EXCMA. SRA. DRA. LESLIE C. GRIFFITH

Answering speech

Pedro Clarós, M.D., Ph.D.
Numerary Academic of The Royal European Academy of Doctors

Mr. Chairman of the Royal European Academy of Doctors.
Distinguished Academics
Ladies and Gentlemen

It gives me great pleasure to introduce Dr. Griffith, the new honorary member of this Royal European Academy of Doctors, who will talk on **"Sleep: Insights into human behavior from the study of insects",** which will include concepts concerning the sleep mechanism, insomnia and the latest investigations being carried out in this field.

Prior to her lecture, I would just like to make some considerations and pose a few questions.

Dr. Griffith is a neuroscientist. She is Professor of Biology, and has carried out a great deal of research on sleep behavior in humans, but there are still a lot of unknown factors; both in the results obtained and in the insomnia therapies applied.

Sleep and the need to sleep is vitally important for the human being. Not doing so is harmful to health, given the wide range of negative effects involved.

We, specialist in otorhinolaryngology, treat "Sleep Apnea Syndrome", specifically by carrying out diagnosis and surgical treatment, because its consequences are very serious.

On the other hand, the opposite effect, i.e. insomnia, is also a problem, and many drugs are used to combat it, with varying results, as not all cases of insomnia are the same.

Keep in mind that 30% of the population suffers from insomnia and that a further 30% suffers from sleep apnea, meaning that 60% of the population has trouble sleeping or can barely sleep at all.

Dr. Griffith's research on the human sleep mechanism has involved the use of the Drosophila Melanogaster fly in an effort to understand better the human brain and the sleep mechanism.

Drosophila Melanogaster, also known as the vinegar fly, wine fly or fruit fly, is a Diptera of the Drosophilidae family, very often used for clinical research.

Why is this insect used in genetic experiments?

The reason for this is that Drosophila Melanogaster has a reduced number of chromosomes (4 pairs) and a very short lifecycle (2-3 weeks), which facilitates research.

It is also due to the fact that 50% of this fly's protein sequences have mammalian homologs.

The results have been applied to the study of several human medical conditions such as Parkinson's and Alzheimer's disease, as well as in the study of aging mechanisms, oxidative stress, immunity, diabetes, cancer, and drug abuse.

Is all sleep like human sleep?

In human beings, we know that we can study sleep by measuring the brain's electrical activity using an EEG (Electroencephalogram), but how can we do so with flies? The mechanisms that produce the states of wakefulness and sleep are not yet well known, but a whole cocktail of neurotransmitters is involved. One of these present in humans is GABA (Gamma-Aminobutyric acid), and it

seems also to be important in the fly. GABA is the chief inhibitory neurotransmitter in the mammalian central nervous system. It plays the main role in reducing neuronal excitability throughout the nervous system. In humans, GABA is also directly responsible for the regulation of muscle tone.

Although in chemical terms it is an amino acid, GABA is rarely referred to as such in the scientific or medical communities, because the term "amino acid," used without a qualifier, by convention refers to the alpha amino acids, which GABA is not, nor is it considered to be incorporated into proteins.

In vertebrates, GABA acts on inhibitory synapses in the brain by binding to specific transmembrane receptors in the plasma membrane of both pre and postsynaptic neuronal processes. This binding causes the opening of ion channels to allow the flow of either negatively charged chloride ions into the cell or positively charged potassium ions out of the cell. This action results in a negative change in the transmembrane potential, usually causing hyperpolarization.

Neurons that produce GABA as their output are called GABAergic neurons, and have chiefly inhibitory action at receptors in the adult vertebrate. GABA exhibits both excitatory and inhibitory actions in insects, mediating muscle activation at synapses between nerves and muscle cells, and also the stimulation of certain glands. While GABA is an inhibitory transmitter in the mature brain, its actions are primarily excitatory in the developing brain. Thus, GABA is the major excitatory neurotransmitter in many regions of the brain before the maturation of glutamatergic synapses.

Exogenous GABA does not penetrate the blood–brain barrier; it is synthesized in the brain. It is synthesized from glutamate using the enzyme L-glutamic acid decarboxylase (GAD) and pyridoxal phosphate (which is the active form of vitamin B6) as a cofactor. This process converts glutamate, the principal excitatory neurotransmitter, into the principal inhibitory neurotransmitter (GABA). GABA is converted back to glutamate by a metabolic pathway called the GABA shunt.

Drugs that act as allosteric modulators of GABA receptors (known as GABA analogues or *GABAergic* drugs) or increase the available amount of GABA typically have relaxing, anti-anxiety, and anti-convulsive effects.

GABA enhances the catabolism of serotonin into N-Acetylserotonin (the precursor of melatonin) in rats. It is thus suspected that GABA is involved in

the synthesis of melatonin and thus may exert regulatory effects on sleep and reproductive functions.

Therefore, research and manipulation of this neurotransmitter will increase our understanding in the treatment of the sleep-regulating Peptidergic neuron.

As you will read, Dr. Griffith's manuscript is based on a very interesting study. I will now give a brief account of Dr. Griffith as a person.

Laudation of the new Academic.

Leslie Claire Griffith was born in Michigan and raised in a small town on the shores of Lake Superior. She attended The Massachusetts Institute of Technology (MIT) and obtained a Bachelor of Science in Life Sciences (BS) degree in 1981. She became interested in research while studying there, and worked for three years on a project designed to understand the effects of methylxanthines such as caffeine on the hypothalamic / pituitary axis. After receiving her Bachelor of Science, she went on to Stanford where she completed her MD PhD (1987) in Pharmacology. Her thesis was entitled: "**Regulation of Neuronal Function by Calcium-dependent Phosphorylation**". The complex biochemistry of this enzyme suggested that it might be an integral part of the molecular machinery underlying memory formation, and Dr. Griffith decided to adapt to a genetic model organism, *Drosophila*, in order to test these ideas.

After a short post-doctorate period with Dr. Ralph Greenspan at the Roche Institute of Molecular Biology in Nutley, NJ, 1988-1992, studying the role of calcium / calmodulin-dependent protein kinases in neuronal function and behavior in *Drosophila Melanogaster,* she joined Brandeis University in 1992 as Assistant Professor, at the Department of Biology and Volen Center for Complex Systems, where her research has expanded from biochemical to behavioral and systems levels. She served as Chair from 2009-2012, and is currently the Nancy Lurie Marks Professor of Neuroscience and the Director of the Volen Center for Complex Systems, an interdisciplinary center that serves as a focus for Neuroscience at Brandeis.

She has participated in numerous national and international scientific committees, grant review panels and editorial boards.

Another important aspect of Dr. Griffith's career is her long list of Scholarship Grants.

In her publication list, I have counted more than 60 publications between the years 2004 and 2012 and many of them in high-impact factor medical journals.

She has received a lot of HONORS AND AWARDS in the course of her career:

National Merit Scholar

Phi Beta Kappa, M.I.T.

NSF Predoctoral Fellowship, 1981-1984

Pharmaceutical Manufacturers Association Foundation Advanced Predoctoral Fellowship, 1985-1987

NRSA Postdoctoral Fellowship, 1989-1992

Rothschild Fellowship, Hoffman-LaRoche, 1989-1992

Alfred P. Sloan Research Fellowship, 1994-1996

Klingenstein Research Fellowship, 1994-1997

Whitehall Foundation Grant, 1995-1997

NIH Career Development Award, 1996-2001

McKnight Technological Innovation Award 1999-2000

Scholar in residence, Neural Systems and Behavior, Marine Biological Labs, Woods Hole 2007

Meyerhof Lecturer, Max-Planck Institute, Heidelberg, 2008

Society for Neuroscience Presidential Lecture, 2008

Nancy Lurie Marks Professor of Neuroscience, 2012-present

Benzer Lecture, CSH Neurobiology of *Drosophila* meeting, 2015

Mr. Chairman, Distinguished Academics, Ladies and Gentlemen:

On behalf of all those present, I would like to welcome you most warmly to this European Academy.

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Leslie Griffith, M.D., Ph.D.
Professor of Biology
Nancy Lurie Marks Professor of Neuroscience
Director, Volen National Center for Complex Systems
Biochemistry of Behavior
Massachusetts Institute of Technology, S.B.
Stanford University, Ph.D.
Stanford University, M.D.

I would like to thank the Academy for this great honor, and I would like to present a taste of the type of work done in my lab.

We spend 1/3 of our lives sleeping, yet we do not really know why. But it is clear that if you do not sleep, bad things happen. There are both physical and mental consequences that require us to sleep. Today I will tell you a bit about sleep in general then give you some examples of research in my lab that is address how brains generate sleep states.

Why is understanding how sleep is regulated important? Insomnia affects approximately 1/3 of the American population; over 60 million people, and lifestyle-induced sleep disorders from shiftwork and jet lag are rampant. Acute sleep deprivation can cause severe cognitive effects. Chronic sleep deprivation is associated with increased risk for cancer, diabetes, heart disease and mental illness. Our healthcare system's ability to deal with promoting sleep and perhaps more importantly suppressing or reversing the effects of sleep deprivation are inadequate.

One of the reasons that we do not have optimal sleep therapies is that insomnia is not a homogenous disorder. There is sleep onset insomnia, sleep maintenance insomnia and terminal insomnia. Mild insomnia is often treated with behavioral or environmental changes. For severe or chronic insomnia the therapies center on drugs that enhance $GABA_A$ transmission, but none of these drugs have selective effects on onset vs. maintenance. Their differential use is based on solely on their pharmacokinetics: short half life drugs are used for onset insomnia, while longer acting drugs that have serious residual daytime sleepiness effects are used for maintenance and terminal insomnia.

What I want to convince you of in my talk today is that insects can be useful model systems for studying complex human behaviors like sleep. Research in Drosophila is shedding new light on both how sleep is generated and may help us design therapeutic interventions to treat human disorders.

I am sure many of you have heard about the virtues of invertebrate model systems, but I think it is worthwhile to think about not only their potential, but what they have already contributed to our understanding of the brain. Genetics provides an unbiased way of looking at the molecular basis of a behavior. 60% of known human disease genes have an insect ortholog. Flies are being used not only to understand pathophysiology of disease, but they are being used to screen for small molecule therapeutics for a variety of diseases. Genetics in invertebrate model systems has also provided the molecular scaffolding on which our understanding of mammalian brain function is based.

But my talk today will not be about gene discovery, it will be about how to use the simplified structure of the insect nervous system to take apart and understand at the cellular and circuit level how the brain can generate sleep. The main takeaway point is that simple systems that can generate complex behaviors have a lot to offer us in terms of understanding how the brain works.

Any discussion of sleep has to start with a definition of the salient characteristics of the behavior. There are a number of criteria that have been established to differentiate sleep from other quiescent states like quiet rest, coma or death. First, sleep is rapidly reversible. It occurs on a daily basis at a predictable time of day due to the influence of the circadian clock on the process. For us and for flies, our consolidated sleep occurs primarily at night. Sleep is able to be differentiated from quiet rest by the fact that during sleep arousal thresholds increase- it takes more light, more shaking, louder sound to rouse a sleeping animals than an animal who is awake but immobile. Sleep is also distinguished

from wake by gross patterns of electrical activity. In humans we measure this with EEG, in flies with a microelectrode. Lastly, and very importantly, sleep is homeostatically regulated. Homeostasis is the ability of a system to adjust itself to maintain a state of constancy. For sleep there is a brain sensor that detects how much sleep you get in a night and if it is not enough, it increases sleep drive to generate extra sleep the next day. This extra sleep is called "rebound" or "recovery" sleep. For an animal to be considered to have a sleep state, all these criteria must be met. And, as is true for humans, flies meet all these criteria and can be considered to have a genuine sleep state.

So how do we study fly sleep? Because we cannot carry out EEG on flies due to size, we must use the absence of locomotor activity as a proxy for sleep. We can monitor locomotor activity in either a tube with an IR beam or we can monitor sleep directly, by videotaping flies. Because sleep is regulated by circadian rhythms, we always entrain our flies to a 12h:12h light dark cycle.

The data from both methods of assessing sleep are analyzed similarly. Sleep is defined as greater than 5 min of immobility based on the direct observations by a number of groups that have shown that 5 min of immobility correlates with a decrease in sensory responsiveness and in changes brain activity as measured by field electrodes. This criterion is an effective way to differentiate quiet waking from sleep. We are not simply looking at changes in locomotor activity, we are looking at a state change and I will show you some of the data that demonstrate this directly later in my talk.

The first problem we wanted to get at in flies was the issue of state transitions: how do we fall asleep? I indicated that I am going to try to convince you that we can learn things relevant to human control of sleep from flies, so I want tell you a bit about what we know about human sleep first.

We know a lot about the neural systems that generate sleep in humans from lesion and pharmacological work in other mammals. Sleep is controlled by a complex collection of wake-promoting neurons that have diffuse projections to cortical and sensory areas of the brain. These wake-promoting cells can be shut off by a relatively small group of neurons that include GABAergic neurons of the ventrolateral preoptic area. The details of the process are not well understood and the circuits are massively complex and involve millions of neurons.

Sleep in humans and all other animals in which it has been described involves a distinct state change: the brain is either awake or it is asleep. Being in

an intermediate state has distinct disadvantages in the real world. This has led to the system being very "switch-like" in its control.

In mammals the switch is controlled by GABAergic inhibition of arousal centers. Does the fly have a GABAergic sleep switch? As in humans, GABA is the major inhibitory neurotransmitter in the *Drosophila* CNS- but mutants that lack GABA are not viable. How do we ask the question more precisely?

Traditional methods for looking at the role of a particular chemical in a neural circuit have involved pharmacologically altering activity, neuronal ablation and electrophysiological recordings from neurons. We know that by altering the activity of neurons that are embedded in a circuit, we can alter the output of that circuit. These approaches have been very powerful, but they are usually at least one step removed from the behavior that is being studied. Luckily in the fly we have a variety of genetic tools that can let us ask about the role of GABA in more subtle and precise ways.

In the fly we can apply the same logic, using alteration of circuit component activity to identify and characterize the function of candidate neurons. Because we are doing this genetically instead of with drugs or electrodes, we can manipulate specific neurons (e.g. ones that make GABA) and ask in the intact animal does this change sleep. The genetics, which I can describe later if any one is interested, allow us to manipulate basically any neuron or sets of neurons we would like. This is an incredibly powerful system.

But getting back to the question at hand: is there a GABAergic sleep switch in flies? To ask this we expressed a gene in GABAergic neurons that would decrease the ability of those neurons to generate action potentials. This would serve to turn down their activity. The result of this manipulation was that animals slept less and took longer to fall asleep.

This result was exciting to us since it meant that the basic neurochemical processes that underlie sleep in humans might be conserved in flies. In follow up studies that I will not show you, we determined that one of the main targets of these GABAergic sleep promoting neurons was a set of wake-promoting neurons in the circadian clock. The reciprocal connectivity of these circuit element strongly suggest that the organization of the sleep circuit in mammals derives from an ancient strategy.

In the intervening years, we have turned our attention to another important issue around sleep: how is the decision to sleep influenced by other internal and external states? Sleep is costly in terms of lost opportunities and if an animal is starving the need to find food has to eclipse the need to sleep. How does the brain do this integration? One common theme is to use neuromodulators, like peptide hormones, to tune circuits to reflect the animals internal state.

To address the question of the role of peptides in sleep, we carried out a genetic screen to look at the effects of activation of specific peptidergic circuits on sleep. Out of over 20 neuron types screened, the biggest effects by far came from activation of neurons containing sNFP. Using a temperature-activated transgene that causes neurons to fire, we showed that release of sNFP caused animals to fall immediately and profoundly asleep. This was a reversible state, since reducing the temperature to stop neuronal firing allowed animals to wake up and resume normal activity. Subsequent studies showed that this peptide targets many different behavioral circuits including those involved in feeding and memory formation. This discovery highlights the power of this system to provide insight into the molecular basis of behavior, insights that hopefully will lead to new ways to understand and treat human sleep disorders.

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Trabajos aportados por la nueva Académica de Honor

Reorganization of Sleep by Temperature in *Drosophila* Requires Light, the Homeostat, and the Circadian Clock

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SUMMARY

Increasing ambient temperature reorganizes the *Drosophila* sleep pattern in a way similar to the human response to heat, increasing daytime sleep while decreasing nighttime sleep. Mutation of core circadian genes blocks the immediate increase in daytime sleep, but not the heat-stimulated decrease in nighttime sleep, when animals are in a light:dark cycle. The ability of per^{01} flies to increase daytime sleep in light:dark can be rescued by expression of PER in either LNv or DN1p clock cells and does not require rescue of locomotor rhythms. Prolonged heat exposure engages the homeostat to maintain daytime sleep in the face of nighttime sleep loss. In constant darkness, all genotypes show an immediate decrease in sleep in response to temperature shift during the subjective day, implying that the absence of light input uncovers a clock-independent pro-arousal effect of increased temperature. Interestingly, the effects of temperature on nighttime sleep are blunted in constant darkness and in cry^{OUT} mutants in light:dark, suggesting that they are dependent on the presence of light the previous day. In contrast, flies of all genotypes kept in constant light sleep more at all times of day in response to high temperature, indicating that the presence of light can invert the normal nighttime response to increased temperature. The effect of temperature on sleep thus reflects coordinated regulation by light, the homeostat, and components of the clock, allowing animals to reorganize sleep patterns in response to high temperature with rough preservation of the total amount of sleep.

INTRODUCTION

Both internal states and environmental factors can affect sleep. Disruptions of sleep architecture in individuals suffering from circadian disorders and in blind individuals have been well documented [1, 2]. In general, circadian rhythms schedule wakefulness and activity at specific times of day depending on whether the animal is diurnal or nocturnal. Light has a dual role in the regulation of sleep-wake cycles. First, it is the major Zeitgeber (time giver) that entrains and synchronizes the circadian system. Second, light regulates locomotor activity. The latter effects of light are independent of the circadian system and are observed even in animals without a functional circadian clock [3]. Similar to light, temperature also acts as a Zeitgeber to the circadian system and has circadian-independent effects on sleep-wake cycles [4]. In contrast, homeostatic sleep drive promotes sleep as a function of accumulated wakefulness and, as such, dissipates during sleep [5]. The regulation of sleep-wake cycles by the balance between homeostatic, environmental, and circadian forces has important implications for human health.

The fruit fly, *Drosophila melanogaster*, is an ideal model for dissection of the relationships between genes, environment, and behaviors [6]. As in mammals, sleep in *Drosophila* is associated with reduced sensory responsiveness, is under circadian and homeostatic regulation [7, 8], and is associated with altered oscillatory brain activity [9–11]. Researchers have identified a number of genes [12], circuits [13], and biological processes [14, 15] that regulate fly sleep. However, the mechanisms underlying the regulation of *Drosophila* sleep by temperature have not been extensively investigated.

Temperature has been shown to have a plethora of potentially sleep-related effects in both flies and humans. In flies it has been shown to effect locomotor activity at mid-day, during siesta [16, 17] and sleep itself [18]. The level of splicing of the circadian clock gene *period* is associated with this modulation [19, 20]. In humans, increased ambient temperatures correlate with decreases in REM [21], and optimal temperatures have been suggested to improve nighttime sleep [22]. Interestingly, thermoregulation has also been shown to influence human sleep patterns [23, 24]; people suffering from insomnia have aberrant core body temperature at bedtime compared to normal sleepers [25]. Here, we show that in flies too sleep patterns are sensitive to temperature and investigate the processes that mediate this effect. We find that both the daytime and nighttime effects of an acute increase in temperature in normal light:dark (LD) conditions are gated by daytime light exposure, but only the effects of

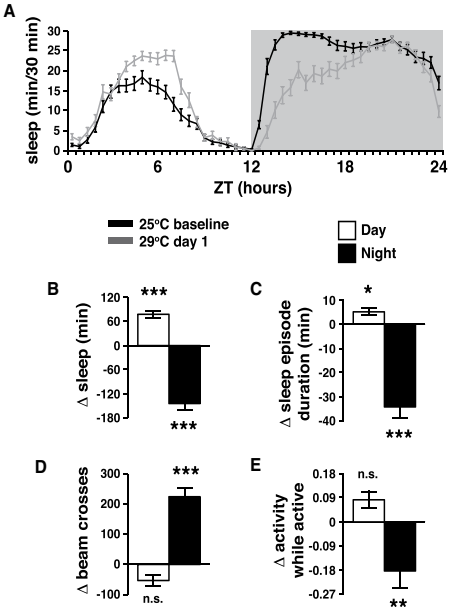

Figure 1. Temperature Shift Induces Sleep Restructuring in Wild-Type Flies

(A) Baseline sleep per 30 min comparing 25°C (black line) to 29°C (gray line) after a temperature shift at ZT0 in wild-type *Canton S* flies (n = 35).

(B–E) Changes from baseline in light and dark periods are shown for the following sleep parameters: (B) total sleep, (C) mean sleep episode duration, (D) total number of beam breaks, and (E) activity level while awake (beam breaks/min active). Data in (B)–(E) are presented as means ± SEM and 25°C compared to 29°C using type 2, two-tail Student's t test. *p < 0.05, **p < 0.005, ***p < 0.0005; n.s., not significant.

See also Figures S1 and S2A–S2D.

temperature on daytime sleep are regulated by circadian clock genes. Prolonged exposure to high temperature engages the homeostat to further shift sleep into the daytime. In constant light (LL) or constant darkness (DD), temperature has acute and chronic effects on sleep that are independent of the circadian clock.

RESULTS

Acute Increases in Temperature Alter Sleep Architecture and Distribution

Previous studies have shown that increasing ambient temperature from 25°C to 29°C affects circadian regulation of locomotor activity by decreasing daytime locomotion and increasing nighttime locomotion [16, 26]. This change was interpreted as arising solely from changes in clock regulation of motor function, and it was unknown how much of this activity change is due to changes in sleep. To evaluate the effect of temperature on sleep, we assayed the sleep pattern of wild-type *Canton S* female flies in a (12 hr:12 hr) light:dark cycle for 3 days at 25°C using beam break data (DAM system, Trikinetics), defining a sleep bout as a period of >5 min of inactivity [27]. On day 4, animals were shifted to 29°C at the start of the light period (ZT0). Figure 1A shows a comparison of sleep at 25°C to sleep on the first day after the shift to 29°C in wild-type flies. Daytime sleep increased on average by more than 1 hr in wild-type flies at 29°C, compared to baseline, while nighttime sleep is decreased by over 2 hr during the temperature elevation (Figure 1B), resulting in a rough preservation of total sleep after the temperature shift. Similar results were seen with males, though the magnitude of daytime changes was limited by the high baseline level of male daytime sleep (Figure S1).

Changes in the amount of sleep were accompanied by changes in its structure. Daytime sleep became more consolidated, with sleep episode duration increasing by 7 min (Figure 1C). Conversely, nighttime sleep became more fragmented with a significant decrease (over 30 min shorter) in mean episode duration (Figure 1C). These data suggest temperature is altering sleep structure as well as its day/night distribution. This supports the idea that temperature has a primary effect on sleep and that these effects may be upstream of temperature-dependent changes in locomotion. The chronic effects of this temperature shift (assessed on day 3 after the shift) were similar (Figures S2A–S2C).

Consistent with previous reports [16, 26], we observed a decrease in the number of daytime beam crosses at 29°C, and an increase in the number of beam crosses at night both immediately after the shift and after prolonged exposure to higher temperature (Figures 1D and S2D). To determine whether the observed changes in locomotor activity were due to hyperlocomotion or to differences in arousal, we measured the average level of activity exclusively during wake periods. We found that the intensity of locomotor activity was not significantly altered during daytime waking, but that it was significantly decreased during nighttime wake periods (Figures 1E and S2E). This is the opposite of what would be expected if increased temperature was simply causing hyperlocomotion, indicating that the observed changes in activity at night are due to arousal changes affecting sleep and not hyperlocomotion.

Acute Upregulation of Daytime Sleep, but Not Nighttime Sleep, by Heat Requires Core Clock Proteins

The apportioning of sleep between day and night is in part a function of the circadian clock. To determine whether temperature-dependent redistribution of sleep required a fully functional clock, we evaluated the effects of temperature shifts in circadian mutants by testing flies carrying loss of function alleles of the core clock genes *period* (per^{01}) and *timeless* (tim^{01}) and flies mutant for *cryptochrome* (cry^{OUT}), whose gene product conveys light information to the clock [28]. Baseline sleep was measured at 25°C in LD, and then animals were shifted to 29°C at the start of the light period (ZT0). In per^{01}, tim^{01}, and the $per^{01};tim^{01}$ double mutants, elevated temperature failed to elicit a significant increase in daytime sleep on the day after temperature shift (Figures 2A, 2B, 2D, and 2F), indicating that the molecular clock is required. In cry^{OUT} mutants, however, elevated temperature elicited a normal increase in daytime sleep on the day of the shift (Figures 2C and 2F) suggesting that CRY is not necessary for

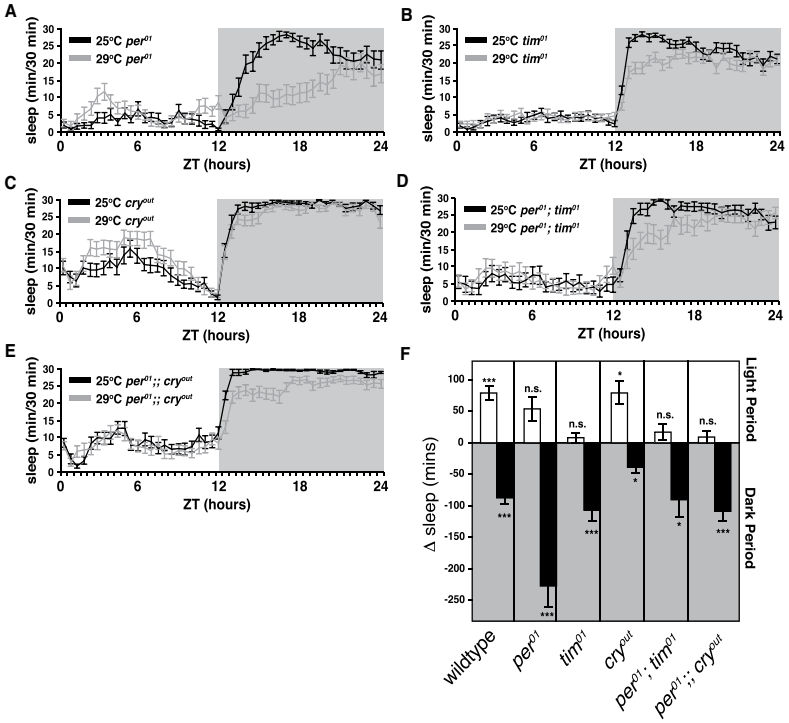

Figure 2. Temperature Shift at ZT0 Fails to Increase Siesta in Circadian Mutants

(A–E) Sleep plots for each genotype comparing 25°C baseline sleep (black line) to first day after a ZT0 29°C temperature shift (gray line).

(F) Changes in total daytime (white bars) and nighttime (black bars) sleep, 25°C versus 29°C, for wild-type and circadian mutants are shown. *Canton S* wild-type, n = 37; per^{01}, n = 20; tim^{01}, n = 33; per^{0};tim^{01}, n = 17; cry^{OUT}, n = 26; per^{0};cry^{OUT}, n = 28. Data are presented as means ± SEM and are compared within genotype (25°C versus 29°C) using Student's t test. *p < 0.05, **p < 0.005, ***p < 0.0005; n.s., not significant.

See also Figure S2F.

the daytime heat response. These results imply that temperature-dependent increases in daytime sleep require the molecular clock.

The effects of temperature on nighttime sleep were not reduced by mutations in *per* or *tim*, indicating that the ability of temperature to modulate nighttime sleep does not require a functional clock. Notably, however, mutations in *cry*, almost completely eliminated the nighttime decrease in sleep. This raises the possibility that light during the previous day may gate the ability of high temperature to inhibit nighttime sleep.

Interestingly, the inability of circadian clock mutants to mount a daytime increase in sleep is gone after longer exposure to high temperatures. Figure S2F shows data from the third day after ZT0 shift (daytime sleep is elevated on the second day as well; data not shown). This suggests that some other process is being rapidly engaged to compensate for these mutants' inability to mount an increase in daytime sleep. One possible scenario was that sleep loss during the night was stimulating a homeostatic increase in the following day's sleep. This would result in homeostatically generated rebound sleep masking the circadian mutant phenotype after the first day of exposure.

Heat-Induced Suppression of Nighttime Sleep Can Induce Homeostatic Rebound Sleep the following Day

To test the idea that homeostatic processes compensate for loss of clock-dependent daytime sleep, we needed to first determine whether heat-induced nighttime sleep loss could drive daytime rebound sleep. We shifted wild-type flies to 29°C at ZT12 at the beginning of the dark period to assess both the acute effect of high temperature on nighttime sleep and the effect on the subsequent day's sleep. Wild-type flies shifted to 29°C at the beginning of the dark period displayed significantly reduced nighttime sleep followed by an increase in sleep the following day that was independent of ambient temperature during the rebound period (Figures 3A and S3). The decrease in nighttime sleep after a ZT12 shift implies that the effect of temperature on nighttime sleep is

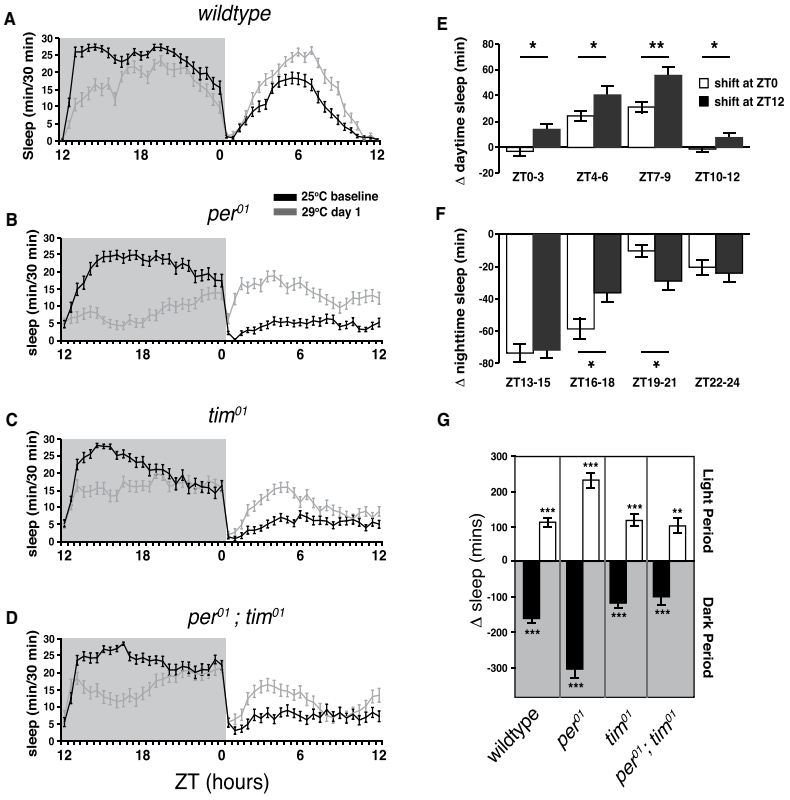

Figure 3. Homeostatic Rebound from Temperature-Induced Sleep Loss is Intact in Circadian Mutants

(A–D) Sleep plots comparing sleep at baseline (25°C black line) to sleep after a shift to 29°C at ZT12 (gray line) for wild-type and mutant animals. Shaded background area indicates dark period, ZT12-ZT24.

(E) Comparison of sleep changes in 3-hr bins during the day for wild-type animals experiencing a ZT0 shift (white bars, data from Figure 1) or a ZT12 shift (black bars). Data are presented as means ± SEM and ZT0 compared to ZT12 for each bin using Student's t test.

(F) Comparison of sleep changes in 3-hr bins during the night as in (E).

(G) Changes in total daytime (white bars) and nighttime (black bars) sleep after a 25°C to 29°C temperature shift at ZT12 for wild-type and circadian mutants are shown.

Data are presented as means ± SEM and are compared within genotype (25°C versus 29°C) using Student's t test. *Canton S* wild-type, n = 35; $per^{0,1}$, n = 40; tim^{01}, n = 44; per^{0};tim^{01}, n = 37. *p < 0.05, **p < 0.005, ***p < 0.0005; n.s., not significant.

See also Figures S3 and S4.

direct and does not require increased temperature during the previous light period. As shown for the ZT0 shift in Figure 1, changes in the distribution of sleep were accompanied by changes in sleep structure; mean episode duration increased during the day and decreased at night (Figure S4A). Locomotor activity during daytime waking periods was unchanged and actually decreased at night, suggesting that, as with ZT0 shift, the overall changes in activity were a result of changes in arousal and not changes in locomotion (Figure S4B).

Consistent with homeostasis having a large role in the generation of excess daytime sleep when temperature elevation is prolonged, the change in daytime sleep during the first 24 hr after a shift was larger when the 29°C regimen was started at ZT12 (the beginning of the dark period) than if it was started at ZT0 (53% increase for the ZT12 shift compared to 37% for the ZT0 shift p = 0.02 Student's t test). Visual comparison of Figures 1A and 3A suggests that, in addition to the difference in the amount of extra sleep, the distribution across the day induced by the temperature shift is different depending on when the shift occurred. To examine this quantitatively, we calculated the change in sleep (baseline 25°C versus 29°C) for 3-hr windows across the day (Figures 3E and 3F). Indeed, shifting flies at the

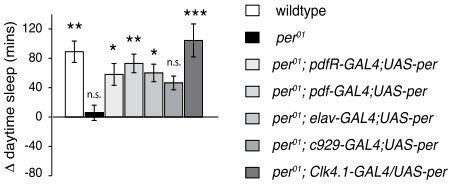

Figure 4. PER Expression in LNvs or DN1 Circadian Cells Is Sufficient for Recovery of Heat-Induced Siesta

Change in total daytime sleep after a shift from 25°C to 29°C at ZT0 is shown. GAL4 control lines (all on a per^{01} background) were compared to experimental rescue lines containing the same GAL4 and *UAS-per16* transgenes during the light period. Data are presented as means ± SEM and analyzed using type 2, two-tail, Student's t test. *p < 0.05, **p < 0.005, ***p < 0.0005; n.s., not significant. *Canton S* wild-type, n = 24; per^{01}, n = 49; per^{01};*pdfR2-GAL4/+;UAS-per16*, n = 26; per^{01};*pdf-GAL4/+; UAS-per16/+*, n = 25; per^{01};*elav-GAL4/+; UAS-per16/+*, n = 25; per^{01};*c929-GAL4/+; UAS-per16/+*, n = 22; per^{01}; *Clk4.1GAL4/UAS-per16*, n = 17 (male flies were assayed for this genotype). See also Figure S5.

start of the light period (ZT0) does not produce an increase in sleep in the ZT0–ZT3 window, whereas shifting flies the previous night (at ZT12) induces an immediate increase in total sleep in the ZT0–ZT3 period the following day. Importantly, rebound sleep has been shown to occur most profoundly in the beginning of the day [7]. This implies that the overall higher levels of daytime sleep observed in wild-type animals at 29°C in the first day after a ZT12 shift are due to a combination of sleep rebound caused by the negative effect of high temperature on the previous nighttime sleep plus a direct effect of temperature on late daytime sleep. A further implication is that in wild-type flies, when temperature is chronically elevated, the increase seen in daytime sleep will come from two separate processes: rebound sleep from nighttime sleep loss and a direct, clock-dependent, effect of temperature on daytime sleep. In contrast, the magnitude and temporal distribution of changes in nighttime sleep are almost identical after ZT0 and ZT12 shifts during both ZT13–ZT15 and ZT22–ZT24 windows (Figure 3F), suggesting little homeostatic component to nighttime sleep changes.

Homeostatic Changes in Sleep after Temperature Shift Are Intact in Clock Mutants

Since daytime sleep was relatively unchanged by a ZT0 shift in per^{01} and tim^{01} mutants, we were interested in determining whether they could mount a homeostatically driven increase in daytime sleep after a ZT12 shift or whether they were refractory to all sleep-inducing processes during the light period. We found that these circadian mutants were able to increase sleep during the subsequent light period after a ZT12 shift (Figures 3B–3D), consistent with a previous study showing that per^{01} mutants were able to generate homeostatic sleep after mechanical sleep deprivation [29]. This suggests that while temperature alone cannot induce an increase in daytime sleep in the absence of a PER or TIM in LD, homeostatic drive is still able to increase sleep during the light period. The direct effects of homeostatic rebound and temperature are therefore mechanistically separate, with only the latter requiring these clock proteins. This strongly supports the notion that the recovery of daytime sleep in mutants on the second and third days after a ZT0 shift is due to engagement of the homeostat. Notably, nighttime sleep was decreased in all genotypes regardless of when the shift occurred (Figure 3G).

Table 1. Expression Patterns and Rhythm Effects of GAL4 Lines Used for *UAS-per* Rescue of Daytime Sleep

	Expression Pattern		
GAL4 Line	Clock Cell Expression	Non-clock Expression	Rhythmicity in DD
elav-GAL4	all [31]	panneuronal [31]	yes [32, 33]
pdf-GAL4	all sLNvs and lLNvs [34]	none [34]	yes [35]
pdfR-GAL4	subsets of all clock groups including sLNvs and DN1ps [36]	ellipsoid body, optic lobes [36]	unknown
c929-GAL4	lLNvs [37]	peptidergic neurons [37]	no [35]
Clk4.1-GAL4	subset of DN1ps [38]	none [38]	no [39]

For expression patterns, the reference to the original description of the line is listed. For locomotor rhythmicity in DD, results from papers containing data on rescue of per^{01} with *UAS-per* are described along with the reference. There are no published data on rescue of DD rhythms for *pdfR-GAL4>UAS-per*.

PER Expression in sLNv or DN1 Cells of the Clock Circuit Is Sufficient to Allow Heat-Induced Increases in Daytime Sleep

To determine the site of action of PER in generating temperature-dependent increases in daytime sleep, we expressed *per* in specific cell types in per^{01} flies using the GAL4/UAS system [30]. We assayed sleep in LD before and after a ZT0 25°C to 29°C shift. Both *UAS-per* and each individual GAL4 on the per^{01} mutant background were assessed as controls and daytime sleep change in these lines was not significantly different from the per^{01} mutant alone (Figure S5A). Figure 4 shows that daytime heat response is rescued to a statistically significant level when PER is expressed panneuronally (*elav-GAL4*) or under control of *pdf-GAL4*, *pdfR-GAL4*, or *clk4.1-GAL4*. All lines, controls and experimental, showed a decrease in nighttime sleep when shifted to 29°C in LD (Figures S5B and S5C).

The GAL4 lines used for rescue and their expression patterns are listed in Table 1. Interestingly, there is no single neuron type that rescues daytime sleep regulation by temperature in LD. The best rescue is with *clk4.1-GAL4*, which has very limited expression, only labeling a subset of dorsal clock neurons, the DN1ps [38]. DN1ps have previously been implicated in integration of temperature and light signals [39–41] and are also present in *elav-GAL4* and *pdfR-GAL4*. The three lines other than *clk4.1-GAL4* that rescue (*elav-GAL4*, *pdf-GAL4*, and *pdfR-GAL4*) have overlapping expression only in the small and large ventrolateral clock neurons (sLNvs and lLNvs). *c929-GAL4*, which has a statistically insignificant increase in daytime sleep, expresses in lLNvs, but not sLNvs, suggesting that sLNvs are more important for the circuitry that allows heat to increase daytime sleep in LD. These results imply that the presence of a

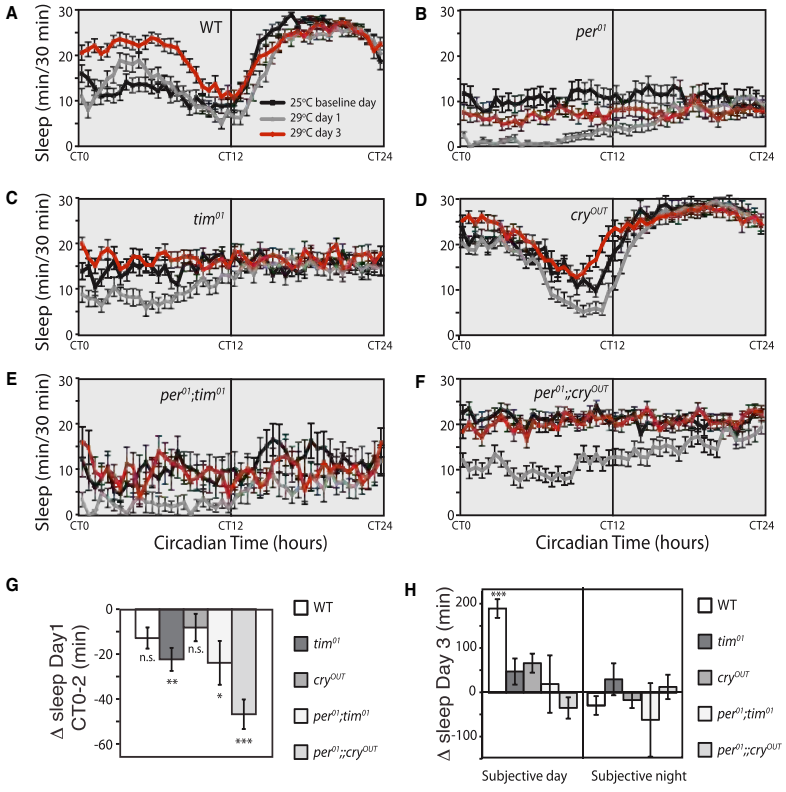

Figure 5. Temperature Shifts in Constant Darkness

Animals were entrained in LD at 25°C and released into constant darkness (DD) for 3 days before temperature was increased to 29°C at CT0.

(A–F) Sleep plots for the 25°C baseline (black line), day 1 after 29°C shift (gray line) and day 3 after 29°C shift (red line) are shown for the indicated genotypes. Day 1 shows acute effects including entrainment effects, while day 3 shows steady state, after entrainment artifacts have resolved. *Canton S* wild-type, n = 38; per^{01}, n = 42; tim^{01}, n = 25; per^{0};tim^{01}, n = 13; cry^{OUT}, n = 28; per^{0};cry^{OUT}, n = 38. Data for per^{01} are from an independent experiment run on a different day and included to show qualitative changes are similar.

(G) Acute change in sleep between CT0 and CT2 for indicated genotypes run concurrently. 25°C baseline is compared to day 1 at 29°C.

(H) Chronic change in sleep for indicated genotypes run concurrently. 25°C baseline is compared to day 3 at 29°C.

All comparisons were done using Student's t test, and data are presented as mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005; n.s., not significant. In (H), only wild-type showed a significant change in sleep; all others were n.s.

functional clock in either sLNv or DN1 is sufficient to allow temperature to increase daytime sleep.

Notably, locomotor rhythms are not rescued in all lines that rescue the ability of heat to increase daytime sleep (Table 1). This suggests that the role of PER in regulation of temperature responses of daytime sleep is not due to its effects on locomotor rhythms, but rather reflects a separate role for the molecular clock, and for specific neurons of the clock circuit, in regulation of sleep by temperature. The finding that both sLNv and DN1p expression can rescue could suggest redundancy, but may reflect reciprocal connectivity between these cell types; cycling in LNvs likely drives cycling in DN1ps and vice versa (see the Discussion).

Light Regulates the Ability of Heat to Alter Sleep

The fact that the major difference between wild-type and clock mutants is their ability to respond to heat during the light period suggested that light itself may have a role in regulating temperature-dependent changes in sleep structure and distribution. To look at the role of light, we tested wild-type and mutant flies in constant darkness (DD) or constant light (LL). Animals were entrained at 25°C in LD for 3 days and then released into constant conditions. On the third day of DD or LL, the temperature was increased to 29°C at CT0, and data were collected for an additional 3 days. Figures 5A–5F (DD) and 6A–6F (LL) show sleep plots demonstrating baseline sleep (25°C, black lines),

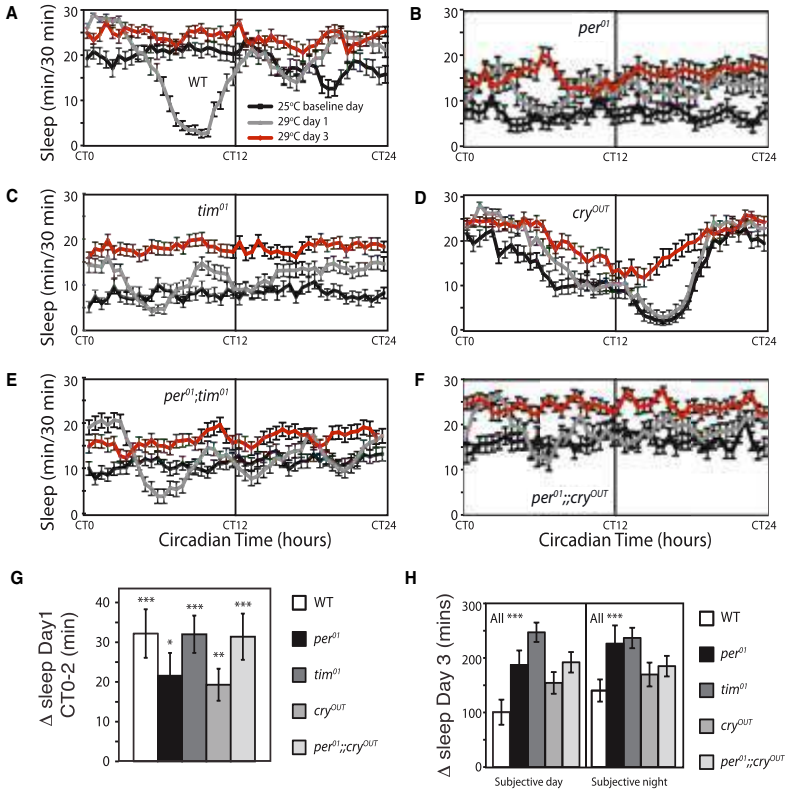

Figure 6. Temperature Shifts in Constant Light

Animals were entrained in LD at 25°C and released into constant light (LL) for 3 days before temperature was increased to 29°C at CT0.

(A–F) Sleep plots for the 25°C baseline (black line), day 1 after 29°C shift (gray line) and day 3 after 29°C shift (red line) are shown for the indicated genotypes. Day 1 shows acute effects including entrainment effects, while day 3 shows steady state, after entrainment artifacts have resolved. *Canton S* wild-type, n = 39; per^{01}, n = 34; tim^{01}, n = 42; per^{0};tim^{01}, n = 65; cry^{OUT}, n = 37; per^{0};cry^{OUT}, n = 37. Data for per^{0};tim^{01} are from an independent experiment run on a different day and included to show qualitative changes are similar.

(G) Acute change in sleep between CT0 and CT2 for indicated genotypes run concurrently. 25°C baseline is compared to day 1 at 29°C.

(H) Chronic change in sleep for indicated genotypes run concurrently. 25°C baseline is compared to day 3 at 29°C.

All comparisons were done using Student's t test, and data are presented as mean ± SEM*p < 0.05, **p < 0.005, ***p < 0.0005; n.s., not significant. In (H), all genotypes were ***.

immediate temperature effects (first day after shift to 29°C, gray lines), and steady-state temperature effects (third day after shift to 29°C, red lines). Because a temperature shift presents a potential entrainment signal, the acute effects of the shift in constant light conditions are more complex than in LD where cycling light is the dominant entrainment signal. Animals that are arrhythmic in DD or LL show a single cycle change in activity but eventually revert to arrhythmicity in the face of constantly elevated temperature. For this reason, we examined both the immediate and chronic effects of temperature for all experiments in which light was held constant. The acute window was very short (CT0–CT2) in order to avoid the locomotor artifacts produced by entrainment processes.

In contrast to the increase in daytime sleep seen in LD, wild-type animals in DD show a small immediate decrease in sleep after a CT0 shift. This is quantified in Figure 5G for the first 2 hr following the shift (CT0–CT2). Sleep during subjective night is still decreased, similar to LD. This suggests that, in the absence of light, the default acute response to heat is a decrease in sleep. Does this default pathway require clock proteins? Clock mutants assayed in DD also exhibited an immediate decrease in sleep at the start of a CT0 heat treatment (Figure 5G). Clockless flies

therefore also responded to an acute increase in heat during the subjective day with a "night-like" sleep suppression.

We also examined the effects of temperature at steady state by comparing sleep from baseline to the third day after the CT0 shift (Figure 5H). Notably, the effects of temperature are relatively minor in the absence of light. For circadian mutants that are arrhythmic in DD, sleep in both the subjective day and night either did not change or was reduced. Only flies with intact *per* and *tim* genes (wild-type and cry^{OUT}) were able to mount increases in sleep in the subjective day in the absence of light. The significant decrease in the ability of heat to cause changes in sleep during the subjective night further supports the idea that, in LD, light during the preceding day enhances the ability of heat to decrease sleep at night.

Heat Increases Sleep in Constant Light

We next wanted to assess the effects of constant light on the ability of temperature to regulate sleep. This manipulation renders wild-type flies arrhythmic since the clock is essentially "frozen" in a ZT12-like state. TIM levels are low and do not cycle [42], while PER in heads continues to cycle for at least 2 days [43]. Flies lacking CRY, however, remain rhythmic in LL [44, 45]. To examine the role of these proteins, we assayed the effects of LL temperature shift in per^{01}, tim^{01}, and cry^{OUT} flies. Flies were shifted to 29°C on the third day of LL. The immediate effect of temperature shift on wild-type and mutant flies was to increase sleep (Figures 6A–6F). Figure 6H quantifies the difference in the first 2 hr after the temperature increases (CT0–CT2). This window was chosen to capture direct effects of temperature before the entraining effects of the shift (which are notable as changes in locomotion on the day of the shift). At steady state on the third day after the CT0 shift, when there is no longer an entrainment artifact, all genotypes show large increases in sleep in both the subjective day and night (Figure 6H). These changes are independent of a functioning clock, since they occur in wild-type flies, and do not require circadian genes.

Temperature Effects on Sleep Architecture Reflect an Integration of Environment and Internal State

These data support the idea that there are three separate processes regulating temperature-dependent changes in sleep and its architecture (Figure S6). The first is a default pathway that acutely decreases sleep in response to increased temperature. This pathway does not require the molecular clock and is used in wild-type flies only in the dark. This process is enhanced at night by light during the preceding day, and this hysteresis serves to link nighttime behavior to conditions present the day before. The second process is light dependent and can suppress the effects of the default pathway and actively promote sleep during the day. This requires *per* and *tim* and is mediated by an LNv-DN1p subcircuit of the clock. The third player in the heat response is only important when temperature is chronically elevated. Loss of nighttime sleep activates the homeostat to generate rebound sleep the next day. This serves as a second mechanism for linking changes in daytime and nighttime sleep. The sensors required for heat effects are unknown, but recent studies have suggested that both dTrpA1 and Ir25a are important in communication of temperature information to clock and activity circuits [46, 47].

DISCUSSION

Sleeping at the right time of day is paramount to species survival. At 25°C in LD, wild-type flies display a midday siesta that peaks at ZT6, and they obtain their most consolidated sleep at night. We find that increased temperature reorganizes the distribution of sleep and that the effects of temperature shifts on daytime and nighttime sleep are mechanistically different. At 29°C, flies sleep less at night and more during the daytime. For a poikilothermic organism, environmental temperatures acutely affect physiology; there may be less chance of desiccation if activity is shifted to the nighttime during warm periods. Sleep patterns in homeotherms are also sensitive to environmental conditions; both sleep latency and episode duration are altered by heat [24].

Our results indicate that the main sleep parameter altered by temperature shift is sleep episode duration (Figures 1C, S2C, and S4A). Drawing parallels to human sleep behavior is enticing; we can all relate to the predicament of having difficulties in falling and staying asleep on hot summer nights. It is also common in tropical climates for local populations to partake in midday siesta at the hottest part of the day.

Acute Regulation of Daytime Sleep by Temperature in LD Requires PER in a Subset of Circadian Clock Cells

In LD, animals with a functional clock (wild-type and cry^{OUT}) increase daytime sleep in response to an acute increase in temperature. In contrast, mutants lacking either of the core circadian transcription factor proteins PER or TIM are unable to mount an immediate daytime increase in sleep with temperature. To determine whether the phenotype of these mutants was due to their role in the clock, we rescued per^{01} by expression of *UAS-per* in different neuronal populations (Figure 4). We found that immediate heat-induced daytime sleep was restored by expressing PER in either sLNv or DN1p neurons of the clock circuit, indicating that an intact clock in either of these neuron types is sufficient.

The implication of these data is that there is a reciprocally connected sLNv-DN1p subcircuit within the circadian clock circuit controlling the daytime sleep response to temperature. The DN1ps, or the neurons they target, have been shown to be under both photic and thermal regulation-integrating light and temperature inputs to control a variety of behaviors [38, 39, 41, 48]. Significantly, sLNvs have been shown to regulate the activity of DN1ps via release of the neuropeptide PDF [38, 39, 49], and the speeds of the clocks in these two cell types appear to be tethered [32]. The fact that PER expression in either of these cell types is sufficient to rescue daytime sleep effects suggests their functional interaction is a critical feature of sleep regulation by temperature. The ability of this subcircuit to allow temperature regulation of daytime sleep in the absence of locomotor rhythms (Table 1) indicates that sleep is an independent output of the clock rather than a downstream consequence of clock-driven locomotor activity.

Increases in Daytime Sleep after Prolonged Temperature Elevation Are Due to a Combination of Clock-Driven and Homeostatic Processes

While circadian mutants were unable to generate excess daytime sleep immediately after a temperature shift, they were

indistinguishable from wild-type by the second day of high temperature exposure. This is due to the ability of these mutants to generate normal rebound sleep after heat-dependent nighttime sleep loss (Figures 3 and S3). This implies that, in wild-type animals, the ability of increased temperature to stimulate daytime sleep relies on two mechanistically separable processes: a clock-gated temperature sensing subcircuit and homeostatic rebound sleep stimulated by heat-dependent nighttime sleep loss (Figure S6).

Regulation of Nighttime Sleep by Temperature in LD Requires Light and CRY

Nighttime sleep is directly decreased by high temperature, and, while this decrease does not require a functioning clock, it is significantly influenced by light during the preceding day. This can be seen by comparison of wild-type nighttime sleep changes in LD and DD conditions (Figures 2F and 5H). This permissive effect of daytime light for suppression of nighttime sleep appears to be partially mediated by CRY, since animals carrying a *cry* null allele have a blunted heat response during the night in LD: both cry^{OUT} mutants and $per^{01};cry^{OUT}$ double mutants have a decreased heat-dependent suppression of nighttime sleep compared to wild-type and per^{01} alone (Figure 2F). This argues that CRY and daytime light are important to setting the amount of sleep the following night or its sensitivity to temperature. The importance of light for sculpting nighttime sleep has been seen previously in the response of animals to activation of dopaminergic pathways [50]. In this study, light was shown to induce expression of an inhibitory dopamine receptor that acted to blunt the wake-promoting effects of dopamine on nighttime sleep. Cycling light conditions can therefore produce a hysteresis that links nighttime sleep to conditions during the preceding day, coordinating the effects of temperature over the course of the 24-hr day (Figure S6). These effects would be predicted to be absent in constant darkness.

Regulation of Sleep by Temperature in Non-cycling Light Conditions Is Clock Independent

Because cycling light clearly had effects that influenced nighttime sleep, it was important to look at constant conditions to identify the underlying processes invoked by light. In the absence of light (DD), an acute increase in temperature immediately decreased sleep regardless of circadian genotype (Figure 5). In wild-type and cry^{OUT} flies, which have an intact clock, this reduction was transient and sleep levels increased within a few hours to a level higher than that seen at 25°C similar to what is seen during the light period in LD. For circadian mutants, the decrease lasted about 12 hr and likely reflects the ability of a temperature shift to produce a single cycle of "entrained" behavior with low subjective daytime sleep and higher subjective nighttime sleep.

Prolonged elevation of temperature in flies with a cycling clock in DD produced effects similar to those seen in LD: slightly higher daytime sleep and slightly lower nighttime sleep with rough preservation of total sleep. Mutants that do not have a clock kept chronically in DD at 29°C had total sleep levels similar to levels at 25°C. This could reflect a homeostatic response to the sleep disrupting effects of heat, or a rundown of the wake-promoting process, perhaps due to the lack of cycling light. The ability of these genotypes to show pronounced decreases in nighttime sleep in LD further supports a role for light during the preceding day in promoting the effects of heat in the dark.

While light at night is an arousal signal for most diurnal animals, maintaining animals in constant light (LL) has a paradoxical sleep-promoting effect for temperature shifts in all genotypes tested. This increase in sleep is seen both acutely, in the first few hours after the temperature increase (Figure 6G), and chronically, several days after the increase in temperature (Figure 6H). The effect of heat in the long term is essentially identical during the subjective day and subjective night in LL. Interestingly, this sleep-promoting effect of light does not require a clock. While similar to what happens during the day in LD, the heat-dependent increase in sleep in LL must be mechanistically distinct. Constant light is known to affect the abundance of a number of clock-related proteins (notably CRY and TIM), and it is possible that differences in the steady-state levels of these proteins account for the differences in clock requirement, though this remains to be tested.

Our data indicate that the mechanisms by which environmental (light and temperature) and internal (circadian clock and homeostatic drive) pressures shape sleep structure are highly coordinated. The fact that temperature exerts effects that are gated by light and modulated by the molecular clock suggests that there is a hierarchical organization to sleep circuits. Using *Drosophila*, a model in which many of these processes are well defined both genetically and at the circuit level to study such problems will allow us to untangle these complex relationships and achieve a more complete understanding of sleep regulation.

EXPERIMENTAL PROCEDURES

Strains and Fly Rearing

Fly cultures were kept at 25°C in 12-hr:12-hr light/dark conditions (LD) on cornmeal, yeast, sucrose, and agar food. *period* mutants were obtained from both Toshi Kitamoto's laboratory (University of Iowa) and Michael Rosbash's Laboratory (Brandeis University). *timeless* and *cryptochrome* mutants were from Jeff Hall's laboratory (Brandeis University). *UAS-per16* was generously provided by Patrick Emery (University of Massachusetts Medical School). GAL4 lines were obtained from Michael Rosbash and then put onto a per^{01} background.

Behavioral Assays

Flies were collected under CO_2 anesthesia 1- to 2-day-old post-eclosion (to allow time for mating of the females), placed in food vials in sets of 30–50 flies each, and placed in behavioral incubator for 2 days of LD. Female flies were used for all experiments except where noted. Flies were loaded into sleep tubes and entrained for 3 days in LD before proceeding into a given experiment (see below). Behavioral assays and analysis were carried out as previously described [36]. For all experiments, flies were placed individually in glass locomotor-monitoring tubes with standard media (5% sucrose, 2% agar). Locomotor activity was monitored in 1-min bins using the *Drosophila* Activity Monitoring System (TriKinetics). Sleep was defined as 5 consecutive minutes of inactivity. Sleep parameters were calculated and graphed using MATLAB software [27]. In all experiments (except where noted in Figures 5 and 6) in which different genotypes are compared, data are from flies collected and run concurrently. Activity while active (Figures 1E, S2, and S4B) is a measure of the intensity of locomotor activity and is independent of sleep. It is calculated by measuring the number of beam breaks during periods during which the fly is awake. Hyperlocomotor flies would be expected to show an increase in this metric, while hypoactive flies would show a decrease.

Temperature Shifts

Flies was assayed under LD conditions for 3 days at 25°C (to obtain a stable baseline), and then the incubator was set to 29°C for 2–3 days starting at either the beginning of the dark period (ZT12), or at the beginning of the light period (ZT0).

Light Affects

Flies were entrained for 3 days in LD at 25°C and subsequently transferred to constant darkness, remaining at 25°C for 3 days of baseline. After the third full day of DD at CT0, the incubator was shifted to 29°C for 2–3 days. Likewise, for constant light, flies were entrained for 3 days in LD at 25°C and subsequently transferred to constant light, remaining at 25°C for 3 days of baseline. On the third full day of LL at CT0, the incubator was shifted to 29°C for 2–3 days.

Statistical Analysis

Data were analyzed as described in figure legends using JMP software v.5.0.1.2 for the PC and Macintosh (SAS Institute) and using Microsoft Office Excel.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.02.011.

AUTHOR CONTRIBUTIONS

Conceptualization, K.M.P. and J.L.A.R.; Investigation, K.M.P., J.L.A.R., N.C.D., and S.K.; Writing – Original Draft, K.M.P.; Writing – Review & Editing, K.M.P., J.L.A.R., N.C.D., and L.C.G.; Supervision, L.C.G.; Funding Acquisition, L.C.G.

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A single pair of neurons links sleep to memory consolidation in *Drosophila melanogaster*

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Abstract Sleep promotes memory consolidation in humans and many other species, but the physiological and anatomical relationships between sleep and memory remain unclear. Here, we show the dorsal paired medial (DPM) neurons, which are required for memory consolidation in *Drosophila*, are sleep-promoting inhibitory neurons. DPMs increase sleep via release of GABA onto wake-promoting mushroom body (MB) α'/β' neurons. Functional imaging demonstrates that DPM activation evokes robust increases in chloride in MB neurons, but is unable to cause detectable increases in calcium or cAMP. Downregulation of α'/β' $GABA_A$ and $GABA_BR3$ receptors results in sleep loss, suggesting these receptors are the sleep-relevant targets of DPM-mediated inhibition. Regulation of sleep by neurons necessary for consolidation suggests that these brain processes may be functionally interrelated via their shared anatomy. These findings have important implications for the mechanistic relationship between sleep and memory consolidation, arguing for a significant role of inhibitory neurotransmission in regulating these processes.
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Introduction

Accumulating evidence suggests that sleep plays a role in promoting the consolidation of memory (***Stickgold, 2005***; ***Diekelmann and Born, 2010***; ***Mednick et al., 2011***; ***Abel et al., 2013***; ***Rasch and Born, 2013***). Sleep deprivation following an associative learning task impairs consolidated memory in *Drosophila*, rodents, and humans whereas sleep immediately after a learning task actually improves consolidated memory across the same broad range of organisms (***Ganguly-Fitzgerald et al., 2006***; ***Donlea et al., 2011***; ***Rasch and Born, 2013***; ***Diekelmann, 2014***). It is not, however, clear exactly how sleep promotes memory consolidation: it may simply be a permissive state generated by other brain regions that prevents sensory interference with memory circuits, or alternatively the memory circuitry itself may actively participate in sleep promotion as an integral aspect of the consolidation process. To begin to probe these issues, we have investigated the role of the dorsal paired medial (DPM) neurons, which are critical to memory consolidation in *Drosophila melanogaster*, in the regulation of sleep.

The *Drosophila* learning and memory circuitry has been well characterized and provides an excellent system in which to study cellular interactions between sleep and memory consolidation. The mushroom bodies (MBs) are a set of ca. 5000 neurons in the *Drosophila* brain, organized into five distinct lobular neuropils, which are required for odor memory acquisition, consolidation, and retrieval. Although the anatomy involved in memory consolidation in mammals is highly complex and distributed, in the fly it is quite compact: the DPM neurons, a single pair of neurons innervating all of the MB lobes, are the mediators of consolidation for odor memories (***Waddell et al., 2000***; ***Keene et al., 2004***, ***2006***; ***Yu et al., 2005***; ***Krashes and Waddell, 2008***). Like mammals, *Drosophila* consolidates memories at the systems level. Critical memory information is transferred from short-term storage in neurons required for initial acquisition to anatomically and physiologically distinct long-term storage

eLife digest Sleep affects memory: if you do not sleep well after a learning task, chances are you will not be able to recall whatever you tried to learn earlier. This is seen in almost all animals ranging from the fruit fly *Drosophila,* to mice and humans. However, the precise details of how memory and sleep are connected remain unclear.

Drosophila is an excellent model for teasing out the connections between memory and sleep. This is because its brain has a simple and well-studied memory region that contains a pair of nerve cells called the dorsal paired medial neurons. These neurons enable memories to be stored for the long term. Here, Haynes et al. asked whether these neurons can also affect sleep, and if so, how.

The experiments show that the dorsal paired medial neurons promote sleep in fruit flies. The neurons release a signaling molecule called GABA, which is detected by a type of neighboring 'mushroom body' neuron that usually promotes wakefulness. This leads to increases in the levels of chloride ions in the mushroom body neurons, but no change in the levels of calcium ions and a molecule called cAMP, which indicates that GABA inhibits these cells. Flies that have lower levels of two receptor proteins that detect GABA sleep less than normal flies.

Haynes et al.'s findings suggest that dorsal paired medial neurons deactivate their neighbors to promote sleep in fruit flies. This result was unexpected because current models of memory formation propose that dorsal paired medial neurons can activate the mushroom body neurons. Understanding how inhibiting mushroom body neurons influences memory will require researchers to reassess these models.

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sites (***Yu et al., 2005***; ***Krashes et al., 2007***; ***Wang et al., 2008***; ***Cervantes-Sandoval et al., 2013***; ***Dubnau and Chiang, 2013***). DPM neurons, along with the α′/β′ subset of MB neurons, are required for early phases of this memory information transfer (***Keene et al., 2004***, ***2006***; ***Krashes et al., 2007***; ***Krashes and Waddell, 2008***).

The MB memory circuit has also been implicated in the regulation of sleep by a number of studies (***Joiner et al., 2006***; ***Pitman et al., 2006***; ***Yuan et al., 2006***; ***Yi et al., 2013***). Loss of MB $5HT_{1A}$ receptors (***Yuan et al., 2006***) as well as alterations in MB PKA activity (***Joiner et al., 2006***) and neurotransmitter release (***Pitman et al., 2006***) have been shown to affect sleep in *Drosophila* in a lobe-specific manner. Mutation of the *amnesiac (amn)* gene, which encodes a putative neuropeptide expressed in DPM neurons (***Waddell et al., 2000***), results in fragmented sleep and impaired sleep rebound following deprivation, suggesting a role for these cells (***Liu et al., 2008***). While the molecular and cellular requirements for sleep and memory clearly overlap, whether the circuit that regulates sleep is identical to that required for memory is not clear and this is a question that bears directly on the functional interrelationship between sleep and memory consolidation.

The primary question addressed in this study is the role of the DPM neurons and their outputs in regulation of sleep. The DPM contribution to memory consolidation had been suggested to occur due to the release of acetylcholine (ACh) (***Keene et al., 2004***) and the product of the *amn* gene (***Waddell et al., 2000***) enhancing MB potentiation via an excitatory feedback loop (***Yu et al., 2005***; ***Keene and Waddell, 2007***) similar to what has been proposed to occur in the mammalian hippocampus (***Hebb, 1949***; ***Hopfield, 1982***; ***Amit, 1989***; ***Treves and Rolls, 1994***; ***Battaglia and Treves, 1998***; ***Lisman, 1999***). Recently, however, DPM release of serotonin (5HT) has been shown to promote anesthesia resistant memory, a form of consolidated memory, by acting on $G\alpha_i$-coupled $5HT_{1A}$ receptors in the α/β lobes of the MBs (***Lee et al., 2011***). The involvement of a potentially inhibitory receptor, $5HT_{1A}$, in consolidation suggests that a simple positive feedback model for consolidation is unlikely to be completely correct, and it highlights the fact that there is currently no information on the functional nature of the synapses between DPM neurons and MBs. An understanding of this synapse is critical for elucidating DPM's role in sleep. To address this aspect of DPM function, we have investigated the nature of their connection to the MBs.

Here, we show that the DPM neurons promote sleep via the release of 5HT and the inhibitory neurotransmitter GABA. We find that DPM activation results in inhibitory chloride influx into post-synaptic MB neurons and find no evidence that DPM neuron activation has an excitatory effect on post-synaptic MB neurons. We suggest a model in which post-synaptic MB α′/β′ neurons are wake-promoting, and

inhibition by DPM neuron GABA and 5HT release during memory consolidation results in increased sleep. These findings provide new insight into the functional relationship between sleep and memory consolidation, and suggest an important role for inhibitory neurotransmission in regulating these processes.

Results

DPM activity promotes sleep

In order to determine whether DPMs play a role in regulating sleep, we acutely activated these neurons by driving the warmth-sensitive cation channel, dTrpA1 (***Hamada et al., 2008***) with *NP2721-GAL4*, a driver with relatively specific and strong DPM expression (***Figure 1—figure supplement 1***). A temperature shift at ZT0 from 22°C, a temperature at which dTrpA1 is inactive, to 31°C, where it is open and can depolarize DPMs, produced an immediate and dramatic increase in sleep (***Figure 1A***). dTrpA1 activation with a weaker, but even more specific DPM driver line, *VT64246-GAL4*, also resulted in immediate and significant increases in sleep (***Figure 1—figure supplement 2A,C***) indicating the effect is due to activation of DPM neurons and not other neurons in the *NP2721-GAL4* expression pattern. Activating DPMs with dTrpA1 did not alter the level of locomotor activity during waking periods (light period of first day: $P_{GAL4} = 0.57$, $P_{UAS}<0.0001$ and for the dark period of first day: $P_{GAL4} = 0.5$, $P_{UAS} = 0.13$), suggesting that this treatment does not cause locomotor impairment. Additionally, video recordings at 0-2 min and >2 hr after DPM activation at 31–32°C show that flies are immediately arousable by gentle tapping and have normal geotaxis and locomotion, consistent with DPM activation inducing a sleep state rather than paralysis or locomotor dysfunction (***Video 1***).

Upon cessation of dTrpA1 activation, after 2 days of activation at 31°C, flies showed decreased sleep. The negative sleep rebound after release is consistent with the presence of strong compensatory homeostatic mechanisms counteracting excessive sleep (***Shang et al., 2013***) and/or excessive DPM activity. Since DPM neurons are normally activated in the first 0–3 hr following training (***Yu et al., 2005***; ***Cervantes-Sandoval and Davis, 2012***), it is unlikely that these neurons would ever naturally exhibit such high levels of activity for the length of time we have imposed artificially. The immediate increase in sleep upon dTrpA1 activation is most likely to be indicative of normal DPM function. The idea that DPMs can act acutely is in agreement with the literature that shows there is a temporally circumscribed window during which they are required for consolidation (***Keene et al., 2004***, ***2006***; ***Yu et al., 2005***) and with the finding that a short period (45 min) of TrpM8 activation of DPM neurons following a learning task rescues age-induced memory impairment (***Tonoki and Davis, 2012***).

Since activation of DPM neurons acutely induces sleep in flies, we wanted to determine if DPM activity also played a role in the maintenance of baseline sleep. In order to assess this, we used the *NP2721-GAL4* line to drive expression of a temperature-sensitive, dominant negative Dynamin, Shibire^{ts} (Shi^{ts}) to block vesicle recycling in DPMs. Following a shift at ZT0 from the permissive temperature of 18°C, to the restrictive temperature of 31°C, flies showed a small but significant decrease in levels of nighttime sleep relative to the baseline sleep of each genotype at 18°C (***Figure 1B1***). Because the temperature shift protocol induced changes in the baseline nighttime sleep of control flies (compare 'baseline' and 'recovery' days in panel B1) we asked if the ability of DPM inhibition to decrease nighttime sleep was independent of baseline by doing a second round of temperature shift (***Figure 1B2***). We found that DPM inhibition decreased sleep regardless of the starting baseline. We obtained a similar result using another DPM line, *C316-GAL4* to drive Shi^{ts} (***Figure 1—figure supplement 3***). Additionally, when the weaker, but cleaner, *VT64246-GAL4* driver line was used with the temperature-sensitive repressor, *Tubulin-GAL80^{ts}*, to produce acute expression of the hyperpolarizing potassium channel Kir2.1 in DPM neurons nighttime sleep was also reduced (***Figure 1—figure supplement 2B,D***).

These data demonstrate that sleep loss after inhibition of DPM activity is both cell-specific and independent of the particular method used to suppress DPM activity. The limitation of the DPM loss-of-function phenotype to nighttime sleep implies there is a baseline function of DPM activity that occurs even in isolated animals in a relatively stimulus-poor environment, but the small magnitude of these changes suggests that DPMs are not the major driver of baseline sleep. The DPM-dependent gain-of-function experiments with dTrpA1, however, indicate that significant changes in both nighttime and daytime sleep can be produced with acute activation of DPM neurons, as might perhaps naturally occur secondary to some type of experience.

The DPM neurons are coupled via gap junctions to second pair of neurons innervating the MB, the anterior paired lateral (APL) neurons (***Wu et al., 2011***). It was possible that sleep gains resulting

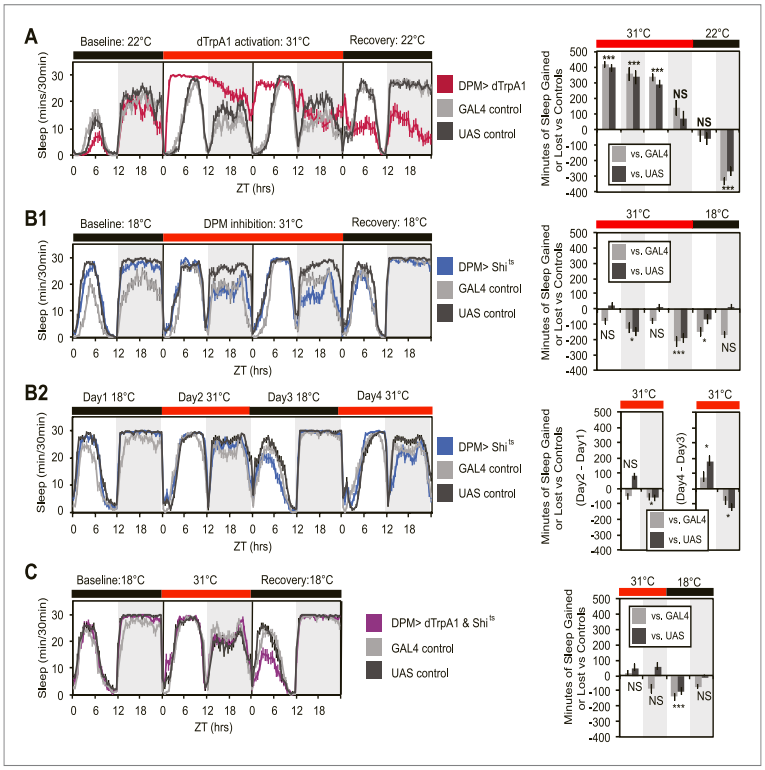

Figure 1. DPM activity and synaptic release are sleep promoting in a cell-autonomous manner. (**A**) Flies exhibit large gains in sleep when DPM neurons are activated with dTrpA1 at 31°C (*w-; NP2721-GAL4/ UAS-dTrpA1-II*). Compensatory sleep loss is apparent during recovery following 2 days of dTrpA1 activation. (**B1**) Flies show small but significant sleep loss when DPM synaptic release is inhibited with Shi^{ts} after shift to 31°C (*w-; NP2721-GAL4; 20xUAS-IVS-Syn21-Shi^{ts}*). Continuing sleep loss is apparent during the first 12 hr of recovery following Shi^{ts} inhibition of DPM synaptic release. (**B2**) Sleep loss can be seen over multiple cycles of temperature shift when DPM synaptic release is inhibited with Shi^{ts} (*w-; NP2721-GAL4; 20xUAS-IVS-Syn21-Shi^{ts}*). For quantification in **B2**, day 1 was used as a baseline to calculate day 2 sleep changes and day 3 was used as a baseline to calculate day 4 sleep changes. (**C**) Sleep gains resulting from dTrpA1 activation are fully blocked when DPM synaptic release is inhibited with Shi^{ts} at 31°C (*w-; NP2721-GAL4/ UAS-dTrpA1-II; 20xUAS-Tts-Shi^{ts}*). Left plots show sleep in 30-min bins during a baseline day (22°C for dTrpA1 alone, 18°C for Shi^{ts} or combined UAS experiments), followed by 1–2 days of DPM hyperactivation or inhibition (31°C) and 1 day of recovery (22°C for dTrpA1 alone, 18°C for Shi^{ts} or combined UAS experiments). Right plots show a quantification of the 30-min data in 12-hr bins across 1 or 2 days of heating and 1 day of recovery. Sleep change is quantified as the minutes of sleep gained or lost by the experimental genotype in comparison to either the *UAS* or *GAL4* control genotypes during heating and recovery periods. Grey shading indicates the dark period/night, red bars indicate increased temperature. All data are presented as mean ± SEM where * represents $p < 0.05$, ** $p < 0.001$, and *** $p < 0.0001$ using the Mann-Whitney-Wilcoxon rank sum test. Calculation of sleep gain or loss and statistics are described in the 'Materials and methods' section.

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The following figure supplements are available for figure 1:

Figure supplement 1. Comparison of DPM-expressing *GAL4* lines used in experiments.

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Figure supplement 2. DPM activity regulated by a different *GAL4* insertion is also sleep-promoting.

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Figure supplement 3. Vesicle release from DPMs promotes consolidated nighttime sleep.

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Figure supplement 4. An additional UAS transgene does not prevent dTrpA1-induced sleep gains.

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Video 1. DPM > dTrpA1 activation induces sleep, but not locomotor impairment. Groups of ten individual female flies with DPM*(NP2721)> dTrpA1* (center), *UAS-dTrpA1* (left) or DPM *(NP2721)-GAL4* (right) were kept at 31°C for 2 hr before video recording. Flies with DPM driven dTrpA1 expression show normal locomotion when gently tapped (0:00:01), but quickly assume a stationary resting position after ~30 s undisturbed (0:00:35), whereas control flies remain awake and continue to explore the environment. All flies were anesthetized with CO_2, counted, and sorted into groups of ten 1 day prior to video recording and kept on food at 22°C prior to heating. Flies were heated at 31°C on food for 2 hr and flipped to empty vials just prior to recording.
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from dTrpA1-mediated activation of DPMs were not due to neurotransmitter release from DPMs themselves, but instead were a secondary result of gap–junction coupled APL activation and neurotransmitter release. In order to distinguish between these possibilities, we coexpressed dTrpA1 and the temperature-sensitive Dynamin mutant, Shi^{ts}, in DPM neurons. Since Shi^{ts} protein and mRNA are unlikely to pass through gap junctions, DPM-Shi^{ts} expression should prevent neurotransmitter release in a cell-autonomous manner from DPM, but not affect APL neurons. Thus, if dTrpA1-mediated sleep gains are the result of DPM, but not APL neurotransmitter release, they should be blocked by the coexpression of Shi^{ts} in DPMs at high temperature. Conversely, if APL neurotransmitter release is responsible for sleep gains resulting from DPM activation, DPM expression of Shi^{ts} should have no effect on dTrpA1-evoked sleep. We found that coexpression of Shi^{ts} completely blocked activity-induced sleep gains (***Figure 1C***). This was not due to dilution of GAL4-mediated expression since the coexpression of a neutral second UAS transgene (UAS-GCaMP6) did not block dTrpA1-stimulated sleep (***Figure 1—figure supplement 4***). This suggests that sleep from dTrpA1-mediated DPM activation is the result of release of neurotransmitter from DPMs, not APLs.

Thus, we find that DPMs are capable of acutely promoting sleep and have an additional role in mediating baseline sleep during the night in stimulus-poor conditions (single flies in sleep tubes). How this function of DPMs is regulated is unknown. Given their role in memory consolidation, however, it is likely DPMs are chiefly active in acute sleep regulation when they are recruited to promote sleep following stimulus-rich experiences such as learning.

α'/β' activity promotes wakefulness

MB α′/β′ neurons are thought to be a key postsynaptic target of DPM neurons (***Keene et al., 2006***; ***Krashes et al., 2007***; ***Pitman et al., 2011***). Both DPM and α′/β′ activity are required during the memory consolidation period 0–3 hr after training for the storage of subsequent 24 hr long-term memory (***Krashes and Waddell, 2008***). Recently, multiple groups have posited that *Drosophila* experiences a form of systems consolidation, similar to that of mammals, in which memories are transferred from a set of neurons serving as a short-term storage site (e.g., the hippocampus in mammals, and γ and α′/β′ lobes in flies) to a different set of neurons which store the memory in a more stable long-term state (e.g., the cortex in mammals, α/β lobes and MB output neurons in flies) (***Cervantes-Sandoval et al., 2013***; ***Dubnau and Chiang, 2013***). Since systems consolidation in *Drosophila* requires DPM and α′/β′ activity and is known to be promoted by sleep in other organisms, we reasoned that α′/β′ activity may also play a role in promoting sleep. While it has been shown previously that the *Drosophila* MB can promote sleep (***Joiner et al., 2006***; ***Pitman et al., 2006***; ***Yi et al., 2013***), a specific role for the α′/β′ lobes has not been reported.

To address this issue, we acutely activated these neurons with an MB-restricted version of the α′/β′ driver line *c305a-GAL4* and the warmth-sensitive cation channel, dTrpA1. If DPM neurons act to excite α′/β′, as postulated by models of consolidation, we would expect this manipulation to increase sleep. Instead, we see a strong decrease in nighttime sleep. This α′/β′–dependent nighttime sleep loss remained stable throughout 48 hr of dTrpA1 activation and was accompanied by increasing daytime sleep loss which continued even after release from dTrpA1 activation (***Figure 2A,C***). This unusual pattern exactly matches the phenotype seen in flies expressing Shi^{ts} in DPM neurons. Thus, DPM and α′/β′

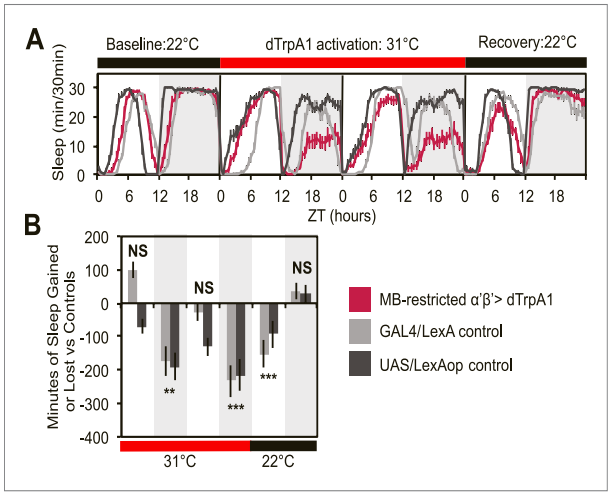

Figure 2. MB α′β′ neuron activity promotes wakefulness. The α′β′ *c305a-GAL4* driver line was crossed to *UAS-dTrpA1-II* (with *c305a* expression restricted to the MB) to determine effects on sleep of α′β′ activation. (**A**) shows sleep in 30-min bins during a baseline day (22°C), followed by 2 days of DPM hyperactivation (31°C) and 1 day of recovery (22°C). (**B**) shows minutes of sleep gained or lost by the experimental genotype in comparison to either the *UAS* or *GAL4* control genotypes during heating and recovery periods. MB-restricted genotypes are: 1). *UAS-dTrpA1, ptub>GAL80>/c305a-GAL4; MB-LexA/LexAop-Flp*, 2). *c305a-GAL4; MB-LexA* (GAL4/LexA control), and 3). *UAS-dTrpA1, ptub>GAL80>; LexAop-Flp* (UAS/LexAOP control). Grey shading indicates the dark period/night, red bars indicate increased temperature. All data are presented as mean ± SEM where * represents $p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$ using the Mann-Whitney-Wilcoxon rank sum test. Calculation of sleep gain or loss and statistics are described in the 'Materials and methods' section.

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activity have opposing roles in the regulation of sleep: DPM activity promotes sleep whereas α′/β′ activity is wake-promoting. These data suggest that DPM neurons may inhibit MB neurons.

DPMs contain 5HT and GABA, but not ACh or dopamine

The shared requirement for activity during memory consolidation (***Krashes et al., 2007***; ***Krashes and Waddell, 2008***) as well as the extensive physical connectivity as determined by membrane-localized GRASP (***Pitman et al., 2011***) strongly suggests that DPM and MB neurons are synaptically connected. Given the lack of information on the functional nature of the connections, we set out as a first step to determine what neurotransmitters are present in DPM neurons. Colocalization of mCD8-GFP expression in DPM cell bodies with staining against a panel of neurotransmitters shows that DPM neurons contain both GABA (***Figure 3A***) and 5HT (***Figure 3—figure supplement 1A***). DPM cell bodies also stain positively for Gad1 (***Figure 3C***), the GABA synthetic enzyme. We found no evidence for expression of ChAT, the ACh synthetic enzyme (***Figure 3C***), or tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis (***Figure 3—figure supplement 1B***). These combined results suggest that the DPM neurons release GABA and 5HT, but not ACh or dopamine.

Although GABA release is known to be inhibitory, there are both stimulatory and inhibitory 5HT receptors in the *Drosophila* brain and it is unknown whether $G\alpha_s$-coupled 5HT receptors, such as 5HT7, are expressed in the MBs. To test whether 5HT could be stimulatory in the MBs, we used *5HT7-GAL4* (***Becnel et al., 2011***) to drive expression of Epac1-camps (EPAC) (***Nikolaev et al., 2004***; ***Shafer et al., 2008***), a FRET-based cyclic nucleotide sensor. There was no expression evident in the MBs, although there was strong fluorescence in the central complex as reported previously (***Becnel et al., 2011***). We bath-applied 5HT and saw increased cAMP in the labeled cells of the ellipsoid body, confirming the efficacy of the drug as well as the positive coupling to cyclase in 5HT7-GAL4+ cells

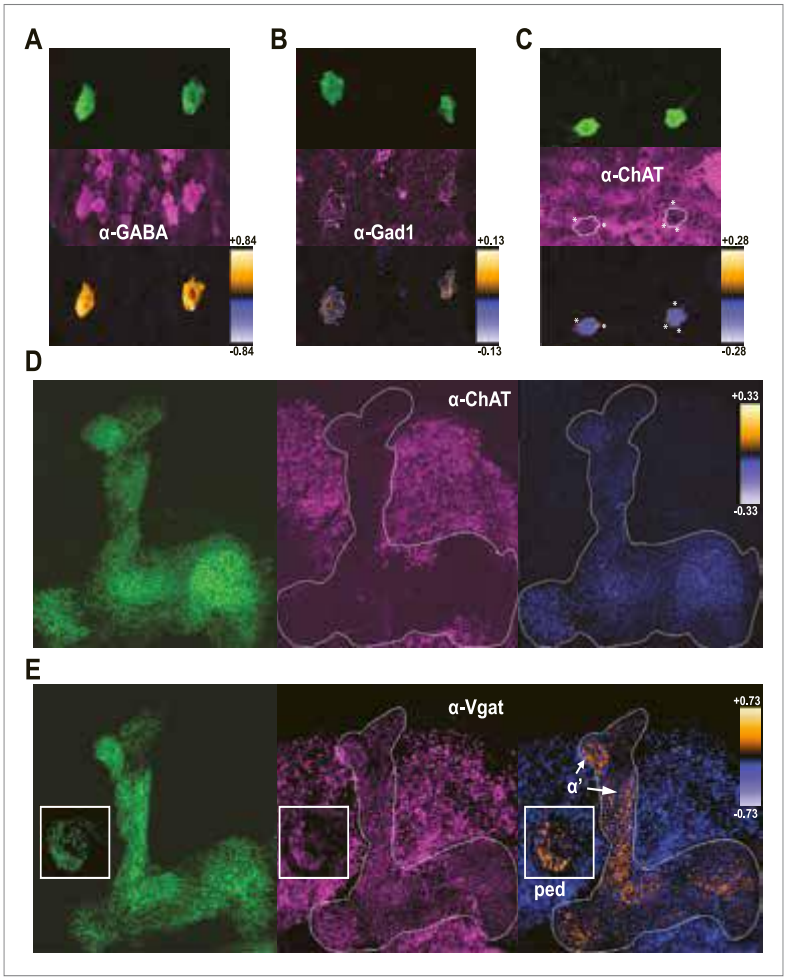

Figure 3. DPMs are GABAergic, but not cholinergic. (**A–C**) Top: *VT64246-GAL4* was used to drive expression of membrane-localized mCD8-GFP in DPM cell bodies, which was visualized with an anti-GFP antibody. Middle: brains were stained using antibodies against (**A**) GABA (N = 11/11 cell bodies with positive staining), (**B**) Gad1 (N = 12/13 cell bodies with positive staining), and (**C**) ChAT (N = 11/11 cell bodies had no staining). Although a number of neighboring ChAT-positive cell bodies cross over the periphery of the DPM cell bodies resulting in very localized correlation between channels (* in image), the DPMs do not show a general colocalization with anti-ChAT. Bottom: ICA was used to visualize the relative colocalization between DPM>GFP and transmitter staining in pairs of DPMs. (**D**–**E**) Left: *VT64246-GAL4* was used to drive expression of a presynaptic marker, BRP-short-GFP, in DPM projections to the MB. Middle: brains were stained with antibodies against (**D**) ChAT (N = 16/16 MB lobe sets with negative staining) and (**E**) VGAT, with insets showing the MB peduncles (N = 10/10 MB lobe sets with positive staining). Right: ICA was used to build false color maps of relative colocalization between DPM>Brps-GFP and transmitter staining in DPM projections. For ICA, orange indicates colocalization/correlation of pixel intensities between channels (PDM>0) and purple indicates a lack of colocalization/anticorrelation of pixel intensities between channels (PDM<0) relative to the scale shown for each image (see 'Materials and methods' for further details). '**α**'' indicates the MB **α**' lobe and 'ped' indicates peduncles, shown in the inset.

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Figure 3. Continued on next page

Figure 3. Continued

The following figure supplements are available for figure 3:

Figure supplement 1. DPMs are serotonergic, but not dopaminergic.

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Figure supplement 2. MB neurons do not express stimulatory serotonin receptors.

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(***Figure 3—figure supplement 2***). Because the lack of *5HT7-GAL4* expression in MB does not necessarily mean there is not a stimulatory 5HT receptor expressed there, we also bath-applied 5HT to brains with EPAC driven by *MB247-lexA* to determine if there was an excitatory response from this structure. We saw no increase in cAMP (***Figure 3—figure supplement, 2***). Because EPAC may not be not effective at reporting inhibition (e.g., if there is no basal activation of cyclase), we cannot rule out inhibitory effects of 5HT via $5HT_{1A}$, which is known to be expressed in MBs (***Yuan et al., 2006***; ***Lee et al., 2011***).

DPMs project throughout the MBs and have both pre- and postsynaptic markers comingled in all lobes (***Waddell et al., 2000***; ***Wu et al., 2013***) To investigate where DPM GABA might be released, we examined colocalization of a DPM-expressed presynaptic marker, Bruchpilot-short-GFP (***Schmid et al., 2008***; ***Fouquet et al., 2009***) with immunostaining against VGAT, the vesicular GABA transporter. Colocalization was prominent in the MB α′/β′ lobes and MB peduncles (***Figure 3E***). This is consistent with a role of DPM neurons in inhibiting α′/β′ neurons in order to promote sleep and opens up the interesting possibility that there may be branch-specific neurotransmission from DPM neurons (***Yu et al., 2005***; ***Cervantes-Sandoval and Davis, 2012***; ***Samano et al., 2012***). We found little to no colocalization between DPM presynaptic sites and the cholinergic marker ChAT (***Figure 3D***), again suggesting that DPM neurons do not release ACh.

DPM activation has an inhibitory effect on MB neurons

These results suggest that DPM neurons might be inhibitory rather than excitatory, and are inconsistent with a role for DPM neurons in directly enhancing potentiation. To test the sign of the connection, we first used functional imaging techniques to determine if DPM activation could stimulate postsynaptic MB neurons (***Figure 4A***). We expressed the mammalian ATP-gated P2X2 receptor (***Lima and Miesenbock, 2005***; ***Yao et al., 2012***) in DPMs using *NP2721-GAL4* or *c316-GAL4*, and activated these cells by applying ATP to dissected adult *Drosophila* brains. We first confirmed that bath-applied ATP was sufficient to activate the P2X2 receptors in DPMs by co-expressing genetically-encoded fluorescent sensors and using functional imaging to observe changes in fluorescence indicating a response. Using *c316-GAL4* to drive *UAS-GCaMP3.0* (***Tian et al., 2009***), *UAS-Arclight* (***Cao et al., 2013***), and *UAS-Synapto-pHlorin* (SpH) (***Meisenbock et al., 1998***), we found that P2X2-mediated stimulation effectively activated DPM neurons, evoking increases in intracellular calcium, membrane voltage, and vesicle fusion, respectively (***Figure 4B***). It should be noted that these responses were observed in the DPM projections to the MBs, not the DPM cell bodies, demonstrating that this technique successfully activates the DPMs and causes them to release neurotransmitter from their projections onto downstream targets in the MB neuropil. We also co-expressed P2X2 receptors and *UAS-GCaMP3.0* in the DPMs using *NP2721-GAL4* to confirm that this technique was effective with the weaker driver (***Figure 4—figure supplement 1A***).

To determine the effect of DPM activation on the MBs, we expressed P2X2 receptors in DPMs using the *UAS/GAL4* binary expression system and either GCaMP3.0 or EPAC in the MBs using the *lexA/lexAop* binary expression system to observe changes in intracellular calcium or cAMP, respectively (***Figure 4A***). We found that activation of DPM neurons had no excitatory effects on the MBs when using either *NP2721-GAL4* (***Figure 4—figure supplement 1B,C***) or the stronger *c316-GAL4* with *eyeless-GAL80* and *MB-GAL80* to restrict expression to DPM neurons (***Figure 4C,D***). To confirm that these negative results were not due to lack of drug efficacy or some systematic problem, positive controls (P2X2 and GCaMP3.0 or EPAC expressed in the same cell type) were performed concurrently with every experiment (data not shown). We also did a separate set of experiments using dTrpA1 and a temperature step to activate DPM neurons, but failed to see any MB calcium responses (data not shown).

Although we demonstrated that P2X2 is capable of activating DPM neurons and causing vesicle fusion, we needed to rule out the possibility that MBs were simply incapable of responding to excitatory

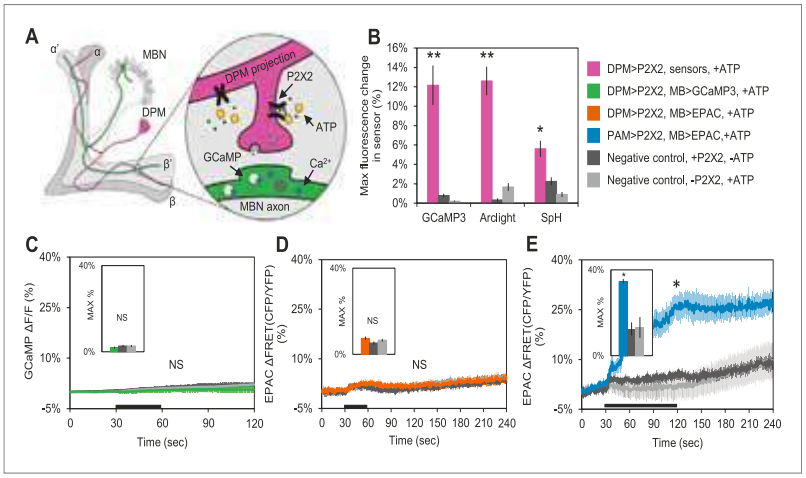

Figure 4. DPM activation has no excitatory effect on the MBs. (**A**) Schematic of genetic set-up for functional imaging experiments. P2X2 receptors were expressed in the DPM neurons, and fluorescent sensors (GCaMP shown) were expressed in the MB neurons. (**B**) Bath-applied ATP is effective at activating DPMs expressing P2X2 receptors. Mean maximum percentage change in GCaMP3.0 (*w-, eyeless-GAL80; UAS-GCaMP3.0/+; c316-GAL4/UAS-P2X2*, N = 9 with *UAS-P2X2* transgene, 8 without [9, 8], $p < 0.001$ by Mann–Whitney U test), Arclight (*w-, eyeless-GAL80; UAS-Arclight/+; c316-GAL4/UAS-P2X2*, N = [11,6], $p < 0.001$ by Mann–Whitney U test), and Synapto-pHluorin (*w-, eyeless-GAL80; UAS-Synapto-pHluorin/+; c316-GAL4/UAS-P2X2*, N = [11, 6], $p = 0.002$ for Mann–Whitney U test) fluorescence in horizontal DPM neuron projections in response to 30 s perfusions of 2.5 mM ATP. (**C**–**E**) Black bar denotes time of perfusion of 2.5 mM ATP or vehicle. Insets are histograms summarizing the mean maximum percentage change in fluorescence of the respective sensor. The *eyeless-GAL80* and *MB247-GAL80* transgenes were used to restrict GAL4 driven expression to DPMs. (**C**) Mean GCaMP3.0 response traces of *w-, eyeless-GAL80; lexAop-GCaMP3.0/MB247-GAL80; c316-GAL4, MB247-lexA/UAS-P2X2* (green), or without the *UAS-P2X2* transgene (grey), to 30 s perfusion of 2.5 mM ATP or vehicle (black). N = [10, 8], $p > 0.05$ for Kruskal–Wallis one-way ANOVA, histogram values are 1.9 ± 0.5% (green), 2.6 ± 0.5% (black), 2.7 ± 0.5% (grey). (**D**) Mean EPAC response traces of *w-, eyeless-GAL80; lexAop-EPAC/MB247-GAL80; c316-GAL4, MB247-lexA/UAS-P2X2* (orange), or without the *UAS-P2X2* transgene (grey), to 30 s perfusion of 2.5 mM ATP or vehicle (black). N = [10, 8], $p > 0.05$ for Kruskal–Wallis one-way ANOVA, histogram values are 7.4 ± 0.8% (orange), 5.3 ± 0.8% (black), 6.6 ± 0.7% (grey). (**E**) MBs respond to excitatory inputs. Mean EPAC response traces of *w-; R58E02-lexA/lexAop-P2X2; MB247-GAL4/UAS-EPAC* (blue), or without the *lexAop-P2X2* transgene (grey), to 90 s perfusion of 2.5 mM ATP or vehicle (black). N = [9, 5], $p < 0.001$ for Mann–Whitney U test, histogram values are 34.6 ± 3.1% (blue), 12.5 ± 1.2% (black), 13.2 ± 4.8% (grey). (**C**–**E**) Traces represent ROIs taken from horizontal sections of MB lobes. (**C**–**D**) ROIs were also taken from the vertical lobes and no change in fluorescence was seen (data not shown).

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The following figure supplements are available for figure 4:

Figure supplement 1. DPM activation has no excitatory effect on the MBs.

DOI: 10.7554/eLife.03868.014

Figure supplement 2. Bath-applied carbachol (CCh) evokes an excitatory response in MBs.

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synaptic inputs. We first examined the response of the MBs to bath-applied carbachol (CCh), a cholinergic agonist. Tetrodotoxin (TTX) was present to block action potentials so that we could isolate direct effects on MBs. We observed robust increases in calcium and cAMP (***Figure 4—figure supplement 2***), indicating that MB neurons are capable of responding to ACh in an excitatory manner.

To show that MBs could respond to activation of upstream stimulatory neurons using the P2X2 technique, we expressed P2X2 receptors in the PAM cluster of dopaminergic neurons using *R58E02-lexA* and EPAC in the MBs using *MB247-GAL4*. Previous reports have demonstrated that the PAM cluster of dopamine neurons signals to the MBs by activation of $G\alpha_s$-coupled receptors (***Liu et al., 2012***). As expected, we observed an increase in cAMP in the MBs in response to PAM neuron activation (***Figure 4E***), indicating that MBs are capable of responding to stimulatory inputs.

These results clearly demonstrate that DPM neurons are not excitatory; however, these fluorescent sensors may not be effective at reporting inhibition unless there is some basal level of activity in the

circuit. Therefore, to determine if DPM activation has an inhibitory effect on MBs, we used P2X2 receptors to activate the DPMs and expressed the fluorescent intracellular chloride sensor SuperClomeleon (***Grimley et al., 2013***) in the MBs. We found that DPM activation evoked an increase in chloride in the MBs which could be almost completely blocked by bath-application of picrotoxin (***Figure 5A***). To determine if these results could be caused by DPM GABA release, we bath-applied GABA and observed similar MB SuperClomeleon responses in the presence of TTX, which could be completely blocked by picrotoxin (***Figure 5B***). These results demonstrate that DPM neurons inhibit the MBs via activation of $GABA_A$ receptors.

GABA and 5HT mediate the sleep-promoting effects of DPMs

To determine if GABA- and/or 5HT-mediated inhibition was playing a role in the ability of DPMs to promote sleep, we manipulated transmitter in DPM neurons and receptors in α′/β′ neurons. In order to assess whether DPM GABA release promotes sleep we expressed *VGAT-RNAi* to knock down the vesicular GABA transporter, using two different DPM lines, *c316-GAL4* and *NP2721-GAL4*. Knockdown of VGAT in DPM neurons results in the loss of a large proportion of nighttime sleep (***Figure 6A,B***). Nighttime sleep loss is not due to hyperactivity since flies exhibit normal levels of nighttime activity while awake when compared to controls (for *c316-GAL4* with *VGAT-RNAi* $P_{GAL4} = 0.83$, $P_{UAS} = 0.62$ and for *NP2721-GAL4* with *VGAT-RNAi* $P_{GAL4} = 0.84$, $P_{UAS} = 0.69$).

As noted above, DPM neurons are coupled via gap junctions to the Anterior Paired Lateral (APL) neurons (***Wu et al., 2011***), which densely innervate the MB and synthesize the neurotransmitters GABA (***Liu and Davis, 2009***) and octopamine (***Wu et al., 2013***). Small molecules including neurotransmitters can pass through gap junctions (***Vaney et al., 1998***) and, for some subtypes of mammalian connexins, even RNAi fragments can pass (***Valiunas et al., 2005***). This raised the possibility that DPM sleep phenotypes could be dependent on APL-synthesized GABA and that manipulation of VGAT in DPMs might be indirectly acting by inhibition of APL GABA packaging. In order to assess this, we expressed *VGAT-RNAi* using three different APL *GAL4* lines: *GH146, NP5288, NP2631* (***Figure 6—figure supplement 1***). We never saw sleep loss with *NP2631-GAL4*. With *GH146-* and *NP5288-GAL4*, we saw weak nighttime sleep loss. When it was observed, nighttime sleep loss due to APL-driven

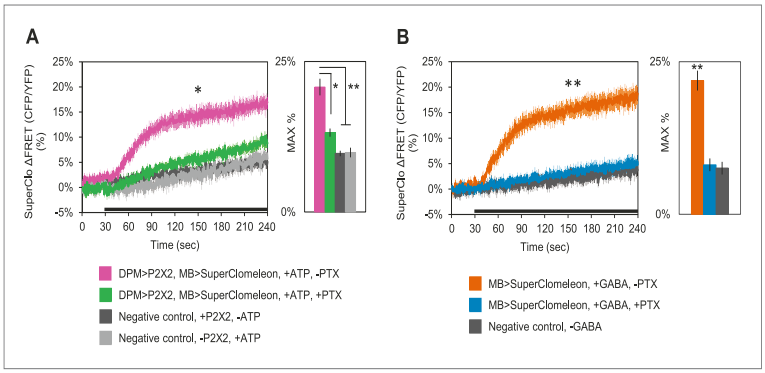

Figure 5. DPM activation has an inhibitory effect on the MBs. (**A**) DPM activation evokes a chloride increase in MBs via that can be reduced by picrotoxin (PTX). Mean SuperClomeleon response traces of *w-, eyeless-GAL80; lexAop-SuperClomeleon/MB247-GAL80; c316-GAL4, MB247-lexA/UAS-P2X2* to perfusion of 2.5 mM ATP alone (pink, N = 12) or in bath of 10 μM PTX (green, 10). Negative controls: mean response to ATP without *UAS-P2X2* transgene (grey, 8), or vehicle (black, 12). $p < 0.001$ between pink and negative controls, $p = 0.001$ between pink and green, $p < 0.01$ between green and negative controls for Mann–Whitney U test. Histogram values are 20.8 ± 1.4% (pink), 13.2 ± 0.7% (green), 9.7 ± 0.5% (black), 9.9 ± 0.8% (grey). (**B**) Bath-application of GABA in the presence of TTX evokes a chloride increase in MBs that can be blocked by PTX. Mean SuperClomeleon response traces of *w-; lexAop-SuperClomeleon/+; MB247-lexA/+* to perfusion of 1.5 mM GABA alone (orange, 8) or with 10 μM PTX (blue, 8), in 1 μM TTX bath. Negative control: Mean response to vehicle 1 μM TTX bath (black, 8). $p < 0.001$ for Mann–Whitney U test. Histogram values are 21.9 ± 1.6% (orange), 8.1 ± 1.1% (blue), 7.6 ± 1.0% (black). (**A**–**B**) Black bar denotes time of perfusion. Histograms summarize the mean maximum percent change in fluorescence of SuperClomeleon. Traces represent ROIs taken from vertical sections of MB lobes. ROIs were also taken from the horizontal lobes and similar results were seen (data not shown).
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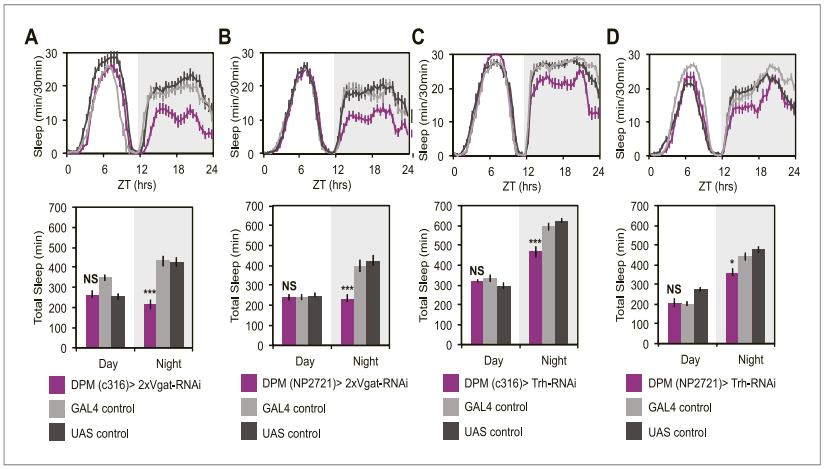

Figure 6. DPM GABA and 5HT promote nighttime sleep. DPM expression of VGAT was reduced by combining two copies of *UAS-VGAT-RNAi* with each of two different DPM-GAL4 drivers, *c316-GAL4* (**A**) and *NP2721-GAL4* (**B**). Expression levels of TRH where reduced in DPMs by driving *UAS-Trh-RNAi* with each of two different DPM-GAL4 drivers, *c316-GAL4* (**C**) and *NP2721-GAL4* (**D**). Top: shows total sleep in 30-min bins averaged across 3 days. Bottom: shows the same data quantified in 12-hr day/night bins. In all cases, a decrease in VGAT or 5HT synthetic enzymes (TRH) in DPMs resulted in nighttime sleep loss, with no change in nighttime activity while awake, although increases in daytime activity while awake were often apparent. Grey shading indicates the dark period/night. All data are presented as mean ± SEM where * represents $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$ using the Mann-Whitney-Wilcoxon rank sum test. Statistics are described in the 'Materials and methods' section.

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The following figure supplement is available for figure 6:

Figure supplement 1. APL GABA can sometimes promote nighttime sleep.

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expression of VGAT-RNAi was generally accompanied by an increase in activity while awake indicating an effect on locomotion; this is never seen with VGAT-RNAi in DPMs. Thus while APL GABA may be weakly sleep-promoting, its effects are qualitatively and quantitatively distinct from GABA released from DPMs. This also implies that the contribution of APLs to phenotypes seen after DPM activation or hyperpolarization is likely to be minimal. Along these lines it is also important to note that driving Shi^{ts} in DPMs reduces baseline sleep and completely blocks dTrpA1-induced sleep gains. These manipulations, using DPM-expressed Shi^{ts}, would not be expected to influence APL activity in any way, again supporting the idea that DPMs promote sleep in a distinct and cell-autonomous manner.

While there are two known types of GABAergic neurons innervating the MB, DPMs are likely the sole source of MB lobe 5HT (***Lee et al., 2011***). MB expression of inhibitory, $G\alpha_i$-coupled $5HT_{1A}$ receptors are necessary for anesthesia-resistant memory (***Lee et al., 2011***), and are also known to promote sleep (***Yuan et al., 2006***). 5HT from DPMs, however, has not actually been shown to promote sleep. Knockdown of 5HT synthesis in DPM neurons with RNAi targeted against tryptophan hydroxylase (Trh) using the most strongly expressing DPM line, *c316-GAL4*, results in a significant loss of nighttime sleep (***Figure 6C***). This nighttime sleep loss is also apparent, but milder, with *Trh-RNAi* being driven by the somewhat weaker *NP2721-GAL4* (***Figure 6D***). No difference is seen in nighttime activity during waking periods vs controls (for *c316-GAL4* with *Trh-RNAi* $P_{GAL4} = 0.09$, $P_{UAS} = 0.17$) indicating the sleep loss is not an artifact of hyperactivity.

DPM GABA acts on α′/β′ lobes to promote sleep

GCaMP, EPAC and Arclight experiments demonstrate a lack of excitatory transmission from DPM neurons to the MBs and SuperClomeleon experiments demonstrate that the DPMs are capable of inhibiting MB neurons. The wake-promoting phenotype of MB α′/β′ neurons as well as a shared temporal role in memory consolidation suggest these neurons could be the targets of sleep-promoting DPM

GABA release. In order to test this possibility, we expressed RNAi against *Drosophila* GABA receptors in MB α′/β′ neurons. It has previously been shown that the *Drosophila* ionotropic $GABA_A$ receptor, Rdl, is highly expressed in all lobes of the MBs (***Liu et al., 2007***). Consistent with the phenotype of DPM VGAT knockdown, we observe decreased nighttime sleep with knockdown of either Rdl (***Figure 7A***), or $GABA_B$-R3 (***Figure 7B***) in MB α′/β′ neurons. In both cases sleep loss is the result of a decrease in the duration of nighttime sleep episodes. Knockdown of Rdl results in less total sleep loss since an increase in the total number of nighttime sleep episodes partially compensates for the decrease in mean sleep episode duration. Importantly, concurrent expression of the *MB-GAL80* transgene, which blocks GAL4-mediated expression of receptor RNAis, greatly suppresses sleep loss and fragmentation phenotypes showing that the effects are specific to the MB α′/β′ lobes (***Figure 7—figure supplement 1***). Interestingly, experiments to determine the lobe-specific role of $5HT_{1A}$ receptors suggest that 5HT acts generally in the MB, not just on the α′/β′ lobes (data not shown), suggesting that these two transmitters may play somewhat different roles at the circuit level in sleep and memory consolidation.

Discussion

The inhibitory neurotransmitter, GABA, is known to promote sleep in both mammals (***Rasch and Born, 2013***) and *Drosophila* (***Agosto et al., 2008***), but specific sleep-promoting GABAergic neurons have not been identified in the fly. Additionally, it is known that sleep promotes memory consolidation in *Drosophila* (***Donlea et al., 2011***) and other animals (***Rasch and Born, 2013***; ***Diekelmann, 2014***), but it is unclear how sleep and memory circuits interact to facilitate memory consolidation. Here we find a shared anatomical locus of memory consolidation and GABAergic sleep-promotion in the DPM neurons. We show that α′/β′ neurons, postsynaptic targets of the GABAergic DPM processes, are wake-promoting. The specific involvement of neurons required for memory consolidation (and their memory-relevant post-synaptic targets) in the regulation of sleep suggests that generation of sleep by activation of learning circuits is an intrinsic property of the circuit, not an extrinsically imposed phenomenon. Further, our finding that the memory-consolidation specific DPM neurons are inhibitory suggests that inhibitory neurotransmitters may play an as-of-yet uncharacterized role in memory consolidation in *Drosophila*.

The role of DPM vs APL neurons in regulation of sleep

Previous studies on the role of GABA in the *Drosophila* learning circuit have focused on the role of the APLs, a pair of GABAergic neurons which densely innervate the MBs and are coupled to DPMs by gap junctions. APL GABA has been shown to inhibit acquisition (***Liu and Davis, 2009***), perhaps by acting at the level of olfactory coding (***Lin et al., 2014***). APLs have also been shown to be critical for a labile component of anesthesia-sensitive intermediate-term memory but not for consolidation to long-term memory (***Pitman et al., 2011***). Because of the gap junction coupling, it was formally possible that GABA found in DPMs could be coming from APLs and that sleep loss due to DPM VGAT-RNAi expression was the result of reductions in APL VGAT levels (***Vaney et al., 1998***; ***Valiunas et al., 2005***). It was also possible that phenotypes seen after manipulation of DPM electrical activity were secondary to changes in APL activity. However, a number of lines of evidence suggest that DPM neurons are intrinsically GABAergic and sleep-promoting independent of APLs. First, we find that DPMs stain positively for the GABAergic markers Gad1 and VGAT, meaning that DPMs intrinsically possess the ability to synthesize and release GABA. Second, we find that direct expression of *VGAT-RNAi* in APL neurons has a relatively minor effect on sleep as compared to phenotypes seen with expression in DPMs, indicating that GABA endogenous to DPMs is a more significant regulator of sleep than GABA from APLs. Third, we find that sleep loss due to VGAT-RNAi expression in APLs, when it is seen, is accompanied by increases in nighttime activity while awake, which is never seen with DPM-driven VGAT-RNAi expression. This indicates that although APL GABA may promote sleep in its own right, the APL VGAT-RNAi sleep loss phenotype is distinct from that of DPMs. Fourth, expression of Shi^{ts} in DPMs, a manipulation that should have no effect on APL activity since it does not alter the electrical properties of DPMs, results in the same nighttime sleep loss as DPM-driven *VGAT-RNAi* and Kir2.1. Fifth, coexpression of Shi^{ts} along with dTrpA1 in DPMs, a manipulation that should not affect APL neurotransmitter release, results in a complete blockade of activation-induced sleep gains. The most parsimonious explanation for all of these data is that the relevant effect of these manipulations is a change in transmitter release specifically from DPMs and that this bidirectionally modulates sleep. Thus, while it remains possible that APL neurons are modestly sleep-promoting in their

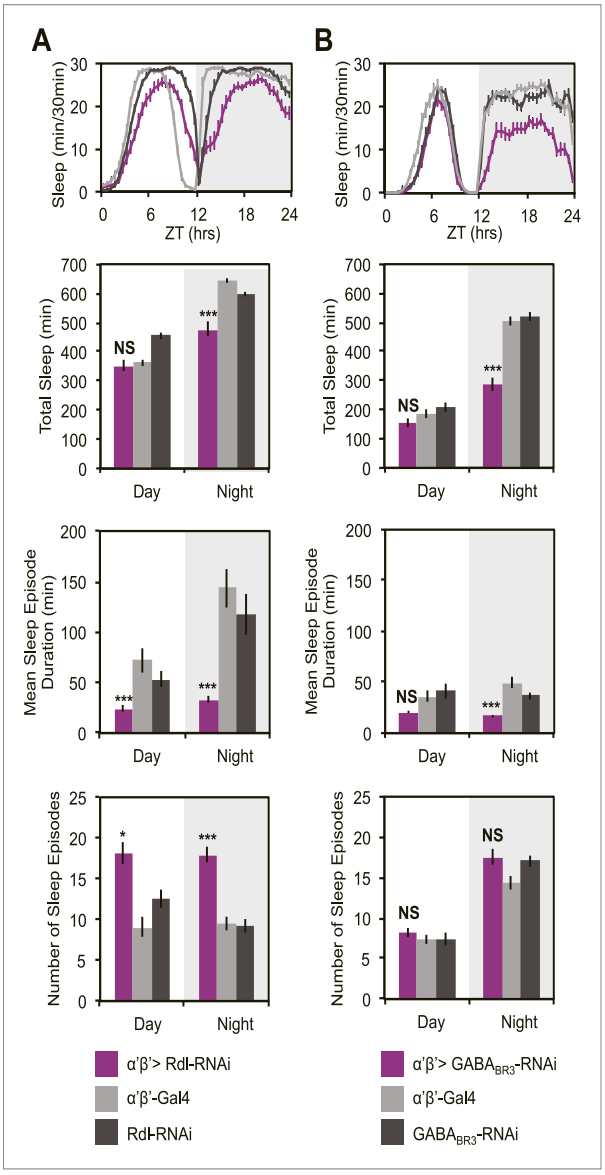

Figure 7. MB α′β′ GABA receptors promote nighttime sleep. *c305a-GAL4* was used to drive expression of *Rdl-RNAi* (**A**) or *GABA$_{BR3}$-RNAi* (**B**) in the α′β′ neurons. Top: shows total sleep in 30-min bins averaged across 3 days. Middle and bottom plots: show 3-day means of total sleep, mean sleep episode duration and number of sleep episodes quantified in 12-hr day/night bins. **α′β′>*Rdl-RNAi*** causes mild sleep loss and increases in nighttime sleep

Figure 7. Continued on next page

Figure 7. Continued

fragmentation, whereas α′β′>*GABA$_{BR3}$-RNAi* causes greater reductions in total sleep due to a decrease in the average sleep episode length. Grey shading indicates the dark period/night. All data are presented as mean ± SEM where * represents $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$ using the Mann-Whitney-Wilcoxon rank sum test. Statistics are described in the 'Materials and methods' section.

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The following figure supplement is available for figure 7:

Figure supplement 1. Sleep loss resulting from *Rdl/GABA$_{BR3}$-RNAi* is primarily due to MB α′β′ expression.

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own right, we find strong evidence for an independent and significant role in regulation of sleep by DPM neurons.

Evidence for DPM inhibition, but not excitation of α′/β′ lobes

Our finding that DPM neuron activation has an inhibitory effect on post-synaptic MB neurons is in apparent disagreement with models of memory consolidation that posit recurrent excitatory feedback between DPM and α′/β′ neurons (***Yu et al., 2005***; ***Keene and Waddell, 2007***). Our observations using SuperClomeleon demonstrate that DPMs evoke a chloride increase in MB neurons, but we did not see decreases in calcium, membrane voltage or cAMP after DPM activation using fluorescent sensors specific for those cellular parameters. This failure is not surprising for two reasons. First, in order to see an inhibitory response it is likely that there has to be some activity or tone in the system. In cases where MB inhibition has been seen with GCaMP it is always in context of a temporally controlled acute activation of the system (***Lei et al., 2013***; ***Lin et al., 2014***; ***Masuda-Nakagawa et al., 2014***). Second, the nature of our DPM activation (bath application of ATP) would make it difficult to see small inhibitory changes over noise in averaged data since our activation of cells is not cleanly time-locked due to differences in diffusion of ATP into the brain between experiments. This is particularly critical with a sensor like Arclight where the expected hyperpolarization induced by inhibition might only be a few millivolts as opposed to 10–40 mV for depolarization by an action potential.

An additional critical test of the sign of this synapse is to ask if there is evidence of inhibition in the functional output of the circuit. Our finding that DPM and α′/β′ activity have opposite roles in the regulation of sleep to the extent that suppression of DPMs with either Shi^{ts} or Kir2.1 almost exactly phenocopies the increasing levels of nighttime sleep loss that results from dTrpA1 activation of α′/β′ strongly suggests an inhibitory connection. Further, we find that decreases in DPM VGAT result in similar nighttime sleep loss phenotypes as α′/β′ Rdl or $GABA_BR3$ knockdown. Thus, suppression of DPM synaptic release (Shi^{ts}), DPM electrical activity (Kir2.1) and DPM GABA release (*VGAT-RNAi*) all result in nighttime sleep loss phenotypes nearly identical to dTrpA1 activation of α′/β′ or loss of α′/β′ GABA receptors. All of these results are consistent with a model in which DPMs act to inhibit α′/β′ neurons by release of GABA.

Inhibition of MBs and sleep

MBs are a sensory integration center in the insect brain. They have been shown, using many different behavioral paradigms, to be critical nodes for attention and arousal (***Xi et al., 2008***; ***van Swinderen et al., 2009***; ***Chow et al., 2011***). Paying attention to the right features of one's environment, whether naively or as a learned response, has high survival value. The fact that MBs are important sites of plasticity is likely related to this role in attention. The linkage of attention and arousal to MB output is consistent with our finding that suppression of arousal-promoting MB subsets increases sleep.

The involvement of DPMs, a neuron type previously believed to function exclusively as a regulator of memory consolidation, in control of sleep raises several interesting questions. First, are DPMs the only sleep-inducing regulators of MB activity? This seems unlikely, since memory consolidation is but one function of the MBs and there are other behavioral situations in which an animal might want to modulate MB-regulated arousal such as during courtship/aggressive behaviors (***Baier et al., 2002***; ***Sakai and Kitamoto, 2006***), or more generally during the integration of internal and external cues and decision making (***Zhang et al., 2007***; ***Neckameyer and Matsuo, 2008***; ***Krashes et al., 2009***; ***Donlea et al., 2012***; ***Bracker et al., 2013***). A second question is whether DPMs might also have a role in regulating MB output in contexts other than during memory storage. Our data suggest a role for DPMs in maintaining basal levels of nighttime sleep indicating that they may be responsive to other types of

input. An understanding of the regulation of DPMs and their in vivo activity patterns will be required to gain insight into these issues.

Inhibition in memory consolidation

A potential role for GABA-mediated inhibition in memory consolidation is novel. Long-term memory storage in mammals is believed to involve a transfer of information from one brain region to another. In *Drosophila*, it is associated with sequential potentiation of activity in specific MB neuropils. Elegant studies using conditional inhibition of transmitter release have provided a temporal ordering of transfer (***Keene and Waddell, 2007***; ***Cervantes-Sandoval et al., 2013***; ***Dubnau and Chiang, 2013***) leading to the idea that the role of DPM neurons is to facilitate the movement of memory from α′/β′ lobes, one initial site of memory storage, to a more permanent home in α/β lobes and perhaps other neurons (***Chen et al., 2012***). Given the clear requirements for molecular pathways associated with synaptic potentiation, and the need for synaptic transmission from both DPMs and α′/β′ neurons after acquisition, the view that information transfer involves a positive feedback loop between these cell types makes sense.

The data presented here, however, strongly suggest that the DPM neurons are inhibitory. It is clear that potentiated output from α′/β′ neurons is required for consolidation, so how could their inhibition facilitate this process? Our findings suggest DPMs are unlikely to participate directly in the excitatory arm of a recurrent feedback loop. However, both models and physiological data related to mammalian cortical/hippocampal recurrent feedback circuits involved in the maintenance of stable memory states also require the presence of inhibition (***Buzsaki and Chrobak, 1995***; ***Hasselmo et al., 1995***; ***Battaglia and Treves, 1998***; ***Chance and Abbott, 2000***). Interestingly, the coordination of excitatory activity amongst diverse functional sub-circuits in the mammalian hippocampus and neocortex is regulated by the activity of broadly-projecting, gap-junction coupled inhibitory neurons/networks (***Freund and Antal, 1988***; ***Gibson et al., 1999***; ***Tamas et al., 2000***; ***Baude et al., 2007***; ***Jinno et al., 2007***) which have been proposed to potentially control the timing of memory replay events during sleep/memory consolidation (***Viney et al., 2013***). The DPM-APL network may represent an analogous set of neurons in *Drosophila* which function to coordinate the temporal stabilization, gating and transfer of different memory stages between different sub-circuits within the MBs. While APL neurons have been shown to broadly inhibit recurrent feedback from all MB Kenyon cells to all MB Kenyon cells (***Lin et al., 2014***), it has been proposed that DPMs may impose a directionality on internal MB feedback which would allow for memory transfer and consolidation (***Yu et al., 2005***). Although, inhibition was not previously considered for such a role, it may be capable of coordinating the timing of prime lobe output in a way that is not possible via excitation. Thus, our data are not inconsistent with the presence of an excitatory recurrent feedback loop within the MB α′/β′ lobes, but rather provide information that constrains future models in a new way and suggests new possibilities for the role of DPMs that may not have been considered previously.

What kind of role could inhibitory neurons play? While α′/β′ neurons need to be active during acquisition, DPMs do not and this temporal difference suggests some testable possibilities for the role of DPM-mediated inhibition in consolidation. One idea is that temporally-regulated inhibition of potentiated α′/β′ output could serve as a way of sharpening the transition of memory from one neuropil to another by suppressing activity in the brain area from which information has already been transferred. A second possibility is that a period of inhibition during consolidation is a way of preventing new, potentially interfering, information from being encoded in α′/β′ before the first memory is transferred to α/β. A third possibility is that the function of inhibition is actually to provide precisely timed rebound excitation to α′/β′. This would not have been seen in our imaging experiments due to the slow kinetics of ATP washout, but could actually result in feedback excitation of MB neurons.

All of these models imply that there is a very tight temporal ordering of activity within the circuit, with DPM neurons suppressing α′/β′ neuron activity in a narrow window either before or after their output function has been completed. It is important to note that none of these ideas is inconsistent with the demonstrated requirement for α′/β′ activity during the post-training consolidation period. GABA- and 5HT-mediated inhibition is not equivalent to the action of Shi^{ts}, which completely shuts off neurotransmission. Inhibition is often modulatory rather than switch-like and can even be compartment-specific—for example, it could serve to alter the ratio of activity in α′/β′ to that in α/β. More precise mapping of the connectivity and branch-specific activity in the MB neuropil will be required to develop a more detailed model.

Coupling sleep and memory consolidation at the cellular level

Although investigators have speculated that memory consolidation and sleep interact, an actual understanding of how they are related at the circuit level has been elusive. This study is the first demonstration of a cellular- and circuit-level mechanism for the coupling of sleep and consolidation. The fact that memory and sleep are behaviorally linked even in the insect (**Ganguly-Fitzgerald et al., 2006**; **Donlea et al., 2011**) is evidence of the evolutionary importance of coupling these two processes. The simplicity of the cellular mechanism in *Drosophila*, using a single pair of neurons to carry out both functions, provides an example of how coupling can occur in a small nervous system and suggests a template for understanding it in larger brains.

Materials and methods

Fly stocks

Fly stocks were raised on modified Brent and Oster cornmeal-dextrose-yeast agar media (**Brent et al., 1974**) Per batch: 60 l H_2O, 600 g Agar, 1950 g flaked yeast, 1451 g cornmeal, 6300 g dextrose, 480 g NaKT, 60 g $CaCl_2$, 169 g Lexgard dissolved in ethanol. Flies were raised under a 12:12 light:dark cycle at 25°C except for animals carrying UAS-dTrpA1 which were raised at 22°C or flies carrying either *pTub-GAL80^{ts}* or *UAS-IVS-Syn21-Shi^{ts}* which were raised at 18°C. *UAS-P2X2* (**Lima and Miesenbock, 2005**), *lexAop-P2X2*, *lexAop-Epac1-camps (1A)*, *lexAop-GCaMP3.0* (**Yao et al., 2012**), and *UAS-Epac1-camps(55A)* (**Shafer et al., 2008**), flies were kindly provided by Dr Orie Shafer. *UAS-Arclight* (**Cao et al., 2013**) was a gift from Dr Michael Nitabach, and the *5HT7-GAL4* flies (**Becnel et al., 2011**) were a gift from Dr Charles Nichols. The following lines have also been previously described: *20xUAS-IVS-GCaMP6M* (**Akerboom et al., 2012**), *UAS-Synapto-pHlorin* (SpH) (**Meisenbock et al., 1998**), *UAS-GCaMP3.0* (**Tian et al., 2009**), *UAS-dTrpA1* (chromosome 2 insertion site) (**Hamada et al., 2008**), *UAS-Rdl-RNAi 8-10J* (**Liu et al., 2007**, **2009**), *20xUAS-IVS-Syn21-Shi^{ts}* (**Pfeiffer et al., 2012**), *UAS-mCD8-GFP* (**Lee and Luo, 1999**), *UAS-Brp-short-GFP* (**Schmid et al., 2008**; **Fouquet et al., 2009**), *UAS-Kir2.1* (**Baines et al., 2001**), *c316-GAL4* (**Waddell et al., 2000**), *c305a-GAL4* (**Krashes et al., 2007**), *NP2721-GAL4* (**Wu et al., 2011**), *VT64246-GAL4* (**Lee et al., 2011**), *MB247-GAL4* (**Zars, 2000**), *MB247-lexA* (**Pitman et al., 2011**), *R58E02-lexA* (**Liu et al., 2012**), *MB-GAL80* (**Thum et al., 2007**), *eyeless-GAL80* (**Chotard et al., 2005**), *ptub-GAL80^{ts}* (**McGuire et al., 2003**), *ptub>GAL80>* (**Gordon and Scott, 2009**), *LexAOP-Flp* (**Shang et al., 2008**), and *GH146-GAL4*, *NP5288-GAL4*, *NP2631-GAL4* (**Tanaka et al., 2008**). The following RNAi lines were obtained from the VDRC (**Dietzl et al., 2007**): *VGAT-RNAi* (X-stock #45917), *VGAT-RNAi* (II- stock #45916), *Trh-RNAi* (II-#105414), *GABA$_B$R3-RNAI* (III-#50176). The following RNAi lines have been functionally verified previously: *Rdl-RNAi* (**Liu et al., 2007**), *GABA$_B$R3-RNAi* (**Dahdal et al., 2010**).

The genetic intersectional method used to restrict expression to the MB is described in **Shang et al. (2008)**. MB restriction to the prime lobes with *c305a* is shown in **Perrat et al. (2013)**.

SuperClomeleon flies

13xLexAOP-IVS-Syn21-SuperClomeleon expressing flies were generated using the SuperClomeleon construct designed by **Grimley et al. (2013)**. Gateway cloning (Invitrogen) was used to create the entry vectors pDonr221-13xLexAOP and pDonrP2rP3-Syn21-SuperClomelon-p10. A modified version of pBPGUw (**Pfeiffer et al., 2008**), pBPGUw-R1R3-p10 was used for gateway recombination and injection into flies where it was targeted to the attP40 landing site on the second chromosome. pBPGUw-R1R3-p10 was generated from the following modifications to pBPGUw: removal the DSCP (Drosophila Synthetic Core Promoter), replacement of the attR2 Gateway recombination site with attR3, and replacement of the weaker Hsp70 terminator with the stronger p10 terminator sequence (**Pfeiffer et al., 2012**).

The entry vector, pDonr221-13XLexAOP, was generated by PCR and Gateway cloning of the 13XLexAOP-Hsp70 TATA-IVS sequence from pJFRC19 (**Pfeiffer et al., 2010**), into the pDonr221 Gateway entry vector. The pDonrP2rP3-SuperClomeleon entry vector was generated by PCR and Gateway cloning of the SuperClomeleon sequence from pUC19-SuperClomeleon (**Grimley et al., 2013**) into the pDonrP2rP3 Gateway entry vector. Primers for the pDonr221 construct were designed by fusing Gateway attB1 and attB2 sequences upstream and downstream, respectively, of the 13xLexAOP and IVS sequences.

Primers for pDonrP2rP3-SuperClomeleon were designed by fusing Gateway attB2r and attB3 sequences upstream and downstream, respectively, of the SuperClomeleon sequence. To enhance

expression, the 21bp Syn21 sequence (**Pfeiffer et al., 2012**) was added to the forward primer just upstream of SuperClomeleon. All PCRed and ligated sequences were verified by sequencing before injection into flies **Table 1**.

Behavioral analysis

Individual virgin female flies were housed separately in 65 mm × 5 mm glass tubes (Trikinetics, Waltham, MA) containing 5% agarose with 2% sucrose. Parafilm with pinholes poked in it was used to cover the open end of each tube. 2- to 5-day old flies were entrained under standard light–dark conditions, with a 12 hr light phase and followed by 12 hr dark phase for 3–4 days prior to collection of data for sleep analysis. Locomotor activity was collected with DAM System monitors (Trikinetics) in 1 min bins as previously described (**Agosto et al., 2008**). Sleep was defined as bouts of uninterrupted inactivity lasting for five or more minutes (**Hendricks et al., 2000**; **Shaw et al., 2000**). Sleep/activity parameters (total sleep, mean sleep episode duration, maximum sleep episode duration, number of sleep episodes, and activity while awake) were analyzed for each 12-hr period of light or dark conditions and averaged across 3 days. Sleep analysis was conducted using an in-house Matlab program described previously (**Donelson et al., 2012**). Since we found that some, but not all sleep data were not normally distributed, we chose to use the less powerful, but more conservative Mann–Whitney/Wilcox ranked sum test rather than ANOVA and Tukey post-hoc tests, which assume data are normally distributed. As the Mann–Whitney/Wilcox ranked sum test is a pairwise test, this generates two p values, one for experimental vs the UAS control and one for the experimental vs GAL4 control line. In all figures only the most conservative/numerically greatest p value is reported.

For temperature-shift experiments, flies expressing either Shi^{ts} or *Tub-GAL80*^{ts} were raised at 18°C, whereas flies expressing dTrpA1 were raised at 22°C. In all cases, baseline data were recorded at the respective rearing temperature (18°C or 22°C) and compared to sleep at the activation (dTrpA1) or suppression (Tub-$GAL80^{ts}$ and Shi^{ts}) temperature of 31°C. The effect of heat on sleep is highly sensitive to genotype. In order to assess heat-induced changes in sleep, first, the baseline sleep of each fly at either 18°C or 22°C was subtracted from sleep of the same fly at 31°C and this difference was averaged together across flies of the same genotype. Following baseline subtractions for each genotype, average GAL4 or UAS values were then subtracted from average experimental group values to obtain minutes of sleep gained or lost vs controls.

Immunohistochemistry

For immunostaining, a standard fixation and staining protocol was used. Briefly, brains were dissected in ice-cold PBS and were fixed immediately after dissection for 15 min at room-temperature in 4% paraformaldehyde (vol/vol). Brains were incubated in PBS containing 0.5% Triton X-100, 10% NGS and primary or secondary antibodies for one night each with 3 × 15 min washes between each incubation. Brain samples were then mounted using Vectashield and were visualized by a Leica TCS SP5 confocal microscope with a 20×, 40×, or 63× objective lens. All images were taken sequentially to prevent bleed-through between channels. For colocalization, either mouse anti-GFP (1:200, Roche Applied Biosciences) or rabbit anti-GFP (1:1,000, Invitrogen A11122) was used together with transmitter-specific primary antibodies as follows: rabbit anti-dVGAT (1:400, (**Fei et al., 2010**), a kind gift from Dr D.E. Krantz), rabbit anti-GABA (1:200, Sigma; Cat. No. A2052), rabbit anti-Gad1 (1:500, kind gift from Dr FR Jackson), mouse anti-choline acetyltransferase (ChAT) (1:200, code 4B1 Developmental Studies Hybridoma Bank; [**Takagawa and Salvaterra, 1996**; **Yasuyama et al., 1996**]),

Table 1. Primer sequences

Primers (5′-3′)	Sequence (Gateway sequences are in caps and vector sequences are lower case)
attB1-13xLexAOP forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTATgcatgcctgcaggttactgtac
attB2-IVS reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTAggccgcctgaagtaaaggataag
attB2r-Syn21-SuperClomeleon forward	GGGGACAGCTTTCTTGTACAAAGTGGAAAA*CTTAAAAAAAAAAAT*CAAAat ggtgagcaagggcgagg,
attB3-SuperClomeleon reverse	GGGGACAACTTTGTATAATAAAGTTGCttaaagcttcttgtacagctcgtccatg

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rabbit anti-serotonin (1:1,000, Sigma S5545), and mouse anti-tyrosine hydroxylase (TH) (1:500, Immunostar 22,941). Alexa Fluor 488 and 635 anti-mouse or anti-rabbit secondary antibodies (1:200, Invitrogen) were used to visualize staining patterns. Alexa 488 was always used to label GFP so that any residual endogenous GFP fluorescence would be of a similar wavelength as the dye and would not bleed through to the 633 wavelength channel.

Image processing and intensity correlation analysis (ICA)

All image processing was done using the freely available FIJI (IMAGEJ) software and plugins (***Schindelin et al., 2012***). Background was subtracted from all confocal stacks prior to further processing. All images are sums or maximum intensity Z-projections of the relevant confocal slices. Quantification of cells with positive/negative staining was done by visually comparing colocalization of GFP and antibody staining in multiple individual Z-slices. Cell bodies/MB lobe sets where high background prevented interpretation of staining were excluded. For the images presented in ***Figure 3***, Intensity Correlation Analysis (ICA) was also performed to assess spatial colocalization of staining between channels (***Li et al., 2004***). Rather than only comparing visible overlap of the absolute fluorescence intensity in each channel (red plus green equals yellow), which is subject to viewer bias and differences in staining intensity between channels, this method determines whether changes in staining intensity covary or are correlated between channels. This provides an objective and spatially specific representation of colocalization. ICA analysis generates a correlation/colocalization value for each pixel defined by the Product of the Differences from the Mean (PDM) that is, PDM= (red intensity-mean red intensity) × (green intensity – mean green intensity). PDM values for each pixel can then be visualized as an image showing positive intensity correlation (PDM>0) and negative intensity correlation (PDM<0). Relative PDM value scales are shown on each figure generated from ICA analysis.

Functional fluorescence imaging

Adult hemolymph-like saline (AHL) consisting of (in mM) 108 NaCl, 5 KCl, 2 $CaCl_2$, 8.2 $MgCl_2$, 4 $NaHCO_3$, 1 NaH_2PO_4-H_2O, 5 trehalose, 10 sucrose, 5 HEPES; pH 7.5 (***Wang, 2003***) was used to bathe the brain, as previously described (***Wang, 2003***; ***Shang et al., 2011***). For SuperClomeleon experiments, the AHL pH was increased to 7.7 to optimize response magnitude. Test compounds adenosine 5′-triphosphate magnesium salt (ATP), carbamoylcholine chloride (CCh), picrotoxin (PTX), and γ-aminobutyric acid (GABA) were purchased from Sigma–Aldrich (St Louis, MO), serotonin hydrochloride (5HT) was purchased from Tocris Bioscience (Minneapolis, MN), and tetrodotoxin (TTX) was purchased from Abcam Biochemicals (Cambridge, England). ATP, CCh, and TTX were dissolved in milliQ water and frozen as aliquot stocks, which were then prepared for experiments by dilution in AHL. PTX was dissolved and frozen in DMSO aliquots, which were diluted in AHL for experiments. All solutions used in experiments with PTX were prepared with the same percentage of DMSO. GABA and 5HT were dissolved directly in AHL immediately prior to the experiment, and 5HT was kept in light-shielded containers to prevent degradation.

Imaging experiments were performed using a naked brain preparation. Flies were anesthetized on ice, and brains were dissected into cool AHL. Dissected brains were then pinned to a layer of Sylgard (Dow Corning, Midland, MI) silicone under a small bath of AHL contained within a recording/perfusion chamber (Warner Instruments, Hamden, CT). Brains expressing GCaMP3.0, Arclight, and SpH were allowed to settle for 5 min after dissection to reduce movement. These brains were then exposed to fluorescent light for approximately 30 s before imaging to allow for baseline fluorescence stabilization, while brains expressing the FRET sensors Epac1-camps or SuperClomeleon were exposed an extra 5 min to minimize differences in photobleaching rates between the CFP and YFP fluorophores, as YFP has been described to photobleach more slowly than CFP (***Shafer et al., 2008***; ***Pirez et al., 2013***). Perfusion flow was established over the brain with a gravity-fed ValveLink perfusion system (Automate Scientific, Berkeley, CA). ATP, CCh, GABA, or 5HT were delivered by switching perfusion flow from the main AHL line to another channel containing diluted compound after 30 s of baseline recording for desired durations followed by a return to AHL flow. To control for the effects of switching channels, a vehicle control trial was performed by switching to another line containing AHL for the same duration as the experimental trial.

Imaging was performed using an Olympus BX51WI fluorescence microscope (Olympus, Center Valley, PA) under an Olympus x40 (0.80W, LUMPlanFI) or x60 (0.90W, LUMPlanFI) water-immersion objective, and all recordings were captured using a charge-coupled device camera (Hamamatsu ORCA

C472-80-12AG). For GCaMP3.0, Arclight, and SpH imaging, we used the following filter set (Chroma Technology, Bellows Falls, VT): excitation, HQ470/x40; dichroic, Q495LP; emission, HQ525/50m. For EPAC and SuperClomeleon, a 86002v1 JP4 (436; Chroma Technology) excitation filter was used, and emitted light from the CFP and YFP flurophores was separated using a splitter (Photometrics DV^2 column) with the emissions filters D480/30m and D535/40m (Photometrics, Tucson, AZ), which allowed for simultaneous collection from both fluorescence channels. Frames were captured at 2 Hz with 4× binning for either 2 min or 4 min using μManager acquisition software (***Edelstein et al., 2010***). Neutral density filters (Chroma Technology) were used for all experiments to reduce light intensity to limit photobleaching.

Although there are tools for temporally controlled activation such as dTrpA1 or Channelrhodopsin (ChR2) that are well-characterized, we utilized P2X2 receptors for the majority of our experiments. Activation wavelengths for ChR2 overlap with those of many fluorescent sensors such as GCaMP and EPAC, so ChR2 could not be used in this circuit due to the close proximity of the cells and processes. Applying heat to activate dTrpA1 causes changes in refractive index, which disrupt focus, necessitating manual focus correction in the cases where we used this technique.

Regions of interest (ROIs) were selected within the horizontal or vertical lobes of the MBs or, in the case of DPM neurons, over the horizontal projections to the MBs. Figures depict responses in horizontal lobes or projections; however, similar results were observed in the vertical lobes when noted in the figure captions. For recordings using GCaMP3.0, Arclight, and SpH, ROIs were analyzed using custom software developed in ImageJ (***Schindelin et al., 2012*** and National Institute of Health, Bethesda, MD). Briefly, the percent change in fluorescence over time was calculated using the following formula: $\Delta F/F = (F_n - F_0)/F_0 \times 100\%$, where F_n is the fluorescence at time point n, and F_0 is the fluorescence at time 0. For GCaMP3.0 and SpH, maximum fluorescence change values were determined as the maximum percentage change observed for each trace over the entire duration of each imaging experiment. For Arclight, because increases in voltage are represented as decreases in fluorescence, maximum fluorescence change values were determined as the minimum percentage change. Maximum values for each group were then averaged to calculate the mean maximum change from baseline.

For recordings using EPAC or SuperClomeleon, ROIs were analyzed using custom software developed in MATLAB (The MathWorks, Natick, MA). This analysis package is provided in ***Source Code 1***. Briefly, identical ROIs were selected from both the CFP and YFP emissions channels, and the fluorescence resonance energy transfer (FRET) signal (YFP/CFP ratio) was calculated for each time point and normalized to the ratio of the first time point. The relative cAMP changes were determined by plotting the normalized CFP/YFP ratio (percentage) over time. As with GCaMP3.0 and SpH, the average maximum percent change values were determined as the mean maximum values for each group.

Statistical analyses were performed using MATLAB (The MathWorks). A Kruskal–Wallis one-way ANOVA was used to determine statistical significance between the experimental group and the two negative controls. In cases in which there was significance in the ANOVA, a Mann–Whitney U test (also known as Wilcoxon rank-sum test) was used to determine the significance between the experimental group and each negative control. In all figures only the most conservative/numerically greatest p value is reported. Results are expressed as means ± standard error of the mean (SEM).

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Additional information

Competing interests

LCG: Reviewing editor, *eLife*. The other authors declare that no competing interests exist.

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Additional files

Supplementary file

• Source code 1. Custom software developed in MATLAB.
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Short Neuropeptide F Is a Sleep-Promoting Inhibitory Modulator

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SUMMARY

To advance the understanding of sleep regulation, we screened for sleep-promoting cells and identified neurons expressing neuropeptide Y-like short neuropeptide F (sNPF). Sleep induction by sNPF meets all relevant criteria. Rebound sleep following sleep deprivation is reduced by activation of sNPF neurons, and flies experience negative sleep rebound upon cessation of sNPF neuronal stimulation, indicating that sNPF provides an important signal to the sleep homeostat. Only a subset of sNPF-expressing neurons, which includes the small ventrolateral clock neurons, is sleep promoting. Their release of sNPF increases sleep consolidation in part by suppressing the activity of wake-promoting large ventrolateral clock neurons, and suppression of neuronal firing may be the general response to sNPF receptor activation. sNPF acutely increases sleep without altering feeding behavior, which it affects only on a much longer time scale. The profound effect of sNPF on sleep indicates that it is an important sleep-promoting molecule.

INTRODUCTION

Although animals need to coordinate sleep with food intake and/or metabolism, the relationship is quite complex. Hunger acutely suppresses sleep in flies and humans (Keene et al., 2010; MacFadyen et al., 1973), and sleep need is antagonistic to foraging/feeding behaviors. However, sleep is also essential for maintaining normal feeding patterns, body mass, and metabolism (Howell et al., 2009; Knutson and Van Cauter, 2008). Neuropeptide Y (NPY) plays a central role in regulating both sleep and feeding in rats and humans. Although NPY receptors are potential drug targets for obesity treatment (Dyzma et al., 2010; Yulyaningsih et al., 2011), their regulation of sleep is not well understood and may be state-dependent. Moreover, injection of NPY into different brain regions led to either sleep promotion or suppression in rats, depending on the site of injection and dosage (Dyzma et al., 2010), while repetitive intravenous injection of NPY promoted sleep in young men (Antonijevic et al., 2000).

Flies express two NPY-like peptides, NPF and sNPF, which bind to NPFR1 and sNPFR, respectively (Garczynski et al., 2002; Mertens et al., 2002; Vanden Broeck, 2001). Both receptors are structurally similar to vertebrate neuropeptide Y2 receptors (Garczynski et al., 2002; Mertens et al., 2002). In the adult, NPF is expressed predominantly in two pairs of neurons (Wen et al., 2005), whereas sNPF is broadly expressed in multiple brain regions, including the mushroom body (MB), the pars intercerebralis (PI), the central complex (CC), and some clock neurons (Johard et al., 2009; Nässel et al., 2008). NPF has been shown to be important for feeding in larvae (Shen and Cai, 2001; Wu et al., 2003), alcohol sensitivity (Wen et al., 2005) and context-dependent memory retrieval in adults (Krashes et al., 2009). The major function of sNPF has been proposed to be the regulation of feeding and metabolism in adults (Hong et al., 2012; Lee et al., 2004, 2008, 2009; Root et al., 2011). Although it has also been shown to modulate the fine-tuning of locomotion (Kahsai et al., 2010), a role for sNPF in sleep has not been identified.

In a screen to test the role of different peptidergic neurons in the adult brain, we identified sNPF-expressing neurons as potently sleep promoting. We found that the s-LNv clock neurons are part of this sNPF-expressing sleep-promoting circuit, and the wake-promoting l-LNvs are a postsynaptic target. sNPF has very different effects on feeding circuits, suggesting that the role of sNPF in feeding is more indirect and unrelated to its acute sleep-promoting effects.

RESULTS

Activation of sNPF Neurons Rapidly Increases Sleep Independent of Changes in Locomotion

To test the role of different subsets of adult brain peptidergic neurons in sleep (Figure 1A), we used the warmth-activated dTRPA1 cation channel to acutely activate nine different peptidergic neuron classes (Hamada et al., 2008). Because environmental light affects *Drosophila* sleep behaviors (Shang et al., 2011), we assayed activation under both light-dark (LD) and dark-dark (DD) conditions. To our surprise, most peptidergic neurons, including those that express NPF, do not affect sleep under either condition (Figure 1A). Neurons expressing DMS or DILP only affect sleep in LD conditions, and neurons expressing SIFa or CCAP only in DD conditions. This context-dependence may indicate that these neurons only affect sleep indirectly by changing other internal physiological states.

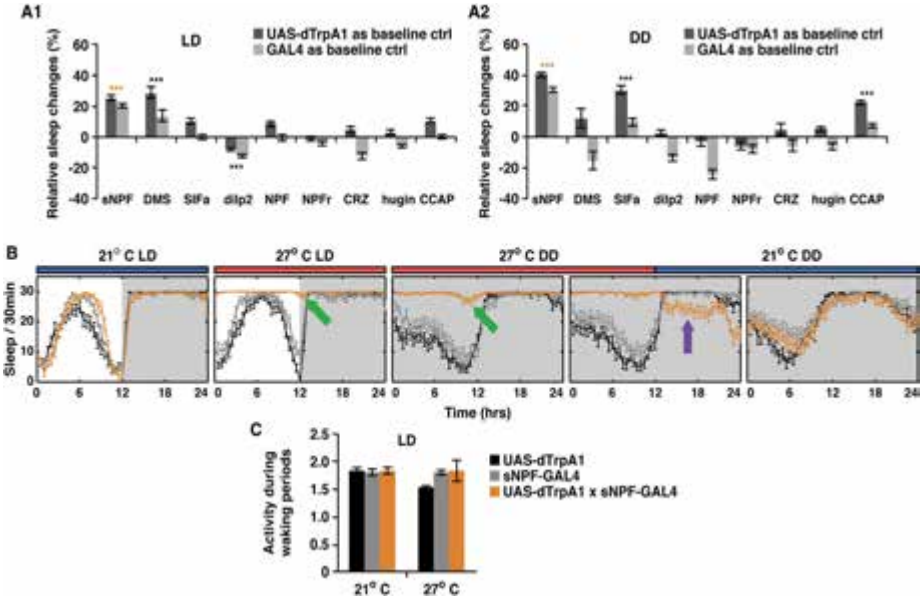

Figure 1. Activation of sNPF-Expressing Neurons Promotes Sleep Independent of Changes in Locomotion

(A) A dTRPA1 screen identified neurons expressing sNPF as a potent sleep-promoting system when activated in fly brains in both LD (A1) and DD (A2). All experiments were repeated at least three times except for *hugin-GAL4* (two repeats). Calculation of relative sleep changes and statistical analyses are described in the Experimental Procedures. Most driver lines did not show significant effects on sleep pattern or sleep time. DMS-expressing neurons promoted sleep in LD while sIFamide and CCAP neurons promoted sleep in DD. *sNPF-GAL4* is the only driver line that dramatically increased total sleep and altered sleep pattern in both LD and DD. n = 52 for *sNPF-GAL4:dTRPA1* flies.

(B) Sleep analysis showed that heat-induced firing of sNPF-expressing peptidergic neurons dramatically and rapidly (within 1 hr) increased quiescent state in both LD and DD. Release from activation was followed by an immediate negative sleep rebound (purple arrow). The green arrows indicate flies still showed circadian-related locomotor evening peak around ZT and CT12 (see Figure S1 for locomotor analyses).

(C) Waking activity between controls and experimental groups were compared using the DAM system, measured as average number of beam crosses per minute. All the genotypes show similar activity at 21°C and heat activation of sNPF neurons led to no significant change of activity level while awake.

Data are presented as mean ± SEM; $^{*}p < 0.01$, $^{**}p < 0.001$, and $^{***}p < 0.0001$ are significant differences from the control group (ANOVA with Tukey post hoc test, described in the Experimental Procedures). See also Figure S1 and Movies S1 and S2.

The only condition-independent cell group was the short-form NPY-like peptide sNPF, which led to a dramatic increase in quiescence in both LD and DD conditions (Figures 1A and 1B). High-resolution computer video tracking confirmed the results obtained with the DAM system (Donelson et al., 2012; Figure S6 available online). Upon inactivation of dTRPA1 at 21°C, these effects were rapidly reversed, and the mean duration of quiescent episodes decreased (Figure 1B; Figure S6B). Remarkably, flies slept even less after reversal of dTRPA1 activation, i.e., they manifested negative sleep rebound (Figure 1B, purple arrow; Figure S6B green arrows), suggesting that the observed quiescence is really sleep and that this state can be homeostatically regulated in both directions (Hendricks et al., 2000; Shaw et al., 2000).

Importantly, excess quiescence is not due to a loss of ability to engage in locomotor activity. Quantitative analysis of single fly behavior showed similar or even slightly higher locomotor activity during periods when flies were awake (Figure 1C; Figure S6B). We also directly tested locomotion within 2 hr after the temperature shift by tapping vials and assaying negative geotaxis behavior. Although at this time point *sNPFGAL4:dTRPA1* flies already showed increased quiescence due to activation of sNPF-expressing neurons, they still rapidly climbed to the top of the vials when stimulated (Movie S1). However, most flies were not responsive to the initial tap compared with controls 3 days after the shift (data not shown). Consistent with an increased sensory/arousal threshold, these flies required several taps but then climbed with a similar speed (Movie S2). Moreover, flies with activated sNPF neurons still exhibited spontaneous circadian-related locomotor activity in both LD and DD conditions (Figure 1B, green arrows; see Figure S1 for locomotor analyses). This indicates that the locomotor circuitry is intact because it can be accessed by the circadian clock. Finally, release from dTRPA1 activation reversed the increased

locomotor activity observed during waking periods (Figure S6B, blue arrows). This indicates that the negative sleep rebound is not due to an increased intensity of locomotor activity.

sNPF Is Necessary for Sleep Maintenance

To investigate if the activity of sNPF neurons is required to maintain normal sleep, we silenced these neurons by coexpressing the Kir2.1 potassium channel (Nitabach et al., 2002). To restrict the silencing to adults, *tubulin-GAL80^{ts}* was used to block expression of *UAS-kir2.1* until adulthood (McGuire et al., 2003). After a temperature shift to release the GAL80 block, sleep levels and the mean duration of sleep bouts during the daytime were significantly reduced (Figure 2A). In contrast, control flies manifested a temperature-driven increase in daytime sleep.

Given the potency with which the sNPF-expressing neurons promote sleep, their activity should be regulated if they are part of normal sleep-promoting circuits. Because GABAergic neurons can function in wake promotion by $GABA_A$-mediated suppression of sleep-promoting regions (Y.S. and M.R., unpublished data; P. Haynes and L.C.G., unpublished data; Hassani et al., 2009), we knocked down the *Rdl* $GABA_A$ receptor in sNPF neurons. This indeed led to significant increases in both daytime and total sleep time as well as to longer sleep bouts, suggesting that sNPF neuron activation is normally attenuated by $GABA_A$ signaling (Figure 2B).

To determine if the sleep-promoting function of these neurons is due to the release of sNPF itself, we examined *sNPF^{c00448}* mutant flies, which have a more than 50% reduction of sNPF mRNA (Lee et al., 2008). These flies showed significant reductions of both daytime and nighttime sleep (Figure 2C). Sleep was also fragmented because the mean duration of sleep bouts was reduced by more than 50% (Figure 2C). Sleep was sensitive to gene dosage because heterozygous flies had intermediate phenotypes compared to control and homozygous flies (Figure 2C), although statistical significance for the gene dosage effect was only reached in the case of nighttime sleep. Sleep was also assayed in animals with an adult-specific knockdown of sNPF mRNA in adult brains. Both total sleep and nighttime sleep were significantly reduced (Figure 2D). Daytime sleep was also more fragmented, although the amount of daytime sleep was unaffected (Figure 2D). While the dTrpA1 activation strategy cannot rule out a role for a cotransmitter, these two independent methods of reducing sNPF levels also support a sleep-promoting function for the sNPF peptide in the adult fly.

Activation of sNPF Neurons Suppresses Sleep Homeostasis during Mechanical Deprivation

To test the idea that sNPF neuron activation might affect the sleep homeostat and therefore interfere with the effects of mechanical sleep deprivation (SD), we activated these neurons during traditional mechanical SD. The SD protocol was standard, with the exception that *sNPFGAL4:dTRPA1* and control strains were heated to 27°C during the 12 hr of deprivation to activate sNPF neurons and then returned to 21°C after the deprivation, at the end of the night (Figure 3). During the mechanical SD, the *sNPFGAL4:dTRPA1* strain appeared indistinguishable from the control strains, i.e., it manifested no sleep during these 12 hr as expected. This reflects true locomotor arousal because DAM data from unconscious or dead flies can be distinguished from live moving animals (Figure S2). Remarkably, however, this strain produced much less sleep rebound or recovery sleep after the deprivation than the control strains upon the return to 21°C. This can be seen both in comparisons of recovery day sleep levels of SD flies to non-SD flies (Figure 3B) and by comparison of the sleep of SD flies on the recovery day to their own pre-SD day sleep levels (Figure 3C). We conclude that activation of sNPF neurons during mechanical SD caused at least a partial sleep-like state, which was invisible with standard locomotor activity monitoring but could be interpreted by the sleep homeostat as genuine sleep. Another possibility is that sNPF provides a direct signal to the homeostat to indicate that sleep has occurred.

sNPF Promotes Nighttime Sleep through the s-LNv-to-l-LNv Circuit

sNPFGAL4 drives strong expression in many brain regions, including the MB, the PI and the FSB in the CC (Johard et al., 2008). We used multiple strategies to test the involvement of the MB because it is a previously identified sleep-promoting region (Joiner et al., 2006; Pitman et al., 2006) Addition of a *MB-GAL80* transgene blocked the expression of dTRPA1 in the MB without affecting the sleep-promoting effect of *sNPFGAL4:dTRPA1* flies (Figures S3B and S3E). The converse experiment, activation of just the MB-specific subset of *sNPF-GAL4* cells using an intersectional strategy (Shang et al., 2008), also did not produce a sleep phenotype (Figure S3E). Although the PI has also been shown to be involved in sleep (Crocker et al., 2010; Foltenyi et al., 2007), direct activation of subsets of the PI did not significantly increase total sleep in a state-independent manner (Figure 1A: DILP2-, CRZ-, SIFa-, and DMS-GAL4). Additionally, subdivision of the sNPF-GAL4 pattern with different GAL80s demonstrated no correlation of the sleep phenotype with PI or MB expression (Figures S3C–S3E). Therefore, most sleep-promoting sNPF neurons likely reside outside these two regions.

We then assayed PDF-expressing clock neurons. The 8 PDF+ small ventral lateral neurons (s-LNvs) are labeled by *sNPF-GAL4* and have recently been shown to express sNPF (Johard et al., 2009). Moreover, sNPF mRNA is one of the most strongly cycling transcripts in the s-LNvs (Kula-Eversole et al., 2010). As the 10 neighboring cells, the PDF+ wake-promoting large ventral lateral neurons (l-LNvs), express little or no sNPF (Johard et al., 2009; Kula-Eversole et al., 2010), RNAi knockdown of sNPF using *pdf-GAL4* should be specific for s-LNvs. Two independently generated RNAi lines against *sNPF* produced small but significant decreases in nighttime sleep without affecting daytime sleep (Figure 4A). Nighttime sleep bout length was also ~10%–50% shorter than control strains (Figure 4A). Therefore, sNPF within s-LNvs promotes normal nighttime sleep.

The wake-promoting l-LNvs express sNPFR (Kula-Eversole et al., 2010; Nitabach and Taghert, 2008). Because *c929GAL4* expresses in the l-LNvs (and in multiple other peptidergic cells) but not in the s-LNvs (Shang et al., 2008), we used *c929GAL4, tubulin-GAL80^{ts}* and *UAS-sNPFR-DN* to downregulate sNPF signaling (Lee et al., 2008) in adult cells. *tubulin-GAL80^{ts} ; c929GAL4:UAS-sNPFR-DN* flies had no detectable change in

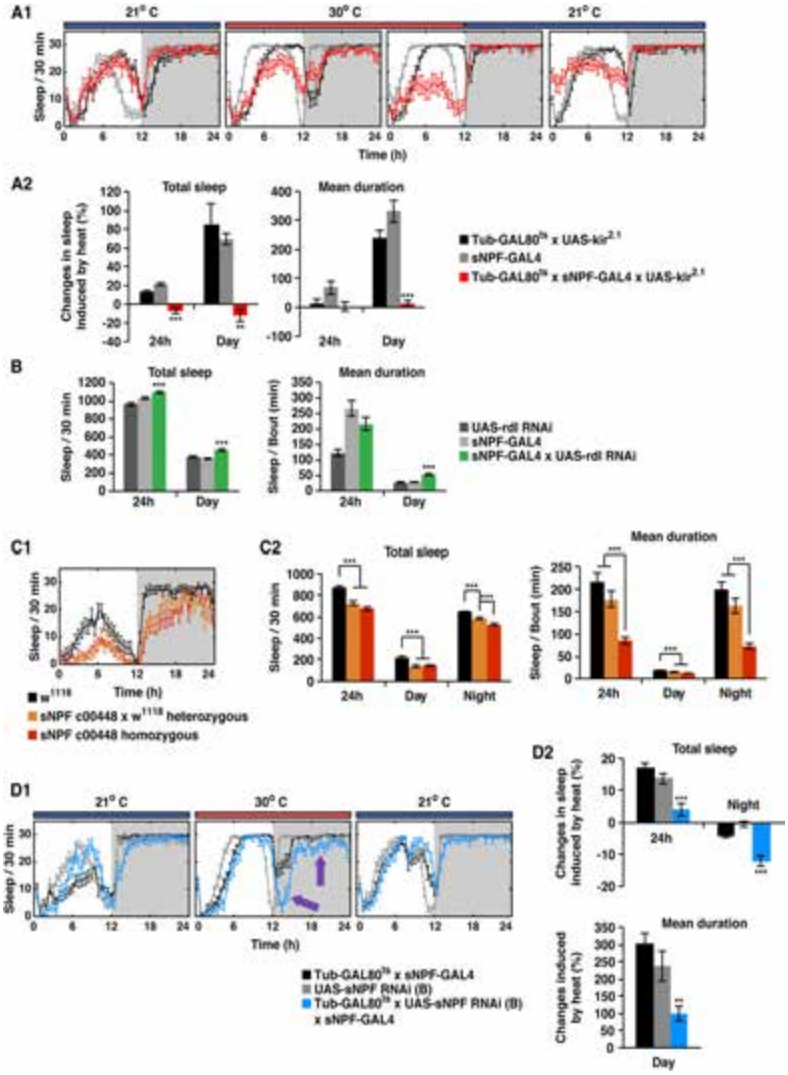

Figure 2. sNPF Is Required for Maintaining Sleep

(A) sNPF-expressing neurons are required for maintaining sleep. (A1) A *tubGAL80^{ts}* transgene was used to block the expression of *UAS-kir2.1* in *sNPF-GAL4* neurons at 21°C. The GAL80 protein was inactivated at 30°C, allowing the expression of *Kir2.1* mRNA driven by *sNPF-GAL4* in adult brains. Temporally controlled silencing of the sNPF neurons induced by heat led to a decrease of total as well as daytime sleep. The sleep loss was rapidly reversible once the GAL80 protein was reactivated at 21°C. (A2) Quantitative data for the heat-induced sleep loss and changes in mean bout duration. The calculation for heat induced sleep changes is described in the Experimental Procedures.

(B) Sleep-promoting sNPF neurons are suppressed by GABA through *Rdl* GABAA receptors in the daytime. The total 24 hr sleep time and daytime sleep for each genotype are shown, as well as mean bout duration.

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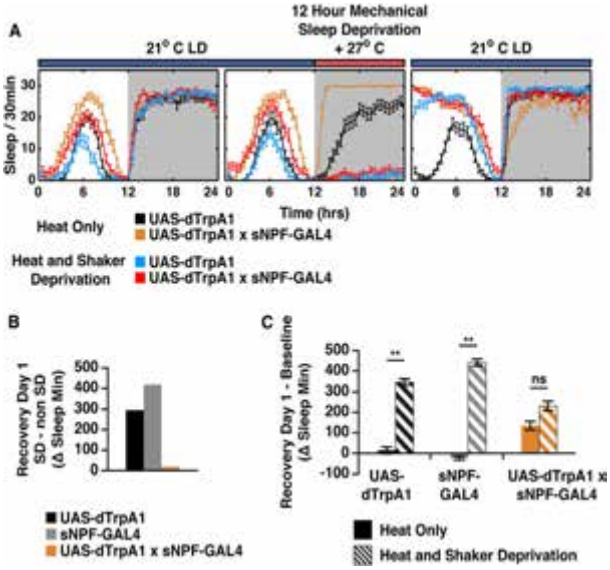

Figure 3. sNPF Regulates the Response to Sleep Deprivation.

(A) Sleep plots of control and *sNPF-GAL4*-driven dTRPA1. Animals were maintained at 21°C and baseline sleep recorded. At ZT12 of the sleep deprivation (SD) day, temperature was increased to 27°C for 12 hr ± mechanical SD. Flies were returned to 21°C to allow recovery sleep to occur. Animals with activated sNPF neurons that did not receive mechanical SD showed increased sleep, while controls showed a temperature-dependent decrease in sleep during the heat treatment.

(B) Amount of recovery sleep in SD flies was quantitated by comparison to sleep of siblings that were heated but did not receive SD. sNPF neuron activation significantly reduced recovery sleep.

(C) Recovery sleep was quantitated by comparison of each group (heat only, no SD, and heat +SD) to its previous day's sleep. Flies with activated sNPF neurons slept more than control flies even without SD, perhaps due to sleep inertia. Recovery sleep in SD flies was significantly different in flies with activated sNPF neurons (**$p < 0.001$, ANOVA, posthoc Tukey test shows only the UAS and GAL4 control heat alone are not significantly different from each other). Pairwise comparisons were all significantly different ($p < 0.0001$ for UAS and GAL4 controls, $p = 0.0019$ for the experimental cross). $n \geq 36$ for each genotype and condition. Data are presented as mean + SEM. See also Figure S2.

total sleep time or mean duration of sleep episodes at 21°C. After shifting to 30°C for 3 days, however, flies exhibited notable nighttime sleep fragmentation compared with parental controls (Figure 4B; for quantitative data, see Figure 4C). The effect on sleep consolidation was fully reversible after shifting back to 21°C (Figure 4B). Remarkably, addition of the *pdf-GAL80* transgene to flies carrying *c929GAL4* and *UAS-sNPFR-DN* strongly blocked the effects of *UAS-sNPFR-DN* (Figure 4D), indicating that most if not all of the sNPF effect on sleep from the diverse peptidergic neurons labeled by the *c929GAL4* driver is due to the l-LNvs.

sNPF Neuromodulation Is Predominantly Inhibitory

We used functional imaging to address the cellular mechanisms by which sNPF affects neuronal function. Flies carrying *pdf-GAL4:UAS-EPAC* express the FRET-based cAMP reporter in both l-LNvs and s-LNvs (Shang et al., 2011). Because we previously showed that the l-LNvs receive synaptic inputs from dopaminergic neurons, with bath application of 100 μM DA evoking a dramatic increase in cAMP (Shang et al., 2011), we used subsaturating concentrations of DA and coapplication sNPF. Twenty micromoles of DA with 20 or 80 μM sNPF suppressed cAMP responses in the l-LNvs compared with application of 20 μM DA alone (Figure S4A). Although these data suggest that the balance between sNPF and DA signaling in the l-LNvs affects nighttime sleep consolidation, the effect of sNPF did not always reach statistical significance. Moreover, sNPF alone did not alter FRET (Figure S4B).

To address the mechanism by which sNPF regulates neuronal function in a more general way, we assayed the electrophysiological effects of the sNPF via its receptor, sNPFR in larval central neurons. The *OK371-GAL4* driver was combined with *UAS-sNPFR* to ectopically express sNPFR in larval motor neurons. Perfusion of 20 μM sNPF reduced the firing response to current injection (Figure 5A; ANOVA, $F_{(1,15)} = 10.504$, $p = 0.005$). The shift in the input-output function of the neuron

(C) sNPF-deficient flies sleep less than genetic background control flies. (C1) The sleep plot in LD for control w^{1118}, heterozygous flies, as well as homozygous $sNPF^{c00448}$ flies from one experiment is shown. The heterozygous flies were F1 progeny from w^{1118} (the genetic background line) crossed to $sNPF^{c00448}$. (C2) Quantitative analysis shows that reduction of sNPF led to less total sleep and decreased mean bout duration in both daytime and nighttime. The effect is dose-dependent, i.e., the heterozygous flies slept less than the control flies but more than the homozygous flies.

(D) Transient knockdown of sNPF in the sNPF-expressing neurons led to nighttime sleep loss. (D1) A $tubGAL80^{ts}$ transgene was used to block the expression of *UAS-sNPFRNAi* in the *sNPF-GAL4* neurons at 21°C. GAL80 protein was inactivated at 30°C, allowing the expression of *UAS-sNPFRNAi* driven by *sNPF-GAL4*. Transient knockdown of sNPF induced by heat led to significant decrease of total as well as nighttime sleep (purple arrows). The sleep loss was rapidly reversible once the GAL80 protein was reactivated at 21°C. (D2) Quantitative data for the heat-induced sleep loss. The calculation for heat-induced sleep changes is described in the Experimental Procedures.

Data are presented as mean ± SEM; *$p < 0.01$, **$p < 0.001$, and ***$p < 0.0001$ are significant differences from the control group (ANOVA with Tukey post hoc test, described in the Experimental Procedures). See also Figure S3.

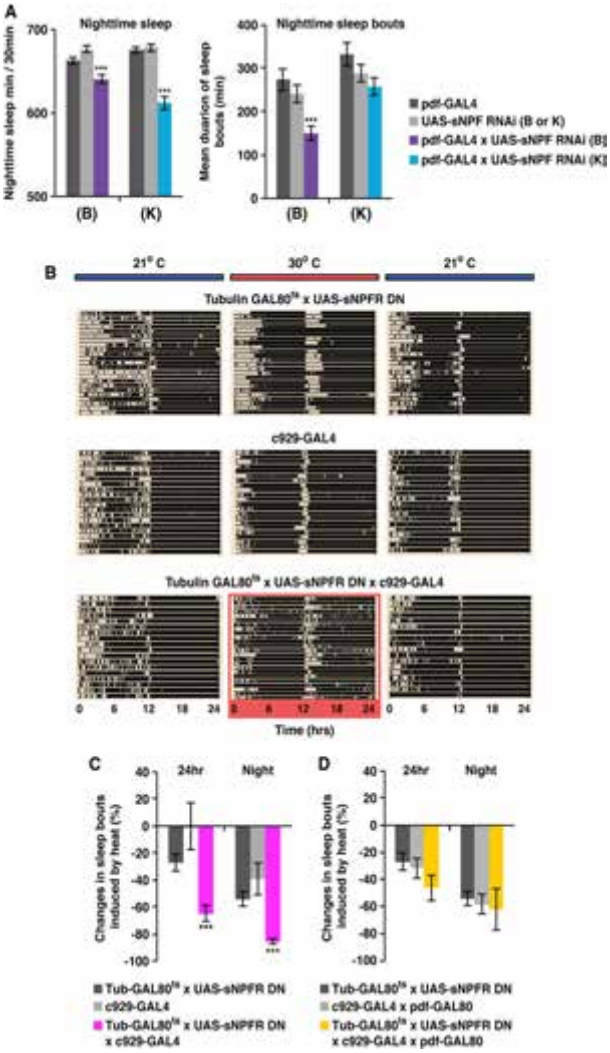

Figure 4. sNPF Promotes Nighttime Sleep through the s-LNv-to-l-LNv Circuit

(A) *pdf-GAL4*-driven knockdown of sNPF using two independently generated RNAi lines in s-LNvs led to sleep suppression at night. *pdf-GAL4* only drives *UAS* expression in the l-LNvs and s-LNvs (Renn et al., 1999). Because the l-LNvs do not express sNPF (Johard et al., 2009; Kula-Eversole et al., 2010), the knockdown of *sNPF* mRNA using *pdf-GAL4* should be specific for s-LNvs. More than 60 flies were tested in three trials. Nighttime sleep for the control and experimental lines are shown (left). Both RNAi lines led to significant decrease of nighttime sleep. The mean duration for the sleep episode at night for each genotype is shown (right). *UAS-sNPF-RNAi (B)* also affected sleep consolidation at night.

(B) Transient expression of *UAS-sNPFR-DN* in the c929+ cells caused sleep fragmentation. Raster plots of the sleep-wake pattern of individual flies in LD are shown for each genotype. Each row represents a single fly. Black bars are sleep episodes and white bars are wake episodes. A *tubGAL80^{ts}* transgene was used to block the expression of *UAS-sNPFR-DN* in the *c929-GAL4* neurons at 21°C. The *tubulin-GAL80^{ts},UAS-sNPFR-DN;c929-GAL4* flies showed a sleep pattern similar to control genotypes (top two blocks of rasters). Expression of *UAS-sNPFR-DN* in the *c929-GAL4* neurons was induced by shifting the temperature to 30°C (highlighted in red). Heat did not induce detectable changes in control strains, while severe sleep fragmentation was observed in the experimental flies. The fragmentation phenotype was rapidly reversed once the GAL80 protein was reactivated by reducing the temperature to 21°C.

(C) Quantitative analysis shows that the mean duration of 24 hr sleep as well as nighttime sleep episodes were significantly reduced in the *tubulin-GAL80^{ts},UAS-sNPFR-DN;c929-GAL4* flies at 30°C.

(D) Defects in sleep consolidation are due to the reduction of sNPFR signaling in the l-LNvs. The sleep fragmentation phenotype was rescued by a *pdf-GAL80* transgene, which blocks the expression of *UAS-sNPFR-DN* in the l-LNvs.

Data are presented as mean ± SEM; *p < 0.01, **p < 0.001, and ***p < 0.0001 are significant differences from control group (ANOVA with Tukey post hoc test, described in the Experimental Procedures). See also Figure S3.

was associated with a significant (p = 0.002) hyperpolarization in resting membrane potential, typically occurring within 1-2 min of treatment onset (Figure 5C). Vehicle had no effect (Figures 5B and 5D) and effects were completely dependent on expression of the sNPFR (C.G.V. et al., unpublished data). Taken together with the strong sleep-promoting effect of sNPF firing, hyperpolarization and inhibition of firing may be the general response to sNPF. This is consistent with the fact that NPY, the mammalian analog of sNPF, is primarily inhibitory (van den Pol, 2012).

sNPF Has Different Effects on Sleep and Feeding Circuits

The inhibitory nature of sNPF action in nonfeeding neurons contrasts sharply with its published role in feeding pathways. For example, sNPF enhances the responsiveness of olfactory

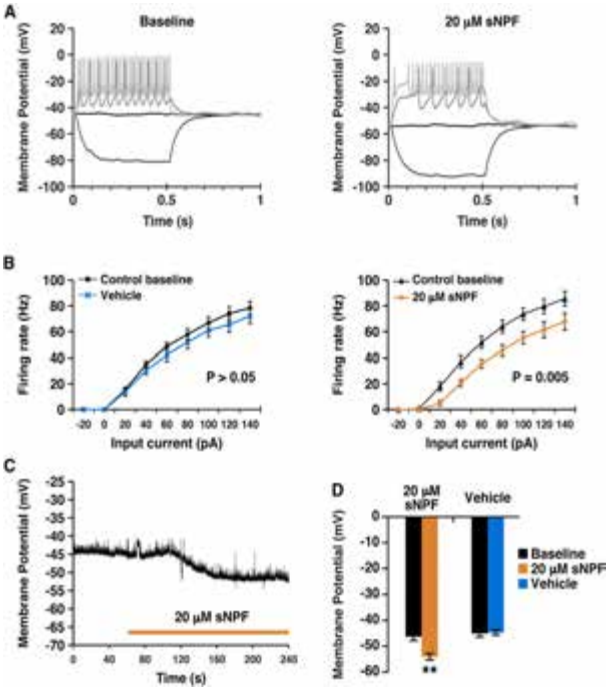

Figure 5. sNPF Reduces Neuronal Resting Membrane Potential and Suppresses Action Potential Generation

(A) Example of current clamp recordings made from third instar larval motor neurons expressing sNPFR before (left) and after (right) treatment with sNPF. Current was injected to depolarize the neuron and elicit action potentials. Only selected sweeps of the current injection protocol are shown for clarity.

(B) Quantification of current clamp data, plotting firing rate as a function of input current (F/I curve). Vehicle treatment (n = 6) in flies expressing sNPFR did not cause a significant change in F/I curve (left). sNPF (n = 11) caused a significant rightward shift in the curve, showing that more current was required to elicit the same spike rate after sNPF treatment (right). Data are plotted as mean ± SEM and significance calculated by one-way ANOVA.

(C) An example trace showing a typical hyperpolarization response to sNPF treatment. Duration of treatment is indicated by bar.

(D) Quantification of the effects of sNPF and vehicle treatment on resting membrane potential. Numbers in parentheses represent the number of animals in each condition.

Data are presented as mean ± SEM. ** represents $p < 0.001$, t test. See also Figures S4 and S5.

receptor neurons (ORNs), which promotes foraging (Root et al., 2011), and sNPF has been shown to directly activate cyclase in the neuronal BG2-c6 *Drosophila* cell line (Hong et al., 2012). We therefore examined the effect of sNPF on DILP cells, which respond to octopamine (OA) and have functions in feeding as well as sleep (Crocker et al., 2010; Lee et al., 2008). Although sNPF alone did not evoke detectable changes in FRET (data not shown), coapplication of 20 or 80 µM sNPF with subsaturating concentrations of OA (10 µM) evoked large increases of cAMP (Figures S5A and S5B), consistent with the excitatory effects of sNPF on the ORNs (Root et al., 2011). This is a direct effect because it was not blocked by TTX (Figure S5C).

However, transient downregulation of sNPFR signaling in DILP cells did not affect sleep under starvation conditions, i.e., sleep was inhibited indistinguishably from control strains (Figure 6). This is despite the fact that *dilp2-GAL4;UAS-sNPFR-DN* flies show defects in metabolism and growth (Lee et al., 2008). We therefore suggest that the modest effect of DILP cell activation with dTRPA1 on sleep under LD conditions (Figure 1A) and the imaging results (Figure S5) may reflect a role of DILP cells in metabolism rather than a direct modulation of sleep circuitry (c.f. Erion et al., 2012).

To further address the role of sNPF in fly feeding, we assayed the location of flies within behavior tubes subsequent to dTRPA1-mediated activation of sNPF cells. The temperature shift caused the flies to spend more time at the end of the tube containing food (Figure 7). Notably however, the onset of the location change or "food dwelling" was dramatically delayed from the onset of sleep by almost 12 hr (compare orange and green lines in Figure 7C). This was due to a very slow accumulation of flies at the food (Figure 7C). Moreover, a temperature downshift led to rapid awakening, whereas food dwelling persisted for at least 2 days after dTRPA1 heat activation had ceased. In contrast to the slow effects on food dwelling, activation of dopaminergic neurons (*THGAL4:UAS-dTRPA1*) led to an immediate onset of food dwelling (Figure S7).

A predominant effect of sNPF neuronal activation on sleep rather than feeding was also observed in groups of flies housed in vials (Movie S1). At high temperature, almost all of these flies avoided food and stayed at the top half of the vials, consistent with previous observations that sleep occurs preferentially away from food (Donelson et al., 2012). Parental control flies in contrast were frequently observed at the bottom of the vials, either near or on the food (Movie S1).

Importantly, the total percentage time of the population at the food in the behavior tubes (green line) matched exactly the percentage of flies that have visited the food (blue line) during the period when sNPF neurons are active (Figure 7C). This indicates that the low level of locomotor activity of these flies (Figure S1) is used predominantly to go to the food, where they remain. The most parsimonious interpretation is that hypersomnolent flies are unable to feed properly and eventually become hungry or even starved, resulting in an increased drive to find

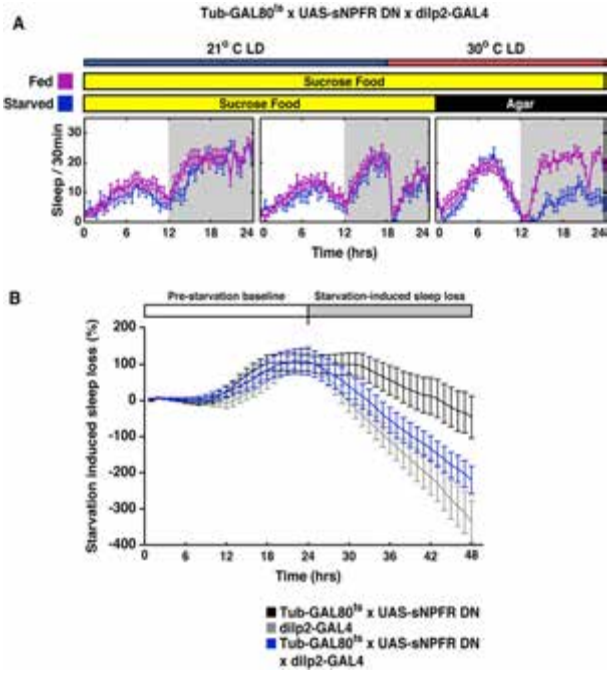

Figure 6. Inhibition of sNPFR Signaling in DILP Cells Does Not Affect Starvation-Induced Sleep Suppression

(A) Starved *tubulin-GAL80^{ts},UAS-sNPFRDN; dilp2-GAL4* flies (blue curve) slept less compared to nonstarved siblings (purple curve). GAL80 was inactivated for 6 hr by shifting the temperature to 30°C at ZT18 and flies transferred onto agar or agar/sucrose food at ZT0. Sleep was monitored for 24 hr. Starvation reduced sleep, indicating that sNPF signaling is not involved in starvation-induced wakefulness.

(B) The experimental flies (blue line) and the parental controls (black and gray lines) showed similar sleep loss in response to starvation. The baseline sleep for each genotype was collected for 3 days before heat inactivation of GAL80. The starvation-induced sleep loss (%) = (sleep during the starvation period − baseline sleep)/baseline sleep %.

Data are presented as mean ± SEM.

food. This suggests that the observed changes in food-related behavior may be predominantly a result of the dramatic increase in sleep by sNPF.

DISCUSSION

We have presented several independent lines of evidence indicating that sNPF acutely increases sleep and alters sleep homeostasis. This is because release of animals from sNPF neuron activation after several days of hypersomnolence resulted in a transient decrease in sleep or negative sleep rebound. Moreover, activation of sNPF neurons during mechanical sleep deprivation blunted the rebound sleep following the deprivation. This suggests that sNPF might alter the internal perception of sleep state during the deprivation despite an apparently behaviorally awake state. It also suggests that sNPF might directly modulate the sleep homeostat.

The most potent in vivo manipulations of sNPF function, mutation of the sNPF gene and strong activation of sNPF neurons with dTRPA1, affect daytime as well as nighttime sleep levels. These manipulations also strongly alter sleep bout duration, a measure of consolidation, in the opposite direction to the sleep duration effects. More limited manipulations of sNPF signaling (cell-specific downregulation of sNPF levels or of sNPF signaling) indicate that sNPF is most important for promoting sleep at night. It also affects the structure of daytime sleep, a function of sNPF circuitry normally suppressed during the day by wake-promoting GABAergic neurons, acting via $GABA_A$ receptors. Suppression of excitability with Kir2.1 likely mimics this daytime GABAergic function. These results in aggregate suggest that sNPF action differs depending on the time of day, a result that supports the idea that daytime and nighttime sleep may be regulated by different circuitries.

The role of sNPF in promoting more consolidated sleep is consistent with a general antiarousal function. As in mammals, *Drosophila* arousal can be measured electrophysiologically (van Alphen et al., 2013; van Swinderen and Andretic, 2003), but the most straightforward measure of arousal state is behavioral, and sleep fragmentation is indicative of a less stable, more easily aroused state. The main neurochemical previously implicated in fly arousal is DA (Andretic and Shaw, 2005), and l-LNvs play a prominent role in the arousal circuitry (Lebestky et al., 2009; Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008).

Although the imaging assays indicated that sNPF alone did not lead to significant cAMP changes in the l-LNvs, it mildly suppressed the activation effect of DA on the l-LNvs (Figure S4). As one subset of clock neurons in the sleep circuit releases sNPF and promotes sleep at night and an adjacent subset responds to sNPF and suppresses nighttime sleep, sNPF may be used by the s-LNv-to-l-LNv pathway to coordinate the timing of sleep with other circadian behaviors. Indeed, sNPF mRNA is a potent cycling mRNA in s-LNvs (Kula-Eversole et al., 2010). Importantly, the electrophysiological assays in larval central neurons suggest that inhibition of neuronal firing may be a general feature of sNPF function and relevant to other sleep centers in addition to the clock neurons.

sNPF and other sleep-relevant neuromodulators like DA are likely to act at multiple sites in the brain given the major state

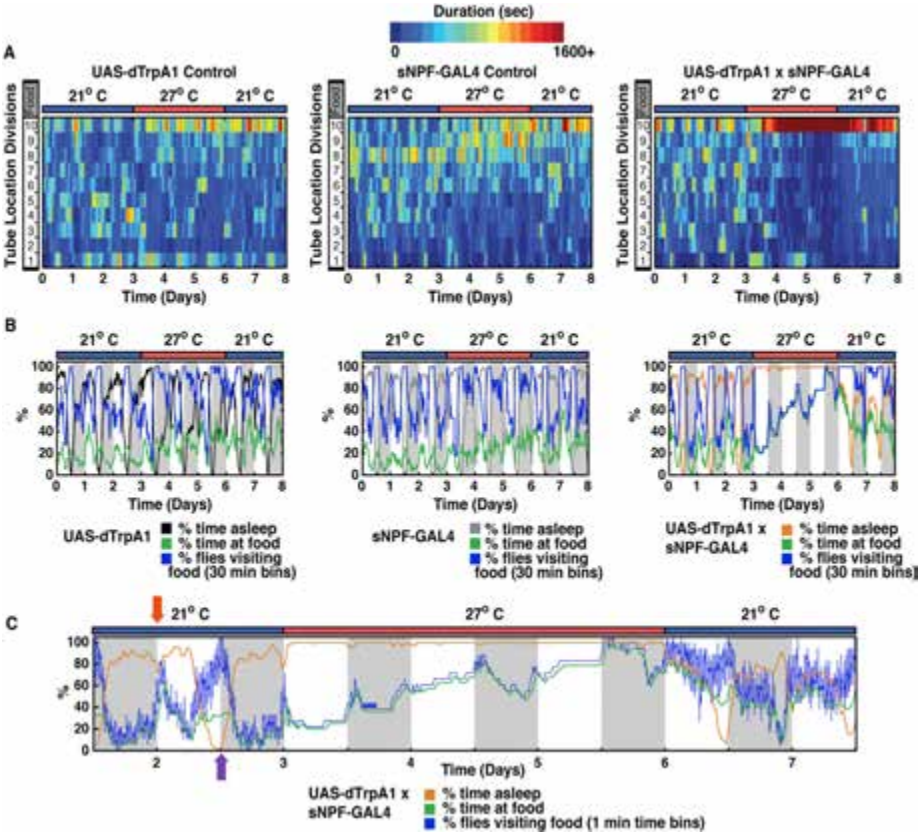

Figure 7. Activation of sNPF Neurons Alters Sleep and Food Preference on Different Time Scales

(A) Sleep, locomotor activity, and position relative to food were monitored by computer video tracking (Donelson et al., 2012). In location heat plots, dark blue indicates flies spent no time at a particular location while dark red indicates flies spent more than 1,600 s at that location. The x axis time and temperature and the *y* axis indicates the location of each genotype within the behavioral tubes relative to food (location 10). Both parental control lines only showed slight increases in the time they spent on food upon heating, while the *sNPFGAL4:UAS-dTRPA1* flies spent significantly more time on the food after heat activation. Sleep plots and sleep parameters for this data set are shown in Figure S6.

(B) Plots of percent time asleep, percent time at food, and percent flies visiting food for experiments in (A). Control lines show a modest change in food dwelling with increased temperature.

(C) Expanded time scale for experimental fly data in (B). During the morning peak of activity at 21°C when animals go to food they stay there (red arrow; blue line and green line overlap). During the evening peak of activity, flies visit food often but do not stay. This likely reflects "patrolling behavior" (purple arrow; blue line higher than green line). Heat-induced acute firing of sNPF-expressing neurons dramatically and rapidly (within 1 hr) increased sleep to maximal levels (orange line). The excessive sleep reversed within 1 hr after inactivation by shifting temperature to 21°C. In contrast to the rapid effect on sleep, stimulation of sNPF-expressing neurons caused a very slow accumulation of flies at the food that was not rapidly reversible. Flies remained closer to the food for at least 2 days after dTRPA1 inactivation. $n \geq 17$ for each genotype.

See also Figure S7.

change effected by the sleep/wake transition. This expectation also reflects the modest effects of sNPFR manipulation within l-LNvs on total sleep time. Moreover, fan-shaped body neurons have recently been shown to be important for DA-mediated arousal (Liu et al., 2012; Ueno et al., 2012). The ability of these neuromodulators to act on many circuits may allow for more flexible integration of sleep with other behaviors and with other external and internal factors.

An important influence on sleep is metabolic state (Penev, 2007). Indeed, sNPF facilitated the OA-to-DILP circuit, which may reflect its role in sleep/wake, feeding and/or metabolic regulation (Figure S5). However, the wake-promoting effect of activating the DILP pathway is context-dependent, occurring only in LD (Figure 1A). Moreover, acute activation of octopaminergic neurons by dTRPA1 only mildly affects sleep and also in a condition-dependent manner (data not shown), and feeding animals with octopamine only significantly suppresses total sleep after 2–3 days of exposure (Crocker and Sehgal, 2008). Although long-term activation of octopaminergic neurons leads to long-lasting increases in food dwelling (N.C.D. and L.C.G., unpublished data), these effects contrast sharply with the rapid and condition-independent effects seen with acute increases in dopamine signaling (Shang et al., 2011). Dopaminergic neurons have also been shown to be a critical part of NPF-regulated changes in satiety and response to food (Inagaki et al., 2012; Krashes et al., 2009), and activation of these neurons indeed led to an immediate onset of food dwelling, which reversed rapidly upon dTRPA1 inactivation (Figure S7). As expected, tracker analysis shows that these food-dwelling flies also sleep very little, indicating that dopamine affects both sleep and feeding rapidly. These effects contrast with the slow effects on food dwelling by sNPF neuronal activation.

The simplest interpretation of this slow food-dwelling response is that it is secondary to a more primary effect of sNPF on sleep. Indeed, a slow buildup in hunger or even starvation as a consequence of too much sleep is a simple explanation consistent with most if not all of our data. Behavioral effects as a secondary consequence of some other more direct effect is also our interpretation of many of the sleep effects of activation of peptidergic neurons shown in Figure 1, in which only sNPF robustly increased sleep, i.e., under both LD and DD conditions. We therefore suggest that a necessary condition for serious consideration of a molecule as behavior-relevant is a rapid response, which is also relatively condition independent. Dopamine as a wake-promoting molecule and now sNPF as a sleep-promoting molecule meet these criteria.

EXPERIMENTAL PROCEDURES

Fly Stocks

Flies were raised on standard medium and 12 hr light:dark cycles (we used fluorescent light and the light intensity was 1,600 ± 400 lux). Flies carrying *UAS-dTRPA1* or *tubGAL80^{ts}* were raised at 21°C.

The *UAS-dTRPA1, UAS-rdlRNAi,* and *UAS-Epac1-cAMP* (50A, II) flies were kindly provided by Drs. Paul Garrity (Brandeis University), Ron Davis (Scripps Florida), and Paul Taghert (Washington University). *UAS-sNPFR-DN* (X) and *UAS-sNPFRNAi (K)* were provided by Dr. Kweon Yu. The RNAi line was validated using immunoblotting (Lee et al., 2004) and immunohistochemistry (Kahsai et al., 2010). The dominant negative receptor competes with sNPFR for G protein binding and was functionally validated in multiple assays (Lee et al., 2008). *UAS-sNPFRNAi (B), UAS-kir2.1, and tubGAL80^{ts} (X)* were ordered from Bloomington stock center. Dr. Paul Taghert also provided *DMSGAL4, SIFaGAL4, CCAPGAL4, CRZGAL4,* and *huginGAL4* used in Figure 1. *NPFGAL4 and NPFRGAL4* in Figure 1 were obtained from Dr. Ping Shen (University of Georgia). *sNPFGAL4* flies (NP6301; order number 113901) were ordered from the *Drosophila* Genetic Resource Center (DGRC), Kyoto Institute of Technology, Kyoto, Japan. *dilp2GAL4* were kindly provided by Dr. Ulrike Heberlein (Janelia Farm). *pdf-GAL4*/CyO or *dilp2GAL4* flies were used to express the UAS transgenes in the PDF- expressing l-LNvs or DILP cells in fly brains, respectively.

Behavioral Analysis

Individual flies were housed separately in 65 mm × 5 mm glass tubes (Trikinetics, Waltham, MA) containing 5% agarose with 2% sucrose. Two- to 5-day old flies were collected and entrained under standard light-dark conditions, with a 12 hr light phase and followed by 12 hr dark phase for 3–4 days. We first entrained adult flies at 21°C for 3–4 days. We then activated dTRPA1 or inactivated the GAL80 proteins by shifting the temperature to 27°C or 30°C for 3 days. This will either activate the neurons expressing the dTRPA1 or turn on the expression of *UAS* transgenes. Finally, we inactivate the dTRPA1 or reactivated the GAL80 proteins by shifting the temperature back to 21°C to test if the effects are reversible. For Figure 1B, the lights were turned off permanently upon heat activation. The temperature was then returned to 21°C in DD to inactivate the dTRPA1 channel.

The sleep-like resting state is defined as no movement for 5 min (Hendricks et al., 2000; Shaw et al., 2000). Total sleep measures the amount of sleep per 24 hr and the mean duration measures the average length of sleep episodes (Agosto et al., 2008). The behavioral pattern of each fly was monitored either by an automated method (DAM System, Trikinetics) or by a video-based tracking application (Donelson et al., 2012). The latter recorded the exact location of each fly every second for the 8 days of the experiment. Using this more direct method to record activity, we were able to gain a higher data resolution as well as analyze the preference for food location shown in Figures 7, S6, and S7. While DAM records beam breaks, the tracking system used in this experiment was able to detect movements of as little as 1.5 mm. In short, 3- to 5-day-old female flies were loaded into the same sleep-tubes used for DAM. The tubes were capped with parafilm on both ends to prevent visual obstruction and were placed onto a piece of white paper, which afforded a high visual contrast to the fly. The flies were then placed under a video camera (Logitech Quickcam for Notebooks) connected to the computer running the tracking software. A red compact fluorescent light allowed for continuous recording during the flies' 12 hr dark period. The tracker data were transformed from coordinate data into DAM-style 1 and 30 min data files and analyzed as described previously. For sleep deprivation studies, DAM monitors were mounted on a Trikinetics plate attached to a VWR vortexer and shaken for 2 of every 10 s for 12 hr.

Calculation of the Relative Sleep Changes and Statistical Analysis

Sleep time as well as the effect of heat on sleep is highly sensitive to genotype. We therefore needed to subtract the heat-induced changes occurring in the parental controls. We first calculated the heat-induced percentage change in sleep (SI) for each genotype, which is SI = (sleep time 30°C − sleep time 21°C)/sleep time 21°C%; Figures 2A, 2D, 4C, and 4D). To simplify the data presentation, we then calculated the relative sleep change (ΔSI), which is ΔSI = SIexp − SIctrl (Figure 1A). The SI of the experimental group was compared with the two control parental groups using the ANOVA with Tukey post hoc test. *$p < 0.01$, **$p < 0.001$, and ***$p < 0.0001$ are significant differences from both control groups. Error bar represents SEM.

Functional Imaging

For Figures S4 and S5A, live FRET imaging was performed as described previously (Shang et al., 2011). Briefly, 3- to 6-day-old entrained male flies were dissected in ice-cold adult hemolymph-like medium (AHL; Wang et al., 2003); 600 μl room temperature (RT) AHL was added to the imaging chamber. An individual brain was then placed in the chamber. We used an Olympus BX51WI microscope with a CCD camera (Hammamatsu Orca C472; 80–12 AG). The acquisition system for this setup allows for simultaneously recording both channels. The 86002v1 JP4 excitation filter (436, Chroma) as well as two-channel, simultaneous-imaging system from Optical Insights with the D480/30 m and D535/40 m emission filters were used. EPAC expressed in l-LNvs was excited with 50 ms pulses of light using CFP filters and fluorescent signals emitted by the l-LNvs or DILP cells were imaged every 5 s by an epifluorescent microscope using a 40× objective. The software Volocity (Perkin Elmer) was used for acquisition, and the CFP and YFP images were recorded simultaneously. Under these conditions, we determined that the baseline fluorescent

signal in l-LNvs stabilized after imaging the neurons for 150 frames. We were then able to obtain reliable responses induced by 10 μM forskolin (data not shown).

Octopamine and dopamine were purchased from Sigma and a stock solution (10 mM) was freshly prepared in H_2O before the imaging (Cayre et al., 1999). sNPF was purchased from Polypeptide, San Diego, CA. 2mM stock solution in DMSO was vacuum dried and stored at −20°C. The baseline images were collected for 50 s before applying drugs to the brain. The mean intensity of CFP or YFP of a nonfluorescent brain region next to the l-LNvs or the DILP cells was first subtracted from that of l-LNvs or DILP cells. The YFP/CFP ratio for each time point was then calculated and normalized to the ratio of the first time point, before drug application. The relative cAMP changes were determined by plotting the normalized CFP/YFP ratio (%) over time. We also determined the average fluorescence change (area under the "relative cAMP change" curve) by calculating an average CFP/YFP ratio increase from 100 s to 200 s.

For Figure S5C, experiments were performed in a different configuration with a different drug delivery method. This is the likely source of differences in OA effective dose and duration of effect. Optical signals from an Olympus BX51WI microscope were recorded using a back-illuminated CCD camera. A 45 ms exposure stimulated the FRET-based EPAC sensor, and CFP and YFP emissions were collected using a splitter (Photometrics). A 60x, 0.9 NA water immersion lens (Olympus LUMPlanFI) was used, and images were acquired at 1 Hz with the software Volocity (PerkinElmer), with 4× binning. Filters used for cAMP imaging were: excitation 86002v1 JP4 filter (436, Chroma), and emission D480/30 m and D535/40 m (Optical Insights). Offline data analysis was performed using ImageJ (US National Institutes of Health) and Matlab (Mathworks). To limit bleaching, a 25% neutral density filter (Chroma) was used for all experiments, and brains were pre-exposed to 436 nm blue light twice for 90 s followed by another 60 s, with a 5 s off period between. Imaging experiments were performed in male progeny from crosses of *w;dilp-GAL4* x *w;;UAS-EPAC-55A*. After collection, flies were raised to 20–23 days of age at 25°C on a 12:12 light/dark cycle with lights on at 9 am. All imaging was carried out during the light period. Each brain was dissected in ice-cold 0 mM Ca^{2+} Modified A solution containing (in mM) 118 NaCl, 2 NaOH, 2 KCl, 4 MgCl2, 22.3 sucrose, 5 trehalose, 5 HEPES, pH 7.15, and mOsm 281, and was transferred to an RC-26 chamber on a P1 platform (Warner Instruments) and pinned in Sylgard (Dow Corning). The brain was then perfused with AHL by gravity feed at 3–4 ml/min. Switching between solutions was achieved using a three-way valve solenoid (Cole-Parmer) under manual control. All Recordings lasted 240 s, with 30 s of baseline before 60 s of drug treatment before washout with control AHL. TTX (Tocris) was stored as a 100 μM stock at −20°C and was used at 1 μM. TTX was added to all AHL solutions so that brains were TTX-treated throughout the light pre-exposure and the entire recording. Octopamine (Sigma) was made fresh daily to 50 mM in water and was kept wrapped in foil on ice until dilution to 200 nM in AHL. sNPF was prepared as described above, and was used at 40 μM. The FRET signal (CFP/YFP ratio) for each time point was calculated and normalized to the ratio of the first baseline time point. The relative cAMP changes were determined by plotting this normalized CFP/YFP ratio (%) over time. Relative cAMP values were averaged from 90 to 150 s, and resulting response averages were compared between OA+TTX and OA+TTX+sNPF groups using a t test assuming unequal variances.

Electrophysiology

Flies were raised at 25°C. To drive expression of transgenes in larval motor neurons, the *OK371-GAL4* driver line was used (Bloomington stock 26160). The UAS-sNPFR line was generated in the Yu lab (Lee et al., 2008). sNPF-1 (H-AQRSPSLRLRF-NH2) was commercially synthesized (PolyPeptide). sNPF was stored as powder at RT, and as then dissolved in DMSO at 20 mM. Aliquots were desiccated using a SpeedVac (Savant) and were stored at −20°C.

Third instar larvae were dissected and pinned in Sylgard (Dow Corning) in 0 mM Ca^{2+} Modified A solution (see above). The brain was removed and pinned, and the preparation was perfused with adult hemolymph (AHL), containing (in mM) 108 NaCl, 5 KCl, 2 CaCl2, 8.2 MgCl2, 4 NaHCO3, 1 NaH2PO4, 5 trehalose, 10 sucrose, 5 HEPES, pH 7.5, and mOsm 265 (based on Wang et al. 2003). Protease XIV (Sigma-Aldrich) treatment (0.5%–1% w/v) was used to dissolve the glial sheath, allowing access to the motor neurons. Patch electrodes were (in mm) 1.2 OD × 0.9 ID × 100 l (Friedrick & Dimmock), and were pulled and fire-polished to achieve a resistance of 3–7 MΩ. Pipettes were loaded with internal solution as per Choi et al. (2004), containing (in mM) 20 KCl, 0.1 CaCl2, 2 MgCl2, 1.1 EGTA, 120 K-Gluconate, 10 HEPES, pH 7.2, and mOsm 280. Signals were acquired using the Axopatch 200B amplifier and Clampex (Molecular Devices). Current-clamp pulses were 500 ms in duration, and stepped in 20 pA increments from −20 to +140 pA, with a 10 s interpulse interval. sNPF was bath applied at 20 μM, and current-clamp recordings and effects on resting membrane potential were carried out after 5 mls of treatment.

Statistical analysis was carried out using JMP, Version 7 (SAS Institute) and SPSS (IBM), Version 19. The change in resting membrane potential from baseline to posttreatment was calculated for each recording, and this value was compared between sNPF and vehicle treatments using a t test assuming unequal variances. A one-way repeated-measures ANOVA was used to analyze the change in firing rate following drug treatment, with treatment as a between-subject factor and input current as a within-subject factor. No significant interaction between drug treatment and input current was observed, but drug treatment caused a significant overall effect on firing rate across all input currents.

Immunocytochemistry

The protocol has been described (Shang et al., 2008). Briefly, fly heads were fixed in 4% paraformaldehyde/0.008% PBS-Triton X-100 for 1 hr at 4°C. Paraformaldehyde fixed samples were washed for 1 hr in 0.1% PBS- Triton X-100 at RT and then dissected in PBS. Fixed brains were washed twice, 10 min each, in 0.5% PBS-Triton X-100 at RT and then blocked in 10% goat serum with 0.5% PBS-Triton X-100 for 1 hr at RT. Brains were incubated with primary antibody (anti-GFP) at 4°C overnight, then washed three times and incubated in secondary antibodies (Molecular Probes) at 1:500 dilution for 1 hr at RT. Brains were washed three times and resuspended in mounting solution (Vectashield, Vector Laboratories). Brain samples were depicted with a Leica TCS SP2 confocal microscope.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.07.029.

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Supplemental Information

Short Neuropeptide F Is a Sleep-Promoting Inhibitory Modulator

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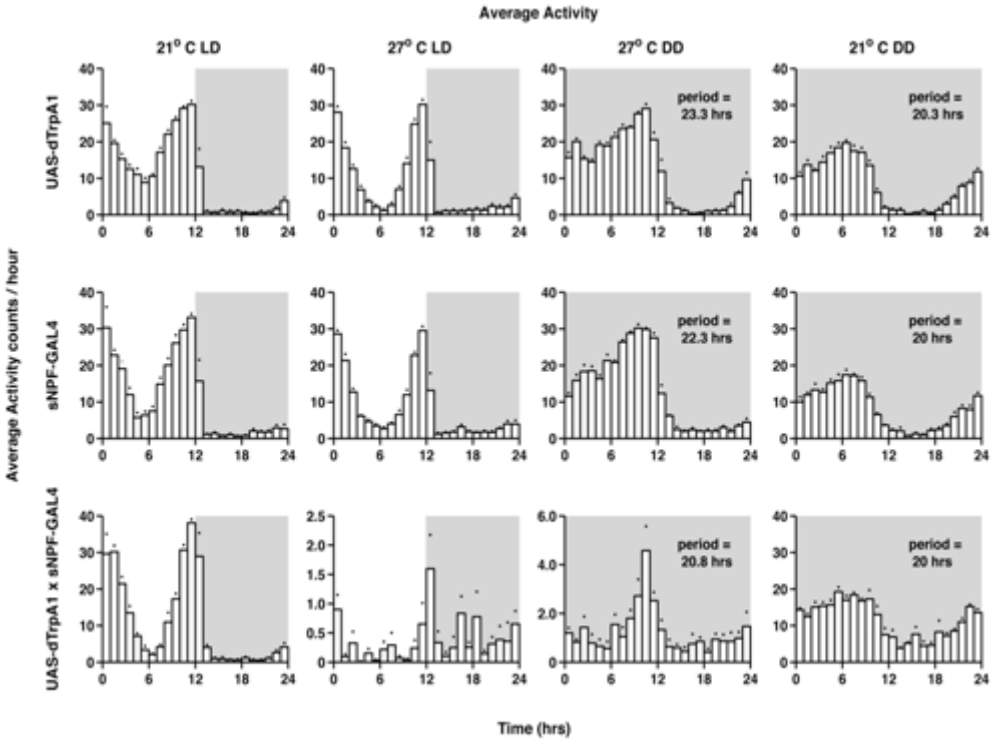
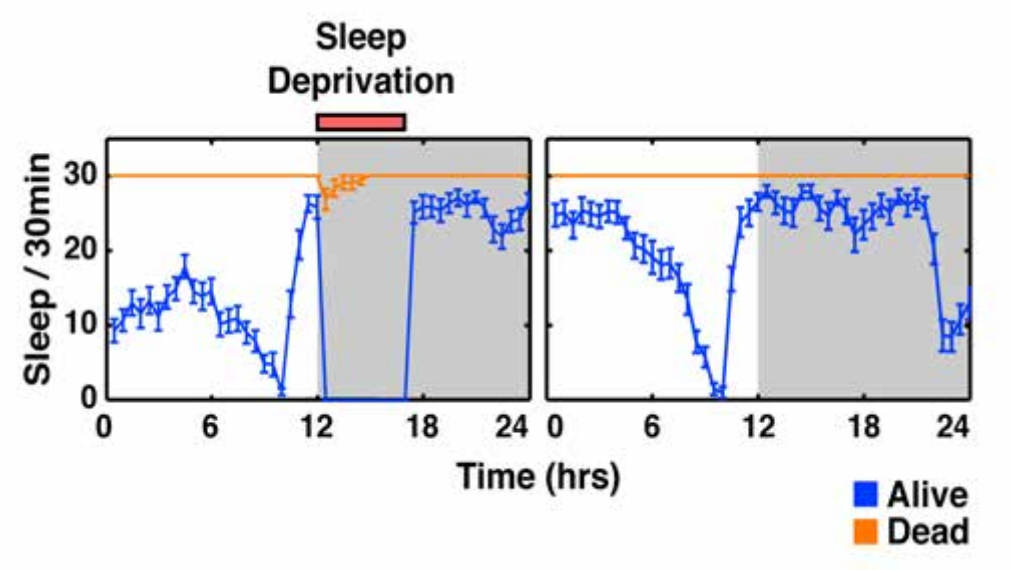
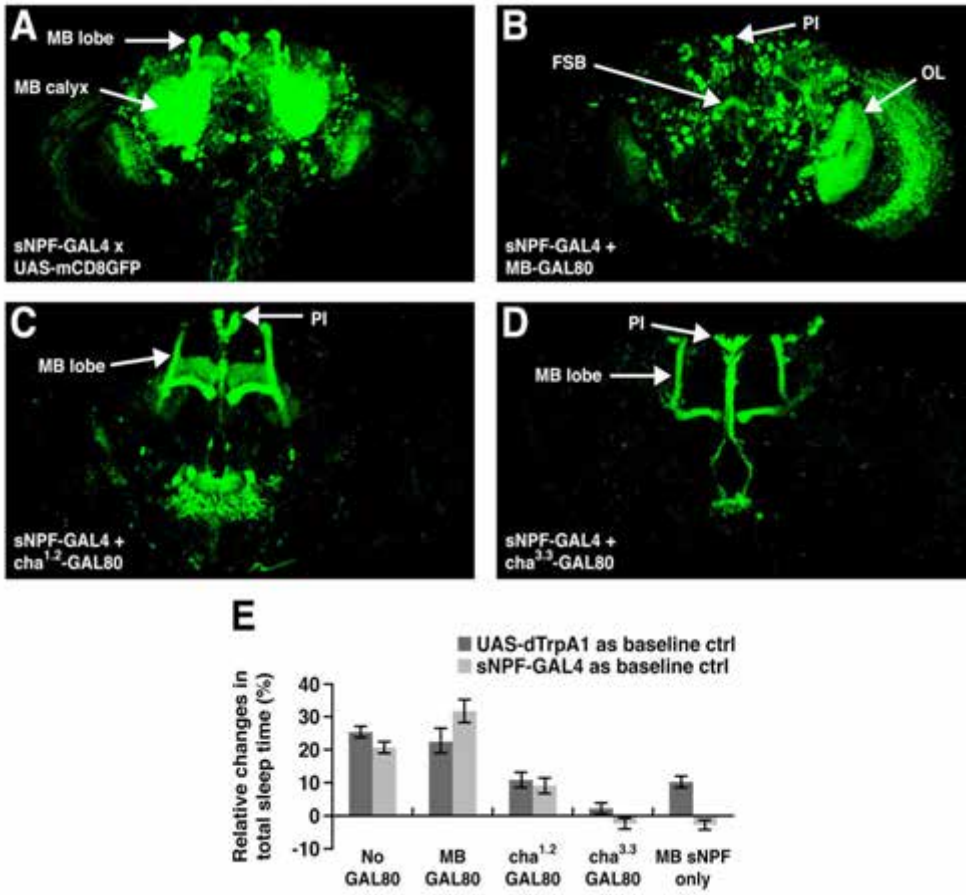
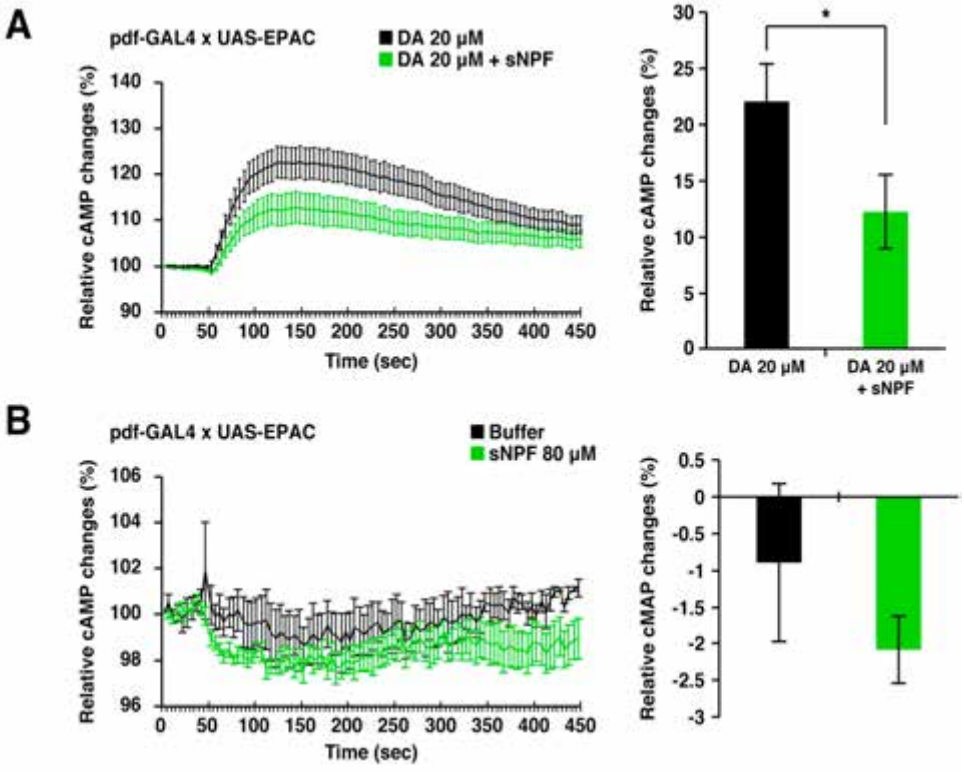
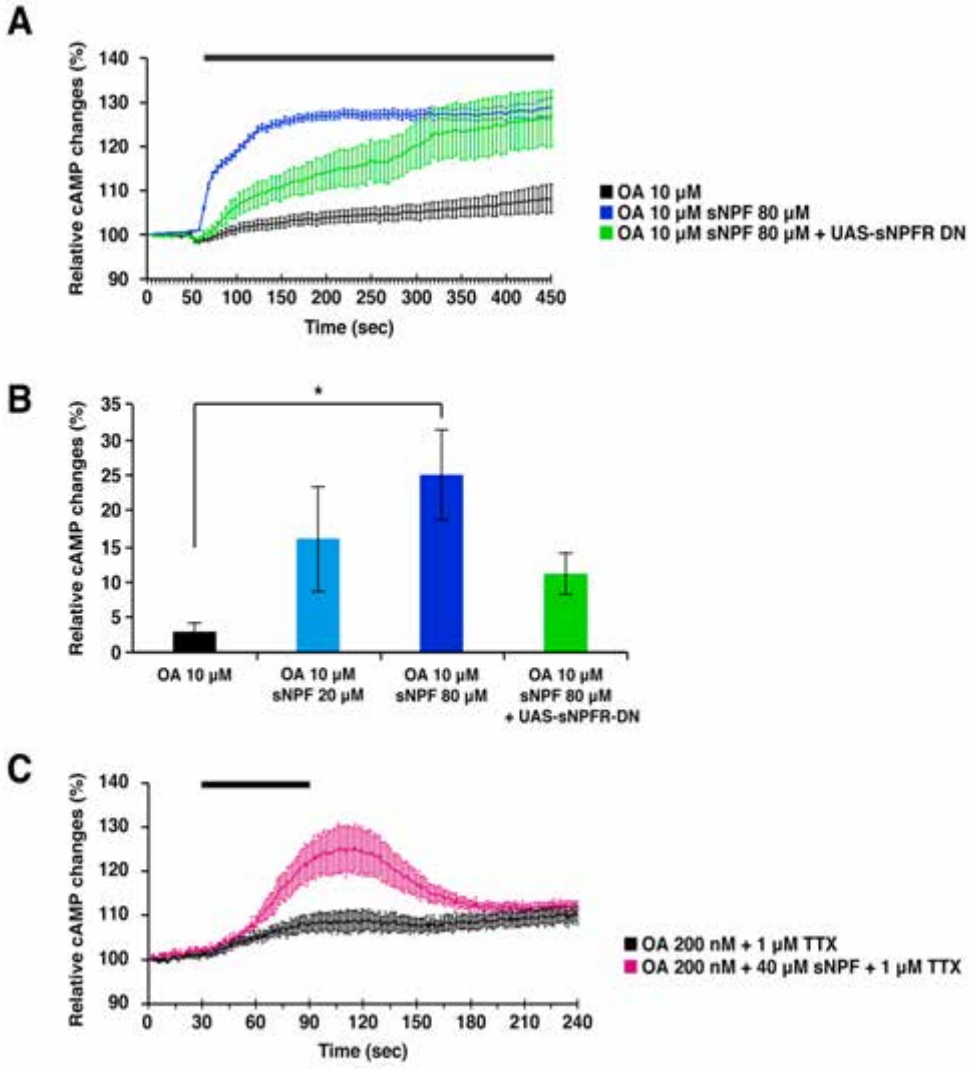
Supplemental Figure 1

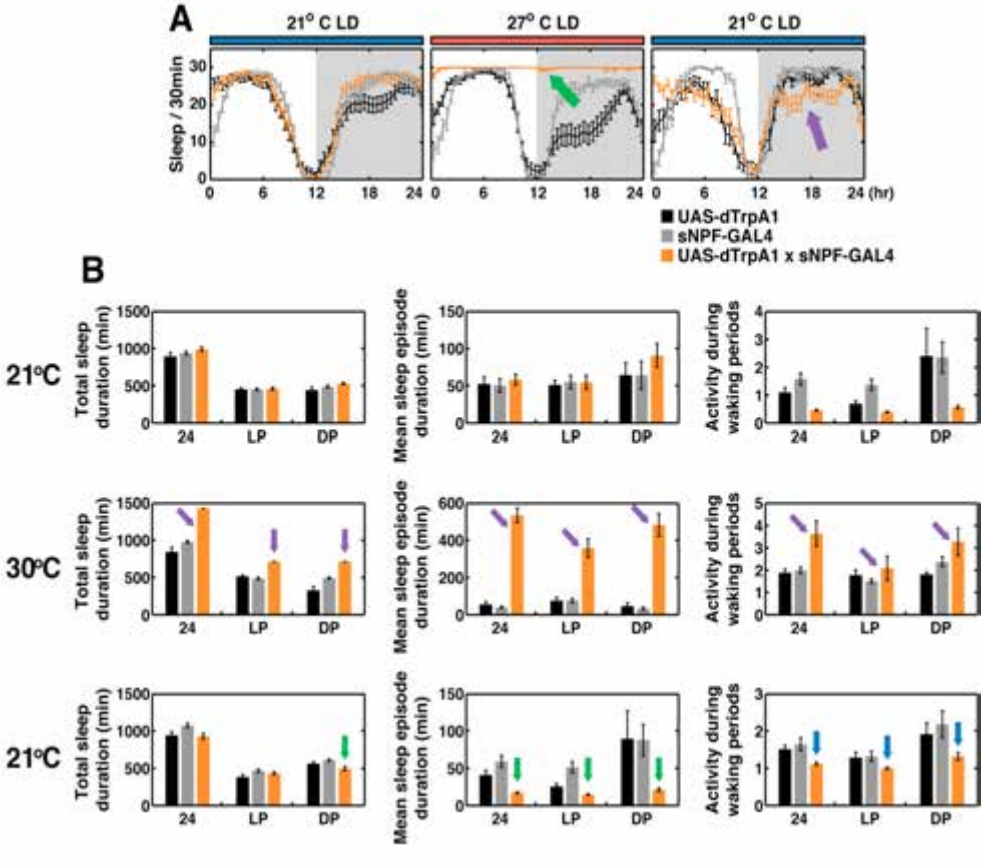


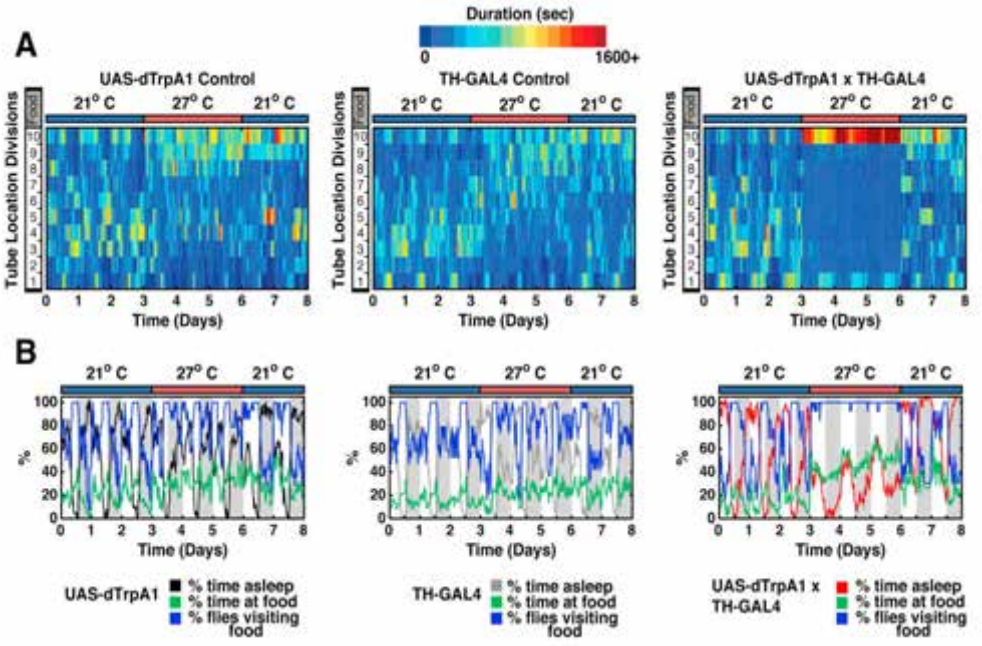

Sleep
Deprivation
Sleep / 30min
30
20
10
0
0
6
12
18
24
0
6
12
18
24
Time (hrs)
Alive
Dead


A
MB lobe
MB calyx
sNPF-GAL4 x
UAS-mCD8GFP
B
PI
FSB
OL
sNPF-GAL4 +
MB-GAL80
C
PI
MB lobe
sNPF-GAL4 +
cha1.2-GAL80
D
PI
MB lobe
sNPF-GAL4 +
cha3.3-GAL80
E
UAS-dTrpA1 as baseline ctrl
sNPF-GAL4 as baseline ctrl
Relative changes in total sleep time (%)
40
30
20
10
0
-10
No GAL80
MB GAL80
cha1.2 GAL80
cha3.3 GAL80
MB sNPF only


A
pdf-GAL4 x UAS-EPAC
DA 20 µM
DA 20 µM + sNPF
Relative cAMP changes (%)
Time (sec)
Relative cAMP changes (%)
DA 20 µM
DA 20 µM + sNPF
B
pdf-GAL4 x UAS-EPAC
Buffer
sNPF 80 µM
Relative cAMP changes (%)
Time (sec)
Relative cMAP changes (%)


A
Relative cAMP changes (%)
140
130
120
110
100
90
0 50 100 150 200 250 300 350 400 450
Time (sec)
OA 10 μM
OA 10 μM sNPF 80 μM
OA 10 μM sNPF 80 μM + UAS-sNPFR DN
B
Relative cAMP changes (%)
35
30
25
20
15
10
5
0
*
OA 10 μM
OA 10 μM sNPF 20 μM
OA 10 μM sNPF 80 μM
OA 10 μM sNPF 80 μM + UAS-sNPFR-DN
C
Relative cAMP changes (%)
140
130
120
110
100
90
0 30 60 90 120 150 180 210 240
Time (sec)
OA 200 nM + 1 μM TTX
OA 200 nM + 40 μM sNPF + 1 μM TTX


A
21° C LD
27° C LD
21° C LD
Sleep / 30min
0
10
20
30
0 6 12 18 24 0 6 12 18 24 0 6 12 18 24 (hr)
UAS-dTrpA1
sNPF-GAL4
UAS-dTrpA1 x sNPF-GAL4
B
21°C
30°C
21°C
Total sleep duration (min)
Mean sleep episode duration (min)
Activity during waking periods
24
LP
DP


A
Duration (sec)
0
1600+
UAS-dTrpA1 Control
TH-GAL4 Control
UAS-dTrpA1 x TH-GAL4
21° C
27° C
21° C
Tube Location Divisions
food
Time (Days)
B
%
% time asleep
% time at food
% flies visiting food
UAS-dTrpA1
TH-GAL4
UAS-dTrpA1 x TH-GAL4

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. (related to Figure 1) Circadian analysis of sNPF flies indicates that they have normal circadian rhythms even during periods of increased sleep due to sNPF neuron activation. DAM data from Figure 1 were analyzed using Matlab scripts developed by the Hall/Rosbash labs. Columns show temperature and lighting conditions (21^{o}C LD and 27^{o}C LD are three day averages, while 27^{o}C DD and 21^{o}C DD are two day averages). Rows are arranged by genotype. Flies of all genotypes and in all conditions showed statistically significant rhythms. While total activity was reduced in flies with activated sNPF neurons, these flies still had appropriate peaks of activity in DD indicating that they are capable of locomotor activity and that their clocks are not impaired.

Figure S2. (related to Figure 3) DAM analysis during mechanical sleep deprivation can discriminate between dead and live, moving, flies. To determine if the lack of sleep in flies with activated sNPF neurons during mechanical sleep deprivation was due to actual arousal or simply due to unconscious flies being tossed across the infrared beam, we compared DAM profiles of live and dead flies. *Canton S* wild type female flies, either alive or freshly freeze-killed, were loaded into the end of the DAM tubes farthest from the food to avoid non-specific sticking and subjected to 6 h of mechanical SD at ZT12. Dead flies only occasionally crossed the beam during mechanical SD, while live flies that have been woken up have high levels of beam crossing reflecting their aroused state. This supports the idea that during mechanical SD, flies with activated sNPF neurons behaviorally awake and moving of their own volition.

Figure S3. (related to Figures 2 and 4) sNPF+ MB and PI neurons do not contribute to the sleep promoting effects of sNPF. A. *sNPF-GAL4* reflects the expression of *Drosophila* sNPF and drives strong expression in the MB, PI, FSB and a subset of the clock circuit (Nassel et al., 2008)**. B.** *MBGAL80* completely blocked the expression of GFP in the MB. **C.** The combination of *cha1.2GAL80* and *sNPF-GAL4* allows the expression of GFP in subsets of sNPF neurons, including some of the MB neurons, PI (arrows), and neurons located on the ventral side of the brain. **D.** The combination of *cha3.3GAL80* and *sNPF-GAL4* almost completely blocks expression outside the MB and PI. **E.** Induced firing of the entire, as well as subsets of, sNPF-expressing neurons outside the MB and PI promotes sleep and the magnitude of the effect allows correlation with expression pattern. Genotypes for X-axis labels are as follows: No GAL80 = *sNPF-GAL4/UAS-dTRPA1*; MB GAL80 = *sNPF-GAL4/UAS-dTRPA1;MBGAL80*; cha$^{1.2}$ GAL80 = *sNPF-GAL4/UAS-dTRPA1;cha1.2GAL80*; cha$^{3.3}$ GAL80 = *sNPF-GAL4/UAS-dTRPA1;cha3.3GAL80*; MB sNPF only = *sNPF-GAL4/UAS-dTRPA1,tub>GAL80>STOP;MBLexA/ LexAopFLP* (only sNPF within MB will express dTRPA1). The addition of *MBGAL80*, while completely eliminating MB expression, did not reduce the strong sleep-promoting effect caused by *sNPF-GAL4* activation and activation of sNPF neurons within the MB did not

cha3.3GAL80 also did not have much effect on total sleep although both MB and PI were labeled. For dark gray bars, *UAS* flies were used as control for baseline subtraction and for light gray bars, *sNPF-GAL4* flies were used as the baseline control.

Figure S4. (related to Figure 5) sNPF can suppress dopamine (DA) signaling in l-LNvs. A. sNPF suppressed the DA induced cAMP increase in l-LNvs. *pdf-GAL4* was used to drive the expression of the cAMP reporter, EPAC, in the l-LNvs. Co-application of 20 µM DA and either 20 or 80 µM sNPF induced weaker responses in the l-LNvs compared with DA alone. Quantitative analysis shows that the presence of sNPF reduced the DA induced responses in the l-LNvs. The average fluorescence change (area under the "relative cAMP change" curve) was determined by calculating an average CFP/YFP ratio increase from 100 s to 200 s. The effects of application of 20 µM sNPF were not statistically distinguishable from those of 80 µM sNPF, so data sets were pooled. Data are presented as mean $\pm$ SEM (*$p < 0.05$ by Student's t-test; N = 10 for DA alone and 13 for DA + sNPF). **B.** Bath application of sNPF alone did not significantly change the cAMP in the l-LNvs. The l-LNvs showed similar cAMP responses to bath application of 80 µM sNPF compared with buffer. Data are presented as mean $\pm$ SEM and were not significantly different (N = 3 for buffer and 5 for sNPF).

Figure S5. (related to Figure 5) sNPF enhances the responses of DILP cells to octopamine (OA). *dilp-GAL4* was used to drive expression of the cAMP reporter, EPAC in the DILP cells. sNPF enhanced OA-induced increases of cAMP in DILP cells. Data are presented as mean $\pm$ SEM. **A.** Co-application of 10 µM OA and 80 µM sNPF induced much stronger responses in the DILP cells compared with OA alone. Bar above graph indicates time of drug application. Co-expression of sNPFR-DN partially blocked the effect. **B.** Quantified data from panel A. The average fluorescence change (area under the "relative cAMP change" curve) was determined by calculating an average CFP/YFP ratio increase from 100 s to 200 s. Only OA + 80 uM sNPF was significantly different from OA alone (ANOVA with Tukey posthoc test *$p < 0.05$; N = 10, 5, 7 and 6 respectively). **C.** The response to sNPF was direct, since inclusion of TTX in the bath did not block the ability of sNPF to enhance octopamine effects. Bar above graph indicates time of drug application. The average fluorescence change (area under the "relative cAMP change" curve) was determined by calculating an average CFP/YFP ratio increase from 90 s to 150 s. OA + sNPF was significantly different from OA alone ($p < 0.05$, t-test; N = 4 for OA and 5 for OA + sNPF).

Figure S6. (related to Figure 7) High resolution video tracking shows that activation of sNPF expressing neurons promotes sleep, independent of changes in locomotion. A. Sleep plots are shown for each genotype. Pre-heat activation: All 3 genotypes show similar sleep pattern at 21°C. During activation, the *sNPF-GAL4:dTRPA1* flies slept more, though they still show activity at the time of the circadian evening peak (green arrow). After inactivation, the *sNPF-GAL4:dTRPA1* flies slept less than controls at night, a reverse sleep

Total sleep time (1st column), mean sleep episode duration (2 column) and activity during waking (3 column) for each genotype during each temperature phase are compared. Activation of sNPF neurons causes an increase in total sleep in both light and dark periods as well as an increase in sleep episode duration (purple arrows). After return to 21°C, inverse sleep rebound is apparent (green arrows). Locomotor activity during wake periods for *sNPF-GAL4:dTRPA1* flies is slightly increased compared with controls upon dTRPA1 activation (orange arrows). The level of activity of the *sNPF-GAL4:dTRPA1* flies during wake periods was reduced during recovery (blue arrows). Differences in the distribution of sleep in light and dark compared to Figure 1 reflect differences in lighting conditions between the DAM and tracker incubators.

Figure S7. (related to Figure 7) Activation of dopaminergic neurons causes a rapid onset of food dwelling which is rapidly reversible. A. Heat plots show the duration for each genotype at each location. Dark blue indicates flies spent no time at that location while dark red indicates flies spent more than 1,600 s at the location. The x-axis indicates time at different temperatures and the y-axis indicates the location of each genotype within the behavioral tubes relative to food (location 10). Both parental control flies only showed slight increases in the time they spent on food upon heat activation, while the *TH-GAL4:UAS-dTRPA1* flies spent significantly more time on the food immediately after heat activation. N $\geq$ 15 for each genotype. **B.** Comparison of %time asleep, %time at food and % flies visiting food from the experiment shown in panel A. Activation of dopaminergic neurons causes a rapid decrease in sleep and a sleep rebound after inactivation of dTRPA1 (red line). Activation of dTRPA1 also caused a rapid (within 1 h) increase in the propensity of flies to reside near the food (green line). The %flies visiting food is very high suggesting that these animals are hyperlocomotor i.e. visit food a lot but do not stay there. Recovery from both the sleep and food localization effects occurred rapidly after dTRPA1 inactivation. These data show that food dwelling after activation of sNPF neurons is not simply a consequence of inactivity since flies with activated dopaminergic neurons are more active and awake than controls and still show a tendency to prefer to be near food. The time course of this effect is also very different from that of activation of sNPF neurons, being much more rapid both in onset and recovery.

SUPPLEMENTAL REFERENCES

Nassel, D.R., Enell, L.E., Santos, J.G., Wegener, C., and Johard, H.A. (2008). A large population of diverse neurons in the Drosophila central nervous system expresses short neuropeptide F, suggesting multiple distributed peptide functions. BMC neuroscience *9*, 90.

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