UNIVERSITY OF CALIFORNIA SAN DIEGO

Nf1 functions in sleep homeostasis neurons for circadian rhythms and memory

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by

Tianhao Qiu

Committee in charge:

Professor William Joiner, Chair
Professor Chih-Ying Su, Co-Chair
Professor Cory Matthew Root

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The Thesis of Tianhao Qiu 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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Master of Science in Biology

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Professor William Joiner, Chair
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The sleep/wake cycle is thought to be controlled by two main processes: a circadian clock that regulates timing of arousal and a sleep homeostat that regulates duration of arousal according to sleep need. It is unknown though how these two processes work together: whether they work in parallel or functionally interact with each other. This gap in conceptual understanding is due

in part to the dearth of information about the nature of the sleep homeostat as well as the neuroanatomy responsible for transmitting time-of-day information from the circadian clock network to the rest of the brain.

In unpublished data, my lab has shown that a cluster of neurons in the antennal mechanosensory and motor center (AMMC) functions in both processes. In this thesis I describe the contribution that expression of the Neurofibromatosis 1 (Nf1) gene within these neurons makes to the sleep/wake cycle. By utilizing various Gal4>RNAi combinations, I found that Nf1 is required AMMC neurons for aversive associative memory and required in a GABAergic subset of these neurons for circadian locomotor rhythmicity. In genetic epistasis experiments I also found that deficits in both behaviors can be rescued by suppressing Insulin Receptor-Ras signaling. Collectively, my results illustrate a specific molecular pathway for the circadian and memory functions controlled by Nf1 and indicate that sleep need, circadian timing, and memory are linked by neurons in the AMMC.

Introduction

The sleep/wake cycle has long been considered to be based on the influential two-process model. According to this model, homeostatic and circadian processes function in tandem to determine the timing and duration of sleep. Specifically, the circadian process encodes the time-of-day information and promotes wakefulness. The homeostatic process, on the other hand, keeps track of sleep need and promotes sleep after extended waking [1]. However, little is known about how these two processes interact with each other on the circuit and molecular levels or how they contribute to the well-established connection between consolidated sleep/wake cycles and memory formation [2].

The neurofibromatosis 1 (Nf1) gene may represent a link between these processes. Loss-of-function mutations in this gene lead to disrupted sleep/wake cycles and deficits in cognition in human patients [3-6]. In addition, null mutations in Nf1 cause locomotor arrhythmicity and learning deficit in Drosophila [7,8]. These data suggest that Nf1 functions in Drosophila and humans in similar ways. Intriguingly, despite demonstrating disrupted sleep/wake cycle, Nf1 mutants show normal cycling of key circadian proteins such as period (PER) and timeless (TIM) in central clock networks, indicating that Nf1 functions in an output pathway of the circadian clock network [7].

Thus, identifying novel sites of function for Nf1 may be important for several reasons: 1. It could potentially bridge the gap between the circadian and homeostatic processes since Nf1 is suggested to function in an unknown circadian output pathway that may interact with the sleep homeostat according to the two-process model. 2. It could link Nf1-mediated phenotypes to specific cellular mechanisms. 3. It could shine light on the well-known connection between consolidated sleep and memory in specific neural circuits [2].

To address these goals my lab previously performed a screen for neurons in which Nf1 is required for circadian locomotor activity. Two drivers from the screen, 42D11-Gal4 and 53D10-

Gal4, were identified as causing locomotor arrhythmicity when coupled to a UAS-Nf1 RNAi (unpublished data). When the expression patterns of these drivers were compared, a small cluster of neurons in the AMMC was identified as an apparent region of overlap and thus a likely locus for Nf1 function **(Figure S2).** Interestingly the same drivers were independently identified in a screen for neurons that satisfy several expected features of a locus for sleep homeostasis. Their activity is sufficient to promote sleep **(Figure S3a,b)**; their activity is required for rebound sleep following sleep deprivation **(Figure S3c);** and their activity is upregulated during the rebound period **(Figure S3d)**. Notably at least some of these expectations are not satisfied by drivers that have been used to implicate the central complex in sleep homeostasis [10, 11]. Most importantly, we found that blocking the synaptic output of the central complex with these drivers has no effect on rebound sleep **(Figure S1)**. Thus, I hypothesize that the same cluster of neurons in the AMMC that seems to function as an output pathway of the circadian clock also contributes to the sleep homeostat. In this thesis I refine the mapping of the AMMC neurons involved in these processes and demonstrate that they are GABAergic neurons. I also demonstrate that the same AMMC neurons require Nf1 for aversive associative memory.

At the molecular level, previous research has shown that some circadian and memory phenotypes of Nf1 mutants flies are mediated by adenylate cyclase/PKA pathway and Ras/MAPK pathways [7-9]. However, it is unclear whether all neurons that utilize Nf1 for circadian and memory functions utilize the same signaling pathways. With drivers that localize Nf1-mediated phenotypes I was in a fortuitous position to address this question. To do so I manipulated cAMP/PKA and Ras/MAPK signaling pathways with the 53D10 and 42D11 drivers to either phenocopy or rescue deficits caused by Nf1 knockdown. I found that instead of adenylate cyclase/PKA, the Ras/MAPK signaling pathway is coupled to Nf1-mediated locomotor rhythmicity as well as memory formation. Furthermore, I showed that Insulin receptor was coupled to the Ras signaling controlled by Nf1 in

the same neurons. Collectively these results suggest a close relationship between normal circadian function and memory formation.

In summary my result shows that: 1. Nf1 functions in a likely locus for sleep homeostasis, thus suggesting that circadian signaling and sleep need are integrated at this site in the brain. 2. Nf1-mediated circadian and memory function are both coupled to the Insulin Receptor-mediated Ras signaling pathway.

Section I. Nf1 is required for locomotor rhythmicity in sleep homeostat neurons

Results

I first confirmed that Nf1 does not function in clock neurons by knocking down Nf1 in neurons labeled by pdf-Gal4 and tim Gal4, which label the core oscillator and nearly all neurons in the circadian clock network, respectively [12]. As expected, I found that locomotor rhythmicity is not affected **(Figure 1a)**. However, when knocking down Nf1 with 42D11-Gal4 or 53D10-Gal4, I found that locomotor rhythmicity is disrupted, indicating that Nf1 is required for normal circadian function in sleep homeostat neurons **(Figure 1a,b)**. These data suggest that neurons that comprise the putative sleep homeostat also function as a previously unidentified clock output pathway.

I also examined the sleep phenotypes of Nf1 knockdown in 42D11 and 53D10 neurons. The sleep/wake patterns of flies were measured over 24 hours as previously described [13, 14]. I found that there was a significant decrease in both daytime and nighttime sleep in 42D11 flies. Nf1 knockdown in 53D10 neurons led to slightly but not significant decrease in LD Sleep **(Figure 1c,d)**. It is not clear what is responsible for this discrepancy, but it is possible that Nf1 affects baseline sleep in some neurons targeted by 42D11-Gal4 but not 53D10-Gal4.

(a) (b)

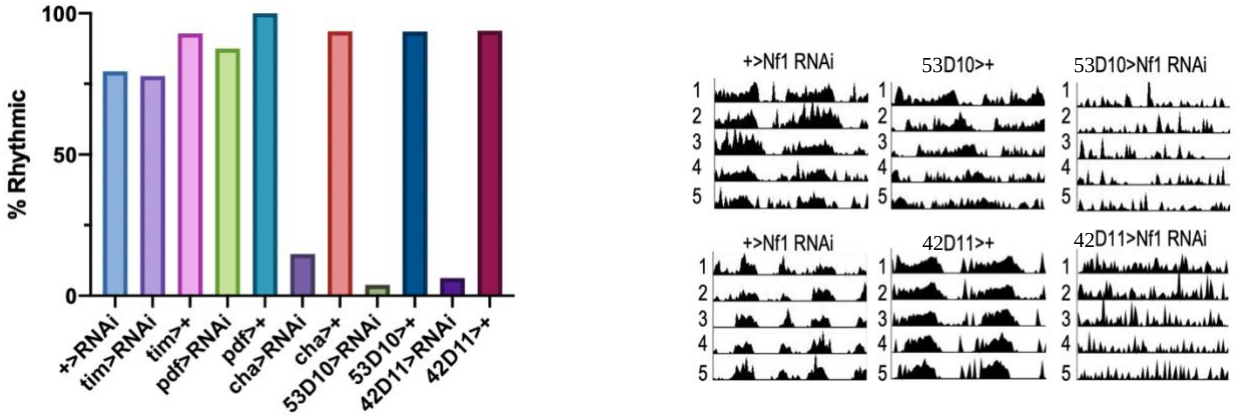


Figure 1a,b. Circadian Phenotypes of Nf1 knockdown in control and 42D11/53D10 Gal4 flies (a) Percentage of flies that were rhythmic in constant darkness. n ≥ 9 for each group. (b) Activity profiles of control and flies with Nf1knockown in 42D11/53D10 Gal4-labeled neurons over six days.

(c) (d)

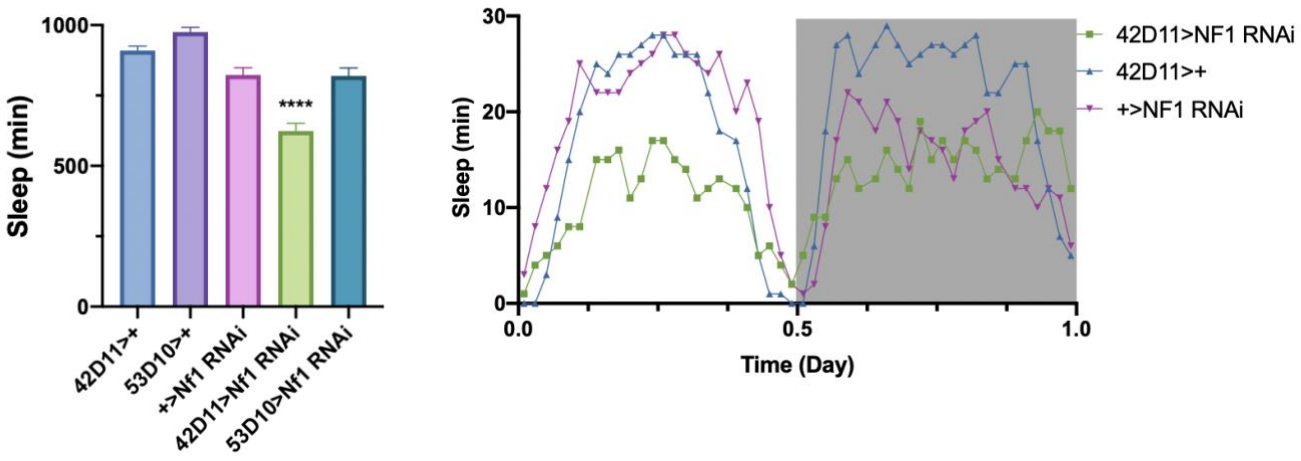
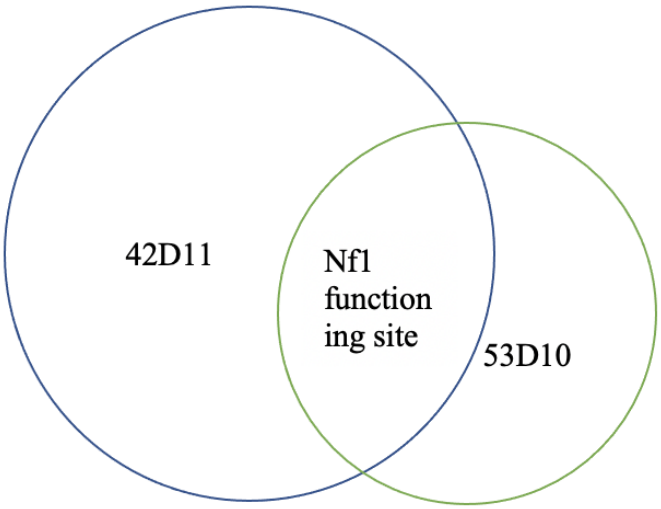


Figure 1c,d. Sleep Phenotypes of Nf1 knockdown in control and 42D11/53D10 Gal4 flies. (c) Total amount of daily sleep for each genotype in minutes. ****p<0.0001 by Tukey Test following one-way ANOVA. n ≥ 54 for each group. (d) Example sleep trace of 42D11>Nf1 RNAi flies and control. Shaded area indicated dark cycle.

42D11 and 53D10 Gal4-labeled neurons appear to overlap in the AMMC region of fly brains (**Figure S2**) and are sufficient and necessary for sleep homoeostasis **(Figure S3)**. To confirm that the two drivers overlap in neurons in which Nf1 is required for locomotor rhythmicity Nf1 RNAi was genetically combined with 42D11-Gal4 flies and then crossed to either 42D11- or 53D10-Gal80. If Nf1 is indeed functioning only in the region in which the two drivers have overlapping expression, then both Gal80s should reduce Gal4 activity and thus restore locomotor rhythmicity to Nf1 knockdown flies **(Figure 1e)**. As expected, both groups demonstrated complete rescue of the circadian phenotype **(Figure 1f)**. Since 53D10-Gal4 has a more restricted expression pattern compared to 42D11-Gal4, I chose to focus on 53D10 knockdown flies for the remaining experiments in this section.

(e)



(f)

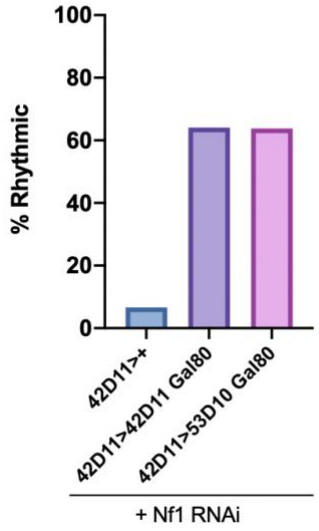


Figure 1e,f. Circadian Phenotypes of Nf1 RNAi rescued by 42D11-Gal80 and 53D10-Gal80 (e) Venn Diagram showing proposed functioning site of Nf1 in the overlapped region of two drivers. (f) Percentage of flies that were rhythmic when expressing 42D11-Gal80 vs 53D10 Gal80 n ≥ 39 for each group.

Recently my lab has attempted to further subdivide AMMC neurons labeled by the 53D10 driver according to classical neurotransmitter identity using RNAscope. These efforts have revealed that 53D10 neurons consist of at least three overlapping subgroups: cholinergic, glutamatergic and GABAergic neurons. More specifically, most lateral AMMC neurons are cholinergic; most medial AMMC neurons are glutamatergic; and overlapping with both subsets are GABAergic neurons **(Figure S4)**. Previous research has shown that glutamate in this region functions as an inhibitory neurotransmitter of GABAergic neurons in the AMMC [15]. To determine if 53D10 neurons might be mediating glutamatergic signaling to regulate the sleep/wake cycle I crossed 53D10-Gal4 to various RNAi's encoding glutamate receptor subunits. Consistent with a role for local glutamatergic neurons in regulating GABAergic neuron-mediated arousal, I found that knockdown of GluCl, a glutamate-gated chloride channel, increased sleep substantially above control levels **(Figure S5).** Based on these results, I hypothesize that 53D10 glutamatergic neurons promote arousal by inhibiting nearby sleep-promoting GABAergic neurons. Consistent with this hypothesis, thermogenetically activating the GABAergic subset of 53D10 neurons

selectively caused an increase in sleep **(Figure S6)**.

Next I asked whether the sleep-promoting GABAergic 53D10 neurons are responsible for Nf1's circadian function. To achieve this goal, I blocked knockdown of Nf1 in 53D10 neurons with cha-Gal80, VGlut-Gal80 and Gad-Gal80. I found that only Gad-Gal80 rescued the locomotor rhythmicity suggesting that Nf1 was only required in the GABAergic subset of 53D10-labeled neurons for normal circadian function **(Figure 1g).** Notably, knocking down Nf1 in all cholinergic neurons disrupted locomotor rhythmicity as well. However, 53D10-Gal80 could not rescue the disrupted locomotor rhythmicity of those flies **(Figure 1h)**. These results suggest that Nf1 regulates circadian rhythms through at least two sites of action: the GABAergic 53D10 neurons I have described and cholinergic neurons that have yet to be identified.

(g) (h)

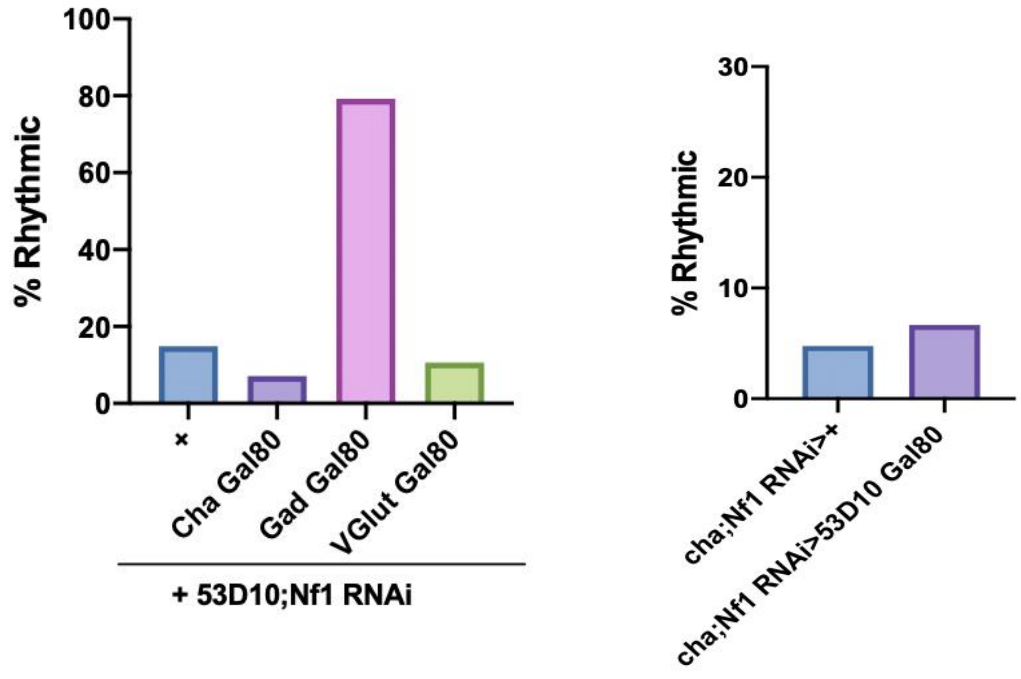


Figure 1g,h. Circadian and Sleep Phenotypes of 53D10>Nf1 RNAi rescued by GAD Gal80s.
(g) Percentage of flies that were rhythmic in Nf1 mutant flies expressing Cha/Gad/VGlut Gal80. n ≥ 42 for each group.
(h) Percentage of flies that were rhythmic in cha;Nf1 RNAi flies expressing 53D10 Gal80. n≥21 for each group.

Discussion

In the first set of experiments, I verified that Nf1 does not function in neurons that comprise the circadian clock network. Rather, it functions to regulate normal locomotor rhythmicity and sleep in neurons required for sleep homeostasis, which are labeled by 42D11- and 53D10-Gal4 drivers. I confirmed that the two drivers label the same neurons using 42D11/53D10 Gal80. Since the two drivers appear to have overlapping expression only in a small cluster of neurons in the AMMC, these neurons appear to be the locus at which circadian arousal and sleep need is integrated.

My follow-up experiments showed that Nf1 is only required in a GABAergic sleep-promoting subset of 53D10 neurons for normal circadian rhythms. Thus, the circadian function of Nf1 in 53D10 neurons maps to GABAergic neurons but not glutamatergic or cholinergic neurons, suggesting a functional role based on neurotransmitter identities.

Mapping of the circadian function of Nf1 to GABAergic 53D10 neurons is significant for another reason as well. The widely accepted two-process model for sleep/wake control establishes distinct roles for the clock and the sleep homeostat in regulating arousal, but presumably the signals must be integrated somewhere in the brain. As I showed, the sleep-promoting effect of GABAergic 53D10 neurons presumably underlies the sufficiency and necessity of 53D10 neurons as a whole for sleep homeostasis. Since a circadian function of Nf1 maps to the same neurons, this suggests that the sleep homeostat is coincident with an output of the circadian clock. That is, arousal signals driven by sleep need and circadian function appear to be integrated in GABAergic 53D10 neurons.

I also showed that these neurons lie immediately adjacent to glutamatergic neurons, and that knockdown of GluCl throughout 53D10 neurons increases sleep. Since local glutamatergic neurons have previously been shown to inhibit GABAergic neurons in the AMMC, I further propose that local glutamatergic neurons inhibit sleep-promoting GABAergic neurons by activating GluCl in the latter to permit waking [15]. Future experiments will be required to test

this hypothesis more directly and to determine whether glutamate release or GluCl activity might be a focal point for sleep/wake control.

Section II. Nf1's circadian function in 53D10 neurons is carried out by Insulin-Receptor Mediated Ras Signaling Pathway

Results

Previous research indicates that NF1 in Drosophila activates adenylate cyclase/PKA signaling and suppresses Ras/MAPK signaling [7-9]. I asked whether Nf1's circadian function in 53D10 neurons utilizes one or both proposed pathways **(Figure 2a)**. To address this question, I crossed UAS-Nf1 RNAi;53D10-Gal4 flies to various RNAi lines encoding previously reported elements of the two pathways. I expected to rescue rhythmicity by phenocopying the wildtype function of NF1 – i.e. by either elevating cAMP/PKA signaling or by suppressing Ras/MAPK pathway signaling.

(a)

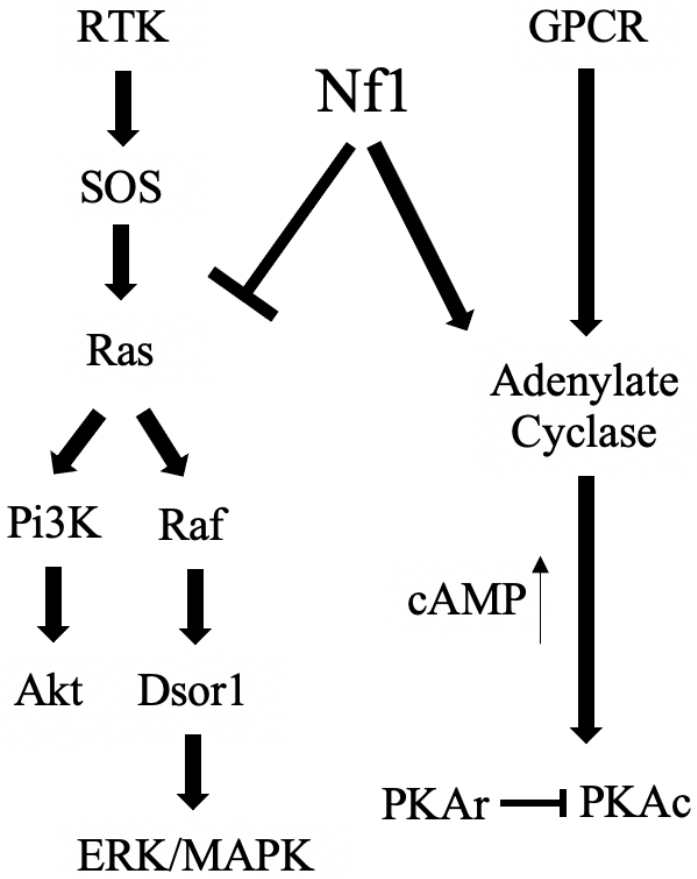
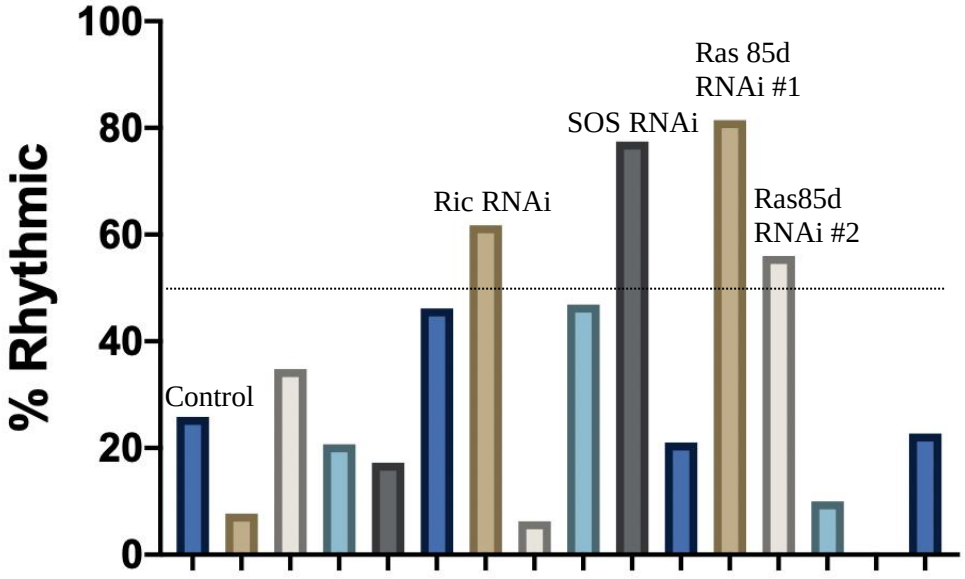


Figure 2a. Possible Molecular Mechanism of Nf1 regulating circadian function. Two different pathways are regulated by Nf1 in this model: Nf1 suppresses Ras/MAPK pathway way and activate adenylate cyclase/PKA pathway.

I found that among all the RNAi's I tested, those that targeted SOS, Ric and Ras 85d stood out for their rescue of locomotor rhythmicity **(Figure 2b)**. All three genes are positive regulators of Ras signaling [16]. In contrast, restoring cAMP/PKA signaling by knocking down regulatory subunits of PKA (PKAr) failed to rescue locomotor rhythmicity in Nf1-knockdown flies **(Figure 2b)**. These results suggest that NF1's ability to suppress Ras but not its ability to elevate PKA signaling is necessary for normal circadian function in 53D10 neurons.

(b)



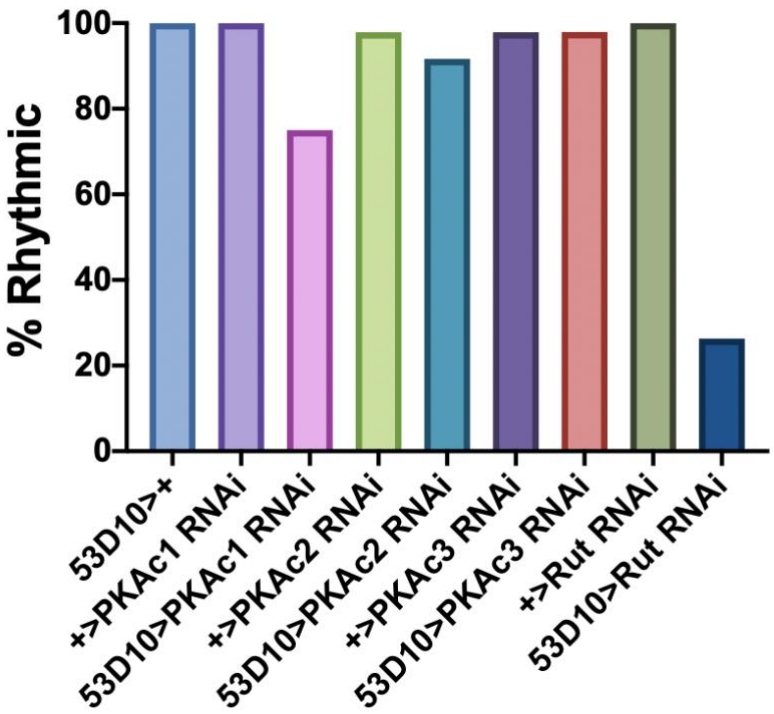
+ :	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rala RNAi:	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rap1 RNAi #1:	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Rap1 RNAi #2:	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Rap2l RNAi:	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Rhebb RNAi:	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Ric RNAi:	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
GEF RNAi:	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Rgl RNAi:	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
SOS RNAi:	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Ras64b RNAi:	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Ras85d RNAi #1:	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Ras85d RNAi #2:	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
PKAr1 RNAi:	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
PKAr2 RNAi #1:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
PKAr2 RNAi #2:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

+ 53D10>Nf1 RNAi

Figure 2b. Circadian rhythmicity is restored to Nf1 knockdown animals by reducing SOS or Ras signaling in 53D10 neurons. Percentage of flies that were rhythmic upon manipulating cAMP/PKA and Ras signaling in 53D10 neurons. Groups above dashed lines mean more than 50% of the flies of the same genotype were rhythmic, indicating strong rescue of Nf1's circadian phenotype.

To further test the contribution of each pathway, I attempted to phenocopy Nf1-driven arrhythmicity by reducing PKA or by elevating Ras signaling in 53D10 neurons. Specifically, if Nf1 suppresses Ras or activates adenylate cyclase/PKA signaling to regulate locomotor rhythmicity, then overexpressing components of Ras or knocking down components of adenylate cyclase/PKA signaling pathway should disrupt locomotor rhythmicity, just like depleting neurons of Nf1. As expected, I found that knocking down catalytic subunits of PKA (PKAc) had no clear effect on rhythmicity **(Figure 2c)**. In contrast, I found that overexpressing Ras64b or Raf (a known effector of Ras) disrupted locomotor rhythmicity **(Figure 2d)**. These results further indicate that the circadian phenotype coupled to Nf1 is carried out through Ras, but not PKA signaling, in 53D10 neurons.

(c)



(d)

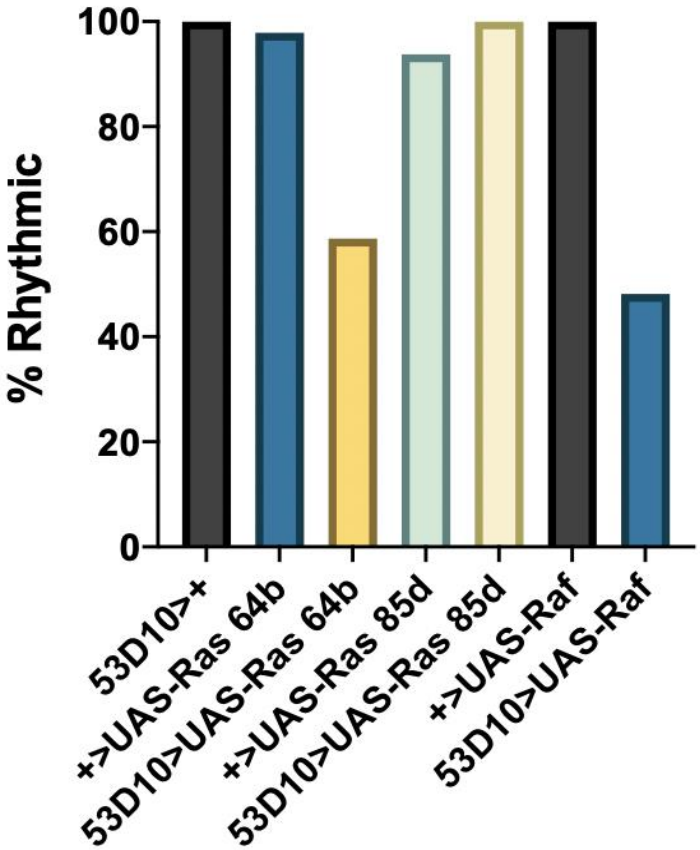
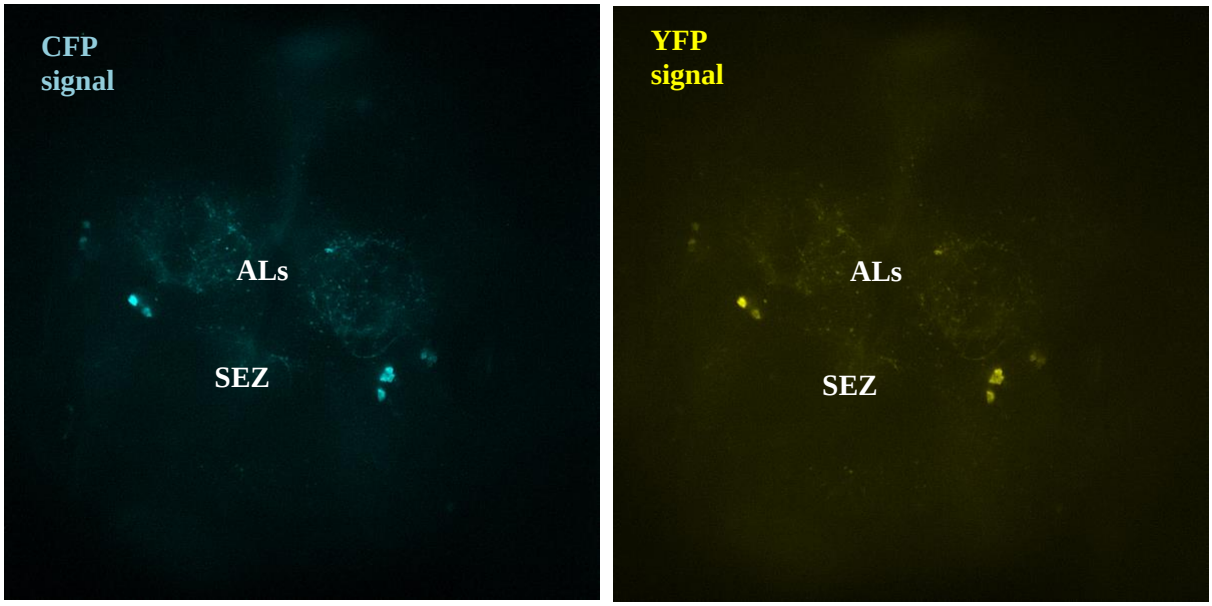


Figure 2c,d. Circadian arrhythmicity caused by Nf1 depletion is phenocopied by elevating Ras but not by reducing PKA signaling in 53D10 neurons. (c) Percentage of flies that were rhythmic upon knocking down catalytic subunits of PKA. n ≥ 19 for each group. (d) Percentage of flies that were rhythmic when overexpressing Ras or Raf. n ≥27 for each group.

Intriguingly, knockdown of Rut, which encodes adenylate cyclase, did induce strong locomotor arrythmia **(Figure 2c)**. However, I suspect this phenotype was independent of the Nf1 pathway since evidence mentioned above was strongly against the possibility that Nf1 was coupled to adenylate cyclase/PKA signaling. To test this possibility, I collaborated with another lab member to conduct ratiometric cAMP imaging in animals expressing EPAC in 53D10 neurons. Our results showed that Nf1 knockdown in 53D10 neurons has very minimal effect on the YFP/CFP ratio, indicating no change in cAMP **(Figure 2e,f)**. Thus, I conclude that the circadian function of Nf1 in 53D10 Gal4-labeled neurons was mediated through Ras but not PKA signaling pathway.

(e)



(f)

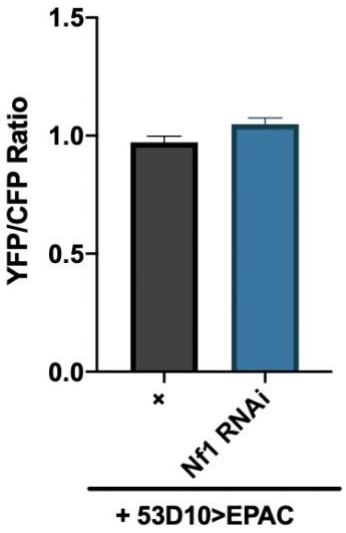
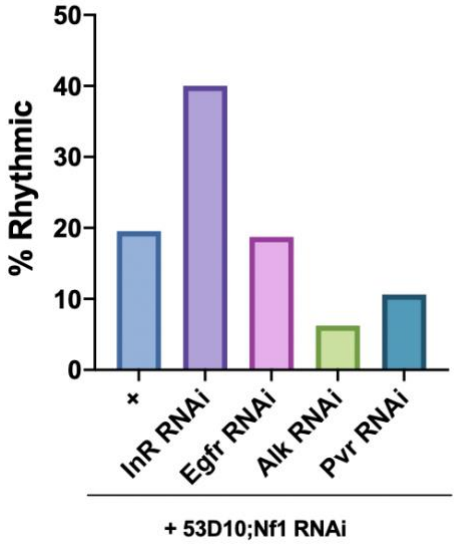


Figure 2e, f. EPAC imaging results of 53D10 neurons with and without Nf1. (e) Examples of EPAC imaging of 53D10 Gal4-labeled neurons under CFP & YFP channels. (f) Average YFP/CFP ratio for 53D10>EPAC +/- Nf1 RNAi. There was no significant difference after applying unpaired t-test.

Next, I tried to find the receptor that mediated the Ras signaling pathway regulated by Nf1 in 53D10 neurons. Ras signaling pathway has long been shown to be coupled with receptor tyrosine kinases, and there are approximately 20 receptor tyrosine kinases (RTKs) encoded by the Drosophila genome [17]. Based on the fact that knocking down SOS or Ras 85d could rescue the Nf1-related circadian disruption, I chose several RNAi lines that have been shown to target RTKs

upstream of Ras signaling components [17]. I hypothesized that if any of these RTKs couple to the Ras signaling pathway regulated by Nf1, then knocking down these genes should at least partially attenuate the activation of Ras signaling and thus rescue the locomotor rhythmicity in Nf1-deficient flies. My results show that depleting 53D10 neurons of insulin receptor, but not Egfr, Alk or Pvr could partially rescue the disrupted locomotor rhythmicity caused by Nf1 knockdown in 53D10 neurons **(Figure 2g)**. In contrast to other RTKs, the insulin receptor in Drosophila has been shown to recruit Pi3K (phosphoinositol 3-kinase) to activate Ras signaling pathway [17]. Thus, I also tried to restore rhythmicity to Nf1-depleted flies by knocking down several types of Pi3K's. I found that Pi3k92E, which has previously been proposed to couple with insulin receptor, indeed partially rescues the locomotor rhythmicity [17] **(Figure 2h)**. I also tried knocking down several downstream components of Ras signaling and found that Dsor1 (downstream of Raf1) also rescues the locomotor rhythmicity **(Figure 2h)**. Overall, these results show that NF1 must suppress insulin receptor-mediated Ras signaling in 53D10 neurons for normal circadian locomotor rhythmicity.

(g)



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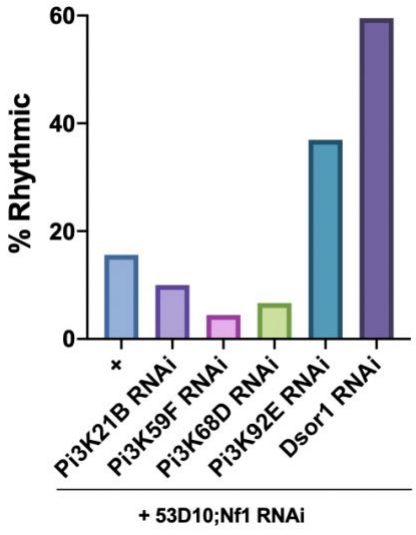


Figure 2g, h. Circadian rhythmicity is restored to Nf1-depleted animals by knocking down insulin receptor pathway components in 53D10 neurons. (g) Percentage of 53D10>Nf1 RNAi flies that were rhythmic after co-expressing receptor tyrosine kinase RNAi. $n \geq 30$ for each group. (h) Percentage of 53D10>Nf1 RNAi flies that were rhythmic after co-expressing different Pi3K/Dsor1 RNAi's. $n \geq 30$ for each group.

(i)

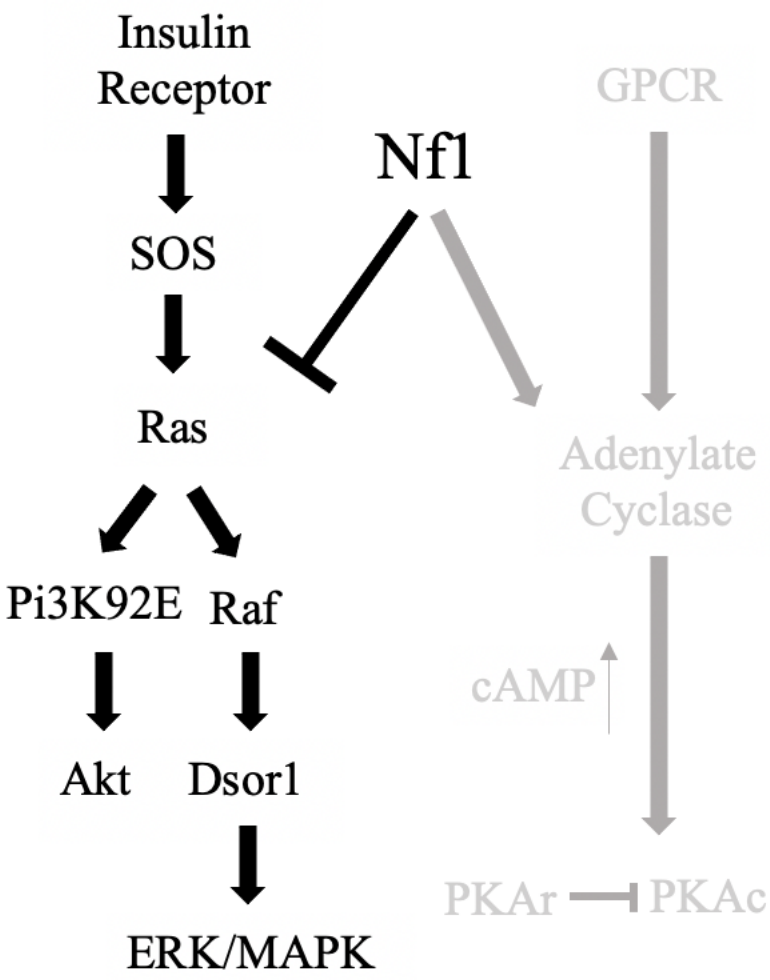


Figure 2i. Proposed molecular mechanism of Nf1 regulation of circadian function in 53D10 neurons. A modified version of figure 2a based on experimental results. On the left side, through Nf1 the suppression of Insulin receptor-mediated Ras signaling pathway was proposed. On the right side, the adenylate cyclase/PKA signaling pathway was turned gray since no evidence showed Nf1 functioned through that pathway in 53D10 neurons.

Discussion

In this set of experiments, I utilized RNAi screening and neural imaging to determine which signaling pathway was necessary for the Nf1-regulated circadian function in 53D10 neurons. My results show that Nf1 was primarily coupled to the insulin receptor-mediated Ras signaling pathway but not adenylate cyclase/PKA signaling pathway **(Figure 2i)**. One caveat of these experiments was that insulin receptor only partially rescued the locomotor rhythmicity in Nf1-deficient flies compared to knocking down SOS and other components of Ras signaling pathway. Although this limited rescue could be attributed to a weak RNAi, another possible interpretation is that there might be other RTKs that additionally contribute to this pathway. It was quite surprising that knockdown of Nf1 did not change cAMP levels in 53D10 neurons since positive coupling between NF1 and adenylate cyclase has been described by others [18]. My results suggest that the Nf1 might have different molecular functions based on the requirements of the particular cells in which it is expressed.

My finding that Nf1 suppresses insulin receptor signaling indicates potential metabolic roles for 53D10 neurons as well as integrating arousal signals from the circadian clock and sleep homeostat. Previous research has shown that insulin signaling pathway is not only important for metabolism but also affects sleep intensity [19, 20]. The potential interaction between sleep and metabolism in sleep homeostat neurons thus would be a valid suggestion for future direction.

Section III. Nf1 in 53D10 neurons contributes to associative memory using signaling pathways that resemble those involved in Nf1-regulated circadian rhythms

Results

Sleep and arousal states are known to be an important factor in associative memory. Since Nf1 in 53D10 neurons is required for a normal sleep/wake cycle, and since Nf1 is known to be important for learning and memory in flies, I hypothesized that Nf1 in 53D10 neurons might be required for associative memory [8]. To test this hypothesis, I used an assay called the Proboscis Extension Reflex (PER) [21, 22]. In this assay, flies receive repetitive training to associate the appetitive tastant fructose with the bitter taste of quinine. Wildtype control flies form such associative memory after the training and thus stop extending their proboscis when presented with fructose alone in the following test session **(Figure 3a)**. After three consecutive training sessions, control flies with normal associative memory show a decreasing curve of response rate toward fructose. Test score refers to response rate in the final test session, and lower test score suggests higher degree of memory formation and retention.

Two types of graphs are used to demonstrate the result of the PER assay. There is a line graph showing the response rate starting from pre-test to three training session and ending at test session, which outlines the learning curve of each genotype. Flies with normal associative memory typically show a steep slope, indicating their quick learning during the early training session. The bar graph instead just shows the response rate in the final test round to indicate degree of learning and memory.

I first tried the PER assay on flies with Nf1-knocked down in either 42D11 or 53D10 neurons. Both experimental groups showed more or less similar decline in response rate through the progression of training session compared to control groups **(Figure 3b,d)**. However, in the final test round, both Nf1-knockdown groups had significantly higher test scores compared to control, indicating a much smaller proportion of flies with intact gustatory associative memory **(Figure 3c,e)**.

This result suggests that Nf1 knockdown in 42D11/53D10 neurons indeed leads to a memory deficit, much like has been observed in Nf1 mutant mammalian models [3,4,8]. Interestingly, the relatively normal progression curve in the training session suggests that Nf1 knockdown affects maintenance of aversive memory instead of memory formation.

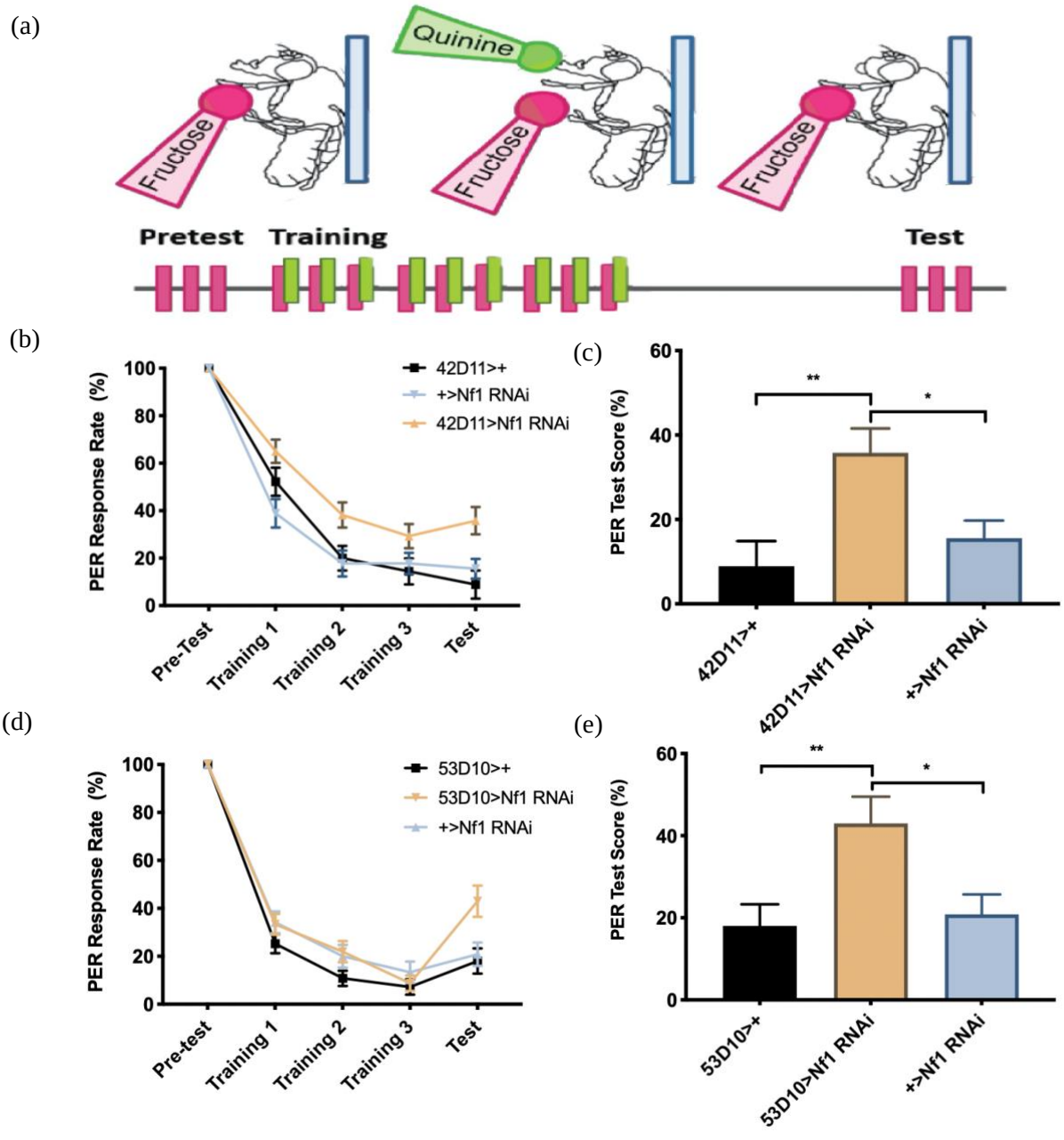


Figure 3a-e. Memory phenotypes of Nf1 knockdown in 42D11/53D10 neurons. (a) Graphical protocol for the associative memory test based on proboscis extension reflex (PER). (b) Learning curve of 42D11>Nf1 RNAi flies and controls showing their response rate at each session of the memory test. (c) Final test score of 42D11>Nf1 RNAi flies and controls. (d) Learning curve of 53D10>Nf1 RNAi flies and controls. (e) Final test score of 53D10>Nf1 RNAi flies and controls. **p<0.01/*p<0.05 by Tukey Test following one-way ANOVA. n ≥ 30 for each group.

In the previous section I showed that Nf1 is required for locomotor rhythmicity in AMMC neurons at the intersection of expression of the 42D11 and 53D10 drivers. Therefore, I hypothesized that Nf1's contribution to gustatory associative memory might map to the same neurons. To test this hypothesis, I combined 42D11>Nf1 RNAi or 53D10>Nf1 RNAi with either 42D11-Gal80 or 53D10-Gal80. I expected that if Nf1 is required at the intersection of 42D11 and 53D10 expression then both Gal80s should rescue the memory deficits of both Gal4>Nf1 RNAi combinations. Indeed, compared to Gal4>RNAi alone, both Gal80 groups rescued the memory phenotype with normal learning curve and low response rate during the final test round **(Figure 3f,g)**. This result suggested that the memory phenotype of Nf1 knockdown was also restricted in the overlapped neurons of both regions.

(f) (g)

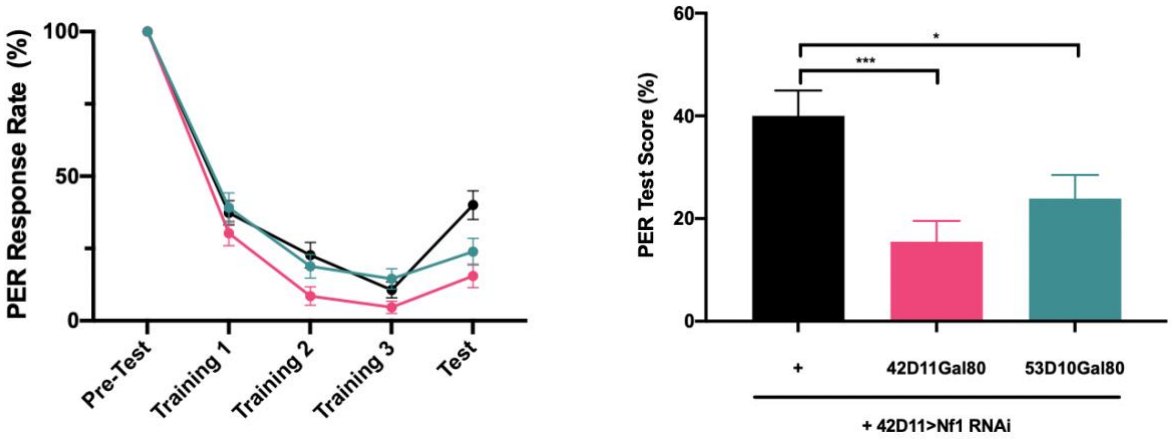


Figure 3f, g. Memory Phenotypes of Nf1 RNAi rescued by 42D11/53D10 Gal80. (f) Learning curve of Nf1 RNAi; 42D11 flies expressing 42D11/53D10 Gal80. (g) Final test score of Nf1 RNAi; 42D11 flies expressing 42D11/53D10 Gal80. ***p<0.001/*p<0.05 by Tukey Test following one-way ANOVA. n ≥ 43 for each group.

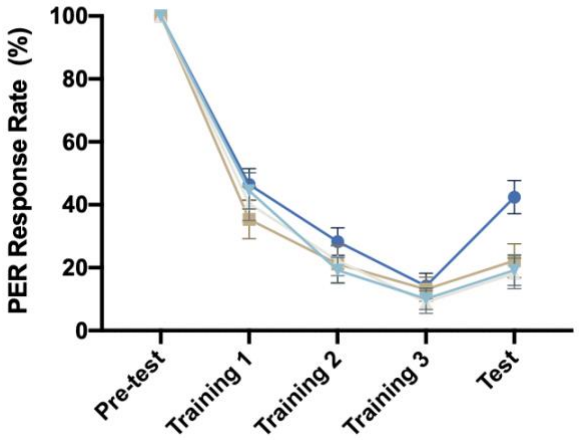
To make sure that the phenotypes I just measured were truly due to deficits in associative memory, rather than taste discrimination, I performed an additional experiment. I crossed Nf1 RNAi to elav-Gal4, a pan-neuronal driver, and then applied taste discrimination test to these flies and their genetic controls. As shown in Table 1, there was no difference in taste preference, suggesting intact taste sensation in Nf1-knockdown flies.

Table 1. Taste discrimination test results for Nf1-knockdown flies and controls. Each group of flies was placed on fructose-containing gels with two distinct color dyes while quinine was added to only one of them. The color of flies' abdomens was used to indicate taste preferences. There was no effect of color on results, so data from the two colors was combined.

elav;dicer>+		dicer>Nf1 RNAi		elav;dicer>Nf1 RNAi	
Fructose	Quinine + Fructose	Fructose	Quinine + Fructose	Fructose	Quinine + Fructose
57 (100%)	0 (0%)	51 (100%)	0 (0%)	60 (100%)	0 (0%)

Next, I investigated the cellular mechanism for Nf1's memory function. Because of the more restricted expression pattern of 53D10-Gal4, I used this driver for the following experiments. For reasons explained in the previous section, I again chose to focus on Ras and adenylate cyclase/PKA signaling pathways [7-9]. Since disrupted sleep/wake has long been suggested to cause memory deficit, I focused on RNAi's from the previous section that rescued circadian function of Nf1-depleted animals. Just as I described for circadian rhythms in the last section, I found that SOS, Ric and Ras RNAi's also rescued the memory deficit of Nf1-depleted animals during the test round of PER **(Figure 3h, i)**. These results indicate that NF1 suppresses Ras signaling to facilitate short-term gustatory associative memory. In additional experiments I also knocked down rut with the 53D10 driver, since I previously found that this disrupts locomotor rhythmicity. However, 53D10>rut RNAi flies had normal associative memory using the PER assay **(Figure 3j, k).** Combining these two results together, it appears that Nf1 in 53D10 neurons uses the same Ras signaling pathway for its regulation of both circadian rhythms and short-term memory.

(h)



(i)

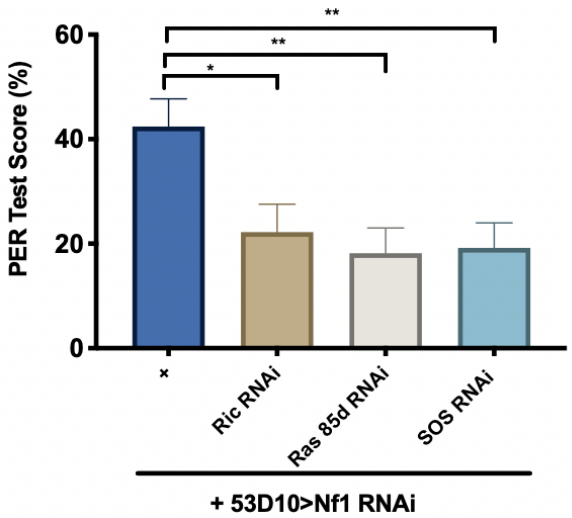
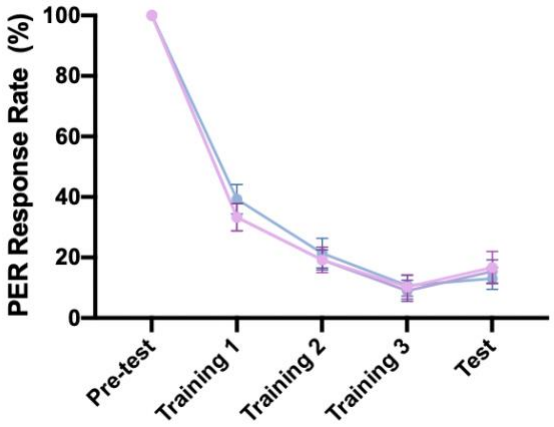


Figure 3h, i. Memory phenotypes of Nf1 RNAi rescued by SOS and Ras RNAi. (h) Learning curve of Nf1 RNAi;53D10 flies co-expressing Ric, Ras or SOS RNAi. (i) Final test score of Nf1 RNAi;53D10 flies expressing Ric, Ras or SOS RNAi. **p<0.01, *p<0.05 by Tukey Test following one-way ANOVA. n = 33 for each group.

(j)



(k)

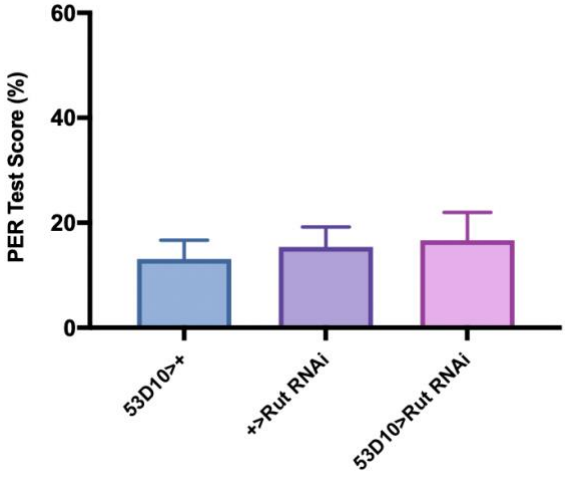


Figure 3j, k. Normal memory phenotypes of 53D10>Rut RNAi flies. (j) Learning curve of flies depleted of rut in 53D10 neurons. (k) Final test scores of flies in j. No significant difference by Tukey Test following one-way ANOVA. n ≥ 26 for each group.

To further confirm this conclusion, I tried to knock down upstream and downstream components indicated in previous section that were necessary for Nf1-regulated locomotor rhythmicity in order to determine whether that would also rescue the short-term memory deficit. I found that Dsor1 knockdown significantly lowered the response rate in the final test round.

Knockdown of Pi3k92E or InR also lowered the response rate of 53D10>Nf1 RNAi flies, though these results were not statistically significant **(Figure 3l, m)**. Overall, the data suggest that Nf1 regulates both circadian rhythm and associative memory through suppression of insulin receptor mediated Ras signaling pathway in 53D10 neurons.

(l) (m)

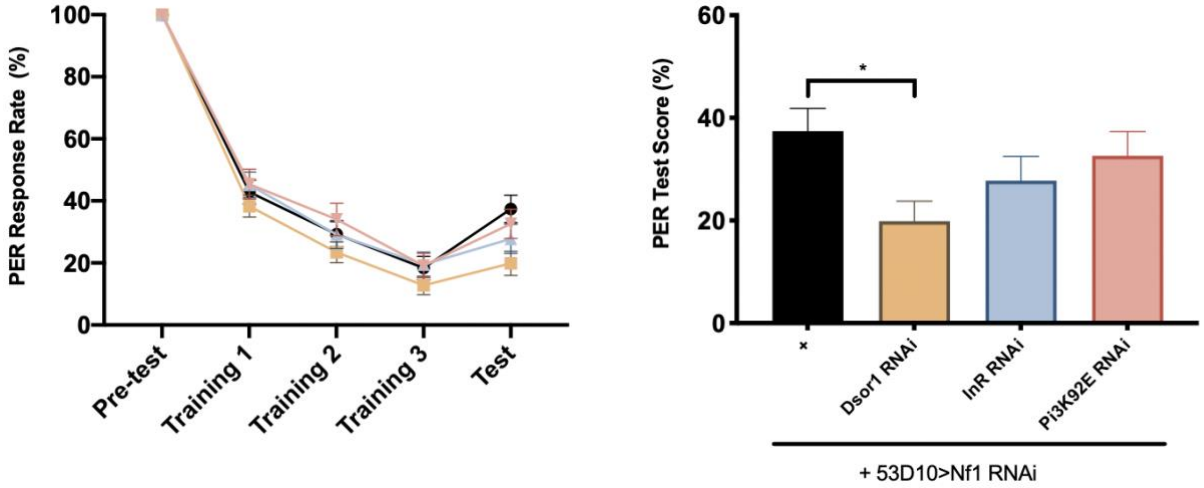


Figure 3l, m. Memory deficits of 53D10>Nf1 RNAi flies are rescued by knockdown of Dsor1. (l) Learning curve of Nf1 RNAi;53D10 flies co-expressing RNAi's for Dsor1, InR or Pi3k92E. (i) Final test score of flies in l. *$p<0.05$ by Tukey Test following one-way ANOVA. $n \geq 47$ for each group.

Discussion

In this section, the contribution of Nf1 to short-term memory formation was investigated using a gustatory associative memory assay. My results show that Nf1 knockdown in 53D10 neurons indeed led to inability to maintain aversive associative memory. My results also indicate that suppression of insulin receptor-mediated Ras signaling pathway is a mechanism by which Nf1 regulates both circadian locomotor rhythmicity and memory functions.

Another interesting finding from my experiments is that knocking down adenylate cyclase (rut) in 53D10 neurons disrupted sleep/wake cycles but had no effect on associative memory of flies. This indicates that despite the well-established requirement for consolidated sleep to facilitate certain forms of memory, the two phenotypes can be dissociated under certain conditions. In fact, surprisingly memory formation seemed to be better correlated with circadian rhythmicity than sleep in my experiments.

Another interesting finding from my experiments is that Nf1 expression is required in the AMMC for associative memory. Although previous results from flies and mammals have previously shown that Nf1 is required for cognition, in flies this was thought to be due to Nf1 expression in the mushroom bodies, a major locus for sleep/wake control and memory formation [8, 23-25]. Although it is possible that Nf1 is required in both brain loci for associative memory, unpublished data from my lab indicates that NF1 is poorly expressed at best in mushroom bodies [W. Joiner, personal communication]. Additional experiments will be needed to resolve this discrepancy. The anatomical location and functions of these AMMC sleep homeostat neurons resembled local interneurons of antennal lobe [15]. Imaging data supported this by showing these neurons sent projection to antennal lobe [Unpublished data]. It is possible that Nf1 regulated memory in these neurons through their modulation of antennal lobe projection neurons, which further projected to mushroom body [31].

Section IV. Conclusion and Future Directions

Nf1 has been suggested to function in a clock output pathway and to play a role in associative memory [7,8]. My data suggest that these two functions may be linked due to a requirement for Nf1 expression in a small cluster of neurons in the AMMC that is labeled by the 42D11 and 53D10 drivers. Motivated by the two-process model of sleep/wake regulation and an apparent labeling of neurons involved in sleep homeostasis by 42D11-Gal4 and 53D10-Gal4, I knocked down Nf1 using the same drivers. My results show that indeed Nf1 functions in a cluster of AMMC neurons labeled by both drivers to regulate locomotor rhythmicity and sleep. Collectively these data suggest that the sleep homeostat is part of a novel clock output pathway. I further investigated the subpopulations of these AMMC neurons defined by their neurotransmitter identities and found that Nf1 functions in a GABAergic subset for locomotor rhythmicity. However, unpublished data from my lab indicate that the GABAergic subset also contributes to sleep homeostasis. Thus, my findings add an intriguing twist to the two-process model by demonstrating integration of signals from both the circadian clock and the sleep homeostat within a single brain locus.

One potential caveat for this claim could be that these sleep homeostat neurons were not sensing and coding time of day information, which means they only played a regulatory role on the clock output. To rule out that possibility, future experiments should express reporters in these sleep homeostat neurons and utilize neural imaging to investigate cell activity across 24 hours. Cycling of cell activity across the day would be a strong evidence against that possibility.

After identifying a specific locus for Nf1's function, I tried to figure out the cellular mechanism for normal circadian rhythm. Based on previous research, I tested Ras and PKA signaling pathways utilizing RNAi knockdown and UAS overexpression of signaling pathway components. My results show that Nf1 is coupled to the insulin receptor-mediated Ras signaling pathway but not to the adenylate cyclase/PKA signaling pathway. Further EPAC imaging confirmed this conclusion

by showing Nf1 knockdown did not affect cAMP levels in 53D10 neurons. Overall, my experiments provide ample evidence for a specific cellular pathway for Nf1 to regulate locomotor rhythmicity.

The Ras signaling pathway has been shown to play important roles during developmental periods [17]. To address whether Nf1 functions in adulthood to regulate circadian rhythms or whether it regulates development of neural circuitry required for locomotor rhythmicity, future experiments should restrict Nf1 RNAi expression in selected developmental/adult stages to determine the exact time window for its effect.

Lastly, I showed that Nf1 knockdown could also lead to an associative memory deficit using the PER assay, thus confirming previously published results that reduced Nf1 function causes learning and memory deficits [8]. Specifically, I showed that Nf1 knockdown in 42D11/53D10 neurons impairs the maintenance of gustatory associative memory. Furthermore, I found that Nf1 uses the insulin receptor-mediated Ras signaling pathway to regulate both circadian and memory functions.

Collectively, my results demonstrate that Nf1 functions in a neural circuit involved in regulating sleep homeostasis, circadian rhythms and associative memory, and I provide a detailed signaling pathway for the last two functions. My results also highlight the important cognitive role played by neurons involved in integrating arousal signals by the circadian clock and sleep need.

Supplemental Figures

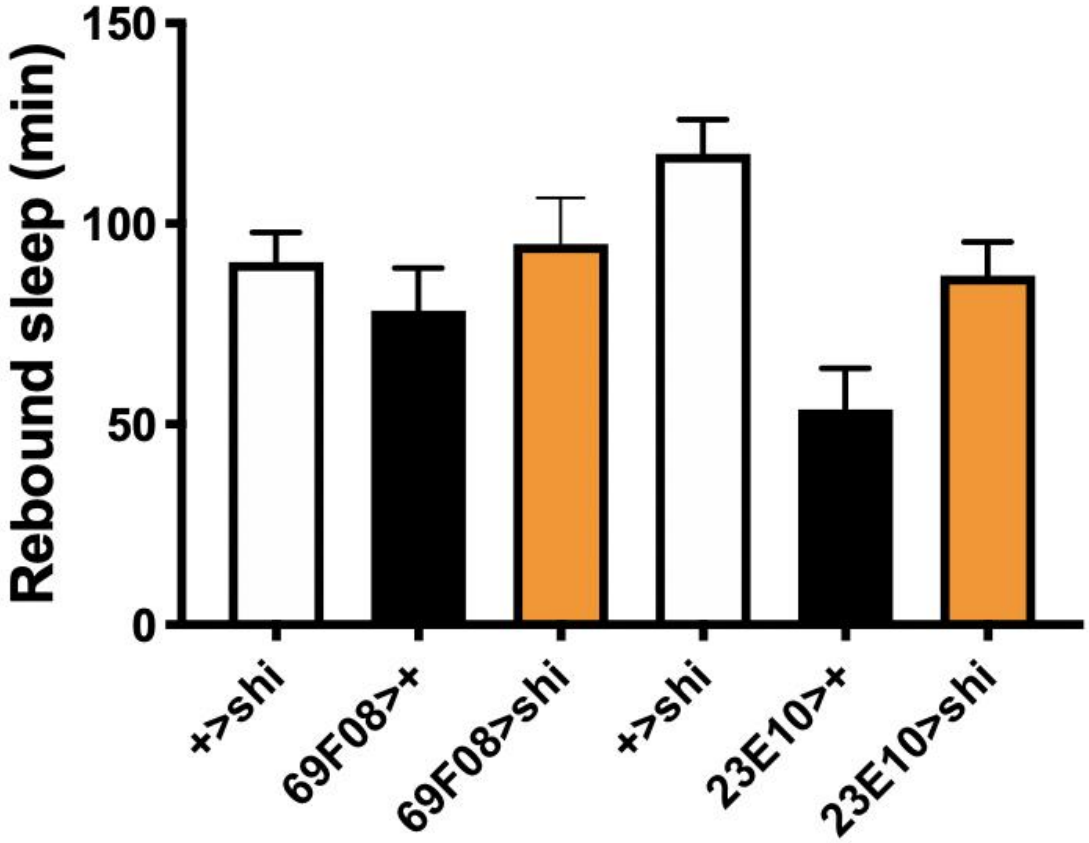
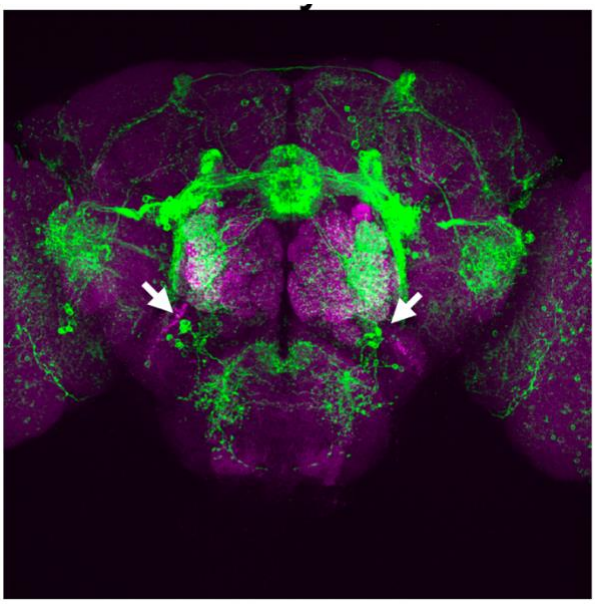


Figure S1. Rebound sleep is unaffected when synaptic output is blocked from the ellipsoid body or the dorsal fan-shaped body of the central complex with temperature-sensitive dominant-negative shibire. Animals were deprived of sleep by mechanical agitation from ZT20-24 at 20 C, and rebound sleep was measured immediately afterward from ZT0-6 at 30 C. No significant reduction in rebound sleep was observed in experimental groups relative to controls. 69F08-Gal4 targets the ellipsoid body, and 23E10-Gal4 targets the dorsal fan-shaped body.

(a)

42D11>myr-GFP



(b)

53D10>myr-GFP

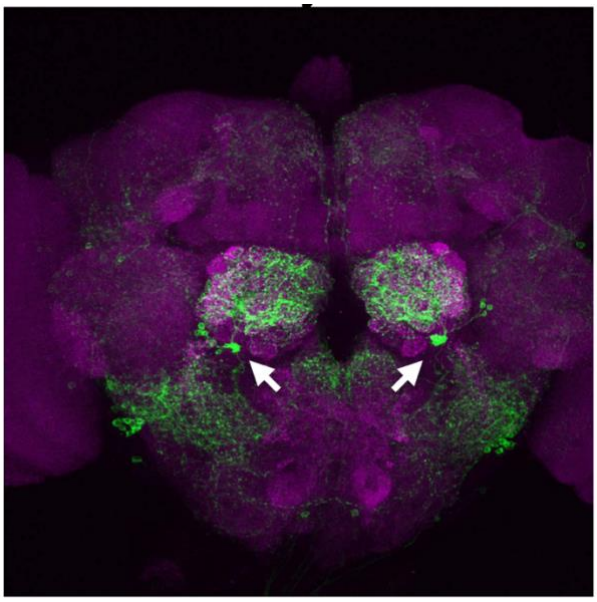


Figure S2. Expression Patterns of 42D11- and 53D10-Gal4 Drivers in the Fly Brain. (a-b) Expression patterns of 42D11 and 53D10 Gal4 drivers, respectively. White arrowheads mark relevant cell bodies. Anti-GFP staining is green.

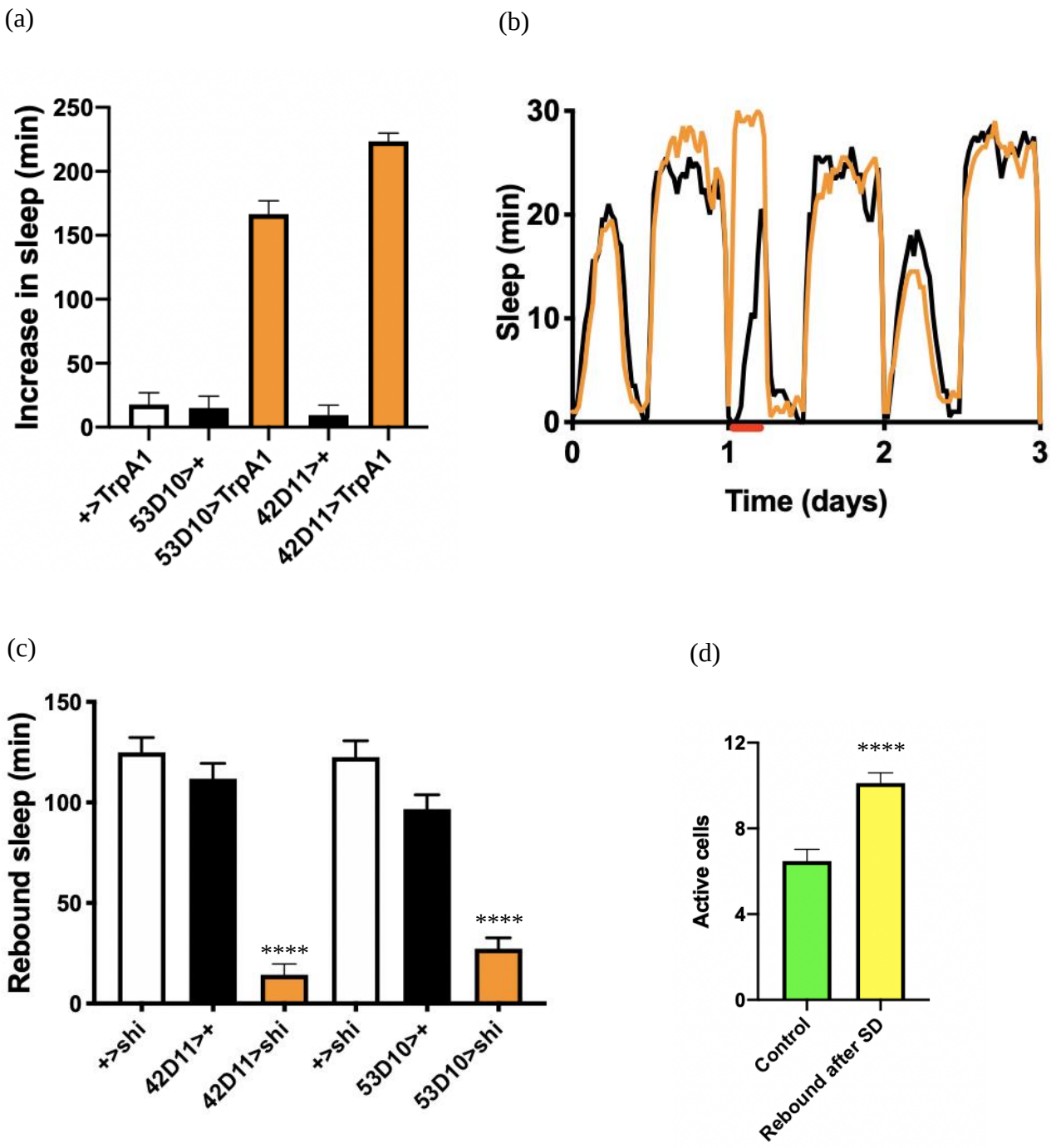


Figure S3. Experiments demonstrating 53D10 Gal4-labeled neurons were sufficient and necessary for sleep homeostasis. (a) Using TrpA1 to thermogenetically active 42D11/53D10 Gal4 labeled neurons significantly increase the amount of sleep. ****p<0.0001 by Tukey Test following one-way ANOVA. n ≥ 25 for each group. (b) Example sleep trace of 53D10>TrpA1 flies (Orange) and control (Black). Red line indicated the time window when TrpA1 channel was being activated by elevated temperature. (c) Expressing shibire in 42D11/53D10 Gal4-labeled neurons block the rebound sleep after mechanically depriving the sleep of flies. ****p<0.0001 by Tukey Test following one-way ANOVA. n ≥ 94 for each group. (d) More 53D10 neurons were activated based on GCaMP6s signals during the rebound sleep following 6-hour sleep deprivation. ****p<0.0001 by Tukey Test following one-way ANOVA. n≥17 for each group.

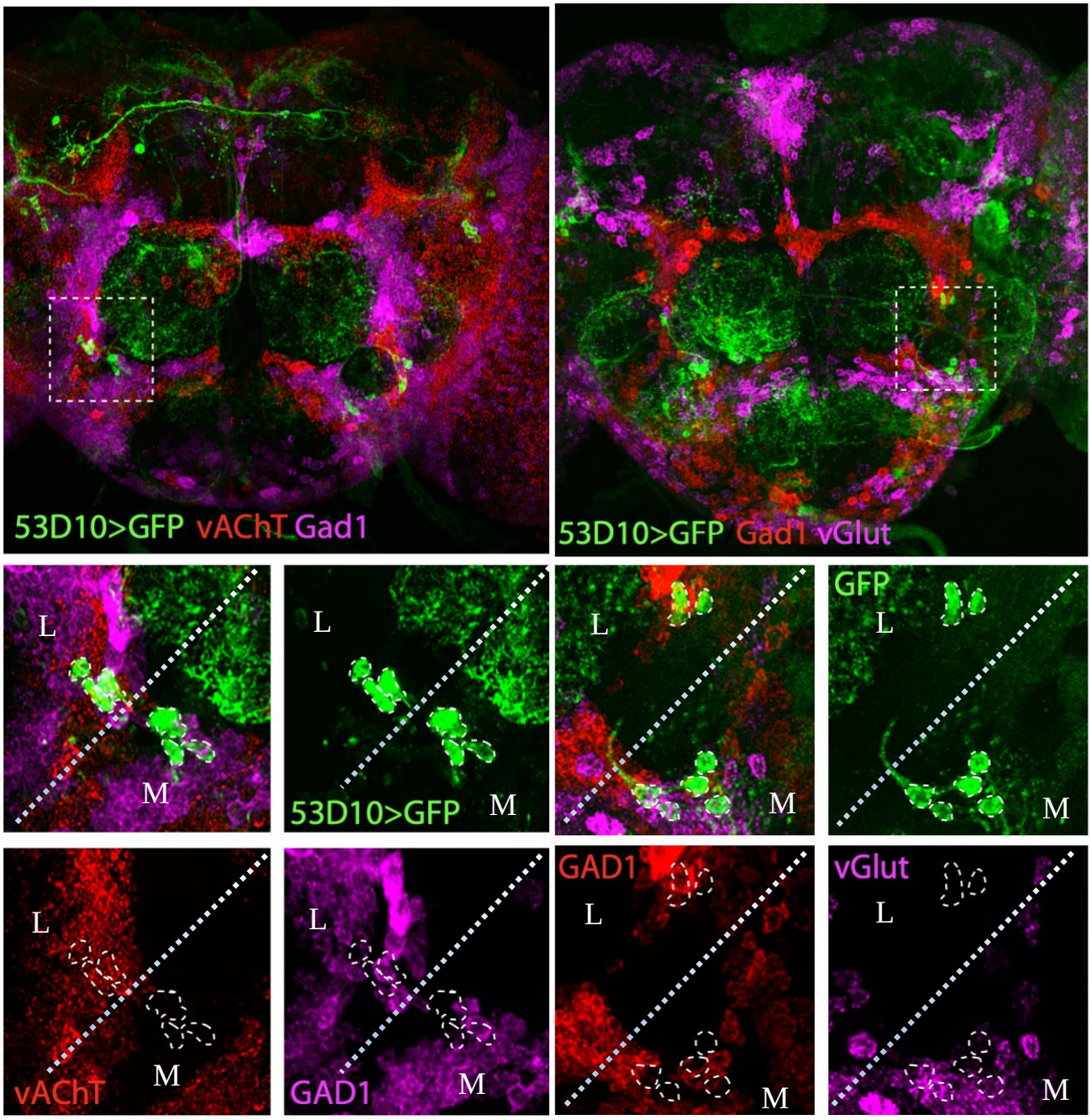
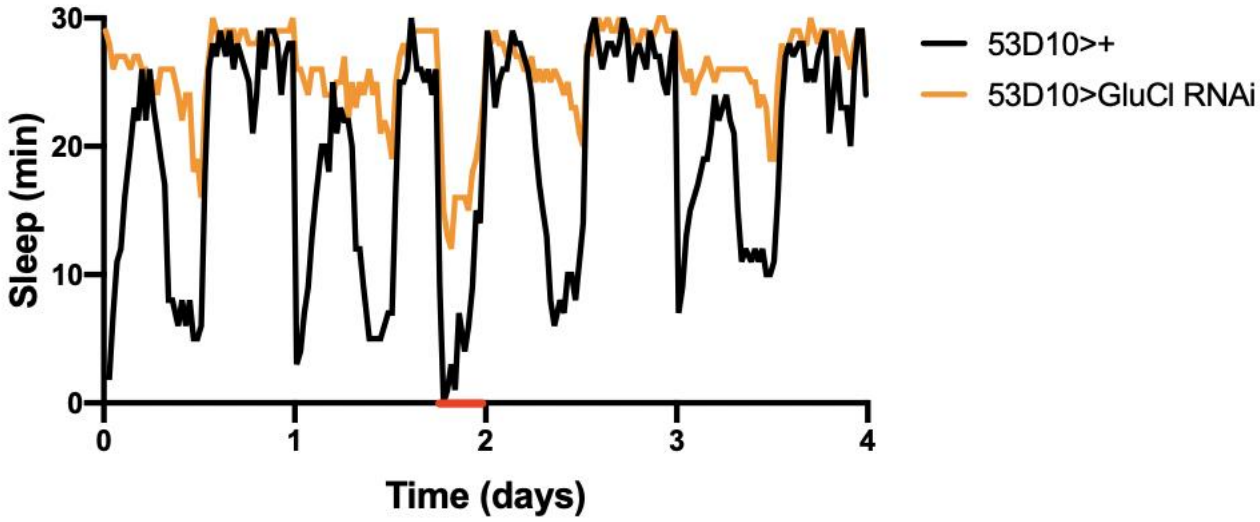
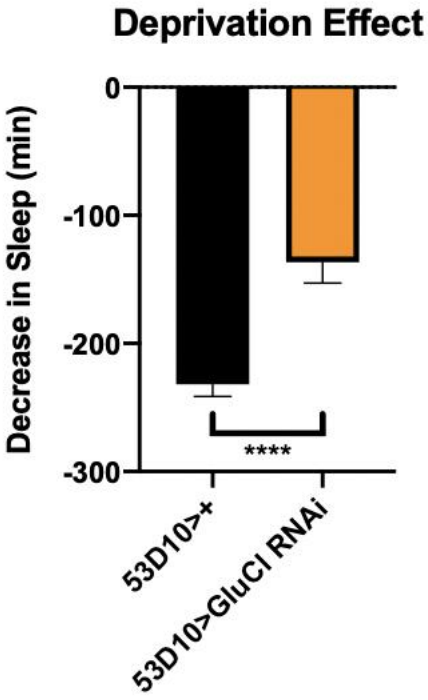


Figure S4. Expression Patterns of neural subsets of 53D10-Gal4 Drivers in the Fly Brain. RNAscope imaging showing GABAergic subset of 53D10 neurons partially overlapped with Cholinergic and Glutamatergic subset neurons. M and L indicates medial and lateral subsets of 53D10 neurons, respectively.

(a)



(b)



(c)

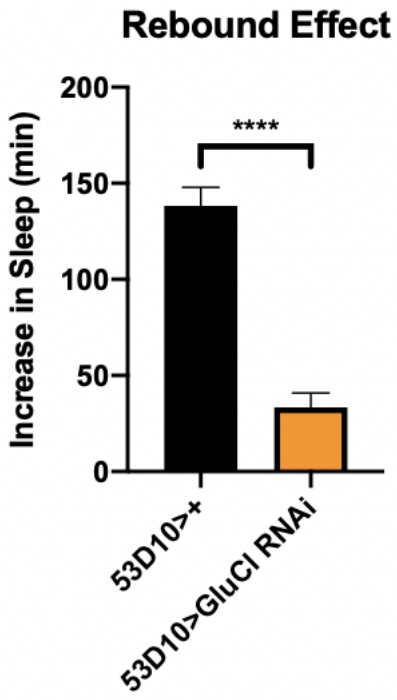


Figure S5. Sleep phenotypes of GluCl knockdown in 53D10-Gal4-labeled neurons. (a) Example sleep trace of 53D10>GluCl RNAi flies (Orange) and control (Black). Red line indicated the sleep deprivation window. (b) Amount of sleep decrease during deprivation period compared to previous day. (c) Amount of sleep increase (rebound) during the 6-hour window after deprivation compared to previous day. ****$p<0.0001$ by Tukey Test following one-way ANOVA. $n \geq 23$ for each group.

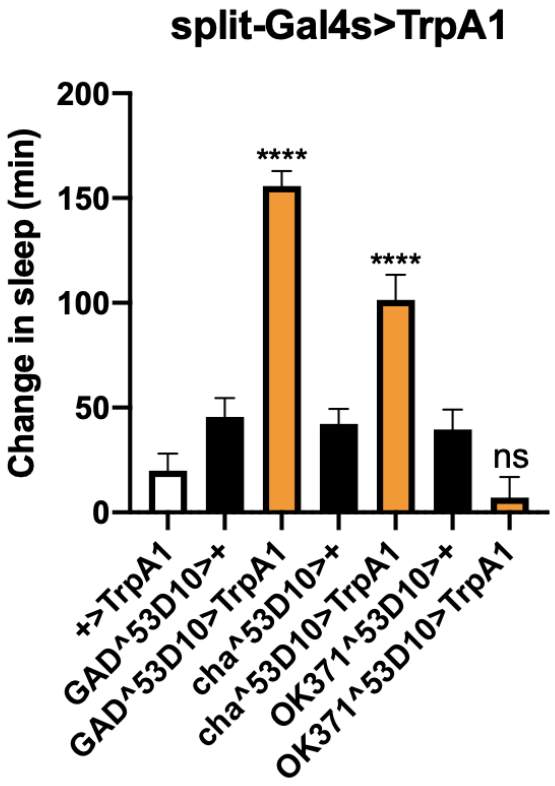


Figure S6. Sleep/Wake-promoting effect of different subset of 53D10-Gal4-labeled neurons by their neurotransmitter identities. (a) Sleep-promoting effect of GABAergic/Cholinergic subset instead of Glutamatergic subset utilizing Split Gal4 and TrpA1 activation. Expressed TrpA1 channels were activated from ZT0 to ZT6 at 29ºC. Change in sleep was quantified in that 6-hour window compared to previous day. ****$p<0.0001$ by Tukey Test following one-way ANOVA. $n \geq 20$ for each group.

Materials and Methods

Fly Stocks and Transgenic Fly Lines

Most Gal4 drivers and RNAi lines were obtained from the Bloomington Stock Center, with stock numbers listed in brackets: 42D11-Gal4 [50156], 53D10-Gal4 [45347], pdf-Gal4 [25031], tim-GAL4 [80941], 69F08-Gal4 [39499], 23E10-Gal4 [49032], Nf1 RNAi [53322], SOS RNAi [34833], Ras64b RNAi [29318], Ras85d RNAi [29319, 34619], Ric RNAi [82973], Rala RNAi [34375], Rap1 RNAi [35047, 57851], Rap2l RNAi [51840], Rhebb RNAi [33966], PDZ-GEF RNAi [28928], Rgl RNAi [28938], Raf RNAi [55863, 55679], PKAr1 RNAi [52906], PKAr2 RNAi [34983, 53930], Pi3K21B RNAi [38991], Pi3K59F RNAi [64011], Pi3K92E RNAi [61182], InR RNAi [51518], Pvr RNAi [37520], Egfr RNAi [60012], Alk RNAi [27518], Dsor1 RNAi [34830], Rut RNAi [80468], GluClalpha RNAi [53356], PKAc1 RNAi [57743], PKAc2 RNAi[55859], PKAc3 RNAi [55860], UAS-Raf [2033], UAS-Ras 64b [2025], UAS-Ras 85d [4845], UAS-EPAC-camps [25407], UAS-GCaMP6s [91990], UAS-shi^ts [66600] .

Wildtype (w^{1118} iso31), flies were described previously [11]. Cha-Gal4 and Cha-Gal80 were provided by Dr. Toshi Kitamoto [26,27]. VGlut-Gal80 was provided by Dr. Julie Simpson. Gad-Gal80 was was generated by cloning of the 3.1 kb promoter of the Gad-Gal4 driver [28], fusing it to Gal80 in the pattB vector, and phiC31-mediated recombination and insertion into the attP2 site on the third chromosome. To generate Cha/42D11/53D10-Gal4;Nf1 RNAi lines, Gal4 drivers were combined with Nf1 RNAi on the second chromosome. To generate 42D11/53D10-Gal80 lines, promoters of the 42D11 and 53D10 drivers were cloned, fused to Gal80 in the pattB vector, and inserted into the attP2 site on the third chromosome by phiC31-mediated recombination. UAS-TrpA1 was provided by Dr. Paul Garrity. UAS-shi^ts chromosome 2 line was provided by Dr. Gerry Rubin [29]. To generate GAD/Cha/OK371;53D10 split Gal4 lines, 53D10-DBD was generated by cloning the 53D10 promoter from stock # 45347, fusing it to Gal4 DBD in pattB,

followed by phiC31-mediated targeting to VK00027 on chromosome 3. GAD-AD and Cha-AD were generated by cloning the GAD and Cha promoters, fusing them to the Gal4 AD in pattB, followed by phiC31-mediated targeting to attP2. OK371-AD was provided by Dr. Chi-Hon Lee [30].

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:2hr 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 [13, 14].

Circadian Measurements

Two- to seven-day-old male flies were loaded into glass tubes containing 5% sucrose and 2% agarose and then measured for sleep/wake patterns using the Drosophila Activity Monitoring System (Trikinetics). After two days of 12hr:2hr light/dark cycle at 25ºC, flies went through six days of dark/dark cycle. Locomotor Rhythmicity was quantified by Sleep Lab programmed by Dr. William Joiner using MATLAB.

Aversive Taste Memory Assay

The Proboscis Extension Reflex assay was performed as previously described [22] 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 22-26 hours and glued onto glass slides 2 hours before experiments. Starvation time varied based on the health condition of each genotype but were kept consistent for each experiment. Flies that consistently extended their proboscis when giving fructose passed the pre-test and were given three rounds of training trials. After training, fructose alone was given during the test trial. Sucrose was presented

after that to verify flies have the normal taste sensation and 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 put in a plastic vial containing 5% agarose gel. Half of the geal contained 100mM fructose and the other half contained mixture of 100mM fructose and 10mM quinine. Each half of agarose gel was randomly dyed with red or blue food dye. Colors of dye were reversed in repeated experiments. Flies stayed in the vial for gel feeding for 3 hours in the dark. After that, their abdomens were examined for color to determine their preference toward what kinds of gel.

Statistics

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

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