ARTICLE

A mechanosensory pathway to the *Drosophila* circadian clock

Alekos Simoni[1,2†], Werner Wolfgang[1†], Matthew P. Topping[3], Ryan G. Kavlie[4], Ralf Stanewsky[1,5], Joerg T. Albert[3,4,5]**

[1] School of Biological and Chemical Sciences, Queen Mary, University of London, London, E1 4NS, UK

[2] Present address: Department of Life Science, Division of Cell & Molecular Biology, Imperial College London, South Kensington, London, SW7 2AZ, UK

[3] Centre for Mathematics and Physics in the Life Sciences and Experimental Biology (CoMPLEX), University College London, Gower Street, London, WC1E 6BT, UK

[4] The Ear Institute, University College London, 332 Gray's Inn Road, London, WC1X 8EE, UK

[5] Department of Cell and Developmental Biology, University College London, London WC1E 6DE, UK

*To whom correspondence should be addressed. Email: r.stanewsky@ucl.ac.uk and joerg.albert@ucl.ac.uk

†These authors contributed equally.

Abstract:
Circadian clocks attune the physiology of virtually all living organisms to the diurnal cycles of their environments. In metazoan animals, multiple sensory input pathways have been linked to clock synchronization with the environmental cycle (entrainment). Extrinsic entrainment cues include light and temperature. We show that (12h:12h) cycles of vibration and silence (VS) are sufficient to synchronize the daily locomotor activity of wildtype *Drosophila melanogaster*. Behavioral synchronization to VS cycles required a functional clock and functional chordotonal organs and was accompanied by phase-shifts of the daily oscillations of PERIOD protein concentrations in brain clock neurons. The feedback from mechanosensory, and particularly proprioceptive, organs may help an animal to keep its circadian clock in sync with its own, stimulus-induced activities.

One Sentence Summary
Mechanical entrainment of the *Drosophila* circadian clock is mediated by sensory feedback from proprioceptors.

Main Text
The neurocellular network that adjusts an organism's physiological needs to the diurnal fluctuations of its environment is summarily referred to as the *circadian clock* (*1*). The tasks associated with the operation of circadian clocks are computationally challenging. In metazoan animals, clock synchronization requires integration of inputs from different sensory modalities, of which light and temperature changes provide major cues.

Chordotonal organs (ChOs) have been linked to temperature entrainment of the circadian clock in adult flies (*2*). ChOs are internal mechanoreceptors mediating proprioception and the detection of air- and substrate-borne vibrations (*3, 4*). If signaling from ChOs provides sensory input for the entrainment of the fly's circadian clock to temperature cycles (*2*), we reasoned that exposure to a rhythmic mechanical stimulus (see Fig. 1A,B for stimulus details) that excites the fly's ChOs (see Fig. S1 for response details) might phenocopy temperature entrainment (*5*) and be sufficient to synchronize the clock, and clock-controlled locomotor behavior. To test this, we first entrained adult wildtype flies to 12h:12h light-dark (LD) cycles (*6*). Flies were then transferred to constant darkness (DD) and constant temperature (*7*). One group remained in silence to serve as controls (Fig. 1C, top), whereas a second group was exposed to 12h:12h vibration:silence (VS) cycles (Fig. 1C, bottom). In the first, 4-day long, VS regime (VS1), vibration onset was delayed by 6h from light onset in the preceding LD cycles (L+6h). In a second, 5-day long, vibration regime (VS2), vibration onset was then delayed by another 6h (thus now L+12h). At the end of VS2, the flies were released into the final free running (FR) conditions, that is darkness and silence, in which they were kept for another 5 days (Fig. 1C).

During VS1, wildtype flies showed an initial activity peak after vibration onset, which decreased throughout the remaining vibration part (see Figs. 1C and S2). During VS2, flies again showed increased activity immediately after vibration onset, which declined rapidly (Figs. 1C,D and S2). In contrast to VS1, flies now also exhibited increased activity several hours before vibration onset, reminiscent of anticipatory behavior in LD cycles (Figs 1D and S2). To quantify the behavioral activity occurring before vibration onset (i.e., the anticipatory activity component), we determined the ratio of the activity in the 4h time window before the V-phase and the total activity in the S-phase (cf ref. (*8*)). The resulting entrainment index (EI) revealed that anticipatory activity was significantly increased (Fig. 2B). To further probe whether the activity patterns during the VS cycles resulted from a clock-controlled synchronization of behavioral activity, as indicated by the EI calculation, we conducted a phase analysis of the activity peaks in the final free running conditions between flies exposed to VS cycles and controls (Fig. S3) (*7, 9*). The free running activity peaks were in phase with those of the last VS cycle, demonstrating that the circadian clock driving these rhythms had indeed been stably synchronized (see Figs. 1C,D and 2C). In the control group, activity peaks free-ran from the synchronized phase set during the initial LD cycle and hence occurred significantly earlier (mean difference: 4.9h, $P < 0.001$, Watson-Williams-Stevens test; Table S1; see also Figs. 1C,D 2C, S3) than those of the experimental group.

Not all flies synchronized their activity to the vibration cycles (Fig. S4). We therefore assessed each fly's synchronization by inspecting individual actograms without any knowledge about the experimental treatments of the particular fly under investigation. This 'observer-blind' analysis revealed that, across 8 independent experiments, about 53% of all flies (n=312) synchronized to vibration cycles (Table S1). The reasons for this incomplete synchronization are unclear. The vibration stimulus used across our experiments

Page 526 →

was chosen for its experimental reliability (i.e. easy quantifiability and reproducibility, (*7*)) and was not optimized for behavioral efficacy. Two-frequency vibration as used in this study may only excite a certain fraction of the animals' ChOs and other, spectrally more complex, stimuli might prove behaviorally more efficient. Indeed, ablating the animal's antennae, which changes the stimulus perceived by the flies, was already sufficient to increase synchronization rates to about 74% (Figs. 2B,C and S5; Table S1).

To test whether behavioral entrainment to VS cycles requires a functional clock, we performed the above experiment in the background of the per^{01} mutation - a loss-of-function allele of the central clock gene *period* (*10*). On average, per^{01} mutant flies displayed higher activity during the silent phase and lower activity during the vibration phase (Fig. S6); but individual actograms revealed a 'noisy' activity pattern during the entire vibration part compared to constitutively high- or low- activity in silence (Fig. S4). The per^{01} flies did not show anticipatory behavior during VS cycles (Fig. S6), suggesting that this anticipation requires a functional clock. We tried to rescue the VS entrainment in per^{01} flies by introducing a *period* construct (*13.2*) that restores behavioral and molecular rhythms (*8, 11, 12*). EI calculation and 'observer-blind' actogram classification showed that per^{01} *13.2* flies synchronized to the VS cycles (Table S1, Figs. 2A,2B,S4 and S6) with the phase difference between the VS-exposed and control flies differing significantly ($p<0.01$, Fig. S3). Although on average per^{01} *13.2* flies show a 24h period (Table S1), the silent control flies do show a lengthened period during the part of the experiment corresponding to VS2 (DD days 8-12 in panel A of Fig. 2). They thereby acquire a later phase compared to the VS-exposed flies before entry into the final 5 experimental days (used to calculate the phase differences between silence and VS-exposed flies); as a result, the phase relation between the activity curves of experimental and control flies is reversed as compared to CantonS flies (Figs. 2C, S3).

To explore the molecular requirements of vibration-dependent entrainment, we tested flies with mutations in *tilB* (*13*) and *nocte* (*14*), two genes important for both temperature synchronization of the fly's circadian clock and structural integrity of ChOs (*2, 13*). Flies carrying either the loss-of-function allele $tilB^{1}$ or the hypomorphic allele $nocte^{P}$ failed to synchronize to vibration cycles and instead free-ran throughout the experiment (Figs. 2,S5,S7 and Table S1).

If VS cycles synchronize clock controlled behavior, they should also synchronize the oscillations of clock gene products in the neurons driving this behavior. We therefore compared the free-running bioluminescence oscillations in brain clock neurons of two groups of flies initially synchronized to the same LD cycle and expressing a PER-LUCIFERASE (PER-LUC) fusion protein in subsets of their clock neurons (*8, 15*): Experimental flies that had been exposed to 12h:12h VS cycles and control flies that had not been exposed to VS cycles (Fig. 3). Both experimental and control flies displayed circadian oscillations of PER-LUC protein levels in constant conditions (τ=22.7h±0.2h, Fig. 3). In flies exposed to VS cycles, however, the phase of the molecular oscillation was shifted compared to that of flies kept in silence. The observed

Page 527 →

molecular phase shift was sensitive to the phase of the VS entrainment regime: If the VS cycles were delayed ($\Delta t_{[vib]}$=+6h) relative to the initial LD entrainment, the molecular oscillations appeared to be shifted to the left, with the peaks occurring ~5h before those of the free-running control group (Fig. 3, left); if the VS cycles were advanced ($\Delta t_{[vib]}$=-6h), however, the peaks appeared shifted to the right, occurring ~3.5h after those of the controls (Fig. 3, right). Thus, mechanical stimulation can change the phase of the *Drosophila* molecular clock and function as Zeitgeber.

The phenotypes of *nocte*^{P} and *tilB*^{1} mutants, together with the crucial role of chordotonal organs for both mechanical and temperature-dependent circadian entrainment suggest that both modes of entrainment share a common molecular, cellular, and potentially mechanistic, basis. When directly comparing the activities of wildtype flies synchronized to the two different Zeitgebers, the activity peaks in relation to the entraining stimuli acquired similar phases (Fig. S2). At the end of two phase-delayed temperature cycles (TCs), wildtype flies exhibited their main activity peak at the late cold/early warm phase, whereas after comparable VS cycle shifts the main activity peak occurred during the late silent or early vibration phase (Figs. S2,1C,D).

Our results reveal a mechanosensory input pathway to the fly's circadian clock that requires signaling from ChOs. Although external, diurnally fluctuating mechanical stimuli can also act as *extrinsic* Zeitgebers, a circadian pattern of mechanoreceptor activation will inevitably result from every locomotor activity that is patterned in a circadian way. ChOs act as proprioceptors and they are located at almost every joint of the insect body (*3*). The summary output of an animal's ChOs

Page 528 →

would thus be a faithful monitor of its overall (locomotor) activity.

Circadian clocks respond to light and numerous other factors (*16, 17*) including, for example, social interactions (*18, 19*), drug administration (*20*), temperature (*21*) or feeding protocols (*22*), forcing the question of how these are being integrated to compute a central clock time. The finding that - in flies - proprioceptor activation, which accompanies all forms of locomotor behavior, can re-set the clock, offers a potential solution to this problem: All environmental stimuli that lead to changes in the animal's behavior would also impact on the clock by the concomitant changes in proprioceptor activation. A proprioceptive clock entrainment would automatically weight environmental stimuli according to their respective ability to generate locomotion, which is a good indicator of the stimuli's evolutionary importance. An animal's activity can indeed affect its circadian clock (*23, 24*) and some non-photic influences, such as certain drugs, only affect the clock if the animal is free to move (*25*). Proprioceptive feedback to the clock offers a mechanism for such activity-dependent clock entrainment.

Figure legends

Fig. 1. Synchronization of *Drosophila* locomotor activity by vibration-silence (VS) cycles. (**A**) Experimental set-up showing a *Drosophila* activity monitor (DAM) mounted on top of a bass loudspeaker (BLS). Vibrations were monitored using an accelerometer (ACC). (**B**) Example of vibratory stimulus sequence played in loop (bottom: acceleration, middle: velocity, top: displacement). (**C**) (top) Locomotor activity (actogram) of wildtype (CantonS; n=15) control flies that were exposed to initial 12h:12h, light-dark (LD) cycles, followed by complete darkness (DD). (bottom) Actogram of experimental flies (CantonS; n=15) that were exposed to initial 12h:12h, LD cycles, followed by two phase-delayed 12h:12h, Vibration-Silence (VS) cycles (VS1-VS2) in DD, followed by DD without VS. Grey areas: darkness, yellow areas: light, white areas: vibration in darkness. The final 5 days in DD were used to compare the phase of the peak activity during free-run (FR) between experimental and control group. Circadian reference time is given relative to the initial LD entrainment (Lights ON=0). (**D**) Histograms showing the daily activity averages during three different phases for experimental flies (LD, VS2, FR, bottom row; n=75) and control flies (LD, DD, FR, top row; n=72). Error bars represent SEM.

Fig. 2. Requirement of a functional clock and chordotonal organs for synchronization to VS cycles. (**A**) Average locomotor actograms (top: control flies, bottom: experimental flies) for *per*^{01}, *per*^{01} *13.2*, *tilB*^{1}and *nocte*^{P} mutant flies. See Table S1 and (B) for quantification. For stimulus sequence and actogram shadings see Fig. 1. (**B**) Entrainment Index: Quantification of behavioral activity anticipating the V-onset as a measure for entrainment (see text for details). Red dotted line indicates random distribution of activity in the 4h window (*= p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, One-way ANOVA, see ref. (*7*) for details). (**C**) Average locomotor activities for final 5 days in free-running (FR) conditions of experimental flies exposed to VS cycles (vibrated, red) and control flies not exposed to VS cycles (free-running, grey). Solid lines represent sinusoidal fits to the average locomotor data (circles: control flies, triangles: experimental flies). To facilitate appreciation of phase differences, both data and corresponding fit values have been normalized to the maximum of the fit function. The arrhythmic activities of *per*^{01} flies have been normalized to their respective mean value. For raw behavioral data of all genotypes in (C), see panel (A) and Figs. 1C,S4 and S7. CantonS: n=75 (vibrated), n=72 (FR); CantonS w/o antennae: n=28 (vibrated), n=30 (FR); *per*^{01}: n=32 (vibrated), n=32 (FR); *per*^{01} 13.2: n=21 (vibrated), n=22 (FR); *tilB*^{1}: n=24 (vibrated), n=25 (FR); *nocte*^{P}: n=16 (vibrated), n=16 (FR). Error bars represent SEM.

Fig. 3. Phase shifts of PERIOD protein oscillations in central clock neurons caused by rhythmic mechanical stimulation. (**A**) Schematic representation of the environmental conditions before flies were transferred to the bioluminescence counter. For the delay and advance experiment, flies were transferred at ZT20 and ZT8, respectively, relative to the previous vibration onset. For colors and shadings referring to light and stimulus conditions see Fig. 1. (**B**) Normalized bioluminescence activity of free-running 8.0-*luc* flies-, which express a PER-LUC fusion construct in a subset of the clock neurons (*7, 8, 15*) - after exposure to VS cycles (red) and control flies not exposed to VS (grey). VS cycles that were delayed ($\Delta t_{[vib]}$=+6h; experimental flies: n=15, controls: n=11) relative to the initial LD entrainment lead to an advance of the molecular oscillations (left), whereas VS cycles that were advanced ($\Delta t_{[vib]}$=-6h; experimental flies: n=10, controls: n=8) relative to the initial LD entrainment lead to a delay (right) compared to PER-LUC expression of control flies not exposed to VS stimuli. Bioluminescence data was de-trended prior to fitting of a sinusoidal model (solid lines, see ref. (*7*) and Fig. S8 for details). Both data and corresponding fit values have been normalized to the maximum of the fit function to highlight the phase difference. Two additional delay ($\Delta t_{[vib]}$=+6h; experimental flies: n=17, controls: n=25) and one advance ($\Delta t_{[vib]}$=-6h; experimental flies: n=9, controls: n=10) experiments were performed and molecular phase shifts in the same direction as shown in Fig 3 were observed (data not shown). Error bars represent SEM.

References and Notes

1. S. Panda, J. B. Hogenesch, S. A. Kay, *Nature* **417**, 329 (May, 2002).
2. H. Sehadova *et al.*, *Neuron* **64**, 251 (Oct, 2009).
3. L. H. Field, T. Matheson, in *Advances in Insect Physiology, Vol 27*. (Academic Press Inc, San Diego, 1998), vol. 27, pp. 1-228.
4. M. J. Kernan, *Pflugers Archiv-European Journal of Physiology* **454**, 703 (Aug, 2007).
5. F. T. Glaser, R. Stanewsky, *Cold Spring Harb. Symp. Quant. Biol.* **72**, 233 (2007).
6. M. J. Hamblen-Coyle, D. A. Wheeler, J. E. Rutila, M. Rosbash, J. C. Hall, *J. Insect Behav.* **5**, 417 (1992).
7. Materials and methods are available as supplementary materials on Science Online.
8. C. Gentile, H. Sehadova, A. Simoni, C. H. Chen, R. Stanewsky, *Curr. Biol.* **23**, 185 (Feb 4, 2013).
9. J. D. Levine, P. Funes, H. B. Dowse, J. C. Hall, *Bmc Neurosci* **3**, (Jan 18, 2002).
10. R. J. Konopka, S. Benzer, *Proc Natl Acad Sci U S A* **68**, 2112 (Sep, 1971).
11. Y. Citri *et al.*, *Nature* **326**, 42 (Mar 5-11, 1987).
12. D. M. Zerr, J. C. Hall, M. Rosbash, K. K. Siwicki, *J Neurosci* **10**, 2749 (Aug, 1990).
13. R. G. Kavlie, M. J. Kernan, D. F. Eberl, *Genetics* **185**, 177 (May, 2010).
14. F. T. Glaser, R. Stanewsky, *Curr Biol* **15**, 1352 (Aug 9, 2005).
15. S. Veleri, C. Brandes, C. Helfrich-Forster, J. C. Hall, R. Stanewsky, *Curr. Biol.* **13**, 1758 (Oct 14, 2003).
16. D. A. Golombek, R. E. Rosenstein, *Physiological Reviews* **90**, 1063 (Jul, 2010).
17. N. Mrosovsky, *Biol. Rev. Camb. Philos. Soc.* **71**, 343 (Aug, 1996).
18. R. E. Mistlberger, D. J. Skene, *Biol. Rev.* **79**, 533 (Aug, 2004).
19. J. D. Levine, P. Funes, H. B. Dowse, J. C. Hall, *Science* **298**, 2010 (Dec, 2002).
20. F. W. Turek, S. Loseeolson, *Nature* **321**, 167 (May 8, 1986).
21. W. F. Zimmerman, C. S. Pittendrigh, T. Pavlidis, *J. Insect Physiol.* **14**, 669 (1968).
22. K. A. Stokkan, S. Yamazaki, H. Tei, Y. Sakaki, M. Menaker, *Science* **291**, 490 (Jan, 2001).
23. N. Mrosovsky, P. A. Salmon, *Nature* **330**, 372 (Nov 26, 1987).
24. F. van Oosterhout *et al.*, *PLoS ONE* **7**, e39693 (2012).
25. N. Mrosovsky, P. A. Salmon, *Chronobiol. Int.* **7**, 35 (Jan 1, 1990).

Acknowledgments

This work was supported by grants from the BBSRC (BB/H001204/1 to R.S. and BB/G004455/1 to J.T.A.), the HFSP (to J.T.A) and the EU FP6 Integrated Project 'EUCLOCK' (to R.S). M.P.T. received funding from the EPSRC (EP/F500351/1). Additional data, including raw data, are presented in the supplementary materials. The authors thank CoMPLEX students W. Ashworth and M. Ransley for their support and P. Dayan from the Gatsby Computational Neuroscience Unit at UCL for valuable discussions.

Supplementary Materials

www.sciencemag.org
Materials and Methods
Figs. S1 to S10
Table S1
References (26-31)