Neuronal reactivation during post-learning sleep consolidates long-term memory in

*Drosophila*

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Abstract

Animals consolidate some, but not all, learning experiences into long-term memory. Across the animal kingdom, sleep has been found to have a beneficial effect on the consolidation of recently formed memories into long-term storage. However, the underlying mechanisms of sleep dependent memory consolidation are poorly understood. Here, we show that consolidation of courtship long-term memory in *Drosophila* is mediated by reactivation during sleep of dopaminergic neurons that were earlier involved in memory acquisition. We identify specific fan-shaped body neurons that induce sleep after the learning experience and activate dopaminergic neurons for memory consolidation. Thus, we provide a direct link between sleep, neuronal reactivation of dopaminergic neurons, and memory consolidation.
Introduction

Animals guide their behavior in part based on the memories of past learning experiences. Temporally, these memories can be either short- or long-lasting; short-term memory (STM) shapes future animal behavior within seconds, minutes to hours after a learning experience, whereas long-term memory (LTM) holds the learned information for hours, days or even a lifetime. STM is thought to rely on protein synthesis-independent covalent modifications and strengthening of existing synaptic connections, whereas LTM is believed to reflect protein synthesis-dependent structural changes in specific synapses\(^1\). Depending on the salience or duration of the learning experience, some STMs can be transformed into LTMs through the process of memory consolidation\(^2\).

Numerous studies in vertebrates suggest that sleep has a beneficial effect on memory in various learning tasks\(^3,4\). Studies in humans and rodents have shown that an insufficient amount of sleep impairs cognitive functions such as memory formation and retention, while an ample amount of sleep after learning experience supports memory storage\(^5,6\). In addition, several studies have found that animals, including humans, exhibit an enhanced amount and quality of sleep after experiencing a novel or enriched environment\(^7,8,9\). One hypothesis is that sleep plays an active role in consolidation of newly acquired memories into long-term storage, that are critical for the animal future actions. To date, however, a mechanistic understanding of sleep function in this selective memory consolidation remains elusive.

Over the last two decades, studies of sleep and LTM have expanded from the almost exclusive studies in mammals to other animals, including insects. Sleep is now well-documented in *Drosophila*. Sleep in *Drosophila* exhibits almost all the hallmarks of sleep in mammals, including homeostatic regulation, diminished behavioral responsiveness, and the existence of different sleep stages as characterized by distinct electrophysiological signatures\(^10-12\). Recently, a link between sleep and LTM has been established: sleep deprivation before and after learning impairs mnemonic performance\(^13-15\), whereas sleep induction enhances memory formation and
retention in wild-type and restores memory in memory mutant flies. Moreover, flies that were exposed to a socially enriched environment, including courtship learning, exhibit an increased amount of sleep.

Courtship conditioning has been extensively exploited to study both memory and sleep in Drosophila. Courtship conditioning is an ethologically relevant form of complex learning whereby male flies learn to associate the outcome of their own behavior with multisensory cues presented by females during courtship. Naive Drosophila males eagerly court both virgin and mated females, which are generally receptive and unreceptive, respectively. However, after being repeatedly rejected by mated females, males become less likely to court other mated females. Like with other types of learning, the resulting memory can last from minutes to days depending on the training protocol.

We previously established that activity of the dopaminergic aSP13 neurons (DAN-aSP13s) is necessary and sufficient for STM acquisition via the dopamine receptor DopR1 in the γ neurons of the mushroom body known as Kenyon Cells (γKCs), a neuropil in the Drosophila central brain critical for memory formation. Moreover, we recently demonstrated that the activity of the same DAN-aSP13s is also essential for the consolidation of courtship STM to LTM. This requirement is observed in a discrete time window after learning, and it is mediated via the dopamine receptor DopR1 in the γKCs.

In this study, we examine the mechanisms of DAN-aSP13 post-learning activation. We show here that DAN-aSP13s are activated during sleep after courtship experience that induces LTM. We present evidence that the specific class of sleep-promoting neurons in the fan-shaped body (FB), a neuropil previously implicated in sleep regulation, activates DANs-aSP13 in the discrete time window after learning to consolidate courtship LTM.
Results

DAN-aSP13 neurons are activated during post-learning sleep

To investigate the role of sleep in post-learning activation of DAN-aSP13s, and hence LTM consolidation, we first asked whether DAN-aSP13s are active in freely behaving males after a prolonged experience with mated females, which induces LTM. To monitor activity of DAN-aSP13s for several hours in unrestrained males we employed a luminescence-based transcriptional reporter of neuronal activity\textsuperscript{22}. Using specific DAN-aSP13s GAL4 driver\textsuperscript{23} (Figure 1-figure supplement 1A), we expressed luciferase exclusively in DAN-aSP13s under the control of multimerized binding sites for the neuronal activity regulated gene Lola and FLP recombinase target sites (FRT) for its activity dependent cell specific expression (\textit{MB315B-GAL4>UAS-FLP, Lola(FRT)stop(FRT)LUC})\textsuperscript{24}. We measured luminescence as a proxy for neuronal activity in males that had undergone prolonged training with mated females and in naïve males without prior courtship experience. As a control we used males after prolonged training with virgin females which does not induce memory when tested 24 hours later with mated females (our unpublished results). For training, single males were paired with a single recently-mated or virgin female for 6 hours. Afterwards, trained, naïve and control males were individually transferred to a 96-well luminescence reading plate. Luminescence measurements were taken every 15 minutes over 16 hours beginning at a common starting time for all males, at the hour 7 from the onset of training. Notably, flies that had undergone training with mated females displayed a gradual increase of the luminescence signal, which was significantly different from naïve males between 7-10 hours from the onset of training (Figure 1A). In contrast, control males that had undergone training with virgin females also displayed an increase in luminescence however, it was identical to that in naïve males (Figure 1-figure supplement 1B). That specific time window of DAN-aSP13 activation after onset of training with mated females was preserved in males that were trained in a later circadian time during the day (Figure 1-figure supplement 1C). Together, these data show that DAN-aSP13s are activated in a specific time window after learning experience that leads to LTM.
It was previously shown that flies that had been subjected to social enrichment, including courtship experience, display an increased amount of daytime sleep\textsuperscript{13}. To determine whether Drosophila males sleep in the time period when DAN-aSP13s are activated, we measured the amount of sleep in males after prolonged courtship training and in naïve males. Sleep was analyzed with Drosophila Activity Monitors, and periods of inactivity lasting for at least 5 minutes were classified as sleep\textsuperscript{25,26}. Both trained and naive wild-type males showed a significant amount of daytime sleep, particularly during hours 7-10 when DAN-aSP13s are active. Importantly, males that had undergone training for LTM slept significantly more in this time window in comparison to naïve males (Figure 1B). Both groups displayed the same amount of sleep throughout the night.

Notably, enhancement of post-training sleep occurs between 7-10 hours from the start of training regardless of the circadian time of training. Males that were trained for LTM in the afternoon instead of the usual morning session, displayed an increased amount of sleep between 7-10 hours after onset of training (Figure 1-figure supplement 1D). They also had a normal LTM when tested 24 hours later (Figure 1-figure supplement 1E, Figure 1-figure supplement 2A). To evaluate their memory, we used automated video analysis to derive a courtship index (CI) for each male; CI is defined as the percentage of time over a 10-minute test period during which the male courts the female. Memory is represented as a suppression index (SI), which is the relative reduction in the median courtship indices of trained versus naïve males: SI=$100\times[1-\text{CI}_{\text{train}+}/\text{CI}_{\text{train}-}]$\textsuperscript{19}. In contrast, males that were trained for 1-hour to induce STM did not exhibit an increased amount of sleep (Figure 1-figure supplement 1F). Together, these data suggest that only training that can induce LTM leads to sleep enhancement between 7-10 hours after the start of training.

To investigate whether the enhancement of post-training sleep is caused by learning or a prolonged intense activity such as courtship towards mated female, we monitored sleep pattern and courtship memory of mutants for the dopamine receptor DopR1 that do not form
courtship memory due to impairment in memory acquisition$^{19}$. DopR1 mutant males, although they underwent the same courtship experience and courted mated females as vigorously or more than the wild-type males during 6-hour training (Figure 1-figure supplement 1G) they neither displayed an increased amount of post-training sleep (Figure 1C) nor formed LTM (Figure 1-figure supplement 1H, Figure 1-figure supplement 2B). Together, these data show that learning is essential for the sleep enhancement in the specific time window after training.

Sleep after learning is necessary for LTM consolidation

To determine whether post-learning sleep is necessary for LTM consolidation, we deprived flies of sleep during specific time intervals after training and tested their memory 24 hours later. Single wild-type males were trained with mated females for 6 hours and immediately after were exposed to intermittent gentle mechanical perturbation spanning 2-hour intervals. Naïve males were sleep deprived during the same time periods.

Control males that were trained with mated females and allowed to sleep, and males that were sleep deprived after training between 9-11 and 10-12 hours had normal SIs of approximately 30-40%. However, males that were deprived of sleep between 7-9 and 8-10 hours had SIs indistinguishable from 0 (Figure 2A, Figure 2-figure supplement 2A). Deprivation of sleep during the night did not impair LTM significantly, except for a small but statistically-significant effect between 14-16 hours (Figure 2-figure supplement 1A, Figure 2-figure supplement 2E). These results show that sleep deprivation between 7-9 hours after the start of training blocks LTM, and thus post-learning sleep is necessary for LTM consolidation.

Previously, we had shown that silencing of DAN-aSP13s in a discrete time window after training impairs LTM$^{21}$. To test whether the temporal effect of sleep deprivation mimics the effect of DAN-aSP13s silencing, we expressed specifically in DAN-aSP13s (Figure 2-figure supplement 1B) a temperature-sensitive inhibitory form of dynamin shibire (shi$^{ts}$)$^{27}$ (VT005526-LexA>LexAop-shi$^{ts}$), which blocks synaptic transmission at 32°C but not at 22°C. We silenced DAN-aSP13s at 2-hour intervals after a 6-hour training session. Inhibition of DAN-aSP13s between 7-9 or 8-10
hours, but not between 9-12 hours, after the onset of training abolished LTM. Control males, in which DAN-aSP13s remained functional throughout the assay, had a normal SI between 30-40% (Figure 2B, Figure 2-figure supplement 2B). These results show that sleep and DAN-aSP13 activity are essential for memory consolidation in the same time window after training.

Enhancement of sleep consolidates LTM

To investigate whether sleep can consolidate STM into LTM, we tested whether artificial enhancement of sleep would lead to memory consolidation. We trained males for 1 hour, which does not generate LTM, and then induced sleep at various time intervals afterwards. Since activation of the FB neurons induces sleep, we expressed the thermosensitive cation channel TrpA1 (open at 30°C and closed at 20°C) in FB neurons (104y-GAL4>UAS-TrpA1) and monitored sleep for 24 hours. Consistent with previous reports, males in which FB neurons were activated slept significantly more than males that were kept at 20°C and the empty-GAL4 control males (pBDP-GAL4>UAS-TrpA1) (Figure 2- figure supplement 1C).

To induce sleep precisely with 2-hour temporal resolution after a 1-hour training session, we used the optogenetic activator CsChrimson. We found that activating FB neurons (104y-GAL4>UAS-CsChrimson) in the period between 5-7 hours after the onset of training led to a SI of ~30%, which is similar to that obtained after LTM training (Figure 2C, Figure 2-figure supplement 2C). In contrast, males activated at other time intervals or not activated at all failed to consolidate LTM and had SIs that were indistinguishable from 0. This temporal window was identical to that observed for DAN-aSP13s activation to consolidate LTM (VT005526-LexA>LexAop-CsChrimson) (Figure 2D, Figure 2-figure supplement 2D). When we activated FB neurons (104y-GAL4>UAS-CsChrimson) while silencing DAN-aSP13s (VT005526-LexA>LexAop-Shi) between 5-7 hours after the onset of training we did not observe LTM (Figure 2C, Figure 2-figure supplement 2C). Taken together, these data show that enhancement of sleep after a learning experience that induces STM can consolidate it to LTM, in a manner that requires activation of DAN-aSP13s. This LTM consolidation is a specific effect of DAN-aSP13s activation after training since activation of DAN-aSP13s (VT005526-LexA>LexAop-CsChrimson) in naïve
males between 5-7 hours when they normally display a significant amount of sleep does not induce courtship suppression towards mated females and thus “courtship LTM” (Figure 2-figure supplement 1D).

**FB neurons provide excitatory input to DAN-aSP13 neurons**

To test whether FB activation excites DAN-aSP13s, we optogenetically activated FB neurons (104y-GAL4>UAS-Chrimson88) and monitored calcium levels in DANs located in the protocerebral anterior medial (PAM) cluster in explant brains (R58E02-LexA>LexAop-GCamP6s). The PAM cluster of DANs consists of multiple neuronal classes, including a class of DAN-aSP13s, which is thought to respond to rewarding stimuli and promote their avoidance (Figure 3-figure supplement A). Since the expression pattern of 104y-GAL4 is not exclusive to the FB, to activate FB neurons selectively, we used a digital mirror device (DMD) to target the illumination activating Chrimson to specific layers in the FB. 104y-FB neurons form three layers of projections: dorsal (dFB), medial (mFB) and ventral (vFB). To test the targeted illumination, we expressed in 104y neurons both Chrimson88 and GCamP6s (104y-GAL4>UAS-Chrimson88>UAS-GCamP6s) and selectively activated one of two layers of the FB (dFB or vFB) while monitoring calcium levels in the presence of tetradoxotoxin (TTX). TTX inhibits propagation of action potentials, and thus calcium signals should only be observed in the projections located in the targeted layer. Upon activation of either dFB or vFB layers, a robust calcium response was observed only in the activated layer, implying that this local activation was highly restricted (Figure 3A).

We next activated all FB layers using targeted illumination and monitored calcium responses in DAN-aSP13s (104y-GAL4>UAS-Chrimson88, R58E02-LexA>LexAop-GCamP6s). Local activation of FB neurons elicited an excitatory response in DAN-aSP13s (Figure 3B). Given that the FB has a multilayered organization, we next aimed to identify the specific layer of the 104y-FB expression pattern that provides excitatory input to DAN-aSP13s. We individually activated all three 104y-FB layers and monitored calcium levels in DAN-aSP13s. Surprisingly, activation of the dFB layer, which has been recently implicated in homeostatic sleep regulation, resulted in...
a mixture of small inhibitory and excitatory responses (Figure 3C), whereas activation of the vFB
layer induced excitatory responses in DAN-aSP13s (Figure 3D). Activation of the mFB had no
effect on DAN-aSP13 activity (Figure 3- figure supplement B). These data show that neurons
that project to the vFB and potentially the dFB layers provide an excitatory input onto DAN-
aSP13s and may thus have a role in LTM consolidation.

vFB neurons promote sleep and activate DAN-aSP13s

To identify the specific neurons in the 104y-GAL4 line that project to the different FB layers, we
performed multicolor flip-out experiments\cite{36}. We identified two distinct neuronal populations
that project to either the dFB or vFB layers (Figure 4- figure supplement A). Next, we searched
the Janelia\cite{37} and Vienna Tiles (VT)\cite{38} collections for specific GAL4 driver lines with expression in
these two cell types. We identified one sparse GAL4 line with expression in the dFB neurons
(R23E10) and one in the vFB neurons (VT036875). Since the expression pattern of the VT03687-
GAL4 line also includes a class of DAN-β’1 neuron, we combined this line with PAM-GAL80 to
restrict its expression to vFB neurons only (VT036875-GAL4, R58E02-GAL80). In addition, we
generated a split line (SS057264-GAL4)\cite{39,40} with the expression restricted to the vFB neurons
only (Figure 4- figure supplement B, C, D, E).

As previously reported, thermogenetic activation of dFB over a 24-hour time period resulted in
an increased amount of sleep (R23E10-GAL4>UAS-TrpA1) (Figure 4A). Sleep increase was
displayed mainly during day time since males sleep almost continuously during night. We found
that activation of vFB (VT036875-GAL4>UAS-TrpA1; VT036875-GAL4>UAS-TrpA1, R58E02-
GAL80; SS057264-GAL4>UAS-TrpA1) also robustly induced day time sleep (Figure 4B, C, D) in
comparison to the genetic control (pBDP-GAL4>UAS-TrpA1) (Figure 4-figure supplement G).
Loss of night time sleep observed upon prolonged vFB activation with VT036875-GAL4 (Figure 4
B) was likely caused by co-activation of wake promoting DAN-β’1 neurons\cite{41} (Figure 4-figure
supplement C). Accordingly, males upon activation with the same line while silencing DAN-β’1
neurons displayed a higher level of both day and night sleep (Figure 4 C, Figure 4-figure
supplement D). Similarly, acute optogenetic activation of the dFB and vFB neurons with
CsChrimson for 1 hour also significantly enhanced the amount of sleep (\(R23E10\text{-GAL4>UAS-CsChrimson}\); \(VT036875\text{-GAL4>UAS-CsChrimson}\); \(VT036875\text{-GAL4>UAS-CsChrimson, R58E02-GAL80}\); \(SS057264\text{-GAL4>UAS-CsChrimson; 104y-GAL4>UAS-csChrimson}\)) in comparison to the genetic control (\(pBDP\text{-GAL4>UAS-CsChrimson}\)) but to a lesser degree when DAN-\(\beta^+\)1 neurons were co-activated (Figure 4-figure supplement F).

To test whether these neurons provide an excitatory input on DAN-aSP13s, we activated either dFB (\(R23E10\text{-GAL4>UAS-Chrimson88}\)) or vFB (\(VT036875\text{-GAL4>UAS-Chrimson88; VT036875-GAL4>UAS-Chrimson88, R58E02-GAL80; SS057264-GAL4>UAS-Chrimson88}\)) neurons and monitored calcium levels in DAN-aSP13s. Since specific DANs innervate MB lobes in well-defined discrete areas, we used a broad PAM-DAN GAL4 driver (\(R58E02\text{-LexA>LexAop-GCamP6s}\)). To monitor activity specifically in DAN-aSP13 upon activation of FB neurons we focused on the region at the tip of the MB\(\gamma\) lobe (Figure 3-figure supplement A). Activation of dFB neurons did not elicit calcium changes in DAN-aSP13s (Figure 4A) however, activation of vFB neurons elicited a robust increase of calcium levels in DAN-aSP13s (Figure 4B, C, D). These data show that neurons in the vFB provide excitatory input onto DAN-aSP13s.

**vFB neurons mediate LTM consolidation**

Consistent with the functional connectivity results, optogenetic activation of dFB neurons (\(R23E10\text{-GAL4>UAS-CsChrimson}\)) between 5-7 hours after a 1-hour training period did not lead to LTM consolidation. However, activation of either a combination of dFB and vFB neurons (\(104y\text{-GAL4>UAS-CsChrimson}\) or just the vFB neurons (\(VT036875\text{-GAL4>UAS-CsChrimson; VT036875-GAL4>UAS-CsChrimson, R58E02-GAL80; SS057264-GAL4>UAS-CsChrimson}\)) in that same time window fully consolidated STM to LTM (Figure 5A, Figure 5-figure supplement A). In addition, silencing of dFB neurons (\(R23E10\text{-GAL4>UAS-Shi^{ts}}\)) in the period between 7-10 hours after the onset of a 6-hour training period did not affect LTM persistence, while silencing of a combination of dFB and vFB or just the vFB neurons (\(VT036875\text{-GAL4>UAS-Shi^{ts}; SS057264-GAL4>UAS-Shi^{ts} and 104y\text{-GAL4>UAS-Shi^{ts}}}\)) in the same time window strongly impaired LTM consolidation (Figure 5B, Figure 5-figure supplement B). These results show that a class of
sleep-promoting neurons in the vFB layer of the 104y-FB expression pattern is necessary and sufficient to consolidate LTM during post-learning sleep. dFB neurons, although they promote sleep, appear to have no role in courtship LTM consolidation.

Discussion

The activity of DAN-aSP13s, which is essential for courtship memory acquisition, is also necessary during a discrete post-learning time window for LTM consolidation. Because neuronal reactivation occurs during sleep in rodents, we hypothesized that post-learning activation of DAN-aSP13s involves a sleep-dependent mechanism. Using behavioral analysis and neuronal activity monitoring and perturbation approaches, we have shown here that DAN-aSP13s display an increased activity in freely behaving animals during sleep after a prolonged learning experience. We demonstrated that this sleep is necessary for LTM consolidation, and it can be mediated by a specific class of sleep promoting neurons in the ventral layer of the FB (vFB). These vFB neurons consolidate courtship LTM in a discrete time window and provide an excitatory input to DAN-aSP13s. Thus, the data we present here provide a causal link between sleep promoting neurons in the vFB, post-learning activation of dopaminergic neurons, and LTM consolidation.

Based on our data, we propose the following model for sleep-dependent consolidation of courtship LTM in Drosophila (Figure 6). During a prolonged learning experience, γKCs and DAN-aSP13s are repeatedly activated by olfactory and behavioral cues presented by an unreceptive female, respectively. Whereas prolonged wakefulness leads to an increase in homeostatic sleep drive in ellipsoid body neurons that in turn is conveyed to dFB, we hypothesize that an extended learning experience generates a learning-dependent sleep drive that is transmitted to vFB. In turn, the vFB neurons enhance sleep after learning and provide an excitatory input back on DAN-aSP13s. We believe that one potential site of a learning-dependent sleep drive are the MB neurons since they have been implicated in both memory formation and sleep regulation. Dopamine released upon DAN-aSP13 reactivation stimulates molecular processes...
Dopaminergic pathways are thought to convey information about whether an experience is rewarding or punishing and thus, worth remembering\textsuperscript{7,45}. Post-learning neuronal activity of the dopaminergic hippocampal inputs from the Ventral Tegmental Area (VTA) has been implicated in the consolidation of fear memory in rodents. Interestingly, this activity is critical during a discrete time window after learning\textsuperscript{46}. Post-learning activity of the VTA dopamine neurons has been also implicated in the reactivation during sleep of the hippocampal cells involved earlier in encoding of the spatial experience\textsuperscript{47}. Here we show that post-learning activation of DAN-aSP13s mediates the consolidation of courtship LTM in \textit{Drosophila}. We propose that reactivation during sleep of the dopamine neurons that were previously active during memory acquisition ensures that spurious experiences are not admitted into LTM storage and thus only experiences that are either sufficiently salient or persistent become long-lasting memories. Specifically, reactivation of DAN-aSP13s during post-learning sleep enhances reactivation of the $\gamma$KCs and cognate MBON-M6, which together with DAN-aSP13s form a recurrent circuit necessary for courtship memory acquisition\textsuperscript{48}. We consider two hypotheses to account for this selectivity. During sleep, vFB neurons might selectively reactivate only the relevant DANs, or alternatively, they might activate all DANs but only the relevant subset is able to consolidate LTM. The selective-reactivation model would require some marker to distinguish which DANs were activated, whereas the selective-consolidation model would require a marker in the synapses of the $\gamma$KCs that were earlier active during memory acquisition, for example translational regulator Orb2, which regulates translation upon neuronal activity during LTM consolidation\textsuperscript{21}.

Activity of dopaminergic neurons regulates sleep-wake states in animals, including flies\textsuperscript{49}. Artificial activation of DAN-aSP13s has been shown to increase wakefulness\textsuperscript{41}. In contrast, the data we present here imply that activation of vFB neurons, although they activate DAN-aSP13s, do not promote wakefulness. These results suggest that activation of DAN-aSP13s by vFB neurons during sleep is qualitatively different from a direct optogenetic or thermogenetic
activation used in previous studies. One potential explanation is that post-learning sleep involves activation of the vFB circuit, which provides both excitatory stimulus to DAN-aSP13s and inhibitory input to motor neurons. Another possibility is that post-learning sleep activates distinct downstream dopamine pathways that do not affect wakefulness.

We have identified here a class of sleep-promoting neurons in the ventral layer of FB that are distinct from the well-studied sleep-promoting neurons in the dorsal layer of FB, which regulate sleep homeostasis\textsuperscript{16,50,51}. Given that vFB neurons enhance sleep and activate DAN-aSP13s for LTM consolidation, whereas dFB neurons are neither necessary nor sufficient for LTM consolidation, we hypothesize that dFB and vFB neurons promote distinct components of sleep that have different functions. Homeostatic sleep is thought to facilitate memory encoding by downscaling synaptic weights and clearing metabolites from the brain accumulated during wakefulness\textsuperscript{52-54}. In contrast, the function of experience-dependent sleep might be to facilitate memory consolidation by strengthening synaptic connections that were engaged earlier during memory acquisition\textsuperscript{55}. Thus, the co-operation of homeostatic- and experience-dependent sleep would facilitate optimal conditions for learning new information and, if appropriate, incorporating it into long-term storage.

Recent studies have implied that sleep in flies, as in humans and rodents, exhibits sleep stages characterized by distinct electrophysiological signatures\textsuperscript{11,12}. Interestingly, the signature of sleep that is induced by activation of the dFB neurons seems to have a simpler oscillatory pattern, recorded by local field potentials, than sleep that is induced by activation of the FB neurons comprising both dFB and vFB neurons\textsuperscript{11}. Thus, these data support our hypothesis that dFB and vFB neurons promote sleep with different properties and likely different functions.

It is thought that sleep evolved in animals that are capable of complex learning which requires selective attention\textsuperscript{56}. Courtship learning is a multisensory form of learning that requires selective attention of a male to associate multiple learning cues presented by the mated female with the outcome of his own behavior\textsuperscript{56}. Accordingly, studies in bees have shown that sleep
affects a complex form of learning such as spatial memory but has no role in the simple learning paradigm of proboscis extension\textsuperscript{57}. Hence, it would be interesting to investigate whether post-learning sleep is involved in the consolidation of other types of memory in \textit{Drosophila}, such as the well-studied Pavlovian olfactory associative learning whereby animals associate an individual learning cue with a behavioral contingency.

In this work, we establish a functional link between a novel class of sleep-promoting neurons in the FB, post-learning reactivation of dopaminergic neurons and consolidation of courtship LTM. Moreover, our data suggest that sleep promoting vFB neurons mediate a learning-dependent regulation of sleep that is distinct from the homeostatic control which is facilitated by dFB neurons\textsuperscript{43}. Thus, we uncover a causal link between sleep-mediated neuronal reactivation and LTM consolidation in \textit{Drosophila}. In addition, we establish courtship LTM in \textit{Drosophila} as a tractable model to investigate the mechanisms that link learning-dependent sleep, neuronal reactivation and LTM consolidation.

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**Competing Interests**

The authors declare that no competing interests exist.

**Figure Legends**

**Figure 1.** DAN-aSP13 neurons are activated during sleep
(A) (left) Luminescence of DAN-aSP13 neurons expressing Lola-LUC reporter (MB315B-GAL4>UAS-FLP; Lola>stop>LUC) normalized to luminescence of the genetic control (UAS-FLP; Lola>stop>LUC). Mean luminescence of the wild-type males trained with mated female in single pair assays as indicated (red, n=42) and naïve males (black, n=40) is shown as a solid line with SEM indicated as thin vertical lines. (right) Total luminescence in experienced and naïve males between 7-10 hours. P value is for Ho Luc_{exp}=Luc_{naive}; *** p<0.001. Student T-test.

(B) (left) Sleep profile of the wild-type males that were trained for 6 hours with mated females (red, n=16) and naïve males (black, n=16). Sleep time was plotted in 30-minute bins. (right) Total sleep of the experienced and naïve males between 7-10 hours. P value is for Ho Sleep_{exp}=Sleep_{naive}; ** p<0.01. Student T-test.

(C) (left) Sleep profile of the dopR1 mutants that were trained for 6 hours with mated females (red, n=16) and naïve males (black, n=16). Sleep time was plotted in 30-minute bins. (right) Total sleep of the experienced and naïve males between 7-10 hours. P value is for Ho Sleep_{exp}=Sleep_{naive}; ns p>0.05. Student T-test.

Schematic of the experimental set-up in A, B and C indicates 12-hour light and dark periods (white and black areas) and time of training and test (blue shading).

The following figure supplements are available for Figure 1

Figure 1- figure supplement 1

(A) Expression pattern of MB315B-Gal4 line

(B) Luminescence of DAN-aSP13 neurons expressing Lola-LUC reporter (MB315B-GAL4>UAS-FLP; Lola>stop>LUC) normalized to luminescence of the genetic control (UAS-FLP; Lola>stop>LUC). Mean luminescence of the wild-type males trained in single pair assays as indicated with virgin female (red, n=24) or naïve males (black, n=20) shown as a solid line with SEM indicated as thin vertical lines.

(C) Luminescence of DAN-aSP13 neurons expressing Lola-LUC reporter (MB315B-GAL4>UAS-FLP; Lola>stop>LUC) normalized to luminescence of the genetic control (UAS-FLP; Lola>stop>LUC). Mean luminescence of the wild-type males trained in single pair assays as
indicated with mated female (red, n=22) and naïve males (black, n=19) is shown as a solid line with SEM indicated as thin vertical lines.

**D** Sleep profile of the wild-type males that were trained for 6 hours with mated females as indicated (red, n=16) and naïve males (black, n=16). Sleep time was plotted in 30-minute bins.

**(E)** SIs of wild type males trained as indicated with a mated female in single pair assays and tested 24 hours later. P value is for Ho SI=0; *** p<0.001, ns p>0.05. Permutation test.

**(F)** Sleep profile of the wild-type males that were trained for 1 hour with mated females (red, n=16) and naïve males (black, n=16). Sleep time was plotted in 30-minute bins.

**(G)** CIs of the wild-type or dopR1 mutant males during 6-hour training in single pair assay w a mated female. P value is for Ho Cl_{wild-type} = Cl_{DopR1}; *** p<0.001. Mann-Whitney-Wilcoxon test.

**(H)** SIs of the wild-type or dopR1 mutant males tested 24 hours after training for 6 hours with mated females. P value is for Ho SI=0; *** p<0.001, ns p>0.05. Permutation test.

**Figure 1- figure supplement 2**

**(A)** Courtship indices (CIs) of the wild-type males trained in single pair assays with a mated female as indicated in Figure 1-figure supplement 1E and Table S1.

**(B)** Courtship indices (CIs) of males of the indicated genotypes trained in single pair assays with a mated female as indicated in Figure 1-figure supplement 1H and Table S3.

**Supplementary file 1** Supplementary Tables

**Supplementary file 2** Fly genotypes

**Figure 2. Sleep after learning is necessary and sufficient for LTM consolidation**

**(A)** SIs of the wild-type males tested 24 hours after training for 6 hours with a mated female and sleep deprived at indicated time periods after training (dark purple bars). SI of the wild-type control males that were allowed to sleep (green bar). P value is for Ho SI=0; *** p<0.001, ** p<0.01, * p<0.05, ns p>0.05. Permutation test.

**(B)** SIs of males after training for 6 hours with a mated female and DAN-aSP13s silenced with shi^{ts} at indicated time periods (dark blue bars). SI of the control males with DAN-aSP13s active (green bar). P value is for Ho SI=0; *** p<0.001, ** p<0.01, * p<0.05, ns p>0.05. Permutation test.
(C) SI of males after training for 1 hour with a mated female and 104y neurons activated with csChrimson at indicated time periods (orange bars). SI of the control males with 104y neurons not activated (green bar). SI of the wild-type males with 104y neurons activated and DAN-aSP13s silenced between 5-7 hours after training (blue bar). P value is for Ho SI=0; *** p<0.001, ns p>0.05. Permutation test.

(D) SI of males after training for 1 hour with a mated female and DAN-aSP13 neurons activated with csChrimson at indicated time periods (orange bars). SI of the control males with DAN-aSP13 not activated (green bar). P value is for Ho SI=0; ** p<0.01, ns p>0.05. Permutation test.

The following figure supplements is available for Figure 2

Figure 2- figure supplement 1

(A) SI of the wild-type males after 6-hour training with a mated female and sleep deprived during night at indicated time periods (dark purple bars). SI of the control males which were not deprived of sleep (green bar). P value is for Ho SI=0; *** p<0.001, ** p<0.01, * p<0.05, ns p>0.05. Permutation test.

(B) Expression pattern of VT005526-LexA line

(C) (upper panel) Sleep profile of males (104y-GAL4>UAS-TrpA1) with 104y neurons activated (30°C, red, n=16) and not activated (20°C, black, n=16). (lower panel) Sleep profile of the genetic control males (pBDP-GAL4>UAS-TrpA1) at 30°C (red, n=32) and 20°C (black, n=32). Sleep time was plotted in 30-minute bins. White and black areas indicate 12-hour light and dark periods, respectively.

(D) CIs of the naïve and experienced males trained for 1 hour with a mated female and DAN-aSP13 activated as indicated. Ho CI_{control} = CI_{naive act} ns p>0.05; Ho CI_{naive act} = CI_{trained act} ***p<0.001. Mann-Whitney-Wilcoxon test.

Figure 2- figure supplement 2

(A) Courtship indices (CIs) of the wild-type males trained in single pair assays with a mated female and sleep deprived as indicated in Figure 2A and Table S4.

(B) Courtship indices (CIs) of males trained in single pair assays with a mated female and DAN-aSP13s silenced as indicated in Figure 2B and Table S6.
(C) Courtship indices (CIs) of males trained in single pair assays with a mated female and sleep induced as indicated in Figure 2C and Table S7.

(D) Courtship indices (CIs) of males trained in single pair assays with a mated female and DAN-aSP13s activated as indicated in Figure 2D and Table S8.

(E) Courtship indices (CIs) of males trained in single pair assays with a mated female and sleep deprived as indicated in Figure 2-figure supplement 1A and Table S5.

Supplementary file 1 Supplementary Tables

Supplementary file 2 Fly genotypes

Figure 3. FB neurons provide an excitatory input to DAN-aSP13

(A) (left) Expression pattern of 104y-GAL4>UAS-Chrimson88-tdTomato with depicted dFB (magenta) and vFB (blue) layers for local activation with DMD. (right) Excitatory response of dFB (magenta) or vFB (blue) layers in the presence of 20uM tetrodotoxin (TTX) upon activation of dFB or vFB, respectively. The calcium response pattern evoked by stimuli was calculated by the correlation of determination.

(B) (left) Expression pattern of 104y-GAL4>UAS-Chrimson88-tdTomato with depicted 104y FB neurons (orange) for local activation with DMD. (right) Normalized calcium levels (dF/F) in DAN-aSP13 upon local activation of 104y FB neurons (104y-GAL4>UAS-Chrimson88-tdTomato, R58E02-LexA>LexAop-GCamP6s). DAN-aSP13 activity in individual flies is shown in colored thin lines and the mean trace is shown in a thick orange line with SEM indicated by shaded area.

(C) (left) Expression pattern of 104y-GAL4>UAS-Chrimson88-tdTomato with depicted dFB layer (magenta) for local activation with DMD. (right) Normalized calcium levels (dF/F) in DAN-aSP13 upon local activation of dFB (104y-GAL4>UAS-Chrimson88-tdTomato, R58E02-LexA>LexAop-GCamP6s). DAN-aSP13 activity in individual flies is shown in colored thin lines and the mean trace is shown in a thick magenta line with SEM indicated by shaded area.

(D) (left) Expression pattern of 104y-GAL4>UAS-Chrimson88-tdTomato with depicted vFB layer (blue) for local activation with DMD. (right) Normalized calcium levels (dF/F) in DAN-aSP13 upon local activation of vFB (104y-GAL4>UAS-Chrimson88-tdTomato, R58E02-LexA>LexAop-GCamP6s). DAN-aSP13 activity in individual flies is shown in colored thin lines and the mean trace is shown in a thick blue line with SEM indicated by shaded area.
(B, C, D): Red line indicates the time of the light stimulus. P value in all panels represents the probability that the mean dF/F of pre-stimulation (10 sec) and the mean dF/F during stimulation has the same median across flies (tested by Wilcoxon rank sum test, sample size indicated with n value).

The following figure supplement is available for Figure 3

Figure 3-figure supplement

(A) Expression pattern of the broad PAM-DANs driver (R58E02-LexA>LexAop-GCaMP6s).

Manually defined DAN-γ5 (DAN-aSP13), DAN-γ4 and DAN-γ3 are depicted in white dashed circles.

(B) (left) Expression pattern of 104y-GAL4>UAS-Chrimson88-tdTomato with depicted mFB layer for local activation with DMD (yellow). (right) Normalized calcium levels (dF/F) in DAN-aSP13 upon local activation of mFB (104y-GAL4>UAS-Chrimson88-tdTomato, R58E02-LexA>LexAop-GCaMP6s). DAN-aSP13 activity in individual flies is shown in colored thin lines and the mean trace is shown in a thick yellow line with SEM indicated by shaded area.

Red line indicates the time of the light stimulus. P value represents the probability that the mean dF/F of pre-stimulation (10 sec) and the mean dF/F during stimulation has the same median across flies (tested by Wilcoxon rank sum test, sample size indicated with n value).

Supplementary file 2 Fly genotypes

Figure 4. Sleep promoting vFB neurons activate DAN-aSP13

(A) (left) Sleep profile of males (R23E10-GAL4>UAS-TrpA1) upon activation of dFB neurons (red, n=16) and control males with dFB neurons not activated (black, n=16). Sleep time was plotted in 30-minute bins. White and black areas indicate 12-hour light and dark periods, respectively.

(right) Normalized calcium levels (dF/F) in DAN-aSP13 upon activation of dFB (R23E10-GAL4>UAS-Chrimson88, R58E02-LexA>LexAop-GCaMP6s). DAN-aSP13 activity in individual flies is shown in colored thin lines, and the mean trace is shown in thick magenta line with SEM indicated by shaded area.
(B) (left) Sleep profile of males (VT03687-GAL4>UAS-TrpA1) upon activation of vFB neurons (red, n=16) and control males with vFB neurons not activated (black, n=16). Sleep time was plotted in 30-minute bins. White and black areas indicate 12-hour light and dark periods, respectively. (right) Normalized calcium levels (dF/F) in DAN-aSP13 upon activation of vFB (VT03687-GAL4> UAS-Chrimson88, R58E02-LexA>LexAop-GCamP6s). DAN-aSP13 activity in individual flies is shown in colored thin lines, and the mean trace is shown in thick blue line with SEM indicated by shaded area.

(C) (left) Sleep profile of males (VT036875-GAL4>UAS-TrpA1, R58E02-GAL80) upon activation of vFB neurons (red, n=16) and control males with vFB neurons not activated (black, n=16). Sleep time was plotted in 30-minute bins. White and black areas indicate 12-hour light and dark periods, respectively. (right) Normalized calcium levels (dF/F) in DAN-aSP13 upon activation of vFB (VT036875-GAL4>UAS-Chrimson88, R58E02-GAL80, R58E02-LexA>LexAop-GCamP6s). DAN-aSP13 activity in individual flies is shown in colored thin lines, and the mean trace is shown in thick blue line with SEM indicated by shaded area.

(D) (left) Sleep profile of (SS57264-GAL4>UAS-TrpA1) males upon activation of vFB neurons (red, n=16) and control males with vFB neurons not activated (black, n=16). Sleep time was plotted in 30-minute bins. White and black areas indicate 12-hour light and dark periods, respectively. (right) Normalized calcium levels (dF/F) in DAN-aSP13 upon activation of vFB (SS57264-GAL4>UAS-Chrimson88, R58E02-LexA>LexAop-GCamP6s). DAN-aSP13 activity in individual flies is shown in colored thin lines, and the mean trace is shown in thick blue line with SEM indicated by shaded area.

(A-D) (right panels) Red line indicates the time window of the light stimulus. P value represents the probability that the mean dF/F of pre-stimulation (10 sec) and the mean dF/F during stimulation has the same median across flies (tested by Wilcoxon rank sum test, sample size indicated with n value).

The following figure supplement is available for Figure 4

Figure 4-figure supplement
Three single FB neural cell types were manually traced in MCFO data: dFB (magenta), vFB (blue) and mFB (grey).

Confocal images registered to standard brain of four FB lines in figure 4 and superimposed on the standard brain. (B) R23E10-GAL4 (C) VT036875-GAL4 (D) VT036875-GAL4, 58E02-GAL80 (E) SS57264 driving UAS-myRFP or UAS-Chrimson88-tdTomato. (F) Total amount of sleep of the experienced and naïve males quantified per 30-minute time period upon activation with CsChrimson (activated, red, n=8-9 and not activated, black, n=8-9).

Sleep profile of the genetic control males (pBDP-GAL4>UAS-TrpA1) (30°C, red, n=48) and (20°C, black, n=48). Sleep time was plotted in 30-minute bins. White and black areas indicate 12-hour light and dark periods, respectively.

Supplementary file 1 Supplementary Tables
Supplementary file 2 Fly genotypes

Figure 5. Sleep promoting vFB neurons are sufficient and necessary for LTM consolidation.

(A) SIs of males of indicated genotypes tested 24 hours after 1-hour training with a mated female and activation at the specific time interval with CsChrimson (orange bars). SI of control males with relevant neurons not activated (green bar). P value is for Ho SI=0; *** p<0.001, ** p<0.01, * p<0.05, ns p>0.05. Permutation test.

(B) SIs of males of indicated genotypes tested 24 hours after training for 6 hours with a mated female and silencing with shiTs at the specific time interval (dark blue bars). SI of wild type control males with DAN-aSP13 active (green bar). P value is for Ho SI=0; *** p<0.001, ** p<0.01, * p<0.05, ns p>0.05. Permutation test.

The following supplement is available for Figure 5

Figure 5- figure supplement

(A) Courtship indices (CIs) of males trained in single pair assays with a mated female and with FB neurons activated as indicated in Figure 5A and Table S9.

(B) Courtship indices (CIs) of males trained in single pair assays with a mated female and with FB neurons silenced as indicated in Figure 5B and Table S10.

Supplementary file 1 Supplementary Tables
Supplementary file 2 Fly genotypes

Figure 6. Post-learning activation of DAN-aSP13 neurons mediates LTM consolidation
(A) The MBγ and DAN-aSP13s are repetitively activated during 6-hour training by the olfactory and behavioral cues presented by a mated female, respectively.
(B) Males display an enhanced amount of sleep after training for LTM. Enhanced sleep is mediated by the vFB neurons in response to a learning induced sleep drive while the remaining amount of sleep is regulated by dFB neurons in response to homeostatic sleep drive. Only vFB neurons activate DAN-aSP13s. Dopamine released as a result of DAN-aSP13s activation stimulates molecular processes in the γKCs neurons that involve synthesis of new proteins essential for LTM memory persistence.
(C) Subsequently, experienced males suppress their courtship towards mated females for 24 hours or longer.

Supplementary File 1-Table S1. LTM does not depend on the circadian time of training
Suppression indices (SIs) of naïve (train-) and experienced (train+) males of the indicated genotypes according to Figure 1-figure supplement E, tested in single-pair assays with mated females as trainers and testers. Courtship indices (CIs) are shown as median of n males and dispersion of the data as interquartile range (IQR). P values determined by permutation test for the null hypothesis that learning equals 0 (H₀: SI = 0) or for the null hypothesis that experimental and control males learn equally well (H₀: SI = SIᶜ).

Supplementary File 1-Table S2. dopR1 mutant court mated females more than wild-type males
Courtship indices (CIs) of naïve males of the indicated genotypes according to Figure 1-figure supplement G during 6-hour training with mated female are shown as median of n males and dispersion of the data as interquartile range (IQR). P values determined by permutation test for the null hypothesis that CIs of both groups are equal (H₀: CI₀ = CI₀⁺).

Supplementary File 1-Table S3. dopR1 mutant males do not learn
Suppression indices (SIs) of naïve (train-) and experienced (train+) males of the indicated genotypes according to Figure 1-figure supplement H, tested in single-pair assays with mated females as trainers and testers. Courtship indices (CIs) are shown as median of $n$ males and dispersion of the data as interquartile range ($IQR$). $P$ values determined by permutation test for the null hypothesis that learning equals 0 ($H_0: SI = 0$) or for the null hypothesis that experimental and control males learn equally well ($H_0: SI = SI_c$).

**Supplementary File 1-Table S4. Day-time sleep deprivation between 7-9 hours impairs LTM**

Suppression indices (SIs) of naïve (train-) and experienced (train+) males of the indicated genotypes, sleep deprived as denoted in Figure 2A, tested in single-pair assays with mated females as trainers and testers. Courtship indices (CIs) are shown as median of $n$ males and dispersion of the data as interquartile range ($IQR$). $P$ values determined by permutation test for the null hypothesis that learning equals 0 ($H_0: SI = 0$) or for the null hypothesis that experimental and control males learn equally well ($H_0: SI = SI_c$).

**Supplementary File 1-Table S5. Night-time sleep deprivation does not impair LTM**

Suppression indices (SIs) of naïve (train-) and experienced (train+) males of the indicated genotypes, sleep deprived as denoted in Figure 2-figure supplement A, tested in single-pair assays with mated females as trainers and testers. Courtship indices (CIs) are shown as median of $n$ males and dispersion of the data as interquartile range ($IQR$). $P$ values determined by permutation test for the null hypothesis that learning equals 0 ($H_0: SI = 0$) or for the null hypothesis that experimental and control males learn equally well ($H_0: SI = SI_c$).

**Supplementary File 1-Table S6. Silencing of DAN-aSP13 between 7-9 hours impairs LTM**

Suppression indices (SIs) of naïve (train-) and experienced (train+) males of the indicated genotypes with DAN-aSP13 silenced as denoted in Figure 2B, tested in single-pair assays with mated females as trainers and testers. Courtship indices (CIs) are shown as median of $n$ males and dispersion of the data as interquartile range ($IQR$). $P$ values determined by permutation
test for the null hypothesis that learning equals 0 (H\(_0\): SI = 0) or for the null hypothesis that experimental and control males learn equally well (H\(_0\): SI = SI\(_c\)).

Supplementary File 1-Table S7. Sleep induction between 5-7 hours consolidates STM to LTM
Suppression indices (SIs) of naïve (train-) and experienced (train+) males of the indicated genotypes and sleep induced as denoted in Figure 2C, tested in single-pair assays with mated females as trainers and testers. Courtship indices (CIs) are shown as median of \(n\) males and dispersion of the data as interquartile range (IQR). P values determined by permutation test for the null hypothesis that learning equals 0 (H\(_0\): SI = 0) or for the null hypothesis that experimental and control males learn equally well (H\(_0\): SI = SI\(_c\)).

Supplementary File 1-Table S8. DAN-aSP13 activation between 5-7 hours consolidates LTM
Suppression indices (SIs) of naïve (train-) and experienced (train+) males of the indicated genotypes with DAN-aSP13 activated as denoted in Figure 2D, tested in single-pair assays with mated females as trainers and testers. Courtship indices (CIs) are shown as median of \(n\) males and dispersion of the data as interquartile range (IQR). P values determined by permutation test for the null hypothesis that learning equals 0 (H\(_0\): SI = 0) or for the null hypothesis that experimental and control males learn equally well (H\(_0\): SI = SI\(_c\)).

Supplementary File 1-Table S9. Activation of vFB neurons between 5-7 hours consolidates LTM
Suppression indices (SIs) of naïve (train-) and experienced (train+) males of the indicated genotypes with vFB activated as denoted in Figure 5A, tested in single-pair assays with mated females as trainers and testers. Courtship indices (CIs) are shown as median of \(n\) males and dispersion of the data as interquartile range (IQR). P values determined by permutation test for the null hypothesis that learning equals 0 (H\(_0\): SI = 0) or for the null hypothesis that experimental and control males learn equally well (H\(_0\): SI = SI\(_c\)).

Supplementary File 1-Table S10. Silencing of vFB neurons between 7-10 hours impairs LTM
Suppression indices (SIs) of naïve (train-) and experienced (train+) males of the indicated genotypes with vFB silenced as denoted in Figure 5B, tested in single-pair assays with mated females as trainers and testers. Courtship indices (CIs) are shown as median of n males and dispersion of the data as interquartile range (IQR). P values determined by permutation test for the null hypothesis that learning equals 0 (H₀: SI = 0) or for the null hypothesis that experimental and control males learn equally well (H₀: SI = SIₑ).

**Supplementary File 2. Specific fly genotypes used in all main and supplementary figures.**

**Materials and methods**

**Key resource table**

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**Drosophila culture conditions**

Flies for behavioral experiments were reared in vials with standard cornmeal food at 25°C, or as indicated, at 60% humidity in a 12h light:12h dark cycle. Detailed information regarding specific strains and genotypes is provided in the Key resources table section.

**Behavior**

Courtship conditioning was performed as described\(^58\). Briefly, solitary males aged for 5–6 days after eclosion were placed for training (during day time as indicated) in food chambers for 1 hour (STM) or 6 hours (LTM) either with (trained) or without (naïve) a single mated female. After training each male was recovered, allowed to rest for 30 minutes or 24 hours and tested with a fresh mated female. Tests were performed in 10-mm diameter chambers and videotaped for 10 minutes (Prosilica GT cameras, Allied Vison Technologies). Automated video analysis was used to derive a courtship index (CI) for each male, defined as the percentage of time over a 10-min test period during which the male courts the female. Memory was calculated as a suppression index (SI) that is a relative reduction in the mean courtship indices of trained (CI\(^+\)) versus naïve (CI\(^-\)) populations: SI=100*[1-CI\(^+\)/CI\(^-\)].

To monitor sleep over a 24-hour time period, male flies were individually inserted into 65-mm glass tubes containing standard fly food, loaded into TriKinetics *Drosophila* activity monitors (DAM), and housed under 12h light:12h dark schedule\(^59\). Periods of inactivity lasting at least 5 minutes were classified as sleep. Total 24-hour sleep quantity (day time and night time sleep) was extracted from DAM system as described\(^59\).

To monitor sleep upon acute induction with csChrimson, individual males were reared at 25°C on retinal supplemented (0.1 mM) cornmeal medium in darkness for 4-5 days after eclosion.
For sleep induction single males were placed in 10-mm diameter behavioral chambers in a temperature and illumination-controlled box and videotaped (Prosilica GT cameras, Allied Vision Technologies). The amount of sleep was scored manually with periods of inactivity lasting at least 5 minutes being considered as sleep.

For sleep deprivation, flies were subjected to intermittent mechanical perturbation method while housed in TriKinetics DAM monitors. Flies received mechanical perturbations on a horizontal shaker with a total cycle of 15s/min delivered in eight pulses of 1-3 s each occurring intermittently at random times.

Courtship assays: a MATLAB script\textsuperscript{60} (permutation test) implemented in\textsuperscript{19} was used for statistical comparison of SIs between two groups. Briefly, the entire set of courtship indices for both naïve and trained flies were pooled and then randomly assorted into simulated naïve and trained groups of the same size as the original data. A SI was calculated for each of 100,000 randomly permutated data sets, and P values were estimated for the null hypothesis that learning equals 0 ($H_0$: $SI = 0$) or for the null hypothesis that experimental and control males learn equally well ($H_0$: $SI = SI_c$).

Luminescence Assay

Luminescence assay for detecting neuronal activity in freely behaving adult flies was modified from\textsuperscript{22}. Flies were starved on filter soaked in water overnight prior to training. They were transferred to fresh chambers with filter paper containing 40 mM Luciferin (GOLDBIO) in a 2% sucrose solution. For luminescence measurements after training, flies were placed into 96-well plates (Greiner Bio-one) with 40 mM Luciferin in a 2% sucrose solution. CLARIOstar microplate reader (BMG Labtech) was used for luminescence detection. Luminescence was measured every 15 minutes over 16 hours. Relative Luminescence (R. Luminescence) was calculated by dividing the luminescence of the experienced and naïve groups by the luminescence of the genetic controls (males with luciferase reporter only, fed with luciferin) at every measurement time point.
Optogenetic activation in intact animals

Single housed male flies were reared at 25°C on retinal supplemented (0.1 mM) cornmeal medium in darkness for 4-5 days after eclosion. For sleep induction or memory consolidation specific classes of neurons were optogenetically activated in behavior chambers housed in a temperature and illumination-controlled box. During activation, the behavior chamber was illuminated with 617 nm LEDs (Red-Orange LUXEON Rebel LED—122 lm; Luxeon Star LEDs, Brantford, Ontario, Canada) with a 3 mm thick diffuser between the LED and flies. The LED was driven by a customized linear current controller. 20HZ red light was used to activate neurons with intensity varying from 20 uW/mm² to 70 uW/mm². The LED board was cooled by a customized liquid cooling system to maintain the temperature in the chamber.

Optogenetic activation in explant brains

Single housed male flies were reared at 25°C on retinal supplemented (0.1 mM) cornmeal medium in darkness for 4-5 days after eclosion. Brains of the immobilized flies on ice were dissected out in saline (103 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 4 mM MgCl₂, 26 mM NaHCO₃, 1 mM NaH₂PO₄, 8 mM trehalose, 10 mM glucose, 5 mM TES, bubbled with 95% O₂/5% CO₂) and mounted anterior up on the cover slip in a Sylgard-lined dish in a 20°C saline bath. Brains were imaged with a resonant scanning two-photon microscope with near-infrared excitation (920 nm, Spectra-Physics, INSIGHT DS DUAL) and a 25x objective (Nikon MRD77225 25XW). The microscope was controlled by ScanImage 2015.v3 (Vidrio Technologies). Images of brain volume were acquired with ~157 μm x 157 μm field of view at 512 x 512 pixels resolution. Each frame and each volume was sampled by 42 frames with 3 μm per step, at approximately 1 Hz volume rate. Times series of volume images were acquired and the excitation power for calcium imaging ~12 mW.

For the optogenetic activation, the light-gated ion channel Chrimson88 was activated with a 660 nm LED (M660L3 Thorlabs) coupled to a digital micromirror device (DMD) (Texas Instruments DLPC300 Light Crafter) and combined with the imaging light path using a FF757-DiO1 dichroic (Semrock). On the emission side, the primary dichroic was Di02-R635 (Semrock),
the detection arm dichroic was 565DCXR (Chroma), and the emission filters were FF03-525/50 and FF01-625/90 (Semrock). Photostimulation light was delivered in a pulse train that consisted of six 8 s or 15 s pulses (100% duty cycle during each pulse) with a 30 s inter-pulse interval. The light intensity was ~0.6 mW/mm², as measured using Thorlabs S170C power sensor.

All image data were analyzed off-line. Region of interest (ROI) of DAN-aSP13 at mushroom body γ5 compartment was manually defined in Fiji or CircuitCatcher (a customized python program by Daniel Bushey) and the average GCaMP signal intensity within the DAN-aSP13 ROI was taken as the calcium activity of DAN-aSP13. Time series calcium activity of DAN-aSP13 (f(t)) was extracted from the image data and then analyzed with customized Matlab (MathWorks®) programs. The normalized Calcium activity of DAN-aSP13 dF/F is defined as:

\[
dF/F = \frac{f(t) - F_0}{F_0}
\]

where the f(t) is the calcium signal intensity and the F0 is the mean F of the first 10 s of the image session before optogenetic activation. dF/F traces of six stimulation were aligned to the LED onset and averaged to represent the DAN-aSP13 activity upon neuron activation. To determine the connectivity from the activated neuron to DAN-aSP13, the mean dF/F during 10s pre-stimulation and the mean dF/F during stimulation were taken as baseline activity and stimulated activity in a fly. Groups of baseline activities and stimulated activities of different flies were tested with student’s test or Wilcoxon rank sum test to determine if optogenetic activation had evoked significant calcium activity changes in DAN-aSP13 against the hypothesis that the baseline activity and stimulated activity were the same level. p value smaller than 0.05 was taken as the criteria of connectivity (either inhibitory or excitatory).

**Correlation of determination**

Correlation of determination \((r^2)\) was used to measure the correlation between the time series calcium trace \((f(t))\) of each voxel and a predicted model. Here we used the voltage driving the stimulation LED \((V(t))\) as the model.

The calcium trace of each voxel was firstly normalized to z-score, calculated with the following function
\[ Z(t) = \frac{f(t) - \mu}{\sigma} \]

Where \( \mu \) is the mean and \( \sigma \) is the standard deviation estimated of the f(t).

Then by a linear fit of the model V(t), we have the predicted calcium response \( Z_p(t) \).

Correlation coefficient \( (r) \) between \( Z(t) \) and \( Z_p(t) \) was calculated by:

\[
r = \frac{\text{cov}(Z(t), Z_p(t))}{\sigma Z \cdot \sigma Z_p}
\]

where \( \text{cov}(Z(t), Z_p(t)) \) is the covariance of \( Z(t) \) and \( Z_p(t) \), \( \sigma Z \) and \( \sigma Z_p \) are the standard deviation of \( Z(t) \) and \( Z_p(t) \).

The correlation of determination \( (R^2) \) was simply the square of the \( r \). \( R^2 \) is by definition in the range of 0 to 1. The higher the value is, the higher is the correlation (both positive and negative) between a voxel’s calcium trace and stimuli. The \( r^2 \) indexes of a whole brain volume are projected to a 16-bit image stack, which demonstrates the calcium response pattern evoked by stimuli.

**Multicolor Flip-out (MCFO)** was performed according to the protocol described in\(^{36}\).

**Immunostaining** was performed according to a protocol described in\(^{48}\).

**References**


Figure 1—figure supplement 1
A

![Box plot for training and CI%](image)

B

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Training</th>
<th>CI [%]</th>
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</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0-6h</td>
<td></td>
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<tr>
<td>dopR1</td>
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<tr>
<td>dopR1</td>
<td>0-6h</td>
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</table>

Figure 1– figure supplement 2
Figure 2

A

Control
7-9
8-10
9-11
10-12

B

Control
7-9
8-10
9-11
10-12

C

Control
1-3
3-5
5-7
7-9
9-11

D

Control
1-3
3-5
5-7
7-9
9-11

Hours after onset of training

SI [%]

0 6 24

0 6 24

0 6 24

0 6 24

Training
Sleep deprivation

Training
DAN-aSP13 silencing

Training
DAN-aSP13 silencing

Training
DAN-aSP13 activation

Hours after onset of training

SI [%]

**

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Figure 2– figure supplement 1

A

B

VT05526-LexA>LexAop-myrGFP

C

104y-GAL4>UAS-TrpA1

Day

Night

Sleep [min/30min]

0 5 10 15 20 25 30

ZT [hours]

0 6 12 18 24

30°C

20°C

pBDP-GAL4>UAS-TrpA1

Day

Night

Sleep [min/30min]

0 5 10 15 20 25 30

ZT [hours]

0 6 12 18 24

30°C

20°C

D

DAN-aSP13

Training

Activation

- -

- 5-7h

+ 5-7h

CI [%]

0 20 40 60 80 100

ns

***
Figure 2– figure supplement 2
Figure 3
**Figure 3 – figure supplement**

**A**

![Image of green fluorescence with labeled area](image1)

**B**

![Image of brain region with labeled mFB](image2)

Graph showing time vs. dF/F [%] with lines representing different conditions, with p=0.21 (n=7).
**Figure 4**

(A) dFB (R23E10)

(B) vFB (VT036875)

(C) vFB (VT036875, 58E02-GAL80)

(D) vFB (SS57264)
Figure 4– figure supplement

A 104y

B R23E10

C VT036875

D VT036875, R58E02-GAL80

E SS057264

F

G

pBDP-GAL4>UAS-TrpA1

Day

Night

Sleep [min/30 min]

0 5 10 15 20 25 30

0 12 24

ZT [hours]

0 5 10 15 20 25 30

30°C 20°C

1 dFB (R23E10)
2 vFB (VT036875)
3 vFB (VT036875, PAMGAL80)
4 vFB (SS57264)
5 all FB (104y)
6 empty GAL4 (pBDP)
Figure 5
Figure 5—figure supplement 1
Long-term courtship memory (24 hour)

Homeostatic sleep drive

dFB

vFB

DAN

aSP13

MB output neuron

Memory acquisition (0-6 hours)

mated female

MBγ KCs

Memory consolidation (7-9 hours)

Homeostatic sleep drive

Learning-dependent sleep drive

Sleep

Protein synthesis in MBγ KCs

Long-term courtship memory (24 hour)

mated female

courtship suppression

Figure 6