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# Light exposure during development affects physiology of adults in *Drosophila melanogaster*

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Light is one of most important factors synchronizing organisms to day/night cycles in the environment. In *Drosophila* it is received through compound eyes, Hofbauer-Buchner eyelet, ocelli, using phospholipase C-dependent phototransduction and by deep brain photoreceptors, like Cryptochrome. Even a single light pulse during early life induces larval-time memory, which synchronizes the circadian clock and maintains daily rhythms in adult flies. In this study we investigated several processes in adult flies after maintaining their embryos, larvae and pupae in constant darkness (DD) until eclosion. We found that the lack of external light during development affects sleep time, by reduction of night sleep, and in effect shift to the daytime. However, disruption of internal CRY-dependent photoreception annuls this effect. We also observed changes in the expression of genes encoding neurotransmitters and their receptors between flies kept in different light regime. In addition, the lack of light during development results in decreasing size of mushroom bodies, involved in sleep regulation. Taking together, our results show that presence of light during early life plays a key role in brain development and affects adult behavior.

## KEYWORDS

clock, sleep, dopamine pathway, histamine, octopamine, cryptochrome, mushroom bodies

## Introduction

Light plays a key role in the synchronization of animal behavior to cyclical changes in environmental conditions, it acts as an arousal signal for diurnal animals and promotes sleep in nocturnal ones (Redlin, 2001). *Drosophila* is a very useful model for sleep/activity research since it exhibits a clear daily activity pattern. Under LD12:12 (12 h of light and 12 h of darkness) conditions it shows two peaks of activity, morning and evening, while in the middle of the day and night it has resting time called siesta and sleep, respectively. This rhythmic behavior is regulated by brain sleep centers, glial cells, as well as by different clusters of clock cells (Grima et al., 2004; Stoleru et al., 2004; Picot et al., 2007; Cusumano et al., 2009; Zhang et al., 2009; Yao and Shafer, 2014; Chatterjee et al., 2018; Guo et al., 2018; Schlichting et al., 2019). Sleep in flies is defined as at least 5 min of total inactivity

with elevated sensory threshold and specific body posture (Shaw, 2000). Daytime (siesta) and nighttime sleep, which are the deepest around noon, and at midnight, respectively constitute total sleep. Siesta is usually less deep and has shorter single sleep episodes as well as lower arousal threshold comparing to nighttime sleep (Hendricks et al., 2000; Huber et al., 2004).

Sleep is regulated by several neurotransmitters: promoting sleep as serotonin and gamma-aminobutyric acid (GABA) (Yuan et al., 2005, 2006; Chung et al., 2009; Haynes et al., 2015; Qian et al., 2017) and wake-promoting as dopamine, octopamine, and histamine (Friggi-Grelin et al., 2003; Hamasaka and Nässel, 2006; Pantazis et al., 2008; Mao and Davis, 2009; Crocker et al., 2010; Liu et al., 2012; Ueno et al., 2012; Oh et al., 2013; Sitaraman et al., 2015; Alejevski et al., 2019) and those playing a dual role depending on target cells (acetylcholine, glutamate) (Buchner et al., 1986; Yasuyama and Salvaterra, 1999; Sinakevitch-Pean et al., 2001; McCarthy et al., 2011; Raghu and Borst, 2011; Takemura et al., 2011; Liu and Wilson, 2013; Mauss et al., 2014, 2015; Tomita et al., 2015; Robinson et al., 2016). In addition, there are neurotransmitters regulating clock network activity: Pigment-dispersing factor (PDF) and short Neuropeptide F (sNFP), which play a role in the integration of clock and sleep (Shang et al., 2013; Kunst et al., 2014).

Dopamine is a monoamine synthesized from tyrosine or phenylalanine mainly in a set of specific neurons in the presence of tyrosine hydroxylase encoded in *Drosophila* by the *ple* gene. Once produced and released into synaptic cleft it can bind to specific receptors on postsynaptic cells in the following structures involved in sleep regulation: Dop1R1 in neurons of mushroom bodies, ellipsoid body and fan-shaped body, Dop1R2 in neurons of mushroom bodies and DD2R in three pairs of dorsal neurons in the ventral nerve cord and sleep promoting protocerebral bridge interneurons (Friggi-Grelin et al., 2003; Mao and Davis, 2009; Liu et al., 2012; Ueno et al., 2012; Sitaraman et al., 2015; Tomita et al., 2021).

The another neurotransmitter involved in light transmission and sleep regulation is histamine. It is released mostly by the retina photoreceptors and synthesized by histidine decarboxylase (Hdc). One of its receptor HisCl1 is expressed in photoreceptors and clock neurons, and the other one—Ort receptor—in the lamina and medulla interneurons (Hamasaka and Nässel, 2006; Pantazis et al., 2008; Oh et al., 2013; Alejevski et al., 2019).

Octopamine is synthesized by anterior paired lateral neurons (APL) using tyramine beta hydroxylase (Tbh) and its signal is transmitted to mushroom bodies through Oamb receptor (Crocker et al., 2010).

All these neurotransmitters control activity of specific neurons located in structures called sleep centers. The most important ones are mushroom bodies (MB) and central complex in the central and dorsal brain (Joiner et al., 2006; Pitman et al., 2006; Donlea et al., 2011; Liu et al., 2012; Guo et al., 2016). The mushroom body is formed by Kenyon cells (Kc), and can be divided into morphologically distinct parts: calyx, which

contains synapses between Kc and projection neurons, and five output lobes ( $\alpha$ ,  $\alpha'$ ,  $\beta$ ,  $\beta'$ ,  $\gamma$ ), composed of synapses between Kc, mushroom body output neurons and dopaminergic neurons (Aso et al., 2014). Output signals from multiple sensory pathways are received by calyx and then transferred to the lobes. MB integrate and process also visual signals coming from medulla, lobula and posterior lateral protocerebrum (Morante and Desplan, 2008; Vogt et al., 2016; Li et al., 2020).

Light input is received by the visual system: retinal photoreceptors, Hofbauer-Buchner eyelet and ocelli through the rhodopsin-dependent phototransduction pathway (Busto et al., 1999; Hassan et al., 2000). All photoreceptors use histamine to communicate with downstream neurons (Hamasaka and Nässel, 2006), but some of them use also acetylcholine as a neurotransmitter, for example eyelets which contact directly with clock cells (Pollack and Hofbauer, 1991; Yasuyama and Meinertzhagen, 1999; Helfrich-Förster et al., 2002; Malpel et al., 2002; Damulewicz et al., 2020). In addition, light is detected by deep brain photoreceptors, Cryptochrome (CRY) and Quasimodo (QSM), which are mostly involved in clock synchronization (VanVickle-Chavez and Van Gelder, 2007; Chen et al., 2011). Moreover, rhodopsin 7, which is involved in non-visual light signaling, regulates morning activity and time of siesta (Kistenpfennig et al., 2017).

In larvae light activates CRY located in clock neurons (four LNvs and DN1s) and photoreceptors of the Bolwig organ (BO), which contains rhodopsin 5 or rhodopsin 6 (Malpel et al., 2002; Mazzoni et al., 2005) and communicates directly with the larval pacemaker to synchronize this oscillator to changes in environmental light conditions (Keene et al., 2011; Klarsfeld et al., 2011). Although larvae do not show circadian behavior, their clock is entrained by light which can affect the clock in adult flies. It has been shown that a single light pulse during embryonal or postembryonic development can synchronize activity of adult flies kept in constant darkness (Sehgal et al., 1992; Zhao et al., 2019).

The role of light in the sleep regulation is already well described, however, little is known about the link between light exposure during development and sleep amount in adult life. In this study, using *Drosophila* as a model, we showed that lack of light in early life affects behavior of adult flies.

## Material and methods

### Flies

The following strains of *Drosophila melanogaster* were used: wild type CantonS, *norPA*<sup>7</sup> mutant (BDSC, no. 5685), *cry*<sup>01</sup> mutant (Dolezelova et al., 2007), OK107-Gal4 (kindly donated by P. Menegazzi, Wurzburg University), UAS-mCD8::GFP (BDSC, stock no. 5137).

Flies were maintained on a standard cornmeal medium at constant temperature 25°C. The control group was kept under LD12:12 (12 h of light and 12 h of darkness) regime during development and adulthood (group described as LD/LD), while experimental flies were kept in constant darkness (DD) during development (embryonal, larval and pupal stage) (group described here as DD/DD). Because sleep assay was measured on the second day of LD12:12 conditions, we added the additional group called DD/LD, in which flies exposed to DD during development were transferred for 2 days to LD12:12.

## Behavioral assays

Locomotor activity was recorded using *Drosophila* Activity Monitoring System (DAMS, Trikinetics, Waltham) for 5 days in LD12:12 and 6 days in DD. Activity was counted every 1 min and analyzed in Excel using “Befly!” software (Department of Genetics, Leicester University). Lomb—Scargle normalized periodogram was used to determine rhythmic flies; individuals with period below 10 (confidence level 0.05) were regarded as arrhythmic. Flies, which did not survive until the end of experiments were removed from analyses. Every experiment was repeated three times, at least 60 flies in total were used.

Sleep analysis was performed on the second day of LD12:12, and sleep was recorded as at least 5 min of a fly immobility.

## Immunohistochemistry

Males from the strain OK107 > GFP were collected at ZT1 (1 hour after lights-on in LD 12:12) or CT1 (in DD), their heads were fixed in 4% paraformaldehyde and brains were isolated. GFP fluorescence of this strain is strong enough to skip additional staining steps.

For TH staining wild type flies were used. Fixed brains were washed in PBS and 0.2% PBST (PBS with Triton X), then unspecific antigens were blocked with Normal Goat Serum (NGS) for 1 h. Samples were incubated with mouse anti-TH antibodies (1:1000, Immunostar) for 3 days at 4°C. Then, brains were washed three times in 0.2% PBST and incubated for 2 h with goat anti-mouse antibodies conjugated with Cy3 (1:500, ThermoFisher). After washing in 0.2% PBST, brains were mounted in Vectashield medium (Vector) and examined with a Zeiss Meta 510 Laser Scanning Microscope.

The size of the brain and GFP-labelled mushroom bodies lobes was measured using ImageJ software (Area measurements). To avoid changes in body size, dependent on overcrowding, the same number of parental flies was used for every group.

The TH immunofluorescence intensity was measured for specific dopaminergic cells: PPL1, PAM and PPM3 because these cluster are known to innervate sleep centers. Area of every single

cell was selected manually and mean grey value was measured using ImageJ software.

Experiments were repeated 3 times and each repetition was composed of 10 brains per group.

## Gene expression analysis by qPCR

For gene expression analysis we divided the experimental flies into two groups—first was continuously kept in constant darkness (DD/DD), the second one was moved to LD12:12 for 2 days (DD/LD) to provide light input and synchronize flies. All groups were collected at the same time—ZT1 (1 hour after lights-on for control and flies transferred to LD12:12) and CT1 (for flies kept in DD). At least 20 heads were cut off and the total RNA was isolated using a TriReagent (Invitrogen). 1 µg of the total RNA was used to prepare cDNA with the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and random primers. cDNA (diluted 1:10) was used for SybrGreen qPCR (KapaBiosystems) analysis. The specific primers (the specificity was controlled with Primer BLAST and gel electrophoresis) used for the reaction are listed in [Table 1](#). Expression level was calculated by relative standard curve. The reference gene used was *rpl32*. Experiments were repeated 5 times.

## Statistical analysis

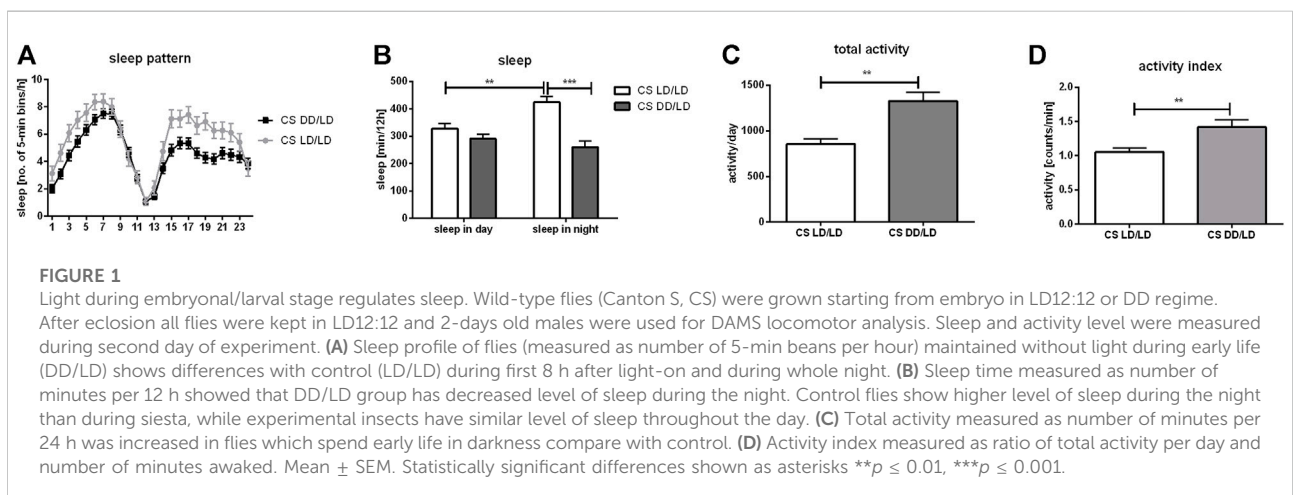
GraphPad Prism software was used for statistics and making graphs. Outliers were removed using Grubbs’ test (GraphPad online software). Shapiro-Wilk’s test was used to check normality in distribution. Data were analyzed using one-way ANOVA with post-hoc Tukey’s test or *t*-test to detect statistically significant differences between groups.

## Results

Light exposure during development is not necessary to maintain rhythmicity in behavior, but it affects activity/sleep ratio. It has been shown that larvae kept in DD can be easily synchronized to light:dark regime, which suggests that their clock develops normally. Our data confirmed these results because flies which were kept in DD during development and transferred to LD12:12 after eclosion were perfectly synchronized and after moving back to DD they were able to maintain 24 h rhythmicity. We focused our research on the other aspect of behavior – activity and sleep amount and pattern. We observed that flies from the experimental group (DD/LD) were more active than the control (LD/LD)

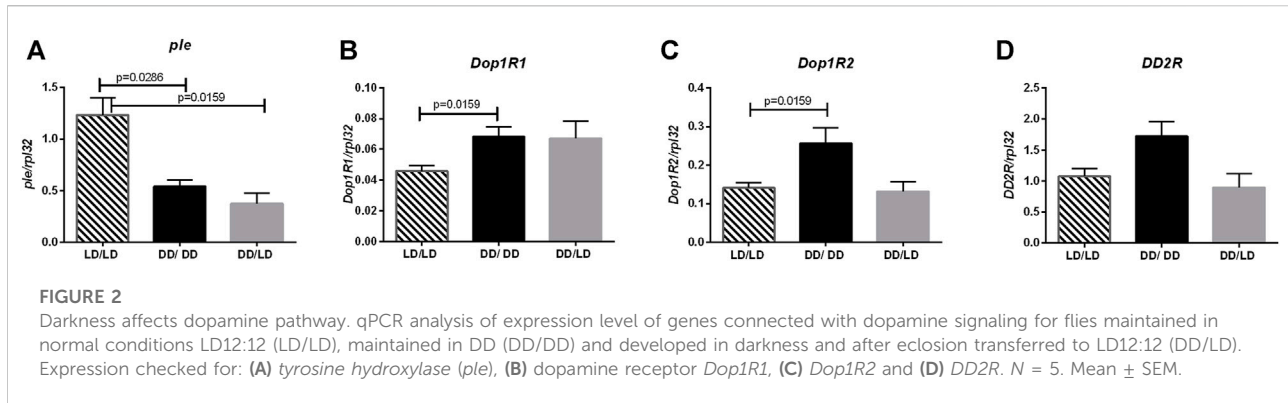
TABLE 1 Primer sequences used for qPCR.

Gene	Primer sequence	Product length [bp]
<i>Dop1R1</i>	For: GGATACAATAGTTGGCATCTTCCT Rev: ACGAGGCCAGGAACAGATTG	123
<i>Dop1R2</i>	For: GGTGTCGATAACTCCAGCGT Rev: GTCGATAGCGTCGAGTCGTT	101
<i>DD2R</i>	For: TGTCGATAACTCCAGCGTCG Rev: TCGATAGCGTCGAGTCGTTG	98
<i>ple</i>	For: ATTGCATTGTGCAGGCTCAG Rev: ATTCCTCCAAGGGCAGGTTC	96
<i>HisCl1</i>	For: GCGCCATGAAGTTTGAGTCC Rev: ACGGGATTTGCAGGGTGTAG	106
<i>Ort</i>	For: CCACACCACGGATGACTTGA Rev: TCATTTCCGGATGAGGGCCAC	99
<i>Oamb</i>	For: TTCCAAGGGCATCGGTTCTC Rev: CCTGCTGGTTTGATCCTCGT	80
<i>Hdc</i>	For: TCAAGCGTGCATTTCATCAG Rev: TACACAGATACTTGCCGAGC	136
<i>Tbh</i>	For: CGATGGACTTCGCCTATCA Rev: CAGATCGGTGGCGAATTAT	137
<i>Pdf</i>	For: GGCCACTCTCTGTGCGTATC Rev: ATTAGCTCCGAGTTGCGCTT	84
<i>Pdfr</i>	For: ACGGCCGTGTTACAAGCCG Rev: GGAGAGGCAGAGGCCACGA	124
<i>sNPF</i>	For: ACCGCCTATGATGACCTCCT Rev: CGTACAGTCCGTGTAAGTCG	115
<i>sNPF-R</i>	For: TGCCATCGATCGGTAAGTTCG Rev: GGGCTATCAGCCAGATGCTC	98
<i>rpl32</i>	For: ATGCTAAGCTGTGCGACAAATG Rev: GTTCGATCCGTAACCGATGT	107



( $p = 0.0052$ ), activity index was increased ( $p = 0.0025$ ) and their sleep amount during the night, measured in minutes, was decreased ( $p = 0.0202$ ), while siesta was not changed ( $p =$

$0.0723$ ) (Figure 1). Moreover, control flies had longer sleep during the night than during the day, while experimental ones had similar sleep level all over the day.



## Neurotransmission is affected by the lack of light during development

Sleep is regulated by several neurotransmitters released by specific neurons and recognized by receptors on target cells. Because we observed increased activity and decreased sleep level in the experimental flies, we expected to see changes in the expression of wake-promoting factors. The most important ones are dopamine, histamine and octopamine, as well as PDF and sNPF which affect the clock network. To investigate how light during development regulates these pathways we decided to check changes in the mRNA level of both, neurotransmitters and their receptors in heads. We compared mRNA level of selected genes in adult flies from three different groups: flies which were maintained during the development and as adults in DD (group DD/DD), flies developing in DD and transferred to LD12:12 as adults, which is similar to the experiment for sleep analysis (group DD/LD) and control flies developing and kept in LD12:12 after eclosion (group LD/LD).

First, we investigated effect of light during development on dopamine signaling. Our results showed that dopamine synthesis was decreased in flies developing in constant darkness (Figure 2A, LD/LD vs. DD/LD  $p = 0.00159$ , LD/LD vs. DD/DD  $p = 0.0286$ ), which suggests that light during development is necessary to maintain high level of dopamine signaling. This result was not expected, as dopamine is wake-promoting factor, and knowing that experimental flies are more active we predicted to observe higher dopamine synthesis. However, the opposite situation was observed for *Dop1R1*, which was expressed at higher level in the groups DD/LD and DD/DD (Figure 2B, LD/LD vs. DD/LD  $p = 0.1905$ , LD/LD vs. DD/DD  $p = 0.0159$ ). The level of *Dop1R2* mRNA was increased in the group DD/DD, but it was decreased after moving flies to LD12:12 (Figure 2C, LD/LD vs. DD/LD  $p > 0.9999$ , LD/LD vs. DD/DD  $p = 0.0159$ ). Finally, *DD2R* showed the pattern of expression similar to *Dop1R2*, but the observed changes were not statistically significant (Figure 2D, LD/LD vs. DD/LD  $p = 0.06857$ , LD/LD vs. DD/DD  $p = 0.0635$ ). The obtained results

suggest more complex, cell-type specific regulation of dopamine signaling.

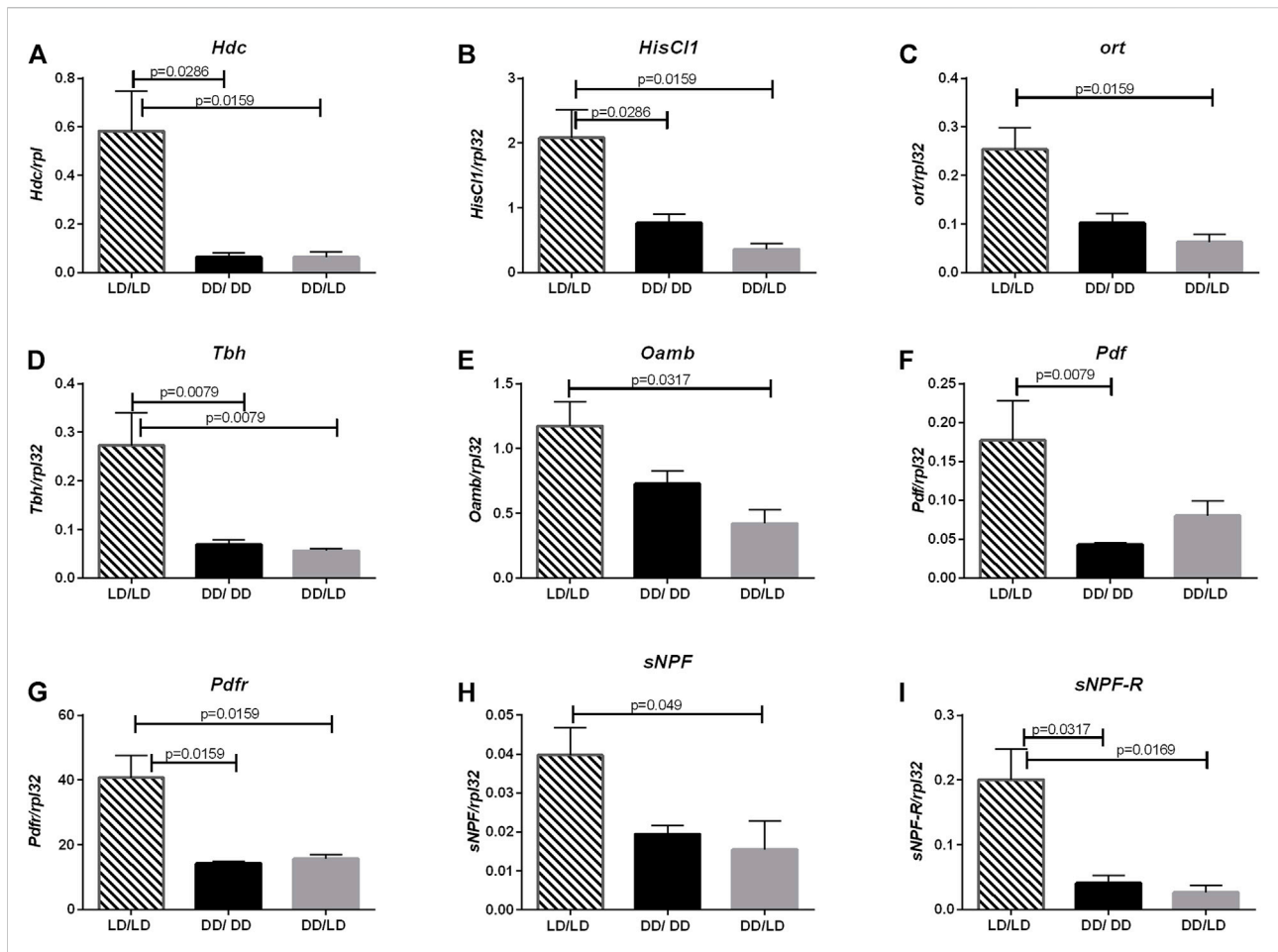
The another neurotransmitter involved in light transmission and sleep regulation is histamine. The expression pattern of *Hdc* (LD/LD vs. DD/LD  $p = 0.0079$ , LD/LD vs. DD/DD  $p = 0.0079$ ), *HisCl1* (LD/LD vs. DD/LD  $p = 0.0159$ , LD/LD vs. DD/DD  $p = 0.0286$ ) and *ort* (LD/LD vs. DD/LD  $p = 0.01159$ , LD/LD vs. DD/DD  $p = 0.0571$ ) in our experiment was similar, with decreased level in flies kept in DD during development (Figures 3A–C).

The next pathway affected by light conditions during development was octopamine. Light during the development decreased the expression level of both, *Tbh* (LD/LD vs. DD/LD  $p = 0.0079$ , LD/LD vs. DD/DD  $p = 0.0079$ ) and *Oamb* (LD/LD vs. DD/LD  $p = 0.0317$ , LD/LD vs. DD/DD  $p = 0.1143$ ), however, flies developing in DD and then transferred to LD had the lowest amount of transcript (Figures 3D,E).

Finally, we checked the expression level of two neurotransmitter genes known to be involved directly in the circadian control of sleep: sNPF and PDF and their receptors, sNPF-R and PDFR. All of them had the similar pattern—in flies developing in DD their expression level was decreased, and after transferring adults to LD, the level of mRNA did not change significantly (Figures 3F–I, for *Pdf* LD/LD vs. DD/LD  $p = 0.0952$ , LD/LD vs. DD/DD  $p = 0.0079$ , for *Pdfr* LD/LD vs. DD/LD  $p = 0.0159$ , LD/LD vs. DD/DD  $p = 0.0159$ , for *sNPF* LD/LD vs. DD/LD  $p = 0.049$ , LD/LD vs. DD/DD  $p = 0.0635$ , for *sNPF-R* LD/LD vs. DD/LD  $p = 0.0169$ , LD/LD vs. DD/DD  $p = 0.0317$ ).

## Photoreception affects synthesis of neurotransmitters

Flies receive light signal through different pathways. The most important is the retinal phototransduction pathway, which requires phospholipase C (PLC), encoded by the gene *norPA*. *NorPA* mutants are almost blind, however, they still have partial light sensitivity through deep brain photoreceptors. Because we did not observe significant changes in gene expression between



**FIGURE 3** Lack of light changes neurotransmitters signaling level. qPCR analysis of expression level of genes connected with histamine, octopamine, PDF and sNPF signaling for flies maintained in normal conditions LD12:12 (LD/LD), maintained in DD and developed in darkness (DD/DD) and after eclosion transferred to LD12:12 (DD/LD). Expression checked for: (A) *histidine decarboxylase (Hdc)*, (B) receptor *HisCl1*, (C) receptor *Ort*. (D) *Tbh* and (E) receptor *Oamb*, (F) *Pdf*, (G) *Pdfr*, (H) *sNPF* and (I) *sNPF-R*. *N* = 5. Mean ± SEM.

wild type flies kept in DD/DD and DD/LD conditions, and to avoid impact of light input received by additional photoreceptors, we perform this experiment for LD/LD and DD/DD groups only. Flies with *norpA* mutation developing in LD12:12 conditions, showed normal activity profile and they were able to maintain rhythmicity in DD. However, similarly to CS, *norpA* mutants, developing in DD, were more active than control in LD12:12 ( $p = 0.0003$ ), but activity index was not changed ( $p = 0.8724$ ). Their pattern of sleep was changed and the total amount of sleep was decreased during the day ( $p = 0.0001$ ) and night ( $p = 0.0039$ ) (Figure 4).

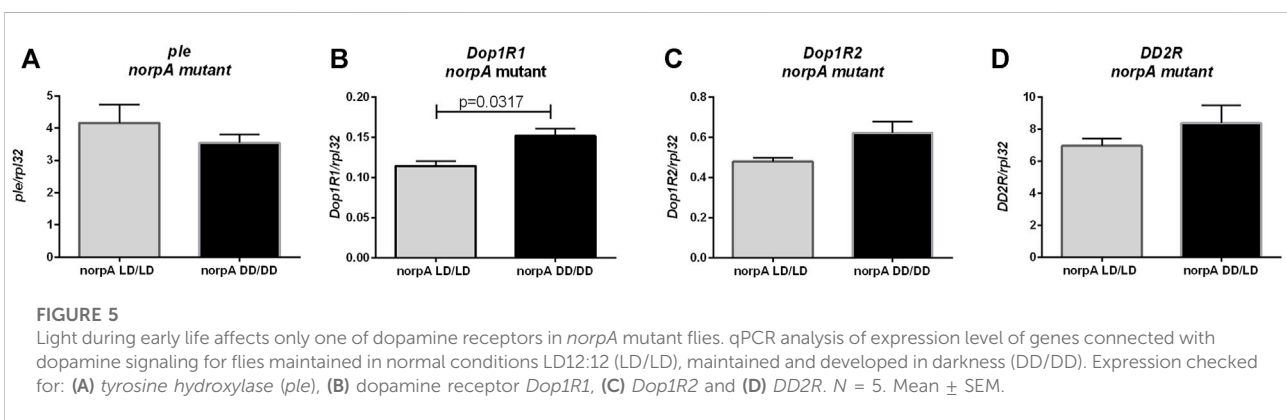
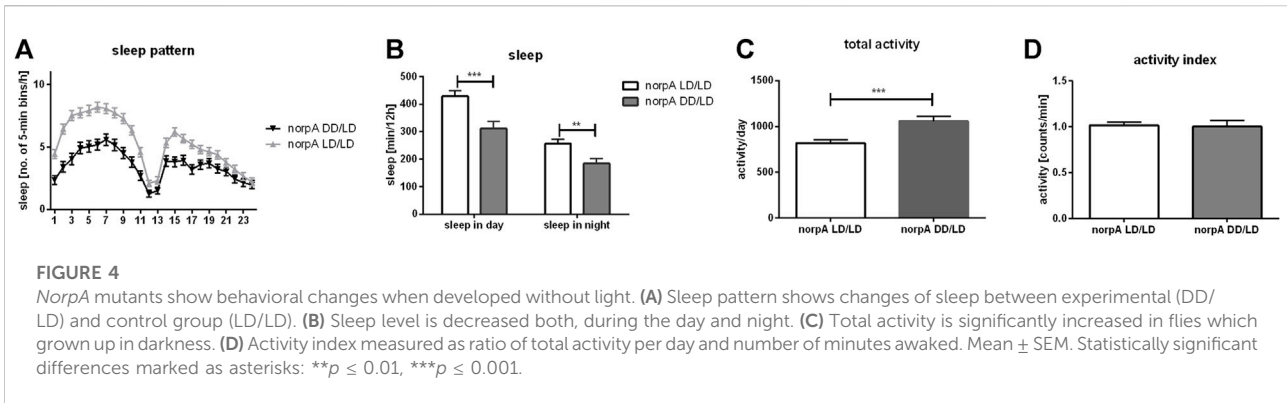
Having in mind that photoreception through the retina photoreceptors can affect neurotransmission level in the brain we checked gene expression of the previously selected genes in head homogenates from *norpA* mutants.

In dopamine pathway we found changes only in *Dop1R1* expression between *norpA* mutants developing in LD12:12 and

DD ( $p = 0.0317$ ), while tyrosine hydroxylase ( $p = 0.6905$ ), *Dop1R2* ( $p = 0.0952$ ) and *DD2R* ( $p = 0.3095$ ) encoding genes were expressed at the similar level in both groups (Figure 5). This result suggests that light during embryonic and postembryonic development affects *Dop1R2* and *ple* expression in PLC-dependent manner.

Histamine synthesis (*Hdc* gene expression) was decreased in *norpA* mutants growing without light (Figure 6A,  $p = 0.0079$ ), but mRNA levels of *HisCl1* (Figure 6B,  $p = 0.8413$ ) and *ort* genes of histamine receptors (Figure 6C,  $p = 0.4206$ ) were unchanged. However, other neurotransmission pathways checked previously (octopamine, PDF and sNPF) seem do not to be PLC-dependent, as *Tbh* (Figure 6D,  $p > 0.9999$ ), *Oamb* (Figure 6E,  $p = 0.2222$ ), *Pdf* (Figure 6F,  $p = 0.8413$ ), *Pdfr* (Figure 6G,  $p = 0.1508$ ), *sNPF* (Figure 6H,  $p = 0.0556$ ) and *sNPF-R* (Figure 6I,  $p > 0.9999$ ) mRNAs had similar level in LD/LD and DD/DD groups in *norpA* mutants.

To examine effect of light detected by deep brain photoreceptors, which can affect neurons directly, we used



*cry<sup>01</sup>* mutants. Surprisingly, we did observe neither differences in activity ( $p = 0.9137$ ), activity index ( $p = 0.5335$ ), sleep pattern nor its level (for day  $p = 0.874$ , for night  $p = 0.334$ ) between flies reared in LD and DD (Figure 7). This result indicates that CRY is the most important light photoreceptor during development.

Moreover, we did not observe changes in *ple* (Figure 8A,  $p = 0.4$ ), *Dop1R1* (Figure 8B,  $p > 0.9999$ ) nor *DD2R* expression (Figure 8D,  $p = 0.0571$ ), but *Dop1R2* mRNA level was increased in flies reared in DD, similarly to wild-type flies (Figure 8C,  $p = 0.0266$ ). Histamine (*Hdc*, Figure 9A,  $p = 0.309$ , *HisCl1*, Figure 9B,  $p = 0.4$ , *Ort*, Figure 9C,  $p = 0.2857$ ), octopamine (*Tbh*, Figure 9D,  $p = 0.8413$ , *Oamb*, Figure 9E,  $p = 0.1143$ ), PDF (*Pdf*, Figure 9F,  $p = 0.3095$ , *Pdfr*, Figure 9G,  $p = 0.5476$ ) and sNPF (*sNPF*, Figure 9H,  $p = 0.0571$ , *sNPF-R*, Figure 9I,  $p = 0.5476$ ) signaling components were also not changed.

### Light affects development of mushroom bodies and dopaminergic input to mushroom bodies

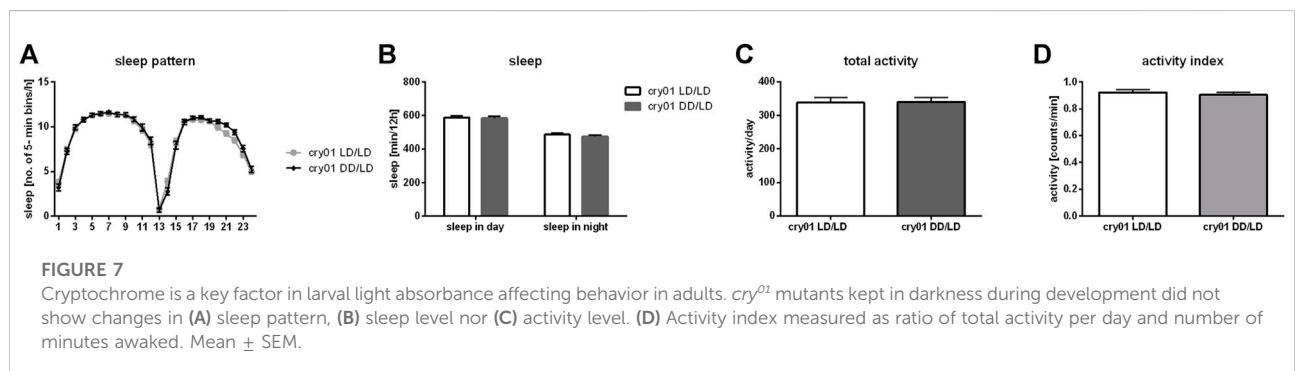
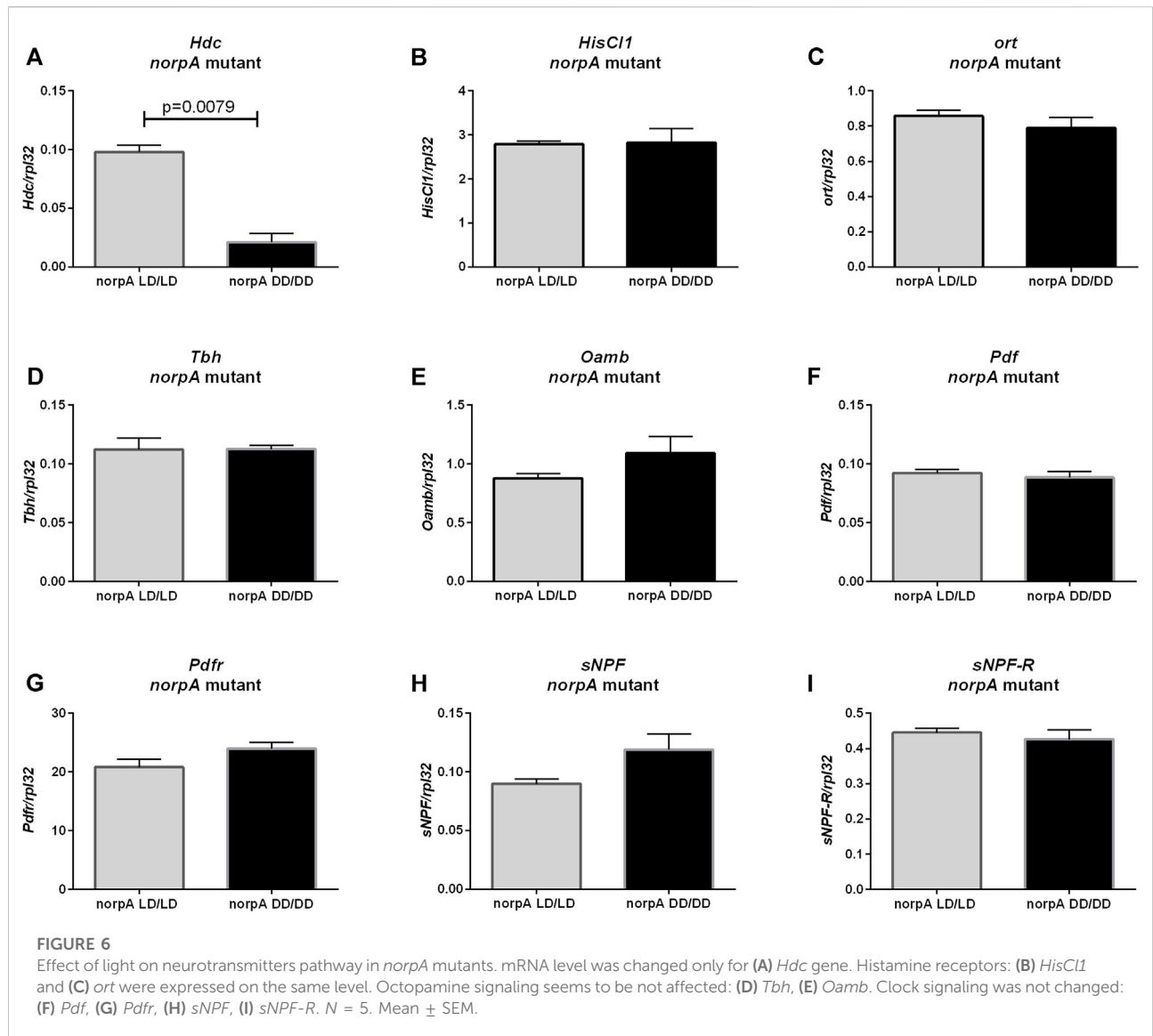
Sleep is controlled by many different cell types, but the most important ones are sleep centers located in the mushroom bodies

(MB). Because we observed the strong effect of light during development on sleep level in adult flies, we wondered whether it can be correlated with MB size changes. We used transgenic cross OK-107-Gal4 with UAS-mCD8::GFP to visualize MB. We reared flies in LD or DD, respectively and collected brains of 2-days old males. We measured brain size as well as mushroom bodies of every single individual and found that flies kept in DD had smaller MB ( $p < 0.0001$ ), while the brain size was not changed ( $p = 0.4483$ ) (Figures 10A–C).

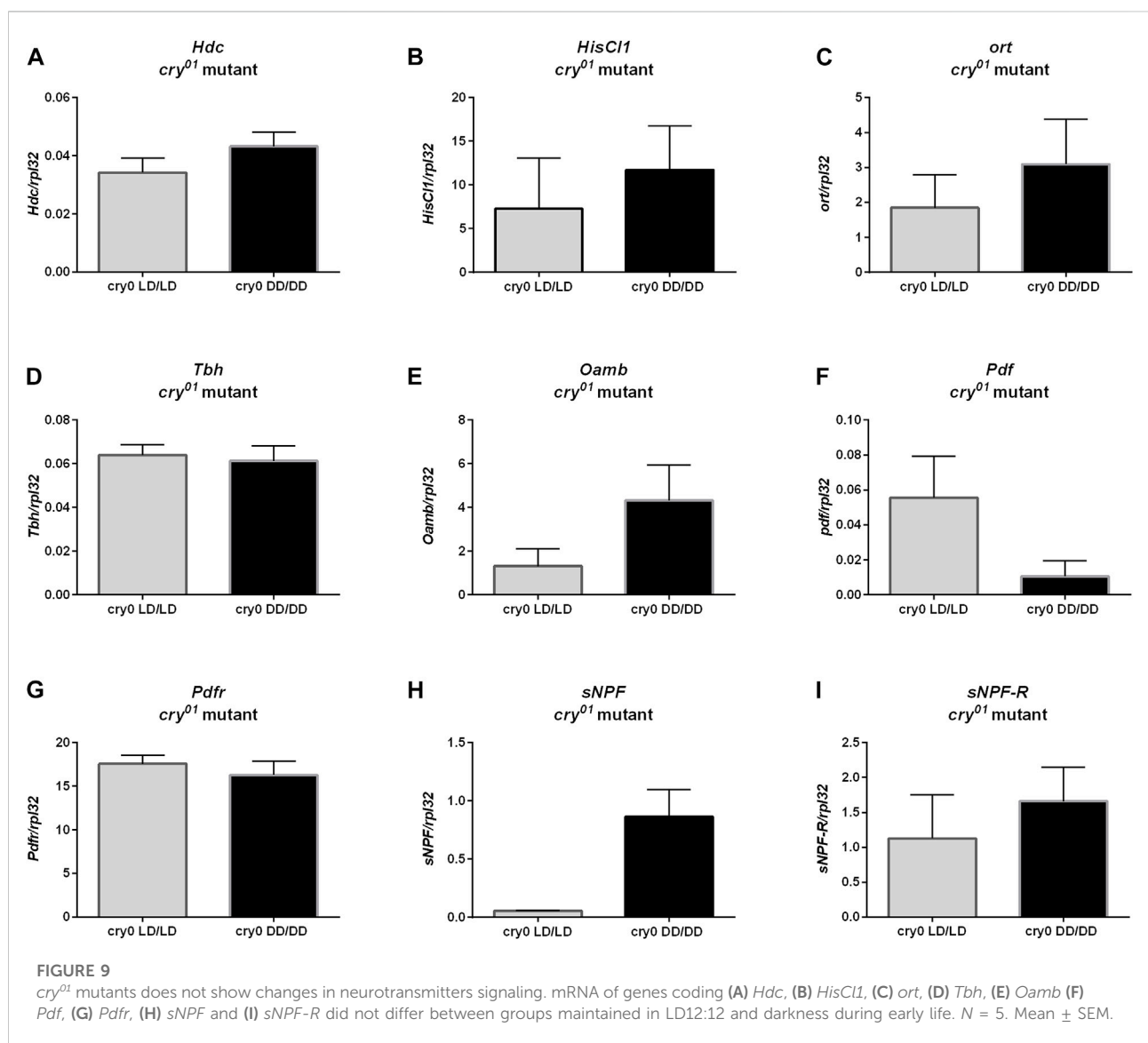
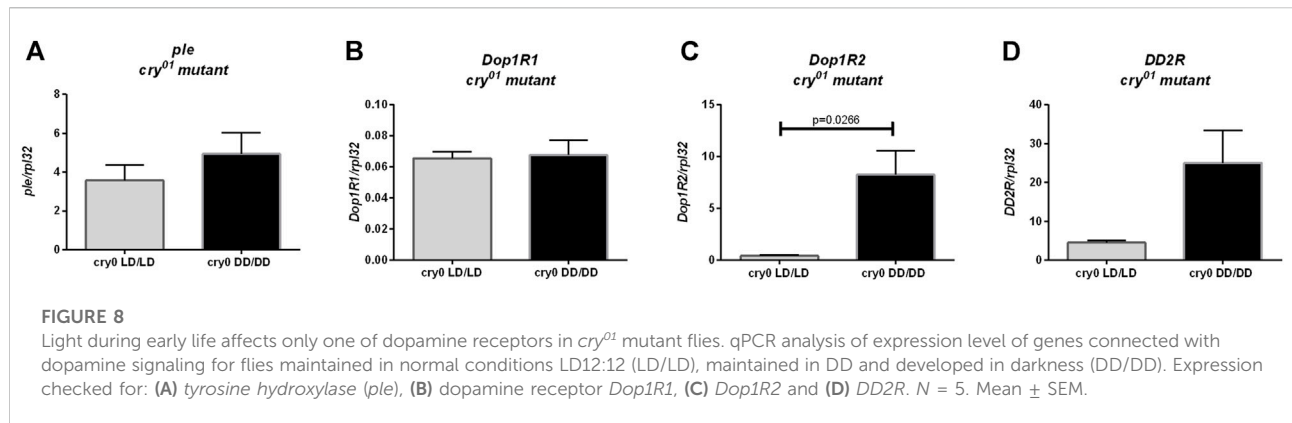
Additionally, we checked tyrosine hydroxylase immunofluorescence in specific cell clusters innervating MB. We found that flies kept in DD during development have lower expression of TH in PPL1, PPM3 and PAM neurons, however, transferring flies to the light:dark conditions restored normal level of the enzyme, but in the case of PPM3 the amount of TH was higher than in the control group (Figures 10D–G).

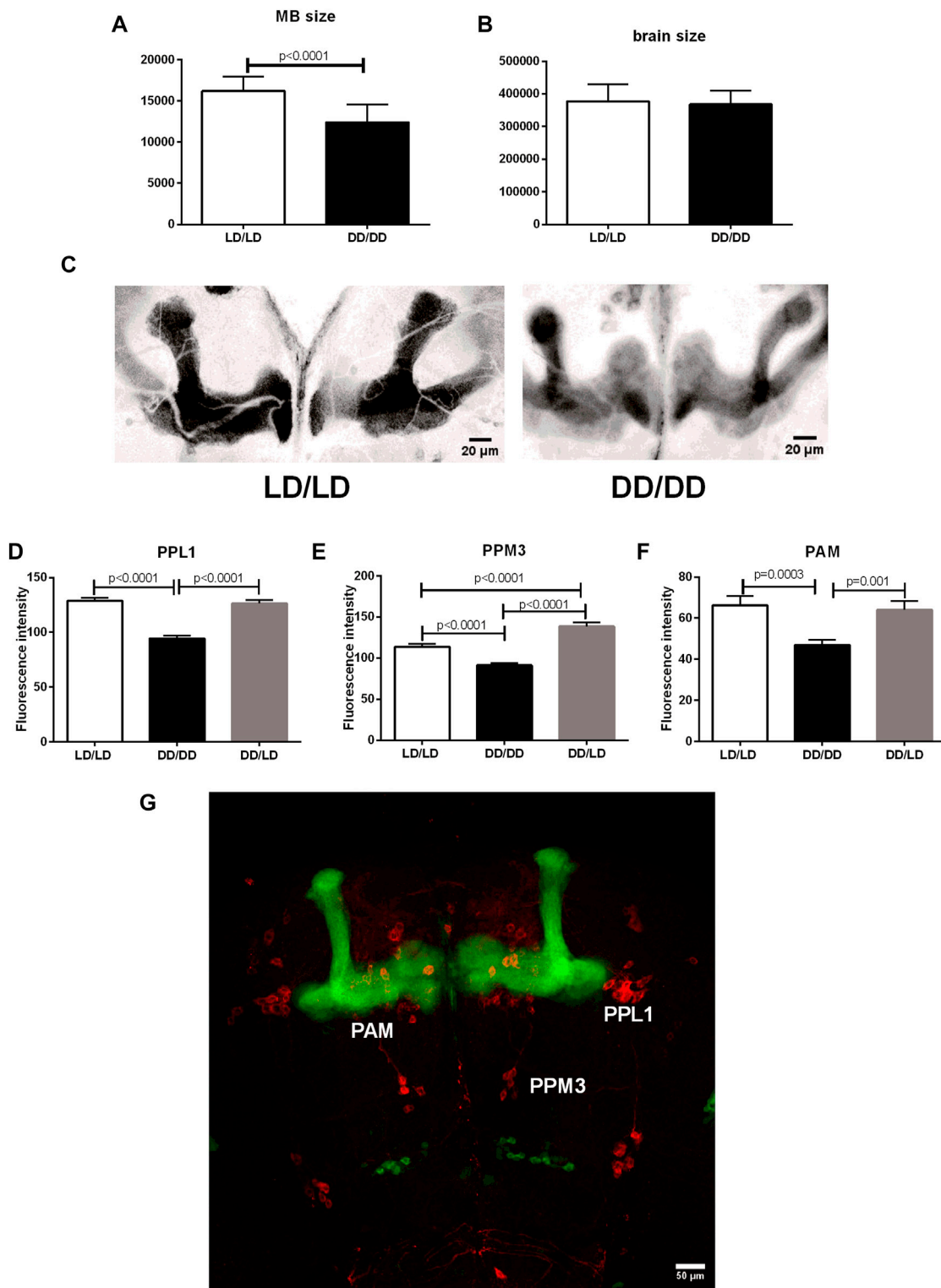
### Discussion

Sleep is one of most important functions of organism. During resting time many processes occur, like memory consolidation, wound healing, etc. Sleep is regulated by internal signals coming









**FIGURE 10**

Mushroom bodies development and its dopaminergic input depends on light during early life. **(A)** The size of MBs is significantly decreased in flies from DD/DD group, while **(B)** the whole brain size was not changed. **(C)** Representative confocal images of MB from flies developed in LD and DD conditions. Images showed here were modified: selected area was cropped, 8-bit image was color inverted and contrast was enhanced. **(D–G)**: Tyrosine hydroxylase immunofluorescence signal measured in specific dopaminergic cell clusters innervating sleep centers showed decreased level in DD/DD group. DD/LD group showed TH level similar to the control. Total number of brains used for experiments: 30 per group. Mean ± SEM. **(G)**: confocal image of the brain - OK107 > GFP strain has visible mushroom bodies (green), anti-TH immunostaining (red) marks dopaminergic clusters.

from the circadian clock and sleep centers, but it is also responsive to external signals, mostly light inputs. Sleep regulation is very complex and requires multicellular communication and homeostasis. Light is a well-known arousal factor, however, mid-day siesta is promoted during the most intense light exposure.

Mechanisms affecting sleep have already been investigated carefully focusing on different aspects, however, little is known about the role of light during development in the regulation of sleep. Larvae have the circadian clock, which can be synchronized by environmental light. Adults eclosing from pupae, which during postembryonic development were reared in constant light or constant darkness, can be easily synchronized by light and they are able to keep normal period of locomotor activity in DD (Sheeba et al., 2002; Zhao et al., 2019). Moreover, single 12 h light pulse in the first instar larva is sufficient to keep the daily rhythmicity in adult life. It has been shown that this entrainment can be observed in an individual fly, however, the whole population is not synchronized, meaning that each fly has different phase of the rhythm. This indicates that the endogenous clock starts to tick independently of light and goes continuously until adulthood (Sehgal et al., 1992; Zhao et al., 2019). The larval time-memory, results from cyclic *per* and *tim* expression in the pacemaker in the brain (Kaneko et al., 1997). However, in peripheral oscillators PER is expressed during embryogenesis and postembryonic development, and its level increases during development, but there are no daily cycling of PER before adulthood (Zhao et al., 2019). A light pulse given to larvae causes phase shift of the rhythm in adults and there are differences in the magnitude and direction of shifts between wild type and *per<sup>r</sup>* mutants (Kaneko et al., 2000). The mechanism of this process depends on changes in the larval molecular clock induced by light received by both photoreceptive pathways: CRY- and rhodopsin-dependent (Kaneko et al., 2000). Having in mind these larval time-memory aspects, it was not surprising that flies used in our experiments showed normal pattern of activity in adult life and they were able to keep rhythmicity in constant darkness with the period length similar to control. However, total level of activity was increased in insects reared in DD, and their activity index was higher, which means that they were more active during waking time. They also slept less, especially during the night when compared to flies developing in normal LD12:12 conditions. Interestingly, sleep was decreased during first 8 h of the day and during the night starting 3 h after lights-off, while during the rest of the time, the number of sleep bins was similar to the control group. Flies were already synchronized to LD12:12 regime, as morning and evening peaks of activity, amplitude of the rhythm and time of sleep were the same in both groups. Similar changes, higher activity and decreased sleep time, were observed in *norPA* mutants, however, activity index was not changed and sleep was affected in both during the day and night. This indicates that light perception through photoreceptors is only partially responsible for the observed changes in this behavior. On the other hand, flies lacking functional CRY did not show any changes in activity and

sleep profile and level, which indicates that CRY is a key factor affecting observed changes.

The obtained results suggest that our experimental flies had the normally working circadian clock, but some pathways regulating wakefulness were enhanced. Our first goal was to check dopamine, as it is well-known wake-promoting factor (Andretic et al., 2005). Surprisingly, wild-type flies reared in DD showed reduced expression level of *ple*, encoding tyrosine hydroxylase (TH)—key enzyme in dopamine synthesis, and in effect also dopamine level in the brain most probably was decreased. Since the inhibition of dopamine-releasing neurons causes hyperactivity in response to mechanical startle (Friggi-Grelin et al., 2003), it is possible that also our experimental flies having lower dopamine level could be more active after light changes, as we observed most severe changes in behavior in hours after lights-on and lights-off. Moreover, our flies were probably stressed by transferring them from DD to LD12:12, so they could be also more sensitive to light, and all of this can reduce dopamine level (Neckameyer and Weinstein, 2005). On the other hand, the group DD/DD had lower *ple* expression level, even without any light input. To investigate this problem in details, we focused on dopaminergic neurons innervating the central complex (PPM3) and mushroom bodies (PPL1 and PAM), the structures hosting a sleep center (Mao and Davis, 2009). Immunofluorescence intensity measurements showed that the lack of light during development decreased TH level in these clusters, however transferring flies to LD rescued this effect. It means that light can directly enhance dopamine synthesis in specific cells, which can affect sleep control. Dysregulation of the balance in dopamine production between different clusters may enhance stress and in effect promote wakefulness.

Although our experimental flies showed decreased dopamine synthesis, at the same time they had increased *Dop1R1* and *Dop1R2* expression. This support our findings that dopamine signaling is changed in specific target neurons. *DopR* mutants showed increased sleep level (Lebestky et al., 2009), which supports our data, that shorter sleep time is correlated with increased *DopR* expression. However, *DopR* regulates Repetitive Startle-induced Hyperactivity (ReSH) and sleep in the opposite way, acting on specific cells (Lebestky et al., 2009). Wake-promoting effect of dopamine, but not ReSH, occurs mostly in clock neurons (Parisky et al., 2008; Shang et al., 2008; Lebestky et al., 2009). The main pacemaker cells, l-LNvs, are wake-promoting neurons during the day (Parisky et al., 2008; Shang et al., 2008), however, they do not play this role in constant darkness (Shang et al., 2008), as their firing rate is enhanced by the acute light exposure (Sheeba et al., 2008a; 2008b). This group of cells is sensitive to dopamine, which increases cAMP level and causes depolarization of cell membrane, but it does not affect firing rates of the l-LNvs (Shang et al., 2011; Fernandez-Chiappe et al., 2020). Moreover, decreasing of *Dop1R2* expression in s-LNvs decreases nighttime sleep (Fernandez-Chiappe et al., 2020). Dopamine signaling to l-LNvs is suppressed by light, and in effect, sleep during the night is more sensitive to

dopamine than during the day, and this effect is even stronger in constant darkness (Shang et al., 2011). Our data suggest that *Dop1R2* expression can be inhibited by light directly, as flies developing and maintained continuously in DD showed higher level of *Dop1R2* mRNA than flies reared in DD and transferred to LD (DD/LD group) or the control flies developing in LD12:12 (LD/LD group). On the other hand, *ple* and *Dop1R1* expression seems to be continuously inhibited by the lack of light during development, while *DD2R* expression level is not affected by light conditions. The results obtained for *cry<sup>01</sup>* and *norpA* mutants are very interesting, since they show that gene expression of the dopamine pathway in the group DD/LD strictly depends on photoreception—*Dop1R1* is CRY-dependent, while *Dop1R2* is PLC-dependent, and *ple* is both CRY and PLC dependent. The fact, that dopamine acts through Cryptochrome, and both of them are required for nocturnal increased activity was shown also by other authors (Kumar et al., 2012). However, only *Dop1R1* seems to be related to the disruption of night-time sleep in flies from DD/DD group, as its enhanced expression follows behavioral changes in wild-type and *norpA* mutants, while *cry<sup>01</sup>* kept in the same light regimes does not change behavior nor *Dop1R1* level.

In case of histamine, the main neurotransmitter used by retina photoreceptors, it has been shown that it is involved in the regulation of sleep/wake cycles through *HisCl1* located on clock neurons (Oh et al., 2013; Alejevski et al., 2019). Flies maintained in DD during development had lower expression of *Hdc* gene, which encodes a key enzyme in histamine synthesis. Moreover, gene expression of both known receptors *HisCl1* and *Ort* was decreased. Histamine has been identified as a wake-promoting agent however our experimental flies showed decreased histamine and increased both activity time and activity during waking. Light effect on histamine synthesis seems to be precisely regulated by Cryptochrome, but not by PLC, while expression of *HisCl1* and *ort* receptor genes are regulated by both PLC and CRY.

Another neurotransmission pathway involved in sleep and affected by darkness during the postembryonic development was octopamine. Here, again, in flies reared in DD we observed lower expression of *Tbh* (a key enzyme involved in octopamine synthesis) and octopamine receptor gene *Oamb*, which indicates that this signaling pathway was decreased in adults. It was previously shown that octopamine regulates activity/sleep cycle in larvae, since the activation of *Tdc*-expressing neurons decreases sleep time, while *Tbh* and *Oamb* mutations or silencing increases sleep time (Szuperak et al., 2018). Octopamine is also a wake-promoting agent in adult flies, which forces l-LN<sub>v</sub> cells to release PDF during the day (Oh et al., 2014). However, it has been shown that sleep regulation using this neurotransmitter does not involve mushroom bodies, although they express octopamine receptors (Crocker and Sehgal, 2008). Octopamine signaling inhibition in DD applied during development, seems to depend on both CRY and PLC, because it was not changed in

*cry<sup>01</sup>* and *norpA* mutants. However, our results suggest that the observed changes in mRNA levels of *cry<sup>01</sup>* and *norpA* do not drive sleep inhibition during the night.

Neurotransmitters involved in the regulation of the clock mechanism are very interesting in the context of sleep and light. The main clock neurotransmitter is PDF and its function in sleep regulation is very complex. It inhibits dopaminergic arousal neurons PPM3 or activates DN1p, which in turn inhibits tubular bulbar cells (Tubu). PDF released from l-LN<sub>v</sub>s is also responsible for nocturnal activity (Sheeba et al., 2008a) and light-dependent arousal (Shang et al., 2008). It is possible that the lack of light during development causes defects in the l-LN<sub>v</sub> excitability, which in turn affects cell physiology, neurotransmitter production, and in effect sleep time. This is supported by the fact, that light-induced firing-rate of l-LN<sub>v</sub>s depends on CRY, and is not observed in *cry* mutants (Emery et al., 2000; Sheeba et al., 2008b; Fogle et al., 2011). Decreased *Pdf* and *Pdfr* expression observed in our experimental flies may cause weaker inhibition of PPM3 and, in effect, higher total activity.

The second neurotransmitter present in the circadian clock neurons, called sNPF is broadly expressed in the brain, including sleep centers (Nässel et al., 2008; Johard et al., 2009) and its role in the sleep regulation is well documented (Shang et al., 2013). sNPF expressed in s-LN<sub>v</sub>s shows circadian changes in abundance and promotes normal night-time sleep (Kula-Eversole et al., 2010; Shang et al., 2013), which matches with our results. We observed that expression of PDF and sNPF, as well as their receptors is regulated by light during development in PLC and CRY-dependent manner. However, these changes do not affect behavior.

Comparing wild-type flies with mutants used in the study we can conclude that the exposure to DD during development affects sleep during the night, reduces its time and increases activity time, with lower locomotor activity during wake. This indicates that experimental flies spent more time being active, but their activity level was similar to that in the control. Observed changes are regulated mostly by increasing *DopR1* expression. Changes in the expression of other gene studied seems do not to be connected with the observed behavioral phenotype because we did not observe them in *norpA* mutants which show nocturnal sleep deficits similar to wild-type flies. However, *norpA* mutants showed increased total activity time with no changes of activity index, which indicates that they were more and longer active than blind flies reared in LD.

The last part of our work showed that lack of light during the development affects the size of mushroom bodies. Flies kept in DD had similar body size, their brain size was also not changed, but the area of MB was significantly decreased. It was previously shown that larvae in DD have reduced number of ganglion mother cells (GMCs) derived from mushroom bodies neuroblasts, similarly to *per<sup>01</sup>* and *cry<sup>01</sup>* mutants kept in LD12:12 (Dapergola et al., 2021). In addition, NBs size was smaller than in control, and their transcriptional activity was decreased. In effect, the size of larval brain was decreased too. Surprisingly, the size of adult brain was normal (Dapergola et al., 2021), which was

also confirmed in our study. As it was shown that development of mushroom bodies is affected by light conditions, we decided to check what happens with this sleep-controlling structure in the adult brain. Our results showed that the MB development was constantly affected, as in DD they did not grow to the normal size. It supports an idea that light is necessary for proliferation of specific cell types in the brain. However, since we did not measure Kenyon cells number, it is possible that size changes are the consequence of decreased number of Kc cells or their processes and arborizations, which build MB lobes. Because the total size of the brain was not changed, other brain areas should be enlarged, however we did not have methods to check this interesting issue. The question which part of the brain or which cell types can compensate this difference in size reminds open for further investigations. What is interesting, sleep deprivation also affects NB number (Szuperak et al., 2018). It has been shown that 3 h of mechanical sleep deprivation in the second instar larvae inhibits neuroblasts proliferation in about 15%, and this effect can be recovered after sleep rebound (Szuperak et al., 2018). One of the explanations of the smaller size of MB in flies developing in DD might be that, lack of light decreases their sleep time during larval stage, which in turn affects NBs proliferation rate. This indicates how important is light, clock and sleep in early stages of brain development.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

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## Author contributions

MD designed the study, performed the experiments, analyzed data and wrote the manuscript. AT performed the experiments, EP contributed to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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