UNIVERSITY OF CALIFORNIA SAN DIEGO

Sleep and Memory Regulation through Cholinergic and Dopaminergic Signaling in *qvr/sss* Neurons

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in

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by

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The Thesis of Veronica Qiao Lin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

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by

Veronica Qiao Lin

Master of Science in Biology

University of California San Diego, 2020

Professor William Joiner, Chair
Professor Jing Wang, Co-Chair

Sleep is known to affect memory, but the exact relationship is unclear. My thesis aims to elucidate this relationship and identify key contributors to the regulation process. I studied the

correlation between sleep and memory phenotypes in sss^{P1} and DAT^{fmn} fly mutants via the *Drosophila* Activity Monitoring system and the Proboscis Extension Reflex assay; rescued both phenotypes by various Gal4>UAS and Gal4>RNAi combinations; and identified several molecules that play important roles in the regulation of sleep and memory. I established that healthy sleep behavior is positively correlated with intact memory function and chronic sleep deprivation is linked to memory deficits. By studying sss^{P1} and DAT^{fmn} mutants, I found that both QVR/SSS and DAT are required for regular sleep and memory functions and 11E12, 12C10, piezo, and 109(2)80 neurons are important loci for the sleep and memory effects of *qvr/sss*.

In these *qvr/sss* neurons, I found that knocking down either Dop1R2 or Dα3 is sufficient to rescue the phenotypes of sss^{P1} and DAT^{fmn} mutants. The results suggest that Dop1R2 and Dα3 may function in parallel in *qvr/sss* neurons, and dopaminergic and cholinergic signaling may converge onto these *qvr/sss* neurons to regulate sleep and memory. Further, Dα3 is regulated by two molecules with opposing effects; QVR/SSS inhibits Dα3 activity while dNACHO promotes it. Collectively my data suggest that sleep and memory functions are regulated by *qvr/sss* neurons by coordinated dopaminergic and cholinergic mediation.

Introduction

Sleep is known to affect memory, but the exact relationship between the two processes remains poorly understood. In mammals, two main explanations have been proposed: the synaptic homeostasis hypothesis (SHY), and the system consolidation hypothesis. According to SHY, during wakefulness new information is acquired from the environment, leading to net synaptic potentiation across the brain. Such waking plasticity consumes energy and would eventually lead to saturation of synaptic strength and learning capacity if continued unabated. Thus, according to SHY, sleep has evolved to downscale synaptic strength across the brain, thus keeping net synaptic strength within a range that is sustainable and biologically meaningful. This is thought to be possible because synchronized slow wave oscillations that occur during NREM sleep homeostatically downregulate synaptic strength. According to SHY, although this downregulation occurs globally across the brain, the relative differences between synapses are preserved. As a result, the total synaptic weight is reduced, weak potentiations are nullified, and strong potentiations persist [1].

On the other hand, the system consolidation hypothesis states that memories are selectively reactivated and actively consolidated during sleep. The two-stage model of memory proposes that memories are encoded and stored in two separate places during waking, and the memory representations are reactivated and redistributed during sleep. During wakefulness, memory traces are encoded in the hippocampus as a “fast-learning, temporary store” and in parallel, in the neocortex as a “slow-learning, long-term store”. During sleep, the hippocampal memory traces are repeatedly reactivated to drive concurrent reactivation of the neocortical memory store, strengthening this long-term store. The neocortex then adapts the new labile memories and integrates them into the pre-existing network of long-term memories [2].

One major weakness of both SHY and the system consolidation hypothesis is that they have not been rigorously tested at the molecular or genetic level, and indeed no molecular mechanism has been proposed to support them. This weakness is partly a product of the overall dearth of data available about molecules that play an essential role in sleep and/or memory. It is simply difficult to isolate testable circuits and perturb single genes or molecules in mammals. On the contrary, *Drosophila melanogaster*, commonly known as the fruit fly, allows scientists to study isolated neuronal networks and manipulate gene expressions. This easy-to-maintain and fast-to-generate organism offers numerous unique genetic tools that have been used to study many processes, including sleep and memory [3, 4]. Like in mammals, the fruit fly shares conserved hallmarks of sleep including decreased brain arousal and responsiveness, changes in electrical activity in the brain, rapidly reversible behavioral quiescence, and homeostatic control of duration [5]. Furthermore, neurotransmitters and pharmacological manipulations exert similar effects on sleep in flies and mammals. Namely, aminergic and cholinergic signaling promotes arousal while GABAergic signaling suppresses it [6-10]; antihistamines and GABAergic agonists promote sleep while caffeine inhibits it [9-12]. In addition, sleep is known to be required for learning and memory across species [13-15]. Studies conducted in the fruit fly offer important insights into on the role sleep plays on learning and memory. Previous studies have shown that sleep deprivation leads to memory impairment in flies [16] and memory consolidation can be induced by increasing sleep [17]. These studies confirmed that there is a strong correlation between sleep loss and impaired memory [18]; but the exact relationship between these processes and the underlying mechanism remain unclear. In my study, I attempt to elucidate this relationship and identify molecules and/or pathways responsible for its regulation. The molecular conservation and genetic tractability of the fruit fly make it highly suitable for my

study; the unique tools it offers are utilized to investigate the relationship between sleep and memory.

My study focused on two previously identified short-sleep mutants: sss^{P1} and DAT^{fmn}. Previous studies in my lab have shown that the *quiver/sleepless* (*qvr/sss*) gene is required for sleep. The QVR/SSS protein promotes sleep at least in part by antagonizing nicotinic acetylcholine receptors (nAChRs) to reduce cholinergic synaptic transmission [19]. Specifically, the QVR/SSS protein forms a stable complex with the Dα3 subunit of nAChRs and directly suppresses nAChR activity. In sss^{P1} mutants this brake on synaptic activity is removed, leading to reduced sleep, which can be restored by RNAi knockdown of Dα3 in *sss*-expressing neurons or pharmacological antagonism of nAChRs [19]. Another gene that is required for sleep is the dopamine transporter (*DAT*). Normally, presynaptic DAT clears dopamine from synaptic clefts and terminates intercellular signaling. In DAT^{fmn} mutants, however, DAT cannot effectively reuptake dopamine. As a result, excess dopamine in the synaptic cleft increases downstream signaling, causing the mutants' hyperactivity and low sleep [20].

Since QVR/SSS and DAT are both required for sleep, and sleep seems to be required for memory, I hypothesized that both QVR/SSS and DAT would also be required for memory. I confirmed this hypothesis in my thesis using a combination of short-sleeping sss^{P1} and DAT^{fmn} mutants. I further hypothesized that if the sleep and memory functions of each gene are functionally related then each phenotype might map to the same neurons. To test this hypothesis, I identified Gal4/UAS-transgene combinations that could rescue both phenotypes for each mutant. In the process of mapping each gene's effects I unexpectedly also found evidence that DAT-regulating neurons and *qvr*/*sss* neurons may comprise two distinct parts of a shared neural circuit. Specifically, my data suggest that the dopamine receptor Dop1R2 functions to mediate

postsynaptic dopamine signaling in *qvr/sss* neurons to promote waking. These results stand in stark contrast to previously published data indicating a more prominent role for Dop1R1 (also called DopR, DopR1, and DA1) functioning in the dorsal fan shaped bodies (dFSBs) to suppress waking [21-22]. Another study has suggested that the Dop1R2 is the key mediator of dopamine effects in the dFSBs [23]; my data recognize the prominent role of Dop1R2 but suggest the primary cellular location Dop1R2 functions to regulate sleep and memory is in the *qvr/sss* neurons, not in the dFSBs.

Section I. Identifying Memory Phenotype in Low-Sleep Drosophila Mutants

Results

I first examined and compared the sleep phenotypes of wildtype control animals to sss^{P1} and DAT^{fmn} mutants using the Drosophila Activity Monitoring system. The sleep/wake patterns of flies were measured over 24 hours as previously described [12]. As expected, wildtype animals displayed the typical crepuscular sleep/wake pattern, showing low daytime sleep, a midday siesta, and high nighttime sleep. In contrast, the sss^{P1} and DAT^{fmn} mutants displayed almost no midday siesta and significantly lower nighttime sleep **(Figure 1a)**. Quantification of total sleep time shows significant reduction in sss^{P1} and DAT^{fmn} mutants compared to controls, confirming previously published observations [20, 24] **(Figure 1b)**.

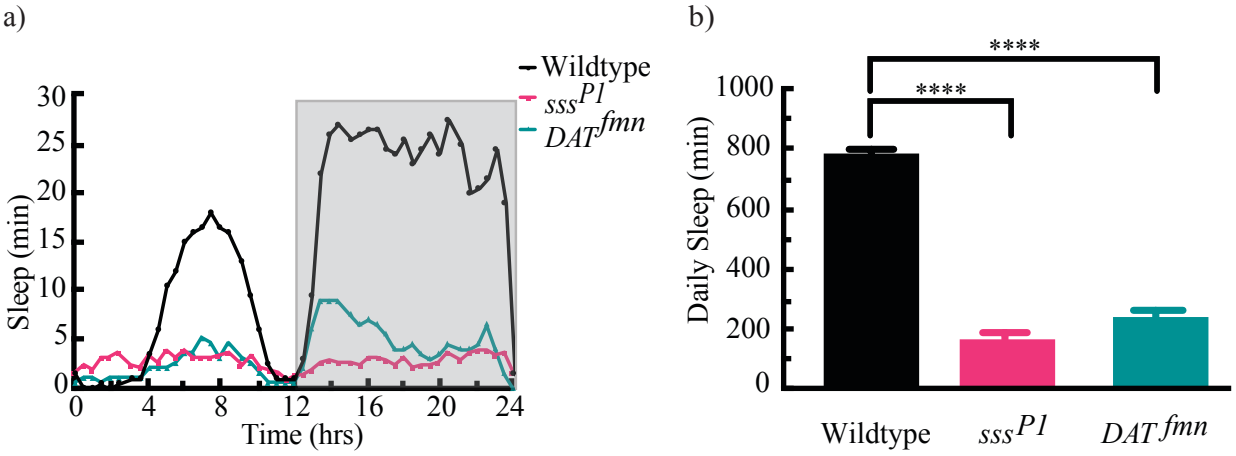


Figure 1a,b. Sleep Phenotypes of Wildtype, sss^{P1}, and DAT^{fmn} flies. The representative sleep profiles of wildtype flies, sss^{P1} mutants, and DAT^{fmn} mutants are shown. (a) Sleep traces of the flies over a period of 24 hours. (b) Total amount of daily sleep for each genotype in minutes.

Since sleep is thought to be important for learning and memory, I next assessed whether low-sleeping sss^{P1} and DAT^{fmn} mutants have deficits in associative memory. In a modified Proboscis Extension Reflex (PER) assay [25], flies were repeatedly trained to associate the bitter taste of quinine with the appetitive tastant fructose. Flies that remember this association subsequently stop extending their proboscis when presented with fructose alone **(Figure 1c)**.

With repeated training wildtype flies are thus expected to show a decreasing response rate with a low final test score. Lower test score indicates higher degree of learning and memory. In contrast, mutant flies with impaired learning and memory are expected to maintain a high response rate in the final test.

The results of the PER assay are presented in two types of graphs, a line graph showing the learning curve and a bar graph showing the final test score. The line graph shows the animals' progression of learning at each stage, from the pre-test to the training trials to the final test score. Control flies typically showed a steep slope as they quickly learned and reached a low score at the final test. Meanwhile, sss^{P1} and DAT^{fmn} mutants typically showed a much slower decline in response rates and did not achieve a low test score **(Figure 1d).** The bar graph shows the quantification of the final test score, which is an indication of successful associative learning and memory retention. Control flies typically had a low test score of around 20%, indicating that ~80% of animals retained the association between quinine and fructose. In contrast, sss^{P1} and DAT^{fmn} mutants had high test scores of ~85% and ~50%, respectively, indicating that a much smaller fraction of these flies than wildtype controls exhibited gustatory associative memory **(Figure 1e).** For the sake of simplicity, in future sections bar graphs quantifying total daily sleep and final PER test scores will be shown without the accompanying behavioral profiles.

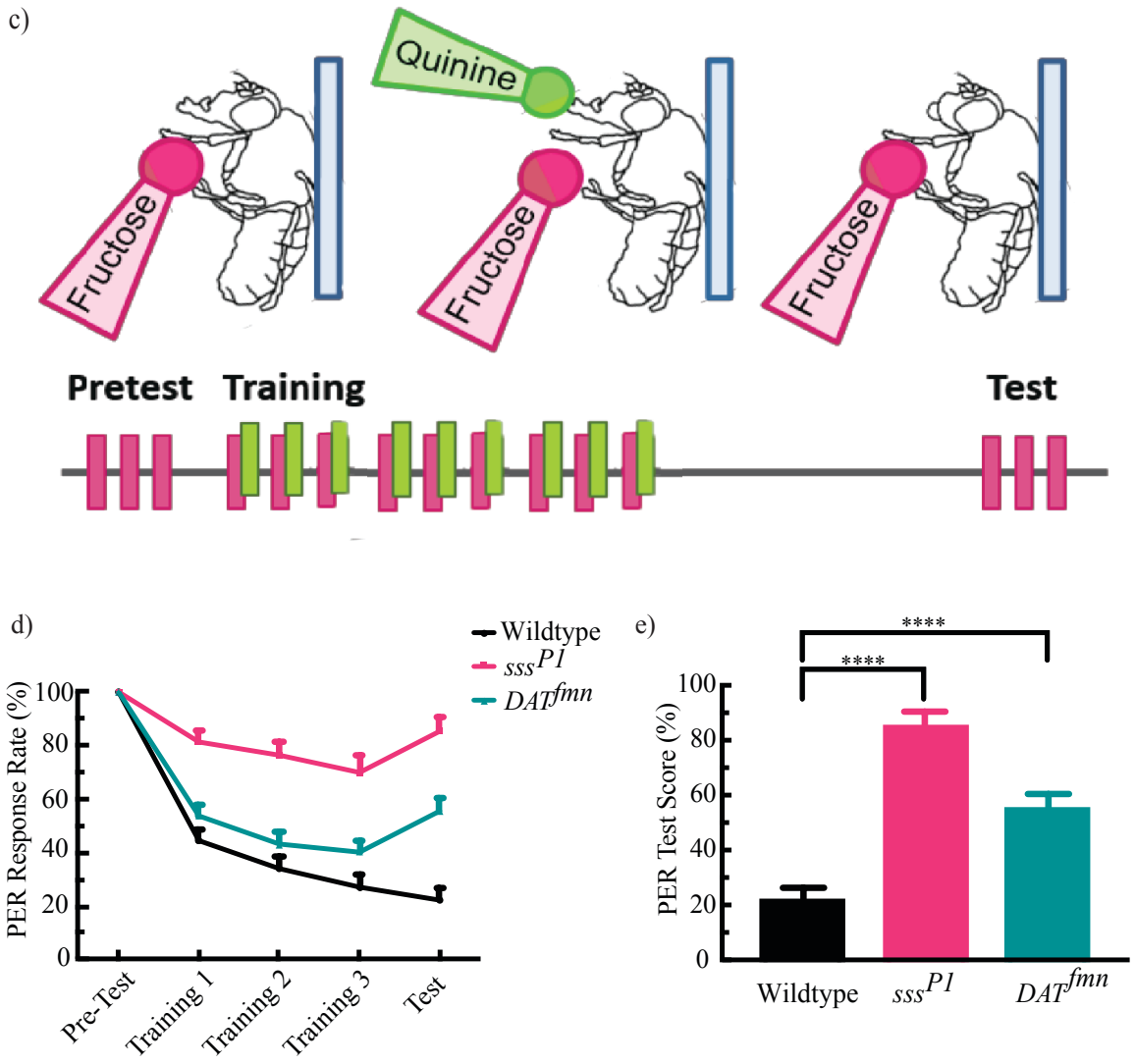


Figure 1c,d,e. Memory Phenotypes of Wildtype, *sss*^{P1}, and *DAT*^{fmn} Flies. The wildtype flies (n=48), *sss*^{P1} mutants (n=41), and *DAT*^{fmn} mutants (n=54) were tested for their ability to learn to make the association between appetitive stimulus and aversive taste. (c) Graphical scheme of the Proboscis Extension Reflex (PER) assy. (d) The flies' learning curve showing their response to stimulus at each stage of the training in percentages. (e) The flies' final test scores in percentage.

The Taste Discrimination Test **(Table 1)** verified the intact sensory perception of the mutants and confirmed that the phenotypic differences were attributed to learning and memory instead of taste defects. All flies tested correctly identified the appetitive stimulus fructose.

Table 1. Taste Discrimination Test Results for Wildtype and Mutant Flies. Wildtype (n=171), sss^{P1} mutants (n=50), and DAT^{fmn} mutants (n=52) were placed on food with two distinct color-labeled areas containing fructose alone or fructose with bitter quinine. The colors of their abdomen were examined to indicate taste preferences.

Wildtype		sss^{P1} Mutants		DAT^{fmn} Mutants	
Fructose	Quinine	Fructose	Quinine	Fructose	Quinine
171 (100%)	0 (0%)	50 (100%)	0 (0%)	52 (100%)	0 (0%)

Discussion

In the first set of experiments, I investigated the relationship between sleep and memory in animals with loss-of-function mutations in two functionally distinct genes. I hypothesized that a healthy sleep behavior would be positively correlated with intact memory, and that chronic lack of sleep would be associated with memory deficits, regardless of the underlying genetic lesion. My results are consistent with this hypothesis. Wildtype flies displayed a typical crepuscular sleep pattern as well as a high total sleep time. These flies consistently performed well on the learning and memory task. They demonstrated the ability to rapidly learn from the training trials and to retain the learned association.

In contrast, both sss^{P1} and DAT^{fmn} mutants exhibited low sleep and impaired gustatory associative memory. Chronic sleep deprivation is thus positively correlated with memory defects, and both QVR/SSS and DAT are required for normal sleep and memory. The memory phenotype of sss^{P1} mutants was more profound than that of DAT^{fmn} mutants, despite the fact that both

genotypes had roughly equivalent sleep phenotypes. Thus, *qvr/sss* and *DAT* may impact memory through effects on sleep-dependent as well as sleep-independent processes.

Two general models could explain sleep-dependent effects of *qvr/sss* and *DAT* on memory. In the first model, prior sleep could precondition the brain for proper memory formation. In this model, any mutation that disrupts sleep would be expected to disrupt memory, regardless of the molecular defect or neuroanatomical locus of the mutation's effect. This model would be difficult to test directly in flies. In the second model, memory could require prior sleep because the molecular and neuroanatomical pathways involved in both processes are linked. Arguing against this latter model, *qvr/sss* and *DAT* reduce cholinergic and dopaminergic signaling, respectively [19-20]; they do not genetically interact [W. Joiner, personal communication]; and they are thought to regulate sleep due to effects on distinct sets of neurons in the brain [20, 26]. However, these differences could still be reconciled with the second model if distinct *qvr/sss* neurons and *DAT* neurons were pre- and post-synaptic partners within a common circuit involved in sleep regulation. Evidence presented below supports this hypothesis.

Section II. Mapping Effects of *qvr/sss* to Neurons Involved in Sleep and Memory

Results

The Gal4-UAS system [27] was used to introduce a functional copy of the *qvr/sss* gene into subsets of neurons in otherwise deficient sss^{P1} mutants. This allowed us to map in which neurons *qvr/sss* expression is sufficient for sleep and/or associative memory. In each experiment crosses were performed to generate two controls, sss^{P1};Gal4/+ and sss^{P1};UAS-*sss*/+, and one experimental group, sss^{P1};Gal4/UAS-*sss*. Thus, if the Gal4 driver expresses in neurons in which *qvr*/*sss* is sufficient for sleep and/or memory, then only the experimental group should exhibit normal behavior.

The first driver I tested was *sss*-Gal4, which is known to rescue the sleep deficit of sss^{P1} mutants when combined with UAS-*sss* [19]. This phenotype was confirmed, as shown in **Figure 2a**. Flies of the same genotypes were then tested in the PER memory assay. Both mutant controls showed high response rates at around 85%, consistent with the absence of *qvr/sss*. In contrast, restoring *qvr/sss* expression using *sss*-Gal4/UAS-*sss* significantly reduced this response rate, though not fully to wildtype levels **(Figure 2b)**. These results confirm that under conditions of broad expression of *qvr/sss*, such as provided by the *sss*-Gal4 driver, sleep and memory phenotypes are indeed positively correlated.

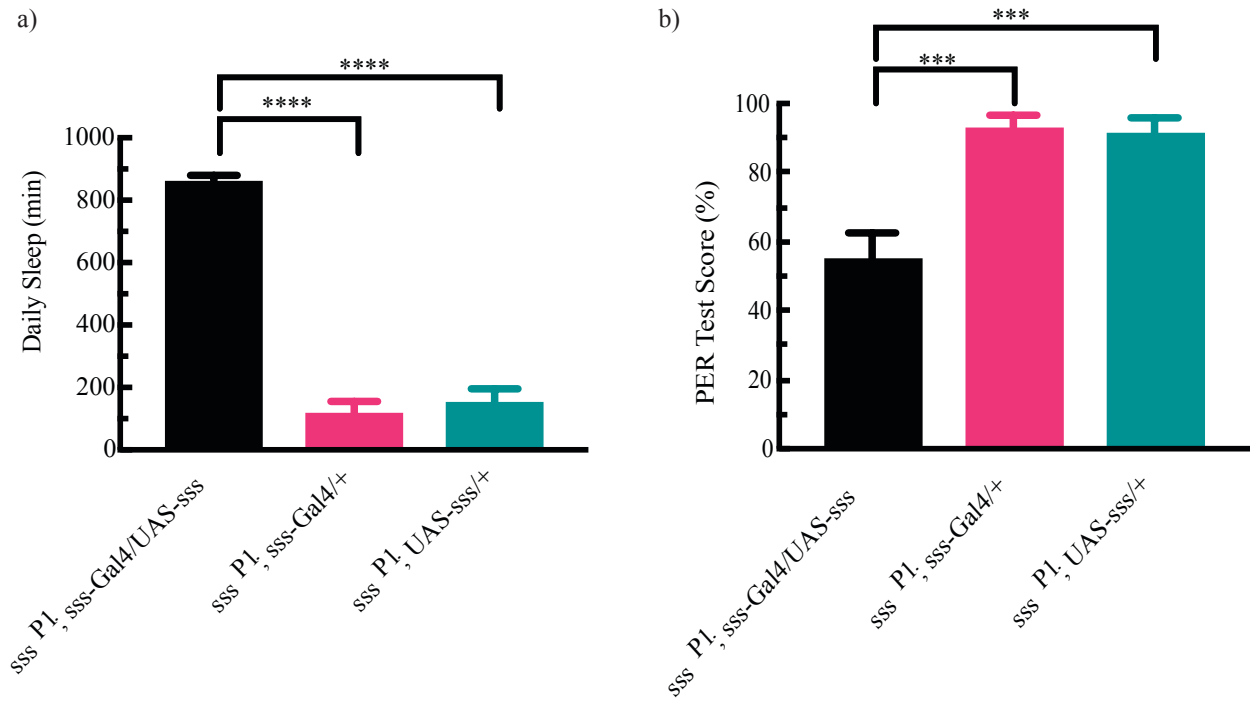


Figure 2a,b. Sleep and Memory Phenotypes of *sss*^{P1} Rescues by *sss*-Gal4>UAS-*sss*. (a) The sleep behaviors of *sss*-Gal4>UAS-*sss* (n=24), *sss*-Gal4/+ (n=12), and UAS-*sss*/+ (n=15) were recorded in the sleep monitor. The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (b) The *sss*-Gal4>UAS-*sss* (n=24), *sss*-Gal4/+ (n=12), and UAS-*sss*/+ (n=15) flies were tested in the PER assay and the test scores are shown in percentages.

Next I examined how closely the relationship between the two phenotypes is conserved using additional Gal4 drivers with varying degrees of expression throughout the brain. Two such drivers are 11E12- and 12C10-Gal4. These drivers were previously identified in a screen for neurons involved in sleep homeostasis [W. Joiner, personal communication]. As shown in **Figure 2c-f**, both drivers rescued the sleep and memory deficits of *sss*^{P1} mutants. To determine if overlapping or non-overlapping neurons were involved in each driver's effects we generated a split Gal4 line capable of expressing the *qvr/sss* transgene only at the overlap between the two parental drivers. In this case as well, the split Gal4 line was able to rescue both total sleep time and associative memory **(Figure 2g,h)**.

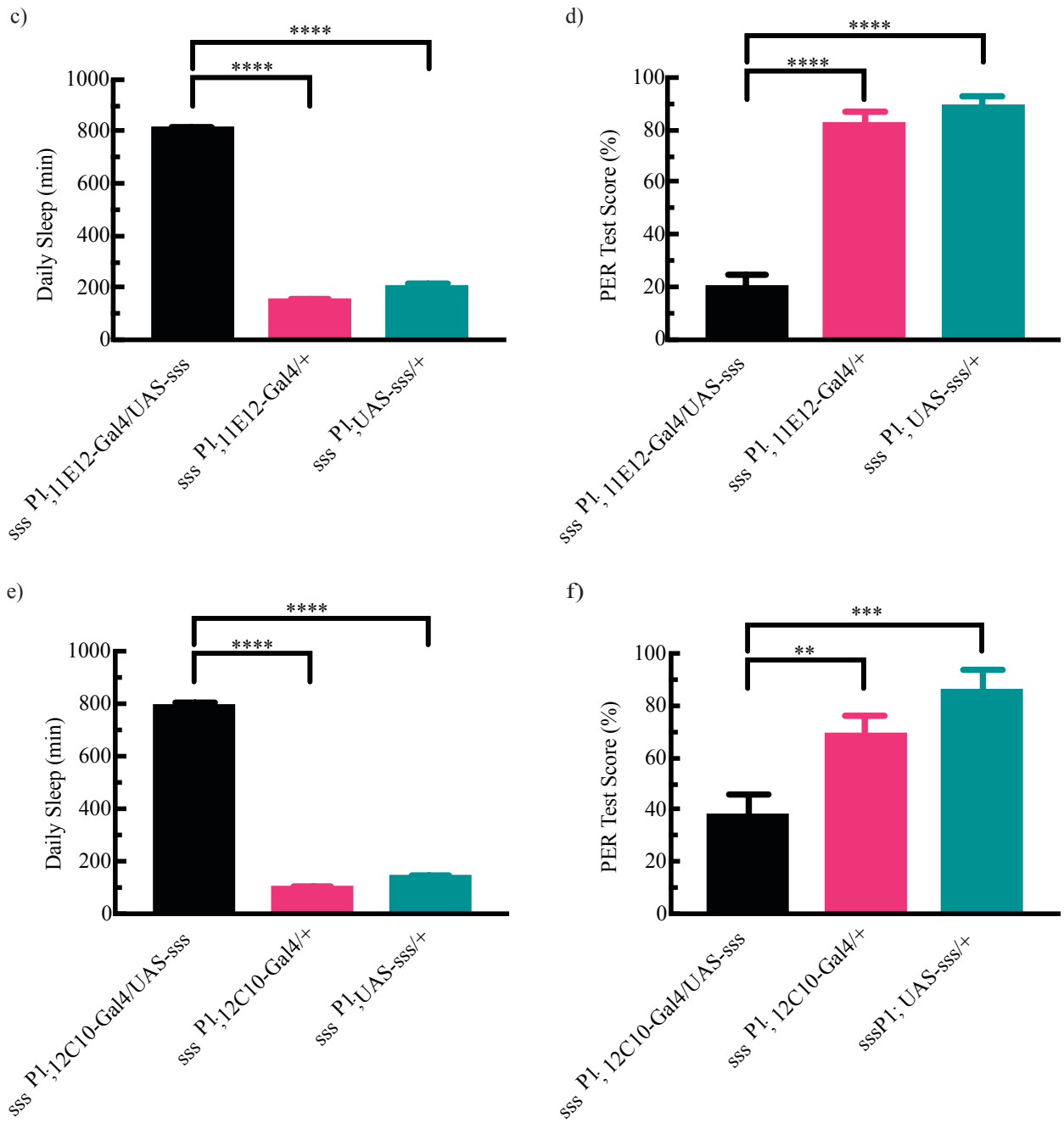


Figure 2c-f. Sleep and Memory Phenotypes of *sss*^{P1} Rescues by 11E12-Gal4>UAS-*sss* and 12C10-Gal4>UAS-*sss*. (c) The sleep behaviors of 11E12-Gal4>UAS-*sss* (n=40), 11E12-Gal4/+ (n=40), and UAS-*sss*/+ (n=32) were recorded in the sleep monitor. The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (d) The PER test scores of 11E12-Gal4>UAS-*sss* (n=54), 11E12-Gal4/+ (n=43), and UAS-*sss*/+ (n=22) are shown in percentages. (e) The sleep behaviors of 12C10-Gal4>UAS-*sss* (n=40), 12C10-Gal4/+ (n=32), and UAS-*sss*/+ (n=49) were recorded in the sleep monitor. The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (f) The PER test scores of 12C10-Gal4>UAS-*sss* (n=31), 12C10-Gal4/+ (n=28), and UAS-*sss*/+ (n=19) are shown in percentages.

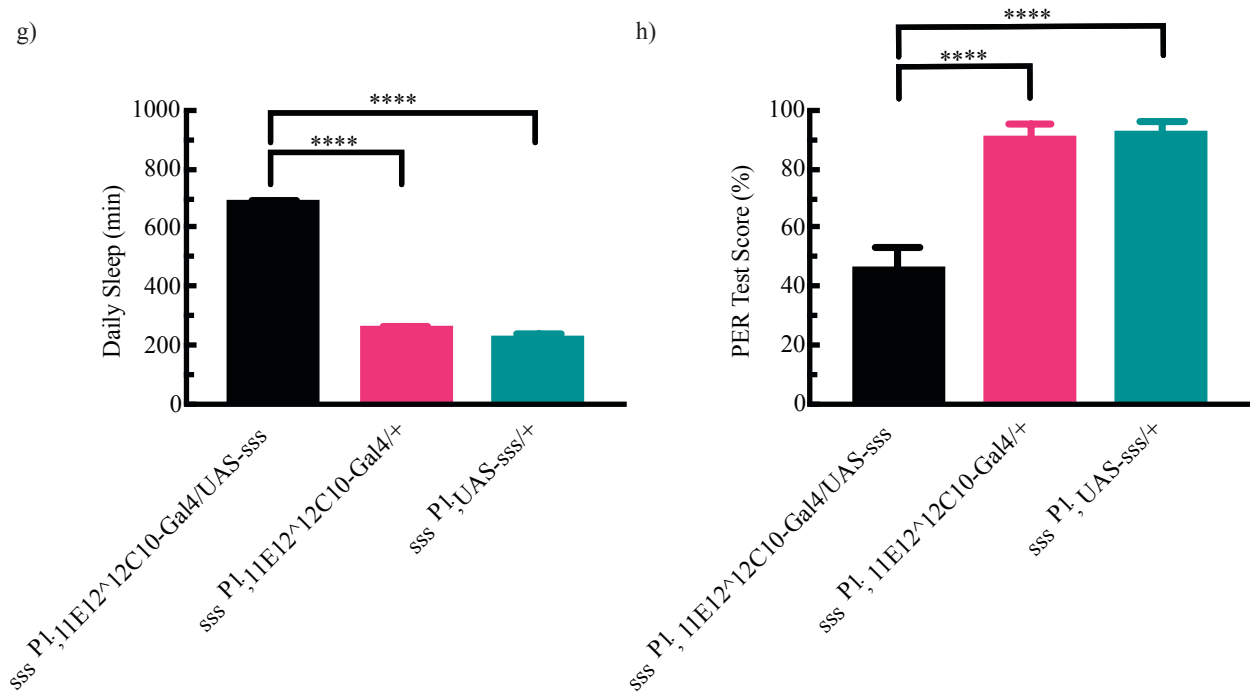


Figure 2g,h. Sleep and Memory Phenotypes of *sss*^{P1} Rescues by 11E12^12C10-Gal4>UAS-*sss*. (g) The sleep behaviors of 11E12^12C10-Gal4>UAS-*sss,* 11E12^12C10-Gal4/+, and UAS-*sss*/+ were recorded in the sleep monitor (n=69, 50, 42 respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (h) The PER test scores of 11E12^12C10-Gal4>UAS-*sss,* 11E12^12C10-Gal4/+, and UAS-*sss*/+ are shown in percentages (n=39, 28, 31 respectively).

Two additional Gal4 drivers that I tested also rescued the sleep and memory deficits of *sss*^{P1} mutants. These were 109(2)80-Gal4 and piezo-Gal4 **(Figures 2i-l)**. Interestingly, just as with 11E12-Gal4 and 12C10-Gal4, these drivers were also previously identified in a screen for neurons involved in sleep homeostasis [W. Joiner, personal communication]. This correlation suggested that perhaps additional drivers with more restricted expression that emerged from the same screen, such as ppk-Gal4 [25], might be sufficient to rescue the phenotypes of *sss*^{P1} mutants as well. However, this was not the case [W. Joiner, personal communication].

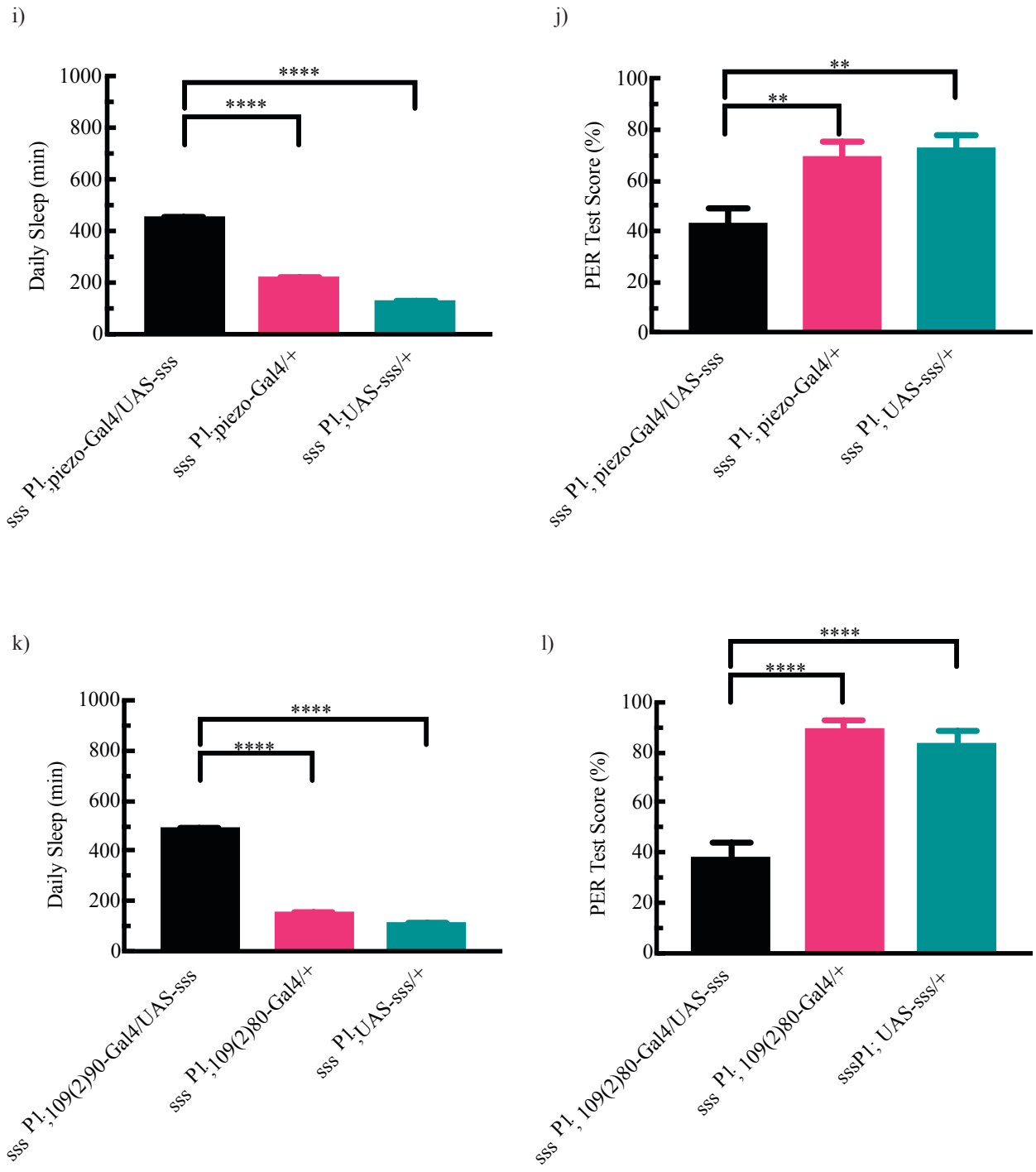


Figure 2i-l. Sleep and Memory Phenotypes of *sss*^{P1} Rescues by piezo-Gal4>UAS-*sss* and 109(2)80-Gal4>UAS-*sss*. (i) The sleep behaviors of piezo-Gal4>UAS-*sss*, piezo-Gal4/+, and UAS-*sss*/+ were recorded in the sleep monitor (n=31, 31, 32 respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (j) The rescue effect of piezo-Gal4>UAS-*sss* on memory phenotype in *sss*^{P1} mutants are shown in PER percentages (n=37, 37, 35 respectively). (k) The sleep behaviors of 109(2)80-Gal4>UAS-*sss*, 109(2)80-Gal4/+, and UAS-*sss*/+ were recorded in the sleep monitor (n=40, 31, 35, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (l) The rescue effect of 109(2)80-Gal4>UAS-*sss* on memory phenotype in *sss*^{P1} mutants are shown in PER percentages (n=37, 37, 35 respectively).

Discussion

To test whether the effects of *qvr/sss* on sleep and memory map to the same neurons, I restored expression of *qvr/sss* to different neurons in sss^{P1} mutants. I found that drivers with purportedly broad expression, such as *sss*-, 11E12- and 12C10-Gal4s, as well as drivers I expected would have more restricted expression, such as 11E12^12C10-, piezo-, and 109(2)80-Gal4s, all rescued both phenotypes. Interestingly, all these drivers have previously been shown by my lab to label neurons capable of eliciting sleep homeostasis following thermogenetic activation. This suggests that *qvr/sss* may be required in a subset of these neurons to enable proper functioning of the homeostat, thus leading to drastically reduced sleep in sss^{P1} mutants.

It is also interesting that 109(2)80 and piezo neurons seem to be important neuronal loci for the sleep and memory effects of *qvr/sss*. These neurons are thought to be involved in mechanosensation. Thus, perhaps in the absence of *qvr/sss* these neurons provide tonic mechanosensory arousal to the brain, which keeps animals awake. However, I cannot rule out the possibility that these drivers may also express in the central nervous system where *qvr/sss* may also be relevant. To distinguish between these possibilities future studies may need to examine the overlapping expression patterns of at least several of the drivers above that rescue the sss^{P1} behavioral phenotypes.

Section III. Identification of the Dopamine Receptor and the Neurons in Which it Functions to Mediate the Sleep and Memory Deficits of *DAT^{fmn}* Mutants

Results

The *DAT^{fmn}* mutants have defects in sleep and memory functions due to excessive dopamine signaling. There are four dopamine receptors in flies (Dop1R1, Dop1R2, Dop2R, and DopEcR), each of which could contribute to these phenotypes. Previous studies have suggested that Dop1R1 in the fan-shaped body is required to reduce sleep in *DAT^{fmn}* mutants [21-22]. To test this hypothesis we knocked down Dop1R1 using the same fan-shaped body driver, 104y, that was used in previous studies. We found that sleep was not significantly restored to *DAT^{fmn}* mutants **(Figure 3a)**. We next proceeded to knock down each of the four dopamine receptors in *DAT^{fmn}* mutants using the broad-expressing 11E12-Gal4 driver that rescued sleep and memory deficits in *sss^{P1}* mutants when coupled to UAS-*sss*. We found that while sleep was significantly restored with knockdown of Dop1R1, the effect was very weak **(Figure 3b)**. Furthermore, the same genetic manipulation failed to restore memory significantly in *DAT^{fmn}* mutants **(Figure 3c).** Although other explanations are possible (see Discussion), the most parsimonious interpretation of these data is that Dop1R1 is not a major contributor to dopamine-dependent regulation of sleep and memory deficits in *DAT^{fmn}* mutants.

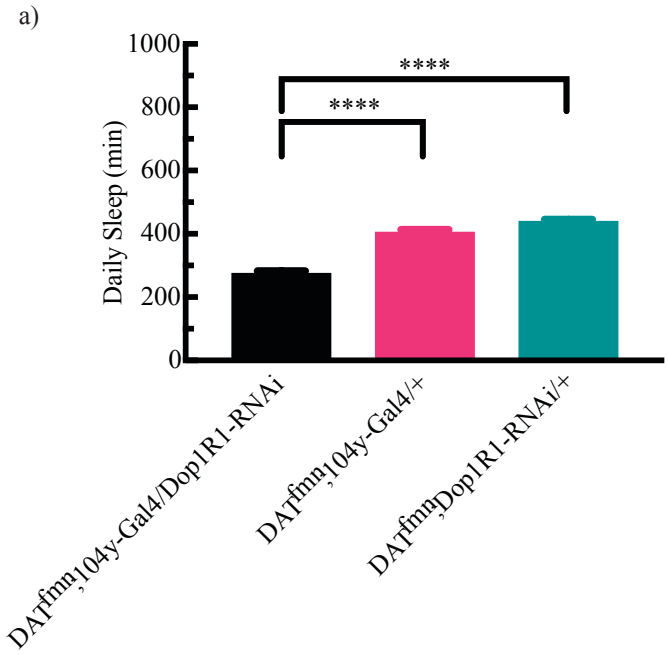


Figure 3a. Sleep and Memory Phenotypes of *DAT^{fmn}* Rescues by 104y-Gal4>Dop1R1-RNAi. The sleep behaviors of 104y-Gal4>Dop1R1-RNAi, 104y-Gal4/+, and Dop1R1-RNAi/+ flies were recorded in the sleep monitor (n=16, 16, 23, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes.

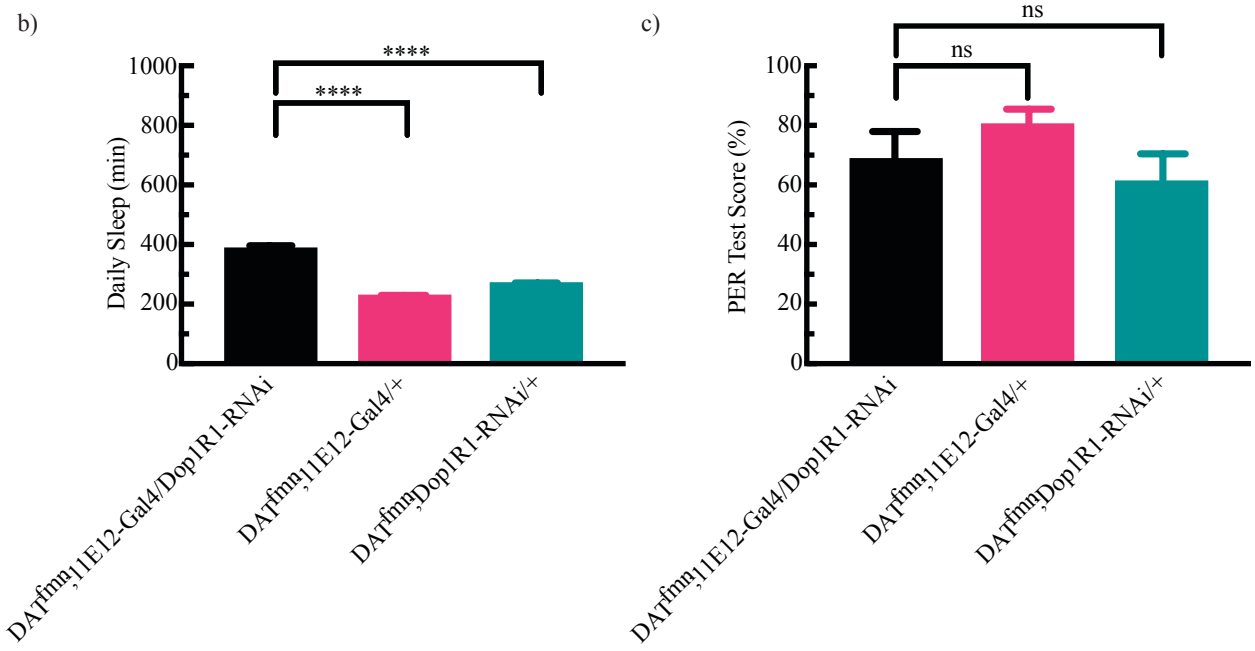


Figure 3b,c. Sleep and Memory Phenotypes of *DAT^{fmn}* Rescues by 11E12-Gal4>Dop1R1-RNAi. (b) The sleep behaviors of 11E12-Gal4>Dop1R1-RNAi, 11E12-Gal4/+, and Dop1R1-RNAi/+ were recorded in the sleep monitor (n=31, 63, 50, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (c) The rescue effect of 11E12-Gal4>Dop1R1-RNAi on memory phenotype in *DAT^{fmn}* mutants are shown in PER percentages (n=17, 19, 18, respectively).

Qualitatively similar results were also obtained for the knockdown of Dop2R and DopEcR. In both cases reducing expression of the particular dopamine receptor in question using the broadly expressing 11E12-Gal4 driver led to a significant but small increase in sleep and no restoration of associative memory in DAT^{fmn} mutants **(Figures 3d-g).** In contrast, using the same driver and either of two RNAi's against Dop1R2 led to a large, significant rescue of sleep and associative memory in DAT^{fmn} mutants **(Figure 3h-k)**. The simplest interpretation of these results is that Dop1R2 is the major mediator of dopamine-mediated waking and the deficit in associative memory that occurs with chronically elevated dopamine signaling.

Next we tested other drivers that rescued sleep and memory deficits in sss^{P1} when combined with UAS-*sss*. Surprisingly, piezo-Gal4-driven knockdown of Dop1R2 rescued the sleep and memory deficits in DAT^{fmn} mutants **(Figures 3l-o).** Notably, the piezo-Gal4 driver expresses much more sparsely than 11E12-Gal4. Furthermore, like 11E12-Gal4, piezo-Gal4 does not appear to label the fan-shaped body **(Figure S1)**. Thus, like *qvr*/*sss*, Dop1R2 seems to function in a limited number of neurons outside the fan-shaped body to regulate sleep and associative memory.

Furthermore, we knocked down Dop1R2 in the 11E12 neurons in sss^{P1} mutants and also achieved significant rescue of the sleep and memory phenotypes **(Figure 3p,q).** That is, eliminating the receptor responsible for the phenotypes of DAT^{fmn} mutants reversed the effects caused by the lack of QVR/SSS in sss^{P1} mutants. This result suggests that signaling in both DAT^{fmn} and sss^{P1} mutants shares at least part of a common neural circuit that regulates arousal and memory.

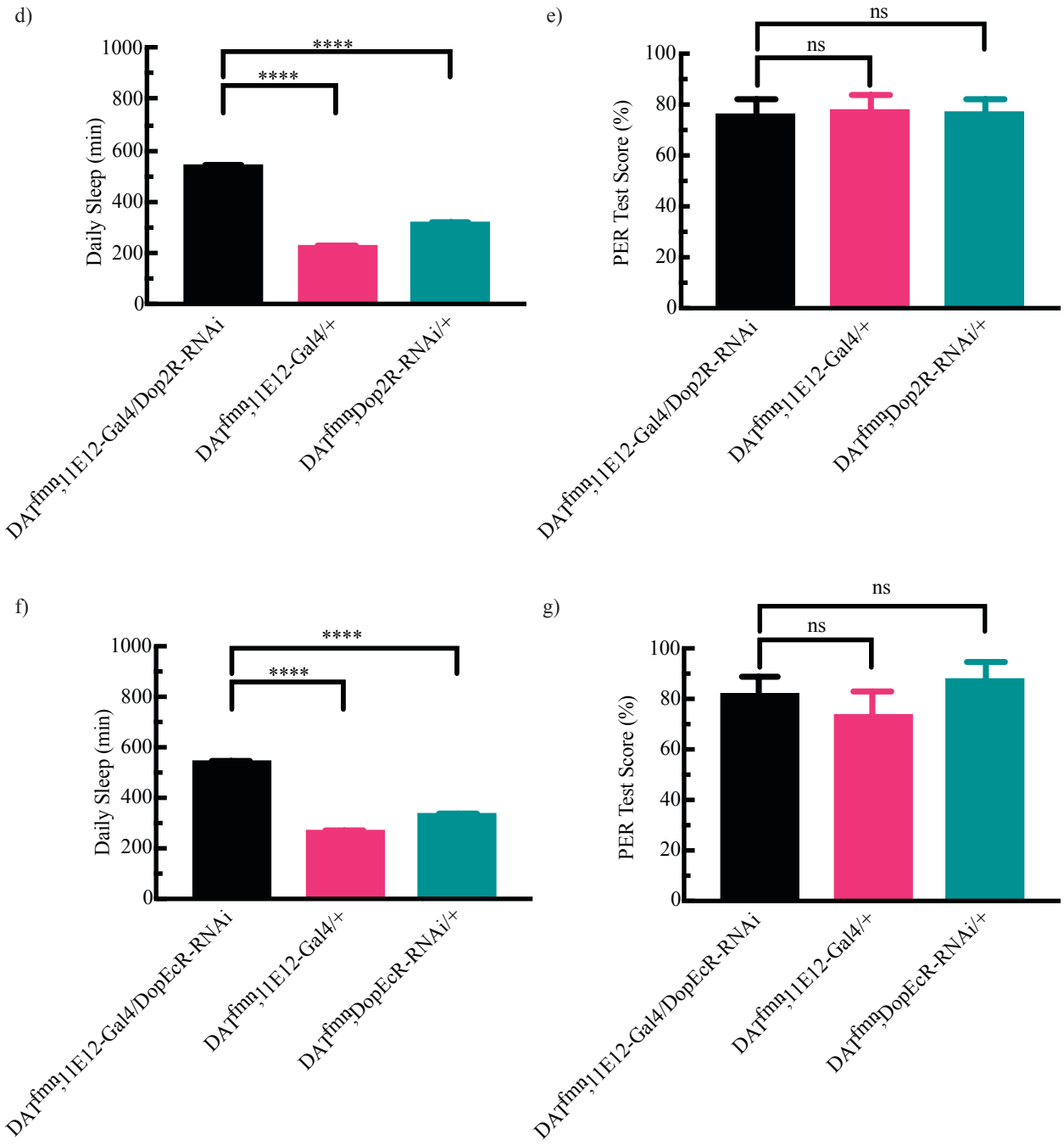


Figure 3d-g. Sleep and Memory Phenotypes of *DAT^{fmn}* Rescues by 11E12-Gal4>Dop2R-RNAi and 11E12-Gal4>DopEcR-RNAi. (d) The sleep behaviors of 11E12-Gal4>Dop2R-RNAi, 11E12-Gal4/+, and Dop2R-RNAi/+ were recorded in the sleep monitor (n=31, 28, 32, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (e) The rescue effect of 11E12-Gal4>Dop12R-RNAi on memory phenotype in *DAT^{fmn}* mutants are shown in PER percentages (n=34, 32, 33, respectively). (f) The sleep behaviors of 11E12-Gal4>DopEcR-RNAi, 11E12-Gal4/+, and DopEcR-RNAi/+ were recorded in the sleep monitor (n=37, 42, 48, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (g) The rescue effect of 11E12-Gal4>DopEcR-RNAi on memory phenotype in *DAT^{fmn}* mutants are shown in PER percentages (n=11, 10, 11, respectively)

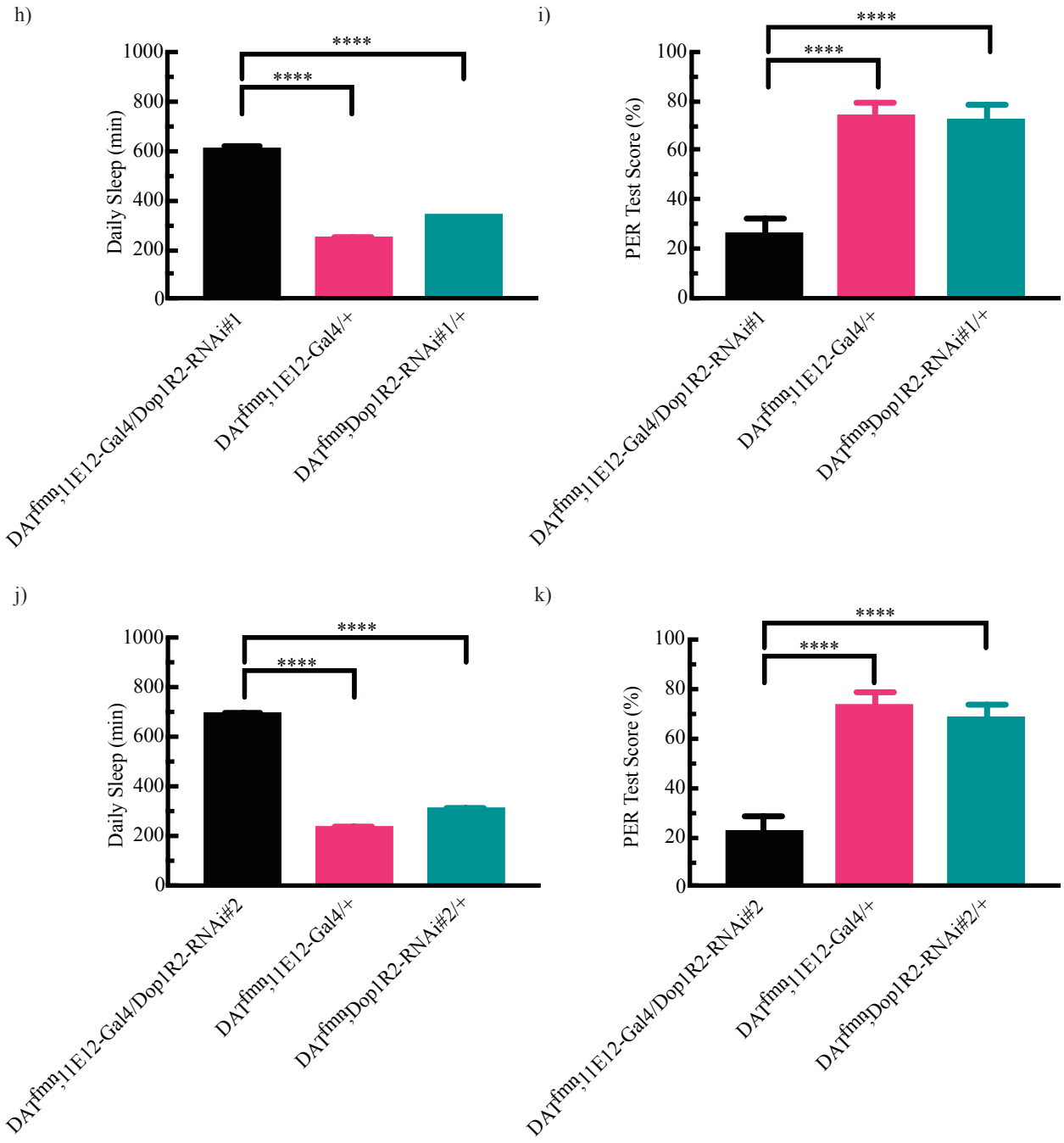


Figure 3h-k. Sleep and Memory Phenotypes of *DAT^{fmn}* Rescues by 11E12-Gal4>Dop1R2-RNAi #1 and #2. (h) The sleep behaviors of 11E12-Gal4>Dop1R2-RNAi#1, 11E12-Gal4/+, and Dop1R2-RNAi#1/+ were recorded in the sleep monitor (n=35, 59, 62, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (i) The rescue effect of 11E12-Gal4>Dop1R2-RNAi#1 on memory phenotype in *DAT^{fmn}* mutants are shown in PER percentages (n=32, 32, 34, respectively). (j) The sleep behaviors of 11E12-Gal4>Dop1R2-RNAi#2, 11E12-Gal4/+, and Dop1R2-RNAi#2/+ were recorded in the sleep monitor (n=31, 48, 43, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (k) The rescue effect of 11E12-Gal4>Dop1R2-RNAi#2 on memory phenotype in *DAT^{fmn}* mutants are shown in PER percentages (n=30, 33, 34, respectively).

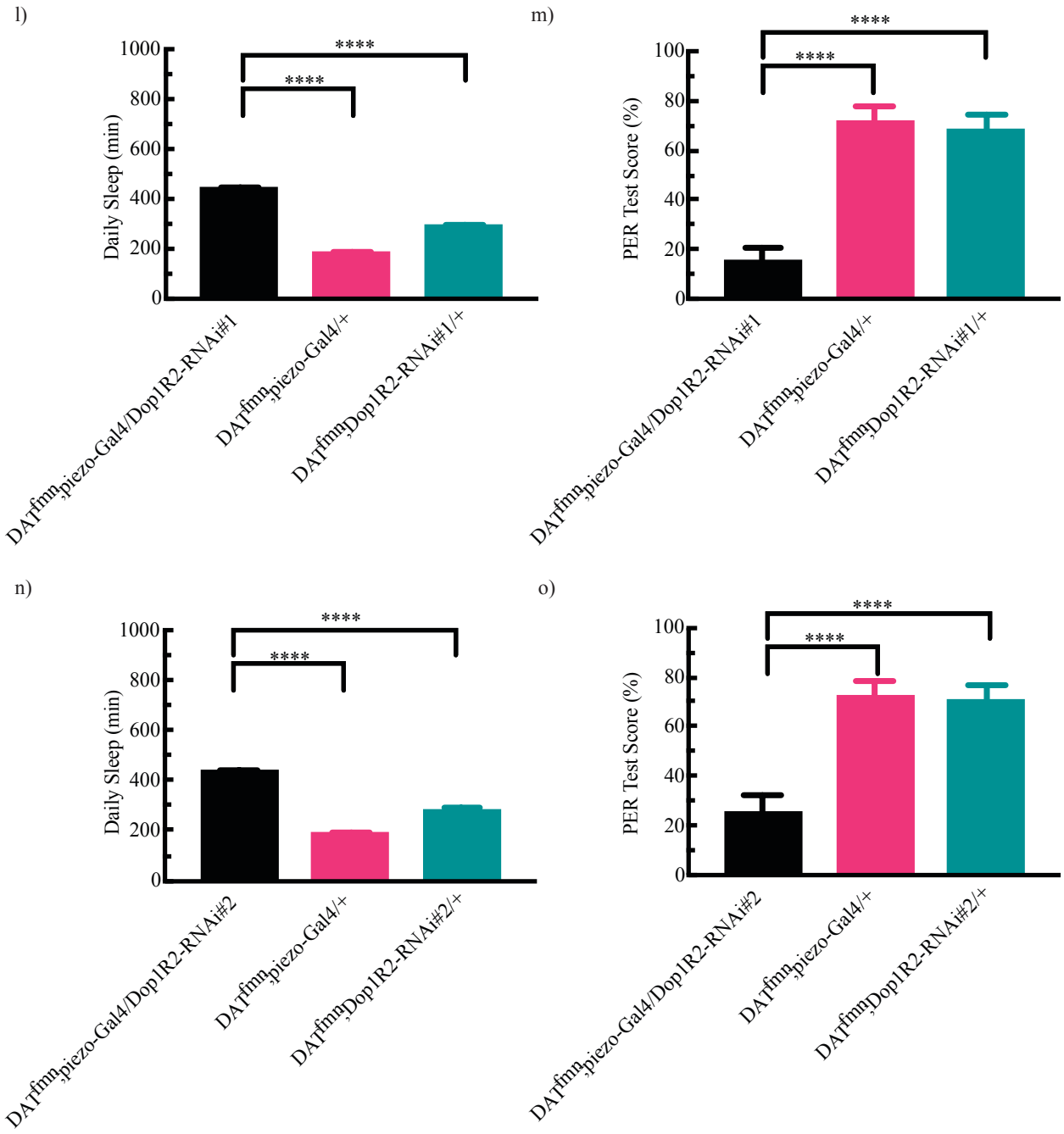


Figure 3l-o. Sleep and Memory Phenotypes of *DAT^{fmn}* Rescues by piezo-Gal4>Dop1R2-RNAi #1 and #2. (l) The sleep behaviors of piezo-Gal4>Dop1R2-RNAi#1, piezo-Gal4/+, and Dop1R2-RNAi#1/+ were recorded in the sleep monitor (n=35, 44, 47, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (m) The rescue effect of piezo-Gal4>Dop1R2-RNAi#1 on memory phenotype in *DAT^{fmn}* mutants are shown in PER percentages (n=31, 34, 31, respectively). (n) The sleep behaviors of piezo-Gal4>Dop1R2-RNAi#2, piezo-Gal4/+, and Dop1R2-RNAi#2/+ were recorded in the sleep monitor (n=30, 48, 37, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (o) The rescue effect of piezo-Gal4>Dop1R2-RNAi#2 on memory phenotype in *DAT^{fmn}* mutants are shown in PER percentages (n=28, 25, 25, respectively).

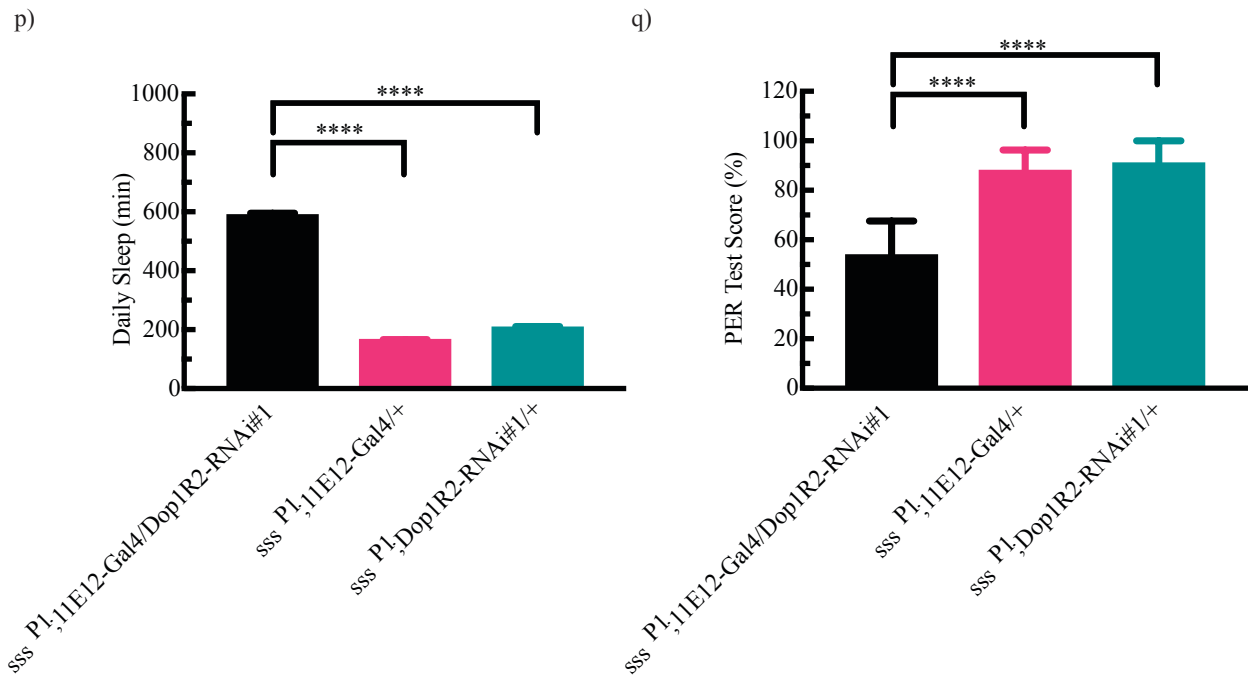


Figure 3p,q. Sleep and Memory Phenotypes of *sss*^{P1} Rescues by 11E12-Gal4>Dop1R2-RNAi #1. (p) The sleep behaviors of 11E12-Gal4>Dop1R2-RNAi#1, 11E12-Gal4/+, and Dop1R2-RNAi#1/+ were recorded in the sleep monitor (n=36, 44, 37, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (q) The rescue effect of 11E12-Gal4>Dop1R2-RNAi#1 on memory phenotype in *sss*^{P1} mutants. The PER test scores are shown in percentages (n=10, 8, 7, respectively).

Discussion

My earlier experiments established that *DAT*^{fmn} mutants have deficits in both sleep and associative memory. The phenotypes of the *DAT*^{fmn} mutants are attributed to a loss of function mutation in the presynaptic dopamine transporter (DAT), which is expected to lead to high levels of synaptic dopamine and thus excessive signaling through postsynaptic dopamine receptors. Several previous reports suggested that expression in the dFSBs of one such receptor, Dop1R1, is responsible for the low sleep of *DAT*^{fmn} mutants [21-22]. However, we were unable to rescue this phenotype by knocking down Dop1R1 with the 104y driver that was used in published studies. This suggests that Dop1R1 may not be the receptor mediating the downstream effects of

the *DAT* mutation and that the dFSBs may not be involved in the regulatory pathway. Another study suggested that Dop1R2 regulates sleep by modulating Shaker and Sandman potassium channels in the dFSBs [23]. However, we did not see any effects on sleep by manipulating these potassium channels in the dFSBs [W. Joiner, personal communication]. To confirm that the dFSBs are indeed not involved in this regulatory circuit, further experiments need to be conducted to see the effects of knocking down Dop1R2 with the 23E10-Gal4 driver used in the study.

In a more unbiased approach we knocked down each of the four dopamine receptors in flies with the broadly expressing 11E12-Gal4 driver and tested whether sleep and memory could be restored in DAT^{fmn} mutants. We found that reducing signaling through Dop1R1, Dop2R or DopEcR had small effects on sleep but no effect on memory. Thus these dopamine receptors may make small contributions to dopaminergic regulation of sleep, but they are likely not involved in the neural circuit that regulates both sleep and memory. Only Dop1R2 seems to be involved in this coordinated function since only knockdown of this receptor restored sleep nearly to wildtype levels and significantly rescued associative memory. I also found that knockdown of Dop1R2 with sparsely expressing drivers such as piezo-Gal4 significantly restored sleep and associative memory to DAT^{fmn} mutants. In Section II I demonstrated that reintroduction of *qvr*/*sss* into neurons labeled by these same drivers can rescue the sleep and memory deficits of sss^{P1} mutants. The collective results suggest that DAT^{fmn} reduces sleep and associative memory by over-activating Dop1R2 in an as-yet unidentified locus outside the fan-shaped body where *qvr*/*sss* also functions to regulate the same behaviors. In the next section I will describe efforts to characterize additional molecules that may function in the same neurons.

Section IV. Contribution of Additional Molecules to Neurons that Regulate Sleep and Associative Memory

Results

Experiments described in previous sections demonstrated that the Gal4>UAS-*sss* combinations that restore sleep and associative memory to sss^{P1} mutants involve the same drivers as the Gal4>Dop1R2 RNAi combinations that restore sleep and associative memory to DAT^{fmn} mutants. Collectively these data suggest that QVR/SSS and Dop1R2 may function in the same neurons to regulate both sleep and memory formation. However, the functional relationship between QVR/SSS and Dop1R2 remains unclear. In this section I describe two additional molecules, Dα3 and dNACHO, and their possible roles in sleep and memory regulation in sss^{P1} and DAT^{fmn} mutants.

Previous work in my lab demonstrated that QVR/SSS forms a stable complex with and likely reduces the activity of Dα3 nAChRs to promote sleep. Since my results from previous sections suggested that Dop1R2 functions in the same neurons as *qvr/sss* to promote waking, I hypothesized that Dα3 is a terminal effector of either a linear pathway or two converging pathways involving opposing effects of Dop1R2 and QVR/SSS. To test this hypothesis I assayed sleep and associative memory in sss^{P1} and DAT^{fmn} mutants in which Dα3 was knocked down with different drivers. I found that both phenotypes could be rescued in sss^{P1} mutants using 11E12^12C10 split-Gal4, piezo-Gal4 and 109(2)80-Gal4 **(Figures 4a-f).** Similarly, I found that both phenotypes could also be rescued in DAT^{fmn} mutants using 11E12-Gal4 and piezo-Gal4 **(Figures 4g-j).** Thus, knocking down Dα3 was phenotypically equivalent to restoring *qvr/sss* and reducing Dop1R2 in targeted neurons of sss^{P1} and DAT^{fmn} mutants, respectively. A possible interpretation of these data is that Dα3 functions downstream of QVR/SSS and modulates the

effects of acetylcholine in a limited population of neurons involved in regulating sleep and associative memory; in the same set of neurons, Dop1R2 functions in parallel to Dα3 to mediate the effects of dopamine.

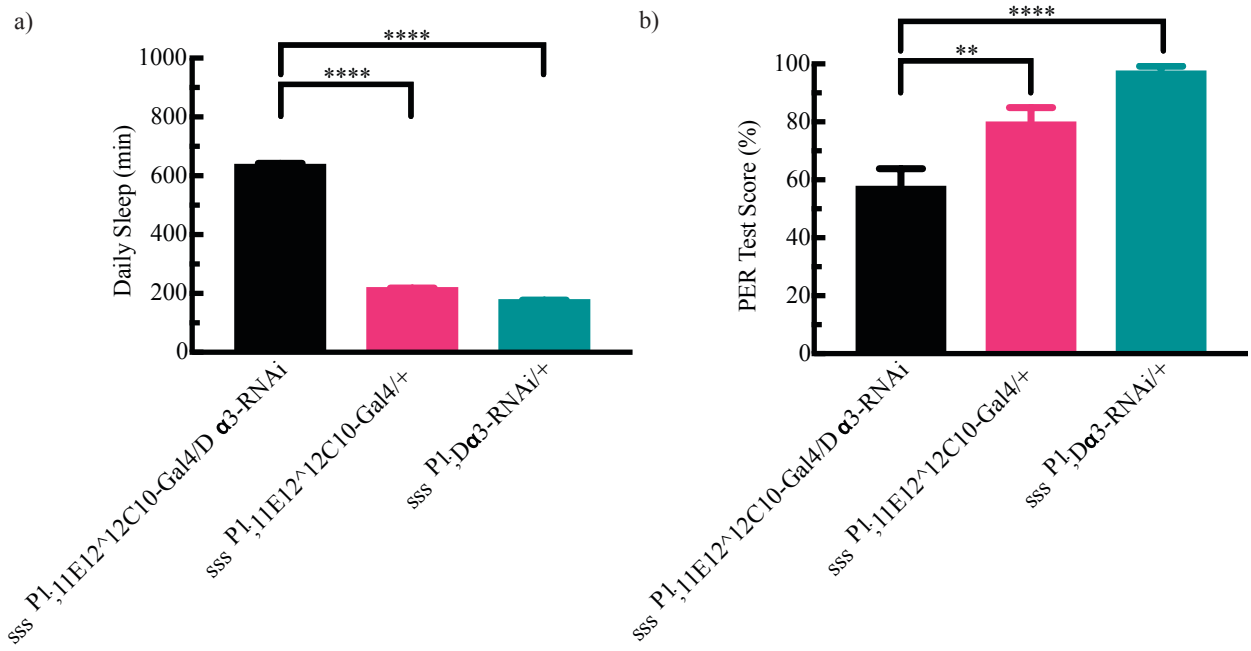


Figure 4a,b. Sleep and Memory Phenotypes of *sss*^{P1} Rescues by 11E12^12C10-Gal4>Dα3-RNAi.
(a) The sleep behaviors of 11E12^12C10-Gal4>Dα3-RNAi, 11E12^12C10-Gal4/+, and Dα3-RNAi /+ were recorded in the sleep monitor (n=29, 47, 45, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (b) The rescue effect of 11E12^12C10-Gal4>Dα3-RNAi on memory phenotype in *sss*^{P1} mutants are shown in percentages (n= 39, 34, 34, respectively).

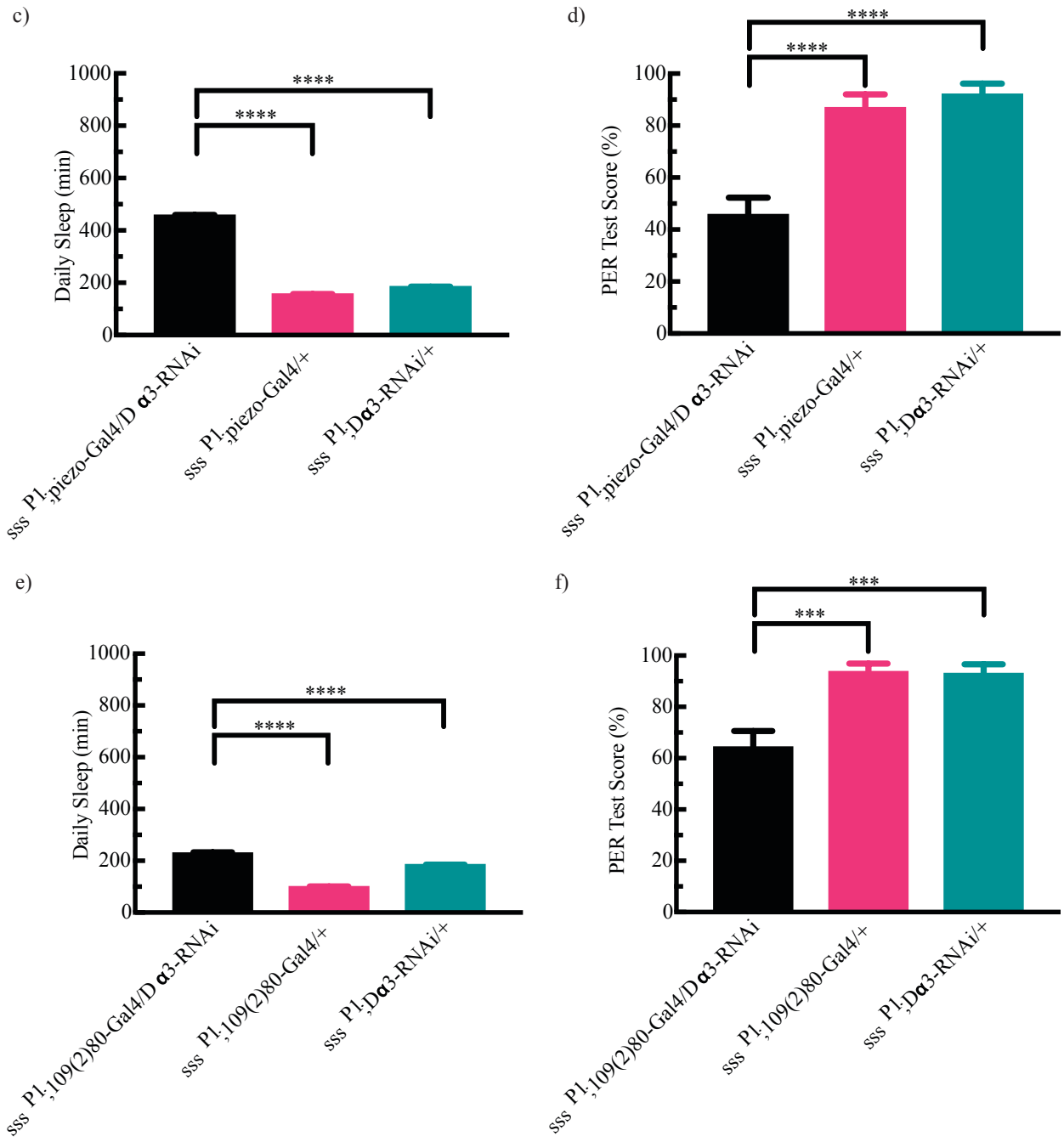


Figure 4c-f. Sleep and Memory Phenotypes of *sss*^{P1} Rescues by piezo-Gal4>Dα3-RNAi and 109(2)80-Gal4>Dα3-RNAi. (c) The sleep behaviors of piezo-Gal4>Dα3-RNAi, piezo-Gal4/+, and Dα3-RNAi /+ were recorded in the sleep monitor (n=47, 43, 56, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (d) The rescue effect of piezo-Gal4>Dα3-RNAi on memory phenotype in *sss*^{P1} mutants are shown in PER percentages (n=36, 32, 32, respectively). (e) The sleep behaviors of 109(2)80-Gal4>Dα3-RNAi, 109(2)80-Gal4/+, and Dα3-RNAi/+ were recorded in the sleep monitor (n=33, 37, 56, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (f) The rescue effect of 109(2)80-Gal4>Dα3-RNAi on memory phenotype in *sss*^{P1} mutants are shown in PER percentages (n=37, 35, 36, respectively).

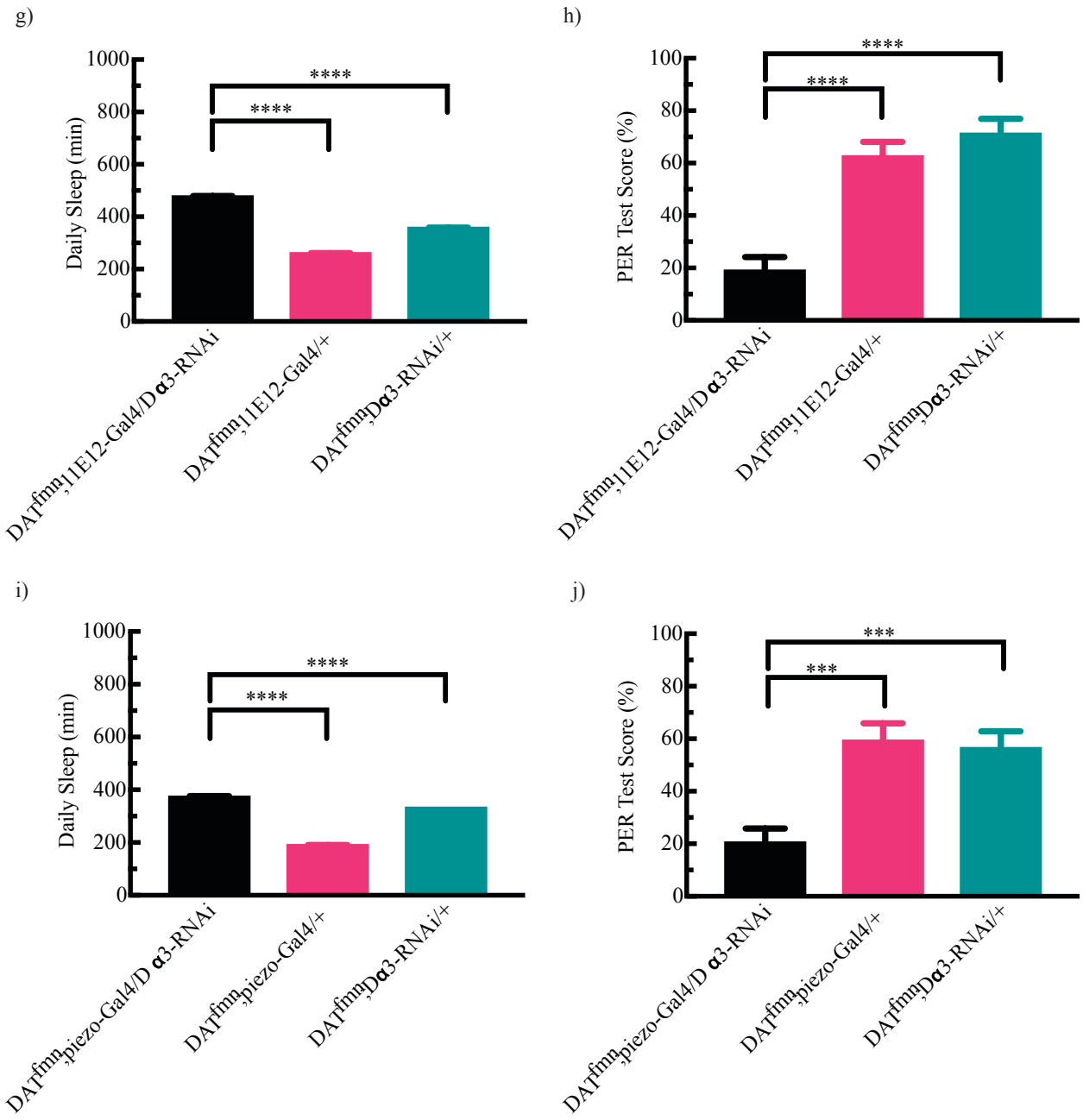


Figure 4g-j. Sleep and Memory Phenotypes of *DAT^{fmn}* Rescues by 11E12-Gal4>Dα3-RNAi and piezo-Gal4>Dα3-RNAi. (g) The sleep behaviors of 11E12-Gal4>Dα3-RNAi, 11E12-Gal4/+, and Dα3-RNAi /+ were recorded in the sleep monitor (n=41, 43, 40, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (h) The rescue effect of 11E12-Gal4>Dα3-RNAi on memory phenotype in *DAT^{fmn}* mutants shown in percentages (n=32, 31, 31, respectively). (i) The sleep behaviors of piezo-Gal4>Dα3-RNAi, piezo-Gal4/+, and Dα3-RNAi/+ were recorded in the sleep monitor (n=36, 48, 43, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (j) The rescue effect of piezo-Gal4>Dα3-RNAi on memory phenotype in *DAT^{fmn}* mutants are shown in percentages (n=33, 35, 35, respectively).

Recently in mammals another protein called NACHO has been shown to promote assembly and activity of nAChRs [28-29]. My lab cloned the fly ortholog, called dNACHO, and showed that it has the same function as its mammalian counterpart (data not shown). Since dNACHO seems to function in opposition to QVR/SSS, we hypothesized that reducing dNACHO might lower Dα3 activity and thus compensate for Dα3 disinhibition in sss^{P1} mutants. To test this hypothesis we knocked down dNACHO in sss^{P1}/sss^{P2} transheterozygotes and assayed sleep in these animals. Consistent with a role for dNACHO opposing the function of QVR/SSS, knockdown of dNACHO restored sleep to *sss* mutants **(Figure 4k).** Furthermore, we found that knockdown of dNACHO in the same neurons restored sleep and associative memory to DAT^{fmn} mutants **(Figure 4l-o).** Collectively these data support the hypothesis that dNACHO antagonizes the function of *qvr/sss* by promoting Dα3 activity in wake-promoting neurons that are also involved in associative memory.

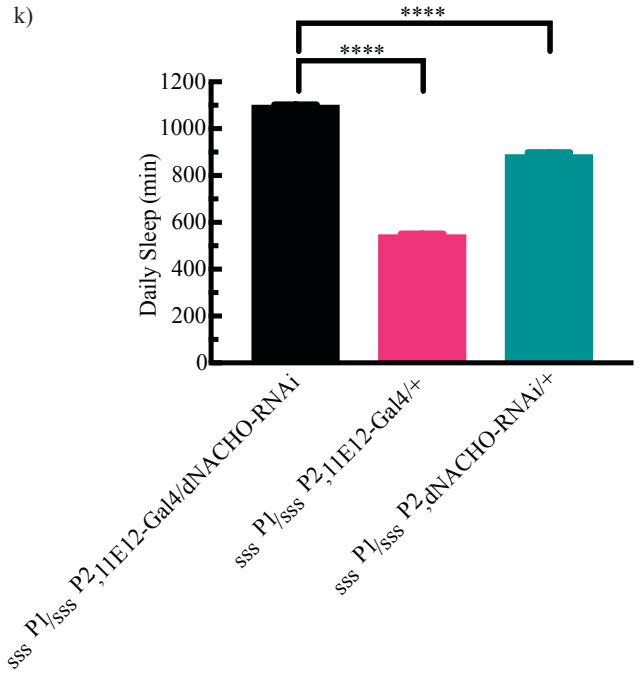


Figure 4k. Sleep Phenotype of sss^{P1}/sss^{P2} Transheterozygote Rescues by 11E12-Gal4>dNACHO-RNAi. The sleep behaviors of sss^{P1}/sss^{P2} ;11E12-Gal4>dNACHO-RNAi and its controls were recorded in the sleep monitor (n=18, 21, 8, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes.

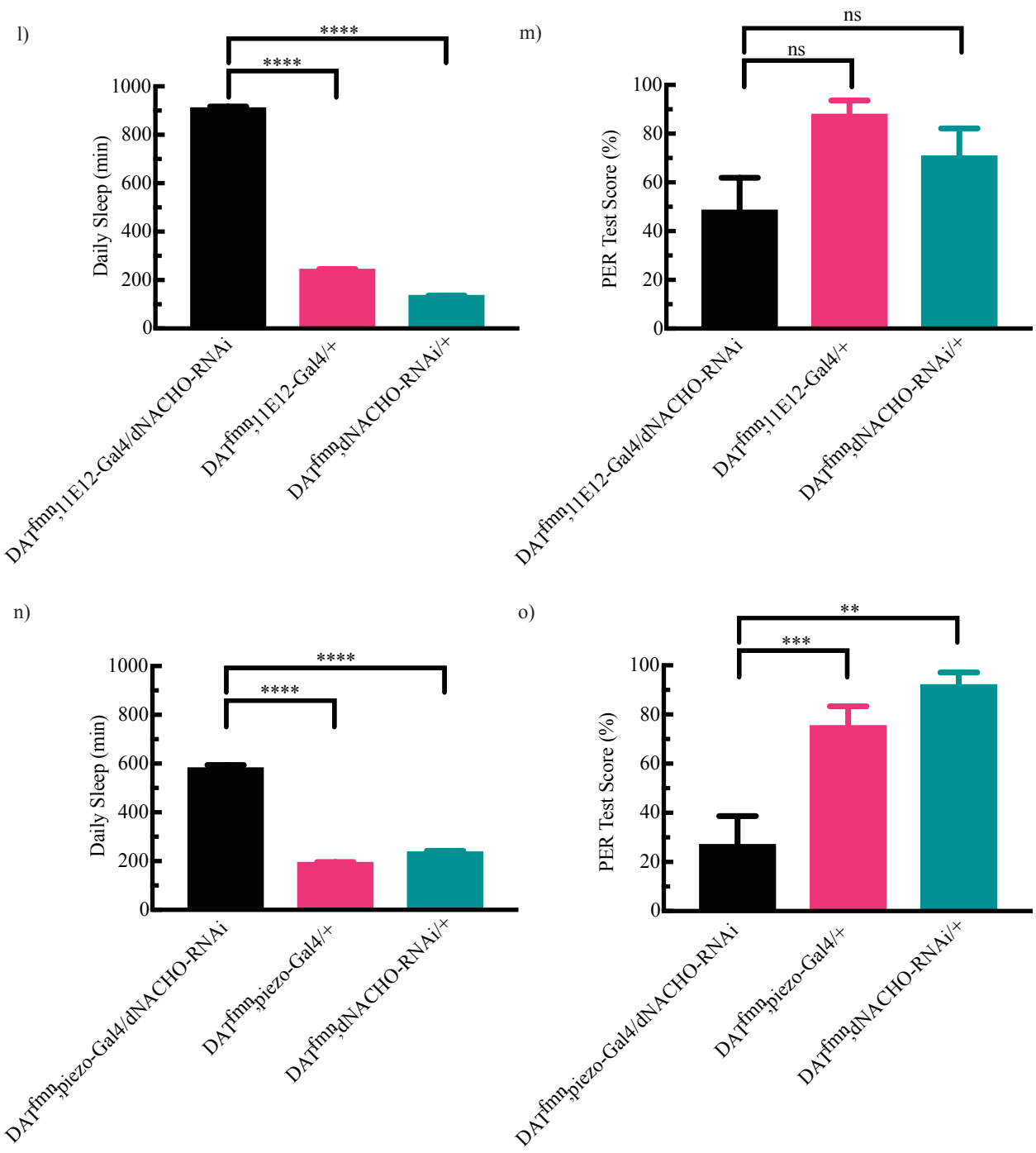


Figure 4l-o. Sleep and Memory Phenotypes of *DAT^{fmn}* Rescues by 11E12-Gal4>dNACHO-RNAi and piezo-Gal4>dNACHO-RNAi. (l) The sleep behaviors of 11E12-Gal4>dNACHO-RNAi, 11E12-Gal4/+, and dNACHO-RNAi/+ were recorded in the sleep monitor (n=14, 31, 31, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (m) The rescue effect of 11E12-Gal4>dNACHO-RNAi on memory phenotype in *DAT^{fmn}* mutants are shown in PER percentages (n=9, 8, 9, respectively). (n) The sleep behaviors of piezo-Gal4>dNACHO-RNAi, piezo-Gal4/+, and dNACHO-RNAi/+ were recorded in the sleep monitor (n=12, 24, 23, respectively). The rescue effect on the sleep phenotype is shown as the total amount of daily sleep in minutes. (o) The rescue effect of piezo-Gal4>dNACHO-RNAi on memory phenotype in *DAT^{fmn}* mutants are shown in PER percentages (n=10, 8, 8, respectively).

Discussion

Evidence suggests that QVR/SSS reduces the levels and activity of Dα3 nAChRs at the cell surface. Thus, in sss^{P1} mutants, Dα3 nAChRs are thought to be disinhibited, leading to elevated cholinergic-driven arousal [19]. However, it is not known where in the nervous system QVR/SSS-Dα3 interactions impact sleep/wake behavior. Here I have demonstrated that inhibition of Dα3 by QVR/SSS is likely to be cell-autonomous since the same drivers rescue sleep and memory in sss^{P1} mutants when combined with either UAS-sss (Section I) or UAS-Dα3 RNAi (this section). In the same neurons I have shown that Dα3 is likely involved in the hyperdopaminergic impairment of sleep and memory since I rescued these phenotypes in DAT^{fmn} mutants with piezo-Gal4>Dα3-RNAi, which also rescued sss^{P1} mutants. Furthermore, knocking down either Dop1R2 (Section III) or Dα3 (this section) in *qvr/sss* neurons achieved rescue effects of sleep and memory in both sss^{P1} and DAT^{fmn} mutants, suggesting that Dop1R2 and Dα3 are likely functioning in parallel as terminal effectors in these neurons to regulate sleep and memory. The *qvr/sss* neurons may serve as the converging point of distinct cholinergic and dopaminergic pathways onto a shared neural circuit.

I also described for the first time a phenotypic function for dNACHO. Consistent with its proposed role as a facilitator of nAChR assembly, I found that knocking it down led to similar behavioral effects as knocking down Dα3. Specifically, I was able to rescue the phenotypes of both sss^{P1} and DAT^{fmn} mutants. These results thus support the hypothesis that dNACHO functions as a molecular counterbalance to QVR/SSS. They also support the hypothesis I proposed earlier that a critical pathway in the two impaired behaviors I have been studying consists of hyperdopaminergic signaling interfacing with hypercholinergic signaling.

Section V. Conclusion and Future Directions

In my study, I identified the memory phenotype of short-sleep sss^{P1} and DAT^{fmn} mutants and established the correlation between chronic sleep deprivation and memory deficits. Since both QVR/SSS and DAT are required for normal sleep and memory phenotypes, I hypothesized that these functionally related genes or their downstream effectors might map to the same neurons and thus might be involved in a common neural circuit. Using various Gal4>UAS-*sss* combinations, I identified populations of neurons that are sufficient in restoring both sleep and memory functions to sss^{P1} mutants. Drivers with broad expressions (*sss*-, 11E12- and 12C10-Gal4s) and those whose expressions are more restricted (11E12^12C10-, piezo-, and 109(2)80-Gal4s) all successfully rescued both phenotypes. I further hypothesized that there might exist a small subset of neurons that are sufficient and necessary for normal sleep and memory functions. One future direction is to identify these neurons by targeting the overlapping populations among some of these drivers and investigating their ability to rescue the phenotypes. In addition, the specific roles of piezo and 109(2)80 neurons can be further examined. It is possible that these neurons known to be responsible for mechanosensation also play a role in the central nervous system to help regulate sleep and memory. Examining the overlapping expression patterns between these drivers and others that also rescued the sss^{P1} behavioral phenotypes may help elucidate this relationship.

Next I examined each of the four dopamine receptors and identified Dop1R2 as the key mediator of dopamine signaling that causes the phenotypes in DAT^{fmn} mutants. Using various Gal4>RNAi combinations, I found that knocking down Dop1R2 in 11E12- and piezo-Gal4 driven neurons rescued both sleep and memory phenotypes in DAT^{fmn} mutants. Contrary to previous studies, *qvr/sss* neurons, not dFSBs neurons, seem to be the cellular location that

Dop1R2 functions to regulate sleep and memory. Future experiments are needed to identify the locus outside the dFSBs in which Dop1R2 exerts its effects where *qvr*/*sss* also functions to regulate the same behaviors.

More importantly, knocking down Dop1R2 in 11E12 neurons in sss^{P1} mutants also rescued the sleep and memory phenotypes. The elimination of excessive dopaminergic signaling by Dop1R2 reversed the effects of elevated cholinergic signaling caused by the lack of QVR/SSS in sss^{P1} mutants. This suggests that signaling pathways in DAT^{fmn} and sss^{P1} mutants share at least part of a common neural circuit that regulates sleep and memory. In the same neurons, I found that knocking down Dα3 nAChRs also rescued the sleep and memory functions in both sss^{P1} and DAT^{fmn} mutants. This suggests that Dα3 is likely another terminal effector in the shared regulatory network and signals downstream effects in parallel with Dop1R2. I also identified the role of dNACHO as a molecular counterbalance to QVR/SSS. Phenotypically equivalent results of restoring *qvr/sss* can be achieved by knocking down dNACHO, reducing Dα3, or eliminating Dop1R2 in both sss^{P1} and DAT^{fmn} mutants. Collectively, these results suggest that hyperdopaminergic and hypercholinergic signaling interfaces in *qvr/sss* neurons and the proposed relationships between the molecules involved are summarized in **Figure 5.**

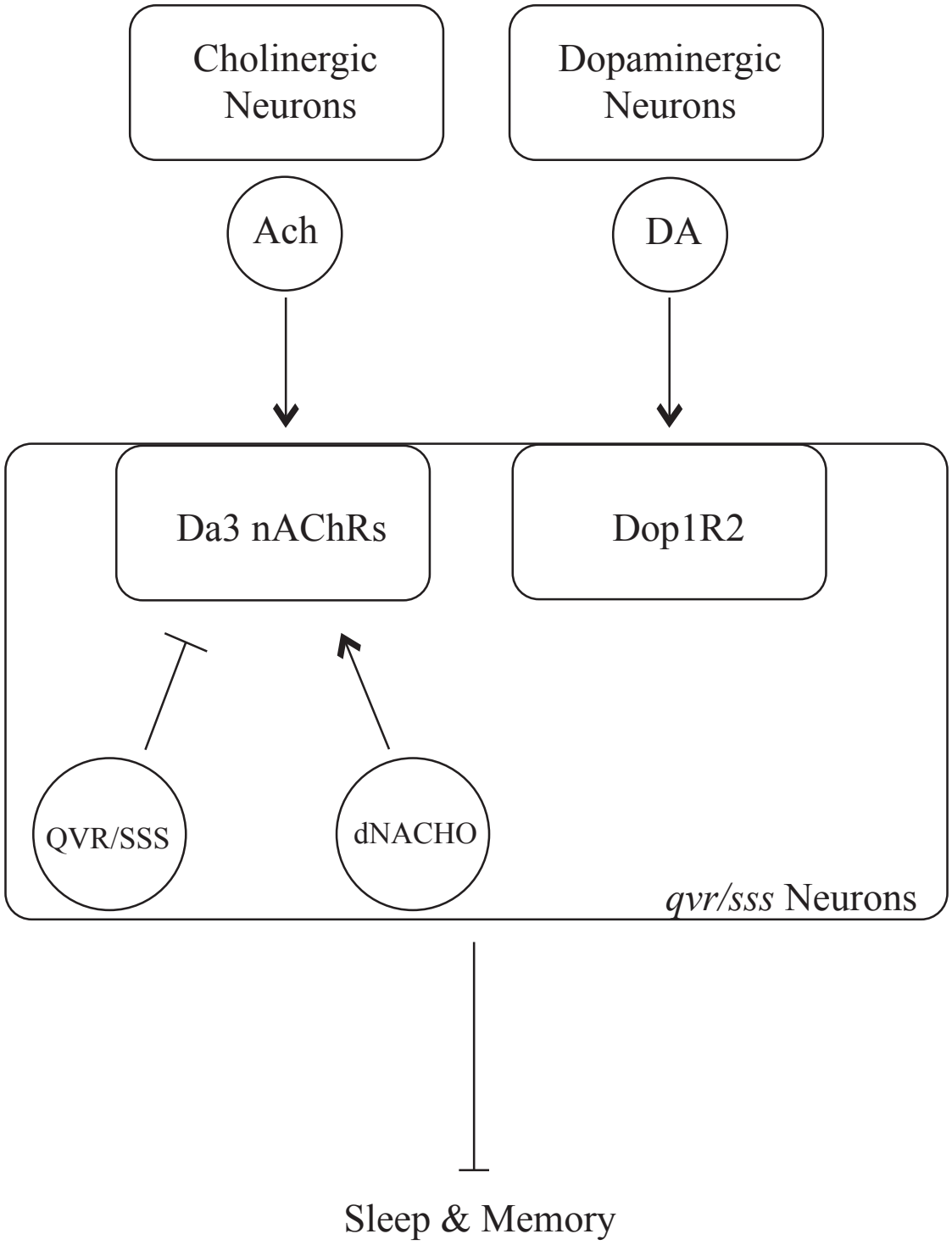
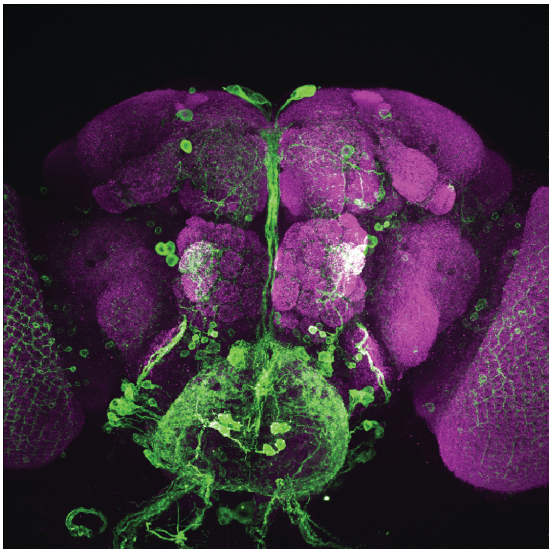


Figure 5. Proposed Molecular Mechanism of Sleep and Memory Regulation. The model proposes that the two terminal effectors, Dα3 nAChRs and Dop1R2, function in parallel in *qvr/sss* neurons to regulate sleep and memory. QVR/SSS and dNACHO have opposing effects in that QVR/SSS downregulates cholinergic signaling through Dα3 while dNACHO upregulates it.

Collectively, my data strongly suggest that the neuroanatomical pathways involved in sleep and memory processes are linked and that dopaminergic and cholinergic signaling converge in *qvr/sss* neurons, whose activities are tightly coupled to control sleep and short-term memory. *DAT* neurons and *qvr/sss* neurons may be pre- and post-synaptic partners within this shared circuit involved in sleep and memory regulation. However, the exact relationship between cholinergic signaling and dopaminergic signaling in these neurons remain unsolved. It is possible that Dα3 and Dop1R2 gate the neurons in a coordinated fashion and together maintain an optimal level of downstream signaling; dysfunction in either one may disturb this balance and subsequently cause the mutants' phenotypes. This hypothesis is supported by the results that the knockdown of Dα3 reverts the effects of hyperdopaminergic signaling in DAT^{fmn} mutants while the knockdown of Dop1R2 rescues the effects of hypercholinergic signaling in sss^{P1} mutants.

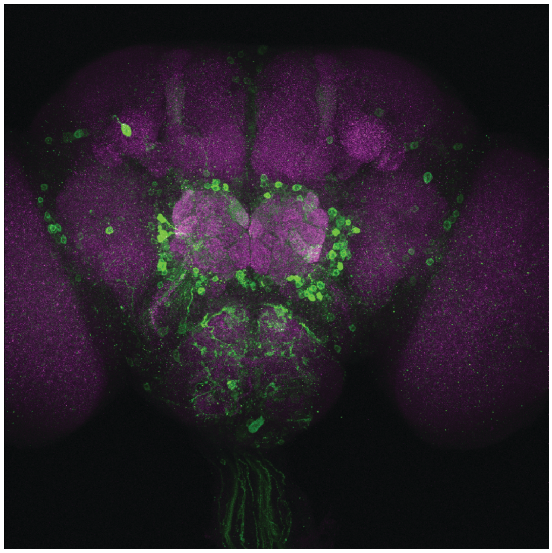
Supplemental Figures

a)



11E12CD8-GFP

b)



piezoCD8-GFP

Figure S1. Expression Patterns of 11E12- and piezo-Gal4 Drivers in the Fly Brain. Gal4 drivers were coupled to UAS-CD8-GFP (Gal4>GFP) and the nervous systems of dissected flies were examined by confocal microscopy. Expression patterns of 11E12>CD8-GFP (a) and piezo>CD8-GFP are shown. Anti-GFP staining is shown in green; neuropil stained with anti-nc82 is shown in magenta. Brain images were analyzed using Fiji [30] (SciJava ecosystems).

Materials and Methods

Fly Stocks and Transgenic Fly Lines

Wildtype (w^{1118} iso31), sss^{P1}, sss^{P2}, *sss*-Gal4, UAS-*sss* and DAT^{fmn} flies were described previously [17, 23]. Piezo-Gal4 and 109(2)80-Gal4 were gifts from Ardem Patapoutian and Yuh Nung Jan, respectively. Other Gal4 drivers and RNAi lines were obtained from the Bloomington Stock Center, with stock numbers listed in brackets: 11E12-Gal4 [45014], 12C10-Gal4 [45028], Dop1R1 RNAi [55239], Dop2R RNAi [26001], DopEcR RNAi [31981], Dop1R2 RNAi #1 [51423], Dop1R2 RNAi #2 [65997], Da3 RNAi [27671], dNACHO RNAi [65942]. The 11E12^12C10 split Gal4 line was generated using PhiC31-mediated targeted recombination of 11E12-AD into attP2 and 12C10-DBD into VK00027.

Sleep Measurements

Two- to seven-day-old female flies were loaded into glass tubes containing 5% sucrose and 2% agarose and entrained on a 12hr : 12hr light : dark cycle at 22°C for two days prior to measurement of sleep/wake patterns using the Drosophila Activity Monitoring System (Trikinetics). Sleep was defined as 5 min of inactivity and was measured as previously described [11].

Aversive Taste Memory Assay

The Proboscis Extension Reflex assay was performed as previously described [19] with minor modifications. Briefly, two- to seven-day-old female flies were entrained on a 12hr : 12hr light : dark cycle at 22°C for two days prior to experiments. Flies were starved for 24 hours and glued onto glass slides 2 hours before experiments. Flies that consistently extended their

proboscis at fructose presentation passed the pre-test and were given three rounds of three fructose-quinine pairings. After training, fructose alone was presented at the final test rounds. Glucose was presented post-test to validate the flies' ability to extend their proboscis.

Taste Discrimination Assay

Two- to seven-day-old female flies were entrained on a 12hr : 12hr light : dark cycle at 22°C for two days prior to experiments. At ZT0, flies were transferred to a plastic vial containing 5% agarose gel with a different tastant on each half, either 100mM fructose or a mixture of 100mM fructose and 10mM quinine. Each half disc of agarose gel was dyed with either red or blue food dye; colors were reversed in repeated experiments. Flies were allowed to feed for 3 hours in the dark and the coloration of their abdomens were examined.

Statistics

Bar graphs depict mean ± SEM. One-way ANOVA with multiple comparisons was performed for significance. All statistical tests were performed on GraphPad Prism 8.0 for Mac.

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