Environmental light is required for maintenance of long-term memory in *Drosophila*

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Abstract

Long-term memory (LTM) is stored as functional modifications of relevant neural circuits in the brain. However, it remains poorly understood how the consolidated memory is maintained for a long period. Here, we show that in *Drosophila*, universally available environmental light plays a critical role in LTM maintenance by reactivating the memory modulator cAMP response element-binding protein (CREB) via neuropeptide signaling. LTM is impaired when flies are kept in constant darkness during the memory maintenance phase. Because light activates the brain neurons expressing the neuropeptide Pigment-dispersing factor (Pdf) through the brain photoreceptors, we examined the possible involvement of Pdf in LTM maintenance and found that temporal knockdown of Pdf during the memory maintenance phase impairs LTM in light–dark cycles. Furthermore, we demonstrated that the Pdf receptor (Pdfr) and CREB are required in the memory center for LTM maintenance and Pdfr regulates light-dependent transcription via CREB.

Introduction

A newly formed memory is initially labile, but under certain circumstances, it is consolidated into a more stable long-term memory (LTM). Previous studies using various animal species have shown that activation of specific transcription factors, such as the cAMP response element-binding protein (CREB), and the corresponding *de novo* protein synthesis are essential for memory consolidation (Alberini, 2009; Kandel, 2012; Lee et al., 2008; Yin and Tully, 1996). Once consolidated, LTM requires continual maintenance for its long-term storage and subsequent recall, because memory traces gradually decay owing to molecular turnover and cellular reorganization. Similarly to memory consolidation, transcriptional activation and *de novo* protein synthesis are required for LTM maintenance (Bekinschtein et al., 2007; Fioriti et al., 2015; Hirano et al., 2016; Majumdar et al., 2012). For example, the maintenance of hippocampal long-term potentiation (LTP) and spatial memory in mice is dependent on the prion-like translational regulator CPEB3 (Fioriti et al., 2015). Similarly, LTM maintenance in *Drosophila* also requires Orb2, a *Drosophila* homolog of CPEB (Majumdar et al., 2012). Furthermore, transcriptional regulations through CREB and its coactivator CRTC play crucial roles in LTM maintenance in the *Drosophila* memory center, namely, the mushroom bodies (MBs) (Hirano et al., 2016), suggesting that memory consolidation and maintenance share some of the same molecular mechanisms (Bekinschtein et al., 2007). An obvious and important question is how transcriptional activation and the following protein synthesis are triggered during the memory maintenance phase. Unlike transcriptional and translation activation involved in memory consolidation, those associated with LTM maintenance cannot be directly controlled by the stimuli that induce memory formation, because there is a significant time separation between stimulus-induced memory formation and the memory maintenance process, and LTM should be continually maintained. However, the molecular and cellular underpinnings of active LTM maintenance that is transcription- and translation-dependent still remain elusive.

Earth's rotation generates the daily cycle of day and night, and the rhythmic light–dark (LD) cycles have a significant impact on animal behavior and physiology. In animals, light is not only essential for acquiring information for image-forming vision in nature but also acts as a powerful modulator of brain functions such as circadian entrainment, hormone secretion, sleep-wake cycles, mood, and cognitive functions (Altimus et al., 2008; Crocker and Sehgal, 2010; Fernandez et al., 2018; LeGates et al., 2012; Vandewalle et al., 2009). Using the diurnal fruitfly *Drosophila melanogaster*, here, we found that LTM was severely impaired in flies kept in constant darkness (DD) after memory consolidation. Thus, LTM maintenance is light-dependent. In *Drosophila*, light activates photoreceptors in the brain neurons

expressing the Pigment-dispersing factor (Pdf), a neuropeptide, and increases their spontaneous firing rate (Fogle et al., 2011; Ni et al., 2017; Sheeba et al., 2008). We found that the brain photoreceptors in Pdf-expressing neurons (hereafter, Pdf neurons) and the Pdf receptor (Pdfr) in the *Drosophila* memory center are essential for light-driven LTM maintenance. We further showed that light activates CREB-dependent transcription in MBs and that Pdfr in MBs regulates light-dependent CREB transcription. Overall, our results demonstrate for the first time that environmental light, which is available daily to all animals under normal conditions, plays a critical role in LTM maintenance by reactivating the evolutionarily conserved memory modulator CREB via neuropeptide signaling.

Results

Light is essential for LTM maintenance

To investigate whether lighting conditions affect LTM in *Drosophila*, the courtship conditioning assay was carried out (Griffith and Ejima, 2009; Keleman et al., 2012; Sakai et al., 2004; Siegel and Hall, 1979). In this assay, males receive stressors from nonreceptive mated females (e.g., sexual rejection) to block successful mating (conditioning) (Lee et al., 2017), and memory is subsequently observed as experience-dependent courtship suppression toward virgin females. One hour conditioning generates short-term memory (STM), which persists for at least 8 h, whereas 7 h conditioning induces LTM, which persists for at least 5 days (d) (Ishimoto et al., 2009; Sakai et al., 2012; Sakai et al., 2004). The courtship activity of naïve and conditioned males was quantified using a courtship index (CI); subsequently, a memory index (MI) was calculated to quantify courtship memory (see Materials and methods). When males were conditioned for 7 h in light or darkness and the conditioned males were subsequently kept under LD cycle**s** until the test, they showed lower courtship activity on d 5 (five days after conditioning) than naïve males, and there was no significant difference in MI between these flies [Figure 1A; (1) and (2)], indicating that conditioning in darkness has no adverse effects on LTM. However, when flies were conditioned in light and then kept in DD after the conditioning and before the test, LTM was severely attenuated [Figure 1A; (3)]. DD for two consecutive days after conditioning was sufficient to impair LTM [Figure 1A; (6)], but not DD for only one day [Figure 1A; (4) and (5)], indicating that flies cannot maintain their LTM when DD lasts more than two days. In constant light (LL), the *Drosophila* circadian clock does not work normally, and flies show arrhythmic locomotor activity (Qiu and Hardin, 1996). When flies were kept in LL after conditioning, their LTM was intact [Figure 1A; (7)], as previously reported (Sakai et al., 2004). Furthermore, the LTM of several clock mutants except for *period* (*per*)

mutants is intact (Sakai et al., 2004). Thus, light input, but not the circadian clock, is necessary for LTM maintenance.

Sleep plays an important role in the consolidation of *Drosophila* courtship memory (Donlea et al., 2011). However, it remains unclear whether an abnormal sleep phenotype is attributable to disturbed LTM maintenance of flies kept in DD. To examine whether light conditions affect *Drosophila* sleep (see Materials and methods), sleep amount was measured in flies kept in LD and DD (Figure 2A). The mean amount of daytime sleep of flies kept in DD was lower than that of flies kept in LD (Figures 2A–C). However, when flies in LD were slightly deprived of sleep to adjust the amount of daytime sleep to the level of that in DD (Figures 2D–F), their LTM maintenance was not attenuated (Figure 2G). Thus, LTM impairment induced by DD does not simply result from the reduced amount of sleep.

Expression of brain photoreceptors in Pdf neurons is necessary for LTM maintenance in LD

Because light directly activates Pdf neurons through circadian photoreceptors [Rhodopsin 7 (Rh7) and Cryptochrome (Cry)] (Fogle et al., 2011; Ni et al., 2017; Sheeba et al., 2008), Pdf neurons may regulate light-driven LTM maintenance. To examine whether Rh7 and Cry are required for LTM, *Rh7* and *cry* null mutant flies ($Rh7^{1}$ and cry^{01}, respectively) were used. Since memory on d 1 (one day after conditioning) was intact in $Rh7^{1}$ and cry^{01} flies after 7 h conditioning (Figure 1B), *Rh7* and *cry* are dispensable for memory consolidation. Unlike the memory on d 1, $Rh7^{1}$ and cry^{01} flies showed memory impairment on d 5 (Figure 1C), suggesting that Rh7 and Cry are required for keeping LTM more than one day. We next sought to determine the temporal requirement of brain photoreceptors for LTM maintenance. For this purpose, we performed temporal knockdown of *Rh7* or *cry* in Pdf neurons using the TARGET system (McGuire et al., 2003) and RNA interference (RNAi) technology. The effectiveness of *Rh7* and *cry* RNAi was confirmed by qRT-PCR (Figures 3A and B). To knockdown *Rh7* during memory consolidation, maintenance or test phase, the temperature was raised to 30 °C for 24 h during three experimental periods (Figures 1D–G): starting at 24 h before the end of conditioning, 48–72 h after conditioning (memory maintenance phase), and 24 h before the test initiation. When flies were kept for 24 h before the test initiation at the restrictive temperature (RT), *Rh7* should remain suppressed during the 10 min test. In *Rh7* knockdown flies, LTM was impaired only when the temperature was shifted during the memory maintenance phase (Figure 1F). Temporal induction of *cry* RNAi in Pdf neurons did not affect LTM (Figures 1D–G). This is probably because temporal *cry* knockdown was insufficient to impaire LTM

since nonconditional knockdown of *cry* in Pdf neurons (*Pdf*-GAL4 / UAS-*cry* RNAi) resulted in severe memory impairment on d 5, as was observed in cry^{01} flies (Figure 1H).

In *Rh7* knockdown flies, the 48–72 h RT exposure resulted in impaired LTM, indicating that *Rh7* expression is required during the maintenance phase. However, the 24 h RT exposure immediately before the test did not affect LTM. When flies were exposed to RT for 48–72 h, the targeted genes should remain knocked down even after flies are returned to the permissive temperature (PT). As a result, functions of the target genes should be suppressed for over 24 h during the maintenance phase. In contrast, when flies were exposed to RT for 24 h immediately before the test, the genes should be suppressed during the maintenance phase for 24 h or less. As shown in Figure 1A, the length of time in DD is the critical factor for LTM defect: 1-d DD had no effect whereas 2-d DD led to LTM impairment. Thus, it is most likely that only long-term (>24 h) suppression of the light-Pdf pathway results in impaired LTM. These results support the conclusion that brain photoreceptors are necessary for LTM maintenance and Pdf neurons regulate light-driven LTM maintenance.

Pdf expression is critical for LTM maintenance in LD

To demonstrate that the Pdf signaling pathway regulates light-driven LTM maintenance, we performed temporal knockdown of *Pdf* in Pdf neurons (Figures 4A–D). The effectiveness of *Pdf* RNAi was confirmed by qRT-PCR (Figure 3C) and immunostaining using an anti-Pdf antibody (Figures 3F and G). LTM was impaired only when *Pdf* was knocked down during the memory maintenance phase (Figure 4C), indicating that *Pdf* expression is required for LTM maintenance. Since memory on d 1 was intact in Pdf^{01} null mutant flies after 7 h conditioning (Figure 4E), Pdf is dispensable for memory consolidation. Unlike the memory on d 1, Pdf^{01} mutant flies showed memory impairment on d 5, which was rescued by *Pdf* expression (Figure 4F), indicating that Pdf plays a critical role in LTM maintenance.

We next used two additional GAL4 drivers, c929 and *R18F07* to further investigate neuronal cell types involved in Pdf-mediated LTM maintenance. Pdf neurons form two clusters, small lateral ventral neurons (s-LNvs) and large lateral ventral neurons (l-LNvs) (Dubowy and Sehgal, 2017). c929 drives the expression of GAL4 in peptidergic neurons including l-LNvs but not s-LNvs (Shimada et al., 2016; Taghert et al., 2001), and *R18F07* drives GAL4 expression in all s-LNvs and only weakly in one of the l-LNvs (Figure 4G). *Pdf* knockdown in c929-positive neurons impaired LTM, but not that in *R18F07*-positive neurons (Figure 4H). Taken together, it is most likely that Pdf expression in l-LNvs is essential for LTM maintenance.

Activity of Pdf neurons regulates light-dependent LTM maintenance

We next examined whether the temporal activation of Pdf neurons induced by the temperature-sensitive cation channel TrpA1 can compensate for the DD-dependent LTM impairment (Figures 5A–D). When Pdf neurons were activated in *Pdf*-GAL4/UAS-*TrpA1* flies within each subjective day or night in DD for 2 d after conditioning, LTM was maintained for 5 d in *Pdf*-GAL4/UAS-*TrpA1* flies (Figures 5C and D). Under the same temperature-shift conditions, GAL4 and UAS control flies still showed LTM impairment (Figures 5C and D). Thus, this finding indicates that the activation of Pdf neurons during either a subjective day or night is sufficient to rescue the LTM impairment.

Consistently, the electrical silencing of Pdf neurons during the memory maintenance phase attenuated LTM in LD (Figures 5E and F). Furthermore, l-LNv-specific electrical silencing (see Materials and methods) also impaired LTM (Figures 5G and H), indicating that Pdf in l-LNvs plays a predominant role in LTM maintenance. Taken together, these results support the conclusion that the light-inducible activation of l-LNvs is essential for LTM maintenance.

Pdfr in MB α/β neurons regulates LTM maintenance

Because Pdfr activation increases cAMP levels (Hyun et al., 2005; Mertens et al., 2005) and MBs are the critical brain structures for learning and memory (Davis, 2011; Dubnau and Chiang, 2013), it is possible that Pdf activates Pdfr in MBs and drives LTM maintenance. However, previous studies using *Pdfr*-GAL4 lines, in which GAL4 is fused to the upstream regulatory sequences of *Pdfr*, or an anti-Pdfr antibody did not indicate Pdfr expression in MBs (Im and Taghert, 2010; Mertens et al., 2005). In contrast, a recent study using RNA sequencing revealed that *Pdfr* is expressed in MBs (Crocker et al., 2016). In this study, we generated a GAL4 knock-in fly strain, *Pdfr::2A::GAL4*, in which a transgene containing the 2A peptide sequence and GAL4 was inserted immediately upstream of the stop codon of *Pdfr* (Figures 6A and B). In *Pdfr::2A::GAL4* flies, GAL4 was expected to be expressed in the cells endogenously expressing *Pdfr*. mCherry::NLS signals driven by *Pdfr::2A::GAL4* were detected in Per-expressing clock neurons (Figure 6C). Furthermore, reporter gene expression driven by *Pdfr::2A::GAL4* partially colocalized with that driven by MB-LexA (Figure 7A). Several Kenyon cells with the colocalization of *Pdfr::2A::GAL4* and MB-LexA were identified [5 ± 0.3 (mean ± SEM) cells in one hemisphere, $N = 6$]. In the MB-LexA line, LexA is specifically expressed in all lobes (Figure 7A). However, considering that LexA-positive neurons in this line are only a subset of all MB neurons, the

number of Kenyon cells with the colocalization of *Pdfr::2A::GAL4* and MB-LexA should be only a part of the all Pdfr-positive MB neurons. In contrast to the *Pdfr::2A::GAL4* line, when the previously generated *Pdfr*-GAL4 line was used, we did not detect the colocalization of *Pdfr*-GAL4 and MB-LexA in the Kenyon cells (Figure 7B). To examine whether Pdfr is expressed in the MB α/β core region, we used another LexA line (*R58F02*-LexA) with LexA expression in the MB α/β core region (Figure 7C). When this line was used, the Kenyon cells with the colocalization of *Pdfr::2A::GAL4* and *R58F02*-LexA were also detected (4 ± 1.0 cells in one hemisphere; $N = 5$). However, no colocalization of GAL4 and LexA was detected ($N = 5$) when the *R19B03*-LexA line with weak LexA expression in the MB α/β core region was used (Figure 7D). These findings indicate that Pdfr is indeed expressed in MB α/β core region.

To examine whether the Pdfr is required for LTM, we performed knockdown of *Pdfr* in Pdfr-expressing neurons. The effectiveness of *Pdfr* RNAi was confirmed by qRT-PCR (Figure 3D, E). LTM was attenuated when *Pdfr* was knocked down in *Pdfr::2A::GAL4*-positive neurons (Figure 8A). Furthermore, *Pdfr*-null mutant flies ($Pdfr^{5304}$) also showed severe LTM impairment (Figure 8B), which was rescued by *Pdfr* expression when an MB-GAL4 line, OK107, was used (Figure 8B). However, when the previously generated *Pdfr*-GAL4 line was used, the impairment was not rescued (Figure 8B). Using an anti-Per antibody, we confirmed that GAL4-positive neurons in OK107 did not include Per-positive clock neurons (Figure 9A). Thus, Pdfr expression in MB neurons is essential for LTM maintenance.

LTM was also attenuated when *Pdfr* was knocked down in MB α/β or γ neurons (Figures 8C and D). In contrast, targeted expression of *Pdfr* RNAi in Pdf neurons, GAL4-positive neurons in the previously generated *Pdfr*-GAL4 line, and MB α'/β' neurons did not affect LTM (Figures 8C and D). The temporal knockdown of *Pdfr* in MB γ neurons during conditioning or the memory maintenance phase did not affect LTM (Figures 8E–G). However, temporal *Pdfr* knockdown in MB α/β neurons during the memory maintenance impaired LTM (Figure 8G). In the MB α/β GAL4 line used in this experiment, GAL4-positive neurons did not include Per-positive clock cells (Figure 9B). Thus, Pdfr in MB α/β neurons is likely involved in intracellular signaling necessary for LTM maintenance; however, it remains unclear whether Pdfr in MB γ neurons plays an acute physiological role in LTM.

CREB activity in MB α/β neurons is necessary for LTM maintenance

A *Drosophila* homolog of CREB (CrebB) is required for the consolidation and maintenance of olfactory memory (Hirano et al., 2016; Yin and Tully, 1996). Our previous studies demonstrated that the consolidation of courtship memory is also regulated by CrebB (Ishimoto et al., 2009; Sakai et al., 2004).

Pdf released in response to light may activate Pdfr, increase cAMP levels, and subsequently enhance CrebB transcription in MB α/β neurons. Consistent with this idea, LTM was attenuated when a repressor isoform of CrebB (CrebB-B; also known as dCREB2-b) was expressed in MB α/β neurons during conditioning or the memory maintenance phase (Figures 10A–C), indicating that CrebB expression in MB α/β neurons is required for both the consolidation and maintenance of LTM. On the other hand, CrebB expression in MB γ neurons during conditioning, but not the maintenance phase, attenuated LTM (Figures 10A–C), suggesting that CrebB in MB γ neurons is required only for memory consolidation.

Pdfr is essential for light-dependent CrebB activity in MB α/β neurons

To determine whether light and Pdfr activate CrebB transcription in MBs, we used the CRE>mCherry::STOP>*luciferase* (*luc*) reporter, which is a *luc*-based reporter gene under the control of CrebB-binding sites (CRE) (Tanenhaus et al., 2012). Using this reporter construct, in which the mCherry::STOP sequence is flipped out in the presence of the GAL4-induced recombinase Flippase (FLP), we can measure the MB-specific transcriptional activity of CrebB with MB-GAL4. The induction of CrebB-B driven by a heat-shock promoter severely attenuated the CRE-dependent Luc activity in MBs (Figure 11A), demonstrating that this reporter can reliably be used as an indicator of CrebB activity. When naïve flies were kept in LD, robust CrebB activity was detected in MB α/β neurons, but not in MB γ neurons (Figure 11B). When they were kept in DD for 1 d, CrebB activity in MB α/β neurons was reduced by approximately 50% compared with that in control flies (Figure 11B). On the second day of DD, it was further reduced (Figure 11B), indicating that CrebB activity in MB α/β neurons is light-dependent. *Pdfr* knockdown induced an approximately 50% reduction in CrebB activity, and CrebB activity was severely attenuated in MB α/β neurons with the *Pdfr* null mutant background (Figure 11B). Furthermore, activation of Pdf neurons by TrpA1 increased CrebB activity in MB α/β neurons (Figure 11C). Thus, the Pdf/Pdfr signaling pathway is essential for light-driven CrebB activity in MB α/β neurons. Next, the effect of 7 h conditioning on CrebB activity was examined. CrebB activity in MB α/β and γ neurons increased immediately after 7 h conditioning (Figure 11D). This result is consistent with a previous report (Ishimoto et al., 2009). However, no conditioning-dependent increase in CrebB activity was observed during the memory maintenance phase (Figure 11E).

Discussion

In nocturnal mice, an ultradian LD cycle condition induces spatial learning defects, although such an aberrant light condition does not impair the molecular clock or sleep amount (LeGates et al., 2012). The ultradian LD cycle also attenuates hippocampal long-term potentiation (Fernandez et al., 2018). In contrast, a short pulse of white light during the night before learning enhances long-lasting fear memory through the activation of hippocampal p21-activated kinase 1 (Shan et al., 2015). In humans, who are naturally diurnal, prior exposure to orange light promotes working memory, indicating that light also modulates human cognitive brain functions (Chellappa et al., 2014). Thus, regardless of the nocturnal or diurnal nature of animals, lighting conditions can positively or negatively modify the acquisition or consolidation of memories (Cajochen et al., 2011; Chellappa et al., 2014; LeGates et al., 2012; Shan et al., 2015). However, to the best of our knowledge, it has never been studied whether environmental light affects LTM maintenance. In this study using *Drosophila*, we demonstrated for the first time that environmental light is an essential factor for the appropriate maintenance of LTM.

Here we found that the brain photoreceptors Cry and Rh7, which are essential for light-dependent activation of Pdf neurons (Fogle et al., 2011; Ni et al., 2017; Sheeba et al., 2008), play important roles in LTM maintenance. *Drosophila* has three light-sensing organs: the compound eyes, ocelli, and Hofbauer–Buchner (H–B) eyelets. Besides the brain photoreceptors, compound eyes also play key roles in light entrainment of the circadian clock (Helfrich-Förster et al., 2002), and l-LNvs receive visual information via the compound eyes (Muraro and Ceriani, 2015). Furthermore, the H–B eyelets are also important for circadian photoreception, and axons of H–B eyelet photoreceptors project to the circadian pacemaker neurons including l-LNvs (Li et al., 2018). Although it remains unclear whether visual information via the compound eyes or H–B eyelets is also important for LTM maintenance, light inputs from photoreceptors in *Drosophila* light-sensing organs may also partially contribute to light-driven LTM maintenance.

The *Drosophila* Pdf neuropeptide regulates various biological phenomena such as circadian behavioral rhythms, light-driven arousal, geotactic behavior, rival-induced prolonged mating, and sex pheromone biosynthesis (Helfrich-Förster et al., 2000; Kim et al., 2013; Krupp et al., 2013; Mertens et al., 2005; Renn et al., 1999). In the present study, we found that *Drosophila* has a light-driven memory maintenance system, and that the Pdf signaling pathway regulates this system. Pdf expression was essential for LTM maintenance in LD (Figure 4). LTM was impaired when flies were kept in DD for 2 d after 7 h conditioning (Figure 1), and the activation of Pdf neurons was sufficient to rescue the LTM impairment induced by DD (Figure 5). Moreover, the electrical silencing of Pdf neurons impaired LTM in LD (Figure 5). Considering that light activates Pdf neurons through brain photoreceptors (Fogle et al.,

2011; Ni et al., 2017; Sheeba et al., 2008) and Rh7 and Cry in Pdf neurons are necessary for light-driven LTM maintenance (Figure 1), it is most likely that light-inducible Pdf released from Pdf neurons regulates LTM maintenance. Our study using a GAL4 knock-in fly, *Pdfr::2A::GAL4*, revealed that Pdfr is expressed in MB neurons (Figure 7). We further confirmed that Pdfr in MB α/β neurons is necessary for LTM maintenance (Figure 8G) and Pdfr expression in MB α/β neurons is necessary for the light-driven transcription through CrebB, which is essential for LTM maintenance (Figures 10 and 11). Taken together, our study shows that this light-driven transcription system via the Pdf/Pdfr/CrebB pathway in MB α/β neurons regulates LTM maintenance in *Drosophila*.

The targeted expression of a CrebB repressor in MB α/β neurons during 7 h conditioning impaired LTM, as was observed in that in MB γ neurons (Figure 10), indicating that CrebB-dependent transcription in both MB α/β and γ neurons is necessary for memory consolidation. In addition, the targeted expression of a CrebB repressor in MB α/β neurons during the memory maintenance phase also impaired LTM (Figure 10), whereas, that in MB γ neurons did not (Figure 10). It has been reported that MB γ neurons are necessary for 1 d memory of courtship conditioning (Kruttner et al., 2015). Thus, consolidated courtship memory may last in MB γ neurons only for 1–2 d at most, whereas longer-lasting LTM seems to be established and stored in MB α/β neurons. In contrast to the MB γ neurons, Pdfr expression and CrebB activity in MB α/β neurons are essential for LTM maintenance, and the light-dependent activation of CrebB transcription was evident in MB α/β neurons, indicating that 5 d memory is maintained in MB α/β neurons through the Pdf/Pdfr/CrebB pathway in LD. Thus, long-lasting LTM (> 1 d memory) seems to be established and stored in MB α/β neurons.

When flies were kept in DD for two consecutive days, LTM was impaired (Figure 1) and CrebB activity in MB α/β neurons was severely attenuated (Figure 11). However, DD for only 1 day was not sufficient to impair LTM (Figure 1) but it induced 50% reduction in CrebB activity (Figure 11). Thus, when CrebB activity in MB α/β neurons is severely attenuated, LTM maintenance may break down. Can Pdfr be the only factor that regulates CrebB activity in MB α/β neurons? Pdf/Pdfr signaling should play a role in the regulation of CrebB activity in MB α/β neurons because a *Pdfr* null mutation severely attenuated CrebB activity (Figure 11). However, it remains possible that signaling pathways other than the Pdf/Pdfr signaling pathway also partially contribute to CrebB activity in MB α/β neurons.

In contrast to LTM maintenance, LTM formation occurred in darkness (Figure 1). This finding is consistent with the previous report that STM after 1 h conditioning under a dim red light, which blocks visual input, is intact (Joiner and Griffith, 1997). Although we found that CrebB activity during

conditioning was necessary for memory consolidation (Figure 10), *Pdfr* in MBs was not (Figure 8). Considering that flies were able to establish LTM when they were conditioned in darkness (Figure 1), it is likely that light-independent CrebB transcription without using Pdfr in MBs plays an important role in memory consolidation (Figure 12A). Unlike memory consolidation, LTM maintenance requires a light-driven transcription system via the Pdf/Pdfr/CrebB pathway in MB α/β neurons (Figure 12B). In naïve males, however, light can also increase the CrebB transcription activity in MB α/β neurons (Figure 11). In naïve males, it is most unlikely that this light-driven transcription system provides proteins that are required for LTM maintenance. Thus, it may play a role in innate brain functions other than LTM maintenance. Assuming that this hypothesis is true, how are proteins that are required for LTM maintenance provided in only conditioned males? Although it remains unclear, repetitive exposure to stressors using mated females during courtship conditioning may trigger the change in the target genes of CrebB in the light-driven transcription system, and such a system in MB α/β neurons may provide gene products that are required for maintaining consolidated LTM in a courtship conditioning-dependent manner.

In nature, animals learn much from their experience throughout the day. Through their experience, LTM should be formed and maintained for a long period. If environmental light, which is available daily to all animals in nature, can be used for transcriptional activation in the brain, such a light-driven transcription system is considered reasonable and effective for continually providing *de novo* protein synthesis required for LTM maintenance. When diurnal Nile grass rats were housed under dim LD cycles, 24 h spatial memory is impaired, and this lighting condition inhibits the expression of brain-derived neurotrophic factors and dendritic spine density in the hippocampus (Soler et al., 2018). Although it is unknown whether the rapid forgetting in the Nile grass rats under dim LD cycles results from the inhibition of *de novo* protein synthesis required for LTM maintenance, light-dependent *de novo* protein synthesis in the memory center may be conserved in many animal species. As is observed in *Drosophila*, in mammals, repeated exposure to stressors also induces a long-lasting reduction of male sexual motivation (Hawley et al., 2011; Hawley et al., 2013). It is thus interesting to examine whether environmental light and the evolutionarily conserved memory modulator CREB also play critical roles in the maintenance of such depressed sexual motivation in mammals including humans.

Materials and methods

Fly stocks

All flies were raised on glucose-yeast-cornmeal medium in 12:12 LD cycles at 25.0 ± 0.5 °C (45–60% relative humidity). Virgin males and females were collected without anesthesia within 8 h after eclosion. Fly stocks used for this study are as follows: wild-type Canton-S (CS), *Pdf*^{01} (BL26654), *Pdfr*^{5304}(BL33068), *cry*^{01}(Provided by Dr. Taishi Yoshii), *Rh7*^{1} (BL76022), *Pdf*-GAL4 (BL6900), c929 (BL25373), *R14F03* (BL48648), *R18F07* (BL47876), OK107 (BL854), MB-LexA (provided by Dr. Minoru Saitoe), *R58F02*-LexA (Gift from Dr. Yoshinori Aso), *R19B03*-LexA (BL52537), *R61G12*-LexA (BL52685), *R41C10* (BL50121), *R55D03* (BL47656), *R19B03* (BL49830), c305a (BL30829), *nSyb*-GAL4 (BL51941), UAS-*FLP* (BL4539), UAS-*Kir2.1::eGFP* (BL6596), UAS-*TrpA1* (BL26263), UAS-*mCD8::GFP* (BL5137), UAS-*mCherry::NLS* (BL38424), UAS-*mCD8::RFP* (BL32218), UAS-*cry* RNAi (NIG3772R-3), UAS-*Rh7* RNAi (VDRC109688), UAS-*Pdf* RNAi (VDRC4380), UAS-*Pdfr* RNAi (VDRC42724), UAS-*CrebB-B* (also known as UAS-*dCREB2-b*, BL7219), *tub*-GAL80^{ts} (BL7017), LexAop2-*FLPL* (BL55820), LexAop2-*mCD8::GFP* (BL32203), LexAop-*TrpA1* (provided from Dr. Mani Ramaswami), *hs-CrebB-B* (also known as *hs-dCREB2-b*) (Sakai et al., 2004; Yin et al., 1994), *Pdfr*-GAL4 (provided by Dr. Leslie C. Griffith), UAS-*Pdf* (provided by Dr. Taishi Yoshii), UAS-*Pdfr* (provided from Dr. Taishi Yoshii), UAS-*luc* RNAi (provided by Dr. Kanae Ando), UAS>STOP>*Kir2.1::eGFP* (provided by Dr. David J. Anderson), and CRE>mCherry::STOP>*luc* (provided by Dr. Jerry C. P. Yin). *Pdfr::2A::GAL4* was generated by the targeted integration of a 2A-GAL4 cassette (Diao and White, 2012) into the *Pdfr* locus, such that it replaces the stop codon of the RA isoform of the *Pdfr* transcripts. The 2A-GAL4 cassette contained a *3xP3-RFP* transgene (Horn et al., 2000) flanked by loxP sites as a selection marker, which was removed by Cre expression after the transgenic lines were established. All lines for behavior experiments except for *cry*^{01}, *Pdf*^{01}, *Pdfr*^{5304}, *Rh7*^{1}, UAS-*Pdfr*, UAS-*luc* RNAi, LexAop2-*FLPL*, and *Pdfr::2A::GAL4* were outcrossed for at least five generations to *white*^{1118} flies with the Canton-S (CS) genetic background.

Courtship conditioning assay

The courtship conditioning assay was carried out as previously described (Sakai et al., 2004) with some modifications. Unreceptive mated females were prepared as “trainers” one or two days before they were used for courtship conditioning. In this conditioning, a virgin CS female and a male (3–6 d old) were placed in an acrylic courtship chamber (15 mm in diameter × 3 mm in depth) for copulation. For LTM, a 3–5 d old male was placed with a mated female (4–7 d old) in a conditioning chamber (15 mm in diameter, 5 mm in depth) containing food for 7 h either with (conditioned) or without (naïve) a single

premated female (7 h conditioning). If males remated with mated females during conditioning, we discarded such males after conditioning. After 7 h conditioning, only flies showing courtship behaviors toward the mated female but not copulating successfully were transferred to a glass tube with food (12 mm in diameter × 75 mm in depth) and kept in isolation for 1 or 5 days until the test. The test was performed using a freeze-killed virgin female in a test chamber (15 mm in diameter, 3 mm in depth). All procedures in the experiments were carried out at 25 ± 1.0 °C (45–60% relative humidity) except for the temperature shift experiments. Male courtship behaviors of individual flies were calculated manually as a courtship index (CI), defined as the percentage of time spent in performing courtship behaviors during a given observation period (10 min). We first measured CI in conditioned and naïve males ($CI_{Conditioned}$ and $CI_{Naïve}$), and then mean $CI_{Naïve}$ was calculated. To quantify courtship memory as previously reported (Lee et al., 2017), memory index (MI) was calculated using the following formula: MI = (mean $CI_{Naïve}$ - mean $CI_{Conditioned}$) / mean $CI_{Naïve}$. In the statistical analysis of CI, the Kolmogorov–Smirnov test was used to determine whether the data are normally distributed. When the data were not distributed normally, we carried out the log transformation of the data. When the basic data or transformed data are normally distributed, Student's *t*-test was used for comparisons. When the basic data and transformed data are not distributed normally, we used the Mann–Whitney *U* test for comparisons. The computer software IBM SPSS Statistics 22 (IBM Japan, Ltd.) was used for these tests. In the statistical analysis of MI, the permutation test with 10000 random permutations was used. The free statistical package R was used for these tests (Koemans et al., 2017).

Lighting conditions in courtship conditioning

When courtship conditioning was performed in a dark place, conditioning chambers were placed in a temperature-regulated (25.0 ± 0.5 °C) light-tight incubator (MIR-254, Sanyo Electric Co., Ltd.). To check whether mating occurs during conditioning, we observed the flies for 10 sec every 30 min by opening the incubator door. To determine whether lighting conditions affect memory maintenance, conditioned and naïve flies were kept in a light-tight incubator (MIR-254, Sanyo Electric Co., Ltd.) for one, two, or four days after 7 h conditioning (see Figure 1A).

Temporal activation of Pdf neurons

The temperature-sensitive cation channel TrpA1 was used to activate Pdf neurons (Hamada et al., 2008). For the activation of Pdf neurons, during two days of DD (the second and third days after 7 h

conditioning), *Pdf*-GAL4/UAS-*TrpA1* flies were kept at 30 °C for 8 h within each subjective day [circadian time (CT) 0–8] or night (CT 12–20). However, DD-dependent LTM impairment was not improved. Since it is possible that activation of Pdf neurons under this condition is insufficient for LTM maintenance, the flies were kept at 34 °C for 8 h within each subjective day (CT 0–8) or night (CT 12–20). UAS-*TrpA1*/+ and *Pdf*-GAL4/+ flies were used as the control.

Temporal gene expression using TARGET system

The *tub*-GAL80^{ts} transgene used in the TARGET system (McGuire et al., 2003) encodes a ubiquitously expressed, temperature-sensitive GAL4 repressor that is active at the permissive temperature (PT, 25 °C) but not at the restrictive temperature (RT, 30 or 32 °C). By using UAS-*Rh7* RNAi, UAS-*cry* RNAi, UAS-*Pdf* RNAi, or UAS-*Pdfr* RNAi combined with the TARGET system, we knocked down *Rh7*, *cry*, *Pdf*, or *Pdfr* in GAL4-positive neurons at RT (30 °C), but not at PT. In these experiments, we shifted PT to RT and vice versa during the three experimental phases (Figures 8E–G): 24 h before the end of conditioning, 48–72 h after conditioning, and 24 h before the initiation of the test. Furthermore, to drive the expression of a UAS-*CrebB-B* construct in MBs during a specific temporal phase, the TARGET system was also used. In *CrebB-B* experiments, we shifted PT to RT (30 °C) and vice versa during the two experimental phases (Figures 10A–C): 10 h before the end of conditioning, 48–72 h after conditioning. To drive the expression of a UAS-*Kir2.1* construct in Pdf neurons during the memory maintenance phase using the TARGET system, flies were kept at RT (32°C) for 48–72 h after conditioning. Subsequently, they were kept at PT.

l-LNv-specific electrical silencing

To assay whether l-LNv-specific electrical silencing affects LTM, two binary gene expression systems (GAL4/UAS and LexA/LexAop) combined with flippase (FLP/FRT) were used. Using this system, the specific target gene is expressed in GAL4- and LexA-coexpressing neurons. *R61G12*-LexA and *R14F03*-GAL4 lines were used in the experiments. We confirmed that *Kir2.1::eGFP* is expressed in all l-LNvs but not s-LNvs (Figure 5H). Thus, in *R61G12*-LexA/LexAop-*FLPL*; UAS>STOP>*Kir2.1::eGFP/R14F03* flies, according to the l-LNvs-specific expression of Kir2.1, only l-LNvs will be electrically silenced.

Real-time quantitative reverse transcription PCR (qRT-PCR)

A PicoPure RNA Isolation Kit (KIT0204, Thermo Fisher Scientific) was used for collecting total RNA from three whole brains in each genotype. cDNA was synthesized by the reverse transcription reaction using a QuantiTect Reverse Transcription Kit (#205311, QIAGEN). Real-time quantitative PCR was carried out using the THUNDERBIRD SYBR qPCR Mix (QPS-201, TOYOBO) and a Chromo 4 Detector (CFB-3240, MJ Research). The primer sequences (custom-made by Eurofins Genomics) used for qRT-PCR were as follows: *Pdf*-Forward, 5'-ATCGGGATCTCCTCGACTGG-3'; *Pdf*-Reverse, 5'-ATGGGCCCAAGGAGTTCTCG-3'; *Pdfr*-Forward, 5'- CGTCTCATGCAGCAGATGGG-3'; *Pdfr*-Reverse, 5'-TAAGGGCGAACAGGGAGAGG -3'; *Rh7*-Forward, 5'-GCAAGGACCACGACAAGCAC-3'; *Rh7*-Reverse, 5'- GCTCAGGTCGTACTTGTCCC-3'; *cry*-Forward, 5'-CTCGCCGATAAGGATCAGGG-3'; *cry*-Reverse, 5'-ATGTCCTGCAACGAGTCCAG-3'; *rp49*-Forward, 5'-AAGATCGTGAAGAAGCGCAC-3'; *rp49*-Reverse, 5'-TGTGCACCAGGAACTTCTTG-3'. The expression level of each mRNA was normalized by that of *rp49* mRNA. The average of the normalized mRNA expression level in control flies was calculated using data from 4–6 independent assays. The expression level of each mRNA was normalized by that of *rp49* mRNA. The average of the normalized mRNA expression level in control flies was calculated using data from 4–6 independent assays. The mean (± SEM) ratio was calculated using data from 4–6 independent assays. The Kolmogorov–Smirnov test was used to estimate whether the data are normally distributed. When the basic data or log-transformed data were normally distributed, Student's *t*-test or one-way ANOVA followed by post-hoc analysis using Scheffe's test was used. When the basic data and log-transformed data were not distributed normally, the Kruskal–Wallis test followed by the rank-sum test for multiple pairwise comparisons was used. The computer software IBM SPSS Statistics 22 (IBM Japan, Ltd.) was used for these tests.

Luciferase assay

To test whether light or Pdfr activation promotes CrebB activity in MBs, the Luciferase (Luc) reporter was used (Tanenhaus et al., 2012). Luc should be expressed in MBs in the combination of CRE>mCherry::STOP>*luc* reporter, MB-GAL4, and UAS-*FLP*. Three MB-GAL4 lines (*R41C10*, *R55D03*, and *R19B03*) were used in these experiments. *In vitro* Luc activity was measured using a Luciferase Assay System (E1501, Promega). Three adult male heads were collected into a 1.5 ml Eppendorf tube and homogenized in 50 µl of Glo Lysis Buffer (E266A, Promega) at Zeitgeber time (ZT) 0–2. After centrifugation, 10 µl of the resulting supernatant and 50 µl of Luciferin solution were used to

analyze Luc activity. The luminescence of each sample was measured using a luminometer (GloMax 20/20, Promega) and normalized to total protein concentration using a Protein Assay Kit (#5000006, Bio-Rad). The UAS-*FLP* transgene used in this study displayed leaky expression of Luc (211–279 relative luminescence unit (RLU/mg). Thus, first, in the control line (UAS-*FLP*/CRE>mCherry::STOP>*luc*), the mean normalized luminescence (mean $L_{control}$) was calculated. CrebB activity index (CAI) (RLU/mg) was defined as the difference between the "mean $L_{control}$" and the measured luminescence in each sample in each genotype, and finally, we calculated the mean CAI. To examine whether CrebB activity increases after 7 h conditioning, we measured the CAI in each sample using conditioned or naïve flies ($CAI_{Conditioned}$ and $CAI_{Naïve}$), and then mean $CAI_{Naïve}$ was calculated. Finally, we calculated the conditioning-dependent CrebB activity using the following formula: conditioning-dependent CrebB activity = ($CAI_{Conditioned}$ - mean $CAI_{Naïve}$) / mean $CAI_{Naïve}$. To examine whether induction of the CrebB-B inhibits CrebB activity, we used UAS-*FLP*/*hs-CrebB-B*; CRE>mCherry::STOP>*luc*/*R19B03* flies. For the heat-shock treatment, 3–4 d old male flies were grouped as 20 flies per a food vial. Flies were heat-shocked at 32°C for three days. Luc activity was measured immediately after the heat-shock treatment at ZT 0–2. To examine whether activation of Pdf neurons increases CrebB activity in the MBs, we used UAS-*FLP*/*R61G12*-LexA; CRE>mCherry::STOP>*luc* LexAop-*TrpA1*/*R41C10* flies. For the activation of Pdf neurons by TrpA1, the 3–5 d old males were grouped as 20 flies per a food vial. Flies were heat-shocked at 32°C during two days of DD (the second and third days after transfer). Luc activity was measured at ZT 0–2 after flies were returned to normal LD cycle. The Kolmogorov–Smirnov test was used to estimate whether the data are normally distributed. Because the basic data were normally distributed, Student's *t*-test was used for comparisons of two means, and one-way ANOVA followed by post-hoc analysis using Schefe's test was carried out for multiple pairwise comparisons. The computer software IBM SPSS Statistics 22 (IBM Japan, Ltd.) was used for these tests.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Mabuchi et al., 2016; Shimada et al., 2016). For Pdf staining, brains were stained with a mouse anti-Pdf antibody (PDF C7-s, Developmental Studies Hybridoma Bank at the University of Iowa, 1:200), and Alexa Fluor 568 anti-mouse IgG (A11004, Thermo Fisher Scientific) was used as the secondary antibody (1:1000). For GFP staining, brains were stained with a rabbit anti-GFP antibody (A11122, Thermo Fisher Scientific, 1:200), and Alexa Fluor 488

anti-rabbit IgG (A11008, Thermo Fisher Scientific, 1:1000) was used as the secondary antibody. For Per staining, brains were stained with a rabbit anti-Per antibody (sc-33742, Santa Cruz Biotechnology, 1:250), and Alexa Fluor 568 anti-rabbit IgG (A11011, Thermo Fisher Scientific) was used as the secondary antibody (1:1000). Fluorescence signals were observed under a confocal microscope [C2^{+} (Nikon) or LSM710 (Zeiss)].

Quantitative analysis of Pdf immunoreactivity in l-LNvs

To examine whether temporal knockdown of *Pdf* in l-LNvs by the TARGET system inhibits Pdf immunoreactivity, *Pdf*-GAL4/UAS-*Pdf* RNAi; +/*tub*-GAL80^{ts} flies were used. *Pdf*-GAL4/+; +/*tub*-GAL80^{ts} flies were used as the control. After eclosion, all flies were kept for 3–6 days at PT, and then the temperature was shifted to RT at ZT 8. After 24 h, the temperature was shifted again to PT. Subsequently, we performed brain dissection for antibody staining 1 h after the temperature shift to PT (ZT 9). A confocal image stack of the brain hemisphere containing l-LNvs was Z-projected into several sequential sections. Z-sections were collected at 1 μm intervals. The signal intensity of Pdf immunoreactivity was quantified in a manually set region of interest of the cell body region in each l-LNv using the NIS elements Ar (Nikon). In all samples, image data were acquired under identical conditions. When the basic data or log-transformed data were distributed normally, Student's *t*-test was used. When they were not normally distributed, we performed the Mann–Whitney *U* test.

Sleep analysis

Single male flies (2–3 d old) were introduced into glass tubes (3 mm in diameter × 75 mm in length) containing fly food, and the glass tubes were set in a MB5 MultiBeam Activity Monitor (Trikinetics) to monitor the locomotor activity of individual flies. In this system, 17 independent infrared beams per glass tube were used to detect fly movement. When a fly repositions from one beam to the next, it was counted as one beam-crossing. Flies were acclimated in the glass tubes for 3 days in LD cycles at 25 °C before measuring sleep amount. Locomotor activity data were collected at 1-min intervals for 5 days and analyzed with a Microsoft Excel-based program as previously described (Kume et al., 2005). Sleep was defined as behavioral inactivity for 5 min or more (Huber et al., 2004). Total sleep amount during the day or night was analyzed as previously described (Shimada et al., 2016).

The Kolmogorov–Smirnov test was used to examine whether the data are normally distributed. For comparisons of two groups, when the basic data or log-transformed data were normally distributed,

Student's *t*-test was used. When they were not normally distributed, we performed the Mann–Whitney *U* test. In multiple group analysis of data, basic data and log-transformed data were not distributed normally. Thus, we performed nonparametric ANOVA (Kruskal–Wallis test) followed by the rank-sum test for multiple pairwise comparisons. The computer software IBM SPSS Statistics 22 (IBM Japan, Ltd.) was used for these tests.

Sleep deprivation

To deprive flies of sleep, a MB5 MultiBeam Activity Monitor with glass tubes containing single naïve or conditioned flies was horizontally shaken using a shaker (NJ-022NS, Nissin). The shaker was placed inside a temperature-regulated incubator (MIR-254, Sanyo Electric Co., Ltd.). The shaking speed was set to 200 rpm. On the 2nd and 3rd day after 7 h conditioning, the MB5 MultiBeam Activity Monitor was shaken for 20 sec per 3 min during only the daytime. All experiments were carried out in 12:12 LD cycles at 25.0 ± 0.5 °C.

Acknowledgments

We thank Yuto Kurata and Yushun Sato for technical assistance. We also thank Toshiro Aigaki for helpful discussions. This work was supported by JSPS KAKENHI Grants.

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Figure legends

Figure 1. Light and brain photoreceptors are essential for LTM maintenance

Box-and-whisker plots for a set of CI data show 20th, 25th, 75th, and 80th centiles. In the box-and-whisker plots, the black square in each box indicates the mean, the line in each box is drawn at the median, white boxes show naïve males, and gray boxes show conditioned males. The Mann–Whitney *U* test was used for comparisons of CI. The permutation test with 10000 random permutations was used for comparisons of MI among experimental conditions or genotypes. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant; *N*, sample size in each box. **(A)** LTM was measured under various lighting conditions. Wild-type males were used in the experiments. Males were tested on d 5 after 7 h conditioning. A schematic drawing of lighting conditions in courtship conditioning is shown on the left. The white box indicates day (light) and the black box indicates night (dark). The gray box indicates that experiments were conducted in darkness during the daytime. For MI, asterisks indicate a comparison between the control (1) and test groups. **(B)** Memory on d 1 in wild-type (WT), cry^{01}, and $Rh7^{1}$ flies. **(C)** Memory on d 5 in WT, cry^{01}, and $Rh7^{1}$ flies. **(D–G)** Temporal knockdown of *Rh7* or *cry* in Pdf neurons using the TARGET system. The upper panel shows a schematic drawing of temperature shift experiments using the temperature-sensitive GAL4 repressor *tub*-GAL80^{ts}. All flies were raised at 25°C. PT, permissive temperature (25°C); RT, restrictive temperature (30°C). **(D)** All experiments were performed at PT (25°C). **(E)** Flies were kept at RT for 24 h before the end of conditioning. **(F)** Flies were kept at RT for 48–72 h after 7 h conditioning. **(G)** Flies were kept at RT for 24 h before the test. **(H)** *Pdf*-GAL4/+, UAS-*cry* RNAi/+, and *Pdf*- GAL4/UAS-*cry* RNAi flies were used. For MI, the asterisk shows a comparison between F_1 flies and GAL4 control, and the hashmark shows a comparison between F_1 flies and UAS control. ###, $P < 0.001$

Figure 2. Total sleep amount in darkness decreases in comparison with that in light.

(A–F) Sleep amount was measured for 5 d using wild-type males. Data are presented as mean ± SEM. **(A)** Continuous sleep amounts of control (black line) and experimental flies (magenta line). In control flies, sleep amount was measured in LD. In experimental flies, sleep amount was measured for two days in LD, which was subsequently shifted to DD for two days, and then back to LD. $N = 31$ in black line; $N = 30$ in magenta line. **(B)** Total daytime sleep amounts in control (black bars) and experimental flies (magenta bars). *, $P < 0.05$; ***, $P < 0.001$; NS, not significant. $N = 31$ for black bars; $N = 30$ for magenta bars. Student's *t*-test or the Mann–Whitney *U* test was used for comparisons. **(C)** Total nighttime

sleep amounts in control (black bars) and experimental flies (magenta bars). N = 31 for black bars; N = 30 for magenta bars. Student's t-test was used for comparisons. NS, not significant. **(D)** Continuous sleep amounts in naïve (deep blue line) and conditioned flies (orange line). N = 30 for deep blue line; N = 31 for orange line. Flies were deprived of sleep during the daytime on the 2nd and 3rd days after 7 h conditioning. SD, sleep deprivation. **(E, F)** Nonparametric ANOVA (Kruskal–Wallis test) followed by post-hoc analysis using the rank-sum test was carried out for multiple pairwise comparisons. Bars with the same letter indicate values that are not significantly different ($P > 0.05$). N = 30 for deep blue bars; N = 31 for orange bars. **(E)** Total daytime sleep amounts in naïve (deep blue bars) and conditioned flies (orange bars). **(F)** Total nighttime sleep amounts in naïve (deep blue bars) and conditioned flies (orange bars). **(G)** Memory on d 5 was measured using sleep-deprived flies. Control flies were kept in LD without sleep deprivation. In the box-and-whisker plots, the white boxes indicate naïve males and the gray boxes indicate conditioned males. The Mann–Whitney U test was used for comparison of CI. The permutation test with 10000 random permutations was used for comparisons of MI among genotypes. *, $P < 0.05$; **, $P < 0.01$; N, sample size in each box.

Figure 3. Real-time qRT-PCR analysis and quantitative analysis of Pdf immunoreactivity. **(A)** Analysis of *Rh7* mRNA expression level. The pan-neuronal driver *nSyb*-GAL4 was used for the induction of *Rh7* RNAi. Mean ± SEM was calculated from five replicates. **(B)** Analysis of *cry* mRNA expression level. *nSyb*-GAL4 was used for the induction of *cry* RNAi. Mean ± SEM was calculated from four replicates. **(A, B)** One-way ANOVA followed by post-hoc analysis using Scheffe's test was carried out for multiple pairwise comparisons. *, $P < 0.05$; NS, not significant. **(C)** Analysis of *Pdf* mRNA expression level. *Pdf*-GAL4 was used for the induction of *Pdf* RNAi. Mean ± SEM was calculated from five to six replicates. Nonparametric ANOVA (Kruskal–Wallis test) followed by the rank-sum test for multiple pairwise comparisons was used. *, $P < 0.05$; NS, not significant. **(D, E)** Analysis of *Pdfr* mRNA expression level. *nSyb*-GAL4 (D) or MB-GAL4 OK107 (E) was used. Mean ± SEM was calculated from six (D) or four (E) replicates. **(D)** One-way ANOVA followed by post-hoc analysis using Scheffe's test was carried out for multiple pairwise comparisons. *, $P < 0.05$; NS, not significant. **(E)** F_1 flies between UAS-*Pdfr* RNAi and MB-GAL4 (OK107) lines were used. Student's t-test was used for comparisons between F_1 and GAL4 control flies. *, $P < 0.05$. **(F, G)** Quantitative analysis of Pdf immunoreactivity in l-LNvs. Adult brains were dissected at ZT 9. In the box-and-whisker plots, white boxes indicate *Pdf*-GAL4/+; *tub*-GAL80^{ts}/+ flies and gray boxes indicate *Pdf*-GAL4/ UAS-*Pdf* RNAi; *tub*-GAL80^{ts}/+

flies. **(F)** All experiments were carried out at PT (25°C). N = 26 for white box; N = 27 for gray box. Student's t-test was used for statistical analysis. NS, not significant. **(G)** Flies were kept at RT (30°C) for 24 h. N = 50 for white box; N = 38 for gray box. The Mann–Whitney U test was used for statistical analysis. ***, $P < 0.001$.

Figure 4. Pdf expression is essential for LTM maintenance

(A–F, H) Males were tested on d 1 or d 5 after 7 h conditioning. In the box-and-whisker plots, the white boxes indicate naïve males and the gray boxes indicate conditioned males. The Mann–Whitney U test was used for comparisons of CI. The permutation test with 10000 random permutations was used for comparisons of MI. *****, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ##, $P < 0.01$; NS, not significant; N, sample size in each box. **(A)** All experiments were performed at PT (25°C). **(B)** Flies were kept at RT for 24 h before the end of conditioning. **(C)** Flies were kept at RT for 48–72 h after 7 h conditioning. **(D)** Flies were kept at RT for 24 h before the test. **(A–D)** memory on d 5 in *Pdf*-GAL4 / UAS-*Pdf* RNAi; *tub*-GAL80^{ts}/+ flies and control flies (*Pdf*-GAL4 /+; *tub*-GAL80^{ts}/+). **(E)** Memory on d 1 in UAS-*Pdf* / *Pdf*-GAL4; Pdf^{01}/Pdf^{01} flies and control flies (UAS-*Pdf* /+; Pdf^{01}/Pdf^{01}). **(F)** Memory on d 5 in UAS-*Pdf* / *Pdf*-GAL4; Pdf^{01}/Pdf^{01} flies and control flies. **(G)** Confocal section images at Pdf neuron level of the adult brain. Triangles, l-LNvs; arrows, s-LNvs. Scale bars represent 50 μm. UAS-*mCherry::NLS*/*R18F07* flies were used. Magenta, mCherry::NLS; green, Pdf. **(H)** Knockdown of *Pdf* in l-LNvs and/or s-LNvs using three GAL4 drivers (*Pdf*-GAL4, c929, and *R18F07*). For MI, asterisks indicate a comparison between F_1 and GAL4 control flies, and hashmarks indicate a statistical comparison between F_1 and UAS control flies.

Figure 5. Activity of Pdf neurons is required for LTM maintenance

(A–D) Temporal activation of Pdf neurons compensates for DD-dependent LTM impairment. *Pdf*-GAL4/+, UAS-*TrpA1*/+, and *Pdf*-GAL4/UAS-*TrpA1* males were used. For MI, asterisks indicate a comparison between F_1 (GAL4/UAS) and GAL4 control flies, and hash marks indicate a statistical comparison between F_1 and UAS control flies. **(A)** All experiments were performed at PT. **(B)** On the 2nd and 3rd days after 7 h conditioning, flies were kept in DD. **(C)** On the 2nd and 3rd days after 7 h conditioning, flies were kept at 34°C during the period between CT 0 and CT 8. **(D)** On the 2nd and 3rd days after 7 h conditioning, flies were kept at 34°C during the period between CT 12 and CT 20. **(E, F)** UAS-*Kir2.1*/*Pdf*-GAL4; *tub*-GAL80^{ts}/+ flies were used. UAS-*Kir2.1*/*tub*-GAL80^{ts} flies were used as the

control. **(E)** All experiments were carried out at PT (25°C). **(F)** Flies were kept at RT (32°C) for 48–72 h after 7 h conditioning. **(G)** l-LNv-specific silencing impairs LTM. *R61G12*-LexA/LexAop-*FLPL*; UAS>STOP>*Kir2.1*/*R14F03* flies were used. For MI, asterisks indicate a comparison between l-LNv-silenced flies and the LexA/LexAop control (*R61G12*-LexA/LexAop-*FLPL*), and the hashmark indicates a comparison between l-LNv-silenced flies and the GAL4/UAS control (UAS>STOP>*Kir2.1*/*R14F03*). **(A–G)** The Mann–Whitney *U* test was used for comparisons of CI. The permutation test with 10000 random permutations was used for comparisons of MI. *****, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.05$; ##, $P < 0.01$; NS, not significant; *N*, sample size in each box. **(H)** Confocal section images at the level of Pdf neurons of the adult brain. Scale bars represent 10 μm. Triangles, l-LNvs; arrows, s-LNvs.

Figure 6. GAL4-expressing neurons in the adult brain in *Pdfr::2A::GAL4* flies

(A, B) *Pdfr::2A::GAL4*; UAS-*mCD8::GFP*/+ flies were used for visualizing GAL4-positive cells. Brains were stained with an anti-GFP antibody. Stacked confocal images of the adult brain showing an anterior (A) or posterior view (B). Scale bars, 100 μm. **(C)** *Pdfr::2A::GAL4*; *UAS-mCherry::NLS*/+ flies were used for visualizing GAL4-positive cells. Adult brains were dissected at ZT 0–2, in which flies were entrained in more than 3 LD cycles. All brains were stained with an anti-Per antibody. Magenta, mCherry::NLS; green, Per. Partially stacked or sectional images of specific clusters of clock neurons (DN1s, LNds, l-LNvs, and s-LNvs). Rectangles, DN1s; triangles, LNds or l-LNvs; arrows, s-LNvs. Scale bars, 50 (DN1s) or 10 μm (LNds, l-LNvs, and s-LNvs).

Figure 7. Pdfr::2A::GAL4-positive MB neurons

(A) Confocal image of whole brain and sectional images at the level of Kenyon cells in MBs. *Pdfr::2A::GAL4*; UAS-*mCherry::NLS*/+ ; MB-LexA LexAop-*mCD8::GFP*/+ flies were used to visualize Pdfr-positive neurons (magenta, mCherry::NLS) and MB neurons (green, mCD8::GFP). The small window located in the upper left in each picture shows an enlarged image of a single cell enclosed in a small white square. Scale bars, 5 (enlarged images), 50 (Kenyon cells), or 100 μm (whole brains). **(B)** UAS-*mCherry::NLS/ Pdfr*-GAL4*;* MB-LexA LexAop-*mCD8::GFP/*+ flies were used. **(C)** *Pdfr::2A::GAL4*; UAS-*mCherry::NLS*/ LexAop-*mCD8::GFP* ; *R58F02*-LexA/+ flies were used. Triangles show the colocalization of *Pdfr::2A::GAL4* and *R58F02*-LexA. **(D)** *Pdfr::2A::GAL4*;

UAS-*mCherry::NLS*/ LexAop-*mCD8::GFP* ; *R19B03*-LexA/+ flies were used. **(A–D)** Brains were stained with an anti-GFP antibody.

Figure 8. Pdfr expression in MB α/β neurons is essential for LTM maintenance

The Mann–Whitney *U* test was used for comparisons of CI. The permutation test with 10000 random permutations was used for comparisons of MI among genotypes. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.05$; ##, $P < 0.01$; NS, not significant; *N*, sample size in each box. **(A)** Memory on d 5 in *Pdfr::2A::GAL4*/UAS-*luc* RNAi (control) and *Pdfr::2A::GAL4*/UAS-*Pdfr* RNAi flies. **(B)** Memory on d 5 in $Pdfr^{5304}$; *Pdfr*-GAL4/+; UAS-*Pdfr* /+ and $Pdfr^{5304}$; UAS-*Pdfr* /+; OK107/+ flies. **(C)** *Pdfr* RNAi was driven by *Pdf*-GAL4, *Pdfr* -GAL4, MB α/β -GAL4 (*R41C10*), MB γ-GAL4 (*R55D03*), and MB α'/β'-GAL4 (c305a). For MI, asterisks indicate a comparison between F_1 and GAL4 control flies, and hash marks indicate a statistical comparison between F_1 and UAS control flies. **(D)** Stacked confocal images of the adult brain. *R41C10*/ UAS-*mCD8::GFP*, *R55D03*/UAS-*mCD8::GFP*, and c305a/UAS-*mCD8::RFP* flies were used. Scale bars represent 100 μm. **(E–G)** Temporal knockdown of *Pdfr* using MB-GAL4 lines. All flies were raised at 25°C. The temperature was shifted during two experimental periods. **(E)** All experiments were performed at PT (25°C). **(F)** Flies were kept at RT (30°C) for 24 h before the end of conditioning. **(G)** Flies were kept at RT for 48–72 h after 7 h conditioning.

Figure 9. GAL4-expressing neurons in the adult brain in OK107 and *R41C10*.

(A, B) Brains were stained with an anti-Per antibody and anti-GFP antibody. Magenta, Per; green, mCD8::GFP. Adult brains were dissected at ZT 0–2, in which flies were entrained in more than 3 LD cycles. Partially stacked images of specific clusters of clock neurons (DN1s, LNds, and LNvs). Stacked confocal images showing DN1s, LNds or LNvs. Triangles, LNds or l-LNvs; arrows, s-LNvs. Scale bars, 50 μm (DN1s) or 10 μm (LNds and LNvs). **(A)** OK107/ UAS-*mCD8::GFP* flies were used. **(B)** *R41C10*/ UAS-*mCD8::GFP* flies were used.

Figure 10. Temporal expression of CrebB repressor in MB neurons

(A–C) The Mann–Whitney *U* test was used for comparisons of CI. The permutation test with 10000 random permutations was used for comparisons of MI. For MI, asterisks indicate a comparison between the UAS control and test groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant; *N*, sample

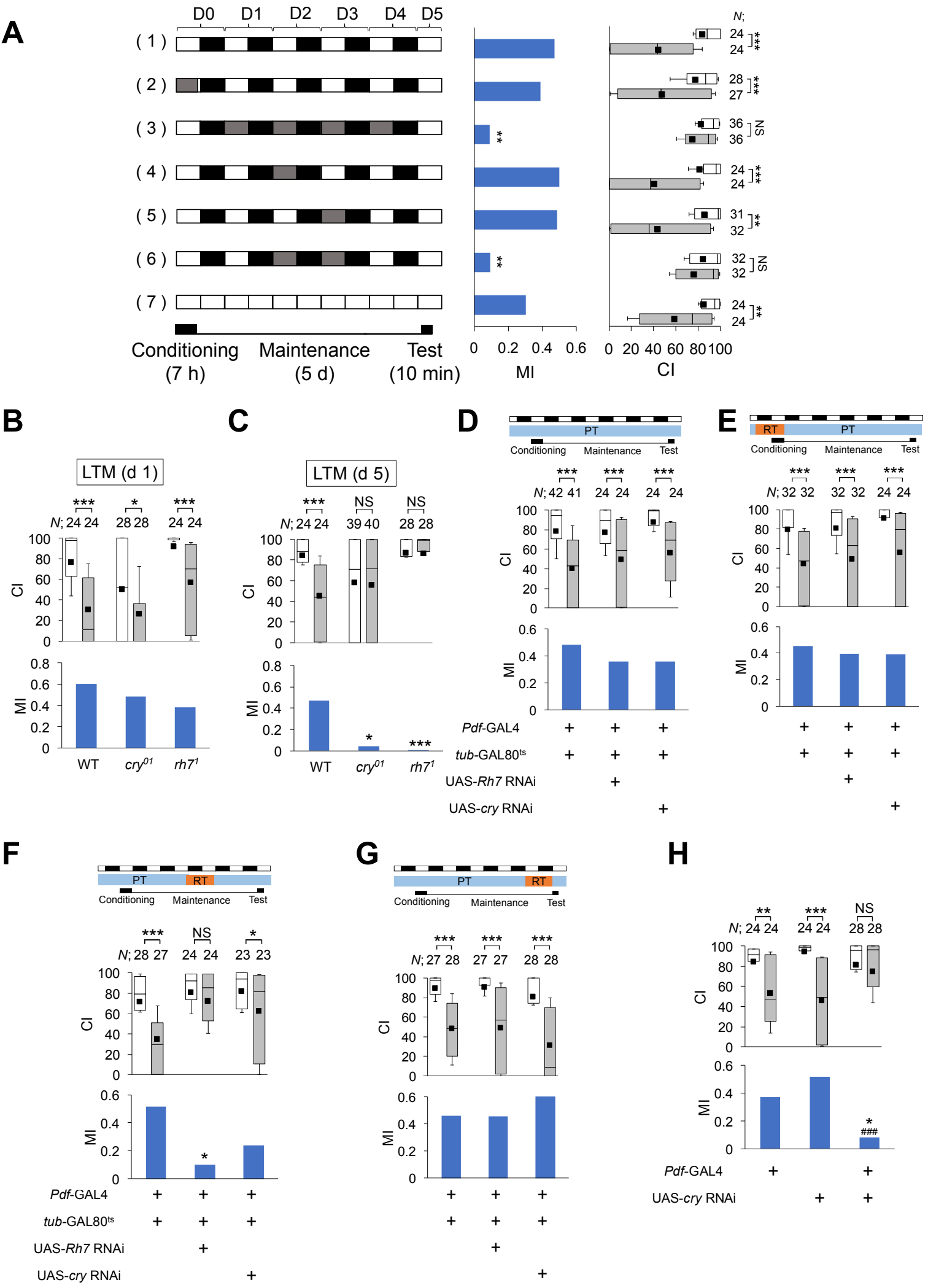
size in each box. All flies were raised at 25°C. The temperature was shifted during two experimental periods. **(A)** All experiments were performed at PT (25°C). **(B)** Flies were kept at RT (30°C) for 10 h before the end of conditioning. **(C)** Flies were kept at RT for 48–72 h after 7 h conditioning.

Figure 11. Light-dependent transcriptional activity of CrebB via Pdfr in MB α/β neurons

(A) Using UAS-*FLP*/*hs-CrebB-B*; CRE>mCherry::STOP>*luc*/*R19B03* and UAS-*FLP*/+; CRE>mCherry::STOP>*luc*/*R19B03* flies, we examined whether induction of the CrebB repressor CrebB-B inhibits CrebB activity. N = 9–10 for each bar. In heat-shock treatment, males (3–4 d old) were kept at 32°C for three days. Luc activity was measured immediately after the heat-shock treatment. Error bars indicate SEM. Student's *t*-test was used. ***, $P < 0.001$. **(B)** CrebB activity in MB α/β or γ neurons was measured in LD or DD using MB α/β-GAL4 (*R41C10*) or MB γ-GAL4 (*R55D03*). Samples were prepared between ZT 0 and ZT 2. N= 8–13 in each bar. Error bars indicate SEM. One-way ANOVA followed by post-hoc analysis using Scheffe's test was used. Bars with the same letter indicate values that are not significantly different ($P > 0.05$). **(C)** UAS-*FLP*/*R61G12*-LexA; CRE>mCherry::STOP>*luc* LexAop-*TrpA1*/*R41C10* and UAS-*FLP*/*R61G12*-LexA; CRE>mCherry::STOP>*luc* /*R41C10* flies were used. N = 5–6 for each bar. For activation of Pdf neurons by TrpA1, males (3–5 d old) were kept at 32°C during two days of DD. Error bars indicate SEM. Student's *t*-test was used. **, $P < 0.01$. **(D, E)** Conditioning-dependent CrebB activity in MB α/β or γ neurons was measured immediately after 7 h conditioning **(D)** and on the 2nd day after 7 h conditioning **(E)**. Student's *t*-test was used. ∗, $P < 0.05$; NS, not significant. N= 6–9 in each bar.

Figure 12. A possible model of light-driven memory maintenance system.

(A) During courtship conditioning, males receive stressors from mated females, and CrebB-dependent transcription activity increases. Consequently, memory is consolidated. **(B)** During the memory maintenance phase, light directly activates l-LNvs, and then Pdf is released. The released Pdf activates Pdfr in MB α/β neurons, and promotes CrebB-dependent transcription required for LTM maintenance.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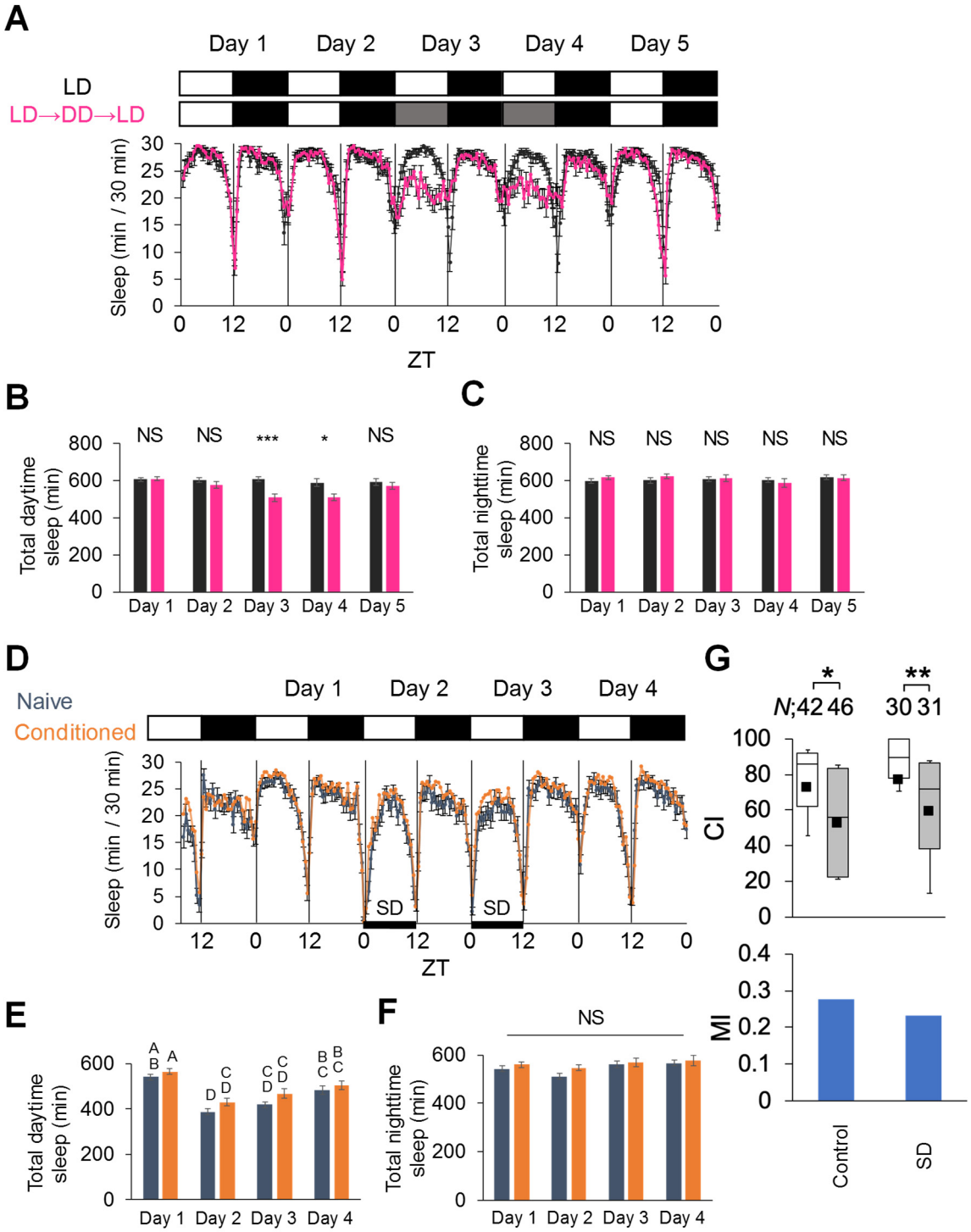
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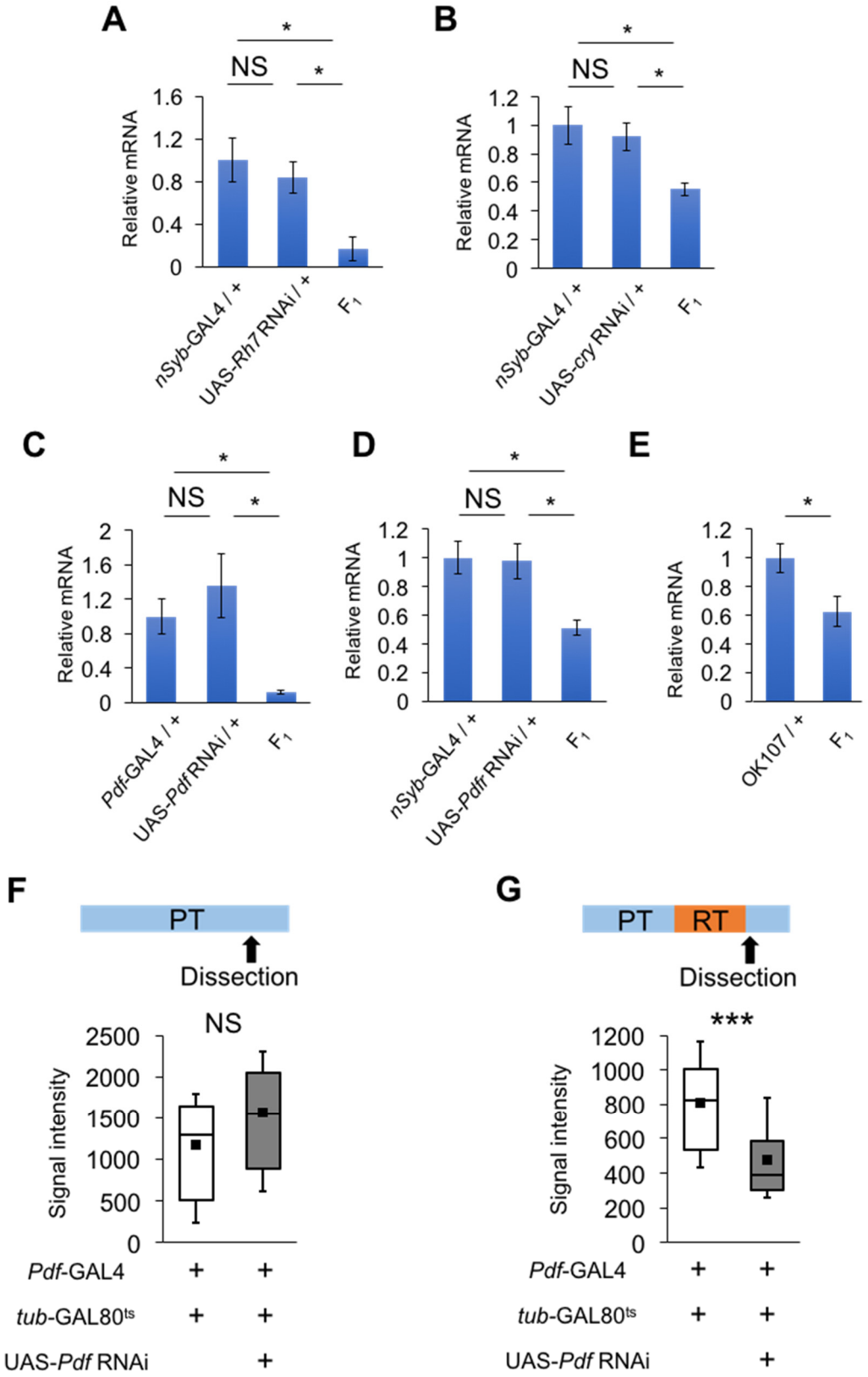
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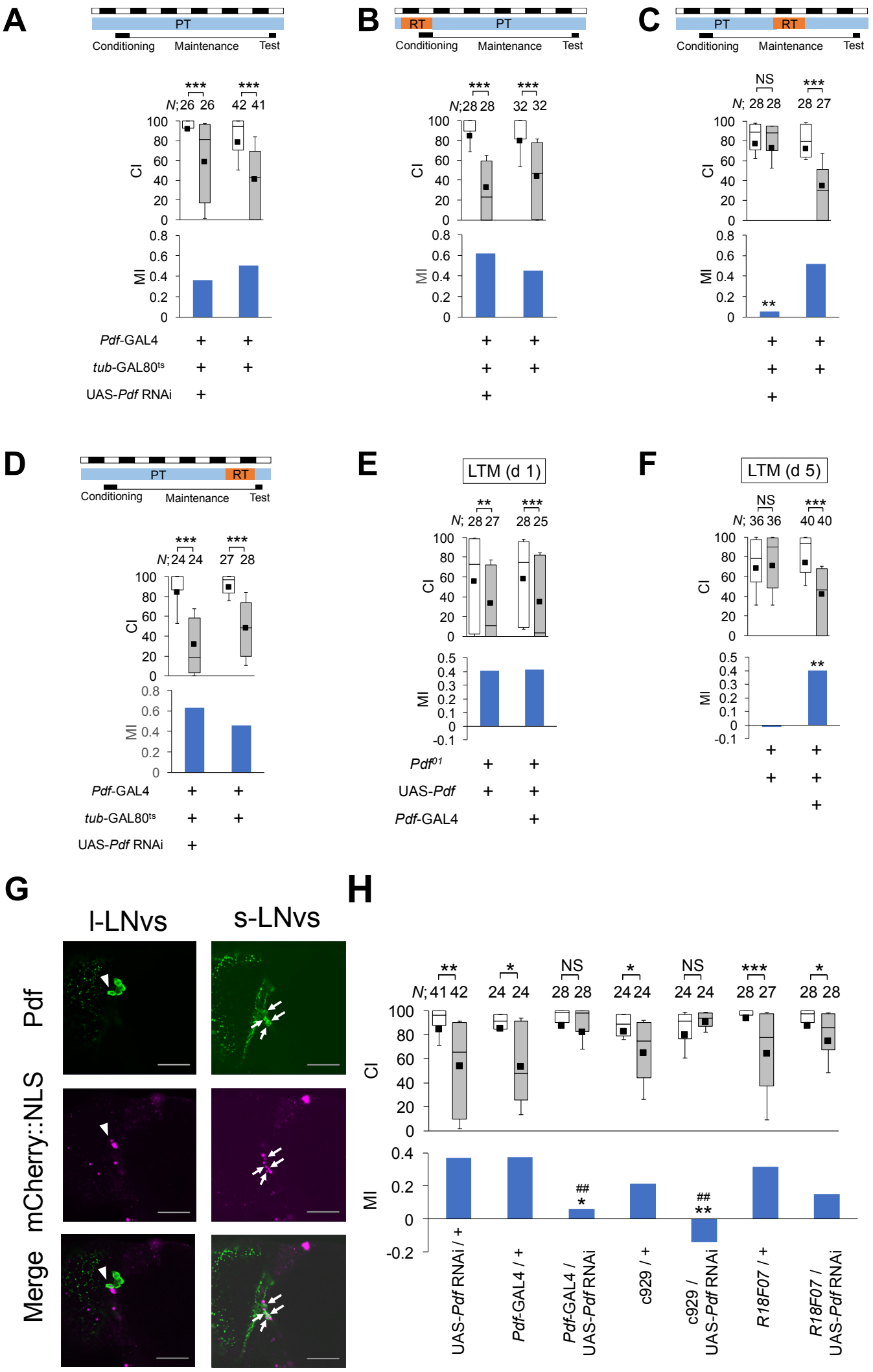
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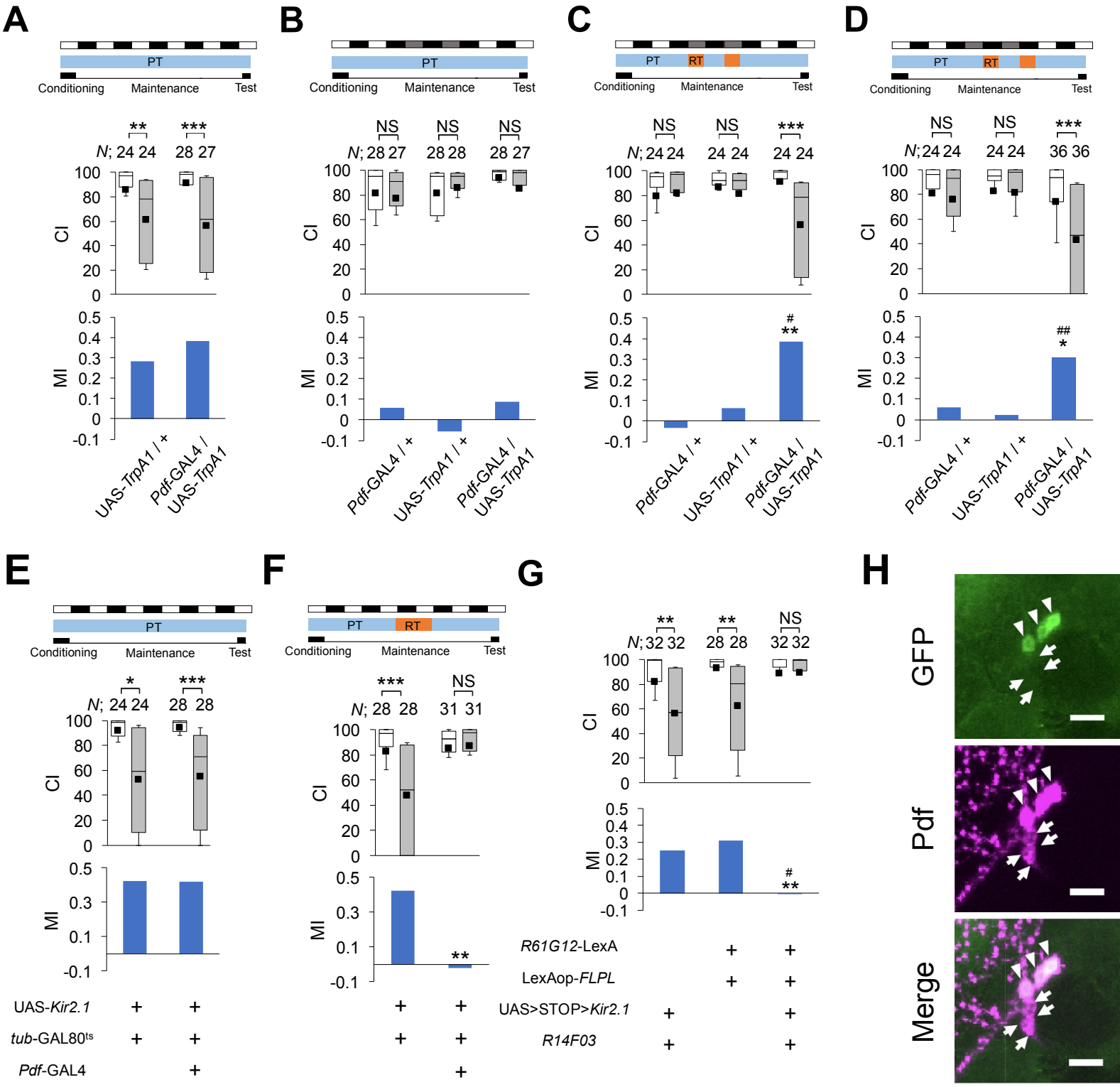
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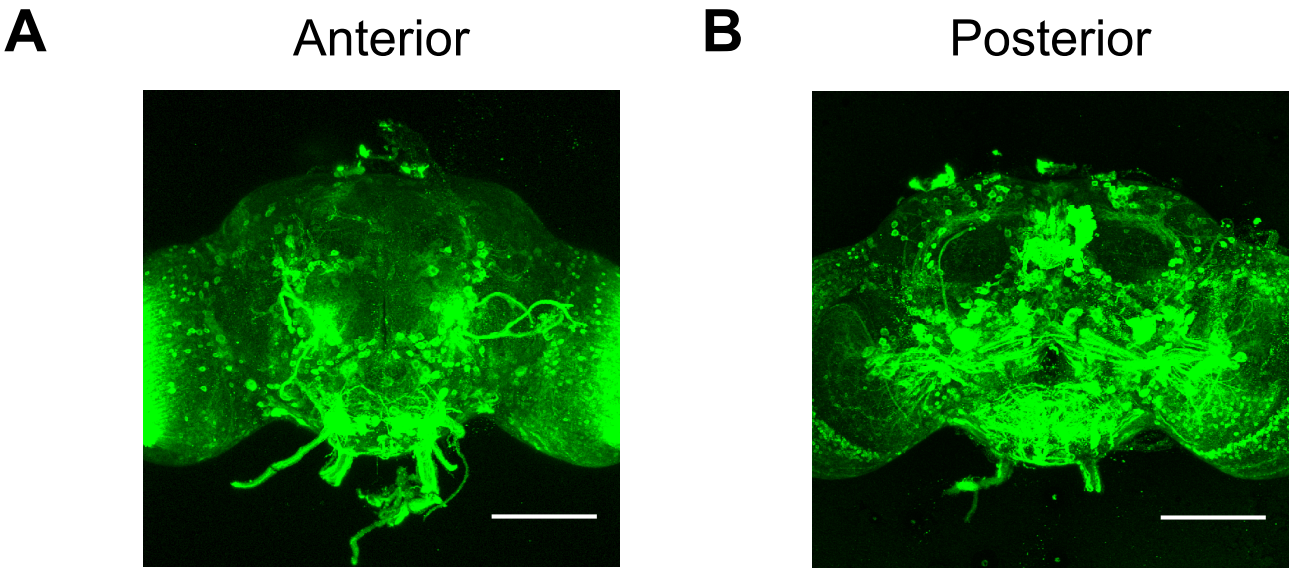

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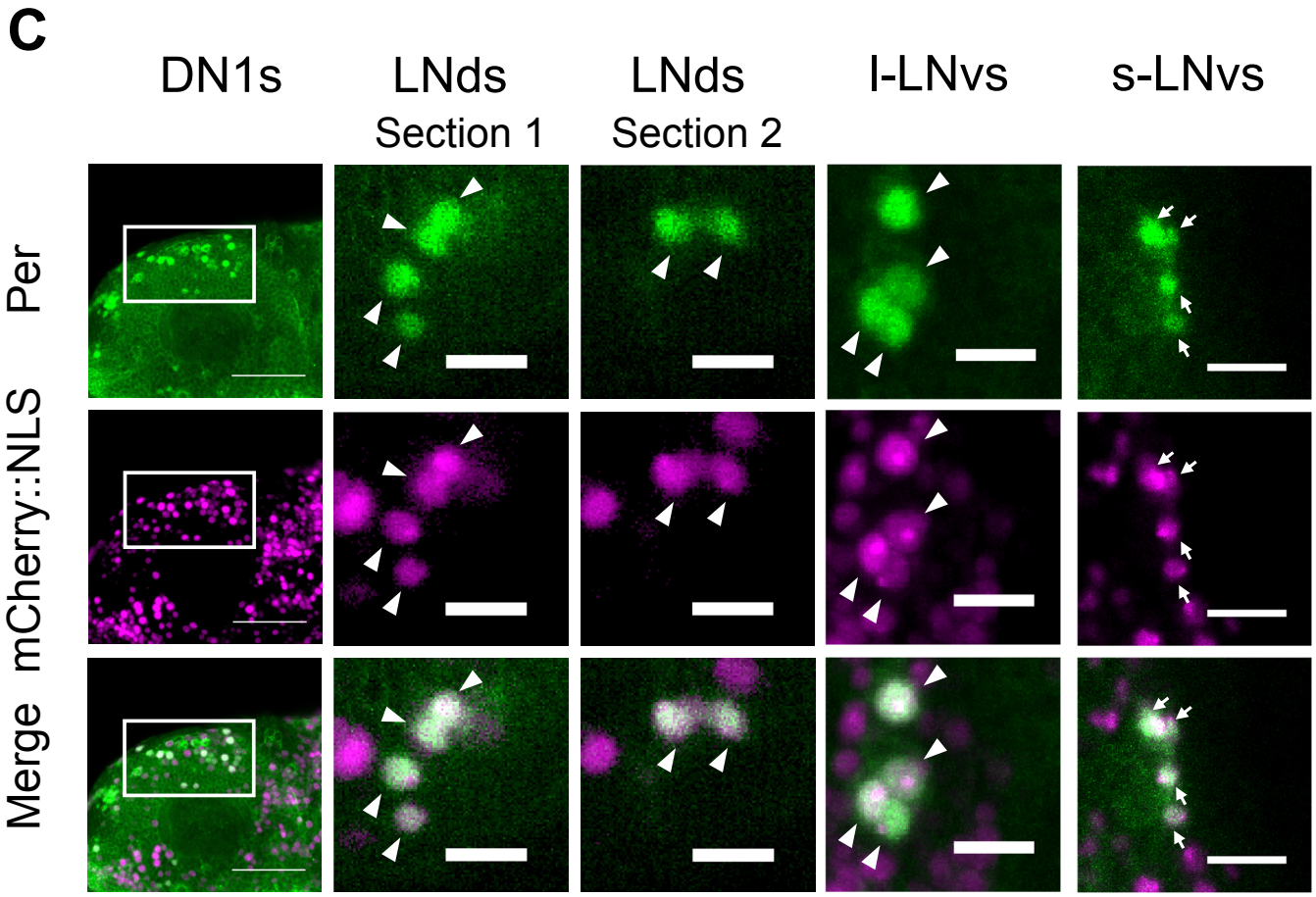

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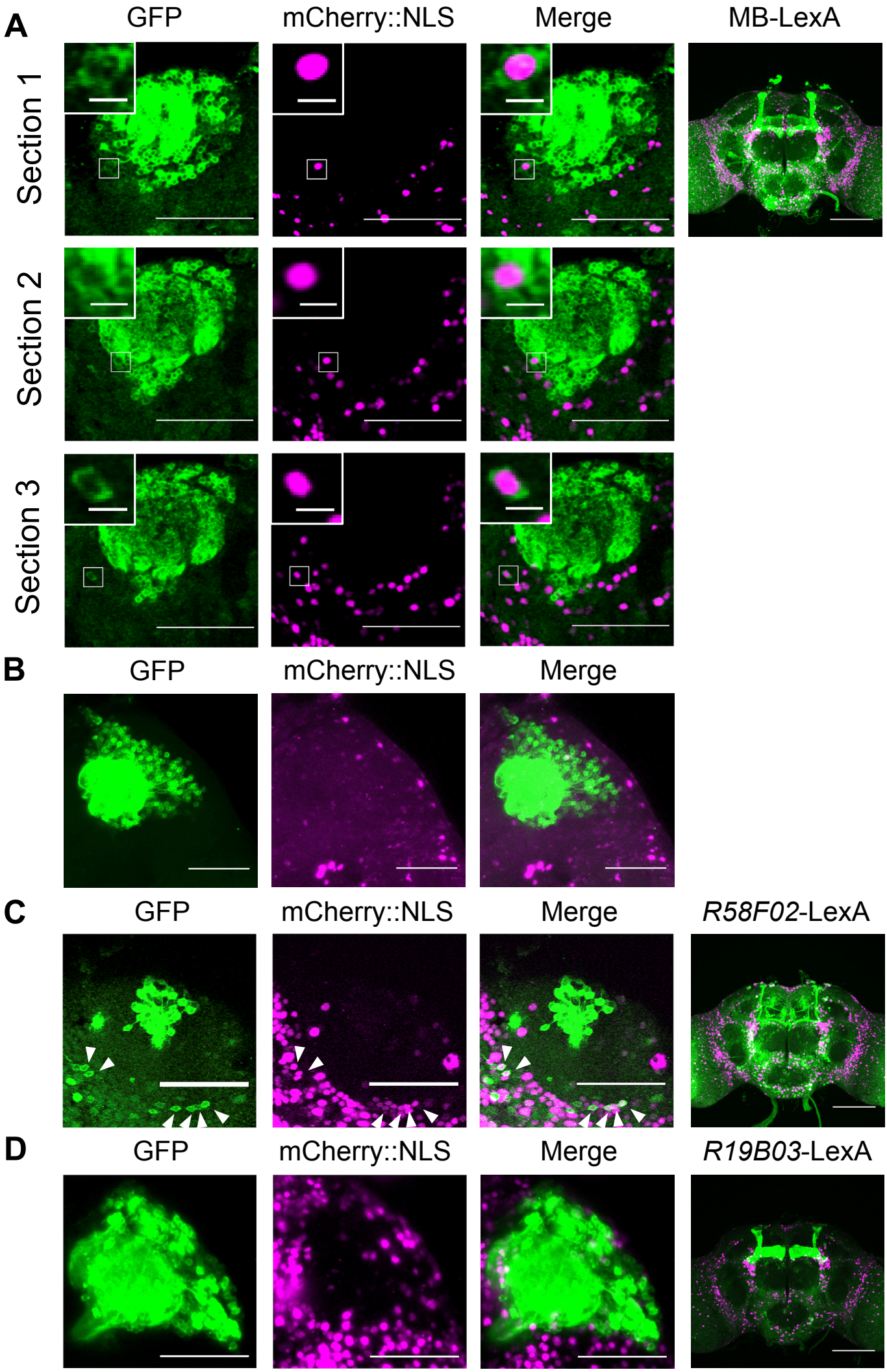
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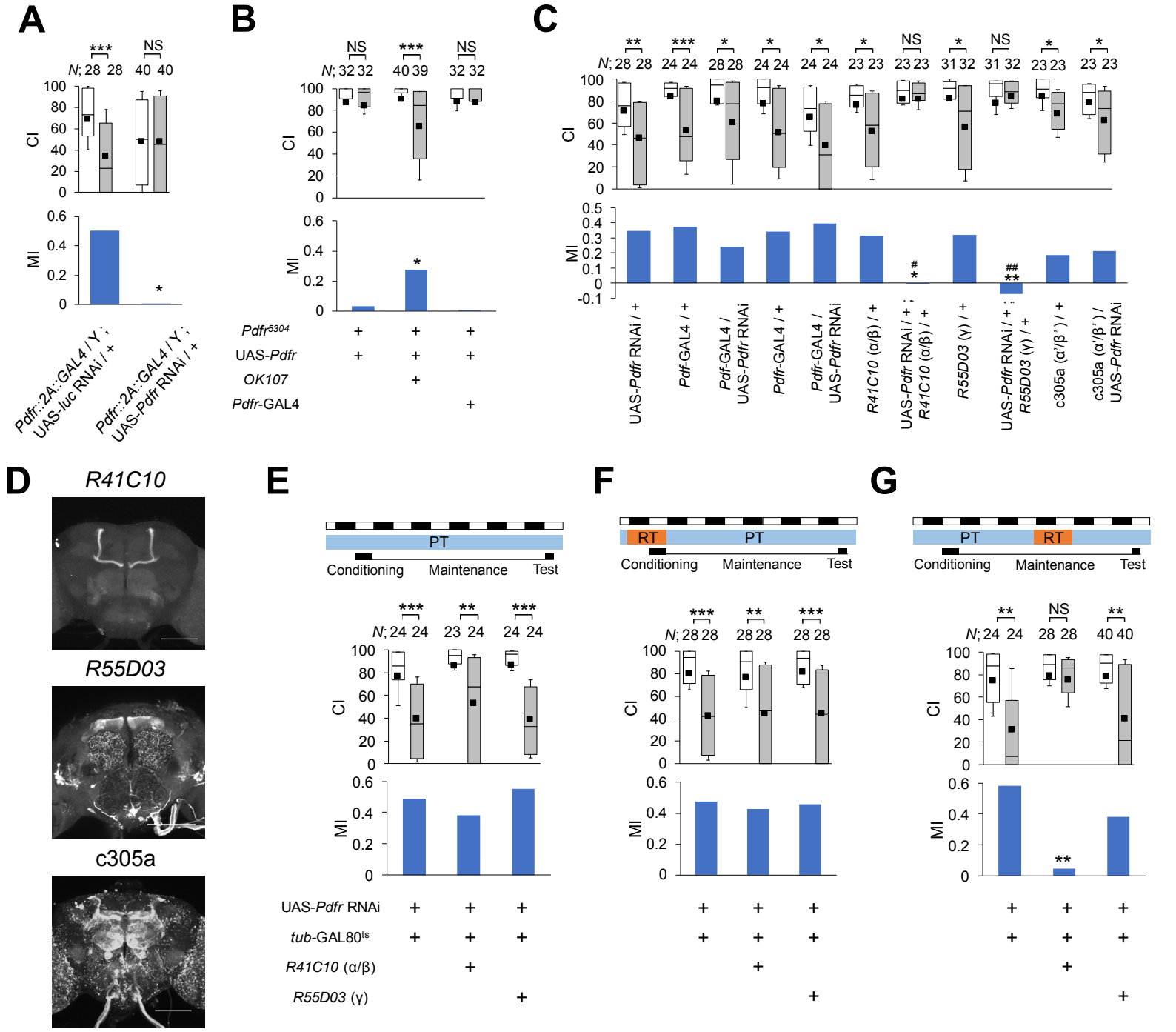
Figure 8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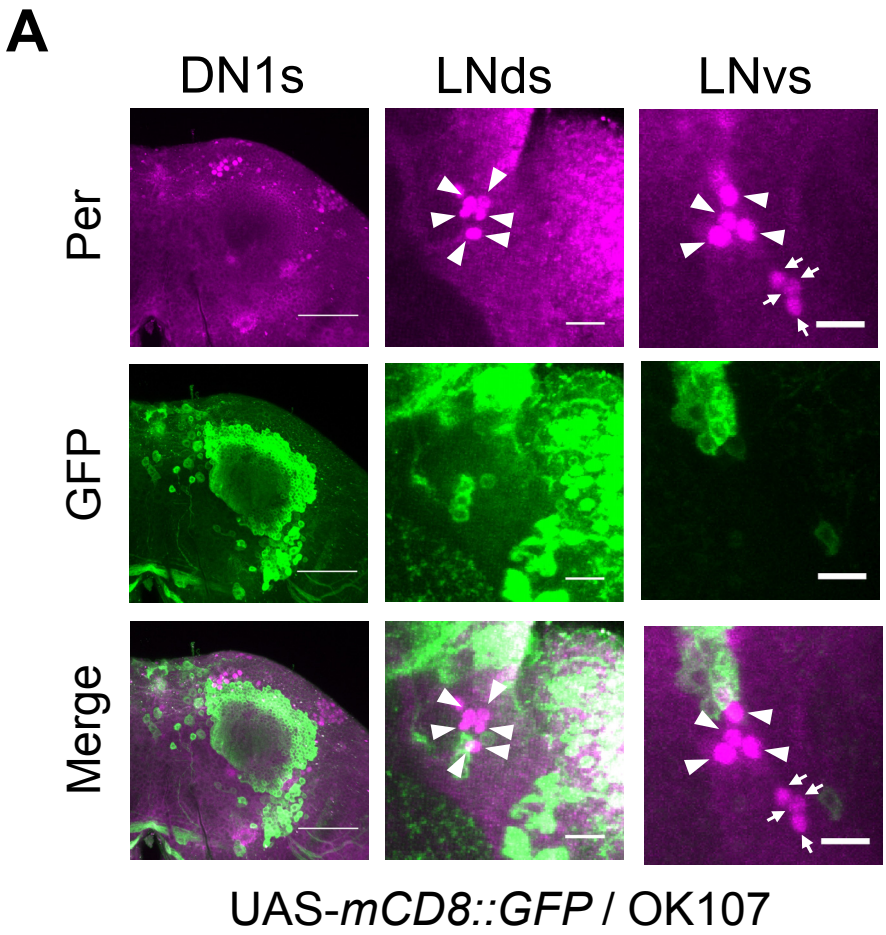
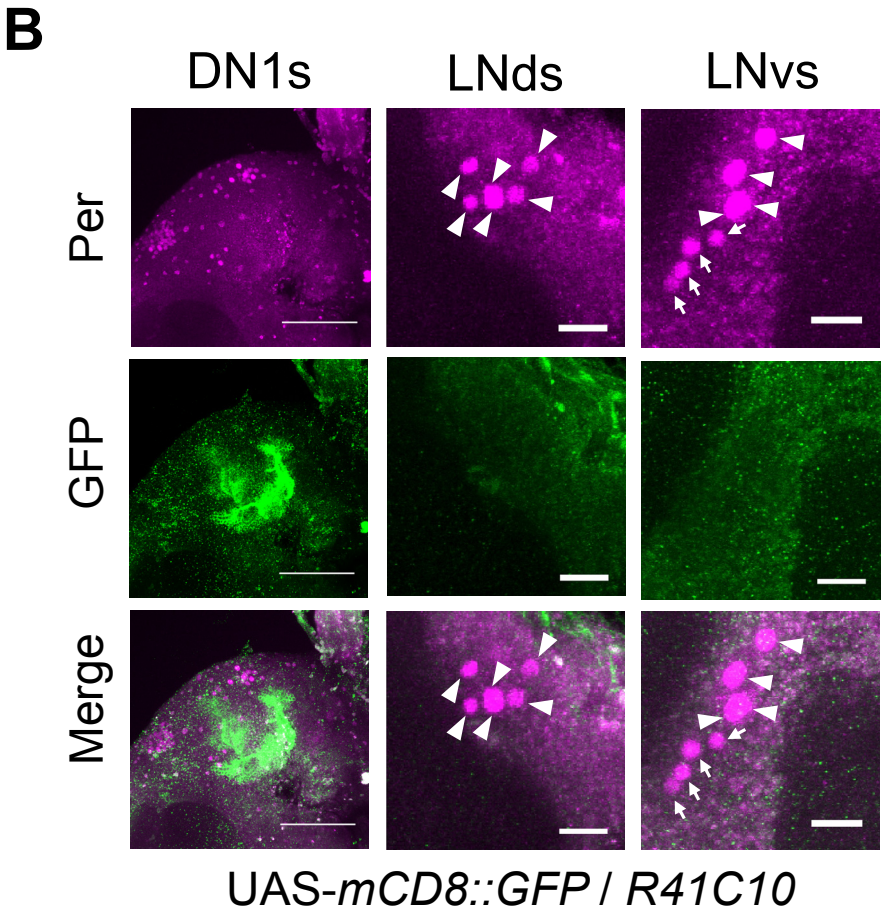
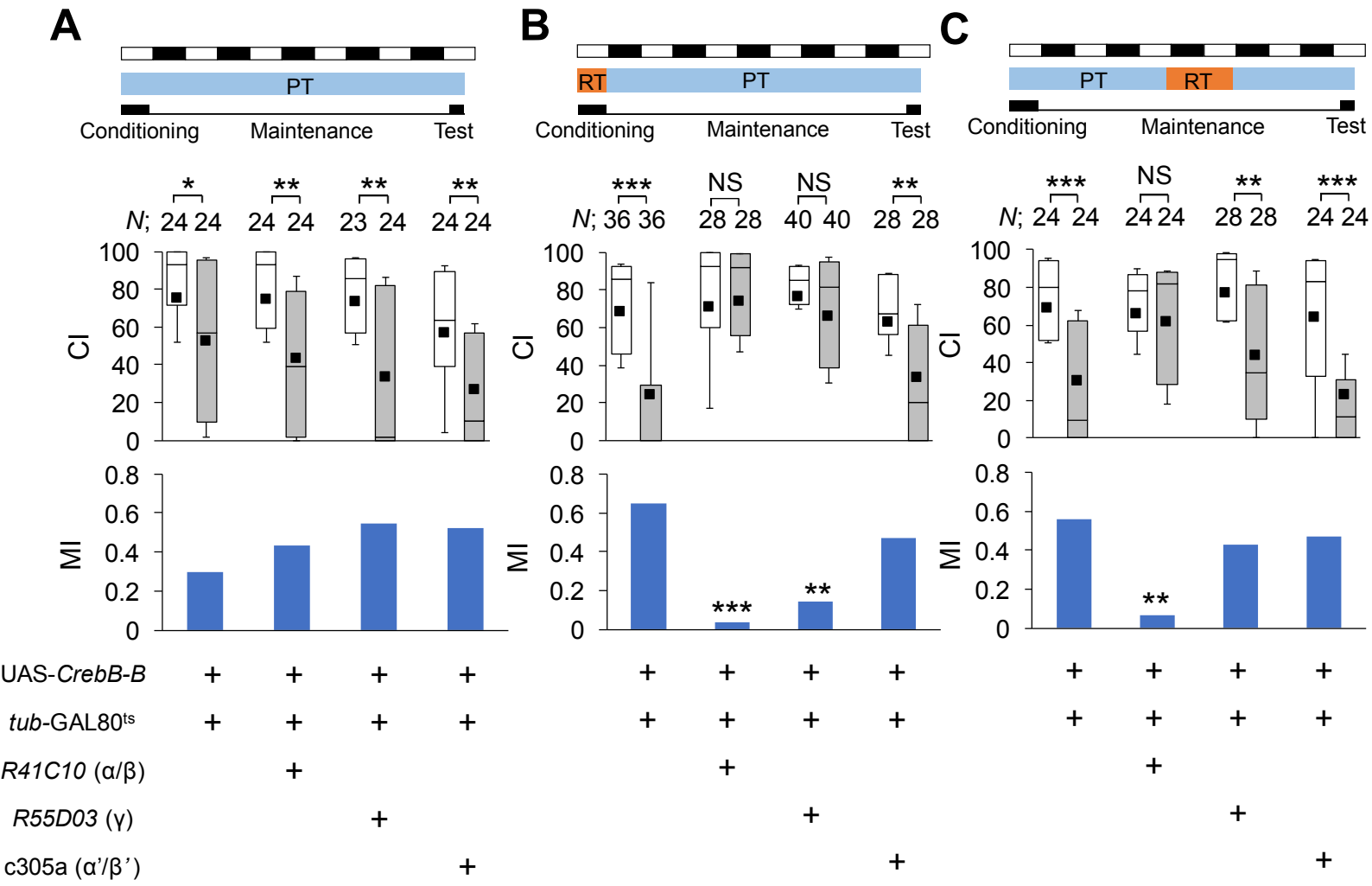
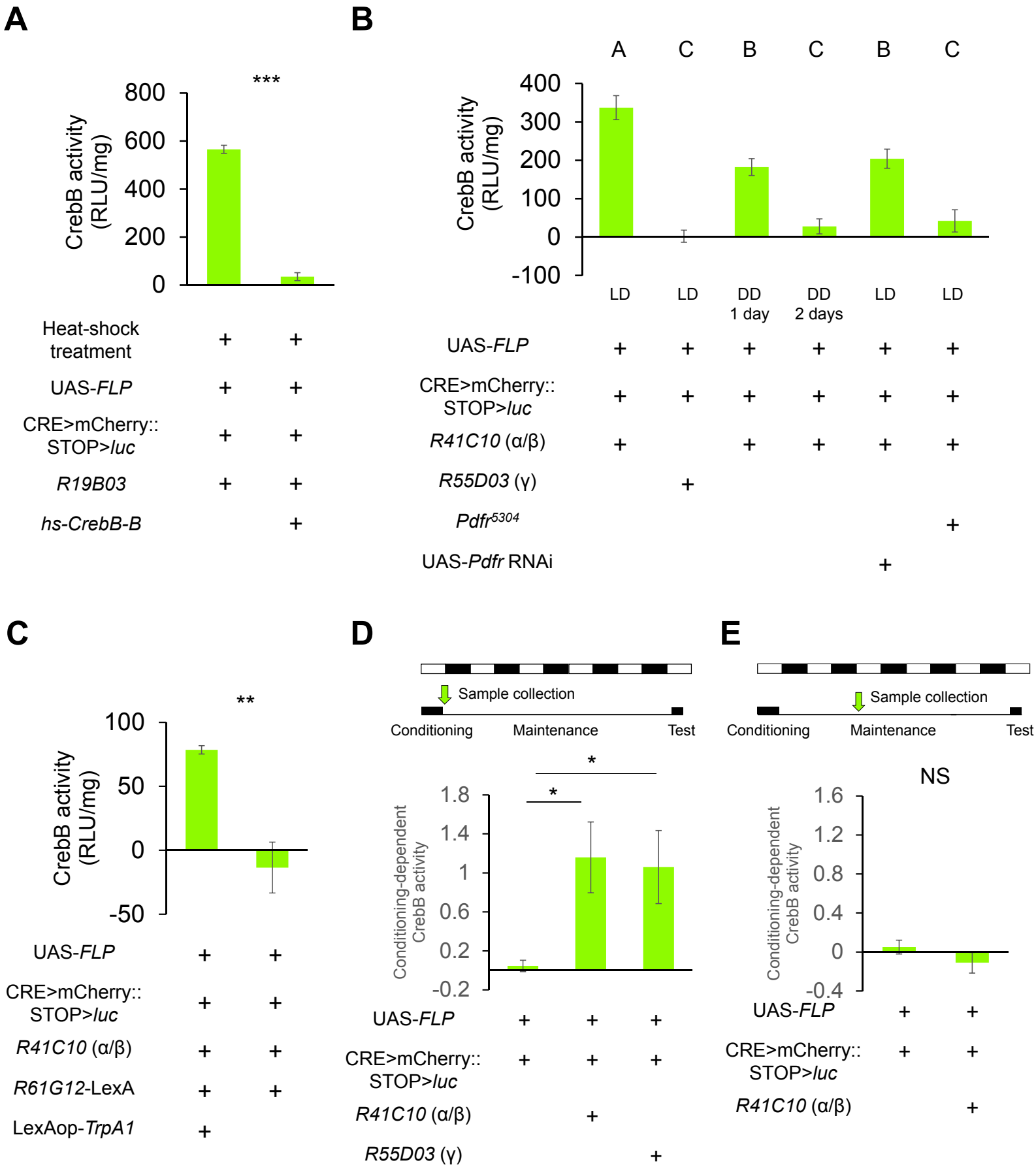
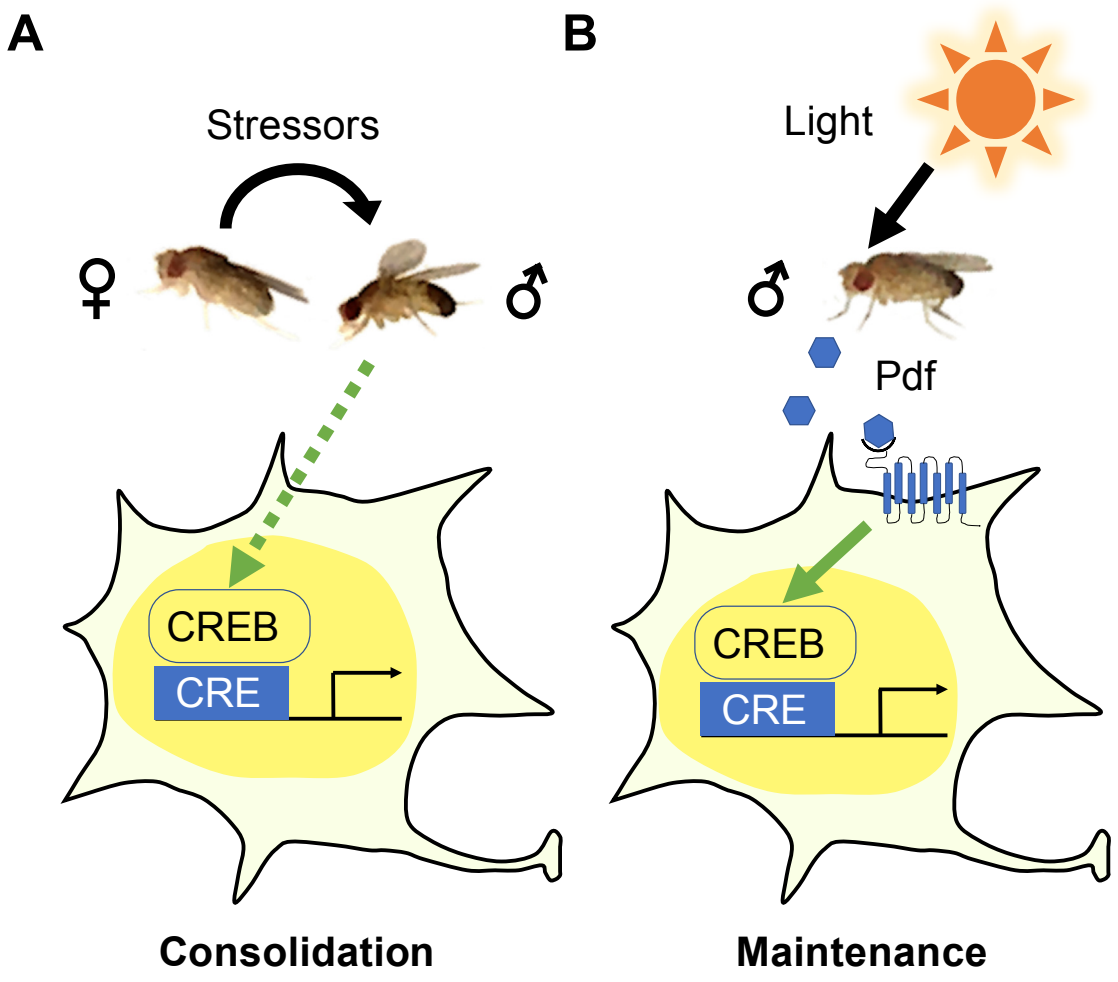
OK107
Pdfr-GAL4
UAS-Pdfr RNAi / +
Pdf-GAL4 / +
Pdf-GAL4 / UAS-Pdfr RNAi
Pdfr-GAL4 / +
Pdfr-GAL4 / UAS-Pdfr RNAi
R41C10 (α/β) / +
UAS-Pdfr RNAi / + ; R41C10 (α/β) / +
R55D03 (γ) / +
UAS-Pdfr RNAi / + ; R55D03 (γ) / +
c305a (α'/β') / +
c305a (α'/β') / UAS-Pdfr RNAi
PT
RT
Conditioning
Maintenance
Test
UAS-Pdfr RNAi
tub-GAL80ts
R41C10 (α/β)
R55D03 (γ)


A
DN1s
LNds
LNvs
Per
GFP
Merge
UAS-mCD8::GFP / OK107


B
DN1s
LNds
LNvs
Per
GFP
Merge
UAS-mCD8::GFP / R41C10


A
PT
Conditioning
Maintenance
Test
B
RT
PT
Conditioning
Maintenance
Test
C
PT
RT
Conditioning
Maintenance
Test
CI
MI
N; 24 24 24 24 23 24 24 24
N; 36 36 28 28 40 40 28 28
N; 24 24 24 24 28 28 24 24
UAS-CrebB-B
tub-GAL80ts
R41C10 (α/β)
R55D03 (γ)
c305a (α'/β')


A
***
CrebB activity (RLU/mg)
800
600
400
200
0
Heat-shock treatment + +
UAS-FLP + +
CRE>mCherry::STOP>luc + +
R19B03 + +
hs-CrebB-B +
B
A C B C B C
CrebB activity (RLU/mg)
400
300
200
100
0
-100
LD LD DD 1 day DD 2 days LD LD
UAS-FLP + + + + + +
CRE>mCherry::STOP>luc + + + + + +
R41C10 (α/β) + + + + +
R55D03 (γ) +
Pdfr5304 +
UAS-Pdfr RNAi +
C
**
CrebB activity (RLU/mg)
100
50
0
-50
UAS-FLP + +
CRE>mCherry::STOP>luc + +
R41C10 (α/β) + +
R61G12-LexA + +
LexAop-TrpA1 +
D
Sample collection
Conditioning Maintenance Test
*
*
Conditioning-dependent CrebB activity
1.8
1.4
1
0.6
0.2
-0.2
UAS-FLP + + +
CRE>mCherry::STOP>luc + + +
R41C10 (α/β) +
R55D03 (γ) +
E
Sample collection
Conditioning Maintenance Test
NS
Conditioning-dependent CrebB activity
1.6
1.2
0.8
0.4
0
-0.4
UAS-FLP + +
CRE>mCherry::STOP>luc + +
R41C10 (α/β) +


A
Stressors
♀
♂
CREB
CRE
Consolidation
B
Light
♂
Pdf
CREB
CRE
Maintenance