Calcium Reporters and Optogenetic Introduce Novel Way to Monitor Real-time Neuronal Activity and Locomotor Activity in *Drosophila*

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ABSTRACT

Calcium Reporters and Optogenetic Introduces a Novel Way to Monitor Real-time Neuronal and Locomotor Activities in *Drosophila*

A thesis presented to the Department of Biology

Brandeis University
Waltham, Massachusetts

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*Drosophila* circadian rhythms are regulated by an endogenous circadian clock located in about 150 pacemaker neurons in the fly brain. These pacemakers express key circadian proteins such as PERIOD (PER) and TIMELESS (TIM). Here we utilized a newly developed calcium reporter to real-time monitor the neuronal activity of key circadian pacemakers. As expected, we found that the master circadian pacemakers, morning cells, exhibited a self-sustained calcium oscillation that peaked around lights on and was maintained in constant darkness. This cycling pattern was affected by light conditions and more interestingly, by mating. Virgins showed a calcium cycling pattern similar to that observed in males while mated females exhibit an increased amplitude and anti-phase calcium oscillations, which may be related to egg-laying behavior. In addition, we exploited a video recording assay combined with an optogenetic tool to simultaneously record and manipulate fly behavior. We verified that this assay can faithfully record fly locomotor patterns and activate the key circadian pacemakers to produce different behavior outputs. Although this work was done in collaboration with Dr. Fang Guo, my specific contributions were to 1) recognize the importance of mating to female behavior, 2) build and fine-
tune the LED-video system for neuron stimulation and 3) begin the analysis of wake-promoting neurons with this system. The important tools described here will allow us to make the underlying links between neuronal circuits and different behaviors in *Drosophila* and ultimately construct the neuronal network at the cellular level.
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INTRODUCTION

Nowadays people mainly rely on external, unnatural clocks such as cell phones and alarm clocks for timing cues. However, our body is under the control of an intrinsic timer that is encoded in our genome. This timer, known as a circadian rhythm, is a biological process that oscillates about every twenty-four hours and repeats independently of external cues. It controls daily behavioral and physiological oscillations that occur over the course of the day. The circadian clock is driven by pacemaker cells in the mammalian suprachiasmatic nucleus and central clock neurons in insects such as Drosophila (1, 2). These evolutionarily conserved cycles enable organisms to respond to and anticipate daily changes in the external environment and serve to coordinate in time processes at the molecular, cellular, tissue, and systems levels. In Drosophila, these processes include sleep/wake behavior, metabolism, learning and memory, egg laying, and courtship (3). Moreover, the disruption of the circadian clock has been implicated in several metabolic, sleep, neurological, and neurodegenerative disease such as Alzheimer’s disease and insomnia in humans (4, 5), further emphasizing the importance of the circadian clock to organismal health and survival.

In the Drosophila brain, about 150 clock neurons interact through many delicate and complex cell-signaling pathways to produce the output generated by the circadian clock (6). Signaling between these neurons is a crucial element in the regulation of the circadian clock. Of these neurons, approximately 20 are responsible for producing the distinct morning and evening peak activities that Drosophila are known to exhibit (7).

Intracellular circadian mechanisms include transcriptional feedback loops involving at least five genes that generate the coordinated cycling of the molecular pacemaker: PERIOD (PER), TIMELESS (TIM), CLOCK (CLK), CYCLE (CYC), and DOUBLETIME (DBT) (8, 9). Among
these proteins, the molecular feedback system in *Drosophila* consists of four key proteins: PER, CYC, CLK, and TIM. These proteins are homologs of mammalian PERIOD1, PERIOD2, BMAL1 (corresponding to CYC) and CLOCK. There is no true mammalian homolog of TIM, which is replaced by mammalian CRY1 and CRY2 in the core feedback loop (2).

1. **Regulating the circadian gene expression in a feedback loop**

   There are two main feedback loops that regulate the circadian clock in *Drosophila*. The primary loop consists of the PER/TIM heterodimer, and the secondary feedback loop involves the expression of VRI and PDP1. The principle mechanisms of the clock revolve around the activity of CLK and CYC (see Supplementary Figure 1). The CLK and CYC heterodimer promotes the expression of *per* and *tim* by binding to E boxes on the DNA. After PER and TIM are translated, these proteins undergo post-translational modification in the cytoplasm. They form a heterodimer that enters the nucleus to bind to the CLK/CYC heterodimer, releasing the heterodimer from the core clock gene promoter. The expression of PER and TIM eventually repress their own expression as more CLK/CYC heterodimers are removed from the promoter (10-15). At the same time, PER stability is increased upon binding with TIM to form a heterodimer, which participates in negative feedback to repress *per* and *tim* transcription. The heterodimer was also phosphorylated by kinases to increase the stability of the PER/TIM heterodimer. When CRY is activated by light, PER and TIM degradation starts, resulting in the release of the CLK/CYC heterodimer. Consequently, released CLK/CYC can once again promote the expression of PER and TIM (Supplementary Figure 1). The overall cycle constitute the up and down regulation of PER and TIM over a twenty-four hour cycle (15).

   In addition to the principle feedback loop involving CLK/CYC activation of *per* and *tim* transcription, there is an additional loop involving the transcription of Par domain protein (Pdp1)
and vrille (vri) (Supplementary Figure 1). Unlike PER and TIM, VRI and PDP1 do not directly interact with the CLK/CYC heterodimer. Instead clock proteins regulate the circadian clock on the transcriptional level with VRI representing \textit{clk} expression and PDP1 promoting \textit{clk} expression (16). CLK/CYC drives the expression of Pdp1 and vri, PDP1 then activates \textit{clk} and \textit{cry} transcription at a time opposite of when \textit{per} and \textit{tim} expression occurs. VRI, which accumulates faster than PDP1, represses \textit{clk} and \textit{cry}, reinforcing the timing of circadian feedback loops.

2. Neuronal network of the circadian clock

In animals, a relatively small number of neurons constitute the central clock or the circadian pacemaker that transmits rhythmic information to target organs and tissues by electric or chemical signals, such as neurotransmitters and hormones. In \textit{Drosophila}, 150 neurons in the central clock works delicately with each other to maintain homeostasis and a twenty-four hour period. These 150 neurons are divided into two distinct groups: lateral neurons (LNs) and dorsal neurons (DNs) (see Supplementary Figure 2) (17).

Lateral neurons (LNs) located between the central brain and the optic lobes generate robust adult behavioral rhythms. Lateral neuronal cells that contain circadian genes are divided into three clusters: six neurons located relatively dorsally (LNds); four to five more ventrally located neurons with large somata (large LNvs); four to five ventrolateral neurons with relatively small somata (small LNvs); and lateral posterior neurons (LPNs) (17).

\textit{Drosophila} shows bimodal peak in a twenty-four hour period: one in the morning and one in the night (36). The activity/sleep pattern in flies is also controlled by these pacemakers. s-LNvs play an important role in circadian locomotor rhythms (17). The s-LNvs and l-LNvs with an exception of 5\textsuperscript{th} s-LNv are commonly denoted as PDF-cells because they are the only neurons in the brain that express the neuropeptide PDF (PIGMENT DISPERING FACTOR) (18). The PDF-
cells likely target areas near the Pars Intermedia, a brain region that influences growth and sleep (19). L-LNvs coordinate light driven arousal and subsequent behavior in flies (20). LNds control the evening activity peak while s-LNvs and l-LNvs control the morning peak in 12-hour light and 12-hour dark (LD) conditions with the exception of the 5th s-LNv, which contributes to the evening peak instead of the morning peak (21). The LNs are known as the pacemaker cells since they were shown to be necessary and sufficient to drive behavioral rhythmicity in the absence of external time cues.

Dorsal Neurons (DN) can be divided into three groups of DNs, the DN1s, DN2s, and DN3s. The role of the DNs is not yet fully understood, but they are believed to regulate the response to environmental stimuli such as temperature and light (22). DN1 encompasses a large and heterogeneous group of clock neurons. The DN1s consist of anterior and posterior clusters (DN1ps and DN1as). Additionally, the DN1ps can be either CRY positive or negative (23). The DN1s and DN3s are glutamatergic and are believed to provide input to the s-LNvs (24).

As mentioned above, two groups of central brain circadian neurons appear particularly important for behavior rhythms. The four PDF-expressing small ventrolateral neurons (s-LNvs) dictate morning activity as well as rhythmicity in constant darkness (25-27). This feature, free running locomotor activity rhythms, has caused the s-LNvs to be considered the major fly pacemaker neurons. A less well-defined set of cells directs evening activity. These neurons (E cells) also dictate circadian behavior in constant light and include PDF negative 5th s-LNv (28). The PDF positive neurons play a dominant role in dictating the circadian clock (29). Flies lacking PDF expression ($pdf^{01}$) will show arrhythmic behavior in constant darkness (DD) (30).

*Drosophila* shows bimodal peak in a twenty-four hour period: one in the morning and one in the night (31). The activity/sleep pattern in flies is also controlled by circadian neurons.
However, the real neuronal activity of pacemakers has not been investigated in vivo in flies contrast to the SCN in mammals. The size limitation of *Drosophila* makes it impossible to place electrical probe into flies head. And using a voltage sensor or calcium reporter in dissected brains did not provide information in living animals. To solve this problem, we employed the newly generated calcium-dependent transcription activator CaLexA to drive the expression of Luciferase (LUC) in discrete neurons and assayed flies in a standard top counter machine (36). Sustained neural activity induces nuclear import of the chimeric transcription factor LexA-VP16-NFAT, which in turn drives Luciferase (bioluminance) reporter expression in activated neurons (33). Using this system, circadian neuronal activity and related molecular signal pathway involved in alteration can be rigorously investigated.

In parallel with the CaLexA neuronal activity assay, optogenetic was used to manipulate circadian neural activity in freely moving animals. Recently described, Red-shifted Channel Rhodopsin (CsChrimson) allowed temporal control of the activity of adult flies at wavelengths that are not thought to interfere with normal visual function and can also penetrate the fly cuticle (34). This tool provided an opportunity to control neural activity with millisecond precision without interfering with the flies’ natural activity. By expressing CsChrimson in the circadian and sleep neurons, we can investigate the altered circadian neuronal activity in a non-invasive way.
CHAPTER ONE

Using Calcium Reporter to Monitor Real-Time Neuronal Activity of *Drosophila*

BACKGROUND

*Drosophila* circadian rhythms are controlled by multiple oscillators that are regulated by the clock. The circadian clock relies on several groups of neurons that express the key circadian protein. Although the molecular mechanisms of circadian rhythms are well known, how the circadian clock guides these neurons’ activities to generate a structured rhythmic output remains a mystery.

To measure the clock gene expression within the living animal, firefly luciferase can be used as real-time reporter gene. Luciferase enzyme activity is reliably linked to gene expression rhythms and can be monitored noninvasively by assaying luminescence via bioluminescence recorder like TOPCOUNT assuming that the enzyme substrate Luciferin is made available to the cells of interest. Furthermore, the luciferase protein is sufficiently unstable to allow for the detection of circadian rhythms generated at the transcript level. In order to assay real-time neuronal activity of *Drosophila*, both bioluminescence reporter and calcium reporter, CaLexA (Calcium-dependent nuclear import of LexA), were combined to record neuronal activity of flies indirectly through calcium levels.

CaLexA is a novel activity reporter system that utilizes nuclear factor of activated T cells (NFAT), a transcription factor that is imported into the nucleus in an activity-dependent manner. The CaLexA system targets a modified NFAT to specific neuronal populations (33). Its import into the nucleus upon sustained depolarization induces the expression of the reporter gene. The flies are fed with Luciferin. By expressing CaLexA system to specific circadian neuron, we can
monitor its neuronal activity in living flies. With this new tool, real-time activities of key circadian neurons can be monitored in several days.
RESULTS

CaLexA-LUC was expressed within three groups of circadian neurons, PDF, DN1P, and DvPdf. Three separate fly lines, one expressing DvPdf-CaLexA-LUC, and the other one expressing DN1P-CaLexA-LUC were entrained to 12:12 light: dark cycle at 25 °C for 2 days before flies were loaded to the 96-well plate for TOPCOUNT assay (Figure 1.1). TOPCOUNT assay lasted for 5-7 days depending on the assay definition.

PDF is expressed in l-LNV and s-LNVs. These neurons are the morning cells. DN1P are in the downstream of PDF neurons. Lastly, DvPdf is expressed in l-LNv, s-LNv, and LNds. These neurons are known for its role in morning and evening oscillations (see supplementary figure 2). However, no one has monitored their neural activities in living flies rather than in dissected brains.

All two groups show bimodal peaks as predicted. There is one peak during the daytime and one peak during the nighttime (Figure 1.2). The master circadian pacemakers, morning and evening cells, maintained a self-sustained calcium oscillation which peaks around lights on. This data is consistent with the arousal-promoting function of these neurons. DvPdf-CaLexA-LUC males also show higher calcium level during the day time and lower during the night time when fly fall asleep. As the downstream circadian neurons, DN1ps also exhibit same neural activity pattern (data not shown). To figure out if the neural activity pattern is controlled by the endogenous circadian clock, DvPdf-CaLexA-LUC flies were tested under constant darkness. Entrained flies were first monitored under constant darkness for three days and then were returned to 12:12 LD cycle. Under constant darkness, flies were able to keep their calcium oscillations similar with what we observed in LD (Figure 1.2). This indicates that the neural
activity pattern of both morning and evening neurons is under the control of their endogenous circadian clock, which may be involved in maintaining rhythmicity in constant darkness.

We also wanted to examine the change in calcium oscillation with in circadian neurons when circadian clock is destroyed by constant light. We observed that neuronal oscillation period gets lengthened from ~24 hr to ~27 hr and the amplitude dampens. DvPdf-CaLexA-LUC flies gradually became arrhythmic in the following LL days. Therefore, DvPdf neurons cannot keep 24hr neuronal oscillation period with the deconstruction of endogenous clock (Figure 1.3).

Moreover, calcium cycling pattern can be affected by mating in females. After mating in female flies, many biological changes occur in their body. Among those changes, one of them is the egg-laying behavior. Virgin female and mated female were tested at the same time and found that virgin females show similar neuronal oscillation peaks like males; however, mated females exhibited different circadian oscillation pattern comparing to virgins and males (Figure 1.4). Mated females had a significantly bigger peak around the lights-off time. This anti-phase of neuronal oscillation is observed in DN1P and DvPdf. More interestingly, the sudden increase is almost exactly on the exact time of the lights off. This result indicate that there could be a correlation between egg-laying behavior and mating.
DISCUSSION

The classical *Drosophila* bimodal LD behavior pattern occurs in male and is controlled by a dual M-E oscillator system in the fly brain. Although female flies have the same set of circadian cells with no anatomical differences from males, they have a very different behavioral pattern. However, M-E oscillator system might control both male and female behavior pattern. After mating, females show different locomotor and neuronal behavior that are triggered by male specific sex peptide injected with sperm. Furthermore, DvPdf virgins showed similar calcium oscillation pattern as male (data not shown). Once females mated, their calcium oscillation pattern dramatically changed. This sudden neuronal activity might be the cause of a behavioral change. Also, the change in mated female’s calcium oscillation can be due to the egg-laying instinct to increase the survival of the progeny. It has been reported that females have a peak of egg-laying around the night time (35). Thus, the endogenous clock might control M-E oscillation to cue the fly the ideal time for egg-laying. In the future, DvPdf-CalexA-LUC virgins will be mated with males without sex sexptide to see if oscillation change still occurs.

In summary, calcium reporters are powerful tool to real-time record neuronal activity in living flies for several days (Figure 1.1). This tool also can be combined with other techniques such as optogenetic method to investigate the circadian neural circuit connection.
Characterization of the real-time CaLexA-LUC assay. The small panel in the solid black box pictorially depicts the basic principle of CaLexA-LUC: increased calcium levels in activated neurons will trigger translocation of the CaLexA fusion protein from the cytoplasm to the nucleus. CaLexA will then bind to the LexAop to express Luciferase. Flies expressing CaLexA-LUC were loaded to the plates, which were translated to TOPCOUNT plate reader every hour to record LUC activity.
Figure 1.2 – Calcium Oscillation Pattern of DvPdf in LD and DD

Without the external cues such as light, flies have to rely on their endogenous circadian clock to stay in sync. Constant darkness (DD) reveals that calcium oscillation is under circadian clock control. In DD, DvPdf-CaLexA-LUC keeps its calcium oscillation in sync with the entrained 12:12 LD cycles. DD is indicated by the grey shaded box and blue outlined box indicate the LD period. Grey and black alternating stripe shows theoretical 12:12 LD cycle. Red arrows indicate the night time anticipation peak.
Figure 1.3 – Constant Light Lengthens Period and Dampens Amplitude

Constant Light (LL) disrupts the circadian clock in flies. Without the destruction of circadian clock, calcium oscillation in DvPdf neurons gradually become arrhythmic and their period lengthens. As pointed out by the red arrow, the amplitude gradually decrease as LL period lengthens. Also, DvPdf-CaLexA-LUC males slowly lose their sync with entrained 12:12 LD cycle (left panel). The evening peak starts later in the night time as fly stays longer in LL condition.
Figure 1.4 – Post-mating Switch of Calcium Oscillating Pattern in Mated Female Flies

Averaged bioluminescence level of *CLK4.1M-GAL4 (DN1p driver)>CaLexA-LUC* males (red) and females (grey) was plotted. Shaded background depicts the dark periods. Before mating, virgin females and male flies show similar calcium oscillating pattern: big peak during the day time and a small peak during the night time. However, mated female circadian neurons’ calcium levels increase at lights off and decrease throughout the day. Mated female and male create an anti-phase.
CHAPTER TWO
Combination of Video Recording and Optogenetic

BACKGROUND

In the first chapter, neuronal activity of circadian neurons was recorded in freely moving flies in a 96-well plate format. Moreover, we were curious if flies kept in 96-well plate exhibited the known locomotor behavior pattern recorded in the classical behavior monitoring method, the *Drosophila* Activity Monitor (DAM). To satisfy this curiosity, a video recording system was installed to monitor fly behavior and locomotor activity in the 96-well plate.

As background, most labs use the standard *Drosophila* Activity Monitor (DAM) to record locomotor activity and sleep. DAM records activity from individual flies maintained in sealed tubes placed in activity monitors. An infrared beam directed in the midpoint of each tube records an activity each time a fly crosses the beam. However, this system has many disadvantages. It creates blind spots on the edges of the tubes where the fly could move a bit without being detected. In addition, DAM boards can only record 32 flies at a time. Also, the sheer size of the boards makes it difficult to combine external accessories such as LEDs for optogenetic. Consequently, we have created a long-term video recording system that uses standardized 96-well plate to record locomotor activity and visualize the behavior of individual flies.

In addition to the 96-well plate, we added LEDs for optogenetic stimulation. Using 96-well plate allows us to increase the sample size by three compared to a DAM board. Furthermore, 96-well plate is one-fourth the size of a DAM board, allowing a great saving of space and more accessibility to the samples. The flat surface and the compact wells of the 96-well plate also allows us to more uniformly illuminate LED to ensure that each wells gets even amount of LED light
pulse. This even illumination is harder to achieve in DAM behavior tubes because tube is round and big compared to individual well. Lastly, the video recording system decreases the amount of labor tremendously as there is no need to prepare behavior tubes or caps. In summary, the video recording system is more powerful than the classical DAM system, due to its higher throughput and small flat surface area to manipulate fly behavior by using LEDs.

This chapter is focused on the pioneering experiments that were done to figure out assay definition for LED experiments. Stimulation experiments were done with LED light pulses to investigate how neurons respond differently to different voltages. All-trans-Retinal (ATR) fed flies were loaded in the 96-well plate and then a one hour of LED light pulse was used to stimulate the flies expressing CsChrimson. Experiments were done using three different strands of neurons: DN1P-GAL4, Dvpdf-GAL4, and TH-GAL4. These three GAL4 lines were crossed with UAS-CsCrimson, which can be activated by 627nm red LED light. However, I focused more on the last two strains, as they were the ones I had the most to do with and the first one is submitted for publication.
RESULTS

This *Drosophila* behavioral activity is different between males and females. Males exhibit morning (M) activity followed by a strong siesta, which is followed by robust evening (E) activity. Females in contrast show very little M activity before the lights-on event. They also manifest a fairly constant high activity level during the daytime. Consequently, females have a quite modest siesta. This also affects E activity, as it is less distinct in females than in males due to the higher levels of preceding activity. As mentioned previously, mated females show dramatically different calcium oscillation pattern compared to the males and virgins. Here we show that video recording can reveal the same locomotor activity pattern like DAM—bimodal activity peak in males and prolonged daytime activity in females (Figure. 2.1).

To test how flies response to different neurons activation, we combined this assay with optogenetic to stimulate these neurons by using the LEDs. Compared to the classical temperature sensitive *dTrpA1* activation method, the optogenetic provides a fast manipulation, a precise control of stimulation and no abnormal activities evoked by temperature pulse. *UAS-CsChrimson* flies with different GAL4 lines were crossed and entrained for two days in ATR food before the experimental day. On the third day, flies were exposed to one hour LED light pulse with different voltages. Our data first proves the accuracy and the capacity of video recording system. Similar to classical DAM data, video recording also shows both morning and evening peaks accurately. In figure 2.2A, males keep siesta while females have higher locomotor activity during the mid-day.

In order to define the assay, flies were entrained for two days in ATR food and were LED light pulsed with different voltages (0.1V, 0.5V, 1V, 2V, and 3V) during the day time to find the most effective stimulation for each neuron type. For comparison, the flies expressing
CsChrimson in sleep promoting neuron, DN1s, and two activity promoting neurons including E cells, as well as dopaminergic neurons were tested. *DvPdf >CsChrimson* flies show increased activity at >1V LED stimulation (Figure 2.4C). In addition to having an optimal stimulation at >1V during the daytime (Figure 2.4C), *DvPdf >CsChrimson* has highest activity peak at 1V during the night time (Figure 2.6C).

Moreover, activating different circadian neurons have different responses. Control flies were affected very little by the LED light pulse (Figure 2.5A). While *DNIP >CsChrimson* flies show lowest activity, *DvPdf >CsChrimson* flies show highest activity level (Figure 2.5B-C). This is consistent with our hypothesis since *DNIP-GAL4* is mostly a sleep promoting neuron and *DvPdf-GAL4* is an activity-promoting neuron. Additionally, *DNIP >CsChrimson* have the best response to LED light intensity in range from 1V to 2V. Also, we can see that 0.1V is not an effective light intensity for any neurons during the night time (Figure 2.6). Higher voltage (3V) also cause reduced activity in control flies. Thus, the optimal effective voltage range for these neurons is from 1V – 2V.

We also observed that different neurons have different post-stimulation response (Figure 2.2B). *TH-GAL4 >CsChrimson* flies (dopaminergic neurons) were light pulsed for one hour at ZT 14. Even though it was a one hour LED light pulse, the activity-promoting effect lasted throughout the whole night. As the light intensity increased, the post- stimulation response became longer and greater. This response may reflect the overwhelming amount of dopamine released into the synaptic cleft; thus it takes a longer time to retake dopamine and return to homeostasis. In comparison, we can see that when another activity promoting E cell is activated, the DvPdf evening cells, the flies exhibited an increased activity during the LED period. After
the light LED stimulation, the flies quickly fell asleep. This observation could be due to the evening cell specific homeostatic regulation after neuronal firing (red arrows in Figure 2.2C).

Furthermore, during the LED light stimulation period, we found that TH-GAL4>CsChrimson flies have different response comparing to DvPdf-GAL4> CsChrimson flies. TH-GAL4>CsChrimson fly’s activity was jerky and more frantic (Figure 2.7 and supplementary video). Their wings were extended out and looked as if they are rolling over. These behaviors could be the sign of too much dopamine triggered by the LED. Unlike TH-GAL4>CsChrimson flies, DvPdf-GAL4> CsChrimson flies just increased their movement speed during LED period.
DISCUSSION

We have just started to reveal the tip of the iceberg on *Drosophila* optogenetic and video recording assays. I plan to do more experiments to figure out the optimal conditions. We have not yet explored the relationship between voltage and frequency. For example, a higher frequency might compensate for a lower voltage. There are so many possibilities to try to create a reference guide, and these may vary from one neuron type to another. In addition, the coding for the Arduino control board is not most suitable for the parameters used in our experiment. Currently, there is an error in the code that does not allow certain frequencies to be combined with certain voltages. This prevents us to fully explore the depth of the parameters. Thus, I will edit the coding to be more effective with fewer limitations.

As shown here, different circadian neurons react differently with LED light pulse. Activity-promoting neurons such as *TH-GAL4* and *DvPdf-GAL4* can increase activity of the flies during day time and night time. During the day time, the activity promoting neurons might have lower range of effectiveness since flies are already active during the day (Figure 2.4). However, during the night time, LED might be more effective than daytime since flies are less active during the night time especially in *DvPdf-GAL4* and *TH-GAL4* flies (Figure 2.2-3). Also, *DN1s* is known as sleep-promoting neurons. However, recently, it was known that *DN1P-GAL4* might have two different roles depending on the circadian time. At late night and early morning, DN1s can act as an activity-promoting neuron. In reverse, DN1s can also act as a sleep-promoting neuron in late day and early night (paper submitted). My findings confirm that *DN1P-GAL4* can be a activity-promoting neuron during the dawn (data not shown).

Some neurons could be overwhelmed with the LED light stimulation and will be sensitive to the light intensity. For example, *TH-GAL4>CsChrimson* have a long last effect after
the LED light pulse when voltage is higher than 0.5V (Figure 2.2B). This could be the result of
abundance of dopamine in synaptic cleft and the flies do not know what to do with it. Thus, flies
might go frantic and look unhealthy due to its sudden change in its chemistry. Same with the

*DvPdf-GAL4*, once the neuron fires extensively, it needs to refuel and recover to be ready to
depolarize and fire again. Thus, after the LED light pulse, *DvPdf-GAL4* flies try to return to their
homeostasis by going to sleep.

Overall, we determined that video recording system with LEDs can be used as a method to
record fly locomotor activity and stimulate circadian neurons to immediately see a targeted
response. This new method allows a lot of potential findings in circadian neurons, sleep, and many
more behavioral paradigms.
Figure 2.1 – Scheme of Video Recording and Optogenetic Strategy

Flies expressing CsChrimson were placed in 96-well plate and were recorded with a camera without an infrared filter (left panel). The 850nm back light provides illumination for recording in both light and dark periods. A 627nm LED was carefully positioned to ensure uniform irradiation. Assay definition was controlled by an Arduino UNO board. Representative data from a video recording of male and female activity (middle panel) and sleep (right panel) in LD are shown.
Flies were entrained for two days in ATR food. Red LED at 627nm was pulsed at ZT 14 for one hour. The red shaded box indicates these LED pulses (A–C). From left to right, different voltages (0.5V, 1V, 2V) were used in each day. The yellow shaded box represents the post-LED response period (B). The red arrow points at Dvpdf-Gal4 >UAS-Crimson flies’ low activity after the LED light pulse (C). Black and white stripe represents the 12:12 light and dark cycle.
Figure 2.3 -- Different Groups of Fly Response as a Function of LED Voltage at ZT14

Control flies remain their low activity at different light intensity (A). Control flies show normal behavior during early night time (B). DvPdf flies are sensitive light intensity. In DvPdf flies, light intensity has a linear relationship with locomotors activities. Error bars represent SEM.
Figure 2.4 – Different Groups of Fly Response as a Function of LED Voltage During day Time

The behavior response of control group (A), DN1p group (B) and DvPdf group (C) to different voltage of LED light pulses during the daytime. DN1P seems to promote sleep during the daytime (B). DvPdf has less responsive to LED light intensity since their activity is high during the daytime already. After certain amount of locomotors activity, there is a threshold to its maximum locomotors activity. Error bars represent SEM.
Figure 2.5 – Different Groups of Fly React Differently to LED Light Stimulation

The behavior response of activating different neurons at lower voltage (0.5 V) (A) and higher voltage (2V) (B) during the daytime. Flies react differently to LED light stimulation. W118>UAS-Crimson remains its activity at normal activity level at 20. While DN1P showing the lowest activity since DN1P promote sleep. And Dvpdf has the highest amount of activity since it is an activity promoting neuron. ** indicates p<0.001 and error bars represent SEM.
Figure 2.6 – Different Groups of Fly Response as a Function of LED Voltage during Night Time

The behavior response of control group (A), DN1p group (B) and DvPdf group (C) to different voltage of LED light pulses during the nighttime. DN1P flies show more effectiveness with increase voltage (B). Best light intensity for DvPdf flies are 1V as seen in other figure as well. Please note that 0.5V LED stimulation cannot trigger strong behavior change during the nighttime. Error bars represent SEM.
Figure 2.7 – Snapshot of TH-Gal4>UAS-CsCrimson Behavior

The snapshot of video recording with 96-well plate during the LED light pulse period. TH-Gal4>UAS-CsCrimson flies show this unusual wing extension behavior. When LED is irradiated to them, their wings extend out and flies start to move around frantically. The gender and genotype is labeled.
METHODS AND MATERIALS

Fly Strains

For bioluminescence assay, *UAS-CaLexA-LUC* was developed by Guo Fang. It was crossed with *DvPdf-GAL4* and *Clk4.1m-GAL4*. Flies were cultured on a standard cornmeal/agar medium supplemented with yeast. The adult flies were entrained in 12:12 light-dark (LD) cycles at 25°C.

For optogenetics experiments, *UAS-CsCrimson* virgins was crossed with *TH-GAL4*, and *DN1P-GAL4, Dvpdf-GAL4* males. Flies were cultured on a standard cornmeal/agar medium supplemented with yeast. The cross was cultured on a standard cornmeal/agar medium mixed with all trans-Retinal (Sigma Aldrich) in 1:250 ratio with dried yeast. Vials were covered in aluminum foil to prevent any light from degrading the drug, all trans-Retinal. The adult flies were mated and were entrained in 12:12 light-dark (LD) cycles at 25 °C.

Feeding of Retinal

All trans-Retinal powder (Sigma Aldrich) was dissolved in alcohol to prepare a 100mM stock solution for CsChrimson experiments (37). 100 µl stock solution was diluted in 25ml 5% sucrose and 1.8% agar medium to prepare 400 µM all trans-retinal (ATR) food. Newly enclosed flies from standard cornmeal/agar medium were transferred to ATR food for at least 2 days prior to optogenetics experiments. Flies from standard cornmeal/agar medium with ATR were also placed to ATR food in 96-well plate for 2 days prior to optogenetics experiments to entrain the flies.
**Optogenetics and Video Recording System**

The behavior setup for the optogenetics and video recording system is schematized in Figure 2.1 in chapter 2. ATR fed flies were loaded to white 96-well Microfluor 2 plates (Fisher) containing 5% sucrose and 1.8% agar food with or without 400uM ATR. Food was cooled in 4°C cold room and flies were placed in each well to entrain the flies in 12:12 LD cycle. The back light was supplied by an 850 nm LED board (Smart vision lights) located under the plate. Two sets of high power LEDs (627 nm) mounted on heat sinks (4 LEDs per heat sink) were symmetrically placed above the plate to provide most even amount of light stimulation among the plate. The angle and height of LEDs were adjusted to allow uniform illumination. The voltage and frequency of red light pulses were controlled by an Arduino UNO board (Smart Projects, Italy). The whole circuit was described in (38) but it was modified to fit the needs of the experiments. Fly behavior was recorded by a web camera (Logistic C910 HD 720p). IR filter from web camera was removed to enable night vision. To reduce potential computer and software crash, time-lapse software was used to capture snapshots in 10 second intervals. Fly movements were calculated by Pysolo software and transformed into a MATLAB readable file from compiled snapshots (39). The activity and sleep analysis were performed with a signal-processing toolbox implemented in MATLAB (MathWorks) or in Microsoft Excel. The LD cycle and temperature was controlled by incubator and the light intensity was maintained to entrain flies without activating CsChrimson.

**In vivo luciferase assays – TOPCOUNT Assay**

To monitor bioluminescence activity in living flies, we used previously described protocols (40). White 96-well Microfluor 2 plates (Fisher) were loaded with 5% sucrose and
1.8% agar food (to prevent larva growth) containing 20mM D-luciferin potassium salt (GOLDBIO). Each well consisted of 200 µl of 1.8% agar food and 50 µl of 1.8% agar food with D-luciferin potassium salt. D-luciferin potassium salt was diluted with distilled water. Individual flies expressing CaLexA-LUC were first anaesthetized with CO₂ and then transferred to the wells. Flies were contained by using adhesive transparent seal (TopSeal-A PLUS, Perkin Elmer) to cover the plate. Seals were poked with 2 to 3 times to allow sufficient amount of air flow. Plates were loaded into a stacker in a TopCount NXT luminescence counter (Perkin Elmer). Assays were carried out in an incubator under 12:12 light dark conditions. Luminescence counts were collected for 5-7 days. The raw data were analyzed in MATLAB and with Microsoft Excel. Experiments were repeated at least three times with similar results.
A.1 SUPPLEMENTARY FIGURES

Supp. Figure 1. —Molecular feedback loops of Circadian rhythm in Drosophila melanogaster

(Left) CLOCK/CYCLE heterodimer acts as transcriptional activator (positive element) for period (per) and timeless (tim) genes. The heterodimer of PER/TIM is phosphorylated in the cytoplasm in the presence of specific kinases, and the phosphorylated complex then acts as inhibitor for its own transcription (negative element). (Right) The VRI and PDP1 proteins regulate the levels of CLK/CYC complex, which in turn are regulated by CLK/CYC. Thus, CLK/CYC heterodimer appears to be an important component that connects the two loops and is important for sustaining molecular oscillations. The protein Cryptochrome (CRY) has been implicated in the light entrainment pathways of the Drosophila molecular clock.
Supp. Figure 2. – Overview of Circadian Neuronal Network

A basic overview of clock-gene-expressing neurons and their neurochemical characterization. The different colors of the neurons indicate the peptides/proteins that are expressed in those cells. Namely Cryptochrome (Cry) in yellow, Pigment dispersing factor (PDF) in green, short Neuropeptide F (sNPF) in blue, Neuropeptide F (NPF) in red, ion transport peptide (ITP) in gray, choline acetyltransferase (Cha) in purple and the IPN-amide in orange. Cells with unknown peptidergic content are colored in black. Aborization of PDF is indicated in green.

Supplementary Video – Time-lapse of One Hour LED-Light Pulse at Night Time

Column 1,2,11, and 12 are empty. Column 3-4 are UAS-CsCrimson male and female in order. UAS-CsCrimson was used as a control. Column 5-6 are DN1P>CsChrimson male and female. Column 7-8 are DvPdf>CsChrimson male and female. Column 9-10 are TH-GAL4>CsChrimson male and female. Control flies are constantly active. While DN1P>CsChrimson flies falls asleep. Both DvPdf>CsChrimson and TH-GAL4>CsChrimson are actively moving. However, there is a difference in the movement. DvPdf>CsChrimson flies move more freely while TH-GAL4>CsChrimson flies move in jerking motions with their wings extended.
REFERENCE

18. Helfrich-Förster C. (1995) The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian


