Investigating the circadian function of potential neuropeptide CG17777 in *Drosophila*

Master’s Thesis

Presented to

The Faculty of the Graduate School of Arts and Sciences
Brandeis University
Graduate Program in Molecular and Cell Biology
Michael Rosbash, Advisor

In Partial Fulfillment
of the Requirements for the Degree

Master of Science
In
Molecular and Cell Biology

by

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February 2020
ABSTRACT

Investigating the circadian function of potential neuropeptide CG17777 in Drosophila

A thesis presented to the Graduate Program in Molecular and Cell Biology

Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts

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Rhythmic behaviors regulated by the circadian clock are essential for living creatures and Drosophila is an ideal model in which to study them. About 150 neuron control circadian rhythms housed in the Drosophila brain, and they rely on molecules like neuropeptides to communicate with each other to be synchronous. Only seven neuropeptides have been identified within clock neurons so far. CG17777 is a candidate novel neuropeptide that is expressed in the circadian network including the LNds, DN1as and possibly the LNvs and DN1s. CRISPR deletion of CG17777 showed hyperactivity specifically during the day. Knockdown of CG17777 using RNAi in the central clock or eyes might be responsible for a higher activity in the evening period. Thus, we concluded that CG17777 might represses daytime activity in Drosophila. Interestingly, when knocking down CG17777 in two of the LNds cells, the flies showed less daytime activity. CG17777 might play different roles in different neurons. Also, circadian period of CG17777 knockdown or CRISPR deletion files is normal, so CG17777 may not regulate periods alone.
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Introduction

Organisms from bacteria to humans possess circadian rhythms like feeding, sleep and reproduction. These rhythms are regulated by the circadian clock which receives environmental signals such as light and temperature to adapt to the light–dark cycles (LD) then generate a near-24-hr period. These rhythms persist in the absence of any external cues with the almost same period. As a well-studied model in circadian field, *Drosophila* exhibit a bimodal activity pattern with one bout of morning activity close to lights-on and one evening peak near the lights-off under standard 24hr light conditions (12hr light and 12hr dark) (Figure 1).

The circadian clock, also known as the molecular circadian oscillator, relies on two delayed transcriptional negative feedback loops composed of core genes to regulate rhythmic behavior and physiology. About 150 clock neurons in *Drosophila* brain expressing clock genes and they are defined by expression of core circadian proteins including Clock (CLK), Cycle (CYC), Period (PER), and Timeless (TIM) (Houl et al. 2006; Kaneko and Hall. 2000). In the primary feedback loop, CLK and CYC form a transcriptional activator complex that binds to the promoters of *per* and *tim* as well as many other genes to regulate their transcription (Figure 2; Yu et al. 2006; Taylor and Hardin 2008). PER and TIM proteins are translated then assembled to form a transcriptional repressor complex.
The PER/TIM complex is imported into the nucleus where it represses the activity of CLK/CYC complex (Yu et al. 2006; Abruzzi et al. 2011). The degradation of TIM is induced by light, thus dawn converts PER/TIM complexes to nuclear PER that persists for 4–6 hr (Adrian et al, 2000). The CLK/CYC complex is released from PER/TIM to initiate a new round of transcriptional activity and closes the feedback loop. These events happen with a 24 hours periods. In the second feedback loop, CLK/CYC complex mediates the transcriptional activity of vrille (vri) and par domain protein 1 (pdp1ε), which are translated into protein PDP1ε and VRI. PDP1ε promotes the transcription of Clk and cyc, whereas VRI inhibits it so the two proteins together to make sure it is rhythmic Clk transcription (Cyran et al. 2003). There is also a core clock gene clockwork orange (cwo) that cycles like PER and temporally represses CLK/CYC activity like PER/TIM (Kadener et al. 2007; Lim et al. 2007; Matsumoto et al. 2007). Clk and cyc, along with the five CLK/CYC direct target genes-per, tim, vri, pdp1ε, and cwo are considered core clock genes and act as a molecular oscillator to maintain molecular circadian rhythms of the Drosophila molecular clock. Loss-of-function mutations in any of these core genes generate arrhythmic behavior in constant darkness.

The 150 neurons in the circadian network are divided into seven neuronal clusters according to their anatomical location (Yoshii T et al., 2012; Tataroglu et al., 2014). There are three dorsal neuron groups: DN1s, DN2s and DN3s and four lateral neuron groups (LNs) including the lateral posterior neurons (LPN; 3 neurons), dorsal lateral neurons (LNds; 6 neurons), the small

![Figure 2. Transcriptional negative feedback loops in the core clock. CYC and CLK activate transcription of per/tim while PER and TIM block the activity of CLK/CYC. CLK/CYC also mediates transcription of vri and pdp1ε which in turn promotes the transcription of Clk and cyc (Zheng and Sehgal, 2008).]
ventral lateral neurons (s-LNvs; 5 neurons) and the large ventral lateral neurons (l-LNvs; 4 neurons)(Figure 1). The s-LNvs are also known as morning cells (M-cells) and are sufficient to drive morning anticipation and can be easily identified as they express the neuropeptide pigment dispersing factor (PDF). The LNds and the fifth s-LNv are regarded as evening cells (E-cells) as they drive the evening peak (Grima et al., 2004; Stoleru et al., 2004).

These different neuronal clusters need to respond to signals from the environment and rely on neuropeptides and neurotransmitters to maintain the normal function of circadian clock within the circadian network. Neurotransmitters are chemicals which quickly transmit signals from a neuron to a target cell through a synapse and produce a short-term response. Neuropeptides are relatively large and are derived from about 90 amino acids inactive precursors which contains a signal sequence that initiates its journey into secretory pathway. During export, the signal sequence from the neuropeptide precursor is removed to produce the active peptide. After the neuropeptides are synthesized inside the neuron, they are packaged into LDCV (large dense core vehicle) and released in the synapses. Ca2+ ions are used for the release of LDCVs. Neuropeptides help with signal transduction within the circadian network that control behaviors such as sleep and locomotor activity. However, due to large number, huge diversity, low expression, and signaling complexity, signaling pathways of most neuropeptides remain largely unknown.

So far, only seven neuropeptides have been identified in the circadian clock. The neuropeptide pigment dispersing factor (PDF), as mentioned above, is expressed in the LNvs. It helps with the synchronization of most of the circadian neurons, and is necessary to maintain rhythmicity in constant darkness (Renn et al, 1999)(Peng et al, 2003)(Lin et al, 2004). The l-LNvs also express neuropeptide F (NPF) (Hermann et al, 2012), and the s-LNvs also express short NPF (sNPF) (Lohard et al, 2009). Previous studies showed that *Drosophila* NPF regulates adult feeding...
and sleep-wake behavior, and sNPF is a sleep-promoting inhibitory modulator (Chung et al., 2017)(Shang et al., 2013). The 5th s-LNv and five of the six LNds express three neuropeptides: NPF, sNPF, and ion transport peptide (ITP) (Hermann et al., 2012 & 2014) (Lohard et al., 2009). Neuropeptides IPNamide and CCHamide1 are expressed in the DN1as (Shafer, 2006) (Fujiwara et al., 2018). The neuropeptide diuretic hormone 31 (Dh31) is synthesized from five of the DN1ps and may have some effect on sleep (Kunst et al., 2014).

In order to identify more neuropeptides, RNA-sequencing (RNA-seq) was exploited to profile the transcriptomes of three groups Drosophila clock neurons: LNvs, LNds plus 5th s-LNv and DN1s (Abruzzi et al., 2017). We found that the transcript of CG17777 is highly enriched in all three neuron groups and cycles with different phases in the LNvs and DN1s (Abruzzi et al., 2017). These transcripts peak in the early morning and are lowest during the mid-day in LNvs, but they are lowest in early morning and peak at mid-day in DN1s (Abruzzi et al., 2017). More recent single-cell sequencing showed that CG17777 is highly expressed and the transcripts cycles with a peak in the early morning and becomes lowest at mid-day in LNds and DN1as (D. Ma and D. Przybylski, unpublished). NeuroPID (http://neuropid.cs.huji.ac.il) and NeuroPred (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py) were used to test whether circadian expressed genes exhibit typical characteristics of neuropeptides (Abruzzi et al., 2017). NeuroPID was used to identify neuropeptide precursors including the signal peptides and NeuroPred was used to predict the potential cleavage sites and their possibility. Analysis of the putative neuropeptide precursors of CG17777 indicated that CG17777 shares some similar characteristics with PDF, containing a signal peptide at the N-terminal and cleavage sites (K. Abruzzi, personal communication).
The function of CG17777 is poorly understood. Some studies showed that the level of CG17777 mRNA has some relationship with ion channels and perhaps neuron firing. One study showed that CG17777 levels are downregulated upon knockdown of the endoplasmic reticular calcium sensor, \emph{dStim}. \emph{dStim} is a principal component of Store-Operated Calcium Entry (SOCE) which is used to refill the endoplasmic Ca\textsuperscript{2+} stores (Richhariya, 2017). In another paper, researchers used NachBac, a low activation threshold voltage-gated Na\textsuperscript{+} channel from bacteria to increase LNv excitability in the evening. They found that CG17777 mRNA upregulated about 11-fold change in LNvs expressing NachBac at CT15 (NachBac15) and 6-fold in NachBac15>WT15 in per01 background (Mizrak et al, 2012). These papers suggested that CG17777 might be regulated by ion channels and respond to firing of some neurons.

In this thesis, we examined the locomotor behavior of the CG17777 CRISPR deletion and CG17777 RNAi knockdown flies. CRISPR deletion of CG17777 showed hyperactivity specifically during the day. Knockdown of CG17777 using RNAi in the central clock or eyes showed a similar behavior with a higher activity in the evening period. After knocking down CG17777 in two of the LNds cells, however, the flies showed less daytime activity. Thus,
CG17777 might play different roles in different neurons. Also, circadian period of CG17777 knockdown or CRISPR deletion files is normal, so CG17777 may not regulate periods alone.
Methods

Fly stocks and maintenance

*Drosophila melanogaster* strains were reared on standard cornmeal-agar medium, with added yeast in standard LD 12:12 condition at 25°C. Young adult flies (3-4 days old) were used in all experiments. The source of fly strains is indicated in the Key Resources Table.

DNA Cloning and Plasmid Generation

The 5×UAS-CG17777 plasmid construct was generated using a full-length CG17777 cDNA clone. CG17777 gene was PCR amplified using a forward primer KA1422 containing an *Xho*I site and a reverse primer KA1423 containing an *Xba*I site. The amplified fragment was ligated into the pJFRC5 transformation vector via Gibson assembly. 5×UAS-CG17777::GFP plasmid construct were generated in the same way except the reverse primer is KA1424 which contains a *BamHI* site this time.

Four plasmids are needed in SEAP assay. One experimental plasmid pMT-CG17777-AP, one positive control plasmid pMT-BiP-AP, one negative control plasmid pMT-AP and one pMT-GFP plasmid. In order to make experimental plasmid pMT-CG17777-AP, CG17777 gene was PCR amplified using a primer KA1316 containing *EcoR*I site from MIP plasmid (bKA169) and a primer KA1317. The gene of AP was PCR amplified from pEC1A14 using a primer KA1207 containing an *Xho*I site and a primer KA1391. First, I did an overlap PCR to ligate CG17777 gene fragment
and AP gene fragment. Then I ligated this combined fragment into pMT/V5-His A vector by T4 DNA ligase. Sequencing from the construct showed that the AP gene had been ligated successfully but there is a ~100 bp deletion in the CG17777 gene ORF. Then I took this plasmid that has deletion as the backbone and digested it with EcoRI and BamHI to linearize it. The plasmid was finally constructed by a three-piece Gibson Assembly ligating CG17777 gene, ap gene and the backbone. The control plasmids were made by other members in our lab. The sequence of primers is indicated in Table S1.

S2 cell transfection and Secreted alkaline phosphatase (SEAP) assay

SEAP assay is to determine whether CG17777 can be secreted. The assay was performed in S2 cells. The positive control plasmid, the negative control plasmid and the experimental plasmid were cloned from pMT/V5-His vector. This vector contains the inducible metallothionein promoter (pMT) that is activated by addition of copper sulfate. The N-terminal signal sequence from the insect BiP gene can guide the recombinant fusion protein through the secretory pathway of S2 cells into the culture medium. The positive control plasmid contains the gene sequence of insect BiP gene and human AP (alkaline phosphatase). The negative control contains the AP gene only. The experimental plasmid contains the cDNA sequence of CG17777 fused to the DNA of N-terminus of AP. If AP is secreted into the culture medium, the mix of the medium and a solution from SEAP kit will turn blue. The positive control is to make sure that the SEAP system works well. The negative control is to make sure that AP itself cannot be secreted. Thus, the blue signal that get from the experimental group is caused by a signal sequence in CG17777 driving the secretion of the CG17777-AP fusion.
At first, I transfected 2ul of each plasmid DNA using PEI (protocol was adapted from Josh Lepson in our lab) into S2 cells (2.5 x 10^6) maintained in 2.5 ml of S2 medium with 10% FBS per well of a 6-well plate. After inducing with copper for 24hr, medium was centrifuged at 300g for 5 min and mixed 20 ul supernatant with 200 ul solution from KPL BluePhos Microwell phosphatase substrate system (SeraCare Life Sciences, Inc No: 5120-0061). Later, I co-transferred each plasmid DNA with pActin-GFP to monitor the transfection efficiency.

Immunostaining

Flies were entrained for three days before collecting at respective time points. Whole flies were fixed in PBS with 0.5% Triton X-100 (PBS-T) for 2hr and 40min at room temperature while rotating. Fixed flies were washed in PBS with 0.5% Triton X-100 (PBS-T) and then fly brains were dissected in PBS-T, blocked in 10% normal goat serum (NGS in 1×PBST; Jackson Immunoresearch) for an hour at room temperature and later incubated with primary antibodies-monoclonal anti-CG17777 (guinea pig, 1:1000 dilution) at 4°C for three nights. After washing with PBST, the brains were incubated with either Alexa Fluor 488-conjugated anti-mouse (1:500 dilution in 10% NGS). Brains were then washed and mounted in 50% glycerol. The slides were viewed on a Leica SP5 confocal microscope with a 20× objective.

Locomotor Behavior Assay

We used Drosophila Activity Monitoring (DAM) system (Trikinetics, Waltham, MA, USA) to record the times of beam crosses of the flies in one-minute intervals. Flies first underwent 5 days of entrainment under 12:12 light: dark (LD) then tested under 12:12 dark: dark (DD) conditions for additional five days. For the sleep assay, flies were monitored for 5 days of 12:12 LD. The flies were put individually in 65×5×5 mm glass tubes that are bisected with an infrared
beam. If the fly breaks the beam, the system would record this as an activity event. Flies who do not move for more than 5 min were regarded as in a sleep state. For the determination of the evening peak timing under long days, the flies were first incubated under 8 days of standard 12:12 LD cycle then transferred to 20:4 LD for three additional days. The first day of locomotor activity was not used when doing analysis. The data is analyzed by Sleep and Circadian Analysis MATLAB Program (S.C.A.M.P.), excel, or ShinyR. The temperature in the incubators is 25°C.
Progress and Results

Characterization of CG17777 as a secreted neuropeptide by SEAP assay

Computational approaches suggested that CG17777 had characteristics of a neuropeptide including an N-terminal signal sequence and cleavage sites. To test whether CG17777 was secreted, I performed secreted alkaline phosphatase (SEAP) assay in S2 cells. BiP-alkaline phosphatase (AP) (positive control). AP alone (negative control) and CG17777 fused to AP were cloned under the control of the metallothionine promoter that can be induced using copper. These plasmids were transfected into S2 cells and half of the samples were induced with copper. If alkaline phosphatase (AP) is secreted in the media, it can be detected using a colorimetric assay; blue color indicates the presence of AP. As shown in Figure 4, the positive control generated an induction dependent blue signal within 30 min. However, neither the negative control nor the experimental sample turned blue. We have been unable to replicate this result due to technical difficulties but this preliminary experiment suggests that CG17777 is not sufficient to drive secretion of AP in S2 cells.

Figure 4. SEAP assay showed that CG17777 does not secreted. Row 1-3: Culture medium from S2R+ cells expressing a fusion of AP with the CG17777, a fusion of AP with the immunoglobulin binding protein (BiP; positive control), or alkaline phosphatase (negative control). Row 4 is the medium only control. The four panels show the result at 0min, 10min, 20min and 30min respectively. + and - indicate with or without induction via Cu2+SO4.
CRISPR deletion of CG17777 lead to less daytime sleep and hyperactivity.

The molecular clock regulates daily locomotor rhythms as well as impacting sleep. If CG17777 plays an important role in the circadian neurocircuit, deletion or overexpression of CG17777 may lead to changes in activity rhythms and/or sleep. We assayed behavior and sleep in two fly lines in which CG17777 was deleted using CRISPR (two separate isolates: deletions 9 and 10) as well as a control line from the same genetic background. The circadian period was normal in CRISPR deletion flies (Figure S1). Both CG17777 deletion isolates showed increased activity during the day especially during evening anticipation (Figure 5A and 5B). As sleep and activity are intrinsically related, it was not surprising that CG17777 deletion flies also showed an approximately 2-fold reduction in the daytime sleep (Figure 5C, compare red and pink to the control-black). As a consequence, total daily sleep was also decreased (Figure 5D).

Figure 5. CRISPR deletion of CG17777 led to hyperactivity during the day. (A and B) Normalized average activity profiles (A) and average sleep profiles (B) for CRISPR CG17777 deletion isolates #9, #10 and CRISPR deletion background flies under standard 12:12 LD conditions. (C) Normalized averaged actograms CRISPR CG17777 deletion isolates #9, #10 and CRISPR deletion background flies under long photoperiods 20:4 LD conditions. (D) Mean day activity. (E) Average day sleep. In this figure and all the others, error bars represent SEM. White and dark boxes indicates the respective light and dark phases. *p<0.001, one way ANOVA.
The increase in evening locomotor activity observed in the CG17777 deletion lines could be due to a change in the timing of the evening peak or simply hyperactivity of the flies. To distinguish between these two possibilities, we performed a behavior assay under long photoperiod conditions (20:4 LD), under which the evening peak is dissociated from lights-off transition at ZT20. In wild-type flies, the evening peak timing would be adjusted to match the new lights-off timing. If CG17777 affects evening peak timing, the E-peak in deletion flies would be slow to adjust to the new timing. Both CRISPR deletion flies and background flies exhibited the maximum E-peak at around ZT14 while the wild-type strain (w1118) adjust to the new lights-off timing and had a maximum E-peak at around ZT16 (Figure 5E). This shows that both the CG17777 deletion flies and their wild-type fly in the same background are deficient in evening peak timing. It also suggests that the 12:12 LD differences between the CRISPR deletion of CG17777 and the background control are likely to be hyperactivity instead of a problem with E-peak timing.

**Modulating CG17777 in circadian neurons results in sleep and activity phenotypes**

CG17777 is not only expressed in brains but also highly expressed in somatic tissues like eyes, the second segment of antenna and testis. In order to determine where CG17777 acts to promote decreased activity, we knocked down CG17777 using two RNAis (296-2 and 395-5) with TIM-GAL4 (expressed in clock neurons, glia and optic lobe), CLK856-GAL4 (expressed in all clock neurons not optic lobe) and Dv-pdf-GAL4 drivers (expressed in LNvs and LNds). To test whether overexpression of CG17777 has a phenotype, we also generated a 5×UAS-CG17777 transgenic flies (see Methods) and examined overexpression using TIM-GAL4, CLK856-GAL4, and Dv-pdf-GAL4 drivers. CG17777 knockdown with TIM-GAL4 showed lower activity first in the day, then the activity goes up quickly just before the lights off and formed a higher E-peak (p<0.001, Figure 6A and 6B). The phenotype observed in CG17777 knockdown flies using
TIM-GAL4 driver is limited to male flies with no effect on female flies. The period length is normal compared to the two controls (Figure S1).

Figure 6. RNAi knockdown of CG17777 in tim-expressing cells led to more activity in day. (A and B) Normalized average activity (A) of male flies: 395-5 RNAi CG17777 knockdown, RNAi only control and the driver only control in all clock neurons mediated by TIM-GAL4 under standard 12:12 LD conditions. (C and D) Mean day activity or sleep. *p<0.001, one way ANOVA.

Then we asked whether knockdown of CG17777 in the glia or neurons is responsible for the phenotype. We performed CG17777 knockdown using TIM-GAL4; REPO-GAL80 and REPO-GAL4 driver. Both knockdown with two different drivers showed normal daytime activity and normal E-peak. TIM-GAL4, REPO-GAL80 mediated CG17777 knockdown showed normal averaged daytime activity but a statistically significant wider and lower E-peak (p<0.05, Figure 7A). The mean activity was decreased indicated by a smaller E-peak when CG17777 was knocked down using REPO-GAL4 (p<0.001, Figure 7B), suggesting that knocking down CG17777 in glia
may not contribute to the significant E-peak observed in CG17777 knockdown with TIM-GAL4. The period length is normal as well (Figure S1). In addition, only a few cells in glia expressing Figure 7. CG17777 in clock neurons or eyes might associate with the decreased activity observed in tim-GAL4>RNAi knockdown. (A and B) Normalized average activity profiles for flies of the following genotypes: TIM-GAL4/REPO-GAL80 >395-5 RNAi, TIM-GAL4/REPO-GAL80 only, REPO-GAL4>395-5 RNAi, REPO-GAL4 only and 395-5 RNAi only under standard 12:12 LD conditions. (C and D) Mean activity of each genotype. *p<0.05, one way ANOVA.
Figure 8. RNAi knockdown of CG17777 in split evening cell led to less daytime activity. (A and B) Normalized average activity for 395-5, 296-2 RNAi knockdown (A) and 105327 knockdown (B) with Split E-GAL4 and control flies under standard 12:12 LD conditions. (C and D) Mean day activity. (E-G) Normalized average sleep for 395-5, 296-2 RNAi knockdown (F and G) and 105327 knockdown (E) with Split E-GAL4 and control flies under standard 12:12 LD conditions. (H and I) Average day sleep. *p<0.001, one way ANOVA.
CG17777 when using Scope (http://scope.aertslab.org/#/d7a1233f-4af3-4b39-9afb-f701550b04e6/*/welcome)(Kristofer et al, 2018). The RNAi knockdown experiments using TIM-GAL4/REPO-GAL80 and REPO-GAL4 had only been done once.

Single-cell sequencing showed that CG17777 is highly expressed in the circadian neuronal network in both the LNds and the DN1as (D. Ma and D. Przybylski, unpublished). To test whether CG17777 expression in these neurons is important for circadian rhythms or sleep, we preformed CG17777 knockdown in two different LNd-specific drivers: LNd-GAL4 (expressed in all LNd cells) and Split E-GAL4 (expressed in two LNd cells) as well as a DN1a specific driver: DN1a-GAL4. Although two of the three replicates of CG17777 knockdown using Split E-GAL4 led to less daytime activity and more daytime sleep (Figure 8A and 8B), down regulating CG17777 in all three LNd cells looks normal (p<0.001; Figure 9). We also overexpressed CG17777 using Split E-GAL4 but activity was normal (p>0.1) (data not shown). There is no change in period length of transgene flies using LNd-GAL4 and Split E-GAL4 (Figure S1). In addition, knockdown of CG17777 with DN1as resulted in less daytime activity, which is a similar trend as knockdown using Split E-GAL4 (P<0.001; Figure 10).

![Figure 9.](image-url)
Figure 10. Knockdown of CG17777 in DN1a cells lead to less daytime activity. (A and B) Normalized average activity of flies: 105327 RNAi CG17777 knockdown, RNAi only control and the driver only control in all clock neurons mediated by DN1a-GAL4 under standard 12:12 LD conditions. (C) Mean activity per day in LD. (D-E) Average sleep in LD. *p<0.001, one way ANOVA.
Discussion and Future Directions

To learn about the circadian function of a potential neuropeptide CG17777 which was discovered in RNA-seq data, we modulated CG17777 levels to see how it affects *Drosophila* circadian rhythms and sleep. The CG17777 CRISPR deletion flies showed hyperactivity during the day (Figure 5). This suggests that CG17777 may function to suppress daytime activity. However, due to the high expression of CG17777 in somatic tissues, we are not sure where this phenotype comes from. The RNAi knockdown of CG17777 using TIM-GAL4 showed hyperactivity in the form of a higher E-peak which is similar to the CRISPR deletion flies (Figure 6). It seems likely that this phenotype is due to CG17777 in the circadian network or the eyes, as TIM-GAL4/REPO-GAL80 showed less daytime activity instead of hyperactivity. To rule out CG17777 function in the eyes, knockdown CG17777 in the eyes using GMR-GAL4 and/or TIM-GAL4/GMR-GAL80 needs to be performed.

Furthermore, knockdown of CG17777 in two LNds led to less daytime activity and more daytime sleep while knockdown of CG17777 in three LNds led to normal activity (Figure 10). It could be possible that CG17777 in the two LNd cells could promote E-peak while CG17777 in the third LNd cells suppress E-peak. In other words, CG17777 from these two group serves opposite function to generate a normal E-peak. To further test this model, we could make a LNd-GAL4/Split E-GAL80 to drive RNAi knockdown. However, it is more likely that LNd driver (Dv-pdf-gal4, pdf-GAL80) is weaker than Split E driver. Overexpression experiments and knock down experiments using LNd-GAL4, DN1a-GAL4, REPO-GAL4 and TIM-GAL4/REPO-GAL80 have only been done once and still need replicates.
Previous studies examining the role of the neuropeptide ITP in the circadian network showed that ITP knockdowns had no phenotype unless the major neuropeptide in the circadian network, PDF, was also knocked down (Hermann and Christiane, 2014). Inspired by this experiment, we also want to test the behavior of double mutants of CG17777 and PDF.

One critical experiment that has not yet been completed is a test of whether the phenotypes we observed for CG17777 are due to its role in development. To test this, we would need create an inducible driver such as a gene switch (GSG) driver under the control of the TIM or a LNd-specific promoter (Sun et al., 2013)(McGuire et al., 2004). Alternatively, we could utilize a GAL80-temperature sensitive allele to repress the GAL4 driver during development.

As mentioned in the introduction, several studies suggest that CG17777 expression may be linked to neuronal firing (Mizrak et al, 2012) (Richhariya, 2017). To test this hypothesis, we entrained two groups of flies (CLK856-GAL4>dTripA1::EGFP) at 20°C for 2 days, then fired CLK856 driven neurons by transfer one group of flies into 30°C incubator at ZT14 and incubated for an hour. RNA-sequencing libraries have been made from both the control and experimental flies and should be sequenced in the coming weeks. We will examine the changes of mRNA level of CG17777 and other related genes.
### Appendix

#### Supplementary Table 1: List of Fly Stocks

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig monoclonal anti-CG17777</td>
<td></td>
</tr>
<tr>
<td><em>D. melanogaster: w1118</em></td>
<td>Helfrich-Förster lab</td>
</tr>
<tr>
<td><em>D. melanogaster: TIM-GAL4</em></td>
<td>Laboratory of Jeff Hall, USA</td>
</tr>
<tr>
<td><em>D. melanogaster: Clk856-GAL4</em></td>
<td>Laboratory of Orie Shafer, USA</td>
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<tr>
<td><em>D. melanogaster: UAS-CG17777 RNAi</em></td>
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<td><em>D. melanogaster: Dv-pdf-GAL4</em></td>
<td>Laboratory of Jae Park, USA</td>
</tr>
<tr>
<td><em>D. melanogaster: split E cell GAL4 (MB122B-GAL4)</em></td>
<td></td>
</tr>
<tr>
<td><em>D. melanogaster: Control KK</em></td>
<td>VDRC ID: 60100 KK</td>
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<td><em>D. melanogaster: UAS-CG17777 RNAi 296-2 (Background Control is 36303 KK)</em></td>
<td>Laboratory of Michael Rosbash, USA</td>
</tr>
<tr>
<td><em>D. melanogaster: UAS-CG17777 RNAi 395-5 (Background Control is 36303 KK)</em></td>
<td>Laboratory of Michael Rosbash, USA</td>
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<tr>
<td><em>D. melanogaster: Control 36303 KK</em></td>
<td>Bloomington Stock Center</td>
</tr>
<tr>
<td><em>D. melanogaster: UAS-CG17777 overexpression</em></td>
<td>Laboratory of Michael Rosbash, USA</td>
</tr>
<tr>
<td><em>D. melanogaster: CRISPR deletion CG17777 line 9 &amp; 10 (different isolates)</em></td>
<td>Laboratory of Michael Rosbash, USA</td>
</tr>
<tr>
<td><em>D. melanogaster: DN1a-GAL4 (R23E05-Gal4 tsh-GAL80)</em></td>
<td></td>
</tr>
<tr>
<td><em>D. melanogaster: LNd-GAL4 (Dv-pdf-GAL4 PDF-GAL80 R78G02-GAL4)</em></td>
<td></td>
</tr>
</tbody>
</table>
Supplementary Table 2: List of Primer Sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>KA1316</td>
<td>CGAGAATTCTGATGTTCTTTGCGTGTTCTGT</td>
</tr>
<tr>
<td>KA1317</td>
<td>GAAGTCCGGGTCTCTCTCTCCAATGGAGTACTGCCTCGGCGCCCGCTAGAAGCGTACTGATGGG</td>
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<tr>
<td>KA1297</td>
<td>ATCATCCAGTGAGAGAGGAG</td>
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<td>KA1391</td>
<td>ACTCTCGAGGTACCGGTTGCGCGCGTTCCGCTG</td>
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<tr>
<td>KA1422</td>
<td>TACTTCAGGGCCGCCTGAGATGCTCTGTGCTGTGTTTCGTCA</td>
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<tr>
<td>KA1423</td>
<td>GCTTTCTCTTACTCATGGATCGTGAAGCCGTAGTGCCATG</td>
</tr>
<tr>
<td>KA1424</td>
<td>GAAGCTACTCTCTCACAAAGATCCTAGTAGAAGCCGTAGTGCCATG</td>
</tr>
</tbody>
</table>

Supplementary Figure 1: Circadian period of RNAi knockdown or CRISPR deletion flies is normal
Supplementary Figure 2. CG17777 antibody is not specific.

One pervious situ data in our lab showed that CG17777 protein localized in LNds and immunostaining study showed that is visible only in the LNvs. In order to test the specificity of the antibody of CG17777, we conducted immunostaining using CRISPR deletion CG17777 isolates 9 and 10 and background flies. Several replicates showed that the l-LNvs cells of both CRISPR deletion CG17777 isolates and background flies were stained by CG17777 antibodies.

Figure 4. Immunostaining of CG17777 CRISPR deletion fly brains and control. About 4 brains were tested in each type and in both cases. Red circles showed the location of stained l-LNv cells which were zoomed in on the right.
Supplementary Figure 3: Replicates of CRISPR deletion of CG1777
References:


Hermann, Christiane. (2014). The role of the neuropeptides NPF, sNPF, ITP and PDF in the circadian clock of Drosophila melanogaster.