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## ***translin* is required for metabolic regulation of sleep**

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### **Summary**

Dysregulation of sleep or feeding has enormous health consequences. In humans, acute sleep loss is associated with increased appetite and insulin insensitivity, while chronically sleep-deprived individuals are more likely to develop obesity, metabolic syndrome, type II diabetes, and cardiovascular disease. Conversely, metabolic state potentially modulates sleep and circadian behavior; yet, the molecular basis for sleep-metabolism interactions remains poorly understood. Here, we describe the identification of *translin* (*trsn*), a highly conserved RNA/DNA binding protein, as essential for starvation-induced sleep suppression. Strikingly, *trsn* does not appear to regulate energy stores, free glucose levels, or feeding behavior suggesting the sleep phenotype of *trsn* mutant flies is not a consequence of general metabolic dysfunction or blunted response to starvation. While broadly expressed in all neurons, *trsn* is transcriptionally upregulated in the heads of flies in response to starvation. Spatially restricted rescue or targeted knockdown localizes *trsn* function to neurons that produce the tachykinin-family neuropeptide Leucokinin. Manipulation of neural activity in Leucokinin neurons revealed these neurons to be required for

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starvation-induced sleep suppression. Taken together, these findings establish *trsn* as an essential integrator of sleep and metabolic state, with implications for understanding the neural mechanism underlying sleep disruption in response to environmental perturbation.

## Results and Discussion

In humans, sleep and feeding are tightly interconnected, and pathological disturbances of either process are associated with metabolism-related disorders. Acute sleep loss correlates with increased appetite and insulin insensitivity, while chronically sleep-deprived individuals are more likely to develop obesity, metabolic syndrome, type II diabetes, and cardiovascular disease [1–3]. Conversely, in humans and rodents internal metabolic state potentially modulates sleep and circadian behavior [4–6]. Despite the widespread evidence for interactions between sleep loss and metabolic dysfunction, little is known about how these processes integrate within the brain.

To address this question, we sought to identify integrators of sleep and metabolic state in the fruit fly, *Drosophila melanogaster*. Knockdown of genes from randomly selected RNAi lines was achieved by expression of UAS-RNAi under the control of the neuron-specific GAL4 driver, *n-Synaptobrevin-GAL4* (*nSyb-GAL4*) [7, 8]. Following 24 hours of baseline sleep measurements on food, sleep was measured during 24 hours starvation on agar and the change in sleep was calculated as previously described [9]. Starvation-induced sleep suppression was reduced in flies with neuron-specific knockdown of the RNA/DNA binding protein *translin* (*trsn*) (Figure 1A). To confirm the effect of *trsn*-RNAi on sleep, we tested two additional RNAi transgenes. All three RNAi lines showed similar phenotypes; *trsn* knockdown flies slept similarly to control flies on food, while sleep loss resulting from starvation was reduced or absent (Figure 1B-E). Targeted knockdown of *trsn* in the fat body (yolk-GAL4) or muscle (24b-GAL4), two tissues involved in energy storage, showed normal sleep suppression in response to starvation (Figure S1A) supporting the notion that *trsn* functions primarily in neurons to regulate sleep.

In *Drosophila*, starvation induces hyperactivity in addition to sleep loss [10–12]. To determine whether *trsn* also regulates the hyperactivity response to starvation, we analyzed waking activity in fed and starved *trsn* knockdown flies. Neuronal knockdown of *trsn* had no effect on waking activity in fed flies, but reduced starvation-induced hyperactivity (Figure S1B). These findings are consistent with the notion that *trsn* does not modulate sleep or activity in the fed state, but is required for both sleep and locomotor changes that result from starvation.

To validate that the sleep phenotype in *trsn* knockdown flies was not due to off-target effects of RNAi, we measured sleep in flies with a mutation in the *trsn* locus. Both male and female flies with a P-element insertion in the *trsn* locus (*trsn*<sup>EP</sup>) or the excision allele (*trsn*<sup>null</sup>) are viable [13] and exhibit reduced sleep suppression during starvation (Figure 1F-H and S1C-D), phenocopying flies with neuron-specific RNAi knockdown. The waking activity of *trsn*<sup>null</sup> flies phenocopies RNAi knockdown flies under fed conditions, while starvation-induced hyperactivity is blunted or absent in *trsn* mutants (Figure S1E).

A number of systems have been developed for high-resolution video tracking that may provide a more accurate measure of sleep compared to infrared-based monitoring systems [14–17]. Tracking analysis revealed that *w<sup>1118</sup>* control, but not *trsn<sup>null</sup>* flies, suppress sleep during starvation, confirming that the results obtained using infrared tracking are not an artifact of the sleep acquisition system (Figure S1F). Taken together, these findings indicate starvation-induced sleep suppression and locomotor activity are reduced in *trsn* mutant flies.

Starved flies utilize glucose and fatty acids to maintain metabolic homeostasis, and the availability of these energy sources may regulate sleep. To determine the energy source required for normal sleep, we fed flies the glycolysis inhibitor 2-Deoxyglucose (2-DG) [18], or the carnitine palmitoyltransferase antagonist, etomoxir, an inhibitor of fatty acid  $\beta$ -oxidation [19]. Treatment with both of these drugs has been used extensively in mammals, and these inhibitors have similar effects on fly metabolism [20, 21]. Flies were fed standard food laced with 400mM 2-DG or 25 $\mu$ M etomoxir and monitored for sleep to determine whether the breakdown products of glucose or triglyceride stores (or both) contribute to reduced sleep during starvation. Flies fed 2-DG, but not etomoxir, significantly reduced sleep, suggesting that reduced glucose availability or the energy derived from its metabolism, rather than fatty acids, contribute to sleep suppression (Figure 1I; and data not shown). When *trsn* mutant flies were subjected to the same protocol, no changes in sleep were observed with 2-DG feeding (Figure 1I). The finding that *trsn* mutant flies are insensitive to sleep regulation in response to both acute food deprivation and pharmacological perturbation of energy utilization, suggests *trsn* is critical for the integration of sleep and metabolic state.

It is possible that the reduced ability of *trsn* mutants to suppress sleep during starvation stems from a general inability to modulate sleep in response to environmental or pharmacological disruption. To test this, sleep rebound was determined by mechanically shaking flies at 3-4 minute intervals for 12 hours during the night (ZT12-ZT24) and measuring sleep for 12 hours (ZT0-ZT12) the following day. Sleep-deprived *trsn<sup>null</sup>* flies showed a significant increase in daytime sleep that was not present in undisturbed controls (Figure S1G). The sleep rebound in *trsn<sup>null</sup>* flies was comparable to *w<sup>1118</sup>* control flies indicating that *trsn* is dispensable for the homeostatic response to mechanical sleep deprivation (Figure S1G). In addition to mechanical deprivation, numerous pharmacological agents including the stimulant caffeine and free-radical inducing agent paraquat, disrupt sleep in flies [22, 23]. Both *w<sup>1118</sup>* control and *trsn<sup>null</sup>* flies significantly reduced sleep when fed food laced with caffeine (Figure S1H) or paraquat (Figure S1I), supporting the notion that the loss of starvation-induced sleep suppression in *trsn* mutant flies does not result from a generalized inability to suppress sleep.

Flies with enhanced energy stores do not suppress sleep or increase activity in response to starvation [11, 21]. *Drosophila* primarily stores energy as triglycerides and glycogen, and prolonged food-deprivation results in depletion of both stores. To test the possibility that *trsn* mutant flies do not suppress sleep when fasted due to increased energy stores, we measured triglyceride and glycogen levels using colorimetric assays standardized to total protein level [24, 25]. No differences in glycogen, triglyceride or free glucose levels were observed between fed or 24 hour starved *trsn<sup>null</sup>* flies and *w<sup>1118</sup>* controls (Figure S2A-C), indicating

that the loss of starvation-induced sleep suppression in *trsn* mutant flies is not due to an increase in energy stores.

Many metabolism-related genes regulate both sleep and feeding [26], raising the possibility that *trsn* is generally required for hunger-dependent behaviors. To determine whether *trsn* modulates reflexive food acceptance response, we measured the Proboscis Extension Reflex (PER) of flies starved for 24 hours prior to behavioral testing (Figure 2A) [27, 28]). Total PER response did not differ between starved *trsn*<sup>null</sup> and *w*<sup>1118</sup> flies to sucrose concentrations of ranging from 1 to 1000 mM (Figure 2B), or 5% yeast extract (Figure 2C), indicating that *trsn* is dispensable for reflexive feeding. To measure food consumption, we provided flies with 100mM sucrose or 5% yeast extract in the Capillary Tube Feeding (CAFÉ) assay (Figure 2D) [29]. Flies were starved for 24 hours prior to the start of the assay and consumption was measured over 12 hours. No differences in total consumption of 100 mM sucrose or 5% yeast extract was detected between control and *trsn*<sup>null</sup> flies (Figure 2E). To quantify feeding over a shorter timeframe, the blue-dye assay was used to determine the quantity of food consumed in fed and 24 hour starved flies over a 30 minute period [30]. No differences between control and *trsn*<sup>null</sup> flies were detected in overall consumption in the fed or starved state, indicating that *trsn* does not regulate acute food consumption (Figure 2F-G). Taken together, three independent feeding assays indicate that *trsn* does not regulate feeding behavior during the starved state.

In *Drosophila*, *trsn* is expressed in the brain throughout development [31]. To determine whether *trsn* is acutely regulated in response to sleep or feeding state, we measured *trsn* transcript levels by quantitative PCR in flies that were previously starved or sleep-deprived. *trsn* was expressed at low-levels in the heads and bodies of fed flies and was specifically upregulated in the head following 24 hours of starvation (Figure 3A). No changes in *trsn* transcript were detected after 12 hours of mechanical sleep deprivation, suggesting the upregulation of *trsn* expression is not a generalized response to stress or environmental perturbation (Figure 3B). To confirm that TRSN protein is increased in response to starvation, we performed immunohistochemistry on brains immunostained with anti-TRSN. Quantification of whole-brain fluorescence confirmed that TRSN protein is increased in response to starvation (Figure 3C-D). In agreement with previous findings, TRSN signal is below detection in *trsn*<sup>null</sup> mutants and dramatically reduced in *nSyb-GAL4>trsn-IR* flies confirming the antibody specifically labels TRSN (data not shown and [13]). Counterstaining with the neuronal marker Embryonic Lethal Abnormal Vision (ELAV) revealed that TRSN and ELAV are expressed in all neurons during the fed and starved states (Figure 3E), suggesting the observed changes in protein levels are not due to altered protein localization. Together, these data suggest that at the RNA and protein levels, *trsn* is increased in response to starvation.

The finding that *trsn* is upregulated in response to starvation raises the possibility that it functions acutely to modulate sleep. RNAi targeted to *trsn* was acutely induced in three-day old animals using the GeneSwitch system. Flies were fed food laced with 0.25mM RU486, and sleep was measured on food and agar. Adult-specific pan-neuronal knockdown with all three RNAi lines under regulation of *elav*-Switch impaired sleep-suppression compared to genotype matched controls not fed RU486 or genetic controls lacking the *trsn*<sup>IRi</sup> transgene

([32, 33] Figure 3F and S3). These findings, coupled with the upregulation of *trsn* in response to starvation, provide evidence that *trsn* is required during adulthood for the integration of sleep and metabolic state.

We next sought to identify neurons where *trsn* functions to modulate sleep. Peptidergic neurons are critical regulators of many behaviors, including sleep and feeding [34–36]; therefore, we screened GAL4 lines labeling defined populations of peptidergic neurons or neurons previously shown to regulate sleep. We identified the Leucokinin (LK) neurons, where knockdown of *trsn* reduced sleep modulation in response to starvation. LK has been implicated in a host of fly behaviors including feeding and water homeostasis, locomotion, and olfactory behavior [37, 38]. Driving membrane tethered CD8::GFP with LK-GAL4 labeled a single large neuron in the lateral horn and three pairs of neurons in the subesophageal zone ([38]; Figure 4A). Immunostaining brains of LK-GAL4 flies driving nuclear GFP (UAS-GFP.nls) revealed that the LK-GAL4 neurons that are co-labeled by TRSN antibody (Figure 4B). In addition, all three *trsn-IR* lines impaired starvation-induced sleep suppression when expressed under the control of LK-GAL4 (Figure 4C), whereas restoration of *trsn* specifically in LK-GAL4 neurons, or in all neurons with *nSyb*-GAL4, rescued starvation-induced sleep suppression to control levels (Figure 4D and S4A). Therefore, *trsn* function in LK neurons is essential for starvation-induced sleep loss.

To further examine the role of LK neurons in sleep regulation we blocked synaptic release from LK neurons and measured sleep in fed and starved flies [38, 39]. Chronic blockade of synaptic release in LK neurons with tetanus toxin (TNT) impaired starvation-induced sleep suppression compared to control flies expressing an inactive form of TNT (UAS-IMP-TNT) or genetic controls harboring only a single transgene ([40] Figure 4E and S4B). In fed conditions, silencing of LK neurons increased sleep compared to controls that approached significance, raising the possibility that these neurons are wake promoting (Figure S4B). To examine the effects of acutely silencing LK-GAL4 neurons, the dominant-negative form of the GTPase *Shibire* (*Shi*<sup>TS1</sup>) was expressed in LK neurons and sleep was measured in both fed and starved flies during the night period [41]. Flies expressing *Shi*<sup>TS1</sup> in LK labeled neurons failed to suppress sleep at the non-permissive temperature of 31°C (Figure 4F and S4C-E). Control and experimental groups did not suppress sleep at 22°C due to the lower temperature and shortened duration of the assay (Figure S4C,D). Therefore, LK neurons are acutely required for modulation of sleep in response to starvation, supporting the notion that *trsn* function in LK neurons is essential for the integration of sleep and metabolic state.

Taken together, we have identified *trsn* as an essential regulator of sleep-metabolism interactions. While many genes have been identified as genetic regulators of sleep or metabolic state, multiple lines of evidence indicate that *trsn* functions as a unique integrator of these processes. *trsn* is not required for the homeostatic increase in sleep following mechanical deprivation or response to stimulants, suggesting *trsn* is not generally required for acute modulation of sleep. Further, *trsn*-deficient flies display normal feeding behavior, indicating that it is not required for modulation of behavior in response to food deprivation. Finally, energy stores in *trsn* mutant flies are normal indicating that the starvation-induced sleep suppression phenotype is not due to increased nutrient storage. These results provide evidence that *trsn* is not required for the perception of starvation or the general induction of

hunger-related behaviors, but is required for the induction of wakefulness in the absence of food.

While *trsn* is broadly expressed in the *Drosophila* nervous system, we localize the function of *trsn* in metabolic regulation of sleep to LK-expressing neurons. Targeted knockdown of *trsn* in LK neurons disrupts metabolic control of sleep, while restoring *trsn* to LK neurons rescues sleep regulation in *trsn* mutants. In addition to regulating sleep, ablation of LK neurons reduces meal number, while increasing consumption during individual feeding bouts, suggesting a role in feeding behavior [38]. LK is expressed in the subesophageal zone, the insect taste center, and in modulatory neurons within the lateral horn, raising the possibility that the sleep and feeding phenotypes associated with LK mutations or manipulation of LK neurons may localize to distinct brain regions [38]. It is possible that the same populations of LK neurons regulate meal frequency and sleep or distinct neurons modulate each process. Combinatorial genetic approaches to manipulate subsets of GAL4-labeled neurons in combination with recent advances in behavioral analysis of meal frequency may allow for the localization of LK neurons involved in each behavioral process [42–44].

In addition to its known role in the synthesis of non-coding RNA, TRSN physically associates with Translin- Associated Protein X (TRAX) [45, 46]. TRSN and TRAX are essential components for the RNA induced silencing complex (RISC), suggesting a role in post-transcriptional gene silencing through the generation of small RNAs. *trsn* knockout mice have diminished forebrain monoamine levels, indicating that *trsn* may serve to regulate neurotransmitter synthesis [47]. Further investigation of the mechanistic relationship between *trsn* and neural regulation of sleep will provide a framework to study the molecular properties and neural networks that are associated with interactions between sleep and metabolic state.

## Experimental Procedure

### *Drosophila* maintenance and fly stocks

Flies were grown and maintained on standard food (New Horizon Jazz Mix, Fisher Scientific). Flies were maintained in incubators (Powers Scientific; Dros52) at 25°C on a 12:12 LD cycle, with humidity set at 55-65%. The background control line used in this study is the *w<sup>1118</sup>* fly strain, and all experimental fly strains including *trsn<sup>EP</sup>* and *nSyb-GAL4* were outcrossed for 5-6 generations into this background. The *nSyb-GAL4* line was a generous gift from Dr. Julie Simpson (UCSB). For further genotype information see Supplemental Methods.

### Sleep and feeding analysis

Unless otherwise noted, fly activity was monitored using *Drosophila* Activity Monitors (DAM2; Trikinetics, Waltham, MA) as previously described [48]. Female flies were briefly anesthetized using CO<sub>2</sub> within 1 hour of lights on at Zeitgeber Time on at (ZT0) and placed into plastic tubes containing standard fly food. All flies were given at least 22 hours to



recover from anesthesia prior to behavior experiments. For detailed description of all behavioral paradigms, see Supplemental Methods

### Pharmacological manipulation

For pharmacological manipulation of glucose and fatty acid utilization, flies were loaded into tubes containing standard fly food. Following a 24 hour acclimation period, flies were transferred at ZT0 into tubes containing standard fly food (control), food laced with either 400mM 2-DG, 25 $\mu$ M etomoxir, or 400mM 2-DG and 25 $\mu$ M etomoxir and sleep was measured for an additional 24 hours. For GeneSwitch experiments, a 100mM stock solution of RU486 (Sigma, St. Louis) was added to fly food or 1% agar solution to a final concentration of 0.25mM RU486. For further details, see Supplemental Methods.

Paraquat dichloride (Sigma, St. Louis) was dissolved directly into 1% agar with 5% sucrose and poured into plates to obtain a 1mM concentration of paraquat. To test the effect of caffeine on sleep, caffeine (Sigma, St. Louis) was dissolved in melted fly food and poured into plates to a concentration of 4mg/mL. Further details are provided in Supplemental Methods.

### Protein, glycogen, and triglyceride measurements

Assays for quantifying triglyceride, glycogen and protein content of flies were performed as previously described [24, 25]. Further details are provided in Supplemental Methods.

### qPCR and immunohistochemistry

Flies were collected 5-7 days after eclosion. Ten or more flies were separated into fed and starved groups and were flash frozen. Total RNA was extracted from fly heads using the QIAGEN RNeasy Tissue Mini kit according to the manufacturer's protocol. RNA samples were reverse transcribed using iScript (Biorad), and the generated cDNA was used for real-time PCR (Biorad CFX96™, SsoAdvanced™ Universal SYBR® Green Supermix qPCR Mastermix Plus for SYBRGreen I) using 1.7ng of cDNA template per well and a primer concentration of approximately 300nM. Specific primer details are provided in Supplemental Methods.

### Statistical Analysis

Statistical analyses were performed using InStat software (GraphPad Software 5.0 Inc.) or IBM SPSS 22.0 software (IBM, Somers, NY, USA). For analysis of sleep, we employed a one or 2-way ANOVA followed by a Tukey's posthoc test. For PER experiments each fly was sampled three times with the same stimulus. The response was binary (PER yes/no), and these three responses were pooled for values ranging from 0-3. The Kruskal-Wallis test (nonparametric ANOVA) was performed on the raw data from single flies and Dunn's Multiple Comparisons test was used to compare different groups. For the capillary feeding assay, 30-60 flies were used per tube and 4-20 tubes per group were tested. The Wilcoxon signed rank test (non-parametric) with two-tailed p-value was used to test significance on single groups.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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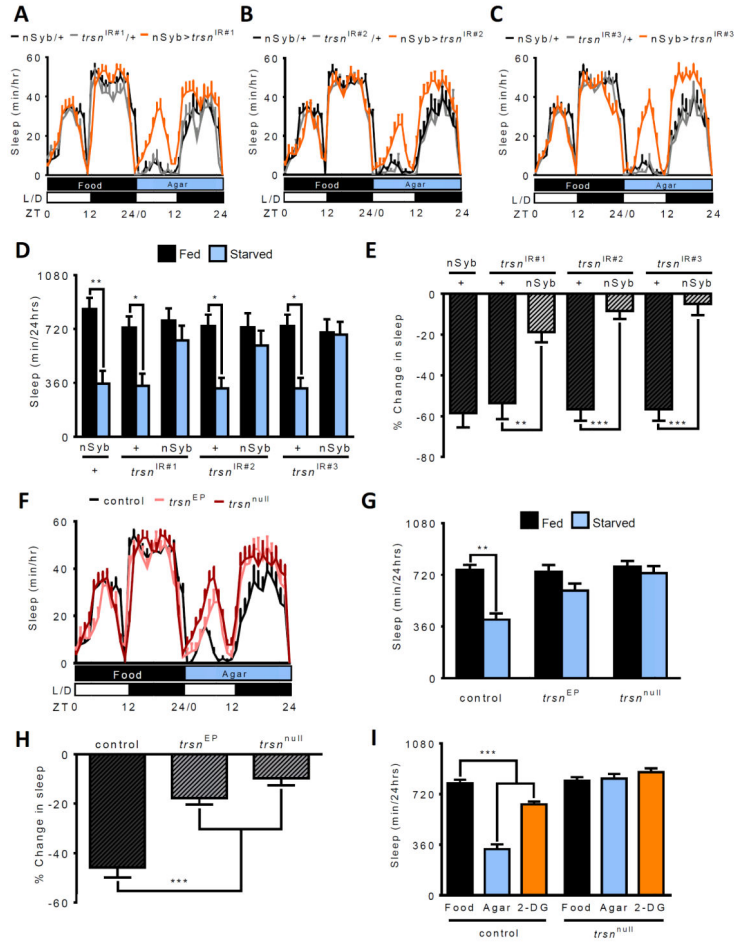


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### Highlights

- Flies deficient for *translin* fail to integrate sleep and metabolic state.
- *translin* does not regulate stress response, metabolic function, or feeding.
- *translin* functions in Leucokinin neurons to regulate sleep.
- Silencing of Leucokinin neurons abolishes starvation-induced sleep suppression.



### Figure 1. *trsn* is required for metabolic regulation of sleep

**A-C.** Sleep profile for hourly sleep averages over a 48 hour experiment. Flies are on food for day 1, then transferred to agar for day 2. Sleep does not differ between any of the groups for day 1. The *trsn* knockdown groups (*nSyb>trsn*, orange) sleep more than *nSyb-GAL4/+* (black) and *trsn<sup>IR/+</sup>* controls (grey) during day 2 (starved). **D** Control flies (*nSyb-GAL4/+* and *trsn<sup>IR/+</sup>*) sleep significantly more on food (black) than when starved (blue, N = 36; P<0.001) while no significant differences in sleep duration are observed in flies where *nSyb-GAL4* drives expression of *trsn<sup>IR#1</sup>* (N=45; P>0.98), *trsn<sup>IR#2</sup>* (N=45; P>0.99), or *trsn<sup>IR#3</sup>* (N=36; P>0.98). **E.** Quantifying the percentage change in sleep between fed (day 1) and starved (day 2) states reveals significantly greater sleep loss in *nSyb-GAL4/+* controls (*nSyb-Gal4/+* vs *trsn<sup>IR#1/+</sup>*, N = 38; P>0.95; *nSyb-Gal4/+* vs *trsn<sup>IR#2/+</sup>*, N = 39; P>0.99; *nSyb-Gal4/+* vs *trsn<sup>IR#3/+</sup>*, N = 37; P>0.99) compared to all three lines with neuronal expression of *trsn<sup>IR#1</sup>* (N = 38; P<0.01), *trsn<sup>IR#2</sup>* (N = 39; P<0.001) and *trsn<sup>IR#3</sup>* (N = 36; P<0.01). **F.** Sleep profile over 48 hours reveals that sleep in *trsn<sup>EP</sup>* and *trsn<sup>null</sup>* does not differ from *w<sup>1118</sup>* control flies on food. Both *trsn<sup>EP</sup>* and *trsn<sup>null</sup>* mutant flies sleep more than control flies on agar. **G.** Sleep is significantly reduced in starved control flies (N = 54; P<0.001), while sleep differences are not significant in *trsn<sup>EP</sup>* (N=69; P>0.23) or *trsn<sup>null</sup>* flies (N=58; P>0.98). **H.** Percentage sleep loss is also significantly reduced in *trsn<sup>EP</sup>* and *trsn<sup>null</sup>* mutants compared to controls (N = 54; P<0.001). **I.** In control flies, sleep is significantly reduced in flies on agar

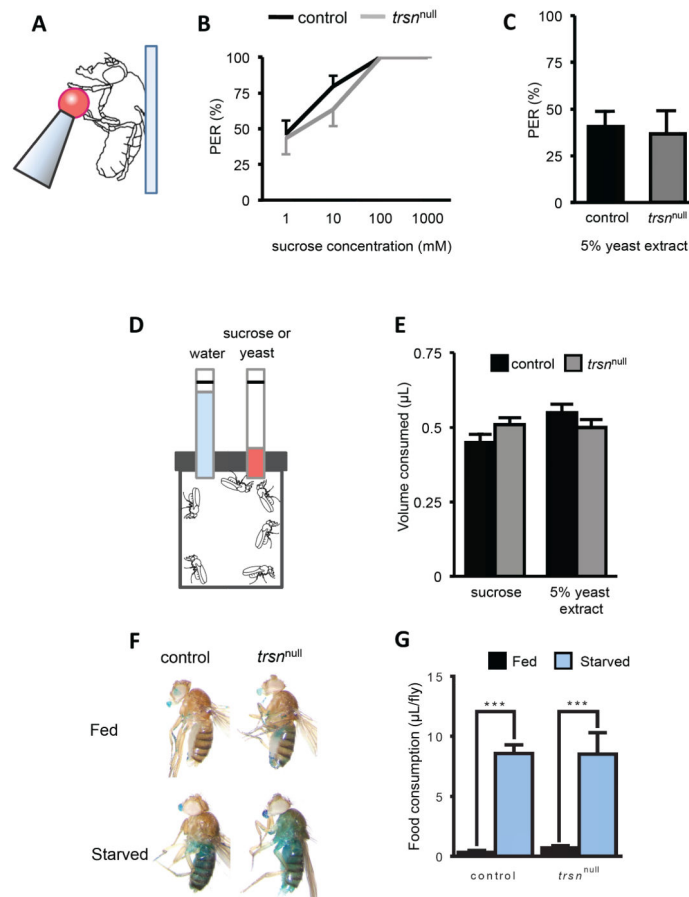
(blue; N=44; P<0.001) or food laced with 2-deoxyglucose (2-DG; orange) (N = 64; P<0.001), compared to flies fed standard food (black). No differences are detected between flies fed standard food compared to agar or 2-DG in *trst*<sup>null</sup> mutants (N = 38; P>0.70). Bars for % change in sleep are mean  $\pm$  SEM by one-way ANOVA. All other bars are mean  $\pm$  SEM; P<0.01, \*\*; P<0.001, \*\*\* by 2-way ANOVA. See also Figure S1.

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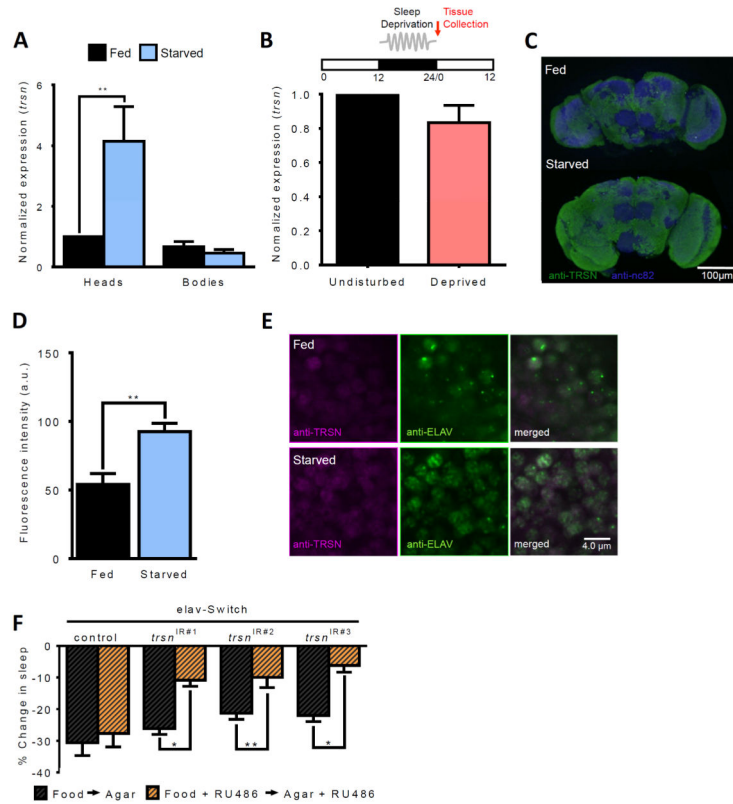
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### Figure 2. Starvation-induced feeding is normal in *trsn* mutant flies

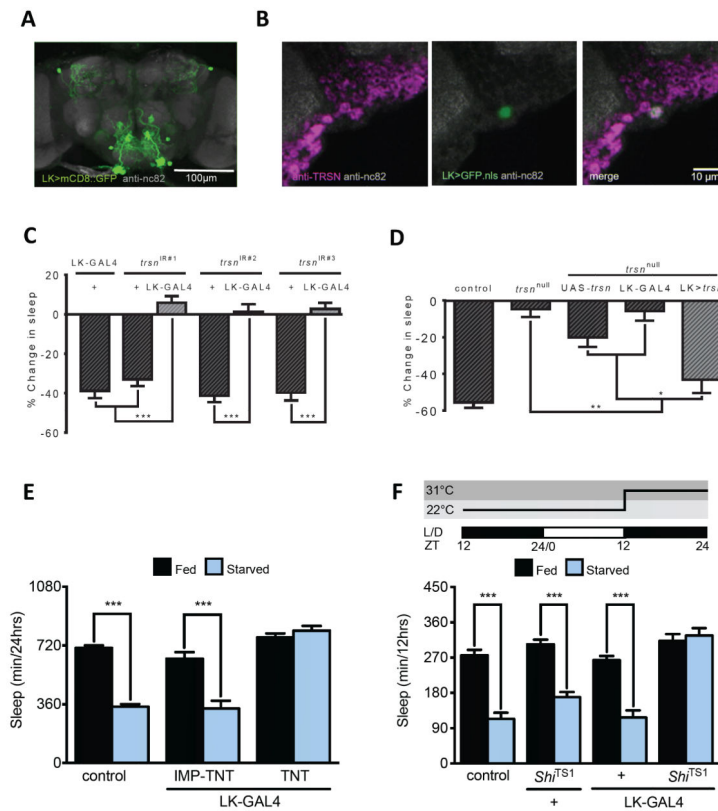
**A.** Diagram of the proboscis extension reflex (PER) assay. Tastant is supplied to the tarsi of a tethered female fly. **B, C.** No significant differences in PER are detected between control (black) and *trsn*<sup>null</sup> mutants (grey) to increasing concentrations of sucrose (N = 10; 1mM,  $P > 0.84$ ; 10mM,  $P > 0.21$ ; 100mM and 1000mM  $P > 0.95$ ) (B) or 5% yeast extract (N=18;  $P > 0.98$ ) (C). **D.** Diagram of the Capillary Feeder Assay (CAFÉ) assay. Flies are presented with one capillary containing 100mM sugar or 5% yeast extract and a second containing water. **E.** No significant differences in sucrose (left bars, N=4;  $P > 0.34$ ) or yeast (right bars, N=4;  $P > 0.18$ ) were detected between control and *trsn*<sup>null</sup> flies when presented with each tastant. **F.** Starved or fed flies are placed on food containing blue dye for 30 minutes and consumption is measured. Representative images of flies following the assay show increased consumption in starved control and *trsn*<sup>null</sup> mutants compared to fed controls. **G.** Quantification of food intake reveals a significant increase in starved controls and *trsn*<sup>null</sup> flies compared to fed flies from each genotype (N = 26;  $P < 0.001$ ). No differences were observed between genotypes in the fed (N = 29;  $P > 0.99$ ) or starved (N = 26;  $P > 0.99$ ) states. All bars are mean  $\pm$  SEM;  $P < 0.001$ , \*\*\* by 2-way ANOVA. See also Figure S2.





### Figure 3. Spatial and temporal localization of *trsn* function

**A.** Expression of *trsn* is upregulated in the heads (N 14;  $P < 0.01$ ) but not bodies of  $w^{1118}$  control flies (N 14;  $P > 0.99$ ) following 24 hours of starvation. **B.** *trsn* transcript does not differ in heads between flies sleep-deprived for 12 hours from ZT12-ZT24 and undisturbed controls (N=3;  $P > 0.17$ ). Red arrow denotes point of tissue collection. **C, D.** Immunohistochemistry for whole-brain TRSN protein (B). Neuropils are labeled by NC82 for reference (magenta) and anti-TRSN (green) is observed throughout the brain. Whole-brain TRSN protein quantification of fluorescence intensity revealed TRSN is increased in starved flies compared to fed control (N 6;  $P < 0.002$ ) by paired t-test. **E.** Immunostaining for anti-TRSN (magenta) and the neuronal marker anti-ELAV (green) reveals colocalization between TRSN and ELAV proteins in brains of fed (upper) and starved (lower) flies. Depicted is a representative section from the dorsomedial central brain, near the lateral horn region. Scale bar denotes 4 µm. **F.** Percentage sleep loss in experimental flies treated with RU486 (orange bars) or controls without drug treatment (black bars). Sleep suppression is significantly reduced in elav-Switch>*trsn*<sup>IR#1</sup> flies (N 36;  $P > 0.031$ ), elav-Switch>*trsn*<sup>IR#2</sup> (N 68;  $P < 0.011$ ) and elav-Switch>*trsn*<sup>IR#3</sup> (N 34;  $P < 0.041$ ) flies fed RU486 compared to non-RU486-fed controls. There is no effect of RU486 feeding in flies harboring the elav-Switch transgene alone (N 39;  $P > 0.99$ ). All other bars are mean  $\pm$  SEM;  $P < 0.05$ , \*;  $P < 0.01$ , \*\*; by 2-way ANOVA. See also Figure S3.



#### Figure 4. *trsn* functions in Leucokinin neurons to regulate sleep

**A.** Whole-brain confocal reconstruction of LK-GAL4>mCD8::GFP. GFP-expressing neurons (green) labeled the subesophageal zone and dorsal protocerebrum. The brain was counterstained with the neuropil marker nc82 (grey). Scale bar denotes 100  $\mu$ m. **B.** Immunostaining for anti-TRSN (magenta) in the brain of LK-GAL4>UAS-GFP.nls reveals TRSN localizes to neurons labeled by LK-GAL4 (white). Depicted is a representative 2  $\mu$ m section from the lateral horn region. Scale bar denotes 10  $\mu$ m. The neuropil marker anti-nc82 (grey) is used as background. **C.** Knockdown of *trsn* in LK-GAL4 neurons alone reduces starvation-induced sleep suppression in all three *trsn*<sup>IR</sup> lines compared to control flies harboring a UAS-*trsn*<sup>IR</sup> transgene alone (N = 52; P<.001) or LK-GAL4 transgenes alone (N = 64; P<.001). **D.** Expression of UAS-*trsn* under LK-GAL4 control in the background of a *trsn*<sup>null</sup> mutation restores starvation-induced sleep suppression compared to flies harboring either UAS-*trsn* (N=87; P<.05); or the GAL4 lines alone (N=79; P<.01). No significant differences were detected between LK rescue and *w*<sup>1118</sup> control flies (N = 38; P>.10). **E.** Starvation-induced sleep suppression is abolished in flies expressing TNT in LK-GAL4 neurons (LK-GAL4>UAS-TNT, Fed vs Starved N=39; P=0.96) while controls expressing inactive UAS-IMP-TNT suppress sleep (Fed vs. Starved: LK-GAL4>UAS-IMP-TNT, N=33, P<.001). Sleep duration on food does not differ significantly between LK-GAL4>UAS-TNT and UAS-IMP-TNT flies (N = 34, P>.06). **F.** Flies were transferred to agar at ZT9 then sleep was measured at 31°C on food (black) or agar (blue) over the 12hr night (ZT12-ZT24). Genetic silencing of LK-GAL4 abolished starvation-induced sleep suppression (LK-GAL4>UAS-*Shi*<sup>TS1</sup>, Fed vs Starved, N = 40, P>.98) while control flies robustly suppressed

sleep (Fed vs Starved: control, N 79,  $P < 0.001$ ; UAS- *Shi*<sup>TS1</sup> /+, N 42,  $P < 0.0001$ ; LK-GAL4/+, N 51,  $P < 0.002$ ). No differences were observed between genotypes in the fed state (Fed vs Fed: control vs LK-GAL4>UAS-*Shi*<sup>TS</sup>,  $P > 0.72$ ; UAS-*Shi*<sup>TS</sup>/+ vs LK-GAL4>UAS-*Shi*<sup>TS</sup>,  $P > 0.98$ , LK-GAL4/+ vs LK-GAL4>UAS-*Shi*<sup>TS</sup>,  $P = 0.07$ ). All columns are mean  $\pm$  SEM;  $P < 0.01$ , \*\*;  $P < 0.001$ , \*\*\* by 2-way ANOVA. See also Figure S4.