Brief Change in Dopamine Activity during Consolidation Impairs Long-Term Memory via Sleep Disruption

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SUMMARY

Sleep disturbances are associated with poor long-term memory (LTM) formation, yet the underlying cell types and neural circuits involved have not been fully decoded. Dopamine neurons (DANs) are involved in memory processing at multiple stages. Here, we show that brief activation of protocerebral anterior medial DANs (PAM-DANs) or inhibition of a pair of dorsal posterior medial (DPM) neurons during the first few hours of memory consolidation impairs 24 h LTM. Interestingly, sleep deprivation elevates the neural activity of PAM-DANs and DPM neurons, and brief thermos-activation of PAM-DANs or inactivation of DPM neurons results in sleep loss and fragmentation. Pharmacological rescue of sleep after this manipulation restores LTM. A specific subset of PAM-DANs, PAM-α1 that synapse onto DPM neurons specify the microcircuit that links sleep and memory. PAM-DANs, including PAM-α1, form functional synapses with DPM neurons mainly via Dop1R1 receptor to inhibit DPM. Our data suggest that the post-training activity of PAM(-α1)-DPM microcircuit, especially during memory consolidation, plays an essential role in maintaining the sleep necessary for LTM consolidation, providing a new cellular and circuit basis for the complex relationship between sleep and memory.

Key Words: dopamine, PAM neurons, DPM neurons, memory consolidation, long-term memory, sleep, fragmentation, basal activity, *Drosophila*

INTRODUCTION

Memory consolidation is a time-dependent process which, post-encoding allows maturation of those initially formed but unstable memories (Dudai et al., 2015; McGaugh, 2000). Neurons undergo a cascade of events that actively remodel the connections at both synaptic and system levels to consolidate newly formed memories (Tonegawa et al., 2018). Systems consolidation, referring to the biological interaction between brain regions or circuits, results in establishment of an information flow for

the neuronal ensemble during memory transformation (Dubnau and Chiang, 2013). Sleep, as one of the most important physiological processes that affects memory, has been shown to influence several different memory stages. Sleep deprivation (SD) before training compromises memory formation, while SD post-learning impairs memory consolidation (Dag et al., 2019; Li X, 2009; Spira et al., 2014). The neural replay of memories during sleep, in particular, has been considered to be part of the active process of memory consolidation (Klinzing et al., 2019). However, the neural mechanisms that bridge sleep and memory consolidation are still largely unclear.

The fruit fly *Drosophila melanogaster* is a valuable model for uncovering the mechanistic underpinnings of memory, and studies in this organism have contributed to revealing the nature of the representations of memory traces formed in associative conditioning paradigms (Aso and Rubin, 2020; Adam and Griffith, 2021). Classical olfactory conditioning, which stems from studies on Pavlov's dogs (Pavlov, 1927), forms a memory of a conditioned stimulus (CS, an odour) as a pleasant or an unpleasant prediction of an unconditioned stimulus (US, a reward or a punishment) after pairing the CS with the US (Quinn WG et al., 1974; Tempel B L et al., 1983; Tully T et al., 1985). Multiple types of neurons have been identified involved in distinct memory processes. Dopamine neurons (DANs), as an indispensable part of the US pathway, convey reinforcement signals to spatially segregated subdomains of the mushroom body (MB) to form appetitive or aversive short-term memories (STM) (Aso et al., 2012; Burke et al., 2012; Liu et al., 2012). In addition to their role in STM formation, DANs also participate in different phases of LTM (Placais et al., 2012). The different subsets of DANs are morphologically and functionally distinct (Aso et al., 2014a; Mao and Davis, 2009). Persistent activity in PPL1-γ1pedc and -γ2α'1 subtypes interferes with STM and anesthesia-resistant memory (ARM), and promotes forgetting (Berry et al., 2015; Berry et al., 2012; Placais et al., 2012). PPL1-α2α'2 and PPL1-α3 mediate aversive LTM formation with persistent post-training activity (Feng et al., 2021). A delayed post-training activity in aSP13-DANs is required for LTM consolidation of

courtship learning (Kruttner et al., 2015). The function of many of other subsets remains uncharacterized.

The MB, a multi-functional unit analogous to the hippocampus in mammals, encodes and integrates CS and the US information in a compartmentalized manner. Dopamine (DA) released from DANs acts directly on the MB intrinsic neurons via DA receptors (Kim et al., 2007; Qin et al., 2012). Functional observations revealed that ongoing activity in α'β' neurons is required to maintain memory during the first hour after training (Krashes et al., 2007). In addition, MB αβ core neurons have been shown to act as a gate to permit LTM consolidation (Huang et al., 2012). These data imply that during memory consolidation, a DA signal cascade recruits multiple subsets of neurons to exert sequential modulation of these functional loops. Upstream of the MB, two pairs of neurons, dorsal paired medial (DPM) neurons and anterior paired lateral (APL) neurons, have been well characterized for their requirement in memory consolidation (Keene et al., 2006; Keene et al., 2004; Waddell S et al., 2000; Zhang et al., 2013). In addition to memory processes, DA also regulates wakefulness and sleep by acting on distinct downstream targets (Donlea Jeffrey M., 2011; Kume et al., 2005; Tomita et al., 2021; Ueno et al., 2012; Van Swinderen and Andretic, 2011). Subsets of PAM-DANs have been suggested to signal wakefulness and project to wake-promoting compartments of the MB (Driscoll et al., 2021). Furthermore, DPMs have been shown to promote sleep via GABA signaling (Haynes et al., 2015). It is unknown about whether memory consolidation and dynamic sleep changes are bridged by persistent activity of DAN-DPM connections.

In this study, we show that PAM neurons form inhibitory connections on DPM neurons mediated mainly via the Dop1R1 receptor. Interestingly, activation of PAM neurons or inactivation of DPM neurons during the first few hours of memory consolidation results in an impairment of LTM. Furthermore, we observed a sleep reduction and fragmentation after brief disruption of these neurons. One the flip side, the neural activity of PAM and DPM were elevated upon sleep deprivation, suggesting

that this microcircuit is responsive to sleep status. Finally, we identified a specific subset of PAM neurons that are sufficient and necessary to modulate memory consolidation via sleep regulation, and characterized their functional connection with DPM neurons as well as their dynamic neural activity post-training. Maintenance of basal activity of DANs during memory consolidation has a profound effect on LTM, through influencing sleep in an internal state-dependent manner.

RESULTS

PAM neurons form functional synapses and inhibit downstream DPM neurons

PAM neurons project to the horizontal lobes of the MB where they participate in formation of appetitive memory (Burke et al., 2012; Liu et al., 2012) (Figure 1A), and a pair of DPM neurons intensively innervates all lobes of the MB and is important for consolidation of memory (Keene et al., 2006; Yu et al., 2005) (Figure 1A). The electron microscope (EM) connectome database have shown the synaptic connection from PAM to DPM neurons with 4709 synapses (Scheffer et al., 2020; Zheng et al., 2018; Plaza et al., 2022). To determine if PAM and DPM neurons sequentially function together to regulate distinct memory processes, we first needed to determine how they were connected. We investigated the connectome-predicted structural and functional connection between PAM and DPM neurons using GFP Reconstitution Across Synaptic Partners (GRASP) and activity-dependent GRASP (Macpherson et al., 2015; Feinberg et al., 2008). Signals from GRASP and active GRASP, which detect physical proximity of neurons, were detected in the horizontal lobes of the MB in flies with split-GFP fragments (spGFP1-10/syb::spGFP1-10 and CD4::spGFP11) expressing in PAM neurons labeled by R58E02-GAL4 or -LexA and DPM neurons labeled by L0111-LexA or eyeless-GAL80;MB-GAL80;c316-GAL4, respectively, showing that PAM neurons likely to connect to DPM neurons functionally (Figure S1A). Further, using a restricted *trans*-Tango, a technique to probe the synaptic connections between GAL4-labeled upstream neurons and LexA-labeled targeted neurons (Sun et al., 2022), we

confirmed that DPM neurons are the downstream target of PAM neurons (Figures 1B and S1C).

To investigate the functional connection from PAM to DPM neurons, we expressed $P2X_2$ receptors in PAM neurons using R58E02-LexA, and GCaMP6f in the DPM neurons using VT064246-GAL4, and found that direct activation of PAM neurons by bath application of ATP resulted in a strong inhibition of DPM activity (Figure 1C). To understand which dopamine receptor mediates this inhibition, we knocked down different dopamine receptors in DPM neurons using VT046004-GAL4, and imaged EPAC in DPM, an indicator of cAMP levels, after application of dopamine. Knockdown Dop1R1 receptors in DPM neurons significantly rescued the dopamine-induced decrease in cAMP, whereas knockdown Dop1R2 and DopEcR trended to further decrease the cAMP level (Figure 1D). These data suggest that dopamine released from PAM neurons acts on DPM mainly via Dop1R1 receptors, but that other receptors may also participate in modulating the neural activity in a complex way. Our observations suggested the hypothesis that PAM neurons may be involved in memory consolidation via a direct synaptic connection with DPM neurons.

PAM and DPM neurons both influence memory consolidation

To understand the biological function of the inhibitory connection from PAM to DPM neurons, we wanted to ask whether PAM and DPM neurons sequentially function in distinct memory processes or whether they are part of a single pathway. We first confirmed their roles in learning or consolidation, employing an appetitive olfactory conditioning paradigm which is able to induce LTM with a single session of training (Krashes and Waddell, 2008). Consistent with our previous work, activation of PAM neurons paired with an odour in non-starved and starved flies was sufficient to induce immediate appetitive memory (Figure S1D) (Liu et al., 2012). Blocking the PAM neurons during training using shibire^{ts1} (shi^{ts1}), which inhibits neuronal output at high temperature (Krashes et al., 2009), resulted in 24 h appetitive memory defects (Figure S1E). DPM neurons have been found an essential role in memory consolidation during

a critical window for consolidation (Cervantes-Sandoval and Davis, 2012; Keene et al., 2006; Waddell S et al., 2000; Yu et al., 2005). Inactivation of DPM neurons after training for 2.5 h resulted in 24 h memory deficit (Figures 1E and S1F), but no effect on 24 h memory when they were blocked before and during training (Figure S1G). Since PAM neurons can inhibit DPM neurons, we wondered whether PAM neurons participate in the consolidation process and used conditional activation/inactivation to probe their role. Activation of PAM neurons expressing dTrpA1 (Hamada et al., 2008) driven by R58E02-GAL4 for 1 h after training, resulted in an impairment of 24 h memory (Figure 1F). Without activation, *R58E02-GAL4/UAS-dTrpA1* flies and their genetic controls formed normal 24 h memory (Figure S1H). Blocking the PAM neuronal activity by shi^{ts1} at restrictive temperature of 32 °C after training for 2.5 h failed to disrupt 24 h memory (Figure S1I), suggesting that activity of PAM neurons is necessary in the consolidation window or that heterogeneity in the effects of subsets of PAMs masks any phenotype.

Increased activity in PAM neurons results in reduced and fragmented sleep

It has been suggested that one of the important functions of DPM in consolidation is its ability to regulate sleep (Haynes et al., 2015). To understand whether the impairments in LTM that occurred with activation of PAM neurons after training were attributable to sleep disruption, we monitored sleep under starvation and normal feeding conditions before (LP1), during and 24 h after activation/inactivation (DP1 and LP2) using the Drosophila Activity Monitor (DAM2) System. We measured sleep amount, the number of sleep episodes and P(wake), a parameter which reflects sleep depth (Liu et al., 2019; Wiggin et al., 2020). Sleep data were analyzed in one-hour bin to assess dynamic changes from the time course of starvation treatment before training to the time when the 24 h memory tests were done in the memory experiments (Figures 2A and 2E). Activation/inactivation periods were applied in the same time windows that reliably disrupted LTM 1 h for the PAM neurons and 2.5 h for the DPM neurons. Interestingly, a reduction in sleep occurred a few hours after activation of PAM neurons (Figures 2A

and 2B), possibly due to a reduced number of episodes (Figure 2C) with an increased possibility of waking (Figure 2D). Although LTM impairment was similar in the memory experiments, unlike the sleep change after activation of PAM neurons, inactivation of DPM neurons resulted in a stronger, long-lasting sleep reduction and fragmentation that was delayed until the dark period after inactivation (Figures 2E-2H and S2A). Unexpectedly, inactivation of PAM neurons led to a sleep change similar to that seen with inactivation of DPM neurons (Figure S2B), suggesting that perhaps a specific subsets of PAM neurons was important for linking memory consolidation and sleep via modulation of DPM. The weak effect of activation of the large R58E02-GAL4+ group of PAM neurons on sleep could be due to masking of the role of a PAM subgroup.

Appetitive LTM expression requires that the animal be motivated by hunger (Liu et al., 2012) and starvation is known to suppresses sleep (MacFadyen U.M. et al.,1973; Keene et al., 2010). To determine whether the memory defect and sleep disruption are linked to a certain internal state, we also analyzed the sleep effects of manipulation of PAM and DPM neurons under a no starvation condition. Without starvation, activation of PAM neurons led to a significant decrease in total sleep during the activation period, and also to a significant reduction in the early night (Figure S2C), suggesting a complex role for PAM neurons in regulation of sleep. Inactivation in the fed condition of PAM neurons (Figure S2D) or DPM neurons (Figures S2E-S2F) produced almost no change in sleep pattern compared to genetic controls before, during and after manipulation. Taken together, these observations again raise the possibility that subtypes of PAM neurons act onto DPM neurons in a context-dependent manner.

The complex effects of activating and inactivating the PAM/DPM microcircuit suggested that maintenance of basal activity PAM neurons after training may be critical for consolidation. We expressed GCaMP7s in R58E02-GAL4+ neurons and evaluated the normalized fluorescence level at two time points. In line with the prediction, the GCaMP level in the CS+US trained group did not differ from the untrained group right

after training (Figures S3B and S3C) or an hour after training (Figures S3D and S3E), suggesting that maintenance of the basal activity of PAM neurons be an important early phase of consolidation. Consistent with previous observations (Yamagata et al., 2016), both CS alone and US alone presentation suppressed the basal activity of PAM neurons (Figures S3B and S3C).

The finding that post-training SD can block LTM consolidation (Ganguly-Fitzgerald et al., 2006; Donlea et al., 2011; Dag et al., 2019), coupled with our observation that increased PAM activity or blocked DPM activity during consolidation impairs LTM, let us to speculate that the memory impairment caused by sleep disturbance may be due to abnormal neural activity in this microcircuit. To test this hypothesis, we examined the post-training GCaMP signal in these neurons after SD compared to a no SD condition. Training increases signal in PAM neurons and decreases signal in DPM neurons, and 1 h SD significantly increases the GCaMP levels of both PAM neurons and DPM neurons (Figures 2I-2K), supporting at causal link between neural activity in this microcircuit and sleep during consolidation to 24 h memory.

The PAM-α1 subset of PAM neurons contributes to linking memory consolidation and sleep

Two distinct subsets of PAM neurons have been identified to signal reward for STM and LTM (Yamagata et al., 2015). LTM-PAM neurons are labeled by R15A04-GAL4, innervating the MB subdomains of β2, α1, β'1, γ5 and pedunculus (Figure 3A), and STM-PAM neurons labeled by R48B04-GAL4, innervating β'2, β1, and γ1-5 (Figure 3C). To address which subsets of PAM neurons are involved in the process of consolidation, we employed the same single session of appetitive conditioning protocol, activated these neurons after training for 1 h, and examined their 24 h memory. We found that activation of LTM-PAM neurons impaired 24 h memory (Figure 3B). In contrast, activation of STM-PAM neurons did not affect 24 h memory (Figure 3D). Thus, LTM-PAM neurons participate in the process of memory consolidation.

To identify which subset(s) of LTM-PAM neurons are involved in the regulation of memory consolidation, we tested split-GAL4 drivers labeling more specific groups. MB213B-, MB025B-, MB032B- and MB315C-GAL4+ neurons innervate to β1/β2, β'1, β'2 and γ5 subdomains, respectively (Figure 3E). MB299B-GAL4+ (which labels 24 PAM neurons) and MB043B-GAL4+ (which labels 16-18 PAM neurons) label the α1 subdomain (Figure 3E). We trained flies expressing dTrpA1 under control of each of these drivers, +/- 1 h activation after training, and tested 24 h memory. Intriguingly, only activation of MB299B-GAL4+ and MB043B-GAL4+ neurons impaired 24 h memory compared to their no-activation groups (Figure 3F). These data indicate that elevated activity of PAM-α1 neurons during consolidation impedes LTM.

To further clarify the requirement of PAM-α1 neurons in memory consolidation, we either activated PAM-α1 neurons or blocked the output of PAM-α1 neurons for 1 h or 2.5 h after training, and tested 24 h memory of the experimental flies along with their genetic controls. Activation of both MB299B-GAL4 and MB043B-GAL4 labeling PAM-α1 neurons resulted in an impairment of 24 h memory (Figures 3G and S4A). Blockade of MB299B-GAL4+ but not MB043B-GAL4+ neurons also resulted in 24 h memory defect (Figures 3H and S4B), perhaps reflecting a difference in the number or identity of the PAM-α1 neurons in each line.

PAM-α1 neurons have been shown to be important for LTM formation, memory retention and updating memory (Aso and Rubin, 2016; Huetteroth et al., 2015; Ichinose et al., 2015; Yamagata et al., 2016). To clarify if their action is specific to consolidation or to forgetting or retrieval, we activated and/or blocked PAM-α1 neurons labeled by MB299B-GAL4 prior to and or during the 24 h memory test. Activation of PAM-α1 prior to retrieval impaired 24 h memory (Figure S4C), but activation and inactivation of PAM-α1 neurons during retrieval did not affect 24 h memory (Figures S4D and S4E). These data suggest that PAM-α1 neurons may interact with the active forgetting process during a specific window, but they are not required for retrieval. In summary, our

findings indicate that the normal activity of PAM-α1 neurons after training is critical to maintain the processing of memory consolidation.

To investigate whether 24 h memory defects were attributable to disrupted sleep upon changed activity of PAM-α1 neurons, we recorded and analyzed sleep. Neither activation nor inactivation in fed flies had a major effect on sleep (Figures S4F-S4I). To our surprise, activation of PAM-α1 neurons labeled by MB299B and MB043B under starvation condition, reduced sleep amount mainly during DP1 (Figures 3I and S5A), reminiscent of DPM suppression. The night after activation of PAM-α1 neurons, flies exhibited a significantly increased number of episodes and P(wake) (Figures 3I and S5A). Inactivation of starved MB299B neurons had little effect on sleep, but inactivation of PAM-α1 labeled by MB043B produced a strong reduction and fragmentation of sleep under starvation (Figures S5B and S5C), which might be due to 6-8 neurons difference between the two drivers. In aggregate, these data demonstrate that a brief increase in activity of PAM-α1 neurons induces reduced and fragmented sleep. The 24 h memory defect resulting from this change of PAM-α1 activity during consolidation is possibly due to a delayed effect on nighttime sleep, and is completely dependent on internal starvation status.

A specific PAM-α1-DPM inhibitory microcircuit binds memory consolidation and sleep via maintaining basal activity post-learning

Using a broadly-expressing driver that captures 148/137 PAM neurons of each hemisphere, we showed that PAM-DANs form inhibitory synapses onto the DPM neurons (Figure 1). To determine if the connections between the PAM-α1 subset and DPM neurons followed the same rules as the aggregate, we determined the connectivity of this subset. From the EM connectome database (Scheffer et al., 2020; Zheng et al., 2018) and analyzed the connectivity using NeuPrint (Plaza et al., 2022), we found that 14 PAM-α1 neurons of the right hemisphere form 11, 12, 13, 12, 16, 16, 12, 17, 21 and 17 synapses with the right DPM neuron, 5 out of 14 PAM-α1 neurons form a single

synapse and one PAM-α1 neuron form 4 synapses with the left DPM neuron, based on the Hemibrain connectome dataset (Figure 4A).

To clarify the functional connection between PAM-α1 and DPM neurons, we expressed $P2X_2$, ATP receptors in PAM-α1 neurons labeled by MB299B and GCaMP6f in the DPM neurons using VT064246-LexA (III), and confirmed an inhibition from PAM-α1 to DPM neurons by observing a significant decrease in GCaMP signal after application of ATP *in vitro* (Figure 4B).

We also wanted to know if PAM-α1 and DPM activity changed post-learning, so we asked if PAM-α1 and DPM GCaMP signal changed at different time points after appetitive olfactory conditioning. We observed higher activity in PAM-α1 and DPM neurons during the daytime than the nighttime, and a significantly increased activity of PAM-α1 but not DPM one hour after the conditioning compared to the unconditioned group (Figures 4C-4E). These data suggest that PAM-α1 neurons respond differently to training than the aggregate of PAM neurons.

While it is clear that these two neuron types are connected, it was still possible that they were functioning in sleep and LTM via parallel independent pathways. If this was the case, we would predict that the effects of inactivating both neuron groups at the same time might additively decrease sleep. To determine if this was the case, we simultaneously inactivated these neurons by expressing shi^{ts1} in both cell groups, and found sleep reduction and fragmentation statistically the same as when each of them was inactivated separately under starvation (Figures 4F and S5D). This result supports a model of a PAM-DPM microcircuit which link sleep and memory consolidation. Lastly, to cement the link between sleep disruption by manipulation of this circuit and memory impairment, we employed a pharmacological method. Rescue of the sleep disturbance produced by activating PAM-α1 for an hour by feeding of gaboxadol (also called THIP), a GABA agonist that promotes sleep (Dissel et al., 2015) was sufficient to also rescue memory consolidation (Figures 4G and 4H). Taken together, these results

support a mechanistic link between PAM-α1-DPM microcircuit activity in the post-learning time window and both memory consolidation and sleep.

DISCUSSION

PAM-DANs have been known for the formation of short-term/long-term memories, and DPM neurons for memory consolidation and sleep. In the present study as summarized in Figure 4I, we find that these two types of MB extrinsic neurons form functional inhibitory synapses mainly via Dop1R1 receptor, and this novel circuit plays an essential role in bridging LTM via dynamic sleep change. We demonstrate that a brief disruption of this circuit, in particular of a subset of PAM neurons (PAM-α1) during the critical memory consolidation window, results in an impairment of LTM. Worth noticing, sleep loss and fragmentation occur hours later after the brief disruption of this circuit, under starvation. Thus, maintenance of basal activity of PAM-α1-DPM microcircuit during memory consolidation has a profound effect on LTM, through influencing sleep under a certain internal state.

Mushroom body DANs are not just a valence signal

DANs can have multiple roles at different times in the memory process. PAM-α1 is one of many cell types of DANs that required for memory formation (Yamagata et al., 2021; Yamagata et al., 2015). We find that not only suppressed activity but also elevated activity of PAM-α1 neurons right after training disrupt memory consolidation, approving of its role in consolidation (Ichinose et al., 2015). PAM-α1 neurons form a recurrent loop with MBON-α1 neurons (Aso et al., 2014b; Ichinose et al., 2015), and the recurrent DAN/MBON networks are likely to be important in allowing this to work. Several subsets of DANs have been shown to be involved in forgetting, PPL1-γ1pedc (MP1) and PPL1-γ2α’1 (MV1) for permanent forgetting (Berry et al., 2012), and PPL1-α2α’2 for transient forgetting (Sabandal et al., 2021). In addition, Aso and Rubin (2016) demonstrated that writing and updating memories recruit different rules across distinct MB compartments. Memory consolidation is a dynamic time window from hours after

learning but may continue for weeks (Roesler McGaugh, 2010), and a bunch of studies have suggested that sleep deprivation is important during memory consolidation but not retrieval (Krishnan et al., 2016; Manassero et al., 2022). Our results do not rule out a role for PAM-α1 in active forgetting process but it is clearly not required for retrieval (Figures S4D-S4F), adding a new layer of complexity of multiple functions within a single type of neurons.

Sleep interacts with mushroom body DANs in multiple ways

Sleep has been thought to be indispensable to facilitate memory consolidation process, so as to convert the initially unstable memory into stable long-term memory during sleep (Diekelmann and Born, 2010; Girardeau and Lopes-Dos-Santos, 2021; Klinzing et al., 2019). Besides the amount of sleep, accumulating evidence have pointed out that sleep structure is responsible for memory processing (Kjaerby et al., 2022; Lipinska and Thomas, 2019; Liu et al., 2019; Rolls et al., 2011). Sleep structure can be evaluated by the number of episodes as well as P(wake). The number of episodes reflects a frequency of awakenings, and P(wake) is the probability of transitioning from a sleep to an awake state, that reflects as the threshold for arousal (Liu et al., 2019; Wiggin et al., 2020). In the present study, we found that a brief disruption of neural activity of a specific microcircuit results in reduced and fragmented sleep, with a consequent impairment of LTM, providing a new circuit base for facilitating behavioral and neuronal plasticity.

DPM neurons process memory consolidation via promoting sleep, likely via GABA inhibition of wake-promoting MB-α'β' neurons (Donlea Jeffrey M et al., 2011; Haynes et al., 2015), and this circuit also seems particularly important for consolidation (Krashes et al., 2007). The PAM-α1-DPM microcircuit in the present work unlocks an upstream modulation of MB circuits for linking memory and sleep. There are "forgetting" DANs (such as PPL1-γ2α'1) whose activity has been shown to be suppressed by sleep (Berry et al., 2015). This PPL1-γ2α'1 subset, and other MB DANs (including PAM-α1) have been identified as wake promoting neurons (Donlea et al.,

2011; Driscoll et al., 2021; Sitaraman et al., 2015). Maybe this is one of the ways the recurrent network processes sleep. Perhaps the activation of PAM-α1 decreases sleep and that allows PPL1-γ2α'1 to cause forgetting? A model would be needed in the future. In spite of the fact that MB circuits have been elegantly investigated, the complexity at the systems scale remains puzzling, and how these circuits enable consolidation during sleep still needs further effort in the future.

Internal state dictates circuit configuration for consolidation

Internal state gates many of interactions and can suppress or enhance the role of DAN (Liu et al., 2017). For appetitive LTM, flies need to be starved, which is known to suppress sleep. But the animal's state during consolidation can recruit different circuitry at both the KC level and the DAN level (Chouhan and Sehgal, 2022). Our paper provides yet another example of this. Disrupted activity of PAM-α1-DPM microcircuit negatively impacts on both sleep and memory under starvation status. The fact that there is no effect on sleep in fed flies is really interesting. How this happens will be a very important future direction.

DA receptor coupling is more diverse than previously appreciated

In the present study, we observed that activation of PAM-DANs or direct application of DA result in an inhibition of DPM neurons. Unexpectedly but interestingly, the mechanism of DPM suppression is mediated via Dop1R1, suggesting that there is also going to be heterogeneity at this level of mechanism to- cells may couple the same receptor to different signaling pathways. In *Drosophila*, Dop1R1 (also as DUMB) and Dop1R2 (as DAMB) are classified as dopamine D1-like family, and reported to be coupled to Gs that leads to increases in cAMP (Fernandez-Chiappe et al., 2020; Karam et al., 2020; Zhuang et al., 2021). Consistently, knockdown of Dop1R2 in the DPM neurons tended to enhance the DA-induced inhibition in our experiment that similar to DopEcR knockdown, though statistically not significant, suggesting a Gs coupled effect. However, an interesting observation is that Dop1R2 seems to be involved in a DA-dependent synaptic suppression between the MBs and the MBONs (Aso et al., 2014b;

Cohn et al., 2015; Feng G, 1996; Han KA, 1996; Hattori et al., 2017). Another example of this is DA action on the dorsal fan-shaped body. It increases cAMP via Dop1R1 but this increase causes inhibition of the neuron (Ueno et al., 2012). This means that mutants are never going to explain the action of DA on every cell. Anyhow, whether and how Dop1R1 mediates DA signaling cascades in a bidirectional manner still requires future investigation.

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AUTHOR CONTRIBUTIONS

CL designed and supervised research; LY, LW, TDW, XS, WY, HL, and CL performed research; LY, LL, ZL, ZM, FG, LCG and CL analyzed and interpreted data; LY, FL, LCG and CL wrote the manuscript and all authors reviewed the manuscript.

DECLARATION OF INTERETS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. PAM neurons form functional synapses and inhibit downstream DPM neurons. (A) The schematic and expression patterns of PAM and DPM neurons. PAM neurons labeled by R58E02-LexA are visualized by GFP (green), and DPM neurons labeled by eyeless-GAL80;MB-GAL80;c316-GAL4 are visualized by RFP (magenta). (B) Schematic of restricted *trans*-Tango for direct synaptic contacts between PAM-GAL4 (R58E02) labeled upstream neurons and DPM-LexA (VT064246) labeled targeted postsynaptic to PAM neurons. Anti-GFP-labeled neurons are the downstream of PAM neurons. Anti-RFP-labeled neurons are nondownstream neurons. Scale bar: 20 μm for all images. (C) Functional connection between PAM neurons and DPM neurons. Averaged GCaMP traces and quantification of DPM neurons in response to activation of $P2X_2$ expressing PAM neurons by application of 2.5 mM ATP. n = 22-25. (D) Averaged cAMP traces and quantification of DPM neurons in response to DA with the presence of 2.0 mM TTX when Dop1R1, Dop1R2 and DopEcR were knocked down. n = 11 for all groups. (E) Schematic of innervations of DPM neurons on all lobes of MB (left). Inactivation of DPM neurons for 2.5 h after training disrupts 24 h memory (right). (F) Schematic of innervations of PAM neurons on the horizontal lobes of MB, including subdomains of γ1-5, β'2, β1-2 and α1 (left). Activation of PAM neurons for 1 h after training disrupts 24 h memory (right). Red line: 30 °C; blue line: 32 °C. n.s.: not significant; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$. Bar graph are represented as mean ± SEM with individual values. See also Figure S1, and Tables S1 and S3.

Figure 2. Increased activity of PAM neurons results reduced and fragmented sleep. (A) Timeline for sleep recording with activation of PAM neurons for 1 h before, during and after activation till supposed 24 h memory test. (B-D) Total sleep, number of episodes, and P(wake) (probability of transition from a sleep to an awake status) in 1 h bin for activation of PAM neurons. (E) Timeline for sleep recording with inactivation of DPM neurons for 2.5 h before, during and after activation till supposed 24 h memory

test. (F-H) Total sleep, number of episodes, and P(wake) in 1 h bin for inactivation of DPM neurons. Red line: 30 ℃; blue line: 32 ℃; LP1: light period on day 1; red box: activation of neurons for 1 h; DP1 (grey box): dark period on day 1; LP2: light period on day 2; ZT: Zeitgeber time. n = 14-18. (I) Paradigms of GCaMP levels of target neurons 1 h post-training or no-training under sleep deprivation (SD+) or no sleep deprivation (SD-) conditions. (J-K) Representative images (left) and quantification (right) of GCaMP and mtdTomato of PAM neurons (J) and DPM neurons (K) under different conditions. n = 12-16 for PAM and n = 8-17 for DPM. See also Figures S2-S3, and Table S3.

Figure 3. The PAM-α1 subset of PAM neurons contributes to linking memory consolidation and sleep. (A, C) The expression pattern of LTM-PAM and STM-PAM neurons labeled by R15A04-GAL4 and R48B04-GAL4, respectively. Upper panels, cell body; middle panels, projections on the MB; lower panels, schematic of the projections on the horizontal lobes. (B, D) The paradigm of 24 h memory by activation of LTM/STM-PAM neurons for 1 h after training. Activation of R15A04-GAL4+ neurons impairs 24 h memory (B). Activation of R48B04-GAL4+ neurons has no effect on 24 h memory (D). n = 15-21. (E) The expression patterns of specific subtypes of LTM-PAM neurons labeled by split-GAL4 lines: MB299B and MB043B (α1), MB213B (β1/β2), MB025B (β'1), MB032B (β'2), MB315C (γ5). (F) Left panel, activation and no-activation of subtypes of LTM-PAM neurons. Right panel, activation of MB299B and MB043B which project to α1 subdomain impairs LTM compared to the no-activation group. n = 5-12. (G) Flies with activation of MB299B-GAL4+ neurons after training for 1 h exhibited impaired 24 h memory compared to their genetic controls. n = 14-17. (H) Flies with blockade the output of MB299B-GAL4+ neurons after training for 2.5 h exhibited an impairment of 24 h memory compared to genetic controls. n = 11-14. (I) Upper panel: the paradigm for sleep recording under starvation.

Lower panel: total sleep, number of episodes, and P(wake) in 1 h bin for activation of PAM-α1 neurons. n = 22-30. See also Figures S4 and S5, and Tables S1 and S2.

Figure 4. A specific PAM-α1-DPM inhibitory microcircuit accesses memory consolidation and sleep via maintaining basal activity post-learning. (A) Characterization of anatomical connections of PAM-α1-DPM microcircuit. Left panel: 3D view of expression patterns of PAM-α1 neurons (colored) and DPM neurons (grey) from NeuPrint. Right panel: synaptic connections between 14 PAM-α1 neurons and 2 DPM neurons with their identity number based on the Hemibrain connectome dataset. (B) Activation of $P2X_2$ expressing PAM-α1 neurons labeled by MB299B by application of ATP reduced GCaMP levels in DPM neurons labeled by VT064246-LexA. n = 10-18. (C) Paradigm for neural activity tests of PAM-α1 neurons at multiple time points post training. (D-E) Left panel: representative images of GCaMP and mtdTomato of PAM-α1 (D) and DPM (E). Right panel: quantification of the ratio of fluorescence intensity between the trained and untrained groups at 4 different times. n = 8-27 for PAM-α1; n = 12-24 for DPM. (F) Simultaneous silence of PAM-α1-DPM microcircuit resulted in sleep reduction and fragmentation. n = 36-40. (G) Sleep profiles of before, during and after activation of PAM-α1 neurons till supposed 24 h memory test with/without 0.1 mM gaboxadol (THIP) treatment. Application of THIP rescued sleep reduction induced by activation of PAM-α1 neurons. n = 38-42. (H) THIP rescued 24 h memory deficit induced by 1 h activation of PAM-α1 neurons during consolidation. n = 15-17. (I) A working model of PAM-alpha1-DPM microcircuit in linking sleep and memory. Red filled circle: activated; blue filled circle: inactivated. See also Figure S5, and Tables 1 and 3.

STAR★METHODS

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Chang Liu (chang.liu3@siat.ac.cn).

Materials Availability

This study did not generate new unique reagents. Further information and requests for resources should be directed to and will be fulfilled by the lead contact.

Data and Code Availability

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code. The URL of the code used in this paper are listed in the Key Resources Table.

Any additional information required to analyze the data reported in this paper is available from the lead author upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly stocks

All flies were reared on standard cornmeal food at 25 ℃ and 60% relative humidity on a 12 h/12 h light-dark cycle. Flies expressing dTrpA1 and shi^{ts1} used in temperature-shift experiments were raised at 23 ℃. Flies for memory and sleep experiments allowed to freely mate. 5-10 days old and 5-7 days old adult flies were used at the start of memory and sleep experiments, respectively. Lines of *w;;R58E02-GAL4* (BDSC 70226), *w;;R15A04-GAL4* (BDSC 48671), *w;;R48B04-GAL4* (BDSC 50347) , *MB025B-GAL4* (BDSC 68299), *MB213B-GAL4* (BDSC 68273), *MB032B-GAL4* (BDSC 68251), *MB315C-GAL4* (BDSC 68316), *MB299B-GAL4* (BDSC 68310), *MB043B-GAL4* (BDSC 68304), *UAS-mCD8::GFP; Pin/Cyo* (BDSC 5136) , *w;UAS-dTrpA1* (BDSC 26263), *w;;UAS-shibire^{ts1}* (BDSC 44222), *w;R58E02-LexA* (BDSC

52740), *eyeless-GAL80;MB-GAL80/Cyo;c316-GAL4/TM6B* (BDSC 30380), *UAS-CD8::RFP,LexAop-CD8::GFP* (BDSC 302229), *LexAop-nSyb:spGFP1-10,UAS-CD4:spGFP11;MKRS/TM6B* (BDSC 64315), *UAS-EPAC* (BDSC_78802), *UAS-dcr2* (BDSC_24650), *LexAop-P2X*$_2$ (BDSC_76030), *UAS-GCaMP6f* (BDSC_42747), *UAS-CD4-spGFP1-10* (BDSC_93016), *LexAop-CD4-spGFP11* (BDSC_93019), *UAS-DopEcR RNAi* (BDSC_31981), *UAS-P2X*$_2$ (BDSC_91223) and *LexAop-GCaMP6f* (BDSC_44277) were ordered from Bloomington Drosophila Stock Center (Bloomington, IN, USA). *VT064246-GAL4/TM2* (VDRC_v204311), *UAS-Dop1R1 RNAi* (VDRC_v107058), *UAS-Dop1R2 RNAi* (VDRC_v3392) were ordered from Vienna Drosophila Resource Center (BDRC, Vienna, Austria). The following lines have been previously described: *UAS-mtdTomato;UAS-GCaMP7s and trans-Tango,QUAS-FLP;LexAop-FRT-RFP-FRT-GFP* (Sun et al., 2022). *VT046004-GAL4* was one of Vienna Tile GAL4 lines (Meadows, 2015), but not available anymore (the FlyBase ID is: FBti0172378). For behavioral experiments, w^{CS}*,UAS-dTrpA1* or *w;;UAS-shi*^{ts1} virgin female flies were crossed to male flies of GAL4 lines. Virgin female w^{CS} flies were crossed with GAL4 or UAS parental lines as genetic controls.

The *VT064246-LexA* transgenic lines were based on VT064246-GAL4. The 2296 bp promotor fragment was amplified from genomic DNA of wild-type flies using the same primers as used for VT064246-GAL4 (forward primer: 5'-AATACGAGAGCGCTCACTAC-3' and reverse primer: 5'-TCTTATGGACGGCGA GGAG-3'). This fragment was then cloned into enhancer-LexA vector (Fungene Biotech, http://www.fungene.tech) using NotI and AscI (Thermo Fisher Scientific) restriction sites. The sequence was confirmed by sequencing using primers as follow: VT064246-3F: 5'-AATACGAGAGCGCTCACTAC-3' and reverse lexA-5R: 5'-TCTTATGGACGGCGAGGAG-3'. The plasmids were inserted into attP40 (25C6 of chromosome 2) site and attP2 (68A4 of chromosome 3) site using attp PhiC31 mediated recombination (Fungene Biotech).

METHOD DETAILS

Behavioral analysis

Memory assays

F1 generation of mixed genders in a group were trained and tested together. All training was carried out under the condition of dim red light and all tests were performed in darkness.

4-methylcyclohexanol (MCH) and 3-octanol (OCT) diluted in mineral oil to 10% were used as conditioned odours. Flies were trained with/without starvation. Flies were starved for 30-46 h when each genotype exhibits a mortality rate of 10%-20% when flies started to be tested.

The paradigm to induce reward immediate memory by activation of rewarding DANs was performed as described previously (Liu et al., 2012). Briefly, a group of about 50 flies were loaded into the training tubes and accommodated for 1 min. Two odours were presented at 30 ℃ and 23 ℃ for 1 min reciprocally, and tested the choices between the two odours for 2 min. 30 ℃ was applied either with the first presented odour or the second one (Figure S1D).

For 24 h sucrose-odour memory, a single training session of sucrose paired with an odour for 2 min was employed (Figure S1E, add all other figures using this protocol). Blockade of the output of neurons by shi^{ts1} during the training phase, flies were placed at 32 °C 2.5 h prior to training. Manipulation of the activity of target neurons during memory consolidation phase included: 1) activation for 1 h by dTrpA1 at 30 ℃; and 2) blockade for 2.5 h by shi^{ts1} at 32 °C.

A performance index (PI) was calculated by the difference between flies chose the odour paired with an unconditioned stimulus (US: high temperature or sucrose) and flies chose the other odour without US, divided by the total number of flies. A learning index (LI) was then calculated as the averaged two PIs from two reciprocal groups multiplied by 100. Half of LIs were from the first odour paired with US, and the other

half of LIs from the second odour paired with US to minimize the bias of the conditioning order.

Sleep assays

Regular sleep tubes contained food of 2% agar with 5% sucrose, and the starvation sleep tubes contained only 2% agar. A female fly was anesthetized with CO_2, and then loaded into a 65 mm × 5 mm glass tube which was then placed into a *Drosophila* Activity Monitoring (DAM2, Trikinetics, Waltham) board. DAM boards were then loaded into an incubator with a 12 h:12 h light-dark cycle at 60% relative humidity, and connected to DAM system. After 2 days of entrainment, flies were recorded for another 2 days for sleep analysis. Starvation time of each genotype was consistent as in the memory tests. Flies were transferred to starvation sleep tubes between Zeitgeber Time (ZT) 23 and ZT2. The environment temperature was increased to 30 ℃ for 1 h to activate the neurons expressing dTrpA1, or to 32 ℃ for 2.5 h to inactivate the neurons expressing shi^{ts1} at ZT5/7, and then shifted the temperature back to 23 ℃ for another 24 h. Sleep parameters including total sleep, number of sleep episodes, and P(wake) were analyzed in 1 h bin. Sleep files acquired from DAM system were processed with DAMFileScan113X (TriKinetics Inc, Waltham, MA), and SCAMP 2019v2 using MATLAB_R2021b (MathWorks, Natick, MA). The probability of transition from a sleep to an awake state P(wake) was analyzed as previously described (Wiggin et al. 2020 PNAS). The MATLAB scripts for analysis is accessed in GitHub at https://github.com/Griffith-Lab/FLy_Sleep_Probability.

For sleep with an application of gaboxadol hydrochloride (known as THIP, Cat# 85118-33-8, Sigma-Aldrich), flies were activated for 1 h at ZT7 in 2% agar starvation tubes, and then transferred to sleep tubes containing THIP (0.1mg/ml) in 2% agar at ZT12 for 20 h.

Immunohistochemistry experiments

Both male and female flies aged 5-7 days were used for immunostaining. Fly brains were dissected in Schneider's Drosophila Medium (1×) (Cat# 21720-024, Gibco).

Dissected brains were fixed in 4% paraformaldehyde (PFA, Cat# 50-00-0, GHTECH) for 20 min at room temperature, and then washed in 1× phosphate buffered saline (PBS) containing 0.5% Triton X-100 (PBS-T) at 4 ℃ for 10 min for 3 times. After that, the brains were incubated in primary antibodies with 5% normal goat serum (Cat# 0005-000-121, Jackson) at 4 ℃ overnight. Primary antibodies used for visualization of GFP and RFP (including restricted *trans*-Tango) were: chicken anti-GFP (1:200, Cat# 13970, Abcam) and rabbit anti-DsRed (1:200, Cat# 632496, Clontech); primary antibodies used to detect GRASP signals was mouse monoclonal anti-GFP (1:200, Cat# 11814460001, Roche Applied Science). Samples were then washed in PBS-T at 4 ℃ for 10 min for 3 times, incubated in secondary antibodies at 4 ℃ overnight. Secondary antibodies (1:200) included: Alexa Fluor 488 (goat anti-chicken, Cat# A11039, Invitrogen), Alexa 568 (goat anti-rabbit, Cat# A11011, Invitrogen), Alexa Fluor 633 (goat anti-mouse, Cat# A-21052, Invitrogen). Samples were washed in PBS-T at 4 ℃ for 10 min for 3 times, and mounted with Vectashield (Cat# H-1000, VECTOR). Images were acquired with a Zeiss LSM 900 confocal microscope with 40× oil objective. Confocal Z-stacks were acquired in 2 μm, and analyzed using a free and open source FIJI (https://fiji.sc).

Intracellular calcium measurements

For neural activity of PAM neurons, starved flies experienced different conditions at 23℃： 1) a single session of odour (CS) and sucrose (US) training, 2) no exposure to odour and sucrose, 3) only exposure to two odours without sucrose as training protocol, and 4) only exposure to sucrose without odours as training protocol. Heads were removed from the flies and sampled right after conditioning or 1 h after conditioning, and then fixed in 4% PFA at room temperature for 30 min, and then dissect.

For post-learning neural activity of PAM and DPM neurons, starved flies were tested: 1) training with sleep deprivation (SD, 4 s/20 s for 1 h); 2) training without SD; 3) no-training with SD, and 4) no-training without SD. Heads were removed from the flies and sampled 1 h after conditioning or vehicle experience.

For dynamic neural activity of PAM-α1 and DPM neurons post-training, starved flies were either trained or untrained and tested GCaMP levels at 4 different time points: right after training, 1 h, 9 h, and 15 h post training when brains were sampled.

Primary antibodies used for visualization of GCaMP and mtdTomato were: chicken anti-GFP (1:200, Cat# 13970, Abcam) and rabbit anti-DsRed (1:200, Cat# 632496, Clontech). Secondary antibodies were Alexa Fluor 488 (goat anti-chicken, Cat# A11039, Invitrogen) and Alexa 568 (goat anti-rabbit, Cat# A11011, Invitrogen).

Images from the anterior-to-posterior of the MB lobes of PAM/PAM-α1 projections and cell bodies of DPM neurons were acquired using the same protocol as immunohistochemistry experiments, and set to the same offset for comparisons. Terminal regions of PAM/PAM-α1 neurons on the MB lobes and cell bodies of DPM neurons were outlined as the region of interest (ROI) for quantification. Averaged fluorescent levels of the ROIs of the GCaMP channel and tdTomato channel were obtained from the Z-stacks using FIJI, respectively. Normalized GCaMP intensity to mtdTomato intensity were used for the comparison among different conditions.

***In vitro* calcium imaging**

Functional imaging experiments of neurons were performed on both male and female flies aged 5-7 days. Adult hemolymph-like saline (AHL) composed with (Concentration, mM): NaCl 108; KCl 5; NaH_2PO4 1; $MgCl_2.6H_2O$ 8.2; $CaCl_2.2H_2O$ 2; $NaHCO_3$ 4; trehalose 5; sucrose 10; and HEPES 5. Adenosine 5′-triphosphate magnesium salt (ATP, Cat# A100885, Aladdin) was dissolved in AHL immediately prior to the experiment to the final concentration of 2.5 mM. Tetrodotoxin (TTX, Cat# 554412, Sigma-Aldrich) and dopamine (DA, Cat# 73483, Sigma-Aldrich) were made freshly to final concentrations of 1 μM and 1 mM, respectively.

Flies of genotypes *w^{1118};R58E02-LexA/+;VT064246-GAL4/LexAop-P2X$_2$,UAS-GCaMP6f*, *w^{1118};UAS-dcr2/+;VT046004-GAL4/UAS-EPAC*, *w^{1118};UAS-dcr2/UAS-Dop1R1.RNAi;VT046004-GAL4/UAS-EPAC*, *w^{1118};UAS-dcr2/UAS-Dop1R2.RNAi;VT046004-GAL4/UAS-EPAC*, *w^{1118};UAS-dcr2/UAS-DopEcR.RNAi;VT046004-GAL4/*

UAS-EPAC and *w^{1118};UAS-P2X$_2$,LexAop-GCaMP6f/R58E02-p65.AD;VT064246-LexA /R37E10-GAL4.DBD* were employed. Flies were anesthetized on ice, and brains were dissected in AHL (PH = 7.5) at room temperature. Dissected brains were then pinned to a layer of Sylgard silicone (Dow Corning, Midland, MI) in a perfusion chamber containing AHL. Drugs were applied though a gravity-fed ValveLink perfusion system that allowed a switch from one channel to the other (Automate Scientific, Berkeley, CA).

For functional connectivity experiments, after 30 s of baseline recording with perfusion of AHL, ATP was delivered for 90 s. As a control condition, AHL was perfused for 30 s and switched to a second channel of AHL for another 90 s. Brains expressing the GCaMP6f were imaged using an Mshot MF43-N fluorescence microscope (Mshot, Guangdong, China) under an Olympus ×40 (0.80W, LUMPlanFL N) water-immersion objective, and recordings were captured using a camera of Hamamatsu ORCA-Flash4.0 LT+. Images were captured using HCimage Live software. Regions of interests (ROIs) were selected in the MB of PAM/PAM-α1 and cell bodies of DPM neurons. ROIs were analyzed using FIJI. The percent change in fluorescence over time was calculated using $\triangle F / F = (F_n - F_0) / F_0 \times 100\%$ as we previously described (Liu et al., 2019). F_n is the fluorescence at time point n, and F_0 is the fluorescence at time 0. Averaged fluorescence change values were quantified for periods of 0-30 s and 31-120 s, respectively.

For EPAC experiments, brains were exposed to fluorescent light for 5 min to reduce the photobleaching rates between the CFP and YPF. Also, TTX was applied for 5 min prior to the recording to block voltage-dependent sodium channels and throughout the entire recording period. After 30 s of baseline recording, DA was delivered for 120 s and switched back to TTX again for another 30 s. Brains expressing the FRET sensor EPACcamps1 were imaged using an Olympus BX51WI fluorescence microscope (Olympus, Center Valley, PA) under an Olympus ×40 (0.8W, LUMPlanFI) water-immersion objective, and recordings were captured using a charge-

coupled device camera (Hamamatsu ORCAC472-80-12AG). Images were captured using μManager acquisition software (Edelstein et al., 2010). ROIs analysis was the same as previously described using custom software developed in MATLAB (Liu et al., 2019). Identical ROIs were selected from both the CFP and YFP channels, and calculated the ratio of normalized CFP/YFP to the first 20 frames of baseline recording for each time point. The maximum change values were determined for each group during drug perfusion period (31-150 s) and TTX washout period (151-240 s) for the following quantification.

QUANTIFICATION AND STATISTICA LANALYSIS

All the raw data were analyzed using the Prism 9.0 software (GraphPad). For most memory experiments, datasets with normal distribution, a one-way ANOVA followed by Sidak's multiple comparisons test was applied. For those did not have a normal distribution, nonparametric statistics, Kruskal-Wallis, followed by Dunn's multiple comparisons test was applied. For induced appetitive memory experiment (Figure S1D), two-way ANOVA followed by Tukey's multiple comparisons test was applied. Furthermore, in the experiments to compare the activation group to the no-activation group (Figure 3F), an unpaired t test or nonparametric test was applied based on data distribution. For sleep experiments, a two-way ANOVA followed by Dunn's multiple comparisons test was applied to determine statistical significance between the experimental groups and the control groups for each 1 h bin. All data were presented as mean ± standard error (SEM). A statistically significant difference was set to $p < 0.05$. Detailed statistical analysis of each experiment was summarized in Supplemental Tables 1-3.

Supplemental information

Brief Change in Dopamine Activity during Consolidation Impairs Long-Term Memory via Sleep Disruption

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Figure S1. Related to Figure 1.

The connection between PAM and DPM neurons. (A) Fragment of spGFP1-10 and nSyb-GFP1-10 is expressed in R58E02-GAL4 and LexA+ neurons, and fragment of spGFP11 is expressed L0111-LexA and eyeless-GAL80;MB-GAL80;c316-GAL4+ neurons, respectively. Reconstituted GFP signals are shown as puncta (green). The mushroom body is outlined with dashed lines. (B) Newly generated VT064246-LexA (II) and VT064246-LexA (III) recapitulate expression pattern of DPM neurons labeled with VT064246-GAL4. (C) The control of restricted *trans*-Tango. Signals of downstream target of DPM neurons was not detected without PAM driver R58E02-GAL4. (D) Left panel: the paradigm of 1 min association of activation of neurons with odours for training. M: MCH; O: OCT. Red line: 30 ℃; black line: 23 ℃. Right panel: activation of PAM neurons pairing with an odour in non-starved and starved flies are sufficient to induce 2 min appetitive memory. n = 16 for all groups. (E) The paradigm of a single session training with 2 min association of sucrose (Green) and an odour, and the paradigm of 24 h appetitive memory with blocking the output of neurons for 2.5 h before and during training at 32 ℃ (left). Inactivation of PAM neurons during training impaired 24 h memory (Right). n = 8-10. (F) Inactivation of DPM neurons labeled by VT064246-GAL4 after training for 2.5 h impaired 24 h memory. n = 28-30. (G) Inactivation of DPM neurons during training did not impair 24 h memory. n = 6-9. (H) Flies form 24 h appetitive memory without activation of R58E02-GAL4+ neurons. n = 14-18. (I) Inactivation of PAM neurons after training for 2.5 h did not impair 24 h memory. n = 14-15. n.s.: not significant, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$. See also Supplemental Tables 1 and 3.

Figure S2. Related to Figure 2.

Sleep effects with manipulation of neural activity of PAM or DPM neurons. (A) Inactivation of DPM neurons labeled by VT064246-GAL4 with starvation resulted in a decreased and fragmented sleep after activation at night, but almost no effect on P(wake). n = 11-37. (B) Inactivation of PAM neurons labeled by R58E02-GAL4 under

starvation resulted in a significant decrease of sleep at early night, with increased number of episodes and P(wake). n = 33-39. (C) Activation of PAM neurons without starvation resulted a reduction of sleep at early night, as well as a slight decrease in number of episodes, but an increase in P(wake). n = 39-42. (D) Inactivation of PAM neurons had no effect on sleep without starvation. n = 22-27. (E) Inactivation of DPM neurons labeled by eyeless-GAL80;MB-GAL80;c316-GAL4 without starvation had barely no effects on sleep. n = 35-47. (F) Inactivation of DPM neurons labeled by VT064246-GAL4 had mild effect in sleep at early phase after inactivation without starvation. n = 27-36. Sleep data were presented in 1 h bin. White box: light period; red box: activation period; grey box, dark period; solid fill: starvation; patterned fill: no starvation. See also Supplemental Table 3.

Figure S3. Related to Figure 2.

Neural activity of PAM neurons under different conditions. (A) Protocol for examination of the change in GCaMP level of PAM neurons at two time points under 4 conditions: 1) train with 2 min appetitive conditioning paradigm; 2) experience in the vehicle conditioning tubes; 3) experience of two odours only; and 4) experience of sucrose only. Grey arrows, sample right after conditioning; black arrows, sample 1 h after conditioning. (B-C) PAM neurons exhibited similar GCaMP level between the trained group (Group 1) and untrained group (Group 2) right after conditioning. Presentation of sucrose only (Group 4) suppressed the GCaMP level compared to the trained and untrained group. n = 8-10. (D-E) No significant difference of GCaMP level among all groups after 1 h of conditionings. n = 8-10. GCaMP level was normalized to reference mtdTomato level. See also Supplemental Table 1.

Figure S4. Related to Figure 3.

The PAM-α1 subset of PAM neurons contributes to linking memory consolidation and sleep. (A) 1 h activation of PAM-α1 neurons labeled by MB043B after training for 1 h impaired 24 h memory. n = 16-20. (B) Inactivation of PAM-α1 neurons labeled by

MB043B after training for 2.5 h did not affect 24 h memory. n = 16-25. (C) 1 h activation of PAM-α1 neurons labeled by MB299B prior to and during the test impaired 24 h memory. n = 11-17. (D) Activation of PAM-α1 neurons labeled by MB299B during test did not affect 24 h memory. n = 18-23. (E) Inhibition of PAM-α1 neurons labeled by MB299B prior to and during the test for 30 min did not affect 24 h memory. n = 9-10. (F-G) Sleep profiles of total sleep, the number of episodes, and P(wake) with 1 h activation of MB299B (F) or MB043B (G) before, during and after activation. Sleep was not affected without starvation. n = 28-31. (H) Inactivation of MB299B for 2.5 h without starvation had no effects on sleep. n = 30-37. (I) Inhibition of MB043B for 2.5 h under a no starvation condition resulted significant reduction in total sleep, a mild increase of number of episodes during the dark period, and no effect on P(wake). n = 30-39. See also Supplemental Tables 1 and 3.

Figure S5. Related to Figures 3-4.

The PAM-α1 subset of PAM neurons contributes to linking memory consolidation and sleep. (A) Activation of PAM-α1 neurons labeled by MB043B for 1 h reduced total sleep, increased the number of episodes and P(wake) especially during the dark period. n = 23-30. (B) Inactivation of PAM-α1 neurons labeled by MB299B with starvation had no effects on sleep. n = 33-39. (C) Inactivation of PAM-α1 neurons labeled by MB043B with starvation significantly reduced total sleep, and increased both the number of episodes and P(wake), especially during the dark period. n = 30-39. (D) Simultaneous silence of PAM-α1-DPM microcircuit without starvation had little effect on sleep and number of episodes. n = 29-31. Sleep data were presented in 1 h bin. See also Supplemental Table 3.

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Figure 1. PAM neurons form functional synapses and inhibit downstream DPM neurons .

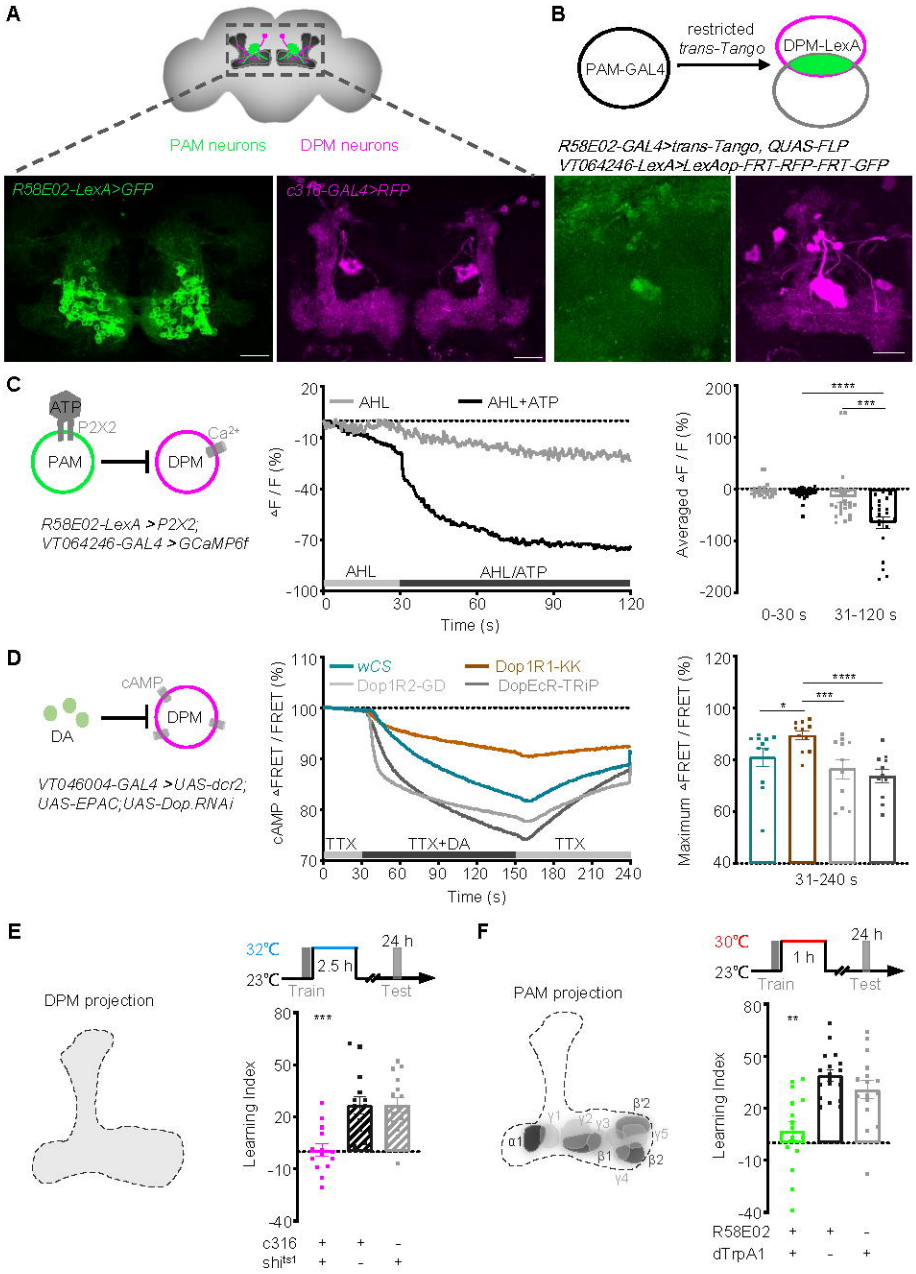

Figure 2. Increased activity of PAM neurons results reduced and fragmented sleep.

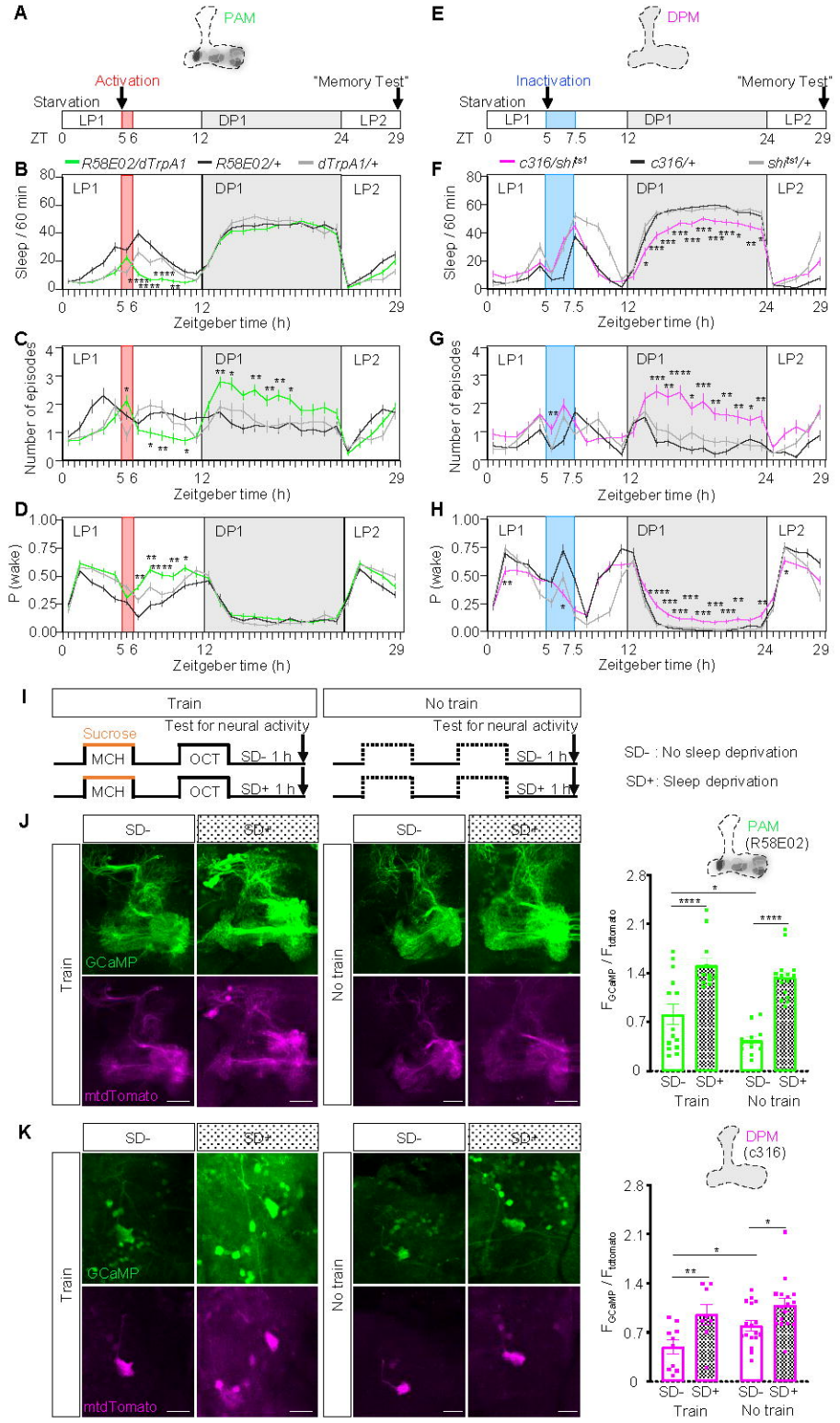

Figure 3. The PAM-α1 subset of PAM neurons contributes to linking memory consolidation and sleep.

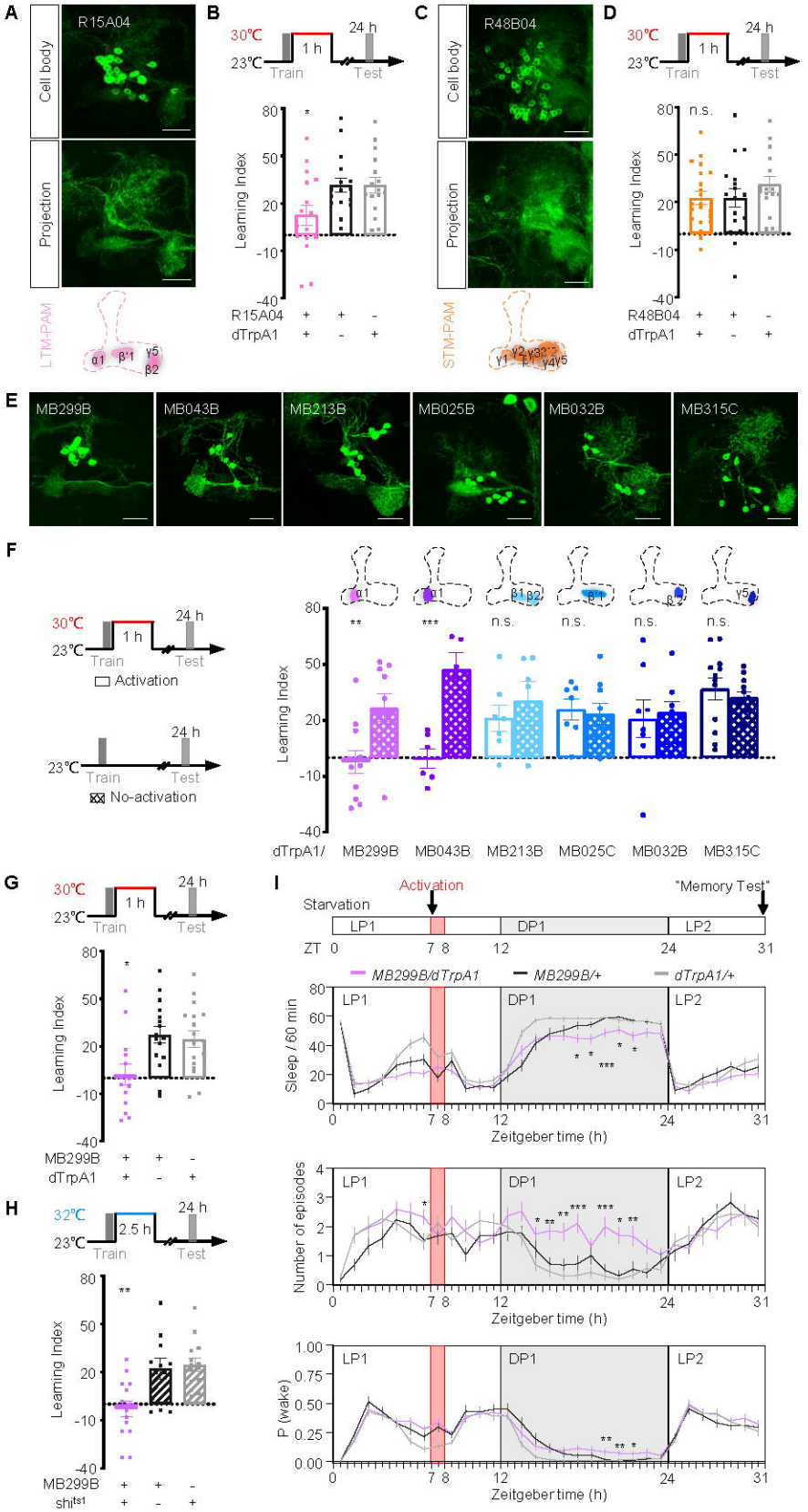

Figure 4. A specific PAM-α1-DPM inhibitory microcircuit binds memory consolidation and sleep via maintaining basal activity post-learning

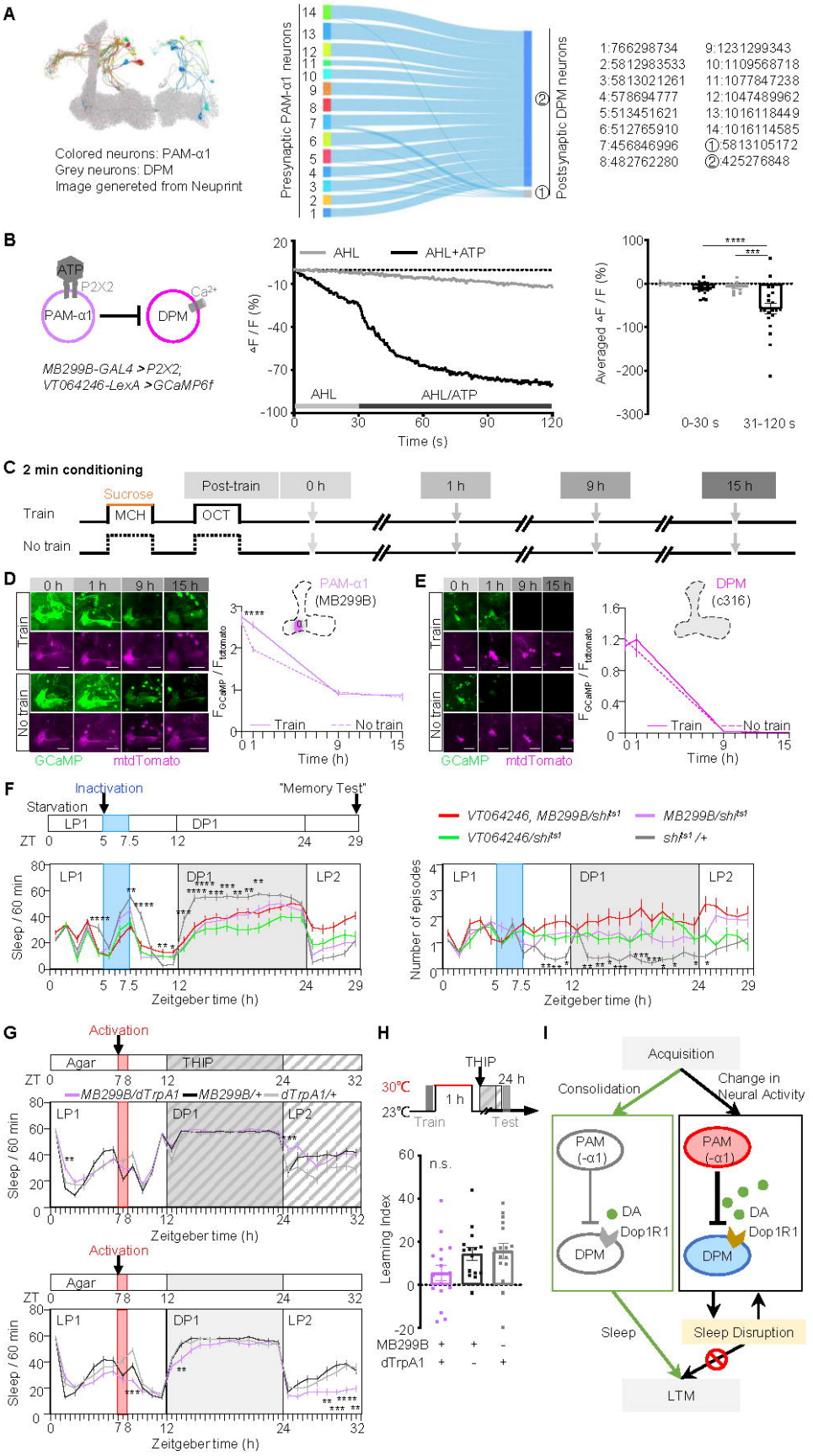

Figure S1. Related to Figure 1.

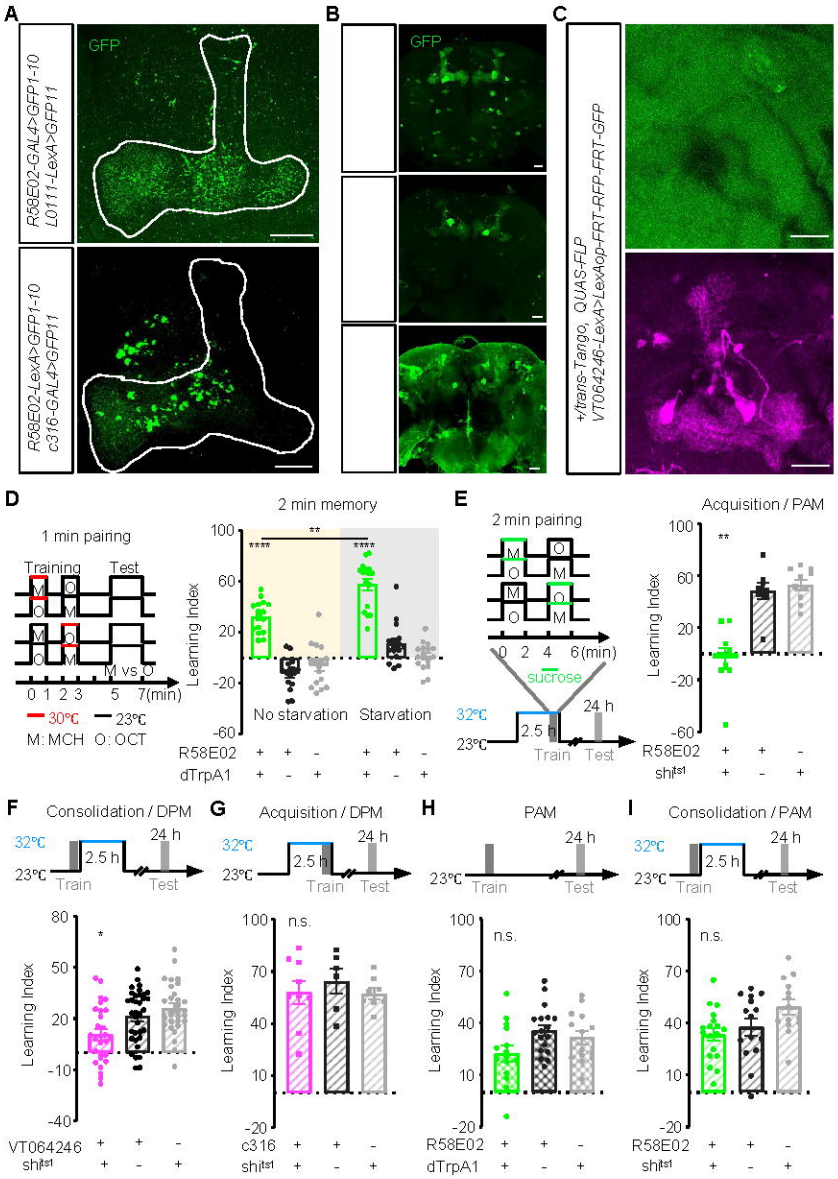

Figure S2. Related to Figure 2.

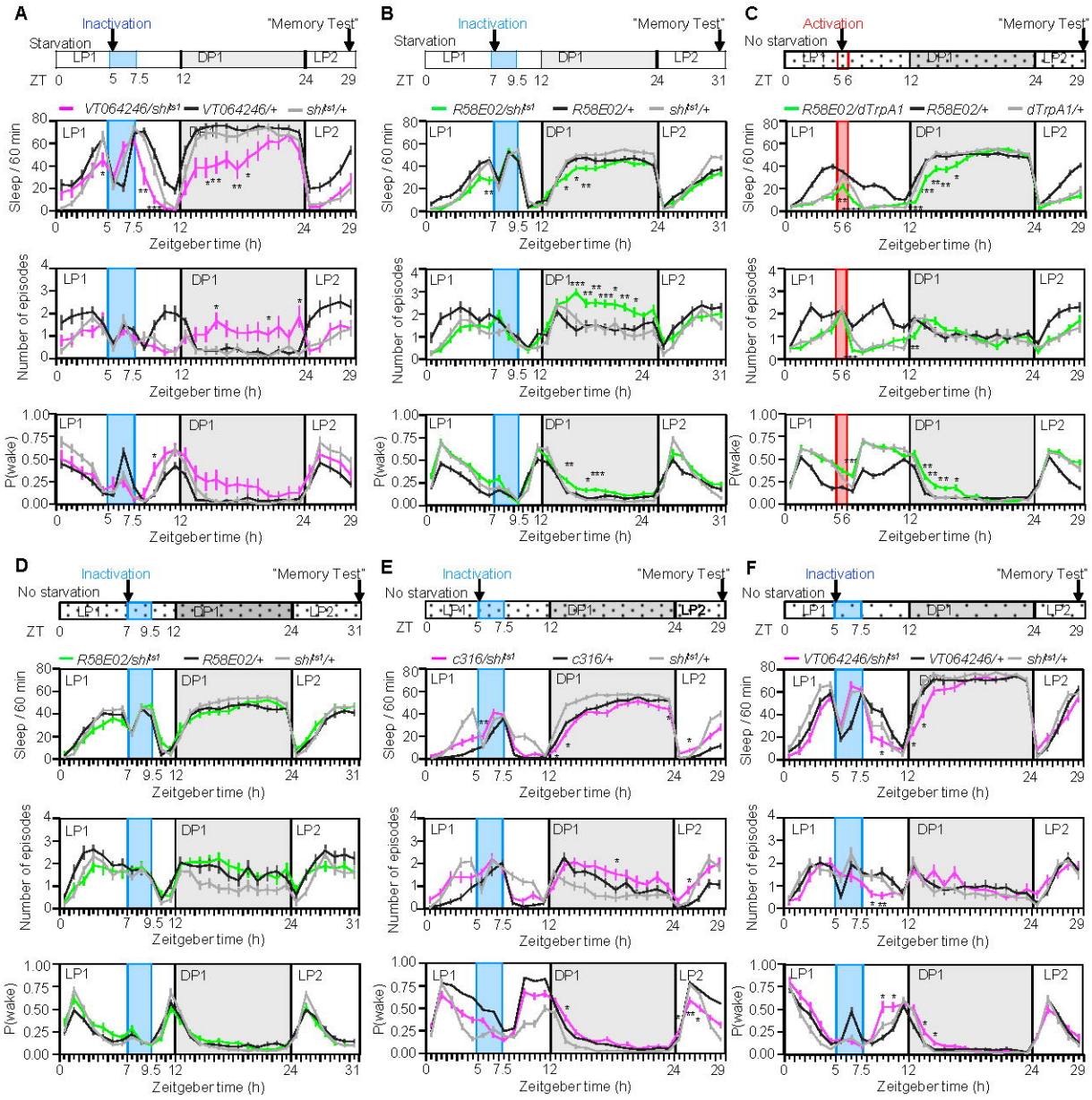

Figure S3. Related to Figure 2.

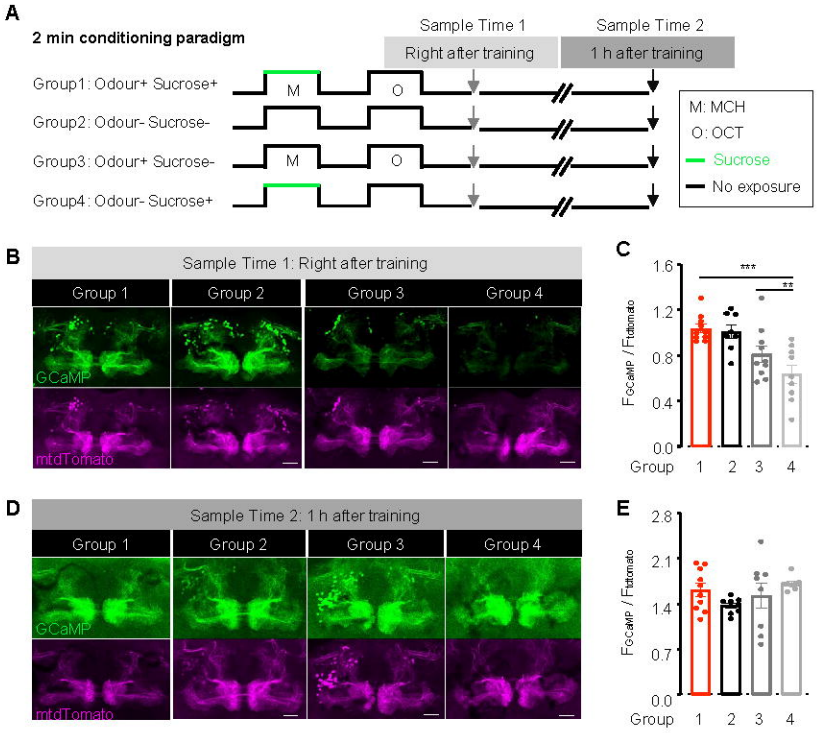

Figure S4. Related to Figure 3.

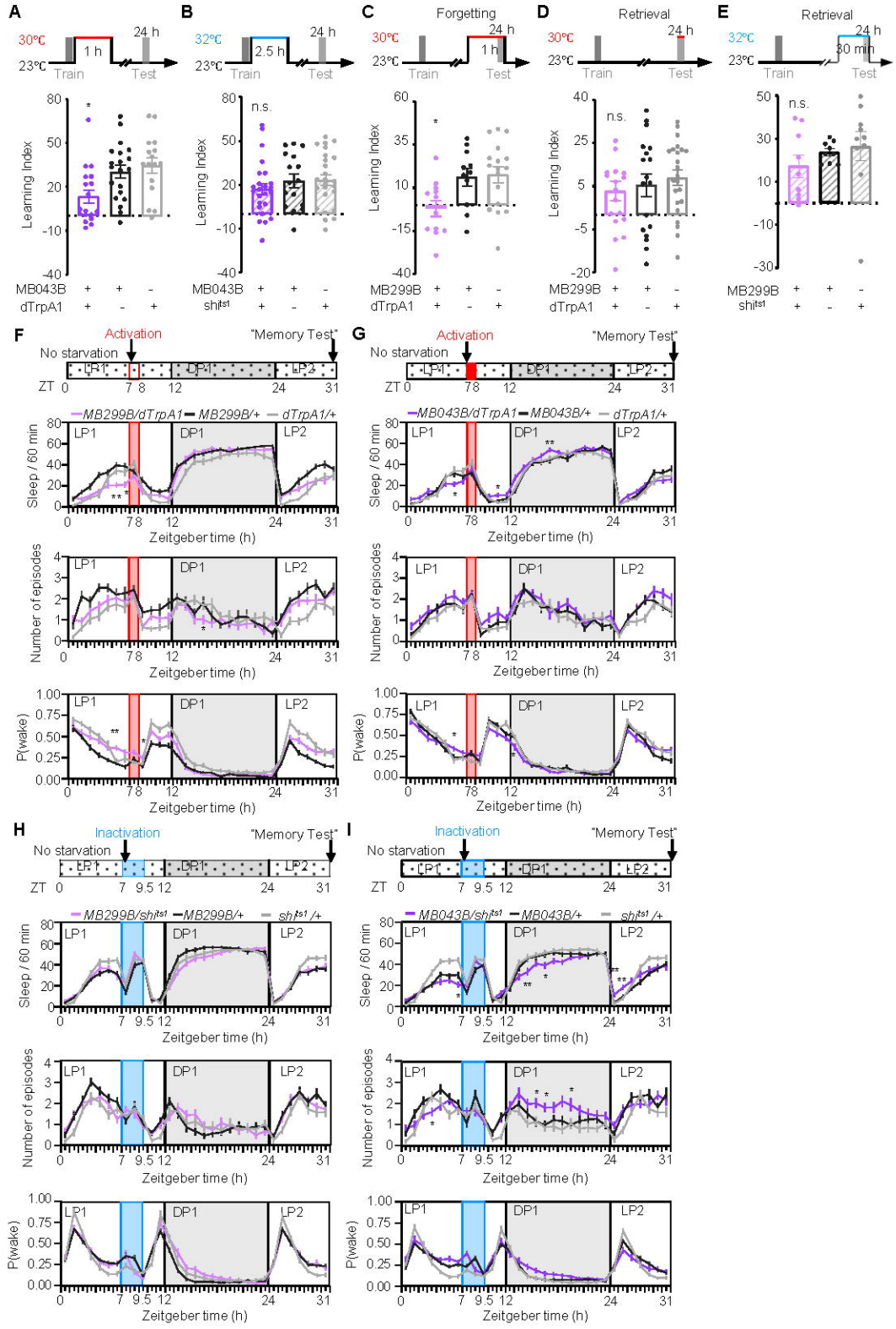
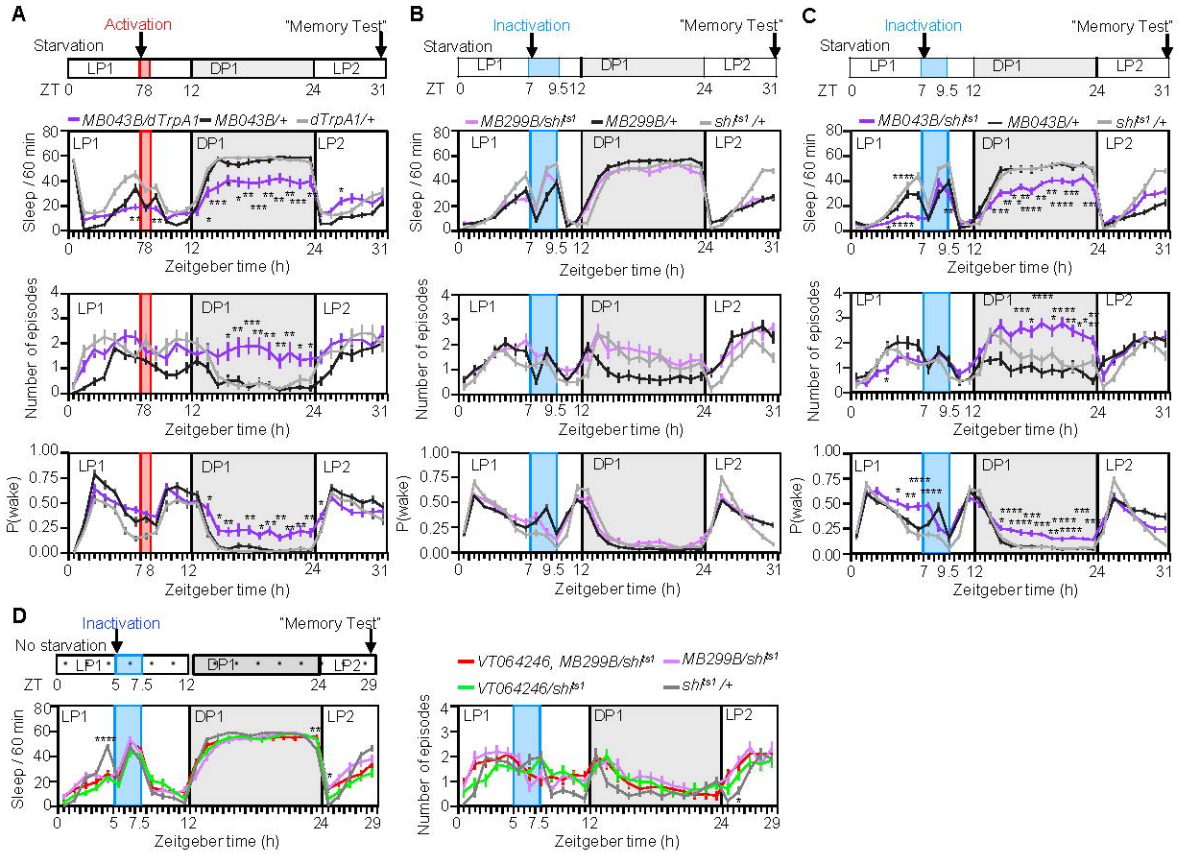
Figure S5. Related to Figures 3 and 4.