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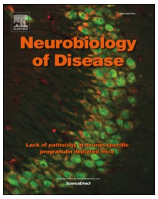

Alzheimer's disease-associated tau alters *Drosophila* circadian activity, sleep and clock neuron electrophysiology

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia, which is associated with an enormous personal, social and economic burden worldwide. However, there are few current treatments with none of them targeting the underlying causes of the disease. Sleep and circadian rhythm defects are not only distressing symptoms of AD and other tauopathies and are a leading cause of care home admission but are also thought to accelerate AD pathology. Despite this, little is understood about the underlying causes of these behavioural changes. Expression of the 0N4R isoform of tau has been associated with AD pathology and we show that expressing it in the *Drosophila* clock network gives rise to circadian and sleep phenotypes which closely match the behavioural changes seen in human AD patients. Tauopathic flies exhibited greater locomotor activity throughout the day and night and displayed a loss of sleep, particularly at night. Under constant darkness, the locomotor behaviour of tau-expressing flies was less rhythmic than controls indicating a defect in their intrinsic circadian rhythm. Current clamp recordings from wake-promoting, pigment dispersing factor (PDF)-positive large lateral ventral clock neurons (l-LNvs) revealed elevated spontaneous firing throughout the day and night which likely underlies the observed hyperactive circadian phenotype. Interestingly, expression of tau in only the PDF-positive pacemaker neurons, which are thought to be the most important for behaviour under constant conditions, was not sufficient or even necessary to affect circadian rhythmicity. This work establishes *Drosophila* as a model to investigate interactions between human pathological versions of tau and the machinery that controls neuronal excitability, allowing the identification of underlying mechanisms of disease that may reveal new therapeutic targets.

1. Introduction

Alzheimer's disease (AD) is a major challenge to modern science and healthcare. Due to prolonged life expectancy, the prevalence of the disease is set to increase in the coming decades, with treatments still limited in number and effect. Symptoms are diverse and highly distressing and debilitating. Many of those suffering with AD display altered sleep patterns, including a tendency to be awake more at night and sun-downing, wherein their symptoms of confusion and wandering are exacerbated in the evening (McCurry et al., 1999; Vitiello et al., 1992; Volicer et al., 2001). Sleep disruption in community-dwelling AD patients is a primary reason for institutionalisation (Pollak and Perlick, 1991). An intact circadian clock is also important for proper memory function (Gerstner and Yin, 2010), positing circadian clock impairment as a central pathology in AD. Post-mortem examination of brains of those with AD has shown degeneration of the suprachiasmatic nucleus (SCN), the locus of the mammalian circadian clock. Moreover, SCN dysfunction has been documented in transgenic murine models of AD (Sterniczuk et al., 2010) and tauopathy (Stevanovic et al., 2017).

Tauopathies are a set of diseases that include AD, Pick's disease, progressive supranuclear palsy and chronic traumatic encephalopathy. Tauopathy is a key histopathological feature of AD (Arendt et al., 2016). The post-mortem AD brain contains elevated levels of the microtubule-associated protein tau (MAPT), which is hyper-phosphorylated and aggregated in neurofibrillary tangles (NFTs) and neuropil threads (Arriagada et al., 1992). Tau tangles are correlated with

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Abbreviations: Alzheimer's disease, (AD); Pigment dispersing factor, (PDF); Large lateral ventral clock neurons, (l-LNv); Suprachiasmatic nucleus, (SCN); 12 h light: 12 h dark conditions, (LD); Continuous darkness, (DD); *Drosophila* Activity Monitors, (DAM); Rhythmicity statistic, (RS); Day/night activity, (D/N); Zeitgeber time, (ZT); Resting membrane potential, (RMP); Spontaneous firing rate, (SFR); Action potential, (AP); Afterhyperpolarisation amplitude, (AHP); Input resistance, (R_{in})

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neuronal and synaptic loss associated with cognitive decline in AD. Although the tau aggregates are usually cytoplasmic, their spread can occur by an activity dependent trans-synaptic mechanism between connected brain regions (Wu et al., 2016). In mammals, tau is encoded by the *MAPT* gene which is subject to alternative splicing, giving rise to six major tau isoforms. These isoforms are categorised depending on the number of N-terminal domains (0 N, 1 N or 2 N) and C-terminal repeats (3R or 4R). 4R isoforms are upregulated, with concomitant down-regulation of 3R isoforms, in the AD brain (Yasojima et al., 1999).

Mice expressing human 0N4R tau exhibit behavioural dysfunction and neurophysiological changes, including elevated neuronal activity, which precedes neurodegeneration (Sterniczuk et al., 2010; Wittmann et al., 2001). What is more, tauopathic mice display changes in circadian behaviour including being more active during light periods resulting in perturbations in the rhythmic expression of clock-controlled genes in the SCN (Stevanovic et al., 2017). The sleep-wake cycle also regulates brain interstitial fluid tau in mice and cerebrospinal fluid tau in humans. Tau levels increased during the day, while sleep deprivation caused a further increase and furthermore also increased tau seeding and spreading pathology (Holth et al., 2019).

Little is known about the consequences of tau expression on *Drosophila* circadian behaviour. The expression of Aβ42, another key pathological protein in AD, reduced and fragmented sleep (Tabuchi et al., 2015), as well as reducing the robustness of circadian rhythms (Chen et al., 2014). It has been shown that enhancing sleep in tau-expressing *Drosophila* attenuates memory deficits (Dissel et al., 2017). Broad neuronal expression of human AD genes was required to cause these circadian and sleep phenotypes, with more restricted expression having little or no effect (Chen et al., 2014). Together this suggests sleep disruption is a vital determinant of disease trajectory, though the precise nature and mechanism of this sleep disruption remains unknown.

Drosophila is a useful organism for probing clock dysfunction induced by human tau. Their circadian clock network of ~75 pairs of neurons has been extensively studied, leading to the identification of neurons which promote wakefulness, large lateral ventral neurons (l-LNvs; (Parisky et al., 2008)) and neurons important for behaviour in constant conditions (small LNvs; (Helfrich-Forster, 1998)). Monitoring the locomotor activity of *Drosophila* is a non-invasive, high-throughput method of probing the intactness of the animal's circadian clock.

In the present study we used the compact and genetically tractable nervous system of *Drosophila* to examine the consequences of human 0N4R tau expression on locomotor behaviour, sleep and clock neuron electrophysiology. Flies expressing tau throughout their clock network exhibited changes in their circadian behaviour, including reduced night time sleep. Electrophysiological recordings from the wake-promoting large LNvs revealed elevated spontaneous firing. We propose that tau potentiates the activity of arousal neurons, shifting the balance between wakefulness and sleep.

2. Methods and materials

2.1. Fly stocks

Flies were bred on standard food mixture at 25 °C under 12 h light: 12 h dark conditions (LD). *CSw-* flies (provided by Dr. Scott Waddell, University of Oxford, UK) were crossed with *GAL4* and *UAS* lines to generate control genotypes. *UAS-human tau-0N4R* flies were provided by Dr. Linda Partridge (University College London, UK) *and UAS-human tau-2N4R* were provided by Dr. Efthimios Skoulakis (Alexander Fleming Biomedical Sciences Research Center, Greece). *tim-GAL4*, *PDF-GAL4, tim-GAL4; PDF-GAL80* flies were from Dr. Ralf Stanewsky (University of Münster, Germany), *uas-GFP* was a gift from Dr. Mark Wu (John Hopkins University, US)*, elav-GAL4* (III; BL8760) and *repo-GAL4* (BL7415) were from the Bloomington stock center.

2.2. Drosophila activity monitoring

Individual male flies aged 2–5 days were placed in 65 mm tubes with a small amount of standard food. Locomotor activity was monitored using *Drosophila* Activity Monitors (DAMs, Trikinetics Inc.), which count the number of times the fly breaks an infrared beam intersecting the tube in a given time bin. DAMs were housed in a 25 °C and 70% relative humidity incubator. Day/night activity and sleep was measured by maintaining the flies in a 12 h LD cycle for 5 days. To determine circadian rhythms in locomotor behaviour, flies were then released into constant darkness (DD) for 7–9 days, during which their locomotor activity was measured.

Sleep was quantified in Matlab using SCAMP scripts (Donelson et al., 2012). Locomotor activity was collected in 1 min bins and sleep was defined as five or more minutes of inactivity (Hendricks et al., 2000; Shaw et al., 2000). Sleep parameters analysed were total sleep, maximum sleep episode duration, mean sleep episode duration, number of sleep episodes and sleep latency (time to first sleep episode following a light transition) for day- and night-time, averaged over 5 days.

Circadian rhythm and day/night activity analysis was performed in Matlab using the Flytoolbox (Levine et al., 2002). Locomotor activity (collected in 30 min bins) data were low-pass Butterworth filtered to eliminate periodicities under 4 h (diurnal noise). Autocorrelation analysis of the activity record was performed to identify any rhythmicity in the data. Rhythmicity is quantified by the rhythmicity statistic (RS), defined as the ratio between the height of the third peak on the autocorrelogram (between 16 and 32 h) and the absolute value of the 95% confidence interval (dependent on the number of data points obtained). Behavioural rhythmicity was defined by convention as an RS ≥ 1.5 (Hodge and Stanewsky, 2008). An estimate of the period of a behavioural rhythm was also obtained from autocorrelation analysis and only flies deemed rhythmic were used to calculate period.

Day/night activity (D/N) index was used to highlight differences in locomotor activity between day and night in LD, and subjective day and subjective night in DD, to complement other analyses (Julienne et al., 2017; Kumar et al., 2012):

$$D/N\ index = \frac{Day\ time\ activity - night\ time\ activity}{Day\ time\ activity + night\ time\ activity}$$

2.3. Electrophysiology

Whole cell current-clamp recordings of large lateral ventral neurons (l-LNv) were performed as described previously (Buhl et al., 2016; Julienne et al., 2017). *tim-GAL4; uas-mCD8-GFP* (*tim; GFP*) was used to drive transgene expression and visualize the clock neurons by their expression of GFP. l-LNv were identified by their position, morphology and size.

Briefly, whole brains of male flies were acutely dissected, cleaned and held ventral side up using a custom-made anchor and superfused with extracellular solution containing (in mM): 101 NaCl, 1 $CaCl_2$, 4 $MgCl_2$, 3 KCl, 5 glucose, 1.25 NaH_2PO_4 and 20.7 $NaHCO_3$ at pH 7.2. Neurons were visualised with a 63× lens on an upright Zeiss microscope (Examiner Z1, Carl Zeiss Microscopy GmbH, Jena, Germany). Recordings were made using 8–12 MΩ borosilicate glass electrodes filled with (in mM): 102 K-gluconate, 17 NaCl, 0.94 EGTA, 8.5 HEPES, 0.085 $CaCl_2$, 1.7 $MgCl_2$ or 4 MgATP and 0.5 NaGTP at pH 7.2. Data were acquired with an Axon Multiclamp 700B at 20 kHz and low-pass Bessel filtered at 10 kHz, digitised with an Axon DigiData 1440A and recorded using pClamp 10 (Molecular Devices, Sunnyvale, CA, USA).

The liquid junction potential was calculated as 13 mV and subtracted from all membrane voltages. Resting membrane potential (RMP) and spontaneous firing rate (SFR) were measured after the recording had stabilised for 1–3 min. Input resistance was calculated by injecting hyperpolarising current pulses and measuring subsequent changes in membrane potential. Neuronal excitability was determined by injecting 500 ms depolarising current pulses (0–40 pA, 2 pA increments) and manually counting the resulting spikes. The action potential peak value, afterhyperpolarisation amplitude (AHP) and the width at half maximal amplitude were measured in relation to RMP from averages of 10 spikes for each recording. For comparing the spike shapes, each averaged spike form was normalised to its peak value.

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Inc.). All data were scrutinised to check they met the assumptions of parametric analyses and non-parametric, rank-based alternatives were used where appropriate. Details of statistical tests used are in figure legends. Statistical levels are denoted as following $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. *Expression of tau in the clock elevated locomotor activity throughout the day and night and reduced sleep*

To investigate early clock-specific effects of tau in *Drosophila*, human *tau 0N4R* was expressed throughout the entire clock network of young (2–5 days old) flies using *tim-GAL4* (Buhl et al., 2016). This is comparable to the age used in previous studies that allow early behavioural and physiological effects of AD genes to be characterised prior to widespread neurodegeneration (Chen et al., 2014; Kosmidis et al., 2010) as well as before the progressive loss of circadian rhythms, sleep and clock neuron electrophysiology we have documented in wildtype animals (Curran et al., 2019).

Control (*tim* / + and *tau* / +) animals displayed well-characterised day/night behaviour under a 12 h light:dark cycle (LD; Fig. 1 and Table S1) with greater activity during the day than at night. Flies showed a typical siesta period around noon between the two activity peaks, which occurs once in the morning and once in the evening, as well as anticipation of the light transitions (lights on: zeitgeber time (ZT) = 0, lights off: ZT = 12). Tau-expressing flies (*tim* > *tau*), on the other hand, were found to exhibit greater total activity during both the day and night ($F_{2,102} = 22.75$, $p < 0.0001$, Fig. 1A, B). *tim* > *tau* animals also exhibited a reduced day/night difference in locomotor activity (D/N index) compared to control flies ($F_{2,102} = 21.12$, $p < .0001$, Fig. 1C).

Given that tau-expressing flies displayed an AD-relevant day/night phenotype, we went on to examine the sleep parameters of these animals. Tau-expressing flies demonstrated sleep disturbance particularly at night-time (Fig. 2 and Table S2). It can be seen from the sleep profile (Fig. 2A) that tau-expressing flies appear to sleep later during the night, wake up earlier in the morning and sleep less in the afternoon. *Tim* > *tau* flies slept for less time in total at day and night than control flies ($F_{2,204} = 37.57$, $p < .0001$, Fig. 2B). The number of sleep episodes was different at day-time only compared to *tau* / + controls which slept more often than *tim* / + controls ($F_{2,204} = 8.17$, $p < 0.001$, Fig. 2C) and both the maximum ($F_{2,204} = 16.68$, $p < .0001$, Fig. 2D) and average sleep episode duration ($F_{2,204} = 6.36$, $p = 0.002$, Fig. 2E) was decreased in tau expressing flies at night-time. Additionally, the night-time sleep latency (time it takes to get to sleep) of *tim* > *tau* flies was much longer than that for control flies ($F_{2,204} = 11.7$, $p < 0.0001$, Fig. 2F) which would also contribute to a loss of sleep. It is also noteworthy that, as was the case for locomotor activity in LD, *tim* > *tau* flies displayed no day/night difference in sleep parameters, other than sleep latency.

3.2. *Tau weakens the circadian rhythm of locomotor behaviour*

Human AD patients often have circadian rhythm defects, which not only result in disrupted sleep but also in perturbed eating patterns and body temperature rhythms (Scarmeas et al., 2007). Monitoring locomotor activity in the absence of external cues (i.e. in continuous darkness, DD) is a fast and non-invasive method of assessing the integrity of the circadian clock and the strength of the circadian rhythm using *Drosophila*.

Flies showed entrainment after three days of LD but their locomotor behaviour diverged upon transition into DD (Fig. S1). Control flies maintained clear differences in activity between subjective day and night for five days in DD (Fig. 3A, left and right panel). However, there was much less distinction between subjective day and night in the activity of *tim* > *tau* flies (Fig. 3A, middle panel). Autocorrelation analysis revealed a decrease in rhythmicity statistic (RS) by roughly one-third in tau-expressing flies compared to control counterparts

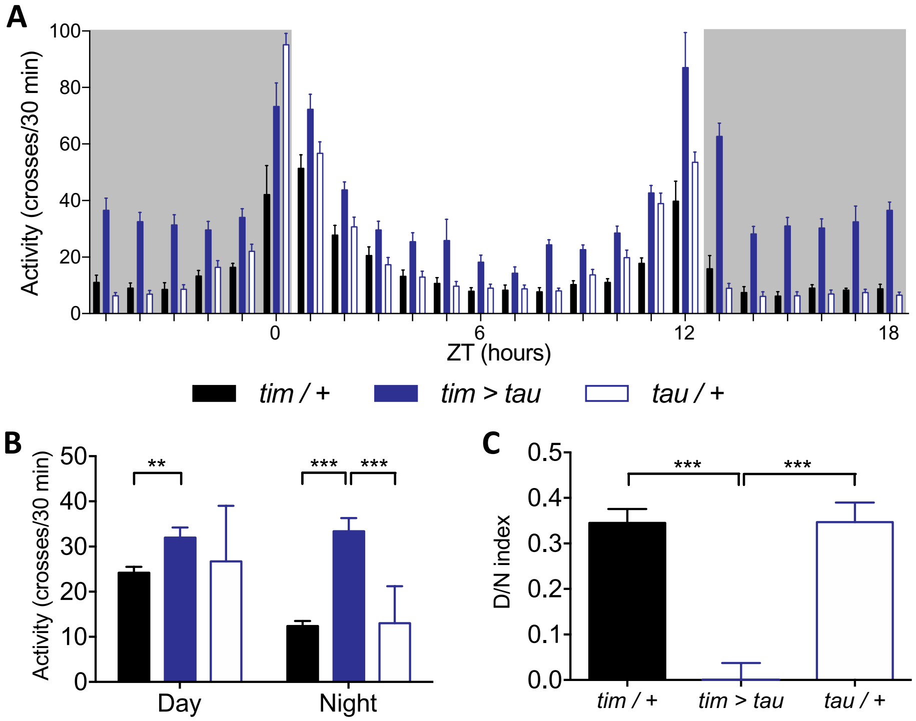

Fig. 1. Tau-expressing flies exhibited elevated locomotor activity throughout the day and night. A, Tauopathic flies (*tim* > *tau*, solid blue bars) were more active than controls (*tim* / + in black and *tau* / + in open blue bars) at most times, especially in the evening and during the night. B, Average locomotor activity of *tim* > *tau* flies across the day and night was elevated compared to control animals (two-way ANOVA with Tukey's *post-hoc* test). C, Tau-expressing flies showed no difference in activity in the day compared to at night (D/N index), while control flies were more active during the day than at night (one-way ANOVA with Holm-Sidak's *post-hoc* test). Mean, standard error of the mean (SEM), n (*tim* / +) = 38, n (*tim* > *tau*) = 24, n (*tau* / +) = 43 flies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

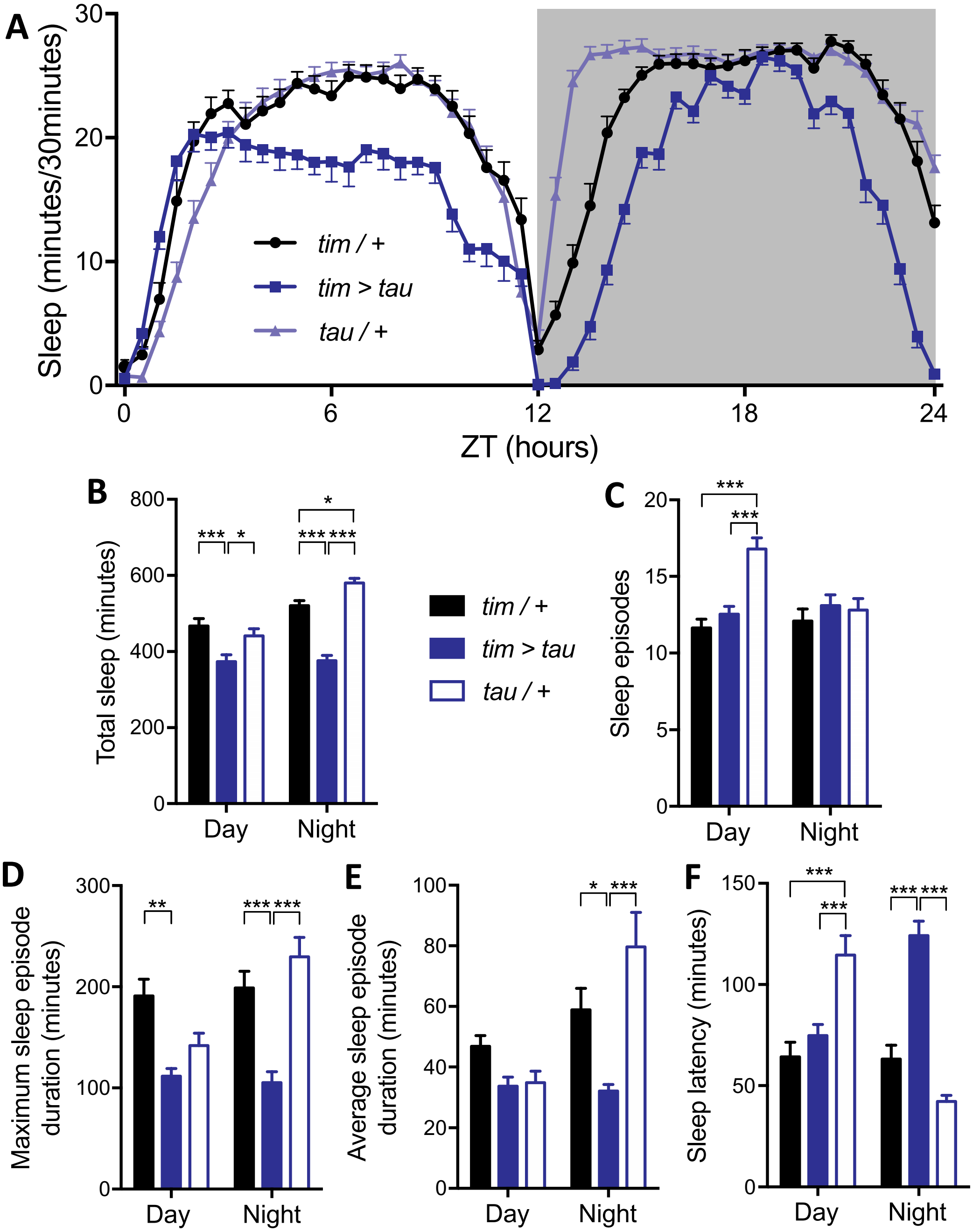

Fig. 2. Tau perturbed sleep. A, Sleep plot (minutes of sleep per 30 min) for *tim* control (black circles), tau-expressing (blue squares) and *tau* control (light blue triangles) animals shows a reduction in sleep in tau-expressing flies in both the day (white left side of the plot) and night (grey-shaded side of the plot) phase of the LD cycle. B, Total sleep duration was different between genotypes showing a sleep loss for *tim* > *tau* flies both during day and night. C, Expression of tau did not affect the number of sleep episodes. D and E, Tau-expressing flies show a night-time-specific reduction of maximum and average sleep episode duration. F, Latency to sleep was prolonged at night by tau expression. All data analysed by two-way ANOVA with Bonferroni's *post-hoc* test. Mean, SEM, n (*tim* / +) = 38, n (*tim* > *tau*) = 24, n (*tau* / +) = 43 flies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

($F_{2,82} = 13.87$, $p < 0.0001$, Fig. 3B and Table S3). 96% and 95% of control genotype flies were rhythmic (RS ≥ 1.5) compared to 58% of the tau-expressing flies (Table S3). To complement this analysis, the difference in gross activity between subjective day and night (D/N index) was also calculated. While control flies upheld a robust difference in locomotor activity for seven days in DD, *tim* > *tau* flies showed no such distinction ($F_{2,82} = 72.96$, $p < .0001$, Fig. 3C). These changes in behavioural rhythmicity were accompanied by no change in free-running period of rhythmic flies compared to *tim* / + controls but to *tau* / + controls that maintained a shorter period ($F_{2,64} = 5.45$, $p = 0.007$, Fig. 3D and Table S3).

3.3. Elevated spontaneous firing rate of l-LNvs in flies expressing tau throughout the clock network

Previous work has demonstrated that the large LNvs are wake-promoting and are important for the regulation of activity and rest in *Drosophila* (Parisky et al., 2008; Sheeba et al., 2008). To probe whether the phenotype we observed in tau-expressing flies was due to perturbed physiology of these neurons, we performed current-clamp recordings from l-LNvs in control (*tim; GFP* / +) and *tim; GFP* > *tau* flies during the day (ZT1–3) and night (ZT13–15; Fig. 4A).

Confirming previous reports (Buhl et al., 2016; Sheeba et al., 2008),

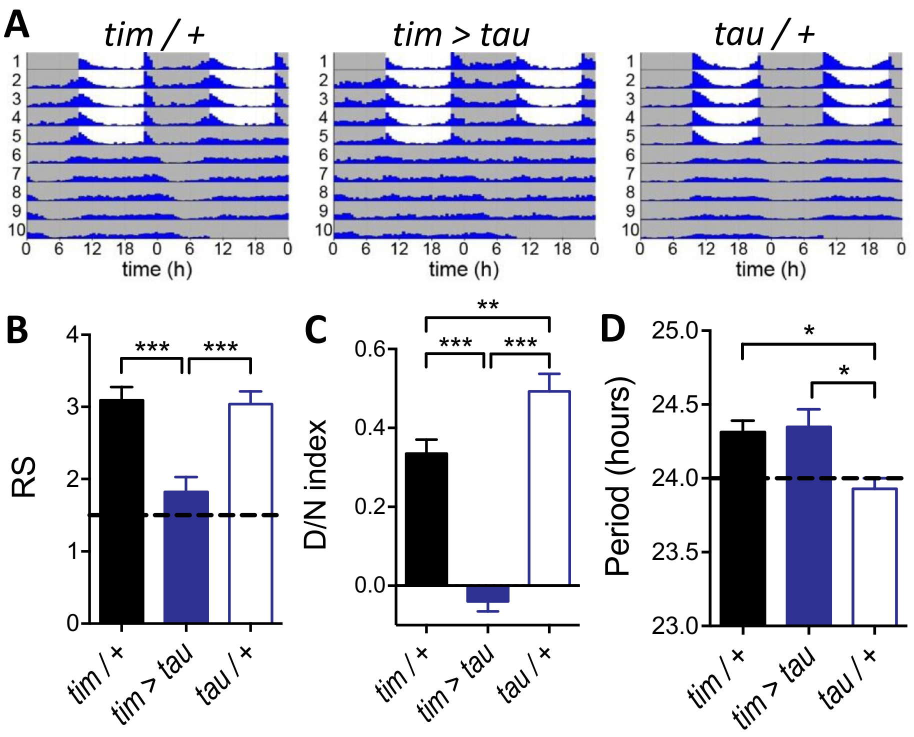

Fig. 3. Clock-specific tau expression disrupted locomotor behaviour in DD. A, Averaged double-plotted actograms of *tim / +*, *tim > tau* and *tau / +* flies for the first 5 days in LD (alternating white and grey-shaded portion of plot) and then released in constant conditions (DD, continuous shaded portion of plot). B, Rhythmicity of locomotor behaviour in DD (or rhythmicity statistic (RS)) was reduced by tau expression in the clock. C, Control animals maintained a robust day/night (D/N index) difference in locomotor activity in DD, but tau-expressing flies showed no distinction between subjective day and night. D, However, tau did not alter the period of rhythmic behaviour in DD. All data analysed by one-way ANOVA with Tukey's *post-hoc* tests. Mean, SEM, n (*tim / +*) = 28, n (*tim > tau*) = 38, n (*tau / +*) = 19 flies.

l-LNvs show a more depolarised resting membrane potential (RMP) during the day compared to night in wild-type animals (Fig. 4B and Table S4), which was also observed in *tim; GFP > tau* flies. However, RMP was even more depolarised in tau-expressing neurons than control neurons, particularly at night time. Spontaneous firing rate (SFR) also cycles between an elevated, more active day state and suppressed activity at night in both experimental groups. Again, the SFR of l-LNvs in tau-expressing flies was significantly greater during both the day and night compared to control flies. Input resistance (R_{in}), a function of the number of open ion channels in the plasma membrane, was also greater during the day than at night in wild-type animals. R_{in} of l-LNvs in tau-expressing flies did not display this cycling, maintaining the same magnitude during the day and night due to lower day time R_{in}. l-LNv excitability ($F_{+40\ pA}$), measured by injecting depolarising currents, did not change between day and night and was not different in control and tau-expressing neurons. We found that the duration of spontaneous action potentials in control neurons was reduced at night compared to day time, the expression of tau removed this day/night difference in action potential duration. We found no other day/night difference in action potential properties (Fig. 4C).

3.4. Expression of tau in the LNvs alone is not sufficient nor necessary to impair circadian rhythms in locomotor behaviour

Given the change in action potential firing observed in the l-LNvs of *tim > tau* flies, we sought to resolve whether tau can disrupt circadian rhythms when expressed in only the PDF-positive neurons that comprise of both the s- and l-LNvs. The actograms of *PDF / +* and *PDF > tau* flies show sustained day/night differences in locomotor activity following the transition to DD (Fig. 5A and B). Autocorrelation analysis confirmed this observation as the genotypes displayed indistinguishable RS values corresponding to rhythmic behaviour and a similar proportion of flies remained rhythmic throughout DD (Table S3). *PDF > tau* flies were found to have a slightly shorter period than control animals (Table S3), although this difference is so small (13.8 ± 3.2 min, $p = 0.0137$) that we believe it is improbable that this represents a meaningful change in behaviour.

On the other hand, the expression of tau in all clock neurons and associated glia (*tim-GAL4*) except the LNvs (*PDF-GAL80*) yielded flies which exhibited reduced behavioural rhythmicity in DD (Fig. 5C and D), confirmed by a reduction of RS compared to control animals (Table S3). Furthermore, only 61% of *tim; PDF-GAL80 > tau* flies were able to maintain rhythmicity in DD, compared to 100% of controls (Table S3). This change in rhythmicity was accompanied by a reduction in period of 43.2 ± 6.6 min ($p < 0.0001$, Table S3). Expressing tau in all glial cells (*repo-GAL4*) resulted in pupal lethality and therefore these flies could not be tested for circadian defects. Pan-neuronal (*elav*^{c155}*-GAL4,* a *Gal4* enhancer trap in the *elav* gene on the X chromosome) expression of *tau* 0N4R or *tau* 2N4R was also lethal (late embryonic lethal). However, expression of *tau* 0N4R with the weaker *elav-Gal4* (an *elav* promoter element upstream of Gal4 inserted in the 3rd chromosome) allowed adult progeny that resulted in fewer rhythmic flies expressing the *tau* 0N4R (53%) and the *tau* 2N4R (66%) isoforms and a reduction in RS similar to *tim > tau* 0N4R levels (Fig. 5E and F; Table S3).

4. Discussion

Little is known about the mechanisms underlying sleep and circadian dysfunction in AD and other tauopathies. These defects are a primary reason for those with AD requiring institutional care (Logsdon et al., 1998), with symptoms being distressing for patients and carers alike. Knowledge of the cellular underpinnings of these symptoms is a prerequisite for the design of rational treatments which are currently lacking in clinical practice. Here we demonstrate that tau, a major pathological component of AD, causes an AD-relevant phenotype when expressed in the central clock neurons of *Drosophila* and thereby establishes the fly as a behavioural and electrophysiological model for studying human tauopathies.

While the greatest risk factor for AD may be age, here we study the effects of tau on young flies because previous *Drosophila* studies overexpressing human amyloid-β (Aβ42) in clock neurons (Chen et al., 2014) and human 0N4R tau in mushroom body neurons (Kosmidis et al., 2010) demonstrate behavioural changes prior to neurodegeneration already at this age. Furthermore, most disease-gene-expressing

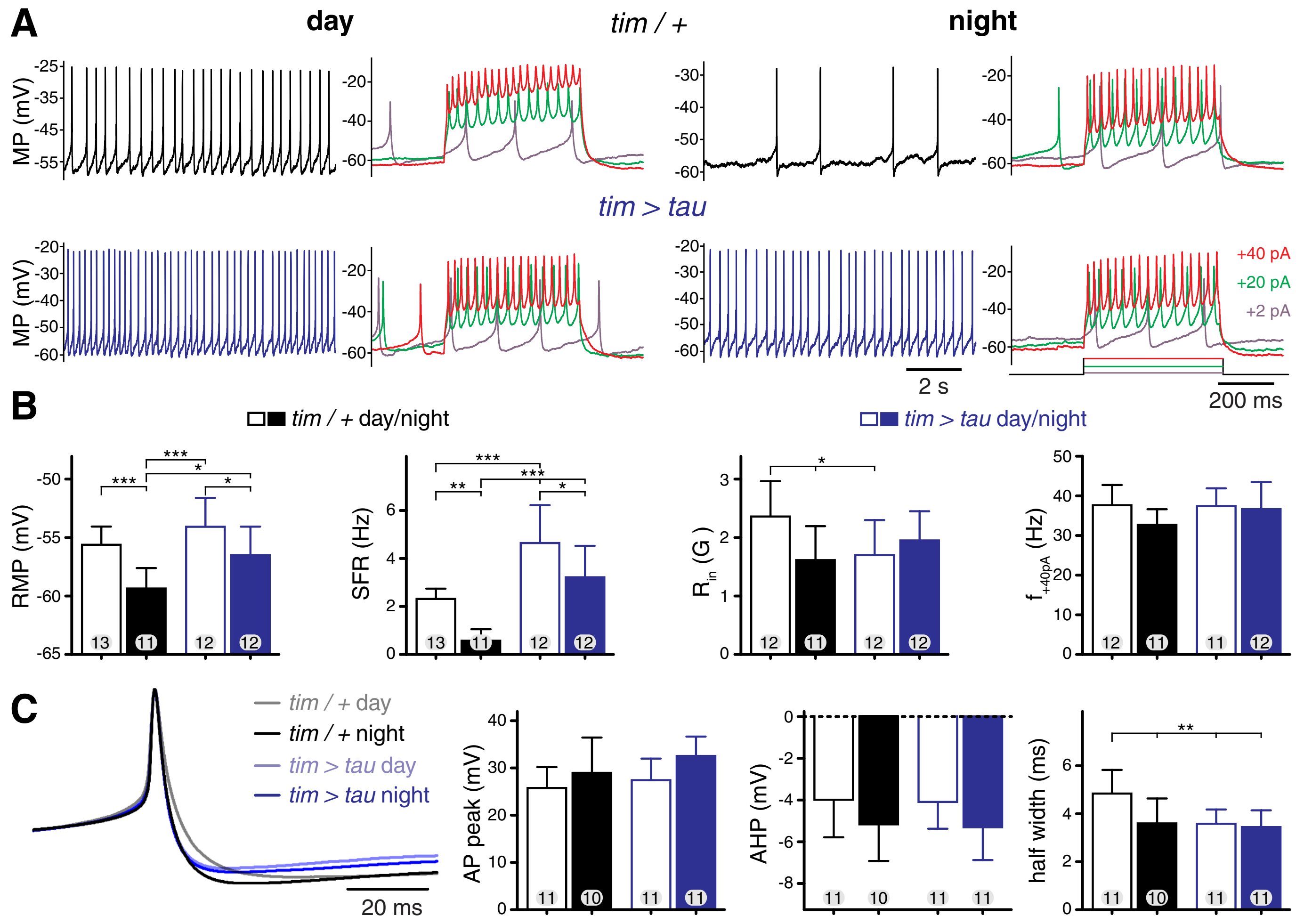

Fig. 4. Expression of tau in the central clock perturbed electrophysiological properties of l-LNvs. A, Spontaneous activity (left panels) and response to current injection (right panels, colour-coded as indicated) of control and tau-expressing l-LNvs during the day (ZT1–3, left side) and night (ZT13–15, right side). B, Quantification of electrophysiological parameters of control (black bars) and tau-expressing (blue bars) l-LNvs during the day (open bars) and night (filled bars). The day/night difference in resting membrane potential (RMP) and spontaneous firing rate (SFR) is present in the tau-expressing flies but with an overall elevated firing frequency. The day/night difference seen in the wild-type input resistance (R_{in}) is absent in the tau neurons while excitability (frequency of spikes resulting from a 500 ms 40 pA depolarising current (f_{+40pA})) is not affected. C, Average action potential (AP) waveforms of normalised spikes (left). Action potential peak and afterhyperpolarisation amplitude (AHP) shows a small day/night difference for both control and tau mutant spikes. APs in wild-type neurons are wider during the day but this difference in half-width is absent in the mutants. Mean, error bars are standard deviation (SD), n, indicated in bars; one-way ANOVA with Tukey's *post-hoc* tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flies have a reduced viability and shortened life span, and because the transgenes are expressed throughout development, we can detect tau related changes in behaviour and physiology even in young flies. Therefore, we study early functional changes to understand early symptoms of the disease, revealing new mechanisms and potential drug targets for early interventions before the irreversible death of the neurons.

Tau-expressing flies were more active during the day and night compared to controls. Locomotor activity was robustly potentiated around the times of light transitions, particularly in the evening (Fig. 1). Elevated activity is commonly observed in humans with AD as they tend to wander more (Logsdon et al., 1998). Further, human AD subjects, and those with other tauopathies, sleep for less time at night (Aldrich et al., 1989; Vitiello and Borson, 2001), as did tau-expressing *Drosophila* (Fig. 2). Sleep loss in tau-expressing flies was mainly characterised by a delayed sleep onset and was more pronounced during the late night, implicating dysfunction of the PDF-positive LNvs as they are key regulators of night time sleep (Gmeiner et al., 2013; Parisky et al., 2008). The prolonged sleep latency following lights-off seems to mimic the sundowning syndrome observed in humans, wherein their symptoms are exacerbated in the evening, resulting in, amongst other behaviours, increased wandering and resistance to sleep (Aldrich et al., 1989). The similarity in behaviour between tau-expressing flies and humans with AD implies tau could have a similar effect on the central clocks in flies and humans, validating *Drosophila* as a model for this behaviour.

In alignment with our data, rTg4510 mice, which express the frontal temporal lobe dementia (FTD)-associated P301L tau mutant, were more active during the day than their control littermates (Stevanovic et al., 2017). Moreover, in constant darkness, tau-expressing mice displayed a ~1 h prolongation of behavioural period, demonstrating a central clock defect (Stevanovic et al., 2017). We observed no change in behavioural period in *tim > tau* flies (Fig. 3), however, this difference could be due to the peculiarities of different model organisms, differential effects of wild-type and P301L tau or, indeed, the expression patterns of the driver lines used (CaMKIIα driver used in rTg4510 mice expresses at a high level in the entire forebrain and is not clock neuron specific).

Others have found that flies pan-neuronally expressing the Arctic mutant of Aβ42 (arc Aβ42), another important model of AD pathology, slept for less time during the day and night, with sleep being more

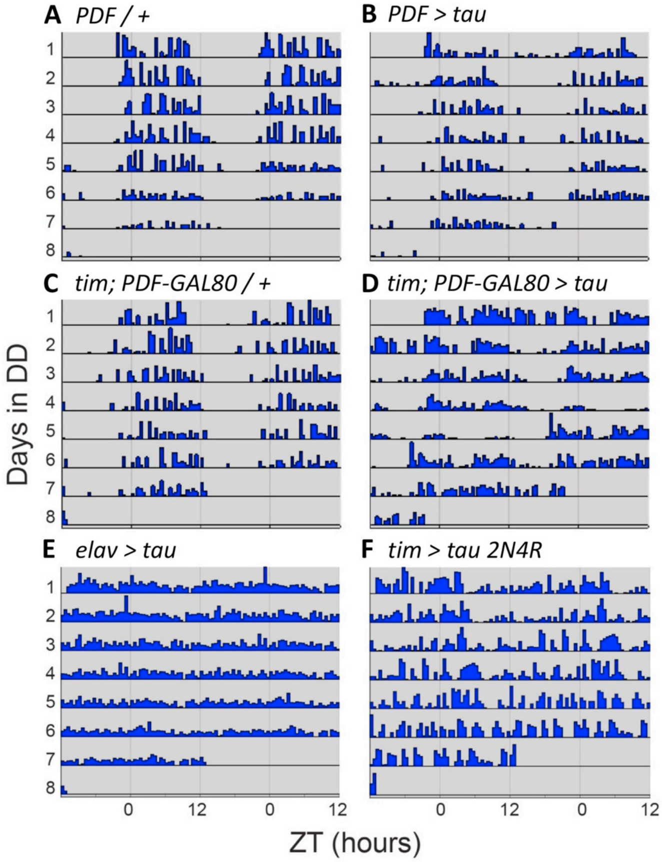

Fig. 5. Expression of tau in the LNvs is not necessary or sufficient to disrupt circadian locomotor behaviour. Exemplar double-plotted actograms of a *PDF* / + (A), *PDF* > *tau* (B), *tim; PDF-GAL80* / + (C), *tim; PDF-GAL80* > *tau* (D), *elav* > *tau* (E) and *tim* > *tau 2N4R* (F) fly in DD. This shows flies that organise their activity into roughly 12 h of activity followed by 12 h of inactivity, hence no behavioural effect of tau expression in the PDF-positive clock neurons while driving tau expression in all clock neurons except the PDF-positive cells or in all neurons resulted in decreased locomotor rhythmicity with activity occurring at similar level throughout the 24 h period. Expression of the 2N4R tau isoform produced similar arrhythmicity as the 0N4R tau isoform. Rhythmicity statistics (see Table S3) analysed with one-way ANOVA with Tukey's *post-hoc* tests.

fragmented (Tabuchi et al., 2015) and here we have observed similar behavioural changes with pan-neuronal expression of tau (Fig. 5). The highly toxic oligomerising tandem Aβ42 (TAβ42) construct, but not Aβ42 or arc Aβ42, caused behavioural arrhythmia when expressed in the *Drosophila* clock but only in aged animals (Chen et al., 2014). As both 0N4R and 2N4R tau expression in the clock neurons caused arrhythmia in young animals (Figs. 3 and 5), it seems that tau is more toxic to these cells than Aβ42. This could also be because tau is more correlated to AD neurodegeneration than Aβ (Holth et al., 2019; Spillantini and Goedert, 2013) or tau toxicity is via an intracellular mechanism while Aβ toxicity is via extracellular plaques (Selkoe and Hardy, 2016) and the Gal4/UAS system, which is intracellular, may be better suited to initiating tau toxicity. However, neither TAβ42 (Chen et al., 2014) nor tau was sufficient to impair behavioural rhythmicity when expressed in only the PDF-positive LNvs, which only total 18 in number (Kula-Eversole et al., 2010).

The small LNvs are major drivers of DD rhythmicity (Helfrich-Forster, 1998), but expression of tau in these neurons is not required or sufficient to drive changes in circadian locomotor behaviour (Fig. 5). Perhaps the disruption of circadian behaviour by tau arises from effects outside of the DD pacemaker, as has been suggested for TAβ42 pathology (Chen et al., 2014) and in Huntington's disease (Pallier et al., 2007). It was found that TAβ42 expression by *tim-GAL4; PDF-GAL80* disrupted PDF cycling in s-LNv terminals, despite these cells not being directly affected by TAβ42 expression (Chen et al., 2014). It has also been shown that tau has differential effects on different cells types. For example, CA1, but not CA3, pyramidal neurons in 3xTg mice displayed augmented L-type calcium current density (Wang and Mattson, 2014). Further, network neuronal properties in the dorsal, but not ventral, medial entorhinal cortex were disrupted in rTg4510 mice (Booth et al., 2016). As such, it is not unreasonable to assert that tau could have differential effects on *Drosophila* neurons, as is the case in transgenic mice. Driving tau expression with *tim; PDF-GAL80* would still result in glial cell, in addition to dorsal clock neuron, expression of tau. Disruption of glial cell function has been posited as an important component of AD pathology (Dzamba et al., 2016) and expression of tau in all glial cells resulted in pupal lethality. However, tau expression in glia might not be necessary since pan-neuronal expression of tau using *elav-GAL4* produced a similar arrhythmic phenotype. Tau pathology has also been shown to spread trans-synaptically via an activity dependent release mechanism (Spires-Jones and Hyman, 2014; Wu et al., 2016), whereby the electrical hyperactivity we saw due to clock-wide expression of tau, could result in propagation of pathological tau to downstream neurons. Likewise, although tau is predominantly intracellular, it can be released into the extracellular space, in a manner which is increased by neuronal excitability, with sustained neuronal hyperexcitability such as in sleep deprivation, increasing tau spread and pathology (Holth et al., 2019; Pooler et al., 2013; Wu et al., 2016). Therefore, it is possible that, while tau expression in the LNvs has no behavioural correlate, expression of tau elsewhere in the clock has deleterious effects on these cells, leading to changes in circadian behaviour.

In humans, brain regions downstream of the SCN are frequently affected in AD. Rhythmic production and release of melatonin by the pineal gland is disrupted (Skene et al., 1990; Wu and Swaab, 2005) despite there being no evidence for typical AD-like histopathology in this brain region (Pardo et al., 1990). As the major afferents to the pineal gland arise in the SCN, it is likely that a clock defect underpins changes in melatonin rhythms. Indeed, this highlights dysfunction imparted on a brain region due to defects in surrounding areas.

Our electrophysiological data offer an insight into the mechanism underlying tau-induced behavioural impairment. Tau expression in the clock resulted in elevated spontaneous firing of the l-LNvs, an ablation of day/night cycling in input resistance and a more depolarised resting membrane potential (particularly at night), with no effect on excitability (Fig. 4). Hippocampal and cortical pyramidal neurons from transgenic rTg4510 mice exhibited greater firing rates than neurons from wild-type animals (Crimins et al., 2012), implying the cellular mechanisms underlying tau pathology could be conserved between flies and rodents.

The large LNvs are a wake-promoting component of the circadian and sleep circuits (Parisky et al., 2008). Elevated spontaneous firing of these cells would, therefore, be expected to lead to elevated locomotor activity and to suppress sleep. During the day, input resistance was decreased by tau expression, suggesting more open channels conducting depolarising currents, which would lead to elevated firing rates. Depolarised night-time resting membrane potential in tau-expressing flies would increase responsiveness to endogenous inputs, potentiating firing. Interestingly, the observed increase in firing does not come with an increase in general excitability of these neurons suggesting a potential non-cell-autonomous mechanism. Additionally, the effects of tau on neuronal physiology may not be limited to the LNvs but result in an overall increase in activity of the whole clock network by an unknown mechanism that needs further investigation. Ablation of the day/night cycling of input resistance by tau could suggest disruption of the circadian cycling of ion channel expression, trafficking or function in the large LNvs, or could reflect changes in synaptic drive onto these neurons.

We are overexpressing the human version of the microtubule associated protein tau which encodes 4C terminal repeats that mediate aggregation of the protein. This is thought to lead to a pathological aggregation, dissociation from microtubules and disrupted trafficking of proteins to axonal and dendritic membranes. Because we see a change in the electrical properties of clock neurons (Fig. 4), it is likely that tau overexpression is disrupting the normal circadian regulation of membrane trafficking of ion channels. Potassium channels, for example, have been proposed to mediate day/night differences in clock neuron activity (Belle et al., 2009; Buhl et al., 2016; Feng et al., 2018; Flourakis et al., 2015), with increases in intrinsic excitability via downregulation of potassium channels being implicated in AD (Allen et al., 2017; Kidd et al., 2006; Ping et al., 2015; Tabuchi et al., 2015). The change in action potential duration between control and tau-expressing neurons further suggests a perturbation of ion channel function by tau. However, future experiments will need to demonstrate how overexpression of tau disrupts the microtubule mediated trafficking of potassium channels to the membrane which could result in the change in neuronal activity, shift in resting membrane potential and duration of action potential repolarisation recorded here.

Expression of transgenes which induce neuronal hyperactivity, such as *Shaw-RNAi* or *NaChBac* (Buhl et al., 2016; Hodge, 2009; Hodge and Stanewsky, 2008; Nitabach et al., 2005; Parisky et al., 2008), in the LNvs yielded flies with a strikingly similar phenotype to that observed here in *tim* > *tau* flies, with hyperexcitation of large LNvs reducing sleep time and increasing sleep latency across the day and night (Artiushin and Sehgal, 2017). Given tau's detrimental effects on clock neurons other than the LNvs (see Fig. 5), the observed phenotype is probably the result of interrupted communication between clock components, leading to perturbation of the output from the clock to its downstream targets.

In summary, we show that expression of a pathological variant of the AD causal gene tau in the *Drosophila* clock network results in circadian and sleep defects similar to those seen in patients with the disease. The wake-promoting large LNv clock neurons of these flies showed increased firing and depolarised membrane potentials, potentially underlying this hyperactive behavioural phenotype and, consequently, disruption of circadian behaviour. The central DD pacemaker small LNv neurons appear less susceptible to tau pathology per se and may be perturbed due to disruption of neighbouring cells. This perturbation shifts the clock network into a persistently waking state.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.104507.

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