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

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Abstract

How animals maintain proper amounts of sleep yet still be flexible to changes in the environmental conditions remains unknown. Here we showed that environmental light suppresses the wake-promoting effects of dopamine in fly brains. A subset of clock neurons, the 10 large lateral-ventral neurons (l-LNvs), are wake-promoting and respond to dopamine, octopamine as well as light. Behavioral and imaging analyses suggested that dopamine is a stronger arousal signal than octopamine. Surprisingly, light exposure not only suppressed the l-LNv responses but also synchronized responses of neighboring l-LNvs. This regulation occured by distinct mechanisms: light-mediated suppression of octopamine responses is regulated by the circadian clock, whereas light regulation of dopamine responses occurs by upregulation of inhibitory dopamine receptors. Plasticity therefore alters the relative importance of diverse cues based on the environmental mix of stimuli. The regulatory mechanisms described here may contribute to the control of sleep stability while still allowing behavioral flexibility.

INTRODUCTION

Animal sleep is responsive to external signals like light and social environment [1–4]. Sleep is also modulated by internal signals like 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 multiple internally generated arousal signals, including dopamine, octopamine, the circadian clock-related neuropeptide PDF, etc [5–8]. Light is a prominent arousal signal for diurnal animals like flies. However, wakefulness does not scale

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linearly with light intensity, nor does light work in isolation. For example, flies as well as humans are prone to sleepiness in the middle of the day, often accompanied by a nap or siesta [9,10]. This indicates that light effects on the brain and on sleep circuitry are likely to be complex and integrated with other sleep-relevant signals.

Relevant to 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 10 large lateral-ventral neurons (l-LNvs; 5 on each side of the brain), as potently wake-promoting [4,8,11]. Importantly, 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 showed 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, which 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 10 l-LNvs have related neurons nearby, the 8 small LNvs (s-LNvs). s-LNvs express neuropeptide, PDF, which 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 FRET based cAMP reporter driven by this driver [15] revealed robust responses evoked by octopamine in the l-LNvs but not the s-LNvs [16]. This is consistent with the fact that mRNAs for two octopamine receptors, OAMB and OA2, are enriched 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 mammals as well as flies [5]. We first showed that a 12hr light exposure suppresses dopamine-mediated wake promoting effects, i.e., sleep in the dark was more inhibited than sleep in the light by dopaminergic neuron firing. Since 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 showed that the l-LNvs receive synaptic inputs from dopamine and octopamine neurons. However, dopamine appears to be a stronger arousal signal than octopamine in fly brains, at least for flies raised under basal 12:12 light-dark conditions. By comparing the l-LNv responses evoked by dopamine or octopamine under different light conditions, we showed that light suppresses 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 as well as different internal sleep-regulating 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.

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METHODS AND MATERIALS

Fly Stocks

Standard medium, 12hr light:dark cycles, and 23–25°C were used to raise flies. The *pdf-Gal4* (X) and *UAS-Epac1-cAMP* (50A and 55A) flies were kindly provided by Paul Taghert at Washington U, St. Loius. *pdf-Gal4*/Cyo flies were used to express the EPAC sensor in the PDF-expressing l- and s-LNvs in fly brains. *UAS-dD2R-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 days before imaging. We used fluorescent light and the light intensity was 1600 ± 400 l×. 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 days of entrainment and continued housing the flies in the constant darkness. The flies kept in constant darkness for less than 24 hr were then dissected in the red light to avoid light exposure.

Behavioral analysis

Individual flies were housed separately in 65mm × 5mm glass tubes (Trikinetics, Waltham, MA) containing 5% agarose with 2% sucrose. 2–5 day old flies were collected and entrained under standard light-dark conditions, with a 12hr light phase and followed by 12hr dark phase for 3–4 days.

To test the effect of heat-induced firing by dTrpA1 channels, we entrained flies in standard light-dark conditions at 21 °C for 3–5 days, and then raised the temperature of the incubator to 27 °C or 30 °C at ZT12 for 2–3 days (Fig. 1). For Fig. 1C, the lights were turned off permanently upon the heat activation. The temperature was then returned to 21 °C to inactivate the dTrpA1 channel.

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 %. We then calculated the relative sleep change (ΔSI), which is $\Delta SI\ \% = SI_{exp} - SI_{ctrl}$ (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 1hr on ice in 4% PFA. Brains were incubated in primary antibodies for two nights at 4°C and secondaries for one night at 4°C. Sequential staining was used to prevent the Alexa-488 anti-mouse from reacting with the rat anti-PDF. Brains were sequentially incubated with 4 antibodies washing between each in the following order: 1.) mouse anti-GFP monoclonal

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(Roche), which stains GRASP reconstituted GFP only, but not either GFP fragment alone; 2.) Alexa 488 anti-mouse; 3.) rat anti-PDF; 4.) cy3 anti-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 in Shafer et al, 2008 with some modifications [16]. Briefly, 3–6 day old entrained male flies were dissected in ice cold adult hemolymph-like medium (AHL) [32]. 400 µl room temperature AHL was added to the imaging chamber. An individual brain was then placed in the chamber. To avoid brain floating, a small piece of nylon was attached to the bottom of the chamber with grease. Individual brain was then inserted under the nylon. EPAC expressed in LNvs was excited with 50ms pulses of light using CFP filters. To avoid light-induced effects, two 25mm 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 ET436/20×, 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 set up was used that consisted of an Olympus BX51WI microscope with a CCD camera (Hammamatsu Orca C472—80-12AG). The acquisition system for this set up was slightly different and allow 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 D480/30m and D535/40m emission filters were used. 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 LNvs stabilized after imaging the neurons for 150 frames. We were then able to obtain reliable responses induced by 10µM foskolin (data not shown).

Octopamine and dopamine were purchased from Sigma and a stock solution (10mM) 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 (10mM) was prepared in DMSO and 500 µM (+)-Butaclamol hydrochloride was prepared in H_2O [24]. The stock solutions were stored at −20 °C. 100uM Pergolide mesylate salt, was used to induce the cAMP responses in the LNvs. To block the dopamine induced responses, brains were pre-incubated with 50 µM antagonist, (+)-Butaclamol hydrochloride, for 15min 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 15min 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- or s-LNvs. The background is the mean intensity of a non-fluorescent brain

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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 100s to 445s.

Automated data analysis and statistical analysis

Each video has two channels (YFP and CFP, at a resolution of 512×512 pixels) that are preprocessed as the following. First a Gaussian kernel (9×9 pixels) is 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 is 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 is modeled as a Gaussian distribution. A threshold representing 99.9% background population is 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 is generated to include those pixels that are 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 (10 frames) is applied to each pixel in the foreground mask. A reference image is generated for each channel by averaging the images recorded in the pre-drug administration period (the first ten frames). This reference image is then used to normalize all images in the same channel.

Both the image intensity and the temporal dynamics are utilized to segment cells into clusters. The initial segmentation is 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 within a cell or more frequently within a cluster of cells. Therefore, 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 is computed as the ratio CFP/YFP, which is normalized by its response in the reference image. The mean and the standard deviations of all the pixels in a segment were calculated. The temporal response of an image segment is then computed as the mean of the temporal response of all pixels in it.

The difference between 2 different segments was then compared to the distribution of differences between background patches. If none of the initial segments are different with a p-value <= 0.005, the segments will be merged into a single cluster. If the standard deviation of this cluster is less than 0.05, these segments or cells will be considered as a homogenous group. Segments with differences in the top 0.5% (p-value: 0.005) are considered as heterogeneous class. The null distribution (supplemental Fig. 2) used for comparing the

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temporal responses of two segments is built using 10,000 background patches (16×16 pixels) randomly selected from 10 brains. The difference between the temporal responses of two segments, $a=[a_1,a_2,\ldots,a_T]$ and $b=[b_1,b_2,\ldots,b_T]$, is calculated as the Euclidean distance between them:

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

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 standard deviation between two segments, *p* and *q*, is $\max_{1\le t\le T}[\sigma(\{p_t^m\}_{m=1}^M,\{q_t^n\}_{n=1}^N)]$ 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

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

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

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

RESULTS

The wake promoting effect of dopaminergic neurons is suppressed by 12 hr light exposure

Since 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 *THGal4:UAS-dTrpA1* fly lines to activate dopaminergic neurons under light-dark or constant darkness conditions and tested the resulting behavioral effects. The dTrpA1 channel allows an acute activation for 2–3 days of adult brain neurons using a shift to warm temperature [4,21]. Unlike the chronic activation done 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 *THGal4* dramatically suppressed total sleep followed by an increased amount of sleep during recovery the next day (Fig. 1A-D). In contrast, activation of octopaminergic neurons in *Tdc2Gal4:UAS-dTrpA1* fly lines using the same temperature protocol had no detectable effect on total sleep in either condition (supplemental Fig. 1 and data not shown for constant darkness conditions). Since chronic stimulation of these neurons using a sodium channel led to mild decrease of total sleep [6], we speculated that stronger activation of octopaminergic neurons in adult brains may be necessary to produce significant

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total sleep effects. We then used 30 °C to activate octopamine neurons and observed a slight decrease of total sleep as well as nighttime sleep (supplemental Fig. 1).

Since activation of dopaminergic neurons produced stronger effects, we decided to focus on the interaction between light and dopamine. Only nighttime sleep was dramatically 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 light-dark conditions (compare Δ Total sleep in light-dark and constant darkness in 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 is more affected than nighttime sleep in light-dark (compare Δ Nighttime sleep in Table 1 in light-dark and constant darkness conditions), suggesting the 12hr of light exposure in light-dark conditions also suppressed dopamine-mediated wake promoting effects in the nighttime.

The wake promoting l-LNvs receive synaptic input from dopaminergic neurons

To further investigate the circuitry mechanisms underlying this light effect on dopamine responsiveness, we focused on the l-LNv clock neurons; they 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 large LNvs than in small 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 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 6 brains 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 4 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 octopaminergic as well as dopaminergic neurons and l-LNvs.

Individually labeled PPL2 dopaminergic neurons have previously been shown to project to the area containing the LNvs [22,23]. In order to evaluate potential presynaptic dopaminergic projections in the vicinity of the LNv dendrites, we stained *TH-Gal4* driven *UAS-mCD8-GFP* with anti-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).

Since split-GFP analysis with the pdf driver does not *a priori* distinguish between the l- and s-LNvs and may also label non-synaptic 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

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PDF+ 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- or s-LNvs within the same hemisphere (for details, see **Methods and Materials** and supplemental Fig. 2).

We observed a strong decrease in FRET ratio (YFP/CFP) in l-LNvs upon bath application of dopamine, indicating that the relative cAMP level in these cells increased dramatically (Fig. 3A, plotted as CFP/YFP ratio). We reached a similar conclusion using *pdfGal4: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, show very weak responses (Fig. 3A and Supplemental Fig. 3B), similar to their weak response to octopamine (Supplemental Fig. 3A)[16]. Moreover, 100 μM dopamine induced stronger responses in the l-LNvs than 100 μM octopamine 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]. 100uM pergolide mesylate induced a significant increase in cAMP in the l-LNvs (Fig. 3C). Moreover, pre-incubation with 50uM antagonist, (+)-Butaclamol hydrochloride [24], almost completely blocked the ability of dopamine to stimulate cAMP production (Fig. 3C).

The much stronger dopamine response of l-LNvs compared to s-LNvs is consistent with the receptor mRNA distribution [16]. To further test if the dopamine-induced responses are cell-autonomous, we applied 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, compare colored curves with grey curves) 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 as well as octopamine neurons.

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

The wake promoting effects of l-LNvs are “plastic,” i.e., they are effective in standard 12hr: 12hr 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.

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 significant cAMP increase in both subjective day and subjective night (Fig. 4A-C). The increased cAMP response to dopamine therefore appears light-sensitive but time-insensitive; both daytime and nighttime responses to dopamine are negatively regulated by the 12hr light exposure of light-dark conditions. On the other hand, octopamine responses are both light and time-sensitive. l-LNvs from subjective night were

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more sensitive to octopamine than those from subjective day. In other words, the 12hr light exposure specifically suppressed the nighttime response (Fig. 4D-F).

We also compared the response of individual l-LNvs within the same hemisphere and observed heterogeneous responses during the first day of constant darkness rearing. In the most extreme case, the difference between the responses of all 4 l-LNvs is statistically significant (supplemental Fig. 4A, $p<0.005$). We therefore classified the brain responses into 4 categories based on heterogeneity (supplemental Fig. 4B-C): brains with homogenous responses; brains with 2 types of responses; brains with 3 types of responses; brains in which all 4 l-LNvs showed different responses. Types 3 and 4 were only observed in samples from constant darkness rearing, indicating that the 12hr light exposure also made the dopamine and octopamine-evoked responses more homogeneous among neighboring l-LNvs within 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-mediated suppression of octopamine-induced responses is regulated by the clock

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} are 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. 5DF). In other words and unlike in wild-type flies, we observed day-night difference in per^{01} flies (Fig. 5F, $p<0.05$). Therefore, 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 (Supplemental Fig. 7). Daytime responses appear more stable, i.e., less affected by either light or the clock (see Discussion).

To test if 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 (supplemental Fig. 5). Therefore, the reduced octopamine sensitivity at night observed in the per^{01} mutant is likely due to the absence of the circadian clock.

Light regulates dopamine responses by upregulating 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 co-express stimulatory D1-like receptors (D1Rs) and inhibitory D2-like receptors (D2Rs) [26]. D1Rs

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modulate neurons by increasing intracellular cAMP, whereas D2Rs antagonize cAMP signaling. Therefore, D2Rs play an important role in 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 (D1R) [27], whereas dD2R is the only known inhibitory receptor in the fly genome [28]. Moreover, the dD2R is highly enriched in l-LNvs compared with the neighboring s-LNvs [16].

To investigate whether the LNvs use dD2R to buffer the effectiveness of dopamine activation, we used *pdf-Gal4* to drive the expression of a *UAS-dD2R-RNAi* in the l-LNvs and s-LNvs. Knockdown of dD2R dramatically increased the dopamine-induced cAMP response in l-LNvs in light-dark conditions (Fig. 6A, C), indicating that they indeed co-express inhibitory as well as excitatory dopamine receptors and that dD2R is gating 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 dD2R knockdown group now showed similar responses in light-dark compared to constant darkness conditions. As a control, we imaged the s-LNv responses to dopamine in the dD2R-RNAi flies and observed no detectable effects (Supplemental Fig. 6), consistent with the fact that these cells express much lower levels of dD2R than l-LNvs [16]. Taken together, the data suggest that light-dark conditions lead to an upregulation of the dD2R 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 10 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 show here that they are downstream targets of dopaminergic neurons. They not only form membrane contacts with dopaminergic neurons but also respond to dopamine by increasing cAMP levels. This presumably reflects the fact that l-LNvs express stimulatory receptors for these neurotransmitters. The response is largely cell-autonomous, because they still respond to dopamine in the presence of TTX (Fig. 3). We also showed that the responses are likely to be specific to dopamine because they are blocked by a dopamine antagonist and can be induced by a dopamine agonist (Fig. 3).

These cells also receive direct synaptic input from octopaminergic neurons (Fig 2; 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 requires 2–3 days to suppress sleep and the 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.

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The s-LNvs are neighbors of the l-LNvs and are key pacemaker neurons in *Drosophila.* In contrast to the l-LNvs, s-LNvs show 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 dD2R inhibitory receptors, which also explains why the dD2R knockdown did not lead to a detectable cAMP increase in s-LNvs in response to dopamine application (supplemental Fig. 6).

Light has a profound impact 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]. Here we show that the 12hr light exposure of standard light-dark housing conditions has a profound impact 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. The microarray data indicate that transcription of the octopamine receptor OA2 peaks around ZT12, whereas that for OAMB peaks around ZT6 [16]. Since imaging analysis showed 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 to octopamine, the dopamine-mediated responses of l-LNvs are time-insensitive and are not affected by *per01* mutation (Fig. 4–5). However, light exposure suppresses the l-LNv dopamine responses at all times of day, nighttime as well as daytime. As downregulation of dD2R is sufficient to mimic the responses of flies reared in constant darkness and D2R-RNAi had no effect in constant darkness, light exposure apparently upregulates dD2R activity to dampen dopamine responsiveness in light-dark conditions. This implies that there are light-stimulated changes in either dD2R gene expression or regulation, such as a modification of the dD2R receptor or its downstream targets. Light may also downregulate stimulatory D1R signaling pathways in concert with the upregulation of dD2R, although our results suggest that expression of dD2R 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 (supplemental 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 12hr light exposure suppresses the ability of dopamine and octopamine to stimulate l-LNvs suggests that they do not simply sum different arousal signals. Rather, they are integrated and perhaps scaled depending on conditions, suggesting a link to behavioral flexibility. Light appears in this scenario to be a dominant signal, as its presence during the day reduces the ability of internal

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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 like those described here may also serve the fly brain to preserve sleep stability. 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, i.e., the circuit organization allows the activity of sleep-relevant neurons to be maintained within 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 within a group, for example between different individual l-LNvs. It will not be surprising if additional integration mechanisms will also be 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, i.e., for sleep-wake homeostasis, as well as for appropriate responses to emergency circumstances.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Ganguly-Fitzgerald I, Donlea J, Shaw PJ. Waking experience affects sleep need in Drosophila. Science. 2006; 313:1775–1781. [PubMed: 16990546]
2. Ho KS, Sehgal A. Drosophila melanogaster: an insect model for fundamental studies of sleep. Methods Enzymol. 2005; 393:772–793. [PubMed: 15817324]
3. Keene AC, et al. Clock and cycle Limit Starvation-Induced Sleep Loss in Drosophila. Curr Biol. 2010
4. Shang Y, Griffith LC, Rosbash M. Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. Proc Natl Acad Sci U S A. 2008; 105:19587–19594. [PubMed: 19060186]
5. Andretic R, van Swinderen B, Greenspan RJ. Dopaminergic modulation of arousal in Drosophila. Curr Biol. 2005; 15:1165–1175. [PubMed: 16005288]
6. Crocker A, Sehgal A. Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. J Neurosci. 2008; 28:9377–9385. [PubMed: 18799671]

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7. Crocker A, Shahidullah M, Levitan IB, Sehgal A. Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior. Neuron. 2010; 65:670–681. [PubMed: 20223202]
8. Parisky KM, et al. PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. Neuron. 2008; 60:672–682. [PubMed: 19038223]
9. Hendricks JC, et al. Rest in Drosophila is a sleep-like state. Neuron. 2000; 25:129–138. [PubMed: 10707978]
10. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. Correlates of sleep and waking in Drosophila melanogaster. Science. 2000; 287:1834–1837. [PubMed: 10710313]
11. Sheeba V, et al. Large ventral lateral neurons modulate arousal and sleep in Drosophila. Curr Biol. 2008; 18:1537–1545. [PubMed: 18771923]
12. Donlea JM, Ramanan N, Shaw PJ. Use-dependent plasticity in clock neurons regulates sleep need in Drosophila. Science. 2009; 324:105–108. [PubMed: 19342592]
13. Sheeba V, Gu H, Sharma VK, O'Dowd DK, Holmes TC. Circadian- and light-dependent regulation of resting membrane potential and spontaneous action potential firing of Drosophila circadian pacemaker neurons. J Neurophysiol. 2008; 99:976–988. [PubMed: 18077664]
14. Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell. 1999; 99:791–802. [PubMed: 10619432]
15. Shafer OT, et al. Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of Drosophila revealed by real-time cyclic AMP imaging. Neuron. 2008; 58:223–237. [PubMed: 18439407]
16. Kula-Eversole E, et al. Surprising gene expression patterns within and between PDF-containing circadian neurons in Drosophila. Proc Natl Acad Sci U S A. 2010; 107:13497–13502. [PubMed: 20624977]
17. Shakiryanova D, Levitan ES. Prolonged presynaptic posttetanic cyclic GMP signaling in Drosophila motoneurons. Proc Natl Acad Sci U S A. 2008; 105:13610–13613. [PubMed: 18765813]
18. Feinberg EH, et al. GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems. Neuron. 2008; 57:353–363. [PubMed: 18255029]
19. Gordon MD, Scott K. Motor control in a Drosophila taste circuit. Neuron. 2009; 61:373–384. [PubMed: 19217375]
20. Crocker A, Sehgal A. Genetic analysis of sleep. Genes Dev. 2010; 24:1220–1235. [PubMed: 20551171]
21. Hamada FN, et al. An internal thermal sensor controlling temperature preference in Drosophila. Nature. 2008; 454:217–220. [PubMed: 18548007]
22. Chiang AS, et al. Three-dimensional reconstruction of brain-wide wiring networks in Drosophila at single-cell resolution. Curr Biol. 2011; 21:1–11. [PubMed: 21129968]
23. Mao Z, Davis RL. Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. Front Neural Circuits. 2009; 3:5. [PubMed: 19597562]
24. Sugamori KS, Demchyshyn LL, McConkey F, Forte MA, Niznik HB. A primordial dopamine D1-like adenylyl cyclase-linked receptor from Drosophila melanogaster displaying poor affinity for benzazepines. FEBS Lett. 1995; 362:131–138. [PubMed: 7720859]
25. Hall JC. Systems approaches to biological rhythms in Drosophila. Methods Enzymol. 2005; 393:61–185. [PubMed: 15817287]
26. Bonci A, Hopf FW. The dopamine D2 receptor: new surprises from an old friend. Neuron. 2005; 47:335–338. [PubMed: 16055058]
27. Han KA, Millar NS, Grotewiel MS, Davis RL. DAMB, a novel dopamine receptor expressed specifically in Drosophila mushroom bodies. Neuron. 1996; 16:1127–1135. [PubMed: 8663989]
28. Hearn MG, et al. A Drosophila dopamine 2-like receptor: Molecular characterization and identification of multiple alternatively spliced variants. Proc Natl Acad Sci U S A. 2002; 99:14554–14559. [PubMed: 12391323]

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29. Nitabach MN, Sheeba V, Vera DA, Blau J, Holmes TC. Membrane electrical excitability is necessary for the free-running larval Drosophila circadian clock. J Neurobiol. 2005; 62:1–13. [PubMed: 15389695]
30. Wijnen H, Naef F, Boothroyd C, Claridge-Chang A, Young MW. Control of daily transcript oscillations in Drosophila by light and the circadian clock. PLoS Genet. 2006; 2:e39. [PubMed: 16565745]
31. Helfrich-Forster C, et al. Development and morphology of the clock-gene-expressing lateral neurons of Drosophila melanogaster. J Comp Neurol. 2007; 500:47–70. [PubMed: 17099895]
32. Wang JW, Wong AM, Flores J, Vosshall LB, Axel R. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. Cell. 2003; 112:271–282. [PubMed: 12553914]
33. Cayre M, Buckingham SD, Yagodin S, Sattelle DB. Cultured insect mushroom body neurons express functional receptors for acetylcholine, GABA, glutamate, octopamine, and dopamine. J Neurophysiol. 1999; 81:1–14. [PubMed: 9914262]
34. Gervasi N, Tchenio P, Preat T. PKA dynamics in a Drosophila learning center: coincidence detection by rutabaga adenylyl cyclase and spatial regulation by dunce phosphodiesterase. Neuron. 2010; 65:516–529. [PubMed: 20188656]
35. Soille, P. Morphological image analysis: principles and applications. Secaucus, NJ, USA: Springer-Verlag New York, Inc.; 2003.
36. Meyer F. Topographic distance and watershed lines. Signal Processing. 1994; 38:13.

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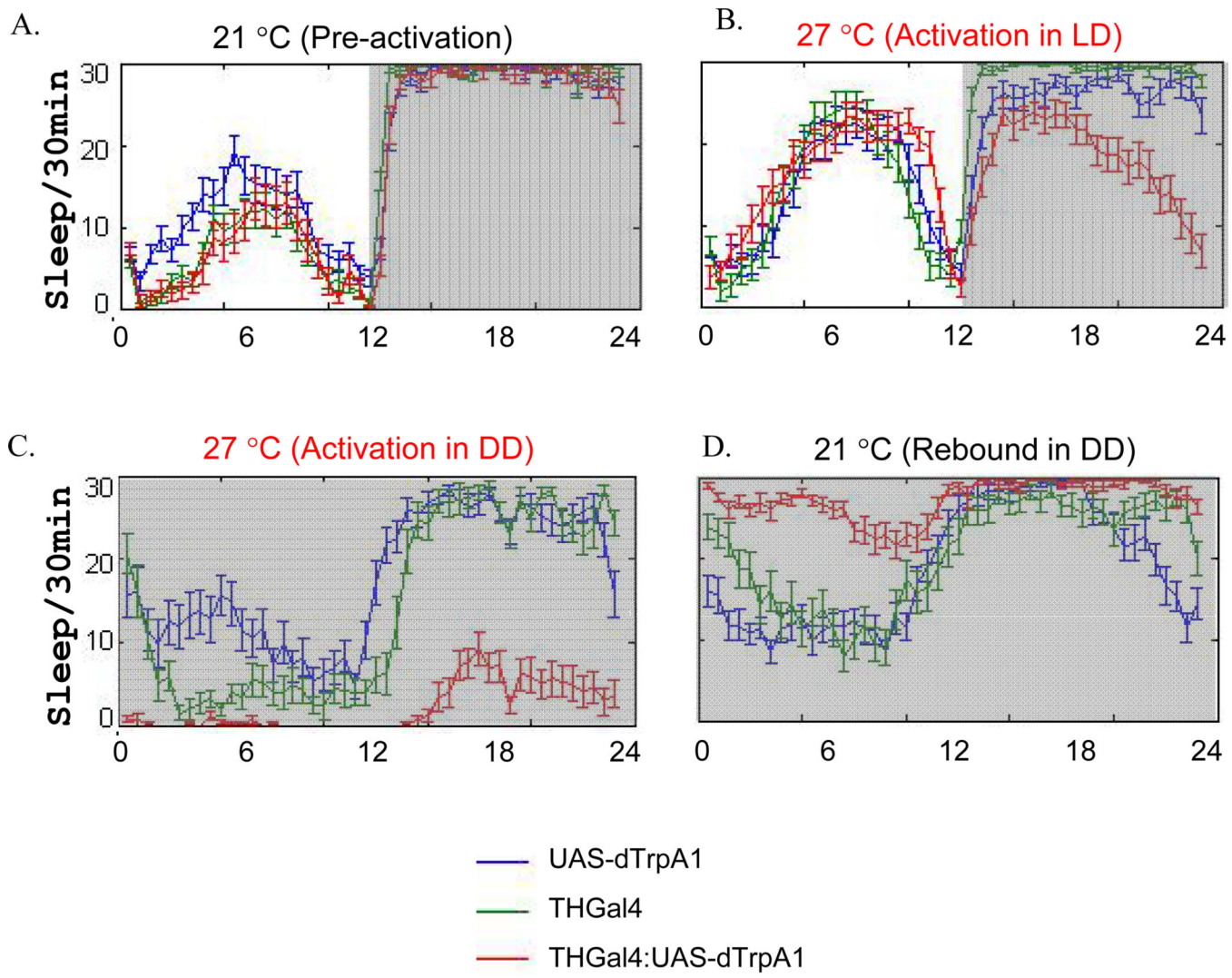

Fig. 1. Light suppressed the wake promoting effects of dopamine

A-D. Induced firing of dopaminergic neurons dramatically decreased sleep during the dark period in light-dark or constant darkness 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 almost entirely absent. In A-D, *TH-Gal4* driven expression of *dTrpA1* was used to transiently increase the activity of dopaminergic neurons when the temperature was raised from 21 °C to 27 °C at the beginning of the night. The behavior was monitored for 3 days either in light-dark or constant darkness at 27 °C before returning to 21 °C. For simplicity, only one day of data from each condition is shown. The data was collected from control *UAS-dTrpA1* (blue), control *TH-Gal4* (green), and *TH-Gal4:UAS-dTrpA1* (orange).

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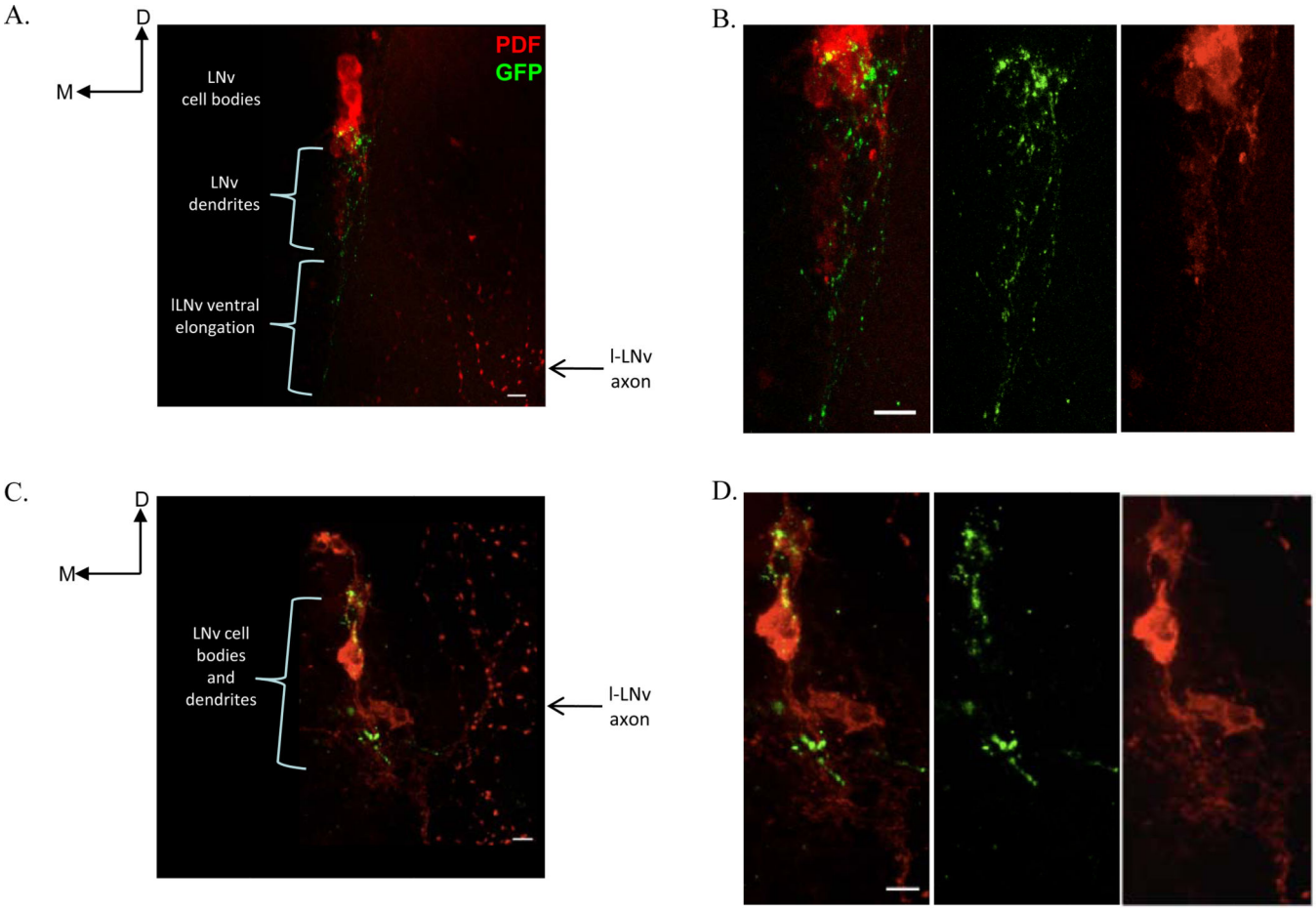

Fig. 2. The l-LNvs form membrane contacts with dopaminergic and octopaminergic neurons

A-B. Membrane tethered GFP fragment CD4::spGFP1-10 was expressed in most of the dopaminergic neurons with *TH-Gal4* and CD4::spGFP11 was expressed in l- and s-LNvs with pdfLexA. Green is GFP staining and red is PDF staining. **A.** The fine fibers in the ventral elongation are likely to be the dendrites of the l-LNvs [31]. Reconsitituted 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 side of the brain, respectively. **B.** An image with higher magnification shows the reconstituted GFP signals around the LNv cell body and dendritic area. Note, the anti-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 anti-PDF staining in the dendritic areas. **C-D.** Membrane tethered GFP fragment CD4::spGFP1-10 was expressed in most of the octopaminergic neurons with *Tdc2-Gal4*. Reconsitituted GFP signals were also detected around the LNv cell bodies and dendritic area (N=10). Scale bar is 10 μM.

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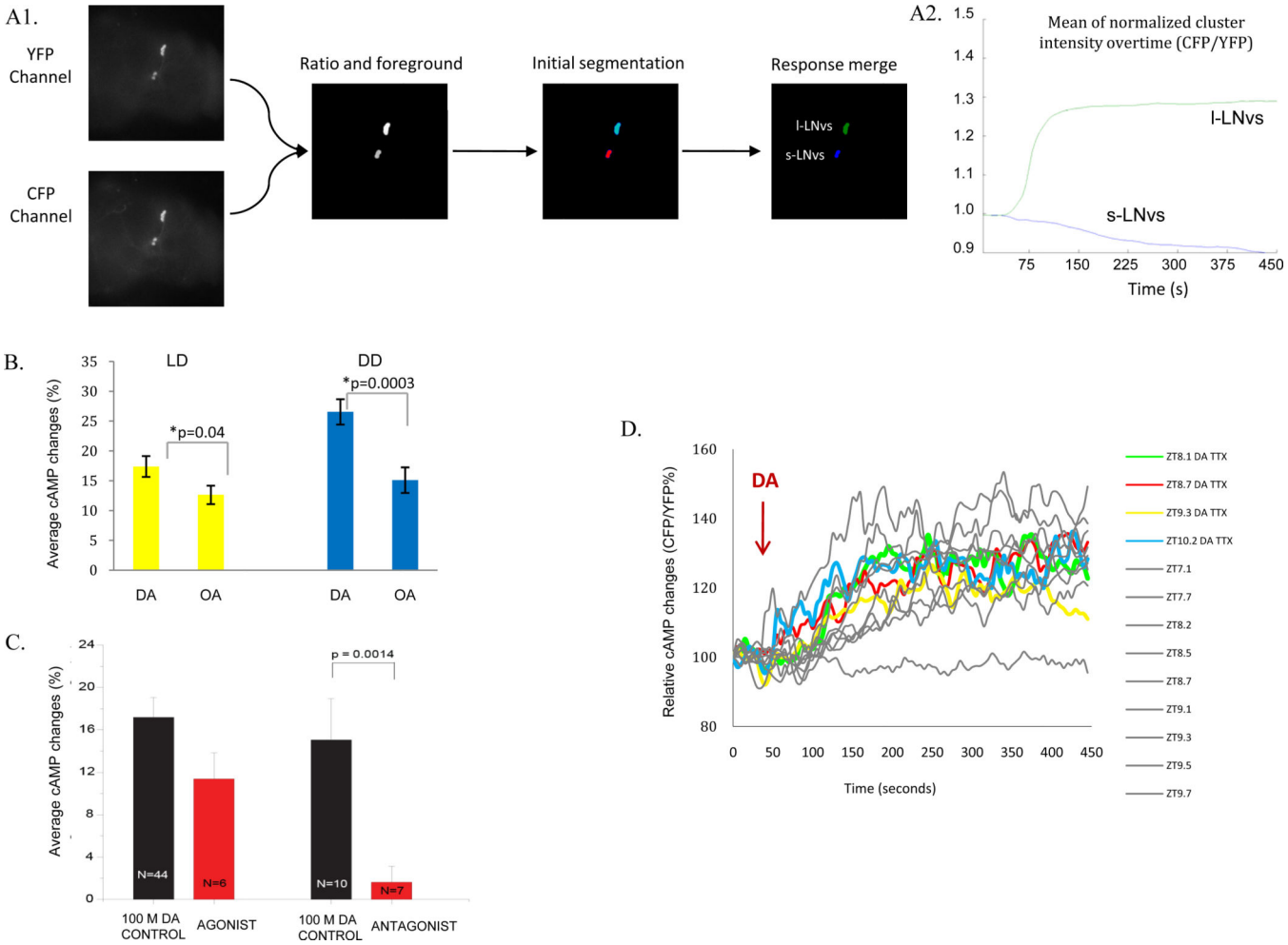

Fig. 3. The l-LNvs responded to dopamine or octopamine application by increasing cAMP

A. Example showing how FRET images were processed using an automated method as described in METHODS and MATERIALS. Briefly, each video has two channels (YFP and CFP). The responses of a cell to a drug can be computed as the mean of its CFP/YFP ratios, which are normalized by signals captured under the untreated condition. Cells without statistically significant response differences over time are 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 application induced stronger responses in the l-LNvs compared with octopamine. (Left, flies reared in light-dark conditions were used for imaging. Right, flies reared in constant darkness day 1 were used). **C.** The responses can be induced by a dopamine agonist and are 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 100s to 445s. Error bar represents SEM. A dopamine agonist, 100uM 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, 50uM (+)-Butaclamol hydrochloride. **D.** dopamine-induced responses are cell-autonomous; the l-LNv response to dopamine in both the presence and absence of TTX was

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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, TTX was added to the acutely dissected brains before bath application of dopamine. Grey curves, responses were recorded without TTX.

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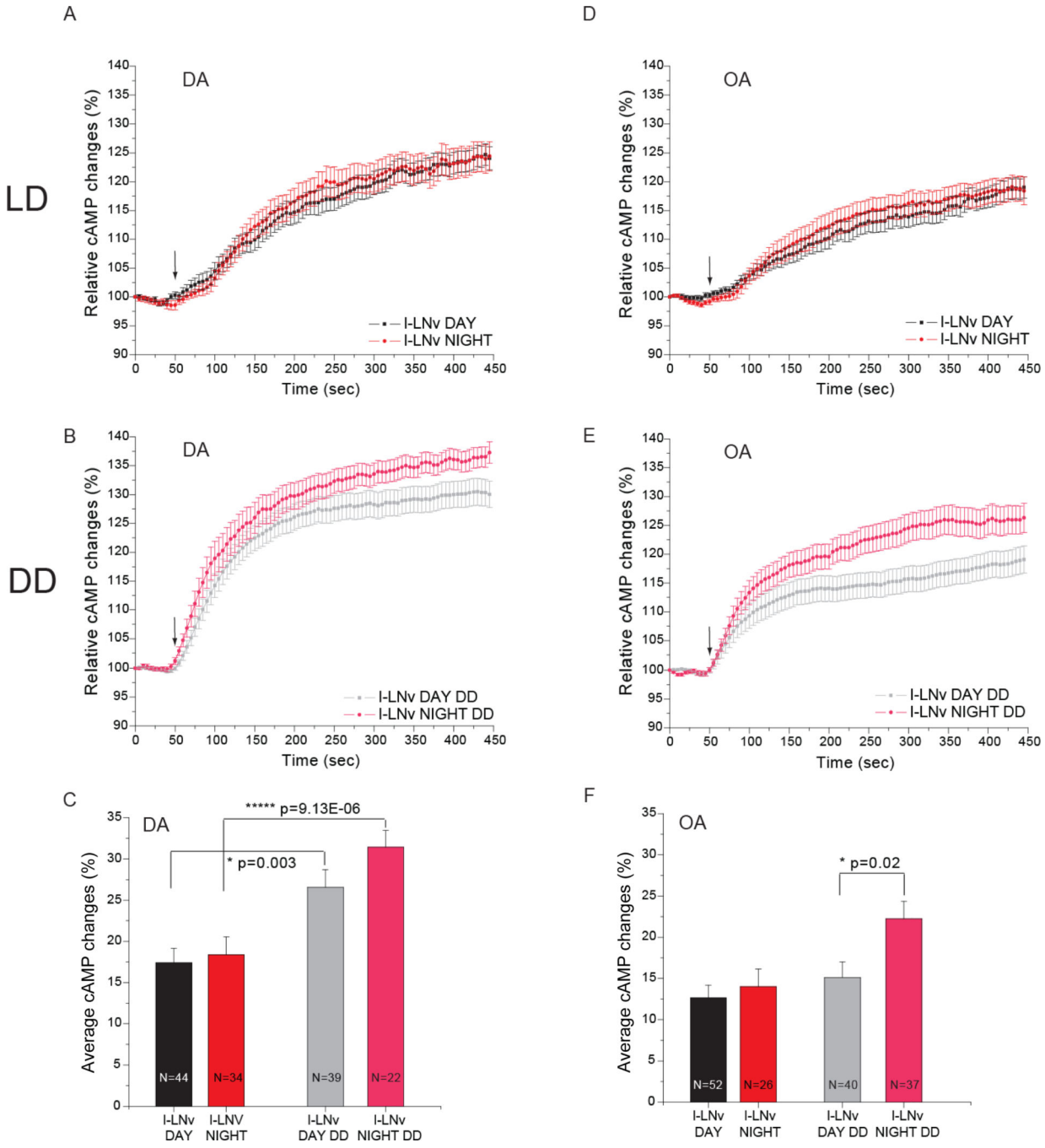

Fig. 4. 12hr light exposure suppressed the responses of l-LNvs to dopamine or octopamine

A-C. Light exposure suppressed the l-LNv responses to dopamine. Flies were housed in light-dark conditions (**A**) or constant darkness conditions (**B**) and the response to dopamine during daytime or subjective day is compared with that during nighttime or subjective night. **C.** Summary of the relative changes of cAMP shown in A and B. The l-LNv responses to dopamine during the day/subjective day versus the night/subjective night are not significantly different within either light-dark or constant darkness conditions. However, comparison between light-dark and constant darkness conditions showed that the responses

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of the l-LNvs to dopamine in constant darkness are 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. Flies were housed in light-dark conditions (**D**) or constant darkness conditions (**E**) and the response to octopamine during daytime or subjective day is 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. **F.** Summary of the relative changes in cAMP shown in D and E. The responses to octopamine during daytime, nighttime, or subjective daytime were similar while the l-LNvs from subjective night were more sensitive to octopamine. p are significant difference from control groups (student's t-test). Error bar represents SEM.

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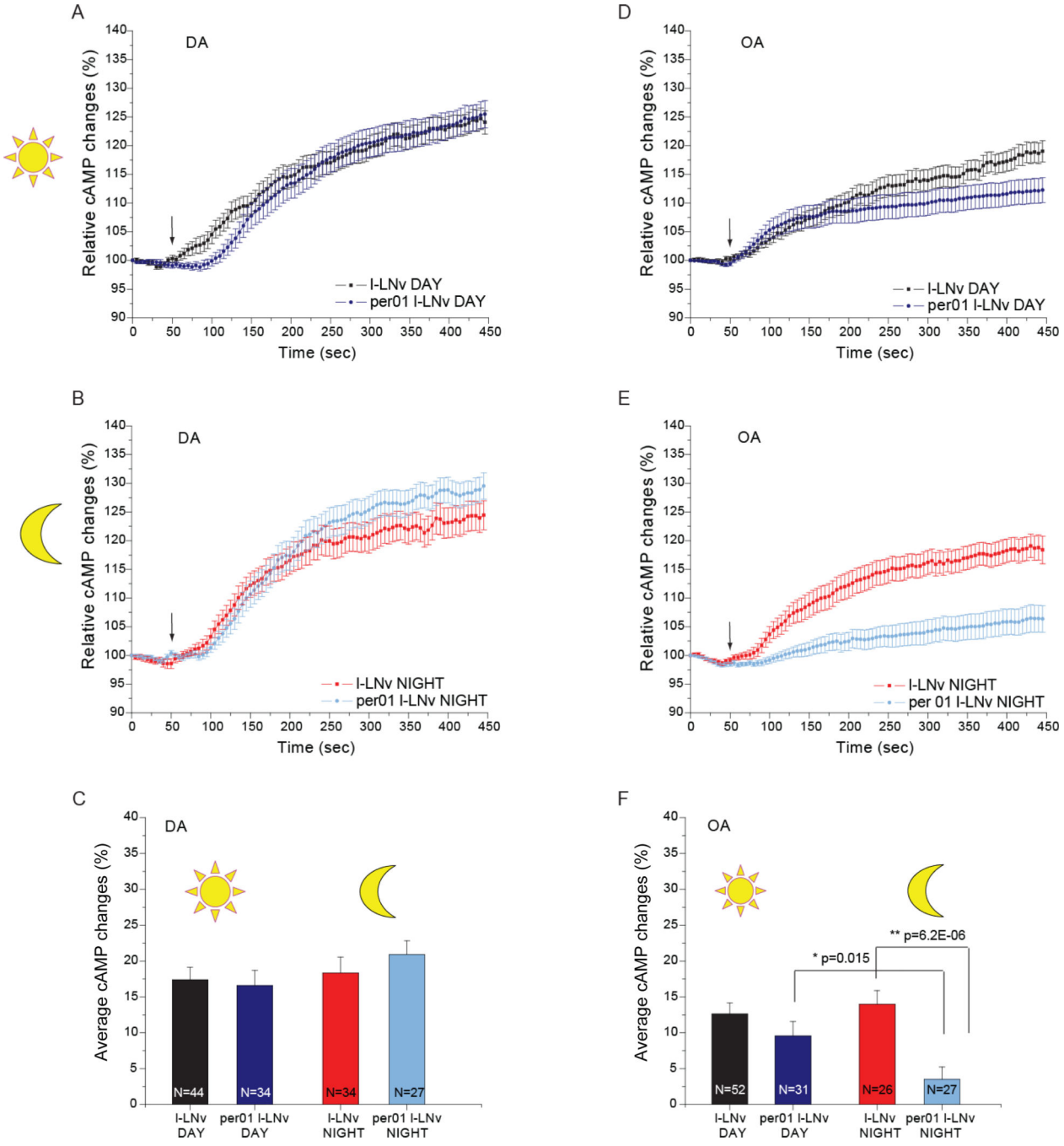

Fig. 5. The circadian clock (PER) specifically promotes octopamine-induced responses in l-LNvs at night. A-C

The l-LNv responses to dopamine were not affected by PER. The daytime (**A**) and nighttime responses (**B**) are plotted separately. The dopamine-induced responses of the l-LNvs from control brains are compared with those from *per*^{01} mutant flies. **C.** Summary of the relative changes of cAMP shown in A and B. The responses to dopamine were not affected by *per*^{01} mutation. **D-F.** *PER* positively regulates octopamine evoked responses by l-LNv at night. Flies were housed in light-dark conditions and the daytime (**D**) and nighttime responses (**E**)

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are plotted separately. The octopamine-induced responses of the l-LNvs from control brains are compared with those from per^{01} mutant flies. **F.** Summary of the relative changes of cAMP shown in D and E. The responses to octopamine during daytime were not affected by per^{01} mutation (left), while the nighttime responses were dramatically decreased in the per^{01} mutants (right).

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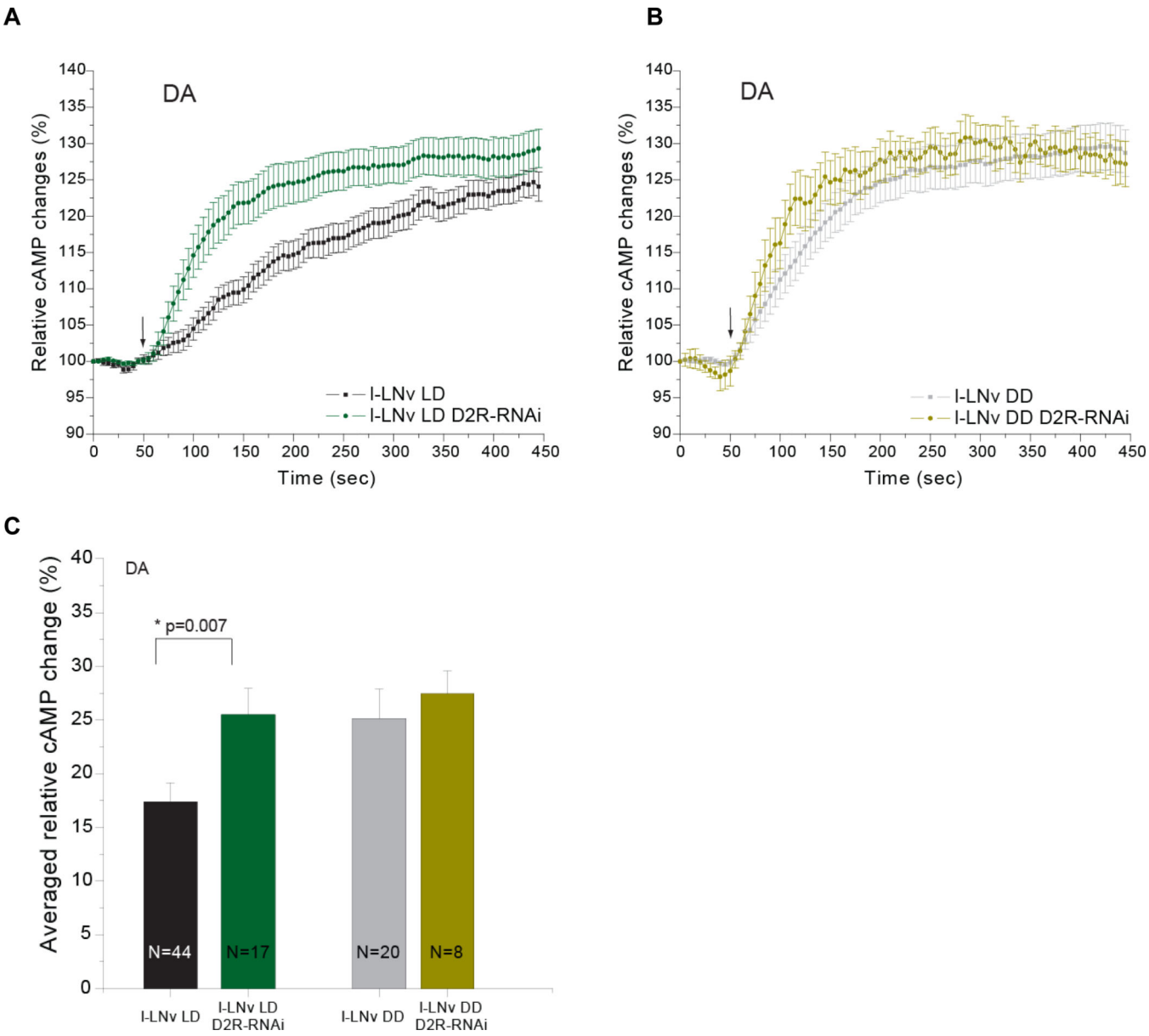

Fig. 6. Light suppressed dopamine responses by upregulating inhibitory dopamine receptors

A-B. dD2R negatively regulates dopamine evoked responses in the l-LNvs. **A.** The l-LNv response to dopamine in light-dark conditions was dramatically increased by knocking down dD2R expression in the l-LNvs. The dopamine-induced responses of the l-LNvs from control brains are compared with those from dD2R-RNAi knockdown mutant flies. **B.** The l-LNv response to dopamine in constant darkness conditions was not affected by knocking down dD2R expression in the l-LNvs. The dopamine-induced responses of the l-LNvs from control brains are compared with those from dD2R-RNAi knockdown mutant flies. **C.** Summary of the relative changes of cAMP shown in A and B. The responses in constant darkness are comparable with those in dD2R knockdown mutants in light-dark conditions.

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Table 1

Experimental conditions	**Light-dark conditions**		**Constant darkness**	
Control used for subtraction	***UAS-dTrpA1***	***THGal4***	***UAS-dTrpA1***	***THGal4***
Δ Total sleep (%)	−15.4±2.5%	−29.5±2.5%	−48.9±4.4%	−55±4.4%
Δ Daytime sleep (%)	N.S.		−46.2±8.5%	−235.5±8.5%
Δ Nighttime sleep (%)	−29.4±2.5%	−34±2.5%	−51.1±4.2%	−52±4.2%

N.S. – Not statistically significant; see Methods and Materials for calculation of the relative change in sleep time.