Sleep-promoting neurons remodel their response properties to calibrate sleep drive with environmental demands

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Abstract

Falling asleep at the wrong time can place an individual at risk of immediate physical harm. However, not sleeping degrades cognition and adaptive behavior. To understand how animals match sleep need with environmental demands, we used live-brain imaging to examine the physiological response properties of the dorsal fan-shaped body (dFB) following interventions that modify sleep (sleep deprivation, starvation, time-restricted feeding, memory consolidation) in Drosophila. We report that dFB neurons change their physiological response-properties to dopamine (DA) and allatostatin-A (AstA) in response to different types of waking. That is, dFB neurons are not simply passive components of a hard-wired circuit. Rather, the dFB neurons intrinsically regulate their response to the activity from upstream circuits. Finally, we show that the dFB appears to contain a memory trace of prior exposure to metabolic challenges induced by starvation or time-restricted feeding. Together, these data highlight that the sleep homeostat is plastic and suggests an underlying mechanism.

Introduction

The importance of sleep is highlighted by the observation that it is evolutionarily conserved despite directly competing with all motivated waking behaviors [1,2]. Not only does sleep compete with foraging, eating, and mating [3–6], for example, high sleep drive may be maladaptive in many circumstances since falling asleep could place the individual in danger of immediate physical harm [7,8]. On the other hand, sleep plays a critical role in learning and memory, supports adaptive behavior, and facilitates creative insight [9–12]. Together, these observations suggest that it will not be possible to fully understand sleep’s function without knowing how sleep circuits calibrate sleep drive with motivational states.
In recent years, a great deal of progress has been made dissecting circuits that regulate motivated behavior in flies [13–17]. Typically, the properties of a circuit are examined as a function of one internal state. For example, sleep circuits are evaluated after sleep loss [18–20], feeding in response to starvation or high dietary sugar [21,22], thirst following water deprivation [23], mating after social isolation [24], etc. However, since internal states can promote conflicting goal-directed behaviors, recent studies have begun to evaluate how circuits regulate competing activities such as feeding and sleep [5,25–29], thirst and hunger [23], sweet and bitter taste [30], hunger and mating [31], and thirst versus hunger relevant memory [23], for example.

A common theme that has emerged from these studies has been that a specific deprivation-state differentially activates a subset of peptidergic neurons that then modulate classic neurotransmitter systems, frequently dopamine (DA), to alter downstream circuits and thus motivated behavior [31–33]. For example, water deprivation preferentially activates a subset of peptidergic neurons that inhibit specific dopaminergic neurons to alter thirst-relevant memory [34]. In this model, the competition between state-specific peptidergic neurons will determine which motivated behavior will be expressed. An open question, however, is whether the neurons receiving peptidergic or dopaminergic input are constrained such that their responses are determined by upstream signals, or on the other hand, whether they can change their own response properties over time to influence a given outcome.

Sleep-promoting R23E10 neurons are well suited to investigate these relationships. R23E10 neurons are modulated both by dopaminergic neurons and allatostatin-A (AstA)-expressing circadian-neurons [19,23,25–27,35,36]. AstA-expressing neurons promote sleep by releasing glutamate onto R23E10 neurons [25]. AstA is functionally similar to galanin and has been implicated in both feeding and sleep [27,37]. Sleep-promoting R23E10 neurons comprise a sleep switch and are an integral part of the sleep homeostat [19,26]. The intrinsic properties of R23E10 neurons have been evaluated in the context of sleep loss where it seems they monitor redox processes as an indicator of energy metabolism [19,38,39]. Thus, R23E10 neurons may serve as command neurons of sorts that can integrate stimuli from competing internal states to gate information flow [40,41]. Both the input and the output connections to R23E10 are well documented [25,26,35,36]. Thus, the focus of this study is to evaluate sleep-promoting R23E10 neurons following challenges that modulate motivational states.

In this study, we identify independent sets of heterogeneous sleep-promoting neurons that change their responses to DA and AstA following challenges that influence sleep. In addition, we identify a wake-promoting effect of AstA on R23E10 neurons suggesting that the co-release of inhibitory AstA with excitatory glutamate may attenuate the overexcitement of R23E10 neurons during high sleep drive and allow animals to maintain wakefulness in dangerous or life-threatening conditions. Finally, we find that both time-restricted feeding and acute starvation enhance subsequent waking by remodeling the expression of DA receptors in sleep-promoting neurons. Together, these data provide new insights into the interaction between internal states and sleep regulation.

Results

Neurons projecting to the dorsal fan-shaped body are modulated by allatostatin

Fan-shaped body (dFB)-projecting R23E10 neurons are an important component of the sleep homeostat [19,38,39]. Sleep-promoting dFB neurons are believed to be inhibited by wake-promoting dopaminergic neurons and activated when glutamate is released from sleep-promoting AstA-expressing neurons [25,35,36]. Surprisingly, the role of AstA on R23E10 neurons has not been investigated. Thus, we used behavioral genetics and live-brain imaging to characterize
the effects of AstA and DA on R23E10 neurons. The expression pattern of R23E10 neurons is shown in Fig 1A. Expressing the temperature-sensitive transient receptor potential cation channel (UAS-TrpA1) in R23E10 neurons and raising the temperature from 25°C to 31°C for 6 h increased sleep (Fig 1B and 1C). The parental controls do not show an increase in sleep during the exposure to 31°C (S1A Fig). Since many neuromodulators act through second messenger signaling cascades, we expressed the cyclic adenosine monophosphate (cAMP) sensor, UAS-Epac1-camps, in R23E10 neurons and used high-throughput live-brain imaging to monitor neuronal responses to bath applied DA (Fig 1D) [42,43]. As seen in Fig 1E, DA increases...
cAMP levels in dFB neurons as previously reported [36]. It is important to note that while R23E10>GFP identifies approximately 14 neurons/hemisphere, R23E10>UAS-Epac1-camps reliably labeled approximately 8 neurons/hemisphere (S1B Fig). Thus, we have confirmed that R23E10 neurons are sleep-promoting and respond to DA.

Given that R23E10 neurons are downstream of AstA-expressing neurons [25], we evaluated the response properties of R23E10 neurons to AstA and 14 other compounds that can influence motivational states [44]. As seen in Fig 1F and 1G, AstA reduced cAMP levels in R23E10 neurons. Previous studies indicate that AstA is an inhibitory peptide [45]. Individual traces for Fig 1G are plotted in S2A Fig. Consistent with the cAMP responses, knocking down a battery of neuropeptide receptors in R23E10 neurons using RNA interference (RNAi) did not substantially modify sleep (S2B Fig). To determine whether the effects of AstA on R23E10 neurons are direct, we incubated brains in tetrodotoxin (TTX), which inhibits the firing of action potentials. TTX does not prevent the AstA-mediated cAMP response of R23E10 cells (Fig 1H, green trace). To further confirm a direct role of AstA, we used RNAi to knock down the AstA-R1 receptor in R23E10 neurons. As seen in Fig 1H (red trace), the cAMP response triggered by application of AstA is attenuated. Quantification of these effects is shown in Fig 1I. Importantly, R23E10 neurons responded normally to DA when AstA-R1 levels are knocked-down indicating that the reduced response to AstA is not the result of a nonfunctional neuron (S2C Fig).

To better understand the relationship between AstA and R23E10 neurons, we used immunohistochemistry to evaluate the overlap between AstA and AstA-GAL4. As previously reported, AstA-GAL4 does not fully recapitulate the AstA expression pattern within the projections to the dFB (Fig 1J–1J") [37]. Nonetheless, AstA-GAL4 is expressed near the dendritic fields of R23E10 suggesting a physical connection between the 2 group of neurons (Fig 1K–1K") [25]. To evaluate functional connectivity of the AstA-GAL4, R23E10 circuit, we expressed the P2X2 activator [46] in AstA-GAL4 neurons while measuring cAMP with LexAop-Epac in R23E10-LexA neurons. As seen in Fig 1L and 1M, perfusion of ATP that activates P2X2, leads to a reduction of cAMP levels in R23E10 cells, mimicking the effect of AstA on R23E10 neurons reported above (Fig 1F). No changes in cAMP signaling were observed in parental controls perfused with ATP but that do not express P2X2 indicating the effects are specific to the activation of AstA-GAL4 neurons (Fig 1L, red trace). Thus, we show that the cAMP response of R23E10 neurons to AstA is similar to that seen when AstA-expressing cells are activated.

The sleep-promoting effects of AstA-expressing neurons have been shown to be due to the release of glutamate onto R23E10 neurons [25]. Because AstA is an inhibitory neuropeptide [44,45], we would predict the impact of AstA on R23E10 neurons would be wake promoting and that knocking down AstA-Rs would thus increase sleep. To test this hypothesis, we evaluated sleep after knocking down AstA-R1 or AstA-R2 in R23E10 neurons. As seen in Fig 2A and 2B and 2E and 2F, total sleep is increased when AstA-R1 or AstA-R2 is knocked down in R23E10 neurons, suggesting that the dFB is under consistent allatostatnergic inhibitory tone. Importantly, sleep is also more consolidated during the day (Fig 2C and 2G); sleep consolidation is also increased at night (S3A and S3B Fig). Furthermore, the latency to fall asleep at night is reduced (S3C and S3D Fig). The increase in sleep is not due to unhealthy or sick flies since waking activity is not reduced compared to parental controls (Fig 2D and 2H). To rule out off-target effects of the single RNAi line, we tested 3 additional, independent AstA-R1 RNAi lines and found that sleep is significantly increased in each line (S3E Fig). Given that increasing the activity of AstA neurons has been reported to increase sleep, but the R23E10>AstA-RRNAi data indicate that AstA provides a wake signal, we asked whether our results might be due to unknown environmental factors in our laboratory. To evaluate this possibility, we utilized 2 lines (AstA-GAL4 and R65D05-LexA) that express in AstA-expressing
clock neurons and increase sleep [25]. As seen in S3F Fig, both AstA-GAL4-dTrpA1 and R65D05-LexA-LexAop-dTrpA1 lines substantially increased sleep consistent with previous reports [25]. Because AstA is an inhibitory neuropeptide, and similar mammalian receptors signals mainly through the Gi pathway [47], we hypothesized that the AstA receptors might be coupled to inhibitory G protein subunits. To test this hypothesis, we used RNAi to knockdown specific G protein subunits in sleep-promoting dFB neurons. As seen in Fig 2I, knocking down $Go^{\alpha47A}$ had no effect on sleep, while knocking down $Gi^{\alpha65A}$ using 2 different RNAi lines significantly increased sleep. Furthermore, independently knocking down $\beta$ and $\gamma_1$ subunits in sleep-promoting R23E10 neurons also increased sleep (Fig 2I). Thus, these data suggest that the inhibition of dFB neurons can be achieved via coupling with inhibitory G proteins.

**Sleep-promoting dFB neurons are diverse**

dFB neurons have been hypothesized to gate different aspects of sleep such as locomotion, sensory thresholds, etc. [26,48]. This hypothesis suggests that the dFB could be comprised of independent sets of sleep-promoting neurons that each respond to distinct environmental challenges. If this were to be the case, then we should be able to identify novel dFB projecting GAL4 lines. We obtained 12 GAL4 lines from the Flylight collection that were selected to match the expression pattern of the original dFB sleep-promoting GAL4 lines (104y and C5).
We activated these neurons by expressing UAS-TrpA1 as described above. Surprisingly, only 1 driver, R55B01 increased sleep similarly to R23E10 when compared to siblings maintained at 25˚C (Figs 3A and 3B and S4A). The parental controls do not show an increase in sleep during the 6-h exposure to 31˚C (S1 Fig). Similar results were obtained when expressing the sodium bacterial channel, UAS-NaChBac (S4B Fig). As seen in Fig 3C, R55B01 project to the dFB neuropil (blue arrow) and have cell bodies located in the same anatomical region in the brain as R23E10 neurons. To determine potential overlap between R23E10 and R55B01, we conducted co-labeling experiments by expressing RFP with GAL4/UAS and GFP with LexA/LexAop in the same fly. As seen in S5C–S5C” and S5D Fig R23E10-LexA is similar, but not identical, to the expression pattern of R23E10-GAL4. Importantly, R23E10-LexA and R55B01-GAL4 only share approximately 4 neurons in common (S5A–S5A” and S5B Fig). As with R23E10 neurons, R55B01 > UAS-Epac1-camps labels fewer dFB projecting neurons (approximately 8 neurons) compared to R55B01 > GFP. Similar to R23E10 neurons, R55B01 > UAS-Epac1-camps show robust responses to AstA (Fig 3D). Moreover, AstA positive staining is co-localized with the GFP positive processes of R55B01 neurons (S5E–S5E” Fig).
contrast to R23E10 neurons, the response of R55B01 neurons to DA is either reduced or absent (Fig 3E). A box plot comparing the range of responses of R23E10 and R55B01 neurons to DA is shown in Fig 3F. Given the robust responses of R55B01 neurons to AstA, we evaluated sleep after knocking down AstA-R1 or AstA-R2. As seen in Fig 3G and 3H, sleep is increased upon knocking down either AstA-R1 or AstA-R2 in R55B01 neurons. Quantification of sleep architecture in R55B01> AstAR1RNAI and R55B01> AstAR2RNAI can be found in S6 Fig. Thus, we have identified an additional sleep-promoting driver that projects to the dFB, has little overlap with R23E10 neurons, and is less responsive to DA.

**R23E10 neurons differentially integrate sleep-relevant stimuli**

Together with the literature, these data identify 2 major wake-promoting signals that impact R23E10 neurons, AstA and DA [35,36]. Although both signals are inhibitory, they derive from neuronal circuits with opposite functions; AstA is released from sleep-promoting neurons while DA is released from wake-promoting neurons [25,35,36]. Given these divergent roles, we hypothesize that AstA and DA will operate largely independently. To test this hypothesis, we used the live-brain imaging approach described above (Fig 1D) and evaluated the response of R23E10 neurons to either DA or allatostatin during conditions that alter sleep drive. Because R55B01 neurons respond less to DA, we focused solely on R23E10 neurons; R55B01 neurons will be studied later. We first evaluated the response properties of R23E10 neurons in unperturbed flies under conditions characterized by large changes in sleep time (e.g., ontogeny, gender, individual differences). We then evaluated the response properties of R23E10 neurons after experimental interventions that modulate sleep drive (e.g., sleep loss, starvation, training that induces long-term memory) [3,28,50–53] (Fig 4).

As with humans, sleep is highest in young flies and then stabilizes in early adulthood. Interestingly, R23E10 neurons are less responsive to DA in 0- to 1-day-old flies compared to 6- to 8-day-old mature adults; no changes were observed in response to AstA (Fig 4A and 4B). The response properties of R23E10 neurons to DA were similar in 6- to 8-day-old flies and 30- to 38-day-old flies S7 Fig. In contrast to age, we did not observe any changes in the response properties of R23E10 neurons in male and female flies despite large sexual dimorphisms in sleep behavior [52,54]. We have previously shown that individual differences in sleep time can be exploited to evaluate sleep regulation and function [55]. Importantly, our previously published data indicate that spontaneously short-sleeping flies appear to experience both high wake-drive and high sleep drive simultaneously [56]. With that in mind, we evaluated the response properties of R23E10 neurons in spontaneously short-sleeping R23E10> UAS-Epac1-camps (i.e., total sleep time less than 400 min) compared to normal sleeping siblings (800 to 960 min sleep). As seen in Fig 4C, R23E10 neurons appear to be under stronger inhibitory tone from AstA in spontaneous short sleepers compared to normal sleeping siblings. Although one would predict a stronger response of R23E10 neurons to the wake-promoting effects of DA, short-sleeping flies were less responsive to DA (Fig 4D). Future studies will be needed to determine whether incongruent responses of R23E10 neurons to AstA and DA reveal underlying deficits in sleep regulation.

We next asked whether distinct experimental interventions that modulate sleep drive would alter the physiological response properties of R23E10 neurons in similar or dissimilar ways. Both sleep deprivation and extended periods of starvation are followed by a compensatory increase in sleep [3,28,57]. To evaluate how sleep disruption would influence R23E10 neurons, flies were sleep deprived for 12 h or starved for 18 h and compared to untreated siblings. As seen in Fig 4E, cAMP responses to AstA are increased following sleep deprivation but were unchanged in response to DA (Fig 4F). Surprisingly, the response of R23E10 neurons to either
AstA or DA were not changed while being starved (Fig 4G and 4H and see below). In contrast to sleep deprivation and starvation, social enrichment induces plasticity in specific neural circuits to increase sleep without exposing flies to sleep loss [51,58]. Changes in sleep following social enrichment have been mapped to pigment-dispersing factor (PDF)-expressing clock neurons [58]. Interestingly, AstA signaling is modulated by PDF [27]. Thus, to evaluate the impact of social rearing, we housed flies in a socially enriched environment (50 flies/vial) for 5 days and evaluated cAMP responses compared to isolated siblings. As seen in Fig 4I, R23E10 neurons of socially enriched or isolated flies show similar responses to AstA. However, DA responses of R23E10 cells are strongly reduced by social enrichment (Fig 4J). These data demonstrate that disparate behavioral manipulations that increase sleep drive in different ways induce independent physiological responses of R23E10 neurons to AstA and DA.

Sleep-dependent memory consolidation has been linked to both the ventral and the dFB [10,59]. Thus, we asked whether R23E10 neurons would modify their physiological responses to a training protocol that induces LTM [9]. As seen in Fig 4K, responses of R23E10 neurons to AstA are not different following courtship conditioning. However, training resulted in a dramatic reduction in the response of R23E10 neurons to DA (Fig 4L). Interestingly, the effect of training on DA responses could still be observed 24 h after the end of the training only returning to baseline 48 h later suggesting long-term plastic changes in R23E10 neurons (S8 Fig). To determine whether the changes in cAMP were due to nonspecific effects of courtship or to memory consolidation, we evaluated cAMP levels following a massed training protocol consisting of a single 3-h session that does not result in LTM formation [10]. As seen in S8 Fig, massed training did not alter the responses of R23E10 cells to DA. Furthermore, no changes in responses to DA were found when training was followed by 4 h of sleep deprivation S8 Fig. To further evaluate the role of the dFB in courtship memory, we used CaLexA (calcium-dependent nuclear import of LexA) to see if R23E10 neurons might show sustained activity following a spaced training protocol [60]. R23E10 flies expressing CaLexA were exposed to a training protocol consisting of 3 × 1 h individual pairings of a naïve male with a non-receptive female target separated by a rest period of 1 h. As seen in Fig 4M, sleep is increased following training compared with non-trained siblings consistent with previous reports [51,58]. R23E10 neurons show higher GFP signal in trained animals when assessed the following morning compared with their naïve counterparts indicating that sleep-promoting neurons are more active following courtship memory training (Fig 4N and 4O for quantification). Together, these data indicate that the response properties of R23E10 neurons display long-lasting changes following protocols that induce LTM.
Prior feeding experience alters the recruitment of DA receptors to modulate sleep

Initial studies indicated that the wake-promoting effects of DA on dFB neurons are mediated by Dopamine 1-like receptor 1 (Dop1R1) [35,36,61]. However, the role of Dop1R1 has been called into question [19]. We have recently shown that the constellation of receptors expressed on a neuron can be altered by starvation to include a receptor that is not typically present [3]. Specifically, our data suggest that the new receptor is recruited to amplify wake-promoting signals in clock neurons to allow animals to engage in adaptive waking behaviors. To determine whether the phenomenon of recruiting new wake-promoting receptors to a neuron will generalize to the dFB, we re-examined the role of Dop1R1 in R23E10 neurons. In addition, we also evaluated R55B01 neurons because these cells have a limited response to DA. We hypothesized that if Dop1R1 is needed to support waking following a metabolic challenge, knocking it down would manifest as an increase in sleep. Since 18 h of starvation did not change the response properties of R23E10 neurons (Fig 4G and 4H), we used time-restricted feeding to safely impose an alternate challenge of longer duration [62–64]. The time-restricted feeding protocol is shown in Fig 5A. Flies are only given access to food between 8 AM and 5 PM for a total of 7 days (restricted). After time-restricted feeding, flies are placed into Trikinetics tubes where they were allowed to eat ad lib while sleep is evaluated. Siblings that were maintained in vials with standard food available ad lib and flipped at the same times as their restricted counterparts served as treatment controls (Fig 5A). Consistent with previous reports [19,36], knockdown of Dop1R1 in R23E10 neurons did not alter sleep in flies that were able to feed ad lib (Fig 5B and 5D and 5E). Similarly, no changes in sleep were observed in untreated R55B01>Dop1R1RNAi flies (Fig 5C–5E). However, both R23E10>Dop1R1RNAi and R55B01>Dop1R1RNAi flies displayed dramatic increases in sleep following 7 days of time-restricted feeding compared to their R23E10/+, R55B01/+, and Dop1R1RNAi/+ parental controls (Fig 5F–5I). Changes in sleep during time-restricted feeding are shown in S9 Fig. These data are consistent with the hypothesis that, under certain circumstances, a new receptor can be recruited to amplify wake-promoting signals.

Time-restricted feeding is characterized by defined intervals of feeding and fasting. Thus, during time-restricted feeding, Dop1R1 may be recruited to R23E10 neurons to either support waking during starvation or to support waking when food is available. To distinguish between these possibilities, we monitored sleep in R23E10>Dop1R1RNAi and R55B01>Dop1R1RNAi flies and their parental controls during baseline, during 18 h of starvation, and for 3 days after being placed back onto food (Fig 6A and 6B). As above, no changes in baseline sleep were observed while knocking down Dop1R1 in either R23E10 or R55B01 neurons compared to parental controls (Fig 6A–6D). Moreover, R23E10>Dop1R1RNAi and R55B01>Dop1R1RNAi flies exhibited similar sleep patterns to parental controls during starvation (S10A Fig). However, during recovery following 18 h of starvation, an increase in sleep was observed in both R23E10>Dop1R1RNAi and R55B01>Dop1R1RNAi flies compared to their respective parental controls (Fig 6A and 6B). It is important to highlight that sleep in the experimental lines only diverged from their parental controls after several hours, or more, of recovery (Fig 6A and 6B arrows). Quantification of sleep during recovery day 1 and recovery day 2 is shown in S10B and S10C Fig. Sleep stabilized in both R23E10>Dop1R1RNAi and R55B01>Dop1R1RNAi flies on the third day of recovery and remained elevated for several days thereafter (Fig 6A, 6B and 6E). Thus, these data indicate that the Dop1R1 is recruited to R23E10 and R55B01 neurons to support waking behavior during recovery from starvation.

To gain additional insight into the physiological impact of starvation, we used live-brain imaging to evaluate the responses of R23E10>UAS-Epac1-camps; UAS-Dop1R2RNAi and
R55B01 > UAS-Epac1-camps; UAS-Dop1R2RNAi to DA under baseline and on recovery day 2 from starvation. While both Dop1R1 and Dop1R2 couple to Gas, Dop1R2 appears to also activate Gαs [65]. We hypothesized that knocking down Dop1R2 would reduce the response of R23E10 neurons to DA under baseline conditions and that the neurons would respond to DA following 2 days of recovery from starvation.

R55B01 > UAS-Dop1R2RNAi flies displayed an increase in sleep under baseline conditions consistent with previous reports [19]. As seen in Fig 7A and 7B, R23E10 > UAS-Epac1-camps; UAS-Dop1R2RNAi neurons did not show a strong response to DA under baseline conditions. Although much smaller than that observed for R23E10 neurons, R55B01 > UAS-Epac1-camps; UAS-Dop1R2RNAi neurons did respond modestly to DA after 2 days of recovery from starvation.
starvation (Fig 7C and 7D). While we cannot exclude a role of other DA receptors (e.g., Dopamine/Ecdysteroid receptor) for the observed changes, when viewed with the sleep experiments shown above (Figs 5 and 6), these data suggest that during recovery from starvation the constellation of DA receptors in R23E10 and R55B01 changes and most likely includes the recruitment of Dop1R1. These data provide new insights into the mechanisms used by sleep circuits to link internal states and prior waking history with sleep need.

Discussion

In this work, we ask whether the activity of sleep-promoting dFB neurons reflects the summation of their upstream inputs in a winner-take all strategy or if they can change their own response properties to influence how environmental demands alter behavioral state. Although one possibility does not preclude the other, our data indicate that both time-restricted feeding
and approximately 18 h of starvation, alter the constellation of DA receptors expressed by R23E10 and R55B01 neurons. These results emphasize that the ability of upstream circuits to alter behavior will not only depend upon the strength of the incoming signals but also the recent historical context of R23E10 and R55B01 neurons themselves. In that regard, it important to note that while sleep competes with all motivated waking behavior, the proper regulation of motivated behaviors degrade in the absence of sleep [50,66–74]. Together, these observations suggest that it will not be possible to fully understand sleep’s function without knowing how sleep-promoting neurons alter their functional properties to prioritize conflicting motivational states.

The dFB is comprised of independent sets of sleep-promoting neurons

Previous studies indicate that the dFB can independently gate different aspects of sleep [26]. Moreover, the expression of the gap junction innexin6 in R23E10 neurons is required to gate sensory thresholds and sleep time [48]. Together, these data suggest the presence of independent sets of dFB neurons. Although we screened through a refined set of hand-selected GAL4 lines that project into the dFB in a manner similar to 104y-GAL4 and C5-GAL4 [10,49], we only identified 1 additional GAL4 line, R55B01, that reliably increased sleep when crossed with UAS-dTrpA1. Similar results were obtained when activating the neurons using the bacterial sodium channel UAS-NaChBac. This latter result is relevant given that sleep is modulated by temperature and that GABAergic neurons projecting onto dFB regulate temperature-dependent changes in sleep [75,76]. We also identified dFB projecting GAL4 lines that increased waking. However, the precise role that these GAL4 lines play in waking awaits
additional inquiry. Nonetheless, these data suggest that sleep-promoting dFB neurons are limited in number. It should be noted that the extent of the overlap between R23E10 and R55B01 remains unclear as the R23E10-LexA driver does not fully recapitulate the R23E10-GAL4 expression patterns (S5 Fig). Although it may be possible to subdivide R23E10 and R55B01 neurons further, our data suggest that there are at least 2 independent sets of sleep-promoting neurons that have projections into the dFB (see below for additional discussion). In mice, sleep-promoting neurons are also diverse as assessed by their role in regulating behavior and their projection patterns [77].

Allatostatin inhibits sleep-promoting neurons

Although AstA-expressing neurons are believed to promote sleep by releasing glutamate onto R23E10 neurons [25], our data identify a wake-promoting effect of AstA on R23E10 neurons. Specifically, we find that knocking down either the AstA-R1 or AstA-R2 in R23E10 or R55B01 neurons using several independent RNAi lines results in a substantial increase in sleep. The observation that knocking down either the AstA-R1 or AstA-R2 in R23E10 neurons increases sleep, suggests that R23E10 neurons are under constant inhibitory tone from AstA and that both receptors play an important role in modulating sleep in untreated flies. In addition, we report that the application of 1 mM ATP to R23E10>UAS-Epac1-camps; AstA-GAL4>P2X2 neurons reduces cAMP levels similarly to that observed with application of AstA. Although our data appear to be in conflict with that of Ni and colleagues, who provide evidence that AstA-GAL4 provide an excitatory drive onto R23E10 neurons, we hypothesize that the co-release of inhibitory AstA with excitatory glutamate may attenuate the overexcitement of R23E10 neurons during high sleep drive and allow animals to maintain wakefulness in dangerous or life-threatening conditions.

In support of this hypothesis, a growing body of evidence indicates that sleep and wake-regulating neurons co-release different transmitters and neuropeptides to better match sleep need with environmental demands [78]. Indeed, it has been suggested that antagonistic neurotransmitter co-release may both prevent excessive excitation of postsynaptic targets and increase the flexibility of neuronal networks [45,78,79]. For example, the co-release of GABA by histaminergic tuberomammillary nucleus is believed to prevent histamine-induced overexcitement of downstream circuits and thereby better support normal amounts of waking [80]. Similarly, the co-release of galanin by noradrenergic neurons is believed to prevent overexcitement of locus coeruleus [81]. Although the co-expression of AstA and glutamate has been established [25,82], our data reveal a new mechanism for how incompatible motivational drives can be regulated.

An alternate possibility to explain the wake-promoting effects of AstA on R23E10 and R55B01 neurons may be that R23E10 and R55B01 are, in fact, a heterogeneous sets of neurons that also include wake-promoting neurons and/or neurons whose primary role is to promote active sleep at the expense of deep sleep [83]. Active sleep is best observed following 24 h of sleep deprivation and is associated with dramatic reductions in the response to external sensory stimuli. If AstA were to inhibit a subset of R23E10 neurons that promote active sleep, one would predict an increase in deep sleep. The diversity in the responses to DA seen in both R23E10 and R55B01 neurons (Fig 3F) further supports the hypothesis that these GAL4 lines express in heterogeneous set of neurons with different functions; a previous study has found similar heterogeneity in R23E10 neurons [38]. Alternatively, it is possible that AstA does not inhibit the R23E10 neurons per se but rather alters temporal spike patterns to favor active sleep or deep sleep in a manner similar to that observed in clock neurons [84]. Indeed, AstA has been shown to alter the spike-timing precision of mechanoreceptor afferents in Carcinus...
maenas and Galanin, alters spontaneous spike firing in rodents [85,86]. These possibilities will be explored in future studies.

It is important to note that neuropeptides are notoriously pleiotropic and also work as neurohormones [44]. Thus, it is possible that additional sleep- and wake-promoting circuits downstream of AstA-GAL4 or 65D05-GAL4 will be found elsewhere in the brain. It is interesting to note that neurons expressing the mouse galanin, which is functionally similar to AstA, also regulate conflicting behaviors [77]. Specifically, galanin-expressing neurons that project to the tuberomammillary nucleus promote sleep while galanin-expressing neurons that project to the medial amygdala promote parental behaviors [77,87,88]. Thus, it will be important for future studies to discern how AstA and galanin circuits regulate competing activities in other circuits.

R23E10 neurons differentially encode arousal signals

Increased sleep drive may be maladaptive in many circumstances since falling asleep could place the individual in danger of immediate physical harm [7,8]. Increased sleep also competes with important waking behaviors such as foraging, eating, and mating [3,4]. However, sleep plays a critical role in learning and memory, supports adaptive behavior, and facilitates creative insight [9–12]. How do sleep-promoting neurons distinguish between competing drives? Our live-brain imaging data suggest the intriguing possibility that R23E10 neurons can distinguish between different types of waking and change their response properties accordingly. Specifically, R23E10 neurons selectively decrease their response to the wake-promoting effects of DA following conditions that promote plasticity. In contrast, R23E10 neurons increase their response to the wake-promoting effects of AstA during conditions when sleep drive is high but the expression of sleep might be dangerous (e.g., during sleep deprivation). Finally, R23E10 neurons appear to contain a memory trace of starvation as evidenced by the increase inhibitory tone conveyed by the recruitment of Dop1R1. It should be emphasized that we did not use TTX when evaluating the response properties of R23E10 neurons to different types of waking. As a consequence, it is possible that the difference in the intrinsic release of AstA and DA during these manipulations modified the response of dFB neurons. Such a possibility will be evaluated in future studies. Nonetheless, R23E10 neurons are plastic and can utilize AstA and DA in very different ways to favor specific behavioral outcomes.

As mentioned, increasing inhibitory signals onto sleep-promoting neurons during sleep deprivation may be an adaptive response that allows animals to stay awake despite increasing sleep drive. Indeed, lesioning galanin neurons in the preoptic area were found to reduce sleep rebound in zebrafish [89] and mice [90]. However, AstA is also reported to be a satiety signal that limits feeding [27,32,37,91]. The effects of AstA on feeding seem to be in conflict with the studies linking sleep deprivation to increased food intake [92–95]. Nonetheless, recent reports have identified complex interactions between hunger and satiety signals that may be nutrient specific and can be modified by Hebbian plasticity [32,34,40,44]. Thus, it is reasonable to expect that sleep deprivation must differentially activate neurons regulating a variety of motivated behaviors including satiety and sleep drive. Indeed, these data indicate that sleep deprivation may be harnessed as a tool to further explore how internal states impact neuronal circuits regulating conflicting goal-directed behaviors [30].

R23E10 neurons support long-term memory

Interestingly, the ventral fan-shaped body (vFB) promotes sleep and regulates the activity of Dopaminergic aSP13 neurons (DAN-aSP13s) to consolidate long-term memory as assessed using courtship conditioning [59]. Surprisingly, activating R23E10 neurons does not alter the
activity of DAN-aSP13s neurons suggesting that the dFB may not be involved in courtship memory [59]. It should be noted, however, that \textit{R23E10} neurons regulate the activity of other sets of arousal promoting neurons including the MV1 Dopaminergic neurons and octopaminergic arousal neurons [25,96]. These data suggest that the dFB and vFB may play distinct roles in different memory assays. Nonetheless, our data clearly indicate that \textit{R23E10} neurons change their response properties following training that induces LTM but not massed training. Importantly, the changes in \textit{R23E10} neurons only return to baseline 48 h after training. It is possible that \textit{R23E10} neurons play an important, yet indirect, role in courtship memory. That is, activating \textit{R23E10} neurons strongly suppresses the response to external sensory stimuli [48,83]. Thus, the activity of \textit{R23E10} neurons may protect sleep by limiting the opportunity for external stimuli to wake the animal up. Under this scenario, dFB and vFB neurons would work in concert to carry out sleep functions.

In addition, we find that courtship conditioning increases the activity of \textit{R23E10} neurons as assessed by \textit{CaLexA}. Consistent with this observation, spaced-training reduced the inhibitory effects of DA on \textit{R23E10} neurons. Importantly, massed-training, which only induced short-term memory, does not alter the response of \textit{R23E10} neurons to DA. A previous report has shown that the ectopic expression of \textit{Dop1R1} in dFB neurons reduces sleep and fails to rescue courtship conditioning memory in \textit{Dopamine transporter} mutants (\textit{fmn}) [36]. Together, these data emphasize that reducing Dopaminergic signaling to the dFB neurons is important for long-term memory following courtship conditioning.

\section*{Internal state remolds dopamine receptors in sleep-promoting neurons}

The arousal promoting properties of DA projections to the dFB are well established [19,25,35,36,61]. However, which DA receptor is responsible for the increased waking remains controversial. Initial results, using the ectopic expression \textit{Dop1R1} and live-brain imaging implicated \textit{Dop1R1} [35,36]. Nonetheless, RNAi knockdown of \textit{Dop1R1} in dFB neurons did not alter sleep [35]. A subsequent study demonstrated that activation of \textit{Dop1R2} results in a transient hyperpolarization of dFB neurons followed by a lasting suppression of excitability lasting minutes; knocking down \textit{Dop1R2} but not \textit{Dop1R1} altered sleep [19]. Our data replicate previous RNAi studies that have failed to observe a change in sleep following the expression of \textit{Dop1R1RNAi} in dFB neurons in untreated flies. Indeed, no changes in FRET signal were observed upon the bath application of DA to untreated \textit{R23E10>UAS-Epac1-camps; Dop1R2RNAi} or \textit{R55B01>UAS-Epac1-camps; Dop1R2RNAi} flies further supporting the role of \textit{Dop1R2} in baseline sleep regulation.

However, in the context of time-restricted feeding and starvation, knocking down \textit{Dop1R1} in \textit{R23E10} or \textit{R55B01} neurons resulted in substantial increases in sleep. We hypothesize that both time-restricted feeding and starvation place energy-saving sleep drive in conflict with a metabolic signal that facilitates motivated waking behavior. Increased wake drive would allow the animals to be more alert and productive during their primary wake period. Under these circumstances, \textit{Dop1R1} would be recruited to provide additional inhibitory tone to sleep-promoting \textit{R23E10} and \textit{R55B01} neurons during the light period. Interestingly, starvation results in the recruitment of the \textit{pigment-dispersing factor receptor} (\textit{Pdfr}) into wake-promoting \textit{large ventrolateral neurons} (\textit{lLNvs}) to facilitate waking at the end of the biological day [3]. Whether other sleep- and wake-promoting circuits are regulated in a similar fashion is an open question that will require additional inquiry.

\section*{Conclusions}

Given the important roles that DA and AstA play in regulating motivated behaviors, including sleep, we studied their impact on a subset of sleep-promoting neurons. Our data reveal that
AstA is wake-promoting and likely serves to maintain waking during periods of high sleep drive. In addition, our data reveal that time-restricted feeding or 18 h of starvation recruits the Dop1R1 to sleep-promoting neurons to maintain wakefulness during the day. These results are consistent with our previous data showing that the wake-promoting lLNvs recruit the Pdfr following sleep loss to facilitate waking and that wing-cut reactivates a developmental sleep circuit [3,97]. The ability of sleep- and wake-promoting neurons to alter their own physiology, including the recruitment of a new receptor, provides important clues into sleep regulation and function.

Supporting information

S1 Fig. Changes in sleep in parental controls following 6 h at 31˚C. (A) The % change from baseline sleep at 25˚C seen following switch to 31˚C between 9 AM and 3 PM. (B) Confocal image of R23E10>UAS-Epac1-camps. Underlying data is in S1 Datasheet. (TIF)

S2 Fig. R23E10 neuropeptide RNAi screen for Daytime Sleep. (A) Individual cAMP responses of R23E10 neurons expressing UAS-Epac1-camps shown as % change in FRET ratio during exposure to crustacean cardioactive peptide (CCAP), Drosophila myosuppressin (DMS), allatostatin C (astA C), proctolin, TPAEDFMRFamide, corticotropin-releasing factor-like diuretic hormone 44 (DH44), Tachykinin 1, Tachykinin 3, short neuropeptide F (sNPF), adipokinetic hormone (AKH), corazonin, and melatonin. (B) Daytime sleep in female 5-day-old flies expressing RNAi lines for the depicted neuropeptide receptors using R23E10-GAL4 and their parental controls (n = 14–16 flies/genotype). To be significant, the experimental lines must be significantly different from both the GAL4/+ (red line) and the UAS/+ (white bar) parental controls. Error bars are SEM. Underlying data is in S1 Datasheet. (TIF)

S3 Fig. Expressing AstA-R1-RNAi or AstA-R2-RNAi in R23E10 neurons alters sleep architecture. (A, B) Nighttime sleep bout duration is increased in UAS-Dcr2, R23E10-GAL4/+ > AstA-R1RNAi/+ and UAS-Dcr2,R23E10-GAL4/+ > AstA-R2RNAi/+ experimental flies compared with UAS-Dcr2, R23E10-GAL4/+ , AstA-R1RNAi/+ , and AstA-R2RNAi/+ parental controls (n = 16 condition, *p < 0.05, modified Bonferroni test). (C, D) Sleep latency is shortened in UAS-Dcr2, R23E10-GAL4/+ > AstA-R1RNAi/+ , and UAS-Dcr2,R23E10-GAL4/+ > AstA-R2RNAi/+ experimental flies compared with UAS-Dcr2, R23E10-GAL4/+ , AstA-R1RNAi/+ , and AstA-R2RNAi/+ parental controls (n = 16 condition, *p < 0.05, modified Bonferroni test). (E) Total sleep is increased when levels of AstA-R1 is decreased in R23E10 sleep-promoting neurons using 3 additional independent RNAi lines (n = 16 condition, *p < 0.05, modified Bonferroni test). (F) Sleep is increased in AstA-GAL4 > UASdTrpA1 and 65D05-Lex-A > LexAopdTrpA1 flies at 31˚C compared with siblings maintained at 25˚C; parental controls did not show an increase in sleep at 31˚C (n = 14–16/condition and genotype, *p < 0.05, modified Bonferroni test). Underlying data is in S1 Datasheet.
modified Bonferroni test). Error bars represent SEM. Underlying data is in S1 Datasheet.

S4 Fig. Screen of Janelia-GAL4 lines that express in the dorsal fan-shaped body in a pattern similar to that observed with 104y-GAL4 and C5-GAL4. (A) Despite similar anatomical profiles, most dorsal fan-shaped body drivers do not reliably impact sleep when expressing UAS-Transient receptor potential cation channel A1 and raising the temperature to 31°C (n = 14–16 flies/genotype). The data for R55B01-dTrpA are the same as in Fig 3A. (B) R23E10>UAS-NaChBac and R55B01>UAS-NaChBac sleep more than R23E10/+ , UAS-NaChBac/+ , and R55B01/+ parental controls. Error bars represent SEM. Underlying data is in S1 Datasheet.

S5 Fig. Anatomy of R23E10 and R55B01 neurons. (A) Representative confocal stack focusing on the area containing cell bodies of a R23E10-LexA>LexAop-GFP, R55B01-GAL4>UAS-RFP fly brain stained with anti-GFP antibody, (A’) anti-RFP antibody (magenta) and (A”) a merged image. Yellow arrows on the merge image indicate cells that express both GFP and RFP. (B) Quantification of the number of cells expressing only GFP, only RFP or both GFP and RFP. (C) Representative confocal stack focusing on the area containing the cell bodies of a R23E10-LexA>LexAop-GFP, R23E10-GAL4>UAS-RFP fly brain stained with anti-GFP antibody, (C’) anti-RFP antibody (magenta), and (C”) a merged image. (D) Quantification of the number of cells expressing only GFP, only RFP or both GFP and RFP. (E) Representative confocal stack of a using R55B01-GAL4>UAS-GFP brain stained with anti-GFP antibody, (E’) anti-AstA antibody (magenta), and (E”) a merged image. Error bars represent SEM. Underlying data is in S1 Datasheet.

S6 Fig. Expressing AstAR1RNAi or AstAR2RNAi R55B01 neurons alters sleep architecture. Sleep parameters in UAS-Dcr2, R55B01-GAL4/+, AstA-R1RNAi/+ , UAS-Dcr2, R55B01-GAL4/+ >AstA-R2RNAi/+ experimental flies and both UAS-Dcr2, R55B01-GAL4/+ and AstA-R1RNAi/+ , AstA-R2RNAi/+ control flies for: (A, B) total sleep, (C, D) nighttime sleep bout duration, (E, F) nighttime sleep latency, (G, H) daytime sleep bout duration, and (I, J) counts/waking minute; (n = 16/condition, *p < 0.05, modified Bonferroni test). Error bars represent SEM. Underlying data is in S1 Datasheet.

S7 Fig. Response of R23E10 neurons to Dopamine is not changed in older flies. Response of R23E10 neurons in young (0–1 day old), adult (6–8 day old), and old (30–38 day old) flies in response to Dopamine (3e−5 M) (n = 15, 14, 6 cells). The data are expressed as amplitude % change; data for young and adult flies are taken from data in Fig 4B. Error bars represent SEM. Underlying data is in S1 Datasheet.

S8 Fig. Space-trained flies vs. naïve controls. Space-trained flies vs. naïve controls. The reduction of DA responses remained significant 24 h after the end of the training but not 48 h post-training. A Massed training courtship protocol that does not induce LTM had no significant effect on amplitude of DA response in R23E10 neurons; 4 h of sleep deprivation did not alter DA responses in R23E10 neurons (n = 12–25 cells, *p < 0.05). Error bars represent SEM. Underlying data is in S1 Datasheet.

S9 Fig. Time-restricted feeding changes sleep-promoting neurons. (A) Sleep profiles in R23E10>Dop1R1RNAi, flies and their parental controls R23E10/+ , and Dop1R1RNAi/+ during
baseline, 7 days of time-restricted feeding and 5 days of recovery (n = 24–26 flies/group); gray arrow indicates disruption in data collection. (B) Daytime sleep is increased following time-restricted feeding in R23E10>Dop1R1RNAi compared to both parental controls. A Genotype (2) x Time (13) ANOVA revealed a Genotype X Time interaction: F[2,24] = 5.5, p = 9.99E-16; *p < 0.05, modified Bonferroni test. (C) Sleep profiles in R55B01>Dop1R1RNAi flies and their parental controls R55B01/+, and Dop1R1RNAi/+ during baseline, 7 days of time-restricted feeding and 5 days of recovery (n = 24–30 flies/group) (Dop1R1-RNAi flies are the same as in A, B); gray arrow indicates disruption in data collection. (D) Daytime sleep is increased following time-restricted feeding in R23E10>Dop1R1RNAi compared to both parental controls. A Genotype (2) x Time (13) ANOVA revealed a Genotype X Time interaction: ANOVA F[2,24] = 7.87, p = 9.99E-16; *p < 0.05, modified Bonferroni test. Underlying data is in S1 Datasheet.

S10 Fig. Starvation alters recovery sleep. (A) During starvation, sleep in R23E10>Dop1R1RNAi, R55B01>Dop1R1RNAi flies is not consistently above or below R23E10/+; Dop1R1RNAi/+ or R55B01/+ parental controls (n = 13–16 flies/group; ANOVA F[2,36] = 2.2, p = 0.12 and ANOVA F[2,38] = 15.4, p = 1.2E-05, *p < 0.05, modified Bonferroni test). (B) Daytime sleep is not increased in R23E10>Dop1R1RNAi or R55B01>Dop1R1RNAi flies compared to both parental controls R23E10/+; Dop1R1RNAi/+ or R55B01/+ on recovery day 1 (ANOVA F[2,36] = 4.8, p = 0.02 and ANOVA F[2,38] = 0.5, p = 0.58, *p < 0.05, modified Bonferroni test). (C) Sleep is increased in R23E10>Dop1R1RNAi and R55B01>Dop1R1RNAi flies compared to R23E10/+; Dop1R1RNAi/+ or R55B01/+ parental controls on recovery day 2 (ANOVA for Genotype F[2,36] = 14.25, p = 2.75E-05 and ANOVA for Genotype F[2,38] = 8.35, p = 0.0009 for R23E10 and R55B01, respectively. Underlying data is in S1 Datasheet.

S11 Fig. Expressing Dop1R2-RNAi in R23E10 neurons increases sleep. (A) Sleep is increased in R23E10>Dop1R2RNAi, flies compared to parental controls (n = 16 flies/group; 3 (genotype) X 24 (hour) repeated measures ANOVA reveals a significant interaction F[46,1012] = 7.15, p = 0.14E-13). Underlying data is in S1 Datasheet.

S1 Data. Data underlying Fig 1. (XLSX)

S2 Data. Data underlying Fig 2. (XLSX)

S3 Data. Data underlying Fig 3. (XLSX)

S4 Data. Data underlying Fig 4. (XLSX)

S5 Data. Data underlying Fig 5. (XLSX)

S6 Data. Data underlying Fig 6. (XLSX)

S7 Data. Data underlying Fig 7. (XLSX)
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Sleep promoting neurons calibrate sleep drive with environmental demands

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