Interaction between circadian neurons and sleep homeostat to regulate sleep/wake cycles in *Drosophila melanogaster*

A Thesis

Submitted for the Degree of

Doctor of Philosophy

By

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To

Evolutionary and Organismal Biology Unit

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Declaration

I hereby declare that the thesis entitled "**Interaction between circadian neurons and sleep homeostat to regulate sleep/wake cycles in *Drosophila melanogaster***" submitted towards the fulfillment of the Ph.D degree 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.

Sheetal Potdar

Place : Bangalore

Date : 15th March, 2018

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CERTIFICATE

This is to certify that the work described in this thesis entitled "**Interaction between circadian neurons and sleep homeostat to regulate sleep/wake cycles in *Drosophila melanogaster***" is the result of studies carried out by Ms. Sheetal Potdar in the Behavioural Neurogenetics Laboratory, 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.

Prof. Sheeba Vasu

Associate Professor

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List of publications

1. **Potdar, S. and Sheeba, V.** (2013). Lessons From Sleeping Flies: Insights from *Drosophila melanogaster* on the Neuronal Circuitry and Importance of Sleep. ***J. Neurogenetics*.** (1-2): 23-42.
   A part of this article has been reused with permission in Chapter 1.

2. **Potdar, S., Daniel, D.K., Thomas, F.A., Lall, S. and Sheeba, V.** (2018). Sleep deprivation negatively impacts reproductive output in *Drosophila melanogaster*. ***J. Exp. Biol.*** Doi: 10.1242/jeb.174771.
   This article has been reused with permission in Chapter 4.
   Author contributions: Conceptualization, S.P. and V.S.; Methodology, S.P. and V.S.; Investigation, S.P., D.D. F.A.T. and S.L.; Writing – Original Draft, S.P. and V.S.; Writing – Review & Editing, S.P. and V.S; Funding Acquisition, V.S.; Resources, V.S.; Supervision, V.S.

3. **Potdar, S. and Sheeba, V.** Wakefulness is promoted during daytime by PDFR signalling to dopaminergic neurons in *Drosophila melanogaster*. ***eNeuro. In press.***

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Synopsis

Sleep is a period of quiescence that is widespread in the animal kingdom and while its quantity and quality may differ between different animals, certain characteristic features remain conserved across taxa. These include electrophysiological signatures such as reduced overall brain activity and muscle tone. Additionally, mammals and birds have characteristic waves of activity of specific frequencies which define different stages of sleep such as Rapid Eye Movement (REM) and non-REM sleep. However, in animals lacking complex nervous systems where such features may not be present and/or detection of such features maybe technically difficult, several behavioural features are used to characterize sleep. These include species-specific posture during sleep, preference for certain sites to sleep, reduced responsiveness to sensory cues and increased arousal thresholds. Most importantly, sleep is regulated by a sleep homeostat, which regulates how *much* and how *well* an animal sleeps, and by circadian clocks, which are internal time-keeping mechanisms that regulate the timing of sleep and wake behaviours. How the two processes overlap and interact in their regulation of sleep is a question that remains unanswered and I sought to address this question in my studies on sleep/wake cycles in fruit flies *Drosophila melanogaster* that form a part of this thesis. A small part of my thesis also addresses the question of significance of sleep by examining the relationship between sleep and reproductive output in female fruit flies.

The two-process model of sleep regulation was proposed almost thirty-five years ago to explain sleep regulation in rats, and was later used to explain sleep regulation in humans as well. It posits that sleep is regulated by a homeostatic process that builds up sleep pressure during wake and discharges during sleep; and a circadian

process which determines the timing of occurrence of sleep and wake. This model implicitly assumes an interaction between these two processes, and while several studies using mammalian model systems have tried to understand the nature of this interaction, a clear picture is yet to emerge.

Several features of sleep are recapitulated in *Drosophila* and therefore it serves as an excellent model system to study many behavioural, neuronal and molecular aspects of sleep/wake cycles. Almost two decades of research on *Drosophila* sleep has resulted in the uncovering of distinct neuronal circuits underlying sleep and wakefulness. Thus, higher centres of the *Drosophila* brain such as the central complex structures like the Ellipsoid Body (EB) and the dorsal Fan-shaped Body (dFB) are shown to be parts of the sleep homeostat; and circadian clock neurons such as the large ventral lateral neurons (l-LN_v) are shown to promote wakefulness. Yet, so far, none of the homeostatic structures have been shown to affect circadian clock properties, and neither have circadian neurons been shown to affect sleep homeostatic features, thereby suggesting independent regulation of sleep/wake cycles by homeostat and circadian clocks. Furthermore, whether the two-process model can be used to explain sleep regulation in simpler organisms such as invertebrates remains as yet untested. Thus, as a first step to address these questions, I examined the possibility and nature of interaction between the sleep homeostat and circadian clocks in *Drosophila melanogaster*. The two-process model and its assumptions and implications, neuronal circuits controlling sleep and wake in *Drosophila* and a review of studies conducted in *Drosophila* to address the significance of sleep are discussed in detail in the introductory chapter, Chapter 1.

In order to probe the nature of interaction between sleep homeostat and circadian clocks, my first approach was to examine if sleep homeostatic features can be

modulated by circadian clocks and if functioning of the sleep homeostat can affect circadian clock properties. In animals with large brains that enable recording neuronal activity through electroencephalograms (EEGs) while the animals are freely moving and sleeping, it is easy to obtain homeostatic markers such as delta power of slow wave sleep (a well-characterized sleep homeostatic feature). However, recording brain activity of flies requires a setup where the flies are tethered that may not allow them to freely move and sleep. Mapping the levels of other molecular markers such as NMDA receptors, Shaker and Sandman Potassium (K^+) channels that are shown to encode sleep pressure from freely moving and sleeping flies is difficult due to technical limitations. Thus, examining behavioural markers of the sleep homeostat is the best method available in order to investigate the homeostat-circadian clock interaction. I examined whether properties of one of the most fundamental features of the sleep homeostat – recovery or rebound sleep, varies across time of day. I found that depriving flies of sleep through mechanical perturbation at different time intervals, results in differential recovery of sleep both in terms of quantity and quality – sleep lost at certain time intervals is not recovered at all, whereas sleep lost at certain other time intervals is recovered only in terms of quality, while sleep lost during the middle of the night is recovered both quantitatively and qualitatively. Intriguingly, functioning of the sleep homeostat at different times of day does not change any core circadian clock properties such as clock period, robustness and phase. Taken together these results suggest that circadian clocks and sleep homeostat indeed interact and perhaps in a unidirectional manner. These experiments and their results are discussed in detail in Chapter 2.

My next approach was to probe sleep homeostatic roles for different subsets of circadian clock neurons. I found that activity of a subset of 3-5 dorsal lateral neurons (LN_d) represents a sleep deprived state as their activation alone results in sleep loss

which is recovered upon removal of the activation. Interestingly, functional circadian clocks within the LN_d are not required to modulate sleep recovery, thereby indicating that these neurons lie at the interface of clocks and sleep homeostat as they have independent functions in both the processes. The results of these experiments are presented in detail in Chapter 3.

My third and final approach was to examine if any of the known sleep homeostatic neurons are downstream of the wake-promoting subset of Pigment-dispersing-factor (PDF) expressing ventral lateral neurons (LN_v) of the circadian clock network. In a screen using the down-regulation and over-expression of the gene encoding PDF receptor (*pdfr*), I found that a subset of dopaminergic neurons respond to PDF to promote wakefulness during the day. Moreover, LN_v and dopaminergic neurons form synaptic contacts, and PDFR signaling to the dopaminergic neurons results in their inhibition during the day-time thereby promoting wake. I propose that these dopaminergic neurons that respond to PDFR signaling are sleep-promoting and that during the day when PDF levels are high they are inhibited to promote wake and during night when PDF levels are low their inhibition is reduced, thereby leading to sleep. Further, in a previous study it was found that dopaminergic neurons arborize to dFB, EB and the learning and memory centre, mushroom body, thus uncovering a unique circadian – homeostat pathway. The results of these experiments are detailed in Chapter 3.

While sleep research over several years has uncovered features unique to sleep or sleep-like states, the adaptive significance of this phenomenon remains unclear. Although reproductive deficits are associated with lifestyle induced sleep deficiencies, how sleep loss affects reproductive physiology is poorly understood, even in model organisms. I aimed to bridge this gap by impairing sleep in female fruit flies and

testing its effect on egg output. I found that sleep deprivation by feeding caffeine or by mechanical perturbation results in decreased egg output. Transient activation of wake-promoting dopaminergic neurons decreases egg output in addition to sleep levels, thus demonstrating a direct negative impact of sleep deficit on reproductive output. Similarly, loss-of-function mutation in dopamine transporter *fumin* (*fmn*) leads to both significant sleep loss and lowered fecundity. This demonstration of a direct relationship between sleep and reproductive fitness indicates a strong driving force for the evolution of sleep. Results pertaining to this section are detailed in Chapter 4.

In summary, my studies reveal important characteristics about the organization of homeostat and circadian clocks in regulation of sleep/wake cycles in *Drosophila melanogaster*. I find that the two-process model can be used to explain sleep regulation in *Drosophila*, as I demonstrate using three different approaches that the primary assumption of the model of interaction between the homeostatic and clock processes is met. Furthermore, by showing different amount and intensity of recovery sleep occurring as a result of sleep deprivation at different times of the day, I uncover presence of different stages of sleep in *Drosophila* through behavioural means, thereby building upon previous studies that have demonstrated these stages using electrophysiology and other behavioural features of sleep homeostat. Moreover, my studies also reveal two independent pathways, one involving the circadian neurons LN_d as putative sensors of sleep need, and another involving PDF^+ LN_v to dopaminergic neurons as regulators of day-time wakefulness. Additionally, I find that sleep deprivation by different methods decreases reproductive fitness in female fruit flies. The role of sleep in maintaining brain homeostasis has been extensively examined with the prevailing idea being that sleep has evolved to serve restorative functions within the nervous system. My results connecting sleep to reproductive health underscores a

broader restorative function for sleep encompassing other systems and thereby supporting the idea that perhaps sleep may have evolved to serve functions not pertaining to the brain exclusively, unraveling the potential to discover sleep-like states in organisms lacking organized nervous systems.

Chapter 1

Regulation and significance of sleep

1.1.What is sleep?

Organisms perform many behaviours that are essential for their survival, among which sleep is perhaps the most paradoxical one. This is because, sleep which is a behaviour that is characterized by reduced activity and reduced responsiveness to the sensory world, results in increased risk of predation and reduction in time available for evidently more beneficial activities such as foraging, feeding, courting and mating. Yet, it is clear that sleep exists to serve vital functions because till date, no animal has been found that either can do without sleep or manage to not accrue negative effects of sleep loss (Cirelli and Tononi, 2008). While questions have been raised about such a simplified reasoning for assigning functional significance to sleep (Eban-Rothschild et al., 2016), one cannot possibly argue against the logic of Allan Rechtschaffen's rather spectacular statement – "If sleep does not serve an absolutely vital function, it is the biggest mistake evolutionary process ever made." (Rechtschaffen, 1971).

Sleep is so essential that several organisms have evolved ways to sleep "on the job". Dolphins and whales have the ability to sleep uni-hemispherically, such that one half of their brain is asleep and displaying slow wave brain activity characteristic of sleep, while the other half displays electrical activity associated with wakefulness (Cirelli and Tononi, 2008; Lyamin et al., 2008). Nevertheless it is important to note that in such animals Rapid Eye Movement (REM, discussed later) sleep stage does not occur, and therefore certain stages of sleep may require whole brain participation (Mascetti, 2016). Some birds such as male pectoral sandpipers display "microsleeps" throughout the day especially during the fertile phase of the females, so that they can compete for longer durations (Lesku et al., 2012). Ducks typically line up in rows such that the ones in the middle shut both eyes while the "sentinel" birds at the end sleep uni-hemispherically and keep an eye out for predators in a literal sense (Rattenborg et al.,

1999). Other examples including cows chewing their cud while displaying a non-Rapid Eye Movement (NREM, discussed later) stage of sleep (Ruckebusch, 1972), great frigate birds sleeping while flying (Rattenborg et al., 2016) and ostriches sitting in a vigilant pose during NREM stage of sleep (Lesku et al., 2011) point toward the indispensable facet of sleep.

Sleep in its broadest form can be defined as physical quiescence accompanied by reduced responsiveness to the sensory world (Campbell and Tobler, 1984). However, sleep is different from mere rest or simply the opposite of activity, as sleeping individuals display elevated arousal threshold, viz. a higher intensity of stimulus is required to elicit a response from a sleeping individual as compared to when the same individual is awake (Hendricks et al., 2000b). It is characterized by additional behavioural attributes such as immobility, maintenance of a typical posture, exhibiting a preference for the site of sleep, and accompanied by stereotypical behaviours like yawning and so on (Campbell and Tobler, 1984). These behavioural criteria have been defined since 1913 (Pieron, 1913) and has aided in the behavioural characterization of sleep in several invertebrates such as octopus (Lafont, 1870) and insects (Fiebrig, 1912; Rau, 1916). The opposite of sleep, arousal or wakefulness, makes animals more active, heightens their awareness of the surroundings and increases their ability to respond to various sensory cues (van Swinderen and Andretic, 2003). These two seemingly opposite processes are timed to perfection by an internal timekeeping mechanism that allows organisms to choose their temporal niches depending upon their daily needs (Ascoff, 1966).

The internal timing systems, or circadian (Latin, ‘circa’ – about, ‘diem’ – day) clocks, apart from keeping time using cues from the cycling environment, also function as natural alarm clocks, by ensuring that animals wake and sleep at the correct time of

day (Franken and Dijk, 2009). Moreover, if the animal is deprived of a certain fraction of its normal duration of sleep, it is found to compensate for the sleep loss by sleeping longer, thereby displaying homeostatic control. If sleep deprivation is intense, then the animal is found to fall asleep even at a time when it is normally active. This phenomenon of making-up for lost sleep – sleep rebound is a very important feature in the behavioural characterization of sleep. Certain physiological signatures also characterize sleep, most important of which are the electrophysiological correlates. In most mammals and birds, electrical activity of the entire brain as measured by electroencephalograms (EEGs), cycles between fast active firing during waking and Rapid Eye Movement (REM) sleep which as its name suggests is also accompanied by rapid jerky movements of the eye. Slow waves and spindles during deep sleep, or Non-Rapid Eye Movement is yet another sleep stage (NREM; (Steriade et al., 2005)). In fact, NREM sleep is often divided into four more stages based on proportion of Slow Wave Activity (SWA) i.e. EEG power in the range of 0.5-4.5 Hz and spindles, i.e. EEG oscillations in the range of 12-14 Hz – typically, in NREM stages 3 and 4, occurrence of SWA is at its highest (Dijk et al., 1993; Rechtschaffen, 1968), and thus sleep during these stages is termed Slow Wave Sleep (SWS). Apart from the electrophysiological properties, other physiological attributes of sleep include a reduction in core body temperature and slowing down of metabolic processes during NREM and instabilities in heart beat rate and breathing rate during REM sleep (Iber, 2007).

1.2.Models of sleep/wake regulation

The question of how sleep is regulated has been a long-standing one. Ancient Greek physician Hippocrates thought sleep is induced when blood retreated into the inner reaches of the body, while Aristotle believed that waking warmed up the brain through warm vapors rising by digestion, and condensation of these vapors caused the

brain to cool down which would result in blood flow into the heart to induce sleep (Hammond, 1902). While Aristotle was obviously wrong in thinking about the heart's role in sleep induction, his basic view of a warmed up or "switched-ON" brain during wakefulness and a general shutting OFF of the brain during sleep was a popular opinion even in the late 19th century, according to William C. Dement – the famous American physician considered as the father of sleep medicine (Dement, 1998). Perhaps because of the Austrian psychoanalyst Sigmund Freud's obsession with explaining the causation of dreams with innate, instinctive and suppressed behaviours, sleep research especially in human patients was focused more on dreams rather than viewing it as an independent physiological state during the early 1900s. Nevertheless, with the advent of recording methods such as EEGs (described first in humans by Hans Berger in 1929) and interest among physicians to conduct sleep, EEG, electrooculogram (EOG) and electromyogram (EMG) recordings all through the night (also called polysomnography), the formal description and characterization of sleep as a physiological process commenced. Even though two of the earliest pioneers of sleep research, Eugene Aserinsky and Nathaniel Kleitman, began their studies with the intention of finding a neurological correlate for dreaming – which they did (Aserinsky and Kleitman, 1953; Dement and Kleitman, 1957), Dement's interest in REM sleep soon facilitated sleep research in becoming independent of dream research (Dement, 1998).

REM and NREM sleep typically alternate 3-4 times throughout the night and occur in approximately 80-120 minute intervals. Thus, sleep can be considered to consist of an ultradian rhythm of alternating REM and NREM cycles. To explain the mechanistic details of this rhythm, a model based on neurophysiological signatures obtained empirically was proposed whose basic principle is similar to the Lotka-

Volterra model, such that alternation between REM and NREM sleep was predicted to occur by reciprocal interaction between self-inhibitory and self-excitatory cell populations in the brain stem (Hobson, 1986; Massaquoi and McCarley, 1992; McCarley and Hobson, 1975). This model represented an attempt to understand the composition of sleep, and the idea of reciprocal interaction has also been somewhat extended to sleep/wake regulation by reciprocal inhibition and disinhibition of ascending arousal systems and the sleep-promoting ventrolateral preoptic nucleus (VLPO) to promote wakefulness and sleep, that gave rise to the classical flip-flop model (Saper et al., 2005).

While the EEG characteristics of sleep and their anatomical origins were of interest to certain researchers, some researchers were intrigued by how qualitative features of sleep could emerge from these EEG signatures. In fact, sleep intensity was correlated with proportion of slow waves in brain activity way back in 1937 (Blake and Gerard, 1937). Furthermore, reduced responsiveness to stimuli was observed in subjects with the onset of SWS (Loomis A.L., 1937). Moreover, arousal thresholds for pain and acoustic stimuli were higher during SWS than during stage 2 NREM sleep (Goodenough et al., 1965). Importantly, after sleep deprivation, amount of SWS increased during sleep recovery (Moses et al., 1975; Nakazawa et al., 1978; Webb and Agnew, 1971) and upon prolonged sleep during night or due to day-time napping SWS was reduced (Feinberg et al., 1980; Karacan et al., 1970). These results indicate a homeostatic process at play – i.e. a mechanism which regulates maintenance of appropriate levels of sleep.

On the other hand, several chronobiologists (chronobiology – study of biological rhythms) were interested in modeling sleep/wake regulation as a rhythmic behaviour since many studies had shown that sleep drive is a rhythmic process. For

instance, in a 3 day sleep deprivation study, it was found that self-reported fatigue in subjects shows a circadian rhythm (Åkerstedt, 1977). Jim Enright fashioned a simplistic single-oscillator model that he proposed could arise from a population of loosely coupled individually weakly rhythmic neurons, whose synchronous activity corresponded to the active phase of the animal, and trough of the oscillation brought about sleep (Enright, 1980). Often, core body temperature rhythms were used as "hands of the clock", in other words, as a marker of the phase of the internal clock, and it was shown to get desynchronized with sleep/wake cycles, such that each of these rhythms would run with their own period where external cycles could not entrain (or synchronize with a stable phase relationship) the circadian oscillator (Aschoff et al., 1967). Thus, it was hypothesized that sleep/wake cycles are due to the combined action of two oscillators – a temperature rhythm-controlling and a sleep/wake-controlling one (Wever, 1975). This two-oscillator model was further developed as consisting of van der Pol oscillators, and sleep was predicted to occur during two-thirds of the cycle when the phase of one of the two oscillators is below the average phase (Kronauer et al., 1982). While these circadian oscillator models were rich in their description of circadian clock properties and functions of entrainment, free-running behaviour and precision, these models were inadequate to explain sleep/wake regulation since they lacked an explanation for homeostatic control of sleep.

It was Alexander Borbély who brought the two processes of homeostasis and circadian clocks together in a coherent model to explain sleep/wake regulation initially using data from rats (Borbély, 1982), and with formalizations, a quantitative model was put forth by Serge Daan, Domien Beersma and Borbély to explain features of sleep/wake regulation in humans (Daan et al., 1984). In the original model, Borbély proposed that two processes regulate sleep propensity, i.e. the drive to sleep – a

homeostatic process S which is wake-dependent, and a wake-independent circadian process C. The fundamental idea of the model is that as the animal remains awake; the propensity to sleep regulated by the wake-dependent process S begins to rise. With sleep onset and maintenance of sleep through the night, sleep propensity decreases (Figure 1.1A, red curve). The parameter values regarding process S were obtained from SWS characteristics. Thus, wake-dependent component of sleep propensity is thought to asymptotically rise with wakefulness, and exponentially fall with occurrence of sleep. Moreover, the rising phase is much longer than the falling phase (Figure 1.1A). Process C is regulated by the circadian oscillator which refers to the inherent rhythm in sleep propensity that does not depend upon the occurrence of sleep (wake-independent sleep propensity, Figure 1.1A, blue curve). Thus, net sleep propensity is thought to be a difference between its levels due to S and C, i.e. wake-dependent and wake-independent components. On the basis of rhythms in vigilance and core body temperature, circadian oscillation of sleep propensity was modeled to be a sine wave with its peak in the middle of the day and trough in the middle of the night. Thus, even though wake-independent sleep propensity due to C was high in the afternoon, sleep is not initiated because wake-dependent sleep propensity is low. During night, just before the onset of sleep, sleep propensity due to S is high, and that due to C is at an intermediate level, such that the difference between S and C is high and sleep occurs at the highest point of difference (Figure 1.1A, solid black line). Next morning, just before wake, sleep propensity due to S is low and due to C is also low and thus sleep gets terminated and wake occurs. Given the assumption of two processes regulating sleep and wake, this model was quite unimaginatively called the two-process model of sleep/wake regulation (Borbély, 1982).

Figure 1.1

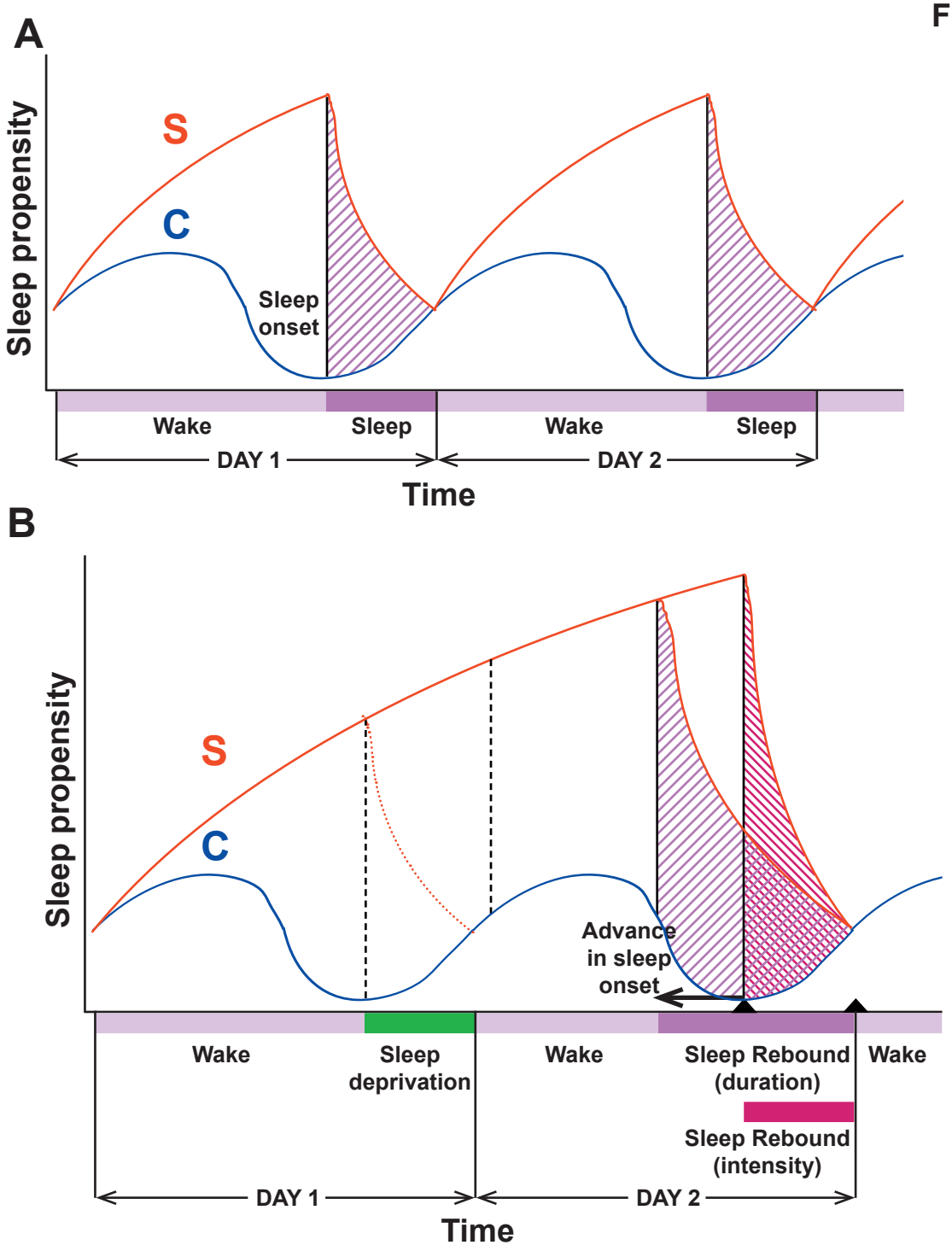


Figure 1.1. Schematic representation of the two-process model. (A) Process S - the sleep homeostat, regulates wake-dependent sleep propensity, i,e. this propensity to sleep builds up with time spent awake and is discharged as sleep occurs (red curve). Circadian process C regulates daily cycling of wake-independent sleep propensity (blue curve). According to the two-process model, the two processes interact such that sleep (purple shaded bar) begins when the difference between the values of sleep propensities brought about by process S and C is maximum (solid vertical black line), also known as the preferred bed-time of the animal. This ensuing sleep duration is represented by purple hatched lines. Wake (light purple shaded bar) is said to begin when this difference reaches a minimum value. (B) During sleep deprivation (green bar), the wake-dependent sleep propensity continues to rise (red solid curve), instead of discharging (red dotted curve). Wake state is maintained due to process C-regulated sleep propensity being in the rising phase, such that difference between sleep propensities brought about by S and C is not as much to initiate sleep (DAY 2, dashed vertical black line). With the process C-regulated sleep propensity entering the descending phase, sleep can begin, and in this case, lost sleep in terms of duration is recovered (purple shaded bar, purple hatched lines) as sleep onset is advanced (black arrow). If sleep onset and wake onset (black triangles) occur at expected times of the day, then more intense sleep occurs since process S-regulated sleep propensity must be discharged from a higher value, and hence at a faster rate (dark pink shaded bar, hatched lines).

The two-process model in its simplest form could explain different phenomena observed during sleep. For instance, under sleep deprivation, process S-driven sleep propensity was thought to continue to increase (Figure 1.1B), as a result of which sleep could get induced even during the day when animals should be typically awake. An interesting prediction emerged due to the nature of circadian oscillation of sleep propensity. During the early part of the day, the process C-driven oscillation is in the rising phase, while in the later part of the day, it is in the descending phase. Thus, even though process S-driven sleep propensity is high, the difference between S and C driven sleep propensity is higher during the later part of the day, because of which recovery sleep is more likely to occur during the later part of the day (Figure 1.1B). Yet another prediction owing to the exponential nature of decay of sleep propensity is that duration of recovery sleep is similar for sleep deprivation of different durations. What is expected to change is the recovery sleep intensity, as the level of process S-driven sleep propensity would be higher with longer sleep deprivation durations (Figure 1.1B).

The two-process model, while adequate in many ways, was modified several times in order to explain different aspects of sleep characteristics and patterns in novel experimental conditions (Borbély and Achermann, 1992; Borbély and Achermann, 1999). One important feature that Borbély's 1982 model could not explain was the internal desynchronization often seen in non-entrained rhythms of humans resulting in phase jumps and circabidian (~48 h rhythm) sleep patterns (Czeisler et al., 1980; Wever, 1979). A modified version of the two-process model which was a culmination of Daan and Beersma's circadian gating model (Daan and Beersma, 1984) and Borberly's 1982 model was put forth. The basic principle of this model was similar to Borbély's original model with the difference being that two circadian oscillations were thought to oscillate in parallel to determine sleep onset and sleep termination times.

The sleep propensity determined by process S oscillated between these two cycles, and its amplitude, interval and period could be varied. Under low amplitudes, this modification allowed the replication of circabidian sleep patterns and when noise was added to the threshold oscillations, phase jumps could be replicated (Daan et al., 1984). An important difference in the combined model, also called the "somnostat" model for its similarities with a thermostat model for temperature regulation, is that the threshold oscillations are left-skewed sine waves. Because such left-skewed waves were unlikely to be found physiologically, Arcady Putilov equated the thermostat analogy to the somnostat model and in his model, the threshold cycles were now 24 h sine waves, which led to an interesting prediction that the interaction between process S and C is continuous rather than occurring only at discrete intervals at the thresholds (Putilov, 1995). Yet another modification led to what is called the three-process model, which included the parameter sleep inertia (W) to account for sleepiness changes during shiftwork (Folkard and Åkerstedt, 1992). Other modifications concerning REM-NREM cycles within sleep, REM sleep homeostasis, and brain regional differences in EEG power spectra have also been discussed in detail (Borbély and Achermann, 1992; Borbély and Achermann, 1999; Borbély et al., 2016).

1.3.Empirical evidence for two-process model of sleep/wake regulation in mammalian sleep

One of the main assumptions of two-process model is that circadian oscillation of sleep thresholds and wake-dependent sleep propensity are independently regulated. Indeed, it has been found that lesions in the Suprachiasmatic Nucleus (SCN), a set of ~20,000 neurons in the hypothalamus identified as the site of circadian clocks (Moore and Eichler, 1972; Stephan and Zucker, 1972), do not affect recovery sleep (Mistlberger et al., 1983; Tobler et al., 1983). Moreover, sleep deprivation also does not affect clock

phase or free-running period (Borbély et al., 1982). Furthermore, it was possible to manipulate core body temperature and melatonin rhythms without affecting sleep intensity as measured by SWA (Dijk et al., 1989; Dijk et al., 1987b). These results show that circadian and sleep homeostatic mechanisms originate independently and are regulated independent of each other. However, sleep propensity is assumed to be regulated by both processes S and C, and according to later modifications of the model, it is brought about through the interaction of S and C (Borbély and Achermann, 1999). In fact, in one of the original papers of the two-process model, Daan and colleagues have speculated the modulation of circadian process by the homeostat, as sleep/wake cycles would influence active light-selecting behaviours (Daan et al., 1984). This expectation now has ample empirical evidence in mammals – nocturnal rats and mice as well as diurnal Sudanian grass rats show altered phase-shifts in response to light pulses (Challet et al., 2001; Jha et al., 2017; Mistlberger et al., 1997; van Diepen et al., 2014). On the other hand, there seems to be some evidence to suggest that circadian phase may alter SWA after prolonged waking (Deboer et al., 2007). Moreover, many studies have shown that several mutations in core clock genes show defects in REM and NREM EEG (reviewed in (Franken and Dijk, 2009)). Furthermore SCN electrical activity has been shown to be affected by NREM sleep deprivation – it increases during NREM deprivation and decreases during NREM recovery (Deboer et al., 2007; Deboer et al., 2003). These kinds of interactions and mutual influences of S and C are discussed in detail elsewhere (Borbély et al., 2016).

Most of the sleep homeostat related predictions of the model have received considerable attention in the mammalian sleep field. The model predicts that wake-dependent sleep propensity increases with waking. SWA, one of the main markers of process S both for deriving parameter values to use in simulations of the model, as well

as for gaining insights in terms of estimating variables, has been found to increase during waking, with the observation that day-time naps following increasing times spent awake consist of increasing proportion of SWA (Dijk et al., 1987a). SWA and SWS was greater during afternoon naps as compared to morning naps (Knowles et al., 1990). Other predictions of the model including effect of increased day-time sleep on night-time sleep, effect of repeated partial sleep deprivation on SWA etc. and the empirical data for the same are discussed in detail elsewhere (Borbély and Achermann, 1992). An interesting prediction, as mentioned before, pertains to the recovery sleep intensity and duration. Borbély very eloquently argued that sleep intensity, and not duration is recovered after sleep deprivation. The findings that almost 100 hours of sleep deprivation in one case and a spectacular case of ~260 hours of sleep deprivation recovering only about 10-15 hours of sleep in both cases can be explained by this prediction of the model (Blake and Gerard, 1937; Gulevich et al., 1966).

1.4.*Drosophila* as a model system for studying sleep

Much of what we know about mechanisms underlying sleep is as a result of studies on model organisms – both vertebrate (Siegel, 2005) and invertebrate (Miyazaki et al., 2017; Zimmerman et al., 2008a). Vertebrate model systems such as mice and rats have always been attractive as they are closer to humans in terms of their phylogenetic relationship (Hedges, 2002). The fruit fly *Drosophila melanogaster* is a favorite model organism for the neurogenetic dissection of many behaviours as its minimal genetic redundancy enables researchers to target candidate genes effectively and obtain clear, strong phenotypes (Sokolowski, 2001; St.Johnston, 2002). Moreover, tremendous potential exists in the usage of modern genetic tools and techniques that give an exceptional control over spatio-temporal expression of certain genes (Venken et al., 2011). Thus, when two seminal papers by the Sehgal and Tononi groups in the year

2000 demonstrated that rest in flies resembles sleep in mammals, it heralded a new chapter in sleep research (Hendricks et al., 2000a; Shaw et al., 2000). Indeed, both papers using different techniques were able to demonstrate in flies, all the behavioural characteristics that are attributable to sleep – physical inactivity, specific posture associated with sleep, site preference for sleep, elevated arousal threshold, regulation via both circadian clock and homeostatic processes (Hendricks et al., 2000a; Shaw et al., 2000). Additionally, fly sleep was responsive to sleep-inducing and sleep-inhibiting agents in much the same way as mammalian sleep was (Hendricks et al., 2000a; Shaw et al., 2000). Just like in mammals, flies also sleep more as young adults (Kayser et al., 2014; Koh et al., 2006; Shaw et al., 2000). Moreover, the finding that frequency of oscillations of EEG-like Local Field Potentials (LFPs) in flies differs depending upon whether they were asleep or awake (Nitz et al., 2002; van Swinderen et al., 2004) established *D. melanogaster* as a potent model organism to study sleep.

The utility of *Drosophila* in studying sleep (Donelson et al., 2012; Gilestro, 2012; Huber et al., 2004) and the simplicity with which it can be quantified (Andretic and Shaw, 2005; Huber et al., 2004) have yielded mutants that show certain sleep defects and initial studies have favored the forward genetic approach. In flies, sleep is defined as a period of immobility that lasts for longer than five minutes (Hendricks et al., 2000a; Shaw et al., 2000). This definition was arrived at based on extensive studies, which showed that disruption of such sleep requires a higher arousal threshold, thus distinguishing it from mere inactivity (reviewed in (Shaw, 2003)) and is now the accepted definition for estimating fly sleep using the standard *Drosophila* Activity Monitor (DAM) devised by Trikinetics Incorporated (Waltham, MA, USA). There are video recording-based methods which also complement DAM recording system and in fact are considered better for studying sleep as several other features of sleep such as

site-specificity, inactive rest and micro-movements can be captured (Faville et al., 2015; Zimmerman et al., 2008b).

The fact that no single gene mutation has been uncovered in any organism which completely abolishes sleep suggests that sleep is a complex behaviour influenced by many different genes (Crocker and Sehgal, 2010). Typically, sleep is intrinsically highly variable even within a somewhat genetically homogenous population and can be easily modified by subtle environmental variations (Parisky et al., 2016; Zimmerman et al., 2012), as a result of which researchers enforce stringent criteria in screening for sleep mutant phenotypes, such as sleep levels falling outside of two standard deviations away from the population mean (Cirelli, 2009). Most researchers now use a standard protocol of back-crossing sleep mutants to genetically homogeneous *Iso*31 flies in order to study mutant flies in similar genetic backgrounds. Many excellent reviews have discussed the genetic control of sleep and mutations which give rise to sleep disorders (Cirelli, 2009; Harbison et al., 2009; Sehgal and Mignot, 2011; Shaw and Franken, 2003; Tomita et al., 2017). Here I review the complexity of neuronal circuitry underlying *Drosophila* sleep and the insights on the most intriguing question in sleep research - why do we sleep?

1.4.1. Neuronal circuitry underlying sleep in *Drosophila*

1.4.1.1.Mechanisms of sleep homeostatic control

Several important discoveries regarding homeostatic control of sleep have been made in mammalian model systems because of the relative ease with which EEGs can be used to obtain a reliable homeostatic marker in the form of SWA. Moreover, apart from SWA, the accumulation of adenosine as a result of breakdown of Adenosine Tri-Phosphate (ATP) during waking, and its clearance during sleep has also been

considered a very good indicator of mammalian sleep homeostatic process (Porkka-Heiskanen et al., 2002; Porkka-Heiskanen et al., 1997). Nonetheless, the technical challenge presented by the tiny size of *Drosophila* has been overcome in part by tethering flies, yet allowing them free movement on a suspended foam ball, and simultaneously recording LFPs from whole brain. This kind of a setup, while hard to achieve, has yielded tremendous insights into the electrophysiological sleep structure of *Drosophila*. Indeed, it was shown that the power of brain activity in the 11-80 Hz range was reduced during sleep, and importantly was even further reduced during night-time sleep (van Alphen et al., 2013) showing that night-time sleep is deeper than day-time sleep. Yet another study from the same group showed that flies with alleles of the same gene show different electrophysiological sleep signatures (Faville et al., 2015). Importantly, lower power of the LFPs was associated with higher arousal thresholds and reduced number of brief awakenings (i.e. activity interspersed within two inactive counts), both of which are behavioural markers of sleep homeostat (van Alphen et al., 2013). Moreover, 7-10 Hz oscillatory waves were observed in LFPs during sleep, but not during wake; at the beginning of the sleep bout, such waves were found to be higher in proportion than during the middle of a sleep bout suggesting occurrence of different sleep stages (Yap et al., 2017). Indeed, it was also shown that sleep recovery intensity as measured by responses to thermal stimuli, number of brief awakenings and sleep continuity scores was dependent upon prior time spent waking (Huber et al., 2004). Another study showed that a nicotinic acetylcholine receptor subunit (encoded by *redeye* gene) showed sleep drive-dependent expression (Shi et al., 2014) suggesting a molecular marker of sleep homeostasis. Thus, these results show that sleep in flies is also under homeostatic control in a similar way as in mammals (Allada et al., 2017).

In mammals, EEG signatures are a combined effect of oscillatory electrical waves of activity occurring in the neurons of thalamus and cortex (Pace-Schott and Hobson, 2002). Thus it is not surprising that two neuropil regions that are considered the higher centres of the fly brain emerge as the sites of fly sleep homeostat. Central complex (CC), which consists of protocerebral bridge, fan-shaped body (FB), ellipsoid body (EB) and noduli (NO) are the higher centers regulating behaviours such as walking (Martin et al., 1999), flying (Ilius et al., 1994) and the processing of visual cues (Pan et al., 2009) and dorsal FB (Donlea et al., 2014) and EB (Liu et al., 2016) have been shown to have sleep homeostatic functions.

When a thermogenetic screen using heat-activated transient hyper-excitation by TRPA1 (Transient Receptor Potential, (Hamada et al., 2008)) was conducted to elucidate regions that induce sleep, it was found that *ExFl2* neurons that project to dorsal FB (dFB, Figure 1.2A) are sleep-promoting (Donlea et al., 2011). Strikingly, it was found that rise in sleep pressure through sleep deprivation causes an increase in the membrane excitability of these neurons projecting to the dFB, and after a period of recovery, membrane excitability returned to baseline levels (Donlea et al., 2014). Furthermore, wake-promoting effects of dopamine (Andretic et al., 2005; Kume et al., 2005; Lebestky et al., 2009) are mediated by inhibiting dFB neurons – both Posterior Proptocerebral Lateral (PPL1) and Medial (PPM3) subsets of dopamine neurons are implicated in this inhibition to promote wakefulness (Liu et al., 2012; Ueno et al., 2012). Interestingly, when dFB neurons are depolarized, i.e. when they are in an ON-state, they are found to produce A-type voltage-dependent Potassium (K^+) currents (Pimentel et al., 2016), ion channels for which are encoded by the *Shaker* gene which is known to function in membrane repolarization during action potential firing (Jan and Jan, 2012). On the other hand, K^+ leak currents, channels for which are encoded by

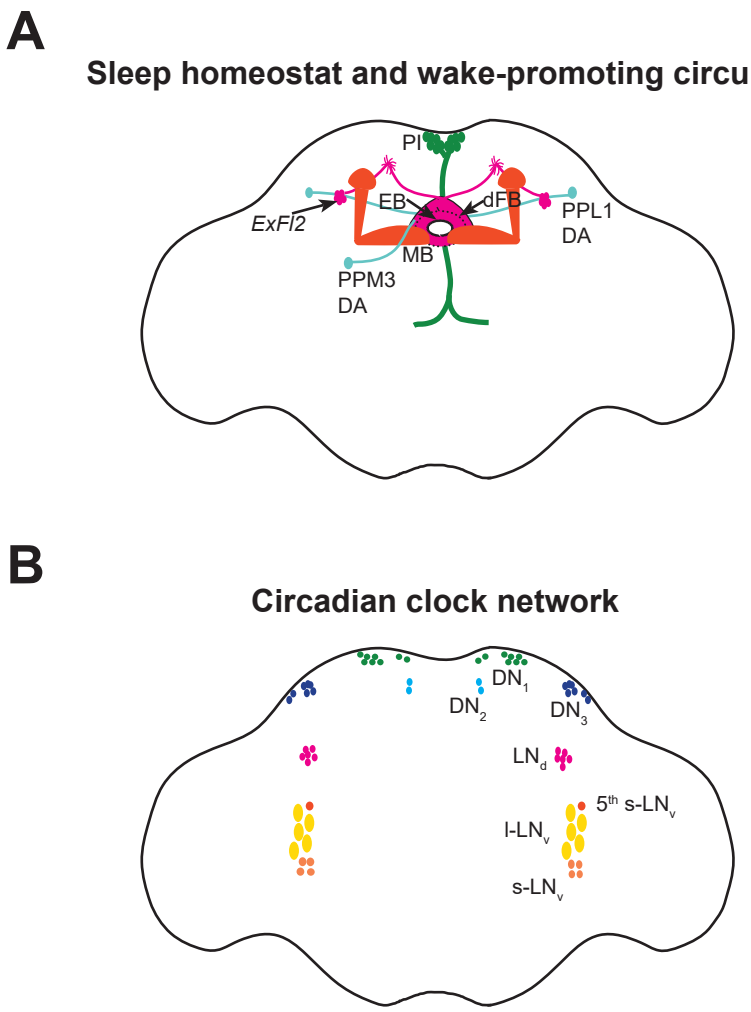


Figure 1.2. Diagrammatic representation of the Drosophila sleep homeostat and circadian network. The *Drosophila* adult brain is schematized to represent sleep homeostatic and circadian clock neurons. (A) Mushroom Body (MB), Ellipsoid Body (EB) and *ExFl2* neurons that project to the dorsal Fan-shaped Body (dFB) are known sleep homeostatic substrates. Additionally, PPM3 and PPL1 dopamine (DA) neurons project to the dFB to modulate wakefulness. Neurons within the Pars Intercerebralis (PI) are wake-promoting, though their role in the sleep homeostatic function remains unknown. (B) Bilaterally distributed subsets of circadian clock neurons are subdivided into dorsal (DN) and lateral (LN) groups on the basis of their location. The dorsal neurons are further subdivided into three groups - the DN_1, DN_2 and DN_3. The LNs are subdivided into PDF^+ large and small ventral lateral neurons (l-LN_v and s-LN_v) and PDF^- 5^{th} s-LN_v and dorsal lateral neurons (LN_d).

Sandman, help in maintaining the membrane at a more hyperpolarized state and this non-A type current was predominant in the dFB OFF-state (Pimentel et al., 2016). Indeed, previous studies had shown that mutations in genes encoding subunits of the *Shaker* K^+ channel (Bushey et al., 2007; Cirelli et al., 2005a) as well as *sleepless* (*sss*) (Koh et al., 2008) where the levels of the *Shaker* (*Sh*) protein was significantly reduced due to impaired distribution of *Shaker* K^+ channels in the membrane, caused reduced sleep and impaired sleep homeostasis (Wu et al., 2010). The neuronal substrate for the action of *Shaker*-dependent K^+ current was not clear; however, now it appears that reduction in *Shaker*-dependent K^+ current could cause dFB neurons to be locked in an OFF-state, thereby inhibiting sleep. It is hypothesized that sleep deprivation would result in internalization of *Sandman* channels and allow *Shaker*-dependent A-type K^+ currents, such that dFB neurons would increase their membrane excitability (Donlea, 2017), be switched ON, and promote sleep, and Rho-GTPase activating protein encoded by *crossveinless-c* (Donlea et al., 2014) appears to be a good candidate in mediating this function. Serotonin (5-HT) could also modulate dFB membrane excitability as knockdown of 5-HT2B receptors in dFB results in impaired sleep recovery (Qian et al., 2017).

In yet another screen designed to identify neurons that encode sleep drive, transiently hyper-excitation of R2 ring neurons of the EB (Figure 1.2A) did not result in sleep loss, however, when the membrane properties were returned to baseline levels the next day, it resulted in increased sleep reminiscent of a sleep rebound (Liu et al., 2016). This rather unexpected case was thought to be possible only if the R2 ring neurons were encoding sleep drive, and indeed, using imaging of intracellular Ca^{2+} levels which is a good indicator of neuronal activity, it was found that R2 ring neuronal activity mimicked sleep drive – in sleep deprived conditions, neuronal activity was much

higher, which returned to baseline levels after sleep recovery (Liu et al., 2016). Moreover, due to rise in sleep need, R2 ring neurons also actively increase the number of glutamatergic NMDA receptors that also results in increase in pre-synaptic active sites. Most importantly, EB R2 ring neurons activate dFB neurons to increase sleep levels during sleep recovery. These results indicate that sleep drive is encoded by synaptic plasticity occurring in the sleep drive "sensor" R2 ring neuron circuit which directly acts on the sleep-promoting "effector" dFB neurons. Furthermore, EB R2 ring neurons activate dFB neurons, which act on helicon neurons of the CC to promote sleep, which in turn modulate R2 ring neuronal activity (Donlea et al., 2018). This recurrent interaction between EB and dFB neurons led to the conclusion that the *Drosophila* sleep homeostat behaves like a "relaxation" oscillator, which, excitingly, process S of the two-process model has often been likened to (Moore-Ede and Czeisler, 1984).

Another higher brain region implicated in sleep homeostatic processes is the olfactory information integration as well as learning and memory centre, mushroom body (MB; (Heisenberg, 2003), Figure 1.2A). This was a result of efforts to find the neural correlates of sleep modulated by cAMP signaling and PKA activity since sleep was shown to be inversely related to cAMP levels (Hendricks et al., 2001). After extensive screening of 21 different fly lines targeting different subset of neurons in the MB, differential effects on sleep were observed, suggesting that MB consists of both sleep-inhibiting and sleep-promoting neurons (Joiner et al., 2006; Pitman et al., 2006). This is quite astonishingly demonstrated by the findings that ablating mushroom body on the whole results in increase in activity (Joiner et al., 2006), yet Ca^{2+} imaging from tethered behaving flies showed reduced neuronal activity in Kenyon cells (KCs, neurons that make up the MB) during sleep as compared to during wake (Bushey et al.,

2015). These results were extended when it was discovered that within the MB, wake-promoting and sleep-promoting circuits that innervate in overlapping regions exist (Sitaraman et al., 2015a; Yi et al., 2013). For instance, KCs whose axons arborize in α'/β' lobes of the MB, promote wake (Aso et al., 2014a; Aso et al., 2014b), and their electrical activity is reduced during sleep deprivation. These neurons specifically communicate the wake-promoting signals to glutamatergic MB output neurons (MBONs) (Sitaraman et al., 2015a). Interestingly, glutamatergic MBONs also are intrinsically wake-promoting and decrease electrical activity in response to sleep deprivation. Such a functionally streamlined circuit also exists for sleep promotion - γ_d KCs promote sleep and show increased electrical activity in response to sleep deprivation, and project to cholinergic MBONs that are also sleep-promoting and also encode sleep need. Yet, there is another circuit headed by γ_m KCs which are sleep-promoting but do not respond to sleep deprivation at all. In fact, it was also shown that wake-promoting dopaminergic neurons also innervate onto wake-promoting KCs and form yet another wake-promoting microcircuit (Sitaraman et al., 2015b). Thus, not only are these circuits functionally different in terms of sleep and wake, but also certain sleep-promoting circuits are functionally different in that, one circuit is involved in maintaining baseline sleep levels, while another is involved in generating recovery sleep (Aso et al., 2014a; Aso et al., 2014b; Sitaraman et al., 2015a). The idea of the existence of distinct neuronal circuits, some for maintaining baseline sleep and others for promoting recovery sleep is consistent with the finding that there are very few dedicated wake-promoting neurons (cholinergic) whose activity results in increased wakefulness followed by sleep recovery when deactivated, while there are other neurons such as dopaminergic and octopaminergic neurons which encode baseline wake

levels such that their deactivation does not result in recovery sleep (Seidner et al., 2015).

Octopamine in flies functions as a wake promoting substance in a manner similar to norepinephrine in mammals (Roeder, 1999). Wild type flies behaved like sleep deprived flies when administered octopamine externally (Crocker and Sehgal, 2008) showing increased activity in the presence of octopamine and sleep rebound when administration was ceased. Yet, electrical silencing and hyper-excitation of octopaminergic neurons brought about increase and decrease in total sleep levels respectively without causing sleep rebound (Crocker and Sehgal, 2008). When a detailed analysis was done using MARCM (Mosaic Analysis with Repressible Cell Marker), a technique that enabled only a subset of octopaminergic neurons to be hyper-excited, octopamine-modulated wake-promoting behaviour was shown to be mediated by a subset of neurons called the ASM cells that communicate to Pars Intercerebralis (PI, Figure 1.2A) neurons located in the medial protocerebrum (Crocker et al., 2010). Interestingly, EGFR (Epidermal Growth Factor Receptor) signaling-mediated sleep-promotion is undertaken by a subset of PI neurons that do not show an overlap with the octopamine-mediated wake-promoting PI neurons (Foltenyi et al., 2007). Moreover, a neuropeptide SIFamide and its receptor also promote sleep by acting on PI neurons (Park et al., 2014). Thus, PI also consists of both wake-promoting and sleep-promoting regions that are modulated by and signal through different pathways, although the question of it functioning as a sleep homeostat is unexplored.

1.4.1.2.Circadian control of sleep and wake

Circadian clocks in *Drosophila* are involved in timing various rhythmic behaviours such as activity/rest, adult emergence, mating, responsiveness to olfactory

and gustatory stimuli, egg-laying and many more in accordance with external time (Allada and Chung, 2010). The underlying machinery that enables circadian clocks to keep time and oscillate with about 24 h period consists of auto-regulatory transcriptional translational feedback loops (TTFLs) consisting of positive and negative elements (Cyran et al., 2003; Glossop et al., 1999; Zheng and Sehgal, 2008). In *D. melanogaster*, the TTFL comprises positive regulators CLOCK (CLK) and CYCLE (CYC) that activate the transcription of components of the negative limb *period* (*per*) and *timeless* (*tim*), whose protein products bind to CLK-CYC and turn-off their own transcription (Collins and Blau, 2007; Glossop et al., 1999). This system while being self-sustained in the absence of external time cues can become synchronized to external light/dark (LD) cycles as TIM is labeled for degradation by blue light-activated CRYPTOCHROME (CRY; (Dissel et al., 2004; Dubruille and Emery, 2008)). In concordance with evidence from mammals that circadian clocks modulate sleep, (Landgraf et al., 2012; Wisor et al., 2002) canonical clock gene mutants in *Drosophila* also display defective sleep. Mutations in *per* and *tim* do not alter the total amount of sleep duration in constant dark (DD) conditions, however, they re-distribute sleep throughout the 24 h duration as opposed to consolidated sleep and wake events in wild type flies (Hendricks et al., 2000a; Shaw et al., 2000). Mutations in *clk* and *cyc* (Kim et al., 2002; Kumar et al., 2012; Lu et al., 2008) also have effects on sleep even in 12:12 h LD cycles as these flies become more active during the night.

In *Drosophila*, the circadian clock circuit is well mapped and consists of about 150 neurons grouped into six subsets on the basis of their anatomical location (Kaneko and Hall, 2000; Sheeba, 2008). They are the dorsal neurons (DN_1, DN_2, DN_3; Figure 1.2B), the lateral dorsal neurons (LN_d; Figure 1.2B), large and small ventral lateral neurons (l-LN_v and s-LN_v; Figure 1.2B) and the lateral posterior neurons (LPN), among

which the s-LN_v are the most important clock neurons that govern the period and phase of activity/rest rhythms as well as oscillations of circadian proteins in other circadian clock neurons under constant darkness (Lin et al., 2004; Nitabach et al., 2006; Wülbeck et al., 2008). Eight out of 10 s-LN_v secrete a neuropeptide called Pigment Dispersing Factor (PDF) that maintains synchrony within the clock circuit in the absence of environmental cues (Lin et al., 2004; Renn et al., 1999). Flies with loss-of-function mutations in *Pdf* and the gene encoding its receptor *Pdfr* show increased levels of sleep both during the day and night under LD cycles, and even in DD (Chung et al., 2009) apart from loss of clock output. These defects are seen even in flies with ablated LN_v (Chung et al., 2009); in fact because of the phenotypes shown by Pdf^{01}, $Pdfr^{0}$ and LN_v-ablated flies, PDF is considered as a wake-promoting molecule whose function is similar to orexin in mammals (Sehgal and Mignot, 2011), mutations in which, lead to narcoleptic symptoms such as difficulty in maintaining wakefulness. However, recently it was shown that PDF can have sleep-promoting effects by acting on a subset of Allatostatin A neurons (Chen et al., 2016), a neuropeptide previously known for its role in feeding behaviour (Hergarden et al., 2012). Thus, it appears that PDF has differential effects on sleep/wake behaviour depending upon the identity of downstream neurons.

Among the circadian neurons, the l-LN_v, which are also PDF^+, have by far, the greatest influence on sleep, although roles for other clock neurons are now being discovered. When the PDF^+ LN_v are hyper-excited, flies show increased levels of wake and decreased levels of sleep, especially during the night (Sheeba et al., 2008a). l-LN_v neurons alone are sufficient for this nocturnal behaviour as hyper-exciting l-LN_v in the absence of functional s-LN_v leads to similar amount of nocturnal hyperactivity as seen when both LN_v are hyper-excited (Sheeba et al., 2008a). In addition, hyper-exciting s-LN_v alone does not lead to nocturnal hyperactivity (Shang et al., 2008). The levels of

sleep decrease seen is directly proportional to the number of hyper-excited l-LN_v (Shang et al., 2008). Furthermore, this increased level of wakefulness with hyper-excited LN_v is modulated by the levels of light (Shang et al., 2008). Thus, l-LN_v are light-activated neurons that sense light through CRY (Fogle et al., 2011; Sheeba et al., 2008b) and promote wakefulness most likely through the action of PDF. Another study showed that *Clk* mutants also exhibit nocturnal activity associated with elevated levels of dopamine and elevated CRY in the l-LN_v (Kumar et al., 2012), although CRY by itself does not have any role in sleep regulation. Moreover, l-LN_v likely receive synaptic inputs from the photoreceptor cells in the compound eyes, as the dendrites of l-LN_v arborize richly in the optic lobes as seen by staining with antibody against PDF (Helfrich-Förster, 1995; Kaneko and Hall, 2000). Furthermore, l-LN_v have been shown to act downstream of dopaminergic neurons based on synaptic connections between the two (Shang et al., 2011). Moreover, isolated l-LN_v increase their cAMP levels in response to exogenously applied dopamine and octopamine (Shang et al., 2011). Wake-promoting effects of histamine also seem to be mediated through the LN_v (Oh et al., 2013). Parisky and colleagues (2008) reported that $GABA_A$ receptor RDL (resistance to dieldrin; (Agosto et al., 2008)) is expressed in circadian neurons LN_v. Moreover, reduced amount of total sleep and increased sleep latency was observed when *rdl* was downregulated in them (Parisky et al., 2008). A subsequent study showed that anti-RDL staining was exclusively localized to the l-LN_v (Chung et al., 2009). In fact, anti-RDL was found to stain l-LN_v projections to optic lobes and accessory medulla, suggesting that inhibition by GABA to decrease wakefulness is specific to the l-LN_v. Indeed, it was found that WAKE (WIDE AWAKE) upregulates the expression of *rdl* and increases its localization to cell membrane specifically in the

l-LN_v during dusk, thereby increasing l-LN_v sensitivity to GABAergic inhibition in order to promote sleep onset (Liu et al., 2014) during transition to night.

There is a lack of clarity regarding the role of s-LN_v in sleep/wake regulation; one study found that 1-2 s-LN_v promote sleep through the action of short Neuropepetide F (sNPF) which also affects sleep homeostasis (Shang et al., 2013), though another study reported the role of sNPF on sleep hoemostasis in the mushroom body (Chen et al., 2013). Yet another study suggested that s-LN_v inhibit l-LN_v through sNPF during night to suppress wakefulness (Oh et al., 2014). Another subset of clock neurons that have a prominent role to play in sleep/wake regulation are the DN_1s. DN_1s were found to receive PDF inputs specifically during late night in order to increase the production of a neuropeptide DH31 (Diuretic hormone) that suppresses sleep (Kunst et al., 2014). A sleep-promoting role was also found for DN_1s, as activating these neurons increased day-time sleep and this was found to be brought about by glutamatergic inhibition of neurons modulating "evening" peak of activity (Guo et al., 2016). Thus, it is clear that both wake-promoting and sleep-promoting roles for different subsets of circadian neurons are emerging from these studies.

1.4.1.3.Organization of the sleep/wake circuit

One of the major assumptions of the two-process model was that circadian and homeostatic processes would be independently regulated. So far, the anatomical substrates for sleep homeostat and circadian clocks are found to be distinct in mammals (Mistlberger et al., 1983) and flies, as outlined in the previous sections. According to the model, sleep propensity is regulated by both process S and process C, and whether the two processes interact or independently regulate sleep propensity is not specified in the model. Under special circumstances, it was predicted that there would be some

feedback of process S on process C, and indeed, as noted in section 1.3, there is now evidence for interdependence of process S and process C in mammalian sleep regulation. Thus, in mammals, it appears that the two processes interact functionally to regulate sleep propensity. Investigating if this is true for sleep/wake regulation in *Drosophila* is an important question and this is one of the major questions addressed in my thesis.

Most of the mutants that have sleep homeostatic defects have so far been shown to not have circadian clock defects (Cirelli et al., 2005a; Koh et al., 2008; Kume et al., 2005). Interestingly however, core clock gene *tim*, but not *per* was implicated in sleep homeostasis based on the observation that loss of function mutation of *tim* (tim^{01}) abolished sleep rebound after sleep deprivation, which could be rescued by a transgenic copy of full-length *tim* (Hendricks et al., 2000a). In addition to *tim*, mutation in *cyc*, another core clock gene, leads to impaired sleep rebound and death ~ 10 hrs after sleep-deprivation along with reduced expression of heat shock and stress response genes (Shaw et al., 2002). Importantly, the response to heat shock is not affected by the mutation, suggesting that *cyc* is important for sleep homeostasis (Shaw et al., 2002). Moreover, two recent studies show possibly different subsets of DN_1s to be indirectly communicating with the sleep homeostatic R2 neurons of the EB (Guo et al., 2018; Lamaze et al., 2018). Thus, until recently, the evidence for a direct link between sleep homeostatic and circadian clock elements in order to regulate timing and quality of sleep has been limited.

A recent study has demonstrated the involvement of *Cyclin A* (*CycA*) and its regulator (both important cell cycle proteins) in sleep homeostasis, and interestingly, most *CycA* expressing neurons were found to be located near subsets of circadian neurons (Rogulja and Young, 2012). Moreover, *taranis (tara)*, a gene encoding a

transcriptional coactivator, shows circadian clock defects when mutated, and also regulates sleep by acting on 12-14 *CycA* expressing Pars Lateralis neurons (Afonso et al., 2015). Interestingly, TARA was found to act in cholinergic neurons as well in order to promote sleep, and a subset of wake-promoting cholinergic neurons also are involved in "sensing" sleep deprivation states (Seidner et al., 2015). Yet another pathway that could be at the intersection of clocks and sleep homeostat is the *insomniac*/*Cullin-3* pathway. A forward genetic screen designed to select aberrant sleep phenotypes yielded a mutant called *insomniac* (Stavropoulos and Young, 2011)). As the name suggests, the behavioural manifestations of this mutation includes reduced sleep consolidation and levels as well as reduced sleep homeostatic response (Pfeiffenberger and Allada, 2012; Stavropoulos and Young, 2011). The protein product of *inc* functions as an adaptor of *Cullin3* ubiquitin ligase complex, which is involved in protein degradation pathways (Stavropoulos and Young, 2011) and has been discovered to modify core components of the circadian clock machinery in order to maintain about 24 h rhythmicity (Grima et al., 2012). The same protein degradation pathway being involved in both sleep homeostasis (through *inc*) and modifying core clock proteins (through *Cullin3*) underscores the possibility of *inc* modulating sleep levels through the action of Cullin-3 on circadian clock genes. These are interesting prospects which can help in understanding mechanistic links between sleep homeostat and circadian clocks that eventually results in sleep/wake regulation.

Although several studies have attempted to understand the exact neuroanatomical correlates of sleep in *Drosophila*, many other questions persist. It appears that several separate circuits exist that modulate sleep and wake; however whether they interact with one another to produce timed sleep is worth investigating. In other words, does sleep regulation occur at a global level, or are individual circuits

independently causing synaptic plasticity at a local neuronal level, which together result in additive effects on sleep at the level of behaviour? None of the circuits when tampered with completely abolish sleep; they only affect certain aspects of sleep. Nonetheless, the fact that several different circuits modulate this single behaviour of sleep suggests that sleep serves essential functions that have led to genetic and circuit-level redundancy even in the fly.

1.4.2. Why do we sleep?

This question, by far, is the hardest to answer. While appearing deleterious on many counts, the fact that sleep has persisted over the course of millions of years of natural selection in a wide variety of organisms suggests that it may have several benefits. Fruit flies selected for insomnia-like behaviour for about 60 generations displayed reduced life span, increased levels of triglycerides and fatty acids, hyperactivity and upregulation of metabolism and neuronal activity-associated genes (Seugnet et al., 2009). The results of this study mirror similar results seen in mice, where organisms chronically deprived of sleep show reduced life span and skin lesions among other defects (Everson, 1995; Rechtschaffen et al., 1983). So far, among many hypotheses proposed for the importance of sleep, the three most widely accepted and prominent ones are as follows - synaptic downscaling and memory consolidation, reducing energy demands and restoration of vital cellular components (Mignot, 2008). While none of these explain all the features of sleep convincingly, a number of studies in flies provide evidence in support of these hypotheses with the discovery of various factors involved in the above mentioned processes that also differentially regulate day-time and night-time sleep (Ishimoto et al., 2012).

The hypothesis that sleep is a physiological state during which synaptic downscaling mostly occurs was proposed as one of the ways in which sleep affects synaptic homeostasis (Tononi and Cirelli, 2003). In brief, this hypothesis suggests that synaptic potentiation and thus memory formation occur during wakefulness, whereas synaptic downscaling and clearance of irrelevant memories – and thus strengthening of relevant ones, occurs during sleep. In addition, it also proposes that sleep serves a universal, essential function of homeostatically controlling synaptic plasticity – that is restoring molecules and conditions to baseline levels needed for optimum learning during wakefulness (Frank, 2012; Tononi and Cirelli, 2012). Several studies have gathered evidence in support of this controversial (Frank, 2012) hypothesis. For instance, various mutants of the cAMP signaling pathway such as *rutabaga* (*rut*) and *dunce* (*dnc*) have been shown to have both sleep and memory defects (Hendricks et al., 2001). Additionally, *amnesiac* mutants which are defective in intermediate-level or middle-term memory also show fragmented sleep and defective sleep initiation (Liu et al., 2008). Dorsal paired medial (DPM) neurons which are important for memory consolidation are also shown to be sleep-promoting (Haynes et al., 2015). Interestingly, memory mutants *rut* and *dnc* were able to improve their short term and long term memory scores after sleep was induced in them through three independent methods (Dissel et al., 2015). Moreover wild type flies that were kept in a socially 'enriched' condition, i.e. in groups or pairs, slept more when compared with similar flies kept in isolation (Ganguly-Fitzgerald et al., 2006; Lone et al., 2016). Interestingly, teaching flies to not court unresponsive females decreased courtship levels, however, when males were sleep deprived immediately after the training session, they 'unlearned' the experience and showed similar courtship levels as that exhibited during training.

Indeed, increase in sleep due to social experience was found to be modulated by genes associated with Long-Term Memory (LTM; (Ganguly-Fitzgerald et al., 2006)).

In conjunction, it was observed that certain synaptic markers are expressed when flies are sleep deprived and their levels of expression are dependent upon the extent of deprivation (Gilestro et al., 2009). When synapses were examined in different behavioural states – flies that slept normally had decreased number of spiny dendritic boutons and decreased branch length of processes, whereas those that were awake or sleep-deprived had opposite features. These results were consistently obtained in three different circuits thus establishing the universality of this phenomenon within the fly brain (Bushey et al., 2011). Additionally, social enrichment increased the number of spiny boutons and number of synapses in the LN_v terminals, thus increasing the need to sleep and thereby the need to restore the synaptic markers to baseline levels (Bushey et al., 2011; Donlea et al., 2011). These results were accompanied by behavioural experiments that showed that memory formation was facilitated with induction of sleep by hyper-exciting the sleep-promoting dFB neurons (Donlea et al., 2011)). Interestingly, increasing sleep after learning suppresses active forgetting mechanisms thereby improving memory retention (Berry et al., 2015). These results together suggest that experience during waking facilitates formation of memories, which are strengthened, and unwanted synapses are pruned during sleep that leads to remembrance of important experiences, thereby priming to imbibe from new experiences during the next waking event.

Increasing evidence points towards a role for sleep in global restorative functions, and not just restricted to the brain as suggested by the synaptic homeostatic hypothesis. Another reason for occurrence of sleep is given by the energy reduction hypothesis, according to which sleep evolved in order to reduce the energy

requirements of organisms. Consistent with this, in humans, energy expenditure as measured from oxygen consumption is lower during sleep than during wakefulness, and consequently sleep deprivation increases energy expenditure whereas sleep recovery decreases it (Jung et al., 2011). In conjunction with these results, it has been shown that starved flies sleep less (Thimgan et al., 2010); in mammals sleep deprivation is also accompanied by increased feeding (Rechtschaffen and Bergmann, 2002). Indeed, microarray analysis found that the glial-specific gene *anachronism* and certain lipid metabolism genes are upregulated during sleep. Additionally, wakefulness was associated with upregulation of genes involved in the NAD-reducing pentose phosphate shunt (Cirelli et al., 2005b). These results can be interpreted keeping in mind the ecology of the animals; an organism that has not been able to obtain food for a long time would rather spend its remaining time and energy in foraging for food as opposed to indulging in sleep. This is well-supported by the findings that neuronal activity of the mammalian arousal-promoting hypocretin/orexin neurons is modulated by extracellular levels of glucose, leptin, ghrelin (Yamanaka et al., 2003), lactate (Parsons and Hirasawa, 2010) and intracellular levels of ATP (Liu et al., 2011), all of which are indicators of energy balance.

While the 'orexin-like' PDF^+ LN_v neurons in *Drosophila* have not yet been implicated in a similar metabolic state-dependent modulation of wakefulness, PDF^- circadian neurons influence starvation-mediated sleep suppression, especially through the action of *Clk* (Keene et al., 2010). In addition, *foraging* (*for*), which codes for a cGMP-dependent protein kinase (PKG) and determines the larval feeding strategy of roving (for^R allele) or sitting (for^s allele), is also implicated in sleep in both flies and worms (Dabbish and Raizen, 2011; Raizen et al., 2008). Studies to understand the dietary regulation of sleep-wake cycles have been undertaken, and results suggest that

excessive yeast in the food leads to sex-specific effects on both day-time and night-time sleep (Catterson et al., 2010). The nutritional quantity also determines sleep characteristics – food with high levels of sucrose induced fragmented sleep, this kind of fragmentation was not obtained with increasing protein content (Linford et al., 2012). Additionally, *fumin* (*fmn*; gene encoding dopamine transporter) mutants that showed reduced sleep levels but normal lifespan (Kume et al., 2005) now showed reduced lifespan on a high-calorie diet (Yamazaki et al., 2012). Interestingly, c-Jun N-terminal Kinase (JNK), a signaling molecule regulating the insulin-producing pathway in the PI, was shown to function in MB to regulate sleep levels and lifespan depending upon dietary conditions (Takahama et al., 2012). Another signaling pathway that is important for regulating blood pressure and electrolyte content in mammals includes the angiotensin-converting enzyme whose *Drosophila* homologue ACER, is implicated in sleep consolidation especially during the night (Carhan et al., 2011). Taken together, quite a few studies have been able to establish the link between sleep and metabolism, providing a genetically tractable model system to study metabolic impairments due to sleep loss and health issues such as obesity-linked sleep defects in a comprehensive way.

Glycogen levels were found to have a diurnal pattern during the 24 hr cycle, with a peak immediately following the peak in rest period, while sleep deprivation resulted in overall decrease in glycogen levels (Zimmerman et al., 2004). Interestingly, sleep duration was positively correlated with glycogen stores in males and triglyceride stores in females (Harbison and Sehgal, 2008). However, these results should be considered with the caveat that sleep loss by mechanical disturbance in both males and females led to decrease in glycogen levels accompanied by increase in triglyceride levels, leaving the possibility that change in energy stores is a stress-related response

(Harbison and Sehgal, 2009). Nonetheless, these results suggest that sleep deprivation, stress-related or not, can affect energy stores, providing empirical evidence for the hypothesis that sleep evolved in order to replenish cellular components. Microarray studies done on the mouse cerebral cortex and hypothalamus – brain structures important in regulating sleep, revealed upregulation of genes involved in synthesis of macromolecules (Mackiewicz et al., 2007). The alleviation of various cellular and physiological stresses by sleep can also be considered as a restorative function of sleep. Indeed, the mRNA level of a molecular chaperone protein BiP (Immunoglobulin Binding Protein) that is also an indicator of Endoplasmic reticulum (ER) stress increases during sleep loss and decreases as sleep is recovered (Naidoo et al., 2007). In addition, heat shock proteins are expressed with sleep deprivation, the inability to do so results in death of cyc^{01}flies within 10 h of sleep deprivation (Shaw et al., 2002). NfκB *relish*, a gene important for inflammatory immune responses was shown to be important in sleep homeostasis (Williams et al., 2007). Indeed, when flies were injected with *Escherichia coli* or were injured without causing sepsis, in addition to mounting an immune response, they also displayed increased sleep levels during morning hours, which was absent in flies lacking *relish*, and which was rescued with the transgenic expression of *relish* in the fat bodies (Kuo et al., 2010). Furthermore, FMRFamide, another neuropeptide was shown to be involved in modulating sleep recovery due to heat stress (Lenz et al., 2015). Thus, the fact that a wide variety of genes involved in stress response and physiology have some effects on sleep points towards a restorative function of sleep.

Sleep is also modified by context, as it was shown that flies tend to sleep lesser during the night when they are coupled with individuals of the opposite sex which does not result in sleep rebound (Beckwith et al., 2017; Lone and Sharma, 2012). This

context-dependent regulation of sleep drive which evidently does not need to be recovered was seen exclusively in males and was mediated by a subset of octopamine neurons (Machado et al., 2017). Moreover, apart from sleep duration being dependent on presence of conspecifics of opposite sex, nutritional requirements and maintenance of behavioural states are dependent upon gender – females need to forage more both for feeding and laying eggs. Accordingly, mated females have been shown to sleep less during the day when they are presumed to be foraging for food in order to lay eggs; a change brought about by the reception of the male sex peptide via the ejaculate during copulation (Isaac et al., 2010).

While it is clear that considerable evidence has been garnered in favor of each of the hypotheses discussed above, it is quite evident that sleep itself has not evolved to serve any one function. Substantial overlap exists between the processes that govern each of them, as studies in *Drosophila* have shown that sleep has restorative functions in modulating synaptic conditions, mounting stress responses and regulating energy reserves. For instance, genes like *brummer* which are involved in lipid metabolism also affect sleep in addition to showing an effect on sleep deprivation-associated learning deficits (Thimgan et al., 2010). In addition, circadian gene *cyc* has been shown to affect both the restorative function of sleep as well as modulate metabolic and sleep changes with respect to food availability (Keene et al., 2010; Shaw et al., 2002). Indeed, these overlaps can provide bases for examining and establishing *Drosophila* as a potent model organism to study various diseases (Sehgal and Mignot, 2011), symptoms of which include sleep disturbances. For example, *Drosophila* has also been found to display certain symptoms of restless-leg syndrome (Freeman et al., 2012) and demonstrated to be a useful model to study Angelman's syndrome, symptoms of which includes mental retardation and sleep disturbances in humans (Wu et al., 2008a).

1.5. Concluding remarks

The model organism *D. melanogaster* has offered fresh insights in what can be considered as one of life's paradoxes – sleep. Throughout, I have emphasized sleep as a behaviour that is regulated by multiple interacting pathways, and has effects on multiple functions, which also overlap to a certain extent. Thus, recovery during sleep, brought about by homeostatic mechanisms, is not just restricted to restore balance only in the brain, but also in other organ systems. Thus, it is a unique behaviour that has an immense contribution to the well-being of the organism. Nonetheless, while sleep confers myriad advantages to organisms, it still remains unclear as to what and how selection pressures molded the evolution of the behavioural state which we today call sleep. Indeed, it has recently been shown that sleep deprivation in young adult fruit flies results in impaired courtship behaviours (Kayser et al., 2014) and aggressive behaviours which in turn results in reduction of mating opportunities (Kayser et al., 2015). However, to address this question of significance of sleep more directly, in a part of this thesis I have examined the effect of sleep deprivation on egg output

A question that has not received much attention pertains to the primitive forms of sleep displayed by our ancestors. With fruit flies having served as an important model organism in the study of sleep and amenable to perform large-scale artificial selection studies, it would be indeed worthwhile and insightful to probe the evolutionary significance of sleep in these organisms. Another fruitful approach would be to take the comparative outlook – one could uncover potentially interesting features and functions of sleep by comparing its structure among closely related species. For instance, differences in sleep bout number and average sleep bout length between related species can go a long way in establishing correlations between habitat and dietary differences and sleep requirements, physiological adjustments and sleep maintenance and

spatiotemporal niche selection and sleep timing and placement preferences. Indeed, an ongoing study in our laboratory has indicated sleep structure differences between two closely related species *D. melanogaster* and *D. ananassae* (Prabhakaran and Sheeba, 2012). In addition, moving towards the study of sleep in more natural conditions, rather than in the laboratory can yield fresh insights on the functional aspects of sleep (Aulsebrook et al., 2016), as has recently been exemplified by examining activity/rest rhythms (Lu et al., 2006; Menegazzi et al., 2012; Prabhakaran and Sheeba, 2013; Vanin et al., 2012) under semi-natural conditions. Thus, the credibility of *Drosophila* in studying sleep is unquestionable and further studies that are directed towards unraveling the organization of the sleep circuit can increase its potential to be exploited in solving the many mysteries of sleep.

The two-process model is a 'terrific example', as pointed out by Arthur Winfree (Moore-Ede and Czeisler, 1984) of how mathematical models can help in gaining insights about fundamental processes. This model works well because it combines the most essential aspects of sleep/wake cycle in a comprehensive manner and without introducing too many parameters, manages to capture most of the empirical observations about sleep. Even though several new modifications continue to be made in order to explain the myriad nitty-gritty of mammalian sleep, this model is very useful in trying to establish a universal process for sleep regulation. Thus, a major part of work outlined in my thesis examines *Drosophila* sleep in the context of the two-process model, so as to take the first steps towards gaining insights into general principles of invertebrate sleep regulation. Moreover, in any animal, a homeostatic and circadian component for sleep must be defined in order for that animal to be deemed to perform sleep in accordance with behavioural criteria for the definition of sleep. Therefore, the principal components for testing the model will always be present in any animal that

sleeps. Thus, as more and more organisms get caught napping, a universal feature of sleep regulation is bound to emerge if considered within the framework of two-process model, which would help in understanding the evolutionary origins of sleep regulation.

Chapter 2

Nature of interaction between sleep homeostat and circadian clocks in *Drosophila melanogaster*

2.1. Introduction

How the two diametrically opposite behavioural states of sleep and wake (Saper et al., 2005) are regulated is an important question and as mentioned in the previous chapter, the two-process model has gained traction over the past three decades (Borbély, 1982; Daan et al., 1984). Essentially, according to the model, a homeostatic variable S indicative of sleep debt or pressure, builds up in response to the animal remaining awake, while getting discharged as sleep sets in. The circadian process C regulates the timing of sleep, such that sleep is initiated when sleep debt reaches a certain threshold (Figure 1.1A). Thus, an implicit assumption of the model is that these two processes interact with each other in order to set the threshold for sleep (and wake) and thereby regulate sleep and wake.

In mammals, it has been shown that in mice and rats with lesions in the Suprachiasmatic nucleus (SCN), site of the mammalian circadian clocks which are internal timekeeping mechanisms, Rapid Eye Movement (REM) and non-REM (NREM) sleep duration is not impaired (Ibuka et al., 1980; Tobler et al., 1983). Moreover, SCN-lesioned rats are also able to show sleep recovery, a sleep homeostatic feature, after sleep deprivation (Mistlberger et al., 1983) which suggests that SCN is not the site of the sleep homeostat. However, in a series of studies in which human participants were subjected to forced desynchrony and constant routine protocols, it was shown that circadian amplitude of various behavioural factors such as mood, cognitive performance and alertness were reduced upon sleep deprivation suggesting that sleep deprivation can impact circadian clock driven rhythms (Boivin et al., 1997; Dijk and Czeisler, 1994; Dijk and Czeisler, 1995; Dijk et al., 1992). Interestingly, mutations in mammalian core clock genes such as *Bmal1*, *Npas2* and *Cry1* and *Cry2* results in altered levels of sleep as well as impaired responses to sleep deprivation suggesting

effects of circadian genes on sleep homeostatic features (Dudley et al., 2003; Laposky et al., 2005; Wisor et al., 2002). Furthermore, changes in sleep pressure through sleep deprivation also results in altered electrical activity of SCN neurons (Deboer et al., 2007; Deboer et al., 2003) and altered expression of clock genes in the cerebral cortex (Wisor et al., 2008). Together, these studies provide ample evidence for circadian clocks affecting sleep homeostatic features in mammals. On the other hand, it has been shown that sleep deprivation also results in changes in circadian clock properties. In Syrian hamsters, sleep deprivation in the middle of the day (i.e. sleep period) resulted in phase advances of activity/rest rhythm and decreased *c-fos* expression in the SCN (Antle and Mistlberger, 2000). Moreover, phase shifts in response to light cues at different times of day are also attenuated due to sleep deprivation in mice and hamsters (Challet et al., 2001; Mistlberger et al., 1997; van Diepen et al., 2014). Recently, this finding was also extended in a diurnal mammalian species of Sudanian grass rats and it was reported that sleep deprivation resulted in increased phase-shifting responses to light cues (Jha et al., 2017). Thus, in mammals, sleep/wake regulation seems to occur through distinct circadian and homeostatic processes, while influencing properties and functions of one another.

In *Drosophila melanogaster*, electrophysiological correlates have been recorded (Nitz et al., 2002; van Swinderen et al., 2004) using which "deep" stages of sleep in *Drosophila* have been uncovered recently (van Alphen et al., 2013; Yap et al., 2017), these measures cannot be extensively used to study homeostatic features of sleep because of technical difficulties. Therefore, in order to address questions pertaining to sleep homeostat in invertebrates, behavioural markers such as sleep rebound, arousal thresholds, number of brief awakenings and responsiveness to sensory stimuli have been used (Faville et al., 2015; Huber et al., 2004). Moreover, a pleiotropic role for

core circadian clock gene *cycle* (*cyc*) in sleep homeostasis (Shaw et al., 2002) has been shown, and circadian modulation of sleep/wake properties have also been studied (Huber et al., 2004; van Alphen et al., 2013).

According to the two-process model, sleep propensity brought about by the sleep homeostat is dependent upon prior sleep (wake-dependent sleep propensity), whereas circadian clocks generate a rhythmic wake-independent sleep propensity. Whether these two processes impinge upon one another and act concertedly to generate sleep propensity, or directly and independently regulate the wake-dependent and independent aspects of sleep propensity is a question that remains unanswered. While it is clear that they interact in order to regulate sleep propensity in mammals, it is necessary to address this question in other animal species so as to assess the universality of sleep/wake regulation in order to begin to understand its functional significance. Thus, while research in the past in *Drosophila* and other invertebrate model systems has uncovered mechanistic details about sleep/wake regulation (Artiushin and Sehgal, 2017), extensive study within the framework of the two-process model to examine fundamental features of sleep/wake regulation has been lacking. In this study, I focused on testing the primary assumption of the two-process model – interaction between homeostatic and circadian clock components of sleep/wake regulation in fruit flies. Specifically, I addressed how circadian clocks regulate the cycling of sleep homeostatic features, for which I used several markers. I found that quantity and quality of recovery sleep is dependent upon time of day during which sleep deprivation is imposed. Moreover, a functional circadian clock is necessary to bring about time-dependent sleep rebound. I found that circadian properties of period and phase do not change with sleep deprivation at any time of the day, suggesting a one-way influence between clocks and sleep homeostat. These results point to the conclusion that sleep homeostat and

circadian clocks interact in a manner by which they together influence sleep propensity as proposed by the two-process model of sleep/wake regulation.

2.2. Materials and methods

2.2.1. Fly strains. All flies were reared on standard cornmeal medium in LD 12:12 at 25 °C. All genotypes except $per^{0}w$ and clk^{JRK} were back-crossed to *Iso*31 (w^{1118}, BDSC # 5905) for at least 5 generations. *CCM* (Chrono Control Merged) flies are an outbreeding population maintained in the lab (Gogna et al., 2015) and per^{s} and per^{l} have been back-crossed to this background for five generations (Manishi Srivastava, Vishwanath Varma, Abhilash Lakshman, Vijay Kumar Sharma and Vasu Sheeba; unpublished). All genotypes and their sources are tabulated in Appendix 1.

2.2.2. Sleep recording and deprivation. 3-5 day old virgin male flies (unless otherwise stated) were housed individually in glass tubes (3mm inner diameter, 5 mm outer diameter, 65 mm length) with corn meal medium or 5% sucrose-2% agar medium on one end and cotton plug on the other. Activity counts every minute were obtained by recording from *Drosophila* Activity Monitoring (DAM) system (Trikinetics, Waltham, MA, USA), which works on the beam-breaking principle, such that whenever the fly moves along the middle of the glass tube, it breaks an infra-red beam passing through the middle of the tube and this is recorded as an activity count. Flies were recorded in controlled environments of either Light/Dark cycles of 12 hours each (LD 12:12, ~300-500 lux during light phase) or constant darkness (DD) at 25 °C (unless otherwise stated) in incubators (DR-36VLC8 Percival Scientific Inc., Perry, IA, USA). Flies were subjected to sleep deprivation with the help of a vortexer (Multitube vortexer, VWR, Radnor, PA, USA) which rotates the plate on which DAM monitors were securely clamped with the help of a custom-made clamp (Vortexer Mounting

Plate, Trikinetics), at a force of 1-g (500 RPM, 3.6mm eccentric orbit). The vortexer was programmed using a light controller (LC4, Trikinetics) to rotate for 10 seconds every minute for either 4 hour or 12 hour duration. For arousal threshold experiments, vortexer was used to give mechanical stimuli where 1-g force was applied just once for either 1, 5 or 10 seconds at different time-points. For sleep deprivation experiments, flies were recorded for a period of three days, with first day used to obtain baseline levels of sleep (BS) and second day used to perform sleep deprivation (SD) at indicated time intervals. In another experiment, sleep deprivation, for the duration of 4 hours was followed by a brief light pulse (2 mins, ~500 lux) one hour after the deprivation had been stopped. This experiment was conducted on the first day of DD following entrainment to previously imposed LD cycles. For experiments assessing effects of deprivation on circadian clock properties, flies were initially recorded in DD for a period of 7 days; on the 8^{th} day, flies were transferred into fresh food tubes and were given sleep deprivation at different time windows, and then were recorded for a period of 7 days post-deprivation.

2.2.3. Data analysis. Sleep in *Drosophila* is defined as inactivity spanning for five or more minutes. Sleep duration was analyzed with the help of custom-made excel spreadsheets as well as the widely used software pySolo (Gilestro and Cirelli, 2009). Percent sleep lost during a particular window, for example ZT 0-4 (Zeitgeber Time, where ZT 0 refers to lights-ON), was calculated as (sleep during SD at ZT 0-4 – BS sleep during ZT 0-4) / (BS sleep during ZT 0-4) *100. Percent sleep gained during a particular window, for example ZT 0-4, was calculated as (sleep during interval after SD i.e. ZT 4-8 – BS sleep during ZT 4-8) / (BS sleep during ZT 4-8) *100. Similar calculations were performed for sleep deprivation at other time windows. For arousal thresholds, percent responses were calculated as percentage of sleeping flies which

responded to mechanical stimulus by increasing their activity in the five minutes after the stimulus. Brief awakening was defined as an event where the fly was inactive initially, but became active for a short period i.e. 1 minute, and then resumed inactivity. Thus, if inactivity count is represented by 1 and activity count is represented by 0, then a 24 hour data set binned at 1 minute interval was scanned for occurrence of 1,0,1 events every hour or every 4 hours, and numbers of such events were noted as brief awakenings per hour or brief awakenings per 4 hours. This was achieved with the help of a custom-written MATLAB (Mathworks, Inc., Natick, MA, USA) code. Circadian clock properties of period and robustness were obtained from Chi-square periodogram using ClockLab (Actimetrics, Wilmette, IL, USA), where amplitude of periodogram was considered as robustness of rhythm. To estimate phase of rhythms, activity offsets calculated from actograms were used as phase markers. Change in period was calculated by subtracting period obtained 7 days post-deprivation from period obtained 7 days pre-deprivation. Similarly, change in robustness was calculated by using amplitude of periodogram. For change in phase, average phase of last four days post-deprivation was subtracted from average phase of last four days pre-deprivation. Data analyses were done independently in experiments repeated multiple times. One run among replicates was arbitrarily chosen as a representative run only if all runs showed similar results.

2.2.4. Statistical analysis. For % sleep lost, % sleep gained and sleep latency after light pulse, two-way ANOVA with time window and treatment as fixed factors followed by *post-hoc* Tukey's Honest Significant Differences (HSD) test was conducted. For responses to brief light pulse, two-way ANOVA with time-point (before, during and after pulse) and treatment as fixed factors followed by *post-hoc* Tukey's HSD test was conducted. For number of brief awakenings, change in period,

phase and robustness, one-way ANOVA with time (for brief awakenings) or treatment (for change in period, phase and robustness) as fixed factor followed by *post-hoc* Tukey's HSD test was conducted. Significance level was set at $p < 0.05$. Details of statistical analyses are given in results and Appendix 2.1.

2.3. Results

2.3.1. Quantity of sleep rebound depends upon time of sleep deprivation. In order to study *Drosophila* sleep/wake cycles within the framework of the two-process model, it was important to first understand how circadian clocks modulate sleep propensity. To do so, I examined sleep propensity as measured by two behavioural markers in constant conditions in the absence of environmental time cues. Previously, arousal thresholds have been used as a marker of sleep pressure in flies, with the caveat that the same individual flies were given mechanical stimuli to elicit behavioural responses at different times of a LD 12:12 cycle as well as DD, and it was found that arousal thresholds were higher during the night (Faville et al., 2015; van Alphen et al., 2013). I subjected different sets of flies to mechanical stimuli lasting either 1, 5 or 10 seconds at different times of the day both in the presence and absence of time cues (LD 12:12 and DD). As expected, arousal thresholds were lower during day as compared to night (Figure 2.1A); interestingly we obtained a similar pattern even in constant darkness (Figure 2.1B). During the day and subjective day, responses to stimuli in terms of increase in activity are higher even for stimuli lasting for the shorter durations of 1 and 5 seconds, whereas higher percentage of response is seen only when stimuli last for 10 seconds during middle of night (ZT 16) and subjective night time-points (CT 16 and CT 20, Figure 2.1A-B). At some time-points (such as ZT 4 and CT 4), stimulus duration for the longest duration, i.e. 10 seconds did not elicit the strongest response. This could

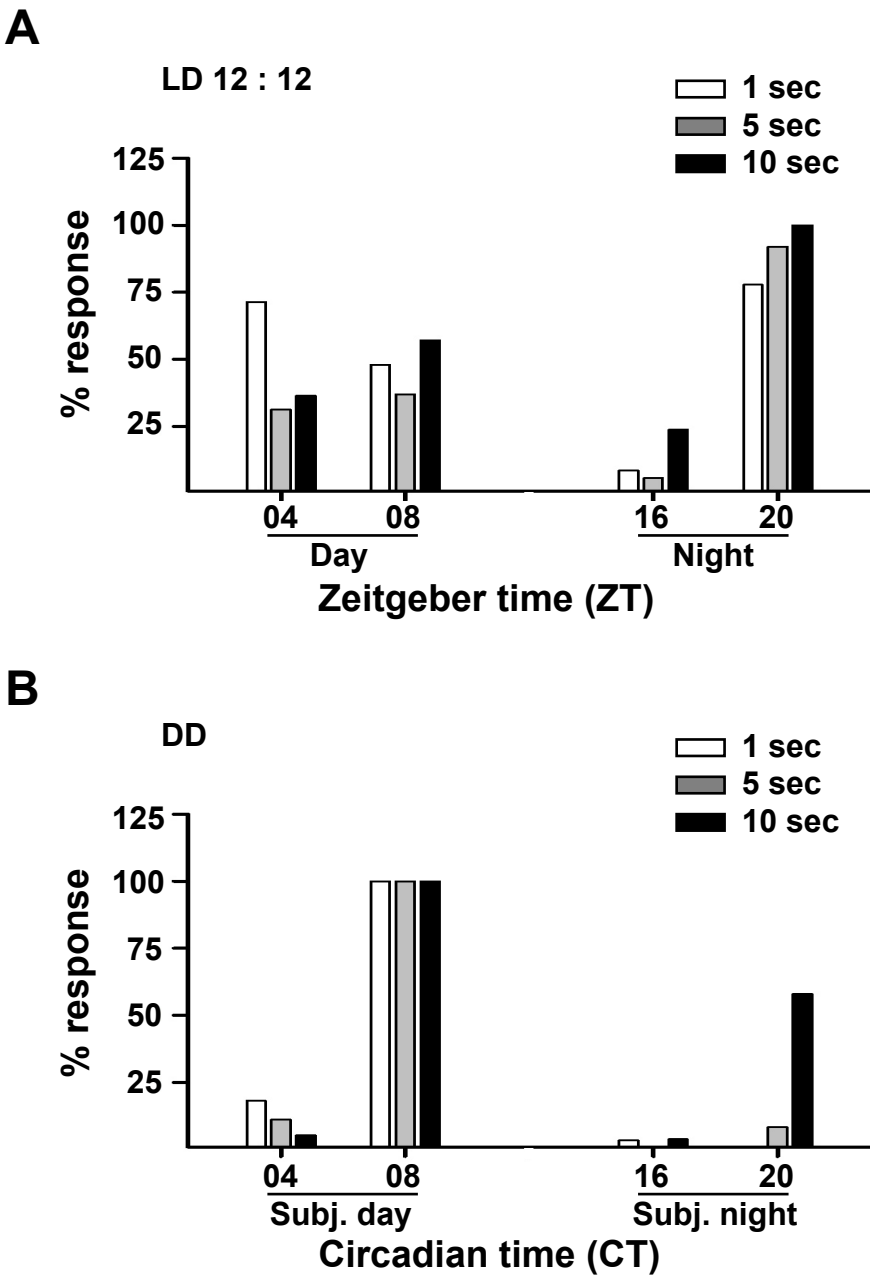


Figure 2.1. Arousal thresholds vary across time of day. Percentage of sleeping flies (w^{1118}) that responded with increase in activity levels to mechanical stimuli lasting either 1, 5 or 10 seconds at different time-points in (A) LD 12:12 and (B) DD conditions. At ZT 0 and 12, as well as CT 0 and 12, majority of the flies were awake and hence % response could not be obtained. *n* between 22-32 flies. Experiment in both regimes conducted once.

be due to low resolution of data acquired from DAM system. Indeed, in another study in which video recording was conducted to measure sleep and activity of flies, the expected result of highest stimulus intensity eliciting strongest response was seen (van Alphen et al., 2013). Nevertheless, the diurnal as well as circadian pattern of higher arousal thresholds during the night remains consistent across studies.

Yet another marker of sleep pressure is sleep intensity as measured by number of brief awakenings, which are defined as activity counts (typically for a minute) interspersed within two inactive intervals (also lasting a minute each), and which has previously been used as a measure of sleep intensity in mice, rats, humans and flies (De Gennaro et al., 2001; Franken et al., 1991; Huber et al., 2000; Huber et al., 2004; van Alphen et al., 2013). Furthermore, number of brief awakenings can be considered as a marker of sleep continuity or consolidation, and thus as an output of the sleep homeostat, since by definition, maintenance of sleep/wake levels is a function of the sleep homeostat. In conditions where organisms do not receive time cues from the environment, such as in DD, sleep continuity could still be cycling due to the sleep homeostat functioning as an hourglass. To test this hypothesis, I recorded wild type flies as well as flies lacking functional clocks and flies with clocks running with altered speeds and quantified the number of brief awakenings across time of day on three consecutive days in DD. I found that wild type w^{1118} flies show higher number of brief awakenings during subjective day and there is a sharp dip during subjective night (Figure 2.2). In fact, across three circadian cycles, number of brief awakenings varies across time in a similar manner. On the other hand, in two of the circadian clock mutants tested that render the clocks dysfunctional (cyc^{01} and *timeless*, tim^{01}), number of brief awakenings does not cycle across the circadian day (Figure 2.2). Intriguingly, flies carrying a mutation in *period* gene that shortens their free-running period, per^{s}

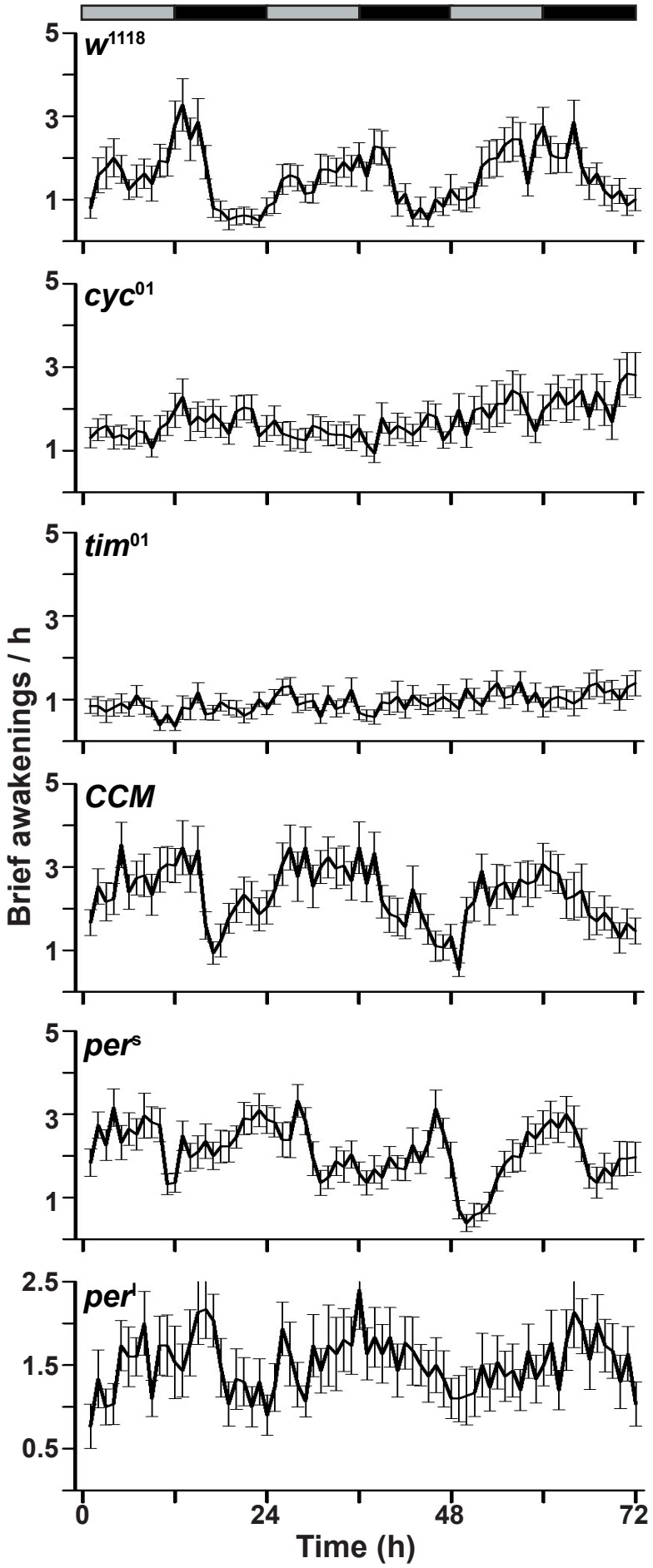
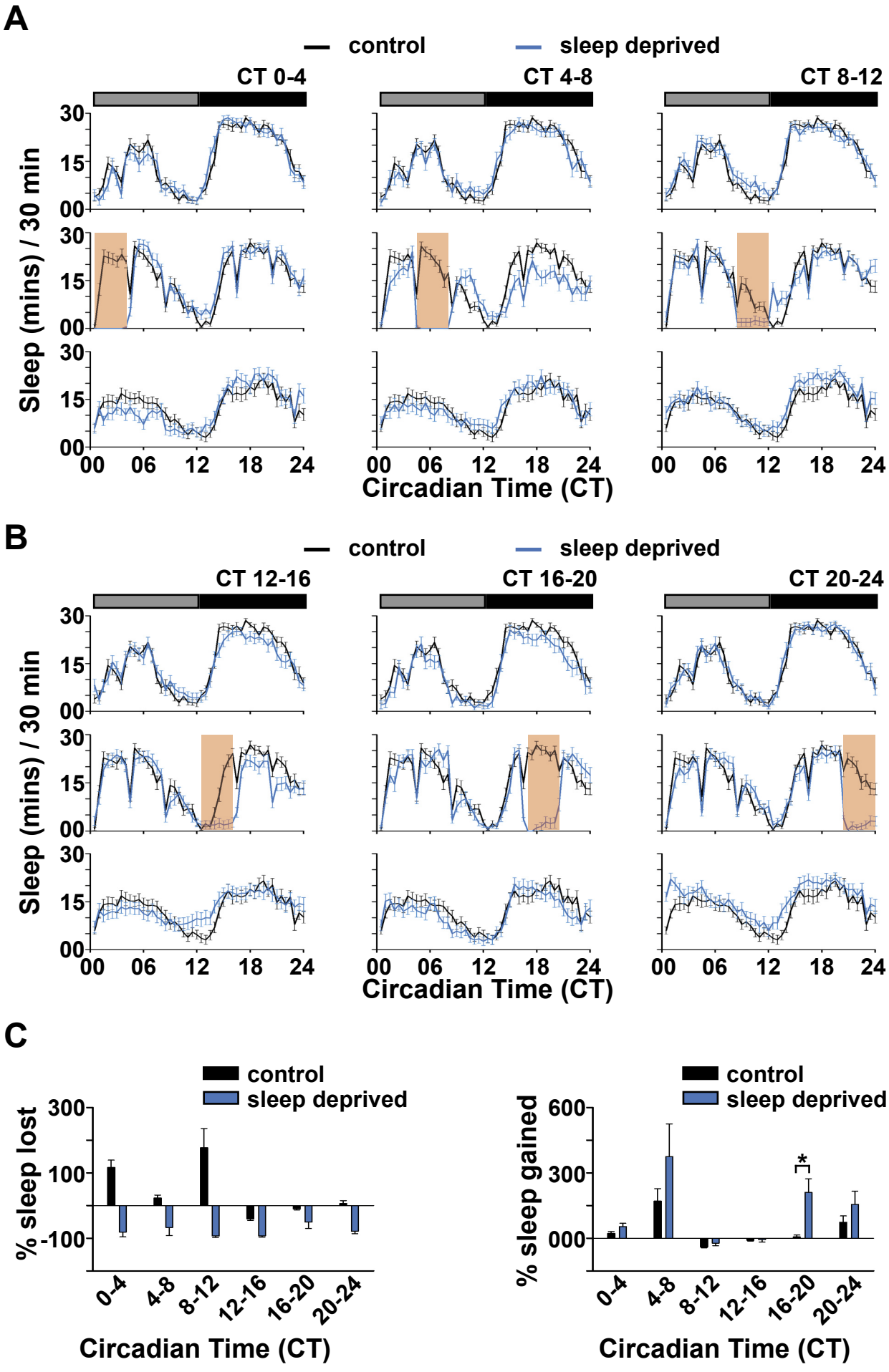


Figure 2.2. Number of brief awakenings varies across circadian day. Number of brief awakenings per hour across time is plotted for three consecutive days in DD as indicated by grey (subjective day) and black (subjective night) bars on the top. Wild type w^{1118} (n = 29 flies) and *CCM* (n = 30 flies) flies show rhythmic variation in number of brief awakenings on all days. Circadian clock mutants cyc^{01} (n = 32 flies) and tim^{01} (n = 31 flies) do not show rhythmic variation in number of brief awakenings on any day. per^{s} (n = 31 flies) and per^{l} (n = 30 flies) flies show altered period and amplitude in number of brief awakenings rhythms. y-axis scale of the brief awakenings graph of per^{l} is different so as to aid in visualization of the low-amplitude rhythm. Experiment conducted once.

(Konopka and Benzer, 1971), show four cycles of number of brief awakenings whereas their background control *CCM* flies (Gogna et al., 2015) show three cycles in three days. Indeed, in long period per^{l} mutants (Konopka and Benzer, 1971), the number of cycles does not differ, though the amplitude of cycling of number of brief awakenings is reduced (Figure 2.2) which could be due to long periods being often associated with less robust rhythms (Dowse and Ringo, 1987). These results suggest that cycling in sleep continuity (which can be considered an output of the sleep homeostat) requires the presence of circadian clocks in order to cycle with appropriate period and amplitude.

In mammals, typically, slow wave activity (SWA) during NREM sleep, which are composed of EEG slow waves in the 0.5-4.5 Hz frequency range, is a robust indicator of wake-dependent sleep propensity and therefore can act as a reliable marker of the sleep homeostat (Steriade et al., 2005). It is possible and relatively easier to record changes in SWA while simultaneously changing sleep states by depriving sleep and inducing sleep recovery. Moreover, measures such as melatonin levels and body temperature can reveal phases of the underlying circadian clocks, thereby enabling independent observations of distinct effects of sleep homeostat and circadian clocks on sleep/wake cycles. However, in invertebrates, behavioural arousal is an output of both circadian clocks as well as the sleep homeostat and thus, distinguishing distinct effects of these processes becomes challenging. Nevertheless, certain features are typically homeostat-specific or clock-driven, for instance, sleep recovery is strictly a sleep homeostat feature, and therefore can be used to measure wake-dependent sleep propensity; while period, phase and robustness of activity/rest rhythms are clock-driven properties which can reveal properties of wake-independent sleep propensity. Therefore, in order to test the interaction between the two processes, I tested the effect of circadian clocks on wake-dependent sleep propensity and examined the nature of

Figure 2.3



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rebound sleep when flies were deprived of sleep at different phases of the circadian cycle.

I subjected wild type (widely used *Iso*31 or w^{1118}) flies to sleep deprivation by mechanical perturbation with the help of a vortexer at different time intervals for 4 hours. These flies were previously entrained to Light/Dark cycles of 12 hours each (LD 12:12) and were then transferred to constant darkness (DD) for three days. The first day in DD served as a day of recording baseline sleep levels following which sleep deprivation was imposed on different sets of flies at different times on the second day. I observed that sleep deprivation at each of the different time windows of 4 hours each, resulted in sleep rebound when measured during the next 4-5 hours. This recovery did not continue till the third day of recording (Figure 2.3A-B). I defined % sleep lost as the amount of decrease in sleep during sleep deprivation relative to sleep levels during that same interval on the baseline day. Sleep deprivation imposed at different time windows resulted in similar percent of sleep loss, all of which were different from their respective undisturbed controls (Figure 2.3C-left, two-way ANOVA, $F_{5, 339} = 12.07$, $p < 0.00001$). In undisturbed control flies, apart from CT 0-4 (Circadian Time, where CT 0 refers to start of day as determined by lights-ON time of previously imposed LD cycle) and CT 8-12 when sleep levels seemed to have increased, sleep levels were not different on the second day compared to the baseline day (Figure 2.3C-left). % sleep gained was defined as the amount of sleep gained during the subsequent 4 hour time

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Figure 2.3. Time of sleep deprivation determines sleep rebound quantity. (A) Sleep per 30 minutes across time of day in three consecutive days in DD. On second day, sleep deprivation treatment (orange shading) was imposed for 4 either during early (CT 0-4), middle (CT 4-8) or late (CT 8-12) subjective day. (B) Sleep per 30 minutes across time of day with sleep deprivation occurring on second day either during early (CT 12-16), middle (CT 16-20) or late (CT 20-24) subjective night. Control flies remain undisturbed throughout. (C) Percent sleep lost during the window of sleep deprivation and percent sleep gained in the 4 h window after sleep deprivation indicate that sleep is lost similarly in all time windows, but sleep gained is significantly higher than controls only for deprivation during CT 16-20. $*p < 0.05$. Error bars are SEM. *n* between 27-32 flies for all time-point and treatment combinations. Experiment conducted three times, data from a single experiment.

Window following the sleep deprivation window relative to sleep during the same interval on the baseline day. Thus, % sleep gained for sleep deprivation during CT 0-4 was obtained by calculating sleep increase during CT 4-8 (immediately after sleep deprivation) normalized to sleep during this interval (CT 4-8) on the baseline day. I found that % sleep gained is significantly different from day-to-day changes in sleep levels (as seen from % sleep gained for undisturbed controls) only when sleep was deprived during CT 16-20 (Figure 2.3C-right, two-way ANOVA, $F_{5, 355} = 1.96$, $p = 0.08$; since time window * treatment interaction was marginally significant, *post-hoc* Tukey's test was conducted which showed $p < 0.05$ for comparison between control and sleep deprived flies at CT 16-20). While it appears that % sleep gained was also high for deprivation during CT 4-8, this was not significantly different from day 1 to day 2 changes in sleep level during the subsequent interval (CT 8-12) in control flies (Figure 2.3C-right). These results show that depending upon the circadian phase, similar levels of sleep deprivation elicit different homeostatic response – sleep deprivation during middle of subjective night results in significant sleep recovery in the subsequent 4 hours, whereas sleep deprivation at other times of the subjective day and night do not result in significant sleep rebound.

2.3.2. Quality of sleep rebound depends upon time of sleep deprivation. Sleep recovery after deprivation can occur either in terms of increase in sleep duration or sleep intensity (Borbély, 1982). Typically, number of brief awakenings is expected to be lower when sleep intensity is higher. To test if sleep deprivation at different time windows in DD results in differential intensity of recovery sleep, I quantified the mean number of brief awakenings for 4 hours after the sleep deprivation interval in w^{1118} flies. I found that in the 4 hours following sleep deprivation, the mean number of brief awakenings was reduced as compared to control flies only when sleep deprivation was

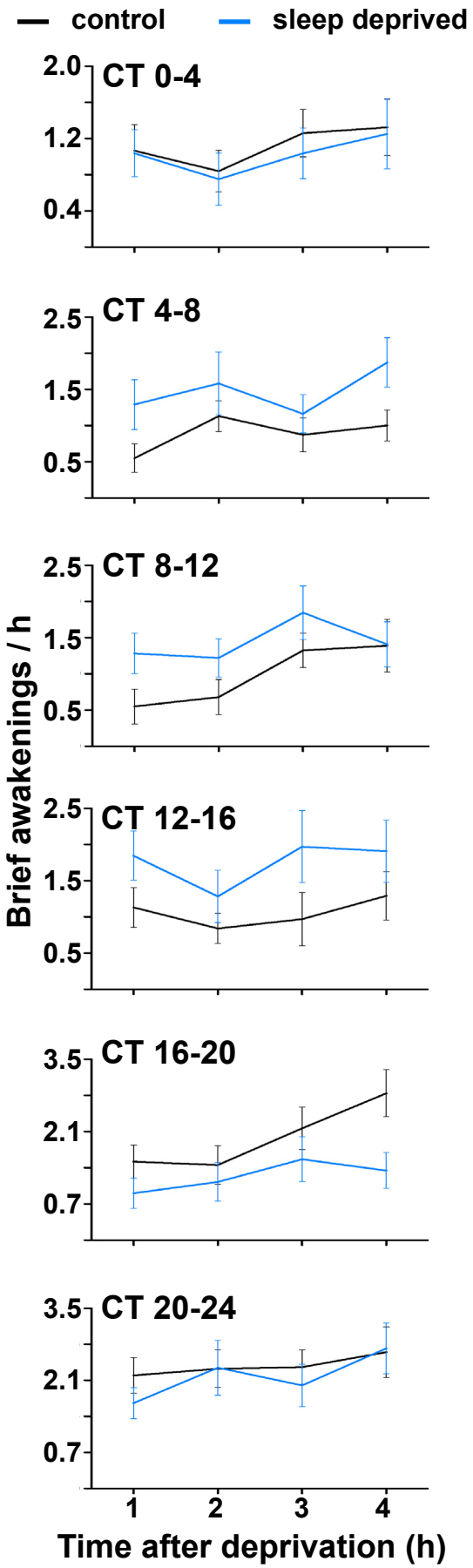


Figure 2.4. Number of brief awakenings does not alter with sleep deprivation during different time windows. Brief awakenings per hour plotted for four consecutive hours after sleep deprivation during indicated time windows for undisturbed control and sleep deprived w^{1118} flies. All other details are as in Figure 2.3.

imposed at CT 16-20, however, this reduction was not statistically significant (Figure 2.4, two-way ANOVA, $F_{3, 244} = 0.9$, $p = 0.44$). Moreover, I also found that sleep deprived flies did not show any reduction in number of brief awakenings during sleep recovery after 12 hours of sleep deprivation the previous night (data not shown). Thus, number of brief awakenings did not reveal differences between intensity of recovery sleep due to deprivation at different circadian phases.

I used another approach to estimate recovery sleep intensity whereby I assessed behavioural responses in terms of activity counts in response to exposure to brief light pulses (lasting 2 minutes) delivered one hour into recovery sleep following sleep deprivation at different time windows. Thus, flies previously entrained to LD cycles were subjected to sleep deprivation during different time intervals on the first day of DD, and light pulses were given at CTs 5, 9, 13, 17, 21 and 25 (or CT 1 of day 2 in DD). As seen before, sleep deprivation during CT 0-4, 4-8, 16-20 and 20-24 appear to result in increased sleep following sleep deprivation, whereas deprivation during CT 8-12 and CT 12-16 does not seem to result in increased levels of sleep after deprivation (Figure 2.5A). Importantly, both sleep deprived and control flies respond to brief light pulses with a sudden decrease in sleep, however the extent to which sleep dips, especially among the sleep deprived flies changes with time of day (Figure 2.5A). Furthermore, undisturbed control flies respond to the brief light pulses at all time points (except at CT 13) with a significant increase in activity in the 5-min interval after the light pulse, as compared to the 5-min interval before it (Figure 2.5B, Appendix 2.1A). Importantly, flies sleep deprived during CT 4-8, 12-16 and 20-24 also show an increase in activity in response to the light pulse given an hour later, however, these responses are not as high as that of undisturbed controls (Figure 2.5B-top middle, bottom left and right panels, Appendix 2.1A). Furthermore, flies deprived of sleep during CT 0-4 and

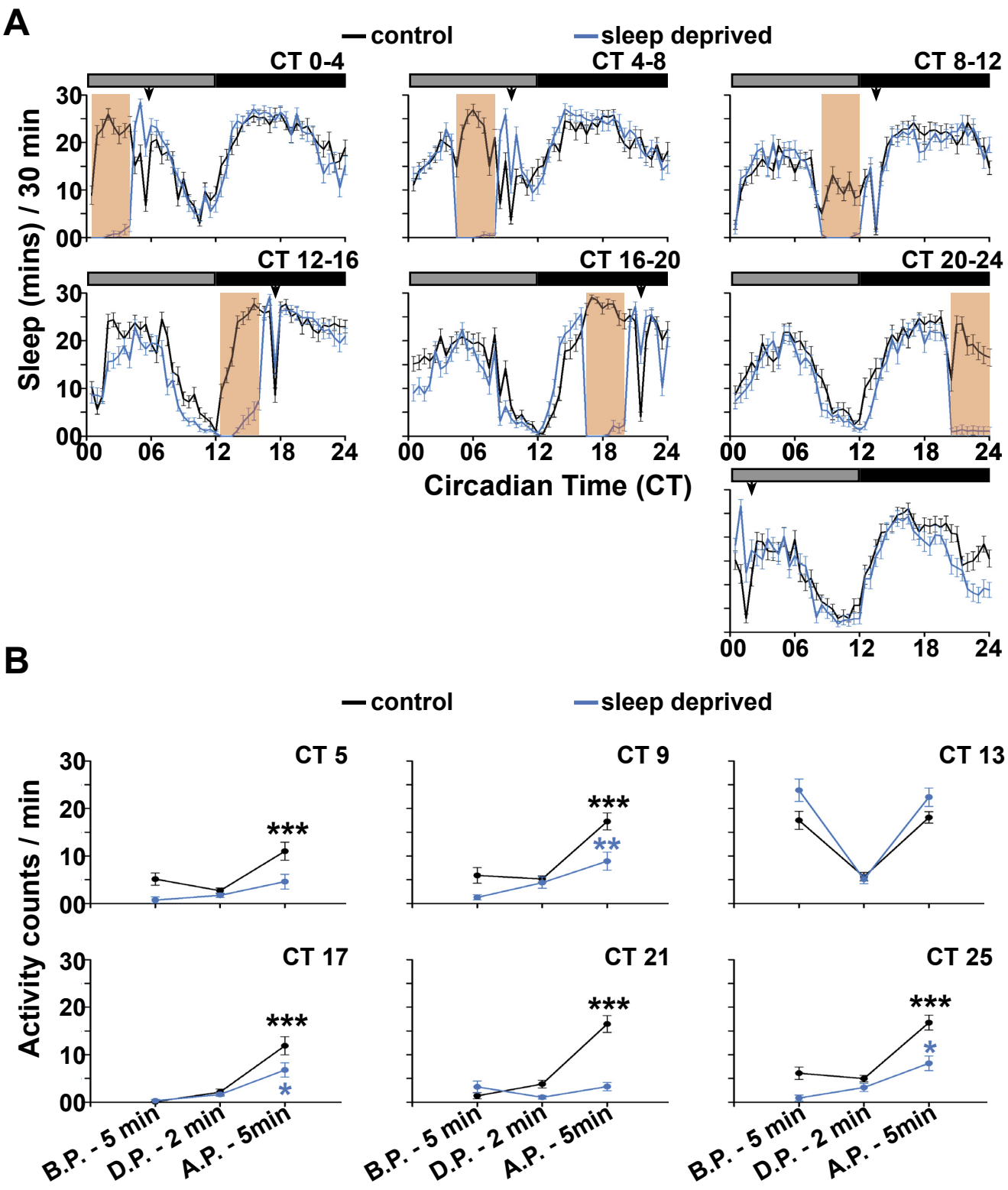


Figure 2.5. Time of sleep deprivation determines sleep rebound quality. (A) Sleep per 30 minutes across time of day for w^{1118} flies deprived of sleep during indicated time windows along with their respective controls. Orange shading indicates timing of 4 h of sleep deprivation. Arrowheads indicate timing of brief light pulse lasting 2 minutes. For deprivation during CT 20-24, light pulse delivered at CT 25, i.e. CT 1 of next day (second graph). (B) Activity levels for five minutes before (B.P.), two minutes during (D.P.) and five minutes after (A.P.) light pulse for flies deprived during indicated time windows along with respective controls. Asterisks indicate significant increase in five minute activity after light pulse compared to five minute activity before light pulse. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$. *n* between 30-32 flies for all time-point and treatment combinations. All other details as in Figure 2.3. Experiment conducted once.

CT 16-20 do not respond to the light pulse as they fail to show an increase in activity following light pulses at CT 5 and CT 21 respectively (Figure 2.5B-top left, bottom middle panels, Appendix 2.1A). However, at CT13, because the activity level prior to the light pulse itself is high, that light pulse does not result in further increase in activity in both control and flies deprived of sleep during CT 8-12 (Figure 2.5B-top right, Appendix 2.1A). Moreover, flies sleep deprived at different time windows also show different sleep latencies after the light pulse, with flies deprived of sleep during CT 4-8, 8-12 and 12-16 taking as much time as the undisturbed controls to initiate sleep (Figure 2.6A, two-way ANOVA, $F_{5, 368} = 2.64$, $p < 0.05$), whereas flies sleep deprived during CT 0-4, 16-20 and 20-24 took lesser time to initiate sleep after the light pulse as compared to their undisturbed controls (Figure 2.6A). Interestingly, when a similar experiment at four different time windows was conducted in female flies, flies deprived of sleep during CT 4-8, CT 12-16 and CT 16-20 showed similar sleep latencies as their controls, while those deprived of sleep during CT 0-4 took lesser time to fall asleep after light pulse as compared to their controls (Figure 2.6B, two-way ANOVA, $F_{3, 236} = 3.73$, $p < 0.05$). However, when the sleep latencies of female flies deprived of sleep during CT 16-20 were compared with their controls alone, they showed a significant decrease (Student's two-tailed *t*-test, $T_{0.05, 2, 51} = 2.85$, $p < 0.05$). Thus, to a certain degree, recovery sleep intensity of female flies is similar to that seen in male flies. These results point toward the conclusion that recovery sleep intensity also differs depending upon time of sleep deprivation.

2.3.3. Functional circadian clocks are necessary for time-of-day-dependent sleep rebound. Given that mutations in core clock genes impair some aspects of sleep homeostat in mice (reviewed in (Landgraf et al., 2012)) and my own results of time-dependent homeostatic responses, I next asked whether any sleep homeostatic features

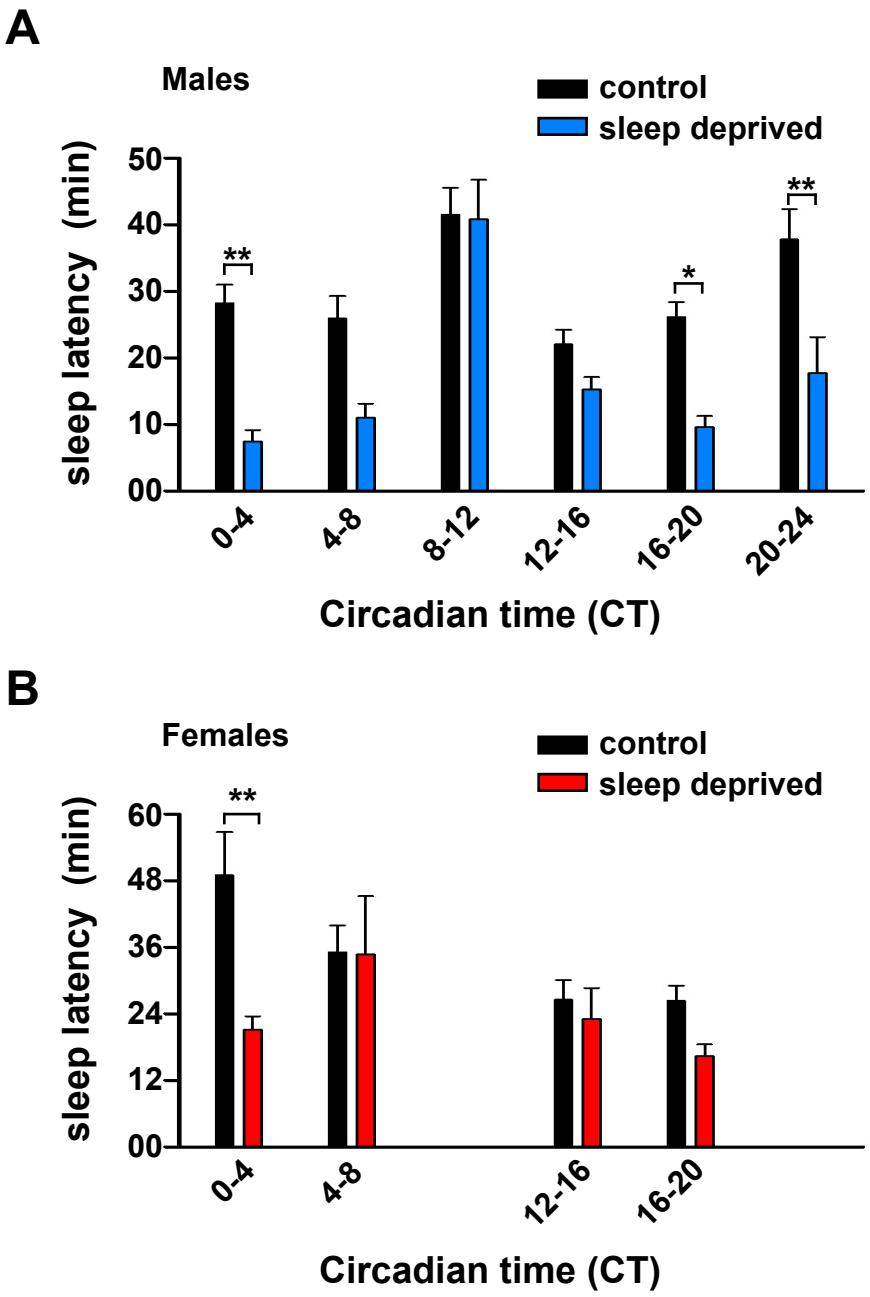


Figure 2.6. Sleep latency after light pulse differs among flies deprived of sleep during different time windows. Amount of time taken after brief light pulse given one hour after sleep deprivation during indicated time windows for control and sleep deprived (A) males and (B) females. In females, experiment was conducted during only four time windows. *n* between 28-32 flies in experiments with females for all treatment and time-point combination. All other details are as in Figure 2.5.

are disrupted in circadian clock mutants. As mentioned earlier, in flies, mutation in *cyc* results in excessive sleep recovery following sleep deprivation (Shaw et al., 2002), whereas mutation in *tim* was shown to result in decreased amount of sleep rebound in response to sleep deprivation (Hendricks et al., 2000a). I subjected cyc^{01} and tim^{01} flies as well as their background control w^{1118} flies to 12 h of sleep deprivation during the night (dark phase of LD 12:12) and found that while all genotypes lose similar amounts of sleep (75-85%), they recover different amounts. Sleep deprived w^{1118} flies show increased sleep for almost 6-8 h during the subsequent day and recover almost 75% sleep as compared to the undisturbed controls (Figure 2.7A-B, Student's two-tailed *t*-test, $T_{0.05,\ 2,\ 36} = -2.99$, $p < 0.005$). Unlike previous studies, we find that both cyc^{01} and tim^{01} flies recover lost sleep, albeit to different levels – cyc^{01} flies sleep more for about 6 h in the subsequent day and recover about 25% sleep as compared to undisturbed controls (Figure 2.7A-B, Student's two-tailed *t*-test, $T_{0.05,\ 2,\ 42} = 5.41$, $p < 0.00001$), while tim^{01} flies sleep more for about 4-5 h during the next day and recover about 66% sleep as compared to their undisturbed controls (Figure 2.7A-B, Student's two-tailed *t*-test, $T_{0.05,\ 2,\ 43} = -3.32$, $p < 0.005$). The reduced level of sleep rebound could be explained by higher baseline level of day-time sleep in undisturbed controls of both the mutants, thereby resulting in seemingly lower sleep rebound. Contrary to previous reports, the amount of recovery sleep seen in cyc^{01} flies is lower than that in w^{1118} flies, and tim^{01} flies do show a significant sleep rebound which is comparable to w^{1118} flies.

Since the sleep homeostat builds up and discharges sleep pressure as the animal remains awake and falls asleep respectively, i.e. sleep pressure itself is cycling, it is possible that sleep homeostat itself could be functioning like an hour-glass and generating this rhythm, especially in the presence of environmental time cues. To address this possibility, I next asked how mutations in core clock genes that result in the

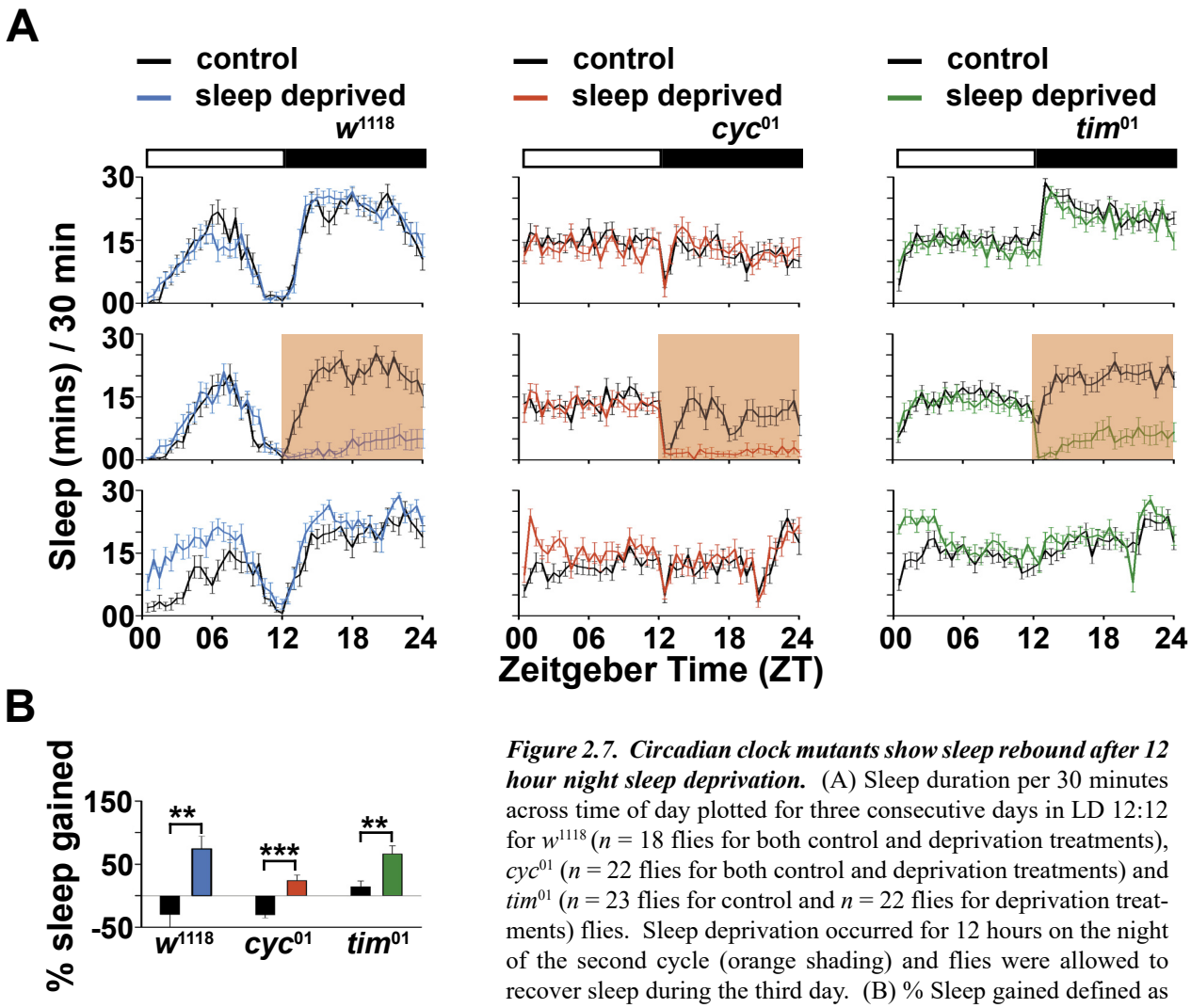


Figure 2.7. Circadian clock mutants show sleep rebound after 12 hour night sleep deprivation. (A) Sleep duration per 30 minutes across time of day plotted for three consecutive days in LD 12:12 for w^{1118} (n = 18 flies for both control and deprivation treatments), cyc^{01} (n = 22 flies for both control and deprivation treatments) and tim^{01} (n = 23 flies for control and n = 22 flies for deprivation treatments) flies. Sleep deprivation occurred for 12 hours on the night of the second cycle (orange shading) and flies were allowed to recover sleep during the third day. (B) % Sleep gained defined as amount of increase in sleep on the third day during ZT 0-6 relative to sleep levels during the same interval on BS day plotted for different genotypes. Black and coloured bars represent change in sleep levels for controls and sleep deprived flies respectively. *p < 0.05, **p < 0.005, ***p < 0.0005. Experiment conducted once.

disruption of the functional clock affect sleep pressure as measured by amount of sleep rebound across time of the day in LD 12:12 cycles. I also examined number of brief awakenings across time of day as a marker of sleep continuity as mentioned before. In wild type w^{1118} flies with intact sleep homeostat and a ticking circadian clock, the sleep/wake cycle is rhythmic (Figure 2.8A) and importantly, the number of brief awakenings varies across time of the day. The number of brief awakenings during the day is higher than during the night (Figure 2.8B, Appendix 2.1B). Furthermore, as seen in DD (Figure 2.3), even in a cyclic environment, quantity of sleep rebound varies depending upon time of sleep deprivation – in LD 12:12, sleep deprivation in the late night window (ZT 20-24) alone results in significant sleep rebound in the subsequent 4 hours (Figure 2.8C, Appendix 2.1C). In cyc^{01} flies, which have been previously shown to be night-active (Kumar et al., 2012), sleep was equally distributed between day and night (Figure 2.8A). Importantly, the number of brief awakenings as well as sleep rebound do not vary across time of day in the cyc^{01} flies (Figure 2.8B-C, Appendix 2.1B-C). Interestingly, clk^{JRK} flies, another mutant which was previously found to be night-active (Kumar et al., 2012), showed lower sleep during night as compared to day, yet the number of brief awakenings during day was significantly higher than those during night (Figure 2.8A-B, Appendix 2.1B). However, even though the temporal profile of number of brief awakenings is similar to w^{1118}, clk^{JRK} flies do not show significant sleep rebound due to deprivation at any of the time windows (Figure 2.8C, Appendix 2.1C). Importantly, tim^{01} flies show lesser sleep during day as compared to night (Figure 2.8A), higher number of brief awakenings during day as compared to night (Figure 2.8B, Appendix 2.1B), yet fail to show significant sleep rebound due to sleep deprivation during any time interval (Figure 2.8C, Appendix 2.1C). Just like tim^{01} flies, $per^{0}w$ flies also show differences in levels of sleep and brief awakenings between

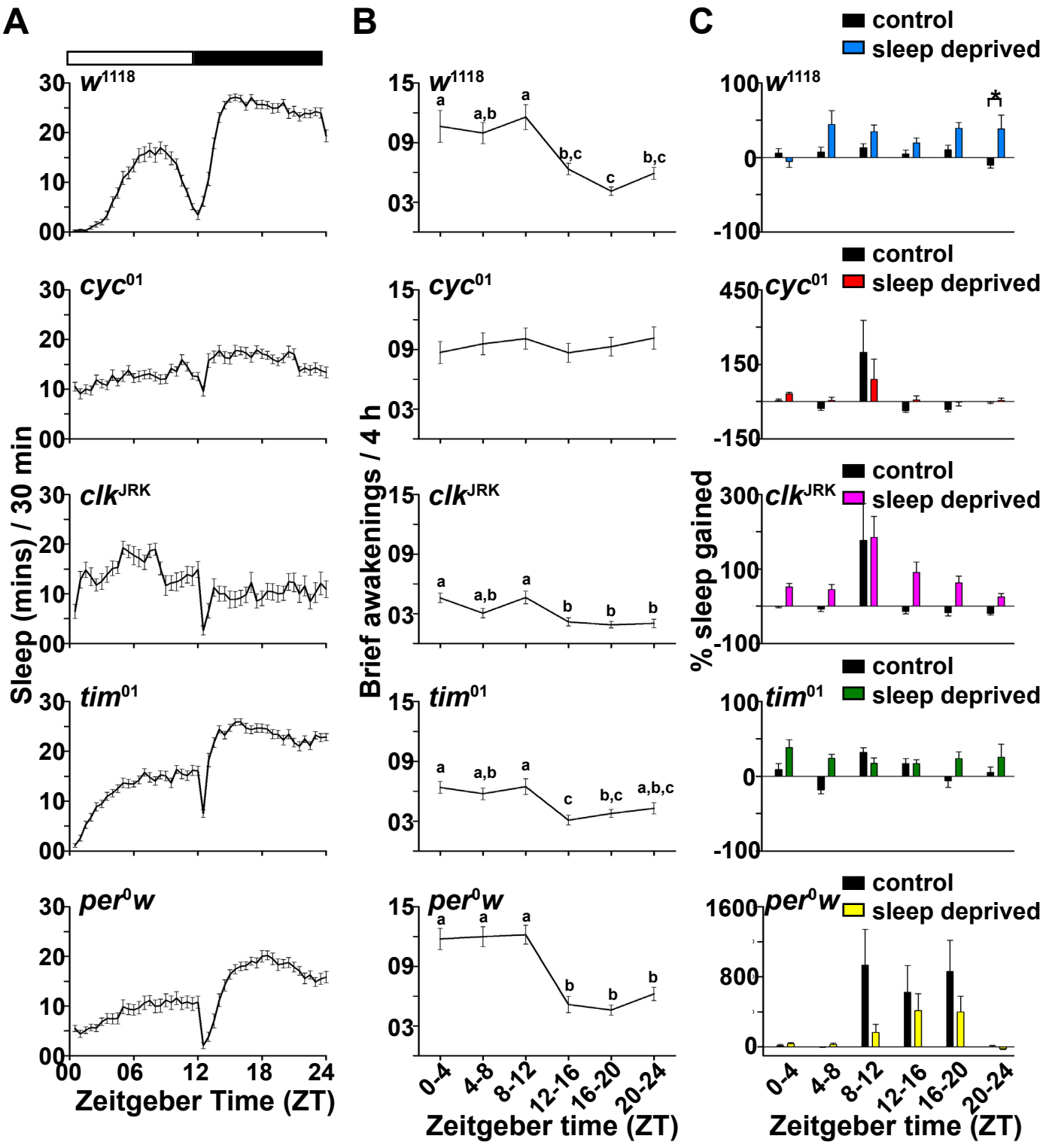


Figure 2.8. Circadian clock mutants show impairments in sleep homeostatic properties. (A) Sleep per 30 minutes across time of day, (B) number of brief awakenings at different time windows and (C) sleep gained due to deprivation at different time windows under LD 12:12 in wild type w^{1118} flies and circadian clock mutants cyc^{01}, clk^{JRK}, tim^{01} and $per^{0}w$. Number of brief awakenings higher during day windows versus during night windows in all but cyc^{01} flies. clk^{JRK} flies show low-amplitude but significant cycling in number of brief awakenings. Different letters indicate significant differences across time windows. (C) Percent sleep gained in subsequent 4 h after deprivation in indicated time windows. Only w^{1118} show significant sleep rebound when deprived during ZT 20-24. Clock mutants do not show time-of-day-dependent response to sleep deprivation. Both clk^{JRK} and $per^{0}w$ flies show high amount of day-to-day changes in sleep, perhaps owing to their genetic backgrounds. Experiment conducted two times for w^{1118}, cyc^{01} and tim^{01}; and one time for clk^{JRK} and $per^{0}w$. $n = 16$ flies for all genotypes and time-point and treatment combinations. All other details are as in Figure 2.3.

day and night (Figure 2.8A-B, Appendix 2.1B). These results suggest that in two of the four clock mutants (*tim*^{01} and *per*^{0}*w*) sleep/wake profile reflects the way in which sleep continuity (as assessed by number of brief awakenings) builds up and gets discharged and this is very similar to the wild type flies. Yet, neither of the mutants show a significant sleep rebound in response to deprivation at any of the 4 h time windows across the 24 h cycle (Figure 2.8C). This suggests that the sleep homeostat is unable to sense sleep deprivation occurring for a short duration of 4 hours in these mutants. However, the sleep homeostat can sense and respond to deprivation for a longer duration of 12 hours at least in *tim*^{01} flies (Figure 2.7). In *clk*^{JRK} flies however, while number of brief awakenings builds up and gets discharged similar to wild type flies (albeit with a smaller amplitude), the sleep/wake profile is reversed in comparison, which suggests a defect perhaps at the level of output pathways. Furthermore, in *cyc*^{01} flies, number of brief awakenings, sleep rebound and sleep/wake do not cycle across the day suggesting that sleep pressure of *cyc*^{01} flies does not cycle across the day thereby re-inforcing this gene's pleiotropic effect on sleep homeostat (Shaw et al., 2002). Taken together, in flies, dysfunctional circadian clocks can affect the sleep homeostat, albeit to varying degrees.

2.3.4. Sleep deprivation during any time window does not alter circadian clock properties. Given that circadian clock mutations result in changes in sleep homeostatic properties, I next asked how the sleep homeostat might affect circadian clock properties. I subjected flies to sleep deprivation during different times of the circadian day and assessed how core clock properties such as period, phase and robustness (as estimated from amplitude of periodogram) changed from the pre-deprivation to the post-deprivation days. I found that neither period (Figure 2.9A, top, one-way ANOVA, $F_{3, 92} = 1.5$, $p = 0.22$; bottom, one-way ANOVA, $F_{3, 79} = 1.15$, $p = 0.33$), nor robustness

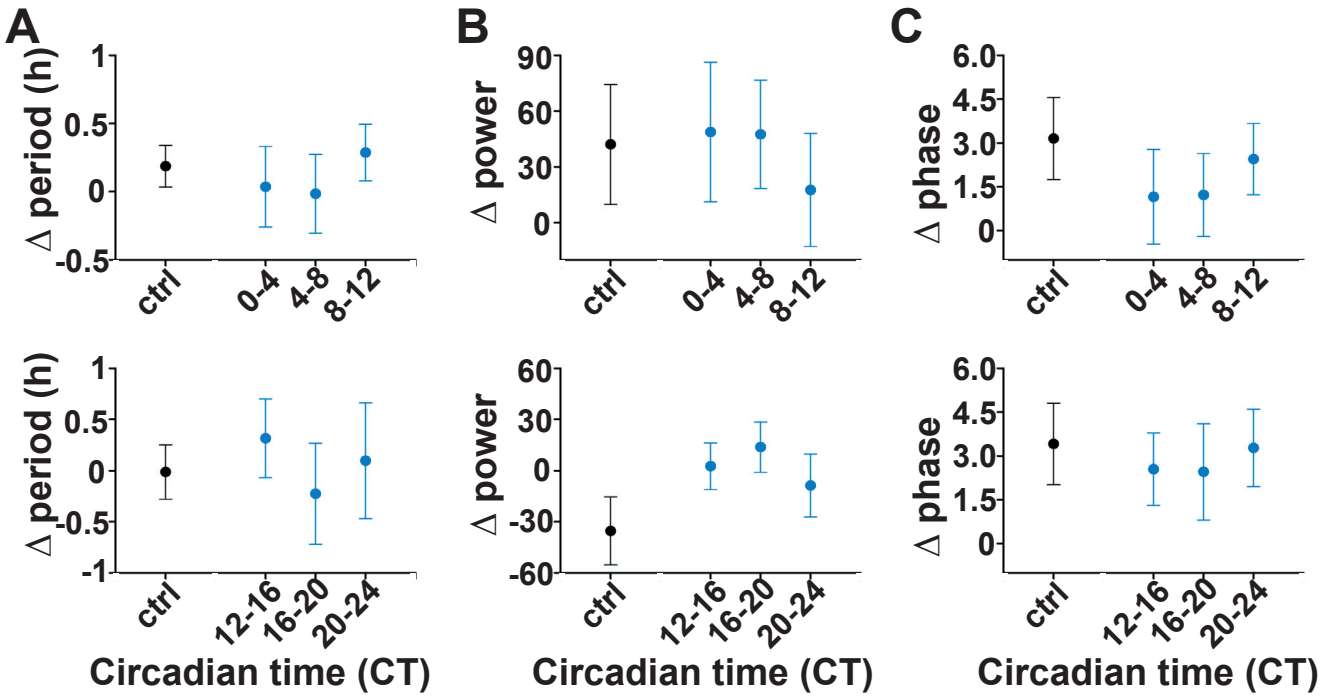


Figure 2.9. Circadian clock properties show no changes due to sleep deprivation during different times of day. Change in (A) period, (B) power and (C) phase from pre-deprivation interval to post-deprivation interval plotted for undisturbed controls as well as flies sleep deprived during indicated time windows. Period and power obtained from Chi-square periodogram. Average offset phases of last four days within pre- and post-deprivation intervals used for calculating change in phase. Error bars are 95% confidence intervals. Change in power of controls and flies deprived during CT 12-16 and CT 16-20 are significantly different; however, this was not replicated in another experiment. *n* between 19-28 flies. Experiment conducted two times, data from a single experiment. Top and bottom panels are data from different experiments. All other details are as in Figure 2.3.

(Figure 2.9B, top, one-way ANOVA, $F_{3, 92} = 0.95$, $p = 0.42$; bottom, one-way ANOVA, $F_{3, 75} = 6.02$, $p < 0.005$; however this result was not replicated) nor phase (Figure 2.9C, top, one-way ANOVA, $F_{3, 92} = 1.99$, $p = 0.12$; bottom, one-way ANOVA, $F_{3, 79} = 0.53$, $p = 0.66$) changed due to deprivation during any of the time windows any more or less from the undisturbed controls. This shows that, in flies, sleep deprivation for short duration of 4 hours does not affect circadian clock properties, suggesting that sleep homeostat may not feedback on to the circadian clock. Furthermore, it suggests that perhaps the sleep homeostat does not influence the wake-independent sleep propensity generated by the circadian clocks, since core clock properties do not change with sleep deprivation for 4 h intervals.

2.4. Discussion

Since the time of its conception, the two-process model has served as an excellent framework to study sleep/wake regulation and research spanning three decades has established the model as the underlying mechanism of sleep/wake regulation in mammalian species (Borbély et al., 2016). Since invertebrate sleep research is relatively new, the two-process model has not been as popular in addressing questions based on mechanism of sleep/wake regulation in invertebrates as in mammals. Here, I examined the nature of interaction of circadian clocks and sleep homeostat in order to address one of the assumptions of the two-process model by using behavioural criteria to probe the underlying sleep pressure of flies. I have used a previously reported measure for sleep intensity – number of brief awakenings as readout of the sleep homeostat. Number of brief awakenings is higher during the day than during the night in wild type flies as well as in all circadian clock mutants except cyc^{01}. I have also used recovery sleep as a marker of sleep propensity dependent upon

prior wake time, which according to the two-process model is strictly a homeostatic feature. I found that while wild type flies tend to recover sleep depending upon time of sleep deprivation either in the presence or absence of external time cues, none of the circadian clock mutants tested show time-dependent sleep rebound response, suggesting that the homeostatically regulated sleep propensity in circadian clock mutants is impaired. Different clock mutants have similar effects on circadian clock properties, but affect the sleep homeostat in different ways, suggesting that these genes are perhaps involved in different levels of the sleep/wake organization.

In mammals, different stages of sleep can easily be distinguished on the basis of cortical oscillations recorded from the whole brain, and we can make inferences about the intensity of sleep on the basis of power of oscillations comprising the slow wave activity (Steriade et al., 2005). In the recent past, local field potentials recorded from tethered flies whose movement on a foam ball can also be tracked to gauge behavioural states, has provided useful insights about the presence of multiple sleep stages in *Drosophila* (van Alphen et al., 2013; Yap et al., 2017). Here I found that both homeostatic features of sleep rebound quality and quantity depend upon circadian phase during which sleep deprivation is imposed. Specifically, sleep deprivation during middle of subjective night (CT 16-20) results in sleep rebound both in duration and intensity, whereas sleep deprivation during early subjective day (CT 0-4) and late subjective night (CT 20-24), results in sleep rebound only in terms of intensity (Figures 2.3, 2.5, 2.6). Sleep deprivation during middle of subjective day (CT 4-8) also results in sleep rebound through intensity but to a lesser extent (Figures 2.5, 2.6). Sleep deprivation either during late subjective day (CT 8-12) or early subjective night (CT 12-16) does not result in sleep rebound either through duration or intensity (Figures 2.3, 2.5, 2.6). These results suggest that flies inherently go through different levels of sleep

depth, which occur depending upon time of day. Thus, the "deepest" stage of sleep occurs during early subjective day and mid-subjective night, when sleep is most indispensable, whereas sleep during mid- and late subjective day as well as early subjective night is of a lesser intensity as it is less indispensable. Sleep during other times maybe of an intermediate depth. These findings are in contrast to the definition of deep sleep on the basis of electrophysiological recordings, according to which deep sleep occurs mostly during early night (Faville et al., 2015; van Alphen et al., 2013). Here, we provide a new behavioural criterion to measure different stages of fly sleep, specifically indispensable sleep, i.e. an intense sleep stage that when lost causes more of a sleep rebound. In accordance with this criterion, we find that circadian clock mutants do not show different stages of indispensable sleep.

The behavioural criteria that I have employed to measure sleep pressure are consistent with previous documentation of activity rhythm features of *Drosophila* in both LD and DD conditions (reviewed in (Allada and Chung, 2010)). For instance, sleep during time windows around the evening peak of activity, both in LD and DD (ZT or CT 8-12 and 12-16) is minimal in amount, and if it occurs, then it is of poor quality. This is because, apart from not making up for lost sleep during these intervals, flies also display high number of brief awakenings and low arousal thresholds. In fact, at ZT 12 and CT 12, I hardly ever found sleeping flies; and if there were, they would respond to either mechanical stimuli of any duration or brief light pulse with high levels of activity. Several interesting features about sleep intensity at different time intervals of the day are revealed from these behavioural criteria. For example, sleep during early subjective day is of higher intensity as compared to middle subjective day. While several flies are awake during CT 0, at CT 4 very few flies respond to mechanical stimuli, and at CT 5, very few flies sleep deprived during early subjective day respond

to a brief light pulse. On the other hand, at CT 8, all flies recorded respond to mechanical stimuli, and at CT 9, most of the flies sleep deprived during middle of subjective day (CT 4-8) respond to light pulse. However, in LD conditions, sleep during early and middle of day are similar in intensity as there are no differences in number of flies responding to mechanical stimuli at either time-points. Yet another intriguing feature is sleep intensity during late night. It appears that flies are sleeping less intensely as the arousal thresholds at late night is lower (ZT 20) than it is at middle of the night (ZT 16), yet sleep deprived during ZT 20-24 gets maximally recovered. Perhaps sleep during this interval consists of both "dispensable" and "indispensable" stages and further experimentation within this interval will shed more light. It would also be fruitful to examine the electrophysiological features as an intermediate depth of sleep has a greater probability of occurring during this time window as can be predicted from these behavioural data.

In mammals, there exists extensive evidence for sleep homeostat and circadian clocks interacting and influencing each other and their functions (Borbély et al., 2016; Landgraf et al., 2012). Here, I showed that circadian clocks influence the cycling of wake-dependent sleep propensity, which under the two-process model is completely under homeostatic control. Furthermore, consistent with a previous study (Shaw et al., 2002), I also found that a circadian clock gene has pleiotropic effects on sleep homeostatic properties. Thus, even though circadian clock cogs and gears can impinge upon the sleep homeostat, these effects are modulatory in nature. Furthermore, in contrast to what was shown in mammals, in flies, circadian clocks perhaps do not receive feedback from the sleep homeostat, although the evidence pointing to this conclusion is limited. Nevertheless, it agrees well with previous reports in which it was shown that certain mutant flies with decreased sleep need do not have circadian clock

defects (Cirelli et al., 2005a; Koh et al., 2008; Kume et al., 2005). From an adaptive viewpoint, this suggests that *Drosophila* circadian clocks, are protected against changes in sleep states, whereas the responses of the sleep homeostat depends upon time of the day. Our results suggest that, perhaps different sleep functions may be achieved at different times of the day, thereby making sleep at certain times more vulnerable to deprivation. Nonetheless, my study focusing on the two-process model of sleep/wake regulation in flies, which to the best of my knowledge is the first of its kind, has revealed interesting features about the nature of sleep and its regulation in *Drosophila*, by relying on behavioural criteria of sleep. I believe that further studies combining molecular, electrophysiological and other behavioural signatures not examined here such as sleep-specific posture, can result in significant advances in the understanding of organization and regulation of sleep in invertebrates.

Chapter 3

Putative pathways from circadian clock to sleep homeostat in *Drosophila melanogaster*

3.1. Introduction

In the previous chapter, I showed that sleep homeostat is influenced by the circadian clocks as sleep deprivation at different times of the day had varying effects on producing a sleep rebound. Here, I asked how circadian clocks can modulate the sleep homeostat at the level of neuronal circuits in the *Drosophila* brain. Since the discovery of sleep behaviour of *Drosophila melanogaster* being similar to mammalian sleep in several aspects (Hendricks et al., 2000a; Shaw et al., 2000), many pathways and neuronal circuits involving sleep homeostat and circadian clocks have been uncovered. Genes such as *minisleep* (*mns*) and *hyperkinetic* (*hk*) encoding subunits of Shaker potassium channel function in the sleep homeostat (Bushey et al., 2007; Cirelli et al., 2005a). More recently, central complex structures such as dorsal fan-shaped body (dFB) (Donlea et al., 2014) and the ellipsoid body (EB) (Liu et al., 2016) have been shown to function as effector and modulator of the sleep homeostat respectively. Meanwhile, mutations in core circadian clock genes such as *Clock* (*clk*) and *Cycle* (*cyc*) have been shown to cause impaired timing of sleep and they tend to become nocturnal (Kumar et al., 2012). The circadian neuropeptide Pigment Dispersing Factor (PDF) and its receptor (PDFR) are involved in relaying wake-promoting signals from the circadian pacemaker ventral lateral neurons (LN_vs) (Chung et al., 2009; Parisky et al., 2008; Sheeba et al., 2008a) in response to light input (Shang et al., 2008) as well as dopamine and octopamine (Shang et al., 2011). While it has been suggested that the EB may be the downstream target of this wake-promoting PDF/PDFR signaling, the evidence in favour of the same is limited (Parisky et al., 2008).

In the recent past, in the quest to uncover output pathways of the circadian clocks that help in timing of sleep/wake cycles, a few dedicated circuits have been mapped. Most notably, sleep is suppressed at the end of the night by the action of PDF

on PDFR^{+} Dorsal Neuron 1 (DN_1) group of the circadian network which in turn secrete the wake-promoting neuropeptide Diuretic Hormone 31 (DH31) (Kunst et al., 2014). On the other hand, timing of sleep onset at the beginning of night is a function of increased inhibition of wake-promoting large LN_v (l-LN_v) by GABA (Liu et al., 2014). Furthermore, yet another group showed that DN_1s through glutamate modulate day-time siesta and night-time sleep by inhibiting the morning (small LN_v, s-LN_v) and evening (dorsal lateral neurons, LN_d) activity controlling circadian neurons (Guo et al., 2016). While this work was in progress, two studies showed that a subset of DN_1s project to the Tubular Bulbar neurons in the Anterior Optic Tubercle region, which in turn make synaptic contacts with the ring neurons of the EB, thereby underlining a pathway linking circadian clocks to a known sleep homeostatic centre (Guo et al., 2018; Lamaze et al., 2018).

Here, I addressed the question of modulation of sleep homeostasis by circadian timing at the neuronal circuitry level by adopting a two-pronged approach. My first approach was to probe sleep homeostatic roles for different subsets of circadian clock neurons. I found that activity of a subset of 4-6 lateral neurons represents a sleep deprived state as their activation alone results in sleep loss which is recovered upon removal of the activation. Furthermore, I found that circadian clocks within these neurons are not essential for carrying out their homeostatic role; therefore, they lie at the interface of clocks and sleep homeostat, as they have distinct independent functions in both processes. My next approach was to examine if any of the known sleep homeostatic neurons were downstream of the wake-promoting subset of PDF expressing LN_vs of the circadian clock network by altering the levels of *pdfr* expression in the sleep homeostat EB (Liu et al., 2016) and sleep-promoting dFB neurons (Donlea et al., 2011). My screen for putative downstream targets also included several other

subsets of neurons – namely, circadian neurons that are known to express *pdfr* (Hyun et al., 2005; Im and Taghert, 2010; Lear et al., 2005; Mertens et al., 2005) subsets of mushroom body (MB) neurons that are sleep- or wake-promoting (Cavanaugh et al., 2016; Joiner et al., 2006; Pitman et al., 2006), wake-promoting pars intercerebralis (PI) (Foltenyi et al., 2007) as well as aminergic neuronal groups, most of which are reported to be wake-promoting (Crocker et al., 2010; Kume et al., 2005). Strikingly, I found that a subset of dopaminergic neurons respond to changes in *pdfr* expression by changing the levels of day-time sleep – increasing *pdfr* levels decreases day-time sleep and vice-versa. Moreover, PDF^{+} and dopaminergic neurons were found to form synaptic contacts with one another, along with the possibility of the former inhibiting the latter. Indeed, in a previous study it was found that dopaminergic neurons communicate with sleep homeostatic EB and dFB neurons. Thus, my results uncover two putative pathways that represent the anatomical correlates of circadian clock – sleep homeostat interaction.

3.2. Materials and methods

3.2.1. Fly strains. Fly strains used along with their source information is listed in Appendix 1. Briefly, flies were maintained on standard cornmeal medium under standard 12:12 hour Light/Dark (LD 12:12) cycles at 25 °C. All flies used for sleep measurements except *UAS Clk DN* and *UAS Cyc DN* have been back-crossed to the standard *Iso*31 or w^{1118} (BDSC # 5905) background for at least five generations. *Dvpdf GAL4*, $Pdfr^{5304}$, $Pdfr^{3369}$, *UAS Pdfr RNAi*, *UAS dicer*, *UAS Pdfr* and *TH GAL4* have been back-crossed to w^{1118} for 7 generations.

3.2.2. Sleep assays. 3-6 day old mated females were individually housed in glass tubes (65 mm length, 3 mm inner diameter) with sucrose medium (5% sucrose and 2% agar)

on one end and cotton plug on the other and activity was recorded in DAM2 monitors (*Drosophila* activity monitoring system, Trikinetics, Waltham, MA, USA). Mated females were used as per convention as well as because they show marked differences in their day-time and night-time sleep levels, and measuring their sleep on a sucrose medium did not result in interference from larval activity as it was seen that the eggs laid did not hatch into larvae. The DAM system works on the principle that whenever a fly crosses the middle of the tube, it breaks an infra-red beam which is detected by infra-red sensors and recorded as activity counts. Flies were housed in light and temperature controlled environments with 12 hours of light (~300-500 lux) and 12 hours of darkness (LD 12:12) at 25 °C in incubators (MIR-273, Sanyo, Osaka City, Osaka, Japan; DR-36VLC8 Percival Scientific Inc., Perry, IA, USA) for a period of 3 days. Activity was binned at 1 minute intervals and sleep parameters such as day-time and night-time sleep duration, bout length and number and activity per waking minute were estimated using PySolo (Gilestro and Cirelli, 2009), while sleep profiles and sleep latency were obtained from a custom-made Microsoft Excel spreadsheet template. Here too, as in Chapter 2, data analyses of experiments that were repeated multiple times were done independently. One run among replicates was arbitrarily chosen as a representative run only if all runs showed similar results.

3.2.3. Statistical Analysis. For experiment using *dTRPA1*-mediated (*Drosophila* Transient Receptor Potential A1) heat activation of clock neurons, percentage change in sleep was calculated as (total sleep during 21 °C – total sleep during 29 °C)/ (total sleep during 21 °C) *100. Percentage change in day-time sleep was calculated as difference between day-time sleep of experimental flies and parental control (*GAL4* or *UAS* control) flies normalized to day-time sleep of parental control flies. For comparison of sleep parameters, one-way ANOVA with genotype as fixed factor followed by *post-hoc*

Tukey's Honest Significant Difference (HSD) test was conducted. For comparison of GFP fluorescence intensity, two-way ANOVA with genotype and time-point as fixed factors followed by *post-hoc* Tukey's HSD test was conducted. Significance level for all tests was set at $p < 0.05$. Details of statistical analyses are given in results and Appendix 2.2.

3.2.4. Immunocytochemistry. Adult brains were dissected in ice-cold Phosphate Buffered Saline (PBS) and fixed immediately for 30-40 minutes in 4% paraformaldehyde. Fixed brains were blocked in 10% horse serum for 1 hour at room temperature and 6-9 hours at 4 °C, followed by incubation with cocktail containing primary antibodies for 48 hours. The primary antibodies used were anti-GFP (chicken, 1:2000, for GFP labeling and *CaLexA* measurements, Invitrogen #A10262), anti-PDF (mouse, 1:5000, DSHB, PDF C7), anti-MYC (mouse, 1:1000, Cell Signalling Technology, #9B11), anti-TH (rabbit, 1:1000, Invitrogen #P21962), anti-GFP (mouse, 1:500, for GRASP, Sigma-Aldrich #G6539) and anti-PDF (rabbit, 1:30,000, M. Nitabach and T.C. Holmes). Following 7-8 serial washes with 0.5% Triton X in PBS (0.5% PBT), brains were incubated with appropriate secondary antibodies for 24 hours. Secondary antibodies were used at a concentration of 1:3000 and they were anti-chicken-Alexa Fluor 488 (Invitrogen, #A11039), anti-mouse-Alexa Fluor 546 (Invitrogen, #A11003), anti-mouse-Alexa Fluor 488 (Invitrogen, #A11001) and anti-rabbit-Alexa Fluor 546 (Invitrogen, #A11035). Brains were washed with 0.5 % PBT and mounted on glass slides in 7:3 glycerol:PBS medium. Confocal images were taken on Zeiss LSM 880 (with Airyscan, ZEISS, Oberkochen, Germany) microscope either with 20X, 40X (oil immersion) or 63X (oil immersion) objectives.

3.3. Results

3.3.1. "Evening" neurons are involved in sleep homeostatic function. Given that circadian clock mutations resulted in alterations of sleep homeostatic properties as seen in the previous chapter (Figure 2.8), I asked whether any of the neurons within the circadian clock network play a role within the sleep homeostat. To do so, I transiently hyper-excited different subsets of clock neurons by expressing the heat-activated *Drosophila* Transient Receptor Potential A1 (*dTRPA1*) channel (Hamada et al., 2008) under the control of different *GAL4* drivers. I found that overall sleep levels were significantly lower than both *GAL4* and *UAS* parental control flies when all lateral neurons (PDF^+ s-LN_v and l-LN_v; PDF^- 5^{th} s-LN_v; dorsal lateral – LN_d neurons, Figure 1.2B) were hyper-excited under the control of *Dvpdf GAL4* (Figure 3.1A, one-way ANOVA, $F_{2, 87} = 27.67$, $p < 0.00001$). When a subset of DN_1 neurons were hyper-excited under the control of *Clk 4.1M GAL4*, sleep levels were reduced compared to the *GAL4* controls only (Figure 3.1A, one-way ANOVA, $F_{2, 88} = 22.04$, $p < 0.00001$). Even though previous studies have shown that PDF^+ neurons are wake-promoting (Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008a), when these neurons are hyper-excited, overall levels of sleep are not affected (Figure 3.1A, one-way ANOVA, $F_{2, 78} = 2.85$, $p = 0.06$). This is because only night-time sleep level is reduced while day-time sleep remains unaffected when PDF^+ neurons are hyper-excited (Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008a). This suggests that under *Dvpdf GAL4* driver, hyper-excitation of LN_d and 5^{th} s-LN_v causes reduction of sleep, since hyper-exciting PDF^+ neurons alone under the *Pdf GAL4* driver has negligible effect on overall sleep levels. Importantly, when the temperature was reduced to 21 °C such that the neurons were now no longer hyper-excited, I observed an increase in day-time sleep for about 12 hours (Figure 3.1C) suggestive of sleep rebound only when neurons under *Dvpdf*

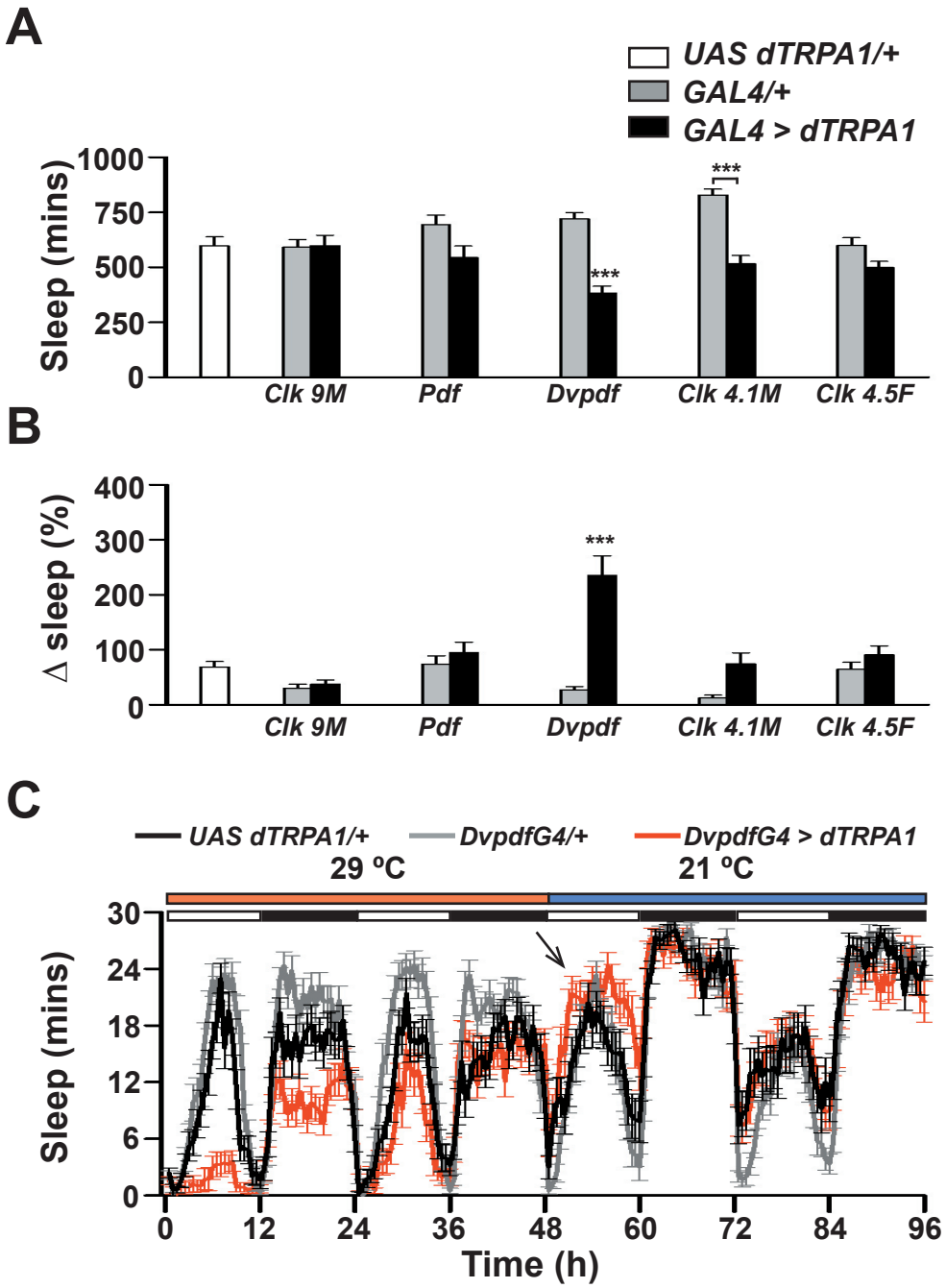


Figure 3.1. Lateral neurons play a role in sleep homeostatic function. (A) Total sleep levels of flies with different subsets of clock neurons hyper-excited using heat-acitvation in LD 12:12 at 29 °C. All lateral neurons labelled by *Dvpdf GAL4* when hyper-excited, show significant decrease in total sleep as compared to both *UAS dTRPA1/+* and *Dvpdf GAL4/+* parental controls. DN_1s labelled by *Clk 4.1M GAL4* upon hyper-excitation show decreased sleep as compared to *Clk 4.1M GAL4/+* controls. (B) Change in sleep when temperature changes from 29 °C to 21 °C. Removal of hyper-excitation of lateral neurons results in significant increase of sleep. (C) Sleep per 30 minutes under LD 12:12 for two days each at 29 °C (orange bar) and 21 °C (blue bar). White and black bars denote light and dark phases of LD 12:12. Sleep is reduced in *Dvpdf GAL4 > UAS dTRPA1* flies at 29 °C. Arrow indicates increase in sleep of *Dvpdf GAL4 > UAS dTRPA1* flies while temperature decreased from 29 °C to 21 °C. Sleep/wake profiles of control flies remain unaltered with change in temperature. *n* between 23-32 flies. Experiment conducted two times with similar results, data from a single experiment. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$. Error bars are SEM.

GAL4 were previously hyper-excited (Figure 3.1B, one-way ANOVA, $F_{2, 87} = 25.35$, $p < 0.00001$). This significant increase in sleep upon decrease in temperature was not observed when the other *GAL4* drivers including *Pdf GAL4* were used (Figure 3.1B, one-way ANOVA, $F_{2, 78} = 0.76$, $p = 0.47$), once again highlighting the role of LN_d and 5^{th} s-LN_v which have previously been shown to modulate evening peak of activity under standard LD 12:12 cycles (Grima et al., 2004; Rieger et al., 2006; Stoleru et al., 2004; Yao et al., 2016; Yao and Shafer, 2014). This suggests that, LN_d and 5^{th} s-LN_v encode a "sleep deprivation" state, as their hyper-activity results in sleep loss and restoring activity levels to normal conditions results in a significant sleep rebound.

3.3.2. Functional circadian clocks within the lateral neurons are not required in sleep homeostatic function. So far, I have shown that certain sleep homeostatic features are altered due to mutations in core clock genes in the previous chapter (Figure 2.8), and that the LN_d and 5^{th} s-LN_v neurons within the circadian network encode a sleep deprivation state (Figure 3.1). I next asked if a ticking molecular clock within these neurons is required in sleep homeostatic function of these neurons. To address this, molecular clocks within the lateral neurons (under *Dvpdf GAL4* driver) were disrupted by expressing dominant negative (DN) form of either *clk* or *cyc*, such that the over-expressed mutant forms bind to the native wild type CLK or CYC proteins and render them dysfunctional. I found that sleep rebound due to disrupted circadian clocks within the lateral neurons either due to dysfunctional CLK or CYC has no effect on the extent of sleep recovery due to deprivation for the entire duration of 12 hours during night (Table 3.1). Furthermore, I found that neither the distribution of sleep and wake nor number of brief awakenings show significant alterations from parental control flies (Figure 3.2A-B, Appendix 2.2A), contrary to what was observed in cyc^{01} flies (Figure 2.8A-B). While there are slight differences in the manner in which number of brief

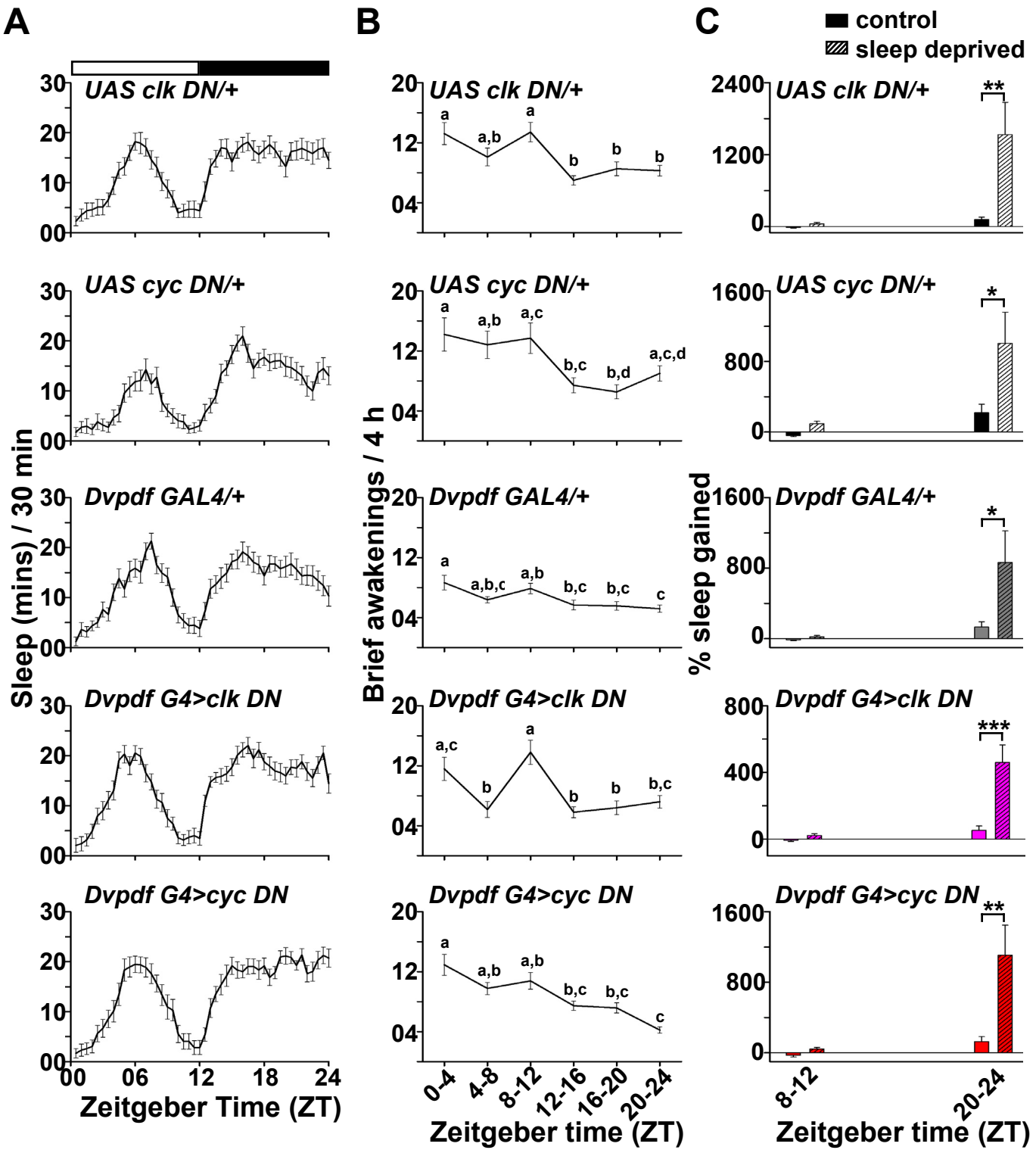


Figure 3.2. Dysfunctional circadian clocks within lateral neurons do not affect sleep homeostatic features. (A) Sleep per 30 minutes across time of day, (B) number of brief awakenings at different time windows and (C) sleep gained due to deprivation during ZT 8-12 and ZT 20-24 time windows under LD 12:12 in *UAS clk DN/+*, *UAS cyc DN/+*, *Dvpdf GAL4/+*, *Dvpdf GAL4 > UAS clk DN* and *Dvpdf GAL4 > UAS cyc DN* flies. Sleep/wake profiles of experimental flies resemble that of parental control flies. Number of brief awakenings is higher during day than night in all genotypes, although the specific time intervals may vary in different genotypes. Different letters indicate significant differences across time windows. Sleep is gained when deprived during ZT 20-24, but not during ZT 8-12 in all genotypes including experimental *Dvpdf GAL4 > UAS clk DN* and *Dvpdf GAL4 > UAS cyc DN* flies. *n* between 20-24 flies. Experiment conducted two times, data from a single experiment. All other details are as in Figure 3.1.

awakenings vary across the day, in both the sets of experimental flies, number of brief awakenings is higher during day than night (Figure 3.2B, Appendix 2.2A). Interestingly, when the parental control and experimental flies were sleep deprived during late day (ZT 8-12) and late night (ZT 20-24) intervals as in the previous chapter, significant recovery sleep was obtained for all flies only when they were sleep deprived during the late night window (Figure 3.2C, Appendix 2.2B). Sleep deprivation during late day has negligible effect on the sleep levels in the subsequent 4 hour window in all genotypes (Figure 3.2C), which is similar to what was observed in wild type flies and different from what was observed in circadian clock mutants (Figure 2.8C). These results suggest that a ticking circadian clock within the lateral neurons is not required to carry out the sleep homeostat function, as disrupting molecular rhythms within these neurons does not impair any of the sleep homeostatic features tested.

Table 3.1.

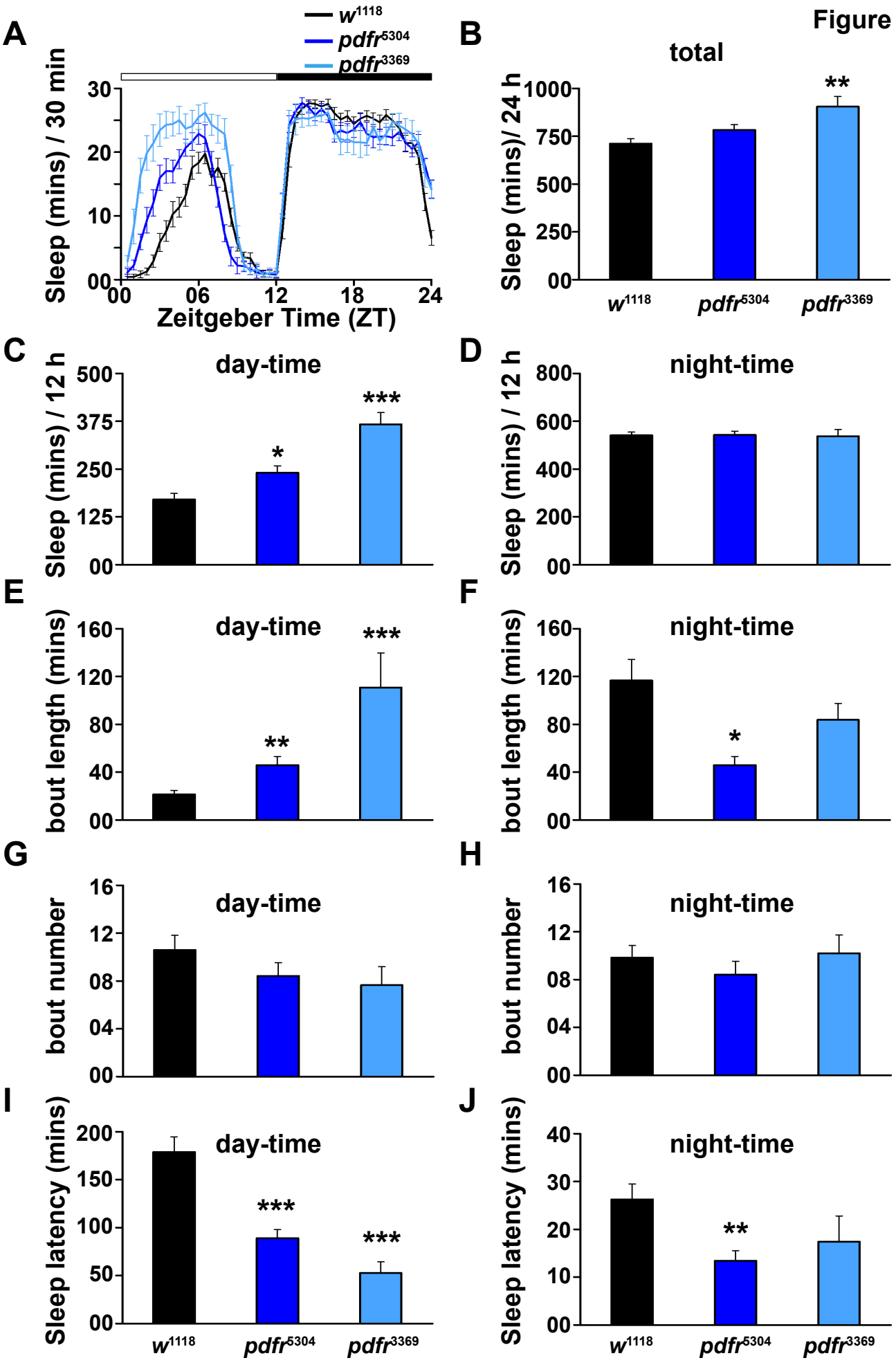
Genotype	*n* (flies)	% sleep gained
UAS clk DN/+	23	39.4 ± 10.5
UAS cyc DN/+	14	146.9 ± 43.2
Dvpdf GAL4/+	24	31 ± 7.6
Dvpdf GAL4 > UAS clk DN	24	39.5 ± 13.4
Dvpdf GAL4 > UAS cyc DN	16	53.3 ± 17.9

Table 3.1. Amount of sleep recovery after 12 hours of night sleep deprivation. % sleep recovery for indicated genotypes calculated as increase in 12 hour day-time sleep post deprivation relative to day-time sleep levels during baseline day of recording (no disturbance). *UAS cyc DN/+* flies showed unusually high amount of recovery sleep perhaps owing to their background. All other genotypes gain similar amounts of sleep after 12 hours of sleep deprivation during the night.

3.3.3. PDFR signaling promotes wakefulness specifically during the day. In order to find more circadian clock – sleep homeostat pathways, my next approach was to find

if known sleep homeostatic structures are downstream of wake-promoting circadian clock PDF^+ LN_v neurons. To establish a phenotype on the basis of which, my screen to uncover downstream targets of PDFR signaling (and thus PDF^+ circadian neurons) could be designed, I examined two previously established loss-of-function mutants of the *pdfr* gene – $pdfr^{5304}$ and $pdfr^{3369}$. A previous study had reported that both day-time and night-time sleep of these mutants is significantly higher than that of background control flies (Chung et al., 2009). However, I found that both mutants after backcrossing to the widely used *Iso31* (w^{1118}) background for 7-8 generations exhibited significantly higher sleep only during the day-time under LD12:12 cycles at 25 °C (Figure 3.3A, C, w^{1118} *vs* $pdfr^{5304}$, Student's two-tailed *t*-test, $T_{0.05,\ 2,\ 46} = -2.93$, $p < 0.05$; w^{1118} *vs* $pdfr^{3369}$, Student's two-tailed *t*-test, $T_{0.05,\ 2,\ 38} = -6.33$, $p < 0.00001$). Total sleep is significantly higher than the controls only in one of the mutants (Figure 3.3B, w^{1118} *vs* $pdfr^{5304}$, $T_{0.05,\ 2,\ 46} = -1.92$, $p = 0.06$; w^{1118} *vs* $pdfr^{3369}$, $T_{0.05,\ 2,\ 38} = -3.68$, $p < 0.005$). However, night-time sleep of the *pdfr* mutants was not different from that of the controls (Figure 3.3D, w^{1118} *vs* $pdfr^{5304}$, $T_{0.05,\ 2,\ 46} = -0.09$, $p = 0.93$; w^{1118} *vs* $pdfr^{3369}$, $T_{0.05,\ 2,\ 38} = 0.13$, $p = 0.9$). These differences in sleep were not due to differences in activity levels (Figure 3.4A-B). Although the activity per waking minute is significantly lower for one of the mutants (Figure 3.4B, w^{1118} *vs* $pdfr^{5304}$, $T_{0.05,\ 2,\ 46} = 2.58$, $p < 0.05$; w^{1118} *vs* $pdfr^{3369}$, $T_{0.05,\ 2,\ 38} = 1.91$, $p = 0.06$) as compared to the control, this observation was not seen in replicate experiments using the same genotypes (data not shown). Furthermore, the increase in day-time sleep seen in the *pdfr* mutants is also seen during subjective day-time, when these flies are transferred to constant darkness (DD) at 25 °C (Figure 3.4C-D, w^{1118} *vs* $pdfr^{5304}$, $T_{0.05,\ 2,\ 41} = -5.51$, $p < 0.00001$; w^{1118} *vs* $pdfr^{3369}$, $T_{0.05,\ 2,\ 35} = -5.24$, $p < 0.00001$). Moreover, we confirmed that back-crossing has not resulted in loss of the *pdfr* mutation by the finding that behavioural

Figure 3.3



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phenotype of the advanced evening activity peak (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005) is reproduced under LD 12:12 (Figure 3.4A). Taken together, these data suggest that absence of functional *pdfr* results in increased sleep duration specifically during the day.

While the quantity of day-time sleep has increased in the *pdfr* mutants, the quality of day-time sleep is also different as these *pdfr* mutants sleep longer within a typical sleep bout during the day-time (Figure 3.3E, w^{1118} *vs* $pdfr^{5304}$, $T_{0.05,\ 2,\ 46} = -3.23$, $p < 0.005$; w^{1118} *vs* $pdfr^{3369}$, $T_{0.05,\ 2,\ 38} = -4.17$, $p < 0.0005$). However the number of such sleep bouts is not different in all three genotypes (Figure 3.3G, w^{1118} *vs* $pdfr^{5304}$, $T_{0.05,\ 2,\ 46} = 1.27$, $p = 0.21$; w^{1118} *vs* $pdfr^{3369}$, $T_{0.05,\ 2,\ 38} = 1.43$, $p = 0.16$). These results suggest that sleep is more consolidated during the day-time in the absence of functional *pdfr*. During the night average sleep bout length is significantly lower in $pdfr^{5304}$ than the control (Figure 3.3F, $T_{0.05,\ 2,\ 46} = 2.15$, $p < 0.05$), whereas it is comparable to the control in the case of $pdfr^{3369}$ (Figure 3.3F, $T_{0.05,\ 2,\ 38} = 1.26$, $p = 0.22$), and number of sleep bouts during the night of both mutants is similar to that of the control (Figure3.3H, w^{1118} *vs* $pdfr^{5304}$, $T_{0.05,\ 2,\ 46} = -1.24$, $p = 0.22$; w^{1118} *vs* $pdfr^{3369}$, $T_{0.05,\ 2,\ 38} = -0.2$, $p = 0.84$). Interestingly, both mutants take lesser time to fall asleep after lights-ON (Figure 3.3I,

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Figure 3.3. Loss-of-function mutants of pdfr display higher sleep duration during the day. (A) Amount of time spent sleeping estimated every 30 min as a function of time-of-day averaged across 3 cycles. Both $pdfr^{5304}$ (n = 22 flies) and $pdfr^{3369}$ (n = 14 flies) sleep more during the day-time as compared to w^{1118} (n = 26 flies). Night-time sleep of *pdfr* mutants is similar to that of w^{1118} flies. White and black bars on top indicate 12 h of day and 12 h of night, respectively. (B) Total sleep over the 24 h cycle of $pdfr^{3369}$ flies is significantly increased as compared to w^{1118} flies, whereas that of $pdfr^{5304}$ is not different from w^{1118} flies. (C) Day-time sleep of both *pdfr* mutants is significantly higher than that of w^{1118} flies, whereas (D) night-time sleep of both *pdfr* mutants is similar to that of w^{1118} flies. (E) Average length of sleep bouts during the day is higher in both *pdfr* mutants as compared to w^{1118}, while (F) average length of sleep bouts during the night in only $pdfr^{5304}$ mutants is lower than that of w^{1118}. Average number of sleep bouts of the *pdfr* mutants is comparable to that of w^{1118} both during (G) day and (H) night. Time taken to fall asleep (I) after lights-ON is lower in $pdfr^{5304}$ and $pdfr^{3369}$ mutants as compared to w^{1118} flies and (J) after lights-OFF is lower only in $pdfr^{5304}$ as compared to w^{1118}. Asterisks indicate levels of significance obtained from performing Student's two-tailed *t*-tests for both mutants comparing each of them to w^{1118} independently. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Error bars are SEM. Results representative from two independent experiments.

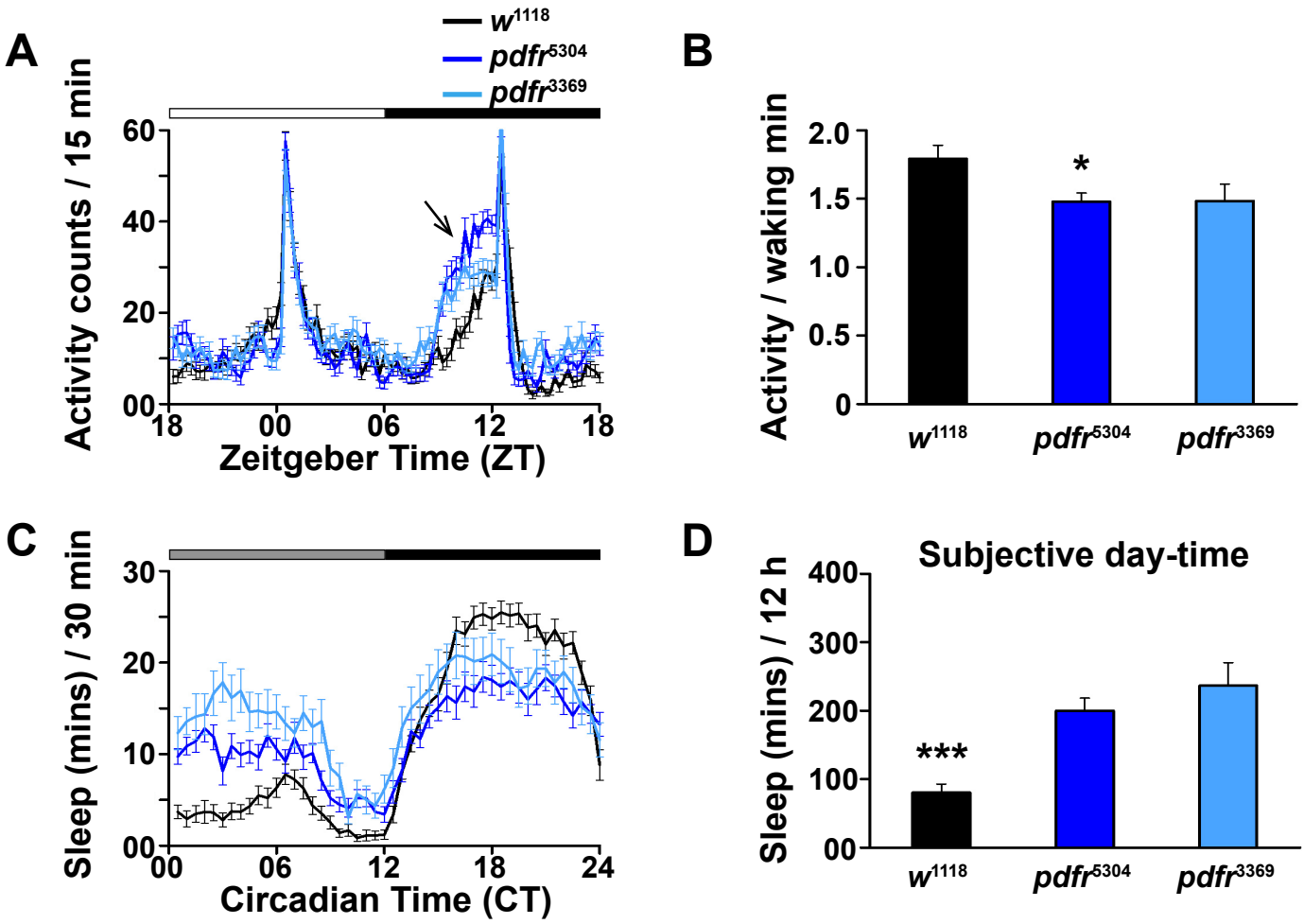

Figure 3.4. Activity in LD 12:12 and sleep behaviour in DD of pdfr mutants. (A) Activity counts of male flies of w^{1118}, $pdfr^{3369}$ and $pdfr^{5304}$ for every fifteen minutes across time of the day shows that evening peak of $pdfr^{3369}$ and $pdfr^{5304}$ flies is advanced in phase as compared to that of w^{1118} flies (arrow). (B) Activity counts per waking minute of female flies whose sleep/wake patterns are described in Figure 3.3 is significantly lower in $pdfr^{5304}$ flies as compared to w^{1118} flies in this particular run (not observed across independent runs). (C) Sleep per 30 minutes on first day of DD plotted against time of day, where CT 0 refers to onset of day in the previous LD 12:12 cycle. (D) Amount of sleep in the first 12 hours of first day in DD shows higher amount of sleep during subjective day-time for $pdfr^{5304}$ and $pdfr^{3369}$ as compared to w^{1118} flies. All other details are as in Figure 3.3.

w^{1118} *vs* $pdfr^{5304}$, $T_{0.05,\ 2,\ 46}$ = 4.16, $p < 0.00001$; w^{1118} *vs* $pdfr^{3369}$, $T_{0.05,\ 2,\ 38}$ = 5.3, $p < 0.00001$), whereas only $pdfr^{5304}$ falls asleep sooner than w^{1118} after lights-OFF (Figure 3.3J, w^{1118} *vs* $pdfr^{5304}$, $T_{0.05,\ 2,\ 46}$ = 3.18, $p < 0.005$; w^{1118} *vs* $pdfr^{3369}$, $T_{0.05,\ 2,\ 38}$ = 0.77, $p = 0.45$). Given that absence of *pdfr* leads to increased sleep duration as well as consolidated sleep and makes flies sleep sooner especially during the day-time, these results corroborate the previously established role for PDFR signaling mediated by the PDF^{+} neurons in wake-promoting effects (Chung et al., 2009; Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008a) while highlighting a greater effect on day- time sleep compared to night.

3.3.4. Screen for downstream targets of PDFR signaling. Previous studies that have characterized the expression pattern of *pdfr* using different antibodies against PDFR and/or promoter-mediated expression of cellular tags such as *myc* have revealed *pdfr* expression in a subset of circadian clock neurons, Pars Intercerebralis (PI), Ellipsoid Body (EB) and about 50 as yet uncharacterized non-clock cells (Hyun et al., 2005; Im and Taghert, 2010; Lear et al., 2005; Mertens et al., 2005; Parisky et al., 2008). Therefore, on the basis of the predicted expression pattern of *pdfr* and potential sites in the vicinity of PDF projections, as well as those that function in sleep/wake regulation, I altered expression of *pdfr* in a total of 26 *GAL4* lines including distinct subsets of circadian clock neurons, mushroom body, PI, central complex and some neurotransmitter/peptide systems. My interest was to identify driver lines whose targets responded with *both* an increase in day-time sleep upon down-regulation and a decrease in day-time sleep upon over-expression of *pdfr*. Moreover, to rule out non-specific effects on day-time sleep of either the *GAL4* or *UAS* parental line, it was required that the experimental flies be significantly different as compared to both parental controls in order to be considered as a hit.

Quite surprisingly, down-regulation and/or over-expression in subsets of circadian clock neurons, which had previously been reported to modulate activity/rest rhythms in LD as well as in DD (Im and Taghert, 2010) did not show an effect on day-time sleep (Figure 3.5). While down-regulation of *pdfr* in ~12-14 DN_{1p}s using *Clk 4.1M GAL4* (Zhang et al., 2010) resulted in a significant increase in day-time sleep as compared to both parental controls (Figure 3.5A, Appendix 2.2C), over-expression of *pdfr* in the same subset of neurons did not result in a corresponding decrease in day-time sleep (Figure 3.5B, Appendix 2.2D). Moreover, down-regulation of *pdfr* in almost all $PDFR^+$ clock neurons using *Pdfr (B) GAL4* (Im and Taghert, 2010) resulted in an increase in day-time sleep but this was significantly different only from the *UAS* parental control (Figure 3.5A, Appendix 2.2C). Over-expression of *pdfr* using the same driver however resulted in significant decrease in day-time sleep only as compared to the *GAL4* control (Figure 3.5B, Appendix 2.2D). Moreover, when *pdfr* was down-regulated and/or over-expressed in a different combination of essentially the same cluster of circadian clock neurons (*Cry GAL4-39*; (Klarsfeld et al., 2004)), consistent effects on day-time sleep were not observed (Figure 3.5, Appendix 2.2C-D). These results together lead to the interpretation that circadian clock neurons may not be major downstream targets of PDFR signaling that regulates day-time wakefulness.

In a recent study it was found that PDF^+ neurons communicate with DN_1s, which then communicate with $DH44^+$ (Diuretic Hormone 44) PI neurons that brings about rhythmic locomotor activity under DD conditions (Cavanaugh et al., 2014). Given my finding that DN_1s are most likely not the downstream targets of PDF^+ neurons for sleep regulation, I next asked if the PI neurons were direct recipients of PDF signals for modulation of day-time sleep. Barring a few non-specific parental effects on sleep, none of the 5 PI-specific *GAL4* drivers I screened, showed any

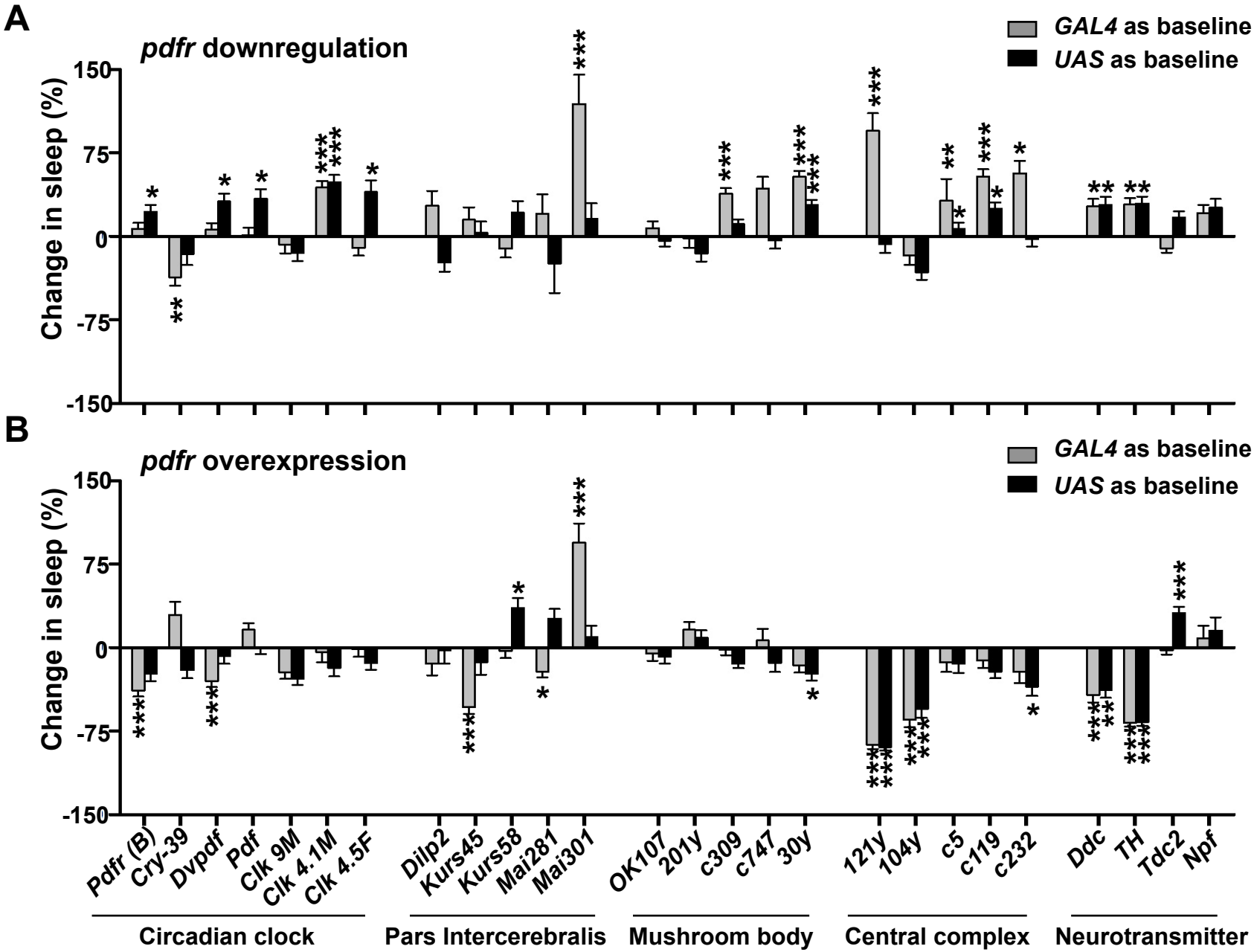

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Figure 3.5

significant effects on day-time sleep when *pdfr* was down-regulated and/or over-expressed (Figure 3.5, Appendix 2.2C-D). Thus, though PI neurons appear to be anatomically well-placed to receive PDF signals, my finding suggests that they are not required for sleep regulation by the PDF^{+} neurons. In the light of these results and previous findings that PI neurons modulate sleep and wake levels (Crocker et al., 2010; Foltenyi et al., 2007), it appears that the PDF signaling and PI neurons are in different pathways of sleep and wake regulation.

Given that *pdfr* is expressed in the EB, and the suggestion that they could be the output neurons of PDF effects on sleep and wake levels (Parisky et al., 2008), *pdfr* was down-regulated and over-expressed using *GAL4* drivers that distinctly label the EB. I found that down-regulation of *pdfr* using *c119 GAL4* led to an increase in day-time sleep (Figure 3.5A, Appendix 2.2C), however it was not accompanied with a corresponding decrease in day-time sleep upon over-expression of *pdfr* (Figure 3.5B, Appendix 2.2D). Another *GAL4* driver targeting the EB did not show these effects on day-time sleep upon down-regulation and over-expression of *pdfr* (c232 *GAL4*, Figure 3.5, Appendix 2.2C-D). Specifically targeting the sleep homeostatic R2 neurons of the EB using more restricted drivers (such as *R69F08 GAL4*; (Liu et al., 2016)) could still

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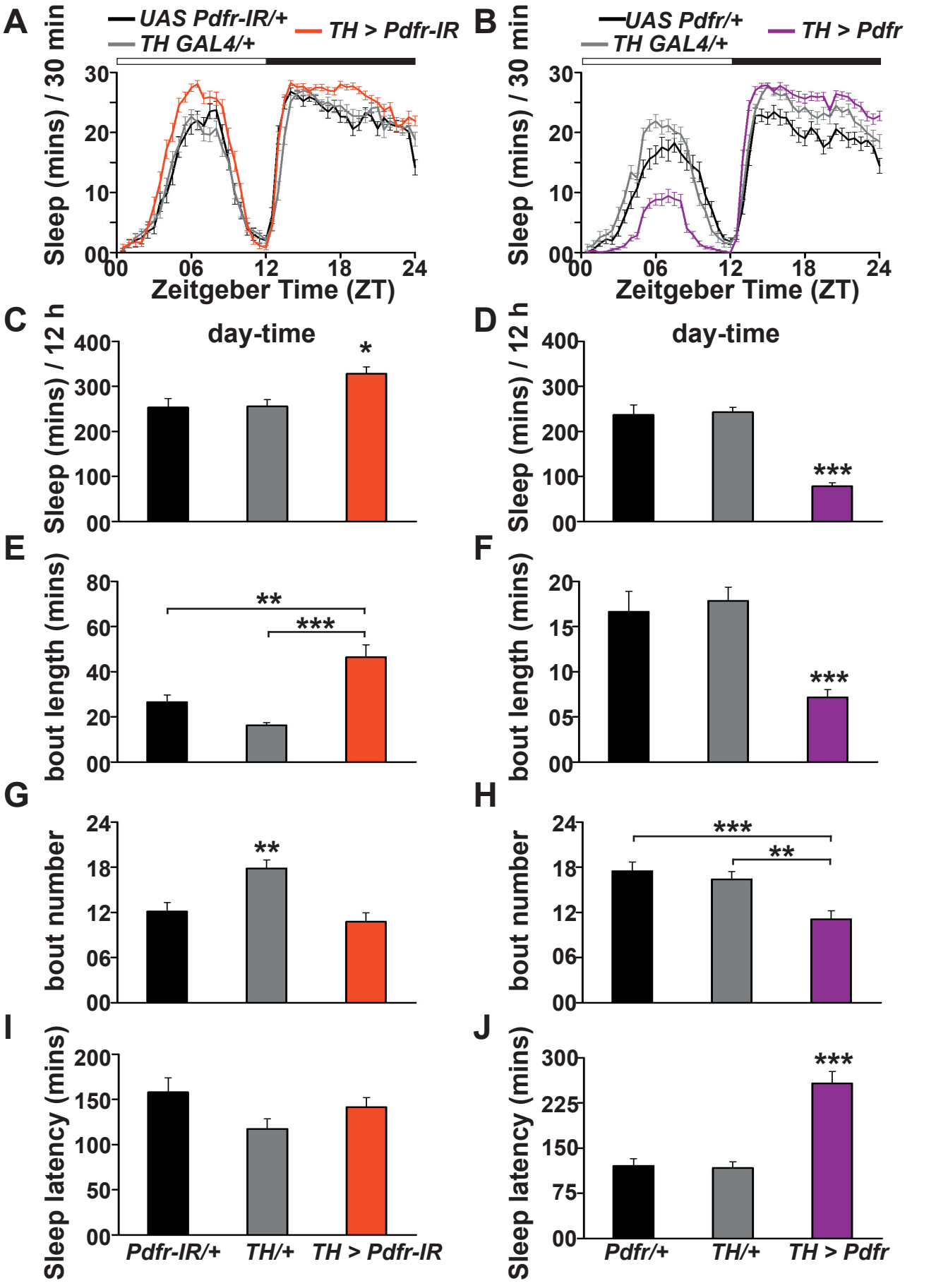
Figure 3.5. Screen to identify downstream targets of PDFR signaling. (A) Down-regulation of *pdfr* using *UAS pdfr RNAi; UAS dcr* and (B) over-expression of *pdfr* using *UAS pdfr* crossed with *GAL4* lines expressed in subsets of circadian clock neurons, Pars Intercerebralis, mushroom body, central complex and major neurotransmitter systems. Bars represent percentage increase (positive values) or decrease (negative values) in day-time sleep of experimental flies with respect to that of *GAL4* (grey) and *UAS* (black) parental controls. Asterisks above the bars indicate level of significance when a one-way ANOVA with genotype as factor followed by *post-hoc* Tukey's test was done on raw day-time sleep levels. Several lines when used to down-regulate *pdfr* show a significant increase in day-time sleep, but do not show a corresponding decrease in day-time sleep when *pdfr* is over-expressed (*Clk 4.1M GAL4*, *30y GAL4*, *c5 GAL4*, *c119 GAL4*), whereas a few lines show a significant decrease in day-time sleep when *pdfr* is over-expressed, but no corresponding increase in day-time sleep is seen when *pdfr* is down-regulated (*121y GAL4*, *104y GAL4*). However, in 2 lines (*Ddc GAL4*, *TH GAL4*), when *pdfr* is down-regulated, there occurs a significant increase in day-time sleep; and when *pdfr* is over-expressed, there occurs a significant decrease in day-time sleep. All other details are as in Figure 3.3. For all genotypes, *n* between 24-32 flies. Results representative from at least two independent experiments for all drivers.

be instructive. Nonetheless, my results with *c119* and *c232 GAL4*s suggest that EB may not be downstream of PDFR signaling in sleep and wake modulation.

Im and Taghert (2010) reported that in addition to circadian clock neurons, PI and EB, there are about 50 cells in the brain that are $PDFR^{+}$. I hypothesized that these 50 cells could potentially be any one of the mushroom body and/or fan-shaped body cells, neurons of which are implicated in sleep regulation (Donlea et al., 2014; Donlea et al., 2011; Joiner et al., 2006; Pimentel et al., 2016; Pitman et al., 2006; Sitaraman et al., 2015a; Sitaraman et al., 2015b) and which may lie in the vicinity of projections of the PDF^{+} s-LN_v neurons. Not so surprisingly, none of the *GAL4* lines labeling either mushroom body or fan-shaped body showed significant and opposite effects on day-time sleep upon down-regulation and over-expression of *pdfr* (Figure 3.5, Appendix 2.2C-D). Interestingly, however, 4 *GAL4* drivers showed strong significant effects on day-time sleep upon either down-regulation or over-expression of *pdfr* only. Out of these, when *pdfr* was down-regulated using the *30y GAL4* which labels the α/β lobes strongly and the rest of the mushroom body weakly (Aso et al., 2009), day-time sleep was significantly higher as compared to both *GAL4* and *UAS* controls (Figure 3.5A, Appendix 2.2C). Interestingly, over-expression of *pdfr* using broader fan-shaped body drivers such as *121y GAL4* and *104y GAL4* resulted in decrease of day-time sleep (Figure 3.5B, Appendix 2.2D). However, similar results were not obtained with a more restricted driver (*c5 GAL4*) for FB thereby revealing non-specific effects of the over-expression using the broad driver.

I next focused on a few neurotransmitter/peptide clusters that have previously been known to regulate sleep and wake such as dopamine, serotonin, octopamine and Neuropeptide F (NPF; (Crocker and Sehgal, 2008; He et al., 2013; Kume et al., 2005;



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Yuan et al., 2006)). Surprisingly, when *pdfr* was down-regulated in serotonergic and dopaminergic neurons using *Ddc GAL4*, as well as dopaminergic neurons alone using *TH GAL4*, day-time sleep was significantly higher than the parental controls (Figure 3.5 A, Appendix 2.2C). Moreover, when *pdfr* was over-expressed using the same *GAL4* drivers, day-time sleep was significantly lesser than the parental controls (Figure 3.5B, Appendix 2.2D). However, there was no significant effect of either down-regulating or over-expressing *pdfr* in either NPF^{+} or octopaminergic neurons on day-time sleep. Taken together, these results suggest that dopaminergic neurons are the most likely candidate for being the downstream targets of PDFR signaling in order to modulate day-time sleep and wake levels.

3.3.5. PDFR signaling to dopaminergic neurons promotes day-time wakefulness. I next examined the sleep/wake behaviour of flies with down-regulated or over-expressed *pdfr* in dopaminergic neurons in further detail. While down-regulation (DR) of *pdfr* led to an increase in day-time sleep and over-expression (OEX) of *pdfr* in dopaminergic neurons decreased day-time sleep (Figure 3.6A-D, one-way ANOVA, DR, $F_{2, 89} = 6.53$, $p < 0.005$; OEX, $F_{2, 88} = 43.81$, $p < 0.00001$), interestingly both manipulations of *pdfr* expression levels led to an increase in night-time sleep (Figure 3.6A-B, Figure 3.7C-D; DR, $F_{2, 89} = 12.48$, $p < 0.0005$; OEX, $F_{2, 88} = 43.91$, $p < 0.00001$). However, these

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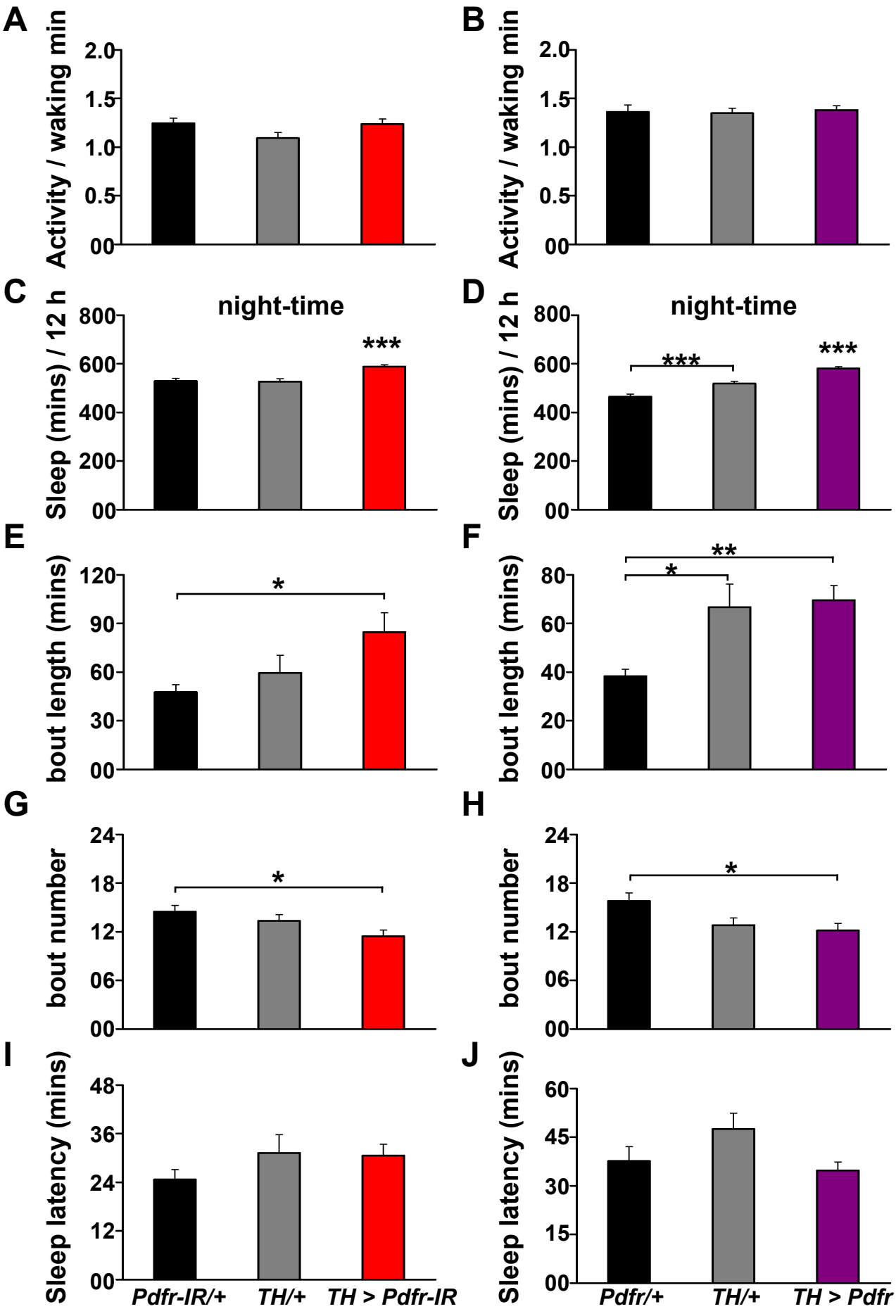
Figure 3.6. Quantity and quality of day-time sleep changes with changing pdfr expression levels in dopamine neurons. Sleep duration for every 30 mins of an average LD12:12 cycle (A) when *pdfr* is down-regulated and (B) when *pdfr* is over-expressed in dopaminergic neurons. (A) Day-time sleep as well as night-time sleep is increased in *TH GAL4 > UAS Pdfr RNAi; UAS dcr* (n = 32 flies) as compared to *TH GAL4/+* (n = 31 flies) and *UAS Pdfr RNAi/+; UAS dcr/+* (n = 29 flies) controls, whereas (B) day-time sleep is decreased but night-time sleep is increased in *TH GAL4 > UAS Pdfr* (n = 28 flies) as compared to *TH GAL4/+* (n = 28 flies) and *UAS Pdfr/+* (n = 30 flies) controls. (C) Day-time sleep duration, (E) average sleep bout length, (G) average number of sleep bouts and (I) latency to fall asleep after lights-ON for *TH GAL4 > UAS Pdfr RNAi; UAS dcr* flies compared to controls. (D) Day-time sleep duration, (F) average sleep bout length, (H) average sleep bout number and (J) latency to fall asleep after lights-ON for *TH GAL4 > UAS Pdfr* flies compared to controls. Asterisks indicate significance levels obtained from one-way ANOVA with genotype as factor followed by *post-hoc* Tukey's test. All other details are as in Figure 3.3. Results representative from four independent experiments.

Figure 3.7



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differences in sleep levels were not as a result of changes in activity levels (Figure 3.7A-B, DR, $F_{2, 89} = 2.55$, $p = 0.08$; OEX, $F_{2, 88} = 0.09$, $p = 0.9$). Not only was the day-time sleep increased when *pdfr* was down-regulated in dopaminergic neurons, but the average sleep bout length was significantly longer as compared to both controls (Figure 3.6E, $F_{2, 89} = 16.45$, $p < 0.00001$), although the number of sleep bouts was not different from the *UAS* control (Figure 3.6G, $F_{2, 89} = 10.33$, $p < 0.0005$). Interestingly, the flies with down-regulated *pdfr* in dopaminergic neurons took the same amount of time to fall asleep after lights-ON as the controls (Figure 3.6I, $F_{2, 89} = 2.59$, $p = 0.08$). Flies with over-expressed *pdfr* in dopaminergic neurons displayed shorter average sleep bouts during the day-time (Figure 3.6F, $F_{2, 88} = 13.78$, $p < 0.00001$) as well as lesser number of such sleep bouts (Figure 3.6H, $F_{2, 88} = 9.45$, $p < 0.0005$). Unlike the *pdfr* down-regulated flies, those with over-expressed *pdfr* in dopaminergic neurons took longer to fall asleep after lights-ON (Figure 3.6J, $F_{2, 88} = 28.96$, $p < 0.00001$). Night-time sleep in both manipulations of *pdfr* expression levels was different from the controls only in terms of quantity, not in quality since sleep bout length and number were not affected (Figure 3.7E-J, sleep bout length: DR, $F_{2, 89} = 3.72$, $p < 0.05$; OEX, $F_{2, 88} = 6.24$, $p < 0.005$; sleep bout number: DR, $F_{2, 89} = 3.98$, $p < 0.05$; OEX, $F_{2, 88} = 4.24$, $p < 0.05$. Note that these significant differences are because of differences between genotypes as

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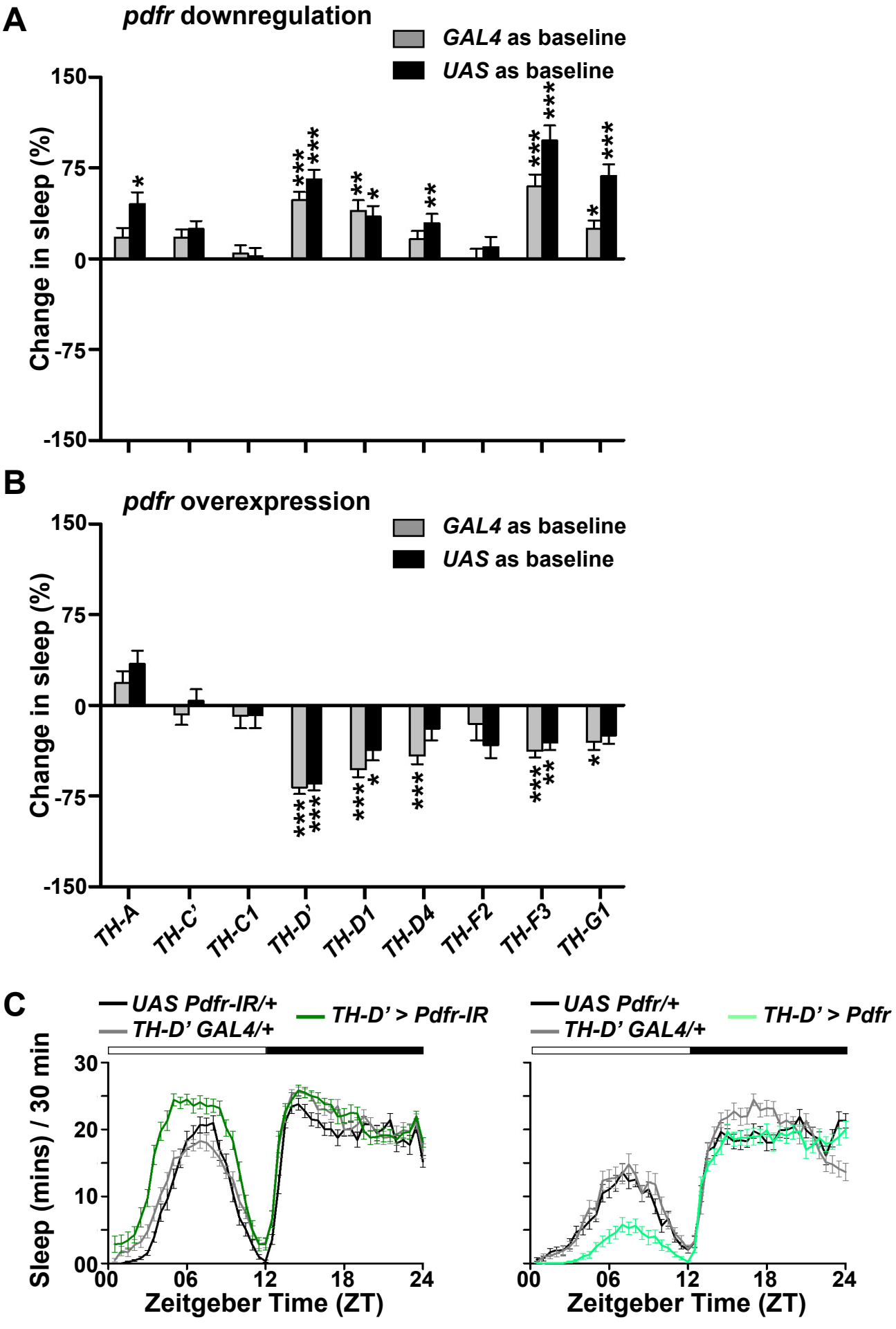
Figure 3.7. Sleep and activity levels of flies with altered pdfr expression in dopaminergic neurons. Activity counts per waking minute of (A) *TH GAL4 > UAS Pdfr RNAi; UAS dcr* and (B) *TH GAL4 > UAS Pdfr* are not different from their respective parental controls. Night-time sleep of (C) *TH GAL4 > UAS Pdfr RNAi; UAS dcr* and (D) *TH GAL4 > UAS Pdfr* are significantly higher than their respective parental controls. Average length of sleep bout during night of (E) *TH GAL4 > UAS Pdfr RNAi; UAS dcr* is significantly higher than only *UAS Pdfr RNAi/+; UAS dcr/+* control flies, whereas (F) that of *TH GAL4 > UAS Pdfr* is significantly higher than both parental controls. Average number of sleep bouts during night of (G) *TH GAL4 > UAS Pdfr RNAi; UAS dcr* and (H) *TH GAL4 > UAS Pdfr* are significantly higher than only *UAS Pdfr RNAi/+; UAS dcr/+* and *UAS Pdfr* control flies respectively. Sleep latency after lights-OFF of (I) *TH GAL4 > UAS Pdfr RNAi; UAS dcr* and (J) *TH GAL4 > UAS Pdfr* are not different from their respective parental controls. All other details are as in Figure 3.6.

Figure 3.8



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shown in Figure 3.7. Night sleep latency : DR, $F_{2, 89} = 1.1$, $p = 0.34$; OEX, $F_{2, 88} = 2.81$, $p = 0.07$). Thus, these results lead to the hypothesis that decreasing PDFR signaling to dopaminergic neurons increases day-time sleep, while increasing PDFR signaling to dopaminergic neurons suppresses day-time sleep and makes it fragmented, as well as delays its onset, suggesting that PDFR signaling to dopaminergic neurons is necessary for initiating and maintaining day-time wakefulness.

3.3.6. PDFR$^+$ PPM3 neurons modulate day-time wakefulness. Dopaminergic neurons labeled on the basis of reactivity to antibody against Tyrosine Hydroxylase (anti-TH), which is the rate-limiting enzyme for dopamine synthesis, are divided into several subsets based on their anatomical location (Mao and Davis, 2009). There are two subsets present in the anterior brain (PAM and PAL – Protocerebral Anterior Medial and Lateral) and five subsets in the posterior brain (PPM and PPL – Protocerebral Posterior Medial and Lateral; PPM1-3 and PPL1-2). Of these, two previous studies have implicated a pair of bilaterally located PPL1 neurons (Liu et al., 2012) and a unilateral PPM3 neuron (Ueno et al., 2012) in promoting wakefulness through the inhibition of sleep-promoting dFB. I asked if the PDFR signaling is acting upon either or both of these subsets to promote wakefulness specifically during the day. I used the previously created and characterized *GAL4* drivers (*TH-A*, *C'*, *C1*, *D'*, *D1*, *D4*, *F2*, *F3* and *G1*) targeting different subsets of dopaminergic neurons (Liu et al.,

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Figure 3.8. Screen to identify the subset of dopaminergic neurons that are downstream of PDFR signaling. Down-regulation of *pdfr* using *UAS pdfr RNAi; UAS dcr* and (B) over-expression of *pdfr* using *UAS pdfr* crossed with *GAL4* lines expressed in different subsets of dopamine neurons. Down-regulation and over-expression of *pdfr* using only *TH-D'*, *TH-D1* and *TH-F3 GAL4* lines leads to significant and opposite effects on day-time sleep. In all three lines, down-regulation of *pdfr* leads to increase in day-time sleep, whereas over-expression of *pdfr* leads to decrease in day-time sleep. For all genotypes, $n > 24$ flies. All other details are as in Figure 2. (C) Sleep duration for every 30 mins of an average LD12:12 cycle of *TH-D' GAL4> UAS Pdfr RNAi; UAS dcr* ($n = 32$ flies) compared to *TH-D' GAL4/+* ($n = 31$ flies) and *UAS Pdfr RNAi/+; UAS dcr/+*($n = 31$ flies) controls (left) and *TH-D' GAL4> UAS Pdfr* ($n = 32$ flies) compared to *TH-D' GAL4/+* ($n = 32$ flies) and *UAS Pdfr/+* ($n = 32$ flies) controls (right). Day-time sleep is increased with down-regulation, whereas it is decreased with over-expression of *pdfr* in a subset of dopaminergic neurons driven by *TH-D' GAL4*. All other details are as in Figure 3.3. Results representative from at least two independent experiments.

TH GAL4 > UAS GFP

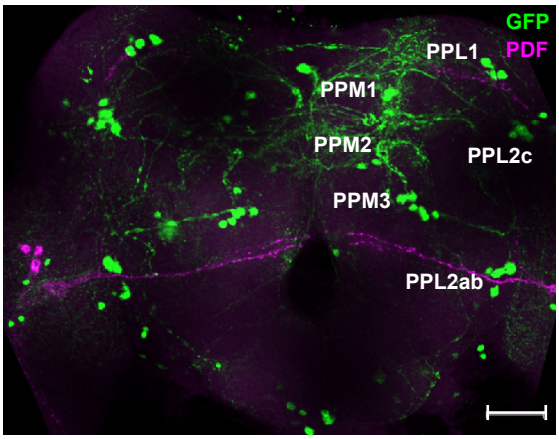
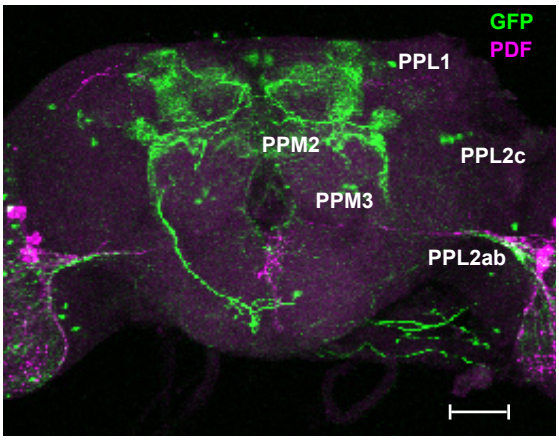
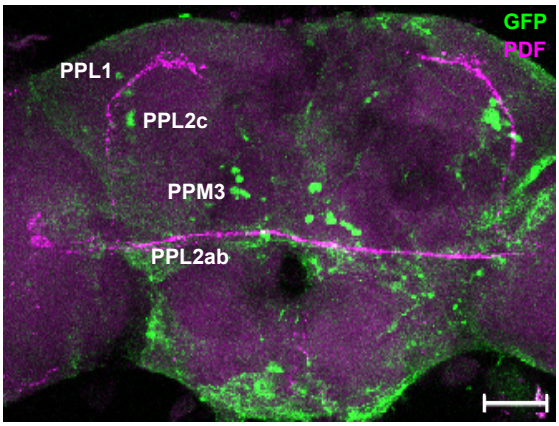


Figure 3.9. Expression pattern of dopaminergic drivers. Expression of GFP under *TH GAL4*, *TH-D'*, *TH-D1*, *TH-D4*, *TH-F2*, *TH-F3*, *TH-G1 GAL4* drivers label different subsets of Posterior Protocerebrum Lateral (PPL1-2) and Medial (PPM1-3) neurons and their projections when posterior parts of the brains are imaged. Brains are co-stained with PDF for visualization of LN_v and their projections. Scale bars are 20 µm.

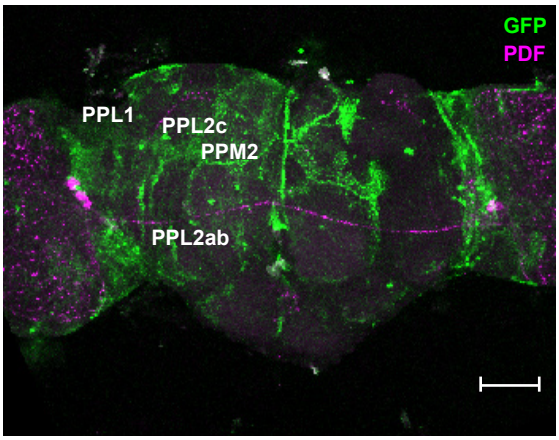
TH-D' GAL4 > UAS GFP



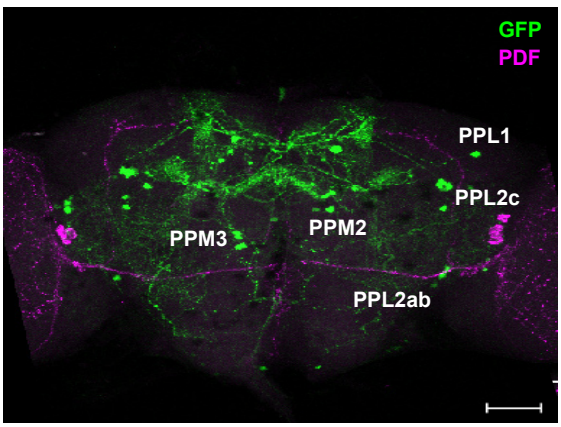
TH-D1 GAL4 > UAS GFP



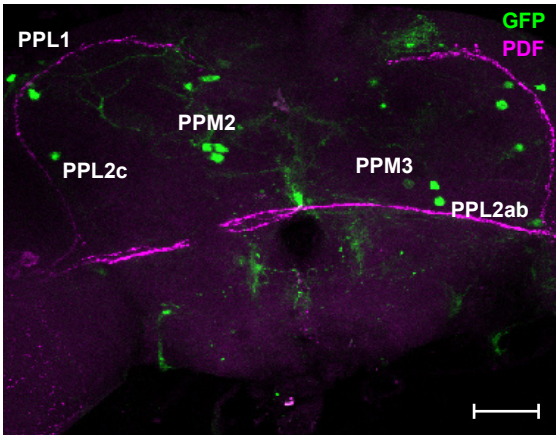
TH-D4 GAL4 > UAS GFP



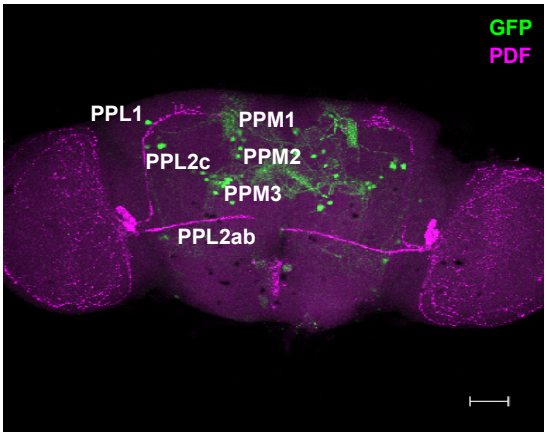
TH-F2 GAL4 > UAS GFP



TH-F3 GAL4 > UAS GFP



TH-G1 GAL4 > UAS GFP



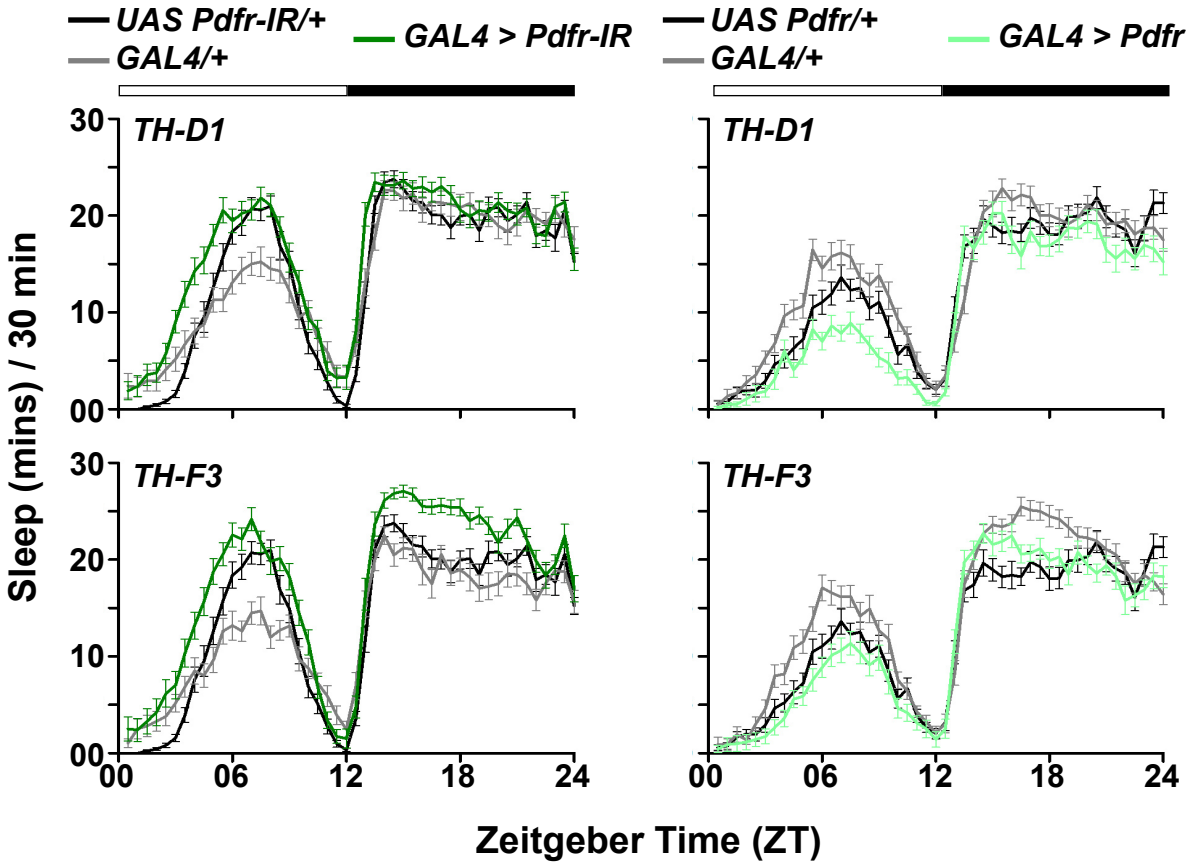


Figure 3.10. Sleep profiles of flies with altered pdfr expression in subsets of dopaminergic neurons. Sleep duration for every 30 mins averaged across 3 days of LD12:12 cycles shows increased day-time sleep with down-regulation (left) and decreased day-time sleep with over-expression (right) of *pdfr* using both *TH-D1 GAL4* (top) and *TH-F3 GAL4* (bottom) drivers. For all genotypes, *n* between 24-32 flies. All other details are as in Figure 3.3.

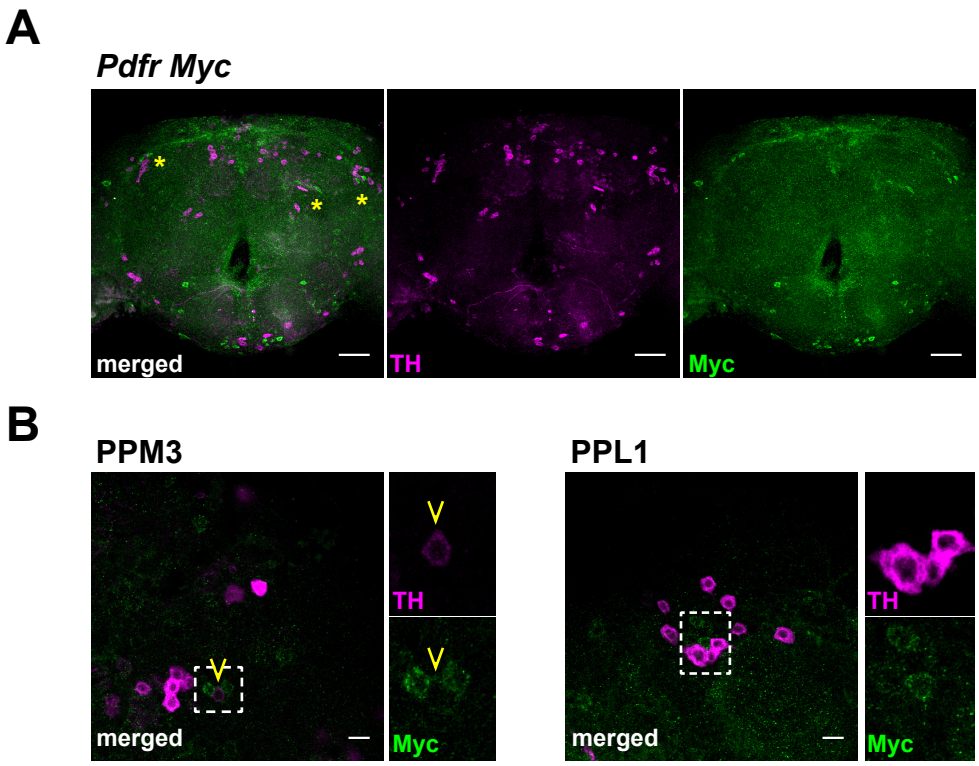


Figure 3.11. Putative overlap between PDFR⁺ and TH⁺ neurons. (A) *Pdfr myc* flies co-labelled with antibodies against MYC and TH reveal TH⁺ and MYC⁺ cells in the regions marked by the asterisks. (B) These regions contain TH⁺ neurons of the PPM3 subset (left), of which one neuron shows faint MYC⁺ signal (as indicated by the arrowheads) and two neurons of the PPL1 subset (right) which lie close to but do not overlap with MYC⁺ cell bodies. All scale bars are 20 µm.

2012). When *pdfr* was down-regulated or over-expressed using the *TH-A GAL4* which does not drive expression in any of the dopaminergic neurons (Liu et al., 2012), expectedly no difference in the day-time sleep levels was seen (Figure 3.8A-B, Appendix 2.2E-F), thus implying no non-specific *GAL4* effects. When *pdfr* down-regulation or over-expression was specifically targeted to the anterior dopamine subsets PAM & PAL by using the *TH-C'* and *TH-C1 GAL4* drivers, no changes in day-time sleep were observed (Figure 3.8A-B, Appendix 2.2E-F) thus ruling out the involvement of PAM and PAL subsets in receiving PDFR signaling and promoting day-time sleep. The *TH-D*, *F* and *G* drivers are expressed in different subsets of PPM2, PPM3, PPL1 and PPL2 neurons (Figure 3.9; (Liu et al., 2012)). Upon applying the same stringent criteria as before I found that down-regulation and over-expression of *pdfr* under the control of *TH-D'*, *D1* and *F3* drivers result in significant and opposite changes in day-time sleep as compared to both parental controls (Figure 3.8A-C, Figure 3.10, Appendix 2.2E-F). Thus, neurons belonging to PPL1, PPL2 and PPM3 subsets that are common to *TH-D'*, *D1* and *F3* drivers but not expressed by *TH-D4*, *F2* and *G1* drivers are the likely downstream targets of PDFR signaling important in modulating day-time wakefulness.

In order to identify the dopaminergic neurons that receive signals from PDF, I used the previously described *Pdfr myc* line (Im and Taghert, 2010) where *myc* is fused to the *Pdfr* gene, such that labeling MYC labels most $PDFR^+$ neurons including clock neurons and about 50 as yet uncharacterized non-clock neurons. Adult brains of *pdfr myc* flies were co-stained with antibodies against TH and MYC and examined for any overlap that may exist between TH^+ and $PDFR^+$ neurons. I found that 1-2 $PDFR^+$ neurons always lie in the vicinity of PPL1 and PPM3 subset of dopaminergic neurons (Figure 3.11A; n = 22 hemispheres). Upon careful examination, I found that in 3 out of

11 brains visualized, there was 1 PPM3 neuron in each hemisphere that was both TH^+ and $PDFR^+$ (Figure 3.11B-left). The low number of brains showing TH^+ and $PDFR^+$ PPM3 neurons could be because of high background and low affinity of the anti-MYC antibody. However, in none of the brains was there any overlap between PPL1 TH^+ and $PDFR^+$ neurons, although they were quite close to each other (Figure 3.11B-right). Thus, I can only conclude that perhaps 1 PPM3 neuron per hemisphere may express the PDF receptor.

3.3.7. PDF^+ and TH^+ neurons form synaptic contacts in sLN_v axons. Based on my results with altering *pdfr* levels that show that dopaminergic neurons are downstream targets of PDFR signaling, I next examined the nature of communication between PDF^+ and dopaminergic neurons. Because PDF is a neuropeptide, it can have long-range non-synaptic effects on downstream neurons expressing the PDF receptor (Nassel and Winther, 2010). I carried out a GRASP (GFP Reconstitution Across Synaptic Partners) experiment which relies on two independent binary systems allowing the expression of two membrane-bound GFP fragments in different sets of neurons, such that GFP is reconstituted and fluoresces only when the fragments are present at synaptic distances (Gordon and Scott, 2009). A similar experiment done previously had shown the presence of synapses between PDF^+ and dopaminergic neurons in the LN_v dendrites (Shang et al., 2011). However, here I asked if synapses between PDF^+ and dopaminergic neurons occur specifically in the region of LN_v axons since I wished to examine postsynaptic targets of PDF. Adult brains of flies in which dopaminergic neurons expressed GFP1-10 fragment and PDF^+ neurons expressed GFP11 fragment were co-stained with anti-GFP antibody that specifically labels reconstituted GFP and anti-PDF to visualize the LN_v projections. Reconstituted GFP signal was specifically detected in the ascending part of the dorsal projection of sLN_v (Figure 3.12A) which is

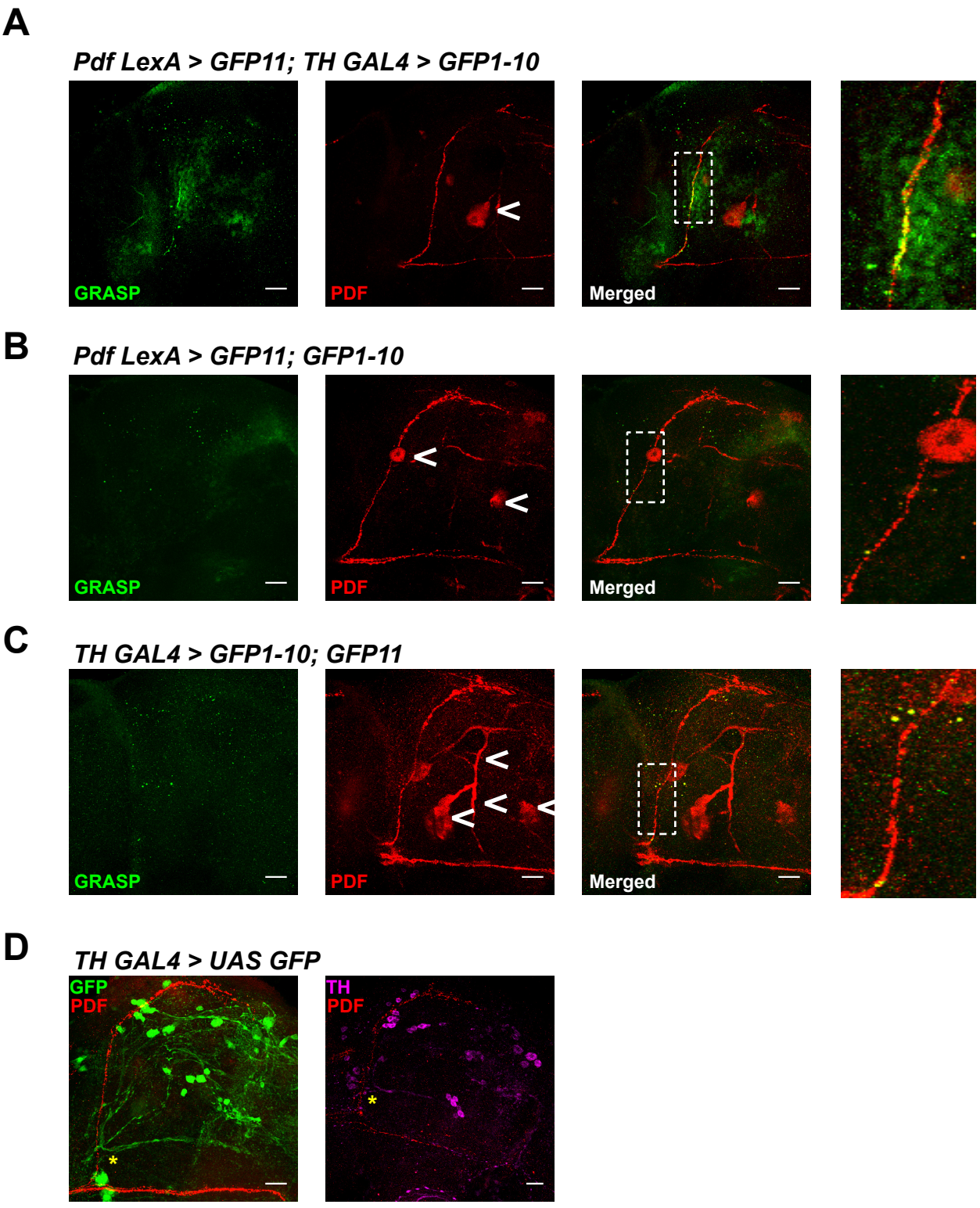
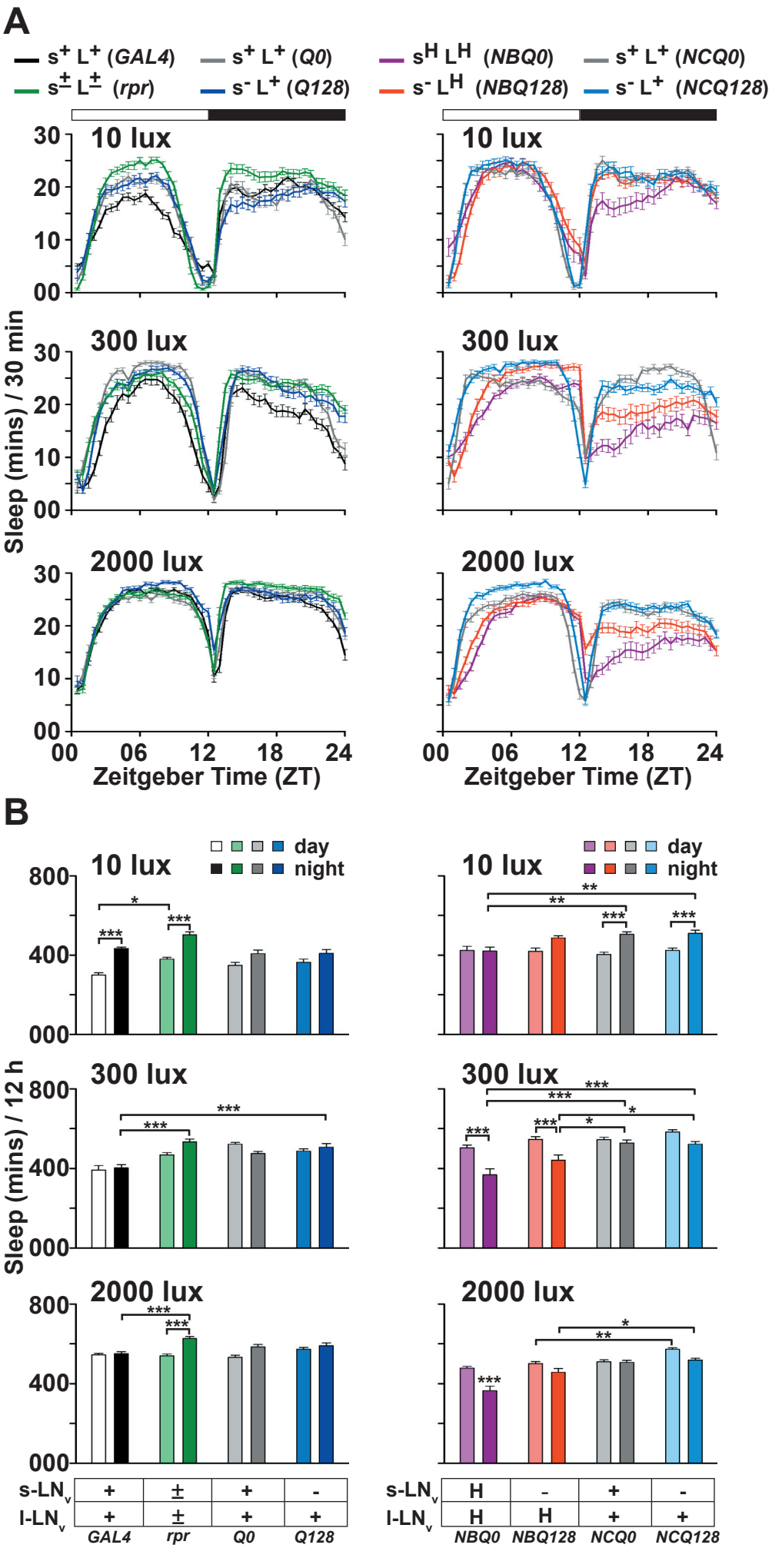


Figure 3.12. Anatomical connections between TH⁺ and PDF⁺ neurons. (A) Reconstituted GFP (GRASP) signal was detected in brains of flies expressing *LexAop CD4::spGFP11* under *Pdf LexA* control and *UAS CD4::spGFP1-10* under *TH GAL4* control ($n = 22$ hemispheres). GRASP signal colocalized with ascending portion of s-LN$_v$ dorsal projections labelled with antibody against PDF. Highlighted region is magnified in the right-most panel. Control flies lacking expression of (B) *spGFP1-10* ($n = 16$ hemispheres) and (C) *spGFP11* ($n = 16$ hemispheres) do not show GRASP signal. Results representative from two independent experiments. Arrowheads indicate non-specific staining. (D-left) Expression of GFP using *TH GAL4* and co-labelling PDF and (D-right) using antibodies against TH and PDF in wild type flies reveal dopaminergic projection in the vicinity of ascending portion of s-LN$_v$ dorsal projection as indicated by asterisks. Scale bar is 20 μm.

an axonal process. However, when either fragment was individually driven in the PDF^+ neurons or dopaminergic neurons alone, no GFP signal was detected (Figure 3.12B-C) showing that the antibody does not recognize individual fragments of GFP. This shows that PDF^+ and dopaminergic neurons form synaptic connections especially in the axonal projections of s-LN_v, thus bolstering my finding that dopaminergic neurons are downstream of PDFR signaling. Furthermore, in brains with dopaminergic neurons labeled with promoter driven GFP (*TH GAL4 > UAS GFP*), as well as with anti-TH, a dopaminergic projection lying close to the ascending dorsal projection of s-LN_v was found (Figure 3.12D, asterisk).

3.3.8. Auxiliary role of sLN_v in mediating wake-promoting effects of l-LN_v. While previous studies suggest a negligible role for the s-LN_v in the sleep/wake circuit (Chung et al., 2009; Shang et al., 2008), s-LN_v have been proposed to have a secondary role in promoting wake-mediating effects of l-LN_v (Parisky et al., 2008; Potdar and Sheeba, 2012). To explore their role further, I made use of previously reported toxic version of Huntingtin protein expression (*Htt Q128*, referred to as *Q128*, non-toxic form referred to as *Q0*) to selectively render s-LN_v dysfunctional (Sheeba et al., 2010) while simultaneously changing the electrical properties of the remaining l-LN_v by expressing the bacterial sodium channel *NaChBac* (*NB*, (Nitabach et al., 2006)). Sleep patterns of the following genotypes of flies were compared: those in which s-LN_v were dysfunctional but l-LN_v were normally firing (s^- L^+ *Q128* and *NCQ128*), some neurons from both LN_v subsets were ablated ($s^\pm$ $L^\pm$ *rpr*, apoptotic gene *reaper*, (Potdar and Sheeba, 2012)), both LN_v subsets hyper-excited (s^H L^H *NBQ0*) and s-LN_v were dysfunctional but l-LN_v were hyper-excited (s^- L^H *NBQ128*) with their respective controls in which both LN_v were normally firing and functional (s^+ L^+ *Q0* for s^- L^+ *Q128*, s^+ L^+ *GAL4* for $s^\pm$ $L^\pm$ *rpr*, s^+ L^+ *NCQ0* for s^H L^H *NBQ0* and s^- L^+ *NCQ128*, *NC*

Figure 3.13



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refers to *dORKNC1*, which is a non-conducting potassium channel, (Nitabach et al., 2002)). Additionally, $s^- L^+$ *NCQ128* served as control for $s^- L^H$ *NBQ128*. Because l-LN_v wake-promoting effects are primarily mediated by light (Shang et al., 2008), I examined sleep levels of these flies in LD12:12 cycles with different day-time light intensities. Altered levels of day-time sleep were observed only when the LN_v were ablated, but never when they were hyper-excited either completely or partially (Figure 3.13A-B, two-way ANOVA, 10 lux : $F_{7, 478} = 4.5$, $p < 0.0005$; 300 lux : $F_{7, 468} = 8.27$, $p < 0.00001$; 2000 lux : $F_{7, 476} = 13.91$, $p < 0.00001$). Moreover, the increased levels of day-time sleep in $s^{\pm} L^{\pm}$ flies is seen only when the light intensity is low (~ 10 lux), but with increasing light intensity, day-time sleep levels are comparable to $s^+ L^+$ flies, suggesting that remaining LN_v that have not been ablated can modulate day-time sleep effectively especially in the presence of saturating light intensities (Figure 3.13A-B-left). Furthermore, day or night-time sleep levels were not altered when s-LN_v were dysfunctional and l-LN_v were normally firing ($s^- L^+$). Interestingly, the finding that flies with hyper-excited LN_v show unchanged day-time sleep levels even in low light intensity LD12:12 cycles suggests that light-responsive l-LN_v can be saturated in terms of their firing capacity with as low light intensity as 10 lux. However, night-time sleep levels were always significantly lower than controls when both LN_v were hyper-excited

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Figure 3.13. s-LN_v mediate wake-promoting action of l-LN_v. (A) Sleep duration per 30 mins is plotted against time of the day in LD 12:12 of low (10 lux), moderate (300 lux) and high (2000 lux) light intensities. In moderate LD 12:12 (middle) $s^{\pm} L^{\pm}$ (*rpr*) flies sleep significantly more as compared to the $s^+ L^+$ (*GAL4*) control flies at almost all time-points both during the day and night. $s^- L^+$ (*Q128* and *NCQ128*) sleep similarly to their respective $s^+ L^+$ (*Q0* and *NCQ128*) controls during both day and night. $s^H L^H$ and $s^- L^H$ flies sleep significantly lesser than their controls, and $s^H L^H$ flies sleep even lesser than the $s^- L^H$ flies especially during the early part of the night. In both low (top) and high (bottom) LD 12:12 cycles, $s^H L^H$ flies take longer than all other genotypes including $s^- L^H$ flies after lights-OFF to fall asleep.(B) In low (top) LD 12:12 cycles, day-time sleep of $s^{\pm} L^{\pm}$ flies is significantly higher than that of $s^+ L^+$ (*GAL4*) flies, whereas night-time sleep of $s^{\pm} L^{\pm}$ flies is significantly higher than that of $s^+ L^+$ (*GAL4*) flies in moderate (middle) and high (bottom) LD 12:12 cycles. In both low and moderate LD12:12 cycles, night-time sleep of $s^H L^H$ and $s^- L^H$ flies is significantly lesser than their respective controls, but not different from each other. Night-time sleep of $s^H L^H$ is different from its respective control $s^+ L^+$ (*NCQ0*) as well as $s^- L^H$ flies in high light intensity LD 12:12 (bottom).For all genotypes, *n* between 24-32 flies. All other details are as in Figure 3.3.

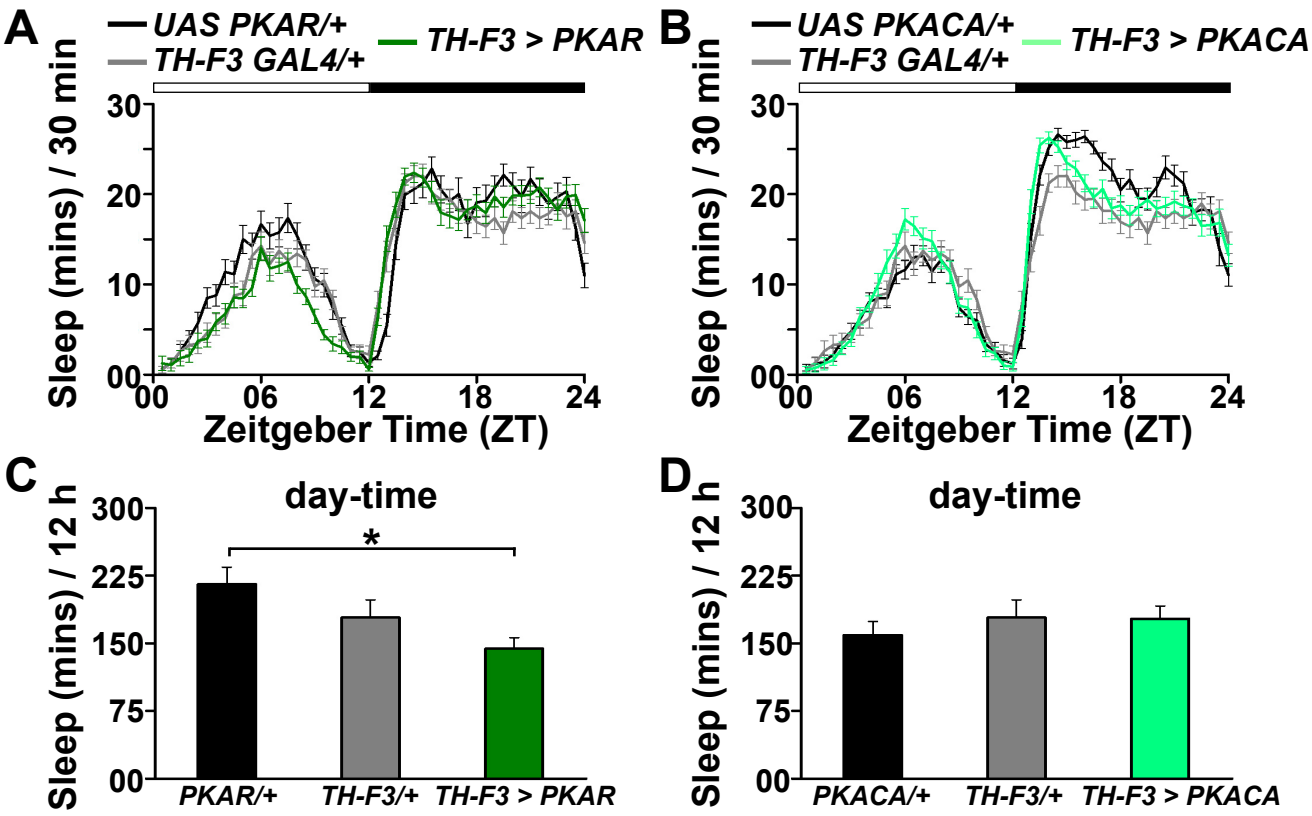

Figure 3.14. Sleep profiles of flies with altered PKA signaling in subsets of dopaminergic neurons. (A) Sleep duration per 30 mins is plotted against time of the day in LD 12:12 for flies with either decreased (*TH-F3 GAL4 > UAS PKAR*) or increased (*TH-F3 GAL4 > UAS PKACA*) PKA signalling in TH-F3^{+} neurons. (B) Day-time sleep of *TH-F3 GAL4 > UAS PKAR* (n = 31 flies) is significantly lower than *UAS PKAR/+* (n = 22 flies) but not different from *TH-F3 GAL4/+* (n = 27 flies), whereas day-time sleep of *TH-F3 GAL4 > UAS PKACA* (n = 32 flies) is not different from both *UAS PKACA/+* (n = 30 flies) and *TH-F3 GAL4/+* (n = 27 flies). All other details are as in Figure 3.3.

(s^H L^H, Figure 3.13A-B-right). Interestingly, night-time sleep levels of flies with dysfunctional s-LN_v but hyper-excited l-LN_v (s^- L^H) is significantly reduced as compared to controls, but always higher than the s^H L^H flies (Figure 3.13A-B-right). In fact, under LD12:12 with low light intensity days (10 lux), night-time sleep levels of s^- L^H flies were comparable with their s^+ L^+ controls (Figure 3.13A-B, right-top). These results validate that l-LN_v modulate wakefulness and further show that functional s-LN_v are required to mediate these effects.

3.3.9. PDFR signaling inhibits PPM3 neuronal activity specifically during the day. Previous studies have shown that binding of PDF to PDFR results in a strong increase of cyclic Adenosine Monophosphate (cAMP) levels (Mertens et al., 2005; Shafer et al., 2008) and moderate increase of intracellular calcium (Ca^{2+}) levels when expressed in HEK293 (Human Embryonic Kidney) cells (Mertens et al., 2005). To test whether cAMP is the secondary messenger involved in mediating wakefulness through PDFR signaling, either the catalytic (*PKACA*) or regulatory (*PKAR*) subunit of cAMP-dependent Protein Kinase A (PKA) which increases or reduces PKA activity respectively were over-expressed using *TH-F3 GAL4*. No significant changes in day-time sleep as a result of increasing or decreasing PKA activity were found (Figure 3.14A-B, D, one-way ANOVA, $F_{2, 86} = 0.44$, $p = 0.64$), although day-time sleep of *TH-F3 GAL4 > UAS PKAR* flies was significantly lower as compared to only the *UAS PKAR* control flies (Figure 3.14C, one-way ANOVA, $F_{2, 77} = 4.41$, $p < 0.05$). This shows that cAMP may not be the secondary messenger responding to PDFR signaling in the TH-$F3^+$ neurons, as changing PKA activity levels has negligible effects on day-time sleep.

To assess the functional importance of the connectivity between PDF^+ and dopaminergic neurons, I next examined intracellular Ca^{2+} levels in dopaminergic

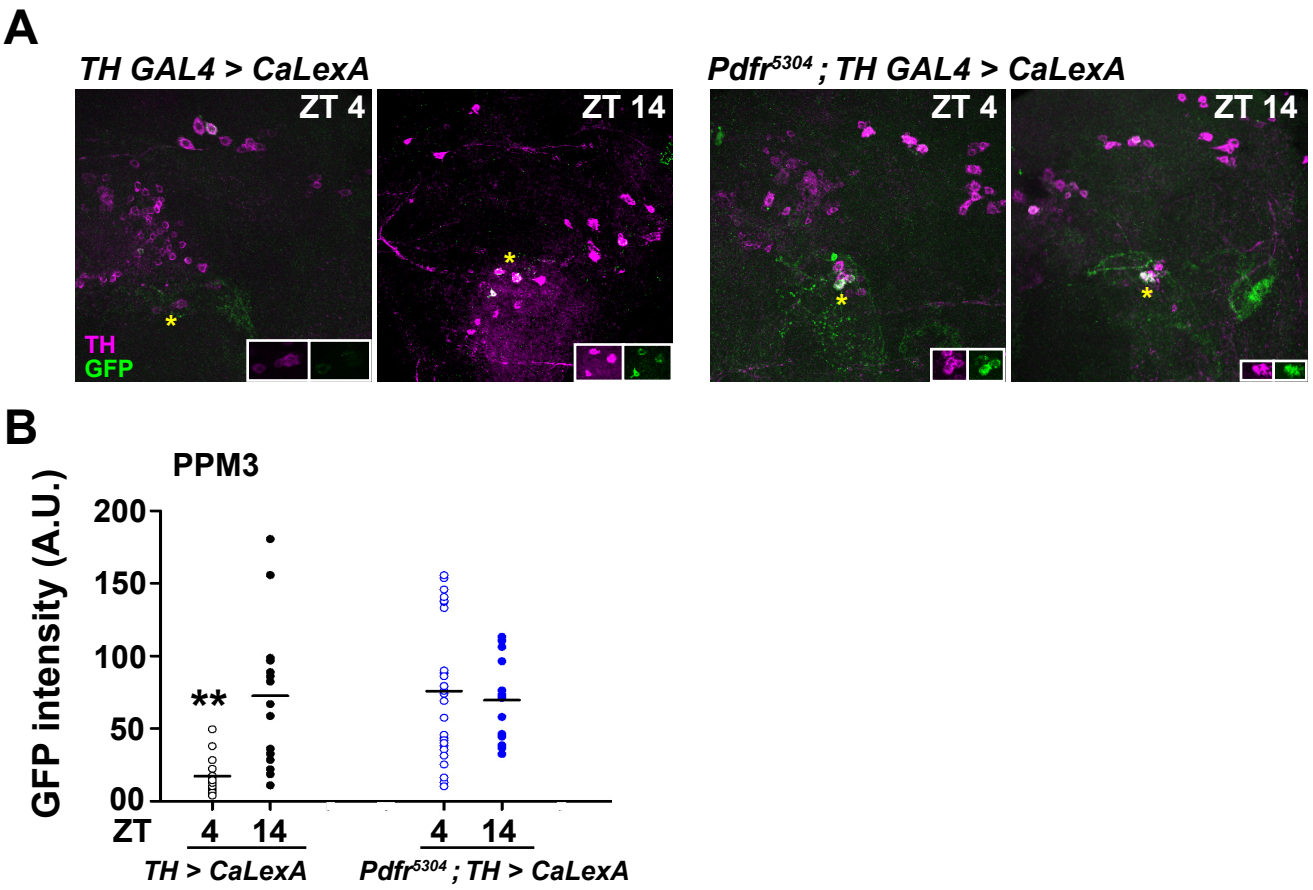

Figure 3.15. Intracellular Ca^{2+} levels in PPM3 neurons lower during day than night but remain similar during day and night in the absence of PDFR. (A) *TH GAL4* expressing *CaLexA* in *WT* and *pdfr*^{5304} backgrounds co-stained with antibodies against TH to mark dopaminergic neurons and GFP to quantify intracellular Ca^{2+} levels at two time-points – ZT4 and ZT14. *CaLexA*-driven GFP^{+} signal was detected at a low level at ZT4 whereas higher intensity at ZT 14 (left panels) in *WT* background. *CaLexA*-driven GFP^{+} signal was detected at similar high level at both ZT4 and ZT 14 (right panels) in *pdfr*^{5304} background. Asterisks indicate PPM3 neurons which are zoomed in inset. (B) Quantification of results in (A) shows significantly lower GFP fluorescence in *TH GAL4 > CaLexA* flies at ZT4 as compared to GFP fluorescence in *TH GAL4 > CaLexA* flies at ZT14, *pdfr*^{5304}*; TH GAL4 > CaLexA* flies at ZT4 and ZT14. All other details are as in Figure 3.3.

neurons at two time-points, one during day (Zeitgeber Time (ZT) 4; 4 hours after lights-ON) and another during night (ZT14) in the presence and absence of functional PDFR (*Pdfr*^{5304} mutant background). To quantify Ca^{2+} levels, I used the recently developed *CaLexA* method which relies on calcium-dependent-nuclear transport of *VP16:LexA* to drive GFP downstream of *LexAop* responder element (Masuyama et al., 2012). When the *CaLexA* transgenes are expressed using the broad dopaminergic driver *TH GAL4*, I found that 1-2 neurons of some dopaminergic subsets notably within the PAL, PPM2 and PPM3 clusters express GFP (Table 3.2A). However, differential expression of GFP depending upon time-point and genotype is observed only in about 1-2 PPM3 neurons per hemisphere (Table 3.2B). In the presence of functional PDFR, at ZT4 when the levels of PDF are also high (Park et al., 2000), the amount of GFP^+ signal seen in the PPM3 neurons is quite low (Figure 3.15A-B, two-way ANOVA, $F_{1, 70} = 10.85$, $p < 0.005$). However, at ZT14 when the levels of PDF are low (Park et al., 2000), amount of GFP^+ signal seen in PPM3 neurons is significantly higher than that at ZT4 (Figure 3.15A-B). To measure the calcium levels in PPM3 neurons in the absence of functional pdfr, I expressed the *CaLexA* transgenes in dopaminergic neurons (using *TH GAL4*) in a *pdfr*^{5304} mutant background. While it would have been worthwhile to measure calcium levels in flies with reduced *pdfr* levels using RNAi-based knock-down, I used the *pdfr*^{5304} mutant background to reduce the transgene load. I found that in the absence of functional PDFR, at both ZT4 and ZT14, the GFP^+ signal is high and not different from each other (Figure 3.15A-B). Importantly, at ZT4, the GFP^+ signal is significantly higher in the absence of functional PDFR than in its presence (Figure 3.15). This shows that PDF acting on PDFR in the PPM3 dopaminergic neurons decreases Ca^{2+} levels specifically during the day-time.

Table 3.2A

Genotype	***TH GAL4 > UAS CaLexA***		***Pdfr^{5304}; TH GAL4 > UAS CaLexA***	
Time-point	**ZT 4**	**ZT 14**	**ZT 4**	**ZT 14**
***n* (hemispheres)**	14	16	13	12
PAL (13)	1.4 ± 0.3	1.3 ± 0.3	2.4 ± 0.4	2.1 ± 0.4
PAM (5)	0.1 ± 0.09	0.4 ± 0.2	0.1 ± 0.1	0
PPM1 (5)[a]	0.1 ± 0.09	0	0	0
PPM2 (8)	2.1 ± 0.5	2.6 ± 0.4	2 ± 0.5	2 ± 0.4
PPM3 (8)	1.1 ± 0.3	1 ± 0.3	2.2 ± 0.4	1.2 ± 0.3
PPL1 (12)	0.3 ± 0.1	0.7 ± 0.3	0.2 ± 0.1	1.5 ± 0.4
PPL2ab (6)	0.07 ± 0.07	0.8 ± 0.3	1.1 ± 0.3	0.8 ± 0.3
PPL2c (4)	0	0	0.08 ± 0.08	0.08 ± 0.08
PPL3 (1)	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.3	0.2 ± 0.1
PPL4 (1)	0	0.06 ± 0.06	0	0

Table 3.2B

Genotype	***TH GAL4 > UAS CaLexA***		***Pdfr^{5304}; TH GAL4 > UAS CaLexA***	
Time-point	**ZT 4**	**ZT 14**	**ZT 4**	**ZT 14**
***n* (hemispheres)**	14	16	13	12
PAL	30.4 ± 4.8	32.3 ± 5.7	50.3 ± 5.9	36.8 ± 6
PAM	27.3 ± 13.8	55.2 ± 19.2	36.7 ± 10.6	0
PPM1	6.4 ± 0.3	0	0	0
PPM2	23.4 ± 5.2	41.3 ± 5.9	36.9 ± 6.7	23.8 ± 4.3
PPM3	17.2 ± 3	72.5 ± 12.1	75.8 ± 8.7	69.6 ± 7.6
PPL1	22 ± 9.9	17.4 ± 3.5	59.2 ± 41.3	22.6 ± 4.6
PPL2ab	15.4	20.2 ± 2.8	49.5 ± 10.9	36.7 ± 5.9
PPL2c	0	0	18.7	33.6
PPL3	6 ± 1.5	23.6 ± 8.6	11.4 ± 1.9	52.2 ± 0.6
PPL4	0	15.8	0	0

Table 3.2. Ca^{2+} levels in dopaminergic neurons. (A) Number of GFP^+ neurons as seen in different dopaminergic subsets (mean ± SEM) in brain hemispheres expressing *CaLexA* under the *TH GAL4* driver in wild type and *pdfr*^{5304} backgrounds during day (ZT 4) and night (ZT 14) time-points. Numbers in parentheses indicate overall number of neurons of different subsets per hemisphere that are *TH GAL4*-positive as seen from data in Mao and Davis, 2009. [a]Number of neurons per brain. (B) GFP^+ fluorescence intensity (mean ± SEM) in different subsets of dopaminergic neurons.

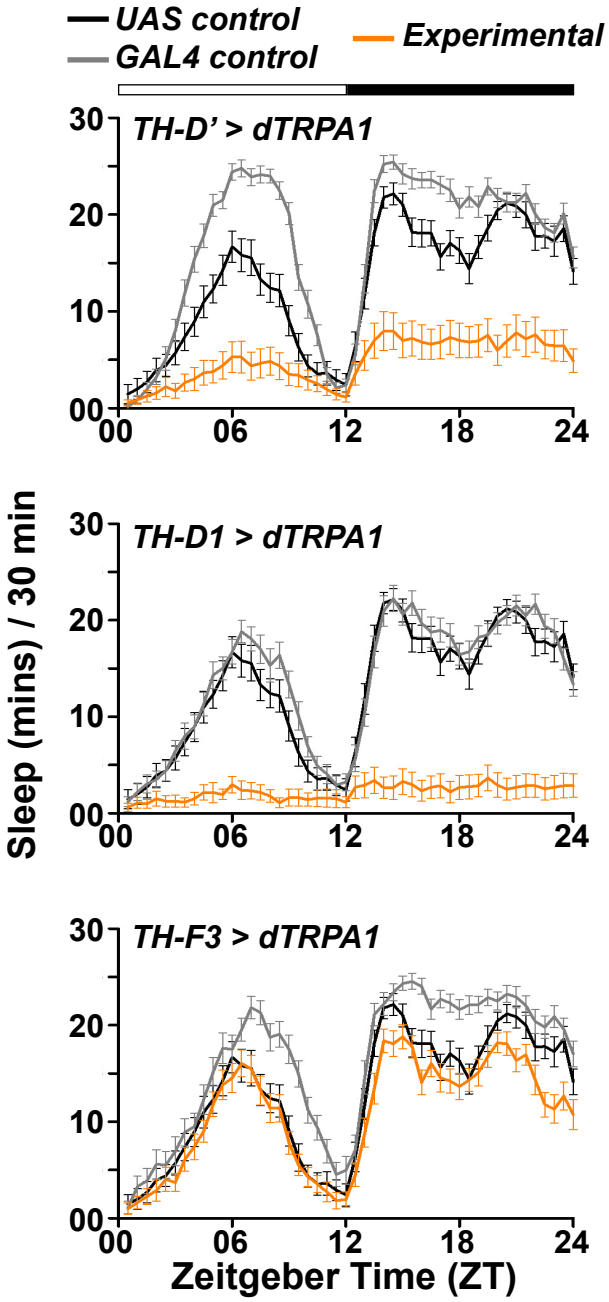

Figure 3.16. Sleep profiles of flies with hyper-excited subsets of dopaminergic neurons. (A) Sleep duration per 30 mins is plotted against time of the day in LD 12:12 at a high temperature of 29 °C for flies with hyper-excited dopamine neurons labelled by the *TH-D'*, *TH-D1* and *TH-F3 GAL4* drivers. Both *TH-D' GAL4 > UAS dTRPA1* (top) and *TH-D1 GAL4 > UAS dTRPA1* (middle) flies sleep lower both during the day and night as compared to their respective controls. *TH-F3 GAL4 > UAS dTRPA1* (bottom) do not differ in their sleep levels either during day or night as compared to both controls. For all genotypes, *n* between 30-32 flies. All other details are as in Figure 3.3.

Given that dopaminergic neurons are wake-promoting, inhibiting them should inhibit wakefulness. Yet, increasing PDFR in PPM3 neurons which, in accordance with the calcium level quantification should cause increased inhibition, results in decreased day-time sleep. Similarly, absence of *pdfr* leads to reduced inhibition (as seen from increased Ca^{2+} levels) of PPM3 neurons, yet behaviourally the flies sleep more during the day-time. This indicates that PDF/PDFR signaling is acting on those PPM3 neurons that are, in effect, sleep-promoting. An alternative possibility could be that PDFR levels are cycling in such a way that they are higher during the night, although it would be peculiar since PDF levels are usually low during the night (Park et al., 2000). Thus, while a majority of dopaminergic neurons may be wake-promoting there may still be 1-2 PPM3 neurons which are $PDFR^{+}$ and which effectively promote sleep. In order to examine this heterogeneity, I expressed the *dTRPA1* channel in different subsets of dopaminergic neurons using *TH-D1*, *TH-D'* and *TH-F3 GAL4* drivers and examined the sleep levels of flies at a low inactivating temperature of 21 °C as well as at a high activating temperature of 29 °C. As reported in an earlier study (Liu et al., 2012), I found that flies sleep lesser both during the day and night when dopaminergic neurons driven by *TH-D1* and *TH-D' GAL4* are hyper-excited (Figure 3.16, one-way ANOVA, *TH-D1*: $F_{2, 76} = 23.16$, $p < 0.00001$; *TH-D'*: $F_{2, 78} = 28.88$, $p < 0.00001$). However, when neurons expressed by the *TH-F3 GAL4* are hyper-excited, flies tend to sleep as much as their *GAL4* and *UAS* parental controls do, especially during the day-time (Figure 3.16, one-way ANOVA, $F_{2, 85} = 2.29$, $p = 0.1$). This can happen only if these neurons do not actually have any effect on sleep, or if they are a heterogeneous group of wake-promoting and sleep-promoting neurons, such that hyper-exciting both leads to a cancellation of effects caused by both groups. Given that *TH-F3 GAL4*-driven

dopaminergic neurons have effects on sleep when *pdfr* levels are altered, these results point toward the possibility of 1-2 $PDFR^{+}$ PPM3 neurons that are also sleep-promoting.

3.4. Discussion

In mammals, it has been shown that mutations in circadian clock genes affect sleep homeostatic features (Landgraf et al., 2012). To a certain extent, from my work in the previous chapter, this is true even for flies. Here, my aim was to find if any of the circadian clock neurons could either perform sleep homeostatic functions, or communicate with sleep homeostatic structures. I found that modulating neuronal firing of the circadian neurons responsible for evening bout of activity (Grima et al., 2004; Rieger et al., 2006; Stoleru et al., 2004; Yao et al., 2016; Yao and Shafer, 2014) results in changes in sleep pressure, as hyper-exciting them causes sleep deprivation but restoring them to baseline levels of firing causes sleep recovery. Additionally, I found that PDF^{+} LN_v regulate day-time wakefulness by communicating with dopaminergic neurons among the PPM3 subset. Interestingly, PPM3 neurons arborize heavily in the EB, dFB and MB regions (Aso et al., 2014a; Liu et al., 2012), all of which have been implicated in performing sleep homeostatic functions (Donlea et al., 2014; Liu et al., 2016; Pimentel et al., 2016; Sitaraman et al., 2015a; Sitaraman et al., 2015b). Thus, my study has uncovered two potential pathways using which circadian clocks may modulate sleep homeostatic properties.

Dopamine is primarily involved in promoting wakefulness (Andretic et al., 2005; Kume et al., 2005) and is known to act on l-LN_v (Shang et al., 2011) as well as inhibit sleep-promoting dFB (Liu et al., 2012; Ueno et al., 2012) to carry out its wake-promoting function. Here, my studies suggest that certain dopamine neurons could be sleep-promoting and through the inhibitory action of PDFR signaling, wakefulness gets

promoted specifically during the day. Interestingly, a previous study has found that dopamine acts on l-LN_v to promote wakefulness (Shang et al., 2011) and I find that PDFR signaling acts on dopamine neurons, suggesting a feed-forward pathway for wake promotion, where dopamine acting on l-LN_v promotes the inhibition of sleep-promoting dopaminergic neurons by PDFR signaling. The identity of dopamine neurons acting on l-LN_v and those responding to PDFR signaling may differ which can be uncovered with further experiments.

Several lines of evidence show that dopaminergic neurons are involved in maintaining wakefulness in mammals (reviewed in (Eban-Rothschild et al., 2018)). Specifically, dopaminergic neurons in the ventral tegmental area were shown to be necessary for maintaining wakefulness and for promoting arousal in the wake of important sensory cues (Eban-Rothschild et al., 2016). In addition, dopaminergic neurons in the dorsal Raphe nucleus were also shown to be involved in promoting wakefulness (Cho et al., 2017; Lu et al., 2006). In fact, a recent study has shown that some dopaminergic neurons within the nucleus accumbens also promote wakefulness (Luo et al., 2018), whereas another study has shown that dopaminergic neurons within this region promote NREM sleep (Qiu et al., 2016). This heterogeneity in the role of dopamine in sleep/wake regulation is reminiscent of what is reported here in this study, with 1 PPM3 neuron possibly being sleep-promoting, and the well-established role of dopamine neurons being wake-promoting (Andretic et al., 2005; Kume et al., 2005; Liu et al., 2012; Ueno et al., 2012). Thus, on the basis of the current study, one could speculate that these dopamine neurons are innervated and modulated by the dorsal medial hypothalamus, the output centre of the SCN (Saper, 2013), as a mechanism for circadian clock control of sleep/wake regulation in mammals.

The role of s-LN_v in modulating sleep and wake has been explored in some detail in the recent years. s-LN_v have also been shown to promote sleep via short Neuropeptide F (sNPF) as well as myoinhibitory peptide (MiP) by inhibiting the wake-promoting l-LN_v (Oh et al., 2014; Shang et al., 2013). Here, I show that PDF^+ s-LN_v make synaptic contacts with dopaminergic neurons (Figure 3.12) and that PDFR signaling inhibits the downstream dopaminergic neurons (Fig 3.15) to promote wakefulness during the day. Moreover, I have shown a secondary role for s-LN_v in modulating wake-promoting effects of l-LN_v. Yet, how this wake-promoting signal which originates in the l-LN_v is relayed to the s-LN_v is not understood. Furthermore, from my screen it is clear that this function is not mediated via PDFR signaling among the LN_v, as down-regulating and over-expressing *pdfr* in s-LN_v (*Clk 9M GAL4* and *Pdf GAL4*) do not result in any sleep defects. Thus, wake-promoting signal from l-LN_v to s-LN_v is independent of PDF while s-LN_v to dopamine wake-promoting signal requires PDFR signaling.

PDFR being a class B1 GPCR (G-protein coupled receptor) utilizes cAMP as its secondary messenger (Kunst et al., 2015; Shafer et al., 2008), although there is evidence for Ca^{2+} also to act as the secondary messenger (Mertens et al., 2005). For most of the functions of PDF including stabilizing core clock proteins such as TIMELESS and PERIOD in different target neurons such as DN_1s and s-LN_v, cAMP is the major secondary messenger (Li et al., 2014; Seluzicki et al., 2014). Moreover, it is thought that different actions of PDF of slowing down and speeding up of morning and evening clock neurons is also mediated by different components of cAMP signaling mechanism (Duvall and Taghert, 2012; Duvall and Taghert, 2013). However, here I show that for the function of regulating wake levels during the day-time, PDFR changes levels of intracellular Ca^{2+} in dopamine neurons with negligible role for cAMP signaling,

suggesting a mechanism by which a neuropeptide that has diverse effects on its downstream targets can modulate different functions independently. I therefore identify a unique subset of downstream targets for PDFR signaling among the dopamine neurons that promote wakefulness depending upon time of day.

Interestingly, in my screen I note that there are several driver lines using which there are significant changes in day-time sleep but with only one type of manipulation of *pdfr* levels (*Clk 4.1M*, *30y*, *104y*, *121y GAL4*). Given that PDF is a neuropeptide which can have long-range non-synaptic effects (Nassel and Winther, 2010), even mis-expressing it (*104y* and *121y GAL4*) in different substrates has resulted in altered day-time sleep levels. Interestingly, because DH31 can also respond to PDFR (Kunst et al., 2015), it is possible that these effects could be mediated by DH31 binding to mis-expressed PDFR. However, I found that this may not be the case as down-regulating DH31-receptor in these regions does not cause changes in sleep levels (data not shown). Perhaps examining the effects of manipulating *pdfr* levels only in the dFB using more restricted drivers (such as *R23E10 GAL4*; (Liu et al., 2016)) as compared to the ones used here could clarify the picture. Nevertheless, I can conclude that in regions previously not known to express *pdfr*, mis-expression of *pdfr* can cause sleep level deficits suggesting that PDF can act in regions which are not direct targets but yet may lie in the vicinity of LN_v projections.

The role of PDF/PDFR signaling is well-known in synchronizing the free-running molecular rhythms in neurons across the circadian network (Lin et al., 2004; Peng et al., 2003; Wu et al., 2008b; Yoshii et al., 2009). PDFR signaling in the "evening" neurons is important for appropriate phasing of the evening bout of activity in Light/Dark cycles (Guo et al., 2016; Yao and Shafer, 2014). While the role of PDF as a wake-signal has been known, here I demonstrate that a subset of dopaminergic

neurons is downstream of the PDF/PDFR signaling. While the PDFR expression is not conclusive, I show that perhaps one PPM3 neuron per hemisphere may express the PDF receptor. Down-regulating *pdfr* in these neurons results in increase of day-time sleep, which is a phenocopy of the sleep behaviour of loss-of-function *pdfr* whole-body mutants. On the other hand, over-expressing *pdfr* in these neurons leads to decrease of day-time sleep specifically. I further show that PDF and dopaminergic neurons make synaptic contacts with each other at the site of the axonal projection of s-LN_v. Moreover, the effect of PDFR signaling on the PPM3 neurons appears to be inhibitory, suggesting that the $PDFR^+$ PPM3 neurons promote sleep. Taken together, I conclude that wake-promoting LN_v make synaptic connections with sleep-promoting dopaminergic neurons and promote wakefulness specifically during the day-time through inhibitory PDFR signaling (Figure 3.17).

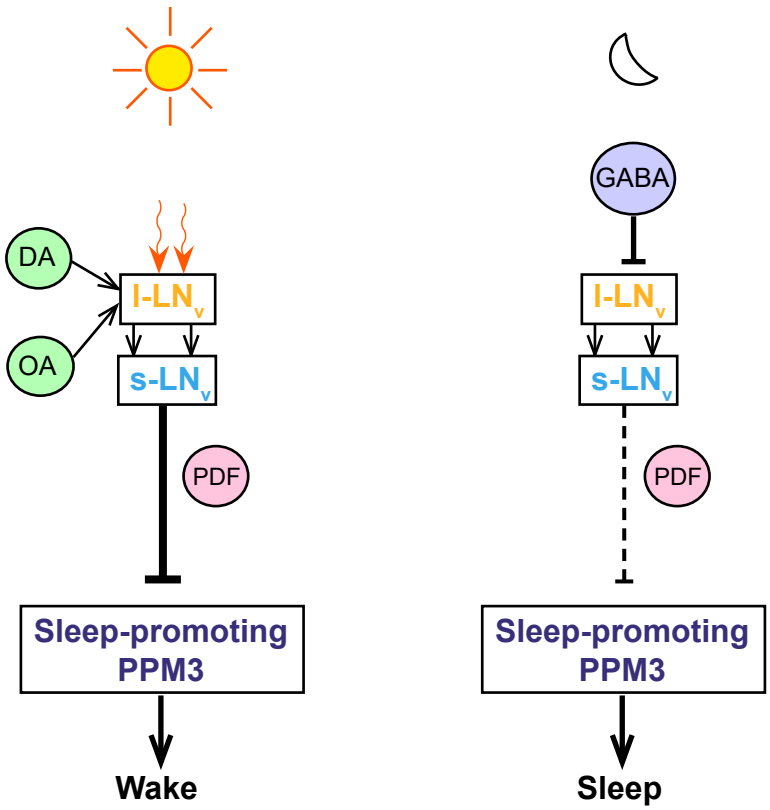

Figure 3.17. Day-time wakefulness and night-time sleep regulation by PDFR signaling to PPM3 neurons. During day, l-LN$_v$ receive various activating cues such as light (Shang et al., 2008), dopamine and octopamine (Shang et al., 2011), which they communicate to s-LN$_v$. s-LN$_v$ express high levels of PDF during the day-time which results in inhibition of sleep-promoting PPM3 neurons (solid line), as a result of which wakefulness is promoted. During night, l-LN$_v$ are inhibited by GABA (Parisky et al., 2008, Liu et al., 2014), therefore s-LN$_v$ do not receive any wake-promoting signal. As a result, reduced PDF expression leads to removal of inhibition of PPM3 neurons, thereby resulting in sleep.

Chapter 4

Sleep deprivation negatively impacts reproductive output in *Drosophila melanogaster*

4.1. Introduction

The rest phase of sleep is remarkably ubiquitous in animals suggesting that sleep is important. While we humans spend a third of our lives sleeping, we do not know *why* sleep is indispensable. Several studies link sleep levels to cognition, mood and emotional states (Krause et al., 2017), as well as physiological health in humans (Mahoney, 2010). When rats are chronically deprived of sleep there are detrimental effects on longevity (Rechtschaffen et al., 1983), skin condition (Everson et al., 1989) and body weight (Everson and Szabo, 2011) accompanied by physiological changes in internal organs (Everson and Szabo, 2009). Thus, sleep positively influences many organ systems in addition to the nervous system.

The genetically tractable model organism *Drosophila melanogaster* exhibits several characteristics of mammalian sleep (Hendricks et al., 2000a; Nitz et al., 2002; Shaw et al., 2000; van Alphen et al., 2013) and sleep deprivation in flies results in deleterious effects similar to those seen in mammals. Mechanically depriving flies of sleep decreases their lifespan (Seugnet et al., 2009; Shaw et al., 2002) and short-sleeping mutants of the Shaker potassium channel have reduced lifespan (Bushey et al., 2010; Cirelli et al., 2005a). However, lifespan by itself is an insufficient indicator of overall fitness of an organism as it can be radically influenced by reproductive output (Sheeba et al., 2000). Since reproductive success is a strong evolutionary driving force, I focused on possible mechanistic links between sleep and reproductive output.

In humans, infertility is often associated with sleep disturbances; however, the complexity of the reproductive system and sleep characteristics in humans makes the analysis of sleep disruption affecting reproductive processes difficult (Kloss et al., 2015). Shift-workers and women who experience frequent jet lag conditions report

sleep disturbances and abnormal menstrual cycles and are at a higher risk of developing pregnancy-related complications (Mahoney, 2010). Chronic sleep deprivation in rats increases spontaneous ejaculations (Andersen and Tufik, 2002) and reduces the number of live sperm (Alvarenga et al., 2015). In mice subjected to light protocols mimicking jet lag and circadian misalignment, reproductive success is hampered (Summa et al., 2012). Circadian clock mutants with defective timing and consolidation of sleep also have reduced reproductive output in flies (Beaver et al., 2002) and mice (Loh et al., 2014). In a recent study in *Caenorhabditis elegans*, it was found that depriving worms of the developmentally regulated sleep-like lethargus state activated a protective response in the endoplasmic reticulum, blocking which caused apoptosis of sperms as well as defects in muscular activity of egg-laying circuit (Sanders et al., 2017). Sleep deprivation alters aggressive behaviour in flies and hampers the chances of mating (Kayser et al., 2015). Most studies show that sleep and reproductive output are associated with one another, without testing the direct effects of sleep on reproductive success. Here, I address this question by impairing sleep in female fruit flies and testing its effect on reproductive output. I found that feeding flies with caffeine or depriving them of sleep by mechanical perturbation, or by decreasing sleep by genetic activation of wake-promoting dopamine neurons all result in decreased egg output. Decreased sleep is associated with decreased egg output for all manipulations. Thus, my study establishes a model system to study the mechanisms underlying relationships between sleep and reproductive processes that underlie fitness.

4.2. Materials and Methods

4.2.1. Fly strains. Fly strains and their sources used for both activity/rest and egg output assays are given in Appendix 1. All the transgenic flies used were back-crossed into the standard w^{1118} background for at least 7 generations.

4.2.2. Activity/rest and egg output assays. For the activity/rest assays, 4-5 day old virgin female flies were initially allowed to mate for a day and then were individually housed in tubes (65 mm length, 3 mm inner diameter) with standard cornmeal food on one end and cotton plug on the other and activity was recorded in DAM2 monitors (*Drosophila* activity monitoring system, Trikinetics, Waltham, MA, USA). The DAM system works on the standard beam-breaking principle where a fly cuts an infra-red beam whenever it moves in the middle portion of the tube, thereby generating activity counts. Activity counts were binned at 1 min intervals to obtain sleep parameters using the software PySolo (Gilestro and Cirelli, 2009). Flies were housed in light and temperature controlled environments with 12 hours of light and 12 hours of dark (LD 12:12) at 25 °C using incubators (MIR-273, Sanyo, Tokyo, Japan; DR-36VLC8 Percival Scientific Inc., Perry, IA, USA). Flies were transferred into fresh food tubes containing either standard food or food containing different concentrations of caffeine (Hi-Media, Bangalore, India) every 12 hours depending upon their treatment. The activity recording assays were run for a period of 6-7 days. First two days represent baseline days of recording, next three days (days 3-5) were the days during which sleep deprivation was given either by caffeine treatment or temperature increase, and the last two days represent the recovery days during which sleep rebound is expected to occur. For specific assays, flies were fed with caffeine either during day or night for a period of 6 days.

The egg output assays were conducted simultaneously along with the activity/rest assays, on a parallel set of flies housed in glass vials (10 cm length, 2.5 cm diameter) containing ~3 ml of cornmeal food with or without caffeine depending upon the treatment. For the egg output assays, a small amount of charcoal (0.8 g/L) was added to cornmeal food to increase the contrast between eggs and food surface, thereby

aiding in egg counting. As before, flies were transferred into fresh food vials every 12 h and the number of eggs laid were counted with the help of a stereo-microscope (SZ160, Olympus, Tokyo, Japan). In the experiment for sleep deprivation by mechanical means, individual flies were housed in tubes (65 mm in length, 5 mm in inner diameter) placed in DAM5 monitors which were then mounted on a vortexer (VWR, Radnor, PA, USA) that was used to mechanically disturb flies either during the day or night. Eggs laid by flies in these tubes as well as by flies that remained undisturbed throughout day or night were then counted for a period of 5 days. Oviposition choice assays were performed by introducing 5 female w^{1118} flies for a period of two or twelve hours on petri-dishes that contained standard cornmeal food on one half and cornmeal food with specific concentrations of caffeine on the other. Data analyses were done independently in experiments repeated multiple times. One run among replicates was arbitrarily chosen as a representative run only if all runs showed similar results.

The CAFE (Capillary Feeder) assay was carried out for a period of 24 hours as described in (Ja et al., 2007). Briefly, individual flies were housed in vials containing 0.5% agar and 5 µL micro-capillaries containing a solution of 5% sucrose, 1% food dye (blue, McCormick, Sparks, MD, USA) and either 0.5 or 1 mg/ml caffeine as the food source. Fresh micro-capillaries were provided after 12 hours and the level of food was noted to indicate food consumption for the 12-h duration. Filled micro-capillaries in vials with no flies served as evaporation controls. The final consumption values were obtained after correcting for evaporation and adding the values for both day and night durations.

4.2.3. Statistical analysis. Oviposition preference for a given food was defined as the percentage of total eggs laid on that food surface. Percentage sleep loss was calculated as percentage decrease in sleep during sleep deprivation days with reference to sleep levels during baseline days. Sleep measures of control and sleep deprived flies were compared using one-way ANOVA with treatment or genotype as a fixed factor followed by *post-hoc* Tukey's Honest Significant Difference (HSD) test with *p*-level set at 0.05. For sleep deprivation using caffeine, average day or night sleep levels were compared using two-way ANOVA with treatment and days (BS, SD or RC) as fixed factors followed by *post-hoc* Tukey's Honest Significant Difference (HSD) test. Egg output data were first tested for normality using a Shapiro-Wilk's W test. For mean number of eggs laid, one-way ANOVA followed by *post-hoc* Tukey's HSD test was conducted if all datasets under consideration were normally distributed. However, even if one of the datasets were not normally distributed, a Kruskal-Wallis test was conducted with *p*-level set at 0.05. For day-to-day comparisons of egg output, two-way ANOVA with treatment and day as fixed factors followed by *post-hoc* Tukey's HSD test was conducted. For day-to-day egg output of *fmn* and *fmn-bg* flies fed with caffeine, three-way ANOVA with genotype, treatment and day as fixed factors followed by *post-hoc* Tukey's HSD test was conducted. Details of statistical analyses are given in results and Appendix 2.3.

4.3. Results

4.3.1. Effect of sleep deprivation on egg output of inbred w^{1118} flies. To assess the impact of sleep deprivation on reproductive output, I first used caffeine to deprive female flies of sleep. Flies were given caffeinated food during the day only (D_{caf}), or during the night only (N_{caf}) or standard cornmeal food during both day and night that acted as controls (Ctrl). To estimate the appropriate concentration of caffeine for the

egg output assay, I quantified the amount of sleep loss in flies with two concentrations (0.5 and 1 mg/ml) based on previous studies (Andretic et al., 2008; Wu et al., 2009) and my pilot experiments. Flies that were fed with food containing 0.5 mg/ml caffeine only during the day (D_{caf}) tend to exhibit less sleep during the day as compared to their own baseline (BS) as well as compared to control flies during caffeine (CAF) days (Figure 4.1A, BS and CAF), although this reduction was not statistically significant (Figure 4.1B, day, two-way ANOVA, $F_{4, 232} = 4.47$, $p < 0.05$). However, these flies showed a rebound increase in day-time sleep upon removal from caffeinated food (Figure 4.1A, RC) which was significantly higher than day-time sleep during BS and CAF (Figure 4.1B-top). Similarly, when flies were provided food containing 0.5 mg/ml caffeine only during the night (N_{caf}, Figure 4.1A-B), their night sleep was significantly reduced as compared to their own BS days as well as control flies during CAF days (Figure 4.1A, BS and CAF; Figure 4.1B, night, two-way ANOVA, $F_{4, 232} = 10.41$, $p < 0.00001$). These data show that caffeine has an immediate effect on sleep – D_{caf} flies show reduced day-time sleep while N_{caf} flies show reduced night sleep. Similar trends of reduced day-time sleep of D_{caf} and reduced night sleep of N_{caf} with respect to BS were found when flies were fed with food containing 1 mg/ml caffeine (Figure 4.2, two-way ANOVA, day : $F_{4, 237} = 2.14$, $p = 0.08$; night : $F_{4, 237} = 4$, $p < 0.005$). Importantly, 0.5 mg/ml is more efficient in decreasing sleep levels (53% day and 49% night sleep loss) as compared to 1.0 mg/ml of caffeine (38 % day and 4 % night sleep loss, Figure 4.1B'). This is likely due to reduced food intake with increasing caffeine content, as a capillary feeder (CAFE) assay (Ja et al., 2007) conducted for a period of 24 hours, showed that flies consumed less quantity of 5% sucrose solution containing 1 mg/ml caffeine (0.55 ± 0.07 µL, $n = 8$ flies) as compared to that containing 0.5 mg/ml caffeine

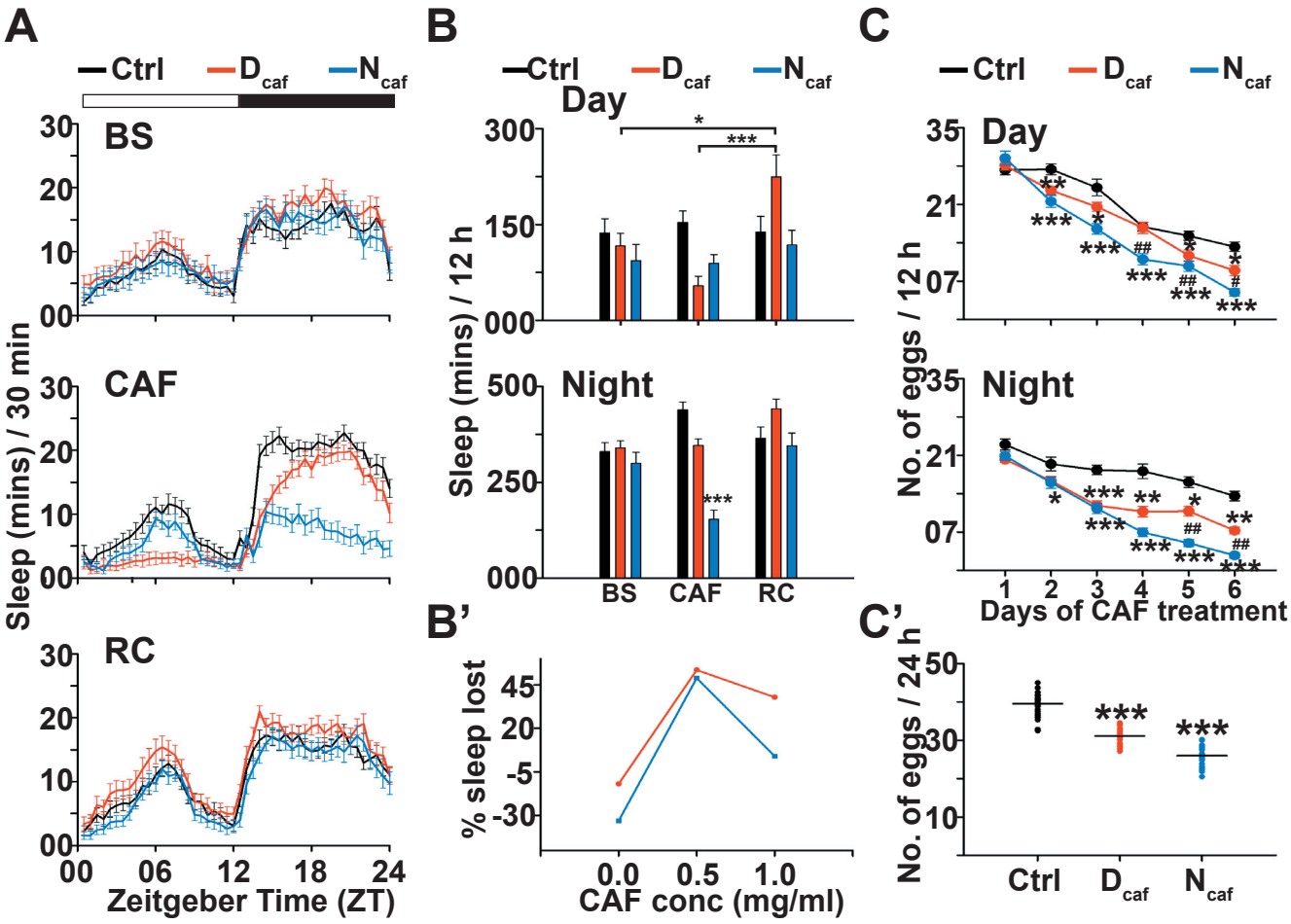

Figure 4.1. Sleep deprivation of w^{1118} flies by caffeine results in decrease of egg output. (A) Sleep in minutes for every half hour over a period of 24 h is shown for w^{1118} flies fed with standard food (Ctrl, n = 28), flies fed with 0.5 mg/ml caffeine only during the day (D_{caf}, n = 25) and only during the night (N_{caf}, n = 24) averaged across two baseline (BS), three caffeine feeding (CAF) and two recovery (RC) days. Horizontal white and black bars on top represent day and night respectively. (B) Day-time (top) and night (bottom) sleep of control, D_{caf} and N_{caf} flies are compared across BS, CAF and RC days. D_{caf} flies show significant increase in day-time sleep during RC days as compared to that during BS and CAF days. N_{caf} flies show significantly lower levels of night sleep during CAF days as compared to that during BS and RC days, as well as night sleep of controls during CAF days. (B') Percentage total sleep loss during CAF days with respect to BS days plotted as function of caffeine concentration shows that sleep loss is higher for caffeine concentration of 0.5 mg/ml during both day and night as compared to a concentration of 1.0 mg/ml. (C) Number of eggs laid by control (n = 25), D_{caf} (n = 24) and N_{caf} (n = 25) flies both during day and night over a period of six days of caffeine (0.5 mg/ml) treatment. * denotes significant differences between either D_{caf} or N_{caf} with control flies, while # indicates significant differences between D_{caf} and N_{caf} flies. (C') Total number of eggs laid averaged across six days of caffeine treatment. D_{caf} flies laid significantly lesser number of eggs as compared to control flies, while N_{caf} flies lay significantly lower number of eggs as compared to both control and D_{caf} flies. The experiment was repeated with similar results (data not shown). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Error bars are SEM.

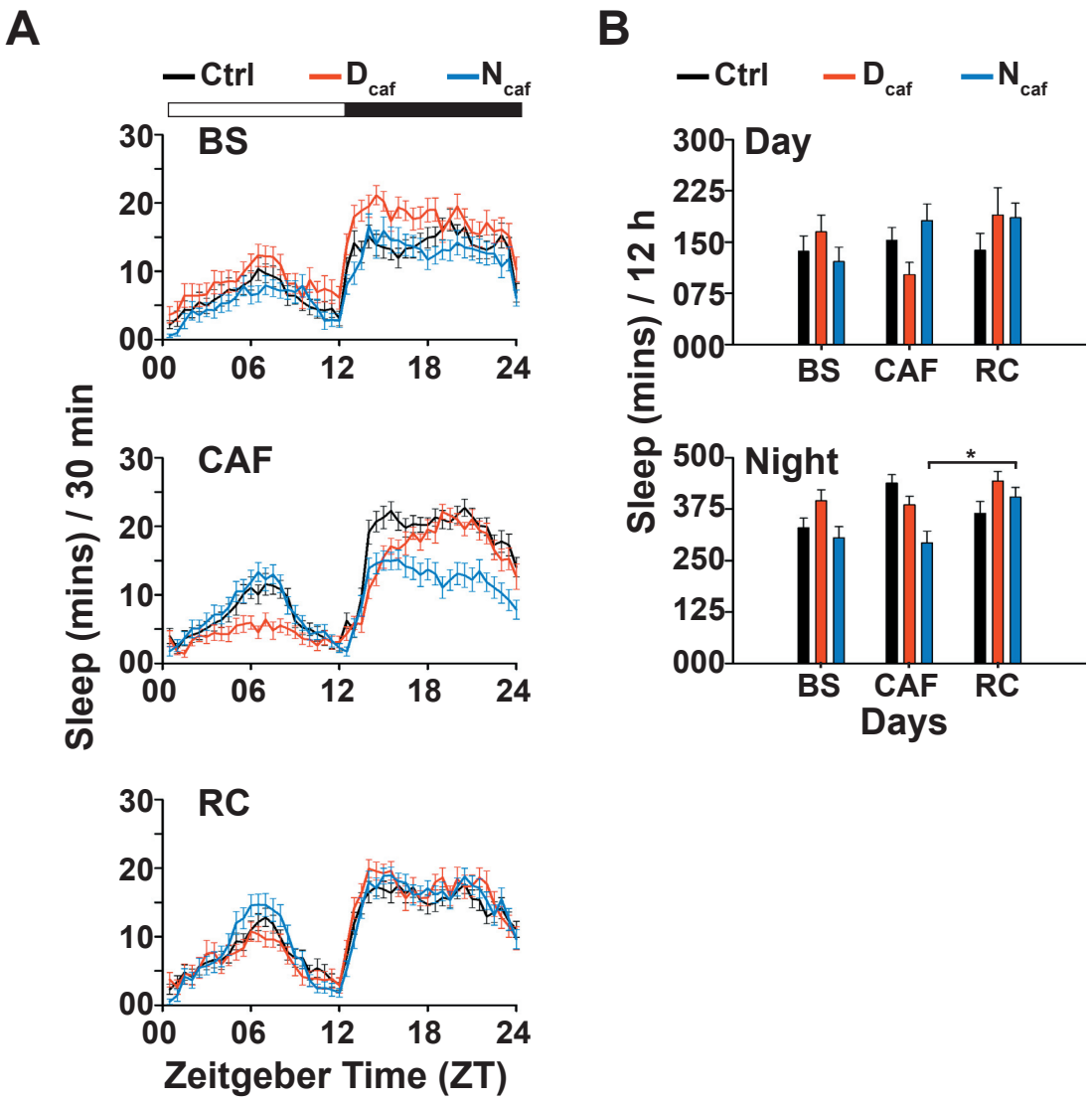

Figure 4.2. Sleep/wake behaviour of flies fed with 1.0 mg/ml caffeine. (A) Sleep in minutes for every half hour over a period of 24 h is shown for w^{1118} flies fed with standard food (Ctrl, $n = 28$), flies fed with 1.0 mg/ml caffeine only during the day (D_{caf}, $n = 29$) and only during the night (N_{caf}, $n = 28$) averaged across two baseline (BS), three caffeine feeding (CAF) and two recovery (RC) days. (B) Day-time (top) and night-time (bottom) sleep of control, D_{caf} and N_{caf} flies are compared across BS, CAF and RC days. Only night-time sleep of N_{caf} flies during CAF and RC days is significantly different from each other. All other details as in Figure 4.1.

(1.05 ± 0.12 µL, n = 8 flies, Mann-Whitney U test, $p < 0.005$) which could in turn result in lesser extent of sleep loss.

Since providing flies with food containing 0.5 mg/ml caffeine during day or night leads to about 50 % reduction in both day-time and night sleep respectively, I next determined how this affects their reproductive output. I subjected 5-day old female flies (mated for one day prior to the start of the experiment) to caffeine treatment only during the day (D_{caf}) or only during the night (N_{caf}). I found that both D_{caf} and N_{caf} flies laid lesser number of eggs as compared to the control flies during day as well as night (Figure 4.1C, Appendix 2.3A), even though on the first day and night the number of eggs laid were comparable suggesting a cumulative effect of caffeine-mediated sleep loss on egg output. N_{caf} flies laid lesser number of eggs as compared to D_{caf} flies also, which was statistically significant on the later days of the treatment (Figure 4.1C, Appendix 2.3A). When the total number of eggs averaged over the 6 days of treatment was compared, D_{caf} flies laid significantly lesser number of eggs as compared to control flies, and N_{caf} flies laid significantly lesser number of eggs as compared to both control and D_{caf} flies (Figure 4.1C', one-way ANOVA, $F_{2, 71} = 156.55$, $p < 0.00001$).

Since it is likely that flies fed with caffeine laid fewer eggs simply because oviposition was inhibited by food containing caffeine, an oviposition preference assay was carried out, where flies were allowed to lay eggs for two hours on a petri dish with half the plate containing standard food and the other half containing 0.5 mg/ml caffeinated food. Flies laid almost equal number of eggs on both halves, suggesting that for food containing caffeine at a concentration of 0.5 mg/ml, flies do not have any ovipositional avoidance (Preference Index $_{caf}$ = 0.49 ± 0.11, chi-square test, $\chi^2 = 0.049$, $p = 0.82$). However, since the egg output assays last for a period of 5-6 days and flies get access to fresh food every 12 hours, yet another oviposition preference assay was

conducted for a longer time course of 12 hours. I found that when given a choice for a longer period of time, flies tend to lay more number of eggs on food containing 0.5 mg/ml caffeine as compared to standard food. Therefore, flies tend to show a significant preference towards caffeine containing food in conditions resembling the egg output assays (Preference Index $_{caf}$ = 0.75 ± 0.1, chi-square test, χ^2 = 99.75, p < 0.0005). Thus, these results suggest that flies lay lesser number of eggs when exposed to caffeine in spite of a preference towards it. Overall, caffeine decreases egg output and flies that lose night-time sleep tend to lay lesser number of eggs than flies that lose day-time sleep.

To confirm the effect of sleep loss on egg output I used a completely different sleep deprivation method. I substituted caffeine with a vortexer-based mechanical perturbation protocol. Since this assay was done in DAM5 monitors with flies housed in glass tubes (65 mm in length, 5 mm in inner diameter) as opposed to the caffeine-fed flies which were housed in standard glass vials (10 cm length, 2.5 cm diameter), the overall number of eggs laid is expected to be significantly fewer in tubes (39 vs 6 for control flies in Figure 4.1C' and 4.3C'). Three sets of flies received either of the following treatments – exposure to mechanical disturbance only during day (D_{dep}), or only during night (N_{dep}) or control (Ctrl) condition with no mechanical perturbation. For the same sets of flies, both sleep levels and egg counts were obtained by transferring flies to fresh tubes every 12 hours for five days. As expected, mechanical disturbance during day reduced day-time sleep and that during night reduced night-time sleep drastically (Figure 4.3A-B). However, only N_{dep} flies recovered this lost night-time sleep during the subsequent days (Figure 4.3B-top, one-way ANOVA, $F_{2, 86}$ = 149.16, p < 0.00001) whereas D_{dep} flies did not recover the lost day-time sleep during subsequent nights (Figure 4.3B-bottom, $F_{2, 86}$ = 344.22, p < 0.00001). Nevertheless,

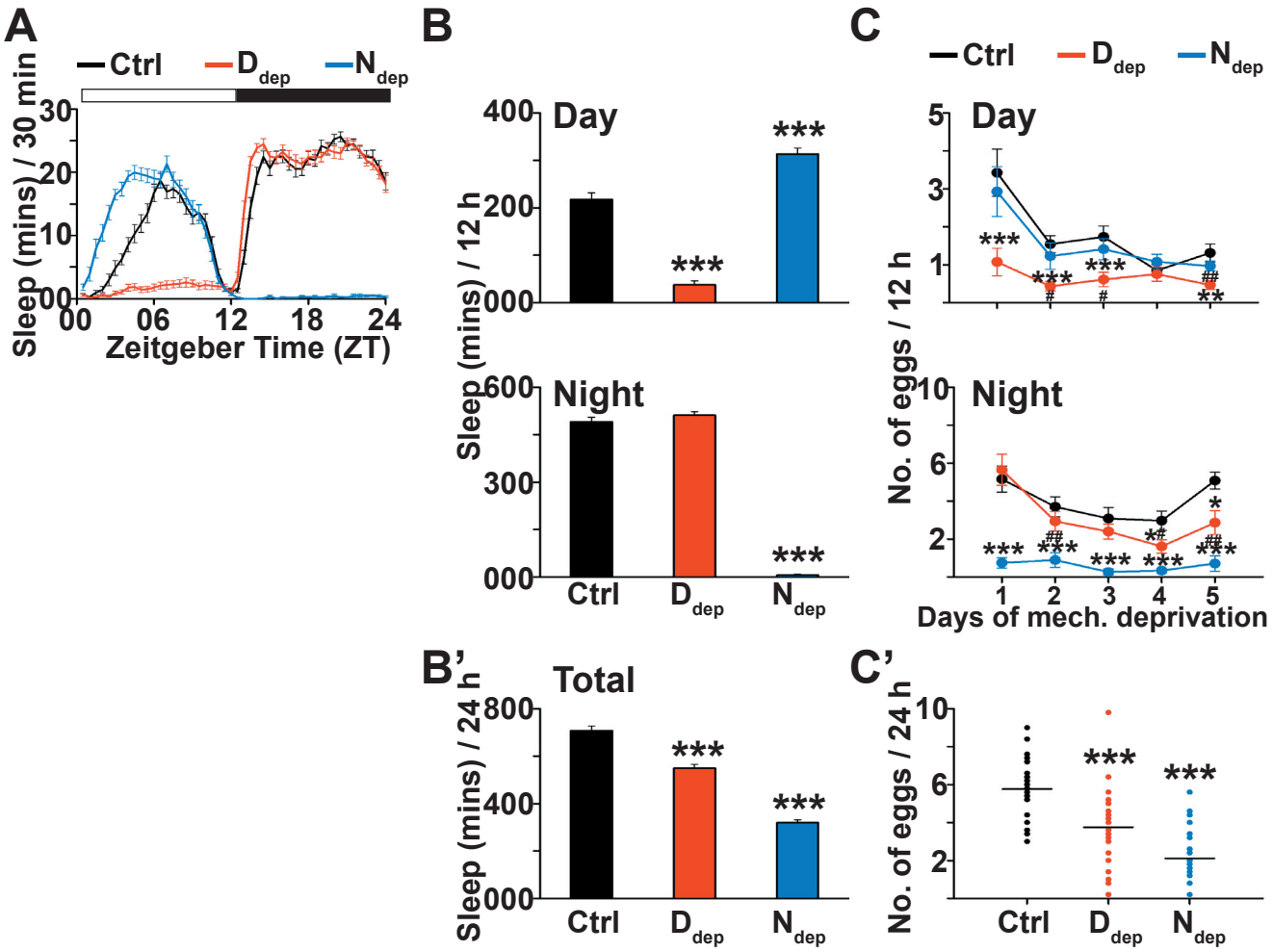

Figure 4.3. Sleep deprivation of w^{1118} flies by mechanical deprivation results in decrease of egg output. (A) Sleep in minutes for every half hour over a period of 24 h averaged across 5 days is shown for control w^{1118} flies (Ctrl, n = 26), flies receiving mechanical disturbance only during the day (D_{dep}, n = 28) and only during the night (N_{dep}, n = 27). (B) Day-time sleep (top) of D_{dep} flies significantly reduced as compared to Ctrl and N_{dep}, whereas that of N_{dep} flies significantly higher than that of Ctrl and D_{dep}. Night-time sleep (bottom) of N_{dep} flies significantly lower than Ctrl and D_{dep} flies. (B') Total sleep of D_{dep} flies is significantly lower than Ctrl and that of N_{dep} flies is significantly lower than Ctrl and D_{dep} flies. (C) Number of eggs laid by control, D_{dep} and N_{dep} flies both during day and night over a period of five days of mechanical deprivation protocol. * denotes significant differences between either D_{dep} or N_{dep} with control flies, while # indicates significant differences between D_{dep} and N_{dep} flies. (C') Total number of eggs laid by Ctrl, D_{dep} and N_{dep} flies averaged across 5 days. D_{dep} flies show significant reduction in number of eggs laid as compared to Ctrl; N_{dep} flies laid even lower number of eggs significantly reduced as compared to both Ctrl and D_{dep} flies. All other details are as in Figure 4.1.

N_{dep} flies lost greater amount of overall sleep as compared to D_{dep} flies (Figure 4.3B', one way ANOVA, $F_{2, 86} = 39.59$, $p < 0.00001$). Importantly, the number of eggs laid by D_{dep} flies was lower than the controls especially during the day-time (Figure 4.3C-top, Appendix 2.3B) and that of N_{dep} flies was significantly lower than the controls during the night (Figure 4.3C-bottom, Appendix 2.3B). Unlike the caffeine-fed flies, the effect of sleep loss due to mechanical deprivation on egg output was evident from the first day of treatment (Figure 4.3C, Appendix 2.3B). Moreover, the average egg output in both D_{dep} and N_{dep} flies was significantly lowered as compared to the control flies (Figure 4.3C', Kruskal-Wallis test, $H_{2, 89} = 24.03$, $p < 0.00001$). Furthermore, N_{dep} flies, which on average lost more sleep, also laid significantly lesser number of eggs as compared to D_{dep} flies (Figure 4.3B', C'). Thus, these results along with similar results obtained with sleep deprivation using caffeine suggest that sleep loss results in reduction in egg output and that sleep loss during the night has a greater detrimental effect on egg output.

4.3.2. Effect of sleep deprivation on reproductive fitness of outbred flies. In the studies described above I used a strain of w^{1118} flies which has been maintained in our laboratory for several years and is likely to harbour loci that have been fixed for certain traits which may have resulted in the above phenotype by chance. Given that reproductive output is a major Darwinian fitness trait, I asked how sleep loss might affect reproductive output in a large, random mating and therefore outbred population of flies which is unlikely to have suffered from similar genetic bottlenecks (*CCM*, (Gogna et al., 2015)). I subjected flies to three different concentrations of caffeine (0.5, 1.0 and 1.5 mg/ml) either only during day or only during night and found that none of the D_{caf} flies lost day-time sleep, whereas all the N_{caf} flies lost similar amounts of night sleep (Figure 4.4A-B, one way ANOVA, day: $F_{6, 156} = 3.24$, $p < 0.005$; night: $F_{6, 156} =$

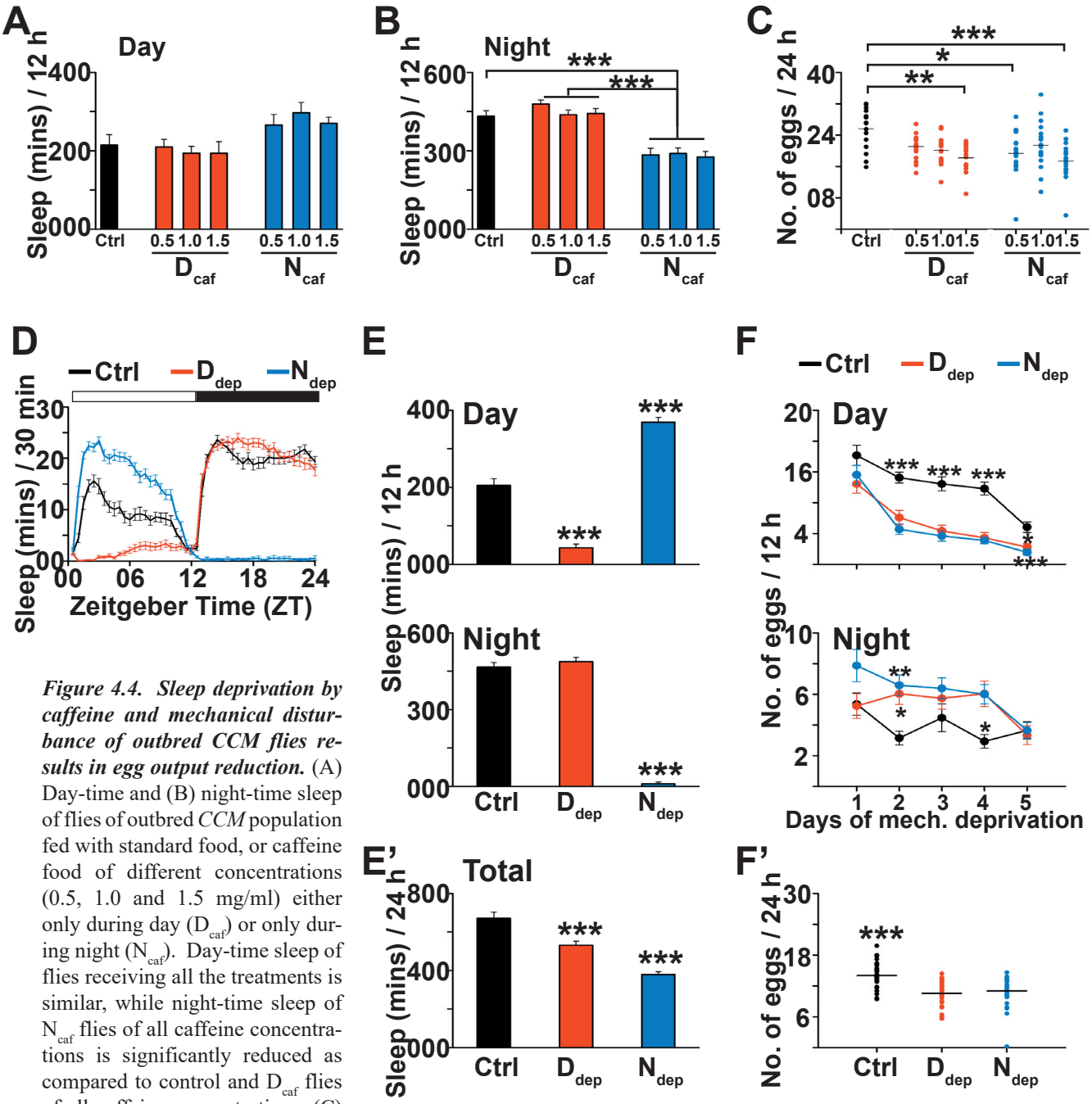

Figure 4.4. Sleep deprivation by caffeine and mechanical disturbance of outbred CCM flies results in egg output reduction. (A) Day-time and (B) night-time sleep of flies of outbred *CCM* population fed with standard food, or caffeine food of different concentrations (0.5, 1.0 and 1.5 mg/ml) either only during day (D_{caf}) or only during night (N_{caf}). Day-time sleep of flies receiving all the treatments is similar, while night-time sleep of N_{caf} flies of all caffeine concentrations is significantly reduced as compared to control and D_{caf} flies of all caffeine concentrations. (C) Total number of eggs laid averaged across 6 days by D_{caf}-1.5 (n = 13), N_{caf}-0.5 (n = 19) and N_{caf}-1.5 (n = 17) flies are significantly reduced as compared to the control (n = 16) flies. D_{caf}-0.5 (n = 17), D_{caf}-1.0 (n = 17) and N_{caf}-1.0 (n = 18) do not differ from the control flies in the number of eggs laid. (D) Sleep in minutes for every half hour over a period of 24 h averaged across five days is shown for control (n = 28) flies of outbred *CCM* population, flies mechanically disturbed during the day (D_{dep}, n = 30) and during the night (N_{dep}, n = 31). (E) During the day (top), D_{dep} flies sleep significantly lower than both control and N_{dep} flies due to mechanical disturbance, N_{dep} flies sleep significantly higher than control and D_{dep} flies indicating sleep rebound due to sleep deprivation during the previous night. During the night (bottom), N_{dep} flies sleep significantly lower than the control and D_{dep} flies due to mechanical perturbation. (E') Total sleep averaged across 5 days of D_{dep} flies is significantly lower than control flies, whereas that of N_{dep} is significantly lower than both control and D_{dep} flies. (F) Number of eggs laid by both D_{dep} and N_{dep} flies is significantly lower than the controls during days 2-5 (top) and they show a trend of increased egg output during night (bottom) which is significantly different from the controls on the 2nd and 4th nights. (F') Total number of eggs laid averaged across five days by both D_{dep} and N_{dep} flies is significantly lower as compared to control flies. All other details as in Figure 4.1. A similar experiment with higher levels of deprivation yielded similar results (data not shown).

19.14, $p < 0.00001$). However, D_{caf} (1.5 mg/ml) flies laid significantly lower number of eggs than the control flies, suggesting that caffeine at a relatively higher concentration can affect egg output even without its effect on day-time sleep (Figure 4.4C, Kruskal-Wallis test, $H_{6, 119} = 23.96$, $p < 0.05$). Moreover, N_{caf} flies receiving 0.5 mg/ml and 1.5 mg/ml caffeine also showed reduced egg output as compared to control flies (Figure 4.4C). These results point toward a direct effect of caffeine on egg output independent of its effect on sleep as well as an indirect effect on egg output through sleep loss. Alternatively, this could also indicate the inability of infrared beam-break-based methods such as DAM system to detect subtle effects of caffeine treatment and also that immobility may not always be the best measure for sleep. Nevertheless, I next increased caffeine concentration and found that even higher caffeine concentrations of 4.0 mg/ml fed during the day did not affect day-time sleep (Figure 4.5A-BS and CAF, 4.5B-top, two-way ANOVA, $F_{4, 127} = 7.13$, $p < 0.00001$), however, when fed during the night, it decreased night sleep (Figure 4.5B-bottom, two-way ANOVA, $F_{4, 127} = 4.37$, $p < 0.05$). With respect to egg output, I found that the total number of eggs laid by D_{caf} and N_{caf} flies was significantly lower than that of the control flies, however, the number of eggs laid by D_{caf} and N_{caf} flies were not statistically different from each other (Figure 4.5C-top, two-way ANOVA, $F_{10, 267} = 2.19$, $p < 0.05$; 4.5C-bottom, two-way ANOVA, $F_{10, 267} = 1.66$, $p = 0.09$, C', Kruskal-Wallis test, $H_{2, 48} = 18.55$, $p < 0.0005$) similar to what was found for lower concentrations of caffeine. Caffeine treatment does not affect the viability of the eggs laid as seen from egg-to-adult survivorship of eggs laid by D_{caf}, N_{caf} (0.5 mg/ml) and Ctrl flies (data not shown). Taken together, these results suggest that caffeine treatment may affect the reproductive fitness directly or indirectly through sleep loss.

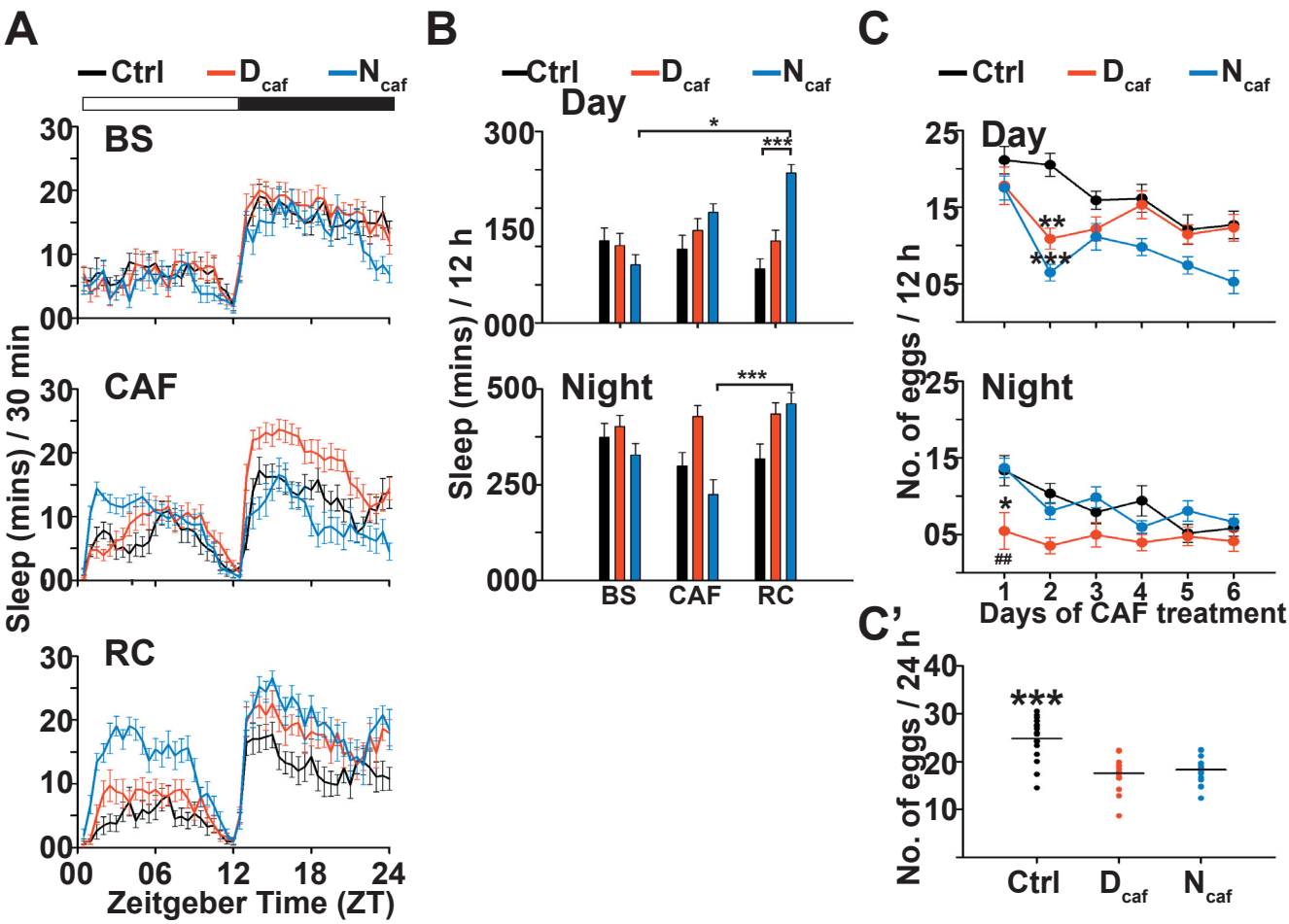

Figure 4.5. Number of eggs laid by outbred flies fed with higher concentration of caffeine. (A) Sleep in minutes for every half hour over a period of 24 h is shown for control flies of outbred *CCM* population fed with standard food (Ctrl, $n = 16$), flies fed with caffeine only during the day (D_{caf}, $n = 16$) and only during the night (N_{caf}, $n = 14$) for caffeine concentration of 4.0 mg/ml averaged across two baseline (BS), three caffeine feeding (CAF) and two recovery (RC) days. Night-time sleep of N_{caf} flies during CAF days is lower than that of controls, and both day-time and night-time sleep of N_{caf} flies is higher than the controls during RC. (B) Day-time sleep levels of control and D_{caf} flies show no differences across different days, whereas those of control and N_{caf} flies significantly differ from each other during RC. Day-time sleep of N_{caf} flies during RC is significantly higher than that during BS. Night-time sleep of N_{caf} flies during CAF and RC days are significantly different from each other other . (C) N_{caf} flies showed a trend of laying lower number of eggs than controls during the daytime (top), which was significant on day 2, while D_{caf} flies showed a trend of laying lower number of eggs during the night (bottom) which was significant on night 1. (C') Total eggs laid by control ($n = 16$), D_{caf} ($n = 14$) and N_{caf} ($n = 18$) flies averaged across six days of caffeine feeding. Control flies laid higher number of eggs as compared to both D_{caf} and N_{caf} flies. All other details as in Figure 4.1.

I next subjected the *CCM* flies to sleep deprivation protocol using mechanical perturbation either during the day only (D_{dep}) or during the night only (N_{dep}). Expectedly, D_{dep} flies lost day sleep and N_{dep} flies lost night sleep which they could recover during subsequent days (Figure 4.4D-E, one way ANOVA, day: $F_{2, 78} = 140.22$, $p < 0.00001$; night: $F_{2, 78} = 705.25$, $p < 0.00001$). Nevertheless N_{dep} flies lost overall greater amount of sleep as compared to D_{dep} flies (Figure 4.4E', one way ANOVA, $F_{2, 78} = 134.77$, $p < 0.00001$). Here too, since the assay was conducted in tubes (see methods), expectedly all flies laid lower number of eggs owing to the decreased surface area of food as compared to the caffeine-feeding experiment where flies were housed in vials. Unlike the mechanically disturbed inbred flies (Figure 4.3C), in the case of outbred flies both D_{dep} and N_{dep} flies lay significantly lower number of eggs as compared to controls during the day (Figure 4.4F-top, Appendix 2.3C) while they both lay higher number of eggs compared to controls during the night (Figure 4.4F-bottom, Appendix 2.3C) starting from day 2. Again, as in the case of caffeine fed outbred flies, with mechanical disturbance also I found that there is a reduction in egg output in D_{dep} and N_{dep} flies as compared to control flies, although there was no difference in egg output between flies experiencing day vs. night sleep disturbance (Figure 4.4F', Kruskal-Wallis test, $H_{2, 81} = 38.27$, $p < 0.00001$). This difference among inbred and outbred flies could be due to different levels of sleep rebound, at least in the case of mechanical deprivation (Table 4.1). However, in yet another assay with mechanically sleep deprived flies, egg output of N_{dep} flies averaged across three days *after* the deprivation protocol was still significantly reduced, while that of D_{dep} flies was comparable to control flies (Figure 4.6, Kruskal-Wallis test, $H_{2, 66} = 9.7$, $p < 0.05$). Therefore, with both caffeine and mechanical disturbance, the resultant sleep deprivation contributed in part to the decrease in egg output of outbred flies.

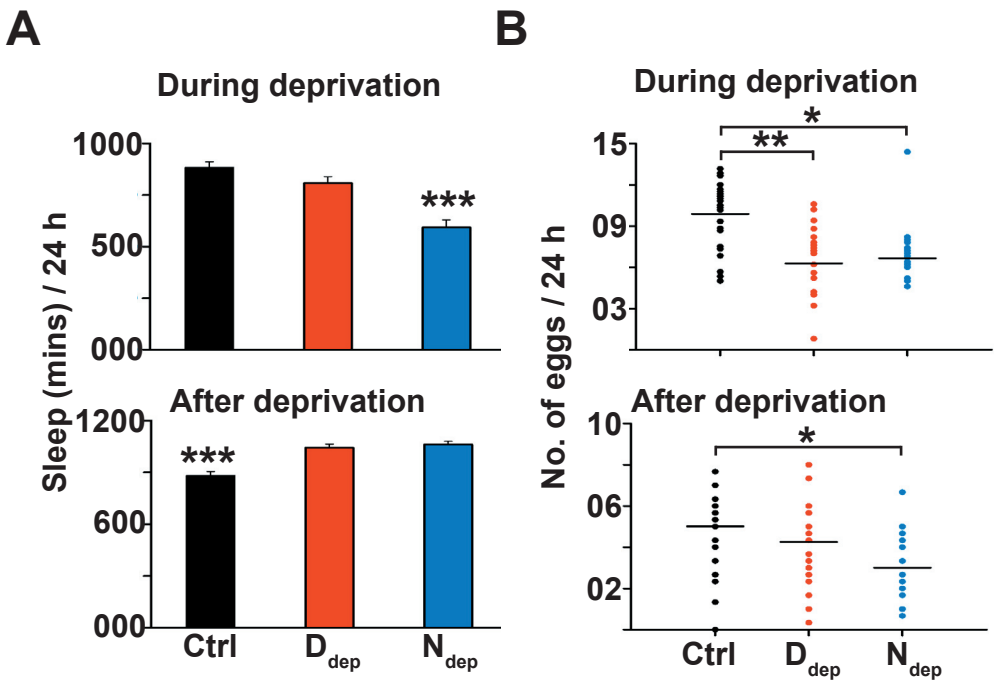

Figure 4.6. Chronic effect of mechanical sleep deprivation on egg output. (A) Total sleep (top) during 6 days of sleep deprivation and (bottom) averaged for 3 days post-deprivation. Sleep of N_{dep} (n = 16) flies is significantly lower than both control (n = 29) and D_{dep} (n = 21) flies during sleep deprivation, whereas both D_{dep} and N_{dep} flies sleep more after deprivation (one-way ANOVA followed by *post-hoc* Tukey's HSD test). (B) Average number of eggs laid (top) during sleep deprivation and (bottom) after sleep deprivation. D_{dep} and N_{dep} flies lay lesser number of eggs as compared to control flies during deprivation, but only N_{dep} flies lay lower number of eggs compared to control flies after deprivation. All other details as in Figure 4.1.

Furthermore, as seen in inbred flies, night sleep loss had greater impact on egg output as compared to day-time sleep loss, though this difference was less discernible and the effect much more subtle in outbred flies. Nevertheless, the finding that flies deprived of sleep during night show high levels of day-time sleep rebound, yet it does not lead to a concomitant rescue of harmful effects on egg output to levels mimicking undisturbed flies, further highlights the notion that sleep during the night is more important.

Table 4.1

Genotype	**% sleep lost**		**% sleep rebound**	
	caffeine	**depriver**	**caffeine**	**depriver**
Inbred (w^{1118})	48.9	98.8	26.1	45.9
Outbred (*CCM*)	34.3	98	23.7	80.2

Table 4.1. Sleep loss and rebound characteristics of night time sleep. Percentage sleep lost during the night and rebound during the subsequent days after caffeine treatment (0.5 mg/ml) and mechanical deprivation for inbred and outbred flies. These values were calculated on the basis of baseline sleep levels for w^{1118}-caffeine flies. For the rest, these values are calculated with respect to sleep levels of control flies set as baseline.

4.3.3. Transient sleep reduction is accompanied by transient reduction in egg output. It is possible that both caffeine feeding and mechanical perturbation could have broad effects on general physiology of the fly. Therefore, I used a third method (genetic) whereby sleep reduction is transient and measured egg output following neural-circuit-driven sleep loss. I used the GAL4-UAS system to express a temperature-sensitive cation channel *Drosophila* Transient Receptor Potential 1 (*dTRPA1*, which opens above temperatures of 27 °C and causes hyper-excitation

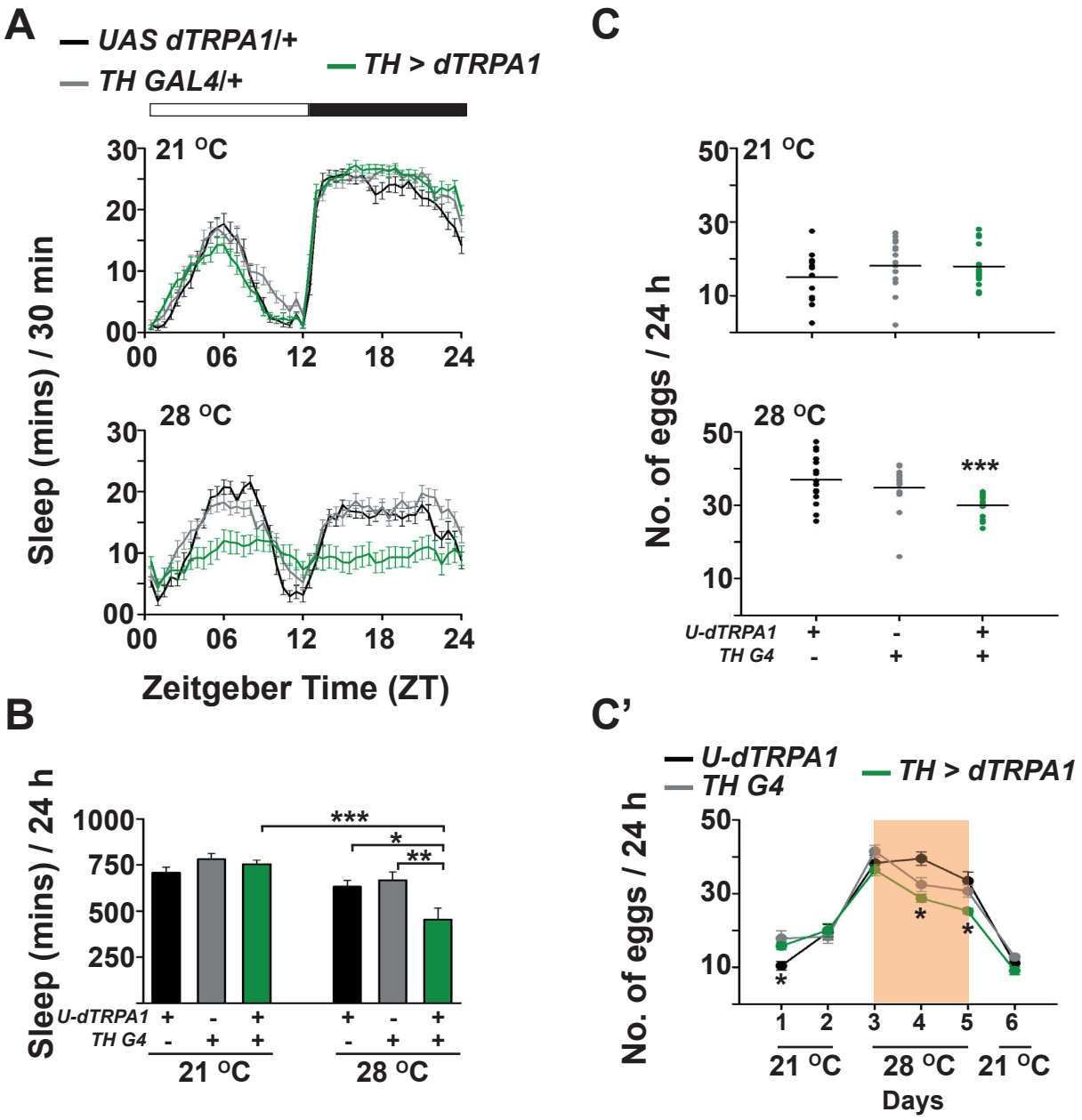

Figure 4.7. Decreasing sleep levels using dTRPA1-based reversible activation of dopaminergic neurons reversibly decreases egg output. (A) Sleep in minutes for every half hour over a period of 24 h averaged across two days at 21 °C (left) and three days at 28 °C (right) is shown for *UAS dTRPA1/+* (n = 29), *TH GAL4/+* (n = 28) and *TH GAL4 > UAS dTRPA1* (n = 32) flies. (B) At 21 °C, total sleep levels of all three genotypes is similar, whereas at 28 °C, *TH GAL4 > UAS dTRPA1* flies sleep significantly lower than *UAS dTRPA1/+* and *TH GAL4/+* flies. (C) Total number of eggs laid averaged across two days at 21 °C (left) is similar across all genotypes, while average number of eggs laid by *TH GAL4 > UAS dTRPA1* (n = 16) flies is significantly lower than *UAS dTRPA1/+* (n = 16) and *TH GAL4/+* (n = 19) flies during the three days at 28 °C (right). (C') Total number of eggs laid on all six days of the assay at different temperatures as indicated. Orange shaded region represents high temperature of 28 °C. *TH GAL4 > UAS dTRPA1* flies laid significantly lower number of eggs as compared to *UAS dTRPA1/+* and *TH GAL4/+* especially on the final two days of 28 °C, while all flies laid similar number of eggs at 21 °C except on day 1 when *UAS dTRPA1/+* flies laid slightly but significantly lower number of eggs. All other details as in Figure 4.1.

(Hamada et al., 2008)), in dopaminergic neurons that have previously been shown to be wake-promoting (Liu et al., 2012; Shang et al., 2011; Ueno et al., 2012). I recorded sleep levels of flies in tubes and egg output in vials exposed to the following regime – two days at 21 °C followed by three days at 28 °C followed by a day at 21 °C under LD 12:12. As expected, at the higher temperature, sleep was reduced both during day-time and night when dopaminergic neurons were activated, whereas the baseline sleep levels of these experimental flies were not different from that of the parental controls at the lower temperature (Figure 4.7A-B, two-way ANOVA, $F_{2,\ 159} = 5.3$, $p < 0.05$). The number of eggs laid by the experimental flies was significantly lower than that of the controls (Figure 4.7C-C', Kruskal-Wallis test, 21 °C: $H_{2,\ 51} = 2.75$, $p = 0.25$, 28 °C: $H_{2,\ 51} = 17.61$, $p < 0.005$, Appendix 2.3D). Indeed, these differences in egg output between experimental and control flies were not seen at the lower temperature of 21 °C (Figure 4.7C-C') when sleep levels were not affected (Figure 4.7A-B), suggesting that transiently reducing sleep levels by activating wake-promoting neurons also resulted in transient reduction of egg output. Since the *TH GAL4* that I have used drives expression in about ~130 dopaminergic neurons (Friggi-Grelin et al., 2003; Mao and Davis, 2009) that could likely comprise a combination of neurons that independently regulate sleep and egg output, I asked if decreasing sleep levels by using a more restricted driver also leads to a decrease in the egg output. I used the *TH-F2 GAL4* driver which targets expression in a restricted subset of ~ 20 dopaminergic neurons and hyper-excitation of these neurons results in decrease in sleep levels ((Liu et al., 2012); Figure 4.8A-B, two-way ANOVA, $F_{2,\ 130} = 16.53$, $p < 0.00001$). Reducing sleep using this driver has a somewhat less dramatic effect on egg output as compared to the broader driver, nonetheless, the number of eggs laid by flies with reduced sleep due to hyper-excited TH-$F2^{+}$ neurons is still lesser than its parental controls (Figure 4.8C-C',

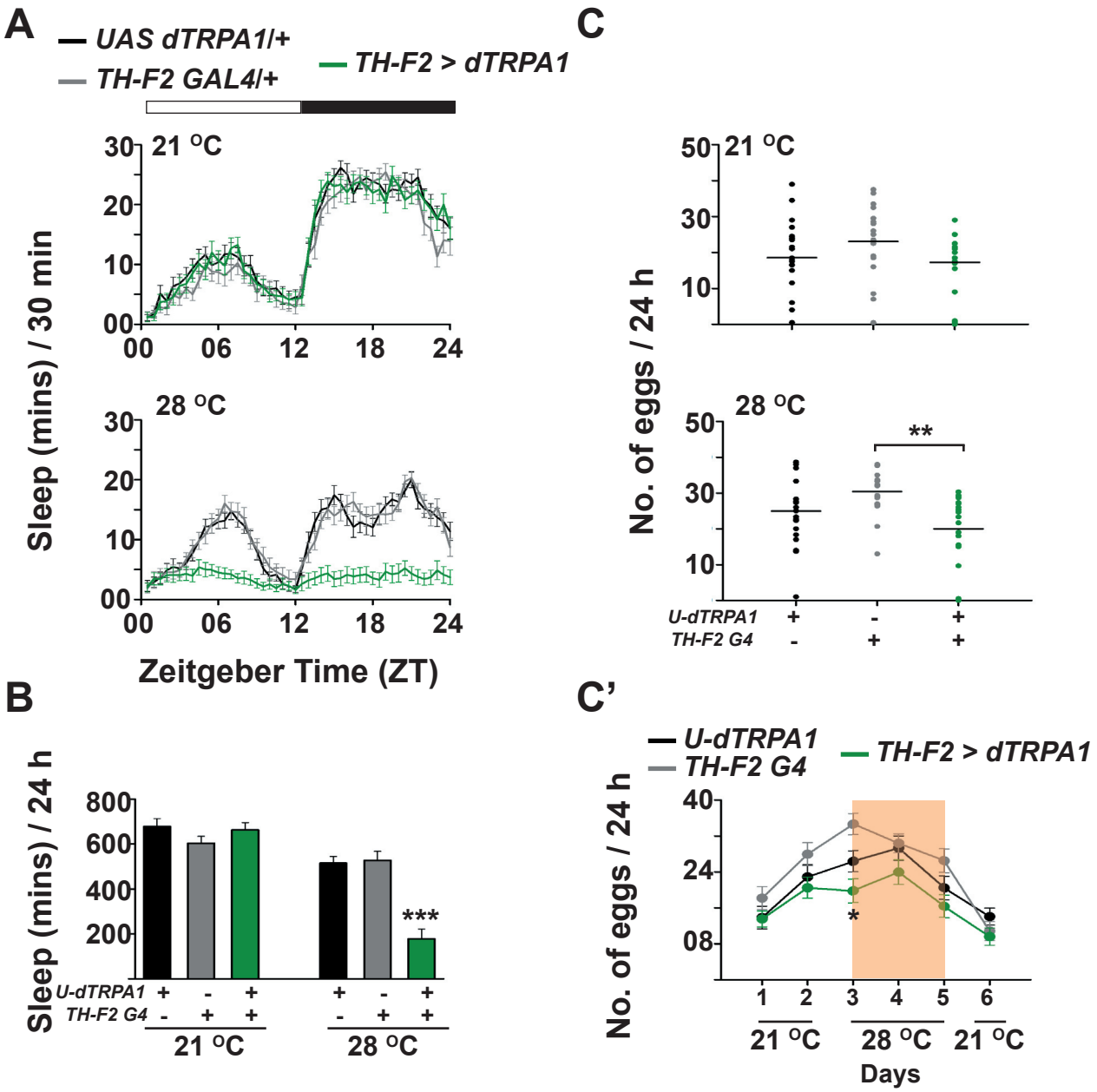

Figure 4.8. Decreasing sleep levels using dTRPA1-based reversible activation of a small subset of dopaminergic neurons reversibly decreases egg output. (A) Sleep in minutes for every half hour over a period of 24 h averaged across two days at 21 °C (left) and three days at 28 °C (right) is shown for *UAS dTRPA1/+* (n = 22), *TH-F2 GAL4/+* (n = 23) and *TH-F2 GAL4 > UAS dTRPA1* (n = 23) flies. (B) At 21 °C, total sleep levels of all three genotypes is similar, whereas at 28 °C, *TH-F2 GAL4 > UAS dTRPA1* flies sleep significantly less than *UAS dTRPA1/+* and *TH-F2 GAL4/+* flies. (C) Total number of eggs laid averaged across two days at 21 °C (left) is similar across all genotypes, while average number of eggs laid by *TH-F2 GAL4 > UAS dTRPA1* (n = 20) flies is significantly lower than *TH-F2 GAL4/+* (n = 18) flies during the three days at 28 °C, but not from *UAS dTRPA1/+* flies (n = 20) (right). (C') Total number of eggs laid on all six days of the assay at different temperatures as indicated. *TH-F2 GAL4 > UAS dTRPA1* flies laid significantly lower number of eggs as compared to *UAS dTRPA1/+* and *TH-F2 GAL4/+* on the first day of 28 °C, while it showed a decreasing non-significant trend on the other two days of 28 °C. All flies laid similar number of eggs at 21 °C. All other details as in Figure 4.7.

Kruskal-Wallis test, 21 °C: $H_{2, 59} = 4.94$, $p = 0.08$, 28 °C: $H_{2, 58} = 11.84$, $p < 0.005$, Appendix 2.3E), though it reaches statistical significance only when compared to the *GAL4* control flies. This suggests that perhaps the *TH GAL4* driver may still drive expression in dopaminergic neurons that affect egg output without necessarily affecting sleep, even though to date no study has shown a direct role for dopamine on egg-laying. Nevertheless, the trend of reduced egg output with reducing sleep occurs even with targeting a smaller subset of neurons and thus taken together, my results suggest that sleep loss leads to reduction in egg output, irrespective of the method of sleep deprivation.

4.3.4. Dopamine transporter mutants show reduced sleep but not reduced egg output in response to caffeine. Given that increasing dopaminergic activity increases wakefulness and decreases egg output, I asked if increasing the amount of dopamine in synaptic clefts also led to decreased egg output. I used flies with loss-of-function mutation in the *fumin* (*fmn*) gene, which codes for dopamine transporter. Mutant *fmn* flies have been reported to show overall reduced sleep and no reduction in lifespan, but the authors did not measure fertility in their study (Kume et al., 2005). I quantified their egg output along with sleep levels and found that the *fmn* flies expectedly showed reduced sleep levels both during the day and night (Figure 4.9A-B-top, two-way ANOVA, main effect of genotype: $F_{1, 141} = 310.85$, $p < 0.00001$), and the egg output of *fmn* flies was drastically reduced as compared to that of the background control flies (*fmn-bg*, Figure 4.9C-C', Student's two-tailed *t*-test, $T_{0.05, 2, 27} = 30.21$, $p < 0.00001$). Since *fmn* flies carry a mutation in the dopamine transporter gene throughout the body, it is likely that this mutation can have fecundity defects independent of sleep. A previous study has shown that *fmn* mutants show a further reduction in sleep when fed with caffeine (Andretic et al., 2008). I asked if the egg output is also further reduced in

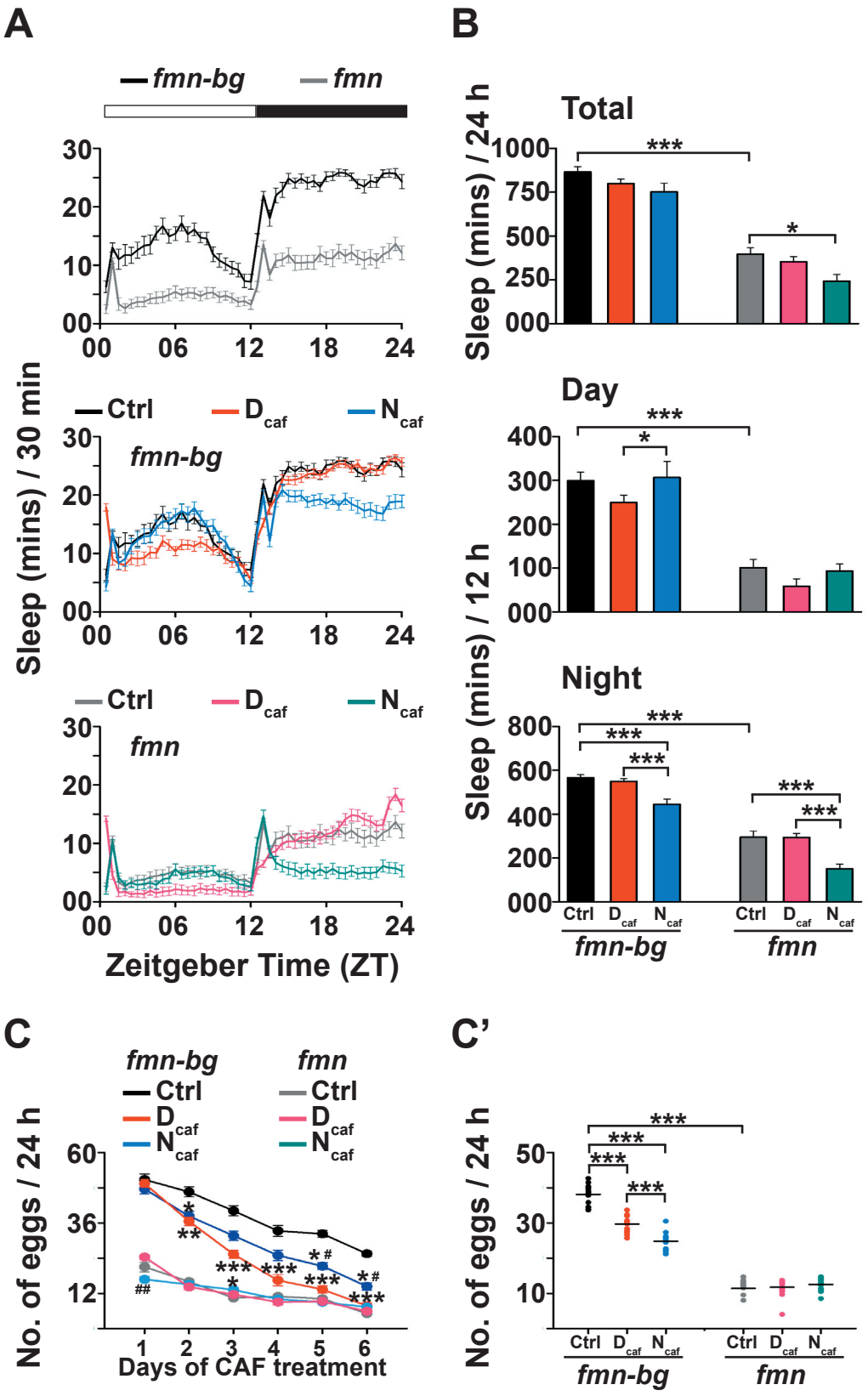

Figure 4.9. fmn flies reduce sleep but not egg output in response to caffeine. (A) Sleep in minutes for every half hour over a period of 24 h averaged across 6 days of *fmn* and *fmn* background control (*fmn-bg*) flies (top), *fmn-bg* flies fed with standard food (n = 17), caffeine food (0.5 mg/ml) only during the day (D_{caf}, n = 28) and only during the night (N_{caf}, n = 26) (middle) and *fmn* receiving control (n = 22), D_{caf} (n = 24) and N_{caf} (n = 28) treatments (bottom). (B) Total sleep levels of *fmn-bg* and *fmn* flies, compared with that of D_{caf} and N_{caf} flies of each genotype (top), daytime sleep (middle) and night sleep (bottom). *fmn* flies sleep significantly lower than the *fmn-bg* flies both during the day and night, thereby leading to overall reduced levels of sleep. Daytime sleep of D_{caf} and N_{caf} flies of the control genotype are significantly different from one another, whereas night sleep of N_{caf} flies is significantly lower than D_{caf} and control flies of the *fmn-bg* genotype. Night sleep of N_{caf} flies is significantly lower than both control and D_{caf} flies of the *fmn* genotype. (C) Total number of eggs laid on all six days of CAF treatment shows that *fmn-bg* D_{caf} and N_{caf} flies laid significantly lower number of eggs than their controls from days 2-6, whereas there was no difference in number of eggs laid by D_{caf}, N_{caf} and controls of *fmn* genotype on any of the days except day 1 (C') Total number of eggs laid averaged over 6 days by *fmn* flies is significantly lower than that of *fmn-bg* flies. D_{caf} flies of *fmn-bg* genotype (n = 14) laid significantly lower number of eggs as compared to its controls (n = 14), while N_{caf} flies of *fmn-bg* genotype (n = 16) laid significantly lower number of eggs as compared to both control and D_{caf} flies. Control (n = 15), D_{caf} (n = 17) and N_{caf} (n = 17) flies of the *fmn* genotype laid similar number of eggs. All other details as in Figure 4.1.

fmn flies fed with caffeine compared to those fed with standard food. *fmn* and *fmn-bg* flies were fed with 0.5 mg/ml caffeine either only during the day or night and it was found that N_{caf} flies of both *fmn* and *fmn-bg* genotypes show reduced levels of night sleep as compared to their respective controls (Figure 4.9B-bottom, two-way ANOVA, main effect of treatment: $F_{2,\ 141} = 27.64$, $p < 0.00001$), whereas D_{caf} flies of both genotypes show reduced levels of day-time sleep (Figure 4.9B-middle, two-way ANOVA, main effect of treatment, $F_{2,\ 141} = 5.05$, $p < 0.05$), even though it does not reach statistical significance. Interestingly, just like the previously used inbred flies of the w^{1118} genotype, the *fmn-bg* which are flies from another inbred line show a statistically significant trend of decreasing number of eggs laid by Ctrl, D_{caf} and N_{caf} flies, in that order (Figure 4.9C, three-way ANOVA, treatment * day interaction effect: $F_{10,\ 522} = 4.24$, $p < 0.0005$, genotype * treatment * day interaction effect: $F_{10,\ 522} = 1.14$, $p = 0.33$; C', Kruskal-Wallis test, $H_{2,\ 44} = 35.25$, $p < 0.00001$). However, flies of the *fmn* genotype receiving the Ctrl, D_{caf} or N_{caf} treatments did not differ in the average number of eggs laid (Figure 4.9C-C', Kruskal-Wallis test, $H_{2,\ 49} = 1.12$, $p = 0.57$). This suggests that while sleep is affected by caffeine treatment in *fmn* flies, egg output is not, suggesting that egg output cannot be reduced by caffeine beyond a threshold due to a floor effect. Alternatively, the *fmn* gene may be involved in caffeine-mediated egg output reduction independent of the caffeine-mediated sleep loss.

4.4. Discussion

My study aimed to understand how sleep affects reproductive output in female fruit flies *Drosophila melanogaster*. I found that feeding flies with caffeine reduces sleep and also reduces egg output in both inbred and outbred strains of flies (Figures 4.1, 4.4). Also, reduced night time sleep can be seen consistently across two 'wild type' strains and perhaps the milder effects of caffeine on day-time sleep results in the

inconsistent effects across strains. Depriving flies of sleep via mechanical perturbation also reduces egg output considerably (Figures 4.3, 4.4). A loss-of-function mutation in dopamine transporter gene that results in reduced sleep (Kume et al., 2005) also results in reduced egg output (Figure 4.9). Most importantly, reducing sleep by transient dopaminergic neuronal activation reduces egg output; removal of this activation results in wild type levels of sleep and egg output (Figure 4.7, 4.8). Thus, these results strongly indicate that it is sleep loss which has a direct detrimental impact on reproductive output. While it is possible that three distinct methods of sleep deprivation all cause a direct negative impact on egg output independent of sleep loss, I believe that it is unlikely, especially considering the transient nature of the genetic manipulation induced sleep loss. It is probable that it is increased activity which could be reducing egg output by causing a conflict between increased activity and the need to stay inactive during the act of egg-laying. However, if this were the case, then the amount of activity during time spent awake would be higher in flies receiving caffeine or deprivation treatments. This occurs when flies are mechanically deprived of sleep, however waking activity does not differ among caffeine-fed and control flies, and this also does not change with transiently activating dopamine neurons (data not shown). This suggests that sleep loss and not increased activity is resulting in decreased egg output. To my knowledge, this is the first study to establish a direct link between sleep and reproductive physiology in *Drosophila melanogaster*.

Egg-laying in *Drosophila* is the final step in a sequence of processes that occur in a co-ordinated manner which include ovulation of eggs into the uterus, mating and subsequent sperm storage in a pair of spermathecae and the seminal receptacle as well as fertilization in the uterus (reviewed in (Bloch Qazi et al., 2003)). Thus, mechanistically, sleep could influence egg output by modulating any combination of

some or all of the above processes. Virgin females also lay a small quantity of unfertilized eggs; therefore by quantifying the egg numbers laid by sleep deprived virgin flies, the question of whether sperm storage gets modulated by sleep levels could be addressed. I found that the fraction of virgin flies laying eggs was reduced when they were deprived of sleep either during the day or night (19% of D_{dep} flies, n = 21, 17.4% of N_{dep} flies, n = 23) as compared to control flies that slept normally (40.7% of control flies, n = 27). This indicates that sleep modulates egg output by affecting steps other than sperm storage, as virgin flies do not store sperm and yet their egg output is reduced upon sleep deprivation. However, a more detailed analysis of ovulation rates, egg hatchability, mature and immature egg numbers and amount of stored sperm will aid in finer dissection of the relationship between sleep and reproductive system.

Reproduction in *Drosophila* is regulated by an array of hormones and fecundity critically depends upon balance in the amounts of Juvenile Hormone (JH) and ecdysone (Soller et al., 1999). Dopamine regulates levels of JH in *Drosophila viridis* (Rauschenbach et al., 2007) thereby indirectly affecting fecundity. Indeed, dopaminergic neuronal circuits are involved in governing oviposition choice, specifically to media containing favourable levels of alcohol (Azanchi et al., 2013). Moreover, it has been also shown that dopamine acts to promote adaptation of *Drosophila sechelia* to a specialist diet of an otherwise toxic fruit, *Morinda citrifolia* by boosting its fecundity (Lavista-Llanos et al., 2014). In a recent study using genome-wide association methods, two genes encoding dopamine receptors (*Dop1R1* and *DopEcR*) in *D. melanogaster* were shown to have pleiotrophic effects on traits associated with ovariole number and sleep parameters (Lobell et al., 2017). Importantly, lowered levels of dopamine during larval stages or immediately after eclosion both have far reaching consequences in terms of decreased egg output and

stalled ovarian development respectively (Neckameyer, 1996). In contrast, I show that a loss-of-function mutation in the dopamine transporter gene which retains dopamine in synaptic clefts reduces sleep and reduces egg output while transient *increase* in dopaminergic activity causes a transient decrease in both sleep and egg output (Figure 4.7). Together these results demonstrate that levels of neuromodulatory substances can have strong dose dependent effects such that both low and high titres can lead to sub-optimal outcomes to the organism (Berridge and Arnsten, 2013).

Caffeine is one of the most widely used psychostimulants in the world and it promotes wakefulness and causes sleep deprivation. With increased precedence in shift work and a general lifestyle favouring delayed bedtimes and decreased night sleep levels, the consumption of caffeine specifically during the night is bound to increase. Here, I show that caffeine consumption and increased night activity decreases sleep and negatively alters egg output in *Drosophila*. While I have shown this effect with female flies, similar trends may also be found in male reproductive output. In conclusion, my results unequivocally show that each method of sleep deprivation, be it chemical, mechanical or genetic, results in sleep loss accompanied with reduction in egg output. For animals that invest in parental care, sleep deprivation may be an inevitable consequence resulting in lowered reproductive output thereby potentially giving rise to a subtle level of parent-offspring conflict or co-adaptation. I conclude that sleep may contribute to reproductive success of organisms, thereby amplifying its propensity to be selected for, over evolutionary timescales.

Conclusions

Drosophila melanogaster has proved to be a valuable model system to study the neuronal and genetic mechanisms underlying several behaviours, including sleep. Over the last eighteen years, sleep research in *Drosophila* has made significant strides in understanding mechanistic details of sleep/wake regulation in invertebrates. However, general principles regarding the organization of distinct neural circuits into a coherent system for the maintenance and timing of sleep/wake behaviours are yet to emerge. As the initial step towards addressing this question, I focused on understanding the relationship between sleep homeostatic mechanisms which control the maintenance of sleep and wake, and circadian clocks which time the onset of sleep and wake. I found that circadian clocks drive timed sleep homeostatic responses, but do not receive any feedback from the sleep homeostatic process. The circadian clock neurons that are involved in timing sleep homeostatic responses remain unknown; although I found that certain clock neurons such as the dorsal lateral neurons (LN_d) play a distinct role in encoding a sleep-loss state. I also found that maintenance of wake during day-time, which can be considered as a sleep homeostatic function, relies upon signaling from a circadian neuropeptide to certain dopamine neurons that also connect to the sleep homeostat. Taken together, these findings suggest that the sleep homeostat is downstream of circadian clocks for daily sleep/wake regulation in *Drosophila*.

The two-process model of sleep regulation, which posits that both circadian and sleep homeostatic processes interact and generate the propensity to sleep (Borbély, 1982), has served as an excellent guiding principle to understand sleep regulation in various mammalian and bird species. Yet, the primary proposition of interaction between circadian and sleep homeostatic processes remained untested in *Drosophila* sleep regulation. In agreement with this, I found that circadian clocks regulate the

quantity of recovery sleep, a sleep homeostatic response, depending upon the time of day during which sleep was deprived. Indeed, such an expectation of circadian phases being able to modulate sleep homeostatic responses was predicted even before *Drosophila* was established as a model system to study sleep (Hendricks et al., 2000b). However, I also found that the sleep homeostat does not influence core circadian clock properties in *Drosophila*. In the two-process model, this was proposed to occur in mammals that have been shown to "select" the time at which they are exposed to light, and therefore, while this prediction may hold true for cave-dwelling and burrowing mammals (Daan et al., 1984), it may not be relevant for *Drosophila*.

According to the two-process model, the thresholds that determine the onset of sleep and wake are set by circadian clocks. One prediction of the model was that rhythmic environmental cues such as light can also set these thresholds. In fact, this prediction is met in my study as certain flies lacking functional circadian clocks (tim^{01}, $per^{0}w$) show robust sleep/wake cycles that are quite similar to wild type flies in the way they vary across the 24 h cycle especially in the presence of cyclic time cues. Yet another prediction was that cyclic cues would time wake-dependent sleep propensity brought about by the homeostatic process, rather than timing the wake-independent sleep propensity due to the circadian process. Indeed, the finding that number of brief awakenings, which is a proxy for the sleep homeostat, cycles across the light/dark cycle in three of the four circadian clock mutants tested (tim^{01}, $per^{0}w$ and clk^{JRK}) agrees well with this prediction.

The original two-process model had proposed that the homeostatic and circadian processes interact with one another only at the transitions between sleep and wake, where circadian process was expected to set the threshold for onset of either sleep or wake (Borbély, 1982). However, modifications to the model led to the prediction of

dynamic and continuous interactions between the two processes (Borbély and Achermann, 1999; Putilov, 1995). Here, I found that sleep deprivation during times when flies are expected to transition from wake to sleep (ZT 12-16 and CT 12-16) did not result in any sleep rebound. However, deprivation during expected transitions from sleep to wake resulted in significant sleep rebound as measured by sleep quantity (ZT 20-24) and quality (CT 0-4). Nevertheless, maximum amount and intensity of sleep rebound was observed when sleep was deprived during the middle of subjective night (CT 16-20). These results indicate that circadian clocks interact with sleep homeostatic mechanisms even during different times and not just during transitions, suggesting that perhaps the interaction occurs continuously.

Among the various subsets of circadian clock neurons that play distinct roles in modulating rhythmic behaviours, I found that the LN_d also are necessary for a sleep homeostatic function. The two-process model posited that the circadian and homeostatic processes while originating independently may still significantly influence one another. Even though the LN_d are circadian clock neurons which are important for a sleep homeostatic function, functional molecular clocks are not required for this homeostatic function. This shows that even in LN_d the expectation of the two processes being independent from one another is fulfilled, as mechanistically the two processes seem to employ different pathways. Another important finding of my study is that signaling through a very important circadian neuropeptide (PDF) and its receptor to dopamine neurons is important for maintenance of wakefulness during the day. Incidentally, the same dopamine neurons have been shown to arborize within known sleep homeostatic structures (Liu et al., 2012; Sitaraman et al., 2015b). This suggests that LN_d and PDF^+ ventral lateral neurons (LN_v) through their action on dopamine neurons represent circadian neurons that have distinct sleep homeostatic roles, thereby

highlighting these neurons as putative regions where circadian clocks may interact with the sleep homeostat.

By definition, homeostasis is any process because of which the system returns to its baseline level. Sleep homeostat is therefore expected to not only maintain levels of sleep and wake, but also return sleep levels to baseline levels due to changes brought about by sleep deprivation. The physiological basis of the sleep homeostatic process has been explored in some detail in *Drosophila* by examining changes in levels of synaptic numbers, lengths and amounts of synaptic proteins, cellular components and macromolecules, as well as overall immune functions due to sleep deprivation. I focused on how sleep loss impacts reproductive physiology and found that sleep deprivation results in reduction in egg output of female flies. This reduction in egg output was also seen with transient activation of wake-promoting dopamine neurons, and egg output levels were rescued when sleep was restored to normal levels. These results are important in the context of impact of sleep deprivation on non-neural tissues and also highlight the role of sleep regulation in overall maintenance of reproductive health.

Several questions regarding organization of sleep/wake circuits remain unanswered and many possibilities remain to be explored. A key prediction of the two-process model not completely tested in this study pertains to changes in core properties of circadian clocks due to the sleep homeostatic process. While I have shown that period, phase and robustness does not change due to sleep deprivation during different time intervals lasting four hours, how longer durations of sleep deprivation affect clock properties remains to be seen. Circadian sensitivity to light cues at different time-points was found to be altered due to sleep deprivation in mice, rats and hamsters (Challet et al., 2001; Jha et al., 2017; Mistlberger et al., 1997; van Diepen et al., 2014). However,

whether circadian sensitivity to photic cues changes due to sleep deprivation even in flies remains to be examined.

Interestingly, another study had found that cholinergic neurons encode a sleep deprivation state, as hyper-exciting those, results in sleep reduction and returning the neuronal activity to baseline levels results in sleep rebound (Seidner et al., 2015). Using the same method with a different *GAL4* driver, I found that neuronal activity of a circadian clock subset of LN_d neurons also encodes sleep state. Interestingly, at least two LN_d neurons have been reported to be cholinergic (Cho et al., 2017); this opens up the possibility of LN_d using acetylcholine as their neurotransmitter to conduct their sleep homeostatic function of conveying the information of sleep state. Another downstream signaling molecule that could be potentially used by LN_d in their sleep homeostatic function is *Cyclin A* as it has been implicated in sleep homeostasis and one or two LN_d are shown to express it (Rogulja and Young, 2012). Short Neuropeptide F (sNPF), expressed by LN_d and implicated in sleep (Shang et al., 2013), as well as the Ion transport Peptide (ITP) expressed by LN_d (Cho et al., 2017) could be the neuropeptides involved in LN_d sleep homeostatic function. Another important question that needs to be addressed is how LN_d encode sleep state, and to which homeostatic structure they convey this information. Further experimentation in this direction will help in understanding mechanisms of sleep homeostasis governed by the action of circadian LN_d neurons. Notwithstanding these unanswered questions, my study has originated a general organizational structure for sleep/wake regulation in *Drosophila melanogaster*, with circadian clocks impinging upon the sleep homeostatic process to not only time the onset of sleep and wake, but also to maintain these opposite states.

References

Afonso, D. J., Liu, D., Machado, D. R., Pan, H., Jepson, J. E., Rogulja, D. and Koh, K. (2015). TARANIS Functions with Cyclin A and Cdk1 in a Novel Arousal Center to Control Sleep in Drosophila. *Curr Biol* **25**, 1717-26.

Agosto, J., Choi, J. C., Parisky, K. M., Stilwell, G., Rosbash, M. and Griffith, L. C. (2008). Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in Drosophila. *Nat Neurosci* **11**, 354-9.

Åkerstedt, T., Froberg, J.E. (1977). Psychophysiological carcadian rhythms in women during 72 h of sleep deprivation. *Waking Sleeping*, 387-394.

Allada, R. and Chung, B. Y. (2010). Circadian organization of behavior and physiology in Drosophila. *Annu Rev Physiol* **72**, 605-24.

Allada, R., Cirelli, C. and Sehgal, A. (2017). Molecular Mechanisms of Sleep Homeostasis in Flies and Mammals. *Cold Spring Harb Perspect Biol* **9**.

Alvarenga, T. A., Hirotsu, C., Mazaro-Costa, R., Tufik, S. and Andersen, M. L. (2015). Impairment of male reproductive function after sleep deprivation. *Fertil Steril* **103**, 1355-62 e1.

Andersen, M. L. and Tufik, S. (2002). Distinct effects of paradoxical sleep deprivation and cocaine administration on sexual behavior in male rats. *Addict Biol* **7**, 251-3.

Andretic, R., Kim, Y. C., Jones, F. S., Han, K. A. and Greenspan, R. J. (2008). Drosophila D1 dopamine receptor mediates caffeine-induced arousal. *Proc Natl Acad Sci U S A* **105**, 20392-7.

Andretic, R. and Shaw, P. J. (2005). Essentials of sleep recordings in Drosophila: moving beyond sleep time. *Methods Enzymol* **393**, 759-72.

Andretic, R., van Swinderen, B. and Greenspan, R. J. (2005). Dopaminergic modulation of arousal in Drosophila. *Curr Biol* **15**, 1165-75.

Antle, M. C. and Mistlberger, R. E. (2000). Circadian clock resetting by sleep deprivation without exercise in the Syrian hamster. *J Neurosci* **20**, 9326-32.

Artiushin, G. and Sehgal, A. (2017). The Drosophila circuitry of sleep-wake regulation. *Curr Opin Neurobiol* **44**, 243-250.

Aschoff, J., Gerecke, U. and Wever, R. (1967). Desynchronization of human circadian rhythms. *Jpn J Physiol* **17**, 450-7.

Ascoff, J. (1966). Circadian activity pattern with two peaks. *Ecology*, 657-662.

Aserinsky, E. and Kleitman, N. (1953). Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. *Science* **118**, 273-4.

Aso, Y., Grubel, K., Busch, S., Friedrich, A. B., Siwanowicz, I. and Tanimoto, H. (2009). The mushroom body of adult Drosophila characterized by GAL4 drivers. *J Neurogenet* **23**, 156-72.

Aso, Y., Hattori, D., Yu, Y., Johnston, R. M., Iyer, N. A., Ngo, T. T., Dionne, H., Abbott, L. F., Axel, R., Tanimoto, H. et al. (2014a). The neuronal architecture of the mushroom body provides a logic for associative learning. *Elife* **3**, e04577.

Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guerin, G., Placais, P. Y., Robie, A. A., Yamagata, N., Schnaitmann, C. et al. (2014b). Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. *Elife* **3**, e04580.

Aulsebrook, A. E., Jones, T. M., Rattenborg, N. C., Roth, T. C., 2nd and Lesku, J. A. (2016). Sleep Ecophysiology: Integrating Neuroscience and Ecology. *Trends Ecol Evol* **31**, 590-599.

Azanchi, R., Kaun, K. R. and Heberlein, U. (2013). Competing dopamine neurons drive oviposition choice for ethanol in Drosophila. *Proc Natl Acad Sci U S A* **110**, 21153-8.

Beaver, L. M., Gvakharia, B. O., Vollintine, T. S., Hege, D. M., Stanewsky, R. and Giebultowicz, J. M. (2002). Loss of circadian clock function decreases reproductive fitness in males of Drosophila melanogaster. *Proc Natl Acad Sci U S A* **99**, 2134-9.

Beckwith, E. J., Geissmann, Q., French, A. S. and Gilestro, G. F. (2017). Regulation of sleep homeostasis by sexual arousal. *Elife* **6**.

Berridge, C. W. and Arnsten, A. F. (2013). Psychostimulants and motivated behavior: arousal and cognition. *Neurosci Biobehav Rev* **37**, 1976-84.

Berry, J. A., Cervantes-Sandoval, I., Chakraborty, M. and Davis, R. L. (2015). Sleep Facilitates Memory by Blocking Dopamine Neuron-Mediated Forgetting. *Cell* **161**, 1656-67.

Blake, H. and Gerard, R. W. (1937). Brain potentials during sleep. *Am J Physiol*, 692-703.

Bloch Qazi, M. C., Heifetz, Y. and Wolfner, M. F. (2003). The developments between gametogenesis and fertilization: ovulation and female sperm storage in Drosophila melanogaster. *Dev Biol* **256**, 195-211.

Boivin, D. B., Czeisler, C. A., Dijk, D. J., Duffy, J. F., Folkard, S., Minors, D. S., Totterdell, P. and Waterhouse, J. M. (1997). Complex interaction of the sleep-wake cycle and circadian phase modulates mood in healthy subjects. *Arch Gen Psychiatry* **54**, 145-52.

Borbély, A. A. (1982). A two process model of sleep regulation. *Hum Neurobiol* **1**, 195-204.

Borbély, A. A. and Achermann, P. (1992). Concepts and models of sleep regulation: an overview. *J Sleep Res* **1**, 63-79.

Borbély, A. A. and Achermann, P. (1999). Sleep homeostasis and models of sleep regulation. *J Biol Rhythms* **14**, 557-68.

Borbély, A. A., Daan, S., Wirz-Justice, A. and Deboer, T. (2016). The two-process model of sleep regulation: a reappraisal. *J Sleep Res* **25**, 131-43.

Borbély, A. A., Tobler, I. and Groos, G. (1982). Sleep homeostasis and the circadian sleep-wake rhythm. In *New perspectives in sleep research*, (ed. M. Chase). Jamaica, New York: Spectrum.

Bushey, D., Huber, R., Tononi, G. and Cirelli, C. (2007). Drosophila Hyperkinetic mutants have reduced sleep and impaired memory. *J Neurosci* **27**, 5384-93.

Bushey, D., Hughes, K. A., Tononi, G. and Cirelli, C. (2010). Sleep, aging, and lifespan in Drosophila. *BMC Neurosci* **11**, 56.

Bushey, D., Tononi, G. and Cirelli, C. (2011). Sleep and synaptic homeostasis: structural evidence in Drosophila. *Science* **332**, 1576-81.

Bushey, D., Tononi, G. and Cirelli, C. (2015). Sleep- and wake-dependent changes in neuronal activity and reactivity demonstrated in fly neurons using in vivo calcium imaging. *Proc Natl Acad Sci U S A* **112**, 4785-90.

Campbell, S. S. and Tobler, I. (1984). Animal sleep: a review of sleep duration across phylogeny. *Neurosci Biobehav Rev* **8**, 269-300.

Carhan, A., Tang, K., Shirras, C. A., Shirras, A. D. and Isaac, R. E. (2011). Loss of Angiotensin-converting enzyme-related (ACER) peptidase disrupts night-time sleep in adult Drosophila melanogaster. *Journal of Experimental Biology* **214**, 680-6.

Catterson, J. H., Knowles-Barley, S., James, K., Heck, M. M., Harmar, A. J. and Hartley, P. S. (2010). Dietary modulation of Drosophila sleep-wake behaviour. *PLoS One* **5**, e12062.

Cavanaugh, D. J., Geratowski, J. D., Wooltorton, J. R., Spaethling, J. M., Hector, C. E., Zheng, X., Johnson, E. C., Eberwine, J. H. and Sehgal, A. (2014). Identification of a circadian output circuit for rest:activity rhythms in Drosophila. *Cell* **157**, 689-701.

Cavanaugh, D. J., Vigderman, A. S., Dean, T., Garbe, D. S. and Sehgal, A. (2016). The Drosophila Circadian Clock Gates Sleep through Time-of-Day Dependent Modulation of Sleep-Promoting Neurons. *Sleep* **39**, 345-56.

Challet, E., Turek, F. W., Laute, M. and Van Reeth, O. (2001). Sleep deprivation decreases phase-shift responses of circadian rhythms to light in the mouse: role of serotonergic and metabolic signals. *Brain Res* **909**, 81-91.

Chen, J., Reiher, W., Hermann-Luibl, C., Sellami, A., Cognigni, P., Kondo, S., Helfrich-Förster, C., Veenstra, J. A. and Wegener, C. (2016). Allatostatin A Signalling in Drosophila Regulates Feeding and Sleep and Is Modulated by PDF. *PLoS Genet* **12**, e1006346.

Chen, W., Shi, W., Li, L., Zheng, Z., Li, T., Bai, W. and Zhao, Z. (2013). Regulation of sleep by the short neuropeptide F (sNPF) in Drosophila melanogaster. *Insect Biochem Mol Biol* **43**, 809-19.

Cho, J. R., Treweek, J. B., Robinson, J. E., Xiao, C., Bremner, L. R., Greenbaum, A. and Gradinaru, V. (2017). Dorsal Raphe Dopamine Neurons Modulate Arousal and Promote Wakefulness by Salient Stimuli. *Neuron* **94**, 1205-1219 e8.

Chung, B. Y., Kilman, V. L., Keath, J. R., Pitman, J. L. and Allada, R. (2009). The GABA(A) receptor RDL acts in peptidergic PDF neurons to promote sleep in Drosophila. *Curr Biol* **19**, 386-90.

Cirelli, C. (2009). The genetic and molecular regulation of sleep: from fruit flies to humans. *Nat Rev Neurosci* **10**, 549-60.

Cirelli, C., Bushey, D., Hill, S., Huber, R., Kreber, R., Ganetzky, B. and Tononi, G. (2005a). Reduced sleep in Drosophila Shaker mutants. *Nature* **434**, 1087-92.

Cirelli, C., LaVaute, T. M. and Tononi, G. (2005b). Sleep and wakefulness modulate gene expression in Drosophila. *Journal of Neurochemistry* **94**, 1411-9.

Cirelli, C. and Tononi, G. (2008). Is sleep essential? *PLoS Biol* **6**, e216.

Collins, B. and Blau, J. (2007). Even a stopped clock tells the right time twice a day: circadian timekeeping in Drosophila. *Pflugers Arch* **454**, 857-67.

Crocker, A. and Sehgal, A. (2008). Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. *J Neurosci* **28**, 9377-85.

Crocker, A. and Sehgal, A. (2010). Genetic analysis of sleep. *Genes Dev* **24**, 1220-35.

Crocker, A., Shahidullah, M., Levitan, I. B. and Sehgal, A. (2010). Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior. *Neuron* **65**, 670-81.

Cyran, S. A., Buchsbaum, A. M., Reddy, K. L., Lin, M. C., Glossop, N. R., Hardin, P. E., Young, M. W., Storti, R. V. and Blau, J. (2003). vrille, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. *Cell* **112**, 329-41.

Czeisler, C. A., Weitzman, E., Moore-Ede, M. C., Zimmerman, J. C. and Knauer, R. S. (1980). Human sleep: its duration and organization depend on its circadian phase. *Science* **210**, 1264-7.

Daan, S. and Beersma, D. G. (1984). Circadian gating of human sleep and wakefulness. In *Mathematical modeling of circadian systems*, (ed. M. C. Moore-Ede and C. Czeisler), pp. 129-158. New York: Raven.

Daan, S., Beersma, D. G. and Borbély, A. A. (1984). Timing of human sleep: recovery process gated by a circadian pacemaker. *Am J Physiol* **246**, R161-83.

Dabbish, N. S. and Raizen, D. M. (2011). GABAergic synaptic plasticity during a developmentally regulated sleep-like state in C. elegans. *J Neurosci* **31**, 15932-43.

De Gennaro, L., Ferrara, M. and Bertini, M. (2001). EEG arousals in normal sleep: variations induced by total and selective slow-wave sleep deprivation. *Sleep* **24**, 673-9.

Deboer, T., Detari, L. and Meijer, J. H. (2007). Long term effects of sleep deprivation on the mammalian circadian pacemaker. *Sleep* **30**, 257-62.

Deboer, T., Vansteensel, M. J., Detari, L. and Meijer, J. H. (2003). Sleep states alter activity of suprachiasmatic nucleus neurons. *Nat Neurosci* **6**, 1086-90.

Dement, W. and Kleitman, N. (1957). The relation of eye movements during sleep to dream activity: an objective method for the study of dreaming. *J Exp Psychol* **53**, 339-46.

Dement, W. C. (1998). The study of human sleep: a historical perspective. *Thorax* **53 Suppl 3**, S2-7.

Dijk, D. J., Beersma, D. G. and Daan, S. (1987a). EEG power density during nap sleep: reflection of an hourglass measuring the duration of prior wakefulness. *J Biol Rhythms* **2**, 207-19.

Dijk, D. J., Beersma, D. G., Daan, S. and Lewy, A. J. (1989). Bright morning light advances the human circadian system without affecting NREM sleep homeostasis. *Am J Physiol* **256**, R106-11.

Dijk, D. J. and Czeisler, C. A. (1994). Paradoxical timing of the circadian rhythm of sleep propensity serves to consolidate sleep and wakefulness in humans. *Neurosci Lett* **166**, 63-8.

Dijk, D. J. and Czeisler, C. A. (1995). Contribution of the circadian pacemaker and the sleep homeostat to sleep propensity, sleep structure, electroencephalographic slow waves, and sleep spindle activity in humans. *J Neurosci* **15**, 3526-38.

Dijk, D. J., Duffy, J. F. and Czeisler, C. A. (1992). Circadian and sleep/wake dependent aspects of subjective alertness and cognitive performance. *J Sleep Res* **1**, 112-7.

Dijk, D. J., Hayes, B. and Czeisler, C. A. (1993). Dynamics of electroencephalographic sleep spindles and slow wave activity in men: effect of sleep deprivation. *Brain Res* **626**, 190-9.

Dijk, D. J., Visscher, C. A., Bloem, G. M., Beersma, D. G. and Daan, S. (1987b). Reduction of human sleep duration after bright light exposure in the morning. *Neurosci Lett* **73**, 181-6.

Dissel, S., Angadi, V., Kirszenblat, L., Suzuki, Y., Donlea, J., Klose, M., Koch, Z., English, D., Winsky-Sommerer, R., van Swinderen, B. et al. (2015). Sleep restores behavioral plasticity to Drosophila mutants. *Curr Biol* **25**, 1270-81.

Dissel, S., Codd, V., Fedic, R., Garner, K. J., Costa, R., Kyriacou, C. P. and Rosato, E. (2004). A constitutively active cryptochrome in Drosophila melanogaster. *Nat Neurosci* **7**, 834-40.

Donelson, N., Kim, E. Z., Slawson, J. B., Vecsey, C. G., Huber, R. and Griffith, L. C. (2012). High-resolution positional tracking for long-term analysis of Drosophila sleep and locomotion using the "tracker" program. *PLoS One* **7**, e37250.

Donlea, J. M. (2017). Neuronal and molecular mechanisms of sleep homeostasis. *Curr Opin Insect Sci* **24**, 51-57.

Donlea, J. M., Pimentel, D. and Miesenbock, G. (2014). Neuronal Machinery of Sleep Homeostasis in Drosophila. *Neuron* **81**, 1442.

Donlea, J. M., Pimentel, D., Talbot, C. B., Kempf, A., Omoto, J. J., Hartenstein, V. and Miesenbock, G. (2018). Recurrent Circuitry for Balancing Sleep Need and Sleep. *Neuron* **97**, 378-389 e4.

Donlea, J. M., Thimgan, M. S., Suzuki, Y., Gottschalk, L. and Shaw, P. J. (2011). Inducing sleep by remote control facilitates memory consolidation in Drosophila. *Science* **332**, 1571-6.

Dowse, H. B. and Ringo, J. M. (1987). Further evidence that the circadian clock in Drosophila is a population of coupled ultradian oscillators. *J Biol Rhythms* **2**, 65-76.

Dubruille, R. and Emery, P. (2008). A plastic clock: how circadian rhythms respond to environmental cues in Drosophila. *Mol Neurobiol* **38**, 129-45.

Dudley, C. A., Erbel-Sieler, C., Estill, S. J., Reick, M., Franken, P., Pitts, S. and McKnight, S. L. (2003). Altered patterns of sleep and behavioral adaptability in NPAS2-deficient mice. *Science* **301**, 379-83.

Duvall, L. B. and Taghert, P. H. (2012). The circadian neuropeptide PDF signals preferentially through a specific adenylate cyclase isoform AC3 in M pacemakers of Drosophila. *PLoS Biol* **10**, e1001337.

Duvall, L. B. and Taghert, P. H. (2013). E and M circadian pacemaker neurons use different PDF receptor signalosome components in drosophila. *J Biol Rhythms* **28**, 239-48.

Eban-Rothschild, A., Appelbaum, L. and de Lecea, L. (2018). Neuronal Mechanisms for Sleep/Wake Regulation and Modulatory Drive. *Neuropsychopharmacology* **43**, 937-952.

Eban-Rothschild, A., Rothschild, G., Giardino, W. J., Jones, J. R. and de Lecea, L. (2016). VTA dopaminergic neurons regulate ethologically relevant sleep-wake behaviors. *Nat Neurosci* **19**, 1356-66.

Enright, J. T. (1980). The timing of sleep and wakefulness. New York: Springer.

Everson, C. A. (1995). Functional consequences of sustained sleep deprivation in the rat. *Behav Brain Res* **69**, 43-54.

Everson, C. A., Bergmann, B. M. and Rechtschaffen, A. (1989). Sleep deprivation in the rat: III. Total sleep deprivation. *Sleep* **12**, 13-21.

Everson, C. A. and Szabo, A. (2009). Recurrent restriction of sleep and inadequate recuperation induce both adaptive changes and pathological outcomes. *Am J Physiol Regul Integr Comp Physiol* **297**, R1430-40.

Everson, C. A. and Szabo, A. (2011). Repeated exposure to severely limited sleep results in distinctive and persistent physiological imbalances in rats. *PLoS One* **6**, e22987.

Faville, R., Kottler, B., Goodhill, G. J., Shaw, P. J. and van Swinderen, B. (2015). How deeply does your mutant sleep? Probing arousal to better understand sleep defects in Drosophila. *Sci Rep* **5**, 8454.

Feinberg, I., Fein, G. and Floyd, T. C. (1980). EEG patterns during and following extended sleep in young adults. *Electroencephalogr Clin Neurophysiol* **50**, 467-76.

Fiebrig, K. (1912). Schlafende Insekten. *Jenaische Zeit Naturwissenschaft*, 315-364.

Fogle, K. J., Parson, K. G., Dahm, N. A. and Holmes, T. C. (2011). CRYPTOCHROME is a blue-light sensor that regulates neuronal firing rate. *Science* **331**, 1409-13.

Folkard, S. and Åkerstedt, T. (1992). A three-process model of the regulation of alertness-sleepiness. In *Sleep, Arousal and Performance: A Tribute to Bob Wilkinson*, (ed. R. J. Broughton and R. D. Ogilvie), pp. 11-26. Boston, Basel, Berlin: Birkhauser.

Foltenyi, K., Greenspan, R. J. and Newport, J. W. (2007). Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in Drosophila. *Nat Neurosci* **10**, 1160-7.

Frank, M. G. (2012). Erasing synapses in sleep: is it time to be SHY? *Neural Plast* **2012**, 264378.

Franken, P. and Dijk, D. J. (2009). Circadian clock genes and sleep homeostasis. *Eur J Neurosci* **29**, 1820-9.

Franken, P., Dijk, D. J., Tobler, I. and Borbély, A. A. (1991). Sleep deprivation in rats: effects on EEG power spectra, vigilance states, and cortical temperature. *Am J Physiol* **261**, R198-208.

Freeman, A., Pranski, E., Miller, R. D., Radmard, S., Bernhard, D., Jinnah, H. A., Betarbet, R., Rye, D. B. and Sanyal, S. (2012). Sleep fragmentation and motor restlessness in a Drosophila model of restless legs syndrome. *Curr Biol* **22**, 1142-8.

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J. and Birman, S. (2003). Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J Neurobiol* **54**, 618-27.

Ganguly-Fitzgerald, I., Donlea, J. and Shaw, P. J. (2006). Waking experience affects sleep need in Drosophila. *Science* **313**, 1775-81.

Gilestro, G. F. (2012). Video tracking and analysis of sleep in Drosophila melanogaster. *Nat Protoc* **7**, 995-1007.

Gilestro, G. F. and Cirelli, C. (2009). pySolo: a complete suite for sleep analysis in Drosophila. *Bioinformatics* **25**, 1466-7.

Gilestro, G. F., Tononi, G. and Cirelli, C. (2009). Widespread changes in synaptic markers as a function of sleep and wakefulness in Drosophila. *Science* **324**, 109-12.

Glossop, N. R., Lyons, L. C. and Hardin, P. E. (1999). Interlocked feedback loops within the Drosophila circadian oscillator. *Science* **286**, 766-8.

Gogna, N., Singh, V. J., Sheeba, V. and Dorai, K. (2015). NMR-based investigation of the Drosophila melanogaster metabolome under the influence of daily cycles of light and temperature. *Mol Biosyst* **11**, 3305-15.

Goodenough, D. R., Lewis, H. B., Shapiro, A., Jaret, L. and Sleser, I. (1965). Dream Reporting Following Abrupt and Gradual Awakenings from Different Types of Sleep. *J Pers Soc Psychol* **2**, 170-9.

Gordon, M. D. and Scott, K. (2009). Motor control in a Drosophila taste circuit. *Neuron* **61**, 373-84.

Grima, B., Chelot, E., Xia, R. and Rouyer, F. (2004). Morning and evening peaks of activity rely on different clock neurons of the Drosophila brain. *Nature* **431**, 869-73.

Grima, B., Dognon, A., Lamouroux, A., Chelot, E. and Rouyer, F. (2012). CULLIN-3 Controls TIMELESS Oscillations in the Drosophila Circadian Clock. *PLoS Biol* **10**, e1001367.

Gulevich, G., Dement, W. and Johnson, L. (1966). Psychiatric and EEG observations on a case of prolonged (264 hours) wakefulness. *Arch Gen Psychiatry* **15**, 29-35.

Guo, F., Holla, M., Diaz, M. M. and Rosbash, M. (2018). A circadian output circuit controls sleep-wake arousal threshold in Drosophila. *bioRxiv*.

Guo, F., Yu, J., Jung, H. J., Abruzzi, K. C., Luo, W., Griffith, L. C. and Rosbash, M. (2016). Circadian neuron feedback controls the Drosophila sleep--activity profile. *Nature* **536**, 292-7.

Hamada, F. N., Rosenzweig, M., Kang, K., Pulver, S. R., Ghezzi, A., Jegla, T. J. and Garrity, P. A. (2008). An internal thermal sensor controlling temperature preference in Drosophila. *Nature* **454**, 217-20.

Hammond, W. A. (1902). Aristotle's Psychology. A Treatise on the Principles of Life (De anima and Parva Naturalia). Translated with introduction and notes. London / New York: Swan Sonnenschein & Co. / The Macmillan Co.

Harbison, S. T., Mackay, T. F. and Anholt, R. R. (2009). Understanding the neurogenetics of sleep: progress from Drosophila. *Trends Genet* **25**, 262-9.

Harbison, S. T. and Sehgal, A. (2008). Quantitative genetic analysis of sleep in Drosophila melanogaster. *Genetics* **178**, 2341-60.

Harbison, S. T. and Sehgal, A. (2009). Energy stores are not altered by long-term partial sleep deprivation in Drosophila melanogaster. *PLoS One* **4**, e6211.

Haynes, P. R., Christmann, B. L. and Griffith, L. C. (2015). A single pair of neurons links sleep to memory consolidation in Drosophila melanogaster. *Elife* **4**.

He, C., Yang, Y., Zhang, M., Price, J. L. and Zhao, Z. (2013). Regulation of sleep by neuropeptide Y-like system in Drosophila melanogaster. *PLoS One* **8**, e74237.

Hedges, S. B. (2002). The origin and evolution of model organisms. *Nat Rev Genet* **3**, 838-49.

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. *Nat Rev Neurosci* **4**, 266-75.

Helfrich-Förster, C. (1995). The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of Drosophila melanogaster. *Proc Natl Acad Sci U S A* **92**, 612-6.

Hendricks, J. C., Finn, S. M., Panckeri, K. A., Chavkin, J., Williams, J. A., Sehgal, A. and Pack, A. I. (2000a). Rest in Drosophila is a sleep-like state. *Neuron* **25**, 129-38.

Hendricks, J. C., Sehgal, A. and Pack, A. I. (2000b). The need for a simple animal model to understand sleep. *Prog Neurobiol* **61**, 339-51.

Hendricks, J. C., Williams, J. A., Panckeri, K., Kirk, D., Tello, M., Yin, J. C. and Sehgal, A. (2001). A non-circadian role for cAMP signaling and CREB activity in Drosophila rest homeostasis. *Nat Neurosci* **4**, 1108-15.

Hergarden, A. C., Tayler, T. D. and Anderson, D. J. (2012). Allatostatin-A neurons inhibit feeding behavior in adult Drosophila. *Proc Natl Acad Sci U S A* **109**, 3967-72.

Hobson, J. A., Lydic, R., Baghdoyan, H. A. (1986). Evolving concepts of sleep cycle generation: From brain centers to neuronal populations. *Behavioral and Brain Sciences*, 371-448.

Huber, R., Deboer, T. and Tobler, I. (2000). Effects of sleep deprivation on sleep and sleep EEG in three mouse strains: empirical data and simulations. *Brain Res* **857**, 8-19.

Huber, R., Hill, S. L., Holladay, C., Biesiadecki, M., Tononi, G. and Cirelli, C. (2004). Sleep homeostasis in Drosophila melanogaster. *Sleep* **27**, 628-39.

Hyun, S., Lee, Y., Hong, S. T., Bang, S., Paik, D., Kang, J., Shin, J., Lee, J., Jeon, K., Hwang, S. et al. (2005). Drosophila GPCR Han is a receptor for the circadian clock neuropeptide PDF. *Neuron* **48**, 267-78.

Iber, C., Ancoli-Israel, S., Chesson Jr., A.L., Quan, S.F. (2007). The AASM manual for the scoring of sleep and associated events: Rules, terminology and technical specifications. Westchester: Illinois: American Academy of sleep medicine.

Ibuka, N., Nihonmatsu, I. and Sekiguchi, S. (1980). Sleep-wakefulness rhythms in mice after suprachiasmatic nucleus lesions. *Waking Sleeping* **4**, 167-73.

Ilius, M., Wolf, R. and Heisenberg, M. (1994). The central complex of Drosophila melanogaster is involved in flight control: studies on mutants and mosaics of the gene ellipsoid body open. *J Neurogenet* **9**, 189-206.

Im, S. H. and Taghert, P. H. (2010). PDF receptor expression reveals direct interactions between circadian oscillators in Drosophila. *J Comp Neurol* **518**, 1925-45.

Isaac, R. E., Li, C., Leedale, A. E. and Shirras, A. D. (2010). Drosophila male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female. *Proc Biol Sci* **277**, 65-70.

Ishimoto, H., Lark, A. and Kitamoto, T. (2012). Factors that Differentially Affect Daytime and Nighttime Sleep in Drosophila melanogaster. *Front Neurol* **3**, 24.

Ja, W. W., Carvalho, G. B., Mak, E. M., de la Rosa, N. N., Fang, A. Y., Liong, J. C., Brummel, T. and Benzer, S. (2007). Prandiology of Drosophila and the CAFE assay. *Proc Natl Acad Sci U S A* **104**, 8253-6.

Jan, L. Y. and Jan, Y. N. (2012). Voltage-gated potassium channels and the diversity of electrical signalling. *J Physiol* **590**, 2591-9.

Jha, P. K., Bouaouda, H., Gourmelen, S., Dumont, S., Fuchs, F., Goumon, Y., Bourgin, P., Kalsbeek, A. and Challet, E. (2017). Sleep Deprivation and Caffeine Treatment Potentiate Photic Resetting of the Master Circadian Clock in a Diurnal Rodent. *J Neurosci* **37**, 4343-4358.

Joiner, W. J., Crocker, A., White, B. H. and Sehgal, A. (2006). Sleep in Drosophila is regulated by adult mushroom bodies. *Nature* **441**, 757-60.

Jung, C. M., Melanson, E. L., Frydendall, E. J., Perreault, L., Eckel, R. H. and Wright, K. P. (2011). Energy expenditure during sleep, sleep deprivation and sleep following sleep deprivation in adult humans. *J Physiol* **589**, 235-44.

Kaneko, M. and Hall, J. C. (2000). Neuroanatomy of cells expressing clock genes in Drosophila: transgenic manipulation of the period and timeless genes to mark the perikarya of circadian pacemaker neurons and their projections. *J Comp Neurol* **422**, 66-94.

Karacan, I., Williams, R. L., Finley, W. W. and Hursch, C. J. (1970). The effects of naps on nocturnal sleep: influence on the need for stage-1 REM and stage 4 sleep. *Biol Psychiatry* **2**, 391-9.

Kayser, M. S., Mainwaring, B., Yue, Z. and Sehgal, A. (2015). Sleep deprivation suppresses aggression in Drosophila. *Elife* **4**, e07643.

Kayser, M. S., Yue, Z. and Sehgal, A. (2014). A critical period of sleep for development of courtship circuitry and behavior in Drosophila. *Science* **344**, 269-74.

Keene, A. C., Duboue, E. R., McDonald, D. M., Dus, M., Suh, G. S., Waddell, S. and Blau, J. (2010). Clock and cycle limit starvation-induced sleep loss in Drosophila. *Curr Biol* **20**, 1209-15.

Kim, E. Y., Bae, K., Ng, F. S., Glossop, N. R., Hardin, P. E. and Edery, I. (2002). Drosophila CLOCK protein is under posttranscriptional control and influences light-induced activity. *Neuron* **34**, 69-81.

Klarsfeld, A., Malpel, S., Michard-Vanhee, C., Picot, M., Chelot, E. and Rouyer, F. (2004). Novel features of cryptochrome-mediated photoreception in the brain circadian clock of Drosophila. *J Neurosci* **24**, 1468-77.

Kloss, J. D., Perlis, M. L., Zamzow, J. A., Culnan, E. J. and Gracia, C. R. (2015). Sleep, sleep disturbance, and fertility in women. *Sleep Med Rev* **22**, 78-87.

Knowles, J. B., Coulter, M., Wahnon, S., Reitz, W. and MacLean, A. W. (1990). Variation in process S: effects on sleep continuity and architecture. *Sleep* **13**, 97-107.

Koh, K., Evans, J. M., Hendricks, J. C. and Sehgal, A. (2006). A Drosophila model for age-associated changes in sleep:wake cycles. *Proc Natl Acad Sci U S A* **103**, 13843-7.

Koh, K., Joiner, W. J., Wu, M. N., Yue, Z., Smith, C. J. and Sehgal, A. (2008). Identification of SLEEPLESS, a sleep-promoting factor. *Science* **321**, 372-6.

Konopka, R. J. and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proc Natl Acad Sci U S A* **68**, 2112-6.

Krause, A. J., Simon, E. B., Mander, B. A., Greer, S. M., Saletin, J. M., Goldstein-Piekarski, A. N. and Walker, M. P. (2017). The sleep-deprived human brain. *Nat Rev Neurosci* **18**, 404-418.

Kronauer, R. E., Czeisler, C. A., Pilato, S. F., Moore-Ede, M. C. and Weitzman, E. D. (1982). Mathematical model of the human circadian system with two interacting oscillators. *Am J Physiol* **242**, R3-17.

Kumar, S., Chen, D. and Sehgal, A. (2012). Dopamine acts through Cryptochrome to promote acute arousal in Drosophila. *Genes Dev* **26**, 1224-34.

Kume, K., Kume, S., Park, S. K., Hirsh, J. and Jackson, F. R. (2005). Dopamine is a regulator of arousal in the fruit fly. *J Neurosci* **25**, 7377-84.

Kunst, M., Hughes, M. E., Raccuglia, D., Felix, M., Li, M., Barnett, G., Duah, J. and Nitabach, M. N. (2014). Calcitonin gene-related peptide neurons mediate sleep-specific circadian output in Drosophila. *Curr Biol* **24**, 2652-64.

Kunst, M., Tso, M. C., Ghosh, D. D., Herzog, E. D. and Nitabach, M. N. (2015). Rhythmic control of activity and sleep by class B1 GPCRs. *Crit Rev Biochem Mol Biol* **50**, 18-30.

Kuo, T. H., Pike, D. H., Beizaeipour, Z. and Williams, J. A. (2010). Sleep triggered by an immune response in Drosophila is regulated by the circadian clock and requires the NFkappaB Relish. *BMC Neurosci* **11**, 17.

Lafont, A. (1870). Journal d'observation sur les animaux matins., (ed.

Lamaze, A., Kratschmer, P. and Jepson, J. E. C. (2018). A sleep-regulatory circuit integrating circadian, homeostatic and environmental information in Drosophila. *bioRxiv*.

Landgraf, D., Shostak, A. and Oster, H. (2012). Clock genes and sleep. *Pflugers Arch* **463**, 3-14.

Laposky, A., Easton, A., Dugovic, C., Walisser, J., Bradfield, C. and Turek, F. (2005). Deletion of the mammalian circadian clock gene BMAL1/Mop3 alters baseline sleep architecture and the response to sleep deprivation. *Sleep* **28**, 395-409.

Lavista-Llanos, S., Svatos, A., Kai, M., Riemensperger, T., Birman, S., Stensmyr, M. C. and Hansson, B. S. (2014). Dopamine drives Drosophila sechellia adaptation to its toxic host. *Elife* **3**.

Lear, B. C., Merrill, C. E., Lin, J. M., Schroeder, A., Zhang, L. and Allada, R. (2005). A G protein-coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. *Neuron* **48**, 221-7.

Lebestky, T., Chang, J. S., Dankert, H., Zelnik, L., Kim, Y. C., Han, K. A., Wolf, F. W., Perona, P. and Anderson, D. J. (2009). Two different forms of arousal in Drosophila are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. *Neuron* **64**, 522-36.

Lenz, O., Xiong, J., Nelson, M. D., Raizen, D. M. and Williams, J. A. (2015). FMRFamide signaling promotes stress-induced sleep in Drosophila. *Brain Behav Immun* **47**, 141-8.

Lesku, J. A., Meyer, L. C., Fuller, A., Maloney, S. K., Dell'Omo, G., Vyssotski, A. L. and Rattenborg, N. C. (2011). Ostriches sleep like platypuses. *PLoS One* **6**, e23203.

Lesku, J. A., Rattenborg, N. C., Valcu, M., Vyssotski, A. L., Kuhn, S., Kuemmeth, F., Heidrich, W. and Kempenaers, B. (2012). Adaptive sleep loss in polygynous pectoral sandpipers. *Science* **337**, 1654-8.

Li, Y., Guo, F., Shen, J. and Rosbash, M. (2014). PDF and cAMP enhance PER stability in Drosophila clock neurons. *Proc Natl Acad Sci U S A* **111**, E1284-90.

Lin, Y., Stormo, G. D. and Taghert, P. H. (2004). The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the Drosophila circadian system. *J Neurosci* **24**, 7951-7.

Linford, N. J., Chan, T. P. and Pletcher, S. D. (2012). Re-patterning sleep architecture in Drosophila through gustatory perception and nutritional quality. *PLoS Genet* **8**, e1002668.

Liu, Q., Liu, S., Kodama, L., Driscoll, M. R. and Wu, M. N. (2012). Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in Drosophila. *Curr Biol* **22**, 2114-23.

Liu, S., Lamaze, A., Liu, Q., Tabuchi, M., Yang, Y., Fowler, M., Bharadwaj, R., Zhang, J., Bedont, J., Blackshaw, S. et al. (2014). WIDE AWAKE mediates the circadian timing of sleep onset. *Neuron* **82**, 151-66.

Liu, S., Liu, Q., Tabuchi, M. and Wu, M. N. (2016). Sleep Drive Is Encoded by Neural Plastic Changes in a Dedicated Circuit. *Cell* **165**, 1347-1360.

Liu, W., Guo, F., Lu, B. and Guo, A. (2008). amnesiac regulates sleep onset and maintenance in Drosophila melanogaster. *Biochem Biophys Res Commun* **372**, 798-803.

Liu, Z. W., Gan, G., Suyama, S. and Gao, X. B. (2011). Intracellular energy status regulates activity in hypocretin/orexin neurones: a link between energy and behavioural states. *J Physiol* **589**, 4157-66.

Lobell, A. S., Kaspari, R. R., Serrano Negron, Y. L. and Harbison, S. T. (2017). The Genetic Architecture of Ovariole Number in Drosophila melanogaster: Genes with Major, Quantitative, and Pleiotropic Effects. *G3 (Bethesda)*.

Loh, D. H., Kuljis, D. A., Azuma, L., Wu, Y., Truong, D., Wang, H. B. and Colwell, C. S. (2014). Disrupted reproduction, estrous cycle, and circadian rhythms in female mice deficient in vasoactive intestinal peptide. *J Biol Rhythms* **29**, 355-69.

Lone, S. R., Potdar, S., Srivastava, M. and Sharma, V. K. (2016). Social Experience Is Sufficient to Modulate Sleep Need of Drosophila without Increasing Wakefulness. *PLoS One* **11**, e0150596.

Lone, S. R. and Sharma, V. K. (2012). Or47b receptor neurons mediate sociosexual interactions in the fruit fly Drosophila melanogaster. *J Biol Rhythms* **27**, 107-16.

Loomis A.L., H. E. N., Hobart G.A. (1937). Cerebral states during sleep as studied by human brain potentials. *J Exp Psychol*, 127-144.

Lu, B., Liu, W., Guo, F. and Guo, A. (2008). Circadian modulation of light-induced locomotion responses in Drosophila melanogaster. *Genes Brain Behav* **7**, 730-9.

Lu, J., Jhou, T. C. and Saper, C. B. (2006). Identification of wake-active dopaminergic neurons in the ventral periaqueductal gray matter. *J Neurosci* **26**, 193-202.

Luo, Y. J., Li, Y. D., Wang, L., Yang, S. R., Yuan, X. S., Wang, J., Cherasse, Y., Lazarus, M., Chen, J. F., Qu, W. M. et al. (2018). Nucleus accumbens controls wakefulness by a subpopulation of neurons expressing dopamine D1 receptors. *Nat Commun* **9**, 1576.

Lyamin, O. I., Manger, P. R., Ridgway, S. H., Mukhametov, L. M. and Siegel, J. M. (2008). Cetacean sleep: an unusual form of mammalian sleep. *Neurosci Biobehav Rev* **32**, 1451-84.

Machado, D. R., Afonso, D. J., Kenny, A. R., Oztu Rk-Colak, A., Moscato, E. H., Mainwaring, B., Kayser, M. and Koh, K. (2017). Identification of octopaminergic neurons that modulate sleep suppression by male sex drive. *Elife* **6**.

Mackiewicz, M., Shockley, K. R., Romer, M. A., Galante, R. J., Zimmerman, J. E., Naidoo, N., Baldwin, D. A., Jensen, S. T., Churchill, G. A. and Pack, A. I. (2007). Macromolecule biosynthesis: a key function of sleep. *Physiol Genomics* **31**, 441-57.

Mahoney, M. M. (2010). Shift work, jet lag, and female reproduction. *Int J Endocrinol* **2010**, 813764.

Mao, Z. and Davis, R. L. (2009). Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. *Front Neural Circuits* **3**, 5.

Martin, J. R., Raabe, T. and Heisenberg, M. (1999). Central complex substructures are required for the maintenance of locomotor activity in Drosophila melanogaster. *J Comp Physiol A* **185**, 277-88.

Mascetti, G. G. (2016). Unihemispheric sleep and asymmetrical sleep: behavioral, neurophysiological, and functional perspectives. *Nat Sci Sleep* **8**, 221-38.

Massaquoi, S. G. and McCarley, R. W. (1992). Extension of the Limit Cycle Reciprocal Interaction Model of REM cycle control. An integrated sleep control model. *J Sleep Res* **1**, 138-143.

Masuyama, K., Zhang, Y., Rao, Y. and Wang, J. W. (2012). Mapping neural circuits with activity-dependent nuclear import of a transcription factor. *J Neurogenet* **26**, 89-102.

McCarley, R. W. and Hobson, J. A. (1975). Neuronal excitability modulation over the sleep cycle: a structural and mathematical model. *Science* **189**, 58-60.

Menegazzi, P., Yoshii, T. and Helfrich-Förster, C. (2012). Laboratory versus nature: the two sides of the Drosophila circadian clock. *J Biol Rhythms* **27**, 433-42.

Mertens, I., Vandingenen, A., Johnson, E. C., Shafer, O. T., Li, W., Trigg, J. S., De Loof, A., Schoofs, L. and Taghert, P. H. (2005). PDF receptor signaling in Drosophila contributes to both circadian and geotactic behaviors. *Neuron* **48**, 213-9.

Mignot, E. (2008). Why we sleep: the temporal organization of recovery. *PLoS Biol* **6**, e106.

Mistlberger, R. E., Bergmann, B. M., Waldenar, W. and Rechtschaffen, A. (1983). Recovery sleep following sleep deprivation in intact and suprachiasmatic nuclei-lesioned rats. *Sleep* **6**, 217-33.

Mistlberger, R. E., Landry, G. J. and Marchant, E. G. (1997). Sleep deprivation can attenuate light-induced phase shifts of circadian rhythms in hamsters. *Neurosci Lett* **238**, 5-8.

Miyazaki, S., Liu, C. Y. and Hayashi, Y. (2017). Sleep in vertebrate and invertebrate animals, and insights into the function and evolution of sleep. *Neurosci Res* **118**, 3-12.

Moore-Ede, M. C. and Czeisler, C. (1984). Mathematical models of the circadian sleep-wake cycle. New York: Raven.

Moore, R. Y. and Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res* **42**, 201-6.

Moses, J. M., Johnson, L. C., Naitoh, P. and Lubin, A. (1975). Sleep stage deprivation and total sleep loss: effects on sleep behavior. *Psychophysiology* **12**, 141-6.

Naidoo, N., Casiano, V., Cater, J., Zimmerman, J. and Pack, A. I. (2007). A role for the molecular chaperone protein BiP/GRP78 in Drosophila sleep homeostasis. *Sleep* **30**, 557-65.

Nakazawa, Y., Kotorii, M., Ohshima, M., Kotorii, T. and Hasuzawa, H. (1978). Changes in sleep pattern after sleep deprivation. *Folia Psychiatr Neurol Jpn* **32**, 85-93.

Nassel, D. R. and Winther, A. M. (2010). Drosophila neuropeptides in regulation of physiology and behavior. *Prog Neurobiol* **92**, 42-104.

Neckameyer, W. S. (1996). Multiple roles for dopamine in Drosophila development. *Dev Biol* **176**, 209-19.

Nitabach, M. N., Blau, J. and Holmes, T. C. (2002). Electrical silencing of Drosophila pacemaker neurons stops the free-running circadian clock. *Cell* **109**, 485-95.

Nitabach, M. N., Wu, Y., Sheeba, V., Lemon, W. C., Strumbos, J., Zelensky, P. K., White, B. H. and Holmes, T. C. (2006). Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. *J Neurosci* **26**, 479-89.

Nitz, D. A., van Swinderen, B., Tononi, G. and Greenspan, R. J. (2002). Electrophysiological correlates of rest and activity in Drosophila melanogaster. *Curr Biol* **12**, 1934-40.

Oh, Y., Jang, D., Sonn, J. Y. and Choe, J. (2013). Histamine-HisCl1 receptor axis regulates wake-promoting signals in Drosophila melanogaster. *PLoS One* **8**, e68269.

Oh, Y., Yoon, S. E., Zhang, Q., Chae, H. S., Daubnerova, I., Shafer, O. T., Choe, J. and Kim, Y. J. (2014). A homeostatic sleep-stabilizing pathway in Drosophila composed of the sex peptide receptor and its ligand, the myoinhibitory peptide. *PLoS Biol* **12**, e1001974.

Pace-Schott, E. F. and Hobson, J. A. (2002). The neurobiology of sleep: genetics, cellular physiology and subcortical networks. *Nat Rev Neurosci* **3**, 591-605.

Pan, Y., Zhou, Y., Guo, C., Gong, H., Gong, Z. and Liu, L. (2009). Differential roles of the fan-shaped body and the ellipsoid body in Drosophila visual pattern memory. *Learn Mem* **16**, 289-95.

Parisky, K. M., Agosto, J., Pulver, S. R., Shang, Y., Kuklin, E., Hodge, J. J., Kang, K., Liu, X., Garrity, P. A., Rosbash, M. et al. (2008). PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. *Neuron* **60**, 672-82.

Parisky, K. M., Agosto Rivera, J. L., Donelson, N. C., Kotecha, S. and Griffith, L. C. (2016). Reorganization of Sleep by Temperature in Drosophila Requires Light, the Homeostat, and the Circadian Clock. *Curr Biol* **26**, 882-92.

Park, J. H., Helfrich-Förster, C., Lee, G., Liu, L., Rosbash, M. and Hall, J. C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. *Proc Natl Acad Sci U S A* **97**, 3608-13.

Park, S., Sonn, J. Y., Oh, Y., Lim, C. and Choe, J. (2014). SIFamide and SIFamide receptor defines a novel neuropeptide signaling to promote sleep in Drosophila. *Mol Cells* **37**, 295-301.

Parsons, M. P. and Hirasawa, M. (2010). ATP-sensitive potassium channel-mediated lactate effect on orexin neurons: implications for brain energetics during arousal. *J Neurosci* **30**, 8061-70.

Peng, Y., Stoleru, D., Levine, J. D., Hall, J. C. and Rosbash, M. (2003). Drosophila free-running rhythms require intercellular communication. *PLoS Biol* **1**, E13.

Pfeiffenberger, C. and Allada, R. (2012). Cul3 and the BTB adaptor insomniac are key regulators of sleep homeostasis and a dopamine arousal pathway in Drosophila. *PLoS Genet* **8**, e1003003.

Pieron, H. (1913). Le Probl~me Physiologique du Sommeil. Paris: Masson.

Pimentel, D., Donlea, J. M., Talbot, C. B., Song, S. M., Thurston, A. J. F. and Miesenbock, G. (2016). Operation of a homeostatic sleep switch. *Nature* **536**, 333-337.

Pitman, J. L., McGill, J. J., Keegan, K. P. and Allada, R. (2006). A dynamic role for the mushroom bodies in promoting sleep in Drosophila. *Nature* **441**, 753-6.

Porkka-Heiskanen, T., Alanko, L., Kalinchuk, A. and Stenberg, D. (2002). Adenosine and sleep. *Sleep Med Rev* **6**, 321-32.

Porkka-Heiskanen, T., Strecker, R. E., Thakkar, M., Bjorkum, A. A., Greene, R. W. and McCarley, R. W. (1997). Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. *Science* **276**, 1265-8.

Potdar, S. and Sheeba, V. (2012). Large Ventral Lateral Neurons Determine the Phase of Evening Activity Peak across Photoperiods in Drosophila melanogaster. *J Biol Rhythms* **27**, 267-79.

Prabhakaran, P. M. and Sheeba, V. (2012). Sympatric Drosophilid species melanogaster and ananassae differ in temporal patterns of activity. *Journal of Biological Rhythms*, In press.

Prabhakaran, P. M. and Sheeba, V. (2013). Insights into differential activity patterns of drosophilids under semi-natural conditions. *J Exp Biol* **216**, 4691-702.

Putilov, A. A. (1995). Timing of sleep modelling: Circadian modulation of the homeostatic process. *Biological Rhythm Research* **26**, 1-19.

Qian, Y., Cao, Y., Deng, B., Yang, G., Li, J., Xu, R., Zhang, D., Huang, J. and Rao, Y. (2017). Sleep homeostasis regulated by 5HT2b receptor in a small subset of neurons in the dorsal fan-shaped body of drosophila. *Elife* **6**.

Qiu, M. H., Yao, Q. L., Vetrivelan, R., Chen, M. C. and Lu, J. (2016). Nigrostriatal Dopamine Acting on Globus Pallidus Regulates Sleep. *Cereb Cortex* **26**, 1430-9.

Raizen, D. M., Zimmerman, J. E., Maycock, M. H., Ta, U. D., You, Y. J., Sundaram, M. V. and Pack, A. I. (2008). Lethargus is a Caenorhabditis elegans sleep-like state. *Nature* **451**, 569-72.

Rattenborg, N. C., Lima, S. L. and Amlaner, C. J. (1999). Facultative control of avian unihemispheric sleep under the risk of predation. *Behav Brain Res* **105**, 163-72.

Rattenborg, N. C., Voirin, B., Cruz, S. M., Tisdale, R., Dell'Omo, G., Lipp, H. P., Wikelski, M. and Vyssotski, A. L. (2016). Evidence that birds sleep in mid-flight. *Nat Commun* **7**, 12468.

Rau, P., Rau, N. (1916). The sleep of insects: An ecological study. *Annals of the Entomological Society of America*, 227-274.

Rauschenbach, I. Y., Chentsova, N. A., Alekseev, A. A., Gruntenko, N. E., Adonyeva, N. V., Karpova, E. K., Komarova, T. N., Vasiliev, V. G. and Bownes, M. (2007). Dopamine and octopamine regulate 20-hydroxyecdysone level in vivo in Drosophila. *Arch Insect Biochem Physiol* **65**, 95-102.

Rechtschaffen, A. (1971). The control of sleep. In *Human Behavior and its control* (ed. W. A. Hunt), pp. 75-92. Cambridge, MA: Schenkman.

Rechtschaffen, A. and Bergmann, B. M. (2002). Sleep deprivation in the rat: an update of the 1989 paper. *Sleep* **25**, 18-24.

Rechtschaffen, A., Gilliland, M. A., Bergmann, B. M. and Winter, J. B. (1983). Physiological correlates of prolonged sleep deprivation in rats. *Science* **221**, 182-4.

Rechtschaffen, A., Kales, A. (1968). A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects. Bethesda, MD: U. S. National Institute of Neurological Diseases and Blindness, Neurological Information Network.

Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C. and Taghert, P. H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. *Cell* **99**, 791-802.

Rieger, D., Shafer, O. T., Tomioka, K. and Helfrich-Förster, C. (2006). Functional analysis of circadian pacemaker neurons in Drosophila melanogaster. *J Neurosci* **26**, 2531-43.

Roeder, T. (1999). Octopamine in invertebrates. *Prog Neurobiol* **59**, 533-61.

Rogulja, D. and Young, M. W. (2012). Control of sleep by cyclin A and its regulator. *Science* **335**, 1617-21.

Ruckebusch, Y. (1972). The relevance of drowsiness in the circadian cycle of farm animals. *Anim Behav* **20**, 637-43.

Sanders, J., Scholz, M., Merutka, I. and Biron, D. (2017). Distinct unfolded protein responses mitigate or mediate effects of nonlethal deprivation of C. elegans sleep in different tissues. *BMC Biol* **15**, 67.

Saper, C. B. (2013). The central circadian timing system. *Curr Opin Neurobiol* **23**, 747-51.

Saper, C. B., Scammell, T. E. and Lu, J. (2005). Hypothalamic regulation of sleep and circadian rhythms. *Nature* **437**, 1257-63.

Sehgal, A. and Mignot, E. (2011). Genetics of sleep and sleep disorders. *Cell* **146**, 194-207.

Seidner, G., Robinson, J. E., Wu, M., Worden, K., Masek, P., Roberts, S. W., Keene, A. C. and Joiner, W. J. (2015). Identification of Neurons with a Privileged Role in Sleep Homeostasis in Drosophila melanogaster. *Curr Biol* **25**, 2928-38.

Seluzicki, A., Flourakis, M., Kula-Eversole, E., Zhang, L., Kilman, V. and Allada, R. (2014). Dual PDF signaling pathways reset clocks via TIMELESS and acutely excite target neurons to control circadian behavior. *PLoS Biol* **12**, e1001810.

Seugnet, L., Suzuki, Y., Thimgan, M., Donlea, J., Gimbel, S. I., Gottschalk, L., Duntley, S. P. and Shaw, P. J. (2009). Identifying sleep regulatory genes using a Drosophila model of insomnia. *J Neurosci* **29**, 7148-57.

Shafer, O. T., Kim, D. J., Dunbar-Yaffe, R., Nikolaev, V. O., Lohse, M. J. and Taghert, P. H. (2008). Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of Drosophila revealed by real-time cyclic AMP imaging. *Neuron* **58**, 223-37.

Shang, Y., Donelson, N. C., Vecsey, C. G., Guo, F., Rosbash, M. and Griffith, L. C. (2013). Short neuropeptide F is a sleep-promoting inhibitory modulator. *Neuron* **80**, 171-83.

Shang, Y., Griffith, L. C. and Rosbash, M. (2008). Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. *Proc Natl Acad Sci U S A* **105**, 19587-94.

Shang, Y., Haynes, P., Pirez, N., Harrington, K. I., Guo, F., Pollack, J., Hong, P., Griffith, L. C. and Rosbash, M. (2011). Imaging analysis of clock neurons reveals light buffers the wake-promoting effect of dopamine. *Nat Neurosci* **14**, 889-95.

Shaw, P. (2003). Awakening to the behavioral analysis of sleep in Drosophila. *J Biol Rhythms* **18**, 4-11.

Shaw, P. J., Cirelli, C., Greenspan, R. J. and Tononi, G. (2000). Correlates of sleep and waking in Drosophila melanogaster. *Science* **287**, 1834-7.

Shaw, P. J. and Franken, P. (2003). Perchance to dream: solving the mystery of sleep through genetic analysis. *J Neurobiol* **54**, 179-202.

Shaw, P. J., Tononi, G., Greenspan, R. J. and Robinson, D. F. (2002). Stress response genes protect against lethal effects of sleep deprivation in Drosophila. *Nature* **417**, 287-91.

Sheeba, V. (2008). The Drosophila melanogaster circadian pacemaker circuit. *J Genet* **87**, 485-93.

Sheeba, V., Fogle, K. J. and Holmes, T. C. (2010). Persistence of morning anticipation behavior and high amplitude morning startle response following functional loss of small ventral lateral neurons in Drosophila. *PLoS One* **5**, e11628.

Sheeba, V., Fogle, K. J., Kaneko, M., Rashid, S., Chou, Y. T., Sharma, V. K. and Holmes, T. C. (2008a). Large ventral lateral neurons modulate arousal and sleep in Drosophila. *Curr Biol* **18**, 1537-45.

Sheeba, V., Gu, H., Sharma, V. K., O'Dowd, D. K. and Holmes, T. C. (2008b). Circadian- and light-dependent regulation of resting membrane potential and spontaneous action potential firing of Drosophila circadian pacemaker neurons. *J Neurophysiol* **99**, 976-88.

Sheeba, V., Sharma, V. K., Shubha, K., Chandrashekaran, M. K. and Joshi, A. (2000). The effect of different light regimes on adult life span in Drosophila melanogaster is partly mediated through reproductive output. *J Biol Rhythms* **15**, 380-92.

Shi, M., Yue, Z., Kuryatov, A., Lindstrom, J. M. and Sehgal, A. (2014). Identification of Redeye, a new sleep-regulating protein whose expression is modulated by sleep amount. *Elife* **3**, e01473.

Siegel, J. M. (2005). Clues to the functions of mammalian sleep. *Nature* **437**, 1264-71.

Sitaraman, D., Aso, Y., Jin, X., Chen, N., Felix, M., Rubin, G. M. and Nitabach, M. N. (2015a). Propagation of Homeostatic Sleep Signals by Segregated Synaptic Microcircuits of the Drosophila Mushroom Body. *Curr Biol* **25**, 2915-27.

Sitaraman, D., Aso, Y., Rubin, G. M. and Nitabach, M. N. (2015b). Control of Sleep by Dopaminergic Inputs to the Drosophila Mushroom Body. *Front Neural Circuits* **9**, 73.

Sokolowski, M. B. (2001). Drosophila: genetics meets behaviour. *Nat Rev Genet* **2**, 879-90.

Soller, M., Bownes, M. and Kubli, E. (1999). Control of oocyte maturation in sexually mature Drosophila females. *Dev Biol* **208**, 337-51.

St.Johnston, D. (2002). The art and design of genetic screens: : Drosophila melanogaster. *Nature Reviews: Genetics* **3**, 176-188.

Stavropoulos, N. and Young, M. W. (2011). insomniac and Cullin-3 regulate sleep and wakefulness in Drosophila. *Neuron* **72**, 964-76.

Stephan, F. K. and Zucker, I. (1972). Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci U S A* **69**, 1583-6.

Steriade, M., Kryger, M. H., Roth, C. and Dement, W. C. (2005). Brain electrical activity and sensory processing during waking and sleep. In *Principles and practice of sleep medicine*, vol. 4 (ed. M. H. Kryger C. Roth and W. C. Dement), pp. 101-119. Philadelphia: Elsevier Saunders.

Stoleru, D., Peng, Y., Agosto, J. and Rosbash, M. (2004). Coupled oscillators control morning and evening locomotor behaviour of Drosophila. *Nature* **431**, 862-8.

Summa, K. C., Vitaterna, M. H. and Turek, F. W. (2012). Environmental perturbation of the circadian clock disrupts pregnancy in the mouse. *PLoS One* **7**, e37668.

Takahama, K., Tomita, J., Ueno, T., Yamazaki, M., Kume, S. and Kume, K. (2012). Pan-neuronal knockdown of the c-Jun N-terminal Kinase (JNK) results in a reduction in sleep and longevity in Drosophila. *Biochem Biophys Res Commun* **417**, 807-11.

Thimgan, M. S., Suzuki, Y., Seugnet, L., Gottschalk, L. and Shaw, P. J. (2010). The perilipin homologue, lipid storage droplet 2, regulates sleep homeostasis and prevents learning impairments following sleep loss. *PLoS Biol* **8**.

Tobler, I., Borbély, A. A. and Groos, G. (1983). The effect of sleep deprivation on sleep in rats with suprachiasmatic lesions. *Neurosci Lett* **42**, 49-54.

Tomita, J., Ban, G. and Kume, K. (2017). Genes and neural circuits for sleep of the fruit fly. *Neurosci Res* **118**, 82-91.

Tononi, G. and Cirelli, C. (2003). Sleep and synaptic homeostasis: a hypothesis. *Brain Res Bull* **62**, 143-50.

Tononi, G. and Cirelli, C. (2012). Time to be SHY? Some comments on sleep and synaptic homeostasis. *Neural Plast* **2012**, 415250.

Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S. and Kume, K. (2012). Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. *Nat Neurosci* **15**, 1516-23.

van Alphen, B., Yap, M. H., Kirszenblat, L., Kottler, B. and van Swinderen, B. (2013). A dynamic deep sleep stage in Drosophila. *J Neurosci* **33**, 6917-27.

van Diepen, H. C., Lucassen, E. A., Yasenkov, R., Groenen, I., Ijzerman, A. P., Meijer, J. H. and Deboer, T. (2014). Caffeine increases light responsiveness of the mouse circadian pacemaker. *Eur J Neurosci* **40**, 3504-11.

van Swinderen, B. and Andretic, R. (2003). Arousal in Drosophila. *Behav Processes* **64**, 133-144.

van Swinderen, B., Nitz, D. A. and Greenspan, R. J. (2004). Uncoupling of brain activity from movement defines arousal States in Drosophila. *Curr Biol* **14**, 81-7.

Vanin, S., Bhutani, S., Montelli, S., Menegazzi, P., Green, E. W., Pegoraro, M., Sandrelli, F., Costa, R. and Kyriacou, C. P. (2012). Unexpected features of Drosophila circadian behavioural rhythms under natural conditions. *Nature* **484**, 371-5.

Venken, K. J., Simpson, J. H. and Bellen, H. J. (2011). Genetic manipulation of genes and cells in the nervous system of the fruit fly. *Neuron* **72**, 202-30.

Webb, W. B. and Agnew, H. W., Jr. (1971). Stage 4 sleep: influence of time course variables. *Science* **174**, 1354-6.

Wever, R. (1975). The circadian multi-oscillatory system of man. *Int J Chronobiol* **3**, 19-55.

Wever, R. (1979). The circadian system of man. Berlin: Springer-Verlag.

Williams, J. A., Sathyanarayanan, S., Hendricks, J. C. and Sehgal, A. (2007). Interaction between sleep and the immune response in Drosophila: a role for the NFkappaB relish. *Sleep* **30**, 389-400.

Wisor, J. P., O'Hara, B. F., Terao, A., Selby, C. P., Kilduff, T. S., Sancar, A., Edgar, D. M. and Franken, P. (2002). A role for cryptochromes in sleep regulation. *BMC Neurosci* **3**, 20.

Wisor, J. P., Pasumarthi, R. K., Gerashchenko, D., Thompson, C. L., Pathak, S., Sancar, A., Franken, P., Lein, E. S. and Kilduff, T. S. (2008). Sleep deprivation effects on circadian clock gene expression in the cerebral cortex parallel electroencephalographic differences among mouse strains. *J Neurosci* **28**, 7193-201.

Wu, M. N., Ho, K., Crocker, A., Yue, Z., Koh, K. and Sehgal, A. (2009). The effects of caffeine on sleep in Drosophila require PKA activity, but not the adenosine receptor. *J Neurosci* **29**, 11029-37.

Wu, M. N., Joiner, W. J., Dean, T., Yue, Z., Smith, C. J., Chen, D., Hoshi, T., Sehgal, A. and Koh, K. (2010). SLEEPLESS, a Ly-6/neurotoxin family member, regulates the levels, localization and activity of Shaker. *Nat Neurosci* **13**, 69-75.

Wu, Y., Bolduc, F. V., Bell, K., Tully, T., Fang, Y., Sehgal, A. and Fischer, J. A. (2008a). A Drosophila model for Angelman syndrome. *Proc Natl Acad Sci U S A* **105**, 12399-404.

Wu, Y., Cao, G. and Nitabach, M. N. (2008b). Electrical silencing of PDF neurons advances the phase of non-PDF clock neurons in Drosophila. *J Biol Rhythms* **23**, 117-28.

Wülbeck, C., Grieshaber, E. and Helfrich-Förster, C. (2008). Pigment-dispersing factor (PDF) has different effects on Drosophila's circadian clocks in the accessory medulla and in the dorsal brain. *J Biol Rhythms* **23**, 409-24.

Yamanaka, A., Beuckmann, C. T., Willie, J. T., Hara, J., Tsujino, N., Mieda, M., Tominaga, M., Yagami, K., Sugiyama, F., Goto, K. et al. (2003). Hypothalamic orexin neurons regulate arousal according to energy balance in mice. *Neuron* **38**, 701-13.

Yamazaki, M., Tomita, J., Takahama, K., Ueno, T., Mitsuyoshi, M., Sakamoto, E., Kume, S. and Kume, K. (2012). High calorie diet augments age-associated sleep impairment in Drosophila. *Biochem Biophys Res Commun* **417**, 812-6.

Yao, Z., Bennett, A. J., Clem, J. L. and Shafer, O. T. (2016). The Drosophila Clock Neuron Network Features Diverse Coupling Modes and Requires Network-wide Coherence for Robust Circadian Rhythms. *Cell Rep* **17**, 2873-2881.

Yao, Z. and Shafer, O. T. (2014). The Drosophila circadian clock is a variably coupled network of multiple peptidergic units. *Science* **343**, 1516-20.

Yap, M. H. W., Grabowska, M. J., Rohrscheib, C., Jeans, R., Troup, M., Paulk, A. C., van Alphen, B., Shaw, P. J. and van Swinderen, B. (2017). Oscillatory brain activity in spontaneous and induced sleep stages in flies. *Nat Commun* **8**, 1815.

Yi, W., Zhang, Y., Tian, Y., Guo, J., Li, Y. and Guo, A. (2013). A subset of cholinergic mushroom body neurons requires Go signaling to regulate sleep in Drosophila. *Sleep* **36**, 1809-21.

Yoshii, T., Wulbeck, C., Sehadova, H., Veleri, S., Bichler, D., Stanewsky, R. and Helfrich-Förster, C. (2009). The neuropeptide pigment-dispersing factor adjusts period and phase of Drosophila's clock. *J Neurosci* **29**, 2597-610.

Yuan, Q., Joiner, W. J. and Sehgal, A. (2006). A sleep-promoting role for the Drosophila serotonin receptor 1A. *Curr Biol* **16**, 1051-62.

Zhang, L., Chung, B. Y., Lear, B. C., Kilman, V. L., Liu, Y., Mahesh, G., Meissner, R. A., Hardin, P. E. and Allada, R. (2010). DN1(p) circadian neurons coordinate acute light and PDF inputs to produce robust daily behavior in Drosophila. *Curr Biol* **20**, 591-9.

Zheng, X. and Sehgal, A. (2008). Probing the relative importance of molecular oscillations in the circadian clock. *Genetics* **178**, 1147-55.

Zimmerman, J. E., Chan, M. T., Jackson, N., Maislin, G. and Pack, A. I. (2012). Genetic background has a major impact on differences in sleep resulting from environmental influences in Drosophila. *Sleep* **35**, 545-57.

Zimmerman, J. E., Mackiewicz, M., Galante, R. J., Zhang, L., Cater, J., Zoh, C., Rizzo, W. and Pack, A. I. (2004). Glycogen in the brain of Drosophila melanogaster: diurnal rhythm and the effect of rest deprivation. *Journal of Neurochemistry* **88**, 32-40.

Zimmerman, J. E., Naidoo, N., Raizen, D. M. and Pack, A. I. (2008a). Conservation of sleep: insights from non-mammalian model systems. *Trends Neurosci* **31**, 371-6.

Zimmerman, J. E., Raizen, D. M., Maycock, M. H., Maislin, G. and Pack, A. I. (2008b). A video method to study Drosophila sleep. *Sleep* **31**, 1587-98.

Appendices

Appendix 1

Fly strains	Source
w^{1118}	BDSC # 5905
clk^{JRK}	BDSC # 24515
cyc^{01}	Amita Sehgal
$per^{0}w$	Todd Holmes
tim^{01}	Amita Sehgal
CCM	Laboratory population
per^{s} (backcrossed to *CCM*)	Vijay Kumar Sharma
per^{l} (backcrossed to *CCM*)	Vijay Kumar Sharma
$Pdfr^{5304}$	BDSC # 33068
$Pdfr^{3369}$	BDSC # 33069
Pdfr Myc	Paul Taghert
fmn	Kazuhiko Kume
2202CS (*fmn-bg*)	Kazuhiko Kume
Pdfr (B) GAL4	Paul Taghert
Cry-39 GAL4	Todd Holmes
Dvpdf GAL4	Michael Rosbash
Pdf GAL4	Todd Holmes
Clk 9M GAL4	Fumika Hamada
Clk 4.1M GAL4	BDSC # 36316
Clk 4.5F GAL4	BDSC # 37526
OK107 GAL4	NCBS
201y GAL4	BDSC # 4440, NCBS
c309 GAL4	BDSC # 6906, NCBS
c747 GAL4	BDSC # 6494, NCBS
30y GAL4	BDSC # 30818
Dilp2 GAL4	Amita Sehgal
Kurs45 GAL4	Gunter Korge
Kurs58 GAL4	Gunter Korge
Mai281 GAL4	Gunter Korge
Mai301 GAL4	Gunter Korge
121y GAL4	BDSC # 30815
104y GAL4	NCBS
c5 GAL4	BDSC # 30839
c119 GAL4	BDSC # 30824
c232 GAL4	BDSC # 30828
Ddc GAL4	BDSC # 7009
TH GAL4	BDSC # 8848
Tdc2 GAL4	BDSC # 9313, NCBS
Npf GAL4	Charlotte Helfrich-Förster
TH-A GAL4	Gaiti Hasan and Mark Wu

Fly strains	**Source**
TH-C' GAL4	Gaiti Hasan and Mark Wu
TH-C1 GAL4	Gaiti Hasan and Mark Wu
TH-D' GAL4	Gaiti Hasan and Mark Wu
TH-D1 GAL4	Gaiti Hasan and Mark Wu
TH-D4 GAL4	Gaiti Hasan and Mark Wu
TH-F2 GAL4	Gaiti Hasan and Mark Wu
TH-F3 GAL4	Gaiti Hasan and Mark Wu
TH-G1 GAL4	Gaiti Hasan and Mark Wu
Pdf LexA	Michael Rosbash
LexAop spGFP11/Cyo;UAS spGFP1-10/TM6B	Amita Sehgal
LexAOp CD8 GFP-2A-CD8GFP;UAS mLexA VP16 NFAT,cdc1(H-2,LexAOpCD2GFP/TM6,Tb	BDSC # 66542, NCBS
UAS dTRPA1	BDSC # 26263, NCBS
UAS clk DN	BDSC # 36319
UAS cyc DN	BDSC # 36317
UAS GFP AH2	BDSC # 6874
UAS Pdfr RNAi	VDRC, KK/110677
UAS dicer	BDSC # 24651
UAS Pdfr	Paul Taghert
UAS NachBac	Todd Holmes
UAS dORKNC1	Todd Holmes
UAS Htt Q0A	Todd Holmes
UAS Htt Q128c	Todd Holmes
UAS reaper	Paul Taghert
UAS PKAR	Daniel Kalderon
UAS PKACA	Daniel Kalderon

Appendix 1. Fly strains used and their sources are tabulated. BDSC – Bloomington *Drosophila* Stock Centre, Bloomington, IN, USA; NCBS – National Centre for Biological Sciences, Bangalore, India; VDRC – Vienna *Drosophila* Resource Centre, Vienna, Austria. *TH*-subset *GAL4*s that were generated in Mark Wu's (Johns Hopkins University, Baltimore, MD, USA) lab were obtained from Gaiti Hasan (NCBS, Bangalore, India). Other sources – Amita Sehgal (University of Pennsylvania, Philadelphia, PA, USA), Todd Holmes (University of California, Irvine, CA, USA), Vijay Kumar Sharma (JNCASR, Bangalore, India), Paul Taghert (Washington University, St. Louis, MO, USA), Kazuhiko Kume (Nagoya city University, Nagoya, Japan), Michael Rosbash (Brandeis University, Waltham, MA, USA), Fumika Hamada (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA), Gunter Korge (Freie Universität Berlin, Berlin, Germany), Charlotte Helfrich-Förster (Universität Würzburg, Würzburg , Germany), Daniel Kalderon (Columbia University, New York, NY, USA).

Appendix 2.1

Appendix 2.1A

Time-point	F-statistic	*p*
CT 5	$F_{2,186}$ = 2.63	0.08
CT 9	$F_{2,186}$ = 3.7	< 0.05
CT 13	$F_{2,177}$ = 2.3	0.1
CT 17	$F_{2,186}$ = 3.85	< 0.05
CT 21	$F_{2,186}$ = 26.96	< 0.00001
CT 25	$F_{2,183}$ = 4.15	< 0.05

Appendix 2.1A. Two-way ANOVA with time (before, during and after light pulse) and treatment as fixed factors conducted for light pulse given at each of the indicated time-points. $F_{(a-1)(b-1),\ (N-k)}$, where a and b are number of factor levels of the two factors, N is the total number of replicates and k refers to total number of groups. F-statistic and *p*-level of the time * treatment interaction are indicated. For CT 5, *post-hoc* Tukey's test was conducted as interaction was marginally significant. This table refers to Figure 2.5B.

Appendix 2.1B

Genotype	F-statistic	*p*
w^{1118}	$F_{5,186} = 8.88$	< 0.00001
cyc^{01}	$F_{5,156} = 0.38$	0.86
clk^{JRK}	$F_{5,90} = 7.34$	< 0.00001
tim^{01}	$F_{5,144} = 6$	< 0.0005
$per^{0}w$	$F_{5,138} = 18.41$	< 0.00001

Appendix 2.1B. One-way ANOVA with time window as fixed factor conducted for number of brief awakenings per 4 h interval for indicated genotypes. $F_{(a-1),\ (N-k)}$, where a is number of factor levels, N is the total number of replicates and k refers to total number of groups. F-statistic and *p*-level of the main effect of time window are indicated. This table refers to Figure 2.8B.

Appendix 2.1C

Genotype	F-statistic	*p*
w^{1118}	$F_{5,180} = 2.23$	0.05
cyc^{01}	$F_{5,173} = 0.95$	0.45
clk^{JRK}	$F_{5,163} = 0.6$	0.7
tim^{01}	$F_{5,176} = 3.05$	< 0.0.5
$per^{0}w$	$F_{5,153} = 1.35$	0.24

Appendix 2.1C. Two-way ANOVA with time window and treatment as fixed factors conducted for % sleep gained due to sleep deprivation during different time windows. $F_{(a-1)(b-1),\ (N-k)}$, where a and b are number of factor levels of the two factors, N is the total number of replicates and k refers to total number of groups. F-statistic and *p*-level of the time window * treatment interaction are indicated. For w^{1118}, *post-hoc* Tukey's test was conducted as interaction was marginally significant. This table refers to Figure 2.8C.

Appendix 2.2

Appendix 2.2A

Genotype	F-statistic	*p*
UAS clk DN/+	$F_{5,138}$ = 6.19	< 0.0005
UAS cyc DN/+	$F_{5,126}$ = 4.44	< 0.005
Dvpdf GAL4/+	$F_{5,186}$ = 4.58	< 0.005
Dvpdf GAL4 > UAS clk DN	$F_{5,132}$ = 8.32	< 0.00001
Dvpdf GAL4 > UAS clk DN	$F_{5,186}$ = 11.77	< 0.00001

Appendix 2.2A. One-way ANOVA with time window as fixed factor conducted for number of brief awakenings per 4 h interval for indicated genotypes. $F_{(a-1),\ (N-k)}$, where a is number of factor levels, N is the total number of replicates and k refers to total number of groups. F-statistic and *p*-level of the main effect of time window are indicated. This table refers to Figure 3.2B.

Appendix 2.2B

Genotype	F-statistic	*p*
UAS clk DN/+	$F_{1,85}$ = 6.51	< 0.05
UAS cyc DN/+	$F_{1,83}$ = 3.09	0.08
Dvpdf GAL4/+	$F_{1,88}$ = 3.39	0.07
Dvpdf GAL4 > UAS clk DN	$F_{1,89}$ = 11.21	< 0.005
Dvpdf GAL4 > UAS clk DN	$F_{1,88}$ = 7.5	< 0.05

Appendix 2.2B. Two-way ANOVA with time window and treatment as fixed factors conducted for % sleep gained due to sleep deprivation during different time windows. $F_{(a-1)(b-1),\ (N-k)}$, where a and b are number of factor levels of the two factors, N is the total number of replicates and k refers to total number of groups. F-statistic and *p*-level of the time window * treatment interaction are indicated. For *UAS cyc DN/+ and Dvpdf GAL4/+*, *post-hoc* Tukey's tests were conducted as interactions were marginally significant. This table refers to Figure 3.2C.

Appendix 2.2C

	Down-regulation of *pdfr*	
Driver	**F-statistic**	***p***
Pdfr (B) GAL4	$F_{2,80}$ = 3.66	< 0.05
Cry-39 GAL4	$F_{2,86}$ = 5.89	< 0.005
Dvpdf GAL4	$F_{2,82}$ = 3.59	< 0.05
Pdf GAL4	$F_{2,91}$ = 6.18	< 0 005
Clk 9M GAL4	$F_{2,66}$ = 0.89	0.41
Clk 4.1M GAL4	$F_{2,77}$ = 8.87	< 0.0005
Clk 4.5M GAL4	$F_{2,67}$ = 5.52	< 0.05
Dilp2 GAL4	$F_{2,54}$ = 2.47	0.09
Kurs 45 GAL4	$F_{2,73}$ = 0.43	=0.65
Kurs 58 GAL4	$F_{2,79}$ = 2.9	= 0.06
Mai 281 GAL4	$F_{2,85}$ = 5.03	< 0.05
Mai 301 GAL4	$F_{2,82}$ = 12.38	< 0.0005
OK 107 GAL4	$F_{2,72}$ = 0.98	= 0.38
201y GAL4	$F_{2,88}$ = 2.02	= 0.14
c309 GAL4	$F_{2,87}$ = 8.89	< 0.005
c747 GAL4	$F_{2,75}$ = 4.43	< 0.05
30y GAL4	$F_{2,87}$ = 21.39	< 0.00001
121y GAL4	$F_{2,80}$ = 13.24	< 0.0005
104y GAL4	$F_{2,69}$ = 3.87	< 0.05
c5 GAL4	$F_{2,69}$ = 6.37	<0.005
c119 GAL4	$F_{2,85}$ = 12.29	< 0.0005
c232 GAL4	$F_{2,89}$ = 6.69	< 0.005
Ddc GAL4	$F_{2,90}$ = 4.87	< 0.05
TH GAL4	$F_{2,89}$ = 6.53	< 0.005
Tdc2 GAL4	$F_{2,64}$ = 4.3	< 0.05
Npf GAL4	$F_{2,91}$ 2.8	= 0.07

Appendix 2.2C. One-way ANOVA with genotype as fixed factor conducted for day-time sleep of flies with down-regulation of *pdfr* in indicated drivers. $F_{(a-1),\ (N-k)}$, where a is number of factor levels, N is the total number of replicates and k refers to total number of groups. F-statistic and *p*-level of the main effect of genotype are indicated. Specific differences between genotypes determined after *post-hoc* Tukey's tests and indicated as asterisks in Figure 3.5A.

Appendix 2.2D

	Over-expression of *pdfr*	
Driver	**F-statistic**	***p***
Pdfr (B) GAL4	$F_{2,88}$ = 6.6	< 0.005
Cry-39 GAL4	$F_{2,86}$ = 5.2	< 0.05
Dvpdf GAL4	$F_{2,89}$ = 9.82	< 0.0005
Pdf GAL4	$F_{2,82}$ = 2.53	0.09
Clk 9M GAL4	$F_{2,81}$ = 3.64	< 0.05
Clk 4.1M GAL4	$F_{2,78}$ = 1.6	0.2
Clk 4.5M GAL4	$F_{2,85}$ = 1.6	0.2
Dilp2 GAL4	$F_{2,63}$ = 0.69	0.5
Kurs 45 GAL4	$F_{2,93}$ = 34.93	< 0.00001
Kurs 58 GAL4	$F_{2,93}$ = 6.37	< 0.005
Mai 281 GAL4	$F_{2,91}$ = 13.46	< 0.00001
Mai 301 GAL4	$F_{2,89}$ = 11.44	< 0.0005
OK 107 GAL4	$F_{2,80}$ = 0.49	0.61
201y GAL4	$F_{2,88}$ = 1.55	0.22
c309 GAL4	$F_{2,89}$ = 1.92	0.15
c747 GAL4	$F_{2,81}$ = 1.06	0.35
30y GAL4	$F_{2,89}$ = 4.01	< 0.05
121y GAL4	$F_{2,86}$ = 98.63	< 0.00001
104y GAL4	$F_{2,89}$ = 27.48	< 0.00001
c5 GAL4	$F_{2,71}$ = 1.29	0.28
c119 GAL4	$F_{2,88}$ = 2.58	0.08
c232 GAL4	$F_{2,82}$ = 3.52	< 0.04
Ddc GAL4	$F_{2,84}$ = 8.59	< 0.0005
TH GAL4	$F_{2,83}$ = 13.33	< 0.0005
Tdc2 GAL4	$F_{2,92}$ = 16.2	< 0.00001
Npf GAL4	$F_{2,83}$ = 0.6	0.55

Appendix 2.2D. One-way ANOVA with genotype as fixed factor conducted for day-time sleep of flies with over-expression of *pdfr* in indicated drivers. $F_{(a-1),\ (N-k)}$, where a is number of factor levels, N is the total number of replicates and k refers to total number of groups. F-statistic and *p*-level of the main effect of genotype are indicated. Specific differences between genotypes determined after *post-hoc* Tukey's tests and indicated as asterisks in Figure 3.5B.

Appendix 2.2E

	Down-regulation of *pdfr*	
Driver	**F-statistic**	***p***
TH-A GAL4	$F_{2,87} = 3.82$	< 0.05
TH-C1 GAL4	$F_{2,89} = 0.13$	0.87
TH-C' GAL4	$F_{2,92} = 2.73$	0.07
TH-D1 GAL4	$F_{2,89} = 6.53$	< 0.005
TH-D' GAL4	$F_{2,91} = 26.8$	< 0.00001
TH-D4 GAL4	$F_{2,76} = 4.75$	< 0.05
TH-F2 GAL4	$F_{2,91} = 0.57$	0.57
TH-F3 GAL4	$F_{2,72} = 14.08$	< 0.00001
TH-G1 GAL4	$F_{2,88} = 11.66$	< 0.0005

Appendix 2.2E. One-way ANOVA with genotype as fixed factor conducted for day-time sleep of flies with down-regulation of *pdfr* in indicated drivers. $F_{(a-1),\,(N-k)}$, where a is number of factor levels, N is the total number of replicates and k refers to total number of groups. F-statistic and *p*-level of the main effect of genotype are indicated. Specific differences between genotypes determined after *post-hoc* Tukey's tests and indicated as asterisks in Figure 3.8A.

Appendix 2.2F

	Over-expression of *pdfr*	
Driver	**F-statistic**	***p***
TH-A GAL4	$F_{2,93} = 2.9$	0.06
TH-C1 GAL4	$F_{2,93} = 0.21$	0.8
TH-C' GAL4	$F_{2,93} = 0.46$	0.63
TH-D1 GAL4	$F_{2,91} = 12.21$	< 0.0005
TH-D' GAL4	$F_{2,93} = 18.76$	< 0.00001
TH-D4 GAL4	$F_{2,93} = 9.99$	< 0.0005
TH-F2 GAL4	$F_{2,91} = 2.37$	0.1
TH-F3 GAL4	$F_{2,76} = 13.36$	< 0.0005
TH-G1 GAL4	$F_{2,90} = 4.06$	< 0.05

Appendix 2.2F. One-way ANOVA with genotype as fixed factor conducted for day-time sleep of flies with over-expression of *pdfr* in indicated drivers. $F_{(a-1),\,(N-k)}$, where a is number of factor levels, N is the total number of replicates and k refers to total number of groups. F-statistic and *p*-level of the main effect of genotype are indicated. Specific differences between genotypes determined after *post-hoc* Tukey's tests and indicated as asterisks in Figure 3.8B.

Appendix 2.3

Appendix 2.3A

	Day		Night	
Days	**H-statistic**	***p***	**H-statistic**	***p***
1	$H_{2,74} = 0.9$	0.64	$H_{2,74} = 9.52$	< 0.05
2	$H_{2,74} = 20.19$	< 0.00001	$H_{2,74} = 9.02$	< 0.05
3	$H_{2,74} = 24.48$	< 0.00001	$H_{2,74} = 30.69$	< 0.00001
4	$H_{2,74} = 19.53$	< 0.0005	$H_{2,74} = 33.01$	< 0.00001
5	$H_{2,74} = 19.63$	< 0.0005	$H_{2,74} = 40.58$	< 0.00001
6	$H_{2,74} = 31.74$	< 0.00001	$H_{2,74} = 45.74$	< 0.00001

Appendix 2.3A. Kruskal-Wallis tests conducted for day-time and night-time egg output of w^{1118} flies fed with 0.5 mg/ml caffeine on indicated days. $H_{(a-1),\ df}$, where a is number of groups, df is the degrees of freedom. H-statistic and *p*-level are indicated. This table refers to Figure 4.1C.

Appendix 2.3B

	Day		Night	
Days	**H-statistic**	***p***	**H-statistic**	***p***
1	$H_{2,89} = 3.5$	0.17	$H_{2,89} = 6.47$	< 0.05
2	$H_{2,89} = 27.13$	< 0.00001	$H_{2,89} = 15.72$	< 0.005
3	$H_{2,89} = 27.78$	< 0.00001	$H_{2,89} = 5.88$	0.05
4	$H_{2,89} = 32.03$	< 0.00001	$H_{2,89} = 13.6$	< 0.005
5	$H_{2,89} = 17.37$	< 0.0005	$H_{2,89} = 0.66$	0.72

Appendix 2.3B. Kruskal-Wallis tests conducted for day-time and night-time egg output of w^{1118} flies mechanically sleep deprived on indicated days. $H_{(a-1),\ df}$, where a is number of groups, df is the degrees of freedom. H-statistic and *p*-level are indicated. This table refers to Figure 4.3C.

Appendix 2.3C

	Day		Night	
Days	**H-statistic**	***p***	**H-statistic**	***p***
1	$H_{2,81}$ = 15.72	< 0.0005	$H_{2,81}$ = 36.53	< 0.00001
2	$H_{2,81}$ = 22.27	< 0.00001	$H_{2,81}$ = 21.41	< 0.00001
3	$H_{2,81}$ = 17.83	< 0.0005	$H_{2,81}$ = 31.61	< 0.00001
4	$H_{2,81}$ = 3.03	= 0.22	$H_{2,81}$ = 29.34	< 0.00001
5	$H_{2,81}$ = 12.91	< 0.05	$H_{2,81}$ = 38.74	< 0.00001

Appendix 2.3C. Kruskal-Wallis tests conducted for day-time and night-time egg output of *CCM* flies mechanically sleep deprived on indicated days. $H_{(a-1),\ df}$, where a is number of groups, df is the degrees of freedom. H-statistic and *p*-level are indicated. This table refers to Figure 4.4F.

Appendix 2.3D

Days	**H statistic**	***p***
1	$H_{2,51}$ = 12.18	< 0.005
2	$H_{2,51}$ = 0.04	= 0.98
3	$H_{2,51}$ = 5.3	= 0.07
4	$H_{2,51}$ = 17.01	< 0.0005
5	$H_{2,51}$ = 11.04	< 0.005
6	$H_{2,51}$ = 7.79	< 0.05

Appendix 2.3D. Kruskal-Wallis tests conducted for total egg output of flies with *dTRPA1*-mediated heat-activation of dopamine neurons using *TH GAL4* on indicated days. $H_{(a-1),\ df}$, where a is number of groups, df is the degrees of freedom. H-statistic and *p*-level are indicated. This table refers to Figure 4.7C'.

Appendix 2.3E

Days	**H statistic**	***p***
1	$H_{2,55}$ = 3.17	= 0.2
2	$H_{2,55}$ = 3.55	= 0.17
3	$H_{2,55}$ = 10.85	< 0.005
4	$H_{2,55}$ = 2.2	= 0.33
5	$H_{2,55}$ = 4.57	= 0.1
6	$H_{2,55}$ = 3.18	= 0.2

Appendix 2.3E. Kruskal-Wallis tests conducted for total egg output of flies with *dTRPA1*-mediated heat-activation of dopamine neurons using *TH-F2 GAL4* on indicated days. $H_{(a-1),\ df}$, where a is number of groups, df is the degrees of freedom. H-statistic and *p*-level are indicated. This table refers to Figure 4.8C'.