*. The second feedback loop involves the expression of *Pdp1* and *vri* caused by the CLK/CYC dimer. The VRI protein is rapidly translated and immediately binds to the VRI/PDP1 box in the *Clk* promoter, limiting *Clk* transcription. Later, PDP1 binds to the *Clk* promoter and rescues *Clk* transcription. The third feedback loop involves the E-box-regulated protein CWO with the CLK/CYC dimer. The function of CWO is to compete with the CLK/CYC dimer in binding to E-BOX sites (Foerster, 2017; Ozkaya et al., 2012) (Figure 3).

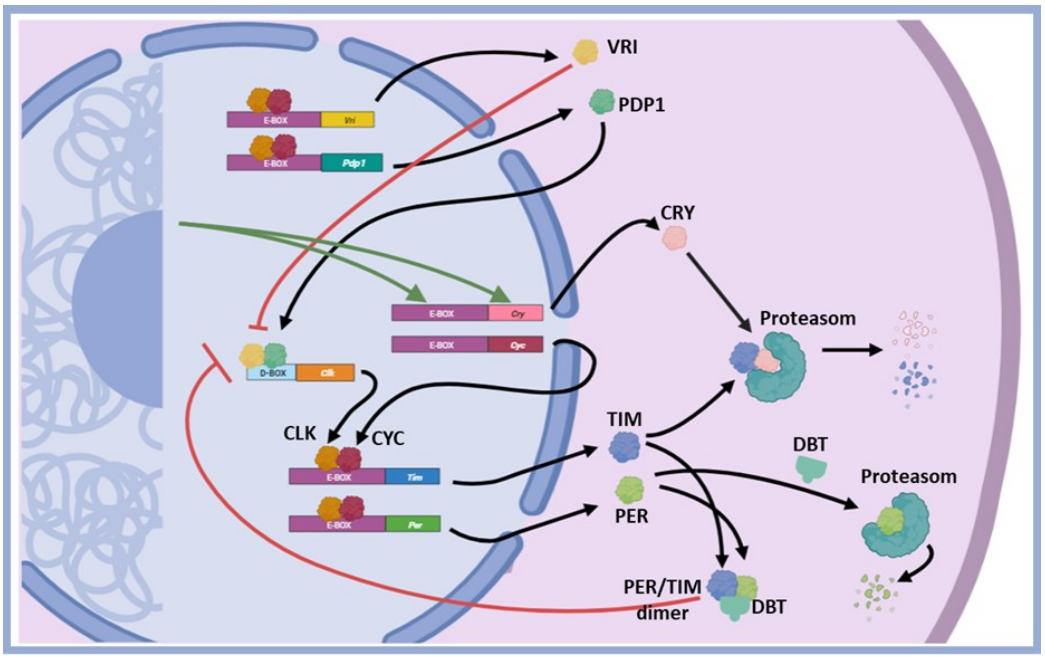

Figure 3: Schematic of transcription-translation loops in clock gene oscillation in the clock cell. First, a CLK and CYC dimer is formed that binds to (E-boxes) in the promoter of the clock genes *per* and *tim*. The PER and TIM proteins form a dimer with the participation of doubletime (DBT), enter the nucleus, and inhibit CLK-CYC activity. The second loop modulates *Clk* gene expression by inducing CLK-CYC dimer transcription of the *vri* and *Pdp1* genes. VRI and Pdp1 compete for the same element in the promoter (D-box) of the *Clk* gene, thereby controlling its transcription. In addition, DBT modulates clock protein activities by phosphorylation and thus regulates protein-protein interactions, nuclear translocation and PER degradation. TIM is degraded in the cell to form a dimer with the CRY protein which is an internal light-activated photoreceptor.

1.2.2 Light synchronization

In the context of animal behavior, light can have different effects. Based on the finding that the TIM protein is degraded in the presence of the light-sensitive protein CRY, a simple model was developed to explain how light synchronizes the clock. This model explains the prolongation of the behavioral period or arrhythmia in LL due to a longer accumulation of the required level of TIM. In addition, the same model can be used to explain the phase shift response that occurs after a light pulse during subjective night. Experiments with a cryb mutant lacking a functional CRY revealed that cryb mutants are unaffected by light pulses and do not show arrhythmia in LL. On the contrary, they are able to entrain LD, although their PER and TIM oscillations are reduced. These oscillations can be rescued in combination with TC, and immunostaining with the anti-CRY antibody showed that some subsets of clock neurons (six DN1, both DN2, DN3s, three LND, and LPN) have little or no CRY expression. These results suggest that more inputs must be involved in light synchronization and that temperature is the second most powerful zeitgeber next to light. The complexity of this system lies at several levels. The variety of mechanisms that receive the light signal, the regulation of this signal at the level of the clock cell, the modulation of the output within the individual oscillators in the brain to the output in the form of a change in the internal environment or a change in behavior. all this is only one side of the coin, because although temperature is a secondary zeitgeber, it is still an important factor that influences and synchronizes the circadian clock (Ozkaya et al., 2012; Marrus et al., 1996; Helfrich-Forster et al., 2001; Stanewsky et al., 1998).Temperature synchronization mechanism in adults

The model of the mechanism of temperature synchronization begins with the perception of temperature changes by peripheral organs, which transmit this information to the brain to specialized subpopulations of clock cells, in which the molecular machinery of the clock is altered. This is followed by the integration of multiple signals from individual subpopulations of clock neurons by neuropeptides, which then relay the resulting output to higher centers responsible for changing the behavior of the whole organism or peripheral organs. Moreover, at all these levels there is a possible combination and variety of as yet unknown inputs, outputs, and modifications that may contribute to the final synchronization. Moreover, the structures and molecules involved in circadian thermoreception are still not well understood.

The first requirement for synchronization by TC is the receipt of information about the ambient temperature or its change. It is likely that peripheral thermosensors are responsible for transmitting temperature information to clock neurons. This has been demonstrated in experiments investigating the role of DN1p in the timing of sleep and activity under constantly changing TC. (Yadlapalli et al., 2018) For correct perception of cooling or warming, it was necessary to use an intact fly, because isolated brains could not be synchronized by TC. (Sehadova et al., 2009). This means that temperature inputs must be located somewhere in the periphery. The first organ identified as potentially sensitive to temperature was the chondrotonal organ (ChO), which is located inside body segments such as the femoral joints, the wing base, the halteres, and the second segment of the auditory Johnston organ (JO) of the antennae. The basic units of the ChO are called scolopidia. Each scolopidium consists of accessory cells (dendritic cells and scolopal cells) that protect and anchor one to three sensory neurons, which are mostly specialized sensory proprioceptors (Tuthill et al., 2016; Matsuo et al., 2013; Mamiya et al., 2018). ChOs are not only specific mechanoreceptors but also are thermoresponsive, which was demonstrated in larvae whose ChOs increased their activity upon both cooling and heating. (Liu et al., 2003). These results were supported by research on the TRP channel INACTIVE (IAV), which is expressed in ChO and is important for cold perception, especially for temperature preference around 17.5 °C. However, this is not the only important factor. Another indispensable gene is nocte (no circadian temperature entrainment), which encodes a large glutamine-rich protein and is widely expressed in sensory organs, including chordotonal organs (Cho) (Sehadova et al., 2009). Nocte1 showed no oscillations during LL and DD with TC, but oscillations were normal at LD and constant temperature. (Glaser and Stanewsky, 2005) Moreover, this mutation leads to the influence of PER and TIM oscillations in clock neurons and disrupts the electrical responses of DN1p clock neurons (Chen et al., 2018). Similarly, iRNA knockdown of the nocte gene specifically in ChO has the same phenotype. Moreover, both nocte1 and iRNA knockdown of ChO showed relevance to the structural function of the whole ChO organ, as both cause deformations, after which mechanosensor function is also disrupted. This implies that the NOCTE protein has a structural rather than a thermosensory function.

Another potential thermoreceptor is expressed in cap cells that connect the dendritic cilia of ChO neurons to the cuticle (Wolfgang et al., 2013). This protein belongs to the TRP family of channels (like IAV) and is called PYRAXIA (PYX). Loss of the pyx gene impairs behavioral synchronization with 16 °C:20 °C temperature cycles at both LL, reducing

oscillations of PER in clock neurons. Despite these defects at the lower end of the *D. melanogaster* physiological temperature optimum, synchronization to warmer temperature cycles, such as 25 °C:29 °C, is normal (Wolfgang et al., 2013). Surprisingly, PYX also protects flies from heat shock at temperatures above 40 °C. Although this gene is expressed in ChOs, it has a more important function for TC synchronization in antennae, as the removal of poorly functioning PYX channels by ablation of antennae improves behavioral synchronization with temperature cycles (Roessingh et al., 2019).

The next family of receptors expressed in ChO are ionotropic glutamate receptors (IRs), which are a diverse group of receptors involved in mechanoreception, gravity, wind, taste, hearing, and finally termoreception (Chen et al., 2015; Sun et al., 2009; Sánchez-Alcaiz et al., 2018). In particular, (IR25a), known to be involved in thermosensation, is expressed in several types of sensory neurons in the ChO, including JO and labellum. In the olfactory system, IR25a works as a co-receptor with different IRs that sense odors (Sánchez-Alcañiz et al., 2018). IR25a loss-of-function mutants show impaired synchronization to temperature cycles, particularly in the synchronization of the DN1 and DN2 subsets of clock neurons. IR25a knockout flies show normal synchronization to temperature cycles with high ranges, but IR25a is specifically required for synchronization to low temperature intervals with an amplitude as low as 2 °C. Unlike wild-type flies, IR25a mutants do not synchronize with shallow temperature cycles in constant darkness (DD) or constant light (LL) and are active throughout the temperature cycle, except for a brief period of reduced activity at the beginning of the warm phase of TC (Chen et al., 2015).

In the context of animal behavior, light can have different effects. Based on the finding that the TIM protein is degraded in the presence of the light-sensitive protein CRY, a simple model was developed to explain how light synchronizes the clock. This model explains the prolongation of the behavioral period or arrhythmia in LL due to a longer accumulation of the required level of TIM. In addition, the same model can be used to explain the phase shift response that occurs after a light pulse during subjective night. Experiments with a cryb mutant lacking a functional CRY revealed that cryb mutants are unaffected by light pulses and do not show arrhythmia in LL. On the contrary, they are able to entrain LD, although their PER and TIM oscillations are reduced. These oscillations can be rescued in combination with TC, and immunostaining with the anti-CRY antibody showed that some subsets of clock neurons (six DN1, both DN2, DN3s, three LND, and LPN) have little or no CRY expression. These results suggest that more inputs must be involved in light synchronization and that temperature is the

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1.2.3 Temperature synchronization

Zimmerman, Pittendrigh, and Pavlidis observed that the circadian rhythms of *Drosophila pseudoobscura* can be entrained by temperature cycles and that heat or cold pulses cause phase shifts whose magnitude depends on the timing of the oscillation in the cycle (Zimmerman et al., 1968). Further studies have shown that the circadian rhythm of *Drosophila melanogaster* can be synchronized by temperature cycles in constant darkness or constant light (Wheeler et al., 1993; Yoshii et al., 2005). Moreover, later studies showed that flies without CRY positive clock neurons are still able to synchronize their circadian clock with TC. These results are consistent with experiments at LD conditions with non-synchronous TC, where wild-type flies have CRY positive neurons that are synchronized, in contrast to LPN and DN2, which are out of phase. Surprisingly, temperature synchronization can be so sensitive that a temperature difference of 2-3°C degrees between day and night within the physiological temperature range (17-29° C) is sufficient to synchronize the circadian clock. Rhythmic temperature fluctuations outside the temperature range that trigger diapause or thermal stress may serve as a strong zeitgeber for entrainment, at least in poikilothermic animals. This sensitivity to temperature fluctuations is surprising because the endogenous free-running clock period is independent of temperature, a phenomenon referred to as temperature compensation (Wheeler et al., 1993; Zimmerman et al., 1968). Temperature compensation is probably a mechanism completely independent of temperature synchronization. Clock cells in the brain are self-compensated and require a signal from peripheral temperature-sensitive cells to synchronize with TC, unlike the head, body, or leg (Sehadova et al., 2009). Moreover, recent studies have shown that not only isolated brains, but also peripheral tissues such as halters or antennae are temperature compensated. Surprisingly, even an isolated brain lacking PDF signaling (pdf01 mutant) shows robust temperature compensation (Versteven et al., 2020). Some polymorphisms that affect temperature compensation have evolved as alternative adaptations to living at different longitudes, e.g., northern populations of *D. melanogaster* have a longer period than southern

populations. Furthermore, the polymorphisms are not just adaptations, but *D. melanogaster* changes its activity pattern through different tim or per splicing variants. (Lamaze et al., 2022; Montelli et al., 2015) In summary, a growing body of evidence suggests that temperature cycles may be a more reliable zeitgeber for animals (including some fly species) that experience summer in polar regions (Harper et al., 2016).

Temperature is also related to the recently identified output of the circadian clock, namely the diurnal variation in preferred temperature, termed the temperature preference rhythm (TPR). *Drosophila* is ectothermic organism, meaning that its body temperature is the same as the ambient temperature, and it tends to choose a preferred temperature to regulate its body temperature. The preferred temperature of *Drosophila* increases during the day and decreases during the night. The TPR persists even under constant light conditions and is controlled by an endogenous circadian clock (Goda et al., 2019). The main clock neurons responsible for regulating TPR are DN2s and DN1a. TRP functions independently of neurons that regulate the rhythm of locomotor activity (Chen et al., 2022).

As mentioned earlier, the isolated brain cannot be synchronized with TC without relying on peripheral heat sensors. Input from the periphery to the central clock is complicated and involves different sensory structures and different families of thermoreceptive molecules (such as IRs, TRP channels, and probably rhodopsins). In addition, different thermoreceptors operate in different temperature ranges, and some receptors can detect temperature changes across the spectrum, such as IR25a (Chen et al., 2015). These mechanisms have evolved to provide a rapid response to sudden temperature changes that could be deleterious, while also using smaller temperature fluctuations between day and night to regulate behavior.

1.2.3.1 Temperature synchronization mechanism in adults

The model of the mechanism of temperature synchronization begins with the perception of temperature changes by peripheral organs, which transmit this information to the brain to specialized subpopulations of clock cells, in which the molecular machinery of the clock is altered. This is followed by the integration of multiple signals from individual subpopulations of clock neurons by neuropeptides, which then relay the resulting output to higher centers responsible for changing the behavior of the whole organism or peripheral organs. Moreover, at all these levels there is a possible combination and variety of as yet unknown inputs, outputs, and modifications that may contribute to the final synchronization.

Moreover, the structures and molecules involved in circadian thermoreception are still not well understood.

The first requirement for synchronization by TC is the receipt of information about the ambient temperature or its change. It is likely that peripheral thermosensors are responsible for transmitting temperature information to clock neurons. This has been demonstrated in experiments investigating the role of DN1p in the timing of sleep and activity under constantly changing TC. (Yadlapalli et al., 2018) For correct perception of cooling or warming, it was necessary to use an intact fly, because isolated brains could not be synchronized by TC. (Sehadova et al., 2009). This means that temperature inputs must be located somewhere in the periphery. The first organ identified as potentially sensitive to temperature was the chondrotonal organ (ChO), which is located inside body segments such as the femoral joints, the wing base, the halteres, and the second segment of the auditory Johnston organ (JO) of the antennae. The basic units of the ChO are called scolopidia. Each scolopidium consists of accessory cells (dendritic cells and scolopal cells) that protect and anchor one to three sensory neurons, which are mostly specialized sensory proprioceptors (Tuthill et al., 2016; Matsuo et al., 2013; Mamiya et al., 2018). ChOs are not only specific mechanoreceptors but also are thermoresponsive, which was demonstrated in larvae whose ChOs increased their activity upon both cooling and heating. (Liu et al., 2003). These results were supported by research on the TRP channel INACTIVE (IAV), which is expressed in ChO and is important for cold perception, especially for temperature preference around 17.5 °C. However, this is not the only important factor. Another indispensable gene is nocte (no circadian temperature entrainment), which encodes a large glutamine-rich protein and is widely expressed in sensory organs, including chordotonal organs (Cho) (Sehadova et al., 2009). Nocte1 showed no oscillations during LL and DD with TC, but oscillations were normal at LD and constant temperature. (Glaser and Stanewsky, 2005) Moreover, this mutation leads to the influence of PER and TIM oscillations in clock neurons and disrupts the electrical responses of DN1p clock neurons (Chen et al., 2018). Similarly, iRNA knockdown of the nocte gene specifically in ChO has the same phenotype. Moreover, both nocte1 and iRNA knockdown of ChO showed relevance to the structural function of the whole ChO organ, as both cause deformations, after which mechanosensor function is also disrupted. This implies that the NOCTE protein has a structural rather than a thermosensory function.

Another potential thermoreceptor is expressed in cap cells that connect the dendritic cilia of ChO neurons to the cuticle (Wolfgang et al., 2013). This protein belongs to the TRP

family of channels (like IAV) and is called PYRAXIA (PYX). Loss of the pyx gene impairs behavioral synchronization with 16 °C:20 °C temperature cycles at both LL, reducing oscillations of PER in clock neurons. Despite these defects at the lower end of the *D. melanogaster* physiological temperature optimum, synchronization to warmer temperature cycles, such as 25 °C:29 °C, is normal (Wolfgang et al., 2013). Surprisingly, PYX also protects flies from heat shock at temperatures above 40 °C. Although this gene is expressed in ChOs, it has a more important function for TC synchronization in antennae, as the removal of poorly functioning PYX channels by ablation of antennae improves behavioral synchronization with temperature cycles (Roessingh et al., 2019).

The next family of receptors expressed in ChO are ionotropic glutamate receptors (IRs), which are a diverse group of receptors involved in mechanoreception, gravity, wind, taste, hearing, and finally termoreception (Chen et al., 2015; Sun et al., 2009; Sánchez-Alcaiz et al., 2018). In particular, (IR25a), known to be involved in thermosensation, is expressed in several types of sensory neurons in the ChO, including JO and labellum. In the olfactory system, IR25a works as a co-receptor with different IRs that sense odors (Sánchez-Alcañiz et al., 2018). IR25a loss-of-function mutants show impaired synchronization to temperature cycles, particularly in the synchronization of the DN1 and DN2 subsets of clock neurons. IR25a knockout flies show normal synchronization to temperature cycles with high ranges, but IR25a is specifically required for synchronization to low temperature intervals with an amplitude as low as 2 °C. Unlike wild-type flies, IR25a mutants do not synchronize with shallow temperature cycles in constant darkness (DD) or constant light (LL) and are active throughout the temperature cycle, except for a brief period of reduced activity at the beginning of the warm phase of TC (Chen et al., 2015).

The last known receptors involved in peripheral termosensing are rhodopsins (1-7RH). However, the mechanism of their function in adult flies is still difficult to understand, but results in larvae of *D. melanogaster* show their importance (Shen et al., 2011). Although we already know some of the genes involved in temperature synchronization, their relationship with the cell clock is still not entirely clear. The neuronal circuit in which the ChO termoreceptors are integrated and their cooperation in temperature sensing remain to be identified; however, many studies provide strong evidence that ChO function in temperature sensing is critical for synchronizing the circadian clock in flies (Figure 4).

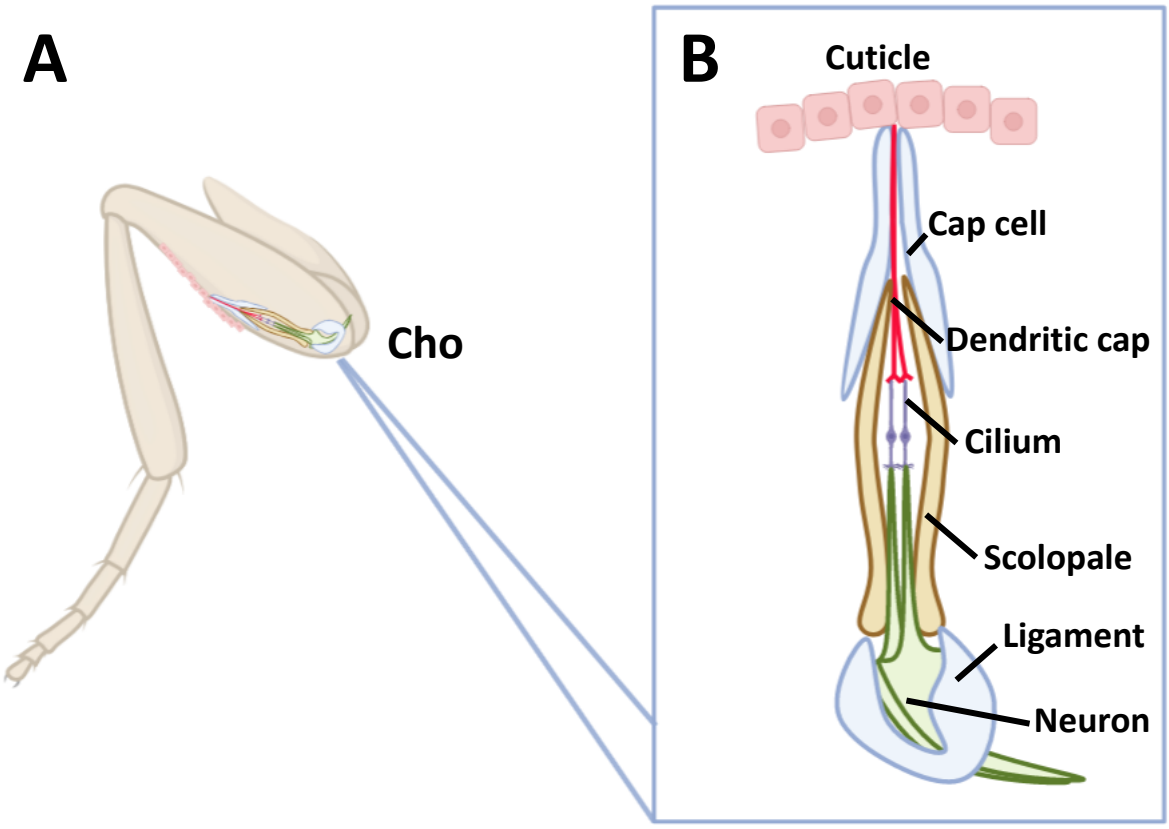

Figure 4: The scheme (**A)** shows the location of the ChO organ in the leg and its basic structural unit, the scolopidium. (**B)** description of the different parts of the scolopidium contains different thermoceptive candidates. The PYX receptor is localized in the cap cell. The *nocte* gene is expressed in the dendritic cup. A number of IR genes (*IR8a, IR25a, IR64a, IR75a, IR75d, IR76a, IR94b and IR100a*) and rhodopsin genes (*rh1* and *rh3-rh7*) are expressed directly in neurons (inspired by George and Stanewsky).

Antennae are other complex sensory organs of *Drosophila*, located on the front of the fly's head between the eyes, and serve their main function as near-field sound receptors. Each antenna consists of an arista and three segments (A1-A3). The individual segments differ from

each other not only in structure but also in function. The A2 segment of antennae is called Johnston's organ (JO), and its internal structure contains the largest chondrotonal organ of the fly. The A3 segment is called the sacculus and is divided into three more chambers. All thermoreceptors known so far are distributed at different frequencies in all these parts of the antenna.

The arista is not only the main organ for sound like hair and sensory bristles, but also an important thermosensor, with three cold and three warm cells at its base. The cold and warm cells respond to temperature changes in the cold and warm ranges. Warm cells respond to a rise in temperature and are inhibited by cooling. Cold cells show activation in response to cooling and are inhibited by heating (Budelli et al., 2019). Warm and cold cells express three different IRs. Some IRs are included as co-receptors in multiple structures (IR93a and IR25a), and the others have a specific function, e.g., (IR21) responds to cooling. In addition to the IRs, hot cells also express the gustatory receptor Gr28b, which triggers rapid temperature avoidance. This supports the hypothesis that Gr28b controls rapid responses to ambient temperature, in contrast to TRPA1, which controls responses to temperature increases in the organism (Ni et al., 2013). From the ultrastructure of aristae, it appears that properly formed membrane lamellae (bossy orthogonal surface substructures - BOSSs) are essential for proper cold cell function. Mutants of the *IR25a* and *IR21a* genes exhibit defects in the structure of BOSS, resulting in the loss of their ability to mediate thermotactic behavior (Budelli et al., 2019). Although it is still unclear whether IRs in the aristae are directly involved in TC synchronization, the lack of thermosensitive cells in the aristae results in altered sleep timing during and impaired acute temperature avoidance. Termosensors between the base of the aristae and sacullus chamber 3 express Brivido 1-3 genes that are sensitive to cooling, but are not involved in temperature avoidance behavior (Gallio et al., 2011; Budelli et al., 2019). The sacculus, a multichambered invagination in the A3 segment, also contains thermoreceptors. Termosensitive receptors (IR25a, IR93a) and huminidy receptors (IR40a and IR68a) are expressed in chambers 1-2. The *IR21a* is expressed near chamber 3 (Benton et al., 2009; Abuin et al., 2011; Frank et al., 2017).

The second segment of the antenae (JO), contains many different receptors. Some of them, such as RH5, RH6 and IR75a, are important for hearing (Senthilan et al., 2012). Scolopidium cells from JO also express PYX and IR25a receptors (Roessingh et al., 2019). However, because a subset of rhodopsin, IR -receptors, and PYX -channels that may contribute to temperature entrainment in resetting the thermal clock were disrupted by a

mutation of the Pyx gene resulting in disruption of TC, and subsequent removal of the antenna resulted in amplification of TC, a direct link of the antenna to circadian clock synchronization cannot be confirmed (Shen et al., 2011). The fact that flies can entrain to temperature cycles even without antennae suggests that other alternative thermosensing circuits exist (Figure 5).

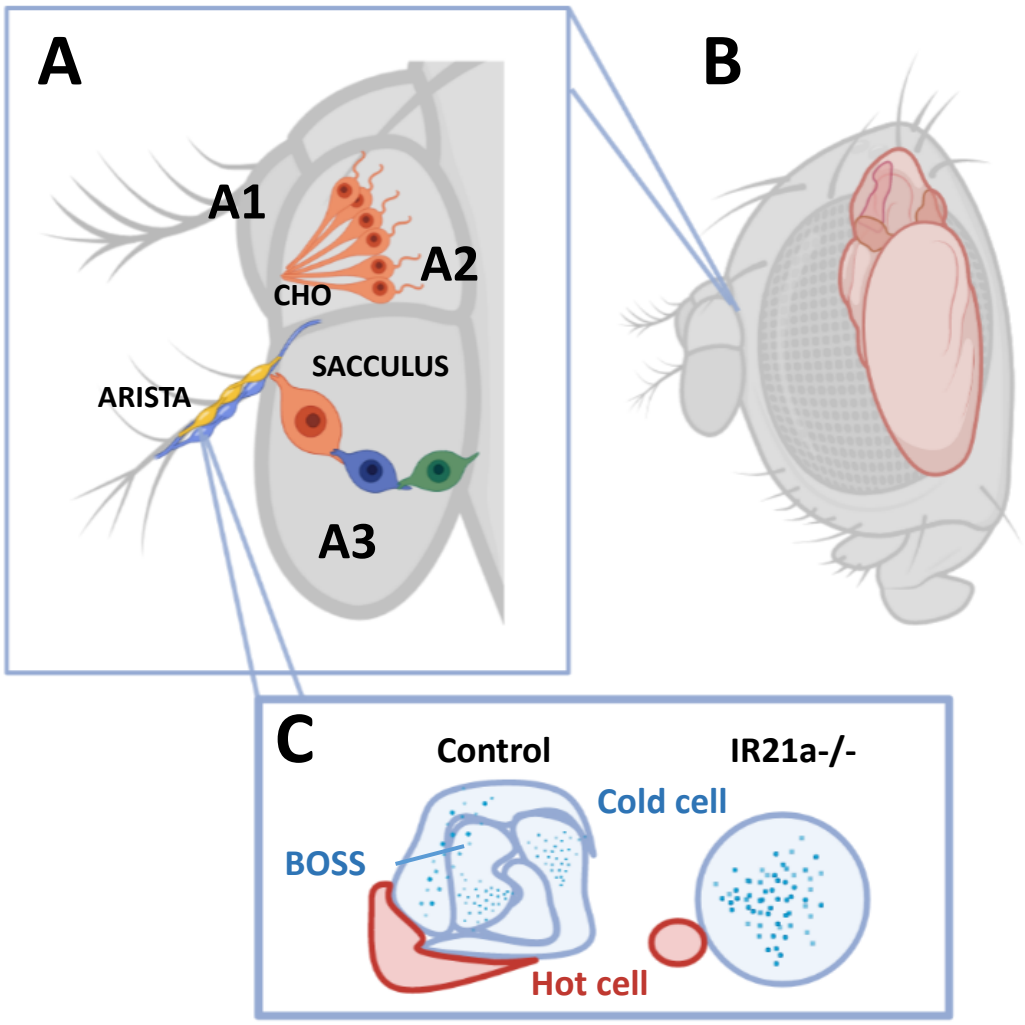

Figure 5: Diagram of the antenna and its parts with the distribution of potential thermoreceptors. (**B)** shows the location of the antenna on the head of *Drosophila*. (**A**) divides the antenna into parts (arista, sacculus, JO and scape), distribution of termosensitive cells. (**C**) shows the effect of the *IR25a* and *IR21a* gene mutations on the ultrastructure of cooling cells.

Although antennal thermal sensors are not the main inputs for temperature entrainment, it is still important to map these antennal circuits and describe their mechanism. Recent studies have shown that receptors project from the antennae to the antennal lobe (PAL) in the same way that olfactory sensory neurons project to aMe (Marin et al., 2020). Neurons in PAL are divided into stereotyped ventroposterior glomerular subdivisions (VP-AL). One of these glomeruli is the hot glomerulus (VP2), which receives information from hot cells in the aristae and whose thermoreceptor is Gr28b.d. Conversely, cold-sensitive thermoreceptors (IR21a) from cold cells in the aristae, cold thermoreceptors from sacculus chamber 1, and AC neurons newly identified thermoreceptors expressing *ir25a* are guided by the activity of TPN-II and terminate not only in the cold glomerulus (VP3) but also directly in DN1 clock neurons. The IR40a receptor projections in the sacculus terminate in VP4, and the IR68a huminidy receptor terminates in VP5. Finally, putative ir40a thermoreceptors in chamber 2 project to VP1. From these glomeruli, projection neurons send information to higher brain regions such as the mushroom body (MB) and lateral horn (LH) of the protocerebrum (Marin et al., 2020) (Figure 6).

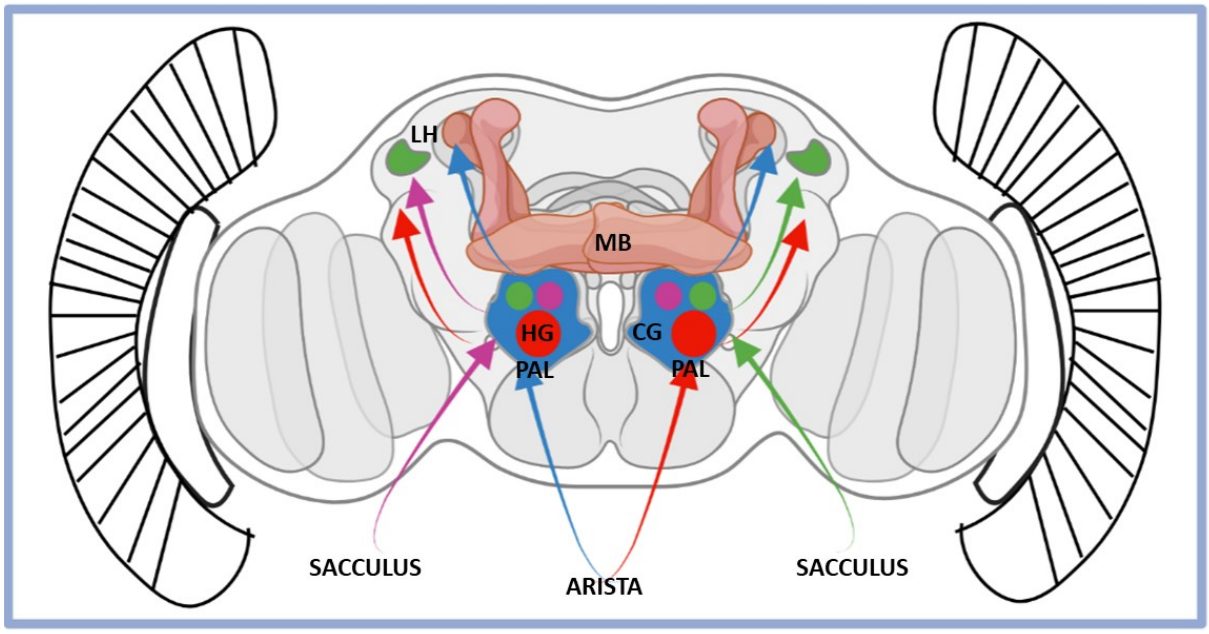

Figure 6: The diagram shows the integration of signals from different parts of the antenna. The green colour shows the path for the dry signal, the purple colour shows the processing of the moisture signal. Red (HG – hot glomerus) shows the warming signal and blue shows the cooling signal (CG–cold glomerus). lateral horn (LH), mushroom body (MB), antennal lobe (PAL).

Moreover, the clock neurons are located between the brain bodies. It is possible that there are direct connections between some thermoreceptors and clock cells in terms of temperature synchronization, but such connections have not yet been described.

One of these connections could be the TRPA1 channel, although its effect on temperature synchronization is questionable. On the other hand, it is important for temperature preference behavior. TRPA1 is affected both directly by temperature and indirectly through the GQ/PLC-B signaling pathway. Although TRPA1 is also expressed in AC neurons and some clock neurons (not confirmed in situ by antibodies), TRPA1 mutants were synchronous with TC in DD (Tang et al., 2017) and two other studies (Hamada et al., 2008 Lee et al., 2013) refuted the need for a link between TRPA1 and temperature cycles across the Drosophila temperature optimum. Despite the known role of TRPA1 in regulating temperature-dependent behaviors, the function of trpa1 in temperature synchronization is still unclear (Lee et al., 2013; Roessingh et al., 2015; Das et al., 2016).

For this reason, the only connection known so far is the signal from TPN-II to DN1 neurons. Movever, in IR25a, nocte1, and pyx mutants, the expression of TIM and PER is altered in DN1, providing strong evidence for the role of the DN1 group in receiving temperature inputs from multiple peripheral thermosensors. (Chen et al., 2015; Roessingh et al., 2019) Moreover, sLNvs receive input from DN1a neurons via CCHamide signaling with the function of timing sleep. This work highlights the importance of input from cold sensors in the antennae for inhibiting DN1a activity and regulating sleep-wake patterns in a cold environment. Another group of clock cells likely involved in temperature synchronization is DN2. Although they do not influence temperature-dependent locomotor behavior, they are directly involved in the regulation of the circadian temperature preference (TPR) rhythm. Interestingly, the TPR rhythm persists under constant light conditions, suggesting that the TPR rhythm and the locomotor activity rhythm are regulated independently. In addition, TPR fluctuates at different time poins of the day, including daytime, the onset of night, and before dawn.

DH31R, the receptor for the diuretic hormone 31, is responsible for mediating TPR in flies. The DH31R mutant exhibits a constant preferred temperature of approximately 27 ◦C during the day and has disrupted diurnal TPR in DD, suggesting that the function of DH31R in diurnal TPR is related to the endogenous circadian clock. Although DH31R is not expressed in DN2s, it is expressed in a subset of DN1s, DN3s, and lLNvs, suggesting that DH31R may be a mediator between subsets of clock neurons by acting downstream of the clock (Goda at al.,2019) (Figure 7).

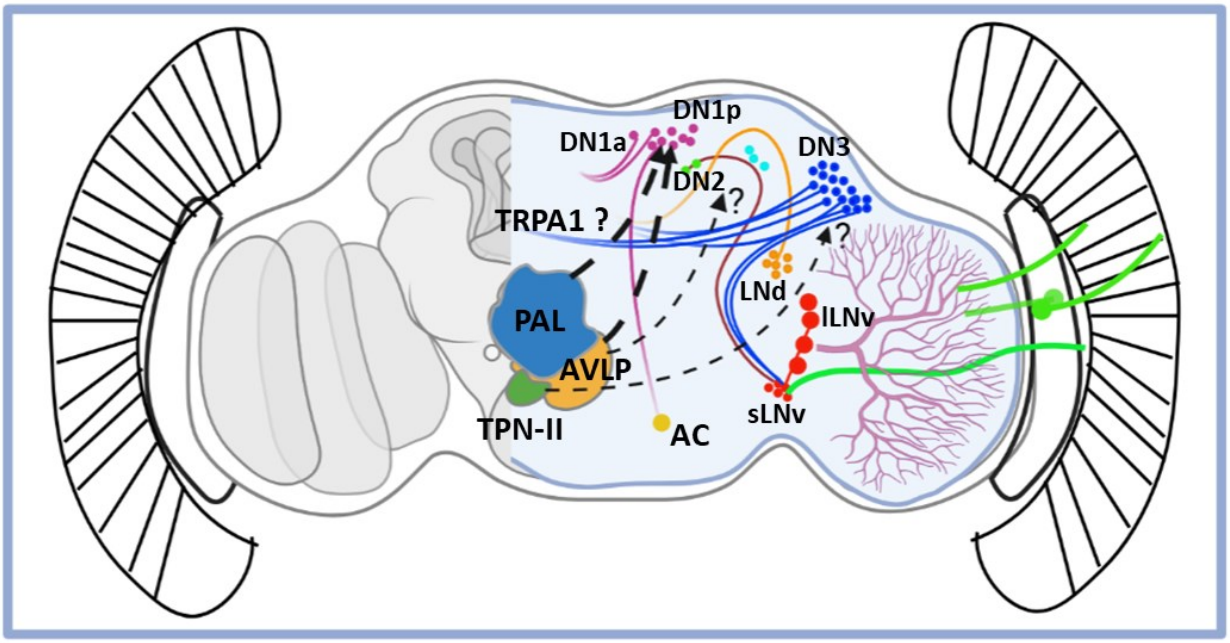

Figure 7: Diagram of the Drosophila brain with individual groups of clock neurons (sLNv,lLNv, LNd, DN3, DN2, DN1a, DN1p) and their integration of mutual signals.

Although some potential thermoreceptors have already been identified, it is still unclear how clock cells integrate these input signals at the cellular level. However, some studies show that TCs affect the expression levels of many genes, including those with circadian functions. (Boothroyd et al., 2007; Anduaga et al., 2019). Temperature affects the expression of the central clock genes per and tim and modulates their amplitude and phase (Shakhmantsir et al., 2019). The splice variant of per intron called dmpi8, increases at cooler temperatures, leading to higher diurnal activity. At the same time, reduced splicing at warm temperatures correlates with delayed onset of nocturnal activity and prolonged inactivity during the day (Majercak et al., 1999). It is not clear how temperature directly modulates the efficiency of dmpi8 splicing. However, experiments with the norpA mutant showed that the norpA mutation mimics cold temperatures and is involved in increasing the splicing efficiency of dmpi8. Increased dmpi8 splicing leads to an increase in PER protein amplitude. Moreover,

cold-enhanced dmpi8 splicing is also co-expressed with the daywake gene (dyw), whose RNA overlaps with dmpi8 at the 3' ends, allowing reciprocal regulation of these genes. Dyw encodes a juvenile hormone-binding protein that reduces siesta behavior (Beckwith et al., 2019). Studies suggest that this per-splicing event is important for behavioral adaptation to hot and long summer days, with less splicing leading to increased siesta and reduced risk of dehydration (Figure 8).

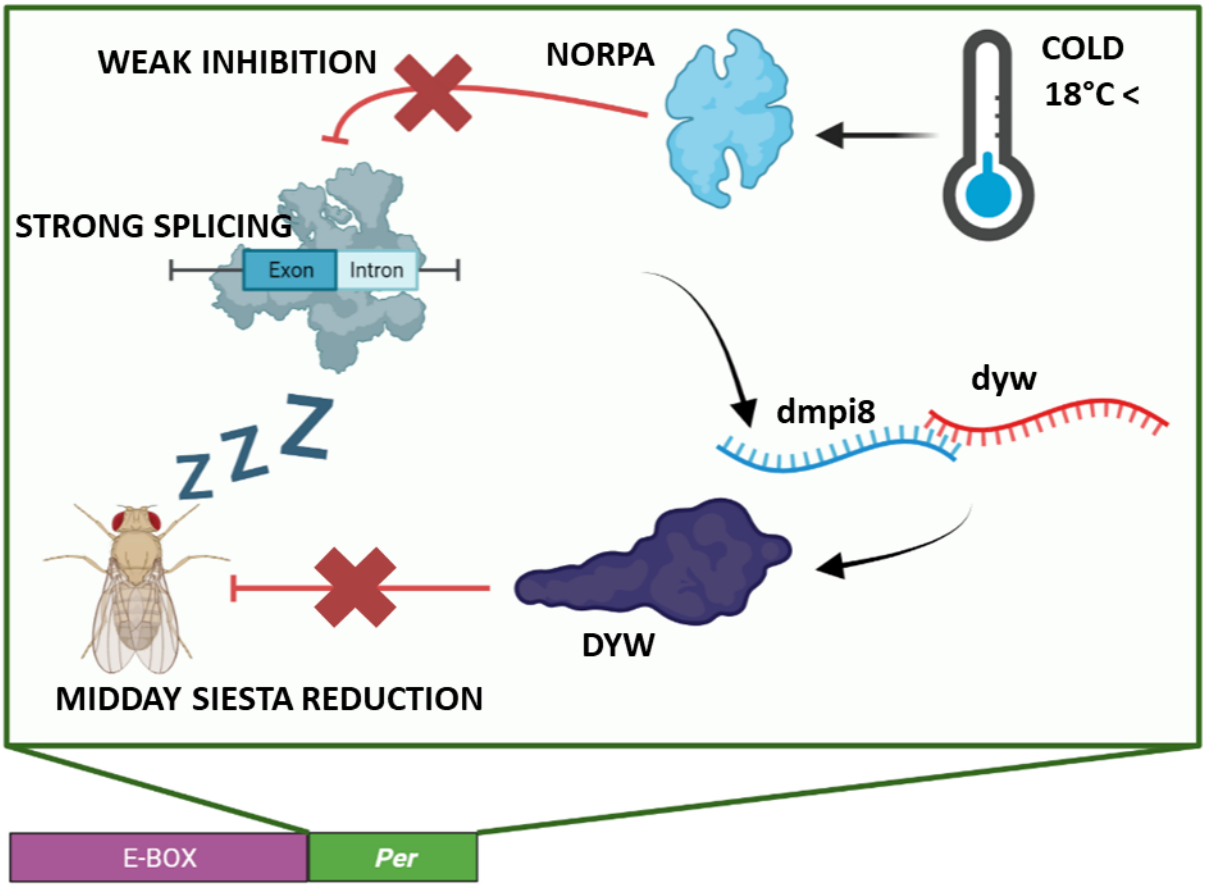

Figure 8: Scheme of cold (18 °C<) alternative splicing per gene. At temperatures below 18 °C, accumulation of no receptor potential A (NORPA) protein negatively affects inhibition of *per* gene splicing. This produces the *dmpi8* variant which, together with *dwy*, induces the production of the DWY protein, which inhibits midday siesta and increases day activity at dusk.

Alternative splicing also occurs in the Tim gene. Considering the 9 predicted variants of alternative splicing, this is a dynamic system surprisingly dependent on environmental temperature. In contrast to the per gene, tim generates different tim transcripts, such as tim-tiny, which reduces the rate of tim RNA, resulting in reduced amounts of the TIM protein under the conditions of 30°C:25°C TC in DD and LL. Its amount increases with increasing temperature (Shakhmantsir et al., 2019). Moreover, at low temperatures (18°C), two different alternative splice variants are prevalent, resulting in two different transcripts (tim-cold and

tim-sc) (Boothroyd et al., 2007; Anduaga et al., 2019). The TIM-COLD variant plays a role in seasonal adaptation to cold temperatures by having an increased affinity of TIM-COLD for CRY (Montelli et al., 2015). TIM-SC binds to but does not stabilize the PER protein, and in tim-sc mutants have same activity pattern in LD 25°C as wild-type flies at 18°C (Anduaga et al., 2019). Accumulation of TIM-SC increases greatly in flies at temperatures below 10°C. In addition, TIM-SC binds and stabilizes a co-transcription factor called EYA under short photoperiods, which is required for efficient transition to female diapause under cold conditions (Anduaga et al., 2019). These splice variants are associated with adaptations to different environmental conditions, such as reproductive dormancy in temperate climates. The role of alternative splicing in daily entrainment to temperature cycles is not well understood, but it is possible that these mechanisms are involved in temperature synchronization and compensation. However, the role of cell-autonomous temperature-dependent clock RNA alternative splicing events in temperature entrainment appears to be minor (Figure 9).

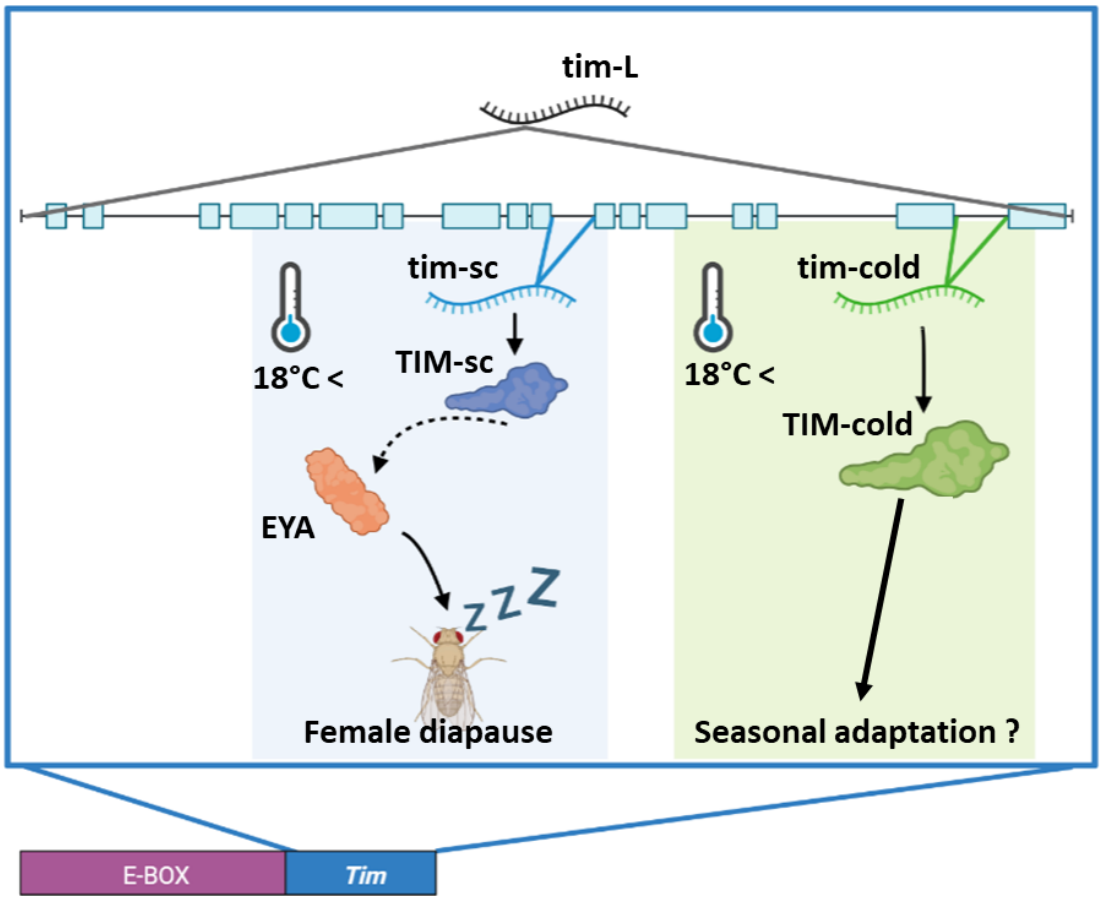

Figure 9: Scheme of cold alternative splicing of the tim gene. At temperatures of 18°C< the splicing pattern of the tim protein changes and the tim-sc and tim-cold variants predominate. The tim-sc variant is able to release the tim01 mutant and is thought to be involved in seasonal adaptation. Similarly, tim-cold affects the accumulation of the protein protein-tyrosine-phosphatase (EYA), which helps induce diapause in female flies (oogenesis).

In summary, temperature synchronization depends mainly on signals from peripheral temperature-sensitive organs (ChO, antenea). However, these organs contain a variety of different thermoactive proteins from different protein groups, and it is still unclear which of these thermoreceptors are involved in temperature synchronization and which in the immediate escape response or internal temperature perception. These processes are likely interconnected and regulated at multiple levels, complicating their clarification. The cooperation of individual thermosensory organs is supported by the fact that direct removal of the ChO or antenna does not lead to complete disruption of TC synchronization. It can be assumed that receptors that respond to temperature changes over longer periods of time are more likely to be involved in temperature synchronization. Receptors of IRs thus seem to be more important for temperature synchronization.

1.2.3.2 Temperature synchronization mechanism in larvae

Temperature synchronization has been studied extensively in adult *D. melanogaster*, but many important insights into temperature perception have come from experiments with larvae. The larval circadian system is much simpler than that of adults and therefore easier to manipulate. Larval temperature perception, as in adults, depends on peripheral organs. Peripheral organs already known are the bilaterally symmetrical olfactory organ (dorsal organ), in which an IR21a, IR25a, and IR93a are expressed. These genes have been shown to mediate cold detection in larval dorsal organs (Knecht et al., 2016, Ni et al., 2016). The dorsal organ also expresses rhodopsins that are important for temperature perception, whether for immediate response to warming or cooling or for selection of preferred temperature. One of these is the rh1 receptor, which is important for the thermotaxic behaviour of third instar larvae after they enter the wandering stage. *Drosophila* larvae respond to temperature fluctuations of a fraction of a degree (Klein et al., 2015), and third instar larvae change their temperature preference from 24 °C to 18 °C. Rh1 is required for temperature preference before the wandering stage (Kwon et al., 2008; Shen et al., 2011). In the wandering stage, there are two other RH5 and RH6 mutants that do not show temperature preferences (Shen et al., 2011). These results are confirmed by an experiment with a mutant in the chromophore, one of the rhodopsin subunits. Such a mutant also lost its temperature preference for 18C late in the third instar. Moreover, rh5 and rh6 are expressed in larval ChO neurons and support the structural integrity of Cho neuron dendrites (Sokabe et al., 2016).

Rhodopsins require TRP channels to function. Drosophila has several of these: two members of the TRPV family, one TRPM, and four TRPAs. TRPVs are activated upon hearing

(Gong et al. 2004). TRPA Painless, which mediates larval nociceptive responses to mechanical stimulation at high temperatures (Tracey et al., 2003). TRPA1 is involved in thermoreception because a mutation of trpA1 prevents larvae from discriminating 18 °C from other temperatures in the comfort range in the mid-third instar (Kwon et al., 2008). In addition, a member of the TRP channel family, IAV, is expressed in ChO, the mutation of which causes disruption of temperature preference in the range above 17 °C.

The IR25a protein is also found in various types of gustatory organs distributed on the head and near the pharynx, including head sensory neurons in the terminal and ventral organs and internal neurons in the dorsal, ventral, and posterior pharynx. The peripheral neurons in the body wall are thermosensitive cells of the peripheral nervous system. In a previous study, these neurons were already identified by *elav* expression and measurements of their activity shifts showed a response to temperature stimuli. From these results, a functional map of these cells and their thermosensitivity was constructed. In contrast to adults, there are other temperature-sensitive body wall neurons in larvae besides ChO, each of which responds differently to temperature stimuli; which of these are involved in temperature synchronization is still unclear. Some of the peripheral thermosensitive cells (Cho/ICH5 and cluster1) showed increased activity during both cooling and warming (Liu et al., 2003; Scholz et al., 2017). On the other hand, previous studies have shown that the larval clock can be synchronized by TC cycles even in completely blind larvae (Malpel et al., 2004). Per-oscillations are also observed in DD with TC cycles in clock cells (DN2, DN1, LNs), with the strongest oscillations occurring in DN2. At the same time, a simultaneous phase of DN2 and LNv can be observed in TC cycles with PER lows at ZT4 and peaks at ZT16. Some results even suggest that thermal synchronization, unlike light synchronization, does not require LNs with PDFs (Picot et al., 2009). However, the link between the circadian clock in TC cycles and behavioral outcomes has not yet been fully described. In addition, the neural circuits that send information from

thermoreceptors to the circadian clock and the function of thermoreceptors in temperature synchronization have not been fully elucidated (Figure 10).

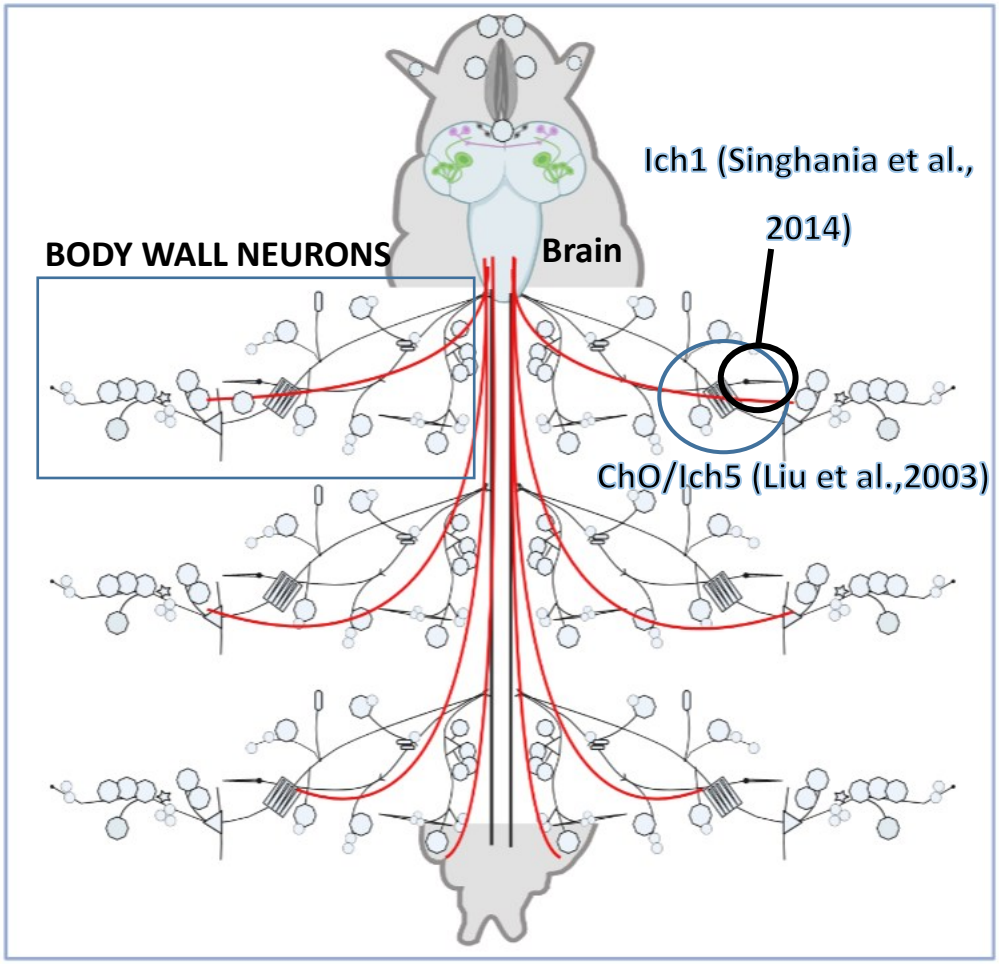

Figure 10 The diagram shows the distribution of sensory organs in the body (body wall peripheral neurons) The blue circle indicates ChO/Ich according to (Liu et al., 2003) and the black circle indicates the more precisely specified ChO/Ich1 unit according to (Singhania et al., 2014). The red lines indicate the signaling of R5 and R6 rhodopsins in the larval body for which their precise termination is not known. Head sensory organs in which the expression of IRs is already known are also indicated (Sánchez-Alcañiz et al., 2018).

2 Aims

1. Identify cells expressing the *IR25a* gene in the body of *Drosophila melanogaster IR25aGal4-UAS-RFP* larvae.
2. Investigate the effect of the *IR25a*-/- mutation on the the ability of circadian clock to synchronized to the low amplitude temperature cycles by investigating daily oscillations in PER protein level and in CO_2 production.
3. Cross *IR25aGal4-UAS-RFP* with *UAS-rpr* flies to initiate apoptosis of *IR25a* expressing cells, and to investigate the effect of *IR25a* elimination on synchronization of the progeny to the low temperature cycles.
4. Examine the effect of *IR25a* elimination of adult *IR25aGal4-UAS-RFP-UAS-rpr* flies to synchronize the locomotor activity to the low temperature cycles.

3 Material and methods

3.1 *Drosophila* stock

Flies were grown in a light/dark (LD) cycle of 12 hr L and 12 hr D at 25 °C on standard corn meal-yeast-agar medium. Depending on the temperature conditions, the developmental time varies between 5 and 7 days to the wandering third instar larval stage, at which the immunostaining experiments were done. *IR25aGAL4-UAS-RFP* strain was used as a control. Transgenic UAS-GAL4 technique was performed to express target *UAS-RFP* transgene reporter only in *IR25aGal4* cells. The following strains used in this study: *UAS-rpr, IR25aGal4-UAS-RFP, IR25-/-* were obtained from the Bloomington *Drosophila* Stock Center (Larsson et al., 2004; BDSC, NIH P40OD018537). For ablation of *IR25a* positive cell *IR25aGal4-UAS-RFP* and *UAS-rpr* were crossed and eliminating of the *IR25a* positive cells were checked under confocal microscope. Successful cross produsing *IR25aGAL4-UAS-RFP* fly stock was verified by loss of RFP signal in the progeny (Figure 11).

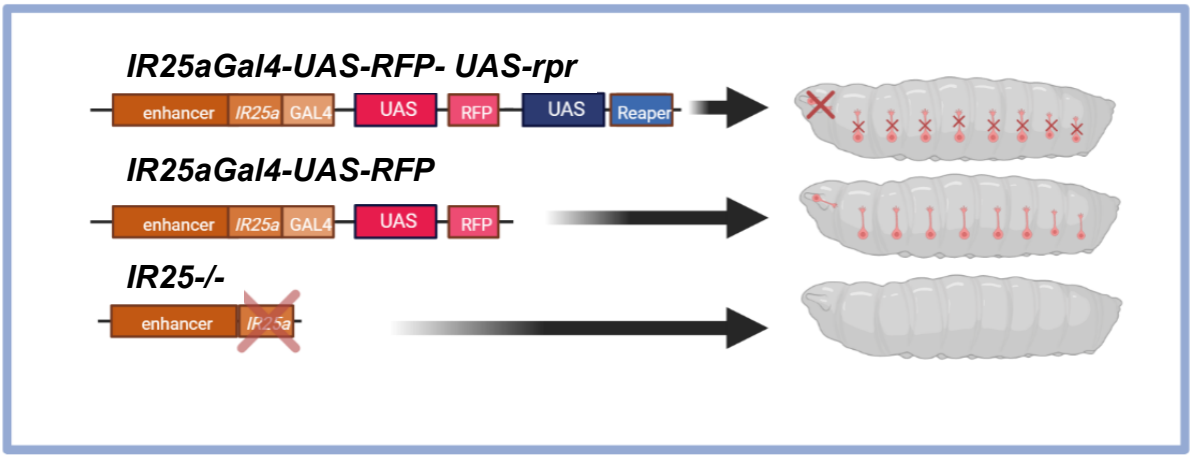

Figure 11: Scheme of individual strains and their genetic background that leads to their phenotypes in RFP expression.

For the light entrainment experiments, larvae were placed in LD conditions at 25 °C. At this temperature, the larvae reached third instar wandering stage in 5 to 6 days. For the TC entrainment experiments, larvae were grown in LD 12:12, and at the beginning of the third instar, they were moved to DD with the low TC 17 °C:19 °C for 5 days before first dissection time. The warm interval has been defined as Zeitberger time 0 (ZT0) to ZT12. Under these conditions, larvae reached third instar wandering stage in 10 days.

3.2 Imunohistochemistry

The principle of this technique is the visualization of a protein using a specific antibody that reacts with the protein epitopes. Indirect immunohistochemical detection, which uses a labeled secondary antibody to detect an unlabeled primary antibody, was used in the experiments.

3.2.1 Primary and secundary antibodies

All experiments were done on whole mounted third-instar larvae or on their brains. The rabbit anti-PER antibody (Stanewsky et al., 1997) was preabsorbed using *per0* embryos, and then was diluted 1:1000 in the blocking solution consisting of 5% goat serum in phosphate buffer (PB) supplemented with 0.3% Triton (PB-T). The rat anti-ELAV antibody (Hybridoma Bank) and the monoclonal mouse antibody nb33 (anti-PDF antibody; Hybridoma Bank) were used in 1:400 and 1:1000 dilution, respectively. Primary antibodies were diluted 1:200 in the blocking solution. Secondary antibodies: goat anti-rabbit antibody conjugated with fluorophore, AlexaFluor 488 nm; goat anti-rat antibody conjugated with fluorophore AlexaFluor 488 nm; and goat anti-mouse antibody conjugated with fluorophore AlexaFluor 647 nm (Molecular Probes) were diluted 1:200 in the blocking solution.

3.3 Immunoflourescence

Immunohistochemical staining was performed as described previously with a few modifications (Lin et al., 2021). *Drosophila* specimens were dissected in PB and fixed in 4% paraformaldehyde for 20 min. After washing in PB-T three times for 10 min, the samples were incubated in the blocking medium for 1 h. Next, the blocking solution was replaced by primary antibodies and incubated for overnight at 4 °C. After washing in PB-T three times for 10 min, the samples were incubated in appropriate secondary antibodies for 1 h. Following incubation with secondary antibodies, the samples were washed in PB-T three times for 10 min and were mounted with Fluoreshield (Sigma-Aldrich).

3.4 Confocal microscopy

The samples were analyzed in the laser confocal microscope (FluoViewTM FV 3000 Olympus), which allowed to obtain Z-stack images with a resolution of 1024 x 1024 pixels. The Imaris software version 6.3.1 (Bitplane, Oxford Instruments) was used for image analysis.

This composite image in TIF format was then graphically modified using brightness and contrast tools in the Adobe Photoshop software (Adobe Systems).

3.5 Software quantification and statistics

Analysis of the intensity of the PER protein immunoreactivity was measured using the FIJI (ImageJ program; Rasband et al., 1997). Individual set of Z-stacks were always calibrated to ensure that the identical scale of mean gray value intensity. Only the nuclear signal was measured, there were 3 measurements performed in each individual cell of every cell clusters. The mean values of each group (DN1, DN2, LNs) within 5 brains were then plotted in graphs. In the R study programne (RStudio Team, 2020), the ANOVA statistical model was used to compare difference between all timepoints. Tukey multiple comparisons of means test was used to compare the intensity of PER staining at each time point tested (RStudio Team, 2020).

3.6 Respirometry

The production of CO2 was measured using the respirometry analysis system MFC-2/TR-SS3/MUX (Sable Systems International, Las Vegas, NV, USA) equipped with LI 7000 CO_2/H_2O analyzer (LI-COR Biosciences, Bad Homburg, Germany). Six groups of 10 larvae were placed in six glass tubes (volume 22 ml) on 0.3 g of larval diet. All tubes were placed inside the incubator (Sanyo) set to constant DD and to the low temperature cycles 17 °C:19 °C (12 h:12 h). Larvae lived inside tubes for approximately 84 h and their CO_2 production was measured in 30 min intervals. During each interval, the system was "closed" for 1.575 sec and then flushed for 225 sec using 75 ml of CO_2 to devoid air from a tank (Linde Gas, Praha, Czech Republic). The CO_2 was removed using CO_2-absorbet soda lime (Elemental Microanalysis, Okehampton, UK). The data analyses in the ExpeData software tool (v.1.2.02, Sable Systems International) were followed by visual inspection of the obtained graphs to searched for a daily rhythmic pattern (Figure 12).

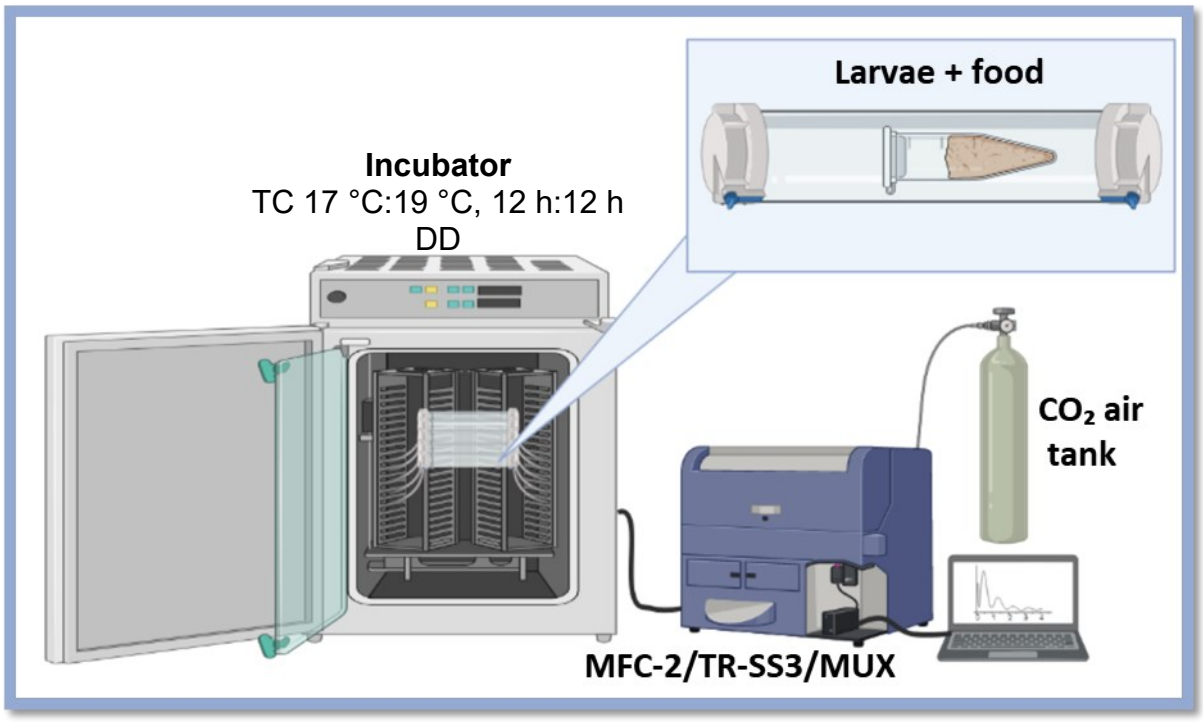

Figure 12: A schema of experimental setup for measuring daily respiration activity in *D. melanogaster* larvae.

3.7 Behavioral analyses

The behavioral analyses were performed on adults of *IR25aGAL4-UAS-RFP-rpr* strain, kept for 4 days in DD with the low TC 17 °C:19 °C. Then two to four days old males were CO_2 anesthetized and transferred under red safelight to 70 mm long tubes containing 5% sucrose in agar, and the tubes were loaded into the DAM2 TriKinetics system (Waltham, MA, United States) entrained to the low TC conditions for 15 days, and released to constant temperature and darkness (DD) for additional 5 days. ActogramJ was used to create double-plotted actograms, that were furhter eye inspected (Schmid et al., 2011). In order to quantify behaviour during temperature cycles, activity from the first five days was plotted in Excel (Office, Microsoft).

4 Results

4.1 Distribution and characterization of neurons expresing *IR25a* gene in *D. melanogaster* larvae

The distribution of *IR25a* gene in the peripheral neural system was determined using the third instar larvae of *IR25aGAL4-UAS-RFP* strain. The confocal microscopy revealed one bilateral cell expressing *IR25a* gene in the lateral region of the frontal plain of each body segment (Figure 12 A, B1, B2). The presence of axonal projection running from the cell body showed the neuronal character of the detected cells. The neurons layed in the medial plane of the lateral segment region and their axons passed throught the body wall to terminated in the dorsal cuticle (Figure 12 D).

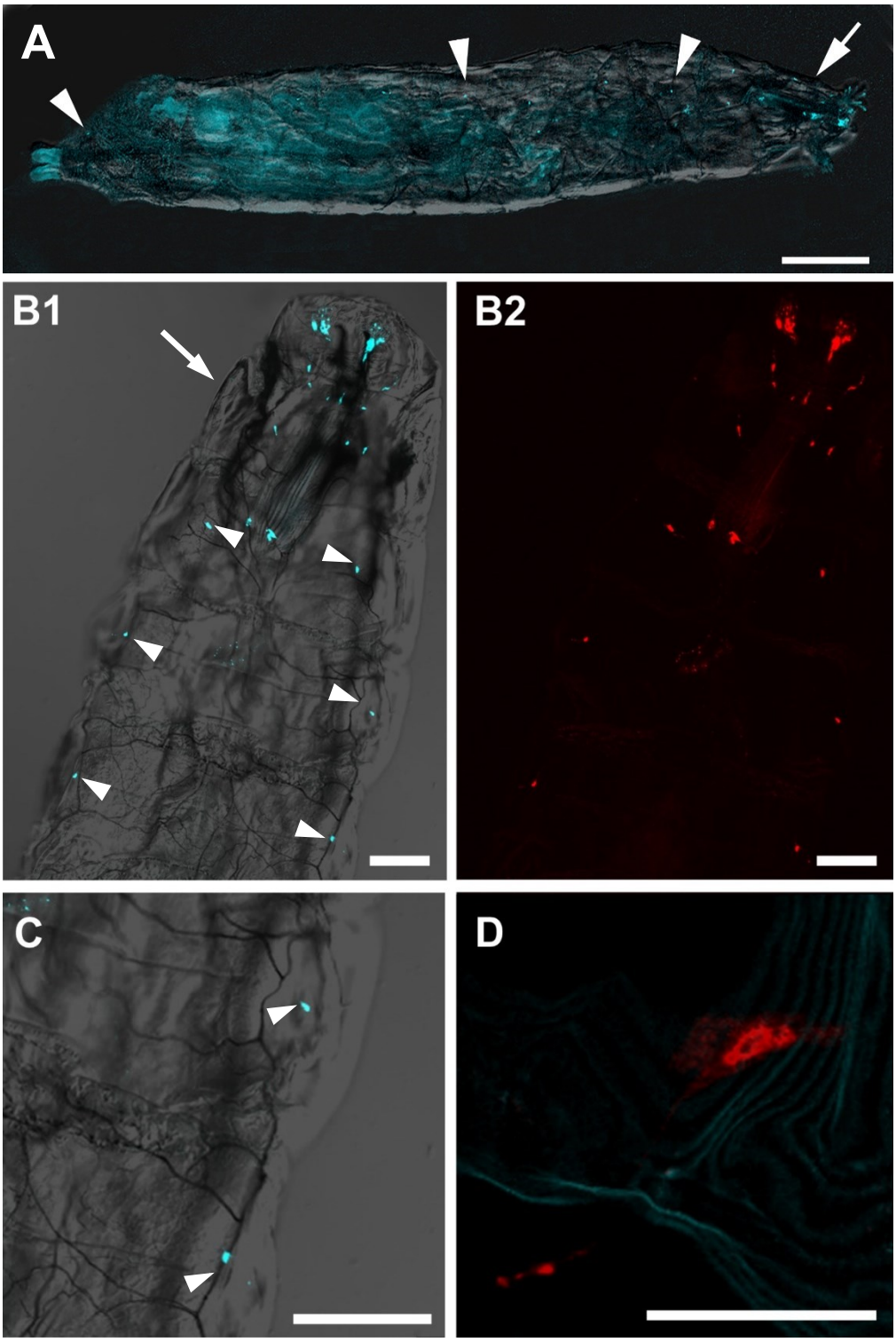

Figure 12: Localization of *IR25a* expressing neurons in the peripheral neuron systém of the *D. melanogaster* third instar larvae. *IR25a* is driving expression of RFP (in red or blue) in *IR25aGAL4-UAS-RFP* strain by Gal4-UAS system. **(A)** An image showing IR25a sensory neurons in entire larval body. The RFP signal in blue is overlaid with transmition light image. **(B1, B2)** The images showing IR25a signal in the superior part of the *IR25aGAL4-UAS-RFP* larvae. Transsmision light image overlaid with RFP signal (B1). RFP signal (B2). Arowheads point to the neurons in the body wall. Arrows show sensory organs on the head and in the pharynx. scale bar in A 500 µm, in B1, B2, C 100 µm, D 50 µm.

In addition, the signal was detected in the head, where the *IR25a* gene was expressed in sensory neurons of the terminal and ventral organs, and in internal neurons of the dorsal, ventral, and posterior pharyng (Figure 13 C). These organs function as chemosensory, termosensory and humidity organs (Sánchez-Alcañiz et al., 2018).

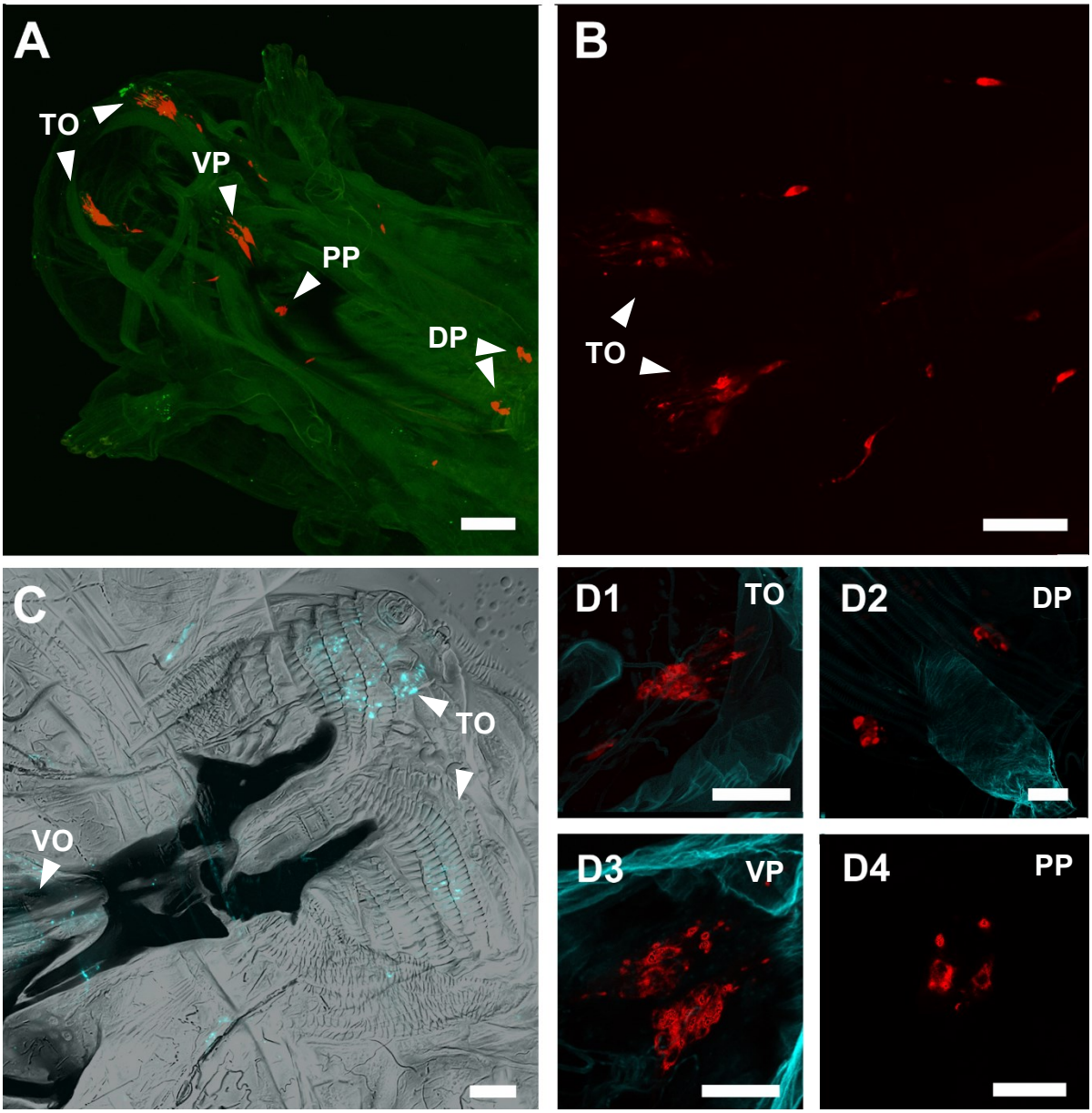

Figure 13: Localization of *IR25a* expressing neurons the in head of the *D. melanogaster* third instar larvae. *IR25a* is driving expression of RFP *IR25aGAL4-UAS-RFP* by Gal4-UAS system. **(A)** An image of the head part with RFP signal in red and anti-ELAV staining in green. **(B)** The head of a larvae with RFP signal. **(C)** Combinated image of transmitted light and RFP expression (in blue) in the sensory head organs. **(D1-D4)** Close-up views of individual IR25a positive head sensory organs. DP – dorsal pharynx organ, PP – posterior pharynx organs, TO – Terminate head organs, VO – ventral head organs, VP – ventral pharynx organs. Scale bar in A, B 50 µm, in C 30 µm, and in D1-D4 10 µm.

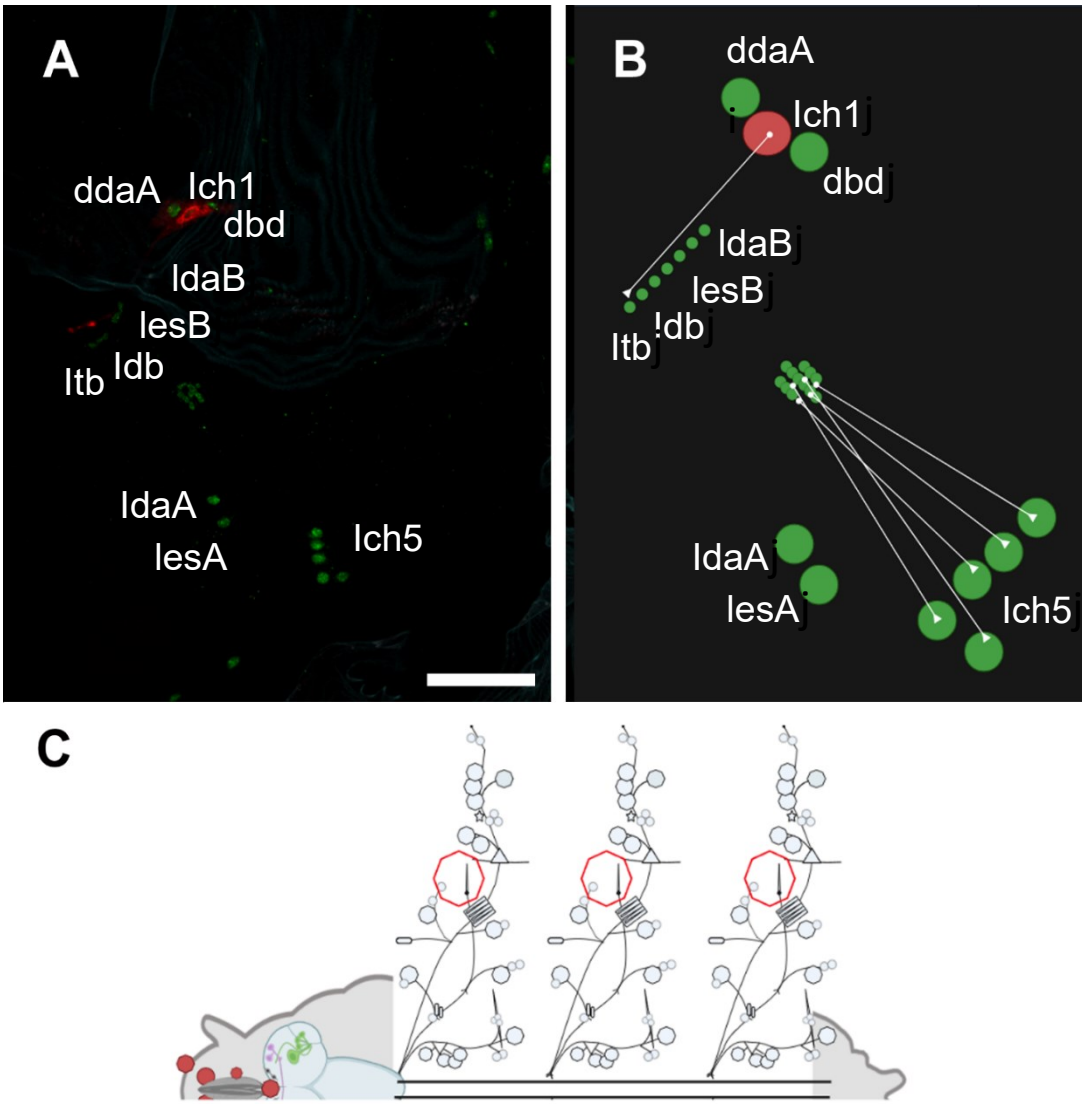

Figure 14: Co-localization of IR25a positive cells with neurons in the peripheral body wall labeled with anti-ELAV antibody. **(A)** The IR25a positive neuron (in red) and Elav positive neurons (in green). **(B)** Space arangme of the body wall neurons. **(C)** A scheme of peripheral neuronal system based on Liu et al., 2003. Note that IR25a neurons are located in a position Ich1 chordotonal neuron. Its position in each segment is marked by red octagones. Scale bar 10 µm

The detailed characterization of *IR25a* positive neurons was based on previous studies (Sánchez-Alcañiz et al.,2018, Liu et al., 2003) that mapped the larval peripheral system neurons using pan-neuronal promoter embryonic-lethal abnormal visual system (*elav*) driving expression of yellow cameleon (YC2.1) by GAL4-UAS system. The network of peripheral neurons was further classified based on their perception characteristics (Grubber et al., 2002). The co-localization of RFP signal with anti-ELAV antibody staining in *IR25aGAL4-UAS-RFP* larvae allow the indetification of *IR25a* positive cells. Based on the position of ELAV-positive neurons in the neuronal system of the body wall and on the cell type, the IR25a positive cells were idetified as a chodotonal organ Ich1 (Figure 14).

4.2 Impact of *IR25a* mutation on synchronization of the larval circadian clock to low TC

To identify if *IR25a* positive neurons affect synchronization of the larval circadian clock to low temperature cycles, the circadian pattern in expression of PER protein and the daily oscillation of CO_2 production were compared in *IR25a-/-* mutants and control larvae kept in DD and low temperature cycles 17 °C : 19 °C. The *IR25aGAL4-UAS-RFP* larvae was served as a control. In both strains, antibody against PER protein stained a group of five lateral neurons (LNvs) and two dorsal neurons (DN2). The group of dorsal neurons located in the *pars intercerebralis* (DN1) were found exclusively in the control larvae. The staining with anti-PDF antibody helped to distinquish the group of LNv neurons. The specificity of PER antibody and the presence of functional circadian pacemaker in *D. melanogaster* larvae was verified by a pilot experiment with *IR25aGAL4-UAS-RFP* larvae conducted under the LD conditions (12 h L : 12 h D) and constant temperature 25 °C (Supplementary Figure 2A). Evaluation of PER protein expression controled by the LD in the course of 24 h was consistent with previously published data (Picot et al., 2009, Supplementary Figure 2).

Over the course of low TC in DD conditions, the level of PER protein in both LNv and DN1 cell clusters oscillated in IR25a-/- mutant with the significantly high expression through entire warm phase and with the low expression during the cold phase (Figure 15 A). The daily oscillation of PER expression in both LNs and DN1 clusters in the control *IR25aGAL4-UAS-RFP* larvae revealed the opposite phase to the phase of PER expression in IR25-/- mutants with the peak in the cold phase (ZT16) and the decline in the warm phase. The circadian oscillation of PER protein level in DN1 neurons, that was not found in IR25-/- mutants, resemble the pattern of PER oscillation in LNv and DN2 groups with the lower expression level in all time points tested (Figures 15 B). To verify the effect of IR25a-/- mutation on the pattern of PER oscillation controlled by low temperature cycles, the profile of PER expression was examined in flies *IR25aGAL4-UAS-RFP-UAS-rpr* were the IR25a positive cells were eliminated by expression of *reaper* gene, that cause their apoptosis. No significant oscillation

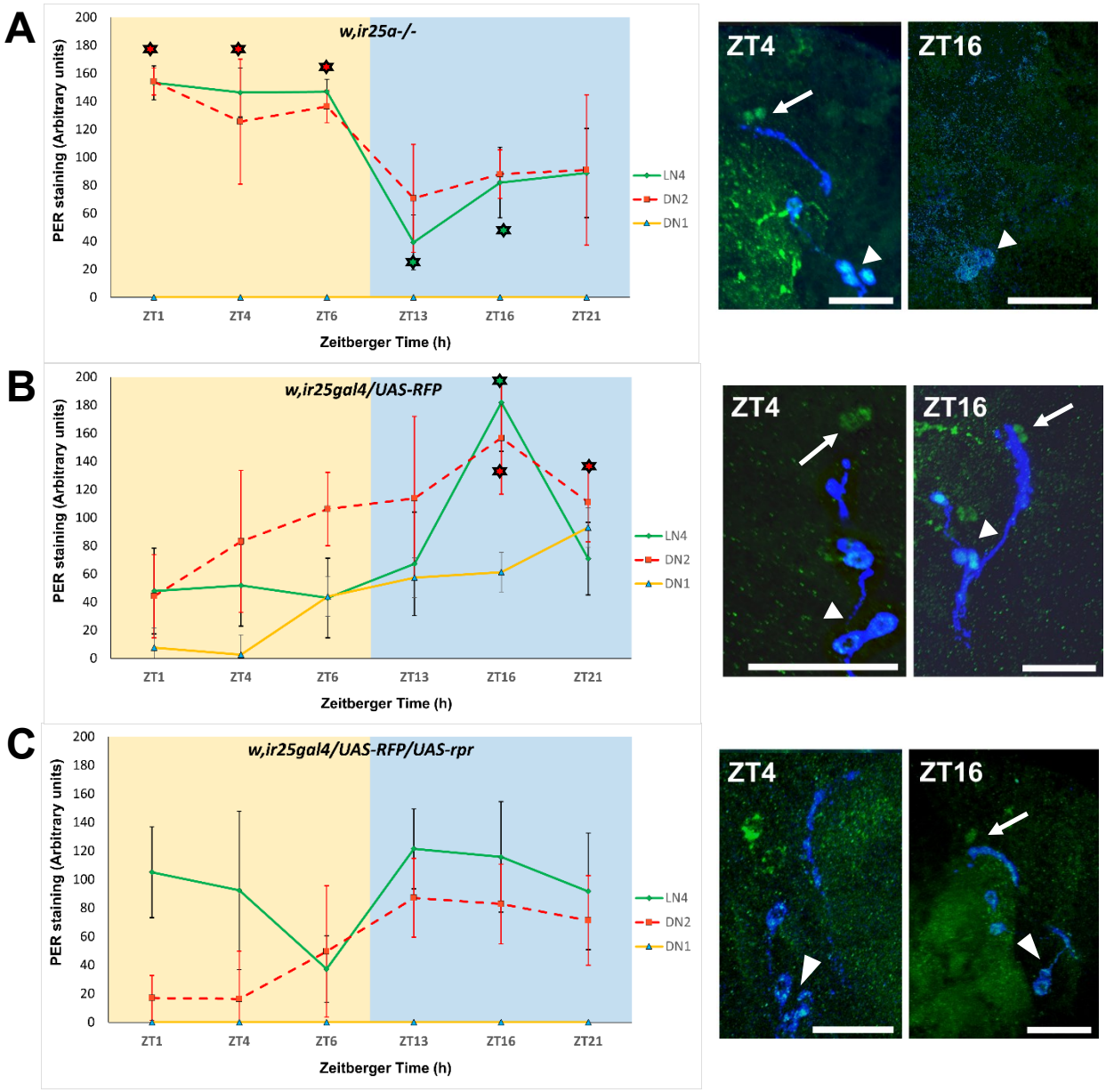

Figure 16: The oscillation of PER protein level in flies kept in the low TC (17 °C/19 °C) and in DD. **(A)** PER staining in the larval brain of *IR25a-/-*mutants. Per levels are significantly different in two groups of clock neurons (DN2 and LNs): p-value = 2.07e-07*** and p-value = 0.00271**. **(B)** PER staining in the brain of *IR25aGAL4-UAS-RFP* larvae. Per levels are significantly different in all three groups of larval clock neurons (DN2, DN1, and LNs), p-value = 0.01203, p-value = 0.03275, and p-value= 4.87e-05***. **(C)** PER staining in the brain of *IR25aGAL4-UAS-RFP-UAS-rpr* larvae. Per levels did not show any significant difference neither in DN2 nor in LNs groups, p-value=0.0613, p-value = 0.8451677. Error bars (STD). On the right there are representative images of anti-PER (in green) and anti-PDF (in blue) staining of DN2 (arrows) and LNs (arrowheads) clock neurons at ZT4 and ZT16 time points. Size bar:10 um

in PER expression was found in either LNv nor DN2 neurons (Figure 15 C). In both LNv and DN2 cells the level of PER protein was moderate during the entire 24 h cycle while PER protein in DN1 was under the detection level in all time points tested.

To investigate how the phenotype of *IR25a-/-* and *IR25aGAL4-UAS-RFP-UAS-rpr* that is phase shift and the disruption of the circadian clock respectively, affects behavioral activity, the daily rhythms in the production of CO_2 was measured. The measurement of respiratory activity was chosen because the larvae live inside the substrate and therefore their locomotion activity cannot be detected.

Third instar larvae were for four days prior the measurment of CO_2 pro٧uction entrained to low TC 19 °C:17 °C and DD conditions. Under these conditions, respiratory activity exhibits circadian oscillations with increased CO_2 production during the warm phase with aticipation of the transition to both the warm and cold phases of the cycle (Figure 16A). Anticipation of temperature changes was surprising given its absence under LD 25°C conditions (Supplementary Figure 2B). IR25a-/- mutant larvae show a loss of anticipation for the cold phase and thus there is a delay in the decline of CO_2 production until the switch of the from warm phase to cold phase (Figure 18B) Anticipation to the temperature phase is preserved. Interestingly, in *IR25aGAL4-UAS-RFP-UAS-rpr* larvae there was a lack of anticipation to both the cold and thermal phases of the TC (Figure 16 C).

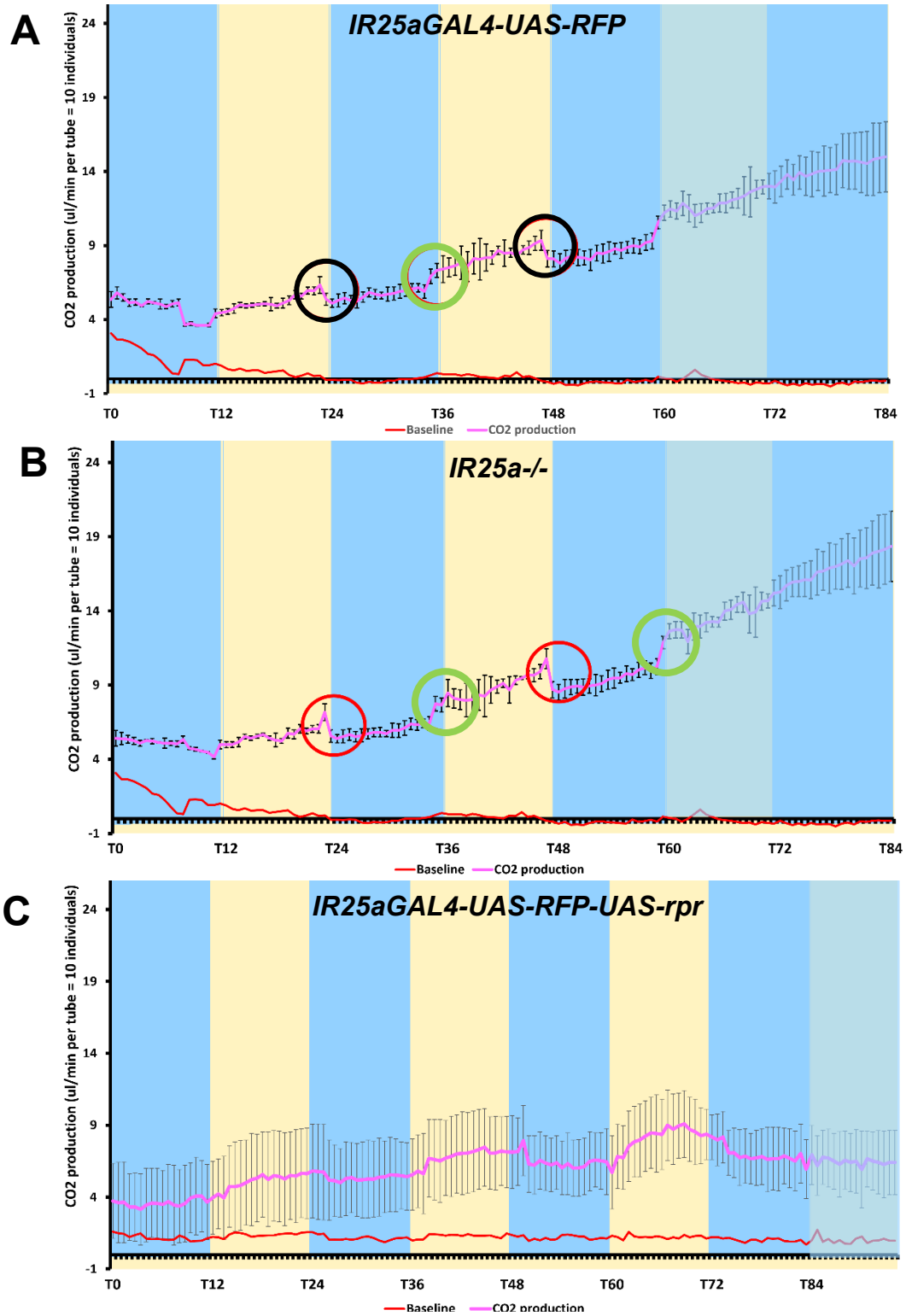

Figure 16: The daily oscillation in CO_2 production in the group of larvae kept in the low TC 17 °:19 °C and in DD for 3.5 days. **(A)** *IR25aGAL4-UAS-RFP*. **(B)** *IR25a-/-* . **(C)** *IR25aGAL4-UAS-RFP-UAS-rpr*. Anticipation to the warm phase of an TC is marked by green circle, anticipation to cold of is marked by black circle. Red circle point to imediate response to change of temperature. Error bars (STD)

4.3 The comparison of behavioral locomotor activity in adults of *IR25aGal4-UAS-RFP-UAS-rpr* and *IR25aGal4UasRFP*

To find out that if the luck of IR25a positive cells produce identical phenotype to phenotype of IR25a-/- mutant described previously (Chen et al., 2015), the monitoring of locomotor activity in condition of low TC 19 °C:17 °C and constant darkness was conducted in three days old *IR25aGAL4-UAS-RFP-UAS-rpr* male adults. he individuals lose the ability to synchronize locomotor activity to the low TC while maintaining a free-running period. The free-running period is seen in the histogram capturing the behavior of one individual over 20 days (an example in Figure 17 A) but due to the different period of the individuals, the graph shows a plot analyzing the entire group of asynchronous individuals unlike, the group of control *IR25aGAL4-UAS-RFP* flies (Figure 17 B).

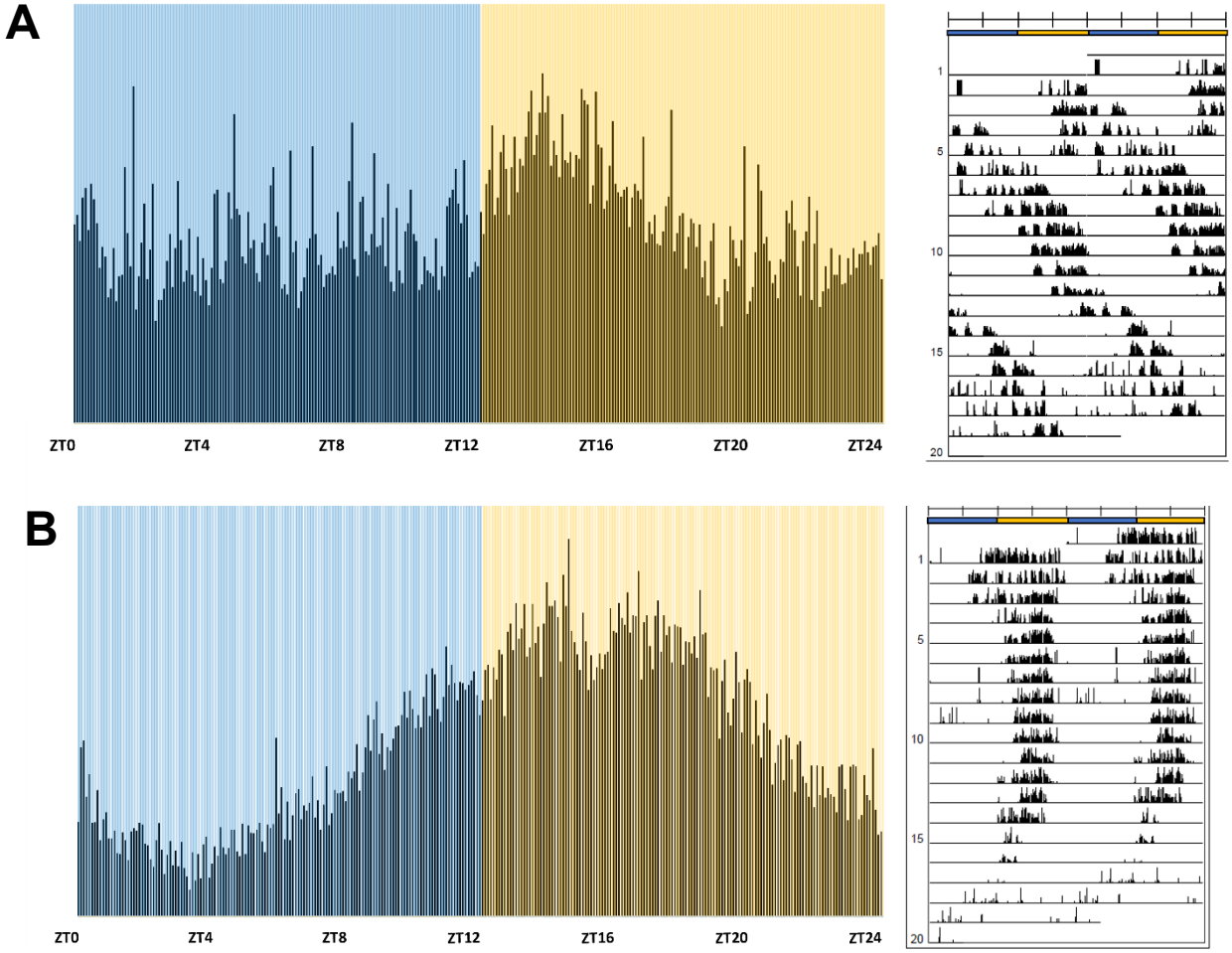

Figure 17: Histograms of locomotor activity in *IR25aGAL4-UAS-RFP* and *IR25aGAL4-UAS-RFP-UAS-rpr* adults kept in the low TC cycles 17 °:19 °C in DD. Left panel represents the average of all flies tested. Right panel shows the actogram of an individual fly. Note the luck of synchronization to the low TC cycles in *IR25aGAL4-UAS-RFP-UAS-rpr* flies.

5 Discussion

The circadian clock of the adult *D. melanogaster* is synchronized to environmental temperature cycles in a same way as most living organisms. Surprisingly, the amplitude could be as low as a difference of 2-3° C and yet still worked as powerful zeitgeber. The key effort of this study is to determine whether also *Drosophila* larvae, that are living under completely different conditions compared to adults, most of them buried in food, have their circadian pacemaker under the control of low TC cycles. Studies on *D. melanogaster* adults have identified a gene, *IR25a*, that plays crucial role in synchronizing organisms to low temperature cycles. This work demonstrates a role for IR25a in synchronizing the circadian rhythms of *D. melanogaster* larvae to low temperature cycles.

In the *Drosophila* third instar larvae, IR25a protein was localized in bilateral neuron in each body segment. In addition, there were several groups of sensory neurons in the head forming sensitive terminal and ventral organs, and rest of them were distributed in the dorsal, the ventral, and the posterior pharyngeal. The localization of IR25a positive cells in the head region is consistent with previously published data (Sánchez-Alcañiz et al., 2018). Sánchez-Alcañiz et al. (2018) mentioned the distribution of IR25a in the body of the larvae but the exact position of IR25a positive cells has not been described. This thesis characterizes IR25a neurons in the larval body wall based on colocalization with anti-ELAV staining, that point on transcriptional active cells (Liu et al., 2003). According to a detailed map of ELAV positive neurons in peripheral cells of the body wall (Liu et al., 2003), IR25a positive neurons could be identified as one of the peripheral chordotonal organs. IR25a neuron laid below the cuticula, which excluded it from the group of external sensory neurons. Within the internal sensory neurons, the absence of dendrites ranks this IR25a belong to chordotonal organ rather than multidendritic neuron. According to Singhania et al. (2014) the peripheral chordotonal organs was divided into four parts (Ich5, Ich, VchB, VchA) Based on our comparative study with ELAV staining showing location of IR25a neuron in distance from Ich5, can be concluded that IR25a is expressed exclusively in the chondrotonal organ neuron Ich1. The entire cluster of the larvae chondrotonal organ was identified as a temperature sensor responding to prolonged temperature changes but was not affected by short temperature pulses (Liu et al., 2003). The localization of IR25a protein in the Ich1 chordotonal neuron, refined the role of the Ich1 in sensing low-amplitude temperature cycles in the range of (17 º C/19 º C). In a close vicinity to Ich1, there are class 4 multidendritic neurons consisting of three neurons (vdaB,

v'ada and ddaC) that co-express TRPA1 and two rhodopsins, Rh5 and Rh6, involved in larval temperature preference (Shen et al., 2011, Grueber et al., 2002).

The shift in the phase of the PER protein circadian oscillation in IR25a mutants in respect to the control flies, suggest the role of IR25 protein in the entrainment to the low temperature cycles. However, the maintaining of the 24 h PER oscillation in IR25a mutant clock neurons, suggest involvement of some other factor/factors controlling the phase of rhythmic PER expression. These factor/factors are most likely co-expressed in IR25a positive cells since induction of their apoptosis by expression of *reaper* gene leads to desynchronization of the daily oscillation of PER protein levels. This hypothesis is supported by disruption of synchronic oscillations in respiratory activity of the group of *IR25aGAL4-UAS-RFP-UAS-rpr* third instar larvae, while the daily rhythms in respiratory activity of IR25a-/- mutant are synchronized. However, in the contrast to the control larvae, the IR25a-/- mutants lost their ability to anticipate the cold phase while still anticipating the onset of the warm phase. Despite the loss of cold anticipation, IR25-/- mutant larvae still performed a synchronous 24h respiration pattern most likely dependent of the onset of the cold phase, that causes an immediate reduction in respiratory activity (Figure 16 B). The loss of the cold phase anticipation could explain the phase shift of the PER protein oscillation.

The *IR25aGAL4-UAS-RFP-UAS-rpr* larvae lost the ability to anticipate even the thermal phase of an TC cycle. They respond to temperature change with a significant delay. In *IR25aGAL4-UAS-RFP-UAS-rpr* larvae lost the ability to synchronize the circadian clock with a low TC (17 º C/19 º C) probably with preservation of the free running period. A free running circadian clock cannot be observed in the larval group. However, the data from adults, which can be monitored individually, showed a free running period in behavioral activity with loss of its synchronization. The loss of synchronization to low TC occurred also in *IR25a-/-* adults (Chen et al., 2015) unlike in *IR25a-/-* larvae, where the period of the circadian clock is preserved but with shifted phase. The ability of *IR25aGAL4-UAS-RFP* to anticipate periodic changes in enviromental stimuli was not observed for LD cycles (Supplementary Figure 2A). This result supports the greater importance of temperature perception in larvae living buried in the substrate.

The fact that in both *IR25a-/-* and *IR25aGAL4-UAS-RFP-UAS-rpr* the PER protein is not expressed in DN1 cells indicates a crucial role of DN1 cells in processing information about low temperature cycles. A key role of DN1 neurons in synchronization to the low

temperature cycle has been demonstrated in adults. Expression of TIM protein v DN1s of IR25a flies is reduced and the its maximum level is shifted to the warm phase. In larvae, there were pointed out the role of DN2 neurons in temperature entrainment of the circadian clock, since they responded very differently to LD and TC (Picot et al.,2009). In those neurons the molecular oscillations of PER protein are in antiphase to other clock neurons whereas in high thermocycles (25 ºC /19 ºC), PER protein in those cells oscillate with similar phase as in other clock neurons. Their phasing in LD is controlled by PDF signaling since DN2 neurons contains PDFR. DN2 are blind with the absence of PDF but they can still synchronize to TC (Picot et al.,2009). Similar pattern in PER oscillation were observed in our experiments (Supplementary figure 2B).

6 Conclusion

Our results showed a role for IR25a protein in the synchronization of larval circadian pacemaker to low temperature cycles. The mutation of the *IR25a* gene leads to the phase shift in the PER protein oscillation and cause disability for anticipation of cold phase in the respiratory activity. The role of IR25a in synchronization to TC is supported by its distribution in the internal receptor, the chordotonal neuron Ich1, located in every body segment and in other receptors in the terminal, ventral organs and on the pharynx. Induced apoptosis of all IR25a positive cell caused desynchronization of the circadian clock to low thermocycles. The crucial rule in the proceeding of temperature entrainment signaling play DN1 and DN2 clock neurons. The synchronization to the TC in larvae might involve other gen/genes expressed in IR25a positive cells (either in all or in their subset), since in mutant larvae, unlike *IR25a-/-* adults, the synchronization to TC is maintained but with a shifted phase. Thus, the molecular mechanism of the perception of low temperature cycles in adults and in larvae of *D. melanogaster* shares similarities in both the genes involved in this signaling pathway and the cells that serve as sensory or executive units, however their molecular mechanisms controlling this signal pathway differ.

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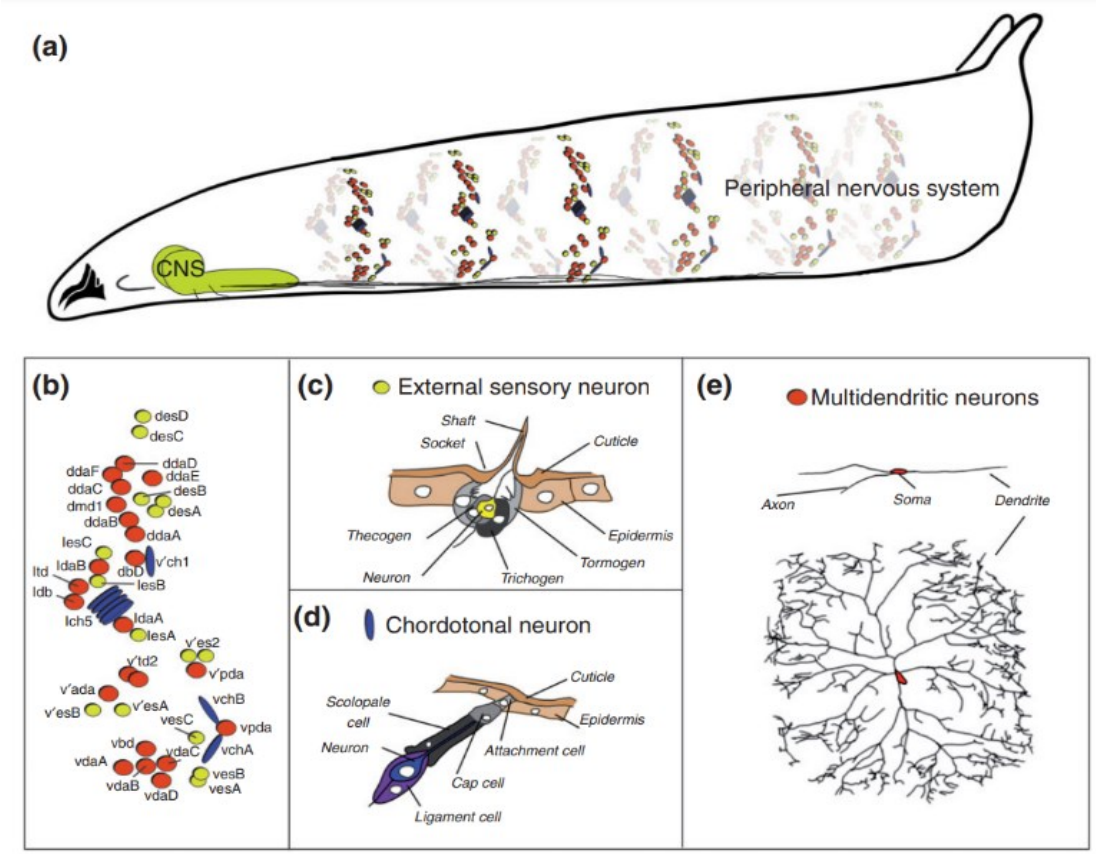
8 Suplementary data

Suplementary Figure 1:



Supplementary Figure 1: Map of neurons of the peripheral neural system in the body wall. Adapted from Singhania et al., 2014.

A. Drawing of a third instar *Drosophila* larva showing sensory elements that comprise the peripheral nervous system. For simplicity, only sensory neurons of a subset of abdominal segments are shown. Bundled sensory axons project to the central nervous system (CNS) that resides in the ventral and anterior part of the larva.

B. Schematic of the arrangement of sensory neurons in a single abdominal hemisegment. External sensory organs are indicated by yellow circles, chordotonal organs by blue ovals, and multidendritic neurons by red circles.

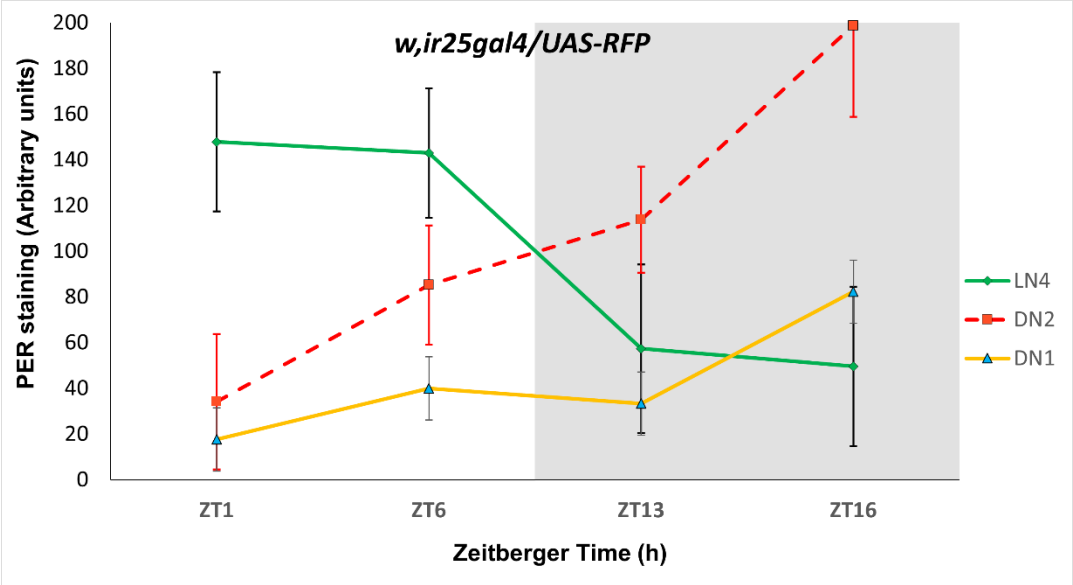
C. Drawing of external sensory organ structure. Names of individual cellular elements are indicated. Drawing adapted, with permission, from Comprehensive Molecular Insect Science. Vol. 1: Hartenstein V. Development of Insect Sensilla. pp. 379–419, 2005.

D. Drawing of chordotonal organ structure. Names of individual cellular elements are indicated. Drawing adapted, with permission, from Comprehensive Molecular Insect Science. Vol. 1: Hartenstein V. Development of Insect Sensilla. pp. 379–419, 2005.

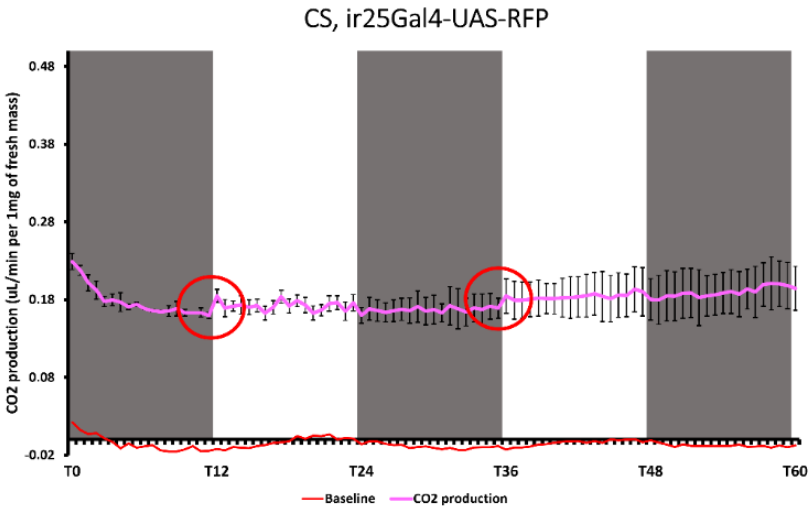
E. Tracings of multidendritic neurons. Two different neurons are shown, the dorsal bipolar dendrite neuron (top), and a class IV nociceptive neuron (bottom). Note the different degrees of dendritic branching shown by the two neurons. Tracing of class IV neuron.

Supplementary Figure 2:

A



B



Supplementary Figure 2: Circadian oscillation of PER protein level (A) and CO_2 production (B) in *IR25aGAL4-UAS-RFP* kept in LD (12 h L : 12 h D) and 25 °C. Please note the absence of anticipation of dark/light transition.