The Role of Temperature Sensitive Ion Channel dTRPA1 in Modulating Rhythmic Activity/Rest Behaviour in *Drosophila melanogaster*

A Thesis

Submitted for the Degree of

Doctor of Philosophy

By

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Dedicated to

Mrs. Anju Das

My Mother

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Declaration

I hereby declare that the matter presented in my thesis entitled "**The Role of Temperature Sensitive Ion Channel dTRPA1 in Modulating Rhythmic Activity/Rest Behaviour in *Drosophila melanogaster*"** is the result of studies carried out by me at the Evolutionary and Organismal Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore, India, under the supervision of Dr. Sheeba Vasu and that this work has not been submitted elsewhere for any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described has been based on the findings of other investigators. Any omission in this regard, which might have occurred by oversight or misjudgment, is regretted.

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Certificate

This is to certify that the work described in the thesis entitled "**The Role of Temperature Sensitive Ion Channel dTRPA1 in Modulating Rhythmic Activity/Rest Behaviour in *Drosophila melanogaster***" is the result of investigations carried out by **Ms. Antara Das** in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

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"The woods are lovely and deep

But I have promises to keep,

And miles to go before I sleep,

And miles to go before I sleep."

-Robert Frost (Stopping by Woods on a Snowy Evening)

List of Publications

1. Das A, Holmes TC, and Sheeba V (2015). "dTRPA1 Modulates Afternoon Peak of Activity of Fruit Flies *Drosophila melanogaster*." PLoS One 7: e0134213.

2. Das A, Holmes TC, and Sheeba V (2016). "dTRPA1 in Non-circadian Neurons Modulates Temperature-Dependent Rhythmic Activity in *Drosophila melanogaster*." J Biol Rhythms 31(3): 272-88. DOI: 10.1177/0748730415627037.

3. Das A and Sheeba V. Book Chapter "Temperature input for diurnally rhythmic behaviours in flies - the role of temperature sensitive ion channels." (*in press*).

4. Das A and Sheeba V. "Role of intracellular signaling pathways in modifying dTRPA1 mediated changes in activity/rest patterns in *Drosophila melanogaster*." (*manuscript in preparation*).

Synopsis

Organisms have evolved complex mechanisms to detect and respond to various sensory stimuli in the environment. Ambient temperature influences development, growth and reproduction of organisms and it plays a vital role in regulating many physiological, biochemical and metabolic processes. Unlike endothermic organisms that maintain a relatively constant internal body temperature, ectothermic organisms actively move towards or away from temperature sources in order to thermo-regulate. In fruit flies, *Drosophila melanogaster*, both the larva and the adult fly exhibit distinct, well characterised responses to innocuous and noxious thermal stimuli.

In this thesis, I focussed on the role of a temperature sensor, dTRPA1 that is predominantly expressed in the brain in contrast to other known peripheral thermosensors in *Drosophila*. Thermo-sensitive dTRPA1 (*drosophila* Transient Receptor Potential –A1) is a member of a subfamily of the large TRP ion channel group called the thermo-TRPs. I describe my attempts to understand how temperature evoked signals from dTRPA1 neurons modify changes in circadian clock controlled activity/rest pattern in flies. I used the GAL4 /UAS binary system to genetically modify fly lines for dTRPA1 expression or alter neuronal function of different subsets of cells and then recorded the activity/rest behaviour of flies under various environmental regimes using the Drosophila Activity Monitor (DAM) recording system (Trikinetics, USA). Since the nature and mechanism of neuronal circuit modifications remain largely conserved across species, the work described in my thesis helps us to understand how thermosensory neuronal signalling modifies behaviour in flies and enables them to adapt to external environmental conditions.

In the introductory chapter (**Chapter 1**), I present a brief survey of the literature about

our current understanding of thermosensation in fruit flies and the known temperature sensors. I discuss the role of thermo-TRP ion channels in fly thermosensation and, in particular, dTRPA1, which is known to be active within a range of temperatures conducive for fly survival. I also talk about circadian clock circuit in *Drosophila* and the well studied clock output - rhythmic activity/rest behaviour in flies. An overview of previous studies demonstrating entrainment of circadian clocks to temperature cycles is also discussed. Further, neuronal circuits that interact with dTRPA1 neurons and signalling pathways that have been proposed to modify neuronal responses to temperature cues are also described.

Rhythmic light/dark (LD) cycles or thermophase/cryophase (TC) cycles can entrain locomotor activity/rest behaviour in flies. I examined the activity/rest behaviour of dTRPA1 null mutants and flies with modified dTRPA1 expression under warm, ambient constant temperature conditions under LD cycles and find that phasing of morning (M) peak of activity is dependent on neuronal signalling from a subset of dTRPA1 neurons. I also explored the role of thermosensitive dTRPA1 in entrainment of circadian clocks to temperature cycles in constant darkness (DD). In contrast to a recent report (Lee and Montell, 2013), my studies show that dTRPA1 is not essential for flies to entrain to temperature cycles in DD (henceforth DD/TC). However, the lack of dTRPA1 results in high mid-thermophase activity under DD/TC that can be rescued by restoring dTRPA1 expression in a subset of dTRPA1 neurons in a mutant background. Further, I also examined the activity/rest patterns of these flies under temperature cycles in constant light (LL/TC), a regime also used to study temperature entrainment by other researchers (Glaser and Stanewsky, 2005; Sehadova et al., 2009). Interestingly, under LL/TC, dTRPA1 null mutants do not phenocopy their behaviour under DD/TC; instead, they show defects in both activity peaks - a significantly diminished M-peak and a significantly advanced evening (E) peak.

Thus, my studies demonstrate that dTRPA1 signalling is crucial for flies to phase their rhythmic activity/rest profiles under different kinds of thermal cues within the activation range of the thermosensitive ion channels – constantly warm LD cycles versus cyclic thermophase in TC in combination with constant darkness or constant light. These studies are described in detail in **Chapter 2**.

Since behavioural activity/rest pattern of dTRPA1 lacking flies was distinct under LD and TC cycles, I also studied their locomotor behavioural patterns under combination of light and temperature time cues. An out-door enclosure within the JNCASR campus, provided us an attractive system to study the rhythmic activity/rest of flies under multiple zeitgebers that cycle in a gradual manner in sharp contrast to rectangular step-up/step-down cycles programmed in laboratory incubators that have been traditionally used to study circadian behaviour of flies. While wild-type flies exhibit an additional peak of activity during the afternoon under such semi-natural (SN) conditions and simulated SN conditions in lab incubators, dTRPA1 null mutants do not show this afternoon or A-peak of activity. Moreover, the amplitude of the A-peak is directly dependent on dTRPA1 expression levels – with low dTRPA1 levels resulting in an attenuated A-peak and vice-versa. My results also show that the dTRPA1-dependent A-peak in wild-type flies is a temperature driven response and is not under the influence of circadian clocks, although earlier studies that reported the occurrence of A-peak in SN conditions claimed that it is indeed a circadian clock controlled behaviour (Vanin et al., 2012; Menegazzi et al., 2012). I discuss the results pertaining to studies in SN or simulated SN conditions and role of dTRPA1 in mediating A-peak in **Chapter 3** of this thesis.

Along with studying behavioural activity/rest patterns in flies with altered dTRPA1 function, I also examined the expression pattern of dTRPA1-positive neurons in the fly brain. My studies show that GFP tagged dTRPA1 cells overlap with a few circadian neurons and

their processes in the adult brain but dTRPA1 expression in these circadian neurons do not influence behavioural changes observed in dTRPA1 null flies under different environmental regimes. Through a series of experiments my studies show that the signals from non-circadian dTRPA1 neurons are predominantly required for mediating changes in activity/rest pattern of flies in response to thermal cues. Immunolabelling of *Drosophila* brains to examine the overlap between circadian clock circuit and dTRPA1 thermosensory circuit and subsequent behavioural assays to dissect out the role of circadian and dTRPA1 neurons in controlling temperature-dependent behaviour in flies is the focus of **Chapter 4** of this thesis.

The mechanisms underlying activation of thermo-TRPs largely remain unknown and both direct and indirect means of activation of dTRPA1 ion channels have been put forward by different research groups. Indirect modes of dTRPA1 activation includes thermal stimuli either reaching dTRPA1 by intracellular signalling cascade via *norpA* encoded Phospholipase C (PLC) or through synaptic communications from neurons expressing another thermo-TRP, Pyrexia. My studies suggest that dTRPA1 signalling in response to thermal cues appear largely to be independent of these putative upstream circuits. Further, I also examined how rhythmic behavioural patterns are altered in flies wherein dTRPA1 synaptic signals have been blocked and attempted to identify synaptic targets of dTRPA1+ve AC neurons. I also explored the possible role of an important intracellular cAMP/PKA signalling pathway; which has been previously shown to be crucial in Mushroom Body (MB) neurons to mediate temperature preference behaviour in flies. MB neurons, along with components of Central Complex (CC) comprise the sensory processing centres in the fly brain. In a preliminary screen involving different fly lines that were genetically modified to reduce cAMP/PKA signalling in MB and CC neurons, I have isolated a driver line that targets neurons which may function in the same downstream pathway as dTRPA1 neurons. In **Chapter 5**, I discuss

the results involving neuronal signalling circuits that function upstream or downstream of dTRPA1 neurons.

In summary, my studies emphasise the importance of dTRPA1 signalling in maintaining rhythmic activity/rest behaviour of flies in response to environmental temperature cues. My results demonstrate that dTRPA1 ion channels and firing properties of these neurons are crucial for flies to phase their circadian clock-controlled locomotor activity/rest pattern to appropriate times of the day. Studies described in this thesis are also able to distinguish between circadian and non-circadian dTRPA1 neurons and show that the signals from the latter group is important to modulate temperature-dependent behaviour in flies. My studies also highlight the fact that depending on the nature (chronic vs acute) and duration (prolonged 12hr *vs*. short 1hr) of the thermal cue for dTRPA1 activation, behavioural responses in flies are distinct. While 12hr-long prolonged exposure to 29 ^{o}C under TC cycles results in *suppression* of activity during middle of the thermophase, an acute 1hr pulse of 32 ^{o}C under SN or SN-like conditions elicits a sharp *rise* in activity. Although we do not yet know how temperature signals are able to activate dTRPA1 ion channels, with the advent of advances in cryo-EM based studies to study ion channel structures (reviewed in Liu and Montell, 2015; Paulsen et al., 2015), gaining insights into the mode of activation of these temperature-sensitive ion channels may not be a distant dream. Future studies that would explore the response of temperature sensitive ion channels to thermal cues in greater detail, while simultaneously examining changes in neuronal activity of the corresponding sensory neurons and gaining insight into their downstream targets, would reveal how signals from thermosensory neurons modify behavioural patterns in a manner that is adaptively advantageous to the organism.

List of Abbreviations

5-HT : 5-hydroxytryptamine (Serotonin)

AC : anterior cell

A-peak : afternoon peak

BDSC : Bloomington Drosophila Stock Center

CRY : Cryptochrome

DAM : Drosophila Activity Monitor

DC : dorsal cell

DL: dorso-lateral cell

dcr : *dicer*

DD : constant darkness

DD/ TC : 12:12 hr symmetric temperature cycles (thermophase 29 ^{o}C : cryophase 21 ^{o}C) under constant darkness

DD+SN : semi-natural conditions in absence of light cues

DD+T_{r32} : ramped or gradually changing temperature cycles with temperature maxima 32 ^{o}C under constant darkness

DN : dorsal neurons (circadian)

DN1 : Dorsal neurons subgroup 1 ; DN1a : Dorsal neuron subgroup 1 anterior cells

DN2 : Dorsal neuron subgroup 2

dTRPA1 : *Drosophila* Transient Receptor Potential

dTRPA1^{SH+} : a subset of dTRPA1 expressing neurons targeted by *dTRPA1^{SH}-GAL4* driver

dTRPA1^{ablated} : Ablation of dTRPA1^{SH+} neurons.

dTRPA1^{hyperexcited} : Electrical activation of dTRPA1^{SH+} neurons via expression of NaChBac1

dTRPA1^{oex} : Overexpression of dTRPA1 expression in dTRPA1^{SH+} neurons

dTRPA1^{RNAi} : RNAi knockdown of dTRPA1 expression in dTRPA1^{SH+} neurons

dTRPA1^{silenced} : Electrical silencing of dTRPA1^{SH+} neurons via expression of Kir2.1

dTRPA1^{rescue} : Restored dTRPA1 expression only in dTRPA1^{SH+} neurons in dTRPA1 null background

GFP : green fluorescent protein

GPCR : G-protein coupled receptor

L_{max} : maximum light intensity

L_r: ramped or gradually changing light intensity cycles

LH : lateral horn

LD : light / dark cycles

LL : constant light

LL/TC : 12:12 hr symmetric temperature cycles (thermophase 29 ^{o}C : cryophase 21 ^{o}C) under constant light

LL_{100} : constant light with light intensity of 100 lux

LN : lateral neurons (circadian)

LN_d : dorso-lateral neurons

LN_v : ventro- lateral neurons

l-LN_v : large ventro-lateral neurons

MB : Mushroom Body

PDF : Pigment Dispersing Factor

PLC : Phospholipase C

PLP : lateral protocerebrum

PKA : Protein Kinase A

PKAR : dominant negative form of PKA

SEM : Standard error of mean

SLPR : superior lateral protocerebrum

SN: semi-natural

SOG : suboesophageal ganglion

SOL : small optic lobes

s-LN_v : small ventro-lateral neurons

T_{max} : maximum temperature

T_r : ramped or gradually changing temperature cycles

$L_r + T_{r28}$: ramped or gradually changing light intensity cycles in combination with ramped temperature cycles with temperature maxima 28 ^oC

$L_r + T_{r32}$: ramped or gradually changing light intensity cycles in combination with ramped temperature cycles with temperature maxima 32 ^oC

$LD+T_r$: ramped or gradually changing temperature cycles under light/ dark cycles

$LL+T_{r32}$: ramped or gradually changing temperature cycles with temperature maxima 32 ^oC under constant light

TC : thermophase / cryophase temperature cycles

TPR : Temperature preference rhythm

TRP : Transient Receptor Potential ion channel family

TTFL : Transcriptional Translational Feedback Loops

ZT : zeitgeber time

Chapter 1

Introduction

1. Introduction

Organisms detect and respond to various sensory stimuli in the environment. Amongst several abiotic factors, appropriate temperature is crucial for optimal development, growth and reproduction of organisms, and plays a vital role in regulating many physiological, biochemical and metabolic processes whereas extreme high or low temperatures can be detrimental to survival. Temperature of a given geographical location depends upon a number of factors such as the latitude, altitude, distance from sea or other water bodies, vegetation, wind conditions etc. Climatic specifications of the region have a large influence on the habitat and geographic distribution of a species. Besides these climatic factors, the rotation of the earth around its own axis changes the temperature in a daily fashion (being hot during the day and cold during the night). Furthermore, the fact that the Earth's axis has an ~ 23 degree tilt in relation to its equatorial or rotational axis gives rise to seasonality which also heavily influences annual changes in temperature.

Possibly, because of the remarkable regularity in changes of many environmental factors in the real word, living organisms have evolved internal time keeping mechanisms (circadian clocks) that help them anticipate daily change in the environment and synchronize their behaviours and physiological processes to those changes. Circadian clocks are also known to help organisms adapt to seasonal changes across the year (Dubruille and Emery, 2008). Since many animals use shelters such as dens, nests or burrows possibly to escape from predators and avoid extreme weather conditions such as hot afternoons and chilly nights in a daily manner, it is quite likely that along with changes in light intensity, cyclic temperature cues are dominant external time cues or '*zeitgebers*'. This may be especially true for deep cave-dwelling and deep oceanic dwelling organisms where light penetration may be limited.

1.1. Temperature entrainment of circadian clocks. Circadian clocks are endogenous time keeping mechanisms that synchronize daily physiological and biochemical processes within organisms to their external environment (Allada and Chung, 2010; Dubruille and Emery, 2008; Peschel and Helfrich-Forster, 2011). Circadian clocks play a crucial role in helping organisms to anticipate daily variation in environmental conditions and schedule their daily behavioural activities such as foraging, sleep-wake cycles, mating etc. during times of the day most conducive for their survival. Circadian clocks are termed as such due to the length of their cycles, which is approximately 24 hours (from Latin '*circa*' = approximately and '*diem'* = a day). They generate rhythmic outputs due to an underlying molecular clockwork that involves **T**ranscriptional **T**ranslational **F**eedback **L**oops (TTFL). In the absence of external time cues, the completion of a cycle of negative feedback mediated transcription-translation process takes place with a period of approximately 24 hours, similar to the behaviours that they control. Over the years many molecules that participate in the generation and sustenance of this clockwork have been discovered and characterised (for details see (Allada and Chung, 2010; Peschel and Helfrich-Forster, 2011). In *Drosophila*, approximately 150 neurons in the brain comprise the circadian clock neuronal circuit within which the circadian oscillations of several clock genes and proteins result in outputs in firing frequency and neurotransmitter release thus modulating rhythmic behaviour and physiology. In nature, the levels of abiotic factors such as light, temperature and humidity exhibit a very precise periodicity of exactly 24 hours and in the presence of such daily time cues (or zeitgebers), circadian clocks can synchronize their internal clock oscillations to the phase of the external time cycle, a process called entrainment. However, circadian clocks are temperature and nutrition compensated; implying that the period of the clock is not altered by physiologically tolerable changes in temperature or quality of food. Thermosensitive splicing of *per* that generates spliced variants is one of the proposed mechanisms by which temperature

compensation is thought to occur (Chen et al., 2007). At the same time, when cycles of low and high temperature are provided, they can entrain circadian clocks in both invertebrates (for review see (Dubruille and Emery, 2008) and peripheral clocks of vertebrate model systems (Buhr et al., 2010). Moreover, short pulses of high temperature can induce phase shifts in circadian clock controlled locomotor activity rhythm in *Drosophila* (Busza et al., 2007; Kaushik et al., 2007) in a time-of-day dependent manner. Hence, the manner in which cyclic thermal cues are perceived by the organism and subsequently impinge on the "central clock" in the brain is of considerable interest.

While our understanding of light entraining pathway has advanced greatly since the discovery of the first clock gene *period* (Konopka and Benzer, 1971), how temperature influences circadian clocks is less understood. In brief, photoentrainment of circadian clocks is mediated through activation of blue-light sensitive circadian photoreceptor CRYPTOCHROME (or CRY) which in turn binds to TIM and marks it for ubiquitin-dependent proteosome degradation (for details see reviews (Dubruille and Emery, 2008; Sehgal, 2004)). In the absence of TIM, PER is hyperphosphorylated via DBT (*doubletime*) kinase which destabilizes PER and leads to proteosomal degradation of PER. Due to decreased PER and TIM levels in the cytoplasm, stable heterodimers of PER and TIM are not formed, PER-TIM heterodimers do not enter the nucleus and are unable to bind to CLK-CYC complex to inhibit their own transcription. Thereafter, CLK-CYC heterodimer complex bind to the promoters of PER and TIM again, beginning another cycle of transcription. Thus, light activates CRY, which in turn leads to TIM degradation, resetting the circadian clocks (Sehgal, 2004).

Several research groups took different approaches to understand how temperature influences circadian clocks. In this endeavour, few thermoreceptors have been identified, subsets of circadian neurons (especially the DNs) have been implicated to be temperature

sensitive (Picot et al., 2009; Zhang et al., 2010), yet the molecular mechanism of temperature entrainment remains elusive. The first study showed that 12:12hr warm /cold temperature cycles with a difference of 1-2 ^{o}C can moderately entrain behavioural and molecular rhythms in flies in constant darkness (DD) (Wheeler et al., 1993). They also showed that flies lacking a functional clock (such as per^{01} flies – loss of function mutation in *per* locus) could not entrain to the above temperature cycles. Another study showed that temperature cycles with a 5 ^{o}C difference is able to entrain flies under constant light (LL) conditions (Tomioka et al., 1998). It was remarkable that flies that were otherwise arrhythmic in LL under a constant temperature, now become rhythmic and can entrain to imposed temperature cycles in LL. Following this, many research groups started studying behaviour of flies under temperature cycles in LL because wild type flies were found to entrain only to 12:12 hr temperature cycles in DD but entrained to a range of T cycles (8-32 hrs) under LL (Yoshii et al., 2002). Two studies from the laboratory of Ralf Stanewsky advanced our understanding of temperature entrainment of circadian clocks in flies. The first study showed that flies with ablated antennae or those carrying *spineless* mutation (antennae transformed into legs) could entrain to temperature cycles thus ruling out role for known peripheral thermoreceptors in circadian entrainment (Glaser and Stanewsky, 2005). At this time the antennal thermoreceptors were the only known thermosensitive receptors. They went on to screen ~800 chemically mutagenised fly strains under temperature cycles in LL and isolated a mutant for the gene *nocte* (no circadian temperature entrainment). In *nocte* mutants, *per* oscillation were dampened as quantified through bioluminescence of *per*-luc construct (luciferase reporter fused downstream of *per* promoter) and locomotor activity/rest rhythm of *nocte* mutants took longer to entrain to temperature cycles than controls (Glaser and Stanewsky, 2005). In a follow-up study from the same group, they found that *nocte* is expressed in chordotonal organs and rectified their previous observation that isolated brain

tissue can entrain to temperature cycles in LL (Sehadova et al., 2009) by demonstrating that while peripheral tissues can autonomously entrain to temperature cycles, brains need signals from chordotonal organs which express *nocte* (Sehadova et al., 2009). Notwithstanding the implication of *nocte* in thermal entrainment of circadian clocks, not much is known about how this protein enables circadian entrainment. *In-vitro* studies suggested that peripheral clocks are autonomously capable of temperature entrainment whereas brain tissue is dependent on signals from the chordotonal organs to reset the clock (Sehadova et al., 2009). This model suggests that non–clock neurons from chordotonal organs send temperature inputs to circadian clock neurons in the brain. This pathway of communication is reminiscent of the one observed for sex-peptide signaling in *Drosophila* (Hasemeyer et al., 2009) where peripheral neurons from reproductive organs send signals to the brain. However, this model which proposed that extra-brain thermal signals from chordotonal organs expressing *nocte* is a prerequisite for temperature entrainment of circadian clocks has been contested by a recent study (Tataroglu et al., 2015) which proposes that a cell-autonomous mechanism for temperature entrainment exists. The recent study proposes that temperature-dependent degradation of TIMELESS (or TIM) via a calpain protease small optic lobes (SOL) dependent degradation pathway in circadian pacemaker neurons resets the clock in response to temperature cues (Tataroglu et al., 2015), defining a cell autonomous mode for temperature entrainment. This is reminiscent of photoentrainment of circadian clocks that involves light activation of CRY which tags TIM with ubiquitin, resulting in proteosomal degradation of TIM which in turn resets the clock. This study also suggests that entrainment of circadian clocks to temperature cycles involves dTRPA1 ion channels that gate Ca^{+2} entry into the cell upon receiving thermal stimulus and triggers SOL-dependent degradation pathway. However, my studies described in this thesis and another study (Roessingh et al., 2015)

demonstrates that dTRPA1 is not required for temperature entrainment in the range of temperatures between 20-29 ^{o}C.

Interestingly, CRY-negative DN2 neurons have been previously shown to be temperature sensitive in larval circadian clock circuitry (Picot et al., 2009). The study showed for the first time that under temperature cycles, phasing of molecular rhythms of circadian clock proteins PER and TIM in different circadian neuronal subgroups is synchronised by DN2 subgroups. In the absence of a functional clock in DN2, molecular output entrained to LD but were disrupted under warm: cold (or thermophase cryophase) cycles (henceforth TC), suggesting that a functional molecular clock in the DN2 subset is a prerequisite for circadian clocks to entrain to TC cycles. In LD cycles, phase of molecular oscillations of clock proteins, PER and TIM, in lateral neurons (LNs) and other dorsal neurons (DN1) were 'almost antiphasic' to the phase of molecular oscillations in DN2 neurons. Importantly, phase of molecular oscillations of clock proteins in DN2 neurons under both LD and TC cycles remain unaltered. In contrast, under TC cycles phase of molecular oscillation in all LNs and DN1 neurons shift by almost 12 hr to match the phase of PER in DN2 neurons (Picot et al., 2009). This suggests that under TC cycles DN2 neurons are the pacemaker neurons in the larval brains and they synchronise the phase of clock proteins oscillations in other clock neuronal subsets.

When exposed to a thermal gradient (also see section below), the preferred temperature range of adult wild-type flies lies between 24-26 ^{o}C (Hamada et al., 2008; Sayeed and Benzer, 1996) whereas larvae prefer much lower temperatures of 18 ^{o}C (Kwon et al., 2008; Rosenzweig et al., 2008). A study proposed that thermoreceptors that inform circadian clocks are likely to function in tandem with other thermosensors that allow flies to orient themselves towards their preferred temperature range (Glaser and Stanewsky, 2005). In the recent past, it has been demonstrated that flies also exhibit a circadian clock controlled

temperature preference rhythm (TPR) (Head et al., 2015; Kaneko et al., 2012) similar to mammalian body temperature rhythms (BTR) (Refinetti and Menaker, 1992). TPR in flies exhibit an almost bimodal rhythm with the preferred temperature being low during morning and high just before lights-off (ZT10-12) and there is another attenuated peak during late night (ZT19-21). This rhythm persists several days in absence of cyclic light cues but clock mutants such as per^{01} and tim^{01} fail to exhibit robust TPR, suggesting that TPR is under clock control and does not require cyclic light conditions. While the study rules out the role of canonical morning (M) cells and evening (E) cells in TPR, it posits that DN2 neurons control TPR in flies during the day (ZT1-2). The authors suggest that TPR remain rhythmic even in LL conditions because it is primarily driven by DN2 neurons which are believed to be temperature responsive neurons and they do not express blue-light sensitive circadian photoreceptor CRYPTOCHROME which is required for photoentrainment in flies.

1.2. Behaviour of flies under multiple time cues in nature: In the past few years, chronobiologists have embraced the idea of studying rhythms under natural conditions (Hut et al., 2002; Menegazzi et al., 2013; Menegazzi et al., 2012; Munoz-Delgado et al., 2004; Prabhakaran et al., 2013; Prabhakaran and Sheeba, 2013; Rotics et al., 2011; Vanin et al., 2012). This offers two major advantages over laboratory-based studies – 1) behaviour of organisms can be studied in an enriched and complex environment with multiple zeitgebers, and 2) zeitgebers such as light temperature and humidity change gradually (changing across 24 hr) in contrast to abrupt changes in zeitgebers under standard, rectangular cycles in laboratory. These studies enable us to analyse behaviour of flies in an environment resembling their natural habitat and simultaneously capture complex, dynamic characteristics of zeitgebers in nature like spectral properties of light, combined effects of different physical cues during transitions (dawn and dusk), rise and fall in humidity levels across the day etc.,

thus providing an easy alternative to replicate all complex properties of light and temperature cues in a laboratory incubator. Moreover, since one cannot measure all of the various environmental factors in nature that influence my studies and focus mainly on how light, temperature and humidity condition vary across the day, these conditions are referred to as semi-natural (SN) conditions. Since my experiments will invariably impose several levels of artificial interventions in spite of conducting studies in an outdoor environment, I will henceforth refer to such regimes as semi-natural conditions.

An interesting feature of activity/rest rhythm of *Drosophila* under SN conditions is the presence of an afternoon or (A) peak of activity (De et al., 2013; Menegazzi et al., 2012; Prabhakaran and Sheeba, 2013, 2014; Vanin et al., 2012). The A-peak of activity could be elicited in laboratory incubators by simulating gradual cycles of light and temperature (Menegazzi et al., 2012; Prabhakaran and Sheeba, 2014; Vanin et al., 2012). This is in sharp contrast to bimodal activity/rest pattern of flies with a clear siesta in the middle of day under laboratory LD or TC cycles. Whether this A-peak of activity was clock-controlled has been arguable, however, two recent studies including one from our group, describing my results on behaviour of flies under semi-natural conditions, has now convincingly shown that A-peak is an acute temperature-dependent response and is mediated by dTRPA1 expression in non-circadian clock neurons (Das et al., 2015; Green et al., 2015). This fits well with the hypothesis that A-peak is an "escape" response of flies to high mid-day temperatures (Menegazzi et al., 2012; Vanin et al., 2012).

Studies under 'natural' habitats have sparked much interest and many different behaviours are being examined such as locomotor activity/rest rhythm (De et al., 2013; Menegazzi et al., 2012; Prabhakaran and Sheeba, 2013), eclosion rhythm (De et al., 2012; Kannan et al., 2012; Prabhakaran et al., 2013), foraging based learning in flies in 'free-flying' greenhouse arenas that mimic natural conditions (Zrelec et al., 2013). Comparative studies of

a few *Drosophila* species under SN conditions across seasons have also revealed a variety of subtle differences in temporal patterns of activity/rest of these sympatric species, while some features of the rhythm are conserved across species (Prabhakaran and Sheeba, 2013, 2014).

I examined the behaviour of *D. melanogaster* under multiple zeitgebers in semi-natural conditions and also simulated natural cycles of light and temperature in laboratory incubators to better understand how these environmental factors affect circadian clock controlled locomotor activity/rest behaviour in a temperature dependent manner. My studies show that the afternoon peak exhibited by wild-type flies is predominantly a temperature-driven stress response and requires dTRPA1 signaling.

1.3. Thermosensation in fruit flies. Metazoans can be broadly classified as endotherms and ectotherms based on the dynamics of their core body temperature with respect to their external environmental temperatures. Endotherms depend on internal physiological processes to maintain a 'set' core body temperature irrespective of outside temperature, through homeostatic processes. Ectotherms on the other hand, continuously change their core body temperature to match that of their external environment. Under very warm or cold external temperature conditions small insects like the fruit fly with their large volume-surface are a ratio will suffer severe consequences if they do not modulate their behaviour to avoid such harsh temperature conditions. Thus, for an ectothermic organism it becomes imperative to be able to detect external temperatures and locomote to avoid harmful temperature zones; this active avoidance of extremes of temperature is referred to as negative thermotactic movement.

1.3.1. Thermosensory organs in *Drosophila.* A robust thermosensory system is a prerequisite for ectotherms to detect changes in the external environment. Most insects are

known to have peripheral thermosensors and the antenna is an important organ known to house thermoreceptors across several insect models such as cockroaches (Heinrich, 2013), dragonflies (Piersanti et al., 2011) locusts (Loftus, 1988), etc. Thermoreceptors are broadly classified as noxious and innocuous. Noxious receptors function in extreme temperature ranges and help organisms to avoid too hot or too cold stimuli. Innocuous receptors function in the ambient temperature ranges to modify behaviour. Both the classes of thermoreceptors are known in flies. Distinct thermosensory organs have been identified in larvae and adults as described below.

Larvae - In fruit fly larvae, two classes of thermosensors have been identified similar to those found in nematodes and vertebrates. One set of thermoreceptors are low temperature sensors and the other responds to noxious temperatures. The larval terminal organs are believed to house the cold sensor whereas the multi-dendritic body wall (md class IV) neurons of abdomen express the hot sensor (for review see (Zars, 2003)). Towards the anterior end of the larvae, the neurons in the terminal organs respond to low temperature stimuli, as blocking synaptic transmission from these neurons abolished larval thermotactic movement towards preferred temperature of 18 ^{o}C (Liu et al., 2003). The cold sensors present in the terminal organs are yet to be identified but dTRPA1 expression has been reported in the anterior part of the head and dTRPA1 ion channels are involved in mediating larval thermotactic movement towards optimal temperature of 18 ^{o}C (Kwon et al., 2008). Also, TRPL and TRP ion channels, belonging to TRPC family, are involved in perception of cool temperatures (Rosenzweig et al., 2008). Larvae show typical nocifensive response by rolling with a corkscrew like movement when touched by a hot probe. Such a response is mediated through abdominal multi-dendritic neurons which express thermosensitive Pyrexia TRPA channels (Lee et al., 2005). Among these multi-dendritic neurons, there are two sets

of neurons responding to distinct temperature ranges (29 ^{o}C – 38 ^{o}C and 38 ^{o}C - 41 ^{o}C or above) (Lee et al., 2005).

Thermosensory neurons in the terminal organs and abdominal multi-dendritic neurons undergo changes in intracellular Ca^{2+} levels in response to warm temperature (Liu et al., 2003; Tracey et al., 2003) suggesting that these neurons become active upon exposure to warmth. Further, larval chordotonal organs also express thermosensors, TRPV ion channel, which enable *Drosophila* larvae to distinguish minor temperature changes; allowing larvae to choose their preferred optimal temperature of 18 ^{o}C over very slightly lower temperatures (Kwon et al., 2010b).

Adults – In the mid 90s, Sayeed and Benzer demonstrated for the first time that the 3rd antennal segments of fruit flies are involved in thermosensation (Sayeed and Benzer, 1996). While unilateral ablation of the 3rd antennal segment did not abolish temperature preference in fruit flies, bilateral ablation of 3rd antennal segments rendered flies temperature insensitive on a thermal gradient (Sayeed and Benzer, 1996). The study suggested that thermoreceptors must be present in the 3rd antennal segment of flies but the identity of these receptors were not known. However, flies lacking both 3rd antennal segments were able to avoid relatively high temperatures, above 31^{o}C (Sayeed and Benzer, 1996). This hinted at the presence of additional thermosensors in the fly that function at these warmer temperatures and aid flies to avoid such extremes of temperature. After almost two decades, a study from the laboratory of Charles Zuker reported the presence of hot and cold neurons at the base of aristae, thus, bringing peripheral thermoreceptors into focus again (Gallio et al., 2011). Aristae have been known to mediate hygrosensory cues but were not believed to be important in thermosensation (Sayeed and Benzer, 1996). Now, both aristae and sacculus of antenna in *Drosophila* have been shown to house thermoreceptors (Gallio et al., 2011). The cold sensors in the aristae express Brivido, an ion channel belonging to TRPP family, described by

a previous study (Montell et al., 2002). So far, three brivido genes (*brv1, 2, 3*) are known in *Drosophila* and specific expression pattern of only *brv1* has been identified in the three aristal neurons and in the sacculus of 3rd antennal segments. Base of the aristae also houses three neurons that express hot thermosensors which were later identified to be a gustatory receptor, Gr28d (Ni et al., 2013). Thus, all six aristal neurons of the fly antenna are involved in thermosensation and distinct neurons respond to hot and cold temperatures. These thermosensory aristal neurons are very sensitive, responding to temperature changes as minute as 0.5 ^{o}C change as seen by quantifying neuronal activity via two-photon calcium imaging (Gallio et al., 2011), implicating that the antenna thermosensors are very crucial in mediating thermosensation in flies.

In fruit flies, the exoskeletal joints in antenna, limb joints, wings have chordotonal organs that primarily function as mechanoreceptors, composed of a group of sensilla that are connected to skeletal tissues (Field and Matheson, 1998). Chordotonal organs were implicated in sending temperature cues for entrainment of circadian clocks in the brain to thermal cycles. Expression of gene *nocte* in chordotonal organs was also shown to be important for temperature entrainment of flies (Sehadova et al., 2009). This suggests that chordotonal neurons must possess thermoreceptors to detect changes in temperature and signaling components to convey themal inputs to downstream neurons. Molecular identity of such thermoreceptors in chordotonal organs is not well known but Iav ion channel belonging to TRPV family, expressed in chordotonal neurons, has been implicated -in larval thermosensation (discussed above).

1.3.2. Temperature insensitive mutants: A number of thermosensory defective mutants have been isolated in *Drosophila*. The mutation might present malfunction in temperature sensitivity at the level of the sensor or transmission of thermal signal. One of the first reports

of temperature insensitive mutants came from the laboratory of Seymour Benzer in 1990s (Sayeed and Benzer, 1996). Among the many mutants screened for a thermal preference, two temperature insensitive mutants were characterized, one was *Bizarre* (*biz*) which was completely insensitive to high temperature and flies randomly distributed themselves in a preference assay gradient reaching upto 45 ^{o}C. Second mutant was *Spineless*^{aristapedia} (*ss*^{a}) which was only partially thermo-insensitive; the mutants randomly distributed themselves on a thermal gradient 18-31.5 ^{o}C, exhibiting no preference for ambient temperatures, but avoided temperatures zones beyond 35 ^{o}C (Sayeed and Benzer, 1996)s. The latter mutant had deformed antennal segment once again suggesting the presence of thermoreceptors in antennae (Sayeed and Benzer, 1996). Biz mutants, on the other hand, had no detectable morphological modifications although it was also insensitive for hygrosensation (Sayeed and Benzer, 1996).

Thermo-TRPs encompass all known temperature sensitive ion channels and belong to a large family of TRP (**T**ransient **R**eceptor **P**otential) ion channels. Thermo-TRPs are selectively active in a defined range of temperature and involved in mediating avoidance of extremely hot or cold temperatures both in larvae and in adult flies (Bellemer, 2015; Montell, 2011). Flies carrying mutation in a given TRP ion channel gene do not become completely temperature insensitive but are unable to respond only to range of temperature that encompasses the functional activation range of the specific TRP protein. This is important because it suggests that fruit flies have a large repertoire of thermoreceptors which function in a defined thermal range and receptors functioning in overlapping or adjacent range of temperatures can compensate for lack of signaling in flies mutants for a certain TRP channel.

1.4. Thermosensory circuit in the adult fly brain. Notwithstanding the discovery of temperature sensitive mutants and our ever-increasing knowledge about thermo-TRP ion

channels, the neural circuitry encoding thermosensation in flies remain poorly understood. Central complex and mushroom body (MB) in flies comprise the higher processing centers in the brain and signal inputs from all other sensory modalities are believed to be integrated in these brain areas. Protocerebral bridge in central complex has been implicated in nociception in adult flies (Xu et al., 2006) but the neural circuit from the thermoreceptor and their corresponding projection neurons are yet to be elucidated.

The first study to systematically trace a spatial map for thermosensation in adult fly brain advocated a labelled-line coding for thermal cues in the central brain (Gallio et al., 2011). This is similar to olfactory labelled-line response of trichoid sensilla, specific for mediating pheromone-dependent courtship behaviour in *Drosophila* (Kurtovic et al., 2007). As mentioned above, six thermosensory neurons in each arista can be functionally divided into cold cells (CC) and hot cells (HC) comprising three neurons each. Cold cells express *brivido* ion channels – *brv1*, *brv2* and *brv3* (Gallio et al., 2011) whereas hot cells (HC) express a gustatory receptor, Gr28b, which is the first instance where a gustatory receptor has been shown to mediate thermal signals (Ni et al., 2013). Projection pattern of these thermosensitive aristal neurons were studied by driving expression of membrane bound GFP under cell specific *GAL4* lines: *NP448 GAL4* (cold cells) and *HC-GAL4* (hot cells) (Gallio et al., 2011). Cold cells were found to map onto a novel area in the brain with arborisation reaching a distinct glomerulus of the antennal lobe, which the authors christened as the 'cold glomerulus' at the lateral margin of **P**roximal **A**ntennal **P**rotocerebrum (PAP, now referred to as **P**osterior **A**ntennal **L**obe or PAL according to revised nomenclature). Neurons expressing hot receptors mapped onto an adjacent but non-overlapping glomerulus of the antennal lobe. This 'hot glomerulus' also receives projections from neurons expressing another thermo-TRP ion channel, dTRPA1 (Gallio et al., 2011; Hamada et al., 2008), which is active between 24-29 ^{o}C (Hamada et al., 2008; Tang et al., 2013). This "hot glomerulus" receives warm thermal

signals from sensory neurons both from periphery (aristal neurons) and from central brain (dTRPA1-expressing neurons), thus, suggesting that this is an important centre for integration of warm thermal signals for higher order processing.

Recently, two reports further dissect thermosensory circuits in the central brain by identifying distinct thermosensory projection neurons (tPNs) through which hot and cold sensations are encoded in the brain network (Frank et al., 2015; Liu et al., 2015). Using a host of genetic labelling techniques now available in fruit flies, Frank and colleagues identified second order tPNs by screening *GAL4* driver lines that made synaptic connections with neurons expressing previously known as thermo-receptors. They found that tPNs predominantly send projections to three centres – calyx of Mushroom body (MB), the lateral horn (LH) and lateral protocerebrum (PLP). These three structures thus form the triangle of temperature processing hub in the fly brain (Frank et al., 2015). Most tPNs were found to be narrowly tuned responding to either hot or cold stimuli, but some were broadly tuned neurons that responded to both hot and cold stimuli. The tPNs were also functionally divided as fast-adapting or slow-adapting neurons depending on the temporal dynamics of spikes elicited by the thermal stimulus (Frank et al., 2015). The other study used similar labelling techniques and electrophysiological recordings to functionally characterise hot and cold tPNs (Liu et al., 2015). They found that majority of cold activated tPNs follow a feed-forward or labelled-line approach to convey cold signals to higher processing centres. However, warm cues are conveyed in a more complex manner - warmth-responsive neurons and broadly tuned warm-cold neurons get direct excitatory signals from warm receptors and indirect excitatory signals from cold inter-neurons, the latter phenomenon has been called “crossover inhibition” (Liu et al., 2015).

In summary, thermoreceptors are housed in the periphery in antennal organ – at the base of aristae and sacculus of the 3rd antennal segment. Thermosensory neurons expressing

distinct hot or cold thermoreceptors and the sensory neurons send their projections to distinct glomeruli in the antennal lobe. Second order projection neurons convey hot and cold signals to the triad of higher processing centres of thermal signals in the brain – MB, LH and PLP. How these hot and cold signals are integrated in these higher order brain regions and the pathways through which signals are sent to motor centres to modify temperature-dependent behavioural responses remain to be understood.

1.5. Role of thermo-TRPs in thermosensation. Transient Receptor Potential (TRP) family of ion channels are a large group of proteins found in evolutionarily diverse phyla from nematodes to humans and function in one or more sensory modalities. In *Drosophila*, 13 classes of TRP channels are known which are further sub-divided into seven major classes based on sequence homology (Liu and Montell, 2015; Montell, 2011). Five of them are clustered in Group1 comprising TRPC, TRPV, TRPM, TRPA, TRPN, and Group 2 has two distantly related members, TRPML and TRPP (Montell, 2011). In vertebrates, members of Group1, TRPM, TRPV and TRPA encode thermo-TRPs whereas only members of TRPA encode thermo-TRPs in flies; however other members such as TRPP (Brivido 1, 2, 3) (Gallio et al., 2011), TRPC (TRPL, TRP) (Rosenzweig et al., 2008) and TRPV (Inactive) (Kwon et al., 2010a) have all been implicated in thermosensation via indirect activation modes such as phospholipase C (PLC) signaling, osmotic stimulation and yet unknown mechanisms (for review see (Bellemer, 2015)). The first *trp* gene was indentified via a *Drosophila* mutant with impaired vision under prolonged exposure to intense light (Cosens and Manning, 1969). The mutants were named so because of their transient phenotype observed under electroretinogram recordings wherein the mutant flies displayed a quick return to baseline within 10-15s upon prolonged exposure to bright light whereas wild-type flies showed a sustained component (reviewed in (Minke, 2010). Subsequent biochemical and

electrophysiological studies demonstrated that TRP proteins are Ca^{+2} permeable transmembrane ion channels. All TRP channels have six transmembrane domains with multiple ankyrin repeats at the N terminal; both N and C termini are cytoplasmic. Combining data on TRP channels across different taxa, several possible mechanisms have been proposed for activation of these channels. Earlier, two modes of thermo-TRP channel activation had been proposed: one is direct activation by temperature signal; second, indirect activation by temperature sensitive G-Protein Coupled Receptor (GPCR) mediated signaling through signaling molecule phospholipase C (PLC) (Montell, 2011).

With the advent of cryo-electron microscopy, studying structures of transmembrane proteins at high resolution has become a reality since it bypasses the hurdle of crystallizing membrane proteins. Vertebrate TRPV and TRPA structures studied at a very high resolution (~ 4 Å) using cryo-EM revealed important details (Cao et al., 2013; Liao et al., 2013; Paulsen et al., 2015). Both the studies used chemical agonists to activate channels and captured snapshots of the channels in different conformations. The studies conclude that both vertebrate TRPV and TRPA are tetramers and S5-S6 domains form the pore region with two restriction gates, composed of two helices, regulating cation permeability. They also suggest that binding of electrophiles induces allosteric modification in the structure of protein thus, activating the channel. Previous studies in vertebrates have also attributed thermal or chemical responsiveness of TRP channels to ankyrin repeat domains (ARD) present at the N-terminus (Cordero-Morales et al., 2011; Jabba et al., 2014). The study on TRPA structure re-emphasises the role of ankyrin repeats playing a crucial role in modifying structure to activate the channel upon binding of ligands (Paulsen et al., 2015). In the wake of these studies and others that demonstrated indirect activation of TRP channels via PLC signaling, Montell and colleagues have proposed that most TRP channels may be activated via mechanical force which changes membrane structure (Liu and Montell, 2015). They suggest that non-

mechanical stimuli such as light, temperature or chemicals via signal transduction can change membrane tension, thereby generating mechanical force, which in turn alters the structure of the channel and enables ion permeability (Liu and Montell, 2015).

Over the last decade a number of TRP channels have been found to be involved in the sensation of temperature. Thermal cues can be broadly divided into nociceptive and innocuous and TRP channels functioning in both types of cues have been identified. In addition to avoiding very high or very low temperatures and being able to choose an intermediate preferred temperature over either extreme, *Drosophila* larvae are capable of discriminating minute temperature differences. TRP vanilloid ion channel (TRPV) encoded by *inactive* (or *iav*) gene is unique to chordotonal neurons and TRPV is necessary for larvae to choose 17.5 ^{o}C over cooler temperatures between 14°C -16°C (Kwon et al., 2010a). Painless and Pyrexia TRP channels are involved in sensing warm nociceptive temperatures whereas TRPL and TRP, belonging to TRPC subfamily have been shown to be required for avoidance of cool temperatures on a thermal gradient in larvae (Rosenzweig et al., 2008). Brivido channels (described above) are involved in sensing cool temperatures in adults (Gallio et al., 2011). In addition, dTRPA1 ion channels have been shown to be involved in sensing warm ambient temperatures in larvae and adults (Hamada et al., 2008; Kwon et al., 2008; Rosenzweig et al., 2005) and interestingly even in nociception (Neely et al., 2011). Activation range of *Drosophila* TRPA1 falls in ambient temperature range and encompasses the preferred temperature range of wild-type flies (24-26 ^{o}C) and these channels have been the subject of my studies.

a) Painless. *Drosophila* Painless (TRPN) is a homolog of vertebrate TRPV1 and is involved in nociception in both larvae (Tracey et al., 2003) and adults (Xu et al., 2006). Painless is a receptor for mediating both noxious mechanical and thermal stimuli and requires intracellular Ca^{+2} levels for activation (Sokabe et al., 2008). Painless mutant larvae do not exhibit

nocifensive responses at 41^{o}C unlike wild-type larvae but display responses to very high temperatures of 53 ^{o}C, indicating that motor function of rolling movement is not impaired in these larvae but only perception of hot temperatures in the range of 40 ^{o}C is lost. Expression of Painless was found in chordotonal neurons and a subset of multi-dendritic body wall neurons in the larvae (Tracey et al., 2003) whereas in adults, *pain-GAL4* driven GFP expression was detected in legs, thoracic ganglia and mushroom bodies (MBs) in the central brain (Xu et al., 2006). However, disrupting parts of MBs did not abolish nocifensive response in flies, indicating that such responses are mediated by other brain regions namely the protocerebral bridge which is part of the Central complex (CC) (Neely et al., 2011; Xu et al., 2006).

b) Pyrexia. Another thermo-TRP member, Pyrexia, was isolated from a behavioural screen involving temperature preference choice. The mutant isolated had reduced tolerance to high temperatures and showed about six time higher incidence of paralysis upon exposure to 40 ^{o}C than wild-type flies (Lee et al., 2005). The gene encoding the mutation was mapped to CG17142, which was found to encode two spliced variants, called Pyr-PA and Pyr-PB and they shared homology with other thermo-TRP proteins, Painless and dTRPA1 (then called as ANKTMI). Although some *pyx* alleles led to abnormal temperature preference behaviour, the major role attributed to Pyx-TRP ion channel is in combating thermal stress. Pyx-TRP channel expressed in heterologous system was found to open in response to ~40 ^{o}C and Pyx-TRP channel activation inhibited high rate of neuronal firing at very high temperatures, thus, preventing temperature induced paralysis. Upon activation, Pyx channel was more permeable to K^{+} than Na^{+} ions and this preference for K^{+} ions has been proposed to the mechanism for electrical silencing of neurons during thermal induced stress (Lee et al., 2005). Pyrexia has broad expression in the peripheral and central nervous system in embryos; larval body wall neurons including multi-dendritic neurons; sensory neurons lying

below eye and thorax bristles, antennae and maxillary palps and proboscis in adults (Lee et al., 2005).

c) dTRPA1 channels. dTRPA1 (fly ortholog of vertebrate TRPA1) is a member of TRP family implicated in sensation of warm ambient temperature in both larvae and adults (Hamada et al., 2008; Kwon et al., 2008; Rosenzweig et al., 2005). dTRPA1 mutant larvae were found to be defective in thermotactic behaviour as they did not actively move towards their preferred temperature (18 ^{o}C) or avoid high temperatures zones (Kwon et al., 2008; Rosenzweig et al., 2005). With respect to larval navigation, different thermosensors mediate directional thermotaxis. dTRPA1 expressed in brain neurons are important for negative thermotaxis (Kwon et al., 2008; Rosenzweig et al., 2005; Rosenzweig et al., 2008) wherein larvae actively show locomotion away from warm temperature areas whereas terminal organ neurons in larvae, expressing GH86, is required for positive thermotaxis (Luo et al., 2010). Similarly, dTRPA1 mutant (*dTRPA1*^{ins}) flies also were unable to avoid warm temperatures on a thermal gradient, choosing 28-30 ^{o}C in contrast to wild-type flies which orient themselves in 24-26 ^{o}C temperature zone (Hamada et al., 2008). Moreover, dTRPA1 has also been implicated in both thermal nociception (Neely et al., 2011) and chemonociception (Kang et al., 2010). RNAi knockdown of dTRPA1 in multi-dendritic sensory neurons in larvae disrupts nociception response (Neely et al., 2011). Similarly, dTRPA1 has been shown to function in gustatory neurons of fly labellum (labral sense organ – LSO) enabling them to avoid ingesting reactive electrophiles (Kang et al., 2010).

Expression of dTRPA1 were found in few neurons in the central brain and neuroendocrine cells of corpus cardiacum immunolabelled with dTRPA1 antibody (Rosenzweig et al., 2005) whereas promoter driven GFP expression was also seen in peripheral neurons in two pairs of neurons at the anterior part of head, adjacent to mouth hooks in larvae (Kwon et al., 2008). In adults, dTRPA1 anti-sera labelled only three pairs of

neurons, anterior cells (ACs), ventral cells (VCs) and lateral cells (LCs) (Hamada et al., 2008); however, promoter driven GFP expression was more widespread (Shih and Chiang, 2011). GFP expression was found in ACs, also in ten other neurons in the brain and in eight neurons in thoracic ganglion (Shih and Chiang, 2011). A subset of dTRPA1-expressing neurons was later reported to overlap with LN_v, LN_d and DN groups of circadian neurons (Lee and Montell, 2013; Yoshii et al., 2015). Among the dTRPA1 neurons, the AC neurons are called the "internal thermosensors" since RNAi knockdown of dTRPA1 expression in these cells disrupts thermal preference behaviours in flies whereas dTRPA1 expression in ACs is sufficient to mediate this behavioural response to choose preferred ambient temperatures on a thermal gradient (Hamada et al., 2008). There have been isolated reports that dTRPA1 expression was also found in gustatory neurons (GRNs) to mediate avoidance of bitter substance (Kim et al., 2010), in labral sense organs (LSO) to mediate chemonociception (Kang et al., 2010) and in olfactory neurons in the antenna to avoid noxious insect repellent vapours (Kwon et al., 2010a).

Voltage clamp studies from dTRPA1 expressed in *Xenopus* oocytes showed that the channel opened in response to warm temperatures above ~27 ^{o}C (Viswanath et al., 2003). In larvae, dTRPA1 ion channels function at ambient temperatures between 18-24 ^{o}C, aiding larvae to choose their preferred 18 ^{o}C against slightly warmer 19-24 ^{o}C (Kwon et al., 2008). Much later, Ca^{+2} imaging from dTRPA1-expressing AC neurons in adult flies demonstrated that these cells have two temperature responsive peaks at 25 and 27 ^{o}C respectively (Tang et al., 2013); however due to technical difficulties in measuring temperature close to tissue sample, the more probable temperature peaks are at 27 and 29 ^{o}C (Tang et al., 2013). The response of AC neurons to higher than 29 ^{o}C have been shown to be dependent on synaptic inputs from Pyrexia neurons in the antenna, as Ca^{+2} signals recorded from AC neurons in *pyx* mutants lacked the second temperature responsive peak at 29 ^{o}C (Tang et al., 2013).

Recently, four isoforms of dTRPA1 were isolated (Kang et al., 2012; Zhong et al., 2012), isoforms A and D are temperature sensitive and function at distinct temperature ranges- dTRPA1-A (24- 29 ^{o}C) and dTRPA1-D (34 ^{o}C and above). Isoforms B and C are not temperature responsive and their functions are not yet completely understood (Zhong et al., 2012).

Besides direct activation by heat, dTRPA1 channels have been proposed to be indirectly activated by signaling cascades. dTRPA1 channels are thought to be activated by PLC signaling downstream of a G protein coupled receptor or GPCR to mediate thermosensation and chemosensation (Kim et al., 2010; Kwon et al., 2008) (described in detail below) and synaptic inputs from Pyrexia neurons have been shown to be required for functioning of dTRPA1-expressing AC neurons at warmer temperatures of ~29 ^{o}C (Tang et al., 2013).

1.6. Thermo-TRPs in temperature entrainment. Drawing parallels from photoentrainment pathways in flies (Dubruille and Emery, 2008), entrainment of circadian clocks to temperature cycles would require inputs from thermoreceptors that would convey information about temperatures in the external environment. In *Drosophila*, both peripheral tissues (chordotonal organs such as antenna) and central brain neurons (dTRPA1-expressing **a**nterior **c**ells or AC) are known to harbour thermoreceptors. Notwithstanding the importance of peripheral thermoreceptors to help flies to avoid extremes of temperatures, the antennal thermoreceptors do not seem to be involved in temperature entrainment as flies with surgically removed antennae or mutant flies with antennae modified into legs are still able to entrain to thermal cycles (Glaser and Stanewsky, 2005). A dedicated circadian thermoreceptor that sends input to circadian clocks to modulate behavioural rhythms is yet to be identified, although a handful of candidate genes have been implicated in temperature

entrainment of circadian clocks. These include *nocte* and to a lesser extent *norpA* (Glaser and Stanewsky, 2005) and two TRP receptors, Pyrexia (Wolfgang et al., 2013) and dTRPA1 (Lee and Montell, 2013). Recently a non TRP receptor, namely an ionotropic receptor, IR25a, has been reported to be involved in mediating entrainment to low-amplitude temperature cycles (Chen et al., 2015). The first member of TRP family to be implicated in temperature entrainment was Pyrexia (Wolfgang et al., 2013). Pyrexia channels were previously known to be activated only at noxious hot conditions of 40 ^{o}C (Lee et al., 2005) but this study showed that Pyx-TRP that are expressed in peripheral chordotonal organs and are involved in entrainment of circadian clocks to temperature cycles in the cool range (warm: cold cycles at 20:16 ^{o}C) but are not required for entrainment to thermal cycles in warm conditions (warm: cold cycles at 29:25 ^{o}C) (Wolfgang et al., 2013). Moreover, restoring *pyrexia* expression in few neurons in *pyx* null mutants lead to only partial rescue of thermal entrainment (Wolfgang et al., 2013). This suggests that other thermoreceptors besides the Pyx-TRP channels send thermal cues to circadian clocks. Future studies that unravel the molecular mechanism of Pyx-TRP activation and neural circuits that modify Pyx-dependent temperature entrainment can possibly explain how Pyx-TRP channel functions at both noxious (40 ^{o}C) and cool ambient temperature (16-20 ^{o}C) ranges.

Another report claimed that dTRPA1 is involved in influencing behaviour under temperature cycles (Lee and Montell, 2013). The authors claimed that dTRPA1 null flies are unable to show stable entrainment under asymmetric 18:6hr warm:cold cycles (29:18 ^{o}C) (Lee and Montell, 2013), however, role of dTRPA1 in temperature entrainment of circadian clocks has since been questioned because even the wild-type flies do not seem to entrain to the asymmetric temperature cycles (also see footnote in (Maguire and Sehgal, 2015)). Moreover, even a non-thermosensitive isoform, dTRPA1-B, is able to rescue the defects of dTRPA1 null mutant flies under imposed temperature cycles, raising questions on the

influence of temperature in determining the mutant phenotype. Studies have shown that dTRPA1 is expressed in circadian clock neurons including three CRY-positive LN_d neurons (Lee and Montell, 2013; Yoshii et al., 2015) and dTRPA1 null mutants have dampened PER oscillations in LN_d subsets (Lee and Montell, 2013) due to which Montell and colleagues propose that dTRPA1 is involved in synchronizing circadian behaviour to temperature cycles.

In Chapter 2 of my thesis, I present results of my studies that suggest that entrainment of circadian clocks to temperature cycles in constant darkness can occur independent of dTRPA1 signaling. Similar conclusions have been put forward by another study (Roessingh et al., 2015).

1.7. Signaling pathways in thermosensation:

1.7.1. Role of GPCR signaling and second messenger system: Light and temperature are the dominant physiological cues in nature and therefore, it is not surprising that signaling cascade of phototransduction and thermosensation may share common entities. Two candidates involved in signaling pathways for photic cues, namely phospholipase C and rhodopsin, have also been implicated in mediating thermosensation in flies. Indirect mode of TRP channel activation has been linked to components of G protein coupled receptor (GPCR) via phosopholipase C (PLC) signaling (Montell, 2011). GPCR signaling pathway is thought to amplify small temperature changes to bring about a vigorous physiological response. In flies, PLC exists as three isoforms which includes NorpA, Small wing (sl) and PLC21c (Singer et al., 1997). PLC encoded by *norpA* is known to be involved in phototransduction and functions downstream of a GPCR and trimeric G protein, Gq (Bloomquist et al., 1988; Scott and Zuker, 1998) and PLC21c is also known to function downstream of G alpha subunit, Go (Dahdal et al., 2010). Further, Gq-PLC signaling have been proposed to function as indirect activators of dTRPA1 ion channels in both thermosensation and chemosensation

(Kim et al., 2010; Kwon et al., 2010a; Kwon et al., 2008). Larvae of *norpA* null mutants exhibit abnormal thermotactic movements on a thermal gradient, unable to avoid warm temperatures, similar to dTRPA1 nulls larvae. Inability of *norpA* larvae to avoid warm temperature can be rescued by restoring *norpA* expression only in dTRPA1-expressing neurons suggesting that *norpA* encoded PLC functions upstream of dTRPA1 signaling mediating thermotactic movement in *Drosophila* larvae (Kwon et al., 2008). Similarly, PLC has been shown to function upstream of dTRPA1 ion channels in gustatory neurons to mediate chemosensation. Expression of dTRPA1 has been detected in a small numbers of gustatory neurons (GRNs) and functional PLC signaling is required for flies to avoid bitter compounds such as aristolochic acid (Kim et al., 2010). dTRPA1 signaling functioning downstream of PLC has also been shown to be required in olfactory receptor neurons in the antenna to activate a Ca^{+2} dependent potassium channel to avoid certain odours such as the insect repellant, citronellal (Kwon et al., 2010a).

Further, a study also showed that *Drosophila* rhodopsin which is a GPCR, encoded by gene *ninaE*, is also involved in thermosensation (Shen et al., 2011). In contrast to wild-type larvae, *ninaE* mutant larvae showed defective thermotactic behaviour and were unable to choose the preferred temperature of 18 ^{o}C over 24 ^{o}C, thus resembling disrupted thermotactic behaviour of *norpA* and *dTrpA1* mutant larvae. RNAi knockdown of *ninaE* in dTRPA1-expressing cells impaired thermal discrimination in larvae across 18 ^{o}C - 24 ^{o}C range, whereas overexpression of *ninaE* in dTRPA1 neurons restored preference for 18 ^{o}C (Shen et al., 2011); suggesting that rhodopsin in dTRPA1 neurons aid in thermosensation in larvae but the mechanism of activation of dTRPA1 ion channels via rhodopsin is not yet known.

A previous study demonstrated that cAMP / PKA signaling in αβ lobes of mushroom bodies (MB) in the fly brain is essential for flies to exhibit thermal preference behaviour (Hong et al., 2008). Fly mutants with altered cAMP levels were not able to choose their

preferred ambient temperatures on a thermal gradient. Flies with increased cAMP levels were unable to avoid warmer temperatures whereas flies with lowered cAMP levels did not avoid cooler temperatures (Hong et al., 2008). Thus, role of GPCRs and second messenger cAMP in modulating thermosensation in flies have been under speculation for quite some time (Montell, 2011, Minke 2011). In my thesis, I describe my studies that aim to elucidate the role of PLC and cAMP signaling in influencing dTRPA1-dependent behavioural patterns under different cyclic environmental regimes.

1.7.2. Role of Neurotransmitters in Thermosensation. Biogenic monoamines play important roles in neuronal communications in both vertebrates and invertebrates. Like mammals, *Drosophila* employ neurotransmitters such as acetylcholine, GABA and glutamate and monoamines like dopamine, serotonin and histamine in many physiological processes (Martin and Krantz, 2014). Biosynthetic pathways of monoamines are also conserved between mammals and flies and there are ever increasing reports that their functions in influencing complex behaviour such as sleep also appear to be conserved ((Nall and Sehgal, 2014), reviewed in (Potdar and Sheeba, 2013)).

a) Dopamine. Dopamine (DA) acting as a neuromodulator for various sensory modalities and also many complex behaviours ranging from locomotion, motivation and reward, learning and memory, addiction, aggression circadian rhythms and sleep (Potdar and Sheeba, 2013; Van Swinderen and Andretic, 2011; Yamamoto and Seto, 2014). Dopamine is also required for normal temperature preference behaviour of *Drosophila* (Bang et al., 2011), which is the ability of flies to move towards their preferred temperature range on a thermal gradient. Impairment of dopamine synthesis or absence of dopamine receptor in flies disrupts thermal preference and affected flies choose cooler temperatures than their preferred ambient temperatures. Electrical silencing of dopaminergic neurons by ectopic expression of

potassium channel blocker (*UAS-Kir2.1*) also shows similar defect in temperature selection. Driving expression of dopamine receptor under mushroom body specific driver, rescued this abnormal thermal preference (Bang et al., 2011). This also adds credence to the role of mushroom body as the 'higher' brain centre in governing temperature mediated behavior in *Drosophila*.

In flies, neurons expressing dopamine have been divided into 13 clusters (Mao and Davis, 2009). A study showed that a subset of PPL1 dopaminergic neurons encode "asymmetric" representation of temperature in the fly brain (Tomchik, 2013). A subset of dopaminergic PPL1 neurons innervating the vertical lobes of mushroom body (MB) is involved in mediating responses to specifically cooler temperatures in flies and these neurons do not respond to warm temperature signals. These responses of dopaminergic neurons were attenuated, but not abolished when antennae and maxillary palps were ablated (Tomchik, 2013). This suggests that thermoreceptors in these peripheral organs and possibly other receptors feed into PPL1 dopaminergic neurons to transmit thermal cues to the higher processing centers in the fly brain.

b) Serotonin. Serotonin (5 hydroxytryptamine or 5HT) is a monoamine neurotransmitter that is known to influence a variety of physiological processes in fruit flies such as circadian rhythm, sleep, aggression, learning and memory, courtship, etc. (Becnel et al., 2011; Johnson et al., 2009, 2011; Nichols, 2007; Yuan et al., 2005). In flies, serotonin has been implicated to function downstream of thermosensitive dTRPA1 neurons (Shih and Chiang, 2011). dTRPA1- positive AC neurons have been shown to secrete serotonin (Shih and Chiang, 2011) and out of the five known classes of serotonin receptors in *Drosophila* (Gasque et al., 2013), neurons expressing serotonin receptor class 1B ($5HT_R$-1B), has been predicted to be downstream of AC neurons (Shih and Chiang, 2011). Further studies that explore the neural connections between serotonin receptors and dTRPA1 channels would improve our

understanding of how temperature cues are processed in the fly brain. In this thesis, I describe my attempts to elucidate how downstream signaling from dTRPA1 neurons may be encoded.

c) Histamine. Another monoamine that has been linked to temperature preference in flies is histamine (Hong et al., 2006). Flies with mutations in histamine pathway showed defective thermal preference behavior (inability of flies to orient themselves towards their preferred temperature range on a thermal gradient). This defective behavior could be induced in wild-type flies by blocking the receptors of histamine using pharmacological agents such as, cimetidine and hydroxyzine (Hong et al., 2006). Conversely, rescue of histamine signaling components in mutant backgrounds could rescue the phenotype, thus emphasizing the role of this pathway in controlling thermal preference related behaviour. Thus, multiple neurotransmitters and signaling pathways have been implicated to function in thermosensation in fruit flies. My studies focuses on signaling pathways that maybe involved mediating thermal cues encoded by dTRPA1 neuronal circuits.

Both larvae and the adult fruit flies *Drosophila melanogaster*, exhibit distinct, well characterised responses to mild and noxious thermal stimuli. Temperature-dependent behavioural changes can be cleverly modelled in flies with the help of sophisticated genetic tools to dissect behavioural responses to thermal cues and study the underlying neuronal circuitry that give rise to such complex behavioural patterns. Additionally, the circadian clock circuitry is well-studied in fruit flies and clock driven rhythmic locomotor activity/rest rhythm provides a robust behavioural read out. I examined the neural circuitry of dTRPA1 flies in mediating temperature dependent modifications in circadian clock controlled activity/rest rhythms.

In this thesis, I describe my attempts to understand how temperature cues modify rhythmic behavioural activity/rest pattern of flies via thermosensitive dTRPA1 signaling.

dTRPA1 expression is predominantly in the brain and the activation range of these ion channels closely overlaps with external temperature that the flies are likely to encounter in their natural habitat. I asked if dTRPA1 sends thermal cues to the circadian clocks which in turn modulate the activity/rest pattern in flies in a temperature-dependent manner. I report that neurons expressing warmth-activated ion channels ***Drosophila*** **Transient Receptor Potential-A1** (dTRPA1) modulate distinct aspects of the rhythmic activity/rest rhythm in response to thermal cues under different environmental regimes. Under light/dark (LD) cycles paired with constantly warm ambient conditions, flies deficient in dTRPA1 expression are unable to phase their morning and evening activity bouts appropriately. Correspondingly, my studies show that the electrical activity of few neurons targeted by *dTRPA1^{SH}-GAL4* driver modulates temperature-dependent phasing of activity/rest rhythm under LD cycles. The expression of dTRPA1 also impacts behaviour responses to temperature cycles combined with constant dark (DD) or light (LL) conditions. My results demonstrate that the mid-day "siesta" exhibited by flies under temperature cycles in DD is dependent on dTRPA1 expression in a small number of neurons that include thermosensory AC neurons. Although a small subset of circadian pacemaker neurons express dTRPA1, my studies show that CRY-negative *dTRPA1^{SH}-GAL4* driven neurons are critical for the suppression of mid-thermophase activity, thus enabling flies to exhibit "siesta".

In contrast to temperature cycles in DD, under temperature cycles in LL, dTRPA1 is not required for exhibiting "siesta" but is important for phasing of evening peak. My studies show that activity/rest rhythms are modulated in a temperature-dependent manner via signals from *dTRPA1^{SH}-GAL4* driven neurons. Taken together, these results emphasise the differential influence of thermoreceptors on rhythmic behaviour in fruit flies in coordination with light inputs.

Further, I also studied the role of dTRPA1 signaling in modifying behaviour of flies under natural conditions where different environmental factors such as light, temperature, humidity etc. change in a gradual manner across the day, which is in contrast to the rectangular zeitgeber cycles mostly used for studies in the laboratory. Under such natural conditions, wild-type flies exhibit an additional peak of activity in the middle of the day. This is referred to as the afternoon peak (A-peak) and is absent under standard laboratory regimes using gated light and temperature cues. Although previous reports identified the physical factors that contribute towards the A-peak there was no evidence for underlying molecular mechanisms or pathways that control A-peak. My studies show that the A-peak is mediated by thermosensitive dTRPA1 signaling as this peak is absent in dTRPA1 null mutants. Further, when natural cycles of light and temperature are simulated in the lab, my results demonstrate that the amplitude of the A-peak is dTRPA1-dependent. Further, my studies distinguishes that although a few circadian neurons express dTRPA1, modulation of A-peak is primarily influenced by non-CRY dTRPA1 expressing neurons. Hence, my studies propose that A-peak of activity observed under SN is a temperature sensitive response in flies that is elicited through dTRPA1 receptor signaling.

The studies described in this thesis reveal that dTRPA1 signaling has opposite effect on behaviour depending on the nature and duration of temperature cues- prolonged exposure to 29 ^{o}C leads to suppression of activity whereas acute 32 ^{o}C for 1 hr induces activity in flies. My studies also delve into the neural circuits that have been proposed to function upstream and/or downstream of dTRPA1. I also explore the role of cAMP/PKA intracellular signaling pathway and how secondary processing centres in the adult fly brain may be involved in modifying behavioural patterns in response to dTRPA1 signaling. In summary, my studies demonstrate that dTRPA1 receptor mediated signaling primarily in non-circadian neurons is crucial for modifying rhythmic behaviour of flies in a temperature-dependent manner.

Chapter 2

dTRPA1 signaling influences rhythmic activity/rest profiles under rectangular light or thermal cycles in *Drosophila melanogaster*

Introduction

In *Drosophila melanogaster*, about 150 clock neurons in the adult brain constitute the circadian circuit and distinct subsets of clock neurons function to convey photic and thermal cues to circadian clocks (Sheeba et al., 2008c). Temperature cycles can entrain circadian clocks (Busza et al., 2007; Glaser and Stanewsky, 2005; Miyasako et al., 2007; Picot et al., 2009; Yoshii et al., 2010; Yoshii et al., 2005; Yoshii et al., 2009) and dedicated thermoreceptors are likely to be involved in this behaviour. In flies, thermoreceptors are mostly present in the periphery - antennae, aristae (Gallio et al., 2011; Sayeed and Benzer, 1996) and chordotonal organs (Sehadova et al., 2009). Six neurons at the base of the aristae has been identified to house both warm and cold receptors (Gallio et al., 2011) and while the molecular identity of these warm receptors remain unknown, the cold receptors are encoded by *brivido* channels, that belong to the large TRPL family. The neuronal circuit from these warm and cold sensing aristal neurons have been traced from the periphery to their respective projection neurons and finally to their arbors in three distinct higher processing centres – mushroom body calyx, lateral horn and lateral protocerebrum (Frank et al., 2015; Liu et al., 2015).

In addition, several well characterised thermoreceptors in *D. melanogaster* belong to the TRP ion channel family (Montell, 2011), among which only two have been implicated in temperature entrainment - Pyrexia (Wolfgang et al., 2013) and dTRPA1 (drosophila Transient Receptor Potential A1) (Lee and Montell, 2013). Besides the thermo-TRPs , IR25- an ionotropic glutamate receptor, has been recently shown to mediate entrainment to low amplitude temperature cycles (Chen et al., 2015). While the photoentrainment pathway in *Drosophila* is well studied there have been few attempts at examining how cyclic thermal cues affect rhythmic behaviour (Dubruille and Emery, 2008). Recently, a molecular pathway

for temperature dependent degradation of TIMELESS (or TIM) has been proposed to occur via a calpain protease- small optic lobes (SOL) (Tataroglu et al., 2015) in circadian pacemaker neurons, defining a cell autonomous mode for temperature entrainment. Such a mechanism is contrary to the proposition that the fly brain is dependent on signals from peripheral chordotonal organs in order to entrain to temperature cycles based on an earlier study (Sehadova et al., 2009). Thus, although several thermosensors have been identified, how thermal stimuli from thermosensitive neurons are conveyed to the circadian clock neurons is not yet clearly understood.

Amongst the thermo-TRPs, the dTRPA1-expressing anterior cells (AC) neurons are considered 'internal thermosensors' in the fly brain (Hamada et al., 2008) and dTRPA1 mutants show deficits in both larval (Kwon et al., 2008; Rosenzweig et al., 2005; Rosenzweig et al., 2008) and adult thermotaxis (Hamada et al., 2008). However, dTRPA1 are not the warm sensors in the aristae since aristal neurons in dTRPA1 null flies were also found to respond to warm temperature stimuli similar to controls (Gallio et al., 2011) and thus, dTRPA1 are unlikely to function in the peripheral thermosensory organs. dTRPA1 ion channels are known to function in the range of 27-29 ^{o}C (Hamada et al., 2008; Tang et al., 2013). Since these temperatures are likely to be encountered by flies on a daily basis, especially in more tropical climates (Prabhakaran and Sheeba, 2013), I examined the role of dTRPA1 in modulating daily rhythmic activity under 1) light/dark cycles at constant warm temperatures and 2) temperature cycles in the context of different light conditions. I asked if the ability of flies to modify their distribution of activity/rest profiles in response to different ambient temperatures is dependent on dTRPA1.

Here, my studies focussed on the role of a small subset of dTRPA1-expressing neurons, targeted by the most conservative and restricted driver, *dTRPA1^{SH}-GAL4* (Hamada

et al., 2008), henceforth (dTRPA1^{SH+} neurons) to understand the contribution of dTRPA1 ion channels in modifying behaviour in response to different temperature stimuli and corroborated my results with the use of other *dTRPA1-GAL4* drivers available. Under 12:12 hr light / dark (LD) cycles, wild-type flies shift locomotor activity into the dark-phase if ambient temperature is constantly warm temperatures whereas flies consolidate their activity to the light-phase under constant cool temperatures (Majercak et al., 1999). I used *dTRPA1^{SH}-GAL4* driver to manipulate dTRPA1 expression or to alter electrical properties of dTRPA1-positive AC neurons. Activation of dTRPA1 modulates the phase of morning and evening activity peaks under LD at constant warm ambient temperature. I also examined activity/rest pattern of flies under 12:12-hr thermal cycles (thermophase -29 ^{o}C :cryophase - 21 ^{o}C or TC 29:21) in constant darkness (DD) where thermophase (29 ^{o}C) is within the range of activation of this ion channel as shown previously (Hamada et al., 2008; Viswanath et al., 2003). Mutational loss of dTRPA1 function renders flies incapable of exhibiting the normal "siesta" under thermal cycles in constant darkness (DD/TC). My studies show that dTRPA1 expression in dTRPA1^{SH+} neurons is crucial in mediating rhythmic siesta of flies under DD/TC. Further, my results show that signals from dTRPA1^{SH+} neurons contribute to phasing of evening activity under TC in constant light (LL). Taken together, the most parsimonious explanation for the above results is that dTRPA1^{SH+} neurons provide sensory inputs to circadian neurons and modulate time of day responses to changes in temperature.

Materials and Methods

Fly strains. All genotypes were reared on standard cornmeal medium under LD (12:12-hr) at 25 ^{o}C. *dTRPA1^{SH}-GAL4* (Hamada et al., 2008), *dTRPA1-GAL4^{48951}* (Green et al., 2015), *TRPA1$^{KI-GAL4}$* (Kim et al., 2010) drivers were used to target subsets of dTRPA1-expressing neurons. Overexpression of dTRPA1 was achieved using *UAS-dTRPA1* (Rosenzweig et al.,

2008) which is expected to express dTRPA1-A isoform (see Supp Fig 5 in (Hamada et al., 2008) , also see (Kang et al., 2010)). *UAS-Dcr2D* (Bloomington Stock Centre) was combined with two *UAS-dTRPA1*^{RNAi} transgenes (VDRC - *UAS-dTRPA1T*$_1^{RNAi}$ #37249 and *UAS-dTRPA1T*$_2^{RNAi}$ #37250) for RNAi knockdown of dTRPA1 in different groups of neurons. Flies expressing *UAS-dcr-2/ UAS dTRPA1T*$_2$ ^{RNAi} under *dTRPA1*^{SH}*-GAL4* have been referred to as *dTRPA1*^{RNAi} flies throughout the text. In RNAi experiments, *dTRPA1*^{SH}*-GAL4/UAS-dcr-2* served as *GAL4* control. Neuronal hyperexcitation was induced by driving expression of voltage gated Na^+ channel *UAS-NaChBac1* (Nitabach et al., 2006; Sheeba et al., 2008a). Apoptosis-inducing transgene *UAS-hid* (Zhou et al., 1997) was used for ablation of neuronal subsets (donation from Michael Rosbash, Brandeis). Flies expressing *UAS-hid* under *dTRPA1*^{SH}*-GAL4* driver, which targets only a subset of dTRPA1 neurons, have been referred to as *dTRPA1*^{ablated} flies. *dTRPA1*^{ins} is a "strong loss of function" mutation (Hamada et al., 2008; Head et al., 2015). *TRPA1*$^{KI\text{-}GAL4}$ (donated by Youngseeok Lee, Kookmin University) was created by ends out homologous recombination which inserted *GAL4* gene at normal translational initiation codon and also deleted 185bp region encoding N-terminal residues (Kim et al., 2010), thus making it a dTRPA1 driver in a null background. Verification of RNAi knockdown or overexpression of different genes in neurons of interests via the *GAL4 / UAS* system has not been carried out due to the following reasons: 1) the number of target neurons in each case are very few and to detect small changes in mRNA expression levels using whole head extracts is cumbersome and often inconclusive; 2) antibodies against the proteins were not available to us in order to estimate expression levels via immunocytochemistry on whole fly brains and to generate the antibodies in the laboratory was not possible due to limitation of resources.

Behavioural assays: Locomotor behaviour of male flies (2-4 day old) was measured using Drosophila Activity Monitors (DAM, Trikinetics, Waltham, MA) in 5-min binning intervals

as described previously (Sheeba et al., 2008b) in incubators (Sanyo, Japan and Percival Drosophila Chambers, USA).

Light/dark cycles: Flies were subjected to (12:12-hr) LD cycles with constant temperatures of 21, 25, 27 or 30 ^{o}C (LD21, LD25, LD27 and LD30) for 6-7-days followed by constant darkness (DD). Free-running period of individual flies was determined using Chi-square periodogram analysis (excluding first two days of DD to avoid transient cycles) with Actimetrics^{TM} Clocklab software (Wilmette, IL). Activity profile was generated by normalising activity counts of individual flies in 15-min bins over total activity across 24-hr and then averaging across five consecutive days. For a given genotype, values were averaged across flies and plotted against time of the day. Phase of morning peak was determined for individual flies by identifying the time point of highest activity between ZT20–02. Wherever possible, the 'true' morning peak was distinguished from the startle response at lights-ON based on the presence of anticipatory activity before lights-ON (Sheeba et al., 2010). I have defined the phase of onset of evening activity as time-point at which post-siesta activity levels reached 20% of maximum daily activity (for individual flies). Phase differences in morning peak or onset of evening activity among genotypes were compared using two-way ANOVA with genotypes and temperature as fixed factors, $p < 0.05$, followed by Tukey's HSD post hoc comparisons.

Temperature cycles: Flies were reared under LD (25 ^{o}C) and adults were kept in LL (21 ^{o}C) for 24 hrs to eliminate phasing effects of previous LD. Flies were then subjected to 12:12-hr temperature cycles in DD (thermophase: cryophase 29:21 ^{o}C - DD/TC) for 9-10-days to allow for stable entrainment, followed by 10-hr phase-shifted TC cycles (advance or delay) for next 13-days and then released into DD 21 ^{o}C for next 7-10-days (or when specified, directly released into DD 21 ^{o}C without phase shift). Flies were considered to have entrained when period of activity rhythm for 7 days in DDTC = 24±0.5hr. Activity counts binned into

15-min intervals were first averaged across 5-days for individual flies and mean across flies for a given genotype was plotted against time of day. Mean activity counts per 2-hr bins were used to compare differences in activity levels among genotypes by performing a two-way ANOVA with genotype and time-points as fixed factors followed by Tukey's HSD test ($p < 0.05$). To estimate the rate of re-entrainment to shifted temperature cycles, offset of evening activity was chosen as phase-marker and the number of transient cycles was calculated for individual flies. Number of transients taken to re-entrain to shifted TC cycles was compared across genotypes using non-parametric Kruskal-Wallis ANOVA followed by multiple comparisons of mean ranks, $p < 0.05$. Free-running period of individual flies were determined using Chi-square periodogram analysis in Clocklab using data for at least 7 days in DD. Morning peak amplitude and evening peak phases were manually determined for individual flies subjected to TC cycles in LL (LL/TC). Statistical differences among genotypes were compared by performing ANOVA, followed by Tukey's HSD ($p < 0.05$).

Lifespan assay: Virgin male flies of w^{1118} and $dTRPA1^{ins}$ flies were divided into 12 vials with corn-meal food; each vial contained 10 virgin males. The vials were kept horizontally in trays in incubators (Sanyo, Japan) under DD/TC (29:21 ^{o}C) with food change every alternate days. Fly counts were recorded everyday till all the flies were dead. Percentage survival per genotype is plotted against time (in days). To estimate difference in average lifespan between genotypes, average lifespan per vial was compared using a student's t-test ($p < 0.05$).

Immunocytochemistry: Ablation of dTRPA1^{SH+} neurons by expressing *UAS-hid* was confirmed as the lack of GFP expression in six out of seven adult fly brains examined using Zeiss epifluorescence microscope (Axio Observer. Z1). Immunocytochemistry was performed on 2-3 day old male adult fly brains as described in detail in Chapter 4. Primary

antibody used was chicken anti-GFP (1:1000, Molecular Probes) and secondary antibody was anti-chicken conjugated with Alexa Fluor 488 (1:2000, Invitrogen). Images were assembled using ZEN 2011 software.

Results

Temperature-dependent modulation of morning and evening activity bouts under LD cycles is mediated by dTRPA1. Changes in ambient temperature alter the pattern of activity of flies under LD cycles (Majercak et al., 1999; Miyasako et al., 2007). Compared to their preferred temperature of 25 ^{o}C (Sayeed and Benzer, 1996), flies exposed to constant low temperature (18 ^{o}C) shift their usual evening activity earlier into the day whereas at constant high temperature (29 ^{o}C) flies shift their evening activity later into the night (Majercak et al., 1999). dTRPA1 channel enables *Drosophila* to choose their preferred temperature both as larvae (Rosenzweig et al., 2005; Rosenzweig et al., 2008) and adults (Hamada et al., 2008; Kaneko et al., 2012; Rosenzweig et al., 2005). I tested whether loss of dTRPA1 alters the waveform of activity/rest rhythm under LD at different ambient temperatures (21, 25, 27 or 30 ^{o}C, henceforth LD21, LD25, LD27 and LD30). Wild-type (w^{1118}) flies display a bimodal activity pattern with similar phase of morning (M)-peak under LD21 and LD25 (Figure 2.1A, left panel). Under LD30, the M-peak is significantly advanced in w^{1118} flies compared to their phase of M-peak under LD21, LD25 and LD27 (Figure 2.1A, black arrow; Figure 2.1B, top panel, one-way ANOVA with regime as factor, $F_{3,105} = 61.1$, $p < 0.05$). Onset of evening activity (E-onset) is delayed under the warmer LD30 condition compared to the cooler LD21, LD25 and LD27 regimes (Figure 2.1A, grey arrow; Figure 2.1B, bottom panel, one-way ANOVA with regime as factor, $F_{3,103} = 128.7$, $p < 0.05$). "Strong loss-of-function" mutant $dTRPA1^{ins}$ flies (Hamada et al., 2008; Head et al., 2015) also exhibit bimodal activity patterns, however, they exhibit no difference in phasing of M-peak or E-onset under LD30

(Table 2.1; Figure 2.1A, right panel; one way ANOVA with regime as factor, $F_{3,90} = 1.98$, $p = 0.12$). Mutant *dTRPA1^{ins}* flies, unlike controls, are unable to shift their M-peak into pre-dawn under warm LD30 conditions (Figure 2.1B, top panel; two-way ANOVA with genotype and regime as factors, $F_{3,195} = 20.4$, $p < 0.05$). Although *dTRPA1^{ins}* flies show delayed phase of E-onset compared to *w^{1118}* under cooler LD21, LD25 and LD27 (Table 2.2; Figure 2.1B), they do not show a further delay under the warm LD30, and in contrast, show a significantly advanced E-onset compared to *w^{1118}* flies (Table 2.2; Figure 2.1B, bottom panel; two way ANOVA with genotype and regime as factors, $F_{3,201} = 44.9$, $p < 0.05$). Wild-type flies delay their E-onset by ~2-hr at LD30 compared to LD25 (Figure 2.1B, bottom panel) and lack of dTRPA1 renders flies incapable of delaying E activity under warm conditions of LD30. The wild-type responses at high temperature for LD30 are consistent with an adaptive response of flies to avoid activity during the day when temperatures are generally highest, which may account for light modulation of the temperature response. My studies on the null mutants reveal that dTRPA1 provides temperature sensory input critical for flies to appropriately phase their activity peaks around the light/dark/light transitions when ambient temperature is warm.

Electrical properties of dTRPA1 neurons influences temperature-dependent modulation of morning and evening activity bouts under LD cycles. dTRPA1 null mutants show wild-type behaviour under LD cycles at cooler temperatures. It is likely that under cool conditions such as LD21, dedicated thermosensors for cool temperature may override the activation of dTRPA1-expressing neurons. I tested this by constitutive expression of a bacterial sodium ion channel (*UAS-NaChBac1*) in a subset of dTRPA1 neurons targeted by *dTRPA1^{SH}-GAL4* driver, henceforth dTRPA1^{SH+} neurons (Figure 2.2A, orange curve). Expression of NaChBac1 depolarises the neurons thus, making dTRPA1^{SH+} neurons fire even at temperatures below the activation threshold of warmth-activated dTRPA1 ion channel.

Subjecting flies expressing *UAS-NaChBac1* in dTRPA1^{SH+} neurons (*dTRPA1^{hyperexcited}* flies) to cool LD21 does not significantly affect activity patterns (Figure 2.2A). Interestingly at both LD25 and LD27, hyperexcitation causes an advancement of M-peak compared to controls (Table 2.1; Figure 2.2A; one-way ANOVA with genotype as factor; LD25- $F_{2,48}$ =13.13; LD27- $F_{2,53}$ =44; $p < 0.05$). For *dTRPA1^{hyperexcited}* flies (orange curves), pre-dawn activity appears to be broader under LD27 than under LD25 (Figure 2.2A).

Warm temperatures can activate thermosensitive dTRPA1 ion channels which in turn mediate firing of dTRPA1-expressing neurons. Hence, overexpression of dTRPA1 via *UAS-dTRPA1* in dTRPA1^{SH+} neurons will also lead to acute activation of these neurons when exposed to warm temperature conditions. I over-expressed dTRPA1 channels using *dTRPA1^{SH}-GAL4* driver (henceforth, *dTRPA1^{oex}*) (Figure 2.2B, dark red curve) and find that it does not significantly alter activity profiles under cool LD21, which is below the threshold temperature for dTRPA1 activation (Figure 2.2B; Kolmogorov-Smirnov test, $p > 0.1$). However, under warmer temperatures of both LD25 and LD27, *dTRPA1^{oex}* flies show a significant advance in the M-peak phase compared to both parental controls (Table 2.1; Figure 2.2B; one-way ANOVA with genotype as factor; LD25: $F_{2,68} = 13.96$; LD27: $F_{2,71} = 35.97$; $p < 0.05$). Thus, artificially enhancing firing of dTRPA1^{SH+} neurons (*dTRPA1^{oex}* or *dTRPA1^{hyperexcited}*) at ambient temperatures of LD25 is sufficient to advance morning activity. This resembles *w^{1118}* at warmer conditions of LD30 showing that these neurons can modulate activity peaks.

On the other hand, electrical silencing dTRPA1^{SH+} neurons via expression of inward rectifier potassium channel, *UAS-Kir2.1* does not affect bimodal activity/rest profiles of flies (*dTRPA1^{silenced}* flies) under different LD cycles – LD21, LD25 and LD27 (Figure 2.3A). Further, dTRPA1 expression levels were downregulated in dTRPA1^{SH+} neurons by

expressing *UAS-dTRPA1*^{RNAi} in combination with *UAS-dcr-2* to enhance the effect of RNAi (Figure 2.3B). RNAi knockdown of dTRPA1 also did not alter activity/rest rhythm of flies under LD cycles tested (Figure 2.3B). Taken together, activation of dTRPA1^{SH+} neurons at lower ambient temperatures of 25 and 27 ^{o}C results in a phenotype similar to response of wild-type flies to warm LD30 conditions indicating that firing properties of these neurons are crucial for flies to modify their behaviour in response to external temperature conditions. However, silencing dTRPA1^{SH+} neurons or reducing dTRPA1 expression levels in these neurons at ambient temperatures do not alter behavioural locomotor rhythms in flies because thermal signals from other dTRPA1-expressing neurons (outside the target of *dTRPA1*^{SH}-*GAL4*) may compensate for lack of signals from dTRPA1^{SH+} neurons.

dTRPA1 is needed for suppression of mid-day activity under temperature cycles in absence of other time cues. Since dTRPA1 encode temperature-sensitive ion channels, I wanted to examine the role of these receptors in temperature entrainment of flies. For this, I subjected *dTRPA1* null mutant lines to 12:12-hr temperature cycles (thermophase: cryophase - 29:21^{o}C under constant darkness or DD, henceforth, DD/TC). To downregulate dTRPA1 levels in a cell-specific manner, I examined two *dTRPA1*^{RNAi} lines expressed under the control of the *dTRPA1*^{SH}*-GAL4* driver, which yielded similar results. Therefore, my results depict only one of them *UAS-dTRPA1T*$_2$^{RNAi} -henceforth, *dTRPA1*^{RNAi}. Flies were subjected to DD/TC for 10-days to enable stable entrainment before imposing a 10-hr phase-delayed DD/TC for 13-days before finally releasing the flies into constant dark conditions at 21 ^{o}C (DD21) (Figure 2.4A). Under temperature cycles-DD/TC, wild-type (*w*^{1118}) flies exhibit bimodal pattern of activity with relative inactivity or siesta in the middle of the warm phase (Figures 2.4A, top panel and 2.5A) whereas null mutants *dTRPA1*^{ins} flies remain active throughout the warm phase of temperature cycles which is clearer before the phase shift in TC cycles (Figures 2.4A, red arrow). Enhanced activity of *dTRPA1*^{ins} and *dTRPA1*^{RNAi} flies during the warm phase

continued even after the 10-hr phase-delay in TC cycles, although some separation between "morning" and "evening" bouts of activity was apparent during the last few days of the shifted cycles (Figures 2.4A). Similar to dTRPA1 null flies, the behavioural afternoon siesta is almost lost in *dTRPA1*^{RNAi} flies driven by *dTRPA1*^{SH}*-GAL4*, which remain active throughout the thermophase (Figure 2.4A, red arrow) in contrast to their parental control flies which resemble w^{1118} flies and exhibit clear siesta during mid-thermophase (Figure 2.4A). Evening offset was used as a phase marker to determine number of transient cycles taken by a given genotype to re-entrain to phase-shifted temperature cycles. The number of transient cycles required for re-entrainment to phase-shifted temperature cycles by *dTRPA1*^{ins} flies is not significantly higher than w^{1118} flies (Figure 2.4B, compare red bars and black bars). Similarly, transient cycles taken by *dTRPA1*^{RNAi} flies were not higher than their parental controls (Figure 2.4B). Similar results were obtained for phase advanced shifts (data not shown) suggesting that signalling via dTRPA1 does not influence the rate of re-entrainment to phase-shifted thermal cycles. My results are bolstered by a recent report that also show that dTRPA1 is not required for circadian entrainment to temperature cycles (Roessingh et al., 2015). Endogenous periodicity of *dTRPA1*^{ins} and *dTRPA1*^{RNAi} flies are also not altered (Table 2.3) indicating that lack of dTRPA1 signals does not affect the circadian clock period.

When the average activity/rest profiles of flies under DD/TC (pre-shift) were compared I find that, as seen in the actograms, wild-type (w^{1118} – black curve) flies exhibit bimodal activity/rest pattern whereas *dTRPA1*^{ins} flies (red curve) exhibit significantly higher activity during mid-thermophase (Figures 2.5A, arrow). The above results have been corroborated by comparing the behaviour of another null mutant, *TRPA1*$^{KI\text{-}GAL4}$ (Figure 2.5A, orange curve). Both null mutants - *dTRPA1*^{ins} and *TRPA1*$^{KI\text{-}GAL4}$ show greater activity compared to w^{1118} control flies during ZT02-08 (mid-thermophase) (Figure 2.5B, two-way

ANOVA, $F_{22,804} = 29.1$; $p < 0.05$; significant differences from w^{1118} within each time interval are indicated by asterisks).

In order to evaluate detrimental effects on dTRPA1 null flies which might be due to enhanced mid-thermophase activity, I also examined the lifespan of male flies under identical temperature cycles in constant darkness (Figure 2.5C). I find that after first 20 days, the survival curve of $dTRPA1^{ins}$ flies (red) fall more steeply than w^{1118} (black) control flies (Figure 2.5C). Null mutant $dTRPA1^{ins}$ flies have a significantly shorter average lifespan of 39.7 days compared to w^{1118} flies that have an average lifespan of 49.95 days (t-test on independent samples, $p < 0.001$). These results suggests that inability of dTRPA1 null flies not to suppress activity during middle of the thermophase under DD/TC is detrimental to their survival as these null mutant flies have a reduced average lifespan than the controls. However, dTRPA1 may influence other physiological processes besides thermosensation driving activity/rest rhythms, hence, one cannot rule out that shortened lifespan of dTRPA1 null flies could be due to a combination of lack of functional dTRPA1 in multiple temperature-dependent and independent pathways.

Downregulating dTRPA1 levels in subset of dTRPA1 neurons renders flies active during mid-thermophase under DD/TC. Similar to dTRPA1 null flies, when dTRPA1 expression is downregulated in neurons targeted by $dTRPA1^{SH}$-*GAL4* (henceforth, $dTRPA1^{RNAi}$ flies), the behavioural afternoon siesta is almost lost and these flies remain active throughout the mid-thermophase (Figure 2.4A- actogram, Figure 2.6A, left panel-blue curve). $dTRPA1^{RNAi}$ flies exhibit higher activity than controls between ZT02-08 (mid-thermophase) under DD/TC (Figure 2.6B, left panel, two-way ANOVA, $F_{33,912} = 13.0$, $p < 0.05$). During the cryophase (below the threshold activation temperature of dTRPA1) there is no detectable difference in activity levels between experimental and control genotypes (Figure 2.6A, left panel).

The loss of siesta phenotype in flies with RNAi knock down of dTRPA1 expression in dTRPA1-expressing neurons has been verified with two more *GAL4* drivers. *TRPA1*$^{KI\text{-}GAL4}$ flies can be used to drive expression of genes under *GAL4* inserted at the translational initiation codon (Kim et al., 2010). RNAi knockdown of dTRPA1 driven in heterozygous null background using *TRPA1*$^{KI\text{-}GAL4}$ /+ flies also leads to enhanced mid-thermophase activity (Figure 2.6A, right panel, blue curve), similar to homozygous *TRPA1*$^{KI\text{-}GAL4}$ null flies (Figure 2.6A, right panel, orange curve). These flies display enhanced mid-thermophase activity which is significantly higher compared to parental controls, between time points ZT4-ZT8 (Figure 2.6B, right panel, two-way ANOVA, $F_{33,984} = 7$, $p < 0.05$). dTRPA1 expression has been knocked down using another driver, *dTRPA1-GAL4*^{48951} (Green et al., 2015). This driver targets a very small number of neurons (Figure 2.7A) and does not include AC neurons in most cases (7/11 brains). RNAi knockdown in these neurons does not result in a loss of siesta (Figure 2.7B), rather RNAi flies show lower activity levels than *GAL4/+* parental controls between ZT0-6 but activity levels are not different from *UAS/+* controls (Figure 2.7B, right panel). Taken together, my results suggest that dTRPA1 in AC neurons is crucial for mediating the siesta phenotype under rectangular temperature cycles. Thus, RNAi knockdown in dTRPA1-expressing neurons - including the "internal thermosensors" AC neurons, targeted either by restricted *dTRPA1*^{SH}*-GAL4* or the broad *TRPA1*$^{KI\text{-}GAL4}$ /+ drivers, phenocopies the loss of mid-day behavioural suppression seen in the *dTRPA1* null mutant flies, suggesting that inhibitory signals from dTRPA1-positive neurons enables suppression of mid-day activity.

Next, I also enhanced the expression of dTRPA1 in dTRPA1^{SH+} neurons and subjected these flies also to DD/TC. Flies driving *UAS-dTRPA1* expression under *dTRPA1*^{SH}*-GAL4* will be henceforth referred to as *dTRPA1*^{oex}. Not surprisingly, my results show that *dTRPA1*^{oex} flies exhibit bimodal activity/rest pattern under DD/TC and have similar

activity levels during mid-thermophase compared to both parental controls (Figures 2.8A, 2.8B). *dTRPA1*^{oex} flies differ from *dTRPA1*^{ins} null flies during mid-day ZT02-06 (Figure 2.8B, right panel; two-way ANOVA, $F_{33,1358} = 25.1$, $p < 0.05$). Altering dTRPA1 expression, specifically in dTRPA1^{SH+} neurons, does not affect endogenous periodicity of activity/rest rhythm when released into DD21 following thermal cycles (Table 2.3) suggesting that dTRPA1 mediated changes in neuronal activity has no effect on circadian clock period. However, alteration of dTRPA1 channel signalling disrupts the normal siesta; thus, causing flies to be maladaptively active at mid-day when temperatures are ordinarily highest and flies are at higher risk for desiccation.

***dTRPA1 expression in a small number of dTRPA1^{SH+} brain cells is both necessary and sufficient to rescue mid-day inhibition of activity under thermal cycles*.** To verify that dTRPA1^{SH+} neurons modulate mid-day activity, I ablated these neurons by driving ectopic expression of *UAS-hid* under *dTRPA1*^{SH}*-GAL4* driver (henceforth *dTRPA1*^{ablated}) and subjected flies to DD/TC. *dTRPA1*^{ablated} flies exhibit weakened siesta especially on initial exposure to DD/TC (Figure 2.9A, left actogram marked by yellow asterisk), although a more normal siesta behaviour becomes apparent after ~ 5 days (Figure 2.9A, left actograms). Thus, *dTRPA1*^{ablated} flies (blue curve) initially phenocopy the null *dTRPA1*^{ins} flies (red curve) (Figure 2.9B) and knockdown *dTRPA1*^{RNAi} flies (compare with Figures 2.6A, 2.6B). Between ZT04-08, *dTRPA1*^{ablated} flies exhibit significantly higher activity compared to controls (Figure 2.9C; two-way ANOVA with genotype and time points as factors, $F_{33,1284} = 25.34$, $p < 0.05$). Thus, my results demonstrate that dTRPA1^{SH+} neurons are necessary for inhibition of mid-day activity. However, during the latter part of TC cycles, *dTRPA1*^{ablated} flies show suppression of activity during siesta (Figure 2.9, left actograms) which may be due to signaling from dTRPA1 neurons outside the target of *dTRPA1*^{SH}*-GAL4* that compensate for lack of inputs from dTRPA1^{SH+} neurons. Alternatively, other thermosensors that are

activated in temperature ranges overlapping with or proximal to dTRPA1 activation temperature may override lack of signals from dTRPA1^{SH+} neurons over time thus, enabling *dTRPA1^{ablated}* flies to display siesta after a few days in DD/TC regime.

To examine whether dTRPA1 expression only in the small number of dTRPA1^{SH+} neurons is sufficient to inhibit mid-day activity under DD/TC, dTRPA1 expression was restored in the dTRPA1^{SH+} neurons in the *dTRPA1^{ins}* null background (*dTRPA1^{rescue}*). This genetic manipulation restores the mid-day siesta. *dTRPA1^{rescue}* flies exhibit wild-type behaviour as shown by clear suppression of mid-day activity in 41 out of 42 flies tested (Figures 2.10A, left actogram; Figure 2.10B - green curve) in contrast to genetic background control flies bearing the null mutation which fail to suppress their mid-day activity efficiently (*dTRPA1^{SH}-GAL4*; *dTRPA1^{ins}* - Figure 2.10A, middle actograms, Figure 2.10B, grey curve, and *UAS-dTRPA1; dTRPA1^{ins}* – Figure 2.10A, right actograms, Figure 2.10B, black dashed curve). However, *dTRPA1^{rescue}* flies also exhibit significantly enhanced nocturnal activity and slightly advanced and smaller morning peak (Figure 2.10A, actograms and Figure 2.10B, green curve) which partially resembles the high temperature response seen in wild-type flies under LD30 (Figure 2.1A), not seen in either of the background controls (Figures 2.10A, 2.10B). The increase in late-cryophase (ZT19-23) activity for *dTRPA1^{rescue}* flies could be partly attributed to the effect of over-expression of dTRPA1 using the *dTRPA1^{SH}-GAL4* driver (compare with Figure 2.8B, blue curve) although this does not explain the apparent temperature hyper-responsive increased early-cryophase activity. Interestingly, enhanced nocturnal activity during late cryophase is restricted to expression of *UAS-dTRPA1* by *dTRPA1^{SH}-GAL4* driver in a null background and does not occur when expressed in a wild-type background (see Figure 2.8B, blue curve).

There are four known isoforms of dTRPA1, out of which two are temperature-sensitive; isoform A and D (Zhong et al., 2012). To determine the molecular specificity for suppression of mid-thermophase activity, I tested expression of either of the thermosensitive isoforms of dTRPA1 (Zhong et al., 2012), *UAS-dTRPA1-A* (activated at 24-29 ^{o}C) or *UAS-dTRPA1-D* (activated at 34 ^{o}C) in $dTRPA1^{ins}$ null background (Figure 2.11A, green curves). Expression of *UAS-dTRPA1-A* in dTRPA1^{SH+} neurons in a $dTRPA1^{ins}$ null background results in suppression of mid-thermophase activity (Figure 2.11A, left panel, green curve). Isoform A driven rescue flies show lower activity levels between ZT0-8 compared to *GAL4/+* control flies in null background and between ZT0-2 and ZT6-8 compared to *UAS/+* control flies in null background respectively (Figure 2.11A, left panel, two-way ANOVA with genotype and time points as factors, Tukey's HSD, $F_{22,696} = 15.06$, $p < 0.05$). The suppression of mid-day activity seen in $UAS\text{-}dTRPA1\text{-}A;dTRPA1^{ins}$ parental control in null background between ZT 2-4 could be attributed to leaky expression of *UAS-dTRPA1-A* transgene (Figure 2.11A, left panel, black dashed curve). Since activation of the D-isoform is expected maximally at much warmer temperatures, it is surprising that *UAS-dTRPA1-D* expression could also induce inhibition of mid-day activity at constant temperatures of 29 ^{o}C (Figure 2.11A, right panel, green curve). Flies with isoform D exhibit significantly lower activity levels between ZT0-8 compared to both parental controls in null background (Figure 2.11A, right panel, two-way ANOVA with genotype and time points as factors, Tukey's HSD, $F_{22,936} = 45.32$, $p < 0.05$). Interestingly, such behavioural rescue in a dTRPA1 null background was reported previously even with the expression of temperature *insensitive* isoform, TRPA1-B (Lee and Montell, 2013). My results confirm that dTRPA1 expression in a small number of dTRPA1^{SH+} brain neurons is critical for inhibition of activity under thermal cycles and either of two temperature-sensitive isoforms can rescue the phenotype of dTRPA1 null flies.

Under temperature cycles with constant light, dTRPA1 influences both morning and evening peaks of activity. Constant light (LL) is known to disrupt circadian clocks (Konopka et al., 1989) but temperature cycles can entrain the circadian clock in LL (Glaser and Stanewsky, 2005; Sehadova et al., 2009). Although *dTRPA1*^{null} flies are unable to suppress mid-day activity in absence of light (DD/TC- Figures 2.4 and 2.5), this defect is not seen under LD (Figure 2.1). I wanted to examine if continuous presence of light could modify this behaviour under TC. For this, flies were subjected to temperature cycles (12:12-hr -29:21 ^{o}C) under LL of 0.1, 1, 10 or 100 lux intensity. Both *w*^{1118} and *dTRPA1*^{ins} flies synchronise their activity/rest rhythm to LL/TC (Figures 2.12A, B) and suppress afternoon activity in contrast to DD/TC. Interestingly, *dTRPA1*^{ins} flies (red) exhibit a reduced morning (M) peak amplitude (two-way ANOVA, with genotype and light intensity as factors, $F_{3, 213} = 0.7$) and a ~ 2-hr advanced evening peak compared to *w*^{1118} (black) under LL/TC across all light intensities tested (Figure 2.12C; two-way ANOVA, $F_{3, 204} = 5.12$, $p < 0.05$). However, restoring dTRPA1 only in dTRPA1^{SH+} neurons in *dTRPA1*^{ins} null background is sufficient to rescue M-peak amplitude and phasing of E-peak under LL/TC at 100 lux light intensity (Figure 2.12D). E-peak occurs after ZT12 in *dTRPA1*^{rescue} flies whereas both parental controls in null background exhibit reduced M-peak (one-way ANOVA, $F_{2,75} = 20.5$, $p < 0.05$) and an advanced E-peak under LL/TC (Figure 2.12D, right panel, one-way ANOVA, $F_{2,75} = 95.8$, $p < 0.05$). Thus, dTRPA1 expression in dTRPA1^{SH+} neurons is needed to appropriately phase morning and evening bouts of activity under LL/TC. Taken together, my study suggests that thermosensitive dTRPA1 ion channels can influence distinct behaviours depending on external environmental conditions.

Discussion

The role of dTRPA1 in thermosensation in *Drosophila* has been extensively studied in the past decade (Barbagallo and Garrity, 2015) but the role of dTRPA1 in conveying thermal inputs to circadian clocks has been suggested only recently (Lee and Montell, 2013). Their study did not conclusively identify dTRPA1 as a circadian thermosensor and left open the possibility of other unidentified thermosensors. My study differs from (Lee and Montell, 2013) in several aspects, most importantly in their choice of asymmetric 18:6hr cycles with 29:18 ^{o}C TC cycles. In my studies, I avoided using such a low cryophase temperature based on my previous observation that 18 ^{o}C greatly suppresses activity, contributes to masking of activity rhythms and an exaggerated startle to the morning transition rather than a gradual anticipation. While they inferred the role of dTRPA1 in entrainment based on the behaviour of flies when shifted from symmetric LD cycles to an asymmetric TC, I used symmetric 12:12hr, 29:21 ^{o}C TC cycles to examine the ability of flies to shift between advanced or delayed TC cycles and did not detect any significant difference in number of transient cycles. Prior to phase shifts, I ensured that flies have entrained to the TC regime with a stable phase of entrainment and a period of 24±0.5h. Hence, I propose that the observed defects in entrainment reported by the previous study may be due to after-effects of LD to TC rather than a difference in circadian entrainment to thermal cycles. Furthermore, it appears that the long-day regime does not entrain even the wild-type flies, as all genotypes appear to free run even in the presence of the TC cycles (Lee and Montell, 2013), Fig.1D-F), also see footnote in (Maguire and Sehgal, 2015).

My studies support the inference that when gated temperature cycles are the only time cues available, inhibitory signals due to dTRPA1 in a subset of neurons (including AC neurons) is both necessary and sufficient to prevent excessive locomotor activity during the

thermophase. I hypothesise that these neurons are the temperature sensitive cells (TS cells) proposed by a previous study to be a set of neurons distinct from the canonical circadian subsets which inhibit activity in the middle of the warm phase under temperature cycles (Busza et al., 2007). My results also depict that the lack of siesta phenotype of dTRPA1 null mutants under temperature cycles can be rescued by expressing either of the two temperature-responsive isoforms of dTRPA1, isoform A or D. These results have been substantiated by another study which also reported the involvement of dTRPA1 in mediating siesta under 20:29 ^{o}C temperature cycles and attributed it to reduced sleep during siesta (Roessingh et al., 2015). However, when dTRPA1 expression is rescued in *dTRPA1*^{ins} background by driving *UAS-dTRPA1* under *dTRPA1*^{SH}*-GAL4* driver, *dTRPA1*^{rescue} flies exhibit an additional feature-high nocturnal and pre-dawn activity. As mentioned earlier, enhanced pre-dawn activity could be contributed by overexpression of dTRPA1 since *dTRPA1*^{oex} flies in a wild-type background also exhibit enhanced pre-dawn activity under identical DD/TC. However, at this time, I do not have an explanation for the delay in the evening peak and enhanced activity during cryophase seen only when expressed in a null background and not in a wild-type background. Based on my results, I posit that a combination of the absence of dTRPA1 in some cells accompanied with overexpression in dTRPA1^{SH+} cells can cause enhanced nocturnal activity in *dTRPA1*^{rescue} flies.

I also attempted to study the behaviour of female flies under temperature cycles in DD but their behavioural patterns have been inconsistent. Out of three independent experiments, only in one instance *dTRPA1*^{ins} female flies resembled their male counterparts and exhibited higher mid-thermophase activity than the female *w*^{1118} flies. In another instance, mid-thermophase activity levels of female *dTRPA1*^{ins} flies were lower than female *w*^{1118} flies whereas in the third instance the activity levels of these two genotypes were not different during mid-thermophase. Future studies that examine the behavioural activity/ rest rhythms

of female flies in more details would be interesting and help us understand if temperature driven behavioural responses are conserved across sexes in flies.

My studies demonstrate that dTRPA1 modulates different aspects of the activity/rest rhythm under LD cycles when temperatures are constantly high and in the range that can activate dTRPA1 signalling. I posit that *dTRPA1*^{ins} flies are unable to sense extreme warm temperatures due to lack of signalling from dTRPA1^{SH+} neurons and therefore are unable to re-distribute their activity into the dark phase of LD30. Inducing electrical activity of dTRPA1^{SH+} neurons (via *UAS-NaChBac* or *UAS-dTRPA1*) advances the phase of M-peak under LD in a temperature-dependent manner suggesting that signals from dTRPA1^{SH+} neurons in the wild-type flies can shift the M-peak under warm ambient temperatures. As electrical hyperexcitation of the dTRPA1^{SH+} neurons at LD21 (Figure 2.2, right panel) does not modify the morning and evening peaks, it appears that at cool temperatures other thermoreceptors participate in modulation of activity patterns, while at moderate or warm temperatures, activation of dTRPA1 neurons has dominant effects on this behaviour such that it mimics the sensation of constant warm conditions.

Immunolabelling studies (described in Chapter 4 of this thesis) show that distal processes of AC neurons partially overlap with dorsal terminals of s-LN$_v$ neurons. Combining my understanding of dTRPA1 circuits from behavioural and anatomical studies I propose that AC neurons are likely candidates to send thermosensory signals to higher centres in the fly brain, thus eliciting temperature-dependent behaviour in flies.

In conclusion, I observe that light influences dTRPA1-dependent behavioural phenotypes. Under LD, when temperatures are in the preferred range (< 29 ^{o}C), dTRPA1 null mutants are able to exhibit bimodal activity/rest patterns like controls and under warm temperatures show phasing defects in M and E activity bouts. Under LD cycles with ambient

temperatures, *dTRPA1*^{ins} flies exhibit bimodal activity/rest pattern with prominent siesta during the afternoon similar to wild-type flies. This is in contrast to their lack of siesta phenotype under temperature cycles in constant darkness (DD/TC). Under DD/TC, the M and E peaks are not significantly affected in the null mutants, whereas null mutants are unable to suppress mid-day activity. Further, under LL/TC, the same mutants are able to suppress mid-day activity but show abnormal phasing of E-peak. In summary, presence of light suppresses afternoon activity resulting in siesta both in LD and LL/TC regime, however, in absence of light, in DD/TC, flies lacking dTRPA1 do not display siesta. Taken together, these results indicate that warmth mediated signalling in the dTRPA1 expressing cells interacts with downstream circadian and motor circuits in a light-dependent manner via yet unknown mechanisms. Thus, my results suggest a crucial role for dTRPA1 receptor mediated signalling primarily in dTRPA1^{SH+} neurons in modifying different aspects of rhythmic behaviour of flies in a temperature-dependent manner.

Table 2.1

Temperature (^{o}C)	Genotype	N	M_{peak} ± S.E.M (hr)
21	***dTRPA1^{SH}-GAL4/+***	32	0.516 ± 0.18
	dTRPA1^{SH}-GAL4/UAS NaChBac1	13	1.0 ± 0.21
	UAS NaChBac1/+	12	0.292 ± 0.25
	dTRPA1^{SH}-GAL4/UAS dTRPA1	28	0.143 ± 0.10
	UAS dTRPA1/+	22	0.511 ± 0.22
	dTRPA1^{SH}-GAL4/UAS dTRPA1^{RNAi}	26	0.442 ± 0.16
	UAS dTRPA1^{RNAi} /+	16	0.312 ± 0.21
	w^{1118}	27	-0.130 ± 0.06
	dTRPA1^{ins}	30	0.108 ± 0.13
25	***dTRPA1^{SH}-GAL4/+***	25	0.09 ± 0.07
	dTRPA1^{SH}-GAL4/UAS NaChBac1	15	0.833 ± 0.22 *
	UAS NaChBac1/+	11	-0.136 ± 0.04
	dTRPA1^{SH}-GAL4/UAS dTRPA1	29	0.966 ± 0.15 *
	UAS dTRPA1/+	17	0.515 ± 0.12
	dTRPA1^{SH}-GAL4/ UAS dTRPA1^{RNAi}	24	0.729 ± 0.21
	UAS dTRPA1^{RNAi} /+	19	0.645 ± 0.18
	w^{1118}	24	0.03 ± 0.04
	dTRPA1^{ins}	20	0.525 ± 0.24
27	***dTRPA1^{SH}-GAL4/+***	31	-0.073 ± 0.07
	dTRPA1^{SH}-GAL4/UAS NaChBac1	14	1.731 ± 0.18 *
	UAS NaChBac1/+	11	0.568 ± 0.28
	dTRPA1^{SH}-GAL4/UAS dTRPA1	28	1.152 ± 0.12 *
	UAS dTRPA1/+	15	0.683 ± 0.19
	dTRPA1^{SH}-GAL4/ UAS dTRPA1^{RNAi}	26	0.577 ± 0.17
	UAS dTRPA1^{RNAi} /+	19	0.434 ± 0.16
	w^{1118}	31	0.121 ± 0.10
	dTRPA1^{ins}	22	0.534 ± 0.14
30	***w^{1118}***	27	1.44 ± 0.13 *
	dTRPA1^{ins}	25	0.215 ± 0.09

Table 2.1: Phase of morning peak under LD cycles across different ambient temperatures. Phase of morning peak was manually calculated for individual flies by subtracting the timing of morning peak of activity for a given fly from time of Lights ON (ZT0). Positive phase values indicate M-peak occurring before Lights ON and negative value indicate M-peak occurring after Lights-ON. Asterisks indicate statistically significant differences in phase of experimental genotypes from both parental controls within a temperature regime whereas *dTRPA1^{ins}* flies have been compared to *w^{1118}* flies (one-way ANOVA, Tukey's HSD, $p < 0.05$). S.E.M = Standard Error of Mean.

Table 2.2

Temperature (^{o}C)	Genotype	N	E_{onset} ± S.E.M (hr)
21	w^{1118}	27	3.14 ± 0.14
	$dTRPA1^{ins}$	30	1.675 ± 0.10 *
25	w^{1118}	23	2.90 ± 0.13
	$dTRPA1^{ins}$	23	1.24 ± 0.11 *
27	w^{1118}	30	1.76 ± 0.09
	$dTRPA1^{ins}$	24	0.85 ± 0.10 *
30	w^{1118}	27	0.54 ± 0.05
	$dTRPA1^{ins}$	25	1.02 ± 0.06

Table 2.2: Phase of onset of evening activity onset under (12:12) LD cycles across different ambient temperatures. Phase of evening onset was determined by taking the difference between timing of evening onset of activity for individual flies and Lights OFF (ZT12) and mean phase values obtained by averaging values across flies. Positive phase values indicate E-onset occurring before Lights OFF and negative value indicate E-onset occurring after Lights-OFF. Asterisks indicate significant differences in mean phase between the two genotypes (two-way ANOVA, Tukey's HSD, $p < 0.05$). S.E.M = Standard Error of Mean.

Table 2.3

Genotype	TC (29:21 °C)		DD 21 °C			
	N	**% Entrained**	***N***	**% Rhythmic**	**Period ± SEM**	**Power ± SEM**
w^{1118}	28	100	26	81	23.56 ± 0.04	321.94 ± 19.74
$dTRPA1^{ins}$	30	97	23	74	23.74 ± 0.08	200.60 ± 11.42
UAS-Dcr /+;UAS- $dTRPA1^{RNAi}$*/+*	31	100	14	71.4	23.57 ± 0.05	223.42 ± 18.76
$dTRPA1^{SH}$*-GAL4/UAS-Dcr ;UAS-*$dTRPA1^{RNAi}$*/+*	28	100	27	66.7	23.65 ± 0.05	192.03 ± 8.47
$dTRPA1^{SH}$*-GAL4/ UAS-dTRPA1*	32	100	31	90.3	23.38 ± 0.06	317.75 ± 9.78
UAS-dTRPA1 /+	32	100	32	93.8	23.38 ± 0.03	233.97 ± 11.30
$dTRPA1^{SH}$*-GAL4/ UAS-Dcr*	32	100	26	46.1	23.77 ± 0.08	180.23 ± 18.14
$dTRPA1^{SH}$*-GAL4/+*	30	100	22	72.7	23.58 ± 0.05	177.38 ± 10.91

Table 2.3: Percentage of entrained flies under TC cycles in DD and free running period values and measure of robustness of rhythm under DD at 21 °C. To determine percentage of entrained flies, periodicity of individual flies were determined by chi-square periodogram analysis across nine-ten days of DD/TC cycles. Flies with periodicity of 24 ± 0.5hr under TC cycles were considered to be entrained. For determining free running period of genotypes, the first day of DD was omitted from analysis. Chi-square analysis was performed on at least seven days of activity under DD.

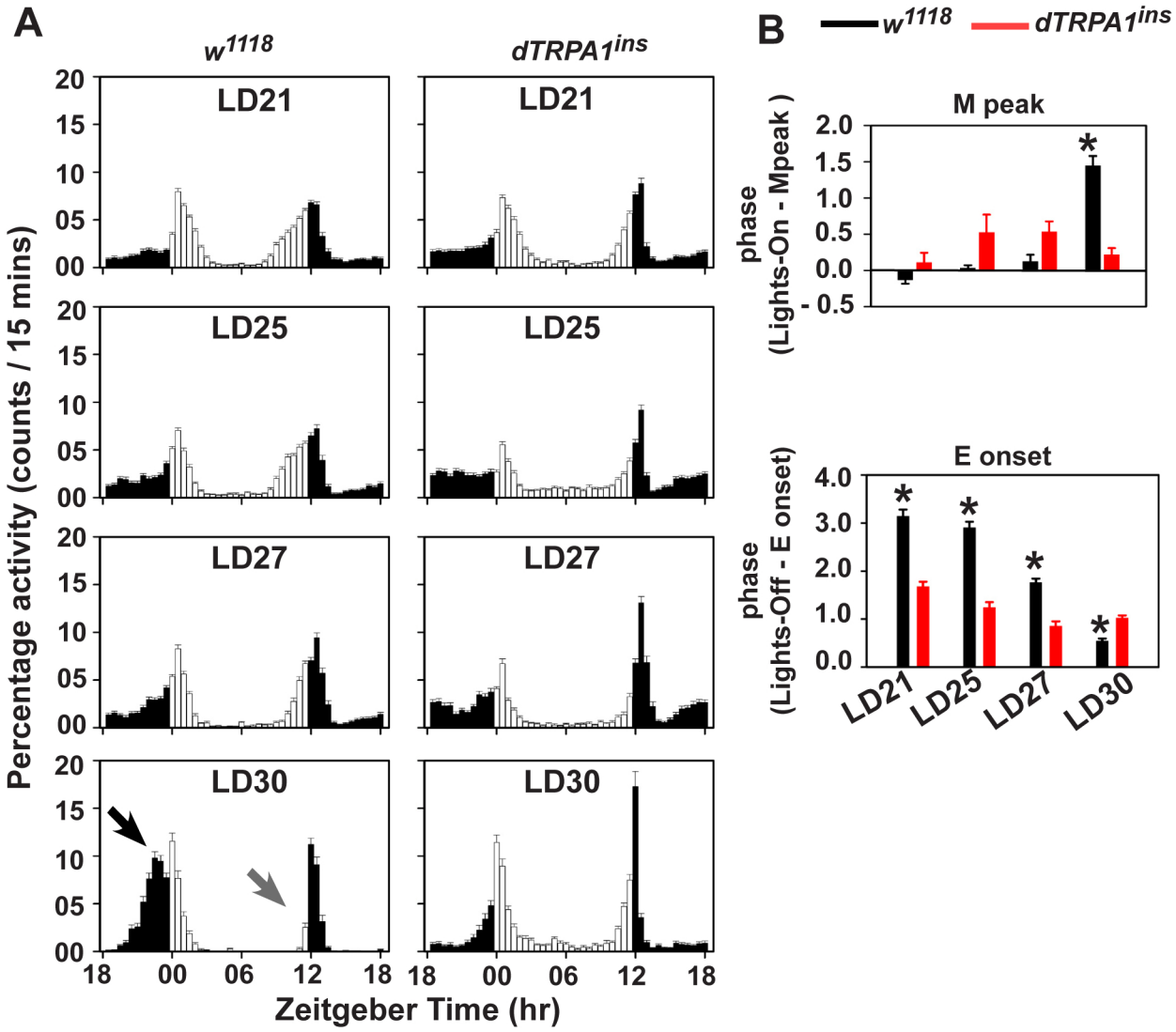

Figure 2.1. dTRPA1 neurons modulate temperature-dependent phasing of morning and evening activity under LD. (A) Wild-type (w^{1118}) and $dTRPA1^{ins}$ flies display rhythmic activity under LD cycles, white bars represent activity in light and black bars in darkness. Under LD30, w^{1118} flies advance their morning (M) peak (black arrow) compared to other regimes- LD21, LD25 and LD27 whereas $dTRPA1^{ins}$ flies do not alter the phase of M peak across LD regimes. Under LD30, w^{1118} flies also delay their evening onset (grey arrow) compared to LD21, LD25 or LD27 regimes but $dTRPA1^{ins}$ flies do not. (B) Phase of M-peak (top) and onset of evening activity (bottom) compared between w^{1118} and $dTRPA1^{ins}$ flies across LD regimes. M-peak of w^{1118} flies is significantly advanced at LD30 compared to phase of their M-peak at lower temperatures and is also different from M-peak phase of $dTRPA1^{ins}$ at LD30. Phase of E-onset in w^{1118} flies is increasingly delayed such that LD21 = LD25 < LD27 < LD30. Under LD21, LD25 and LD27, phase of E-onset is advanced in w^{1118}compared to $dTRPA1^{ins}$ except at LD30, wherein w^{1118} have a delayed phase of E-onset in contrast to $dTRPA1^{ins}$. Asterisks indicate significant differences among genotypes in a given regime. Error bars are SEM.

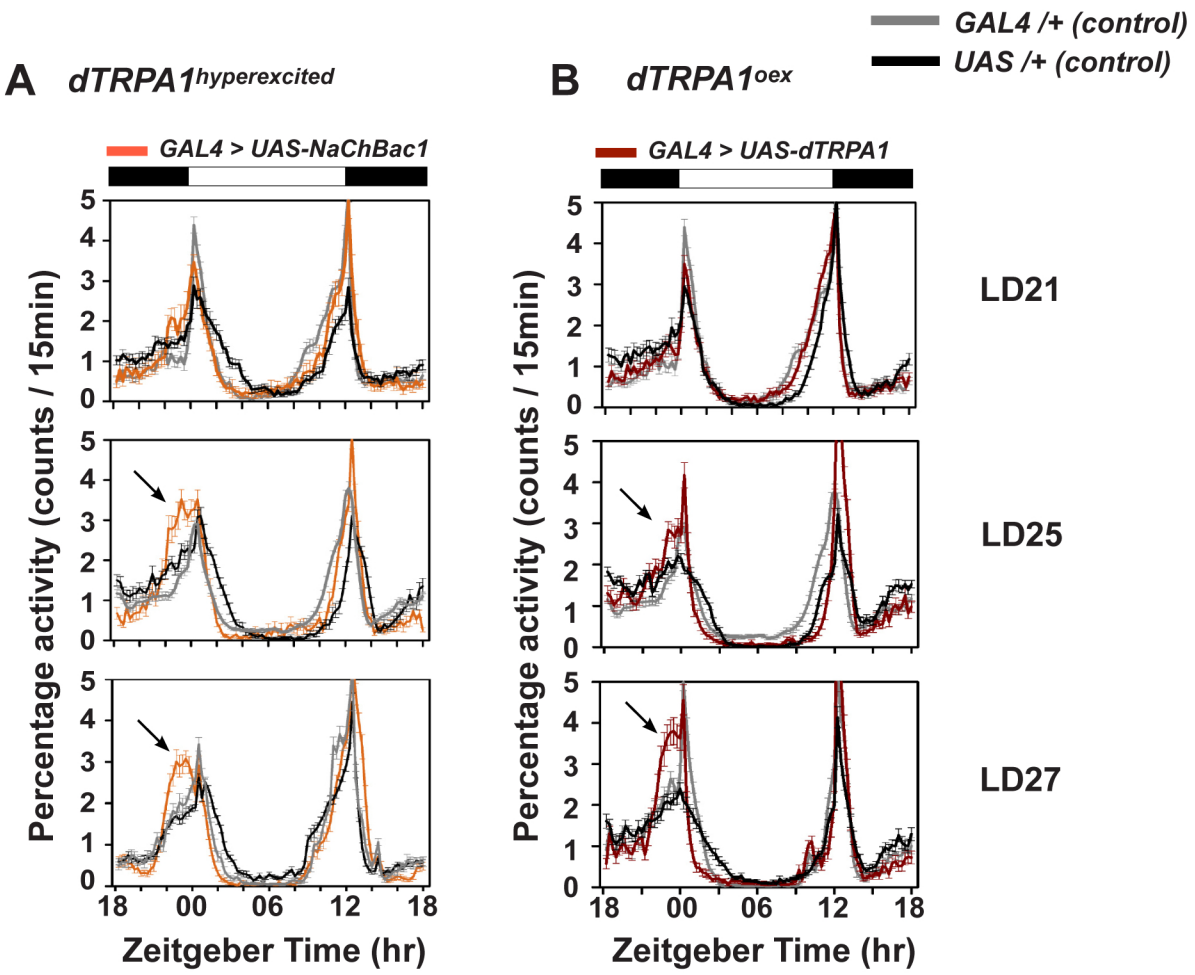

Figure 2.2. Enhanced neuronal firing of dTRPA1^{SH+} neurons at lower temperatures mimics wild-type flies at LD30. (A) Electrical hyperactivation dTRPA1^{SH+} neurons via expression of *UAS-NaChBac1* (*dTRPA1^{hyperexcited}*) causes no significant change under LD21 but at both LD25 and LD27, *dTRPA1^{hyperexcited}* flies advance their M-peak phase under (orange curves, arrows) compared to their respective parental controls. (B) Similarly, neuronal activation dTRPA1^{SH+} neurons by *dTRPA1^{SH}-GAL4* driven over-expression of dTRPA1 (*dTRPA1^{oex}*) at LD21 does not alter bimodal activity/rest rhythms similar to controls but at LD25 and LD27, they exhibit an advance phase of M-peak (arrows). Parental controls are represented in grey curves (*GAL4/+*) and black curves (*UAS/+*). Horizontal white and black bars above the panels represent light/dark phases.

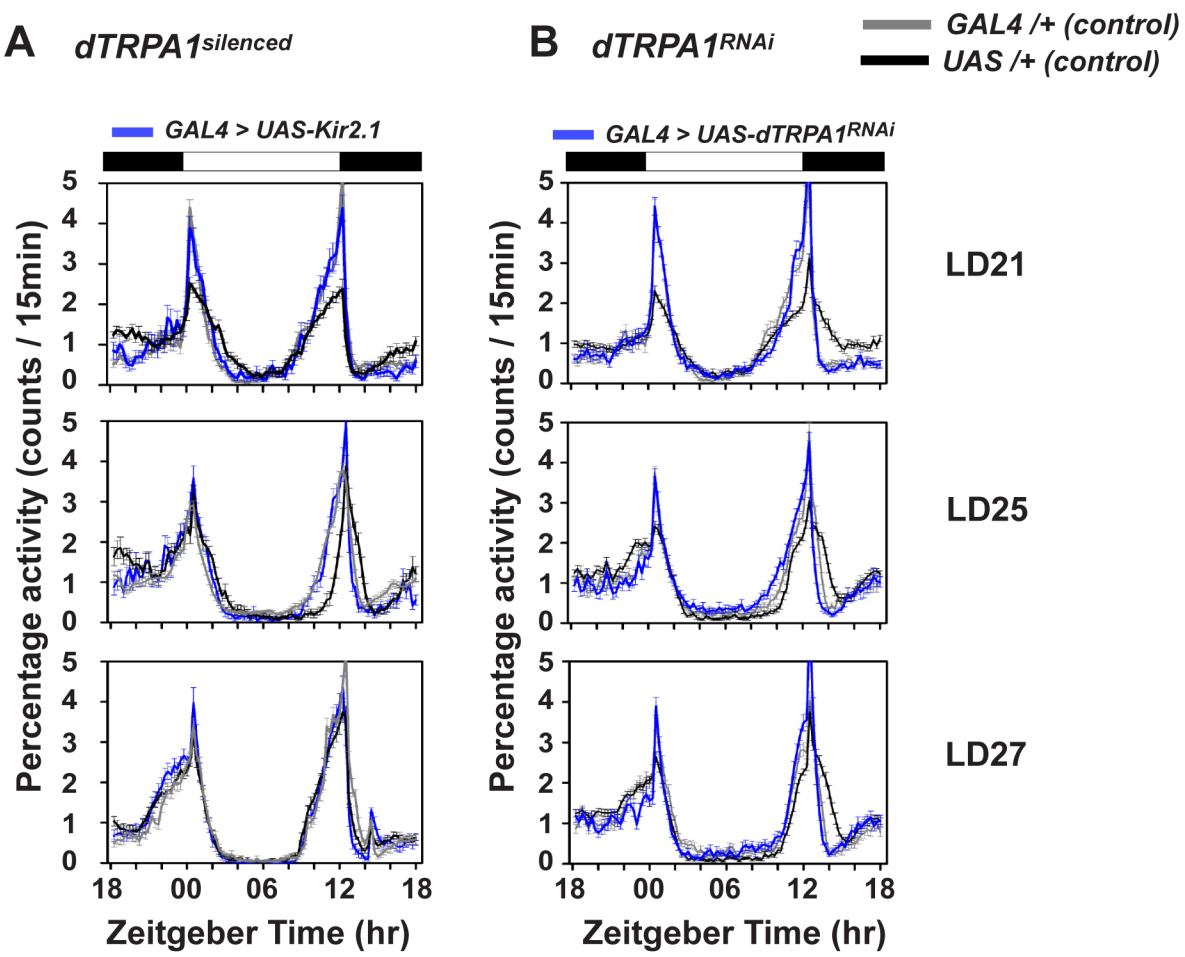

Figure 2.3. Electrical silencing or dTRPA1 RNAi knockdown in dTRPA1^{SH+} neurons does not alter activity/rest rhythms of flies under LD cycles. (A) *dTRPA1^{SH}-GAL4* driven silencing of neurons via expression of *UAS-Kir2.1* does not alter bimodal activity/rest rhythms under different LD cycles. (B) Flies with RNAi knockdown of dTRPA1 in dTRPA1^{SH+} neurons also exhibit bimodal activity/rest rhythms similar to their parental control flies under LD regimes. To enhance the effect of RNAi, *UAS-dcr2* was combined with *UAS-dTRPA1 RNAi* transgene. In RNAi experiments, *dTRPA1^{SH}-GAL4/ UAS-dcr2* served as one of the controls.

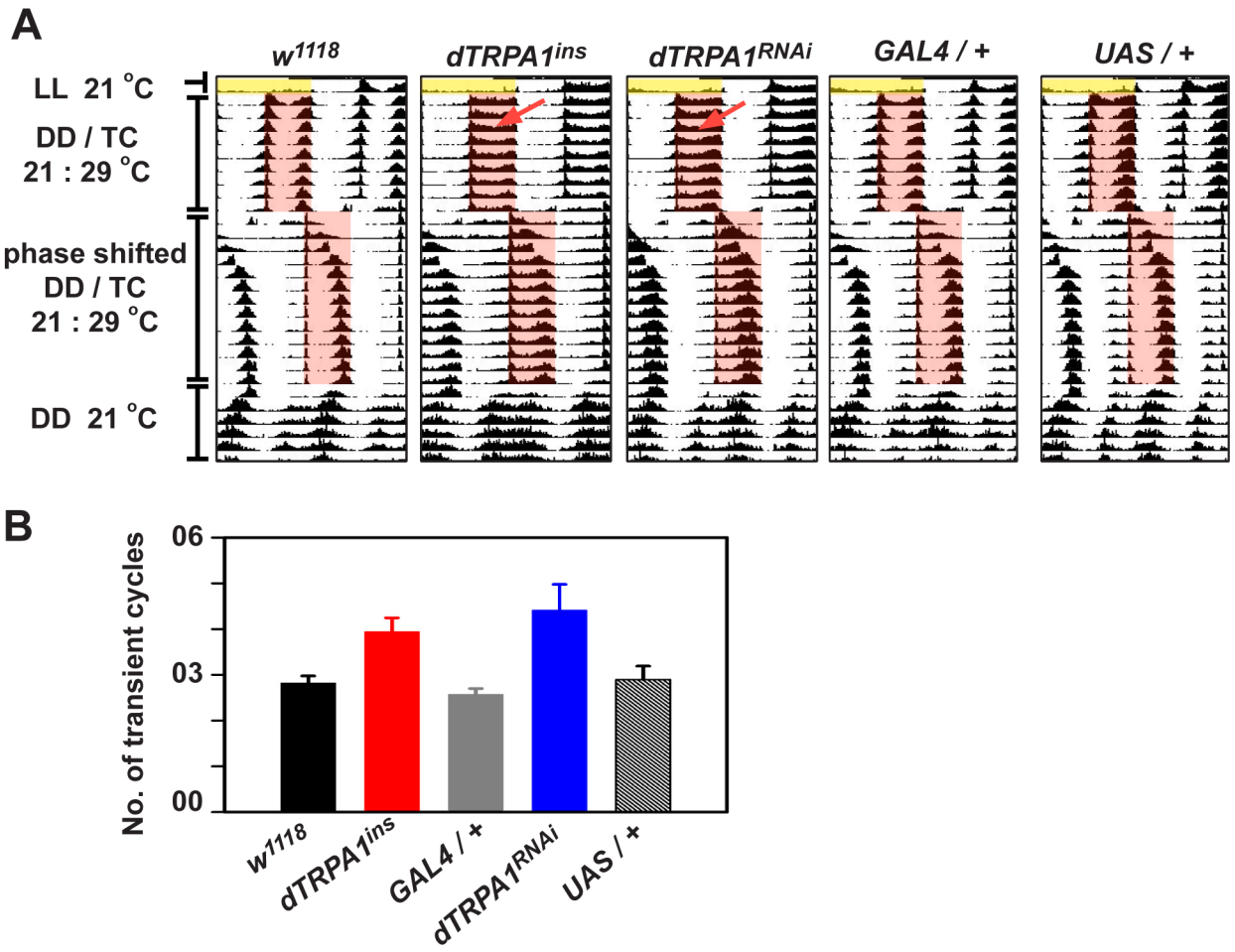

Figure 2.4. Average actograms of flies with altered levels of dTRPA1 expression under temperature cycles. (A) All control genotypes display bimodal activity/rest pattern under 12:12 hr thermophase: cyrophase, TC (29:21 °C) cycles with morning anticipatory activity followed by a startle at onset of thermophase with clear suppression of mid-thermophase activity in both pre-shift and post 10-hr delay shift in TC cycles. *dTRPA1*^{ins} null mutant and *dTRPA1*^{RNAi} flies are active for most part of thermophase (arrows) in pre- and post-shifted TC cycles. (B) Comparison of number of transient cycles required by *dTRPA1*^{ins} (red bars) is not different from wild-type (w^{1118}) control flies (black bars). Similarly, number of transient cycles taken by *dTRPA1*^{RNAi} flies (blue bars) to re-entrain to shifted TC cycles is not different compared to their parental controls in grey and hatched black bars (Kruskal Wallis rank ANOVA, $p < 0.05$).

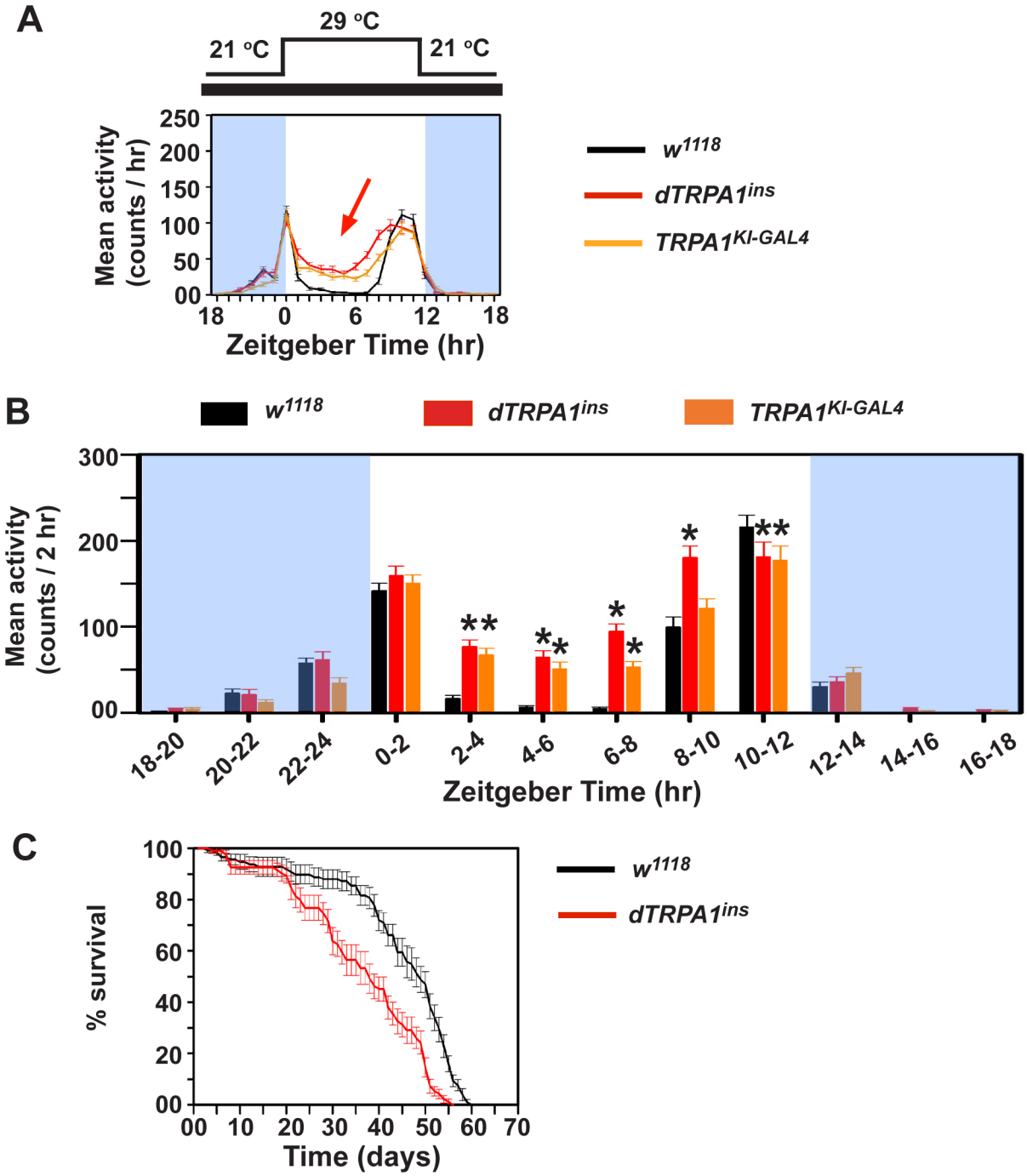

Figure 2.5. dTRPA1 is essential for consolidation of activity/rest rhythm and to inhibit mid-day activity under laboratory rectangular temperature cycles (DD/TC 29:21). (A) Average activity profiles of wild-type and dTRPA1 null mutant flies under temperature cycles (12:12hr) in DD (temperature regime is depicted above the panel). Null mutants *dTRPA1*^{ins} (red) and *TRPA1*$^{KI\text{-}GAL4}$ (orange) exhibit significantly greater mid-thermophase activity (arrow) compared to w^{1118} (black). (B) Comparison of activity levels of w^{1118} (black) and dTRPA1 null flies-*dTRPA1*^{ins} (red) and *TRPA1*$^{KI\text{-}GAL4}$ (orange) binned in 2hr intervals . *dTRPA1*^{ins} flies show significantly higher activity levels between ZT02-10 whereas *TRPA1*$^{KI\text{-}GAL4}$ null flies show significantly greater activity between ZT02-08 when compared to w^{1118} control flies. Blue shaded region represents the cryophase (21 °C). Error bars are SEM. Asterisks indicate significant differences from w^{1118} control flies in panel B (two-way ANOVA, Tukey's HSD, $p < 0.05$). (C) Survivorship curves for w^{1118} (black) and *dTRPA1*^{ins} (red) flies under DD/TC.

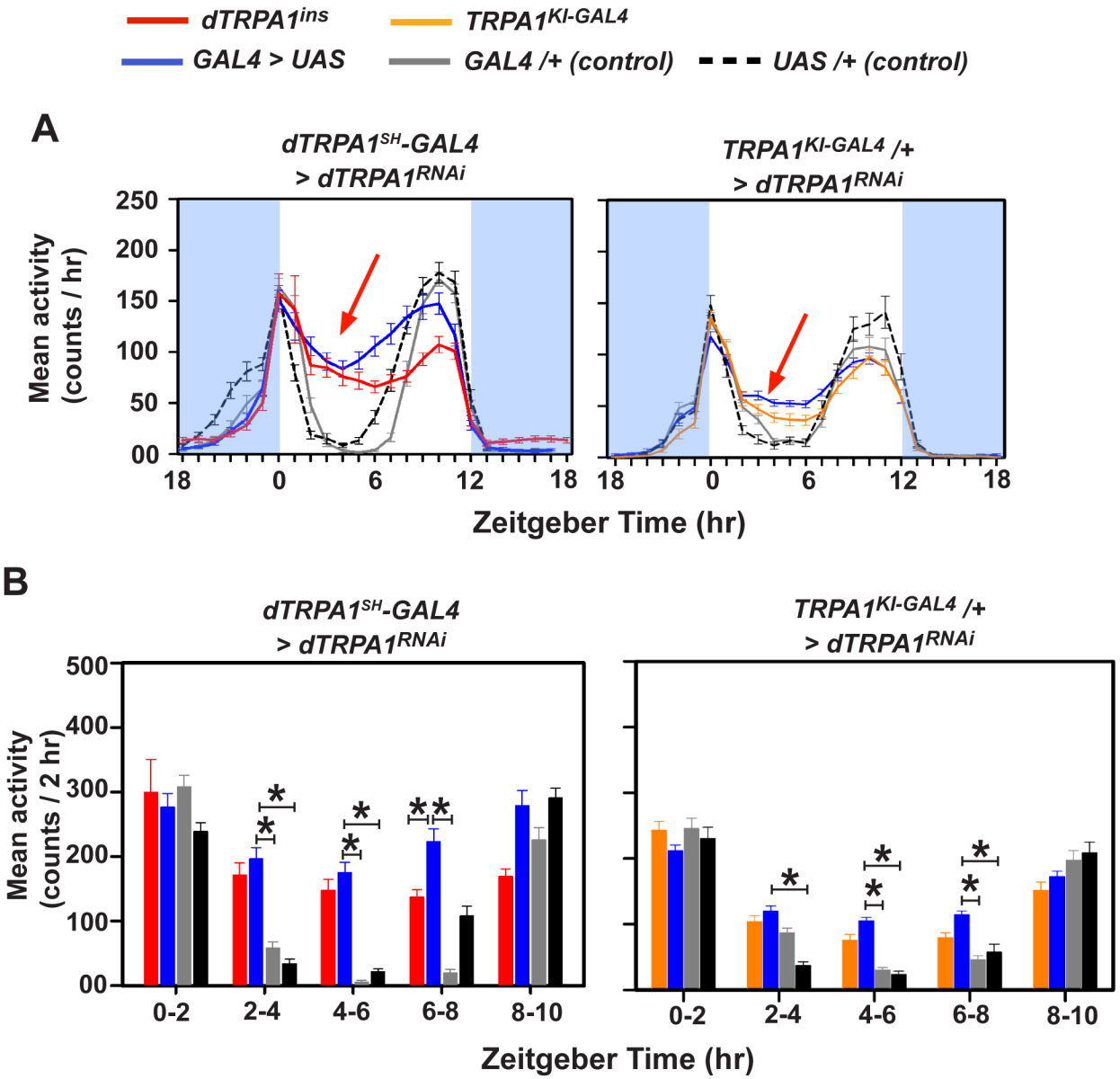

Figure 2.6. RNAi knockdown of dTRPA1 renders flies incapable of suppressing mid-day activity under temperature cycles (DD/TC). (A) Average activity profiles of flies with reduced levels of dTRPA1 expression in neurons targeted by *dTRPA1^{SH}-GAL4* and *TRPA1$^{KI\text{-}GAL4}$/+* and their respective controls under temperature cycles in DD. (A, left) RNAi knockdown of dTRPA1 under *dTRPA1^{SH}-GAL4* driver - *dTRPA1^{RNAi}* flies (blue) exhibit enhanced activity (arrow) during the thermophase similar to *dTRPA1^{ins}* null flies (red). (A, right) Flies with dTRPA1 RNAi knockdown under *TRPA1$^{KI\text{-}GAL4}$/+* driver (blue) also show enhanced mid-thermophase activity, similar to *TRPA1$^{KI\text{-}GAL4}$* mutants (orange). (B) Quantification of daytime activity levels (ZT 0-10) of *dTRPA1^{RNAi}* flies under two *GAL4* drivers with their controls. (B, left) Flies with *dTRPA1^{SH}-GAL4* driven dTRPA1 RNAi (blue) exhibit significantly greater activity levels compared to both parental controls between ZT 02-08 and phenocopy *dTRPA1^{ins}* (red). (B, right) Flies with RNAi knockdown under *TRPA1$^{KI\text{-}GAL4}$/+* driver exhibit significantly greater activity than both controls between ZT04-08, similar to homozygous *TRPA1$^{KI\text{-}GAL4}$* null mutant flies (orange). Shaded regions indicate cryophase (21 °C) in panel A. Error bars are SEM. Asterisks indicate significant differences among indicated genotypes in panel B (two-way ANOVA, Tukey's HSD, $p < 0.05$).

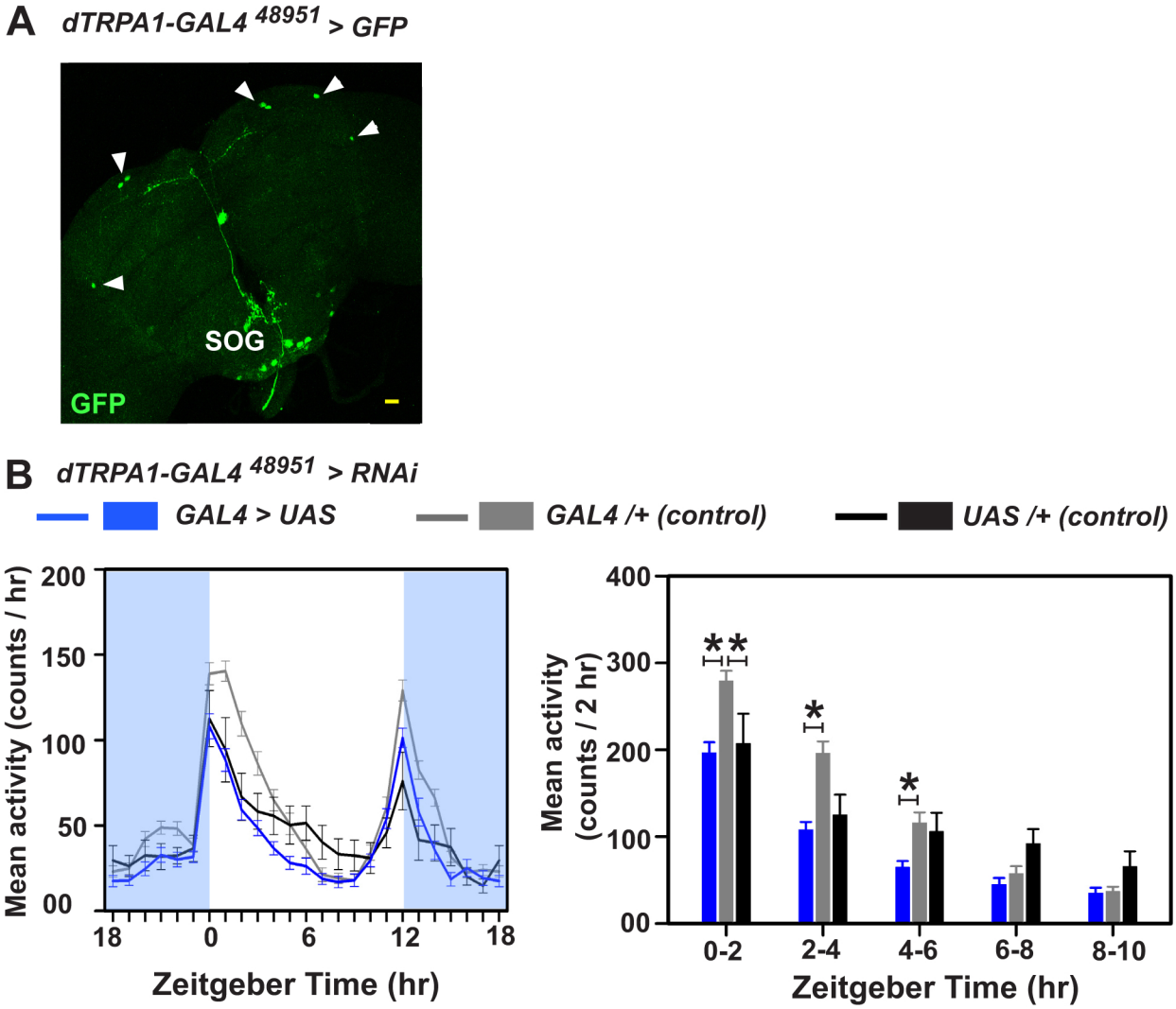

Figure 2.7. RNAi knockdown under *dTRPA1-GAL4*^{48951} has no effect on siesta under DD/TC. (A) Narrow expression pattern of *dTRPA1-GAL4*^{48951} driver as visualised by driving *UAS-2xeGFP* in adult fly brains. In most cases, AC neurons are not labelled whereas a few neurons in the dorsal brain are labelled (arrowheads) and a group of cells are targeted in the suboesophageal ganglion (SOG) region. Scale bar is 20µm. (B, left) dTRPA1 RNAi knockdown under *dTRPA1-GAL4*^{48951} driver (blue curve) has no effect on mid-thermophase activity under DD/TC (29:21 °C). (B, right) Mean activity levels (binned in 2hr intervals) of dTRPA1 RNAi knockdown flies is lower than *GAL4* controls between ZT0-6 but not different from *UAS* controls between ZT0-10. Error bars are SEM. Asterisks indicate significant differences among indicated genotypes (two-way ANOVA, Tukey's HSD, $p < 0.05$). Blue shaded regions represent cryophase (21°C).

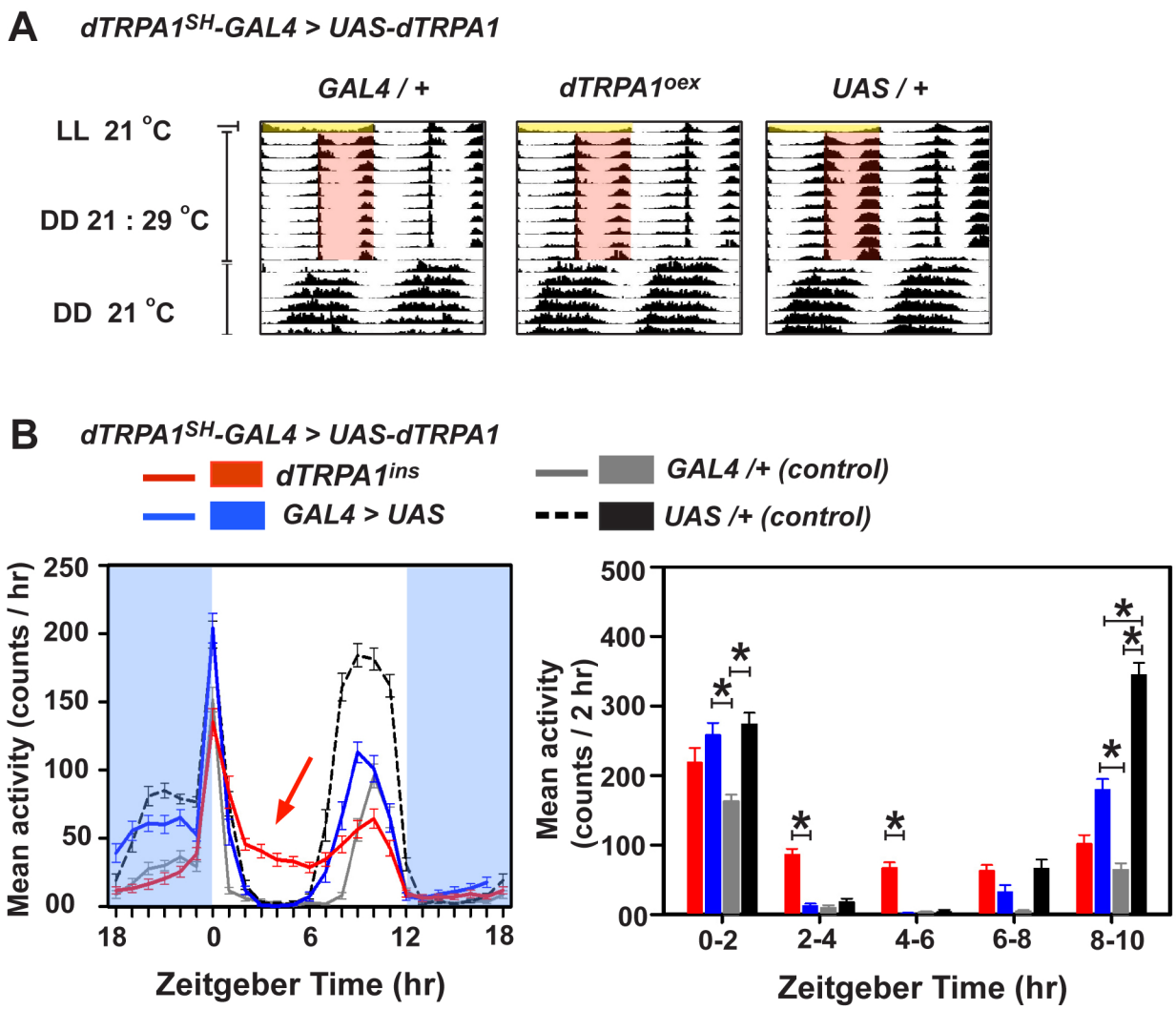

Figure 2.8. dTRPA1 overexpression in neurons targeted by *dTRPA1^{SH}-GAL4* does not modify mid-thermophase activity under DD/TC. (A) Flies are first subjected to LL/21 °C for one day followed by temperature cycles (12:12 hr -29:21 °C) in DD before releasing them in constant conditions (DD/21 °C). *dTRPA1^{oex}* flies inhibit activity during mid-thermophase and resemble their controls. Shaded regions in actograms represent the thermophase (29 °C). (B, left) Flies with dTRPA1 over-expression in dTRPA1^{SH+} neurons do not alter the profile and *dTRPA1^{oex}* flies (blue curve) inhibit their activity during thermophase resembling controls (grey and black dashed curves) whereas *dTRPA1^{ins}* (red curve) flies show enhanced mid-thermophase activity (arrow). (B, right) Comparison of mean activity levels during ZT0-10 binned in 2hr interval show that *dTRPA1^{oex}* flies (blue) do not differ from controls and inhibit mid-day activity. Asterisks indicate significant differences among indicated genotypes (two way ANOVA, Tukey's HSD, $p < 0.05$). All other details same as Figure 2.6.

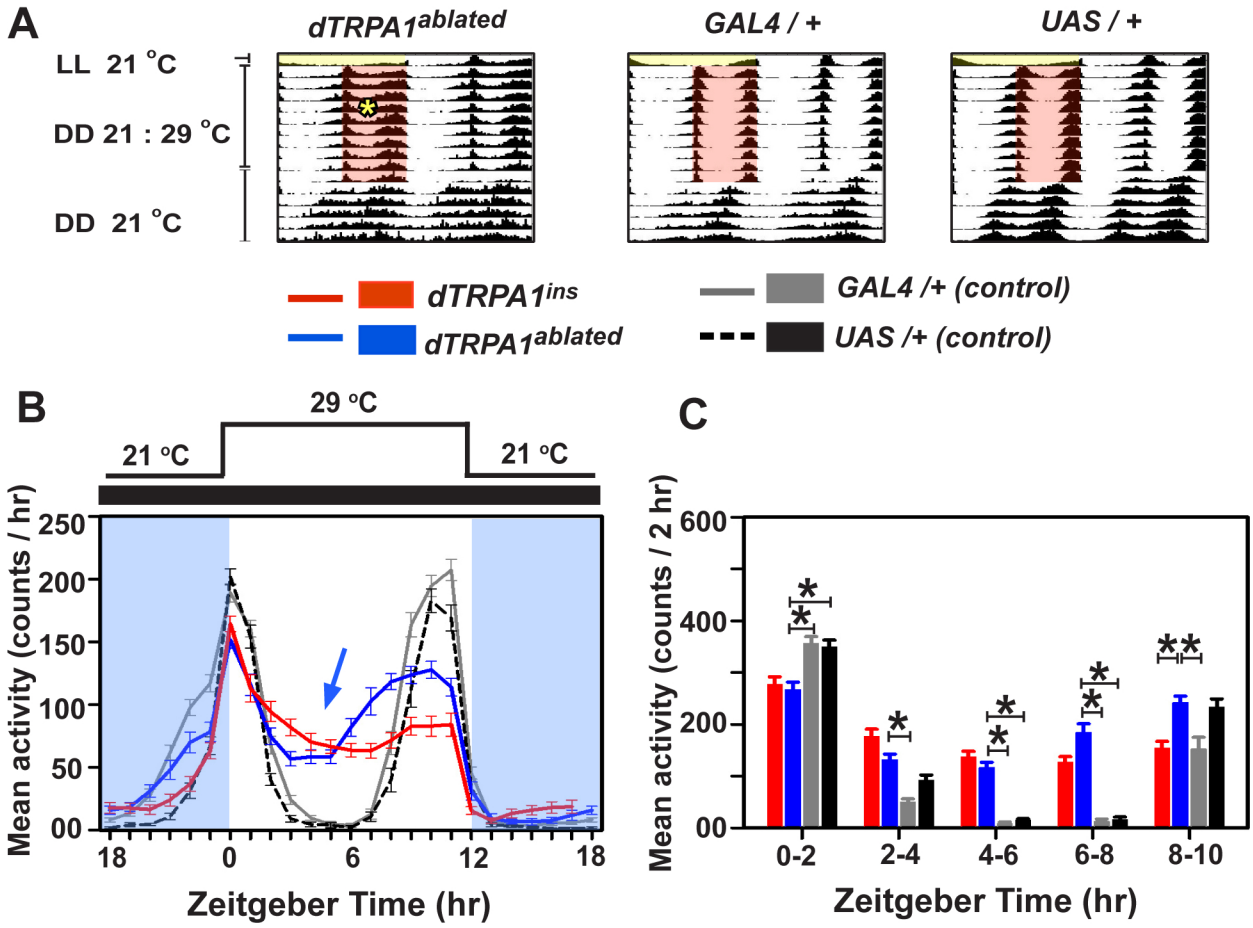

Figure 2.9. Functional dTRPA1 neurons targeted by *dTRPA1^{SH}-GAL4* driver in the fly brain are necessary to inhibit mid-day activity under temperature cycles. (A) Average actograms of flies with ablated dTRPA1^{SH+} neurons (*dTRPA1^{ablated}*) recorded under DD/TC. Control genotypes show bimodal activity with a clear mid-day "siesta" whereas *dTRPA1^{ablated}* flies exhibit increased mid-thermophase activity (yellow asterisk). (B) Average activity profiles of *dTRPA1^{ablated}* flies (blue) phenocopy *dTRPA1^{ins}* (red) and display enhanced mid-thermophase activity (arrow) in contrast to controls flies (grey and black). (C) Mean activity levels binned in 2hr intervals of *dTRPA1^{ablated}* flies (blue) are significantly higher than both controls between ZT 04-08. Mean activity levels of *dTRPA1^{ins}* (red) are shown for comparison. All other details same as Figure 2.6.

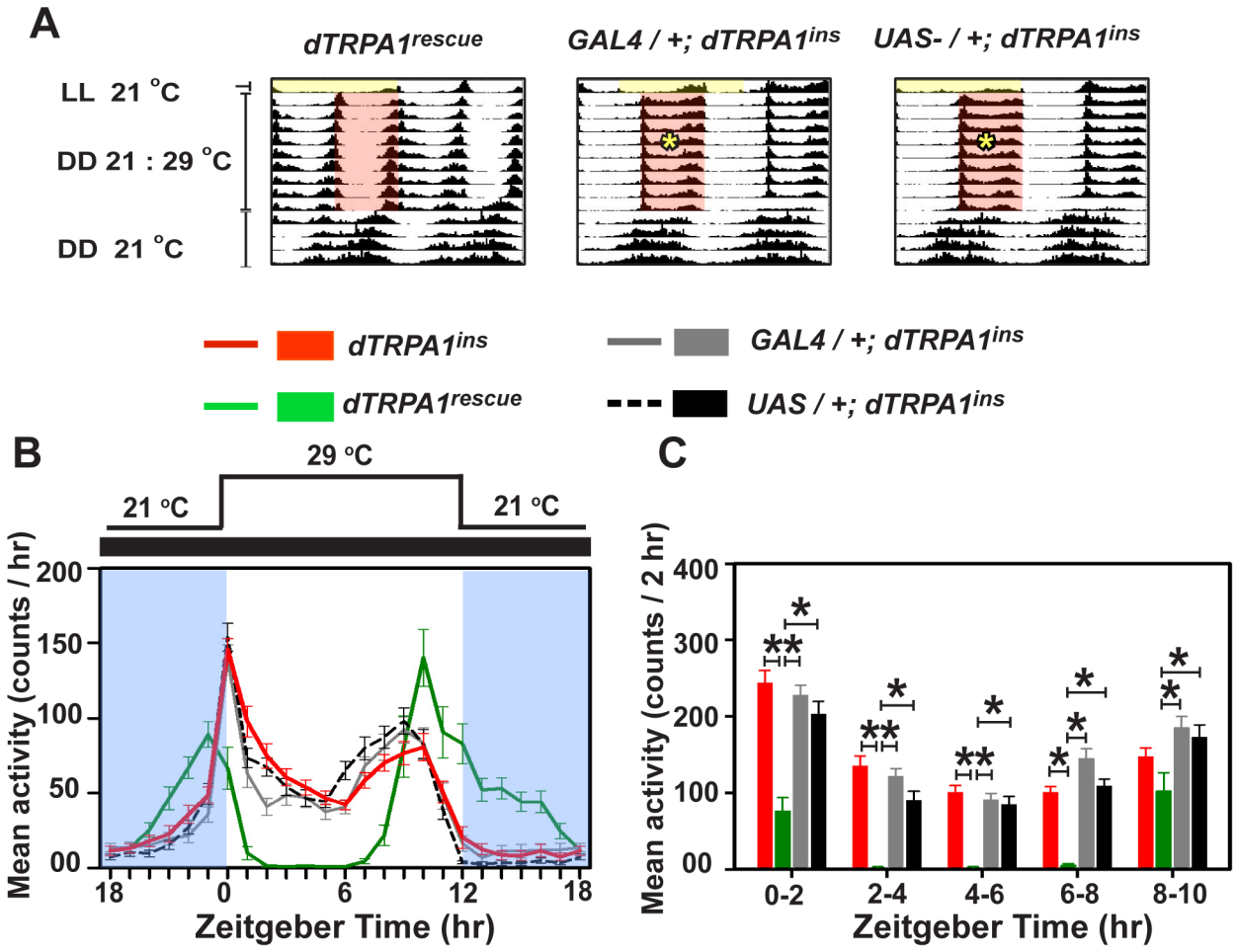

Figure 2.10. Restoring dTRPA1 function in neurons targeted by *dTRPA1^{SH}-GAL4* driver is sufficient for flies to inhibit mid-day activity under temperature cycles. (A) Average actograms of flies with dTRPA1 expression restored in dTRPA1^{SH+} neurons in a null background (*dTRPA1^{rescue}*) recorded under DD/TC. In contrast, controls genotypes in the *dTRPA1^{ins}* genetic background exhibit high mid-day activity (asterisks) resembling *dTRPA1^{ins}* null flies. Shaded regions in actograms represent the thermophase (29 °C). (B) *dTRPA1^{rescue}* flies (green curve) exhibit mid-day "siesta" by suppressing activity during mid-thermophase whereas control flies in null back ground (grey and black curves) exhibit enhanced mid-day activity. (C) Bar graphs show quantification of mean daytime activity levels. *dTRPA1^{rescue}* flies (green) exhibit significantly lower activity between ZT00-08 compared to control flies in null background (grey and black bars) and *dTRPA1^{ins}* null flies (red). All other details are same as Figure 2.6.

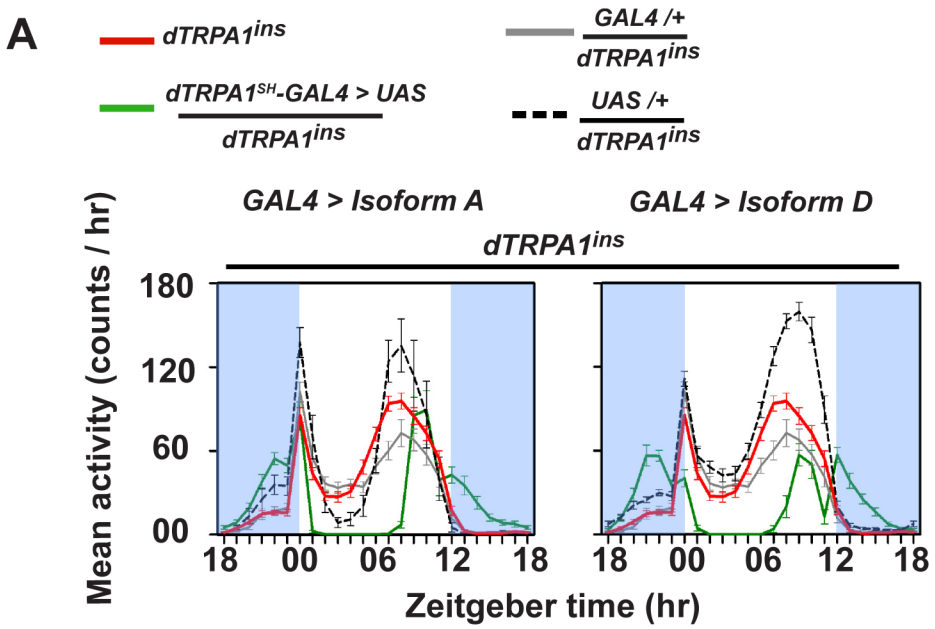

Figure 2.11. Temperature-sensitive isoforms of dTRPA1 rescue the increased mid-thermophase activity of *dTRPA1^{ins}* flies under DD/TC cycles. (A) Average activity profiles of flies driving expression of isoforms; *UAS-dTRPA1-A* (left) or *UAS-dTRPA1-D* (right) under *dTRPA1^{SH}-GAL4* driver in *dTRPA1^{ins}* background show complete suppression of activity during mid-thermophase in DD/TC cycles. The parental controls in *dTRPA1^{ins}* background (grey and black dashed curves) resemble *dTRPA1^{ins}* flies (red) and exhibit increased mid-day activity except for *UAS-dTRPA1-A/+; dTRPA1^{ins}* flies (left, black dashed curve). *UAS* control flies in null background may show suppression of midday activity probably due to leaky expression of the *UAS-dTRPA1-A* transgene.

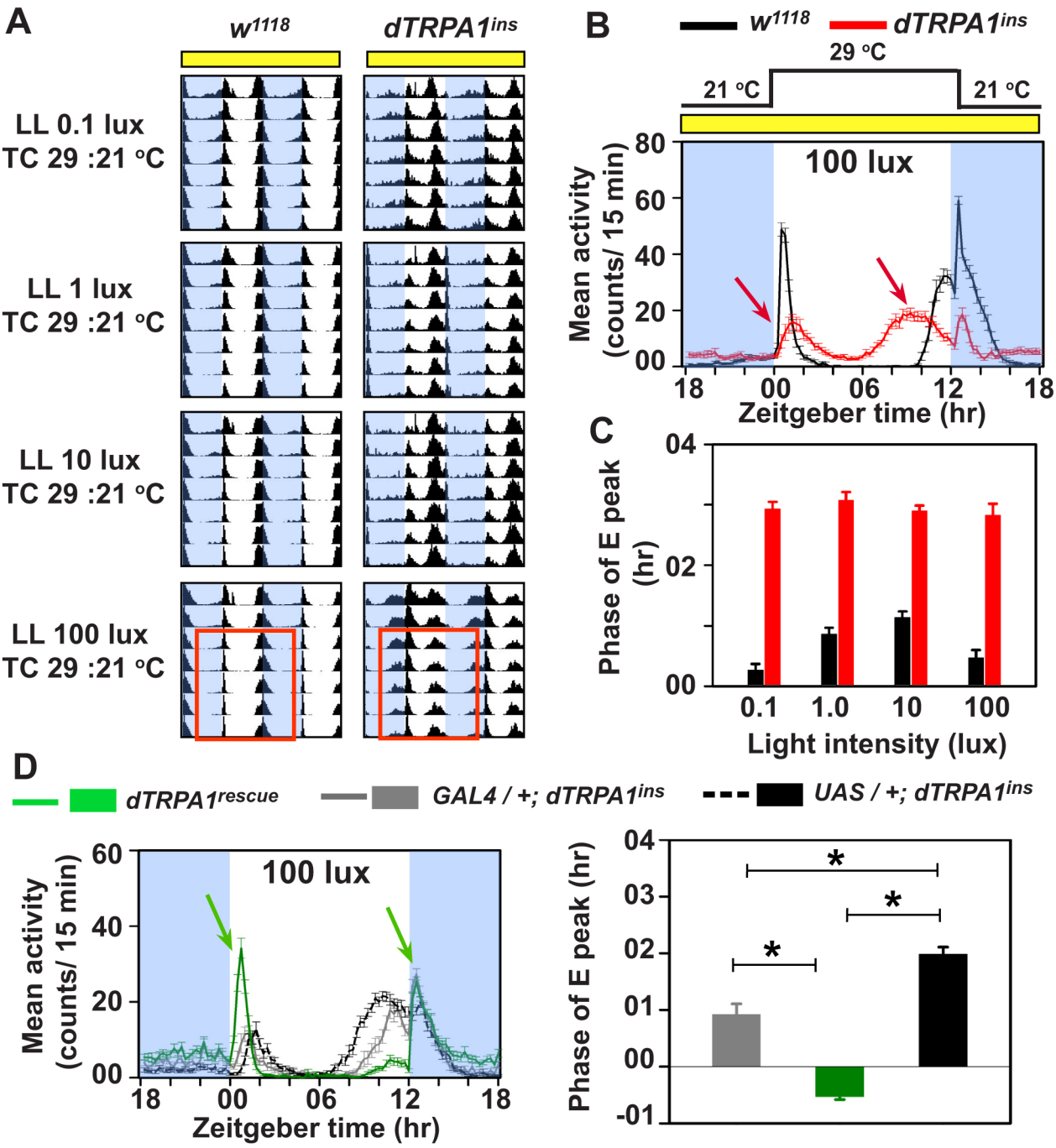

Figure 2.12. Lack of dTRPA1 modifies morning and evening peak under temperature cycles in constant light (LL/TC). (A) Average actograms of wild-type (w^{1118}) flies and $dTRPA1^{ins}$ under temperature cycles (21:29 °C) in LL at different light intensities. Blue shaded region represent cryophase. $dTRPA1^{ins}$ flies advance their E-peak compared to w^{1118} flies under LL/TC across range of light intensities examined. Red boxes indicate the days used for plotting activity profiles in (B). (B) $dTRPA1^{ins}$ flies have a reduced morning (M) peak (arrow) and an advanced E-peak (arrow) compared to w^{1118} flies under LL/TC at 100 lux. (C) $dTRPA1^{ins}$ flies phase their E-peak ~2hr prior to w^{1118} flies within a given light intensity but the E-peak phases of $dTRPA1^{ins}$ flies do not differ among themselves across light intensities. (D, left) Restoring dTRPA1 expression in dTRPA1^{SH+} neurons in a null background (green) rescues M and E peak phenotypes exhibited by $dTRPA1^{ins}$ flies under LL/TC (100lux). Parental controls in null background phenocopy $dTRPA1^{ins}$ flies and exhibit reduced M-peak and an advanced E-peak. (D, right) $dTRPA1^{rescue}$ flies phase their E-peak after ZT12 whereas parental controls in dTRPA1 null background have an advanced E-peak. Statistical comparisons are based on two-way ANOVA, Tukey's HSD, $p < 0.05$. Yellow bars in panel A and B represent constant light (LL) conditions. Error bars are SEM.

Chapter 3

Role of dTRPA1 in modulating afternoon peak of activity of fruit flies *Drosophila melanogaster*

Introduction

During mid-day, high temperature, low humidity and desiccation present considerable challenges to insect survival. A combination of sensory responses modulated by circadian clock-driven processes allow fruit flies - *Drosophila melanogaster* to coordinate their physiological and behavioural processes to the cyclic external environment (Allada and Chung, 2010; Dubruille and Emery, 2008). In recent years, behavioural responses of flies under semi-natural conditions have garnered much attention following the publication of a paper by the Kyriacou and Costa groups (Vanin et al., 2012). In nature, organisms receive multiple time cues or "zeitgebers" in the form of continuously changing environmental variables such as light, temperature, humidity etc. (De et al., 2013; Menegazzi et al., 2012; Vanin et al., 2012) in contrast to laboratory regimes which usually provide gated rectangular cycles of light and/or temperature. Under semi-natural conditions (SN), wild-type flies exhibit an additional peak of activity during the day, the afternoon or A-peak which previous studies have suggested to be a stress response to high temperature and low humidity conditions during noon (De et al., 2013; Menegazzi et al., 2012; Vanin et al., 2012). The first study to observe the occurrence of A-peak under SN conditions (Vanin et al., 2012) suggested that A-peak was circadian clock driven and also proposed that A-peak was a "stress/escape" response of flies to high temperatures. Similar observations were made by another group of researchers and they posit that although circadian clock may inhibit mid-day activity because it is "unproductive" for the flies, extremely high temperatures elicits A-peak under "life threatening" environments (Menegazzi et al., 2012). Another study has suggested that A-peak is circadian clock-independent and elicited due to flies seeking shade within the recording monitors during extremely hot afternoons (De et al., 2013). More recently it was shown that very high light intensity under SN can also elicit A-peak in several wild-caught species of *Drosophila* (Prabhakaran and Sheeba, 2013), and that high mid-day temperatures

simulated in the lab can induce A-peak even under constant light which is believed to disrupt the circadian clock (Prabhakaran and Sheeba, 2014). All of the above studies suggested that temperature has a crucial role in modulating the occurrence of A-peak under SN. I examined the possible role of an important class of thermoreceptor, the warmth activated ion channel dTRPA1 in eliciting the A-peak under SN.

Drosophila possess several thermoreceptors (Gallio et al., 2011; Ni et al., 2013; Sayeed and Benzer, 1996; Sehadova et al., 2009) including a pair of neurons in each brain hemisphere just below the antennae called the anterior cells (AC), which express thermosensitive ion channels dTRPA1 (drosophila Transient Receptor Potential A1) (Hamada et al., 2008). dTRPA1 has been implicated both in chemical nociception (Kang et al., 2010) and thermal nociception (Neely et al., 2011) and it's role in thermosensation has been studied in great detail by several groups (Hamada et al., 2008; Kang et al., 2012; Kwon et al., 2008; Rosenzweig et al., 2005; Rosenzweig et al., 2008; Tang et al., 2013; Zhong et al., 2012). In adults, neurons expressing dTRPA1 ion channels respond to thermal stimuli at two distinct temperatures (25 ^{o}C and 27 ^{o}C) (Tang et al., 2013), similar to the activation range of the channel obtained from whole cell recordings in a heterologous system (24-29 ^{o}C) (Viswanath et al., 2003). Since these temperatures fall in the range that the adult flies are likely to encounter in nature (Prabhakaran and Sheeba, 2013) and because dTRPA1 has wide expression in the adult fly brain, I asked whether dTRPA1 modulates the daily rhythm of activity/rest in *D. melanogaster* in response to temperature cycles under SN.

A previous study has shown that intensity of light can modify phase and amplitude of activity peaks under SN (De et al., 2013). To understand the combinatorial effects of light and temperature on the activity pattern in flies that lacked dTRPA1 function, I recorded the activity of flies under SN, thereby exposing them to an environment highly enriched in terms

of time cues compared to the relatively impoverished environmental cues under standard laboratory conditions. I, further, studied the activity/rest rhythms of flies under controlled simulated SN conditions in the laboratory with gradually changing light and temperature regimes. My results show that *Drosophila* dTRPA1 mediates occurrence of A-peak in flies in a temperature-dependent manner.

Materials and Methods

Fly strains: All genotypes were reared on standard cornmeal medium under 12:12 hr LD and 25 ^{o}C. *dTRPA1^{SH}-GAL4* (Hamada et al., 2008) driver targets a subset of dTRPA1 expressing neurons (~30 cells) in the adult fly brain (henceforth dTRPA1^{SH+}). *TRPA1$^{KI-GAL4}$* (Kim et al., 2010) has a *GAL4* gene inserted in dTRPA1 promoter region making it a dTRPA1 null but can also drive expression under the dTRPA1 promoter. *TRPA1$^{KI-GAL4}$* has a much broader target area (~70 cells) with innervations near the fan-shaped body. *Pdf-GAL4* (Renn et al., 1999) and *cry-GAL4-39* (Klarsfeld et al., 2004) drivers were used to target subsets of circadian neurons. Thermosensor dTRPA1 channel was over-expressed using *UAS-dTRPA1*(Rosenzweig et al., 2008) under *dTRPA1^{SH}-GAL4* driver for heat dependent activation of neurons. *UAS-hid* was used for ablation of neuronal subsets (provided by Michael Rosbash, Brandeis). Null mutants used were *dTRPA1^{ins}* (provided by Paul Garrity, Brandeis University) and *TRPA1$^{KI-GAL4}$* (donated by Youngseok Lee, Kookmin University). There are four known isoforms of dTRPA1 – temperature responsive A (activation range 24-29 ^{o}C) and D (activation ~34 ^{o}C) isoforms and temperature non-responsive, B and C isoforms (Zhong et al., 2012). Here I have used the isoform nomenclature developed by the Montell (Lee and Montell, 2013) and Tracey labs (Zhong et al., 2012). The fly lines for different dTRPA1 isoforms were kindly provided by Paul Garrity and Dan Tracey. The expression pattern of *GAL4* drivers used in this Chapter are described in Table 3.1.

Behavioural assays: All recordings under semi natural (SN) conditions were carried out in DAM2 monitors in an outdoor iron-meshed enclosure(122 x 122 x 122 cm^3) with grids (6 x6 cm^2) situated within the JNCASR campus in Bangalore, India (12^o58', 77^o38'). The iron-meshed enclosure allowed free flow of air but was covered on top with an inclined translucent, plastic sheet and dense canopy to avoid exposure of monitors and flies to direct sunlight. Environmental factors - light, temperature and humidity, were simultaneously monitored using DEnM environmental monitors (Trikinetics). To record the activity of flies under DD+SN, DAM2 monitors were placed in light-tight metal boxes kept in the outdoor enclosure. The boxes were fitted with fans and appropriate vents and baffles to enable air circulation and prevent overheating. DEnM monitors were placed inside the boxes to measure light, temperature and humidity in parallel with locomotor activity measured by DAM2 monitors. To mimic the gradual light and temperature cycles of SN in the laboratory, light intensity and temperature were increased in a step-wise manner either alone or in combination. The details of light and temperature conditions of simulated SN regimes are given in Table 3.2. All laboratory based protocols involving simulated light and/or temperature were carried out in incubators (Sanyo, Japan and Percival Drosophila chambers, USA). Temperature and light steps were applied using available programs of the respective incubators. DEnM placed within the incubators recorded changes in light, temperature and humidity throughout the experiments.

Analysis: Activity counts in 15-min intervals of individual male flies, across at least 5 days were first averaged, following which mean activity counts were averaged across flies of a given genotype and plotted against time of day. In all the figures where *x*-axis denotes time of the day (h), time point 00 hr correspond to 00:00 hrs in the real world, thus, 12 hr corresponds to 12:00 hrs and so on. Whether a particular genotype exhibited A-peak was determined using visual estimation described in detail previously (Prabhakaran and Sheeba,

2014). Briefly, a fly was considered to exhibit an A-peak in SN when there was a gradual increase in activity leading to a peak during mid-day (between 11:00 hrs to 16:00 hrs) and the amplitude of the peak was at least 20% of the highest activity displayed by the fly. A-peak estimation was carried out for each fly from its 15 min binned profiles for a single day. A given genotype was considered to exhibit A-peak only when at least 25% of flies displayed A-peak. Proportion of flies showing A-peak was determined by the above method for each day and maximum light intensity (L_{max}) and maximum temperature (T_{max}) has been tabulated using data recorded in DEnM monitors. Data from multiple experiments was pooled to perform Spearman rank order correlation analysis on proportion of flies displaying A-peak each day with their corresponding values of L_{max} or T_{max} using STATISTICA^{TM}. Under laboratory simulated regimes, mean activity profiles averaged across 5 days for an individual fly was considered for estimating proportion of flies showing A-peak. To estimate A-peak amplitude, percentage activity during 1 hr of T_{max} (32 ^{o}C) of gradual temperature cycles from individual flies obtained in 15 min bins were normalised to sum of activity across 24 hrs; following which the profiles were averaged across 5 days. Thereafter, data across one hour of T_{max} (when temperature was 32 ^{o}C) was binned to estimate percentage A-activity. Percentage activity levels of all flies of a genotype were included to estimate activity during 1hr of T_{max}, irrespective of whether the fly exhibited A-peak, for example dTRPA1 null flies. Significant differences among genotypes for the A-peak amplitude were determined by one-way-ANOVA on arc-sine transformed data, followed by Tukey's HSD, $p < 0.05$. Phase of E-peak was estimated subjectively such that the activity data of individual flies averaged across days showed a gradual rise in activity culminating in a peak. E-peak phase values of individual flies were averaged to obtain mean E-peak phase for a given genotype. Differences in E-peak phase values among genotypes were compared by performing one-way ANOVA, followed by Tukey's HSD ($p < 0.05$).

Results

dTRPA1 is necessary for the A-peak in semi-natural conditions. Recent studies show that wild-type flies exhibit an additional activity peak during mid-day besides the canonical morning and evening peaks under SN (De et al., 2013; Menegazzi et al., 2012; Vanin et al., 2012). Temperature has been shown to be a consistent factor that elicits the A-peak (Prabhakaran and Sheeba, 2013, 2014; Vanin et al., 2012). I studied the behaviour of flies lacking dTRPA1, a thermosensitive ion channel that has been reported to become activated *in vivo* within the range of 24-29 ^{o}C (Viswanath et al., 2003). Locomotor activity of flies under semi-natural conditions (labelled SN on the Figures) was recorded using the outdoor enclosure in JNCASR campus as described in materials and methods section. It has been proposed that under warm conditions in nature, wild-type flies suppress their afternoon activity but relieve such inhibition resulting in an A-peak only when average daytime temperatures are high (Menegazzi et al., 2012; Vanin et al., 2012). Clock mutants enhance their activity in response to warm day time temperatures at a much lower range, thus exhibiting unproductive afternoon activity (Menegazzi et al., 2012). My results confirm that wild-type (w^{1118}) flies exhibit three peaks of activity under SN in multiple independent experiments (see Table 3.3). When the maximum light intensity (L_{max}) reached was either low (325 lux), moderate (680 lux) or high (1759 lux), with temperature maximum (T_{max}) of ~32 ^{o}C w^{1118} flies exhibit all three peaks- in the morning (M), afternoon (A, marked by red arrow) and evening (E) (Figure 3.1, top-left panels, Table 3.3). In contrast, most $dTRPA1^{ins}$ flies with a "strong loss of function" mutation (Hamada et al., 2008; Head et al., 2015) assayed in parallel, display a bimodal activity pattern with no A-peak (Figure 3.1, top-middle panel; Table 3.3). An independently derived dTRPA1 null mutant (Kim et al., 2010), *TRPA1*

$^{KI\text{-}GAL4}$, also does not exhibit an A-peak (Table 3.3) when temperature is highest and humidity is lowest under SN (Figure 3.1, top-right panel). These results indicate that the A-peak is a dTRPA1-dependent response. Previous studies have suggested that the A-peak is temperature dependent (Menegazzi et al., 2012; Prabhakaran and Sheeba, 2013, 2014; Vanin et al., 2012). My studies now provide evidence for underlying role of dTRPA1 ion channels in inducing A-peak.

The proportion of w^{1118} flies that exhibit the A-peak is positively correlated with T_{max} (Spearman's rank order correlations, $r = +0.78$, $p < 0.05$) but no significant correlation is detectable with L_{max}. On the other hand, the small proportion of $dTRPA1^{ins}$ flies that do exhibit the A-peak is correlated with L_{max} ($r = + 0.51$, $p < 0.05$) but not with T_{max}. Similarly, the small proportion of $TRPA1^{KI\text{-}GAL4}$ flies that do display the A-peak is positively correlated with L_{max} (Spearman's rank order correlations, $r = + 0.56$, $p < 0.05$). Proportion of $TRPA1^{KI\text{-}GAL4}$ flies that exhibit the A-peak is also found to be negatively correlated with T_{max} (Spearman's rank order correlations, $r = - 0.61$, $p < 0.05$) probably due to a few days with high light intensity but low T_{max}.

Both the null mutants, $dTRPA1^{ins}$ and $TRPA1^{KI\text{-}GAL4}$ are sensitive to other environmental factors and exhibit small spikes in their activity throughout the day in response to very bright light similar to w^{1118} flies (>1700 lux, Figure 3.1, bottom row). However, under very bright light conditions, w^{1118} flies show two bouts of intense activity during mid-day - one coinciding with L_{max} and distinct from the second that coincides with T_{max} (Table 3.3), illustrating that w^{1118} flies which have intact photosensitivity and thermosensitivity show two distinct peaks of activity in response to temporally separated L_{max} and T_{max}. In contrast, dTRPA1 null flies respond with activity peaks only in response to L_{max} under very bright light conditions (Table 3.3), and presumably, due to compromised thermal sensitivity, do not

respond to T_{max}. Under high light intensity, activity levels are suppressed for all genotypes compared to low light conditions; even at sunrise although light intensity is not as bright as during mid-day (compare Figure 3.1, top vs bottom panels). In addition to light intensity, both temperature and humidity (and possibly other factors) differ between the two experiments under SN. At sunrise, along with high light intensity, humidity is lower by about 20% and temperature is lower by 5 ^{o}C. Hence, I speculate that perhaps light is not the major modulator of activity during sunrise.

Wild-type flies exhibit A-peak even in absence of light cues in SN. To eliminate the effect of light on the A-peak, activity of flies was recorded in light-tight metal boxes kept in the same outdoor enclosure (henceforth, DD+SN). Under DD+SN, where the flies experience daily variation in temperature (T_{max} ~32 ^{o}C) and humidity but not light, w^{1118} flies show a distinct A-peak whereas both null mutants- $dTRPA1^{ins}$ and $TRPA1^{KI\text{-}GAL4}$ do not show the A-peak (Figure 3.2A, Table 3.3). In contrast to SN, under DD+SN, only 60% of control w^{1118} flies show E-peak, while among the mutants 87% of $dTRPA1^{ins}$ and 100% of $TRPA1^{KI\text{-}GAL4}$ exhibit it (Figure 3.2A). Further, w^{1118} flies exhibit a significantly delayed E-peak compared to $dTRPA1^{ins}$ and $TRPA1^{KI\text{-}GAL4}$ (one-way ANOVA with genotype as factor, $F_{2,51} = 78.3$, $p < 0.05$). This may be possibly due to compensatory reduction in activity due to excessive activity at noon. Interestingly, w^{1118} flies do not exhibit A-peak under DD+SN if the T_{max} only reaches 28 ^{o}C and predictably nor does $dTRPA1^{ins}$ null mutant exhibit A-peak under identical conditions (Figure 3.2B).

Flies overexpressing dTRPA1 show enhanced A-peak under SN conditions in DD. In order to determine if signals from dTRPA1 neurons influence occurrence of A-peak, I either ablated a subset of dTRPA1 neurons ($dTRPA1^{SH+}$ neurons) or downregulated dTRPA1 levels in $dTRPA1^{SH+}$ neurons and studied the behaviour of flies under SN and DD+DN conditions.

dTRPA1^{SH+} neurons were ablated using *dTRPA1^{SH}-GAL4* driver (henceforth, *dTRPA1^{ablated}*) and subjected to SN (Figure 3.3A) and DD+SN (Figure 3.3B) where T_{max} reached 32 ^{o}C. Under SN, more than 25 % of *dTRPA1^{ablated}* flies (blue curves) and their parental controls (grey and black curves) displayed small A-peaks (Figure 3.3A, left panels; Table 3.3). However, under DD+SN conditions, it was not possible to determine if individual flies exhibit A-peak due to high activity displayed by flies throughout the day; hence, they have not been mentioned in Table 3.3. Also, under DD+SN, among the control flies only *UAS-hid* control flies exhibit an enhanced E-peak, which could be due to leaky expression of *hid* resulting in loss of cells that influence the E-peak (Figure 3.3B, left panel). Similarly, flies with downregulation of dTRPA1 via RNAi knockdown in neurons targeted by *dTRPA1^{SH}-GAL4* (*dTRPA1^{RNAi}*) also exhibit A-peak under both SN (Figure 3.3A, middle panel; Table 3.3) and DD+SN (Figure 3.3B, middle panel) regimes. These results indicate that while complete lack of dTRPA1 renders flies incapable of exhibiting the A-peak (*dTRPA1^{null}* in Figure 3.1), flies with partial reduction in dTRPA1 expression or ablation of a subset of dTRPA1 neurons targeted by *dTRPA1^{SH}-GAL4* do not phenocopy dTRPA1 nulls (*dTRPA1^{ins}* or *TRPA1$^{KI-GAL4}$*). Thus, my studies show that dTRPA1 ion channels are crucial for flies to exhibit A-peak under harsh conditions whereas dTRPA1^{SH+} neurons do not seem to be essential for A-peak. It is, however, possible that other dTRPA1 neurons, outside the target of *dTRPA1^{SH}-GAL4*, are involved in influencing A-peak behaviour.

I reasoned that if the A-peak depends on dTRPA1 expression in the dTRPA1^{SH+} neurons, A-peak would become amplified upon enhanced expression of dTRPA1 in these thermosensory neurons. dTRPA1 was over-expressed under *dTRPA1^{SH}-GAL4* (henceforth, *dTRPA1^{oex}*) and these flies show A-peak not significantly higher than controls (Figure 3.3A, right panel; Table 3.3). I speculate that the bright light conditions dampened the A-peak

expected of *dTRPA1^{oex}* flies. This hypothesis is supported by tests under DD+SN where *dTRPA1^{oex}* flies show a strikingly exaggerated A-peak with high amplitude (Figure 3.3B, right panel, arrow). Increasing the expression level of dTRPA1 is likely to enhance the firing rate of these neurons at temperatures of 25-27 ^{o}C and higher (Hamada et al., 2008; Tang et al., 2013), which I propose results in the induction of the A-peak. Over-expression of dTRPA1 also delays E-peak of *dTRPA1^{oex}* flies (Figure 3.3B, right panel - arrowhead; one-way ANOVA with genotype as factor, $F_{2,69} = 244.24$, $p < 0.05$).

dTRPA1 expression is required to elicit A-peak under simulated natural conditions.

Activity /rest behaviour of flies under SN conditions can be complex to understand since there are multiple environmental factors that influence their behaviour. Further, as seen above, my results show that even cyclic light cues are blocked (DD+SN conditions), when maximum temperature is ~32 ^{o}C, wild-type flies display A-peak, suggesting that temperature alone can elicit A-peak. However, in natural conditions, influence of other physical factors such as humidity and other unknown factors cannot be ruled out. I wanted to examine if A-peak is predominantly a temperature driven response and if yes, which kind of temperature regime would elicit A-peak in flies. To tease apart the role of light and temperature in influencing A-peak, I studied the activity/rest pattern of flies in response to gradually cycling light and temperature conditions, first in combination and then in isolation. To mimic aspects of natural light and temperature in the laboratory, flies were exposed to gradually changing light intensity cycles or ramped light cycles (L_r) and / or temperature cycles (T_r) by a series of controlled steps using incubators in the laboratory (Figures 3.4 – 3.11). Since the maximum temperature (T_{max}) reached in SN in most experiments was around 32 ^{o}C, the temperature cycles were calibrated to reach a T_{max} = 32 ^{o}C (Figure 3.4A, pink curves). Under such a regime, both light and temperature gradually increased and decreased in-phase (L_r+T_{r32} – in-

phase). Under such simulated conditions in the laboratory, 100 % of wild-type (w^{1118}) flies exhibit the A-peak (Table 3.4) but less than 25 % of $dTRPA1^{ins}$ and $TRPA1^{KI\text{-}GAL4}$ flies exhibit the A-peak (Table 3.4; Figure 3.4A, top-left panel; Figure 3.5A, top row). Henceforth, only $dTRPA1^{ins}$ null flies are shown for comparison with wild-type flies under different regimes unless specified. Further, by comparing percentage activity of flies, I find that $dTRPA1^{ins}$ flies show significantly reduced activity compared to w^{1118} flies during 1hr of T_{max} (Figure 3.4B) which is consistent with my observation that only a small fraction of null mutants exhibit small bouts of activity during T_{max} (Table 3.4). Next, I tested flies in which dTRPA1 expression was either reduced ($dTRPA1^{RNAi}$) or elevated ($dTRPA1^{oex}$) using the $dTRPA1^{SH}$-*GAL4* driver under the simulated L_r+T_{r32} regime in the laboratory where wild-type flies exhibited a clear A-peak (Figure 3.4A, middle and right panels, Table 3.4). Perhaps for technical reasons (e.g. a residual effect of remaining protein expression or incomplete knockdown by RNAi) RNAi knockdown does not phenocopy the null mutant, and $dTRPA1^{RNAi}$ flies exhibit an A-peak (Figure 3.4A, middle panel-blue curve, Table 3.4) and resemble their parental controls (Figure 3.4B; Figure 3.5A, middle row- actograms). Further, overexpression of dTRPA1 also results in an A-peak coinciding with T_{max} (Table 3.4, Figure 3.4A, right panel; Figure 3.5A, bottom row) however, the amplitude of the A-peak of $dTRPA1^{oex}$ flies was not higher than their parental controls in these simulated natural conditions in the laboratory (Figure 3.4B, one-way ANOVA with genotype as factor, $F_{7,196} = 49.6$, $p < 0.05$).

Subsequently the same genotypes were subjected to a similar regime where light and temperature cycles gradually changed over time, except that the T_{max} was 28 ^{o}C (L_r+T_{r28}) (Figure 3.4A, bottom row). Interestingly under this regime, w^{1118} flies also did not exhibit A-peak similar to $dTRPA1^{ins}$ null mutant (Figure 3.4A, bottom left; Figure 3.5A - actograms,

Table 3.4). Similarly, *dTRPA1*^{RNAi} and *dTRPA1*^{oex} flies also do not exhibit A-peak under L_r+T_{r28} and thus, resemble their respective controls (Figure 3.4A, bottom row). The above results suggest that T_{max} beyond 28 ^{o}C is required to elicit A-peak. Thus, when gradually changing light and temperature cycles were provided with T_{max} of 32 ^{o}C, altering the expression levels of dTRPA1 in neurons targeted by *dTRPA1*^{SH}*-GAL4* does not seem to influence the occurrence of A-peak greatly whereas null mutation in dTRPA1 prevented the induction of A-peak.. Hence, my results propose that the A-peak seen under laboratory simulated natural conditions is induced depending on expression levels of dTRPA1.

A-peak exhibited by flies under laboratory simulated natural cycles is predominantly temperature driven. In order to further understand the role of dTRPA1 in mediating A-peak under the combinatorial effects of gradual light and temperature cycles, I first subjected the flies to LD (12:12) at 25 ^{o}C for three days followed by LD in combination with gradual temperature cycles (LD+T_{r32}) such that the T_{max} either occurred 3hr before Lights-OFF (LD+T_{r1}) or 3hr after Lights-ON (LD+T_{r2}) (Figure 3.5B). Wild-type (*w*^{1118}) flies exhibit an A-peak coinciding with T_{max} irrespective of the time of the T_{max} whereas *dTRPA1*^{ins} flies do not display an A-peak in response to T_{max} (Figure 3.5B). Thus, my results confirm that the A-peak seen under SN is a temperature–dependent behaviour modulated by dTRPA1. In addition, A-peak does not appear to be directly circadian clock dependent as it can be induced at different times of the day by changing the phase of T_{max} with respect to LD cycles.

In nature, light intensity often reaches its peak prior to temperature. As seen previously, under SN, wild-type flies exhibit two peaks during the day when light intensity is high - one coinciding with L_{max} and the other T_{max} (Figure 3.1). To further dissociate the potential effects of light and temperature, flies were subjected to a regime, L_r+T_{r32} (out of phase), where light intensity peak (L_{max} ~ 1700 lux) occurred 3hr prior to temperature peak

(T_{max} = 32 ^{o}C) (Figure 3.6A, B). All control genotypes show A-peak coinciding with T_{max} (Figure 3.6A, Table 3.4). However, the laboratory simulated regime could not evoke the corresponding peak with L_{max}, possibly because light intensity profile and other spectral qualities of light present in nature cannot be adequately simulated in the laboratory. As expected, most dTRPA1 null mutants did not show A-peak coinciding with either L_{max} or T_{max} (Figure 3.6A, left panel; Figure 3.6B; Table 3.4) and thus, activity levels during T_{max} is significantly lower in *dTRPA1^{ins}* flies compared to *w^{1118}* flies (Figure 3.6B). *dTRPA1^{RNAi}* flies show an attenuated A-peak (Figure 3.6A, middle panel, Figure 3.6B) and *dTRPA1^{oex}* flies show an enhanced A-peak (Figure 3.6A, right panel, Figure 3.6B) higher than their respective parental control flies (Figure 3.6B, one-way ANOVA followed by Tukey's HSD, $F_{9,250}$ = 83.25, $p < 0.05$) and the A-peak coincided with T_{max} (temperature steps above 28 ^{o}C) and not with L_{max} (Figure 3.6A). Hence, my results suggest that the A-peak seen under simulated natural conditions in the laboratory is dependent on expression levels of dTRPA1.

In a separate experiment, I imposed gradually changing light cycles at a constant temperature of 30 ^{o}C (L_r+T_{30}) on *w^{1118}* and *dTRPA1^{ins}* flies. Interestingly, *w^{1118}* flies advance their morning peak into the dark phase but *dTRPA1^{ins}* flies are unable to do so (Figure 3.6C, one-way ANOVA with genotype as factor, $F_{1,38}$ =159.2, $p < 0.05$). This is reminiscent of their behaviour under LD30 (Chapter 2, Figure 2.1). Thus, it appears that gradually changing temperature cycles reaching a T_{max} above 28 ^{o}C is required to elicit dTRPA1-dependent A-peak in flies. As seen above, temperature conditions ~32 ^{o}C for a short period (about an hr) under a gradually changing temperature cycles either in natural conditions (Figure 3.2A, DD+SN) or simulated temperature cycles (Figure 3.4, L_r+T_r) can induce A-peak in wild-type flies. However, constant warm conditions (30 ^{o}C) is not sufficient to induce A-peak, re-iterating that it is the quality of steady dynamic changes in the temperature conditions in

nature that elicits A-peak in flies. Thus, ramped temperature cycles (T_r) that change gradually and reaches a peak temperature ~32 ^{o}C induces A-peak whereas a constant temperature regime ~30 ^{o}C only shifts morning peak activity but does not result in eliciting A-peak.

A-peak can be elicited by gradual temperature cycles in absence of other time cues. To determine if dTRPA1 activation by temperature is sufficient to elicit the A-peak in the absence of all other time-cues that are normally present in nature, I simulated natural temperature cycles in the laboratory under constant darkness (DD+T_{r32}) such that temperature is the only gradually changing variable. Under DD+T_{r32}, all other genotypes show the A-peak while *dTRPA1^{ins}* flies (red curve) do not (Figure 3.7A, Table 3.4). Further, under DD+T_r, flies of all genotypes show small bouts of activity at every step-increase in temperature, with the most dramatic increase in response to the 32 ^{o}C step. However *dTRPA1^{ins}* flies respond to the increasing steps early in the day, while steps coinciding 30 ^{o}C and 32 ^{o}C have very small responses suggesting the critical need for dTRPA1 channels for this behaviour (Figure 3.7A, top-left panel). Thus, most *dTRPA1^{ins}* flies do not display A-peak under DD+T_{r32} (Table 3.4). Downregulation of dTRPA1 levels in dTRPA1^{SH+} neurons with RNAi results in a small A-peak (Figure 3.7A, top-right panel, Table 3.4) and the amplitude is attenuated compared to controls (Figure 3.7B). *dTRPA1^{oex}* flies show a prominent A-peak and enhanced activity during T_{max} compared to their parental controls (Figure 3.7A, bottom-left panel; Figure 3.7B; Table 3.4). Significant differences in A-peak amplitude are based on statistical tests (one-way ANOVA with genotype as factor, $F_{7,235}$ = 75.4, $p < 0.05$). Similarly, flies with hyperexcited dTRPA1^{SH+} neurons via expression of *UAS-NaChBac1* also exhibit A-peak (Table 3.4) and a delayed evening peak compared to controls (one-way ANOVA with genotype as factor, $F_{2,73}$ = 29.8, $p < 0.05$) but it is

interesting to note that the amplitude of A-peak of *dTRPA1*^{hyperexcited} flies is not higher than controls (one-way ANOVA with genotype as factor, $F_{2,77} = 6.98$, $p < 0.05$) unlike *dTRPA1*^{oex} flies (Figure 3.7A, bottom-right panel). This emphasises the dependence of A-peak on dTRPA1 levels and not mere increased neuronal firing of dTRPA1^{SH+} neurons and the amplitude of the A-peak appears to depend on whether the activation of these neurons is chronic or acute. These results are similar to those obtained under DD+SN and together demonstrate that gradually changing temperature cycles which reach above 28 ^{o}C are necessary and sufficient to induce the dTRPA1 mediated A-peak.

These results were further corroborated by comparing *TRPA1*$^{KI\text{-}GAL4}$ null flies with their heterozygotes (Figure 3.8). These flies have a *Gal4* gene knocked into the *dTrpA1* promoter region, thus, making them mutants for *dTrpA1* gene but additionally allow us to drive expression under GAL4 in native dTRPA1 neurons (Kim et al., 2010). Heterozygous *TRPA1*$^{KI\text{-}GAL4/+}$ flies display A-peak like wild-type control flies (Figures 3.8A, B; Table 3.4). In contrast, homozygous *TRPA1*$^{KI\text{-}GAL4}$ null mutants do not exhibit A-peak (Figure 3.8A, left panel, violet curve, Table 3.4) suggesting the knock-in mutation is recessive and half the amount of wild-type *dTrpA1* expression is sufficient to elicit A-peak. Further, driving dTRPA1 RNAi under *TRPA1*$^{KI\text{-}GAL4}$ in the heterozygous background also does not abolish the occurrence of A-peak (Figure 3.8A, right panel, blue curve) although the amplitude of A-peak activity is significantly reduced compared to controls (Figure 3.8B, one-way ANOVA with genotype as factor, $F_{3,98} = 36.5$, $p < 0.05$).

Under constant light (LL), locomotor activity/rest behaviour of wild-type flies is arrhythmic (Fogle et al., 2015; Fogle et al., 2011; Helfrich-Forster et al., 2001; Stanewsky et al., 1998; Yoshii et al., 2004) but rectangular temperature cycles can entrain the circadian clock even in LL (Glaser and Stanewsky, 2005; Sehadova et al., 2009). To determine if the

dTRPA1 mediated A-peak can be elicited under LL conditions, flies were subjected to gradually changing temperature cycles with T_{max} = 32 ^{o}C (LL+T_{r32}). Although overall activity levels are low, wild-type (*w^{1118}*) flies exhibit a mid-day peak coinciding with T_{max} (Figure 3.9A, left panel; Figure 3.9B; Table 3.4). The null mutant, *$dTRPA1^{ins}$* flies respond to temperature step-ups below the range of dTRPA1 channel activation with small increases in activity but show no A-peak (Figure 3.9A, left panel; Figure 3.9B; Table 3.4). Although *$dTRPA1^{RNAi}$* flies also show a small A-peak coinciding T_{max} in this regime (Figure 3.9A, middle panel), the activity levels during 1hr of T_{max} are lower compared to their parental controls (Figure 3.9B). As expected, *$dTRPA1^{oex}$* flies (blue curve) show a large and significant increase in activity levels compared to controls during the1hr interval when maximum temperature is reached (T_{max}) (Figure 3.9A, right panel; Figure 3.9B). Statistical significance in A-peak amplitude were determined by performing one-way ANOVA with genotype as factor, $F_{7,214} = 77.8$, $p < 0.05$. Thus, my studies posit that expression levels of dTRPA1 modulates the amplitude of temperature-dependent A-peak under temperature cycles even in constant light conditions which otherwise disrupts molecular circadian clock cycling.

Thermosensitive isoform dTRPA1-A can rescue the occurrence of A-peak in a dTRPA1 null background. A recent study has revealed that the *dTRPA1* gene codes for four alternatively spliced isoforms –dTRPA1-A-D, out of which B and C isoforms are not temperature-responsive (Zhong et al., 2012). dTRPA1-A isoform responds to temperature range 24-29 ^{o}C whereas dTRPA1-D activates at 34 ^{o}C (Zhong et al., 2012). Since the isoforms of dTRPA1 are activated at distinct temperature ranges and dTRPA1-dependent A-peak in my experiments is seen around 32 ^{o}C (above the temperature threshold for dTRPA1-A, but below the threshold for dTRPA1-D), I aimed to distinguish which of the temperature

sensitive isoforms of TRPA1 mediates the occurrence of A-peak. I overexpressed temperature-sensitive isoform, *UAS-dTRPA1-A* and *UAS-dTRPA1-D* under *dTRPA1^{SH}-GAL4* and subjected the flies to L_r+T_{r32}, a regime that my previous results have demonstrated to adequately mimic conditions that elicit the A-peak. Overexpressing dTRPA1-A isoform or the higher temperature threshold dTRPA1-D isoform in dTRPA1^{SH+} neurons induces an A-peak (Figure 3.10A, Table 3.5) but the amplitude of A-peak is not different from both their respective parental controls (Figure 3.10B, one-way ANOVA, $F_{4,74} = 20.03$, $p < 0.05$). Next, I attempted to rescue dTRPA1 expression in neurons targeted by *dTRPA1^{SH}-GAL4* in the null *dTRPA1^{ins}* background by either expressing *UAS-dTRPA1-A* or *UAS-dTRPA1-D*. Rescue using *UAS-dTRPA1-A* isoform caused an enhanced A-peak in the *dTRPA1^{ins}* background (Figure 3.11A, left panel; Figure 3.11B, right panel -one-way ANOVA, $F_{4,142} = 66.9$, $p < 0.05$; also see Table 3.5). In contrast, expressing *UAS-dTRPA1-D* isoform in the null background is not able to rescue A-peak phenotype but shows a small bout of activity similar to its parental controls in a null background (Figure 3.11A, right panel, compare green curves with grey and black curves). In this assay, a small fraction of all parental controls in null background displayed A-peak (Figure 3.11A, grey and black curves, Table 3.5). Based on these results, I conclude that the dTRPA1-A isoform is critical for the induction of A-peak under the simulated SN conditions since restoring functional isoform A in neurons targeted by *dTRPA1^{SH}-GAL4* in a null background is sufficient to elicit A-peak.

Discussion

Insects exhibit many physiological adaptations to high temperature and low humidity in nature. In addition to modulating respiration and metabolism, behavioural modulation is an important adaptive response that requires temperature sensing. Several recent studies show that under semi-natural conditions, flies exhibit an additional peak of activity during

mid-day that could be a reflection of an 'escape response' or an 'environmentally modulated circadian' response to hot daytime temperatures (De et al., 2013; Menegazzi et al., 2012; Vanin et al., 2012). Although rhythmic regulation of activity in fruit flies under natural conditions remains far from understood, recent studies suggest that the three activity peaks (M, A and E) are modulated by both environmental factors and circadian clocks (Menegazzi et al., 2012; Vanin et al., 2012), while other studies including previous reports from our group posit that non-clock mechanisms can also induce the A-peak (De et al., 2013; Prabhakaran and Sheeba, 2014). In nature, light and temperature would be expected to modulate activity levels (Prabhakaran and Sheeba, 2013) in a complex fashion.

My study shows that under complex semi-natural conditions of fluctuating light, humidity and relatively high temperature maxima, dTRPA1 mediates the afternoon peak of activity. Although previous studies posit that the A-peak reflects escape responses of flies to high temperature conditions (Menegazzi et al., 2012), the underlying molecular mechanisms that control the A-peak remained unknown till my studies demonstrated a temperature-dependent mechanism for the A-peak response exhibited by flies under SN. I find that *dTRPA1* null mutants do not exhibit the A-peak and that its amplitude is a function of dTRPA1 expression levels. Similar findings have been reported by Kyriacou and Costa group (Green et al., 2015). Dependence of the A-peak on dTRPA1 was further confirmed by studies in the laboratory under controlled simulated natural light and temperature conditions. Female flies were also subjected to simulated natural conditions (L_r+T_{r32}) in parallel to their male counterparts, and I find that female dTRPA1 null flies also do not exhibit A-peak in contrast to female w^{1118} flies which show a prominent A-peak (data not shown). My studies reveal that dTRPA1 receptor-mediated signalling pathway adaptively regulates locomotor activity in response to temperature. Hence, I propose that A-peak of activity observed under SN is a temperature sensitive response in flies that is elicited through dTRPA1 receptor

signalling. This exemplifies yet another of the diverse sensory functions of the evolutionarily conserved TRP family of ion channels (Montell, 2011).

Behaviour of flies under semi-natural conditions has been studied extensively by our research group. In a previously study from the laboratory, our colleagues performed visual observations to examine the behaviour of flies under SN conditions placed in different spatial arenas including glass tubes used for measuring locomotion and also larger arenas such as petri-dishes (De et al., 2013). These studies suggested that flies seek shaded regions in the small glass tubes and that when they are provided larger arenas, they do not show heightened A-activity under similar environmental cycles. Later studies from our laboratory have shown that both light and temperature can induce A-peak (Prabhakaran and Sheeba, 2013). Furthermore, I subjected flies to simulations of natural light and / or temperature cycles in the laboratory and showed that sufficiently high maxima of either light or temperature alone can elicit A-peak (Prabhakaran and Sheeba, 2014). This is verified by my current results which show that under SN conditions, if the light and temperature peaks are clearly separated in time, two distinct A-peaks are produced, one in response to light maximum and the other in response to temperature maximum (Figure 3.1). Since the T_{max} induced A-peak can be elicited at any phase of daytime, it further confirms that it is independent of circadian clock control (Figure 3.5B). Based on our past results and my new data, our current view is that when peak daytime temperatures are relatively low, flies will tend to seek shade whereas when temperatures rise above (~29 ^{o}C) or if light intensities are sufficiently high (~ 3000 lux) then flies show more intense locomotion. Hence, dTRPA1 is critical for this temperature sensitive locomotor activity.

In most cases when temperature rises beyond a certain threshold, almost 100% wild-type flies exhibit A-peak (Tables 3.2 and 3.3), however, in some conditions, for example,

L_r+T_{r32} out-of-phase, only 61% of control *UAS-dTRPA1* flies show A-peak (Table 3.4). This suggests that either the thermal sensation is not conveyed to those cells that eventually control motor activity, or that the neuronal circuits in those flies require to cross a higher threshold in order to produce this behaviour. Under experimental regimes such as DD+SN, DD+T_{r32} and LL+T_{r32}, wild-type flies display a clear A-peak whereas dTRPA1 null flies do not exhibit an A-peak. The above results demonstrates that gradual temperature cycles with adequate contrast in temperature range and with a peak temperature around 32 ^{o}C is sufficient to elicit A-peak response and supports my hypothesis that dTRPA1 mediated A-peak is temperature dependent. However, RNAi knockdown of dTRPA1 or ablating neurons under *dTRPA1^{SH}-GAL4* driver does not completely abolish occurrence of A-peak under SN, DD+SN or simulated SN regimes in the laboratory. The above results can be explained most parsimoniously by the narrow expression pattern of *dTRPA1^{SH}-GAL4* driver that may not encompass the full range of cells in which dTRPA1 is natively expressed. Alternatively it is also likely that gradual changes in temperature profile under SN or simulated SN laboratory protocols activates other thermoreceptors that function in temperature ranges adjacent to dTRPA1 activation. Under such conditions, even flies with attenuated dTRPA1 function (*dTRPA1^{RNAi}* or *dTRPA1^{ablated}* flies) are likely to be able to respond to high temperatures during mid-day. This phenomenon can also be seen in *dTRPA1^{ins}* null flies that show short bouts of activity in response to temperature shifts under LL+T_{r32} (Figure 3.9) and more clearly under DD+T_{r32} (Figure 3.7) while not responding to temperature shifts from 28 -30 ^{o}C or 30 -32 ^{o}C. Further, since *dTRPA1^{ablated}* flies also display A-peak, it is possible that dTRPA1 expression in neurons outside the target of *dTRPA1^{SH}-GAL4* maybe more crucial to elicit A-peak and dTRPA1^{SH+} neurons may not be essential for A-peak.

The A-peak seen under SN or gradually changing light and temperature cycles in the laboratory is likely to be a reflection of the fly's sensation of adverse warm temperatures and its attempts to escape from it. In my studies, flies lacking dTRPA1 are perhaps unable to perceive or respond to temperature changes beyond 28 ^{o}C and consequently do not exhibit the A-peak. Over-expressing dTRPA1 in a small subset of dTRPA1^{SH+} neurons renders the flies acutely thermosensitive. This provides direct evidence for the involvement of dTRPA1 in mediating the A-peak. This specific modification also causes a delay in the phase of the E-peak. Since E-peak is not a mere response to temperature, but a circadian clock-controlled activity, and flies are likely to compensate (homeostatically) for the increased activity of the afternoon with a depression in activity in the evening, I speculate that both factors come into play in modifying E-peak phase in these flies. Chronic activation of these neurons (using *UAS-NaChBac*) did not amplify the A-peak suggesting compensatory or buffering mechanisms in the circuits that regulate the amplitude of this peak. This likely occurs by modulation of downstream neurons that receive multiple inputs from temperature and circadian circuits and future studies may reveal the pathways by which thermosensors influence rhythmic locomotor activity. While RNAi knockdown in dTRPA1^{SH+} neurons or ablation of these neurons does not abolish A-peak, overexpression of dTRPA1 in the same dTRPA1^{SH+} neurons results in enhanced A-peak. Taken together, my studies suggest that dTRPA1^{SH+} neurons are important and overexpression of dTRPA1 in these cells results in major behavioural modification, however, in absence of signals from these neurons, possibly other dTRPA1 neurons are able to override lack of signals from dTRPA1^{SH+} neurons.

While dTRPA1 ion channels have been implicated in one study as mediating thermal nociception (Neely et al., 2011), it has been mostly studied in the context of thermotaxis at a much lower temperature range (24-29 ^{o}C) (Hamada et al., 2008; Kang et al., 2012; Kwon et

al., 2008; Rosenzweig et al., 2005; Rosenzweig et al., 2008; Tang et al., 2013; Zhong et al., 2012). dTRPA1 is an ideal candidate for mediating behavioural changes in response to a range of temperatures more likely to be encountered in the natural habitat of flies where they exhibit diurnal rhythms. Further, the two temperature-responsive isoforms dTRPA1-A and dTRPA1-D have activation ranges of 24-29 ^{o}C and 34 ^{o}C respectively (Zhong et al., 2012). I find that rescue of dTRPA1 function in dTRPA1^{SH+} neurons in *dTRPA1^{ins}* background with A isoform is sufficient to rescue the induction of A-peak, once again suggesting that these neurons modulate A-peak. However, D isoform, that is activated at a temperature range slightly above the T_{max} of my experimental regime does not rescue the A-peak. Future studies that examine the response of flies to gradual temperature cycles at a higher T_{max} (~34 ^{o}C) could shed light on whether isoform D is also able influence amplitude of A-peak when overexpressed under *dTRPA1^{SH} GAL4* or able to rescue A-peak phenotype in a null background. Also, one cannot rule out the possibility that dTRPA1 isoforms are differentially expressed under the *dTRPA1^{SH}-GAL4* driver. However, since the activation range of the dTRPA1-D isoform is higher than T_{max} used in my experiment, I think it is unlikely that the expression levels of the isoforms affect the behavioural phenotype. The above results reiterate my findings that A-peak is predominantly a temperature driven phenomenon and is dependent on dTRPA1-A.

Under SN or even DD+SN conditions, several factors including temperature possibly contribute to the generation of the A-peak. Of these, dTRPA1 seems to be critical, however, the cells in which dTRPA1 must be expressed is not revealed by the *dTRPA1^{SH}-GAL4* driver. It is clear however that its expression in the cells targeted by this driver is sufficient for the behaviour (Figure 3.11). Taken together, I speculate that among the dTRPA1-expressing cells, those targeted by the *dTRPA1^{SH}-GAL4* alone can generate a temperature-dependent

peak. However, eliminating them does not prevent A-peak since there are other cells outside of the target of this driver that are sufficient to elicit the A-peak. Based on my present understanding of dTRPA1 functions I propose that under semi-natural conditions when gradually increasing temperature reaches a mid-day peak above 28 ^{o}C, dTRPA1^{SH+} neurons likely activate downstream neurons that enhance locomotor activity, facilitating escape from stressful conditions. On the other hand, lack of dTRPA1 renders flies unable to sense warm temperatures and hence dTRPA1 null flies do not exhibit A-peak. Therefore, I posit that on hot days, dTRPA1 mediated increased locomotion may enable flies to escape stressfully hot conditions.

Table 3.1

***GAL4* drivers**	**Expression Pattern**
dTRPA1^{SH}-GAL4	Targets a subset of dTRPA1 neurons (~ 30 cells). In text, referred to as dTRPA1^{SH+} neurons
TRPA1^{KI}-GAL4	Targets a larger subset of dTRPA1 neurons (~70 cells)
Pdf-GAL4	Targets circadian ventro-lateral neurons (LN_vs) that produce a neurotransmitter Pigment Dispersing Factor (PDF)
cry-GAL4	Targets subset of circadian neurons expressing blue-light sensitive photoreceptor CRYPTOCHROME (CRY)

Table 3.1. List of *GAL4* drivers described in this chapter and their expression pattern.

Table 3.2

Details of experimental regimes

Regime	Light condition	Temperature condition	Phase difference
L_r +T_{r32} (In-phase)	Gradually changing (ramped) light intensity cycles reaching a peak of 1800 lux	Gradually changing (ramped) temperature from 17 ^oC to 32 ^oC	0 hr (In-phase)
L_r +T_{r32} (out of-phase)	Gradually changing (ramped) light intensity cycles with peak of 1800 lux	Gradually changing (ramped) temperature from 17 ^oC to 32 ^oC	Light peak 3 hr prior to temperature peak
L_r +T_{r28} (In-phase)	Gradually changing (ramped) light intensity cycles reaching a peak of 1800 lux	Gradually changing (ramped) temperature from 17 ^oC to 28 ^oC	0 hr (In-phase)
LL_{100}+T_{r32}	Constant light intensity of 100 lux	Gradually changing (ramped) temperature from 17 ^oC to 32 ^oC	NA
DD+T_{r32}	Constant darkness (DD -0 lux)	Gradually changing (ramped) temperature from 17 ^oC to 32 ^oC	NA
LD+T_{21}	LD (12hr :12hr) with Light-On at 10:00hrs Lights-OFF –at 22:00hrs	Constant temperature of 21 ^oC	NA
LD+T_{r1}	LD (12hr :12hr) with Light-On at 10:00hrs Lights-OFF –at 22:00hrs	Gradually changing (ramped) temperature from 17 ^oC to 32 ^oC with T_{max} at 6 hr after Lights-ON (16:00 hrs)	NA
LD+T_{r2}	LD (12hr :12hr) with Light-On at 10:00hrs Lights-OFF –at 22:00hrs	Gradually changing (ramped) temperature from 17 ^oC to 32 ^oC with T_{max} at 3 hr after Lights-ON (13:00 hrs)	NA
LD+T_{r3}	LD (12hr :12hr) with Light-On at 10:00hrs Lights-OFF –at 22:00hrs	Gradually changing (ramped) temperature from 17 ^oC to 32 ^oC with T_{max} at 6 hr before Lights-ON (04:00 hrs)	NA

NA = not applicable

L_r – ramped light intensity cycles, LL100 – constant light conditions with 100 lux light intensity, LD – 12:12hr light / dark cycles, DD – constant dark conditions

T_{r32} – ramped temperature cycles with maximum temperature step of 32 ^oC, T_{r28} - ramped temperature cycles with maximum temperature step of 28 ^oC, T_{21} – constant temperature of 21 ^oC

T_{r1} T_{r2} T_{r3} – ramped temperature cycles wherein the T_{max} step occurred at different times of the day

L_r+T_{r32} (in phase)

Local time (hr)	Light intensity scale (UML –Percival)	Temperature (^oC)
04:00 – 06:00	0	17
6:00 – 06:15	7	17
06:15 – 06:45	10	17
06:45 – 07:00	10	17
07:00 – 07:30	20	21
07:30 – 08:00	25	23
8:00 – 8:30	30	23
8:30 – 09:00	35	23
9:00 – 10:00	40	26
10:00 – 11:00	50	26
11:00 – 12:00	60	28
12:00 – 13:00	80	30
13:00 – 14:00	100	32
14:00 – 15:00	80	30
15:00 – 16:00	50	28
16:00 – 17:00	30	26
17:00 – 17:30	20	28
17:30 – 17:45	10	28
17:45 – 18:00	7	26
18:00 – 22:00	0	23
22:00 – 04:00	0	21

Note: The same temperature protocol was imposed in $LL_{100}+T_{r32}$ where light was 100 lux throughout. Under $DD+T_{r32}$ regime, the above gradual temperature cycles were imposed under constant darkness.

L_r+T_{r32} (out of phase)

Local time (hr)	Light intensity scale (UML –Percival)	Temperature (^oC)
04:00 – 06:00	0	17
06:00 – 06:15	7	20
06:15 – 06:45	10	20
06:45 – 07:00	15	21
07:00 – 07:30	20	21
07:30 – 08:00	25	21
08:00 – 8:30	30	23
08:30 – 09:00	35	23
09:00 – 10:00	40	23
10:00 – 10:30	50	26
10:30 – 11:00	60	26
11:00 – 12:00	80	26
12:00 – 13:00	100	26
13:00 – 14:00	80	28
14:00 – 15:00	60	30
15:00 – 16:00	50	32
16:00 – 17:00	30	30
17:00 – 17:30	20	28
17:30 – 18:00	15	28
18:00 – 18:30	10	28
18:30 – 19:00	1	26
19:00 – 20:00	0	26
20:00 – 22:00	0	23
22:00 – 04:00	0	21

Table 3.3

Regime	Genotype	N	% flies exhibiting A-peak
SN	**L_{max} 325 lux**		
	w^{1118}	28	53
	$dTRPA1^{ins}$	27	11
	$TrpA1^{1}$	29	3
	L_{max} 680 lux		
	w^{1118}	30	78
	$dTRPA1^{ins}$	15	13.3
	$TrpA1^{KI\text{-}GAL4}$	14	17.6
	L_{max} 1759 lux		
	w^{1118}	28	84.2 (L_{max} -88%; T_{max} -67%)
	$dTRPA1^{ins}$	16	57.8 (L_{max} -91%; T_{max} -13%)
	$TrpA1^{KI\text{-}GAL4}$	20	70.3(L_{max} -100%; T_{max} -16%)
SN	$dTRPA1^{SH}$-GAL4/+	22	54.5
	$dTRPA1^{SH}$-GAL4/ UAS-hid	18	55.5
	UAS-hid/+	15	73.3
	$dTRPA1^{SH}$-GAL4/ UAS-dcr $dTRPA1^{RNAi}$	18	50
	UAS-dcr $dTRPA1^{RNAi}$ / +	14	42.8
	$dTRPA1^{SH}$-GAL4/ UAS-dTRPA1	15	73.3
	UAS-dTRPA1/ +	19	84.2
DD+SN	**T_{max} 32 ^{o}C**		
	w^{1118}	28	85.7
	$dTRPA1^{ins}$	12	NA
	$TrpA1^{KI\text{-}GAL4}$	25	NA

Table 3.3: Percentage of flies from different genotypes exhibiting A-peak across SN and laboratory simulated natural cycles of light and temperature regimes. Under SN conditions, when L_{max} =1759 lux, percentage of flies from each genotype responding with an activity peak either to L_{max} or T_{max} has been shown in brackets. Under DD+SN, distinct A-peaks could not be determined for most genotypes because of high activity exhibited by flies throughout the day. NA = not applicable, L_{max} = maximum light intensity, T_{max} = maximum temperature.

Table 3.4

Regime	Genotype	N	% flies exhibiting A-peak
L_r+T_{r28}	*w^{1118}*	16	6.25
	$dTRPA1^{ins}$	23	4.3
L_r+T_{r32}	*w^{1118}*	28	100
	$dTRPA1^{ins}$	27	14.8
	$TrpA1^{KI\text{-}GAL4}$	26	3.8
	$dTRPA1^{SH}$-GAL4/ UAS-dcr $dTRPA1^{RNAi}$	24	87.5
	$dTRPA1^{SH}$-GAL4/ UAS-dcr	25	100
	UAS-dcr $dTRPA1^{RNAi}$ / +	26	100
	$dTRPA1^{SH}$-GAL4/ UAS-dTRPA1	27	100
	$dTRPA1^{SH}$-GAL4/ +	23	100
	UAS-dTRPA1/ +	28	100
L_r+T_{r32} (out of phase)	*w^{1118}*	30	100
	$dTRPA1^{ins}$	26	11.5
	$dTRPA1^{SH}$-GAL4/ UAS-dcr $dTRPA1^{RNAi}$	26	38.5
	$dTRPA1^{SH}$-GAL4/ UAS-dcr	25	100
	UAS-dcr $dTRPA1^{RNAi}$ / +	28	89.3
	$dTRPA1^{SH}$-GAL4/ UAS-dTRPA1	28	100
	$dTRPA1^{SH}$-GAL4/ +	29	100
	UAS-dTRPA1/ +	26	61.5
$DD+T_{r32}$	*w^{1118}*	30	100
	$dTRPA1^{ins}$	31	22.5
	$dTRPA1^{SH}$-GAL4/ UAS-dcr $dTRPA1^{RNAi}$	28	60.7
	$dTRPA1^{SH}$-GAL4/ UAS- dcr	29	96.5
	UAS-dcr $dTRPA1^{RNAi}$ / +	31	100
	$dTRPA1^{SH}$-GAL4/ UAS dTRPA1	32	100
	$dTRPA1^{SH}$-GAL4/ +	28	96.4
	UAS-dTRPA1/ +	31	90.3
	$dTRPA1^{SH}$-GAL4/ UAS NaChBac1	28	100
	UAS NaChBac1/+	27	100
	$TrpA1^{KI\text{-}GAL4}$ (homozygous)	24	16.7
	$TrpA1^{KI\text{-}GAL4/+}$ (heterozygous)	32	96.7
$LL+T_{r32}$	*w^{1118}*	28	100
	$dTRPA1^{ins}$	24	0
	$dTRPA1^{SH}$-GAL4/ UAS-dcr $dTRPA1^{RNAi}$	25	75
	$dTRPA1^{SH}$-GAL4/ UAS dcr	31	100
	UAS-dcr $dTRPA1^{RNAi}$ / +	30	100
	$dTRPA1^{SH}$-GAL4/ UAS-dTRPA1	29	100
	$dTRPA1^{SH}$-GAL4/ +	28	100
	UAS-dTRPA1/ +	27	100

Table 3.4: Percentage of flies exhibiting A-peak under different laboratory simulated natural cycles of light and temperature protocols.

Table 3.5

Regime	Genotype	N	% flies exhibiting A-peak
$\mathbf{L_r+T_{r32}}$	*dTRPA1^{SH}-GAL4 / UAS-dTRPA1-A*	31	100
	dTRPA1^{SH}-GAL4 / UAS-dTRPA1-D	14	100
	dTRPA1^{SH}-GAL4 / +	30	100
	UAS-dTRPA1-A/ +	32	100
	UAS-dTRPA1-D/ +	29	100
	dTRPA1^{SH}-GAL4 / UAS-dTRPA1-A; dTRPA1^{ins}	20	95
	dTRPA1^{SH}-GAL4 / UAS-dTRPA1-D; dTRPA1^{ins}	25	52
	dTRPA1^{SH}-GAL4 / +; dTRPA1^{ins}	22	31.8
	UAS-dTRPA1-A/ +; dTRPA1^{ins}	16	75
	UAS-dTRPA1-D/ +; dTRPA1^{ins}	18	77.7

Table 3.5: Percentage of flies exhibiting A-peak under gradual cycles of light and temperature when dTRPA1 isoforms are expressed in dTRPA1^{SH+} neurons in wild-type background or in *dTRPA1^{ins}* null background.

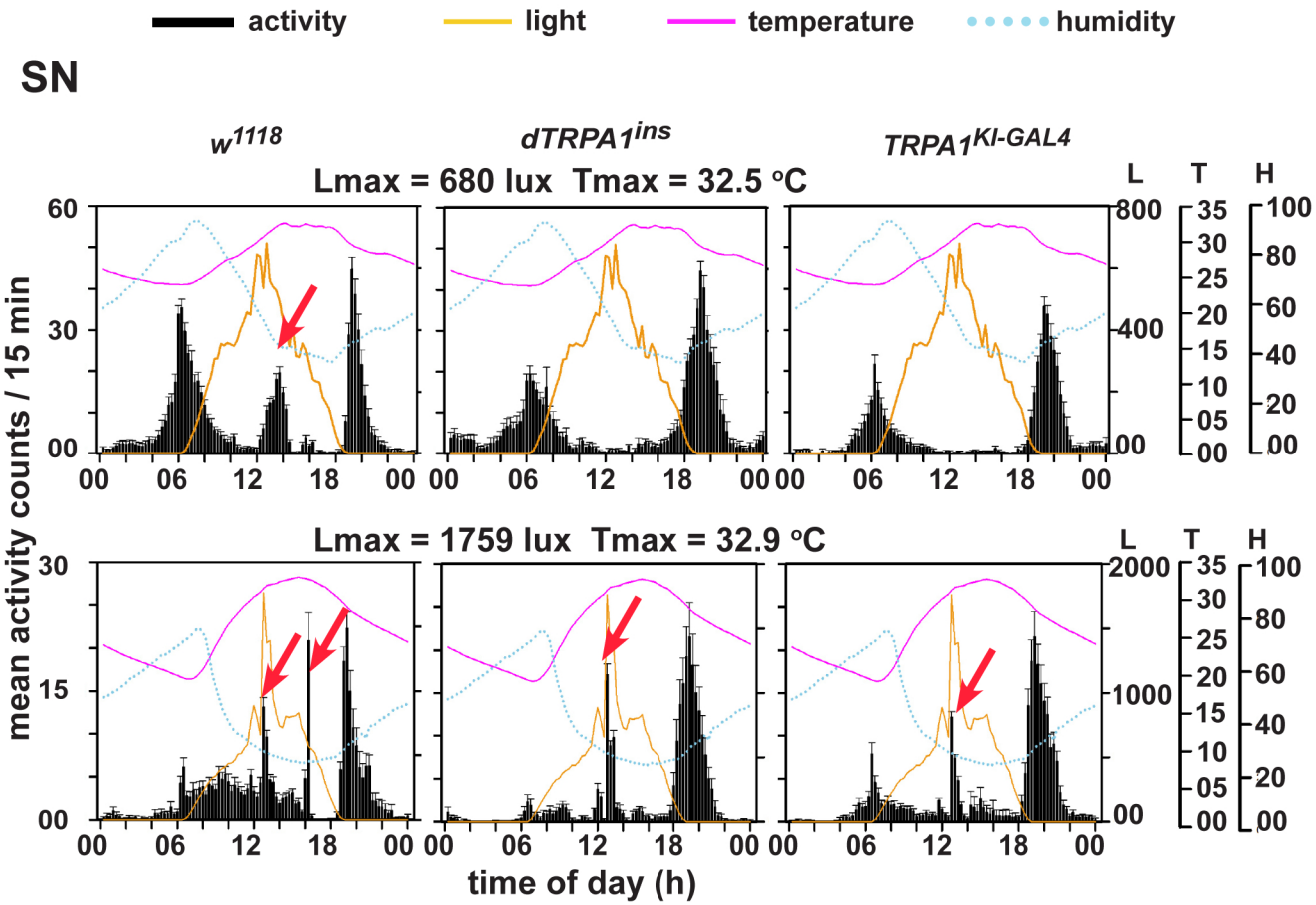

Figure 3.1. Under semi-natural conditions the mid-day A-peak depends upon dTRPA1. (Top panel) Average activity profiles of wildtype flies (w^{1118}) and two dTRPA1 null mutant lines ($dTRPA1^{ins}$ and $TRPA1^{KI\text{-}GAL4}$) under SN conditions (23rd Apr - 29th Apr, 2014) with different light intensities . Under low light (~680 lux) and high temperature, w^{1118} flies show three peaks of activity with distinct A-peak (arrow) whereas mutant flies display bimodal activity lacking the A-peak. (Bottom panel) Under high light (~1760) and high temperature SN conditions (22nd Mar - 26th Mar, 2014), w^{1118} flies show two distinct peaks in the afternoon, one coinciding with L_{max} and another with T_{max} (arrows). Interestingly, dTRPA1 nulls also show an A-peak coinciding with L_{max} (arrow) but not with T_{max}. Error bars are SEM. X-axis denotes local time where 00 hr corresponds to 00:00hr and 12hr corresponds to 12:00hr in 24hr clock format. Three axes on the right represent environmental factors - light (L-lux), temperature (T-degree Celsius) and relative humidity (H-percentage). Arrows indicate A-peak displayed by more than 25% flies in a given genotype.

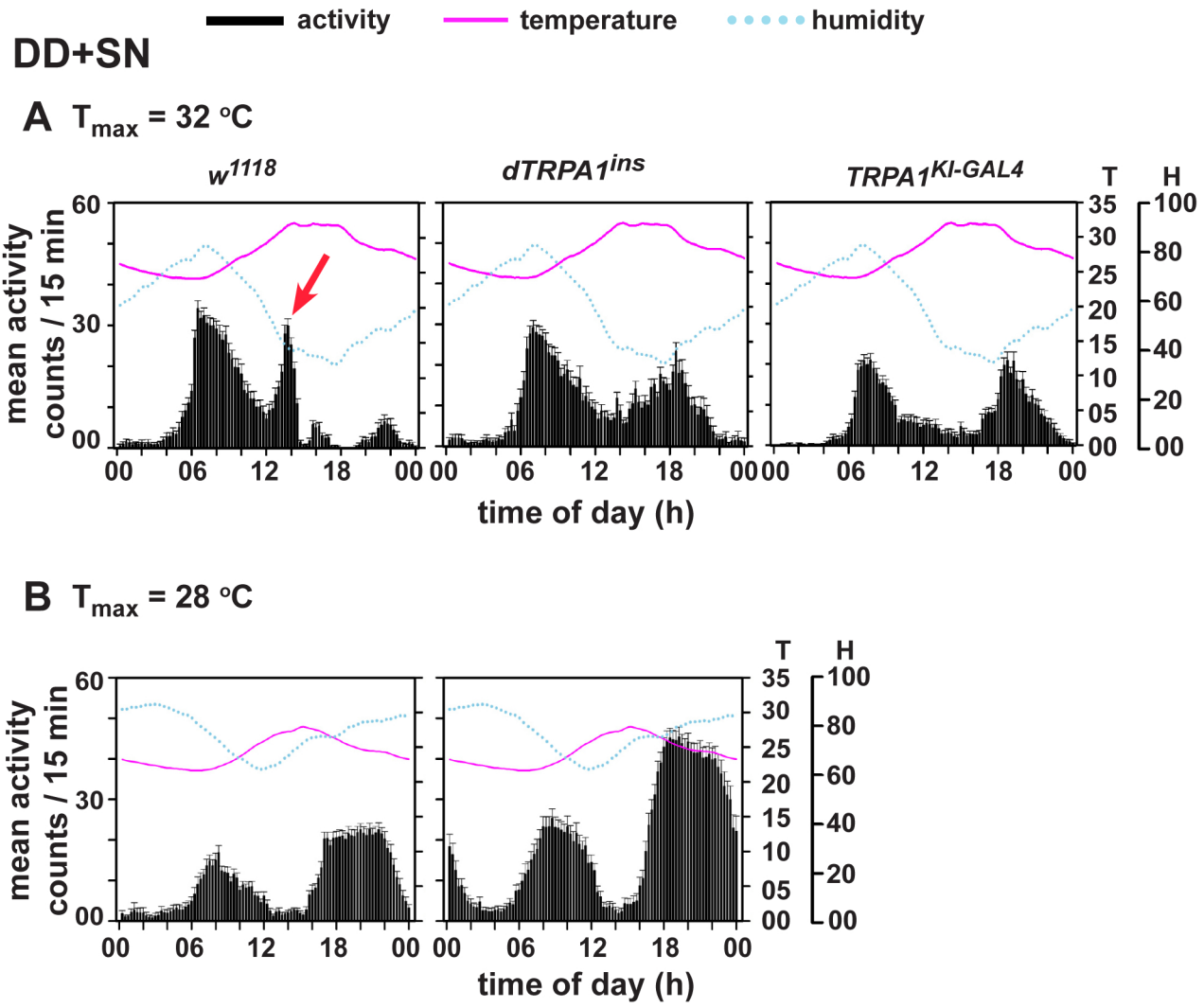

Figure 3.2. Flies display afternoon peak even in absence of light cues under semi-natural conditions (DD+SN). (A) In DD+SN, flies are exposed to gradual cycles of temperature and humidity but not light. When T_{max} was ~ 32 °C in DD+SN, w^{1118} flies display distinct A-peak (arrow) whereas dTRPA1 null flies (*dTRPA1*^{ins} and *TRPA1*$^{KI\text{-}GAL4}$) do not show A-peak. The recording was conducted between 24th April - 28th Apr, 2014. (B) When T_{max} rises only upto 28 °C in DD+SN, even w^{1118} flies do not show A-peak. dTRPA1 null flies also do not exhibit A-peak under identical conditions. Recording for data shown in panel B was conducted between 29th Sep - 4th Oct, 2012. All other details same as in Figure 3.1.

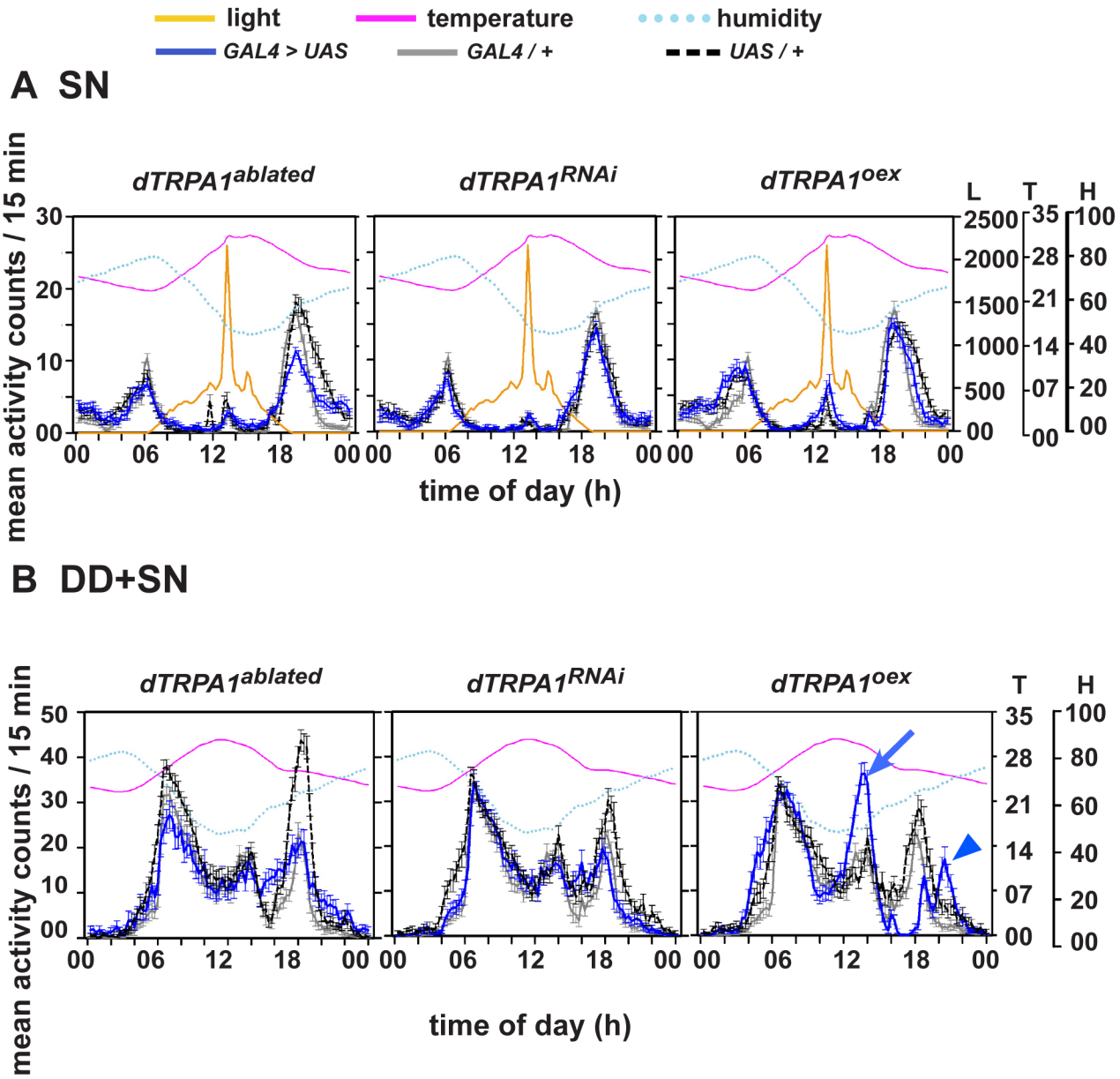

Figure 3.3. Average activity profiles of flies with altered dTRPA1 expression under semi-natural condition. (A, left) Flies with dTRPA1 ablated neurons (*dTRPA1*^{ablated} - blue curve) show A-peak under SN with very high light intensity (~2100 lux), indicating that cells outside the target of *dTRPA1*^{SH}*-GAL4* driver can mediate the occurrence of A-peak. (A, middle) Flies with RNAi knockdown of dTRPA1 (*dTRPA1*^{RNAi} - blue curve) also exhibit A-peak similar to their parental controls under SN. (A, right) Flies with dTRPA1 over-expression (*dTRPA1*^{oex}- blue curve) also show A-peaks under SN similar to their controls. (B, left) Similar to SN conditions, neuronal ablation of dTRPA1^{SH+} neurons or (B, middle) RNAi knockdown in dTRPA1^{SH+} neuronal subset did not abolish the occurrence of the A-peak under DD+SN. (B, right) dTRPA1 over-expression caused dramatic increase in the A-peak (arrow, right panel) under DD+SN and also delayed evening peak (arrowhead). The recoding for data shown in A and B panel was done between 13th May - 18th May, 2014. All other details same as Figure 3.1.

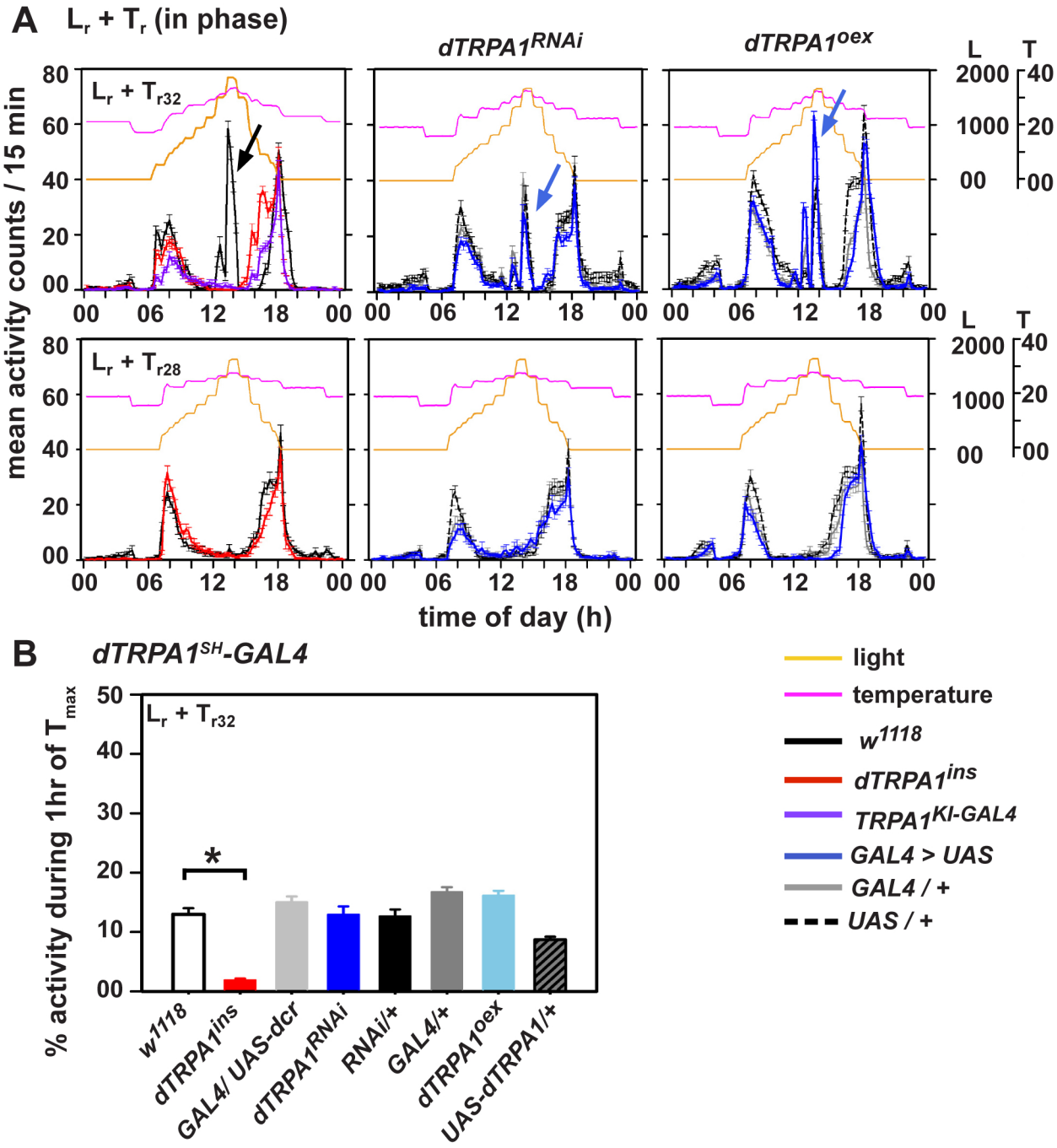

Figure 3.4. Simulating natural temperature profile in the laboratory generates dTRPA1-dependent A-peak. (A, top) Average activity profiles of flies under L_r+T_{r32} (L_{max} and T_{max} occur in phase) wherein wild-type (w^{1118}) and control flies show a prominent A-peak (arrow) but dTRPA1 null flies (*dTRPA1*^{ins}- red curve and *TRPA1*$^{KI\text{-}GAL4}$-violet curve) do not show an A-peak. (A, top-middle) Flies with dTRPA1 RNAi knockdown also display A-peak similar to controls. (A, top-right) Flies overexpressing dTRPA1 show enhanced A-peak (arrow) compared to controls. (A, bottom) Flies under simulated light and temperature protocol with reduced temperature maxima (L_r+T_{r28}). None of the genotypes exhibit A-peak under L_r+T_{r28} where T_{max} reaches only 28 ºC. (B) Percentage activity during 1 hr of T_{max} (32 ºC) when L_{max} and T_{max} occur in-phase. *dTRPA1*^{ins} flies show significantly reduced activity compared to w^{1118} flies. Light and temperature profiles are represented in yellow and pink curves and their corresponding axes are present on the right of the graphs. Time of the day (h) is plotted on X-axis where 00 hr represnts 00:00hr in 24hr clock format. Black arrow indicates that at least 25% of w^{1118} flies exhibited A-peak whereas blue arrow shows that at least 25% of experimental flies exhibited A-peak.

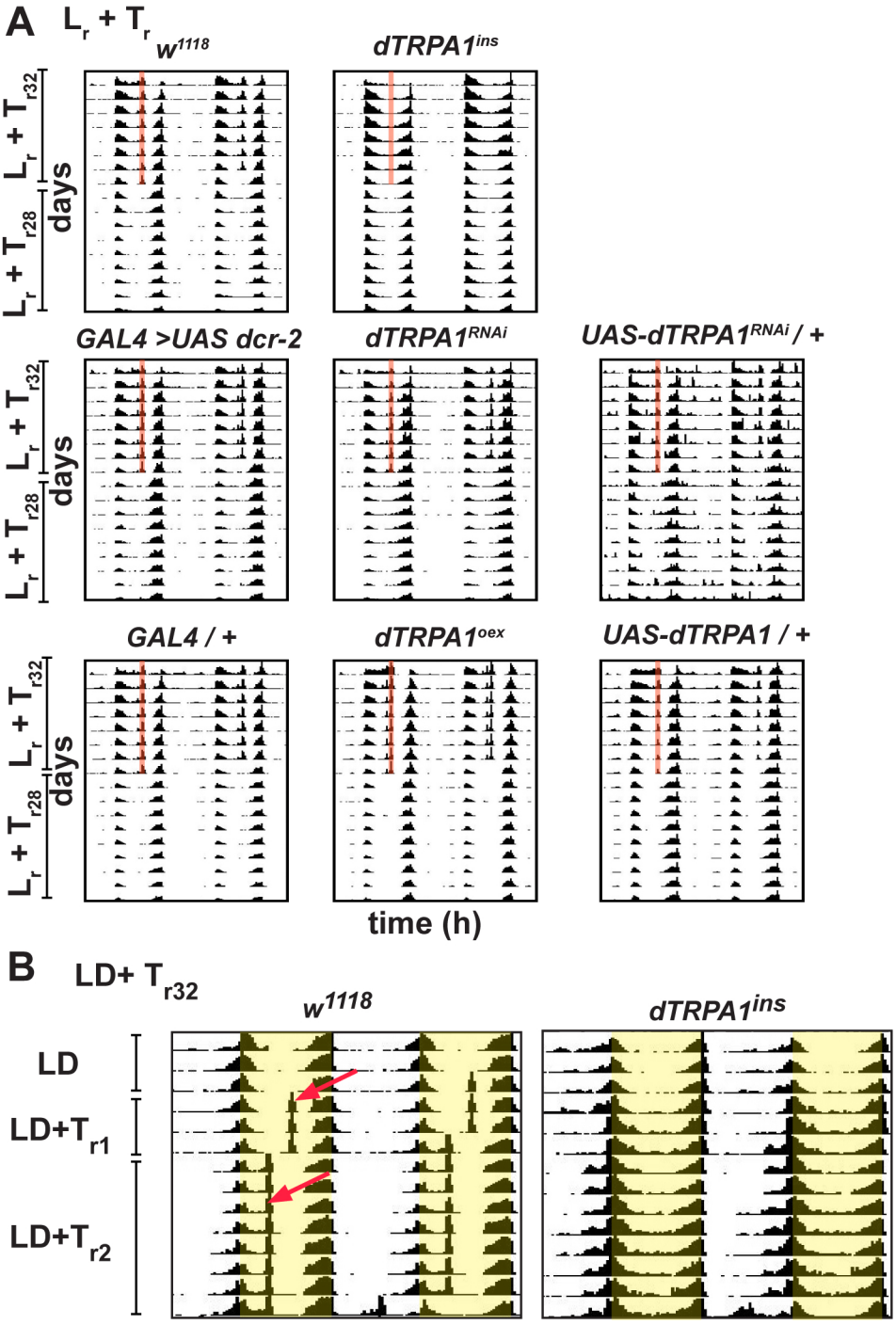

Figure 3.5. A-peak occurrence depends on dTRPA1 levels under simulated natural conditions in the laboratory. (A) Average actograms of flies exposed to L_r+T_{r32} (T_{max} = 32 °C - orange shading) followed by L_r+T_{r28} (T_{max} = 28 °C). Wild-type (*w*^{1118}) flies and all control genotypes show A-peak only during L_r+T_{r32} whereas *dTRPA1*^{ins} do not show a A-peak under either regime. (B) Induction of A-peak at different times of day under LD cycles. Flies were subjected to LD/21 °C for 3 days. Next gradual temperature cycles were imposed with T_{max} at 6hr after Lights-ON ($LD+T_{r1}$) and at 3hr after Lights-ON ($LD+T_{r2}$). *w*^{1118} flies show A-peak coinciding with T_{max} (arrows) whereas *dTRPA1*^{ins} flies did not show any A-peak. Yellow shaded area represents light phase of LD cycles.

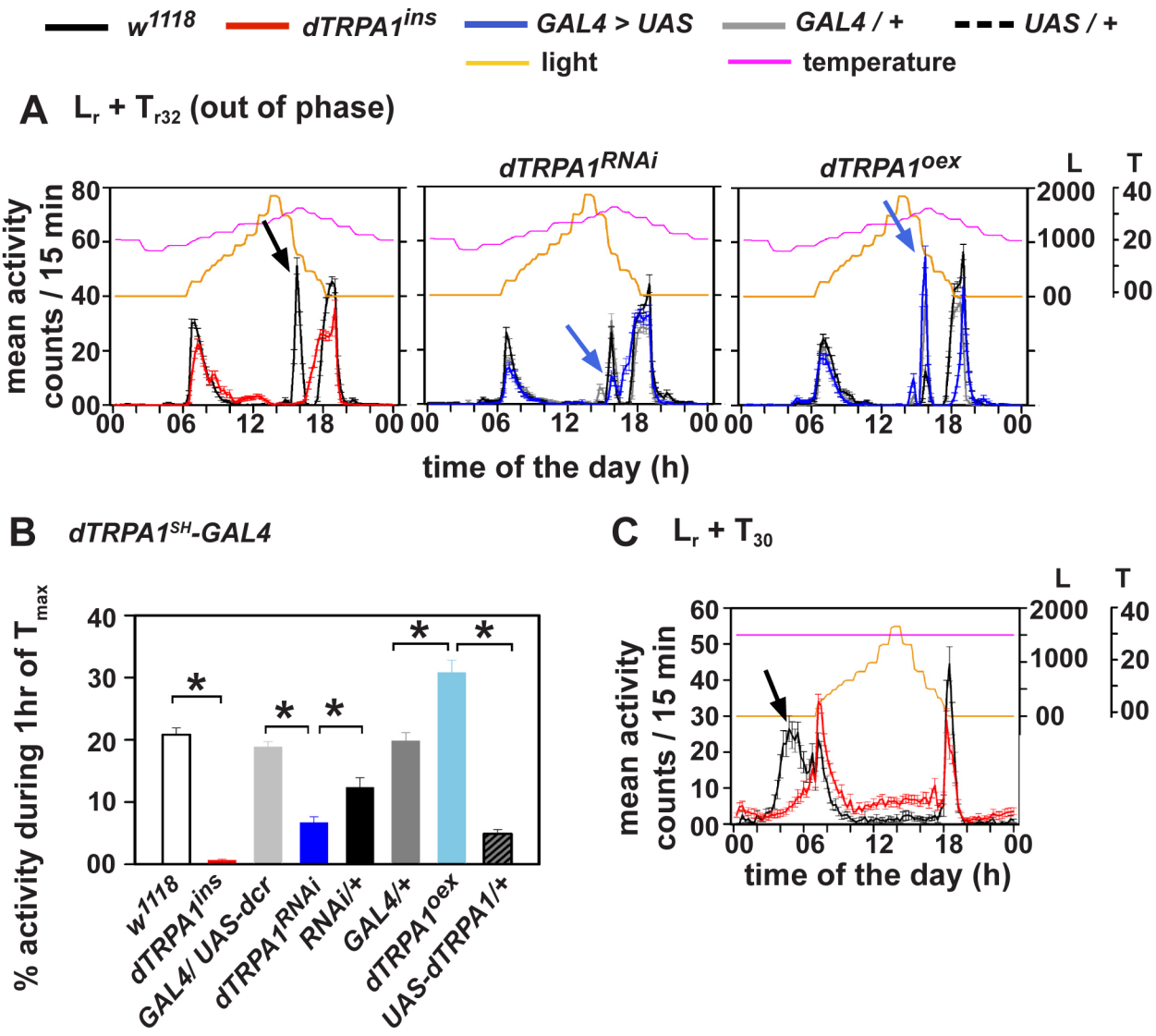

Figure 3.6. Under laboratory simulated out of phase light and temperature cycles, A-peak corresponds to T_{max}. (A, left) Activity/rest profiles depict that all control flies show an A-peak conjugated with T_{max} but not to L_{max} under out of phase L_r+T_{r32} whereas *dTRPA1^{ins}* flies do not show any A-peak. (A, middle) Flies with RNAi knockdown of dTRPA1 show an A-peak but with reduced amplitude compared to controls. (A, right) *dTRPA1^{oex}* flies show an enhanced A-peak compared to controls. (B) Percentage activity during 1 hr of T_{max} (32 °C) under L_r+T_{r32} out of phase. *dTRPA1^{ins}* and *dTRPA1^{RNAi}* flies show significantly reduced activity compared to their respective controls. A-peak amplitude of *dTRPA1^{oex}* flies is significantly higher than controls. (C) Under gradual light cycles (L_r) conditions at a constant temperature of 30 °C, *w^{1118}* flies advance their M-peak into the dark phase but *dTRPA1^{ins}* flies are unable to do so. L_r+T_{30} regime elicits similar response from wild-type flies as LD30 (compare Figure 2.1 from Chapter 2). All other experimental details are same as in Figure 3.4.

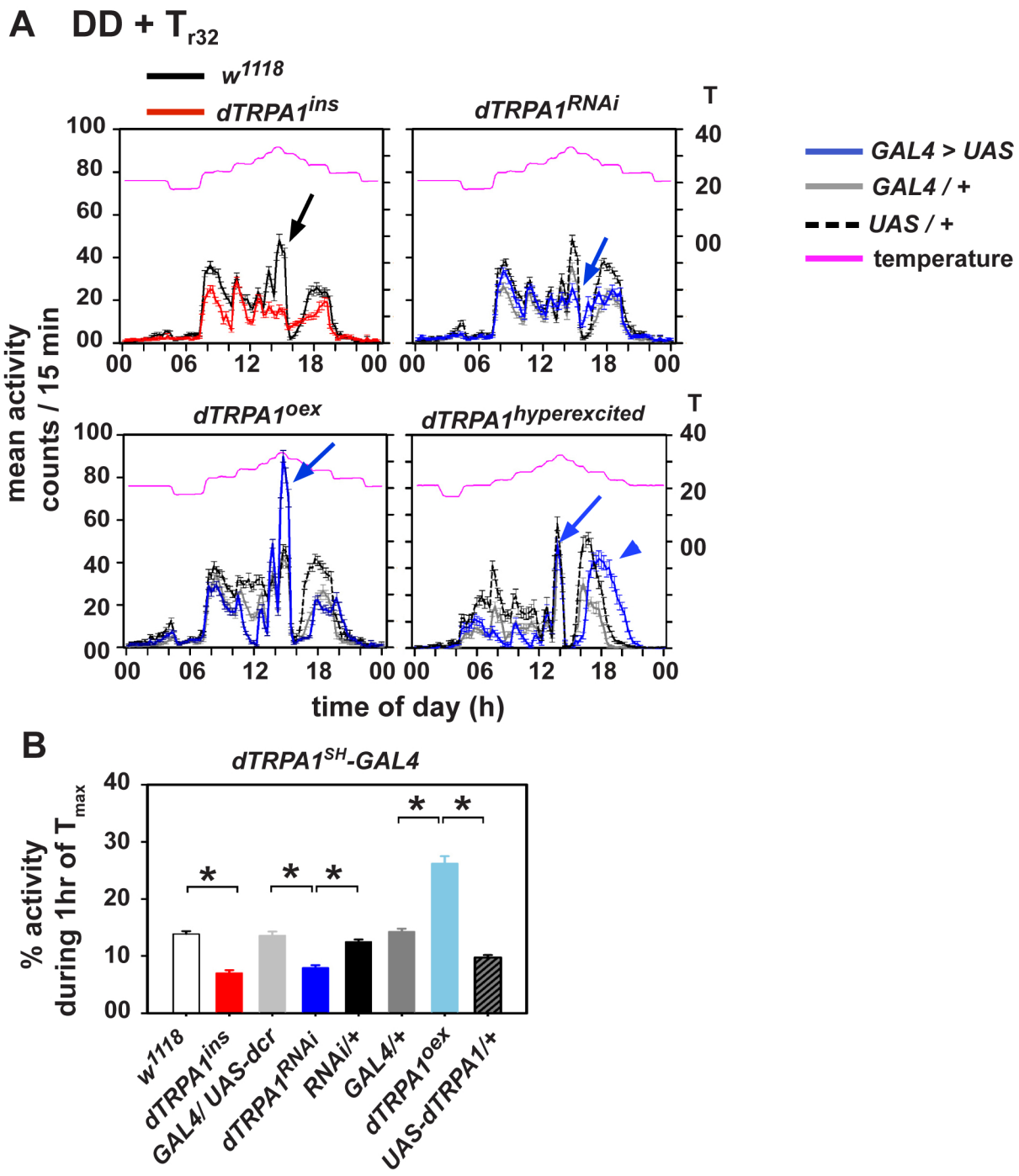

Figure 3.7. dTRPA1-dependent A-peak can be elicited by gradual temperature cycles even in absence of other time cues. (A, top-left) w^{1118} flies display A-peak whereas $dTRPA1^{ins}$ flies do not. (A, top-right) Flies with RNAi knockdown also do not show robust A-peak. (A, bottom-left) $dTRPA1^{oex}$ flies show an enhanced A-peak compared to parental controls. (A, bottom-right) Flies with hyperexcited dTRPA1^{SH+} neurons show A-peak similar to controls. (B) Percentage activity during 1 hr of T_{max} (32 ^{o}C). $dTRPA1^{ins}$ and $dTRPA1^{RNAi}$ flies show significantly reduced activity compared to controls whereas $dTRPA1^{oex}$ flies show higher A-peak activity than controls. All other details same as Figure 3.4.

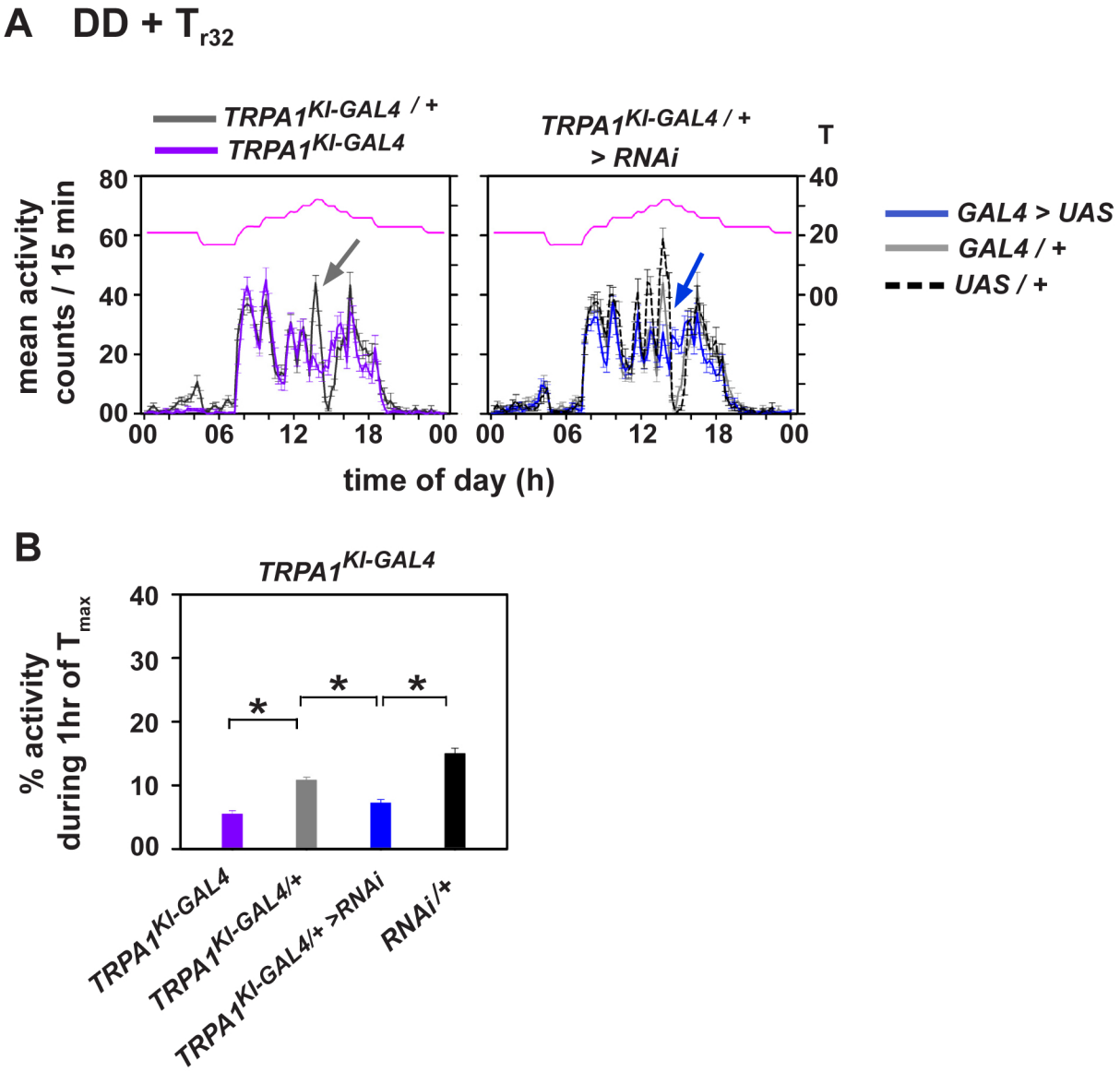

Figure 3.8. dTRPA1-dependent A-peak is also absent in another dTRPA1 mutant, *TRPA1*$^{KI\text{-}GAL4}$ under gradual temperature cycles in DD (DD+T$_{r32}$). (A, left) Heterozygous *TRPA1*^{GAL4}/+ flies (violet curve) show A-peak whereas homozygous *TRPA1*^{GAL4} null flies (grey curve) do not. (A, right) Flies driving dTRPA1 RNAi under *TRPA1*$^{GAL4/+}$ exhibit a small Apeak (blue curve). (B) Percentage activity during 1 hr of T$_{max}$ (32 ^{o}C). Homozygous *TRPA1*^{GAL4} null flies show reduced activity than heterozygous flies during 1hr of T$_{max}$. Flies with RNAi knockdown under *TRPA1*$^{GAL4/+}$ exhibit an Apeak with lower amplitude compared to both parental controls. All other details same as Figure 3.4.

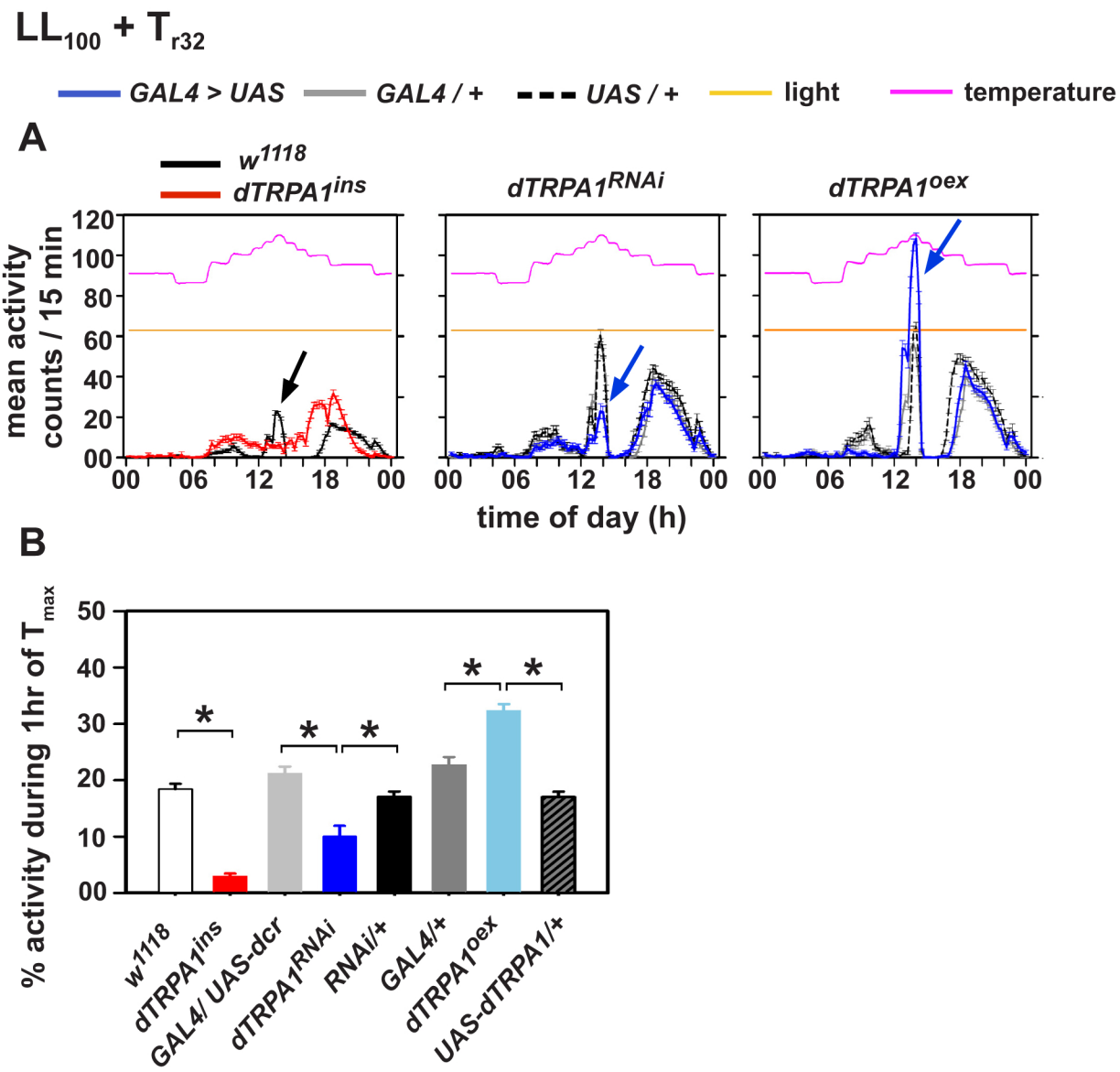

Figure 3.9. dTRPA1-dependent A-peak can be elicited by gradual temperature cycles under constant light. (A) Average activity profiles of flies in simulated natural temperature cycles under constant light (100 lux) (LL_{100}+T_{r32}). (A, left) w^{1118} flies display an A-peak while $dTRPA1^{ins}$ flies do not. (A, middle) $dTRPA1^{RNAi}$ flies show reduced A-peak whereas (A, right) $dTRPA1^{oex}$ flies show an enhanced A-peak compared to their respective parental controls. (B) Percentage activity during 1 hr of T_{max} (32 °C). $dTRPA1^{ins}$ flies showed significantly reduced activity compared to w^{1118} flies. Similarly, $dTRPA1^{RNAi}$ flies exhibit significantly lower activtity compared to controls during T_{max}. $dTRPA1^{oex}$ flies show higher A-peak activity than controls under this regime. All other details same as Figure 3.4.

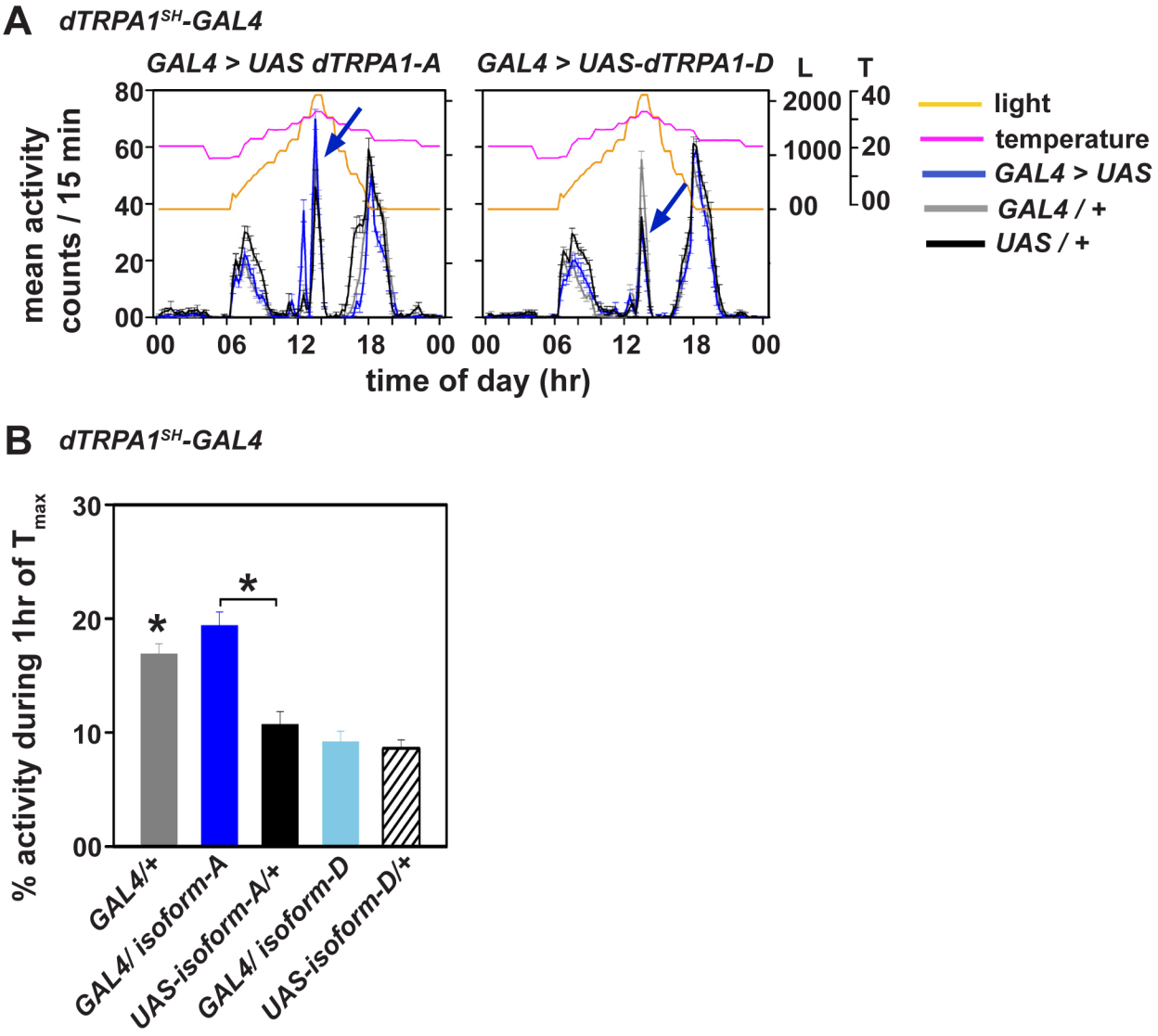

Figure 3.10. Overexpression of dTRPA1 isoforms does not alter A-peak phenotype under gradual light and temperature cycles. (A) Flies overexpressing dTRPA1 isoforms, dTRPA1-A or dTRPA1-D under *dTRPA1^{SH}-GAL4* exhibit A-peak under L_r+T_{r32}. (B) Percentage activity during 1 hr of T_{max} is used to compare amplitude of A-peak. Overexpression of dTRPA1-A (blue bar) leads to an A-peak which higher than *GAL4/+* control (grey bar) but not from *UAS/+* control (black bar) flies. Similarly, flies overexpressing of dTRPA1-D isoforms in dTRPA1^{SH+} neurons exhibits an A-peak which is lower than the *GAL4/+* control (grey bars) but it is not different from *UAS/+* control (hatched bar) flies. A-peak amplitude of *GAL4/+* control (grey bar) is different rest of the genotypes (asterisk). All other details same as Figure 3.4.

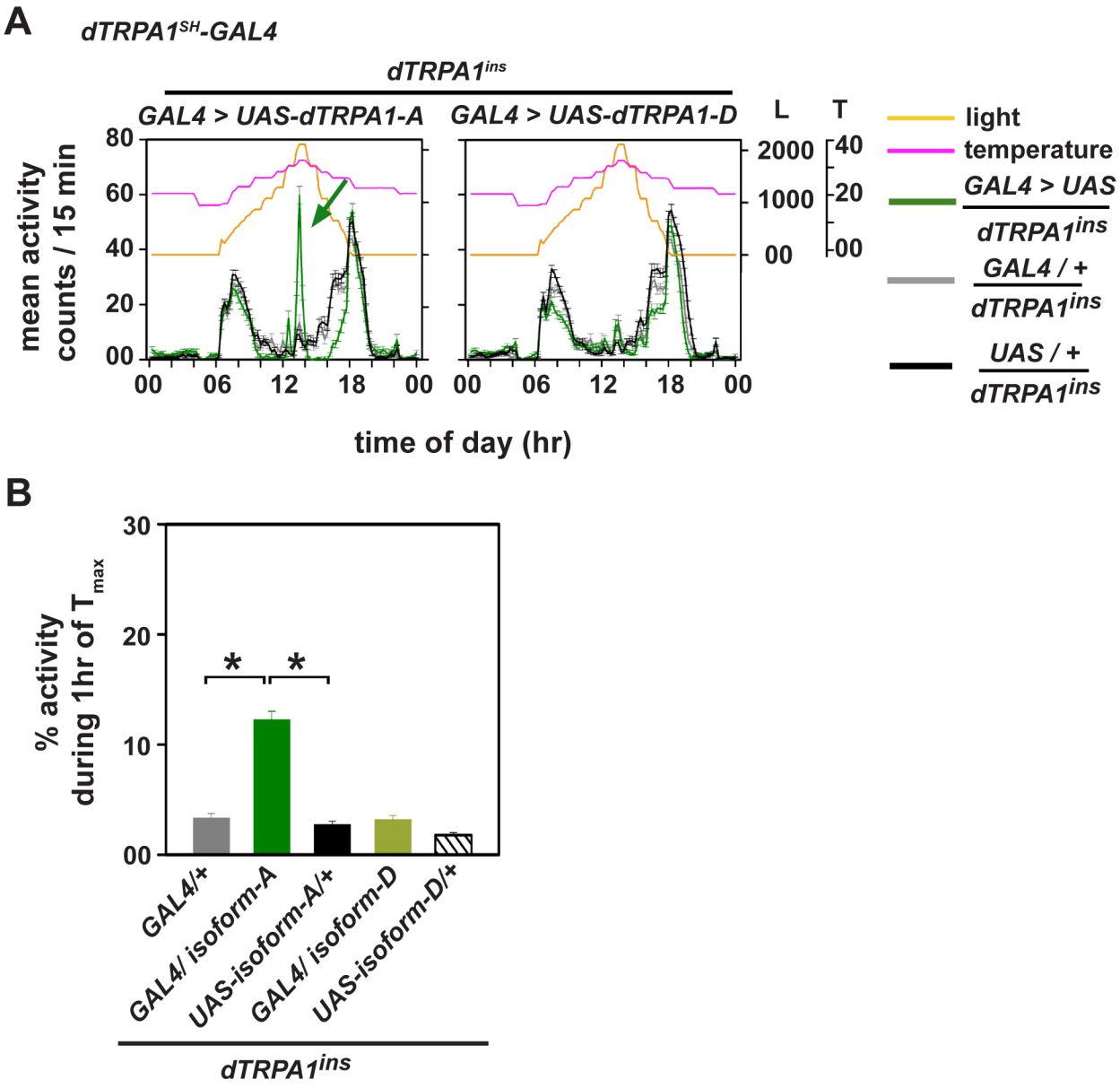

Figure 3.11. dTRPA1-A isoform rescues the mutant phenotype of dTRPA1 null flies under gradual light and temperature cycles. (A) Lack of A-peak of *dTRPA1*^{ins} null mutants is rescued only with expression of dTRPA1-A in a null background but not with dTRPA1-D isoform. (A, left) dTRPA1-A which is known to be active 24-29 ^{o}C elicits an enhanced A-peak similar to *UAS-dTRPA1* mediated overexpression driven under *dTRPA1*^{SH}*-GAL4*. (A, right) Expression of dTRPA1-D isoform in *dTRPA1*^{ins} null background induces a small A-peak similar to parental controls in null background. dTRPA1-D isoformwhich has an activation threshold aroun 34 ^{o}C is not able to rescue occurrence of A-peak in mutant background. (B) Re-storing expression of only dTRPA1-A isoform under *dTRPA1*^{SH}*-GAL4* in *dTRPA1*^{ins} null background is able to elicit an A-peak in flies with higher amplitude compared to their parental controls in null background which have significantly low levels of activity during 1hr of T_{max}. All other details same as Figure 3.4.

Chapter 4

Role of circadian neurons expressing dTRPA1 in influencing temperature-dependent activity/rest rhythms in fruit flies

Introduction

My behavioural data thus far suggests that rhythmic activity/rest profiles of *Drosophila melanogaster* is modified in a temperature-dependent manner by dTRPA1. Also, anomalies in behavioural rhythms due to lack of dTRPA1 signalling in null mutants were restricted to a particular time of the day and altered specific feature of bimodal activity/rest rhythm of fruit flies – mid-day siesta was disrupted in null mutants in temperature cycles in DD (DD/TC), evening peak of activity/rest rhythm was advanced in temperature cycles in LL (LL/TC). A report from another research group, that examined the role of dTRPA1 signalling in mediating behavioural changes to activity/rest rhythms in flies under rectangular temperature cycles, has implicated a circadian clock influence in mediating these changes (Lee and Montell, 2013). This study has proposed that dTRPA1 influences temperature entrainment of circadian clocks since the authors found that dTRPA1 null flies show defects in entraining to asymmetric 18:6 hr (thermophase :cryophase – 18:29 ^{o}C) temperature cycles in DD (Lee and Montell, 2013) in contrast to my observations that dTRPA1 null mutants entrain to 12:12hr temperature cycles in DD. The same study also claimed that dTRPA1 in circadian neurons are responsible for mediating temperature entrainment based on PERIOD oscillations in different circadian neuronal subsets (Lee and Montell, 2013). Further, my studies have shown that under semi-natural (SN) conditions, dTRPA1 null mutants do not exhibit an afternoon peak (or A-peak) in contrast to wild-type flies (Chapter 3, Figure 3.1). Previous studies have proposed that the circadian clock influences A-peak under SN conditions (Menegazzi et al., 2012; Vanin et al., 2012).

To determine whether dTRPA1 in circadian or non-circadian neurons mediate the above behavioural modification observed in my studies, I studied the anatomical distribution of dTRPA1 neurons relative to the circadian neurons and examined

behavioural changes when dTRPA1 expression is altered in circadian neurons. Thermosensory dTRPA1 ion channels have widespread expression in the adult brain and thoracic ganglion in *Drosophila melanogaster* (Shih and Chiang, 2011). While conducting these studies, two reports described overlap between circadian neurons and dTRPA1 using two different *GAL4* drivers- $TRPA1^{KI\text{-}GAL4}$ (Lee and Montell, 2013) and $dTRPA1^{SH}$*-GAL4* (Yoshii et al., 2015). Similar to the above reports I also observed that dTRPA1 expression overlaps with few circadian neurons that I have described in detail below. Next, I either ablated the circadian neurons that express dTRPA1 or altered dTRPA1 expression and examined the behaviours under temperature cycles to determine the role of circadian clock in mediating changes in locomotor activity rhythms seen in dTRPA1 null mutants.

Materials and Methods:

Fly strains: As described previously in Chapter 2, all genotypes were reared on standard cornmeal medium under LD (12:12-hr) at 25 ^{o}C. $dTRPA1^{SH}$*-GAL4* (Hamada et al., 2008), *dTRPA1-GAL4* (Rosenzweig et al., 2005) drivers were used to target subsets of dTRPA1-expressing neurons. *UAS-2xeGFP* was driven under the *GAL4* drivers to visualize the expression pattern of dTRPA1 cells targeted by them. *Pdf-GAL4* (Renn et al., 1999), *cry-GAL4-39* (Klarsfeld et al., 2004) and *tim-GAL4-27* (Lear et al., 2005) were used to target subsets of circadian neurons respectively. For RNAi knockdown of dTRPA1 in different groups of neurons, *UAS-Dcr2D* (Bloomington Stock Centre) was combined with *UAS-*$dTRPA1T_2^{RNAi}$ transgene (VDRC stock number #37250). In all RNAi experiments, $dTRPA1^{SH}$*-GAL4/UAS-dcr-2* served as one of the parental controls. Overexpression of dTRPA1 was achieved using *UAS-dTRPA1* (Rosenzweig et al., 2008) under different *GAL4* drivers. Apoptosis-inducing transgene *UAS-hid* (Zhou et al., 1997) was used for ablation of neuronal subsets (donation from Michael Rosbash, Brandeis). To specifically

manipulate dTRPA1 expressing neurons that do not overlap with circadian neurons, *GAL4* driven gene expression specifically suppressed in circadian neurons by using *Pdf-GAL80* and *cry-GAL80* transgenes in combination with *dTRPA1^{SH}-GAL4*.

Immunocytochemistry: Immunocytochemistry was performed on brains of wandering 3rd instar larva and 2-3-day old adult virgin male flies as described previously (Sheeba et al., 2008). In brief, brains were dissected in ice cold 1X phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 30min at room temperature (RT) with constant shaking. Brain tissue was washed thrice with 0.5% PBT solution (Phosphate buffer saline with 0.5% TritonX-100). Brains were treated with 10% horse serum prepared in 0.5% PBT (blocking solution) for 1hr at RT to block all the antibody binding sites. Additional 6hr incubation with blocking solution was done at 4 ^{o}C with constant shaking for anti-PER staining. Post blocking, brains were incubated with primary antibodies at 4 ^{o}C for 24hr for single antibody staining. When more than one antibody was used, specimens were incubated with primary antibodies for 48 hrs at 4 ^{o}C with constant shaking. Primary antibodies used were chicken anti-GFP (1:1000, Molecular Probes), rabbit anti-PER (1:10,000, donated by Ralf Stanewsky) and mouse anti-PDF (1:5000, DSHB). Secondary antibodies used were Alexa 488 (goat anti-chicken at 1:1500, Molecular Probes), Alexa 546 (goat anti-rabbit at 1:3000, Invitrogen), Alexa 546 (goat anti-mouse at 1:3000, Invitrogen) and Alexa 633 (goat anti-rabbit at 1:3000, Invitrogen). After staining, brains were again washed with 0.5% PBT 6-7 times to remove excess and non-specifically bound antibody and mounted on glass slides in mounting medium (7:3 glycerol:1X PBS). Specimens were imaged under Zeiss epifluorescence microscope (Axio Observer. Z1). Representative specimens were also imaged under Zeiss LSM 700 and Zeiss 510 META confocal microscopes. Images were assembled, and brightness and contrast adjusted using ZEN 2011 software.

Behavioural assays: Locomotor behaviour of male flies (2-4-day old) was measured using Drosophila Activity Monitors (DAM, Trikinetics, Waltham, MA) in 5-min binning intervals in incubators (Sanyo, Japan and Percival Drosophila Chambers, USA) as described previously in Chapter 2 and 3.

Rectangular temperature cycles: Briefly, flies were then subjected to 12:12-hr temperature cycles in DD (thermophase: cryophase 29:21 ^{o}C - DD/TC) for 9-10-days to allow for stable entrainment, and then allowed to free run for 7-8 days under constant darkness at 21 ^{o}C (DD 21). As described in Chapter 2, activity counts binned into 15-min intervals of individual flies were first averaged across 5-days and then activity counts were averaged across flies for a given genotype. These mean activity counts were binned in 1hr intervals was plotted against time of day. Mean activity counts binned in 2hr intervals were used to compare differences in activity levels among genotypes by performing a two-way ANOVA with genotype and time-points as fixed factors followed by Tukey's HSD test ($p < 0.05$).

***Gradual light and temperature cycles*:** As described in detail in Chapter 3, natural cycles of light and temperature were simulated in laboratory incubators (Sanyo, Japan and Percival Drosophila chambers, USA). Light and temperature were gradually changed in short steps using available programs of the respective incubators. DEnM placed within the incubators recorded changes in light, temperature and humidity throughout the experiments. Activity counts in 15-min intervals of individual male flies across at least 5 days were first averaged, following which mean activity counts were averaged across flies of a given genotype and plotted against time of day. A-peak estimation was carried out for each fly as described in Chapter 3. A given genotype was considered to exhibit A-peak only when at least 25% of flies displayed A-peak. Mean activity profiles averaged across 5 days for an individual fly was considered for estimating proportion of flies

showing A-peak in laboratory simulated regimes. To estimate percentage activity (A-peak) during 1 hr of T_{max} at 32 ^{o}C, first activity data from individual flies obtained in 15 min bins were normalised to sum of activity across 24 hrs following which the profiles were averaged across 5 days. Thereafter, activity counts across one hour of T_{max} (when temperature was 32 ^{o}C) was binned to estimate percentage A-activity. Significant differences among genotypes for the A-peak amplitude were determined by one-way-ANOVA on arc-sine transformed data, followed by Tukey's HSD, $p < 0.05$.

Results

dTRPA1 expression in the adult fly brain overlaps with few circadian neurons. My studies demonstrate the location of GFP labelled non-circadian cells targeted by *dTRPA1^{SH}-GAL4* and their anatomical proximity with circadian neuronal circuitry (Figure 4.1, Table 4.1). *dTRPA1^{SH}-GAL4* driven GFP expression was seen in a small number of neurons including two thermosensory AC neurons in each hemisphere and 2-4-cells in the suboesophageal ganglion (SOG) (Figure 4.1A, also see (Hamada et al., 2008; Shih and Chiang, 2011). The AC neurons send dense projections to the dorsal brain where one branch forms ipsilateral arbors while another sends contralateral projections to the opposite hemisphere (Figure 4.1A, also see (Hamada et al., 2008; Shih and Chiang, 2011)). I also detected two distinct cell types previously illustrated (Shih and Chiang, 2011) including a single cell in the anterior-dorsal brain in each hemisphere, which has been referred to as dorsal cell (DC) (Figure 4.1A, B) and 4-6-cells above the superior arch (SA, Figure 4.1A). I also detect a previously undescribed pair of dorsal-lateral cells (henceforth, DL, Figure 4.1A, B) in each hemisphere which appear to have a flattened morphology with cell depth spanning only 1-2 microns. Further, two cells in the ventral side of the brain in the accessory medulla region were also detected (Figure 4.1C, asterisks).

When co-immunostained with antibodies against circadian protein PERIOD and/or PDF, one dTRPA1^{SH+} cell in the accessory medulla was found to overlap with one PER^{+ve} PDF^{+ve} s-LN$_v$ (Figure 4.1D, yellow arrow) and a second dTRPA1^{+} cell also overlapped with the PER^{+ve} PDF^{-ve} 5th s-LN$_v$ (Figure 4.1D, arrowhead, Figure 4.1E-G) (Table 4.1). The dTRPA1/GFP^{+ve} PER^{+ve} PDF^{-ve} 5th s-LN$_v$ also send projections to the dorsal brain that runs parallel to the PDF^{+ve} s-LN$_v$ projections (yellow arrow, Figure 4.1C). In most cases, at least one LN$_d$ neuron overlapped with one dTRPA1^{SH+} dorso-lateral (DL) cell in most of the brain hemispheres examined (8 out of 11, Table 4.1). In two instances, a pair of dTRPA1^{SH+} DL cells overlapped with two LN$_d$ neurons (Figure 4.1D-G, Figure 4.1I, white arrows; Table 4.1). Thus, I find cell bodies of 3-4 pairs of canonical circadian neurons that overlap with *dTRPA1^{SH}-GAL4* driver expression (Figure 4.1J, Table 4.1). This is in congruence with a recent report that has shown that *dTRPA1^{SH}-GAL4* overlaps with few circadian neurons – the 5th s-LN$_v$ (small lateral ventral neuron), three CRY^{+ve} LN$_d$ (lateral dorsal neuron) and one DN1a (dorsal neuron) (Yoshii et al., 2015). While in my studies I do not detect overlap with DN1a, I do see consistent overlap with one s-LN$_v$ neuron.

Moreover, I observe that terminals of dorsal projections of s-LN$_v$ partially overlap with the dense arborizations of thermosensory AC neurons in the superior lateral protocerebrum (SLPR) (Figure 4.1H). In adult brains, the terminals of AC neurons also appear to surround the DN2 cell bodies in the SLPR (Figure 4.1I, yellow arrow). Previously, *TrpA1$^{KI-GAL4}$* driven *UAS-mCD::GFP* expression has been reported to have dense arborization in dorsal protocerebrum (DP) and SOG and send innervations to dorsal fan-shaped body (dFSB) (Lee, 2013).

In a recent study from our group, I report that dTRPA1-expressing cells partially overlapping with dorsal projections of s-LN$_v$ in larval brains (Das et al., 2016). Further,

co-staining 3rd instar larval brains with anti-TRP and anti-PER also revealed that the projections of AC neurons arborise in the vicinity of larval DN2 (Das et al., 2016). Thus, my anatomical data indicate that a subset of circadian neurons in the adult brain could be directly thermosensitive via dTRPA1 which in turn may synchronize rhythms in other clock cells. Close proximity of s-LN_v projections to terminals of AC neurons suggests the possibility of functional connectivity between the circadian and thermosensory circuits in the *Drosophila* brain (Figure 4.1J).

Two other studies have shown the extent of overlap between dTRPA1–expressing neurons and circadian neurons using *TRPA1$^{KI\text{-}GAL4}$* and report overlap with up to 1-2 cells in all of major subsets of circadian neurons, namely, 5th s-LNv (Lee, 2013), LNd, LPN, DN1 DN2 DN3 (Lee and Montell, 2013). However, the image panel showing overlap with DN2 cells appear to be DN1 group rather than DN2 cells (Lee and Montell, 2013). dTRPA1-expressing cells under *TRPA1$^{KI\text{-}GAL4}$* also sends arbors to dorsal fan shaped body (dFSB) and dorsal protocerebrum (DP) (Lee, 2013). Since *dTRPA1^{SH}-GAL4* has a restricted pattern of expression, I wanted to compare the extent of overlap with circadian neuronal subsets with dTRPA1$^+$ neurons by using a broad *GAL4* driver. For this, I examined the expression pattern of a broad driver, *dTRPA1-GAL4* (Rosenzweig et al., 2005), and dTRPA1$^+$ cells were found to overlap with 1-2 circadian neurons both in larval and adult fly brains (Figure 4.2). *dTRPA1-GAL4* driver has a broad expression pattern seen in both larval brains (Figure 4.2A, B) and adult (Figure 4.2C, left panel). In the larval brain, dTRPA1 expression is not present in PDF-positive neurons (Figure 4.2A) but 2-3 larval dorsal neurons (DN) labelled with PERIOD (PER) antibody overlap with GFP-stained dTRPA1 cells (Figure 4.2B). In adult brains also, PDF-positive LN_v are not found to express dTRPA1 (Figure 4.2C, middle panel) but PDF negative, PER-positive 5th s-LN_v overlaps with GFP expression driven by *dTRPA1-GAL4* (Figure 4.2C, right

panel). Co-immunolabelling adult brains with anti-GFP and anti-PER, I find that both DN1a neurons overlap with GFP-positive dTRPA1 neurons (Figure 4.2D), corroborating my observation that larval DN cells co-express dTRPA1 (Figure 4.2B). However, unlike other *GAL4* drivers like *dTRPA1^{SH}-GAL4* and *TRPA1$^{KI-GAL4}$*, this broad driver does not show overlap with LN_d neurons although many GFP^+ cells lie close to the LN_d cluster (Figure 4.2D).

dTRPA1 dependent suppression in mid-thermophase activity is not mediated by circadian neurons expressing dTRPA1. Previously, my studies have shown that flies lacking dTRPA1 exhibit enhanced mid-day activity compared to controls under 12:12hr (thermophase :cryophase - 29:21 ^oC) temperature cycles in constant darkness (DD/TC). Since my anatomical studies show that a small subset of circadian neurons also express dTRPA1, I tested whether dTRPA1 expression in circadian neurons is sufficient to modulate mid-thermophase activity seen under DD/TC. I altered dTRPA1 levels in circadian neurons using different circadian drivers. The *tim-GAL4* driver targets all circadian neurons, *cry-GAL4-39* driver targets LN_v, LN_d and DNs (Klarsfeld et al., 2004) and *Pdf-GAL4* driver (Renn et al., 1999) targets only the LN_v neurons. RNAi knockdown of dTRPA1 driven under these *GAL4* drivers has no effect on mid-thermophase activity when the flies were subjected to DD/TC (Figure 4.3A, B). The experimental flies, *Pdf-GAL4/dTRPA1^{RNAi}*, *cry-GAL4-39/dTRPA1^{RNAi}* or *tim-GAL4/dTRPA1^{RNAi}*, exhibit bimodal activity/rest pattern with complete suppression of activity during middle of the thermophase, resembling their respective parental controls (Figures 4.3A, B). Over-expression of dTRPA1 in *Pdf-GAL4* or *cry-GAL4-39* driven circadian neurons also does not alter the activity/rest profiles of flies under DD/TC (Figure 4.4A, B, left panels). Flies with dTRPA1 over-expression under *tim-GAL4* did not survive to adulthood. Next, I genetically ablated the circadian neuronal subsets and subjected flies to DD/TC. It is

known from previous studies that *UAS-hid* mediated neuronal ablation under *Pdf-GAL4* and *cry-GAL4-39* removes all PDF-positive LN_v (Hamasaka et al., 2010; Renn et al., 1999) and all CRY^+ neurons (Yoshii et al., 2010) respectively. Ablation of circadian neurons by expressing *UAS-hid* under *Pdf-GAL4* and *cry-GAL4-39* drivers also does not modify mid-thermophase activity under DD/TC (Figure 4.4A, B, right panels). However, ablation of CRY-positive neurons reduces the amplitude of the evening peak (Figure 4.4B, top-right panel, arrow) and causes slightly higher nocturnal activity between ZT14-18 (two-way ANOVA, $F_{22,1056} = 43.88$) as observed previously (Busza et al., 2007).

To further dissect the role of dTRPA1 in circadian neurons, I altered the levels of dTRPA1 expression only in non-circadian $dTRPA1^{SH+}$ neurons. To achieve this, I used a *GAL80* transgene to suppress gene expression in circadian neurons only and subjected flies to DD/TC. I combined *dTRPA1^{SH}-GAL4+Pdf-GAL80* and *dTRPA1^{SH}-GAL4+cry-GAL80* to target non-circadian $dTRPA1^{SH+}$ neurons and verified the expression pattern of the same (Figure 4.5). On driving GFP expression under *dTRPA1^{SH}-GAL4+Pdf-GAL80*, I find that no GFP signals are detected in the LN_v cluster but LN_d subsets which are labelled with anti-PER, also show GFP signals (Figure 4.5A, arrow), showing that *Pdf-GAL80* prevents any GAL4-UAS driven gene expression in PDF positive neurons. Similarly, no GFP signals are detected in LN_v and LN_d clusters when *UAS-GFP* is driven under *dTRPA1^{SH}-GAL4+cry-GAL80*, although as expected LN_v neurons get labelled with both anti-PDF and anti-PER and LN_d neurons get labelled with anti-PER (Figure 4.5B). Thus, *cry-GAL80* suppresses gene expression in both LN_v and LN_d clusters, thus, enabling me to target non-circadian dTRPA1 expressing neurons as I have previously detected dTRPA1 expression in both LN_v and LN_d neuronal subsets when driving GFP expression under *dTRPA1^{SH}-GAL4*. First, using *dTRPA1^{SH}-GAL4+Pdf*-GAL80, I altered dTRPA1 expression in all $dTRPA1^{SH+}$ neurons except the LN_v (Figure 4.6A). Flies with

RNAi knockdown of dTRPA1 in all dTRPA1^{SH+} neurons except the LN_v (blue curve) display bimodal activity/rest profile similar to controls (Figure 4.6A, top-left panels). Comparison of activity levels among the genotypes during the thermophase (ZT0-12) show that mid-thermophase activity levels of *dTRPA1^{SH}-GAL4+Pdf*-GAL80 > *UAS-dTRPA1 RNAi* flies is not different from the parental controls (Figure 4.6A, bottom-left panel; two-way ANOVA with genotype and time points as factors, Tukey's HSD, $F_{55,1536} = 6.07$, $p < 0.05$). Similarly, over-expressing dTRPA1 in only in non-PDF dTRPA1^{SH+} neurons also does not alter mid-thermophase activity (Figure 4.6B, right panels, two-way ANOVA with genotype and time points as factors, Tukey's HSD, $F_{55,1536} = 6.07$, $p < 0.05$). Similarly, *dTRPA1^{SH}-GAL4+cry-GAL80* mediated RNAi downregulation only in CRY-negative dTRPA1^{SH+} neurons result in reduced evening activity (Figure 4.6B, right panels) and suppression of mid-day activity similar to controls (Figure 4.6B, bottom-right panel- two way ANOVA, $F_{22,696} = 17.3$, $p < 0.05$).

Unlike RNAi knockdown in dTRPA1^{SH+} neurons which enhances mid-day activity (see Chapter 2, Figure 2.6, left panel, blue curve), restricting RNAi knockdown to only circadian neurons expressing dTRPA1 (Figure 4.3) or to only non-circadian dTRPA1^{SH+} neurons (Figure 4.6A) is not sufficient to elicit enhanced mid-thermophase activity. Taken together, these results suggest that a combinatorial knockdown in both circadian and non-circadian dTRPA1^{SH+} neurons manifests as a loss of mid-day siesta phenotype under DD/TC, while limited knockdown in circadian neurons alone is insufficient to disrupt siesta. I also speculate that the circadian *GAL80* drivers may target important dTRPA1^{SH+} neurons, consequently preventing RNAi knockdown in dTRPA1^{SH+} cells, thus, preventing enhanced mid-thermophase activity. This is also consistent with my finding that upon knock-down of dTRPA1 expression under the *TRPA1$^{KI-GAL4}$* driver, that also includes a large number of circadian neurons (Lee and

Montell, 2013), mid-day activity is enhanced under DDTC (Chapter 2, Figure 2.6A,B, right panels). Thus, it appears that dTRPA1 expression in CRY-positive circadian neurons is not crucial for inhibition of mid-day activity under thermal cycles. Instead, dTRPA1 expression in non-circadian neurons underlies inhibition of mid-day activity under thermal cycles.

dTRPA1 neurons distinct from cells overlapping with CRYPTOCHROME expressing neurons modulate A-peak under gradual light and temperature cycles. My studies described in Chapter 3 of this thesis, demonstrate that under semi-natural (SN) condition or simulated SN conditions in laboratory, temperature driven afternoon or A-peak is dependent on dTRPA1 expression. Next, I wanted to examine the neuronal correlates that influence the occurrence of A-peak under SN or simulated SN conditions and also determine if A-peak is under circadian clock control. Previous reports have suggested that the phasing of onset of afternoon activity is modulated by circadian clocks (Menegazzi et al., 2012; Vanin et al., 2012). I wanted to examine whether the occurrence of A-peak is mediated via dTRPA1signalling from circadian neuronal subsets that overlap with the *dTRPA1^{SH}-GAL4* driver. Therefore, I modified dTRPA1 expression levels in different circadian neuronal subsets with drivers described above. RNAi knockdown of dTRPA1 specifically in PDF-expressing neurons (*Pdf-GAL4*) or CRY-expressing neurons (*cry-GAL4-39*) does not abolish occurrence of A-peak under simulated light and temperature cycles, L_r+T_{r32} (Figure 4.7A, Table 4.2). The amplitude of A-peak displayed by flies with dTRPA1 RNAi in PDF is not different from their parental controls (Figure 4.7B, left panel, one-way ANOVA, $F_{(2,67)} = 0.43$, $p = 0.65$) but, flies with dTRPA1 RNAi in CRY-expressing neurons have a lower A-peak compared to controls (Figure 4.7B, middle panel, one-way ANOVA, $F_{(2,64)} = 20.1$, $p < 0.05$). Further, RNAi knockdown of dTRPA1 expression in pan circadian neurons under *tim-GAL4* also does not affect

occurrence or amplitude of A-peak compared to controls (Figure 4.7A, B, right panels, one-way ANOVA, $F_{(2,57)} = 8.21$, $p < 0.05$; Table 2) undermining the role of dTRPA1 signalling in circadian neurons to influence A-peak.

Next, I overexpressed dTRPA1 in these circadian neurons but flies with overexpression of dTRPA1 under *tim-GAL4* did not survive. Enhancing dTRPA1 expression either in the PDF- expressing LN_v or in CRY-expressing circadian neurons has no effect on A-peak under L_r+T_{r32} (Figure 4.8A, Table 4.2). The amplitude of A-peak is also not affected by driving overexpression of dTRPA1 in PDF neurons (Figure 4.8B, left panel, one-way ANOVA, $F_{(2,85)} = 4.6$, $p < 0.05$) or CRY-positive neurons (Figure 4.8B, right panel, one-way ANOVA, $F_{(2,84)} = 2.1$, $p = 0.12$). To test if dTRPA1 expression in circadian neurons is sufficient to elicit A-peak in flies, I ablated subsets of circadian neurons (Figure 4.9). Neuronal ablation of PDF- positive LN_v neurons or CRY-expressing neurons did not abolish A-peak in flies under L_r+T_{r32} regime (Figure 4.9A, B; Table 4.2); suggesting that circadian neurons expressing dTRPA1 have little influence on occurrence of A-peak under gradual environmental cycles. However, while ablating PDF-positive neurons does not alter the amplitude of A-peak compared to controls (Figure 4.9B, left panel, one-way ANOVA, $F_{(2,72)} = 23.5$, $p < 0.05$), ablating CRY-positive neurons reduces the amplitude of A-peak compared to controls (Figure 4.9B, right panel, one-way ANOVA, $F_{(2,73)} = 47.3$, $p < 0.05$).

As a further verification of this finding, when dTRPA1 levels are downregulated or overexpressed in dTRPA1 neurons except in overlapping LN_v (using *dTRPA1^{SH}-GAL4+Pdf-GAL80*), flies continue to display A-peak under L_r+T_{r32} (Figure 4.10A, Table 4.2) and the amplitude of A-peak is also similar to controls (Figure 4.10B, one-way ANOVA, $F_{(4,122)} = 7.25$, $p < 0.05$), minimizing any role of PDF-positive neurons for mediating an A-peak under L_r+T_{r32}. RNAi knockdown under *dTRPA1^{SH}-GAL4+cry-*

GAL80 also does not affect the A-peak (Figure 4.10C, left panel, Table 4.2) but overexpression of dTRPA1 under *dTRPA1^{SH}-GAL4+cry-GAL80* causes an enhanced A-peak (Figure 4.10C, right panel; Figure 4.10D, one-way ANOVA, $F_{(4,129)} = 48$, $p < 0.05$). This phenotype of enhanced A-peak is reminiscent of flies driving overexpression of dTRPA1 under *dTRPA1^{SH}-GAL4* (*dTRPA1^{oex}* flies) under various gradual light and temperature cycles regimes such as L_r+T_{r32} (out of phase), DD+T_{r32} and LL+T_{r32} (Chapter 3, Figures 3.6-3.8).

These results suggest that non-CRY neurons under *dTRPA1^{SH}-GAL4* are the predominant modulators of enhanced A-peak activity levels. Although some degree of overlap exists between the *dTRPA1^{SH}-GAL4* and circadian pacemaker neurons as suggested by anatomical evidence, my results show that dTRPA1 in these CRY-positive circadian neurons (5^{th} s-LN_v and LN_d) do not play a major role in mediating occurrence of dTRPA1-dependent A-peak and the latter is mostly mediated by non-circadian dTRPA1-expressing cells under the conditions tested. Thus, my studies posit that the non-circadian dTRPA1^{SH+} neurons are the primary modulators of afternoon activity.

Discussion

My anatomical studies show that a few circadian neurons express dTRPA1 and thus are potentially thermosensitive. Based on my results, I propose that communications between dTRPA1 and circadian clock neurons could exist since several interesting juxtaposition of dTRPA1-expressing neurons with circadian neurons were found. Especially, dTRPA1 neurons sending projections to dorsal circadian neurons in the larval brain and to DN2 cell bodies in the adult with partial overlap with dorsal terminals of PDF-positive s-LN_v neurons, all indicate of potential routes of signalling.

However, my results show that circadian neurons expressing dTRPA1 do not influence aspects of activity/rest rhythms elicited by dTRPA1 mediated signalling. This is consistent with my finding that downregulation of dTRPA1 using RNAi in CRY-positive cells or majority of circadian neurons does not influence the ability of flies to exhibit siesta under temperature cycles in constant darkness. Interestingly, downregulation of dTRPA1 under the *dTRPA1^{SH}-GAL4* driver in presence of *cry-GAL80* rescues the mid-day suppression of activity. From my studies, I speculate that effective knockdown of the channel using RNAi occurs only with the *dTRPA1^{SH}-GAL4* driver that targets both circadian and non-circadian dTRPA1 neurons, resulting in a phenotype similar to null mutants. In contrast, prevention of RNAi in CRY-positive subset of dTRPA1^{SH+} neurons (*dTRPA1^{SH}-GAL4+cry-GAL80*) enables inhibition of mid-day activity resembling wild-type flies. However, RNAi only in the CRY-positive dTRPA1^{SH+} neurons (via *cry-GAL4*) is incapable of eliciting a phenotype, suggesting that downregulation of dTRPA1 in both circadian and non-circadian subsets results in enhanced mid-thermophase activity, characteristic of dTRPA1 null flies. Alternatively, the *cry-GAL80* driver could target neurons outside of the *cry-GAL4* driver and within the target of *dTRPA1^{SH}-GAL4* driver, and hence lack of RNAi in that subset of dTRPA1-expressing neurons suppresses mid-day activity. Further, dTRPA1 expression in circadian neurons does not influence occurrence of mid-day siesta under DD/TC as ablating CRY-positive neurons or downregulating dTRPA1 expression in large subsets of circadian neurons (under *tim-GAL4*) does not abolish mid-thermophase activity. Thus, my results indicate that the CRY-negative dTRPA1^{SH+} neurons primarily control suppression of mid-thermophase activity under temperature cycles in DD.

However, one cannot rule out the possibility that dTRPA1-expressing neurons interact with circadian neurons to modulate behaviour under temperature cycles in DD

(DD/TC). One possibility is that AC neurons with partial overlap with dorsal terminals of s-LN_v (Figure 4.1H, K) can perceive differences in temperature via dTRPA1 ion channels and probably convey temporal information to s-LN_v (by being active during warm temperatures and being inactive at cooler temperatures). PDF-positive s-LN_v in turn send projections to CRY-negative DN2 neurons, and synaptic communication between s-LN_v and DN2 have been previously suggested to bring about temperature preference rhythm in flies (Kaneko et al., 2012). AC neurons send extensive projections towards lateral protocerebrum, a brain region which has been recently identified as an important centre for processing thermal signals (Frank et al., 2015; Liu et al., 2015). AC neurons probably send thermosensory signals to higher centres in the fly brain, thus, eliciting temperature-dependent behaviour in flies but the neuronal trajectory encoding thermal signals is yet to be elucidated.

As described previously, A-peak exhibited by wild-type flies under gradual light and temperature cycles is dependent on dTRPA1 expression. Here my studies demonstrate that while complete lack of functional dTRPA1 ion channels abolishes occurrence of A-peak under SN and laboratory simulated natural conditions, a small number of CRY-negative dTRPA1^{SH+} neurons are sufficient to modulate afternoon activity. My results demonstrate that the CRY-negative dTRPA1^{SH+} neurons modulate enhanced A-peak under gradual light and temperature cycles. Specifically, my studies show that *dTRPA1^{SH}-GAL4* driven cells that do not overlap with *cry-GAL4* driver are needed for the A-peak and that increased electrical activity of these neurons can enhance the amplitude of the A-peak. Thus, my results negate a role for the CRY-positive 5th s-LN_v and LN_d that overlap with *dTRPA1^{SH}-GAL4* targeted neurons in mediating A-peak.

In conclusion, among the TRP channels known to mediate thermal signals, dTRPA1 is an ideal candidate for mediating behavioural changes in response to a range of

temperatures more likely to be encountered in the natural habitat of flies where they exhibit diurnal rhythms. My studies show that dTRPA1 in primarily non-circadian neurons play a vital role in mediating A-peak in natural conditions which enable the flies to “escape” stressful environment during afternoon by exhibiting higher activity.

Table 4.1

Antibody	**Hemispheres**		**Number of hemispheres GFP expression**			
Anti-GFP	22			0 cell	1 cell	2 cells
			DC	0	22	NA*
			DL	0	03	19
Antibody	**Hemispheres**	**GFP+ve dTRPA1 cells detected**	**Circadian neuronal subgroups overlapping with dTRPA1**			
				0 cell	1 cell	2 cells
Anti-GFP + Anti-PDF	10	10	**PDF^{+} LN$_v$**	02	05	03
Anti-GFP + Anti-PER	12	12	**LN$_d$**	01	08	03
		11	**LN$_v$**	03	06	02
Anti-GFP + Anti-PER + Anti-PDF	12	11	**LN$_d$**	01	08	02
		12	**s-LN$_v$**	01	10	01
		09	**5th s-LN$_v$**	02	07	NA*

Table 4.1: Number of cells targeted by *dTRPA1SH-GAL4* was quantified by driving expression of *UAS-2xeGFP* and subsequent staining with anti-GFP. Subsets of circadian neurons that overlap with dTRPA1 neurons targeted by *dTRPA1^{SH}-GAL4* driver was compared by counter-staining with antibodies against PER and PDF. DC-dorsal cell, DL-dorsal lateral cell (see Figure 4.1). NA = not applicable.

Table 4.2

Genotypes	N	% flies exhibiting A-peak
Pdf-GAL4/ UAS dcr dTRPA1 ^{RNAi}	28	100
Pdf -GAL4/ UAS dcr	14	100
Pdf -GAL4/ UAS dTRPA1	29	100
Pdf -GAL4/ UAS hid	18	100
Pdf -GAL4/ +	29	100
cry-GAL4-39/ UAS dcr dTRPA1 ^{RNAi}	26	100
cry-GAL4-39/ UAS dcr	13	100
cry-GAL4-39/ UAS dTRPA1	30	100
cry-GAL4-39/ UAS hid	21	100
cry-GAL4-39/ +	27	100
tim-GAL4/ UAS dcr dTRPA1 ^{RNAi}	15	100
tim-GAL4/ UAS dcr	15	100
UAS dcr dTRPA1 ^{RNAi} / +	28	100
UAS dTRPA1/ +	30	100
UAS hid/ +	28	100
dTRPA1^{SH}-GAL4+Pdf-GAL80/ UAS dcr dTRPA1 ^{RNAi}	29	100
dTRPA1^{SH}-GAL4+Pdf –GAL80/ UAS dTRPA1	28	100
dTRPA1^{SH}-GAL4+Pdf –GAL80/ +	15	100
dTRPA1^{SH}-GAL4+cry –GAL80/ UAS dcr dTRPA1 ^{RNAi}	26	96.1
dTRPA1^{SH}-GAL4+cry –GAL80/ UAS dTRPA1	8	100
dTRPA1^{SH}-GAL4+cry –GAL80/ +	29	100
UAS dcr dTRPA1 ^{RNAi} / +	29	100
UAS dTRPA1/ +	31	100

Table 4.2: Percentage of flies from different genotypes displaying A-peak under gradual light and temperature cycles in laboratory (L_r+T_{r32}). For details regarding genotypes, please refer to the Methods section.

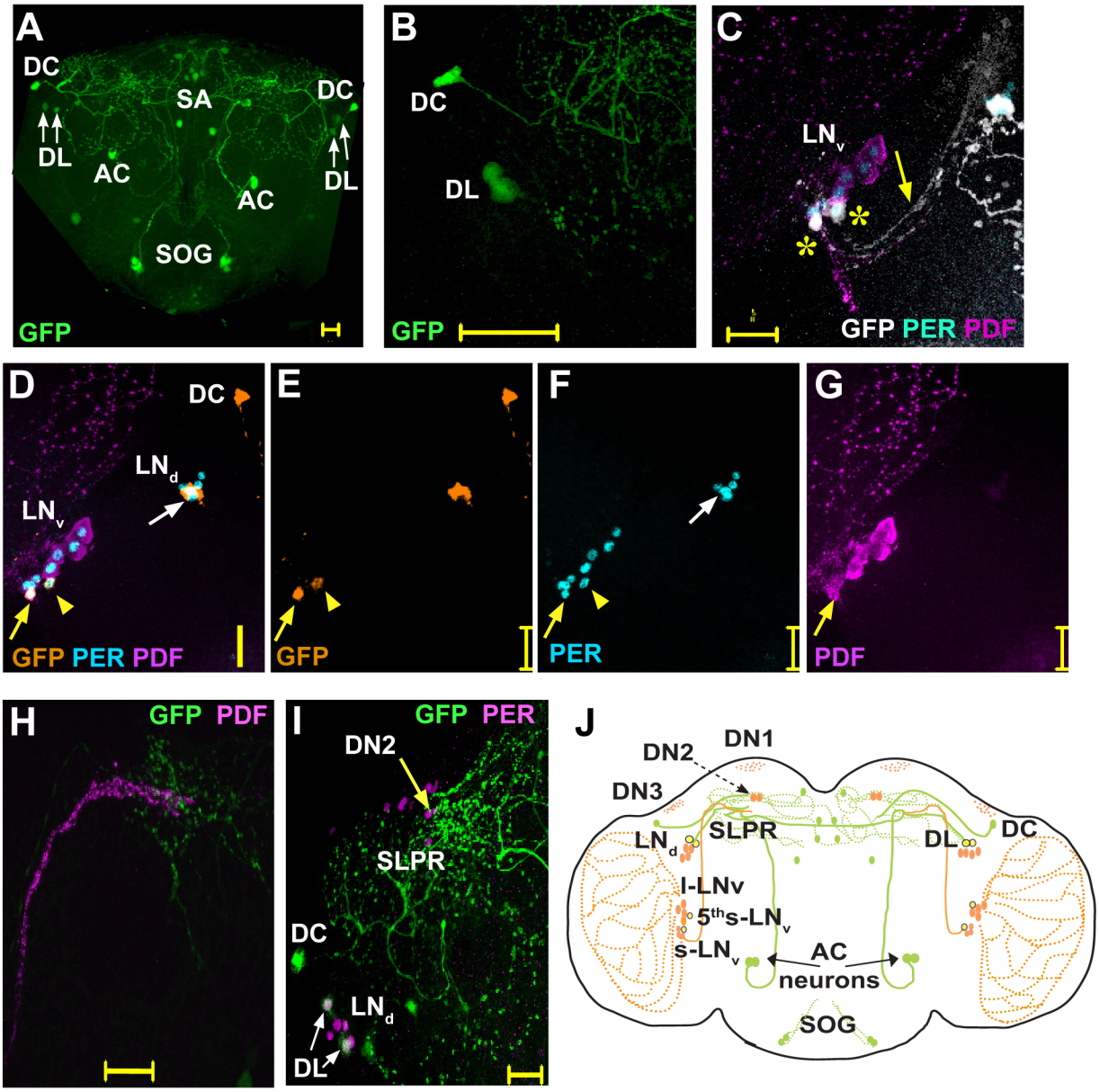

Figure 4.1. Overlap between circadian proteins and dTRPA1 expression in cell bodies and arborisation fields. (A-C) *dTRPA1^{SH}-GAL4* drives relatively restricted GFP expression in two AC neurons, two dorsal lateral (DL, arrows) cells and a solitary cell in the dorsal cell (DC) in each hemisphere; 4-6 neurons above the superior arch (SA) and cluster of 4 cells and their projections in SOG. (B) Magnified view of dorsal brain showing the relative position of DC and DL cells. (C) Co-immunostaining with GFP, PER and PDF antibodies show that two GFP labelled dTRPA1 cells (yellow asterisks) overlap with PER^{+ve} PDF^{+ve} s-LN$_v$ and PER^{+ve} PDF^{-ve} 5th s-LN$_v$. GFP labelled arbors of 5th s-LN$_v$ (yellow arrow) project dorsally parallel to s-LN$_v$ dorsal terminals. (D) Magnified image of lateral brain co-immunolabelled with GFP, PER and PDF show overlap of GFP^{+ve} dTRPA1 neurons with s-LN$_v$ (yellow arrow) and 5th s-LN$_v$ (arrowhead) and two PER^{+ve} LN$_d$ (white arrow). (E-G) Separation of signals of the merged image described in (D) is shown. (H) PDF stained s-LN$_v$ dorsal projections terminate close to dTRPA1^{+ve} AC neurons arbors (green) in SLPR. (I) Dense arborisation of AC neurons terminate in close proximity to PER^{+ve} DN2; two PER^{+ve} LN$_d$ overlap with GFP labelled DL (arrows). (J) Schematic representing relative position of cell bodies and projections of dTRPA1^{+ve} and circadian neurons. Clock neurons co-expressing GFP are marked in yellow – one s-LNv, 5th s-LNv and two LN$_d$ in each hemisphere. AC- anterior cells; DL- dorsolateral cells; DC- dorsal cells, SLPR-superior lateral protocerebrum. All scale bars are 20-µm.

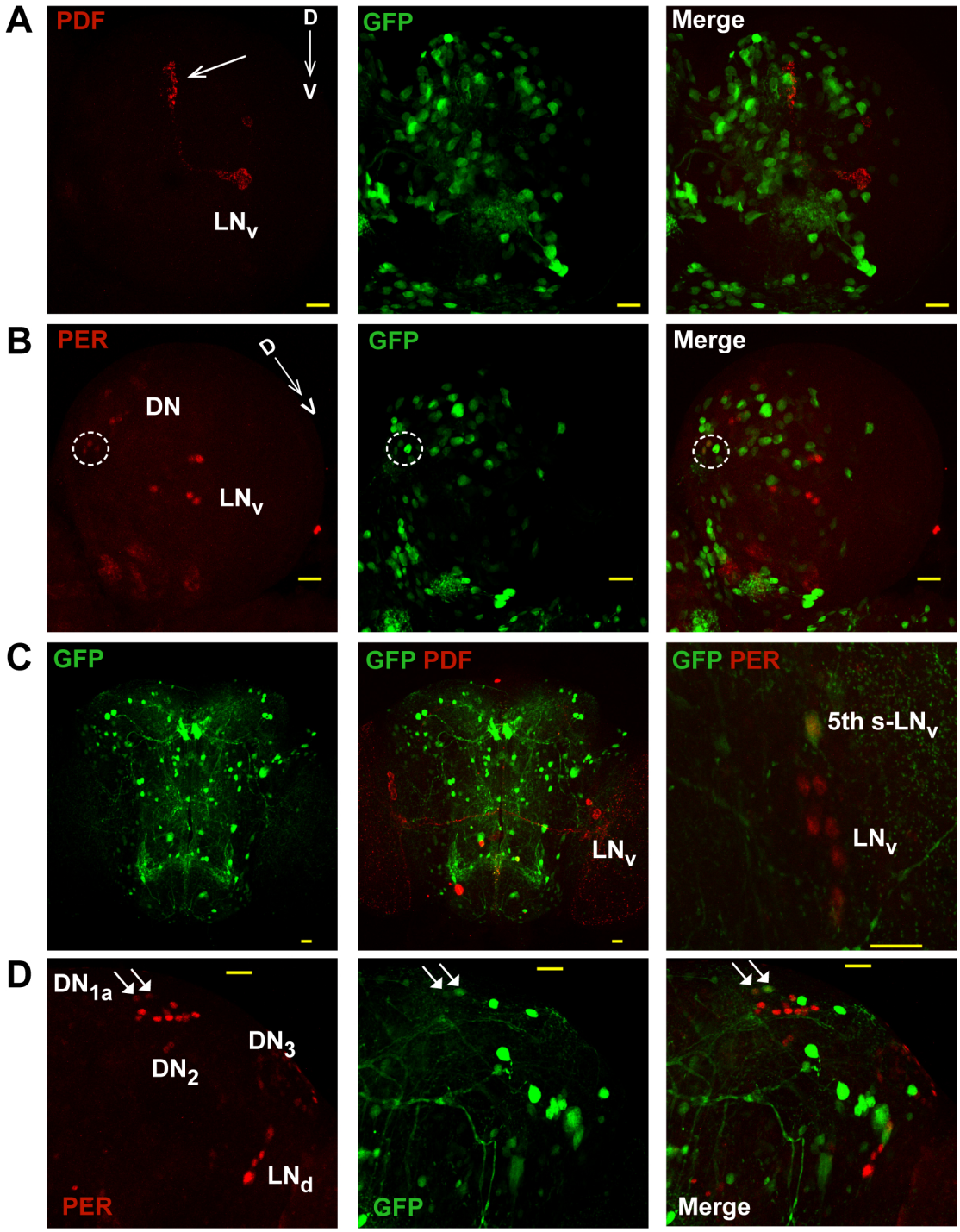

Figure 4.2. Neurons targeted by a broad *dTRPA1-GAL4* driver overlap with circadian neurons in both larval and adult brains. *dTRPA1-GAL4* driven GFP expression and subsequent staining with anti-GFP labels the putative dTRPA1-expressing neurons. Subsequent counter staining with anti-PDF and anti-PER identifies circadian neurons expressing dTRPA1. (A) dTRPA1 neurons do not overlap with PDF neurons in larval brains. (B) Co-immunolabelling with anti-PER (red) shows 2-3 DNs overlapping with GFP^{+ve} dTRPA1 (green) cells (dashed circle). (C, left) Adult fly brain showing broad expression pattern of *dTRPA1-GAL4* driver. (C, middle) dTRPA1 neurons do not overlap with PDF-expressing neurons even in adult fly brain. (C, right) One dTRPA1 neuron overlap with one PER^{+ve} circadian neuron in the LN_v cluster, most likely 5th s-LN_v, because dTRPA1 do not overlap with PDF^{+ve} neurons. (D) Dorsal part of adult fly brain showing overlap of PER^{+ve} DN1a (red) circadian neurons with GFP labelled dTRPA1 cells (green, arrows). D ---> V represent the dorso-ventral axis. Scale bars are 20 µm.

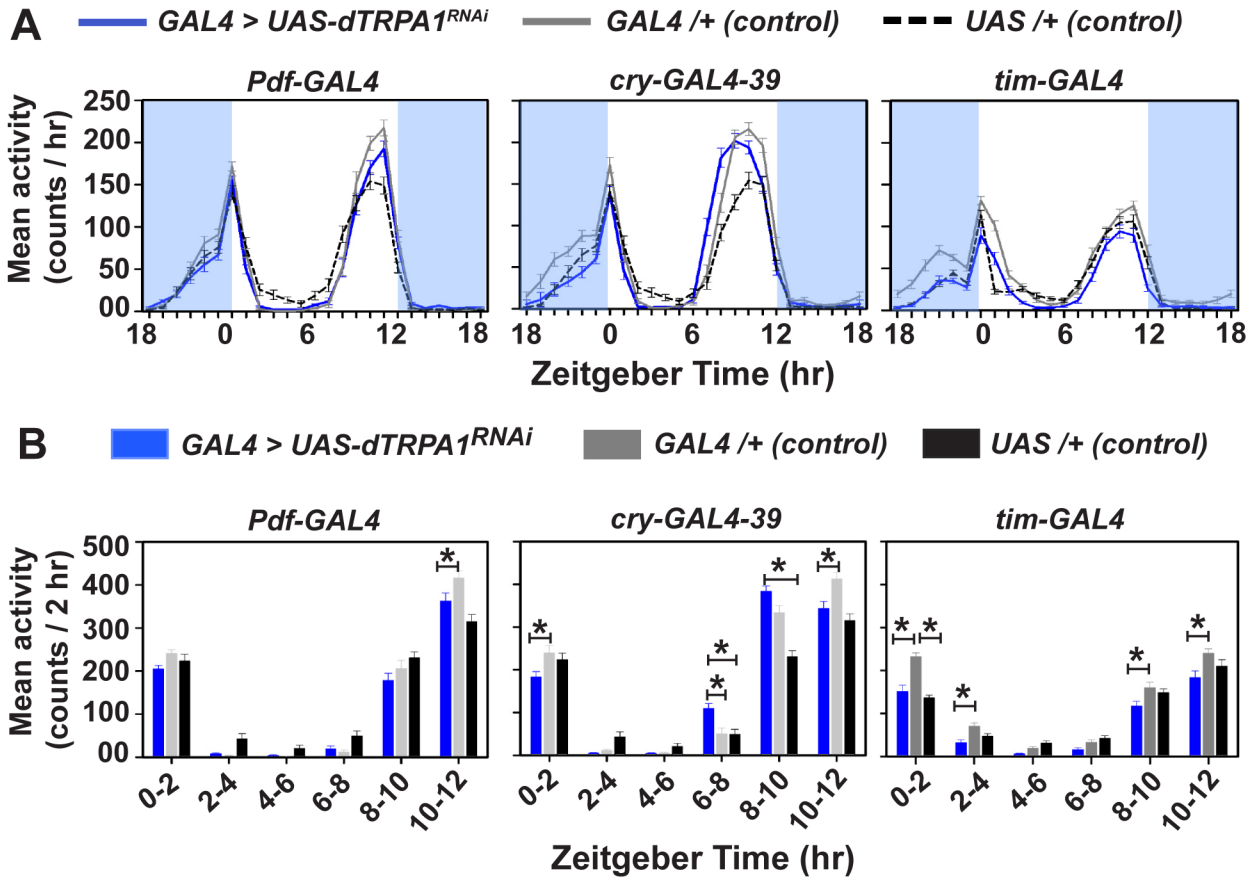

Figure 4.3. RNAi knockdown of dTRPA1 expression in circadian neuronal subsets does not affect mid-thermophase activity under temperature cycles. (A, left) Downregulation of dTRPA1 in PDF-positive neurons (*Pdf-GAL4*), (middle) CRY-expressing neurons (*cry-GAL4-39*) or (right) pan-circadian neurons targeted by *tim-GAL4* does not alter mid-day activity under DD/TC. (B) Quantification of mean activity levels binned in 2hr interval from ZT0 through ZT12 is shown for above genotypes. dTRPA1 RNAi knockdown in different circadian neuronal subgroups- PDF^{+ve} or tim-expressing cells, does not increase mid-day activity under DD/TC. Downregulation of dTRPA1 in CRY^{+ve} neurons increases activity only between ZT6-8. Asterisks indicate significant differences in between indicated genotypes (two-way ANOVA, Tukey's HSD, $p < 0.05$). Shaded region represents the cryophase (21 °C).

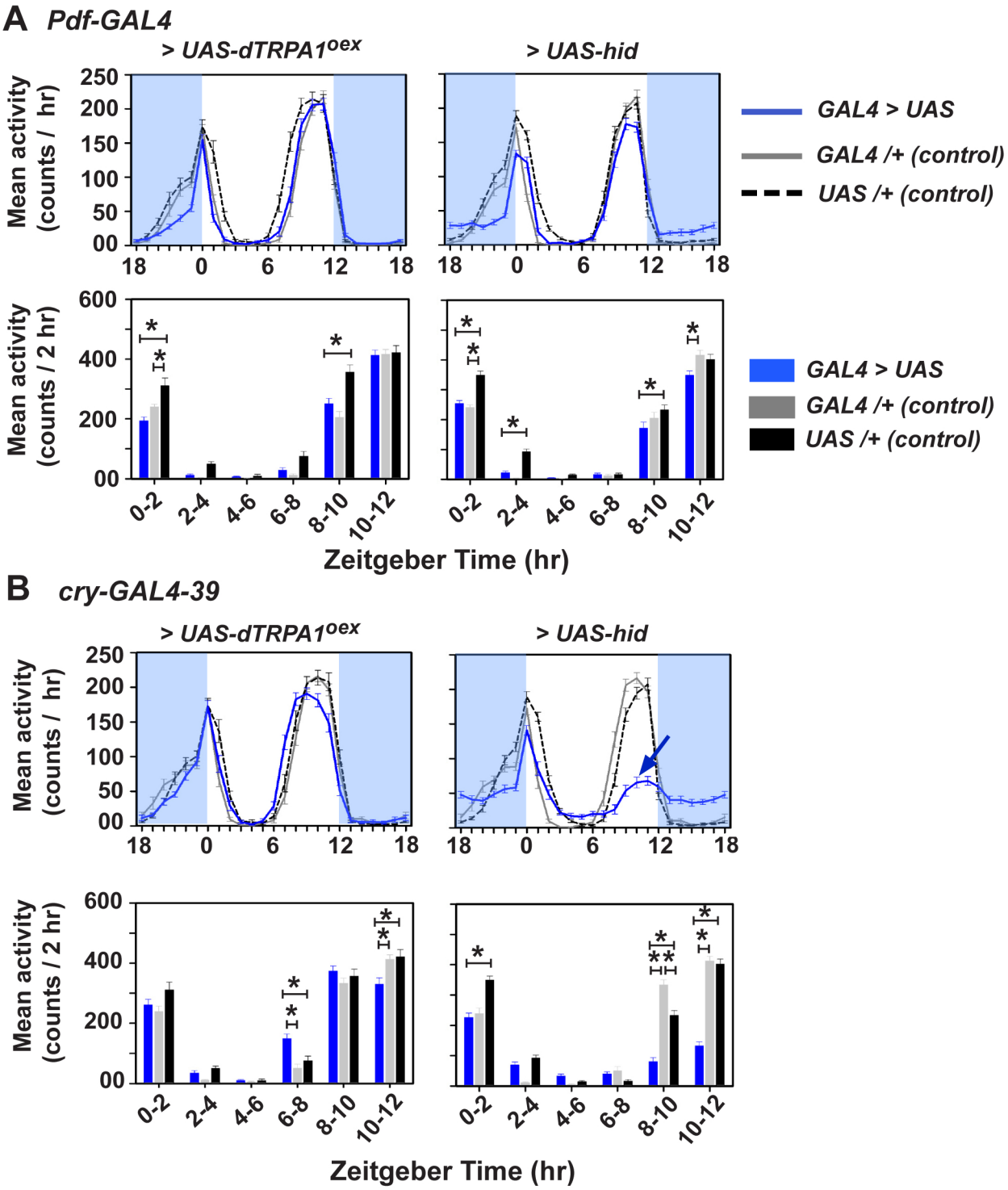

Figure 4.4. Overexpression of dTRPA1 in circadian neurons or neuronal ablation of circadian neuronal subsets does not alter mid-thermophase activity under DD/TC. (A, top) Average activity/ rest profiles of flies with dTRPA1 overexpression in PDF^{+ve} LNv neurons or ablation of PDF neurons. (A, bottom) Comparison of activity levels binned in 2hr intervals show that dTRPA1 overexpression in PDF neurons or ablation of PDF neurons does not effect mid-thermophase activity. (B, left panels) Similarly, overexpression of dTRPA1 in CRY^{+ve} neurons enhances mid-thermophase activity only between ZT6-ZT8 under DD/TC. (B, right panels) Ablating CRY^{+ve} neurons targeted by *cry-GAL4-39* does not affect mid-thermophase activity but reduces the amplitude of evening peak and increases nocturnal activity. Activity levels binned in 2hr intervals were compared by performing two-way ANOVA, Tukey's HSD, $p < 0.05$. All other details same as Figure 4.3.

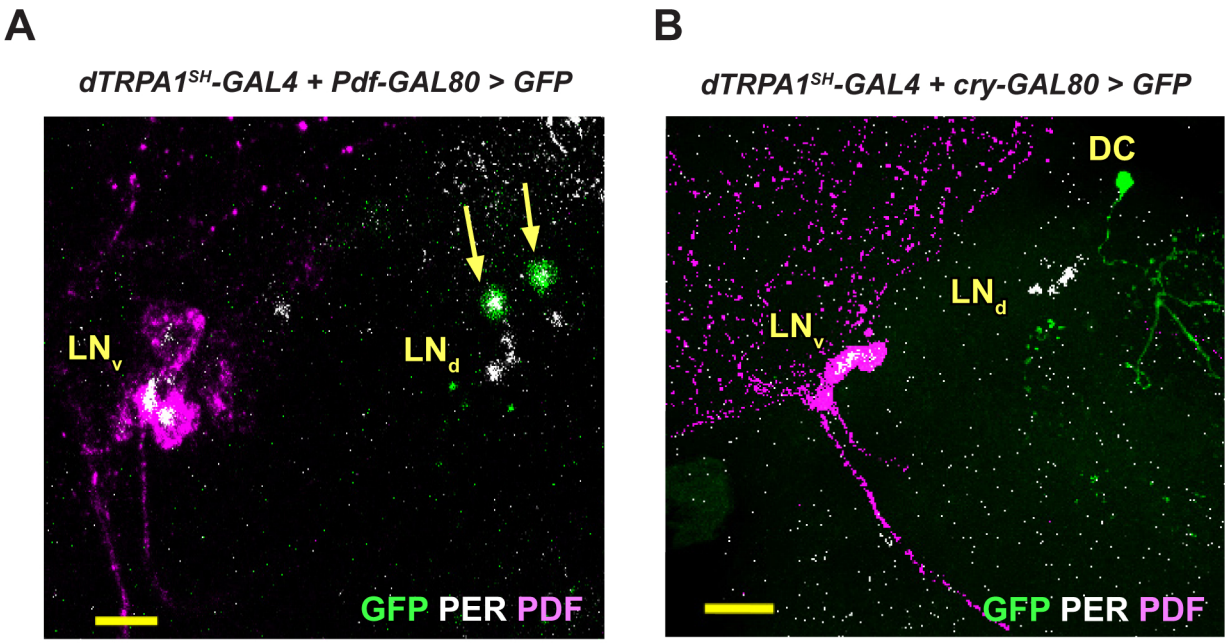

Figure 4.5. Verification of GAL80 mediated suppression of GFP expression with *dTRPA1^{SH}-GAL4* in *Drosophila* adult brains. (A) GFP expression was driven under *dTRPA1^{SH}-GAL4 + Pdf-GAL80* and dissected brains were co-immunolablled with anti-GFP, anti-PER and anti-PDF. LN_v neurons were stained with both PDF and PER and LN_d neurons were labelled with PER. GFP labelled dTRPA1-expressing neurons are seen overlapping with two LN_d neurons (arrows) but no overlap was seen in LN_v cluster. (B) Similarly, GFP expression was driven under *dTRPA1^{SH}-GAL4 + cry-GAL80* and brain tisuues were co-stained with anti-GFP, anti-PER and anti-PDF. *GAL4* driven GFP expression was absent from PDF^{+ve} and PER^{+ve} LN_v neurons and also from PER^{+ve} LN_d cell clusters, although GFP expression is seen in other parts of the brain such as in dTRPA1-expressing dorsal cell (DC) also described in Figure 4.1A, B.
Scale bars = 20μm.

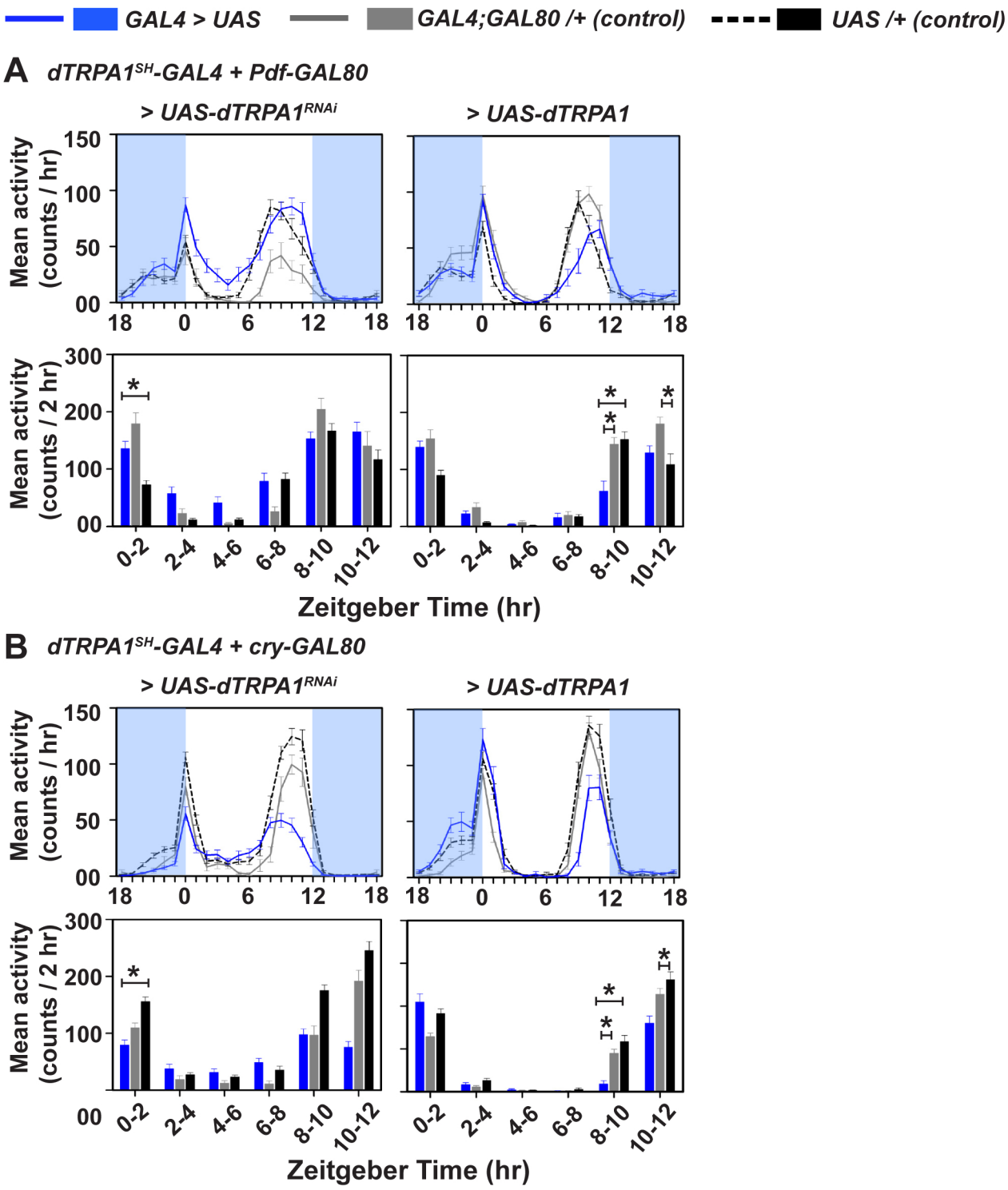

Figure 4.6. Non-circadian dTRPA1 neurons modulate mid-thermophase activity under temperature cycles. (A) RNAi knockdown or overexpression of dTRPA1 in neurons targeted by *dTRPA1^{SH}-GAL4* excluding the overlapping PDF^{+ve} neurons does not modify the activity/rest rhythm of flies. (A, bottom) Mean activity levels binned in 2hr interval from ZT0 through ZT12 is not different among above genotypes. Downregulating dTRPA1 expression in non-PDF, dTRPA1^{SH+} neurons appear to increase mid-thermophase activity but the activity levels are not significantly different from controls. (B) Similarly, RNAi knockdown or over-expression of dTRPA1 in non-CRY dTRPA1-expressing neurons dramatically reduces the amplitude of evening peak (blue curves) but does not alter mid-thermophase activity. (B, bottom) Quantification of mean activity levels binned in 2hr interval from ZT0 through ZT12 is shown for above genotypes show that reducing or increasing dTRPA1 expression in CRY-negative, dTRPA1^{SH+} neurons do not affect mid-thermophase activity. All other details same as Figure 4.3.

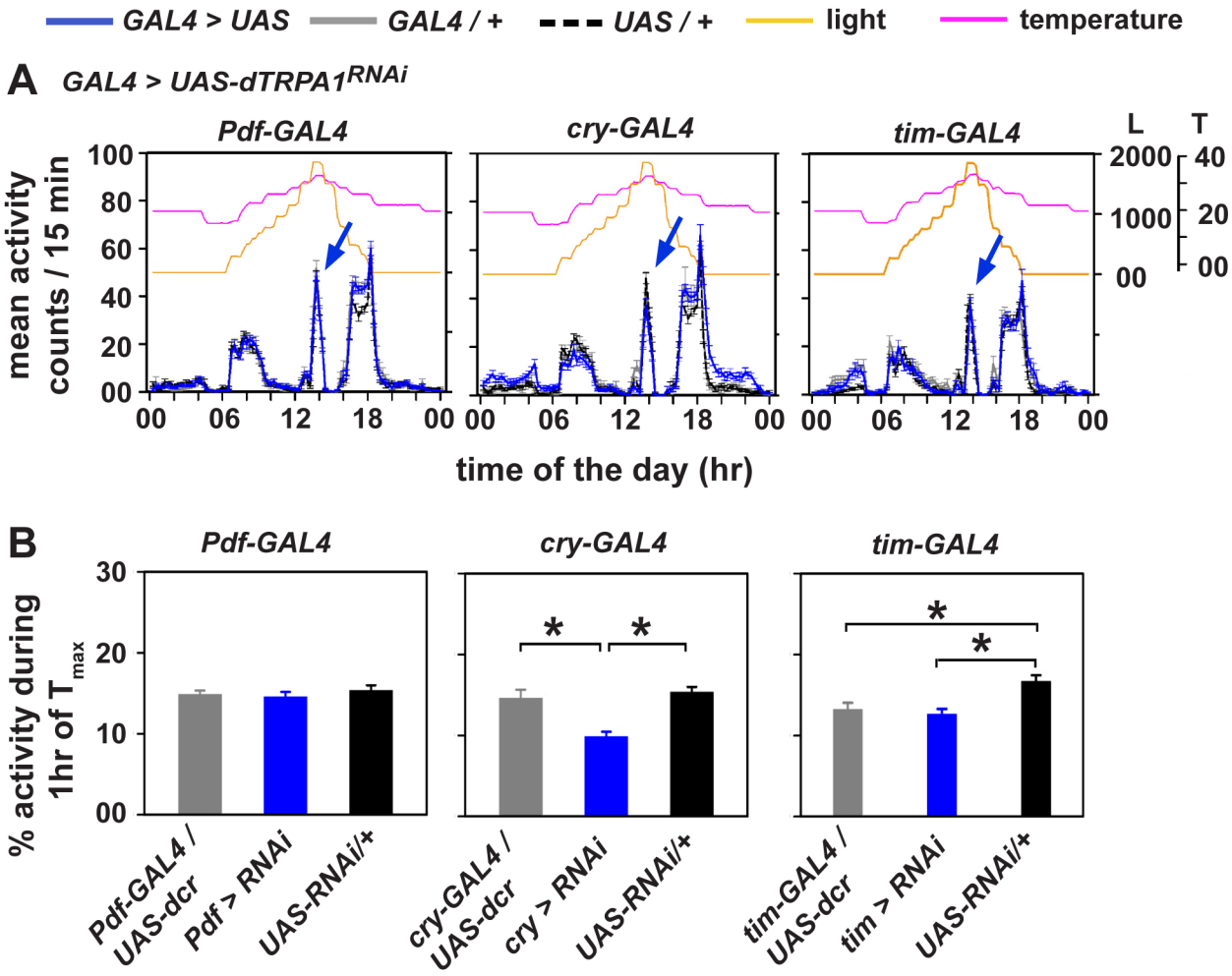

Figure 4.7. RNAi knockdown of dTRPA1 in circadian neuronal subsets does not abolish A-peak under L_r+T_r. (A) Flies with dTRPA1 RNAi in different circadian neuronal subsets driven under *Pdf-GAL4*, *cry-GAL4* and *tim-GAL4* exhibit A-peak (blue curves, arrows) similar to their respective parental controls (grey and dashed black curves). (B) Comparison of A-peak amplitudes among the genotypes described in panel A. A-peak amplitude is reduced when dTRPA1 expression is knocked down only in CRY-expressing neurons, however this reduction is not seen when RNAi knockdown is carried out in pan circadian neurons via *tim-GAL4*. In panel A, two axes on the right represent environmental factors, light (L-lux), temperature (T-degree Celsius). Blue arrows indicate that at least 25% of experimental genotypes exhibited A-peak. Error bars are S.E.M. Asterisks indicate signficant difference in A-peak amplitude among indicated genotypes (one-way ANOVA, Tukey's HSD, $p < 0.05$).

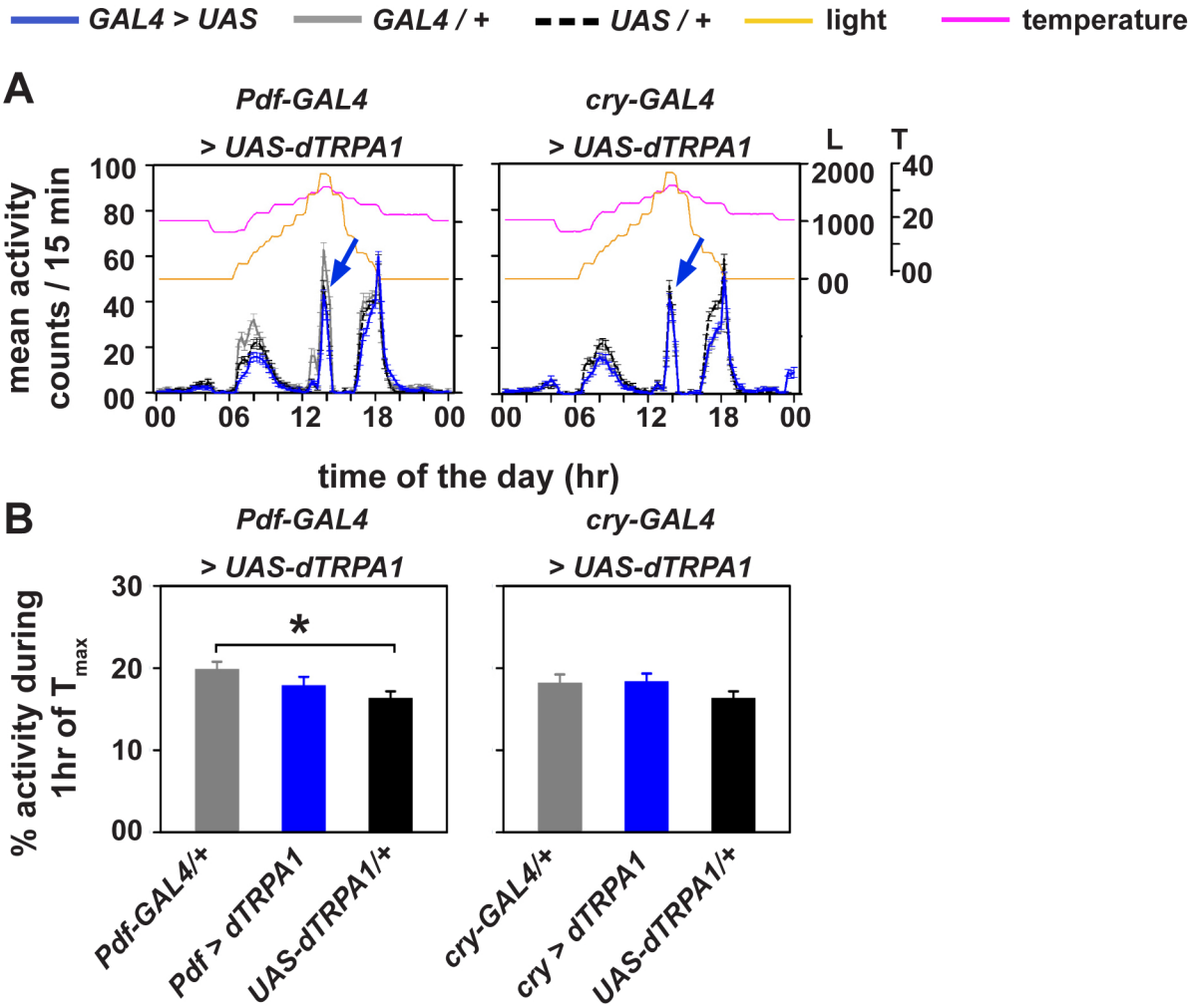

Figure 4.8. Overexpression of dTRPA1 in circadian neuronal subsets does not alter the amplitude of A-peak under gradual light and temperature cycles (L_r+T_{r32}). (A) Flies driving overexpression of dTRPA1 in subsets of PDF-positive neurons or CRY-expressing neurons exhibit A-peak (blue curves, arrows) similar to controls. (B) A-peak amplitude of flies overexpressing dTRPA1 in PDF or CRY-expressing neurons is not different from their respective controls (one-way ANOVA, Tukey's HSD, $p > 0.05$). All other details same as Figure 4.7.

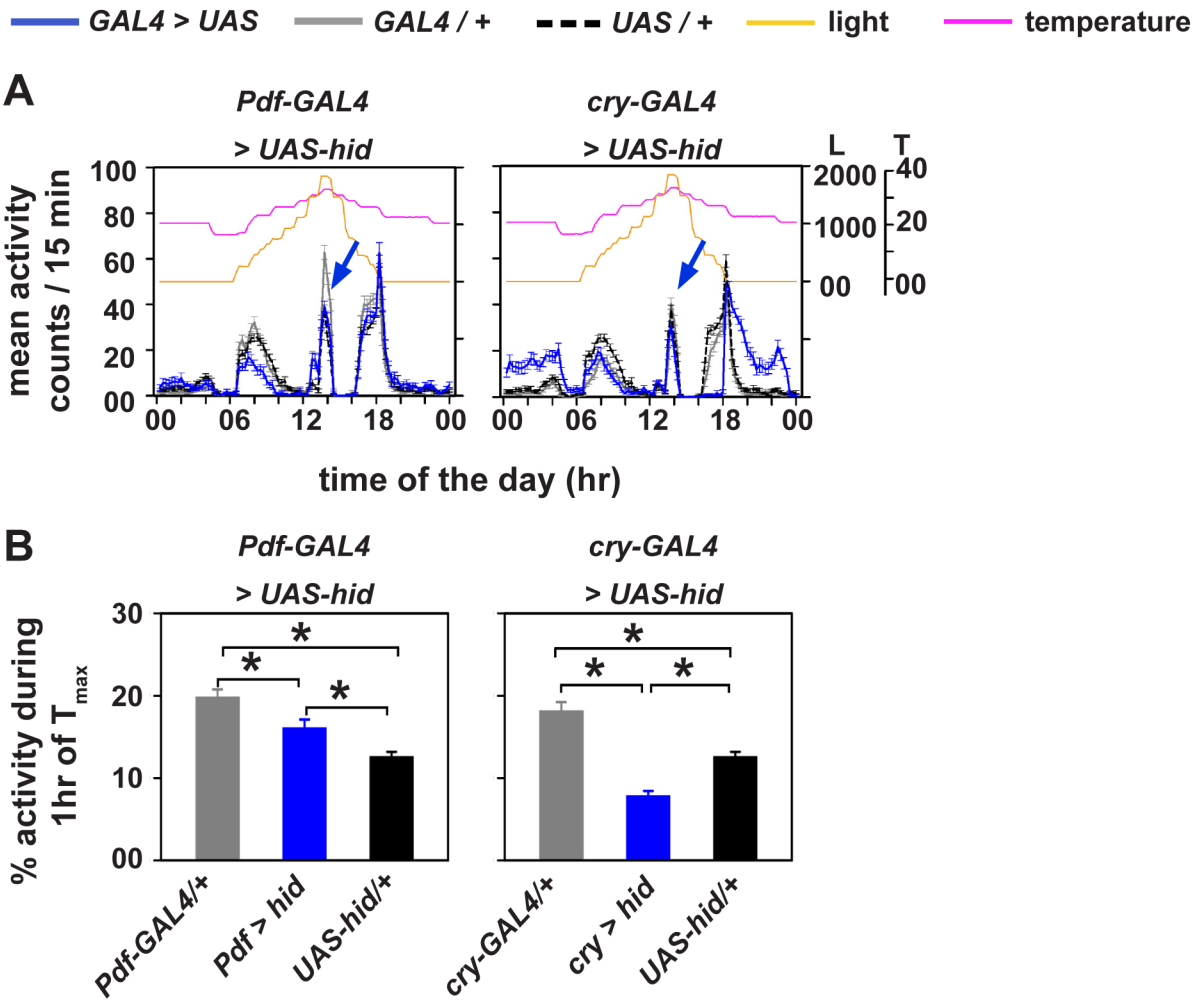

Figure 4.9. Ablation of CRY- expressing neurons reduces amplitude of A-peak under gradual light and temperature cycles (L_r+T_{r32}). (A) Neuronal ablation of PDF^{+ve} LN_v neurons or CRY-expressing neurons has no effect on occurence of A-peak (blue curves, arrows). (B) Comparison of amplitude of A-peak displayed by the genotypes described in panel A, show that flies in which CRY-expressing neurons are ablated, have a reduced A-peak compared to their parental controls (one-way ANOVA, Tukey's HSD, $p > 0.05$). All other details same as Figure 4.7.

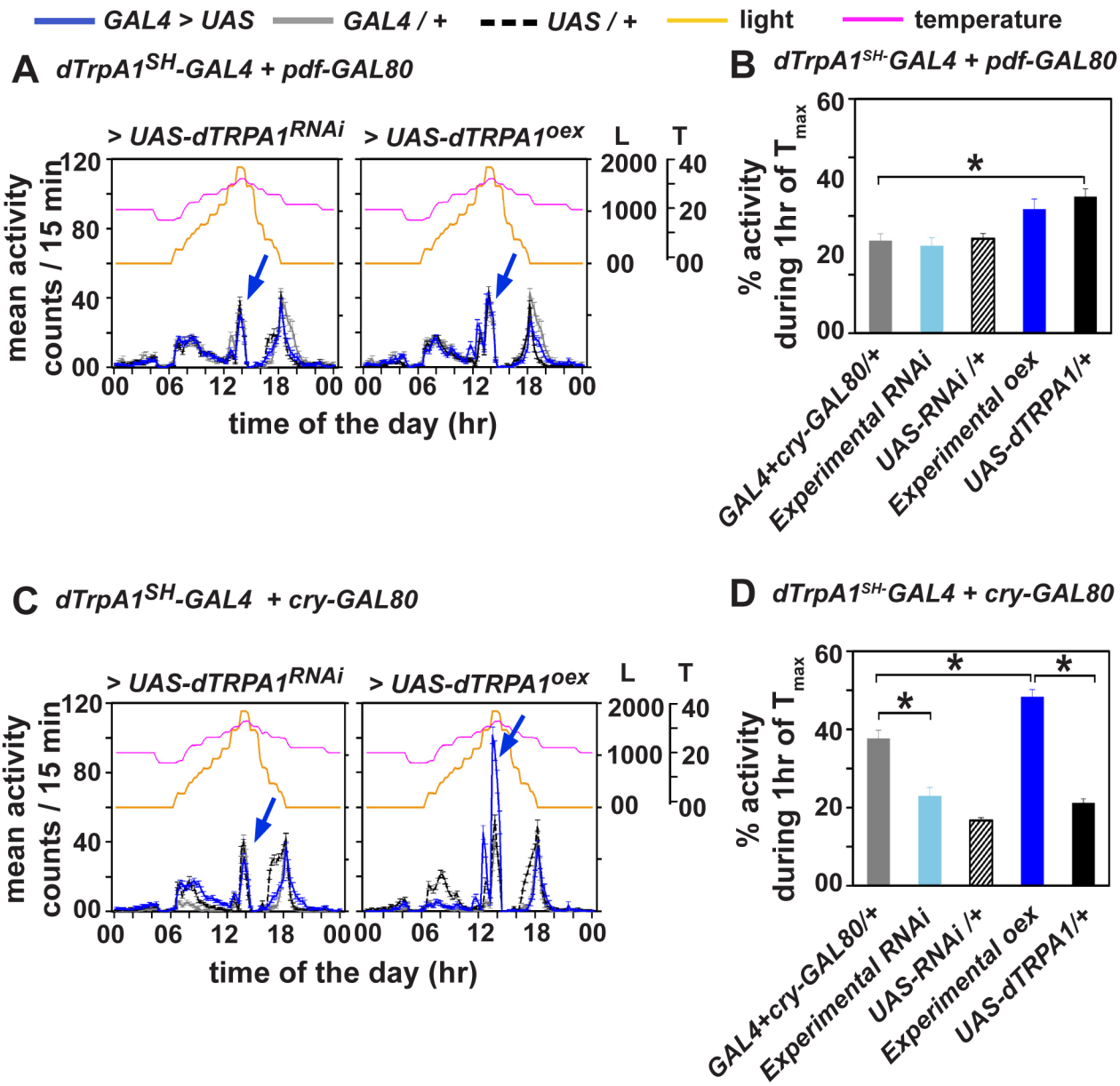

Figure 4.10. CRY-negative dTRPA1 neurons mediate the enhanced A-peak under gradual light and temperature cycles. (A -B) dTRPA1 expression levels in PDF neurons does not influence A-peak under L_r+T_{r32}. (A, left) Flies with either dTRPA1 RNAi knockdown or (A, right) dTRPA1 over-expression in dTRPA1-expressing neurons excluding PDF^{+ve} neurons (*$dTRPA1^{SH}$-GAL4 + Pdf-GAL80*) exhibit an A-peak similar to control flies under L_r+T_{r32}. (B) Comparison of percentage activity during 1hr of T_{max} (32 °C) to estimate difference in A-peak amplitude. Flies with altered dTRPA1 levels in non-PDF, dTRPA1 expressing neurons exhibit A-peaks with similar amplitude as controls. (C, left) Downregulating dTRPA1 levels via RNAi in non-CRY dTRPA1-expressing neurons (*$dTRPA1^{SH}$-GAL+cryGAL80*) does not affect the A-peak . (C, right) Over-expression of dTRPA1 only in non-CRY neurons targeted by *$dTRPA1^{SH}$-GAL4* (*$dTRPA1^{SH}$-GAL4 + cry-GAL80*), leads to enhanced A-peak (blue curve, arrow) compared to controls. (D) Flies over-expressing dTRPA1 in CRY^{-ve}, dTRPA1-expressing neurons show enhanced A-peak compared to controls. Genotype details: Experimental RNAi - *$dTRPA1^{SH}$-GAL4 + GAL80 > UAS-dcr-2; UAS dTRPA1RNAi* and Experimental oex - *$dTRPA1^{SH}$-GAL4 + GAL80 > UAS-dTRPA1*. All other experimental details same as Figure 4.7.

Chapter 5

Study of neural circuits upstream and downstream of dTRPA1 neurons

Introduction

Fruit flies make an excellent model system to study thermosensation and its underlying neural circuitry because of the vast battery of genetic tools available and a large repertoire of behavioural patterns that have a neuronal basis. In a previous study, Sayeed and Benzer demonstrated that antennal thermoreceptors influenced the ability of flies to choose their preferred temperature range (Sayeed and Benzer, 1996). However, the molecular identity of these antennal thermoreceptors remained unknown till recently, when a study from laboratory of Charles Zuker showed that both "hot" and "cold" sensors are present in the antennal organ corroborating Sayeed and Benzer's prediction (Gallio et al., 2011). Further, they showed that the cold sensors are encoded by the gene *brivido* (Italian for shiver) which belongs to the TRPL family of ion channels (Gallio et al., 2011). Another study found that the hot sensors are encoded by gustatory receptors Gr28d (Ni et al., 2013). In a major breakthrough, two independent studies traced the path of individual hot and cold sensory neurons from the antennal cells (periphery) to their projections onto the antennal glomeruli- and identified projection neurons that synapse with the sensory neurons and project to higher processing centres – lateral horn, mushroom body and lateral protocerebrum (Frank et al., 2015; Liu et al., 2015). Thus, the focus of the field has recently shifted towards tracing the neuronal paths that encode the thermal input beginning from sensory neurons to higher processing centres in the brain.

The results described in the previous chapters of this thesis show that signalling from dTRPA1^{SH+} neurons is important for maintaining normal bimodal activity/rest pattern under temperature cycles in the absence of other time cues (DD/TC). Further, the lack of dTRPA1 signalling abolishes the temperature-driven afternoon or (A) peak, which is exhibited by flies under semi-natural conditions (SN) with warm afternoons or

simulated natural temperature cycles in the laboratory when the peak temperature was 32 ^{o}C (DD+T_{r32}). I attempted to identify the synaptic partners of dTRPA1^{SH+} neurons in order to examine the cellular and molecular pathways by which dTRPA1 mediated thermal signals are ultimately conveyed to motor circuits that control behaviour in fruit flies. I focussed on the AC neurons for the following reasons:- my previous results suggested that the AC neurons are among the most critical cells for the mid-day siesta under temperature cycles (see Chapter 2, Figures 2.9, 2.10) and secondly the axonal terminals of AC neurons are very elaborate and distinct (see Chapter 4, Figure 4.1A) and therefore, most accessible to detailed examination.

A previous study has shown that AC neurons are serotonergic and predicted that neurons expressing serotonin receptor 1B are most likely to form synapses with AC neurons (Shih and Chiang, 2011). Furthermore, neurons expressing another TRPA family thermosensor, Pyrexia (Pyx) has been predicted to make synaptic connections with AC neurons (Tang et al., 2013).

First, I attempted to inactivate the neural circuitry that has been previously implicated in functioning upstream of dTRPA1. I examined dTRPA1-dependent behaviour in flies under rectangular or gradual temperature cycles when neuronal signalling from Pyx^{+} neurons was blocked by expressing *UAS-shib^{ts}* under *Pyx-GAL4*. Another signalling pathway that has been proposed to function upstream of dTRPA1 ion channels is *norpA* encoded PLC signalling. I studied *norpA* mutants and flies in which expression of *norpA* is downregulated by RNAi, to examine if lack of signalling from PLC affects dTRPA1 mediated behavioural activity/rest behaviours. Next, I examined the effects of blocking synaptic transmission from dTRPA1^{SH+} neurons. Since AC neurons have been shown to secrete serotonin (Shih and Chiang, 2011), I altered the expression levels of different classes of serotonin receptors to study the behavioural changes arising due to modifying neuronal circuits downstream of dTRPA1^{SH+} neurons.

Disrupting cellular signalling can affect neuronal firing pattern and thus, I also looked at behavioural changes caused due to reduced cAMP signalling in dTRPA1^{SH+} neurons and in higher centres of the fly brain.

Materials and Methods

Fly strains: *Pyx-GAL4* (donated by Fumika Hamada) targets neurons expressing thermosensitive ion channel, Pyrexia. *dTRPA1^{SH}-GAL4* (Hamada et al., 2008) targets a subset of dTRPA1-expressing neurons including the AC neurons. *UAS-shib^{ts}* (Kitamoto, 2001) expresses a temperature-sensitive form of gene *dynamin*, called *shibire*. The mutant form of *shibire* is active at warm temperatures (above 27 ^{o}C) and prevents evoked neurotransmitter release. Consequently, under rectangular temperature cycle regimes (DD/TC – 29:21 ^{o}C) since temperature sensitive *shibire* is active only during the thermophase (29 ^{o}C), synaptic transmission was blocked only for 12hr each day whereas during 12hr of cryophase of DD/TC cycles, synaptic transmission was not blocked. Synaptic transmission was also blocked by expressing tetanus toxin light chain, *UAS-TNT-G* and an inactive form of the toxin (*UAS-TNT-Q*) served as a control (Sweeney et al., 1995). Neurons were hyperexcited by expressing bacterial sodium channel NaChBac (*UAS-NaChBac1* or *UAS-NaChBac4*) (Nitabach et al., 2006), and were electrically silenced via expression of inward rectifying potassium channel *Drosophila* Kir2.1, *UAS-Kir2.1* (Nitabach et al., 2002) (gifts from Todd C. Holmes). Two independent *norpA* RNAi constructs – *UAS-norpA RNAi* (VDRC ID -dna10490 and 105676) were obtained from Vienna Drosophila Resource Centre. Different classes of serotonin receptors were targeted by using *5HT$_R$-1A-GAL4*, *5HT$_R$-7-GAL4* (obtained from NCBS, Bangalore) and *5HT$_R$-1B-GAL4* (Bloomington Stock Centre) drivers. RNAi against 5HT-1B serotonin receptors were driven in respective neurons by expressing *UAS-5HT$_R$-1B RNAi* coupled with *UAS-dcr-2* to enhance RNAi effect. Overexpression of different classes of serotonin

receptors were achieved by driving *UAS-5HT$_R$-1A*, *UAS-5HT$_R$-1B* and *UAS-5HT$_R$-7* under respective *GAL4* drivers. To disrupt the molecular circadian clock in dTRPA1^{SH+} neurons, dominant-negative form of cycle, *UAS-DN-cyc* and *UAS-dBmal1 RNAi* were expressed under *dTRPA1^{SH}-GAL4*. Levels of cAMP (cyclic AMP) was reduced by expressing a dominant negative form of PKA (protein kinase A), *UAS-PKAR*. To target subsets of higher processing centres in the fly brain including mushroom body (MB), pars intercerebralis (PI), I used *121Y-GAL4*, *201Y-GAL4*, *c747-GAL4* (BDSC), *Kurs45-GAL4* (gift from Günter Korge) and *dilp2-GAL4* (gift from Amita Sehgal). Wild-type (*w^{1118}*), mutants fly lines *dTRPA1^{ins}*, *norpA^{45}*, *dunce^{1}* and *rutabaga^{1}* were obtained from Bloomington Drosophila Stock Centre (BDSC). As mentioned in Chapter 2, verification of RNAi knockdown of different genes in neurons of interests has not been carried out due to the following reasons: 1) the number of target neurons in each case are very few and to detect small changes in mRNA expression levels using whole head extracts is likely to be inconclusive; 2) lack of availability of antibodies against the proteins to estimate expression levels via immunocytochemistry on whole fly brains and to generate the antibodies in the laboratory was not possible due to limitation of resources.

Behavioural assays: Activity/rest profiles of adult male flies were recorded in Drosophila Activity Monitors (DAM) system under rectangular light/dark cycles and temperature cycles under gradual temperature cycles as described in Chapter 2 and under gradual temperature cycles as described Chapter 3 respectively.

Statistical analysis: As described previously, raw activity counts of individual flies were first averaged across days and then counts were averaged across flies to obtain mean activity profiles for a given genotype. Activity counts normalised to total activity counts per day for each fly was calculated to obtain percentage activity counts, which were then averaged across days and then across flies to obtain mean percentage activity counts

binned in different time intervals to plot activity/rest profiles. Percentage activity in 2 hr bins were used to compare differences in activity levels by performing a two-way ANOVA with genotype and time points as factors, followed by Tukey's post-hoc comparison, $p < 0.05$. Evening peak phases were manually determined for individual flies and difference from Lights-OFF time was calculated and one-way ANOVA was performed on phase values with genotype as factor, followed by Tukey's post-hoc comparison, $p < 0.05$. Similarly, morning peak phases were determined manually as time of M-peak - time of Lights-ON, and the phase values were used to compare differences among genotypes with one-way ANOVA, followed by Tukey's HSD, $p < 0.05$.

Results

Synaptic transmission from pyrexia neurons is not required for dTRPA1-dependent A-peak under simulated temperature cycles. A previous study has shown that Pyrexia-expressing (Pyx^+) neurons make synaptic contacts with dendrites of dTRPA1-expressing AC neurons (Tang et al., 2013). The same study also recorded Ca^{2+} signals from AC neurons and show that these neurons have two temperature responsive peaks at 25 and 27 ^oC and the latter peak is absent in *pyx* mutants suggesting that Ca^{2+} spike in AC neurons at 27 ^oC is dependent on signals from Pyx^+ neurons. However, the authors also note that due to technical difficulties in recording temperatures from the brain tissue at the site of Ca^{2+} imaging, the actual temperatures at the tissue may have in fact been greater by1-2 ^oC (Tang et al., 2013). Hence, it can be assumed that dTRPA1-dependent activation of AC neurons occurs around ~25-27 ^oC and Pyx-dependent activation of the same neurons occurs at ~27-29 ^oC respectively. I reasoned that if the hypothesis proposed by Tang and colleagues is true that AC neurons are dependent on Pyx^+ neurons for activation at warm ~27-29 ^oC, then blocking synaptic signaling from Pyx^+ neurons during thermophase of DD/TC regime would result in enhanced mid-thermophase activity

and resemble dTRPA1 null flies. I examined the effect of blocking synaptic transmission from Pyx^+ neurons by expressing temperature-sensitive allele of *dynamin*, known as *shibire* - *UAS-shib^{ts}* (Kitamoto, 2001) under *Pyx-GAL4* and subjecting these flies to 12:12hr warm:cold (29:21 ^oC) temperature cycles in constant darkness (DD), henceforth DD/TC. As mentioned previously in materials and methods section, temperature sensitive *shibire* prevents synaptic transmission only at warm temperatures; hence under DD/TC, synaptic blocking of Pyx^+ neurons took place for 12hr everyday only during thermophase (29 ^oC) of DD/TC regime. Blocking synaptic transmission from Pyx^+ neurons during thermophase under DD/TC did not alter activity/rest behaviour under DD/TC (Figure 5.1A; two-way ANOVA, with genotype and time points as factors, $F_{(22,720)} = 59.26$, $p < 0.05$) and *Pyx-GAL4> UAS-shib^{ts}* flies (blue curve) displayed siesta similar to control flies (grey and black curves) (Figure 5.1A). One possible explanation for lack of dTRPA1 null like phenotype could be that blocking synaptic transmission only for 12hr during thermophase of DD/TC regime is not sufficient to elicit "loss of siesta" phenotype. Further, it is possible that dTRPA1 neurons that get activated ~25-27 ^oC (Tang et al., 2012), continue to fire during thermophase (29 ^oC); hence, signals from dTRPA1 neurons are sufficient to suppress mid-day activity and flies exhibited normal siesta (Figure 5.1A). These results suggest that neuronal signals from Pyx^+ neurons do not play a role in influencing mid-day siesta under DD/TC.

Next, flies lacking of signals from Pyx^+ neurons were subjected to a gradually changing (or ramped) temperature regime (T_r) in constant darkness (DD), henceforth DD+T_{r32} instead of rectangular temperature cycles of DD/TC. The temperatures in DD+T_{r32} regime was programmed to mimic gradual temperature cycles as found in nature during the experiments conducted in natural set-up (described in Chapter 3). Previously, my studies have shown that under such conditions, wild-type flies exhibited afternoon (or

A) peak coinciding with 1hr of 32 ^{o}C, which was the peak value (T_{max}) of the temperature cycles imposed (Chapter 3, Figure 3.7). Synaptic transmission from Pyx^{+} neurons was blocked by expressing *UAS-shib^{ts}* or hyperexcited these neurons by constitutive expression of *UAS-NaChBac4* under *Pyx-GAL4* and subjected these flies to DD+T_{r32}. Surprisingly, preventing synaptic transmission from Pyx^{+} neurons has no effect on activity profiles in DD+T_{r32} (Figure 5.1B, left panel) and the amplitude of A-peak in *Pyx-GAL4 > UAS- shib^{ts}* flies is similar to their parental control flies (one-way ANOVA with genotypes as factor, followed by Tukey's HSD, $F_{(2,81)} = 4.12$, $p < 0.05$). Next, Pyx^{+} neurons were electrically hyperexcited by expressing *UAS-NaChBac4* and flies with hyperexcited Pyx^{+} neurons exhibit an A-peak similar to controls under DD+T_{r32} regime (Figure 5.1B, right panel). The amplitude of A-peak of *Pyx-GAL4 > UAS-NaChBac4* flies lies intermediate to their parental control flies; being significantly lower than *GAL4/+* control flies and marginally higher than *UAS-NaChBac4/+* control flies (one-way ANOVA with genotypes as factor, followed by Tukey's HSD, $F_{(2,78)} = 21.7$, $p < 0.05$). Thus, the above results suggest that neuronal signalling from Pyx^{+} neurons is crucial neither for suppression of mid-thermophase activity under DD/TC nor for exhibiting A-peak under DD+T_{r32}.

dTRPA1 mediated behavioural locomotor changes induced by temperature is independent of norpA signalling. Several previous studies that examined the role of dTRPA1 signalling in different behaviours such as larval thermotactic movement or gustatory avoidance of aversive tastants have shown that dTRPA1 neurons require signals from *norpA* encoded phospholipase C (or PLC) (Kim et al., 2010; Kwon et al., 2008). Later, the possibility of an indirect mechanism of dTRPA1 activation via a thermosensitive GPCR-PLC signalling was put forward by Craig Montell (Montell, 2011). Although *norpA* signalling has been suggested to function upstream of dTRPA1,

the mechanism by which *norpA* activates dTRPA1 ion channels remains unknown (Montell, 2011). My previous studies have shown that the temperature-dependent phasing of morning and evening bouts of activity are also mediated by dTRPA1 (Chapter 2, Figure 2.1). In addition, PLC has previously been demonstrated to be important for light input to the circadian clock (Collins et al., 2004). With these results in the background, I tested whether the dTRPA1-dependent siesta under temperature cycles in DD or the advanced morning peak under light/dark cycles at constant temperature of 30 ^{o}C (LD30) conditions require *norpA* signalling upstream of dTRPA1 activation. First, I examined $norpA^{45}$ mutant flies along with controls under LD30 regime. As seen previously (Chapter 2, Figure 2.1), wild-type flies (black curve) advance their morning (M) peak under LD30 whereas $dTRPA1^{ins}$ mutant flies (red curve) do not (Figure 5.2A). On the other hand, $norpA^{45}$ mutants exhibit a reduced M-peak as reported in an earlier study (Collins et al., 2004) and also do not advance the phase of M-peak unlike wild-type flies (Figure 5.2A, one-way ANOVA with genotype as factor, $F_{(2,55)} = 26.9$, $p < 0.05$). They also show a large evening peak compared to w^{1118} flies (Figure 5.2A). Thus, $norpA^{45}$ mutants resemble dTRPA1 null mutants under LD30, both being unable to advance the phase of M-peak. This suggests that *norpA* signalling may play a crucial role in advancing the phase of M-peak under LD30 in wild-type flies. However, since *norpA* encoded PLC plays an important role in photoentrainment and $norpA^{45}$ mutants have a severely reduced morning activity, further studies are required to completely elucidate the role of *norpA* in modulating phasing of M-peak before lights-ON under warm LD30.

Next, *norpA* expression was also downregulated by using two RNAi constructs under $dTRPA1^{SH}$-*GAL4* and subjected these flies along with $norpA^{45}$ mutants to DD/TC (Figure 5.2B, C). As mentioned above, verification of RNAi mediated knockdown of *norpA* levels has not been done due to the reasons stated in materials and methods

section. Unlike *dTRPA1*^{ins} flies which show loss of siesta phenotype under DD/TC (Figure 5.2B, red curve, arrow), *norpA*^{45} mutant flies (green curve) exhibit bimodal activity/rest pattern with clear siesta in middle of the day similar to *w*^{1118} flies (black curve) (Figure 5.2B, top panel). Activity levels of *norpA*^{45} mutant flies binned in 2hr intervals were not different from activity levels of *w*^{1118} flies whereas *dTRPA1*^{ins} flies, as shown previously, show significantly higher activity levels compared to *w*^{1118} flies during mid-thermophase, between ZT02-06, (two-way ANOVA with genotype and time points as factors, followed by Tukey's HSD, $F_{(22,924)} = 22.48$, $p < 0.05$). However, I find that *norpA*^{45} mutants have an advanced E-peak compared to E-peak phase of *w*^{1118} flies (one-way ANOVA with genotype as factor, $F_{(1,43)} = 16.9$, $p < 0.05$). In two separate experiments, *norpA* expression was knocked down in dTRPA1^{SH+} neurons using two independent RNAi constructs and it was found that flies with reduced expression of *norpA* display complete suppression of mid-day activity similar to wild-type flies under DD/TC (Figure 5.2C; two-way ANOVA with genotype and time points as factors, followed by Tukey's HSD, $p < 0.05$, middle panel - $F_{(22,888)} = 36.26$ and bottom panel- $F_{(22,804)} = 20.4$). These results suggest that enhanced mid-thermophase activity displayed by dTRPA1 null mutants is independent of *norpA* signalling. Lack of *norpA* signalling does not phenocopy dTRPA1 null phenotypes under the particular regime of DD/TC, thus negating a role for *norpA* in activating dTRPA1 ion channels indirectly under thermal cycles. Based on my results, I posit that *norpA*-encoded PLC signalling pathway may influence phasing of morning peak under warm LD30 conditions but PLC signalling is dispensable for suppression of mid-day siesta under temperature cycles in constant darkness.

Blocking synaptic transmission from dTRPA1^{SH+} neurons prevents advancement of M-peak phase under LD cycles but has no effect under temperature cycles. I also

attempted to elucidate the neuronal network downstream of dTRPA1^{SH+} neurons. I reasoned that if thermal input from dTRPA1^{SH+} neurons is crucial to suppress activity during mid-thermophase to exhibit siesta, blocking synaptic transmission of dTRPA1^{SH+} neurons and exposing such flies to temperature cycles would render the flies active during mid-thermophase, thus resembling *dTRPA1* null mutants. First, I tried to block synaptic transmission from dTRPA1^{SH+} neurons by expressing *UAS-TNT-G* (Sweeney et al., 1995) under *dTRPA1^{SH}-GAL4* and imposed 12:12hr temperature cycles (29:21 ^{o}C) in constant darkness, DD/TC (Figure 5.3A). Contrary to my expectation, I find that, blocking synaptic communication via expression of tetanus toxin has no effect on activity/ rest profiles of flies subjected to DD/TC (Figure 5.3A, top panel, blue curve) whereas *dTRPA1* null mutants show enhanced mid-thermophase activity (Figure 5.3A, top panel, red curve) as seen previously (Chapter 2 Figure 2.5A,B). As a control for expressing light chain of tetanus toxin (*UAS-TNT-G*), I expressed an inactive form of tetanus toxin, *UAS-TNT-Q* under the same driver (Figure 5.3A, bottom panel). Driving expression of active or inactive forms of TNT has no effect on mid-thermophase activity levels of flies compared to their respective control flies (Figure 5.3A, two-way ANOVA with genotype and time points as factors, Tukey's HSD, $F_{(44,1620)} = 30.29$, $p < 0.05$). However, there have been some previous reports of ineffectiveness of tetanus toxin in preventing synaptic transmission in certain neuronal circuits (Tang et al., 2013; Umezaki et al., 2011). Therefore, one cannot rule out the possibility that the lack of a phenotype in the experimental flies (*dTRPA1^{SH}-GAL4> UAS-TNT-G*) was due to ineffectiveness of TNT-based method of synaptic blocking.

In order to verify the above results, I expressed temperature-sensitive allele of *shibire* (*UAS-shib^{ts}*) under *dTRPA1^{SH}-GAL4*. This mutant allele *shibire* prevents synaptic vesicle recycling under warm temperatures, and this effect can be reversed by lowering

the temperature. Thus, expression of temperature-sensitive *shibire* effectively blocks synaptic transmission in a temperature-dependent manner only during 12 hr of thermophase (29 ^{o}C) under DD/TC regime. However, expression of *UAS-shib^{ts}* in dTRPA1^{SH+} neurons also has no clear effect on activity profiles of flies and mid-day siesta is altered only in a small window of 2 hrs between ZT6-8 when experimental flies (blue curve, arrows) show higher activity than both parental controls (Figure 5.3B, top panel, two-way ANOVA with genotype and time points as factors, Tukey's HSD, $F_{(22,804)} = 7.77$, $p < 0.05$).

Previously, I have tested *dTRPA1^{SH}-GAL4> UAS-shib^{ts}* flies under LD30 regime, because constant warm temperature is believed to activate temperature-sensitive *shibire* and found that *dTRPA1^{SH}-GAL4> UAS-shib^{ts}* flies were unable to advance their morning peak into the dark phase (Figure 5.3B, bottom panel) similar to dTRPA1 null flies under LD30 as seen previously (Chapter 2, Figure 2.1). Flies with *UAS-shib^{ts}* expression in dTRPA1^{SH+} neurons phase their morning (M) peak 0.5±0.1 hr before Lights-ON (Figure 5.3B, bottom panel, blue curve, blue arrow) whereas the parental control flies, *GAL4/+* control (grey curve) and *UAS/+* control (black curve) flies phase their M-peak 1.64±0.19 hr and 2.37±0.09 hr before Lights-ON respectively (Figure 5.3B, bottom panel). In conclusion, flies in which synaptic transmission from dTRPA1^{SH+} neurons is blocked, are unable to shift their M-peak into pre-dawn unlike control flies (one-way ANOVA with genotype as factor, $F_{(2,79)} = 47.7$, $p < 0.05$), thus resembling *dTRPA1^{ins}* mutant flies under LD30 (Chapter 2, Figure 2.1).

Electrical silencing of dTRPA1 expressing neurons enhances mid-thermophase activity, resembling dTRPA1 null mutants under temperature cycles. Altering the firing properties of dTRPA1^{SH+} neurons under DD/TC modifies distinct features of activity/rest profiles of fruit flies. While hyperexciting the neurons delays the evening peak (Figure

5.4A, left panel), silencing the same neurons causes enhanced mid-day activity under DD/TC (Figure 5.4B). Subset of dTRPA1^{+} neurons targeted by *dTRPA1^{SH}-GAL4* were made to drive *UAS-NaChBac1,* a bacterial sodium channel, that has been shown to electrically hyperexcite neurons (Nitabach et al., 2006; Sheeba et al., 2008a). Evening (E) peak of *GAL4* and *UAS* control flies occur at 2.54 ± 0.16hr and 3.38 ± 0.14hr before the end of thermophase respectively. In contrast, *dTRPA1^{SH}-GAL4 > UAS-NaChBac1* flies have a significantly delayed E-peak phase (Figure 5.4A, left panel, orange curve, arrow) that occurs 1.67 ± 0.15hr before the end of thermophase (one way ANOVA with genotype as factor, $F_{(2,64)} = 30.015$, $p < 0.05$). This delay in the E-peak phase is also seen when a broader subset of dTRPA1^{+} neurons (*dTRPA1-GAL4*, (Rosenzweig et al., 2008) overexpress *UAS-dTRPA1* (Figure 5.4A, right panel, dark red curve, arrow). Expression of *UAS-dTRPA1* is known to activate neurons in a temperature-dependent manner (~24-29 ^{o}C) ((Donlea et al., 2011; Parisky et al., 2008) reviewed in (Bellemer, 2015)). During the thermophase, when the temperature is held constant 29 ^{o}C for 12hr, dTRPA1 is expected to increase the firing rate of these neurons. The E-peak occurs about 1.84 ± 0.16hr and about 2.44 ± 0.13hr before ZT12 in *GAL4/+* and *UAS/+* control flies respectively whereas a small percentage of *dTRPA1-GAL4> UAS-dTRPA1* flies show a startle response to warm:cold temperature transition at ZT12 but majority of them phase their E-peak 1.52 ± 0.22hr after ZT12 and thus, their peak is significantly delayed compared to their controls (one-way ANOVA with genotype as factor, $F_{(2,80)} = 145.88$, $p < 0.05$). Thus, electrically exciting a small subset of dTRPA1^{SH+} neurons significantly delay the phase of E-peak, thus mimicking dTRPA1-driven activation of a larger subset of native dTRPA1 neurons under DD/TC.

Electrical silencing of dTRPA1^{SH+} neurons (Figure 5.4B, left panel, blue curve) also leads to significantly enhanced mid-thermophase activity compared to their parental

controls (Figure 5.4B, left panel, black and grey curves). Normalised activity counts binned in 2hr interval are plotted to compare activity levels across time of the day for different genotypes (Figure 5.4B, right panel). Statistical tests (two-way ANOVA, followed by Tukey's HSD, $F_{(22,804)} = 21.85$, $p < 0.05$) show that *dTRPA1^{SH}-GAL4 > UAS-Kir2.1* flies have higher activity during mid-day between ZT2-6 compared to controls and *dTRPA1^{silenced}* flies resemble *dTRPA1^{ins}* null flies (red bars) which have been plotted here for comparison (Figure 5.4B, right panel). Thus, these results demonstrate that dTRPA1 neurons targeted by *dTRPA1^{SH}-GAL4* are very crucial for thermosensation in flies and altering the firing properties of these neurons affect the downstream signalling cascade that results in major behavioural changes as seen in circadian clock-controlled locomotor activity/rest profiles. Taken together, my results imply that the firing properties of a small subset of dTRPA1 neurons are very important to mediate behavioural changes under thermal cycles or light/dark cycles with constant warm ambient temperature conditions.

Overexpression of serotonin receptor (class 1B) in their native neurons enhances mid-day activity under rectangular temperature cycles. Next, I went on to study the neurons downstream of dTRPA1^{+} neurons and identify which neurotransmitters may be involved in communicating thermal inputs from dTRPA1^{+} neurons. I performed a preliminary RNAi screen for potential neurotransmitters (NT) that maybe secreted by dTRPA1^{SH+} neurons. I knocked down expression of NT receptors such as glutamate receptor A (UAS-Glu RA), neuropeptide F (NPFr) and GABA-A1 receptor under DD/TC but no aberrant phenotype was detected by knocking-down these NT receptors (data not shown). Around the same time, a study reported that dTRPA1-expressing AC neurons are serotonergic (Shih and Chiang, 2011) and predicted that serotonin receptor 1B neurons are most likely to form synapses with AC neurons on the basis of anatomical information

present in Flycircuit database (http://www.flycircuit.tw/). I, therefore knocked-down serotonin (5-hydroxytyrptamine or 5HT) receptor 1B in serotonin receptor 1B neurons targeted by *$5HT_R$-1BGAL4* and combined it with *UAS-dcr-2* to enhance the effect of RNAi. These *$5HT_R$-1BGAL4/UAS-dcr-2> UAS-$5HT_R$-1B RNAi* flies were subjected to DD/TC but downregulation of $5HT_R$-1B did not affect activity/rest profiles under temperature cycles (Figure 5.5A, blue curve) and mid-thermophase activity levels were not different from controls (Figure 5.5B).

In addition, I also overexpressed different classes of 5HT receptors using respective GAL4 drivers and the flies were subjected to DD/TC (Figure 5.5B). Flies carrying overexpression of $5HT_R$-1A in neurons targeted by *$5HT_R$-1A-GAL4* (*$5HT_R$-$1A^{oex}$* - blue curve) displayed bimodal activity/rest profile similar to control flies (Figure 5.5B, top panels). Interestingly, overexpression of $5HT_R$-1B in neurons targeted by *$5HT_R$-1B-GAL4* (*$5HT_R$-$1B^{oex}$*- blue curve) led to enhanced mid-day activity and resembled *$dTRPA1^{ins}$* flies (red curve) (Figure 5.5B, middle panels). Comparing normalised activity levels in 2hr intervals show that *$5HT_R$-$1B^{oex}$* flies have significantly low activity compared to controls (grey and black bars) during pre dawn phase (ZT20-24) but after the thermophase starts at ZT0, *$5HT_R$-$1B^{oex}$* flies have higher activity almost throughout the thermophase spanning across ZT0-10 (Figure 5.5B, middle-right panel- two-way ANOVA, Tukey's HSD, $F_{(22,744)} = 36.8$, $p < 0.05$). Similarly, overexpression of $5HT_R$-7 has a mild effect and significantly increases activity between ZT4-6 (Figure 5.5B, bottom-right panel, two-way ANOVA, Tukey's HSD, $F_{(22,732)} = 28.8$, $p < 0.05$).

While downregulating $5HT_R$-1B levels has no effect on activity/rest patterns under temperature cycles in DD, overexpressing the same class of serotonin receptors renders the flies incapable of suppressing mid-day activity and thus, *$5HT_R$-$1B^{oex}$* flies resemble dTRPA1 null flies. However, it is not yet known if other $dTRPA1^{SH+}$ neurons, besides

AC neurons, are also serotonergic. Further studies involving antibody staining against serotonin and its receptors would validate if all dTRPA1^{+} neurons secrete 5HT and which 5HT receptor class is expressed by their synaptic counterparts.

Reducing cAMP levels in dTRPA1^{SH+} neurons leads to enhanced mid-thermophase activity. To rule out the possibility that a functional clock is required within the dTRPA1-expressing neurons to convey temperature signals downstream, I expressed a dominant-negative form of CYCLE (*UAS-DN-cyc)* and also knocked down the levels of expression using *UAS-dBmal1 RNAi* under *dTRPA1^{SH}-GAL4* and subjected these flies to DD/TC (Figure 5.6A). Disrupting the molecular circadian clock with the above genetic manipulations have no effect on dTRPA1-mediated signalling and flies exhibited mid-day siesta similar to *GAL4/+* control flies (two-way ANOVA with genotype and time points as factors, Tukey's HSD, $F_{(22,996)} = 7.96$, $p < 0.05$).

It is known that *Drosophila* TRPA1 ion channels are cationic and gate Ca^{2+} influx in the cells, the latter being an important second messenger molecule which is involved in various cellular signalling pathways. A previous study has shown that reduced cAMP levels in mushroom body (MB) neurons causes disruption of temperature preference behaviour (TPB) in adult flies (Hong et al., 2008). Expression of dominant-negative form of Protein Kinase A (*UAS-PKAR*) effectively reduces cAMP/PKA levels within the cell (Hong et al., 2008). Interestingly, when *UAS-PKAR* was expressed under *dTRPA1^{SH}-GAL4*, the experimental flies (blue curve) exhibit enhanced mid-day activity under rectangular thermal cycles, DD/TC (Figure 5.6B, left panel). Comparison of percentage activity levels binned in 2hr intervals show that flies driving *UAS-PKAR* (Figure 5.6B, right panel, blue bars) exhibit significantly higher activity levels between ZT4-8 (two-way ANOVA, Tukey's HSD, $F_{(22,720)} = 17.65$, $p < 0.05$) compared to their parental controls (Figure 5.6B, right panel, grey and black bars). Activity levels exhibited by flies

with reduced cAMP/PKA levels were similar to *dTRPA1*^{ins} flies (Figure 5.6B, right panel, red bars) that were run in parallel. This clearly indicates that reducing cAMP levels within dTRPA1^{SH+} neurons greatly disrupts downstream signalling and such flies phenocopy dTRPA1 null flies, even though their receptors are intact.

In order to understand the role of cAMP signalling in higher processing centres, dominant negative form of PKA (*UAS-PKAR)* was expressed in different neuronal subsets of MB and PI neurons and subjected the flies to gradual temperature cycles in DD (DD+T_{r32}) which would enable us to observe changes in activity levels of flies in response to small changes to temperatures (Figure 5.7). Simultaneously, I also studied mutants that have increased cAMP (*dunce*^{1} *or dnc*^{1}) and decreased cAMP (*rutabaga*^{1} *or rut*^{1}) levels respectively (Figure 5.7A). Interestingly, I find that *dnc*^{1} mutants show reduced A-peak (Figure 5.7A, left panel dark red curve, one-way ANOVA with genotype as factor, $F_{(2,40)} = 39.82$, $p < 0.05$) whereas *rut*^{1} mutant flies show A-peak only marginally lower than wild-type flies (Figure 5.7A, right panel, blue curve, one-way ANOVA with genotype as factor, $F_{(2,40)} = 39.82$, $p < 0.05$).

I also expressed dominant negative PKA in different subsets of MB neurons (targeted by *121Y-GAL4*, *201Y-GAL4* and *c747-GAL4*) and PI neurons (*Kurs45-GAL4* and *dilp2-GAL4*). Among the different *GAL4* drivers tested, my results show that flies which drive *UAS-PKAR* only under *121Y-GAL4* (which target subsets of MB and PI neurons) show an enhanced A-peak under DD+T_r (Figure 5.7B, top-left panel, one-way ANOVA with genotype as factor, $F_{(10,128)} = 19.07$, $p < 0.05$). Thus, reducing cAMP levels in neurons targeted by *121Y-GAL4* results in an enhanced A-peak that is reminiscent of flies with overexpression of dTRPA1 in dTRPA1^{SH+} neurons under identical regime (Chapter 3, Figure 3.7A, bottom-left panel, B). Taken together, these results suggest that flies with low cAMP/PKA signalling in neurons targeted by *121Y-*

GAL4 is encoded as excitatory signals in the motor circuits downstream thus resulting in an enhanced A-peak under DD+T_{r32}. Reducing cAMP/PKA levels under other *GAL4* drivers that targeted different MB and PI neuronal subsets did not result in any alterations in activity response to different temperature-step changes (Figure 5.7B). This is however, only a preliminary screen and several other neuronal drivers targeting MB and other central complex regions are currently being screened for their role in temperature-dependent activity/rest locomotor rhythms. Future studies that examine flies under DD+T_{r32} regime with increased cAMP levels in different MB neurons will shed light on which neurons are important in conveying thermal input. Moreover, anatomical tracing of terminals of candidate circuits would help determine subsets neurons of higher processing centres interact with dTRPA1 thermosensory circuits and potential interneurons and projection neurons could be identified.

Discussion

I attempted to study the influence of neuronal circuits that have been proposed to function upstream and downstream of dTRPA1 neurons and my studies show very interesting results. Although Pyx^+ neurons make synaptic connections with dTRPA1-expressing AC neurons, signalling from Pyx^+ neurons does not influence behavioural changes which my previous studies have shown to be mediated by dTRPA1, i.e – suppression of mid-thermophase activity under DD/TC (Chapter 2, Figures 2.5, 2.6, 2.9, 2.10) and occurrence of afternoon (or A) peak under gradual temperature cycles (DD+T_r), resembling temperature cycles in nature (Chapter 3, Figures 3.7 and 3.8). It has been suggested that the ability of Pyx^+ neurons to respond to temperature changes in the ambient range 25-27 ^oC is probably due to some other thermosensor expressed by Pyx^+ neurons and not the TRPA channel Pyrexia itself (Barbagallo and Garrity, 2015). However, since blocking synaptic transmission from Pyx^+ neurons also did not result in a

dTRPA1 null like phenotype, I propose that Pyx signalling is not crucial for dTRPA1-dependent behavioural manifestation. My studies also show that *norpA*-dependent PLC pathway also does not seem to influence dTRPA1 signalling since flies carrying *norpA* gene mutation or RNAi of *norpA* in dTRPA1^{SH+} neurons do not phenocopy *dTRPA1^{ins}* null mutants. These results indicate that signals from PLC is not required for mediating changes in circadian clock controlled rhythmic behaviour under the different temperature regimes tested.

The results obtained upon blocking synaptic transmission in the targeted neurons are only partially consistent across regimes. Under DD/TC where warm temperatures are present only for half a day, flies are able to inhibit activity during the warm phase despite *UAS-Shibire* expression. This could be because the synaptic vesicle production during the preceding 12 hours is sufficient for the suppression of activity during the warm phase. Alternately, blockage of synaptic transmission is not complete during the warm phase and small levels of synaptic vesicles are produced which is sufficient for the inhibitory effect. This is in contrast to my previous results, which suggested that dTRPA1 in AC neurons is necessary and sufficient to suppress mid-day activity under temperature cycles (DD/TC). However, under LD cycles, blocking synaptic transmission across dTRPA1^{SH+} neurons by expressing temperature sensitive *shibire* renders the flies unable to advance their M-peak into pre-dawn unlike the control flies. Under LD30, temperature is held constant at 30 ^{o}C and thus, synaptic transmission is blocked across 24hr. As expected, I find that *dTRPA1^{SH}-GAL4 > UAS-shib^{ts}* flies phenocopy dTRPA1 null flies and are unable to shift their M-peak into the dark phase of LD cycles.

Further, my studies emphasise the importance of the electrical properties of dTRPA1 neurons in maintaining locomotor activity rhythms. In addition, dTRPA1^{SH+} neurons are able to fire normally in the absence of a functional molecular circadian clock

in these neurons, suggesting that circadian clocks do not influence heat-dependent activation of dTRPA1^{SH+} neurons. This corroborates my previous results that circadian neurons that express dTRPA1 ion channels do not play a dominant role in influencing behavioural patterns under different experimental protocols (Chapter 4, Figures 4.4, 4.6, 4.9, 4.10). It is more likely that non-circadian dTRPA1 neurons influence activity/rest profiles. Electrical hyperexcitation of small subset of dTRPA1 neurons, i.e., dTRPA1^{SH+} causes a delay in the peak of evening phase under DD/TC, which is similar to when a large subset of dTRPA1 neurons (targeted by *dTRPA1-GAL4*) overexpress *UAS-dTRPA1* and thus, a large subset of dTRPA1^{+} neurons are thermally activated under DD/TC. These results show that activating dTRPA1 neurons is encoded as perceiving warmth and thus the flies continue to remain active beyond the thermophase and phase their evening peak past the onset of cryophase, unlike wild-type flies that phase their E-peak before start of cryophase. On the other hand, electrical silencing of dTRPA1^{SH+} neurons phenocopy *dTRPA1^{ins}* and remain active through mid-thermophase, augmenting the hypothesis that dTRPA1 neurons send inhibitory signals downstream to suppress mid-day activity under DD/TC.

Neurotransmitters play a vital role in neuronal communication and serotonin is the strongest candidate to function in the dTRPA1^{SH+} neuronal subset. A previous study has suggested that serotonin receptor 1B neurons are synaptic partners for dTRPA1-expressing AC neurons (Shih and Chiang, 2011). I hypothesised that if these receptors are downregulated, AC neurons would not be able to convey signals downstream, resulting in enhanced mid-thermophase activity, resembling dTRPA1 null flies. My results discussed above demonstrate that RNAi knockdown of serotonin 1B receptors has no effect on activity /rest rhythms under DD/TC, indicating that perhaps serotonin receptors 1B are not the synaptic partners of AC neurons. Since AC neurons are

serotonergic (Shih and Chiang, 2011), it is possible that some other class of serotonin receptors lie downstream of AC neurons. Furthermore, with our present understanding of AC neurons and their downstream synaptic partners, I do not have an explanation for enhanced mid-thermophase activity exhibited by flies under DD/TC when serotonin receptor levels are overexpressed. Future studies that examine null mutants of different serotonin receptor classes and how AC neurons communicate with serotonin receptors neurons networks may shed light on our understanding of AC neurons and their downstream synaptic neuronal circuit.

My studies also show the role of another signalling molecule, cAMP, in modifying dTRPA1-dependent activity/rest rhythms. My results demonstrate that cAMP signalling is very important within the dTRPA1^{SH+} expressing cells and reduction in cAMP levels in these neurons leads to enhanced mid-thermophase activity under DD/TC, resembling *dTRPA1^{ins}* flies. It is well-known that cAMP is a second messenger molecule and activates PKA downstream of GPCR. dTRPA1 ion channels, upon activation, allows Ca^{2+} to enter the cell, and Ca^{2+} is also known to function as an important second messenger molecule. Further studies that can examine the role of second messenger signalling molecules can elucidate how the thermal stimulus from the dTRPA1 ion channels is encoded and transmitted downstream. Through a preliminary screen, I was also able to isolate a broad central complex driver, *121Y-GAL4*, that target parts of mushroom body, PI neurons etc. and these neurons play a vital role in mediating A-peak, which is believed to be an escape response to avoid stressful, thermal stimuli. Reducing cAMP/PKA signalling under *121Y-GAL4* causes an enhanced A-peak, resembling flies overexpressing dTRPA1 in dTRPA1^{SH+} neurons under identical DD+T_r regime, suggesting that both these neuronal subsets converge into the same downstream signalling pathway.

Future studies that explore the role of signalling molecules such as cAMP in subsets of central complex neurons and trace out the thermosensory circuit from the sensory neurons – projection neurons- higher centre neurons would be very enlightening and advance our understanding of how complex, temperature driven behavioural patterns are encoded in neural circuitry. Presently, very little is known about the neurotransmitters involved in these thermosensory circuits and thus, a systematic screen for these signalling molecules that function in dTRPA1 circuit would be very useful.

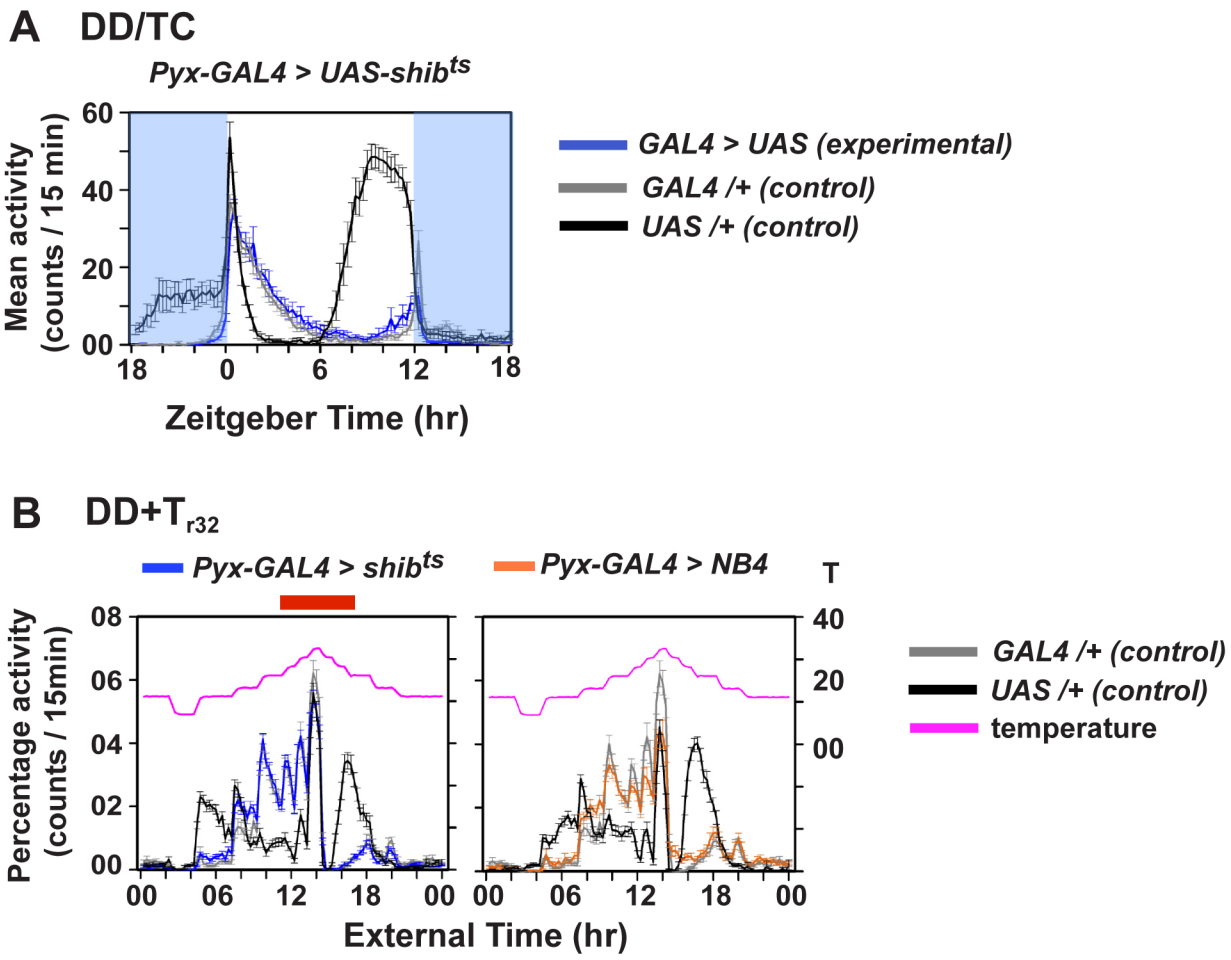

Figure 5.1. Pyrexia neuronal signalling is not essential to elicit dTRPA1-dependent behavioural changes in either DD/TC or DD+T_{r32} (A) Preventing synaptic transmission in Pyx^{+} neurons by expressing *UAS-shib^{ts}* under *Pyx-GAL4* and subjecting flies to DD/TC (29:21 ^{o}C) does not alter mid-thermophase activity. The experimental flies (blue curve) closely resemble *GAL4/+* control flies (grey curve) and exhibit siesta similar to control flies. Blue shaded areas represent cryophase (21 ^{o}C) of thermal cycles. (B) Blocking synaptic transmisson from Pyx^{+} neurons (blue curve) or hyperexciting the Pyx^{+} neurons (orange curve) do not effect the ability of flies to display A-peak under DD+T_{r32} regime. The pink curve represents gradually changing temperature profile imposed under DD+T_{r32} regime. Red line on top of left panel indicates the duration when temperature-sensitive *shibire* is likely to be active.

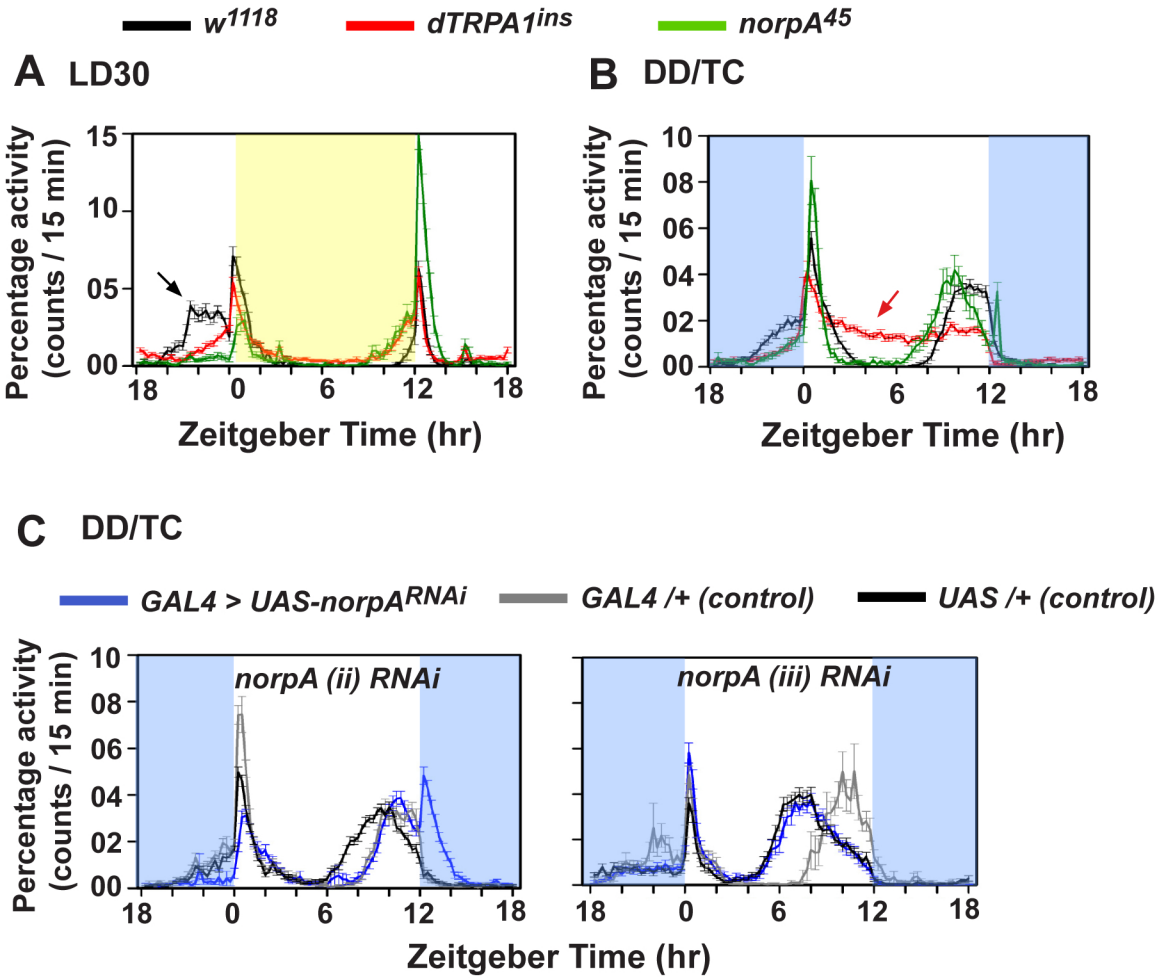

Figure 5.2. Upstream signalling via PLC pathway is not required for dTRPA1-dependent behaviour. (A) Wild-type (w^{1118} - black curve) flies show an advanced M-peak (arrow) that occurs before Lights-ON whereas both $dTRPA1^{ins}$ (red curve) and $norpA^{45}$ (green curve) mutant flies are unable to advance their M-peak and their M-peak occurs coinciding with Lights-ON at ZT0. Yellow shaded area represents light phase of LD cycles. (B) Under temperature cycles (29:21 ºC) in constant darkness, DD/TC, wild-type (w^{1118} - black curve) and $norpA^{45}$ (green curve) mutant flies show bimodal activity/rest pattern with siesta in the middle of the day whereas $dTRPA1^{ins}$ (red curve) show enhanced mid-thermophase activity (arrow). (C, left and right panels) Flies with RNAi knockdown of *norpA* with two independent RNAi constructs (blue curves) also show bimodal activity/rest rhythms under DD/TC resembling their parental controls (grey and black curves). Blue shaded area represent cryophase of DD/TC regime.

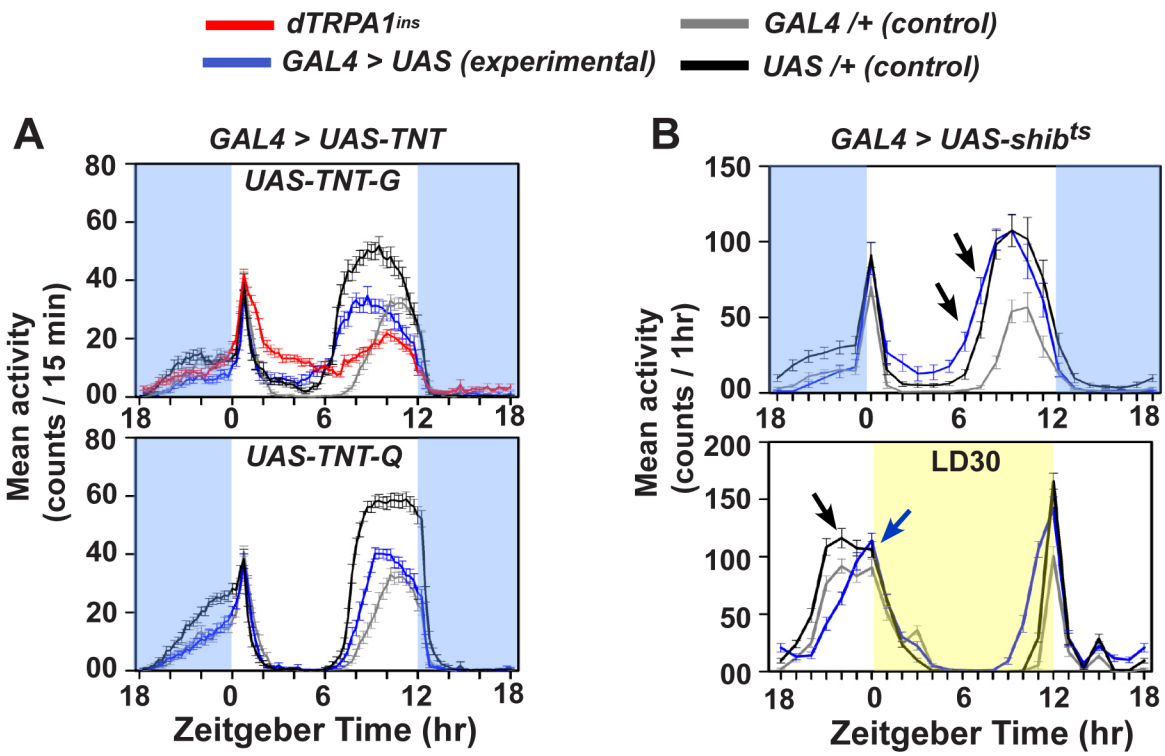

Figure 5.3. Blocking synaptic transmission across dTRPA1^{SH+} neurons has no effect but electrical silencing of same neurons enhances mid-day activity under temperature cycles. (A, top) Blocking synaptic transmisison across dTRPA1^{SH+} neurons by expressing tetanus light-chain toxin (*UAS-TNT-G,* blue curve) does not affect bimodal activity/rest pattern of flies under DD/TC. Loss of mid-day siesta is exhibited by dTRPA1 null flies (*dTRPA1^{ins}* - red curve) is shown for comparison. (A, bottom) Flies expressing a non-toxic form of tetanus toxin (*UAS-TNT-Q,* blue curve) also display bimodal activity/rest profile showing that expression of inactive form of the toxin does not affect behavioural profiles under identical conditions. (B, top) Alternatively, synaptic transmission was transiently blocked only during thermophase (when tempertaure was 29 ^{o}C) by expressing temperature-sensitive, *shibire* but such flies (blue curve) also exhibit normal activity/rest profiles, similar to parental controls except between ZT6-8 (arrows) when experimental flies (blue curve) exhibit higher activity than both parental controls (grey and black curves). (B, bottom) Flies expressing *UAS-shib^{ts}* (blue curve) do not advance their morning (M) peak (blue arrow) under LD30 regime. In contrast, control flies (grey and black curves), phase their M-peak before lights-ON at ZT0 (black arrow). Yellow shaded area represents light phase of LD cycles. Temperature under light/dark cycles is kept constant at 30 ^{o}C.

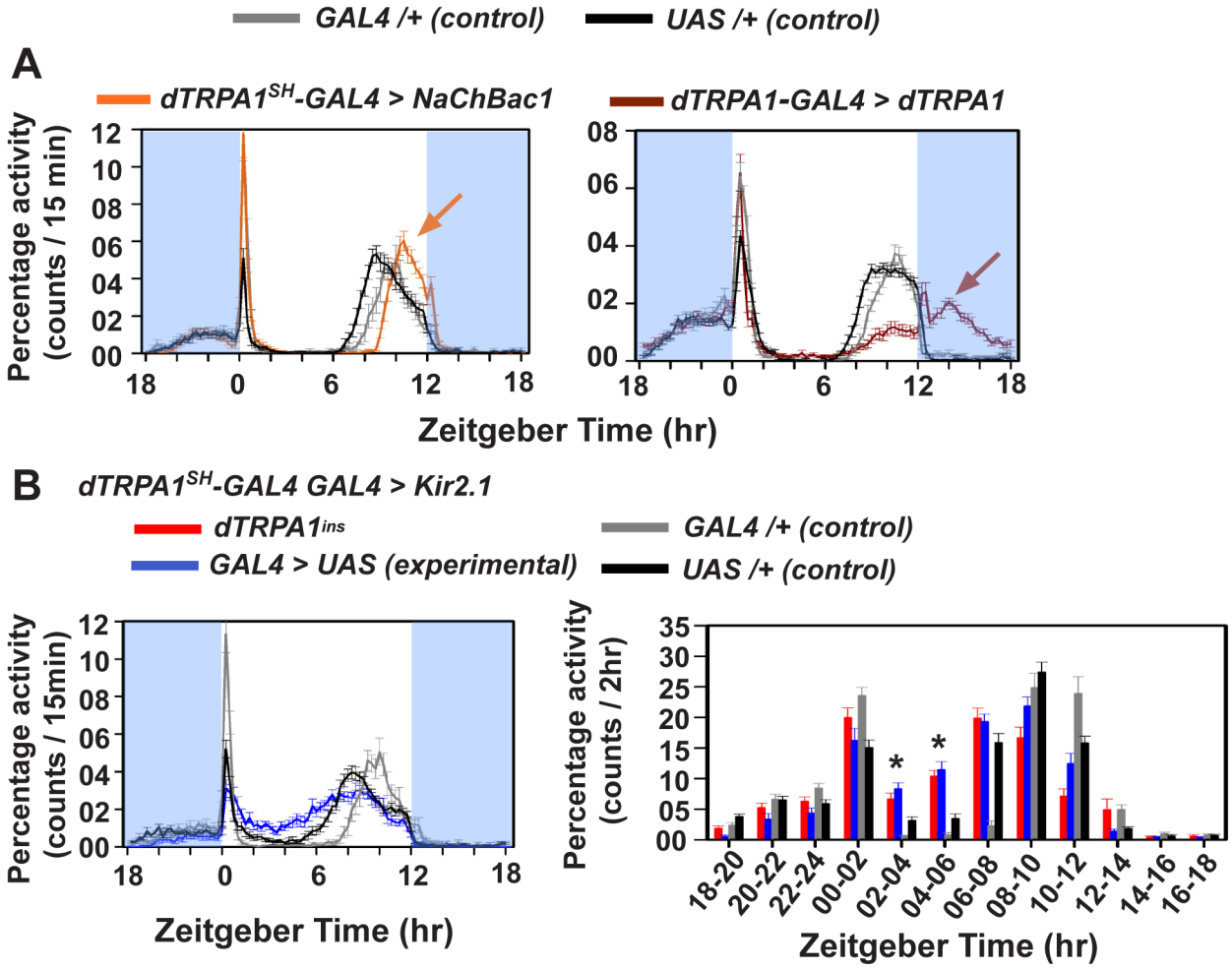

Figure 5.4. Altering electric activity of dTRPA1^{SH+} neurons dramatically modifies locomotor actvity/rest patterns under temperature cycles. (A, left) Electrical hyperexcitation of of a small subset of dTRPA1 neurons (dTRPA1^{SH+} neurons under *dTRPA1^{SH}-GAL4*) via expression of *UAS-NaChBac1* leads to a significantly delayed evening (E) peak (arrow) under DD/TC. (A, right) Under indentical regime of DD/TC, overexpressing dTRPA1 under a broad *dTRPA1-GAL4* driver also causes a significant delay in E-peak (arrow) compared to control flies. (B, left) Electrical silencing of neurons by driving *UAS-Kir2.1* results in enhanced mid-thermophase activity under DD/TC. (B, right) Normalised activity binned in 2hr intervals plotted against time show that *dTRPA1^{SH}-GAL4>UAS-Kir2.1* flies (blue bars) have significantly greater activity levels between ZT2-6 compared to their controls (grey and black bars). *dTRPA1^{ins}* flies (red bars) which also display high mid-thermophase activity is shown here for comparison. Blue shaded area represent cryophase of thermal cycles. Asterisks indicate significant differences in activity levels in experimental flies from both parental control genotypes within a given time interval (two-way ANOVA, followed by Tukey's HSD, $p < 0.05$).

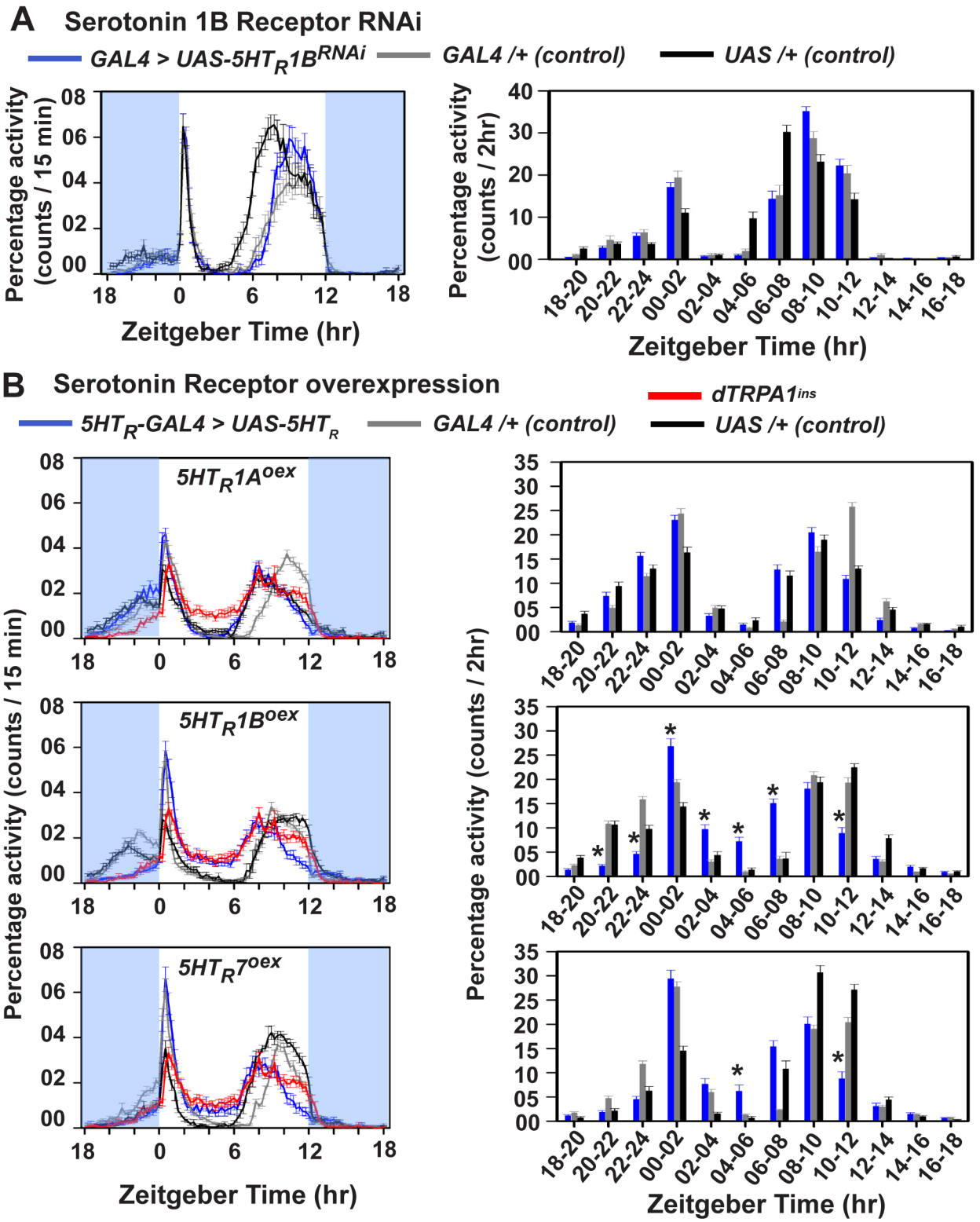

Figure 5.5. Overexpression of serotonin 1B receptors renders flies incapable of mediating mid-day siesta under temperature cycles. (A) RNAi knockdown of 5HT-1B receptor does not alter mid-day activity levels as seen by comparing (left) activity profiles and (right) activity levels in 2hr bins. (B,left) Overexpression of $5HT_R$-1A has no effect on siesta, but overexpressing $5HT_R$-1B causes flies to show enhanced mid-thermophase activity, thus resembling *dTRPA1*^{ins} null flies (red). Overexpressing $5HT_R$-7 mildly enhances mid-day activity between ZT4-6. (B, right) Percentage activity binned in 2hr intervals show that flies overexpressing $5HT_R$-1B or $5HT_R$-7 (middle and bottom panels - blue bars) exhibit enhanced activity compared to controls (grey and black bars). Asterisks indicate that activity levels in a given time interval in experimental genotype is significantly differenent from both parental controls, $p < 0.05$.

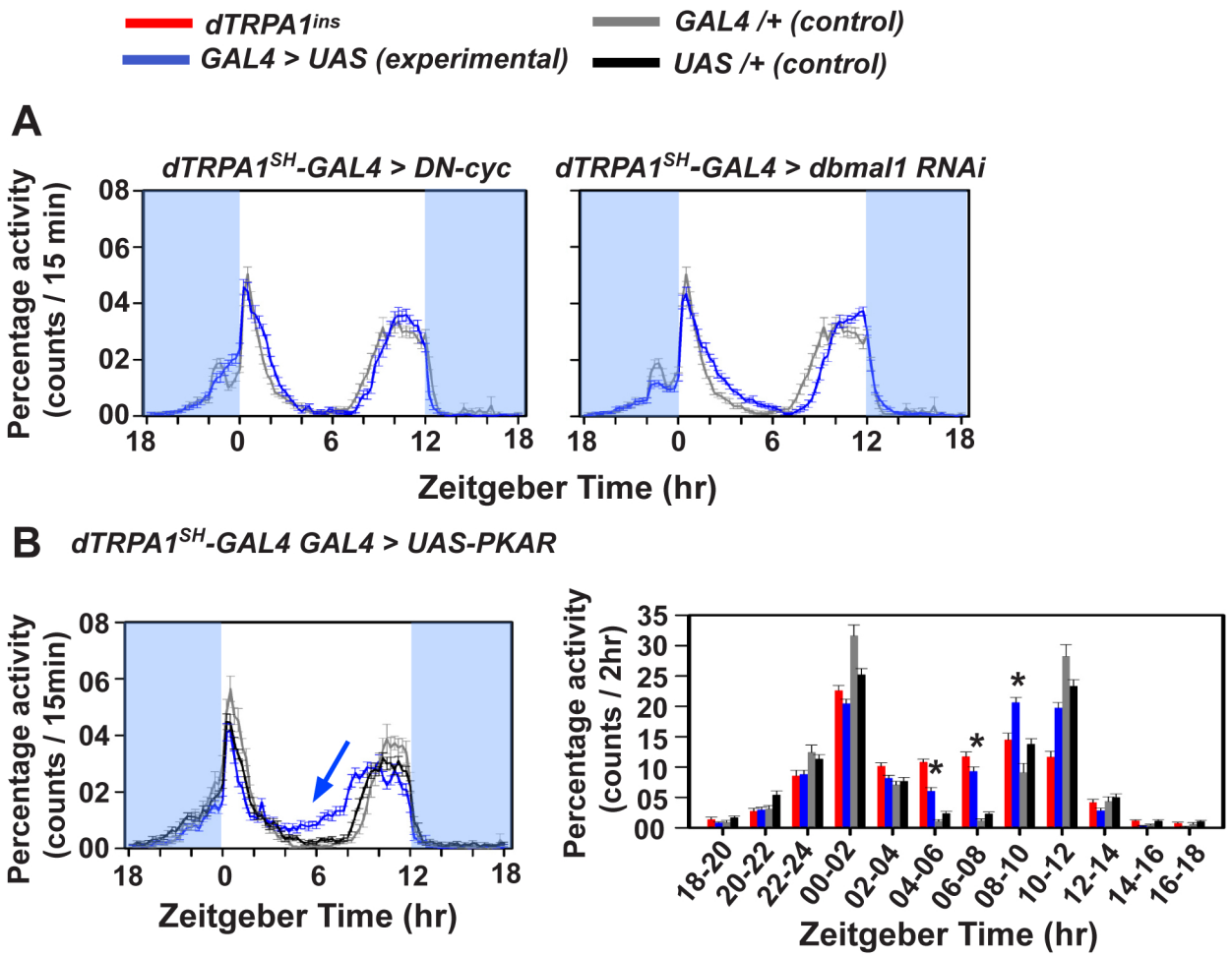

Figure 5.6. Reducing cAMP levels in dTRPA1^{SH+} neurons enhances mid-thermophase activity under temperature cycles. (A) Molecular circadian clock is disrupted in dTRPA1^{SH+} neurons by either expressing (A, left) a dominant-negative form of circadian core protein, CYCLE or (A, right) downregulating *cycle* expression by expressing RNAi. Flies with a dysfunctional clock in dTRPA1^{SH+} neurons (blue curves) exhibit bimodal activity/rest pattern under DD/TC similar to parental control flies (grey curve). (B) Levels of cAMP is reduced in dTRPA1^{SH+} neurons via expression of a dominant-negative form of PKA, *UAS-PKAR*. Flies with reduced cAMP/PKA levels (blue curve) display enhanced mid-thermophase activity (arrow). (B, right) Statistical tests performed on normalised activity levels binned in 2hr intervals show that *dTRPA1^{SH}-GAL4>UAS-PKAR* flies have significantly higher activity compared to control flies (grey and black bars) between ZT4-8. Normalised levels of activity displayed by *dTRPA1^{ins}* flies (red bars) is shown for reference. Blue shaded area represent cryophase of thermal cycles. Asterisks indicate significant difference from both controls, after performing two-way ANOVA, followed by Tukey's HSD, $p < 0.05$.

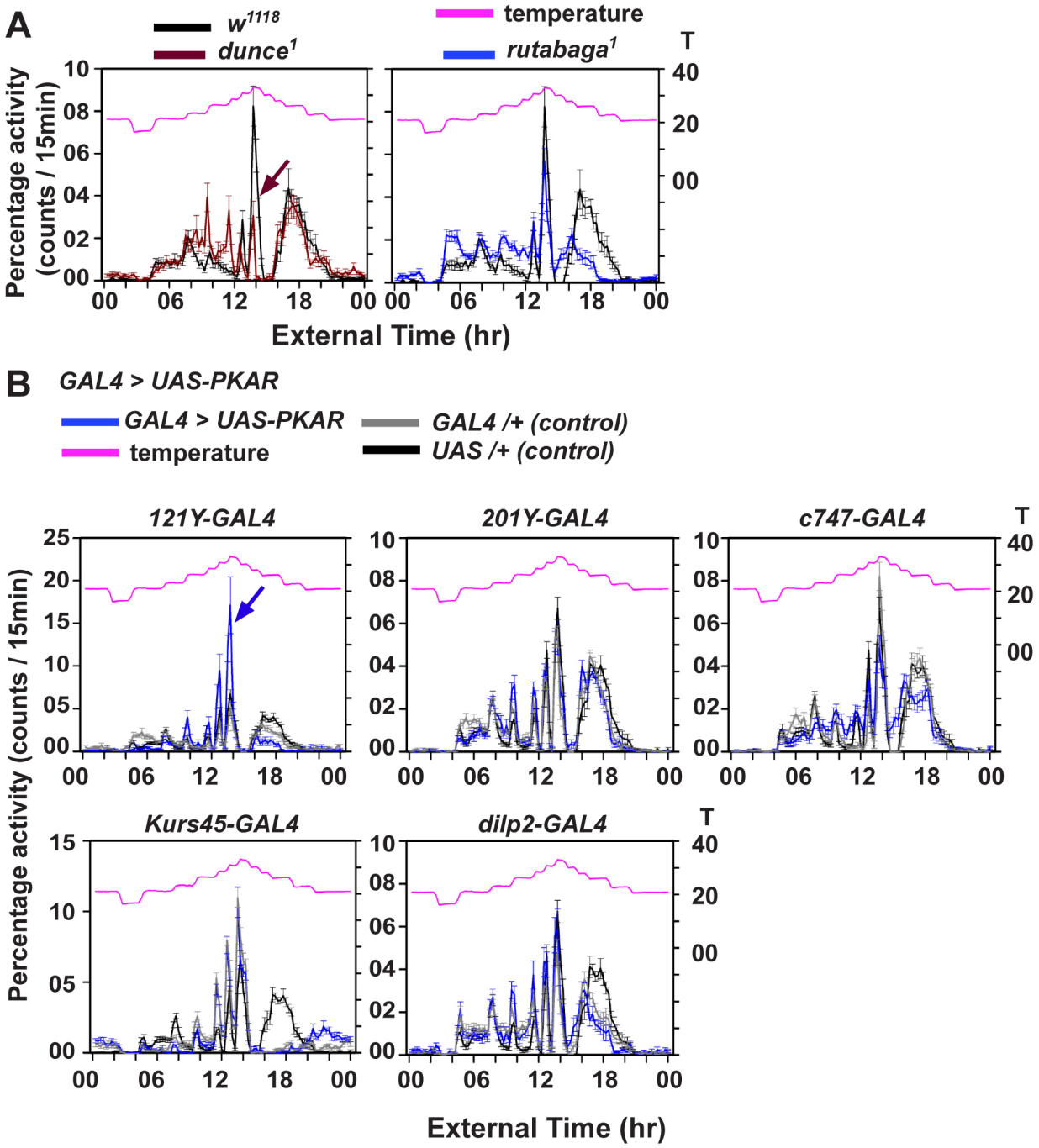

Figure 5.7. Altering cAMP/PKA levels in whole body or in specific subset of MB neurons influence dTRPA1-dependent A-peak under DD+T_{r32} (A) Whole body mutants for increased cAMP levels, *dunce*^{1} flies (dark red curve, arrow), show significantly reduced A-peak under DD+T_{r32} whereas *rutabaga*^{1} flies (blue curve) that have reduced cAMP levels exhibit A-peak similar to wild-type (*w*^{1118} - black curve) flies. (B) A preliminary screen for central complex neuronal subsets with reduced cAMP/PKA levels via expression of *UAS-PKAR*. Flies with reduced cAMP/PKA levels only in neurons targeted by *121Y-GAL4* (blue curve, arrow), which includes subsets of MB and PI neurons, show enhanced A-peak under DD+T_{r32}. All other genotypes show A-peak similar to their respective controls. The pink curve represents gradually changing temperature profile imposed under DD+T_{r32} regime. MB= mushroom body, PI= pars intercerebralis

Summary

Fruit flies, *Drosophila melanogaster*, display rhythmic activity/rest behaviour under cyclic temperature cues. Locomotor activity /rest rhythms are regulated by circadian clocks. In this thesis, I describe my attempts to understand how temperature cues modify rhythmic behavioural activity/rest pattern of flies via thermosensitive dTRPA1 signaling. I modified the neuronal network of dTRPA1 neurons and examined the effects of different environmental time cues on activity/rest behaviour in flies. I also examined if dTRPA1 neurons sends thermal cues to the circadian clocks which in turn modulate the activity/rest pattern in flies in a temperature-dependent manner.

My studies show that neurons expressing warmth-activated ion channels ***Drosophila* Transient Receptor Potential-A1** (dTRPA1) modulate distinct aspects of the rhythmic activity/rest rhythm in response to thermal cues under different environmental regimes. Under light/dark (LD) cycles at constantly warm ambient conditions, flies deficient in dTRPA1 expression are unable to phase their morning and evening activity bouts appropriately (Figure 6.1A, left). dTRPA1 null flies are unable to advance their morning peak into pre-dawn unlike the wild-type flies and my results unequivocally demonstrate that phasing of morning peak is dependent on electrical firing of subset of dTRPA1 neurons targeted by *dTRPA1^{SH}-GAL4* driver (dTRPA1^{SH+} neurons). The expression of dTRPA1 in this subset of neurons is also crucial for driving behaviour responses to temperature cycles in absence of time cues in the form of light/dark (LD) cycles (under constant light (LL) or constant dark (DD) conditions). Similar to LD conditions, dTRPA1 also influences phasing of evening peak under temperature cycles in LL, such that flies lacking dTRPA1 display an advanced phase of evening peak (Figure 6.1A, right) which can be rescued by restoring dTRPA1 expression in dTRPA1^{SH+} neurons. Further, my studies show that the mid-day

"siesta" exhibited by flies under temperature cycles in DD is dependent on dTRPA1 expression in dTRPA1^{SH+} neurons that include thermosensory AC neurons. Although a small subset of circadian pacemaker neurons express dTRPA1, my studies show that most circadian neurons are not essential and that CRY-negative *dTRPA1^{SH}-GAL4* driven neurons are critical for the suppression of mid-thermophase activity, thus enabling flies to exhibit "siesta". Taken together, my studies emphasise the differential influence of dTRPA1 thermoreceptors on rhythmic behaviour in flies in coordination with light inputs.

I also studied the role of dTRPA1 signaling in modifying behaviour of flies under natural conditions where different physical factors such as light, temperature, humidity etc. change in a gradual manner across the day. These environmental conditions are in contrast to the rectangular zeitgeber cycles that have been traditionally used for studies in the laboratory. Under such natural conditions, wild-type flies exhibit an additional peak of activity, referred to as afternoon peak (A-peak). Although several research groups reported the occurrence of A-peak under natural conditions, the underlying molecular mechanisms or pathways that control A-peak was not yet known. Through my studies, I show that the A-peak is mediated is predominantly elicited by temperature cues and is influenced by thermosensitive dTRPA1 signaling. When natural cycles of light and temperature are simulated in the lab, my results demonstrate that the amplitude of the A-peak is dTRPA1-dependent. Although a few circadian neurons express dTRPA1, through my studies, I am able to distinguish that modulation of A-peak is primarily influenced by non-CRY dTRPA1 expressing neurons. Hence, my studies propose that A-peak of activity observed under natural conditions is a temperature sensitive response in flies that is elicited through dTRPA1 receptor signaling.

Based on my current understanding of behavioural responses of flies under different environmental regimes, I find that dTRPA1 signaling has opposite effect on behaviour depending on the nature and duration of temperature cues. Prolonged exposure to 29 ^{o}C

under rectangular temperature cycles leads to suppression of mid-day activity whereas acute 32 ^{o}C for ~1 hr under gradual temperature cycles induces activity in flies, the afternoon peak (Figure 6.1B). These results emphasise the different mechanisms through which the same thermoreceptor dTRPA1 receptor signaling mediates crucial behavioural changes in locomotor activity / rest patterns in flies. To my knowledge, this is the first instance of a thermoreceptor which is known to drive opposite effects on behaviour depending on nature of the thermal stimuli. Further, as seen in Chapters 2 and 3, flies with ablated dTRPA1^{SH+} neurons resemble wild-type flies after few days under temperature cycles and these *dTRPA1^{ablated}* flies do not phenocopy dTRPA1 null flies under semi-natural conditions. These results suggest that either dTRPA1 in neurons outside the target of *dTRPA1^{SH}-GAL4* can compensate for lack of signals from dTRPA1^{SH+} neurons or other thermoreceptors can override lack of dTRPA1 signaling. Non-dTRPA1 thermoreceptors are most likely to belong other TRP channels as most of the known thermoreceptors in flies belong to TRP family or they could belong to ionotropic receptor family (IR); one such member, IR25a, has been recently implicated in temperature entrainment to low amplitude thermal cycles (Chen et al., 2015).

I also examined the neural circuits that have been proposed to function upstream and/or downstream of dTRPA1. dTRPA1-expressing AC neurons are serotonergic but which subclass of serotonin receptor neurons lie downstream of AC neurons are yet to be identified. My results suggest that serotonin receptor 1B neurons are probably not the synaptic partners of AC neurons as suggested by a previous study. Further, my studies demonstrate that cAMP/PKA intracellular signaling in dTRPA1^{SH+} neurons is important for suppression of mid-day activity under temperature cycles. Further, my studies have isolated a subset of MB neurons that influences amplitude of A-peak under gradual temperature cycles, suggesting that these neurons in the secondary processing centres in the adult fly brain may be involved

in modifying behavioural patterns in response to dTRPA1 signaling. Based on my present understanding, a schematic of how dTRPA1 signaling modulates activity / rest rhythms in response to temperature cues has been surmised in Figure 6.2.

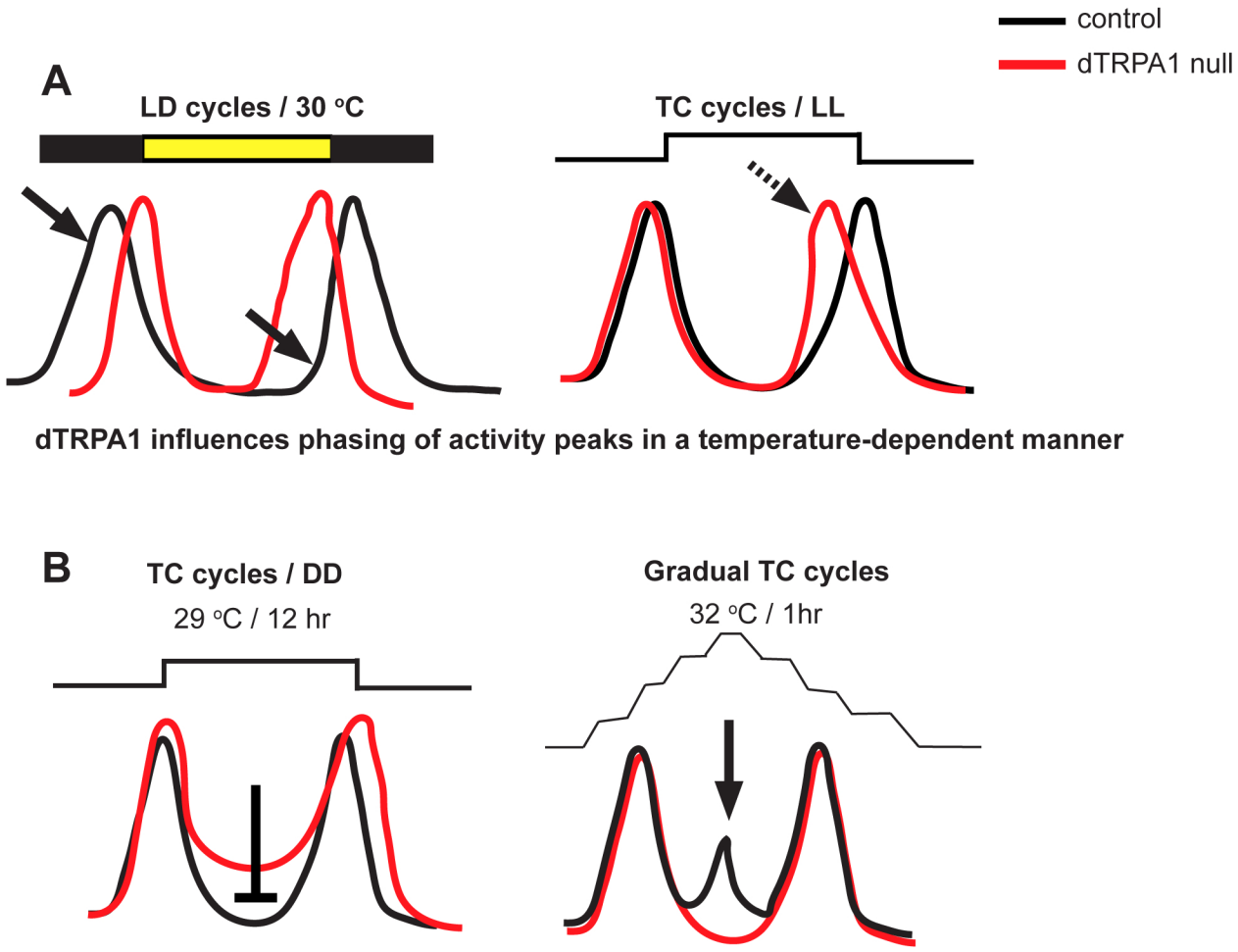

Figure 6.1. dTRPA1 influences activity/rest rhythms in a temperature-dependent manner. (A, left) Wild-type flies (black) advance morning peak and delay evening onset (arrows) under constant warm LD/30 °C conditions whereas dTRPA1 null flies (red) do not, suggesting dTRPA1 signaling influences phasing of activity peaks. (A, right) Under TC cycles in LL, dTRPA1 null flies (red) display an advanced evening peak compared to controls (black), indicating that lack of dTRPA1 signals result in an advance in the evening peak phase (dotted arrow). Thus, dTRPA1 influences phase of morning and evening peaks under LD and LL/TC. (B) dTRPA1 mediates opposite effects on mid-day activity in a temperature-dependent manner. (B, left) Under TC cycles in DD, when thermophase temperature is 29 °C for 12 hrs, dTRPA1 signaling is required to inhibit mid-day activity (control - black) whereas dTRPA1 null mutants (red) do not efficiently suppress mid-day activity. (B, right) In contrast, under gradual TC cycles wherein peak temperature is 32 °C for 1 hr, dTRPA1 mediates occurence of afternoon (or A) peak in wild-type flies (black) whereas dTRPA1 null mutants (red) do not display A-peak.

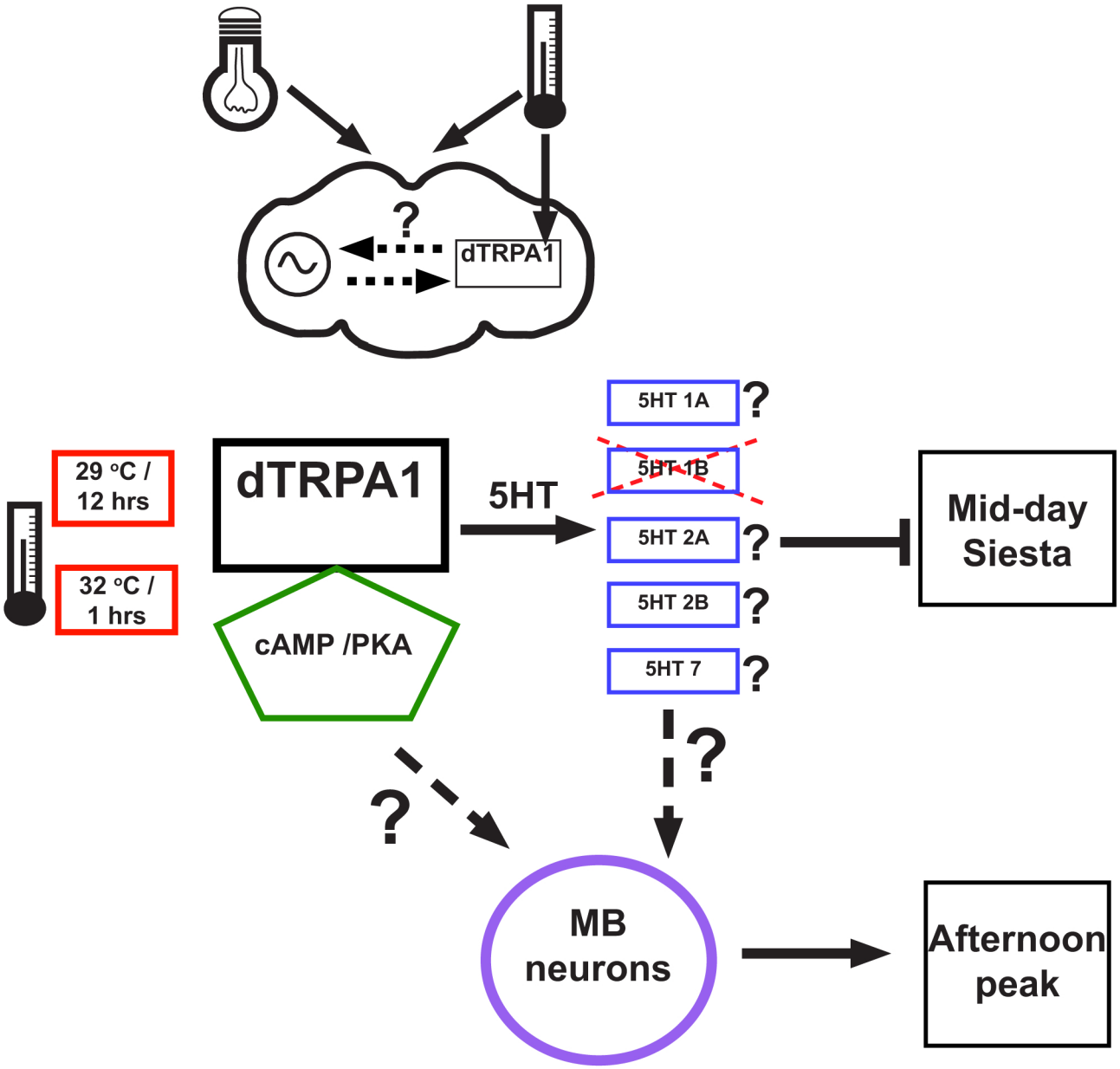

Figure 6.2. Schematic representation of dTRPA1 and downstream signaling that influences activity/rest rhythms in opposite directions. Light and temperature cues synchronise circadian clocks in the fly brain giving rise to rhythmic activity / rest rhythms. Thermosensory dTRPA1 channels are activated by temperature inputs, but how signals from circadian clocks and dTRPA1 neurons interact to modify behaviour remains unlcear. dTRPA1 expressing AC neurons secrete neurotransmitter serotonin and convey thermal signals downstream probably by one of the known serotonin receptor class neurons, except 5HT 1B receptor subclass, which my studies have ruled out. Intracellular cAMP/PKA signaling within dTRPA1^{SH+} neurons is crucial for flies to mediate suppression of activity during mid-thermophase. When a prolonged temperature cue (~29 °C for 12hrs) is given, signals from dTRPA1 via serotonin receptor neurons act directly or indirectly on motor circuits to inhibit activity, thus, resulting in afternoon siesta under temperature cycles. When the temperarure cue is short and acute (32 °C for 1hr), dTRPA1 signaling results in flies exhibitng A-peak. This pathway may involve direct signals to MB neurons or dTRPA1 signals reach MB neurons via serotonin receptor neurons. These signals promote activity and flies display A-peak.

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