Published in final edited form as:

Nature. 2021 January ; 589(7843): 582–585. doi:10.1038/s41586-020-2997-y.

Availability of food determines the need for sleep in memory consolidation

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Summary:

Sleep remains a major mystery of biology, with little understood about its basic function. One of the most commonly proposed functions for sleep is the consolidation of memory[1–3]. However, as conditions like starvation require the organism to be awake and active[4], the ability to switch to a memory consolidation mechanism that is not contingent on sleep may confer an evolutionary advantage. Here, we identify a novel adaptive circuit-based mechanism that enables *Drosophila* to form sleep-dependent and sleep-independent memory. Flies fed after appetitive conditioning needed increased sleep for memory consolidation, but flies starved after training did not require sleep to form memories. Memory in fed flies is mediated by the anterior-posterior α'/β' neurons of the mushroom body (MB), while memory under starvation is mediated by medial α'/β' neurons. Sleep-dependent and sleep-independent memory rely upon distinct dopaminergic neurons and corresponding MB output neurons. However, sleep and memory are coupled such that mushroom body neurons required for sleep-dependent memory also promote sleep. Flies lacking Neuropeptide F display sleep-dependent memory even when starved, suggesting that circuit selection is determined by hunger. This plasticity in memory circuits enables flies to retain essential information in changing environments.

Behavioral plasticity is critical for adaptation in varying environments. For instance, *Drosophila* typically display robust cycles of sleep and wake, but with prolonged starvation, they increase foraging activity at the expense of sleep[4]. Sleep is typically thought to be

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Author Contributions: N.C, P.H, L.G., and A.S. conceived the project. N.C. and A.S. designed all experiments. P.H. conducted pilot sleep deprivation experiments and identified the sleep-promoting role of α'/β'ap neurons, N.C. conducted and analysed all behavioral experiments. N.C. and P.H. conducted and analysed imaging experiments. The manuscript was written by N.C. and A.S.

Competing Interests: The authors declare no competing interests.

Data availability statement: All data shown in the manuscript are included in the Source data files.

required for the consolidation of long-term memory, but, surprisingly, starved flies can still consolidate memory related to food[5]. From an evolutionary standpoint, this facilitates survival as the increased arousal promotes foraging for food and the preserved capability for memory is relevant for obtaining food. However, it raises the intriguing question of whether sleep is dispensable for long-term memory under conditions of starvation. Conversely, what mechanisms are employed to consolidate memory when food is available? Here, we show that a feeding/hunger-dependent adaptive switch drives the recruitment of distinct neural circuit mechanisms to promote appetitive long-term memory formation.

To test if sleep is coupled to appetitive memory formation, starved flies were first trained in an olfactory conditioning paradigm and then sleep was assessed in individual flies kept on either agar or sucrose tubes. Training/conditioning occurred at ZT6 and then sleep was assessed from ZT8-ZT12. We saw no difference in sleep between trained and untrained groups of flies starved postconditioning (Fig.1a). In contrast, trained flies kept in sucrose tubes slept significantly more than untrained controls (Fig.1a). This was also evident in flies starved for only 6h pre-training, as opposed to the standard 18h. (Extended Data Fig.1a). Sleep bout length, a measure of sleep quality, was also significantly longer in trained flies kept on sucrose but not in flies on agar tubes (Extended Data Fig.1b–c). The sleep increase after conditioning was variable across trained groups but was consistently higher in trained flies that exhibited robust memory.

We next evaluated if sleep is required for long-term memory. Flies were starved or fed for 24h after training, after which starved flies were tested immediately while fed flies were re-starved for 30h for robust memory retrieval[5]. Long-term memory in fed flies was protein synthesis-dependent (Extended Data Fig. 2a), as shown previously in starved flies[5]. To assess the need for sleep, groups of trained flies were sleep-deprived via mechanical stimulation. We verified that fed and starved flies display sleep rebound following group deprivation (Extended Data Fig. 2b), and, given that sleep deprivation is typically conducted with fed flies, we ensured effective sleep deprivation of trained starved flies by monitoring them individually (Extended Data Fig. 2c). 6h sleep deprivation of fed flies immediately post-training resulted in significant impairment in long-term memory (Fig. 1b). In contrast, flies starved post-training showed no effect of sleep deprivation on long-term memory (Fig. 1c). Sleep deprivation had a comparable feeding-dependent effect on memory in flies starved for only 6h before training (Extended Data Fig. 2d–e). These results indicate that the role of sleep in memory might not be universal but instead is dependent on feeding. Sleep deprivation initiated 6h post-training had no effect on memory, demonstrating that sleep in a specific time window is relevant for memory formation (Extended Data Fig. 2f).

To determine if the duration before testing influences the need for sleep, flies were starved post-training for 6h, and then fed and re-starved so they could be tested at the same time as flies fed post-training i.e. 54h. These flies displayed long-term memory, which was unimpaired by sleep deprivation during the initial 6h starvation, indicating that post-training duration does not confer sleep-dependence and that feeding immediately post-training is essential for switching to sleep-dependent memory (Extended Data Fig. 2g).

Aversive 24h memory consolidation is not sensitive to sleep deprivation in flies kept in constant light settings[1]. In contrast, we found that flies maintained in constant light demonstrated impaired long-term memory when sleep-deprived and fed, but not if sleep-deprived and starved (Extended Data Fig. 2h–i). Thus, the effect of sleep on appetitive memory formation is independent of environmental light cues. The need for sleep in fed flies was supported by the analysis of a short sleeping mutant. As some short-sleeping mutants were impaired in learning or unable to survive starvation, we focused on the *redeye* (*rye)* mutant[6] and found that *rye* flies demonstrated impaired long-term memory only if they were fed after conditioning (Extended Data Fig. 3a–b). Correspondingly, sleep in these mutants did not increase post-training (Extended Data Fig. 3c). Therefore, feeding acts as an adaptive switch such that it induces sleep-dependent memory formation. Conversely, starvation triggers a distinct consolidation mechanism that is sleep-independent.

A feeding based adaptive switch may require caloric intake. Alternatively, the sweet taste associated with sucrose might be sufficient to induce sleep-dependent memory consolidation. Starved flies kept on arabinose, a non-metabolizable sugar[7,8], post-training formed robust long-term memory, which was sensitive to sleep deprivation (Fig. 1d). Accordingly, post-training sleep was higher and better-consolidated in trained flies kept on arabinose compared to untrained flies (Extended Data Fig. 4). Thus, the sensation of food is sufficient to trigger the formation of sleep-dependent memory.

Starved animals have a high drive for food, raising the possibility that hunger signals, such as Neuropeptide F (NPF)[9,10], contribute to the adaptation to sleep-independent memory. Indeed, starved flies lacking the NPF receptor (*npfr*) demonstrated a substantial increase in sleep quantity and quality post-training (Extended Data Fig. 5a–b) and required sleep for long-term memory (Fig. 1e). Also, knockdown of *npf* in all NPF-positive cells or *npfr* pan-neuronally with RNA interference resulted in sleep-dependent memory in flies starved post-training (Extended Data Fig. 5c–d), supporting the idea that loss of NPF renders flies dependent on sleep for memory consolidation. A switch to sleep-dependent memory may account for the reported memory impairment at 3h post-training in flies with disrupted *npf* signaling[11], as sleep-dependent memory may not be stable at this time-point.

Circuits underlying appetitive memory in flies starved post-training have been identified, so we sought to determine if the same circuits mediate memory in fed flies. The mushroom bodies (MB), a major center of olfactory learning and memory, are assembled into distinct lobes: α/β, α'/β', and γ[12,13], of which α'/β' lobes are particularly important for appetitive memory[5]. We expressed a dominant-negative and temperature-sensitive allele of dynamin (UAS-*shibire*^{ts1}) in α'/β' neurons using a split-GAL4 driver, MB461B[14], trained flies at 25°C and then moved them for 4h to 32°C, the temperature at which *shibire*^{ts1} blocks synaptic transmission. We found that activity in α'/β' lobes in the first four hours, but not 8–12h, post-training is required for long-term memory in both starved and fed settings (Extended Data Fig. 6).

During development, specific projection patterns further divide α'/β' neurons into two subtypes: α'/β'm (medial) and α'/β'ap (anterior/posterior)[14]. To delineate the role of α'/β' subsets in long-term memory we used R35B12 and VT50658 Gal4 lines to target α'/β'ap

neurons while R26E01 and MB370B were utilized to target the α'/β'm subset[14–16]. Blocking neurotransmission in α'/β'm neurons reduced long-term memory performance in starved flies, but not in those that were fed (Fig. 2a, Extended Data Fig. 7a). Conversely, the activity of α'/β'ap cells was needed for long-term memory formation in only fed flies but was dispensable in flies starved post-training (Fig. 2b, Extended Data Fig. 7c).

To determine how the activity of α'/β' subsets is affected by training under fed/starved conditions, we measured calcium using CaLexA[17]. Trained or untrained flies were kept on either a food or agar vial for 4h and then individual fly brains were prepared for imaging. Following training, the GFP/calcium signal in α'/β'ap neurons was reduced in trained-starved flies relative to trained-fed and untrained-starved flies (Fig. 2c). Nevertheless, this decrease in α'/β'ap activity may not be relevant for memory in starved flies as hyperactivating α'/β'ap neurons post-training had no effect on memory performance (Extended Data Fig. 7g–h). In contrast, α'/β'm neurons showed an increase in calcium after training in starved flies and a training-dependent decrease in fed flies (Fig. 2d). Together, our results indicate that food availability influences the selection of neural circuits for the consolidation of appetitive memories.

We next asked if the circuitry for memory in fed flies also affects sleep. Previous work showed that α'/β' neurons drive wakefulness[18,19]. This effect may be mediated by α'/β'm neurons as we found that transient activation of this subset with temperature-induced TrpA1 substantially reduced sleep in flies (Fig. 2e, Extended Data Fig. 8a). Surprisingly, stimulating α'/β'ap neurons resulted in a considerable increase in sleep (Fig. 2e, Extended Data Fig. 8a). We infer that α'/β'm neurons and α'/β'ap neurons have opposing effects on sleep. Disrupting neurotransmission with UAS-*shibire*^{ts1} in α'/β'ap or α'/β'm neurons had no effect on sleep, perhaps because these neurons only influence sleep in specific contexts such as appetitive conditioning (Extended Data Fig. 8b–c).

To determine if the activity of α'/β'ap neurons is required for the sleep increase with appetitive conditioning, we blocked the activity of these neurons post-training. Experimental and control flies showed a significant increase in sleep post-training when kept at 25°C (Fig. 2f, Extended Data Fig. 9a). Blocking α'/β'ap neurotransmission eliminated the increase in sleep as well as in sleep bout length (Fig. 2f–g; Extended Data Fig. 9b), indicating that α'/β'ap activity is required for the post-training sleep increase. In contrast, α'/β'm activity is dispensable for this change in sleep (Extended Data Fig. 9d–g). Given that sleep deprivation affects memory mediated by α'/β'ap neurons, we asked if it also affects activity by sleep-depriving trained flies for 6h, and imaging fly brains for calcium using CaLexA. Sleep loss significantly reduced calcium in α'/β'ap neurons of flies fed post-training (Fig. 2h), but it had no effect in α'/β'm neurons of flies kept starved after training (Extended Data Fig. 9h). The effect of sleep deprivation on the activity of α'/β'ap neurons may account for its effect on impaired long-term memory in fed flies.

The consolidation of appetitive memory requires the activity of dopaminergic neurons (DANs), in particular PPL1 DANs[20]. To identify the relevant DANs in our experimental paradigms, we first tested a split-GAL4 driver line, MB504B, which labels multiple PPL1

DANs[14,21]. UAS-*shibire*^{ts1}/MB504B-Gal4 flies showed impaired long-term memory at the restrictive temperature in both fed and starved settings (Extended Data Fig. 10a–d).

To functionally restrict neurons in the PPL1 cluster, we first used the MB320C line that labels the MB-MP1 DANs[14,21]. The activity of MB-MP1 neurons was required for reward memory consolidation in starved flies, as previously reported[20], but was dispensable for long-term memory in fed flies (Fig. 3a–b, Extended Data Fig. 10g). On the other hand, silencing MB-MV1 (also known as PPL1-γ2α'1) neurons with MB296B impaired memory consolidation in fed but not starved flies (Fig. 3a–b). Thus, as in the case of the α'/β' lobes, different PPL1 DANs are recruited for sleep-dependent and sleep-independent memory.

MB neurons are tiled by individual DANs and corresponding MB output neurons (MBON) to form 15 distinct compartments[14,22]. MBON-γ2α'1 neurons form functional connections with MB-MV1[23,24], so we asked if these are required for memory under fed conditions. Blocking the activity of the MBON-γ2α'1 resulted in a substantial decrease in long-term memory in fed but not starved flies (Fig. 3c–d). Conversely, as previously reported[25], memory under starved conditions requires MBON-γ1pedc neurons, which are connected to MB-MP1 DANs (Fig. 3c–d). MBON-γ2α'1 are sleep-promoting neurons that project back to MB-MV1 DANs to form a recurrent circuit[22–24] and are also functionally connected to α'/β'ap neurons[19]. We propose that the MV1→MBON-γ2α'1→MV1 recurrent circuit acts in conjunction with α'/β'ap neurons to drive sleep-dependent memory formation (Fig. 3e).

Discussion

In a typical appetitive conditioning paradigm, flies are starved after training for memory retrieval, and we show here that when they are fed, they require sleep and use different circuits to form memory. In mammals, the need for sleep varies based upon the type of memory assayed. For instance, in rats and humans sleep is specifically required for hippocampus-dependent memory[26–29]. Here we show that appetitive memory has differential requirements for sleep, and recruits different circuits based upon post-training metabolic conditions. The role we report for NPF indicates that circuit selection is driven by the animal's hunger level, which might be mediated through NPF receptors on MB-MP1 DANs[11]. We speculate that feeding results in the accumulation of catabolic waste products that impose energy demands and thereby trigger a need for sleep and sleep-dependent memory consolidation. However, the switch to such memory does not require actual nutrient intake, as sweet taste is sufficient. Importantly, the circuit required for sleep-dependent memory also promotes sleep after training, thereby coupling sleep and memory. This would be the pathway used under standard conditions, but to survive a food-depleted environment, flies have clearly evolved mechanisms to memorize cues related to food without curtailing wake/foraging activities. Thus, they can form ethologically relevant memories in distinct environmental settings.

Methods

Fly stocks and maintenance

Flies were raised at 25°C and 60% relative humidity on standard cornmeal fly food under 12:12h light:dark cycle. 4–7 days old flies were used for experiments and were transferred to fresh food vials 48h before behavioral tests. Fly population was randomized but was kept age matched in each trial. For food deprivation, flies were kept in empty bottles with a wet cotton plug to prevent desiccation. The following fly lines were ordered from Bloomington stock center: *Npf*-Gal4 (25681), *npfr* mutants (10747), 20XUAS-TTS-shi[ts1]-p10 (66600; referred to as UAS-*shibire*^{ts1} in the text), UAS-*TrpA1* (26263), MB461B (68327), MB370B (68319), MB504B (68329), MB296B (68308), MB320C (68253), MB077B (68283) and MB112C (68263). UAS-*npf-RNAi* (108772), UAS-*npfr-RNAi* (107663) and VT50658 (200166) were ordered from the Vienna *Drosophila* Resource Center. Other fly lines were described previously: *redeye*[6], R26E01[15], R35B12[16], and UAS-*CaLexA*[17]. The background control line was Canton-S (Heisenberg) strain.

Behavior

Appetitive conditioning was performed as described previously[5,30]. In brief, a 4–7 days old mixed-sex population of ~100 flies were starved for either 6h or 18h and then trained at 25°C and 70% relative humidity to associate sucrose (US) with odor A (CS^+), presented in an air stream, for 2 min. A filter paper soaked in 1.5 M sucrose solution and then dried with a blow dryer was used as a US reward. A 30-sec stream of clean air was followed by a presentation of a water-soaked filter paper (blank) plus odor B (CS^-) for 2 min, followed by another 30-sec stream of clean air. In reciprocal experiments, odor B and odor A were presented with sucrose and blank respectively. The odors used in these experiments were: 4-Methylcyclohexanol (MCH) and 3-Octanol (OCT). All odors were diluted in paraffin oil at 1:10 concentration. Odors were presented in 5 mm (MCH) and 3 mm (OCT) diameter cups in the air stream. To block protein synthesis, flies were kept in vials with a filter paper soaked in 35 mM cycloheximide in water for 17h and then given 1h to recover before training as described previously[5,30]. After conditioning flies were either moved to standard fly food or maintained starved for 24h. In experiments assessing the role of sweet taste, flies were kept on 300 mM arabinose in 1% agar after conditioning. Fed flies were restarved for 30h before memory tests. This was determined based on the robustness of memory expression in tests as 24h restarvation was not sufficient but more than 30h restarvation led to a significant number of flies dying. To prolong testing-interval in starved flies, we first kept these flies on agar for 6h after training, the interval in which memory is sensitive to sleep-deprivation, and then fed them overnight for 18h followed by 30h re-starvation before memory tests. Memory was tested by presenting flies in a T-maze with odor A and odor B for 2 min. Performance index (PI) was calculated as the number of flies selecting CS^+ odor minus the number of flies selecting CS^- odor divided by the total number of flies. Each PI is the average of PIs from reciprocal experiments with two odors swapped to minimize non-associative effects.

For appetitive conditioning involving UAS-*shibire*^{ts1} or UAS-*TrpA1*, flies were raised at 21°C. UAS-*shibire*^{ts1} flies were trained at 25°C and 70% relative humidity and then moved

to 32°C (restrictive temperature) for 4h to block neuronal activity. UAS-*TrpA1* flies were kept at 21°C throughout experiments and only moved to 29°C for 4h post-training for temperature-based induction.

For sleep assessment, a mixed population of 4–7 days old male and female flies was introduced in 65 mm glass tubes containing 2% agar and 5% sucrose through an aspirator without anesthesia and loaded into *Drosophila* activity monitors (DAM, Trikinetics system). Locomotor data were collected using DAMsystem3 software and raw data files were analysed with DAMfilescan111. 5 min of inactivity, defined as no beam breaks in the DAM, was classified as sleep[31,32]. Sleep data were analysed using Insomniac 3.0[33]. Experiments to monitor sleep after training involved training a group of starved flies in an appetitive training paradigm and then transferring them individually into locomotor tubes with either sucrose or only agar. Control untrained flies were introduced in the training apparatus and then presented with only sucrose with no odor for 2.5 min followed by 2.5 min of water-soaked filter paper. Training occurred at ZT6 and sleep was assessed from ZT8 onwards in the DAM system due to the time spent in introducing flies into individual tubes and, also, to minimize the effects of handling on sleep. A mounting plate of Trikinetics vortexer was used for mechanical sleep deprivation experiments, which involves horizontal shaking of fly vials for 2 sec within every 20 sec time intervals.

For measuring sleep changes in flies with UAS-*shibire*^{ts1} or UAS-*TrpA1*, flies were raised at 21°C. First, baseline sleep was assessed at 21°C, which was then compared to changes in sleep after TrpA1 based induction at 29°C or *shibire*^{ts1} based inhibition at 32°C. Change in sleep was calculated as the amount of sleep in the first 24h at the restrictive temperature minus baseline sleep at 21°C the previous day. UAS-*shibire*^{ts1} flies were kept at 32°C for 4h after training and then moved to 25°C to assess the role of α'/β' subsets in sleep post-training.

Immunohistochemistry

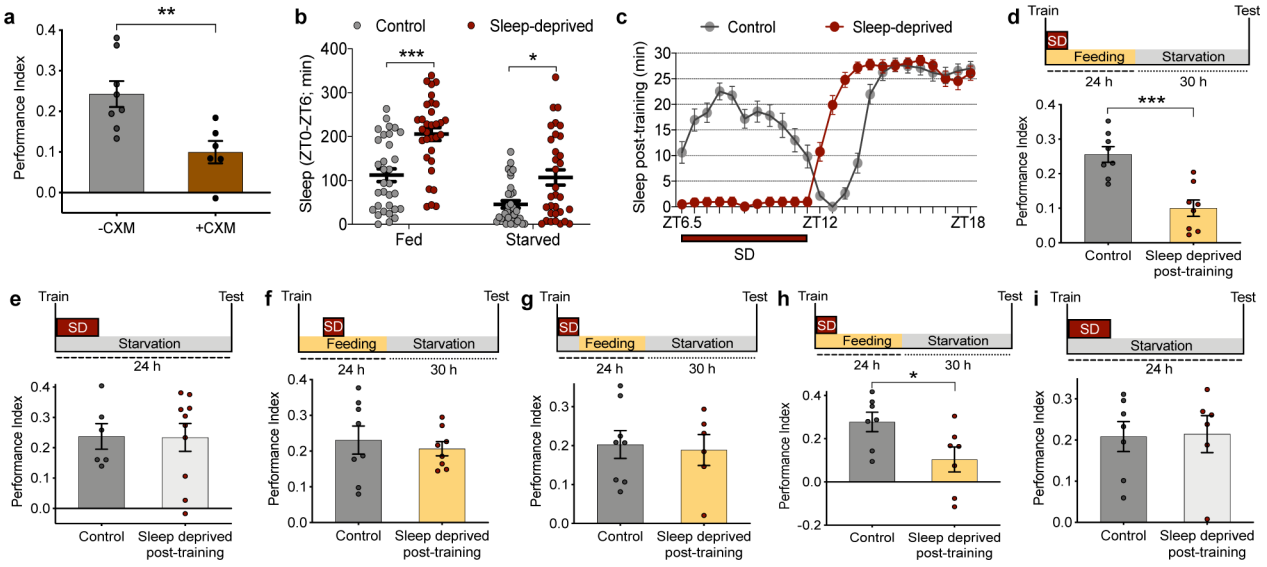
A standard protocol was used for fixation and staining. Briefly, adult fly brains were dissected in cold Phosphate Buffered Saline (PBS) and then fixed in 4% paraformaldehyde (v/v) for 20–30 min at room temperature. Brains were then rinsed in PBS-0.3% Triton-X (PBST) three times, 15 min each. Samples were then incubated with a mixture of 5% Normal Goat and Normal Donkey serum in 10% Bovine Serum Albumin (m/v) and PBST (NGS/NDS) for 1h, and then incubated overnight with primary antibodies in NGS/NDS at 4°C. 15 min PBST wash was then used on the samples seven times followed by incubation with secondary antibodies for 2 h at room temperature in NGS/NDS buffer. Subsequently, another seven repetitions of 15 min PBST wash and a single 15 min PBS wash was performed and then brains were moved into 50% Glycerol. Brains were mounted on slides with anti-fade medium (Vectashield: H1000) and visualized in a Leica TCS SP5 confocal microscope. Primary antibodies used were: Mouse anti-GFP (1:200; Roche Applied Biosciences; Cat. # 11814460001) and Rabbit anti-RFP (1:200; Takara Bio; Cat. # 632475). Following secondary antibodies were utilized: Alexa Fluor 488 Donkey anti-mouse (1:200; ThermoFisher; Cat. # A-21202) and Cy5 Donkey anti-rabbit (1:200; Jackson ImmunoResearch; Cat. # 711-175-152). Mouse anti-GFP primary with secondary Alexa

Fluor 488 Donkey anti-mouse antibodies were used to detect GFP signal from the CaLexA (Calcium-dependent nuclear import of LexA) reporter system. Fiji 2.0 was used for analysing images.

Statistical treatment

The mean and standard error were used to represent data. The sample size is indicated in the respective figure legends and precise 'n' values are depicted in the source data files. The sample size was not determined by any statistical test. Group means are displayed in figures depicting sleep trends. In behavioral experiments, due to the unambiguous nature of measurements blinding was not used. In imaging experiments, investigators were blinded to group allocation during data collection and analysis. GraphPad Prism 8.0 was used to plot graphs and compare independent groups of data. All groups of data were tested for normality using the D'Agostino and Pearson omnibus test. For normally distributed data, a two-sided student's t-test for two groups and one-factor ANOVA followed by Tukey post-hoc test in the case of multiple groups were used for analysis. Also, differences between multiple undisturbed and sleep-deprived groups or trained and untrained groups were assessed using multiple t-tests, followed by Bonferroni correction. For the examination of data with non-Gaussian distribution, a Mann-Whitney test was performed. Statistical significance is demonstrated as ***p<0.001; **p<0.01; *p<0.05.

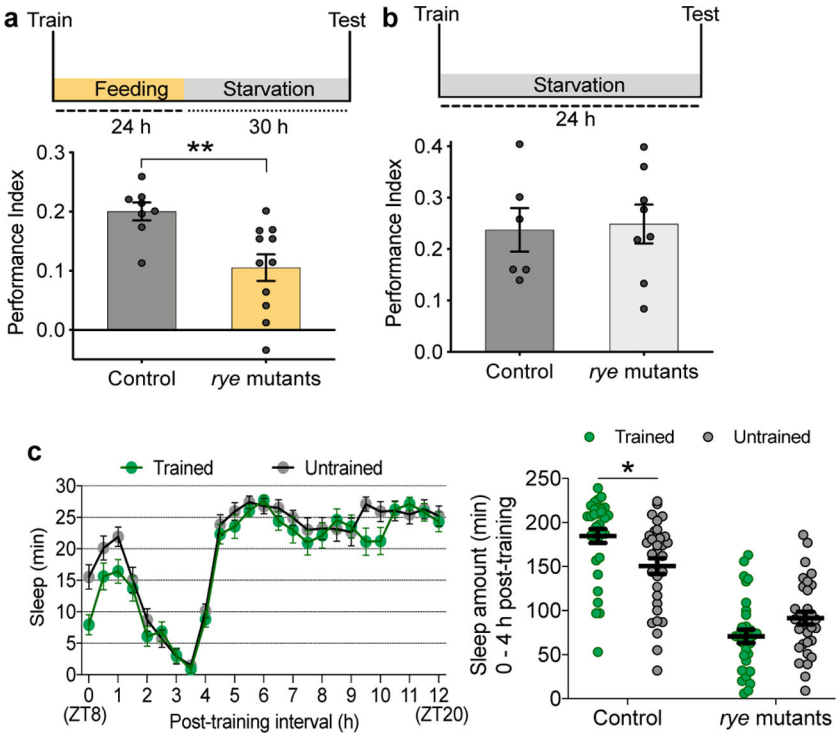
Extended Data



Extended Data Fig. 1. Sleep increases in flies fed after appetitive training

(**a**) Flies starved for 6h prior to training show no difference in sleep between trained and untrained groups. However, moving trained flies into sucrose tubes post-training resulted in a significant increase in sleep compared to untrained controls despite only 6h of pre-training starvation. Sleep was quantified for the ZT8–12 interval (Two-sided t-tests were performed for each condition to compare trained and untrained groups, followed by Bonferroni correction; n=32).

(**b**) and (**c**) Training increases sleep bout length in fed flies but not in starved flies. Flies were trained after 18h (**b**) and 6h (**c**) starvation (Two-sided Mann-Whitney tests were performed for each condition to compare trained and untrained groups; n=32). Data are represented as mean ± s.e.m. Each data point depicts a single fly. Precise 'n' and 'p' values are in the Source data. ***p<0.001; **p<0.01; *p<0.05.



Extended Data Fig. 2. Memory in flies fed after training is sleep and protein synthesis-dependent but independent of light cycles

(**a**) Long-term memory in fed flies is sensitive to cycloheximide based inhibition of protein-synthesis (Two-sided t-test; n≥6).

(**b**) Flies demonstrate substantial rebound sleep when sleep-deprived in a group of about 100 flies in a vial in both fed and starved conditions. Flies were sleep-deprived from ZT12-ZT24 and then introduced individually into locomotor tubes (Two-sided Mann-Whitney tests were performed for each condition to compare undisturbed and sleep-deprived groups; n=32).

(**c**) Starved flies were effectively sleep-deprived when exposed to a mechanical stimulus post-training (n≥31). Flies were starved for 6h and then trained at ZT6 and subsequently introduced into agar locomotor tubes. A mechanical stimulus was applied for 6h after training. A rebound is evident after sleep deprivation.

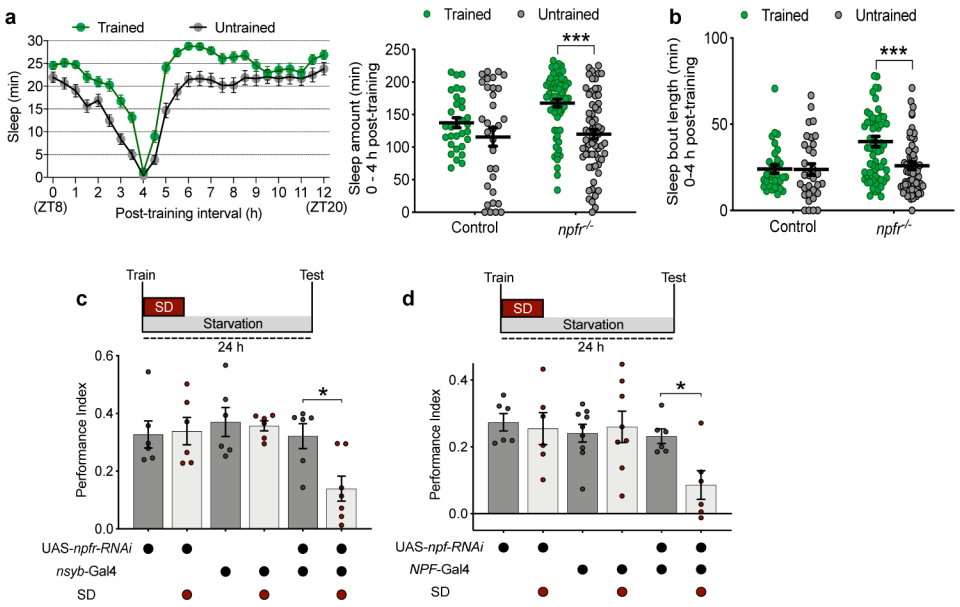
(**d**) Flies starved for only 6h, as opposed to 18h, before training and then allowed to feed showed impaired memory performance when sleep-deprived for 6h post-training (Two-sided t-test; n=8). Sleep post-training was comparable to flies depicted in Extended Data Fig. 1(a).

(**e**) 6h sleep deprivation had no effect on long-term memory in flies kept starved after training. Here, flies were starved for 6h before training (Two-sided t-test; n≥6). Sleep post-training was comparable to flies depicted in Extended Data Fig. 1(a).

(**f**) Sleep deprivation initiated 6h after training had no effect on memory in fed and trained flies (Two-sided t-test; n=8).

(**g**) Long-term memory was resistant to sleep deprivation in flies that were starved after conditioning but then tested after a feeding and re-starvation period (Two-sided t-test; n≥6). Flies were starved (and sleep-deprived) for 6h post-training and then allowed to feed for 18h before 30h restarvation for memory tests.

(**h**) and (**i**) 6h sleep deprivation of flies maintained in constant light affected appetitive long-term memory when they were fed, but not starved, post-training (Two-sided t-test; n≥6). Data are represented as mean ± s.e.m. Each data point in a memory experiment represents a group of flies and in a sleep experiment it depicts a single fly. Precise 'n' and 'p' values are in the Source data. ***p<0.001; **p<0.01; *p<0.05.

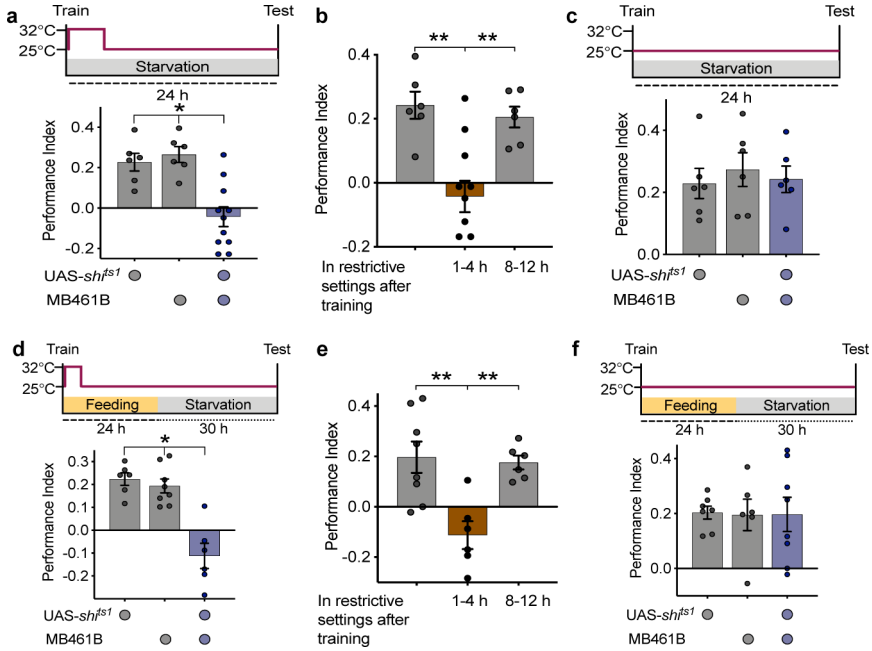


Extended Data Fig. 3. The *rye* mutation affects sleep-dependent memory

(**a**) Long-term memory is substantially lower in satiated short-sleeping *rye* mutants. Background *iso*31 line was used as control (Two-sided t-test; n≥8).

(**b**) *rye* mutants form robust appetitive 24h memory, similar to controls when kept starved (Two-sided t-test; n≥6).

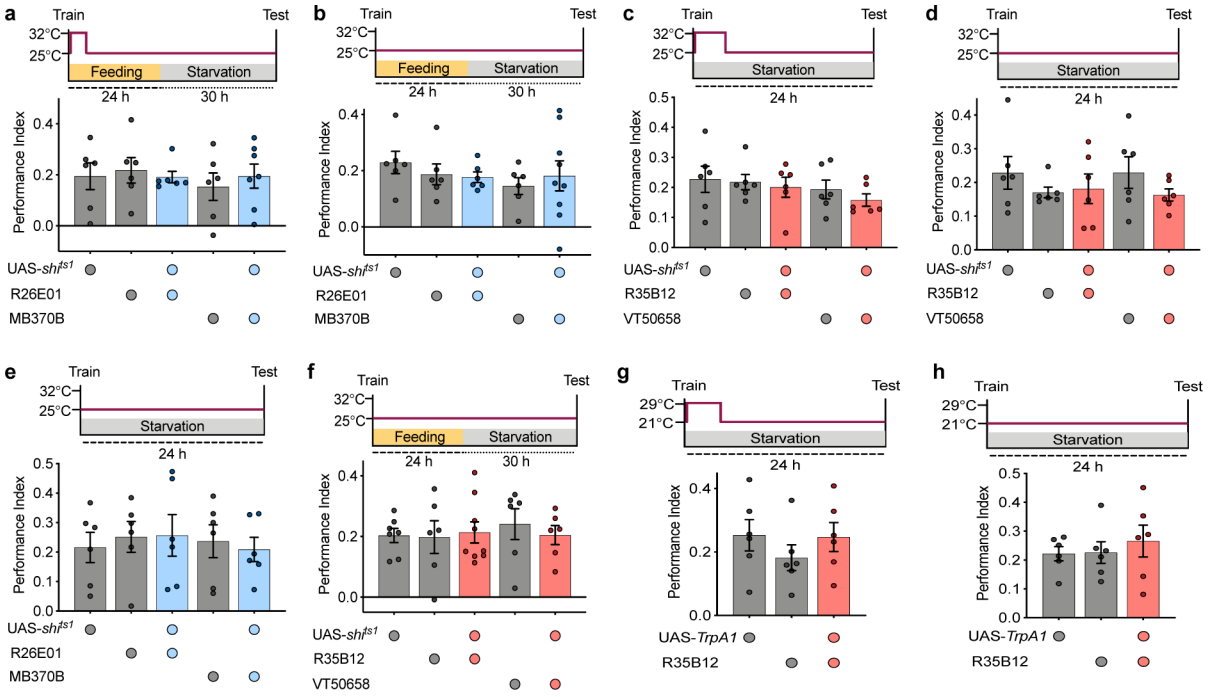
(**c**) Satiated *rye* mutants demonstrate no difference in sleep between trained and untrained groups. Total sleep in the ZT8–12 interval is depicted (Two-sided t-tests were performed for each genotype to compare trained and untrained groups, followed by Bonferroni correction; n≥31). Data are represented as mean ± s.e.m. Each data point in a memory experiment represents a group of flies and in a sleep experiment it depicts a single fly. Precise 'n' and 'p' values are in the Source data. **p<0.01; *p<0.05.



Extended Data Fig. 4. Flies on arabinose demonstrate a significant increase in post-training sleep

(**a**) Trained flies show a substantial increase in sleep relative to untrained flies when kept on arabinose after appetitive conditioning. Sleep was quantified for the 0–4h interval post-training (Two-sided t-test; n≥31).

(**b**) Bout length was considerably higher in trained flies compared to untrained flies when moved to arabinose after training (Two-sided Mann-Whitney test; n≥31). Data are represented as mean ± s.e.m. Each data point depicts a single fly. Precise ‘n’ and ‘p’ values are in the Source data ***p<0.001; **p<0.01.



Extended Data Fig. 5. *npf* signaling is essential for sleep-independent memory in starved flies

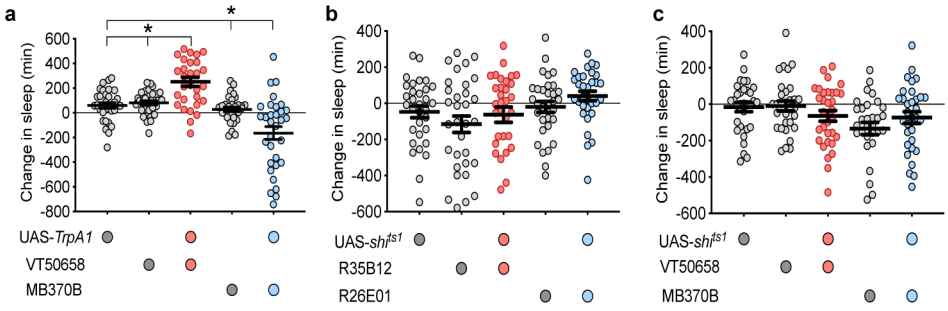
(**a**) Starved *npfr* mutant flies show a substantial increase in sleep post-training compared to untrained flies. *npfr/+* was used as control. Total sleep in the 0–4h interval post-training is depicted (Two-sided t-tests were performed for each genotype to compare trained and untrained groups, followed by Bonferroni correction; n≥32).

(**b**) Bout length was considerably higher in trained and starved *npfr* mutant flies compared to untrained flies. *npfr/+* was used as control (Two-sided Mann-Whitney tests were performed for each genotype to compare trained and untrained groups; n≥32).

(**c**) RNAi knockdown of *npfr* pan-neuronally results in sleep-dependent memory formation in hungry flies. 6h sleep disruption post-training resulted in impaired memory performance in UAS-*npfr*-RNAi/n-syb-Gal4 flies (Two-sided t-tests were performed for each genotype to compare undisturbed and sleep-deprived groups, followed by Bonferroni correction; n≥6).

(**d**) Starved UAS-*npf*-RNAi/NPF-Gal4 flies show lower long-term memory when sleep-deprived for 6h post-training (Two-sided t-tests were performed for each genotype to compare undisturbed and sleep-deprived groups, followed by Bonferroni correction; n≥6).

Data are represented as mean ± s.e.m. Each data point in a memory experiment represents a group of flies and in a sleep experiment it depicts a single fly. Precise ‘n’ and ‘p’ values are in the Source data. ***p<0.001; *p<0.05.



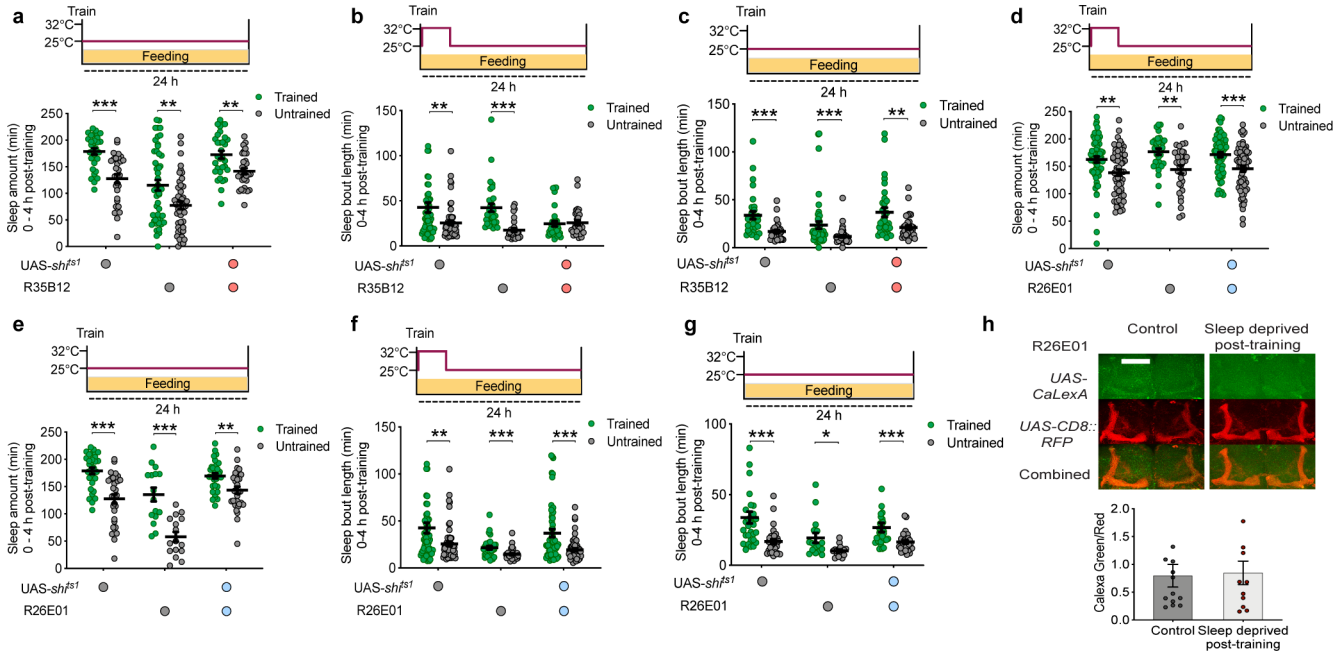
Extended Data Fig. 6. α'/β' neurotransmission is essential for long-term memory under both fed and starved conditions

(**a**) Starved UAS-*shibire*^{ts1}/MB461B flies kept at restrictive settings for 4h immediately after training show impaired long-term memory (n≥6). Restrictive temperature 8–12 hours after training had no effect (**b**) (n=6) (One-factor ANOVA with Tukey post-hoc test).

(**c**) Long-term memory remained unchanged in experimental and control flies when kept at 25°C (One-factor ANOVA with Tukey post-hoc test; n=6).

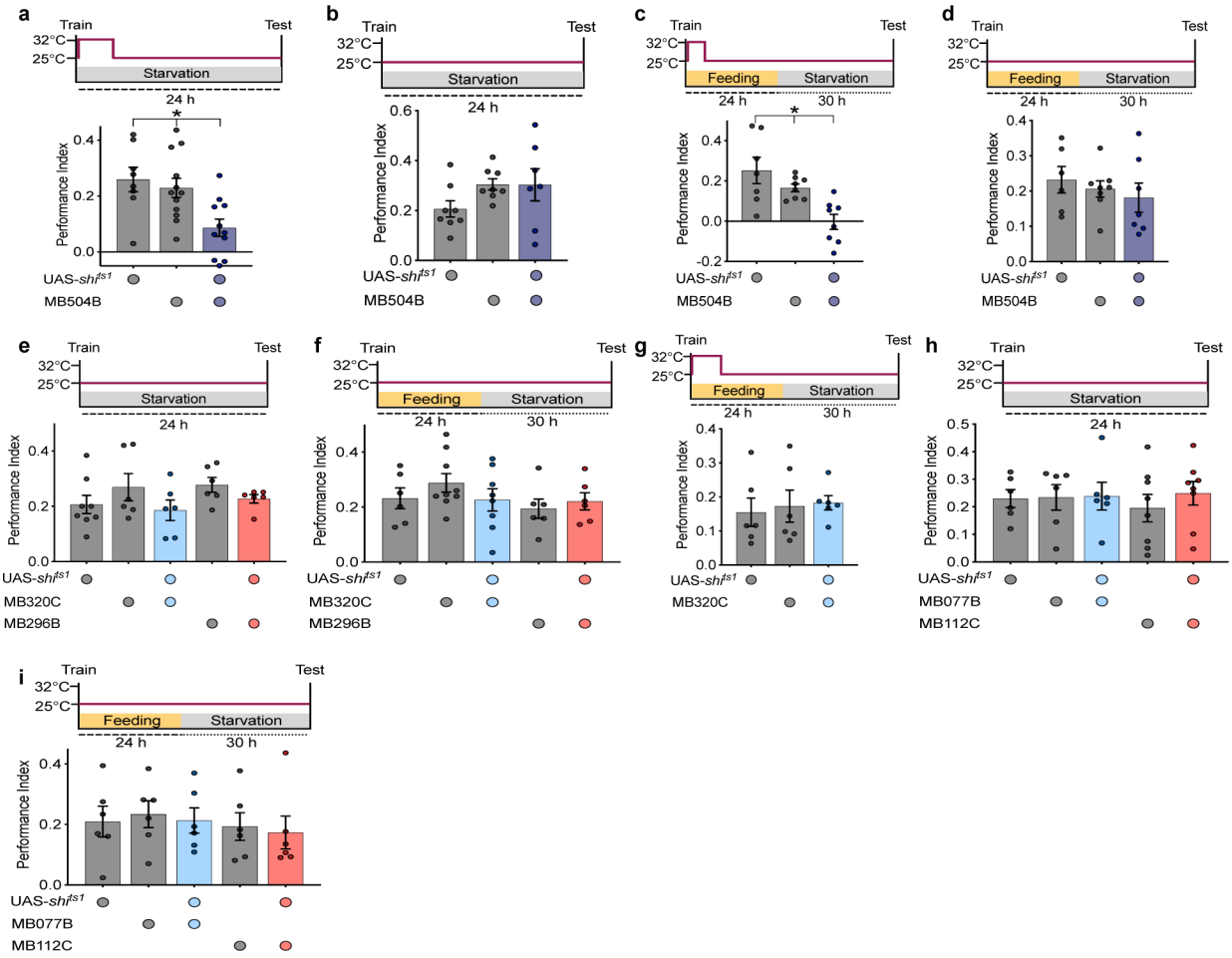
(**d**) and (**e**) Silencing α'/β' neurons immediately after conditioning, but not at hours 8–12, affects long-term memory in fed flies (One-factor ANOVA with Tukey post-hoc test; n≥6).

(**f**) Long-term memory remained intact in UAS-*shibire*^{ts1}/MB461B flies fed after training but maintained at the permissive temperature (One-factor ANOVA with Tukey post-hoc test; n≥6). Data are represented as mean ± s.e.m. Each data point represents a group of flies. Precise 'n' and 'p' values are in the Source data. **p<0.01. Asterisks in (**a**, **d**) indicate a significant difference between experimental and genetic controls



Extended Data Fig. 7. Effects of manipulating the activity of α'/β' subset specific neurons on long-term memory

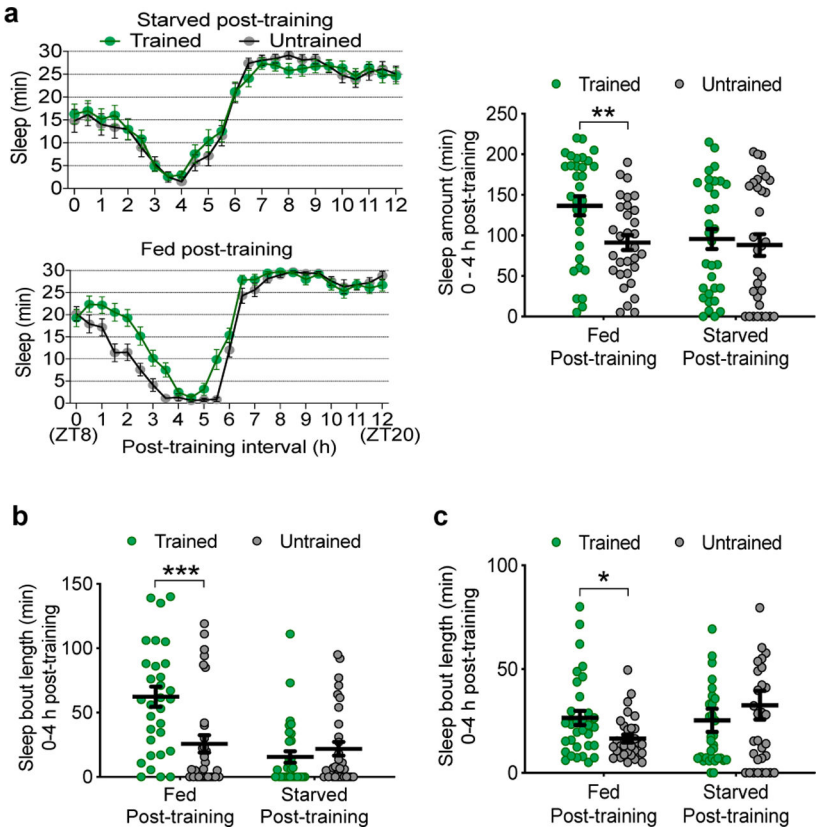
(**a**) and (**b**) Neurotransmission from α'/β'm neurons (UAS-*shibire*^{ts1}/R26E01 and UAS-*shibire*^{ts1}/MB370B) is dispensable for long-term memory in fed flies (n≥6). Temperature controls are depicted in (**b**) (n≥6) (One-factor ANOVA with Tukey post-hoc test).
(**c**) and (**d**) Blocking the activity of α'/β'ap neurons (UAS-*shibire*^{ts1}/R35B12 and UAS-*shibire*^{ts1}/VT50658) for 4h after conditioning in starved flies has no effect on long-term memory (n=6). Long-term memory in experimental and control flies at the permissive temperature, 25°C, is shown in (**d**) (n=6) (One-factor ANOVA with Tukey post-hoc test).
(**e**) *shibire*^{ts} does not affect memory in flies maintained under starvation conditions at the permissive temperature (One-factor ANOVA with Tukey post-hoc test; n=6). Controls related to Fig 2(a).
(**f**) *shibire*^{ts} has no effect on memory in flies maintained on food at the permissive temperature (One-factor ANOVA with Tukey post-hoc test; n≥6). Controls related to Fig. 2(b).
(**g**) Hyperactivation of α'/β'ap neurons (UAS-*TrpA1*/R35B12) for 4h post-training does not affect long-term memory formation in starved flies (One-factor ANOVA with Tukey post-hoc test; n=6).
(**h**) Memory was not affected in UAS-*TrpA1*/R35B12 flies at permissive settings (One-factor ANOVA with Tukey post-hoc test; n=6). Data are represented as mean ± s.e.m. Each data point in a memory experiment represents a group of flies. Precise 'n' and 'p' values are in the Source data.



Extended Data Fig. 8. α'/β' subsets differentially regulate sleep

(**a**) Thermogenetic activation of α'/β'ap neurons (UAS-*TrpA1*/VT50658) results in a considerable enhancement in sleep while flies in which α'/β'm neurons (UAS-*TrpA1*/MB370B) were activated showed a significant decrease in sleep (One-factor ANOVA with Tukey post-hoc test; n≥30).

(**b**) and (**c**) Disabling neurotransmission in α'/β'ap neurons (UAS-*shibire*^{ts1}/R35B12 and UAS-*shibire*^{ts1}/VT50658) or α'/β'm neurons (UAS-*shibire*^{ts1}/R26E01 and UAS-*shibire*^{ts1}/MB370B) had no effect on sleep (One-factor ANOVA with Tukey post-hoc test; n≥31). Data are represented as mean ± s.e.m. Each data point depicts a single fly. Precise 'n' and 'p' values are in the Source data. Asterisks in (**a**) indicate a significant difference between experimental and genetic controls.



Extended Data Fig. 9. The activity of α'/β'ap, but not α'/β'm, neurons is relevant for sleep after conditioning

(**a**) *shibire*^{ts} expression in α'/β'ap neurons has no effect on sleep post-training if flies are maintained at the permissive temperature (Two-sided t-tests were performed for each genotype to compare trained and untrained groups, followed by Bonferroni correction; n≥30). Controls related to Fig. 2(g).

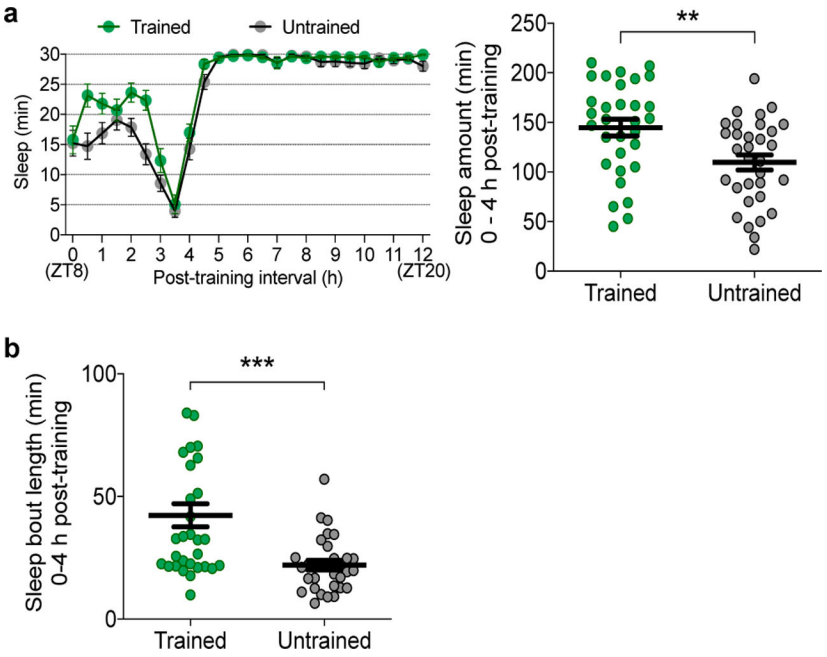
(**b**) and (**c**) A training-dependent increase in sleep bout length was prevented in flies in which α'/β'ap neurons were silenced (n≥31). Temperature controls are shown in (**c**) (n≥30) (Two-sided Mann-Whitney tests were performed for each genotype to compare trained and untrained groups).

(**d**) and (**e**) Trained flies expressing *shibire*^{ts1} in α'/β'm neurons showed an enhancement in sleep even when moved to 32°C for 4h post-training. The total amount of sleep in 0–4h interval after training is quantified (n≥32). Post-training sleep in experimental and control flies at the permissive temperature, 25°C, is shown in (**e**) (n≥16) (Two-sided t-tests were performed for each genotype to compare trained and untrained groups, followed by Bonferroni correction).

(**f**) and (**g**) Silencing α'/β'm neurons does not prevent an increase in sleep bout length after training (n≥32). Temperature controls are shown in (**g**) (n≥16) (Two-sided Mann-Whitney tests were performed for each genotype to compare trained and untrained groups).

(**h**) Calcium/GFP signal in α'/β'm neurons was comparable between control and sleep-deprived flies when kept starved post-training (Two-sided Mann-Whitney test; n≥11). Representative images are shown, two independent experiments; Scale bar, 50 μm. Data are

represented as mean ± s.e.m. Each data point depicts a single fly. Precise ‘n’ and ‘p’ values are in the Source data. ***p<0.001; **p<0.01; *p<0.05.



Extended Data Fig. 10. Effects of manipulating the neurotransmission of PPL1 neurons and MBONs on long-term memory

(**a**) and (**b**) Trained starved flies show lower long-term memory performance when the PPL1 cluster neurons (UAS-*shibire*^{ts1}/MB504B) are silenced for 4h post-training (n≥8). Temperature controls are shown in (**b**) (n≥7) (One-factor ANOVA with Tukey post-hoc test).

(**c**) and (**d**) Silencing PPL1 DANs affects long-term memory performance in flies kept fed after training (n≥7). Temperature controls are shown in (**d**) (n≥6) (One-factor ANOVA with Tukey post-hoc test).

(**e**) Expression of *shibire*^{ts1} in MP1 and MV1 neurons at permissive temperature does not affect memory in flies starved after training (One-factor ANOVA with Tukey post-hoc test; n≥6). Controls related to Fig. 3(a).

(**f**) Permissive temperature control for Fig. 3(b). Expression of *shibire*^{ts1} in MP1 and MV1 neurons does not affect memory in flies kept on food at 25°C after training (One-factor ANOVA with Tukey post-hoc test; n≥6).

(**g**) Blocking the activity of MP1 neurons (UAS-*shibire*^{ts1}/MB320C at restrictive temperature) for 6h after conditioning has no effect on long-term memory in flies kept on food vials after training (One-factor ANOVA with Tukey post-hoc test; n=6).

(**h**) and (**i**) Long-term memory in UAS-*shibire*^{ts1}/MB077B and UAS-*shibire*^{ts1}/MB112C flies was similar to that of genetic controls when kept starved or fed at 25°C (One-factor ANOVA with Tukey post-hoc test; n≥6). Temperature controls related to Fig. 3(c–d). Data are represented as mean ± s.e.m. Each data point represents a group of flies. Precise ‘n’ and ‘p’ values are in the Source data. Asterisks in (**a**, **c**) indicate a significant difference between experimental and genetic controls.

Acknowledgments

We thank the members of the Sehgal laboratory for helpful discussions, and Kevin Lin for input on statistics. We would like to thank Carly Stein and Anna Kolesnik for their assistance in imaging experiments. This work was supported by R01 DK120757 (to AS) and by the HHMI. PH was supported by T32 MH014654. Initial studies were supported by R01 MH067284 (to LCG).

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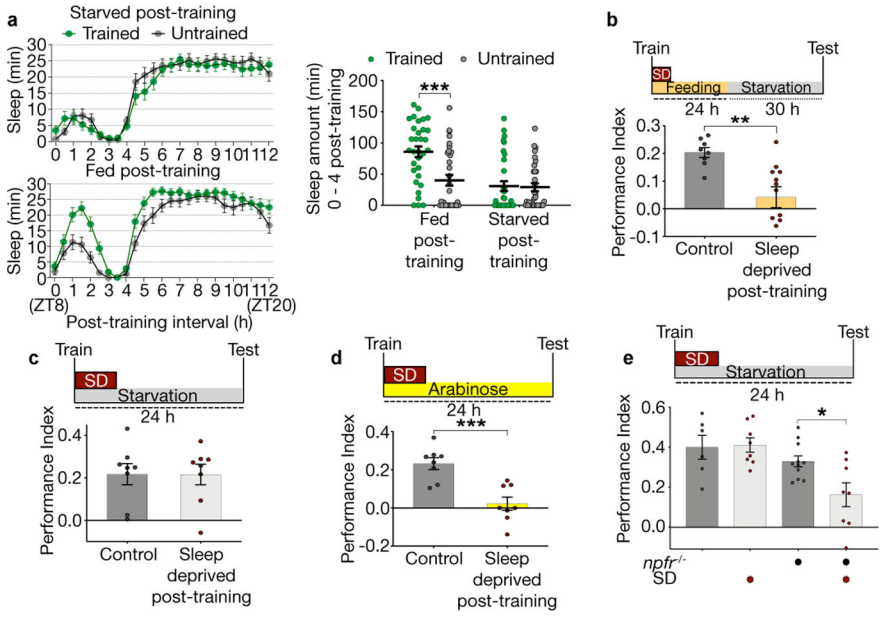

Fig. 1. Flies fed post-training require sleep for memory consolidation

(**a**) Flies trained at ZT6, and thereafter kept in agar tubes, show sleep comparable to untrained flies. In contrast, feeding post-training increases sleep in trained flies compared to controls. Sleep amount was quantified for the ZT8–12 interval (0–4h time-points on the curve) (Two-sided t-tests were performed for each condition to compare trained and untrained groups, followed by Bonferroni correction, n=32)

(**b**) 6h sleep disruption affects long-term memory in flies fed post-training (Two-sided t-test; n≥8). Sleep post-training was comparable to flies depicted in (**a**).

(**c**) 6h sleep deprivation does not affect long-term memory in flies starved post-training (Two-sided t-test; n=8). Sleep post-training was comparable to flies depicted in (**a**).

(**d**) Long-term memory is sensitive to sleep deprivation in flies kept on arabinose post-training (Two-sided t-test; n=8).

(**e**) 6h sleep disruption affects long-term memory in *npfr* mutant flies kept starved post-training. *npfr/+* was used as control (Two-sided t-tests were performed for each genotype to compare undisturbed and sleep-deprived groups, followed by Bonferroni correction; n≥6). Data are represented as mean ± s.e.m. Each data point in a memory experiment represents a group of flies and in a sleep experiment it depicts a single fly. Precise ‘n’ and ‘p’ values are in the Source data. ***p<0.001; **p<0.01; *p<0.05.

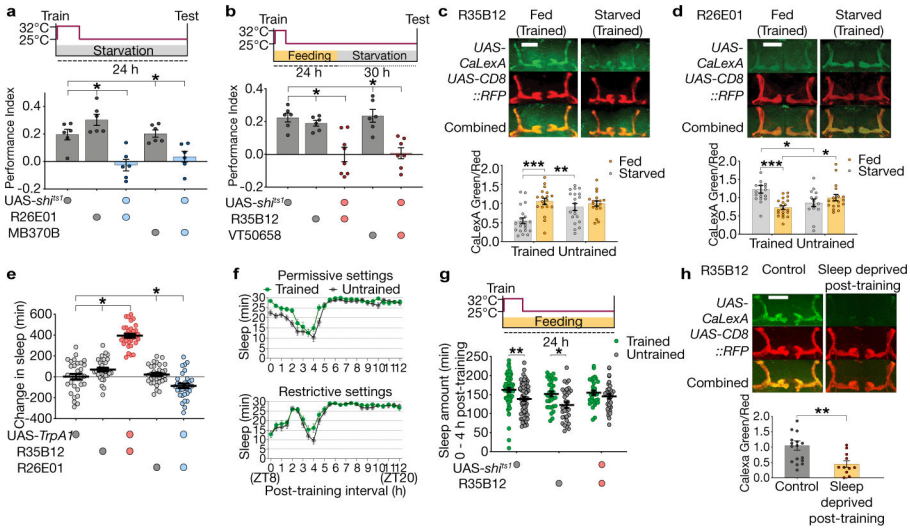

Fig. 2. Distinct α'/β' subsets mediate sleep-dependent and sleep-independent memory

(**a**) Silencing α'/β'm neurons (UAS-*shibire*^{ts1}/R26E01 and UAS-*shibire*^{ts1}/MB370B) affects long-term memory in starved flies (One-factor ANOVA with Tukey post-hoc test; n=6).

(**b**) In fed flies, long-term memory is reduced by the silencing of α'/β'ap neurons (UAS-*shibire*^{ts1}/R35B12 and UAS-*shibire*^{ts1}/VT50658) post-training (One-factor ANOVA with Tukey post-hoc test; n≥6).

(**c**) The GFP signal in α'/β'ap neurons was substantially reduced in trained-starved flies compared to both fed-trained flies and untrained controls (Two-sided Mann-Whitney tests; n≥19).

(**d**) Trained-starved flies demonstrated an increase in α'/β'm activity compared to both fed flies and untrained controls. A significant decrease in calcium/GFP was also observed in trained-fed flies compared to untrained fed flies (Two-sided Mann-Whitney tests; n≥14).

(**e**) Thermogenetic activation of α'/β'ap neurons (UAS-*TrpA1*/R35B12) resulted in a substantial gain in sleep while sleep was reduced significantly when α'/β'm neurons (UAS-*TrpA1*/R26E01) were activated (One-factor ANOVA with Tukey post-hoc test; n=32).

(**f**) UAS-*shibire*^{ts1}/R35B12 flies showed an enhancement in sleep post-training at permissive but not at restrictive settings. Sleep measurements at restrictive settings are shown in (**g**) (Two-sided t-tests were performed for each genotype to compare trained and untrained groups, followed by Bonferroni correction; n≥32).

(**h**) In trained fed flies, CaLexA-based neuronal activity in α'/β'ap neurons was substantially reduced in sleep-deprived flies compared to controls (Two-sided Mann-Whitney test; n≥11).

In (**c**, **d**, **h**), representative images are shown, two independent experiments; Scale bar, 50 μm. Data are represented as mean ± s.e.m. Each data point in a memory experiment represents a group of flies and in a CaLexA imaging and sleep experiment it depicts a single fly. Precise 'n' and 'p' values are in the Source data. ***p<0.001; **p<0.01; *p<0.05. Asterisks in (**a**, **b**, **e**) indicate a significant difference between experimental and genetic controls.

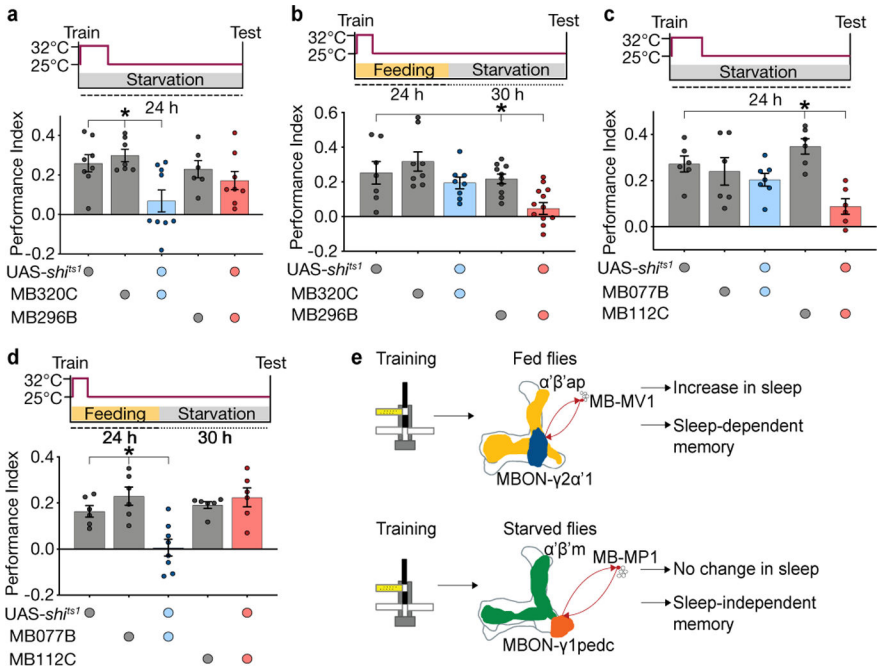

Fig. 3. Feeding drives recruitment of different DANs and MBONs for appetitive memory formation

(**a**) Silencing MB-MP1 (UAS-*shibire*^{ts1}/MB320C), but not MB-MV1 (UAS-*shibire*^{ts1}/MB296B), neurons affects long-term memory in starved flies (One-factor ANOVA with Tukey post-hoc test; n≥6).

(**b**) Neuronal activity in MB-MV1, but not in MB-MP1, DANs is required for long-term memory in flies fed post-training (One-factor ANOVA with Tukey post-hoc test; n≥7).

(**c**) Trained and starved flies show impaired memory when MBON-γ1pedc (UAS-*shibire*^{ts1}/MB112C) neurons are blocked for 4h post-training but remain unaffected if MBON-γ2α'1 (UAS-*shibire*^{ts1}/MB077B) neurons are silenced (One-factor ANOVA with Tukey post-hoc test; n≥6).

(**d**) Long-term memory was lower in fed flies in which MBON-γ2α'1 neurons were silenced post-training (One-factor ANOVA with Tukey post-hoc test; n≥6). Data are represented as mean ± s.e.m. Each data point represents a group of flies. Precise 'n' and 'p' values are in the Source data. Asterisks in (**a-d**) indicate a significant difference between experimental and genetic controls.

(e) Fed flies form sleep-dependent memory which requires activity in α'/β'ap neurons in association with a circuit comprised of MB-MV1 DANs and MBON-γ2α'1. In contrast, α'/β'm neurons with MB-MP1 DANs and MBON-γ1pedc mediate sleep-independent long-term memory in starved flies.