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Brief disruption of activity in a subset of dopaminergic neurons during consolidation impairs long-term memory by fragmenting sleep

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eLife Assessment

This study provides **important** insights into the neural mechanisms linking sleep and long-term memory consolidation. By combining behavioural, genetic, imaging, and connectomic approaches in Drosophila, it identifies a target neural circuit that will be of broad interest to researchers studying sleep, memory, and neural circuits. The evidence supporting the involvement of the identified circuit in the regulation of sleep and memory is **solid** and represents a substantial advance in the field. Nevertheless, there is limited evidence to support the mechanistic claim that this circuit directly links sleep and memory consolidation within the available data, and some results should therefore be interpreted with appropriate caution.

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Abstract

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, using both male and female flies, *Drosophila melanogaster*, we show that, during the first few hours of memory consolidation, disruption of basal activity of a small subset of protocerebral anterior medial DANs (PAM-DANs), by either brief activation or inhibition of the two dorsal posterior medial (DPM) neurons, impairs 24 h LTM. Interestingly, these brief changes in activity using female flies result in sleep loss and fragmentation, especially at night. Importantly, pharmacological rescue of sleep after manipulation restores LTM. A specific subset of PAM-DANs (PAM-α1) that synapse onto DPM neurons specify the microcircuit that links sleep and memory. MBON-α1 also contributes to the integration of sleep and memory by acting as an additional parallel circuit. PAM-DANs, including PAM-α1, form functional synapses onto DPM mainly via two dopamine receptor subtypes. Dop1R1 primarily mediates the link between sleep and memory. This PAM-α1 to DPM microcircuit exhibits a synchronized, transient, post-training change in activity during the critical memory consolidation window, suggesting an effect of this microcircuit on maintaining the sleep necessary for LTM consolidation. Our results provide a new molecular and circuit basis for the complex relationship between sleep and memory.

Introduction

Memory consolidation is a time-dependent process which, during a few hours post-encoding, facilitates maturation of newly formed but unstable memories (McGaugh, 2000; Dudai et al., 2015). 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 (Wu and Liu, 2023). Sleep deprivation (SD) before training compromises memory formation, while SD post-learning impairs memory consolidation (Li et al., 2009; Spira et al., 2014; Dag et al., 2019). 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* has proven to be a valuable model for uncovering the mechanistic underpinnings of memory formation, 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; Adel, 2021). Classical olfactory conditioning, which is exemplified by studies on Pavlov's dogs (Pavlov, 1927), forms a memory of a conditioned stimulus (CS, an odor) as a prediction of a pleasant or unpleasant unconditioned stimulus (US, a reward or a punishment) after pairing of the CS with the US (Quinn, 1974; Tempel, 1983; Tully, 1985).

The MB, a multi-functional unit roughly 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 D1-type 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). Furthermore, a recurrent positive feedback loop between dopaminergic PAM-α1 neurons and the MB output neurons MBON-α1 has been shown to be required for the acquisition and consolidation of appetitive LTM (Ichinose, et al., 2015). These data imply that during memory consolidation, a DA signal cascade recruits circuit components at multiple levels to exert sequential modulation of these functional loops.

While DANs are an indispensable part of the US pathway, they are also morphologically and functionally diverse (Mao and Davis, 2009; Aso et al., 2014a; Mohammad et al., 2024; Huang C et al., 2024). DANs 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) and also participate in several phases of LTM (Placais et al., 2012). DANs can also participate in post-encoding shaping of memories. Persistent activity in PAM-γ4, PPL1-γ1pedc and -γ2α'1 subtypes interferes with STM and anesthesia-resistant memory (ARM), and promotes forgetting (Berry et al., 2012; Placais et al., 2012; Berry et al., 2015; Lee et al., 2025), while PPL1-α2α'2 and PPL1-α3 mediate aversive LTM formation with persistent post-training activity (Feng et al., 2021). 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.

In addition to memory processes, DA also regulates wakefulness and sleep by acting on distinct downstream targets (Kume et al., 2005; Donlea, 2011; Van Swinderen and Andretic, 2011; Ueno et al., 2012; Tomita et al., 2021). Subsets of PAM-DANs have been suggested to signal wakefulness and project to wake-promoting compartments of the MB (Sitaraman et al., 2015; Driscoll et al., 2021).

Additional processing of memory takes place via activation of two pairs of extrinsic neurons, dorsal paired medial (DPM) neurons and anterior paired lateral (APL) neurons, which have been well characterized for their requirement in memory consolidation (Waddell, 2000; Keene et al., 2004; Keene et al., 2006). Furthermore, DPM neurons have been shown to promote sleep via inhibitory signaling (Haynes et al., 2015). The role of the activity of DAN-DPM connections in consolidation as well as the underlying signaling transmission have not been explored.

In this study, we show that PAM neurons form inhibitory connections on DPM neurons. 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 sleep reduction and fragmentation after brief disruption of these neurons. Intriguingly, 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. Maintenance of basal activity of this specific microcircuit during memory consolidation has a profound effect on LTM, through influencing sleep in an internal state-dependent manner. Additionally, we defined the distinct roles of two predominant DA receptors expressed in DPM in mediating sleep and memory processes, respectively. Finally, we characterized additional parallel MBON circuits that contribute to the coupling or segregation of sleep and memory regulation. Together, our findings reveal a microcircuit mechanism by which dopaminergic signaling coordinates sleep and memory consolidation.

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). A pair of DPM neurons intensively innervates all lobes of the MB and is important for consolidation of memory (Yu et al., 2005; Keene et al., 2006) (Figure 1A). The electron microscope (EM) connectome database has shown that PAM and DPM neurons are connected by 4709 synapses (Zheng et al., 2018; Scheffer et al., 2020; Plaza et al., 2022). To determine if PAM and DPM neurons function together to regulate distinct memory processes, we first needed to confirm and characterized the connectome-predicted synapses. To investigate this, we use GFP Reconstitution Across Synaptic Partners (GRASP) and activity-dependent GRASP (Feinberg et al., 2008; Macpherson et al., 2015). 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 1B). 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 labeled by newly generated VT064246-LexA drivers are the downstream target of PAM neurons (Figure 1C-D).

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. As previously described, we used the percent change in fluorescence over time as a ratio to the initial level, $\triangle F/F = (Fn\text{-}F_0)/F_0 \times 100\%$ for quantification (Liu et al., 2019). We found that direct activation of PAM neurons by bath application of ATP resulted in a strong decrease in calcium in DPMs, consistent with inhibition of DPM activity (Figure 2A, see also Table 3). In addition, we conducted *in vivo* functional experiments. Optogenetic activation of PAM neurons using the red light-gated cation channel CsChrimson (Klapoetke NC et al., 2014) resulted in a significant reduction in GCaMP signal in DPM neurons (Figure 2B, see also Table 3). Together, these results provide direct evidence that PAM neurons exert inhibitory control over DPM neurons.

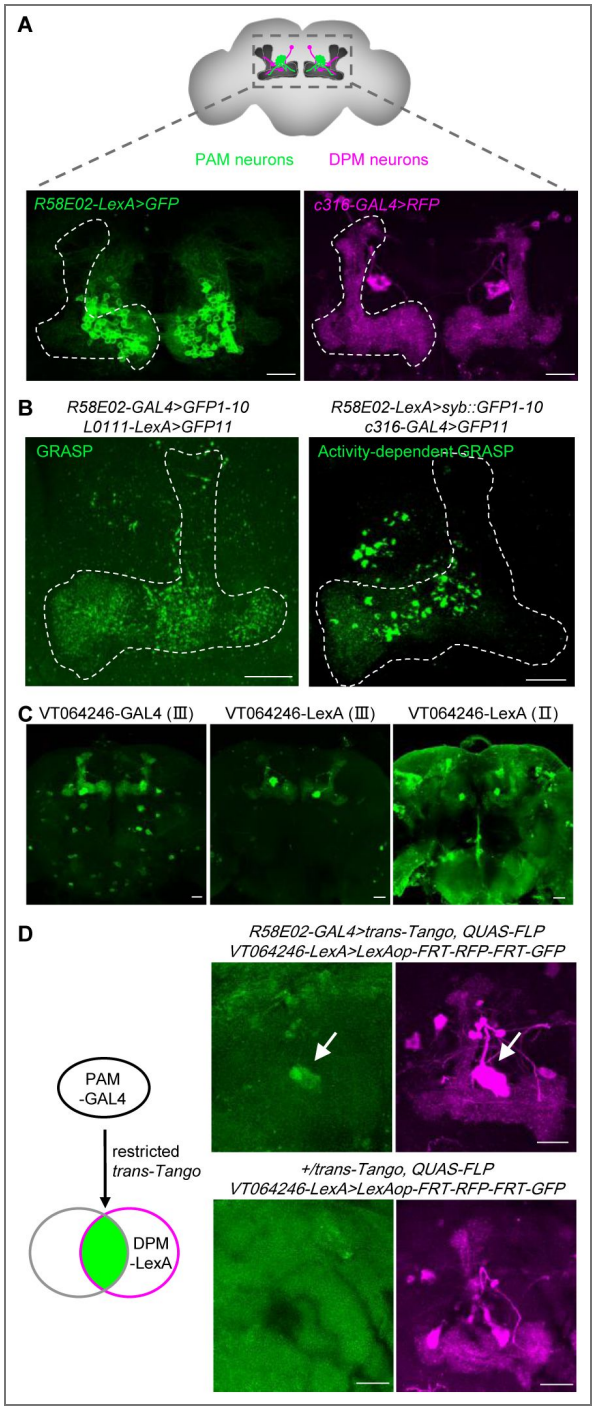


Figure 1. PAM neurons form functional synapses with DPM neurons.

(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) 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. (C) Newly generated VT064246-LexA (II) and VT064246-LexA (III) recapitulate expression pattern of DPM neurons labeled with VT064246-GAL4. (D) 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 non-downstream neurons. Right lower panels, the control of restricted *trans*-Tango. Signals of downstream target of DPM neurons was not detected without PAM driver R58E02-GAL4. Scale bar: 20 μm.

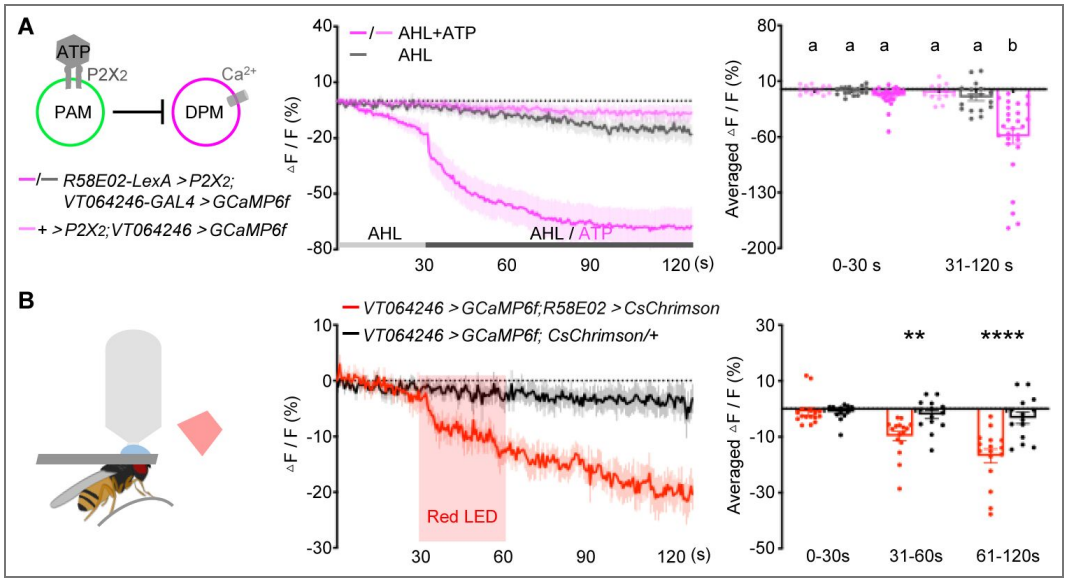


Figure 2. PAM neurons exert direct inhibitory control over DPM neurons.

(A) *Ex vivo* 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 = 12-23. Groups that are statistically no differences using the same letter, while distinct letters denote statistically significant differences between groups. (B) *In vivo* calcium imaging for functional connection between PAM neurons and DPM neurons. Averaged GCaMP traces and quantification of DPM neurons in response to activation of CsChrimson expressing PAM neurons by red light. The red box indicates the 30-s, 2-Hz, 627-nm light pulse. n = 14-16. **p<0.01; ****p<0.0001. Curves and bar graph are represented as mean ± SEM with individual values. See also Table 3.

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 participate 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). 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 3A, see also Table 1), which is consistent with previous findings (Yamagata et al., 2015).

DPM neurons have been found to have an essential role in memory consolidation during a critical window for consolidation (Waddell, 2000; Yu et al., 2005; Keene et al., 2006; Cervantes-Sandoval and Davis, 2012). In accordance with the aforementioned findings, inactivation of DPM neurons by shi^{ts1} at restrictive temperature of 32 °C for 2.5 h after training resulted in a 24 h memory deficit (Figure 3B-C, see also Table 1), but there was no effect on 24 h memory when they were blocked before and during training (Figure 3D, see also Table 1).

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 for 1 h after training resulted in an impairment of 24 h memory (Figure 3E, see also Table 1). Without activation, *R58E02-GAL4/UAS-dTrpA1* flies and their genetic controls formed normal 24 h memory (Figure 3F, see also Table 1). Blocking PAM neuronal activity for 2.5 h after training failed to disrupt 24 h memory (Figure 3G, see also Table 1), suggesting that either continued activity of PAM neurons becomes unnecessary in the consolidation window or that heterogeneity in the subsets of PAM neurons masks any phenotype.

Increased or decreased activity of 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 ask whether the impairments in LTM that occurred with activation of PAM neurons after training might be attributable to sleep disruption, we monitored sleep under starvation and normal feeding conditions for 30 h, a window sufficient for completion of LTM training and testing, with flies which had been entrained to a 12:12 light:dark cycle. Each panel in Figure 4 shows the schedule for the LTM paradigm we utilize to orient the sleep data to the relevant behavioral phase.

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 bins to assess dynamic changes during the course of starvation before the time when training would have occurred through the time when the 24 h memory tests would have been done (Figure 4, see also Table 4). 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, i.e. immediately after when training would have occurred.

Activation of PAM neurons at the time which training occurred caused a reduction in sleep occurred a few hours after the activation of PAM neurons (Figure 4A, upper panel), possibly due to a reduced number of sleep episodes (Figure 4A, middle panel) and an increased possibility of waking (Figure 4A, lower panel). The biggest change, however, was structural- PAM activation significantly increased the number of sleep episodes, which reflects increased wakefulness during the dark period following activation (Figure 4A, middle panel). Although LTM impairment was similar in the memory experiments, the effect of inactivation of DPM neurons resulted in a slightly stronger, long-lasting sleep reduction and fragmentation that was delayed until the dark period after inactivation (Figure 4C-D). However, activation of DPM neurons failed to distrupt sleep and impair LTM (Supplemental Figure 1A-B). Unexpectedly, inactivation of PAM neurons led to a sleep change similar to that seen with inactivation of DPM neurons (Figure 4B), suggesting that

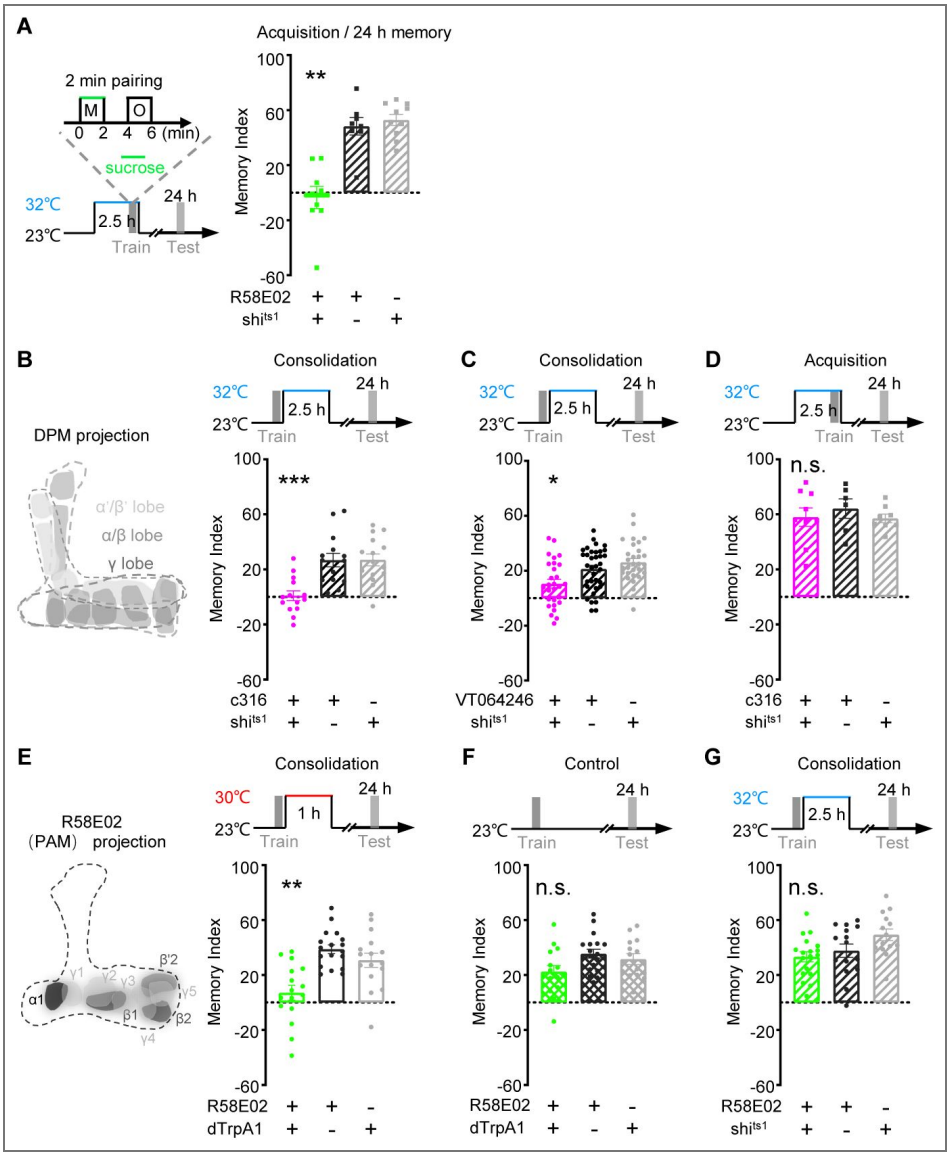


Figure 3. Activation of PAM neurons during consolidation impairs long-term memory, possibly via inhibiting DPM neurons.

(A) The paradigm of a single session training with 2 min association of sucrose (Green) and an odor, 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. (B) 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). (C) Inactivation of DPM neurons labeled by VT064246-GAL4 after training for 2.5 h impaired 24 h memory. n = 28-30. (D) Inactivation of DPM neurons during training did not impair 24 h memory. n = 6-9. (E) Schematic of innervations of R58E02-GAL4 labeling (PAM) neurons on the horizontal lobes of MB, including subdomains of γ1-5, β'2, β1-2 and α1 (left). Different gray scales were for each compartment's visibility, not projection intensity. Activation of PAM neurons for 1 h after training disrupts 24 h memory (right). (F) Flies form 24 h appetitive memory without activation of R58E02-GAL4+ neurons. n = 14-18. (G) Inactivation of PAM neurons after training for 2.5 h did not impair 24 h memory. n = 14-15. Red line: 30 ℃; blue line: 32 ℃. 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 Table 1.

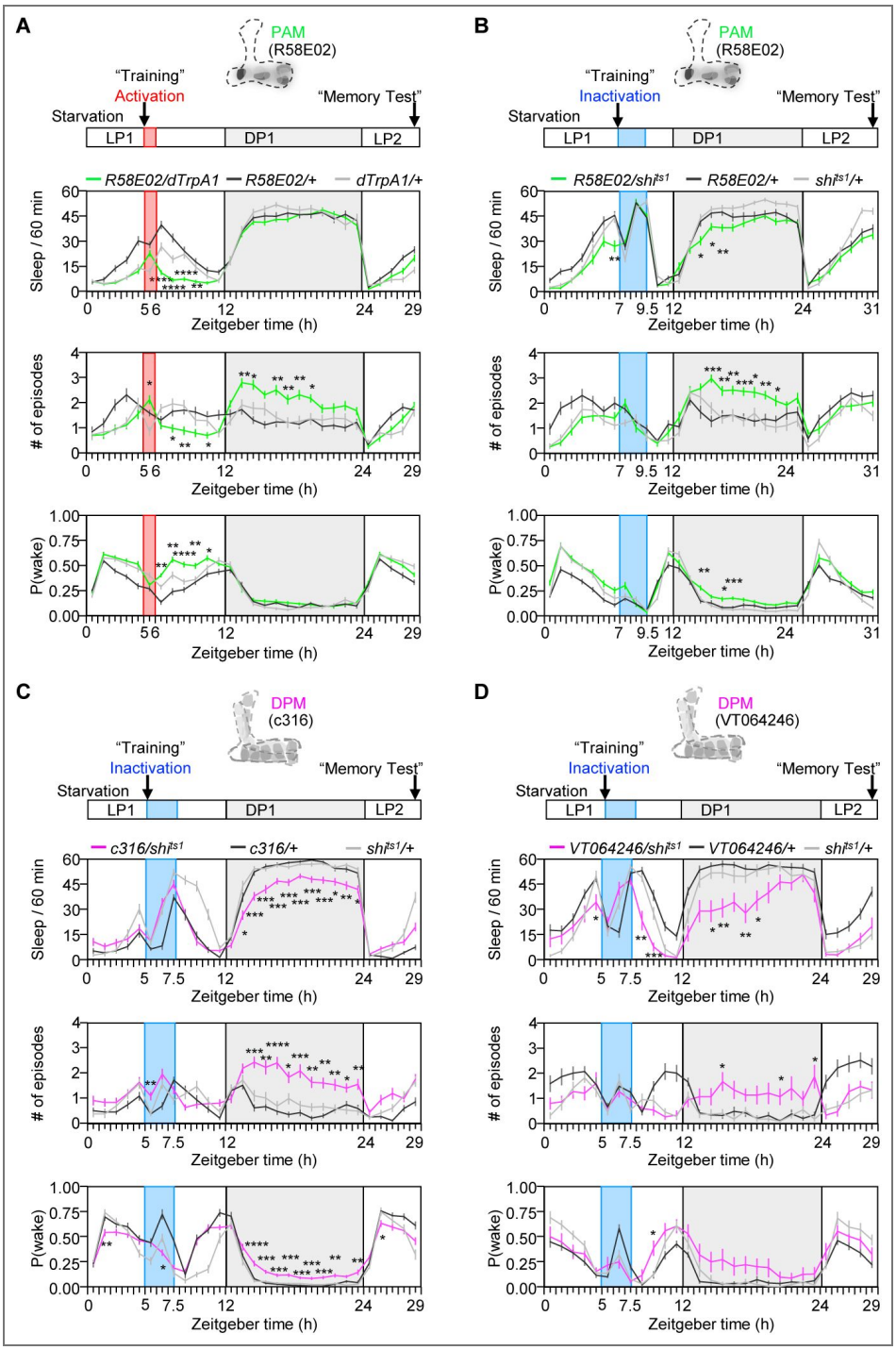


Figure 4. Increased or decreased activity of PAM/DPM neurons results reduced and fragmented sleep.

(A) Timeline for sleep recording with activation of PAM neurons for 1 h before, during and after activation till supposed 24 h memory test. Total sleep (upper panel), number of sleep episodes (middle panel), and P(wake) (probability of transition from a sleep to an awake status) (lower panel) in 1 h bin for activation of PAM neurons. n = 40-43. (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 sleep episodes and P(wake). n = 33-39. (C) Timeline for sleep recording with inactivation of DPM neurons labeled by c316-GAL4 for 2.5 h before, during and after activation till supposed 24 h memory test. Total sleep, number of sleep episodes, and P(wake) in 1 h bin for inactivation of DPM neurons. n = 33-43. (D) 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. 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. See also Table 4.

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 by inter-PAM interactions or DPM-independent effects on sleep.

Appetitive LTM expression requires that the animal be motivated by hunger (Liu et al., 2012) and starvation is known to suppresses sleep (MacFadyen, 1973; Keene et al., 2010; Thimgan 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 (Figure 5). 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 5A, see also Table 4), suggesting a complex and context-dependent role for PAM neurons in regulation of sleep. Inactivation of PAM neurons (Figure 5B, see also Table 4), inactivation or activation DPM neurons (Figure 5C-D, see also Table 4) in the fed condition 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 there may be subtype-specific actions of PAM neurons on DPM neurons. They also demonstrate that their interactions are likely to be context-dependent.

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

The PAM neurons signaling reward for STM and LTM have been shown to be distinct groups that can be labeled separately by R48B04-GAL4 and R15A04-GAL4, except for overlapping expression in the neurons projecting to the γ5 subdomain (Yamagata et al., 2015). Terminals of R15A04-GAL4 labeling (LTM-PAM) neurons are localized in the β2, α1, β'1, γ5 and pedunculus MB subdomains (Figure 6A). Terminals of R48B04-GAL4 labeling (STM-PAM) neurons are localized in the β'2, β1, and γ1-5 MB subdomains (Figure 6C). 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 6B, see also Table 1). In contrast, activation of STM-PAM neurons did not affect 24 h memory (Figure 6D, see also Table 1). 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 send processes to β1/β2, β'1, β'2 and γ5 subdomains, respectively (Figure 6E). We trained flies expressing dTrpA1 under control of each of these drivers, with or without 1 h of activation after training, and tested 24 h memory. Only activation of MB299B-GAL4+ and MB043B-GAL4+ neurons impaired 24 h memory compared to their no-activation groups (Figure 6F, see also Table 2). MB299B-GAL4+ (which labels 12±5 PAM neurons/hemisphere) and MB043B-GAL4+ (which labels 6±2 PAM neurons/hemisphere) primarily express in the α1 subdomain (Figure 6G). These data indicate that elevated activity of PAM-α1 neurons during consolidation impedes LTM.

To further probe the requirement of PAM-α1 neurons captured in MB299B- and MB043B-GAL4s in memory consolidation, we either activated them or blocked their output 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 labelled PAM-α1 neurons resulted in an impairment of 24 h memory (Figure 7A-B, see also Table 1). Blockade of MB299B-GAL4+, but not MB043B-GAL4+ neurons, also resulted in a 24 h memory defect (Figure 7C-D, see also Table 1), perhaps reflecting a difference in the number or identity of the PAM-α1 neurons in each line (Figure 6G). These results are consistent with previous studies employing MB299B-GAL4 (Ichinose et al., 2015; Chouhan and Sehgal, 2022). Since inactivation of the large group of R58E02+ PAM neurons (Figure 3H) also failed to impair 24 h LTM, these results support the idea

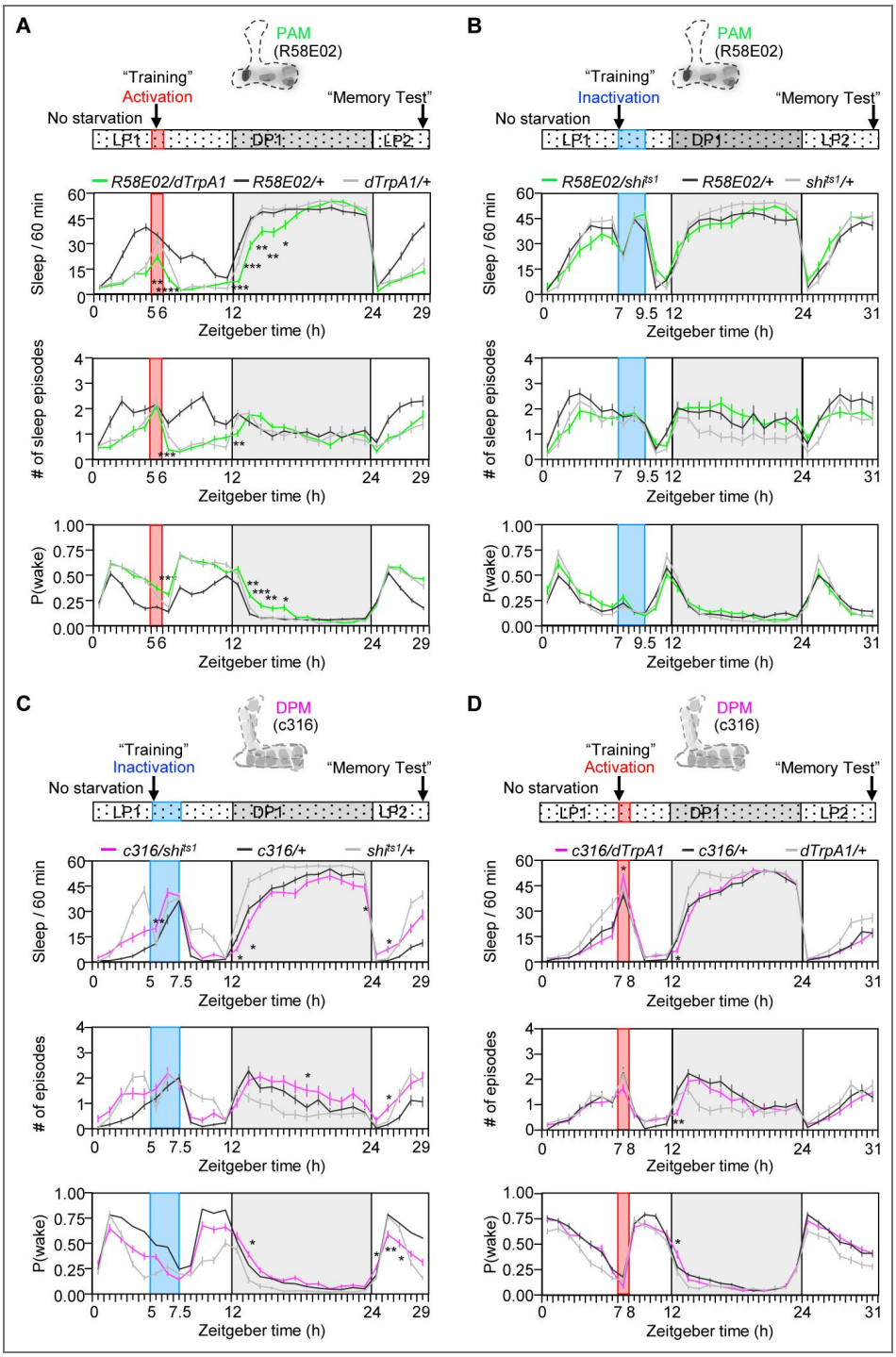
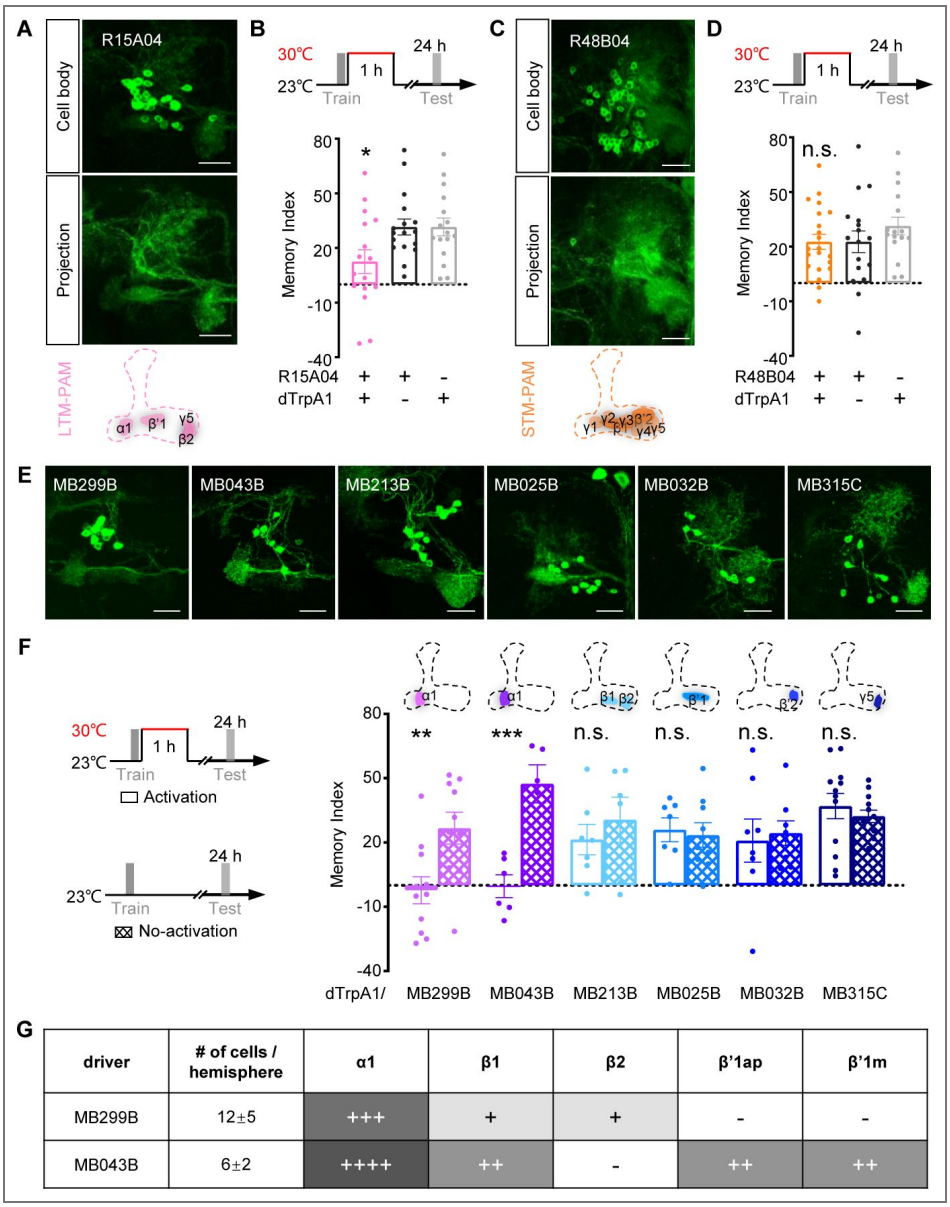


Figure 5. PAM-DPM interactions in sleep regulation are likely to be context-dependent.

(A) Activation of PAM neurons without starvation resulted a reduction of sleep at early night, as well as a slight decrease in number of sleep episodes, but an increase in P(wake). n = 39-42. (B) Inactivation of PAM neurons had no effect on sleep without starvation. n = 22-27. (C) Inactivation of DPM neurons labeled by eyeless-GAL80;MB-GAL80;c316-GAL4 without starvation had barely no effects on sleep. n = 35-47. (D) 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 Table 4.



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driver	# of cells / hemisphere	α1	β1	β2	β'1ap	β'1m
MB299B	12±5	+++	+	+	-	-
MB043B	6±2	++++	++	-	++	++

Figure 6. The PAM-α1 subset of PAM neurons contributes to memory consolidation.

(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. Scale bar: 20 μm. (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). Scale bar: 20 μm. (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) The differences in cell number/identity between the two drivers of MB299B and MB043B. "+" with greyscales indicated the levels of projections of a driver in MB subdomains; "-" indicated no projections. See also Tables 1 and 2.

that the presence of additional non-α1 PAM neurons might suppress PAM-α1 phenotypes. The connectome catalogs both PAM to PAM connections and recurrent PAM to MBON network loops that might underlie some of the differences between these GAL4 lines.

PAM-α1 neurons have been shown to be important for LTM formation, memory retention and updating memory (Huetteroth et al., 2015; Ichinose et al., 2015; Aso and Rubin, 2016; Yamagata et al., 2016). To clarify if their action is exclusive 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 7E, see also Table 1), but activation and inactivation of PAM-α1 neurons during retrieval did not affect 24 h memory (Figure 7F-G, see also Table 1). 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 aggregate, our findings indicate that the normal activity of PAM-α1 neurons after training is critical to maintain the processing of memory during consolidation.

To investigate whether 24 h memory defects were attributable to disrupted sleep secondary to changes in activity of PAM-α1 neurons, we recorded and analyzed sleep. Consistent with a context-regulated role in linking sleep and memory, activation of PAM-α1 neurons labeled by MB299B and MB043B under starvation conditions reduced sleep amount mainly during the night after training (DP1) (Figure 7H, 7J, upper panel, see also Table 4), reminiscent of DPM suppression. The night after activation of PAM-α1 neurons, flies exhibited a significantly increased number of sleep episodes and P(wake) (Figure 7H, 7J, middle and lower panels, see also Table 4). Inactivation of starved MB299B neurons had little effect on sleep, but inactivation of PAM-α1 cells labeled by MB043B produced a strong reduction and fragmentation of sleep under starvation (Figure 7I, 7K, see also Table 4), again suggesting effects of the differences in cell number/identity between the two drivers. In aggregate, these data demonstrate that both brief increases and brief decreases in activity of PAM-α1 neurons can reduce and fragment 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 since neither activation nor inactivation in fed flies had a major effect on sleep (Supplemental Figure 2A-D, see also Table 4).

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 most if not all PAM neurons, 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 follow the same rules as the aggregate, we determined the connectivity of this subset. From the EM connectome database (Zheng et al., 2018; Scheffer et al., 2020) and analysis of the connectivity with 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 8A). To determine 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), respectively, 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 8B, see also Table 3).

Given that memory consolidation and sleep are time-dependent processes, to characterize the dynamics of the PAM-DPM circuit over the course of the consolidation window following associative memory training, we performed 3-hour continuous neural activity recording in freely behaving flies. Specifically, we expressed the Tric-LUC reporter transgene, a calcium-responsive tool that harnesses the interaction between calmodulin and its cognate binding peptides to drive rapid luciferase transcription in a calcium-dependent manner (Gao et al., 2015; Guo et al.,

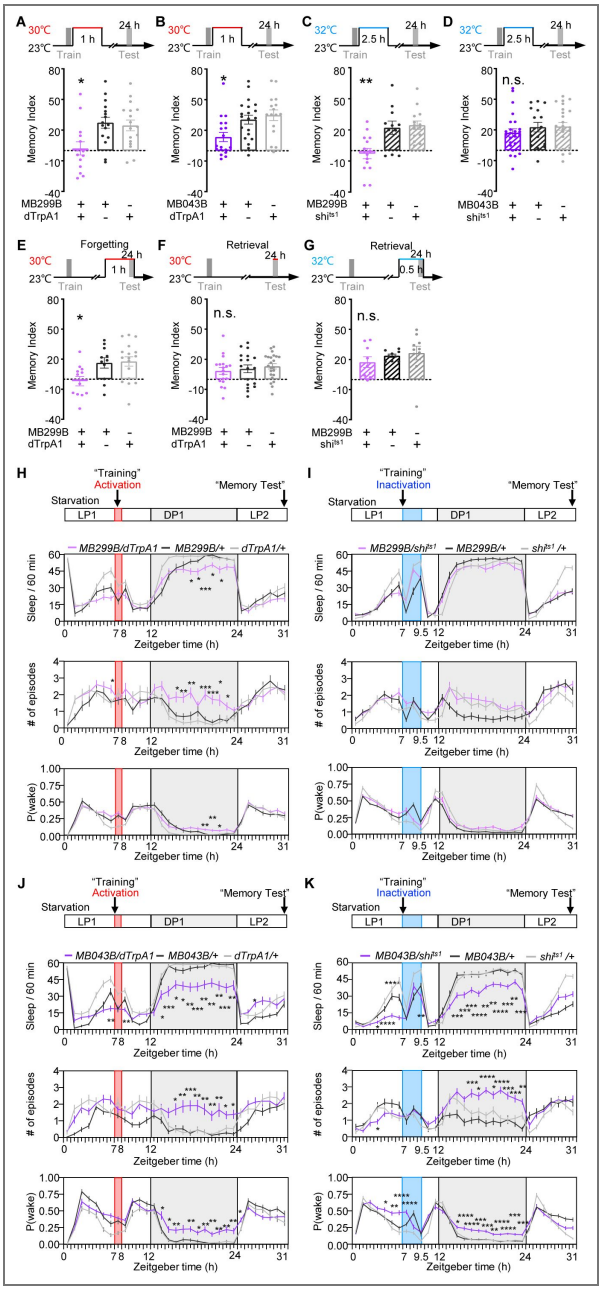


Figure 7. The PAM-α1 subset of PAM neurons contributes to linking memory consolidation and sleep.

(A) 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. (B) Activation of PAM-α1 neurons labeled by MB043B after training for 1 h impaired 24 h memory. n = 16-20. (C) 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. (D) Inactivation of PAM-α1 neurons labeled by MB043B after training for 2.5 h did not affect 24 h memory. n = 16-25. (E) 1 h activation of PAM-α1 neurons labeled by MB299B prior to and during the test impaired 24 h memory. n = 11-17. (F) Activation of PAM-α1 neurons labeled by MB299B during the test did not affect 24 h memory. n = 18-23. (G) 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. (H) Upper panel: the paradigm for sleep recording under starvation. Lower panel: total sleep, number of sleep episodes, and P(wake) in 1 h bin for activation of PAM-α1 neurons labeled by MB299B. n = 22-30. (I) Inactivation of PAM-α1 neurons labeled by MB299B under starvation had no effects on sleep. n = 33-39. (J) Activation of PAM-α1 neurons labeled by MB043B for 1 h reduced total sleep, increased the number of sleep episodes and P(wake) especially during the dark period. n = 23-30. (K) Inactivation of PAM-α1 neurons labeled by MB043B under starvation significantly reduced total sleep, and increased both the number of sleep episodes and P(wake), especially during the dark period. n = 30-39. See also Tables 1 and 4.

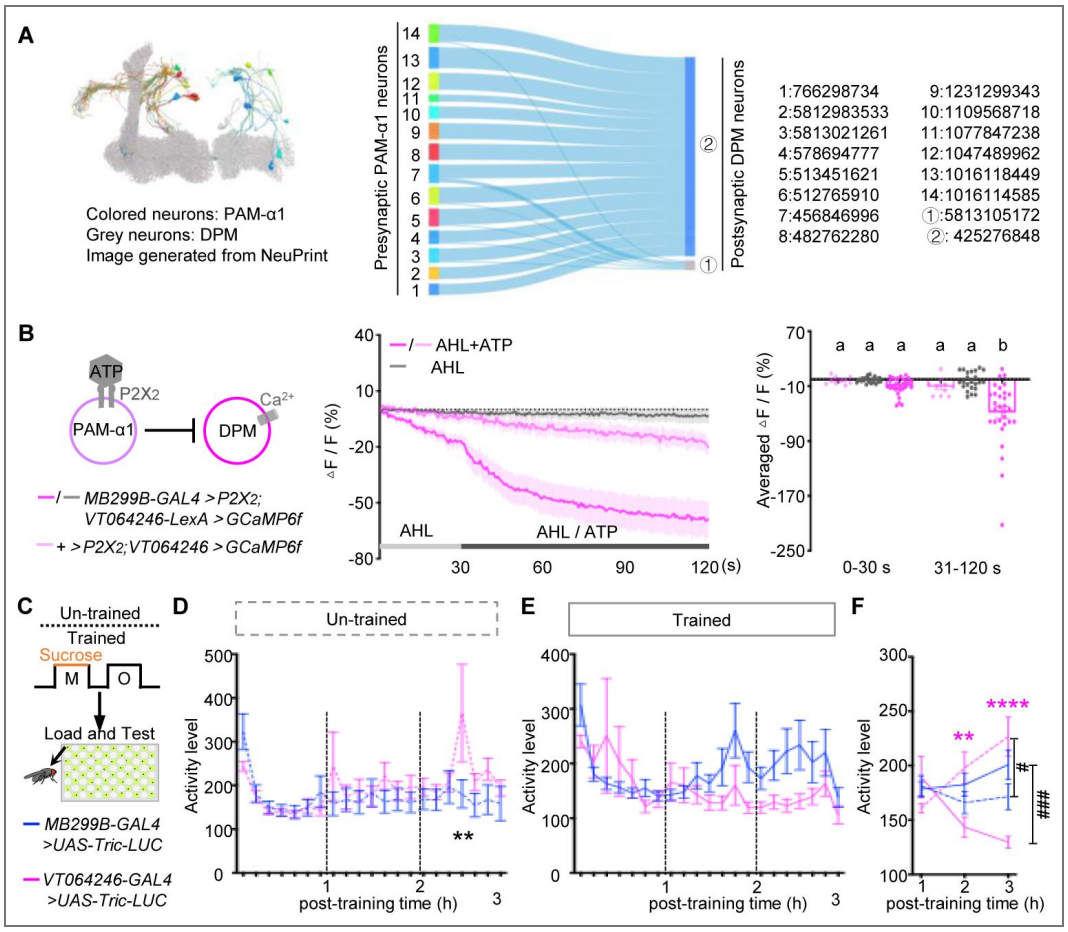


Figure 8. A specific PAM-α1-DPM inhibitory microcircuit maintains dynamic changes in activity post-learning.

(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-F) Paradigm for appetitive olfactory training and vehicle un-trained control, and representative neural activity profiles reflected by Tric-LUC expression in drivers labeling PAM-α1 and DPM neurons. Data were continuously recorded for three hours post-training in freely moving flies. (D-E) Dynamic activity changes of PAM-α1 (blue) and DPM (magenta) neurons under un-trained/trained conditions. Dotted lines represent un-trained groups, and solid lines represent trained groups. n = 20-24. (F) Quantification of the averaged changes in PAM-α1 and DPM neuronal activity at each hour post-training. *: significant difference between the DPM neuron groups under un-trained versus trained conditions; #: significant difference between PAM-α1 and DPM neuron groups. See also Table 3.

2017), in PAM-α1 or DPM neurons. Flies were then subjected to either associative memory training or a no-training control condition, with real-time luciferase levels monitored throughout the recording window (Figure 8C). In the absence of training, both PAM-α1and DPM neurons had a decrease in luciferase signal in the first hour after transfer to monitor tubes, likely reflecting some non-specific acclimation effect (Figure 8D) since this decrease is also seen in trained animals (Figure 8E). Between 1 and 3 h, untrained animals showed similar baseline activity in PAM-α1 and DPM neurons (Figure 8D).

In contrast, associative memory training profoundly reshaped the activity profile of the PAM-DPM microcircuit. During the first hour, activity in PAM-α1 and DPM neurons was similar. During the second hour after training, the activity in the two neurons begins to diverge and becomes quite different as consolidation proceeds (Figure 8E). PAM-α1 neural activity is increased significantly over baseline and DPM neuron activity is robustly decreased (Figure 8E-F). These data suggest that the PAM-α1-DPM microcircuit exhibits delayed neural activity changes following training during the consolidation window, This delay may allow natural memory processes and sleep to be yoked. The fact that both increases and decreases of PAM-α1 activity break this synergy argues that the starvation-dependent resetting of PAM-α1 activity level is a critical feature of the state-dependent function of this circuit.

While it is clear that these two neuron types are connected, it was still possible that they function in sleep and LTM via parallel, independent pathways. If this was true, we would predict that the effects of inactivating both neuron groups at the same time should additively decrease sleep. To determine if this was the case, we simultaneously inactivated these neurons by expressing shi^{ts1} in both cell groups, found sleep reduction, fragmentation, and arousal threshold (P(wake)) statistically the same as when each of them was inactivated separately under starvation (Figure 9A-B, see also Table 4). 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 with feeding of gaboxadol (also called THIP), a GABA agonist that promotes sleep (Dissel et al., 2015) was sufficient to also rescue memory consolidation, as reflected by memory levels comparable to the genetic controls and significantly better than the no-THIP group (Figure 9C-G, see also Table 1, 2 and 4). 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.

Dop1R1 expressed in DPM neurons mediates sleep-memory integration

To define the dopamine receptor (DAR) signaling mechanisms underlying DPM neuron activity and its regulatory roles in sleep and memory, we first characterized the DAR expression profile of DPM neurons. Using a double-labeling assay, we detected robust expression of Dop1R1 and Dop1R2 in DPM neurons, whereas no detectable colocalization was observed for DopEcR and Dop2R (Figure 10A). Dop1R1 and Dop1R2 have been reported to stimulate an increase in intracellular cAMP in the presence of DA (Yamamoto and Seto, 2014). To clarify how these DARs mediate the inhibition of DPM neurons, we knocked down Dop1R1 or Dop1R2 in VT046004-GAL4+ neurons and imaged EPAC, an indicator of cAMP levels in DPMs, after application of dopamine (Figure 10B). Dop1R1 RNAi and Dop1R2 RNAi both efficiently reduced the relative mRNA levels of their target gene (Supplemental Figure 3A-B). Knockdown of Dop1R1 receptors in DPM neurons significantly suppressed the dopamine-induced increase in cAMP (visualized as a decrease in EPAC FRET), whereas knockdown of Dop1R2 trended to further increasing cAMP levels (Figure 10B, see also Table 3). These data confirm that Gαs-coupled Dop1R1 is the primary receptor mediating DA-dependent cAMP elevation in DPM neurons. To further identify the DARs responsible for transducing the DA-induced inhibitory effect on DPM neural activity, we quantified GCaMP signals in DPM neurons with targeted knockdown of individual DARs. Knockdown of either Dop1R1 or Dop1R2 failed to abolish the DA-induced Ca^{2+} decrease (Supplemental Figure 3C-

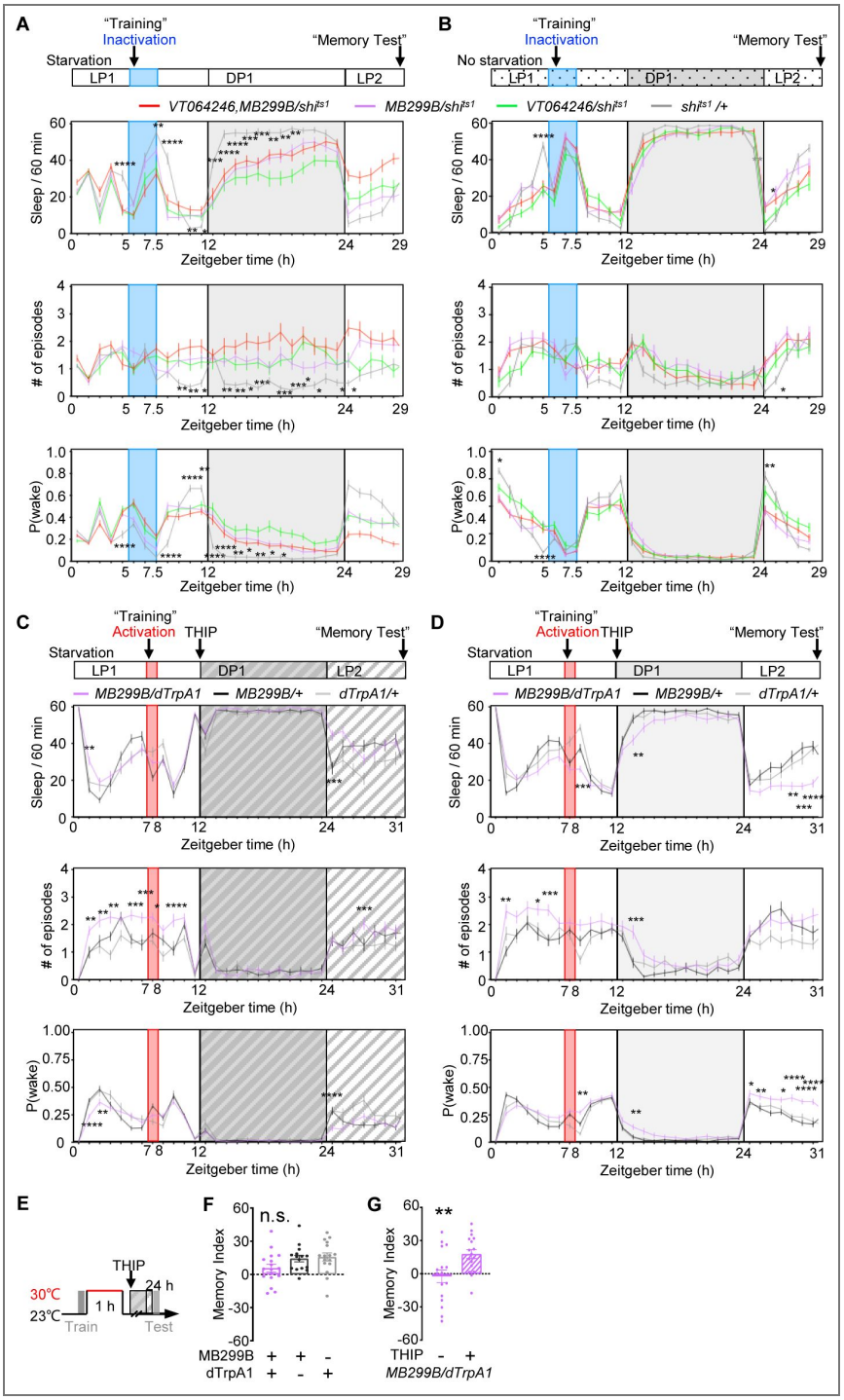


Figure 9. The PAM-α1-DPM inhibitory microcircuit binds memory consolidation and sleep in an internal-state dependent manner.

(A) Simultaneous silence of PAM-α1-DPM microcircuit resulted in sleep reduction and fragmentation, increased possibility of waking. n = 36-40. (B) Simultaneous silence of PAM-α1-DPM microcircuit without starvation had little effect on sleep and the number of sleep episodes and P (wake). n = 29-31. (C-D) 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 (C), n = 37-44 (D). (E) The paradigm of 24 h memory test with activation of PAM-α1 neurons for 1 h after training. (F-G) THIP rescued 24 h memory deficit induced by 1 h activation of PAM-α1 neurons during consolidation compared to genetic controls (F, n = 15-17) or compared to no THIP group (G, n = 17). See also Tables 1, 2 and 4.

E). Because the experiments were done in the presence of TTX to eliminate action potential-dependent release of substances from other cells in the circuit, this suggests that either both receptors cooperate to modulate DPM neural activity, or that there is another (undetected) DAR expressed in DPMs. It is also possible that since RNAi knockdown is never complete, residual Dop1R1/R2 is sufficient to mostly support the calcium-reducing effects of DA, but not the efficient coupling to adenylate cyclase if the downstream pathways are differentially sensitive.

Finally, to dissect the specific contributions of DARs in DPM neurons to sleep and/or memory regulation, we performed sleep monitoring and memory assays in animals with DPM-specific knockdown of distinct DARs (Figure 10C-H). Knockdown of Dop1R1 in DPM neurons resulted in statistically significant sleep reduction, decreased arousal threshold, and impaired 24 h LTM memory with intact learning (Figure 10C-E). In contrast, knockdown Dop1R2 in DPM neuron selectively impaired 24 h LTM memory with no effect on sleep and learning (Figure 10F-H). Collectively, these findings demonstrate that coupling sleep and LTM requires Dop1R1 in DPM neurons through the modulation of both cAMP signaling and neuronal activity, while Dop1R2 specifically mediates LTM regulation, likely through modulating DPM neural activity alone.

MBON-α1 neurons participate in regulation of both memory consolidation and sleep

PAM-α1 has previously been demonstrated to drive appetitive LTM formation and consolidation via a recurrent loop with MBON-α1 (Ichinose et al., 2015). To investigate whether MBON-α1 also participates in sleep regulation, we activated or inactivated MBON-α1 neurons under both starvation and non-starvation conditions (Figure 11A-D). Our results revealed that inhibition of MBON-α1 under both starvation and non-starvation conditions resulted in a significant reduction in sleep and a reduced arousal threshold (Figure 11B, D), suggesting that MBON-α1 participates in regulating sleep in a state-independent manner. However, no significant changes were observed upon activation of MBON-α1 neurons (Figure 11A, C). Moreover, consistent with previous finding, inhibition of MBON-α1 during the memory consolidation phase significantly impaired 24 h LTM (Figure 11F). These results indicate that MBON-α1mediated sleep is necessary for effective memory consolidation. Notably, while activation of MBON-α1 during consolidation phase similarly impaired LTM (Figure 11E), this manipulation did not alter the sleep profile, suggesting a dissociation between MBON-α1's mechanistic roles in sleep regulation and LTM processing. Taken together, these findings reveal a dedicated hierarchical, modular regulatory network that mediates sleep-LTM coupling via an activity-dependent mechanism. Within this network, activation of PAM-α1 acts as an upstream modulator to inhibit the activity of DPM, a downstream integrative hub that coordinates the execution of sleep and memory processes, which is modulated by the recurrent positive feedback loop of PAM-α1-MBON-α1.

Discussion

PAM-DANs have been known to be important for formation of short-term and long-term memories, and DPM neurons for memory consolidation and sleep. In the present study as summarized in Figure 12, we find that these two types of MB extrinsic neurons are connected by inhibitory synapses and play an essential role in bridging LTM stabilization 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 after the brief disruption of this circuit when the flies are starved. Thus, maintenance of basal activity of PAM-α1-DPM microcircuit during memory consolidation has a profound effect on LTM, through influencing sleep when the animal is experiencing a certain internal state. Distinct signaling cascades may be recruited via different dopamine receptors in DPM neurons to integrate or segregate sleep and memory processes. Additional parallel circuits further contribute to linking and specifying the regulatory logic of these processes.

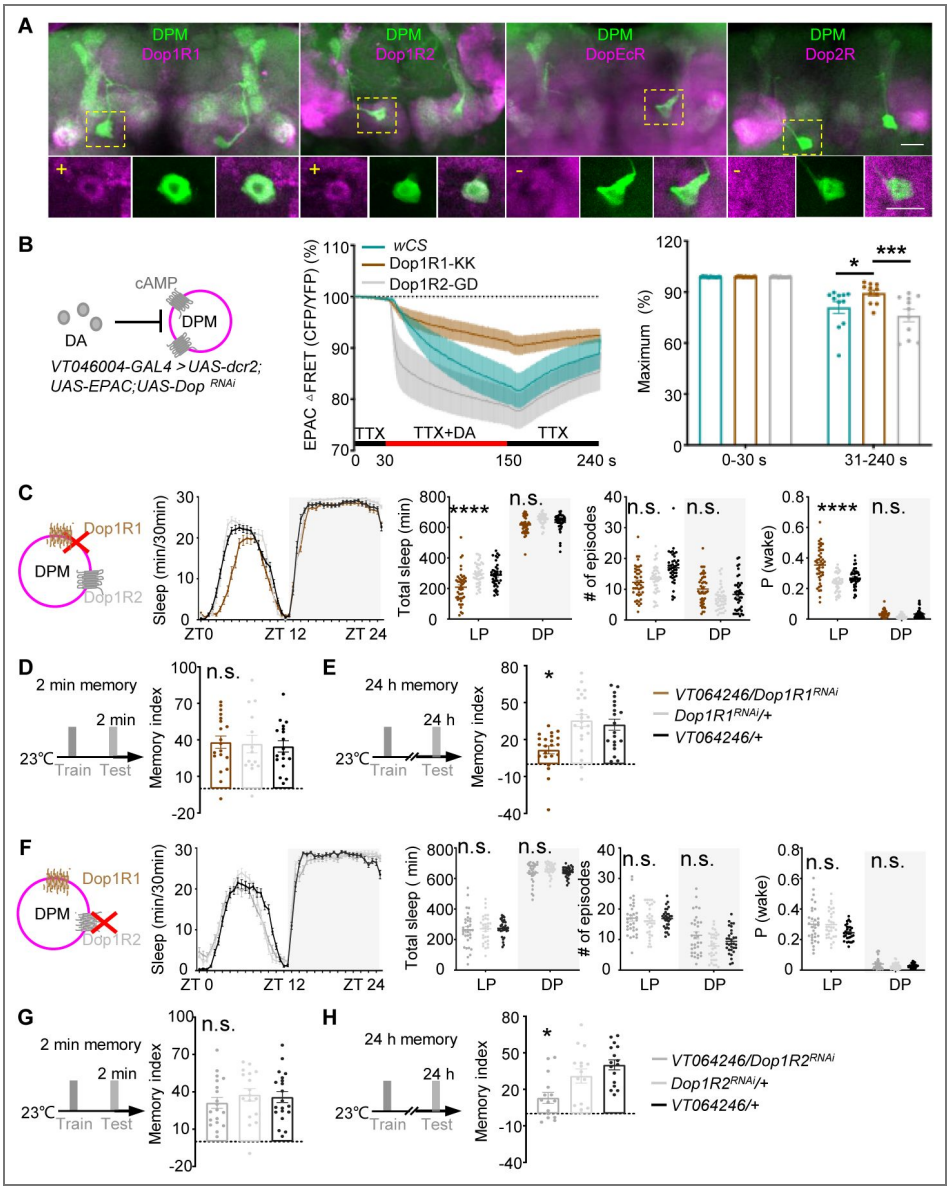


Figure 10. Dop1R1 expressed in DPM neurons mediates sleep-memory integration.

(A) Colocalization of DPM neurons with distinct dopamine receptor subtypes. DPM neurons labeled by VT064246-LexA were visualized by GFP (green), and dopamine receptors labeled by Dop1R1-GAL4, Dop1R2-GAL4, DopEcR-GAL4 and Dop2R-GAL4 were visualized by RFP (magenta). The yellow dashed box outlines the cell body of one DPM neuron, with the upper and lower panels on the right showing the split channels for DPM neurons and the corresponding dopamine receptor subtype (white arrow), respectively. Scale bar: 20 μm. (B) Averaged cAMP traces (middle panel) and quantification (right panel) of DPM neurons in response to DA with the presence of 2.0 mM TTX with or without Dop1R1 or Dop1R2 knockdown. n = 11 for all groups. (C) Knock down Dop1R1 on DPM neurons decreased the total amount of daytime sleep and increased P(wake). n = 40-44. (D-E) Knock down Dop1R1 on DPM neurons had no effect on 2min, but impaired 24 memory. n = 14-19 (D), n = 20-22 (E). (F) Knock down Dop1R2 on DPM neurons, had no effect on sleep. n = 31-32. (G-H) Knock down Dop1R2 on DPM neurons had no effect on 2min, but impaired 24 memory. n = 17-20 (G), n = 15-16 (H). See also Tables 1 and 3.

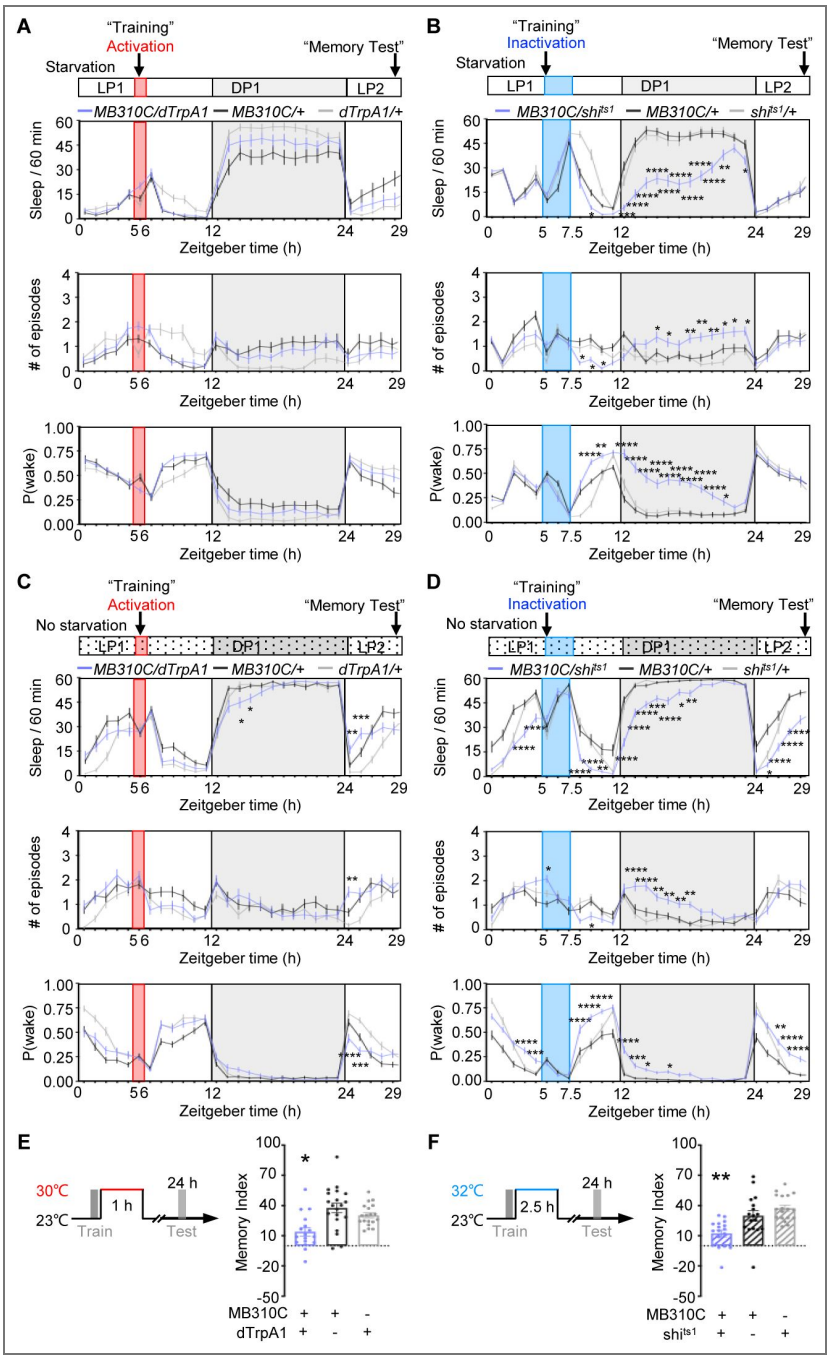


Figure 11. MBON-α1 neurons regulate sleep irrespective of starvation state, and their activity disruption during memory consolidation impairs LTM.

(A, C) Activation of MBON-α1 neurons labeled by MB310C under starvation (A, n = 26-32) or no starvation (C, n = 31 for all groups) condition had no effects on sleep. (B, D) Inactivation of MBON-α1 neurons for 2.5 h significantly reduced total sleep, increased the number of sleep episodes and P(wake) especially during the dark period under starvation (B, n = 37-44) and no starvation (D, n = 33-43) condition. (E) Flies with activation of MBON-α1 neurons after training for 1 h exhibited impaired 24 h memory compared to their genetic controls. n = 17-20. (F) Flies with blockade the output of MBON-α1 neurons after training for 2.5 h exhibited an impairment of 24 h memory compared to their genetic controls. n = 17-18. See also Tables 1 and 4.

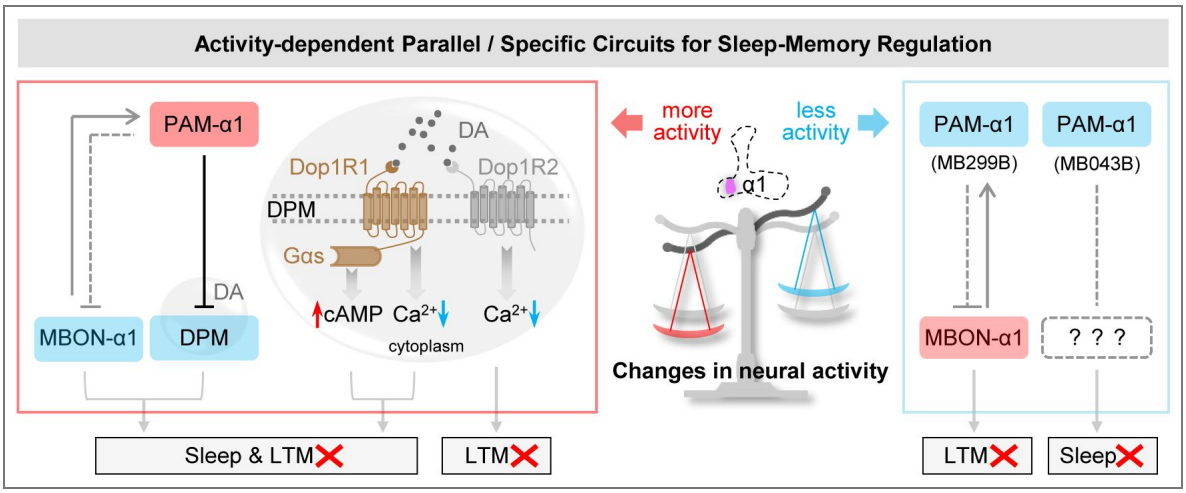


Figure 12. Schematic of activity-dependent parallel and specific regulatory circuits for sleep-memory regulation.

Increased activity of PAM-α1 neurons strongly inhibits DPM, leading to disrupted sleep and impaired LTM. MBON-α1 likely acts as a parallel circuit relative to PAM-α1-DPM pathway, interfering with the coupling between sleep and memory. DA signaling through Dop1R1 receptors triggers an increase in cAMP and a decrease in Ca^{2+}, disrupting both sleep and LTM. By contrast, signaling via Dop1R2 receptors only reduces Ca^{2+} levels, specifically impairing LTM. However, inactivated PAM-α1 may target different downstream targets to separately regulate sleep or memory.

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., 2015; Yamagata et al., 2021). We find that not only suppressed activity but also elevated activity of PAM-α1 neurons right after training can disrupt memory consolidation, supporting a 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 many aspects of memory processing. 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 and McGaugh, 2010), and many 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 processes, but it is clearly not required for retrieval (Figure 7E-G), 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 is thought to be indispensable to memory consolidation process, converting initially unstable memory into stable long-term memory during sleep (Diekelmann and Born, 2010; Klinzing et al., 2019; Girardeau and Lopes-Dos-Santos, 2021; Yan et al., 2025). Besides the amount of sleep, accumulating evidence has suggested that sleep structure can influence memory processing (Rolls et al., 2011; Lipinska and Thomas, 2019; Liu et al., 2019; Kjaerby et al., 2022). In *Drosophila*, sleep structure can be evaluated by the number of sleep episodes as well as P(wake). The number of sleep episodes reflects the frequency of awakenings, and P(wake) is the probability of transitioning from a sleep to an awake state, reflecting 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 resulted in reduced and fragmented sleep, with a consequent impairment of LTM, providing a new circuit basis for the linkage between these two processes.

DPM neurons process memory consolidation via promoting sleep, likely via inhibition of wake-promoting MB-α'β' neurons (Donlea, 2011; Haynes et al., 2015), and this circuit also seems particularly important for consolidation (Krashes et al., 2007). DPM neurons have been recently endowed with new functions, such as a gatekeeper for intrinsic coincidence time windows, and a bridge for mediating multisensory stimulus binding (Okray et al., 2023; Zeng et al., 2023), further revealing a richness in the compartmentalized specificity and plasticity of microcircuits. 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, 2011; Sitaraman et al., 2015; Driscoll et al., 2021). 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. This is a model that could be tested in the future. In spite of the fact that MB circuits have been elegantly investigated, the leap from the microcircuit level to the systems scale remains daunting, and how these circuits enable consolidation during sleep still needs further investigation.

Internal state dictates circuit configuration for consolidation

Internal state, like starvation, gates many processes through molecular mediators and alternative neural circuits (Keene et al., 2010; McDonald, 2010; Lin et al., 2019; Yurgel et al., 2019). DANs contribute robustly to coordination of state-dependent behaviors, such as facilitating

starvation-dependent sugar memory, and mediating starvation-driven food seeking behavior (Krashes et al., 2009; Tsao et al., 2018; Senapati et al., 2019). Driven by context, distinct subsets of DANs exhibit suppressed or enhanced neuronal responses facilitating integration of motivational signals and modulating output behaviors (Liu et al., 2017; Jovanoski et al., 2023). Our present study characterizes another subset of DANs that respond to starvation with an increase in neural activity to provide a context-dependent regulation of behavior.

It is well established that starvation suppresses sleep (MacFadyen, 1973; Thimgan et al., 2010; Melnattur and Shaw, 2019; Keene et al., 2010; He et al., 2020; Oh and Suh, 2023), and our results are consistent with these previous findings: all genotypes exhibited less sleep under starvation than under fed conditions (i.e. Figures 4A, B, 5A, B and 11). With normal appetitive memory training, starvation-induced sleep loss does not necessarily impair memory processing (Thimgan et al., 2010; Chouhan et al., 2021). PAM-α1 neuronal activity is higher in starved, trained flies than in fed or untrained flies (data not shown), suggesting that these neurons act as a critical node for integrating internal motivational and arousal states, as well as conveying positive valance for the normal appetitive memory process, independently of starvation-induced sleep loss. While DPM neurons are less sensitive to starvation, they still exhibit training-induced elevated activity (data not shown), indicating coherent responsiveness to upstream signaling. In the present study, we found that under fed conditions, sleep remained intact even when large changes in neural activity occurred within the PAM(-α1)-DPM circuit; in contrast, under starvation conditions, significant sleep reduction and fragmentation were observed. These observations indicate that starvation may trigger a transition from a physiologically normal brain state to an unstable, abnormally active state, which consequently elicits negative behavioral outputs.

Sleep serves memory consolidation (Brodt et al., 2023; Chandra et al., 2023). Paradoxically, flies need to be starved to express appetitive LTM. But it can be interpretated in a way that the animal's state during consolidation can recruit different circuitry at both the KC level and the DAN level (Chouhan et al., 2021; Chouhan and Sehgal, 2022). Our results provides yet another example of this. Suppressed but not increased activity of MBON-α1 negatively impacts on sleep no matter the animal is starved or not. However, disrupted activity of MBON-α1 during memory consolidation phase impairs appetitive LTM. The activity of the microcircuit we have identified is likely to be key to understanding how sleep need is taken out of the loop during starvation.

An additional interesting feature of the role of PAM-α1 neurons in consolidation of appetitive LTM is that it is very sensitive to the level of activity within this neuron group (Figure 12). Both increases and decreases in activity can block LTM and fragment sleep. The inhibitory nature of the connectivity between PAM-α1 and DPM provides a plausible explanation for the effects of overactivation of PAM-α1: strongly blocking DPM activity would suppress both sleep and consolidation (Waddell, 2000; Keene et al., 2004; Yu et al., 2005; Krashes and Waddell, 2008; Haynes et al., 2015). Furthermore, MBON-α1, provides an additional component which serves as a parallel pathway to suppress sleep and impair LTM modulated by this PAM-α1-MBON-α1 recurrent feedback loop. PAM-α1 may forms an inhibitory pathway on MBON-α1, supported by the same sleep and memory outcomes upon activation of PAM-α1 and inactivation of MBON-α1. How inactivation of PAM-α1 produces a similar 'DPM-inhibition-like' state is more difficult to explain with current data. However, according to the observations of specified modulation on memory or sleep, PAM-α1 relieves its inhibitory control over MBON-α1, leading to MBON-α1 activation which has no effect on sleep; MBON-α1 then functions as a signal amplifier that further exacerbates the reduced activity of PAM-α1, specifically resulting in LTM impairment. Whether this is a direct effect of decreasing the level of stimulation of dopamine receptors that occurs under starvation conditions when PAM-α1 activity increases or if it is an indirect effect mediated by an interneuron will require further investigation. In another case, inactivation of PAM-α1, possibly non-PAM-α1 neurons labeled by MB043B, specifically contributes to the regulation of sleep. Sleep and memory are highly intertwined within this circuit, where distinct neuronal populations exhibit specialized yet interdependent functional roles, with overlapping and divergent regulatory contributions to sleep and LTM. The inherent complexity of this regulatory network thus merits further dedicated investigation in future studies.

Materials and methods

Animals

All flies were reared on standard cornmeal food at 25 °C 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 °C. 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. The following stocks were obtained from the Bloomington Drosophila stock center (BDSC, Indiana University): *w;;R58E02-GAL4* (70226), *w;;R15A04-GAL4* (48671), *w;;R48B04-GAL4* (50347), *MB025B-GAL4* (68299), *MB213B-GAL4* (68273), *MB032B-GAL4* (68251), *MB315C-GAL4* (68316), *MB299B-GAL4*(68310), *MB043B-GAL4* (68304), *elav-GAL4* (458), *UAS-mCD8::GFP; Pin/Cyo* (5136), *w;UAS-dTrpA1* (26263), *w;;UAS-shibire^{ts1}* (44222), *w;R58E02-LexA* (52740), *eyeless-GAL80;MB-GAL80/Cyo;c316-GAL4/TM6B* (30380), *UAS-CD8::RFP,LexAop-CD8::GFP* (302229), *LexAop-nSyb:spGFP1-10,UAS-CD4:spGFP11;MKRS/TM6B* (64315), *UAS-EPAC* (78802), *UAS-dcr2* (24650), *LexAop-P2X$_2$* (76030), *UAS-GCaMP6f* (42747), *UAS-CD4-spGFP1-10* (93016), *LexAop-CD4-spGFP11* (93019), *UAS-Dop1R2^{RNAi}* (51423) (Ni et al., 2011), *UAS-P2X$_2$* (91223) and *LexAop-GCaMP6f* (44277), *UAS-CRTC::GFP/CyO;UAS-mCD8::mCherry -T2A-nls::LacZ in VK00005/TM2* (99656). *VT064246-GAL4/TM2* (v204311), *UAS-Dop1R1^{RNAi}* (v107058) (Scaplen et al., 2020), *UAS-Dop1R2$_{RNAi}$* (v3392) (Dietzl et al., 2007) were ordered from Vienna Drosophila Resource Center (VDRC, Vienna, Austria). *VT046004-GAL4* was one of Vienna Tile GAL4 lines, currently available at Korea *Drosophila* Resource Center (12772). The following lines have been previously described: *trans-Tango,QUAS-FLP;LexAop-FRT-RFP-FRT-GFP* (Sun et al., 2022). *w*;;Dop1R1-GAL4 and w*;;Dop1R2-GAL4* was kindly provided by Prof. J. W. Kim, and *Dop2R-KI-GAL4* was kindly provided by Prof. Yufeng Pan. 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'-AATACGAGAGCG CTCACTAC-3' and reverse primer: 5'-TCTTAT GGACGGCGAGGAG-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'-AATACGAGA GCGCTCACTAC-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).

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 odors. 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.

For all 24 h sucrose-odor memory, a single training session of sucrose paired with an odor for 2 min was employed (Figure 3A). 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 °C; 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 odor paired with an unconditioned stimulus (US: high temperature or sucrose) and flies chose the other odor 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 odor paired with US, and the other half of LIs from the second odor 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). 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

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*).

In vivo Tric-Luciferase assays

5-7 days flies (*MB299B-split-GALl4>Tric-LUC* and *VT064246-GAL4>Tric-LUC*) were collected and pre-fed for 2 days on food containing 5% sucrose, 2% agar and 20 mM D-luciferin potassium salt (Cat# 115144-35-9, Gold Biotechnology). Flies were then transferred to starvation tubes containing 2% agar food supplemented with D-luciferin potassium salt and starved for 22 hours Subsequently, flies underwent either 2 min sucrose-odor pairing training or remained untrained., They were then placed into white 96-well Microfluor 2 plates (Costar) with starvation medium. Luciferase activity from individual flies was recorded using a Microbeta 2 microplate counter (PerkinElmer), with luminescence signals collected continuously for 3 hours.

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};VT 046004-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.

In vivo calcium imaging

All-trans-retinal (ATR) powder (Cat# R2500, Sigma) was dissolved in 100% alcohol to prepare a 100 mM stock solution. For ATR food used in the optogenetic experiments, 240 μl of stock solution was added to 30 ml of 5% sucrose, 1% agar medium to yield final concentration of approximately 800 μM ATR. 3-5 day-old flies (*VT064246-LexA/LexAop-GCaMP6f;R58E02-GAL4/UAS-Cschrimson* and *VT064246-LexA/LexAop-GCaMP6f;+/UAS-Cschrimson,*) were transferred to ATR food and maintained for 3 days prior to optogenetic imaging.

Flies were anesthetized on ice, and their heads were fixed in a custom-made imaging chamber using UV-curable glue. A small window was carefully dissected in the anterior head cuticle under extracellular saline solution AHL. For optogenetic activation, a 630 nm LED was focused onto the

window at an intensity of 1-2 mW. Images were acquired at 1024 × 1024 pixels with a frame rate of 2 Hz. ROIs were analyzed using Fiji. Signal quantification was performed using the same method as for in vitro calcium imaging.

Quantitative PCR

To valid the efficiency of the dopamine receptor knockdown, UAS-RNAi flies were crossed with elav-GAL4 flies. GAL4 and UAS homozygous parental lines were used as genetic controls. For quantitative PCR (qPCR), total RNA was extracted from 40 fly heads per sample using the RNeasy Mini Kit (Cat# 74104, QIAGEN), and cDNA was synthesized using the ReverTra Ace qPCR RT Kit (Cat# FSQ-201, TOYOBO). Three biological replicates were performed for each genotype, with two technical replicates per biological sample.

qPCRs were conducted using the SYBR Green Master Mix (Cat# QPK-201, TOYOBO). Raw fluorescence data were processed using dynamic tube and slope correction via QuantStudio™ Real-Time PCR software. Relative mRNA expression levels were calculated using the comparative CT method, with rp49 as the endogenous reference gene. qPCR primers (0.1 mM each, 5'-3') were used as follows: forward primer for Dop1R1: GCCGCTGTCACTTGTGTGTCAATTGTAG; reverse primer for Dop1R1: ACACCGGCAAAGGTCATCACCAGC; forward primer for Dop1R2: GGCCACCAACT CTCTCATCACCAGC; reverse primer for Dop1R2: AGATTCAGTATGGAGGCGGTGCTG; and forward primer for rp49: GCTAAGCTGTCGCACAAA; reverse primer for rp49, TCCGGTGGGCAGCATGTG.

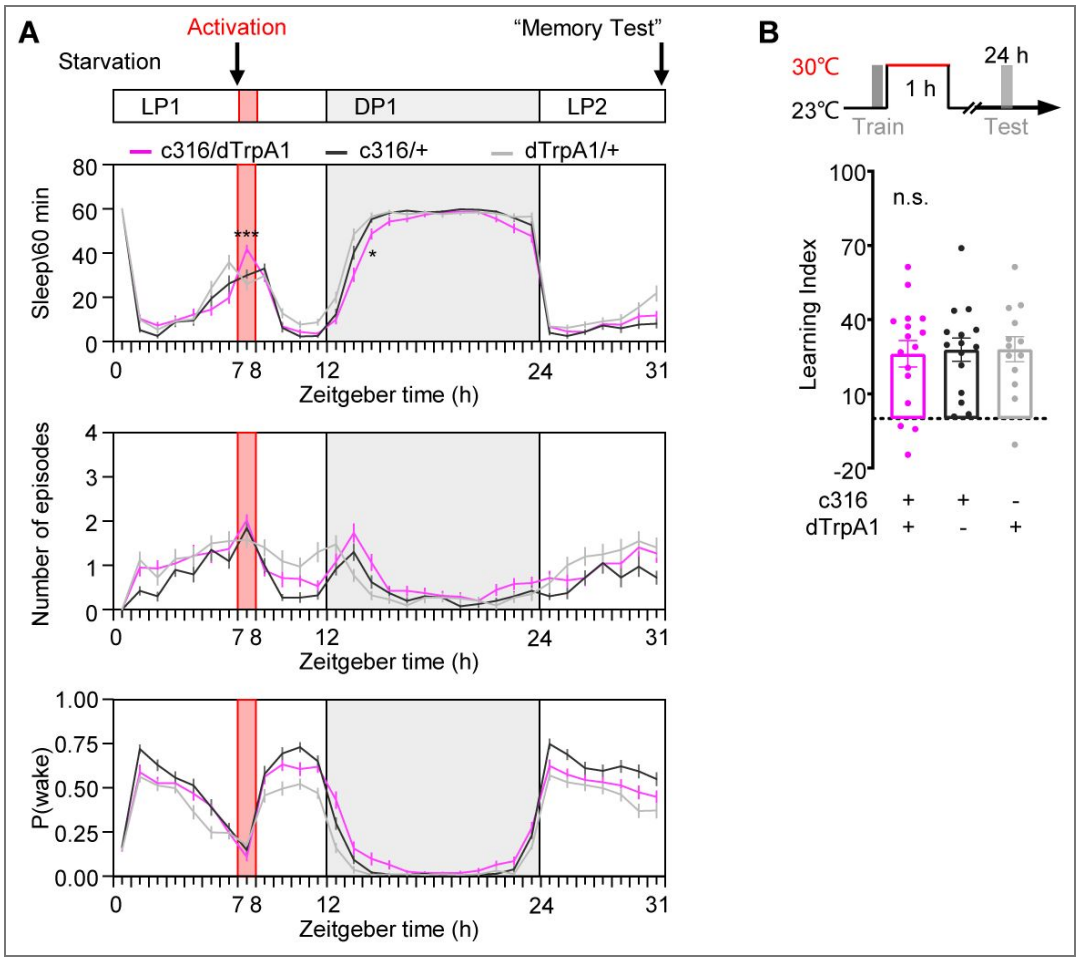
Experimental design and statistical analysis

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. *In vitro/vivo* functional imaging experiments (Figure 2A, B, Supplemental Figure 3C-E and Figure 8B) and Tric-LUC experiments (Figure 8F), two-way ANOVA followed by Tukey's and Sidak's multiple comparisons test were applied. Furthermore, in the experiments to compare the activation group to the no-activation group (Figure 6F), 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 Tables 1-4: Table 1 for one-way ANOVA analysis, Table 2 for unpaired t-test analysis, Table 4 for two-way ANOVA analysis for sleep, and Table 3 for the rest two-way ANOVA analysis.

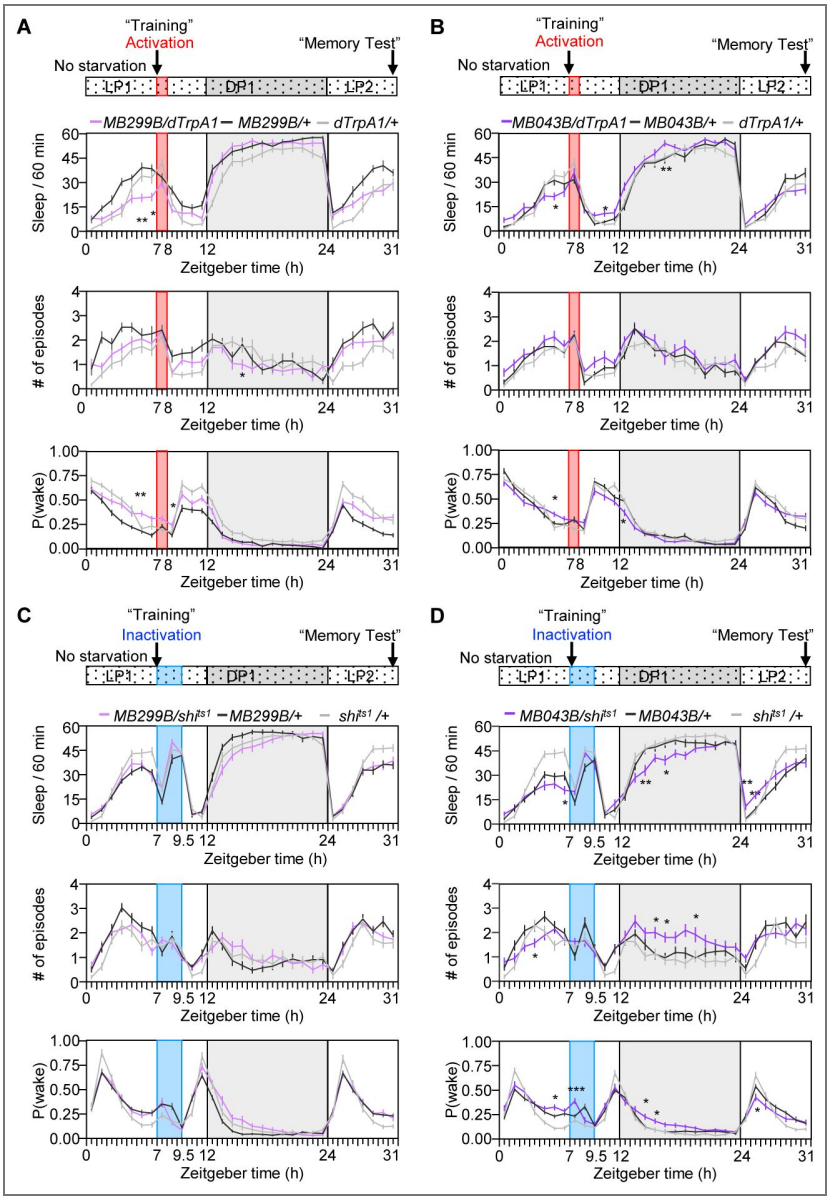
Data availability

All data are contained within the manuscript. Statistical analysis details, including sample size, statistical tests and p values have been provided in Tables 1-4. This work generated fly stocks, which we will distribute on request. Raw data will be distributed on request.

Supplemental figures

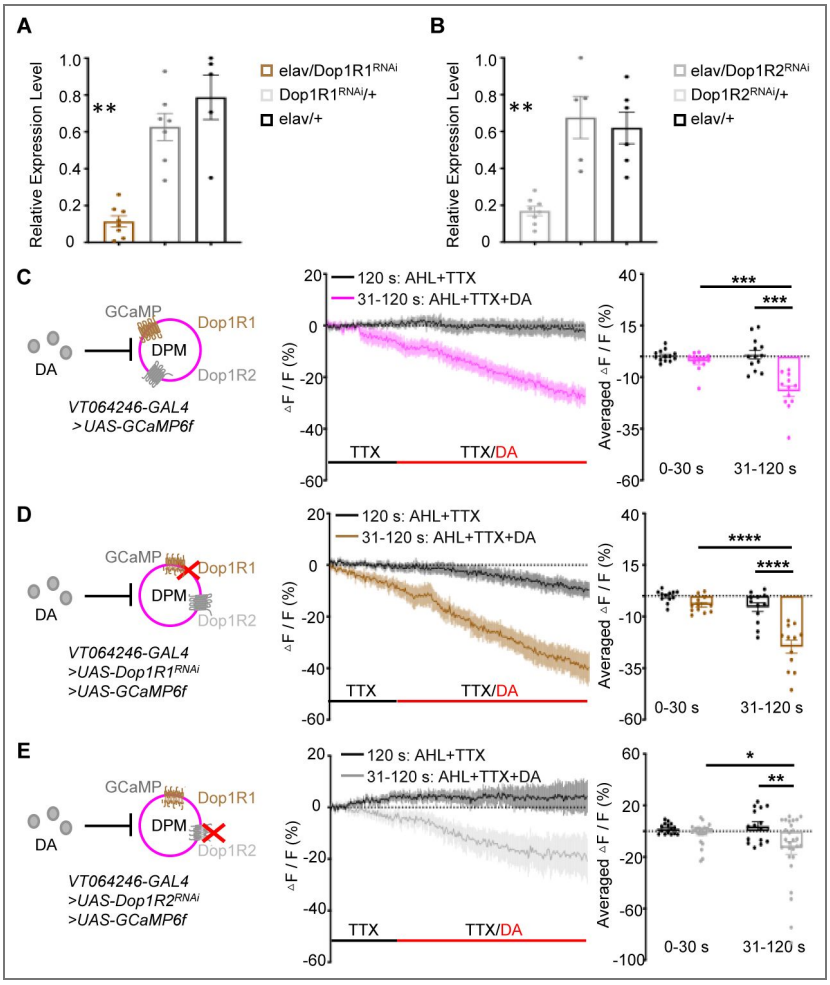


Supplemental Figure 1 related to Figure 4. Activation of DPM neurons increases sleep but has no effect on memory consolidation. (A) Activation of DPM neurons labeled by eyeless-GAL80;MB-GAL80;c316-GAL4 under starvation increased total sleep during the activation period, with little to no effect afterwards. n = 35-42. (B) Activation of DPM neurons for 1 h immediately following training did not affect 24 h memory. n = 13-16. See also Tables 1 and 4.



Supplemental Figure 2 related to Figure 7. The PAM-α1 subset linking memory consolidation and sleep depends on internal starvation status.

(A-B) Sleep profiles of total sleep, the number of sleep episodes, and P(wake) with 1 h activation of MB299B (A) or MB043B (B) before, during and after activation. Sleep was not affected without starvation. n = 28-31. (C) Inactivation of MB299B for 2.5 h without starvation had no effects on sleep. n = 30-37. (D) Inactivation of MB043B for 2.5 h without starvation resulted a significant reduction in total sleep, a mild increase of number of sleep episodes during the dark period, and no effect on P(wake). n = 30-39. See also Table 4.



Supplemental Figure 3 related to Figure 10. Verification of Dop1R1 and Dop1R2 knockdown efficiency and their effects on neuronal activity.

(A-B) Relative mRNA levels of Dop1R1 (A) and Dop1R2 (B) in adult fly heads. Knockdown of either Dop1R1 or Dop1R2 in elav-GAL4 labeling cells resulted in a significant reduction in transcript levels compared to controls. Data are presented as mean ± SEM (n = 5-8). **p < 0.01. (C-E) Averaged GCaMP traces (upper panels) and quantification (lower panels) of DPM neurons in response to DA with the presence of 2.0 mM TTX with or without Dop1R1 or Dop1R2 knocked-down. (C, n = 12-13; D, n = 12 for all groups; E, n = 15-27). See also Tables 1 and 3.

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Additional information

Author Contributions

C. L. designed and supervised research; L.Y., L.W., T.D.W., X.S., W.Y., H.L., and C.L. performed research; L.Y., L.L., Z.L., Y.L., Z.M., F.G., L.C.G. and C.L. analyzed and interpreted data; L.Y., Z.M., Y.L., F.L., L.C.G. and C.L. wrote the manuscript and all authors reviewed the manuscript.

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Additional files

Supplemental Table 1

Supplemental Table 2

Supplemental Table 3

Supplemental Table 4

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Peer reviews

Reviewer #1 (Public review):

Summary:

The authors aim to use state-of-the art behaviour, imaging and connectome techniques to identify the neural interaction between sleep and long-term memory consolidation in the PAM-DPM circuits, a well-known dopaminergic pathway within Drosophila Mushroom Body.

Strengths:

The investigation follows a logical strategy to collect huge dataset of sleep, appetitive memory and live imaging. The authors identified and showed that activation of a PAM subset: alpha-1 reduces sleep quality and memory consolidation in a starvation dependant manner. The author also convincingly demonstrated the corresponding neuronal responses of DPM

neurons following PAM alpha-1 activation, and the positive role of DPM neural activity in sleep and memory consolidation. Moreover, the new data provide TRIC-LUC provided better temporal resolution of neural activity correlates for PAMalpha1-DPM inhibition. Importantly, the author demonstrated that memory loss derived from PAM alpha 1 activation can be partly restored by ectopic sleep enhancement via feeding THIP at the memory consolidation period after training.

Weaknesses:

Although the revised version carries arguments to satisfy the reviewers' concern, the writing is now less cohesive. Crucially an explanation however remains required for the following experimental contradiction: the central observation of the study indicates that PAM alpha1 activation cause DPM inhibition which disrupt sleep and memory consolidation. Therefore, one would expect a reduced PAMalpha1 and increased DPM activities after memory training, but the authors found the opposite is true from now enhanced TRIC-LUC dataset. The authors indicate this data reinforce the inhibitory nature of PAM-alph1-DPM, but it does not explain why such a reduced DPM activity is observed after training.

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Reviewer #2 (Public review):

Summary:

Sleep plays a critical role in memory consolidation, but the neural mechanisms underlying this relationship remain incompletely understood. The authors examined a specific subset of PAM dopaminergic neurons, PAM-α1, and DPM neurons in Drosophila. These neurons have previously been implicated in memory, and DPM neurons have also been linked to sleep. The study explores whether this circuit provides a mechanistic link between sleep and memory consolidation.

Strengths:

The authors report several novel findings. Brief activation or inhibition of PAM-α1 neurons, or brief inhibition of DPM neurons during the first few hours after training, impairs 24-hour LTM. Notably, these brief manipulations disrupt sleep for many hours afterward, particularly during the night. The authors further show that perturbation of PAM-α1 and DPM neurons impairs sleep and appetitive memory consolidation under starvation conditions, and that pharmacological sleep induction during the night rescues the LTM defects. Together, these findings suggest that PAM-α1 and DPM neurons are involved in sleep regulation and LTM consolidation under starvation. These are important observations that advance our understanding of the circuits regulating sleep and memory consolidation.

Weaknesses:

Some claims require additional evidence or clarification.

(1) Previous studies linking impaired memory to reduced sleep have primarily examined conditions involving severe sleep deprivation. In contrast, this manuscript argues that relatively modest decreases in total sleep, accompanied by sleep fragmentation, are sufficient to impair memory consolidation. It remains unclear whether sleep fragmentation of this magnitude is itself critical for LTM consolidation. An independent method for inducing comparably mild sleep loss and fragmentation would be needed to directly test this interpretation.

(2) It is unclear why both activation and inactivation of PAM-α1 neurons produce similar effects on sleep and memory. In addition, MB299B-labeled neurons exert stronger effects on

memory than MB043B-labeled neurons, whereas MB043B-labeled neurons have stronger effects on sleep. If sleep disruption is the primary driver of impaired memory consolidation, a stronger correspondence between the sleep and memory phenotypes might be expected. The authors speculate that MB043B may affect sleep through non-PAM neurons, but without identifying the relevant neurons, this remains speculative.

(3) The complex schematic model (Fig. 12), with parallel circuits and unidentified neuronal groups, underscores the difficulty of interpreting the current data. In the "less activity" arm of the model, distinct circuits are proposed to regulate sleep and LTM, respectively, and DPM neurons are not included. This makes it difficult to reconcile the model with the central claim that the PAM-α1-to-DPM microcircuit links sleep and LTM consolidation.

(4) The TRIC-LUC reporter system is not ideal for resolving dynamic changes in neuronal activity. Activity-dependent Ca^{2+} signaling must first reconstitute the TRIC transcriptional system, which then drives luciferase transcription, translation, and accumulation. The original characterization of TRIC indicates that TRIC signals accumulate and decay over several hours. Thus, the kinetics of the TRIC-LUC reporter should be interpreted cautiously, particularly when inferring transient or precisely timed changes in neuronal activity.

(5) Including data from training under fed conditions would provide a more complete understanding of state-dependent neural activity and would help distinguish starvation-specific effects from more general circuit mechanisms.

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Reviewer #3 (Public review):

Summary:

Understanding the neural circuits that link sleep and memory remains a fundamental challenge in neuroscience. In this study, Lin Yan and colleagues investigate how dopamine signaling in Drosophila regulates long-term memory (LTM) formation in the context of sleep. They identify a specific microcircuit between protocerebral anterior medial dopamine neurons (PAM-DANs) and dorsal paired medial (GABAergic DPM) neurons that modulates memory consolidation. Their findings suggest that disrupting the basal activity of PAM-α1 neurons during early consolidation impairs LTM, with particularly pronounced effects under starvation conditions. Notably, sleep fragmentation caused by this disruption can be pharmacologically rescued, restoring LTM. These results provide compelling evidence how dopamine signaling plays a crucial role in linking sleep and memory, offering new insights into the underlying mechanisms.

Strength:

This study presents a well-executed investigation into sleep-memory interactions, utilizing a combination of connectomics, behavioral assays, functional imaging, and pharmacological manipulations. The authors convincingly demonstrate that the PAM-α1 and DPM circuit interact, highlighting a potential mechanism by which sleep influences memory consolidation. The anatomical and functional dissection of this circuit is of high interest to the field, and the study's integration of sleep and memory processes contributes significantly to our understanding of the role of dopamine in cognitive functions. Additional experiments investigating the contribution of MBON-α1 to the circuit, connectomic analysis together with a dissection of dopamine receptor function further strengthen the proposed circuit motif and its biological relevance.

Weaknesses:

While the study is well designed, presents compelling findings and has been further strengthened by additional experiments, some aspects remain unclear. The role of DPM neurons in memory consolidation seems not yet fully resolved, as different genetic approaches yield variable results. Furthermore, some manipulations impair memory without affecting sleep fragmentation - or vice versa, suggesting that the observed memory deficits cannot be explained solely by impaired sleep-dependent consolidation. It would also have been interesting to discuss potential mechanisms by which dopamine receptor-mediated cAMP signaling could lead to a reduction in Ca^{2+} signals. I am confident that these questions can be addressed in future studies.

Conclusion:

Overall, this study provides valuable new insights into how sleep and dopaminergic circuits interact to regulate memory consolidation in Drosophila and may reveal general principles underlying the neural regulation of memory.

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Author response:

The following is the authors' response to the original reviews

> ***eLife Assessment***
>
> *This study approaches an important topic providing insight into the neuronal circuitry that interconnects memory consolidation and sleep. The data were collected and analysed using a solid methodology, contributing new findings for neurobiologists working on how memories are stored and the roles of sleep. However, the data is incomplete to support the proposed role of the PAM-DPM circuits as the link between sleep state and long-term memory consolidation.*

We sincerely appreciate the editor and reviewers' thoughtful and constructive comments on our study. Your insightful feedback has not only affirmed the significance of our work on the interplay between memory consolidation and sleep, but also provided valuable inputs for improving the clarity, rigour, and impact of our study.

We have carefully addressed all the comments raised by the reviewers and revised the manuscript accordingly. We have also streamlined the paper with the goal of making it more accessible to readers. We feel this revised version strengthens our conclusion that the PAM-DPM circuits as the link between sleep and memory consolidation.

The main improvements in terms of data addition are three complementary sets of circuit-specific experiments:

(1) To better characterize the dynamics of the PAM-DPM circuit following associative memory training, we performed 3-hour continuous neural activity recording in freely behaving flies. This experiment addresses the activity of the microcircuit in a much more relevant time frame than the CRTC data in the previous version of the paper which looked only at the first hour after training. Specifically, we expressed the Tric-LUC reporter gene, a calcium-responsive tool that harnesses the interaction between calmodulin and its cognate binding peptides to drive rapid luciferase transcription in a calcium-dependent manner (Gao et al., 2015; Guo et al., 2017), in PAM-α1 and DPM neurons, respectively. Flies were then subjected to either associative memory training or a no-training control condition, with real-time luciferase levels monitored throughout the recording window.

In the absence of training, both PAM-α1 and DPM neurons displayed similar neural activity over the 3-hour recording period. The first hour was characterized by a synchronous

decrease in activity for both neuron types, with hours 2 and 3 achieving a stable baseline. Since the decrease in the first hour is also seen in the trained condition, we think it is likely a reflection of the animals becoming acclimated to the recording tubes.

Notably, associative memory training profoundly reshaped the activity profile of the PAM-DPM circuit in the LTM consolidation time window. Training induced a mild yet statistically significant elevation in PAM-α1 neural activity specifically during the third hour of recording, while concurrently eliciting a robust reduction in DPM neuron activity over the last two hours (revised Figure 8C-F). These findings not only support the hypothesized role of the inhibitory PAM-α1-DPM circuit in sleep and memory consolidation, but also advance our mechanistic understanding of underlying neural dynamics.

(2) To further support the functional connectivity of the PAM-DPM microcircuit, we conducted *in vivo* experiments to complement the dissected brain prep P2X2 data. Optogenetic activation of PAM neurons in intact flies via the red light-gated cation channel CsChrimson (Klapoetke NC et al., 2014) resulted in a significant reduction in GCaMP signals within DPM neurons (revised Figure 2B). These findings strongly confirm that PAM neurons exert direct inhibitory control over DPM neurons in the intact brain.

(3) Further, we investigated how dopamine signaling to the DPM inhibits its activity, and issue which has not been investigated previously. We conducted a series of experiments:

Firstly, we verified which dopamine receptors (Dop1R1, Dop1R2, DopEcR, and Dop2R) express on the DPM neurons via double-labeling with gene-embedded GAL4 lines. We found that DPM neurons have expression of both Dop1R1 and Dop1R2 (revised Figure 10A).

Secondly, to clarify which receptors on DPM neurons respond to dopamine and how they signal, in addition to EPAC experiments in the first submission, we recorded neural activity changes when we knocked down Dop1R1 and Dop1R2 in DPM neurons. DPM neurons exhibited a significantly reduced GCaMP level with DA application, regardless of whether Dop1R1 or Dop1R2 was intact or knocked down knockdown in comparison to the no-DA control condition (revised Supplemental Figure 3C-E). These data suggest that either residual Dop1R1 and Dop1R2 remaining in the RNAi condition is sufficient or that the two receptors may coordinate to mediate the inhibition of neural activity.

Finally, we investigated the behavioral contributions of Dop1R1 and Dop1R2 in DPM neurons to sleep and memory processes (revised Figure 10C-H). Dop1R1 knockdown resulted in a marked reduction in daytime sleep and a significant impairment of 24 h memory expression. In contrast, Dop1R2 knockdown selectively compromised 24 h memory without affecting sleep.

When integrated with our EPAC assay findings from the initial submission, which demonstrated, that Dop1R1 is the primary receptor mediating dopamine-induced cAMP elevation, these new data collectively delineate a more complex mechanistic framework: dopamine signaling in DPM neurons coordinates the dual regulation of sleep and memory predominantly via Dop1R1. Meanwhile, Dop1R2 are engaged in the selective modulation of memory.

All newly generated experimental datasets, comprehensive statistical analyses, and their corresponding figure panels (revised Figures 2B, 10, 11 and Supplemental Figure 3) have been fully incorporated into the revised manuscript.

In addition to adding the experiments described above, we have reorganized and streamlined the paper. First, the CRTC data have been replaced by the Tric-luc data. The CRTC data were taken in the first hour after training and do not shed light on the bulk of the consolidation window. Since the behavioral and sleep effects we see with manipulation of the PAM/DPM microcircuit all occur with a time delay, examining later times in consolidation is

more relevant. Additionally, the first hour post-training is quite complex since there are sensory changes and STM processes overlaid on the processes we want to study. Second, we have moved the data in Figure 8 to supplemental (revised Supplemental Figure 2) since they are basically a control for the experiments in Figure 7 validating known requirements for appetitive LTM.

We have also substantially expanded the Discussion section to contextualize the PAM-DPM circuit within the broader framework of well-characterized memory-regulatory pathways, such as the intrinsic circuits of the mushroom body, and to explicitly delineate the hierarchical interplay between sleep-dependent synaptic plasticity and LTM consolidation.

We contend that these complementary experimental assays and targeted revisions markedly strengthen the causal evidence underscoring the role of the PAM-DPM circuit as a pivotal regulatory node bridging sleep states and LTM consolidation. We are confident that these revisions essentially address the concerns raised by the reviewers.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The authors aim to use state-of-the art behavior, imaging, and connectome techniques to identify the neural interaction between sleep and long-term memory consolidation in the PAM-DPM circuits, a well-known dopaminergic pathway within Drosophila Mushroom Body.*
>
> *Strengths:*
>
> *From a Drosophila sleep researcher's perspective, the investigation follows a clear and logical strategy to collect a huge dataset of sleep, appetitive memory, and live imaging. The authors clearly identified and showed that activation of a PAM subset: alpha-1 reduces sleep quality and memory consolidation in a starvation-dependent manner. The authors also convincingly demonstrated the corresponding neuronal responses of DPM neurons following PAM alpha-1 activation, and the positive role of DPM neural activity in sleep and memory consolidation. Moreover, the authors applied a new way of sleep statistics to demonstrate hour-by-hour changes between treatment and genotypes. Importantly, the authors demonstrated that memory loss derived from PAM alpha 1 activation can be partly restored by ectopic sleep enhancement via feeding THIP during the memory consolidation period after training.*
>
> *Weaknesses:*
>
> *Two investigatory gaps relate to the misalignment between circuital activity and behaviors, due to the nature of large circuital functional analysis like this. Firstly, the central observation of the study indicates that PAM alpha1 activation causes DPM inhibition which disrupts sleep and memory consolidation. Therefore one would expect a reduced PAMalpha1 and increased DPM activities after memory training, but the authors found that the endogenous CRTC::GFP reported neuronal activity for PAMalpha1 and DPM are both increased after memory training (Figure 9). This can be due to the difficult functional demarcation among the 14 PAMalpha1 projections. Secondly, the authors acknowledged the contradicting finding that memory defect is detected in PAMalpha1 inactivation (Figure 7C), yet suggested a tight link between sleep and memory consolidation; it is clear loss of PAM subset activity can disrupt memory consolidation without affecting sleep (cf Figure 7C and 7I).*

Thank you for your insightful analysis and the relevant possibilities you've raised. We agree that given that memory consolidation and sleep are time-dependent processes, the 1-hour window employed to capture neural activity changes via the CRTC::GFP reporter may not fully reflect the overall dynamics of neural activity in this microcircuit. To better characterize the dynamics of the PAM-DPM circuit in the consolidation window following associative memory training, we performed 3-hour continuous neural activity recording in freely behaving flies. Specifically, we expressed the Tric-LUC reporter gene, a calcium-responsive tool that harnesses the interaction between calmodulin and its cognate binding peptides to drive rapid luciferase transcription in a calcium-dependent manner (Gao et al., 2015; Guo et al., 2017), in PAM-α1 and DPM neurons, respectively. Flies were then subjected to either associative memory training or a no-training control condition, with real-time luciferase levels monitored throughout the recording window.

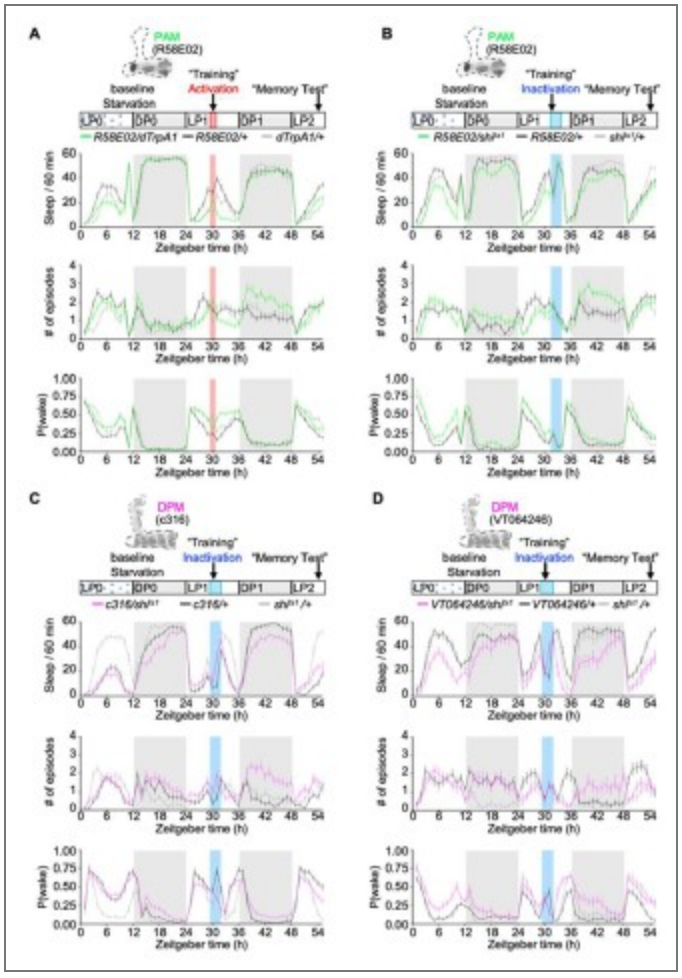
In the absence of training, PAM-α1 neurons displayed stable neural activity over the entire 3-hour recording period. However, associative memory training profoundly reshaped the activity profile of the PAM-DPM circuit. Training induced a mild yet statistically significant elevation in PAM-α1 neural activity specifically during the third hour of recording, while concurrently eliciting a robust reduction in DPM neuron activity over the last two hours (revised Figure 8C-F). These findings not only support to the hypothesized role of inhibitory PAM-α1-DPM circuit in sleep and memory consolidation, but also advance our mechanistic understanding of underlying neural dynamics.

Regarding the second question, the core finding underlying the link between sleep and memory elucidated in the present study lies in the whole PAM-α1-DPM microcircuit rather than the specific DANs alone. MB299B and MB043B, the two split-GAL4 drivers employed to target PAM-α1 neurons, were originally characterized previously (Aso et al., 2014). However, these drivers also exhibit non-specific labeling of additional cells, and we can not rule out the possibility that such off-target labeling may have masked the subtype-specific necessity in sleep or memory processes.

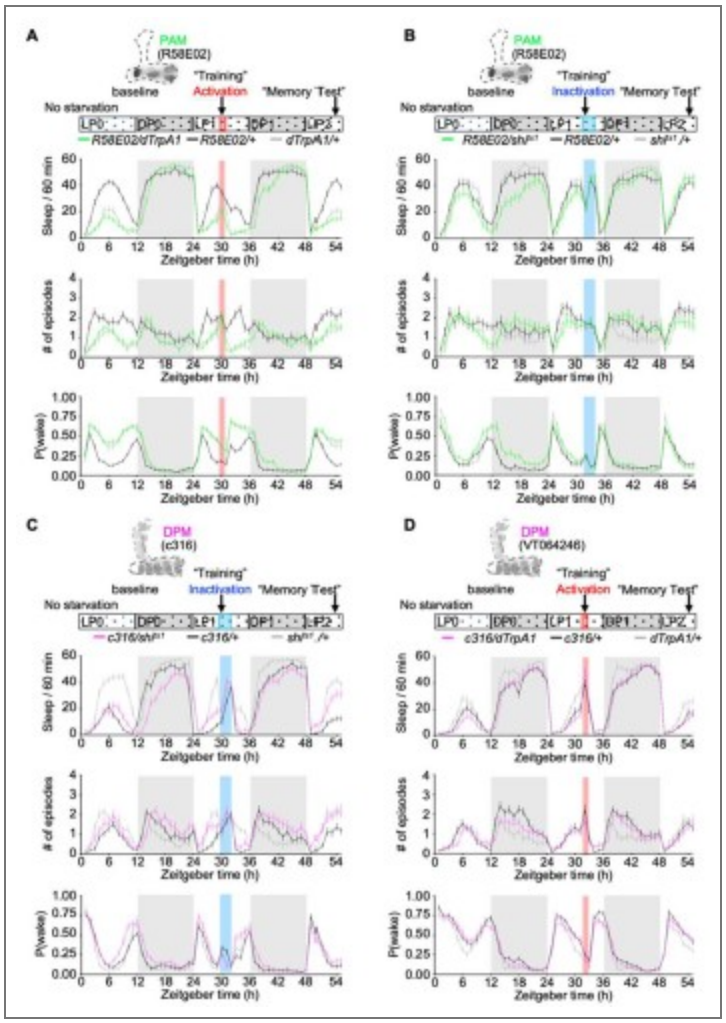
> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Sleep plays a critical role in memory consolidation, but the neural mechanisms underlying this relationship remain poorly understood. The authors present novel findings implicating two small neuronal groups with inhibitory connections, PAM-α1 to DPM, in sleep regulation and LTM consolidation. However, whether the PAM-α1 to DPM microcircuit promotes LTM consolidation through sleep regulation requires further investigation.*
>
> *Strengths:*
>
> *The authors report several novel findings. Brief activation or inhibition of PAM-α1 neurons, or brief inhibition of DPM neurons during the first few hours after training, impairs 24-hour LTM. Notably, these brief manipulations disrupt sleep for many hours afterward, particularly at night. Interestingly, disruption of PAM-α1 and DPM neurons impairs sleep and appetitive memory consolidation only under starvation conditions, and pharmacological induction of sleep during the night rescues the LTM defects. These findings suggest that PAM-α1 and DPM neurons are involved in sleep regulation and LTM consolidation under starvation. These are important findings that advance our understanding of the link between sleep and memory consolidation.*
>
> *Weaknesses*
>
> *Some claims lack sufficient evidence or clarity:*

> *(1) All sleep experiments are conducted under the "training" (temperature-change) condition. While genotypic controls are helpful, additional no-training controls are required to confirm that the observed differences are due to training rather than unknown genotype-related factors. The fact that experimental genotypes exhibit significantly altered sleep even before "training" (e.g., Figs. 7H, J, K, 8A, B, D) highlights the necessity of these controls.*

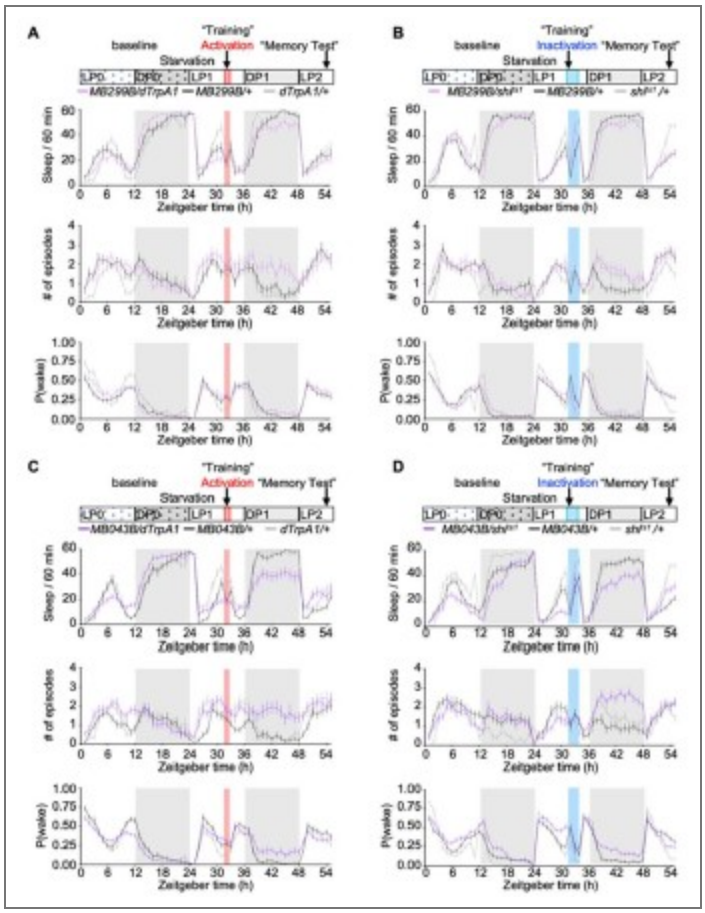
Thank you for raising this important question. We have re-examined the sleep profiles recorded over two acclimation days and one day of baseline sleep, which preceded the implementation of the “training” paradigm (temperature manipulation) and thus served as a valid no-training control. As shown in Author response images 1-4, subtle yet discernible genotype-dependent differences were indeed observed under baseline conditions. However, when animals were subjected to starvation, the experimental manipulations (activation or inactivation of the target cells) elicited marked, statistically significant alterations in sleep patterns that cannot be accounted for by the baseline genotype differences. Collectively, these data confirm that the observed sleep phenotypes are attributable to the “training” intervention, rather than to confounding, pre-existing genotype-related factors.



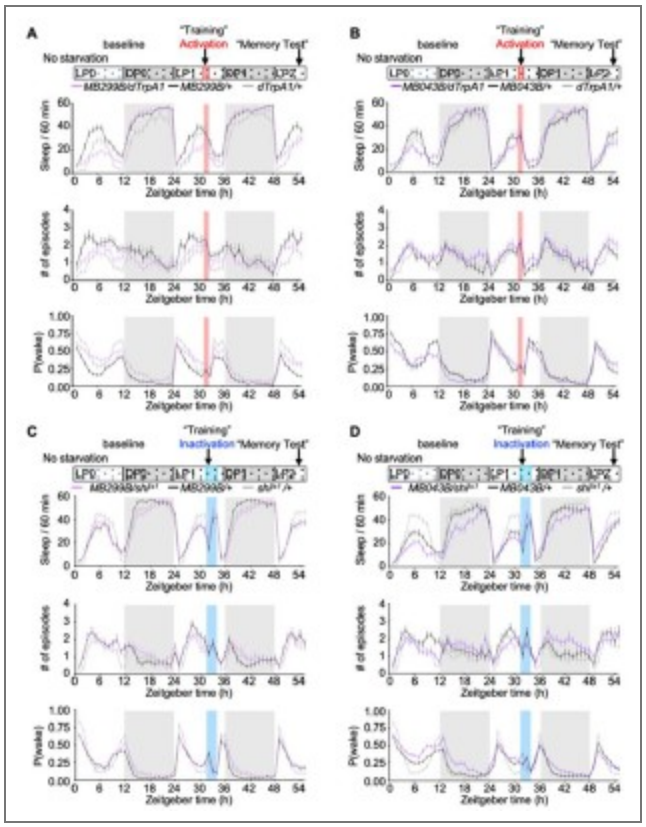
Author response image 1. Baseline and manipulation day sleep profiles following PAM activation and PAM/DPM inactivation under starvation conditions.



Author response image 2. **Baseline and manipulation day sleep profiles following PAM activation and PAM/DPM inactivation under non-starvation conditions.**



Author response image 3. **Baseline and manipulation day sleep profiles following PAM- α1 activation and inactivation under starvation conditions.**



Author response image 4. **Baseline and manipulation day sleep profiles following PAM- α1 activation and inactivation under non-starvation conditions.**

> *(2) Previous studies on disrupted memory due to sleep reduction have primarily examined conditions with severe sleep deprivation. In contrast, this report claims that relatively small decreases in total sleep accompanied by sleep fragmentation are responsible for impaired memory consolidation. It remains unclear whether sleep fragmentation at this level is truly critical for memory consolidation. The authors should cause sleep loss and fragmentation of similar magnitude through other means and determine whether it can impair LTM.*

We appreciate the reviewer's insightful suggestion. While alternative assays for inducing sleep loss or sleep fragmentation are indeed available, this line of investigation lies beyond the core scope of the present study. We will certainly take this valuable suggestion into consideration for the future studies.

> *(3) The authors employed a neural activity reporter to show that starvation increases the basal activity of PAM-a1 but not DPM neurons in untrained flies (Figures 9C-E). They observed small increases in the activity of both neuron groups immediately after training but not one hour later. Given the inhibitory connection from PAM-a1 to DPM, it is unclear why both neuron groups show increased activity after training. Additionally, as the authors acknowledge, it is puzzling how the inactivation of PAM-a1 produces similar effects on sleep and memory as DPM inhibition and PAM-a1 activation. Further experiments are needed to clarify these findings, such as manipulating PAM-a1 activity during the one-hour post-training period and evaluating the effect on DPM activity. Including data from training under fed conditions would provide a more comprehensive understanding of state-dependent neural activity. Even if certain experiments are not feasible, these issues warrant further discussion. It is also important to clarify that the term "synchronized" does not imply single-spike-level synchrony.*

Thank you for raising these critical questions. To deepen our understanding of these issues, we have conducted additional experiments and have incorporated them into the revised manuscript. Below are our specific responses to each of your points:

(1) Regarding the contradiction between "PAM-α1 inhibition of DPM" and a transient increase in the activity of both neurons immediately after training:

PAM/PAM-α1 neurons are well-documented to respond to reward signals (Liu et al., 2012, Ichinose et al., 2015), while DPM neurons have been shown to respond to both olfactory stimuli and electric shocks, and to form delayed olfactory memory traces (Yu et al., 2005). Thus, the concurrent increase in the activity of PAM-α1 and DPM neurons immediately following training is likely a response to the olfactory and/or sucrose stimuli in the assay. Given that memory consolidation and sleep are time-dependent processes, the 1-hour window employed to capture neural activity changes via the CRTC::GFP reporter likely does not fully reflect the overall dynamics of neural activity in this microcircuit. Additionally, this time window overlaps with the period in which the animals are adapting to the new tubes and is likely contaminated with other sensory information.

To better characterize the dynamics of the PAM-DPM circuit following associative memory training, we performed 3-hour continuous neural activity recording in freely behaving flies. Specifically, we expressed the Tric-LUC reporter gene, a calcium-responsive tool that harnesses the interaction between calmodulin and its cognate binding peptides to drive rapid luciferase transcription in a calcium-dependent manner (Gao et al., 2015; Guo et al., 2017), in PAM-α1 and DPM neurons, respectively. Flies were then subjected to either associative memory training or a no-training control condition, with real-time luciferase levels monitored throughout the recording window.

In the absence of training, PAM-α1 neurons displayed stable neural activity over the entire 3-hour recording period. Notably, associative memory training profoundly reshaped the activity profile of the PAM-DPM circuit. Training induced a mild yet statistically significant elevation in PAM-α1 neural activity specifically during the third hour of recording, while concurrently eliciting a robust reduction in DPM neuron activity over the last two hours (revised Figure 9F-I). These findings not only support to the hypothesized role of inhibitory PAM-α1-DPM circuit in sleep and memory consolidation, but also advance our mechanistic understanding of underlying neural dynamics post-training. We have replaced the CRTC data with this more relevant data set.

(2) Regarding the state-dependent neural activity:

We agree that investigating state-dependent neural activity would be an interesting extension of our study. However, this falls beyond the scope of the current study and will be considered in future research. Our primary findings, including sleep disruptions and the associated memory impairments, were specifically observed under starvation conditions, which align with the appetitive memory paradigm employed here. Delving into neural activity changes under non-starvation state would not yield direct evidence to support the core conclusions of the present work, as the study's focus is on the starvation-dependent interplay between sleep, neural circuitry, and appetitive memory consolidation.

(3) Regarding the terminology of "synchronization":

We believe that the use of the term "synchronization" in our study is appropriate. In the context of neural circuitry, synchronization refers to the process by which distinct neurons or neural populations achieve temporal alignment of their activity, a phenomenon that supports neural communication and information integration. In the present work, this specifically describes how PAM-α1 and DPM neurons exhibit phase-related temporal coordination of their activity to regulate the interplay between sleep and memory consolidation.

> *(4) The authors considered that PAM-α1 and DPM might function in parallel, independent pathways for sleep and LTM. They rejected this possibility based on the lack of additive effects when both neuronal groups were simultaneously inactivated. However, they*

> *found that MB299B-labelled neurons exert stronger memory effects than MB043B-labelled neurons, while MB043B neurons have stronger sleep effects. If sleep is a primary driver of memory consolidation, a stronger correlation between memory and sleep effects would be expected. This observation merits further discussion.*

We appreciate the reviewer's constructive suggestions. We have performed additional experiments to explore a well-characterized memory-related PAM-α1 recurrent loop in sleep regulation. The new data, along with further discussion, have been incorporated into the revised manuscript.

The two split-GAL4 drivers (MB299B and MB043B) used to target PAM-α1 neurons were originally characterized previously (Aso et al., 2014). However, these drivers exhibit non-specific labeling of additional neuronal populations, a technical limitation that may have masked the subtype-specific functional requirements of PAM-α1 in sleep and memory processes.

In addition, we assessed sleep and LTM following the thermoactivation of DPM neurons (revised Supplemental Figure 1), and no significant changes were observed in either phenotype.

PAM-α1 has previously been demonstrated to drive appetitive LTM formation and consolidation via a recurrent loop with MBON-α1 (Ichinose et al., 2015). To investigate whether MBON-α1 also participates in sleep regulation, we activated or inactivated MBON-α1 neurons under both starvation and non-starvation conditions. Our results revealed that inhibition of MBON-α1 under both starvation and non-starvation conditions resulted in a significant reduction in sleep and a reduced arousal threshold (revised Figure 11B, D), suggesting that MBON-α1 participates in regulating sleep in a state-independent manner. However, no significant changes were observed upon activation of MBON-α1 neurons (revised Figure 11A, C). Combined with our observation that inhibition of MBON-α1 during the memory consolidation phase also impaired 24 h LTM, these new data indicate that MBON-α1-mediated sleep is necessary for effective memory consolidation. Notably, while activation of MBON-α1 during consolidation phase similarly impaired LTM, this manipulation did not alter the sleep profile, suggesting a dissociation between MBON-α1's mechanistic roles in sleep regulation and LTM processing.

Taken together (see Author response table 1 and the new schematic diagram of revised Figure 12), these findings reveal a dedicated hierarchical, modular regulatory network that mediates sleep-LTM coupling via an activity-dependent mechanism. Within this network, activation of PAM-α1 acts as an upstream modulator to inhibit the activity of DPM, a downstream integrative hub that coordinates the execution of sleep and memory processes via recruiting different signaling cascades mediated by distinct dopamine receptors. MBON-α1, which is likely inhibited by PAM-α1, serves as parallel pathway to suppress sleep and impair LTM. Conversely, inactivation of PAM-α1 relieves its inhibitory control over MBON-α1, leading to MBON-α1 activation; MBON-α1 then functions as a signal amplifier that further exacerbates the reduced activity of PAM-α1, ultimately resulting in LTM impairment. Inactivation of PAM-α1, together with non-PAM-α1 neurons labeled by MB043B, contributes to the regulation of sleep. Sleep and memory are highly intertwined within this circuit, where distinct neuronal populations exhibit specialized yet interdependent functional roles, with overlapping and divergent regulatory contributions to sleep and LTM. The inherent complexity of this regulatory network thus merits further dedicated investigation in future studies.

	Activation		Inhibition	
	sleep	memory	sleep	memory
PAM-α1 (MB299B)	√	√	×	√
PAM-α1 (MB043B)	√	√	√	×
DPM (VT046246)	×	×	√	√
MBON-α1 (MB310C)	×	√	√	√

Author response table 1.

> *(5) Given prior knowledge that PAM neurons are heterogeneous and that the R58E02 driver is broadly expressed, data in Figures 1-5 concerning PAM are outdated. The use of more restricted PAM-α1 drivers from the outset would make the manuscript easier to read and interpret.*

We sincerely appreciate the reviewer's point of view regarding the selection of PAM drivers. While we acknowledge the well-characterized heterogeneity of PAM neurons and the broad expression profile of the R58E02 driver, and fully agree that employing subtype-restricted drivers enhances the precision of functional interpretation, this set of experiments serves as an essential foundational step and logical basis for subsequent subtype-specific investigations and thus merits retention in the manuscript. As detailed above, the more specific drivers also have some drawbacks in terms of additional expression, making the broad driver critical for setting the stage.

> *(6) Some figures lack relevant data, certain experiments are missing necessary controls, and anomalies are present in some data sets.*

We sincerely appreciate the reviewer's detailed suggestions, and we have revised the manuscript comprehensively in accordance with them.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *Understanding the neural circuits that link sleep and memory remains a fundamental challenge in neuroscience. In this study, Lin Yan and colleagues investigate how dopamine signaling in Drosophila regulates long-term memory (LTM) formation in the context of sleep. They identify a specific microcircuit between protocerebral anterior medial dopamine neurons (PAM-DANs) and dorsal paired medial (GABAergic DPM) neurons that modulates memory consolidation. Their findings suggest that disrupting the basal activity of PAM-α1 neurons during early consolidation impairs LTM, with particularly pronounced effects under starvation conditions. Notably, sleep fragmentation caused by this disruption can be pharmacologically rescued, restoring LTM. These results provide compelling evidence that dopamine signaling plays a crucial role in linking sleep and memory, offering new insights into the underlying mechanisms.*
>
> *Strengths:*
>
> *This study presents a well-executed investigation into sleep-memory interactions, utilizing a combination of connectomics, behavioral assays, functional imaging, and pharmacological manipulations. The authors convincingly demonstrate that the PAM-α1 and DPM circuits interact, highlighting a potential mechanism by which sleep influences memory consolidation. The anatomical and functional dissection of this circuit is of high interest to the field, and the study's integration of sleep and memory processes contributes significantly to our understanding of dopamine's role in cognitive functions.*
>
> *Weaknesses:*
>
> *While the study is well-designed and presents compelling findings, some aspects require further clarification. The interpretation of dopamine receptor signaling remains*

> *incomplete, particularly regarding inhibitory pathways. The role of DPM in memory consolidation is not entirely conclusive, as different genetic approaches yield variable results. Additionally, some inconsistencies in neuronal activity patterns and experimental variability, especially regarding sleep patterns or pharmacological rescue, should be addressed to strengthen the mechanistic framework.*
>
> *Conclusion:*
>
> *Overall, this study provides valuable new insights into how sleep and dopamine circuits interact to regulate memory consolidation. While the findings are compelling, addressing the points above-particularly receptor signaling and the specific role of DPM and its activity patterns within the microcircuit would further solidify the study's conclusions.*

We sincerely appreciate the reviewer's constructive feedback and useful suggestions, which have been instrumental in enhancing the rigour and completeness of our study.

To address these points, we have performed a series of additional experiments that we believe strengthen the mechanistic framework of our work. The key new findings are summarized below:

(1) Regarding the dopamine receptor signaling

To define the dopamine receptor (DAR) signaling mechanisms underlying DPM neuron activity and its regulatory roles in sleep and memory, we first characterized DAR expression profile of the DPM neurons. Using double-labeling assay, we detected robust expression of Dop1R1 and Dop1R2 in DPM neurons, whereas no detectable colocalization was observed for DopEcR and Dop2R (revised Figure 10A). Accordingly, we refined our FRET-based EPAC data by removing the DopEcR knockdown group, and now present cAMP changes in DPM neurons following Dop1R1 and Dop1R2 knockdown, in direct comparison with the intact receptor control group (revised Figure 10B). These data conform that Gαs-coupled Dop1R1 is the primary receptor mediating DA-dependent cAMP elevation in DPM neurons.

To further identify the DARs responsible for transducing DA-induced inhibitory effect on DPM neural activity, we quantified GCaMP levels in DPM neurons with targeted knockdown of individual DARs. Knockdown of either Dop1R1 or Dop1R2 failed to abolish DA-induced Ca^{2+} decrease; only Dop1R2 knockdown exhibited a trend toward attenuating this Ca^{2+} decrease (revised Supplemental Figure 3C-E), suggesting that the two receptors cooperate to modulate DPM neural activity.

Finally, to dissect the specific contributions of DARs in DPM neurons to sleep and/or memory regulation, we performed sleep monitoring and memory assays in animals with DPM-specific knockdown of distinct DARs (revised Figure 10C-H). Knockdown Dop1R1 in DPM neurons resulted in statistically significant sleep reduction, decreased arousal threshold, and impaired 24 h LTM memory (revised Figure 10C-E). In contrast, knockdown Dop1R2 in DPM neuron selectively impaired 24 h LTM memory with no effect on sleep (revised Figure 10F-H). Collectively, these findings demonstrate that coupling sleep and LTM requires Dop1R1 in DPM neurons through the modulation of both cAMP signaling and neuronal activity, while Dop1R2 specifically mediates LTM regulation, likely through modulating DPM neural activity alone.

(2) We have additionally characterized the role of MBON-α1 in sleep, which has been previously shown as a PAM-α1-related recurrent feedback loop in the regulation of memory formation and consolidation (Ichinose et al., 2015).

To investigate whether MBON-α1 also participates in sleep regulation, we activated or inactivated MBON-α1 neurons under both starvation and non-starvation conditions (revised Figure 11A-D). Our results revealed that inhibition of MBON-α1 under both starvation and non-starvation conditions resulted in a significant reduction in sleep and a reduced arousal threshold (revised Figure 11B, D), suggesting that MBON-α1 participates in regulating sleep in a state-independent manner. However, no significant changes were observed upon activation of MBON-α1 neurons (revised Figure 11A, C). Moreover, inhibition of MBON-α1 during the

memory consolidation phase significantly impaired 24 h LTM (revised Figure 11E-F). These results indicate that MBON-α1mediated sleep is necessary for effective memory consolidation. Notably, while activation of MBON-α1 during consolidation phase similarly impaired LTM, this manipulation did not alter the sleep profile, suggesting a dissociation between MBON-α1's mechanistic roles in sleep regulation and LTM processing.

Taken together (see Author response table 1 and the new schematic diagram of revised Figure 12), these findings reveal a dedicated hierarchical, modular regulatory network that mediates sleep-LTM coupling via an activity-dependent mechanism. Within this network, activation of PAM-α1 acts as an upstream modulator to inhibit the activity of DPM, a downstream integrative hub that coordinates the execution of sleep and memory processes via recruiting different signaling cascades mediated by distinct dopamine receptors. MBON-α1, which is likely inhibited by PAM-α1, serves as parallel pathway to suppress sleep and impair LTM. Conversely, inactivation of PAM-α1 relieves its inhibitory control over MBON-α1, leading to MBON-α1 activation; MBON-α1 then functions as a signal amplifier that further exacerbates the reduced activity of PAM-α1, ultimately resulting in LTM impairment. Inactivation of PAM-α1, together with non-PAM-α1 neurons labeled by MB043B, contributes to the regulation of sleep. Sleep and memory are highly intertwined within this circuit, where distinct neuronal populations exhibit specialized yet interdependent functional roles, with overlapping and divergent regulatory contributions to sleep and LTM. The inherent complexity of this regulatory network thus merits further dedicated investigation in future studies (See Author response table 1).

We have modified the schematic diagram in the revised manuscript to illustrate the mechanistic framework (revised Figure 12).

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *Here I listed details for potential clarification or further investigation related to the weaknesses:*
>
> *(1) Line 145-147: I suspected the authors used previously verified RNAi lines, but it would be informative to include a citation or their own validation for the effectiveness of these RNAi lines.*

We sincerely appreciate the reviewer's suggestion. As our double-labeling assays confirmed that only Dop1R1 and Dop1R2 are colocalized with DPM neurons (see our responses to the public review from Reviewer #2 and #3), we refined the revised data to focus exclusively on these two DARs. Corresponding revisions have been made to the Materials and Methods, Results and Discussion sections. Additionally, we conducted qPCR analysis to verify the knockdown efficiency of these DARs, providing further support for our findings that Dop1R1 and Dop1R2 are functionally required in DPM neurons for the regulation of sleep and memory (revised Supplemental Figure 3).

> *(2) Line 169: Moving from describing Figure 3A/B to Figure 3C, it is not immediately clear from 3C-H, the authors follow the training paradigm of 3B?*

To enhance clarity, we have added the referenced figure citations in the "Memory assay" section: "For all 24 h sucrose-odour memory, a single training session of sucrose paired with an odour for 2 min was employed (Figure 3A)."

> *(3) Line 179: before the PAM inactivation data are shown in Figure 7, the authors seem to be getting ahead of themselves by stating "suggesting that activity of PAM neurons is necessary for the consolidation window or that heterogeneity in the subsets of PAM neurons masks any phenotype." when the Figure 3 data collectively indicate that "suppression" of PAM is necessary.*

This statement is based on our observation that inactivation of the majority of PAM neurons labeled by R58E02 results in sleep disruption but leaves memory intact, and we stand by this conclusion.

> *(4) Line 186-188: The labelling of DP1 is not entirely aligned between the figures and the text for a reader to follow which time period is described, as DP1 is embedded within the dark phase in the figures.*

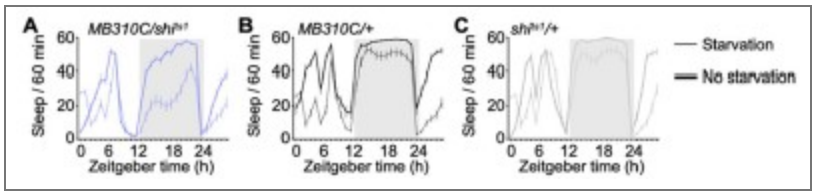
These experiments spanned two consecutive days. LP1 and DP1 denote the light and dark periods on the first day, respectively, whereas LP2 designates the light period on the second day. As only one full dark phase was monitored across the experimental interval, we characterized the relevant phenotype using the general terms dark phase or nighttime, rather than specifying DP1. We thank the reviewer for this thoughtful observation; nonetheless, we consider the original description correct and unambiguous, and thus appropriate for inclusion in the manuscript.

> *(5) Line 321: The statistics for Figure 9 CRTC::GFP measurement is crucial for interpretation but the referring and labelling for this on Figure 9 is poor: it is not apparent which comparisons are indicated. There is inconsistency between Table 1 Figure 9D and Table 3 Figure 9D entries: no significant between train and untrain indicated in Table 1 but it is described as significant in the text and Table 3?*

Thank you for this observation. As described above, we have removed these data from the paper and replaced them with Tric-Luc data that capture the consolidation window more completely.

> *(6) Line 423: The starvation-mediated sleep suppression is not clear in this manuscript, can the author comment on this? The response to this may also alter the summary concept cartoon.*

This is an important point. To directly address the reviewer's question regarding starvation-mediated sleep suppression, we have generated a representative response figure comparing sleep duration under starvation versus non-starvation conditions (Author response image 5). This figure clearly demonstrates that sleep is suppressed under starvation, providing straightforward evidence to address this concern.



Author response image 5. **Examples of starvation-mediated sleep suppression.**

However, the key focus of our study is that changes in neuronal activity disrupt sleep under starvation conditions but not under non-starvation conditions. To emphasize this critical distinction, we have incorporated additional discussion focused specifically on this point.

"It is well established that starvation induces sleep suppression (MacFadyen, 1973; Thimgan et al., 2010; Melnattur and Shaw, 2019; Keene et al., 2010; He et al., 2020; Yangkyun et al., 2022), and our results are consistent with these previous findings: all genotypes exhibited less sleep under starvation than under fed conditions (i.e. Figures 4A-B, 5A-B and 11). Under normal appetitive memory training, starvation-induced sleep loss does not necessarily impair memory processing (Thimgan et al., 2010; Chouhan et al., 2021). PAM-α1 neuronal activity is higher in starved, trained flies than in fed or untrained flies (data not shown), suggesting that these neurons act as a critical node for integrating internal motivational and arousal states, as well as conveying positive valence for the normal appetitive memory process, independently of starvation-induced sleep loss. While DPM neurons are less sensitive to

starvation, they still exhibit training-induced elevated activity (data not shown), indicating coherent responsiveness to upstream signaling. In the present study, we found that under fed conditions, sleep remained intact even when excessive changes in neural activity occurred within the PAM(-α1)-DPM circuit; in contrast, under starvation conditions, significant sleep reduction and fragmentation were observed. These observations indicate that starvation may trigger a transition from a physiologically normal brain state to an unstable, abnormally active state, which consequently elicits negative behavioral outputs."

> *(7) Line 1121: The data points for Figure 9 D-E are surprisingly low considering there are 14 PAMalpha1 labelled, the data presented here indicated potentially only 1-2 neurons were counted per fly brain. Can this contribute to the large variation and the contradiction of PAM's memory-suppressing role?*

We sincerely appreciate the reviewer's critical comments regarding the sample size of labeled PAM-α1 neurons in Figure 9D–E. We have revisited our raw data, incorporated additional brain samples, and reanalyzed the dataset. For this updated analysis, we included all clearly distinguished neurons, excluded overlapping ones, and calculated a single NLI per brain for statistical analysis. The key conclusions remain consistent with those in the original submission, confirming the robustness of the observed phenotype.

Memory consolidation is a time-dependent process. To further elucidate the link between neural activity and behavioral outputs, we performed additional experiments with an extended recording period. A detailed response to this point is provided in the response to public review, and we therefore do not reiterate the details here.

> *(8) Line 345: the effect size and data spread of THIP restored memory is different from the controls in Figure 10, perhaps warranting a more conservative interpretation of the role of sleep in memory consolidation.*

We appreciate this critical comment. We fully agree that the role of sleep in memory consolidation requires cautious interpretation, a point we have integrated into the revised manuscript.

Drug treatment in *Drosophila*, particularly for group-based assays, can introduce substantial variability at both the individual and group levels. To account for this, we employed a statistically valid sample size for our analyses to ensure robust conclusions. While minor quantitative discrepancies exist in the data, this technical consideration does not significantly alter the core conclusions of the study.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) As mentioned in the public review, all data using the broad PAM-DAN driver should be removed. Concerns regarding the experiments involving the broad driver are not included here.*

A detailed response to this point is provided in the response to public review, and we therefore do not reiterate the details here.

> *(2) In GCaMP experiments (Figure 9B), the ΔF/F traces for the AHL and AHL+ATP conditions start diverging before the addition of ATP. The quantification shows they are not significantly different in the first 30 seconds, but the fact that in two separate experiments (2A and 9B), they diverge in the same direction makes me wonder whether the AHL condition is different from the +ATP condition even before the ATP treatment. Also, the traces should include standard errors.*

We observed the same diverging trend in the first 30-second baseline as the reviewer. We reviewed the raw data for each sample and found that this divergence is likely attributable a small number of outliers. Given the absence of a statistically significant difference, this divergence does not affect our conclusions.

We have also added standard errors to the revised figures.

> *(3) Figure 9B. The authors need to show data for a control genotype. +>P2X2; VT064246-LexA > GCaMP6f that does not include MB299B-Gal4 is crucial to demonstrate that expression of P2X2 in PAM-α1 is responsible for the inhibitor effect, as LexA-P2X2 may be leaky.*

One of the UAS-P2X2 lines was found to exhibit leaky expression, so we instead used a non-leaky UAS-P2X2 line for all related experiments. To address the reviewer's comments and further validate our findings, we have added complementary experiments with a control genotype. In addition, we also added a control to confirm the non-leaky expression of LexA-P2X2 under the driver of R58E02-LexA. As shown in revised Figures 8B, application of ATP in the absence of MB299B-GAL4 failed to induce a significant inhibitory effect. These data strongly and convincingly support our conclusion.

> *(4) Figure 9B. Some of the individual data show values lower than -100% ΔF/F0. By definition, ΔF/F cannot be less than -100%, as this would require negative fluorescence, which is physically impossible. The calculation of fluorescence changes using ΔF/F should be carefully reconsidered.*

We thank the reviewer pointing out this potential confusion. We used a standard method of calculating the change in fluorescence over time using $\triangle F/F = (Fn\text{-}F_0) / F_0 \times 100\%$ as we previously described (Liu et al., 2019). Changes of greater than +100% of $\triangle F/F$ would not be unusual, since the reported value is a ratio to the initial level of fluorescence, not a subtraction of the baseline value from the signal (which obviously could not go below 100%). We have included a sentence in the results explaining this (page 7): "As previously described, we used the percent change in fluorescence over time as a ratio to the initial level, $\triangle F/F = (Fn\text{-}F0)/F0 \times 100\%$ for quantification (Liu et al., 2019)." And we have carefully reviewed our raw and processed data and confirmed that our analysis was correct.

> *(5) Figure 2B. The number of UAS transgenes should be controlled, as Gal4 could be diluted with 3 UAS constructs in experimental conditions compared to only 1 UAS construct in controls. Are Dop1R2 and DopEcR significantly different from wt? Why do they present an average ΔF/F in 2A and a maximum in 2B?*

We appreciate the reviewer's careful observations and valuable comments.

As the reviewer noted, the EPAC imaging experiment utilizes three UAS transgenes, which enable Gal4 enhancement via Dicer, targeted manipulation of dopamine receptor expression levels, and neural activity monitoring in DPM neurons. All other imaging experiments in the study employ only one or two UAS transgenes. Given the robustness of the observed phenotypes, the potential dilution effect is not a major concern. Knockdown of Dop1R2 and DopEcR showed no significant differences relative to the WT control group; the maximum values presented in Fig. 2B are included solely to illustrate statistical significance. While the EPAC (CFP/YPF) signal reflects an obvious cAMP elevation, no differences were detected in the averaged signal across groups.

Notably, in the revised manuscript, our double-labeling assays confirmed that only Dop1R1 and Dop1R2 are colocalized with DPM neurons (see our responses to the public review from Reviewer #2). Accordingly, we have refined our data analysis to focus exclusively on these two DARs.

> *(6) Figures 7H, J. Why is almost every MB299B>TrpA1 fly sleeping at ZT0?*

To align the starvation protocol for sleep analysis with that used in the memory assay, MB299B>TrpA1 flies and their genetic controls were transferred to fresh sleep tubes containing starvation food during the ZT0–1 time window. This transfer resulted in no detectable locomotor activity during this period, a pattern indicative of sleep in all flies.

> *(7) The number of episodes and P(wake) should be presented for all sleep data.*

We have added these two parameters as new panels to all relevant sleep figures. The corresponding statistical analyses have also been included in the supplemental tables.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *The study's findings provide compelling insights into the neural circuits connecting sleep and memory and the role of dopamine in general. While the anatomic dissection of the microcircuit and its overall involvement in sleep and memory is convincing and of high interest to the field and beyond, some statements of the study need further clarification, particularly the interpretation of receptor signaling and the role of DPM.*
>
> *Major Points*
>
> *(1) Figure 2: cAMP Imaging and Dopamine Receptor Involvement*
>
> *The authors present calcium and cAMP imaging to support the inhibitory connection between PAM and DPM neurons. While using both sensors is a robust approach, I am not entirely convinced that cAMP imaging is the ideal approach for identifying the dopamine receptors involved. To my knowledge, only Dop1R1 is classically linked to Gs-mediated cAMP signaling. Dop1R2 is typically coupled to Gq (PLC and DAG), while DopEcR is non-canonical and can engage both pathways. Additionally, these receptors are classically excitatory, yet the authors did not analyze Dop2R, the primary inhibitory dopamine receptor - which would represent the most relevant candidate for an inhibitory PAM-DPM connection.*

We have addressed this point in our response to the public comments, so will not reiterate here.

> *While dopamine receptor functions can vary by neuronal context, I would appreciate clarification on the following points:*
>
> *(a) Why was Dop2R not tested? Was it omitted or found to have no effect?*

We sincerely appreciate the reviewer's critical questions. This point has been addressed in our response to the public comments. Briefly, Dop2R is not colocalized with DPM neurons; instead, only Dop1R1 and Dop1R2 are detected in DPM neurons, which is why we focused exclusively on these two receptors in the revised manuscript.

> *(b) Why was cAMP imaging chosen for receptor identification? Was calcium imaging performed, and if so, what were the results?*

This is an excellent point, and we sincerely appreciate the reviewer's valuable input, which has helped to strengthen the logical framework of our analysis on receptor-mediated neural activity. These dopamine receptors are well-characterized as members of the Gas-coupled protein receptor family, and cAMP signaling serves as a reliable readout of their functional activity. To strengthen the logic flow of our analysis on the target inhibitory circuit, we have made the following key revisions to the manuscript: 1) defined the expression profile of dopamine receptors in DPM neurons; 2) refined our cAMP imaging data analyses based on specific receptor subtypes; and 3) assessed DPM neural activity via calcium imaging under conditions of targeted receptor knockdown. For further details, please refer to our response to the public comments.

> *(c) Since the data suggest multiple receptor involvements and complex interactions, I encourage a more detailed discussion of the working hypothesis, particularly regarding the unexpected finding that classically excitatory receptors contribute to an inhibitory connection.*

We appreciate the suggestion to elaborate on our working model. Accordingly, we have revised the schematic diagram and refined the manuscript to clearly illustrate the underlying

mechanistic framework. For further details, please refer to our response to the public comments.

> *(2) Figure 3: DPM Involvement in Memory Consolidation*
>
> *The authors show that PAM activation and DPM inhibition during consolidation impair appetitive LTM. However, the role of DPM is critical. While the c316-GAL4 driver yields strong effects, VT064246 inhibition shows only slight significance, requiring more than twice the sample size of other experiments. Given that c316-GAL4 is not DPM-specific and also labels MB Kenyon cells, I suggest using MB-GAL80 to restrict expression - or commenting on the possibility that other neurons like MB-KCs could directly participate in the phenotype. This is particularly relevant since VT064246 efficiently modulates sleep, indicating that it is generally effective in altering behavior. These issues weaken the claim that DPM plays a crucial role in linking sleep and memory, and should be addressed. Minor comment on this Figure: In the Figure legend, the driver and "n" are not mentioned for 3C, while this is the case for all other panels. Moreover, the DPM schematic only depicts the MB, making it somewhat confusing. DPM innervates the entire MB, still, it would be helpful to shade the DPM projections more distinctly within the MB for clarity.*

We thank the reviewer for the suggestion to improve the precision of our figures.

Regarding the expression specificity concern, in all experiments using c316-GAL4, we had eyeless-GAL80 and MB-GAL80 co-expressed to restrict GAL4-driven expression to DPMs. While complete suppression of expression of MB-KCs was not achievable, we largely eliminated the potential confounding effects from majority of these cells. VT064246-GAL4 is known to exhibit weak expression (Jenett et al., 2011; Haynes et al., 2015), but high relative specificity. Importantly, the overall conclusion derived from experiments using c316-GAL4 with GAL80s and VT064246-GAL4 are consistent, which strongly supports the role of DPM neurons in mediating the link between sleep and memory.

As suggested, we have added sample sizes for all panels and refined the depiction of DPM projections in revised Figure 3C.

> *Minor Comments*
>
> *(1) Introduction:*
>
> *The authors introduce dopamine's role in forgetting but focus on aversive rather than appetitive memories. To avoid confusion, this distinction should be mentioned explicitly (likewise in the discussion). Regarding references: Zhang et al. (line 95) do not discuss DPM or APL. Donlea et al. (line 97) do not cover dopamine - I think Pimentel et al. (2016) would be a more appropriate citation.*

This is a good point. We have removed Zhang et al. (2013) and replaced Donlea et al with Pimentel et al. 2016 as suggested.

> *(2) Figure 9: DPM Activation During Consolidation:*
>
> *The authors show that PAM neurons are activated by starvation and further enhanced by appetitive training. Surprisingly, DPM neurons also increase activity post-training, despite the proposed inhibitory connection between PAM and DPM. The authors state that "PAM-α1-DPM microcircuit exhibits synchronized neural activity changes during the consolidation window" (line 326), yet they do not address this apparent contradiction. If I have not overlooked key information, this should be clarified/addressed e.g. in the discussion.*

This is an excellent point. We have addressed this in our response to point (3) from Reviewer #2 in the public comments, so we will not reiterate it here.

> *(3) Figure 10D/E: THIP Rescue of LTM Deficits:*

> *Some inconsistencies in the THIP rescue experiments need clarification:*
>
> *(a) In Figure 10D, MB299B activation with THIP appears not to significantly restore memory relative to zero, nor to differ from untreated conditions in Figures 7A or 10E.*
>
> *(b) In Figure 10E, MB299B activation +/- THIP shows a much clearer effect.*
>
> *(c) Are Figures 7A, 10E, and 10D independent experiments, or were they conducted together?*
>
> *(d) Should the left bar in 10D and the right bar in 10E be identical? If not, I do not fully understand the discrepancy and suggest discussing the variation.*

Upon revisiting the raw datasets and conducting a one-sample t-test to analyze the group differences, the experimental group in Figure 7A showed no significant difference from the theoretical mean (set at zero). This group also did not differ from the two genetic controls, indicating that the restored memory was comparable to control levels. In Figure 10E, the group with MB299B activation plus THIP treatment exhibited a significant difference from the theoretical mean (one-sample t-test) and from the non-THIP control group, confirming a significant restoration of memory function. Owing to our laboratory relocation, the starvation duration at the new facility was adjusted based on a recalibrated starvation curve; the higher overall 24 h memory index in Figure 10E is likely attributable to a relatively longer starvation period. However, this experimental parameter variation does not alter the study's overall conclusions.

> *(4) Sleep Phenotypes and Starvation Effects:*
>
> *Sleep scores are shown under starvation/fed conditions but not under baseline conditions (without inhibition/activation). Could the authors indicate whether they observe basal starvation-induced sleep changes? The authors frequently state that PAM-DPM effects on sleep are context-dependent, yet mild but significant changes occur under fed conditions. I suggest rewording to clarify that the effect is enhanced in a context-dependent manner rather than strictly context-dependent.*

Starvation-induced sleep reduction is a well-characterised phenotype. Our study focused on the key question of whether altered neuronal activity modulates sleep under innate starvation conditions. Accordingly, all comparisons were made between the experimental and control groups under both starvation and fed conditions. We appreciate the reviewer's suggestion to improve clarity and have revised the text as suggested.

> *(5) Starvation Duration in Methods:*
>
> *The authors use 30-46h of starvation, which is longer than the ~20h typically used in appetitive memory studies. Could the authors explain why such extended starvation times were necessary?*

Determining starvation levels via survival curves is a well-established and relatively objective method, one that has been widely adopted in prior studies. For the memory test, we standardized the total starvation duration for each genotype to the time point at which mortality reached 20%. Owing to inherent differences in to starvation resistance across distinct genotypes, the final starvation durations ranged from 20 hours to 46 hours.

> *(6) Variability in PAM-α1 Sleep Effects:*
>
> *(a) The extent and timing of sleep effects differ across PAM-α1 drivers (e.g. night vs. light-period effects). Could MBON co-targeting by these drivers contribute to the variability?*

We have supplemented additional experiments to investigate the effects of MBON-α1 neurons on 24h memory and sleep. For detailed findings, please refer to our response to your public comments.

> *(b) Even within the same driver, results differ (e.g., Figure 7H vs. 10A). A general comment on these differences would be important, e.g. regarding the relevance of day and night sleep for memory consolidation.*

We sincerely appreciate the reviewer's incisive observation regarding these details. The discrepancy stems from the timing of neuronal activity inhibition, during which a laboratory relocation led to adjustments in starvation duration for memory experiments, which in turn indirectly altered sleep patterns.

> *(c) Technical note: Similar y-axis scales for sleep plots (Figures 10A and B) would make comparison easier.*

We have unified the y-axis scales to the same range.

> *(7) Discussion, Line 376:*
>
> *The phrase "sleep deprivation is important for memory consolidation" is misleading, as it could imply that deprivation aids memory formation. Please clarify.*

We appreciate the reviewer's suggestion. We have revised the text to: "These results demonstrate that preserving unperturbed sleep during the critical memory consolidation window is essential for stabilizing appetitive long-term memory.

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