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The *Drosophila* circadian clock is a variably coupled network of multiple peptidergic units

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Abstract

Daily rhythms in behavior emerge from networks of neurons that express molecular clocks. *Drosophila*'s clock neuron network consists of a diversity of cell types, yet is modeled as two hierarchically organized groups, one of which serves as a master pacemaker. Here we establish that the fly's clock neuron network consists of multiple units of independent neuronal oscillators, each unified by its neuropeptide transmitter and mode of coupling to other units. Our work reveals that the circadian clock neuron network is not orchestrated by a small group of master pacemakers but rather consists of multiple independent oscillators, each of which drives rhythms in activity.

Molecular clocks drive circadian rhythms in animals (1). Most circadian rhythms follow from clocks located in small islands of brain tissue (2) and connections within networks of clock neurons produce a robustness in circadian timekeeping uncharacteristic of rhythms driven by isolated neurons or non-neuronal clocks (3, 4). Here we study the clock neuron network of *Drosophila*, similar to yet simpler than that of mammals (5), to learn how networks of clock neurons produce circadian rhythms.

The *Drosophila* brain contains ~150 clock neurons, of which 11 bilateral pairs of lateral neurons are necessary and sufficient for the insect's normal activity rhythms (6) (Fig. S1). Current models suggest that this network is organized into two coupled oscillators: the pigment-dispersing factor (PDF) expressing lateral neurons that control the morning peak of activity and the remaining lateral neurons that control the evening peak of activity (6, 7) (Fig. S1). The dual-oscillator model predicts that the PDF positive neurons serve as master pacemakers that reset the PDF negative neurons daily, thereby dictating the pace of behavioral rhythms in the absence of environmental time cues (8). We tested this prediction by introducing various clock speed discrepancies between the PDF positive and negative clock neurons.

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The authors declare no conflicting interests.

Supplementary Materials

Materials and Methods

Figures S1-S11 (uploaded separately as single .pdf file)

Supplemental Figure Legends

Tables S1-S7 (uploaded separately as single .docx file)

References (20-36)

The intrinsic speed of the molecular clock can be manipulated through the activity of the kinases Doubletime (DBT) and Shaggy (SGG) (9, 10) (Fig. 1A, Fig. S2, and Table S1). Manipulating these kinases only in the PDF positive clock neurons resulted in a coherent change in clock speed in these neurons (Fig. S3), thereby creating clock speed discrepancies between PDF positive and negative neurons. When these discrepancies were small, activity rhythms were strong and coherent with periodicities determined by the speed of the PDF neurons (Fig. 1B, Figs. S4 and S5, and Table S2). When speed discrepancies were larger, flies displayed variable free-running periods, reduced rhythm amplitudes, and a higher incidence of arrhythmicity (Fig. 1B, Figs. S4 and S5, and Table S2). Flies with large discrepancies often displayed two periodicities simultaneously, one corresponding to the period of the PDF neurons and the other to that of the PDF negative neurons (Fig. 1B, Figs. S4 and S5). In flies lacking PDF receptor (PDFR) the speed of PDF neurons had no influence over activity rhythms (Fig. 1C, Fig. S6, and Table S3), indicating that PDFR signaling is required for PDF neuron control over the network. We conclude that the clock neuron network can produce coherent activity rhythms only when the mismatch between the PDF positive and negative neurons is less than approximately 2.5 hours.

The presence of near 24-hour periodicities despite altered PDF neuron speed suggested that, contrary to the prevailing model, PDF negative clock neurons have independent control of activity rhythms under constant darkness and temperature (DD) (6-8, 11, 12). When we altered the clock speed of PDF negative neurons, flies displayed increased arrhythmicity and desynchronization and reduced rhythm amplitudes (Fig. S7), though the effects were less severe than those seen when PDF positive neurons were manipulated (Fig. 2A, Fig. S8, and Table S4)(8). In the absence of PDFR signaling the PDF negative neurons determined the pace of free-running rhythms (Fig. 2B, Fig. S8, and Table S4).

We hypothesized that the phenotypes caused by large clock speed discrepancies (Fig. 1B and Figs. S4, S5, and S7) were caused by conflicts between PDF positive and negative clock neurons, both of which drive rhythms. PDF neurons alone are sufficient to drive activity rhythms (6). We predicted that in the absence of clocks in PDF negative neurons PDF neurons could coherently drive strong behavioral rhythms at any speed. We restored *period* (*per*) expression only in the PDF neurons of *per⁰¹* mutants (6) (Fig. 2, C and E). When such *per*-rescued PDF neurons overexpressed *DBT^S*, flies displayed a strong ~17 h period and showed improved rhythmicity, coherence and rhythm amplitude relative to *DBT^S* overexpression in a *wild-type* background (Fig. 2, C and F, and Table S5). Such improvements were also apparent for *DBT^L* overexpression in *per*-rescued PDF positive neurons (Fig. S9 and Table S5). We conclude that the ~24 h periodicities displayed by desynchronized *per⁺* individuals with fast- or slow-running PDF neurons (Fig. 2D and Fig. S4) were driven by PDF negative neurons.

PDF signaling is required for the PDF neurons to influence the pace of behavioral rhythms (Fig. 1C and Fig. S6), presumably through the resetting of molecular clocks within PDF negative neurons (8), but only about half of the PDF negative clock neurons are predicted to express PDFR (13, 14). Thus, the limited control of the PDF neurons over activity rhythms might be due to a lack of PDF receptivity among PDF negative neurons. We examined PDF receptivity within the PDF negative lateral neurons, a 5th small ventral lateral neuron (5th s-

LN_v) and six dorsal lateral neurons (LN_{ds}) per hemisphere (Fig. S1). The 5th s-LN_v responds to bath-applied PDF with cAMP increases (15). Using the cAMP sensor Epac1-camps (15), (16) we found that approximately half the LN_{ds} do not display cAMP increases in response to PDF, observing both responding and non-responding LN_{ds} within the same brains (Fig. 3, A to D, and G). Restricting the expression of cAMP sensor to the PDFR⁺ LN_{ds} (14, 17) or the PDFR⁻ LN_{ds} (7), we found that all PDFR⁺ LN_{ds} (18 neurons from 7 brains) displayed cAMP responses to PDF (Fig. 3, E and G), while none of the PDFR⁻ LN_{ds} (11 neurons from 6 brains) responded (Fig. 3, F and G). All LN_{ds} responded to forskolin, an activator of adenylyl cyclases (Fig. 3H) (18). Thus, PDF modulates only subsets of PDF negative neurons.

Given such differential receptivity to PDF, we hypothesized that PDF positive neurons reset the molecular clocks only in subsets of PDF negative lateral neurons. We visualized PERIOD (PER) protein rhythms in the lateral neuron network of control flies and flies with a large clock speed discrepancy, in this case flies with the PDF neurons slowed down through expression of *DBT^L*. We chose this manipulation because the internal desynchronization in these flies was usually not accompanied by arrhythmicity (Table S2). In control flies, the PDF positive and negative lateral neurons all displayed similar phases of PER accumulation on day four of constant darkness (DD4) (Fig. 4, A and D). In contrast, there were differences in PER expression between PDF positive and most PDF negative lateral neurons in flies with slow PDF neurons (Fig. 4, B and E). Only two LN_{ds} per hemisphere were synchronized with the PDF neurons (Fig. 4B). These were the two PDFR⁺ LN_{ds} that express short neuropeptide F (sNPF) (Fig. 4, G to J, and Fig. S10) (19). Synchronization of these two neurons to PDF neurons required PDFR signaling (Fig. 4, C and F). Thus, most PDF negative lateral neurons were not reset by the slow PDF neurons (Fig. 4, B and E). These uncoupled neurons were likely responsible for the wild-type periodicities displayed by these flies (Fig. 1B, Figs. S4, S5, and S11). Two of the PDFR⁺ lateral neurons, a single LN_d and the 5th s-LN_v, both of which express ion transport peptide (ITP) (Fig. S11) (19), were synchronized not with the PDF neurons, but rather with the PDFR⁻ LN_{ds} (Fig. 4, B and E), despite their receptivity to PDF. Thus, the slowed PDF neurons reset only two of the seven PDF negative lateral neurons per hemisphere, despite the fact that four out of seven of these neurons are receptive to PDF, revealing that physiological connections between PDF positive and negative neurons do not insure the coupling of their molecular oscillations.

Our results reveal that the PDF negative lateral neurons consist of at least three functionally and neurochemically distinct oscillatory units: Two pairs of sNPF⁺/PDFR⁺ neurons that are strongly coupled to PDF neurons, two pairs of ITP⁺/PDFR⁺ neurons that are less strongly coupled to PDF neurons, and three pairs of PDFR⁻ neurons that are not directly coupled to PDF neurons (Fig. S11). Each of these oscillatory units is unified by its neuropeptide output and characterized by a distinct mode of coupling to the other oscillatory units (Fig. S11). We conclude that the clock neuron network consists of multiple independent oscillators, each capable of orchestrating bouts of activity (fig S11) and that behavioral rhythms emerge from the interactions of many independent oscillators rather than from a unique group of master pacemakers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. The PDF positive clock neurons coherently set free-running periods via PDF signaling over a limited temporal range

(A to C) Scatter plots of the predominant free-running periods of rhythmic flies overexpressing different forms of *DBT* or *SGG* in both PDF positive and negative clock neurons (driven by *Clk-GAL4*) (A), and only in the PDF positive neurons (driven by *Pdf-GAL4*) of *wild-type* flies (B) or *Pdfr* mutants (C). Circles indicate the highest amplitude free-running period for individual rhythmic flies; lines represent mean $\pm$ SEM. *DBT^S*, *DBT^{Short}*; *SGG^{CA}*, constitutively active *SGG*; *SGG^{WT}*, wild-type *SGG*; *SGG^{Hypo}*, hypomorphic *SGG*; *SGG^{KD}*, kinase-dead *SGG*; *DBT^{WT}*, wild-type *DBT*; *DBT^L*, *DBT^{Long}*. Kruskal-Wallis one-way ANOVA reveals a significant difference among groups in (A) and (B) ($p < 0.0001$ for both), but no significant difference among groups in (C) ($p = 0.4829$).

Fig. 2. The PDF negative clock neurons exert independent control over free-running activity rhythms

(A and B) Scatter plots of the predominant free-running periods of rhythmic flies overexpressing *DBT^S*, *SGG^{Hypo}* or *DBT^L* only in the PDF negative neurons (driven by *Clk-GAL4/Pdf-GAL80*) of wild-type flies (A) or *Pdf^r* mutants (B). Kruskal-Wallis one-way ANOVA reveals a significant difference among groups in both (A) and (B) ($p < 0.0001$ for both). (C) Scatter plots of the predominant free-running periods of rhythmic flies with different compositions of PDF positive and negative clock neurons. Specific genotypes are: *per⁺, Pdf>DBT^S* for “Fast PDF+, Norm PDF-”, *per⁰¹, Pdf>PER* for “*per⁺* PDF+, *per⁰¹* PDF-”, and *per⁰¹, Pdf>PER+DBT^S* for “Fast PDF+, *per⁰¹* PDF-”. (D to F) Representative actograms (upper panels) and χ -square periodograms (lower panels) of individual flies with different compositions of PDF positive and negative neurons under constant darkness. Genotypes are as follows: (D) *per⁺, Pdf>DBT^S*, (E) *per⁰¹, Pdf>PER*, and (F) *per⁰¹, Pdf>PER+DBT^S*.

Fig. 3. PDF modulates only half of the PDF negative dorsal lateral neurons

(A) A representative micrograph showing the dorsal lateral neurons (LN_{ds}) from a *Clk>Epac1-camps* fly brain. Four of the six LN_{ds} (labeled 1-4) were present in the optical section. Scale bar, 5 μm. (B to F) cAMP dynamics of LN_{ds} in response to bath-applied 10⁻⁵ M PDF peptide (green triangles). Responses of 45 LN_{ds} imaged from 13 *Clk>Epac1-camps* brains shown in (B) fell into two classes: responsive LN_{ds} (22/45) that displayed large cAMP increases (> 10% change in CFP/YFP ratio) (C), and non-responsive LN_{ds} (23/45) (< 10% changes) (D). The colored traces in (B to D) are from the LN_{ds} shown in (A) circled with the same color as their plots. All PDFR⁺ LN_{ds} (18 neurons imaged from 7 *Mai179>Epac1-camps* brains) displayed significant cAMP increases in response to PDF application (E). None of the PDFR⁻ LN_{ds} (11 neurons imaged from 6 *Clk/cry-GAL80>Epac1-camps* brains) displayed significant cAMP increases (F). (G) Summary of maximum cAMP responses of LN_{ds} to 10⁻⁵ M PDF. NR: non-responsive LN_{ds} from (D); R: responsive LN_{ds} from (C); PDFR⁺ and PDFR⁻ are from (E) and (F), respectively. The letters 'a' and 'b' denote significantly different groups ($p < 0.0001$), by Kruskal-Wallis one-way ANOVA and Dunn's multiple comparisons test. (H) cAMP responses of LN_{ds} to bath-applied 10⁻⁵ M forskolin, a direct activator of adenylyl cyclases. "All" represents forskolin responses of LN_{ds} recorded from *Clk>Epac1-camps* brains, in which the cAMP sensor was expressed in both PDFR⁺ and PDFR⁻ LN_{ds}. The numbers of neurons and brains examined were: All (16, 5), PDFR⁺ (12, 5), and PDFR⁻ (10, 6). NS: not significant, by Kruskal-Wallis one-way ANOVA and Dunn's multiple comparisons test. For all histograms, data are presented as mean ± SEM.

Fig. 4. Physiological connectivity does not ensure molecular clock coupling in the lateral neuron network

(A to C) Immunostaining of PER protein in PDF positive and negative lateral neurons across different time-points on day 4 of constant darkness (DD4). In *UAS-DBT^L* control fly brains, PER accumulated in the PDF positive (s-LN_vs) and negative (LN_ds and 5th s-LN_v) neurons with the same phase (A). In *Pdf>DBT^L* flies in which the PDF neurons were slowed down, only two LN_ds (marked by yellow arrows) were coupled with the s-LN_vs, displaying a shifted phase of PER cycling relative to the other LN_ds (B). In *Pdfr, Pdf>DBT^L* flies, all PDF negative neurons (LN_ds and 5th s-LN_v) had similar phases of PER cycling, and none were coupled to the uniformly delayed s-LN_vs (C). Scale bars, 5 μ m. (D to F) Quantification of PER immunostaining intensity within lateral neurons of *UAS-DBT^L* flies (D), *Pdf>DBT^L* flies (E), and *Pdfr, Pdf>DBT^L* flies (F). The LN_ds in (E) were divided into two groups based on their phase differences and quantified separately: the two LN_ds coupled to the s-LN_vs were quantified as “LN_d (shifted)” and the others as “LN_d (unshifted)”. (G to J) The two shifted LN_ds express neuropeptide sNPF. LN_ds were co-immunostained for PER and sNPF at CT0 and CT12 on DD4 (CT0 and CT12 correspond to the light-on and light-off times had the 12hr: 12hr light: dark cycles continued). In *UAS-DBT^L* control flies, the two sNPF⁺ LN_ds and four sNPF⁻ LN_ds had similar subcellular PER distribution (G) and

expression levels (I) at each of these time-points. In *Pdf>DBT^L* flies, the sNPF⁺ LN_ds differ in their PER distribution (H, yellow arrows) and intensity (J) from the sNPF⁻ LN_ds. Scale bars, 5 μ m. Asterisks in (G) and (H) indicate sNPF⁺ cells that are not clock neurons (lack of PER expression). The letters 'a' and 'b' in (I) and (J) denote significantly different groups ($p < 0.0001$ for both), by Kruskal-Wallis one-way ANOVA and Dunn's multiple comparisons test. Sample sizes are reported in Table S6 for (D to F), and in Table S7 for (I and J).