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Pattern of distribution and cycling of SLOB, Slowpoke channel binding protein, in *Drosophila*

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Abstract

Background: SLOB binds to and modulates the activity of the *Drosophila* Slowpoke (dSlo) calcium activated potassium channel. Recent microarray analyses demonstrated circadian cycling of *slob* mRNA.

Results: We report the mRNA and protein expression pattern of *slob* in *Drosophila* heads. *slob* transcript is present in the photoreceptors, optic lobe, pars intercerebralis (PI) neurons and surrounding brain cortex. SLOB protein exhibits a similar distribution pattern, and we show that it cycles in *Drosophila* heads, in photoreceptor cells and in neurosecretory cells of the PI. The cycling of SLOB is altered in various clock gene mutants, and SLOB is expressed in ectopic locations in *tim⁰¹* flies. We also demonstrate that SLOB no longer cycles in the PI neurons of *Clk^{irk}* flies, and that SLOB expression is reduced in the PI neurons of flies that lack pigment dispersing factor (PDF), a neuropeptide secreted by clock cells.

Conclusions: These data are consistent with the idea that SLOB may participate in one or more circadian pathways in *Drosophila*.

Background

Five independent groups [1-5] recently conducted genome-wide microarray analyses to identify *Drosophila* transcripts that display circadian oscillations. Each group uncovered *slob* as a robustly cycling RNA transcript. SLOB, Slowpoke binding protein, is a key component of the *Drosophila* Slowpoke/SLOB/Leonardo dynamic protein complex [6]. This complex is thought to affect membrane excitability, as electrophysiological recordings reveal that SLOB binding to the channel results in an increase in channel activity, whereas the addition of Leonardo to the

SLOB/dSlo complex dramatically shifts the channel voltage range of activation to more depolarized potentials [6].

The circadian system consists of an input pathway, a central clock, and an output pathway [7]. The clock itself is comprised of the transcription factors CLOCK (CLK) and CYCLE (CYC), which bind to the promoters of *period* (*per*) and *timeless* (*tim*), inducing their expression. The PER and TIM proteins heterodimerize and feed back to repress activity of CLK/CYC. Although the expression patterns of CLK and CYC are not known, the localization of PER and

TIM has been important with respect to identifying neurons relevant for circadian behavioral rhythms [8]. This molecular loop must transduce signals to surrounding cells to generate rhythmic behavior [9]. Altering membrane excitability is a key mechanism for transducing neuronal information, and thus it is reasonable to suspect that the molecular feedback loop might communicate with ion channels.

Ion channels may be under direct transcriptional control of the clock genes or modulated by clock-controlled genes [10,11]. Mounting evidence supports the importance of electrical activity for the propagation of circadian oscillations. For example, electrical silencing of clock neurons through targeted expression of potassium channels stops the oscillation of PER and TIM proteins and causes arrhythmicity in flies [12]. The cyclic release of neuropeptides from clock cells [13] may be a direct consequence of a rhythmic fluctuation in membrane potential [14]. Diurnal modulation of pacemaker potentials and calcium current, intracellular calcium levels and NMDA-evoked calcium currents have all been observed within a mammalian central clock, the suprachiasmatic nucleus (SCN) [11,15,16]. In addition, microarray screens have detected the cycling transcripts of ion channels such as *Shaker*, *trpl* and *slowpoke* [1,3], and flies mutant in *slowpoke* have weak locomotor rhythms [1]. These observations suggest that ion channels and their modulators may participate in circadian regulation.

We explored a role for SLOB in the circadian system and found that SLOB protein cycles in *Drosophila* heads during both light/dark and constant darkness conditions. SLOB oscillates in at least two discrete areas of the fly head, the photoreceptor cells and the PI neurons. The photoreceptors have their own peripheral circadian oscillator [17], whereas the PI neurons, large neurosecretory neurons, are suspected to play a role in the output pathway that drives rest:activity rhythms (Kaneko and Hall, 2000). Our results reveal differential effects of clock mutations on SLOB expression and cycling in these two regions. There is a significant decrease in SLOB levels in the PI neurons of *Pdf⁰¹* flies, thus implicating PDF as an upstream regulator of SLOB. SLOB also no longer cycles in the PI neurons of *Clk^{jurk}* flies, supporting the idea that SLOB is a clock controlled protein. Together with the observation that flies overexpressing SLOB exhibit a breakdown of rest:activity patterns, these data are consistent with the idea that SLOB participates in circadian rhythms.

Results

SLOB protein cycles in *Drosophila* heads

To determine whether SLOB protein cycles, we performed western blot analysis on *Drosophila* head lysates from wild type Canton S (CS) or yellow-white (*y w*) flies entrained

for three days in 12:12 hour light/dark cycles (LD). We found that SLOB protein cycles (Figure 1A) with a peak at Zeitgeber Time (ZT) 10–14, and a trough around ZT 2 (ZT 0 = lights on; ZT 12 = lights off in a 12 hour:12 hour light/dark cycle).

To ensure that rhythms of SLOB expression are circadian and not driven only by external LD cycling we analyzed SLOB protein cycling in conditions of constant darkness. Flies were entrained for three days in LD, and then placed in constant darkness (DD) for two days. SLOB protein still cycles under these conditions, with a slightly dampened amplitude, but with the same overall phase (Figure 1B). A similar decrease in amplitude has been described for oscillations of other clock genes in DD [18,19].

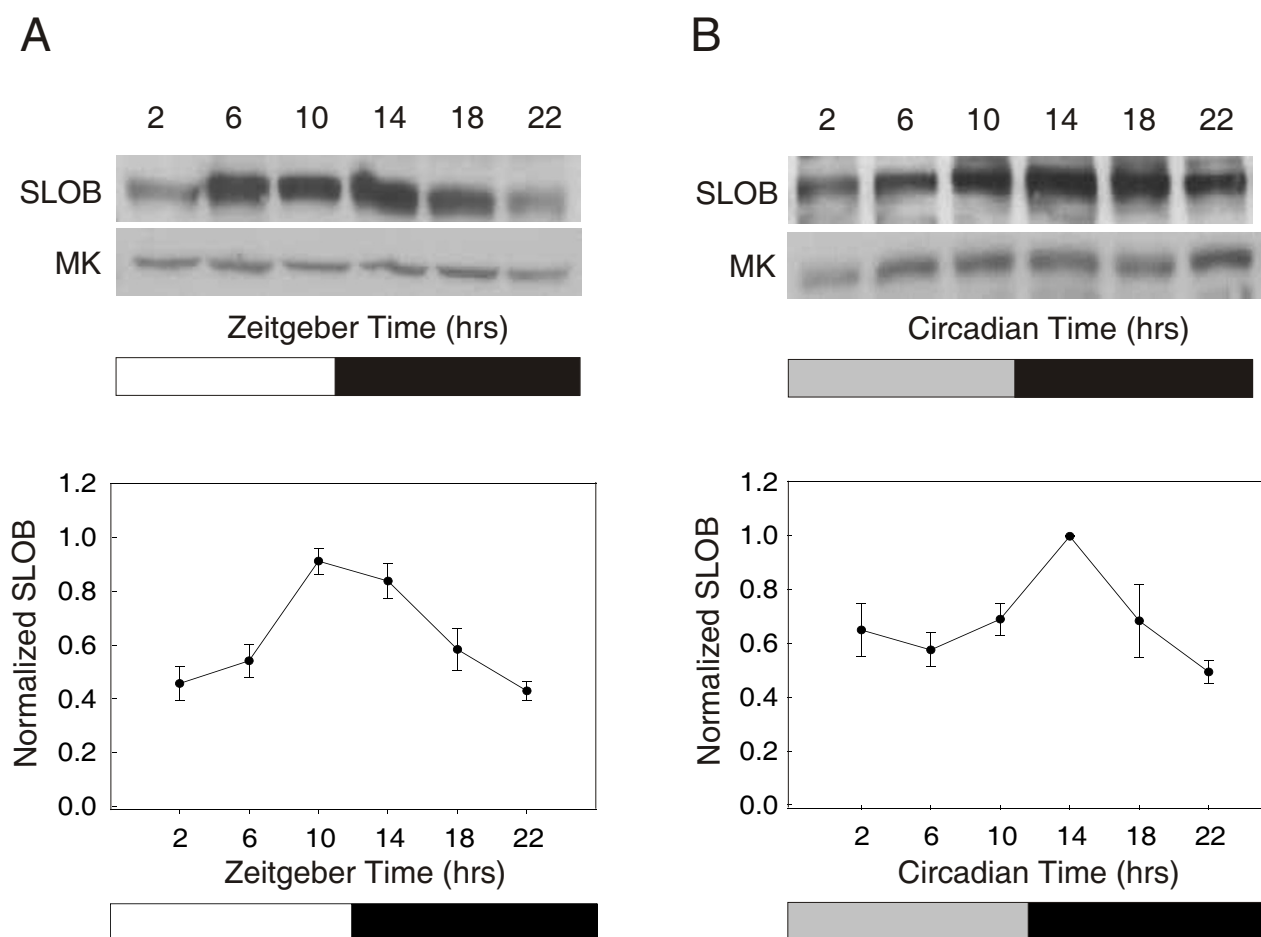
Expression of SLOB protein is influenced by clock genes

To determine if the clock genes control the cycling of SLOB, we assayed its expression in different mutant backgrounds. *Clk^{jurk}* flies, flies mutant for CLK, were entrained in LD cycles for three days and then collected at six time points. Some flies were transferred from LD to DD for two additional days and likewise collected at six time points. Western blot analysis of *Drosophila* heads shows that SLOB protein continues to cycle in *Clk^{jurk}* adults in LD, with a phase and amplitude similar to wild type (Figure 2A). In DD, the western blot analysis shows SLOB expression to have no discernable peak indicating that it does not cycle (Figure 2B). Claridge-Chang et al. [2] noted that *slob* RNA is down regulated in the *Clk^{jurk}* mutant, suggesting that CLK acts as a transcriptional activator of *slob* expression. We did not observe a dramatic decrease in protein levels in *Clk^{jurk}* flies; the level was between the trough and peak levels seen in wild type flies.

The cycling of SLOB was also analyzed in *per⁰¹* and *tim⁰¹* mutants during LD (Figure 2A) and DD (Figure 2C,2D). In both mutant lines, SLOB continues to cycle in LD with a phase similar to that of wild type (Figure 2A). SLOB may be regulated by a direct light-dependent mechanism, obviating the need for these clock genes in LD. As in the case of *Clk^{jurk}*, there appears to be either dampened or no protein cycling in *per⁰¹* flies under DD conditions (Figure 2C). However, in *tim⁰¹* flies SLOB still cycles, but there is a shift in the phase of the oscillation in DD (Figure 2D). Instead of peaking at Circadian Time (CT) 10–14, SLOB now peaks at CT 2. This suggests that the regulation of SLOB by TIM may be different from that by PER, which is not unprecedented [20].

Expression pattern of *slob* transcript in *Drosophila* heads

To determine the expression pattern of *slob* transcript, we performed *in situ* hybridizations on cryosections of *Drosophila* yellow-white (*y w*) fly heads. Digoxigenin-labeled antisense and sense RNA probes of *slob* were used on

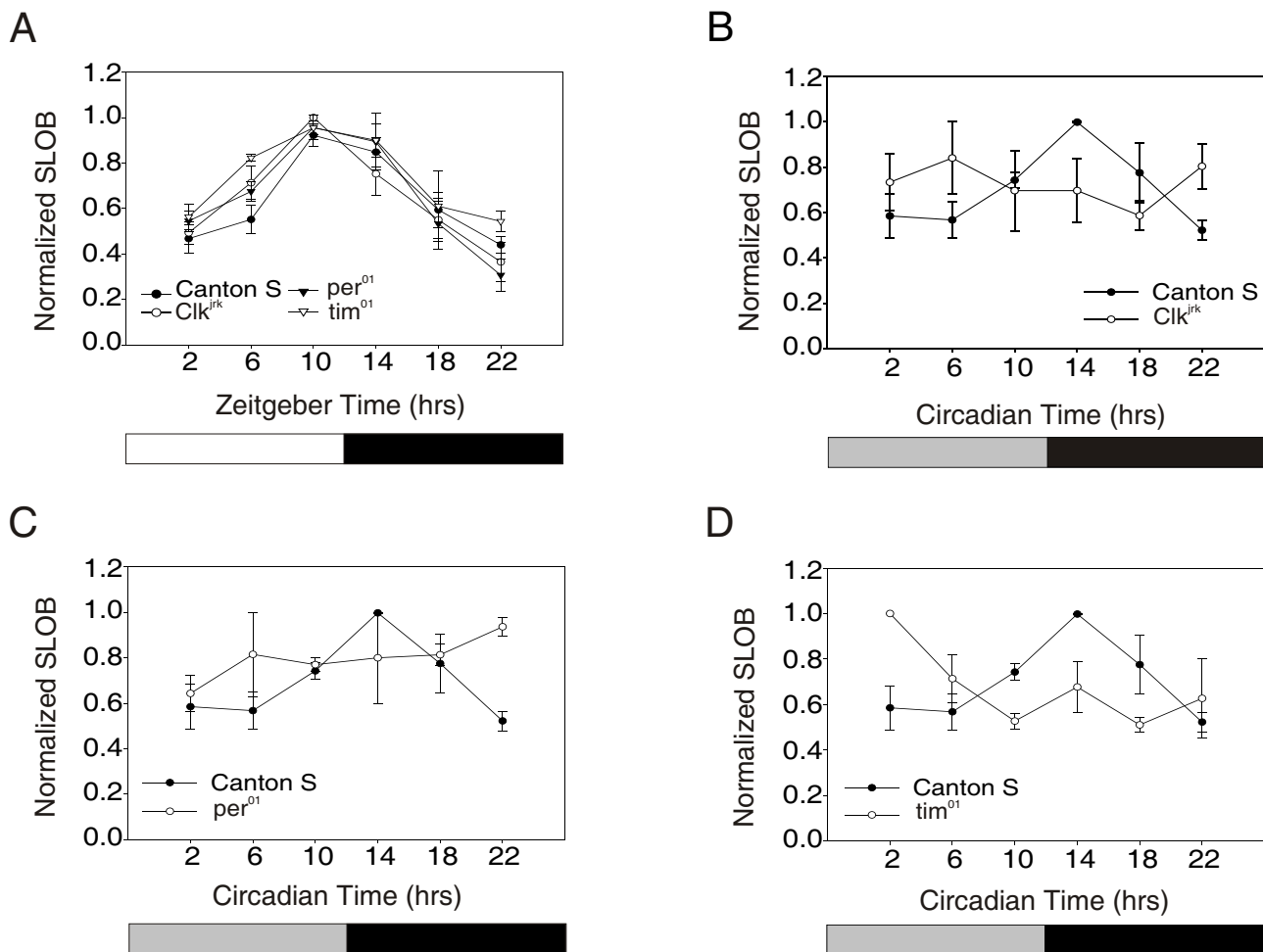
**Figure 1**

SLOB protein cycles in both LD and DD. **(A)** Canton S flies were entrained in LD for three days and collected at six time points over a 24 hour period. Fly head lysates were prepared and an anti-SLOB antibody was used to identify the 58 kDa band representing SLOB on the top western blot. The bottom blot was probed for MAPK, the protein level of which does not cycle [37] and was used as a loading control. SLOB oscillation has a trough at ZT 2 and a peak between ZT 10–14. The graph below represents the mean \pm SEM for each time point from a minimum of three independent experiments. **(B)** Canton S flies were entrained in LD for three days and transferred to constant darkness. They were collected on the second day of DD at six time points, and SLOB cycling was determined as in **(A)**.

frontal sections and the antisense revealed a widespread distribution of *slob*. Expression of *slob* was detected in the brain cortex (Figure 3A), photoreceptors, lamina and medulla (Figure 3B) and the PI region of the brain (Figure 3C). A similar pattern of transcript distribution, particularly in the photoreceptors, optic lobes and brain cortex, is seen for *timeless*, *clock* and *cycle* [21]. Figure 3D,3E,3F shows the respective areas of Figures 3A,3B,3C using the sense RNA probe for *slob*.

SLOB is expressed in the photoreceptors and the brain

Previously, we demonstrated that SLOB protein is present at the neuromuscular junction of *Drosophila* larvae [6]. In order to locate SLOB protein in the adult fly, frontal head sections and wholemounts of the brain were prepared from Canton S and γw flies. Immunostaining of sections reveals intense staining in the nuclei of the photoreceptor cells and the basal cells of the eye (Figure 4A). The brain wholemounts reveal very bright and discrete cytoplasmic staining of 6–8 PI neurons, with widespread but less intense staining elsewhere in the optic lobe and brain cortex (Figure 4B,4C). Although the overall patterns of

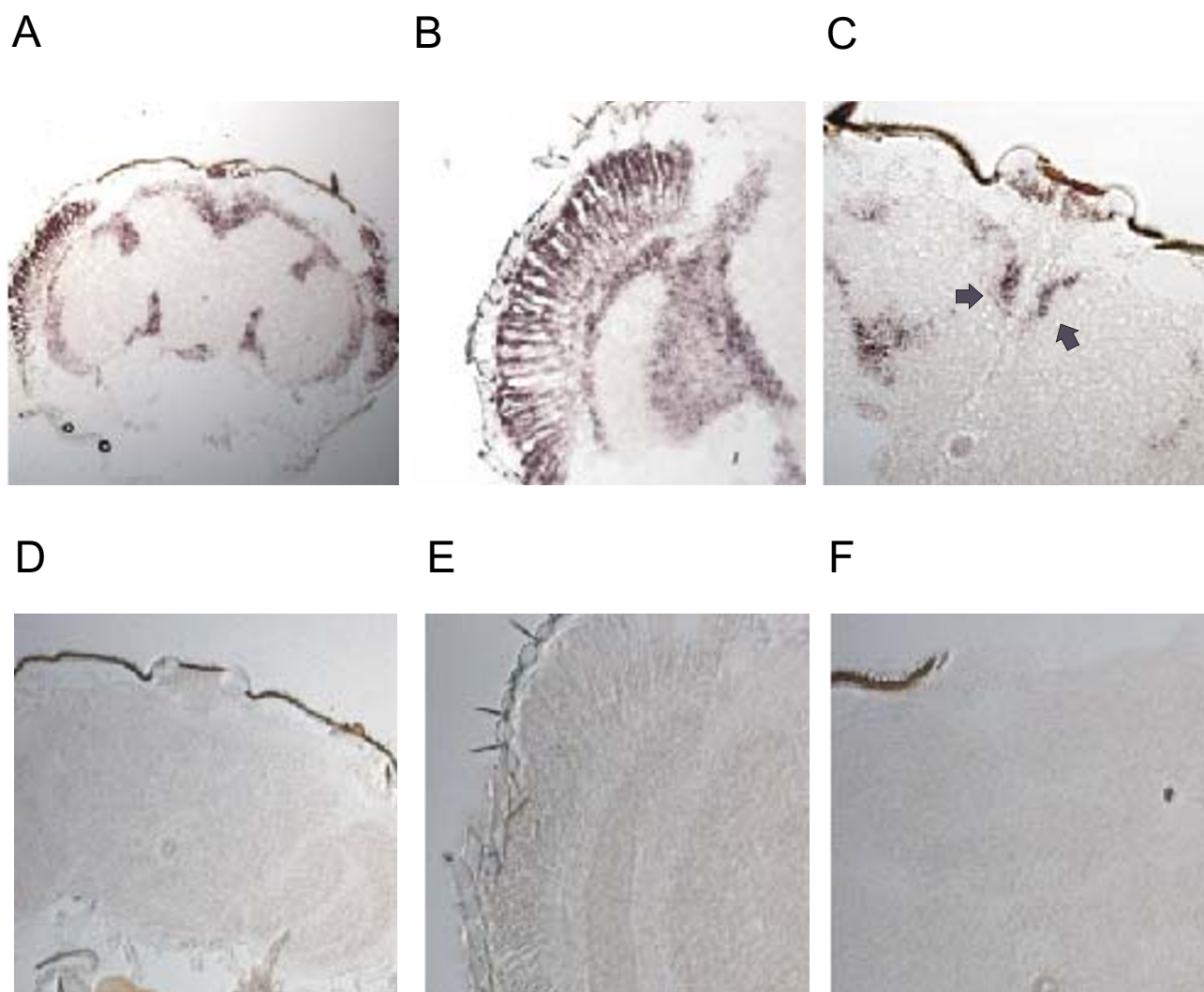
**Figure 2**

SLOB cycling is altered under DD conditions in clock mutants. **(A)** Western blot analysis of SLOB cycling in Canton S, *per⁰¹*, *tim⁰¹* and *Clk^{irk}* flies under LD conditions. Oscillations in all flies are in phase with one another. The graphs represent the mean \pm SEM for each time point from a minimum of three experiments for each genotype. **(B, C)** Western blot analysis of Canton S, *Clk^{irk}* and *per⁰¹* flies in DD conditions. There is no obvious cycling of SLOB in *Clk^{irk}* and *per⁰¹* flies. **(D)** Western blot analysis of Canton S and *tim⁰¹* flies in DD conditions. There is a shift in the phase of SLOB cycling in *tim⁰¹* flies. The peak is no longer at CT 14 but at CT 2. The same Canton S data are illustrated in panels **(B-D)**.

distribution of mRNA and protein are comparable, there are apparent differences in the abundance of protein relative to that of mRNA in some regions. This may simply be due to the histological differences between head sections (Figure 3) and brain wholemounts (Figure 4B), or to the different detection methods used. It is also possible that SLOB protein does not accumulate everywhere the mRNA is found. SLOB protein is also expressed in other head tissues such as the antenna and proboscis (data not shown).

Siegmund and Korge [22] performed a large scale analysis of peptidergic neurons of *Drosophila* larvae. Their study

identified three distinct subsets of PI neurons (PI 1-3) that innervate the corpora cardiaca (a glandular tissue) and the aorta. To determine which subset of PI neurons is SLOB positive we used four of their enhancer trap lines. *Mai 301*, *Kurs 58* and *Kurs 45 GAL4* lines express *GAL4* exclusively in subsets PI-1, PI-2 and PI-3, respectively. *Mai 281 GAL4* expresses *GAL4* in two subsets, PI-2 and PI-3. We crossed these *GAL4* flies to a *UAS-GFP* transgenic line, and found by immunostaining that SLOB is expressed exclusively in subset PI-3 (Figure 4E,4G). SLOB does not colocalize with GFP from the enhancer fly lines that express GFP only in subsets PI-1 or -2 (Figure 4D,4E).

**Figure 3**

Distribution of *slob* mRNA. Frontal sections of *Drosophila* heads were probed with digoxigenin labeled RNA probes for *slob*. **(A)** Low magnification to survey entire head section. Antisense *slob* probes hybridized to the photoreceptors, optic lobe, PI neurons and the surrounding brain cortex. **(B)** High levels of expression detected in the photoreceptors, lamina and medulla. **(C)** *slob* message appears in the PI region. The arrows point to the dorsal medial PI neurons. **(D-F)** Digoxigenin labeled sense probes of *slob* reveal no significant labeling. The three panels correspond to the head sections of **(A-C)**.

Figure 4H highlights components of the circadian system in order to illustrate the location of the PI neurons in relation to other clock gene expressing cells. The lateral neurons (LNs) as mentioned before consist of three clusters, a cluster of cells located dorsally (LN_ds), and two other ventrally located clusters differing in the size of their somata, large LN_vs and small LN_vs. Another group of clock gene expressing cells are the dorsal neurons (DNs), which consists of three subsets as well, DN₁₋₃. This wholemount was immunostained for SLOB, PER and PDF. The LNs and

DNs are immunostained red indicating the presence of PER. Projections from the LN_vs are green due to PDF immunostaining. The PI neurons are the SLOB positive green cells.

The phase of SLOB protein cycling is different in the photoreceptor and PI neurons

Immunohistochemistry was used to determine whether SLOB cycles in the photoreceptor and PI neurons. Flies were entrained for three days in LD and collected at four

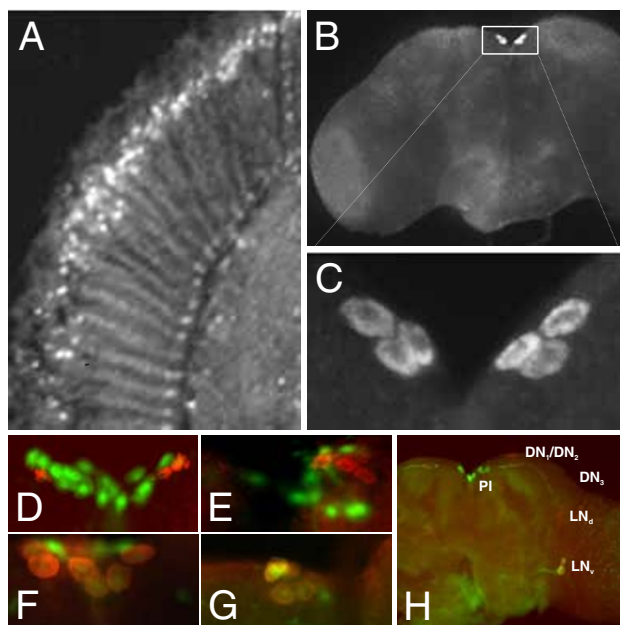


Figure 4

SLOB is expressed prominently in photoreceptor cells and PI neurons. **(A)** Head frontal section from *y w* (wild type) flies immunostained for SLOB protein. SLOB is expressed in the nuclei of the photoreceptor cells. **(B)** Brain wholemounts from *y w* flies immunostained for SLOB protein. SLOB is expressed prominently in the PI neurons of the protocerebrum (box), as well as elsewhere in the brain. **(C)** Enlargement of the boxed area in **(B)** to illustrate the cytoplasmic localization of SLOB in the PI neurons. Typically 6–8 PI neurons are SLOB positive. **(D–G)** GAL4 flies, specific for the three subsets of PI neurons, were crossed to UAS-GFP flies and immunostained for SLOB. **(D)** *Mai 301* GAL4 is expressed in PI-1 neurons [22], shown by GFP expression. UAS-GFP tends to have a nuclear localization. SLOB positive PI neurons are immunostained with Texas Red, and SLOB has a cytoplasmic expression pattern. SLOB does not localize within PI-1 neurons. **(E)** *Kurs 58* GAL4 is expressed in PI-2 neurons. SLOB is not found within the PI-2 neurons. **(F)** *Mai 281* GAL4 is expressed in two of the three subsets of PI neurons (PI-2 and PI-3). SLOB is localized within some of these PI neurons and because SLOB was not found within PI-2 this suggests that the SLOB positive subset is PI-3. **(G)** *Kurs 45* GAL4 is expressed only in PI-3 neurons. SLOB positive cells colocalize with the GFP expressing PI-3 cells. **(H)** Brain wholemount from *y w* flies highlighting components of the circadian system – the small ventral lateral neurons (LN_v), the dorsal lateral neurons (LN_d), the dorsal neurons (DN_{1–3}) and the pars intercerebralis neurons (PI). The wholemount was immunostained for SLOB, PER and PDF. The red component is PER staining; the green staining in the PI neurons corresponds to SLOB, and the green staining elsewhere is PDF. Previous staining in *Pdf⁰¹* flies reveals no PDF in the PI neurons (data not shown). PDF filled projections end close to, but appear to stop short of, the PI neurons. The projections of the DN (not visible) may synapse on the PI neurons.

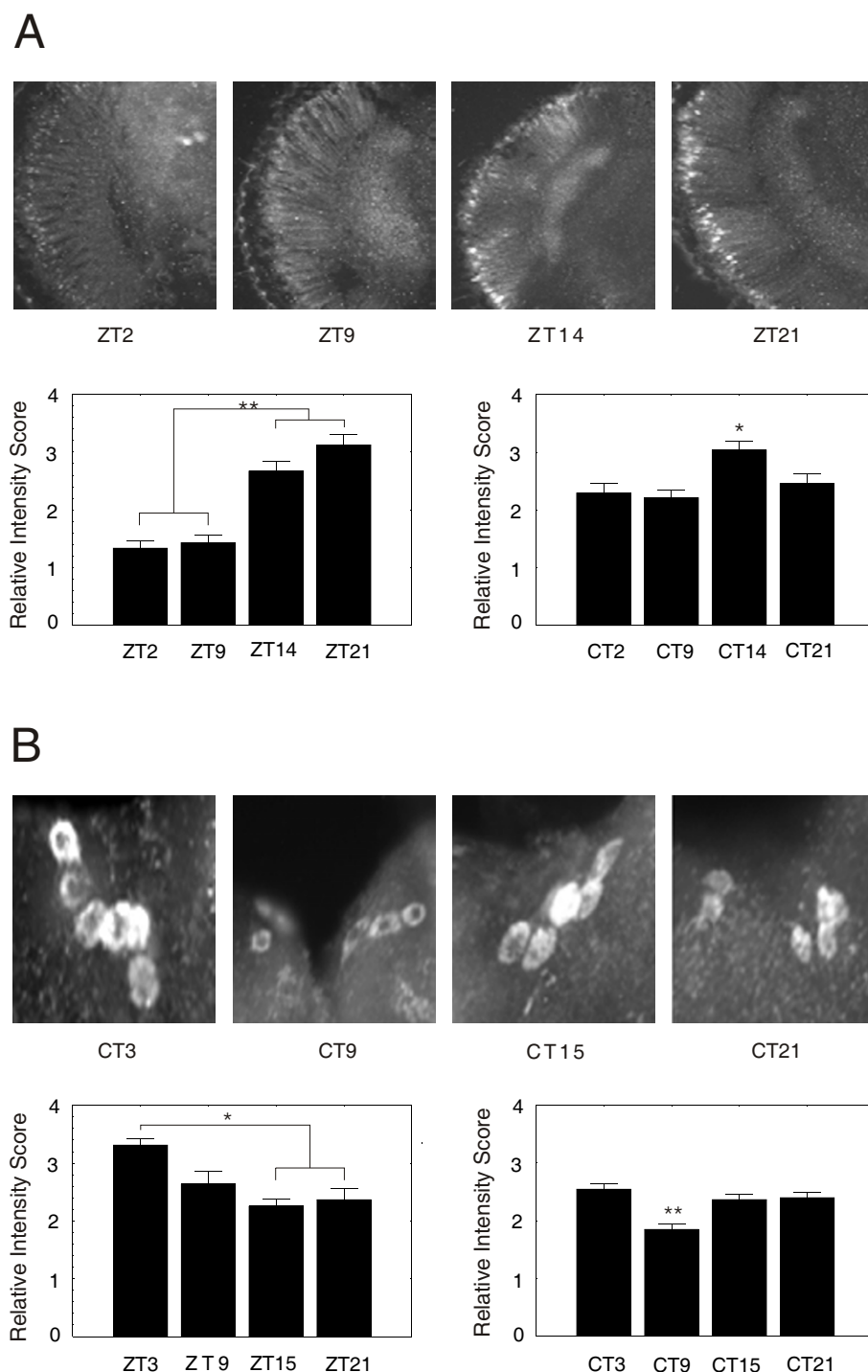
time points; others were then transferred to DD for two additional days and likewise collected at four time points. Antibody against SLOB was used on whole head sections and wholemounts of the brain. We find that SLOB protein cycles in the nuclei of the photoreceptor neurons with a trough at ZT 2 and a peak at ZT 14–21 (Figure 5A). During constant darkness there is a peak at around CT 14 (Figure 5A). This correlates with western blot data collected from the entire *Drosophila* head (see Figure 2). Intriguingly, in the PI neurons, SLOB cycles with a peak at ZT 3 and a trough at ZT 15–21 (Figure 5B), out of phase to the oscillation in the photoreceptor neurons. SLOB continues to oscillate during constant darkness conditions in both the photoreceptor and PI neurons (Figure 5A,5B).

Ectopic expression of SLOB in *tim⁰¹* flies

We noticed an increase in SLOB protein levels, at all time points examined by western blot, in *tim⁰¹* flies. To locate where the increase in SLOB occurs, we stained *tim⁰¹* heads with anti-SLOB antibody, and found considerable SLOB expression in the optic lobe, specifically in the outer edges of the lamina (Figure 6A). Samples shown here are at ZT 14. Expression in these regions is much more limited in the wild type controls. No increases in SLOB expression were detectable in the PI neurons of *tim⁰¹* flies (Figure 6B). Since SLOB protein normally appears to be expressed at relatively low levels in the optic lobe, we infer that the loss of TIM leads to elevated or ectopic expression of SLOB in specific cells. We cannot exclude the possibility that the SLOB signal from *tim⁰¹* flies seen on western blots represents other head tissues as well. This elevated expression of SLOB in *tim⁰¹* flies further demonstrates the regulation of SLOB expression by the clock genes.

SLOB is regulated by a circadian output molecule, PDF

The neuropeptide, PDF, accumulates at dorsal axon terminals of the small LN_vs in a cyclic fashion, indicative of regulated release from these terminals [13]. After two to three days in constant darkness, the majority of *Pdf* null flies are behaviorally arrhythmic supporting a role for PDF in clock output [23]. The location of SLOB in PI neurons makes it a candidate for an output molecule. To determine whether SLOB is a component of the output pathway, we analyzed SLOB expression in the PI neurons of a clock mutant, *Clk^{irk}*, and the output mutant, *Pdf⁰¹*. Flies were entrained and collected at four time points. Western analysis of head lysates during DD indicated that SLOB cycling is dampened or absent in *Clk^{irk}* flies (Figure 3B). Similarly, SLOB cycling in DD is diminished in the PI neurons in *Clk^{irk}* flies (Figure 7A). This suggests that CLK is at least indirectly required for SLOB oscillation in the PI neurons. Wholemounts of brains from *Pdf⁰¹* flies reveal a decrease in SLOB expression and dampened cycling in the PI neurons (Figure 7B), consistent with a role for SLOB downstream of the PDF-secreting neurons.

**Figure 5**

SLOB cycles in the photoreceptor cell and the PI neurons. *y w* flies were entrained in LD for three days and collected at four time points over a 24 hour period. Some flies, after three days entrainment, were transferred to DD for two additional days. At least 5 heads were assayed per time point. **(A)** Head frontal sections were immunostained for SLOB. The bars depict mean \pm SEM staining intensity scores (* $p < 0.05$, ** $p < 0.001$). The left bar graph is for LD and the right is for DD. SLOB cycling in the photoreceptors closely follows that of the western blots for both LD and DD. **(B)** Brain wholemounts from *y w* flies immunostained for SLOB. SLOB cycles in the PI neurons with an altered phase compared to the photoreceptors. The left bar graph is for LD and the right is for DD. Statistical analysis was done as in **(A)**.

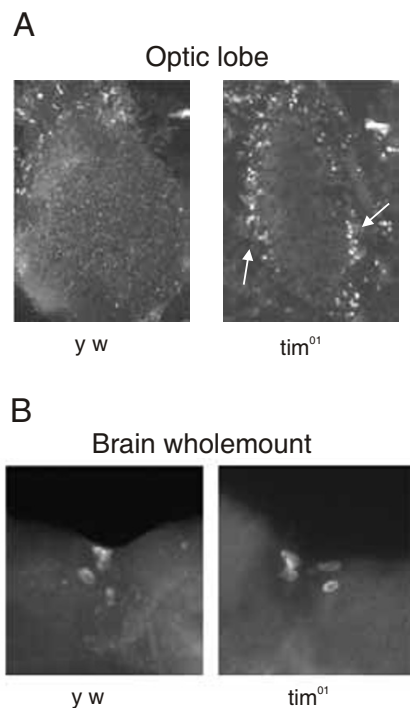


Figure 6
Elevated or ectopic expression of SLOB in *tim⁰¹* flies. **(A)** Head sections from *y w* and *tim⁰¹* flies immunostained for SLOB. There is greater expression of SLOB in the optic lobe in *tim⁰¹* flies. The arrowheads point to the outer edges of the lamina. **(B)** Brain wholemounts from *y w* and *tim⁰¹* flies immunostained for SLOB. There is no change in SLOB expression within the PI neurons of the *tim⁰¹* flies.

Overexpression of SLOB alters locomotor activity

To determine whether manipulation of SLOB levels can alter behavioral rhythms during constant darkness conditions, we created two transgenic UAS (upstream activation sequence) lines. One line, *UAS-slob*, expresses a wild type version of the SLOB protein whereas the second, *UAS-mtslob*, expresses a mutant form of SLOB that renders it unable to bind Leonardo [6]. The UAS lines were crossed to *elav^{C155}* (panneuronal), *GMR* (eye) and *Kurs 45* (PI-3) *GAL4* drivers. Flies were entrained for three days in LD cycles and then monitored for rest:activity in DD for 14 days. Progeny of the crosses between *UAS-slob* or *UAS-mtslob* and *elav^{C155}GAL4* are robustly rhythmic for the first seven days, followed by a breakdown in their rhythms during the last seven days (Figure 8A). This breakdown is not seen in control lines. Thus, the lines that overexpress SLOB exhibit a decrease in the strength of rhythmicity determined by their Fast Fourier Transform (FFT) value during the last seven days, as compared to the control lines (Figure 8B). Overexpression of either form of SLOB

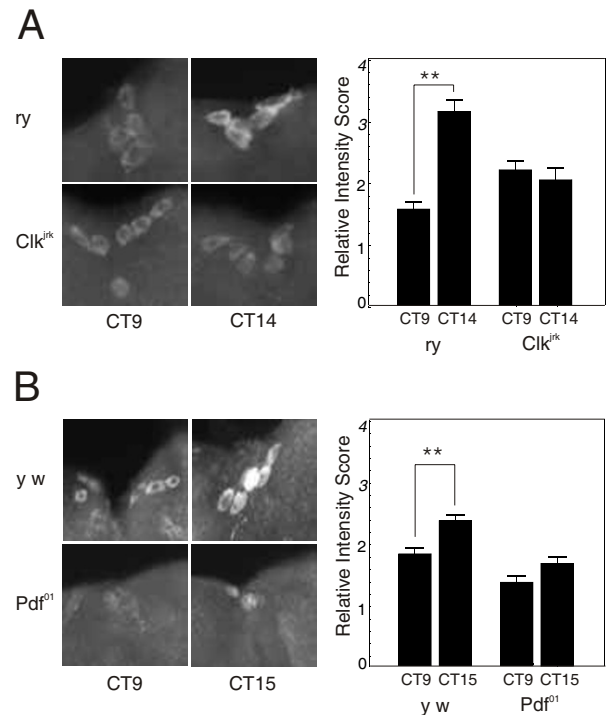
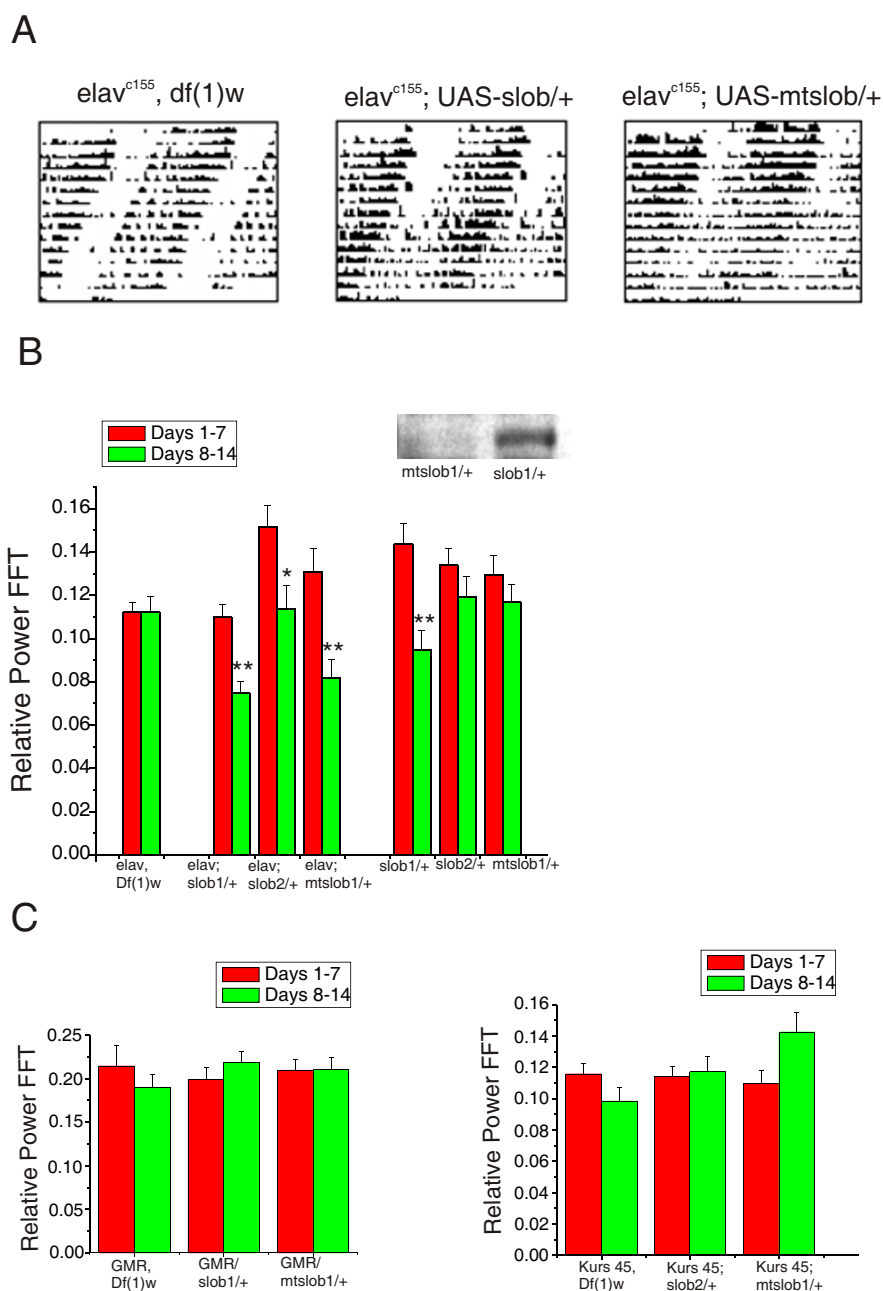


Figure 7
SLOB expression is altered in *Clk^{irk}* and *Pdf⁰¹* flies. **(A)** Brain wholemount from wild type *rosy* (*ry*) and *Clk^{irk}* flies. Two time points, taken on the second day of DD, show that SLOB does not cycle in the PI neurons of the *Clk^{irk}* flies. The accompanying bar graph illustrates relative intensity scores. Data were analyzed as described in Figure 5. **(B)** Brain wholemount from wild type *y w* and *Pdf⁰¹* flies. Two time points, taken on the second day of DD, demonstrate the decrease in SLOB expression and dampened cycling in the PI neurons of *Pdf⁰¹* flies. PDF appears to positively regulate SLOB protein. The bar graph represents relative intensity scores.

protein results in the breakdown of rhythms suggesting that Leonardo binding is not required for this phenomenon. There is some breakdown in rhythmicity in *UAS-slob1/+* flies (Figure 8B), but this is most likely due to leaky expression of the *UAS-slob* transgene (inset in Figure 8B). However, breakdown in rhythmicity does not occur in the *UAS-mtslob/+* flies (Figure 8B), and western blot analysis indicates no such leaky expression of the *UAS-mtslob* transgene (inset in Figure 8B). Fly lines carrying either the *GMR* or *K45* driver along with *UAS-slob* show no decrease in rhythmicity (Figure 8C), suggesting that SLOB overexpression in the eye or PI-3 may not be the cause of the behavioral breakdown. These data indicate that ectopic SLOB expression is capable of altering a behavioral rhythm produced by the circadian system.

**Figure 8**

Overexpression of SLOB alters behavioral rhythms. *Elav*^{c155}, *GMR* and *Kurs 45* GAL4 flies were crossed to *UAS-slob*, *UAS-mtslob* and *Df(1)w* flies. Progeny were entrained for three days in LD cycles and then transferred to a locomotor monitoring device for 14 days in DD. **(A)** Actograms representing control, *UAS-slob* and *UAS-mtslob* lines crossed to *elav*^{c155}. There is an apparent breakdown of rhythmicity after 7 days in flies overexpressing wild type and mutant SLOBs (middle and right panels). **(B)** Summary of behavioral data for *elav*^{c155} flies crossed to control and *UAS* lines. The FFT value is a measure of rhythmic strength and is plotted in the bar graph for the first and second week periods per fly line. Using Student's *t* test, all overexpressing SLOB lines exhibit significant breakdown of rhythms during the second seven days (**p* < 0.05 and ***p* < 0.001, respectively). Controls show no significant breakdown except for one line, *UAS-slob* 1/+. However, as demonstrated by the inserted western blot, this line expresses transgenic SLOB in the absence of a GAL4 driver, whereas the *UAS-mtslob* 1/+ line does not. The blot was probed with an anti-HA antibody that detects only transgenic SLOB. Levels of MAPK were used as loading controls (data not shown). **(C)** Summary of behavioral data for *GMR* and *Kurs 45* GAL4 flies crossed to various lines. No change in rhythmicity is evident when SLOB is overexpressed only in the *Drosophila* eye or PI neurons.

Discussion

We report here that the dSlo binding protein, SLOB, cycles in *Drosophila* heads. Microarray analyses reported *slob* transcript cycling with a peak at either ZT 15 [2] or at CT 11 [3]. We show by western analysis that SLOB protein peaks at ZT 10–14/CT 14, consistent with an earlier peak for the RNA. Under LD conditions, SLOB continues to cycle in *per*⁰¹, *tim*⁰¹ and *Clk*^{jk} flies in phase with the oscillation of wild type flies. It was noted in one of the recent microarray studies [2] that there is a cluster of genes, named the apterous cluster, that shows rhythmicity in these three mutants during LD. This cluster has a characteristic peak at ZT 17 and includes such proteins as transcription factors, synaptic regulators and transporters. The genes in this group may be regulated not only by the circadian clock, but also by a light-dependent mechanism. However in DD, where light is no longer a factor, we find that SLOB does not cycle in *Clk*^{jk} and *per*⁰¹ and exhibits an altered phase in *tim*⁰¹ flies. One might expect *tim*⁰¹ and *per*⁰¹ flies to give similar results, but *tim*⁰¹ flies may not be true genetic nulls [24]. Consistent with the observation that SLOB does not cycle in *Clk*^{jk} flies during DD (Figure 2B) are microarray data from Ueda et al. [5] indicating that *slob* levels do not change in *Clk*^{jk} flies, and other microarray data from McDonald and Rosbash [3] demonstrating that *slob* levels are at mid-point in the *Clk*^{jk} mutants. Taken together these data demonstrate clearly that clock genes regulate *slob* mRNA and protein expression.

Within *Drosophila* adult heads, *slob* mRNA is present in the photoreceptors, optic lobe, the neurosecretory cells of the PI and the surrounding brain cortex. We also find prominent immunostaining for SLOB protein in the photoreceptor cells and the PI neurons. *In situ* hybridization experiments with larval brain revealed *slob* RNA in an area of the brain close to PDF-filled projections of the lateral neurons [2]. This is consistent with our findings of *slob* transcript in the PI neurons and surrounding cortex.

The *Drosophila* eye expresses many of the major circadian genes, and is thought to contain an autonomous oscillator that presumably regulates an eye-specific function [17]. In addition, the eye contributes to photic entrainment of the pacemaker LN cells [25]. Interestingly, the SLOB binding partner, Slo is also expressed in the visual system including the eye, lamina, medulla and lobula [26]. Ceriani et al. [1] have demonstrated that the RNA levels of both *slob* and *slo* cycle in phase in both LD and DD. The dSlo protein was also shown by western blot to cycle and peak at ZT 20 [1]. This correlates with the cycling of SLOB in the photoreceptors, where SLOB peaks at ZT 14–21 (Figure 5A).

The PI region lies directly beneath the root of the ocellar nerve. The PI neurons have large, 15 µm diameter, cell bodies, and their axons project along the median bundle and then bifurcate [27]. One of the branches proceeds ventrally and arborises in the dorsal tritocerebrum region, below the oesophagus. The other branch moves in a posterior direction and enters the cardiac recurrent nerve in the oesophageal canal. The PI neurons have an extensive network of endoplasmic reticulum and contain secretory granules, suggesting that they are neurosecretory cells [27]. In insects, peptidergic neurons of the central nervous system regulate the synthesis of developmental hormones. The PI neurons, in particular, have been implicated in hormone production and release in various insects [28,29]. Three subsets of PI neurons have been identified. We have identified the subgroup PI-3 to be the SLOB positive subset of PI neurons. Among the hormones identified in the PI neurons is insulin, and it has been proposed that the release of insulin into the hemolymph is essential for growth control and carbohydrate homeostasis [30]. We have confirmed that the SLOB positive PI neurons are also insulin positive (data not shown).

The photoreceptors and the PI neurons express oscillating SLOB protein and intriguingly, the rhythms in the two neuronal types are not in phase with each other. The mechanisms responsible for these phase differences are not known. PER and TIM are expressed in the lateral neurons in the central brain, in glial cells of the optic lobes, and in the photoreceptor cells [7]. PER and TIM protein have not been shown to be expressed in the PI neurons, although Kaneko and Hall [31] found that there is expression of *GAL4* driven by the *per* promoter in PI neurons. The photoreceptors in contrast have all the traditional clock genes that might contribute to SLOB cycling [32–34]. The presence of these genes in the eye, but not in the PI, may contribute to the phase differences. In addition, it is reasonable to expect that there will be molecular differences in circadian regulation in different cell types. Furthermore, the subcellular localization of SLOB is different between the two areas (Figure 4A,4C). SLOB appears to be primarily cytoplasmic in the PI neurons, and nuclear in the photoreceptors.

How is SLOB regulated in the PI neurons? Interestingly, the PI neurons have been associated with behavioral rhythmicity and it has been hypothesized that this involves hormone release [35]. In fact, the PI neurons of *Teleogryllus commodus* (crickets) have long been hypothesized to serve as a region of coupling between the circadian pacemakers and behavioral rhythms [36]. A pathway for *Drosophila* proposed by Kaneko and Hall [31] suggests that oscillatory signals of the "master pacemaker" in the small LN_{vs} first modulate the oscillatory mechanism or neuronal activity operating within neurons in the

dorsal region. The DNs send their oscillatory signals to the PI, which may lead to rhythmic neurosecretory peptide release. It has been observed in the larval CNS that projections of the DNs terminate near the midline in the PI region [31]. These DNs express both PER and TIM and hence may send robust oscillatory signals to downstream targets such as PI neurons. Interestingly, two neurons of the DN group express PER and TIM cycling antiphase to the other DNs and LNvs [31]. Regardless of the precise role of the DNs, it is clear that this dorsal region of the brain is important for rest:activity rhythms. For example, PDF release and MAPK activity cycle specifically in this region, and both participate in behavioral rhythmicity [13,37].

Clock mutants alter either SLOB protein oscillation or levels in both the eye and PI neurons. One striking observation is the ectopic or elevated expression of SLOB in the lamina of *tim⁰¹* flies. This suggests that TIM negatively regulates SLOB. Western analyses show that upregulation of SLOB does not occur in *per⁰¹* flies. Ectopic expression of PER occurs in *double-time (dbt)* flies that are mutant for a casein kinase 1 ϵ involved in PER turnover [38]. The interpretation in that case is that PER is synthesized in many cell types where its expression is normally undetectable due to destabilization by the kinase. A similar mechanism may account for elevated expression of SLOB in *tim⁰¹* flies. The observation that *tim⁰¹* alters SLOB expression while *per⁰¹* does not could suggest a pathway for slob regulation that is independent of PER. We also found that SLOB fails to cycle in the PI neurons of *Clk^{jk}* flies.

Perhaps most intriguing is the decrease of SLOB in the PI neurons of *Pdf⁰¹* flies. The oscillation of PDF is restricted to the dorsal projections emanating from the lateral neurons [13]. Dorsal terminals of the LN_s express abundant PDF early in the morning, which is indicative of a block in its release. Thus, PDF release is low during the day while SLOB is at its trough, consistent with PDF being a positive regulator of SLOB. In Figure 4H we see that PDF expressing terminals do not appear to contact the PI neurons. As discussed above, we hypothesize that PDF termini affect the DN, or alternatively, other neurons of the dorsal region, which in turn communicate with the PI neurons. *Clk^{jk}* flies lack PDF in the small ventral LN_s, but still express it in the large LN_s [13]. This may account for the difference in the phenotype of *Clk^{jk}* and *Pdf⁰¹* mutants and would suggest a role for the large LN_s in SLOB regulation.

The molecular oscillations of the circadian clock proteins result ultimately in behavioral rhythmicity [24]. Our data demonstrate that the panneuronal expression of SLOB causes a delayed breakdown of rhythms. Breakdown after several days is characteristic of some circadian output

mutants such as *Pdf⁰¹* flies [13]. Likewise, panneuronal overexpression of PDF results in a delayed disruption of rhythms, and overexpression of PDF in the PI neurons results in a shortened period and an advance of the morning peak [39]. *disconnected (disco)* flies, which lack LN_s, also become arrhythmic only after several days in DD [40].

Using *GAL4* drivers that direct overexpression of SLOB specifically to the eye or PI neurons, we found no obvious circadian locomotion phenotype. It is possible that these drivers are not strong enough, compared to the panneuronal driver *elav^{C155}*. We note that the overexpression of PER and TIM with the *tim-GAL4* driver results in a more severe phenotype than with *per-GAL4*, even though the expression pattern of the two drivers is similar [24], possibly because the *per* promoter is weaker [41]. Alternatively, the specific drivers we used may not target all the behaviorally-relevant SLOB positive neurons in the eye and PI region. Any SLOB positive neurons that are not overexpressing SLOB are therefore wild type, and this may prevent rhythmicity breakdown. Similar explanations have been proposed for the lack of a phenotype when PER and TIM are overexpressed by the *pdf-GAL4* driver although both genes cause arrhythmia when their overexpression is driven by the more widely expressed *tim* and *per-GAL4* drivers [24,42].

The widespread distribution of SLOB in the eye and brain suggests that other cells, in addition to or instead of the photoreceptor and PI neurons, may account for SLOB's apparent role in behavioral rhythms. The panneuronal overexpression of SLOB in other SLOB-expressing cells in the brain cortex may explain the rhythmic breakdown. Alternatively, ectopic expression of SLOB in neurons involved in locomotor rhythms might also account for the altered rhythmicity.

Conclusions

In this study, we have demonstrated that SLOB protein cycles in a circadian fashion in *Drosophila* heads. SLOB oscillates in two discrete areas of the fly head, the photoreceptor cells and the PI neurons. Our results reveal differential effects of clock mutations on SLOB expression and cycling in these two regions. There is a significant decrease in SLOB levels in the PI neurons of *Pdf⁰¹* flies, thus implicating PDF as an upstream regulator of SLOB. Along with the observation that flies overexpressing SLOB exhibit a breakdown of rest:activity patterns, these data suggest that SLOB is a clock controlled protein.

We showed previously that SLOB, along with dSlo and Leonardo, participates in a dynamic regulatory complex in presynaptic nerve terminals. Leonardo binding is dynamically regulated by phosphorylation of SLOB by the

calcium/calmodulin-dependent protein kinase II (CAMKII) [6]. Intriguingly, CAMKII has recently been implicated in circadian rhythmicity in vertebrates [43], and it is tempting to speculate that this regulatory complex participates in the fly circadian output pathway. Not only do *dslo* mutants have weak rhythms [1,44], but *leonardo* mutants have defects in behavior, synaptic transmission and plasticity [45,46]. Our data are consistent with the hypothesis that SLOB participates in circadian rhythmicity by regulating synaptic function and membrane excitability.

Methods

Fly stocks and germ line transformation

D. melanogaster strains Canton S (wild type), *γ w*, *ry*, *tim⁰¹*, *per⁰¹*, *Clk^{rk}*, and *Pd^{f01}* and transgenic fly strains were raised at 25°C on standard *Drosophila* medium. *slob* cDNA was cloned into a pUAST vector and P-element-mediated transformation was performed as described previously [47]. The transformed lines were crossed to either *elav^{C155}* (provided by Leslie Griffith), *GMR* (provided by Konrad Zinsmaier) or *Mai 281*, *Mai 301*, *Kurs 45*, and *Kurs 58* *GAL4* (provided by Gunter Korge).

In situ hybridizations

The *slob* RNA antisense and sense probes were synthesized using the DIG RNA Labeling Mix (Boehringer Mannheim). The sequence used for the RNA probes was made from basepairs 1142–1441 of the *slob* transcript. *In situ* hybridization on adult 12 μm head sections were done according to the protocols found at <http://www.rockefeller.edu/labheads/vosshall/protocols.php> with slight modifications. All hybridizations and washes were done at 55°C. Sections are developed in the dark for 3 days.

SLOB antibody purification

A GST-SLOB fusion protein was used to immunize rabbits as described previously [48]. Polyclonal antibodies specific to SLOB were generated by purifying the serum using a combination of CNBr conjugated GST and CNBr conjugated GST-SLOB columns.

Western blotting and quantitation

Flies were entrained and collected in LD and DD conditions at four hour intervals. Fly heads were lysed in 1% CHAPS, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 120 mM NaCl, 50 mM KCl, 2 mM DTT and protease inhibitors (1 mM PMSF, 1 μg/mL each aprotinin, leupeptin, and pepstatin A (SIGMA)). Protein concentration was determined using the BioRad DC Protein Assay. 100 μg of protein was loaded on 4–15% polyacrylamide gradient gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk in TBST (0.1% Tween 20 in Tris-buffered saline), the blots were probed with the appropriate primary and secondary antibodies. Enhanced

chemiluminescence detection system (Amersham) was used to visualize the proteins. Film exposures of western blots were scanned using Bio Rad Molecular Analyst. The level of SLOB at each time point was calculated as the SLOB signal minus the background in each lane. The blots were stripped and reprobed with anti-MAP Kinase (Sigma). The ratio of SLOB to MAP Kinase was normalized and averaged between several westerns.

Immunohistochemistry

Brain whole mount: Flies were entrained in LD for three days and then transferred to DD. Fly heads were collected at given time points, fixed in 4% paraformaldehyde (PFA), and the brains were dissected and kept in cold PBS, and subsequently blocked with 6% normal donkey serum in phosphate-buffered saline (PBS)/0.3% Triton X-100 for one hour. Samples were then incubated with primary antibody at a dilution of 1:400 overnight at 4°C. After washing in PBS/0.3% Triton X-100 three times for 30 min each at room temperature, samples were incubated with the appropriate secondary antibody (Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rabbit IgG and Texas Red dye-conjugated AffiniPure Donkey Anti-Rabbit IgG from Jackson ImmunoResearch) at a dilution of 1:500 in 3% normal donkey serum in PBS/0.3% Triton X-100 for 1 hour at room temperature, and washed in PBS three times for 30 minutes each. Brains were mounted onto slides with mounting medium (Vector H-1200). Wholemounts were visualized using fluorescence microscopy on a Leica DMIRE2.

Section: Flies were collected at given time points, mounted with mounting medium, and sectioned at 12 μm using a cryostat (Leica 3450). Sections were fixed with 4% PFA, washed in PBS/0.3% Triton X-100, and blocked with 6% normal donkey serum in PBS/0.3% Triton X-100 for 1 hour, and subsequently incubated with primary antibody in 6% normal donkey serum in PBS/0.3% Triton X-100 overnight at 4°C. The sections were then washed and incubated with secondary antibodies as described above.

The staining intensity of brain whole mounts and sections was assessed by blind scoring. A subjective intensity scale from zero to four was used, with zero being undetectable and four being maximal. Statistical analysis of average staining intensity scores was done using ANOVA and the Tukey HSD test.

Behavioral analysis

Flies aged from 1–5 days were entrained for three days in 12 hr light/dark cycles at 25°C and then kept in constant darkness for 14 days. Activity was monitored by using the Trikinetics system. Individual flies were analyzed for rhythmicity based on their by chi-square periodogram

and Fast Fourier Transform (FFT) values [24]. The analyses were performed using ClockLab software.

Authors' contributions

AMJ performed the SLOB antibody purification, western blotting and quantitation, immunohistochemistry, *in situ* hybridization and behavioral analysis. XZ carried out immunohistochemistry and scoring analysis. DAA and AS participated in the immunohistochemistry and western blotting. AMJ and IL drafted the manuscript. AMJ, XZ, YZ, IL and AS conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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