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Systems/Circuits

Inputs to the Sleep Homeostat Originate Outside the Brain

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The need to sleep is sensed and discharged in a poorly understood process that is homeostatically controlled over time. In flies, different contributions to this process have been attributed to peripheral ppk and central brain neurons, with the former serving as hypothetical inputs to the sleep homeostat and the latter reportedly serving as the homeostat itself. Here we re-evaluate these distinctions in light of new findings using female flies. First, activating neurons targeted by published ppk and brain drivers elicits similar phenotypes, namely, sleep deprivation followed by rebound sleep. Second, inhibiting activity or synaptic output with one type of driver suppresses sleep homeostasis induced using the other type of driver. Third, drivers previously used to implicate central neurons in sleep homeostasis unexpectedly also label ppk neurons. Fourth, activating only this subset of colabeled neurons is sufficient to elicit sleep homeostasis. Thus, many published contributions of central neurons to sleep homeostasis can be explained by previously unrecognized expression of brain drivers in peripheral ppk neurons, most likely those in the legs, which promote walking. Last, we show that activation of certain non-ppk neurons can also induce sleep homeostasis. Notably, axons of these as well as ppk neurons terminate in the same ventral brain region, suggesting that a previously undefined neural circuit element of a sleep homeostat may lie nearby.

Key words: **homeostasis; neural circuit; sleep**

Significance Statement

The biological needs that sleep fulfills are unknown, but they are reflected by the ability of an animal to compensate for prior sleep loss in a process called sleep homeostasis. Researchers have searched for the neural circuitry that comprises the sleep homeostat so that the information it conveys can shed light on the nature of sleep need. Here we demonstrate that neurons originating outside of the brain are responsible for phenotypes previously attributed to the proposed central brain sleep homeostat in flies. Our results support a revised neural circuit model for sensing and discharging sleep need in which peripheral inputs connect to a sleep homeostat through previously unrecognized neural circuit elements in the ventral brain.

Introduction

It is unknown how the need to sleep is sensed and discharged to maintain average rest at constant levels over time. This mysterious process, called sleep homeostasis, has traditionally been studied by depriving animals of sleep and then measuring how much sleep they recover, or rebound, afterward. Using this operational definition, we previously established that in fruit flies only rare arousal-promoting neurons are tightly coupled to the sleep homeostat such that rebound sleep ensues when wake-inducing neuronal activity subsides. This observation led us to conclude that such "privileged" arousal-promoting neurons represent rare inputs to the sleep homeostat rather than a core part of the homeostat itself. In our previous study, we mapped some of these inputs to ppk neurons, whose cell bodies and sensory dendritic fields reside throughout the peripheral nervous system of the adult body and project axons into the CNS to transmit sensory information to unknown postsynaptic effector circuits (Seidner et al., 2015).

Despite their privileged ability to regulate sleep need, ppk neurons do not obviously connect to the central complex (Seidner et al., 2015), which has been proposed to function as the sleep homeostat in the brain. The central complex is composed of the fan-shaped body (FB), the ellipsoid body (EB), the protocerebral bridge, and two noduli (Strausfeld and Hirth, 2013; Pfeiffer and Homberg, 2014). A growing body of literature suggests that the FB contributes to sensing and/or discharging sleep need (Donlea et al., 2011, 2014;

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Pimentel et al., 2016; Donlea et al., 2018; Kempf et al., 2019). However, several additional studies support a role for the EB (Liu et al., 2016; Ki and Lim, 2019; Raccuglia et al., 2019). Central to these latter studies is the report that stimulating part of the EB elicits rebound sleep directly without prior sleep deprivation. This finding led to the proposal that electrical discharge from the EB constitutes discharge of the sleep homeostat (Liu et al., 2016). This proposal was based on two important interpretations. The first was that rebound sleep was directly driven by the stimulation, and the second was that the EB was responsible for the rebound sleep.

In the present study, we test both interpretations and come to different conclusions. In particular, we show that previous findings implicating the EB in sleep homeostasis can be explained by the activity of ppk neurons, which drive periods of extended waking that are then followed by rebound sleep. We also show that among ppk neurons, those in the legs are sufficient to drive sleep homeostasis.

Materials and Methods

Fly stocks. *Drosophila melanogaster* were grown at room temperature (20–22°C) on standard cornmeal media with yeast. All animals were outcrossed at least five times into a w^{1118} *iso31* genetic background. Fly lines used in this study were provided as follows: ppk-Gal4, ppk-Gal80 (2× insertions), UAS-TrpA1, UAS-Kir2.1, and ppk-DBD were from a previous study (Seidner et al., 2015). Lines obtained from the Bloomington Stock Center included the following: 30G03-Gal4 (49 646), 69F08-Gal4 (39 499), 58H05-Gal4 (39 198), C584 (30 842), 52B10-Gal4 (38 820), UAS-smGFP-HA,LexAop-smGFP-V5 (64 092), UAS-RedStinger (8546), and LexAop-2xhrGFPnls (29 954 and 29 955). UAS-shi^{ts} was provided by Gerry Rubin (Janelia Research Campus, Ashburn, VA), and 30G03-AD was provided by Mark Wu (Johns Hopkins University, Baltimore, MD).

Additional transgenic fly lines were generated by targeted insertion using PhiC31 integration after injection (Rainbow Transgenics), including ppk-LexA and ppk-AD into attP2, 20B01-DBD into VK00027, and LexAop-TrpA1 into VK00033 (see immediately below for cloning details).

Molecular biology. For ppk-LexA and ppk-AD, the *ppk* promoter sequence was subcloned into the entry vector pENTR1A (Life Technologies) as previously described (Seidner et al., 2015). The *ppk* promoter sequence was then inserted into the pBPLexA_p65Uw or pBPp65ADZpUw plasmid (Pfeiffer et al., 2010) via Gateway recombination. LexAop-TrpA1 was generated by replacing the myristoylated-GFP in pJFRC19-13XLexAop2-IVS-myr-GFP [plasmid # 26224, Addgene (https://www.addgene.org/26224/); RRID:Addgene_26224] with TrpA1 cDNA (a gift from Paul Garrity (Brandeis University, Waltham, MA)) using XhoI and XbaI sites. For 20B01-DBD, the 20B01 promoter fragment was PCR amplified from genomic DNA using the following primers: 5′-CGCCGTGTGCCAAAAATCCATGTGA-3′; and 5′-CGCCCGTGCTCGTTTGACAGTTGTA-3′. PCR products were subcloned into the gateway mENTRY vector (Thermo Fisher Scientific) at the HindIII and XbaI (blunted) restriction sites to generate 20B01-mENTRY. This entry vector was recombined with the pBPp65ADZpUw plasmid via Gateway recombination to generate 20B01-DBD.

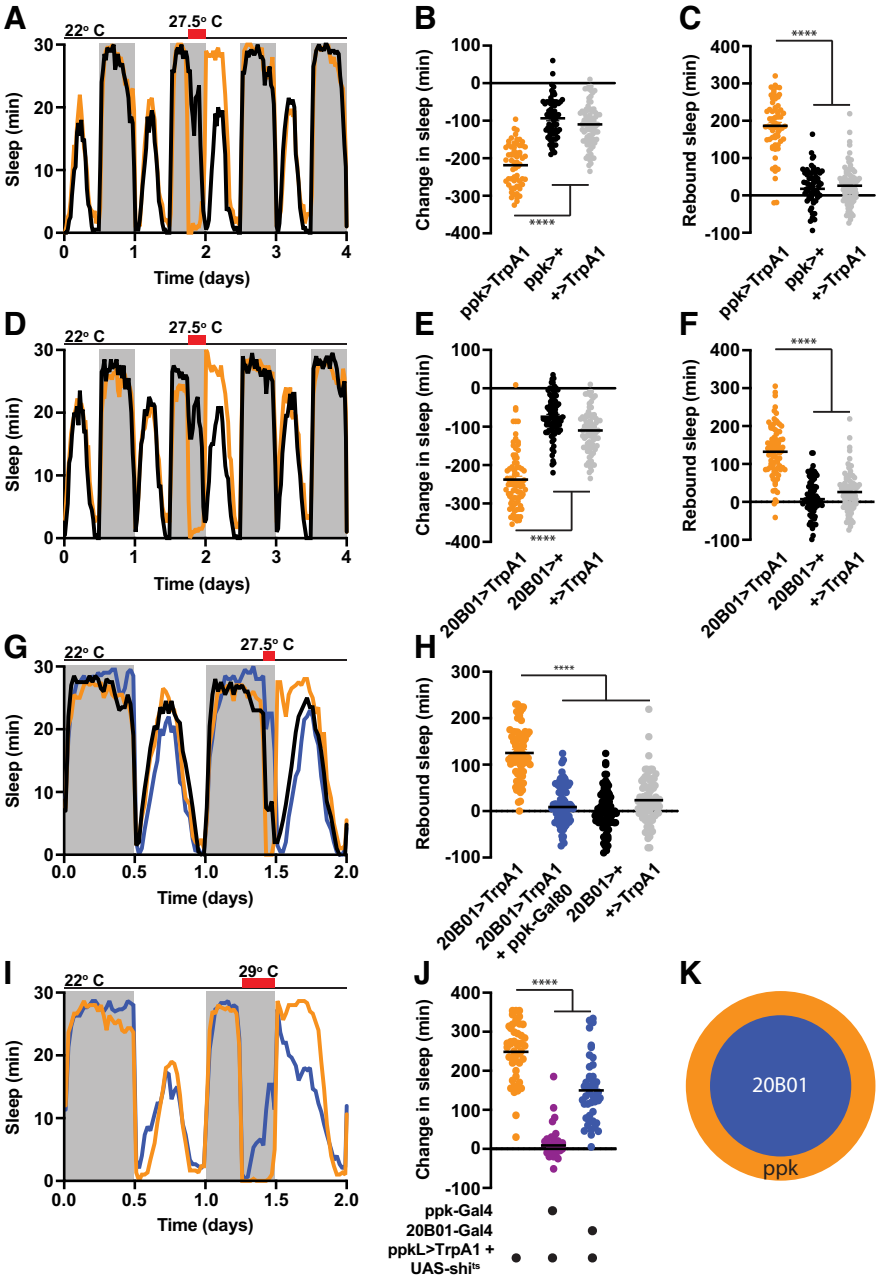
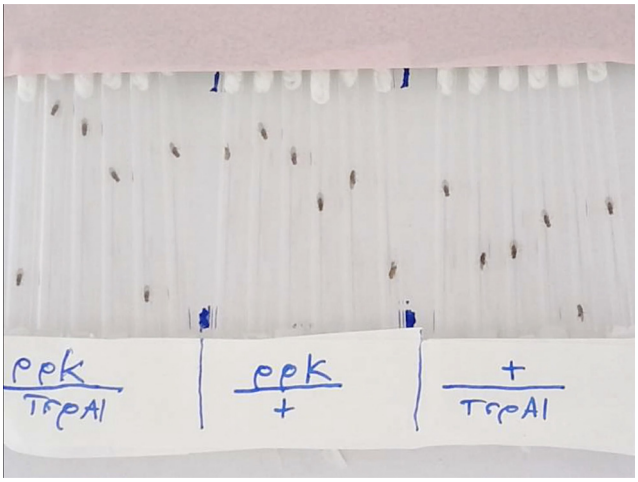


Figure 1. The 20B01 driver targets a subset of ppk neurons whose activity promotes waking and subsequent rebound sleep. ***A–F***, Thermogenetic activation of ppk neurons (***A–C***) or 20B01 neurons (***D–F***) from ZT18 to ZT24 causes waking followed by rebound sleep (***A***, ***D***, $N = 31–32$; ***B***, ***C***, ***E***, and ***F***, $N = 79–80$; ***B***: $F_{(2,204)} = 97.03$, $p < 0.0001$; ***C***: $F_{(2,204)} = 157.7$, $p < 0.0001$; ***E***: $F_{(2,237)} = 141.2$, $p < 0.0001$; ***F***: $F_{(2,237)} = 107.9$, $p < 0.0001$). ***G***, ***H***, ppk-Gal80 blocks rebound sleep elicited by thermogenetically activating 20B01 neurons from ZT22 to ZT24 (***G***, $N = 31–32$; ***H***, $N = 63–80$; ***H***: $F_{(3,315)} = 97.61$, $p < 0.0001$). ***I***, ***J***, Blocking synaptic transmission in 20B01 neurons reduces rebound induced by thermogenetically activating ppk neurons from ZT18 to ZT24 (***J***: $N = 47–48$; $F_{(2,121)} = 25.11$, $p < 0.0001$). ***K***, Diagram suggesting that behaviorally relevant 20B01 neurons represent a subset of ppk neurons. Baseline temperature is 22°C for all panels, and heat pulses (red bars) are 27.5°C (***A–H***) or 29°C (***I***, ***J***). ****$p < 0.0001$ by one-way ANOVA with Tukey's multiple-comparison post-test.



Movie 1. Ppk>TrpA1-driven sleep homeostasis. Ppk>TrpA1 animals and their genetic controls were heat pulsed to 29° C from ZT18 to ZT24 and then subsequently filmed at ZT1. Only thermogenetically sleep-deprived ppk>TrpA1 animals exhibit behavioral manifestations of rebound sleep. [View online]

Table 1. Labeling of ppk neuronal cell bodies in peripheral body parts

	Legs	Wings	Antennae	Proboscis	Thorax	Abdomen
ppk	+	+	+	+	+	+
20B01	+	–	–	–	–	–
30G03	+	+	+	+	–	+
69F08	+	–	–	–	–	–
52B10	–	–	–	–	–	–

Plus signs represent fluorescent Gal4>DsRed-nls colabeling with ppk-LexA>GFP-nls in at least one cell in at least two-thirds of samples examined. Minus signs represent the absence of colabeled cells. $N > 6$ for each driver and each body part.

Behavioral assays. Sleep measurements were performed as previously described using 5 min of inactivity as a proxy for sleep (Seidner et al., 2015; Robinson et al., 2016). Briefly, 1- to 5-d-old female flies were loaded into glass tubes containing 5% sucrose and 2% agarose. Animals were entrained for 2 d on a 12 h light/dark cycle before recording behavior. Sleep/wake patterns were measured using the *Drosophila* Activity Monitoring System (TriKinetics), and custom software was written in MATLAB (MathWorks). Thermogenetic sleep deprivation was performed using a baseline temperature of 22°C for at least 48 h before delivery of a 2–12 h nighttime heat pulse at either 27.5°C or 29°C, ending at zeitgeber time 24 (ZT24). Following the heat pulse, ambient temperature was returned to 22°C, and rebound was measured as subsequent sleep from ZT0 to ZT6 minus sleep during the equivalent baseline period 24 h earlier. Walking speed during the heat pulse was calculated as beam crossings divided by time spent awake.

Immunohistochemistry. Immunostaining of whole-mount brains and thoracic ganglia was performed as previously described (Seidner et al., 2015; Robinson et al., 2016). Briefly, 3- to 5-d-old female brains ($N > 6$ for each genotype) were dissected in cold PBS and fixed in 4% PFA for 1 h at room temperature. After brief washes in PBS + 0.3% Triton X-100 (PBST), brains were blocked in 5% normal donkey serum (The Jackson Laboratory) in PBST for 2 h. Brains were incubated for 1–2 nights at 4°C with 1:1000 rabbit anti-V5 (Thermo Fisher Scientific), or 1:100 rat anti-HA (Roche) and 1:50 mouse anti-nc82 (Developmental Studies Hybridoma Bank). After brief washes in PBST, brains were incubated overnight at 4°C in 1:1000 Alexa Fluor 488 anti-rabbit (Thermo Fisher Scientific), 1:1000 Alexa Fluor 568 anti-rat (Thermo Fisher Scientific), and 3:1000 Alexa Fluor 633 anti-mouse (Thermo Fisher Scientific) before a final set of washes. Brains were mounted in Vectashield (Vector Laboratories) before imaging at 40× magnification on a Leica SP5 or Nikon Eclipse Ti2-E confocal microscope at 1 μm intervals and reassembled for maximum projection using Fiji (SciJava ecosystems).

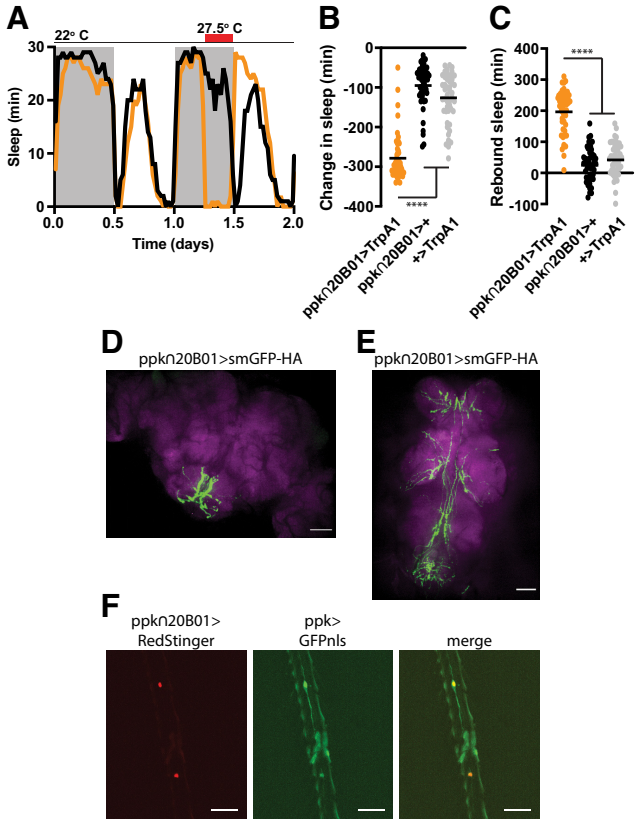


Figure 2. Activation of leg neurons colabeled by the ppk and 20B01 drivers can elicit sleep homeostasis. ***A–C***, Thermogenetic activation of neurons targeted by ppk∩20B01 split-Gal4 is sufficient to cause rebound sleep (***A***, $N = 16$; ***B***, ***C***, $N = 48$; ***B***: $F_{(2,141)} = 132.2$, $p < 0.0001$; ***C***: $F_{(2,141)} = 125.3$, $p < 0.0001$). Baseline temperature is 22°C, and heat pulses (red bars) are 27.5°C from ZT18 to ZT24. ***D***, ***E***, The ppk∩20B01 driver labels processes but no obvious cell bodies in the brain (***D***) or ventral nerve cord (***E***). Dorsal is top; ventral is bottom for each image. ***F***, The ppk∩20B01 driver labels two ppk-positive cell bodies in the leg. Scale bars: ***D–F***, 50 µm. $N > 6$ for each genotype in ***D–F***. All images are maximum projections. ****$p < 0.0001$ by one-way ANOVA with Tukey's multiple-comparison post-test.

Visualization of peripheral neuronal labeling. Labeling of ppk neurons in the peripheral nervous system was accomplished by crossing animals bearing Gal4 drivers of interest to animals containing LexAop-2xhrGFPnls,UAS-RedStinger;LexAop-2xhrGFPnls,ppk-LexA. Legs of progeny were dissected in cold PBS, mounted in Vectashield (Vector Laboratories), and imaged at 20× magnification on a Nikon Eclipse Ti2-E confocal microscope. For each genotype, at least six female flies were used.

Experimental design and statistical analysis. Statistical comparisons were performed using GraphPad Prism version 8 (GraphPad Software). One-way ANOVA with Tukey's multiple-comparison post-test and Pearson correlation were used to calculate *p*-values for experimental versus control groups. Exact *p*-values are reported in figure legends unless $p < 0.0001$. Bar graphs are presented as the mean ± SEM.

Each behavioral experiment was repeated independently two to six times. Each immunohistochemistry experiment was repeated independently at least twice. In the figure legends, *N* reflects the total number of animals per genotype for all pooled replicates. In most replicates, all genotypes were nearly equally represented. In a few cases, animals from one genotype were lacking for a particular paired genotype in an individual replicate.

Results

ppk neurons in the legs drive waking and subsequent rebound sleep

We previously demonstrated that activating ppk neurons elicits acute waking and subsequent rebound sleep (Seidner et al., 2015).

In the present study, we reproduced this behavior by stimulating ppk neurons for 6 h at night using heat-activatable transgenic TrpA1 channels (ppk>TrpA1; Fig. 1*A–C*). Other possible interpretations do not account for what we refer to as rebound sleep since we have shown it is rapidly reversible by mechanical agitation (Seidner et al., 2015). Therefore, it cannot be explained by seizure activity or paralysis. Filming during the rebound period confirmed that ppk>TrpA1 animals are quiescent, with normal body posture, no twitching, and no obvious preference for proximity to the food (Movie 1).

We also previously demonstrated that ppk neurons have no cell bodies in the brain or ventral nerve cord but send behaviorally relevant axonal projections into the suboesophageal zone (SEZ; Seidner et al., 2015). In the present study, we sought to determine the sources of those axons. To achieve this goal, we initially coupled the ppk driver to a DsRed reporter and examined expression in peripheral body parts. We found labeling of cell bodies in the legs, wings, antennae, proboscis, thorax, and abdomen (Table 1).

To determine which of these cell types contributes to sleep homeostasis, we continued our previous screen for cells that function like ppk neurons. As we found with ppk-Gal4, coupling a driver called 20B01-Gal4 to UAS-TrpA1 (20B01>TrpA1) allowed us to thermogenetically deprive animals of sleep, which subsequently elicited strong rebound sleep (Fig. 1*D–F*). To determine the extent to which behaviorally relevant 20B01 cells coincide with ppk neurons, we performed two experiments. In the first experiment, we expressed the Gal4 repressor, Gal80 (Lee and Luo, 1999), under the control of the ppk promoter (ppk-Gal80) and thermogenetically activated neurons in 20B01>TrpA1 animals. We found that 2 h of stimulation was sufficient to elicit robust rebound sleep, so we used this short duration in experiments involving ppk-Gal80 to minimize artifacts that might otherwise emerge because of adaptation or prolonged heat effects. Consistent with ppk neurons contributing to 20B01-driven sleep/wake behavior, we found that ppk-Gal80 blocked rebound sleep in 20B01>TrpA1 animals (Fig. 1*G*,*H*).

In the second experiment, we asked whether blocking synaptic transmission in neurons targeted by the 20B01 driver could reduce sleep homeostasis induced by activating ppk neurons. For this experiment, we targeted dominant-negative temperature-sensitive shibire (Poodry and Edgar, 1979) to 20B01 neurons using the Gal4/UAS system (20B01>shi^{ts}) while simultaneously expressing TrpA1 channels in ppk neurons using the LexA/LexAop system (ppkL>TrpA1; Lai and Lee, 2006). In these experiments, we returned to a stimulus duration of 6 h to test whether ppk-driven arousal was also affected. We found that blocking synaptic transmission with 20B01>shi^{ts} reduced, but did not eliminate, ppkL>TrpA1-driven sleep deprivation and rebound sleep (Fig. 1*I*,*J*).

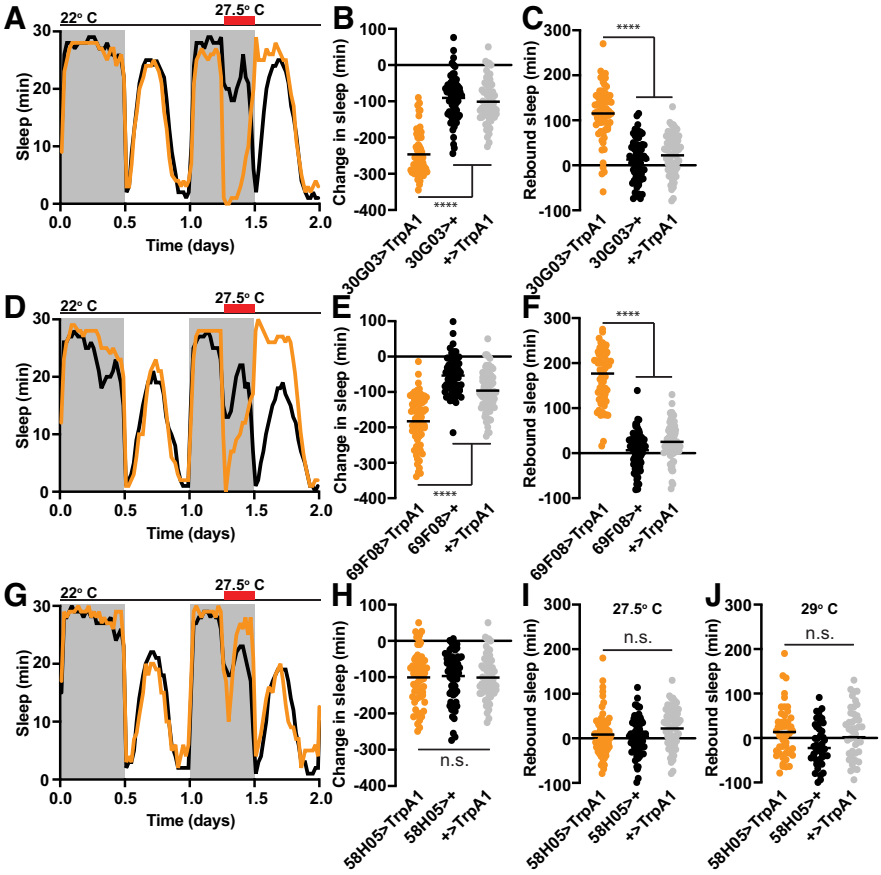


Figure 3. Activation of neurons targeted by EB drivers causes sleep deprivation before rebound sleep. ***A–F***, Thermogenetic activation of 30G03 or 69F08 neurons for 6 h at 27.5°C causes sleep deprivation (***A***, ***B***, ***D***, ***E***), which is followed by rebound sleep (***C***, ***F***) on the cessation of stimulation (***B***, ***C***: $N = 62–64$; ***B***: $F_{(2,185)} = 131.0$, $p < 0.0001$; ***C***: $F_{(2,185)} = 74.82$, $p < 0.0001$; ***E***, ***F***: $N = 66–76$; ***E***: $F_{(2,216)} = 79.21$, $p < 0.0001$; ***F***: $F_{(2,21)} = 242.7$, $p < 0.0001$). ***G–J***, Rebound sleep does not occur after 6 h of thermogenetically activating 58H05 neurons at 27.5°C ($N = 63–64$; ***G–I***) or at 29°C (***J***, $N = 47–48$; ***H***: $F_{(2,186)} = 0.068$, $p = 0.9338$; ***I***: $F_{(2,186)} = 2.467$, $p = 0.0876$; ***J***: $F_{(2,139)} = 4.489$, $p = 0.0129$). In all panels, the baseline temperature is 22°C and heat pulses occur during periods denoted by red bars. *$p < 0.05$ and ****$p < 0.0001$ by one-way ANOVA with Tukey's multiple-comparison post-test.

Collectively, these two experiments suggest that 20B01 labels a behaviorally relevant subset of ppk neurons (Fig. 1*K*). To test this hypothesis more directly, we generated a transgenic split-Gal4 line (Luan et al., 2006) using enhancer fragments from the ppk and 20B01 drivers. Then we crossed this new line to controls or to UAS-TrpA1 animals, collected progeny, and applied a transient heat pulse to thermogenetically activate ppk∩20B01>TrpA1-labeled neurons. We found that stimulating experimental animals for 6 h at night was sufficient to cause waking and to induce subsequent rebound sleep the next morning (Fig. 2*A–C*). Thus, the 20B01 driver labels a subset of ppk neurons that can activate the sleep homeostat.

To confirm the absence of behaviorally relevant ppk∩20B01 neurons in the CNS, we first expressed a nonfluorescent variant of GFP under control of the ppk∩0B01 driver (Nern et al., 2015). Then we performed immunohistochemistry on fixed, dissected brains and ventral nerve cords, and imaged them by confocal microscopy. As expected, we found that the ppk∩20B01 driver labeled only processes, including several that prominently terminate in the ventral brain (Fig. 2*D*,*E*).

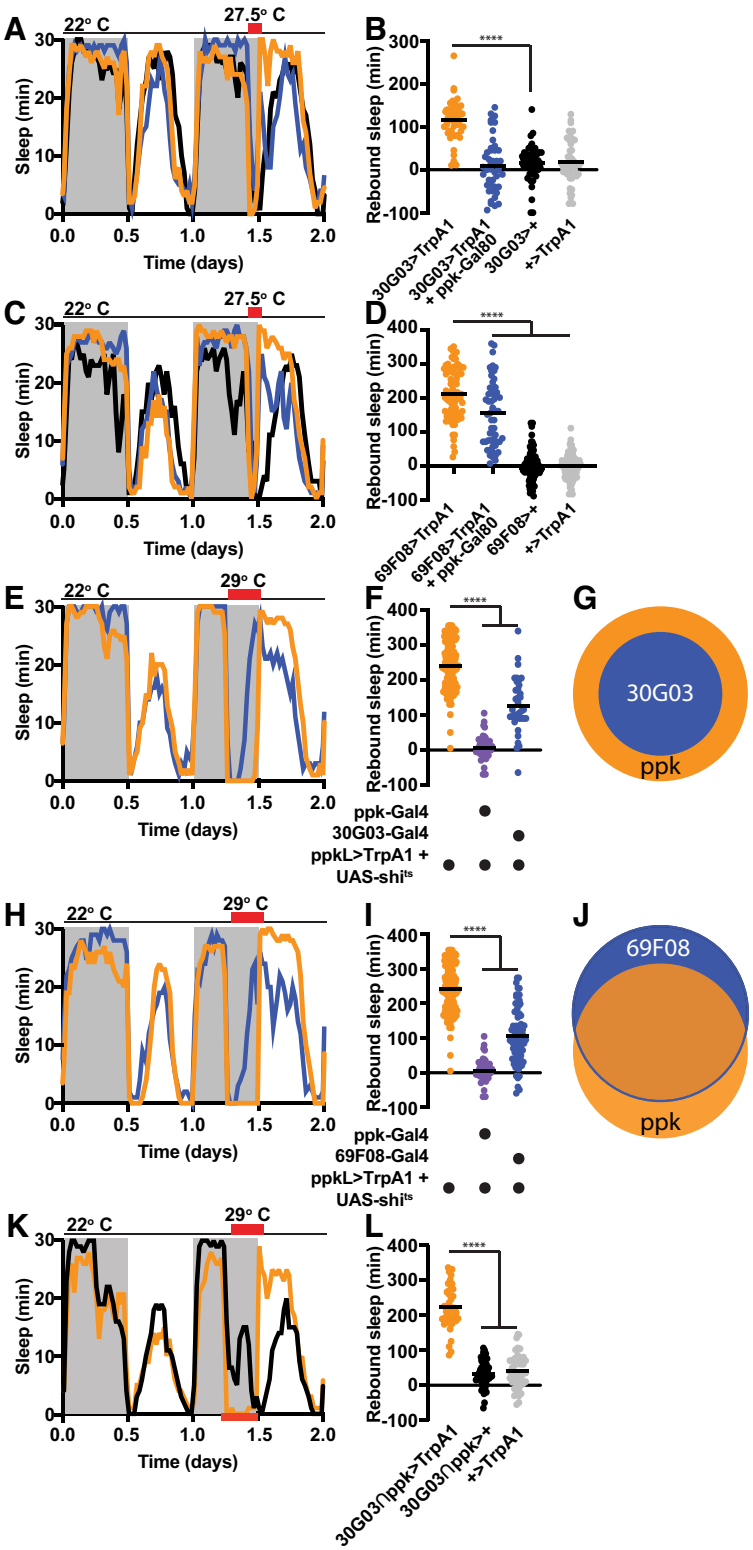


Figure 4. Functional overlap between EB and ppk drivers. ***A–D***, Rebound sleep elicited by thermogenetic activation of 30G03 (***A***, ***B***) or 69F08 neurons (***C***, ***D***) is suppressed by ppk-Gal80 (***A***, ***C***, $N = 16$; ***B***, ***D***, $N = 47–93$; ***B***: $F_{(3,185)} = 47.79$, $p < 0.0001$; ***D***: $F_{(3,316)} = 251.5$, $p < 0.0001$). ***E***, ***F***, Blocking synaptic transmission in 30G03 neurons reduces rebound sleep induced by thermogenetic activation of ppk neurons ($N = 32–74$, $F_{(2,139)} = 150.4$, $p < 0.0001$). ***G***, Diagram suggesting that behaviorally relevant 30G03 neurons represent a subset of ppk neurons. ***H***, ***I***, Blocking synaptic transmission in 69F08 neurons reduces rebound induced by thermogenetic activation of ppk neurons ($N = 43–58$, $F_{(2,144)} = 161.5$, $p < 0.0001$). ***J***, Diagram suggesting that behaviorally relevant 69F08 neurons overlap with ppk neurons. ***K–L***, Thermogenetic activation of neurons targeted by 30G03∩ppk split-Gal4 is sufficient to cause rebound sleep (***K***, $N = 16$; ***L***, $N = 46–48$; $F_{(2,138)} = 217.8$, $p < 0.0001$). In all panels, the baseline temperature is 22°C and heat pulses (red bars) occur for 2 h at 27.5°C (***A–D***) or 6 h at 29°C (***E–L***). ****$p < 0.0001$ by one-way ANOVA with Tukey's multiple-comparison post-test.

Next, we asked where in the peripheral nervous system the cell bodies of behaviorally relevant neurons might be located. To address this question, we expressed a nuclear-targeted DsRed (Barolo et al., 2004) under the control of 20B01-Gal4 and a nuclear-targeted GFP expressed under control of ppk-LexA, and we examined dissected peripheral body parts for colabeled cells. As shown in Table 1, the only location that met this criterion was the legs, each of which showed colabeling of two cells. To confirm this finding, we performed the same experiment using ppk∩20B01 as the driver. Examples of colabeled cell bodies are shown in Figure 2*F*. Thus, activity of ppk neurons in the legs is sufficient to drive waking and sleep need.

Thermogenetic activation of neurons targeted by EB drivers indirectly triggers sleep homeostasis

Next, we compared the functions of ppk neurons, which appear to be inputs to the sleep homeostat (Seidner et al., 2015), and EB neurons, which have been proposed to serve as the homeostat itself (Liu et al., 2016). This latter proposal arose from a screen of various Gal4/UAS-TrpA1 combinations that could elicit rebound sleep after thermogenetic stimulation of different populations of neurons for 12 h. In that study, three Gal4 drivers with ostensibly overlapping expression in the EB—30G03, 69F08, and 58H05—were used for most experiments. The authors reported that activating EB-targeted TrpA1 channels led to a persistent increase in sleep during and after the stimulation period, as if the homeostat were being activated by neuronal discharge of the EB rather than by prior waking (Liu et al., 2016). Since rebound sleep has historically been characterized as a compensatory response to sleep deprivation, we examined the unusual response to neuronal stimulation in more detail. We found that 6 h of thermogenetic activation at 27.5°C elicited subsequent rebound sleep with two of the published EB drivers, 30G03 and 69F08, as expected (Fig. 3*A*,*C*,*D*,*F*). However, contrary to previously published results, we observed sleep deprivation during the stimulation period (Fig. 3*A*,*B*,*D*,*E*). Furthermore, we were unable to elicit any change in sleep with 6 h of thermogenetic activation using the third driver, 58H05 (Fig. 3*G–I*), even at 29°C (Fig. 3*J*). These findings are difficult to reconcile with the conclusion by Liu et al. (2016) that direct stimulation of EB neurons experimentally bypasses endogenous

arousal mechanisms to mimic discharge of the homeostat and thus promotes rebound-like sleep. Instead, our findings are more consistent with standard models in which the sleep homeostat discharges only after sustained signaling by arousal-promoting neurons.

Peripheral ppk neurons are a behaviorally relevant subset of cells targeted by EB drivers

Since stimulating ppk neurons labeled with ppk or EB drivers elicits similar behaviors, namely, extended waking followed by rebound sleep, we asked whether ppk neurons might underlie effects on sleep homeostasis previously attributed to EB neurons. To address this question, we first thermogenetically activated neurons in 30G03>TrpA1 or 69F08>TrpA1 animals in which Gal4 expression was blocked in ppk neurons with ppk-Gal80. As with related experiments in Figure 1*G*, we used a stimulation period of 2 h to minimize potential artifacts from the heat pulse. Consistent with ppk neurons contributing to sleep behaviors attributed to the EB, we found that ppk-Gal80 reduced rebound sleep in both 30G03>TrpA1 and 69F08>TrpA1 animals. Whereas the effect was complete when ppk-Gal80 was combined with 30G03>TrpA1, the reduction in sleep homeostasis was incomplete when ppk-Gal80 was combined with 69F08>TrpA1 (Fig. 4*A–D*). These results suggest that ppk neurons encompass all the behaviorally relevant 30G03 neurons but only some of behaviorally relevant 69F08 neurons.

In a related set of experiments, we then asked whether blocking synaptic transmission in neurons targeted by EB drivers could reduce sleep homeostasis induced by activating ppk neurons. We found that blocking synaptic transmission with 30G03>shi^{ts} or 69F08>shi^{ts} reduced ppkL>TrpA1-driven sleep deprivation and rebound sleep (Fig. 4*E–I*). These data cannot easily be attributed to a circuit in which wake-promoting ppk neurons and sleep-promoting EB neurons are distinct components. If that were true, then blocking the synaptic output of EB neurons should increase waking. However, in the present experiment ppk-driven waking was decreased by 30G03>shi^{ts} or 69F08>shi^{ts} (Fig. 4*E*,*H*). Thus, a more parsimonious interpretation of these results is that both EB drivers target a subset of peripheral ppk neurons, and this subset is sufficient to trigger sleep homeostasis (Fig. 4*G*,*J*).

To test this hypothesis, we first generated a transgenic split-Gal4 line using enhancer fragments from the 30G03 and ppk drivers. Then we crossed this new line to controls or to UAS-TrpA1 animals, collected progeny, and thermogenetically activated 30G03∩ppk neurons for 6 h at night. We found that 30G03∩ppk>TrpA1 animals were effectively sleep deprived during this period and showed strong rebound sleep the next morning relative to controls (Fig. 4*K*,*L*).

These results indicate that the EB drivers label a subset of peripheral ppk neurons that can activate the sleep homeostat. To address where in the nervous system the EB and ppk drivers exhibit overlapping expression, we expressed two different epitope-tagged nonfluorescent variants of GFP under the control of EB and ppk drivers in the same animals (Nern et al., 2015). Then we performed immunohistochemistry on fixed, dissected brains and imaged them by confocal microscopy. We found that the 30G03-Gal4 and 69F08-Gal4 drivers labeled a subset of the same neuronal processes that were labeled by ppk-LexA (Fig. 5*A*,*B*).

Next, we asked where cell bodies of behaviorally relevant ppk neurons might be located. To address this question, we expressed nuclear-targeted DsRed under the control of either the 30G03 or 69F08 driver, and we expressed a nuclear-targeted GFP under the control of the ppk promoter. Then we looked for colabeled cells in dissected peripheral body parts. Although we observed overlap between 30G03-Gal4 and ppk-Gal4 labeling in various locations, we found that the two drivers colabeled only two cells in the legs (Fig. 5*C*,*D*, Table 1) similar to what we observed for overlapping expression of 20B01 and ppk drivers (Fig. 2*F*). Thus, despite the designation of 30G03-Gal4 and 69F08-Gal4 as central EB drivers, these drivers appear to target peripheral ppk neurons that can induce sleep homeostasis.

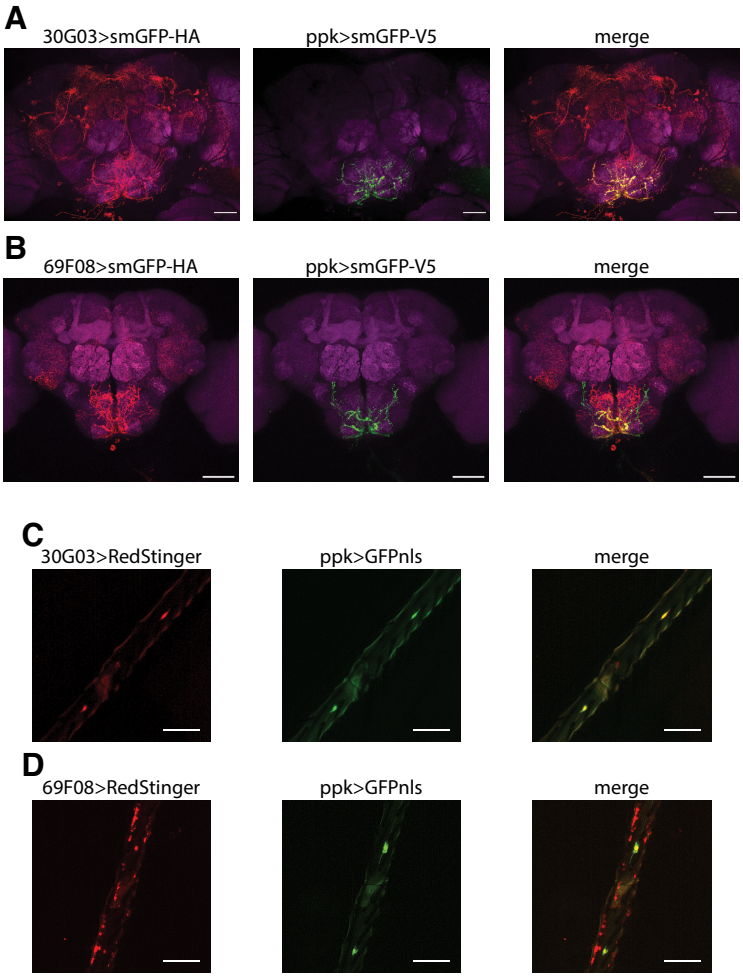


Figure 5. EB and ppk drivers exhibit overlapping expression patterns. ***A***, ***B***, Processes labeled by 30G03 and ppk drivers overlap in the SEZ. ***B***, Processes labeled by 69F08 and ppk drivers also overlap in the SEZ. Dorsal is top; ventral is bottom for each image. ***C***, The 30G03 driver labels peripheral neurons moderately, and two of these are ppk-positive cell bodies in the leg. ***D***, The 69F08 driver labels many peripheral neurons, including two ppk-positive cell bodies in the leg. Scale bars: ***A–D***, 50 μm. All images are maximum projections. $N > 6$ for each genotype.

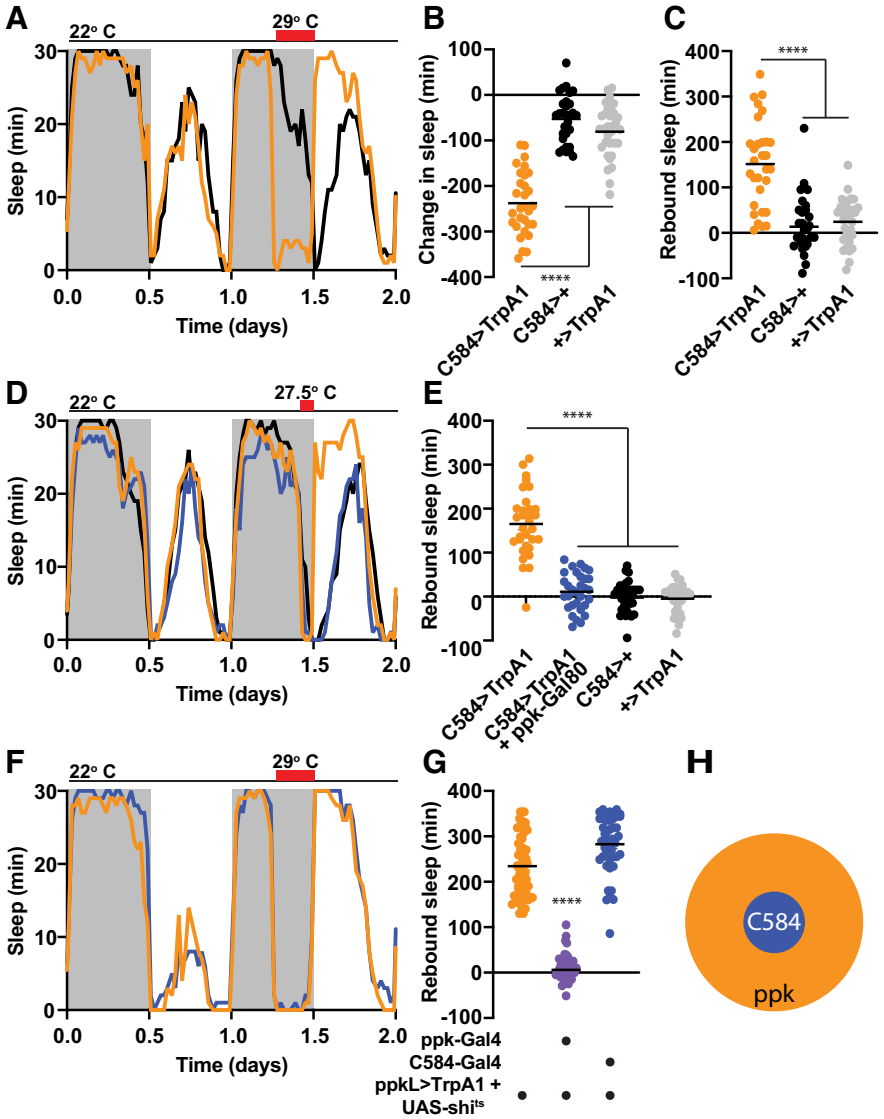


Figure 6. Functional overlap between C584 and ppk drivers. ***A–C***, Thermogenetic activation of C584 neurons causes sleep deprivation that is followed by rebound sleep ($N = 30$; ***B***: $F_{(2,89)} = 82.85$, $p < 0.0001$; ***C***: $F_{(2,89)} = 33.5$, $p < 0.0001$). ***D***, ***E***, Rebound sleep elicited by thermogenetic activation of C584 neurons is suppressed by ppk-Gal80 (***E***: $N = 31–32$; $F_{(3,123)} = 90.23$, $p < 0.0001$). ***F***, ***G***, Blocking synaptic transmission in C584 neurons does not affect rebound sleep induced by thermogenetic activation of ppk neurons ($N = 43–63$, $F_{(2,148)} = 281.2$, $p < 0.0001$). ***H***, Diagram suggesting that behaviorally relevant C584 neurons represent a subset of ppk neurons. In all panels, the baseline temperature is 22°C and heat pulses (red bars) occur for 2 h at 27.5°C (***D***, ***E***) or 6 h at 29°C (***A–C***, ***F***, ***G***). ****$p < 0.0001$ by one-way ANOVA with Tukey's multiple-comparison post-test.

Sleep homeostasis-inducing C584 neurons are also a subset of peripheral ppk neurons

Very few other drivers have been reported to label neurons whose activity is sufficient to trigger sleep homeostasis. One exception is C584 (Dubowy et al., 2016). We found that, like ppk neurons, nighttime activation of neurons in C584>TrpA1 animals caused sleep deprivation followed by rebound sleep the next morning (Fig. 6*A–C*). Therefore, we asked whether C584, like EB drivers, labels ppk neurons that are ultimately responsible for effects on sleep homeostasis. To address this question, we thermogenetically activated neurons in C584>TrpA1 animals expressing ppk-Gal80 to suppress Gal4-driven TrpA1 expression in ppk neurons. Consistent with a role for ppk neurons in mediating C584-driven sleep homeostasis, we found that ppk-Gal80 abolished rebound sleep (Fig. 6*D*,*E*).

C584 has been reported to express broadly throughout the nervous system, and the subset of its labeled neurons that affect sleep homeostasis are unknown (Dubowy et al., 2016). To determine whether the C584 driver labels a subset or superset of behaviorally relevant ppk neurons, we blocked synaptic transmission in C584 neurons with shi^{ts} while attempting to elicit sleep homeostasis by thermogenetically activating ppk neurons with ppkL>TrpA1. We found that C584>shi^{ts} was unable to reduce rebound sleep in these animals (Fig. 6*F*,*G*). We also searched for overlapping expression of reporters of C584 and ppk drivers, but we were unable to find such cells, possibly because C584 expression is weak in them. Nonetheless, collectively our results suggest that ppk neurons encompass behaviorally relevant C584 cells, whereas C584 neurons represent only a subset of ppk cells that can trigger sleep homeostasis (Fig. 6*H*).

Some inputs to the sleep homeostat function independently of ppk neurons

Like ppk-Gal4 and C584, 52B10-Gal4 also targets arousal-promoting neurons capable of eliciting sleep homeostasis (Fig. 7*A–C*; Liu et al., 2016). Therefore, we asked whether ppk neurons represent a behaviorally relevant subset of 52B10-labeled cells. To address this question, we tested whether ppk-Gal80 could limit sleep homeostasis in 52B10>TrpA1 animals. We found that thermogenetic activation of neurons in these animals caused rebound sleep that was unaffected by the suppression of Gal4 activity in ppk neurons (Fig. 7*D*,*E*).

We also asked whether blocking synaptic transmission in 52B10 neurons could reduce the effects of ppkL>TrpA1-driven sleep homeostasis. To address this question, we compared sleep deprivation and rebound sleep in ppkL>TrpA1 animals alone versus animals that additionally expressed 52B10>shi^{ts}. We found that blocking synaptic transmission had no effect on ppk-driven sleep deprivation and reduced ppk-driven rebound sleep by only ~10% (Fig. 7*F*,*G*). Thus, 52B10 and ppk neurons function largely independently in contributing to sleep homeostasis (Fig. 7*H*).

We also coupled the expression of two different nonfluorescent GFP reporters to the 52B10 and ppk drivers and looked for colabeling of cells in the same animals. As expected based on the behavioral results described above, we found that the two drivers are expressed in independent populations of neurons (Fig. 8*A*,*B*). In common with ppk neurons, however, 52B10-labeled neurons include axons that enter the brain from the periphery and terminate in the SEZ. This commonality suggests that the SEZ may receive sensory wake-promoting signals and transmit them to

postsynaptic effectors, at least some of which may contribute to increased sleep drive.

During thermogenetically induced waking, none of the genotypes we examined showed signs of major fatigue that could lead to the subsequent period being misattributed to rebound sleep. Nonetheless, we asked whether walking speed during sleep deprivation is correlated with rebound sleep. As shown in Figure 9*A*, we found that there was no correlation. In fact, we found that walking speeds were sometimes lower in genotypes that exhibited rebound sleep than in genotypes that exhibited none. In summary, ppk neurons appear to represent a superset of 30G03 and C584 neurons, to overlap with 69F08 neurons, and to function largely independent of 52B10 neurons. A model of these relationships is depicted in Figure 9, *B* and *C*.

Discussion

We previously demonstrated that ppk neurons have the rare ability to couple sustained waking to increased sleep need. As a result, we proposed that ppk neurons have a privileged role in activating the sleep homeostat. However, we did not indicate where the homeostat might be located (Seidner et al., 2015). It was subsequently proposed by Liu et al. (2016) that a long-lasting increase in sleep could be elicited by direct activation of neurons targeted by EB drivers, thus suggesting that the EB might represent the elusive sleep homeostat. However, this conclusion was based on several interpretations that our present findings call into question.

The first interpretation involved equating thermogenetic activation of neurons with direct discharge of the homeostat based on the finding that the activation protocol caused rebound sleep without sleep deprivation. But if arousal were driven at high intensity, any expected induced waking might have been overcome by rapid activation of the sleep homeostat. Our results support this hypothesis: at lower-intensity stimulation (i.e., at 27.5°C instead of 29°C used by Liu et al., 2016), two of the three published EB>TrpA1 combinations first caused sleep deprivation, which was then followed by rebound sleep. The third published EB>TrpA1 combination we tested (58H05>TrpA1) was unable to elicit rebound sleep after 6 h of neuronal activation at low or high intensity. Collectively, these results are not consistent with direct stimulation and discharge of a sleep homeostat residing in the EB. Instead, they more closely resemble outcomes of typical sleep deprivation experiments in which sustained arousal causes a slow activation of the sleep homeostat that results in rebound sleep when the arousing stimulus ends.

The second interpretation by Liu et al. (2016) that our results call into question was that consensus expression of three behaviorally relevant drivers in the EB means that the EB functions in the behavior. However, in the original study, the expression of the drivers outside the brain was not examined (Liu et al., 2016). In fact, our present results clearly show that two of the three main drivers in the previous study, 30G03 and 69F08, express in ppk neurons, whose activation alone is sufficient to elicit sleep homeostasis. Collectively, these findings weaken the case for the EB as a source of sleep homeostasis and suggest that ppk neurons could be responsible for many of the behavioral effects of previously reported EB>TrpA1 combinations. We tested this

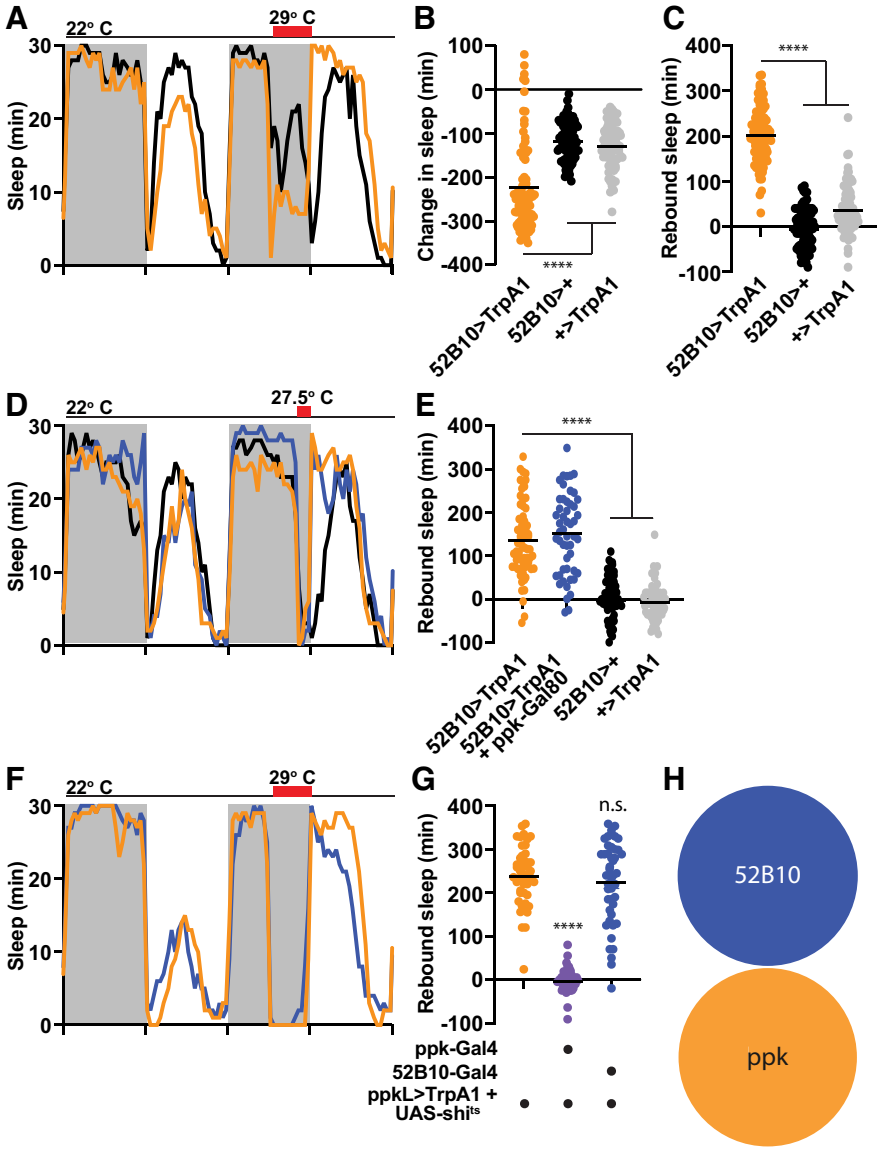


Figure 7. 52B10 and ppk drivers label distinct populations of neurons that are each capable of eliciting sleep homeostasis. ***A–C***, Thermogenetic activation of 52B10 neurons causes sleep deprivation that is followed by rebound sleep ($N = 79$, $F_{(2,232)} = 317.5$, $p < 0.0001$). ***D***, ***E***, Rebound sleep elicited by thermogenetic activation of 52B10 neurons is unaffected by ppk-Gal80 ($N = 51–64$, $F_{(3,230)} = 85.27$, $p < 0.0001$). ***F***, ***G***, Blocking synaptic transmission in 52B10 neurons has little effect on sleep deprivation or rebound sleep induced by thermogenetic activation of ppk neurons ($N = 44–46$, $F_{(2,133)} = 171.3$, $p < 0.0001$). ***H***, Diagram suggesting that behaviorally relevant 52B10 neurons are distinct from ppk neurons. In all panels, the baseline temperature is 22°C and heat pulses (red bars) occur for 2 h at 27.5°C (***D***, ***E***) or 6 h at 29°C (***A–C***, ***F***, ***G***). ****$p < 0.0001$ by one-way ANOVA with Tukey's multiple-comparison post-test.

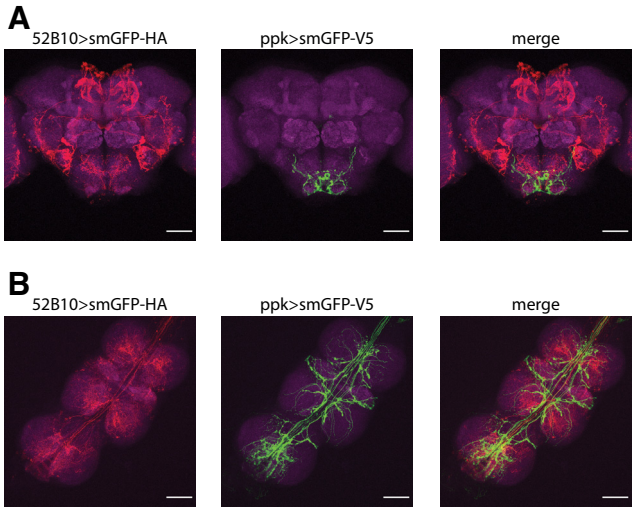


Figure 8. 52B10 and ppk drivers exhibit nonoverlapping expression patterns. ***A***, Processes labeled by 52B10 and ppk drivers show no overlap in the brain. ***B***, Processes labeled by 52B10 and ppk drivers also show no overlap in the ventral nerve cord. Dorsal is top for ***A*** and top right for ***B***; ventral is bottom for ***A*** and bottom left for ***B***. Scale bars, 50 μm. $N > 6$ for each genotype.

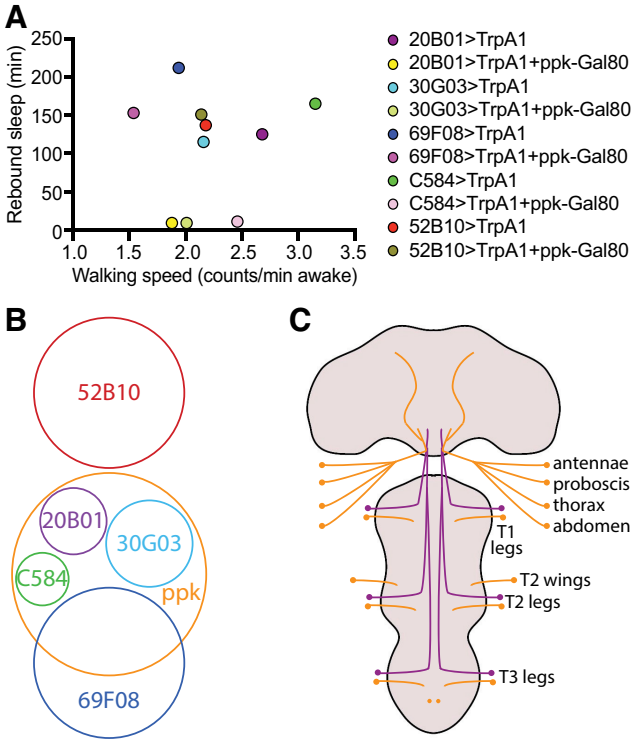


Figure 9. Proposed spatial relationships between behaviorally relevant neurons labeled by tested drivers. ***A***, Walking speed during thermogenetically induced waking is not correlated with rebound sleep. Symbols represent average values from the same populations of animals that were depicted in previous figures (heat pulses from ZT22 to ZT24). $R^2 = 0.01$ and $p = 0.80$ by Pearson correlation. ***B***, Venn diagram of neurons targeted by Gal4>TrpA1 combinations that elicit sleep homeostasis. ***C***, Behaviorally relevant subset of ppk neurons that are labeled by 20B01 or EB drivers. Neurons exclusively labeled by the ppk driver are in orange; relevant colabeled neurons are in purple.

possibility in several ways using a circuit epistasis-based approach. In these experiments, we found that sleep homeostasis was reduced by preventing ppk neurons from being simultaneously activated along with EB neurons, or by blocking synaptic transmission with EB drivers while activating ppk neurons. We also found that sleep homeostasis could be elicited by activating only the ppk neurons that were labeled by the 30G03 EB driver. These results are not consistent with EB and ppk drivers labeling neurons that function independently. Instead, they support a model in which ppk neurons underlie arousal processes that increase the homeostatic drive to sleep.

If the EB hypothesis for sleep homeostasis is suspect, then where might a brain locus that encodes sleep need be located in flies? One possibility is the FB, which several studies have suggested is necessary or sufficient for sleep (Donlea et al., 2011, 2014; Liu et al., 2012; Kottler et al., 2013; Ni et al., 2019). If this is indeed the case, then it is unclear how the sleep homeostat is regulated by ppk neurons, which do not terminate near the FB (Seidner et al., 2015). As yet, unidentified interneurons could provide the missing link to such a hypothetical circuit. But another possibility can be inferred by shared features of ppk and 52B10 neurons. Notably, each set of neurons can independently activate the sleep homeostat, and axonal projections of each terminate in the SEZ. This commonality suggests that the sleep homeostat could lie immediately postsynaptic to both sets of neurons, either in the SEZ or immediately dorsal to it in the antennal mechanosensory and motor center (AMMC). Recently, several studies in flies have suggested that neurons terminating in the AMMC can directly promote sleep (Öztürk-Çolak et al., 2020; Lone et al., 2021). Thus, this brain region may be more important for integrating arousal cues and converting them to sleep need than previously recognized. A sleep homeostat in the AMMC would also explain why vibratory stimuli, among all sensory stimuli, are used most effectively for sleep deprivation and rebound experiments in flies. It is also possible that distinct sleep homeostats could exist in both the FB and ventral brain, each of which responds to different types of arousal cues. While we have no evidence one way or the other, we note that one recent study suggests that neurons in the ventral nerve cord may contribute to sleep phenotypes previously attributed to the FB (Jones et al., 2022).

In summary, our results suggest that the majority of published drivers used to trigger sleep homeostasis likely elicit their behavioral effects by targeting neurons whose cell bodies lie outside the brain. It remains to be determined where these different neurons originate, but our data suggest that at least some of them are ppk neurons in the legs. Surprisingly little is known about the functions of ppk neurons in adults other than a role for an abdominal subset in egg-laying behavior (Häsemeyer et al., 2009; Yang et al., 2009; Gou et al., 2014; Lee et al., 2016; Wang et al., 2020). However, evidence suggests that ppk neurons also serve as nociceptors or proprioceptors, at least in larvae (Ainsley et al., 2003; Tracey et al., 2003; Xiang et al., 2010; Zhong et al., 2010; Neely et al., 2011; Hwang et al., 2012; Ohyama et al., 2013; Gorczyca et al., 2014; Guo et al., 2014; Mauthner et al., 2014; Terada et al., 2016; Jang et al., 2018). Our findings thus raise the possibility that sensory feedback from leg movement functions as a proxy for waking and that this information is transduced into increased sleep need by the sleep homeostat. While this possibility is appealing in its simplicity, it raises the question of why activation of many other types of arousal-promoting neurons fails to induce rebound sleep (Seidner et al., 2015) since ppk neurons would be expected to provide feedback to the CNS whenever the fly is moving and thus presumably awake. Although we do not have a definitive answer, we previously showed that some arousal-promoting neurons appear to inhibit the sleep homeostat (Seidner et al., 2015). As we

suggested at the time, situations may exist in nature where acute survival of an animal depends on suppressing sleep need. Thus, the privileged nature of ppk neurons in sleep homeostasis may be predicated on the absence of simultaneous activity from arousal-promoting neurons required for acute survival.

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