Current Biology 22, 2114–2123, November 20, 2012 ©2012 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2012.09.008

Article

Two Dopaminergic Neurons Signal to the Dorsal Fan-Shaped Body to Promote Wakefulness in *Drosophila*

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Summary

Background: The neuronal circuitry underlying sleep is poorly understood. Although dopamine (DA) is thought to play a key role in sleep/wake regulation, the identities of the individual DA neurons and their downstream targets required for this process are unknown.
Results: Here, we identify a DA neuron in each PPL1 cluster that promotes wakefulness in *Drosophila*. Imaging data suggest that the activity of these neurons is increased during wakefulness, consistent with a role in promoting arousal. Strikingly, these neurons project to the dorsal fan-shaped body, which has previously been shown to promote sleep. The reduced sleep caused by activation of DA neurons can be blocked by loss of DopR, and restoration of DopR expression in the fan-shaped body can rescue the wake-promoting effects of DA in a *DopR* mutant background.
Conclusions: These experiments define a novel arousal circuit at the single-cell level. Because the dorsal fan-shaped body promotes sleep, these data provide a key link between wake and sleep circuits. Furthermore, these findings suggest that inhibition of sleep centers via monoaminergic signaling is an evolutionarily conserved mechanism to promote arousal.

Introduction

The transition from sleep to wakefulness is a key behavior, with significant implications for an animal's survival. In mammals, studies over the past several decades have indicated the importance of specific wake-promoting nuclei [1]. However, these nuclei consist of heterogeneous groups of neurons and contain many individual cells [1, 2], making it difficult to determine the precise cellular circuits regulating sleep in mammals. In contrast, fruit flies have a simpler nervous system, and *Drosophila* has emerged as a powerful model system in which to study the molecular and cellular basis of sleep [3, 4]. Studies of sleep in *Drosophila* have highlighted the importance of neuronal excitability and various signaling pathways for sleep regulation [5–10]. In addition, multiple lines of evidence suggest that dopamine (DA) promotes wakefulness in *Drosophila*. Pharmacological manipulation of DA levels can affect sleep [11], and activation of DA neurons decreases sleep time [12]. In addition, mutations in the DA transporter *DAT/fumin*, which increase synaptic DA levels, cause significant reductions in sleep time and an increase in arousal [13, 14]. However, it is not known whether DA signaling is generally needed to induce wakefulness or whether specific DA neurons perform this function.

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In this study, we provide evidence that, among monoaminergic transmitters, DA is the primary mediator of arousal in *Drosophila*. Analyses of novel Gal4 lines with expression in subsets of PPL1 and PPM3 cells suggest that a single DA neuron from each PPL1 cluster projects to the dorsal fan-shaped body to promote wakefulness. Imaging data using a surrogate marker for chronic neuronal activity suggest that these DA neurons are more active during the day and during wakefulness, consistent with a role for these neurons in promoting arousal. Because the dorsal fan-shaped body has recently been shown to promote sleep [15], our data suggest that arousal centers in *Drosophila* may directly regulate sleep-promoting centers. Finally, our data indicate that DopR is the DA receptor responsible for arousal in *Drosophila* and that it is specifically required in the fan-shaped body for this process. These studies thus define a novel arousal circuit and provide a key link between wake and sleep centers in *Drosophila*.

Results

Dopamine Signaling Is the Major Monoaminergic Pathway Promoting Wakefulness in *Drosophila*

In *Drosophila*, there are five biogenic amine pathways, dopamine (DA), octopamine (OA), tyramine (TA), serotonin (5HT), and histamine (HA); DA and OA signaling have been shown to promote wakefulness, whereas 5HT signaling has been suggested to promote sleep [11, 13, 14, 16, 17]. To examine the effects of activating these monoaminergic neurons on sleep and wakefulness, we used different Gal4 lines to drive expression of dTrpA1, a heat-inducible nonselective cation channel that can be used to depolarize neurons to trigger neurotransmitter release [18]. We used the previously characterized *TH-Gal4* [19], *Tdc2-Gal4* [20], and *TRH-Gal4* [21] lines to drive expression in DA, OA/TA, and 5HT neurons, respectively (to our knowledge, there is no available HA-specific Gal4 driver). Using gentle activation conditions (27°C) with these different Gal4 drivers, we found that *TH-Gal4/UAS-dTrpA1* flies exhibited the strongest reduction in sleep (Figures 1A and 1B). As described previously, the percent decrease in sleep with activation of DA neurons was greater during the night than during the day (see Figure S1A available online) [12]. In contrast, activation of OA/TA neurons (using *Tdc2-Gal4*) resulted in a moderate reduction in nighttime sleep, whereas activation of 5HT neurons (using *TRH-Gal4*) had no significant effect on nighttime sleep (Figure 1B). No significant decreases in nighttime sleep were observed for any of the lines in the absence of heat induction of dTrpA1 (Figure 1C). We next examined the effects of increasing monoaminergic signaling on sleep architecture. Activation of DA neurons caused a decrease in nighttime sleep bout duration and sleep bout number (Figures S1B and S1C). In contrast, activation of OA/TA neurons decreased sleep bout duration but did not affect sleep bout number (Figures S1B and S1C). The effects of activating DA neurons on sleep amount and sleep architecture could be suppressed with *TH-Gal80* [22], which inhibits Gal4 function in DA neurons (Figures 1B and S1A–S1C). To further assess the relative importance of DA signaling for

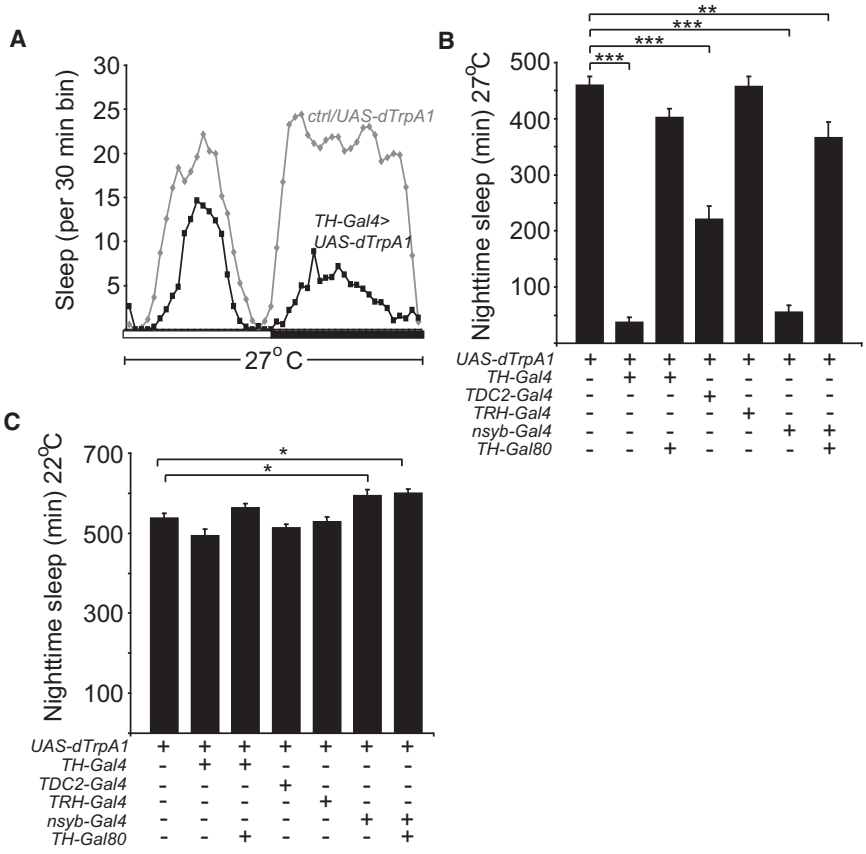

Figure 1. Dopamine Signaling Is a Key Arousal Pathway in *Drosophila*

(A) Sleep profile of *UAS-dTrpA1/+* (gray diamonds) versus *TH-Gal4/UAS-dTrpA1* (black squares) flies at 27°C in 12:12 LD, plotted in 30 min bins. White and black horizontal bars denote light and dark periods, respectively.

(B and C) Nighttime sleep amount at 27°C (B) and at 22°C (C) plotted for *UAS-dTrpA1/+* (n = 54), *TH-Gal4/UAS-dTrpA1* (n = 36), *TH-Gal80/+; TH-Gal4/UAS-dTrpA1* (n = 66), *TDC2-Gal4/+; UAS-dTrpA1* (n = 37), *TRH-Gal4/UAS-dTrpA1* (n = 42), *nsyb-Gal4/+; UAS-dTrpA1/+* (n = 50), and *nsyb-Gal4/TH-Gal80; UAS-dTrpA1/+* (n = 34) flies. Error bars represent SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

wakefulness in *Drosophila*, we compared the effects of gentle activation of most neurons (*nsyb-Gal4*) versus most neurons except DA neurons (*nsyb-Gal4*, *TH-Gal80*) using dTrpA1. Remarkably, the dramatic reduction in nighttime sleep seen with activation of all neurons was largely reversed when Gal4 function was suppressed in DA cells (Figure 1B). Taken together, these data suggest that, among monoamines, DA signaling is the primary mediator of arousal.

The Key Arousal-Promoting DA Cells Reside in PPL1 and/or PPM3

In order to study the specific DA cells that function in arousal, we set out to generate restricted DA Gal4 drivers. There are six major clusters of TH (tyrosine hydroxylase)-positive neurons in each hemisphere. Two are located anteriorly (PAM and PAL) and four posteriorly (PPL1, PPL2, PPM1/2, and PPM3) (Figure S2B) [19, 23]. An ~11 kb genomic *TH* fragment was previously used by Friggi-Grelin et al. [19] to generate *TH-Gal4*, which drives expression in nearly all DA neurons except the PAM subgroup. This study also suggested that the introns of the *TH* gene contain the key enhancers for promoting TH expression [19]. Thus, we generated seven transgenic Gal4 driver lines containing different regions of the *TH* genomic locus, most of which include different combinations of introns of *TH* (Figure S2A).

To determine which DA neurons are captured by these different restricted drivers, we used these drivers to express GFP-nls and immunostained for GFP and TH. The results of these experiments are summarized in Figure S2A and Table S1. Three Gal4 lines (*TH-A*, *TH-B*, and *TH-E*) did not drive expression in any DA cells (data not shown). In contrast, two drivers (*TH-C* and *TH-D*) exhibited largely nonoverlapping TH^+ expression and together recapitulated most of the *TH-Gal4* expression pattern. The *TH-C* lines drive expression in most of the PPM1/2 and all PPL2 cells, as well as three of five PAL cells, whereas the *TH-D* lines drive expression in almost all of the PPL1 and PPM3 cells and one to four of the PPM1/2 neurons (Figure S2A and Table S1). With one exception, discussed below, these expression patterns for *TH-C* and *TH-D* were consistently observed in multiple insertions (data not shown). The *TH-F* and *TH-G* lines demonstrate expression in the PPM1/2, PPM3, PPL1, and PPL2 subgroups but are more variable in the number of DA cells expressed within each subgroup (Table S1). Some nonspecific expression (i.e., in non-TH^+ cells) was observed in the *TH-C*, *D*, *F*, and *G* lines, as is often the case with promoter-Gal4 constructs (data not shown). Thus, because the *TH-C* and *TH-D* constructs seemed to be most useful in dividing the TH^+ expression pattern, we made another version of those constructs by generating deletions in the original ~11 kb *TH-Gal4* construct (Figure S2A). *TH-C′* and *TH-D′* showed expression in TH^+ subsets similar to that of *TH-C* and *TH-D*, respectively, but exhibited little non-TH^+ expression (Figures 2A–2L; Figures S2C–S2J). As expected, expression in TH^+ cells in the *TH-C′* and *TH-D′* lines was suppressed by the addition of *TH-Gal80* (Figures 2D and 2H; Figures S2F and S2J). Finally, all of the restricted *TH* drivers, like the original *TH-Gal4* line [19], expressed poorly in the PAM cluster. In summary, these data confirm that the key enhancers driving expression in DA neurons (except the PAM cluster) are in the introns and also establish novel Gal4 lines driving expression in specific subsets of DA neurons.

To identify the groups of DA neurons regulating sleep/wake, we activated the neurons in the *TH-C1*, *TH-C′*, *TH-D1*, and *TH-D′* drivers using dTrpA1. Activation of the neurons in the *TH-D1* and *TH-D′* driver lines resulted in a profound decrease in nighttime sleep as compared to activation of neurons in the *TH-C1* and *TH-C′* driver lines and controls (Figures 2M and 2N). As before, these effects were more pronounced during the night compared to the day, and there was no significant decrease in nighttime sleep seen in these lines when tested at 22°C (Figures S2M and S2N). As expected, *TH-Gal80* reversed the decrease in sleep amount in the *TH-D1* and

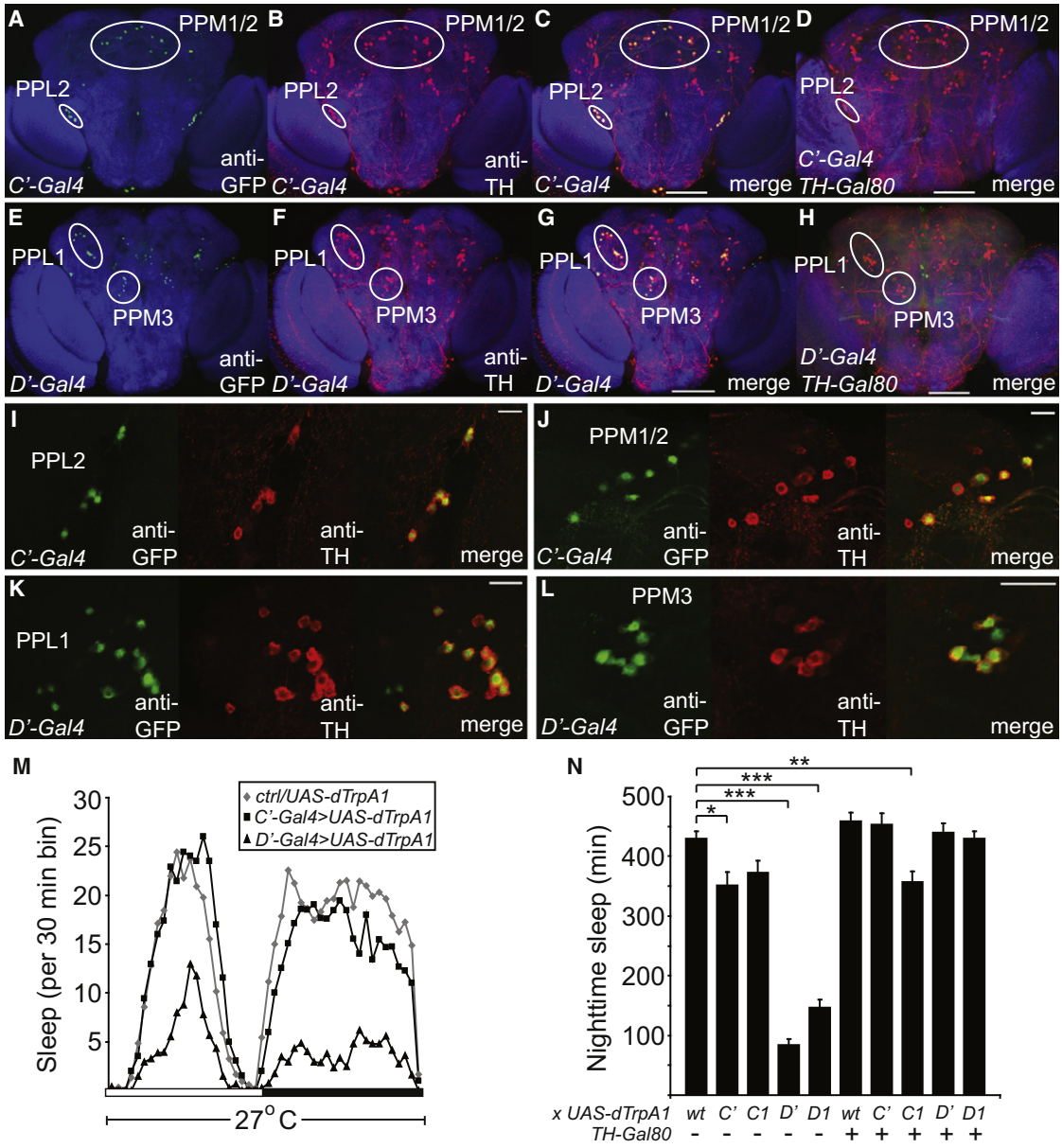

Figure 2. Analysis of Novel DA Gal4 Drivers Suggests that the PPL1 and/or PPM3 Clusters Mediate the Arousing Effects of DA

(A–H) Maximum-projection confocal images of the posterior half of whole-mount adult brains of flies expressing GFP-nls driven by *TH-C′-Gal4* (A–D) or *TH-D′-Gal4* (E–H) in the absence (A–C and E–G) or presence (D and H) of *TH-Gal80*. Scale bars represent 100 μm.

(I–L) Single higher-magnification confocal sections showing PPL2 (I) and PPM1/2 (J) in *TH-C′-Gal4* flies expressing GFP-nls, and PPL1 (K) and PPM3 (L) in *TH-D′-Gal4* flies expressing GFP-nls. Brains were immunostained with nc82, anti-GFP, and anti-TH antibodies. Scale bars represent 20 μm.

(M) Sleep profile of *UAS-dTrpA1/+* (gray diamonds), *TH-C′-Gal4/+; UAS-dTrpA1/+* (black squares), and *TH-D′-Gal4/+; UAS-dTrpA1/+* (black triangles) flies at 27°C in 12:12 LD, plotted in 30 min bins. White and black horizontal bars denote light and dark periods, respectively.

(N) Nighttime sleep plotted for *UAS-dTrpA1/+* (n = 63), *TH-C′-Gal4/+; UAS-dTrpA1/+* (n = 27), *TH-C1-Gal4/+; UAS-dTrpA1/+* (n = 38), *TH-D′-Gal4/+; UAS-dTrpA1/+* (n = 40), *TH-D1-Gal4/+; UAS-dTrpA1/+* (n = 46), *TH-Gal80/+; UAS-dTrpA1/+* (n = 44), *TH-C′-Gal4/TH-Gal80; UAS-dTrpA1/+* (n = 32), *TH-C1-Gal4/TH-Gal80; UAS-dTrpA1/+* (n = 49), *TH-D′-Gal4/TH-Gal80; UAS-dTrpA1/+* (n = 41), and *TH-D1-Gal4/TH-Gal80; UAS-dTrpA1/+* (n = 51) flies. *wt* denotes background control. Error bars represent SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

TH-D′ lines, suggesting that the DA neurons in these drivers are responsible for this effect (Figure 2N). As with the original *TH-Gal4*, activation of DA cells using *TH-D1* and *TH-D′* caused a decrease in sleep bout duration and number at night as compared to controls (Figures S2K and S2L).

To address whether the reduction in sleep in the *TH-D1>dTrpA1* flies results from changes in arousal threshold, we exposed these flies, as well as *TH-C1>dTrpA1* flies, to graded light pulses at zeitgeber time (ZT) 16. Activation of the neurons in the *TH-D1* driver resulted in a significant reduction in arousal threshold as compared to activation of neurons in the *TH-C1* driver or controls (Figure S2O). Together, these data suggest that the cell groups unique to the *TH-D1* and *TH-D′* drivers (PPL1 and PPM3) are more important than

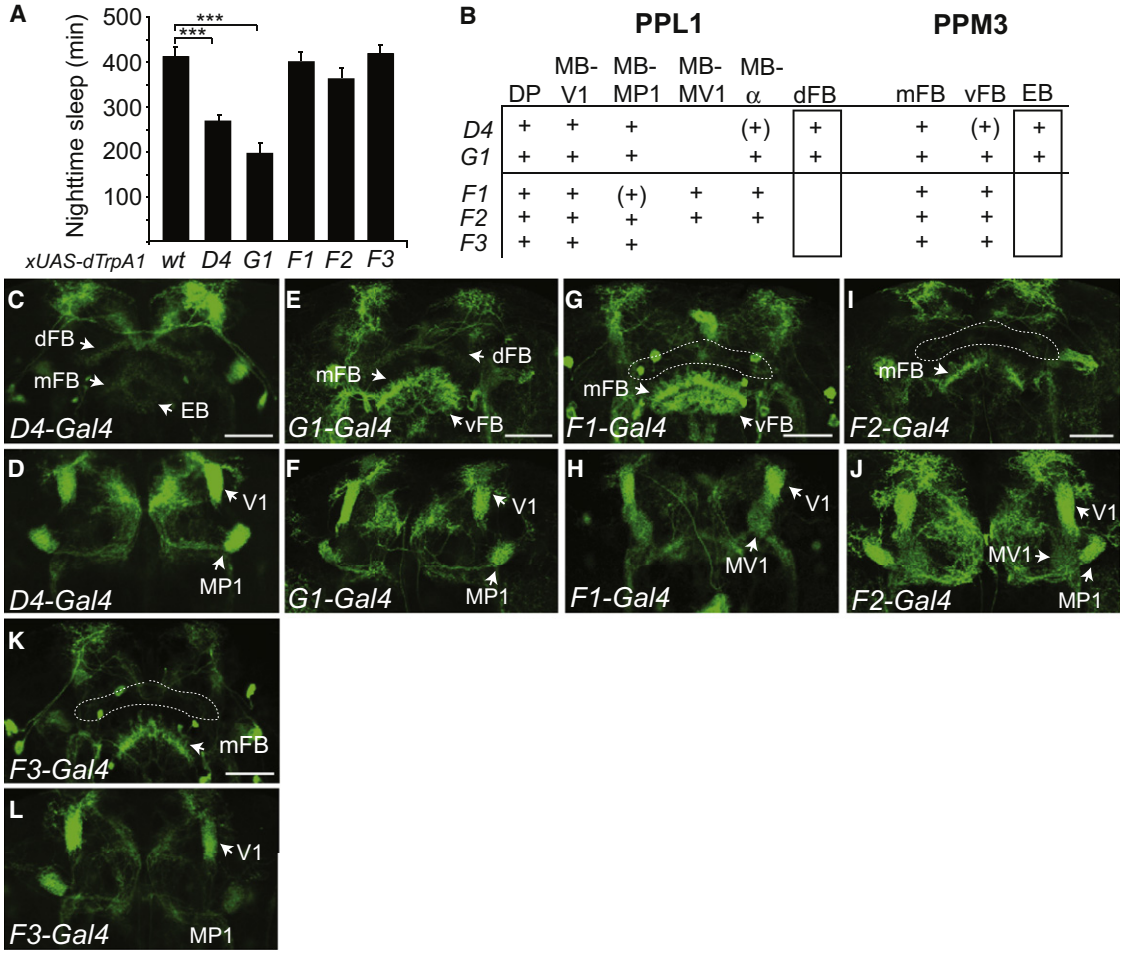

Figure 3. Activation of Gal4 Drivers with Restricted Expression in PPL1 and/or PPM3 Suggests that DA Cells Projecting to the Dorsal Fan-shaped Body or Ellipsoid Body Promote Wakefulness

(A) Nighttime sleep for *UAS-dTrpA1*/+ (n = 56), *TH-D4-Gal4/UAS-dTrpA1* (n = 71), *TH-G1-Gal4/UAS-dTrpA1* (n = 40), *TH-F1-Gal4/UAS-dTrpA1* (n = 62), *TH-F2-Gal4/UAS-dTrpA1* (n = 51), and *TH-F3-Gal4/UAS-dTrpA1* (n = 42) flies at 27°C. *wt* denotes background control. Error bars represent SEM; ***p < 0.001.

(B) Identities of the projections from the PPL1 and PPM3 clusters for the *TH-D4*, *TH-G1*, *TH-F1*, *TH-F2*, and *TH-F3* lines, with naming as described in Table S1. "+" denotes presence of projection, and "(+)" denotes faint staining for projection.

(C–L) Confocal images of brains immunostained with anti-GFP of *TH-D4* (C and D), *TH-G1* (E and F), *TH-F1* (G and H), *TH-F2* (I and J), or *TH-F3* (K and L) driving expression of *UAS-mCD8-GFP; UAS-mCD8-GFP*. Images for (C), (E), (G), (I), and (K) and (D), (F), (H), (J), and (L) consist of maximum projections of an ~4 μm section through the middle or anterior third of the adult brain to highlight the central complex and mushroom bodies, respectively. dFB, EB, mFB, vFB, V1, MP1, and MV1 indicate projections for the dorsal fan-shaped body, ellipsoid body, middle fan-shaped body, ventral fan-shaped body, and the V1, MP1, or MV1 regions of the mushroom bodies, respectively. Dotted lines outline the location of the dFB in (G), (I), and (K). Scale bars represent 50 μm.

other DA subgroups for promoting wakefulness and that this effect is mediated by an increase in arousal.

A Single DA Neuron in Each PPL1 Cluster Projects to the Dorsal Fan-Shaped Body to Promote Wakefulness

To investigate whether individual DA neurons in the PPL1 and/or PPM3 clusters promote arousal, we searched for additional restricted Gal4 drivers. We identified three *TH-F* lines, one *TH-G* line, and one *TH-D* line (*TH-D4*) exhibiting expression in a subset of PPL1 cells and PPM3 cells (Table S1). We next used dTrpA1 to activate the neurons in these driver lines. Two of the lines, *TH-D4* and *TH-G1*, exhibited a significant reduction in nighttime sleep and sleep bout duration compared to controls (Figures 3A and S3A), which could be reversed with *TH-Gal80* (Figures S3B and S3C). No significant differences were observed for sleep bout number or daytime sleep amount for these lines as compared to controls (Figures S3D and S3E). Therefore, these data suggest that specific neurons within PPL1 and/or PPM3, but not the entire subgroups, promote wakefulness.

In order to identify the specific DA neurons in PPL1 and PPM3 that regulate arousal in these restricted drivers, we first classified different DA cells of the PPL1 and PPM3 clusters. To do this, we studied the projection patterns of individual neurons of the *TH-Gal4* driver using images from the FlyCircuit database [24]. Using these images, we could distinguish nine types of PPL1 cells and three types of PPM3 cells with distinct projection patterns (Table S2). We confirmed these classifications by immunostaining our Gal4 lines driving *UAS-mCD8-GFP* with anti-GFP and anti-TH antibodies (data not shown), and also by comparing them with analyses from previous studies [23, 25–29]. We next characterized the projection

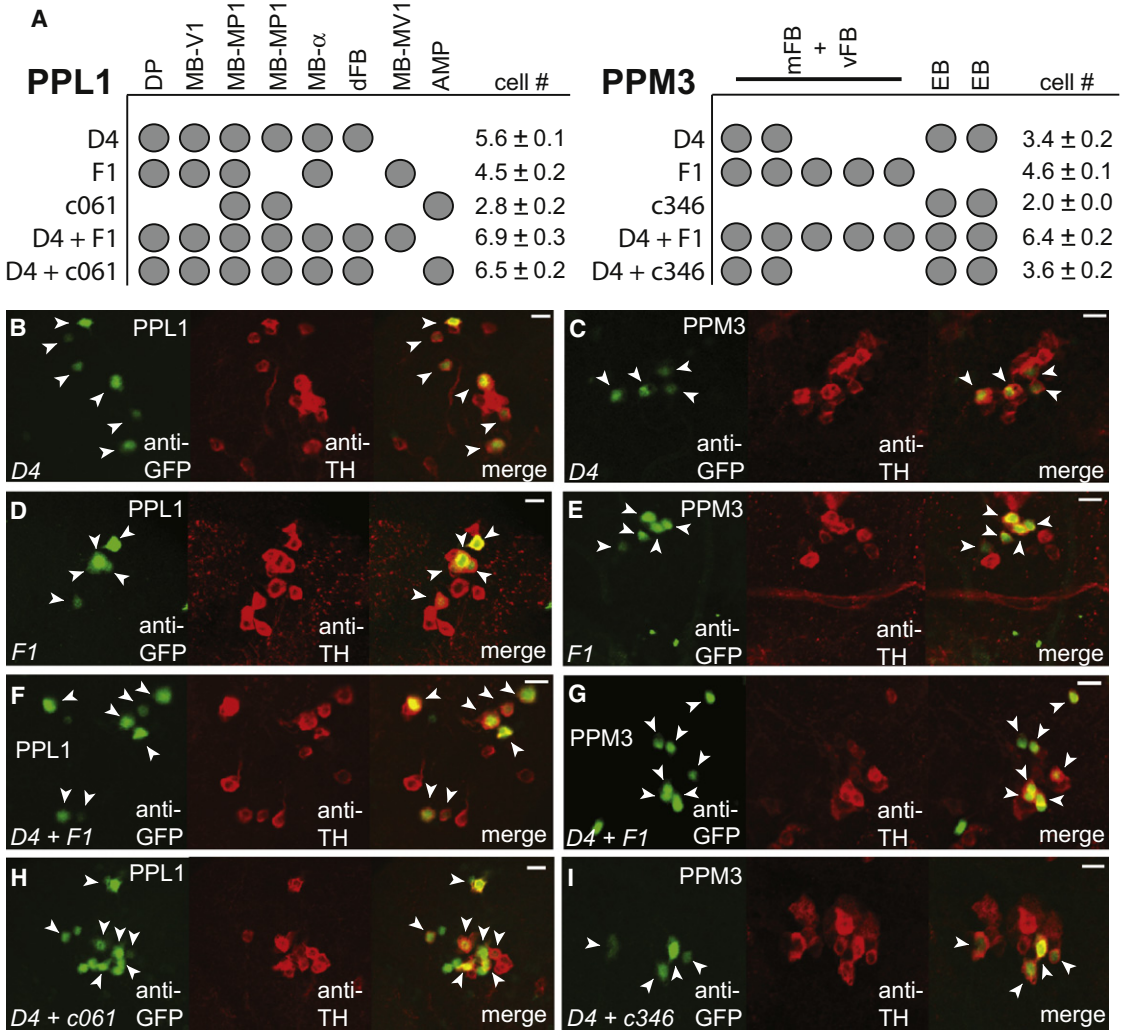

Figure 4. A Single Neuron from the PPL1 Subgroup, which Projects to the Dorsal Fan-Shaped Body, Promotes Wakefulness

(A) Identities of the TH$^+$ cells in the PPL1 and PPM3 clusters in the indicated Gal4 driver lines. Mean cell counts for PPL1 and PPM3 are shown for *TH-D4-Gal4* (n = 13 hemispheres), *TH-F1-Gal4* (n = 14), *c061-Gal4* (n = 5), *c346-Gal4* (n = 3), *TH-D4-Gal4/TH-F1-Gal4* (n = 11), *c061-Gal4; TH-D4-Gal4* (n = 8), and *c346-Gal4; TH-D4-Gal4* (n = 9) flies expressing GFP-nls.

(B–I) Single confocal sections showing the TH$^+$ cells in the PPL1 or PPM3 clusters for the indicated driver lines expressing GFP-nls. Arrowheads indicate GFP+ TH$^+$ cells. Scale bars represent 10 μm.

patterns of the TH$^+$ cells in the five Gal4 lines with restricted expression in PPL1 and PPM3 (Figures 3C–3L; summarized in Figure 3B). In comparing the projection patterns of the two lines that significantly promote arousal (*TH-D4* and *TH-G1*) to those of the three lines that do not, there were two classes of neurons that clearly differed: PPL1 neurons that project to the dorsal fan-shaped body (dFB), and PPM3 neurons that project to the ellipsoid body (EB) (Figure 3B). These data suggest the possibility that these two classes of neurons may be wake-promoting DA cells.

Because *TH-D4* contains fewer TH$^+$ cells than *TH-G1* does, we focused on *TH-D4* for further analysis. To further identify the individual wake-promoting DA neuron (or neurons) in PPL1 or PPM3, we carried out cell-counting experiments (Figures 4B–4I; summarized in Figure 4A). In *TH-D4*, there are five distinct projection patterns (Figure 3B), which are all suppressed by *TH-Gal80* (Figures S4A and S4B). However, counting of TH$^+$ cells in the PPL1 cluster in *TH-D4>GFP-nls* flies revealed six total cells (Figure 4B), suggesting that one of the classes contains two cells. Because there are known to be two MB-MP1 neurons [27, 29], we suspected that *TH-D4* may contain two neurons of this class. We thus generated flies carrying both *TH-D4* and *c061-Gal4*, which has previously been shown to express in three TH$^+$ cells in the PPL1 cluster (two MB-MP1 neurons and one MB-AMP neuron) (Figure S4C) [27, 29]. In *c061-Gal4; TH-D4>GFP-nls* flies, we counted a total of seven cells in PPL1 (Figure 4H). Because *TH-D4* contains six cells in PPL1 and *c061-Gal4* contains two MB-MP1 cells in PPL1, these data suggest that only the MB-AMP cell is added to the *TH-D4* pattern and that there are two MB-MP1 neurons in *TH-D4*. We next examined the effects of activating the two MB-MP1 neurons and one MB-AMP neuron in *c061-Gal4; MB-Gal80*; we used *MB-Gal80* to remove mushroom body (MB) expression in this driver, as done previously [27]. We found that activation of these neurons does not significantly promote wakefulness, suggesting that the MB-MP1 neurons

in *TH-D4* are unlikely to be wake-promoting DA cells (Figures S4E and S4F).

To determine which of the other DA cells in PPL1 might promote wakefulness, we next compared the cellular projections of *TH-D4* with *TH-F1* (which does not promote arousal). There are five projection patterns (Figure 3B) and five TH$^+$ cells in PPL1 (Figure 4D) in *TH-F1* flies, indicating that each TH$^+$ cell can be identified (Figure 4A). We generated flies carrying both *TH-D4* and *TH-F1* in combination with *UAS-GFP-nls*. Counting the GFP+ cells in the PPL1 cluster in these flies revealed a total of seven cells (Figure 4F). Because there are a total of six TH$^+$ PPL1 cells in *TH-D4*, the only cell added by the presence of *TH-F1* is MB-MV1. Thus, the neurons shared between these two lines (DP, MB-V1, and MB-α) are unlikely to promote arousal. Given that the only cell in PPL1 that is unique to the *TH-D4* driver as compared to the *TH-F1* and *c061-Gal4* drivers is the dFB neuron, these data suggest that the PPL1-dFB neuron promotes wakefulness.

Next, because we previously found that the restricted drivers promoting arousal also contained projections to the EB, we compared the PPM3 neurons in *TH-D4* with *c346-Gal4*, which drives expression in a limited subset of neurons, including two PPM3 neurons that project to the EB [28] (Figure S4D). Activation of the two PPM3 neurons in the *c346-Gal4* driver did not significantly promote wakefulness (Figures S4E and S4F). To examine whether the PPM3 EB neurons in *TH-D4* are included in the *c346-Gal4* driver, we combined these two lines to drive expression of GFP-nls. *TH-D4* alone contains four TH$^+$ cells in PPM3 (Figure 4C) and combined with *c346-Gal4* also contains a total of four TH$^+$ cells in PPM3 (Figure 4I). Thus, these data suggest that there are two PPM3 EB neurons in *TH-D4*, which are also present in *c346-Gal4*, and that these neurons are not critical for promoting arousal.

Finally, we investigated whether the other PPM3 neurons projecting to the middle fan-shaped body (mFB) or ventral fan-shaped body (vFB) in *TH-D4* may promote wakefulness. *TH-F1* contains a total of five PPM3 neurons projecting to either the mFB or the vFB (Figure 4E). Combining *TH-D4* with *TH-F1* revealed a total of seven PPM3 TH$^+$ neurons (Figure 4G). These data indicate that the two TH$^+$ PPM3 cells in *TH-D4* are contained within the *TH-F1* expression pattern and suggest that these neurons do not promote wakefulness. We next asked whether loss of function of the neurons in *TH-D4* causes increased sleep. We electrically silenced neurons in the *TH-Gal4*, *TH-D4*, and *TH-F1* drivers in an inducible fashion using *UAS-Kir2.1; tub-Gal80ts*. Kir2.1 is an inwardly rectifying K+ channel that hyperpolarizes the cells in which it is expressed [30]. Inhibition of the neurons in *TH-Gal4* and *TH-D4* resulted in increased sleep as compared to controls and *TH-F1*, consistent with our neuronal activation data (Figures S4G and S4H). Taken together, these data suggest that the pair of PPL1 DA neurons projecting to the dFB promote wakefulness in *Drosophila*.

Imaging Experiments Using ANF-GFP Suggest that the Activity of PPL1-dFB Neurons Is Increased during Wakefulness

If the PPL1-dFB neurons promote wakefulness, then one would predict that these neurons would be more active during the day than during the night. To investigate this, it would be ideal to use a transgenic reporter of chronic neuronal activity, and so we looked for tools that we could potentially adapt for this purpose. A *UAS-ANF-GFP* transgenic line has previously been generated and used in *Drosophila* to monitor neuropeptidergic vesicle trafficking [31, 32]. These flies express rat atrial natriuretic factor (ANF) fused to GFP, under UAS control. Excitation of neurons expressing ANF-GFP induces release of vesicles containing this reporter and a decrease of GFP fluorescence from the terminal; this reduction of GFP signal could potentially be used as a readout for chronic neuronal activity [31, 32]. Neuropeptides are often coexpressed with classical neurotransmitters [33], and we thus asked whether we could express ANF-GFP in DA neurons and whether this would label synaptic terminals. We used *TH-D4-Gal4* to drive *UAS-ANF-GFP* and *UAS-myr-RFP* (to normalize the GFP signal) and found that GFP signal could be readily detected in the terminals of DA cells of this driver, including the PPL1-dFB, PPL1-MP1, and PPL1-V1 neurons (Figure 5A). Next, we examined whether this signal was releasable with activation of these DA neurons, by using dTrpA1 expressed in the same neurons. As shown in Figures 5A and 5B, the GFP/RFP signal decreased with activation of these neurons. Of note, the decrease in GFP/RFP signal was particularly dramatic for the V1 projections, which may reflect an increased capacity of these particular neurons for neuropeptide release.

Using ANF-GFP as a readout, we investigated the activity of PPL1-dFB neurons under physiological conditions. We examined GFP/RFP signal in dFB, MP1, and V1 projections near the beginning of the day (ZT2) and the night (ZT14). There was an ~43%, ~20%, and ~39% reduction in GFP/RFP signal from ZT2 to ZT14 in the dFB, MP1, and V1 projections, respectively (Figures 5C and 5D). These data suggest that, during the day, the activity of multiple DA neurons is increased. We next asked whether these changes are dependent on the presence of light, by performing the experiment in constant darkness (DD). In the absence of light, there was an ~24% and ~27% reduction in GFP/RFP signal from circadian time (CT) 2 to CT14 in the dFB and V1 projections, respectively, whereas there was no significant change in the MP1 projections (Figure 5E). Together, these data suggest that the PPL1-dFB and PPL1-V1 neurons are more active during the day, when flies are awake, compared to during the night.

To address whether these changes are circadian- or sleep state-dependent, we deprived flies of sleep during the night (ZT12–ZT24) and measured GFP/RFP signal at ZT0. As compared to control nondeprived flies, sleep-deprived flies exhibited an ~21% and ~24% reduction in GFP/RFP signal in the dFB and MP1 projections, respectively, whereas there was no significant change in the V1 projections (Figure 5F). These data suggest that PPL1-dFB and PPL1-MP1 neurons, but not PPL1-V1 neurons, are active when flies are forced to stay awake during the night. Together, these data suggest that among these three groups of PPL1 neurons, only the PPL1-dFB neurons are more active both during the day and during wakefulness, consistent with a role in promoting arousal.

DopR in the Fan-Shaped Body Is Required for DA-Mediated Arousal

We next set out to determine the downstream target of the PPL1-dFB neurons that promote wakefulness. There are four DA receptors in *Drosophila*: DopR, DopR2, D2R, and DopEcR [34–37]. To examine which DA receptor is required for DA-mediated arousal, we activated DA neurons using *TH-Gal4* and *UAS-dTrpA1* in $DopR^{f02676}$, $DopR2^{MB05108}$, $D2R^{f06521}$, and $DopEcR^{c02142}$ mutant backgrounds, respectively. $DopR^{f02676}$ and $DopEcR^{c02142}$ have previously been shown to severely reduce DopR and DopEcR levels,

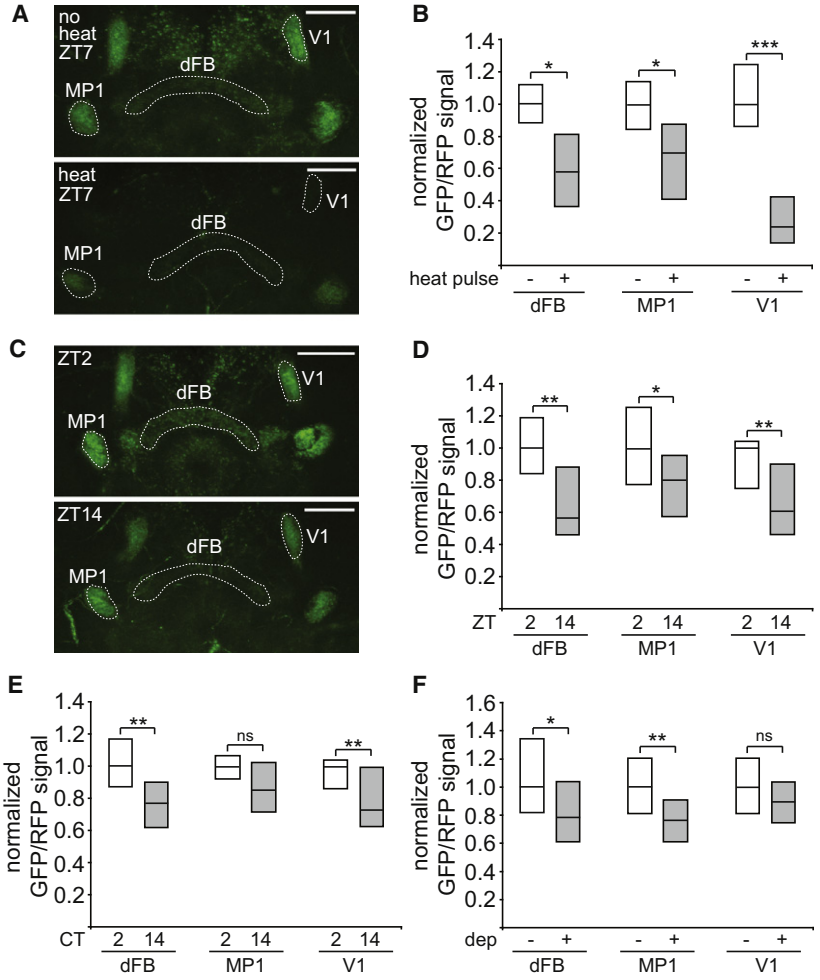

Figure 5. Imaging Experiments Using ANF-GFP Suggest that the PPL1-dFB Neurons Are More Active during the Day and during Wakefulness

(A) Whole-mount brains from flies expressing ANF-GFP, myr-RFP, and dTrpA1 under control of *TH-D4-Gal4*. Images consist of maximum projections of a 12 μm section through the middle of the brain to highlight the mushroom body and central complex projections, with either no heat pulse ("no heat") or 5 hr 32°C heat pulse ("heat") from ZT2–ZT7. dFB, MP1, and V1 indicate projections to the dorsal fan-shaped body or MP1 and V1 regions of the mushroom bodies, respectively. Dotted lines highlight these projections. Scale bars in (A) and (C) represent 50 μm.

(B) GFP/RFP signal for these flies is represented as simplified box plots, where the line inside the box indicates the median and the top and bottom lines represent the 75th and 25th percentiles, respectively. n = 7 for no heat pulse and n = 12 for heat pulse conditions.

(C) Whole-mount brains from flies expressing ANF-GFP and myr-RFP under control of *TH-D4-Gal4* at ZT2 or ZT14.

(D–F) GFP/RFP signal is shown as simplified box plots (as in B) at ZT2 (n = 20) and ZT14 (n = 21) (D), CT2 (n = 15) and CT14 (n = 20) (E), or no sleep deprivation (no dep, n = 22) and sleep deprivation from ZT12 to ZT24 (dep, n = 31) (F) conditions.

*p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

respectively [38, 39]. For the *DopR2*^{MB05108} and *D2R*^{f06521} mutant lines, we performed quantitative PCR analysis and found that these mutants exhibited an ~14% and ~74% reduction in transcript levels, respectively (Figure S5). As shown in Figures 6A and 6B, only loss of DopR fully suppressed the marked reduction in sleep observed when DA neurons are activated. Similarly, *DopR*^{f02676} markedly suppressed the increased wakefulness seen when flies are fed L-3,4-dihydroxyphenylalanine (L-dopa) to enhance DA signaling (Figure 6C). These data suggest that DopR is the main DA receptor required for promoting wakefulness.

The *104y-Gal4* driver was recently used to show that the dFB neurons in this driver regulate sleep [15]. Thus, we next examined whether DopR expression in the FB using *104y-Gal4* is sufficient to rescue the wake-promoting effects of DA in *DopR*^{f02676} flies. Because this transposon itself contains UAS sequences and is inserted in the 5′ nonessential portion of the gene, it can directly be used, in combination with a Gal4 driver, for tissue-specific rescue of *DopR* [28, 38]. When DopR expression was driven in the FB by *104y-Gal4* in the *DopR*^{f02676} mutant background, the reduced sleep caused by DA signaling was significantly restored, suggesting that DA signaling to the FB is necessary for promoting wakefulness (Figure 6C). Because there are non-FB neurons in the *104y-Gal4* expression pattern, we repeated these experiments with an additional driver (*c205-Gal4*) that also expresses in the dFB and obtained similar results. This rescue was specific, because these effects were not observed when DopR was expressed in MB using *OK107-Gal4* (which expresses in all lobes of the MB) or in EB using *c547-Gal4* (which expresses in R2/R4m ring neurons, where PPM3 neurons project) [28] (Figure 6C).

It was previously shown that activation of the FB neurons in the *104y-Gal4* driver results in an increase in sleep time [15]. Because our data suggest that the PPL1-dFB neurons promote arousal by signaling to the dFB, DA signaling to the dFB would be predicted to inhibit the activity of the neurons in *104y-Gal4*. In order to address whether inhibition of the FB neurons of the *104y-Gal4* driver would decrease sleep time, we used *104y-Gal4* to drive expression of *UAS-dORK1ΔC* [40]. dORK1ΔC is a "leak" K^+ channel, which electrically silences neurons [40]. *104y-Gal4>UAS-dORK1ΔC* flies exhibited reduced sleep as compared to controls (Figure 6D). DopR is a D1-type DA receptor [41], which, when activated, stimulates adenyl cyclase activity and increases cAMP levels. Thus, to mimic the effects of activating DopR in the dFB, we expressed mC*, a constitutively active catalytic subunit of cAMP-dependent kinase (PKA), in the dFB using *104y-Gal4*. Increasing PKA activity in the neurons in the *104y-Gal4* driver resulted in a significant decrease in sleep time (Figure 6D), consistent with a model whereby increasing DopR activity and increasing cAMP signaling in the dFB inhibit the function of these neurons, resulting in reduced sleep time.

Discussion

In *Drosophila*, DA has been suggested to play an important role not only in sleep and arousal but also in courtship behaviors, addiction, learning and memory, and appetite/taste

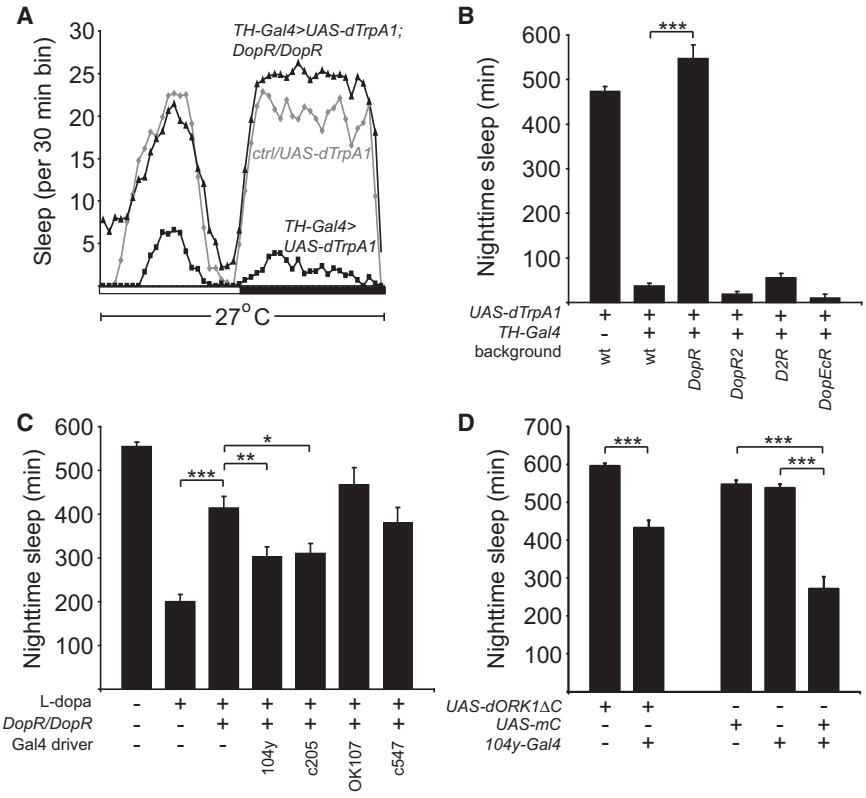

Figure 6. DopR Mediates the Arousing Effects of DA and Is Required in the Fan-Shaped Body to Promote Wakefulness

(A) Sleep profile of *UAS-dTrpA1/+* (gray diamonds), *TH-Gal4/UAS-dTrpA1* (black squares), and *TH-Gal4, DopR/UAS-dTrpA1, DopR* flies (black triangles) at 27°C in 12:12 LD, plotted in 30 min bins. White and black horizontal bars denote light and dark periods, respectively.

(B) Nighttime sleep for *UAS-dTrpA1/+* (n = 62), *TH-Gal4/UAS-dTrpA1* (n = 81), *TH-Gal4, DopR/UAS-dTrpA1, DopR* (n = 31), *TH-Gal4, DopR2/UAS-dTrpA1, DopR2* (n = 31), *D2R;;TH-Gal4/UAS-dTrpA1* (n = 41), and *TH-Gal4, DopEcR/UAS-dTrpA1, DopEcR* (n = 32) flies at 27°C.

(C) Nighttime sleep for control flies not fed L-dopa (n = 64) and control (n = 145), *DopR/DopR* (n = 69), *104y-Gal4/+; DopR/DopR* (n = 82), *c205-Gal4, DopR/DopR* or *c205-Gal4, DopR* (n = 67), *DopR/DopR; OK107/+* (n = 26), and *c547-Gal4, DopR/DopR* (n = 32) flies fed 4 mg/ml L-dopa at 25°C.

(D) Nighttime sleep for *UAS-dORK1ΔC/+* (n = 59), *104y-Gal4/+; UAS-dORK1ΔC/+* (n = 35), *UAS-mC*/+* (n = 62), *104y-Gal4/+* (n = 31), and *104y-Gal4/UAS-mC** (n = 37) flies at 25°C.

Error bars represent SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

[11, 13, 14, 26, 27, 38, 39, 42, 43]. Recent work has identified specific DA neurons in the PPL1 cluster (which are distinct from the dFB-projecting neurons identified in this study) and in the PAM cluster that play an important role in appetitive and olfactory memory [26, 27, 29, 44, 45]. These studies, along with our data, support the notion that individual DA cells, with their distinct targets, play important and qualitatively different roles in animal behavior. Our data suggest that a single DA neuron in each PPL1 subgroup, projecting to the dFB, significantly promotes wakefulness. However, because we did not directly activate single DA neurons, it is possible that these PPL1-dFB cells act in concert with other DA neurons to promote arousal. Indeed, there may be additional wake-promoting DA neurons, because activation of the neurons in *TH-Gal4* causes a stronger phenotype than that seen with activation of the *TH-D4* driver.

Next, to address the physiological regulation of these PPL1-dFB neurons, we used ANF-GFP as a reporter to monitor chronic neuronal activity. The sensitivity of using ANF-GFP to monitor chronic neuronal activity may be somewhat limited, because the ANF-GFP signal cannot be fully depleted even with supraphysiologic neuronal excitation. In addition, because neuropeptides and classical neurotransmitters are processed differently and have different kinetics of release during synaptic transmission, the ANF-GFP decrease may not completely correlate with DA release. Nonetheless, our data suggest that only the PPL1-dFB neurons are consistently active during the day under LD and DD conditions and during forced wakefulness at night, suggesting a specific role for these neurons in arousal. Although *UAS-ANF-GFP* has previously been used to study neuropeptide trafficking and release [31, 32], this work represents, to our knowledge, the first use of this tool to monitor chronic neuronal activity. Given the lack of a transgenic chronic neuronal activity reporter in *Drosophila*, this approach could potentially be useful for measuring neuronal activity on longer timescales (e.g., hours) in other types of neuronal circuits.

We also show that DopR is likely the sole DA receptor responsible for promoting wakefulness and that it is specifically required in the FB for this function. Because the dFB has recently been shown to promote sleep [15], our study provides an important link between wake and sleep circuits in *Drosophila*. The notion that a wake-promoting neuron might act on and inhibit a sleep-promoting center is reminiscent of the "flip-flop switch" model described in mammals, whereby wake-promoting nuclei (e.g., acetylcholinergic and monoaminergic nuclei) and sleep-promoting nuclei (e.g., ventrolateral/median preoptic nuclei, VLPO/MnPO) inhibit each other. In this mutually inhibitory relationship, when one side of the switch becomes more active, it suppresses the other side of the switch, facilitating a rapid and complete transition between wake and sleep [1]. In this model, the dFB in flies may be the analog of VLPO/MnPO in mammals. Because our data suggest that DopR is specifically required in the FB to promote arousal, our model predicts that the PPL1-dFB DA neurons signal to DopR in the FB and inhibit the function of downstream neurons. DopR is a D1-type DA receptor, which has adenyl cyclase activity [46]. In flies, it is unclear whether DopR signaling depolarizes or hyperpolarizes target neurons, but evidence from primary cultured *Drosophila* neurons suggests that activation of DopR can inhibit neurotransmission [47]. Finally, we find that expressing *UAS-mC** to simulate potential downstream effects of elevated cAMP in the FB mimics the effects of electrically inhibiting the FB and is consistent with a model whereby increased DopR activation and subsequent cAMP signaling inhibit the dFB in order to promote wakefulness.

As the circuit map for sleep/wake regulation becomes more delineated, important questions regarding the regulation of the DA circuitry can be addressed. As in mammals, arousal circuits in flies are likely to be regulated by influences such as light, circadian, and homeostatic inputs [48]. For example, a recent study implicated light as modulating the arousing effects of DA specifically in PDF (pigment-dispersing factor) neurons [12]. In addition, DA is required for circadian entrainment to dim light, and cycling of DA expression may play a role in circadian rhythm strength [49]. Ultimately, unraveling the sleep/wake circuitry in flies should lead to a better understanding of how external stimuli and circadian and homeostatic influences regulate arousal at the cellular and molecular level.

Experimental Procedures

Behavioral assays and immunostaining were generally performed as described previously [7]. Details of behavioral assays, imaging experiments, fly strains used, and molecular biology constructs and assays are available in the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.09.008.

Acknowledgments

We thank Fred Wolf, Serge Birman, Hugo Bellen, Paul Garrity, Amita Sehgal, Chris Potter, Tim Lebestky, Ed Kravitz, and the Bloomington Stock Center for fly stocks. We also thank Taro Ueno and Kazuhiko Kume for communication of unpublished results. This work was funded by NINDS grant K08NS059671 and a Burroughs Wellcome Fund Career Award for Medical Scientists (M.N.W.).

Received: July 23, 2012
Revised: August 21, 2012
Accepted: September 5, 2012
Published online: September 27, 2012

References

1. Saper, C.B., Fuller, P.M., Pedersen, N.P., Lu, J., and Scammell, T.E. (2010). Sleep state switching. Neuron *68*, 1023–1042.
2. Tanaka, C., Ishikawa, M., and Shimada, S. (1982). Histochemical mapping of catecholaminergic neurons and their ascending fiber pathways in the rhesus monkey brain. Brain Res. Bull. *9*, 255–270.
3. Hendricks, J.C., Finn, S.M., Panckeri, K.A., Chavkin, J., Williams, J.A., Sehgal, A., and Pack, A.I. (2000). Rest in *Drosophila* is a sleep-like state. Neuron *25*, 129–138.
4. Shaw, P.J., Cirelli, C., Greenspan, R.J., and Tononi, G. (2000). Correlates of sleep and waking in *Drosophila melanogaster*. Science *287*, 1834–1837.
5. Cirelli, C., Bushey, D., Hill, S., Huber, R., Kreber, R., Ganetzky, B., and Tononi, G. (2005). Reduced sleep in *Drosophila* Shaker mutants. Nature *434*, 1087–1092.
6. Koh, K., Joiner, W.J., Wu, M.N., Yue, Z., Smith, C.J., and Sehgal, A. (2008). Identification of SLEEPLESS, a sleep-promoting factor. Science *321*, 372–376.
7. Wu, M.N., Joiner, W.J., Dean, T., Yue, Z., Smith, C.J., Chen, D., Hoshi, T., Sehgal, A., and Koh, K. (2010). SLEEPLESS, a Ly-6/neurotoxin family member, regulates the levels, localization and activity of Shaker. Nat. Neurosci. *13*, 69–75.
8. Allada, R., and Siegel, J.M. (2008). Unearthing the phylogenetic roots of sleep. Curr. Biol. *18*, R670–R679.
9. Foltenyi, K., Greenspan, R.J., and Newport, J.W. (2007). Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*. Nat. Neurosci. *10*, 1160–1167.
10. Stavropoulos, N., and Young, M.W. (2011). *insomniac* and *Cullin-3* regulate sleep and wakefulness in *Drosophila*. Neuron *72*, 964–976.
11. Andretic, R., van Swinderen, B., and Greenspan, R.J. (2005). Dopaminergic modulation of arousal in *Drosophila*. Curr. Biol. *15*, 1165–1175.
12. Shang, Y., Haynes, P., Pírez, N., Harrington, K.I., Guo, F., Pollack, J., Hong, P., Griffith, L.C., and Rosbash, M. (2011). Imaging analysis of clock neurons reveals light buffers the wake-promoting effect of dopamine. Nat. Neurosci. *14*, 889–895.
13. Kume, K., Kume, S., Park, S.K., Hirsh, J., and Jackson, F.R. (2005). Dopamine is a regulator of arousal in the fruit fly. J. Neurosci. *25*, 7377–7384.
14. Wu, M.N., Koh, K., Yue, Z., Joiner, W.J., and Sehgal, A. (2008). A genetic screen for sleep and circadian mutants reveals mechanisms underlying regulation of sleep in *Drosophila*. Sleep *31*, 465–472.
15. Donlea, J.M., Thimgan, M.S., Suzuki, Y., Gottschalk, L., and Shaw, P.J. (2011). Inducing sleep by remote control facilitates memory consolidation in *Drosophila*. Science *332*, 1571–1576.
16. Crocker, A., and Sehgal, A. (2008). Octopamine regulates sleep in *Drosophila* through protein kinase A-dependent mechanisms. J. Neurosci. *28*, 9377–9385.
17. Yuan, Q., Joiner, W.J., and Sehgal, A. (2006). A sleep-promoting role for the *Drosophila* serotonin receptor 1A. Curr. Biol. *16*, 1051–1062.
18. Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in *Drosophila*. Nature *454*, 217–220.
19. Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., and Birman, S. (2003). Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. J. Neurobiol. *54*, 618–627.
20. Cole, S.H., Carney, G.E., McClung, C.A., Willard, S.S., Taylor, B.J., and Hirsh, J. (2005). Two functional but noncomplementing *Drosophila* tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. J. Biol. Chem. *280*, 14948–14955.
21. Alekseyenko, O.V., Lee, C., and Kravitz, E.A. (2010). Targeted manipulation of serotonergic neurotransmission affects the escalation of aggression in adult male *Drosophila melanogaster*. PLoS ONE *5*, e10806.
22. Sitaraman, D., Zars, M., Laferriere, H., Chen, Y.C., Sable-Smith, A., Kitamoto, T., Rottinghaus, G.E., and Zars, T. (2008). Serotonin is necessary for place memory in *Drosophila*. Proc. Natl. Acad. Sci. USA *105*, 5579–5584.
23. Mao, Z., and Davis, R.L. (2009). Eight different types of dopaminergic neurons innervate the *Drosophila* mushroom body neuropil: anatomical and physiological heterogeneity. Front. Neural Circuits *3*, 5.
24. Chiang, A.S., Lin, C.Y., Chuang, C.C., Chang, H.M., Hsieh, C.H., Yeh, C.W., Shih, C.T., Wu, J.J., Wang, G.T., Chen, Y.C., et al. (2011). Three-dimensional reconstruction of brain-wide wiring networks in *Drosophila* at single-cell resolution. Curr. Biol. *21*, 1–11.
25. Tanaka, N.K., Tanimoto, H., and Ito, K. (2008). Neuronal assemblies of the *Drosophila* mushroom body. J. Comp. Neurol. *508*, 711–755.
26. Claridge-Chang, A., Roorda, R.D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., and Miesenböck, G. (2009). Writing memories with light-addressable reinforcement circuitry. Cell *139*, 405–415.
27. Krashes, M.J., DasGupta, S., Vreede, A., White, B., Armstrong, J.D., and Waddell, S. (2009). A neural circuit mechanism integrating motivational state with memory expression in *Drosophila*. Cell *139*, 416–427.
28. Kong, E.C., Woo, K., Li, H., Lebestky, T., Mayer, N., Sniffen, M.R., Heberlein, U., Bainton, R.J., Hirsh, J., and Wolf, F.W. (2010). A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in *Drosophila*. PLoS ONE *5*, e9954.
29. Aso, Y., Siwanowicz, I., Bräcker, L., Ito, K., Kitamoto, T., and Tanimoto, H. (2010). Specific dopaminergic neurons for the formation of labile aversive memory. Curr. Biol. *20*, 1445–1451.
30. Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. J. Neurosci. *21*, 1523–1531.
31. Rao, S., Lang, C., Levitan, E.S., and Deitcher, D.L. (2001). Visualization of neuropeptide expression, transport, and exocytosis in *Drosophila melanogaster*. J. Neurobiol. *49*, 159–172.
32. Shakiryanova, D., Tully, A., Hewes, R.S., Deitcher, D.L., and Levitan, E.S. (2005). Activity-dependent liberation of synaptic neuropeptide vesicles. Nat. Neurosci. *8*, 173–178.

33. Hökfelt, T., Broberger, C., Xu, Z.Q., Sergeyev, V., Ubink, R., and Diez, M. (2000). Neuropeptides—an overview. Neuropharmacology *39*, 1337–1356.
34. Feng, G., Hannan, F., Reale, V., Hon, Y.Y., Kousky, C.T., Evans, P.D., and Hall, L.M. (1996). Cloning and functional characterization of a novel dopamine receptor from *Drosophila melanogaster*. J. Neurosci. *16*, 3925–3933.
35. Han, K.A., Millar, N.S., Grotewiel, M.S., and Davis, R.L. (1996). DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. Neuron *16*, 1127–1135.
36. Hearn, M.G., Ren, Y., McBride, E.W., Reveillaud, I., Beinborn, M., and Kopin, A.S. (2002). A *Drosophila* dopamine 2-like receptor: Molecular characterization and identification of multiple alternatively spliced variants. Proc. Natl. Acad. Sci. USA *99*, 14554–14559.
37. Srivastava, D.P., Yu, E.J., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., Smith, T., and Evans, P.D. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel *Drosophila* G-protein-coupled receptor. J. Neurosci. *25*, 6145–6155.
38. Lebestky, T., Chang, J.S., Dankert, H., Zelnik, L., Kim, Y.C., Han, K.A., Wolf, F.W., Perona, P., and Anderson, D.J. (2009). Two different forms of arousal in *Drosophila* are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. Neuron *64*, 522–536.
39. Inagaki, H.K., Ben-Tabou de-Leon, S., Wong, A.M., Jagadish, S., Ishimoto, H., Barnea, G., Kitamoto, T., Axel, R., and Anderson, D.J. (2012). Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. Cell *148*, 583–595.
40. Nitabach, M.N., Blau, J., and Holmes, T.C. (2002). Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. Cell *109*, 485–495.
41. Gotzes, F., Balfanz, S., and Baumann, A. (1994). Primary structure and functional characterization of a *Drosophila* dopamine receptor with high homology to human D1/5 receptors. Receptors Channels *2*, 131–141.
42. Kaun, K.R., Azanchi, R., Maung, Z., Hirsh, J., and Heberlein, U. (2011). A *Drosophila* model for alcohol reward. Nat. Neurosci. *14*, 612–619.
43. Marella, S., Mann, K., and Scott, K. (2012). Dopaminergic modulation of sucrose acceptance behavior in *Drosophila*. Neuron *73*, 941–950.
44. Plaçais, P.Y., Trannoy, S., Isabel, G., Aso, Y., Siwanowicz, I., Belliart-Guérin, G., Vernier, P., Birman, S., Tanimoto, H., and Preat, T. (2012). Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in *Drosophila*. Nat. Neurosci. *15*, 592–599.
45. Berry, J.A., Cervantes-Sandoval, I., Nicholas, E.P., and Davis, R.L. (2012). Dopamine is required for learning and forgetting in *Drosophila*. Neuron *74*, 530–542.
46. Huang, Y.Y., and Kandel, E.R. (1995). D1/D5 receptor agonists induce a protein synthesis-dependent late potentiation in the CA1 region of the hippocampus. Proc. Natl. Acad. Sci. USA *92*, 2446–2450.
47. Yuan, N., and Lee, D. (2007). Suppression of excitatory cholinergic synaptic transmission by *Drosophila* dopamine D1-like receptors. Eur. J. Neurosci. *26*, 2417–2427.
48. Saper, C.B., Cano, G., and Scammell, T.E. (2005). Homeostatic, circadian, and emotional regulation of sleep. J. Comp. Neurol. *493*, 92–98.
49. Hirsh, J., Riemensperger, T., Coulom, H., Iché, M., Coupar, J., and Birman, S. (2010). Roles of dopamine in circadian rhythmicity and extreme light sensitivity of circadian entrainment. Curr. Biol. *20*, 209–214.