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Age-dependent changes in clock neuron structural plasticity and excitability are associated with a decrease in circadian output behaviour and sleep

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Abstract

Ageing has significant effects on circadian behaviour across a wide variety of species, but the underlying mechanisms are poorly understood. Previous work has demonstrated the age-dependent decline in behavioural output in the model organism *Drosophila*. We demonstrate this age-dependent decline in circadian output is combined with changes in daily activity of *Drosophila*. Ageing also has a large impact on sleep behaviour, significantly increasing sleep duration whilst reducing latency. We used electrophysiology to record from large ventral lateral neurons (l-LNv) of the *Drosophila* circadian clock, finding a significant decrease in input resistance with age, but no significant changes in spontaneous electrical activity or membrane potential. We propose this change contributes to observed behavioural and sleep changes in light-dark conditions. We also demonstrate a reduction in the daily plasticity of the architecture of the small ventral lateral neurons (s-LNv), likely underlying the reduction in circadian rhythmicity during ageing. These results provide further insights into the effect of ageing on circadian biology, demonstrating age-related changes in electrical activity in conjunction with the decline in behavioural outputs.

1. Introduction

Circadian rhythms describe the near 24-hour cycle in behaviour and physiology, driven by the circadian clock, that allow organisms to anticipate daily changes in their environment. Circadian clocks in animals are fundamental biological components responsible for the control of large aspects of physiology and behaviour, ranging from the sleep-wake cycle to rhythms in blood pressure (Roenneberg and Merrow, 2016). Remarkably, the fundamental molecular basis of the intracellular clock is well conserved from *Drosophila* to mice and humans (Allada and Chung, 2010). The health consequences of circadian misalignment as a result of our modern lifestyles are dramatic, with links to cancer, depression and sleep disorders (Menet and Rosbash, 2011; Samuelsson et al., 2018; West and Bechtold, 2015). With increasing human lifespans and an ageing population, understanding how circadian rhythms change during the ageing process is of growing interest and medical relevance, with the population aged over 60 years old set to more than double by 2050 (UN, 2015).

It is well established that elderly individuals have increasing difficulties sleeping at night and have and increase in daytime sleep episodes combined with generally going to sleep and waking up earlier (Kondratova and Kondratov, 2012). The daily cycles of hormone levels, body temperature and the sleep-wake cycle, are modified with age in humans causing disruption in behaviour, and resultant reduction in the strength of the clock (Hofman and Swaab, 2006). Furthermore, circadian sleep-wake disorders are more prevalent in older individuals (Kim and Duffy, 2018).

Using *Drosophila* offers numerous advantages for investigating how ageing affects the circadian clock, not least the strong history of circadian research in the model organism, genetic tractability, short lifespan (50-80 days), rapid generation time as well as clearly defined and manipulatable neural circuits. Genetic analysis of circadian behaviours has identified genes involved in generating rhythmic transcription translation feedback loops comprising the molecular clock of *Drosophila* (Allada and Chung, 2010; Hardin, 2011; Tataroglu and Emery, 2015), which in turn control a wide-range of physiological and cellular responses, likely through rhythmic control of output genes.

The *Drosophila* central clock consists of 150 dispersed but connected circadian neurons that are classified by their anatomical location, projection pattern and the expression of clock genes (Peschel and Helfrich-Förster, 2011). They function as a network to drive rhythmic behaviour (Top and Young, 2017). Examples of outputs from the molecular clock are the circadian remodelling of the projections from the s-LNv clock neurons to the dorsal protocerebrum (Fernández et al., 2008) and circadian modulation of the firing frequency and membrane potential of clock neurons (Cao and Nitabach, 2008; Flourakis et al., 2015; Sheeba et al., 2007). Under laboratory conditions using a 12:12 hr light:dark (LD) cycle, *Drosophila* display morning and evening peaks in locomotor activity with anticipation activity prior to the transitions of light-on and lights-off, with constant darkness (DD) resulting in free-running activity with a period of around 23.8 hours (Dubowy and Sehgal, 2017).

The LNv neurons produce the neuropeptide pigment dispersing factor (PDF) which acts to synchronise activity throughout the clock circuit (Shafer and Yao, 2014). PDF acts through the PDF receptor which has broad expression in the circadian network (Im and Taghert, 2010), with rhythmic synaptic release of the PDF neuropeptide required for maintaining circadian rhythmicity under constant conditions . The PDF neurons have been termed the 'morning' cells due to the absence of the morning (but not evening) peak of activity in flies either having mutations in the *pdf* gene or lacking the PDF neurons (Renn et al., 1999). Another group of clock neurons, the ~~Cry+~~ LNds (dorsal lateral neurons) as well as the PDF-negative 5th s-LNvs, have been termed the "evening" cells as they are necessary for the evening anticipation activity (Grima et al., 2004; Stoleru et al., 2004).

In *Drosophila* ageing has been shown to cause reduced and weakened circadian activity in behaviour, associated with declining levels of PDF (Umezaki et al., 2012). Disruption of the clock has also been shown to accelerate ageing, in flies lacking a functional clock (Hendricks et al., 2003; Krishnan et al., 2012; Vaccaro et al., 2017), or keeping flies under mismatched lighting conditions (Klarsfeld and Rouyer, 1998; Pittendrigh and Minis, 1972; Vaccaro et al., 2016). Studies on how ageing affects the molecular clock have reported conflicting results: it has been found to remain

robust in aged flies (Luo et al., 2012), and to significantly decline in strength with age (Rakshit et al., 2012). To date no studies have been published on the effect of ageing on the electrical activity of clock neurons in *Drosophila*. 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., 2015, 2012).

To investigate the effect of ageing on circadian rhythms we took advantage of the *Drosophila* model that allows systematic monitoring of circadian and sleep behaviour simultaneous from flies across the range of lifespan. Furthermore, we determine the effect of ageing at the neuronal activity level by making patch-clamp recordings from the large-LNv clock neurons from young and aged flies.

2. Materials and methods

2.1. Fly Strains

The following fly stocks and their original sources were used, *Pdf::RFP* (Ruben et al., 2012), *iso31* (Ryder et al., 2004), *Pdf-Gal4* (Bloomington stock centre, #6900) (Park and Hall, 1998) and *UAS-mCD8::GFP* (Bloomington Stock Centre, #5137).

All flies were reared on a standard medium based upon the following recipe; 10L 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. Flies for ageing were collected and flipped onto fresh food every 5 days and maintained in an incubator at 25°C and humidity of 55-65% with a 12:12 LD cycle.

2.2. Circadian behaviour analysis

Locomotor activity of individual male flies (aged 1, 8, 15, 22, 29, 36, 43 and 49 days old) was measured using the *Drosophila* Activity Monitoring (DAM) system (DAM2, Trikinetics Inc, USA). Flies were transferred into DAM tubes after reaching the desired age and were maintained for 5 days under 12:12 LD conditions, followed by constant darkness. The first 7 days of DD activity was used for circadian analysis, with period and rhythmicity analysis performed in MATLAB using the Flytoolbox (Levine et al., 2002).

2.3. Anticipation index analysis

The morning and evening anticipation indexes were calculated from the activity of flies across the 5 days of LD activity. Morning anticipation was calculated as previously described (Harrisingh et al., 2007; Zhang and Emery, 2013). 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.

2.4. Sleep analysis

Sleep data were analysed using the Sleep and Circadian Analysis MATLAB Program (S.C.A.M.P.) (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 were analysed across the 24-hr period, the 12 hr 'light phase', and the 12 hr 'dark phase'. Sleep is visualised by plotting the mean amount of sleep in a 30 min bin against the time of day, averaged for the 5 days of the experiment. From the raw data of sleep amounts and time, a series of measurements of sleep are calculated, including; 'total sleep duration' – sum of all sleep episodes, 'number of sleep episodes' – count of all sleep episodes, 'mean sleep episode duration' – average sleep duration (in mins) and 'sleep latency' – the time to the first sleep episodes (in mins).

2.5. Electrophysiological recording of clock neurons

Whole-cell current clamp recordings were performed as previously described (Buhl et al., 2016; Chen et al., 2015). For visualisation of the l-LNvs, *Pdf::RFP* flies were used (Ruben et al., 2012), which is a transgenic fusion of the *Pdf* promoter and *mRFP1* that specifically labels the LNv neurons. Adult male flies were maintained under a 12:12 LD cycle, and recordings were made at either ZT7-9 (day condition) or ZT19-21 (night condition), where ZT0 corresponds to lights-on.

Firstly, flies were anaesthetised using CO_2, before decapitation, and the brain removed by acute dissection with fine forceps in extracellular saline solution containing (in mM): 101 NaCl, 1 $CaCl_2$, 4 $MgCl_2$, 3 KCL, 5 glucose, 1.25 NaH_2PO_4, 20.7 $NaHCO_3$ at pH 7.2. The photoreceptors, lamina and as much membrane as possible were removed and whole brains were transferred to a recording chamber (ALA scientific) filled with extracellular solution and stably held ventral side up using a custom-built wire harp. Cells were visualised using an Axio Examiner Z1 (Zeiss) using a 63x water immersion objective, l-LNvs were identified using 555 nm light generated using a Colibri Examiner light source (Zeiss). All recordings were performed at room temperature (20-22°C) using thick-walled borosilicated glass electrodes (1B150F-4, World Precision Instruments) ranging in resistance from 10-16 MΩ filled with intracellular solution containing (in mM): 102 K-gluconate, 0.085 $CaCl_2$, 17 NaCl, 0.94 EGTA, 8.5 HEPES, 4 Mg-ATP and 0.5 Na-GTP at pH 7.2. Data were recorded using an Axon Multiclamp 700B amplifier, digitised with an Axon Digidata 1440 (sample rate 20 kHz, 10kHz Bessel filter) and recorded using pClamp (10.5: Molecular Devices, CA, USA). Chemicals were acquired from Sigma (Poole, UK).

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 minutes. Membrane input resistance (R_{in}) was calculated by injecting hyperpolarising current

steps from -40 pA to +5 pA in 5 pA steps and measuring the resulting voltage change. Neuronal excitability was measured by injecting a 500 ms long positive current pulse in 2 pA increments with increasing amplitude up to +40 pA and manually counting the resulting spikes.

2.6. Immunohistochemistry and analysis

Flies were briefly anaesthetised using CO_2 and swiftly decapitated and heads immediately placed into phosphate-buffer saline (PBS) containing 4% PFA (Image-iT™ Fixative Solution, Thermo Fisher Scientific # R37814) and 0.008% Triton X-100 (Sigma) and fixed for 45 min at room temperature. For all steps, 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 blocked in 5% normal goal serum (NGS, Thermo Fisher Scientific # 50197Z) for 30 min at room temperature. Brains were then incubated with primary antibodies in 5% NGS, at 4°C for 36 hr 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 three hours at room temperature, and then overnight at 4°C. Brains were rinsed in 0.1 % PBT, followed by three 20 min washes in PBT, 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 hard set medium (Vector Laboratories). The 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).

Table 1 - Antibodies used and sources

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

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. We used a 20x glycerol immersion objective (HC PL APO CS2, Leica) and obtained confocal stacks with a 2 µm step size and 512 x 512 pixels. The obtained 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. Statistical analysis

All statistical analyses were performed using Graphpad Prism 7 (Graphpad Software, USA), with an α level of $p < 0.05$ considered significant. Data for ageing experiments showed non-normal distribution and were plotted as the median with interquartile range, the non-parametric alternative Kruskal-Wallis test was used, with post-hoc analysis conducted using Dunn's test. For ageing data, statistical comparisons between groups were compared to the D1 group.

For electrophysiological and imaging experiments, groups were compared using two-way ANOVA with Tukey's multiple comparisons test, with 'age' and 'time of day' as factors.

3. Results

3.1. Ageing caused a weakening in circadian behavioural output and lengthening of the free-running period

To address the impact of ageing on the circadian clock we used *Drosophila* to conduct a comprehensive behavioural analysis of circadian activity of male flies at 1-week intervals during the ageing process. Flies were first kept for 5 days in a 12hr:12hr light-dark (LD) cycle and showed normal diurnal behaviour, with morning and evening peaks of activity (Figure 1A). Flies were then maintained in constant darkness and showed typical free-running behaviour. In agreement with previously reported work (Rakshit et al., 2012; Umezaki et al., 2012) we found that ageing resulted in a significant decline in the strength of circadian locomotor activity under free-running conditions (Figure 1B, $p=0.0001$, Kruskal-Wallis statistic = 27.17), with a steep decline in flies aged 36 days and older, and a significant lengthening of the period of the observed behavioural activity (Figure 1C, $p<0.0001$, Kruskal-Wallis statistic = 95.3). We also found there was a significant age-related reduction in total locomotor activity (Figure 1D, $p<0.0001$, Kruskal-Wallis statistic = 37.89).

3.2. Ageing alters daily activity structure in light-dark conditions and reduces anticipatory behaviour

Given that there was a reduction in the amount of locomotor activity in older flies (Figure 1D), we sought to further examine how the daily structure of activity under normal LD conditions was altered by the ageing process. Some of the hallmark features of daily activity of male flies recorded using the *Drosophila* Activity Monitoring (DAM) system are the morning and evening peaks in locomotor

activity (Dubowy and Sehgal, 2017) and anticipation of the light-dark transition (see Figure 2A). Both the peaks of activity and anticipation behaviour are affected by manipulations of the circadian system (Lear et al., 2009). We investigated the effect of age on morning and evening anticipation by first normalising locomotor activity for an individual fly to be the percentage of the daily total (Figure 2B). The anticipation index was then quantified as the proportion of an individual fly's daily 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/night (Harrisingh et al., 2007). Older flies showed significant reductions in morning anticipation index compared to young flies ($p<0.0001$, Kruskal-Wallis statistic = 28.93), and a slight reduction in evening anticipation index ($p<0.0001$, Kruskal-Wallis statistic = 32.45) (Figure 2C).

3.3. Ageing alters the daily structure of sleep

Given that there is a strong connection between the circadian clock and sleep, we sought to also investigate how sleep is altered by age. Sleep analysis was performed for the five days under a LD cycle at the start of the circadian experiment, with sleep classified under the common convention of periods of immobility longer than five minutes in duration (Hendricks et al., 2000; Shaw et al., 2000).

The daily structure of sleep in older male flies was noticeably different to that of young flies (Figure 3A) with a visible increase in the amount of daytime sleep and a shift towards sleep earlier in the day. Quantification of total sleep showed a significant effect of age ($p=0.0006$, Kruskal-Wallis statistic = 25.7) (Figure 3B). Looking only at sleep during the daytime (Figure 3C) there was a significant increase with age ($p<0.0001$, Kruskal-Wallis statistic = 33.83), however there was no effect of age on night-time sleep ($p=0.31$ Kruskal-Wallis statistic = 8.253) (Figure 3D). Measuring the latency of sleep after the LD transitions demonstrated a significant reduction in the speed at which older flies started sleeping both during the day ($p<0.0001$, Kruskal-Wallis statistic = 102.7) (Figure 3E) and night ($p<0.0001$, Kruskal-Wallis statistic = 61.33) (Figure 3F). Analysing the parameters of sleep episodes, we found that there was a significant increase in the number of sleep episodes with age ($p<0.0001$,

Kruskal-Wallis statistic = 58.79) (Figure 3G) and a significant difference in mean sleep episode duration ($p<0.0001$, Kruskal-Wallis statistic = 32.56), (Figure 3H).

3.4. Electrical properties of clock neurons are altered by ageing

We have demonstrated that the ageing process causes significant changes to the behavioural outputs of the circadian clock circuit of *Drosophila* and therefore set out to investigate if these were underpinned by changes in clock neuronal activity. To measure the effects of ageing on clock neurons we made recordings from the prominent wake-promoting l-LNv arousal, the most accessible and well-studied group of clock neurons in *Drosophila* (Buhl et al., 2016; Cao and Nitabach, 2008; Parisky et al., 2008; Sheeba et al., 2008). Recordings were made during the day and at night in explant brain preparations made from young (day (d) 1-5) and middle-aged (d28-35) flies (Figure 4) and measured the electrophysiological properties of these cells (Figure 5). Recordings from flies older than 35 days were limited due to the technical difficulties making stable recordings from aged neurons, with older brains being more difficult to dissect cleanly and difficulties to achieve good seals and access due to changes in the older membranes, making them more rigid and unhealthy.

As previously reported, young l-LNvs showed a strong day-night difference in both their spontaneous firing rate (SFR) and membrane potential (MP) (Cao and Nitabach, 2008; Chen et al., 2015; Sheeba et al., 2007), but the response to an injected current pulse or the input resistance did not differ significantly between day and night (Buhl et al., 2016) (Figure 5). Here we report that the diurnal modulation of SFR and MP are maintained in the l-LNvs recorded from 28-35 day old flies, with no difference found in the response to a current injection between young and aged flies. Interestingly we report a significant decrease in the input resistance of aged l-LNvs (Figure 5D), indicative of an increase in overall conductance across the membrane.

3.5. s-LNv terminal remodelling is reduced by ageing

It has previously been demonstrated that the dorsal projections of the s-LNv neurons show daily remodelling in complexity under clock control (Fernández et al., 2008). To test if this was still occurring in older flies, day/night changes in PDF terminal morphology were measured in flies aged 30 days (Figure 6). Using the previously published protocol (Fernández et al., 2008) we found that the remodelling was no longer a significant feature in aged brains. There was a significant overall effect of time of day ($p=0.0045$, two-way ANOVA, $F(1,24)=9.822$) but no effect of age ($p=0.5286$, two-way ANOVA, $F(1,24)=0.4088$). Multiple comparisons tests showed the magnitude of the day-night difference was reduced in older flies and no longer being significantly different between day and night ($p=0.6138$, Tukey's test, DF=24, $q=1.741$) (Figure 6C).

4. Discussion

Ageing is known to have a significant impact on circadian behaviour but what effect this has at a neuronal level is poorly understood. In this study we have conducted a systematic analysis of the effect of ageing on circadian behaviour and related this to electrical activity of l-LNv clock neurons finding a significant reduction in the input resistance of aged neurons.

Our behavioural experiments 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). In addition, we go further by using a systematic approach to monitor flies at 1-week intervals across the ageing process and show that there is an age-dependent decrease in rhythm strength (Figure 1B) and an equivalent increase in period length with age (Figure 1C). Mouse experiments have found that ageing results in a lengthening in period in both behavioural activity (Turek et al., 1995; Valentinuzzi et al., 1997) and in molecular rhythms in the SCN (Nakamura et al., 2015).

We further sought to investigate how the daily structure of activity under light-dark conditions is altered by ageing, by quantifying changes in morning and evening anticipatory activity (Figure 2). We found that there was a significant effect of age on both the morning and evening anticipation indexes (Figure 2C), with a greater reduction in the morning peak. LNv 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). 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, and supporting a hypothesis that reduced PDF signalling with age underlies the weakening of behavioural rhythmicity.

The l-LNv neurons are involved in promoting arousal (Chung et al., 2009; Sheeba et al., 2008) and regulating sleep and latency during the early night (Liu et al., 2014). We made use of the DAM recording system to monitor sleep under light-dark conditions, using the widely accepted definition of sleep as period of immobility greater than 5 mins (Shaw et al., 2000). Ageing is known to cause changes in the sleep profile across many organisms including mice (Valentinuzzi et al., 1997), non-human primates (Zhdanova et al., 2011) and humans (Moraes et al., 2014). Previous *Drosophila* studies on the effects of ageing on sleep have reported that sleep becomes more fragmented with age (Koh et al., 2006; Vienne et al., 2016), showing a similar increase in sleep episode number and decrease in mean sleep episode duration compared to our results (Figure 3G & 3H).

Electrical silencing of LNv neurons causes deficits in free-running clock behaviour (Depetris-Chauvin et al., 2011), demonstrating a link between electrical activity and behaviour. Most electrophysiological studies use young flies aged between 3 and 7 days for recordings (Cao and Nitabach, 2008), with a limited amount of recordings made from 25 day old flies only looking at the active firing properties of the neurons (Sheeba et al., 2007). Here we report the effect of ageing on l-

LNv neuronal activity and electrical properties. We perform whole-cell patch clamp recordings from young and aged neurons and report no major differences in the observed spontaneous activity of l-LNv neurons (Figure 4). Further analysis of the electrical properties of l-LNv neurons showed that there was a significant effect of age in reducing the input resistance, which surprisingly did not affect spontaneous firing rate, membrane potential or excitability (Figure 5). We propose the age-related changes in l-LNv properties are linked with the observed changes in activity and sleep during light-dark conditions.

There are multiple possible explanations for a decrease in input resistance without changing the active properties of the neurons. One hypothesis would be the involvement of chloride (Cl^-) channels, which could become open and decrease resistance without changing the membrane potential, alternatively, the observed reduction in input resistance could result from age-related changes in the composition of ion channels in the membrane, with future experiments needed to evaluate between potential hypotheses. The l-LNv 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). Conversely, knocking down the *Rdl* gene in the PDF neurons reduces sleep, again suggesting GABA regulates sleep through the LNvs and *Rdl* receptor function (Chung et al., 2009). Therefore, it is possible that during ageing there is an increase in GABA activation through *Rdl* in the l-LNvs, causing increased Cl^- conductance. This increase in Cl^- conductance may contribute to the observed reduction in input resistance recorded and also drive the increase in sleep duration and decreased sleep latency in aged flies.

Studies of ageing on electrical activity of mouse clock neurons found no effect of age on input resistance but reveal a reduction in the difference between day and night firing rates (Farajnia et al.,

2012), showing differences of the effects of ageing between different clock neurons in *Drosophila* and mouse.

Our electrophysiological experiments were limited to the l-LNvs so we can only link the changes in neuronal properties we observed to the changes in morning activity and sleep in LD conditions as the l-LNv do not maintain molecular oscillations in DD (Grima et al., 2004), although it is possible that similar changes in neuronal properties are occurring in the s-LNvs where molecular oscillations do persist in constant conditions. We sought to investigate changes to the s-LNv neurons, namely the remodelling of the s-LNv dorsal projections. Analysis of the branching of the s-LNv projections demonstrated that the day-night difference in complexity is reduced by ageing (Figure 6), indicating changes in the distribution of the PDF release network in older flies. Given the role of PDF in regulating the activity of different groups of clock neurons, namely through excitation of dorsal clock neurons (Seluzicki et al., 2014), changes in PDF signalling would contribute to changes in the clock network as a whole. The s-LNv neurons are known to be important for maintaining behavioural rhythmicity under constant conditions and we propose this weakening of s-LNv terminal remodelling underlies the age-related weakening in circadian locomotor behaviour.

Our study builds upon the existing literature demonstrating an age dependent decline in circadian behavioural outputs, and importantly links this to changes in the electrophysiological properties of clock neurons. Further work is necessary to fully understand what the implications of these changes are for the circadian clock network as a whole and if similar changes are occurring in other groups of clock neurons in *Drosophila*.

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Figure 1. Locomotor activity at different timepoints in the ageing process. (A) Top panel - group activity profiles during LD from wild type flies. Middle-panel - double plotted actograms of representative individual flies are shown, activity scaled to individual maximum. Flies were maintained for 5 days of LD before being maintained in constant darkness (DD). In the actograms white represents day, grey background represents darkness. Bottom panel – group activity profile during the 5th day of DD (DD5). (B) Circadian behaviour weakens with age as measured by the rhythmicity statistic. (C) Ageing causes lengthening of period in wild type flies. (D) Average daily locomotor activity during LD is significantly reduced by ageing. On the x axis D signifies days after eclosion. '***' represents $p<0.001$ as determined using the Kruskal-Wallis test with Dunn's post hoc test, data plotted as median with error bars representing the interquartile range.

Figure 2. Morning and evening anticipation reduce with age in wild type flies. (A) Schematic of morning and evening anticipation index. (B) Normalised daily group activity plots of 1, 15, 29 and 43-day old flies. (C) Quantification of anticipation index shows that the morning anticipation index is significantly reduced by age, and that evening anticipation is slightly reduced. N=20-32 for each group, '*' represents $p<0.05$, '**' - $p<0.01$, '***' - $p<0.001$ as determined using the Kruskal-Wallis test with Dunn's post hoc test, data plotted as median with error bars representing the interquartile range.

Figure 3 Ageing alters the daily structure of sleep in *Drosophila*. (A) Daily sleep profile of groups of male flies aged 1, 29 and 43 days old, average across 5 days. Shaded area represents the 95% confidence interval. (B – H) Quantification of sleep parameters for flies aged D1, 8 ,15, 22, 29, 36, 43 and 49 days, flies were monitored in parallel under identical conditions. Error bars plot the median and interquartile range with '*' representing $p<0.05$, '**' - $p<0.01$, '***' - $p<0.001$ with statistical testing performed by Kruskal-Wallis test with Dunn's multiple comparisons. (B) Mean total sleep duration (C) Mean daytime sleep (D) Mean night-time sleep (E) Latency to sleep after lights on (F)

Latency to sleep after lights off (G) Number of sleep episodes (H) Sleep episode duration (see methods for definitions).

Figure 4 Electrophysiological characterisation of l-LNv clock neurons. Membrane potential and spontaneous activity (left panels) and firing response to a current pulse (right panels, colour-coded as indicated) of wild type l-LNvs from young (d1-5) and aged (d28-35) flies recorded at day (ZT 7-9) and night (ZT 19-21).

Figure 5. Quantitative analysis of electrophysiological properties of l-LNv clock neurons from young (d1-5) and middle-aged (d28-35) flies in day and night conditions. (A) Analysis of input resistance (R_{in}) showed a highly significant effect of age ($p<0.0001$) and an effect of time of day ($p<0.005$) but no interaction. (B) Analysis of the spontaneous firing rate (SFR) showed a significant effect of time of day ($p <0.0001$) but no effect of age. (C) Analysis of the membrane potential (MP) values showed a significant effect of time of day ($p<0.0001$). (D) Analysis of the responses to an injected current pulse (f_{+40pA}) showed no significant effects. Data were analysed using two-way ANOVA with Tukey's multiple comparisons test, error bars show the mean ± SEM. Each data point represents a single l-LNv neuron.

Figure 6. Daily reorganization in the PDF terminals is reduced by ageing. (A) *pdf>mCD8-GFP* wild type brains dissected at ZT2 and ZT14. Brains were stained with anti-GFP (green) and anti-PDF (magenta) antibodies. Scale bar = 50 µm. (B) Schematic depiction of how the quantification of the PDF axonal branching was carried out. (C) The total number of intersections between the concentric rings and the axonal projections was quantified and showed daily remodelling. Error bars show mean ± SEM, statistical analysis performed by two-way ANOVA with Tukey's multiple comparisons test. N>6 for all groups, quantification was performed on the dorsal projections originating from a group of s-LNv neurons.