Dopamine signaling in wake promoting clock neurons is not required for the normal regulation of sleep in *Drosophila*

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Declarations

Author contributions

FF-C performed and analyzed the patch-clamp recordings, CH-L performed and analyzed the cAMP imaging and the behavioral experiments with permanent dopamine receptor knockdown, AP performed the behavioral experiments with conditional dopamine receptor knockdown, NL did the statistical analysis, MH and MS performed the histology, OTS, NIM and CH-F designed the study and supervised the experiments, CH-F analyzed the behavioral experiments with conditional dopamine receptor knockdown and wrote the paper with contributions from CH-L, OTS, NIM and MS.

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Abstract

Dopamine is a wakefulness promoting neuromodulator in mammals and fruit flies. In *D. melanogaster*, the network of clock neurons that drives sleep/activity cycles comprises both wake and sleep promoting cell types, indicating that the sleep-wake circuitry is intimately linked to the circadian clock. The large and small ventrolateral neurons (l-LNvs and s-LNvs) have been identified as wake-promoting neurons within the clock neuron network. The l-LNvs are innervated by dopaminergic neurons, and earlier work proposed that dopamine signaling raises cAMP levels in the l-LNvs and thus induces excitatory electrical activity (action potential firing), which results in wakefulness and inhibits sleep. Here, we test this hypothesis by combining cAMP imaging and patch-clamp recordings in isolated brains. We find that dopamine application indeed increases cAMP levels and depolarizes the l-LNvs, but surprisingly, it does not result in increased firing rates. Down-regulation of the excitatory dopamine receptor, Dop1R1 in the l- and s-LNvs, but not of Dop1R2, abolished the depolarization of l-LNvs in response to dopamine. This indicates that dopamine signals via Dop1R1 to the l-LNvs. Down-regulation of Dop1R1 or Dop1R2 receptors in the l- and s-LNvs does not affect sleep. Unexpectedly, we find a moderate decrease of daytime sleep with down-regulation of Dop1R1 and of nighttime sleep with down-regulation of Dop1R2. Since the l-LNvs do not utilize Dop1R2 receptors and the s-LNvs respond also to dopamine, we conclude that the s-LNvs are responsible for the observed decrease in nighttime sleep. In summary, dopamine signaling in the wake-promoting LNvs is not required for daytime arousal, but likely promotes nighttime sleep via the s-LNvs.

Keywords: wakefulness, sleep, clock neurons, dopamine, cAMP, patch-clamp recording
Significance statement

In insect and mammalian brains, sleep promoting networks are intimately linked to the circadian clock, and the mechanisms underlying sleep and circadian timekeeping are evolutionarily ancient and highly conserved. Here we show that dopamine, one important sleep modulator in flies and mammals, plays surprisingly complex roles in the regulation of sleep by clock containing neurons. Dopamine inhibits neurons in a central brain sleep center to promote sleep and excites wake-promoting circadian clock neurons. It is therefore predicted to promote wakefulness through both of these networks. Nevertheless, our results reveal that dopamine acting on wake promoting clock neurons promotes sleep, revealing a previously unappreciated complexity in the dopaminergic control of sleep.
Introduction

The fruit fly Drosophila melanogaster has become a powerful and widely-used model system for sleep research (reviewed by Cirelli, 2009; Dubowy and Sehgal, 2017; Helfrich-Förster, 2018). As in mammals, the sleep-like state of Drosophila is associated with reduced sensory responsiveness and reduced brain activity (Nitz et al., 2002; van Swinderen et al., 2004), and is subject to both circadian and homeostatic regulation (Hendricks et al., 2000; Shaw et al., 2000). Furthermore, as in mammals, dopamine and octopamine (the insect functional homolog to noradrenaline) promote arousal in fruit flies (Andretic et al., 2005; Kume et al., 2005; Lima and Miesenböck, 2005; Wu et al., 2008, Lebestky et al., 2009; Crocker et al., 2010; Riemensperger et al., 2011), and GABA promotes sleep (Agosto et al., 2008; Gmeiner et al., 2013). Dopamine is most probably the strongest wake-promoting neuromodulator in fruit flies (reviewed by Birman, 2005). Hyperactive and sleepless fumin mutants carry a mutation in the dopamine transporter, which transports released dopamine back into the dopaminergic neurons (Kume et al., 2005). The fumin mutation results in a hypomorphic transporter, which leads to permanently high dopamine levels that continue to activate dopamine receptors on the postsynaptic neurons. Similar wake-promoting and sleep-reducing effects are observed when dopaminergic neurons are excited (Lima and Miesenböck, 2005; Wu et al., 2008, Shang et al., 2011; Liu et al., 2012; Ueno et al., 2012). Conversely, mutants deficient for tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis in the nervous system, have reduced dopamine levels and increased sleep throughout the day (Riemensperger et al., 2011).

In D. melanogaster the mushroom bodies (Joiner et al., 2006; Pitman et al., 2006; Yuan et al., 2006), the pars intercerebralis (Foltenyi et al., 2007; Crocker et al., 2010) and lateralis (Chen et al., 2016), the fan-shaped body of the central complex (Liu et al., 2012; Ueno et al., 2012; Pimentel et al., 2016; Donlea et al., 2018) have been identified as brain regions that regulate sleep. In addition, the Pigment-Dispersing Factor (PDF)-expressing large and small ventral Lateral Neurons (l-LN_s and s-LN_s), which belong to the circadian clock neurons have
been identified as wake-promoting neurons within the flies circadian clock neuron network (Parisky et al., 2008; Sheeba et al., 2008a; Shang et al., 2008; Lebestky et al., 2009; Guo et al., 2016; Guo et al., 2018; Potdar and Sheeba, 2018; Liang et al., 2019).

The l-LNvs respond to both dopamine and octopamine through increases in cAMP, but the responses to dopamine are clearly stronger (Shang et al., 2011). Furthermore, the l-LNvs are directly light sensitive and promote arousal and activity in response to light, especially in the morning (Shang et al., 2008; Sheeba et al., 2008b; Fogle et al., 2011). Despite the strong responses of the l-LNvs to dopamine and their proposed role in controlling arousal, it is not known how dopamine-signaling to the l-LNvs increases wakefulness and inhibits sleep. Receptivity to dopamine in the s-LNvs has not been previously addressed. Here, we down-regulated the activating D1-like dopamine receptors Dop1R1 and Dop1R2 in the wake promoting l- and s-LNvs and examined the consequences on intracellular cAMP levels, resting membrane potential, and electrical firing rate in the electrophysiologically accessible l-LNvs. Moreover, we analyzed the behavioral consequences of Dop1R1/Dop1R2 knock-down in the l- and s-LNvs on sleep and activity rhythms. As expected, we find that the knockdown of Dop1R1 reduces cAMP and electrophysiological responses to dopamine in the l-LNvs, confirming that dopamine signals via Dop1R1 receptors. Unexpectedly, we find that the down-regulation of the excitatory Dop1R1 receptor slightly decreases daytime sleep, suggesting that dopamine signaling via Dop1R1 to the LNvs usually promotes daytime sleep rather than wakefulness. Finally, we find that dopamine also likely signals to the s-LNvs via Dop1R2 receptors, and that the down-regulation of these receptors decreases night-sleep. Collectively, these results cast doubt on the currently held view of LNvs as dedicated wake-promoting neurons, and suggest a more complex regulation of sleep by these important clock neurons.
Material and Methods

Fly stocks

Flies were raised on Drosophila food (0.8 % agar, 2.2 % sugar-beet syrup, 8.0 % malt extract, 1.8 % yeast, 1.0 % soy flour, 8.0 % corn flour and 0.3 % hydroxybenzoic acid) at 25 °C under a 12 h:12 h light:dark (LD) cycle and transferred to 20 °C at an age of ~3 days.

To visualize TH-positive (dopaminergic) and the PDF-positive neurons we used TH-Gal4 (Friggi-Grelin et al., 2002) to drive UAS-10xmyrGFP in dopaminergic neurons and stained with anti-GFP and anti-PDF. For visualizing presynapses of dopaminergic neurons and postsynapses of PDF neurons, we expressed the vesicle marker synaptotagmin::GFP (UAS-sytII::GFP; Bloomington) under control of TH-Gal4 in dopaminergic neurons and a GFP labeled postsynaptic protein - the Down syndrome cell-adhesion molecule (UAS-dscam::GFP; Wang et al. 2004) - under control of Pdf-Gal4 in PDF neurons. To visualize the spatial vicinity of dopaminergic and PDF fibers we used Split-GFP imaging (= GFP Reconstitution Across Synaptic Partners (GRASP); Feinberg et al., 2008): yw;pdf-LexA/LexAop-GFP11;TH-Gal4/UAS-GFP1-10 flies were used to express the GFP11 fragment in the PDF-expressing LN vs and the GFP1-10 fragment in dopaminergic neurons, respectively. yw;pdf-LexA/LexAop-GFP11;TM6B.Tb/UAS-GFP1-10 flies were used as controls.

In order to down-regulate the different dopamine receptors in all clock neurons or only in the PDF neurons (s-LN,s and l-LN,s), we used Clk856-Gal4 (Gummadova et al., 2009) or Pdf-Gal4 (Park et al., 2000), respectively to either express UAS-Dop1R1RNAi (no. 31765, Bloomington stock center), UAS-Dop1R2RNAi (no. 26018, Bloomington stock center) or UAS-D2RRNAi (no. 26001, Bloomington stock center) alone, or to simultaneously express UAS-Dop1R1RNAi and UAS-Dop1R2RNAi. The flies with the relevant Gal4 and UAS constructs (crossed with UAS-dicer2 flies) were taken as controls. In addition, we used an inducible Gal4 version, termed GeneSwitch (GS) (Osterwalder et al., 2001), under the control of the Pdf promotor (Depetris-Chauvin et al., 2011) to down-regulate Dop1R1 or Dop1R2 receptors in the PDF.
neurons only during adulthood of the flies. GS is a fusion between the Gal4 binding, the NFκb activation and the human progesterone receptor ligand-binding domains, which is expressed in the pattern dictated by the desired promoter but remains transcriptionally silent in the absence of RU486 (RU), an analog of progesterone. RU was mixed to the food of the adult flies in the Trikinetics monitors (see below). In all experiments UAS-Dicer2 (no. 60012, Vienna Drosophila RNAi Center, Wien, Austria) was expressed additionally to enhance the effect. For simplicity we will call the experimental flies Clk856>Dop1RxRNAi, Pdf>Dop1RxRNAi or PDF-GS>Dop1RxRNAi, where the ‘x’ stands for the relevant dopamine receptor. Their sleep and activity profiles will always be depicted in red, while the relevant control flies are shown in black.

For imaging experiments the above described Clk856-Gal4 or Pdf-Gal4 line was used to express the ratiometric cAMP sensor UAS-Epac1-camps (Nikolaev et al., 2004), UAS-dicer2 and the RNAi-constructs for different dopamine-receptors (see above).

**Immunostaining and microscopy**

For immunostaining, whole-mount brains were fixed in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 2 hours at RT, followed by 4 washes in PBS containing 0.3 % TritonX-100 (PBT). They were blocked in 5 % normal goat serum (NGS) in PBT. Subsequently, the specimens were incubated in the primary antibody solution overnight at 4 °C. The primary antibody solution contained GFP antibody (raised in rabbit, Molecular Probes, A11122; dilution 1:1000) and PDF antibody (monoclonal mouse C7 antibody; Developmental Studies Hybridoma Bank at the University of Iowa; dilution 1:100). After rinsing in PBT, fluorescence conjugated secondary antibodies (Alexa-Fluor® Dyes, Molecular Probes, Carlsbad, CA) were applied overnight at 4 °C. The stained brains were finally embedded in Vectashield and scanned with a Confocal Microscope (Leica TCS SPE, Wetzlar, Germany).
**Ex vivo live-cAMP imaging**

Flies were well entrained to a LD 12:12 cycle and imaging always took place during the light phase of the LD cycle (between ZT2 and ZT8). For imaging, flies were anesthetized on ice and brains were dissected in cold hemolymph-like saline (HL3; Stewart et al., 1994) and mounted at the bottom of a plastic petri dish in HL3.Brains were allowed to recover from dissection for at least 10 min prior to imaging. An epifluorescent imaging setup (VisiChrome High Speed Polychromator System, ZEISS Axioskop2 FS plus, VisiTron Systems GmbH) with a 40x dipping objective (ZEISS 40x/1.0 DIC VIS-IR) was used for all imaging experiments. Neurons were localized using GFP-optics and were identified according to their position in the brain. Regions of interest were defined on single cell bodies in the Visiview Software (version 2.1.1, VisiTron Systems GmbH). Time-lapse frames were acquired with 0.2 Hz for 12 min, exciting the CFP fluorophore of the ratiometric cAMP sensor with light of 405 nm. Emissions of CFP and YFP were detected separately by a CCD-camera (Photometrics, CoolSNAP HQ, VisiTron Systems GmbH) with a beam splitter. After measuring baseline CFP and YFP levels for ~100 s, pharmacological treatments were bath applied drop-wise using a pipette. HL3 application served as negative control and 10 µM NKH477 (an activator of all adenylate cyclases) as positive control. Dopamine and SKF38393 (a DopR1 agonist) were diluted in HL3 and were applied in an end concentration of 1 mM and 0.1 mM, respectively. For Tetrodotoxin (TTX)-treatments, brains were incubated in 2 µM TTX in HL3 for 20 min prior to imaging and dopamine was diluted in 2 µM TTX in HL3 for the application. Inverse Fluorescence Resonance Energy Transfer (iFRET) was calculated according to the following equation: 

\[ iFRET = \frac{\text{CFP}}{\text{YFP} - \text{CFP} \times 0.357} \]  

(Shafer et al., 2008). Thereby, CFP and YFP are background corrected raw fluorescence data and 0.357 was determined as the fraction of CFP spillover into the YFP channel in our imaging setup, which had to be subtracted from YFP fluorescence. Finally, iFRET traces of individual neurons were normalized to base line levels and were averaged for each
treatment. For quantification and statistical comparison of response amplitudes of each
treatment or genotype, maximum iFRET changes were determined for individual neurons.

Ex vivo patch-clamp electrophysiology

Three to nine days-old female flies were anesthetized with a brief incubation of the vial on ice,
brain dissection was performed in external recording solution which consisted of (in mM): 101
NaCl, 3 KCl, 1 CaCl2, 4 MgCl2, 1.25 NaH2PO4, 5 glucose, and 20.7 NaHCO3, pH 7.2, with an
osmolarity of 250 mmol/kg (based on saline solution used by Cao and Nitabach, 2008). After
removal of the proboscis, air sacks and head cuticle, the brain was routinely glued ventral side
up to a sylgard-coated coverslip using a few microliters of tissue adhesive 3 M Vetbond. The
time from anesthesia to the establishment of the recordings was approximately 20 minutes
spent as following: l-LNvs were visualized by red fluorescence in Pdf-RFP flies (which express a
red fluorophore under the Pdf promoter, Ruben et al., 2012) using an Olympus BX51WI upright
microscope with 60X water-immersion lens and ThorLabs LEDD1B and TK-LED (TOLKET S.R.L,
Argentina) illumination systems. Once the fluorescent cells were identified, cells were
visualized under IR-DIC using a DMK23UP1300 Imaging Source camera and IC Capture 2.4
software. l-LNvs were distinguished from s-LNvs by their size and anatomical position. To allow
the access of the recording electrode, the superficial glia directly adjacent to l-LNvs somas was
locally digested with protease XIV solution (10 mg/ml, SIGMA-ALDRICH P5147) dissolved in
external recording solution. This was achieved using a large opened tip (approximately 20 µm)
glass capillary (pulled from glass of the type FG-GBF150-110-7.5, Sutter Instrument, US) and
gentle massage of the superficial glia with mouth suction to render the underling cell bodies
accessible for the recording electrode with minimum disruption of the neuronal circuits. After
this procedure, protease solution was quickly washed by perfusion of external solution.
Recordings were performed using thick-walled borosilicate glass pipettes (FG-GBF150-86-7.5,
Sutter Instrument, US) pulled to 7-8 MΩ using a horizontal puller P-97 (Sutter Instrument, US)
11 and fire polished to 9-12 MΩ. Recordings were made using a Multiclamp 700B amplifier controlled by pClamp 10.4 software via an Axon Digidata 1515 analog-to-digital converter (Molecular Devices, US). Recording pipettes were filled with internal solution containing (in mM): 102 potassium gluconate, 17 NaCl, 0.085 CaCl₂, 0.94 EGTA and 8.5 HEPES, pH 7.2 with an osmolarity of 235 mmol/kg (based on the solution employed by Cao and Nitabach 2008). Gigaohm seals were accomplished using minimal suction followed by break-in into whole-cell configuration using gentle suction in voltage-clamp mode with a holding voltage of -60 mV. Gain of the amplifier was set to 1 during recordings and a 10 kHz lowpass filter was applied throughout. Spontaneous firing was recorded in current clamp (I=0) mode. Analysis of traces was carried out using Clampfit 10.4 software. For action potential firing rate calculation the event detection tool of Clampfit 10.4 was used. Perfusion of external saline in the recording chamber was achieved using a peristaltic pump (Ismatec ISM831). After 3 min of recording basal conditions, 10 ml of Dopamine (1 mM) prepared in external saline were perfused, this lasted approximately 3 minutes. Dopamine was then washed out with external saline perfusion during 10 minutes. For basal condition, the number of action potentials on the last minute before Dopamine application was counted. For Dopamine condition, the number of action potentials was counted on the last minute of Dopamine perfusion. For wash out condition, the number of action potentials was counted on the last minute of the recording. In all cases, the firing rate in Hz was calculated by dividing the number of action potentials over 60 seconds. The membrane potential was assessed during the same periods for each condition. All recordings were performed during the time-range of ZT6 to ZT9.

Recording of sleep and activity

Locomotor activity of male 3-7 days old flies was recorded as described previously (Hermann-Luibl et al., 2014) using Drosophila Activity Monitors by TriKinetics. The fly tubes were fixed by a Plexiglas frame in such a way that the infrared beam crossed each fly tube at a distance of
~3 mm from the food. The food consisted of 4% sugar in agar. For the gene-switch experiments, RU486 (mifepristone, Sigma) was dissolved in 80% ethanol and mixed with the food to a final concentration of 200 mg/ml. In the controls the same amount of ethanol (vehicle) was added to the food. Flies were monitored for 9 days in 12 h:12 h light-dark cycles 12:12 (LD 12:12) with a light intensity of 100 lux at 20°C and then released into constant darkness (DD). Recording days 3-7 in LD were used for sleep and activity analysis.

Sleep analysis was performed with a custom-made Excel Macro (provided by T. Yoshii; Gmeiner et al., 2013; Hermann-Luibl et al., 2014). Sleep was defined as the occurrence of 5 consecutive recording minutes without interruption of the infrared-beam within the TriKinetics monitor. For average daily sleep profiles, sleep was calculated in 1-hour-bins and averaged over the 5 selected days for each single fly and genotype. Furthermore, the total amount of sleep was averaged over the 5 days, as well as the amount of sleep during the light phase and the dark phase and the average sleep bout duration. Every experiment was repeated at least twice and at a minimum 30 flies of each genotype were used for the analysis.

The same 5 days of recordings used for sleep evaluation were also analyzed for fly activity. Daily average activity profiles were calculated for each fly as described in Schlichting and Helfrich-Förster (2015). From these, the total activity (number of infrared-beam crosses) of every fly during the entire day, the dark-phase and the light-phase were calculated and plotted for each genotype. An activity index (the average of beam crosses per active minute) was also calculated but not shown, since it correlated with the total activity. The free-running period of each fly was determined from the recordings in DD to judge whether down-regulating the dopamine receptors changed the speed of the circadian clock.

**Statistics**

Statistical analyses of sleep and activity data were performed using the R environment (v3.5.3). Data were tested for normal distribution with a Shapiro-Wilk normality test (p>0.05). The three
data groups “whole day”, “day” and “night” were tested separately. If any group wasn’t
normally distributed the whole dataset was handled as not normally distributed. In this case
the Mann–Whitney U test was used. A T-test was used for normally distributed data in case of
variance homogeneity (Levene’s test, p>0.05). Period length was tested for statistically
significant influences of dopamine receptor RNAi and RU treatment by a two-way ANOVA
followed by a post-hoc test with Bonferroni correction. Statistical tests on live imaging data
were also done with the R environment. We compared the Epac1-camps inverse FRET ratio
between vehicle and test compounds and used the Wilcoxon signed rank test with Bonferroni
correction for multiple comparisons of maximum changes. Exceptions are stated in the figure
legends. Electrophysiological data (membrane potential and firing rate) was analyzed with
Kruskal-Wallis non-parametric test, the alpha parameter was 0.05 and the post hoc test used
the Fisher’s least significant difference criterion. Bonferroni correction was applied as the
adjustment method.
Results

Dopaminergic neurons are presynaptic to the ventrolateral clock neurons (l-LN,s and s-LN,s) that arborize in the accessory medulla

Both, the s-LN,s and l-LN,s express the neuropeptide PDF and send dendrites into the accessory medulla (AME) - the insect clock center (Helfrich-Förster, 1995; Helfrich-Förster et al., 2007). These neurons are thought to be wake-promoting: their activity coincides with the morning peak of wakefulness (Liang et al., 2019), and their optogenetic excitation, along with other lateral neuron types, reduces sleep (Guo et al., 2018). The s-LN,s project into the dorsolateral brain and are there connected to other clock neurons and several neurons downstream of the clock that control activity and sleep (reviewed in King and Sehgal, 2020). The l-LN,s are conspicuous clock neurons with wide arborizations in the ipsilateral and contralateral optic lobe and connections between the brain hemispheres (Helfrich-Förster et al., 2007). In the AME, their neurites overlap with those of dopaminergic neurons (Hamasaka and Nässel, 2006; Shang et al., 2011). Microarray studies show that they express genes encoding the excitatory dopamine receptors Dop1R1, Dop1R2, and DopEcR) and the inhibitory dopamine D2R, in addition to the excitatory octopamine receptors OAMB and OA2 (Kula-Eversole et al., 2010; Shang et al., 2011). The AME of Drosophila can be subdivided into two parts: a central part and a ventral elongation (Fig. 1). Whereas the central part is innervated by several clock neurons including the PDF-positive small ventrolateral neurons (s-LN,s), the ventral elongation only receives fibers from the l-LN,s (Helfrich-Förster et al., 2007; Schubert et al., 2018). Previous studies already suggested that the PDF-fibers in the ventral elongation of the AME are predominantly postsynaptic (of dendritic nature) (Helfrich-Förster et al., 2007) and in close vicinity to dopaminergic fibers (Shang et al., 2011; Fig. 1a), but whether the dopaminergic fibers were of presynaptic nature was unclear. By expressing the vesicle marker Synaptotagmin (SytI/II)::GFP in the TH-Gal4-positive (dopaminergic) neurons and the postsynaptic marker Dscam::GFP in the Pdf-Gal4-positive neurons we show here that this is
indeed the case (Fig. 1). Prominent Syt I/II::GFP staining was present in TH-Gal4-positive fibers that are aligned along the ventral elongation (Fig. 1c) and Dscam::GFP was strongly localized in the PDF fibers of the entire ventral elongation of the AME (Fig. 1d). Using GRASP imaging, we confirmed previous results that PDF- and TH-Gal4-positive fibers have contact in the central part of the AME and its ventral elongation (Shang et al., 2011): reconstituted GFP signals were present in both parts of the AME (Fig. 1b), whereas no reconstituted GFP signals were detected in control flies. In summary, we show here that the dopaminergic neurons are presynaptic to the l-LNvs and s-LNvs.

Dopamine signals to different clock neurons

It was shown previously that dopamine application to isolated brains elevates cAMP levels in the l-LNvs (Shang et al., 2011). We confirmed this result and extended it to the other clock neurons that have arborizations in the central part of the AME, i.e. the s-LNvs, the dorsolateral neurons (LNds) and the anterior dorsal neurons 1 (DN1s) (Helfrich-Förster et al., 2007; Schubert et al., 2018). The l-LNvs showed the strongest responses to dopamine, which were even higher after blocking synaptic transmission by TTX, suggesting that inhibitory signals from other interneurons usually reduce the cAMP response to dopamine (Fig. 2a). Significant responses to dopamine that persisted under TTX were also present in the LNds (Fig. 2b) and the DN1s (Fig. 2c). The s-LNvs also exhibited significantly increased cAMP levels after dopamine application; but these cells are hard to image, because they are very small and often located underneath the l-LNvs, so that their responses cannot be unequivocally separated from those of the l-LNvs. Therefore, we could only image a few of them without application of TTX (Fig. 3).

Next, we tested whether these cAMP responses were mediated by Dop1R1 or Dop1R2 receptors. Knockdown of Dop1R1 by RNAi in all clock neurons, reduced cAMP responses in the l-LNvs (Fig. 4a, d), the DN1s (Fig. 4c, f) and the LNds (Fig. 4b, e), whereas the down-regulation of Dop1R2 appeared to reduce cAMP levels in all neuron clusters slightly but not significantly (Fig.
4a-c). Notably, the cAMP signals in the LNds were quite variable when Dop1R1 or Dop1R2 were
down-regulated; some neurons still responded to dopamine, while others did not (Fig. 4e). The
same applies for the DN1s knockdown of Dop1R1; half of the cells responded, the other half
did not (Fig. 4f). However, with knockdown of Dop1R2, only two of the measured 22 DN1 cells
did not respond to dopamine (Fig. 4f). Altogether, this suggests that some LNds and DN1s
express Dop1R1 and others Dop1R2. Consistent with this hypothesis the simultaneous down-
regulation of Dop1R1 and Dop1R2 abolished the responses to dopamine in all evaluated
neurons (Fig. 4). Down-regulation of the inhibitory dopamine receptor D2R, slightly increased
the responses to dopamine in the l-LNvs (Fig. 4a, d) and the LNds (Fig. 4b, e); but in contrast to
a previous study (Shang et al., 2011) this increase was not significant. To make sure that the
neurons were able to increase their cAMP levels in our setup, we measured cAMP levels in
responses to NKH477, an adenylyl cyclase activator, and found that they all responded (Fig. 5).
In summary, our results show that the responses to dopamine are predominantly
mediated by Dop1R1 receptors in the l-LNvs and DN1s and by Dop1R1 and Dop1R2 receptors in
the LNds. As described above, we could not identify the relevant Dop1R1 receptors of the s-
LNvs, because these cells were hidden by the l-LNvs or just located too close to them, which
prevented a successful imaging in all the preparations with down-regulated Dop1R receptors.

381 Effects of Dop1R1 and Dop1R2 down-regulation in the clock neurons on sleep

To study the consequences of reduced dopamine signaling in the LNv clock neurons on sleep,
we first down-regulated the activating Dop1R1 and Dop1R2 receptors in all clock neurons
(using Clk856-Gal4). We did not see any significant changes in sleep pattern (Fig. 6a), total
sleep, or sleep during day and night, nor on sleep bout duration (Fig. 6b) with down-regulation
of each of the receptors alone or down-regulation of both receptors simultaneously. However,
the activity level during the day was significantly reduced by down-regulation of each of the
two dopamine receptors alone or in combination (Fig. 6c, d). Furthermore, in the case of
Dop1R2 down-regulation, activity during the night was significantly increased (Fig. 6d). The free-running period in constant darkness did not change when dopamine receptors were knocked down, only the power of the rhythm was decreased slightly by knockdown of both dopamine receptors simultaneously (Table 1).

Since among all clock neurons the s-LN_s and l-LN_s have been the ones with the most prominent role in sleep and arousal regulation, we decided to repeat Dop1R1 and Dop1R2 receptor down-regulation more specifically using the *Pdf-Gal4* driver. The l- and s-LN_s collectively produce the first daily peak of wakefulness (Renn et al., 1999; Grima et al., 2004; Stoleru et al., 2004; Rieger et al., 2006; Potdar and Sheeba, 2018; Liang et al., 2019) and the l-LN_s mediate light driven arousal (Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008a; Lebestky et al., 2009). We repeated Dop1R1 and Dop1R2 receptor down-regulation in these neurons using the *Pdf-Gal4* driver. Once again, the general sleep pattern was not affected by the down-regulation (Fig. 7a), but total sleep and mean sleep bout duration were significantly reduced after all manipulations (down-regulation of Dop1R1 or Dop1R2 and simultaneous down-regulation of both receptors) (Fig. 7b). Closer inspection revealed that Dop1R1 down-regulation reduced sleep significantly during the day, whereas Dop1R2 down-regulation reduced sleep significantly both during the day and night, as did the down-regulation of both receptors simultaneously. The effects of dopamine receptor down-regulation on activity levels were mixed. We did not observe any effects on daytime activity, but nighttime activity was slightly but significantly increased by Dop1R2 receptor knockdown and knockdown of both receptors (Fig. 7c, d). We did not observe any effects on the period or the power of the free-running rhythms in DD (Table 1). In summary, these results suggest that reduction in dopamine signaling in the LN_s has no effect on the speed of the clock. However, dopamine signaling unexpectedly appears to increase sleep via Dop1R1 receptors during the day and via Dop1R2 receptors during the day and the night. These results should be treated with caution because...
they were achieved by constitutive knockdown of dopamine receptors, which may cause
developmental effects.

To assess possible developmental effects of Dop1R1 or Dop2R1 knockdown on the PDF
neurons, we repeated our LN knockdown experiments using GeneSwitch (GS) (Depetris-
Chauvin et al., 2011). Feeding flies the progesterone derivative RU (dissolved in ethanol) only
during adulthood restricted the expression of RNAi constructs to the adult stage. We used two
types of controls. (1) Pdf-GS>uas-Dop1Rx fed with ethanol alone served as controls for Pdf-
GS>uas-Dop1Rx flies fed with RU (Fig. 9). (2) Pdf-GS and uas-Dop1Rx flies, in which the
dopamine receptors were not down-regulated and which were fed either with ethanol alone
or with RU, served as controls for the effect of RU (Fig. 8). In the latter, we did not find any
systematic difference in activity and sleep between the RU and ethanol-fed flies (Fig. 8). Only
in Pdf-GS controls did we find that nocturnal activity was significantly decreased during the last
few hours of the night after feeding RU. In the experimental animals (with dopamine knock-
down), the differences between controls and permanent Dop1R2-knockdown during the day
disappeared when this receptor knocked-down conditionally, suggesting that these were
caused by developmental effects. Nevertheless, the significant reduction in daytime sleep after
Dop1R1 knockdown and the reduction of night sleep after Dop1R2 knockdown persisted (Fig.
9a, b). Furthermore, the conditional down-regulation of dopamine receptors increased activity
during the day and the night (Fig. 9c, d). Since the effects of conditional dopamine receptor
down-regulation were in the same direction as the constitutive receptor down-regulation and
in the opposite direction of RU feeding (Fig. 8) in Pdf-GS controls, we conclude that these are
specific and indeed caused by down-regulation of the dopamine receptors in the PDF neurons.

We observed a highly significant period-lengthening effect of RU application in Pdf-GS
controls and all the crosses with the Pdf-GS strain (Table 1), which has been reported in the
past (Depetris-Chauvin et al., 2011; Frenkel et al., 2017). Therefore, we conclude that
conditional dopamine receptor down-regulation itself does not affect the free-running period,
which is in line with the results obtained via permanent dopamine receptor knockdown.

Dopamine depolarizes the l-LN\(_s\) via Dop1R1, but does not increase their firing rate

When observed electrophysiologically using whole-cell patch clamp, the l-LN\(_s\) fire spontaneous action potentials in bursting or tonic modes (e.g. Cao and Nitabach, 2008; Sheeba et al., 2008b; Depetris-Chauvin et al., 2011; Fogle et al., 2011; Muraro and Ceriani, 2015). As reported previously, when whole-cell patch clamp recordings are performed in the morning and established rapidly after brain dissection (Muraro and Ceriani, 2015), all l-LN\(_s\) fire action potentials in the bursting mode (Fig. 10). To further explore the role of dopamine on the physiology of l-LN\(_s\), we bath-applied dopamine across control l-LN\(_s\) (Fig. 10a), and in l-LN\(_s\) in which Dop1R1 (Fig. 10b) or Dop1R2 (Fig. 10c) had been down-regulated using RNAi constructs driven by the Pdf-Gal4. Control and Dop1R2\textsubscript{RNAi} l-LN\(_s\) displayed robust depolarizations upon 1 mM dopamine application (Fig. 10a, c, and d). In contrast, we observed significantly reduced dopamine induced depolarization when Dop1R1 expression was down-regulated (Fig. 10b and d). This result is consistent with cAMP imaging experiments (Fig. 4) and supports the hypothesis that dopamine responses in l-LN\(_s\) are mainly mediated by the Dop1R1 receptor.

Although we observed a small trend toward a decrease in firing rate upon dopamine application, this was not statistically significant (Fig. 11). These results suggest that, in l-LN\(_s\), dopamine plays a modulatory role as it depolarizes the membrane without significantly changing the firing rate. Thus, dopamine might make the l-LN\(_s\) more sensitive to excitatory inputs.
Discussion

All tested clock neurons respond to dopamine

Here we show that dopamine acts broadly on the neurons of the *Drosophila* clock network that have neurites in the AME, a neuropil that is invaded by presynaptic terminals of dopaminergic neurons. All of these clock neurons responded to dopamine with increases in cAMP. The responses of the I-LNₜs and DN₁s were almost completely blocked by down-regulation of Dop1R₁ receptors but not significantly by down-regulation of Dop1R₂ receptors, whereas the responses of some LNₙs were blocked by down-regulation of Dop1R₁ and others by down-regulation of Dop1R₂ receptors. Dopamine responses of all LNₙs cells were eliminated by simultaneous down-regulation of both receptors. This indicates that the LNₙs employ different activating dopamine receptors.

Since the electrophysiological and cAMP responses of the I-LNₜs were not blocked by down-regulating Dop1R₂ receptors we conclude that these neurons employ only Dop1R₁ receptors. Unfortunately, we could not assess the nature of the Dop1R receptors in the s-LNₙs, but we hypothesize that these employ Dop1R₂ receptors for the following reason: the down-regulation of Dop1R₂ receptors in the s-LNₙs and I-LNₜs significantly reduces the flies' nighttime sleep. Since the I-LNₜs appear not to utilize Dop1R₂ receptors this effect is most likely mediated by the s-LNₙs.

Dopamine signaling on the s-LNₙs appears to promote sleep

Multiple lines of evidence are consistent with a wake promoting role for the s-LNₙs (e.g. Liang et al., 2019). We were therefore surprised to find that the knockdown of the excitatory dopamine receptor Dop1R₂ produce decreases in nighttime sleep. We note here that the s-LNₙs have been shown to promote sleep during the entire day via PDF-signaling to the AllatostatinA (AstA) positive ‘PLP’ neurons (Chen et al., 2016), which were recently shown to be identical with the Lateral Posterior clock neurons (LPNs) (Ni et al., 2019). Optogenetic
excitation of the LPNs promotes sleep (Guo et al. 2018) and glutamatergic and AstA neurites provide excitatory inputs on to the sleep promoting dorsal fan-shaped body (Donlea et al., 2011; Liu et al., 2012; 2016; Ueno et al., 2012; Pimentel et al., 2016; Ni et al., 2019). Thus, our results, along with previous work, suggest that: 1) the role of the s-LNvs in the control of sleep is more complex than previously acknowledged, 2) dopamine likely increases cAMP levels in the s-LNvs via Dop1R2, 3) the s-LNvs excite the sleep promoting LPNs, which subsequently activate the dorsal fan-shaped body neurons leading to sleep. Thus, down-regulation of Dop1R2 receptors in the s-LNvs would therefore be predicted to reduce sleep, which fits to our observations and is consistent with the literature. However, we also must acknowledge the possibility that the s-LNvs might promote both sleep and wakefulness at different times. Recent work on the DN1p class of clock neurons showed that the temporal codes of firing in these cells shape sleep (Tabuchi et al. 2018), suggesting that some clock neurons can switch between sleep and wake promoting modes through changes in their patterns of firing. The same may prove true of the s-LNvs.

Dopamine signaling on the l-LNvs is not wake-promoting

The l-LNvs were reported to be strongly wake-promoting (Sheeba et al., 2008a; Chung et al., 2009; Shang et al., 2011), but it was not clear if dopamine-signaling was responsible this effect. Here, we could not detect wake-promoting effects of dopamine signaling on the PDF neurons. In contrast, down-regulation of the excitatory Dop1R1 and Dop1R2 receptors in these neurons (along with the s-LNvs) slightly increased wakefulness. Night-sleep decreased after knockdown of Dop1R2 receptors, while day-sleep decreased after knockdown of Dop1R1 receptors. Our physiological observations make it clear that and Dop1R1 receptors are expressed by the l-LNvs. This evidently speaks against a wake-promoting role of dopamine signaling to l-LNvs.

The present study supports the findings of Ueno et al. (2012) who found that the ablation of the l-LNvs did not eliminate the strong arousal effects of dopamine, thereby
suggesting that dopamine does not drive the wake-promoting role of the l-LNvs. In fact, our results suggest a moderate sleep-promoting effect of dopamine signaling on the l-LNvs, despite of the fact that dopamine depolarizes the l-LNvs, potentially making them more excitable. Glutamate, GABA, and histamine inhibit the l-LNvs (Cao and Nitabach, 2008; Schlichting et al., 2016). While GABAergic inputs to l-LNvs have a clear role in the promotion of sleep (Agosto et al., 2008; Parisky et al., 2008; Chung et al., 2009; Gmeiner et al., 2013), such a role has not yet been demonstrated for histamine or glutamate. Other putative silencing neuromodulators of the l-LNvs are glycine (Frenkel et al., 2017) and serotonin (Yuan et al., 2005, 2006), but how these different signals interact to regulate the l-LNvs’ command over wakefulness is still an open question.

Our study does not call into question the wake-promoting role of the l-LNvs. The ablation of the l-LNvs increases sleep, which demonstrates that their wake-promoting influence exceeds their sleep-promoting one (Chung et al., 2009). Furthermore, the l-LNvs are electrically the most active during the day when the flies are awake (Sheeba et al., 2008b; Shang et al., 2011) and the electrical hyperexcitation of the l-LNvs increases activity at night and disrupts nocturnal sleep (Sheeba et al., 2008a). Thus, the l-LNvs are firing during the day, thereby promoting daytime wakefulness, and their firing is decreased at night when flies maintain their deepest sleep. The wake promoting neuromodulators octopamine and acetylcholine act on l-LNvs (Kula-Eversole et al., 2010; Muraro and Ceriani, 2015). But the result described above, lead to the surprising conclusion that dopamine does not act wake-promoting neuromodulator of the l-LNvs.

In any case, the sleep-promoting role of dopamine via the l-LNvs is moderate when compared to the sleep-promoting effects of the fan-shaped body neurons that lack dopaminergic input (Liu et al., 2012; Ueno et al., 2012). Thus, dopamine signaling via the fan-shaped body has a stronger impact on sleep than dopamine signaling via the l-LNvs or the s-LNvs. The precise role played by dopaminergic inputs to l-LNvs and their modulatory effect on
the integration of the multiple excitatory and inhibitory afferences received by these important arousal neurons awaits further research.

Dopamine has different effects on the fan-shaped body neurons and the PDF neurons

Dop1R1 and Dop1R2 receptors have already been implicated in the control of sleep in previous studies. Lebestky et al. (2009) showed that the rescue of Dop1R1 receptors in the l-LNₜₛ of Dop1R1 mutants can partially rescue the flies’ normal sleep pattern, which fits our observation that the l-LNₜₛ utilize Dop1R1 receptors. Liu et al. (2012) and Ueno et al. (2012) showed that dopaminergic neurons signal via Dop1R1 receptors on neurons in the fan-shaped body whereas Pimentel et al. (2016) demonstrated a role of Dop1R2 receptors in the fan-shaped body. Here we suggest that dopamine signals via Dop1R2 receptors on the s-LNₜₛ. Although the PDF neurons and the fan-shaped body neurons respond to dopamine via the same activating receptors and in both cases via an increase in cAMP levels, the electrical responses of the neurons to dopamine appear to be different.

In the fan-shaped body neurons, the increase of cAMP leads to an upregulation of the voltage-independent leak current K⁺ channel “Sandman” and its translocation to the plasma membrane (Pimentel et al., 2016). Consequently, the fan-shaped body neurons switch to long-lasting hyperpolarization (OFF state), which keeps the fruit flies awake. The Rho-GTPase-activating protein Crossveinless-c locks the fan-shaped body neurons in the OFF state (Donlea et al., 2014) until unknown mechanisms flip the neurons back to the ON state. Thus, Dop1R1/2 receptors silence neurons in the fan-shaped body via the increase of cAMP levels (Liu et al., 2012; Ueno et al., 2012; Pimentel et al., 2016).

Our results indicate a very different effect of Dop1R1 receptor signaling in the l-LNₜₛ. The neurons depolarized in response to dopamine and this effect was blocked after knockdown of Dop1R1 receptors. Thus, dopamine excites the l-LNₜₛ as predicted, but does not increase their firing rate. The main effect of dopamine perfusion in our ex-vivo preparation was
a robust and reversible depolarization of the membrane, which should make l-LNₜₜ more sensitive to excitatory inputs. Thus, the effect of dopamine on the l-LNₜₜ may be context-dependent. Lebestky et al. (2009) aroused the flies by repetitive air puffs and found that dopamine reduced the flies’ hyperactivity in response to this excitation, while it increased spontaneous nocturnal activity. Both effects were mediated via Dop1R1 receptors. Although Lebestky et al. (2009) traced the dopamine effects on startle-induced hyperactivity to the central complex, we cannot exclude that similar mechanisms work in the l-LNₜₜ. Therefore, it will be most interesting to study the effects of Dop1R1 receptor knock-down in the l-LNₜₜ on sleep and activity of flies in the context of stimulus-induces arousal, to test not only the role of dopaminergic inputs to l-LNₜₜ in the context of basal sleep-wake activity, but also in the context of environmentally stimulated arousal or in the presence of challenges to the sleep homeostat, such as in the generation of a sleep rebound phenomenon after a night of sleep deprivation.

In summary, dopamine appears to have different modulatory effects on the fan-shaped body neurons and the PDF neurons - inhibiting the former and exciting the latter. In both cases, dopamine signaling increases sleep, though in different ways and to different degrees. Dopamine signaling to the fan-shaped body is strongly sleep promoting, while dopamine signaling to the PDF neurons is weakly sleep promoting and, in case of the l-LNₜₜ, perhaps dependent on the arousal state of the flies.
References


Helfrich-Förster C (1995) The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of Drosophila melanogaster. Proc Natl Acad Sci USA 92:612-616. https://doi.org/10.1073/pnas.92.2.612


Table 1. Rhythmic parameters of the free-running rhythms under constant darkness (DD)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rhythmic</th>
<th>Period (± SD)</th>
<th>Relative Power (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAS-dicer2;clk856-Gal4;UAS-Dop1R1RNAi</td>
<td>94%</td>
<td>23.82 ± 0.41</td>
<td>3950.63 ± 1145.38</td>
</tr>
<tr>
<td>UAS-dicer2;clk856-Gal4;UAS-Dop1R2RNAi</td>
<td>100%</td>
<td>23.93 ± 0.40</td>
<td>4637.78 ± 1399.28</td>
</tr>
<tr>
<td>UAS-dicer2;clk856-Gal4;UAS-Dop1R1R2RNAi</td>
<td>84%</td>
<td>23.83 ± 0.44</td>
<td>2687.00 ± 772.08*</td>
</tr>
<tr>
<td>UAS-dicer2;clk856-Gal4</td>
<td>97%</td>
<td>24.03 ± 0.46</td>
<td>3709.97 ± 1146.97</td>
</tr>
<tr>
<td>UAS-dicer2;Pdf-Gal4;UAS-Dop1R1RNAi</td>
<td>97%</td>
<td>23.93 ± 0.33</td>
<td>6496.61 ± 1652.04</td>
</tr>
<tr>
<td>UAS-dicer2;Pdf-Gal4;UAS-Dop1R2RNAi</td>
<td>97%</td>
<td>24.09 ± 0.41</td>
<td>5315.03 ± 2031.54</td>
</tr>
<tr>
<td>UAS-dicer2;Pdf-Gal4;UAS-Dop1R1R2RNAi</td>
<td>97%</td>
<td>24.00 ± 0.31</td>
<td>3752.74 ± 1485.88</td>
</tr>
<tr>
<td>UAS-dicer2;Pdf-Gal4</td>
<td>100%</td>
<td>24.31 ± 0.34</td>
<td>6966.94 ± 1699.23</td>
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<tr>
<td>UAS-dicer2;;UAS-Dop1R1RNAi</td>
<td>100%</td>
<td>23.70 ± 0.42</td>
<td>4812.78 ± 1702.03</td>
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<td>UAS-dicer2;;UAS-Dop1R2RNAi</td>
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<td>23.75 ± 0.26</td>
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<td>UAS-dicer2;;UAS-Dop1R1R2RNAi</td>
<td>100%</td>
<td>23.66 ± 0.34</td>
<td>4135.06 ± 1298.63</td>
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<td>UAS-dicer2;Pdf-GS;UAS-Dop1R1RNAi + Eth</td>
<td>100%</td>
<td>23.74 ± 0.35</td>
<td>2833.94 ± 720.94</td>
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<td>UAS-dicer2;Pdf-GS;UAS-Dop1R1RNAi + RU</td>
<td>100%</td>
<td>24.18 ± 0.54**</td>
<td>2668.28 ± 365.25</td>
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<tr>
<td>UAS-dicer2;Pdf-GS;UAS-Dop1R2RNAi + Eth</td>
<td>94%</td>
<td>23.84 ± 0.34</td>
<td>3886.97 ± 978.24</td>
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<tr>
<td>UAS-dicer2;Pdf-GS;UAS-Dop1R2RNAi + RU</td>
<td>97%</td>
<td>24.87 ± 0.52**</td>
<td>2865.97 ± 461.19</td>
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<td>23.76 ± 0.24</td>
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<td>UAS-dicer2;Pdf-GS + RU</td>
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<td>24.46 ± 0.42**</td>
<td>4332.97 ± 894.48</td>
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<tr>
<td>UAS-dicer2;;UAS-Dop1R1RNAi + Eth</td>
<td>100%</td>
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<td>3686.97 ± 978.24</td>
</tr>
<tr>
<td>UAS-dicer2;;UAS-Dop1R1RNAi + RU</td>
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<td>23.89 ± 0.25</td>
<td>3608.44 ± 830.90</td>
</tr>
<tr>
<td>UAS-dicer2;;UAS-Dop1R2RNAi + Eth</td>
<td>100%</td>
<td>23.81 ± 0.30</td>
<td>3433.91 ± 859.93</td>
</tr>
<tr>
<td>UAS-dicer2;;UAS-Dop1R2RNAi + RU</td>
<td>100%</td>
<td>23.81 ± 0.36</td>
<td>3526.28 ± 582.67</td>
</tr>
</tbody>
</table>

* significant differences (p<0.05) in power between flies with down-regulated dopamine receptors in all clock neurons in comparison to the relevant controls.

** highly significant differences (p<0.01) after RU application in the Pdf-GeneSwitch (GS) experiments.
Figure 1. Staining of whole-mount brains showing the spatial vicinity of dopaminergic neurites (visualized with TH-Gal4) and neurites from the PDF-positive LNvs in the accessory medulla of one hemisphere. All pictures are overlays of 2 µm thick confocal stacks. (a) Medulla (ME) and accessory medulla (AME) labeled with anti-PDF (magenta) and anti-GFP (TH-Gal4; UAS-10xmyrGFP, green) (overlay of 10 confocal stacks). TH-Gal4 and PDF overlap in the central part (CE) and ventral elongation (VE) of the AME. I-LNvs, PDF-positive large ventrolateral neurons; s-LNvs, PDF-positive small ventrolateral neurons. (b) GFP Reconstitution Across Synaptic Partners (GRASP) between Pdf-Gal4 neurons and TH-Gal4 neurons. GRASP signals are found in the CE and VE of the AME (overlay of 6 confocal sections). (c) Expression of the presynaptic marker Synaptotagmin::GFP (SytI/II::GFP) in the TH-Gal4 neurons (GFP; green) and co-staining against PDF (magenta) (overlay of 3 confocal stacks). GFP-positive vesicles (arrowheads) are present along the PDF-positive fibers in the VE. (d) Expression of the postsynaptic marker Dscam::GFP (green) in the Pdf-Gal4-positive I-LNvs and co-staining with anti-PDF (magenta) (overlay of 3 confocal stacks). The PDF-positive fibers in the VE of the AME are predominantly dendritic. Scale bars = 20 µm in a and b, and 10 µm in c and d.
Figure 2. *Ex vivo* live-cAMP imaging on *Drosophila* clock neurons. (a-c) Mean inverse FRET traces of I-LN\(_v\), LN\(_d\) and DN\(_1\) clock neurons of *clk856*\(\rightarrow\) *Epac1* flies. Error bars (grey) represent SEM and short black bars indicate application of the different solutions: HL3 = buffer (negative control), DA (= 1 mM dopamine), DA+TTX (= 1 mM DA + 2 µM Tetrodotoxin) and SKF\(_{38393}\) (= 0.1 mM Dop1R1-agonist), respectively. (d-f) Quantification of maximum inverse FRET changes for each single neuron (dots in Box Plots) of each treatment. Black horizontal lines in the Box Plots represent the median, different letters indicate significant differences. Cells of all three neuronal clusters respond with robust and significant increases in cAMP levels upon application of DA and DA+TTX compared to negative controls, indicating a direct neuronal connection between dopaminergic neurons and clock neurons. Application of the Dop1R1-agonist SKF also significantly increased cAMP levels in all three clusters of clock neurons (f).
Figure 3. Ex vivo live-cAMP imaging on Drosophila s-LNv neurons. (a) Mean inverse FRET traces of s-LNv clock neurons of clk856>Epac flies. Error bars (grey) represent SEM and short black bars indicate application of negative control (HL3) or 1 mM dopamine (DA). (b) Quantification of maximum inverse FRET changes for each single neuron (dots in Box Plots) of each treatment. Black horizontal lines in the Box Plots represent the median, different letters indicate significant differences. s-LNvs significantly responded to DA with an increase in cAMP. In this case the Mann–Whitney U test was used for pairwise comparison of maximum changes.
Figure 4. Ex vivo live-cAMP imaging on Drosophila clock neurons expressing RNAi-constructs against different Dopamine receptors. (a-c) Mean inverse FRET traces of I-LN\textsubscript{v}, LN\textsubscript{d} and DN\textsubscript{1} clock neurons of clk856>dicer2, Epac1, XRNAi flies. The X stands for ‘wildtype’ (+) or the relevant dopamine receptor RNAi lines: R1 = Dop1R1RNAi, R2 = Dop1R2RNAi, D2R = D2RNAi and R1/R2 = Dop1R1RNAi/Dop1R2RNAi. Error bars (grey) represent SEM and short black bars indicate application of negative control (HL3) or 1 mM dopamine (DA). (d-f) Quantification of maximum inverse FRET changes for each single neuron (dots in Box Plots) of each treatment. Black horizontal lines in the Box Plots represent the median, different letters indicate significant differences. DN\textsubscript{1} neurons responded significantly to application of DA, except when Dop1R1 or Dop1R1/R2 were knocked down. I-LN\textsubscript{v} neurons lacked the responses to dopamine when both dopamine receptors (Dop1R1/R2) were knocked down. Responses of the LN\textsubscript{d} were not different from negative controls when either Dop1R1 or Dop1R2 or both (Dop1R1/R2) were knocked down.
Figure 5. *Ex vivo* live-cAMP imaging on *Drosophila* clock neurons expressing RNAi-constructs against different dopamine receptors (*clk856>dicer2,Epac1;Dop1RX* RNAi flies). (a) Mean inverse FRET traces of l-LNv clock neurons with down-regulated Dop1R1 (*Dop1R1RNAi*). The same set of neurons (5 neurons from 2 brains) was first subject to 1 mM dopamine (DA) application showing no response and afterwards to application of 10 µM of the adenylate-cyclase activator NKH477, which evoked an increase in cAMP. (b) Mean inverse FRET traces of the same l-LNv, LN_d and DN_1 clock neurons shown in Fig. 4a, b, c (bottom) expressing *DopR1RNAi/DopR2RNAi* after application of NKH477. Error bars (grey) represent SEM and short black bars indicate application of negative control (HL3) or 1 mM dopamine (DA).
Figure 6. Sleep and activity in clock neuron specific dopamine-receptor knockdown flies. Dop1R1, Dop1R2 or both were knocked down using clk856-Gal4. (a) Average daily sleep profiles of experimental flies (red, clk856>Dop1R1RNAi) and respective Gal4 and UAS controls (pooled in black; controls were not significantly different from each other and were pooled in a single). (b) Box Plots of sleep parameters (total sleep in hours during the entire 24 h period, during the day and the night; same color code as in a). The median, upper and lower quartiles as well as upper and lower extremes plus the single data points are plotted. No significant differences were observed between experimental flies and controls in any of the three cases. (c) Average activity profiles of the same flies that are depicted in a. The flies with down-regulated dopamine receptors were always less active during day as compared to the controls. (d) Box Plots of total activity during the entire 24 h period, during the day and the night. Significant differences are indicated by asterisks (* p<0.05; ** p<0.01; *** p<0.001). The numbers of tested flies are indicated in (a) and (c).
Figure 7. Sleep and activity in PDF-neuron specific dopamine-receptor knockdown flies. Dop1R1, Dop1R2 or both were knocked down using Pdf-Gal4. (a) Average daily sleep profiles of experimental flies (red, Pdf>Dop1RRNAi) and respective Gal4 and UAS controls (pooled in black; both controls showed significantly more sleep than the flies with dopamine receptor knockdown; therefore they were pooled). (b) Box Plots of sleep parameters as shown in Fig. 6. Flies showed significantly less total sleep and shorter sleep bouts, when either Dop1R1 or Dop1R2 or both were knocked down in the PDF-neurons. Knockdown of Dop1R1 decreased daytime sleep, whereas knockdown of Dop1R2 and simultaneous knockdown of both receptors decreased day- and night-time sleep. (c) Average activity profiles of the same flies that are depicted in a. The flies with down-regulated Dop1R2 receptor were more active than the controls. (d) Box Plots of total activity during the entire 24 h period, during the day and the night. Significant differences are indicated by asterisks (* p<0.05; ** p<0.01; *** p<0.001). The numbers of tested flies are indicated in (a) and (c).
Figure 8. Sleep and activity in control flies fed with RU dissolved in ethanol or only with ethanol. (a) Average daily sleep profiles of flies fed with RU in ethanol (red) and flies fed only with ethanol (black). (b) Box Plots of sleep parameters. (c) Average activity profiles of the same flies that are depicted in (a). (d) Box Plots of total activity during the entire 24 h period, during the day and the night. Significant differences are indicated by asterisks (* p<0.05; ** p<0.01; *** p<0.001). Feeding of RU affected sleep and activity marginally. Only Pdf-Gal4 flies fed with RU slept significantly more and were less active in the night than flies fed only with ethanol. The numbers of tested flies are indicated in (a) and (c).
Figure 9. Sleep and activity in flies with conditional dopamine-receptor knockdown in the PDF-neurons (with Pdf-GS). (a) Average daily sleep profiles of experimental flies (red, Pdf-GS>Dop1R1RNAi fed with RU in ethanol) and control flies (black, Pdf-GS>Dop1R1RNAi fed with ethanol). (b) Box Plots of sleep parameters. Flies showed significantly less total sleep when either Dop1R1 or Dop1R2 or both were knocked down in the PDF-neurons. Knockdown of Dop1R1 decreased daytime sleep, whereas knockdown of Dop1R2 decreased nighttime sleep. Sleep bouts were only significantly affected after knockdown of the Dop1R2 receptor. (c) Average activity profiles of the same flies that are depicted in (a). The flies with down-regulated dopamine receptors were generally more active than the controls. (d) Box Plots of total activity during the entire 24 h period, during the day and the night. Significant differences are indicated by asterisks (* p<0.05; ** p<0.01; *** p<0.001). The numbers of tested flies are indicated in (a) and (c).
Figure 10. Dop1R1 receptor mediates l-LNv responses to dopamine. (a), (b), (c). Representative traces of whole-cell patch clamp recordings during basal conditions (perfusion of external saline, left panels), DA (perfusion of 1 mM dopamine, middle panels) and wash out (perfusion of external saline, right panels). (a) Control group, Pdf-Gal4,UAS-dicer;pdf Red>. (b), Dop1R1RNAi group, Pdf-Gal4,UAS-dicer2;pdf Red>UAS-Dop1R1RNAi. (c), Pdf-Gal4,UAS-dicer2;pdf Red>UAS-Dop1R2RNAi. (d). Boxplots showing the value of membrane potential in mV for the same genotypes in each condition (basal, DA, wash). Kruskal-Wallis non-parametric test with Bonferroni correction was applied for statistical analysis. The alpha parameter was 0.05. Different letters indicate significant differences. Control, n=8. DopR1RNAi, n=9. Dop1R2RNAi, n=6.
Figure 11. Boxplots showing the value of firing rate (number of action potentials per second) obtained in whole-cell patch clamp configuration under three different conditions (basal, Dopamine, wash) for Pdf-Gal4,UAS-dicer;pdf Red>+(Control, left panel), DopR1RNAi group, Pdf-Gal4,UAS-dicer;pdf Red>UAS-Dop1R1RNAi (DopR1RNAi, middle panel), Pdf-Gal4,UAS-dicer;pdf Red>UAS-Dop1R2RNAi (Dop1R2RNAi, right panel). Kruskal-Wallis non-parametric test with Bonferroni correction was applied for statistical analysis. The alpha parameter was 0.05. No statistically significant differences were found (same letter indicate no significant differences). Control, n=8. DopR1RNAi, n=9. Dop1R2RNAi, n=6.