UNIVERSITY OF CALIFORNIA SAN DIEGO

Integration of Dopaminergic and Cholinergic Signaling Regulates Sleep and Associative Memory

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2022

The Thesis of Asyria Holland is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

First and foremost, I would like to give my biggest gratitude to my Principal Investigator and my Thesis Advisor, William (Bill) Joiner, for all the help and guidance he wonderfully provided to me throughout my academic career here at UC San Diego. I would not be in the position I am in now today if it were not for Bill's patience and kindness. I would also like to thank everyone in my lab for all their support. I want to thank Veronica Lin, Melin Wu, and Joydeep De for providing me the necessary data I needed to complete my thesis. And I would also like to thank Tianhao Qiu for teaching me how to properly conduct my assays and for giving me feedback on how I can improve my technique.

Section I, II, and III contains unpublished material that was graciously provided by Veronica Lin. The thesis author was the primary researcher and author of these sections.

Section II contains unpublished images that was graciously provided by Joydeep De. The thesis author was the primary researcher and author of this section.

ABSTRACT OF THE THESIS

Integration of Dopaminergic and Cholinergic Signaling Regulates Sleep and Associative Memory

by

Asyria Holland

Master of Science in Biology

University of California San Diego, 2022

Professor William Joiner, Chair
Professor Cory Root, Co-Chair

It is unclear why sleep is required for associative memory. However, this relationship is conserved across evolution, thus supporting the hypothesis that a core function of sleep is to facilitate memory formation. In this thesis I shed light on this relationship by studying the roles of two established sleep-facilitating genes, *DAT* and *sss*, in facilitating gustatory short-term associative memory. First, I show that loss-of-function mutations in *DAT* and *sss* reduce this form of memory. Second, I show that *DAT* mutants require Dop1R1 and Dop1R2

dopamine receptors, whereas *sss* mutants require Da3 nicotinic acetylcholine receptors, to suppress sleep and associative memory. Third, I show that these receptors appear to function in the same neurons, thus suggesting that hyperdopaminergic signaling by *DAT* mutants and hypercholinergic signaling by *sss* mutants converge to suppress sleep and associative memory. Fourth, I show that suppressing hyperdopaminergic signaling restores sleep and memory to animals with hypercholinergic signaling and vice-versa, which suggests that the two pathways have additive effects on a common endpoint in target neurons. Fifth, I show that associative memory can also be disrupted by targeting a particularly pathogenic form of amyloid beta, a putative causative agent in Alzheimer's disease, to the same neurons in which Dop1R2 and SSS/Da3 function to facilitate sleep and memory. And sixth, I show that amyloid beta may genetically interacts with and appears to coopt signaling by SSS/Da3 to interfere with associative memory. These last two results may be analogous to the recently published finding that amyloid beta downregulates the SSS homolog Ly6h, which leads to hypercholinergic signaling in neurons involved in mammalian memory formation.

INTRODUCTION

The relationship between sleep and associative memory formation is unclear. Two competing theories attribute opposite effects of sleep on memory. The synaptic homeostasis hypothesis (SHY) argues that sleep is required to reduce synaptic strength across the brain to compensate for net synaptic potentiation that occurs during waking (Tononi and Cirelli, 2020). In contrast, the system consolidation hypothesis argues that sleep is required to potentiate synaptic strength to convert labile memories formed during waking into long-term memories that are resistant to degradation (Diekelmann and Born, 2010). There is evidence to support both hypotheses. However, mechanistic data for both are lacking. Regardless, the requirement for at least certain forms of sleep to form stable memories is highly conserved, which suggests that a major function of sleep may be to enable forms of synaptic plasticity that underlie memory formation.

It would be useful to identify neural circuits and molecules that link sleep and memory so that the underlying cellular mechanisms could be better understood. This information has been hard to decipher in mammals, but it may be tractable in fruit flies. These organisms are easy to manipulate genetically, and they have fast regeneration times, thus facilitating identification of neural circuits that control behaviors. Furthermore, fruit flies exhibit short- and long-term memory, and they show conserved features of sleep regulation, including control by neurotransmitter systems and even molecules that have similar roles in mammals (Joiner, 2016). Importantly, the requirement of sleep for associative memory is also apparent in flies, just as in mammals. For example, in unpublished findings my lab has shown that chronically sleep deprived animals with loss-of-function mutations in either the *sleepless* (*sss*) or the dopamine transporter (*DAT*) gene have impaired gustatory associative memory. It is unclear whether these

behavioral deficits are due to the loss of sleep alone or due to other functional effects of disrupted *sss* and *DAT* genes. However, forced sleep deprivation for even 6 hrs by gentle mechanical agitation also causes the same memory deficits in wild-type animals. Thus, the *sss* and *DAT* genes may be useful tools for probing the relation between sleep and memory in flies.

The *sss* and *DAT* genes produce two important proteins that may be involved in regulating both sleep and memory through changes in cholinergic and dopaminergic signaling, respectively. The protein SLEEPLESS (SSS) is anchored to the outer leaflet of the plasma membrane, where it can form a stable complex with and inhibit Da3 subunit-containing nAChRs, thereby reducing cholinergic synaptic transmission to promote sleep (Koh et al., 2008; Wu et al., 2014). In contrast, DAT is located on the presynaptic terminals of dopaminergic neurons where it removes dopamine from the synaptic cleft to limit postsynaptic signaling and promote sleep (Kume et al., 2005; Ueno et al., 2012).

To address the potential roles of *sss* and *DAT* in sleep and memory formation, I utilize two loss-of-function mutations, sss^{P1} and DAT^{fmn}, in my thesis. Dysfunctional SSS encoded by sss^{P1} cannot bind to D∝3 to limit cholinergic synaptic transmission (Wu et al., 2014), and dysfunctional DAT encoded by DAT^{fmn} cannot extrude synaptic dopamine to limit dopaminergic synaptic transmission (Kume et al., 2005; Ueno et al., 2012). Interestingly, sleep can be restored to both sss^{P1} and DAT^{fmn} mutants with the nAChR antagonist, mecamylamine (MCA) ((Wu et al., 2014) and data not shown). This result suggests that the neural circuits in which SSS and DAT function to regulate sleep converge somewhere in the CNS where nAChR activity is critical for arousal. A potential target of MCA is D∝3. Broadly knocking down this nAChR subunit restores sleep to sss^{P1} mutants (Wu et al., 2014). Together, these findings suggest that D∝3 is an effector of elevated signaling in sss^{P1} mutants, that hyperactivation of D∝3 reduces sleep, and that

hypercholinergic and hyperdopaminergic arousal pathways terminate in Da3 signaling. However, the locus within the CNS where these processes occur is unknown. Furthermore, it is unknown whether these processes apply selectively to sleep or whether they also contribute to memory formation. **One goal of my thesis is to address this question.**

One locus at which SSS and DAT might regulate sleep and memory is the dorsal fan-shaped body (dFB). The dFB has been reported to mediate hyperdopaminergic sleep loss in DAT^{fmn} mutants (Liu et al., 2012; Ueno et al., 2012). However, the driver that was used for these mapping studies expresses in other areas of the nervous system, so the actual locus of control is unclear. A sleep-regulating locus for the *sss* gene has never been identified. **A second goal of this thesis is to determine these loci and thus potentially to provide a cellular context in which the relationship between sleep and memory might be further studied.** Although previous work in my lab helped restrict which neurons might be important for the function of *sss*, progress was limited by reliance on drivers with broad expression. In my thesis, I expand on these studies by identifying additional useful drivers based on their ability to restore normal sleep and memory to sss^{P1} or DAT^{fmn} mutants.

Little is also known about molecular mechanisms that link sleep to memory. However, one molecule that seems to be important for both processes is dopamine, at least in flies. Signaling by dopamine through one of its receptors, Dop1R1, has been shown to be required for associative memory in multiple studies (Placais et al., 2013; Kruttner et al., 2015; Berry et al., 2018; Dag et al., 2019). Two additional studies implicated the same receptor in promoting waking (Liu et al., 2012; Ueno et al., 2012), but another study suggested Dop1R2 was instead involved (Pimentel et al., 2016). Thus, it is unclear which pathways are actually coupled to

dopaminergic regulation of sleep and memory. **A third major goal of this thesis is to elaborate on those pathways and to determine their relationship to signaling by SSS/Da3**.

The need for sleep in memory formation also likely explains in part why disorders that disrupt sleep often negatively impact memory. A prime example is Alzheimer's disease (AD), in which accumulation of amyloid beta (Aβ) in extracellular plaques or hyperphosphorylated tau in neurofibrillary tangles drives disease progression, including loss of synapses, loss of memory and neurodegeneration (Sahoo et al., 2020). Many studies have documented the correlation between sleep and AD, with several landmark studies going further and arguing for a causal relationship. These include the findings that release of soluble Aβ in the interstitial space depends on endogenous neuronal activity, with extended wakefulness leading to an overall increase in neuronal firing and thus to elevated Aβ levels (Cirrito et al., 2005; Kang et al., 2009; Bero et al., 2011; Huang et al., 2012; Roh et al., 2012); and the discovery of the glymphatic system in the brain, which clears extracellular Aβ preferentially during sleep (Mendelsohn and Larrick, 2013; Xie et al., 2013). Despite its simpler brain anatomy and the absence of additional genetic factors that may contribute to AD, fruit flies have emerged as a viable model organism in which to study AD phenotypes and their relation to sleep (Tue et al., 2020). For example, several studies have shown that increasing sleep or its consolidation ameliorates memory loss caused by transgenic expression of human Aβ, its precursor protein, or human tau (Dissel et al., 2017; Kaldun et al., 2021). Although the underlying mechanisms are unknown, these findings underscore the conserved importance of sleep to AD pathology as well as the potential utility for exploiting the power of fruit fly genetics to study this relationship.

To better understand this relationship, this thesis will also address the potential role of Aβ in perturbing memory formation via effects on sleep-regulating neurons. This question is

particularly relevant to SSS considering that the mechanism by which this molecule functions to regulate sleep – namely inhibiting D∝3 nAChRs – is strikingly similar to a process my lab has previously shown to be deranged by Aβ in mammalian neurons - namely inhibition of alpha7 nAChRs by the mammalian SSS homolog, Ly6h (Wu et al., 2021). Thus**, a fourth major goal of this thesis will be to determine whether the signaling pathways used by SSS in sleep-regulating neurons are affected by Aβ to perturb memory formation.**

SECTION I. Low-Sleeping Mutants have Impaired Memory due to Hypercholinergic or Hyperdopaminergic Signaling

Results

Acute sleep deprivation causes impairment of memory, which is restored only when animals can undergo homeostatic recover sleep (rebound) (Seidner et al. 2015). Thus, we hypothesized that chronic sleep deprivation would cause the same impairment. We tested this hypothesis using sss^{P1} and DAT^{fmn}, two loss-of-function mutations that have been reported to exhibit low sleep due to hypercholinergic and hyperdopaminergic signaling, respectively. As expected, we found that both mutants showed drastically reduced sleep compared to wildtype controls **(Fig. 1a, b)**. To test whether low sleep in these mutants could potentially impair cognition, we used a gustatory short-term associative memory assay (Seidner et al. 2015). This assay involves presenting a droplet of fructose solution to flies' legs. Flies have taste receptors there which, when stimulated, send a signal to the brain telling the fly to extend its proboscis in expectation of receiving a sweet reward. However, if the flies are simultaneously presented with a droplet of bitter tasting quinine, then they learn to associate this aversive tastant with the appetitive stimulus. As a result, after several training sessions they learn to suppress their proboscis extension reflex (PER) such that subsequent presentation of the appetitive tastant fructose alone during the testing period fails to elicit PER **(Fig. 1c)**. We found that whereas wildtype flies had low PER scores, corresponding to strong gustatory associative learning and memory retention, sss^{P1} and DAT^{fmn} mutants had high PER scores, thus indicating that these mutants had poor short-term gustatory associative memory **(Fig. 1d, e)**. An alternative explanation would be that both mutants were impaired in their ability to distinguish the appetitive and aversive tastants; however, this seems unlikely. When presented with a choice

between food containing fructose and food containing quinine, both mutants chose the former 100% of the time, just like wildtype animals (data not shown). Thus, the results from our memory assay were not confounded by sensory deficits in our sleep mutants.

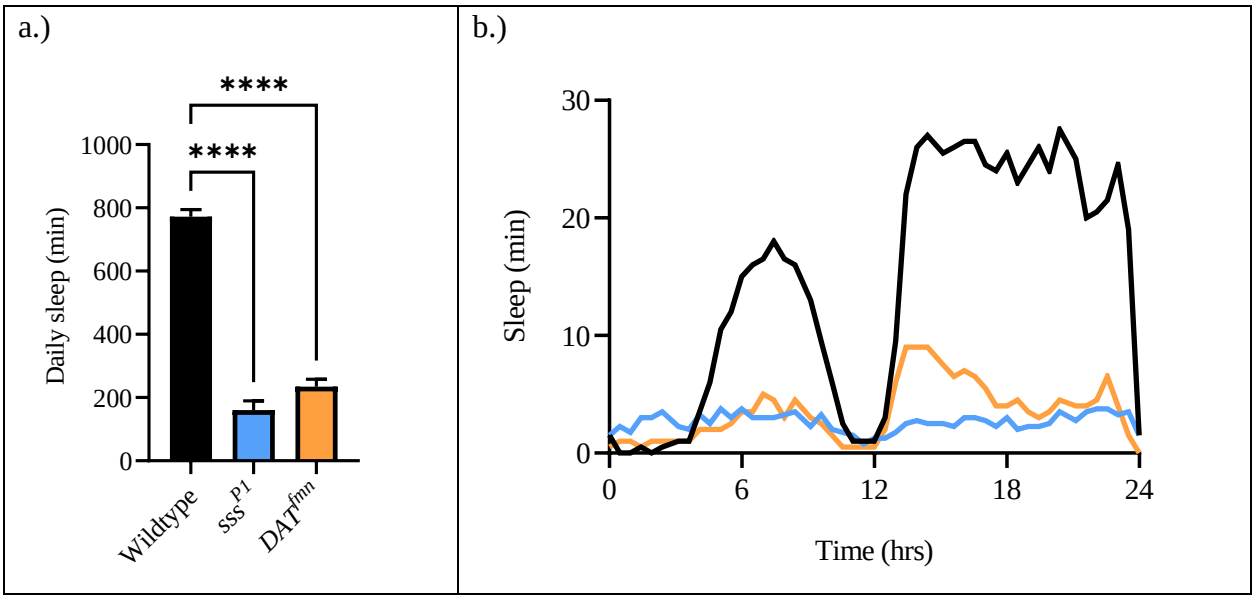

Figure 1a, b. Sleep phenotype in Wildtype, *sss*^{P1} and *DAT*^{fmn} mutant flies. Sleep profiles of Wildtype, *sss*^{P1} mutant and *DAT*^{fmn} mutant flies are presented. (a) Total number of minutes of daily sleep for each genotype was recorded. (b) Sleep profiles of mutant and wildtype flies across 24 hours. Figure provided by Veronica Lin.

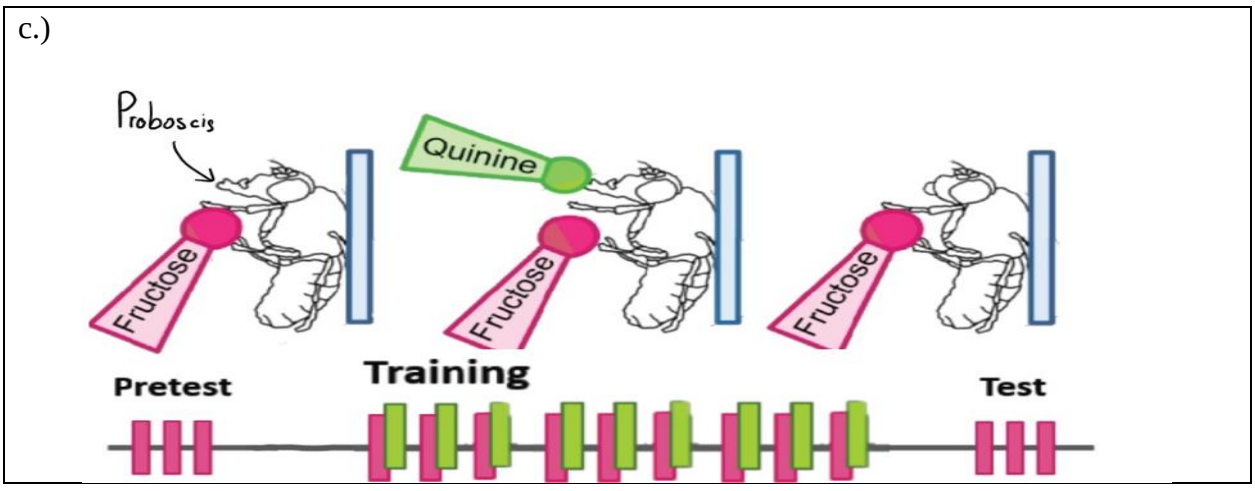

Figure 1c. **Graphical scheme of the Proboscis Extension Reflex (PER) assay.** This assay is composed of pretest (fructose only; pink), Training 1, 2, and 3 (fructose and quinine; pink and green, respectively), and test (fructose only; pink), to which each trial records the responses of three attempts of proboscis extension reflex per animal. Modified from figure in Seidner et al., 2015.

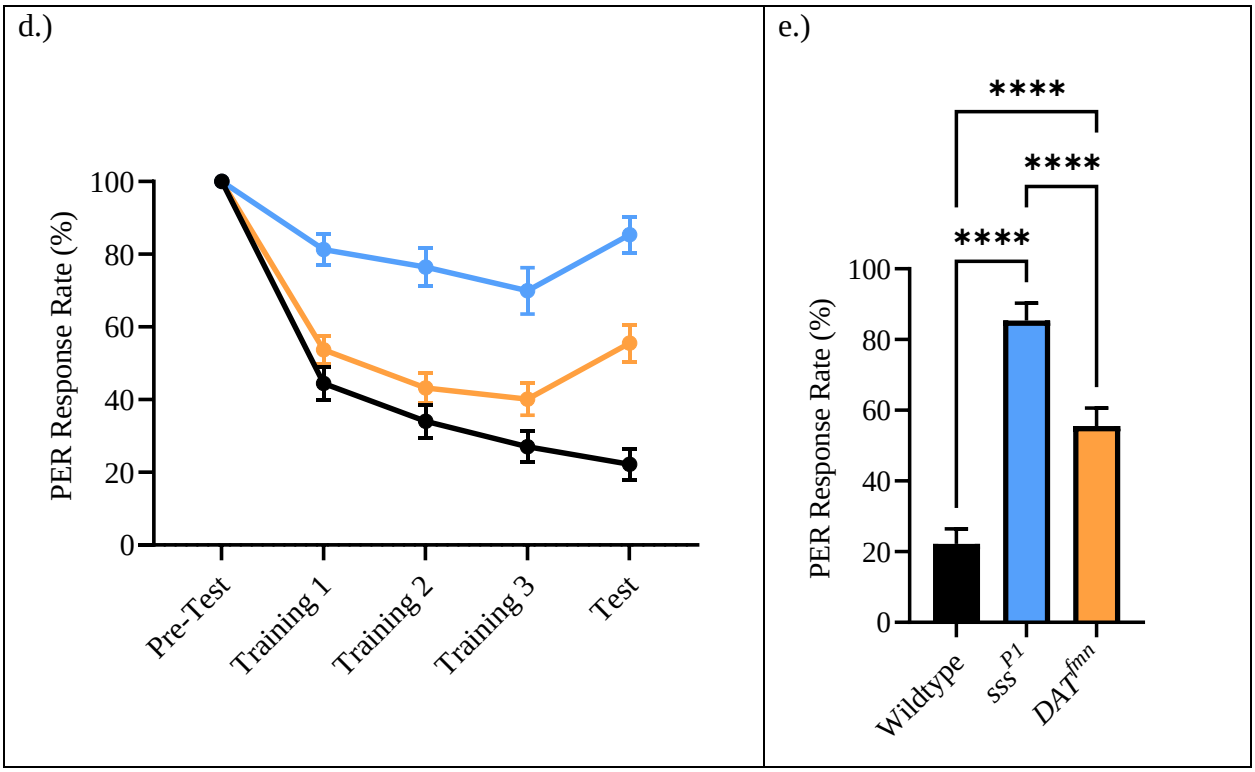

Figure 1d, e. Memory phenotype in Wildtype, *sss*^{P1} and *DAT*^{fmn} mutant flies. The memory phenotype of Wildtype (n=48), *sss*^{P1} mutant (n =41) and *DAT*^{fmn} mutant (n=54) flies were tested using the gustatory PER assay. (d) The learning curve of the flies PER response to the stimulus at each trial of the assay in percentages. (c) The final test scores of the flies PER responses to the stimulus in percentages. Figure provided by Veronica Lin.

SSS forms stable complexes with and inhibits nAChRs in heterologous expression systems, and broad reduction of Dα3 restores normal sleep to *sss*^{P1} mutants. Thus, SSS has been proposed to inhibit Dα3 to permit sleep, and *sss*^{P1} mutants are thought to release nAChRs from this inhibition to prevent sleep (Wu et al., 2014). Consistent with these mechanisms, we found that memory was partially restored to *sss*^{P1} mutants when Dα3 expression was reduced (i.e. *sss*^{P1} compared to Dα3; *sss*^{P1} double mutants) **(Fig. 1f)**. In summary, SSS seems to act as a brake on cholinergic signaling to promote sleep and short-term associative memory.

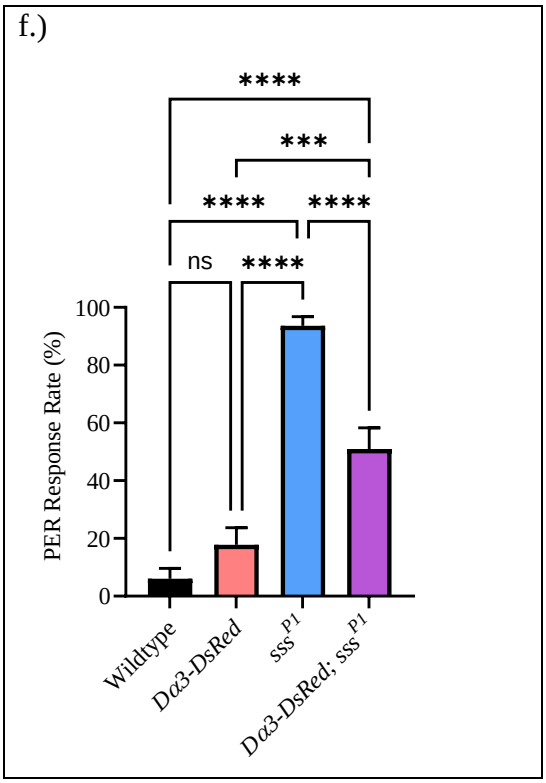

Figure 1f. **Reducing Dα3 restores memory to *sss*^{P1} mutants.** Dα3-DsRed;*sss*^{P1} double mutants have better gustatory associative memory than *sss*^{P1} mutants alone (n=22-34 per genotype). Figure provided by Veronica Lin.

In contrast, there is disagreement about potential effectors of dopamine-driven waking processes that could affect memory. For example, two studies partially restored sleep to *DAT*^{fmn} mutants by reducing Dop1R1 signaling, whereas another study increased sleep in otherwise wildtype animals by reducing Dop1R2 signaling. To determine which receptor might be responsible for impaired memory in *DAT*^{fmn} mutants I examined *DAT*^{fmn};Dop1R1 and *DAT*^{fmn};Dop1R2 double mutants. I found that memory was partially restored in either double mutant relative to *DAT*^{fmn} alone **(Fig. 1g)**. Thus, both Dop1R1 and Dop1R2 appear to be required for hyperdopaminergic-induced short-term associative memory deficits.

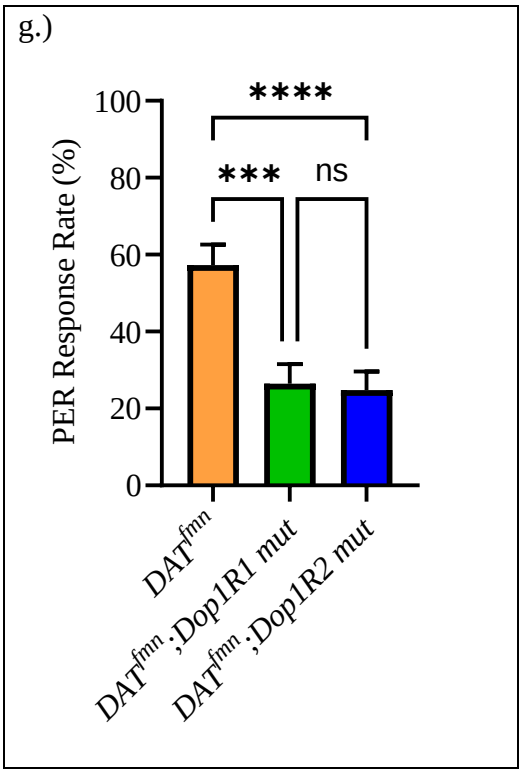

Figure 1g. **Reducing Dop1R1 or Dop1R2 restores memory to DAT^{fmn} mutants.** *DAT^{fmn};Dop1R1* and *DAT^{fmn};Dop1R2* double have better gustatory associative memory than *DAT^{fmn}* mutants alone (n=39 for each genotype).

Discussion

The first experiments examined two loss-of-function mutants, *sss^{P1}* and *DAT^{fmn}*, that are known to induce sleep loss to test whether they also exhibit an impairment of associative short-term memory. The hypothesis was that chronic loss of sleep also induces a deficit in memory. Our results aligned well with our hypothesis, with the two fly mutants exhibiting a loss of sleep and memory compared to wildtype flies. At this point, however, it is unclear whether the memory defects of the mutants are indirect – i.e. because the loss of sleep impairs memory – or due to the direct roles of SSS and DAT in both sleep and memory formation.

We further investigated the functional relationship between SSS and Dα3 regarding cholinergic regulation of sleep and associative memory. By reducing Dα3 and thus nAChR activity, we were able to overcome the memory impairment of *sss^{P1}* mutants. This result

confirms previous findings that SSS normally inhibits Dα3 and suggests that Dα3 disinhibition is responsible for sleep and memory deficits in sss^{P1} mutants. We also investigated which dopamine receptor, Dop1R1 or Dop1R2, may be involved in the impairment of memory in DAT^{fmn} mutants. We found that loss-of-function mutations in either receptor were sufficient to overcome memory deficits of DAT^{fmn} mutants. Thus, it appears that Dop1R1 and Dop1R2 are both required to mediate hyperdopaminergic memory loss. Together, these findings suggest that SSS acts as a negative regulator of Dα3 and that Dα3 cholinergic signaling and Dop1R1/Dop1R2 dopaminergic signaling must be restrained to promote sleep and memory.

The results obtained in this section help us better understand the potential molecular pathways that contribute to sleep and associative memory. Specifically targeting a particular receptor helps us determine which potential effectors are responsible for the behavioral effects of overactive cholinergic and dopaminergic signaling. In the next section, we will elaborate on these pathways and test if they function in the same cells.

Acknowledgements

Section I contains unpublished material that was graciously provided by Veronica Lin. The thesis author was the primary researcher and author of this section.

SECTION II. Hypercholinergic Signaling in *sss^{P1}* Mutants and Hyperdopaminergic Signaling in *DAT^{fmn}* Mutants Converge on the Same Neural Circuit to Regulate Sleep and Associative Memory

Results

Our ability to rescue sleep deficits in *DAT^{fmn}* and *sss^{P1}* mutants with the nAChR blocker mecamylamine (MCA; Wu et al., 2014 and data not shown) led us to hypothesize that dopaminergic and cholinergic contributions to increased arousal converge at the same locus in the brain. Dop1R1 signaling within the dFB has been reported to be responsible for the sleep phenotype of *DAT^{fmn}* (Liu et al., 2012; Ueno et al., 2012). We tested this hypothesis by knocking down Dop1R1 with the dFB driver that was used in these reports, 104y. We found that neither sleep nor memory was restored to *DAT^{fmn}* mutants with the driver/RNAi combination we used **(Fig. 2a, b)**. However, we were able to partly restore sleep and memory to *DAT^{fmn}* mutants using the same RNAi in combination with tsh-Gal4, which expresses abundantly in many cells of the CNS, but notably not in the dFB **(Fig. 2c, d; Fig. 2w)**. These results suggest that our RNAi is effective but that the locus at which hyperdopaminergic signaling through Dop1R1 reduces sleep and memory lies outside the dFB.

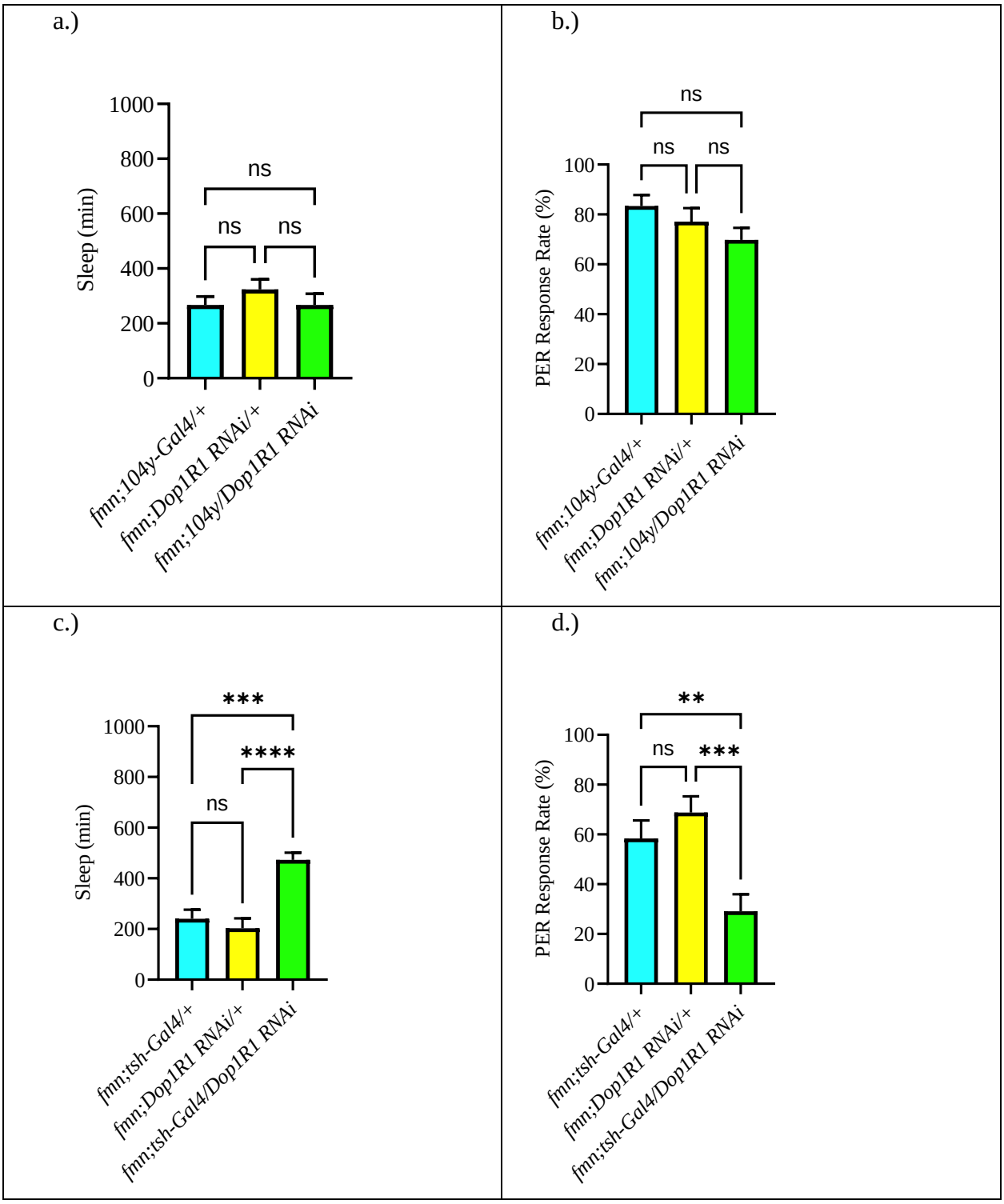

Figure 2a-d. Knockdown of Dop1R1 with tsh-Gal4 but not with a dFB driver restores sleep and memory to *DAT^{fmn}* mutants. (a,b) Knockdown of Dop1R1 in the dFB with 104y does not restore sleep (a) or memory (b) to *DAT^{fmn}* mutants (n=32). (c,d) Knockdown of Dop1R1 with tsh-Gal4 restores sleep (c) and memory (d) to *DAT^{fmn}* mutants (n=32).

We also attempted to map where hyperdopaminergic signaling through Dop1R2 impairs sleep and memory. We found that we could restore very little sleep to *DAT*^{fmn} mutants by knocking down Dop1R2 with tsh-Gal4 **(Fig. 2e)**. Thus, in this context Dop1R2 mainly functions in different neurons than Dop1R1. Instead, we rescued sleep deficits in *DAT*^{fmn} mutants by knocking down Dop1R2 with 11E12-Gal4 and piezo-Gal4 **(Fig. 2f, h)**, and we rescued the memory deficits of *DAT*^{fmn} mutants by knocking down Dop1R2 with 11E12-, piezo- and 39A11-Gal4 **(Fig. 2g, i, j)**. Unfortunately, none of these drivers express in such a restricted manner that it's clear where their expression overlaps and therefore where Dop1R2 is likely to function. However, they all express in the ventral fly brain **(Fig. 2w-z)**.

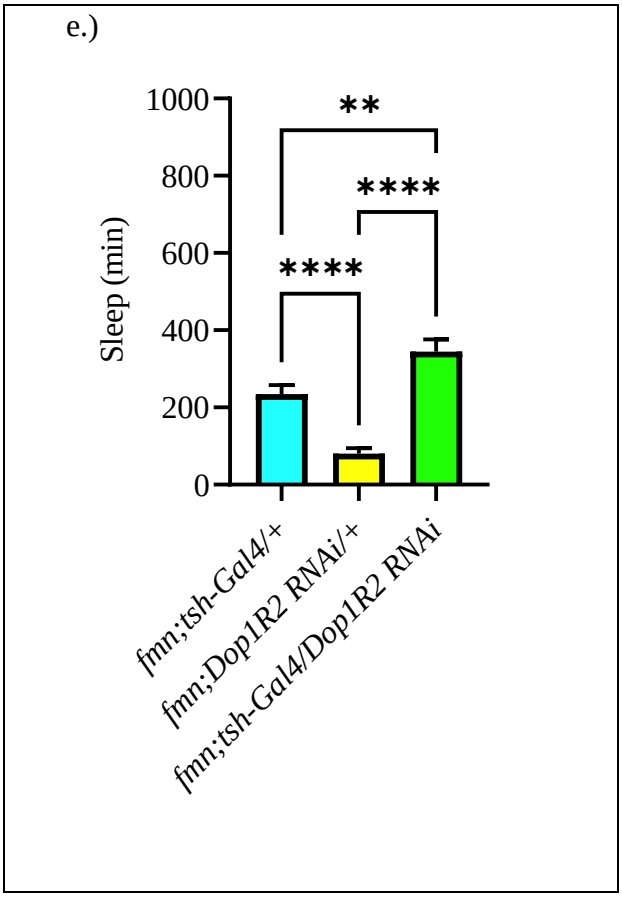

Figure 2e. Knockdown of Dop1R2 with tsh-Gal4 has only minor effects on sleep in *DAT*^{fmn} mutants. Minutes of sleep recorded for each genotype (n= 32, 32, 18, respectfully).

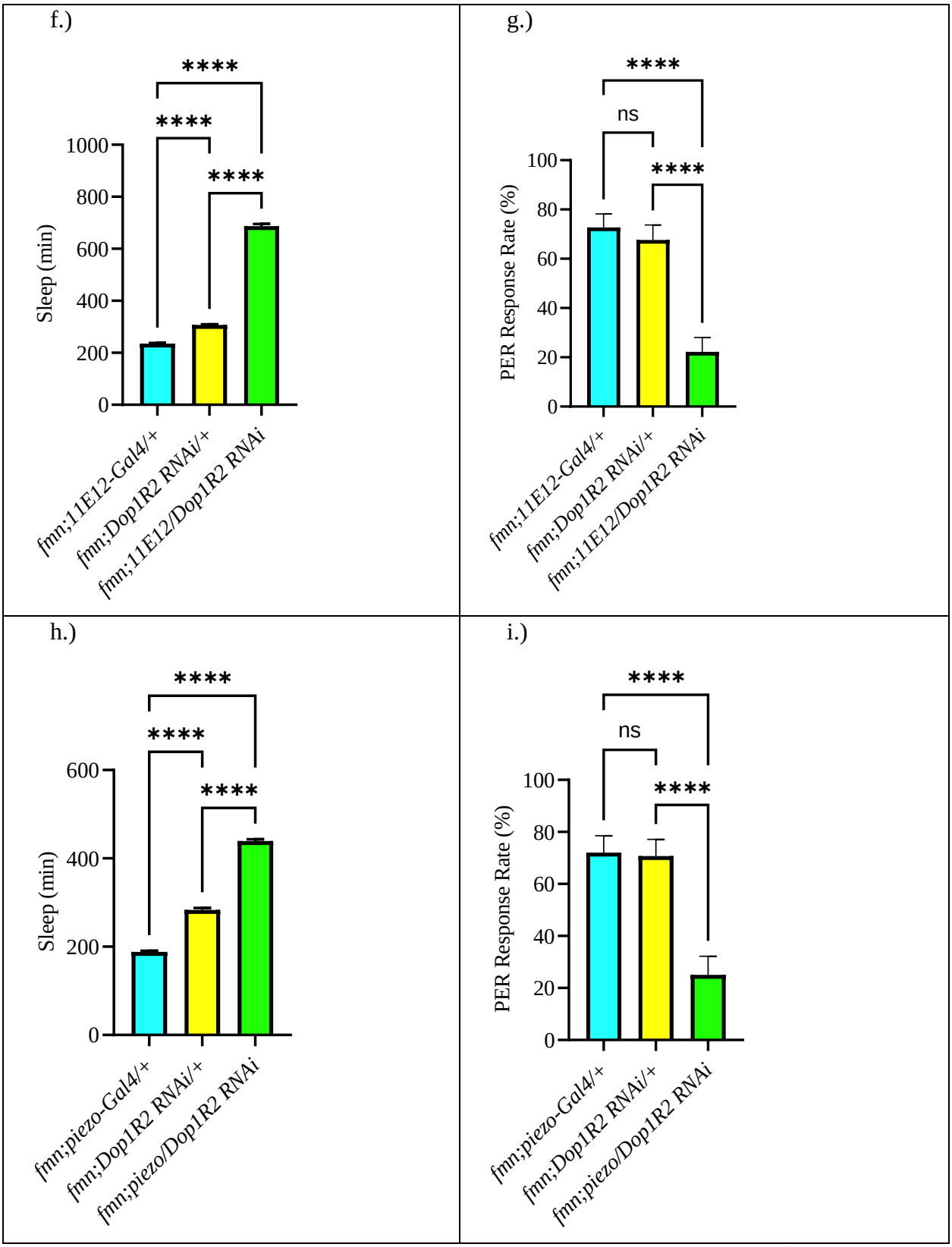

Figure 2f-j. Knockdown of Dop1R2 with specific drivers rescues sleep and memory in *DAT^{fmn}* mutants. Sleep and memory phenotypes of *DAT^{fmn}* mutants upon knockdown of Dop1R2 with 11E12-Gal4 (f, g) and piezo-Gal4 (h, i); n=25-48. Panels f-i were provided by Veronica Lin.

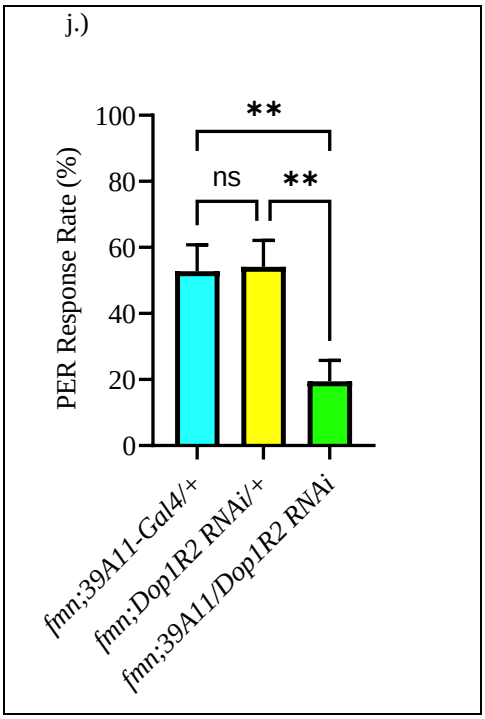

Figure 2f-j. Knockdown of Dop1R2 with specific drivers rescues sleep and memory in *DAT*^{fmn} mutants (Cont.)
Sleep and memory phenotypes of *DAT*^{fmn} mutants upon knockdown of Dop1R2 with 39A11-Gal4 (j); n=25-48.

The sleep phenotype caused by reduced SSS expression has not been mapped within the fly nervous system. We attempted to do so using multiple RNAi's against *sss*, including several we generated. However, even 90% knockdown throughout the nervous system was insufficient to reduce sleep (data not shown). Therefore, we attempted to restore sleep to *sss*^{P1} mutants by expressing a *sss* transgene under the control of different Gal4 drivers, and then we tested successful combinations for restoration of memory. Previous studies that have taken similar approaches for sleep have identified only broadly expressing drivers, such as cha-Gal4 (Wu et al., 2010). We were also unable to identify useful drivers with highly restricted expression. However, the drivers we identified were still informative. In fact, the three drivers that rescued Dop1R2-dependent sleep and memory in *DAT*^{fmn} mutants also rescued sleep **(Fig. 2k, m, o)** and memory in *sss*^{P1} mutants **(Fig. 2l, n, p)**. Thus, SSS appears to function in the same neurons as Dop1R2, and these neurons lie outside the dFB.

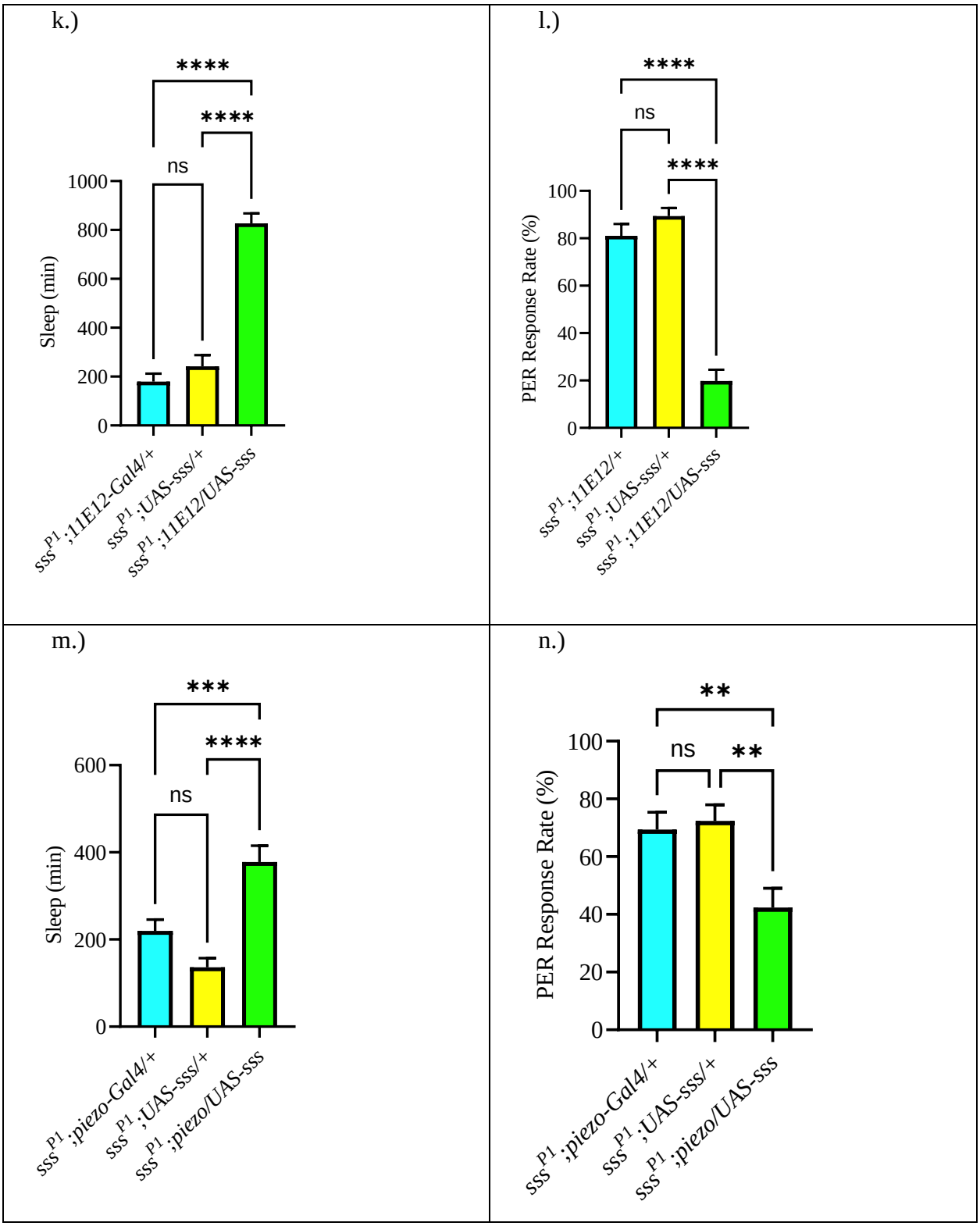

Figure 2k-p. Restoring *sss* expression to *sss^{P1}* mutants in select neurons rescues sleep and memory. Sleep and memory phenotypes of *sss^{P1}* mutants are overcome by restoring *sss* expression with 11E12-Gal-4 (k, l) and piezo-Gal4 (m, n); n=22-54. Panels k-n provided by Veronica Lin.

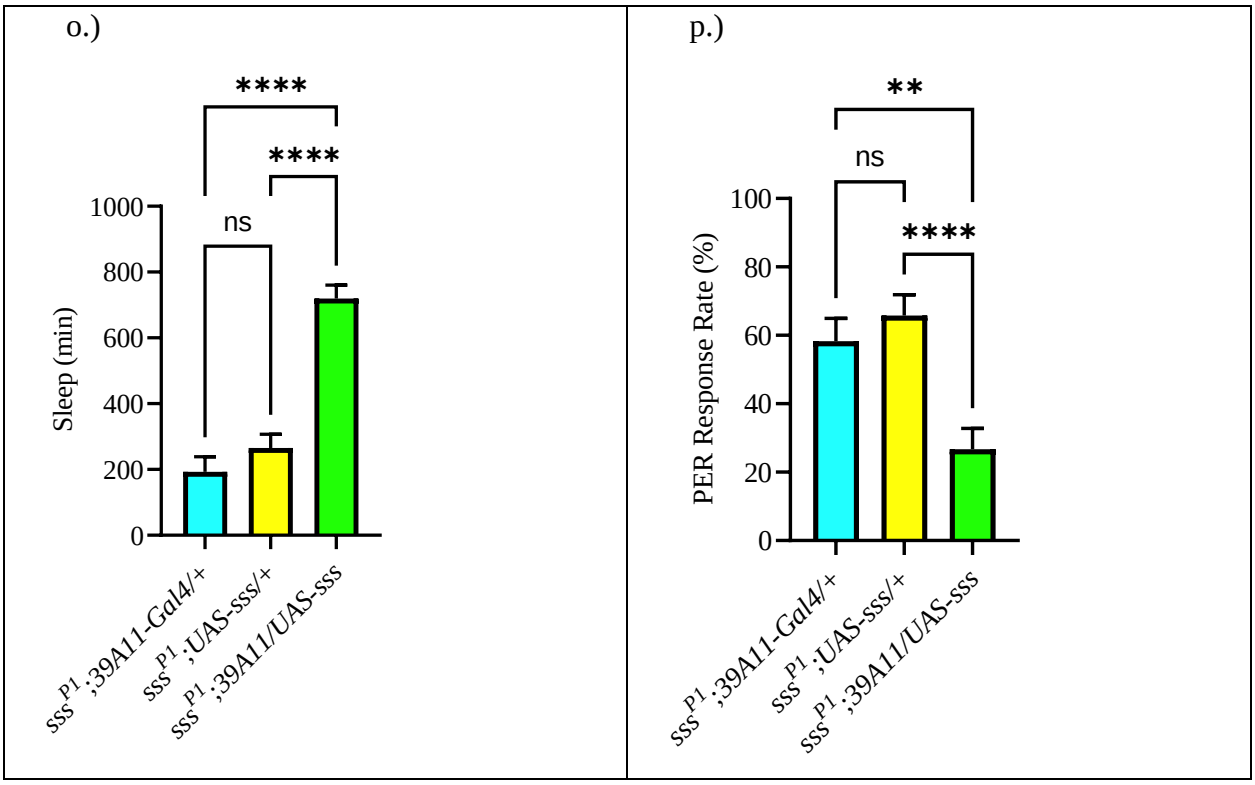

Figure 2k-p. Restoring *sss* expression to *sss*^{P1} mutants in select neurons rescues sleep and memory (Cont.) Sleep and memory phenotypes of *sss*^{P1} mutants are overcome by restoring *sss* expression with 39A11-Gal4 (o, p); n=22-54.

SSS has been shown to limit signaling through Dα3 -containing nAChRs, and this effect is required for normal sleep (Wu et al., 2010) and gustatory associative memory **(Fig. 1f)**. Thus, we hypothesized that Dα3 and SSS regulate these behaviors due to their functions in the same neurons. To test this hypothesis, we attempted to restore sleep and memory to *sss*^{P1} mutants by knocking down Dα3 with the drivers used in Gal4/UAS rescue experiments in the preceding paragraphs. Consistent with our hypothesis, we found that sleep and gustatory associative memory deficits in *sss*^{P1} mutants were both rescued by reducing Dα3 expression with 11E12- and piezo-Gal4 **(Fig. 2q-t).**

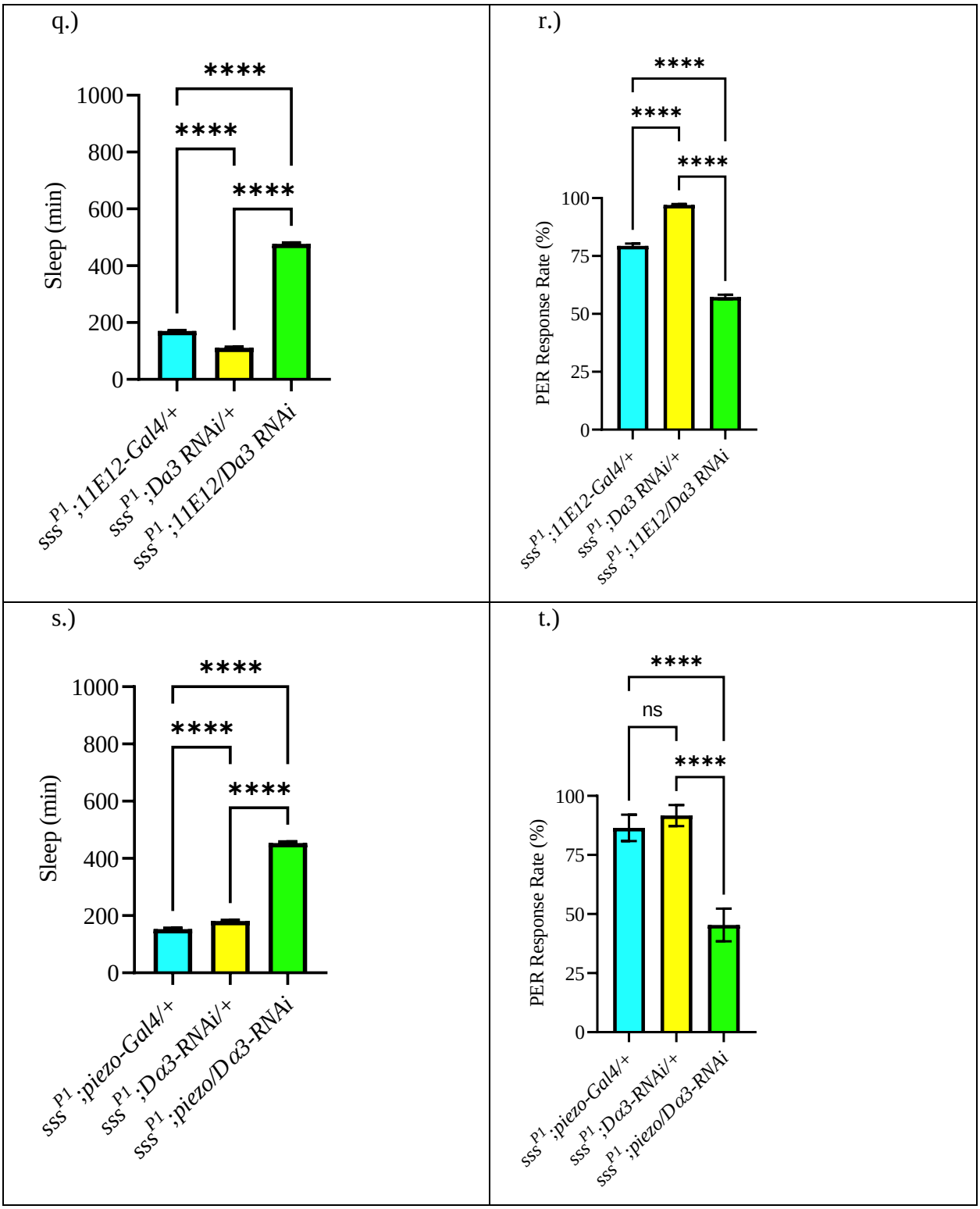

Figure 2q-t. Reducing Dα3 expression in *sss*^{P1} mutants rescues sleep and memory. Sleep and memory defects of *sss*^{P1} mutants can be overcome by reducing Dα3 expression with 11E12-Gal4 (q, r) and piezo-Gal4 (s, t); n-32-63. Figure provided by Veronica Lin.

Our results suggest that SSS limits Dα3 signaling in flies just as a SSS homolog called Ly6h limits α7 nAChR signaling in mammals (Wu et al., 2021). We hypothesized that conservation of this pathway extends to NACHO, which opposes the function of Ly6h (Wu et al., 2021). Thus, according to our hypothesis, Drosophila NACHO (dNACHO) would be expected to oppose the function of SSS. Extending this hypothesis further, we reasoned that knocking down dNACHO should be functionally equivalent to restoring *sss* expression to sss^{P1} mutants. That is, it should limit signaling through nAChRs. Thus, in the case of sss^{P1} mutants where nAChRs appear to be disinhibited, knockdown of dNACHO should restore sleep and associative memory. We tested this hypothesis using sss^{P1}/ sss^{P2} transheterozygotes rather than sss^{P1} mutants since the former retain some SSS that could be opposed by dNACHO to facilitate Dα3 assembly. Consistent with our hypothesis, we found that knockdown of dNACHO with 11E12-Gal4 restored sleep and gustatory associative memory to sss^{P1}/ sss^{P2} mutants **(Fig. 2u, v)**. In summary, dopamine signaling through Dop1R2 and acetylcholine signaling through Dα3/SSS/dNACHO in the same neurons account for the behavioral effects of DAT^{fmn} and sss^{P1} mutants, respectively.

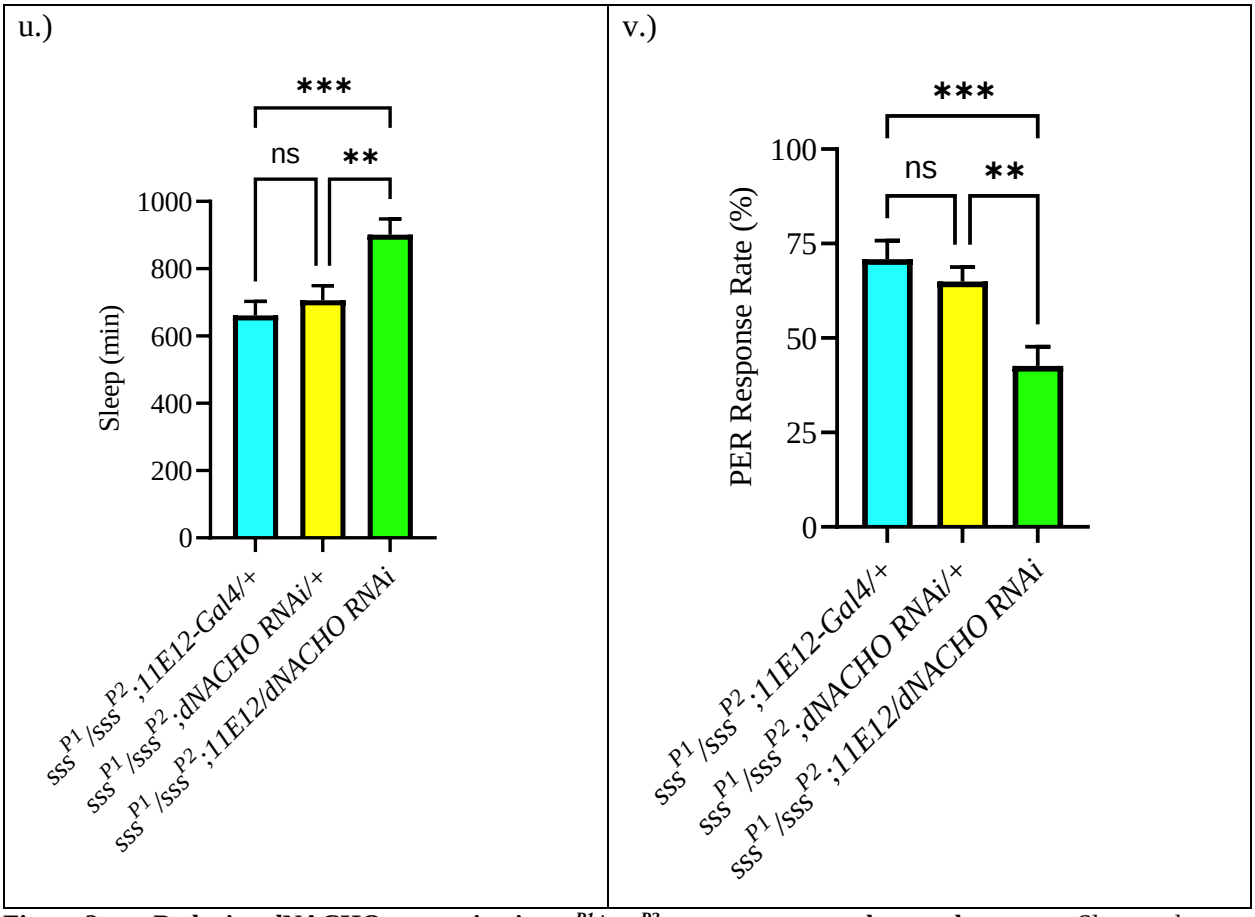

Figure 2u, v. Reducing dNACHO expression in *sss^{P1}/ sss^{P2}* mutants rescues sleep and memory. Sleep and memory defects of *sss^{P1}/ sss^{P2}* mutants can be overcome by reducing dNACHO expression with 11E12-Gal4.; n=16-85

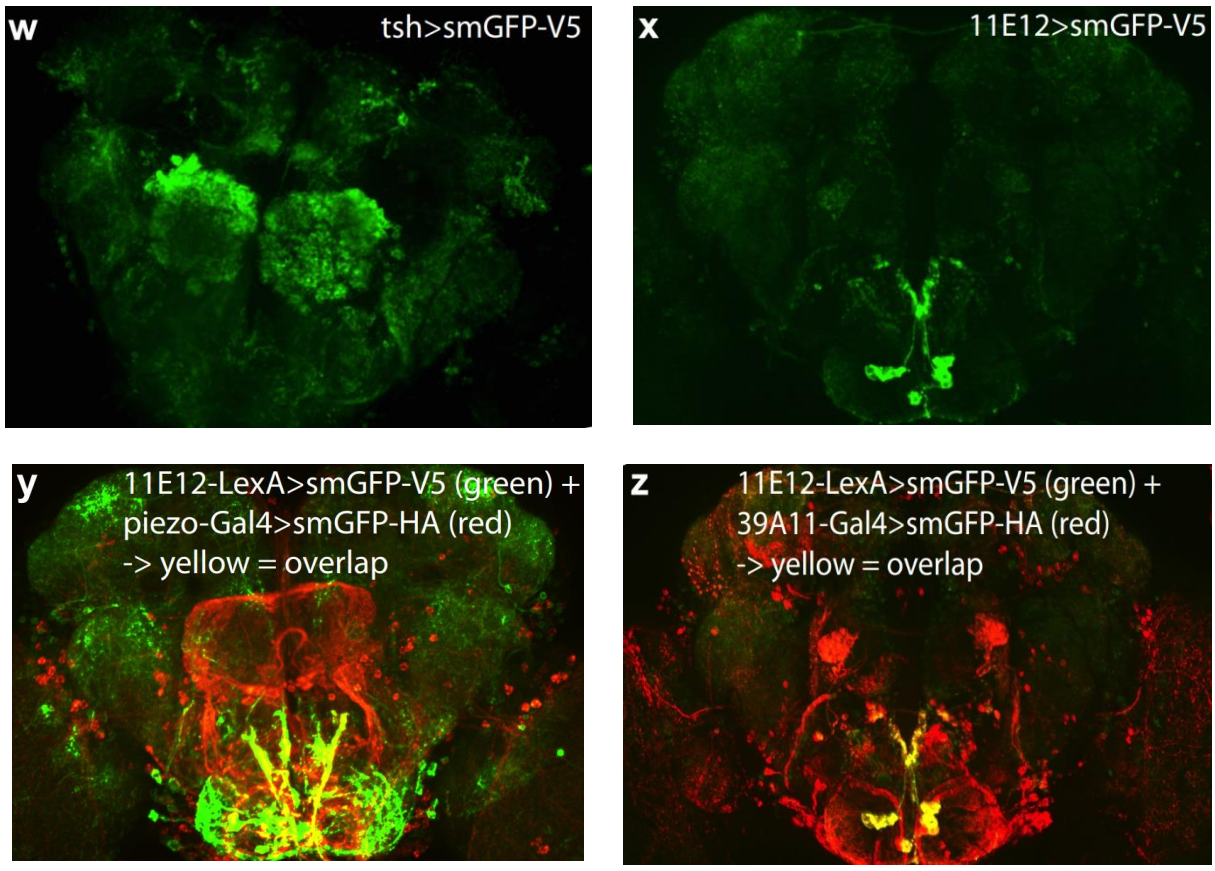

Figure 2w-z. Behaviorally relevant Gal4 expression patterns. Dissected brains were stained with antibodies against GFP reporters expressed under the control of the following drivers: (w) tsh-Gal4, (x) 11E12-LexA, (y) 11E12-LexA and piezo-Gal4, (z) 11E12-LexA and 39A11-Gal4. Images provided by Joydeep De.

Discussion

Collectively, our data are consistent with the hypothesis that hypercholinergic signaling in sss^{P1} mutants and hyperdopaminergic signaling through Dop1R2 in DAT^{fmn} mutants converge on a shared neural circuit to regulate sleep and associative memory. Since the three drivers we identified that label this circuit do not express in the dFB, our data also suggest that both Dop1R2 and *sss* function in other areas of the CNS to promote sleep. Interestingly, our three drivers all label cell bodies and processes in the ventral fly brain. Furthermore, piezo-Gal4 and 39A11-Gal4 express under control of enhancer elements for genes involved in mechanosensation (piezo and nompC). Thus, we hypothesize that hyperdopaminergic and hypercholinergic signaling in DAT^{fmn} and sss^{P1} mutants leads to prolonged activation of a sensory circuit, possibly involved in mechanosensation, that promotes arousal.

Acknowledgements

Section II contains unpublished material and images that was graciously provided by Veronica Lin and Joydeep De, respectively. The thesis author was the primary researcher and author of this section.

SECTION III. Dopaminergic and Cholinergic Pathways Genetically Interact to Regulate Sleep and Associative Memory

Results

Historically, *DAT^{fmn}* and *sss^{P1}* have been considered to affect different signaling pathways due to their impact on distinct neurotransmitter systems. However, this assumption has not been tested rigorously. In previous sections of this thesis, I demonstrated that Dop1R2 and Dα3/SSS/dNACHO converge on the same neurons to regulate sleep and gustatory associative memory. In this section, I address the degree to which these pathways converge and interact within arousal/memory-regulating neurons. I test whether reducing cholinergic signaling through Dα3/SSS/dNACHO can overcome the hyperdopaminergic effects of *DAT^{fmn}*, and whether reducing dopaminergic signaling through Dop1R2 can overcome the hypercholinergic effects of *sss^{P1}* on sleep and gustatory associative memory.

Based on my lab's previous finding that the nAChR antagonist MCA can restore sleep to both *sss^{P1}* and, unexpectedly, *DAT^{fmn}* mutants ((Wu et al., 2014) and data not shown), I hypothesized that Dop1R2 lies upstream and Dα3 lies near a terminal endpoint in a pathway involving postsynaptic dopamine and acetylcholine signaling. According to this hypothesis, reducing acetylcholine signaling through Dα3 nAChRs in the appropriate neurons would thus be expected to restore sleep and memory to *DAT^{fmn}* mutants. To test this hypothesis, we first knocked down Dα3 with the 11E12- and piezo-Gal4 drivers (previously described in Section II). As expected, both Gal4>Dα3 RNAi combinations rescued the sleep and/or memory deficits of *DAT^{fmn}* mutants **(Fig. 3a-d)**. To test our hypothesis further, we also knocked down the nAChR chaperone, dNACHO, with the same drivers. Again, consistent with our hypothesis, we found that sleep and memory were improved in *DAT^{fmn}* mutants **(Fig. 3e-h)**. For each phenotype, all the

effects were significant except for rescue of sleep by piezo>Dα3 RNAi and rescue of memory by 11E12>NACHO RNAi, where results were trending positive but fell below the level of statistical significance.

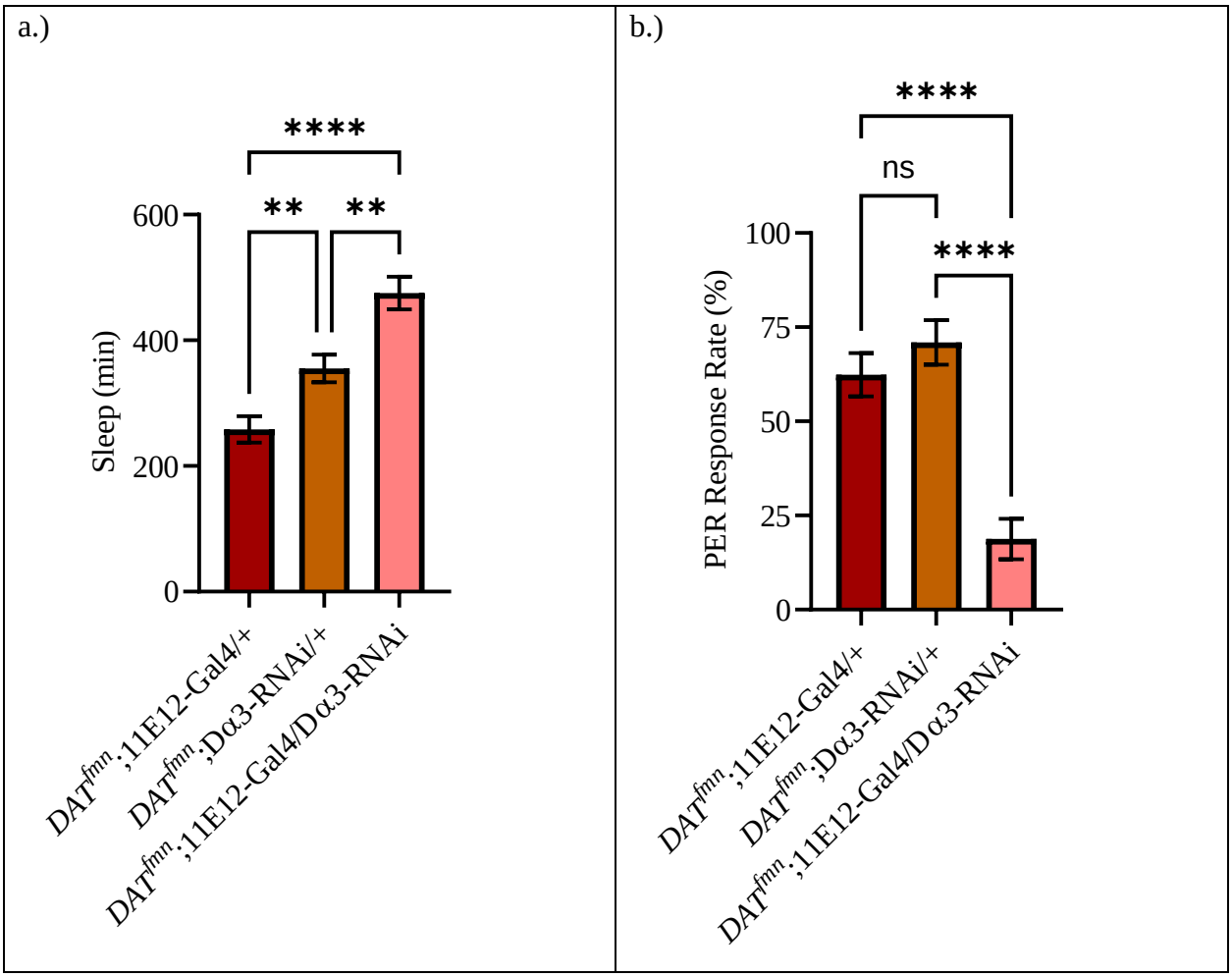

Figure 3a, b. Reducing Dα3 expression with 11E12-Gal4 rescues sleep and memory deficits of *DAT^{fmn}* mutants. Knockdown of Dα3 with 11E12-Gal4 rescues sleep (a) and memory (b) in *DAT^{fmn}* mutants, n=31-43. Figure provided by Veronica Lin.

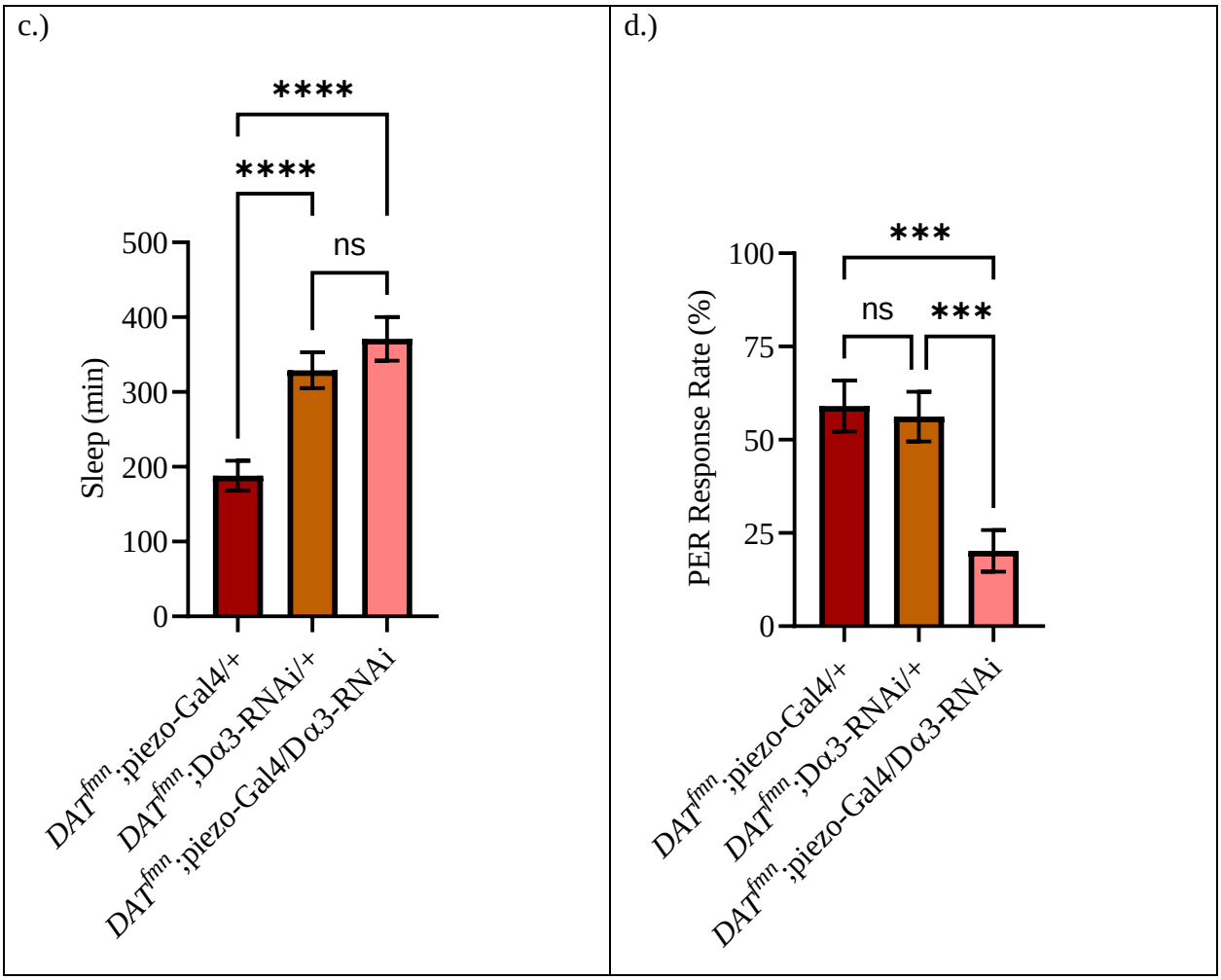

Figure 3c, d. Reducing Dα3 expression with piezo-Gal4 rescues memory deficits of *DAT^{fmn}* mutants. Knockdown of Dα3 with piezo-Gal4 rescues memory (d) but not for sleep (c) in *DAT^{fmn}* mutants; n=33-48. Figure provided by Veronica Lin.

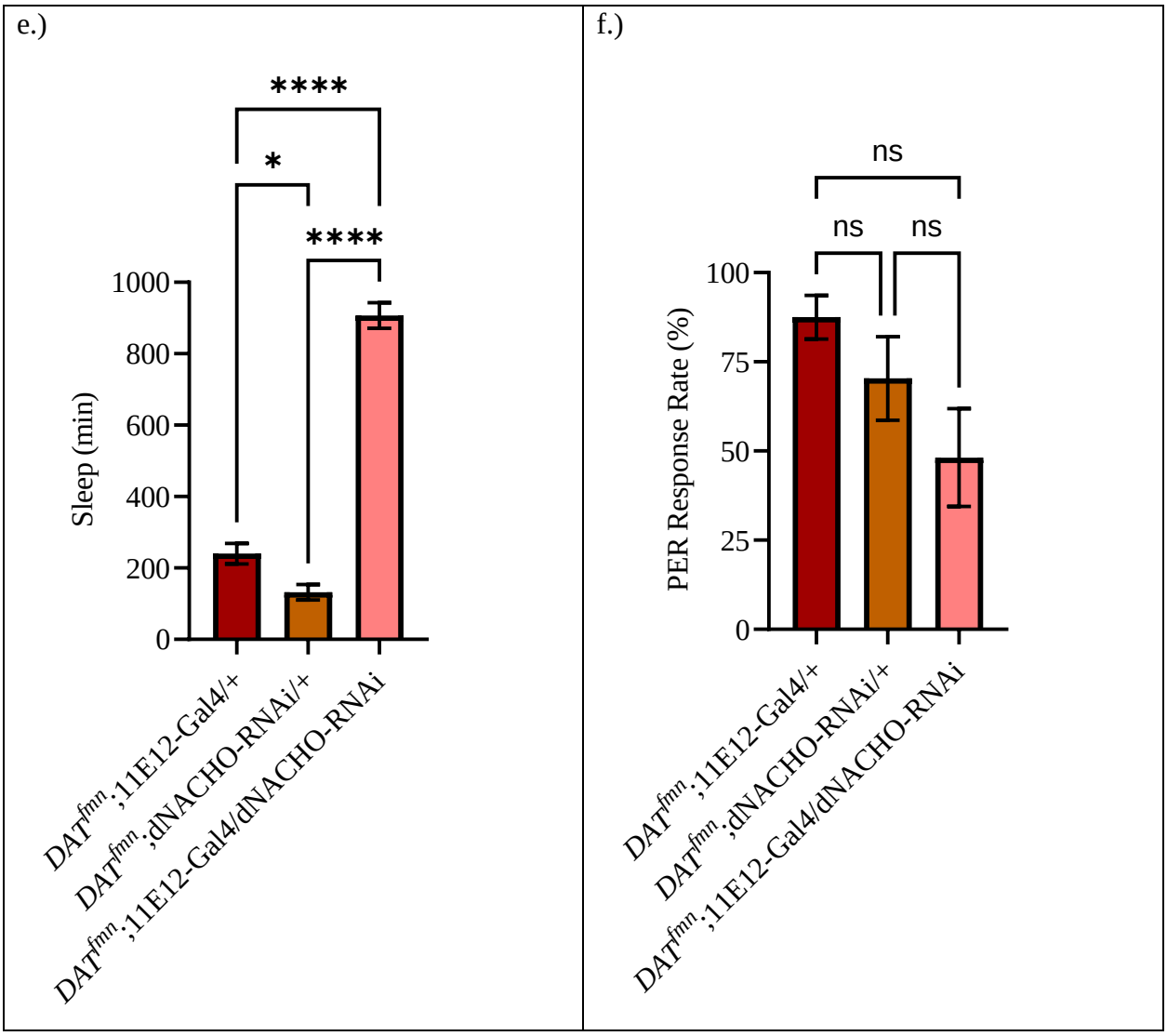

Figure 3e, f. Reducing dNACHO expression with 11E12-Gal4 rescues sleep in *DAT^{fmn}* mutants. Knockdown of dNACHO with 11E12-Gal4 rescues sleep (e) but not memory (f) in *DAT^{fmn}* mutants; n=8-31. Figure provided by Veronica Lin.

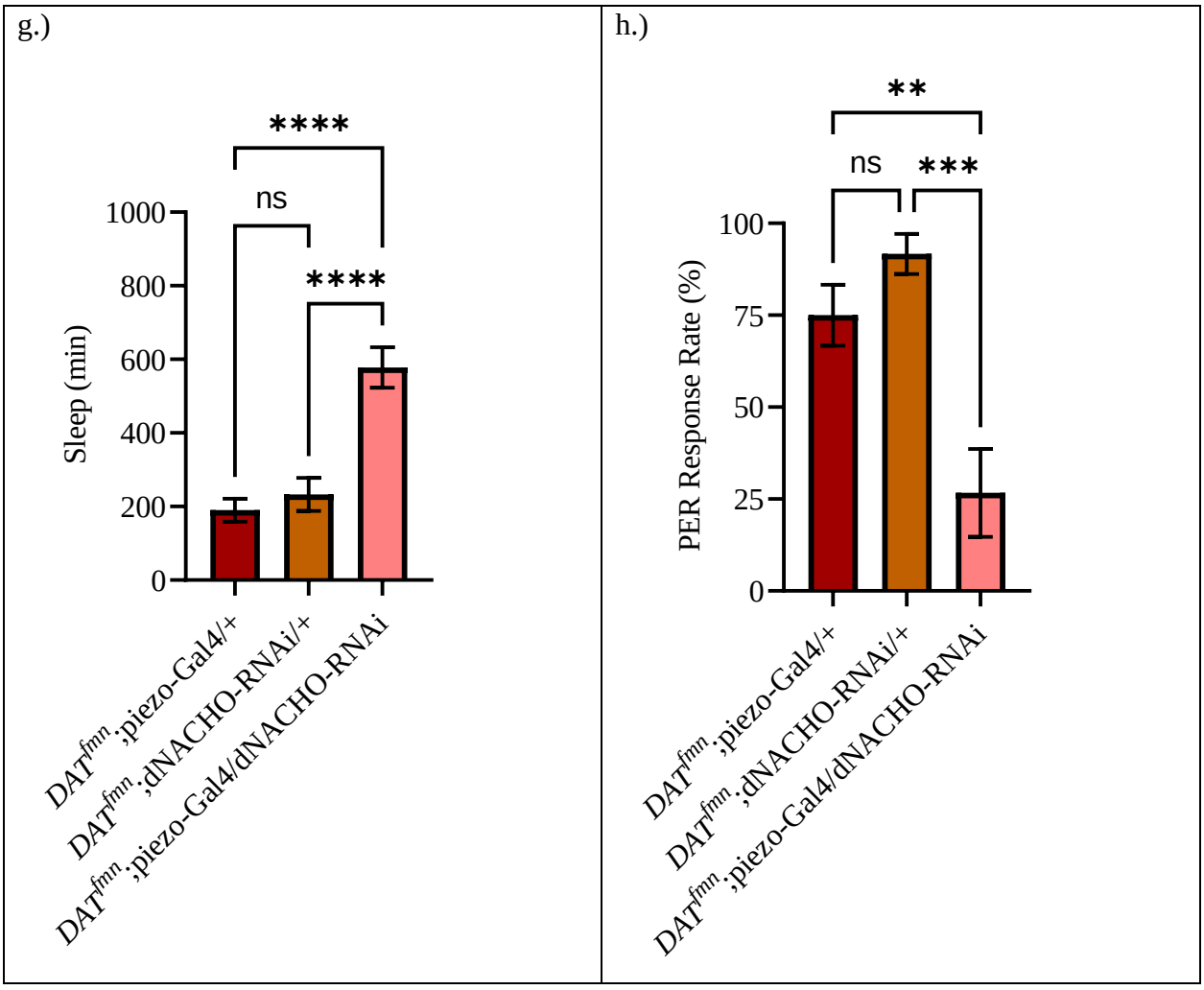

Figure 3g, h. Reducing dNACHO expression with piezo-Gal4 rescues sleep and memory in *DAT^{fmn}* mutants. Knockdown of dNACHO with piezo-Gal4 rescues sleep (g) and memory (h) in *DAT^{fmn}* mutants; n=8-24. Figure provided by Veronica Lin.

On their own, these results support our model for sleep/memory regulating neurons in which Dα3 is positioned functionally downstream of Dop1R2 signaling. However, we can't rule out the hypothesis that Dop1R2 and Dα3 contribute independently to basal neuronal activity such that excess activity by either receptor shifts neuronal activity outside the physiological range, and thereby, impairs behavior. If this were the case, then reducing activity of either receptor should compensate for excess activity by the other. To test this alternative hypothesis, we knocked down Dop1R2 with the 11E12-Gal4 driver in *sss^{P1}* mutants. We found that reducing Dop1R2 signaling indeed compensated for hypercholinergic signaling and restored both sleep and memory to *sss^{P1}*

mutants **(Fig. 3i, j).** Although the effect on memory did not reach the level of statistical significance, this was probably because the sample size was limiting since the effect was quite striking. Collectively, these results support the alternative hypothesis that Dop1R2 and Dα3/SSS/dNACHO function in parallel pathways whose effects sum to regulate behavior.

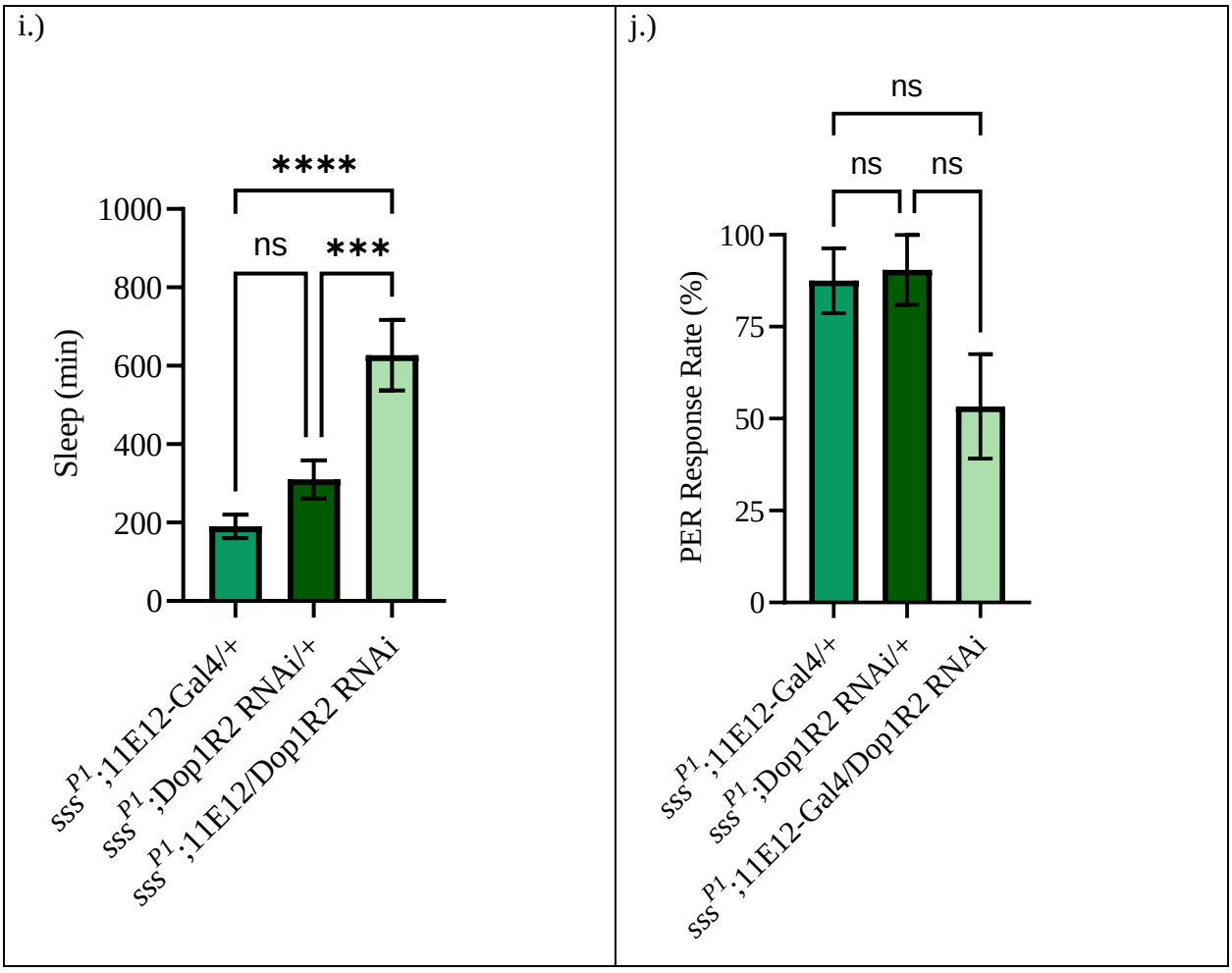

Figure 3i, j. Reducing Dop1R2 expression with 11E12-Gal4 rescues sleep in *sss^{P1}* mutants. Knockdown of Dop1R2 with 11E12-Gal4 rescues sleep (i) but not memory (j) in *sss^{P1}* mutants; n=7-31.

Discussion

DAT^{fmn} and *sss^{P1}* mutants increase dopaminergic and cholinergic signaling, respectively, and these pathways appear to converge in the same cells, as indicated in the previous section. In the current section, I tested whether the two pathways are independent, merge in series, or run in

parallel until merging at a nexus for both. I found that the sleep and memory phenotypes of both *DAT*^{fmn} and *sss*^{P1} mutants could be rescued by knocking down Dα3 or dNACHO. These results suggest that the two pathways cannot be strictly independent. I also found that both mutants could be rescued by knocking down Dop1R2. Thus, reducing signaling by either pathway ameliorates the phenotypes cause by excessive signaling in both pathways. This result suggests that the two pathways do not merge in series but instead likely converge together at a terminal point in common to both, possibly at the level of action potential firing or synaptic transmission. Unfortunately, several experiments that could have helped reinforce this interpretation were not carried out with sufficient statistical power. Our interpretation would also benefit from recapitulation of our results with additional drivers, such as 39A11-Gal4, which was described in the previous chapter. Despite these caveats, our knockdown results using 11E12-Gal4 are consistent across phenotypes and neurotransmitter signaling pathways, thus supporting our interpretation. A merging of the two pathways would also explain why *DAT*^{fmn} and *sss*^{P1} mutants have approximately equivalent, profound defects in sleep and memory. However, it seems unlikely that the two pathways merge in cell types that affect other phenotypes, such as sensitivity to oxidative stress and lifespan, both of which are negatively impacted by *sss*^{P1}, but not *DAT*^{fmn} mutants (Kume et al., 2005; Koh et al., 2008).

Acknowledgements

Section III contains unpublished material that was graciously provided by Veronica Lin. The thesis author was the primary researcher and author of this section.

SECTION IV. Aβ May Coopt the SSS Signaling Pathway to Impair Associative Memory

Results

Aβ peptide is a putative causative agent in Alzheimer's disease (AD) (Sahoo et al., 2020). My lab previously showed that oligomers of Aβ cause downregulation of Ly6h in cultured hippocampal neurons, leading to disinhibition of α7 nAChRs and thus reducing the threshold at which basal cholinergic signaling becomes neurotoxic (Wu et al., 2021). This mechanism may be relevant to Alzheimer's disease since Ly6h is reduced in the temporal cortex of AD patients in proportion to disease severity (Wu et al., 2021). Pan-neuronal expression of Aβ in Drosophila has also been shown to reduce associative memory, thus mimicking one of the hallmarks of advanced AD (Tue et al., 2020). However, the specific cell types and molecular mechanisms that are involved are unknown.

Because Ly6h is a mammalian homolog of SSS, and reduction of SSS impairs associative memory in flies, I hypothesized that Aβ impairs memory in flies by reducing SSS. To test this hypothesis, I performed two experiments using a particularly pathogenic mutant form of Aβ called Arctic. First, I tested whether expressing Arctic impairs memory in the same neurons in which sleep, and memory are regulated by SSS (and Dop1R2). I found that overexpressing Arctic under control of the 11E12 driver impaired associative memory compared to the corresponding genetic controls **(Fig. 4a)**. While the 39A11 driver showed a trend toward an impairment of associative memory **(Fig. 4b)**. Second, I tested whether Arctic and SSS genetically interact in 11E12- or 39A11-Gal4-expressing neurons. Specifically, I tested whether reducing SSS enhances the memory phenotype of Arctic-expressing animals. Consistent with my hypothesis, I found that memory in each Gal4>Arctic combination showed a trend toward further degradation in sss^{P1} heterozygotes, though in neither case was this trend significant **(Fig. 4c, d)**.

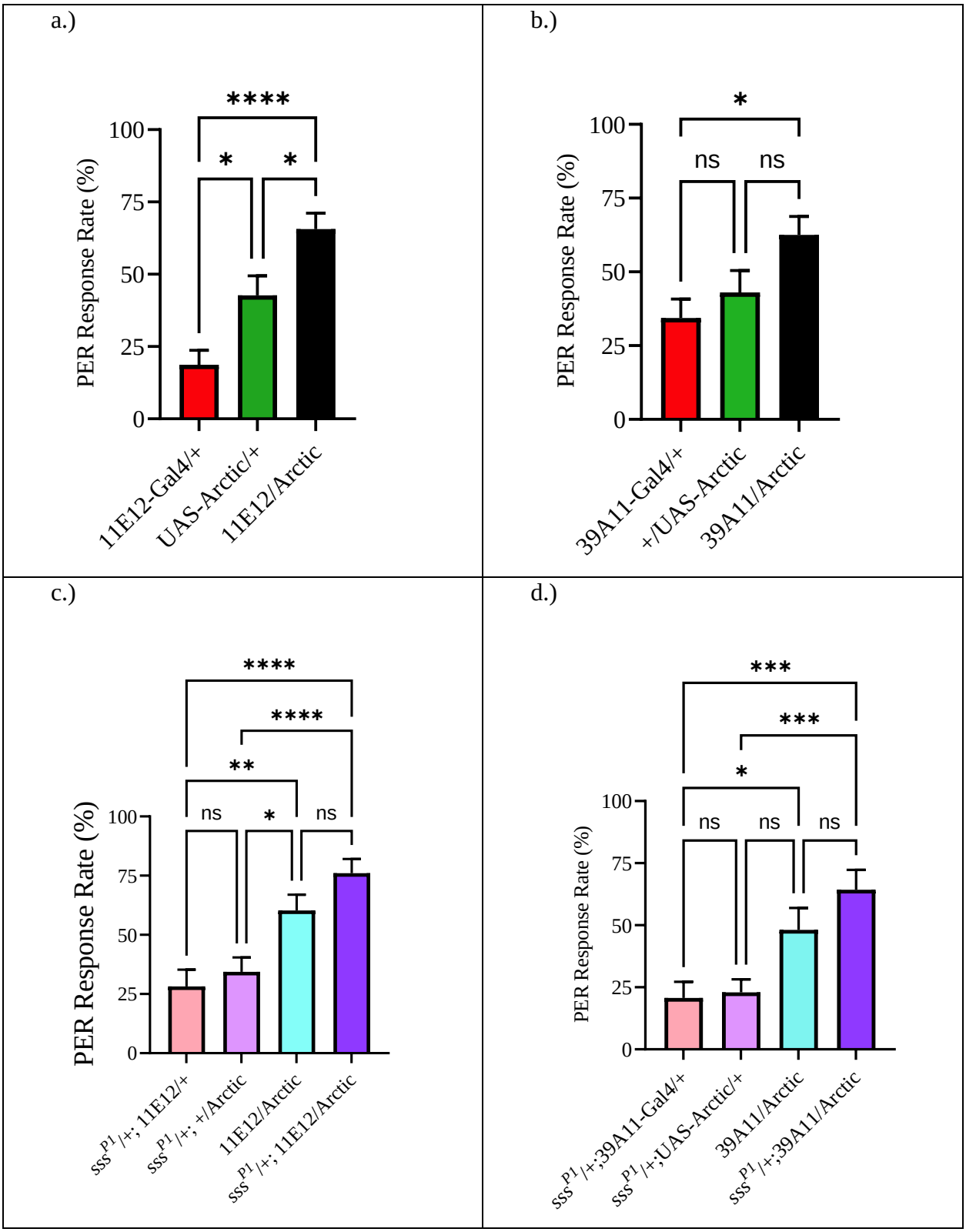

Figure 4a-d. **Arctic may impair memory.** (a,b) 11E12>Arctic (a) impairs associative memory, but not for 39A11>Arctic (b). (c,d) Memory loss in 11E12>Arctic (c) and 39A11>Arctic (d) is exacerbated when SSS levels are reduced in sss^{P1} heterozygotes; n=29-33.

My lab has recently demonstrated that Aβ-driven reduction in Ly6h disinhibits nAChRs to contribute to neurodegeneration in AD (Wu et al., 2021). As I just described, Aβ genetically interacts with SSS, a homolog of Ly6h, and loss of SSS disinhibits nAChRs in flies (Wu et al., 2014), just as loss of Ly6h disinhibits nAChRs in mammals. Based on these similarities, I hypothesized that Aβ-driven memory loss in flies requires nAChR signaling. Since my lab previously showed that SSS forms a stable complex with and inhibits Dα3 nAChRs, I further hypothesized that Aβ causes memory deficits in flies by upregulating Dα3. To test this hypothesis, I determined whether reducing Dα3 expression restores memory to Arctic-expressing flies. I found that knockdown of Dα3 does lead to improved associative memory in otherwise memory-impaired 11E12>Arctic animals **(Fig. 4e)**.

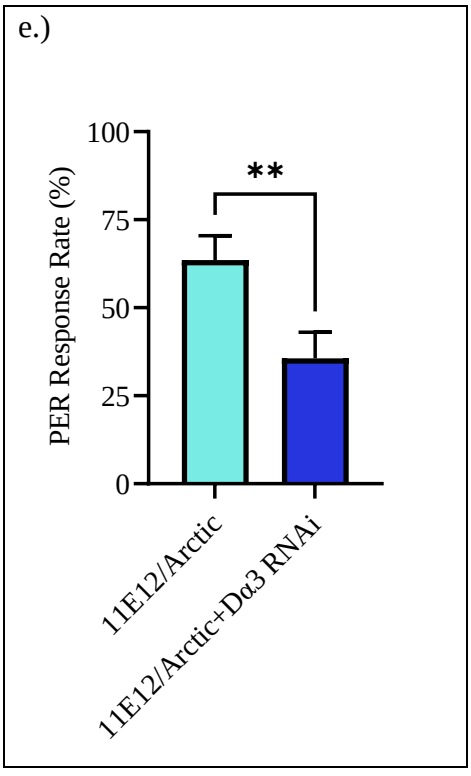

Figure 4e. Memory loss can be partially rescued in 11E12>Arctic flies by knocking down Da3.
11E12/Arctic+Da3 RNAi partially rescued memory when compared to 11E12/Arctic alone; n=23-32.

Discussion

In this section, I showed that overexpressing Arctic impairs associative memory, and it does so through effects in the same neurons in which SSS and Dop1R2 also function to regulate memory. These results suggest that Arctic might co-opt a pathway in which SSS or Dop1R2 function. I tested this hypothesis in two ways. First, I looked for a genetic interaction between the *sss* gene and Arctic. While the results were not statistically significant, they were trending in this direction and would likely have reached this outcome if I had time to test more animals. Second, I confirmed that knocking down the SSS effector, Dα3, could rescue memory loss caused by Arctic.

My results suggest that SSS functions in a similar pathway to its mammalian homolog, Ly6h, namely by inhibiting nAChRs. Further, like Ly6h, which functions in the hippocampus and neocortex, SSS functions in neurons in the fly brain that are essential for associative memory. These results may explain why I also found that Aβ seems to coopt the SSS pathway to perturb memory in flies, much as Aβ downregulates the SSS homolog Ly6h in mammals (Wu et al., 2021). Discovery of this conserved pathway could open new possibilities for probing how Aβ causes neuronal dysfunction and ultimately memory loss. The genetic tractability of flies may aid in this discovery process with the ultimate goal of identifying therapeutics to treat the progression of AD in patients.

MATERIALS AND METHODS

Fly Stocks and Transgenic Fly Lines

Wildtype (w^{1118} iso31), sss^{P1}, sss^{P2}, UAS-*sss* and DAT^{fmn} flies were described previously (Donlea et al., 2011; Pimentel et al., 2016). Piezo-Gal4 and tsh-Gal4 were gift from Ardem Patapoutian and Julie Simpson, respectively. Other Gal4 drivers, RNAi lines and mutants were obtained from the Bloomington Stock Center, with stock numbers listed in brackets: 11E12-Gal4 [45014], 39A11-Gal4 [50034], Dop1R1 RNAi [55239], Dop1R2 RNAi [65997], Da3 RNAi [27671], dNACHO RNAi [65942], Dop1R1 mut [84714], Dop1R2 mut [84715].

Sleep Measurements

One- 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 over 24-48 hrs using the Drosophila Activity Monitoring System (Trikinetics). Sleep was defined as 5 min of inactivity and was measured using custom Matlab software as previously described (Hendricks et al., 2000).

Aversive Taste Memory Assay

A modified version of the Proboscis Extension Reflex assay was performed to test short-term gustatory associative memory by repeatedly training flies to associate an aversive stimulant, quinine, with an appetitive stimulant, fructose. Flies that remember this association subsequently stop extending their proboscis when presented with fructose alone and thus produce a low test score (Seidner et al., 2015).

Briefly, one- to seven-day-old female flies were entrained on a 12hr:12hr light:dark cycle at 22°C for two days. Flies were then starved at ZT10, and the next day they were glued onto glass slides at ZT8. Two hours later they were administered a pretest consisting of a droplet of 10 mM fructose presented repeatedly to one leg. If they failed to extend their proboscis to each of three presentations, then they were excluded from the remainder of the experiment. If they passed this pretest, then they moved on to subsequent training. In each round a droplet of 10 mM fructose was simultaneously presented to a leg while a droplet of 1 mM quinine was presented in front of and accessible to the proboscis. This process was performed three times in rapid succession for each of three rounds of training, with a short pause introduced in between rounds to prevent desiccation by allowing flies to drink water. After the third training period, a droplet of fructose alone was presented to a leg three times, and the frequency with which each animal responded by extending its proboscis was recorded. Afterward a droplet of 1 M sucrose was presented to a leg of each fly to confirm the ability to extend the proboscis.

For experiments involving Arctic the assay was modified slightly. In these experiments only two training periods were used; the time period between each was extended to 30 min; and the time period between the second training period and the test was extended to 1 hr.

Statistics

All bar graphs present the average +/- SEM. One-way ANOVA with Tukey's multiple comparison post-test was used to test for significant differences. GraphPad Prism 9.0 was used to perform all statistical tests.

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