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Imaging analysis of clock neurons reveals light buffers the wake-promoting effect of dopamine

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How animals maintain proper amounts of sleep yet remain flexible to changes in environmental conditions remains unknown. We found that environmental light suppressed the wake-promoting effects of dopamine in fly brains. The ten large lateral-ventral neurons (l-LNvs), a subset of clock neurons, are wake-promoting and respond to dopamine, octopamine and light. Behavioral and imaging analyses suggested that dopamine is a stronger arousal signal than octopamine. Notably, light exposure not only suppressed l-LNv responses, but also synchronized responses of neighboring l-LNvs. This regulation occurred by distinct mechanisms: light-mediated suppression of octopamine responses was regulated by the circadian clock, whereas light regulation of dopamine responses occurred by upregulation of inhibitory dopamine receptors. Plasticity therefore alters the relative importance of diverse cues on the basis of the environmental mix of stimuli. The regulatory mechanisms described here may contribute to the control of sleep stability while still allowing behavioral flexibility.

Animal sleep is responsive to external signals such as light and social environment[1–4]. Sleep is also modulated by internal signals such as the circadian clock and changes in sleep pressure. The latter reflects, for example, prolonged periods of wake or sleep. Because insomnia and hypersomnia often accompany aging and other health problems, it is important to understand how the brain and its sleep circuitry integrate and prioritize diverse sleep-relevant signals, internal as well as external.

Drosophila sleep is modulated by several internally generated arousal signals, including dopamine, octopamine and the circadian clock–related neuropeptide pigment-dispersing factor (PDF)[5–8]. Light is a prominent arousal signal for diurnal animals such as flies. However, wakefulness does not scale linearly with light intensity, nor does light work in isolation. For example, both flies and humans are prone to sleepiness in the middle of the day, often accompanied by a nap or siesta[9,10]. This indicates that effects of light on the brain and on sleep circuitry are likely to be complex and integrated with other sleep-relevant signals.

Concerning how light affects sleep circuitry, we and others have previously investigated the role of clock neurons in sleep regulation. These studies identified a subset of the clock circuit, the ten l-LNvs (five on each side of the brain), as being potently wake-promoting[4,8,11]. Notably, they only promote wakefulness during the light phase of standard light-dark conditions and have no effect when flies are reared in constant darkness[4]. In addition, a recent study found that these cells may even mediate social enrichment–induced increases in daytime sleep need[12]. l-LNvs therefore contribute to sleep regulation as part of a 'plastic' circuit that is important for animals to adapt to their environment. Its physiological basis is largely unknown, except that the l-LNvs increase their firing rate in response to acute light exposure[13].

The ten l-LNvs have related neurons nearby, the eight small LNvs (s-LNvs). s-LNvs express a neuropeptide, PDF, that helps the s-LNvs keep time in the dark and contributes to their function as master clock neurons[14]. Because l-LNvs also express PDF[14], both cell groups can be specifically labeled with a *Pdf-Gal4* driver line. Indeed, brain imaging with a fluorescence resonance energy transfer (FRET)-based cyclic AMP reporter driven by *Pdf-Gal4* (ref. 15) revealed robust responses evoked by octopamine in the l-LNvs, but not the s-LNvs[16]. This is consistent with the enrichment of mRNAs for two octopamine receptors, OAMB and OA2, in l-LNvs relative to their expression in s-LNvs[16].

To extend our previous studies, we investigated how light interacts with other arousal systems in fly brains. Dopamine is a highly potent wake-promoting signal in both mammals and flies[5]. We first found that a 12-h light exposure suppressed dopamine-mediated wake promoting effects; that is, sleep in the dark was more inhibited than sleep in the light by dopaminergic neuron firing. Given that the l-LNvs express dopamine receptors and are the only known wake-promoting neurons modulated by light in fly brains, we decided to focus on understanding the functionality of this circuit node. By combining the split-GFP approach with functional brain imaging using a FRET-based cyclic nucleotide reporter[15,17–19], we found that the l-LNvs receive synaptic inputs from dopamine and octopamine neurons. However, dopamine appeared to be a stronger arousal signal than octopamine in fly brains, at least for flies raised under basal 12-h:12-h light-dark conditions. By comparing the l-LNv responses evoked by dopamine or octopamine under different light conditions, we found that light suppressed both dopamine- and octopamine-induced cAMP responses in the l-LNvs. The data suggest that these neurons are an integration center for the external arousal signal light and different internal sleep-regulating

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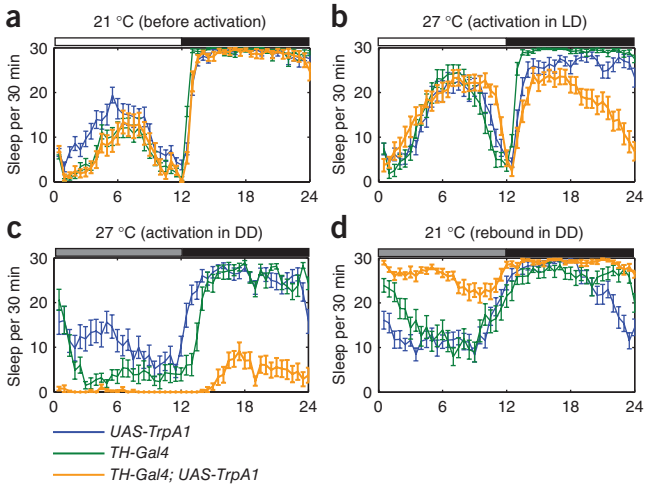

Figure 1 Light suppressed the wake-promoting effects of dopamine. (**a**–**d**) Induced firing of dopaminergic neurons markedly decreased sleep during the dark period in light-dark (LD) or constant darkness (DD) conditions followed by a sleep rebound the following day when firing was returned to normal levels. In constant darkness, sleep was even more severely suppressed, with both subjective daytime and nighttime sleep being almost entirely absent. *TH-Gal4*–driven expression of *TrpA1* transiently increased the activity of dopaminergic neurons when the temperature was raised from 21 to 27 °C at the beginning of the night. The behavior was monitored for 3 d in either the light-dark condition or constant darkness at 27 °C before returning to 21 °C. For simplicity, only 1 d of data from each condition is shown. The data were collected from control *UAS-TrpA1*, control *TH-Gal4* (green) and *TH-Gal4; UAS-TrpA1* flies. Error bars represent s.d.

cues. We propose that the opposing effects of environmental light and dopamine may allow this simple circuit to buffer expected fluctuations in dopamine release from presynaptic partners. This ability to generate condition-dependent plastic responses to various arousal cues may allow animals to maintain proper sleep levels while still being responsive to environmental changes.

RESULTS

Light suppresses the wake-promoting effect of dopamine

Given that both dopamine and octopamine have been shown to promote wakefulness in *Drosophila*[5,6,20], we set out to investigate how the external arousal signal, light, interacts with these two internally generated signals. This was addressed by activating dopaminergic or octopaminergic neurons in adult brains under different entrainment conditions.

We first used *TH-Gal4; UAS-TrpA1* fly lines to activate dopaminergic neurons under light-dark or constant darkness conditions and tested the resulting behavioral effects. The TrpA1 channel allows an acute activation of adult brain neurons for 2–3 d using a shift to warm temperature[4,21]. Unlike the chronic activation carried out previously[6], this manipulation should cause few developmental effects. To this end, we heated flies to 27 °C to mildly activate dopaminergic neurons in either light-dark or constant darkness conditions. Stimulation of dopaminergic neurons using *TH-Gal4* markedly suppressed total sleep and then increased the amount of sleep on the next day during recovery (**Fig. 1a**–**d**). In contrast, activation of octopaminergic neurons in *Tdc2-Gal4; UAS-TrpA1* flies using the same temperature protocol had no detectable effect on total sleep in either condition (**Supplementary Fig. 1** and data not shown for constant darkness conditions). Given that chronic stimulation of these neurons using a sodium channel led to a mild decrease of total sleep[6], we speculated that stronger activation of octopaminergic neurons in adult brains may be necessary to produce significant total sleep effects. We then used 30 °C to activate octopamine neurons and observed a slight decrease in the amount of total sleep as well as nighttime sleep (**Supplementary Fig. 1**).

As activation of dopaminergic neurons produced stronger effects, we decided to focus on the interaction between light and dopamine. Nighttime sleep was markedly suppressed by dopaminergic neuron activation in light-dark conditions, whereas daytime was not affected (**Fig. 1b** and **Table 1**). Moreover, total sleep in constant darkness was consistently more sensitive to dopaminergic neuron activation than total sleep in the light-dark condition (**Table 1**). In constant darkness, sleep in both subjective day and night was suppressed by dopamine activation (**Fig. 1c** and **Table 1**). Moreover, sleep in the subjective night was more affected than nighttime sleep in the light-dark condition (**Table 1**), suggesting the 12 h of light exposure in light-dark conditions also suppressed dopamine-mediated wake-promoting effects in the nighttime.

Table 1 Induced firing of dopaminergic neurons markedly suppresses sleep

Experimental conditions	Light-dark conditions		Constant darkness	
Control used for subtraction	*UAS-TrpA1*	*TH-Gal4*	*UAS-TrpA1*	*TH-Gal4*
Change in Total sleep (%)	−15.4 ± 2.5%	−29.5 ± 2.5%	−48.9 ± 4.4%	−55 ± 4.4%
Change in Daytime sleep (%)	N.S.		−46.2 ± 8.5%	−235.5 ± 8.5%
Change in Nighttime sleep (%)	−29.4 ± 2.5%	−34 ± 2.5%	−51.1 ± 4.2%	−52 ± 4.2%

N.S., not statistically significant ($P > 0.05$); see Online Methods for calculation of the relative change in sleep time. ± values are s.e.m.

The wake-promoting l-LNvs respond to dopamine

To further investigate the circuitry mechanisms underlying this light effect on dopamine responsiveness, we focused on the l-LNv clock neurons, which are the only neurons in fly brains known to promote wakefulness in the light phase[4]. Dopamine receptor mRNAs (*DopR*, *DopR2* and *D2R*) are present in purified l-LNvs. Moreover, all three mRNAs have quite high l-LNv:s-LNv ratios[16], suggesting that dopamine receptors are more abundant in l-LNvs than in s-LNvs.

To assay for synapses between dopaminergic neurons and l-LNvs, we first used the split-GFP system to detect possible membrane contacts between these two classes of neurons[18,19]. The membrane-tethered GFP fragment construct *CD4::spGFP1-10* was driven by *TH-Gal4*, which labels most dopaminergic neurons, and *CD4::spGFP11* was driven by *pdf-LexA*, which labels l-LNvs and s-LNvs. In all six of the brains that we imaged, reconstituted GFP signals were detected around the LNv cell body and dendritic areas, but not in the optic lobe where the axons of the l-LNvs are located (**Fig. 2a**,**b**). A lack of GFP puncta elsewhere in the brain, as well as a complete lack of GFP signal in four control brains indicates that this punctate staining near the LNv cells bodies is, in fact, reconstituted GFP. This method also detected membrane contacts between octopaminergic neurons labeled by *Tdc2-Gal4* and the l-LNvs (**Fig. 2c**,**d**), indicating contacts between l-LNvs and both octopaminergic and dopaminergic neurons.

Individually labeled PPL2 dopaminergic neurons have been shown to project to the area containing the LNvs[22,23]. To evaluate potential presynaptic dopaminergic projections in the vicinity of the LNv dendrites, we stained *TH-Gal4*–driven *UAS–mCD8-GFP* brains with antibody to PDF to label both dopaminergic and LNv neurons. Dense arborizations of the PPL2 dopaminergic neurons were visible near the LNv dendritic area (data not shown).

As split-GFP analysis with the *Pdf* driver does not a priori distinguish between the

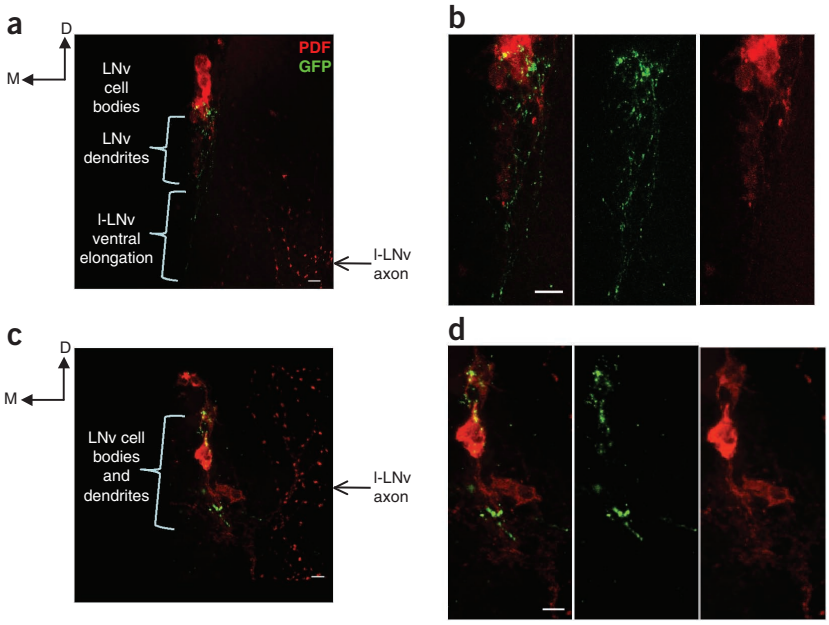

Figure 2 The l-LNvs form membrane contacts with dopaminergic and octopaminergic neurons. (**a**,**b**) The membrane-tethered GFP fragment construct *CD4::spGFP1-10* was expressed in most dopaminergic neurons with *TH-Gal4*, and *CD4::spGFP11* was expressed in l- and s-LNvs with *Pdf-LexA*. Green, GFP; red, PDF. The fine fibers in the ventral elongation in **a** are likely to be the dendrites of the l-LNvs[31]. Reconstituted GFP signals were detected around the LNv cell bodies and dendritic area but not in the optical lobe around the axons of the l-LNvs ($n = 6$). The diagram indicates the orientation of the brain. D and M indicate the dorsal and medial sides of the brain, respectively. An image with higher magnification shows the reconstituted GFP signals around the LNv cell body and dendritic area (**b**). Note that the PDF staining in the dendritic areas is very weak because the dendrites do not likely contain much of the PDF peptide, resulting in GFP that does not appear to colocalize well with PDF staining in the dendritic areas. (**c**,**d**) The membrane-tethered GFP fragment construct *CD4::spGFP1-10* was expressed in most octopaminergic neurons with *Tdc2-Gal4*. Reconsitituted GFP signals were also detected around the LNv cell bodies and dendritic area ($n = 10$). Scale bars represent 10 μm.

l-LNvs and s-LNvs and may also label nonsynaptic contacts, we used functional imaging to assay synaptic inputs from dopaminergic neurons to LNvs. Flies carrying *Pdf-Gal4* and *UAS-EPAC* transgenes specifically express the FRET-based cAMP reporter EPAC in both sets of PDF-positive cells, l-LNvs and s-LNvs[15]. We applied dopamine to acutely dissected brains and determined the effects on cAMP levels. We also developed an image-processing method to automatically compare the temporal responses of individual l-LNvs or s-LNvs in the same hemisphere (for details, see Online Methods and **Supplementary Fig. 2**).

We observed a strong decrease in FRET ratio (yellow fluorescent protein (YFP)/cyan fluorescent protein (CFP)) in l-LNvs on bath application of dopamine, indicating that the relative cAMP level in

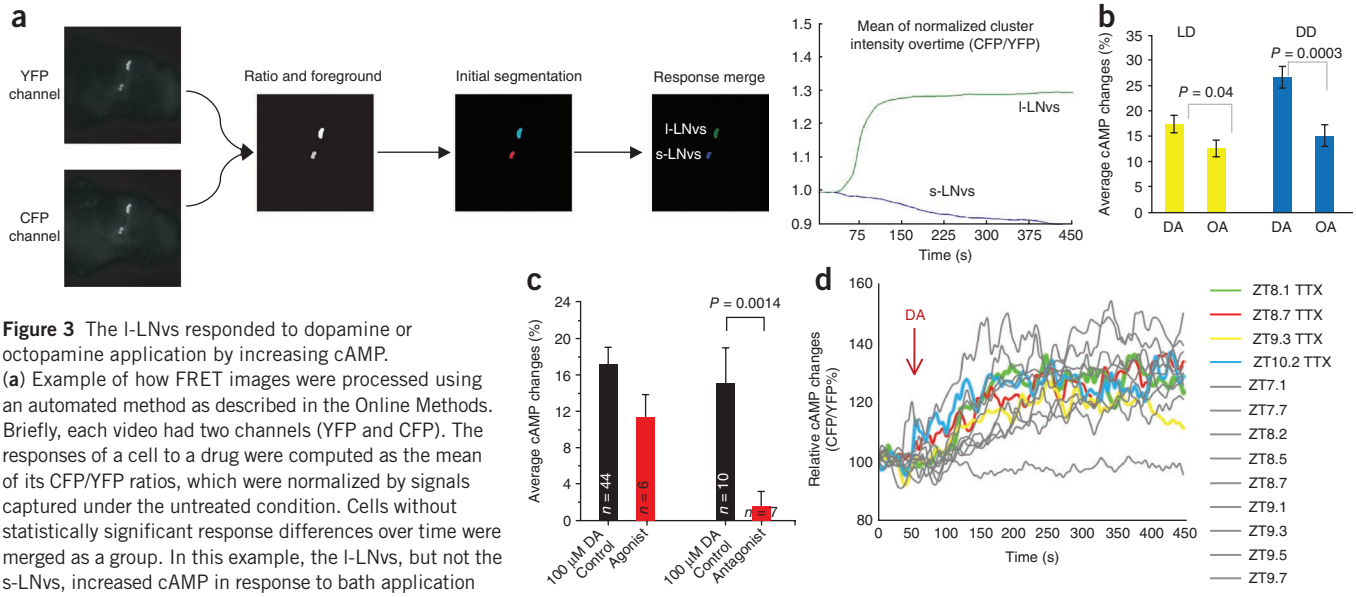

Figure 3 The l-LNvs responded to dopamine or octopamine application by increasing cAMP. (**a**) Example of how FRET images were processed using an automated method as described in the Online Methods. Briefly, each video had two channels (YFP and CFP). The responses of a cell to a drug were computed as the mean of its CFP/YFP ratios, which were normalized by signals captured under the untreated condition. Cells without statistically significant response differences over time were merged as a group. In this example, the l-LNvs, but not the s-LNvs, increased cAMP in response to bath application of dopamine. (**b**) Dopamine (DA) application induced stronger responses in the l-LNvs than octopamine (OA). Left, imaging of flies reared in light-dark conditions (LD); right, flies reared in constant darkness day 1 (DD). (**c**) The responses could be induced by a dopamine agonist and were blocked by a dopamine antagonist. The average fluorescence change (area under the relative cAMP change curve) was determined by calculating an average CFP/YFP ratio increase from 100 to 445 s. Error bar represents s.e.m. Does this apply to error bars in b as well? Please state explicitly. A dopamine agonist, 100 μM pergolide mesylate, also induced an increase of cAMP in the l-LNvs with an effect only slightly less than dopamine alone. The l-LNv dopamine-induced cAMP response was almost completely blocked following a 15-min pre-incubation with a dopamine antagonist, 50 μM (+)-butaclamol hydrochloride. (**d**) Dopamine-induced responses were cell autonomous; the l-LNv responses to dopamine in both the presence and absence of TTX were indistinguishable. The l-LNvs increased cAMP level in response to bath application of dopamine in light-dark conditions. Responses of individual brain samples from different times of the day are shown. The relative cAMP changes are calculated as the normalized CFP/YFP ratio. Each curve represents the average cAMP response of all the visible l-LNvs in one hemisphere. The average cAMP responses from 13 brains are shown. Colored curves, with TTX added to the acutely dissected brains before bath application of dopamine. Gray curves, responses recorded without TTX.

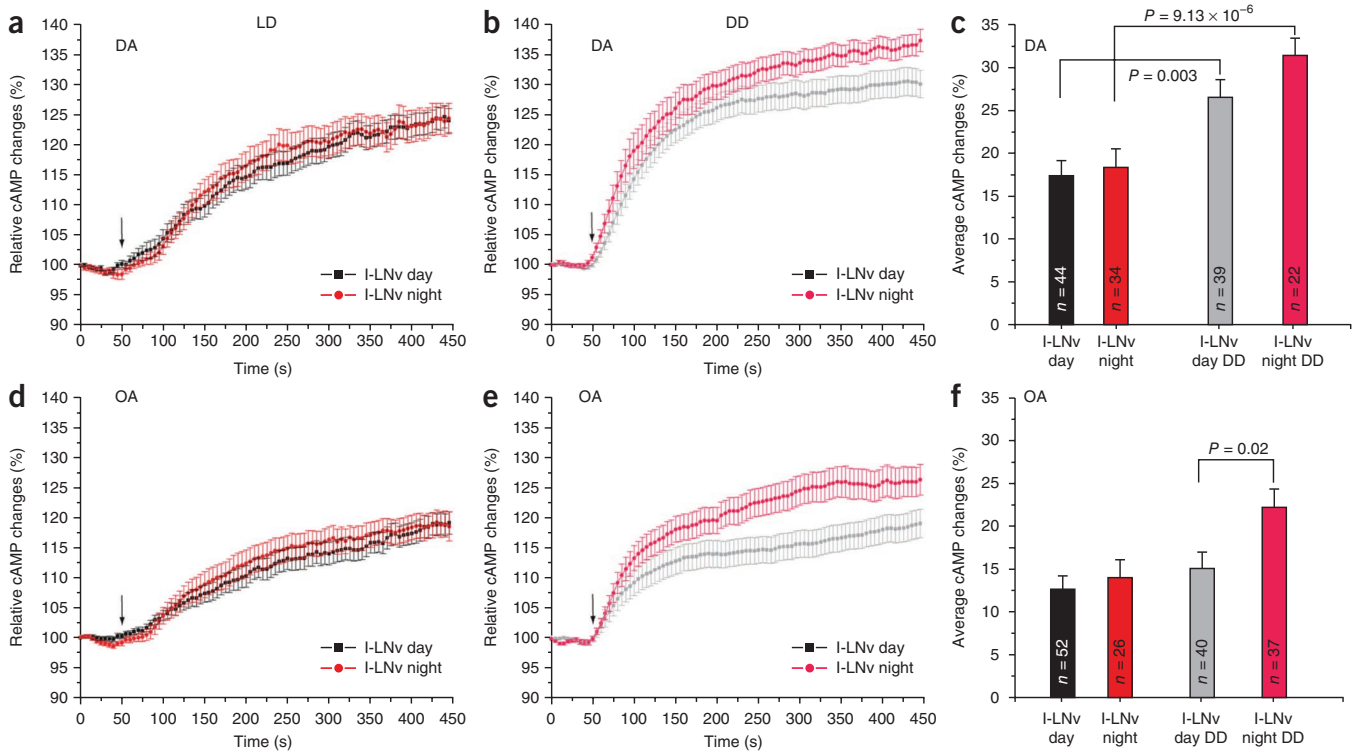

Figure 4 12-h light exposure suppressed the responses of l-LNvs to dopamine or octopamine. (**a**–**c**) Light exposure suppressed the l-LNv responses to dopamine (DA). Flies were housed in light-dark conditions (**a**) or in constant darkness conditions (**b**) and the responses to dopamine during daytime or subjective day were compared with that during nighttime or subjective night. A summary of the relative changes of cAMP (**a**,**b**) is shown in **c**. The l-LNv responses to dopamine during the day/subjective day versus the night/subjective night were not significantly different in either light-dark (LD) or constant darkness (DD) conditions. However, comparison between light-dark and constant darkness conditions showed that the responses of the l-LNvs to dopamine in constant darkness were much stronger during both the subjective day and subjective night than the responses at the same circadian times in light-dark conditions. (**d**–**f**) Daytime light exposure suppressed the nighttime l-LNv responses to octopamine (OA). Flies were housed in light-dark conditions (**d**) or constant darkness conditions (**e**) and the responses to octopamine during daytime or subjective day were compared with that during nighttime or subjective night. Note that the response amplitude of l-LNvs from subjective day in constant darkness was similar to that of daytime in light-dark conditions. A summary of the relative changes in cAMP (**d**,**e**) is shown in **f**. The responses to octopamine during daytime, nighttime or subjective daytime were similar, whereas the l-LNvs from subjective night were more sensitive to octopamine. *P* values indicate significant difference from control groups (Student's *t* test). Error bars represent s.e.m.

these cells increased markedly (**Fig. 3a**). We reached a similar conclusion using *Pdf-Gal4; UAS-EPAC* flies in a *yw* genetic background, suggesting that the connection between dopaminergic neurons and l-LNvs is not strain specific (data not shown). s-LNvs, in contrast, showed very weak responses (**Fig. 3a** and **Supplementary Fig. 3b**), similar to their weak response to octopamine (**Supplementary Fig. 3a**)[16]. Moreover, dopamine (100 μM) induced stronger responses in the l-LNvs than octopamine (100 μM) in both light-dark and constant darkness conditions (**Fig. 3b**). Combined with the behavioral results, this suggests that dopamine is a stronger arousal signal than octopamine in fly brains.

To further test the specificity of the dopamine-induced responses, we applied a dopamine agonist to dissected brains[24]. Pergolide mesylate (100 μM) induced a substantial increase in cAMP in the l-LNvs (**Fig. 3c**). Moreover, pre-incubation with antagonist, (+)-butaclamol hydrochloride (50 μM)[24], almost completely blocked the ability of dopamine to stimulate cAMP production (**Fig. 3c**).

The much stronger dopamine response of l-LNvs compared with s-LNvs is consistent with the receptor mRNA distribution[16]. To further test whether the dopamine-induced responses are cell-autonomous, we applied tetrodotoxin (TTX) to the dissected brains before bath application of dopamine and still observed robust responses. They showed no statistical difference from the non-TTX responses (**Fig. 3d**), except that the non-TTX groups showed a slightly higher variation. We conclude that the l-LNvs receive direct synaptic inputs from dopaminergic neurons. Taken together with our previous study[16], we conclude that the l-LNvs, but not the s-LNvs, are targets of dopamine and octopamine neurons.

Light suppresses dopamine-mediated cAMP increases in the l-LNvs

The wake-promoting effects of l-LNvs are 'plastic', that is, they are effective in standard 12-h:12-h light-dark conditions, but not in constant darkness[4]. To understand how environmental changes affect the physiology of this circuit node, we reared flies in either light-dark or constant darkness and assayed the differences in the l-LNv response to dopamine or to octopamine[21].

We first compared the response amplitude to dopamine between light-dark and constant darkness rearing. Although we did not observe day-night difference in the l-LNv response, constant darkness rearing caused a substantial cAMP increase in both subjective day and subjective night (**Fig. 4a–c**). The increased cAMP response to dopamine therefore appeared to be light sensitive, but time insensitive; both daytime and nighttime responses to dopamine were negatively regulated by the 12-h light exposure of light-dark conditions. On the other hand, octopamine responses were both light and time sensitive. l-LNvs from subjective night were more sensitive to octopamine than those from subjective day. In other words, the 12-h light exposure specifically suppressed the nighttime response (**Fig. 4d–f**).

We also compared the response of individual l-LNvs in the same hemisphere and observed heterogeneous responses during the first

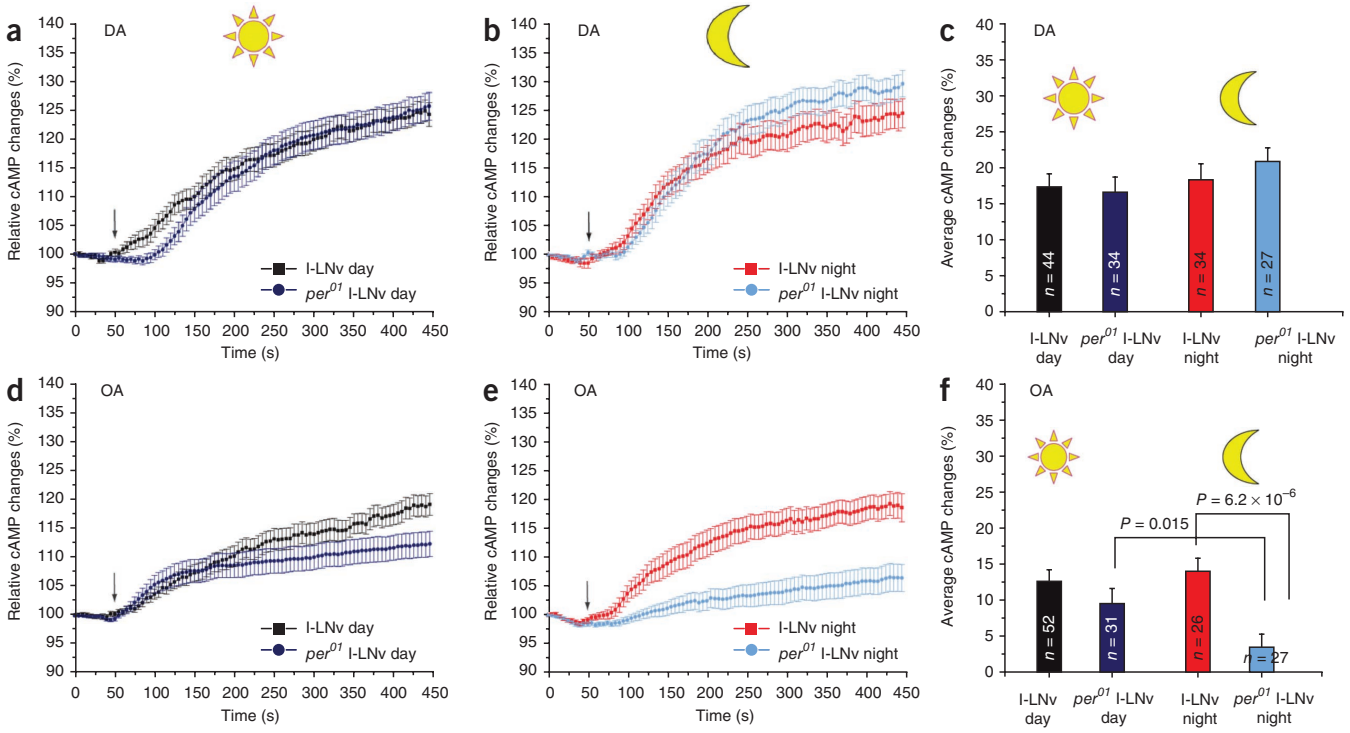

Figure 5 The circadian clock (PER) specifically promotes octopamine-induced responses in l-LNvs at night. (**a**–**c**) The l-LNv responses to dopamine were not affected by PER. The daytime (**a**) and nighttime (**b**) responses are plotted separately. The dopamine-induced responses of the l-LNvs from control brains were compared with those from per^{01} mutant flies. A summary of the relative changes of cAMP (**a**,**b**) is shown in **c**. The responses to dopamine were not affected by per^{01} mutation. (**d**–**f**) PER positively regulated octopamine-evoked responses by l-LNv at night. Flies were housed in light-dark conditions and the daytime (**d**) and nighttime (**f**) responses are plotted separately. The octopamine-induced responses of the l-LNvs from control brains were compared with those from per^{01} mutant flies. A summary of the relative changes of cAMP (**d**,**e**) is shown in **f**. The responses to octopamine during daytime were not affected by per^{01} mutation (left), whereas the nighttime responses were markedly decreased in the per^{01} mutants (right). Error bars represent s.e.m.

day of constant darkness rearing. In the most extreme case, the difference between the responses of all four l-LNvs was statistically significant ($P < 0.005$; **Supplementary Fig. 4a**). We therefore classified the brain responses into four categories on the basis of heterogeneity (**Supplementary Fig. 4b**,**c**): brains with homogenous responses, brains with two types of responses, brains with three types of responses and brains in which all four l-LNvs showed different responses. The third and fourth types were only observed in samples from constant darkness rearing, indicating that the 12-h light exposure also made the dopamine- and octopamine-evoked responses more homogeneous among neighboring l-LNvs in the same hemisphere. We speculate that the synchronization among the neighboring l-LNvs may allow the l-LNvs to produce relatively stable output in light-dark conditions (also see Discussion).

Light and clock regulate octopamine-induced responses

As the l-LNvs are also part of the clock circuit, we asked whether the circadian clock plays a role in regulating their responsiveness to arousal signals. per^{01} flies carry a null mutation in the core clock gene *period* and therefore lack a functioning circadian clock[25]. Because dopamine-induced responses are time insensitive, they may not be regulated by the clock. Consistent with this prediction, the dopamine-induced FRET responses in per^{01} were comparable to those in control brains (**Fig. 5a**–**c**).

However, l-LNvs from per^{01} flies were much less responsive to octopamine than controls during the night, although their responses were similar to control flies during the day (**Fig. 5d**–**f**). In other words and unlike in wild-type flies, we observed day-night difference in per^{01} flies ($P < 0.05$; **Fig. 5f**). Thus, the normal nighttime increase in l-LNv octopamine responsiveness during constant darkness is regulated by two opposing factors: it is increased by the circadian clock and decreased by the light phase of a normal light-dark cycle. Daytime responses appeared to be more stable, that is, less affected by either light or the clock (see Discussion).

To determine whether the phenotype observed in per^{01} mutants is specifically caused by loss of clock function, we tested the octopamine-induced responses of the l-LNvs in flies in a non-circadian mutant. Flies without a functional *yellow* gene show rhythmic behavior in constant darkness conditions and should therefore have a normal clock. The l-LNvs from this strain showed day-night response patterns to octopamine, similar to control strains (**Supplementary Fig. 5**). Thus, the reduced octopamine sensitivity at night that we observed in the per^{01} mutant is likely a result of the absence of the circadian clock.

Light upregulates inhibitory dopamine receptors

How then does light suppress the l-LNv cAMP responses to dopamine? Dopamine activates both stimulatory and inhibitory receptors, and many mammalian brain neurons coexpress stimulatory D1-like receptors (D1Rs) and inhibitory D2-like receptors (D2Rs)[26]. D1Rs modulate neurons by increasing intracellular cAMP, whereas D2Rs antagonize cAMP signaling. Thus, D2Rs are important for gating cellular responses to dopamine and are involved in many neurological and psychological disorders[26]. Both *Drosophila* DopR and DopR2 belong to the D1-like stimulatory receptor subfamily[27], whereas *Drosophila* D2R is the only known inhibitory receptor in the fly[28]. Moreover, D2R is highly enriched in l-LNvs compared with the neighboring s-LNvs[16].

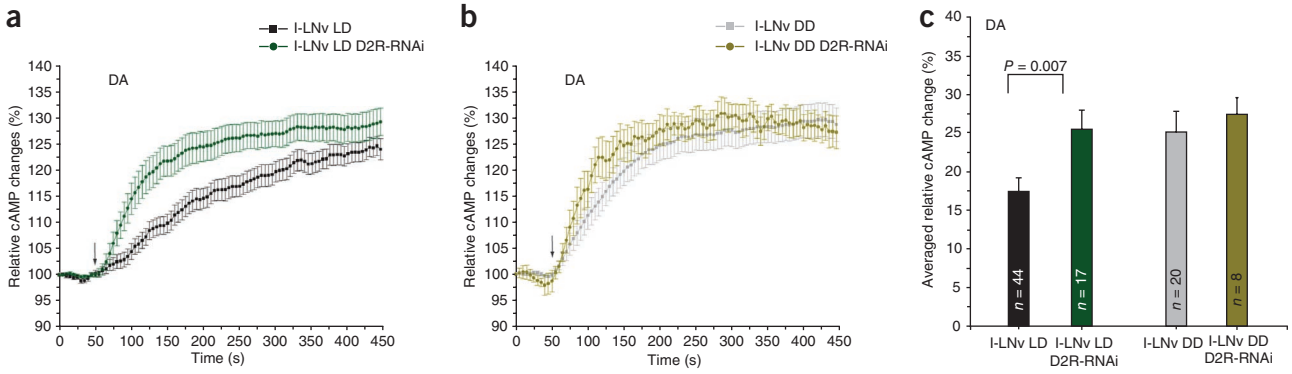

Figure 6 Light suppresses dopamine responses by upregulating inhibitory dopamine receptors. (**a**,**b**) D2R negatively regulated dopamine-evoked responses in the l-LNvs. (**a**) The l-LNv response to dopamine in light-dark conditions was markedly increased by knocking down D2R expression in the l-LNvs. The dopamine-induced responses of the l-LNvs from control brains were compared with those from D2R-RNAi knockdown mutant flies. (**b**) The l-LNv response to dopamine in constant darkness conditions was not affected by knocking down D2R expression in the l-LNvs. The dopamine-induced responses of the l-LNvs from control brains were compared with those from D2R-RNAi knockdown mutant flies. (**c**) Summary of the relative changes of cAMP shown in **a** and **b**. The responses in constant darkness were comparable with those in D2R knockdown mutants in light-dark conditions. Error bars represent s.e.m.

To investigate whether the LNvs use D2R to buffer the effectiveness of dopamine activation, we used *Pdf-Gal4* to drive the expression of a *UAS-D2R* RNA interference (RNAi) in the l-LNvs and s-LNvs. Knockdown of D2R markedly increased the dopamine-induced cAMP response in l-LNvs in light-dark conditions (**Fig. 6a**), indicating that they indeed coexpress inhibitory as well as excitatory dopamine receptors and that D2R gates the dopamine response. In contrast, the knockdown in flies housed in constant darkness conditions showed no effect on cAMP increases (**Fig. 6b**,**c**). Moreover, the D2R knockdown group now showed similar responses in light-dark compared with constant darkness conditions. As a control, we imaged the s-LNv responses to dopamine in the D2R-RNAi flies and observed no detectable effects (**Supplementary Fig. 6**), consistent with the fact that these cells express much lower levels of D2R than l-LNvs[16,24]. Taken together, these data suggest that light-dark conditions lead to an upregulation of the D2R inhibitory signaling pathway in the l-LNvs, which counter-balances the activation effect of dopamine.

DISCUSSION

Light buffers the effectiveness of dopamine-mediated wake-promoting effects in *Drosophila*. Daytime sleep is relatively insensitive to dopamine activation, whereas nighttime sleep in light-dark conditions is sensitive, but less so than nighttime sleep in constant darkness conditions. The ten l-LNvs, a subset of clock neurons, are the only neurons known to be part of the light-mediated wake-promoting circuits in fly brains. We found that they are downstream targets of dopaminergic neurons. They not only formed membrane contacts with dopaminergic neurons, but also responded to dopamine by increasing cAMP levels. This presumably reflects the fact that l-LNvs express stimulatory receptors for these neurotransmitters. The response was largely cell-autonomous, as they still responded to dopamine in the presence of TTX (**Fig. 3**). We also found that the responses are likely to be specific to dopamine, as they were blocked by a dopamine antagonist and could be induced by a dopamine agonist (**Fig. 3**).

These cells also receive direct synaptic input from octopaminergic neurons (**Fig. 2** and data not shown for octopamine in the presence of TTX). Dopamine is likely a stronger arousal signal than octopamine in fly brains, at least for flies raised in standard light-dark conditions. An identical stimulation of octopamine neurons in adult brains only mildly suppressed total sleep, an effect that was also considerably smaller than previously reported[6]. This previous study used a sodium channel to constitutively stimulate octopamine neurons[29]. Combined with the fact that feeding flies with octopamine also required 2–3 d to suppress sleep and that nighttime sleep was still affected even after octopamine was removed[7], we suggest that chronic activation of octopaminergic neurons may require a reconfiguration of neural circuits to produce strong behavioral effects.

The s-LNvs are neighbors of the l-LNvs and are key pacemaker neurons in *Drosophila*. In contrast with the l-LNvs, s-LNvs showed very weak responses to dopamine or octopamine in light-dark conditions, likely reflecting the fact that mRNAs for these receptors are much more abundant in l-LNvs than in s-LNvs[16]. This even includes the dopamine D2R inhibitory receptors, which also explains why D2R knockdown did not lead to a detectable cAMP increase in s-LNvs in response to dopamine application (**Supplementary Fig. 6**).

Light has a profound effect on animal behavior. For example, extensive light-driven cyclic gene expression has been detected in *Drosophila*[30]. The l-LNvs are also reported to increase their firing rate in response to acute light exposure, especially during early morning[13]. We found that the 12-h light exposure of standard light-dark housing conditions has a profound effect on l-LNv physiology. Light-dark rearing not only mitigates the stimulating effects of both dopamine and octopamine, but also synchronizes cell responses. One possible function for synchronization is that the l-LNv responses are more stable when synchronized (see below). Although l-LNvs from light-dark–reared flies are less sensitive to both dopamine and octopamine than those from constant darkness–reared flies, the two signaling pathways are differentially regulated.

Octopamine-mediated responses are time sensitive in constant darkness, and octopamine activation at night is promoted by the clock, but inhibited by prior light exposure. Microarray data indicate that transcription of the octopamine receptor OA2 peaks around zeitgeber time 12 (ZT12), whereas that for OAMB peaks around ZT6 (ref. 16). Given that our imaging analysis indicated that maximum nighttime l-LNv responses to octopamine require the clock (**Fig. 5**), it is possible that the translation or activities of these receptors, or the expression of signaling molecules downstream of these receptors, peaks at night.

In contrast with octopamine-mediated responses, the dopamine-mediated responses of l-LNvs were time insensitive and were not affected by the per^{01} mutation (**Figs. 4** and **5**). However, light exposure suppressed the l-LNv dopamine responses at all times, both nighttime and daytime. Given that downregulation of D2R is sufficient to mimic the responses of flies reared in constant darkness and that D2R RNAi

had no effect in constant darkness, light exposure apparently upregulates D2R activity to dampen dopamine responsiveness in light-dark conditions. This implies that there are light-stimulated changes in either *D2R* gene expression or regulation, such as a modification of the D2R receptor or its downstream targets. Light may also downregulate stimulatory D1R signaling pathways in concert with the upregulation of D2R, although our results suggest that expression of D2R can account for most of the reduction in responsiveness. Given that there are no known inhibitory receptors for octopamine, the l-LNvs must use a different mechanism to effect light-mediated modulation of octopamine responsiveness (**Supplementary Fig. 7**). For example, light may downregulate stimulatory octopamine receptors. Nonetheless, a common theme is that light inhibits the ability of these two chemicals to stimulate the l-LNvs. The fact that the 12-h light exposure suppressed the ability of dopamine and octopamine to stimulate l-LNvs suggests that they do not simply sum different arousal signals. Instead, they are integrated and perhaps scaled depending on conditions, suggesting a link to behavioral flexibility. In this scenario, light appears to be a dominant signal, as its presence during the day reduces the ability of internal signals to stimulate arousal. However, the l-LNvs use a number of mechanisms, including the circadian clock to integrate signals and produce appropriate responses. The surprisingly weak behavioral effects of acute stimulation of octopamine neurons raises the possibility that there are other circumstances (age, nutritional or reproductive status) in which these inputs become more important.

Because animals must maintain a proper quality and quantity of daily wake and sleep time, counter-balancing mechanisms such as those described here may also serve to preserve sleep stability in the fly brain. For example, the opposing effects of environmental light and dopamine may allow the l-LNvs and perhaps other arousal-sleep–relevant neurons to buffer unexpected fluctuations in light intensity and/or dopamine release from presynaptic partners; that is, the circuit organization allows the activity of sleep-relevant neurons to be maintained in a physiological range with a relatively stable output. We imagine that only exceptional circumstances would take precedence over sleep-wake stability; for example, by modulating the ratio of stimulatory and inhibitory dopamine receptors. Our data suggest that modulation could also occur by altering the synchronization of individual cells in a group, such as between different individual l-LNvs. It will not be surprising if additional integration mechanisms are also important for the l-LNvs to generate appropriate signals to downstream circuits, both to maintain optimal sleep at night and optimal wakefulness during the day, that is, for sleep-wake homeostasis, and for appropriate responses to emergency circumstances.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Y.S. conceived the project. Y.S., P. Haynes, N.P. and F.G. performed the experiments. K.I.H., J.P. and P. Hong developed the algorithm for the automated imaging analysis. Y.S., L.C.G. and M.R. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Fly stocks. Flies were raised on standard medium with 12-h light:dark cycles at 23–25 °C. The *pdf-Gal4* (X) and *UAS-Epac1-cAMP* (50A and 55A) flies were kindly provided by P. Taghert (Washington University). *pdf-Gal4*/Cyo flies were used to express the EPAC sensor in the PDF-expressing l-LNvs and s-LNvs in fly brains. *UAS-D2R-RNAi* (II) flies were obtained from VDRC.

We typically entrained day 1–2 male flies at 25 °C in standard light-dark conditions for 3–4 d before imaging. We used fluorescent light at an intensity of 1,600 ± 400 lx. To test the effect of different environmental conditions on the physiological responses of the LNvs, we turned off the lights of the incubators at ZT0 after 3 d of entrainment and continued housing the flies in the constant darkness. The flies were kept in constant darkness for less than 24 h and were then dissected in the red light to avoid light exposure.

Behavioral analysis. Individual flies were housed separately in 65-mm × 5-mm glass tubes (Trikinetics) containing 5% agarose with 2% sucrose (wt/vol). We collected and entrained 2–5-d-old flies under standard light-dark conditions, with a 12-h light phase and followed by 12-h dark phase for 3–4 d.

To test the effect of heat-induced firing by *Drosophila* TrpA1 channels, we entrained flies in standard light-dark conditions at 21 °C for 3–5 d and then raised the temperature of the incubator to 27 °C or 30 °C at ZT12 for 2–3 d (**Fig. 1**). For **Figure 1c**, the lights were turned off permanently after the heat activation. The temperature was then returned to 21 °C to inactivate the TrpA1 channel.

Both sleep time and the effect of heat on sleep are 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 (sleep time at 30 °C – sleep time at 21 °C) / sleep time at 21 °C. We then calculated the relative sleep change (ΔSI), which is $\Delta SI = SI_{experimental} - SI_{control}$ (**Table 1**).

Split-GFP imaging. *w-/yw; pdf-LexA, LexAop-GFP11/+; UAS-GFP1-10 /* (*TH-Gal4* or *Tdc2-Gal4*) flies were used to express the GFP11 fragment in the PDF-expressing LNvs and the GFP1–10 fragment in dopamine or octopamine neurons, respectively. *w-/yw; pdf-LexA/LexAop-GFP11; TM6B.Tb/UAS-GFP1-10* flies were used as controls, and no reconstituted GFP signals were detected around the LNv cell bodies or dendritic areas. For immunostaining, a standard fixation protocol was used. Briefly, the brains were fixed immediately after dissection for 1 h on ice in 4% paraformaldehyde (vol/vol). Brains were incubated in primary antibodies for two nights at 4 °C and secondary antibodies for one night at 4 °C. Sequential staining was used to prevent the Alexa-488 antibody to mouse from reacting with the rat antibody to PDF. Brains were sequentially incubated with four antibodies, washing between each, in the following order: mouse monoclonal antibody to GFP (Roche), which stains GRASP-reconstituted GFP, but not either GFP fragment alone, Alexa 488 antibody to mouse, rat antibody to PDF, and cy3 antibody to rat (Jackson). Brain samples were visualized by a Leica TCS SP2 confocal microscope and all images were taken sequentially.

Brain imaging. Live FRET imaging was performed as described previously with some modifications[16]. Briefly, 3–6-d-old entrained male flies were dissected in ice-cold adult hemolymph-like medium (AHL)[32]. We added 400 µl of 20–25 °C AHL to the imaging chamber. An individual brain was then placed in the chamber. To avoid brain floating, we attached a small piece of nylon to the bottom of the chamber with grease. Individual brains were then inserted under the nylon. EPAC expressed in LNvs was excited with 50-ms pulses of light using CFP filters. To avoid light-induced effects, two 25-mm neutral density filters (Chroma), 1.3 and 0.6, were used to further block the arc lamp light. Fluorescent signals emitted by LNvs were imaged every 5 s by an epifluorescent microscope using a 20× objective on a Zeiss microscope (Intellegent Imaging Innovations). The images were collected with either a CFP or YFP filter. The CFP-2432A filter from Semrock and the Chroma 9052 ET CFP/YFP FRET cube with exciter ET436/20x, dichroic T455LP, and emitter ET535/30m were used. A shutter system was used to control the rotation of the filter sets. SLIDEBOOK 4.1 software (Intelligent Imaging Innovations) was used for imaging analysis. For a subset of the experiments, a different setup was used that consisted of an Olympus BX51WI microscope with a CCD camera (Hammamatsu Orca C472—80-12AG). The acquisition system for this setup was slightly different and allowed for simultaneously recording both channels. The 86002v1 JP4 excitation filter (436, Chroma) as well as a two-channel, simultaneous-imaging system from Optical Insights with the D480/30m and D535/40m emission filters were used. Volocity software (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 LNvs stabilized after imaging the neurons for 150 frames. We were then able to obtain reliable responses induced by 10 µM foskolin (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[33]. Dopamine agonist, pergolide mesylate salt, and antagonist, (+)-butaclamol hydrochloride, were purchased from Sigma. A stock solution of pergolide mesylate salt (10 mM) was prepared in DMSO and 500 µM (+)-butaclamol hydrochloride was prepared in H_2O (ref. 24). The stock solutions were stored at −20 °C. Pergolide mesylate salt (100 µM) was used to induce the cAMP responses in the LNvs. To block the dopamine induced responses, brains were pre-incubated with 50 µM (+)-butaclamol hydrochloride for 15 min before applying 100 µM dopamine. TTX was purchased from Sigma and a stock solution (100 µM) was prepared in H_2O. The final concentration was used at 1 µM[34]. Brains were pre-incubated in 1 µM TTX for 15 min before adding dopamine or octopamine.

The baseline images were collected for 50 s before applying 100 µM of octopamine or dopamine to the brain. The background first was subtracted from the mean intensity of CFP and YFP over l-LNvs or s-LNvs. The background is the mean intensity of a nonfluorescent brain region next to the LNvs. The YFP/CFP ratio for each time point was 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 to 445 s.

Automated data analysis and statistical analysis. Each video had two channels (YFP and CFP, at a resolution of 512 × 512 pixels) that are pre-processed as the following. First, a Gaussian kernel (9 × 9 pixels) was applied to reduce noise in each individual image in each channel. The microscope stage undergoes some vibration during image acquisition and it caused dissected brains to move slightly. To remove the mechanical movements of the dissected brain during imaging, a two-step registration was applied to align images in two channels. In the first step, the first frames of both channels were registered against each other. In the second step, the rest of the frames in each channel were registered to the first one in the respective channel using a mutual information–based method (Artyushkova, K.; Automatic Image Registration using (Normalized) Mutual Information for users of IP toolbox, http://www.mathworks.com/matlabcentral/fileexchange/4145-automatic-image-registration-using-normalized-mutual-information-for-users-of-ip-toolbox) After registration, the background signal in each frame was modeled as a Gaussian distribution. A threshold representing 99.9% background population was selected to detect the foreground that was refined by morphological image-processing operations[35]. To obtain a robust foreground detection result, a final foreground mask was generated to include those pixels that were detected as foreground in more than 70% of the time in the whole video. To account for the noise over time, a temporal median filter (ten frames) was applied to each pixel in the foreground mask. A reference image was generated for each channel by averaging the images recorded in the period before drug administration (the first ten frames). This reference image was then used to normalize all images in the same channel.

Both the image intensity and the temporal dynamics were used to segment cells into clusters. The initial segmentation was computed using the watershed transform[36] of the gradient of the reference image from the YFP channel. In many cases, the gradient may have large variations in a cell or more frequently in a cluster of cells. Thus, cells may be over-segmented. This type of over-segmentation can be resolved by merging segments with statistically indistinguishable differences in their temporal responses. The response of a pixel in an image frame was computed as the ratio CFP/YFP, which was normalized by its response in the reference image. The mean and the s.d. of all the pixels in a segment were calculated. The temporal response of an image segment was then computed as the mean of the temporal response of all pixels in it.

The difference between two different segments was then compared to the distribution of differences between background patches. If none of the initial

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segments were different with a $P \leq 0.005$, the segments were merged into a single cluster. If the s.d. of this cluster was less than 0.05, these segments or cells were considered as a homogenous group. Segments with differences in the top 0.5% ($P = 0.005$) were considered to be a heterogeneous class. The null distribution (**Supplementary Fig. 2**) used for comparing the temporal responses of two segments was built using 10,000 background patches (16 × 16 pixels) randomly selected from ten brains. The difference between the temporal responses of two segments, $\mathbf{a} = [a_1, a_2, \ldots, a_T]$ and $\mathbf{b} = [b_1, b_2, \ldots, b_T]$, was calculated as the Euclidean distance between them

$$D(\mathbf{a}, \mathbf{b}) = \sqrt{\sum_{t=1}^{T} (a_t - b_t)}$$

where a_t and b_t are the responses of segments a and b at time t, and T is the total number of frames in a video. The maximum s.d. between two segments, p and q, is given as $\max_{1 \leq t \leq T} [\sigma(\{p_t^m\}_{m=1}^M, \{q_t^n\}_{n=1}^N)]$, where p_t^m is the response of the m^{th} pixel in segment p in frame t, M is the number of pixels in segment p, q_t^n is the response of the n^{th} pixel in segment q in frame t, N is the number of pixels in segment q, and

$$\sigma\left(\{p_t^m\}_{m=1}^M, \{q_t^n\}_{n=1}^N\right) = \sqrt{\frac{1}{M+N}\left(\sum_{m=1}^M (p_t^m - \bar{x})^2 + \sum_{n=1}^N (q_t^n - \bar{x})^2\right)}$$

$$\bar{x} = \frac{1}{M+N}\left(\sum_{m=1}^M p_t^m + \sum_{n=1}^N q_t^n\right)$$

Finally, the temporal responses of the remaining clusters can be taken to show drug effects.

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