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Sleep restores behavioral plasticity to *Drosophila* mutants

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SUMMARY

Given the role that sleep plays in modulating plasticity, we hypothesized that increasing sleep would restore memory to canonical memory mutants without specifically rescuing the causal molecular-lesion. Sleep was increased using three independent strategies: activating the dorsal Fan Shaped Body (FB), increasing the expression of *Fatty acid binding protein* (*dFabp*) or by administering the GABA-A agonist 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol (THIP). Short-term memory (STM) or Long-term memory (LTM) was evaluated in *rutabaga* (*rut*) and *dunce* (*dnc*) mutants using Aversive Phototaxic Suppression (APS) and courtship conditioning. Each of the three independent strategies increased sleep and restored memory to *rut* and *dnc* mutants. Importantly, inducing sleep also reverses memory defects in a *Drosophila* model of Alzheimer's disease. Together these data demonstrate that sleep plays a more fundamental role in modulating behavioral plasticity than previously appreciated and suggests that increasing sleep may benefit patients with certain neurological disorders.

INTRODUCTION

While the function of sleep remains a mystery, theories on sleep function, including synaptic downscaling [1], memory consolidation [2, 3], developmental maturation [4–6], removing undesirable neuronal interactions [7] and even many theories on sleep restoration [e.g. [8, 9]], require that sleep must influence aspects of plasticity in the brain. Plasticity, refers to the process of modifying the connectivity between neurons and neuronal circuits. Importantly,

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neuronal plasticity also includes alterations in functional connectivity in which distinct components of a neuronal circuit can be dynamically substituted and reconfigured in response to an individual's environment and historical context [10]. Thus, while some of the theories on sleep function appear on the surface to be contradictory, together they all indicate that modulating plasticity may be a fundamental property of sleep.

With this in mind, we set out to test the hypothesis that sleep could reverse cognitive deficits in two canonical memory mutants, the adenylyl cyclase mutant *rutabaga* (*rut*) and the *phosphodiesterase* mutant *dunce* (*dnc*). Although both *rut* and *dnc* were originally identified using aversive olfactory conditioning [11, 12], mutations in both genes show deficits in a surprisingly wide variety of behavioral assays [13–24] and are also deficient in several aspects of neuronal plasticity [25–30]. In addition, we evaluated a *Drosophila* model of familial Alzheimer's disease to assess the potential use of sleep as a therapeutic treatment for certain neurological disorders.

RESULTS

Characterization of a sleep promoting compound in flies

To evaluate whether sleep might restore STM to memory mutants, we considered multiple independent approaches of inducing sleep in flies. Although genetic tools that increase sleep are available, pharmacological methods to increase sleep are currently lacking [31, 32]. Thus, we began by evaluating the sleep promoting properties of several compounds including ethanol (10%), the gamma-aminobutyric acid GABA-B agonist SKF97541 (40μM), the vesicular monoamine transporter inhibitor reserpine (20μM) and the GABA-A agonist 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol (THIP (0.1mg/mL). As seen in Figure 1A, these compounds significantly increases quiescence in wild-type *Canton-s (Cs)* female flies. Identifying a compound that increases sleep but does not also produce negative side-effects is non-trivial [33, 34]. To determine whether pharmacologically induced quiescence could improve or impair STM we evaluated performance using an operant visual learning paradigm, the APS [13, 35]. In the APS, flies are individually placed in a T-maze and allowed to choose between a lighted and darkened chamber over 16 trials. During 16 trials, flies learn to avoid the lighted chamber that is paired with an aversive stimulus (quinine, and humidity in non-thirsty flies [36]). The performance index is calculated as the percentage of times the fly chooses the dark vial during the last 4 trials of the 16 trial test. We found that quiescence induced by 10% ethanol, 40μM SKF97541 and 20μM reserpine also produced deficits in STM when assessed using APS; no alterations in STM were observed for flies maintained on 0.1mg/mL of THIP (Figure 1B). To determine whether higher doses of THIP might disrupt performance, STM was evaluated in *Cs* flies after receiving a 5-fold increase in the dose of THIP (0.5mg/mL); performance was not impaired (data not shown). Similarly, lower doses of SKF97541 and the γ-hydroxybutyric acid (GHB, a GABA-B agonist) precursor 1,4-butanediol [37], which are only able to modestly alter quiescence, still produced deficits in performance (data not shown). Thus, of the compounds evaluated only the GABA-A agonist THIP did not disrupt STM.

Is the quiescence induced by THIP really sleep? To answer this question we evaluated whether THIP-induced quiescent episodes met the historical criteria for identifying sleep

[38]. Female *Cs*, w^{1118} and *Oregon-R (Ore-R)* flies were maintained on 0.025mg/mL, 0.05mg/mL and 0.1mg/mL of THIP. As seen in Figure 1C and Figure S1A, THIP increased quiescence in a dose-dependent fashion. The increase in quiescence is characterized by an increase in the consolidation of quiescent bouts during the day (Figure S1B). Importantly, THIP does not impair locomotor activity (Figure S1C). Next we evaluated arousal thresholds and rapid reversibility [31, 39]. As seen in Figure S1D, flies rapidly awake in response to a strong perturbation. THIP fed flies also displayed increased arousal thresholds (Figure S1E). To determine if quiescence induced by THIP was homeostatically regulated, vehicle-fed and THIP-fed *Cs* flies were sleep deprived for 12 h. As seen in Figure S1F, THIP-fed flies displayed a sleep rebound similar to their vehicle fed siblings. Thus, the quiescence induced by THIP meets the historical criteria for sleep [40, 41].

While it is important to meet the behavioral criteria for sleep, it is equally important to determine whether a period of quiescence can play a role in molecular and physiological processes previously shown, or hypothesized, to be the domain of sleep [31]. Thus, we evaluated transcripts previously shown to be modulated by sleep and waking in flies including *Amylase*, transcripts associated with synaptic function, and those involved in the immune response [42–45]. As seen in Figure 1D, sleep deprivation increases these transcripts; conversely increasing sleep with THIP reduces them. Similarly, sleep deprivation increases synaptic proteins, including DISCS-LARGE (DLG)[44], while sleep induced by THIP reduces DLG protein levels (Figure 1E). To confirm that THIP was not producing a state incompatible with sleep, we evaluated its effects on lifespan. As seen in Figure S2A, lifespan was not altered in flies chronically maintained on THIP. Finally, we recorded local field potentials (LFPs) from flies during spontaneous sleep and sleep following THIP administration to determine if THIP was inducing aberrant brain activity patterns. As seen in Figure 1F, THIP does not result in abnormal brain activity and THIP feeding does not alter brain activity during waking (Figure S2B). Importantly, THIP-induced sleep resembles spontaneous sleep in flies: it is associated with a uniform decrease in spectral power across all frequencies (Figure 1G; Figure S2B,C)[46]. These data favor the interpretation that THIP-induced sleep shares molecular and physiological characteristics with spontaneous sleep.

Previous studies have shown that hypnotics that do not distort electrophysiological signals may nonetheless impair plasticity [33]. Thus, we asked whether THIP-induced sleep would provide some of the same functional benefits as sleep. We have shown that a single 3-hour training protocol (Massed Training, MT) is insufficient to produce LTM in a courtship conditioning assay [31]. However, when MT is followed by 4 h of genetically induced sleep flies exhibit an LTM [31]. Therefore, we exposed naïve adult *Cs* male flies to MT and then fed them either vehicle or 0.1mg/mL of THIP for 4 h. Courtship was tested in all groups 48 h after training (Figure 1H). Vehicle-fed flies did not change their courtship following MT resulting in a low Performance Index (PI) (Figure 1I, black) [31]. However, increasing sleep by placing flies on THIP for 4 h immediately following training significantly reduced courtship yielding a significantly higher PI than vehicle-fed siblings (Figure 1I, white). To determine whether a 4 h period of quiescence following MT would be sufficient to induce LTM, we placed flies on the GABA-B agonist SKF97541 for 4 h using the same protocol.

As seen in Figure 1J, inducing quiescence with SKF97541 following MT does not result in LTM. Importantly, no differences in sleep were observed in either THIP-fed or SKF97541-fed flies at the time of testing indicating that the differences in LTM are unlikely due to persistent changes in sleep (Figure S2D). Thus, sleep during THIP administration provides the same functional benefits to LTM as genetically induced sleep [31].

To investigate how THIP modulates sleep in flies, we used an RNA interference strategy to knock down each of the six *Drosophila* GABA receptors. *Drosophila* express three ionotropic GABA-A receptors, *resistance to dieldrin* (*Rdl*), *Ligand-gated chloride channel homolog 3* (*Lcch3*), and *GABA and glycine-like receptor of Drosophila* (*Grd*), and three metabotropic GABA-B receptors (*GABA-BR1*, *GABA-BR2* and *GABA-BR3*) [47, 48]. We screened several GAL4 lines and found that knockdown of *Lcch3* and *Grd* using *BG380-GAL4; UAS-Dcr2* and *UAS-Dcr2;30y–GAL4* drivers attenuated the sleep-promoting effects of THIP (Figure S3A,B,C); knocking down GABA receptors in *BG380* and *30y* expressing cells does not modify baseline sleep (Figure S3D). The efficacy of the RNAi mediated knockdown is shown in Figure S3E. Importantly, knockdown of *Lcch3* in *BG380-GAL4* expressing cells prevented LTM following THIP administration (Figure S3F–H). These data suggest that THIP induces sleep through the *Lcch3* and *Grd* receptors. Alternatively, reducing GABA receptor signaling may result in excitation of the CNS which could overcome potential depressant effects of THIP independently of its effects on a specific GABA receptor.

Inducing sleep in *rutabaga* mutants restores STM and LTM

Before determining whether sleep could restore STM in *rutabaga* mutants, we asked whether THIP-induced sleep would enhance STM in wild-type flies in the APS. As seen in Figure 2A, performance is remarkably consistent in several common background strains including *Cs*, w^{1118}, *Ore-R*, ry^{506} and *Berlin* flies (Figure 2A). Importantly, THIP-induced sleep does not enhance performance further (Figure 2A). THIP does not affect photosensitivity or quinine sensitivity, two important sensory modalities that might influence performance in the APS (Table S1). Thus, THIP does not produce super-learning flies and does not alter waking sensory thresholds when tested in diverse genetic backgrounds.

Can THIP-induced sleep reverse performance impairments in *rutabaga* mutants (rut^{2080} and rut^{1}) compared to their vehicle-fed siblings? Both rut^{2080} and rut^{1} displayed normal sleep and each mutant allele increased sleep in response to THIP (Figure S4 A – C). As seen in Figure 2B, vehicle-fed rut^{2080} flies exhibit STM deficits. However, STM is restored in rut^{2080} siblings following 2 days of THIP-induced sleep (Figure 2B). To determine whether the improvements in performance were due to increases in sleep *per se* or due to non-specific effects of the drug, rut^{2080} males were sleep deprived while on THIP. We assessed food intake during sleep deprivation by placing flies on blue dye to confirm that they continued to consume THIP. Consistent with previous reports, food intake did not differ from non-sleep deprived controls (data not shown) [49]. Importantly, THIP did not restore STM in the absence of sleep (Figure 2B). THIP did not alter photosensitivity or quinine sensitivity in rut^{2080} mutants indicating that the improved performance in the APS is not due

to changes in sensory thresholds (Table S1). To determine if the improved STM seen in *rut*^{2080} flies was unique to this mutation, we evaluated an additional *rutabaga* mutant allele (*rut*^{1}). As seen in Figure 2C, 2-days of THIP-induced sleep restored STM in *rut*^{1} flies when compared to their vehicle-fed siblings; no improvements in STM were seen in the absence of sleep. THIP did not alter photosensitivity or quinine sensitivity in *rut*^{1} mutants (Table S1). Thus, THIP-induced sleep can restore STM to the adenylyl cyclase mutant *rutabaga*.

Our experiments were designed to evaluate the effects of sleep-induction on age-matched siblings when compared to their vehicle-fed controls. However, we wished to know whether sleep could benefit an individual fly. Thus, we evaluated STM in individual male *rut*^{2080} flies tested on two trials spaced 2 days apart. As seen in Figure 2D, only 20% (2/10) of *rut*^{2080} mutants display STM during trial-1 and their performance was similar during trial-2. Since repeated trials do not improve STM in individual *rut*^{2080} flies, we evaluated STM before and after sleep induction in an independent cohort of flies. As seen in Figure 2E, 0% (0/9) of vehicle-fed *rut*^{2080} mutants exhibited STM during trial 1 indicating that theses flies were impaired. However, 77% (7/9) of *rut*^{2080} flies displayed STM after 2-days of THIP-induced sleep (Figure 2E, mean ± SEM shown in Figure 2F). Thus, THIP-induced sleep can restore STM to individual *rutabaga* mutants.

Finally, we used an RNAi approach to knockdown *rutabaga* in adult flies using the GeneSwitch system [50]. As seen in Figure 2G,H, RU486 (RU)-fed parental controls exhibited normal STM compared to vehicle-fed siblings (veh); 2 additional days of THIP administration (RU$^{0.1T}$, veh$^{0.1T}$) did not enhance STM further. In contrast, *DaGsw/+>UAS-rut*^{RNAi}*/+* flies fed RU for 2 days exhibited impaired STM compared to vehicle-fed siblings (Figure 2I). Importantly, the STM deficits were reversed when *DaGsw/+>UAS-rut*^{RNAi}*/+* flies were maintained on RU for 2 days and then switched to food containing RU and 0.1mg/mL THIP for an additional two days (RU$^{0.1T}$) (Figure 2I). THIP did not alter photosensitivity or quinine sensitivity in *DaGsw/+>UAS-rut*^{RNAi}*/+* or their parental controls (Table S1).

How long must flies sleep before they display an improvement in STM , and how long do the STM improvements persist? We evaluated performance in *rut*^{2080} males after sleep was induced for 48 h, 24 h and 12 h. As seen in Figure 2J, *rut*^{2080} males require 24 h of sleep before they exhibit STM. When *rut*^{2080} males were maintained on THIP for 48 h, they maintained their improved STM 48 h after being removed from THIP even though sleep had returned to baseline (Figure 2K, Figure S5A). These data indicate that flies require a certain amount of sleep to restore brain function and that the benefits persist for several days.

To rule out the possibility that the improvement in STM was not related to sleep, we used an alternate strategy to increase sleep by genetically activating the sleep-promoting dorsal Fan Shaped body neurons in *rut*^{2080} mutants. *rut*^{2080} was combined with *104y–GAL4* and *C5-GAL4* as well as to *UAS-NaChBac*, a bacterial sodium channel that increases neuronal excitability [31, 51]. *rut*^{2080}*;104y/+>UAS-NaChBac/+* males displayed increased sleep compared to their parental controls (data not shown). Importantly, STM is impaired in parental controls (Figure 2L black bars). In contrast, when sleep was enhanced by activating the FB, both *rut*^{2080}*;104y/+>UAS-NaChBac/+* and *rut*^{2080}*;C5/+>UAS-NaChBac/+* males

displayed intact STM (Figure 2L). To determine whether the improved STM was due to chronic changes in neuronal activity during development, we increased sleep in adults by expressing the temperature-sensitive *Transient receptor potential cation channel* (*UAS-TrpA1*) using *104y-GAL4* and raising the temperature from 25°C to 31°C [31]. Parental controls showed impaired performance at 25°C and these impairments persisted when the temperature was raised to 31°C for 24 h (Figure 2M). Normal sleeping rut^{2080}*;104y–GAL4/+>UAS-TrpA1/+* males at 25°C also showed impaired STM. However, inducing sleep for 24 h restored STM in rut^{2080}*;104y–GAL4/+>UAS-TrpA1/+* compared to their siblings maintained at 25°C (Figure 2M). Neither photosensitivity nor quinine sensitivity are altered by activation of the *104y-GAL4* and *C5-GAL4* expressing neurons, indicating that the improved STM is not attributable to changes in sensory thresholds (Table S1). Thus, inducing sleep using an independent approach allows *rutabaga* mutants to regain brain functions supporting STM.

We wished to know whether other sleep-promoting genetic-manipulations might also be used to restore memory in rut^{2080} flies. Curiously, few long-sleeping mutants have been evaluated for memory and we did not wish to use long-sleeping flies with memory impairments [52, 53]. Fortunately, overexpressing *fatty acid binding protein* (*dFabp*) increases daytime sleep and supports LTM [32]. *dFabp* flies contain a heat-shock inducible transgene that can be used to manipulate its expression [32]. Since *dFabp* flies are in the *w(isoCJ1)* background strain, we first evaluated their sleep and STM at 20°C and after being placed at 30°C for 2 days. As seen in Figure 2N, *w(isoCJ1)* flies maintained at 20°C displayed similar amounts of daytime sleep and exhibited normal STM scores compared to their siblings placed at 30°C. *dFabp/+* flies displayed an increase in daytime sleep when maintained at 30°C (Figure 2O). Importantly, *dFabp/+* flies displayed normal STM at 20°C and STM did not improve further when housed at 30°C for 2 days (Figure 2O). As expected, daytime sleep was increased in rut^{2080}*;;dFabp/+* flies housed at 30°C compared to their siblings maintained at 20°C (Figure 2P). Moreover, rut^{2080}*;;dFabp/+* flies displayed STM deficits at 20°C (Figure 2P). However, when sleep was increased for 2 days by shifting the flies to 30°C, rut^{2080}*;;dFabp/+* displayed normal STM (Figure 2P). As seen in Figure 2P, in the absence of sleep rut^{2080}*;;dFabp/+* flies maintained at 30°C exhibited impaired STM. Neither photosensitivity nor quinine sensitivity are altered by temperature or expression of *dFabp*, indicating that the improved STM is not due to changes in sensory thresholds (Table S1). Thus, sleep can be induced to restore STM to rut^{2080} mutants using three independent strategies (i.e., THIP, dFB activation and *dFabp* expression).

We have previously shown that sleep supports LTM using courtship conditioning [23, 31, 54, 55]. Thus, we asked whether THIP-induced sleep would restore LTM to rut^{2080} mutants. Naïve male rut^{2080} flies were exposed to pheromonally-feminized *Tai2* males using a protocol consisting of three one-hour training sessions, each separated by one hour (spaced training , ST); flies were evaluated for memory 48 h after training. When sleep was increased for 48 h following training rut^{2080} did not exhibit memory as evidenced by a lack of courtship suppression (data not shown). The failure of post-training sleep to improve memory is consistent with the observation above that rut^{2080} flies require at least 24 h of sleep prior to testing to restore STM (Figure 2J). To test the hypothesis that sleep is required

prior to training, we maintained *rut*^{2080} flies on 0.1mg/mL THIP 2 days prior to and 24 h following training. Flies were not on THIP during training but were returned to THIP following training to minimize interference resulting from a negative rebound which can last for a few hours following removal from THIP (Figure 2Q,R). Consistent with previous reports, vehicle-fed *rut*^{2080} siblings did not exhibit LTM (Figure 2S, black) [56]. However, when flies are administered THIP for 2 days prior and 24 h following training, they display normal LTM (Figure 2S). Thus, sleep can restore both STM and LTM to *rut*^{2080} mutants.

Inducing sleep in *dunce* mutants restores STM and LTM

rutabaga and *dunce* mutants show similar behavioral deficits when evaluated using a variety of independent assays, including APS and courtship conditioning [13, 15, 21, 24]. However, *rutabaga* mutants exhibit reduced cAMP levels, fewer synaptic boutons and deficits in neurotransmission while *dunce* mutants have elevated cAMP levels, increased numbers of synaptic boutons and increased neurotransmitter release [28–30, 57]. Given that *rutabaga* and *dunce* mutants induce opposing outcomes on important components of synaptic plasticity, it seems unlikely that sleep would be able to restore memory to *dunce* mutants. To test this hypothesis, we evaluated STM in *dnc*^{1} mutants. *dnc*^{1} mutants exhibit normal sleep and respond to THIP with an increase in sleep (Figure S4 A – C). As previously reported, *dnc*^{1} mutants exhibit impaired STM (Figure 3A) [13]. Surprisingly, STM was restored in *dnc*^{1} mutants following THIP-induced sleep when compared to vehicle-fed siblings (Figure 3A). No improvement in STM was observed in *dnc*^{1} flies maintained on THIP when they were sleep deprived (Figure 3A). As with *rut*^{2080}, THIP-induced sleep can restore STM to individual *dnc*^{1} mutants (Figure 3C , D). *dnc*^{1} mutants had normal quinine sensitivity and photosensitivity and these metrics were not altered by THIP (Table S2). To confirm the *dnc*^{1} results, we knocked down *dunce* using RNAi. As seen in Figure 3E, RU-fed *DaGsw/+>UAS-dnc*^{RNAi}*/+* flies exhibited impaired STM compared to vehicle-fed siblings (veh); the STM impairments were reversed following 2 days of THIP administration (RU$^{0.1T}$). Neither RU nor THIP altered STM in *UAS-dnc*^{RNAi}*/+* parental controls (Figure S5B, Figure 2G). Importantly, neither RU nor THIP altered sensory thresholds (Table S2). Thus, the STM deficits observed in *dnc*^{1} and *DaGsw/+>UAS-dnc*^{RNAi}*/+* flies were reversed following THIP-induced sleep.

To determine whether genetically-increased sleep and sleep induced by activating *dFabp* could also rescue STM deficits in *dnc*^{1} mutants, *dnc*^{1} was combined with *104y–GAL4*, *C5-GAL4* and *UAS-NaChBac* as well as *dFabp*. As seen in Figure 3F, *dnc*^{1}*;104y/+*, *dnc*^{1}*;C5/+* and *dnc*^{1}*;UAS-NaChBac/+* controls exhibited STM deficits. In contrast, both experimental lines (e.g. *dnc*^{1}*;104y/+>UAS-NaChBac/+* and *dnc*^{1}*;C5/+>UAS-NaChBac/+*) displayed intact STM compared to parental-controls (Figure 3F). STM was similarly restored when sleep was increased in adult *dnc*^{1}*;104y/+>UAS-TrpA1* flies maintained at 31°C for 24 h compared to their siblings maintained at 25°C (Figure 3G, right). In addition, STM was restored in *dnc*^{1}*;;dFabp/+* flies when sleep was increased by placing them at 30°C for 2 days compared to siblings maintained at 20°C; no improvements in STM were observed in the absence of sleep (Figure 3 H , I). Neither photosensitivity nor quinine sensitivity is altered by activation of the *104y–GAL4* and *C5-GAL4* expressing neurons or by expression of *dFabp* (Table S2). Together these data indicate that inducing sleep using either of three

independent strategies (e.g., pharmacology, FB activation or the expression of *dFabp*), can restore STM to *dnc*^{1} mutants.

To determine how long *dnc*^{1} flies must sleep before they display an improvement in STM we evaluated performance in *dnc*^{1} males after sleep was induced for 48 h, 24 h and 12 h with THIP administration. In contrast with *rut*^{2080}, only 12 h of sleep was required to restore STM in *dnc*^{1} mutants (Figure 3J). However, whereas *rut*^{2080} mutants maintained STM for 48 h after being removed from THIP, the improved performance was only observed in *dnc*^{1} mutants for 24 h, a time when sleep had returned to baseline (Figure 3K, Figure S5A). Thus, while sleep similarly benefits both *rut*^{2080} and *dnc*^{1} mutants, the time courses differ.

Can THIP-induced sleep restore LTM to *dnc*^{1} mutants as assessed using courtship conditioning? *dnc*^{1} flies were maintained on 0.1mg/mL THIP 2 days prior to and 24 h following ST (Figure 3L). Consistent with previous reports, vehicle-fed *dnc*^{1} flies did not exhibit LTM (Figure 3M, black bars) [21, 24]. However, when *dnc*^{1} siblings are administered THIP for 2 days prior and 24 h following training, they display normal LTM (Figure 3M, white bars). Thus, sleep can restore LTM to *dnc*^{1}mutants.

Silencing the FB prevents THIP from restoring STM

To further rule out non-specific effects of THIP, we asked whether silencing the FB would prevent THIP from restoring STM. Previous reports have shown that reducing the excitability of the FB reduces sleep [58]. As seen in Figure 4A, silencing the FB by expressing the inward rectifier K+ channel, Kir2.1, also reduces sleep in a *rutabaga* mutant background. Importantly, while both *rut*^{2080}*;104y/+* and *rut*^{2080}*;UAS-Kir2.1/+* parental controls responded to 0.1mg/ml of THIP with an increase in sleep, THIP did not increase sleep in *rut*^{2080}*;104y/+>UAS-Kir2.1/+* flies (Figure 4B). Importantly, when both *rut*^{2080}*;104y/+* and *rut*^{2080}*;UAS-Kir2.1/+* parental controls are maintained on vehicle they display deficits in STM which are reversed by THIP-induced sleep (Figure 4C). In contrast, both vehicle-fed and THIP fed *rut*^{2080}*;104y/+>UAS-Kir2.1/+* flies display performance deficits (Figure 4C). Neither photosensitivity, nor quinine sensitivity are modulated by silencing the FB neurons (Table S3). Thus, THIP does not restore memory independently from its effects on sleep.

Sleep increases synaptic proteins in *rut*^{2080} mutants

The synaptic homeostasis hypothesis argues that synapses are increased during waking and reduced during sleep [59]. Interestingly, the synaptic homeostasis model is largely based upon observations made in animals that clearly possess the full suite of plasticity related-molecules as well as intact synaptic machinery. Thus, while the hypothesis continues to garner support in intact animals [23, 31, 44, 60], we wished to know what role sleep might play in *rut*^{2080} and *dnc*^{1} flies that have clear deficits in important components of synaptic plasticity [28, 57]. Consistent with data presented above, THIP-induced sleep reduces DLG protein levels in *Cs* flies (Figure 1E, Figure 4D). THIP-induced sleep produced differential effects in *rut*^{2080} flies which have been reported to have reduced synapses [30]. THIP-induced sleep did not influence DLG levels in *dnc*^{1} mutants (Figure 4D). If THIP-induced sleep restores STM to *rut*^{2080} mutants by increasing synapses, then it should be possible to

use genetics to increase synapses and restore STM in a *rutabaga* mutant background without increasing sleep. The *arouser* mutant ($aru^{8.128}$) is known to have an increased number of synaptic terminals in both the larva and adult fly and also display memory impairments [61, 62]. As seen in Figure 4E, both $aru^{8.128}$/+ and rut^{2080};$aru^{8.128}$/+ flies show increased levels of DLG protein compared to rut^{2080} controls. Thus, $aru^{8.128}$ can be used to increase synaptic markers in rut^{2080} mutants. Are the changes in DLG protein levels associated with changes in STM? As seen in Figure 4F, both rut^{2080} and $aru^{8.128}$/+ mutants display impaired STM in the APS as expected [13, 16, 62]. In contrast, rut^{2080};$aru^{8.128}$/+ flies display STM. $aru^{8.128}$/+ and rut^{2080};$aru^{8.128}$/+ displayed normal photosensitivity and quinine sensitivity indicating that the change in performance cannot be explained by changes in sensory thresholds (Table S3). Interestingly, no differences in sleep time were observed between rut^{2080}, $aru^{8.128}$/+ and rut^{2080};$aru^{8.128}$/+ flies (data not shown). To further explore the role of *arouser* in restoring STM to *rutabaga* mutants, we used an RNAi approach to knockdown *arouser* in adult animals using a validated RNAi line and the GeneSwitch system [61]. As seen in Figure 4G, RU-fed *DaGSw/+>UAS-*aru^{RNAi}/+ flies displayed STM impairments compared to vehicle-fed siblings. These data provide a confirmation of the $aru^{8.128}$/+ data shown in Figure 4F and are consistent with previous reports of STM deficits in *aru* mutants [62]. Since THIP-induced sleep did not alter DLG protein levels in dnc^{1} mutants, we hypothesized that knocking down *aru* in the dnc^{1} mutant background would not restore STM. Indeed, both vehicle-fed and RU-fed dnc^{1};*DaGSw/+>UAS-*aru^{RNAi}/+ flies displayed deficits in STM (Figure 4H). Importantly, STM was fully restored when we knocked down *aru* in adult rut^{2080} mutants (Figure 4I). Similar to the results obtained with the mutant, knocking down *aru* using RNAi did not change sleep time nor alter photosensitivity or quinine sensitivity (data not shown and Table S3).

Sleep can restore performance in *Drosophila* models of Alzheimer's disease

To determine whether sleep can be used to reverse cognitive deficits in a *Drosophila* model of Alzheimer's disease, we evaluated LTM in young and old *Presenilin* mutants. Mutations in *Presenilin* have been linked to early onset familial Alzheimer's disease in humans [63], and previous studies have shown that the age-dependent cognitive deficits associated with mutations in *Presenilin* can be modeled in *Drosophila* [64]. As seen in Figure 5 A,E, young *Presenilin* mutants (*PsnB3/+*, *PsnC4/+*) display normal sleep profiles and exhibit intact LTM as assessed using courtship conditioning (Figure 5 B , F). Importantly, 30-day old *PsnB3/+* and *PsnC4/+* mutants respond to THIP with an increase in sleep (Figure 5 C , G). Thirty-day old *PsnB3/+* and *PsnC4/+* flies had impaired LTM consistent with previous reports (Figure 5 D , H) [64]. Thus, 28-day old *PsnB3/+* and *PsnC4/+* flies were placed onto 0.1mg/mL THIP 2 days prior to and 24 h following training. As seen in Figure 5 D,H, THIP-induced sleep was able to reverse deficits in LTM in this Alzheimer's model.

DISCUSSION

Our results demonstrate that sleep can restore brain functions supporting both short-term and long-term memory in two classic plasticity mutants, *rutabaga* and *dunce*. The improvements in performance were not specific to the methods used to increase sleep since they were observed using three independent approaches (activation of the FB, expressing *dFabp* and

pharmacology) and were not observed in the absence of sleep. Moreover, neither pharmacologically-induced sleep nor genetically-induced sleep altered quinine sensitivity or photosensitivity indicating that the recovery in STM is not due to changes in sensory thresholds. This latter interpretation is further supported by the observation that sleep can restore LTM using courtship conditioning, an assay utilizing a more complex set of sensory modalities than the APS. Thus, our data uncover an unexpected level of behavioral plasticity that can be modulated by sleep and which may not be readily accessible to the waking brain.

Surprisingly, while sleep promoting compounds were first used in flies over a decade ago [40, 41], the pharmacology of sleep in *Drosophila* remains poorly understood. Thus, while early studies showed that psycho-stimulants increased waking, sleep promoting compounds have been difficult to identify [35, 65, 66]. Indeed, the role of GABA in sleep regulation has relied heavily upon genetic manipulations, rather than pharmacology, and has largely implicated the involvement of the *Rdl* receptor in the wake-promoting clock neurons [67, 68]. To our knowledge, the GABA-A agonist THIP is the first pharmacological agent identified that can support sustained increases in sleep in flies and which also exhibits shared molecular, physiological and functional characteristics with both spontaneous sleep and genetically enhanced sleep. These sleep-promoting effects in flies are consistent with the THIP-induced increase in slow wave sleep and sleep maintenance in humans [69]. Moreover, our data provide the first indication that sleep can be modulated by alternate GABA-A receptors *Lcch3* and *Grd*.

Nonetheless, one might ask whether the improved performance that is seen in memory mutants following THIP administration is due to sleep *per se* or to non-specific actions of THIP on neuronal excitability. Two lines of evidence indicate that the cognitive enhancement is due to sleep. First, while sleep deprived memory mutants continue to eat and thus ingest THIP similar to non-sleep deprived controls, no improvements in memory are observed in the absence of sleep. Second, THIP does not restore memory when the FB is silenced by expressing *UAS-Kir2.1*. Third, and most importantly, memory deficits are also reversed when sleep is induced in the absence of drug by genetically activating the FB or when expressing *dFabp*. The ability to enhance sleep using three independent research strategies, pharmacology, FB-activation and expression of *dFabp*, signifies that it is sleep, not the method used for inducing sleep, that is responsible for the observed improvements in performance.

Our data demonstrate that sleep can improve cognitive performance in mutant flies without rescuing the underlying genetic lesion. Interestingly, several studies have found that manipulating the environment can similarly reverse deficits of mutants without restoring the specific genetic lesion. For example, flies mutant for *arouser* display increased ethanol sensitivity which can be reversed by social isolation [61]. Flies lacking the male-specific *fruitless* gene (*fruM*) will court if they have been grouped with other flies for several days [70]. Mutations in the foraging gene (for^{s2}) have impaired STM, but these deficits can be reversed following a brief period of starvation [54]. Finally, circuit specific deficits in LTM as assessed using courtship conditioning can be reversed when the same flies are evaluated in the absence of visual input [71]. Together these data emphasize that a variety of

environmental conditions can restore behavior even in the context of an underlying genetic lesion.

Cognitive impairments associated with aging and neurodegenerative disorders are frequently accompanied by alterations in sleep physiology and architecture [72, 73]. These data have led to the hypothesis that improving sleep might be beneficial for slowing or attenuating cognitive deficits [72]. Our data showing that increasing sleep can reverse cognitive deficits in a *Drosophila* model of Alzheimer's disease supports previous hypotheses and suggest that under the appropriate circumstances, increased sleep may benefit patients with certain neurological disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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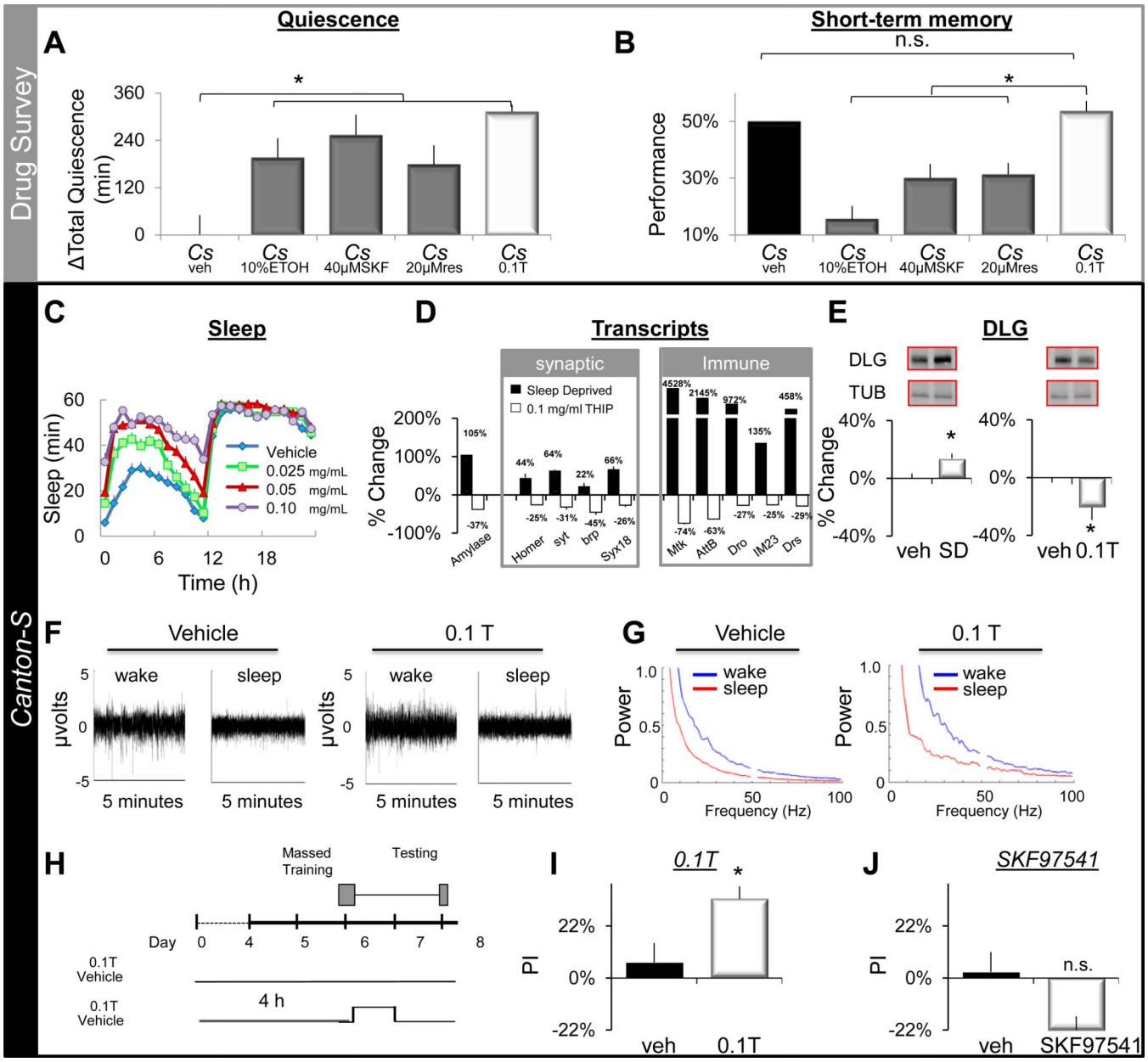

Figure 1. THIP induces sleep in *Drosophila*

(A) *Cs* females were maintained on vehicle (veh), 10% ethanol (ETOH), 40 μM of the GABA-B agonist SKF97541, 20μM of the vesicular monoamine transporter inhibitor reserpine (res), or 0.1mg/mL of the GABA-A agonist THIP (0.1T) for 48 h. Compared to vehicle-fed controls, *Cs* flies maintained on ETOH, SKF97541, res and THIP showed significant increases in Daytime quiescence ANOVA $F_{[3,99]} = 12.9$; $p= 3.35^{E-7}$; the data are presented as difference from vehicle fed controls (ΔDaytime Sleep). *p<0.05 modified Bonferroni test, n=14–30 flies/group. **(B)** Short-term memory was significantly impaired in SKF97541, reserpine and ETOH fed *Cs* flies but was unchanged in flies fed THIP, ANOVA $F_{[3,25]} = 27.6$; $p= 4.21^{E-8}$; *p<0.05 modified Bonferroni test, n=5–9 flies/genotype. **(C)** THIP increases quiescence (min/h) in a dose-dependent manner in *Cs* flies. Data are

presented as sleep in minutes/hour. Repeated measures ANOVAs reveals a significant Dose (4) X Hour (24) interaction (*Cs:* $F_{(69,1265)}$=5.15, p= 9.99^{E-16} n=23–30/group). **(D)** Relative transcript levels of *Amylase*, *Homer*, *Synaptotagmin (syt)*, *bruchpilot (brp)*, *Syntaxin18 (syx18)*, *Metchnikowin (Mtk)*, *Attacin-B (AttB)*, *Drosocin (Dro)*, *Immune induced molecule 23 (IM23)*, and *Drosomycin (Drs)* are upregulated following 12 h of sleep deprivation and reduced following 48 h of THIP (0.1T) feeding. **(E)** DISCS-LARGE (DLG) levels are significantly increased following 12 h of sleep deprivation (left) but reduced by 48 h of THIP treatment as revealed by Western blots (right) (n=3, 6 brains /group). **(F)** Representative traces of local field potentials from individual vehicle-fed (Left) and THIP-fed (right) flies during waking and quiescence. **(G)** Representative power spectra during waking and sleep from the flies presented in 1F: vehicle-fed (left) and THIP-fed fly (right). **(H)** Schematic of the training protocol. **(I)** *Cs* flies maintained on vehicle (veh) post-training do not have an LTM (black bars) while flies whose sleep was increased with THIP for 4 h immediately following training resulted in an LTM, (white bars); Krustal-Wallis, p= 0.008, n=16–20 flies/group, Performance Index (PI). **(J)** No memory is detected when *Cs* flies are fed either veh (black bars) or SKF97541 (white bars) following training; n=17–20 flies/group. Error bars, s.e.m.;*P<0.05.

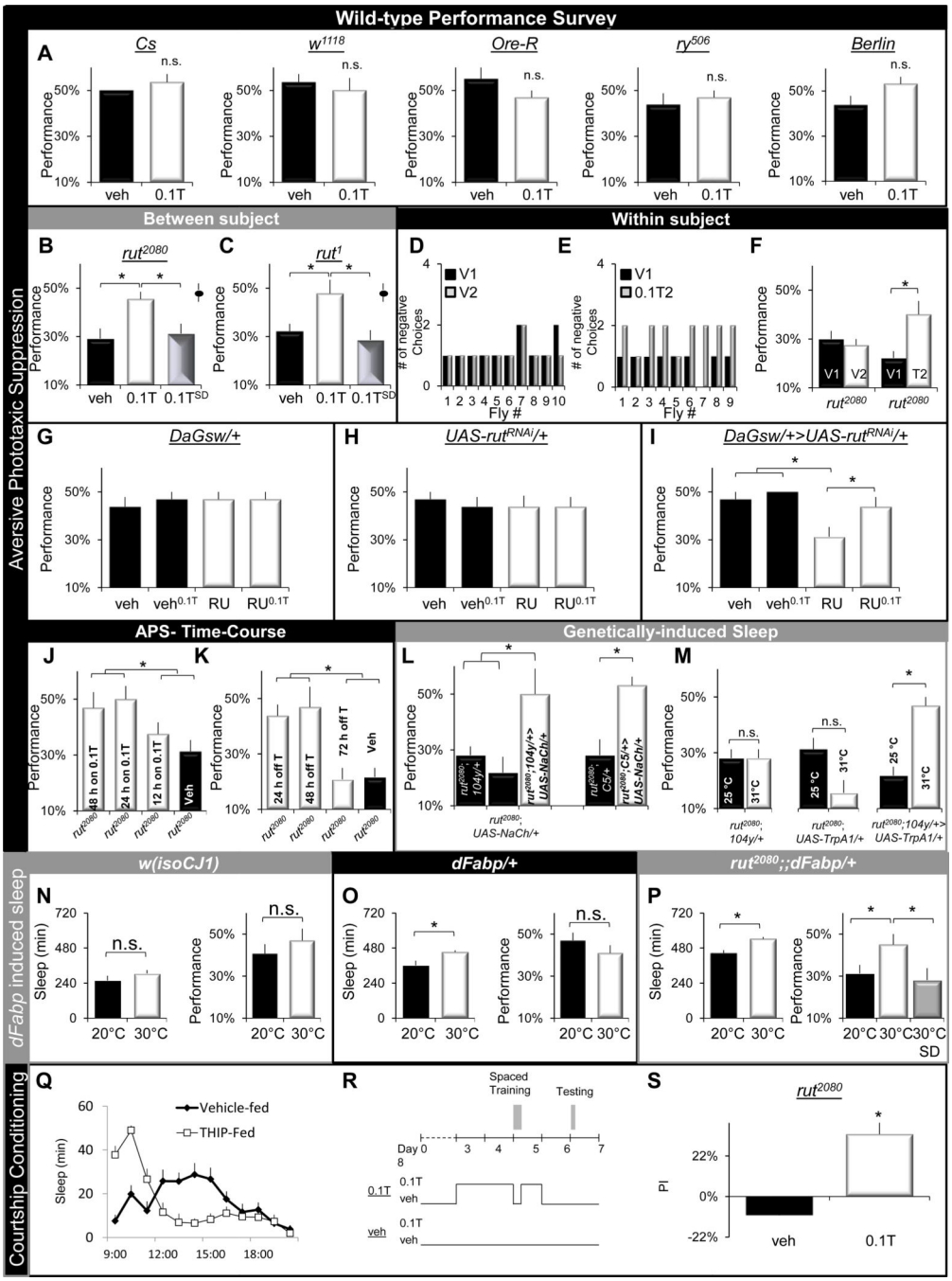

Figure 2. Inducing sleep in *rutabaga* mutants restores short term memory and long term memory

(A) No improvement in STM is observed in 3–5 day old *Cs*, w^{1118} , *Ore-R*, ry^{506} or *Berlin* flies maintained on 0.1mg/mL of THIP compared to vehicle-fed controls. A 5 (Genotype) x 2 (Veh, THIP) ANOVA failed to identify any main effects nor a Genotype X Drug interaction, $F_{[4,69]}=1.4$,$p=0.22$; (n=8/group); nonsignificant (n.s.) modified Bonferroni test. **(B,C)** rut^{2080} and rut^{1} mutants exhibit deficits in STM (veh) which are reversed following 48 h of sleep induced by THIP (0.1T); mutants maintained on THIP but sleep deprived are

learning impaired ($0.1T^{SD}$) (n=>8/group). One way ANOVA for rut^{2080} $F_{[2,21]} = 4.09$; p=0.03 and for rut^{1} $F_{[2,21]} = 5.35$; p=0.01;*P<0.05, modified Bonferroni test. For comparison, the  symbol indicates wild-type performance. **(D)** Individual rut^{2080} maintained on vehicle reliably choose the lighted vial on two trials spaced two days apart (V1 and V2). **(E)** Individual rut^{2080} flies showed performance decrements while on vehicle (V1) and these decrements were reversed following 2 days of THIP-induced sleep (0.1T2). **(F)** Mean performance scores ± SEM for rut^{2080} maintained on vehicle (V1, V2) or switched from vehicle (V1) to THIP for 2-days (T2); paired t-test, *p<0.05. **(G,H)**. Neither RU nor THIP influence STM in *DaGsw/+* or rut^{RNAi}/+ parental controls; main effect for RU ($F_{[1,28]} = 0.21$; p=0.64, and $F_{[1,28]} = 0.16$; p=0.69, respectively), and THIP($F_{[1,28]} = 0.21$; p=0.64, $F_{[1,28]} = 0.16$; p=0.69, respectively). **(I)** RU disrupts STM in *DaGsw/+>UAS-*rut^{RNAi}/+ flies; main effect for RU ($F_{[1,28]} = 11.06$; p=0.002). THIP restores STM to RU fed *DaGsw/+>UAS-*rut^{RNAi}/+ flies (RU^{01T}); main effect for THIP ($F_{[1,28]} = 6.6$; p=0.02); n=8 flies/group, *P<0.05, modified Bonferroni test. **(J)** STM impairments are reversed in rut^{2080} mutants after 24 h, but not 12 h, of THIP-induced sleep, One way ANOVA $F_{[3,29]} = 3.0$; P=0.04; n>=8 flies/group, *P<0.05, modified Bonferroni test. **(K)**rut^{2080} mutants continue to exhibit STM for 48 h after being removed from THIP, One way ANOVA $F_{[3,33]} = 8.4$; P=0.0002; n>=8 flies/group, *P<0.05, modified Bonferroni test. **(L)**rut^{2080}*;104y–GAL4/+>UAS-NaChBac/+* and rut^{2080}*;;C5-GAL4/+>UAS-NaChBac/+* lines display normal STM; in contrast, performance is impaired in all parental controls, One way ANOVA $F_{[4,33]} = 7.01$; $p=3.380^{E-004}$, *P<0.05, n=8 flies/group, modified Bonferroni test. **(M)**rut^{2080}*;104yGAL4/+>UAS-TrpA1/+* flies display normal STM following sleep induction for 24 h at 31°C compared to siblings maintained at 25°C; STM remains impaired in parental controls at 25°C and 31°C. A 3(genotype) X 2 (temperature) ANOVA revealed a significant genotype X temperature interaction $F_{[2,42]} = 16.4$; $p= 5.39^{E-06}$, *P<0.05, n=8 flies/group, modified Bonferroni test. **(N)** *w (isoCJ1)* background controls exhibit similar daytime sleep at both 20°C and 30°C; p>0.05, ttest, n=16 flies/condition. *w (isoCJ1)* flies display similar performance scores in the APS at 20°C and after being maintained at 30°C for 2 days ; p>0.05, ttest, n=8 flies/condition. **(O)** *dFabp/+* flies sleep more at 30°C than at 20° consistent with previous reports; *p<.05, ttest, n=15–16 flies/condition. Increasing sleep by placing *dFabp/+* flies at 30°C for two days does not improve STM; p>0.05, ttest, n=8–10 flies/condition. **(P)** Placing rut^{2080}*;;dFabp/+* at 30°C increases sleep compared to siblings maintained at 20°C, *p<0.05, ttest, n=15–16 flies/condition. At 20 °C, rut^{2080}*;;dFabp /+* exhibit STM impairments which are reversed when sleep is increased by placing flies at 30°C; the improvements in STM are not observed in the absence of sleep (30°C SD). One way ANOVA for condition :$F_{[2,25]} = 3.4$; p=0.05, *p<0.05 modified Bonferroni test, 8–10 flies/condition. **(Q)** Flies were maintained on vehicle or THIP for 2 days. THIP-fed flies removed from THIP and placed onto normal food at 10am sleep less than vehicle-fed controls (n=16). **(R)** Schematic of the protocol used for courtship conditioning. **(S)** No change in the Performance Index (PI) is observed in vehicle-fed rut^{2080}mutants following training; in contrast increasing sleep with 0.1T results in LTM; Krustal-Wallis p=0.007. n=16–20 flies/group. Error bars, s.e.m.;*P<0.05.

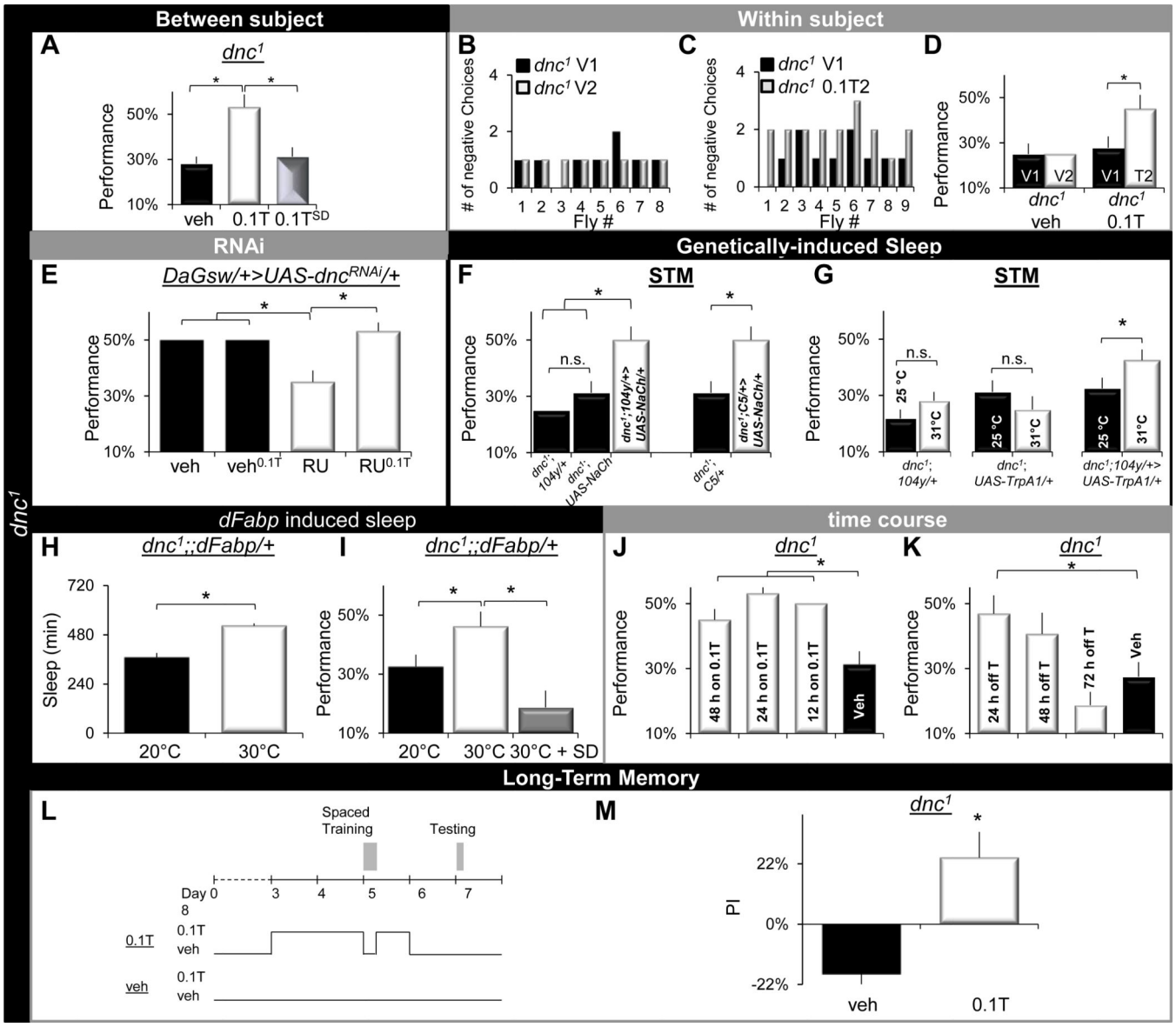

Figure 3. Inducing sleep in *dunce* mutants restores short term memory and long term memory

(A) *dnc*^{1} mutants exhibit deficits in STM (veh) which are reversed following 48 h of THIP-induced sleep (0.1T); mutants maintained on THIP but sleep deprived are learning impaired (0.1T^{SD}) (n=>8/group). One-way ANOVA $F_{[2,21]} = 9.5$; p=0.001; *P<0.05, modified Bonferroni test. **(B)** Individual *dnc*^{1} flies maintained on vehicle exhibit disrupted STM when tested on two trials spaced two days apart (V1 and V2). **(C)** Individual vehicle-fed *dnc*^{1} flies showed impaired STM which is reversed following 2 days of THIP-induced sleep (0.1T2). **(D)** Mean performance scores ± SEM for *dnc*^{1} maintained on vehicle (V1, V2) or switched from vehicle (V1) to THIP for 2-days (T2); paired t-test, *p<0.05. **(E)** RU-fed *DaGsw/+>UAS-dnc*^{RNAi}*/+* flies display impaired STM that is reversed by 48 h of THIP-induced sleep (RU$^{0.1T}$); vehicle-fed flies on and off THIP (veh$^{0.1T}$, veh) display normal STM; A 2(Vehicle, RU) x 2 (Vehicle, THIP) ANOVA yields a significant interaction $F_{[1,30]} = 10.13$; p=0.003; n=8 flies/group, *P<0.05, modified Bonferroni test. **(F)***dnc*^{1}*;104y–GAL4/+>UAS-*

NaChBac/+ and *dnc^{1};;C5-GAL4/+>UAS-NaChBac/+* lines display normal STM; in contrast, performance is impaired in all parental controls, One way ANOVA $F_{[4,35]} = 8.75$; $p = 5.26^{E-05}$, *P<0.05, n=8 flies/group modified Bonferroni test. **(G)** *dnc^{1};104yGAL4/+>UAS-TrpA1/+* flies display normal STM following sleep induction for 24 h at 31°C compared to siblings maintained at 25°C; STM remains impaired in parental controls at 25°C and 31°C, main effect for Genotype $F_{[2,45]} = 6.2$; p= 0.004, n=8 flies/group *P<0.05, modified Bonferroni test. **(H)** *dnc^{1};;dFabp /+* sleep more at 30°C than their siblings maintained at 20°C, *p<0.05, ttest, n=15–16 flies/ condition. **(I)** When *dnc^{1};;dFabp /+* flies are maintained at 20°C, they display impairments in STM; these impairments are reversed when sleep is increased for 2 days by placing the flies at 30°C. Importantly no improvements in STM are observed in the absence of sleep. A oneway ANOVA yielded a significant effect for condition $F_{[2,30]} = 7.5$; p=0.002 modified Bonferroni Test ,n=8–12 flies /condition.**(J)** STM impairments are reversed in *dnc^{1}* mutants after 12 h of THIP-induced sleep, One-way ANOVA $F_{[3,30]} = 5.99$; P=0.002; n>=8 flies/ group, *P<0.05, n=8 flies/group modified Bonferroni test. **(K)** *dnc^{1}* mutants continue to exhibit STM for 24 h after being removed from THIP, One-way ANOVA $F_{[3,30]} = 5.06$; P=0.003; n>=8 flies/group, *P<0.05, modified Bonferroni test. **(L)** Schematic of the protocol used for courtship conditioning. **(M)** No change in the Performance Index (PI) is observed in vehicle-fed *dnc^{1}* mutants following training; in contrast increasing sleep with 0.1T results in LTM; Krustal-Wallis p=0.026, n=16–20 flies/group. Error bars, s.e.m.;*P<0.05.

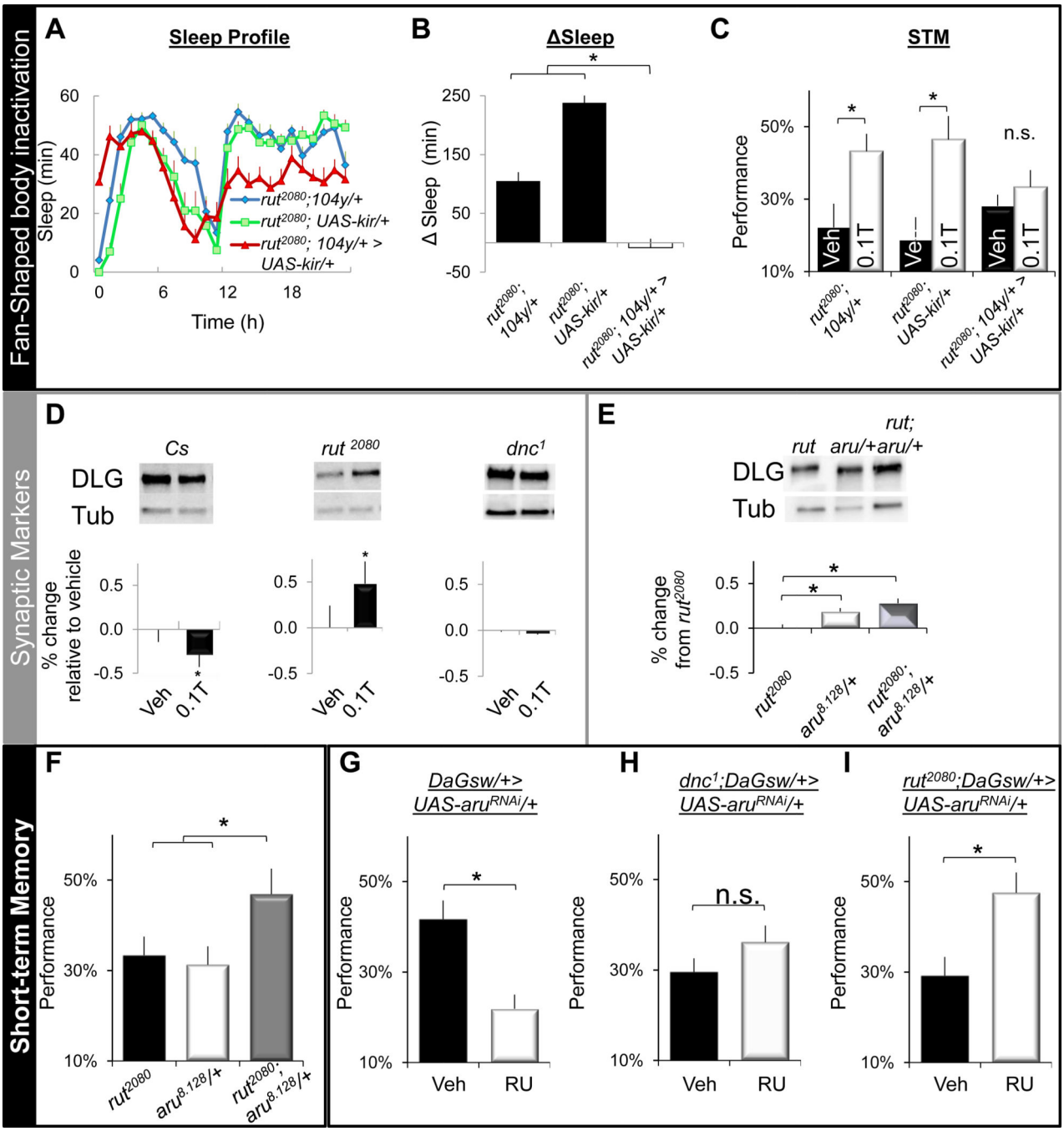

Figure 4. THIP requires the Fan Shaped body to increase sleep

(A) Expressing *UAS-Kir2.1* in *104y–GAL4* expressing cells disrupts sleep in a rut^{2080} mutant background. Both rut^{2080}*;104y/+* and rut^{2080}; *UAS-Kir2.1/+* parental controls sleep normally. A 3(genotype) X 24 (Time) ANOVA revealed a significant Genotype X Time interaction $F_{[46,966]} = 6.68$; $p=9.99^{E-016}$ consistent with previous reports (n=14–16 flies/group). **(B)** THIP does not result in an increase in Daytime sleep in rut^{2080}*;104y/+> UAS-Kir2.1/+* flies; while both rut^{2080}*;104y/+* and rut^{2080}; *UAS-Kir2.1/+* parental controls increase sleep as expected. ΔSleep is calculated by subtracting sleep in THIP fed flies from

vehicle-fed siblings. A One way ANOVA for Genotype: $F_{[2,43]} = 76.2$; $p=7.24^{E-15}$, *p<0.05 modified Bonferroni test, n=15–16 flies /group. **(C)** THIP does not restore STM to *rut^{2080};104y/+> UAS-Kir2.1/+* flies but returns STM to normal in parental controls that increase their sleep. A 3(Genotype) X 2 (Drug) ANOVA revealed differential responses to THIP: $F_{[1,49]} = 15.98$; $p=2.14^{E-004}$, *p<0.05 modified Bonferroni test, n=8–12 flies /group. **(D)** THIP (0.1mg/ml) treated *Cs, rut^{2080}* and *dnc^{1}* flies and their vehicle-fed siblings were collected for Western blot analysis (n=4 brains/condition). Experiments were run in triplicate, a representative blot is shown. The graphs are the quantification (mean ± SEM) expressed as % change relative to vehicle (t-test *, p < 0.05). **(E)** Compared to *rut^{2080}*, both *aru$^{8.128}$/+* and *rut^{2080}; aru$^{8.128}$ /+* mutants exhibit a significant increase in DLG protein, ttest *p<.05. **(F)** Single mutants for either *rut^{2080}*or *aru$^{8.128}$/+* display impairments in STM (black and white bars, respectively); however, *rut^{2080};aru$^{8.128}$/+* flies (gray bar) have normal STM. *p<0.05 ttest, n=8–9 flies/genotype. **(G)***DaGsw/+>UAS-aru^{RNAi}/+* flies fed RU486 (RU)display significant memory impairments compared to vehicle fed controls (Veh); *p<0.05, ttest. **(H)** Knocking down *aru* using *DaGsw* does not restore STM in a *dnc^{1}* mutant background p>0.05, ttest n=8 flies/group. **(I)** Vehicle–fed *rut^{2080};DaGsw/+>UAS-aru^{RNAi}/+* flies display STM impairments while RU-fed siblings exhibit STM; *p<0.05, ttest, n=8 flies/group. Error bars, s.e.m.;*P<0.05.

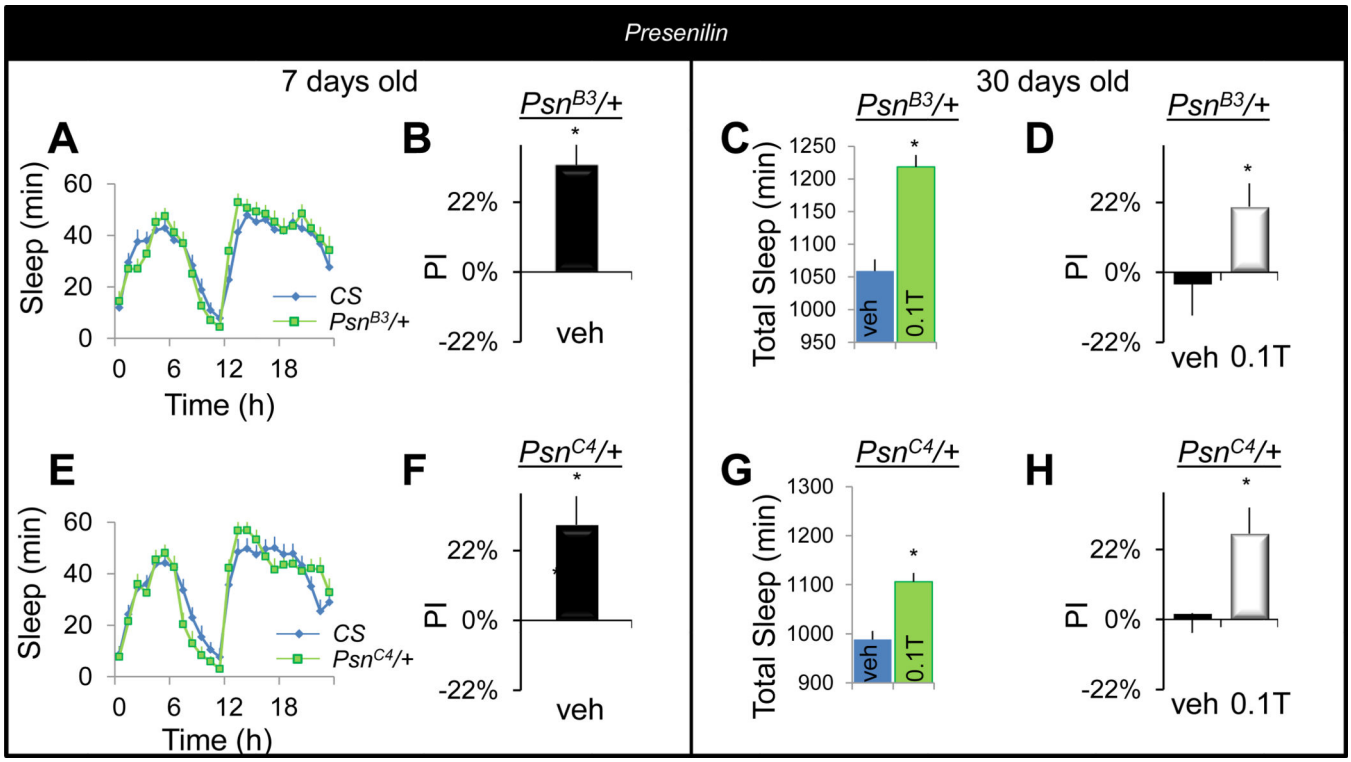

Figure 5. Sleep Induction fully restores LTM to *Presenilin* mutants

(A,E) Young 7-d old Psn^{B3}/+, Psn^{C4}/+ and *Cs* flies, show similar sleep profiles. **(B,F)** Young Psn^{B3}/+ (n=16/naïve and n=14/trained) and Psn^{C4}/+ (n=10/naïve and n=11/trained) flies display normal LTM as assessed using courtship conditioning; Krustal-Wallis p=0.007 Performance Index (PI). **(C,G)** 30 day old Psn^{B3}/+ and Psn^{C4}/+ flies increase sleep in response to 0.1T. **(D,H)** No LTM is observed in vehicle-fed 30-d old Psn^{B3}/+ (n=16 for both groups) and Psn^{C4}/+ (n=22/naïve and n=27/trained) flies after spaced training (black bars). Increasing sleep with 0.1T results in LTM in 30-d old Psn^{B3}/+ (n=16 for both groups) and Psn^{C4}/+ flies (n=15/naïve and n=21/trained); white bars. Error bars, s.e.m.;*P<0.05.