Genetic and neuronal mechanisms governing the sex-specific interaction between sleep and sexual behaviors in Drosophila

Dandan Chen¹, Divya Sitaraman²,³,⁴, Nan Chen², Xin Jin³, Caihong Han¹, Jie Chen¹, Mengshi Sun¹, Bruce S. Baker², Michael N. Nitabach²,³,⁵,⁶ & Yufeng Pan¹,⁷

Animals execute one particular behavior among many others in a context-dependent manner, yet the mechanisms underlying such behavioral choice remain poorly understood. Here we studied how two fundamental behaviors, sex and sleep, interact at genetic and neuronal levels in Drosophila. We show that an increased need for sleep inhibits male sexual behavior by decreasing the activity of the male-specific P1 neurons that coexpress the sex determination genes fruM and dsx, but does not affect female sexual behavior. Further, we delineate a sex-specific neuronal circuit wherein the P1 neurons encoding increased courtship drive suppressed male sleep by forming mutually excitatory connections with the fruM-positive sleep-controlling DN1 neurons. In addition, we find that FRUM regulates male courtship and sleep through distinct neural substrates. These studies reveal the genetic and neuronal basis underlying the sex-specific interaction between sleep and sexual behaviors in Drosophila, and provide insights into how competing behaviors are co-regulated.
A fundamental tenet of biology is that organisms sense their environment, and, in response to sensory inputs, alter their physiology and behavior in ways that may be beneficial to the organism. When an organism is faced with more than one stimulus in the context of distinct behavioral states, multiple decision-making processes are involved in making appropriate behavioral choice. Behavioral choice in the context of internal state and external stimuli has been studied in both invertebrates and vertebrates, but how competing behaviors interact at the genetic and neuronal levels to ensure appropriate decision-making is still poorly understood.

Drosophila melanogaster, like other animals, engages in adaptive innate behaviors such as reproduction and sleep, and the molecular and neuronal mechanisms underlying these behaviors have been intensively studied for decades (see reviews on courtship and sleep). In males, the courtship behavior is largely controlled by the fruitless (fru) and doublesex (dsx) genes. The male-specific proteins (FRUM) derived from the P1 promoter of the fru gene (fruM) are necessary for innate courtship, and are sufficient for some aspects of courtship. The sex-specific products of the dsx gene (DSXM in males and DXXM in females) are involved in experience-dependent courtship in the absence of FRUM and courtship intensity and sing song production in the presence of FRUM. Male and female DXXM are expressed in a dispersed subset of ca. 2000 and 700 neurons, respectively, which partially overlap.

In the last two decades Drosophila has emerged as a promising model to study the molecular and circuit basis of sleep regulation. Many efforts have been made to identify the neuronal substrates controlling sleep behavior in flies, e.g., the mushroom bodies, mushroom body output neurons, the Fan-shaped body, and the DN1 circadian clock neurons. Although sleep is a sexually dimorphic behavior, the sex-specific mechanisms of sleep regulation remain unknown.

Using the amenability of Drosophila as a model system for genetic, behavioral, and physiological approaches, we sought to explore the interaction, at various levels, between sexual and sleep behaviors, in order to understand how these competing behaviors are co-regulated to ensure appropriate behavioral choice.

In this study, we show that sleep and sexual behaviors interact in a sex-specific manner. Sleep-deprived male flies display reduced courtship to females, but sleep-deprived female flies are equally receptive to courting males. Furthermore, sexually aroused males have poor sleep, but aroused females sleep more. We further identify the neural substrates involving the male-specific fruM-expressing P1 neurons and dsx-expressing P1 neurons and fruM-expressing DN1 neurons that control such sex-specific interaction between sex and sleep. Specifically, we find that the male-specific P1 courtship command neurons are inhibited by sleep deprivation (SD), and they control sleep in male flies by forming mutually excitatory synaptic connections with sleep-regulating DN1 neurons. Our studies also identify the key genes that control sexually dimorphic sleep behavior in male and female flies paving way for future studies. Together, our results provide a novel framework for investigating genetic and neuronal mechanisms governing the interaction between sleep and sexual behaviors.

Results

SD inhibits male courtship. To determine whether sleep and sexual behavior in flies influence one another, we first asked whether SD alters the display of sexual behaviors. Wild-type Canton-S male and female flies were sleep-deprived by intermittent mechanical perturbation, and then assayed for male courtship (Fig. 1a) and female receptivity (Supplementary Fig. 1), respectively. As reported in a recent study, we found that wild-type male flies that were sleep-deprived by mechanical perturbation for 12 h during nighttime (ZT12 to ZT0 of the subsequent day, ZT0 is lights-on, and ZT12 is lights-off in 12 h:12 h light-dark condition) do not show obvious courtship deficits (Fig. 1a). However, we found that courtship by male flies that were sleep-deprived for the same amount of time (12 h), but from ZT16 to ZT4, was significantly reduced (Fig. 1a). Furthermore, SD of males for 16 h, either from ZT8 to ZT0 or from ZT12 to ZT4, severely impaired male courtship (Fig. 1a). Males that were sleep-deprived for 16 h have reduced walking speed indicative of increased sleep need, but they do move around during the 10-min test (Supplementary Fig. 2). These results indicate that both duration and time-of-day when male flies are sleep-deprived play a critical role in sleep-loss-induced courtship deficits.

To investigate whether SD-induced courtship deficit in male flies is a general effect of mechanical perturbation, we deprived males of sleep for 8 h from ZT20 to ZT4, using the same amount of mechanical perturbation with the above 16-h SD (30 s/min shaking for 8 h vs. 15 s/min shaking for 16 h). The 16-h SD severely reduced male sleep and induced sleep rebound after SD (Fig. 1b, c); in contrast, the 8-h SD reduced sleep but did not induce sleep rebound (Fig. 1d, e). Consistent with this, the 8-h SD male flies court much more than the 16-h SD male flies (Fig. 1f). These data suggest that it is the prolonged sleep loss, rather than other possible effects of mechanical perturbation, that impairs male courtship.

Unlike males, pre-mating behaviors in females are less demanding and include slowing down and stopping to allow copulation. In contrast to the above results in males, wild-type females that were sleep-deprived for 12 (ZT16 to ZT4), 16 (ZT12 to ZT4), or 20 (ZT8 to ZT4) hours did not significantly reduce their receptivity to courting males (Supplementary Fig. 1). Taken together, these results indicate that SD suppresses sexual behavior in male flies, but not in female flies.

Sex-promoting neurons regulate sleep. To further address the relationship between sleep and sex, we asked whether manipulating neurons that involved in sexual behaviors would affect sleep in either sex. It has been shown that a subset of P1-activated (Fig. 1) males is a general effect of mechanical perturbation, we deprived males of sleep for 8 h from ZT20 to ZT4, using the same amount of mechanical perturbation with the above 16-h SD (30 s/min shaking for 8 h vs. 15 s/min shaking for 16 h). The 16-h SD severely reduced male sleep and induced sleep rebound after SD (Fig. 1b, c); in contrast, the 8-h SD reduced sleep but did not induce sleep rebound (Fig. 1d, e). Consistent with this, the 8-h SD male flies court much more than the 16-h SD male flies (Fig. 1f). These data suggest that it is the prolonged sleep loss, rather than other possible effects of mechanical perturbation, that impairs male courtship.

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Supplementary Fig. 1 for the effect of SD on female sexual behavior.

The 16-h SD results in 468-min sleep loss during SD and induces post-SD sleep rebound. The start point of baseline was chosen at the same ZT the day before SD. n = 32. ***p < 0.001, one-way ANOVA.

Activity-dependent regulation of courtship and sleep by P1. To further study how P1 neurons regulate courtship and sleep in males, we used different temperatures (25.5, 27, 28.5, and 30 °C) to obtain differential activation of P1 neurons, and assay male courtship (wing extension in solitary males) and sleep. Interestingly, we found that P1 activation driven by the splitGAL4 at 27, 28.5, and 30 °C inhibits male sleep at similar levels (Fig. 3a–d), while activation at 28.5 and 30 °C, but not 27 °C, induces wing extension (Fig. 3i–l). We used the other P1 intersectional driver (LexAop2-FlpL/R71G01-LexA; UAS>stop>TNT/dsxGAL4) and found the same phenotype, except that P1 activation at 27, 28.5, and 30 °C inhibits male sleep at different levels (Fig. 3a–d), which may be due to different populations and/or numbers of P1 neurons targeted by these two methods. These results clearly demonstrate that a lower level of P1 activation is sufficient to inhibit sleep, but a higher level of P1 activation is required for courtship promotion.

As the locomotor activity measured in the beam assays used for sleep analysis is not very sensitive to changes in velocity, we analyzed the locomotor activity of single flies during P1 activation using video recordings over 24 h. We found that P1 activation using the above two drivers at 27 °C slightly increases velocity by ~50% (Fig. 3m), indicative of sleep inhibition, while P1 activation at 28.5 and 30 °C dramatically increases velocity by over five times (Fig. 3m), indicative of increased courtship drive.

ACh release by P1 neurons is required for sleep regulation. The above results indicate that P1 neurons inhibit male sleep, but we...
still do not know the neurotransmitter that P1 neurons release to regulate sleep. Thus, we selectively knocked down neurotransmitters using RNA interference (RNAi) in P1 neurons while activating these neurons via dTRPA1. Activating P1 neurons alone (UAS-dTrpA1/+; P1-splitGAL4) severely reduced male sleep, but independently knocking down three genes (Nsf2, Syx8, and unc-13), which are known to be required for neural transmission, in P1 neurons fully restores male sleep (Fig. 4a). Furthermore, knocking down acetylcholine (VACHT and Ace), but not serotonin (Trh), dopamine (DAT and Ddc), octopamine
**(Fig. 3)** Activity-dependent regulation of courtship and sleep by P1. 

**Density profile of males with P1 neurons activated at indicated temperatures, as well as control males.** 

- **a**, **b**, **c**, **d**: Sleep profiles of males with P1 neurons activated at indicated temperatures, as well as control males. 
  - **a**: Sleep (h) at 21.5, 25.5, and 21.5°C. 
  - **b**: Sleep (h) at 21.5, 27, and 21.5°C. 
  - **c**: Sleep (h) at 21.5, 28.5, and 21.5°C. 
  - **d**: Sleep (h) at 21.5, 30, and 21.5°C. 

**Velocity of males with P1 neurons activated at indicated temperatures over 24 h of video tracking.** 

- **e**, **f**, **g**, **h**: Velocity of males with P1 neurons activated at indicated temperatures, as well as control males. 
  - **e**: 25.5°C. 
  - **f**: 27°C. 
  - **g**: 28.5°C. 
  - **h**: 30°C. 

**Percentage of males with P1 neurons activated at indicated temperatures shows wing extension over 24 h of video tracking.** 

- **i**, **j**, **k**: Percentage of males with P1 neurons activated at indicated temperatures shows wing extension over 24 h of video tracking. 
  - **i**: 27°C. 
  - **j**: 28.5°C. 
  - **k**: 30°C. 

**Percentage of males with P1 neurons activated at indicated temperatures shows wing extension.** 

- **l**, **m**, **n**: Percentage of males with P1 neurons activated at indicated temperatures shows wing extension. 
  - **l**: 25.5°C vs. 27°C vs. 28.5°C vs. 30°C. 
  - **m**: 25.5°C vs. 27°C vs. 28.5°C vs. 30°C. 
  - **n**: 25.5°C vs. 27°C vs. 28.5°C vs. 30°C.

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**P1 neurons regulate sleep through fma**.positive DN1 neurons. 

We hypothesized that the activation of P1 neurons might function through known sleep circuitry to suppress sleep. To test this hypothesis, we performed “neuronal epistasis” experiments in which we simultaneously activated P1 neurons with dTRPAl and blocked synaptic outputs of candidate downstream sleep-regulating neurons with Shibire31 (Fig. 5a, b). We selected a set of LexA driver lines (Supplementary Fig. 6) targeting candidate neurons that have been shown to be involved in sleep: mushroom body Kenyon cells (R14H06-LexA, R35B12-LexA, and R4E04-LexA), mushroom body output neurons (R12C11-LexA, R14C08-LexA, R24H08-LexA, R25D01-LexA, and R71D08-LexA), PAM dopaminergic neurons (R58E02-LexA), fan-shaped body neurons (R23E10-LexA and R84C10-LexA), and DN1 circadian clock neurons (R18H11-LexA). Blocking synaptic outputs of any of these neurons with Shibire31 does not significantly affect sleep (Fig. 5b), although activating many of these neurons affects sleep26, 27. This could be a result of basal activity of these neurons already being relatively low, as has been observed for other sleep-regulating neurons33, or differences in strength of expression of driver lines.

On the basis of this screen of putative downstream effectors of P1 neurons, we find that silencing specifically the synaptic
reveals that the Dh31-expressing DN1s are electrically active. Scale bars, 50 µm.

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outputs of a subset of dorsal clock neurons: DN1 neurons partially reverses the sleep deficit induced by activating P1 neurons (Fig. 5d), suggesting that P1 neurons suppress sleep at least in part by activating DN1 sleep-regulating circadian clock neurons. These sleep changes are not due to differences of general locomotor activity between the permissive (21.5 °C) and restrictive (29.5 °C) temperatures (Supplementary Fig. 7).

DN1 neurons express FRU_M and regulate courtship activity rhythms. Furthermore, DN1 neurons are known to suppress sleep through secretion of the wake-promoting neuropeptide diuretic hormone 31 (Dh31)27. Functional imaging of DN1 neurons using genetically encoded fluorescence voltage indicator reveals that the Dh31-expressing DN1s are electrically active before dawn (ZT 22) as compared to late in the day (ZT10), supporting the hypothesis that DN1 activity awakens the fly27. We also tested whether the P1–DN1 circuit mediates courtship by activating P1 neurons and inhibiting DN1 neurons as described above and found that, unlike sleep, P1 activation induced courtship behaviors were not suppressed by DN1 inhibition (Supplementary Fig. 8).

Following up on the neuronal epistasis experimental results and a role for P1-DN1 circuit in sleep suppression, we sought to determine whether the DN1 clock neurons are synaptically downstream of P1 neurons. Double-labeling (data not shown) and brain registration (Fig. 5e and Supplementary Movie 1) of DN1 and P1 neurons suggest that DN1 neurons are not directly connected with P1 neurons. Indeed, there is no GRASP (GFP reconstitution across synaptic partners) signal between DN1 and P1 neurons (Fig. 5f), suggesting indirect polysynaptic connection or no connection between P1 and DN1 neurons. To test the possibility of indirect poly-synaptic connectivity, we transiently activated P1 neurons and recorded Ca2+ signals from DN1 neurons. We found that activation of P1 neurons expressing ATP-dependent depolarizing ion channel P2X246 with a puff of 10 mM ATP induced robust calcium responses in the cell bodies and projections of DN1 neurons (R18H11-LexA/LexAop2-GCamp6m; R15A01-GAL4/UAS-P2X2; Fig. 6a–c and Supplementary Movie 2). DN1 calcium responses were not observed in brains that lack P2X2 expression in P1 neurons (R18H11-LexA/LexAop2-GCamp6m; R15A01-GAL4/+; Supplementary Movie 3). These results demonstrate that DN1 neurons are indeed synaptically downstream of P1 neurons and, based on the above anatomical evidence, this synaptic connectivity is indirect.

We tested whether the DN1 neurons feedback into the P1 neuronal cluster and found that activating the DN1 neurons activates the P1 neurons measured by an increase in Ca2+ response (Fig. 6d–f and Supplementary Movie 4), which may be a mechanism for rhythmic control of courtship by DN1 neurons reported previously31, although we found that activating DN1 neurons via dTRP1 does not significantly change courtship (Supplementary Fig. 8). These data provide direct evidence for mutually excitatory interactions between DN1 and P1 neurons that support a positive feedback circuit model that likely underlies persistence of arousal states associated with sleep-suppression and courtship. Thus, the reciprocal interactions between P1 and DN1 neurons link the sleep and courtship drive and are critical to the behavioral choice. On the basis of the above results we propose that activity of P1 neurons is directly influenced by the sleep-controlling DN1 neurons and that P1 activity is central to the sleep–sex behavioral switch.

P1 activity is suppressed by SD. To test whether the activity of P1 neurons is directly modulated by sleep need, we directly measured spontaneous neural activity of these neurons in sleep-deprived and sleep-replete flies as described33. We expressed the genetically encoded fluorescent voltage indicator ArcLight47 using the above P1-splitGAL4 driver, and imaged spontaneous activity of the lateral junction region of P1 neurons in males (Fig. 7a). We found that P1 activity is significantly reduced in SD males as compared to sleep-replete controls that were imaged in parallel (Fig. 7b, c). We also analyzed these results using comparisons between peak or maximal ΔF/ΔF0 and found significant differences (Supplementary Fig. 9). Furthermore, wing extension by P1-activated males is significantly reduced by SD (Supplementary Fig. 10). These data strongly support the observation that SD males have reduced courtship possibly resulting from diminished activity of the P1 courtship command neurons. Furthermore, activating P1 neurons with dTRP1 in SD males restores male courtship (Fig. 7d). These data further support the hypothesis that reduced activity of DN1 neurons27 in sleepy males reduces excitatory input into P1 neurons, thereby preventing the flies from engaging in wake-associated social behaviors. As P1 neurons receive inputs from multiple sensory inputs, it is likely that sleep deprivation also modulates activity of non-DN1 inputs into the P1 neurons. Thus, P1 neurons regulate...
male courtship by integrating external courtship stimuli and internal sleep needs.

**Sex-specific control of sleep by fruitless and doublesex.** The above results indicate that selection of sleep and sexual behaviors is sexually dimorphic, but we also note that the sleep or sexual behaviors *per se* are sexually dimorphic. Further, P1 and DN1 neurons identified in regulation of the behavioral choice between sleep and courtship express sex-specific genes *fru* and/or *dsx.* To elaborate the role of these genes in behavioral choice, we sought to understand the role of sexually dimorphic genes in individual behaviors. Although it has been well studied how *fru* and *dsx* control sex-specific sexual behaviors, whether they also control sex-specific sleep is unclear.

Male flies sleep more than females. We investigated whether such sexually dimorphic sleep amounts of male and female flies are controlled by the sex determination master gene *transformer* (*tra*), and its two downstream target genes *fru* and *dsx* (Fig. 8a). Knockdown of *tra* in the nervous system using pan-neuronal c155-GAL4 to drive expression of RNAi from UAS-traIR increases female sleep to match that of male flies, while parental control females (c155/+ and UAS-traIR+/+) sleep significantly less than males of the same genotype (Fig. 8b, c). This indicates that sexually dimorphic sleep quantity is indeed controlled by the sex determination pathway.

We then tested the role of the *dsx* and *fru* branches in sleep regulation. We found that females expressing RNAi targeting *dsx* pan-neuronally (c155>UAS-dsxIR) sleep even slightly more than males of the same genotype, while parental control females sleep less than males (Fig. 8b, c). We also found that females pan-neuronally expressing microRNAs targeting *fru* (c155>UAS-fruMi) sleep as much as males of the same genotype, while control females expressing a scrambled version (UAS-fruMiScr) sleep less than males (Fig. 8b, c). These results further support the role of sex determination genes in regulating sleep.

To further investigate how *dsx* and *fru* regulate sexually dimorphic sleep, we tested multiple combinations of *dsx* and *fru* alleles. We found that for two *dsx* null genotypes (dsx*683–7058* and dsx*649–9625*) and a masculinized line (dsx*683–7058/dsx*M) that expresses dsx*M* regardless of sex, the amounts of male sleep are similar to parental control males (Fig. 8d); but the amounts of female sleep are significantly greater than in parental control females (Fig. 8e). Furthermore, the increment of sleep in *dsx* null females is specific to daytime sleep, which contributes to the majority of sexual dimorphic sleep (Supplementary Fig. 11). In contrast, for all five *fru* null genotypes we tested, the amounts of male sleep are significantly lower than that in parental control males (Fig. 8f), while the amounts of female sleep are similar to what is observed in parental control females (Fig. 8g). The decrement of sleep in *fru* null males is not specific to daytime or nighttime (Supplementary Fig. 11).
FRUM differentially regulates courtship and sleep. It has been proposed that FRUM specifies a neuronal circuitry that is dedicated for male courtship behavior\textsuperscript{13,14}. As we found that FRUM also regulates male sleep, we set out to identify the neuronal substrates where FRUM functions to mediate sleep. We used the microRNA, as mentioned above, to target fru\textsuperscript{M} in subsets of fru\textsuperscript{M} neurons, and a scrambled version as control. Knockdown of FRUM in all fru\textsuperscript{M} neurons (fru\textsuperscript{GAL4}) significantly decreases male sleep, but driving the microRNA in glia cells (repo-GAL4), or a subset of P1 neurons (R71G01 and R15A01), that are crucial for male courtship, did not affect male sleep (Fig. 9a, b). Knockdown of FRUM in all dsx neurons (dsx\textsuperscript{GAL4}) only slightly altered male sleep (reduced by ~6%, Fig. 9a, c). However, we observed significant decrement of male sleep when knocking down FRUM in MB Kenyon cells (R13F02, R19F03, and R76D11, Fig. 9a, d, e), or DN1 neurons (R18H11, Fig. 9a, f), all of which express FRUM (Supplementary Fig. 13). These sleep changes are not due to general locomotor activity differences as indicated by activity during the wake phase (Supplementary Fig. 14).

On the basis of our neuronal epistasis and functional imaging experiments, the DN1 neurons seem critical to the male-specific sex–sleep selection mechanism. We investigated the precise mechanism by which FRUM\textsuperscript{M} regulates sleep in the DN1 neurons. We recently found that the neuropeptide Dh31 functions in DN1 neurons to regulate fly sleep\textsuperscript{27}, thus we asked whether FRUM\textsuperscript{M} might regulate sleep through Dh31 in DN1 neurons. We tested sleep in males with FRUM\textsuperscript{M} knocked down in DN1 neurons (R18H11/UAS-fruMi\textsuperscript{F}) in the background of Dh31 mutants. We found that FRUM\textsuperscript{M}-mediated sleep loss is dependent on Dh31, as a single copy of a Dh31 allele (Dh31\textsuperscript{KG09001}) already attenuates the sleep loss, and a combination of Dh31 alleles (Dh31\textsuperscript{KG09001}/Df(Dh31)) almost abolishes the sleep loss (Fig. 9a, g). Thus, FRUM\textsuperscript{M} promotes male sleep in DN1 neurons by regulating Dh31 levels and/or secretion.

To investigate how FRUM\textsuperscript{M} regulates both male courtship and sleep, we then tested courtship behavior of the above FRUM\textsuperscript{M} knocked down males, and found that knocking down FRUM\textsuperscript{F} in dsx-expressing neurons severely impairs male courtship, but knocking down FRUM\textsuperscript{M} in MB neurons or Dh31-expressing DN1 neurons does not affect male courtship (Fig. 9h). Thus, FRUM\textsuperscript{M} functions in distinct neural substrates to regulate male courtship (e.g., fru\textsuperscript{M} and dsx overlapping P1 neurons) and male sleep (e.g., MB and DN1 neurons).

Discussion

Neural networks integrate external sensory cues, past experience, and internal states to control key behavioral decision-making. How these neural networks support behavioral choice critical for reproduction and survival at both the individual and species level is poorly understood. Here we focused our attention on identifying and characterizing the molecular and neural basis of reciprocal control of sleep and reproductive behaviors. The neuronal mechanism we have uncovered involves the P1 neurons implicated in courtship decision-making and the DN1 neurons, a part of the core clock and sleep circuit in suppressing sleep. Recently, the activity of P1 neurons was shown to be modulated by excitatory and inhibitory inputs from gustatory and olfactory

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**Fig. 6** P1 and DN1 neurons form mutually excitatory connections. a Movie still of calcium imaging of DN1 cell bodies after P1 activation via P2X\textsubscript{2}. b Averaged traces of ΔF/F\textsubscript{0} of DN1 neurons after P1 activation. c Peak fluorescence changes (ΔF/F\textsubscript{0}) of DN1 neurons after P1 activation. n = 6 for each, ***p < 0.001. Unpaired t-test. d Movie still of calcium imaging of P1 neurites in the lateral protocerebrum region after DN1 activation via P2X\textsubscript{2}. e Averaged traces of ΔF/F\textsubscript{0} of P1 neurons after DN1 activation. f Peak fluorescence changes (ΔF/F\textsubscript{0}) of P1 neurons after DN1 activation. n = 10 for each, **p < 0.01. Unpaired t-test. Error bars indicate SEM.
systems and thus processing multisensory information controlling behavioral output relevant to male courtship. In addition to the gustatory and olfactory input into the P1 neurons, a subset of dopaminergic neurons in the anterior superior medial protocerebrum has also been implicated in modulating P1 activity. Interestingly, the dopamine activity and its influence on P1 neurons are dependent on mating history. P1 activity is also modulated by housing conditions (e.g., single-housed vs. group-housed).

The male-specific P1 neurons function as the key courtship command neurons that trigger a spatial and temporal pattern of motor neuron activity specific to courtship behaviors. We find that this higher-order processing within the P1 neurons is altered as a result of sleep and wake states as evidenced by decreases in spontaneous activity of the P1 neurons after SD. We also found that P1 neurons could be activated by sleep-controlling DN1 neurons. Additionally, activating P1 neurons (gain of function) decreases sleep while inhibiting P1 neurons (loss of function) increases sleep. These data show that P1 neurons represent an important sleep-regulating locus in the male fly brain.

While the neural circuitry downstream of P1 neurons important in producing courtship behaviors is well understood, the circuit mechanisms by which P1 neurons produce wake behavior are intriguing. Using P2X2 to activate P1 neurons and GCamp6m to visualize DN1 activity, we found that DN1 neurons are functionally downstream of P1 neurons, although they do not have such a direct synaptic connection. To ensure that this specific connection underlies the wake-promoting phenotype of P1 neuron activation, we simultaneously activated the P1 neurons while inhibiting the DN1 clock neurons and observed a strong decline in the wake-promoting phenotype of P1 activation. This effect was not phenocopied by silencing other key sleep-regulating centers of the fly brain including mushroom body and central complex neurons.

Our results demonstrate that P1 and DN1 neurons form a positive feedback loop and support persistent neural activity to sustain extended phases of arousal necessary for social behaviors like courtship. The role of P1-DN1 mutual excitatory circuit as the mechanistic basis of interaction between sleep and courtship drive is further supported by the finding that P1 activity is low in sleepy flies as compared to sleep-replete controls. The P1 neural node activity is highly dynamic and is influenced by sensory inputs and social experiences, which could further underlie the reduced neural activity of P1 neurons in sleep-deprived flies. It is also interesting to note that our induced activation studies of P1 neurons using dTRPA1 show that low levels of activation are sufficient to suppress sleep, but a higher level of activation is required for courtship promotion.

Fig. 7 SD decreases P1 activity. a Optical recording of membrane activity in the lateral junction (circled) of P1 neurons in control (non-deprived) males and sleep-deprived males. b SD of the optical signal was plotted, with mean ± SEM. n = 10 for each. **p < 0.01, unpaired t-test. c Power spectrum was computed using fast Fourier transform with 0.2 Hz bin width. Power of the non-deprived group is significantly greater than the sleep-deprived group (two-way ANOVA with repeated measures). d Activating P1 neurons with dTRPA1 overcomes courtship suppression by SD. n = 24 for each. ***p < 0.001, unpaired t-test. NS, not significant. Error bars indicate SEM.
At the molecular level, we find that acetylcholine release from the P1 neurons is critical to sleep regulation such that inhibiting synthesis or release of Ach from the P1 neurons suppresses the wake behavior induced by P1 activation.

Interestingly, the reciprocal regulation of sleep and sexual behaviors is different in male and female flies, leading to sex-specific interaction of these behaviors. Note that P1 neurons express FRUM and DSXM, and are male-specific, while DN1 neurons also express FRUM. Recent studies have also shown that the DN1 neurons are more active in males as compared to females, further supporting our key findings of male-specific courtship–sleep circuitry involving P1-DN1 neuronal connections. The male-specific courtship–sleep interaction mediated by the P1-DN1 circuit is not surprising as both sexual and sleep behaviors per se are sexually dimorphic. Although it has been well documented that male and female flies have different sleep patterns and that female flies sleep less during the daytime as compared to males, the molecular and neural basis of this sexual dimorphism in sleep patterns is unclear. Sex differences in nervous system structure and behaviors are all attributed to sex determination genes tra, fruM, and dsx. We found that these genes also control sexually dimorphic sleep patterns. In particular, FRUM and DSXF differentially regulate male and female sleep, respectively, where FRUM promotes male sleep during both daytime and nighttime, and DSXF inhibits female
During daytime but not nighttime. Furthermore, we found that knockdown of FRUM in MB or DN1 neurons decreases male sleep amount, but leaves male courtship intact; in contrast, knockdown of FRUM in all dsx-expressing neurons severely impairs male courtship, but only slightly alters sleep, suggesting that FRUM functions in distinct neural substrates to regulate male courtship and sleep.

In order to understand the complex relationship between the male-specific P1-DN1 feedback and sexually dimorphic sleep patterns, we probed how FRUM acts on the DN1 neurons in DH31. Here we find evidence that FRUM differentially regulates courtship and sleep. The neural circuit mechanism by which DH31 released from DN1 neurons regulates wake is unknown, but there is evidence of synaptic communication between the DN1 and Pars intercerebralis neurons, another sleep-regulating loci. Further, the pars intercerebralis neurons regulate circadian output by release of another neuropeptide Dh4453, 54.

Previous studies have looked at more generalized decision-making neurons, but here we have identified a novel sex-specific neuronal circuitry for sex–sleep interaction, which depends on sex determination genes that directly influence the neuronal output of key decision-making neurons (Fig. 10). Thus, our findings on genetic and neural circuit mechanisms underlying sex–sleep interaction will have broad implications for studies on decision-making and behavioral choice in higher-order organisms.

**Methods**

**Fly stocks.** Flies were maintained at 22 or 25 °C in a 12 h:12 h light:dark cycle. fru^M^ alleles used in this study include fru^Lexa^, fru^A^, fru^A^; fru^A^, fru^A^; fru^A^, and fru^A^; fru^A^ alleles are dh3^Gal4-Lexa^, dh3^Gal4-A^, dh3^Gal4-A^, and dh3^Gal4-A^ alleles are dh3^Gal4^ and Dh3^Gal4^). BNAI lines are from Tsinghua Fly Center (THFC) at the Tsinghua University. 56. Neurotransmitter GAL4 lines are from Bloomington Stock Center. R12C11, R14C08, R14H06, R15A01, R18H11, R23E10, R24H08, R25D01, R35B12, R41A01, R44E04, R58D02, R71D08, R71G01, and R86C10 are enhancers for Janelia GAL4 (or GAL4-AD, GAL4-DBD) or LexA drivers.57-59 LexAop-Eypl, LexAop-shits1, and pBdGal4^56, UAS-dTrpA1, LexAop-GCam6, UAS-GCam6, UAS-P2X^60, UAS-fruMi, and UAS-fruMiScr. UAS-ArcLight97, UAS-CD4:spGFP1-10 and LexAop-CD4:spGFP1185.
Courtsip-related sensory cues

Sleep need

Positive feedback

FrU4

P2

Male courtship

Lower threshold

FrU4, DH31

Male sleep

Fig. 10 Sex-specific interaction between courtship and sleep. The male-specific P1 neurons integrate external courtship-related sensory cues and internal sleep need, then inhibit male sleep, and promote male courtship in a threshold-dependent manner. P1 neurons form mutually excitatory connections with sleep-controlling DN1 neurons, which might be important for a persistent behavioral state. Furthermore, FrU4 differentially regulates male courtship and sleep in P1 and DN1 neurons. Scale bars, 50 μm.

Brain image registration. The standard brain used in this study is described previously64. Confocal images for R18H1-LexA and P1-splitGal4 were registered onto this standard brain with a Fiji graphical user interface as described previously64, 65.

P2X2 activation and GCamp6m imaging. To gain access to the P1 or DN1 neurons for ATP application, whole-brain explants were placed on 8-mm diameter circular coverslips and placed in a recording chamber containing external solution (103 mM NaCl, 3 mM KCl, 5 mM N-tris methyl-2-aminoethane sulfonic acid, 8 mM trehalose, 10 mM glucose, 26 mM NaHCO3, 1 mM NaH2PO4, 2 mM CaCl2, and 4 mM MgCl2, pH 7.4). For an early experiment (Fig. 6a-c), an ATP electroporation electrode was filled with freshly prepared 10 mM ATP solution and positionned near P1 neurons using a micromanipulator. The ATP was ejected by applying a 25-ms pressure pulse at 20 psi using a picospritzer (Parker Hamlin, Precision Fluidics Division, NH). The picospritzer was triggered by Zeiss image acquisition and processing software Zen pro 2012. Later, we added ATP simply using a pipette, but the final ATP concentration is still 10 mM (Fig. 6d-f). Calcium imaging was performed using Zeiss Axio Examiner Z1 upright microscope with W Plan Apochromat 40× water immersion objective. GCamp6m was excited with a 475-nm LED light source (Colibi, Zeiss) and images were acquired using ORCA-R2 C10600-10B digital charged-couple device (CCD) camera (Hamamastu, Japan) at 3 Hz. The average fluorescence of all pixels for each time point in a defined region of interest (ROI) was subtracted from the average background fluorescence of the same site ROI within the brain region. The resulting fluorescence change value for each time point was defined as Pt % = [(Ft/F0) × F0 × 100], where F0 corresponds to average of 10 frames of background-subtracted baseline fluorescence before ATP application. All images were processed and quantified using Zen and Fiji (Image J).

Arc light imaging. Imaging of freshly dissected brain explants was performed on a Zeiss Axiolab upright microscope with a W Plan Apochromat ×40 N.A. 1.0 water immersion objective (Zeiss, Germany). ArcLight was excited with a 470 nm LED (Zeiss). The objective C-mount image was projected onto the 80 × 80 pixel chip of a NeuroCCD-SM camera controlled by NeuroPlex software (Red-ShiflImaging, Decatur, GA). Images were recorded at a frame rate of 100 Hz, and depicted optical traces were spatial averages of intensity of all pixels within the ROI with signals processed as previously reported87. Statistical analysis and plotting of the data were performed using R. Sleep-deprived flies and sleep-replete controls were dissected within a few minutes of sleep-deprivation and recorded at the same ZT.

Data availability. We declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request.

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Author contributions
D.S., B.S.B., M.N.N., and Y.P. conceived the experiments, interpreted the results, and wrote the manuscript. D.S., D.C., and Y.P. carried out most of the experiments and analyzed data. X.J. carried out the Arclight experiment; N.C. and C.H. helped with sleep experiments; J.C. and M.S. helped with GCamp imaging.

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