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Ageing and the *Drosophila* circadian clock

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A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy in the Faculty of Life Sciences.

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ABSTRACT

It is well established that elderly individuals have increased difficulty sleeping at night combined with falling asleep and waking up earlier. Although these age-related declines in circadian output are clearly observable in activity recordings of laboratory animals, the underlying changes in molecular and neuronal activity remain unknown. The fruit fly, *Drosophila melanogaster*, has long been used as a model for studying the circadian system and for ageing research. In this thesis *Drosophila* was used as a model to study the effect of ageing on circadian and sleep behaviour.

Circadian behaviour was measured using the *Drosophila* Activity Monitoring system, recording activity of flies at various stages of the ageing process, demonstrating a linear decline in rhythm strength with age combined with an increase in period length. Weakened circadian output is combined with significant alterations of diurnal behaviour of *Drosophila*, namely a reduction in morning and evening anticipatory behaviour. Ageing also has a significant impact on sleep behaviour, significantly increasing sleep duration whilst reducing latency, with larger effects observed on day-time sleep.

Age-related changes in neuronal activity were investigated using whole-cell patch clamp electrophysiology to record from large lateral ventral (l-LN_V) clock neurons, finding that ageing was associated with a significant decrease in input resistance, but no significant changes in spontaneous electrical activity or membrane potential. Manipulating the electrical properties of the circadian system by knocking down expression of candidate ion channels in all clock neurons had significant effects on behaviour, linking electrical activity with clock outputs.

The results presented in this thesis demonstrate the suitability of *Drosophila* as a model to interrogate how ageing effects the circadian clock, identifying Alterations in the electrical properties of the l-LN_V neurons may underlie observed changes in diurnal activity and sleep, while decreased remodelling of the s-LN_V neurons can explain weakened circadian behaviour.

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Finally, thank you to all my friends and family for their continuing love and support.

AUTHOR’S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED:.. DATE:.............................

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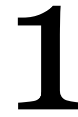
LIST OF ABBREVIATIONS

ANOVA – Analysis of Variance
CLK - Clock
CRY – Cryptochrome
CYC - Cycle
CT – Circadian Time
DD – Constant Darkness
DAM – *Drosophila* Activity Monitor
DN – Dorsal Neuron
GFP – Green Fluorescent Protein
Hr – hour
IQR – Interquartile Range
LD – Light-Dark cycle
$l\text{-}LN_V$ – Large Ventral Lateral Neuron
$s\text{-}LN_V$ – Small Ventral Lateral Neuron
LN_d – Dorsal Lateral Neuron
MP – Membrane Potential
PDF – Pigment Dispersing Factor
PER - Period
RFP – Red Fluorescent Protein
RNAi – RNA interference
RS – Rhythmicity Statistic
SCN – Suprachiasmatic nucleus
SD – Standard Deviation
SEM – Standard Error of the Mean
SFR – Spontaneous firing rate
TIM - Timeless
UAS – Upstream-Activating Sequence
ZT – Zeitgeber Time

GENE AND PROTEIN NOMENCLATURE

Following convention gene symbols are lowercase italicised and protein symbols are uppercase regular type, (e.g. *per*/PER). In *Drosophila* the first letter of the gene symbol is upper case when the mutation for which the gene is named is dominant. The convention used at FlyBase (www.flybase.org) is followed.



CHAPTER 1 INTRODUCTION

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Circadian rhythms describe the near 24-hour rhythms observed in the behaviour, physiology and gene expression of living organisms. There is a growing acceptance of the importance of circadian rhythms in human physiology, with the often-underappreciated influences of the clock becoming most obvious when it is disrupted, for example as a result of jetlag, shift-work, disease or after sleep promoting or suppressing drugs. The global population aged over 60 years old is set to more than double by 2050 (UN 2015; Partridge et al. 2018), and with increasing human lifespans and ageing populations, understanding how circadian rhythms change during the ageing process is of growing interest and medical relevance.

In this PhD I have used the fruit fly *Drosophila melanogaster* to study how the circadian clock is altered by the ageing process. During the course of my PhD, the contribution of *Drosophila* studies in understanding the circadian

clock was recognised when the Nobel Prize in Physiology or Medicine 2017 was awarded to Dr Jeff Hall, Dr Michael Rosbash and Dr Michael Young for "their discoveries of molecular mechanisms controlling the circadian rhythm" (NobelPrize.org 2018). This chapter serves to provide a background about the function and basis of the circadian clock.

1.1 The circadian clock

Life on earth has evolved with exposure to 24-hour rhythms resulting from the planet rotating on its axis once per day, with circadian rhythms found in nearly all species on earth. The evolutionary advantages of having a circadian rhythm and being able to appropriately time physiology and behaviour are such that they are found in organisms ranging from as simple as marine algae, yeast, and plants, to higher species including ourselves (Paranjpe and Sharma 2005). Most circadian clocks are primarily entrained by the external light cycle, but the machinery of the clock is self-sustaining and is capable of maintaining an approximately 24-hour period in the absence of external timing inputs.

In all species the underlying machinery of the clock exists at a biochemical level, in higher organisms consisting of oscillatory networks of clock genes that have an approximately 24-hour cycle. Depending on the complexity of the species, this could all occur in a single cell, or in a large system of clock cells in a dedicated brain region.

No matter the differences in the size and structure of the clock across different species, there are several features of a circadian clock:

- An endogenous rhythm with a near 24-hour period in constant conditions.
- The rhythm is entrainable, e.g. by light.
- The rhythm is temperature compensated.

To give a measure of the importance of the clock, the interplay between the molecular clock and gene expression drives large parts of metabolism (Wijnen and Young 2006), with an estimated 10-20% of the genes in any particular tissue showing cyclical expression (Aguilar-Arnal et al. 2013).

Taking an aggregate view of gene expression across 12 different organs in mice, reveals that 43% of all protein coding genes show circadian rhythms in transcription (Zhang et al. 2014).

1.2 A brief history of circadian research

The first accepted scientific report on circadian rhythms dates from a 1729 submission to the French Royal Academy of Science by Jean-Jacques d'Ortous de Mairan (de Marian 1729). In the report, de Marian reported that the leaves of a heliotrope plant (probably *Mimosa pudica*) maintained a daily rhythm in the opening and closing of leaves, which continued when plants were kept in constant darkness. Biological rhythms in a *Drosophila* behaviour were first identified in 1935 by Dr Hans Kalmus and Dr Erwin Bünning, with the emergence of adult flies from the pupae (eclosion) reliably occurring close to dawn (Bruce and Pittendrigh 1957). Later experiments by Dr Colin Pittendrigh showed that the eclosion rhythm, and therefore the underlying clock, were independent of temperature and controlled by a true biological clock (Pittendrigh 1954).

By the 1950's biological rhythms had started to become more heavily studied in what was termed the field of chronobiology, with the term circadian first used by Dr Franz Halberg in 1959 to refer to daily rhythms (Halberg 1959);

> "The term "circadian" was derived from circa (about) and dies (day); it may serve to imply that certain physiologic periods are close to 24 hours, if not exactly that length. Herein, "circadian" might be applied to all "24-hour" rhythms, whether or not their periods, individually or on the average, are different from 24 hours, longer or shorter, by a few minutes or hours."

In the 1950's Dr Jürgen Aschoff demonstrated that the clock was an innate property and did not require previously exposure to a light-dark cycle by demonstrating that birds raised from the egg in constant darkness and mice reared in constant conditions over multiple generations still showed circadian behaviour (Daan and Gwinner 1998). Aschoff started to study biological rhythms alongside his animal research, and developed the term "Zeitgeber", from the German for "time-giver", to refer to environmental stimuli capable of entraining a circadian clock, most commonly light or

temperature (Aschoff 1960). In laboratory circadian experiments Zeitgeber time (ZT) is commonly used when referring to situations where organisms are kept in conditions with an entraining stimulus. For example, in a 12hour-12hour light-dark cycle, ZT0 would refer to lights-on, while ZT12 would be lights-off.

Likewise, when there is no external cue, time is measured as circadian time (CT), with CT0 the time when lights would have turned on in under the previous entrainment regime, and the length of one unit of circadian time dependent on the period of behaviour such that 1 hour of CT is 1/24 of the period (Jud et al. 2005). When under constant conditions, 'subjective day' is used to refer to when the lights used to be on and 'subjective night' to when the lights used to be off.

In his animal experiments, Aschoff showed that constant light shortens the activity phase of nocturnal animals and lengthens activity in diurnal organisms, what became known as Aschoff's Rule (Pittendrigh 1960). Aschoff's later human experiments into the 1960's and 1970's, making use of a bunker to control environmental cues, provided a huge amount of information about the circadian clock in humans (Aschoff 1965; Aschoff and Wever 1976).

The first genetic mutations affecting rhythmic behaviour, or in fact any behaviour, were famously first identified in *Drosophila* by Dr Seymour Benzer and his then PhD Student Ron Konopka, with three different mutations mapped to a gene on the X-chromosome causing either a loss of behavioural rhythms (per^{01}), a short-period (per^{S}) or a long-period (per^{L}) and the gene involved being termed *period* or *per* (Konopka and Benzer 1971) (see Figure 1-1). This discovery led to *Drosophila* becoming a widely used model for investigating the genetic basis of circadian behaviour and later for discovering the key components of the molecular clock underpinning circadian rhythms.

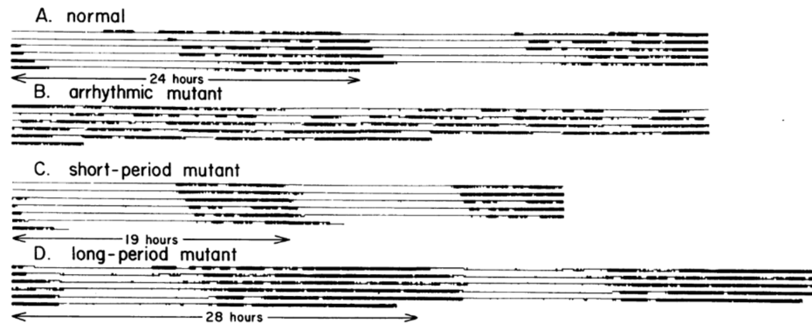


Figure 1-1 Original figure of first clock mutants

The original figure of locomotor activity behaviour of the first clock mutants in constant darkness. (A) The normal behaviour of a wild type fly plotted on a 24-hour time base. (B) Arrhythmic behaviour of a *per* ^{01} fly. (C) Short-period behaviour of a *per* ^{S} fly, plotted on a 19-hour time base. (D) Long-period behaviour of a *per* ^{L} fly, plotted on a 28-hour time base. Figure from (Konopka and Benzer 1971) reproduced with permission of the National Academy of Science.

1.3 Rhythmic behaviour in Drosophila

When *Drosophila* are maintained in laboratory conditions and entrained to a 12hour:12hour light:dark (LD) cycle, flies exhibit characteristic locomotor activity with peaks of activity at morning and evening. Activity starts to build prior to the lights-on and lights-off transition, showing anticipatory behaviour. During the middle of the lights-on period, flies show a reduction in behaviour, with this 'siesta' behaviour much more pronounced in males flies (Figure 1-2A). Interestingly, when flies are kept under more naturalistic conditions consisting of natural lighting and temperature cycles an additional afternoon peak in activity can occur depending on the temperature (Vanin et al. 2012; Green et al. 2015). When flies are then maintained in constant darkness (DD), rhythmic behaviour continues but there is a shrinking of the peaks in activity (Figure 1-2B). Behaviour under constant conditions, i.e. when there is no zeitgeber, is referred to as free-running with the clock allowed to run without any entraining stimulus.

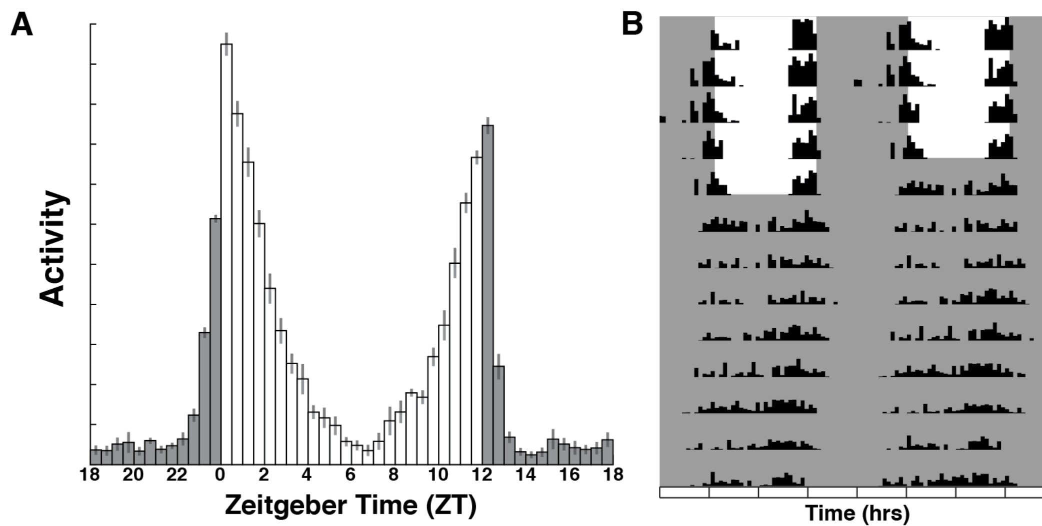


Figure 1-2 Rhythmic behaviour of *Drosophila*

(A) Activity plot of a group of wild type male flies in a 12hr:12hr light-dark (LD) cycle, the bars show mean activity of flies with error bars showing the standard error of the mean. Behaviour during LD shows typical morning and evening peaks of activity, with anticipatory activity prior to light-on and lights-off transitions. (B) Double-plotted activity for an individual wild type male fly housed for five days in LD where activity is mainly concentrated in the lights-on period with the peaks in activity as observed in (A), then free-running behaviour in constant darkness (DD) where behaviour starts to dampen but continues to show a clear active and inactive phase, with a period of just under 24 hrs.

1.4 The molecular clock

At a cellular level the clock consists of a series of molecular oscillators based on transcription-translation feedback loops of a set of core clock proteins (Glossop 2011). As mentioned previously, *period* was the first clock gene identified (Konopka and Benzer 1971), with the majority of the remaining components of the molecular clock first elucidated in *Drosophila*. Later work has demonstrated the molecular clock to be strikingly similar in other species, although the mammalian clock is more complex as a result of a series of gene duplications which increases the number of components.

Identification of the *per* gene and using it to rescue behaviour of *per* ^{01} was done independently in the lab of Dr Michael Young (Bargiello et al. 1984) and by the labs of Dr Jeff Hall and Dr Michael Rosbash (Zehring et al. 1984). Immunohistochemical staining analysis of PER expression was analysed in the brain and the intensity of staining was shown to oscillate with a peak at

night (Siwicki et al. 1988). Next, *per* mRNA was also shown to cycle (with much higher cycling when analysed in sample of only heads), with an approximately six hour delay between the peak of mRNA and protein expression (Hardin et al. 1990). Importantly, the period of the oscillation of *per* mRNA was altered in the per^{S} and per^{L} mutants, and abolished in the per^{01} mutant, suggesting a feedback loop model between the protein and its own RNA (Hardin et al. 1990).

The second circadian gene identified was *timeless* (*tim*), from a genetic screen in the Young lab using a similar approach to Konopka and Benzer but looking at the second and third chromosomes of *Drosophila*. A single arrhythmic mutant was identified in a gene on the second chromosome, *tim*, which like *per* mutants had disrupted rhythms in behaviour and eclosion (Sehgal et al. 1994). The *tim* mutant was demonstrated to block nuclear localisation of the PER protein, and this was shown to be dependent on the presence of a conserved PAS domain (Vosshall et al. 1994), with the PER and TIM proteins were found to interact (Gekakis et al. 1995). Expression of *tim* mRNA was found to be rhythmic, cycling with the same phase and period as *per* mRNA and was dependent on expression of TIM and PER protein (Sehgal et al. 1995). These finding suggested that *per* and *tim* were functioning together to generate a negative feedback loop.

The mechanism by which PER and TIM acted to regulate their own transcription was unknown as the protein sequences of neither contained DNA-binding domains. A genetic screen of mice in the Takahashi lab identified the first mammalian circadian gene *Clock* (*mClock, Circadian Locomotor Output Cycles Kaput*) (Vitaterna et al. 1994), with subsequent cloning showing that it encoded a member of the bHLH (basic helix-loop-helix)–PAS family of transcription factors (King et al. 1997). Work in the Hardin lab found that the *per* promotor contained a transcriptional activator containing an E-Box sequence (CACGTG), which is a binding target of bHLH transcription factors (Hao et al. 1997). Cloning of the *Drosophila* homologue of *Clock,* also called *Clock* (*Clk*), showed its protein, CLK, was required for regulation of *per* and *tim* transcription via E-box binding elements (5' CACGTG 3') in their promoter regions (Allada et al.

1998). A second bHLH-PAS clock gene, *cycle* (*cyc*) was identified which when mutated had the same effect as mutants of *Clk* with a model proposed whereby CLK and CYC form dimers to drive circadian-regulated transcription of *per* and *tim*, and forming the basis of the core molecular clock (Rutila et al. 1998).

The *per-tim* feedback loop is also important for the input of light for entrainment of the clock to the environment. Levels of TIM protein were shown to decrease in response to light, but there was no effect of light on *tim* or *per* mRNA (Myers et al. 1996; Hunter-Ensor et al. 1996). The effect of light on TIM levels was shown to be mediated by *cryptochrome* (*cry*), with the CRY protein a blue-light sensitive photoreceptor, with levels of CRY rapidly decreased during light exposure (Emery et al. 1998; Stanewsky et al. 1998).

Further circadian genes identified in the Young lab were the kinases *double-time* (*dbt*) (Price et al. 1998) and *shaggy* (*sgg*) (Martinek et al. 2001), which through phosphorylation act to stabilise PER and TIM. A second feedback loop of the clock acting through CLK::CYC, was identified containing a transcriptional repressor *vrille* (*vri*) (Blau and Young 1999) and a transcriptional activator *Par domain protein 1* (*Pdp1*) (Cyran et al. 2003).

1.4.1 Operation of the clock

In its simplest form the clock can be considered as a series of transcription-translation feedback loops. At the centre of these loop are CLK and CYC which form a heterodimer CLK::CYC, which acts to initiate transcription of genes with E-box sites in their promoter regions. Transcription of these genes starts a negative feedback loop, promoting transcription of *per* and *tim*, leading to an accumulation of PER and TIM proteins in the cytoplasm. PER::TIM heterodimers must form before translocating to the nucleus, where they bind to and block activity of the CLK::CYC complex, thereby inhibiting their own transcription. This negative feedback combined with degradation of PER and TIM, leads to a decline in PER and TIM levels, relieving the inhibition of transcription and allowing the cycle to begin again, this takes roughly 24 hrs. A second feedback loop acting on CLK, is mediated by VRI and PDP1ε, which respectively act to inhibit and activate

transcription of *Clock*, is important in generating accurate circadian timing (Gallego and Virshup 2007).

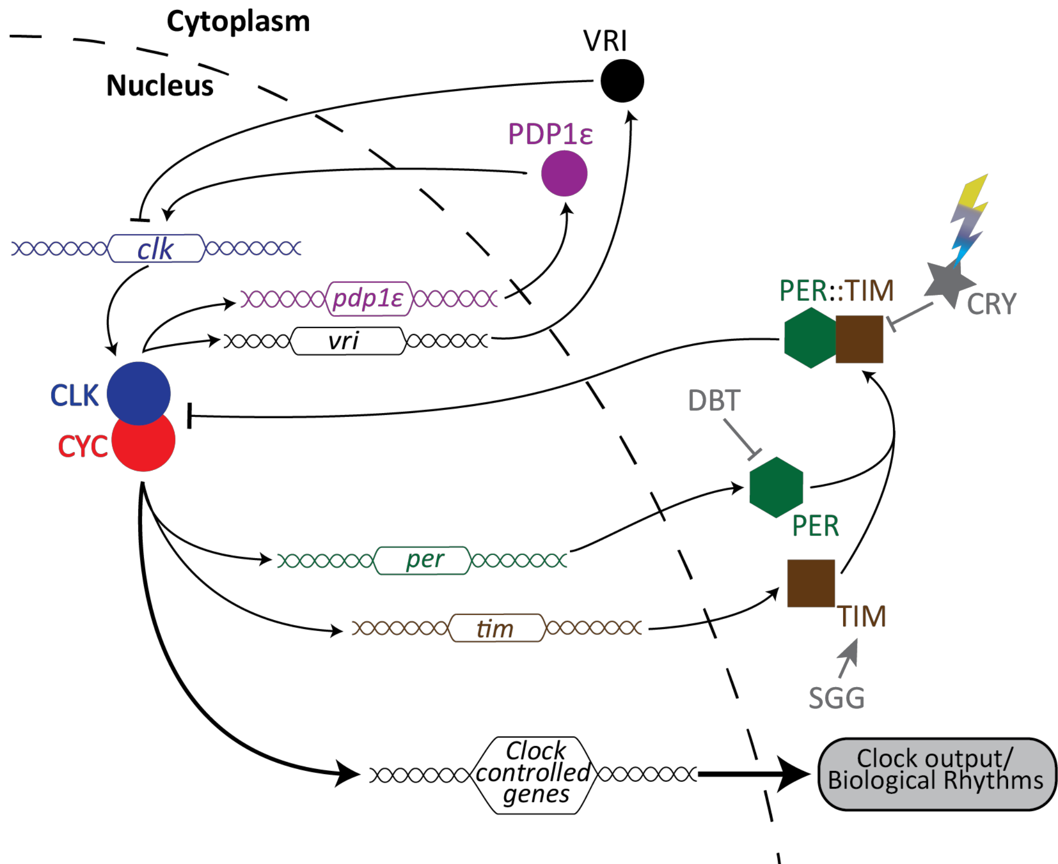


Figure 1-3 The *Drosophila* molecular clock contains multiple transcription-feedback loops

CLK::CYC heterodimers, in the nucleus, drive expression of genes containing E-box promoters sites, namely *per* and *tim*. PER and TIM accumulate in the cytoplasm and form complexes of PER::TIM which translocate to the nucleus and act to block CLK::CYC activity, thereby repressing their own transcription and completing the cycle. CRY acts to reset the clock to external light input, and when activated by light binds to and degrades TIM. A second feedback loop of VRI and PDP1ε acts on the *Clk* gene, to fine-tune the period of the clock. Additionally, phosphorylation of PER and TIM by DBT and SGG act to delay accumulation of PER::TIM dimers and keep the 24-hour cycle.

The period of the clock is regulated by posttranslational phosphorylation and ubiquitination to achieve the circa 24-hour cycle. Doubletime (DBT), an orthologue of casein kinase 1 epsilon (CK1ε) phosphorylates PER in the cytoplasm, while Shaggy (SGG), an orthologue of Glycogen synthase kinase 3 (GSK-3) phosphorylates TIM, targeting the proteins for proteasome-dependent degradation, thereby delaying accumulation of PER and TIM

protein and the formation of PER::TIM complexes. Delayed accumulation of the PER::TIM complex is a key component in determining a 24-hr period. Light input to the molecular clock is mainly through CRY, which causes rapid degradation of TIM when activated by light (Ashmore and Sehgal 2003).

1.4.2 Regulation of output genes

Regulation of circadian expression of output genes of the clock is thought to mainly occur through the aforementioned E-Box sites, which are targets of CLK::CYC (Kyriacou and Rosato 2000; Darlington et al. 2000). More recently, non-E-box sites capable of generating rhythmic expression have been identified in *Drosophila* (Sharp et al. 2017), highlighting a role for other as yet unidentified regulatory pathways.

1.5 The circadian clock

If only considering higher organisms, there are some common features of circadian systems; a central molecular oscillator that maintains a period of approximately 24 hrs, input pathways that allow the clock to be entrained to the environment, and output pathways that drive rhythms in biochemical pathways, physiology and behaviour of the organism (Eskin 1979).

1.5.1 The *Drosophila* circadian clock

In *Drosophila* the central clock is located in the brain and is comprised of a dispersed network of approximately 150 clock neurons, split into different identifiable groups with different circadian functions (Nitabach and Taghert 2008). In terms of comparison, the central clock in the house mouse (*Mus musculus*) consists of ~20,000 clock neurons localised in the suprachiasmatic nucleus (SCN) located at the bottom of the hypothalamus (Piggins and Guilding 2011).

Molecular clocks are also found in many peripheral tissues where they act to control tissue specific rhythms important to function and can either maintain local timing independent of the central clock or can be slave oscillators and receive timing input from the clock (Ito and Tomioka 2016). Examples of peripheral clocks independent of the brain clock are found in the olfactory sensing rhythm of the antenna which does not require a

functioning central clock (Krishnan et al. 1999; Tanoue et al. 2004). Another independent clock is found in the malpighian tubules, the insect equivalent of the kidney, which is cell autonomous and can be directly entrained by light (Giebultowicz and Hege 1997; Giebultowicz et al. 2000). Slave oscillators exist in oenocytes, the sex pheromone producing cells, which receive timing information from the central clock to synchronise pheromone release (Krupp et al. 2008; Krupp et al. 2013).

An interesting peripheral clock is found in fat body tissue (the fly equivalent of adipose tissue and the liver) (Arrese and Soulages 2010). The clock in the fat body regulates a daily rhythm in feeding behaviour that persists in constant darkness (Xu et al. 2008). The fat body clock can be decoupled from the central clock in the brain by time restricted feeding showing a level of local control over the clock (Xu et al. 2011). More recently, work has shown the central clock is necessary for cycling of the molecular clock in the fat body under constant darkness (but not in a light-dark cycle), and that the central clock acts via neuropeptide-F (NPF), to produce cycling of the genes *sex-specific enzyme 1* (*sxe1*) and *Cyp6a21* independently to the local clock (Barber et al. 2016). In an example of conserved evolution, manipulating the mammalian orthologue of NPF in a mouse model, neuropeptide Y, acts to control cycling of cytochrome P450 in the liver (Barber et al. 2016).

1.6 Organisation of the central clock of Drosophila

The location and number of cells in the central clock was determined experimentally using cytological staining for gene products of known components of the molecular clock as well as clock gene promoters expressing fluorescent markers such as Green Fluorescent Protein (GFP) (Kaneko et al. 1997) (Figure 1-4). Clock neurons can largely be divided into two groups by their anatomical location, the lateral neurons and the dorsal neurons (Taghert and Shafer 2006). The lateral neurons number 15-16 cells in each hemisphere and can be classified into three recognised subtypes; the large and small ventrolateral neurons (LN_Vs); the 5th small ventrolateral neurons; and the dorsolateral neurons (LN_ds). The lateral neurons are the

key pacemaker clock neurons which impose the circadian structure on behavioural activity (Vosshall and Young 1995; Frisch et al. 1994).

The dorsal neurons are more numerous, ~50 neurons, and are divided into three groups of dorsal neurons (DN_{1-3}) plus the lateral posterior neurons (LPNs). The dorsal groups of neurons are thought to have more subtle roles in modulating circadian behaviour, for example in integrating both circadian information and environmental signals such as temperature (Zhang et al. 2010; Maguire and Sehgal 2015).

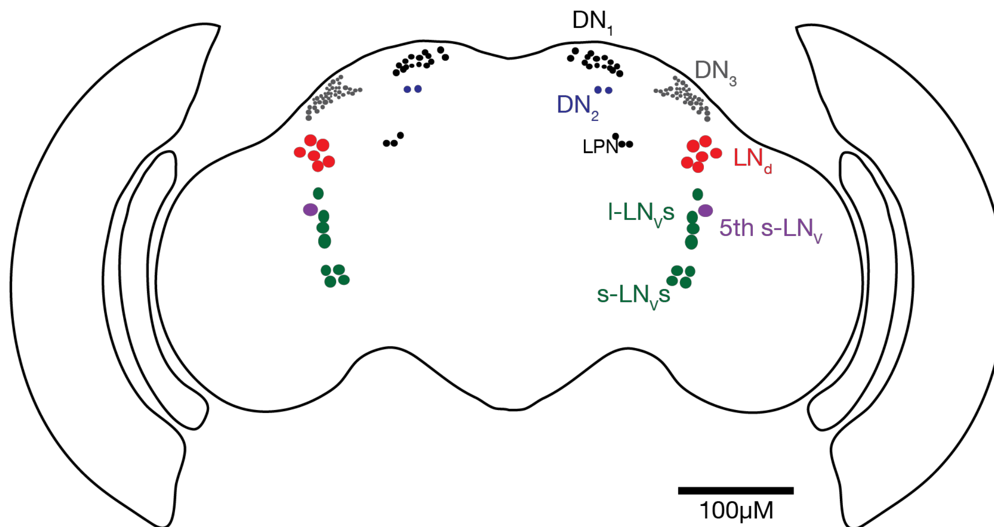


Figure 1-4 Anatomical organisation of clock neurons in the *Drosophila* brain
A diagrammatic representation of the location of the 75 clock neurons in one hemisphere of the *Drosophila* brain. The clusters of neurons are classified into three sets of dorsal neurons (DN_{1-3}) (black/blue/grey) and four groups of lateral neurons (s-LN_V, l-LN_V (red), LN_d and LPN (black)).

There is heterogeneity among the different clock groups, which is reflected in differences in the expression of neurotransmitters, neuropeptides and receptors. Activity of the molecular clock across the different neuronal groups shows approximately the same timing of peak activity, measured by expression levels of the core genes *timeless* (*tim)* (Yoshii et al. 2009) or *per* (Roberts et al. 2015).

1.6.1 The “Morning” cells and “Evening” cells

The LN_V neurons (except the 5th s-LN_V) express the neuropeptide *pigment dispersing factor* (PDF) (Helfrich-Förster 1995; Rieger et al. 2006). PDF

acts to synchronise activity throughout the clock circuit (Shafer and Yao 2014), through PDF-receptor (PDFR), a G-protein coupled receptor, which is expressed in a subset of clock neurons across the network (Im and Taghert 2010). The PDF expressing cells are often termed as the “morning” or “M” cells, as they are necessary for morning anticipation behaviour prior to lights-on in LD, with a pronounced loss of the morning (but not evening) peak of activity in flies with mutations in the *pdf* gene, or lacking the PDF neurons (Renn et al. 1999). The s-LNvs are considered to be the most important neurons for determining behavioural activity rhythms, particularly in DD, with a robust molecular rhythm that persist in DD (Grima et al. 2004; Stoleru et al. 2004).

The LN_Vs send deep projection through the brain, including projections from the s-LN_Vs to the dorsal clock neurons (Helfrich-Förster 1995), with PDF released in a circadian cycle (Park et al. 2000). The axonal terminals of the s-LN_V neurons demonstrate significant circadian remodelling, with higher levels of complexity during the day which reduces at night (Fernández et al. 2008), in addition the amount of PDF in the terminals shows a circadian rhythm of abundance (Park and Hall 1998).

Another group of clock neurons have been identified as being important in the evening peak of activity, and have importance for rhythms in constant light (Stoleru et al. 2007; Picot et al. 2007). This group of “evening” cells consists of the CRY expressing LN_ds and the 5th s-LN_V, with manipulation of the clock in this subset of cells setting the time of the evening peak in LD (Guo et al. 2014; Schlichting et al. 2016).

1.6.2 DN1 neurons and clock outputs

The DN_1 are clock neurons which are downstream synaptic partners of the s-LN_V but also make contact with downstream non-clock neurons cells, suggesting they provide a link between the clock and its outputs (Cavanaugh et al. 2014). The molecular clock of a subset of the posterior DN_1 neurons, the DN_1p neurons, is sufficient for morning anticipation in LD (Zhang et al. 2010; Seluzicki et al. 2014).

The downstream neurons contacted by the DN_1 neurons include the pars intercerebralis (PI) (Cavanaugh et al. 2014), which is considered to be the fly homolog of the mammalian hypothalamus (de Velasco et al. 2007). The PI neurons do not themselves contain the machinery of the clock, but at least some of the cells of the PI, including the insulin producing cells (IPCs) show daily rhythms in electrical activity (Barber et al. 2016). Expression of insulin from the IPCs is important for rhythmic locomotor and feeding rhythms and also links the central clock to a clock in the fat body (Barber et al. 2016). The PI cells also secrete diuretic hormone 44 (DH44), the homolog of corticotropin-releasing factor (CRF), with DH44 also required for robust locomotor rhythms (King et al. 2017). Another molecule produced by the PI is SIFamide, which has been shown to be sleep promoting and provides a link between the circadian and sleep systems (Park et al. 2014).

1.7 The mammalian clock

As briefly mentioned the mammalian clock is located in the SCN, located in the hypothalamus and receiving light input via the retino-hypothalamic tract (Piggins and Guilding 2011). The SCN was identified as the site of the mammalian clock in lesion studies which showed that destruction of the SCN removed the daily rhythm in adrenal corticosterone (Moore and Eichler 1972) and eliminated circadian rhythms in locomotor activity and drinking behaviour (Stephan and Zucker 1972).

The molecular clock in mammals follows the same basic structure of feedback loops found in *Drosophila*, with the main change being a number of gene duplications increasing the complexity. In mammals, the core clock contains three period genes, *Per1-3*, with *timeless* replaced by a non-light sensitive form of *Cryptochrome*, *Cry1-2*. The second loop consists of *Clock* or the paralogue *Npas2* and *Bmal1*, with CLOCK/NPAS2::BMAL1 acting on E-Box sites to drive transcription of clock controlled genes (Ko and Takahashi 2006; Partch et al. 2014).

1.8 The role of electrical activity in the clock

Circadian neurons contain the molecular machinery of the clock and this regulates electrical properties of neuronal cells to drive circadian cycles in action potential firing and membrane potential (Allen et al. 2017). Electrical rhythms of clock neurons are critical for the functioning of the circadian system, and disrupting the firing of SCN neurons results in impairment of circadian activity and drinking behaviour in rodents (Schwartz et al. 1987; Earnest et al. 1991). Likewise, genetically silencing clock neurons in *Drosophila* causes disruption of circadian behavioural outputs (Nitabach et al. 2002).

1.8.1 The role of electrical activity of clock neurons in *Drosophila*

Electrical activity of neurons in the central clock appears to be critical to their function, and the daily differences in daily electrical activity can be considered as a "membrane clock". Disrupting electrical activity of clock neurons, either through mutation or overexpression of ion channels, is reported to prevent clock entrainment and rhythmic behavioural outputs (Ceriani et al. 2002; Nitabach et al. 2002). In particular electrical activity of the LN_Vs has been shown to be crucial for clock function, and specifically disrupting their firing abolishes circadian locomotor activity (Park and Griffith 2006).

The l-LN_Vs are the best characterised of the clock neurons, as their size and location makes them most accessible for electrophysiological recordings. Electrophysiological studies show the electrical activity of the l-LN_Vs demonstrates circadian modulation, with a significantly higher membrane potential and spontaneous firing rate during the subjective day compared to the night (Sheeba et al. 2007; Cao and Nitabach 2008). Complimentary daily rhythms in spontaneous firing rate and membrane potential have also recently been recorded from the DN_1p dorsal clock neurons in electrophysiological experiments (Flourakis et al. 2015), while whole brain calcium imaging suggests there are phase differences in neuronal activity across the groups of neurons which may relate to their behavioural roles (Liang et al. 2016).

1.8.2 Clock neuronal activity during ageing

In mice ageing has been shown to result in reduced amplitude of daily electrical rhythms, measured *in vivo* using multiunit recordings (Nakamura et al. 2011) or from single cells in slice preparations (Farajnia et al. 2012; Farajnia et al. 2015). Electrophysiological experiments from SCN slices from old mice have shown a loss in the day-night differences in membrane potential and firing frequency (Farajnia et al. 2012), and it has recently been shown that there are age related changes in the large-conductance calcium-activated potassium (BK) channels in the SCN, with a loss of circadian modulation of BK expression in old mice (Farajnia et al. 2015).

1.9 The clock and ageing

It is well established that elderly individuals have increasing difficulties sleeping at night and generally wake up earlier than they did previously (Kondratova and Kondratov 2012). The daily cycles of hormone levels, body temperature and the sleep-wake cycle, are modified with age in humans leading to disrupted cycles, and resultant reductions in the strength of the clock as well as phase shifts (Hofman and Swaab 2006).

In humans, ageing is associated with changes in chronotype. Chronotype is a way of measuring morning-evening preference in sleep, i.e. early-bird or night-owl, and can be conducted by a paper or electronic questionnaire rather than the invasive methods needed to determine an individuals' circadian period. Circadian period can be measured in the clinic using forced desynchrony protocol or by measuring of the Dim Light Melatonin Onset (DLMO) both of which require considerable time, equipment and expense. Comparisons of chronotype with DLMO measurements have shown there is strong correlation between chronotype score and circadian period (Kantermann et al. 2015; Kantermann and Eastman 2018), and so chronotype is now often used as a viable proxy for assessing circadian rhythms.

Population studies of chronotype have shown that there is a shift towards later chronotype during adolescence, peaking in late teenage years, before a gradual trend towards an earlier chronotype across the rest of life (Fischer

et al. 2017). In an earlier controlled study of circadian period there was no difference observed between young and old groups (Czeisler et al. 1999), suggesting ageing is altering the relationship between the circadian and sleep-wake systems (Duffy and Czeisler 2002). It is also clear that circadian rhythm sleep-wake disorders become more prevalent in older people (Kim and Duffy 2018). An additional effect of ageing on a circadian output in humans, is a decline in the strength of the hypothalamic pituitary adrenal (HPA) axis with age, as measured by patterns of daily cortisol release (Gardner et al. 2013). The HPA axis is widely involved in control of metabolic processes, immune function and cognitive functions (Gjerstad et al. 2018), and therefore reductions in clock control of HPA function have massive downstream consequences.

Ageing has been shown to have significant effects on the expression of clock genes in humans and rodents. In rodent studies, ageing has been shown to cause reductions in the expression of core clock genes in mice (Weinert et al. 2001) and hamsters (Kolker et al. 2003). Expression of the core clock genes *PER1* and *PER2* in human prefrontal cortex were significantly reduced in amplitude and the phase of expression advanced, measured in post-mortem tissue samples from older samples (>60 years old) compared to younger samples (<40 years old) (C.-Y. Chen et al. 2015). Measurements of peripheral clock gene expression in human blood, have recorded a significant negative correlation between age and expression of *BMAL1* (Ando et al. 2010).

In transcriptomic studies to identify genes that display circadian cycles in expression, results have varied between species, at around 5% in *Drosophila* and 10% in mice (Doherty and Kay 2010; Duffield 2003). Interestingly many more genes can show circadian activity depending on nutritional and metabolic inputs, showing the power of food in entraining the clock (Eckel-Mahan et al. 2013). Forced desynchrony of the clock leads to a reduction in the number of transcripts showing circadian cycling, in mice from 2,032 (~8% of all transcripts) down to only 391 (~1.5%) (Maret et al. 2007), and also in the human blood transcriptome from 6.4% to 1.0% (Archer et al. 2014). Extrapolating from a situation where the clock is weakened during

the ageing process it is logical that the impacts on gene expression will be wide reaching.

1.9.1 The clock in *Drosophila* during ageing

In *Drosophila* ageing has been shown to cause reduced and weakened circadian activity, associated with a decline in PDF levels during ageing (Umezaki et al. 2012). Studies on the effect of ageing on the clock in *Drosophila* show consistently weakened behavioural activity but conflicting results have been reported on the effect of age on the molecular clock, it has been both shown to remain robust in aged flies (Luo et al. 2012), and to significantly decrease in strength (Rakshit et al. 2012). More recently RNA-sequencing has shown the core clock genes remain strongly expressed during ageing (Kuintzle et al. 2017). A recent study using luciferase reporters, has shown that *per* expression in the central clock remains robust with age, but both *per* and *tim* decline in the peripheral clock (Zhao et al. 2018).

Ageing in *Drosophila* is also associated with a significant reduction in CRY expression measured in whole head samples both at the mRNA and protein level (Rakshit and Giebultowicz 2013). Overexpression of CRY throughout the circadian system can reduce the effects of ageing on the decline in other clock proteins and restore behavioural rhythms closer to young levels, as well as increasing median lifespan (Rakshit and Giebultowicz 2013), as can overexpression of PDF in the LN_V neurons (Umezaki et al. 2012).

1.9.2 A dysfunctional clock can cause premature ageing

Associated with the effect of ageing on the clock is the concept that the clock itself may also be a factor in the rate of ageing. Studies in rodents have shown that an SCN transplantation from young to old animals is able to restore behavioural rhythms and even extend lifespan (Hurd and Ralph 1998; Lesnikov and Pierpaoli 1994).

There is also clear evidence for this hypothesis from the fact that animal models of clock mutants often have shortened lifespans (Kondratov et al. 2006). Not having a functional molecular clock has been demonstrated to cause premature ageing in *Drosophila* (Krishnan et al. 2012), with flies

having a mutation in the *period* gene (*per* ^{01}) showing reduced mortality compared to wild type flies. Reduced lifespan is also reported in flies with a null mutation in the clock gene *cycle* (Hendricks et al. 2003). In a mouse clock mutant, with a deficiency in the *Bmal1* gene, animals show accelerated ageing and have increased oxidative stress (Kondratov et al. 2006). Similarly, lifespan of flies is also reduced when the clock is impaired by long exposures to LD cycles either shorter or longer than 24 hours (Pittendrigh and Minis 1972; Klarsfeld and Rouyer 1998). A key role of the clock on the ageing process may lie in circadian control of redox homeostasis, with dysregulation of clock leading to increased oxidative stress and resultant cell damage (Stangherlin and Reddy 2013).

1.10 Sleep in animals

Sleep has become rather generally defined as a reversible state of immobility and greatly reduced sensory responsiveness. The exact function of sleep is still a matter of wide debate, but the ubiquity of sleep across the animal kingdom suggests that it that it plays a critical role in normal physiology, especially considering the increased risk of predation to an organism of reduced sensory perception and the loss of opportunity of being able to eat or mate during sleep (Ly et al. 2018; Allada and Siegel 2008). The importance of sleep to organismal function is best demonstrated by the impairments produce when organisms are deprived of sleep. In the most extreme cases chronic sleep deprivation can cause death, such as in rats chronically deprived of sleep using the disk-over-water method (Rechtschaffen et al. 1983; Rechtschaffen and Bergmann 1995), or in *Drosophila* (Shaw et al. 2002; Cirelli and Tononi 2008).

In mammals, sleep has been separated into different sleep stages which show characteristic differences in electroencephalographic (EEG) recordings. The most clearly defined stages of sleep observed in mammals and birds are non-rapid eye movement (NREM) and rapid eye movement (REM) sleep. EEG signals during wake show low amplitude and fast frequencies. NREM sleep occurs first and is associated with slow frequency EEG, with multiple stages including slow-wave sleep. REM sleep was identified from periods of high eye movement during sleep and is associated

with low amplitude and high frequency EEG (Aserinsky 1996), much like those observed during wake (Scammell et al. 2017). REM sleep is associated with many of the interesting aspects of sleep in humans, most of all dreaming which remains one of the most enigmatic of brain states (Hobson 2009).

1.10.1 Control of sleep

Generally, sleep is thought to be regulated by both a homeostatic drive towards sleep and a circadian drive on wakefulness. These two antagonistic processes form the basis of the two-process model of sleep regulation (Borbély 1982). In the two-process model the homeostatic need for sleep represented by 'Process S' builds during periods of wakefulness and is reduced by sleeping. The circadian drive of wakefulness is represented by 'Process C' and is modelled as a dynamic process which varies across the day, with a time course that can be derived from physiological or behavioural outputs (Borbély et al. 2016) (Figure 1-5). The two processes have been shown to be independent, with SCN lesioned animals lacking circadian rhythms but maintaining sleep homeostasis (Tobler et al. 1983; Trachsel et al. 1992).

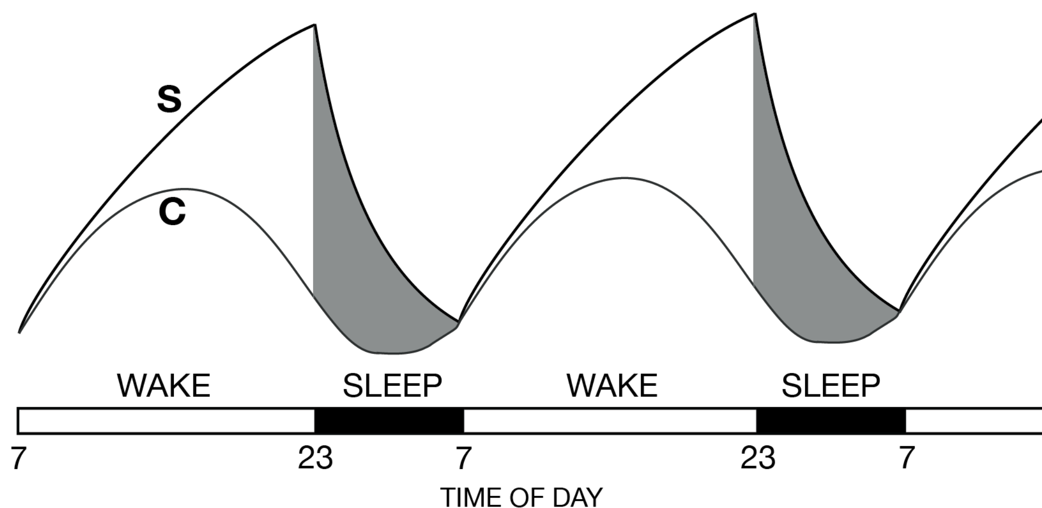


Figure 1-5 The two-process model of sleep regulation

In this model of human sleep regulation, first proposed by (Borbély 1982) describes two antagonistic processes S and C. Process S describes the sleep drive, building during periods of wake and then being reduced by sleeping. Process C describes the wake promoting role of the circadian process which helps to determine correct sleep phase. Process C is high during the day to counteract the sleep promoting effect of Process S and maintain wake.

Process C reduces in the late evening, allowing process S to promote sleep at the appropriate time, thereby gating sleep timing. Figure adapted from (Borbély 1982).

1.10.2 Sleep in other species

Sleep has first often been identified in species in by periods of immobility and has been observed in a wide range of animals, with four main criteria for defining sleep; (i) a species specific posture, (ii) behavioural quiescence, (iii) an elevated arousal threshold and (iv) state reversibility upon stimulation (Campbell and Tobler 1984). Sleep has been demonstrated in the cockroach (Tobler and Neuner-Jehle 1992), honey bee (Kaiser and Steiner-Kaiser 1983; Sauer et al. 2003), zebrafish (Zhdanova et al. 2001; Prober et al. 2006) and in the roundworm *Caenorhabditis elegans* (Raizen et al. 2008). Most recently a sleep-like state has been found in the jellyfish *Cassiopea*, which does not have a central nervous system (Nath et al. 2017), suggesting the evolutionary origins of sleep are even older than previously thought.

1.10.3 Sleep in *Drosophila*

Sleep has become widely studied in *Drosophila* since the first papers were published identifying that *Drosophila* do indeed demonstrate a sleep state (Hendricks et al. 2000; Shaw et al. 2000). Periods of inactivity of longer than five minutes in *Drosophila* demonstrate the hallmarks of sleep in higher organisms, i.e. a reversible state of inactivity, reduced responsiveness to sensory stimuli and homeostatic regulation. In the first study of sleep in *Drosophila*, it was shown that flies chose a preferred sleep location (near the food), became immobile and less responsive to stimuli (Hendricks et al. 2000). The study also demonstrated that sleep showed homeostatic regulation, with a rebound after sleep deprivation and was under clock control. In a hint towards conserved mechanisms of sleep, the study showed that as in mammals, caffeine reduced sleep in *Drosophila* (Hendricks et al. 2000).

Genetic screens for sleep mutants in *Drosophila* have identified short sleeping flies including *minisleep*, which contained a mutation in the *Shaker* ion channel (Cirelli et al. 2005), and *sleepless*, a membrane protein in regulating *Shaker* channels (Koh et al. 2008). More recently a gene involved

more directly in sleep homeostasis was identified from a forward genetic screen, *redeye*, which cycled in expression depending on sleep state and was show to interact with the previous identified *sleepless* gene (Shi et al. 2014).

Further evidence of conserved mechanisms of sleep come from evidence that neurotransmitters appear to play conserved roles across species (Ly et al. 2018). The most commonly used hypnotic drugs used in humans for treating sleep disorders, such as benzodiazepines, act on $GABA_A$ receptor signalling (Harrison 2007). Increasing $GABA_A$ receptor signalling in *Drosophila*, has been shown to both promote sleep and decrease sleep latency (Agosto et al. 2008; Parisky et al. 2008). While in the *sleepless* mutant GABA levels in the brain are only ~30% of wild type, with sleep rescued by restoring GABA (Chen et al. 2015). The neurotransmitter dopamine is known to be highly wake promoting in mammals, and therefore a negative regulator of sleep (Monti and Jantos 2008). One of the first *Drosophila* sleep mutants, *fumin*, was shown to be a dopamine transporter (Kume et al. 2005), while dopaminergic neurons have been shown to inhibit sleep promoting neurons in an area of the brain known as the dorsal fan shaped body (Pimentel et al. 2016; Dissel and Shaw 2016).

As in mammals, sleep in *Drosophila* has associated changes in electrical activity in the brain (Nitz et al. 2002), including different stages of sleep intensity (van Alphen et al. 2013), possibly suggestive of a conserved restorative function for neurons during sleep, such as the homeostatic control of synapses (Tononi and Cirelli 2006). The time to fall asleep, sleep latency, is also regulated by the circadian clock, providing a link between the circadian and sleep systems (Liu et al. 2014).

1.10.4 Ageing and Sleep

It is well established that elderly individuals have increasing difficulties sleeping at night and more daytime sleep episodes combined with generally going to sleep and waking up earlier (Kondratova and Kondratov 2012; Mattis and Sehgal 2016; Skeldon et al. 2016). Furthermore, sleep complaints are highly common in older individuals (Maggi et al. 1998), and there is an increased prevalence of circadian sleep-wake disorders in old age (Kim and Duffy 2018).

Sleep in *Drosophila* has been shown to become more fragmented with age, with shorter more frequent bouts of sleep combined with a decrease in the strength of sleep-wake cycles (Koh et al. 2006). In mice ageing is associated with decrease wakefulness during the active period (Wimmer et al. 2013) and a general increase in sleep in the dark (active) phase (Banks et al. 2015).

1.11 Advantages of using Drosophila

Using *Drosophila* as a model for studying the effect of ageing on the clock offers a number of advantages to other systems. The time-scales involved in *Drosophila* research provides an extraordinary advantage over other model organisms. The lifespan of a laboratory raised fly is approximately two months at 25°C (Linford et al. 2013) (but this is dependent on strain and rearing conditions), with age-related deficits in many behaviours from as early as two weeks of age (Grotewiel et al. 2005; He and Jasper 2014). The *Drosophila* lifecycle is also particularly amenable to genetic experiments with a generation time of approximately 10 days (at 25°C) (Figure 1-6), and with female flies capable of laying up to 100 eggs per day (Stocker and Gallant 2008; Jennings 2011). Working with *Drosophila* also provides advantages in the relative ease of husbandry compared to other laboratory animals, being housed in vials or bottles and using a simple food mixture (see Chapter 2 for rearing methods).

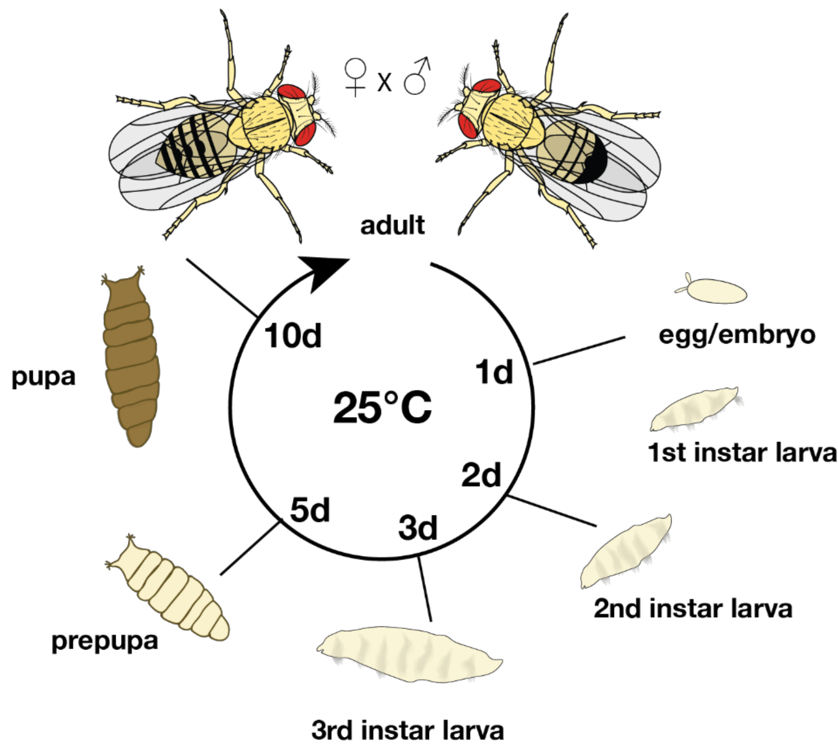


Figure 1-6 Lifecycle of *Drosophila melanogaster*
From laying of an egg to the emergence of adult flies takes approximately 10 days at 25°C. This includes ~24 hrs from embryo to 1st instar larva, ~24 hrs to 2nd instar larva, ~24 hrs to 3rd instar larva, 2-3 days as a prepupa and ~5 days at the pupa stage. Development is dependent on temperature, taking about twice as long at 18°C.

When you compare *Drosophila* ageing studies to those performed in mice, where animals in excess of 500 days old have been used, the costs in time and resources are considerable reduced, as are the ethical considerations. As such this helps replace the need for rodent studies or at very least reduction in rodents (e.g. *Clock* could have been discovered in a *Drosophila* forward genetic screen, and the conservation gene and function in mammals confirmed by using a greatly reduced number of rodents). In keeping with the principles of the 3Rs, with use of *Drosophila* recommended by the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) in the UK.

The wide range of genetic tools available in *Drosophila* allow for very sophisticated manipulations to be made, with the huge array of fly lines already in existence allowing for testing of many possible genes. The *Gal4/UAS* system allows for controlled expression of transgene in a tissue

specific manner (Fischer et al. 1988; Brand and Perrimon 1993). The *Gal4/UAS* system is a two-component expression system taken from yeast that is used to manipulate expression in *Drosophila* and some other model organisms.

In *Drosophila* there are many thousands of lines available from stock centres to target different groups of cells, and drive expression of many transgene. *Gal4* is a transcription activator protein that binds to *UAS* (*Upstream Activation Sequence*) sites to generate transcription of a downstream gene. To generate tissue specific expression of *Gal4*, driver lines are produced using constructs where *Gal4* expression is under control of a native promotor sequence and is restricted to the cells where the native gene would be. Tissue specific expression is then achieved by generating flies expressing both a *Gal4* driver and the *UAS-transgene* construct (Figure 1-7).

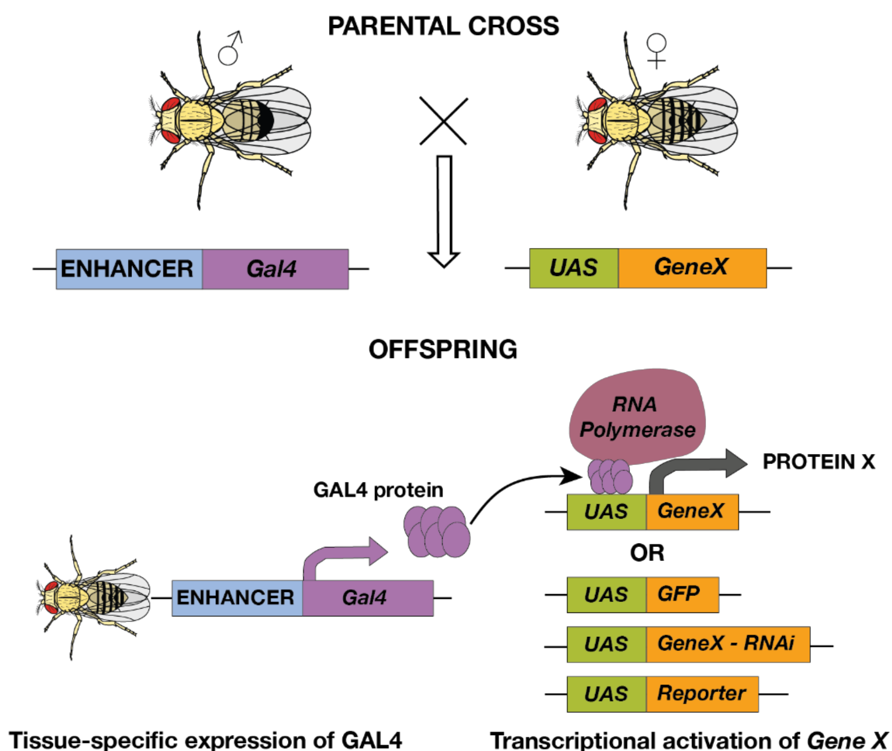


Figure 1-7 The *Gal4/UAS* System

Schematic of the *Gal4/UAS* (Brand and Perrimon 1993). A fly containing the *Gal4* element is crossed to a fly containing a UAS-linked transgene, generating offspring containing both. In the offspring, tissue specific expression of *Gal4* drives expression of the transgene. Schematic inspired by a figure in (Kelly et al. 2017).

In terms of the complexity of the circadian system, the number of neurons involved the circadian network of *Drosophila* is far fewer than in mice and other rodents, 150 in flies compared to 20,000 in mice. Importantly, as the machinery is so well conserved across species, *Drosophila* provides an extremely useful system to understand the ageing process on the level of a clock neuron.

The prevalence of genetic techniques available for use in *Drosophila* has put it at the forefront in the field of optogenetics, using specific wavelengths of light to activate ion channels to activate or silence neurons (Chow et al. 2012; Sidor and McClung 2014). *Drosophila* is also used for visualising neuronal activity with genetically encoded reporters, such as the calcium sensor GCaMP (Nakai et al. 2001; Akerboom et al. 2012), and now genetically encoded voltage indicators (GEVIs) able to detect single action potential events through changes in membrane fluorescence (Jin et al. 2012; St-Pierre et al. 2014). A recently developed GEVI known as *ArcLight* (Cao et al. 2013) has been used to record electrical activity from *Drosophila* brain preparations. Using *ArcLight* could allow for simultaneous recording of multiple cells, and in old brains where dissections are more difficult, may be an easier alternative to recording using patch-clamp electrophysiology. Optical electrophysiology has the potential for allow quicker and less invasive access to the cells of interest, but there is a trade-off compared to the temporal precision and level of information recorded in patch-clamp electrophysiology.

Drosophila is widely used for studies of circadian behaviour, and as such there are readily available systems for behavioural activity recording to allow for high-throughput studies of flies. The *Drosophila* Activity Monitoring (DAM) system makes it very simple to record the activity of individual flies, housed individually in sealed tubes with infrared beams recording each time the movement of a fly breaks the beam. Activity events are binned across a sampling period, and recorded across the duration of the experiment, providing quantification of changes in activity (Rosato and Kyriacou 2006).

1.12 Mathematical modelling of the clock

Modelling the electrical activity of the clock neurons has demonstrated great potential for understanding the role of the different ionic currents involved in control of daily excitability, both generally and in ageing. Previous models have investigated the periodic behaviour of the molecular clock by modelling the interaction of the proteins and their mRNA in the feedback loops. These models exhibit strong agreement with experimental data (Goldbeter 1995; Leloup et al. 1999; Gonze et al. 2003).

Models of the electrical activity of clock neurons have also been developed, for neurons of the mouse SCN. These have reproduced the firing behaviour and explained the role of ionic currents in the SCN clock cells (Belle et al. 2009). This model has been expanded upon to integrate both the membrane potential and calcium concentration, and a mechanism for linking electrical activity to transcriptional activation of the molecular clock in the nucleus (Diekman et al. 2013). Although these previous models of electrical activity on the clock have used the mammalian SCN the underlying biophysical and mathematical concepts are transferrable to *Drosophila* and make a good starting point.

1.13 Thesis aims and structure

The overarching aim of my PhD project was to examine the effects of ageing on circadian rhythms using *Drosophila* as a model. I had a particular interest in the effects of ageing on the electrical activity of the circadian system, given what is already known about the weakening of the behaviour outputs of the clock.

Chapter 2 details the materials and methods used and referred to across the rest of the thesis. Chapter 3 reports the findings from behavioural experiments measuring the impact of ageing on circadian rhythms and sleep. In Chapter 4 the results from electrophysiological experiments from the l-LN_V neurons and from some pilot experiments using an optical reporter of membrane potential. Chapter 5 reports on behaviour experiments investigating manipulation of ion channel expression in the circadian system. Chapter 6 outlines results from using computational

modelling to describe electrical activity of clock neurons, and then to make predictions of the effect of manipulating an ion channel. Chapter 7 concludes the thesis with a discussion of the overall experimental findings and how these fit with the current understanding in the field.

CHAPTER 2 MATERIALS AND METHODS

This chapter outlines the materials and methods used for the experiments performed in the research that contributes to this thesis. Section 2.1 outlines the husbandry conditions that were used for breeding and rearing of flies and details the different fly strains used. Section 2.3 details the circadian rhythm assay and the analysis tools used. Section 2.4 explains the sleep assay. Section 2.5 introduces the electrophysiological methods used for recording the electrical activity of clock neurons. Section 2.6 outlines the approach for immunohistochemistry experiments. Section 2.7 summarises the methods used for studying gene expression. Finally, section 2.8 provides an overview of the standard approaches used for statistical analysis of data.

2.1 Fly Husbandry

Flies were maintained in plastic vials (25 dia. X 95 mm height) or stock bottles (60 mm dia. x 130 mm height) with cotton Flug® closures. Flies were housed in a 12hr:12hr light/dark (LD) cycle, with fly stocks kept in an incubator maintained at 18°C. while experimental flies were kept at 25°C in an environmentally controlled room.

Flies were collected and sorted using CO_2 anaesthesia pads to immobilise flies under a dissecting microscope. Flies could then be sorted into males and virgin females for setting up crosses or identified using known genetic markers. Crosses were setup in vials or bottles using approximately the same number of parent flies, for vials 10 females and 6 males were used, while for bottles crosses used 20 females and 12 males. Adult flies were flipped onto fresh food every 5-7 days.

2.1.1 Fly food

All flies were reared on a standard medium based upon the following recipe; 10 litre batches containing: 400 ml malt extract, 200 ml molasses, 400 g

polenta, 90 g active dried yeast, 50 g soya flour and 35 g granulated agar, with 40 ml of propionic acid (Sigma-Aldrich, #94425) and 100 ml of nipagin (Sigma-Aldrich, #H5501) added after cooling. Food was then dispensed into trays of either vials or bottles. Trays of food were kept covered and allowed to set overnight before sealing with Flug® closures. Trays of food not to be used immediately were refrigerated at 12°C and stored for no longer than a week in order to avoid the food drying out.

2.1.2 Fly stocks

A variety of different fly stocks were used in the different experiments, the various genotypes as well as the sources of the stocks are listed in Table 1.

Targeted expression of genes to specific cells was achieved using the *Gal4/UAS* binary expression system (Brand and Perrimon 1993; Duffy 2002). In the *Gal4/UAS* system, the *Gal4* component targets expression to a specific subset of cells, for example expression of *Pdf-Gal4* is limited to the LN_V clock neurons (20 cells) whereas *tim-Gal4* expresses in all clock neurons (150 cells). The UAS component then contains the transgene to be expressed, for example GFP or RNAi constructs. Parents (virgin) of each line were then crossed together to produce progeny that contain both the *Gal4* and UAS of interest, leading to expression of the transgene in the cells of interest.

Name (chromosome)	Description	Original Reference/Source
Electrophysiology line		
PDF::RFP (II)	Transgenic fusion of PDF promotor and mRFP1, labels LN_V neurons with RFP	Ruben *et al.* 2012 (Ruben et al. 2012)
Wild type stocks		
CS*w*	*Canton S white minus*, wild type control	Prof. Scott Waddell, University of Oxford
iso[31]	Wild type control	Dr Maite Ogueta Gutierrez, University of Münster
Gal4 drivers		
Pdf-Gal4 (II)	Driver line for expression in PDF positive clock neurons	Prof. Ralf Stanewsky, University of Münster
tim-Gal4 (27) (II)	Driver line for expression in all clock neurons	
UAS-expression		
UAS-mCD8::GFP (x, II, III)	Expresses membrane bound green fluorescent protein	Bloomington stock center (BL) #5137
UAS-ArcLight (III)	Expresses the genetically encoded voltage reporter *ArcLight*	BL #51056 (Cao and Nitabach 2008)
RNAi Lines		
UAS-slo^{RNAi} (III)	Knocks down *slo*	BL #26247
UAS-Irk1^{RNAi} (III)	Knocks down *Irk1*	BL #42644
UAS-Ca-α1T^{RNAi} (III)	Knocks down *Ca-α1t*	BL #26251
UAS-Shal^{RNAi} (III)	Knocks down *Shal*	BL #31879
UAS-SK^{RNAi} (III)	Knocks down *SK*	BL #27238
UAS-para^{RNAi} (III)	Knocks down *para*	BL #33923
UAS-Shaker^{RNAi} (II)	Knocks down S*haker*	BL #53347
UAS-Shaw^{RNAi} (III)	Knocks down *Shaw*	Hodge and Stanewsky, 2008 (Hodge and Stanewsky 2008)

Table 2-1 Fly stocks used and sources

A summary of the fly stocks used, with the chromosome of any transgene indicated in brackets. The source of the stock or the original publication they were created in is listed.

2.2 Longevity assay

Male flies, and once-mated female flies, were housed separately in vials of 10 flies, with a total of 100 flies used for each sex. Flies were transferred to fresh vials of food every three days, and the number of flies surviving at that point was recorded. A survival curve of the data was plotted using OASIS (Online Application for Survival Analyses) (Yang et al. 2011), with mean survival calculated using the Kaplan-Meier estimator (Barker 2009).

2.3 Circadian rhythm assay

Circadian rhythms of adult *Drosophila* were assayed by monitoring locomotor activity in free-running conditions, by maintaining flies in conditions of constant darkness to remove the entraining stimulus of light. Locomotor activity was recorded using the *Drosophila* Activity Monitoring (DAM) system (DAM2, Trikinetics), consisting of a series of monitoring platforms connecting to a host computer for data storage.

Each monitor can hold up to 32 tubes, held horizontally, each containing a single adult *Drosophila* with food at one end and a cotton wool closure at the other. The body of the monitor contains a series of infra-red beams that intersect each tube at the midpoint (Figure 2-1). As an individual fly moves along the tube the beam is broken and this is recorded by the monitor. At the end of each recording interval the accumulated counts are sent to the host computer and processed by the DAMSystem3 data collection software.

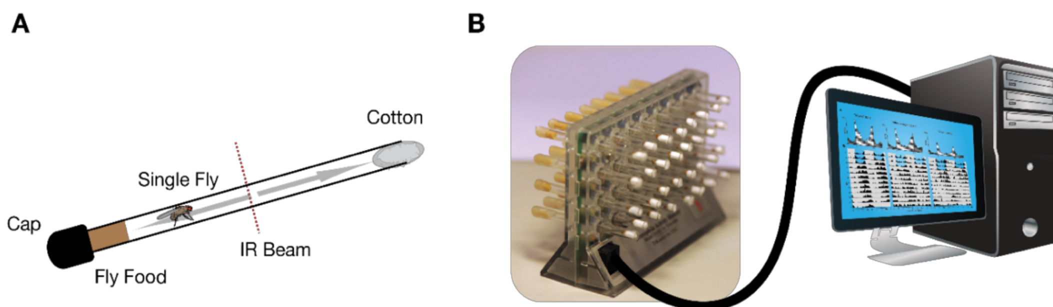


Figure 2-1 Experimental setup of *Drosophila* Activity Monitor (DAM) system
(A) Individual adult *Drosophila* are housed in tubes containing food at one end and sealed with cotton at the other. Tubes are then placed into a DAM monitor, where an infrared beam bisects the tube, recording every time the fly travels along the tube breaking the beam. (B) DAM monitors are connected to a host computer, and at the end of every recording interval the accumulated number of beam breaks is sent the computer and recorded.

Monitoring tubes were autoclaved between uses for cleanliness and to maintain sterility. The standard food recipe was used rather than agar food as in most circadian experiments this was found to be better for maintaining healthy aged flies during monitoring. Tubes were loaded with food the day before experiments were to start, with food being poured into plastic trays while still liquid to a depth of approx. 5 mm and allowed to set. Tubes were

then pressed into the solid food so that they were partially filled at one end (see Figure 2-1). Each tube was sealed with a plastic cap at the food end to prevent desiccation and were stored at 4°C overnight before use.

Flies were loaded into tubes by brief anaesthesia using CO_2, before individual flies were carefully pushed into a tube using a soft paintbrush, with the tube then sealed with a small cotton plug. Tubes were then loaded into monitors, centred so that the detection beam bisects the tube. If necessary, the tubes were secured in place using elastic bands to prevent sliding. Monitors were then connected to the host computer and placed in a light-tight incubator maintained at 25°C, and a humidity level of 55-65%. After setup, proper monitor function was checked in the data collection software. For the initial period of circadian experiments, the incubator was maintained in LD (12hr:12hr light-dark cycle, with lights on from 0900 to 2100), for 5 days before switching to DD (constant darkness) for a further 7 days. Activity counts were recorded as a time series in 30 min binning intervals.

2.3.1 Analysis of circadian rhythms

Raw data from the host computer was processed using the DAMFileScan software to cut the raw files to the period of interest, so that the first bin of each file started at lights on, on the first day. Analysis of circadian data was performed in MATLAB using the Flytoolbox (Levine et al. 2002b), with some modifications which are described in further details below. Any channels that did not record activity for the duration of the experiment were removed from the dataset and not used for analyses.

Circadian rhythms are commonly visualised through activity plots known as actograms, where consecutive days are double-plotted horizontally and vertically, allowing for better observation of rhythmic behaviour across many days in constant conditions (see Figure 2-2A). Locomotor activity can also be plotted as a histogram to analyse behaviour in a 24-hour window.

Rhythm strength is calculated by conventional autocorrelation analysis of the behavioural activity recording under DD conditions (Figure 2-2C). The

activity record is compared to itself, such that at time zero the value is 1 as the record is identical to itself.

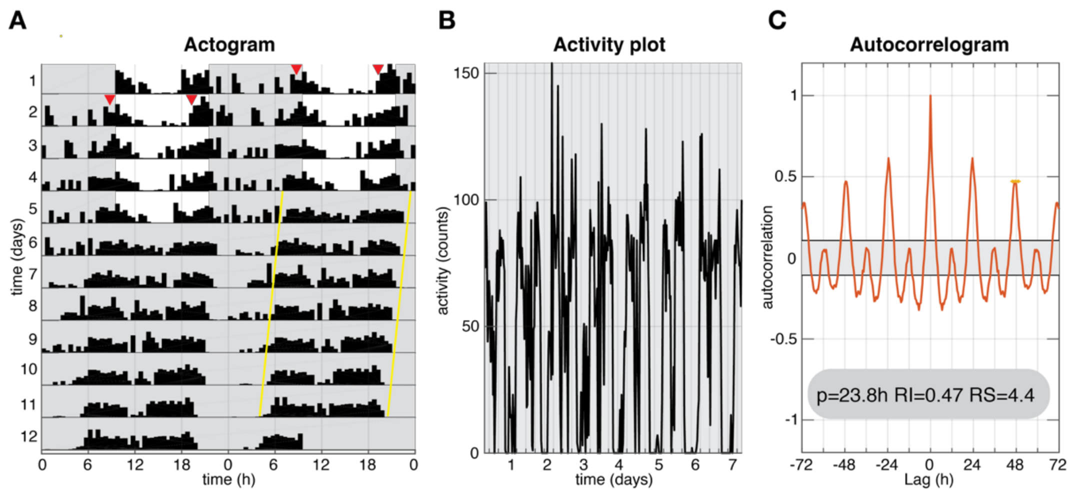


Figure 2-2 DAM Analysis

(A) Locomotor activity recordings are visualised using double-plotted actograms, where consecutive days are plotted both horizontally and vertically. Grey shading indicates where lights were turned off. Red markers on day two indicate morning and evening anticipatory behaviour. (B) Activity traces are filtered using a low-pass Butterworth filter before further analyses. (C) The autocorrelogram generated from the filtered activity trace from (B). The autocorrelogram measures how strongly the activity recording correlates with itself with time. The rhythmicity statistic (RS) is calculated as the ratio of the height of the third peak to the 95% confidence value (shown in grey) and the period calculated from the lag time between peaks.

The Rhythmicity Index (RI) is calculated to give a statistical measure of the rhythm strength, given as the value of the height of the third peak in the autocorrelogram, a higher number indicates stronger rhythmical activity, and is statistically significant if the peak crossed a boundary set by the confidence level. The RI is used to calculate the Rhythmicity Statistic (RS), from the ratio of the RI to the 95% confidence level, such that an RS value >1 indicates a statistical significant rhythm (Levine et al. 2002b; Levine et al. 2002a). Flies with an RS<1 were classified as arrhythmic and were excluded from analyses of circadian period. Circadian period was calculated from the autocorrelation analysis, from the lag between the peaks on the autocorrelogram. Average activity levels were calculated as the average amount of activity per 30-minute time bin across.

2.3.2 Day/Night index

For further analysis of the organisation of daily behaviour the diurnal/nocturnal (D/N) index was calculated as follows:

$$(1.\ 1) \qquad \text{D/N Index} = \frac{\text{Day activity - Night activity}}{\text{Total locomotor activity}}$$

A positive D/N index value indicates preference for activity during the day, and a negative value preference for activity during the night (Kumar et al. 2012).

2.3.3 Anticipation index

The morning and evening anticipation indexes were calculated to quantify the magnitude of the morning and evening peaks in activity. Anticipation indexes were calculated from the activity of flies across the five days of LD activity. Morning anticipation was calculated as previously described (Zhang and Emery 2013; Harrisingh et al. 2007). Briefly, the average activity was calculated as the ratio of activity between ZT21.5-24 compared to ZT17-19.5. Evening anticipation was likewise calculated as the ratio between ZT9.5-12 compared to ZT5-7.5 (see equations (1. **2**(1. 3)

$$(1.\ 2) \qquad \text{Morning Anticipation Index} = \frac{\text{Activity total between (ZT21.5 - ZT24)}}{\text{Activity total(ZT17 - ZT19.5)} + \text{(ZT21.5 - ZT24)}}$$

$$(1.\ 3) \qquad \text{Evening Anticipation Index} = \frac{\text{Activity total between (ZT9.5 - ZT12)}}{\text{Activity total (ZT5 - ZT7.5)} + \text{(ZT9.5 - ZT12)}}$$

2.4 Sleep analysis

Sleep data was analysed using the Sleep and Circadian Analysis MATLAB Program (SCAMP) (Donelson et al. 2012). Individual raster plots of activity were viewed, and flies that had died before the end of the experiment were removed from the data. Data was analysed across the 24-hour period, the 12-hour ‘light phase’, and the 12-hour ‘dark phase’. Sleep was visualised by plotting the mean amount of sleep in a 30-minute bin against the time of day, averaged for the five days of the experiment. From the raw data of sleep amounts and time, a series of measurements of sleep were calculated,

including 'total sleep duration', 'number of sleep episodes', 'mean sleep episode duration' and 'sleep latency' (see Table 2-2).

Parameter	**Description**
Total sleep duration	Sum of all sleep episodes (mins)
Number of sleep episodes	Count of all sleep episodes
Mean sleep episode duration	Average duration of sleep episodes (mins)
Sleep latency	Time to first sleep episode (mins)

Table 2-2 Description of sleep parameters analysed

2.5 Electrophysiological recording of clock neurons

Electrophysiology was performed on flies expressing a fluorescent marker in the PDF-positive clock neurons to allow for visual identification of target cells. Recordings were made between ZT7-9 (where ZT0 indicates lights-ON).

Firstly, flies were anaesthetised using CO_2, before decapitation, and removal of the brain using fine forceps (Dumont #5, Fine Science Tools) under a dissecting microscope (M205-C, Leica), performed in a drop of extracellular electrophysiological solution (detailed below) in a custom-made dissecting dish consisting of a standard petri dish with a lining of Sylgard (DowCorning). The head was grasped by the forceps and the cuticle removed to expose the brain. Next the photoreceptors, lamina, air sacks and trachea were removed, and a small incision made over the position of the neurons of interest. Whole brains were transferred to a recording chamber containing extracellular solution and placed ventral side up, held stable using a custom-built wire harp constructed from a large-bore needle bent into shape strung with fine thread.

2.5.1 Electrophysiological recording methods

The whole-cell configuration of the patch-clamp technique was used to make current-clamp recordings from l-LN_{V}s in whole brains prepared as described above (C. Chen et al. 2015). Electrodes were made on a Flaming/Brown Micropipette puller (Model P-1000, Sutter Instruments,

USA) from fire polished filamented borosilicate glass capillaries (OD 1.50 ID 0.84) (1B150F-4, World Precision Instruments); electrode resistances typically ranged from 10 to 16 MΩ in standard pipette and bath solutions.

The pipette solution was (in mM): 102 K-gluconate, 0.085 $CaCl_2$, 1.7 $MgCl_2$, 17 NaCl, 0.94 EGTA, 8.5 HEPES, ATP (pH 7.2). Extracellular solution contained (in mM): 101 NaCl, 1 $CaCl_2$, 4 $MgCl_2$, 3 KCl, 5 glucose, 1.25 NaH_2PO_4, 20.7 $NaHCO_3$ (pH 7.2, approx. 250 mOsmol/kg) and was used to fill the recording chamber. All chemicals were purchased from Sigma-Aldrich.

Cells were visualised using an Axio Examiner Z1 (Zeiss) using a 63x water immersion objective, l-LN_Vs were identified using 555 nm light generated using a Colibri Examiner light source (Zeiss). A recording pipette was filled with solution using a MicroFil pipette filler (WPI) with a 0.2 µm filter. Positive pressure was applied to the pipette, and the pipette was guided to the target cell using a micromanipulator (Patchstar, Scientifica) and a whole cell gigaOhm seal formed using brief suction or application of a short current pulsing using the zap function (≤200 µs). Pipette offset current was zeroed immediately prior to contacting the cell membrane. Current-clamp measurements were made using the I-clamp circuitry of the MultiClamp 700B amplifier (Axon Instruments).

Data were digitised with a Digidata 1440 (Axon Instruments), connected to a computer running pClamp software (10.5: Molecular Devices, LLC) at a sample rate of 20 kHz and using a 10 kHz Bessel filter. Data was recorded in Clampex (10.6), which was also used to run the current-clamp protocols. Current-clamp recordings were monitored for a stable resting membrane potential and if this deteriorated during an experiment the recording was terminated.

The liquid junction potential was calculated as 13 mV and subtracted *post-hoc* from all the membrane voltages. A cell was included in the analysis if the access resistance was less than 50 MΩ. Membrane potential (MP) and the spontaneous firing rate (SFR) were measured after stabilising for 2–3 min. The membrane input resistance (R_{in}) was calculated by injecting

hyperpolarising current steps and measuring the resulting voltage change. Neuron excitability was measured by injecting a 500 ms long positive current pulse with increasing amplitude up to +40 pA and manually counting the resulting spikes.

2.5.2 Analysis of electrophysiological recordings

Traces from electrophysiological experiments were viewed and analysed using the Clampfit application (10.5 Molecular Devices, LLC.). Neuronal activity was allowed to stabilise for 2-3 mins, before a 120 second section of the trace was selected for analysis. This 120 second trace was then analysed using Threshold Search Event Detection in Clampfit, with the 'baseline' manually fitted for each trace. Neuronal firing activity was calculated based upon the inter-event intervals. Instantaneous frequency was calculated by first converting each event into a frequency (the reciprocal of the inter-event interval) and then averaging these frequencies together.

2.5.3 Optical imaging of clock neuron activity

Optical imaging of electrical activity of neurons was carried out in flies expressing the genetically encoded voltage reporter *ArcLight* (Jin et al. 2012; Gong et al. 2015) in the PDF-expressing clock neurons. *ArcLight* consists of a fusion of the voltage-sensing domain of *Ciona intestinalis* voltage-sensitive phosphatase and the fluorescent protein super ecliptic pHluorin and shows decreased fluorescence in response to membrane depolarisation (Jin et al. 2012).

2.5.4 Imaging setup

Dissection procedure was carried out the same as for electrophysiological recordings and the brain secured by the same method previously described.

Imaging of *ArcLight* fluorescence was performed using either an optiMOS sCMOS camera (QImaging) or an Evolve 128 EMCCD camera (Photometrics). *ArcLight* fluorescence was activated using 470 nm light using a Colibri Examiner with light intensity usually 15%, but this varied between different experiments and different brains. Image stacks were recorded using the µManager software (Edelstein et al. 2010), using the "Multi-D-acquisition" mode to create a time-course image stack. The

imaging region was set to cover the cells of interest, to allow the camera to image at the fastest possible speed and to keep file sizes smaller for analysis. Exposure was set to 2.5 ms to give an approximate imaging speed of 400 Hz.

2.5.5 Imaging analysis

Image-stacks were process using the FIJI distribution of ImageJ (Schindelin et al. 2012). Image-stacks were analysed using the delta-F method. Firstly, an F0 image was created by averaging the first 20 frames of the stack. The 'Image Calculator' tool was then used to divide the image stack by the F0 image, to create a stack of F/F0 images. A region of interest (ROI) was then drawn on the target cell in this F/F0 stack and a time-series of the mean F/F0 values for the ROI recorded.

2.6 Immunohistochemistry

Flies were briefly anaesthetised using CO_2 and swiftly decapitated and heads immediately placed into phosphate-buffered saline (PBS) containing 4% paraformaldehyde PFA (brand) and 0.008% Triton X-100 (Sigma) and fixed for 45 mins at room temperature. For all steps of the protocol tubes were covered by foil to protect tissue from light exposure. Fixed heads were quickly washed twice in 0.5% PBT (PBS with 0.5% Triton X-100), followed by three 20 min washes in PBT, before being dissected in 0.1% PBT. Brains were block in 5% normal goal serum (NGS) for 30 min at room temperature. Brains were then incubated with primary antibodies in 5% NGS, at 4°C for 36 hours on a rotator with tubes upright.

Brains were quickly washed twice in PBT, followed by three 20 min washes in PBT, with tubes upright on a rotator. Brains were then incubated with secondary antibodies in 5% NGS for 3 hours at room temperature, and then overnight at 4°C. Brains were washed in PBT, three times 20 min, and rinsed twice in PBS. Brains were then aligned on a microscope slide, with wells created using imaging spacers (SecureSeal™, Grace Bio-Labs #654002), and then mounted in Vectashield hardset medium (Vector Laboratories). Mounting media was allowed to harden for 30 min at room

temperature, before storage at 4 °C. Coverslip edges were sealed with clear solvent (CoverGrip™, Biotium #23005).

Primary Antibodies	Concentration	Source
Mouse monoclonal anti-PDF	1:200	Developmental Studies Hybridoma Bank, #PDF-C7
Rabbit polyclonal anti-GFP	1:1000	Life Technologies # A11122
Secondary Antibodies		
Alexa Fluor Plus 488 Goat anti-mouse	1:1000	Life Technologies # A32723
Alexa Fluor Plus 555 Goat anti-rabbit	1:100	Life Technologies # A32732

Table 2-3 Antibodies used, concentration and sources

Brains were imaged using a Leica TCS SP8 AOBS confocal laser scanning microscope attached to a Leica DMi8 inverted epifluorescence microscope, equipped with 'hybrid' Gallium arsenide phosphide (GaAsP) detectors with the green channel imaged at 480 – 551 nm and the red at 571 – 650 nm. A 20x glycerol immersion objective (HC PL APO CS2, Leica) was used and confocal stacks were obtained with a 2 µm step size and 512 x 512 pixels. Confocal stacks were analysed using the FIJI implementation of ImageJ (Schindelin et al. 2012). Besides contrast, brightness, colour scheme and orientation adjustments, no further manipulations were made to the images.

To quantify the axonal arbour of the dorsal projections we used an adaptation of the Sholl method (Sholl 1953), as has been previously reported (Fernández et al. 2008). Briefly, using six evenly spaced (10 µm) concentric rings centred at the first branching of the dorsal projections, and counting the number of intersections of each projection with the rings. Scoring was performed blind to the experimental condition.

2.7 Molecular Analyses

2.7.1 Analysis of existing microarray dataset

Previously published molecular investigations on the *Drosophila* clock have focussed on the expression of the components of the molecular clock (Kula-Eversole et al. 2010; Nagoshi et al. 2010). Microarray datasets are available from these studies, with expression data from manually sorted LN_V clock

neurons at four circadian timepoints (ZT0, 6, 12, 18). I went through the available datasets to look at the expression of known ion channels to identify those that show significant cycling across the circadian cycle. I then selected from these ion channels targets to investigate using RNAi knockdown and measurements of circadian and sleep behaviour.

2.7.2 Gene expression analysis

Flies were anaesthetised on ice, and whole brains were removed from 10 flies, per age, per timepoint. RNA was extracted from brain lysates using the Purelink RNA Mini Kit (Ambion, #12183018A). Genomic DNA was removed using the TURBO DNA-free kit (Invitrogen, #AM1907). RNA samples were quantified before cDNA synthesis using a Qubit 3.0 Fluorometer (Invitrogen) using the Qubit RNA HS Assay Kit (Molecular Probes, #Q32852). cDNA was synthesised from 20 ng of extracted RNA using the Superscript VILO cDNA Synthesis Kit (Invitrogen, #11754050). Reverse transcription was performed in a 20 µL reaction, according to the manufacturer's instructions, which used the Superscript enzyme and random primers, with 10 min at 25°C, 60 min at 42°C with the reaction terminated at 85°C for 5 min. cDNA was either immediately used for qPCR or stored at -20°C.

Quantitative PCR (qPCR) was performed using the Taqman Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems, #4324018) according to the manufacturers protocol for 20 µL reactions in a 96-well plate QuantStudio 3 Real-Time PCR System (Applied Biosystems). The thermal cycling procedure consisted of a polymerase activation step at 95°C for 10 min, followed by 40 cycles of PCR consisting of denaturing at 95°C for 15 secs and annealing/extension at 60°C for 1 min. Each 20 µL reaction mix contained 10 µL of master mix, 1 µL of Taqman Assay and 1ng of cDNA in 9 µL of H_2O. Details of the reference and target genes are listed in Table 2-4.

Amplification data were visualised and analysed using the QuantStudio Design and Analysis desktop software (1.2.0, ThermoFisherScientific). Relative gene expression was analysed in the software using the comparative C_T method (Schmittgen and Livak 2008), where the expression values were normalised to a group of reference genes chosen for their stable

expression (Ling and Salvaterra 2011). The C_T (threshold cycle) is the PCR cycle at which the fluorescence crosses a threshold value in the exponential phase of amplification and is inversely related to the amount of transcript detected. In the comparative C_T method, the C_T of the gene of interest is compared to the C_T of the internal control, and then the difference in the CT ($\Delta\Delta$CT) between samples is compared (equation (1.4). The fold change in expression is then calculated by 2 to the power of $\Delta\Delta$CT, as the values are in base-2 since the amount of PCR product doubles each round of the reaction (see equation (1.5).

(1.4) $$\Delta\Delta C_T = [\text{sample A } (C_T \text{ gene of interest- } C_T \text{ internal control}) - \text{sample B } (C_T \text{ gene of interest- } C_T \text{ internal control})]$$

(1.5) $$\text{Fold change} = 2^{-\Delta\Delta C_T}$$

Reference Genes		**Taqman Assay ID #**
Rpl32	*Ribosomal protein L32*	Dm02151827_g1
Target Genes		
per	*period*	Dm01843680_g1
tim	*timeless*	Dm01814239_m1
cry	*cryptochrome*	Dm02149911_m1

Table 2-4 Details of RT-PCR probes used

2.8 Statistical Analyses of data

All statistical analyses were performed using GraphPad Prism 7. Normality of the data was first check using the D'Agostino & Pearson normality test, before deciding what statistical tests to use. Data were plotted as mean with error bars showing standard error of the mean, median with interquartile range, or all individual values were plotted as appropriate.

For standard comparisons of two groups of data, means were compared using a two-tailed unpaired t-test. If the data showed a non-normal distribution, the non-parametric Mann-Whitney test was used. For test of three or more groups of data, one-way analysis of variance (ANOVA) was

used for normally distributed data. Post-hoc comparisons were made using the Tukey's multiple comparisons test. For non-normal data, the non-parametric alternative Kruskal-Wallis test was used, with post-hoc analysis conducted using Dunn's test. Experiments with multiple levels were analysed using 2-way ANOVA with Tukey's multiple comparisons test.

For ageing experiments statistical significance was reported relative to the day 1 group of flies.

Throughout the thesis, results from statistical analyses are reported in the figure legends.

3

CHAPTER 3 THE EFFECTS OF AGEING ON CIRCADIAN BEHAVIOUR AND CLOCK OUTPUTS IN DROSOPHILA MELANOGASTER

This chapter presents the results of experiments to measure the effects of ageing on circadian rhythms and sleep in *Drosophila*. Section 3.1 briefly details the study of circadian rhythms and sleep in *Drosophila*. Section 3.2 outlines the results from a longevity study to give some context to the ages of the flies used in subsequent experiments. Section 3.3 reports the results from experiments investigating how ageing impacts upon behavioural outputs of the circadian clock. Section 3.4 reports age-related changes in the structure of daily activity under light-dark conditions. Section 3.5 extends upon the circadian studies to investigate the effect of ageing on sleep under light-dark conditions. Section 3.6 looks at how sleep behaviour continues under constant darkness. Section 3.7 measures the effect of ageing on circadian neuronal remodelling,. The results of this chapter and its conclusions are presented in sections 3.8 and 3.9.

3.1 Introduction

As introduced in Chapter 1, *Drosophila* is an extremely useful model organism for this study into the effects of ageing on circadian rhythms, not least its long use for the study of circadian research, work from which was recognised by the 2017 Nobel prize (Sehgal 2017).

3.1.1 Circadian behavioural analysis in *Drosophila melanogaster*

Drosophila has been used for many decades for the study of circadian rhythms, and even longer as a model organism for genetic research, with this long history providing a large amount of materials and methods

available for researchers. The best example of this is the *Drosophila* activity monitor (DAM) system as explained in Chapter 2, which is used in this study for monitoring groups of flies during the ageing process to record their behaviour in LD conditions, and then during free-running behaviour in DD.

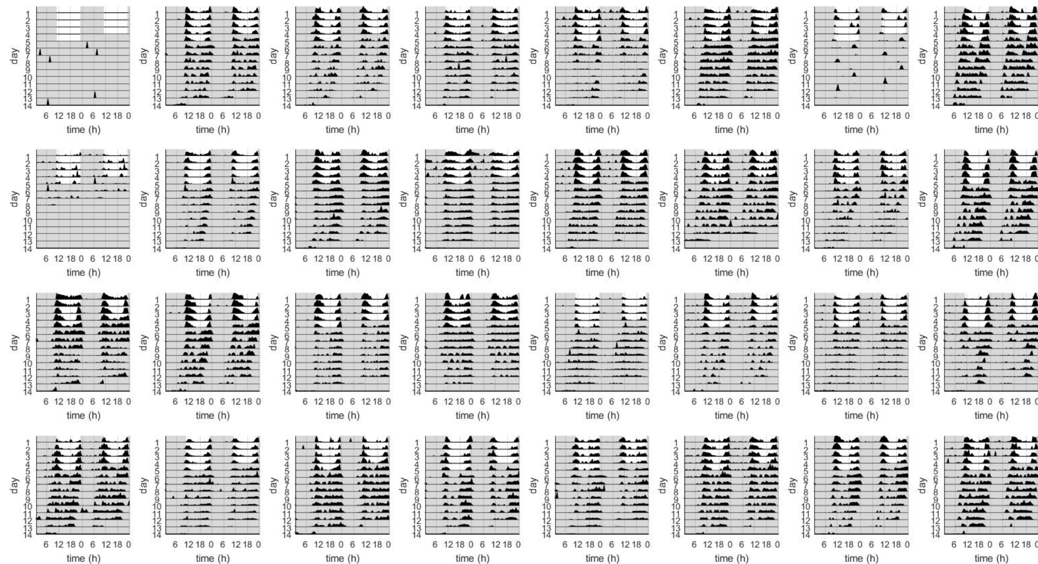


Figure 3-1 Example of the actograms of 32 flies housed in a single DAM monitor

Double-plotted actograms of 32 *iso*[31] wild type flies, shows that within a group of genetically similar and age matched individuals there are a range of behaviours seen, but the overall similarity of the behaviour is similar. White area indicates lights on, grey shading is lights off. Lights are on from 09:00 to 21:00 hrs for 5 days of light-dark, followed by constant darkness. Data is then processed from this form, to remove any flies that died during the experiment, e.g. channels 1, 7 and 9, and is then analysed to look at histograms of activity under the 5 days of LD, and for circadian rhythmicity and period for 7 days of DD.

Each monitor allows for the simultaneous recording of 32 flies, and so for simplicity each monitor was used to record a group of male flies of the same age. Within a group of flies there is an amount of variability that can be observed, and so the flies were analysed separately for their behaviour, and only flies that were classified as rhythmic were used for analysis of circadian period.

Figure 3-1 demonstrates the range of circadian activity recorded from a single DAM monitor. During the five days of light-dark at the start of the experiment flies show typical behaviour of laboratory housed flies, with

morning and evening peaks in activity around the light-dark transitions with a period of less activity during the middle of the day. The regularity of the light-dark cycle during these five days acts as an entrainment cue for the circadian clock

3.1.2 Sleep analysis in *Drosophila*

Sleep in *Drosophila* has been studied since the 1990's, mostly using the DAM system to monitor inactivity, and using the definition of sleep as periods of 5 minutes or longer with no beam breaks (Hendricks et al. 2000; Shaw et al. 2000). In order to make the most of the experimental resources available, the circadian experiments were adapted to combine them with sleep analysis. This simply meant extending the number of days under light-dark conditions from the three that is common for solely circadian studies, to the five used for sleep. Then from the data collected the five days of LD data could also be used for sleep analysis.

3.2 Lifespan of wild type flies under laboratory conditions.

To provide some justification for the classification of *Drosophila* into different age groups, a lifespan assay was performed to calculate the survival of flies maintained under laboratory settings at 25°C using the standard food recipe as described in section 2.1. Flies were segregated by sex and housed in groups of 10 individuals. Groups of flies were flipped onto fresh food every two to three days and the number of deaths scored.

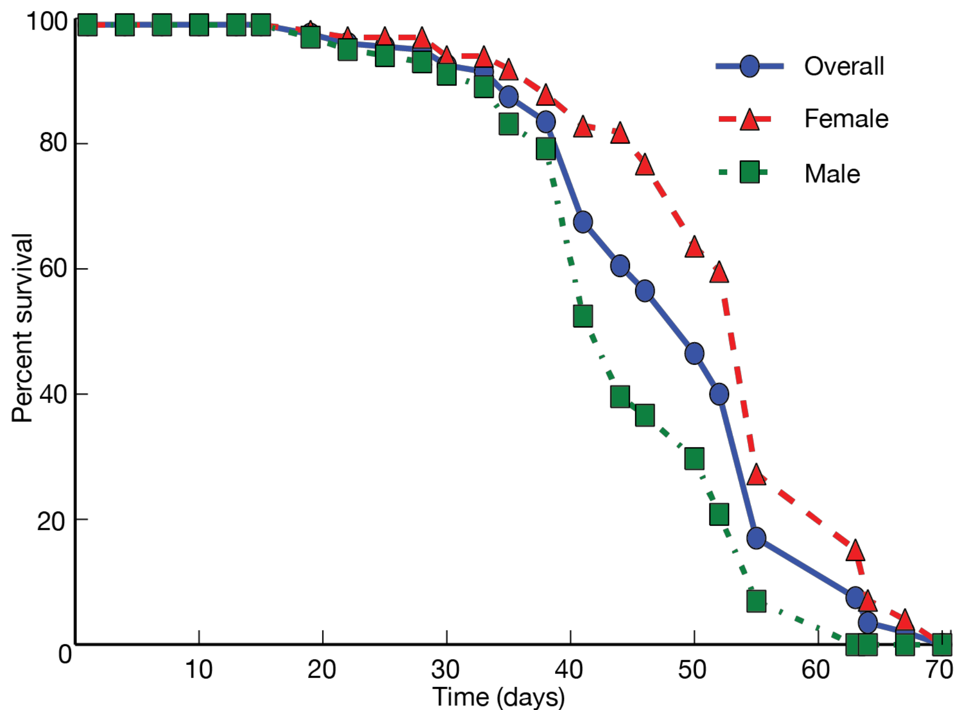


Figure 3-2 Survival curve of group housed wild type (*iso*31) flies

Survival data was plotted, with mean survival calculated using the Kaplan Meier estimator analysis. For male flies the restricted mean survival was 45 days, while for females mean survival was 53 days, with a significant difference in survival between the sexes. Overall mean survival was 49 days.

Results from the survival assay demonstrated that female flies had a higher longevity compared to males. From the longevity assay, the restricted mean survival estimated from the survival curve was 45 days for males and 53 days for females (Figure 3-2). This is consistent with what is widely reported in the literature (Linford et al. 2013) and is supportive of our flies being maintained under healthy conditions.

Using this survival curve and lifespan, a classification of ages was decided for experimental groups with flies less than 10 days post eclosion classified as young, flies aged between 10 – 30 days as middle-aged, and flies older 30 days considered old. For behavioural experiments, only male flies were used for two main reasons. Firstly, male flies are easier to work with for experiments using the DAM system as they will not start laying eggs in the tube which could interfere with recordings. Secondly, there are significant behaviour differences between male and female flies, with most previous

work having been carried out using male flies it is therefore easier to compare results with previous studies.

3.3 Circadian behaviour in Drosophila is weakened by the ageing process.

Locomotor activity recordings using the DAM system have yielded a large amount of data on the changes in circadian activity of *Drosophila* during the ageing process. As flies age their ability to survive in the DAM monitoring tubes seems to decrease, with a much higher proportion of flies failing to survive for the full experiment and having to be discarded from the analysis.

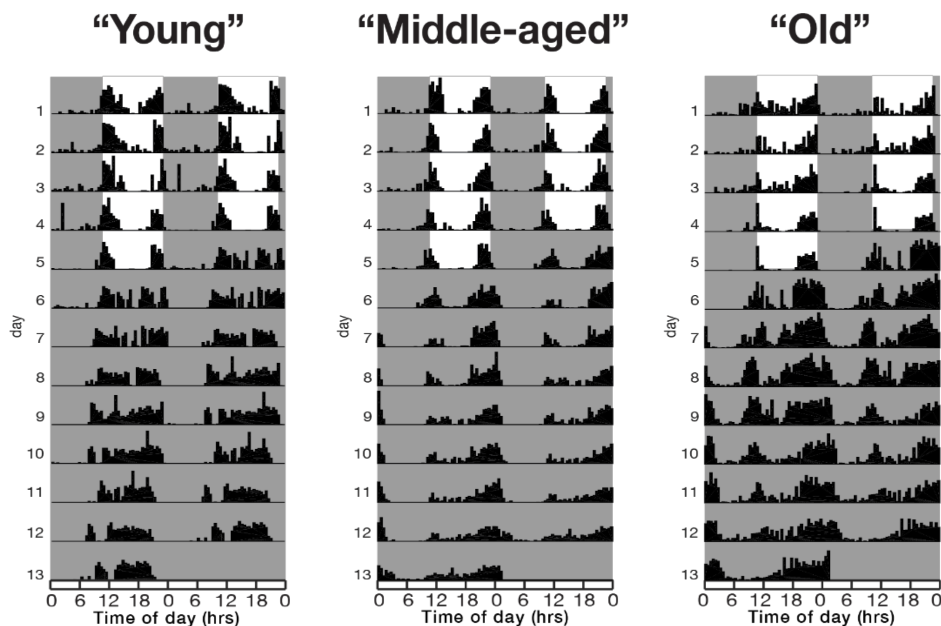


Figure 3-3 Representative actograms of individual young, middle-aged and old wild type flies

Flies age D1, D22 and D36 were maintained in an incubator under 12h:12h light:dark (LD) cycle for 5 days, before continuing under constant darkness (DD). Representative actograms of individual flies. The activity record is double plotted to better visualise circadian activity. During LD lights were on from 0900 to 2100, represented by the light background. Under LD, locomotor activity entrains to the imposed LD cycle with most of the activity occurring in the light phase. After release into DD, the activity free runs with a period of approximately 24 h, reflecting the underlying period of the circadian clock. As flies age the activity under DD becomes more fragmented compared to younger flies.

When maintained under a standard 12:12 LD cycle the vast proportion of flies showed strong entrainment to the lighting conditions, with the majority

of locomotor activity restricted to when the lights were on, with normal morning and evening peaks of activity as well as anticipatory behaviour with increased activity prior to light on or off (Figure 3-3).

Continuing into constant darkness conditions (DD), and allowing the clock to free-run in the absence of any external time cues, significant effects of age were observed on the clock. In young flies the strong behavioural rhythms were maintained, with a clear active and inactive phase, that is maintained for more than a week in DD conditions (Figure 3-3). In contrast flies that were classified as 'middle-aged' or 'old' at the start of the behavioural monitoring period, the rhythms became visibly weaker under DD conditions. This can be observed in how the amplitude of the rhythms flattens and starts to spread out.

3.3.1 The effects of ageing on circadian behaviour

To measure how ageing alters circadian behaviour, locomotor activity under the seven days of DD conditions were analysed using autocorrelation analysis, with the rhythmicity statistic measuring the strength of the observed rhythms and the autocorrelation also giving a numerical estimate of the periodicity.

3.3.2 Number of days used for DD analysis impacts upon rhythmicity statistic values

In the literature the values of RS range widely, and there is not really a consensus of how many days of DD behaviour should be analysed. In order to look how RS changes depending on the number of days used for the autocorrelation analysis, the same analysis pipeline was used and the number of days data used for the analysis varied, with the resultant RS score measured.

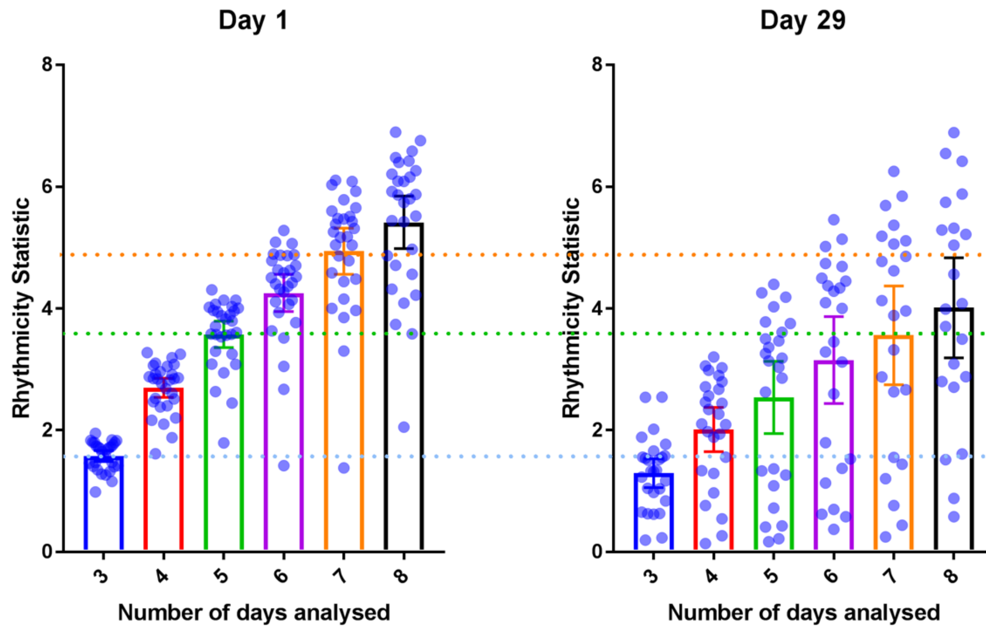


Figure 3-4 Rhythmicity Statistic value increases with number of days used for analysis

Using a different number of days of activity for the analysis gives different values of the rhythmicity statistic, with more days of data giving increased values of RS. Using data from flies of two different ages, Day 1 and Day 29 shows that this effect is consistent between different datasets. Bars show mean ± SEM.

Analysing circadian behaviour over different sized windows of activity demonstrated that increasing the number of days activity used for analysis increased the size of the calculated rhythmicity statistic value. Figure 3-4 shows that using the activity data from the same individual flies but using from three to eight days of activity for the analysis, the RS value increases with longer analysis windows, and the spread of the data points increases.

How the number of days impacts upon the calculated rhythmicity values is important to consider when comparing between different studies which may use different analysis methods. All of the circadian analyses reported in this thesis used seven days of DD activity for consistency and comparability between experiments.

3.3.3 Ageing results in a linear decline in circadian rhythmicity

In order to fully analyse the effects of ageing on the strength of behavioural rhythms, the activity of individual flies was analysed during free-running

behaviour for the first seven days under DD conditions using autocorrelation analysis to calculate the rhythmicity statistic (for details see Methods 2.3).

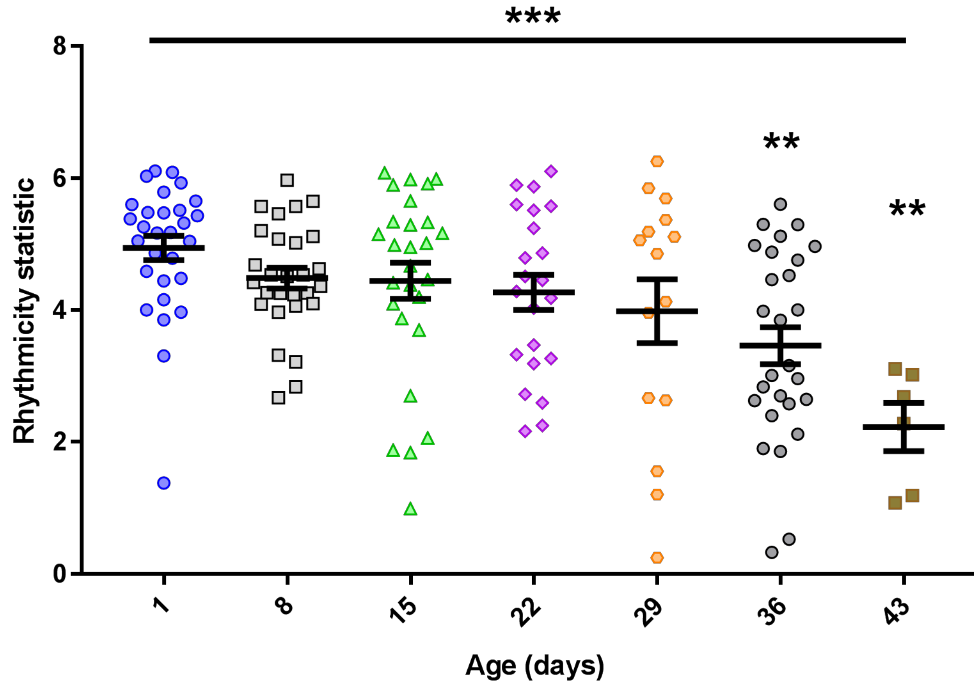


Figure 3-5 Ageing causes a decline in circadian rhythmicity

Circadian rhythm strength was measured by the rhythmicity statistic (RS), with analysis performed across seven days under DD conditions. There is a decline in the RS with age (p=0.0001, Kruskal-Wallis), with a significant difference seen in flies aged 36 and 43 days. Statistical differences tested by Kruskal-Wallis with group significance reported relative to the day 1 group. Error bars show mean ± SEM.

Results of the analysis of circadian rhythm strength show that the rhythmicity statistic decreases with age (Figure 3-5), with the decrease happening from around D15, and becoming statistically significant at D36 and D43. Looking at the individual data points there are still some flies at older ages that can maintain a strong behavioural output, but the group average is reduced by those flies where there is significant circadian disruption.

3.3.4 Ageing results in a lengthening in the free-running period of the circadian clock.

In addition to analysing the strength of the clock, the autocorrelation analysis can also determine the period of the free-running activity of the clock by measuring the lag between behavioural cycles. Firstly, for this analysis those flies that were classified as arrhythmic under the rhythmicity statistic were removed from the dataset for analysis of period.

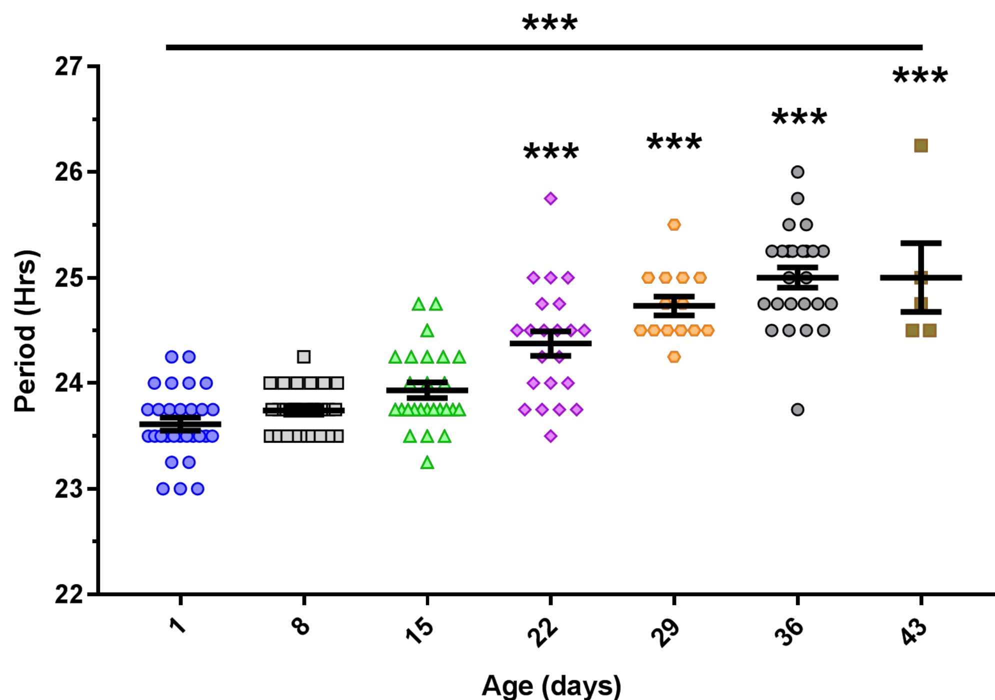


Figure 3-6 Ageing causes a lengthening of the free-running circadian period
Circadian behaviour under DD conditions was analysed for free-running period, with arrhythmic flies excluded from the analysis. There was a significant effect of age on period length ($p<0.0001$, Kruskal-Wallis), with a statistically significant increase observed from day 22 and above. Statistical significance was tested using the Kruskal Wallis test with group significance reported relative to the day 1 group. Error bars show median with interquartile range (IQR).

Analysis of the period of free-running behaviour under DD conditions showed that there is a significant effect of ageing occurring in flies aged D22 and above (Figure 3-6), with the period continuing to lengthen in flies of older ages.

3.4 Under light-dark conditions there is an age-dependent decline in locomotor activity specific to the light-phase

Given that the ageing process is associated with reduced mobility, it is important to consider if any observed differences might be the result of any movement impairments affecting the results. To check this, movement during the five days of light-dark conditions was analysed, and the profile of locomotor activity plotted to look at the effects of ageing.

3.4.1 Total locomotor activity reduces with age

To further look at this the total number of beam breaks across a 24-hr day was calculated, averaged over the five days of monitoring under LD conditions. Even flies aged 43 days at the start of the experiment, the oldest group analysed for circadian behaviour, were capable of recording greater than 500 beam breaks per day. This would seem to indicate that the decline in locomotor movement with age is not related to movement defects severe enough to impact the outcome from the circadian analysis.

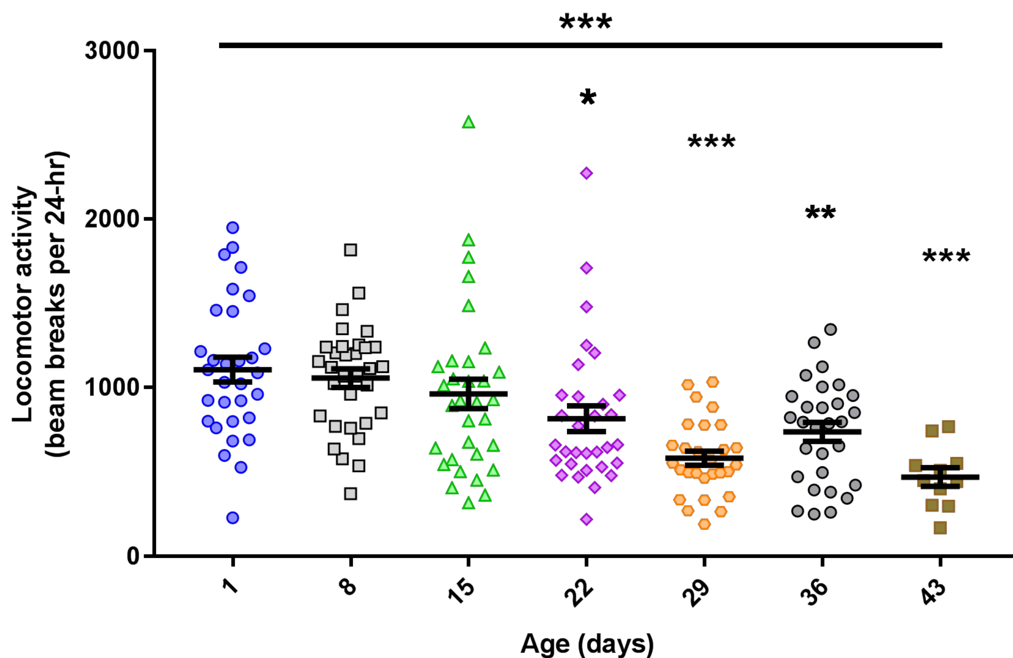


Figure 3-7 Total locomotor output reduces during ageing

There is a significant decline in the total number of beam breaks per day with increasing age measured across the five days of LD ($p<0.0001$, Kruskal-Wallis). Statistical analysis by Kruskal Wallis Test with group significance reported relative to the day 1 group. Error bars show mean ± SEM.

Restricting the analysis to only overall locomotor movement could be masking any time of day effects of ageing on activity. To further investigate this, locomotor activity was analysed separately for the light and dark sections of the LD cycle.

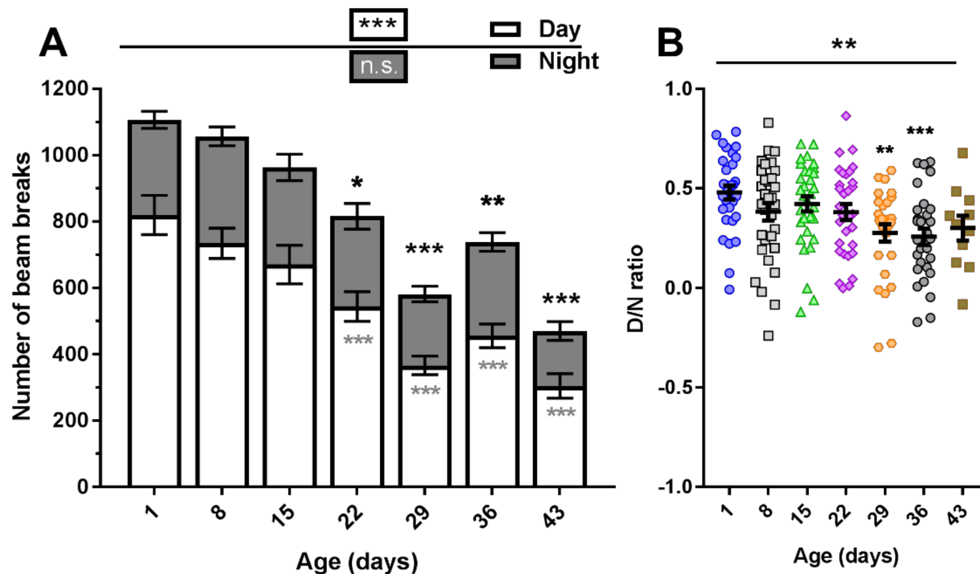


Figure 3-8 Ageing causes a decline in locomotor activity

(A) There is a daytime-specific reduction in the number of beam breaks during the ageing process. In flies older that D22 the number of beam breaks during the light-phase is significantly lower compared to D1. There was no difference observed in the number of dark-phase beam breaks at any age. (B) Age dependent reduction in the D/N ratio, Statistical significance tested by one-way ANOVA. Data plotted as mean ± SEM.

Interesting there was a light-phase specific reduction in the number of beam breaks, suggesting that there might be some differences in the structure of daily activity with age that are not clear from activity histograms (Figure 3-8A). To further analyse the changes in locomotor behaviour, the D/N ratio was calculated, to give a measure of how much of daily activity is occurring during the light-phase. There was an age dependent decline in the D/N ratio (Figure 3-8B) from ~0.5 in D1 flies to ~0.25 from D29 and above, indicating that as flies age they have less of a preference for activity during the day.

3.4.2 *Drosophila* daily activity profile

Drosophila show a typical activity profile under light-dark conditions, where males show high activity in the morning and evening, with

anticipatory activity occurring preceding both the lights-on and lights-off transitions. To measure the impact of ageing on this daily behaviour the structure of daily activity was analysed from flies of different ages.

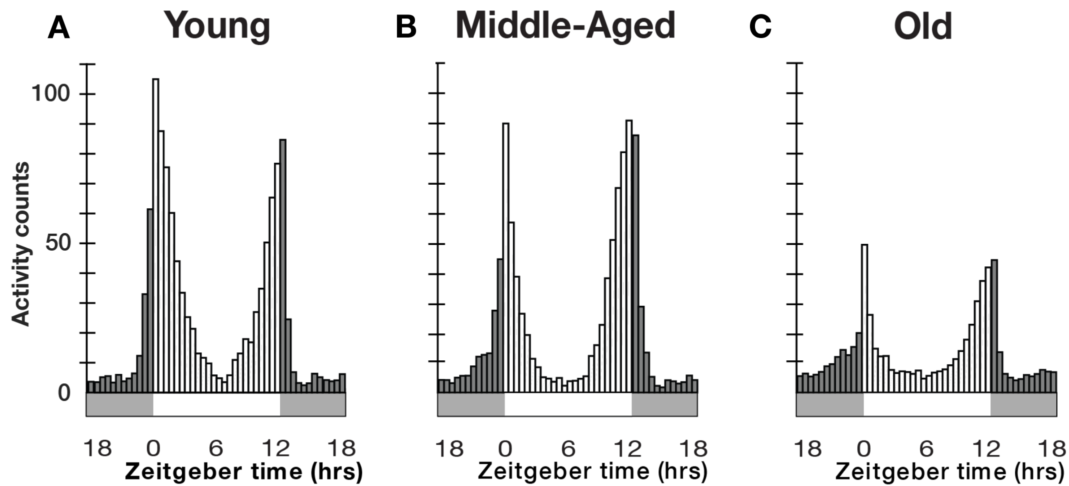


Figure 3-9 Histograms of diurnal activity of young, middle-age and old flies. Plots of groups of *iso*[31] wild type flies averaged over 5 days of LD activity shows that locomotor behaviour is reduced by ageing (A) D1 flies, (B) D15 flies, (C) D29 flies. Old flies still maintain a similar structure of daily activity with morning and evening peaks of activity, but at a reduced level to young and middle-aged flies.

In young flies the histogram of activity (Figure 3-9A) shows that there are strong morning and evening peaks of activity, with anticipatory behaviour in the build-up to both lights on and lights off. Flies that are a few weeks old (Figure 3-9B), show a similar level of activity to young flies, but in flies older than 30 days (Figure 3-9C) there is a clear reduction in the level of locomotor activity, with the morning and evening peaks of activity still clearly visible but at a reduced level.

In order to investigate further how the structure of daily activity of *Drosophila* is altered by the ageing process the morning and evening peaks of behavioural activity were analysed (Figure 3-10). The method for quantifying the strength of the anticipatory activity prior to the light-dark transition was to measuring the size of these peaks in activity, which is under strong clock control and is a good output measure of the clock.

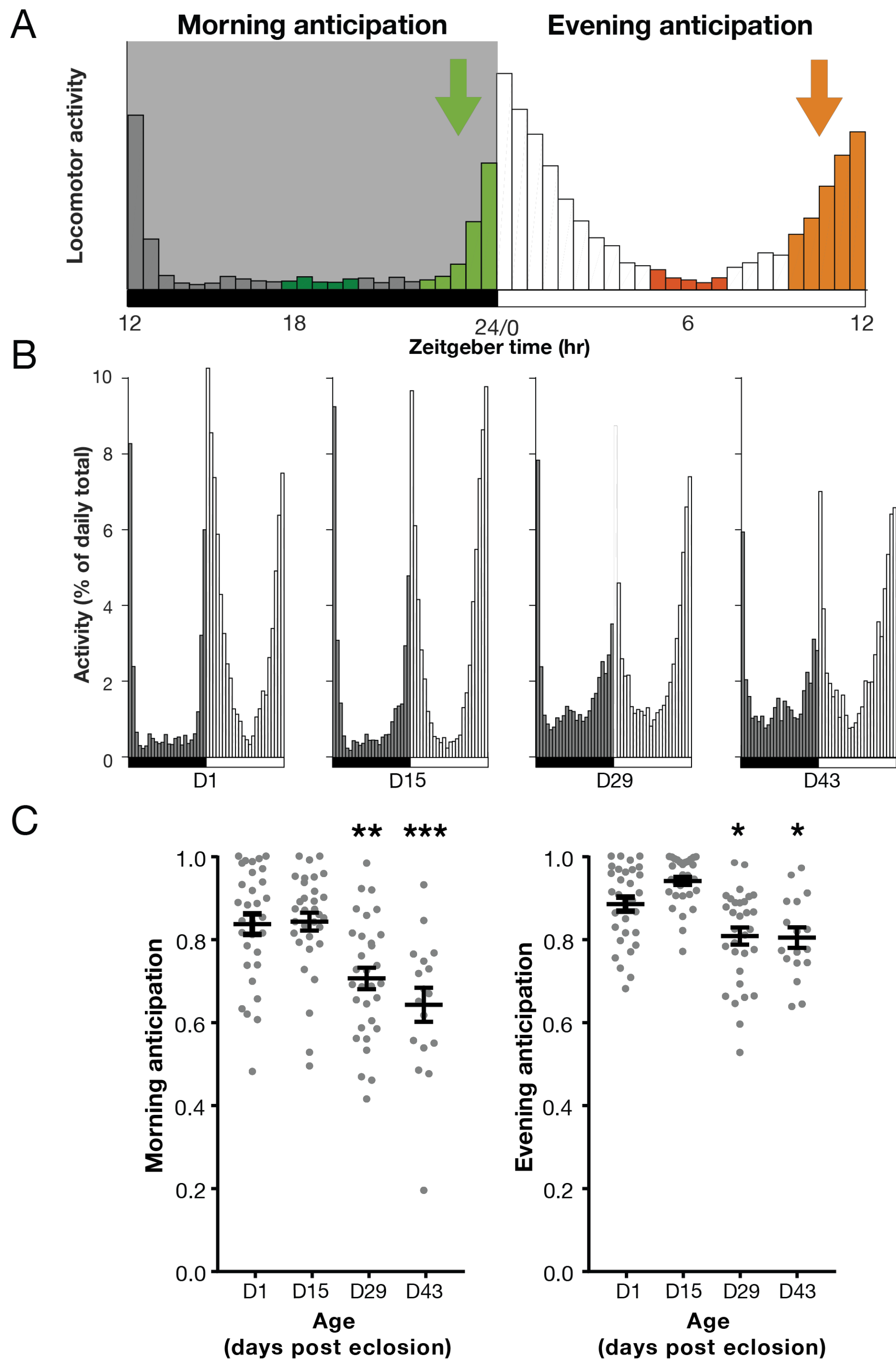


Figure 3-10 Morning and evening anticipation are reduced by ageing
(A) Schematic of morning and evening anticipation index. (B) Normalised daily group activity plots of 1, 15, 29 and 43-day old flies. (C) The morning anticipation index is significantly reduced by age ($p<0.0001$), and evening anticipation is also significantly reduced ($p<0.0001$). N=20-32 for each group, '*' represents $p<0.05$, '**' - $p<0.01$, determined by one-way ANOVA, Tukey post hoc test, and the error bar represent mean ± SEM, group differences compared to D1.

For investigating the activity peaks, activity was first normalised as a proportion of the total daily activity to account for the effects of reduced locomotor output with age as reported in section 3.4. Replotting the histograms of daily activity to be percentage of activity (Figure 3-10B) shows more clearly how the daily activity profile is altered during ageing. In older flies the size of the morning and evening peaks in activity are reduced as a proportion of daily activity, with more activity occurring at night or during the daily siesta.

Quantification of daily activity using the morning/evening anticipation index shows that there is a significant age-dependent reduction in this behavioural index for both morning and evening activity, due to a combination of lower activity peaks and a reduction in the difference between active and inactive phases of activity (Figure 3-10C).

To check differences in activity levels during ageing, the absolute total level of activity measured by beams breaks in the 2.5Hr periods used for calculating the anticipation index was compared (Figure 3-11). Looking at absolute activity levels showed that in the “inactive” windows in the middle of the dark phase or in the daytime siesta, there was no difference in activity levels between flies of different ages. However, when looking at activity in the 2.5Hr anticipatory window, there were significant differences in older flies. This difference was particularly strong in the period of ZT9.5-12 used for calculating evening anticipation. This result shows that absolute activity levels during periods of inactivity were similar between the different aged groups, but that locomotor activity during active periods were impacted during ageing.

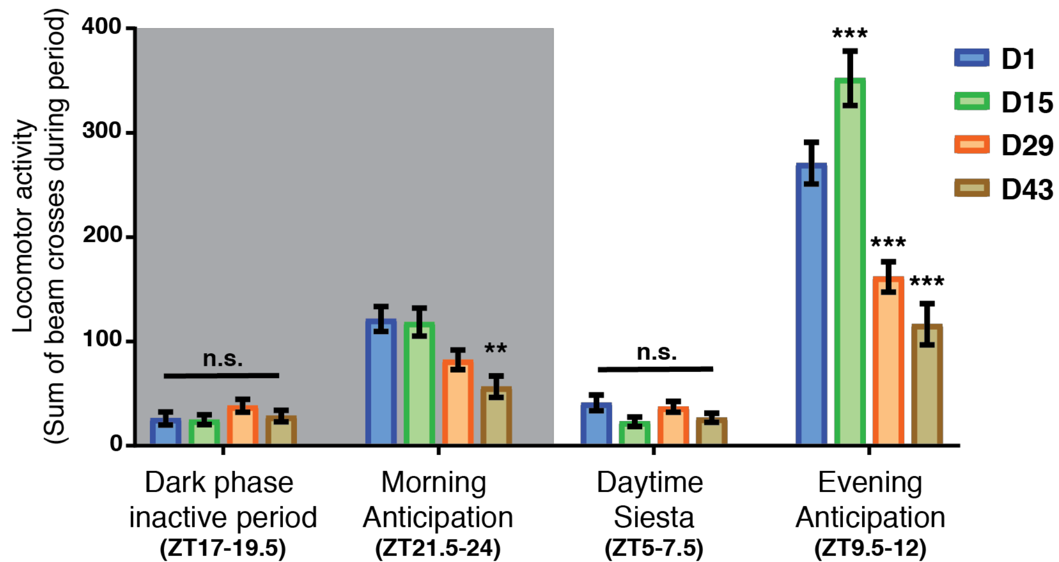


Figure 3-11 Absolute activity levels show there was no effect of age for the "inactive" comparison window but significant differences during the anticipatory window

Absolute total activity for the 2.5Hr windows used for calculating the anticipation index was calculated for the D1, D15, D29 and D43 groups used in the previous analyses. In the “inactive” windows in either the middle of the dark phase or during the daytime siesta there was no effect of age. However, in the “active” anticipatory windows there was a significant effect of age, with a greater effect for morning anticipation, which correlates with the results from Figure 3-11. Data plotted mean ± SEM, with statistical significance tested using 2-way ANOVA, finding effects of age, time of day and an interaction effect, results from multiple comparisons test are reported on the graph.

3.5 Ageing and sleep

Sleep in *Drosophila* is most commonly represented by plotting the duration of sleep in a given 30-minute bin against the time of day. Using the data gathered from the sleep analysis there are a number of different parameters that can be analysed to look at the effects of any manipulation or intervention. Given that there is significant variability in sleep between individual flies, sleep is averaged across each age group and across the five days analysed. As with circadian experiments only male flies were used for sleep experiments.

3.5.1 A *Drosophila* sleep profile

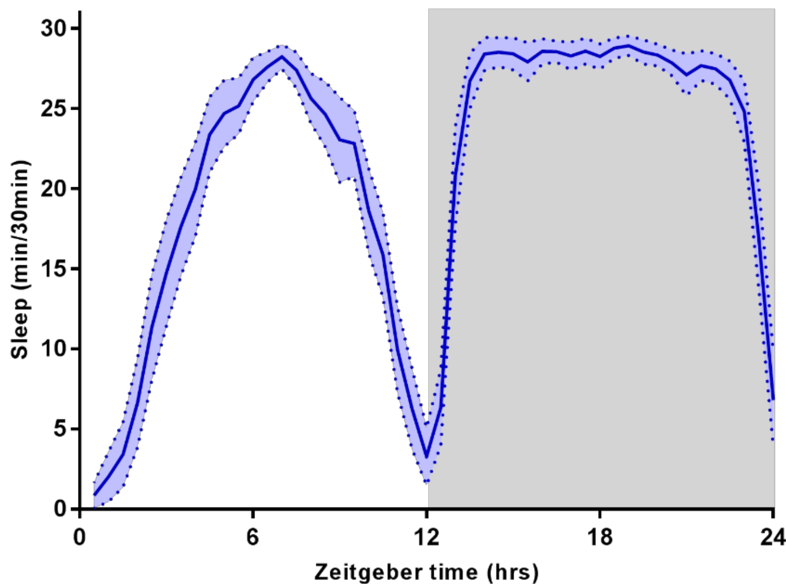


Figure 3-12 A representative daily sleep plot of male wild type *Drosophila*
Sleep is plotted as the total amount of sleep in a 30 min bin plotted against time of day, average over five days of LD. Time is given as zeitgeber time, with 0 indicating when the incubator lights were switch on (09:00). Data plotted as mean with shaded banded representing the 95% confidence interval.

In young male *Drosophila* the sleep plot expectedly mirrors that of the circadian behaviour, with the morning and evening peaks of activity meaning that there is very little to no sleep around lights on and lights off (Figure 3-12). Given that in terms of their activity *Drosophila* are less active when lights are off this is reflected in the sleep profile by high levels of sleep at night, with flies waking up before lights come on.

Looking at group plots is useful to see overall sleep behaviour, but in order to best represent the finer details of sleep it is useful to look at plots of individual flies. Plotting sleep bouts against time as a raster plot displays the variability of sleep across a group of flies the same age (Figure 3-13), showing how the length of the daytime siesta is variable and also showing the sleep comprised of some longer consolidated periods of sleep but also some shorter and more fragmented bouts.

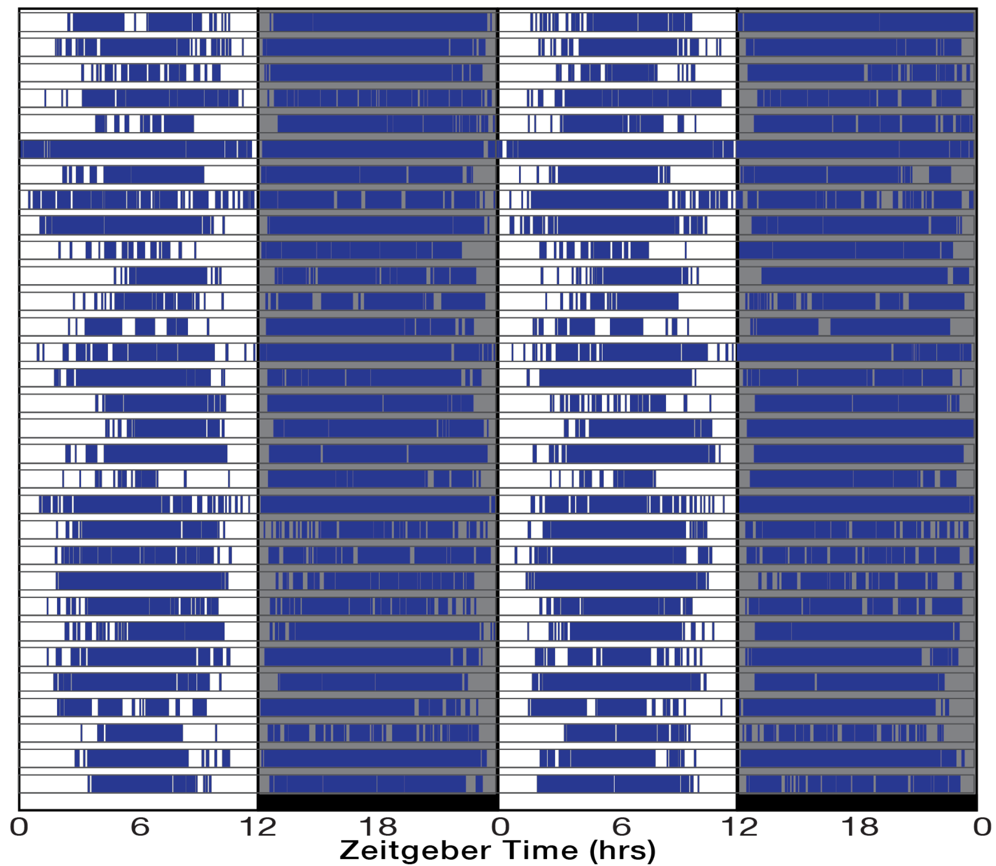


Figure 3-13 Raster plot of sleep episodes of young flies

Each row represents an individual fly, with blue indicating when the fly was classified as sleeping (no movement for >5 min). Sleep is plotted over two days of LD activity with lights on from 0 – 12 hrs zeitgeber time.

3.5.2 Ageing causes significant changes to the daily sleep profile

As with the previous circadian experiments, flies of different ages were compared for their sleep. Comparing between young, middle-aged and old flies, and differences in the sleep profile are clear, with a large increase in day-time sleep in older flies and a shift towards going to sleep earlier in the day.

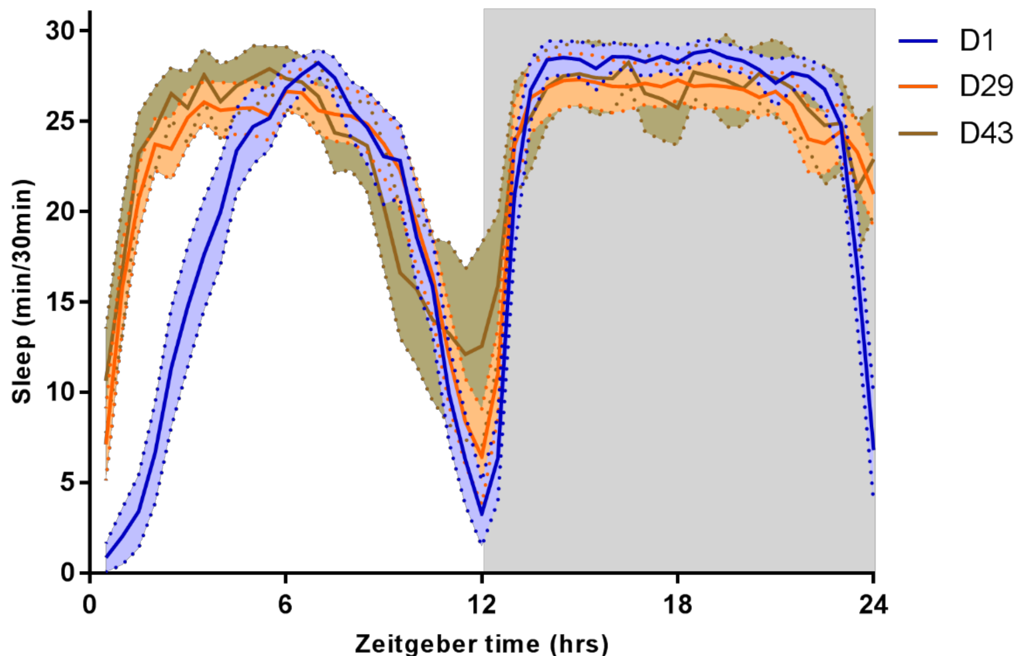


Figure 3-14 Ageing causes large changes to the structure of daily sleep
Groups of flies aged 1, 29 and 43 days were analysed for their sleep across 5 days of 12hr :12hr light-dark cycles. Looking at the daytime sleep it is clear that older flies go to sleep much earlier than young flies but become active again at a similar time near to lights off. Time is given as zeitgeber time, with 0 indicating when the incubator lights were switch on (09:00). Shaded bands represent the 95% confidence interval.

A visual analysis of the daily sleep plot of flies of different ages (Figure 3-14), shows that the structure of sleep is clearly altered by the ageing process, particularly in the period closest to lights on, with older flies sleeping much earlier than young flies. In terms of waking again later in the day, there is little effect of age, and again there is little visible difference in terms of night-time sleep. In order to further interrogate the effects of age of sleep, the duration of sleep can be quantified.

3.5.3 Sleep duration increases with age

The simplest measure is to simply look at the total amount of sleep across a 24-hour period, but this can also be separated between the light and dark phases to look for specific effects on day-time or night-time sleep.

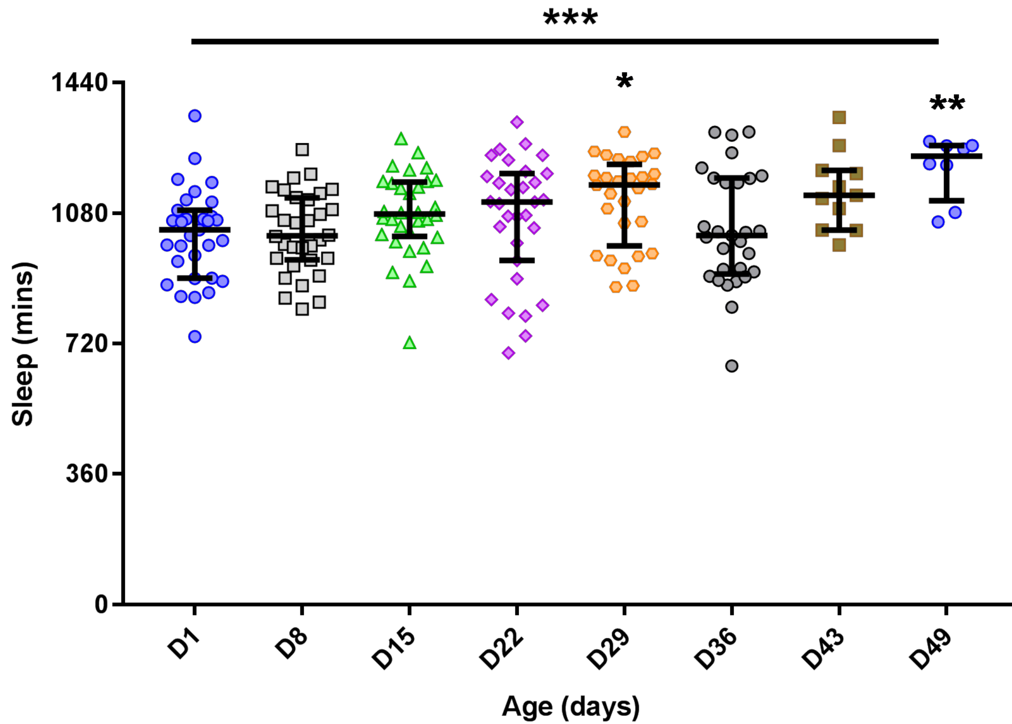


Figure 3-15 Age dependent increase in total sleep duration

Measuring total sleep duration there was an overall effect of age (p=0.0006, Kruskal-Wallis test). Comparing individual groups to D1 flies there was a significant increase at D29 (p=0.0289) and D49 (p=0.0020). Error bars show median with IQR. Statistical difference were tested using Kruskal-Wallis with group significance reported relative to the day 1 group

Simply analysing the total sleep duration is the most direct and crude measure of how ageing affects sleep. Looking across the whole 24-hour day there was a slight but significant increase in total sleep duration (Figure 3-15). Given the clearly observable difference between the sleep profile between day and night, sleep was separated into day-time and night-time sleep and analysed to see if there was a differential effect of age between day and night.

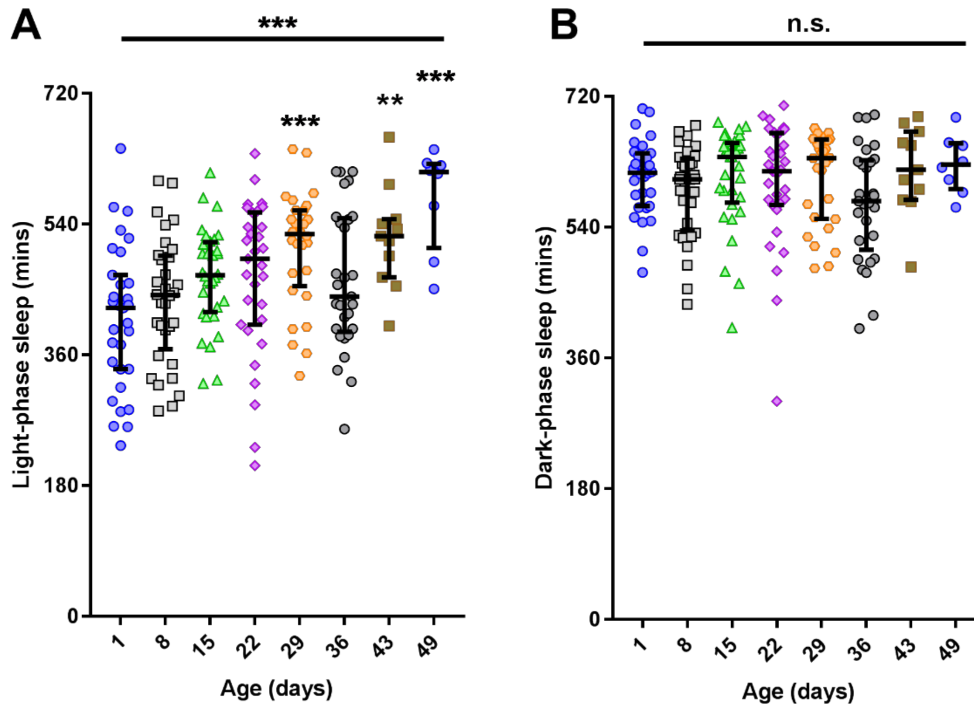


Figure 3-16 Age related increase in sleep duration is specific to daytime sleep
(A) Day-time sleep duration increases with age ($p<0.0001$, Kruskal-Wallis), with a significant difference observed at ages older than D29 relative to the day 1 group (B) No significant effect of ageing on night-time sleep was observed ($p=0.3108$). Error bars show median with IQR.

Looking specifically at daytime sleep, there is an increasing effect of age, with significant differences seen at ages D29 and above (Figure 3-16A). Extending this to look only at night-time sleep, there was clearly no effect of age (Figure 3-16B), with the amount of sleep at night being very consistent at all ages studied.

3.5.4 Sleep latency is significantly shortened by ageing

The most noticeable impact of ageing on the daily sleep profile was the shift in earlier sleeping time during the day for older flies (Figure 3-14). This latency can be quantified by measuring the time from lights on to the first time of recorded sleep, and the same can be done for the latency to lights off.

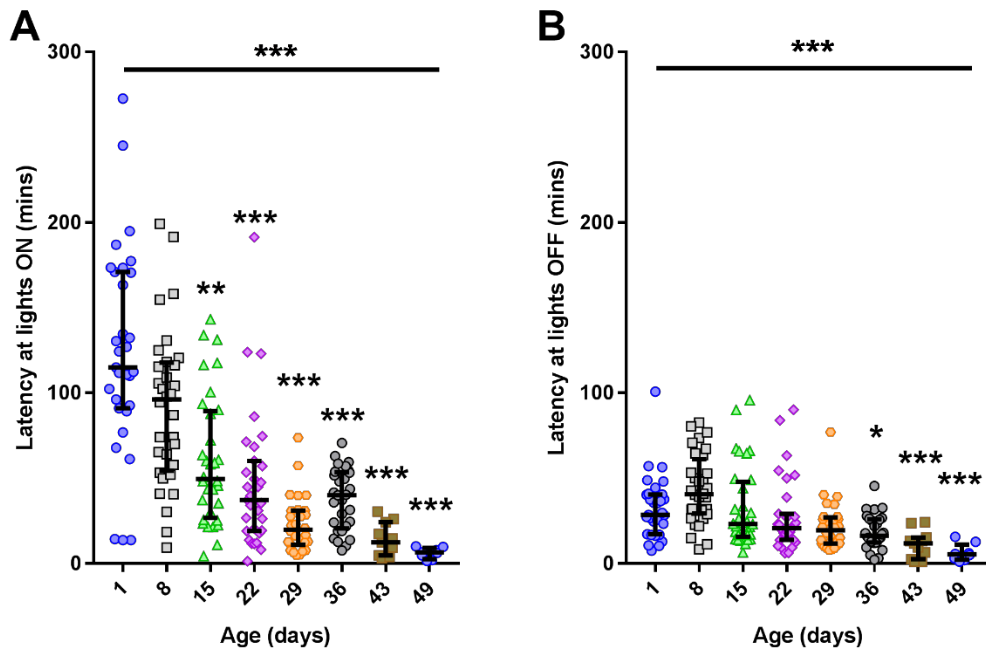


Figure 3-17 Sleep latency is significantly shortened by ageing
(A) Latency at lights on, measuring the delay until the first bout of day-time sleep, is significantly reduced by ageing ($p<0.0001$, Kruskal-Wallis) from as early as D15, with group significance reported relative to the day 1 group. (B) Latency at lights OFF also reduces with age ($p<0.0001$, Kruskal-Wallis), but only significantly so from D36 onwards. Data plotted as median with IQR.

When quantified, sleep latency at both lights on and lights off, significantly reduces with age (Figure 3-17). In terms of latency to lights on, this rapidly decreased with age, from 110 min at D1, to ~50 min by D15, to less than 30 min for flies aged D29 and older. Latency at lights off also shows a significant decrease with age, but the effect is much smaller than that for daytime sleep.

3.5.5 Increased number but slightly shorter sleep bouts

Given that there is an increase in the total sleep duration with age, it is important to look at which sleep parameters are responsible for this change, with there being multiple possible factors which could account for the increase. Investigating the structure of the sleep episodes, shows that while there is a subtle shortening in the length of the average sleep episode (Figure 3-18A), this is more than outweighed by an increase in the number of sleep episodes (Figure 3-18B).

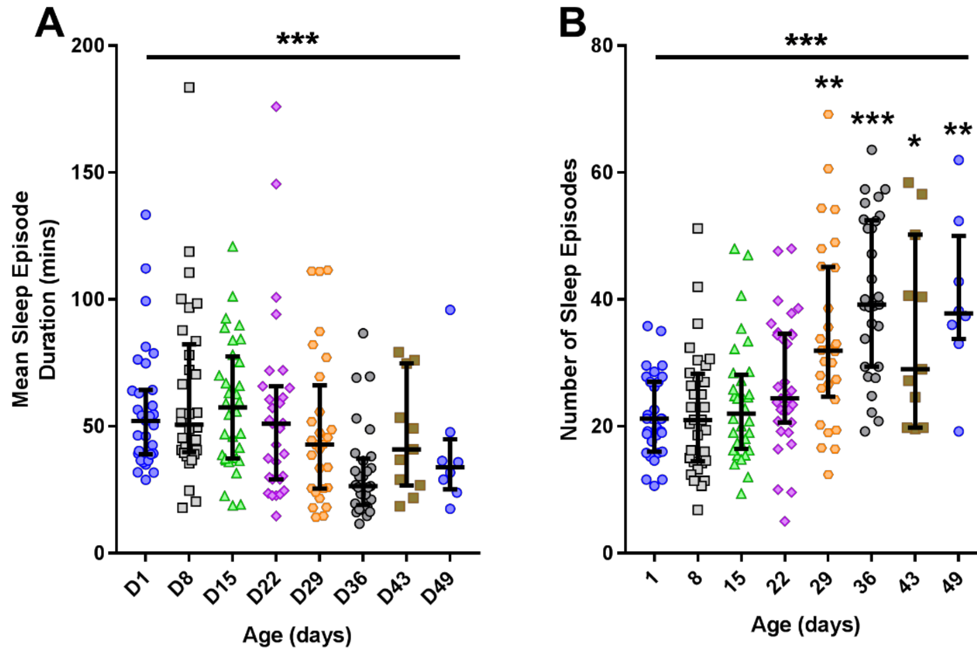


Figure 3-18 Decrease in average sleep episode length is outweighed by an increase in the number of sleep episodes

(A) Mean sleep episode duration shows a significant decrease with increasing age ($p<0.0001$, Kruskal-Wallis). (B) The number of sleep episodes is significantly increased with age ($p<0.0001$, Kruskal-Wallis), with significant effects observed from D22 and older, group significance reported relative to the day 1 group. Data plotted as median with IQR.

3.5.6 Ageing causes a subtle shortening of the longest daytime sleep bout

Recently the longest daytime sleep bout has been proposed as a useful measure given that this long period of daytime sleep is a stable measure (Lamaze et al. 2018). As the name suggests this parameter is simply taken by measuring the length of the longest sleep bout during the daytime siesta.

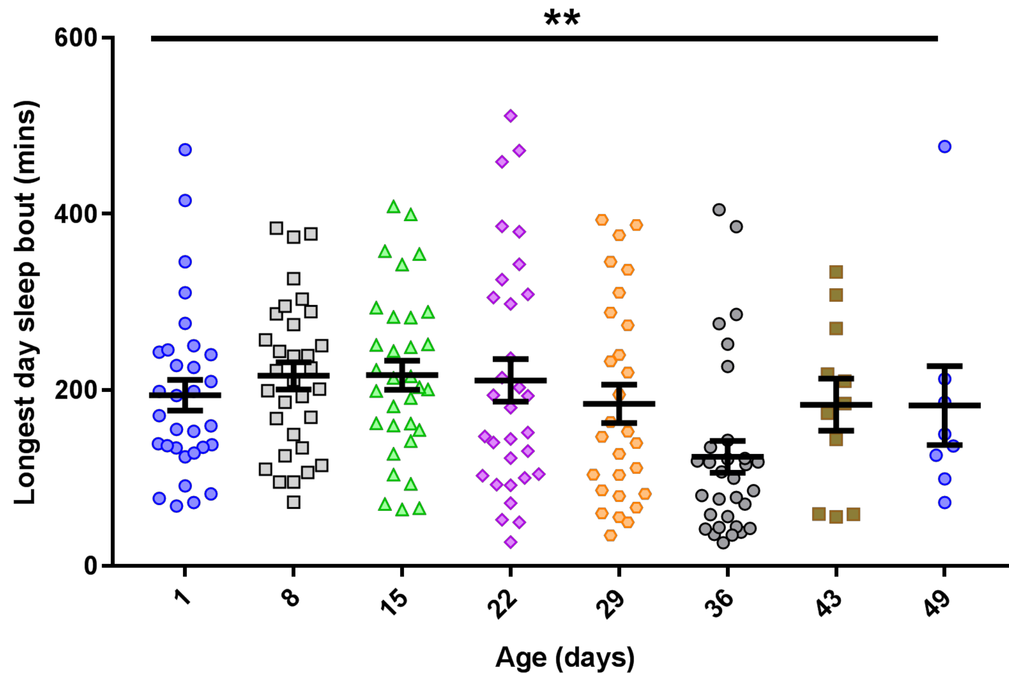


Figure 3-19 Ageing causes a subtle reduction in the longest daytime sleep bout
The duration of the longest sleep bout during the day is a useful sleep parameter, with a small but significant decrease with age (p=0.0017, Kruskal-Wallis test). Error bars represent median with IQR.

Given that the overall duration of day-time sleep increases significantly with age, it might be expected that the length of the longest day-time sleep bout would also increase. Figure 3-19 shows that there is actually a subtle shortening of longest daily sleep bout, which together with the increase in the number of sleep bouts during ageing demonstrated that the sleep-wake cycle becomes more unstable with age.

3.6 Circadian control of sleep under constant conditions is impaired by ageing.

Sleep is controlled both by the homeostatic need for sleep which builds during periods of wake and is reduced by sleep, but also by the circadian gating of the ability to sleep and circadian control over arousal driving periods of wakefulness. Constricting sleep analysis to only LD conditions is only interrogating the behaviour under conditions where the circadian system is synchronised to the zeitgeber provided by the incubator lighting. Looking at sleep during the first few days of DD therefore allows some

ability to investigate sleep parameters under conditions where the underlying control of the circadian system is allowed to act without the entraining effects of the light-dark cycle.

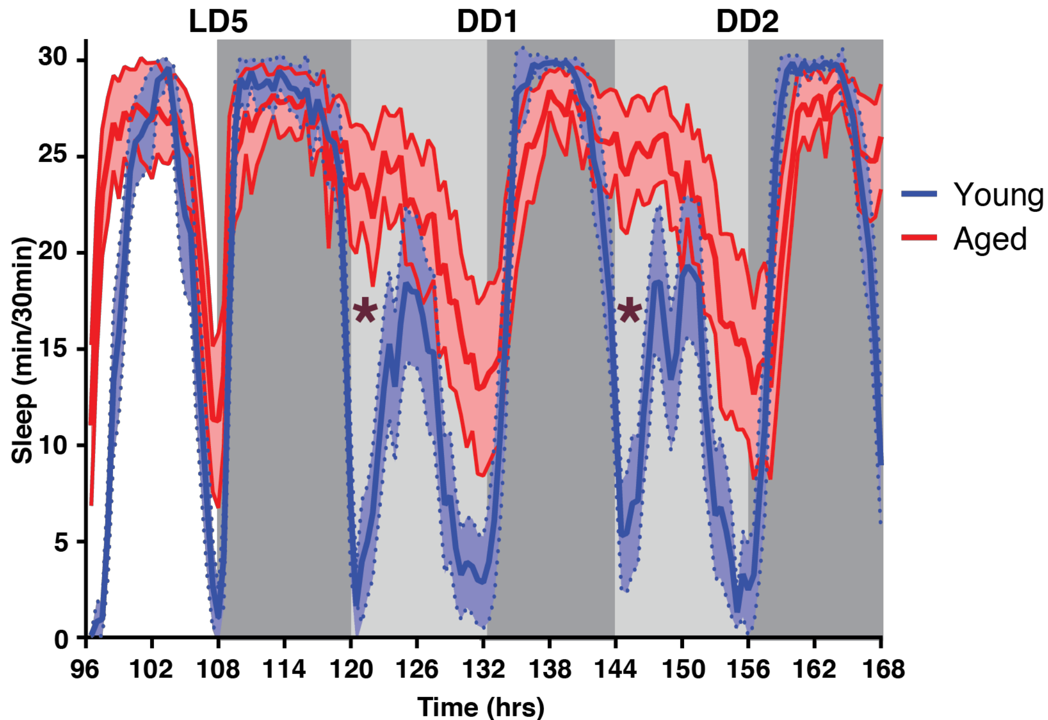


Figure 3-20 Ageing causes loss of morning waking under DD
Plotting the sleep profile of young (D1) and aged (D29) flies from the final day of LD across the first two days of DD shows how there is a large deviation in the sleep pattern in the early "mornings" during DD, indicated by *. Shaded bands represent the 95% confidence interval.

Plotting the sleep profile of day 1 and day 29 flies from the final day of LD conditions across the first two days of DD shows how there are significant divergences in the sleep behaviours of the two groups (Figure 3-20). In order to quantify the effect of DD conditions, the same analysis used for the five days of LD was used to look at sleep parameters separately for the final day of LD (LD5), the first day of DD (DD1) and the third day of DD (DD3).

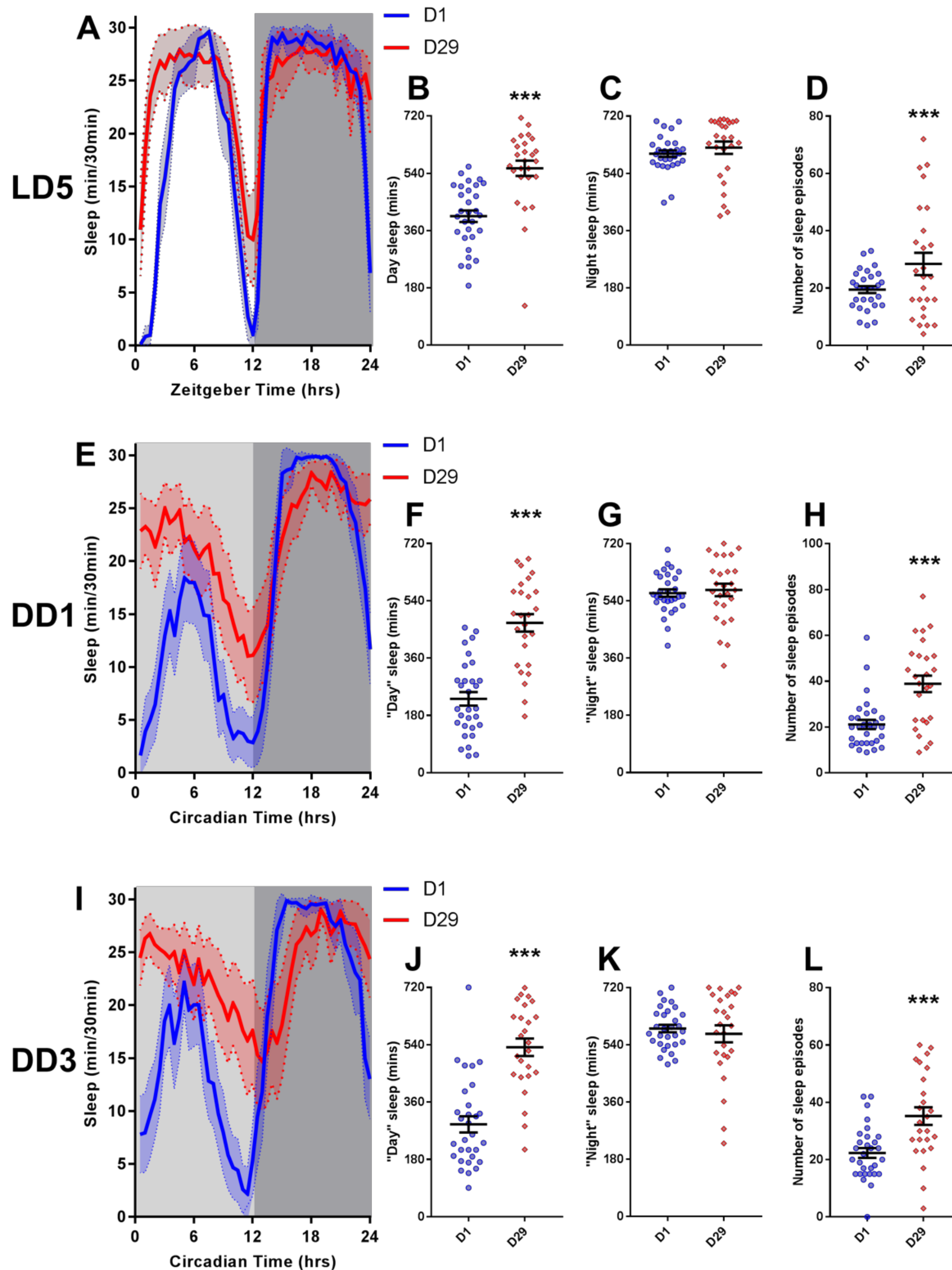


Figure 3-21 Age related effects of sleep persist under DD conditions.
Extending the analysis of sleep behaviour from the five days of LD across to the DD monitoring shows how age-related sleep changes persists under constant conditions. (A-D) Sleep analysis for the fifth day of LD conditions (LD5) shows the difference in sleep profile between D1 and D29 flies, with increased daytime sleep and an increase in number of sleep episodes. (E-H) For the first day of DD (DD1) the sleep plot shows a reduction in daytime sleep for both ages, but the difference between the ages persists. (I-L) For the third day of DD (DD3) the same differences in sleep properties between D1 and D29 are still present. Data plotted as mean ± SEM. Sleep plots show mean with 95% confidence interval. For D1, N = 30, for D29, N = 26. Statistical significance measured using two-tailed unpaired t-test.

Measuring the sleep parameters from LD into DD (Figure 3-21) shows how the differences identified under the five days of LD, namely the increase in day-time sleep duration and sleep episode number with age (Figure 3-16 and Figure 3-18) are still present in DD conditions. Significantly increased day-time sleep was observed in D29 flies compared to D1 flies, at both DD1 and DD3, with significantly more sleep episodes recorded in older flies.

3.7 Measuring how age affects the daily remodelling of PDF-neuron branching

It is well established in the literature that there is a circadian dependent remodelling of the dorsal projections of the PDF neurons (Fernández et al. 2008). This previous work was performed in young flies, and how this remodelling process may be affected by the ageing process has not been investigated. In this section, the same experiment is performed in young and old flies to see if there is any impact of ageing on this remodelling process. In order to compare to this previous study, the same timepoints of ZT2 and ZT14 were used, and flies of 5 and 30 days of age were used to measure the impact of ageing on the circadian remodelling. For imaging of axonal terminals, brains from *Pdf-Gal4>UAS-mCD8::GFP* flies were dissected, processed and counterstained with antibodies against GFP and PDF and imaged using confocal microscopy before quantification using Sholl analysis.

3.7.1 Amplitude of daily remodelling is reduced by ageing

Immunohistochemical analysis of brains from flies dissected at either ZT2 or ZT14 showed strong expression on PDF peptide in both young (D1) and middle-aged (D30) flies. Example images (Figure 3-22) show how the terminals of the LN_V neurons change from a more open state during the early day (ZT2) to a more closed state early at night (ZT14).

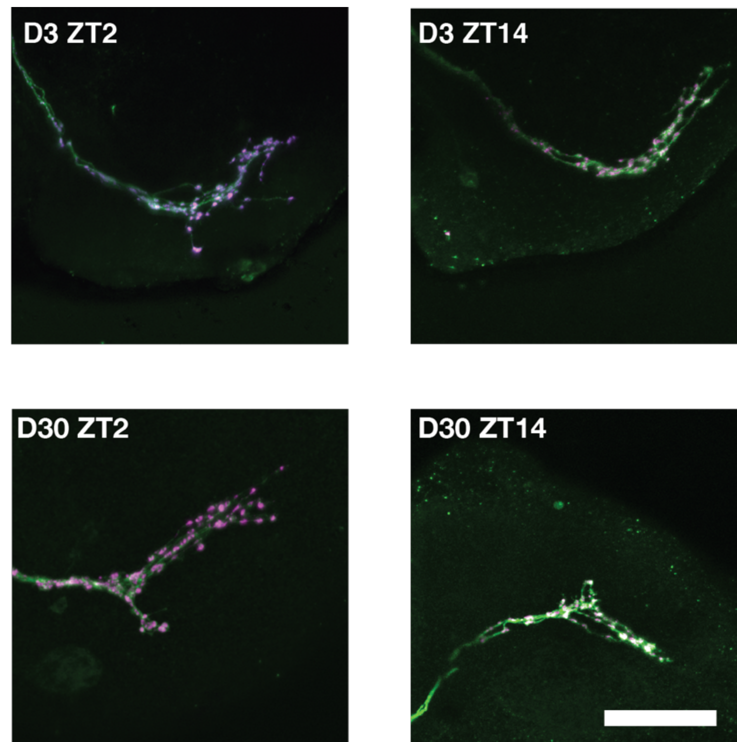


Figure 3-22 Example images of PDF neuronal terminals

Maximum projection of confocal image stacks of the dorsal projections of the s-LNv neurons of *Pdf-Gal4>UAS-mCD8::GFP* flies stained with anti-GFP (green) and anti-PDF (magenta) antibodies. Images show brains from young (D3) and middle-aged (D30) flies dissected either in the early morning (ZT2) and early night (ZT14). Scale bar = 50 µm.

Confocal image stacks of the PDF neuronal dorsal projections (see Figure 3-22 for examples), were traced to produce skeleton images that were used for Sholl analysis to measure the complexity of the branching. The number of times the axonal arbour crosses a series of concentric rings was used as a measure of complexity, with young brains showing significantly more crosses during the early day compared to early night. In middle-aged flies the magnitude of the day-night difference was reduced and there was no longer a significant difference (Figure 3-23).

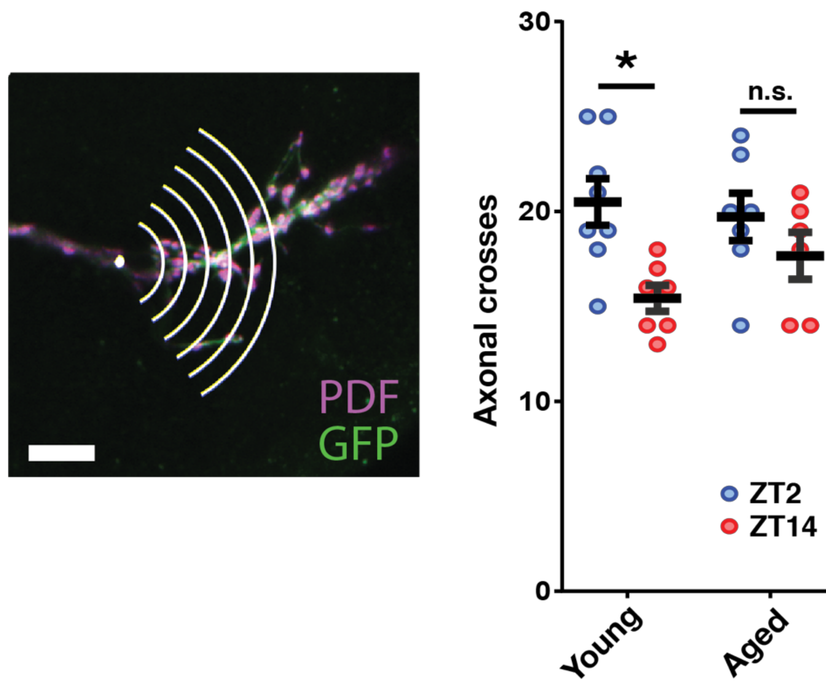


Figure 3-23 Ageing reduces the day-night remodelling of s-LNv neuronal projections

The projections of the s-LNv neurons were quantified using Sholl analysis (left panel) counting the total number of intersections between the concentric rings and the axonal projections, scale bar = 20 µm. In young (D5) flies there was a significant difference between the number of axonal crosses at ZT2 and ZT14. In aged (D30) flies there was still a difference between ZT2 and ZT14 but this was no longer significant. Error bars represent SEM. Statistical significance was tested using a 2-way ANOVA with Tukey's multiple comparisons test.

3.8 Discussion of behavioural monitoring results

The following is a discussion of the results presented in this chapter on the effects of ageing on circadian behaviour and sleep in *Drosophila*. How these results relate to the rest of this thesis are discussed in Chapter 7.

3.8.1 Ageing alters the behavioural output of the *Drosophila* circadian clock

The results presented in section 3.3 demonstrate that there are significant effects of ageing on the circadian system of *Drosophila* monitored by their behavioural outputs when allowed to free-run in constant darkness, i.e. with no *zeitgeber*.

The results presented here are by no means the first study that have investigated the effects of ageing on circadian behaviour in *Drosophila* but these are the most systematic set of experiments that I am aware off. As was mentioned in the general introduction chapter and reiterated at the start of this chapter ageing has been observed to cause significant deficits on the circadian system in humans (Duffy and Czeisler 2002) and in many animals studied in laboratory conditions such as flies (Umezaki et al. 2012) and mice (Valentinuzzi et al. 1997; Banks et al. 2015).

Behavioural experiments presented in this chapter show that there is an effect of ageing on both the strength of circadian clock, with a decrease in the strength of observed rhythms and an increase in the number of arrhythmic flies (Section 3.3.4), but also a lengthening of free-running period in flies that remain rhythmic (Section 3.3.4). The observed age-dependent decline in clock strength is consistent with the findings of previous *Drosophila* research (Koh et al. 2006; Umezaki et al. 2012; Luo et al. 2012). The results presented here go further by demonstrating that weakening of the clock with age is part of a gradual decline that is occurring at a sub-significant level from day 22 and onwards.

The decline in the strength of the clock in free-running conditions becomes significant relatively early in the lifespan of a fly, when other measures of ageing such as mortality rate remain low, and so offers the potential as a useful early indicator and functional measure of the ageing process. When flies were maintained under a light-dark cycle at the start of the experiments, normal behaviour was observed albeit with reducing levels of activity with increasing age, suggesting that it is a weakening of the internal clock that underlies the decline in behavioural outputs rather than any movement defects.

Age-related decline in circadian outputs have been shown in other model organisms, with experiments conducted in mice also identifying clear reductions in the amplitude of circadian locomotor rhythms (Nakamura et al. 2011; Farajnia et al. 2015). By way of comparison to this study, studies in mice have used animals aged up to 24 months of age, again highlighting the

usefulness of *Drosophila* as a model for studying the effects of ageing on circadian rhythms.

3.8.2 Ageing changes the structure of daily activity

Analysis of activity during the five days of LD has revealed how ageing alters the structure of activity and causes a reduction in anticipatory behaviour. Ageing causes a reduction in overall locomotor activity measured by the number of beam breaks recorded by the DAM system, with significant reductions in total movement from D22 and older (Figure 3-8A). This reduction was almost entirely the result of a significantly reduction in only the day-time activity, with no effect of age on night-time activity leading to next investigating in how the daily structure of activity is altered by ageing.

Quantification of these day-night changes in activity was performed using the D/N ratio to measure alterations in the proportion of activity occurring between day and night. The D/N ratio was reduced with age (Figure 3-8B) indicating a reduction in the proportion of activity occurring while the lights are on. Given the crepuscular activity of *Drosophila*, the morning and evening peaks of activity are highly relevant behavioural features and are key components of daily activity.

To measure the effect of ageing on these peaks of activity the anticipatory behaviour in the build-up to the behavioural peak was analysed. Activity peaks were quantified using the anticipation index measuring the proportion of activity occurring in the 2.5 hrs immediately prior to the light-dark transition compared to the 2.5 hrs in the middle of the day or night (see methods). Results from analysis of the morning and evening peaks showed a significant reduction in anticipatory behaviour with age (Figure 3-10C), with a more significant reduction observed for the morning peak.

The LN_V neurons are required for correct morning anticipatory behaviour (Grima et al. 2004; Stoleru et al. 2004) and are an obvious candidate for involvement in an age-related decline in this behaviour. The l-LN_V neurons are also involved in promoting arousal and regulating sleep (Sheeba et al. 2008; Chung et al. 2009; Liu et al. 2014) and provide link between the sleep and circadian systems.

3.8.3 Ageing causes alterations to sleep during diurnal conditions

Observations of diurnal behaviour under a light-dark entrainment regime at the start of the circadian experiments, show that there is a clear decline in the amount of locomotor activity, but that this is particularly clear during the light-phase (Figure 3-8).

In order to investigate sleep changes the experimental design was modified to measure locomotor behaviour in 1-minute bins and extend the period of light-dark to 5 days to allow for analysis of sleep during this period. Analysis of the sleep of groups of male wild type flies during these five days of light-dark showed that ageing has a significant effect on sleep, most prominently during the day-time (Figure 3-14). A study in the early years of *Drosophila* sleep research looking at the impact of ageing found an effect on the strength of sleep-wake cycle with age (Koh et al. 2006). They also found an increase in the number of sleep bouts as reported here but they did not look in detail at other sleep measures and by using 5-min bins for monitoring did not have the same temporal resolution.

The results here demonstrate that the total duration of sleep increases with age (Figure 3-16), across the whole 24-hour period but particularly in the light-phase. This increase in sleep duration was associated with other changes in observed sleep parameters. Sleep is a key output of the circadian clock, with mutations in core clock genes resulting in fragmented sleep (Hendricks et al. 2003), but this can be masked by mutants having normal total sleep amounts.

The larger effect on day-time sleep is interesting because an increase in the amount of day-time sleep is prevalent in human ageing (Kim and Duffy 2018). There was no effect of age on the amount of sleep during the night, but this may be because the amount of sleep at night is already high in young flies and cannot increase further in older flies. One possibility is that the sleep need increases with age, and as such the additional sleep required is occurring during the day.

The increase in the number of sleep episodes with age, combined with a small reduction in sleep episode duration (Figure 3-18), shows that ageing

results in more fragmented sleep, again this is similar to disturbances in sleep found in humans (Kim and Duffy 2018).

3.8.4 Ageing reduces clock outputs

One of the previously identified clock outputs is the daily remodelling of the dorsal projections of the s-LN_V neurons going from a more open state during the day when neuronal excitability is high, to a more closed state at night when excitability is low (Fernández et al. 2008). To test if daily remodelling was still occurring in older flies, day-night changes in PDF terminal morphology was measured in flies aged 30 days (Figure 3-23). Sholl analysis (see methods) show that the magnitude of the difference in complexity between day and night in young flies is reduced in older flies and is no longer a statistically significant difference.

I have used the circadian remodelling of the PDF terminals as a clock output pathway, given that it is impaired in clock mutant flies (Gorostiza and Ceriani 2013). The age-related reduction in the remodelling behaviour provides further evidence of how ageing results in impairments in the circadian system of *Drosophila*. Given the role of PDF release in synchronising difference groups of clock neurons and in driving rhythmic behaviour, an age-related decline in PDF neuronal remodelling has significant implications for reduced output of the circadian system with age.

Some small-scale quantitative PCR experiments were performed to look at how ageing alters clock gene expression. From the experiments I carried out I found that expression of the core clock genes *period*, *timeless* and *cryptochrome* reduce with age. This agrees with what has been reported before by a previous study (Luo et al. 2012), although there is some debate as to. However, this same study also showed that at the protein level the clock still shows strong cycling with age. More recent RNA-sequencing experiments have found that the core clock genes still show strong expression with age (Kuintzle et al. 2017) and a recent study using a luciferase reporter, has demonstrated that the core clock remains robust with age (Zhao et al. 2018).

3.9 Conclusions from behavioural experiments

- Ageing results in a linear decline in the strength of circadian behaviour in *Drosophila* that becomes progressively worse with increasing age.
- Ageing causes a significant lengthening in the free-running period in older flies that maintain behavioural rhythmicity.
- Locomotor activity is reduced in older flies, with a greater reduction observed during the daytime in diurnal recordings.
- Sleep duration is increased with age, with an increase in the number of sleep bouts and a decreased sleep latency.
- Daytime sleep is more significantly affected than night-time sleep.
- Age related changes in sleep continue under constant conditions.
- Circadian remodelling of neuronal architecture is reduced by ageing.
- Expression of core clock genes reduces with age.

Given that the circadian clock system is composed of a group of neurons, the next most obvious step was to investigate how ageing alters activity in clock neurons. This will be the focus of the next results chapter.

4

CHAPTER 4 THE EFFECTS OF AGEING ON THE ELECTRICAL PROPERTIES OF DROSOPHILA CLOCK NEURONS

Chapter 1 introduced the central clock of *Drosophila* as a circuit of approximately 150 neurons that maintain and output timing information. This chapter provides the results for the experiments investigating the effects of ageing on the electrical activity of clock neurons.

Section 4.1 briefly introduces the background to electrophysiological recording. Section 4.2 outlines the experimental approaches taken. Section 4.3 presents the data from patch-clamp electrophysiology experiments of *Drosophila* clock neurons during ageing. Finally, section 4.4 discusses the findings from these experiments.

4.1 Background to electrophysiology recording

Electrophysiology allows the study of the electrical activity of cells. It dates back to the 18th century when Luigi Galvani experimented on frog muscles and realised that electrical activity was a key component of animal physiology (Galvani and Aldini 1797). Recording of electrical activity requires the placing of electrodes into the cell or tissue, and therefore the size of the electrode is a major constraint. Most of the early work made use of the squid giant axon, taking advantage of its massive size to allow for recordings to be made with crude glass electrodes (100 μm in diameter). This allowed recordings of action potentials and measurements of membrane potential to be made (Hodgkin and Huxley 1939). Recording in other tissues clearly required much finer electrodes, and the development of glass micro-pipettes (2 – 5 μm in diameter) allowed for recordings in

skeletal muscle cells, through puncturing of the membrane (Graham and Gerard 1946).

The development of voltage clamp recordings in the squid giant axon by Cole and Marmont, used two electrodes and a feedback circuit to “clamp” the membrane potential at a fixed value and thereby study the flow of current across the membrane (Cole 1949; Marmont 1949). Voltage clamp recordings were used by Hodgkin and Huxley in their experiments describing the currents involved in the action potential and to model the action potential mathematically (Hodgkin et al. 1952; Hodgkin and Huxley 1952b; Hodgkin and Huxley 1952a; Hodgkin and Huxley 1952c).

The next key development was the patch clamp technique (Neher and Sakmann 1976), using the mouth of the pipette to form a tight seal on the cell membrane. In the whole-cell patch clamp technique, after first forming a tight seal on the cell membrane, suction is applied to rupture the membrane within the tip of the electrode whilst maintaining access to the cell (Hamill et al. 1981).

4.1.1 Electrophysiology of Drosophila clock neurons

The electrical activity of the LN_Vs was first shown to be important for their function by genetic manipulations expressing a *Drosophila open rectifier potassium channel* (*dORK*) or human *inward rectifier potassium channel* (*Kir2.1)* to reduce resting membrane potential and silence cells, causing severe defects in circadian rhythmicity and impairments of the molecular clock (Nitabach et al. 2002). As described in Chapter 1, the l-LN_V clock neurons show a diurnal and circadian rhythm in their firing rate and membrane potential (Sheeba et al. 2007; Sheeba et al. 2008; Cao and Nitabach 2008).

Electrophysiological recordings of *Drosophila* neurons are typically more difficult than from mammalian neurons as result of the smaller size of the neurons, with the cell body approximately 5 µm in diameter, and with the cells packed more densely compared to the mammalian brain. The first recordings from *Drosophila* circadian neurons were whole-cell patch-clamp from dissociated l-LN_V neurons, focussing on voltage-gated currents as the

cells did not show spontaneous activity (Lear et al. 2005). Subsequent recordings of l-LN_V in intact brain preparations did not show spontaneous activity, but found circadian modulation of membrane potential (Park and Griffith 2006). Recording conditions were refined for later experiments in which l-LN_V preparations showed spontaneous action potential firing (Sheeba et al. 2007).

As mentioned, recordings from the *Drosophila* adult brain are challenging, and electrophysiological recordings are only routinely performed in a small number of labs (Flourakis and Allada 2015). The small size of the neurons results in poor clamping of the cell leading to recording of membrane potentials more depolarised than their true value (Gouwens and Wilson 2009). The size of *Drosophila* neurons limits access of patch-clamp electrodes to making recordings from the soma, often distant from the location of spike initiation zone of the neuron. Action potentials do not propagate to the soma, and so spiking events recording at the soma in current-clamp are usually much diminished in size (Gouwens and Wilson 2009). As a consequence of these problems, the amplitude of action potentials recorded at the soma are variable between cells types and even between cells, ranging from 10 – 60 mV (Cao and Nitabach 2008; Gouwens and Wilson 2009; Sheeba et al. 2007; Wilson et al. 2004).

4.2 Experimental design for neuronal activity experiments

In order to investigate the impact of ageing on clock neurons I focused on the l-LN_V neurons for electrical recordings, as they are the most well characterised group of clock neurons in terms of their electrical activity and the relative ease of access for making recordings.

Recordings were made using well established protocols (see Chapter 2.7). I used the *PDF::RFP* line of flies, which express a fusion of the *PDF* promotor and *mRFP1*, to fluorescently label and visually identify the LN_V neurons in order to target the patch electrode. Recordings were made from young (D3-5) and middle-aged (D28-35) and aged (D45+) flies, in order to capture the time-points by which circadian behavioural measures start to decline. Recordings were made at time-points during the day and night, looking in current-clamp mode at spontaneous activity and neuronal excitability.

4.2.1 Whole-cell technique

Electrophysiological recordings were performed as described in Chapter 2.7. In current clamp recordings the membrane potential of cells is allowed to freely vary, with the patch-clamp amplifier recording the spontaneous voltage across the cell membrane, or in response to an injection of current.

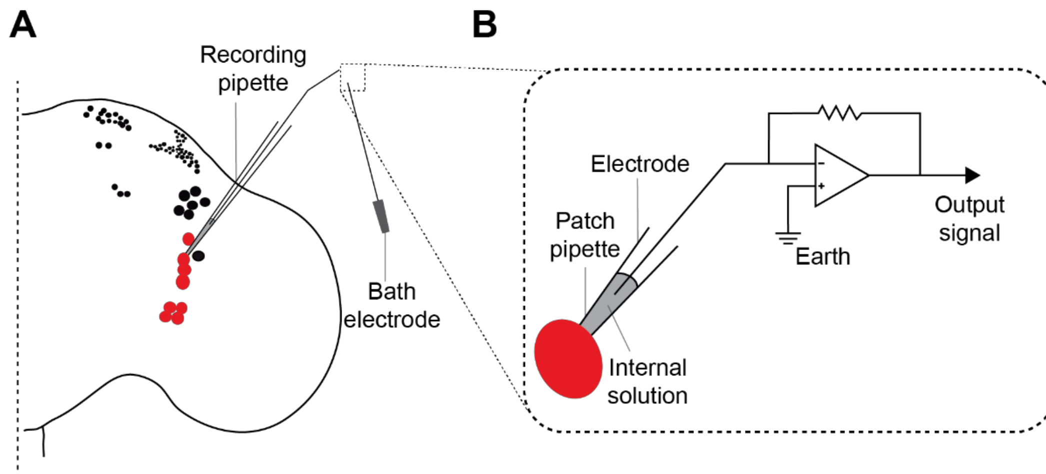


Figure 4-1 Electrophysiology recording diagram

(A) Schematic showing half of the *Drosophila* brain with clock neurons indicated. LNv neurons are shown in red, with an example situation of the recording pipette attached to an l-LNv. (B) Simplified example of the electrophysiological circuit formed when recording electrical activity from clock neurons. A glass pipette allows access to the cell through a patch made in the membrane (cell not to scale). An electrode connected to a highly sensitive amplifier records changes in voltage at the cell membrane. In current clamp, the membrane potential is free to vary, and is recorded by the amplifier. Current can be injected through the patch pipette and changes in membrane potential recorded.

4.3 *The effect of ageing on clock neuron electrophysiology*

To provide some further rationale for the ages of flies used for electrophysiological recordings, a lifespan analysis of the *PDF::RFP* strain of flies that were to be used for electrophysiology was conducted. As described before in section 2.2, flies were housed in vials of 10 and transferred to fresh food every two to three days, with the number of deaths scored and used to conduct a survival analysis (Figure 4-2).

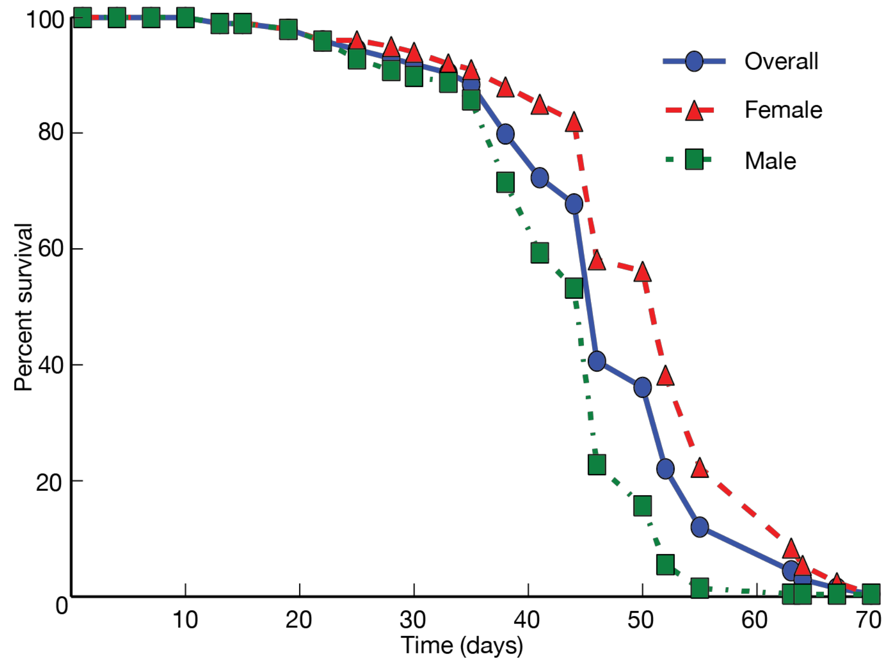


Figure 4-2 Lifespan analysis of *PDF::RFP* flies.
Survival data was plotted using the Kaplan Meier estimator analysis. For male flies the mean survival was 43 days, while for females mean survival was 50 days, with a significant difference in survival between the sexes. Overall mean survival was 46 days.

Results from the survival assay demonstrated the typical situation of females surviving longer than males. From the longevity assay, the restricted mean survival estimate was 43 days for male and 50 days for females (Figure 4-2). These results were similar to the survival rates recorded for *iso*^{31} wild type flies in Chapter 3.2, and the same age groupings can therefore be applied, with flies considered young at <10 days post eclosion, middle-aged from 10 to 30 days and old at >30 days.

4.3.1 Circadian behaviour of *PDF::RFP* flies

Monitoring of behavioural activity of *PDF::RFP* flies in the DAM system showed circadian behaviour was like wild type flies (Figure 4-3). Under LD conditions *PDF::RFP* flies showed typical behaviour with clear morning and evening peaks in activity. Conditions under DD conditions young flies showed circadian behaviour with a period just under 24 hrs, while in aged flies free-running behaviour showed a lengthening of period.

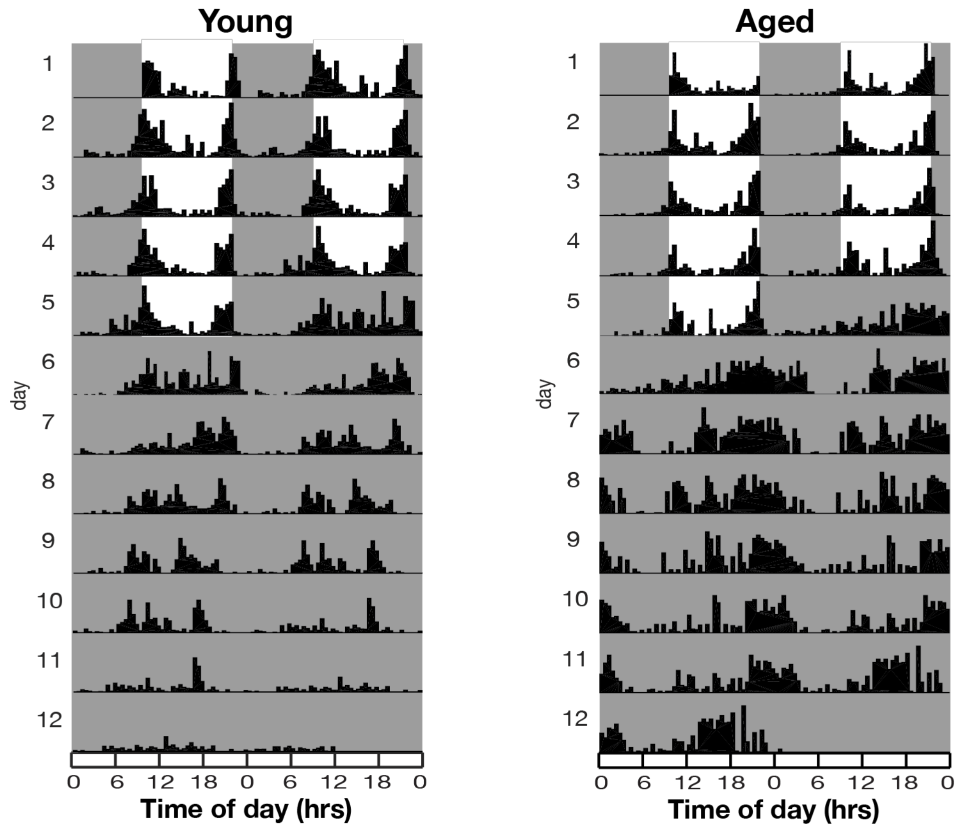


Figure 4-3 Actograms of young and aged *PDF::RFP* flies
PDF::RFP flies show wild type circadian activity, with activity under LD conditions showing clear morning and evening peaks of activity. Under DD conditions young (D1) flies show a free running period just under 24 hrs, while aged (D28) flies have a longer free running period.

4.3.2 Patch-clamp recordings of l-LNv clock neurons

Detailed methods for brain dissection and electrophysiological recordings are listed in section 2.7 of the methods. An example of the electrophysiology setup and the whole brain preparation are shown in Figure 4-4.

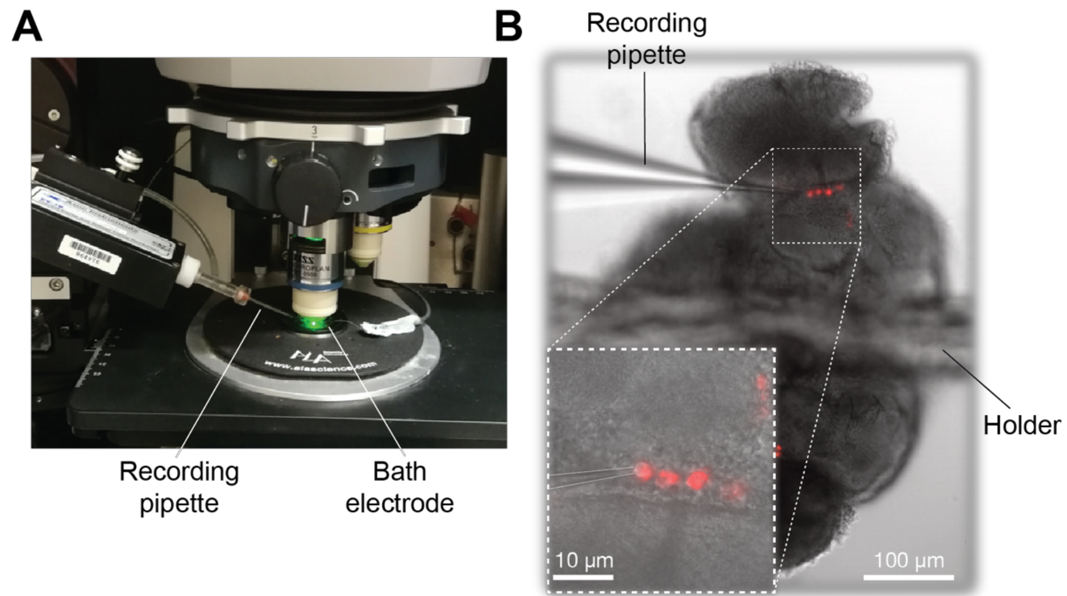


Figure 4-4 Electrophysiology setup for recording from l-LNv neuron in *PDF::RFP* brain

(A) The recording chamber is placed into the microscope and 555 nm green light was used to visualise RFP fluorescence in the brain. The recording pipette is navigated to the area of interest using a micromanipulator. (B) Explant *Drosophila* brain showing *PDF::RFP* expression highlighting the LNvs with red fluorescence and showing the recording pipette in place on an l-LNv cell body (x100 magnification, inset x600 magnification).

4.3.3 Difficulties in recording from aged neurons

It was immediately apparent that making recordings from aged brains was far more difficult than from young brains for a number of reasons. Firstly, the brains of old flies are notably harder to dissect cleanly, with a larger amount of membranous material and fatty deposits requiring more time to dissect. Secondly, and related to this, the cells themselves were more difficult to access due to difficulties in the dissections, and more likely to be covered in remaining membrane which makes forming a good seal and maintaining healthy recordings challenging.

In order to look at differences across the circadian cycle and from the effects of ageing, recorded neurons were measured for spontaneous firing rate, membrane potential, input resistance and the firing response to current. The values for these measures were then compared between neurons recorded from different aged flies.

Current clamp recordings from l-LN_V neurons showed that cells were more active and more depolarised during the day compared to at night, with the difference observed in both young and aged neurons (Figure 4-5).

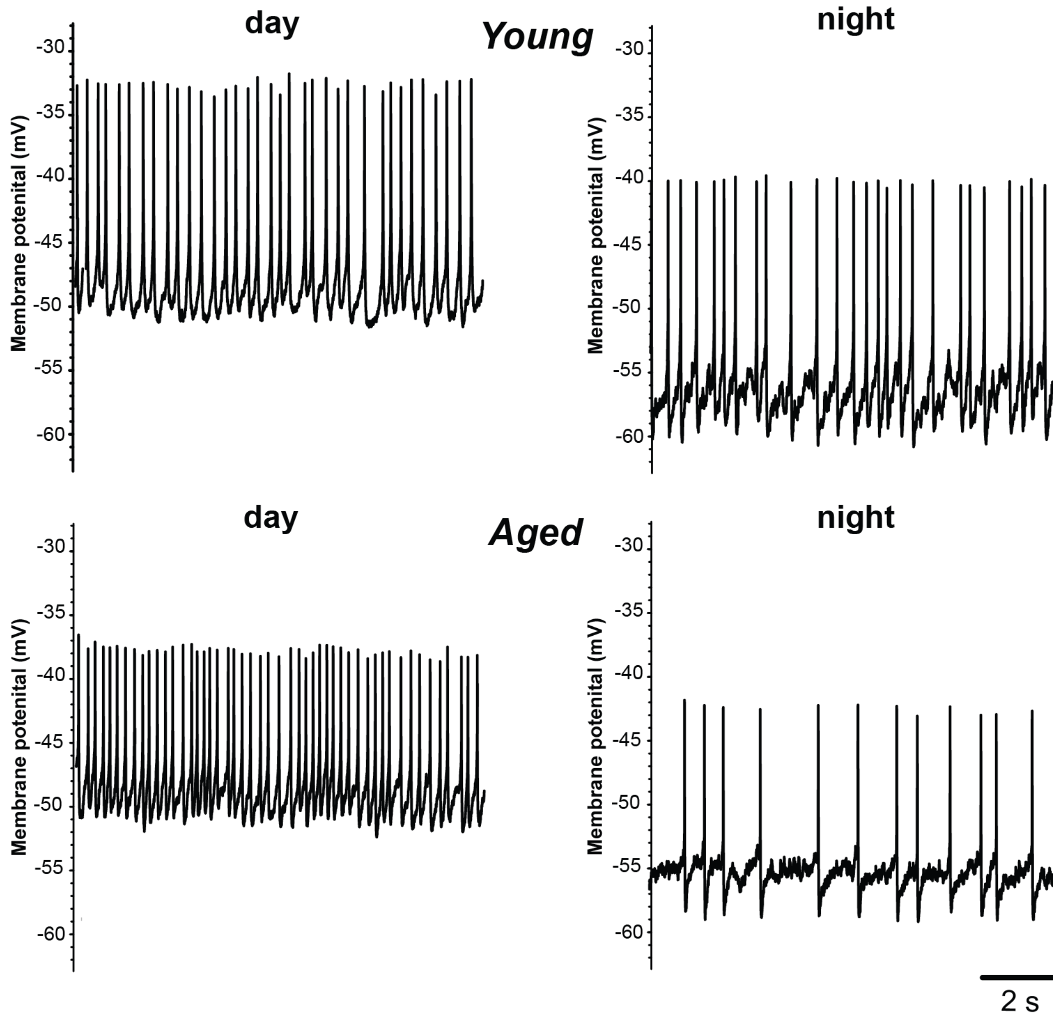


Figure 4-5 Example current clamp recordings at day and night from young and aged l-LN_V neurons

Recording from young (D1-5) and aged (D30-35) l-LN_V neurons during day (ZT8) and at night (ZT20). During the day l-LN_V neuronal activity shows higher firing rate with a more depolarised membrane potential. At night firing rate is reduced and membrane potential is more hyperpolarised.

4.3.4 Analysis of spontaneous activity of l-LN_V neurons

Current clamp recordings made from l-LN_V neurons were analysed to measure the spontaneous firing rate (SFR), which is simply the average number of action potentials fired per second averaged across a 120 second analysis window. In young (D1-5) l-LN_V neurons I found that for recordings

made during the day the mean SFR was 3.27 ± 0.40 Hz compared to 1.01 ± 0.18 Hz at night (Figure 4-6), showing a significant difference in SFR between day and night (Figure 4-6). A higher proportion of cells recorded at night were silent (i.e. they did not produce action potentials in the window of recording).

Moving to recordings of middle-aged neurons (D28 – 35), mean SFR was 4.05 ± 0.53 Hz during the day and 1.01 ± 0.17 Hz at night, again showing a significant difference between day and night. In the old group of flies (>D45), SFR was 1.91 ± 0.69Hz during the day and 2.16 ± 0.65Hz at night, with no significant difference but the N number for the oldest group of flies was limited to four recordings. Analysis of the effect of age using 2-way ANOVA found no significant effect (p=0.59).

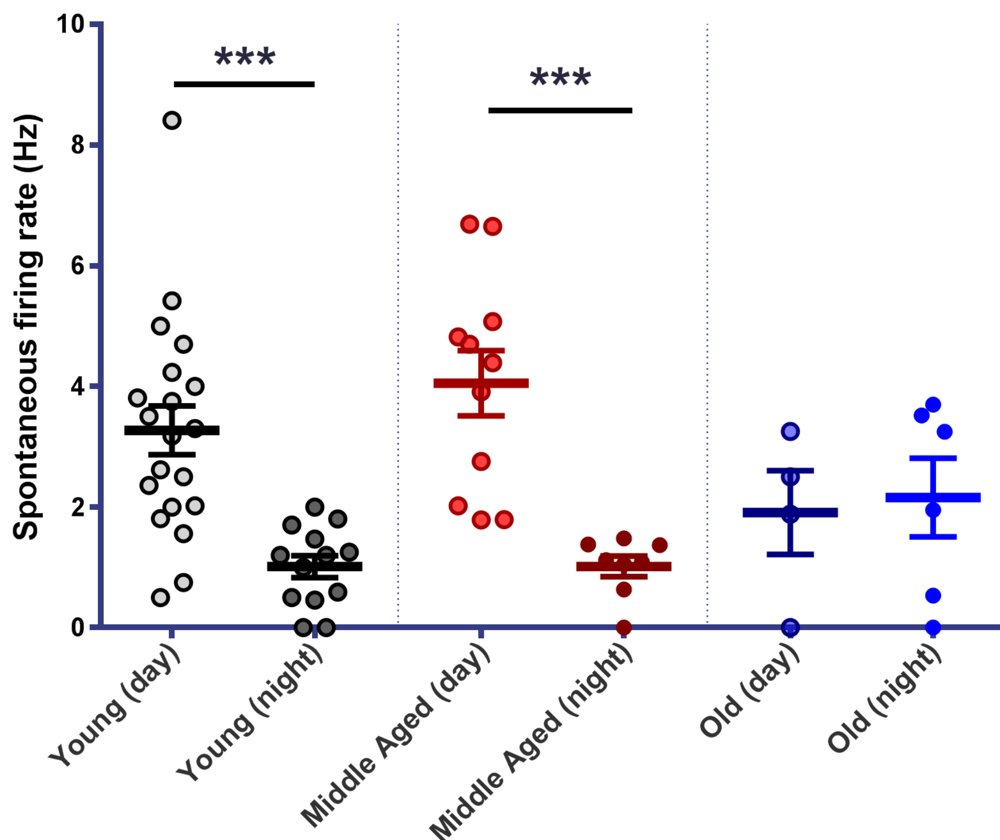


Figure 4-6 Analysis of spontaneous firing rate of l-LN$_V$ neurons during the ageing process

Spontaneous firing rate was measured from l-LN$_V$ neurons at day and night from young (D1-5), middle-aged (D28-35) and old (D45+) flies. Young and middle-aged flies showed a clear day-night difference in firing rate. There was a significant time of day effect

($p<0.001$), but no effect of age ($p=0.5851$). (2-way ANOVA, Tukey's multiple comparison). Error bars indicate mean ± SEM. N = 20, 13, 11, 8, 4 and 6.

4.3.5 Membrane potential persistently demonstrates daily changes in older flies

Membrane potential was calculated by manually fitting a baseline across the recording trace to describe the membrane potential between action potential firing. Measuring young neurons during the day, the membrane potential was -52.3 ± 0.8 mV and at night it was -57.45 ± 0.9 mV, with a significant difference between day and night (Figure 4-7).

In middle-aged neurons, membrane potential during the day was -46.87 ± 1.6mV and at night was -59.13 ± 0.8 mV, again there was a significant day-night difference. In the oldest group of neuron daytime membrane potential was -53.8 mV and at night -58.67 mV, with no significant difference. Analysis for the effect of age found no significant difference ($p=0.096$, 2-way ANOVA).

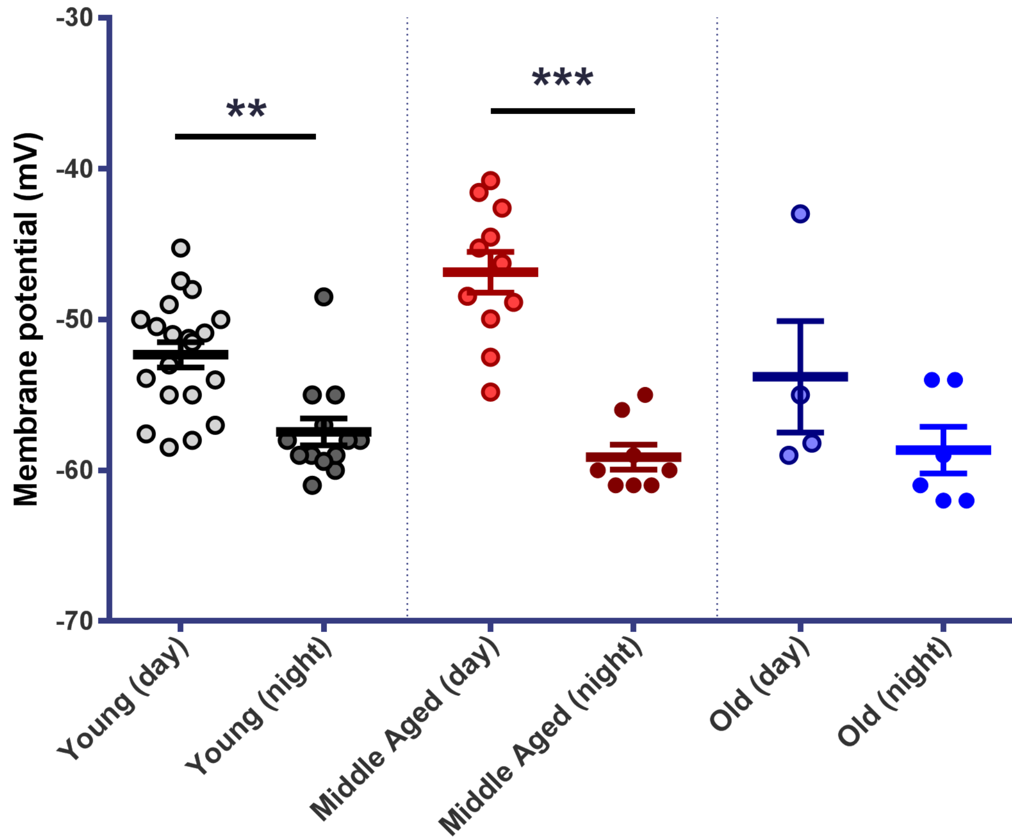


Figure 4-7 Quantification of membrane potential of l-LNv neurons during ageing

Membrane potential was recorded from l-LNv neurons at day and night from young (D1-5), middle-aged (D28-35) and old (D45+) flies. l-LNv neurons demonstrated a day-night difference in membrane potential. There was a significant time of day effect ($p<0.0001$), but not a significant effect of age ($p=0.0956$). (2-way ANOVA, Tukey's multiple comparison). Error bars indicate mean ± SEM. N = 20, 13, 11, 8, 4 and 6.

4.3.6 Excitability of l-LNv neurons

Cellular excitability of clock neurons was measured by injecting depolarising currents into the cell in current clamp mode and measuring the effect on firing rate. Current steps of +2 pA were used, starting from rest at 0 pA and going up to +40 pA, following an established protocol (Julienne et al. 2017). Example traces of neurons from the current injection protocol can be seen in Figure 4-8. Injecting current results in depolarisation of the membrane and results in an increase of action potential firing.

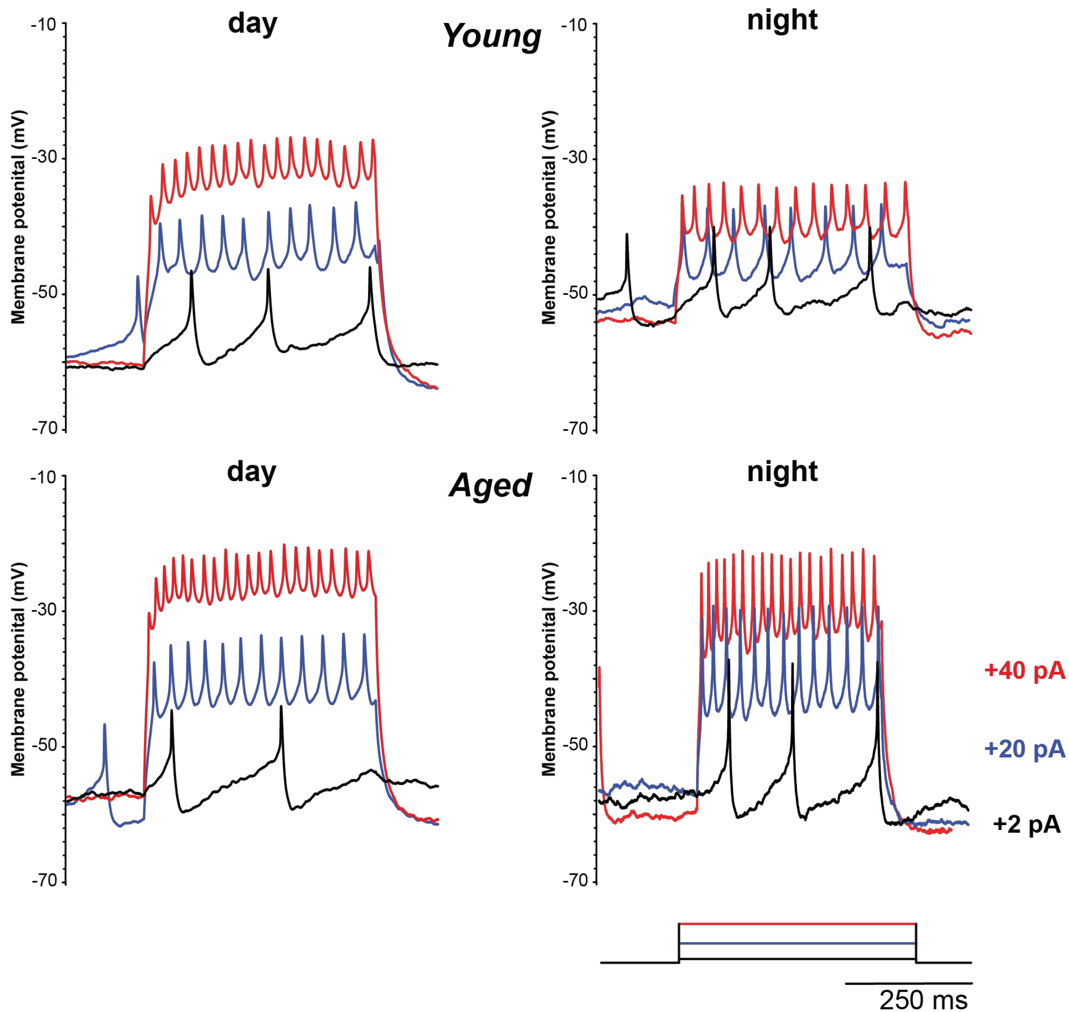


Figure 4-8 Examples of current clamp traces of current injection protocol
The firing response of young (D1-5) and aged (D28-35) l-LNv neurons to current injection during the day (ZT7-9) and at night (ZT19-21). Example traces show the response to a 500 ms current injections of +2 pA, +20 pA and +40 pA.

To quantify the cellular excitability, the firing rate in response to a +40 pA current injection was quantified and compared between neurons of different age recorded at either day or night. Measuring young neurons during the day the firing rate in response to current injection was 34.1 Hz, while at night it was 30.0 Hz, with no significant difference. In middle-age neurons day firing rate was 30.4 Hz and at night it was 33.4 Hz, with no significant difference. Finally, in the oldest group of neurons, firing rate during the day was 25.0 Hz and at night was 27.7 Hz. There was no effect of age when comparing between all the different groups (p=0.25, 2-way ANOVA).

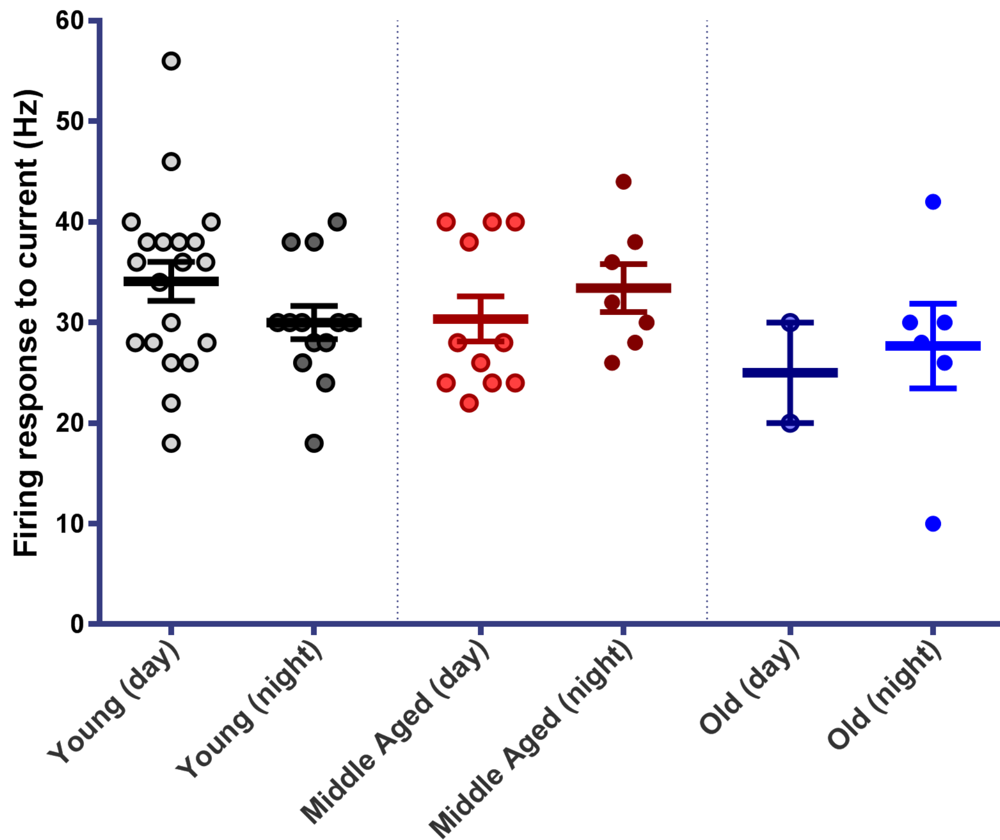


Figure 4-9 Analysis of the induced firing rate of l-LNvs in response to a +40 pA current step

Firing response to a +40 pA current injection was recorded from l-LNv neurons at day and night from young (D1-5), middle-aged (D28-35) and old (D45+) flies. l-LNv neurons demonstrated no effect of time of day (p=0.8376) or age (p=0.2546). (2-way ANOVA, Tukey's multiple comparison). Error bars indicate mean ± SEM. N = 20, 13, 11, 7, 2 and 6.

4.3.7 Neuronal input resistance dramatically decreases with age

Input resistance (R_{in}) is routinely monitored during recordings to assess recording quality, as a drop in R_{in} during a recording is indicative of that neuronal health is falling (Murthy and Turner 2013). R_{in} can be calculated by injected hyperpolarising current into a neuron and measuring the response of the membrane potential (Flourakis and Allada 2015). Hyperpolarising steps from -40 pA to +5 pA in 5 pA intervals were used (Figure 4-10A). The recorded membrane potential to each of the current steps can then be plotted to produce an I-V (current vs voltage) curve (Figure 4-10B) from which the R_{in} can be calculated from as the slope of the

curve. From previous studies the reported range of R_{in} values for l-LN_V neurons is ~ 2 - 4 GΩ (Cao and Nitabach 2008).

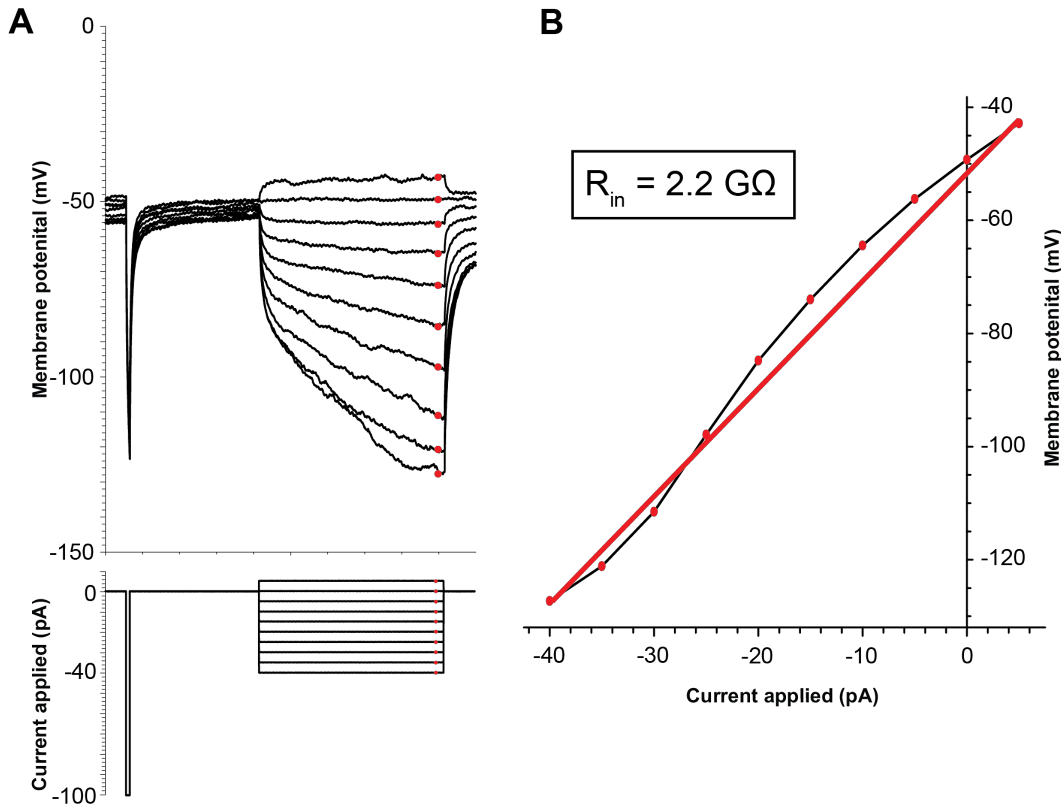


Figure 4-10 Calculation of neuronal input resistance

(A) Input resistance of l-LN_V neurons was measured by the response in membrane potential to steps of current injection. Current from -40 pA up to +5 pA, in +5 pA steps, was injected and the change in membrane potential recorded at the end of the current injection. (B) A current vs voltage (I-V) curve was plotted and the slope of the curve used to calculate the input resistance (R_{in}).

Previous studies of l-LN_V neurons have focused on young neurons and so I was interested in the impact of ageing on the R_{in} of the l-LN_V neurons. Input resistance was recorded as described above at the start of the beginning of a neuronal recording from an l-LN_V. For young neurons the recorded R_{in} during the day was 1.89 GΩ while at night it was 2.34 GΩ, in the range of previously reported values, with no statistical difference between day and night.

For middle-aged neurons there was a reduction in the R_{in} recorded during the day to 1.05 GΩ and to 1.24 GΩ at night, with no day-night difference. For old neurons there was an even further reduction in the R_{in} recorded,

0.62 GΩ during the day and 1.00 GΩ at night, with no statistical day-night difference. Comparing across the groups found a highly significant effect of age on R_{in} ($p<0.0001$, 2-way ANOVA), and a small time of day effect ($p=0.201$).

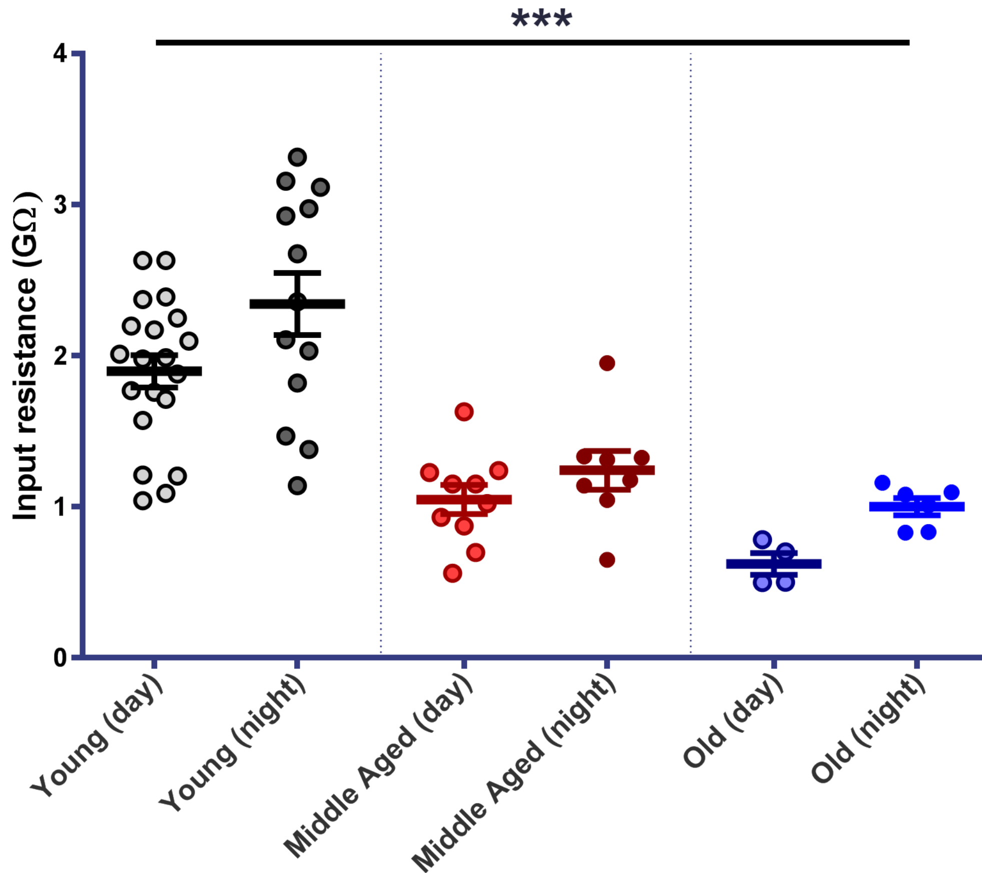


Figure 4-11 Input resistance of l-LNv neurons is significantly reduced by ageing
Input resistance was recorded from l-LNv neurons at day and night from young (D1-5), middle-aged (D28-35) and old (D45+) flies. l-LNv neurons demonstrated a highly significant reduction in input resistance with age ($p<0.0001$), and a small time of day effect ($p=0.0201$). (2-way ANOVA, Tukey's multiple comparison). Error bars indicate mean ± SEM. N = 20, 13, 10, 8, 4, 6.

4.4 Electrical properties of aged neurons are drastically altered

In agreement with previous experiments (C. Chen et al. 2015; Sheeba et al. 2007; Cao and Nitabach 2008), my recordings from young l-LNvs demonstrate a strong day-night difference in both electrical activity measured by spontaneous firing rate (Figure 4-6) and in the membrane

potential of the cells (Figure 4-7). Looking at the properties of the neurons by measuring the response to an injected current pulse (Figure 4-9) or the input resistance (Figure 4-11) did not show a significant day-night difference, in agreement with previous studies (Buhl et al. 2016; Julienne et al. 2017).

When I made recordings from older neurons I found that the active properties of the cells, that is the firing rate and membrane potential, were not significantly altered compared to young cells. I found that the diurnal modulation of spontaneous firing rate and membrane potential were maintained in l-LN_Vs recorded from flies of 30 days old (Figure 4-6 and Figure 4-7). In the oldest group of cells, those aged over 45 days, there might be a loss of this diurnal modulation but the amount of variation in what is a small sample size makes it difficult to make any strong conclusions in this oldest group.

Looking at the passive properties of the neurons, there was no difference found in the response to a +40 pA current injection between young and aged flies (Figure 4-9). Interestingly a significant decrease in the input resistance of aged l-LN_Vs was observed (Figure 4-11), indicative of an increase in the number of open ion channels in the membrane increasing the conductance of the cell.

One possible explanation for an increase in input resistance without changing the active properties of the neurons is involvement of chloride (Cl^-) channels, which could become open and decrease resistance without changing the membrane potential. Previous experiments have demonstrated the importance of daily changes in the Cl^- reversal potential on GABA being either inhibitory or excitatory, both in mammals (Alamilla et al. 2014) and in the l-LN_Vs (Buhl et al. 2016), and a change in the Cl^- reversal potential during the ageing process could help explain how a decrease in input resistance of the l-LN_Vs with age does not causes significant alterations in either membrane potential or electrical activity.

Comparing these findings to what has been done in the mammalian SCN there are some differences. In the aged SCN there is a reduction in the day-

night difference in firing rate by around 50% (Farajnia et al. 2012), with a decreased in day-time electrical activity in old SCN neurons recorded in slice preparations using whole-cell patch clamp. A reduction in the amplitude of daily electrical rhythms has also been recorded *in vivo* using multiunit electrode arrays (Nakamura et al. 2011). The differences in the effects of ageing between *Drosophila* and mice might reflect the differences in the circadian systems of the two model organisms. Maybe the effects of ageing on the *Drosophila* circadian system differ as a result of the more distributed nature of the system and so are more related to changes in the circadian network.

4.5 Conclusions from electrophysiology experiments

- Ageing vastly increases the difficulty of making patch-clamp recordings of *Drosophila* clock neurons.
- Ageing did not significantly alter the active firing properties of $l\text{-}LN_V$ neurons or membrane potential.
- Neuronal input resistance was significantly reduced in the $l\text{-}LN_V$ of older flies, which could be linked to important changes in the expression of ion channels.
- Imaging using the voltage sensor *ArcLight* is a potentially useful tool for measuring neuronal activity, if recordings can be optimised to improve success.

5

CHAPTER 5 A PRELIMINARY RNAi SCREEN OF CANDIDATE ION CHANNELS IMPORTANT FOR THE CONTROL OF CIRCADIAN RHYTHMS AND SLEEP IN *DROSOPHILA*

This chapter details the approaches taken to investigate the importance of various ion channels in the *Drosophila* circadian clock and the results of experiments on manipulating these ion channels.

Section 5.1 introduces some to the ion channels that are present in the circadian neurons of *Drosophila*. Section 5.1.1 takes a look at some of the available datasets from the published literature to identify ion channels that have been demonstrated to show circadian changes in their gene expression. Section 5.1.2 outlines the experimental approach use for manipulation of ion channels and the behavioural experiments performed. Sections 5.2 to 5.9 report the results from these manipulations. Finally, Section 5.10 discusses these findings in context with the literature.

5.1 Manipulating expression of ion channels in clock neurons

Ion channels are the essential electrical components of neurons controlling the electrical properties of neuronal cells, determining their excitability and activity. Therefore, manipulation of ion channel expression or function allows modulation of neuronal and circuit function (Hodge 2009). There is a large amount of homology in the ion channels that are present in *Drosophila* compared to mammals, but the number is reduced. This offers a significant advantage for studying the importance of a particular channel type as there is less redundancy in *Drosophila* compared to mammals.

Analysis of the *Drosophila* genome has identified 145 sequences encoding ion channel subunits (Littleton and Ganetzky 2000). The reduced diversity of ion channels in *Drosophila* provides significant advantages in being able to study the effects of removing specific currents by knocking down a single channel.

Table 5-1 gives some examples of *Drosophila* ion channels and their mammalian equivalent, highlighting how a single channel in flies might translate to an ion channel family containing ten genes in mammals.

Channel type	*Drosophila* gene(s)	Mammalian homologue	Number of genes in mammals
Voltage gated Ca^{2+} channels	*Ca-alpha1T*	$Ca_v3.x$. T-type	3
	Ca-alpha1D	$Ca_v1.x$, L-type	4
	cacophany	$Ca_v2.x$, N/P/Q/R-type	3
Inwardly rectifying potassium channels	*Ir (Irk1)*	$K_{ir}1.1$	1
	Irk2	$K_{ir}2.x$	4
	Irk3	$K_{ir}3.x$	4
Voltage-gated potassium channels	*Shaker, Hk*	$K_v\alpha1.x$	8
	Shab	$K_v\alpha2.x$	2
	Shal	$K_v\alpha3.x$	2
	Shaw, Shawl	$K_v\alpha4.x$	3
	KCNQ	$K_v\alpha7.x$	5
	eag, sei	$K_v\alpha11.x$	3
Ca^{2+}-activated potassium channels	*SK*	SK1-4	4
	slowpoke	$K_{Ca}1.x$	5
Voltage-gated sodium channels	*paralytic*	$Na_v1.1$ - $Na_v1.9$	10

Table 5-1 A selection of *Drosophila* ion channels and their mammalian equivalents.

A list of some of the ion channels present in *Drosophila* shows how for every channel in fruit flies there are usually multiple mammalian homologue genes.

5.1.1 Ion channels of circadian relevance

The explosion of tools for studying gene expression has led to a number of studies investigating which genes display cycling of their expression across the day. Early microarray studies measuring whole brain transcripts found some interesting cycling genes that had links to ion channel function, including *Dlg1* (*discs-large 1*), a protein that colocalises with *Sh* (*Shaker*)

potassium channels and *slowpoke binding protein* (*Slob*) which binds to the calcium activated potassium channel *slowpoke* (Claridge-Chang et al. 2001; Wijnen et al. 2006). More recent studies have identified and demonstrated that a large proportion of the genome cycles across the circadian cycle, with over 2,000 genes showing rhythmic expression in whole-head *Drosophila* samples (Kuintzle et al. 2017).

Previous studies of gene expression specifically in clock neurons have used RNA microarrays to look at expression of components of the molecular clock (Kula-Eversole et al. 2010), using manual sorting of fluorescently labelled LN_V neurons. Analysis of the microarray datasets has identified several ion channels which show significant cycling across the circadian cycle.

The most highly cycling gene was the inwardly rectifying potassium channel (*Ir*), with significant cycling seen in the voltage-gated potassium channels *Shaw* (K_V3) and *Shal* (K_V4) as well as the Ca^{2+}-activated potassium channel *SK*.

Ion Channel		Cycling in l-LN$_V$
Voltage gated Ca+ channels		
Ca-alpha1T	Ca^{2+}-channel protein α_1 subunit	Y
Ca-alpha1D	Ca^{2+}-channel protein α_1 subunit D	N
Ca-beta	Ca^{2+}-channel-protein-β-subunit	N
cac	cacophony, voltage-gated calcium channel	N
Ca^{2+} channels		
nompC	no mechanoreceptor potential C, calcium channel	N
pain	painless, calcium activity	N
Inwardly rectifying potassium channels		
Ir (Irk1)	Inwardly rectifying potassium channel 1, (Kir)	Y
Irk2	Inwardly rectifying potassium channel 2	N
Irk3	Inwardly rectifying potassium channel 3	N
Ork1	Open rectifier K^+ channel 1	N
Voltage-gated potassium channels		
eag	*ether a go-go, potassium channel family.*	N
Elk	*Eag-like K^+ channel*	N
Hk	*Hyperkinetic*	N
KCNQ	*KCNQ potassium channel*	N
sei (seizure)	*seizure, voltage-gated potassium channel*	Y
Sh	*Shaker, voltage-gated potassium channel, Kv1*	N
Shab	*Shaker cognate b, Kv2*	N
Shaw	*Shaker cognate w, Kv3*	Y
Shal	*Shaker cognate l (voltage-dependent A-type K^+ channel), Kv4*	Y
Ca^{2+}-activated potassium channels		
SK	*small conductance calcium-activated potassium channel*	Y
slo	*slowpoke, α subunit of a BK*	N
Slob	*Slowpoke binding protein*	N
Voltage-gated sodium channels		
NaCP60E	*Na channel protein 60E*	N
para	*paralytic, DmNav1, sodium channel*	Y
tipE	*temperature-induced paralytic E, sodium channel regulator*	N
Others		
ine (inebriated)	*SLC6A family of neurotransmitter transporters*	Y
Ncc69	*sodium chloride cotransporter 69*	N
na	*narrow abdomen, sodium leak*	N
Quiver	Trafficking of membrane proteins, including shaker	Y

Table 5-2 Cycling status of selected ion channels identified in l-LN$_{VS}$

Analysis of existing RNA microarray dataset (Kula-Eversole et al. 2010) has revealed a number of ion channels, and associated proteins, which show significant cycling in expression across the circadian cycle.

5.1.2 Experimental approaches to manipulate ion channels

To investigate the importance of the different ion channels outlined in Section 5.1.1 a targeted knockdown strategy using RNA interference (RNAi) was used to knock down the expression of selected channels specifically in

the neurons of the *Drosophila* central clock. This was brought about using the *Gal4/UAS* system (see chapter 2.1.2 for details) (Perrimon et al. 2010), using the *tim-Gal4* driver line to target expression of UAS-driven ds-RNAi constructs designed to target specific mRNA encoding ion channels identified as target genes from the circadian RNA microarray dataset.

Fly lines were acquired from the Bloomington *Drosophila* Stock Center collection of RNAi lines created as part of the Transgenic RNAi Project (TRiP) at Harvard Medical School (http://www.flyrnai.org/TRiP-HOME.html) to create fly RNAi lines covering all *Drosophila* genes (Perkins et al. 2015). The RNAi lines used were second-generation lines from the TRiP collection, which have an increased knock down efficiency without the need for expression of *Dicer* (an enzyme responsible for cleaving ds-RNAi into small fragments), except for *Shaw-RNAi* which was generated and validated in a previous study (Hodge and Stanewsky 2008).

TRiP VALIUM vectors contain a copy of the *vermilion* gene as a selectable marker (flies have brown eyes)(Fridell and Searles 1991), and two 5xUAS sites for driving expression of the RNAi construct. The VALIUM 10 (Ni et al. 2009) vector uses a long double-stranded hairpin design to generate RNAi knockdown, and the more recent VALIUM 20 (Ni et al. 2011) vector uses short hairpin microRNA technology. A list of stocks used is given in Table 5-3.

BDSC #	Gene Target	Genotype	Chr.	Construct
26247	*slo*	y^1 v^1; P{TRiP.JF02146}attP2	3	VALIUM10
42644	*Irk1*	y^1 sc^* v^1; P{TRiP.HMS02480}attP2	3	VALIUM20
26251	*Caα1T*	y^1 v^1; P{TRiP.JF02150}attP2	3	VALIUM10
31879	*Shal*	y^1 v^1; P{TRiP.JF02154}attP2	3	VALIUM10
27238	*SK*	y^1 v^1; P{TRiP.JF02571}attP2	3	VALIUM10
33923	*para*	y^1 sc^* v^1; P{TRiP.HMS00868}attP2	3	VALIUM20
53347	*Sh*	y^1 sc^* v^1; P{TRiP.HMC03576}attP40	2	VALIUM20
55722	*Shaw*	Previously generated and verified RNAi construct (Hodge and Stanewsky 2008)		

Table 5-3 Details of RNAi fly lines used

Full details of gene targets listed in Table 5-2.

Flies expressing the RNAi construct targeting a particular ion channel were then used for circadian behaviour and sleep experiments using the same approach as outlined in Chapter 3. Circadian behaviour and sleep were then compared to control flies containing only the *UAS-RNAi* component crossed with an iso^{31} wild type background.

To further justify the use of the select RNAi lines off-target effect were checked *in silico* using the dscheck online tool (http://dscheck.rnai.jp/), to search for sequence matches across the *Drosophila* genome, simulating the biochemical process of ds-RNAi *in vivo* (Naito et al. 2005). Using this verification tool allows for a small measure of confirmation that chosen lines targeted the selected ion channels without any predicted off target interactions. These RNAi manipulations require independent verification using a combination of multiple alternative RNAi lines (preferable acting at different locations) and molecular analysis to confirm effective knock down of mRNA.

5.1.3 Aims of ion channel manipulations

A large body of work has demonstrated that there are a large number of ion channels which cycle in the daily expression, and daily changes in electrical activity of clock neurons are related to behaviour outputs of the clock. In order to investigate the role of different ion channels in linking electrical activity of clock neurons to behaviour I have used RNAi-mediated knockdown of ion channel mRNA. A subset of ion channels of interest were selected based on analysis of a published micro-array dataset of cycling genes in the LN_V clock neurons and circadian and sleep behaviour measured in RNAi knock down flies.

5.2 Small-conductance calcium-activated potassium (SK) channel

SK channels are potassium channels that are solely activated by increased calcium levels, and are voltage independent, being termed small-conductance due a smaller single-channel conductance (10-20 pS) compared to other ion channels (Adelman et al. 2012). SK is indirectly activated by calcium via binding to calmodulin located as a subunit of the

SK channel itself (Xia et al. 1998; Adelman 2016). SK activation from a calcium influx during the action potential encodes a hyperpolarising current across the neuronal membrane and acts to lower action potential frequency (Faber and Sah 2007).

The *Drosophila* genome contains only a single SK gene (*dSK*) simplifying study of genetic manipulations *in vivo* (Abou Tayoun et al. 2011). Previous work has shown that *Drosophila* SK is broadly expressed through several regions of the CNS including the antennal lobe and mushroom bodies but is particularly highly expressed in the visual system, and in photoreceptors the channel encodes a slow Ca^{2+}-activated K^{+} current with a high similarity to mammalian SK currents (Abou Tayoun et al. 2011).

SK channels have been implicated as important in the circadian and sleep systems, as blocking of SK channels in rodents using apamin causes disruption of the circadian cycle and sleep disturbances (particularly REM sleep) (Gandolfo et al. 1996; Benington et al. 1995). An SK current has been identified in SCN neurons, with blocking of SK channels increasing action potential firing frequency and causing changes in membrane afterhyperpolarisation (Teshima et al. 2003), and more recent work has shown that this SK current may be particular important in *Per1*-expressing SCN neurons that have dramatic daily changes in electrical activity (Belle et al. 2009). In *Drosophila*, *SK* was shown to be a significantly cycling mRNA in clock cells from microarray data, with upregulation at ZT18 compared to ZT0.

5.2.1 Knockdown of SK reduces free-running rhythmicity

Expressing *SK-RNAi* in the circadian clock caused dramatic changes in the daily activity of flies compared to controls (Figure 5-1), with a decrease in day-time activity plus increase in night-time activity. These changes in the structure of daily activity result in large and significant reduction of the D/N index, such that *SK-RNAi* flies display more locomotor activity during the night.

Under constant conditions, there was a significant reduction in the strength of the clock between the SK knock down flies and the controls (3.8 v 4.6 RS), but no effect on period length (23.5 hrs v 23.5 hrs).

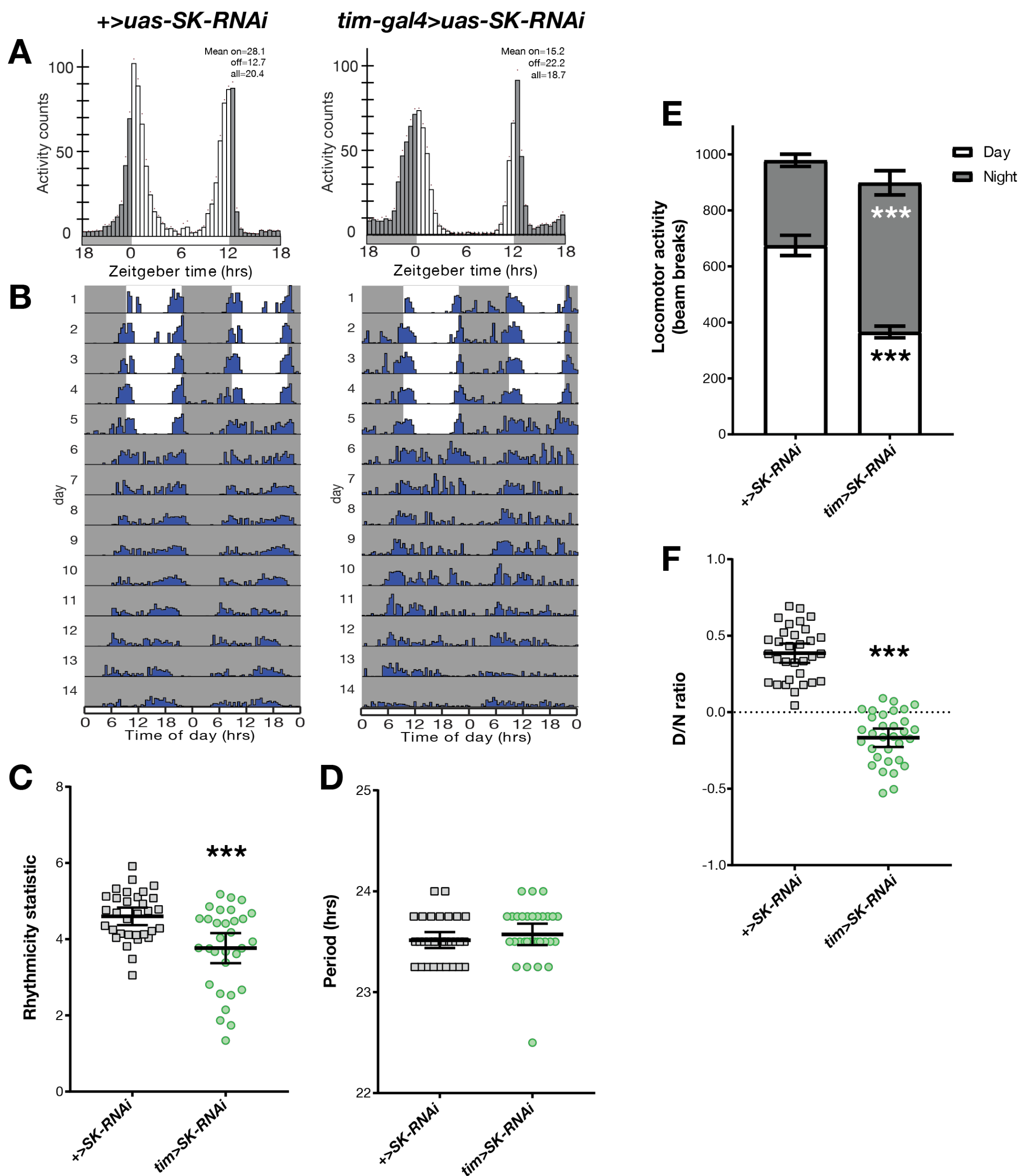


Figure 5-1 Knock down of *SK* reduces the strength of circadian clock outputs
(A) Histograms of daily activity under LD conditions show a reduction in day activity in *SK-RNAi* flies compared to controls. (B) Actograms of representative individual control and *SK-RNAi* flies. (C) *SK-RNAi* flies have reduced circadian rhythmicity under constant conditions. (D) There was no difference in circadian period between *SK-RNAi* and control flies. (E) Total locomotor activity was not altered in *SK-RNAi* flies, but day activity was significantly reduced, and night activity significantly increased compared to controls. (F)

The D/N ratio for *SK-RNAi* flies was massively reduced. Error bars represent mean ± SEM, for period error bars are median with IQR. N = 32 flies for each group. Statistical differences in RS, Locomotor activity and D/N ratio were performed using unpaired two-tailed t-test. Differences in Period were tested using the Mann-Whitney test.

5.2.2 Knock down of SK significantly alters sleep

Sleep in *SK-RNAi* flies was significantly altered compared to controls, both for day-time and night-time sleep. Interestingly the effects were opposite between day and night, with knock down of *SK* resulting in an increase in day-time sleep yet a decrease in night-time sleep.

Sleep latency (e.g. the time to fall asleep) was similarly altered in opposite directions, with a significant reduction in latency to sleep during the day but a striking increase in the latency of night sleep. The parameters of sleep episodes were also significantly altered in *SK-RNAi* flies, with a significant reduction in the number of sleep episodes compared to control flies and a lengthening of the mean duration of individual sleep bouts.

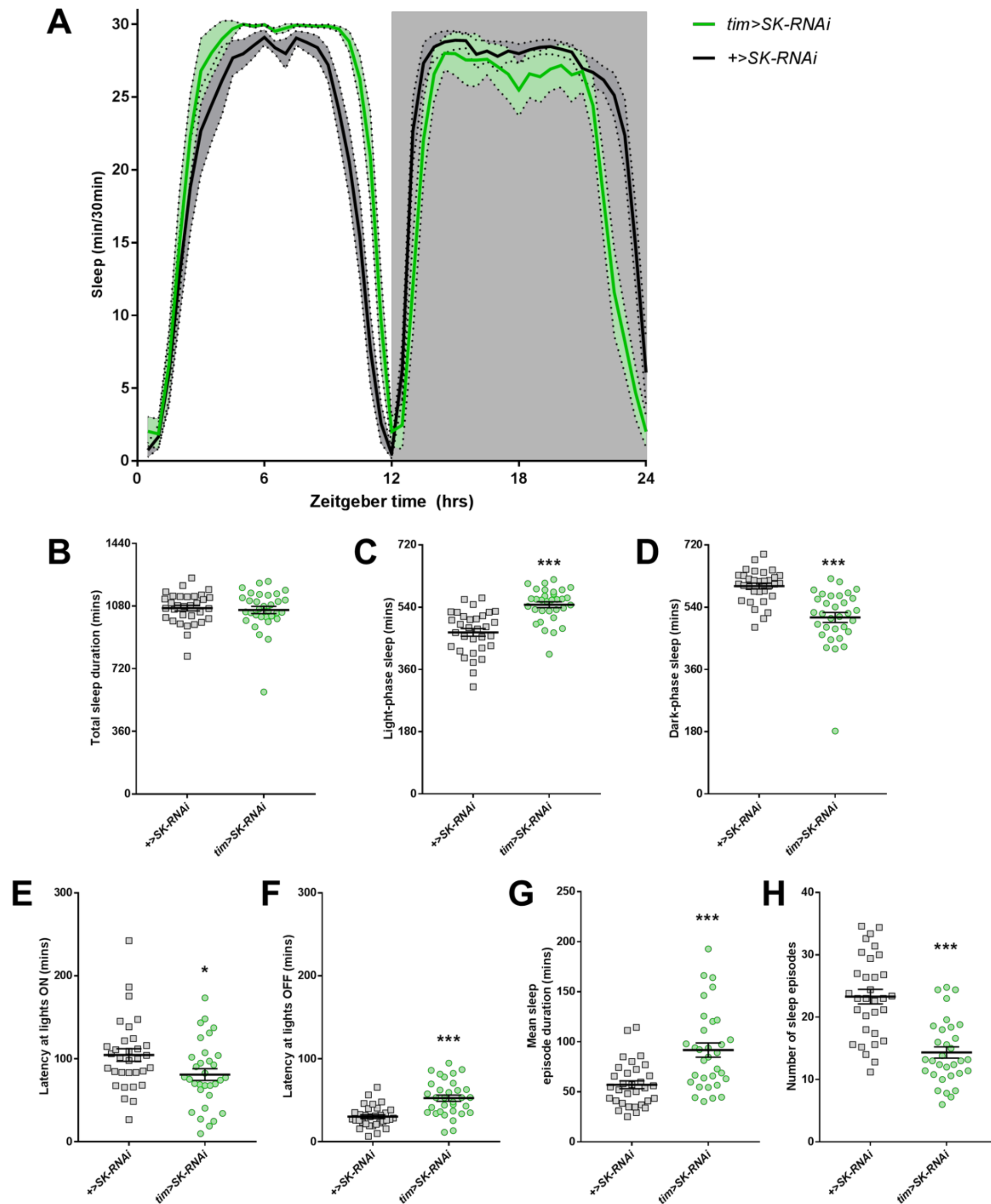


Figure 5-2 Sleep parameters are widely changed by knock down of *SK*

(A) Daily sleep profile of *SK-RNAi* (green) and control (grey) flies, show significant divergence in sleep behaviour during both day and night. (B) Total sleep duration is not altered. (C) Day-time sleep is significantly increased in *SK-RNAi* flies. (D) Night-time sleep is significantly decreased in *SK-RNAi* flies. (E) *SK-RNAi* flies showed slightly reduced latency to sleep at lights-on compared to controls. (F) There is a significant increase in sleep latency at lights-off in *SK-RNAi* flies. (G) The mean sleep episode duration is significantly increased by *SK-RNAi* knock down. (H) *SK-RNAi* flies have a significant reduction in the number of sleep episodes compared to controls. Error bars represent mean ± SEM. N = 32 flies for each group. Statistical differences were tested using two-tailed t-tests.

5.2.3 *SK* knock down alters circadian and sleep behaviour

Manipulating expression of *SK* using RNAi-mediated knock down in the circadian system had large and significant effects on circadian and sleep behaviour. Knocking down *SK* significantly reduced the strength of circadian rhythmicity under constant conditions and had striking effects on locomotor activity during LD conditions with a shift towards flies being more active at night, rather than during the day (Figure 5-1). This shift towards night activity was reflected in significant changes in the sleep behaviour of *SK-RNAi* flies, with a massive reduction in night-time sleep and increase in day-time sleep. Latency, number of sleep episodes and mean episodes duration were all significantly altered as well.

Give that the action of SK channels is to hyperpolarize membranes and thereby reduce firing frequency (Faber and Sah 2007), reducing expression of SK channels with RNAi should cause membrane depolarization and increased firing rates. Experimental evidence for this can be seen in mouse slice experiments, where blocking of SK channels in the SCN results in membrane depolarization (Belle et al. 2009). Mice containing a null-mutation for the *SK2* gene show disrupted sleep behaviour and significant reduction in the power of EEG signatures of NREM sleep (Cueni et al. 2008). More recently *SK* has been demonstrated to be important in control of electrical activity in the *locus coeruleus*, an important sleep-wake centre in the mammalian brain (Matschke et al. 2018), where blocking SK channels increasing firing rate. This experimental evidence from mammals, suggests that clock neurons in *SK-RNAi* flies will be more active, and this may be responsible for the observed behavioural changes.

Expression of *SK* mRNA in wild type LN_V neurons is decreased during the day and elevated at night (ZT18). Given the inevitable delay from mRNA levels to protein levels, the biggest impact on *SK* would be expected to be at the late night. Data from my sleep experiments shows there is very large difference between *SK-RNAi* flies and controls in the late night, with *SK-RNAi* flies waking much earlier prior to lights turning on.

5.3 Ir, Inwardly rectifying potassium channel 1 (Irk1)

Inwardly rectifying potassium (K_{IR}, Irk) channels are comprised of four subunits surrounding an central pore, with each subunit consisting of two-transmembrane-helices (Miller 2000). K_{IR} channels were first identified in skeletal muscle, and were identified for having an “anomalous” potassium current, with a greater flow of potassium into the cells rather than the expected outward current predicted by the Nernst equation (Hibino et al. 2010).

Drosophila has three K_{IR} channels, first identified from their amino acid sequence similar to K_{IR} channels. The first channel to be studied was *Drosophila* inward rectifier (*Dir*), later renamed to *Irk1*, which was found to contain conserved transmembrane regions found in other K_{IR} channels and is expressed throughout embryos and larvae with enriched expression in the brain (MacLean et al. 2002). *Irk1* is considered a classical K_{IR} channel with moderate homology (~44%) to the human Kir2.1 channel, with heterologous expression of *Irk1* in *Drosophila* S2 cells gives rise to prominent inward currents displaying similar rectification properties to mammalian Kir2 channels (Döring et al. 2002).

Irk1 was found to be involved in circadian behaviour in an mRNA GeneChip study showing that *Irk1* mRNA expression in larval pacemaker neurons was upregulated (17-fold changes) in the early night (ZT15) compared to the early day (ZT3) (Ruben et al. 2012). Ruben *et. al.* then used targeted RNAi knockdown of Ir (*Irk1*) and found modest lengthening of the period of circadian locomotor behaviour in adult flies (Ruben et al. 2012). In the RNA microarray dataset that I analysed for circadian expression *Irk1* was shown to be the most highly cycling transcript of all ion channels with a 50 fold change in expression from a low at ZT6 to its high at ZT12 (Kula-Eversole et al. 2010), and so was an obvious candidate channel of interest for my RNAi screen to see if the same circadian effects could be reproduced as in the previous work by Ruben *et al.* and to investigate effects on sleep.

5.3.1 Effects of *Irk1* knock down on circadian behaviour

Expression of RNAi against *Irk1* throughout the circadian clock network had modest effects on circadian behaviour (Figure 5-3). There was no difference in the overall locomotor activity of the flies, but there was a significant difference in the time of day that movement was occurring under LD conditions. There was a reduction in the D/N ratio in *Irk1* knockdown flies compared to controls due to a decrease in day-time activity and increase in night-time activity.

Under constant conditions, there was no significant difference in the strength of the observed free-running locomotor behaviour between the *Irk1* knock down flies and the controls (3.8 v 3.6 RS), but there was a slight lengthening in the circadian period (23.75 hrs v 23.5 hrs).

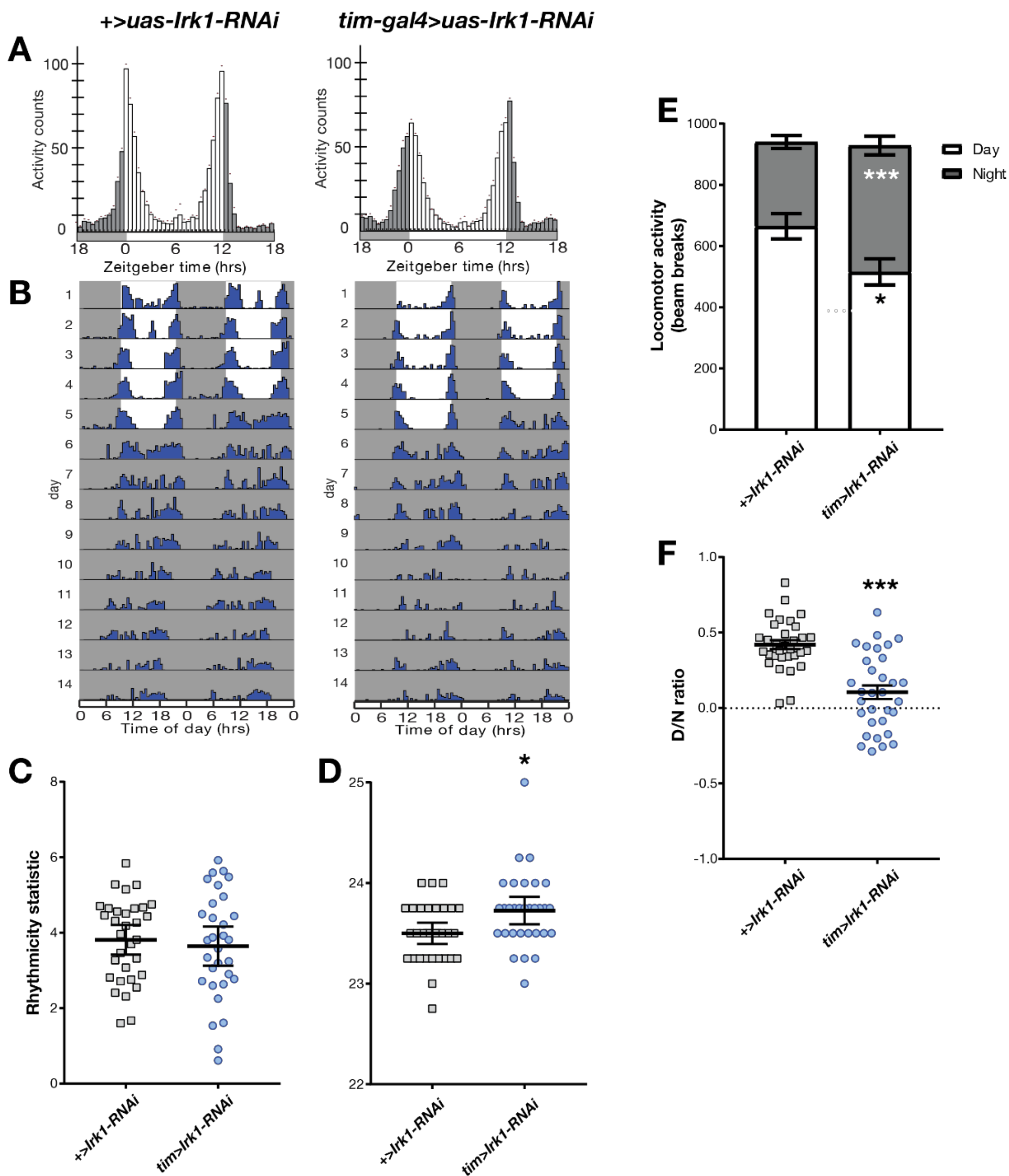


Figure 5-3 Effect of knock down of *Irk1* on circadian behaviour

Locomotor activity of control and *Irk1-RNAi* flies (A) Histograms of daily activity of *Irk1-RNAi* and control flies under LD conditions. (B) Actograms of representative individual control and *Irk1-RNAi* flies. (C) Circadian rhythmicity under constant conditions was not significantly altered in *Irk1-RNAi* flies (D) Circadian period of *Irk1-RNAi* was lengthened compared to controls. (E) Total locomotor activity was not altered in *Irk1-RNAi* flies, but day activity was significantly reduced, and night activity significantly increased compared to controls. (F) The D/N ratio for *Irk1-RNAi* flies was massively reduced. Error bars represent mean ± SEM, for period error bars are median with IQR. N = 32 flies for each group.

5.3.2 *Irk1* knock down alters night-time sleep

Looking at the effect of *Irk1* knockdown on sleep showed only minor changes to night-time sleep behaviour, with a reduction in sleep activity in the late night. Quantification of sleep duration found a significant reduction in night-time sleep in *Irk1-RNAi* flies, but no difference for day-time sleep. Overall sleep duration was slightly lower in *Irk1-RNAi* flies, but this difference was not significant. The changes in sleep duration at night was combined with an increased sleep latency at lights off in *Irk1-RNAi* flies compared to controls. There was no difference in the number of sleep episodes or the mean length of sleep episodes, with the decrease in night sleep a result of the increased latency and earlier wakening of *Irk1-RNAi* flies.

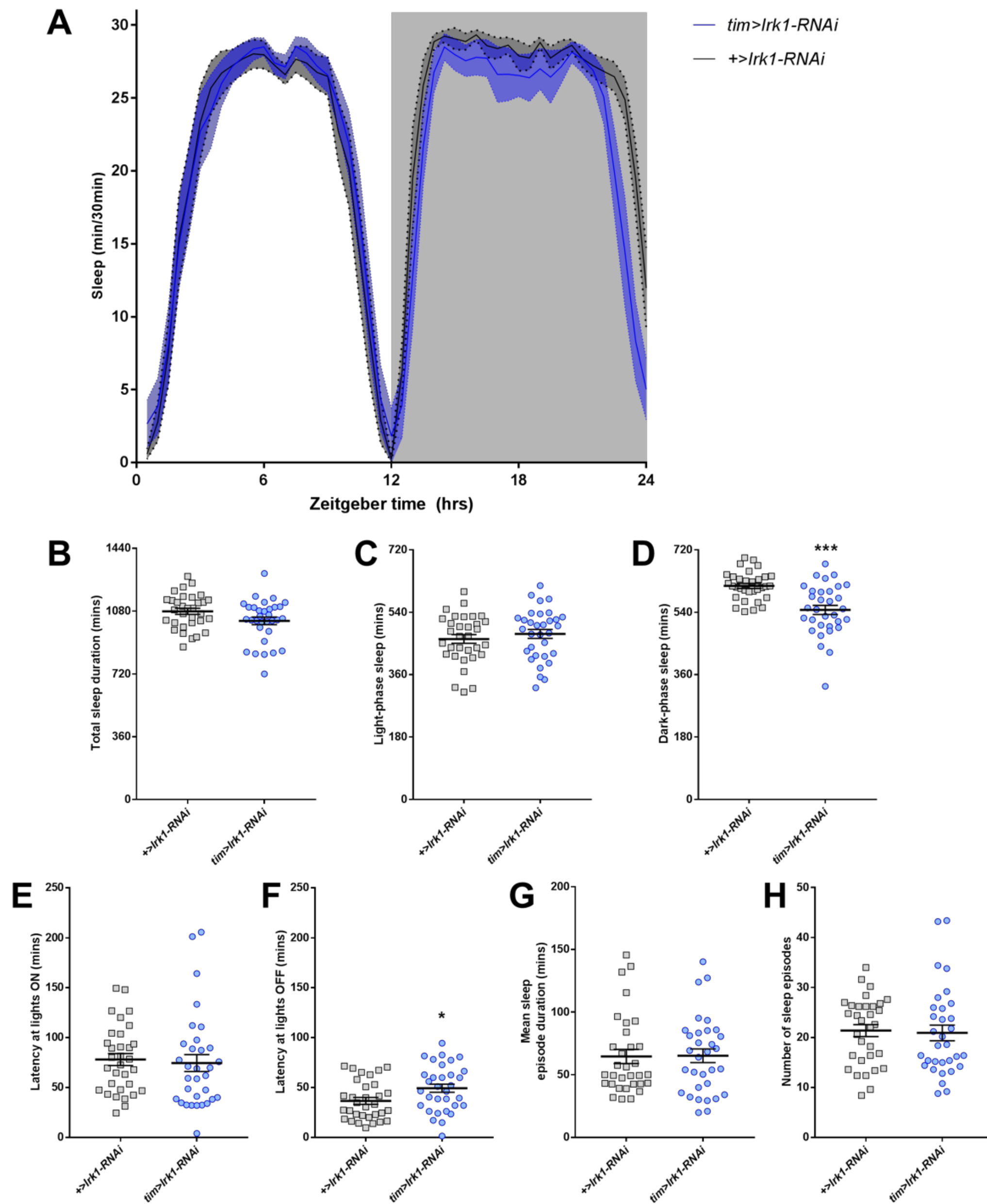


Figure 5-4 Effect of knock down of *Irk1* on sleep behaviour

(A) Daily sleep profile of *Irk1-RNAi* (blue) and control (grey) flies, show day-time sleep is nearly identical between *Irk1-RNAi* and control flies, while there is some difference at late night. (B) Total sleep duration has reduced in *Irk1-RNAi* flies, but not significantly. (C) Day-time sleep was not altered. (D) Night-time sleep is significantly decreased in *Irk1-RNAi* flies. (E) There was no effect on sleep latency at lights-on (F) *Irk1-RNAi* flies had a small but significant increase in latency to sleep at lights-off. (G) and (H) Sleep episode duration and number of sleep episodes were unchanged. Error bars represent mean ± SEM. N = 32 flies for each group.

5.3.3 *Irk1* knock down has limited behaviour effects

Knock down of *Irk1* had limited effects on circadian behaviour, there was no effect on the strength of rhythms and only a slight lengthening of period, which was something of a surprise given that it was the channel with the highest amount of cycling from the microarray dataset. Closer inspection of the data reveals a significant impact on behaviour during LD conditions, with a large increase in night-time locomotor activity.

The day-time sleep profile is almost overlapping between *Irk1-RNAi* flies and controls, during the period when *Irk1* mRNA expression is low. At night, when *Irk1* expression is significantly higher, there was a reduction in sleep in *Irk1-RNAi* flies. Overall, there were only limited effects of the *Irk1* knock down, suggesting that either *Irk1* is not a hugely important ion channel or that other channels are able to compensate for the reduction.

5.4 Ca^{2+}-channel protein α1 subunit T (Caα1t)

Calcium (Ca^{2+}) is an extremely important component of intracellular signalling and has impacts on nearly every aspect of cellular biology from excitability to gene transcription (Clapham 2007). Voltage dependent Ca^{2+} channels (VDCCs) are a group of voltage-gated ion channels predominantly permeable to calcium. When activated by membrane depolarization opening of VDCCs allows Ca^{2+} influx into the cell allowing calcium to act as a second messenger (Catterall 2011). VDCCs channels are highly diverse producing different Ca^{2+} currents depending on their structure and have being classified into five main types; L “long-lasting”, P/Q-type “Purkinje”, N “Neural”, R “residual” and T “transient” – types.

VDCCs are composed of five main subunits (α1, α2, β, γ, and δ), with the α1 determining the type of current mediated by the channel (Catterall 2011). From the microarray dataset, expression of the Ca^{2+}-channel protein α1 T-type subunit (Caα1t) showed significant circadian cycling, with mRNA expression upregulated in the late afternoon and early night compared to the early morning. T-type channels are characterised as low voltage-activated, requiring a smaller membrane depolarization for opening, and

their transient current due to rapid inactivation of the channels (Iftinca 2011).

T-type currents in mammals have been linked to the sleep-cycle, with mice knockouts of a T-type subunit displaying sleep disruption (Lee et al. 2004). Mammalian T-type channels are encoded by three different genes producing the channels $Ca_v3.1 – 3.3$ (Talley et al. 1999), while in *Drosophila* there is only a single gene corresponding to each mammalian family of channels, for the T-type current this is *Caα1t* (*DmαG*) (Littleton and Ganetzky 2000). *Caα1t* knockdown in motor neuron showed that it was a true T-type channel encoding a transient low-voltage activated current (Ryglewski et al. 2012). Recent work expressing the Caα1t channel in human embryonic kidney (HEK) cells found similar channel kinetics to rat $Ca_v3.1$ and supported the previous findings that it is a true T-type channel (Jeong et al. 2016). The study went on to show that *Caα1t* is widely expressed in the *Drosophila* brain and showed that *Caα1t* null mutants sleep more that control flies and this could be replicated by pan-neuronal knockdown of *Caα1t*. Pan-neuronal knockdown of *Caα1t* also resulted in changes in circadian behaviour, with a slight increase in period and decrease in rhythmicity (Jeong et al. 2016).

Given the significant cycling of *Caα1t* mRNA in clock neurons, plus the impact of pan-neuronal knockdown of *Caα1t* on sleep and circadian activity, it is an interesting target for manipulation using RNAi in a clock-specific manner.

5.4.1 *Caα1t* circadian behaviour

Knock down of *Caα1t* throughout the clock had significant effects on circadian behavioural activity. *Caα1t-RNAi* flies displayed significantly reduced locomotor activity compared to control flies, with this reduction specific to day-time activity, there was no difference in night-time activity. This day-time specific reduction in activity resulted in a negative D/N index for *Caα1t-RNAi* flies, with mutant flies showing a nocturnal preference under LD conditions.

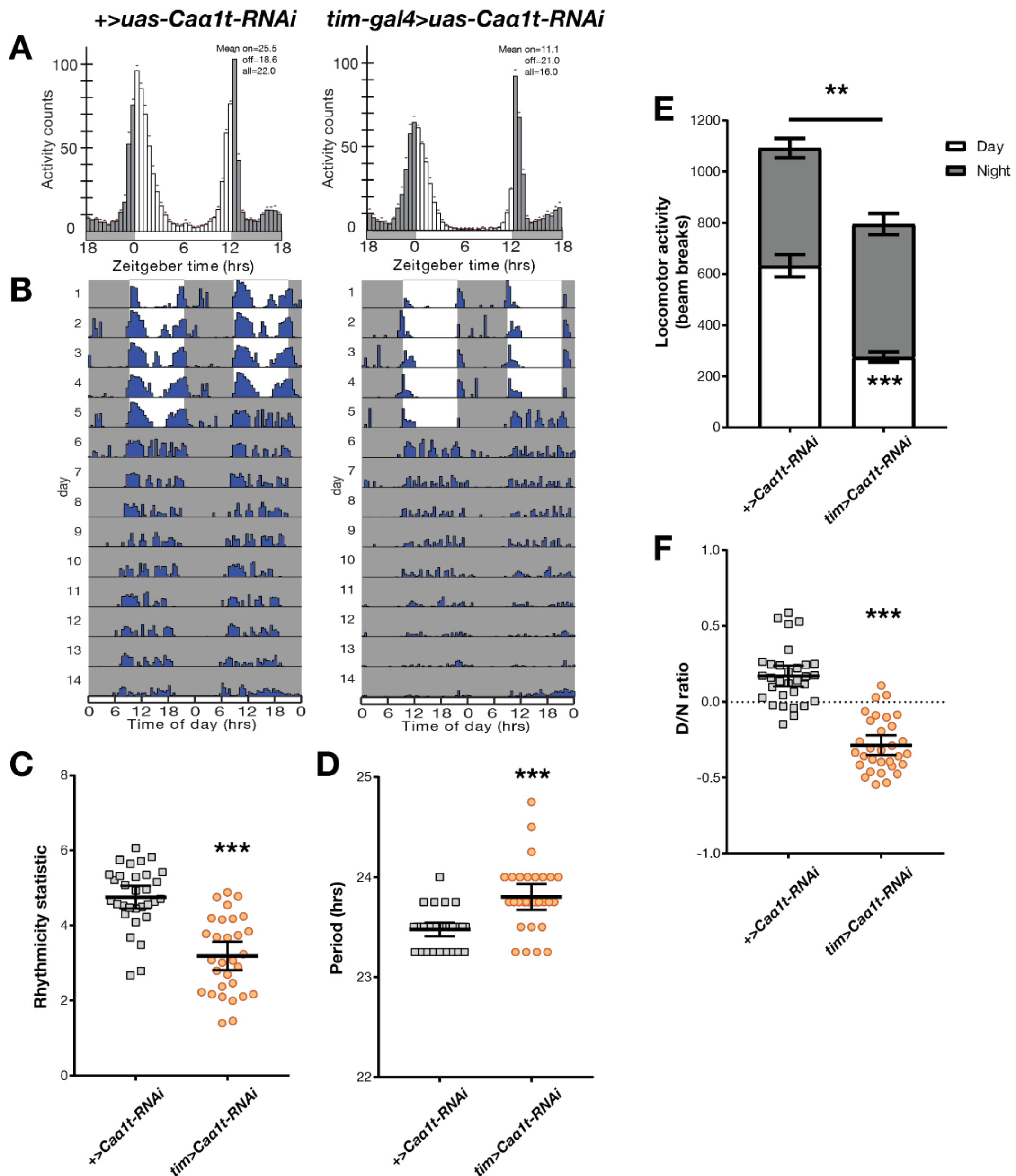


Figure 5-5 Effect of knock down of *Caα1t* on circadian activity

Locomotor activity of control and *Caα1t-RNAi* flies (A) Histograms of daily activity of *Caα1t-RNAi* and control flies under LD conditions. (B) Actograms of representative individual control and *Caα1t-RNAi* flies. (C) Circadian rhythmicity of *Caα1t-RNAi* was greatly reduced. (D) Circadian period of *Caα1t-RNAi* was significantly lengthened compared to controls. (E) *Caα1t-RNAi* had significantly reduced locomotor activity, with a significant reduced of day activity but night activity was not altered. (F) The D/N ratio for *Caα1t-RNAi* flies was significantly reduced. Error bars represent mean ± SEM, for period error bars are median with IQR. N = 32 flies for each group.

Analysing behaviour under constant conditions showed significant effects on both the strength of the clock and the free running period. *Caα1t-RNAi*

flies had a significant reduction in rhythmicity statistic compared to controls, (3.19 v 4.76 RS) and a slight but significant lengthening in period (23.75 hrs v 23.5 hrs).

5.4.2 *Caa1t* knock down significantly alters daytime sleep

Caa1t-RNAi flies showed a significant increase in day-time sleep compared to controls ($p<0.001$), with a particularly noticeable increase in the late afternoon, but no difference in night sleep. This increase in day sleep was also associated with a reduced latency to sleep during the day. Sleep behaviour was also changed in *Caa1t-RNAi* flies with a significant reduction in the number of sleep episodes and increase in mean sleep duration length, such that sleep was consolidated into longer less frequent bouts.

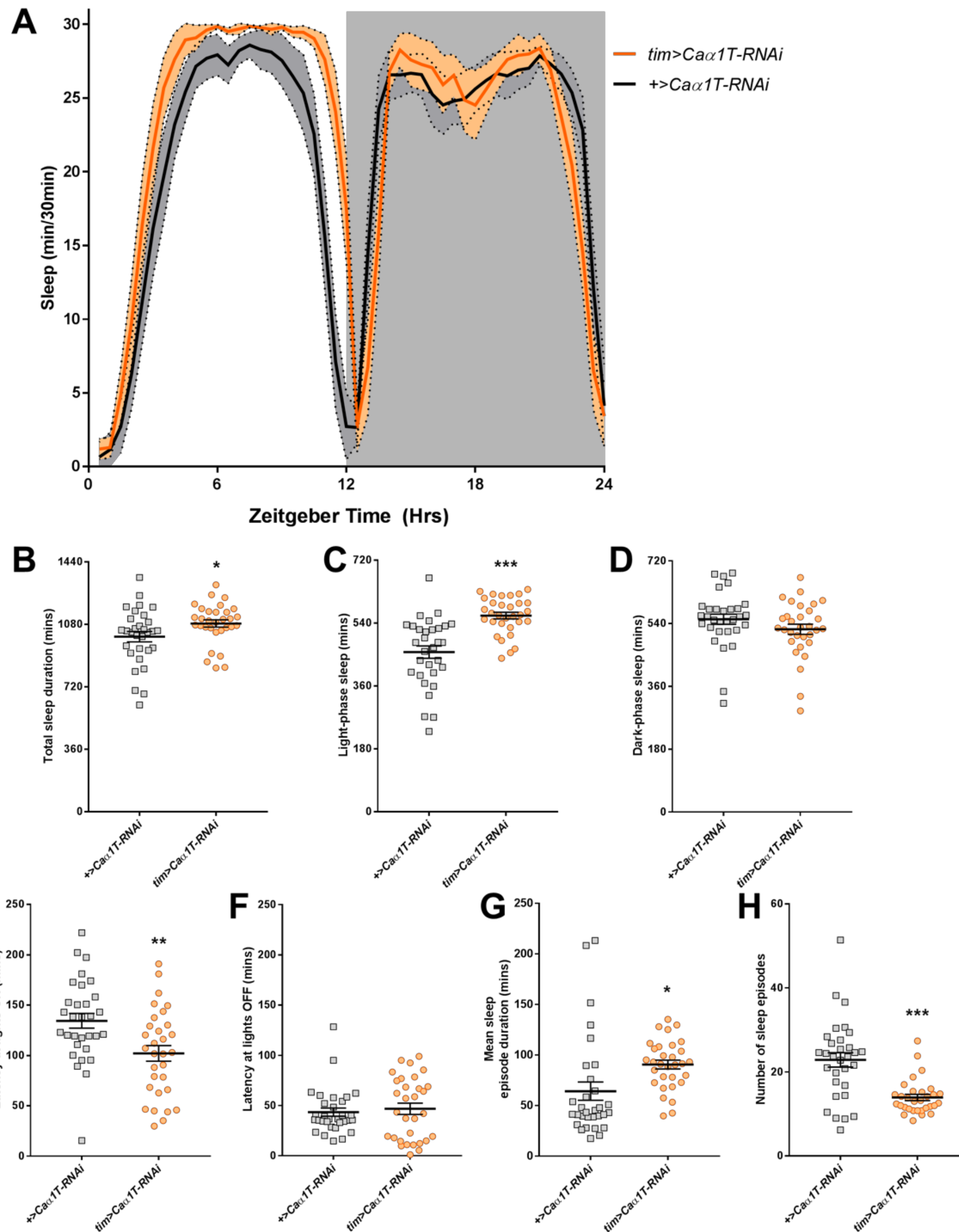


Figure 5-6 Effect of knock down of *Caα1t* on sleep behaviour

(A) Daily sleep profile of *Caα1t-RNAi* (orange) and control (grey) flies, show a divergence in day-time sleep behaviour in *Caα1t-RNAi* flies, but no real difference in night-time sleep. (B) Total sleep duration has increased in *Caα1t-RNAi* flies. (C) Day-time sleep was significantly increased in *Caα1t-RNAi* flies. (D) Night-time sleep was not significantly different. (E) *Caα1t-RNAi* flies showed a significant reduction in sleep latency to lights on. (F) There was no difference in latency at lights off. (G) *Caα1t-RNAi* flies had significantly longer mean sleep episode duration. (H) The number of sleep episodes was greatly reduced in *Caα1t-RNAi* flies compared to controls. Error bars represent mean ± SEM. N = 32 flies for each group.

5.4.3 *Caα1t* knock down weakens the clock and disrupts sleep

Knock down of the *Drosophila* T-type calcium current using RNAi against the *Caα1t* gene had significant effects across both circadian and sleep behaviour. The behaviour output of the clock in constant conditions was weakened in *Caα1t-RNAi* flies with a lengthening of the period. Significant differences were seen in the daily activity of *Caα1t-RNAi* flies under LD conditions with a significant reduction in day-time activity. In terms of sleep behaviour there were clearly observable differences between *Caα1t-RNAi* and control flies, particularly for day-time sleep. Overall sleep duration was slightly increased, but this was the result of a massive increase in day-time sleep only, with no impact on night-time sleep. A previous study using a pan-neuronal knock down of *Caα1t* demonstrated increased sleep at day-time, but did not identify a brain region responsible (Jeong et al. 2016). The results here suggest that expression of the function of *Caα1t* in the circadian system is highly important for its role in modulating sleep.

Expression of *Caα1t* mRNA in l-LN_V neurons is elevated across the day and down at night (Kula-Eversole et al. 2010), and so this could explain the larger effects of *Caα1t-RNAi* knock down on day-time locomotor behaviour and sleep. The T-type current has been shown to be highly circadian regulated in the mammalian SCN (Nahm et al. 2005), with calcium thought to be an important regulator of gene expression and for synchronisation of clock neurons. Here I have shown that specific knock down of *Caα1t* in the circadian system has large effects on behavioural outputs.

5.5 *paralytic*, voltage-gated sodium channel (*para*, $DmNa_V$)

paralytic (*para)* encodes an α-subunit of voltage-gated sodium channels (Na_V) which demonstrates very high amino acid sequence similarity to vertebrate homologues (Loughney et al. 1989). Na_V channels are predominately comprised of a large α-subunit and one or two accessory β-subunits. The α-subunit is the core of the channel containing the pore and is composed of four domains each containing six transmembrane regions and is functional alone and able to conduct Na^+ (Catterall 2000). Na_V channels are highly selective for Na^+, with a small pore just large enough to allow passage of a single Na^+ ion to pass (Shen et al. 2017). Na_V channels

open during changes in voltage across the membrane in the rising phase of the action potential and are key regulators of neuronal excitability (Catterall 2000).

In mammals there are nine different α-subunit proteins (Na_V1.1-1.9) which show high structural homology (Catterall et al. 2005). In contrast in *Drosophila* and in other insects *para* is the only Na_V channel present, but alternative splicing of the *para* locus generates differences in channel properties (Lin et al. 2009). Mutations in the *para* gene are associated with seizure phenotypes and have been used in *Drosophila* as a model for epilepsy (Parker et al. 2011; Kroll et al. 2015). The most closely related mammalian gene to *para* is SCN1A which encodes the Na_V1.1 channel (Malo et al. 1994). Na_V1.1 is expressed in the mammalian suprachiasmatic nucleus, with mice carrying a heterozygous loss of function mutation in the SCN1A gene having a longer period than wild type mice (Han et al. 2012). The role of *para* in the circadian system if *Drosophila* is not well studied. The microarray data showed expression of *para* to be lowest at ZT0 and is increased over the day and early evening.

5.5.1 Effect of knocking down *para* on circadian behaviour

Knock down of *para* across the circadian system caused only subtle changes in the behavioural outputs of the clock. Visualisation of histograms of daily activity under LD and actograms of behaviour did not show any huge differences between control and *para-RNAi* flies (Figure 5-7A). Quantifying circadian behaviour found there was no difference in the strength of the observed free-running activity (Figure 5-7B), but there was a significant lengthening in the period of the behaviour (Figure 5-7C)

Under the light-dark (LD) conditions there was a small shift in the structure of daily activity, with a slight increase in overall locomotor activity in *para-RNAi* flies (Figure 5-7D). When this daily activity was compared using the D/N ratio there was a small decrease in *para-RNAi* flies showing a shift towards more activity during the dark-phase (Figure 5-7E).

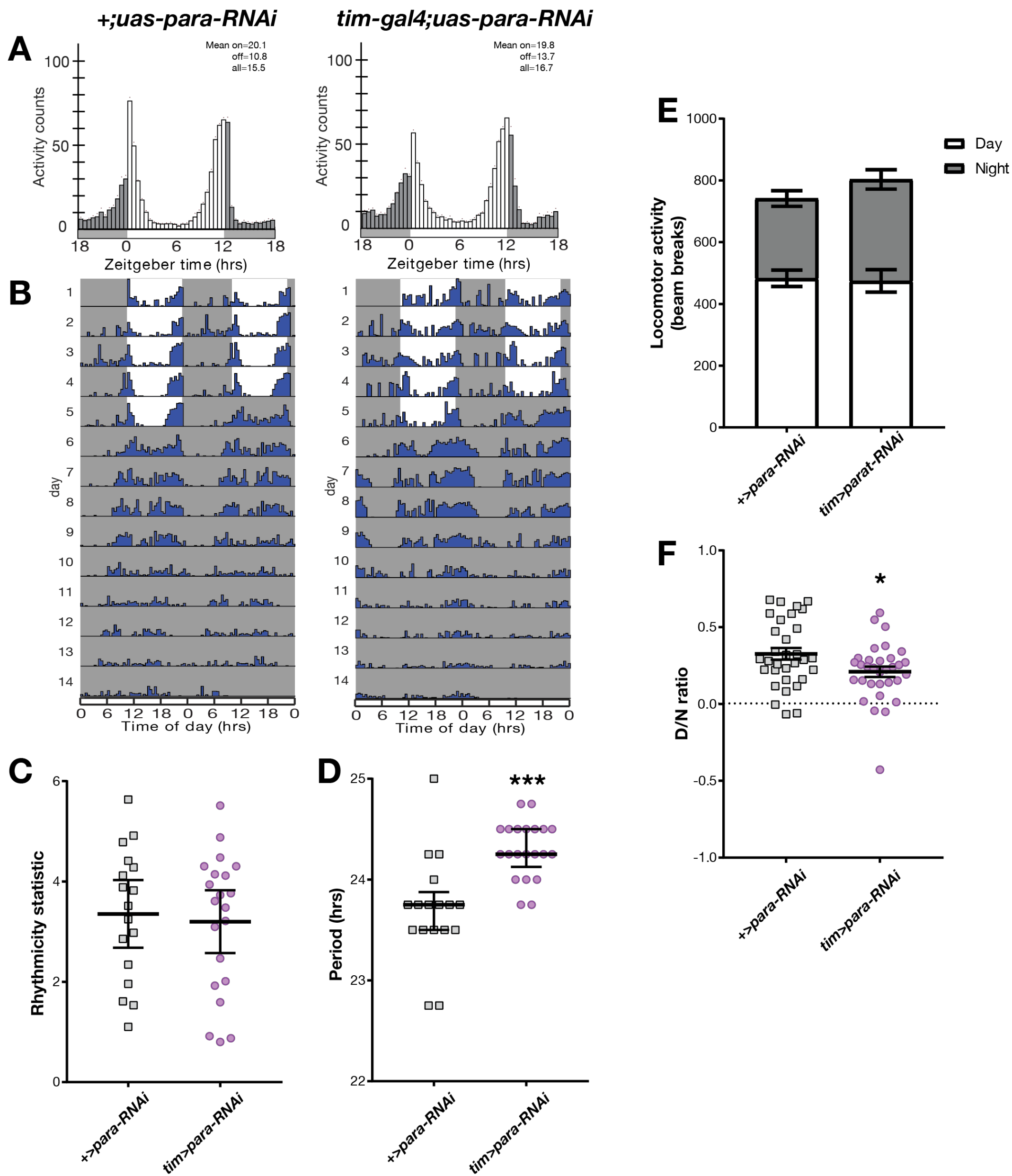


Figure 5-7 Effect of knock down of *para* on circadian activity

(A) Histograms of daily activity of *para-RNAi* and control flies under LD conditions. (B) Actograms of representative individual control and *para-RNAi* flies. (C) Circadian rhythmicity in constant conditions was unaltered by *para-RNAi* knock down (D) Circadian period of *para-RNAi* flies was significantly lengthened compared to controls. (E) Overall locomotor activity was not significantly different between *para-RNAi* and control flies. Night activity was slightly increased, but not significantly. (F) The D/N ratio for *para-RNAi* flies was slightly reduced compared to controls. Error bars represent mean ± SEM, for period error bars are median with IQR. N = 32 for each group.

5.5.2 The role of *para* in sleep

Looking at sleep across five days of light-dark behaviour found that there were no clear differences between control and *para-RNAi* flies. Comparing the daily sleep profile between control and *para-RNAi* flies showed there was nearly no difference with the two plots almost overlapping (Figure 5-8A).

Quantification of sleep parameters showed a small reduction in night-time sleep in *para-RNAi* flies (Figure 5-8C), but all other measures were not significantly different.

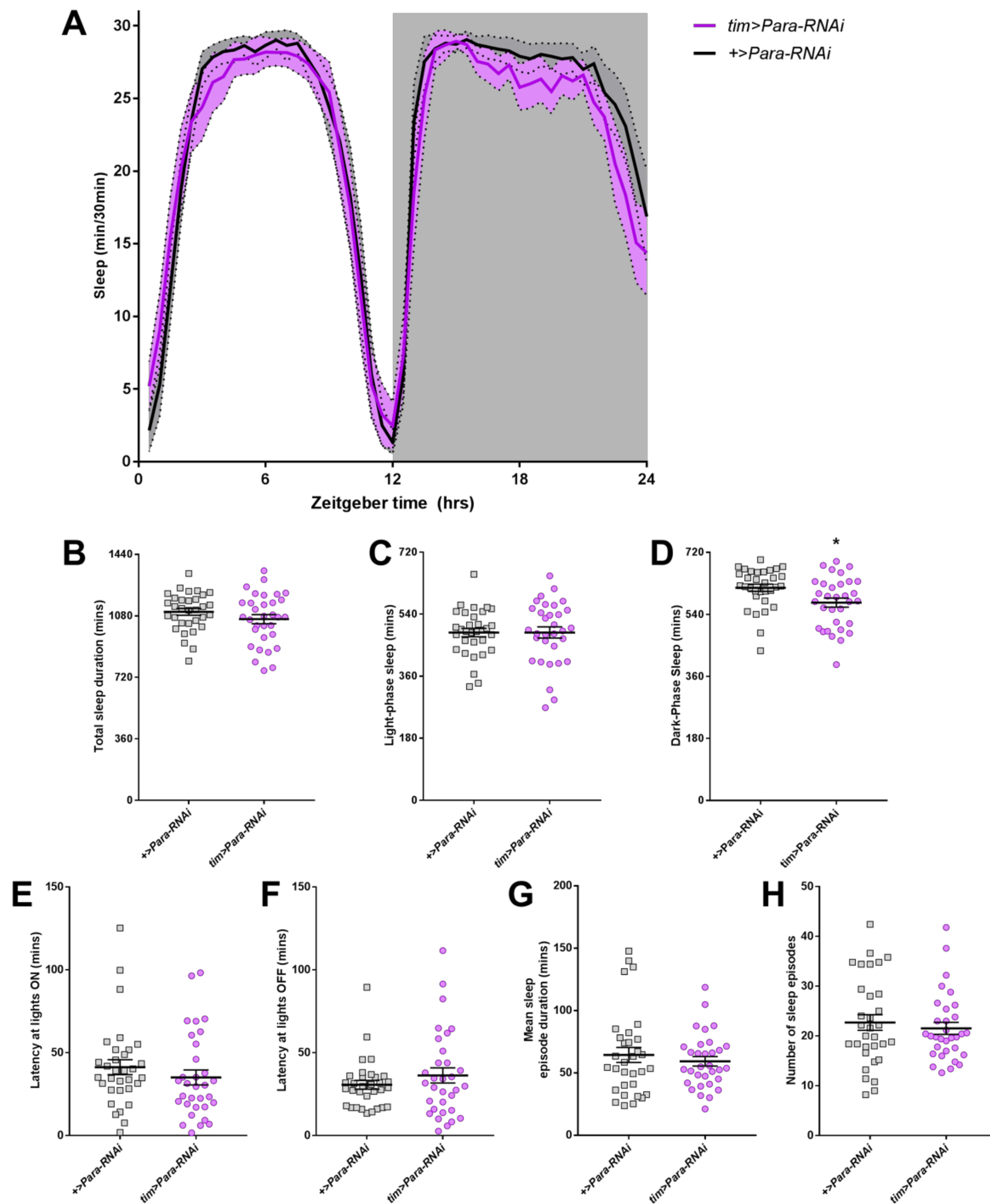


Figure 5-8 Effect of knock down of *para* on sleep behaviour

(A) Daily sleep profile of *para-RNAi* (purple) and control (grey) flies, *para-RNAi* flies showed a very similar sleep profile to control flies, with maybe a slight decrease at the very end of the night. (B) Total sleep duration was not significantly different. (C) Day-time sleep showed no change. (D) Night-time sleep was significantly reduced in *para-RNAi* flies. (E) and (F) Sleep latency showed no difference in *para-RNAi* knock down. (G) and (H) Sleep episode duration and number of sleep episodes were unchanged. Error bars represent mean ± SEM. N = 32 flies for each group.

5.5.3 Knock down of *para* has limited behaviour impacts

Targeted knock down of *para* throughout the circadian system seems to only have limited impact on circadian and sleep behaviour. Using RNAi to knock down the ion channel is unlikely to entirely remove expression of the channel and so it is possible there are still enough sodium channels present for appropriately driving initiation of action potentials in the clock neurons. An incomplete knock down of the channel could produce a situation like the heterozygous SCNA1 mice mutants (Han et al. 2012), where a similar lengthening in circadian period was observed to what I have reported in *Drosophila* here (Figure 5-7).

The limited effects of the *para-RNAi* transgene on both sleep and circadian rhythms suggests that it is not as important as other ion channels, e.g. *para* mRNA expression is about 15% the level of *Shaker* (Kula-Eversole et al. 2010), or that the RNAi knock down is not complete enough to remove enough of the channel to see a full phenotype.

5.6 Shaker, voltage-gated potassium channel

The Shaker (Sh) ion channel was first identified and named from mutations which caused shaking of legs under ether anaesthesia, along with its accessory channels Hyperkinetic (Hk) and ether à go-go (eag) (Kaplan and Trout 1969). Electrophysiological analysis of Drosophila neurons has shown that Shaker encodes the transient A-type K+ current (I_A) (Tanouye and Ferrus 1985; Yao and Wu 1999). Such is the conserved nature of the Shaker gene that the Drosophila sequence was used to predict and identify homologues in other species, including a mouse brain potassium channel which had 72% sequence similarity (Tempel et al. 1988). The mammalian homologue is the $K_V1.1$ family of channels, have broad expression in neurons and skeletal muscle and have an important role in maintaining membrane potential (Gutman et al. 2005).

A *Drosophila* mutagenesis screen of over 9,000 lines identified an extreme short sleeping mutant showing sleep amounts of one third the control levels, termed *minisleep* (*mns*), which was identified as a point mutation in the conserved domain of *Shaker* (Cirelli et al. 2005). In these lines the mutation

was expressed throughout the organism, producing the shaking phenotype and also resulting in a reduced lifespan. *Shaker* currents have been shown to be important in control of sleep homeostasis in neurons of the dorsal fan-shaped body where the shaker current is important for driving sleep, with RNAi against *Shaker* resulting in decreased sleep as in the *minisleep* mutants (Pimentel et al. 2016; Dissel and Shaw 2016).

Here I am expressing *Shaker-RNAi* only in the circadian system and measuring the effect on circadian behaviour and sleep. From the microarray dataset *Shaker* mRNA did not show statistically significant cycling, but expression was at a very high levels and showed increased expression in the early night.

5.6.1 Effect of knocking down *Shaker* on circadian behaviour

Clock specific expression of the *Shaker-RNAi* construct did not drastically alter gross behaviour compared to control flies but did cause a slight increase in the strength of free-running behaviour and a significant lengthening of circadian period (Figure 5-9). Looking at activity under LD conditions, there was no overall difference in locomotor activity between *Shaker-RNAi* flies, but there was a significant increase in night-time activity. The increase in activity at night caused a significant reduction in the D/N ratio of *Shaker-RNAi* flies, with some flies displaying a preference for being active more at night than during the day.

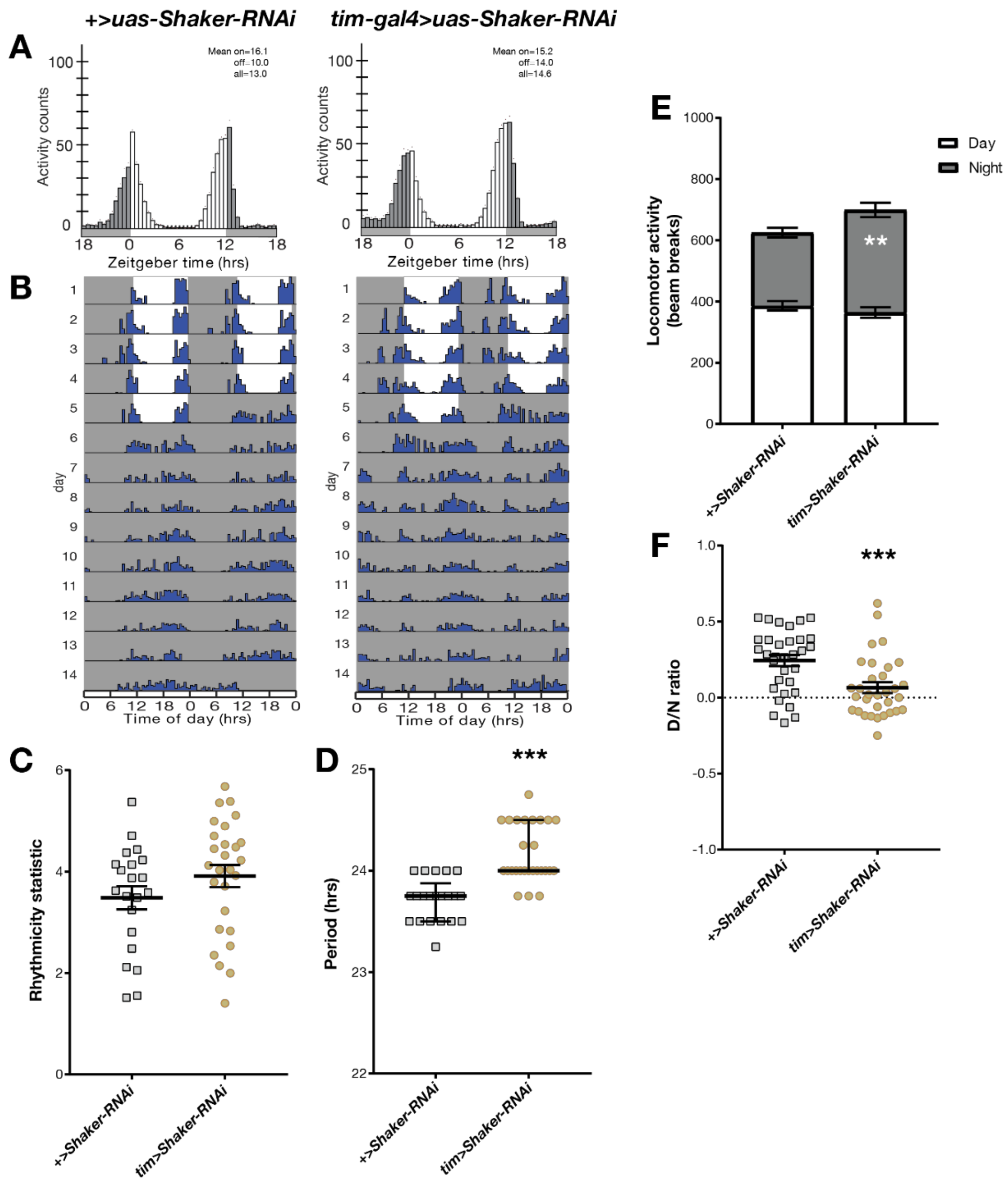


Figure 5-9 Effect of knock down of *Shaker* on circadian activity

Locomotor activity of control and *Shaker-RNAi* flies (A) Histograms of daily activity of *Shaker-RNAi* and control flies under LD conditions. (B) Actograms of representative individual *Shaker-RNAi* and control flies. (C) Circadian rhythmicity was not significantly different (D) Circadian period of *Shaker-RNAi* was significantly lengthened compared to controls. (E) Total locomotor activity of *Shaker-RNAi* flies was not significantly different, there was a significant increase in night locomotor activity (F) The D/N ratio for *Shaker-RNAi* flies was drastically reduced compared to controls. Error bars represent mean ± SEM, for period error bars are median with IQR. N = 32 for each group.

5.6.2 Sleep behaviour in *Shaker-RNAi* flies

Sleep in *Shaker-RNAi* flies showed interesting changes in sleep timing. The total sleep duration across the 24-hour cycle was not altered but breaking this down to day and night sleep showed *Shaker-RNAi* flies had a significant increase in day-time sleep but and an equal decrease in night-time sleep ($p<0.01$) (Figure 5-10). This shift in sleep timing can also be quantified by observed changes in sleep latency in *Shaker-RNAi* flies compared to controls, with a decrease in latency for day sleep and an increase in latency for night sleep. There was no difference in the number of sleep episodes or mean sleep episode length.

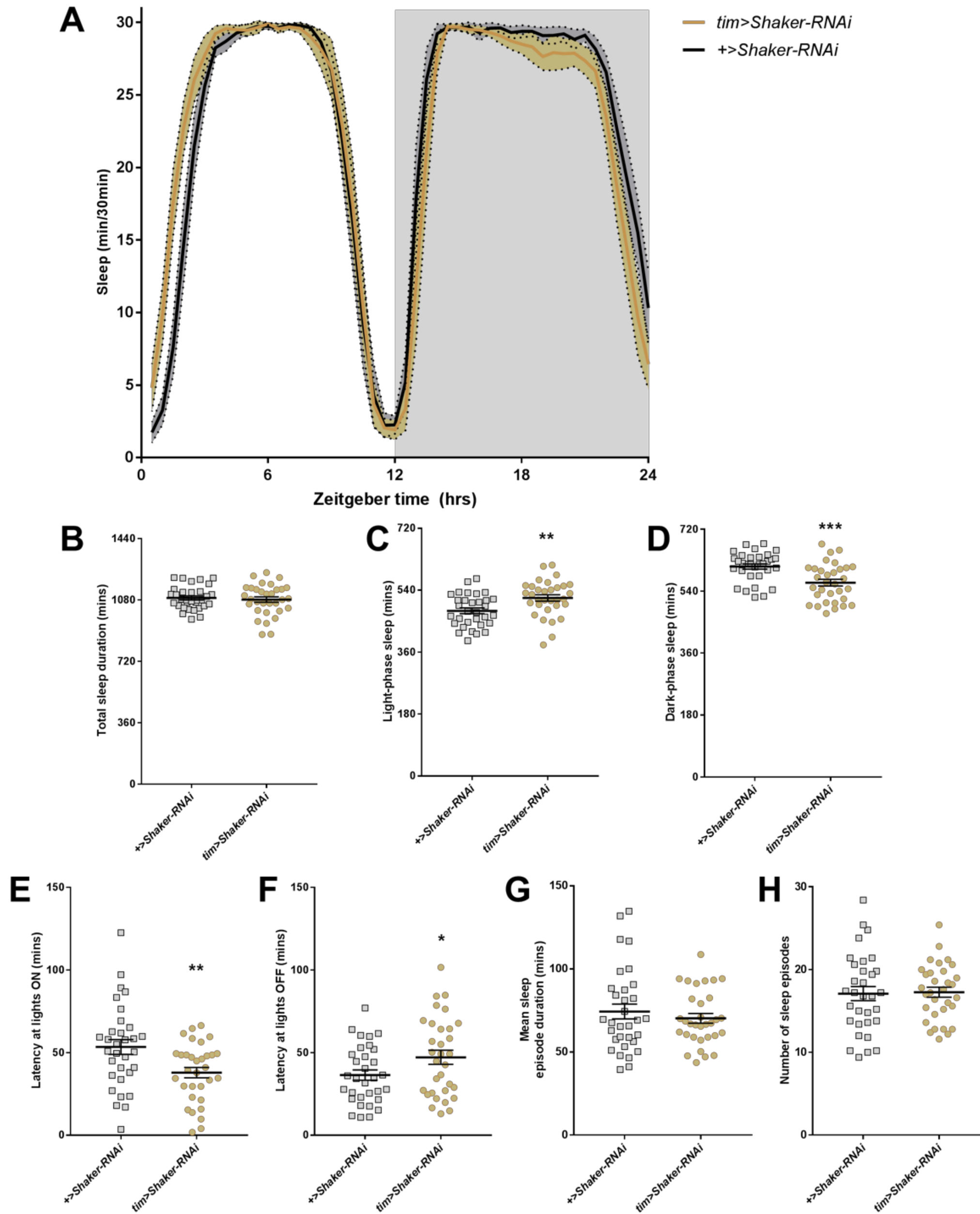


Figure 5-10 Effect of knock down of *Shaker* on sleep

(A) Daily sleep profile of *Shaker-RNAi* (brown) and control (grey) flies, overall sleep behaviour in *Shaker-RNAi* flies looks broadly similar to controls, with a small difference in the early morning. (B) Total sleep duration was not significantly different. (C) *Shaker-RNAi* flies had a significant increase in day-time sleep duration. (D) Night-time sleep was significantly reduced in *Shaker-RNAi* flies. (E) *Shaker-RNAi* flies had a significantly shorted sleep latency at lights on compared to controls. (F) Sleep latency at lights off was slightly longer in *Shaker-RNAi* flies. (G) and (H) Sleep episode duration and number of sleep episodes were unchanged. Error bars represent mean ± SEM. N = 32 flies for each group.

5.6.3 Circadian and sleep behaviour are altered by knocking down *Shaker*

Expressing RNAi against *Shaker* in the circadian system should be expected to bias the neurons against A-type currents, making the neurons more excitable. Given that the total sleep duration is very similar between *Shaker-RNAi* flies and controls, and that the changes in day and night sleep are opposite and equal it seems that the *RNAi* knock down has differential effects at different times of day that might relate to the phasing of *Shaker* expression in a wild type situation.

Somewhat contradictory to this is that the effect of the *Shaker-RNAi* on sleep latency happened in opposite directions. Sleep latency during the day was reduced in the RNAi knock down while for night sleep it was lengthened, which again may relate to when *Shaker* channels are normally active.

5.7 Shal, Shaker cognate I

Of the four potassium channel genes in *Drosophila*, *Shal* is the most highly conserved with 82% amino acid homology to the mouse gene (*mShal*) across the transmembrane region (Pak et al. 1991). *Shal* is similar to *Shaker* in that it is responsible for producing an A-type current, i.e. rapidly activating and inactivating, encoding virtually all of the transient K^+ current in *Drosophila* embryos (Tsunoda and Salkoff 1995), and is normally the main A-type current in neurons (Hodge et al. 2005; Gasque et al. 2005; Ping et al. 2011). *Shal* mRNA showed significant cycling in LN_V neurons from microarray studies, but the expression levels were much lower than for *Shaker* so which channel provides the main A-type current in LN_V neurons is unclear.

The equivalent of *Shal* in mice is the K_V4 family encoded by the KCND gene is widely expressed in the SCN and the current shows a diurnal rhythm that also persists in constant darkness (Itri et al. 2010). Knockdown of $K_V4.2$ or $K_V4.3$ in mouse SCN causes a shortening of circadian periodicity of electrical activity and locomotor output (Granados-Fuentes et al. 2012), and the same has recently been shown for the less well expressed $K_V4.1$ (Hermanstyne et al. 2017). How *Shal* contributes to driving the daily rhythms in electrical

activity of *Drosophila* clock neurons and in turn behavioural activity was unknown at the time of these experiments, but a recent paper has suggested a role of *Shal* in the regulation of sleep onset (Feng et al. 2018).

5.7.1 Circadian behaviour in *Shal-RNAi* flies

Driving knock down of *Shal* in *Drosophila* clock neurons had a significant effect on circadian behaviour resulting in changes in both diurnal behaviour under LD cycles and in free-running activity (Figure 5-11). For circadian monitoring *Shal-RNAi* flies had a significant increase in circadian rhythmicity and a lengthening of free-running period.

During monitoring of behaviour under LD conditions there were significant differences in activity, with *Shal-RNAi* flies demonstrating significantly higher locomotor activity during the night compared to control flies, but no difference in activity during the day. This tendency towards night time activity resulted in a decrease in the D/N ratio value of *Shal-RNAi* flies, identifying an increase in night

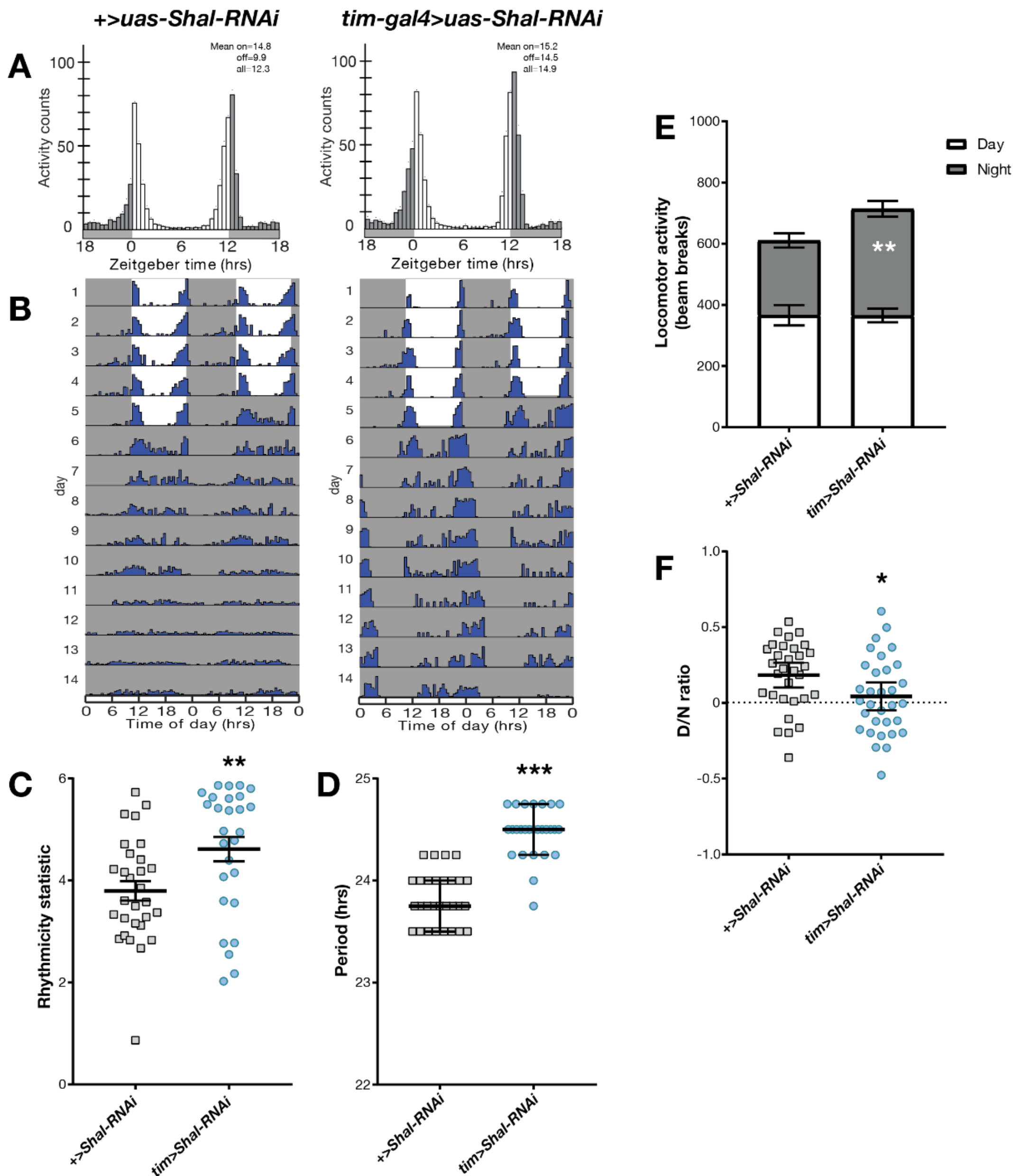


Figure 5-11 Effect of knock down of *Shal* on circadian activity

(A) Histograms of daily activity of *Shal-RNAi* and control flies under LD conditions. (B) Actograms of representative individual *Shal-RNAi* and control flies. (C) *Shal-RNAi* flies showed a significant increase in rhythm strength under DD conditions. (D) Circadian period of *Shal-RNAi* was significantly lengthened compared to controls. (E) Total locomotor activity of *Shal-RNAi* flies was not significantly different, there was a significant increase in night locomotor activity (F) The D/N ratio for *Shal-RNAi* flies was slightly reduced compared to controls. Error bars represent mean ± SEM, for period error bars are median with IQR. N = 32 for each group.

5.7.2 Effect of knocking down *Shal* on sleep

The impacts of knocking down *Shal-RNAi* on sleep behaviour were not huge. Simply looking at the daily sleep plot, sleep activity was very similar to control flies with maybe a slightly longer day-time siesta and slightly shorter night-time sleep. Quantification of sleep duration found that there was no difference in overall, day-time or night-time sleep between *Shal-RNAi* and control flies. Latency to sleep was no different during the day, but there was a slight lengthening at night in knock down flies. More detailed analysis of sleep bout parameters showed that there was a significant reduction in the number of sleep episodes for *Shal-RNAi* flies ($p<0.01$), which seems to be counterbalanced by an increase (but non-significant) in the mean sleep episode length.

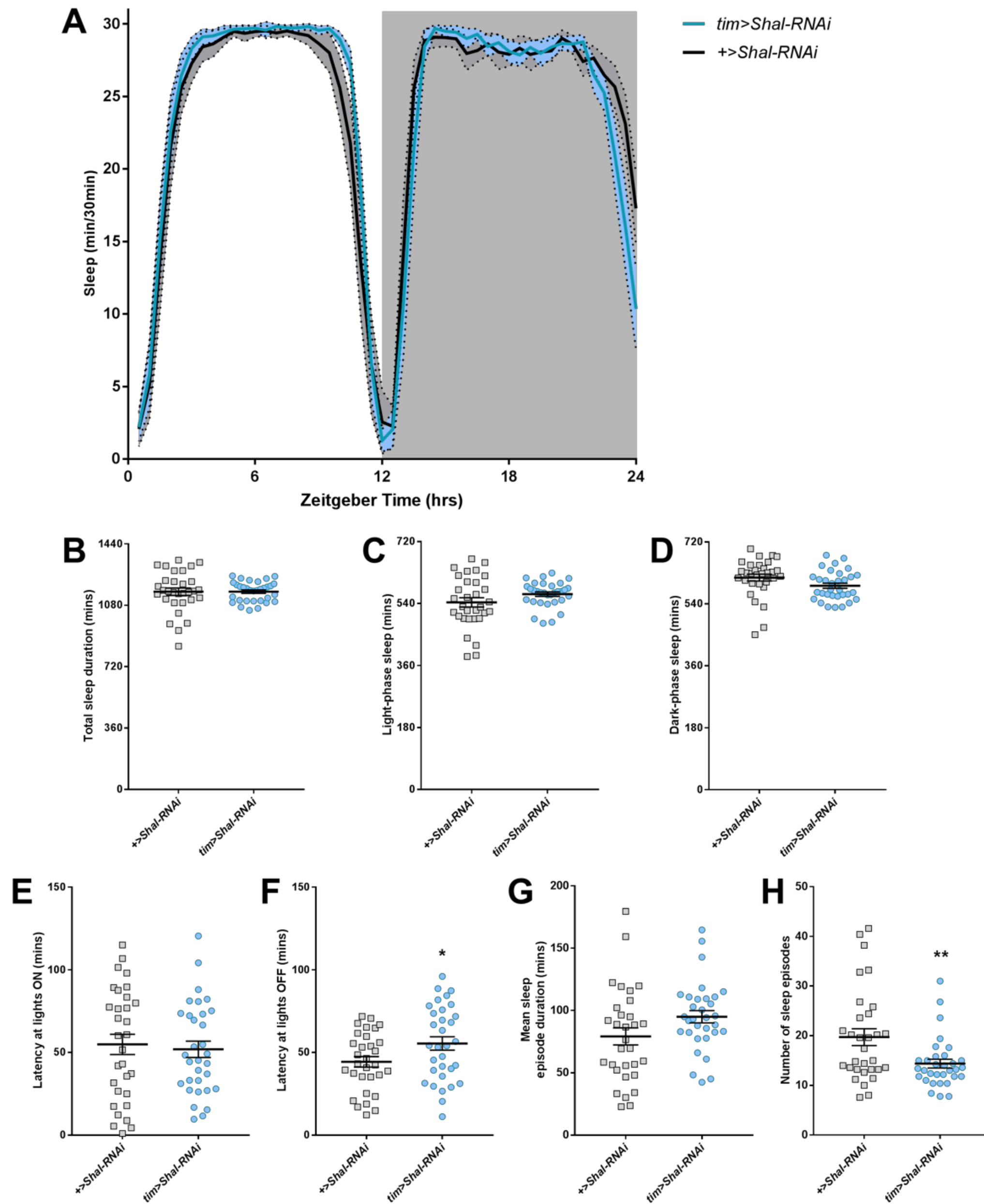


Figure 5-12 Effect of knock down of *Shal* on sleep behaviour

(A) Daily sleep profile of *Shal-RNAi* (cyan) and control (grey) flies. The sleep profile of *Shal-RNAi* flies was highly similar to controls. (B), (C) and (D) Sleep duration was not affected overall, at day or at night. (E) There was no difference in sleep latency at lights on. (F) Sleep latency at lights off was slightly longer in *Shal-RNAi* flies. (G) Mean sleep episode duration was longer in *Shal-RNAi* flies, but the difference was not significant. (H) The number of sleep episodes was significantly lower in *Shal-RNAi* flies. Error bars represent mean ± SEM. N = 32 flies for each group.

5.7.3 *Shal* knock down has significant effects on circadian behaviour

Knocking down *Shal* mRNA throughout the circadian system resulted in significant changes in behaviour both under LD conditions and for free-running in DD. The effect on night-time activity could be linked to the timing of *Shal* mRNA expression in control situations. Normally *Shal* mRNA is highly expressed at ZT12, suggesting that the channel and therefore the current is highly expressed at night. In the RNAi knock down there will be a greater effect of removing this high night expression, and so the effect of *Shal* on reducing excitability will be removed, potentially driving increased electrical and therefore behavioural activity at night.

The effects of knocking down *Shal* on sleep behaviour are much more modest. The largest effects on sleep were a significant reduction in the number of sleep episodes, with a slight increase in mean episode duration, but overall sleep amounts were not different. Together this seems to suggest more stable, consolidated bouts of sleep.

5.8 Shaw, Shaker cognate w

Shaw is a voltage-gated K^+ channel in the *Shaker* family, encoding for a channel that produces a slowly activating and non-inactivating current (Tsunoda and Salkoff 1995). *Shaw* is widely expressed in the *Drosophila* central nervous system, and the channels are thought to contribute to the setting of the resting membrane potential (Hodge et al. 2005). RNAi knock down of *Shaw* in the *Drosophila* circadian clock has previously been shown to reduce the strength of behavioural outputs (Hodge and Stanewsky 2008) and to remove diurnal changes in electrical activity in the LN_V neurons (Buhl et al. 2016).

The mammalian homologue of *Shaw* is the K_V3 family of ion channels, encoded by the genes KCNC1-4 (Gutman et al. 2005). $K_V3.1$ and $K_V3.2$ are widely expressed in the mouse SCN, showing higher expression during the day, and are thought to be important for rhythmic electrical activity (Itri et al. 2005). Mouse double knock outs of $K_V3.1$ and $K_V3.2$ have significant circadian disruption, with fragment behaviour rhythms (Kudo et al. 2011).

I have used the same *Shaw-RNAi* construct as previously published (Hodge and Stanewsky 2008), to examine the effects on circadian behaviour and extend this analysis to sleep. *Shaw* mRNA has been reported to cycle significantly in the LN_V neurons, with higher expression during the early morning and significantly lower expression at night (Kula-Eversole et al. 2010).

5.8.1 *Shaw-RNAi* circadian behaviour

Expressing RNAi against *Shaw* mRNA throughout the circadian clock of *Drosophila* had extremely significant effects on behavioural outputs (Figure 5-13). Circadian free-running activity in *Shaw-RNAi* flies was at a significantly lower strength compared to control flies and the period was significantly longer.

Diurnal activity recorded in LD, showed that *Shaw-RNAi* flies had significantly lower amounts of total locomotor activity, as a result of a significant reduction in activity during the day, activity levels at night were the same as control flies. The reduction in activity during the day, meant that *Shaw-RNAi* flies actually showed more activity at night compared to day, resulting in a negative value for the D/N ratio.

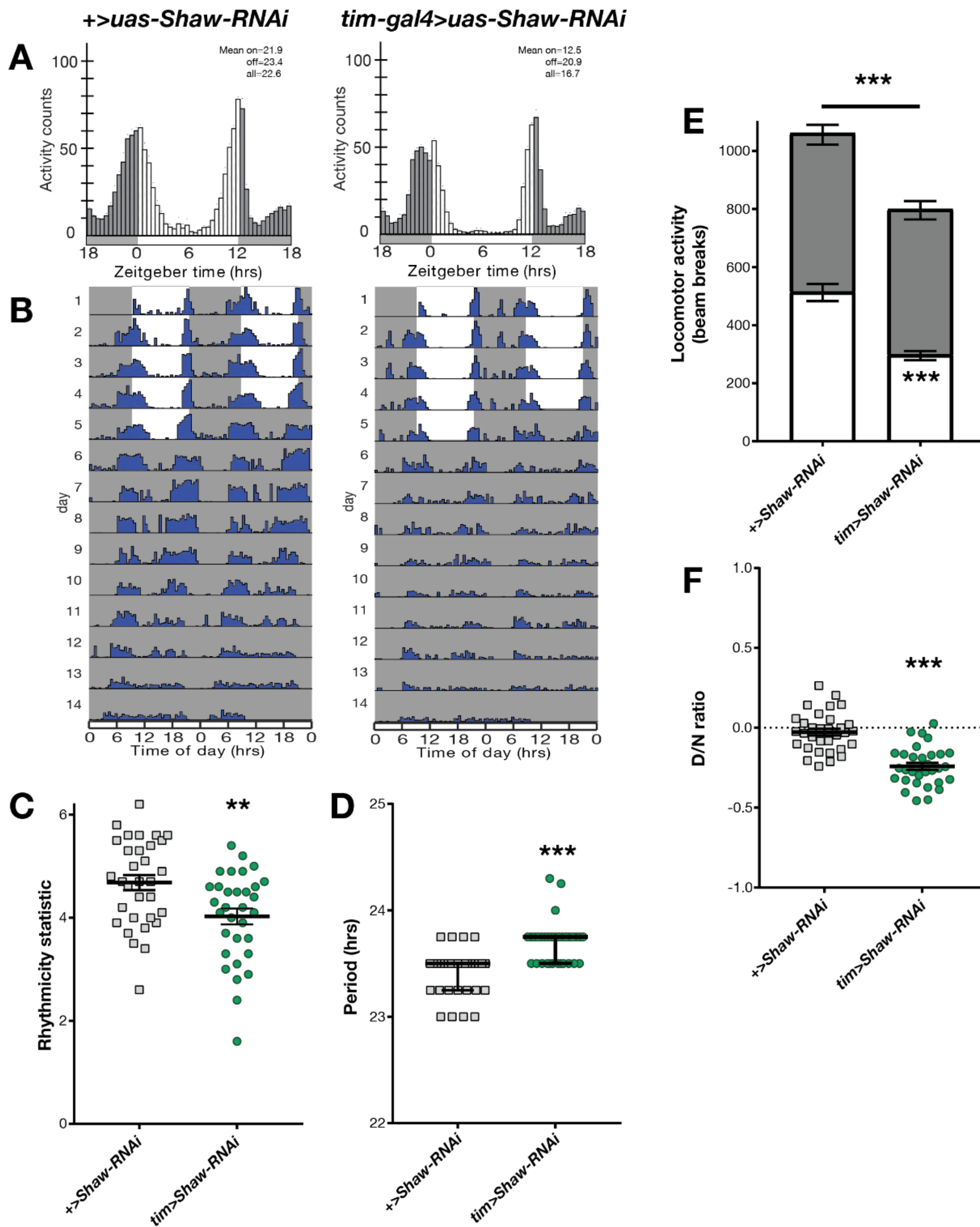


Figure 5-13 Effect of knock down of *Shaw* on circadian activity

(A) Histograms of daily activity of *Shaw-RNAi* and control flies under LD conditions. (B) Actograms of representative individual *Shaw-RNAi* and control flies. (C) *Shaw-RNAi* flies showed a significant decrease in rhythm strength under DD conditions. (D) Circadian period of *Shaw-RNAi* was significantly lengthened compared to controls. (E) Total locomotor activity of *Shaw-RNAi* flies was significantly lower than controls, with a significant decrease in day-time activity but no effect on night-time activity. (F) The D/N ratio for *Shaw-RNAi* flies was slightly reduced compared to controls. Error bars represent mean ± SEM, for period error bars are median with IQR. N = 32 for each group.

5.8.2 *Shaw-RNAi* sleep behaviour

Sleep behaviour of *Shaw-RNAi* flies was significantly altered compared to control flies, which can clearly be seen on the daily sleep plot with significant deviation in sleep profiles between the two groups (Figure 5-14). Total sleep duration was significantly increased in *Shaw-RNAi* flies, as a result of a significant increase in day-time sleep. There was no difference in night-time sleep. In terms of sleep latency, no difference was seen for day-time sleep but there was a significant increase in latency of the first sleep bout at night for *Shaw-RNAi* flies. Significant differences were also observed in the properties of the sleep episodes between control and RNAi flies. *Shaw-RNAi* flies had a significantly reduced number of sleep episodes, with an increase in mean episode duration.

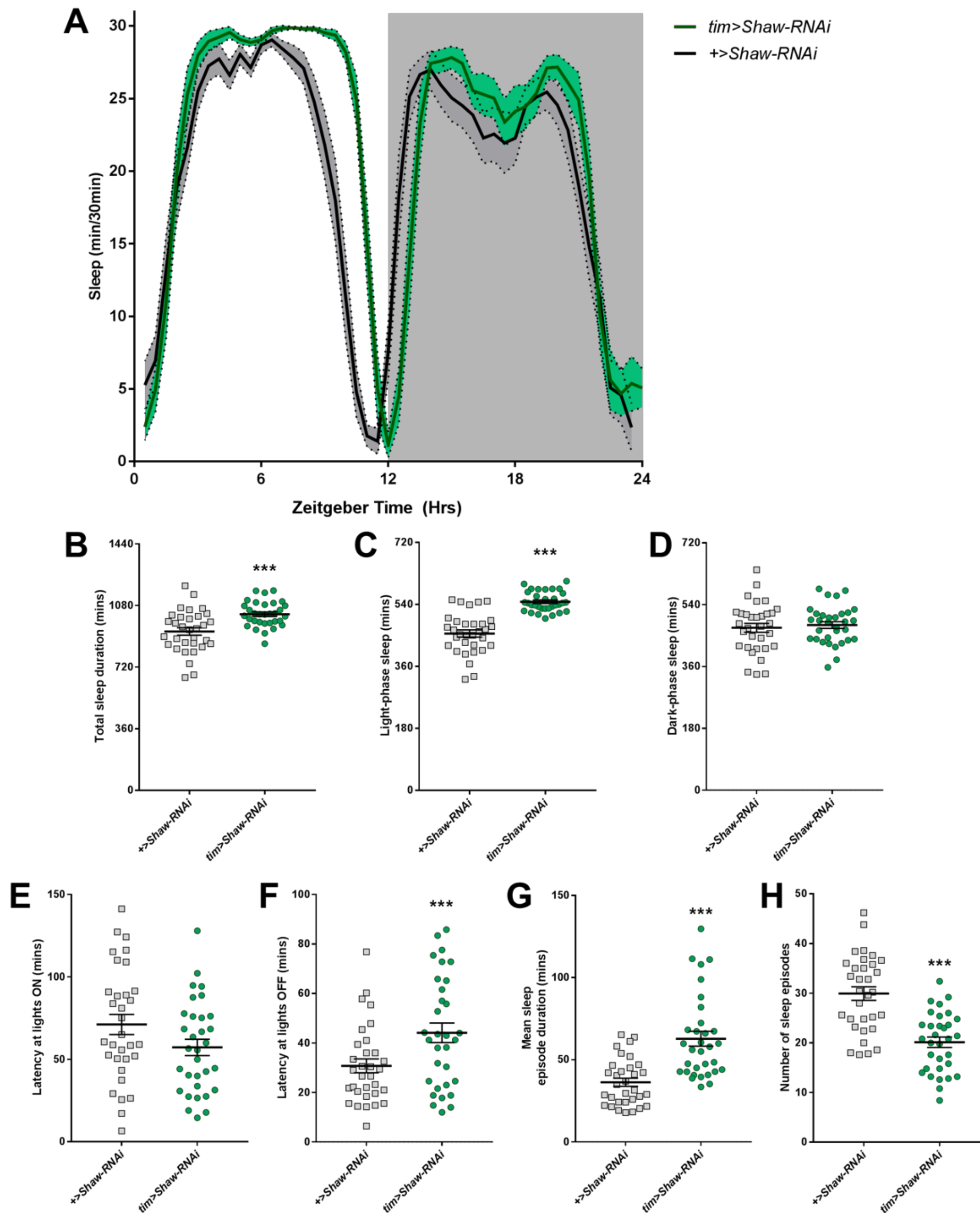


Figure 5-14 Effect of knock down of *Shaw* on sleep behaviour

(A) Daily sleep profile of *Shaw-RNAi* (green) and control (grey) flies. The sleep profile of *Shaw-RNAi* flies was clearly highly altered compared to control flies, with differences most apparent during the day. (B) Total sleep duration was significantly increased in *Shaw-RNAi* flies. (C) The increase in sleep duration in *Shaw-RNAi* flies was highly significant during the day. (D) There was no difference in sleep duration at night. (E) There was no difference in sleep latency at lights on. (F) Sleep latency at lights off was significantly longer in *Shaw-RNAi* flies. (G) Mean sleep episode duration was significant longer in *Shaw-RNAi* flies. (H) *Shaw-RNAi* had a significant reduction in the number of sleep episodes. Error bars represent mean ± SEM. N = 32 flies for each group.

5.8.3 *Shaw-RNAi* effects both circadian and sleep behaviour

Knocking down of *Shaw* in the circadian clock had significant effects on both circadian and sleep behaviour. Diurnal activity was significantly altered, with a large reduction in daytime locomotor activity in *Shaw-RNAi* flies. Given the role of *Shaw* in setting resting membrane potential these changes in activity are likely due to changes in neuronal excitability of subsets of clock neurons. *Shaw-RNAi* l-LN_V neurons have normal wild type like electrical properties during the day (Buhl et al. 2016), which suggests these day-time effects on activity are being mediated by a different part of the circadian network. It has previously been shown that expressing *Shaw-RNAi* in only dorsal clock neurons is sufficient to result in a lengthening of free running period (Hodge and Stanewsky 2008).

In terms of sleep behaviour there was a significant reduction in the amount of sleep in *Shaw-RNAi* flies, a result of a reduction in day-time sleep. This day-time sleep effect likely relates to the same pathway as the reduction in locomotor activity during the day, with *Shaw-RNAi* flies showing less of wake drive during the day consequently moving around less. In a previous study where *Shaw-RNAi* was expressed using the *Pdf-Gal4* driver, flies show decreased sleep (Parisky et al. 2008), again suggesting the changes observed here using the *tim-Gal4* driver are not being mediated by the PDF-expressing clock neurons.

5.9 slowpoke, calcium-activated, voltage activated potassium channel

The *Drosophila* gene *slowpoke* (*slo)* encodes a calcium-activated, voltage-activated potassium channel (K_{Ca}) (Atkinson et al. 1991; Adelman et al. 1992). Mammalian homologues of *slo* were identified from the *Drosophila* channel and were originally termed 'big' potassium (BK) channels due to their large conductance compared to SK channels (Salkoff et al. 2006). *slo*/BK channels are independently gated by calcium and voltage, and are most often opened by an increase in intracellular calcium, either by release from internal stores or from secondary messenger systems (Ghatta et al. 2006). BK channels are widely expressed throughout the nervous system, but in the central brain are involved in neurotransmitter release and maybe

most importantly in repolarization of action potentials in particular the fast phase of the afterhyperpolarization (Latorre et al. 2017).

BK and *slowpoke* have both been widely implicated in the mammalian and *Drosophila* circadian systems. In *Drosophila* the *slowpoke binding protein* (*slob*), a negative modulator of *slo*, was one of the first genes to be identified as showing circadian cycling (Claridge-Chang et al. 2001; Wijnen et al. 2006). At a behavioural level, flies expressing a null mutation of *slo* display significant circadian arrhythmicity and downstream disruption to PDF signalling (Fernandez et al. 2007). In mice, the BK channel is regulated by *Per2* expression and the BK current is essential for regulating the daily cycle in electrical activity of the SCN and for rhythmic behavioural outputs (Meredith et al. 2006; Whitt et al. 2016). Loss of diurnal regulation of the BK current is linked to a loss of circadian rhythmicity (Montgomery et al. 2013) and has been linked to the degradation of circadian behaviour observed during the ageing process in mice (Farajnia et al. 2015).

From the microarray dataset, *slo* and *slob* did not show statistically significant cycling, but their expression did change over the diurnal cycle. Expression of *slo* increased in the late afternoon and at night, with levels of *slob* lower in the afternoon at night, which combined should mean BK channel activity would be higher at night (at least in the LN_V neurons).

5.9.1 Circadian behaviour in *slo-RNAi* flies

Knock down of *slo* across the circadian system had significant effects on diurnal and circadian behaviour (Figure 5-15). Circadian behaviour of *slo-RNAi* flies was significantly altered compared to control flies, there was a significant reduction in the strength of the clock measured by the rhythmicity statistical and a very significant lengthening of period. Under LD monitoring *slo-RNAi* flies had a large and significant reduction in locomotor activity compared to control flies, with activity significantly reduced both during the day and at night. The impact of this reduction in locomotor activity was that *slo-RNAi* flies had a shift towards more activity at night, result in a reduction in the value of the D/N ratio compared to control flies.

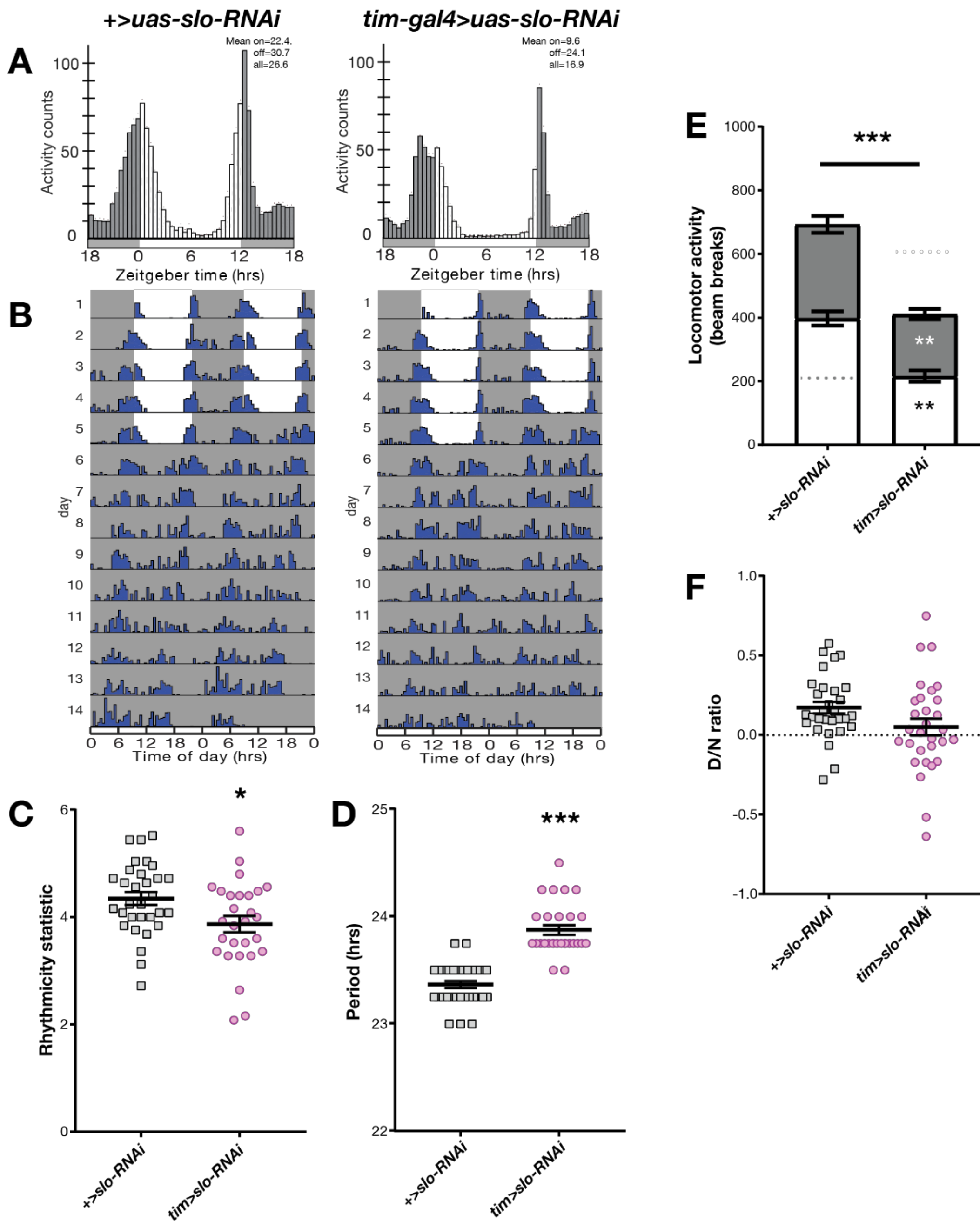


Figure 5-15 Effects of knock down of *slo* on circadian activity

Locomotor activity of control and *slo-RNAi* flies (A) Histograms of daily activity of *slo-RNAi* and control flies under LD conditions. (B) Actograms of representative individual *slo-RNAi* and control flies. (C) Circadian rhythmicity was reduced in *slo-RNAi* flies compared to controls (D) Circadian period of *slo-RNAi* was significantly lengthened compared to controls. (E) Locomotor activity of *slo-RNAi* was drastically altered compared to controls, with significant reduction at day, night and overall. (F) The D/N ratio for *slo-RNAi* was not significantly different. Error bars represent mean ± SEM, for period error bars are median with IQR. N = 32 for each group.

5.9.2 *slo* knock down alters sleep duration

Sleep behaviour of *slo-RNAi* flies under LD conditions was significantly altered compared to control flies. Looking at the daily sleep profile of *slo-RNAi* flies compared to controls (Figure 5-16A), a clear difference can be seen between the two groups with a longer period of day-time sleep in *slo-RNAi* and a higher level of night-time sleep. Quantification of sleep duration found that knock down of *slo* led to a significant increase in total duration, with significant increases in both day-time and night-time sleep. There was also a significant reduction in latency to sleep for both day and night for *slo-RNAi* flies. No difference was found in the mean sleep episode duration or the number of sleep episodes.

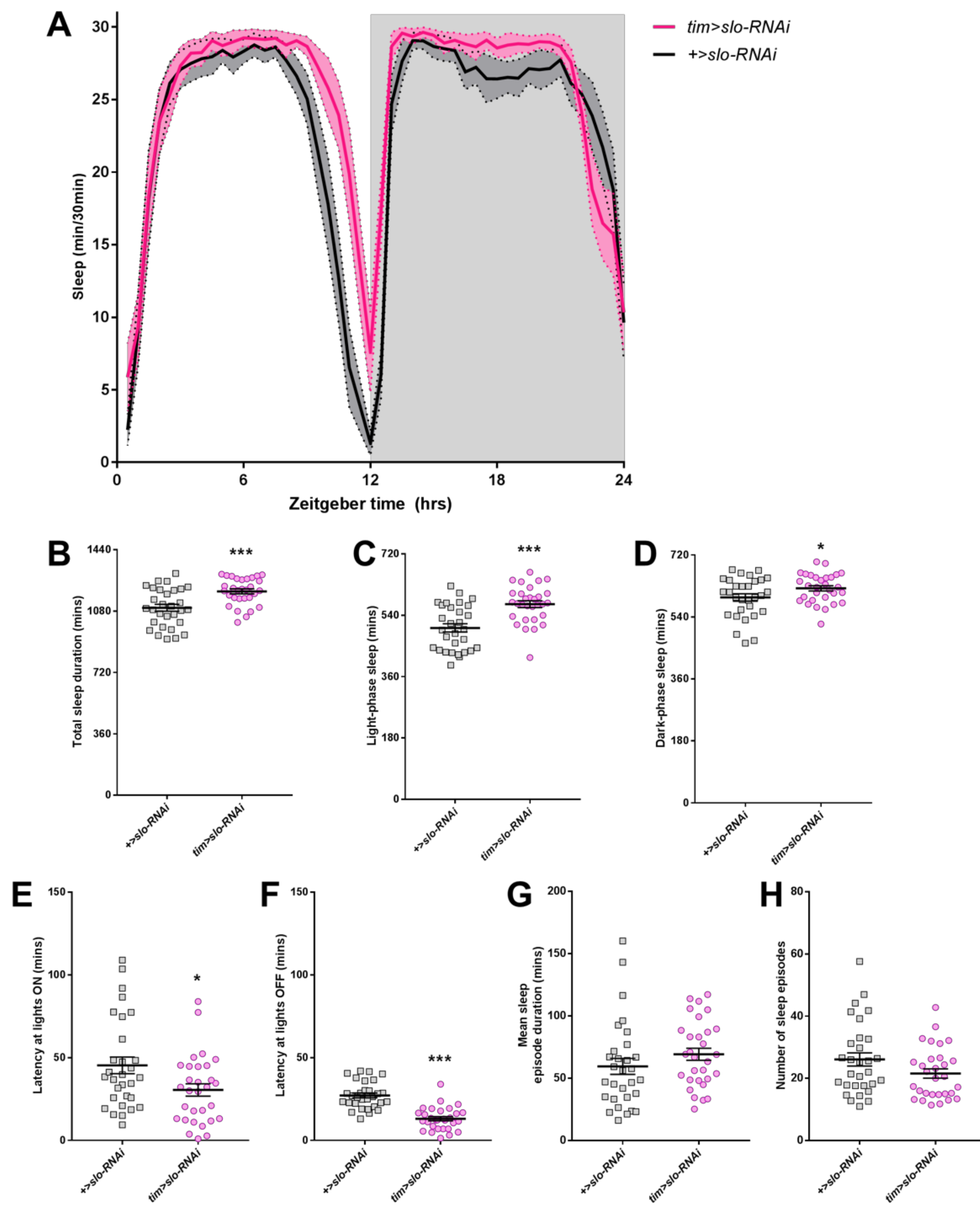


Figure 5-16 Effect of knock down of *slo* on sleep behaviour.

(A) Daily sleep profile of *slo-RNAi* (magenta) and control (grey) flies. The sleep profile of *slo-RNAi* flies shows clear differences compared to control flies, with differences most apparent during the day. (B) Total sleep duration was significantly increased in *slo-RNAi* flies. (C) The increase in sleep duration in *slo-RNAi* flies was highly significant during the day. (D) *slo-RNAi* flies also had increased sleep duration at night. (E) Sleep latency at lights on was reduced in *slo-RNAi* flies. (F) Sleep latency at lights off was significantly shorter in *slo-RNAi* flies. (G) and (H) Sleep episode duration and number of sleep episodes were unchanged. Error bars represent mean ± SEM. N = 32 for each group.

5.9.3 Knocking down *slo* in the clock has significant effect on circadian and sleep behaviour

slo encodes an important ion channel, given its role in membrane repolarisation following action potential and its activation by calcium linking it to other intracellular signalling mechanisms. RNAi-mediated knock down of *slo* across the circadian clock has significant effects on both circadian behaviour outputs and sleep. Circadian behaviour was significantly altered with a reduction in rhythm strength and increase in period. Locomotor activity was also significantly reduced across the day and night. The impacts on sleep were also very significant, with increases in sleep duration at both day and night combined with reductions in sleep latency. Knocking down *slo* and removing its current should be expected to reduce the rate of repolarization after an action potential, thereby increase the time between action potentials and reducing the firing frequency of clock neurons.

5.10 Conclusions from RNAi knockdown experiments

The results in this chapter show that individually manipulating the expression of a single ion channel solely within the circadian system can have large and significant impacts upon circadian behaviour and sleep, with the caveat that the RNAi manipulation require independent verification. As such these results only act as an initial screen of these ion channels and do not claim to report any definitive findings.

The diverse results observed demonstrate the complicated interplay of many ion channels in contributing to overall neuronal activity and output, yet also demonstrate the usefulness of *Drosophila* to study ion channel function where each ion channel family usually only contains a single gene. Table 5-4 summarises the wide range of impacts of manipulation ion channels on both circadian and sleep behaviour.

Ion channel	Circadian			Sleep						
				Sleep Duration			Sleep Latency			
	RS	Period	D/N	Overall	Day	Night	Day	Night	Mean Episode duration	# of episodes
SK	↓***	-	↓***	-	↑***	↓***	↓*	↑***	↑***	↓***
Irk1	-	↑*	↓***	-	-	↓***	-	↑*	-	-
Ca-a1t	↓***	↑***	↓***	-	↑***	-	↓**	-	↑*	↓***
para	-	↑***	↓*	-	-	↓*	-	-	-	-
Shaker	-	↑**	↓***	-	↑**	↓***	↓**	↑*	-	-
Shal	↑**	↑***	↓*	-	-	-	-	↑*	-	↓**
Shaw	↓**	↑***	↓***	↑***	↑***	-	*	↑**	↓***	↑***
slo	↓*	↑***	-	↑***	↑***	↑*	↓*	↓***	-	-

Table 5-4 Summary of the behavioural effects of RNAi knock down of ion channel expression

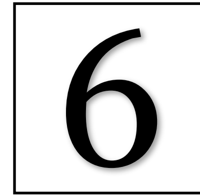
Using the assumption that the larger the impact of knocking down an ion channel the more important it is, it appears that SK has the most wide-reaching impact, affecting strength of the circadian clock and sleep behaviour (section 5.2). Given that all of these ion channels tested show significant cycling in their mRNAs, except *slo*, it should not be too much of a surprise that they have significant impacts of behavioural outputs. In fact, it is somewhat surprising that the effects are not larger.

One caveat that should be added is that for all of these manipulations the RNAi construct is expressed throughout development and so the effects seen may be reduced by compensatory mechanisms, with the expression of other ion channels altered to mediate for the knock down. Previous studies using ectopic expression of mammalian *Kir2.1* to electrically silence clock neurons have demonstrated this results in a loss of behavioural rhythmicity (Nitabach et al. 2002; Nitabach et al. 2005). In a later study using an inducible *Gal4* promotor (*Gal4-GeneSwitch*) to look only at effects silencing clock neurons in adult flies was enough to result in arrhythmic behaviour (Depetris-Chauvin et al. 2011). Using the *Gal4-GeneSwitch* approach in future experiments to only knock down expression of ion channels in adult flies would help to remove any questions about developmental effects.

For example, *Irk1* was the mRNA with by far the highest difference in peak to trough expression yet knocking it down did not have the largest effects. There are two other inwardly rectifying channels, *Irk2* and *Irk3*, in the *Drosophila* genome and it could be that these offer a means of compensating for the reduction in *Irk1*. For the other genes where there are no other homologues in the genome knocking them down has bigger impacts, such as in the case of *SK*.

As shown by the summary of results in Table 5-4, there was a significant effect on increasing period length in knocking down all of the ion channels tested, except for *SK*, and generally there was an effect on significantly reducing the D/N ratio as a result of increased night activity. These results suggest that electrical activity across the clock is a key factor in maintaining appropriate clocks outputs both for DD free-running behaviour, but also for diurnal behaviour in an LD cycle.

My overall conclusion from these RNAi knock down experiments is that the composition of ion channels in clock neurons is extremely important for mediating behavioural outputs. Even manipulating the expression levels of a single gene in the subset of 150 clock neurons can drive dramatic changes in behaviour demonstrating the fine balance of electrical activity in the circadian system. It would be interesting to carry out the same experiments using an inducible promotor to look at the effect of knocking down expression in adults only, or from a certain stage of adulthood, to remove any developmental effects which may be masking the full importance of the role of some of these ion channels.



CHAPTER 6 MODELLING ELECTRICAL ACTIVITY OF CLOCK NEURONS IN *DROSOPHILA*

This chapter describes the results from using mathematical approaches to understand, explain and experiment with electrical properties of clock neurons. Firstly, section 6.1 details mathematical modelling approaches for describing neuronal properties. Section 6.2 summarises some of the previous work on models of circadian neurons. Section 6.3 describes the results from one of the models, and section 6.4 outlines using this model to generate predictions of manipulations of the SK channel, and tests these experimentally using electrophysiology. Finally, section 6.6 summarises the conclusions from computational modelling experiments.

6.1 Modelling of the circadian system

Mathematical modelling approaches to the circadian clock have looked at the internal oscillations of the molecular clock with its transcription-translation feedback loops, in *Drosophila* (Goldbeter 1995; Gonze et al. 2003) and in mammals (Forger and Peskin 2003; Gallego et al. 2006). These models have been used to propose and confirm the role of post-translational mechanisms such as phosphorylation in determining the timing of the internal oscillator (Gallego and Virshup 2007).

Models for the electrical activity of clock neurons have been developed based upon experimental data gathered from electrophysiological recordings of SCN neurons (Choon and Forger 2007; Belle et al. 2009; Diekman et al. 2013). The models are based on the Hodgkin-Huxley model of squid giant axon (Hodgkin and Huxley 1952b), and its description of action potential generation.

6.2 Models of circadian neurons

In a model based upon Hodgkin-Huxley kinetics the neuron is modelled as a single compartment and is treated as akin to an electrical circuit. In this case the cell is modelled as a single spherical/point structure with no spatial elements considered. The lipid bilayer of the neuronal cell is represented by the capacitance parameter (C_m), i.e. the electrical charge stored by the membrane due to the level of ions on both sides of the lipid bilayer. Ion channels are represented in the circuits as resistors, with the conductance of voltage-gated ion channels modelled as dependent on voltage and time. Leak ion channels are simply modelled by a fixed conductance parameter.

One of the first neuronal models of a circadian neuron was the Sim-Forger model (Choon and Forger 2007), which was based on Hodgkin-Huxley equations with parameters fitted to experimental data (Jackson 2004) and showed that small changes in ionic conductance driven by the intracellular clock could drive the circadian modulation in electrical activity. The Sim-Forger model included only three voltage-gated currents (Na^+, K^+ and Ca^{2+}) and a passive leak component.

Diekman expanded upon the model to include calcium dynamics and incorporate a K_{Ca} channel (Diekman et al. 2013). This extended model also separates the leak current into distinct sodium (Na_{leak}) and potassium (K_{leak}) components. A recent model has proposed that the circadian changes in electrical activity are driven by antiphase rhythms in the sodium and potassium leak currents (Flourakis et al. 2015).

The model can be represented schematically and in terms of an electrical circuit (see **Figure 6-1**).

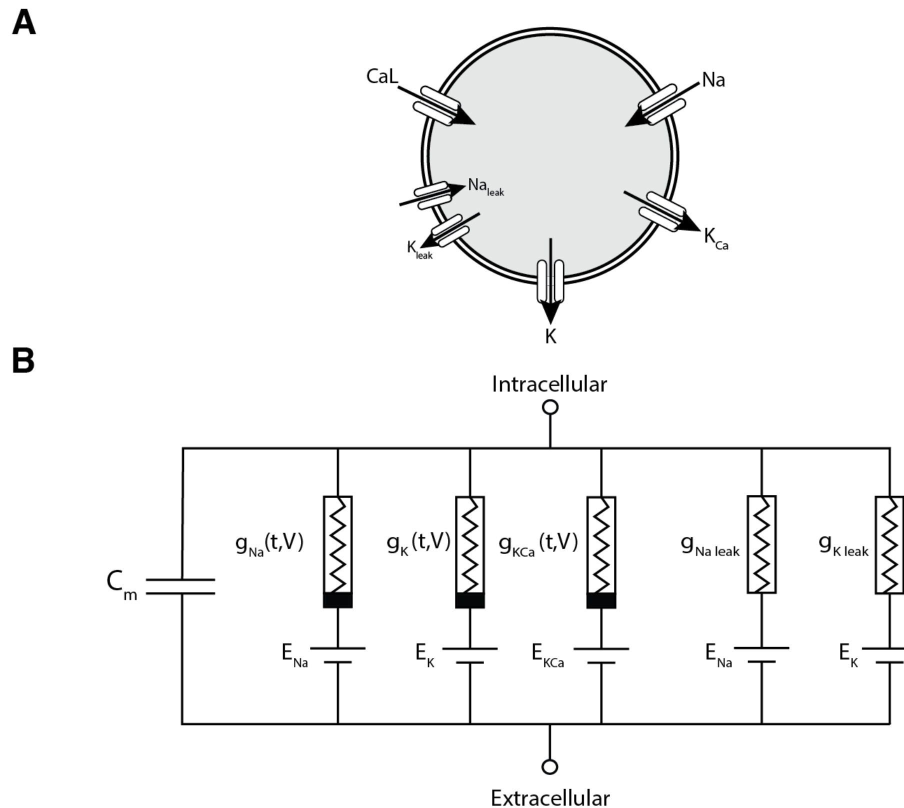


Figure 6-1 Schematic representing a circadian neuron based on the Hodgkin-Huxley modelling framework

(A) In the model the neuron is modelled with a simple sphere with voltage-gated channels for sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}), a calcium-activated potassium channel (K_{Ca}), and Na^+ and K^+ leak channels. (B) Equivalent electrical circuit for the cell in (A), voltage-gated ion channels are represented by conductance dependent on time and voltage, with leak ion channels having a fixed conductance. C_m = cell capacitance, g = conductance, E = reversal potential.

6.3 Implementation of a circadian model

I have implemented computationally the model of an SCN neuron (Diekman et al. 2013) and used this to reproduce the model conditions from the original paper.

The equations unpinning the model are outlined in Figure 6-2, where each group of ion channels is modelled as a current, I, e.g. I_{Na}, with the overall change in membrane potential equal to the sum of the separate ion channels.

$$C_m \frac{dV}{dt} = I_{app} - I_{Na} - I_K - I_{Ca} - I_{KCa} - I_{K\,leak} - I_{Na\,leak}$$

$$= I_{app} - g_{Na} m^3 h(V - E_{Na}) - g_K n^4 (V - E_K) - g_{Ca} r f(V - E_{Ca}) - g_{K_{Ca}} b(V - E_K)$$

$$- g_{K\,leak}(V - E_{K\,leak}) - g_{Na\,leak}(V - E_{Na\,leak})$$

$$\frac{dx}{dt} = \frac{x_\infty(V) - x}{\tau_x(V)} \qquad x = m, h, n, r, f, b$$

$$m_\infty = \frac{1}{1 + \exp(-(V + 35.2)/8.1)} \qquad h_\infty = \frac{1}{1 + \exp((V + 62)/4)}$$

$$n_\infty = \frac{1}{1 + \exp((V - 14)/(-17)))^{0.25}} \qquad r_\infty = \frac{1}{1 + \exp(-(V + 25)/7.5)}$$

$$f_\infty = \frac{1}{1 + \exp((V + 260)/65)} \qquad b_\infty = \frac{1}{1 + \exp(-(V + 20)/2)}$$

$$\tau_m = \exp(-(V + 286)/160) \qquad \tau_h = 0.51 + \exp(-(V + 26.6)/7.1)$$

$$\tau_n = \exp(-(V - 67)/68) \qquad \tau_r = 3.1$$

$$\tau_n = \exp(-(V - 444)/220) \qquad \tau_b = 50$$

Figure 6-2 Equations of the circadian model based upon Diekman et. al 2013
The change in membrane voltage is equal to the sum of all the individual currents (I_{Na}, I_K etc.) for each ionic component, which each has its own set of gating variables described by further equations. C_m = cell capacitance, g = conductance, E = reversal potential, m, h, n, r, f and b are gating variables for the ion channels with τ modelling the time constants for the gating variables.

The parameters used for the model were; C=5.7 pF, I_{app}= 0 pA, E_{Na}= 45 mV, E_K= -97 mV, E_{Na} = -20 mV, E_{leak}= -29 mV, E_{Ca} = 61 mV, E_{Cl} = -60 mV, g_{KCa} = 100 nS, g_{Na} = 229 nS, g_K=14 nS, g_{Ca}=65 nS, g_{Naleak} = 0.14 nS, g_{Kleak} = 0.04nS

The model was computationally implemented in MATLAB as the series of differential equations listed in Figure 6-2, using the Euler-Maruyama method with a time-step of 0.01 ms and the addition of a noise term to reflect the noisy nature of neuronal activity.

6.3.1 Outputs of circadian neuron model

Using the model as described above, differences in firing rate reflecting day and night activity of clock neurons can be achieved by altering the conductances of the sodium and potassium leak channels. In order to achieve a day firing state which has a firing rate of 5 Hz the neuron is modelled to have a high Na^+ leak conductance and low K^+ leak conductance. To shift the model to produce activity of a neuron at night, the Na^+ conductance is reduced, and K^+ conductance is increased, reducing firing rate to 1.5 Hz (Figure 6-3).

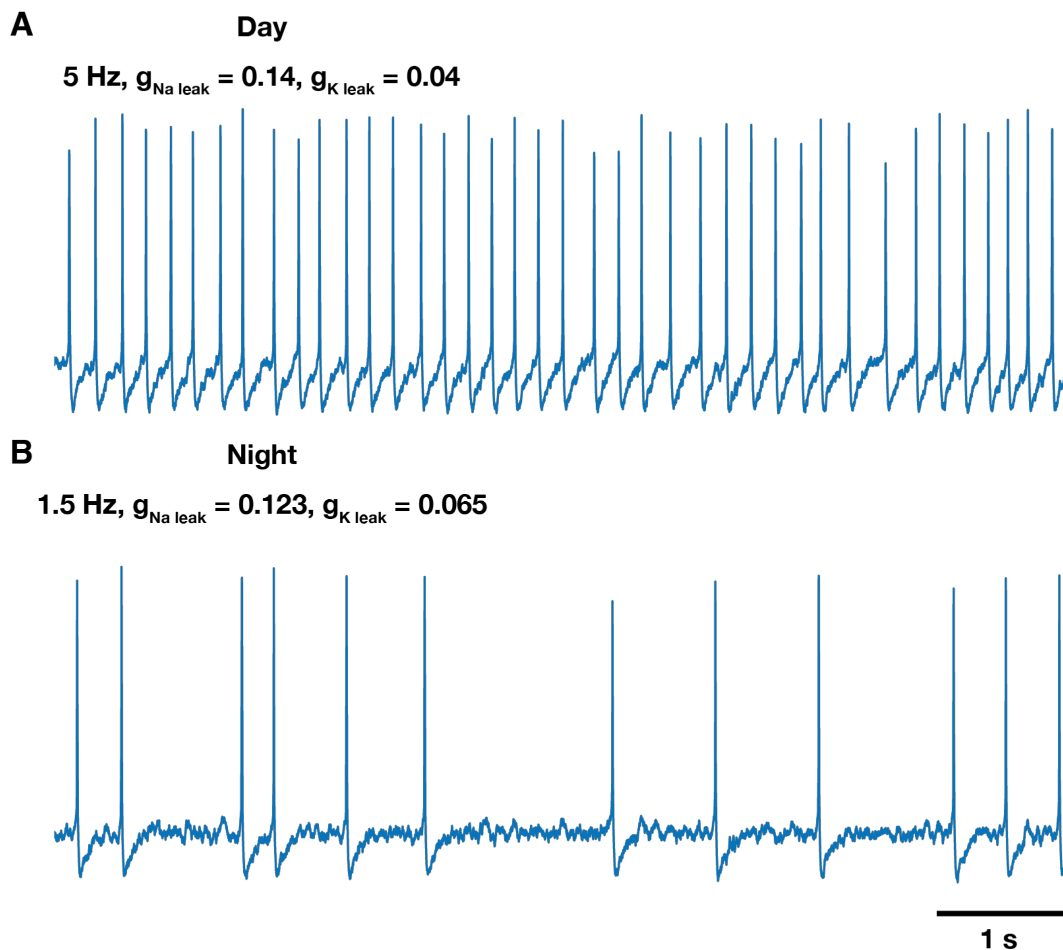


Figure 6-3 Model simulation of day and night firing rates

The model can be used to generate day or night like activity by changing the leak conductance. (A) A day state of 5 Hz can be attained with a Na^+ leak of 0.14 pS and K^+ leak of 0.04. (B) Decreasing the Na^+ leak slightly and increasing the K^+ leak can shift the model into a night state reducing the firing rate to 1.5 Hz.

6.4 SK knockdown – model vs experiments

I decided to focus on the effect of knocking down the calcium activated potassium channel, SK, both in the model and experimentally, in accordance with the findings from my behavioural experiments. In behavioural experiments *SK-RNAi* flies showed significant impacts on circadian and sleep parameters. Circadian rhythmicity was significantly weakened and significant changes to both day-time sleep duration (increased vs controls) and night-time sleep duration observed (decreased vs controls), with a shorter sleep latency during the day and longer sleep latency at night (Section 5.2).

The largest differences in locomotor activity (Figure 5-1) and sleep (Figure 5-2) between *SK-RNAi* flies and controls was observed during the late light-phase (ZT8-11) and so I focused my model manipulations to a day situation and subsequent electrophysiological recordings to this time of day (ZT8-11).

6.4.1 Model predictions from reducing the SK current

To determine the impact of reducing the K_{Ca} current on firing frequency and membrane potential, I used the mathematical model described in section 6.3 and explored the impact of reducing the conductance of the K_{Ca} channel to model the effect of the experimental knock down of the SK channel.

Using the model in its day state predicts a firing rate of 5 Hz (Figure 6-5A), while a 60% reduction in the conductance for the K_{Ca} current component of the model results in a modest increase in firing rate up to 6.5 Hz (Figure 6-5B). A reduction of 60% was used, to model a good knock down efficiency for TRiP RNAi lines (Perkins et al. 2015), although how exactly this would relate to the number of ion channels in the membrane and therefore conductance is unknown.

In the model with reduced K_{Ca} current there were also changes in action potential shape, with a broader shape and less on an after hyperpolarisation, which become clear when the spikes are overlaid (Figure 6-5C).

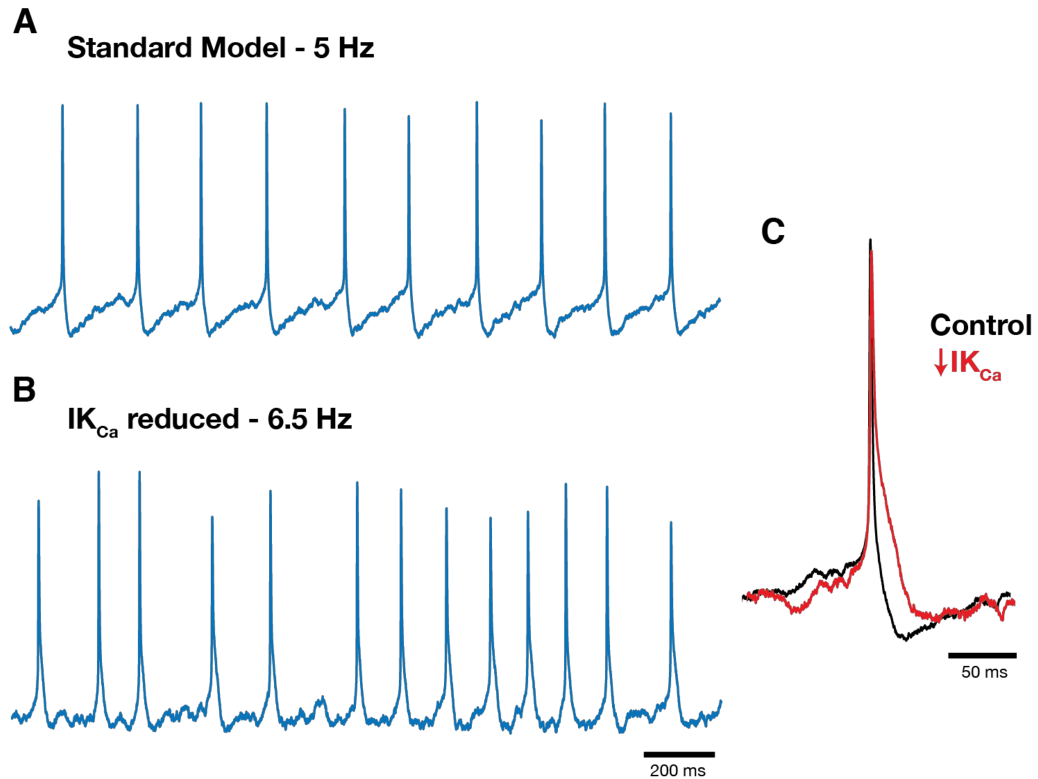


Figure 6-4 Removing K$_{Ca}$ current from model predicts an increase in firing rate

(A) In the standard model the firing rate during the day is 5 Hz. (B) In the modified model with no K$_{Ca}$ current, the firing rate is increased to 6.5 Hz (a 30% increase). (C) There are also changes in the shape of the action potential waveform, becoming broader and with less of an after hyperpolarisation.

6.4.2 Experimental recordings from *SK*-RNAi flies

In order to be able to make recordings from RNAi knock down flies I first had to create a recombined fly line containing the *PDF::RFP* reporter with the *tim-Gal4* promotor to be able to drive expression across the clock circuit and generate more of a behavioural effect. This fly line could then be crossed to the *UAS-SK-RNAi* line to generate flies expressing *UAS*-driven RNAi in all clock neurons and also expressing RFP in the l-LNvs for targeted patch-clamp experiments, and to compared with the behaviour experiments from chapter 5. Recordings were made in the second half of the light-phase in the window of ZT7-9 using the same methods describe in chapter 4. Example traces recorded from control *PDF::RFP* flies, and flies expressing *tim-Gal4>UAS-SK-RNAi* are show below in Figure 6-5.

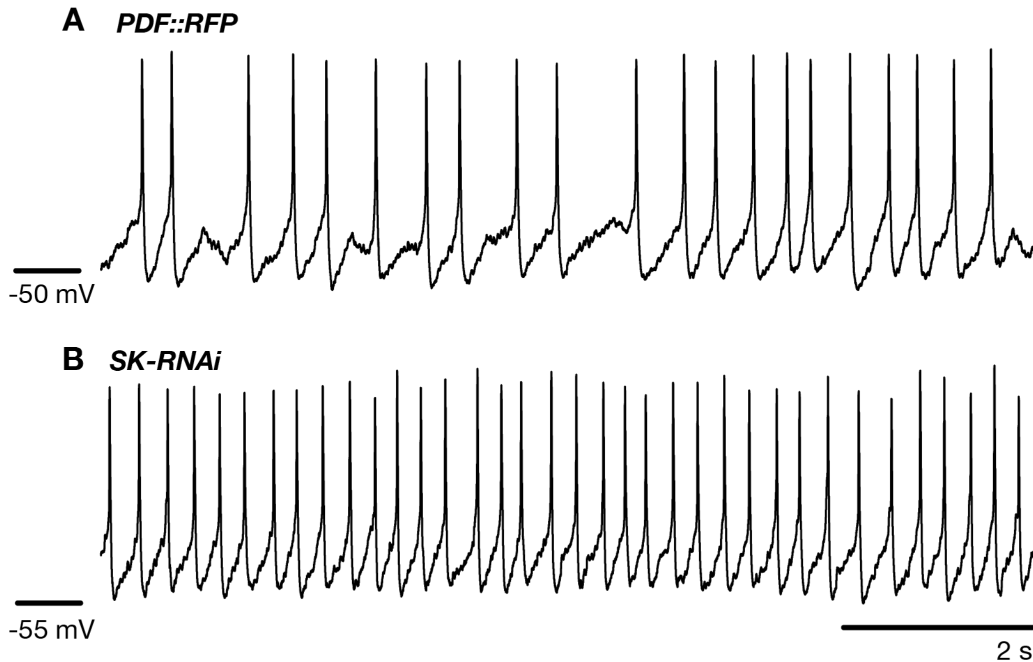


Figure 6-5 *SK*-RNAi l-LNv neurons show increased firing frequency
Example traces of current-clamp electrophysiology recordings at ZT7-9 from control (*PDF::RFP*) and *SK-RNAi* l-LNv neurons. *SK*-RNAi neurons demonstrated an increase in the firing frequency of action potentials.

Analysis of current-clamp recordings from *SK-RNAi* l-LNvs seems to demonstrate some qualitative differences in terms of neuronal activity, with an increase in the firing frequency compared to control neurons. Quantitative analysis of neuronal activity found that there was a slight increase in spontaneous firing rate of *SK-RNAi* neurons (4.96 v 4.08 Hz) but the difference was not significant (p=0.411) (Figure 6-6). Membrane potential of *SK-RNAi* l-LNvs was slightly more hyperpolarised than controls, but there was not a significant difference (p=0.107). The firing response of *SK*-RNAi neurons to a current injection was not altered. There was a significant reduction in the R_{in} of *SK-RNAi* l-LNvs (p=0.0274), which could reflect an increase in the conductance of the membrane, with more open ion channels.

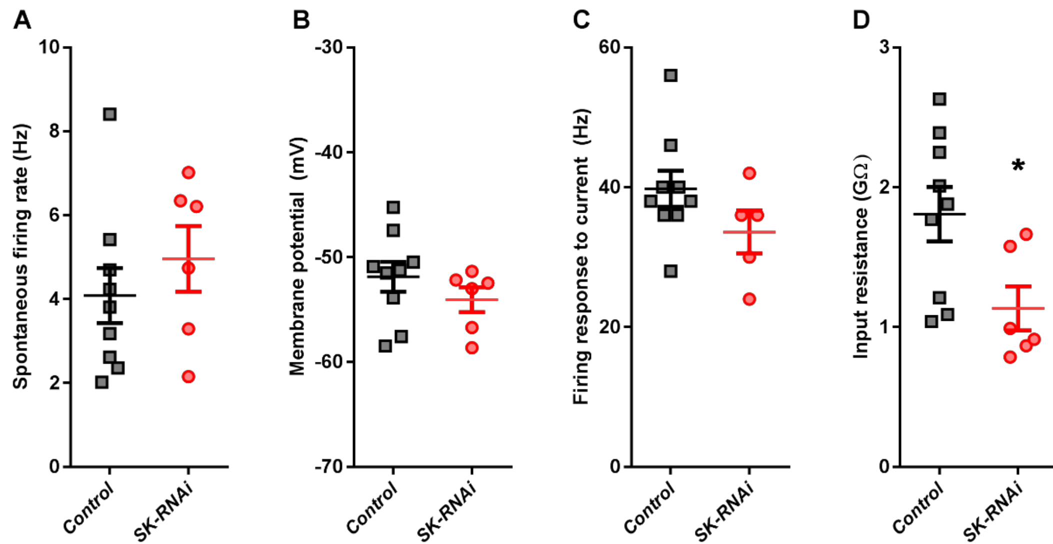


Figure 6-6 Electrophysiological properties of *SK-RNAi* l-LNv neurons
(A) Spontaneous firing frequency of *SK-RNAi* neurons showed a slight increase compared to control neurons, but was not significantly different. (B) Membrane potential was not significantly different in *SK-RNAi* flies. (C) Neuronal excitability of *SK-RNAi* l-LNvs measured by the firing response to a +40 pA current injection, was not significantly altered. (D) Input resistance of *SK-RNAi* l-LNvs was decreased compared to controls (p=0.0274, t-test). N = 11 for controls, N = 6 for *SK-RNAi* flies. Error bars plot mean ± SEM.

Using RNAi to knockdown *SK* mRNA expression across the circadian clock had large and significant effects on sleep and circadian behaviour (Chapter 5.2), and I have also shown that there are consequences for the electrical activity of clock neurons. Patch clamp recordings from *SK-RNAi* l-LNv neurons show an increase in spontaneous firing rate, and a significant reduction in neuronal input resistance.

6.4.3 Using the model to predict the level of *SK* knock down

The computational model can be used to test the effect of different amounts of reduction in the K_{Ca} current on electrical activity. By using different levels of reduction for K_{Ca} conductance and looking at the effect on firing rate in the model and then comparing this to my electrophysiological data, I can predict how much of the K_{Ca} current was removed in the RNAi knock down.

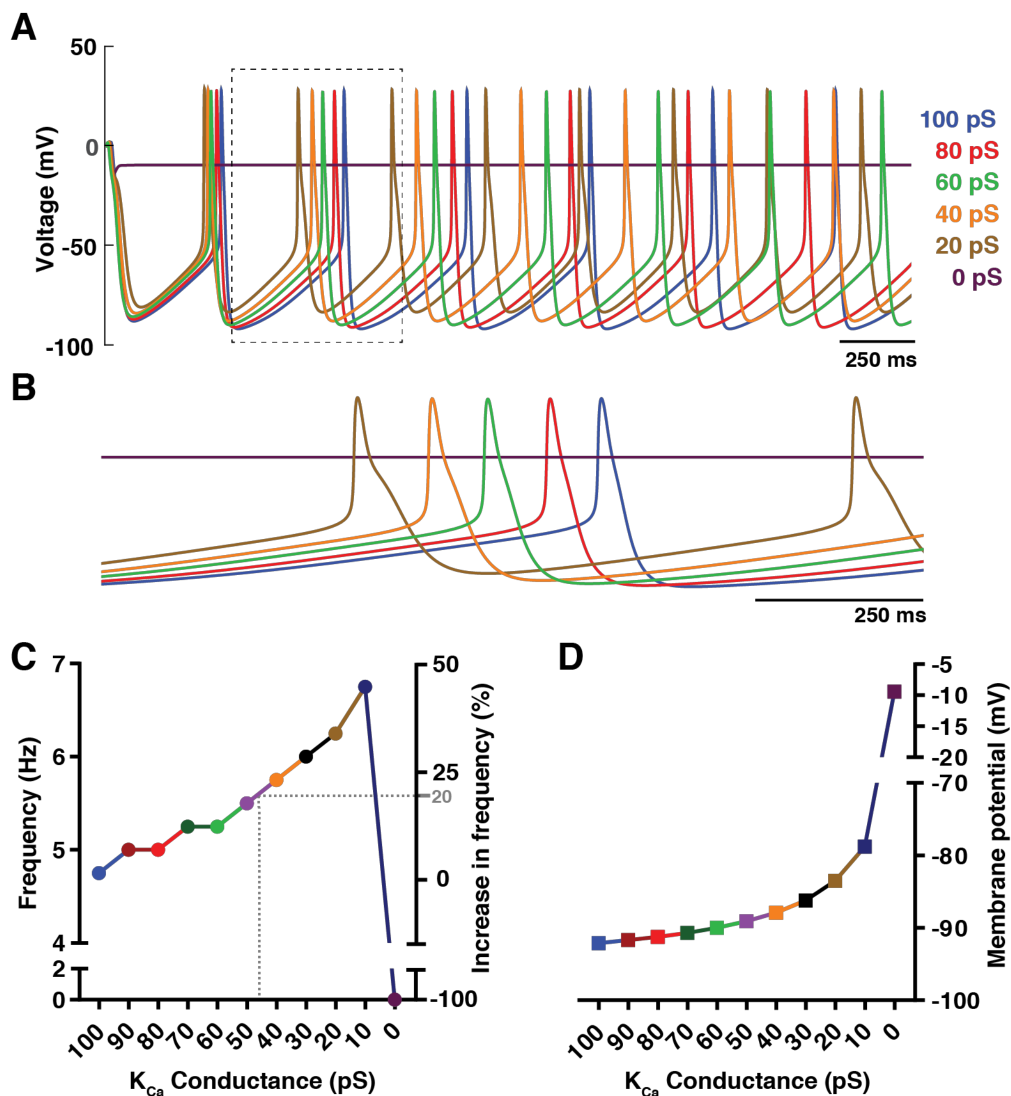


Figure 6-7 Model predictions of decreasing levels of K_{Ca} conductance on firing frequency and membrane potential

(A) Model simulation of electrical activity with K_{Ca} conducatance set at either the original model value of 100 pS, or 80, 60, 40, 20 and 0 pS. (B) Expanded view from (A) shows effect of decreasing KCa conductance on action potential frequency and the shape of the waveform. (C) Action potential frequency increases with decreasing K_{Ca} conductance, until at 0 pS the model is unable to fire action potentials. (D) Membrane potential becomes more depolarised as K_{Ca} conductance is decreased, and at 0 pS when the model does not fire, the membrane potential flatlines to ~-10 mV.

In the experimental recordings the firing rate was increased from 4.08 to 4.96 Hz, an increase of 22%, although this was not statistically significant (p=0.1148) (Figure 6-6). From decreasing the K_{Ca} conductance in the model (Figure 6-7) and comparing to my electrophysiological results, the

equivalent firing rate increase in percentage terms occurs when the conductance is reduced to ~45 pS, or a 55% reduction in conductance, provide some evidence that the RNAi manipulation is effective.

Activation of SK channels is known to reduce action potential frequency (Faber and Sah 2007), with further support for this from experiments in the SCN where pharmacological blocking of SK channels increased firing rate (Teshima et al. 2003), therefore it makes sense reducing the number of SK channels would increase firing rate. A recent modelling paper suggests this is partly due to the role of SK channels in membrane potential repolarisation regulating the activation of voltage-gated sodium channels (Iyer et al. 2017), highlighting how interconnected the function and activity of different ion channels are.

6.5 Predictions from other channel manipulations

Using the model to test the effect of manipulating the K_{Ca} current made a prediction of an increase in firing rate that was partially supported with experimental evidence. This approach can be extended to make predictions of the effects of manipulating other currents in the model.

The two main currents in the model are potassium and sodium, the two main ions involved in action potential generation. In order to test the effects of a reduction in either the potassium or sodium currents the same 50% reduction in conductance as in section 6.4.1 for the K_{Ca} channel predictions was used for the potassium and sodium channels.

Manipulating the potassium conductance and reducing it from 14 pS to 7 pS results in an increase in firing rate from 5 to 7.5 Hz (Figure 6-8B). The same manipulation for the sodium current (a reduction from 229 to 115 pS) results in a model neuron that does not produce action potentials (Figure 6-8C).

These predictions could be tested in future electrophysiological experiments using pharmacological blocking of ion channels and measuring the effect on neuronal activity.

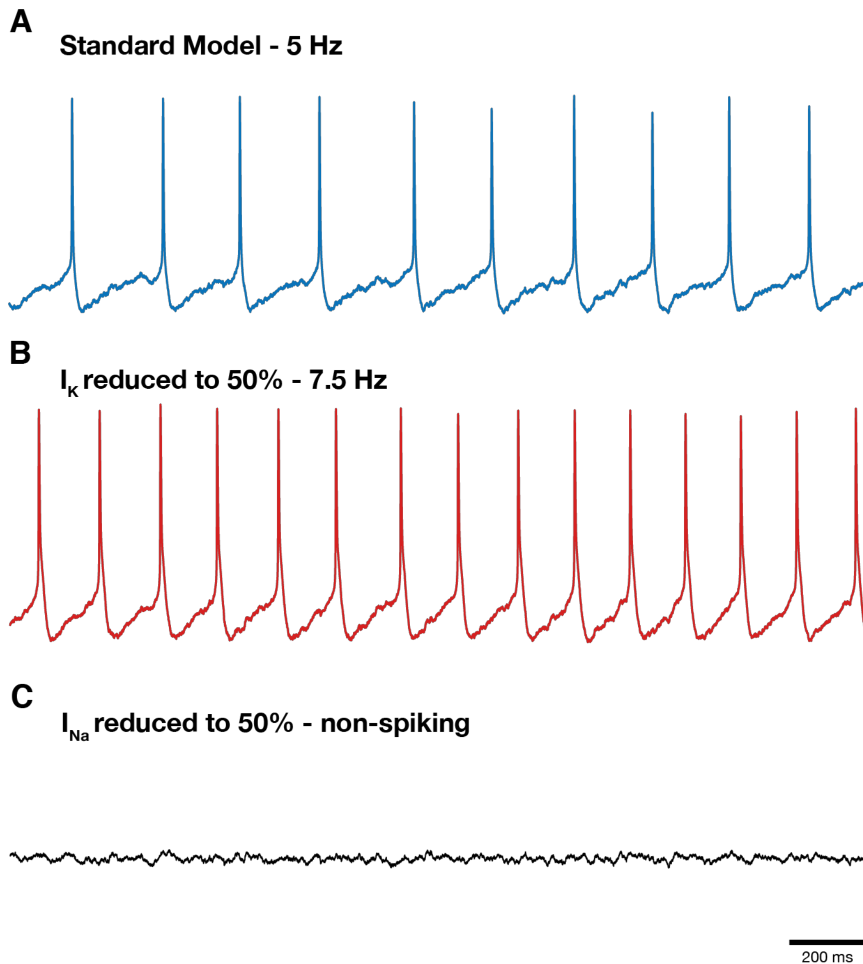


Figure 6-8 Model prediction of altering potassium or sodium conductance
(A) Normal model simulation predicts a firing rate of 5Hz (B) Reducing the potassium conductance by 50% causes a large increase in firing rate to 7.5Hz. (C) Reducing the sodium conductances results in a model neuron that is unable to produce action potentials

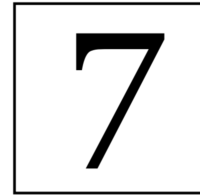
6.6 Conclusions from computational modelling

Using an existing mathematical model from circadian neurons of the SCN (Diekman et al. 2013), I have reproduced the circadian modulation of firing rate in the model output (section 6.3.1). I have then manipulated this model to test the effect of reducing the K_{Ca} current on firing rate to make predictions of the effect of knocking down SK channels in neurons (Figure 6-7), something that I showed had large behaviour effects (chapter 5.2).

Model predictions of increased firing rate were partially confirmed experimentally with electrophysiological recordings from l-LN_Vs (Figure 6-6). The electrophysiological changes in *SK-RNAi* l-LN_V neurons suggest that alternations in the electrical activity contribute to the behavioural

changes observed in the RNAi manipulations in chapter 5. Although I only recorded from the l-LN_V neurons, making the assumption that clock neurons share common electrophysiological characteristics, the model predictions and experimental recordings (Figure 6-4) would suggest that the increase in firing rate would be common across the circadian network.

Extrapolating from this, in the *tim-Gal4*>*UAS-SK*-RNAi flies neuronal activity would be increased across the circadian clock which might in part explain the complex effects on circadian and sleep behaviour observed in chapter 5.2. It would be interesting to make recordings from other neurons in the circadian network to test this prediction. Additionally, the model was used to predict the effects of manipulations of potassium and sodium ion channels, which could be tested experimentally in the future.



CHAPTER 7 GENERAL DISCUSSION

This thesis reports the effects of ageing on the *Drosophila* circadian clock. Chapter 1 introduced the circadian system, with a particular focus on the circadian clock of *Drosophila*, before discussing what is known about how ageing alters the circadian clock. The results presented in chapter 3 demonstrate an age-related decline in circadian and sleep behaviour in *Drosophila*. The results in chapter 4 demonstrated significant changes in the electrical properties of the l-LN_V clock neurons, with chapter 5 reporting the results of genetic manipulation of ion channels on circadian and sleep behaviour. Together these results link changes in the electrical activity and structural remodelling of clock neurons to the changes in behaviour observed during the ageing process, and further support the use of *Drosophila* as a model to study the effects of ageing on circadian biology.

7.1 Motivation and aims of this study

The world's population is ageing at a rapid pace, with a significant proportion of lifespan (~20%) now spent in late-life morbidity (Jagger et al. 2008; Crimmins 2015). A major feature of ageing in humans and other mammals is a weakening in rhythmic behaviours, with circadian and sleep-wake rhythms significantly affected (Kondratova and Kondratov 2012; Giebultowicz and Long 2015). The circadian system has such extensive control of physiology that weakening of the clock with age has many downstream consequences, with circadian disruption also prevalent in many age-related neurodegenerative disorders such as Alzheimer's disease (Videnovic et al. 2014; Musiek 2015). Understanding the role of the circadian system in ageing is therefore of clear medical importance in the treatment and management of the effects of ageing.

The aim of this thesis was to investigate age-related changes in circadian and sleep behaviour in *Drosophila* with a focus on how neuronal activity of clock neurons is altered by ageing.

7.2 Significant decline in clock outputs with age

In chapter 3, behavioural experiments demonstrated that circadian free-running behaviour shows significant changes during the ageing process. These behavioural results complement the work of previous studies in showing that the strength of the free-running behaviour weakens and period lengthens with age (Umezaki et al. 2012; Rakshit et al. 2012), and go further by using a systematic approach to monitor flies at one-week intervals across the ageing process and show that there is an age-dependent decrease in rhythm strength (Figure 3-5) and an equivalent increase in period length with age (Figure 3-6).

Rhythmic behaviour under constant darkness is controlled by the clock in the s-LN_V neurons, with these cells maintaining molecular oscillations in DD (Grima et al. 2004; Stoleru et al. 2005; Roberts et al. 2015). In chapter 3, immunohistochemistry experiments demonstrated a loss of the day-night difference in remodelling of the s-LN_V dorsal terminals (Figure 3-22), and with this a weakening of the outputs of the s-LN_V clock.

The increase in period length observed in older flies is also most likely a result of changes in the clock in the s-LN_V neurons which are known to drive the speed of the molecular clock of the other clock neuron groups (Guo et al. 2014; Yao and Shafer 2014), acting through PDF signalling. A reduction in PDF expression in aged flies has previously been demonstrated (Umezaki et al., 2012), providing further evidence for the importance of PDF in maintaining healthy rhythms with age. Furthermore, in *Pdf* ^{01} mutant flies or in a knock down of the PDF receptor, sleep duration is significantly increased and latency reduced (Parisky et al. 2008). These various results support a hypothesis that reduced PDF signalling with age underlies the weakening of behavioural rhythmicity.

7.3 Diurnal activity is fundamentally altered

One of the major impacts of ageing was a significant alteration in the organisation of daily activity under a light-dark cycle (Figure 1-10). Both morning and evening anticipation activity were significantly reduced in aged flies, with a larger effect of the morning peak of activity, as well as an overall reduction in activity during daytime. l-LN_V neurons are required for correct morning anticipation (Grima et al. 2004; Stoleru et al. 2004) and are obvious candidates for involvement in an age-related decline in this anticipatory behaviour. Morning anticipatory behaviour is also linked to expression of PDF, with *Pdf* ^{01} and *Pdf-RNAi* flies showing significant reductions in morning anticipation (Shafer and Taghert, 2009).

7.4 Ageing significantly alters sleep behaviour

Ageing is known to cause changes in the sleep profile across many organisms including *Drosophila* (Koh et al. 2006), mice (Valentinuzzi et al. 1997), non-human primates (Zhdanova et al. 2011) and humans (Moraes et al. 2014).

Monitoring of sleep under light-dark conditions, using the widely accepted definition of sleep as periods of immobility greater than five minutes in duration (Shaw et al. 2000), showed significant alterations during the ageing process (section 3.5). Daytime sleep was significantly increased, with a reduction in sleep latency, with sleep also becoming more fragmented with age, characterised by more frequent but shorter duration bouts of sleep. Sleep in constant darkness was also significant altered by ageing (Figure 3-19), demonstrating a loss of the circadian sleep drive.

The l-LN_V neurons are known to be wake promoting neurons (Shang et al. 2008; Parisky et al. 2008; Sheeba et al. 2008), and so an increase in sleep duration with age is indicative of a weakening of the l-LN_V output on behaviour. Recently, the LN_V have been shown to have direct connections with the dopaminergic sleep promoting PPM3 neurons, and act to inhibit these neurons during the day (Potdar and Sheeba 2018). The larger effects of day-time sleep during ageing may be suggestive of changes in these connections from the LN_V neurons to sleep centres in the *Drosophila* brain.

7.5 Electrical properties of clock neurons alter with age

Chapter 4 presented results from experiments measuring neuronal activity of *Drosophila* clock neurons finding a significant effect of ageing on the electrical properties of l-LN_V neurons. Electrophysiology experiments using whole-cell patch-clamp recordings from the l-LN_V neurons demonstrated that during ageing there was not an alteration in the firing activity, however there were significant effects on membrane properties as measured by input resistance (Section 4.3).

7.5.1 Linking electrical properties to behavioural changes

One possible explanation for an increase in input resistance without changing the active properties of the neurons is the involvement of chloride (Cl^-) channels, which could become open and decrease resistance without changing the membrane potential. The l-LN_V express the $GABA_A$ receptor *Resistant to dieldrin* (*Rdl*), which when activated by GABA selectively conducts Cl^- through its pore. *Rdl* has important roles in promoting sleep, with a mutation in *Rdl* that causes extended channel openings resulting in increased sleep duration and decreased latency (Agosto et al., 2008; Parisky et al., 2008), while knocking down the *Rdl* gene in the PDF neurons reduces sleep (Chung et al. 2009). GABA is known to change from excitatory to inhibitory across the circadian cycle in the mammalian SCN (Alamilla et al. 2014; Jones et al. 2015), and the same may be a feature in *Drosophila* l-LN_Vs (Buhl et al. 2016). Changes in GABA signalling during ageing could significantly alter clock neuronal signalling and this could explain the observed changes in input resistance, as well as the changes in morning anticipation and sleep.

In chapter 5, RNAi knockdown of ion channels in the clock circuit has identified which currents are more important in controlling circadian and sleep behaviour by measuring the effects of knocking them down. Candidate ion channels were identified from a published RNA microarray dataset of rhythmically expressed genes, with the hypothesis that ion channels of greater importance would have larger effects on behaviour when knocked down. Results from RNAi knockdowns showed how manipulating individual ion channels can have significant effects on circadian and sleep

behaviour (see Table 5-4 for summary), but the effects observed are quite broad. All but one ion channel knockdown increased period length, with the exception the knock down of SK, which suggests that correct timing of the clock is highly dependent on the electrical activity of clock neurons.

7.5.2 Modelling of clock neuronal activity

Chapter 6 present the findings from computational experiments using a model of electrical activity of a clock neuron. The model was used to generate a prediction that knocking down the SK ion channel would increase firing rate (Figure 6-4), a trend towards which was suggested by results from electrophysiological experiments (Figure 6-5). The model prediction of increased firing in SK knockdown neurons can be linked to the behaviour changes of *SK-RNAi* flies, although further work is required to narrow down the neuronal networks involved.

7.6 Future Directions

7.6.1 What underlies the changes in neuronal properties during ageing?

One of the key remaining questions is what ion channels and/or neurotransmitters contribute to the changes in electrical properties of l-LN_V neurons during the ageing process. In order to test the hypothesis that ageing alters GABA signalling through *Rdl*, the next logical experiments would be to characterise the GABA reversal potential in aged neurons using voltage-clamp experiments and focal application of GABA to record the evoked current at different holding potentials.

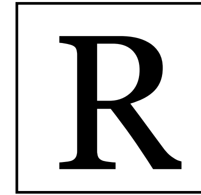
7.6.2 What are the key ion channels in clock control of circadian rhythms and sleep?

In chapter 5, RNAi knockdown experiments demonstrated how altering the electrical properties of clock neurons can have some extremely significant effects on circadian and sleep behaviour. One of these channels, *Shal*, has very recently been linked to a controlling role in sleep the wake-sleep transition at lights-off (Feng et al. 2018), while *Shaker* has been previously identified in sleep control (Cirelli et al. 2005). In this thesis RNAi experiments have identified *SK*, *Caα1t* and *Shaw* as being particularly

important for normal circadian and sleep behaviour, but the reality is that it is likely to be a combination of different ion channels that contribute to overall neuronal control of behaviour.

7.7 Final Conclusions

Ageing has significant and wide-reaching effects on circadian and sleep behaviour in *Drosophila*. Disruption of circadian behaviour in constant conditions can be linked to a loss of circadian remodelling of the s-LN_V neurons and the role of PDF in orchestrating neuronal activity across the circadian network. Changes in the electrical properties of the l-LN_V neurons, namely the significant decrease in input resistance with age, can be linked to the significant changes in daily activity under LD conditions and the large effects of daytime sleep duration and latency. The results from this thesis add to the existing work that has been done on ageing and circadian rhythms in *Drosophila* and suggests neuronal mechanisms to explain the age-related changes in circadian behaviour and sleep.



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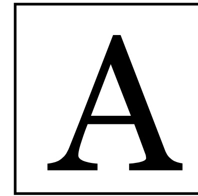

APPENDIX

A.1 Optical-electrophysiology in clock neurons – a pilot study

Although conventional patch electrophysiology has provided us with a good understanding of the electrical activity of clock neurons and is by far the most temporally precise and accurate method of recording electrical activity there are some limiting aspects.

Electrophysiology is a relatively invasive technique that requires close dissection of surrounding tissue in order to allow access of the patch electrode to cell of interest, before requiring direct access to the cell by rupturing of the cell membrane. It is also limited to recording from a small number of neurons, usually one or at most two cells in *Drosophila*, and it would therefore be interesting to be able to record from multiple neurons at the same time in order to look at how neighbouring cells are behaving.

By using an optical approach, the aim was to try and record from multiple l-LN_V neurons at the same time, the advantages including recording activity from multiple cells at the same time and using a less time-consuming dissection approach. The disadvantages compared to patch-clamp electrophysiology are the lower temporal resolution and lack of information regarding membrane potential.

A.1.1 Experimental approach for optical recordings

Optical imaging of electrical activity of neurons was carried out in flies expressing the genetically encoded voltage reporter *ArcLight* (Jin et al. 2012; Gong et al. 2015) in the PDF-expressing clock neurons. *ArcLight* consists of a fusion of the voltage-sensing domain of *Ciona intestinalis* voltage-sensitive phosphatase and the fluorescent protein super ecliptic pHlouorin, and shows decreased fluorescence in response to membrane depolarisation (Jin et al. 2012). Brains were minimally dissected, and

imaging performed as described in section 2.7 of the methods. In initial experiments, conventional patch electrode recordings were performed at the same time to investigate how well optical recordings compared.

A.1.2 An optical recording approach showed limited success

Using the *ArcLight* sensor for recording the electrical activity of clock neurons was occasionally successful, but in the vast majority of attempts nothing of use was recorded from imaging experiments. Frustratingly when imaging was successful recordings showed agreement with the concurrent patch recordings and allowed interrogation of the activity of other neurons in the group. The times that recordings were successful were actually when the plane of focus was slightly above the cells and often when cells did not look well suited for conventional patch-clamp recordings.

A.1.3 Optical recordings show agreement with patch-clamp

Simultaneous recordings made using conventional patch-clamp electrophysiology and optical recordings of *Arclight* fluorescence showed agreement in activity (Figure A-1).

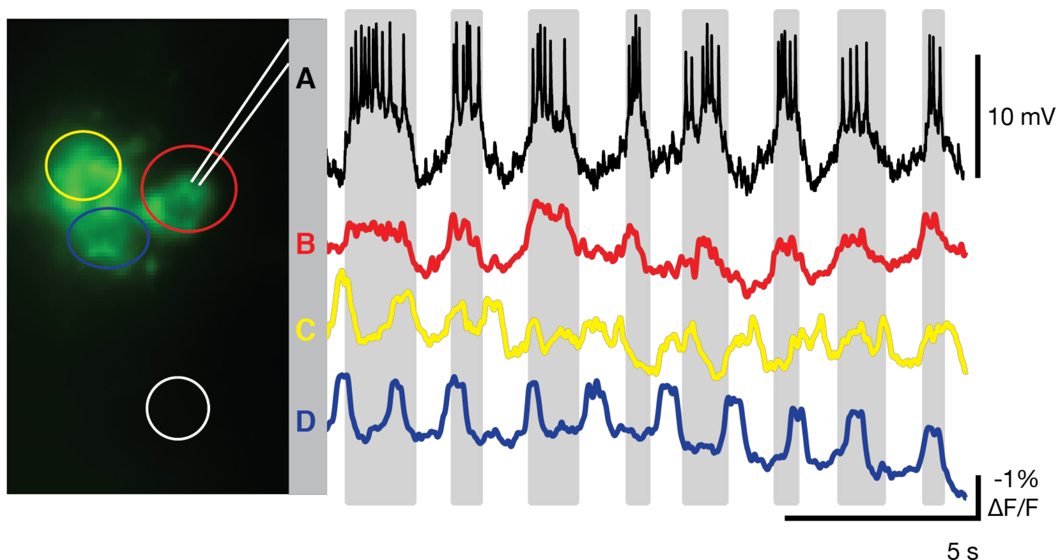

Figure A-1 *Arclight* is a potentially powerful tool for recording simultaneous electrical activity of groups of cells

A grouping of three l-LN_V clock neurons, and the regions of interest used to measure changes in fluorescence from the *ArcLight* reporter during recordings membrane depolarization leading to a decrease in *ArcLight* fluorescence. A simultaneous patch-clamp electrophysiological recording is made from the marked cell. (A) Shows the recorded trace

from current-clamp electrophysiology. (B) Simultaneous *ArcLight* recording of the cell recorded in (A), showing agreement with the electrophysiological data with the shaded area showing where the burst is taking place. (C and D) *ArcLight* recordings of the other two cells in the field of view, showing differences in electrical activity among this group of cells. Recording from D14 fly, at ZT8.

A.1.4 Are there activity differences in neighbouring l-LNvs?

As previously mentioned, one of the main drawbacks of conventional patch-clamp electrophysiology in *Drosophila* is being limited to recording from one cell a time, and so not being able to monitor the activity of cells of the same circadian subtype. Cells in the *Drosophila* brain are often highly specialised and within a subtype might have different functions. Recent work has revealed anatomical differences with the l-LNv subgroup of neurons, with one of the four l-LNv neurons showing a restricted branching pattern (Schubert et al. 2018).

In some of the optical recordings made of l-LNv neuronal activity there appeared to be differences in *ArcLight* fluorescence between cells within the same cluster. For example, in Figure A-2 , two l-LNv cells show tonic firing behaviour and the remaining two show no real activity.

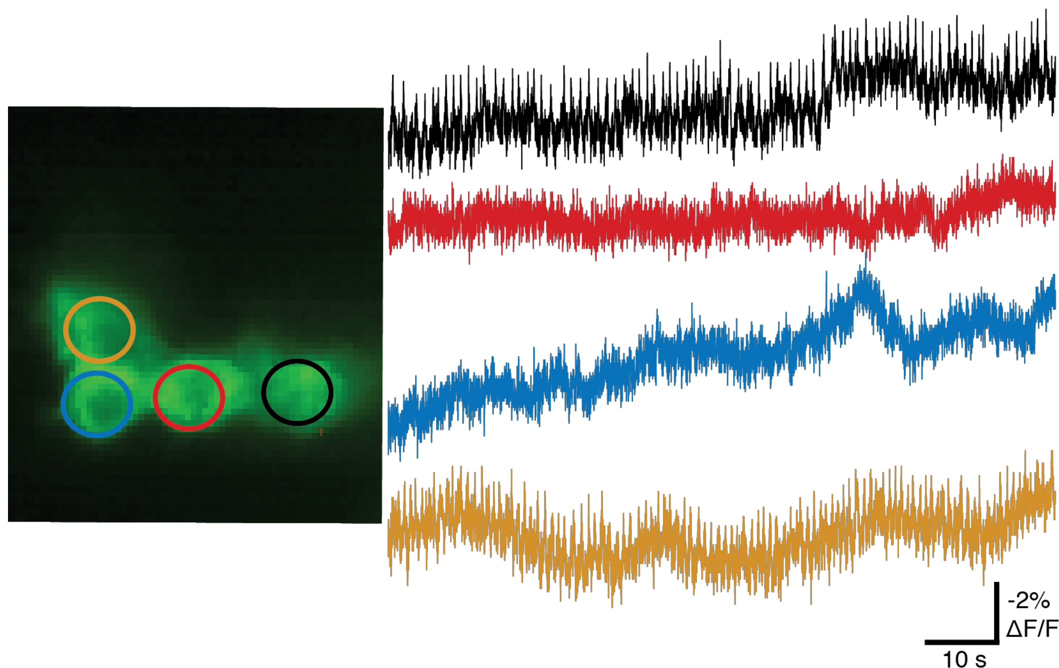

Figure A-2 Purely optical recording of multiple clock neurons shows differences in activity of neighbouring cells

A 60 second recording of four neighbouring l-LNv cells, with the coloured trace corresponding to the same colour region of interest. Neuronal activity seems to show differences between neighbouring cells in the same brain, with the black and yellow traces showing tonic spiking behaviour, and the red and blue trace being seemingly inactive.

A.1.5 Bursting behaviour in *ArcLight* neurons

An issue that I found with recordings from *ArcLight* brains was that a lot of the l-LNv neurons I recorded from exhibited burst firing behaviour, where instead of firing single action potentials they displayed large depolarising events with multiple smaller events (see Figure A-3). Other groups have reported bursting behaviour from l-LNv neurons, but at a low frequency (Sheeba et al. 2007), and it was an extremely rare in recordings from *PDF::RFP* neurons.

Due to the difference in bursting in *ArcLight* and *PDF::RFP* neurons, I hypothesised that this was an issue with having to use blue wavelength light to activate *ArcLight* fluorescence and so also activating the blue-light photoreceptor *Cryptochrome* in the clock neurons being recorded from.

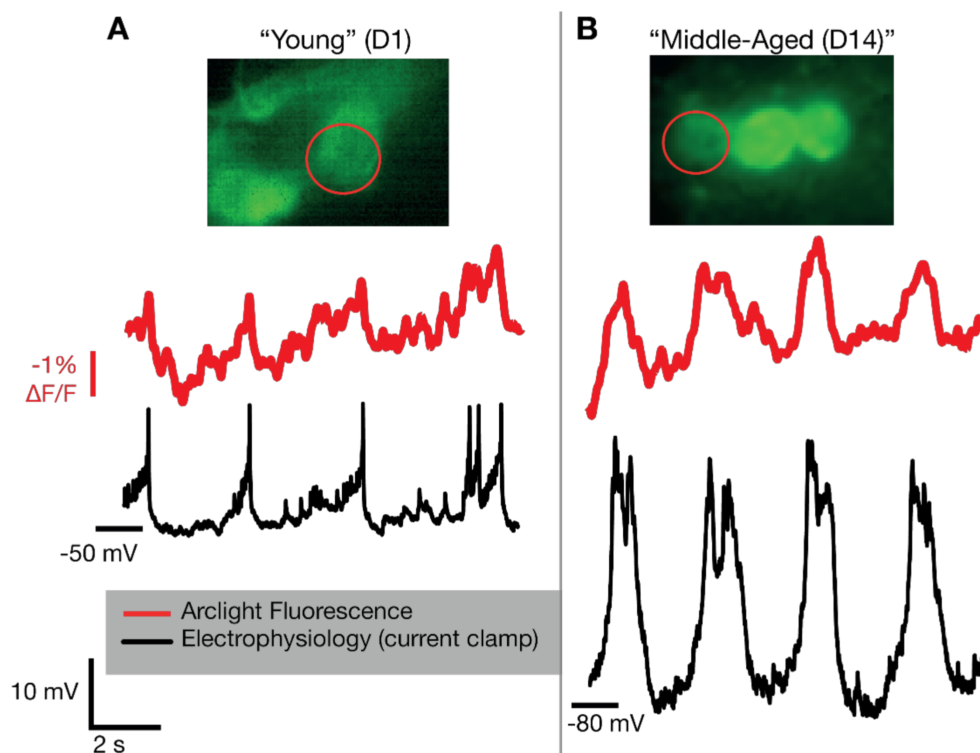

Figure A-3 Bursting behaviour in *Pdf>ArcLight* neurons

(A) A D1 l-LNv displaying tonic single action potential firing that is recorded by both patch-clamp and fluorescence. (B) A bursting D14 l-LNv with large depolarising bursts of electrical activity.

A.1.6 Optical electrophysiology has potential, but is not reliable enough yet

In the first few years I invested a lot of effort in trying to make use of the *ArcLight* reporter for measuring electrical activity of clock neurons, as the technique showed a lot of promise. In the past few years a lot of new voltage reporters have been generated, such as *ASAP* (St-Pierre et al. 2014), *Ace2-mNeon* (Gong et al. 2015) and others (Lin and Schnitzer 2016) which have been shown to work very well in cell lines. However, there have been very few studies which have successfully used them in experimental settings, demonstrating how the technique still needs to be refined before it can be used reliably.

In a circadian context, a major drawback of these sensors is that they require blue-light for their activation, and this seems to have a direct effect on the electrical activity of clock neurons due to its action on cryptochrome when used for long periods for optical recording of membrane voltage. Recent work using brain-wide calcium imaging of the different groups of clock cells in *Drosophila* has shown that the different groups of neurons have differences in the phasing of calcium activity over a daily cycle (Liang et al. 2016), and it would be useful to have similar membrane voltage information.

A.2 Clock gene expression is reduced by ageing in wild type flies.

In order to investigate what might be happening on a cellular level during the ageing process, expression of clock genes was measured using quantitative RT-PCR (qPCR). I only conducted a limited number of gene expression experiments before becoming aware of other major research efforts on the effect of ageing on the circadian transcriptome and focused my efforts on other areas of interest. The results of the experiments I conducted are reported here.

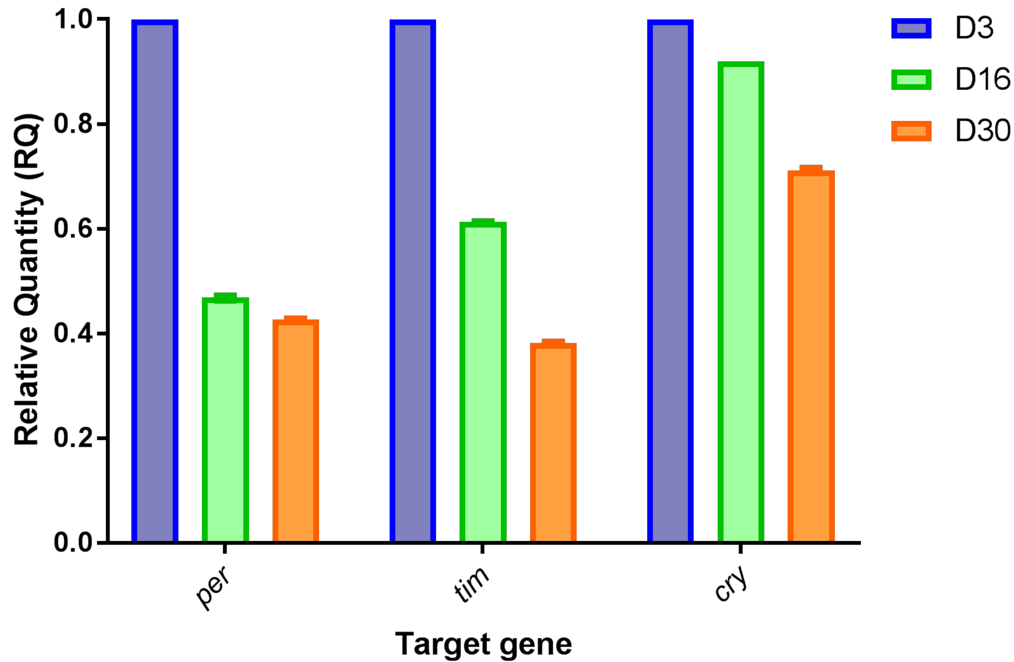

Figure A-4 Ageing causes a reduction in expression of core clock genes *period*, *timeless* and *cryptochrome*

RT-PCR analysis of whole brain samples from wild type flies extracted at ZT1 at ages D3, D16 and D30. Analysis of the relative quantities of *per*, *tim* and *cry* compared to D3 levels (relative to *rpl32*) show reductions in expression of these core clock genes in flies aged D16 and D30.

The results from the qPCR experiments found a reduction in the relative expression of the core clock genes *period*, *timeless* and *cryptochrome* across early and middle-aged flies (Figure A-4).