Title: Sleep and memory consolidation are linked by RNA processing genes in the *Drosophila* mushroom body

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Abstract

Memory consolidation in *Drosophila* can be sleep-dependent or sleep-independent, depending on the availability of food. Different regions of the mushroom body (MB) mediate these two mechanisms, with the ap α'/β' neurons required for sleep-dependent memory consolidation in flies that are fed after training. These neurons are also involved in the increase of sleep after training, suggesting a link between sleep and memory. To better understand the mechanisms underlying sleep and memory consolidation initiation, we analyzed the transcriptome of ap α'/β' neurons one hour after appetitive memory conditioning. A small number of genes were differentially expressed specifically in flies fed after training, but not in trained and starved flies or untrained flies. Knockdown of each of these differentially expressed genes in the ap α'/β' neurons revealed multiple genes that affect sleep, with notable effects observed for Polr1F and Regnase-1, both of which decrease in expression after conditioning. Knockdown of Polr1F, a regulator of ribosome RNA transcription, in adult flies promotes sleep and increases pre-ribosome RNA expression as well as overall translation, supporting a function for Polr1F downregulation in memory consolidation. Conversely, knockdown of Regnase-1, an mRNA decay protein localized to the ribosome, reduces sleep. Given that Regnase-1 knockdown in ap α'/β' neurons affects both sleep-dependent and sleep-independent memory, as well as short-term memory, Regnase-1 likely has an early role in the learning process, which may obscure a later function for its downregulation during

sleep-dependent memory. These findings indicate that changes in RNA processing play a crucial role in triggering post-training sleep and memory consolidation.

Background

Sleep is an optimized state for memory consolidation compared to wake (Rasch and Born, 2013). Indeed, sleep is thought to reorganize and strengthen the neural connections required for novel memory formation and long-term memory consolidation, and sleep disruption leads to impaired memory consolidation (Roselli et al., 2021). Beneficial effects of sleep on memory have also been attributed to post-learning neuronal reactivation (Wagner et al., 2007; Dag et al., 2019).

We recently demonstrated that *Drosophila* switch between sleep-dependent and sleep-independent memory consolidation based on food availability (Chouhan et al., 2021). Flies that are fed after appetitive conditioning show sleep-dependent memory consolidation, which is mediated by the anterior posterior (ap) α'/β' neurons of the mushroom body. On the other hand, flies starved after training display sleep-independent memory mediated by medial (m) neurons of the α'/β' lobes. Neurotransmission from ap α'/β' neurons in the first 4 hours after training in the fed flies is not only required for long term memory but also increased sleep post-training. Activation of ap α'/β' neurons also promotes baseline sleep, supporting the idea that post training sleep is triggered by the same neurons that are required for sleep-dependent memory consolidation. However, how sleep and memory consolidation are coordinated in ap α'/β' neurons in this time window is not understood.

Since both post-training sleep and memory consolidation occur rapidly within a few hours after training, we investigated transient gene expression changes in the ap α'/β' neurons after training to elucidate the interplay of sleep and memory consolidation. Here, we profiled the transcriptome of ap α'/β' neurons 1 hour after flies were fed and trained, as well as under control conditions in which flies were starved and trained or fed and untrained. We knocked down the differentially expressed genes in trained and fed flies and identified two RNA processing genes that affect sleep. Knockdown of one of these, Polr1F, a regulator of ribosomal RNA synthesis, promotes sleep and translation, both of which are required for consolidation of memory in trained and fed flies. Knockdown of Polr1F does not affect memory, which is consistent with downregulation of this gene during memory consolidation. Knockdown of Regnase-1, an mRNA decay protein, reduces sleep and disrupts short and long term memory, suggesting an early role, perhaps in learning. These results suggest that RNA processing is an important mechanism linking sleep to memory.

Results

Transient transcriptome profiling of ap α'/β' neurons after training

We previously demonstrated that flies fed after appetitive memory training exhibit increased sleep and form sleep-dependent memory, which is mediated by ap α'/β' neurons of the mushroom body (MB) α'/β' lobes (Chouhan et al., 2021). To address the mechanisms that mediate increases in sleep and consolidation of memory in ap α'/β' neurons, we assayed gene expression changes in ap α'/β' neurons of flies fed after training in an appetitive conditioning paradigm. We crossed ap α'/β' neuron driver *R35B12-Gal4* (BDSC #49822) with *UAS-nGFP* (BDSC #4775) to label all ap α'/β' neurons with nuclear GFP and collected 5-7 day old F1 progeny subjected to three different conditions: Trained-Fed, Trained-Starved, Untrained-Fed as illustrated (**Figure 1A**). The trained-starved flies served as controls for sleep-dependent changes, while flies that were fed but untrained served as controls for training-dependent changes. After one-hour, 50 mixed sex (25 for each sex) fly brains from each condition were dissected, and 500 GFP+ cells were sorted for bulk-RNA sequencing using the protocol described by Hongjie Li et al (Li et al., 2017).

Our analysis of the bulk RNA-seq data revealed that most genes are not altered in ap α'/β' neurons after one hour, so there are no significant global changes observed among the three conditions. The correlation matrix calculated using the top 75% genes showed high similarity between samples, with most values exceeding 0.9 (**Figure 1 – source data 1**). Nonetheless, we did observe a small subset of genes that were rapidly responsive and differentially expressed between the groups. Principal component analysis (PCA) of these differentially expressed genes (DEGs) indicated that samples

from different conditions are separable, and pathway analysis of PCA indicated that transcription and RNA biosynthetic processes were influenced by the training and feeding paradigm (**Figure 1B**). In total, we identified 59 DEGs, of which 56 were downregulated and only three were upregulated in the Trained-Fed condition compared to the control conditions (**Figure 1C, Figure 1 – source data 1**). Gene ontology (GO) analysis of these 59 genes using FlyEnrichr (https://maayanlab.cloud/FlyEnrichr/) indicated that they encode cellular components of the 90S preribosome, Cajal body, DNA-directed RNA polymerase complex, nuclear euchromatin, and condensed chromosome, consistent with the PCA enrichment (**Figure 1 – source data 1**). Our transcriptome results suggest that ribosome biosynthesis and transcription are the initial changes in ap α'/β' neurons of trained and fed flies.

Two genes expressed differentially in ap α'/β' neurons of trained and fed flies predominately affect sleep.

To investigate if any of the 59 DEGs identified in the ap α'/β' neurons of trained and fed flies affect baseline sleep, we knocked down each of the genes using UAS-RNAi lines and screened for their potential effects on sleep. We used the ap α'/β' neuron constitutive driver R35B12-Gal4 line to drive the RNAi constructs and compared the sleep patterns of knockdown flies with those of R35B12-Gal4 and UAS-RNAi control flies (**Figure 2A, B**). Using a cutoff of a 200 min change in sleep relative to each control group, we identified two genes, Polr1F and Regnase-1, that showed significant effects on baseline sleep. Knockdown of Polr1F, a component of RNA polymerase I complex, led to an increase in sleep. Although Polr1F has not been extensively studied in

Drosophila (Marygold et al., n.d.), its human ortholog hRPA43 is part of the multi-subunit protein complex Pol I that regulates the transcription of ribosomal RNA (Beckouët et al., 2011). Knockdown of Regnase-1, an RNA-binding protein that binds to mRNA undergoing active translation and promotes mRNA decay via its ribonuclease activity, in ap α'/β' neurons resulted in a reduction of both nighttime and daytime sleep (**Figure 2A, B**). Further analysis of sleep architecture revealed that knockdown of Polr1F and Regnase-1 did not significantly impact the total activity of flies, while knockdown of either Polr1F and Regnase-1 resulted in increased and decreased average length of sleep episodes, respectively (**Figure 2 – supplemental figure1**).

Given that these two genes reduce expression rapidly in response to training under fed conditions, we next used the inducible pan-neuronal GeneSwitch driver *nSyb-GeneSwitch (GS)* to determine if restricting knockdown of these two genes to the adult stage recapitulates changes in sleep and/or memory. RU486 (mifepristone) was added to normal fly food and transgene expression is induced when flies are loaded into *Drosophila* Activity Monitor (DAM) glass tubes (Robles-Murguia et al., 2019). We crossed the *nSyb-GS* flies with Polr1F or Regnase-1 RNAi flies and transferred the adult F1 progeny to RU486 tubes to induce expression of the RNAi 3-5 hours before dusk, and continuously monitored sleep for 5 days. Knockdown of Polr1F resulted in immediate inactivity and sleep (**Figure 3A-C**). However, with knockdown of Regnase-1, immediate changes in sleep were not noted (**Figure 3 – supplemental figure 1A-C**). This could be due to potential leakiness of the nSyb-GS, such that it reduced sleep even without RU486 treatment, coupled with the fact that sleep before dusk is already quite low. We also analyzed sleep for the next 3 consecutive days from day 3 to day 5

and found that constitutive pan-neuronal Polr1F knockdown increased sleep while Regnase-1 knockdown seemed to have no effect, again perhaps due to the leakiness of *nSyb-GS.* However, it is also possible that Regnase-1 affects sleep differently in different brain areas (**Figure 3D, E; Figure 3 – supplemental figure 1 D, E**). In general, these results indicate that adult specific knockdown of Polr1F promotes sleep while brain-wide adult-specific knockdown of Regnase-1 has limited effect on sleep.

Knockdown of Regnase-1 affects memory consolidation.

We next evaluated the impact of Polr1F and Regnase-1 knockdown on memory consolidation using our olfactory conditioning paradigm (**Figure 4A**). Starved flies were subjected to training to associate an odor with a reward, and then post-training, they were either kept on food vials for sleep-dependent memory consolidation or kept starved to promote sleep-independent memory consolidation. Memory tests were conducted 24 hours after training for starved flies, while fed flies were restarved for 42 hours before testing, as starvation is necessary for memory retrieval (Krashes and Waddell, 2008). We observed that constitutive knockdown of Polr1F in ap α'/β' neurons did not affect sleep-dependent or sleep-independent memory as memory performance was comparable to that of genetic controls (**Figure 4B-C**). These results were consistent with the fact that Polr1F levels typically decrease during memory consolidation. Monitoring of sleep from ZT8 to ZT12 after training at zeitgeber time (ZT) 6 showed that the post-training increase in sleep was also not affected by Polr1F knockdown in ap α'/β' neurons (**Figure 4E**), suggesting that acute downregulation of Polr1F may not be essential to the post-training increased sleep.

On the other hand, Regnase-1 knockdown in ap α'/β' neurons resulted in a significant decrease in long-term memory performance in both fed and starved flies and eliminated the increase in post-training sleep (**Figure 4 B-E**). These findings suggested that Regnase-1 expression in ap α'/β' neurons is necessary for both sleep-dependent and sleep-independent memory consolidation or that it is required for learning or short-term memory formation. Short-term memory tests confirmed that Regnase-1 knockdown flies performed significantly worse than control flies (**Figure 4F**), indicating that Regnase-1 expression is essential for the increase in post-training sleep and for short-term memory, which precedes both sleep-dependent and sleep-independent long-term memory.

Knockdown of Polr1F promotes translation

Since Polr1F knockdown promotes sleep and a 22 amino acid peptide within Polr1F inhibits ribosomal DNA transcription (Rothblum et al., 2014), we predicted that Polr1F acted as a suppressor for ribosomal DNA transcription, and thus knockdown of Polr1F would enhance the transcription and translation of ribosomal RNA and thereby overall protein synthesis. As Regnase-1 is thought to promote decay of mRNAs undergoing translation, its knockdown, or its downregulation following training, might also be expected to promote translation, or at least the translation of its target mRNAs. The role of protein synthesis in long-term memory consolidation is well-established across organisms (Alberini and Kandel, 2015), and nascent rRNA synthesis was also shown to be induced by training and required for memory consolidation in mouse (Allen et al., 2018). We thus used real-time qPCR of total RNA extracted from vehicle control

and RU-treated fly brains to measure how precursor ribosomal RNA (Pre-rRNA) is affected by knockdown of Polr1F. We found that the pre-rRNA level increased significantly in the RU486 induction (RU+) group compared with vehicle control (RU-) group for *nSyb-GS>polr1F RNAi* flies (**Figure 5A**), indicating Polr1F knockdown results in higher levels of pre-rRNA, which is consistent with studies of Polr1F homologs in yeast cells (Thuriaux et al., 1995; Rothblum et al., 2014).

The increasing ribosomal RNA should help translation and so we also used incorporation of puromycin into newly synthesized peptides as a measure of translation after inducing pan-neuronal knockdown of Polr1F. We observed high levels of translation when Porl1F was knockdown pan-neuronally (RU+) compared to the control (RU-) group (**Figure 5B**), indicating that Polr1F suppresses translation, probably by suppressing ribosomal RNA transcription (Rothblum et al., 2014). Thus, it may need to be downregulated after training to support translation and memory. Given that knockdown of Porl1F enhances rRNA synthesis and sleep, there is a question as to whether global alterations in rRNA synthesis impact sleep. However, feeding of rRNA inhibitor (CX5461) failed to produce rapid sleep phenotypes in both fed and starved flies (**Figure 5 – supplemental figure 1**), suggesting that rRNA synthesis may not directly influence sleep. Using puromycin to address effects of Regnase-1 on translation revealed an insignificant slight increase in translation (data not shown); given that Regnase-1 may specifically affect pre-existing translationally active mRNA or may act only on specific target mRNAs, its effects on de novo translation may not be obvious.

Discussion

The anterior-posterior (ap) α'/β' neurons of the mushroom body make critical and privileged contributions to sleep-dependent memory consolidation and post-training sleep (Krashes et al., 2007; Chouhan et al., 2021), but the mechanisms that link memory and sleep in these neurons are not known. To address this gap, we conducted transcriptomic analysis of ap α'/β' neurons from trained and fed flies to identify genes that change rapidly under conditions that drive sleep-dependent memory. By uncovering two RNA processing genes involved in memory and sleep, our transcriptome profiling of ap α'/β' neurons suggests that genes regulating rRNA transcription and translation are altered in the context of sleep-dependent memory consolidation.

RNA processing genes mediate sleep-dependent memory consolidation and sleep.

Many of the 59 DEGs we identified are implicated in RNA processing. Among them, Polr1F and CG11920 affect ribosomal RNA processing, CG5654 is predicted to be part of the 90S pre-ribosome and involved in endonucleoytic cleavages (Herold et al., 2009), WDR79 encodes a small Cajal body specific RNA binding protein, Nup133 encodes a component of nuclear pore complex, Regnase-1 degrades mRNA, and CG18011, CG17568, Koko, CG11398 and Meics are all involved in RNA polymerase II-specific transcription (Di Giorgio et al., 2017; Rogg et al., 2022). The enrichment of RNA processing and translation genes is consistent with the notion that memory consolidation requires transcription and translation (Seibt and Frank, 2012; Alberini and

Kandel, 2015). More importantly, our findings here indicate that alterations of some of RNA processing genes may impact sleep as well.

Polr1F regulates ribosome RNA synthesis and memory.

Learning-induced changes in gene expression in memory-related neurons are often critical for long-term memory formation (Cavallaro et al., 2002; Hoedjes et al., 2015; Tadi et al., 2015). Our findings with Polr1F implicate changes in Pol I transcription during sleep-dependent memory. Polr1F(Rpa43) is predicted to be part of the RNA polymerase I complex and is involved in DNA-dependent RNA polymerase activity/rDNA transcription in yeast, especially for the initiation of ribosomal RNA (Beckouët et al., 2011; Marygold et al., 2020). As suggested by our data, Polr1F has an inhibitory role in the RNA polymerase I complex, which is also consistent with aforementioned study that found a 22 amino acid peptide within Polr1F can inhibit rDNA transcription (Rothblum et al., 2014). Based upon our finding that inducible knockdown of Polr1F rapidly promotes translation, it is likely that the rapid and dramatic decline of Polr1F after training in fed flies serves to increase *de novo* ribosome RNA synthesis. This is consistent with the report that ribosomal RNA is induced by learning and required for memory consolidation in mice (Allen et al., 2018). While we do not know how knockdown of Polr1F promotes sleep, an attractive possibility is that higher translation is a result of elevated sleep. Sleep is thought to promote translation and it is required for sleep-dependent memory consolidation (Seibt and Frank, 2012; Chouhan et al., 2021). Alternatively, increased translation or rRNA synthesis could promote sleep. However, translation is typically thought of as a consequence of sleep rather than a

cause (Zimmerman et al., 2006). Also, rRNA transcription rates remain constant throughout the day in the liver (Sinturel et al., 2017), although it is still possible that these rates vary in particular regions of the brain and affect sleep. The role of rRNA synthesis in *Drosophila* learning and memory has barely been explored, but our work, together with that of Allen et al. (2018), indicates that the well-known requirement for *de novo* protein synthesis during long-term memory consolidation (Jarome and Helmstetter, 2014) includes increased synthesis of ribosomal RNA and protein.

Rapid Regnase-1 inactivation may affect memory through effects on translation

The role of RNA binding protein Regnase-1 in the innate immune response has been extensively studied (Mino et al., 2015; Mao et al., 2017; Wei et al., 2019). However, our study sheds light on a novel function of neuronal Regase-1 in ap α'/β' neurons on sleep and memory. Regnase-1 is an anti-inflammatory enzyme that inhibits mRNA translation during acute inflammatory responses. It localizes to the ribosomes on the surface of the endoplasmic reticulum (ER) and binds to translationally active mRNAs with specialized stem-loop structures at the 3'UTR (Uehata et al., 2013; Mino et al., 2015). When phosphorylated, Regnase-1 is released from the ER (Tanaka et al., 2019). Because functional Regnase-1 binds and degrades its bound mRNA, Regnase-1 inactivation leads to an increase of its target mRNA (Uehata et al., 2013). The target mRNAs of Regnase-1 in immune cells encode proinflammatory cytokines, which can then be expressed when Regnase-1 is inactivated. However, Regnase-1 has also been reported to modulate cytokines and neuronal injury in the microglia in rats (Liu et al., 2016). Regulation of Regnase-1 is usually rapid and transient, and its rapid response to

277 microenvironmental changes, different pathological states and stress are critical for
278 cellular adaption (Mao et al., 2017).

279 Our study reveals that the expression of Regnase-1 changes after training, and
280 constitutive downregulation of Regnase-1 in ap α'/β' neurons reduces sleep and causes
281 deficits in learning and memory consolidation. We suggest that downregulation of
282 Regnase-1 following training in fed flies may be crucial for the promotion of long-term
283 memory, perhaps by promoting translation of specific transcripts. For instance,
284 downregulation of Regnase-1 after training may release a pool of mRNAs that are
285 normally targeted by it for decay and that are important for consolidation of memory. On
286 the other hand, we find that constitutive loss of Regnase-1 impairs sleep-independent
287 memory and also short-term memory, suggesting that it is required early in the learning
288 and memory process, likely for learning. Interestingly, constitutive knockdown of
289 Regnase-1 also reduces sleep and prevents sleep increase after training. The
290 requirement for sleep is linked most to long-term memory rather than to learning, but
291 increases in neural activity after training and cognitive tasks are thought to increase the
292 need to sleep (Wagner et al., 2007; Diekelmann and Born, 2010), so it is possible that
293 the reduced sleep phenotype of the constitutive Regnase-1 knockdown is related to its
294 role in learning.

295 Our study of local molecular changes in ap α'/β' neurons after training highlights
296 how ribosomal RNA transcription and mRNA translation work in concert during the
297 consolidation of sleep-dependent memory. Neurons are regulated to generate short
298 bursts of boosted transcription and boosted translation, which are required for new
299 protein synthesis. How sleep is involved in this boosted protein synthesis process is

300 unclear, but we suggest that sleep affects several steps of the central dogma, and that
301 deleterious consequences of sleep deprivation are also mediated through effects on
302 rRNA transcription and translation. Overall, our findings indicate the importance of
303 maintaining RNA homeostasis for sleep and memory.

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Conflict of interest statement

The authors declare no competing interests.

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Author contributions

Conceptualization, Y.J.L., N.S.C. and A.S.; Methodology, Y.J.L., N.S.C., S.Z. and R.M. Software, Y.J.L; Validation, Y.J.L., N.S.C., S.Z., R.M., J.S., A.K. and Z.F.Y.; Formal analysis, Y.J.L., N.S.C. and R.M.; Investigation, Y.J.L. and N.S.C.; Resources, Y.J.L., N.S.C., S.Z., R.M., J.S., A.K. and Z.F.Y.; Data Curation, Y.J.L., N.S.C. and R.M.; Writing – Original Draft, Y.J.L. and N.S.C. Writing – Review & Editing, Y.J.L., N.S.C. and A.S. Visualization, Y.J.L., N.S.C. and R.M.; Supervision, Y.J.L., N.S.C. and A.S.; Project administration, A.S.; Funding acquisition, A.S.

Figures

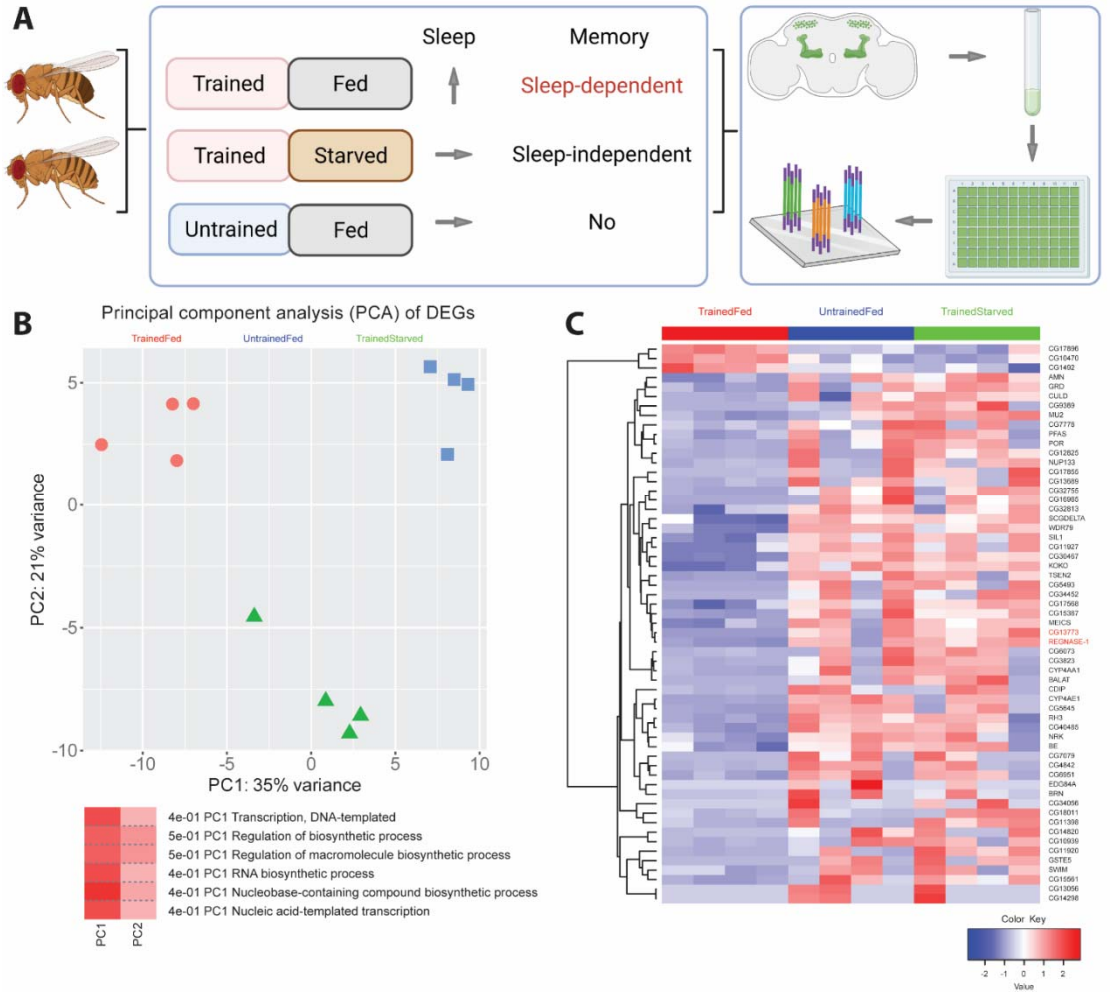

Figure1. Differential gene expression after training in mushroom ap α'/β' neurons. (A) 5-7 day old mixed sex *wCS* flies were exposed to one of the following three conditions: Trained-Fed, Trained-Starved and Untrained-Fed. Only Trained-Fed flies are expected to increase sleep after treatment and thus form sleep-dependent memory (Chouhan et al., 2021). Brain dissection, single cell suspension and cell sorting were used to extract ap α'/β' neurons in each of these three different conditions, and bulk-sequencing of the sorted cells was conducted. (B) We sequenced four samples for each condition and principal component analysis (PCA) analysis of the differentially expressed genes (DEGs) between the three different conditions showed that the samples were separatable from each other and genes responsible for PC1, which accounted for 35% of variance, largely encode proteins involved in transcription and biosynthesis, including RNA biosynthesis, processes. (C) Heatmap of the 59 DEGs including two genes, CG13773 (Polr1F) and Regnase-1, which affect sleep and are the focus of this study. DEGs are identified by DESeq2 with the cutoff of FDR< 0.1 and fold change >1.5.

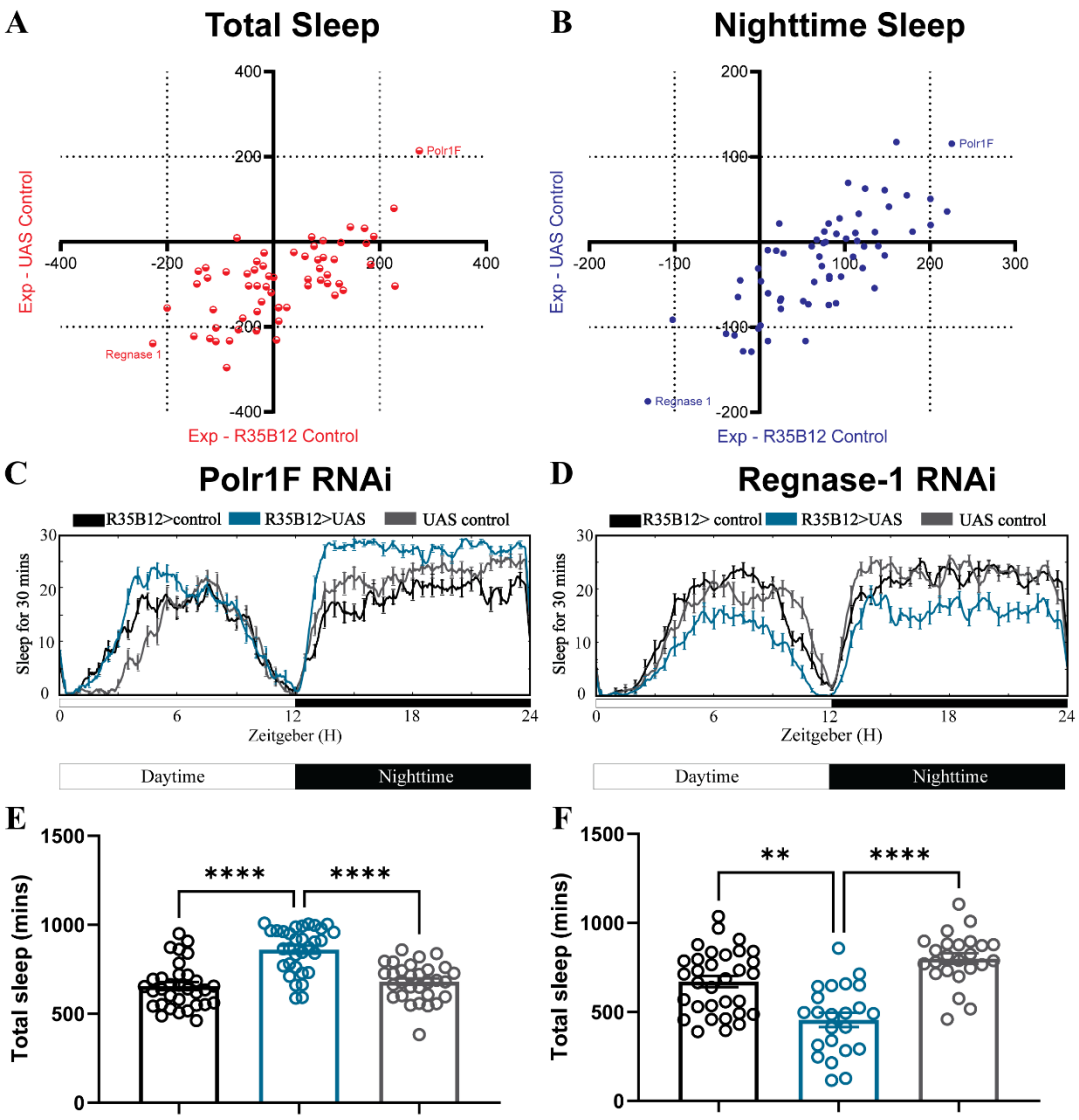

Figure 2. The sleep screen of differentially expressed genes identifies Polr1F and Regnase-1 as sleep-regulating genes. (A-B) Flies carrying the ap α'/β' neuron driver R35B12-Gal4 were crossed with flies carrying UAS-RNAi constructs targeting DEGs identified from RNA-seq analysis. 5-7 days old female F1 progeny were loaded onto Trikinetics DAM monitors to measure their sleep in a 12-hour light: 12-hour dark (12:12 LD) cycle. Mean total sleep (A) and nighttime sleep (B) were calculated by Pysolo and the difference between experimental flies and Gal4 and RNAi controls was calculated separately for each independent experiment; average values comparing each experimental to its Gal4 control (X-axis) and RNAi control (Y-axis) are shown in the plots. Of all the lines screened, knockdown of Polr1F and Regnase-1 had strongest effects on sleep, producing an increase and decrease in sleep respectively. (C-F) show the representative sleep traces of *R35B12-Gal4>polr1F RNAi* flies and *R35B12-Gal4>regnase1 RNAi*. N = 23-32 per genotype from two independent replicates combined are shown in E and F respectively, and bar graphs show mean + SEM. p

453 values for each comparison were calculated using the Kruskal-Wallis test with Dunn's
454 multiple comparisons test. **p<0.01, ***p<0.001, ****p<0.0001.

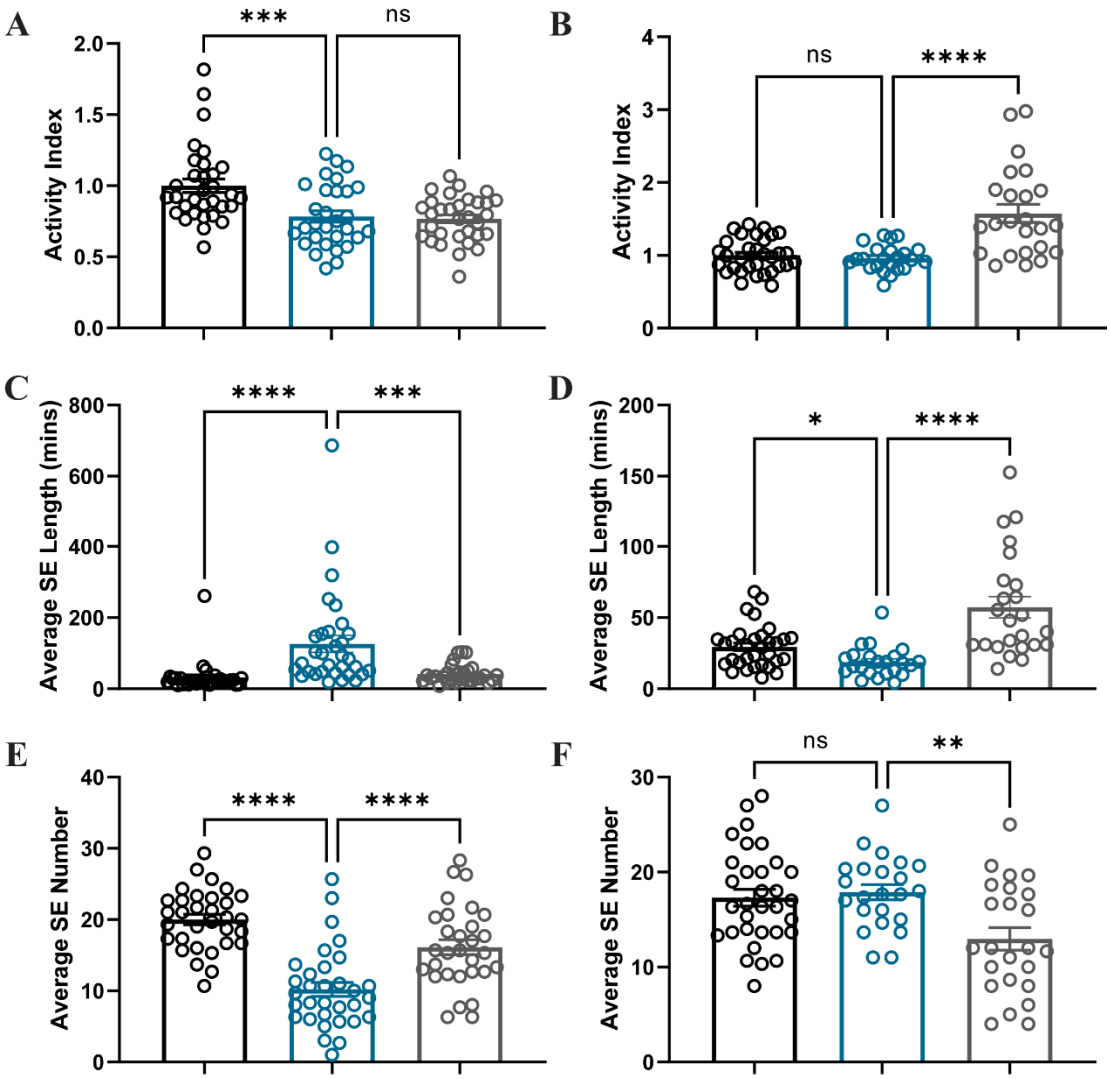

Figure 2 – supplemental figure 1. Effect of Polr1F and Regnase-1 knockdown on activity and sleep architecture. (A-B) Total activity of flies is not altered by knockdown of Polr1F and Regnase-1. One-way ANOVA was used to calculate the p-values for each comparison. (C-D) Knockdown of Polr1F significantly increases the nighttime average sleep episode (SE) length in *R35B12-Gal4>polr1F RNAi* flies, while knockdown of Regnase-1 reduces it in *R35B12-Gal4>regnase1 RNAi* flies. The p-values for each comparison were calculated using the Kruskal–Wallis test with Dunn's multiple comparisons test. (E-F) Knockdown of Polr1F significantly reduces the nighttime average sleep episode (SE) number in *R35B12-Gal4>polr1F RNAi* flies, but it is not significant compared to control groups in *R35B12-Gal4>regnase1 RNAi* flies. The p-values for each comparison were calculated using the Kruskal–Wallis test with Dunn's multiple comparisons test. ns = not significant, $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

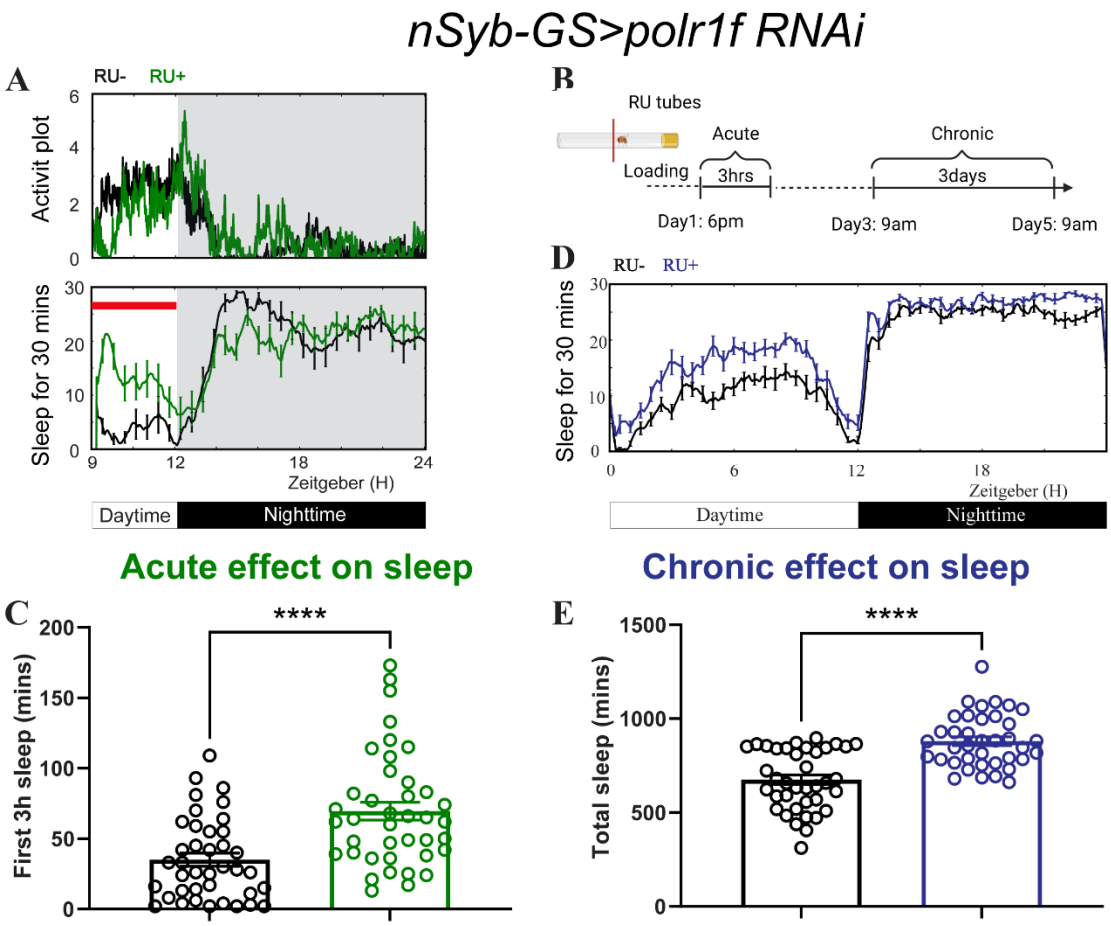

Figure 3. Acute and chronic effects of pan-neuronal knockdown of Polr1F on sleep in adult flies. (A) Representative sleep traces and transient activity plot of flies expressing Polr1F RNAi under the control of an inducible pan-neuronal driver (*nSyb-GS>polr1f RNAi*) with and without RU treatment. (B) Schematic representation of transient and chronic sleep measurements in *nSyb-GS>polr1f RNAi* flies. (C) Quantification of sleep during the first 3 hours (ZT 9-12) after F1 progeny flies were loaded into RU- or RU+ DAM tubes at ZT8-T9. Sleep was measured starting at ZT9. N = 39-40 individual flies per replicate with data from three independent replicates combined. The Mann-Whitney test was used to compare RU+ group and RU- groups. (D) Representative average sleep traces of *nSyb-GS>polr1f RNAi* in the RU- and RU+ DAM tubes for three consecutive days. Chronic sleep effects of pan-neuronal knockdown Polr1F were measured based on sleep data from day 3 to day 5. (E) Quantification of average total sleep of *nSyb-GS>polr1f RNAi* and controls in the DAM tubes from (D). Unpaired t-test was used to compare between RU- and RU+ groups. ****p<0.0001.

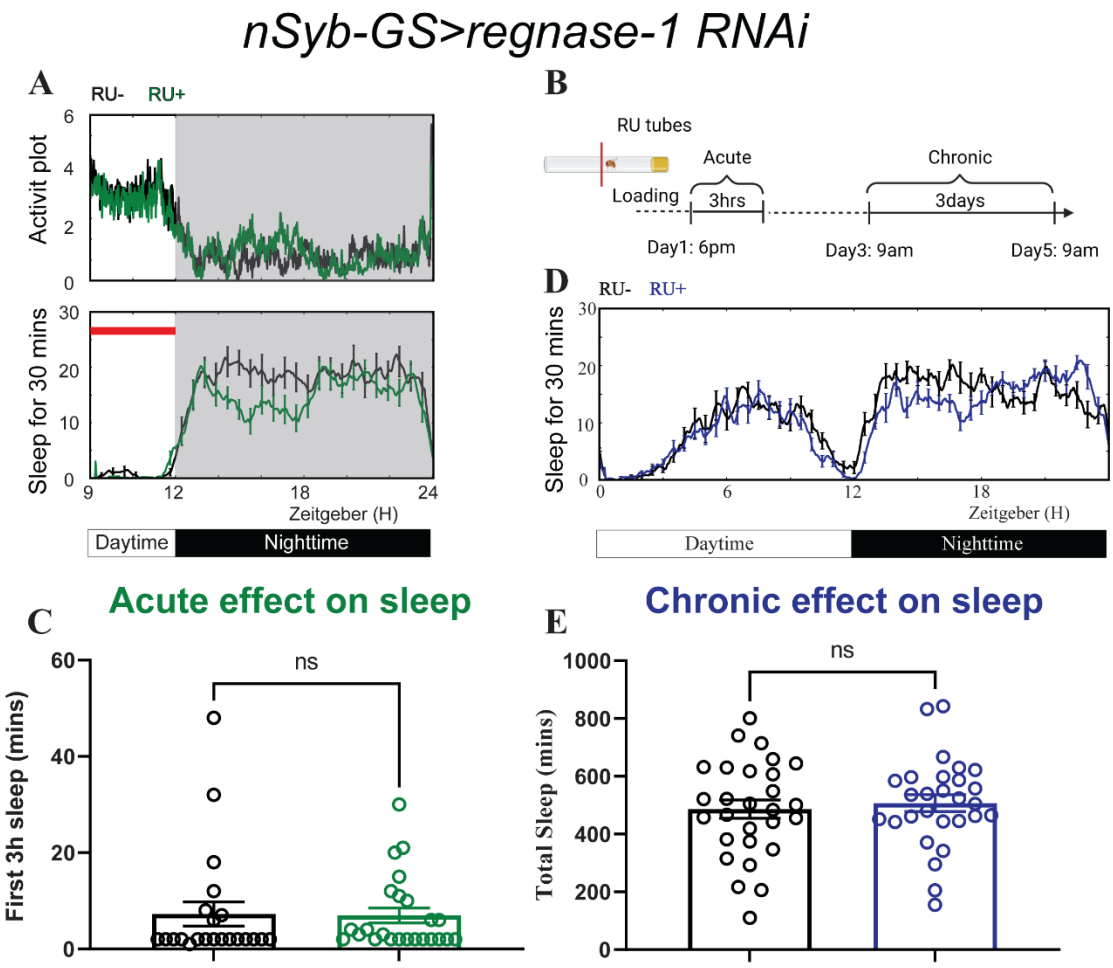

Figure 3 – supplemental figure 1. Acute and chronic effects of pan-neuronal knockdown of Regnase-1 on sleep in adult flies. (A) Representative sleep traces and transient activity plot of flies expressing Rengase-1 RNAi under the control of an inducible pan-neuronal driver (*nSyb-GS>regnase-1 RNAi*) with and without RU treatment. (B) Schematic representation of transient and chronic sleep measurements in *nSyb-GS>regnase-1 RNAi* flies. (C) Quantification of sleep during the first 3 hours (ZT 9-12) after F1 progeny flies were loaded into RU- or RU+ DAM tubes at ZT8-T9. Sleep was measured starting at ZT9. N = 22-24 individual flies per replicate with data from two independent replicates combined. The Mann-Whitney test was used to compare RU+ group and RU- groups. (D) Representative average sleep traces of *nSyb-GS>regnase-1 RNAi* in the RU- and RU+ DAM tubes for three consecutive days. Chronic sleep effects of knockdown of Regnase-1 were measured based on sleep data from day 3 to day 5. (E) Quantification of average total sleep of *nSyb-GS>regnase-1 RNAi* and controls in the DAM tubes from (D). Unpaired t-test was used to compare between RU- and RU+ group. ns = not significant.

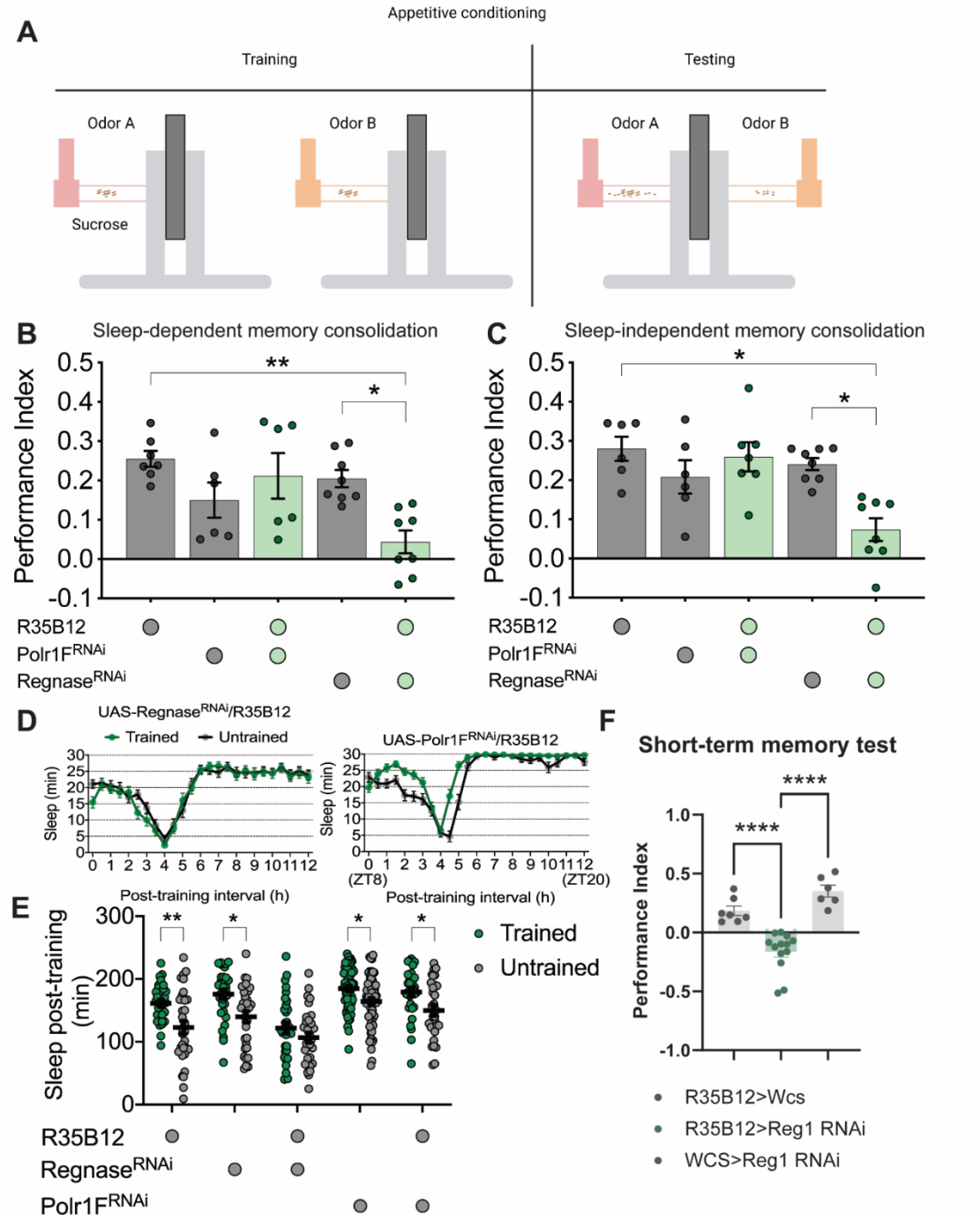

Figure 4. Regnase-1 expression is essential for sleep-dependent and sleep-independent memory. (A) Schematic representation of the memory test protocol. (B, C) Sleep-dependent and sleep-independent memory tests were conducted under fed and starved conditions, respectively. Knockdown of Regnase-1 significantly reduces long-term memory performance in both fed and starved flies. However, knockdown of Polr1F in ap α'/β' neurons does not affect long-term memory performance. N≥6 biological replicates, each replicate containing 100-150 flies. (D, E) Fed UAS-regnase-1-RNAi/+ and R35B12/+ flies exhibit a significant increase in sleep after training, while *R35B12-*

514 *Gal4>regnase-1 RNAi* flies fail to show a comparable increase in post-training sleep.
515 The total sleep in the ZT8-ZT12 interval is shown in (E). Polr1F knockdown in ap α'/β'
516 neurons does not affect the post-training increase in sleep. N≥32. (F) Compared to
517 R35B12-Gal4/+ and +/UAS-Regnase-1 RNAi flies, *R35B12-Gal4>reganse-1 RNAi* flies
518 show a significant decrease in the performance index in short-term memory. N≥6
519 biological replicates, each containing 100-150 flies. ns=not significant, p>0.05, *p<0.05,
520 **p<0.01, ****p<0.0001.

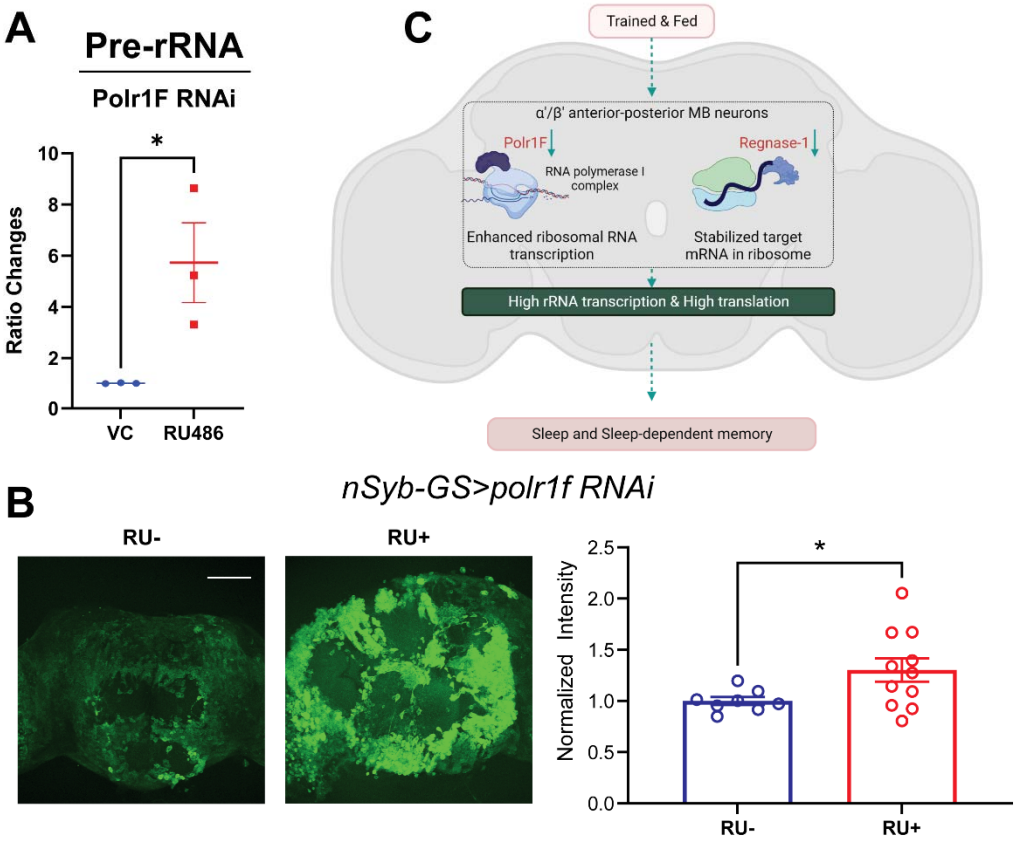

Figure 5. Knockdown of Polr1F results in high translation. (A) The *nSyb-GS>polr1f RNAi* flies exhibit a significant increase in pre-rRNA levels. (B) The ex-vivo puromycin immunostaining assay was used to measure translation in dissected whole brains. The results show that knockdown of Polr1F using the pan-neuronal nSyb-GeneSwitch (GS) system increases translation relative to control flies that were not treated with RU. The normalized mean grayscales from the RU- and RU+ groups are compared using an unpaired t-test. The analysis includes data from 8-11 flies per group, with results from two independent replicates combined. (C) The schematic model illustrates the roles of Polr1F and Regnase-1 in memory consolidation. The genes Polr1F and Regnase-1 are prominently downregulated during memory consolidation in trained and fed flies, respectively. Polr1F is involved in regulating ribosomal RNA synthesis, and its decrease in levels in trained and fed flies promotes sleep and translation. In contrast, Regnase-1 is involved in mRNA decay, and its downregulation during memory consolidation may contribute to the stabilization of mRNAs that encode proteins important for long-term memory formation.

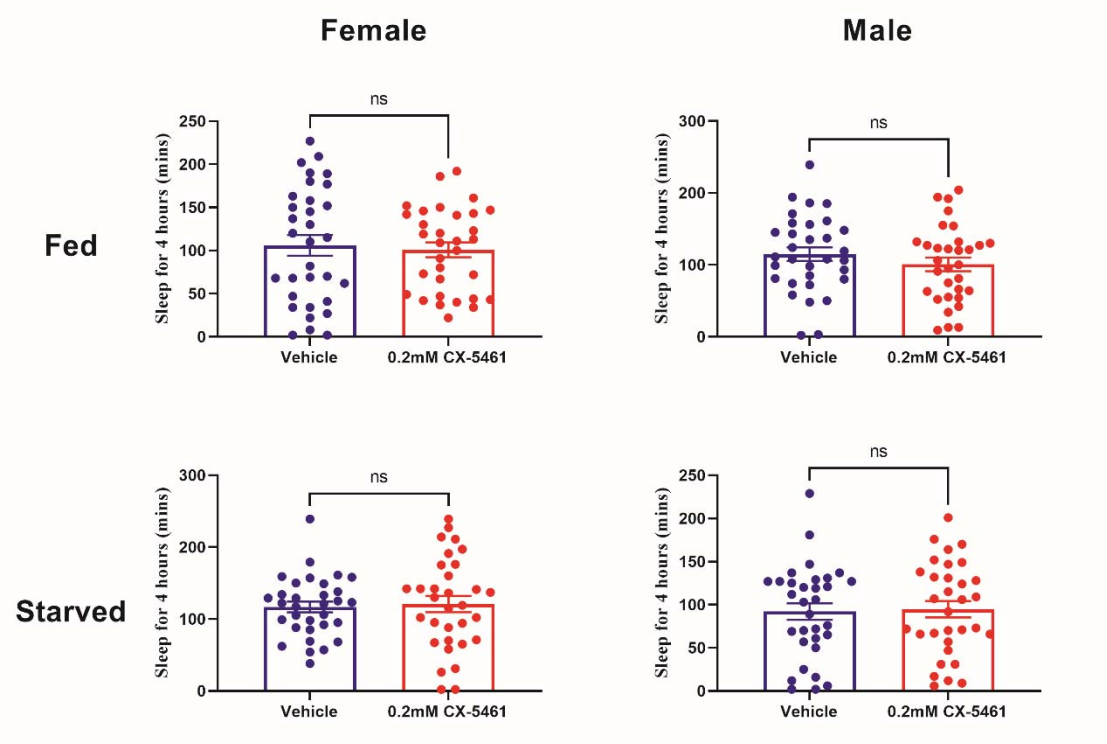

Figure 5 – supplemental figure 1. rRNA inhibitor (CX-5461) feeding does not affect sleep. Feeding of the ribosome RNA inhibitor CX-5461 at a concentration of 0.2mM does not affect sleep in fed or starved flies. Statistical analysis shows that there is no significant difference between CX-5461-fed and control flies. The sample size is N=32 per group from two independent replicates. ns, not significant).

Materials and Methods

Contact for reagent and resource sharing.

Amita Sehgal (amita@pennmedicine.upenn.edu)

Key resource table

REAGENT or RECOURCE	SOURCE	IDENTIFIER
Schneider's medium	Thermofisher	21720024
Papain	Worthington PAP2	LK003178
Liberase	Roche	5401119001
DAPI	Thermofisher	62247
Antibodies		
Anti-Puromycin [3RH11] Antibody	Kerafast	EQ0001
Chemicals, Peptides, and Recombinant Proteins		
Puromycin dihydrochloride	Santa Cruz	Sc-108071A
RNA Polymerase I Inhibitor II, CX-5461	Sigma	5092650001
Critical Commercial Assays		
RNeasy Plus Mini Kit	Qiagen	Item No. 74134
Experimental Models; Organisms/Strains		
white Canton-S (w*CS*)	Laboratory Stocks	
nSyb-GS^{14}	Laboratory Stocks	
R35B12-Gal4	BDSC	49822
UAS-nGFP	BDSC	4775
R26E01-Gal4	BDSC	60510
Oligonucleotides		
Pre-rRNA oligo:		N/A
F: ATG GCC GTA TTC GAA TGG ATT TA	This paper	N/A
R: CTA CTG GCA GGA TCA ACC AGA	This paper	N/A
Polr1F oligos:		N/A
F: TGC TAG AGA ATG GCG AAG C		N/A

R: GGA CTG CCA AAC TTA ATG GAT TT		N/A
Tubulin oligo		N/A
F: CGT CTG GAC CAC AAG TTC GA	(Xu et al., 2008)	N/A
R: CCT CCA TAC CCT CAC CAA CGT	(Xu et al., 2008)	N/A
Software and Algorithms		
GraphPad Prism v9	GraphPad Software	https://www.graphpad.com/
DAMFileScan113	Trikinetics	https://trikinetics.com/
Pysolo	Giorgio Gilestro et al., 2009	https://www.pysolo.net/about/
Adobe Illustrator 2020	Adobe	https://www.adobe.com/
BioRender	BioRender	https://biorender.com/

Fly stock and maintenance

All the stock information of the flies used in this project are listed in the key resource table and flies were reared on the standard cornmeal vials or bottles at 25 °C with 12:12 hours light dark cycle in the preset incubator. The genetic background control used in the paper is White-CantonS (wCS) unless specified.

Behavior measurement in *Drosophila*

We have used both single beam and updated multibeam *Drosophila* activity monitoring (DAM) system from Trikinetics (https://trikinetics.com/) in our experiments. Briefly, 5-7 days old female flies were loaded into 60/90 mm glass locomotor tubes for behavior tests, using DAM2/5H *Drosophila* activity monitors from Trikinetics. 1/15 infrared beams bisect each tube, providing movement (position in multibeam) information of the fly across the tube. Locomotor tubes are loaded with 2% agar with 5% sucrose as fly food on one side, and yarn is put on the other side to restrain the behavior of flies inside the glass tubes. For experiments with the inducible Gene Switch system, 0.5mM RU-486 (mifepristone) was added to the fly food to activate the expression of the transgenes

under the control of UAS. Three constitutive days of data were used for sleep analysis by Pysolo (https://www.pysolo.net/).

Appetitive conditioning

~100 4-7 old flies were starved for 12 hours in *Drosophila* bottles with water -soaked filtered paper and then trained at 25°C and 70% relative humidity to associate sucrose with odor A for 2 minutes, and then a blank with odor B for 2 minutes with 30-second clean air in between. After conditioning, flies were moved back to normal fly food or starved for 1 hour and dissected for subsequent ap cell sorting and RNA-sequencing. For the short-term memory test, flies were tested immediately after conditioning in the same wheel for 2 mins.

To assess post-training sleep, flies were introduced in glass tubes containing 2% agar and 5% sucrose through an aspirator without anesthesia and loaded into the DAM system after training. For long-term memory assessment, trained flies were either kept on food vials for 24 h or were further starved. Starved flies were tested for memory 24 h after training, while fed flies were re-starved for 42 h before memory tests. Memory was tested by giving flies a choice between odor A and odor B for 2 min in a T-maze. 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.

590 **Cell isolation and sorting**

591 Dissected brains are dissociated by following the protocol from (Li et al., 2017). Briefly,
592 brains are dissected in Schneider's medium, and then are placed in a shaker and
593 dissociated in Papain solution, filtered through a 100 μm cell strainer, and re-suspended
594 in Schneider's medium. 500 GFP+ cells from the same conditions were sorted into 96
595 well microplate with lysis buffer from Smart-seq2 HT kit and frozen. We dissected 50
596 brains for each group to ensure enough GFP+ cells. Cell sorting were conducted by
597 either BD FACSMelody or BD FACSAria (BD Biosciences), and dead cells were
598 excluded with 4', 6-diamidino-2-phenylindole (DAPI). Doublets were excluded using and
599 forward scatter (FSC-H by FSC-W) and side scatter (SSC-H by SSC-W). Size of cells
600 was selected by FSC-A by FSC-A and validated for fly neurons using cells from flies
601 expressing nsyb-nGFP. Length of time from tissue harvest to cell collection
602 approximated 4 hours.
603
604 **RNA-seq and data analysis**
605 GFP+ cells were sorted and immediately frozen, then sent to Admera Health
606 (https://www.admerahealth.com/) for RNA extraction, RNA library construction, and
607 sequencing using the Smart-seq2 HT kit. To analyze the RNA-sequence data, we used
608 Hisat2 (http://daehwankimlab.github.io/hisat2/) to map the sequencing data FASTQ files
609 to the fly genome (BDSG6). The alignment results were then counted by LiBiNorm
610 (https://warwick.ac.uk/fac/sci/lifesci/research/libinorm/) using the GENCODE reference
611 genome. Raw count and TPM were used separately in further analysis. Raw count data
612 were analyzed by IDEP v0.95 to identify genes expressed differentially between three

conditions. We filtered out low-expressed genes using a cutoff of CPM>0.5, at least detected in 3 independent samples, and treated missing values as gene median. Regularized log transformation was used to transform raw count data for clustering and PCA. Differentially expressed genes were identified using DESeq2 with an FDR cutoff of 0.1 and minimum fold change of 2.

Puromycin assay and imaging

We developed a puromycin assay to measure the rate and localization of nascent peptide synthesis in the fly brain, and similar method has been described in the fly larvae (Deliu et al., 2017). Fly brains were dissected in Schneiders' medium and incubated with puromycin for 40 min *in vitro* to allow puromycin to incorporate into the newly synthesized peptide. Subsequently, using an anti-puromycin antibody, standard immunostaining protocols were applied to detect the number and position of newly synthesized puromycin-tagged peptides (Aviner, 2020), which provided a measure of translation rate. The brains were then imaged with a 40X oil immersion confocal microscope with a resolution of 1024*1024. The intensity of the images was then measured by FUJI (ImageJ) and the average intensity of the samples was analyzed for comparison.

RNA polymerase I inhibitor II, CX-5461 protocol

CX-5461 was mixed with 2% agar and 5% sucrose to make fly motor tubes at a final concentration of 0.2 mM or 0.6 mM. Flies are loaded into these CX-5461 tubes or

vehicle control tubes 4 hours before light turns off and transient sleep changes are measured.

Quantitative Real-time PCR (qPCR)

10 flies' brains from each group were dissected for RNA extraction using Qiagen's RNeasy Plus Mini Kit. Total RNA was then reverse transcribed to cDNA by using random hexamers and Superscript II from Invitrogen. qPCR was performed using SYBR-green master mix and oligonucleotide information is provided in the Key Resources table. Relative gene expression was analyzed using the ΔΔCt method.

Statistical Analysis

Fly sleep behavioral data extracted from Pysolo was analyzed by GraphPad Prism (https://www.graphpad.com/). Data from different replicates were pooled directly and first tested for normality using D'Agostino-Pearson and Shapiro-Wilk tests. For normally distributed data, unpaired parametric Student's t-test is used for two-sample experiments and one-way ANOVA with Turkey post hoc test for three-sample or more experiments. Non-normally distributed data were analyzed using nonparametric tests, the Mann-Whitney test for two-sample experiments and the Kruskal-Wallis test with Dunn's multiple comparisons test for three or more samples experiments. For all graphs, unless otherwise stated, data are presented as mean and standard error of the mean (SEM) and statistical significance was accepted for p value < 0.05.

Data availability

Sequencing data will be available at GEO. All other data for this study are included in the manuscript and supporting files.

Supplemental information

Supplemental information includes 2 figures and 1 table.