

1 **Simultaneous Monitoring of Behavior & Peripheral Clocks in *Drosophila* Reveals**
2 **Unstructured Sleep in an Alzheimer's Model**

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1 **Abstract**

2

3 Sleep and circadian rhythms are ancient, related phenomena controlled by distinct
4 circuitry, whose appropriate regulation is critical to health. The regulatory machinery
5 underlying sleep homeostasis is ill-defined, but biological clock mechanisms are better
6 understood: from 'clock gene' oscillations to rest/activity cycles. Age- and
7 neurodegeneration-related deterioration in sleep/wake timing was first described in
8 humans decades ago, and has recently been recapitulated in model organisms. To
9 delineate causal relationships between aging, sleep, neuronal function and the molecular
10 clockwork, we developed FLYGLOW, a bioluminescence-based system which allows
11 rest/activity, sleep consolidation and molecular rhythms to be quantified simultaneously in
12 many individual flies over days/weeks. FLYGLOW outperforms existing methods, and
13 allows multiparameter correlational analyses within and between flies. We also show
14 unambiguously that peripheral tissue rhythms free-run independently of the central
15 pacemakers driving behavioural cycles. Using an Alzheimer's fly model we observe a
16 profound disorganization of sleep/wake cycles, phenocopying the human disease.

1 **Introduction**

2 In animals, sleep and wakefulness are regulated by both homeostatic and circadian
3 processes (Cirelli and Bushey, 2008; Wulff et al., 2010). Sleep homeostasis is highly
4 conserved in metazoans, but its fundamental nature is poorly understood compared with
5 the circadian clock. This latter (about daily) biological rhythm, when synchronized with the
6 external environment, allows an organism to anticipate, or resonate with, the varied
7 demands of the day/night cycle, allowing metabolic and behavioral adaptations that are
8 evolutionarily beneficial. The conservation of circadian rhythmicity across the phylogenetic
9 spectrum from cyanobacteria to mammals indicates that these benefits are substantial
10 (Hardin and Panda, 2013; Reddy and O'Neill, 2010). In many organisms however, these
11 benefits are likely lost as circadian regulation of behavior and physiology progressively
12 deteriorates with advancing age (e.g. Luo et al., 2012; Nakamura et al., 2011; Paudel et al.,
13 2010; Rakshit et al., 2012; Tranah et al., 2011; Umezaki et al., 2012); indeed many age-
14 related neurodegenerative disorders in humans, including Alzheimer's Disease (AD)
15 (Hatfield et al., 2004; Volicer et al., 2001; Wulff et al., 2010), are associated with sleep-wake
16 abnormalities from their earliest clinical stages. Moreover a growing number of
17 observations suggest that circadian and sleep deficits may contribute to the pathogenesis
18 of major neurodegenerative disorders (Chen et al., 2014; Coogan et al., 2013; Long et al.,
19 2014; Pallier et al., 2007; Park et al., 2012). Unraveling this putative reciprocal interaction
20 between aging, neuronal (dys)function and circadian sleep/wake cycles is a non-trivial
21 problem however, due to the challenges inherent in performing longitudinal experiments
22 at multiple levels of biological scale in freely behaving individuals.

23
24 At a molecular level the circadian clock machinery is highly conserved from flies, through
25 mice to humans, consisting of a series of interlocked transcription-translation feedback
26 loops observable in every cell (Hardin and Panda, 2013). In mammals at least these
27 rhythms are orchestrated by a central master clock mechanism located within the CNS
28 (Mohawk et al., 2012). The resulting temperature-compensated gene expression rhythms,
29 with an endogenous period of approximately 24 hours, are entrained to the environment
30 via a number of cell-autonomous mechanisms and systemic cues, in response to
31 exogenous signals such as light, feeding or temperature as the clock signal (*zeitgeber*).
32 Such molecular clock oscillations in *Drosophila* may be visualized non-invasively by
33 heterologous expression of firefly luciferase fused with a cycling 'clock protein', such as
34 Period. When the organism is fed the luciferase substrate (luciferin) the level of the fusion
35 protein is reported by the intensity of the emitted light and this signal faithfully reports the

1 cycling of the molecular clock in every cell where it is expressed (Brandes et al., 1996;
2 Plautz et al., 1997b).

3
4 There is much interest in mechanistic relationships within the hierarchy of intracellular,
5 intercellular and behavioral rhythms, for example to what extent peripheral cellular
6 rhythms are merely slave oscillators, tightly coupled to a master central clock which also
7 co-ordinates activity rhythms (Giebultowicz et al., 2000; Hege et al., 1997; Krupp et al., 2013;
8 Mohawk et al., 2012; Myers et al., 2003; Saini et al., 2013; Xu et al., 2008). Our ability to
9 study the relationships between these various oscillations has until now been limited by
10 the need, at least in *Drosophila*, to study molecular and behavioral rhythms in separate
11 groups of organisms. As a result our observations of circadian oscillations are
12 population-based, measuring behavioural or molecular markers not both, and report
13 group parameters such as period, amplitude and rhythm quality with inter-individual
14 differences being reported largely in terms of variance.

15
16 Behaviors such as locomotor activity and sleep are readily observable in a wide range of
17 animals. In *Drosophila*, sleep is distinguished from wakefulness by a decreased sensitivity
18 to arousing stimuli and homeostatically regulated rebound following sleep deprivation
19 (Gilestro et al., 2009; Shaw et al., 2000). For practical purposes fly sleep is defined as a
20 period of immobility exceeding five minutes (Gilestro, 2012; Gilestro et al., 2009).

21
22 In this study we describe for the first time a technique that allows analysis of the
23 relationships between peripheral tissue rhythms, rest/activity cycles (a proxy for the central
24 clock) and sleep consolidation. The characteristics of circadian rhythms in control
25 organisms are compared to those in flies expressing the toxic A β peptide as a model of AD.
26 We do this by making simultaneous real-time measurements of molecular clock dynamics
27 and multiple behaviors simultaneously, over arrays of dozens of individual flies.

28 29 **Materials and Methods**

30 31 **Fly strains and husbandry**

32 All *Drosophila* strains in this study were housed and aged on standard cornmeal food. Flies
33 expressing the E22G (Arctic) variant of amyloid beta peptide 1-42 (A β ₄₂) are used as a
34 model of amyloid toxicity and are described elsewhere (Chen et al., 2014; Crowther et al.,
35 2005). To monitor clock gene expression in control or pan-neuronally A β ₄₂ expressing

1 flies, a new fly strain (*elav-gal4;; XLG-luc2/TM3*) containing *elav-gal4^{C155}* driver and the *period*
2 promoter driven Period-luciferase fusion construct, *XLG-luc2* (Figure 1A and Veleri et al.,
3 2003), were generated and crossed to the *UAS-A β ₄₂* or a background control strain. The
4 following offspring were studied: *elav-gal4;uas-A β ₄₂/+; XLG-luc2/+* and *elav-gal4;; XLG-*
5 *luc2/+*. The control and UAS-A β ₄₂ flies share the same *w¹¹¹⁸* background and both contain
6 *attB* sites derived from phiC31 mediated transformation (Chen et al., 2014)

8 **Fly arena**

9 Individual luciferase expressing flies of required genotypes and ages were housed in a
10 capped glass tube (cap: CAP5-Black; capillary: 5 mm x 65 mm, PGT5x65, Trikinetics. Inc.
11 USA) containing 100 μ l of 1% w/v agar, 5% w/v sucrose and 15 mM (run 1) or 50 mM (run 2)
12 luciferin. These tubes are placed in a microscope slide storage tray (100 slide model,
13 165 mm x 210 mm x 35 mm, Fisher) with card dividers between the tubes (8 mm x 72 mm,
14 480 GSM, Ryman Limited, UK)(Figure 1-figure supplement 1A). Each tray holds up to
15 48 tubes. A customized arena with the mentioned dimension was constructed using
16 black nylon plastics with fixed spacers (Figure 1-figure supplement 1B, distributed by
17 Polygonal Tree, London, <http://polygonaltree.co.uk/>).

19 **Bioluminescence recording**

20 Flies, 20 days post eclosion, were placed in capillary tubes and exposed to a 12-hr light: 12-
21 hr dark (LD) regimen for three days of circadian entrainment prior to being transferred into
22 recording conditions at anticipated dusk (ZT12). Recordings were performed under
23 constant darkness at 26°C over seven days. Bioluminescence was detected using an EM-
24 CCD camera (Hamamatsu Photonics UK Ltd, C9100-14) cooled to -70°C. incorporated
25 within a Cairn Alligator system (Cairn Research Limited, UK). A bright field image was taken
26 before each recording to ensure appropriate focus and tray alignment (Figure 1-figure
27 supplement 1C). Bioluminescence images were recorded with contiguous 5 min
28 integrations over 7 days with camera settings: 4x gain, 200x EM gain (Figure 1B). An
29 example bioluminescence time series is included as a compressed movie, each frame
30 resulting from a 5 minutes exposure (supplementary video). Week-long recordings were
31 made with trays containing 36 control and 32 A β ₄₂-expressing flies. In separate
32 experiments, control flies were loaded individually into the wells of a microtiter plate
33 containing the food-luciferin substrate where their movement was restricted by covering,
34 pierced plastic domes (Stanewsky et al., 1997). The plate was placed in the Cairn Alligator
35 system and recorded in parallel with the tube-based assay condition. The behavior of

1 equivalent flies was also recorded using the DAM system beam-breaking apparatus
2 (Rosato and Kyriacou, 2006) (TriKinetics Inc., MA USA).

3 4 **Image rotation, background subtraction and feature enhancement**

5 The raw data from the camera consisted of the 16 bit photon counts summed over 5 min
6 at a 1024 x 1024 pixel resolution. Pre-processing of the raw data began with an
7 estimation of the intensity of the background signal. This was achieved by averaging the
8 brightness of pixels in a 64 x 64 square at the bottom right of the screen; this average value
9 was then subtracted from all pixel intensities. The next step was the rotation of each
10 frame so that the fly tubes become properly aligned with their long axes parallel to the y-
11 axis of the image. This was achieved by manual location of three marker points (grey
12 dots in corners, Figure 1B) on the tray in which the fly tubes are placed. Once correctly
13 aligned the corresponding tubes in successive rows lie directly above each other on the y-
14 axis. In order to identify where, across the image, each pair of corresponding tubes was
15 located we plotted the profile of the pixel intensities summed along the y-axis. This
16 profile oscillates, with each peak of the summed pixel intensities indicating where the
17 centre of each tube can be found (Figure 1B). A similar process was performed along the
18 y-axis, summing pixel intensities across the image to detect the top and bottom of each
19 row of tubes. Once these steps were complete we assigned a rectangle for each tube
20 that corresponded to its boundary, assuming that each tube was 17 pixels wide. We also
21 assigned a rectangle that corresponds to the dark background between pairs of tubes that
22 is centered on the midpoint between adjacent tubes and was 7 pixels wide. The average
23 pixel intensity of this rectangle was termed the "inter-tube background intensity" and was
24 used in the tube-by-tube background subtraction process described below. At this stage
25 the data was saved as pre-processed "raw data" and was used for all quantitative
26 calculations. To allow sensitive feature detection we also enhanced the contrast and
27 brightness of the images so that flies could be reliably identified despite changes in overall
28 brightness. This process of enhancement used the contrast-stretching transformation
29 algorithm in MATLAB that globally optimizes the dynamic range of pixel intensities. Such
30 processed images were stored as "enhanced data".

31 32 **Detecting resting and moving flies**

33 Hardware issues result in small systematic variations in pixel brightness between regions of
34 the image (vignette effect). To compensate for this we performed a second round of
35 background intensity subtraction. This was done on a tube-by-tube basis using the inter-

1 tube background intensity, calculated as above. We then divided each tube rectangle into
2 4-pixel-high bins along their long axis (y-axis). We summed pixel intensities for each bin
3 and calculated the mean and standard deviation of these values. Bins containing a
4 resting fly were characterized by peaks in the bin intensity profile along a tube (Figure 2,
5 “Enhanced images”); a bin contained a resting fly when maximum pixel intensity of the
6 peak > (Mean tube intensity + Standard Deviation).

7 We then excluded bins that contained resting flies and used the raw data from the
8 remaining bins to recalculate the mean tube intensity (Mean without peaks, Figure 2).
9 For each fly and each frame we partitioned the 300 sec of the camera exposure time
10 between two parameters: i) time spent active and ii) time spent resting. The contribution
11 of activity to the profile was measured by calculating the area of the rectangle between
12 the (Mean without peaks) and the background (Figure 2, yellow areas). The contribution
13 of resting to the profile was measured by calculating the area under each identified peak,
14 bounded by the (Mean without peaks) (Figure 2, purple areas).

15 For each frame:

$$16 \quad \Sigma (\text{area under peaks}) + \text{Area of rectangle below Mean without peaks} = 300 \text{ sec}$$

$$17 \quad \text{Time}_{\text{rest}} = 300 \text{ sec} \times \Sigma (\text{area under peaks}) / \text{Total area}$$

$$18 \quad \text{Time}_{\text{active}} = 300 \text{ sec} \times \text{Area of rectangle below Mean without peaks} / \text{Total area}$$

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20
21 In fewer than five percent of frames, subtraction of the inter-tube background resulted in a
22 negative value for a tube’s mean intensity; this was usually due to artifactually high
23 intensity pixels in the background region. In these circumstances the data was discarded
24 and the values of $\text{Time}_{\text{rest}}$ and $\text{Time}_{\text{moving}}$ interpolated from adjacent time points.

25 26 **Measuring the oscillation of the molecular clock**

27 The oscillation of molecular clock was calculated by summing up the background-adjusted
28 pixel intensity in a rectangle constrained by each tube’s coordinates. This primarily reports
29 cellular circadian rhythms of gene expression in peripheral tissues of the fly.

30 31 **Assigning sleep episodes**

32 *Drosophila* sleep episodes are defined as a period of locomotor inactivity lasting for
33 >300 sec. Having calculated the time spent moving and resting for each frame we
34 defined the presence of a sleep episode in the following way: Firstly, a sleep episode can
35 only be initiated in a frame with a single intensity peak, indicating that the fly was resting

1 in only one position. Secondly, considering the subsequent frames in turn we checked
2 that there was a peak at the same position, indicating that the fly had rested from one
3 frame to the next. Subsequent frames were analyzed sequentially until the resting peak
4 was lost. The component rest times were then summed across the multiple frames; when
5 the total resting time exceeded 300 sec then the episode was defined as sleep. This
6 approach is conservative and does not over-identify brief rests as sleep episodes. Each
7 frame within a sleep episode was assigned a 1 in the binary sleep array; all other frames are
8 assigned a 0 (1=asleep and 0=awake). Using this method, we can robustly assign one of
9 three behavioral states to each frame for each fly; these are “active” (Figure 2A), “active
10 with rest” (Figure 2B) and “sleeping” (Figure 2C).

11 12 **Calculating sleep consolidation**

13 Our novel sleep consolidation index provides an indication of how long a block of sleep
14 lasts. To calculate this index for a particular fly we looped through the binary sleep array
15 and identified blocks of sleep as consecutive episodes separated by wake periods. Each
16 time point within a particular sleep block was assigned with the duration of its
17 encompassing sleep episode (Figure 3).

18 19 **Final data output**

20 The temporal resolution of the data was reduced to 30 min by binning data from
21 consecutive timepoints, similar to the conventional circadian locomotor assay (Chen et al.,
22 2014). For time spent resting or moving, the binned value represented the sum of the
23 constituent timepoints. For sleep consolidation, the binned value was assigned as the
24 mean value of non-zero entries in the sleep consolidation array.

25 26 **Time-series analysis**

27 We adapted the well-established autocorrelation methodology to determine rhythmicity
28 and circadian period using Flytoolbox in the MATLAB environment. To make our analysis
29 comparable to the DAM actimetry system we have limited our observations to 7-days with
30 data considered in 30 min bins. This constrained paradigm permits the convenient use of
31 Rhythmicity Statistic analysis as described by Levine et al. (Levine et al., 2002a). To
32 normalize the exponential decay of bioluminescence intensity with time we employed the
33 detrend filter. We also combined the sliding-window method (3 day window) with the
34 phase determination tool (peakcircleplot, Levine et al., 2002a; Levine et al., 2002b) to
35 quantify differences in circadian phase between time series.

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Results

Simultaneous molecular clock and behavioral measurements: FLYGLOW

Our experimental *XLG-luc2* flies (Figure 1A, I) express a Period-Luciferase fusion protein and produce bioluminescence when fed with luciferin (Figure 1A, II). This light signal is detectable using a sensitive electron-multiplying CCD camera, and effectively reports clock gene expression in peripheral tissues, that is lowest at anticipated dawn (CT0) and highest at anticipated dusk (CT12) (Figure 1A). When constrained within glass capillary tubes (Figure 1A) these flies are considered to exhibit one-dimensional locomotor behavior that is readily quantified by computational image processing. In these experiments one end of the tube was sealed with fly food containing luciferin and the other end closed with cotton wool; these conditions will support a *Drosophila* for more than three weeks. By integrating the bioluminescence across 300 sec exposures for each frame (Figure 1B) we constructed a time-lapse movie representing 7 days of fly behavior (supplementary movie). For the area represented by each tube, within each frame there is sufficient information to directly measure the level of peripheral clock gene expression reported by luciferase activity (number of photons detected *per* region of interest) as well as locomotor activity (spatial distribution of detected photons within that region); from the latter we can reliably infer the duration of fly sleep episodes according to the widely accepted standard (Gilestro, 2012; Gilestro et al., 2009).

In brief the images were processed to detect two patterns of intensity along the length of each capillary tube. Active flies appeared as a smear along the tube (Figure 1A, B, filled arrow) and generated a rectangular area on the intensity profiles (Figure 2, yellow shading). Resting flies generated local bright spots (Figure 1A, B, empty arrow) that were detected as peaks on the profile (Figure 2, purple shading). Contrast-enhanced images were used to detect the bright spots of resting flies; thereafter, raw data, processed only to subtract local background camera signal, was used for all quantitative measures. For each 300 sec frame and for each fly our analysis partitioned time according to the area under the “mean without peaks” value on the intensity profile (yellow) versus the area under the peaks (purple). The sum of all the yellow and purple segments was equivalent to 300 sec for each frame and for each fly.

Because the absolute intensity of the bioluminescence decreased exponentially with time, due to decay of the luciferin, the data was detrended so that the signal had the same mean

1 and amplitude across the 7-day time course (Levine et al., 2002a, b). This peripheral
2 molecular clock signal was then plotted for the population alongside contemporaneous
3 observations of frame-by-frame quantification of activity and sleep (Figure 3A). The
4 conventional sleep binary plot (Figure 3A, sleep binary) depicted total sleep along the time,
5 but failed to demonstrate the sleep episode fluctuation. Further processing of the data
6 allowed a measure of sleep consolidation to be generated (Figure 3B) and facilitated the
7 visualization of circadian oscillation in sleep episode length. The sleep consolidation plot
8 indicates that sleep episodes of over 3 hrs are commonplace in the early part of the
9 subjective night (marked with black bars on x-axis, Figure 3C). Later in the night discrete
10 episodes of 40-50 min are seen but by contrast during the day only shorter naps are seen
11 (<30 min). This pattern is the first clear visualization that *Drosophila* shows daily
12 fluctuation in sleep quality and is reminiscent of the structure of human sleep where deep,
13 slow wave sleep is apparent particularly in the early part of the night whereas rapid eye
14 movement (REM) sleep predominates towards morning (Wulff et al., 2010).

15 16 **Comparison of FLYGLOW with existing experimental paradigms**

17 We then compared the actimetric performance of FLYGLOW with the widely-adopted DAM
18 beam-break-counting apparatus (Rosato and Kyriacou, 2006) (TriKinetics Inc., MA USA). We
19 used two populations of flies, the first were strongly rhythmic control flies and the second
20 pan-neuronally expressed toxic amyloid β peptide - a model for Alzheimer's disease
21 (Figure 4A). Previous work has shown that the $A\beta$ expressing flies show progressive
22 behavioral dysrhythmias as they age despite their central molecular clock remaining
23 relatively intact (Chen et al., 2014). Considering the data for control flies (left panels), as
24 expected the DAM system underestimates locomotor activity as compared to FLYGLOW.
25 In the DAM profile the underlying oscillation in behavior has relatively low amplitude with
26 narrow bursts of beam breaking. By contrast the FLYGLOW profile is less noisy with
27 higher amplitude rhythmic signal, since it misses far fewer false negatives i.e. movement
28 without beam-breaking (consistent with a recent video tracking system, Gilestro, 2012).
29 The $A\beta$ -expressing flies exhibit markedly weaker rhythmicity as compared to controls
30 (right panels), appearing essentially arrhythmic in the DAM system but retaining
31 detectable oscillation in FLYGLOW. The quality of each fly's behavioral rhythmicity may be
32 quantified by calculating a time-delayed auto-correlation statistic (RS value, Levine et al.,
33 2002a). In this case we saw that both DAM and FLYGLOW rank $A\beta$ -expressing flies as less
34 rhythmic than controls ($p < 0.001$ for both techniques, Figure 4B). Notably however the RS
35 values are higher overall for FLYGLOW as compared to DAM ($p < 0.05$) indicating that the

1 new method may be more sensitive at detecting behavioral rhythms than the existing
2 approach. When we examined the distribution of RS values in the population of flies we
3 see that the ability of FLYGLOW to detect very highly rhythmic flies accounts for the bulk of
4 the performance gain (Figure 4C).

5
6 Next we compared FLYGLOW molecular clock detection with the performance of the
7 existing approach in which flies are constrained under small plastic domes within the wells
8 of a 96 well microtiter plate (Stanewsky et al., 1997). We compared our new approach
9 with this current method to exclude the possibility that the extra freedom of movement
10 seen in FLYGLOW could add a systematic artifact of the system: for example the availability
11 of the luciferin substrate might be less in the tubes and result in artifactual changes in
12 bioluminescence. As shown in Figure 4D, the two approaches generate essentially
13 identical bioluminescence traces; moreover the fly-by-fly rhythmicity analysis indicates
14 that the RS values are also very similar.

15 16 **FLYGLOW simultaneous comparison of molecular and behavioral oscillations**

17 Three principal datasets are routinely generated by the FLYGLOW system; these are, i) the
18 molecular clocks in peripheral tissues as reported by total bioluminescence, ii) percent of
19 time spent in locomotor activity and iii) sleep consolidation. These are conveniently
20 presented as mean data for a population of flies (Figure 5A). Control flies aged 23-
21 30 days (left column, n=36) show high amplitude molecular oscillations with gradual
22 dampening (Veleri et al., 2003) that peak in the early part of the subjective night and retain
23 their circadian rhythm throughout the 7-day experiment. For flies expressing the A β
24 peptide (right column, n=32 flies) the molecular rhythms are also well preserved although
25 there is more deterioration from day 4 onwards.

26
27 For control flies, the percent of time spent in activity is also highly circadian. In these
28 controls FLYGLOW can clearly distinguish two activity peaks, the first occurring just before
29 subjective dusk, when flies spend up to 40% of their time moving, and the second just
30 before dawn when they are moving approximately 30% of the time. The locomotor
31 behavior for A β -expressing flies exhibits a low-amplitude, less regular rhythm that can be
32 characterized as a state of continuous restlessness throughout the subjective day and
33 night.

34
35 As expected, the sleep consolidation plot is highest when the locomotor activity is least,

1 with at least a superficial resemblance to the deep sleep (non-REM) that humans tend to
2 manifest earlier in the night. Specifically, the duration of the average sleep episode in the
3 early night, when control flies spend <5% of their time moving, is remarkably high, at more
4 than 2 hrs. During the morning of the subjective day the flies take short naps of up to
5 30 min at a time when they exhibit significant locomotor activity (20-30% time spent
6 moving). Sleep episodes are at their shortest during the late afternoon peak in locomotor
7 activity. The inset indicates that A β -expressing flies (red), similar to patients with AD, have
8 longer sleep episodes than control flies (black) during the subjective day.

9
10 Our qualitative interpretation of population behaviors (Figure 5A) was confirmed
11 objectively by calculating rhythmicity statistic values (RS values). We found that for all
12 three datasets the RS values are significantly higher in control flies than in their A β -
13 expressing counterparts (Figure 5B). Remarkably these changes in rhythmicity do not
14 cause any statistically significant difference in the relative proportions of sleep (sum of
15 rests longer than 5 min), activity and rest (sum of rests shorter than 5 min) (Figure 5C).
16 Thus the differences in sleep relate entirely to its temporal organization and not its total
17 amount.

18
19 The plotting of representative data from individual flies may also be instructive (Figure 5D).
20 For example, many A β flies exhibit chaotic behavior (panels I & II) however it is not
21 uncommon in this context to see ongoing molecular rhythms. At one extreme an
22 individual A β fly may lose rhythmicity in all three signals (panel III) while the majority of
23 control flies remain rhythmic by all three parameters throughout a week long recording
24 (panel IV). Using paired statistics, we found that behavioural rhythms showed greater
25 deterioration in individual A β flies than molecular oscillations (Figure 5B).

26 **Dissecting the mechanistic links between the various rhythms**

27
28 Our single fly measurements permit powerful analysis of correlations between the quality
29 and the period of the three rhythmic datasets. Indeed the simultaneous observation of
30 multiple rhythms within a single fly allows the use of paired statistical tests, something
31 that is not possible when comparing separate population-based measurements.
32 Considering rhythm quality, if the RS values of two rhythms are correlated then one may
33 conclude they are coupled, that is that they share a common clock mechanism. For
34 example when one of the rhythms is robust in a particular fly one expects that the other
35 will also be similarly robust, and vice-versa. Our systematic three-way analysis of RS

1 correlations in control flies showed that there is no evidence for coupling between the
2 peripheral molecular clock and either of the two behavioral phenotypes (Figure 6A: activity,
3 panel I & sleep, panel II). By contrast the RS values of the two behaviors were correlated
4 (panel III, $p < 0.05$), indicating that locomotor and sleep rhythms are both influenced by the
5 same central clock mechanism, but presumably *via* different output pathways.

6
7 A similar three-way comparison of rhythm periods in individual flies, revealed correlations
8 between the peripheral molecular clock and both of the behavioral rhythms (Figure 6B,
9 panels I & II). This is despite the fact that the RS values are not correlated and the periods
10 of the rhythms are not equal. That their periods *are* correlated implies that these
11 oscillations share underlying mechanisms because, in the context of slight variability
12 between individuals, factors that modify the period of one rhythm tend to also vary the
13 period of the correlated rhythm i.e. they are co-variant. Strikingly, the periods of the
14 sleep and activity rhythms do not correlate (panel III), indicating that sleep rhythms are
15 influenced by additional mechanism such as homeostatic regulation and possibly even
16 local brain oscillations in the fly arousal/sleep centers that are not tightly coupled with the
17 central clock neurons (e.g. Kunst et al., 2014; Wu et al., 2008).

18 19 **The peripheral molecular clock and behaviors progressively desynchronize in the** 20 **dark**

21 While the periods of the behavioral rhythms and peripheral bioluminescence rhythm are
22 correlated, surprisingly they are not equal (Figure 6B I & II; average periods for behaviors in
23 magenta, 23.6-23.8 hr & molecular clock in blue, 22.8-22.9 hr). Because the molecular
24 clock oscillates faster than either of the behaviors in constant darkness, we observed the
25 marching of this rhythm across the behaviors during the course of the experiment (Figure
26 6C I & II). In these circumstances, when we plotted the phase difference between the
27 molecular clock and activity (Figure 6D, panel I) we saw a progressive reduction in phase
28 difference as the molecular signal caught up with the behavior. Likewise (panel II) the
29 phase difference increased as the molecular clock progressively moved ahead of the sleep
30 rhythm. By contrast the sleep and activity rhythms had the same period and so
31 maintained a stable phase relationship (panel III).

32 33 **Discussion**

34 Not only do circadian rhythms have profound roles to play in biology, they are also
35 recognized as important factors in human disease. On one hand environmental

1 disruption of circadian rhythms increases the risk of, for example, obesity and diabetes
2 while on the other hand some diseases are accompanied by circadian deficits from an early
3 stage (Rakshit et al., 2014; Reddy and O'Neill, 2010). Neurodegenerative disorders, not
4 least AD, are characterized by sleep-wake abnormalities, indeed it is night-time confusion
5 and wandering that often makes institutional care inevitable in such cases (Wulff et al.,
6 2010). Considering a recent finding that any use of (benzodiazepine) sleeping tablets is
7 linked to a 50% increase in AD risk, it is apparent that circadian deficits become
8 symptomatic early, as part of a dementia prodrome (de Gage et al., 2014). As AD
9 progresses, patients will exhibit marked sleep fragmentation and loss of the normal
10 day/night partitioning of sleep. A consequence of this can be a state of continual
11 restlessness, often worse in the early evening when the syndrome is termed sundowning
12 (Coogan et al., 2013; Hatfield et al., 2004; Volicer et al., 2001).

13
14 *Drosophila melanogaster* is a model organism that phenocopies key aspects of the
15 deterioration in circadian/sleep organization associated with human aging and
16 neurodegeneration. Our inability to longitudinally monitor multiple behavioural and
17 molecular markers in individual animals however, has prevented the full potential of the fly
18 as screening tool from being realized as researchers try to understand the etiology of
19 complex diseases such as AD.

20
21 We have therefore developed FLYGLOW in order to achieve our goal of understanding the
22 causal relationship between molecular clock oscillations, behavioral and sleep rhythms, in
23 health and disease. Our advance relies upon the synergistic optimization of multiple
24 existing technologies. Specifically we used an exquisitely sensitive EM-CCD camera
25 mounted upon a light-tight, environmentally controlled cabinet. The camera collected
26 exposures of a duration that matches the current working definition of fly sleep, and thus
27 by looking at the distribution of the light signal, per tube per exposure, fly movement
28 during each 5 minute window could be robustly inferred. FLYGLOW also relies upon a fly
29 strain expressing a particularly bright, peripherally-expressed clock gene::luciferase
30 reporter, and fed very high luciferin concentrations which are necessary to sustain
31 bioluminescence during sleep (when flies do not eat). Finally the development of new
32 mathematical tools for the analysis of this type of time lapse imaging was essential in
33 allowing us to explore the concept of sleep consolidation in a fly model for the first time.
34 Our spatial tracking combined with measurement of total bioluminescence facilitated the
35 quantification of multiple phenotypic parameters from an array of individual organisms

1 allowing us to assign simultaneous molecular, locomotor and sleep events over a 7 day
2 period. Our approach compares very favorably with current alternative technologies, that
3 are limited by only measuring one of these parameters in any given experiment (sleep,
4 activity, or bioluminescence). This has forced researchers to compare populations of flies,
5 rather than individuals and thus loses the power of intra-individual correlational analyses
6 over time. Importantly FLYGLOW could equally be employed for more broadly based
7 behavioral genetic and drug-based screens, beyond sleep/wake or neurodegeneration
8 research.

9
10 Our initial observation was that, as a population, the flies show complex patterns of sleep
11 and locomotor behavior that remain synchronized in the constant dark. Sleep
12 consolidation reached remarkably high levels in the early night, with episodes of 2-3 hours
13 being commonplace. In flies expressing the $A\beta_{42}$ peptide this high quality “deep” sleep
14 was lost early and instead was partitioned more uniformly across the day, resulting in
15 longer daytime naps. These changes in sleep structure strikingly resemble the deficits
16 seen in patients with AD (Coogan et al., 2013; Wulff et al., 2010). Interestingly there was
17 no overall change in the relative amounts of rest, sleep and activity in the control and $A\beta_{42}$ -
18 expressing flies indicating a change in the structure rather than the amount of sleep
19 between the two groups.

20
21 Remarkably, the molecular clock in peripheral tissues was more robust than its behavioral
22 counterparts. Echoing our previous observations of the central molecular clock
23 becoming uncoupled from behavior in $A\beta_{42}$ -expressing flies (Chen et al., 2014), this study
24 also revealed many examples of flies exhibiting robust molecular oscillations
25 contemporaneously with behavioral arrhythmia. This recurring observation of molecular-
26 behavioral dissociation suggests that one approach to re-entraining disturbed behavioral
27 rhythms may be to enhance the output of the central neuronal clock.

28
29 An important conclusion of this study is that single-fly observations of multiple circadian
30 phenotypes allow us to use RS and period correlations to dissect the causal relationships
31 between circadian oscillations. The ability to use paired statistical tests greatly increases
32 the power of such comparisons. Despite this, we observed no correlation between the RS
33 values for the peripheral molecular clock and either of the behavioral rhythms. This
34 confirms that there is no global peripheral molecular-behavioral coupling in our system as
35 suggested previously by a number of investigators (Giebultowicz et al., 2000; Hege et al.,

1 1997; Plautz et al., 1997a). The contrasting finding that RS values for sleep consolidation
2 and locomotor behavior are indeed correlated indicates that these rhythms are generated
3 by a shared central clock.

4
5 Similar studies of period correlations between rhythms have allowed us to detect
6 oscillations with shared mechanisms despite their having no direct coupling. Thus our
7 observation that there is a period correlation between the peripheral molecular clock and
8 both behaviors is more notable because not only do the periods differ by almost one hour
9 but also because there is no corresponding RS correlation. A corollary of this finding, and
10 one confirmed experimentally, is that the molecular and behavioral signals desynchronise
11 in the dark as the shorter period molecular oscillation marches across the behavioral
12 rhythms. The intriguing absence of a period correlation between sleep and locomotor
13 activity points to more complex regulation of sleep/wake that may respond to other
14 homeostatic and regulatory signals (Cirelli and Bushey, 2008; Gilestro et al., 2009; Myers et
15 al., 2003; Naidoo, 2009). Similar dissociation occurs in humans during forced
16 desynchrony (14 hr:14 hr, L:D) when circadian physiology becomes decoupled from sleep-
17 wake cycles. Such observations however have never been possible in *Drosophila* before.

18
19 **In conclusion we present FLYGLOW: a new broadly applicable approach that for the**
20 **first time allows the causal relationships between molecular and behavioral circadian**
21 **rhythms to be dissected by simultaneous molecular and phenotypic observations in**
22 **individual flies. Since the running costs of our method are low, but is amenable to**
23 **high-throughput genetic screens, we expect it to furnish novel insights into the**
24 **etiology of complex age-related human diseases such as Alzheimer's disease.**

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34 35 **Author contributions**

1 D.C.C., K.-F.C. and J.S.O'N. designed the FLYGLOW strategy. K.-F.C. and J.S.O'N. performed
2 the experiments. E.K. built the image processing/analysis pipeline. K.-F.C., E.K., J.S.O'N.,
3 and D.C.C. analyzed the data. D.C.C. and K.-F.C. wrote the manuscript with crucial inputs
4 from E.K. (Bioinformatics) and J.S.O'N. (Circadian Biology). D.C.C. conducted the project.

5 **Competing financial interests**

6 The authors declare no competing financial interests.

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1 **Figure Captions**

2 **Figure 1.** A *Period-luciferase*-expressing fly allows simultaneous measurement of the
3 molecular clock and behavioral phenotypes. (A) Panel I: *Period-luciferase XLG-Luc2*
4 construct schematic containing period promoter, 5'UTR and full-length period exons and
5 introns. Tss: transcription start site. Panel II: Stylized cartoon to illustrate that wild type
6 flies generate a higher *Period-luciferase* signal and less locomotor activity during the
7 subjective night, resulting in bright stationary spots in the digital image (upper section).
8 During the subjective day the overall magnitude of bioluminescence is reduced and more
9 evenly distributed along the length of the tube due to movement of the fly (lower section).
10 (B) A representative frame from the digital camera. Three circles (grey circles) at the
11 corners of the tray were attached as landmarks for image orientation. Total pixel
12 intensity projections along the y-axis (upper plot) and the x-axis (right plot) were used to
13 calculate the boundaries of each tube in pixels for all images. Active flies (filled arrow)
14 and those at rest (blank arrow) are apparent (see also Figure 2).

15 **Figure 1-figure supplement 1.** Design of fly housing arena. (A) Example image of plastic
16 microscope slide tray, housing glass capillary tubes and the paper spacer. 30 cm ruler is
17 used as scale indicator. (B) Illustration of the customized arena with the fixed spacer and
18 circle markers (see Materials and Methods). (C) Bright field image taken by EM-CCD camera.
19 Three paper circles (grey circles) at the corners of the tray were attached as landmarks for
20 image rotation.

21
22 **Figure 2.** Frame-by-frame measurements of molecular clock and behavioral status. Pixel
23 intensity was plotted and binned into 40 segments along the length of the tube for each
24 image (red boxes). The enhanced images (left column) permitted sensitive feature
25 detection and were used to detect bins containing peaks of intensity: The mean pixel
26 intensity and standard deviation (SD) for all bins in a tube were calculated. Peaks in the
27 enhanced data were assigned when the intensity of a bin exceeded the mean+SD for that
28 tube. Using the raw images the value of the mean bin intensity, excluding bins
29 containing peaks (green boxes), was calculated (the Mean-peak value). The yellow area
30 below this value represents the light emitted by moving flies. The purple area under the
31 peaks represents resting flies. The total of these areas is equivalent to 300 sec.
32 Representative data is presented for frames containing an active fly (A), an active fly
33 exhibiting periods of rest (B) and a sleeping fly (C). The total pixel intensity within a
34 particular tube indicated the level of the *Period-luciferase* construct and hence the status of

1 the molecular clock.

2 **Figure 3.** Time course of molecular and behavioral measurements for a single fly. **(A)** The
3 bioluminescence intensity of a representative single fly during seven days of constant
4 darkness reported the oscillation of the molecular clock (Molecular clock). The percentage
5 of time spent active (Locomotor activity) was calculated and the sleep status (Sleep binary)
6 was assigned to a frame as described in the Materials. **(B)** Each frame containing a fly that
7 had been immobile for over 300 sec was assigned a value of 1 in the binary sleep array.
8 All other frames were assigned a value of 0. The sleep consolidation array contained, for
9 each sleep frame, the total length of the encompassing sleep episode. **(C)** This
10 measurement of sleep consolidation exhibited circadian oscillation. X-axis in (A) and (C)
11 indicates circadian time in hours (Time, h) with subjective day (grey) and night (black)
12 indicated. The recording started from subjective dusk (circadian time=1200 hr).

13
14 **Figure 4.** Comparison of FLYGLOW with conventional methods for population
15 measurements of locomotor activity and molecular clock rhythms. **(A)** The mean
16 locomotor activity for control (n=14) and A β -expressing flies (n=13) was assessed using the
17 DAM system (y-axis: percentage of the maximum beam breaks per 30 minutes) and
18 FLYGLOW (n=20 for control and n=22 for A β -expressing flies). The x-axis represents
19 circadian time in hours (Time, h) with subjective day (grey) and night (black) indicated. The
20 recording started from subjective dusk (circadian time=1200 hr). **(B)** The robustness of
21 locomotor rhythmicity was determined by calculating RS values. Rhythms were
22 significantly stronger in the control flies as compared to A β -expressing flies (2-way ANOVA,
23 p<0.001). The RS value of the FLYGLOW data as a whole was higher than that of DAM (2-
24 way ANOVA, p<0.05). **(C)** The frequency distribution of RS values from FLYGLOW (n=42) is
25 significantly different compared with those from DAM (n=27) (χ^2 -test, p<0.05). FLYGLOW
26 was particularly sensitive at detecting highly rhythmic locomotor behavior (RS>4.0). **(D)**
27 Left panel: Comparing the mean bioluminescence rhythms from FLYGLOW (n=20,
28 magenta) with flies observed in domed 96-well plates (n=8, blue) indicates that the
29 molecular clock signals are essentially identical. Right panel: There are no significant
30 differences between RS values of the molecular clock rhythms between FLYGLOW
31 (magenta) and the classical approach (blue).

32
33 **Figure 5.** Comparing the rhythmicity of control and A β -expressing flies. **(A)** The population
34 means of *XLG-luc2* signal intensity (Molecular clock), percentage of time spent active
35 (Activity) and sleep episode length (Sleep consolidation) were plotted for control (n=36),

1 and A β -expressing (n=32), flies. The insert indicates that daytime sleep episodes were
2 longer in A β -expressing flies (red) as compared to controls (black). **(B)** All three rhythms
3 are significantly less robust in A β -expressing flies (red dots) as compared to controls (black
4 squares) as determined by comparison of RS values by matched two-way ANOVA.
5 Repeated measures one-way ANOVA was used to identify statistical differences in RS
6 values for various pairs of rhythms within each genotype. The same alphabets are
7 indicated for the group with no difference in the RS values ($p>0.05$), e.g. the average RS
8 value of the intensity rhythms (a) in the control flies are the same to those of the sleep
9 rhythms (a), but are different from those of the activity rhythm (b). **(C)** Despite the loss of
10 rhythmicity seen for A β -expressing flies, there were no significant differences in the
11 relative partitioning of time between sleep, activity and rest ($p>0.05$, by χ^2 -test or matched
12 2-way ANOVA). **(D)** Panels I & II: Individual recordings for A β -expressing flies with weak
13 behavioral rhythms (activity in magenta and sleep consolidation in blue) and more
14 robustly rhythmic molecular clock oscillations (black). Panel III: representative A β -
15 expressing fly with non-rhythmic molecular clock and behavioral signals. Panel IV:
16 representative control fly with robust molecular and behavioral rhythms. Sleep
17 consolidation amplitudes are normalized to the longest sleep episode. X-axis in (A) and
18 (D) indicates circadian time in hours (Time, h) with subjective day (grey) and night (black)
19 indicated. The recording started from subjective dusk (circadian time=1200 hr)
20

21 **Figure 6.** RS and period correlation analysis: dissecting causal relationships. **(A)** The paired
22 RS values for the various rhythms were plotted for each fly. Using only control flies there
23 was no correlation between RS values for molecular clock (Intensity) and locomotor
24 activity (panel I) or sleep consolidation (panel II). By contrast the RS values for locomotor
25 activity (Activity) and sleep consolidation (Sleep) rhythms were correlated (panel III,
26 $p<0.05$). Only flies rhythmic for both tested signals were considered, resulting in varying
27 n-values between panels. **(B)** The paired signal periods for the various rhythms were
28 plotted for each fly. Using only control flies we found that the periods of locomotor
29 activity (panel I) and sleep consolidation (panel II) rhythms were both correlated with the
30 period of the molecular clock (both $p<0.05$). By contrast there was no correlation
31 between the periods of the sleep consolidation and locomotor activity rhythms (panel III).
32 The average periods of the behavioral rhythms (magenta) both differed significantly from
33 the shorter molecular clock period (blue). **(C)** The population data for the corresponding
34 pairwise rhythm comparisons were plotted. The molecular rhythm had a shorter period
35 than either behavior rhythm (panels I & II) however the respective behavioral rhythms had

1 very similar periods and retained a fixed phase relationship (panel III). X-axis indicates
2 circadian time in hours (Time, h) with subjective day (grey) and night (black) indicated. The
3 recording started from subjective dusk (circadian time=1200 hr). **(D)** Pairwise
4 observations also allowed the calculation of phase differences between rhythms across the
5 time course. There is a systematic reduction in phase difference as the molecular rhythm
6 “catches up” with locomotor activity (panel I); by contrast the phase difference increases as
7 the molecular rhythm marches away from the sleep consolidation rhythm (panel II).
8 There is no overall change in phase difference when comparing the two behavioral signals
9 (panel III). The phase of each rhythm was calculated for a 3-day window. The start times
10 of each window are separated by half a day. The grey lines connect the half-daily
11 estimates of phase difference for each fly. The red lines indicate linear regressions that
12 have a gradient that is significantly different to zero ($p < 0.01$). The significance of the RS
13 correlations was determined by the *Pearson r* with $p < 0.05$ (A, B). The significance of the
14 correlation between period differences *Paired t-test* with $p < 0.01$ (B).

Figure 1

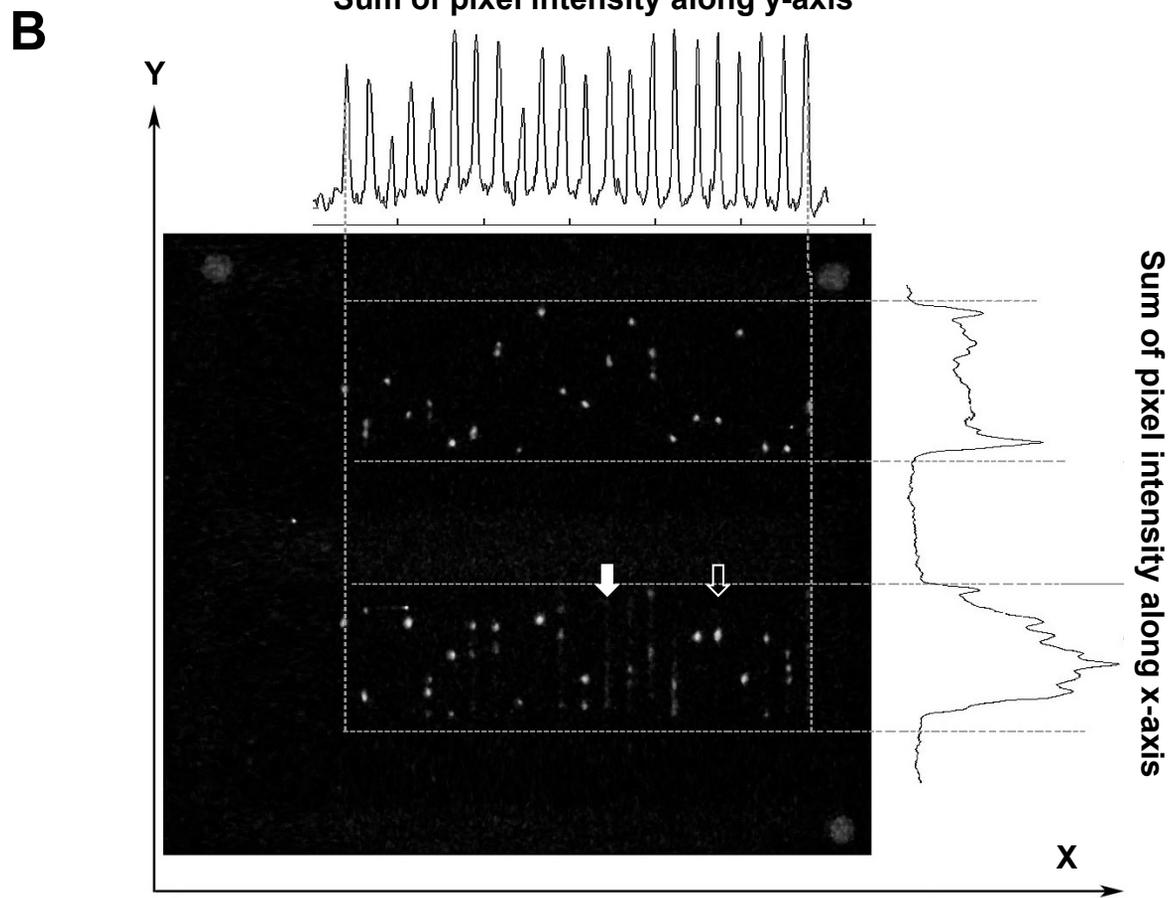
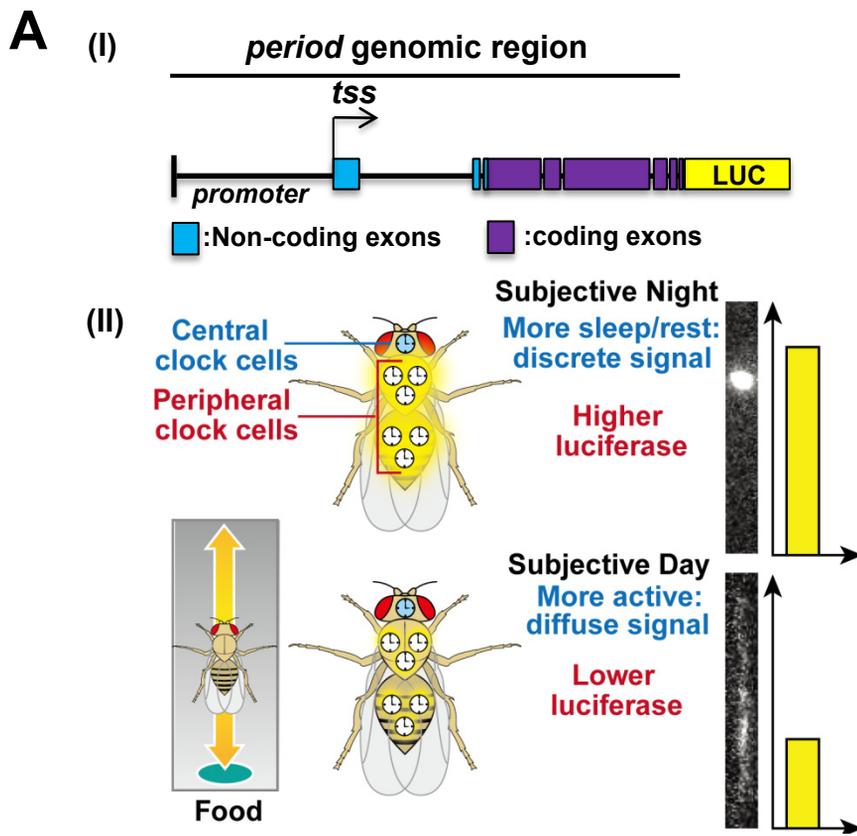
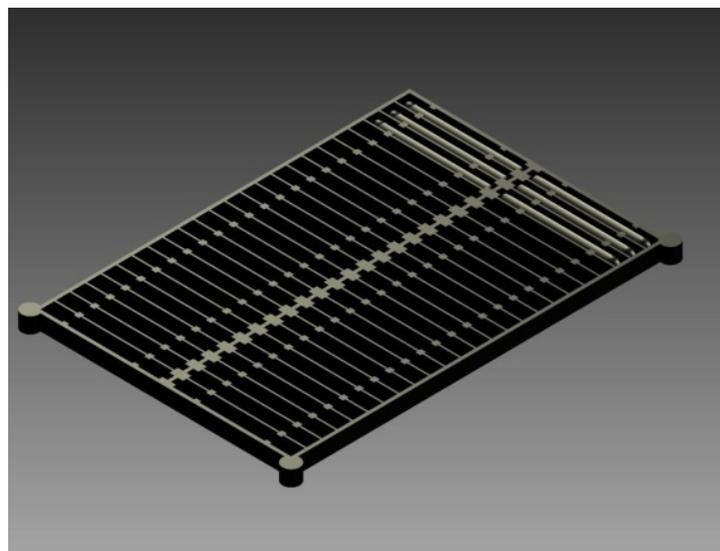


Figure 1-figure supplement 1

A



B



C



Figure 2

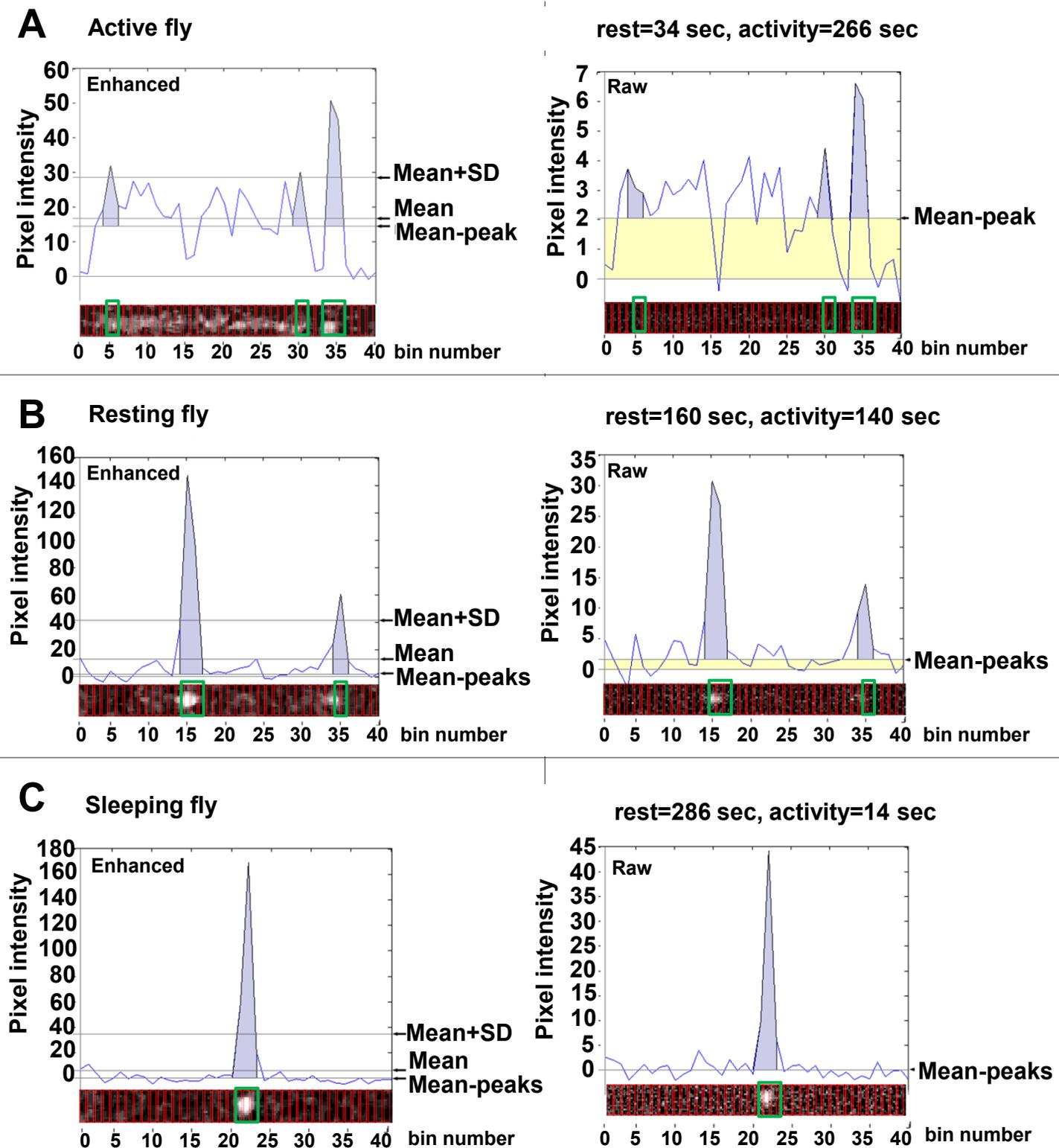
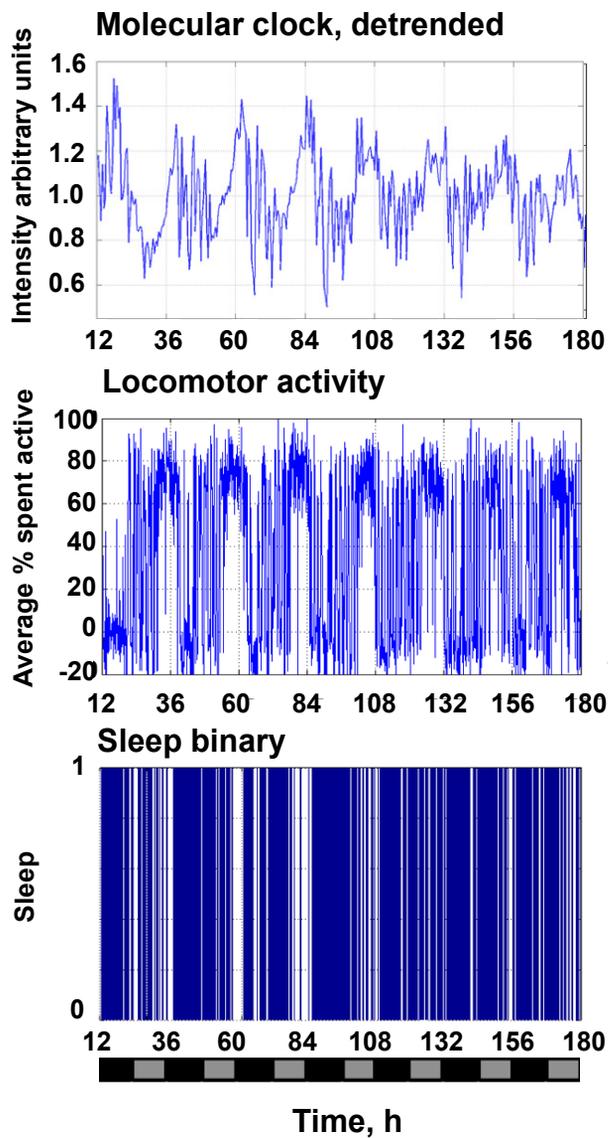
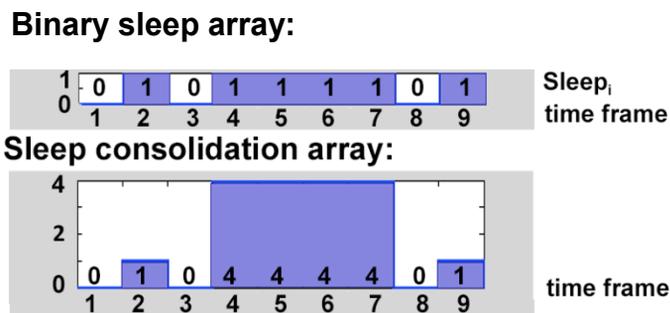


Figure 3

A



B



C

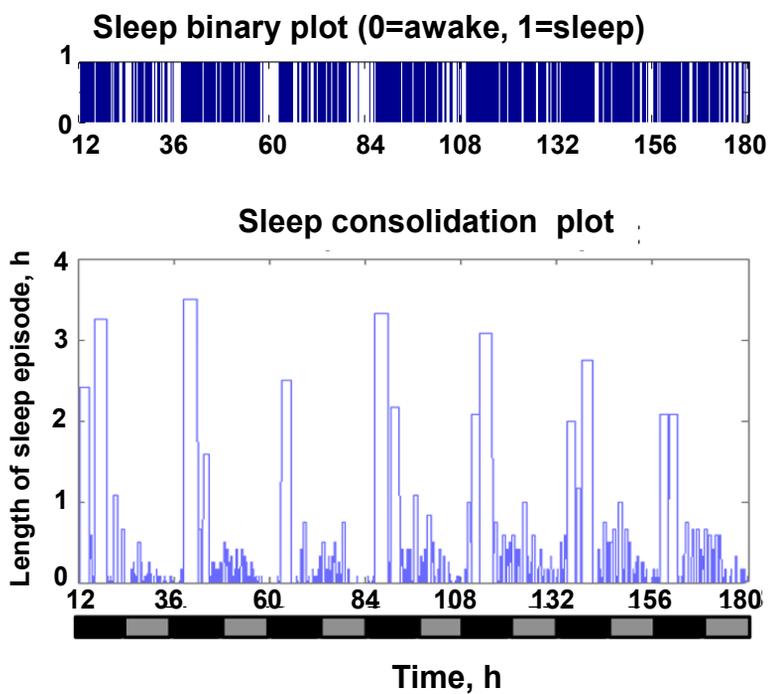


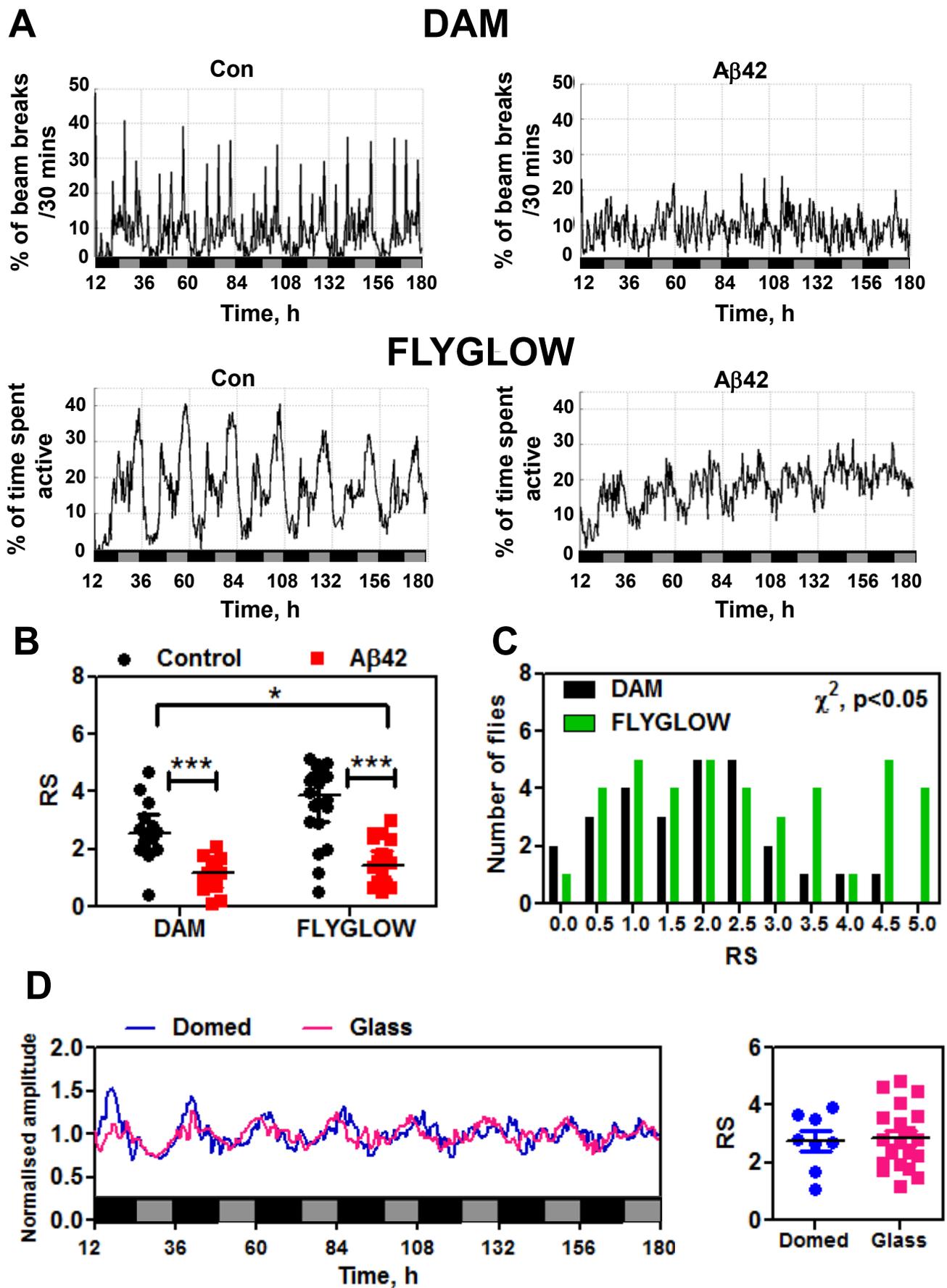
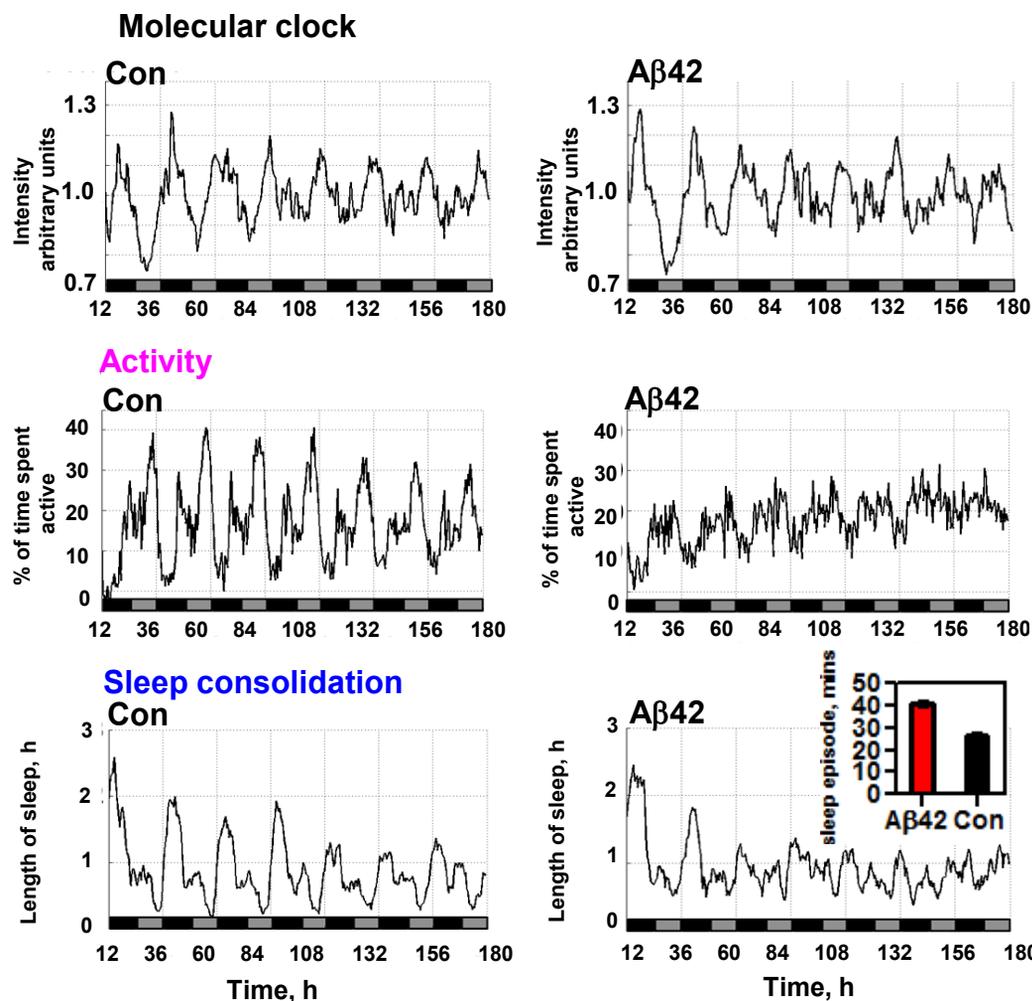
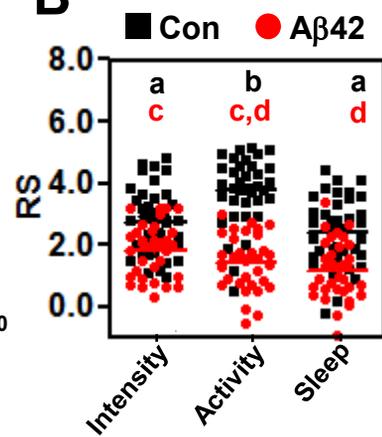
Figure 4

Figure 5

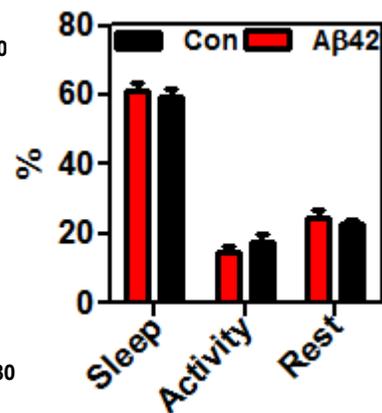
A



B



C



D

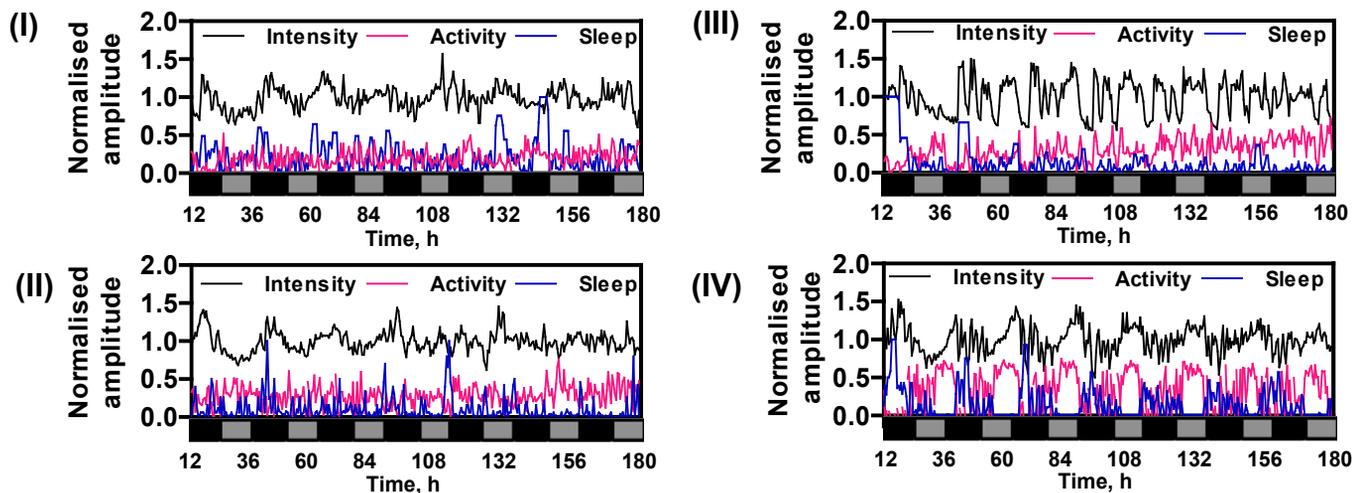


Figure 6

