R1 Neural Circuitry on Ellipsoid Body Promotes Sleep in *Drosophila Melanogaster*

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Abstract

This research identified sleep promoting R1 neurons on *Drosophila* ellipsoid body, but did not locate PPM3 neurons as upstream signal input in this sleep promoting R1 neural circuit. Two GAL4 labeled R1 neuron lines, VT058968-GAL4 and R28E01-GAL4, were selected and identified as sleep promoting ellipsoid body neurons, by utilizing *Drosophila* heat activated transient receptor ion channel, dTRPA1, to activate ellipsoid body R1 neurons (Brody 2015). The research also facilitates the tetanus toxin light chain (TNT) to inhibit the R1 neural activities constantly, and look at locomotor assays on flies (Umezaki et al. 2011). Then we considered dopaminergic PPM3 neurons as the possible candidate for upstream neural input in R1 sleep promoting circuit. PPM3 neurons are dopaminergic neurons and they project to dorsal fan shape body and R2 neurons on ellipsoid body to mediate sleep promotion. The anatomical connectivity between PPM3 neurons and R1 neurons were confirmed by RFP and GFP confocal imaging, and R1 neurons were identified as dopaminergic, as they were activated by dopamine bath in GCamp6f and EPAC assays. Behavioral assays were performed on two GAL4 labeled PPM3 neurons, VT040016-GAL4 and VT024624-GAL4, which were activated by dTRPA1 at sensitive temperature and inhibited by constant expression of TNT. The locomotor assays indicated activated PPM3 neurons participated in sleep promotion during evening and early night time. However, TNT inhibition on PPM3 did not induce sleep reduction or any other sleep phenotype in transgenetic flies. As PPM3 also innervates other sleep promoting circuit, the partial inhibition of PPM3 neurons still have intact sleep promotion circuit in brains. Therefore, PPM3 neurons are still considered as upstream neurons that mediates R1 sleep promoting circuit on ellipsoid body.
Introduction

Sleep is an essential behavior that is conserved widely across animals, and important to memory and learning performance as well as to health in general (Schoofs, De Loof, and Van Hiel 2016). To understand the cellular basis of sleep regulation, multiple studies facilitated the model of Drosophila owing to its highly conserved sleep behaviors compared to mammals and its relatively simple brain structure. There is increasingly evidence supporting the involvement of the central complex in the mechanism of sleep regulation in Drosophila Melanogaster (Donlea et al. 2011).

The insect central complex plays a key role in multiple behavioral and physiological activities, combining various modalities of sensory information and the animal’s past experience to respond to the environment (Wolff, Iyer, and Rubin 2015). The structure of central complex exhibits a precise and sophisticated manner of collaboration, through an intricate collections of neurons connected to neighboring neuropils, and communicate extensively with other regions in central brain, including adjacent lateral complex and the superior protocerebrum (Wolff, Iyer, and Rubin 2015). While various research has been conducted to study the role of central complex in locomotor behavior (Renn et al. 1999), spatial learning and memory (Pan et al. 2009), and flight control (Ilius, Wolf, and Heisenberg 2007), very few research illustrates the relationship between the central complex and sleep and arousal regulation. It has been reported that the fan-shape body actually participates in sleep and arousal regulation through dopaminergic neurons (Ueno et al. 2012). Considering the anatomical and functional connection between ellipsoid body and fan shape body, it is plausible and reasonable to hypothesize that ellipsoid body also involves in the sleep and arousal regulation through dopaminergic neurons.
In order to investigate the position of ellipsoid body in the brain, anatomically examining the internal organization of central complex is necessary. The central complex is comprised of four neuropils across the midline of the brain: the fan-shape body, the ellipsoid body, the protocerebral bridge, and the paired noduli (Renn et al. 1999). Different from other principal neural centers, for instance, the mushroom bodies, antennal lobes, or optics lobes, the central complex spans the sagittal midline and is symmetrically organized (Renn et al. 1999). This symmetry in structure suggests that the central complex is an information layover center in brain, which receives a great amount of information from other adjacent structures. The protocerebral bridge (PB) is the most conserved structure in all to date insects, and is vertically divided into distinct units called glomeruli (Wolff, Iyer, and Rubin 2015). It spans between the calyces of the mushroom bodies and is slightly detached posteriorly from the protocerebral neuropil. The noduli (NO) lie rostral to the PB and constitute the only paired neuropil of the central complex structures (Wolff, Iyer, and Rubin 2015).

The central body, a structure contains both the fan-shaped body (FB) and ellipsoid body (EB), lies more anteriorly, immediately frontally from the medial antennal lobe tracts, connecting the antennal lobes and calyces of the mushroom bodies (Pfeiffer and Homberg 2014). The FSB is posterior to the EB and is the largest of the central complex neuropils, and FSB is subdivided vertically into columns, known as segments (Wolff, Iyer, and Rubin 2015). Along the anterior–posterior axis of the FSB, there are four shells delineated by the positions and extent to which arbors from small field neurons project into these FSB domains (Wolff, Iyer, and Rubin 2015). In Drosophila, the EB is shaped like a torus, and similar to FSB, EB is divided into three anterior-posterior axis, called rings. Along the radius of each ring, neurons are divided into layers in EB (Pfeiffer and Homberg 2014). Anatomically, the EB and FB both are positioned
next to each other anteriorly in central complex. However, we cannot infer the similarity in the functions the EB and FB both serve or the communication between them unless we analyze the innervation and direct and indirect synaptic innervation between them.

On one hand, based on the genetic and morphological features, the majority of the classes of neuron on central body (FSB and EB) were categorized as either large field or small field neurons. Large field neurons arborize within one or more tangential layers of a single central complex subunits. For instance, the single layer of FSB or either ring structure of EB are characterized as one type of large field neurons. They generally connect to other accessory structures within the central complex or adjacent regions. There is an unsettled argument on the number of layers in FB, as one of the research pointed our that this questions will remain unresolved until two prerequisites are met: the features that define a layer, and identification of all the neurons that arborize in the FSB (Wolff, Iyer, and Rubin 2015). The classification of EB, on the contrary, generally reaches to agreement. One major type of large field neurons are R neurons, which constitutes as four ring structures in the EB. The primary neurites of R neurons run in the prominent RF tract. The neurites (projections of neurons) of the R neurons split to send dendrites into the lateral triangles, an region that closely associated with the EB, and send axons into the EB (Renn et al. 1999). Four types of R neurons, R1, R2, R3, and R4, are spatially segregated in the process of the lateral triangles, which is the center for signal input and major innervations.

On the other hand, the majority of the small field neurons interconnect with each other within the substructure or with other substructures within the central complex. In this case, the small field neurons divide the FSB into regular columnar units, or divide the EB into radial elements. The GAL4 driving fluorescence line (Strauss 2002) indicates a set of small field
neurons project through the EB to PB-NO, suggesting collaborated information process from EB to the other parts of central complex. The anatomical connection between EB R1 neurons to FSB small filed neurons further suggest functional connection between this two regions of central complex. However, in most cases, large number of stained neurons cannot accurately be traced to individual axons, preventing the precise assignment to directly innervation or same small field neuron groups.

The screening for dopaminergic neurons was necessary to identify sleep promoting circuit in central complex. GAL4-targeted gene silencing and activation showed that specific tangential neurons of the central complex control activity levels and sleep-wake states, largely through dopamine signaling. It was recently found that a set of dopaminergic neurons projected to the dorsal fan-shaped body actually mediates the sleep and arousal regulation in *Drosophila* (Liu et al. 2012 and Ueno et al. 2012). Previous work indicated the role of dopamine in sleep and arousal through the administration of dopamine inhibitor and enhancer. The results showed that the administration of 3-iodo-tyrosine, a tyrosine hydroxylase inhibitor, increases sleep duration, and administration of psychostimulants that enhance dopamine signaling, such as cocaine or amphetamine, decreases sleep. Other study facilitated the dTRPA1, a heat-inducible nonselective cation channel that can be used to depolarize neurons to trigger neurotransmitter release, to control the dopamine expression in vivo (Liu et al. 2012). In the absence of heat induction of dTRPA1, there is no sleep reduction in dopamine line and control lines, but increasing the temperature results in decrease in night-time sleep bout duration and sleep bout number. Taken together, dopaminergic neurons are the most competitive candidate for sleep and arousal mediation in the central complex. In addition, the percent decrease in sleep with activation of dopamine neurons was greater during the night than during the day, implying the connection
between dopamine mediation and circadian rhythms (Liu et al. 2012). In addition, we will continue to exploit this heat induction of dTRPA1 cation channels to bridge the gap between neural activity and transient change on sleep behaviors.

In order to determine the subset of dopaminergic neurons on EB, we could borrow some insights of the study on dopamine receptors on FSB. One study employed the genetic strategy that used the dopamine transport DAT mutant on FSB (Ueno et al. 2012). The presynaptic DAT clears the released dopamine from the synaptic cleft, and reduced sleep duration of the DAT mutant flies can be attributed to an increase in postsynaptic dopamine signaling. As expectedly, DAT mutant flies has significant decreased sleep phenotype, and the reduction can be rescued by the administration of dopamine inhibitor or conditioned DAT expression (Ueno et al. 2012). Immunohistochemistry also visualizes the DAT expression in wild-type FSB regions and the ellipsoid body, but not in the mutant brain. The activation of dorsal fan-shaped body neurons can simulate the increased sleep phenotype in both control flies and DAT mutant flies, suggesting dorsal fan-shaped body neurons are downstream targets of this sleep regulation circuit. Nevertheless, the role of EB is ignored, even according to the immunohistochemistry results the DAT also partially expressed in ellipsoid body. However, we can hypothesis that EB, as a parallel structure of FSB, also can express dopamine receptors on synapses. In addition, EB and FSB sometimes involve in same complex behavioral function, EB becomes a good candidate for sleep regulating circuitry.

However, there has been a mixed reports on sleep promotion or wakefulness promotion effect on FB, and the controversial theories regarding the sleep regulation in FSB laid another layer of interest to this question. Some research identified dopaminergic neurons in PPL1 cluster that promote wakefulness in Drosophila (Joiner et al. 2006), while others propose dFSB
regulation on sleep/arousal. We found that the D1 dopamine receptor (DA1) in the dorsal fan-shaped body (dFSB) mediates the arousal effect of dopamine in Drosophila. It has also been reported that dopamine regulates the arousal system via a pigment dispersing factor neurons (PDF), so the EB or other part of the central complex could be the downstream neurons receiving signals from PDF neurons and participated in sleep regulation (Ueno et al. 2012). PDF neurons are widely studied in circadian rhythm regulation, their primary targets and downstream neurons remains unclear. Previous findings have shown that DA1, one type of dopamine receptors, in the PDF neurons (lateral ventral neurons) regulates sleep-wake arousal and that DA1 in the ellipsoid body regulates stress-induced arousal (Ueno et al. 2012). However, in the same study, the apoptotic gene hid (also known as wrinkled) to genetically ablated the PDF neurons in flies, the experiment indicates that the excess dopamine expression can still decrease the sleep phenotypes (Ueno et al. 2012). Therefore, the PDF neurons do not eliminate the arousal effect of dopamine and PDF and dopaminergic neurons projected to the FB could have adopted different pathway to regulate sleep. This further implies that the EB and FB could also have different mechanisms in sleep and arousal regulation.

Furthermore, previous research has identified PPM3 as one of the upstream projecting neurons to dFSB (Ueno et al. 2012). When the expression of PPM3 was expressed with FLP with other dopamine neurons in co-colorization, PPM3 along with other dopaminergic neurons were activated in TrapA1 expression neurons after sleep promotion experiments. Another study facilitates the intron of tyrosine hydroxylase (TH) gene to inhibit dopamine expression, and dTrpA1 are used to controllably open the expression of dopamine when temperature rises (Liu et al. 2012). The activation of the TH mutant neurons in PPL1 and PPM3 cells resulted in a significant reduction in arousal threshold as compared to activation of neurons in the other
drivers or controls (Liu et al. 2012). These data suggest that the cell groups unique to PPL1 and PPM3 are more important than other dopaminergic subgroups for promoting wakefulness and that this effect is mediated by an increase in arousal.

It is confirmed that both PPL1 and PPM3 has projections to the FB, and PPM3 are divided into two groups based on projections: one projecting to the FB and one projecting to the EB. In all the cells in the bundle of PPL1 neurons, only a single neuron from the subgroup, which has projections to the dorsal fan-shaped body, has the phenotype of wakefulness promotion (Liu et al. 2012). On the other hand, the groups of neurons in PPM3 that projecting to the dorsal fan-shaped body also has significant sleep reduction in mutants compared to the wild-type controls (Ueno et al. 2012). Those two results indicated that the FB participates in the dopamine regulated sleep and arousal pathway and is the downstream targets of both PPL1 and PPM3 dopaminergic neurons. Some research also pointed out the connection between the mushroom body and FB, as the PPM3 neurons have their arbors close to the vertical lobes of the mushroom body and mushroom body ahs been shown to regulate sleep and memory. However, the results showed that PPM3 neurons did not project directly to the mushroom body, implying the FB can be the major center that process the sleep and arousal information from PPM3 (Ueno et al. 2012). In conclusion, PPM3 has been our primary candidate for upstream signal input to R1 neural circuity in sleep promotion.

As the FB has been studied to play a role in dopamine regulation on sleep and arousal, an important questions arises as to whether the same dopamine neurons, PPL1 or PPM3 or others, can regulate both sleep and arousal mechanism in ellipsoid body. Therefore, behavioral locomotor assays are required to determine whether the activation of PPM3 or any other upstream neurons can directly trigger sleep promotion behaviors. And whether the FB and EB
takes differential functions in sleep and arousal? For instance, the F1 and F5 neurons in the FB and a subset of R neurons, R2 and R4, in the EB are both required for the visual pattern memory (Pan et al. 2009). Nevertheless, among the four learnable pattern parameters in visual memory, large field neurons in the FB are required for the memory for “orientation” and “elevation”, while the EB is involved in the memory for all parameters and can be parameter-independent (Pan et al. 2009).

However, one major barrier that interrupt this hypothesis is that there is no significant evidence supporting the effect of the EB in sleep regulation. The neuron cluster of PPM3 that project to the EB has no significant change in phenotype or DAT expression compared to controls, rising a question that whether the EB actually participated in this arousal circuit (Ueno et al. 2012). Another study using TH mutant showed that the activation of the two PPM3 neurons in the c346-Gal4 driver did not significantly promote wakefulness (Liu et al. 2012). This indicates that the neurons projected to ellipsoid body, even are active during dopamine regulation on sleep and arousal, are not critical in this process. Therefore, it can be excluded that the EB is the downstream target of PPM3 in sleep and arousal regulation. In fact, it is greatly possible that other dopamine neurons, which were not labeled in this MARCM screening in previous study, also affect sleep and arousal, including the neuron cluster that has projections to the mushroom body and the FB (Ueno et al. 2012). Therefore, it is still meaningful to exam the connection between dopaminergic neurons in the EB and arousal and sleep regulation.

Another problem that need to determine the connection of ellipsoid body to the rest of the central complex and other structures. Even though the connections between the central complex and adjacent structures are vast, the majority of input and output projections of the central complex occurs via the pathways of lateral ellipsoid tract and the FB isthmus tract (Pfeiffer and
Homberg 2014). The lateral and medial bulbs, condensations of microglomerular synaptic complexes, are connected to the zone of EB, whereas subfields of the lateral accessory lobes communicate largely with the zone of FB. (Pfeiffer and Homberg 2014). Therefore, we proposed to use double system, UAS/GAL4 and LexA/LexAop system to express upstream neurons and interested R1 neurons in same brain, and perform co-colorization to examine any physical overlap or connection between two clusters of neurons. In addition, functional fluorescence imaging, including GCamp6f calcium dynamic, and EPAC cAMP visualization, can be utilized to determine the neuronal type of EB R1 neurons.

The fan shape body in fruit fly Drosophila has been show to mediate sleep and arousal in many different ways, and there has been a series of approaches reported to examine the sleep promotion function in fan shape body. We focused on the anatomical and functional connection in proposed neural circuitry, that consists of R1 neurons on ellipsoid body and one cluster of fan shape body projecting neurons, PPM3, in sleep promotion behaviors. dTRPA1 and TNT expressing fly stocks have been shown to exhibit sleep promotion phenotypes in behavioral assays, and fluorescence co-colorization and functional fluorescence image confirmed the functional and physiological connectivity between ellipsoid body and PPM3 neurons. Our finding supports a direct sleep promoting neural circuitry that underpins the relationship between sleep regulation and ellipsoid body in Drosophila central complex.

**Methods and Materials**

*Flies Strains*
All genotypes were reared on standard cornmeal medium (containing corn meal, yeast, glucose, wheat germ and agar) under regular light/dark cycle (12:12 h) at 25 °C. VT058968-GAL4 (Kvon et al. 2014; Jefferis) and R28E01-GAL4 (Pfeiffer et al. 2011) drivers on third chromosomes were used to target neurons on R1 ring of Ellipsoid body in *Drosophila*. The w;VT058968-GAL4 and w;R28E01-GAL4 males were crossed with w; dTRPA1 females, and set up recombinant GAL4 driving lines. The desired recombinant lines have dTRPA1 on second chromosomes and GAL4 drivers on third chromosomes, selected based on CyO and Tm6b balancers. The dTRPA1 activated GAL4 drivers on R1 neurons at non-permissive temperature at 30°C. Tsh-GAL80;VT040016-GAL4;Chat 3.3-GAL80 line (Kvon et al. 2014), and Tsh-GAL80;VT024624-GAL4 (Kvon et al. 2014) labeled PPM3 neurons, and also target the subset of dTRPA1-expressing neurons at non-permissive temperature at 30°C. Overexpression of dTRPA1 was achieved using UAS-dTRPA1 (Ueno et al. 2012).

Then fly stocks carrying GAL4 driver transgenes were crossed with UAS-TNT strains (Hekmat-Scafe et al. 2010), and their progenies were selected based on balancers to create transgenetic flies stock with UAS-TNT on second chromosome and different GAL4 drivers on third chromosomes (Hekmat-Scafe et al. 2010).

By crossing and selection based on balancer, we created a line with R92G05-GAL4 on third chromosome and R28E01-LexA on second chromosome. Then the recombinant line was crossed with the line with UAS-RFP on second chromosome and LexAop on third chromosome. The recombinant line has double system, UAS-GAL4 and LexA-LexAop. The double system drive the expression of GFP on R92G05-GAL4 labeled R1 neurons and the expression of RFP on R28E01-LexA labeled PPM3 neurons. All fly stocks were obtained from Bloomington Drosophila stock center or Vietnam Drosophila Center.
Behavioral Assays

**Light/Dark (LD) and constant dark (DD) cycles** This experiment was set for UAS-TNT crossed with GAL4 driver lines to observe the phenotype during inhibition of R1 neurons and PPM3 neurons. The experiment protocols adjusted based on standard Drosophila locomotor assays (Das, Holmes, and Sheeba 2016). The experiments consist of two days entraining at LD (12h: 12h), two days LD conditions, followed by two days constant dark cycles (DD) conditions. The temperature throughout the experiments was constantly maintained at 25°C. Non-virgin female flies were individually housed in glass tubes (length, 65 mm; inside diameter, 3 mm) containing standard fly food at one. Locomotor activity was monitored by recording the number of infrared beam crossings for individual flies in 1 min bins using the Drosophila activity monitoring (DAM) system. Data were continuously collected for six days under LD and DD conditions.

**Temperature shift cycles** This experiment was set for UAS-dTRPA1 crossed with GAL4 driver lines to observe the phenotype during selective activation of R1 neurons and PPM3 neurons. The experiments consist of two days entraining at LD (12h: 12h) condition, and one day LD conditions at permissive temperature (20°C), followed by one day LD cycle at non-permissive temperature (30°C). One day LD recovery at 20°C. After the LD cycle, one-day constant cycle (DD) was performed at 20°C, followed by one day DD cycle at non-permissive temperature (30°C). One day DD recovery at 20°C. Non-virgin female flies were individually housed in glass tubes (length, 65 mm; inside diameter, 3 mm) containing standard fly food at one. Locomotor activity was monitored by recording the number of infrared beam crossings for individual flies in 1 min bins using the Drosophila activity monitoring (DAM) system. Data were continuously collected for five days under LD conditions and three more days at DD condition.
Bath applied dopamine to stimulate R1 neurons

We used the GAL4-UAS system to express a genetically-encoded calcium reporter, GCamp6f, in the adult ellipsoid body. The GAL4 line used to drive reporter expression was R28E01-GAL4 labeled R1 neurons. The region of interest (ROI) on ellipsoid body was selected and imaged in isolated, intact brain with calcium imaging microscopy. 1uM dopamine was applied in the bath for 150 seconds, and washed off by saline bath. After the recovery of fluorescence intensity (f%), another 1uM dopamine bath was applied for 150 seconds. Briefly, the percent change in fluorescence over time was calculated using the following formula: \( \Delta F/F = \frac{(F_n - F_0)}{F_0} \times 100\% \), where \( F_n \) is the fluorescence at time point n, and \( F_0 \) is the fluorescence at time 0.

We then used the the GAL4-UAS system to express a genetically-encoded calcium reporter, FRET based cAMP reporter, EPAC, in the adult ellipsoid body. The GAL4 line used to drive reporter expression was R28E01-GAL4 labeled R1 neurons. The region of ellipsoid body was imaged in isolated, intact brain with microscopy. 1uM dopamine was applied in the bath for 150 seconds, and washed off by saline bath. ROIs were selected from both the CFP and YFP emissions channels, and the fluorescence resonance energy transfer (FRET) signal (YFP/CFP ratio) was calculated for each time point and normalized to the ratio of the first time point. The relative cAMP changes were determined by plotting the normalized CFP/YFP ratio (percentage) over time.

Immunocytochemistry

Protocol was adapted by standard fixation and staining protocol (Haynes, Christmann, and Griffith 2015). Intact fly brains were dissected in ice-cold PBS and were fixed immediately
after dissection for 15 min at room-temperature in 4% paraformaldehyde (vol/vol). Brains were incubated in PBS containing 0.5% Triton X-100, 10% NGS and primary or secondary antibodies for one night each with 3 × 15 min washes between each incubation. Brain samples were then mounted using Vectashield and were visualized by a Leica TCS SP5 confocal microscope with a 20×, 40×, or 63× objective lens. All images were taken sequentially to prevent bleed-through between channels. For colocalization, mouse anti-GFP (1:200, Roche Applied Biosciences) was applied to UAS-GFP/ R92G05-GAL4 line to stain for GFP expression on R92G05-GAL4 labeled R1 neurons, and rabbit anti-RFP (1:1,000, Invitrogen A11122) was used to get colocalization of RFP fluorescence on R28E01-LexA labeled PPM3 neurons. Alexa Fluor 488 and 635 anti-mouse and anti-rabbit secondary antibodies (1:200, Invitrogen) were used to visualize staining patterns. Alexa 488 was used to label GFP on R1 neurons so that any residual endogenous GFP fluorescence would be of a similar wavelength as the dye and would not bleed through to the 633 wavelength channel. Alexa 635 was used to label RFP on PPM3 neurons.

**Confocal Imaging**

Fly brains were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M freshly made phosphate buffer (PB; ph 7.3) for 24 h at 4°C. The fly brains of a line in which the neurons expressing pigment dispersing factor (PDF) were labeled by a Gal4 driven GFP reporter were dissected and fixed. Confocal images were taken using Laser sharp version 3.2 software. Complete series of optical sections were taken with a 40× oil immersion lens at 1 μm intervals. All image processing was done using the freely available FIJI (IMAGEJ) software and plugins. Background was subtracted from all confocal stacks prior to further processing. All images are sums or maximum intensity Z-projections of the relevant confocal slices. Quantification of cells with positive/negative staining was done by visually comparing colocalization of GFP and
Figure 1: 1a VT058968-GAL4 labeled R1 neurons, GFP expression under confocal microscopy. Maximum intensity projections of the full stack are shown without reference channel. Image obtained from FlyLight. 1b, R28E01-GAL4 labeled EB R1 neurons, GFP expression under confocal microscopy. Image obtained from FlyLight.

antibody staining in multiple individual Z-slices. Cell bodies/MB lobe sets where high background prevented interpretation of staining were excluded. For the images presented in Figure 3, Intensity Correlation Analysis (ICA) was also performed to assess spatial colocalization of staining between channels.

Data Analysis

Data were analyzed as described in the figure legends using Matlab 2013. Images were analyzed and edited by the ImageJ software. In all figures, results are expressed as means with standard error of the mean (SEM).

Results

R1 neurons promote sleep with dTRPA1 activation and reduce sleep with TNT inhibition

We primitively screened 30 GAL4 drivers specifically on ellipsoid body(EB) based on behavioral assays, and identified sleep promotion on R1 neurons (data not shown). We selected two GAL4 drivers to perform dTRPA1 assays on VT058968-GAL4 labeled EB R1 neurons and
R28E01-GAL4 labeled EB R1 neurons to perform behavioral locomotor assays. These two drivers specifically mark different clusters of R1 neurons on EB (Figure 1a, 1b). In order to determine whether those GAL4 labeled R1 neurons play a role in promoting sleep, we acutely activated these neurons by driving the warmth-sensitive cation channel, dTrpA1 (Das, Holmes, and Sheeba 2016). Those recombinant flies expressing dTRPA1 on GAL4 driving R1 neurons, and those neurons are activated at non-permissive temperature 30°C, when dTRPA1 opens and depolarized the cells (Das, Holmes, and Sheeba 2016) (Haynes, Christmann, and Griffith 2015). The light/dark cycle locomotor assays were recorded at base line level at 20°C with no neural

![Diagram](image)

Figure 2: Locomotor assays on dTRPA1 activated R28E01-GAL4 at LD cycle (12h:12h). Yellow labeled UAS-dTRPA1/R28E01-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hours, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 2a, 2b, 2c: Baseline phenotype at 20C. 2d, 2e, 2f: dTRPA1 activated state at 30C.
activation, and then at experimental level with rising temperature at 30°C. The control flies were White-CantonS(WCS) line crossed with R28E01-GAL4 line or VT058968-GAL4 line, and WCS crossed with UAS-dTRPA1 line. Both controls were expected to have wild type phenotypes.

In regular LD cycle (12h:12h), flies with inactivated R28E01-GAL4 labeled R1 neurons at 20°C had less sleep compared to wild-type controls (Figure 2a, 2b). When the temperature rise

Figure 3: Locomotor assays on dTRPA1 activated R28E01-GAL4 at constant dark cycle. Yellow labeled UAS-dTRPA1/ R28E01-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hours, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 2a, 2b,2c: Baseline phenotype at 20C. 2d, 2e, 2f: dTRPA1 activated state at 30C.
to 30°C, R28E01-GAL4 line has increased sleep during early morning (morning peak), with an increase in overall sleep duration (Figure 2d, 2e). The sleep promotion was not significant during night time (Figure 2d). The activation of R28E01-GAL4 labeled R1 neurons did not cause any hyperactivity in flies (Figure 2f).

Consistently, activated the R28E01-GAL4 labeled R1 neurons has increased sleep during early morning in constant dark cycle (Figure 3, 3d). Compared to wild-type controls, the sleep promotion in activated R1 neurons was most significant during systematic day time (ZT0-ZT12, Figure 3d, 3e), but we also observed increased sleep during during systematic night time (ZT12-24, Figure 3r). However, the overall sleep during for wide-type controls declines to nearly a half when temperature rise (Figure 3e). Therefore, the temperature may reduce the baseline of overall sleep duration. Nevertheless, the difference between wild-type controls and dTRPA1 GAL4 line still suggests sleep was elevated when we activated R28E01-GAL4 labeled R1 neurons, as the sleep promotion was recovered after the temperature return to baseline at 20°C (Figure 4a, 4b).

We also did heat pulse to stimulate dTRPA1 transiently and observed transient sleep promotion in dTRPA1 transgenic flies (Figure 4d, 4e). The hyperactivity was excluded in the sleep promotion phenotype (Figure 4f). The transient heat pulse confirmed the previous result that R28E01-GAL4 labeled R1 neurons can promote sleep in flies when activated.

To explore the role of R28E01-GAL4 labeled neuron in sleep promotion, we further examined the sleep reduction of GAL4/UAS-TNT transgenic flies (Umezaki et al. 2011) (Umezaki et al. 2011). The GAL4 labeled neurons were blocked by expression of tetanus toxin light chain (TNT) constantly (Umezaki et al. 2011). The locomotor assays of UAS-TNT
Figure 4: 4a, 4b, 4c Locomotor assays recovery on dTRPA1 activated R28E01-GAL4 at constant dark cycle at 20°C. 4d, 4e, 4f Heat pulse on R28E01-GAL4 line on LD cycle (12h:12h) at 20°C. An increase of temperature from 20°C to 30°C at ZT8 was applied to all fly lines, and the temperature maintained for 4 hours, and dropped to 20°C at ZT12. Yellow labeled UAS-dTRPA1/ R28E01-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hour, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity.

Transgenic flies were measured on regular LD cycles and DD cycles to observe the sleep phenotype with wild-type controls. During LD cycle, TNT inhibited flies showed a significant sleep reduction during night time, compared to wild-type (Figure 5a, 5b). During DD cycle, the TNT inhibited flies also had reduced sleep during systemic night time (Figure 5d, 5e), but no
Figure 5 Locomotor assays on TNT constantly inhibited R28E01-GAL4 at 25C. Yellow labeled UAS-dTRPA1/ R28E01-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hours, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 5a, 5b,5c: flies in LD cycle (12h:12h). 5d, 5e, 5f: flies in constant dark DD cycle.

sleep reduction during systemic day time (Figure 5e). However, we also observed a reduction at sleep peak around ZT6 (Figure 5d).

VT058968-GAL4 marked slightly different, and also overlapped, clusters of R1 neurons on EB (Figure 1a), with stronger signal input from other parts of brains. Possibly owing to larger number of neurons marked by this driver, the dTRPA1 activated VT058968-GAL4 has stronger sleep promotion phenotype in behavioral assays (Figure 6). In LD cycle, UAS-dTRPA1/
Figure 6: Locomotor assays on dTRPA1 activated VT058968-GAL4 at LD cycle (12h:12h). Yellow labeled UAS-dTRPA1/VT058968-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 house, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 6a, 6b,6c: Baseline phenotype at 20°C. 6d, 6e, 6f: dTRPA1 activated state at 30°C.

VT058968-GAL4 line showed sleep promotion throughout the day at 30°C. Similar to R28E01-GAL4 line, VT058968-GAL4 line has sleep promotion during early morning (Figure 6d, 6e). However, this line also showed elevated total sleep duration and activity during dark period (Figure 6e, 6f).

In DD cycle, no sleep promoting phenotype was observed after the temperature rise to 30°C (Figure 7d, 7e). Additionally, TNT inhibition on VT058968-GAL4 labeled neurons has no
effect on sleep phenotype (Figure 8d). However, we observed slight reduction on sleep peak at noon around ZT6 in constant dark cycle (DD, figure 8d), and a relative decrease in systemic day time (Figure 8d, 8e).

**Anatomical Connectivity between EB R1 Neurons and PPM3 Neurons**

Previous research indicated the activation of fan shape body (FSB) projecting PPM3 dopamine neuron was sufficient to decrease sleep in Drosophila, suggesting that PPM3 neurons

Figure 7 Locomotor assays on dTRPA1 activated VT058968-GAL4 at constant dark DD cycle. Yellow labeled UAS-dTRPA1/VT058968-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hourse, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 6a, 6b,6c: Baseline phenotype at 20C. 6d, 6e, 6f: dTRPA1 activated state at 30C.
may be the upstream neurons mediating sleep and wakefulness in dorsal fan shape body and ellipsoid body (Artiushin and Sehgal 2017). PPM3 project to dorsal fan shape body (dFSB), noduli (NO), and ellipsoid body (EB) (Ueno et al. 2012). PPM3 neurons are dopaminergic neurons, and they inhibit dFSB and R2 neurons of the ellipsoid body in sleep promoting circuit (Artiushin and Sehgal 2017). However, the projection from PPM3 to ellipsoid body is not limited to R2 neurons.

Figure 8 Locomotor assays on TNT constantly inhibited VT058968-GAL4 at 25C. Yellow labeled UAS-dTRPA1/ VT058968-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hours, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 8a, 8b,8c: flies in LD cycle (12h:12h). 8d, 8e, 8f: flies in constant dark DD cycle. We constructed a recombinant fly strain that expressed GFP on R28E01-LexA labeled R1
EB neurons and RFP on R92G05-GAL4 labeled PPM3 neurons (Figure 9). The transgenic fly stock contains two systems, UAS-GAL4 and LexA-LexAop, to drive the fluorescence expression of two different clusters of neurons. R28E01-LexA expressed in a subset of R1 neurons to drive lexAop-GFP (Figure 9b), and R92G05-GAL4 expressed in one cluster of PPM3 neurons drive the UAS-RFP (Figure 9a). By combining the GFP and RFP fluorescence expression map in brain, we identified anatomical connection between R1 neurons and PPM3 (Figure 9c). R28E01-GAL4 labeled R1 neurons and R92G05-GAL4 labeled PPM3 neurons has overlap at the inner

![Figure 9: Co-colorization of R28E01-GAL4 labeled R1 EB neurons and R92G05-LexA labeled PPM3 neurons under confocal microscope. 9a, LexAop-RFP visualization expressed on R92G05-LexA labeled PPM3 neurons shown as red. 9b, UAS-GFP visualization expressed on R28E01-GAL4 labeled R1 neurons shown as green. 9c, merge image of RFP and GFP visualization. 9d, 9f, single horizontal slide with merged visualization of GFP and RFP. Yellow labeled the peak of overlapping fluorescence detection.](image-url)
Figure 10: GFP expression of GAL4 labeled PPM3 neurons in brain. 10a, VT040016-GAL4 labeled PPM3 neurons, GFP expression under confocal microscopy. Maximum intensity projections of the full stack are shown without reference channel. Image obtained from FlyLight. 10b, R28E01-GAL4 labeled EB R1 neurons, GFP expression under confocal microscopy. Image obtained from FlyLight.

ring of ellipsoid body, as well as the lateral triangle areas (Figure 9c, 9d, 9e). At the peak of signal overlapping, regions with expression of both GFP and RFP, we located the anatomical connectivity between R1 neurons and PPM3 neurons at cell bodies as well as lateral triangle, which is the input signals center of EB (Figure 9c, 9d, 9e). Therefore, PPM3 has anatomical connection to R1 neurons in sleep promoting circuitry.

**TRPA1 Activated PPM3 neurons associate with sleep promotion during evening**

Two specific GAL4 labeled PPM3 line, VT040016-GAL4 and VT024624-GAL4 were selected to test sleep phenotype in locomotor assays. These two GAL4 drivers labeled overlapped but also different clusters of PPM3 neurons (Figure 10a, 10b). Considering relatively weak expression of PPM3 neurons, R92G05-GAL4 labeled line was not used for behavioral
Figure 11: Locomotor assays on dTRPA1 activated VT040016-GAL4 at LD cycle (12h:12h). Yellow labeled UAS-dTRPA1/VT040016-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hour, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 11a, 11b, 11c: Baseline phenotype at 20C. 11d, 11e, 11f: dTRPA1 activated state at 30C. 11g, 11h, 11k, Following recovery period at 20C.
assays (data not shown). UAS-dTRPA1 were crossed with GAL4 lines to achieve selective activation of PPM3 neurons in flies at 30°C. Sleep promotion happened during early morning and evening in GAL4 labeled PPM3 lines with selective dTRPA1 activation but the sleep promotion pattern is not quantitatively consistent with activated R1 neurons results. In addition, both GAL4 labeled PPM3 lines did not show reduction on sleep duration with TNT inhibition.

During LD cycle, we observed sleep promotion in activated VT040016-GAL4 labeled

![Figure 12: Locomotor assays on dTRPA1 activated VT040016-GAL4 at constant dark (DD) cycle. Yellow labeled UAS-dTRPA1/ VT040016-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 house, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 11a, 11b,11c: Baseline phenotype at 20C. 12d, 12e, 12f: dTRPA1 activated state at 30C. 12g, 12h, 12k, Following recovery period at 20C in DD.](image-url)
PPM3 neurons during early morning and evening (Figure 11d, 11e). The sleep promotion in the morning was not significant, but the sleep promotion during evening is significant compared to VT040016-GAL4 lines diminishes, indicating the sleep promotion during evening is completely induced by activation of dTRPA1 on PPM3 neurons.

In DD cycle (Figure 12), when VT040016-GAL4 labeled PPM3 neurons were activated at 30C, the sleep pattern shifted to the right compared to wild-type controls, with no increase in total sleep duration (Figure 12d, 12e). The shift of sleep pattern recovered when the temperature

Figure 13 Locomotor assays on TNT constantly inhibited VT040016-GAL4 at 25C. Yellow labeled UAS-dTRPA1/ VT040016-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hours, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 8a, 8b,8c: flies in LD cycle (12h:12h). 8d, 8e, 8f: flies in constant dark DD cycle.
declined to 20C and PPM3 neurons returned to inactive.

When VT040016-GAL4 labeled PPM3 neurons were constantly inhibited by TNT at 25C, no sleep promotion or suppression was observed in locomotor assays (Figure 13).

Figure 14 Locomotor assays on dTRPA1 activated VT024624-GAL4 at LD cycle (12h:12h). Yellow labeled UAS-dTRPA1/VT024624-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hourse, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 11a, 11b,11c: Baseline phenotype at 20C. 11d, 11e, 11f: dTRPA1 activated state at 30C. 11g, 11h, 11k, Following recovery period at 20C.
We then tested behavioral locomotor phenotype on VT024624-GAL4 line when GAL4 labeled neurons were activated by dTRPA1 at 30C on LD and DD cycle. At 30C on LD cycle (Figure 15). The locomotor assays on dTRPA1 activated VT024624-GAL4 at constant dark DD cycle. Yellow labeled UAS-dTRPA1/VT024624-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hours, systemic light period (ZT0-ZT12), and systemic dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 15a, 15b, 15c: Baseline phenotype at 20C. 15d, 15e, 15f: dTRPA1 activated state at 30C. 15g, 15h, 15k, Following recovery period at 20C.
VT024624-GAL4 line exhibit slightly increase in sleep during early morning and evening in LD cycle (14a, 14d). Similar to VT040016-GAL4 line, the sleep promotion was most significant in the evening around ZT12 (Figure 14d). The overall sleep duration during day time was slightly higher than controls, but not significant (Figure 14e). The sleep promotion can also be recovered to baseline by decreasing the temperature (Figure 14g, 14h).

Figure 16: Locomotor assays on TNT constantly inhibited VT024624-GAL4 at 25°C. Yellow labeled UAS-dTRPA1/ VT024624-GAL4 line, while green and blue labeled wild-type controls. Upper left panel: Averaged sleep counts at interval of 30 minutes. Upper right panel: Total sleep duration at 24 hours, light period (ZT0-ZT12), and dark period (ZT12-ZT24). Measured as indicator of hyperactivity or hypoactivity. 16a, 16b, 16c: flies in LD cycle (12h:12h). 16d, 16e, 16f: flies in constant dark DD cycle.
In constant dark cycle, the sleep promotion only appeared in the evening, with an increase of total sleep duration during systemic day time (Figure 15d, 15e). Therefore, the sleep promotion led by activation of VT024624-GAL4 labeled PPM3 neurons was light independent. Notably, in DD cycle, the combinant line has reduced sleep counts during recovery period at 20C, with no obvious increase in averaged activity (Figure 15). When VT024624-GAL4 labeled PPM3 neurons were constantly inhibited by TNT at 25C, no sleep promotion was observed in locomotor assays (Figure 16). Instead, the sleep duration increased, instead of decrease, when PPM3 neurons were acutely activated by dTRPA1 (Figure 16d).

**Bath-applied Dopamine Activate R1 neurons in vivo**

Previous research indicated PPM3 are inhibitory dopaminergic neurons (Artiushin and Sehgal 2017). In order to allocate dopamine receptors on R1 neurons, we utilized R28E01-GAL4 labeled R1 neurons crossed with UAS-GCamp6f. GCamp6f was expressed under the control of GAL4 driver and the calcium signal indicates the activation of postsynaptic terminals innervating the R1 neurons in EB (Lewis et al. 2015). GCamp6f was measured by Fluorescence Resonance Energy Transfer (FRET) ratio, and the strength of signal also reflects on the brightness of the fluorescence (Figure 17a, 17b, 17c). After 1mM dopamine bath was applied to isolated intact brain, the R28E01-GAL4 labeled R1 neurons were activated with an increase of fluorescence intensity value (Figure 17c) and intensified brightening in live video (images shown in Figure 17a,17b). When the dopamine bath was washed out, the fluorescence intensity declines to baseline and exhibit no increasing trend (Figure 17c). With an interval of 150s, the dopamine bath was reapplied to dissected brain and fluorescence ratio increased accordingly (Figure 17c).
Figure 17 Bath applied dopamine to stimulate R1 neurons in vivo. The region of interest (ROI) on ellipsoid body was selected and imaged in isolated, intact brain with calcium imaging microscope. The R28E01-GAL4 line was crossed with UAS-GCamp and UAS-EPAC, respectively, to obtain Gcamp6f and EPAC expression on R28E01-GAL4 labeled EB R1 neurons. 17a, 17b,17c, 1uM of dopamine solution was applied to naked brain with ROI imaging. The fluorescence intensity was measured and live image of fluorescence intensity was recorded. After 150 seconds, the dopamine bath was washed off by saline solution, and another 1uM dopamine bath was reapplied when the fluorescence intensity was recovered. 17a shown above is ROI before dopamine bath and 17b shown is ROI during dopamine bath. The fluorescence intensity was recorded at 17c. 17d, 1uM dopamine was applied in the bath to R28E01-GAL4 line inserted with EPAC indicator, and washed off by saline bath. ROIs were selected from both the CFP and YFP emissions channels, and the fluorescence resonance energy transfer (FRET) signal (YFP/CFP ratio) was calculated for each time point and normalized to the ratio of the first time point. The relative cAMP changes were determined by plotting the normalized CFP/YFP ratio.
As the calcium concentration indicates the action potential fired by the cell, the results suggest that the R1 neurons were activated by dopamine application.

Another sensor construct that consists of the cAMP-binding protein EPAC was utilized to monitor the cAMP dynamics in R28E01-GAL4 labeled R1 neurons (van der Krogt et al. 2008). cAMP is the indicator for cell metabolic activity. The intact brain of UAS-GAL4 recombinant line was applied with 1mM dopamine bath and FRET ratio was recorded to measure the cell activation. The R28E01-GAL4 labeled R1 neurons were activated with dopamine bath accordingly, and the activation diminished when dopamine was washed out (Figure 17d).

**Discussion**

Based on our results, both VT058968-GAL4 labeled and R28E01-GAL4 labeled EB R1 neurons have sleep promotion phenotype when activated by temperature sensitive dTRPA1. The activation of R1 neurons leads to sleep promotion in early morning and evening, and inhibition of R1 neurons using TNT reduces sleep during day time. Notably, the sleep promotion observed in flies with dTRPA1 activated R1 neurons is independent of light, as LD and DD cycle indicated similar results in VT058968-GAL4 and R28E01-GAL4 line. In addition, the sleep continues to increase during night time when VT058968-GAL4 labeled R1 neurons were activated. This could be explained by larger amount of neurons were expressed in VT058968-GAL4 than R28E01-GAL4 line. Consequently, the TNT inhibition on VT058968-GAL4 labeled R1 neurons could potentially provoked other neural circuit, either upstream or downstream, to compensate for the lack of sleep promoting in R1 neurons. Therefore, the TNT inhibition on VT058968-GAL4 labeled R1 neurons caused insignificant reduction on sleep duration in the morning.
The heat pulse stimulation on dTRPA1/GAL4 R1 neurons in the middle of the day transiently induce sleep promotion in flies. As the controls stays on baseline, the sleep promotion was only observed in R1 neuron activated flies, which implies the direct sleep regulation of EB R1 neurons. Combined with previous dTRPA1 activation and TNT inhibition results, we could confirm that R1 neurons are part of sleep promoting circuit. However, the spontaneous heat pulse also suggests that the sleep promotion in R1 neurons do not specifically happens during morning, because the opening of dTRPA1 channels could instantly promote sleep and the change of temperature in LD and DD cycles was set at ZT0. The sleep promotion could be more closely associated with the time of activation on R1 neurons. R1 neurons may not only activated during morning. Instead, the sleep promotion of R1 neurons possibly may constantly last throughout the day. In order to confirm this hypothesis, future experiments are expected to measure the locomotor activities continually for two or more days at dTRPA1 activated state, and determine

Figure 18: GFP expression of Dop1R1 mimic construct under confocal microscope. Data provided by Junwei Yu, Griffith Neurobiology Lab, Brandeis University.
whether the sleep promotion for the second and following days specifically happens in the morning. However, the activation of dTRPA1 may lead to hyperactivity of the neurons, resulting in other physiological defects or even lethality to flies.

TNT inhibition on GAL4 labeled R1 neurons confirmed our hypothesis on constant sleep promotion by R1 neurons. Because TNT is continually inhibiting the partial activation of R1 neurons, the sleep reduction was observed in different period of the day. In R28E01-GAL4 line, the flies have reduced sleep during night time, whereas in VT058968 line, the sleep reduction was most significant during day time. Two GAL4 drivers could label different clusters of R1 neurons, but in general, the sleep promotion in R1 neurons is not fixed within certain period of the day.

The anatomical innervation of PPM3 neurons on R1 neurons was confirmed by co-colorization of PPM3 and R1 under confocal microscope, utilizing UAS/GAL4 system to express GFP and RFP. The location of overlap is lateral triangle, which is the information input center for ellipsoid body. In addition, the overlap on fluorescence imaging is not parallel, suggesting very high possibility of synaptic connection between two clusters of neurons. Additionally, in vivo bath applied dopamine can activate R1 neurons, visualizing by GCamp6f and EPAC indicators. As previous research only identified PPM3 as dopaminergic neurons, GCamp6f and EPAC results confirm the anatomic connection in PPM3 R1 neural circuit.

However, we do not exclude the possibility that the synaptic transmitters between PPM3 and EB R1 neurons could be other substances. To investigate the subtype of dopamine receptors on R1 neurons, we constructed a Dop1R1 mimic construct that inserted in *Drosophila* genome with
GFP expression\(^1\). Under the confocal microscope, the Dop1R1 is not expressed on ellipsoid body (Figure 18), and we are constructing another DopR2 mimic construct that can express GFP on DopR2 receptors in fly brains. The screening for dopamine receptors on ellipsoid body, or specifically on R1 neurons, could further help us to construct RNAi mutant to target the transmission between PPM3 and R1 neurons, which is necessary and important to specifically identify the sleep phenotype when PPM3-R1 neural circuit is transiently inhibited.

In addition, we have not confirm whether the activation of R1 neurons by dopamine bath is direct stimulation, and whether PPM3 release dopamine or other neurotransmitters to R1 neurons. Therefore, future experiments are expected to activate PPM3 neurons in vivo and measure the GCamp6f and EPAC dynamics on R1 neurons to confirm the temporal order of cell activation.

To investigate the PPM3 R1 neural circuit on behavioral level, we conducted similar locomotor behavioral assays on two GAL4 labeled PPM3 neurons. We found that with activated PPM3 neurons by dTRPA1 showed sleep promotion in the evening, but barely has any sleep promotion during early morning. The dTRPA1 activation results on GAL4 labeled PPM3 neurons further imply that proposed R1 neural circuit mediates sleep promotion throughout the day, instead of limiting to the morning.

The TNT inhibition on PPM3 neurons does not has consistent sleep promoting phenotype. However, many variables also need to be taken into consideration. First, PPM3 regulates sleep via other circuit such as dFSB and R2 neurons on EB. The direct inhibition of PPM3 neurons affects multiple regions, as our PPM3 GAL4 drivers are not specific to R1 neurons. As a result, when VT024624-GAL4 labeled PPM3 neurons was consistently inhibited by TNT, the sleep

\(^1\) Data provided by Junwei Yu.
increases instead of decreases by some compensatory sleep promoting mechanisms. Second, TNT can only partially inhibit the cell activities, but not completely block the PPM3 activation. Therefore, the GAL4 labeled PPM3 lines can still recover most functions by over-activation. Third, the PPM3 upstream input could be activation only. To test our hypothesis, we proposed to use inwardly rectifying potassium channels (Kir) inhibitor to block the neural activity in PPM3 (Nakamura et al. 1998). Nevertheless, Kir2.1 transgene may induce developmental defect and lethality in *Drosophila*, and future experiments may need to consider to suppress the expression of Kir inhibitor during development. For instance, tubulin GAL80 to selective can suppress the expression of Kir inhibitors at low temperature, as tublin GAL80 is expressed ubiquitously and has been shown to potently repress the activity of GAL4 (Wu and Luo 2006). Lastly, we also suspected that PPM3 R1 neural circuit also has an impact on sleep homeostasis. When VT040016-GAL4 labeled PPM3 neurons were activated by dTRPA1 at 30C in constant dark cycle, the flies has a sleep pattern shift instead of sleep promotion phenotype. However, in VT024624-GAL4 labeled PPM3 neurons, both LD cycle and DD cycle showed sleep promotion during similar period. This observation potentially suggests that PPM3 may also participated in other sleep regulation mechanisms, including sleep homeostasis. Additionally, our previous fining that VT024624-GAL4 flies sleep more with TNT inhibition on PPM3 neurons also suggest that PPM3 may promote sleep after sleep deprivation.

In conclusion, we proposed a sleep promoting circuitry that consists of PPM3 neurons as upstream neural inputs and EB R1 neurons as downstream neurons. The connection between PPM3 and R1 neurons are dopamine mediated, with possible synapses at lateral triangles and ellipsoid body inner ring (density of EB cell bodies). According to the behavioral assays, the
PPM3-R1 neural circuitry promote sleep throughout the day and independent of circadian and light cycles.

This research implies opportunities for future directions to research on sleep regulating circuit in *Drosophila* central complex, and reveals the internal complexity of this topic. First of all, sleep regulating circuitry in central complex involves in multiple regions. PPM3 as the upstream neurons project to FSB, NO, and EB. As a result, simple inhibition or genetic knockdown on one cluster of neurons can barely controls for variables, and frequently invoke compensatory systems in sleep regulation to obtain negative outcomes. Secondly, the PPM3-R1 sleep promoting neural circuitry must have undefined other inhibitory or active input to regulate overall sleep behaviors in flies, as the temperature cycle can change the baseline of sleep promotion throughout the experiments. The changes of baseline sleep phenotype were inconsistent, and very hard to generalize. Thirdly, there is no identified functional distinction between sleep promoting in PPM3 projecting dFSB and in PPM3 projecting EB. However, we still consider two neural circuitries have differences in sleep promoting function, or works on different sleep patterns. Therefore, future experiments were expected to explore on other sleep phenotypes, such as sleep homeostasis, to subcategorize various sleep promoting circuits in central complex.
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