Large and Small Ventral Lateral Neurons Play Vital Roles in Circadian and Arousal Circuits in *Drosophila melanogaster*

A Thesis

Submitted for partial fulfillment of the Degree of

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(Biological Science)

By

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To

Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore – 560 064, India

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Dedicated to

My parents

for their unwavering belief and encouragement

DECLARATION

I hereby declare that the thesis entitled "Large and Small Ventral Lateral Neurons Play Vital Roles in Circadian and Arousal Circuits in *Drosophila melanogaster"* submitted towards the partial fulfillment of the M.S-Ph.D degree (Biological Science) is the result of investigations carried out by me at the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India. The work incorporated in this thesis did not form the subject matter of any other thesis submitted by me for any other degree elsewhere.

Due care has been taken to acknowledge the work and findings of other investigators in the light of the present study, keeping in view the practice of reporting scientific observations. Any omission that may have occurred due to misjudgement or oversight is deeply regretted.

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CERTIFICATE

This is to certify that the work described in this thesis entitled "**Large and Small Ventral Lateral Neurons Play Vital Roles in Circadian and Arousal Circuits in *Drosophila melanogaster***" is the result of studies carried out by Ms. Sheetal Potdar in the Behavioural Neurogenetics Laboratory, Evolutionary & Organismal Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560 064, under my supervision, and that the results discussed in the thesis have not previously formed the basis for award of any other diploma, degree or fellowship.

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Contents

Abstract

Neuronal circuits underline various simple and complex behaviours in animals, which are displayed in response to numerous stimuli in the environment. *Drosophila melanogaster* offers several simple but effective tools in the neurogenetic dissection of different behaviours. We have focused on two related neuronal circuits – the circadian clock network and sleep-arousal circuit, and looked to evaluate the roles of two subgroups of circadian clock neurons – the large and small ventral lateral neurons (l-LN_v and s-LN_v) within these circuits.

So far, the l-LN_v have mostly been disregarded or have been given a auxiliary role in the hierarchical arrangement of the *Drosophila* circadian circuit. We aimed to re-evaluate this view by examining the contributions of l-LN_v in governing an important clock function – phasing of locomotor activity peaks. Here, we report that electrical activity of l-LN_v controls phasing of the evening peak of activity across a range of photoperiods. Further, we propose a model in which l-LN_v enable adaptation to seasonal changes by regulating the phase of the evening peak. Thus, our results have revealed a critical role for the l-LN_v in the clock circuit, where it was erstwhile negligible.

In contrast, in the sleep/arousal circuit, the l-LN_v play a major role whereas so far, no known role for the s-LN_v has been reported. Here, we have reprised the role for l-LN_v in the arousal circuit; found novel role for s-LN_v in mediating arousal that is modulated by l-LN_v and identified a downstream target of the LN_v neurons, which is localized in the Pars Intercerebralis, the neuroendocrine centre of the fly brain. Thus, our results underline the flexibility of neuronal function such that certain neurons can play integral roles in otherwise distinct circuits.

Chapter 1

Introduction

A. Animal behaviours are regulated by neuronal circuits

Animals display a repertoire of behaviours in response to various stimuli in their environment. While some behaviours are innate, fixed action patterns that are 'hardwired' in the brain (for example, gull chick's pecking response), others are acquired through experience (Alcock, 2009). For instance, taxi drivers in London are required to memorize the entire road map of London right down to the minutest of the details, in order to gain their license (Woollett and Maguire, 2011). Most behaviours of animals have underlying neuronal mechanisms – or pathways that govern their perception of stimuli and the execution of responses. For instance, the underlying neuronal mechanism for how moths escape being caught by echolocating bats has been mapped to an acoustic circuit consisting of highly specialized A1 and A2 receptor cells that are sensitive to sound in the ultrasonic range (Roeder and Treat, 1961). Similarly, effect of serotonin on a central synapse (Large Giant interneuron) in crayfish is dependent upon an individual's social status and history, thus allowing dominant and subordinate individuals to choose different behavioural paradigms – dominant ones are more aggressive while subordinate crayfish are unwilling to participate in a fight (Yeh et al., 1997). Mice, whose reward pathway neurons are activated upon pressing a lever, become addicted to pressing it in order to stimulate themselves and 'feel' as if they have been rewarded (Olds, 1958). Many day-to-day behaviours that apparently do not seem as dramatic as the afore-mentioned ones are also governed by neuronal clusters in the brain. For instance, sleep/wake cycles, feeding patterns and other rhythmic behaviours in mammals are governed by circadian clocks that reside in the brain in a bilateral structure in the hypothalamus called suprachiasmatic nucleus (Dunlap et al., 2004). The

human language, which is a complex behavioural trait that is quite distinct from the rest of the animal world, is also encoded in the brain although in a much more distributed manner. Vocabulary and speech-related usage is restricted to the Broca's area, while hearing and comprehending words is done in Wernicke's area (Kandel et al., 2000).

Since the 19th century when Franz Joseph Gall propounded the now-extinct concept of phrenology, by which he explained that a specific part of the brain was responsible for bringing about even the most abstract of behaviours (for example, destructiveness, generosity, ideality and hope!), biologists have been interested in deciphering the neural correlates of behaviour (Kandel et al., 2000). Model organisms have been used to dissect fundamental processes underlying behaviours that would have been difficult to study in more complex organisms, including humans. Most model organisms have several useful characteristics, the most important of which are the presence of a simple genetic framework, a fully mapped genome and a fairly simple central nervous system (Hawley and Walker, 2003). While mouse, zebra fish and *C. elegans* have served well in the brain-to-behaviour mapping, substantial number of fundamental discoveries has been made by the use of *Drosophila melanogaster* as a model organism.

B. *Drosophila melanogaster* as a model to study circuits controlling behaviours

Invertebrate model organisms provide unparalleled advantage in the study of neuronal basis of any behaviour, because of their small size, easy maintenance, short development time, uncomplicated genetic make-up and relatively simple nervous systems consisting of less number of neurons. *Caenorhabditis elegans* (Jorgensen and Mango, 2002) and *Drosophila melanogaster* (St.Johnston, 2002) have emerged as

important neurogenetic model organisms in the past two decades or so. *Drosophila melanogaster,* in particular, has been used to study the neuronal and genetic basis of many complex behaviours such as sleep (Sehgal and Mignot, 2011), aggression (Kravitz and Huber, 2003), courtship (Hall, 1994), addiction (Devineni and Heberlein, 2009), circadian rhythms (Allada and Chung, 2010) and many sensory modalities like olfaction, vision, thermosensation, nociception, audition, gustation and so on (Sokolowski, 2001). Additionally, a central nervous system composed of about 100,000 neurons that can be easily manipulated by non-invasive means via genetic methods provides a direct mapping between behaviours and their underlying neuronal circuitry.

It is notable that an innocuous fruit-loving fly can empower a biologist by offering a wide range of choices in manipulating a given neuronal circuit (Venken et al., 2011). One such popular method that enables researchers to target the expression of genes-of-interest in a tissue-specific manner is the bipartite GAL4-UAS system (Brand and Perrimon, 1993). GAL4, which can be expressed under the control of a tissue-specific promoter, is a yeast transcriptional factor that activates the expression of any gene which is downstream of UAS (Upstream Activation Sequence) sequence. This system is one of the easiest ways of unraveling the neuronal circuitry mediating the display of a behaviour-of-interest. Neurons can be ablated, made to over-express or misexpress a gene-of-interest, hyper-excited or silenced and the effects of these manipulations can be examined at the level of behaviour (Holmes et al., 2007). Other applications of this system include studying genes responsible for a behaviour within the context of the neurons underlying the behaviour and visualizing individual neurons that form a circuit (Duffy, 2002). By incorporating GAL80, which is an inhibitor of GAL4, a further level

of tissue-specificity can be achieved such that only subsets of neurons that express GAL4 are manipulated, sparing the ones that co-express GAL4 and GAL80 (White and Peabody, 2009). This kind of finer analysis of underlying neurons can also be performed by combining two binary systems, such as the GAL4-UAS and the LexA transactivator (Szuts and Bienz, 2000) or the Q systems (Potter et al., 2010). These binary systems can be used in conjunction with one another in order to visualize synaptic connections between neuronal subgroups, thus enabling identification of the players in the circuit (Yagi et al., 2010). Various new methods are available that allow for the spatio-temporal control of gene expression, such that circuits are manipulated during a particular window of time. Thus, *Drosophila melanogaster* serves as a rich source of circuit-breaking techniques that enables answering fundamental questions about mechanisms underlying various behaviours.

C. The neuronal circuit that controls the circadian clock in *Drosophila*

Drosophila has enabled the dissection of neuronal circuit underlying the circadian network that has facilitated the examination of mechanistic phenomena governing rhythmic behaviours. Circadian clocks are endogenous, self-sustained time-keeping systems with periodicity of about 24 hr and are present in almost all life forms. These clocks facilitate organisms in timing their physiological and behavioural processes with reference to external time measured by using changes in environmental factors such as light and temperature that take place on a daily basis (Dunlap et al., 2004). Some of the behaviours that are under clock control in *Drosophila* are activity/rest cycles, adult emergence from pupae, olfactory and gustatory responses, egg-laying and mating to name a few (Allada and Chung, 2010).

The molecular mechanisms that generate these self-sustained near 24 hr oscillations are conserved across various taxa and involve transcriptional-translational feedback loops (Young and Kay, 2001). In *Drosophila melanogaster*, the feedback loop is well-characterized and consists of interlocked positive and negative limbs. In brief, *clock* (*clk*) and *cycle* (*cyc*) are two transcriptional activators that turn on the expression of *period* (*per*) and *timeless* (*tim*), whose protein products turn off their own transcription (Yu and Hardin, 2006). Kinases and phosphatases alter the stability of PER and TIM in the cytoplasm, thus delaying their nuclear entry for transcriptional repression, such that this whole cycle gets completed in about 24 hr (Bae and Edery, 2006). TIM, in the presence of light gets labeled for degradation via a blue light sensitive photopigment called CRYPTOCHROME (CRY), such that now the transcription cycles get driven by external light/dark cycles (Zheng and Sehgal, 2008). Thus, endogenous circadian clocks which can run with their own *free-running* period in the absence of time cues, can get *entrained* or synchronized to external environment with a stable phase relationship (Moore-Ede et al., 1982).

The overt behaviour using which much of the neurogenetic basis of circadian clocks has been unraveled is the activity/rest rhythm. In light/dark cycles of 12 hr each (LD 12:12) at a standard temperature of 25 ˚C, flies show a bimodal pattern of activity, with one peak centering around dawn (or lights-ON) and the other around dusk (or lights-OFF). Importantly, the clock-mediated build-up of activity (or anticipation) before the light/dark or dark/light transitions are seen as the markers of presence of a functional circadian clock, as flies carrying mutations in core clock genes do not show any anticipation (Wheeler et al., 1993). Additionally, male flies rest during the night and

also during mid-day, and this rest is very much like mammalian sleep (Helfrich-Forster, 2000). Moreover, just as light can entrain the clock, other cycling cues, such as temperature, can also do so with varying abilities (Sharma and Chandrashekaran, 2005). However, the most direct estimate of the properties of the underlying clock has come from studying activity/rest patterns in constant conditions in the absence of time cues. Various measures from the overt behaviour can be used to gauge the effects of neurogenetic manipulations, such as the presence of a functional circadian clock, its free-running period and the amplitude or the robustness of its cycling when in constant conditions. The activity/rest pattern is monitored by an automated process that involves the breaking of an infra-red beam (manufactured by Trikinetics^{TM}, Waltham, MA, USA). A fly when active, cuts the infra-red beam that passes through a small tube in which it is housed, such that the number of breaks it makes are recorded as activity counts by a computer. The number of counts is an indicator of level of activity. The infra-red beam is constant, but activity can be binned at one or five or 15 min intervals, such that a temporal profile of activity/rest pattern can be obtained for each fly. The availability of this kind of an automated system has facilitated an objective analysis of the functions of various circadian clock neurons.

Circadian clocks in *Drosophila* reside in about 150 bilaterally located neurons which are distinguished from the rest of the CNS by their rhythmic expression of the canonical core clock genes *per* and *tim* (Sheeba, 2008). These neurons are grouped into two main subgroups on the basis of their anatomical location – dorsal neurons (DNs) and lateral neurons (LNs). The dorsal neurons are further subdivided into three subsets – first group is the DN1 consisting of two $DN1_a$ (anterior) and 12-15 $DN1_p$ (posterior)

cells, second group is a couple of DN2 and the third group is about 40 DN3 per hemisphere (Kaneko and Hall, 2000; Helfrich-Forster, 2003). The lateral neurons are clustered into four subsets – dorsal lateral neurons (LN_d), large ventro-lateral neurons (l-LN_v), small ventro-lateral neurons (s-LN_v) and lateral posterior neurons (LPN, Shafer *et al.*, 2006). Another cell that lies close to the l-LN_v and which expresses *per* and *tim* is called the 5^{th} s-LN_v as it is functionally and developmentally different from the rest of the s-LN_v (Helfrich-Forster et al., 2007). Most of these clock neurons, barring the DN2, express *cry* (Klarsfeld et al., 2004), therefore, in principle, they are each capable of entraining to light (Helfrich-Forster et al., 2001). Additionally, the l-LN_v and the s-LN_v produce a neuronpeptide called PIGMENT DISPERSING FACTOR (PDF) which is not expressed by 5^{th} s-LN_v (Kaneko and Hall, 2000). Thus with different subsets expressing different clock-associated genes, neurogenetic dissection of the clock circuit has been possible by the use of some of the methods described above. Some of the main features of the different subsets of clock neurons are outlined below.

Lateral neurons (LNs)

Since the first description of the circadian neurons, the importance of the lateral neurons in bringing about rhythmic behaviours has been validated many times. One of the first studies to do so showed that per expression in LN_v is essential for behavioural and molecular rhythms in constant as well as LD 12:12 conditions (Frisch et al., 1994). Further support to these results came in the form of another study where *per* was expressed under the *glass* promoter in a per^{01} background, such that now *per* was expressed in dorsal and lateral neurons which also rescued the behavioural arrhythmicity. *disconnected* (*disco*) mutants that do not have the clock-relevant lateral

neurons, also showed defects in their free-running rhythms (Helfrich-Forster, 1998). By this time, the identification of pigment-dipersing factor (Helfrich-Forster, 1995) in flies facilitated the further subdivision of lateral neurons into the PDF^+ s-LN_v and l-LN_v and the PDF^- LN_d and 5^{th} s-LN_v (Helfrich-Forster et al., 2007). So far the only known role for the LN_d and the 5^{th} s-LN_v is in governing the ability to anticipate the light/dark or evening transition (Rieger et al., 2006; Johard et al., 2009; Hermann et al., 2012). Another group of lateral neurons, the LPN have been implicated in temperature entrainment along with the DNs (Yoshii et al., 2005).

The most convincing evidence for the involvement of lateral neurons, more importantly the PDF^+ LN_v came in the form of behavioural defects in flies that had ablated LN_v. These flies, like the *pdf*^{01} mutants, showed arrhythmicity in constant dark conditions (DD), especially after spending 2-3 days without time cues (Renn *et al.*, 1999). Additionally, these flies were also defective in their LD 12:12 behaviour, in that they did not show anticipation to dark/light or morning transition, and displayed an evening peak that was phase advanced with respect to lights-OFF (Renn et al., 1999). Furthermore, silencing the PDF^+ neurons also phenocopied the *pdf*^{01} mutants (Nitabach et al., 2002). A recent study, in which the PDF^+ neurons were silenced with an additional temporal control, showed that behavioural arrhythmia persisted so long as the PDF^+ neurons were silenced, the reversal of the silencing resulted in the restoration of activity/rest rhythms in constant darkness (Depetris-Chauvin et al., 2011). This suggests that, the internal mechanism that sets the pace of the clock is intact, the output of the clock affected.

PDF as a synchronizing signal of the clock circuit

The role of PDF as an output molecule was proposed when injections of PDH – pigment dispersing hormone into the cockroach brain was able to cause phase delays (Petri and Stengl, 1997). These results were further backed up by behavioural characterization of pdf^{01}(Renn et al., 1999). In fact, it was seen that *tim* mRNA oscillations were lost in all circadian neurons in constant dark conditions in the absence of functional PDF (Peng et al., 2003). Analysis of the molecular oscillations of the clock neurons of pdf^{01} flies revealed that PER accumulation and subcellular distribution rhythms were desynchronized among s-LN_v and phase advanced in all the LN_d (Lin *et al.,* 2004). This led to the proposition that PDF was not important in giving rise to molecular oscillations but was required to maintain synchrony among neurons within the clock circuit in constant darkness (Lin et al., 2004). Further proof for this role for PDF was lent when three different groups independently discovered the PDF receptor to be a G-protein coupled receptor, similar to the receptor of mammalian clock co-ordinator Vasoactive Intestinal Polypeptide (VIP, (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). Loss-of-function mutations in *pdfr* resulted in a phenotype similar to that of pdf^{01} – behavioural arrhythmicity in constant dark, absence of morning peak and presence of a phase-advanced evening peak (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). Additionally, by monitoring changes in calcium levels in order to assess receptivity to PDF, it was found that all the clock neurons except l-LN_v were responsive to PDF, lending further support to the role of PDF as a synchronizer (Shafer et al., 2008).

Electrical silencing of PDF^+ neurons led to phase advancement of clocks in all PDF^- neurons – consistent with the synchronizing role for PDF (Nitabach et al., 2002; Wulbeck et al., 2008). Similarly, various mutants with altered dorsal and accessory medulla arborizations (where the s-LN_v also send their projections), that either had too little or too much of PDF showed that different neurons responded differently in terms of the effect on the speed of their clocks; some of them sped up, while others slowed down (Y. Wu et al., 2008). Because PDF had complex effects on the neurons that respond to its signaling, it was proposed that PDF maintains phase relationships among clock neurons, such that the network is a multiphasic clock that is able to adjust to dynamic changes in the environment (Yoshii et al., 2009). Indeed, electrical hyper-excitation of LN_v on a short-term basis showed arrhythmicity in DD (Nitabach et al., 2006); but showed re-organization of the circuit after spending longer time in DD (Sheeba et al., 2008d). This was concluded on the basis of the observation that after initial few days of arrhythmicity, complex rhythms began to emerge. By hyper-exciting the PDF^+ neurons in a pdf^{01} background, it was found that the short period component was dependent on PDF, while the long period component was independent of PDF (Sheeba et al., 2008d). An early study that looked at the dorsal projections of the s-LN_v that arborize toward the dorsal protocerebrum found that the intensity of PDF cycles as a function of time of the day (Park et al., 2000), even though the *pdf* mRNA and PDF protein do not cycle in the LN_v cell bodies (Park and Hall, 1998; Park et al., 2000). However, this cycling was not required for maintaining behavioural rhythms in DD or molecular oscillations in other clock neurons (Kula et al., 2006). These studies, taken together suggest that PDF acts differently on different clock neurons, thus enabling the

maintenance of a multi-oscillator network that allows for behavioural adjustment under changing environmental conditions.

Which of the two LN_v subsets sends the PDF signals to the rest of the clock circuit? A further functional distinction between the s-LN_v and the l-LN_v has been made due to differences in their molecular oscillations under DD conditions. Several studies have independently shown that under constant darkness, molecular oscillations in s-LN_v persist robustly, whereas dampen out or remain absent in the l-LN_v (Park et al., 2000; Yang and Sehgal, 2001; Shafer et al., 2002; Lin et al., 2004). This has led to the conclusion that the s-LN_v are more essential than the l-LN_v in maintaining synchrony among the other circadian clock neurons (Park et al., 2000). Altogether, these results establish the role of PDF signaled from the s-LN_v as crucial in the synchronization of the clock circuit in the absence of external time cues.

While the importance of s-LN_v in constant conditions has been proven time and again using both molecular and behavioural means, the relative weights of the two LN_v subset in governing LD behaviour has been contentious. This is best exemplified by the conflicting evidence obtained for determining the substrate for governing morning anticipatory activity. These questions get further obscured by the non-availability of specific drivers that can distinguish between the two subsets. Nevertheless, certain studies have tried to demarcate between the two and have obtained inconsistent results. The earliest study in this direction concluded that the morning anticipatory activity is indeed governed by the s-LN_v (Grima et al., 2004). However, this study used a genetic method by which the authors returned the expression of *per* only in the s-LN_v and not anywhere else in a per^{01} background, such that only the s-LN_v had a functional ticking

clock. This experiment was repeated using a different driver – which while specific to the s-LN_v in the clock circuit, also expresses in other non-circadian neurons (Cusumano et al., 2009). In both these studies, the resulting phenotype was identical, in that the absence of morning anticipatory activity in per^{01} flies was rescued. This led to the conclusion that the s-LN_v alone are necessary and sufficient for generating morning anticipatory activity. These results are in complete disagreement with another study in which the s-LN_v specifically were made dysfunctional by expressing a mutant form of human Huntingtin protein (Sheeba et al., 2010). While a *pdf-GAL4* driver was used to drive this neurodegenerative protein in both the LN_v subsets, it was specific in its action in only the s-LN_v as visualized by lack of PDF or PER expression. Furthermore, the activity/rest behaviour of flies with dysfunctional s-LN_v had become completely arrhythmic in DD. When these flies were subjected to a LD12:12 cycle, surprisingly, they showed the presence of morning anticipatory activity. While the authors were cautious to conclude that this morning anticipatory activity could be as a result of any of the unaffected circadian clock neurons, previous studies that identified the LN_v as the morning neurons lend support to the idea that this anticipatory activity is in fact due to the l-LN_v. Apart from this and another study (Cusumano et al., 2009), no attempt has been made to look into the functional significance of the l-LN_v. Their role has mostly been described in transmitting light information, as their projection patterns suggest that they communicate with the rhodopsin-based compound eyes (Helfrich-Forster and Homberg, 1993; Kaneko and Hall, 2000) and Hofbauer-Buchner eyelets (Malpel et al., 2002). In addition, they have been shown to respond to light by increasing their firing rate, and this response is mediated by CRY (Sheeba et al., 2008b). Recently it was

shown that CRY in l-LN_v has only a light-responsive role; it is not involved in the entrainment of the l-LN_v clock to light (Fogle et al., 2011). The l-LN_v have been ascribed a strong role in the sleep/arousal circuit which is highlighted in a later section.

Dorsal neurons (DNs)

The dorsal neurons, as their name suggests, are located in the dorsal part of the *Drosophila* brain, and all of them send their projections in to the dorsal protocerebrum. The DNs are not as well characterized as the LNs because of lack of GAL4 drivers that target specific DNs exclusively. The DNs were first implicated to play an important role in the clock circuit when a transgenic fusion construct of *per*-luciferase was shown to express *per* rhythmically in only the DNs, and that though this could not sustain rhythms in DD, the evening anticipatory activity was rescued in per^{01} flies by this construct (Veleri et al., 2003). Additionally, it was shown that this behavioural rescue was due to the *per* expression rhythm in the DN3, which was independent of the LN_v. Another study that underlined the importance of DNs looked at *tim* mRNA oscillations after specifically speeding up the clock in certain subsets of neurons by expressing *shaggy*, the protein product of which is a kinase that acts on TIM (Martinek et al., 2001). They found that while speeding the clock in the s-LN_v sped up those in LN_d, DN1 and DN3, speeding up the clock in DN2 sped up the clock in l-LN_v (Stoleru et al., 2005). Importantly, the molecular oscillation in DN2 was found to be anti-phasic to those in s-LN_v during the larval stage both in LD 12:12 and DD (Kaneko et al., 1997), which becomes in-phase in the adult stage, though only in LD 12:12 and for the first two days in DD (Blanchardon et al., 2001; Veleri et al., 2003) . These results taken together led to the proposition that a s-LN_v-independent oscillator is present in the DN2

whose function while unknown, has the ability to dictate the pace of the clock in l-LN_v. In another study, DN1 were shown to be the neurons responsible for rhythmicity in constant light conditions (LL) which normally does not occur in flies (Murad et al., 2007). Because CRY degrades TIM in light conditions, in LL fruit flies show arrhythmic behavior (Wheeler et al., 1993; Busza et al., 2004; Dissel et al., 2004). However, in certain circumstances, for instance in a condition when *per* is overexpressed, it inhibits the CRY pathway and renders flies rhythmic even in LL. This rhythmicity is brought about by the DN1, as observed by the persistent PDP1 oscillations only in the DN1, and none observed in the other clock neurons. This kind of a CRY inhibition is also brought about by other genes such as *morgue* and *kismet* (Dubruille et al., 2009), such that over-expressing the former and knocking down the latter specifically in the DN1 brings about rhythmicity in LL. Further proof of importance of DN1 neurons was unraveled by using different length promoter sequences of *clock* to drive expression of gene-of-interest in specific DNs (L. Zhang et al., 2010; Y. Zhang et al., 2010). Zhang L et al (2010) rescued the expression of *narrow abdomen* (*na*), an ion channel which when present in mutated form causes loss of both morning and evening anticipatory activities, only in a subset of $DN1_p$ neurons and observed a complete behavioural rescue. Interestingly, lengthening the clocks in s-LN_v lengthened those in the $DN1_p$ without particularly affecting the LN_d and 5^{th} s-LN_v clocks leading the authors to conclude that the $DN1_p$ are more sensitive to PDF. Rescuing the expression of *pdfr* specifically in the $DN1_p$ altered the phase of PER accumulation rhythms of both $DN1_p$ and s-LN_v suggesting the existence of some form of a feedback. Another study in the same issue of current biology, not only shared the

last name of the author, but also the $DN1_p$-specific GAL4 driver and found that $DN1_p$ are important in bringing about morning anticipatory activity in the absence of functional clock elsewhere in the circuit, most notably in the LN_v (Y. Zhang et al., 2010). In fact at low light intensity, even the evening anticipatory activity was rescued. However, in a 12:12 temperature cycle, the $DN1_p$ rescued evening anticipatory behaviour, which persisted even in an LD 12:12 at low temperature. These results led the authors to propose that $DN1_p$ are important in assimilating light and temperature cues in a natural environment. Thus it is quite clear that the DNs, like the LNs are quite heterogeneous in their functioning.

While the roles of DNs in bringing about activity/rest behaviour in DD and LD 12:12 have so far been limited, the DNs are implicated in transmitting information about other time cues, especially temperature (Miyasako et al., 2007). In addition, the DN1 neurons have also been shown to govern nocturnal sex drive in male flies (Fujii and Amrein, 2010). These recent studies have facilitated the understanding of an important subset of neurons of the circadian clock circuit.

Organization of the circadian clock circuit – dominance hierarchy versus distributed network

How is the clock circuit organized in *Drosophila*? Is it a hierarchical arrangement, where certain neurons are imperative in the governance of behaviour, with the other neurons performing subordinate roles? Or is it a network of neurons interacting with one another in order to bring about the most apt behaviour in the given environment? Several lines of evidence have been gathered in favour of each of these

two models. These are best exemplified by studies that explored the dual oscillator model as a potential mechanism to explain adaptation activity/rest behaviour to seasonal variations in the environment which is briefly outlined below.

The dual oscillator model, as its name suggests, posits that organisms possess two oscillators – a morning oscillator which follows dawn and an evening oscillator that follows dusk (Pittendrigh and Daan, 1976). This was proposed in order to explain the bimodality in the activity/rest patterns observed in animals, i.e. many of them empirically studied showed distinct morning and evening peaks of activity (Aschoff, 1966). The model suggests that these morning and evening peaks of activity are generated by morning and evening oscillators respectively. Thus, according to the dual oscillator model, as the timing of dawn and dusk changes such that their phase relationship changes, a corresponding alteration in the phase relationship of the dawn-tracking morning and the dusk-tracking evening oscillators leads to altered behavioural patterns across changing seasons (Pittendrigh and Daan, 1976). The tracking of the twilight zones of the day by these oscillators was not proposed to be passive, as according to the model, the two oscillators responded differently to changing light intensities – the morning oscillator sped up and evening oscillator slowed down with increasing light intensity (Pittendrigh and Daan, 1976).

Within the *Drosophila* clock circuit, many studies have localized the morning and evening oscillators to certain subsets of neurons. As mentioned earlier, the morning oscillator was shown to reside in the LN_v (Stoleru et al., 2004), especially the s-LN_v (Grima et al., 2004) by assaying for presence or absence of morning anticipatory activity in LD 12:12; evening oscillator was present in the LN_d, 5^{th} s-LN_v and/or the

DNs (Grima et al., 2004; Stoleru et al., 2004). These results were further accentuated when Stoleru et al. (2007) sped up clocks in different neurons and saw the effect on phasing of morning and evening peaks of activity in different day length conditions that mimicked the environment in varying seasons. They observed that, when the LN_v had a shorter period, the morning and evening peaks of activity were phase advanced in 'winter-like' conditions, while this manipulation had no effect in 'summer-like' conditions. On the other hand, when all clock neurons apart from the LN_v were sped up, both the morning and evening peaks were phase advanced in summer-like conditions, while remaining unaffected in winter-like conditions. This led the authors to propose a dominance-based model, according to which, dominance of morning and evening oscillators changed with changing seasons, such that each was able to dictate key pacemaker properties of the other in different seasons. Parallels were drawn from other studies which showed that s-LN_v were the pacemaking cells in DD (Renn et al., 1999; Lin et al., 2004) while DN1 were the pacemakers in LL (Murad et al., 2007). Importantly, another group that worked on the question of identifying the 'M' and 'E' cells concluded that the morning cells or the s-LN_v were the 'main' oscillator as they seemed to have a bearing on the evening peak as well (Rieger et al., 2006). Recent studies that have focused on characterizing the LN_d have identified GAL4 drivers that can target specific cells within the LN_d and have concluded that the 'E' cells consist of 3-4 LN_d and the 5^{th} s-LN_v (Johard et al., 2009; Hermann et al., 2012).

These results have also been interpreted in a different way to yield another mechanistic explanation with regards the circuit organization. Many researchers feel that rather than oscillators being restricted to specific subsets of clock neurons, the

whole network takes part in order to modulate behaviour (Dubruille and Emery, 2008; Nitabach and Taghert, 2008; Sheeba et al., 2008c). All the manipulations done so far on the clock circuit have yielded differently phased morning and evening peaks – never an absence of the peaks themselves – suggesting that the unaffected part of the clock circuit is capable of generating morning and evening peaks though with altered phases (Rieger et al., 2003; Stoleru et al., 2007; Yoshii et al., 2009; Rieger et al., 2012). The only experiment that has reported the absence of activity peaks is a recent study by Im and Taghert (2011), where a double mutant for *cry* and *pdfr* or *cry* and *pdf* do not show morning and evening peaks across different day lengths. Thus, while CRY and/or PDF-mediated signaling may be necessary to gauge light information and hence peak generation and phase maintenance, the underlying neuronal circuitry responsible for the same is unlikely to be restricted to certain subsets of the clock circuit. A case in point is, as mentioned before, when dysfunctional s-LN_v can no longer produce morning anticipatory activity in LD 12:12, other non-manipulated neurons of the clock circuit (possibly the l-LN_v) step up to produce the same (Sheeba et al., 2010). Additionally, under LD 12:12 in lower temperatures or at standard temperature but high light intensities, functional clocks in the $DN1_p$ group of neurons can rescue morning and/or evening anticipatory activities (Y. Zhang et al., 2010). This result is extremely important because the DNs were never implicated in the 'M' oscillator, yet after receiving different environment cues, they govern morning behaviour, something which was unanimously considered as the sole responsibility of the LN_v (Helfrich-Forster, 2009). Interestingly, in one of the earlier studies, indeed it was observed that certain 'E' cells were able to give rise to the morning anticipatory activity despite the absence of

the traditional 'M' oscillators (Stoleru et al., 2004). Taken together, these results imply that under changing environmental conditions, the circadian clock circuit is fixed as certain roles are assigned to some subsets of cells, but flexible enough to undergo a re-organization if need be, either due to environmental changes or genetic manipulations. These studies are only just the beginning of the understanding of what might be the existent circuit organization of a multiphasic, dynamic and heterogeneous network of circadian clock neurons. Many further cleverer neurogenetic analyses can yield interesting twists and turns to our understanding of the circadian clock circuit of *Drosophila*, which eventually can provide templates for similar experimental designs to answer mechanistic, circuitry-related questions even in the mammals. For instance, questions regarding the anatomical identity of morning and evening cells in the mammals can be addressed which at the moment is undecided (Helfrich-Forster, 2009).

D. The sleep-arousal circuit in *Drosophila*

Sleep is a very important behaviour displayed by a majority of organisms in the animal kingdom with a few exceptions (Lyamin and Chetyrbok, 1992; Lyamin et al., 2004; Allada and Siegel, 2008). While it is as yet unclear as to why organisms spend a considerable amount of time sleeping when they could well be doing some directly beneficial activities, how and when we sleep are relatively well-understood (McNamara et al., 2009). Electrophysiologically sleep is characteristically distinct from waking as seen by different kinds of oscillations recorded from a sleeping v/s waking brain (Kerkhof and Van Dongen, 2010). Behaviourally, sleep is defined as a period of rest or inactivity, when the senses are lowered, so to speak. Arousal threshold, or the intensity of stimulus required to elicit the same degree of response from a sleeping individual

compared to a waking individual is increased (Borbely and Achermann, 1999). Additionally, absence of a bout of sleep makes an individual sleep more the following day, sometimes even at times when the individual is expected to be awake. Apart from sleep rebound, individuals are particular about when and where they sleep, and most importantly about how much they sleep, thus rendering sleep a very complex behaviour governed by both the homeostatic limb and the circadian circuit of the brain (Pace-Schott and Hobson, 2002).

With *Drosophila* being such an important neurogenetic model organism, it indeed was a boon when it was discovered both by video recording and the DAM that resting or quiescent state of *Drosophila* has characteristics similar to mammalian sleep (Hendricks et al., 2000; Shaw et al., 2000). Flies are particular about their site of sleep, timing of sleep and most importantly, exhibit elevated arousal thresholds while sleeping and display sleep rebound upon sleep deprivation. An added bonus was achieved when even the local field potentials recorded from sleeping flies were different from the ones that were awake, thus rendering electrophysiological support to the claim that flies sleep (Nitz et al., 2002). For practical purposes, sleep in *Drosophila* is defined as a period of inactivity for at least five minutes (Cirelli and Bushey, 2008). This definition has facilitated unbiased, objective analysis of a complex behaviour thus unfolding an important chapter in the field of *Drosophila* neurogenetics.

Since the early studies that characterized *Drosophila* sleep, many studies have reported the conduction of forward genetic screens where they have isolated mutants that sleep either more or less than the wild type flies at least by two standard deviations (Cirelli, 2009). Most of these mutants isolated so far have turned out to be mutations in

ion channels, in fact, two of them are related to the Shaker K^+ channel (Cirelli et al., 2005; Bushey et al., 2007), whereas one other is an associated membrane-binding protein that influences the abundance of Shaker (Koh et al., 2008; M. N. Wu et al., 2010). Another novel mutation in a gene encoding an adaptor for Cullin-3 ubiquitin ligase complex, called insomniac has been identified through a forward genetic screen (Stavropoulos and Young, 2011). Other studies that have looked at certain mutants showing impaired sleep are those that have taken the reverse genetic approach. For example, most of the mutations in the players of the cAMP signaling pathway also show sleep deficits (Hendricks et al., 2001). However, the usefulness of *Drosophila* has once again been in the unraveling of the neuronal circuit underlying sleep and arousal, thus giving fresh insights and opportunities to address what functions sleep could serve (Crocker and Sehgal, 2010).

Circadian clock neurons

When *per* and *tim* loss-of-function mutants were assessed for their ability to show sleep rebound, it was seen that while per^{01} flies showed wild type-like rebound, tim^{01} flies failed to so, suggesting a role for *tim* in sleep homeostasis (Hendricks et al., 2000). Out of the core clock genes *per*, *tim*, *cyc* and *clk*, it was flies with mutation in *cyc* that showed the most severe defects in stress response to sleep deprivation. Significant number of cyc^{01} flies died during and after 12 hr of sleep deprivation; the amount of sleep rebound seen in live flies was significantly higher in cyc^{01} flies as compared to wild type flies or to the other clock gene mutants (Shaw et al., 2002). This was found to be due to reduced expression of stress-protective heat shock proteins in

cyc^{01} flies after sleep deprivation. Apart from *cyc* and *tim*, no strong role for any other canonical clock gene has been implied.

With sleep being a clock-controlled phenomenon, the involvement of one or more subsets of clock neurons in the sleep/arousal circuit was expected. Flies with mutations in *pdf* and *pdfr* also show sleep impairments (Chung et al., 2009) and the neurons modulating these effects are the l-LN$_v$. When the LN$_v$ are hyper-excited, the flies show abnormal levels of hyper-activity during the night (Sheeba et al., 2008a). Sleep was reduced to the same extent even after the hyper-excitation of only the l-LN$_v$ (Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008a), although it remained unaffected when only the s-LN$_v$ were hyper-excited (Shang et al., 2008). In fact, the amount of hyper-activity was found to be dependent on how many l-LN$_v$ were hyper-excited (Shang et al., 2008). It appears that the l-LN$_v$ required PDF to mediate this decreased sleep as the phenotype was lost in a *pdf*^{01} background, even though the PDF$^+$ neurons were hyper-excited (Sheeba et al., 2008a). Additionally, the effect of l-LN$_v$ on arousal was found to be light-dependent, with their ablation leading to increased sleep that was most evident in constant light conditions. Importantly, RDL, a GABA receptor that was implicated in sleep, when down-regulated in the PDF$^+$ neurons, led to decreased sleep, whereas, over-expression of the same led to an increase in sleep (Parisky et al., 2008). When antibody against RDL was used to stain adult brains, it was evident that RDL was localized near the optic lobes and accessory medulla, which are also sites where l-LN$_v$ send their projections (Parisky et al., 2008; Chung et al., 2009). While there is some inconsistency with respect to whether RDL is restricted to l-LN$_v$ or not, these studies collectively suggest that GABAergic neurons send inhibitory GABA

signals by which l-LN_v may modulate arousal. GABA is not the only neurotransmitter that is involved in signaling to l-LN_v; recently calcium imaging analysis has revealed that l-LN_v receive dopaminergic and octopaminergic inputs to modulate arousal, especially in the absence of light (Shang et al., 2011). Altogether, it is evident that l-LN_v have a critical role to play in the arousal circuit mostly via PDF, whereas no role has been implicated for the s-LN_v in the arousal circuit so far.

Mushroom body and other central complex regions

Drosophila mushroom bodies are located near the dorsal protocerebrum and are stalk-like structures that receive extensive projections from the underlying antennal lobes (Heisenberg, 2003). These are vital higher level brain regions that control many important behaviours like olfaction (Vosshall and Stocker, 2007), learning and memory (Pascual and Preat, 2001) and sleep (Sehgal and Mignot, 2011). The mushroom bodies consist of cells called Kenyon cells, and depending upon the type of Kenyon cells and when in developmental paradigm they form, the adult mushroom bodies are divided into three pairs of lobes – the α/β, α'/β' and the γ lobes (Heisenberg, 2003). Apart from structural diversity, these different parts of the mushroom bodies are also functionally distinct, including in sleep.

After the discovery that PKA, which is involved in cAMP signaling pathway, is inversely proportional to the amount of sleep (Hendricks et al., 2001), it was a question of where cAMP signaling acts in order to regulate sleep. Joiner and colleagues addressed this question by driving the expression of catalytic sub-unit of PKA in a wide variety of drivers and found that sleep levels were significantly affected in only two

drivers – *201Y GAL4* and *c309 GAL4*. While *201Y GAL4* which drives the expression in the core region of the α/β lobes and γ lobes increased sleep, *c309 GAL4* that has a similar expression pattern to *201Y GAL4* except that it did not drive in the α/β core region decreased sleep. Because the mushroom bodies were found to consist of both sleep-promoting and sleep-inhibiting sites, and the overall effect of mushroom body ablation was that of reduced sleep, the authors proposed a model which suggested that a downstream integrator receives information from both sleep-promoting and inhibiting sites of mushroom bodies; however the default state of this integrator is to promote arousal (Joiner et al., 2006). Another study that had set out to screen for sleep-inhibiting sites by expressing temperature sensitive dynamin protein that blocks synaptic transmission reached similar conclusions about mushroom body containing sleep-inhibiting sites in the α/β and γ lobes (Pitman et al., 2006). Mushroom bodies are also important in promoting sleep via serotonin, as expression of wild type serotonin receptor d5HT1A in sleep-inhibiting sites of mushroom bodies in a d5HT1A mutant background resulted in complete behavioural rescue of increased sleep levels upto wild type levels (Yuan et al., 2006). Another study showed that mushroom bodies are involved in mediating the effects of caffeine in bringing about arousal (Andretic et al., 2008). A recent study has shown that the mushroom bodies are important in the regulation of sleep homeostasis carried out by the Notch signaling pathway (Seugnet et al., 2011).

A recent study which looked to make flies sleep 'on demand' discovered that upon hyper-excitation or heat-activation of neurons expressed by three different GAL4 lines show increased sleep (Donlea et al., 2011). When these GAL4 lines were

examined, the only overlap of expression between these lines was in certain ExFl2 cells that project dorsally to the fan-shaped body of the central complex. While the authors were cautious not to exclude the fan-shaped body in sleep regulation, clearly their results implicate an important role for neurons that project to it in promoting sleep. It was also clear that the ventral fan-shaped body and the ellipsoid body are not involved in the sleep/arousal circuit, as hyper-exciting them did not change sleep levels. However, another study has implicated the role of ellipsoid body in the modulation of stress-mediated arousal via a dopamine receptor (Dop1R); the same receptor is involved in spontaneous nocturnal activity modulated by the l-LN_v (Lebestky et al., 2009). Thus it is clear from these studies that mushroom bodies and regions of the central complex are quite critical to the sleep/arousal circuit and control important components of sleep and arousal.

Pars Intercerebralis

Pars Intercerebralis (PI) is present in the dorsal-most region of the brain and it is an important neuroendocrine region that controls important functions such as growth (Rulifson et al., 2002), reproduction (Terhzaz et al., 2007) and sleep (Sehgal and Mignot, 2011). On the basis of their location, the PI neurons are grouped into three subsets – PI-1, PI-2 and PI-3, on the basis of their expression by certain enhancer trap lines as well as their projection patterns (Siegmund and Korge, 2001). The EGFR (epidermal growth factor receptor) pathway, which was also implicated in mammalian sleep (Kushikata et al., 1998), was also shown to promote sleep via a subset of PI neurons in *Drosophila* (Foltenyi et al., 2007). Another role for PI neurons in sleep is in modulating octopamine-mediated arousal via the OAMB receptor (Crocker et al., 2010).

Octopamine has a sleep-suppressing effect; in fact external administration and subsequent removal of octopamine makes flies exhibit sleep rebound (Crocker and Sehgal, 2008; Crocker et al., 2010). These effects of octopamine are mediated by the PI neurons via the K^+ slowpoke channel, thus stimulating them to fire (Crocker et al., 2010). Importantly, it appears that the effects of octopamine modulated by PI neurons is via cAMP signaling, as seen by the absence of externally delivered octopamine-induced arousal when cAMP signaling was blocked exclusively in the PI neurons. Thus, these results imply that the PI neurons form an important part of the sleep circuit, as it is the only brain region apart from the mushroom body to have been implicated in sleep homeostasis so far.

The importance of sleep

There are many theories as to why sleep is important, the most prominent among them being synaptic downscaling to retain important memories, meeting energy demands and restoration of cellular components (Mignot, 2008). Several lines of evidence have been obtained especially for the first two hypotheses using the fly model. Certain cAMP signaling molecules that play an important role in learning and memory also regulate levels of sleep (Hendricks et al., 2001); another layer of circumstantial evidence is that both occur in the mushroom bodies. Social experiences and learning paradigms are strengthened after a bout of sleep, whereas they become weak after sleep deprivation (Ganguly-Fitzgerald et al., 2006). Changes in synaptic bouton number and structure are associated with sleep – with the number decreasing with sleep and increasing with waking and sleep deprivation (Bushey et al., 2011). These results are accentuated by sleep deficits seen in a *Drosophila fragile X mental retardation*

(*dFMR1*) mutant, which also shows abnormal number of heavily branched synaptic boutons (Bushey et al., 2009). Levels of pre- and post-synaptic proteins were high after waking and low after sleep (Gilestro et al., 2009). Other studies have shown how when flies are starved, their sleep levels reduce (Keene et al., 2010); alternatively flies that are fed with yeast supplements, show sex-specific effects with the males sleeping less and females sleeping more in response (Catterson et al., 2010). Apart from a strong link between sleep and feeding, many metabolic genes are upregulated during sleep (Cirelli, 2005). Stress is also strongly connected with sleep, as seen by sleep deprivation studies on cyc^{01} flies and corresponding decline in their life span associated with reduced heat shock protein expression during sleep deprivation (Shaw et al., 2002). The mRNA levels of an indicator of ER stress BiP is also increased during sleep deprivation which decreases once sleep is recovered (Naidoo et al., 2007). Sleep homeostasis is also affected by a gene which is important in inflammatory responses (Williams et al., 2007). Recently, an important role for neuron-glia interactions mediated by Notch-Delta pathway was implied when it was shown that *bunched*, a negative regulator of *Notch* is involved in sleep homeostasis (Seugnet et al., 2011). In fact, over expression of a dominant form of *notch* in the glia was enough to rescue learning impairments caused due to sleep deficits. Thus, taken together, all these results suggest that *Drosophila* has been helpful in the exploration of possibilities of the importance of sleep apart from making important breakthroughs in organization of the underlying neuronal circuit.

E. The large and small of it – the intersection of clock and arousal circuits at the LN_v

From sections C and D, it is clear that the s-LN_v and l-LN_v have clear, distinct roles in the clock and arousal circuits respectively. Both these neuronal subsets require PDF

to carry about their respective functions, and it appears that the two LN_v groups do not interfere with one another in their functioning capabilities within their dedicated circuits. However, there are many studies that suggest that perhaps the demarcation between the two LN_v subsets is perhaps not that rigid. Thus, in order to make a clear functional distinction between the s- and l-LN_v, a closer examination of their roles in their respective as well as opposite circuits need to be re-evaluated.

The l-LN_v are sidelined in the clock circuit due to their inability to maintain molecular oscillations under constant conditions (Park et al., 2000; Yang and Sehgal, 2001; Shafer et al., 2002; Lin et al., 2004). However, one study has reported that *tim* mRNA oscillations are present in constant darkness for all clock neuronal subsets, including the l-LN_v for up to 8 days (Peng et al., 2003). Importantly, the membrane properties of the l-LN_v (membrane potential, firing frequency) were rhythmic in DD even after 15 days, and even after expression of bacterial sodium channels that constitutively hyper-excited them (Sheeba et al., 2008d). These results point toward an important role for l-LN_v in circuit-wide communication. Another study has indicated a strong role for l-LN_v in bringing about morning anticipatory activity in the absence of s-LN_v (Sheeba, 2008). These results are further supported by a role for l-LN_v in communicating with the 'evening' neurons via PDF especially in the absence of CRY which brings about evening anticipatory activity (Cusumano et al., 2009). Altogether, l-LN_v seem to have an important role to play in the clock circuit that needs further examination. We have sought to do the same by affecting the ability of the l-LN_v to communicate with downstream neurons and examine its effects on activity/rest

behaviour across different photoperiods imitating varying seasons. These results are elaborated in the following chapter.

As far as the sleep/arousal circuit is concerned, while a lot is known about which are the sleep and arousal controlling sites, information regarding their organization is lacking. For instance, how do the arousal controlling l-LN_v, PI neurons and sleep-inhibiting sites of mushroom bodies communicate with one another to bring about arousal? *In situ* hybridization has revealed that both PI and mushroom bodies express PDF receptor (Lear et al., 2005). However, these results were not replicated with antibody staining against PDFR (Hyun et al., 2005; Mertens et al., 2005). Moreover, neuroanatomical studies have revealed that l-LN_v do not project towards the dorsal protocerebrum where these two sites are located; in fact the l-LN_v arborize towards the accessory medulla and optic lobes (Kaneko and Hall, 2000; Sheeba, 2008) . Intriguingly, the s-LN_v apart from sending projections towards the accessory medulla, send one projection towards the dorsal protocerebrum (Kaneko and Hall, 2000; Sheeba, 2008). This opens up the possibility of s-LN_v playing the role of intermediate communicating neurons that connect l-LN_v and rest of the arousal circuit. By manipulating the l-LN_v in the presence of misfiring s-LN_v or dysfunctional s-LN_v, I found that in drastic day lengths, s-LN_v are required for modulating l-LN_v-mediated arousal. These results that point toward a mediator role for s-LN_v are discussed in chapter 3.

Finally, in which region of the brain does the neurotransmitter PDF have the maximum effect? I have examined the neuroendocrine Pars Intercerebralis region as a potential candidate downstream target of PDF+ neurons in bringing about arousal. In

the process, I have identified a subset of PI neurons that could function as an integrating centre by receiving and responding to a myriad of signals from different upstream arousal and sleep promoting neurons. These results are highlighted in chapter 3.

Thus, in totality, I have examined the effective roles of the two LN_v subsets in bringing about activity/rest behaviour by manipulating different limbs of the clock and arousal circuits. I have obtained results that are described in the coming two chapters in detail. Briefly, my studies have revealed an important role for l-LN_v in the clock circuit in bringing about the evening peak at the correct phase depending upon the environment the flies are in. Additionally, I have discovered a secondary, albeit important role for s-LN_v in the arousal circuit. Furthermore, a novel role for PDF in promoting sleep has been uncovered. Finally, I have explored the putative sites that may communicate with the s-LN_v and l-LN_v in order to bring about the final activity/rest behaviour in consultation with both the clock and arousal circuits and have identified a subset of PI neurons that may do the same. Thus, the re-evaluation of the clock and arousal circuits has yielded vital, subsidiary and new roles for the LN_v neurons, and as is the wont of science, more questions than answers.

Chapter 2

Large ventral lateral neurons influence the phase of evening activity peaks across photoperiods in *Drosophila melanogaster*

A. Introduction

The daily cycling of environmental factors such as light and temperature creates a periodic environment for organisms, which synchronizes their metabolic, physiological and behavioural processes to environmental cycles with the aid of endogenous circadian clocks (Allada and Chung, 2010). The cues that provide time information to circadian clocks are numerous, with light being predominant (Dunlap et al., 2004; Sharma and Chandrashekaran, 2005). While in tropical regions, day length or photoperiod remains roughly constant throughout the year, it varies quite dramatically in temperate regions and the poles. Although number of other factors such as temperature and food availability also changes with changing seasons, day length changes are more consistent and robust and can range from approximately 20 hr during summers to about 4 hr during winters. Such variations in the environment can be deleterious if organisms are unable to appropriately anticipate them behaviourally and physiologically.

The dual oscillator model proposed in 1976 offered a probable mechanism by which an animal might cope with such dramatic environmental changes. According to this model, animals are equipped with two mutually interacting oscillators, a morning (M) oscillator that tracks dawn, and an evening (E) oscillator that tracks dusk (Pittendrigh and Daan, 1976). With changing photoperiods, the timing of dawn and dusk varies, resulting in a corresponding change in the phase-relationship between the two oscillators, thus enabling organisms to adjust to varying seasons. The model attempted to provide an explanation for bimodality in activity/rest profiles of many animals (Aschoff, 1966) by positing that the morning and evening oscillators give rise

to the morning and evening peaks respectively, thus rendering the twilight transition periods very crucial (Pittendrigh and Daan, 1976).

The model itself did not propose the existence of structural or anatomical entities in the mammalian hypothalamus to function as 'M' and 'E' oscillators. The authors merely suggested that each of these oscillators could be 'a population of tightly coupled cells', but the question of the exact anatomical identity of these oscillators has been of interest for circadian researchers and remains to be satisfactorily answered in mammals (Helfrich-Forster, 2009). In *Drosophila* seven subsets of circadian clock neurons - the three dorsal neuron subsets (DN1, DN2, DN3) and four lateral subsets - lateral posterior (LPN), large and small ventral lateral neurons (l-LN_v and s-LN_v) and lateral dorsal neurons (LN_d) have been characterized as the neuronal substrates. All the LN_v except 5^{th} s-LN_v secrete neuropeptide Pigment Dispersing Factor (PDF) (Shafer et al., 2006). One of the earliest studies, which designated neuronal correlates to the oscillators, concluded that PDF^+ LN_v and PDF^- CRY^+ (CRYPTOCHROME) LN_d were the morning and evening oscillators respectively (Stoleru et al., 2004). In parallel, a separate group narrowed the morning oscillator location to s-LN_v (Grima et al., 2004). More recently the evening oscillator has been narrowed down to 3-4 NPF^+ LN_d and PDF^- 5^{th} s-LN_v (Hermann et al., 2012). Notably, adaptation to seasonal variation was proposed to be a result of a seesaw in the dominance hierarchy between the M and E cells with each setting the phase of the other in winter and summer conditions respectively (Stoleru et al., 2007).

Other studies have hypothesized that the M oscillator (consisting of the PDF^+ s-LN_v) is the 'main' oscillator, as it also exerts its influence on the evening peak, while

the PDF$^-$ 5th s-LN$_v$ along with CRY$^+$ LN$_d$ form the evening oscillator (Rieger et al., 2006). Indeed, in flies with ablated or electrically silenced LN$_v$ and in *pdf*^{01} mutants, the evening peak is phase advanced compared to controls (Renn et al., 1999; Nitabach et al., 2002). Under DD, oscillation in PER levels (an indicator of a ticking circadian clock) dampens quickly in the l-LN$_v$ (Shafer et al., 2002) suggesting that it is the s-LN$_v$ that regulate PDF signaling to other components of the circadian circuit. Yet another approach showed that speeding up the circadian oscillations in the s-LN$_v$ sped up clocks in LN$_d$, DN1 and DN3, while speeding up the DN2 clocks significantly increased the speed of oscillations in l-LN$_v$ alone suggesting a hierarchical clock circuit in which the l-LN$_v$ occupied a subordinate role (Stoleru et al., 2005). These results have mostly disregarded the role of l-LN$_v$ in the circadian clock in this scheme of a hierarchical clock circuit.

While a hierarchical clock circuit explains some phenomena, a more distributed and plastic clock network has been called for by taking into account mammalian studies (Dubruille and Emery, 2008; Nitabach and Taghert, 2008; Sheeba et al., 2008c). The behaviour of anticipating lights-ON, considered as a hallmark of the M oscillator was shown to persist even in absence of PDF in the cell bodies of s-LN$_v$; the l-LN$_v$ along with other clock neurons were proposed to govern this behaviour (Sheeba et al., 2010). Another study indicated that PDF signaling from the l-LN$_v$ which is independent of the classical circadian clock is essential for E cells to anticipate lights-OFF, especially in the absence of light-sensitive CRY (Cusumano et al., 2009). These results point toward a role for the l-LN$_v$ in the circadian clock network.

In the current study, we have further explored the role of the l-LN_v in the distributed clock network in a variety of environments that approximate varying seasons. We examined the role of l-LN_v in the neuronal circuit by manipulating the membrane properties of these neurons in the absence of s-LN_v. Here, we have probed the role of communication between neurons by manipulating their firing properties or making them dysfunctional, such that the results of potential miscommunication between neurons of the clock circuit are observed as altered activity/rest patterns across varying seasons. This kind of a circuit-breaking approach is more subtle and effective in answering questions regarding functioning of a network of neurons in co-ordination with one another (Holmes et al., 2007) as opposed to the use of whole-body mutations or by eliminating components of the circuit using more harsh methods. Apart from the short and the long day conditions (LD 8:16 and LD 16:8 respectively), we exposed flies to extreme photoperiod conditions (LD 4:20 and LD 20:4) to examine the full extent of seasonality on circadian clock circuit. We were able to assign specific roles to the s-LN_v and l-LN_v in the control of definite components of evening peak in different photoperiod conditions. We observed that the relative importance of the two subgroups of LN_v in phasing the evening peak changes with changing day lengths. Further, we show that the l-LN_v whose role in the circadian circuit was contentious, can influence phasing of the evening activity peak.

B. Materials and methods

Fly strains. Flies were reared on standard corn medium under light/dark cycles with 12 hr light and 12 hr darkness (henceforth, LD 12:12) at 25 ºC and constant humidity (~ 70 ± 15%). To specifically render the s-LN_v dysfunctional, we used the *pdf GAL4* driver -

yw; pdf GAL4 (Renn et al., 1999) to express a toxic form of HTT protein (*w; UAS-HTT-Q128*) consisting of 128 glutamine (Q) repeats (Lee et al., 2004) thus giving the genotype - *yw; pdf GAL4 / UAS-HTT-Q128* and designated as ($s^- L^+$). While *pdf GAL4* can drive the expression of the toxic neurodegenerative protein in the s-LN_v and the l-LN_v, the s-LN_v appear to be specifically targeted as demonstrated by their lack of PDF and PERIOD expression and breakdown of behavioural rhythms in DD (Sheeba et al., 2008a). The reason for this selective degeneration is yet unknown; however it allows us the opportunity to functionally tease apart the two LN_v subsets. The controls for the *pdf Q128* genotype were - *yw; pdf GAL4 / UAS-HTT-Q0;* designated as $s^+ L^+$ with the Q0 construct being a non-toxic form of the polyQ Huntingtin protein (HTT) with no polyQ repeats. Driver control flies *yw; pdf GAL4 / +*, ($s^+ L^+$). With the goal of checking if the number – and not the type of LN_v are important for activity/rest behaviour, we studied *yw; pdf GAL4 / UAS rpr-C14* ($s^{\pm} L^{\pm}$) flies which showed incomplete ablation of the LN_v. To assess the role of l-LN_v, we used - *yw; pdf GAL4 / UAS-HTT-Q128; UAS NaChBac1/ +* ($s^- L^H$) which rendered the l-LN_v alone hyper-excited, as the s-LN_v were made dysfunctional by HTT. Genotype of flies with both hyper-excited s-LN_v and l-LN_v ($s^H L^H$) were - *yw; pdf GAL4 / UAS-HTT-Q0; UAS NaChBac1/ +*). Additional controls *were- yw; pdf GAL4 / UAS-HTT-Q0; UAS dORK-NC1/ +* ($s^+ L^+$) and *yw; pdf GAL4 / UAS-HTT-Q128; UAS dORK-NC1/ +* respectively ($s^- L^+$).

Activity recording. All flies were reared under LD 12:12 at 25 ˚C until they were loaded into locomotor activity tubes to minimize confounding effects of previous entrainment. Locomotor activity/rest profiles of virgin male flies (3-4 day old) of each genotype were recorded separately in *Drosophila* activity monitors (DAM, TriKinetics,

Waltham, MA, USA) under different photoperiods and constant 25 ºC to avoid confounding effects of age. The different photoperiod conditions - standard LD 12:12, short day LD 8:16, very short day LD 4:20, long day LD 16:8 and very long day LD 20:4 were maintained in an incubator (Percival Scientific Inc., Perry, IA, USA) with low light intensity ~ 0.15 W/m^2 (~100 lux) to reduce the typical masking effects seen following lights-ON and -OFF while allowing effective entrainment. Photoperiods were created by symmetrically shrinking or expanding day length from both ends of the day

Analysis of behavioural data. Activity counts collected at 5 min intervals were binned into 15 min to obtain time series of activity data for individual flies of all genotypes. From the 9-day long time series, the first two cycles were excluded for further analysis to eliminate transients. Data for the next seven days were normalized to total activity during each day, which were then averaged across 7 days to obtain average activity profile of each individual fly. Such profiles were then averaged across individual flies of each genotype to obtain the average activity/rest profile of that genotype, which were then smoothed by a moving average of three successive time points (smoothing across 45 min). No other filters or smoothing was done to the data compared to several previous studies that have used non-recursive digital low-pass or Butterworth filters followed by extensive smoothing (Rieger et al., 2003; Rieger et al., 2012), as we felt that such methods tend to obscure some otherwise interesting features of the activity profiles. Normalized actograms of individual flies were batch analyzed to obtain average actograms for each genotype using ClockLab software (Actimetrics ™, Wilmette, IL USA). To compare activity levels across photoperiods, we divided the 24

hr day into 4 hr intervals, and considered lights-ON as ZT 0 in all photoperiods. The total activity in each bin was normalized to total activity during the 24 hr cycle to obtain percent activity in that bin. In the long and short photoperiods, we often found peaks that were mere responses to the light-to-dark or dark-to-light transitions and distinct peaks that were likely to reflect the 'true' morning and evening peaks. These two types of peaks were distinguished based on whether it occurred within 1 hr window following lights-ON or OFF, in which case they were designated as startle peaks. The 'true' peaks in all non-standard photoperiods were assigned based on proximity to lights-ON or OFF transition. In order to objectively determine whether such peaks were present, the average activity counts of flies in each photoperiod were binned into 1 hr intervals. Two-way ANOVA was used to check if any 1 hr bin within an interval of 4-6 hr before or after lights-ON or -OFF transitions was significantly different from its immediate neighbours. When these peaks were broad but showed a characteristic rise and fall in activity, we checked for three successive data points, the extremes of which were significantly different from their neighbours, such that the peak now lay at a time between these three high-value points. Genotypes that met either of these two criteria were considered as displaying non-startle true morning and/or evening peaks. To determine the phase of these peaks, we referred to the 15 min average activity profiles of individual flies and manually scanned within that interval determined by the 1 hr profiles as containing the peak and identified that 15 min bin at which maximum activity count was recorded. The phase values thus obtained for individuals were averaged across flies to obtain phases of the true peaks for each genotype in each photoperiod. We calculated the morning and evening anticipation index in LD12:12

based on Harrisingh *et al.,* (2007). Anticipation indices in photoperiods other than LD 12:12 were not calculated as the activity peaks did not coincide with the transition periods.

Statistical analysis. All statistical analyses were done using STATISTICA ver. 7.0 (StatSoft Inc., Tulsa, OK, USA). Percentage activity data were transformed by taking square root of the arcsine of fraction of activity at each time point before a two-way ANOVA was carried out for 4 hr interval data of all photoperiods with the two factors being genotype and time interval. Tukey's Honest Significant Difference (HSD) test was done for post-hoc analysis. Similarly, for 1 hr activity/rest profiles, data was analysed by a two-way ANOVA with genotype and time interval as fixed factors. 95% confidence intervals for these values were calculated and plotted on the activity/rest graphs to allow for visual hypothesis testing. Morning and evening peak anticipation indices (Harrisingh et al., 2007) of LD 12:12 across genotypes were compared using one-way ANOVA followed by post-hoc Tukey's HSD test. Level of significance was set to $p < 0.05$.

C. Results

Normally firing l-LN$_v$ are necessary for anticipation to lights-OFF under LD 12:12. All control flies with normal s-LN$_v$ and l-LN$_v$ (s^+ L^+ flies) showed bimodal activity under LD 12:12 (Fig 1A-B) with prominent morning and evening peaks with both anticipation and startle components (*GAL4* – black (Fig 1B, top panel), *Q0* – grey (1B, top panel), *NCQ0* – black (1B, lower panel). Anticipation of lights-ON or OFF is an indicator of the presence of a functional circadian clock and is distinct from the 'startle'

response (an abrupt increase in activity counts immediately after lights-ON or lights-OFF) which is independent of known circadian clocks and shown even by arrhythmic circadian-gene null mutants (Wheeler et al., 1993). When both LN_v are partially ablated ($s^{\pm}$ $L^{\pm}$ flies; Fig 8), or only s-LN_v are dysfunctional (s^- L^+) the evening peak was similar to that of s^+ L^+ controls both in terms of anticipation of and startle-due-to lights-OFF (Fig 1A –*rpr* and *Q128* –Fig 1B, top panel green and indigo curves compared to black and grey; evening anticipation -Fig 1D bottom panel). In contrast, the morning anticipation of the $s^{\pm}$ $L^{\pm}$ (*rpr* - green) and s^- L^+ flies *(Q128* – indigo) was significantly lowered compared to their s^+ L^+ control flies *(Q0* – grey) (Fig 1D, upper panel). When both the LN_v groups were hyper-excited (s^H L^H) or only the l-LN_v were hyper-excited (s^- L^H), activity profiles of flies differed from s^+ L^+ controls and they failed to anticipate lights-OFF (Fig 1B lower panel, compare purple and red curves with black curve), although the startle response was similar (Fig 1D lower panel). Consequently, their activity 4 h prior to lights-OFF (ZT 08-12, Fig 1C) and their evening anticipation indices were significantly lower than that of s^+ L^+ control flies and also lower than s^- L^+ and $s^{\pm}$ $L^{\pm}$ flies (Fig 1C and 1D). The s^H L^H and s^- L^H flies also poorly anticipated lights-ON transition, their morning anticipation indices being significantly lower than s^+ L^+ controls (*NCQ0*, Fig 1D, top). In fact, s^H L^H flies were the most severely affected as both morning and evening anticipation indices were significantly lower than both $s^{\pm}$ $L^{\pm}$ and s^- L^+ flies. Expectedly, the overall nighttime activity of s^H L^H and s^- L^H flies was higher than s^+ L^+ controls (*NCQ0* Fig. 1A-C, (Sheeba et al., 2008a), this difference being statistically significant during ZT 16-20 (Fig 1C). Overall, nighttime activity levels of s^- L^H flies appeared to be consistently lower than s^H L^H flies, although this

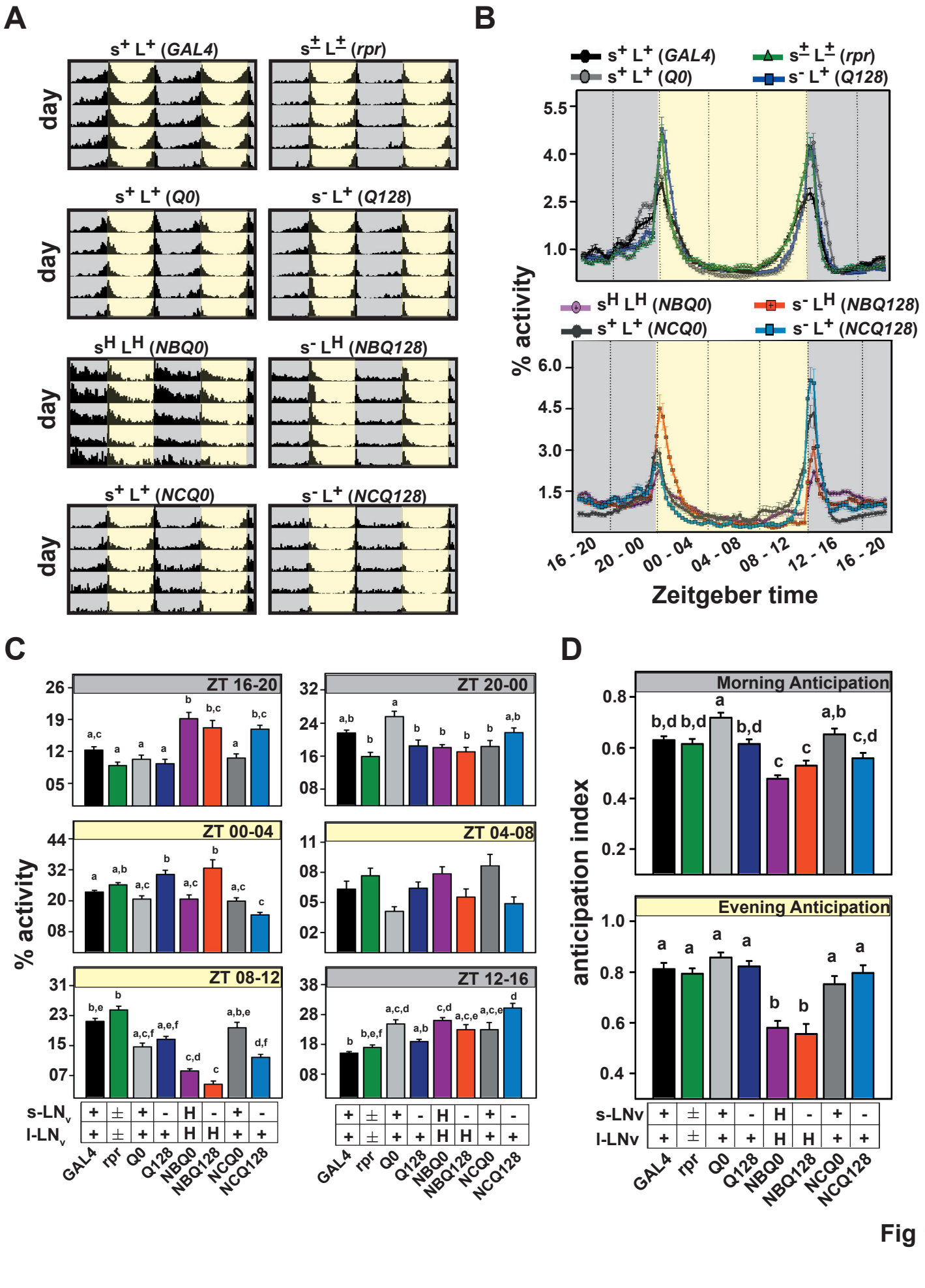
A
s+ L+ (GAL4)
s± L± (rpr)
s+ L+ (Q0)
s- L+ (Q128)
sH LH (NBQ0)
s- LH (NBQ128)
s+ L+ (NCQ0)
s- L+ (NCQ128)
day
B
s+ L+ (GAL4)
s± L± (rpr)
s+ L+ (Q0)
s- L+ (Q128)
sH LH (NBQ0)
s- LH (NBQ128)
s+ L+ (NCQ0)
s- L+ (NCQ128)
% activity
16 - 20
20 - 00
00 - 04
04 - 08
08 - 12
12 - 16
16 - 20
Zeitgeber time
C
ZT 16-20
ZT 20-00
ZT 00-04
ZT 04-08
ZT 08-12
ZT 12-16
% activity
s-LNv
l-LNv
GAL4
rpr
Q0
Q128
NBQ0
NBQ128
NCQ0
NCQ128
D
Morning Anticipation
Evening Anticipation
anticipation index
s-LNv
l-LNv
GAL4
rpr
Q0
Q128
NBQ0
NBQ128
NCQ0
NCQ128

Fig 1

difference was statistically not significant at any time point (Fig 1C). However, the nighttime activity levels of $s^- L^+$ flies of *NCQ128* genotype (Fig 1C, blue) was as high as $s^H L^H$ and $s^- L^H$ flies (Fig 1C, purple and red), probably due to unrelated genetic background effect of the genotype, since *Q128* flies did not show such behaviour (Fig 1C indigo). Thus, these results suggest that absence of s-LN_v function specifically reduces the ability of flies to anticipate lights-ON without particularly affecting the anticipation of lights-OFF. However, hyper-exciting either both the LN_v subsets or only the l-LN_v, reduces anticipation to lights-OFF (Fig 1D lower). While hyper-exciting both the LN_v subsets abolishes anticipation to lights-ON completely, hyper-exciting only the l-LN_v renders the morning anticipation index comparable to flies whose s-LN_v are specifically made dysfunctional leaving normal l-LN_v (compare red and blue bars, Fig. 1D, top panel). Thus, these results indicate that under LD 12:12 cycles, the firing properties of s-LN_v and l-LN_v modulate both the anticipation of - and immediate response to- both lights-ON and -OFF.

Electrical activity of l-LN_v determines phase while that of s-LN_v regulates amplitude of evening peak under short day environments. Having examined the role of the LN_v

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Figure 1. Flies with hyper-excited LN_v show reduced anticipation to L/D transition. (A) Average actograms of LN_v-modified flies are shown along with their respective controls in LD 12:12. The $s^- L^H$ flies (*NBQ128*), show reduced nocturnal activity compared to the $s^H L^H$ flies (*NBQ0*). Actograms of all other genotypes, including the $s^- L^+$ (*Q128*) and $s^{\pm} L^{\pm}$ (*rpr-C14*) resemble that of the $s^+ L^+$ controls (*GAL4*). (B) Normalized activity profiles of four genotypes each averaged across seven days are plotted in top and bottom panels. The vertical dotted lines represent the 4 hr intervals that were considered for quantitative analysis. ZT 0 is considered as lights-ON. The yellow and grey shaded areas in both (A) and (B) denote day and night respectively. (C) Comparison of mean percentage activity in 4 hr time durations across the length of day and night as demarcated by dotted lines in B. Different letters above the bars indicate that the values are significantly different from each other (SEM error). Activity levels of $s^H L^H$ and $s^- L^H$ flies during ZT 8-12 and ZT 16-20 are significantly different from the $s^+ L^+$ (*GAL4*) flies. (D) Anticipation to D/L and L/D transitions as quantified by the anticipation index given in materials and methods. Mean anticipation and SEM are plotted.

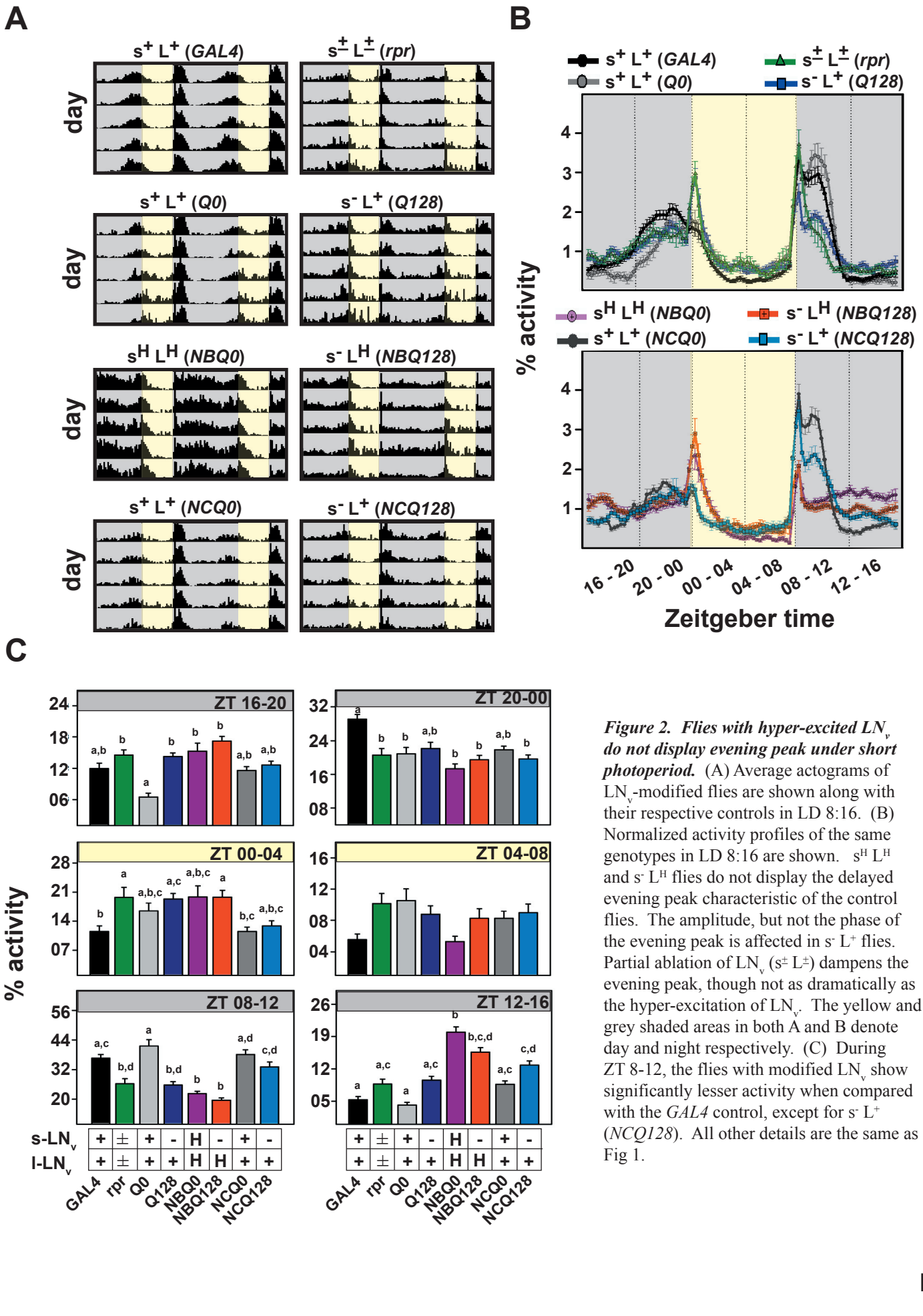


Figure 2. Flies with hyper-excited LN_v do not display evening peak under short photoperiod. (A) Average actograms of LN_v-modified flies are shown along with their respective controls in LD 8:16. (B) Normalized activity profiles of the same genotypes in LD 8:16 are shown. $s^H L^H$ and $s^- L^H$ flies do not display the delayed evening peak characteristic of the control flies. The amplitude, but not the phase of the evening peak is affected in $s^- L^+$ flies. Partial ablation of LN_v ($s^± L^±$) dampens the evening peak, though not as dramatically as the hyper-excitation of LN_v. The yellow and grey shaded areas in both A and B denote day and night respectively. (C) During ZT 8-12, the flies with modified LN_v show significantly lesser activity when compared with the *GAL4* control, except for $s^- L^+$ (*NCQ128*). All other details are the same as Fig 1.

Fig 2

under standard LD 12:12 conditions, we extended our studies to shorter photoperiods where previous studies have implicated a dominant role for s-LN_v in being able to influence the circadian clock of other neurons of the circadian circuit (Stoleru et al., 2007). Under short day LD 8:16, both morning and evening peaks of controls occurred during the dark phase (Fig 2A, 2B - black, grey curves, top panel, Fig 6) with no startle response to lights-ON, while startle to lights –OFF persisted (Fig 2A-B; Fig 2C ZT0-4 and ZT8-12- black and grey bars). In contrast, flies with compromised LN_v such as $s^- L^+$ and $s^{\pm} L^{\pm}$ (Fig 2B, indigo, green curves, top panel), or those with abnormally firing LN_v such as $s^H L^H$ and $s^- L^H$ (Fig 2B, purple and red curves, lower panel) did not show morning peak, although lights-ON startle occurred (Fig 2B). These differences between controls and LN_v-affected flies persisted even in extreme short day LD 4:20 (Fig 3A, 3B, compare black and grey curves with green and indigo in top panel and with purple and red in bottom panel). The $s^- L^+$ and $s^{\pm} L^{\pm}$ flies also showed startle response to lights-OFF, followed by a low-amplitude evening peak about 2 hr after lights-OFF in LD 8:16. However, $s^H L^H$ and $s^- L^H$ flies did not exhibit any evening peak at all (Fig 2B, purple and red curves, Fig 6) as quantified by significantly lowered activity in the 4 hr time interval after lights-OFF (ZT 8-12, Fig 2C purple and red bars); although a small startle response was seen. These results are accentuated under LD 4:20 where $s^- L^+$ flies (*Q128*, indigo curve, Fig 3B top panel and *NCQ128*, blue curve, Fig 3B lower panel) showed a relatively low-amplitude 'true' evening peak, around the same interval (ZT 8-12, Fig 6) as the higher-amplitude evening peaks of the control flies (Fig 3B, black and grey curves, top panel; black curve, lower panel). $s^H L^H$ and $s^- L^H$ flies did not show evening peak of activity (Fig 3B, purple and red curves, lower panel) with activity

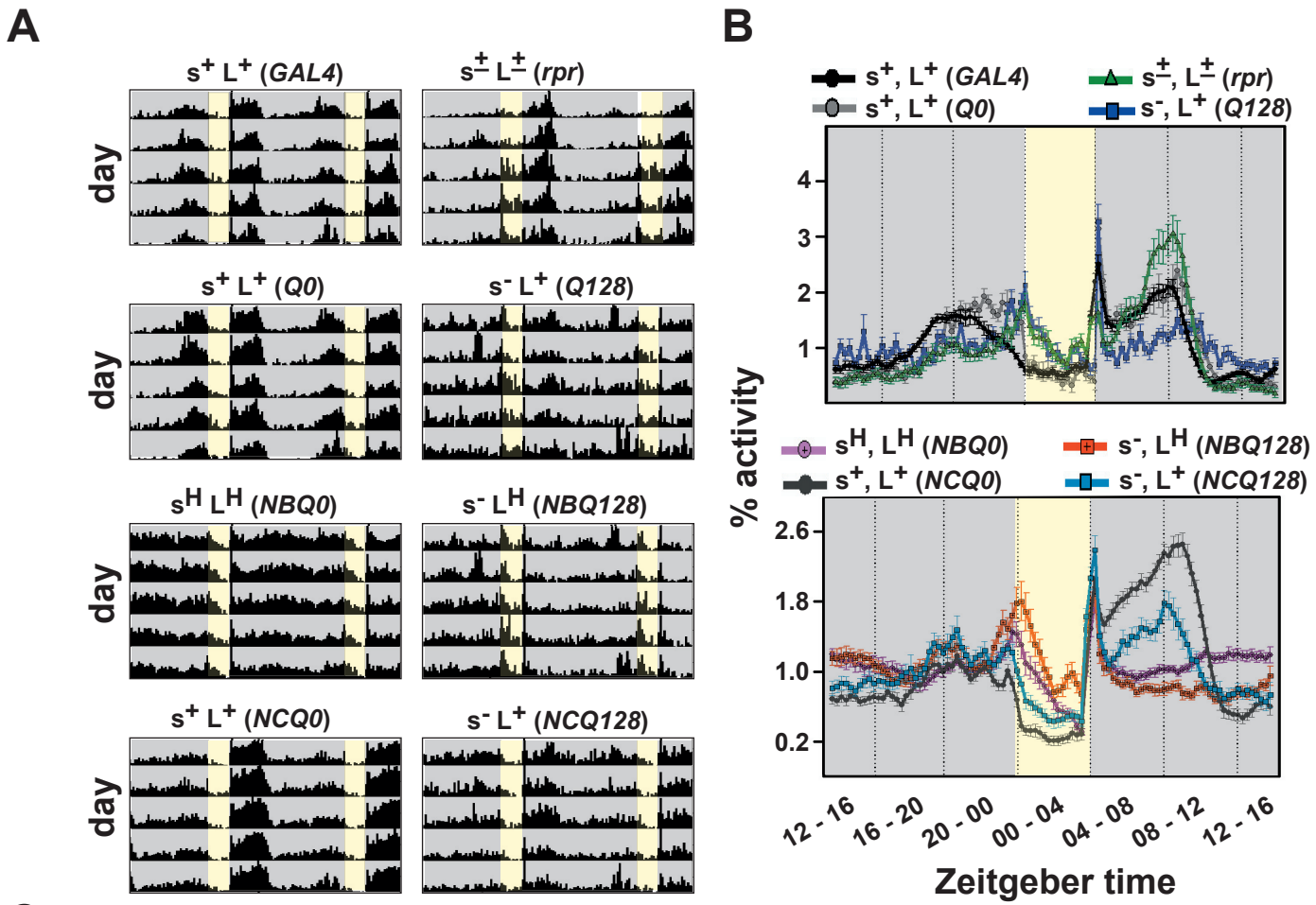


Figure 3. Effects of hyper-excitation of LN_v on activity/rest profile are increased under very short photoperiod. (A) Average actograms of different genotypes showing their activity/rest pattern in LD 4:20. (B) Normalized activity profiles in LD 4:20 show that $s^H L^H$ and $s^- L^H$ flies do not show the delayed evening peak that is seen in the control flies (bottom panel). Flies with partially ablated LN_v ($s^+ L^+$) show only the delayed evening peak, but do not show a morning peak (top panel). As in LD 8:16, flies with specific inhibition of s-LN_v ($s^- L^+$) show decreased amplitude of evening peak, though the phase is unaffected. (C) Activity levels during the ZT 4-8 phase is significantly reduced from their respective controls in all LN_v-manipulated flies, except for *rpr*. All other details are the same as in Fig 1.

remaining similar to that after lights-OFF startle had subsided. Thus $s^H L^H$ and $s^- L^H$ flies showed significant reduction in activity during the 8 hr interval when control flies showed evening peak (ZT 4-8 and 8-12, Fig 3C, compare purple with grey and red with blue bars respectively). However, activity of $s^H L^H$ flies during ZT 8-12 was not significantly different from the *GAL4* and *Q0* control flies (Fig 3C, compare purple with black and green bars) as controls underwent dramatic changes in activity levels with activity peaking around ZT 8 and then dropping steeply (Fig 3B, top panel black and green curves), whereas, activity of $s^H L^H$ flies remained at a constant high level (Fig 3B, bottom panel purple curve). Interestingly, in both these short photoperiods, the differences in the nighttime activity levels between $s^H L^H$ and $s^- L^H$ flies became stark. Visual inspection of actograms indicates that the $s^- L^H$ flies showed less of the abnormal nighttime hyper-activity when compared with $s^H L^H$ flies. A comparative analysis of mean activity levels in 4 hr bins showed that in LD 8:16, the two late-night intervals (ZT 16-20 and 20-0) showed a trend with $s^- L^H$ flies exhibiting greater activity than $s^H L^H$ flies (Fig 2C). This trend was reversed in the early night intervals (ZT 8-12 and 12-16). However, in no interval did the differences between the two genotypes reach statistically significant levels (Fig 2C). These trends were also seen in the five night intervals of LD 4:20, with the differences becoming statistically significant in one interval – in ZT 8-12, where $s^- L^H$ flies had significantly lower activity than $s^H L^H$ flies (Fig 3C). Thus, during short-days, l-LN_v play an important role in enabling the evening peak to occur and to phase it appropriately while s-LN_v determine the amplitude of the evening peak. While the startle to lights-ON can be altered by the membrane properties

of the LN_v, the evening startle is largely unaffected by it, suggesting that this positive masking phenomenon is not modulated by either LN_v.

Enhanced electrical activity of s-LN$_v$ and l-LN$_v$ modulates phasing of evening peak under long day environment. We next tested flies under long days beginning with photophase of 16 hr. Here morning and evening activity peaks were almost completely restricted to the light phase (Fig 4A-B black and grey curves). Furthermore, all flies – including controls – showed a clear morning peak coinciding with light-ON and also startled in response to lights-OFF (Fig 4B, Fig 6). The evening peak was phase advanced with respect to lights-OFF in the controls and in flies where s-LN_v were compromised such as $s^- L^+$, $s^{\pm} L^{\pm}$ and $s^- L^H$ (Fig 4A and 4B, indigo and green curves, top panel, red curve, lower panel). Therefore, s-LN_v are likely not critical for maintenance of the evening peak under long photoperiods. In contrast, $s^H L^H$ flies showed a markedly different activity/rest profile compared to all other genotypes with the evening peak being delayed and occurring in conjunction with lights-OFF with almost symmetric distribution of activity across the dark/light transition (Fig 4A, purple curve, Fig 6). Significantly lower activity is exhibited by $s^H L^H$ flies during ZT 12-16 compared with all other genotypes (including $s^- L^H$) only just beginning to build-up activity, while other flies are at the peak of their activity (Fig 4C- ZT 12-16). Under such long day conditions $s^- L^H$ were able to maintain a phase relationship with lights-OFF similar to control flies. This suggests that under long days, in the absence of s-LN_v, the hyperexcited state of l-LN_v enables the phase of the evening peak to align similar to controls. On the other hand, when both LN_v are hyperexcited their combined action causes the evening peak to be phase-delayed and aligned with the lights-OFF

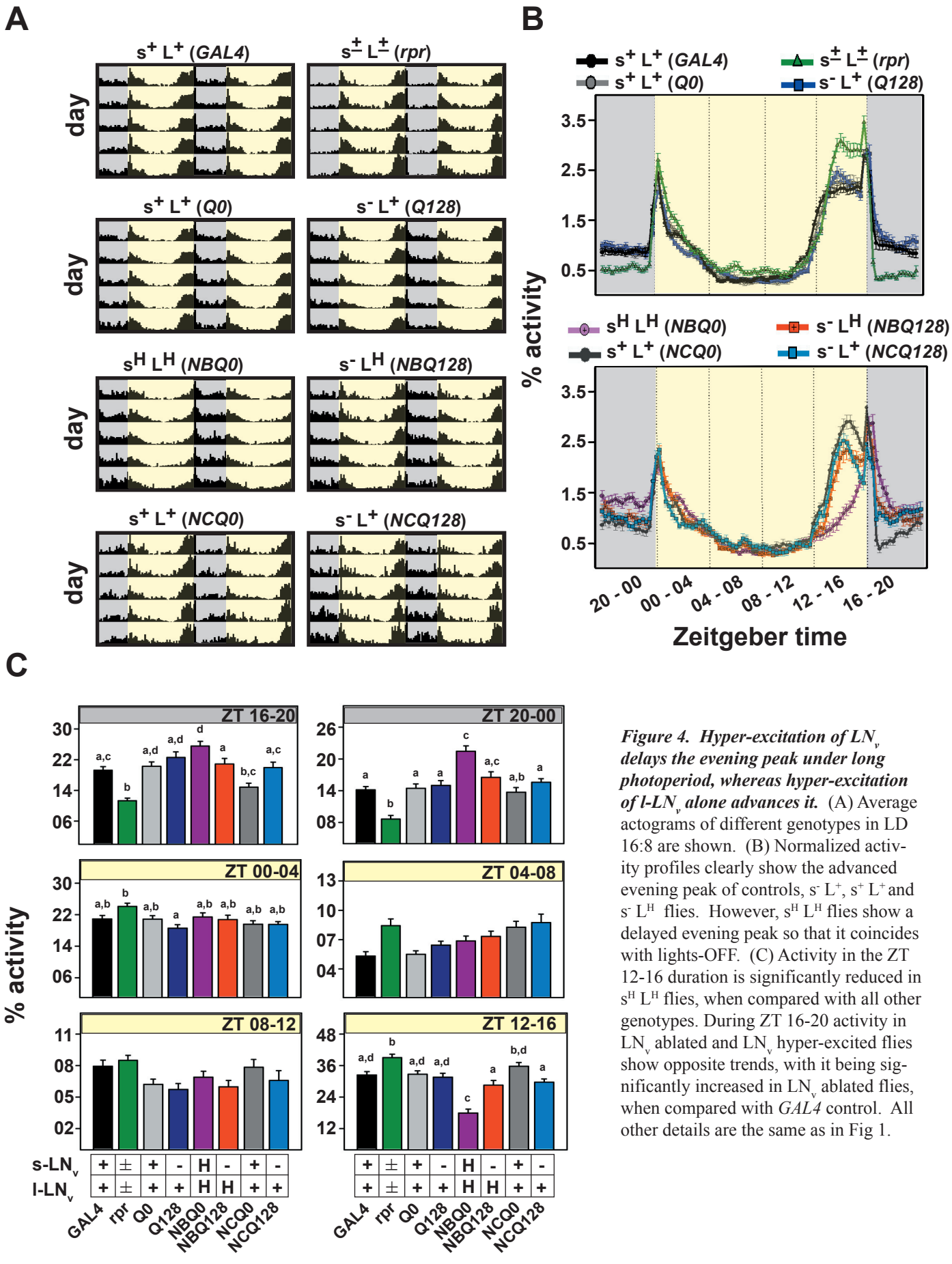


Figure 4. Hyper-excitation of LN_v delays the evening peak under long photoperiod, whereas hyper-excitation of l-LN_v alone advances it. (A) Average actograms of different genotypes in LD 16:8 are shown. (B) Normalized activity profiles clearly show the advanced evening peak of controls, $s^- L^+$, $s^+ L^+$ and $s^- L^H$ flies. However, $s^H L^H$ flies show a delayed evening peak so that it coincides with lights-OFF. (C) Activity in the ZT 12-16 duration is significantly reduced in $s^H L^H$ flies, when compared with all other genotypes. During ZT 16-20 activity in LN_v ablated and LN_v hyper-excited flies show opposite trends, with it being significantly increased in LN_v ablated flies, when compared with *GAL4* control. All other details are the same as in Fig 1.

Fig 4

similar to standard LD 12:12 regime suggesting that the LN_v firing properties are responsible for the phasing of evening peak. With the night being only 8 hr long, in this photoperiod also, $s^H L^H$ flies showed significantly higher activity compared to its controls throughout the night (ZT 16-20, compare purple bar with grey and blue bars Fig 4C). Although the $s^- L^H$ flies continued to exhibit lower nighttime activity compared to $s^H L^H$ flies this difference was significant only in the first half of the night (ZT 16-20, Fig 4C, Fig 6). Taken together these results under long days demonstrate the role of LN_v both in the arousal circuit and in the circadian circuit since their electrical activity can modify both activity levels at night and the phase of the evening peak.

Enhanced electrical activity of the l-LN_v regulates phasing of evening peak under extreme long day condition. Based on the results of the 16:8 hr long day photoperiod we surmised that increased firing of s-LN_v could alter the phasing of the evening activity peak in *Drosophila*, which agrees with previous studies which have used very different approaches (Stoleru et al., 2004; Rieger et al., 2006). Nevertheless, we subjected flies to an even longer photoperiod (LD 20:4) to test their ability to now phase their activity peaks when only s-LN_v or both LN_v are either absent or present in hyper-excited state. Under such extremely long days we found that $s^+ L^+$, $s^- L^+$ and $s^{\pm} L^{\pm}$ flies showed a small morning peak which is most likely a startle response in conjunction with lights-ON and an extremely advanced evening peak, about 4-5 hr prior to lights-OFF (Fig 5A and 5B, Fig 6). Compared to LD 16:8, where only $s^H L^H$ (*NBQ0* genotype) showed tight coupling with lights-OFF (Fig 4B, lower panel), surprisingly, in LD 20:4, the activity/rest profiles of $s^H L^H$ and $s^- L^H$ flies were not different from each other (Fig

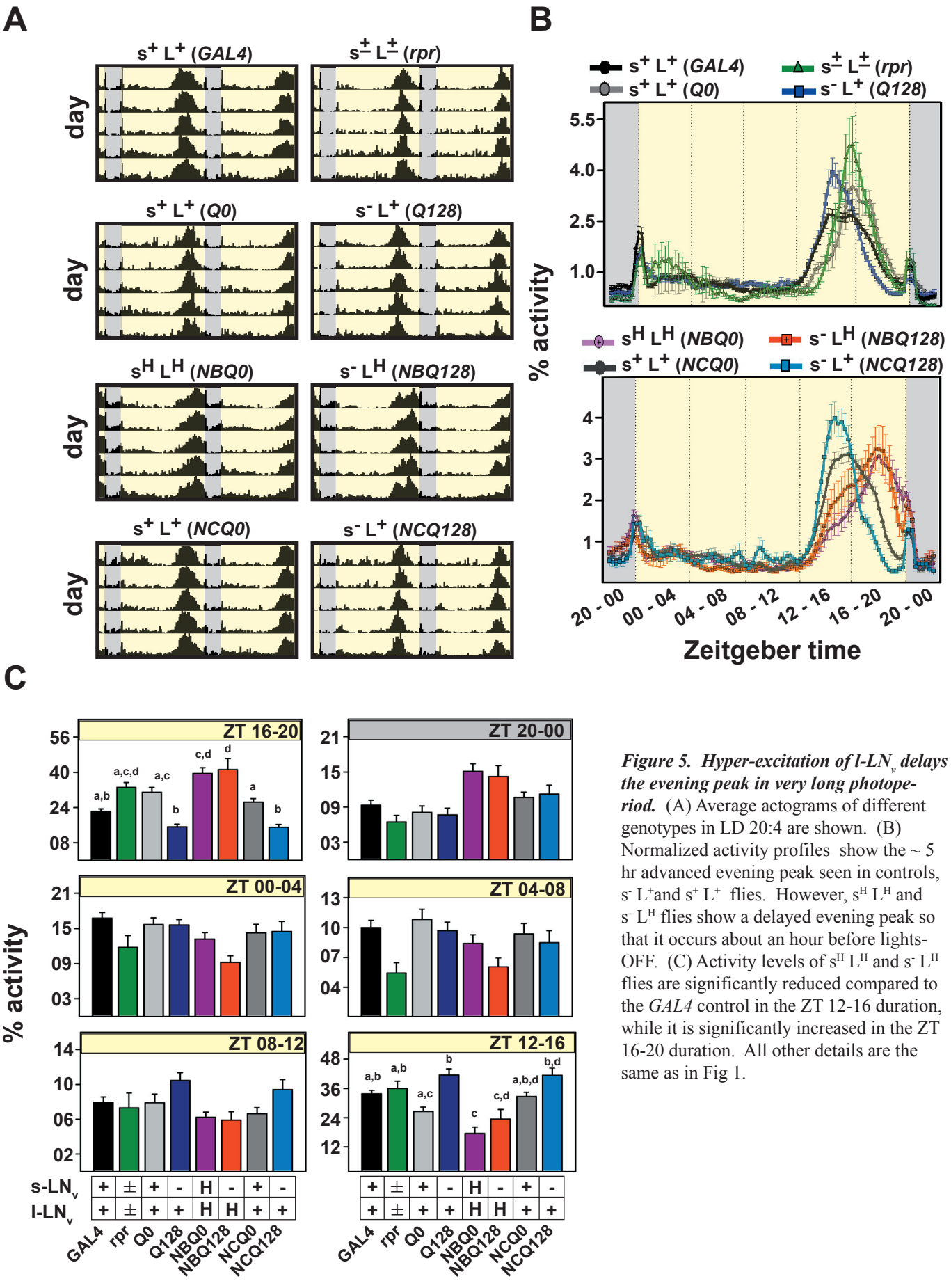


Figure 5. Hyper-excitation of l-LN$_v$ delays the evening peak in very long photoperiod. (A) Average actograms of different genotypes in LD 20:4 are shown. (B) Normalized activity profiles show the ~ 5 hr advanced evening peak seen in controls, s$^-$ L$^+$and s$^+$ L$^+$ flies. However, s^H L^H and s$^-$ L^H flies show a delayed evening peak so that it occurs about an hour before lights-OFF. (C) Activity levels of s^H L^H and s$^-$ L^H flies are significantly reduced compared to the *GAL4* control in the ZT 12-16 duration, while it is significantly increased in the ZT 16-20 duration. All other details are the same as in Fig 1.

Fig 5

5B, lower panel). Yet, they both differed by about 4 hr from the other genotypes in the phase of their evening peak (Fig 5B, Fig 6). Here, flies of both genotypes showed a delayed evening peak, such that it occurred about an hour prior to lights-OFF, while control s^+ L^+ (*NCQ0*) and s^- L^+ (*NCQ128*) both peaked about 4 hr before L/D transition (Fig 5B, lower panel). These differences in phasing of evening peak were reflected in the 4 hr interval analysis, with the activity levels of s^H L^H and s^- L^H flies 4 hr prior to lights-OFF (ZT 16-20) were significantly higher than that of the controls (Fig 5C). In the 4 hr interval beginning 8 hr before lights-OFF (ZT 12-16), the activity levels of s^H L^H flies were significantly lower than the controls (Fig 5C). The activity levels of s^- L^H flies in this interval were not significantly different from the controls, possibly because of within-genotype variance in phasing of evening peak. Indeed, about 59% of these flies showed a peak that occurred 5 hr before lights-OFF (around the same interval as the controls, Fig 6), while the remaining flies showed a peak about an hour before lights-OFF (around the same interval as s^H L^H flies). This result indicates that a fraction of s^- L^H flies were able to align their evening peak even in the absence of s-LN_v, perhaps due to the hyper-excitation of the l-LN_v as in LD 16:8. With the night being only 4 hr long (ZT 20-0), no detectable differences were seen in activity levels in this interval among different genotypes, notably between s^H L^H and s^- L^H (Fig 5C) which showed differences in the 16:8 regime (Fig 4C). Thus under the extremely long photoperiod, the flies seem to require the proper firing properties of both LN_v to appropriately phase their evening activity peaks. The hyper-excitation of LN_v appears to have an overall effect of pushing the evening activity peaks closer to lights-OFF transition especially under long-day photoperiods.

D. Discussion

Previous studies have shown that l-LN_v play an important role in governing morning activity behaviour in LD 12:12 in the absence of s-LN_v (Sheeba et al., 2010). While confirming that anticipation of and startle to lights-ON are conserved in flies lacking functional s-LN_v under LD 12:12, we demonstrate that l-LN_v are important in setting the phase of the evening peak in a wide variety of photoperiods (Fig 7) ranging from extreme short days (LD 4:20) up to extreme long days (LD 20:4). Furthermore, our results suggest that s-LN_v modulate nighttime activity levels, especially during the early part of the night in photoperiods with at least 8 hours of darkness.

While our results also indicate that s-LN_v are important in phasing the morning peak under extremely short day environment, in most other photoperiods they appear to be dispensable (blue and indigo curves Fig 6) for this. We also find that enhanced electrical activity of s-LN_v can reduce the amplitude of the evening peak under short (LD 8:16) and very short days (LD 4:20). In addition, we find that under short days the startle response to lights-ON is highly reduced (Fig 2, 3, 6, black and grey curves). We propose that this is due to the inhibitory action of normally firing s-LN_v, and that this inhibition is lost when there is a partial or complete loss of s-LN_v (Fig 6, indigo and blue curves), suggesting that under short day conditions, they have an inhibitory role on light-mediated immediate increase in activity (which is not revealed in the presence of hyper-excited l-LN_v). Previously, Rieger *et al.*, (2003) have reported separation of startle and non-startle peaks under short days in wild type *Canton-S* flies, which have functional s-LN_v. These studies differ from ours in at least two ways. Firstly, the light intensity used by us is much lower (~100 lux compared to 500 lux). Secondly, there is a

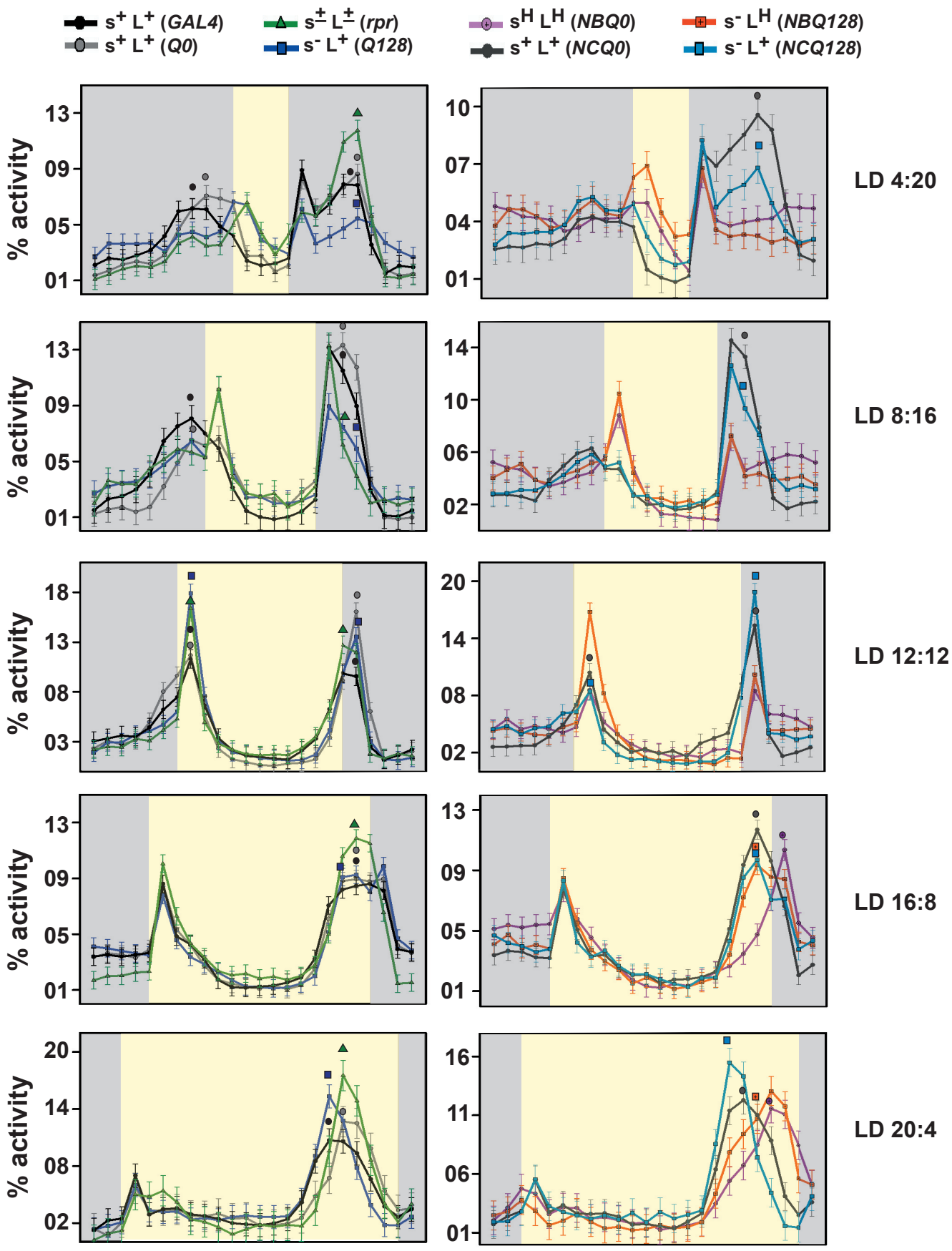

s+ L+ (GAL4)
s+ L+ (Q0)
s± L± (rpr)
s- L+ (Q128)
sH LH (NBQ0)
s+ L+ (NCQ0)
s- LH (NBQ128)
s- L+ (NCQ128)
% activity
LD 4:20
LD 8:16
LD 12:12
LD 16:8
LD 20:4

Fig 6

confounding effect of age in the previous study as the same flies experienced all three different photoperiods. We have also found similar results for the $s^+ L^+$ controls in an experiment when the same flies experienced different photoperiods as they aged (Fig 9). Nevertheless, we conducted experiments with different sets of same-age flies experiencing different photoperiods, to resolve the confounding effects of ageing.

Our results differ from previous studies in that they implicate a secondary role for the s-LN_v in the arousal circuit as activity/rest profiles especially under long nights. They also reveal differences in nighttime activity between flies with hyper-excited small and large LN_v and those with only hyper-excited l-LN_v (Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008a). In fact, in certain cases, the activity levels of flies without functional s-LN_v show increased nighttime activity, although not significantly different from controls (Figs 2 and 3). These results are further explored in chapter 3. Further studies that specifically target the s-LN_v without affecting the l-LN_v would provide a clearer picture regarding the role of these circadian neurons in modulating arousal. We speculate that the s-LN_v neurons form the link between the l-LN_v and the other limbs of the sleep circuit, thus bridging an obvious anatomical gap.

We find that that under short-day environments, the l-LN_v are critical for setting the phase of the evening peak. Irrespective of the status of the s-LN_v, manipulating l-LN_v results in a complete disappearance of the evening peak under extremely short

Figure 6. Comparison of activity/rest profiles of different genotypes across different photoperiod conditions. Activity counts that were binned into 1 hr intervals are plotted as percentage activity normalized to total activity during the 24 hr day. The yellow and grey highlighted regions represent light and dark periods respectively. Error bars represent 95% confidence intervals around the mean. Non-overlapping error bars within the same genotype across different time points in each photoperiod condition were used to determine activity peaks. Presence of peaks in different genotpyes are indicated by corresponding coloured shapes.

days. Two separate previous studies have shown that the phase of evening peak under LD 12:12 can be determined in at least two ways. One study suggests that light input perceived by l-LN_v and communicated via PDF to LN_d and the 5^{th} s-LN_v (evening oscillator) determines the phase of evening peak, even in the absence of functional CRY (Cusumano et al., 2009). However, they show that these oscillators can set the phase of the evening peak even in the absence of PDF using visual inputs perceived via CRY. A second study suggested that CRY was responsible for gating of PDF signaling (L. Zhang et al., 2009). Here we show that under short-day conditions, the membrane properties of l-LN_v critically affects phase of the evening peak. Even under LD 12:12, the firing properties of the l-LN_v exerts its influence on the amplitude of evening peak, since hyper-excitation of l-LN_v alone is enough to reduce amplitude of startle response and anticipation of the evening peak (Fig 1B lower panel; Fig 1C). In addition, our study shows that normal firing of l-LN_v is essential for lights-ON anticipation, thus underlining an important role for l-LN_v in the circadian circuit. Interestingly, a previous study has shown that light information signaled from the compound eyes is important for entrainment to extremely long and short photoperiods, while CRY is specifically required for entrainment to short photoperiods (Rieger et al., 2003). Our results point toward an important role for electrical activity mediated signals from l-LN_v influencing locomotor activity profiles across almost all photoperiods. In support of our claim that l-LN_v communication is important, a recent study showed that flies that are doubly mutant for *pdfr* and *cry*, or alternatively *pdf* and *cry* show activity/rest profiles that are strikingly similar to those in which LN_v are hyper-excited- - they do not display evening peak under short days (Im et al., 2011). Interestingly, *pdfr* single mutants showed a

phase advanced evening peak even under short day condition, suggesting that downstream neurons are capable of producing the evening peak in the presence of functional CRY, albeit with an erroneous phase. However, in absence of both *cry* and *pdfr*, flies became incapable of producing the true evening peak, similar to our finding that upon hyper-excitation of LN_v, s-LN_v influence the amplitude while l-LN_v governs the phase (Fig 6, top 2 rows). Intriguingly, the *pdfr, cry* double mutants did not exhibit a true evening peak even in a long photoperiod, whereas the *pdfr* single mutants displayed a severely phase advanced evening peak. In comparison, in our study the LN_v hyper-excited flies showed a delayed phase of evening peak, whereas flies with dysfunctional s-LN_v, despite hyperexcitation of l-LN_v, showed a phase of evening peak similar to that of the control under LD16:8. Thus, under long day, just as in short day conditions, the communication of the l-LN_v with their target neurons is paramount in the generation of correctly phased evening peak.

Our study provides strong evidence for the existence of a plastic circadian neuronal network by demonstrating that altering the level of activity of the l-LN_v can cause a significant change in the phase of the evening peak under different day lengths. It appears that the circadian neuronal network of *Drosophila* is equipped to deal with varying day lengths by appropriately changing the phase of the evening peak of activity with respect to lights-OFF. However, the phases of the morning and evening peaks do not follow the dawn and dusk transitions in a dedicated fashion as posited by the original dual-oscillator model; instead the phase difference between the coupled morning and evening oscillators is flexible only up to a certain limit. The oscillators become inefficient in tracking dawn and dusk signals under shrinking or expanding

photoperiod beyond a particular limit, as seen in our study and of many others (Rieger et al., 2003; Rieger et al., 2012). We propose that the l-LN_v maintains the phase relationship between oscillators located in s-LN_v and LN_d and 5^{th} s-LN_v across photoperiods (Figure 7). Since l-LN_v are light responsive (Sheeba et al., 2008b) and richly synapse onto the optic lobes (Kaneko and Hall, 2000) we speculate that the l-LN_v represent a group of neurons that are capable of measuring day-length, as they fire continuously in the presence of light. It is possible that with varying day lengths, different subsets of circadian neurons actually respond to the signaling from l-LN_v.

In conclusion, we propose a vitally important role for the l-LN_v in measuring day-length and correspondingly bringing about alteration in the phase of the evening peak. We opine that the circadian clock network consisting of about 150 neurons has distinct, but flexible roles for neurons belonging to each subset, such that according to the environmental condition, they interact to bring about the overt behaviour. Altogether, the significance of l-LN_v in the clock circuit and a secondary role of the s-LN_v in the arousal circuit demonstrate the ability of neurons to transcend circuits and play critical roles apart from their primary functions.

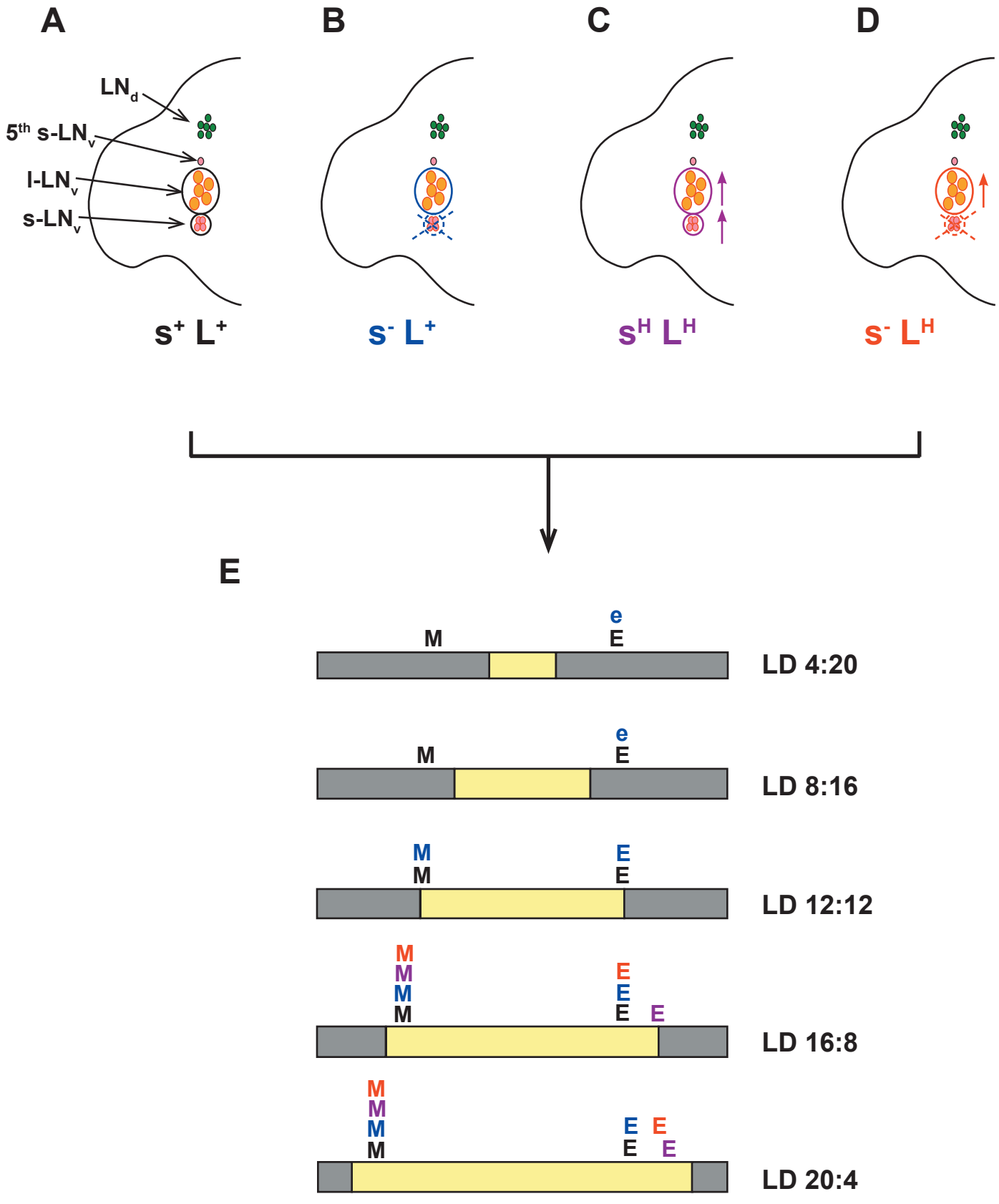

A
B
C
D
LN$_d$
5th s-LN$_v$
l-LN$_v$
s-LN$_v$
s$^+$ L$^+$
s$^-$ L$^+$
s^H L^H
s$^-$ L^H
E
M
e
E
LD 4:20
LD 8:16
LD 12:12
LD 16:8
LD 20:4

Fig 7

Figure 7. A proposed model for the role of lateral neurons in adaptation to seasonal variations. (A-D) Schematic representations of various manipulations to the LN_v – control (A),dysfunctional s-LN_v, normally firing l-LN_v (B), hyper-excited both LN_v (C) and dysfunctional s-LN_v, hyper-excited l-LN_v (D). Solid lines indicate normally firing neurons, while dotted lines indicate dysfunctional neurons. Arrows next to a group of neurons indicate that that group is hyper-excited. (E) Different photoperiod conditions on the right are indicated by the yellow (day) and grey (night) bars. 'M' and 'E' on top of the bars indicate the phases of true morning and evening peaks of genotypes given in A-D. In short photoperiods (LD 4:20 and 8:16), only controls exhibit true morning and evening peaks. $s^- L^+$ flies exhibit a low-amplitude evening peak indicated by 'e' (in blue) at the same phase as the control evening peak ('E'in black). Both $s^H L^H$ and $s^- L^H$ do not display either the morning or the evening peaks indicated by the absence of 'M' and 'E' in purple and red. In LD 12:12, control and $s^- L^+$ flies display anticipation to lights-OFF and thus the true evening peak. Only controls show anticipation to lights-ON and thus the true morning peak. In long day conditions (LD 16:8 and 20:4), all the genotypes show a morning peak close to lights-ON as indicated by variously coloured 'M'. The evening peaks of the controls, $s^- L^+$, $s^- L^H$ flies are phase advanced as compared to that of $s^H L^H$, whose peak is close to lights-OFF especially in LD 16:8. In LD 20:4, the evening peaks of both $s^- L^H$ and $s^H L^H$ flies are delayed when compared with the controls and $s^- L^+$ flies.

A

cell type	number of cells	n*
l-LN_v	2.9 ± 0.2	24
s-LN_v	0.4 ± 0.1	24

*n = 24 brain hemispheres

B

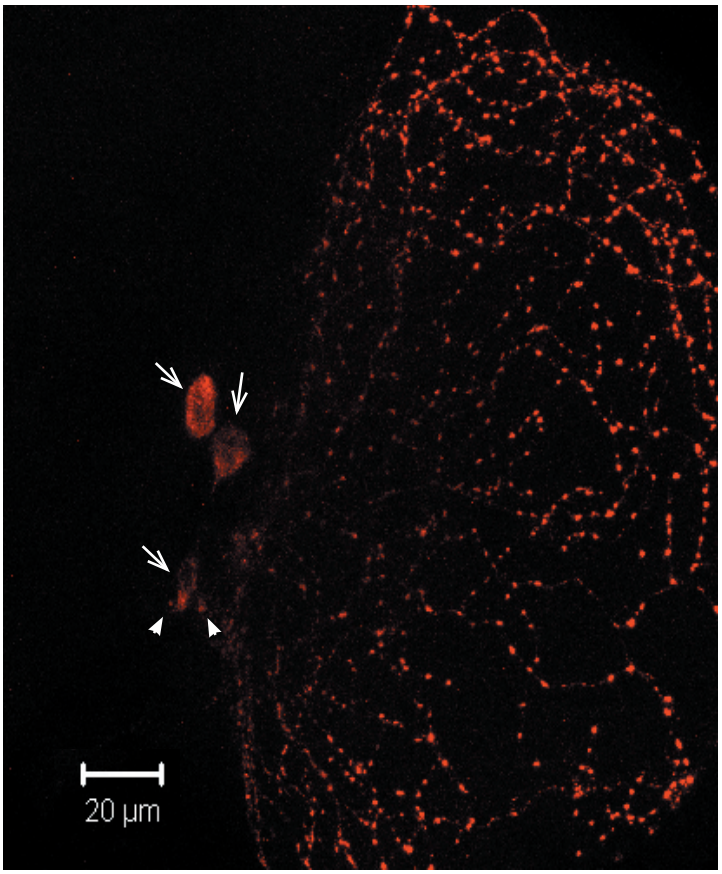


Figure 8. Partial ablation of LNv neurons on driving UAS rprC-14 using pdf GAL4. (A) Table depicting the number of s-LN_v and l-LN_v neurons visualized by staining against PDF in (*n = 24) brain hemispheres of $s^{\pm}$ $L^{\pm}$ flies. Error values are SEM. (B) A 40X brain image of $s^{\pm}$ $L^{\pm}$ flies showing the presence of three l-LN_v (arrows) and two s-LN_v (arrow-heads). All brains were dissected from 3-4 day old s+ L+ flies maintained in LD 12:12 at 25 ˚C between ZT 1-3 and stained using anti-PDF (mouse, 1:3000) as primary antibody and Alexa 546 (anti-mouse, 1:3000) as secondary antibody. The immunocytochemistry protocol followed is as described in Sheeba *et al.,* (2008).

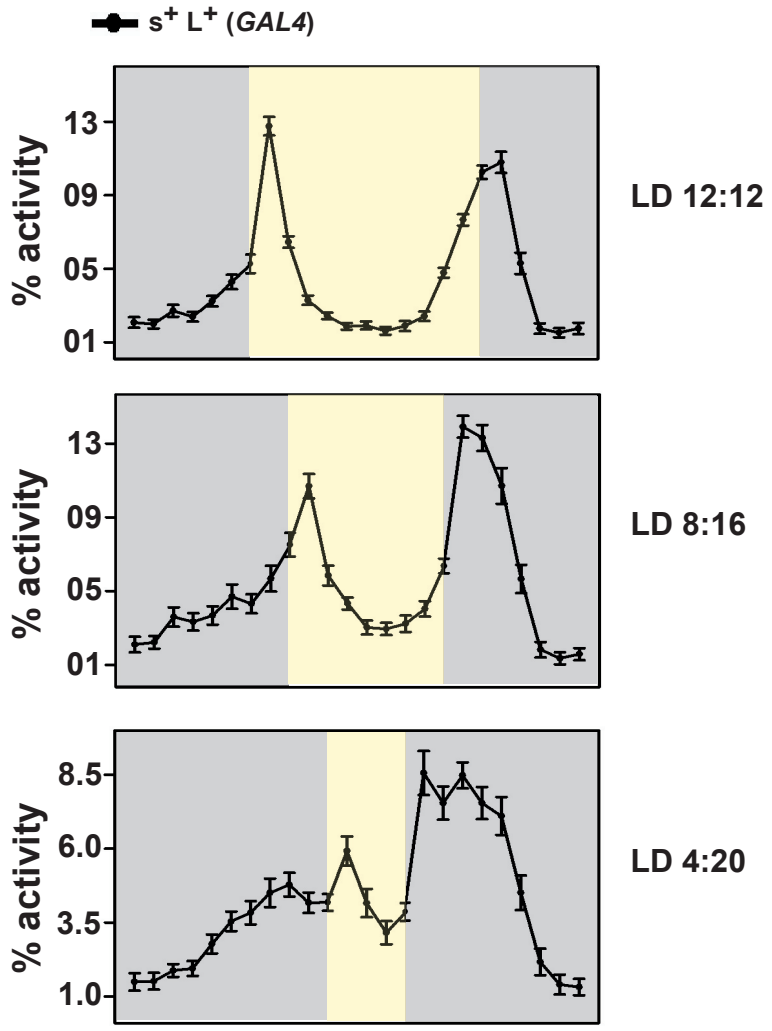


Figure 9. Comparison of activity/rest profiles of the same set of s^+ L^+ (GAL4) flies experiencing decreasing photoperiod conditions. Activity counts that were binned into 1 hr intervals and normalized to total activity during the 24 hr are plotted. Error bars represent SEM. All other details are same as Fig 1. In all the photoperiods, the presence of true non-startle and startle morning and evening peaks can be seen. Startle peaks are seen even in short and very short day conditions that are not seen when different sets of flies experience different photoperiod conditions (Figs 2 and 3). In this assay, 3-4 day old flies were introduced into LD 12:12 at 22 ˚C, and after 7 days, the day length was decreased to create a 8 hr day (LD 8:16), that was further reduced to create a 4 hr day (LD 4:20) after subsequent 7 days in LD 8:16. This plot shows activity/rest profiles in last five days in LD 12:12, LD 8:16 and LD 4:20 as first two days in each of the new condition were transients.

Chapter 3

Different roles for small ventral lateral neurons and a subset of Pars Intercerebralis neurons in modulating sleep and arousal in *Drosophila*

A. Introduction

Sleep is a complex behaviour displayed by most organisms, whose role is not as yet properly understood (Mignot, 2008). Sleep is defined as a period of rest which is distinguished from mere inactivity by various physiological, electrophysiological and behavioural characteristics. The behavioural manifestations of sleep in many vertebrates include physical quiescence, maintenance of a typical posture, exhibiting a preference for the site of sleep, stereotyped behaviours like yawning and circadian control of sleep/wake cycles (Kerkhof and Van Dongen, 2010). Sleeping animals also exhibit elevated arousal threshold, i.e an increased intensity of stimulus is required to elicit the same response from a sleeping animal compared to when it is awake (Cirelli and Bushey, 2008). Furthermore, animals display what is known as sleep rebound, i.e. they sleep more after a period of being deprived of sleep (Cirelli, 2009).

Many model systems that allow for studying molecular, neuronal and genetic substrates of various behaviours like *C. elegans*, *Drosophila melanogaster*, mouse and zebra fish also display either sleep or sleep-like behaviours (Crocker and Sehgal, 2010). *Drosophila melanogaster* exhibit behavioural (Hendricks et al., 2000; Shaw et al., 2000) and electrophysiological (Nitz et al., 2002) signatures of sleep that are quite similar to mammalian sleep. Various neural circuits have been implicated in the control of sleep (Harbison et al., 2009; Sehgal and Mignot, 2011), with different downstream signaling cascades. Mushroom bodies are shown to consist of both sleep-promoting and sleep-inhibiting areas, and they mediate their effects via cAMP signaling (Joiner et al., 2006; Pitman et al., 2006). Additionally, the neuroendocrine centre of fly brain, the Pars Intercerebralis (PI) has been shown to promote sleep via the EGFR pathway (Foltenyi et

al., 2007), whereas they also promote octopamine-mediated arousal (Crocker et al., 2010). Various other higher areas such as the ellipsoid body (Parisky et al., 2008; Lebestky et al., 2009) and fan-shaped body (Donlea et al., 2011) are also shown to be involved in the sleep/arousal pathway.

Circadian large ventrolateral neurons (l-LN_v) that secrete a neuropetptide called Pigment Dispersing Factor (PDF) are implicated in modulating arousal (Sheeba et al., 2008a) by integrating information from various arousal-promoting cues such as light (Shang et al., 2008), dopamine and octopamine (Shang et al., 2011). Furthermore, l-LN_v mediate arousal by acting through PDF, as the night time hyperactivity seen in LN_v hyper-excited flies disappeared in flies with the same manipulation in a pdf^{01} background (Sheeba et al., 2008a). Additionally, the l-LN_v have been shown to receive GABAergic inhibitory signals, in the absence of which they continuously promote arousal (Parisky et al., 2008). Thus far, no role for the l-LN_v has been assigned in promoting sleep, though it was reported that in light/dark cycles of 12:12 hr (LD 12:12), loss of function mutation of *pdf receptor* leads to reduced day time sleep (Chung et al., 2009). In the same study, it was found that RDL, a GABA receptor via which GABAergic signals are perceived by the LN_v are exclusively expressed only in the l-LN_v, suggesting that l-LN_v alone, and not the s-LN_v modulate arousal. Other lines of evidence using different kinds of genetic manipulations have been obtained that also purport this view. Sheeba et al. (2008a) hyper-excited both the subsets of LN_v neurons and found that such flies show decreased night-time sleep, whose levels do not differ from those in which the l-LN_v alone are hyper-excited in the absence of s-LN_v. Additional support came in the form of studies in which different *GAL4* drivers that

drove expression in either the s-LN_v or the l-LN_v alone were used, with the caveat that apart from the respective LN_v subsets, these drivers also targeted some unrelated non-circadian neurons (*c929GAL4*, 300 peptidergic neurons along with l-LN_v, Parisky et al., 2008, Shang et al., 2008; *R6 GAL4*, s-LN_v along with a few non-circadian neurons, Shang et al., 2008). In both these studies, either the l-LN_v or the s-LN_v alone were hyper-excited, and it was observed that flies with hyper-excited l-LN_v showed a decrease in their night-time sleep, whereas flies with hyper-excited s-LN_v did not differ in their night time sleep from the controls. These results together suggest that the l-LN_v are specifically involved in modulating arousal, with little or no involvement of the s-LN_v.

While results obtained by many different groups point towards a negligible role for the s-LN_v in the sleep/arousal circuit, Parisky et al. (2008) obtained circumstantial evidence to argue otherwise. They downregulated *pdfr* in the LN_v neurons and found that both day time and night time sleep were increased in LD 12:12. They reasoned that since l-LN_v are not responsive to PDF (Shafer et al., 2008), the effect of this manipulation on sleep is an effect of manipulating s-LN_v. Indeed, they proposed that PDF signaling from the l-LN_v is perceived by the s-LN_v which then act to signal to the higher motor centres like the ellipsoid body and bring about arousal. This role for the s-LN_v in information transfer between different parts of the sleep circuit is also substantiated by the projection patterns of the two LN_v subsets. Large LN_v project ipsilaterally to the optic lobes to receive light inputs and contralaterally to the other hemisphere, whereas s-LN_v send out rich projections towards the dorsal protocerebrum (Kaneko and Hall, 2000; Sheeba, 2008), that can make contacts with various putative

downstream targets of arousal-promoting l-LN_v (for example, PI). In order to probe the role of s-LN_v in the sleep/arousal circuit and lay to rest speculations about its involvement therein, we sought to analyze sleep parameters of flies with either both the LN_v subsets hyper-excited, or l-LN_v alone hyper-excited under different photoperiod conditions. While this was done to answer a completely different question (see chapter 2), it nevertheless allowed us an opportunity to explore the roles of the two LN_v subsets in governing aspects of sleep and arousal under different environments. We found that, apart from LD 12:12, in all other photoperiod conditions tested, the effect of hyper-excitation of both LN_v subsets was more drastic in terms of sleep reduction than hyper-exciting l-LN_v alone, thus indicating a subsidiary role for s-LN_v in modulating arousal.

Given that so far it has been found that l-LN_v mediate arousal through PDF, we next set out to ask what the target sites of PDF could be for modulating arousal. The PDFR distribution is quite widespread and includes various sites such as circadian neurons, Ellipsoid body, Pars Intercerebralis and mushroom body (Hyun et al., 2005; Im and Taghert, 2010; Lear et al., 2005; Mertens et al., 2005; parisky et al., 2008). In order to examine which of these cells could be crucially involved, we first characterized the sleep abnormalities of flies with loss-of-function mutation in *pdfr* under both LD 12:12 and constant dark (DD) conditions. Next, we chose to downregulate the *pdfr* under the influence of five drivers that primarily drive expression in PI neurons. We specifically chose to target the PI for the following reasons. The PI neurons are implicated in promoting both sleep (Foltenyi et al., 2007) and arousal (Crocker et al., 2010). The dorsal projection of s-LN_v lie very close to where the PI neurons are located, thus allowing for possible contacts between the two (Sheeba, 2008). Additionally, in situ

hybridization of *pdfr* revealed that it was also localized in the PI neurons (Lear et al., 2005). These and other factors such as the PI being the neuroendocrine centre of the fly brain prompted us to specifically target the downregulation of *pdfr* in these neurons. Indeed, we found that downregulating *pdfr* in a subset of about 15-16 neurons in the PI decreased night time sleep in LD 12:12, but this sleep decrement did not persist in DD. However, flies with whole-body mutation in *pdfr* show reduced day-time sleep in LD 12:12, and increased total sleep in DD. Thus, we propose that PDF has complex roles in modulating sleep and arousal and has different effects in different environments.

B. Materials and methods

Fly strains. All strains were reared on standard cornmeal medium in LD 12:12 at 25 ˚C. The s^{+} L^{+} (*GAL4*, *Q0*, *NCQ0*), s^{-} L^{+} (*Q128*, *NCQ128*), $s^{\pm}$ $L^{\pm}$ (*rpr*), s^{H} L^{H} (*NBQ0*) and s^{-} L^{H} (*NBQ128*) flies are as described in chapter 2. *w^{1118} ; dilp2 GAL4; +* (Rulifson et al., 2002), *w^{1118} kurs45 GAL4; +; +*, *w^{1118} ; kurs58 GAL4; +*, *w^{1118} ; mai281 GAL4; +*, *w^{1118} ; mai301 GAL4; +* (Siegmund and Korge, 2001) were all balanced in their third chromosome, except *w^{1118} kurs45 GAL4; +; +* which was balanced on both second and third chromosomes and females of these genotypes were crossed with males of *w^{1118} ; IF/CyO; UAS dcr* (balanced from Bloomington stock number 24651) to yield *w^{1118} ; dilp2 GAL4; UAS dcr*, *w^{1118} kurs45 GAL4; +; UAS dcr*, *w^{1118} ; kurs58 GAL4; dcr*, *w^{1118} ; mai281 GAL4; UAS dcr*, *w^{1118} ; mai301 GAL4; UAS dcr* flies. These female flies were either crossed with males of *w^{1118} ; UAS pdfr IR; +* (VDRC, *CG13758*) in which case the progeny were the experimental flies, or were crossed with males of *w^{1118}* in which case the progeny were *GAL4* controls. To obtain the *UAS* controls, *w^{1118}* females were crossed with males of *w^{1118} ; UAS pdfr IR; +* flies.

Data recording and analysis. 3-4 day old virgin males of all the above crosses as well as those of *pdfr*^{5304} (Bloomington stock number 33068) and *w*^{1118} (Bloomington stock number 5604) were chosen for assaying their sleep/wake cycles in locomotor activity tubes using the *Drosophila* Activity Monitor (DAM) system (Trikinetics, Waltham, MA, USA). Flies were recorded in LD 12:12 at 25 ˚C for five days, after which they were transferred to constant darkness for about 8-9 days in a Sanyo MIR-254 incubator (Sanyo electrical, Osaka, Japan). Activity was recorded at every five min interval and we defined sleep as any period of inactivity for five or more minutes. We used a MicroSoft Excel spreadsheet macro to analyze the number of bouts of five min sleep, as well as when sleep was defined as 15, 30, 45 or 60 min of inactivity. This allowed us to assess sleep consolidation of flies based on number and duration of sleep bouts. We also estimated total time spent sleeping during both day and night. We also plotted average number of five min sleep bouts binned at a half hour interval versus time of the day for all genotypes. This was obtained by first averaging across the five days in LD 12:12 for each individual fly, and then averaging across flies to obtain sleep profiles of all genotypes in LD12:12. For sleep profile in first day of DD, sleep bout number was simply averaged across flies of each genotype. For photoperiods apart from LD 12:12, computed sleep bout numbers only for five min sleep.

Statistical analyses. One way ANOVA was carried out on total five, 15, 30, 45 and 60 min sleep and sleep bout number with genotype as a factor for all the *GAL4* lines tested. Comparison was not made across different *GAL4* lines due to background effects. Within each of the *GAL4* line, comparison was made between *GAL4* control, *UAS* control and experimental line and individual differences between them was revealed by

post-hoc Tukey's HSD test. Two way ANOVA followed by post-hoc Tukey's HSD test was carried out with time of the day and genotypes as factors to compute differences between day-time and night-time sleep across different genotypes. This was done for sleep of all bout lengths for data from flies in LD 12:12 and DD, and only five min sleep data from flies in other photoperiod conditions. All the statistical analyses were done using STATISTICA ver. 7.0 (StatSoft Inc., Tulsa, OK, USA). Level of significance was set to $p < 0.05$.

Immunocytochemistry. Females of all *GAL4* driver lines were crossed with males of *w; UAS 2exe GFP; +* and brains of progeny of these crosses were dissected. Brains of virgin 2-3 day old flies were used to stain with primary antibody against GFP (chicken, 1:1000) and secondary anti-chicken antibody tagged with Alexa-488 dye (1:3000) so as to enhance the signals from GFP. Immunocytochemistry was carried out as given in Sheeba et al., (2008b). In brief, brains dissected from frozen flies in phosphate buffer saline (PBS) were first fixed in 4% formaldehyde for 30 min, washed 3-4 times with 0.5% Triton-X in PBS (0.5% PBT) every 10 min and were blocked with 10% horse serum (in 0.5% PBT) for one hour. Dilutions of both primary and secondary antibodies were made in blocking solution. After blocking, brains were flooded with primary antibody for 24 hr in 4 ˚C, after which they were washed 7-8 times with 0.5% PBT. Brains were next stained with secondary antibody and incubated overnight at 4 ˚C. They were then cleaned and mounted on a slide in 7:3 glycerol: PBS mounting medium and imaged using a Zeiss 510 meta confocal microscope. Confocal stacks were analysed using LSM viewer and about 15-20 planes were stacked together to obtain the final image.

C. Results

s-LN$_v$ are not required for mediating arousal in a standard day of 12 hr of light. In order to assess the importance of s-LN$_v$ in the sleep/arousal circuit, we obtained locomotor activity data from flies subjected to different day length conditions. Data from flies with both LN$_v$ subsets misfiring ($s^H L^H$, NBQ0) and with only l-LN$_v$ ($s^- L^H$) misfiring and their respective controls were subjected to sleep analysis. In a standard day of 12 hr, the $s^+ L^+$ (*Q0* and *NCQ0*) and $s^- L^+$ (*Q128* and *NCQ128*) flies showed comparable number of five min sleep bouts both during the day and night (Fig 1, left panel, compare grey and indigo lines, right panel, compare grey and blue lines). In fact both day time and night time sleep did not differ significantly between the $s^+ L^+$ and $s^- L^+$ flies (Fig 2, left panel, compare grey and indigo hatched and solid bars, right panel, compare grey and blue hatched and solid bars). However, the night time sleep of $s^+ L^+$ (*GAL4*) control was significantly different from that of $s^- L^+$ (*Q128*). This could be due to the lower level of baseline sleep in the $s^+ L^+$ (*GAL4*) control, as this trend of lowered sleep for these flies was observed across all photoperiods. Another example of a genetic background effect is higher level of sleep observed in $s^- L^+$ (*NCQ128*) flies across all photoperiods tested. We sought to resolve this by comparing the $s^- L^+$ *Q128* transgenic flies with more appropriate genetic controls which are the $s^+ L^+$ *Q0* transgenic flies.

$s^{\pm} L^{\pm}$ (*rpr*) flies showed similar five min sleep bout number during the day to the $s^+ L^+$ (*GAL4*) controls, however they differed significantly in their night time sleep duration (Fig 1, left panel, green and black lines, Fig 2, left panel, green solid and black solid bars). Our results are consistent with what was already known for flies with fully

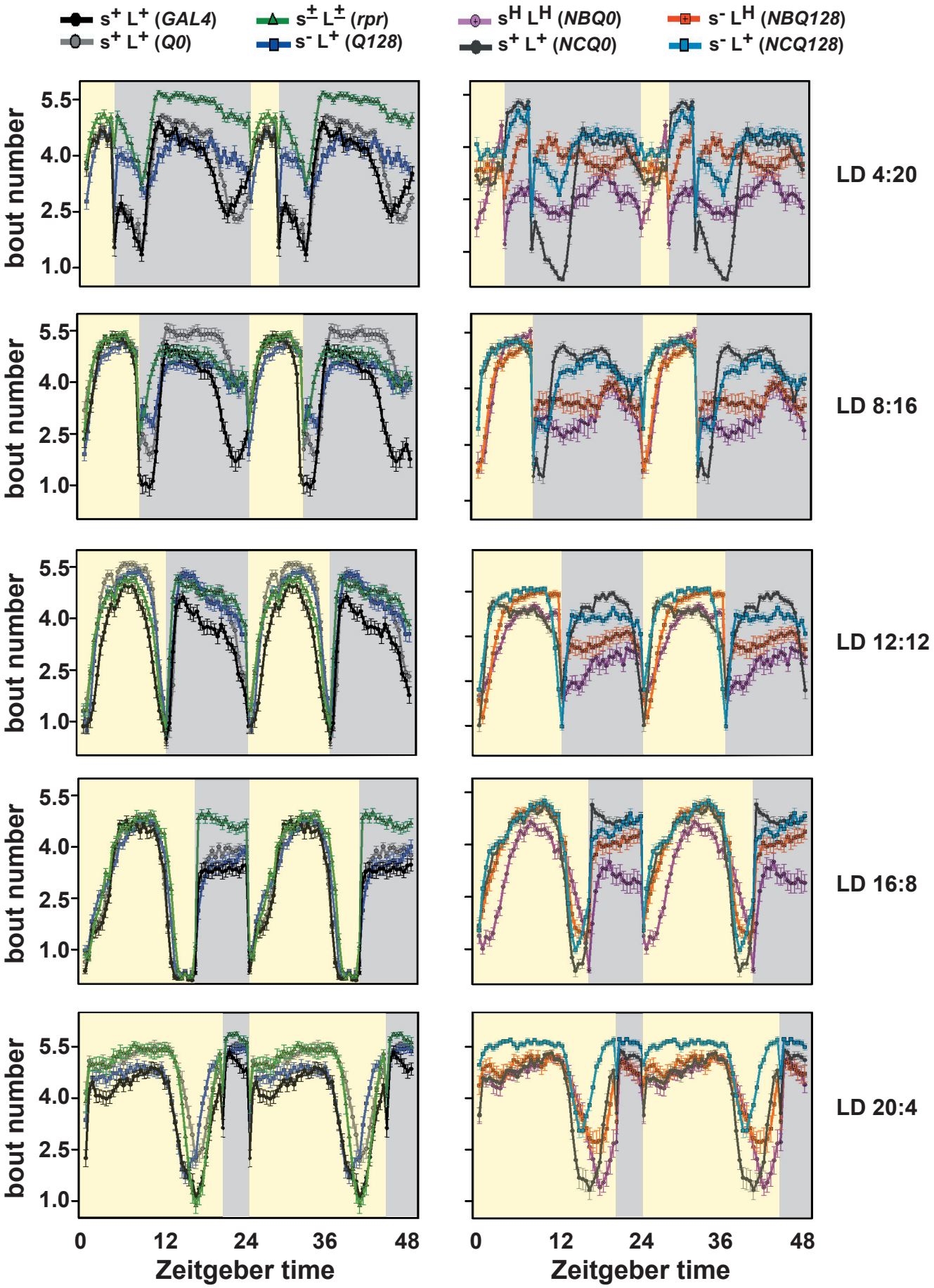

s+ L+ (GAL4)
s+ L+ (Q0)
s± L± (rpr)
s- L+ (Q128)
sH LH (NBQ0)
s+ L+ (NCQ0)
s- LH (NBQ128)
s- L+ (NCQ128)
bout number
5.5
4.0
2.5
1.0
LD 4:20
LD 8:16
LD 12:12
LD 16:8
LD 20:4
0
12
24
36
48
Zeitgeber time

Fig 1

ablated LN_v (*pdf GAL4; UAS hid* in Chung et al., 2009). Thus, our results show that even the loss of one large LN_v and upto three s-LN_v per hemisphere (see Fig 8, chapter 2) can cause significant increase in night time sleep in LD 12:12, thus underlining the importance of LN_v neurons in bringing about arousal.

When both the LN_v subsets were hyper-excited ($s^H L^H$), in a standard day of 12 hr it was observed that night time sleep was significantly reduced when compared with control, whereas, day time sleep remained unaffected, consistent with earlier studies (Fig 1, right panel, compare purple and grey lines, Fig 2, compare purple solid and hatched bars with one another, and with grey solid and hatched bars respectively, Sheeba et al., 2008a). Interestingly, in the sleep profiles it appeared as if the night time sleep of flies with only l-LN_v hyper-excited ($s^- L^H$) was higher than that of the $s^H L^H$ flies (Fig 1, middle right panel, compare red and purple lines). However, these differences did not turn out to be statistically significant (Fig 2, right panel, compare red and purple solid bars) consistent with previous studies (Sheeba et al., 2008a). Indeed, the $s^- L^H$ flies like the $s^H L^H$ flies showed decreased night time sleep that was significantly different from that of both the $s^+ L^+$ and $s^- L^+$ flies (Fig 2, right panel, compare red solid bars with grey and blue solid bars). Moreover, these flies along with the $s^H L^H$ flies were the only ones who showed a significant difference between their day time and night time sleep in this regime (Fig 2, right, purple solid and hatched bars

Figure 1. Night time sleep of LN_v hyper-excited flies is reduced under all photoperiods. Number of five-minute sleep bouts per half hour as a function of time of the day is double-plotted for ease of observation. Yellow and grey shaded areas denote day and night respectively. Error bars denote SEM. $s^+ L^+$ flies show consistent increase in sleep with respect to controls across all photoperiods – night time sleep increases during long nights and day time sleep increases during long days. $s^H L^H$ flies show hyperactivity during the night in all photoperiods except LD 20:4. This night time decrease in sleep is seen even in $s^- L^H$ flies, though the sleep level is higher when compared with $s^H L^H$ flies. Sleep levels of the $s^- L^+$ (*Q128*, left panel; *NCQ128*, right panel) do not seem to differ from their respective controls (*Q0*, left panel; *NCQ0*, right panel) across different photoperiod conditions.

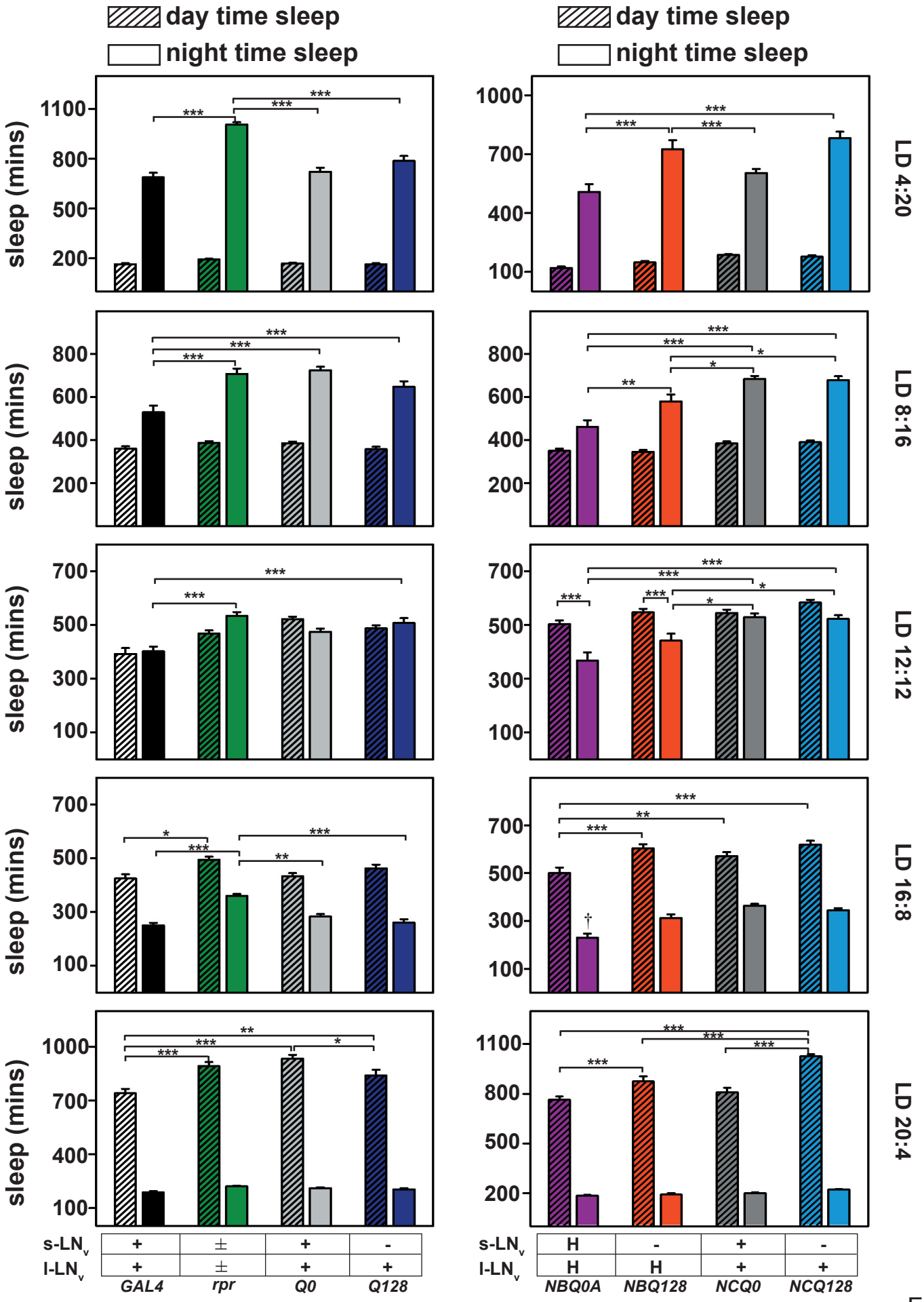

day time sleep
night time sleep
day time sleep
night time sleep
sleep (mins)
LD 4:20
LD 8:16
LD 12:12
LD 16:8
LD 20:4
s-LN$_v$
l-LN$_v$
GAL4
rpr
Q0
Q128
NBQ0A
NBQ128
NCQ0
NCQ128

Fig 2

, red solid and hatched bars). These results reiterate the importance of l-LN_v in the control of arousal and suggest that s-LN_v are not essential for the same.

s-LN_v are required for modulating effect of hyper-excited l-LN_v during different times of day depending upon day length. Sleep analysis of $s^+ L^+$ (*Q0* and *NCQ0*) and $s^- L^+$ (*Q128* and *NCQ128*) flies in short day condition of 8 hr of light did not reveal differences between either their day time or night time sleep levels (Fig 1, top left panel, compare grey and indigo lines, top right panel, grey and blue lines, Fig 2, top left, grey and indigo solid and hatched bars, top right, grey and blue solid and hatched bars). Even in this regime, night time sleep of $s^{\pm} L^{\pm}$ flies was significantly higher than its parental $s^+ L^+$ (*GAL4*) control (Fig 1, top left, green and black lines, Fig 2, top left, green and black solid bars). The decrease in night time sleep of $s^H L^H$ and $s^- L^H$ flies persisted even in this regime and was significantly different from that of their respective control $s^+ L^+$ and $s^- L^+$ flies (Fig 1, top right, purple and grey lines, red and blue lines, Fig 2, top right, purple and grey solid bars, red and blue solid bars). Interestingly, in this regime with the night time being 16 hr long, the night time sleep of $s^H L^H$ flies was significantly lower than that of the $s^- L^H$ flies, suggesting that s-LN_v are required to modulate the effect of hyper-excited l-LN_v in bringing about increased arousal. This is because in the

Figure 2. Flies with only l-LN_v hyper-excited differ from flies with both LN_v subsets hyper-excited in their day-time and night-time sleep. Amount of five min sleep during the day (solid bars) and night (hatched bars) are plotted for different genotypes across different photoperiod conditions. In LD 4:20, 8:16 and 12:12, night time sleep of $s^+ L^+$ flies is significantly increased from their controls; in LD 16:8, both day- and night-time sleep are significantly higher, while in LD 20:4 only day-time sleep is significantly higher than the controls (left panel). Night-time sleep of $s^H L^H$ and $s^- L^H$ flies are significantly lower than that of the controls in LD 8:16 and LD 12:12, in LD 16:8 and 20:4. Day-time sleep of only the $s^H L^H$ flies is significantly reduced compared to controls. In LD 4:20 and 8:16, night-time sleep of $s^H L^H$ is significantly lower than that of $s^- L^H$, in LD 16:8, both night time and day time sleep are significantly lower, whereas in LD 20:4, day-time sleep is significantly reduced from that of $s^- L^H$. Error bars represent SEM. *** $p < 0.0001$, ** $p < 0.001$ and * $p < 0.05$. † in right panel of LD 16:8 denotes that the night-time sleep of $s^H L^H$ is significantly lesser than those of all other flies.

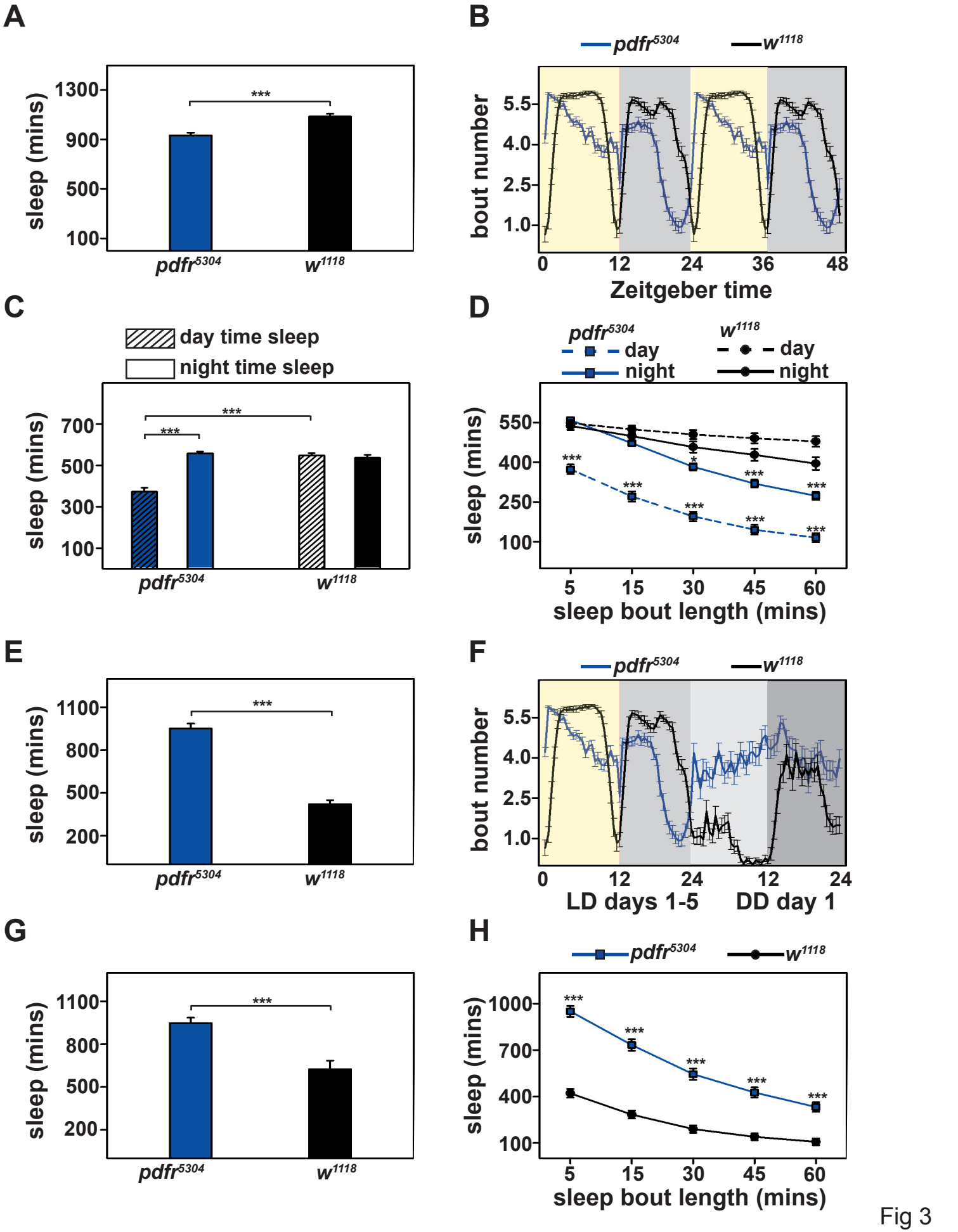
absence of functional s-LN_v but presence of normally firing l-LN_v, sleep levels are not disturbed suggesting that s-LN_v are not necessary for bringing about arousal *per se* in this environment.

In a very short day environment of 4 hr of light, night time sleep of $s^{\pm}$ $L^{\pm}$ flies was significantly higher than all the other genotypes (Fig 1, uppermost left panel, compare green line with black, grey and indigo lines, Fig 2, uppermost left panel, green solid bars with black, grey and indigo solid bars). Additionally, night time sleep of s^{H} L^{H} flies was significantly lower than s^{-} L^{H} flies (Fig 1, uppermost right panel, compare purple line with red line, Fig 2, uppermost right panel, purple solid bar with red solid bar) again suggesting a scenario where s-LN_v mediated arousal-promoting action of l-LN_v. Interestingly, the night time sleep duration of s^{-} L^{H} flies was comparable to that of s^{-} L^{+} flies (Fig 1, uppermost right panel, compare red line with blue line, Fig 2, uppermost right panel, red solid bar with blue solid bar), suggesting that absence of functional s-LN_v was completely able to suppress effect of hyper-excited l-LN_v. However, these results should be viewed with a caveat in mind in that the night time sleep duration of s^{H} L^{H} flies is similar to that of its s^{+} L^{+} controls (NCQ0, Fig 2, uppermost right panel, purple and grey solid bars). While this anomaly can be explained by taking into consideration the high amplitude evening peak of s^{+} L^{+} controls that occurs during the night (Chapter 2, Fig 3B), nevertheless careful analysis of sleep during 4 hr intervals, similar to what was done with percentage activity in the previous chapter (Chapter 2, Fig 3) is desired.

Similar results were obtained with sleep analysis on flies experiencing a long day environment of 16 hr of light with a few interesting exceptions. Both day time and night

time sleep duration of $s^{\pm}$ $L^{\pm}$ flies were significantly higher than that of s^{+} L^{+} control (*GAL4*) flies (Fig 1, bottom left panel, green and black lines, Fig 2, bottom left panel, green and black solid and hatched bars). Similarly, both day time and night time sleep of s^{H} L^{H} flies were significantly different from that of its s^{+} L^{+} controls (*NCQ0*, Fig 1, bottom right panel, purple and grey lines Fig 2, bottom right, purple and grey solid and hatched bars), as well as from that of the s^{-} L^{H} flies (Fig 1, bottom right panel, purple and red lines Fig 2, bottom right, purple and red solid and hatched bars). In fact, in this regime too, the day time and night time sleep of s^{-} L^{H} flies were similar to that of the s^{-} L^{+} flies (Fig 1, bottom right panel, red and blue lines Fig 2, bottom right, red and blue solid and hatched bars), suggesting that the hyper-excitation of l-LN_v had no effect on arousal due to the absence of functional s-LN_v. These results were replicated in a very long day environment, with changes in sleep being restricted to day time sleep only (Figs 1, 2, lowermost panels). In this case however, the day time sleep duration of s^{-} L^{H} flies was significantly lower than that of the s^{-} L^{+} flies, suggesting an incomplete suppression of effect of l-LN_v hyper-excitation caused by absence of functional s-LN_v. Taken together, these results suggest that normally firing l-LN_v are sufficient in modulating arousal in all photoperiods. Additionally, s-LN_v by themselves are not required to promote arousal; however, they are essential in regulating the effects of l-LN_v, even though in certain environments (for example, LD 12:12) the l-LN_v do not require the s-LN_v to bring about arousal.

Loss-of function mutation in pdf receptor has opposite effects on sleep in LD12:12 and DD. Since l-LN_v have been shown to exert its effect on arousal via PDF (Sheeba et al., 2008a; Chung et al., 2009) we sought to explore the putative target sites of PDF. In


A
sleep (mins)
1300
900
500
100
***
pdfr5304
w1118
B
pdfr5304
w1118
bout number
5.5
4.0
2.5
1.0
0
12
24
36
48
Zeitgeber time
C
day time sleep
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700
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550
400
250
100
5
15
30
45
60
sleep bout length (mins)
E
sleep (mins)
1100
800
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200
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pdfr5304
w1118
F
pdfr5304
w1118
bout number
5.5
4.0
2.5
1.0
0
12
24
12
24
LD days 1-5
DD day 1
G
sleep (mins)
1100
800
500
200
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pdfr5304
w1118
sleep (mins)
1000
700
400
100
5
15
30
45
60
sleep bout length (mins)

Fig 3

order to do so, we first characterized mutants for *pdfr* in terms of their sleep parameters in both LD 12:12 and DD, as PDF mediated arousal is light-dependent. We carried out this assay as a first step towards establishing a phenotype using which we could screen for *GAL4* lines as potential candidates of target sites of PDF by downregulating *pdfr* under their influence. *pdfr* mutants ($pdfr^{5304}$, $pdfr^{3369}$) in LD 12:12 do not show the characteristic bimodal activity/rest profile; their morning peak anticipation is impaired and evening peak is advanced in phase (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). Furthermore, the total sleep duration of *pdfr* mutant ($pdfr^{5304}$) in LD 12:12 is significantly lower than its wild type control (w^{1118}, Fig 3A). We also used another *pdfr* mutant line $pdfr^{3369}$ (Bloomington stock number 33069) and observed similar results (data not shown). When sleep bout number was plotted as a function of time of day, it was seen that bout numbers both during day and night was reduced in the $pdfr^{5304}$ flies (Fig 3B). Two way ANOVA revealed that sleep duration only during day time was significantly reduced in $pdfr^{5304}$ flies (Fig 3C, hatched blue and black bars). Additionally, day time and night time sleep of $pdfr^{5304}$ flies were significantly different from one another, with day time sleep being significantly reduced (Fig 3C, hatched and solid bars). Day time sleep was poorly consolidated in the $pdfr^{5304}$ flies when compared with the controls (Fig 3D, compare blue and black dashed lines). Interestingly, though

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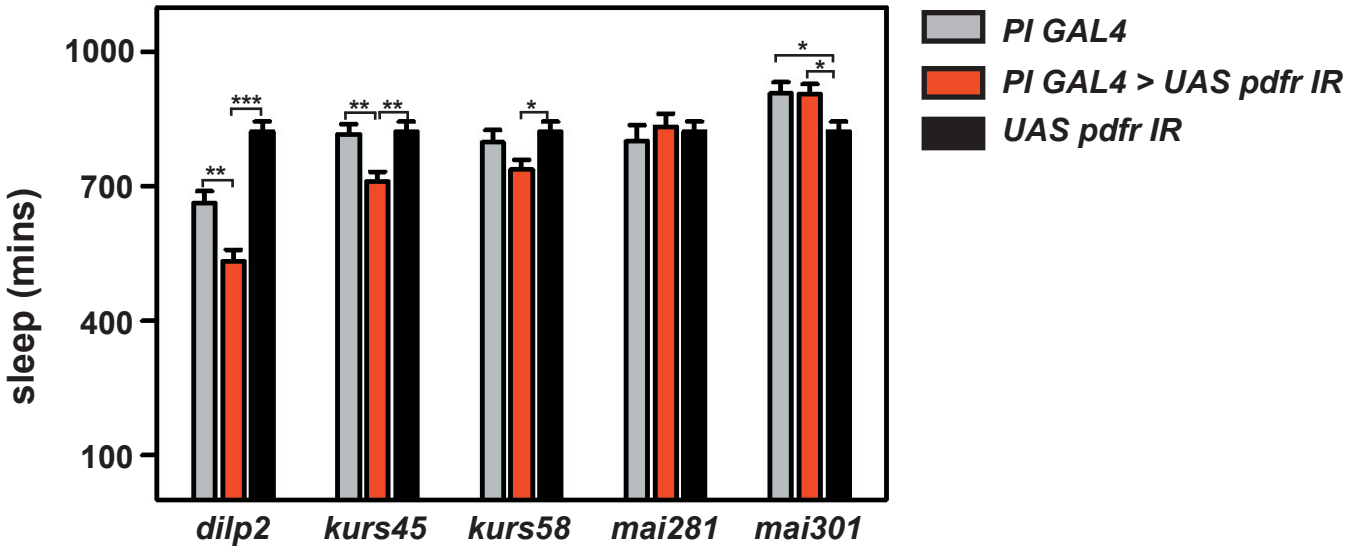
Figure 3. Null mutation in pdfr affects different aspects of sleep in LD 12:12 and DD. (A) Total sleep in LD 12:12 is significantly lowered in $pdfr^{5304}$ mutant flies when compared with wild type w^{1118} flies. (B) Half-hourly sleep profiles suggest that the reduction in overall sleep could be a result of reduction in both day-time and night-time sleep, however, only day-time sleep of mutant flies is significantly lower than that of wild type flies as shown in (C). (D) Mutant flies are unable to consolidate their sleep both during the day and night. (E) Total sleep on first day of DD is significantly higher in mutants as compared with the wild type flies. (F) Mutant flies tend to sleep more than the controls both during subjective day and subjective night and this trend is different from the reduced sleep seen in mutants in LD 12:12. (G) Total sleep averaged over 7 days in DD is also significantly higher in mutants when compared to wild type flies. (H) Consolidation of sleep during first day in DD is not affected by the mutation in *pdfr*; sleep of larger bout lengths is significantly higher in mutants than the wild type flies. All other details are as in fig 2.

A



B

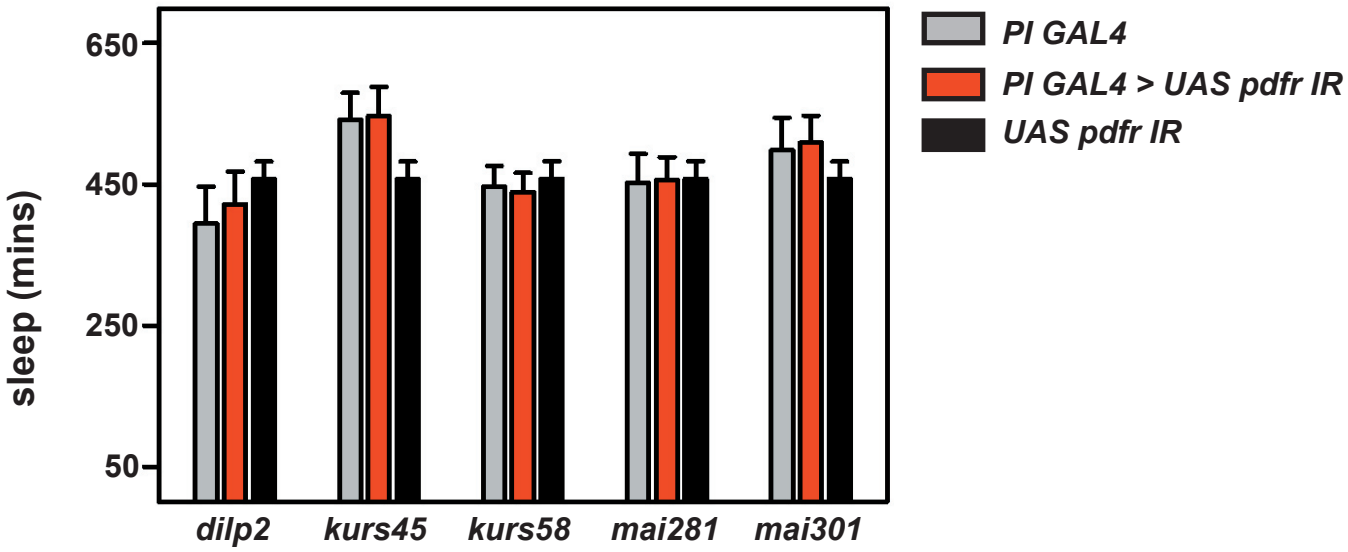


Figure 4. Effect of downregulating pdfr in subsets of PI neurons using different GAL4 drivers. (A) Dowregulating *pdfr* using *dilp2 GAL4* and *kurs45 GAL4* is effective in significantly reducing total sleep in LD 12:12, which is significantly different from both *GAL4* and *UAS* parental controls. (B) Downregulating *pdfr* using any of the five drivers is not effective in increasing overall sleep levels in DD, a phenotype which was observed in *pdfr* mutant flies in DD.

night time sleep duration in *pdfr*^{5304} flies was similar to that of wild type flies, it was fragmented in the mutants as sleep duration defined by bouts of longer time was significantly different from the respective wild type duration (Fig 3D, solid blue and black lines). When these flies were introduced to DD conditions, surprisingly, the trend was reversed. Total sleep duration on first day of DD was significantly increased in *pdfr*^{5304} flies as compared to the wild type controls (Fig 3E). This increment in sleep was found to be due to increase in both subjective day time and subjective night time sleep (Fig 3F, data not shown). These defects in sleep were not specific to the day in question – the enhancement of sleep duration persisted even after 7 days in DD (Fig 3G). Since the trend of sleep differences between *pdfr*^{5304} flies and wild type controls did not change even after changing the bout length of sleep, the *pdfr*^{5304} flies are not impaired in their ability to consolidate their sleep in DD conditions (Fig 3H). Altogether, these data suggest that PDF promotes day-time sleep in LD 12:12, whereas it promotes arousal in subjective night and day in DD conditions, thus modulating different aspects of the sleep/arousal circuit to control overall sleep levels.

Downregulating pdfr in a small subset of PI neurons decreases night time sleep in LD 12:12 while having no effect in DD. Because mutations in *pdfr* had opposite effects on the same behaviour in different environmental conditions, we propounded the hypothesis that PDF has different effects on different sets of downstream neurons. In order to test this hypothesis, we downregulated *pdfr* in different subsets of PI neurons, because this site started out as favourite in our view due to reasons enlisted before. We chose five PI *GAL4* driver lines, four of which had broad expression patterns even in areas outside the PI (data not shown, (Siegmund and Korge, 2001; Jaramillo et al., 2004)

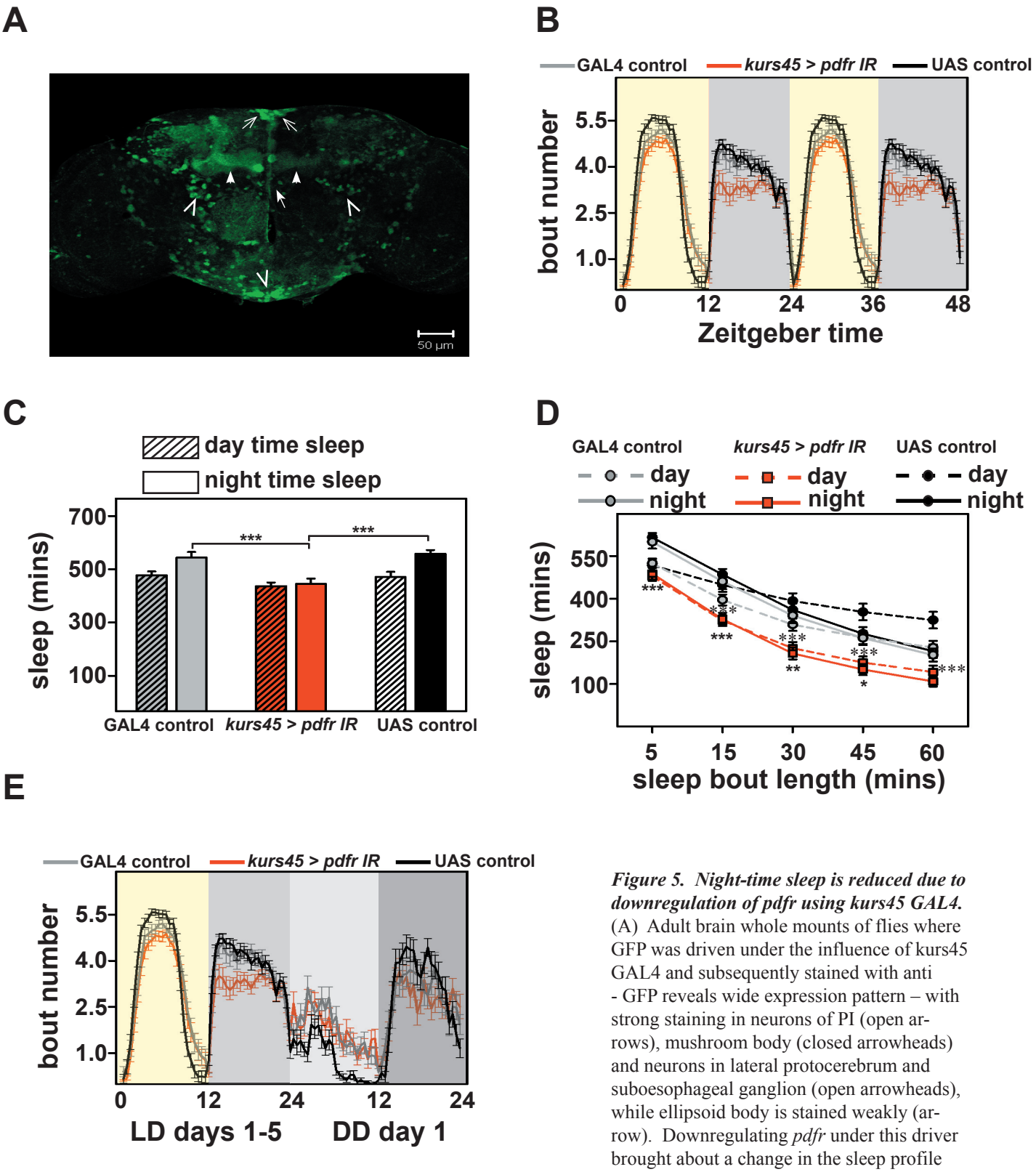


Figure 5. Night-time sleep is reduced due to downregulation of pdfr using kurs45 GAL4. (A) Adult brain whole mounts of flies where GFP was driven under the influence of kurs45 GAL4 and subsequently stained with anti - GFP reveals wide expression pattern – with strong staining in neurons of PI (open arrows), mushroom body (closed arrowheads) and neurons in lateral protocerebrum and suboesophageal ganglion (open arrowheads), while ellipsoid body is stained weakly (arrow). Downregulating *pdfr* under this driver brought about a change in the sleep profile (B), with night-time sleep being significantly lowered in experimental flies when compared with both parental controls (C). (D) Sleep was less consolidated during the day time for experimental flies, as sleep of bout length larger than five minutes was significantly different from both the parental controls. (E), No change was observed in sleep in first day of DD in experimental flies when compared with their parental controls.All other details are as in fig 3.

. Upon downregulation of *pdfr* in the PI and other areas, we assessed the total sleep duration of experimental flies and compared them with both their parental controls both in LD 12:12 and DD conditions. In LD 12:12, downregulation of *pdfr* using two *GAL4* lines – *dilp2 GAL4* and *kurs45 GAL4* showed changes in overall sleep that were significantly different from both the parental controls (Fig 4A). Downregulation of *pdfr* using both of these reduced total sleep duration significantly – a phenotype observed even in the $pdfr^{5304}$ flies. However, these effects did not persist in constant dark conditions, nor did it reverse the direction of change as was seen for $pdfr^{5304}$ flies (Fig 4B). In fact, none of the *GAL4* lines tested showed any significant change in sleep duration compared to parental controls. These results further strengthened our hypothesis that PDF has distinct effects on different areas and in the same areas under different environments.

We next set out to identify the neurons that are targeted by the *kurs45 GAL4* and *dilp2 GAL4* lines in order to identify what neurons are downstream of LN_v in the sleep/arousal circuit. GFP driven under the control of *kurs45 GAL4* driver and subsequent immunocytochemistry revealed that apart from expression in PI neurons, neurons in the mushroom body, ellipsoid body, lateral protocerebrum and suboesophageal ganglion (SOG) are also targeted by this driver line (Fig 5A). Downregulation of *pdfr* in all these neurons brought about a significant reduction in night time sleep duration (Fig 5B, Fig 5C) and even altered consolidation of both day time and night time sleep (Fig 5D). However, these effects did not persist in DD as mentioned before (Fig 5E). These trends were replicated when *pdfr* was downregulated under the control of the narrower *GAL4* driver which targets only a group of 15-16 PI

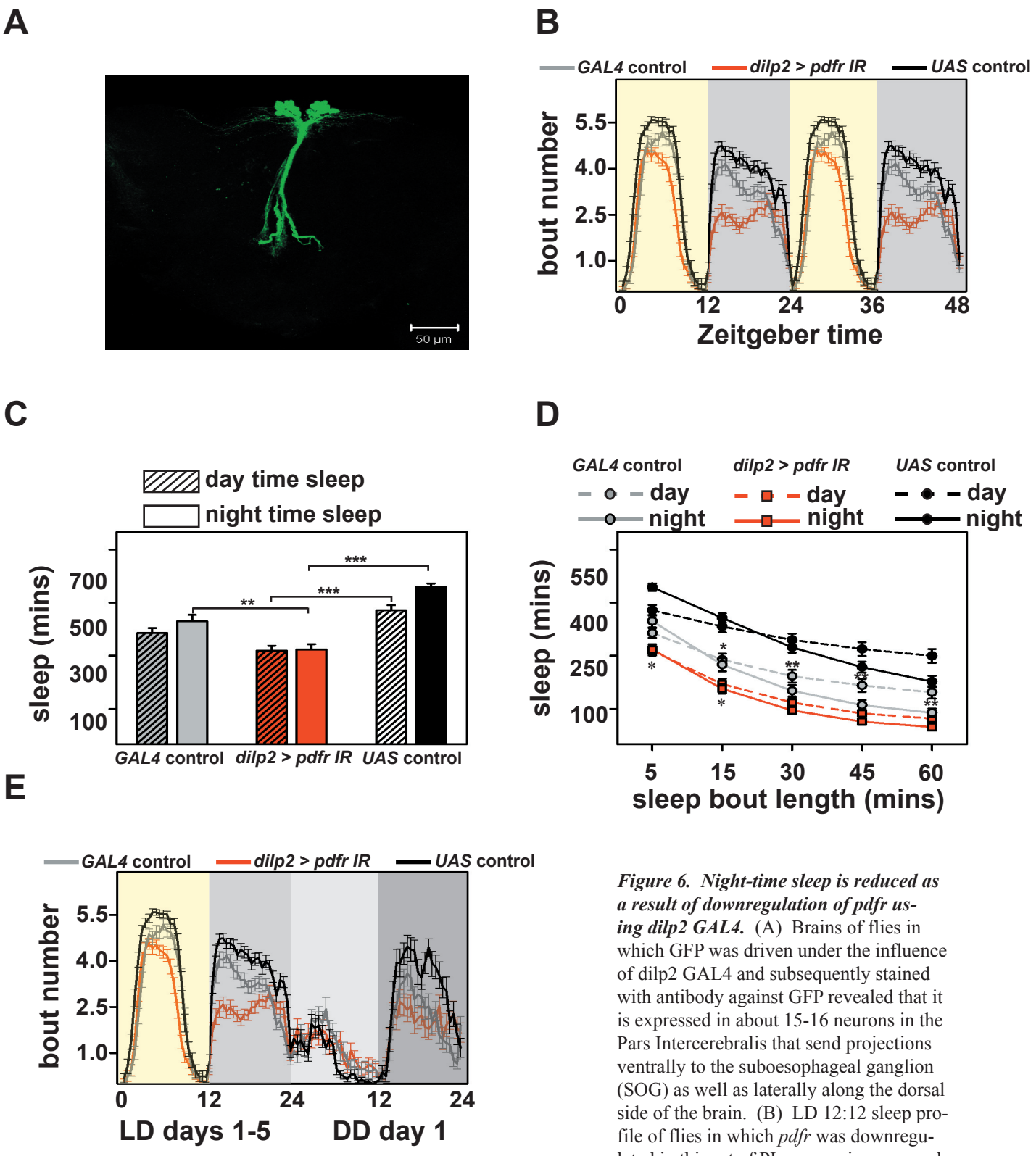


Figure 6. Night-time sleep is reduced as a result of downregulation of pdfr using dilp2 GAL4. (A) Brains of flies in which GFP was driven under the influence of dilp2 GAL4 and subsequently stained with antibody against GFP revealed that it is expressed in about 15-16 neurons in the Pars Intercerebralis that send projections ventrally to the suboesophageal ganglion (SOG) as well as laterally along the dorsal side of the brain. (B) LD 12:12 sleep profile of flies in which *pdfr* was downregulated in this set of PI neurons is compared with its *GAL4* and *UAS* parental controls. Both day-time and night-time sleep are reduced in the experimental flies, however only night-time sleep of experimental flies is significantly different from that of both the controls (C). (D) Day time sleep of bout length larger than five minutes of experimental flies are significantly different from both the controls, whereas in the case of night-time sleep, sleep of all bout lengths are significantly lower than both the controls. (E) Sleep profiles of all three genotypes in first day of DD reveal that sleep is not affected by downregulation of pdfr in this set of PI neurons. All other details are as in fig 3.

neurons that send projections towards the SOG (Fig 6A). The effect of *pdfr* knockdown in these cells alone reduced night time sleep duration (Fig 6B,C) and made day time sleep more fragmented as compared with its controls (Fig 6D), but did not affect sleep in DD (Fig 6E). Given that the overlap of expression between these two lines is only in the PI region, we can conclude that the sleep-promoting effects of PDF at night are mediated by neurons residing in the PI region.

D. Discussion

Previous studies had ruled out the involvement of s-LN_v in the influence of sleep/arousal circuit. We have discovered an auxiliary role for the s-LN_v in sleep/arousal circuit. We have shown that depending upon the day length in the environment, s-LN_v help in modulating the arousal-promoting effects of l-LN_v – during the night time in short days and long nights, and during the day time in long days and short nights. While it is clear that s-LN_v are not essential in bringing about arousal directly, they are required indirectly as they seem to regulate the functioning of l-LN_v. However, our results and others' (Shang et al., 2008; Sheeba et al., 2008a) have made it clear that while s-LN_v are not essential for bringing about arousal, yet they can have modulatory effects. Moreover, studying sleep parameters in different photoperiods allows for mimicking various environmental conditions and facilitates studies in the direction of seasonal affective disorders that are also associated with sleep disturbances.

So far, the l-LN_v have not been implicated in promoting sleep. They have always been shown to contribute towards promoting arousal. We have shown for the first time a role for the PDF^+ LNv neurons in modulating sleep. These conclusions are made on

the basis of the following results. Loss-of-function mutation in *pdfr* leads to decrease in day time sleep as well as its consolidation. This mutation also leads to decrease in night-time sleep consolidation. These results are consistent with previous studies that also showed sleep deficits in *pdfr* and *pdf* mutants (Chung et al., 2009). Downregulating *pdfr* in a subset of PI neurons leads to decrease in night-time sleep and day-time sleep consolidation. These results point toward a role for PDF^{+} neurons in promoting sleep. Taken together, our studies imply that just like in the clock circuit (Wulbeck et al., 2008; Yoshii et al., 2009) even in the arousal circuit, PDF has distinct and opposite effects on different receptive cells.

A previous study had downregulated *pdfr* in PDF^{+} neurons and had concluded that s-LN_v play the role of information mediator between l-LN_v and higher motor centres (Parisky et al., 2008). *pdfr* expression has been explored in a number of studies with differing results – *in situ* hybridization shows localization in PI and mushroom body apart from circadian neurons (Lear et al., 2005), antibodies against PDFR show expression in ellipsoid body (Parisky et al., 2008) and *pdfr GAL4* driven fluorescent proteins also light up in the optic lobes apart from the afore-mentioned areas (Im and Taghert, 2010). The *kurs45 GAL4* line that targets PI, mushroom body and ellipsoid body neurons apart from other neurons that have not been reported to express *pdfr*, which when used to downregulate *pdfr*, showed significant reduction in night time sleep. A more specific line that targets only some cells in the PI also phenocopied flies with downregulated *pdfr* under *kurs45 GAL4*. These results are suggestive of involvement of PI in modulating sleep promotion by PDF. Interestingly, the PI neurons that get targeted by *kurs45 GAL4* and *dilp2 GAL4* each belong to different subsets –

those driven by *kurs45 GAL4* belong to PI-3 neurons, whereas those targeted by *dilp2 GAL4* are a subset of PI-1 neurons (Siegmund and Korge, 2001). However, both these drivers bring about similar behavioural manifestations upon downregulation of *pdfr*. Moreover, other lines that target the same subsets do not show differences in sleep. For example, downregulation of *pdfr* using *mai301 GAL4* that drives expression in PI-1 neurons apart from many other neurons does not bring about any change in sleep in experimental flies (Fig 4). Similarly downregulation of *pdfr* using *mai281 GAL4* that targets PI-2 and PI-3 neurons does not change sleep levels of experimental flies (Fig 4). Thus these results suggest that the PI is a centre with heterogeneous groups of cells that may or may not respond to PDF signaling, or may not respond at all. Further neurogenetic dissection of this neuroendocrine site will yield interesting results that can help in connecting the sleep/arousal circuit in *Drosophila*.

Interestingly, the subset of PI neurons that we discovered as modulating sleep-promoting action of PDF also regulate arousal-promoting action of octopamine (Crocker et al., 2010) via cAMP signaling pathway. While it would be interesting to probe the downstream signaling pathways mediating PDF-associated sleep promotion with EGFR pathway as the frontrunner (Foltenyi et al., 2007), it is clear that this subset of 15-16 PI neurons function as an important downstream integrator and decision making centre that receives and integrates both sleep-promoting and sleep-inhibiting signals from various centres in order to display the correct behaviour given the environment. The close proximity of these neurons to the dorsal projection of s-LN_v and the presence of dorsal neurons of circadian clock circuit in its immediate vicinity suggest that these neurons may receive time of day information from the pacemaker

cells, further substantiating its role as an integrating centre. As the PI has also been implicated in sleep homeostasis (Crocker et al., 2010), this can even be a putative site that links the two limbs of the sleep circuit – hoemostatic and circadian networks.

Thus, in summary, we have shown that s-LN_v do have a small role to play in the arousal circuit. Additionally, we believe that these are involved in regulating l-LN_v action on arousal, though the l-LN_v seem to manage well even without functional s-LN_v. Further, we have shown a sleep-promoting role for PDF; and that it has diverse effects on different neurons depending upon the environment. Importantly, the PDF-mediated sleep promotion is carried out by a subset of PI neurons, which may function as an integrating centre of various conflicting signals to bring about the overt behaviour of sleep/wake cycles in *Drosophila*.

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