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Drosophila circadian rhythms in semi-natural environments; summer afternoon component is not an artifact and requires TrpA1 channels.

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Under standard laboratory conditions of rectangular light dark cycles and constant warm temperature, Drosophila melanogaster show bursts of morning (M) and evening (E) locomotor activity and a ‘siesta’ in the middle of the day. These M and E components have been critical for developing the neuronal dual oscillator model in which clock gene expression in key cells generates the circadian rhythms under semi-natural European summer conditions which replaced the laboratory

The study of laboratory generated circadian locomotor activity patterns of Drosophila, played a critical role in determining how fruity (and mammalian) clocks function. However recent observations of fly activity in the wild challenged many assumptions about how the clock might work. A new prominent summer locomotor component emerged called ‘A’ (afternoon), which replaced the laboratory ‘siesta’. The A component has been criticised by others to be an artifact, but our study here shows that it is genuine and is observed under a variety of simulated natural conditions. The A component is temperature and clock-dependent and is generated by expression of the internal thermosensor TrpA1, revealing a novel pathway for environmental input to the clock.

Reserved for Publication Footnotes

1+ These authors contributed equally to this work.

Significance

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Fig. 1. - Natural simulations in the laboratory support the existence of an A component. A: Flies recorded in the wild (n=9 male HU recorded 28/07/2007, day mean temperature = 27.8°C, max light 542 lux) clearly reveal the additional and major afternoon (A, Black) peak of activity (mean activity ± SEM). B: Standard laboratory conditions at constant 30°C, LD16:8 (32 male HU, LD 18.6:700 lux) reveals no A peak. C: Step-free semi-natural simulation reveals M, A and E peaks of activity, whether measured in tubes using TriKinetics monitors (C, n=32) or virtual beam crossing analysis (D, n=9, plotted as mean ± SEM of 3 replicates), or measured in open field arenas (E, total distance travelled by 4 male and 4 female flies, averaged across 3 replicates).

whereas the more dorsal clock neurons (LNdS and DNs) produce the E component (6, 7).

While Vanin et al focused predominantly on the phases of the major locomotor components under natural lighting and thermal conditions (3), in a similar natural study, Menegazzi et al suggested that although per null mutants look similar in their behavioral phasing to wild-type, the A peak tends to be larger in perr12 mutants (4). These authors suggested that PER normally serves to reduce the amount of ‘inappropriate’ activity that occurs during the warmest part of the day (4). While their results were based on a very small sample of flies on a few days of recordings, they were nevertheless welcomed in that they revealed that possessing a wild-type clock appeared to be behaviorally adaptive compared to having a severely disturbed clock.

Another study performed under semi-natural conditions at tropical latitudes has questioned the validity of the A component (8). These authors suggest instead that A represents a behavioral artifact as a result of flies avoiding the midday sun by sheltering in the shaded part of the glass activity tube where the TriKinetics infra-red detectors are located, leading to inappropriate triggering of the sensor and high activity counts. In apparent support of this model they observed that flies in open field Petri dish arenas did not show an A component under summer conditions, though this interpretation has been criticised (9), in part because Petri dishes are well known to be problematic for Drosophila open field behavioral recordings (10).

Given the interest generated by Vanin et al (3), we have revisited these natural studies and extended them with more sophisticated simulations of natural temperature and light cycles in the laboratory. By using video recordings of fly circadian activity in glass tubes and open field arenas we investigate whether the A component is an artifact. Furthermore, in both the Vanin et al (3) and the Menegazzi et al (4) studies, the classic per mutants were congenic with each other but were compared to three different wild-type strains so genetic background was not controlled. Using congenic controls we re-examine whether we can observe a phenotype for arrhythmic mutants in simulated semi-natural conditions. Finally we study the A peak in a range of photoreceptor and thermoreceptor mutants in order to investigate the underlying genetic and neuroanatomical basis for this novel summer element of circadian behavior.

Results

The A component is not an artifact

Fig 1A shows the locomotor profile of HU wild-type flies using TriKinetics monitors recorded in the wild on an Italian summer’s day with naturally varying temperature and light cycles (max 840 lux, mean temperature 29.7°C). Fig 1B illustrates the results from HU flies in the standard laboratory paradigm at a constant temperature of 30°C in rectangular 700 lux light-dark cycles (LD16:8). The main difference between the two figures is the presence of the A (afternoon) component. By simulating a warm Italian midsummer day using smooth changes in temperature (25-35°C, Fig S1A) and light intensity (max 500 lux, Fig S1B) we were able to induce an activity profile with clear M, A and E components (Fig 1C) very similar to that observed in the wild (Fig S1C-D). As in the wild, the A peak is not prominently expressed with a 20-30°C thermal cycle (Fig S1E-F).

De et al (2013) suggested that the A component is an artifact because on warm sunny days the flies seek the shaded area between the emitter and detector in the TriKinetics DAM2 recording system, thereby over-activating the infrared beam which generates the activity counts. Although Vanin et al recorded their data in completely shaded conditions we addressed this issue by mounting the glass activity tubes from TriKinetics onto an unshaded white background, and recorded infrared video of their
activity under semi-natural conditions (depicted in Fig S2). Using
ActualTrack™ software we simulated a 'virtual' light beam across
the centre of each tube, and counted the number of times flies
crossed this beam. Our results show that monitoring the flies in
this manner results in activity records with clear M, A and E
components (Fig 1D), contradicting the suggestion that the A
component is an artifact of shade within the TriKinetics DAM2
system.

De et al further claimed that observations of flies’ open field
behavior in Petri dishes showed an absence of the A compo-
nent, implying that the A component might only be observed
under the restricted spatial environment of the glass tubes (and
shade) inherent in the TriKinetics system. We recorded the ac-
tivity of groups of four male and four female flies in open field
chambers developed by the Dickinson laboratory (11), and used
ActualTrack™ software to determine the total movement of flies
recorded under infrared light for 5 of every 30 min under un-
shaded semi-natural conditions. Again the results clearly show the
A component as the major part of the locomotor activity profile
under simulated warm summer conditions, with M and E com-
ponents providing smaller contributions (Fig 1E). Consequently,
the A component is observed in TriKinetics monitors, in isolated
glass tubes and in open field chambers (Fig 1A,C-E); indeed De
et al’s incorrect conclusion was based on a misinterpretation of
their own data (see Discussion).

Do arrhythmic mutants show any locomotor phenotypes in
semi-natural conditions?

Menegazzi et al suggested that the amplitude of the A
component could be modulated by clock mutations (4). We re-
interrogated the extensive Vannin et al database (3) by dividing
the data into those segments that represented M activity (02:30
to 08:00), A activity (08:00 to 16:30), and E (16:30 to 22:00) with
night activity (N) falling between 22:00 and 02:30. Data were
expressed as a % total daily activity falling within these seg-
ments and all data were taken from Italian summer recordings be-
tween Jun 19-Sept 3. We selected days in which the maximum temper-
ature exceeded 31°C expecting to observe a strong A response
and correlated each locomotor component with maximum daily
temperature (Fit 2).

Fig 2A shows the relationship of % M activity with the
day into those segments that represented M activity (02:30
to 08:00), A activity (08:00 to 16:30), and E (16:30 to 22:00) with
night activity (N) falling between 22:00 and 02:30. Data were
expressed as a % total daily activity falling within these seg-
ments and all data were taken from Italian summer recordings be-
tween Jun 19-Sept 3. We selected days in which the maximum temper-
ature exceeded 31°C expecting to observe a strong A response
and correlated each locomotor component with maximum daily
temperature (Fig 2).

Fig 4. – The effects of knockdown or overexpression of TrpA1 on the
A peak of locomotor behavior.A: Knockdown of TrpA1 using TrpA1Gal4
is sufficient to recapitulate lack of evening peak. Knockdown: TrpA1Gal4/
TrpA1-IR023461 (n=30), Control: TrpA1Gal4/+ (n=30). B: TrpA1 is
required in neurons to give afternoon peak. Knockdown: elavGal4;
UASDicer2; TrpA1-IR023461 (n=36), Control: +/+TrpA1-IR023461 (n=30),
mutant: TrpA1-IR023461 (n=26). C: timGal4 knockdown of UAS-TrpA1 has
no effect on the A component. Knockdown: UASDicer2, timGal4; UASTrpA1-
IR023461 (n=32), Control: UASDicer2, timGal4; mCherry (n=28), mutant: TrpA1-
IR023461 (n=30). Data scaled to maximum daily peak, mean ±sem.
We further investigated the effects of light and temperature in our semi-natural incubator paradigm by examining the behavior of backcrossed mutant strains (to HU) with restricted abilities to sense their environment.

Under simulated warm summer conditions, mutants with a compromised photo-transduction pathway (left hand panels of Fig 3), either as a result of the morphological loss of photoreceptor cells as in glass 

or the double mutant glass crystal 

(Fig 3A,C), a deficient Phospholipase C-8 as in nopt 

(Fig 3D) or in the cation-specific calcium channel trp 

(Fig 3E), exhibited a relative reduction in the amplitude of the M and E components compared to HU, with a corresponding increase in A (Fig S3), also includes statistical analysis. crystal circadian blue-light photoreceptor mutants in contrast displayed robust M, A and E peaks and under these conditions and were not significantly different from HU (Fig 3B, Fig S3A). We also examined the effect of

mutations in genes known to contribute to temperature sensing in the range 25-40°C (right hand panels of Fig 3), including the Trp channels TAP1, painless and pyrexa, the temperature entrainment mutant nocte, and the gustatory receptor paralogue Gr28b required for rapid negative thermotaxis.

The most dramatic differences observed between the M and E components were significantly suppressed compared to A, and TAP1 mutants in which the A component was largely eliminated (Fig 3F-I, Fig S3).

Reported expression of TAP1 in clock neurons is not required for the A peak

It has been reported that as well as being expressed in a number of brain regions, TAP1 is also expressed within some of the cells that make up each sub-cluster within the LNv and DN clock neurons. Consequently we knocked down TAP1 expression using RNAi using different Gal4 drivers. Knockdown of TAP1 using either a TAP1gal4 (Fig 4A) or the pan-neuronal enhancer trap elavGal4 (Fig 4B) was sufficient to mimic the behavior of TAP1 mutants with a complete lack of A component. However, TAP1 knockdown in clock neurons using timGal4 did not recapitulate the lack of an A peak, even when co-expressing UAS-Dicer2 to enhance the potency of the RNAi (Fig 4C). Thus it would appear that limiting TAP1 knockdown to the clock cells does not reduce the A component.

Discussion

Among several unexpected results of the semi-natural studies of locomotor activity of Vanin et al, the most attention has been generated by the novel finding that flies are highly active under warm natural conditions during the afternoon, giving rise to the A component. This observation was at odds with conventional laboratory studies at constant warm temperatures of 25°C or above that reveal that flies take a siesta in the afternoon, a phenotype that has been associated with per alternative splicing in a number of studies.

De et al proposed that the A component is an artifact of the flies seeking the shaded part of the TriKinetics monitors in which the infra-red detector is situated. According to them, flies ‘fidgeting’ while they are stationary within light beam, generates spurious activity counts. We have shown conclusively using analysis of video recordings in both unshaded glass tubes and in open field arenas that the A component forms a major part of the circadian activity profile under summer conditions, fully consistent with the TriKinetics semi-natural recording of Vanin et al. Our use of the open field arena developed by Simon and Dickinson gave very different results to De et al’s use of Petri dishes. The use of the latter for these kinds of observations is problematic because flies exhibit exploratory responses at the circumference of such chambers, rather than open field behavior, and in doing so
Montell described T'rP'A1 as an important nociceptor for both heat (24) and light (25). Lee & transient receptor potential channel previously implicated as an which the A component was effectively eliminated. TrpA1 is a the most dramatic response was observed in primary effect may be on A, so that under summer conditions, compared to the HU congenic controls, this suggests that their appears that the observation by Menegazzi et al that arrhythmic effect on summer activity is simply due to the flies lacking a clock, is curious that per does not do the same. Consequently, it appears that the observation by Menegazzi et al that arrhythmic mutants may be unable to suppress the A component to the same extent as wild-type (4) may be generally correct, but this effect is significantly modulated by interactions with the genetic background and perhaps by the behavioral paradigm in which it is studied.

In addition, we studied the relative levels of M, A and E in flies carrying mutations in photo- and thermo-reception path- ways. The levels of M, A and E are somewhat interdependent because the individual components contribute to the total activity (or night-time) so as one component is elevated, another may be sup- pressed. Nevertheless, natural summer simulations revealed that glass, trp and norpA and the double mutant glass cry blunted the expression of M and E peaks and led to significant elevation of A (FigS3). These observations resonate with Vanin et al’s (3) results in semi-natural conditions in which the onset of the M component appeared to be a highly temperature-dependent response to the twilights with little clock input. As the absolute levels of A were significantly higher in mutants of trp, norpA and glass cry compared to the HU congenic controls, this suggests that their primary effect may be on M, so that under summer conditions, visual photoreceptor input suppresses the A component. Of the mutants that are known to be implicated in thermal sensing, pxy suppressed both M and E components but left A intact, whereas the most dramatic response was observed in T'rP'A1 mutants in which the A component was effectively eliminated. TrpA1 is a transient receptor potential channel previously implicated as an important nociceptor for both heat (24) and light (25). Lee & Montell described T'rP'A1 expression within each subset of the canonical clock neurons (17) so we determined whether expres- sion of T'rP'A1 in clock cells was required to mediate the A peak.

Down-regulation of T'rP'A1 using the timgal4 driver, enhanced by UAS-Dicer2 had no effect on the amplitude of the A peak so it appears that T'rP'A1 in clock neurons is unlikely to contribute to the A component. TrpA1 expression was initially found to be limited to a few brain cells, the sub-oesophageal ganglion and eight cells in the thoracic ganglion (26, 27). Two pairs of AC neurons expressing TrpA1 appear to be the main internal thermosensors but they also integrate temperature information from peripheral sensors (28). The AC sensors are activated by TrpA1 at ~25°C but a second response is observed at 27°C which is generated by pxy-expressing neurons located in the second antennal segment and which synapse onto the AC neurons (28). Interestingly, when we used the pxy mutant in our behavioral assay, we found no effect on the A component, mirroring the observation that pxy is also not required in a temperature preference assay (28), but we did observe a significant suppression of M and E. Painless is also expressed in the antennae, but again we did not observe any effect on the A component in pain mutants. The rapid warm response peripheral receptor Gr28b(d) which is located in the aristae (16) was also not required for the A component but, like pxy, suppressed M and E. We conclude that the peripheral sensors encoded by pxy, and Gr28b(d) may be involved in setting levels of M and E in circadian locomotor summer responses, but are not relevant to the A component. The circadian temperature entrainment mutant noc4 is also largely irrelevant to the summer locomotor profile, but the effects of norpA which has similar temperature entrainment phenotypes to noc4 are almost certainly due to its role in photoreception (15).

Modulation of the phase of the A component in per mutants has been observed by Vanin et al (3) and under some summer conditions by Menegazzi et al (4). One possible explanation is that in per (and per) mutants, the earlier A phase may simply represent a phase advance in the mutants for sensing the daily increase in temperature (4). As well as the four TrpA1 positive AC neurons that appear to act as internal thermosensors (26, 27), other TrpA1 positive cells also lie in dorsal regions in the vicinity of the DN clock cells (27). It remains to be seen whether any of the non-clock expressing TrpA1 neurons such as the AC or dorsal neurons have direct connections to the clock cells and if so, what the polarity of these interactions might be. It could be imagined that if clock cells send signals to the thermal sensors (or vice versa), then that might generate the phase changes that are observed in the A component in per mutants under natural conditions (3, 4).

In conclusion, the study of semi-natural circadian behavior in D. melanogaster initiated by Vanin et al (3) raised some interesting challenges to the canonical model of the clock developed under strictly artificial laboratory conditions. De et al’s (8) suggestion that the A component is an artefact has been shown to be manifestly incorrect, both by our experiments, and by scrutiny of these authors’ own results which they appear to have badly mis-interpreted. Instead, we suggest that the molecular and physical basis of the A component appears to reside within the TrpA1 internal thermosensory neurons rather than those canonical clock neurons that may express TrpA1 nor the peripheral antennal temper-ature sensors. However all three locomotor components can be modulated by mutation in the photoreceptor and peripheral thermoreceptor pathways and the challenge will be to dissect the neuroanatomical pathways by which these sensors interact with clock cells (6, 7). In conclusion, the study of circadian behavior in semi-natural conditions in mammals (1, 2) and in flies (3, 4), as well as the modelling of natural circadian data (29) can inform and refine the current models of how clocks work at behavioral, ecological, anatomical and molecular levels.

Materials and Methods
Fly strains:

Flies were raised at 25°C in LD12:12 cycles. Congenic male per

and per

mutants were backcrossed for 6 generations to a wt1118 that had itself been previously backcrossed for 10 generations to the wild Houten (HU) strain, isolated from the Netherlands in 2005 and maintained as isofemale lines (30). As per maps very close to w, we followed the per allele in each backcross generation by eye color and confirmed the final strains behaviorally in circadian locomotor assays. After 6 generations of backcrossing into HU, the residual genetic variation is 1/64 or less than 2%. All Gal4 lines had also per

reversion to wt1118 over 4 generations. Other mutant lines first had appropriate chromosomes replaced with those of HU using balancers, and then backcrossed to HU for two further generations before behavioral observations were made.

Outcrossed glass

(31), cry

, npas

(32) and cry

glass

double mutants from existing laboratory stocks, trp

(stock #5692) (33), painless

(34) (stock #27895), Gr288b (stock #24190) and TephA

(stock #26304) were obtained from the Bloomington Drosophila Stock Center.

Note: cry

and pyk

were gifts from Raj Stanovsky (UC, London).

Behavioral observations:

Flies were anaesthetised with CO

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and loaded into the experimental arena with a 10 mm glass bean breaking experiment, male flies were loaded in 10 cm long glass tubes, sealed with maize food and rubber bungs on one end and cotton wool at the other. Open field experiments used groups of four male and four female flies in 12 cm diameter circular arenas (11) with a central core of maize food (Fig 52). Activity arenas were placed into incubators and flies were allowed to recover and entrain to semi-natural conditions for at least 1.5 days before observations were made.

Natural light and temperature simulations:

We used a Memmert IPP500 peltier programmable incubator to smoothly cycle temperature and mimic a midsummer's day in northern Italy. We generated a reference temperature profile by taking the normalised temperature cycle of midsummer recordings (3). Relative daily levels of the M, A and E locomotor components were calculated as in Menegazzi et al (4) to generate a measure of amplitude for each component by dividing up the day and taking the proportion of total daily activity (including night time activity) that fell into each 30 min bin. The spectral composition of the light matched that of natural midsummer light by combining outputs of 6 groups of LEDs with different emission spectra. Temperature was cycled to peak 2.5 h later than the light cycle peak, thereby mimicking natural summer recordings (3). Relative daily levels of the M, A and E locomotor components were calculated as in Menegazzi et al (4) to generate a measure of amplitude for each component by dividing up the day and taking the proportion of total daily activity (including night time activity) that fell into the corresponding daily segments.

Acquisition and analysis of video data:

Activity videos (1280x720 resolution at either 15 or 30 fps) were recorded under infrared lighting using a Logitech c300e webcam, modified to be sensitive only to light >850nm. To assess virtual beam crossings in unshaded conditions, 8, 10 cm glass tubes (same tubes as TriKinetics) were placed horizontally to white background. 3 innate the incubator. The ActualTrackTM software divided the tube into two equidistant zones, and the number of fly movements from one zone into the other was tracked providing a measure of virtual beam crossings. Five minute long videos were recorded every 30 min across a two and a half day period. For all flies on both backgrounds we calculated a daily mean number of SEM for each 30 min bin and for the genotype mean and SEM was calculated. When we re-interrogated our natural data from the Vanin et al study (3), as each day is different in terms of the environmental variation, we calculated the mean daily mean and SEM activity (in 30 min bins) for each group of males. Statistical analysis was performed using Prism 6.05 (GraphPad Software Inc).

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