# Article

# Short Neuropeptide F Is a Sleep-Promoting Inhibitory Modulator

Yuhua Shang,[1,3] Nathan C. Donelson,[1,3] Christopher G. Vecsey,[1] Fang Guo,[1,2] Michael Rosbash,[1,2,*] and Leslie C. Griffith[1,*]

[1]National Center for Behavioral Genomics and Department of Biology, Brandeis University, Waltham, MA 02454, USA
[2]Howard Hughes Medical Institute, Brandeis University, Waltham, MA 02545, USA
[3]These authors contributed equally to this work
*Correspondence: rosbash@brandeis.edu (M.R.), griffith@brandeis.edu (L.C.G.)

## 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; Mac-Fadyen 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.
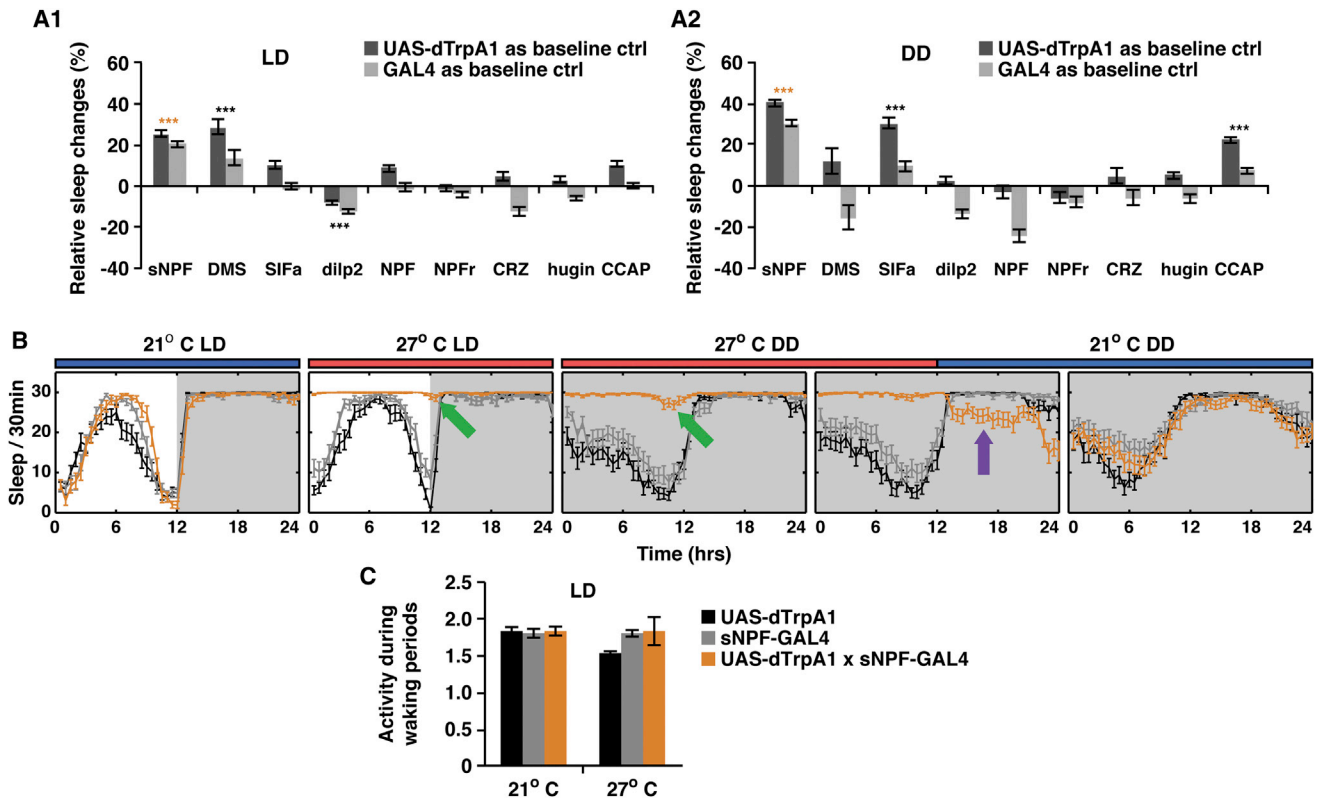
**Figure 1. Activation of sNPF-Expressing Neurons Promotes Sleep Independent of Changes in Locomotion**

(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-GAL80ts* 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-GAL80ts* and *UAS-sNPFR-DN* to downregulate sNPF signaling (Lee et al., 2008) in adult cells. *tubulin-GAL80ts ; c929GAL4:UAS-sNPFR-DN* flies had no detectable change in
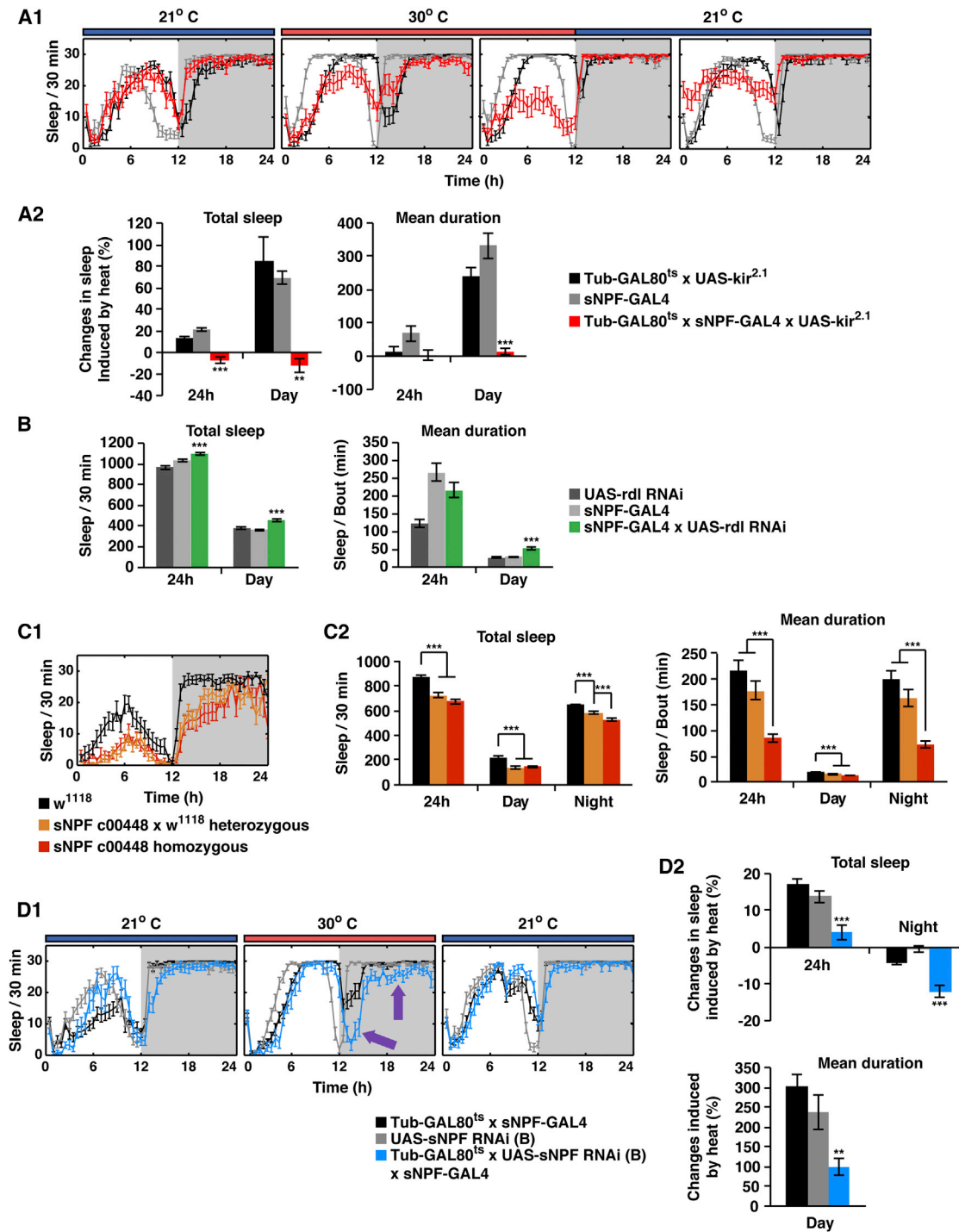
**Figure 2. sNPF Is Required for Maintaining Sleep**

(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.
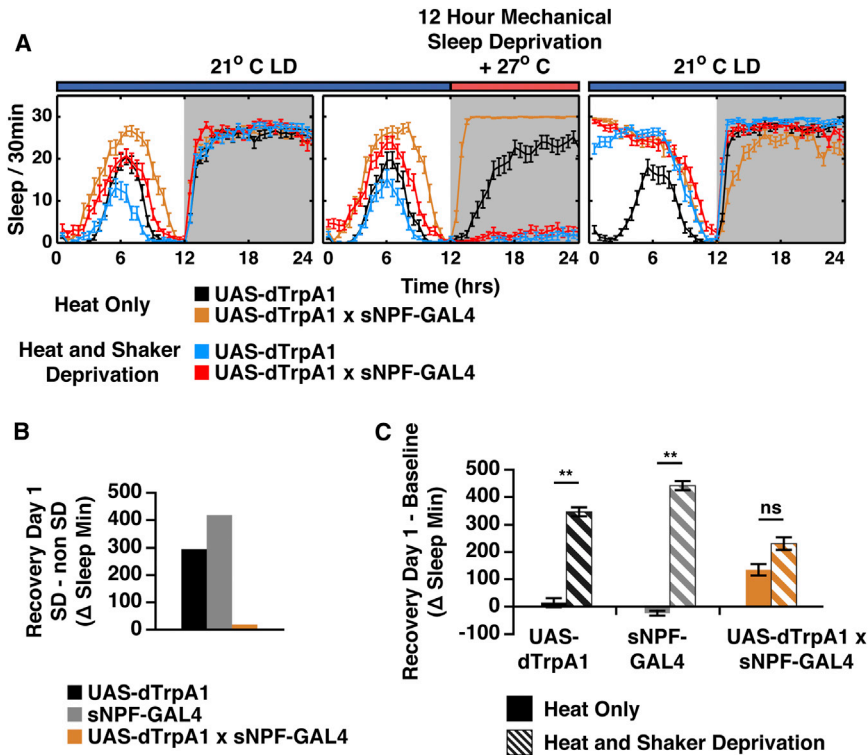
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**Figure 3. sNPF Regulates the Response to Sleep Deprivation.**

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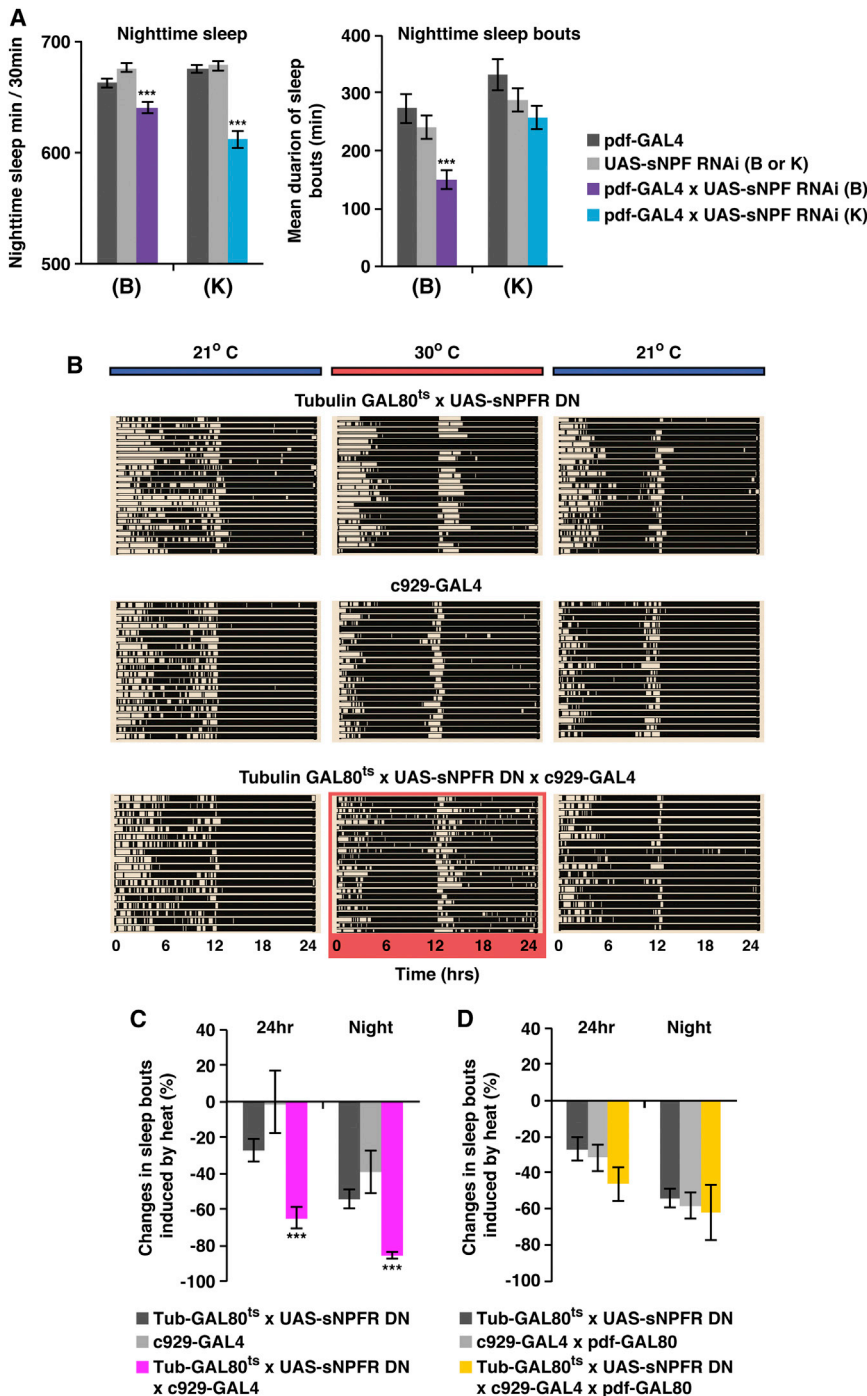
**Figure 4. sNPF Promotes Nighttime Sleep through the s-LNv-to-l-LNv Circuit**

(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 *tubGAL80ts* transgene was used to block the expression of *UAS-sNPFR-DN* in the *c929-GAL4* neurons at 21°C. The *tubulin-GAL80ts,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-GAL80ts,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
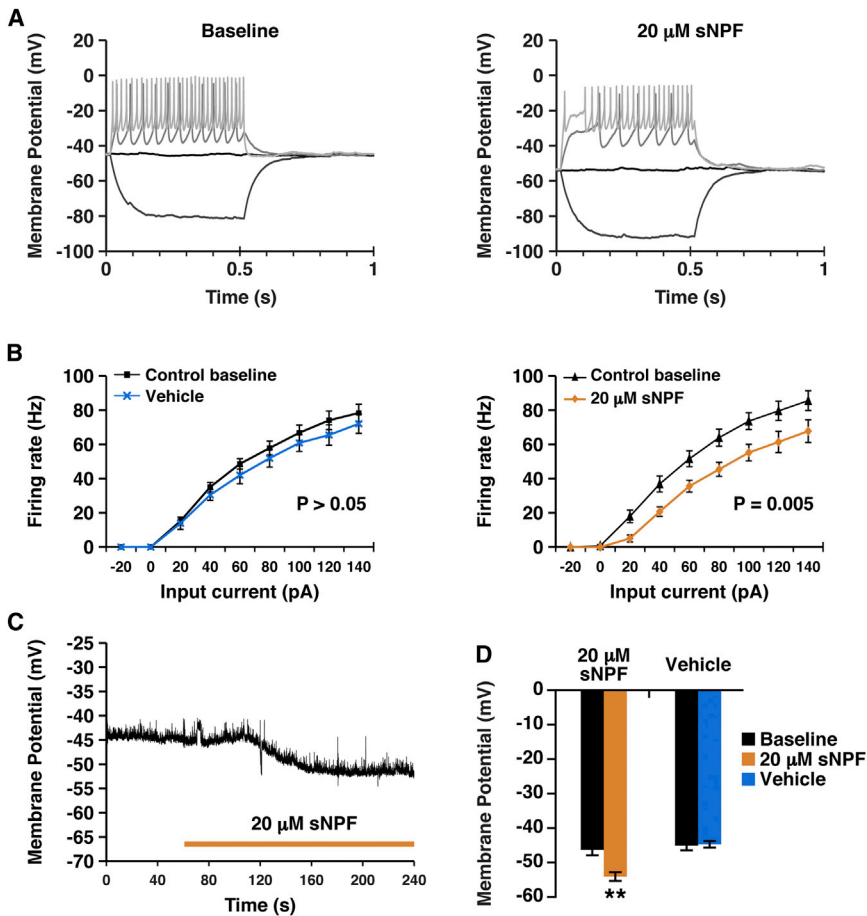
**Figure 5. sNPF Reduces Neuronal Resting Membrane Potential and Suppresses Action Potential Generation**

(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 "fo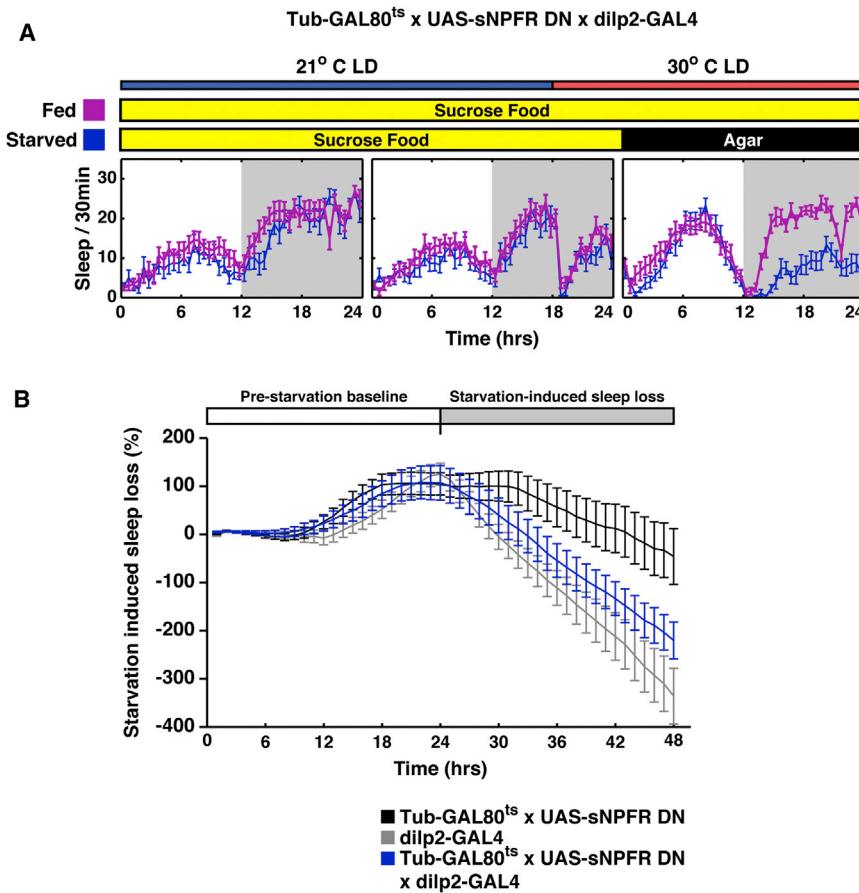od dwelling" was dramatically delayed from the onset of sleep by almost 12 hr (compare orange and green lines in Figure 7C). This was due to a very slow accumulation of flies at the food (Figure 7C). Moreover, a temperature downshift led to rapid awakening, whereas food dwelling persisted for at least 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

## A

**Tub-GAL80^ts x UAS-sNPFR DN x dilp2-GAL4**



**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.

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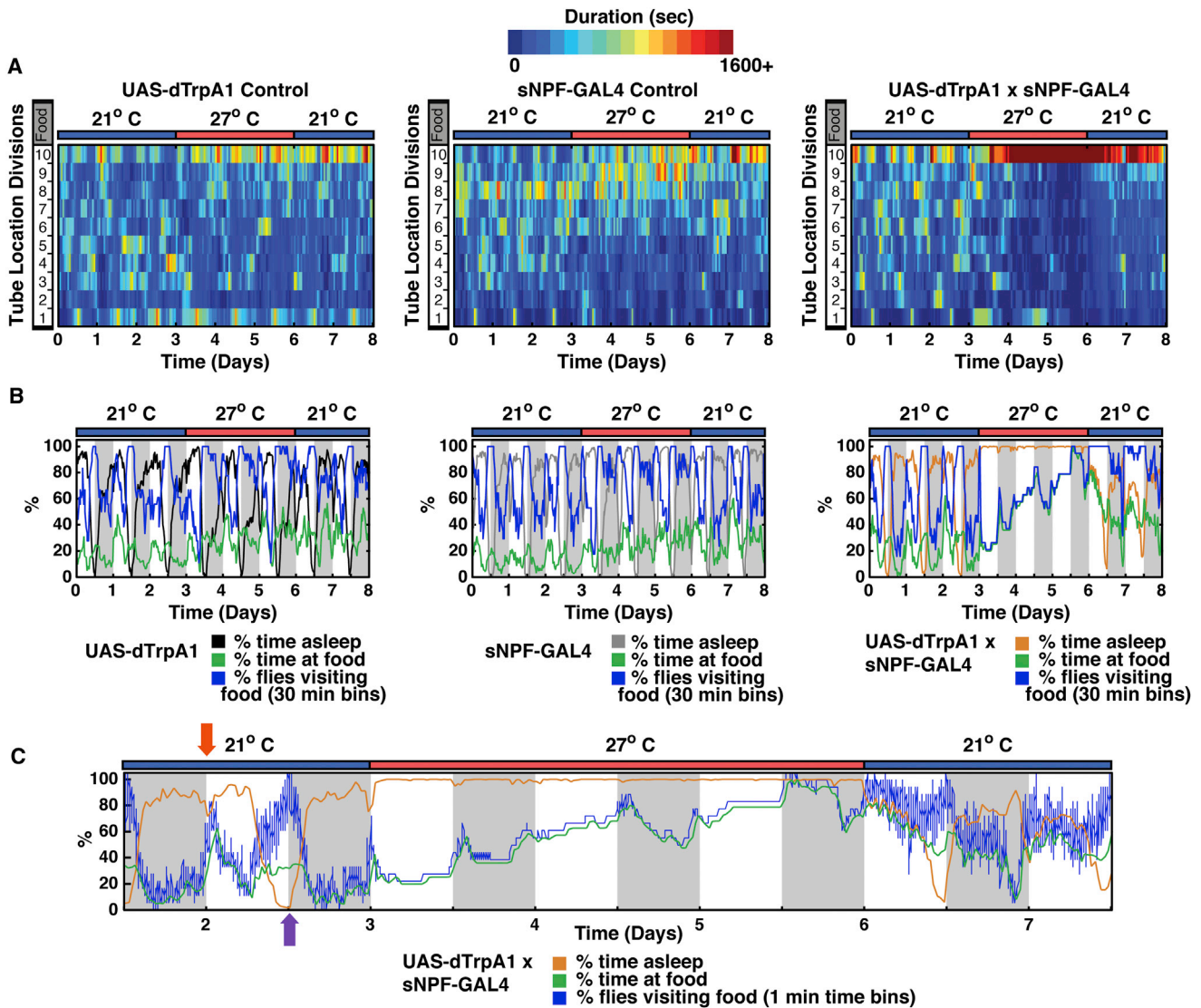
food. This suggests that the observed changes in food-related behavior may be predominantly a result of the dramatic increase in sleep by sNPF.

## 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

**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 ≥ 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 $\mu$M 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 LUMPlanFl) 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 $\mu$M stock at $-20°C$ and was used at 1 $\mu$M. TTX was added to all AHL solutions so that brains were TTX-treated throughout the light preexposure 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 $\mu$M. 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 M$\Omega$. 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 $\mu$M, 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.

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