

# Neuronal *E93* is required for adaptation to adult metabolism and behavior



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## ABSTRACT

**Objective:** Metamorphosis is a transition from growth to reproduction, through which an animal adopts adult behavior and metabolism. Yet the neural mechanisms underlying the switch are unclear. Here we report that neuronal *E93*, a transcription factor essential for metamorphosis, regulates the adult metabolism, physiology, and behavior in *Drosophila melanogaster*.

**Methods:** To find new neuronal regulators of metabolism, we performed a targeted RNAi-based screen of 70 *Drosophila* orthologs of the mammalian genes enriched in ventromedial hypothalamus (VMH). Once *E93* was identified from the screen, we characterized changes in physiology and behavior when neuronal expression of *E93* is knocked down. To identify the neurons where *E93* acts, we performed an additional screen targeting subsets of neurons or endocrine cells.

**Results:** *E93* is required to control appetite, metabolism, exercise endurance, and circadian rhythms. The diverse phenotypes caused by pan-neuronal knockdown of *E93*, including obesity, exercise intolerance and circadian disruption, can all be phenocopied by knockdown of *E93* specifically in either GABA or MIP neurons, suggesting these neurons are key sites of *E93* action. Knockdown of the Ecdysone Receptor specifically in MIP neurons partially phenocopies the MIP neuron-specific knockdown of *E93*, suggesting the steroid signal coordinates adult metabolism via *E93* and a neuropeptidergic signal. Finally, *E93* expression in GABA and MIP neurons also serves as a key switch for the adaptation to adult behavior, as animals with reduced expression of *E93* in the two subsets of neurons exhibit reduced reproductive activity.

**Conclusions:** Our study reveals that *E93* is a new monogenic factor essential for metabolic, physiological, and behavioral adaptation from larval behavior to adult behavior.

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**Keywords** Monogenic factor of obesity; Systemic metabolic failure; Exercise endurance; Circadian rhythm; Neuronal regulation of feeding; Brain rewiring; Adult behavior

## 1. INTRODUCTION

The nervous system integrates environmental and internal cues and generates appropriate physiological and behavioral responses for animals to survive unpredictable and dynamic environments. This physiological and behavioral adaptation often requires a switch between different metabolic states, as best exemplified in insects that go through metamorphosis. Entirely focused on growth, larvae feed constantly to fulfill their metabolic demand, such that their metabolism relies on aerobic glycolysis, similar to cancer metabolism [1–3]. After metamorphosis, however, the focus of an adult switches to reproduction, where the adults execute diverse behavioral paradigms to seek mates, succeed in mating, and produce

progeny. With these multiple tasks to attend, the importance of feeding diminishes in adults. Indeed, many species of moths, such as *Actias luna*, the American moon moth, and most of the *Saturniidae* family to which *A. luna* belongs, do not have a feeding organ in adults, and survive and reproduce solely on the nutrients they preserved as larvae [4].

In *Drosophila*, the switch between growth and metamorphosis is mediated by two essential endocrine signals, Juvenile Hormone (JH) and Ecdysone (20E, a steroid hormone derivative of cholesterol) [5]. JH promotes growth, antagonizing 20E action in metamorphosis, and ensures that larvae grow big enough before they become adults. The balance between these two hormones, which is tightly controlled by the animal's metabolic state and nutritional environment, determines

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Received November 30, 2023 • Revision received April 7, 2024 • Accepted April 9, 2024 • Available online 16 April 2024

<https://doi.org/10.1016/j.molmet.2024.101939>

the timing of development and metamorphosis [6–8]. Metamorphosis itself is a prolonged starvation for over 3 days, and certain metabolically-challenged mutants cannot survive it [1]. In adults, JH and 20E regulate reproduction which also requires an on-off switch upon the females' mating status, suggesting their continuous roles in balancing metabolic states after metamorphosis.

From an RNA interference-based screen designed to discover novel yet conserved metabolic regulators, we identified *E93* (*Eip93F*: Ecdysone-induced protein 93F), an ortholog of mammalian ligand dependent nuclear receptor corepressor (LCoR), and ligand dependent nuclear receptor corepressor-like (LCoRL). In the screen, 70 *Drosophila* orthologs to the mammalian genes enriched in ventromedial hypothalamus (VMH) were selected, individually knocked down in a neuron-specific manner then screened for starvation resistance. We focused on the genes enriched in VMH based on its essential roles in metabolic homeostasis [9].

*E93*, a master regulator of metamorphosis, belongs to the Pipsqueak transcription factor family [10,11] and contains multiple functional domains such as the DNA binding Helix-Turn-Helix (HTH) domain, the CtBP-interacting domain (CtBP-im) and two potential nuclear hormone interaction domains (NR-box) [12,13]. Conserved from *Caenorhabditis elegans* to mammals [14], it regulates neurite pruning in *C. elegans* [13] and metamorphosis in insects, including *Drosophila* [12]. A mammalian ortholog, LCORL, is associated with body size variations and lipid metabolism in mammals [15,16]. In *Drosophila*, *E93* plays an essential role during metamorphosis to suppress expression of larval genes and promote expression of adult genes [17]. The enhancer sites targeted by *E93* continuously change throughout metamorphosis, suggesting that dynamic interactions between *E93* and stage-specific factors allow *E93* to serve as a switch for the transition of development stages [18]. *E93* is also required for termination of neurogenesis in mushroom body neuroblasts, presumably to help the neurons adopt their adult fate [19]. *E93* expression is maintained at low levels until larva L3 puffstage, the beginning of metamorphosis, when expression is induced by ecdysone (20E) [20]. *E93* expression persists in adults mostly in neurons, including subsets of antennal sense organ and olfactory neurons [21], suggesting its roles beyond metamorphosis. Despite its essential roles in this developmental program tightly linked to metabolic transition, whether neuronal *E93* plays any role in adaption to adult metabolism, physiology, and behavior has not been studied. Furthermore, the important sites of *E93* action for them are completely unknown.

Here we report that reduced expression of *E93* in a neuron-specific manner results in hyperphagia and various metabolic and physiological abnormalities. Knockdown of *E93* increases attraction to food, food intake, body weight, and energy stores with impaired exercise endurance and disrupted circadian rhythm. *E93* knockdown in a specific developmental time window caused obesity, suggesting that *E93* action during metamorphosis, the transition from larval phase to adult, is critical for regulation of adult metabolism. The systemic failure of metabolic homeostasis is largely due to *E93*'s action in two populations of neurons: GABA-ergic and myoinhibitory peptide (MIP)-producing neurons (from here, MIP neurons), both of which have been implicated in controlling food intake, metabolism, circadian rhythm, and sleep [22–28]. MIP neuron-specific knockdown of the ecdysone receptor (EcR), the receptor of ecdysone (20E) which acts upstream of *E93* in metamorphosis, partially phenocopies the obesity phenotype of MIP neuron-specific knockdown of *E93*. Additionally,

restoring *E93* only in MIP neurons partially reverses the metabolic abnormalities observed in neuron-specific knockdown of *E93*. Finally, the animals with reduced neuronal *E93* expression fail to exhibit proper adult behavior, prioritizing feeding or resting over mating. Together, our study reveals neuronal *E93* as a novel monogenic regulator in controlling adult metabolism, physiology, and behavior.

## 2. MATERIALS AND METHODS

### 2.1. *Drosophila* strains and growth conditions

The following lines were obtained from the Bloomington *Drosophila* Stock Center: nSyb-GAL4 (#51635), vGlut-GAL4 (#26160), ChAT-GAL4 (#6793), vGAT-GAL4 (#58409), Trh-GAL4 (#38389), Tdc2-GAL4 (#9313), OK107-GAL4 (#854), Akh-GAL4 (#25683), Mip-GAL4 (#51983), Ilp2-GAL4 (#37516), Eth-GAL4 (#51982), Fmrfa-GAL4 (#56837), Lk-GAL4 (#51993), NPF-GAL4 (#25681), phm-GAL4 (#80577), ple-GAL4 (#8848), SifAR-GAL4 (#76670), Tk-GAL4 (#51973), *Eip93F* RNAi (#57868), EcR RNAi (#58286), vGAT RNAi (#41958), and tubP-GAL80ts (#7019). *E93*-RNAi, nSyb-GAL4, mCherry-RNAi lines were outcrossed for five generations to a Berlin background (originally from the Heisenberg lab). Flies were raised on standard cornmeal/molasses food and incubated at 25 °C under a 12 h light/12 h dark cycle.

### 2.2. Quantification of triglycerides (TAG)

Triglycerides and protein were quantified as described by Tennessen et al. [29] with minor modifications. 5 flies were decapitated, collected into 1.5 ml tubes, and kept on dry ice before adding 50 µl of 0.5% PBS-T. They were then homogenized, heated for 5 min at 70 °C, and centrifuged at 14,000 rpm for 3 min at 4 °C. Resulting supernatant was transferred to a new 1.5 ml tube and placed on ice. 200 µl of Infinity Triglyceride reagent (ThermoFisher Scientific, Waltham, MA; catalog no. TR22421) were added to a 96 well plate. 4 µl of supernatant from each sample was transferred to each well in duplicates and the plate was incubated at 37 °C for 30–60 min. Absorbance levels were read at 540 nm.

### 2.3. Quantification of protein

Protein level was quantified using the Pierce BCA Protein Assay kit (ThermoFisher Scientific, Waltham, MA; product no. 23228) and used to normalize triacylglycerides (TAG) and glycogen levels. 4 µl of each sample (collected by the method described above) was added to each well of a 96 well plate. Volume of BCA reagent A was determined using the formula:  $(2 \times \text{number of samples} + 20) \times 100$ . BCA reagent A and B were mixed (A:B = 50:1) and 100 µl of this mixture was added to each well. The plate was incubated at 37 °C for 30 min and absorbance values were read at 562 nm of wavelength.

### 2.4. Quantification of glycogen

Glycogen quantification was performed as described by Tennessen et al. [29] with minor modifications. 2–5 flies per group were decapitated and placed into 1.5 ml tubes before adding 100 µl cold PBS. Samples were homogenized and 10 µl of supernatant were transferred to a new 1.5 ml tube for the protein level measurement. The remaining supernatant was heated at 70 °C for 10 min and then centrifuged at 14,000 rpm for 3 min at 4 °C. 60 µl of resulting supernatant was collected. Amyloglucosidase digestion solution was prepared by mixing 1.5 µl amyloglucosidase enzyme with 5 ml of 100 mM sodium citrate (pH = 5.0). 10 µl of collected supernatant of

the glycogen sample was diluted in 90  $\mu$ l of PBS in a separate 1.5 ml tube. 100  $\mu$ l of amyloglucosidase solution was added and samples were incubated while shaking at 55 °C for 1 h. For quantification of glucose, 20  $\mu$ l of remaining supernatant was added to a 96 well plate in duplicates and were diluted in 60  $\mu$ l PBS. Similarly, 20  $\mu$ l of incubated glycogen sample was added to the plate in duplicates and 100  $\mu$ l prepared GO reagent (o-Dianisidine/Glucose Oxidase/Peroxidase, Sigma Aldrich, St. Louis, MO; GAGO20-1KT) was added to all samples. The plate was incubated at 37 °C for 30–60 min and 10  $\mu$ l of 12 N sulfuric acid were added before absorbance was measured at 540 nm [29].

### 2.5. Photography

Age-matched control and knockdown flies were imaged using an AM Scope camera (AM Scope, MU1003).

### 2.6. Quantification of body weight

A group of five to ten age-matched flies was placed in a pre-weighed 1.5 ml tube. Body weight was calculated as mg per fly. Data was collected from 5 to 10 groups/genotype/sex.

### 2.7. Quantification of wing morphology

The frequency of unexpanded wings was determined by dividing the number of flies with abnormal wings by the total number of flies in the group. Two to five-day old flies were used.

### 2.8. Proboscis extension response (PER) assay

Two-day old flies raised on standard media were transferred to vials containing 0.7% agar after 24 h of fasting. A single fly was transferred by use of an oral aspirator and trapped in a 200  $\mu$ l pipette tip with the head exposed. 1  $\mu$ l of sucrose solution (150 mM) mixed with 3% erioglaucine (Sigma Aldrich, St. Louis, MO; 861146) was placed on the edge of the pipette tip within range of the proboscis. Proboscis extension and duration of feeding were observed for approximately 5 min.

### 2.9. Blue dye feeding assay

For each experiment, two-day old flies of 9–16 were fasted for 24 h on 0.7% agar. To measure baseline food intake without fasting, flies were kept on standard media and allowed to feed *ad libitum*. Feeding vials were prepared by placing a curled strip of filter paper or a pad (Figure 2A) in the center of an empty vial. 400  $\mu$ l of the blue dye food solution (150 mM sucrose + 3% erioglaucine) was pipetted onto the filter paper. Flies were flipped into the vials and allowed to feed for 5 min if previously fasted, and 10 min if previously fed *ad libitum*. Flies were immediately incapacitated on dry ice and feeding frequency was determined by dividing the number of flies with blue bellies by the total number of flies. The flies were then individually placed in 1.5 ml tubes, homogenized in 50  $\mu$ l of water, then centrifuged at 14,000 rpm for 5 min. To measure absorbance, 10  $\mu$ l of supernatant was transferred to a 96 well plate and diluted in 40  $\mu$ l of water. Absorbance was measured at 630 nm.

### 2.10. Quantification of proboscis extension

Flies were placed on dry ice to immediately capture their proboscis positions. The three positions of closed, half open, fully open were determined by the degree of proboscis extension.

### 2.11. Starvation assay

Starvation vials were prepared with 0.7% agar. Age-matched males and virgin females were collected and separately placed into starvation vials in 5 groups of 12 flies/vial for a total of 60 flies/sex/genotype. The

number of deaths in each group was recorded three times a day until all flies were dead. The assay was performed on five separate cohorts of flies totaling 300 flies/sex/genotype.

### 2.12. Endurance and climbing speed assays

The Power Tower exercise platform was used to assess endurance [30,31]. Briefly, eight vials of 20 flies each were placed onto the Power Tower machine and were monitored every 10–15 min for exhaustion. A vial was defined as ‘exhausted’ once less than 20% of the flies in the vial (4 out of 20) were climbing above one centimeter from the top of their food. Each vial was graphed as a separate data point and the average time to exhaustion for a group was compared using a two-way ANOVA (to account for the effects of genotype and sex) with a post-hoc Tukey’s multiple comparisons analysis in GraphPad Prism (GraphPad Software, San Diego, CA). The rapid negative geotaxis assay was used to evaluate climbing speed [30]. Briefly, cohorts of five vials with 20 flies each, were transferred to clear vials mounted in the RING apparatus. Flies were allowed 1 min for assimilation to the new environment. Then the vials were briskly tapped down (to move all flies to the bottom of their vials) and a picture was taken 2 s after the last tap. Six consecutive trials were conducted for each group, generating six pictures. The pictures were processed using ImageJ (U. S. National Institutes of Health, Bethesda, MD) to generate data representing climbing height, then analyzed using 2-way ANOVA with Tukey’s multiple comparisons test.

### 2.13. *Drosophila* activity monitoring (DAM) assay and analyses

Flies were raised on standard media with 12 h light and 12 h dark condition. During the end of the light period, 2-day old flies were placed in the DAM system (TriKinetics Inc., Waltham, MA) where a single fly is loaded in each DAM tube with food on one end. Horizontal locomotor activity was measured by infra-red beam breaks in individual flies under conditions of 12 h light and 12 h dark or in constant darkness for 3 days. Because some *nSyb>E93RNAi* flies became unhealthy after being kept in the tube more than 5 days, we did not continuously record activity under light and dark condition followed by constant darkness. Naïve 2 days old flies were used for each lighting condition. The average number of beam breaks/6 min from 16 to 73 flies of 1–5 cohorts of each genotype and sex were used to generate actograms (ClockLab, Actimetrics, Wilmette, IL). Files that died during 3 days of recording were excluded from the group averaged actogram. Double plotted group averaged actograms were generated from 1 to 3 cohorts for each genotype and sex with 30 min bin and scaled plot (0–120 counts/30 min).

### 2.14. Fertility/receptivity assay

Ten pairs of males and females were placed in the presence of food in a vial and allowed to mate for 24 h. 5–7 days later, the fertility is determined by production of progeny. For female receptivity, 10 to 24 pairs of 2–4-day-old control males (*nSyb>mCherryRNAi*) and 2–3-day-old females (*nSyb>mCherryRNAi* or *nSyb>E93RNAi*) were placed in the presence of food in a vial or in a 12-well plate for 3 h. Then the parents were removed, and the progeny were counted.

### 2.15. Mating (courtship behavior) assay

The 2–5 days old males of each genotype were individually housed for 1–3 nights. The males and females were briefly anesthetized with CO<sub>2</sub>, then each male was paired with a control female. Each pair was placed into each well of a standard 6 well plate either in the presence or absence of food in the center. The flies were given 15–30 min to be fully recovered and then their mating behavior was recorded for

10 min. The number of mating behavior was manually scored when the male exhibited a sequence of two or more components of mating behavior: orienting toward the female, tracking the female, extending his wing and singing to the female, contacting the female, and mating [32,33]. This simple method allows us to monitor the mating behavior of *E93<sup>RNAi</sup>* males based on the behavioral components which do not involve wings.

### 2.16. Temperature sensitive GAL80ts experiments

The fly stock was generated by crossing the tubP-GAL80ts (#7019) to nSybGAL4 (#51635). The tubP-GAL80ts; nSybGAL4 were set up for mating in a bottle with either *UAS-E93<sup>RNAi</sup>* flies or *UAS-mCherry<sup>RNAi</sup>* flies either at 19 °C or at 30 °C. 3–5 days later the parents were removed, and the bottles were kept at the same temperature until wandering larvae appeared. For the constant 19 °C or 30 °C experiments, the bottles were kept at the same temperature and the F1s were collected. For temporal regulation of E93 RNAi at and after the third instar larvae (L3), once wandering larvae appeared, the bottles were moved from the initial temperature to the final temperature; either from 19 °C to 30 °C, or 30 °C to 19 °C. Then the bottles were kept under the new temperature until the flies eclosed. The F1s were collected within 4 h of post-eclosion and kept at the temperature for at least 3 days (72 h) before their body weight and circadian rhythm were measured. Only the F1s that eclosed in first two days at 30 °C and the F1s that eclosed in first three days at 19 °C which should have been L3s when the bottles were moved were used for the measurement of body weight and circadian rhythm.

For temporal regulation of E93 RNAi in adult stage, the flies raised at the initial temperature were collected within 3 h of eclosion then moved to the final temperature (either from 19 °C to 30 °C, or from 30 °C to 19 °C). The flies were kept at the final temperature for at least 3 days before their body weight and circadian rhythm were measured.

### 2.17. Statistical analysis

We examined whether all experimental groups were normally distributed using Sturges' class number and a symmetric distribution. All groups appeared to be normally distributed. F test was used to determine equality of variances for two independent groups. Comparisons between control and test groups were carried out using an unpaired two sample *t*-test with unequal variance. Log-rank test was used to validate the significance in difference in survivals (Figure 2G–H). Two-way ANOVA was used for Figures 1A and 4B. Analysis of endurance and speed (Figure 4) was conducted using a two-way ANOVA (to account for effects of genotype and sex) with post-hoc Tukey's multiple comparisons.

## 3. RESULTS

### 3.1. A starvation-resistance screen of selected *Drosophila* orthologs enriched in the rodent ventromedial hypothalamus (VMH) identified *E93*

To identify new metabolic regulators, we performed an RNAi-based screen of 70 *Drosophila* genes orthologous to the genes enriched in ventromedial hypothalamus (VMH) in mice [34,35] (GSE96627) (Table S1). The fly orthologs were determined based on the previous reports [36]. We focused on VMH because of its essential roles in metabolic homeostasis. Using the GAL4-UAS system, we crossed the line carrying UAS (Upstream Activating Sequence) flanked with an RNAi construct of each gene to a GAL4 line to reduce the expression. To limit the reduction of the gene to the nervous system, we crossed the UAS-RNAi line of each gene to the nSyb-GAL4 driver and reduced the

expression specifically in the neurons. To validate the screen, we used multiple controls: Adipokinetic hormone (Akh), the functional homolog of glucagon in fly whose mutation results in resistance to starvation [37], as a positive control, *sh*, a homolog of *Kcna1* enriched in VMH whose mutation results in reduced lifespan, as a negative control [38]. UAS-GFP and UAS-mCherry<sup>RNAi</sup> were used as controls for UAS and for RNAi, respectively. Neuronal knockdown of *upd1*, the fly leptin, and *NPF* were used as controls for metabolism.

We screened the F1 progeny of the cross between nSyb-GAL4 and the UAS-RNAi of each gene for their survival for long-term starvation on the premise that coping with this extreme metabolic challenge requires the fittest metabolic regulation. Therefore, this screen would allow us to identify genes that contribute to any aspect of metabolic homeostasis by controlling metabolic rates, feeding, reproduction and other related traits. Importantly, the underlying mechanisms and genetic programs for metabolic homeostasis (e.g., insulin signaling) are highly conserved between invertebrates and mammals [39–41].

Reduced expression of *upd1* enhanced starvation survival in both males and females (Figure S1). In addition, GPR155/LYCHOS, an ortholog of *anchor* that was a strong positive hit in the screen, was recently discovered to play an important role in cholesterol signaling to metabolism [42]. These results support that our screen platform was well-tuned to identify a new metabolic regulator. The screen identified *E93* as the strongest hit next to the positive control (Figure S1).

### 3.2. Neuron-specific reduction of *E93* expression increases body weight and energy stores

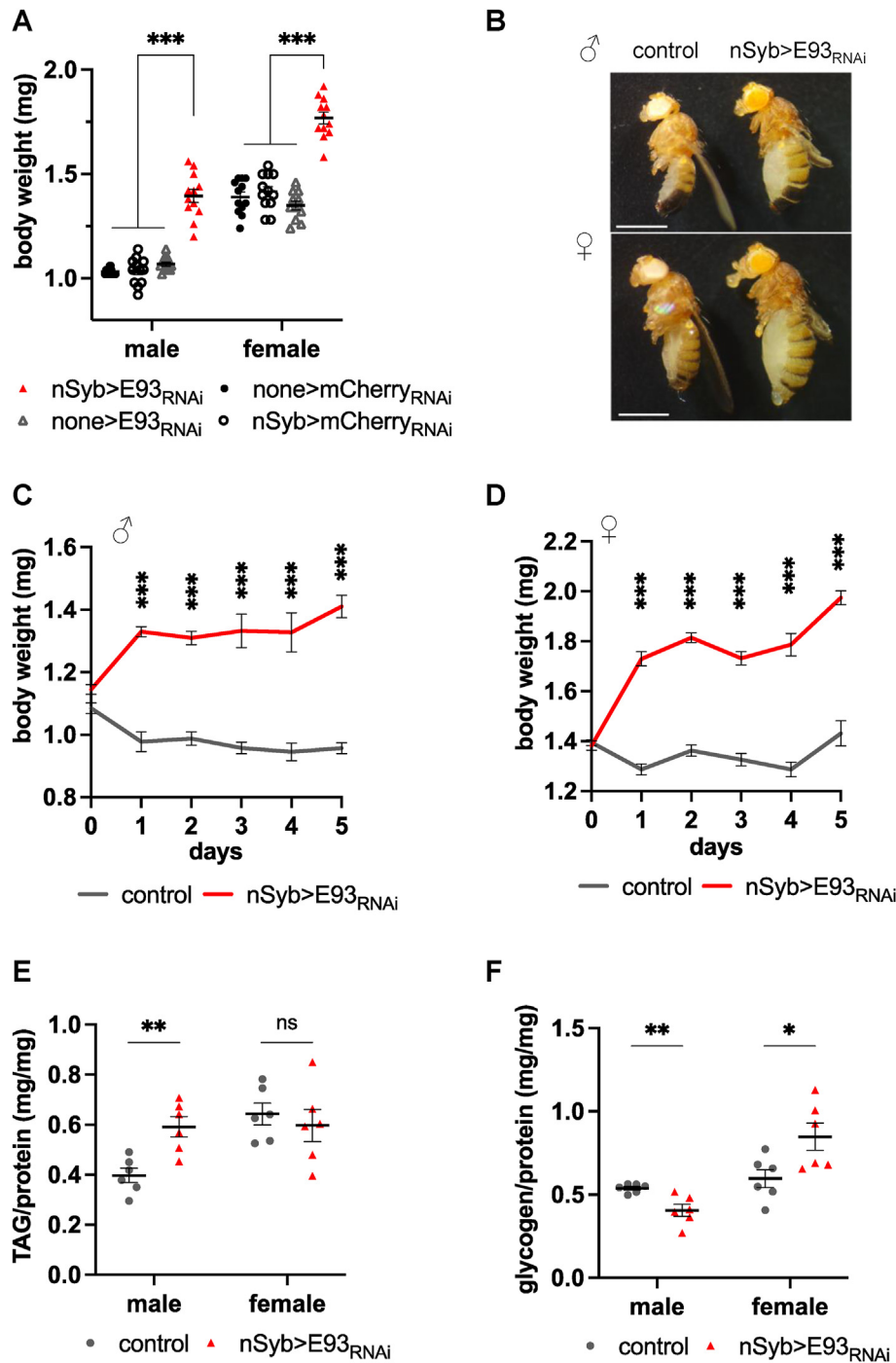
When we reduced the expression of *E93* pan-neuronally using an nSyb-GAL4 driver and a UAS-*E93* RNAi (from here, *nSyb>E93<sup>RNAi</sup>*), the flies became obese; their body weight was increased compared to the three controls of UAS, GAL4, and genetic background (Figure 1A). Because all three controls show very similar body weights, we used either the Berlin K strain carrying UAS-*E93* RNAi without any GAL4 driver (*none>E93<sup>RNAi</sup>*) or *nSyb>mCherry<sup>RNAi</sup>* as a control depending on the experiment. *nSyb>mCherry<sup>RNAi</sup>*, *nSyb>E93<sup>RNAi</sup>* and *non-e>E93<sup>RNAi</sup>* lines were outcrossed to w Berlin for five generations to isogenize the background.

Three days after eclosion, both male and female *nSyb>E93<sup>RNAi</sup>* flies were visibly obese (Figure 1B). The body weight of *nSyb>E93<sup>RNAi</sup>* flies gradually increased each day after eclosion (Figure 1C–D). When we measured abdomen width, both knockdown males and females had increased abdomen size (Figure S2A). Compared to controls, female knockdowns had significantly enlarged abdomens beginning one day after eclosion, but both *nSyb>E93<sup>RNAi</sup>* and controls retained similar numbers of mature eggs ( $14.4 \pm 5.3$ , N = 18, vs  $14.9 \pm 4.3$ , N = 13, Supplementary Methods), indicating that the enlarged abdomen observed in knockdowns was not due to an increased number of retained eggs.

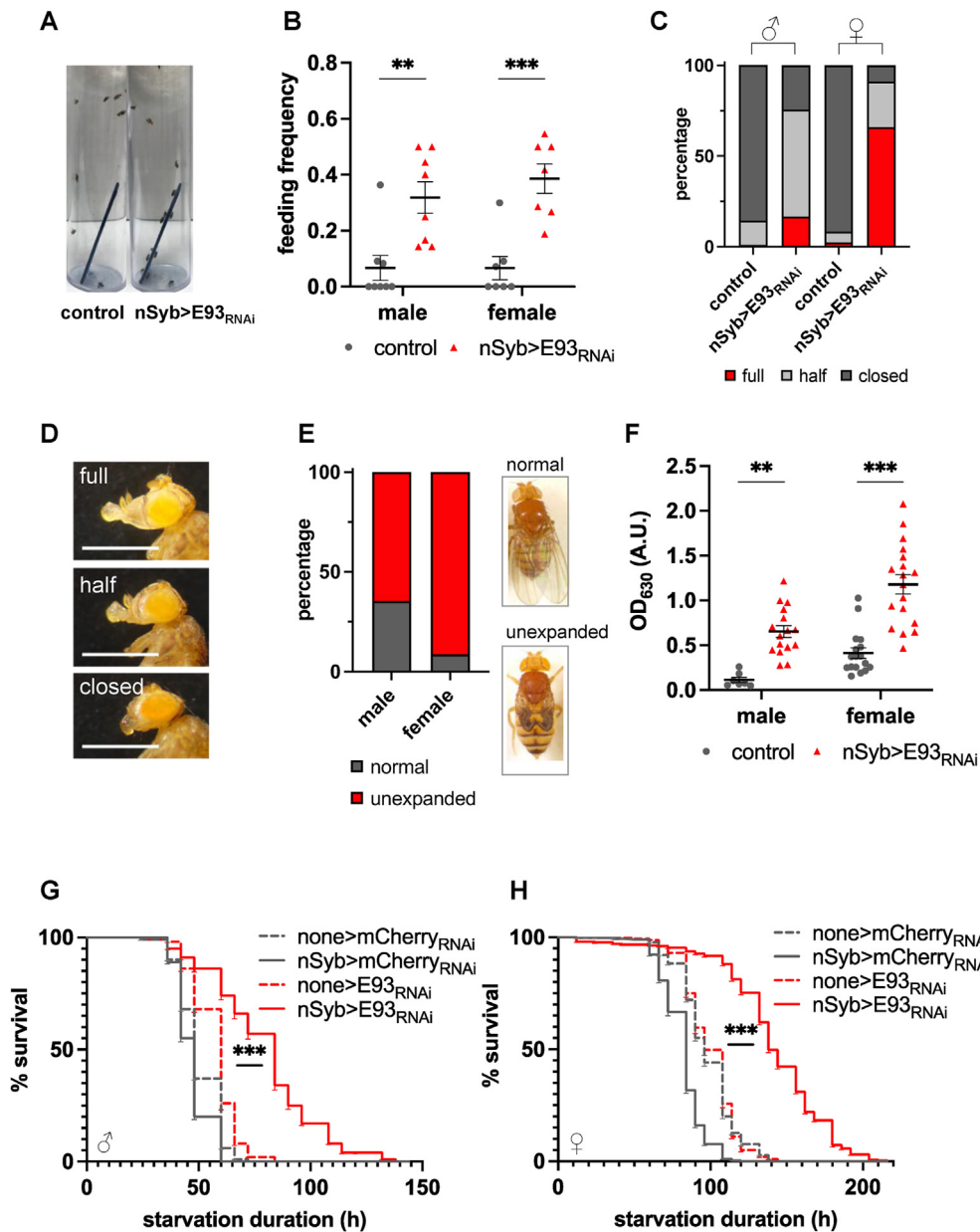
When we measured the levels of triacylglycerides (TAG) and glycogen, we found that males had more TAG, whereas females had more glycogen than controls (Figure 1E–F). These results suggest that neuronal *E93* regulates energy stores and that the energy stores may be regulated in a sexually dimorphic manner.

### 3.3. Neural specific knockdown of *E93* increases food intake

Next, we asked whether the increased body weight and energy stores in *nSyb>E93<sup>RNAi</sup>* flies were due to an increase in food intake. When *nSyb>E93<sup>RNAi</sup>* flies were placed in a vial with a pad containing sucrose solution (150 mM) and blue dye, a higher percentage of *nSyb>E93<sup>RNAi</sup>* flies gathered to the pad than controls (Figure 2A). Consistent with this



**Figure 1: Neuron-specific knockdown of *E93* increases body weight and energy stores.** **A:** Body weights of (3-day old) of males and females of *nSyb>E93<sub>RNAi</sub>* are increased compared to all RNAi only controls (*none>E93<sub>RNAi</sub>* and *none>mCherry<sub>RNAi</sub>*) and the GAL4 driver control (*nSyb>mCherry<sub>RNAi</sub>*). Each data point represents one group of 5 flies. The bars indicate the mean  $\pm$  S.E.M. \*\*\* $p < 0.001$  by two-way ANOVA. **B:** Representative images of males ( $\delta$ ) and females ( $\varnothing$ ) of control (*nSyb>mCherry<sub>RNAi</sub>*) and *nSyb>E93<sub>RNAi</sub>* flies. Scale bar = 1 mm. **C–D:** Time-course of body weight measurements in males (C) and females (D) showing a slight (male) or no (female) difference in initial body weights compared to controls (*none>E93<sub>RNAi</sub>*). Body weights gradually increase in *nSyb>E93<sub>RNAi</sub>* flies compared to controls. Errors are S.E.M. \*\*\* $p < 0.001$  by Student *t*-test. **E:** Triglyceride (TAG) levels are increased in *nSyb>E93<sub>RNAi</sub>* males but not in *nSyb>E93<sub>RNAi</sub>* females when compared to controls (*nSyb>mCherry<sub>RNAi</sub>*). **F:** Glycogen levels are increased in *nSyb>E93<sub>RNAi</sub>* females but not in *nSyb>E93<sub>RNAi</sub>* males when compared to controls (*nSyb>mCherry<sub>RNAi</sub>*). **E–F:** Each data point represents one group of 5 males or one group of 2–5 females. The bars indicate the mean  $\pm$  S.E.M. \* $p < 0.05$ , \*\* $p < 0.005$ , ns = not significant by Student *t*-test.



**Figure 2: Neuron-specific knockdown of *E93* increases feeding frequency, food intake, and starvation survival.** A–B: *nSyb>E93<sub>RNAi</sub>* flies are more attracted to food (A) and show a higher feeding frequency than the control (*nSyb>mCherry<sub>RNAi</sub>*) (B). Each data point represents one experiment of 9–16 flies. The bars indicate the mean  $\pm$  S.E.M. C: *nSyb>E93<sub>RNAi</sub>* flies constantly feed as indirectly measured by % of flies with extended proboscis. N = 144–187 males, 83–100 females. D: Representative images of proboscises in each state of closed, half-open, and fully open. Scale bar = 100  $\mu$ m. E: The % unexpanded wings in *nSyb>E93<sub>RNAi</sub>* flies. N = 82 males, 113 females. F: *nSyb>E93<sub>RNAi</sub>* flies have increased food intake. After 10 min of feeding, the flies were homogenized, and the food intake was measured by absorbance of the blue dye at 630  $\lambda$ . *none>E93<sub>RNAi</sub>* flies were used as controls. Each data point represents one sample. The bars indicate the mean  $\pm$  S.E.M. G–H: Both males (G) and females (H) of *nSyb>E93<sub>RNAi</sub>* survive starvation better than all three controls. B, F: \*\* $p < 0.005$ , \*\*\* $p < 0.001$ , ns = not significant by Student *t*-test. G, H: \*\*\* $p < 0.001$  by log-rank test.

observation, *nSyb>E93<sub>RNAi</sub>* flies showed increased feeding frequency measured by the percentage of total fed flies with blue abdomens (Figure 2B). Furthermore, the majority of *nSyb>E93<sub>RNAi</sub>* flies stayed on the pad throughout the experiment, whereas the control flies quickly ate and left within several seconds (Supplementary Video 1). The proboscis, the feeding organ, of *nSyb>E93<sub>RNAi</sub>* flies was fully extended at a higher frequency in *nSyb>E93<sub>RNAi</sub>* flies than in the control, suggesting that *E93* knockdown flies are feeding more frequently than the controls (Figure 2C–D).

The Capillary Feeding (CAFE) assay is commonly used to assess food intake. It relies on the ability of flies to reach a suspended food source and hang upside down long enough to feed until satiated [43]. As *nSyb>E93<sub>RNAi</sub>* flies have unexpanded wings (Figure 2E), we were unable to perform the CAFE assay reliably. Instead, we modified a blue dye feeding assay and determined the amount of individual food intake by measuring dye absorbance at  $\lambda_{630}$  (see Methods) [44]. Virgin male and female *nSyb>E93<sub>RNAi</sub>* flies fasted for 24 h had increased food intake compared to controls (Figure 2F).

We used the proboscis extension response (PER) assay to further examine the hyperphagia of *nSyb>E93<sup>RNAi</sup>* flies in detail. Flies were individually trapped in a pipet tip and presented with 1  $\mu$ l of sucrose solution containing blue dye under the proboscis [26]. The duration of proboscis extension and the volume of consumed food were measured over 5 min by subtracting the remaining volume from the 1  $\mu$ l originally presented. Although the number of samples we tested with this method was small, we were able to observe a striking difference between *nSyb>E93<sup>RNAi</sup>* flies and controls. All *nSyb>E93<sup>RNAi</sup>* flies maintained extended probosces for the entire 5 min (100%, 5 out of 5) and 80% (4 out of 5) of flies finished 1  $\mu$ l within 5 min. On the other hand, only 40% (2 out of 5) of the control flies had a single bout of feeding lasting 30 s or less and consumed less than 0.4  $\mu$ l (Supplementary Videos 2–3). The remaining 60% did not extend their proboscis to feed at all.

In females, changes in midgut size are often associated with increased food intake when nutritional demand is high [45]. The average diameter of the midgut of *nSyb>E93<sup>RNAi</sup>* females was increased compared to controls, suggesting an increase in food intake (Figure S2B–C). In males increased food intake is often associated with increased defecation rate [46]. *nSyb>E93<sup>RNAi</sup>* males show an increased defecation rate compared to controls, suggestive of increased food intake (Figure S2D). As observed in the screen, both male and female *nSyb>E93<sup>RNAi</sup>* flies survived starvation longer than all the controls (Figure 2G–H), suggesting that their increased energy stores rendered them starvation resistant. Taken together, our data indicate that neuronal *E93* is required to control food intake and energy stores.

### 3.4. *E93* acts mainly in GABA-ergic and MIP-producing neurons

To determine the sites of *E93* action, we screened 17 GAL4 lines by which the expression of the *E93* RNAi construct was limited to subsets of specific classes of neurons and/or endocrine cells. We used glycogen levels in females as a readout, reasoning that the big difference in glycogen levels between *nSyb>E93<sup>RNAi</sup>* and the control would provide us a reliable resolution for detection. The 17 lines include the three classical neurotransmitter-producing neurons: vGlut (glutamate), ChAT (acetylcholine) and vGAT (GABA); three monoamine-producing neurons: ple (dopamine), Tdc2 (octopamine/tyramine) and Trh (serotonin); and the mushroom body specific line, OK107, which is important for learning and memory. The other ten drivers were selected based on their known roles in feeding and metabolism, and include cells producing MIP (myoinhibitory peptide) [26], Akh (Adipokinetic hormone) [47], Eth (Ecdysone triggering hormone) [48], Phm (ecdysone producing cells of the prothoracic glands) [49], Tk (Tachykinin) [50,51], NPF (neuropeptide F) [52], Fmrfa (FMRFamid neuropeptide), Dilp2 (Insulin-like peptide), Lk (Leucokinin), and SIFaR (SIFamide receptor) [53]. To control for the effect of different GAL4s, we used UAS-*mCherry<sup>RNAi</sup>* as a control for each driver. Of note, UAS-*mCherry<sup>RNAi</sup>* (*none>mCherry<sup>RNAi</sup>*) flies had comparable metabolic parameters, including body weight and starvation response, to other controls (Figures 1A and 2G–H).

Flies with a knockdown of *E93* in GABA-ergic neurons or in MIP neurons had increased glycogen levels (Figure S3A). Specific GABA-ergic interneurons are known to play a critical role in inhibiting feeding [22]. Indeed, body weight and feeding frequency were increased in both male and female *vGAT>E93<sup>RNAi</sup>* flies compared to controls (Figure 3A–C). In addition, a higher percentage of *vGAT>E93<sup>RNAi</sup>* males and females extended their probosces than controls (93% of *vGAT>E93<sup>RNAi</sup>* (N = 57) vs. 2% of *vGAT>mCherry<sup>RNAi</sup>* (N = 46)). The abdomen size of the *vGAT>E93<sup>RNAi</sup>* females increased without an increase of the number of retained eggs

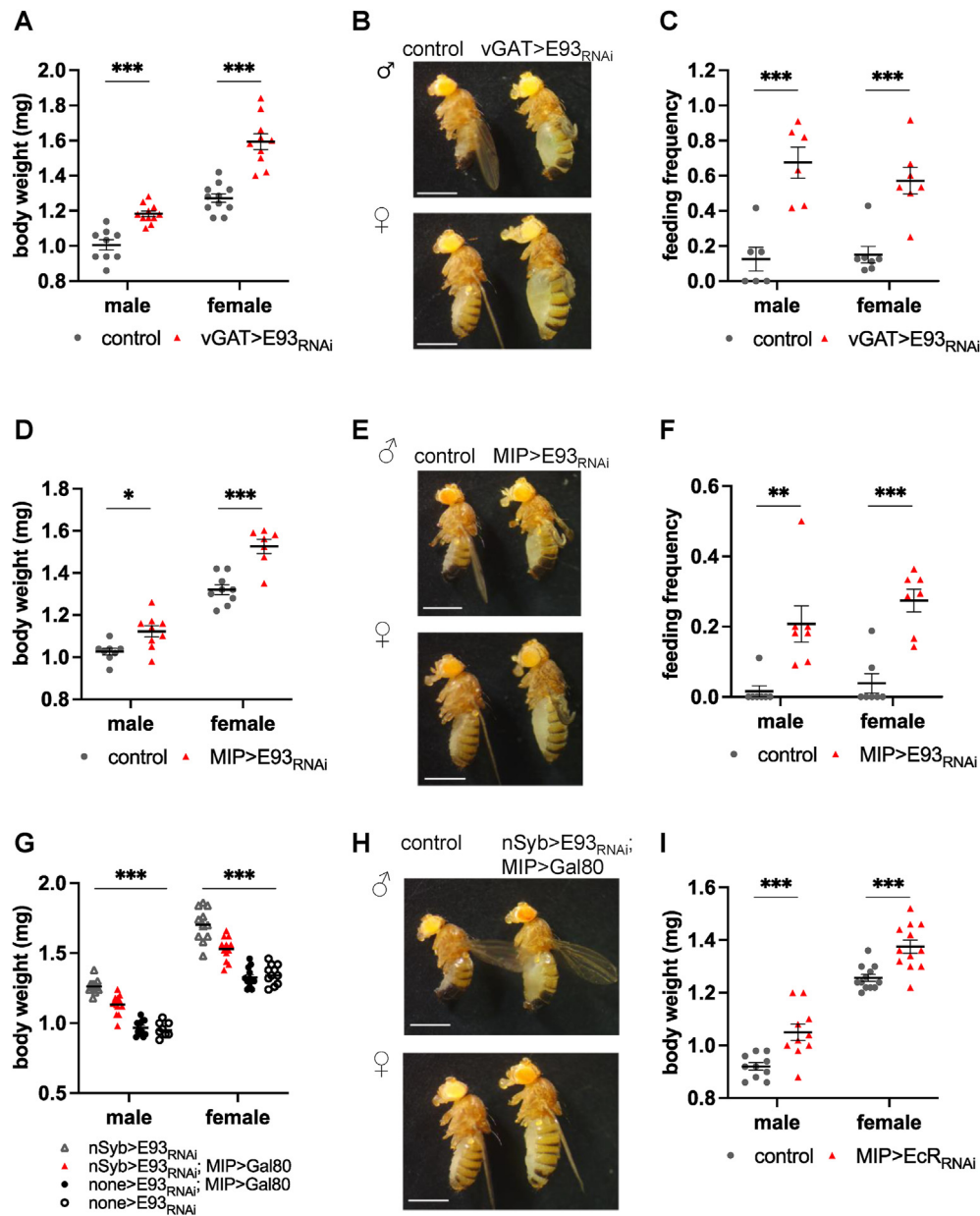
(Figure S3B–C). These results validate the use of glycogen levels as a readout to identify the sites of *E93* action in regulation of food intake and body weight. Similar to *nSyb>E93<sup>RNAi</sup>* flies, *vGAT>E93<sup>RNAi</sup>* flies also exhibited a wing phenotype; 50% of males and 82% of females had unexpanded wings while 100% of controls had normal wings (N = 32 for males, N = 50 for females). These similarities between *nSyb>E93<sup>RNAi</sup>* flies and *vGAT>E93<sup>RNAi</sup>* flies in increased body weight, attraction to food, and unexpanded wings indicate that GABA-ergic neurons are one of the main sites of *E93* action.

Next, we examined flies with *E93* knocked down in MIP neurons. MIP/allatostatin-B homologs are present in almost all animals [54], and serve versatile roles related to metabolism such as feeding and metamorphosis [55]. In *Drosophila*, MIP is expressed in approximately 70 neurons in the areas important for food sensation such as the antennal lobe and subesophageal zone (SEZ), and regulates mating receptivity, satiety, fat storage, and sleep [26–28]. Both males and females of *MIP>E93<sup>RNAi</sup>* are obese and feed more (Figure 3D–F), indicating MIP neurons are also where *E93* acts. The *MIP>E93<sup>RNAi</sup>* females resemble the previously reported phenotypes of the *MIP* mutant females [26]. In addition, *MIP>E93<sup>RNAi</sup>* females have increased abdomen size without changing in the number of retained mature eggs (Figure S3D–E).

To confirm that MIP neurons are a site of *E93* action, we introduced GAL80, a repressor of GAL4, only in MIP neurons in *nSyb>E93<sup>RNAi</sup>* flies. MIP-enhancer-driven GAL80 represses GAL4 driven by *nSyb* in MIP neurons and thus expression of *E93* is restored only in MIP neurons, while *nSyb*-GAL4 continues to drive *E93* RNAi in all other neurons. Introducing GAL80 only in MIP neurons partially but significantly reverses the body weight phenotype of *nSyb>E93<sup>RNAi</sup>* flies (Figure 3G–H), confirming that MIP neurons are one of the sites of *E93* action.

### 3.5. Steroid nuclear receptor controls metabolism in MIP neurons

The steroid hormone, ecdysone, binds to the ecdysone receptor (EcR) to initiate metamorphosis and induce expression of *E93* [11]. Ecdysone and EcR regulate many essential functions not only during metamorphosis but also in adult flies. Ecdysone is secreted mainly from the fat body in males and ovaries in females, and controls reproduction and metabolism [56–58]. To test whether neuronal EcR regulates metabolic homeostasis potentially regulating *E93*, we reduced expression of *EcR* in GABA-ergic neurons and MIP neurons. GABA-ergic neuron-specific knockdown of EcR (*vGAT>EcR<sup>RNAi</sup>*) produces lethality, thus we could not investigate the role of EcR in feeding and metabolism in these cells. However, the MIP neuron-specific knockdown of *EcR* (*MIP>EcR<sup>RNAi</sup>*) partly phenocopied *MIP>E93<sup>RNAi</sup>* showing increased body weight (Figure 3I, Figure S4A), suggesting a genetic interaction of *E93* with *EcR* in MIP neurons in regulation of metabolism. Similar to *nSyb>EcR<sup>RNAi</sup>* females, *MIP>EcR<sup>RNAi</sup>* females had increased abdomen size without changes in the number of retained mature eggs (Figure S4B–C). However, we found that the feeding frequency of *MIP>EcR<sup>RNAi</sup>* was either not different from the controls (Figure S4D, female) or slightly reduced (Figure S4D, male). The mechanisms underlying this dissociation between weight gain and food intake are unknown. One possibility is that *MIP>EcR<sup>RNAi</sup>* flies have altered patterns of food intake and activity (see below), which might have obscured the correct timing for us to measure food intake. Alternatively, as *EcR* plays a significant role from the birth, knockdown of *EcR* in MIP neurons throughout development could result in adaptations or defects that mask the feeding phenotype seen in adult *MIP>E93<sup>RNAi</sup>*. It is also possible that the increased food intake seen in *MIP>E93<sup>RNAi</sup>* might be independent to *EcR*.



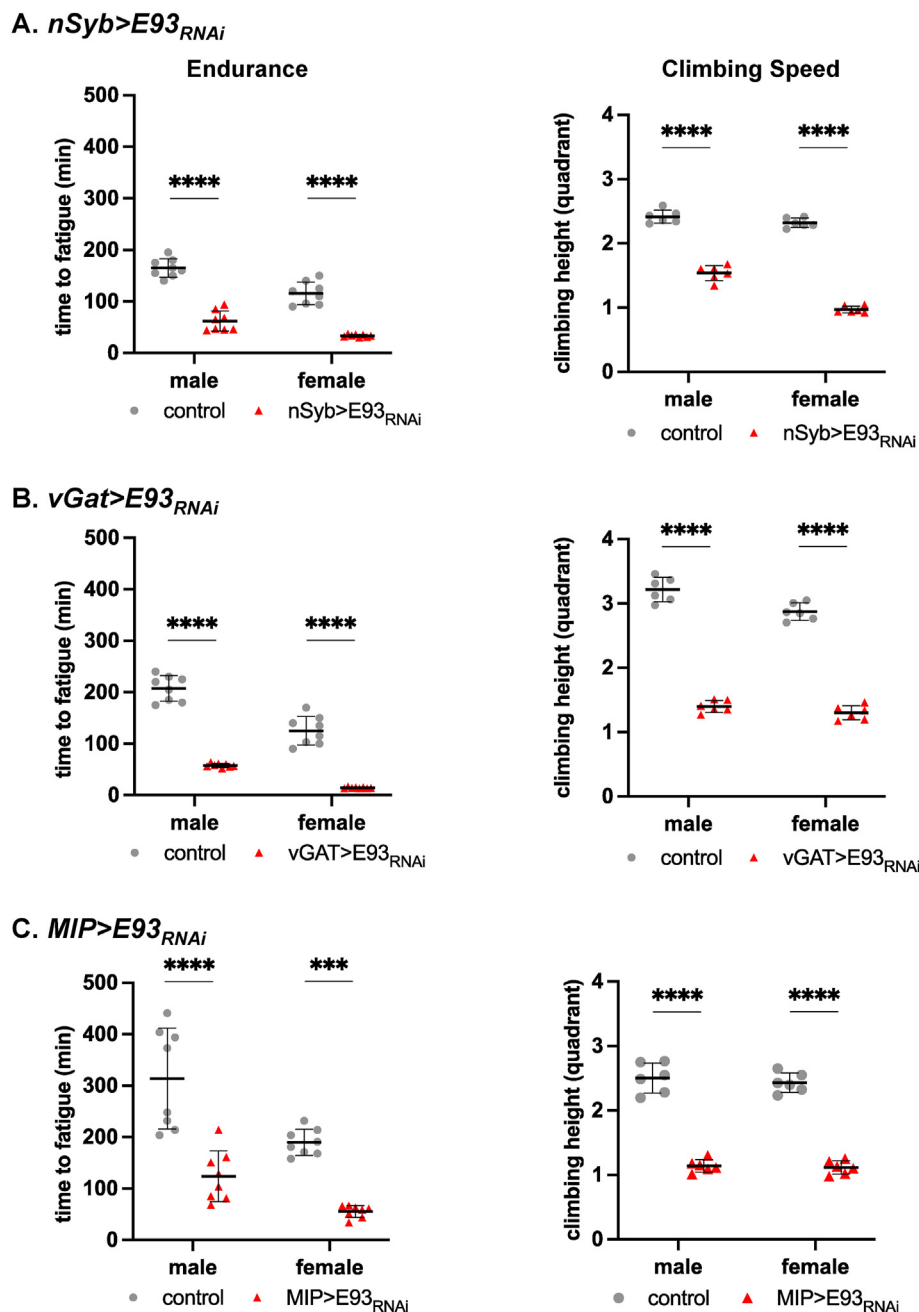
**Figure 3: Loss of *E93* in GABA-ergic and MIP neurons underlies metabolic changes.** **A–C:** *vGAT>E93<sup>RNAi</sup>* flies phenocopies *nSyb>E93<sup>RNAi</sup>* flies with increased body weight (A), obese appearance (B), and increased feeding frequency (C). *vGAT>mCherry<sup>RNAi</sup>* was used as controls. **A:** Each data point represents one group of 5 flies. **C:** Each data point represents one experiment of 9–16 flies. The bars indicate the mean  $\pm$  S.E.M. **B:** Scale bar = 1 mm. **D–F:** *MIP>E93<sup>RNAi</sup>* phenocopies *nSyb>E93<sup>RNAi</sup>* with increased body weight (D), obese appearance (E), and increased feeding frequency (F). *MIP>mCherry<sup>RNAi</sup>* flies were used as controls. **D:** Each data point represents one experiment of 9–16 flies. The bars indicate the mean  $\pm$  S.E.M. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$  by Student *t*-test. **G–H:** *MIP>Gal80* partially but significantly reverses the increased body weight phenotype of *nSyb>E93<sup>RNAi</sup>*. Each data point represents one group of 5 flies. The bars indicate the mean  $\pm$  S.E.M. \*\* $p < 0.005$ , \*\*\* $p < 0.001$  by Two-way ANOVA. **H:** Scale bar = 1 mm. **I:** *MIP>EcR<sup>RNAi</sup>* phenocopies *MIP>E93<sup>RNAi</sup>* with increased body weight. *MIP>mCherry<sup>RNAi</sup>* flies were used as controls. Each data point represents one group of 5 flies. The bars indicate the mean  $\pm$  S.E.M. \*\*\* $p < 0.001$  by Student *t*-test.

GABA is important for controlling food intake [22,23] and is secreted from some MIP neurons [59]. We therefore examined whether GABA produced in MIP neurons contributes to the observed *nSyb>E93<sup>RNAi</sup>* phenotypes. However, no differences in body weight were observed in flies with knockdown of the vesicular transporter of GABA (*vGAT*) in MIP neurons, suggesting that GABA released by MIP neurons might not contribute to the obesity phenotype of *MIP>E93<sup>RNAi</sup>* flies (Figure S4E–F).

### 3.6. Reduced *E93* expression in neurons decreases adult endurance and climbing speed

Studies suggest that metabolic dysregulation can be associated with impaired activity such as climbing [60,61]. We also showed that exercise training improves adult physiology and therefore provides a useful assessment of systemic adaptation of an adult [30]. The obesity phenotype with increased body weight and energy stores led us to ask whether neuronal *E93* was required to maintain wild-type endurance





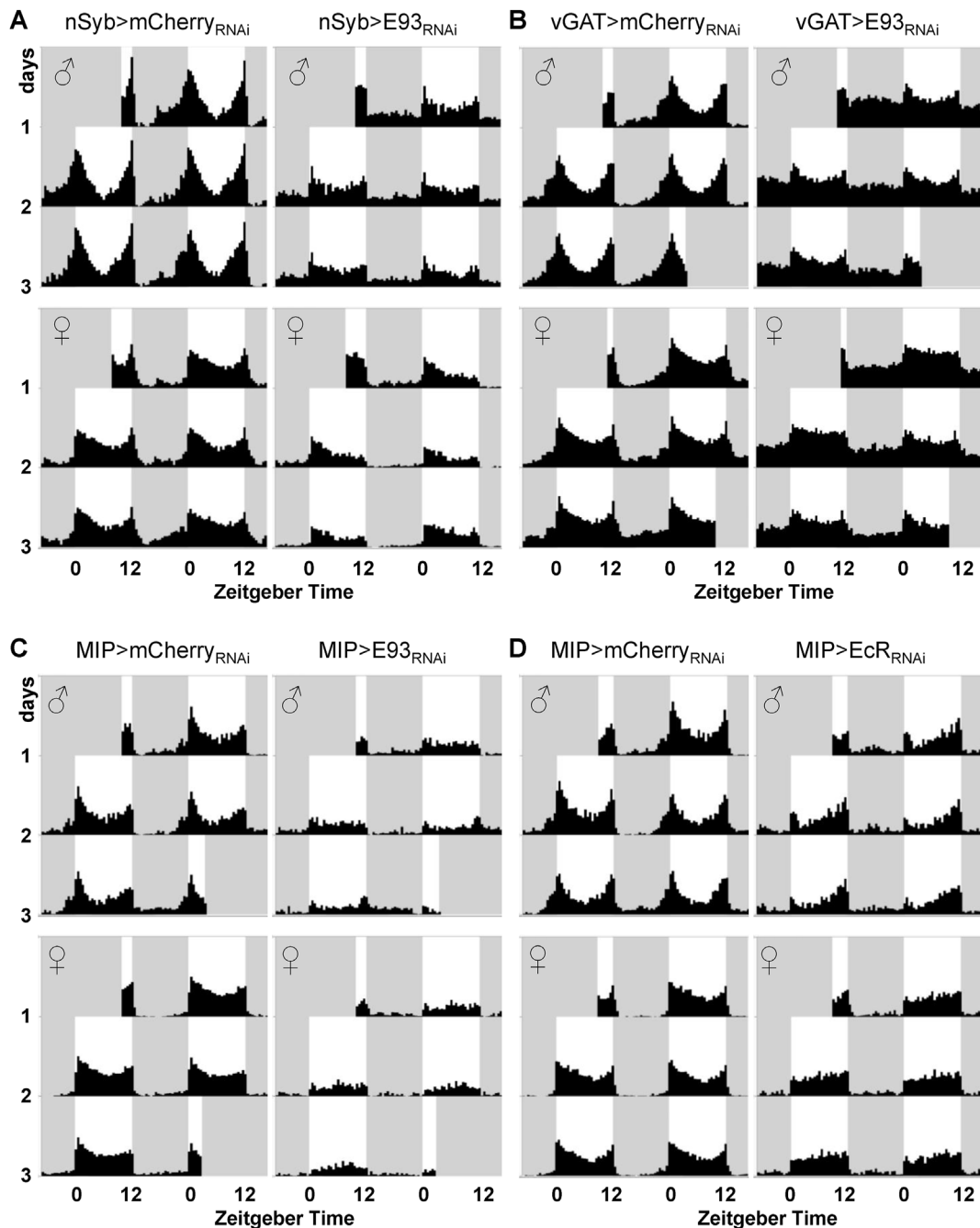
**Figure 4: Reduced *E93* expression in neurons decreases endurance and climbing speed in adult flies.** **A:** Pan-neuronal knockdown of *E93* significantly reduced endurance and climbing speed in *nSyb>E93<sup>RNAi</sup>* male and female flies. **B:** GABAergic neuron-specific knockdown of *E93* significantly reduced endurance and climbing speed in *vGAT>E93<sup>RNAi</sup>* male and female flies. **C:** MIP neuron-specific knockdown of *E93* significantly reduced endurance and climbing speed in *MIP>E93<sup>RNAi</sup>* male and female flies. All controls were generated by crossing the genetic background of the GAL4 line with *E93<sup>RNAi</sup>* (*none>E93<sup>RNAi</sup>*). \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , by two-way ANOVA with post-hoc Tukey's multiple comparisons test.

and speed in induced and involuntary assessments of exercise performance in adults. When we tested male and female *nSyb>E93<sup>RNAi</sup>* flies, using an established paradigm to assess exercise performance [30,31], they showed dramatically reduced endurance and speed (Figure 4A). To determine whether *E93* is required for exercise performance in the same GABA-ergic and MIP neurons where it is required for obesity and feeding phenotypes, we next tested *vGAT>E93<sup>RNAi</sup>* and *MIP>E93<sup>RNAi</sup>* flies. Both males and females of *vGAT>E93<sup>RNAi</sup>* (Figure 4B) or *MIP>E93<sup>RNAi</sup>* (Figure 4C) reduced

endurance and speed to a similar extent as *nSyb>E93<sup>RNAi</sup>* flies (Figure 4A), indicating that *E93* expression in these neurons is critical for adult exercise performance.

### 3.7. Reduced expression of *E93* in neurons alters locomotor activity and disrupts circadian rhythm

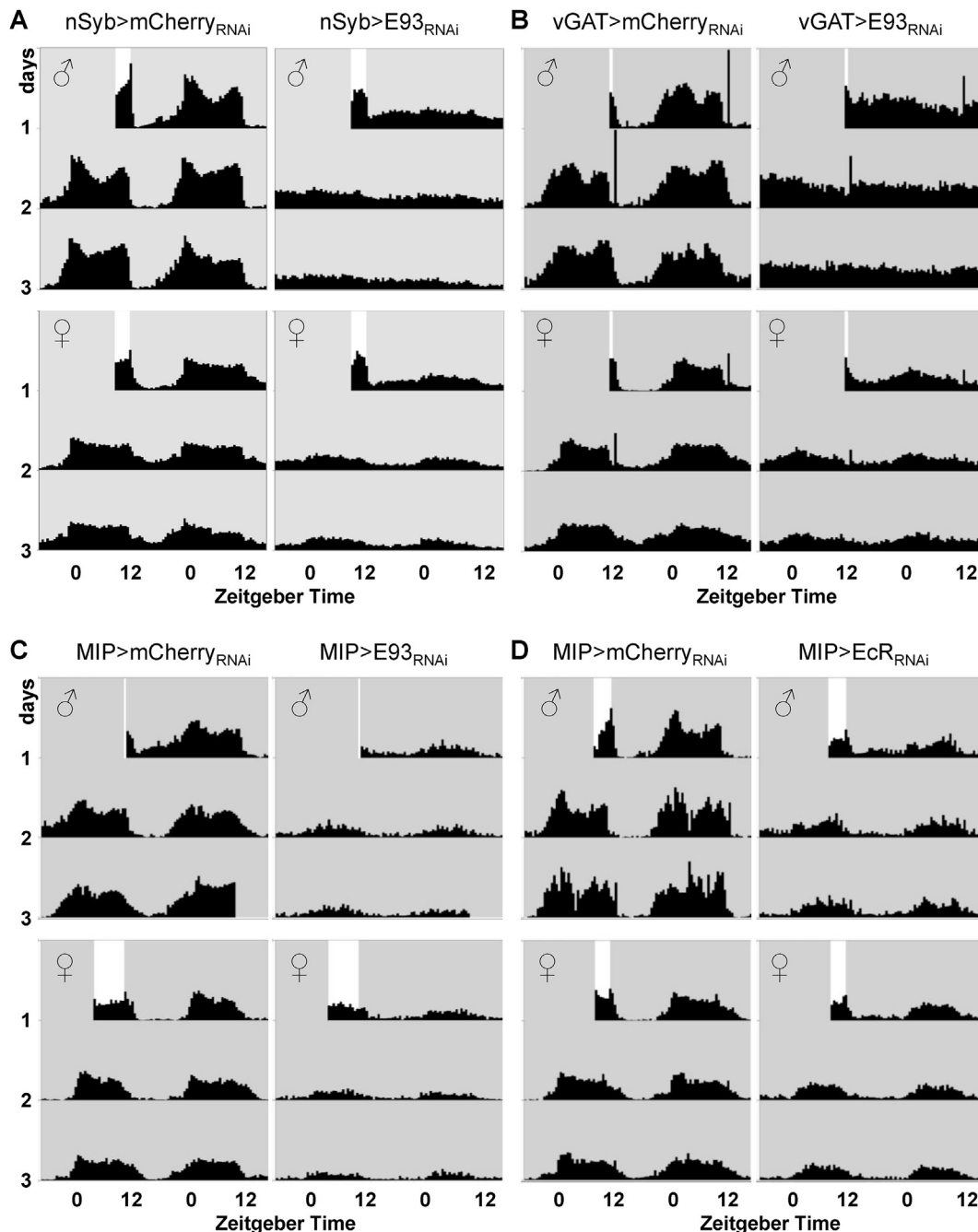
The reciprocal interaction between biological rhythm and metabolism is conserved in many animals and metabolic abnormality is often associated with abnormal locomotive activity [60,62]. For example, *Clock*



**Figure 5: Reduced *E93* expression in neurons attenuates morning and evening circadian anticipation.** Beam breaks were recorded every 6 min from individual fly using the DAM assay system under 12 h light and 12 h dark conditions. Group average double-plotted actograms were generated with 30 min bins and plotted scale 0–120 counts/30 min. The x-axis indicates Zeitgeber time and y-axis indicates days. Control (mCherry RNAi; left) and *E93* or *EcR* knockdown (right) flies are plotted by sex (males (♂): top, females (♀): bottom). White background indicates light phase and gray background indicates dark phase. **A:** Pan-neuronal knockdown of *E93* expression using the nSyb-GAL4 driver. *nSyb>mCherry<sub>RNAi</sub>* males (N = 27, 2 cohorts), *nSyb>mCherry<sub>RNAi</sub>* females (N = 29, 2 cohorts), *nSyb>E93<sub>RNAi</sub>* males (N = 31, 2 cohorts), and *nSyb>E93<sub>RNAi</sub>* females (N = 31, 2 cohorts). **B:** Knockdown of *E93* expression in GABA-ergic neurons. *vGAT>mCherry<sub>RNAi</sub>* males (N = 39, 3 cohorts), *vGAT>mCherry<sub>RNAi</sub>* females (N = 73, 5 cohorts), *vGAT>E93<sub>RNAi</sub>* males (N = 45, 3 cohorts), and *vGAT>E93<sub>RNAi</sub>* females (N = 64, 5 cohorts). **C:** Knockdown of *E93* expression in MIP neurons. *MIP>mCherry<sub>RNAi</sub>* males (N = 31, 2 cohorts), *MIP>mCherry<sub>RNAi</sub>* females (N = 32, 2 cohorts), *MIP>E93<sub>RNAi</sub>* males (N = 32, 2 cohorts), and *MIP>E93<sub>RNAi</sub>* females (N = 32, 2 cohorts). **D:** Knockdown of *EcR* expression in MIP neurons. *MIP>mCherry<sub>RNAi</sub>* males (N = 32, 2 cohorts), *MIP>mCherry<sub>RNAi</sub>* females (N = 28, 2 cohorts), *MIP>EcR<sub>RNAi</sub>* males (N = 30, 2 cohorts), and *MIP>EcR<sub>RNAi</sub>* females (N = 32, 2 cohorts).

mutant mice are obese [63] and high-fat diet disrupts the activity and feeding rhythms [64,65]. Studies in *Drosophila* also reveal evolutionarily conserved mechanisms of circadian rhythm controlled by metabolism [66–69]. When we measured the daytime and nighttime activity of *nSyb>E93<sub>RNAi</sub>* flies using the *Drosophila* Activity Monitoring (DAM)

system, both females and males showed overall reduced activity under 12 h light and 12 h dark condition compared to the controls (Figure 5A). Similar reductions in activity were observed in *MIP>E93<sub>RNAi</sub>* flies (Figure 5C). However, no obvious change in activity was observed in *vGAT>E93<sub>RNAi</sub>* flies and only a modest reduction in activity was



**Figure 6: Reduced *E93* expression in neurons disrupts circadian rhythm.** Group average double-plotted actograms of flies in constant darkness are shown. Flies raised in 12 h light and 12 h dark condition were placed in DAM system during the light phase and released in constant darkness at the time of lights off. The white box indicates the last light cycle before releasing flies in constant darkness. **A:** Pan-neuronal knockdown of *E93* expression using the *nSyb*-GAL4 driver. *nSyb>mCherry<sub>RNAi</sub>* males (N = 45, 3 cohorts), *nSyb>mCherry<sub>RNAi</sub>* females (N = 46, 3 cohorts), *nSyb>E93<sub>RNAi</sub>* males (N = 59, 4 cohorts), and *nSyb>E93<sub>RNAi</sub>* females (N = 61, 4 cohorts). **B:** Knockdown of *E93* expression in GABA-ergic neurons. *vGAT>mCherry<sub>RNAi</sub>* males (N = 26, 2 cohorts), *vGAT>mCherry<sub>RNAi</sub>* females (N = 39, 3 cohorts), *vGAT>E93<sub>RNAi</sub>* males (N = 31, 2 cohorts), and *vGAT>E93<sub>RNAi</sub>* females (N = 44, 3 cohorts). **C:** Knockdown of *E93* expression in MIP neurons. *MIP>mCherry<sub>RNAi</sub>* males (N = 31, 2 cohorts), *MIP>mCherry<sub>RNAi</sub>* females (N = 31, 2 cohorts), *MIP>E93<sub>RNAi</sub>* males (N = 32, 2 cohorts), and *MIP>E93<sub>RNAi</sub>* females (N = 31, 2 cohorts). **D:** Knockdown of *EcR* expression in MIP neurons. *MIP>mCherry<sub>RNAi</sub>* males (N = 31, 2 cohorts), *MIP>mCherry<sub>RNAi</sub>* females (N = 31, 2 cohorts), *MIP>EcR<sub>RNAi</sub>* males (N = 30, 2 cohorts), and *MIP>EcR<sub>RNAi</sub>* females (N = 32, 2 cohorts). Other conditions are the same as the actograms in Figure 5.

observed in *MIP>EcR<sub>RNAi</sub>* flies (Figure 5B and D). Interestingly, the nighttime activity of *vGAT>E93<sub>RNAi</sub>* was elevated (Figure 5B). GABA activity is required to promote sleep by suppressing wake-promoting neurons such as pigment dispersing factor (PDF) producing neurons and monoaminergic neurons such as dopamine or octopamine [25,70].

Therefore, the increased nighttime activity observed in *vGAT>E93<sub>RNAi</sub>* suggests that *E93* could play a role in neuronal GABA activity to control food intake as well as to promote sleep.

Under standard 12 h light and 12 h dark cycles, *Drosophila melanogaster* expresses a bimodal activity pattern with increased

anticipatory activity prior to lights-on (dawn) and lights-off (dusk). Anticipations of dawn and dusk are controlled by different subsets of circadian pacemaker neurons, morning oscillator and evening oscillator, respectively [71–73]. While control flies exhibit stereotypical anticipatory activities, these behaviors are almost absent in *nSyb>E93<sup>RNAi</sup>* flies (Figure 5A), suggesting that autonomous circadian clocks in *E93* knockdown flies could be disrupted. In fact, in constant darkness, the majority of *nSyb>E93<sup>RNAi</sup>* flies show nearly arrhythmic activity patterns (Figure 6A), and only a few flies exhibit low amplitude of rhythms (1 out of 16 knockdown males, 2 out of 15 knockdown females, Figure S5). Similar to *nSyb>E93<sup>RNAi</sup>* flies, *vGAT>E93<sup>RNAi</sup>*, *MIP>E93<sup>RNAi</sup>*, *MIP>EcR<sup>RNAi</sup>* flies show moderate anticipation for lights-on or off (Figure 5B–D), suggesting that their circadian rhythms are also disrupted. *vGAT>E93<sup>RNAi</sup>*, *MIP>E93<sup>RNAi</sup>*, *MIP>EcR<sup>RNAi</sup>* flies also exhibited damped circadian rhythms under constant darkness compared to that of controls (Figure 6B–D). While *nSyb>E93<sup>RNAi</sup>*, *vGAT>E93<sup>RNAi</sup>*, *MIP>E93<sup>RNAi</sup>*, *MIP>EcR<sup>RNAi</sup>* flies all show disrupted circadian rhythm, the disruptions in *nSyb>E93<sup>RNAi</sup>* flies under constant darkness are the most severe.

Together, our results suggest that *E93* in the two subsets of neurons, MIP-producing and GABA-ergic neurons, play an essential role in the regulation of metabolism, locomotive activity, and circadian rhythm all of which are essential for adult fitness.

### 3.8. *E93* action is critical during L3 to adult transition

To determine the timing of *E93* action, we used a GAL80ts line with a tubulin promoter (*tubP*) expressing GAL80ts ubiquitously [74]. GAL80ts is an inhibitor of GAL4, whose activity can be manipulated by temperature. At a permissive temperature (19 °C), GAL80ts is functional and thus inhibits *nSyb*-GAL4 activity in neurons. This blocks neuronal *E93* RNAi and thus *E93* functions normally. At a nonpermissive temperature (30 °C), however, GAL80ts is no longer functional resulting in *nSyb*-GAL4 activation and *E93* RNAi in neurons. To pinpoint the timing of *E93* action, we shifted temperature either at a late larval stage (the third instar larvae L3 and wandering larvae) when *E93* expression is known to be highest [20] or within 4 h after eclosion as an adult. The *GAL80ts; nSyb-GAL4>E93<sup>RNAi</sup>* flies raised at constant 19 °C showed no difference in body weight compared to the control, *GAL80ts; nSyb-GAL4>mCherry<sup>RNAi</sup>* (Figure 7A), whereas *GAL80ts; nSyb-GAL4>E93<sup>RNAi</sup>* flies raised at constant 30 °C became obese (Figure 7B). These results confirmed that the obesity is indeed caused by reduction of *E93* expression. The phenotypes did not change when we shifted the temperature in adults; the obese flies remained obese, and the normal flies remained normal (Figure 7C–D). When we shifted the temperature at the late larva stage, however, *GAL80ts; nSyb-GAL4>E93<sup>RNAi</sup>* flies became obese (Figure 7E). Furthermore, if we raised the flies at 30 °C until L3 then shifted to 19 °C, the flies did not become obese, indicating that the transition beginning from late L3 is the critical period of *E93* action (Figure 7F).

Next, we tested whether the obesity phenotype correlates with other physiological defects such as circadian rhythm. When we examined circadian rhythm of the *GAL80ts; nSyb-GAL4>E93<sup>RNAi</sup>* flies raised at constant 19 °C or 30 °C, under the constant darkness conditions, the activity pattern correlated with the obesity phenotype; the flies raised at 19 °C showed a similar activity pattern to that of the control, whereas the flies raised at 30 °C show disrupted activity pattern (Figure S6A–B). Consistent with that result, the flies raised at 19 °C then moved to 30 °C at L3 stage showed a disrupted activity pattern (Figure S6C), whereas the flies raised at 30 °C then moved to 19 °C at L3 stage did not (Figure S6D). These results indicate that *E93* action at the transition is critical for normal body weight and circadian activity.

### 3.9. Reduced expression of *E93* in neurons reduces reproductive activity

The neuronal knockdown of *E93* results in such a dramatic and systemic failure in the expression of adult metabolism and physiology that we hypothesize that the brain of *nSyb>E93<sup>RNAi</sup>* flies might remain in the larval state focusing on growth and eating instead of on adult behavior such as mating. As shown in Figure 7, *E93* critically functions for regulation of metabolism during larval-to-adult transition when the larval brain develops into the adult brain, supporting this hypothesis. When we examined the courtship behavior in the presence of food [33], the control males exhibited multiple courtship components and sequence within 10-minute recording span, such as following the female, touching her, performing wing dance, and mounting [32] (Figure 8A, Supplementary Video 4, right). However, *nSyb>E93<sup>RNAi</sup>* males did not interact with the female but often went for food (Figure 8A, Supplementary Video 4, left).

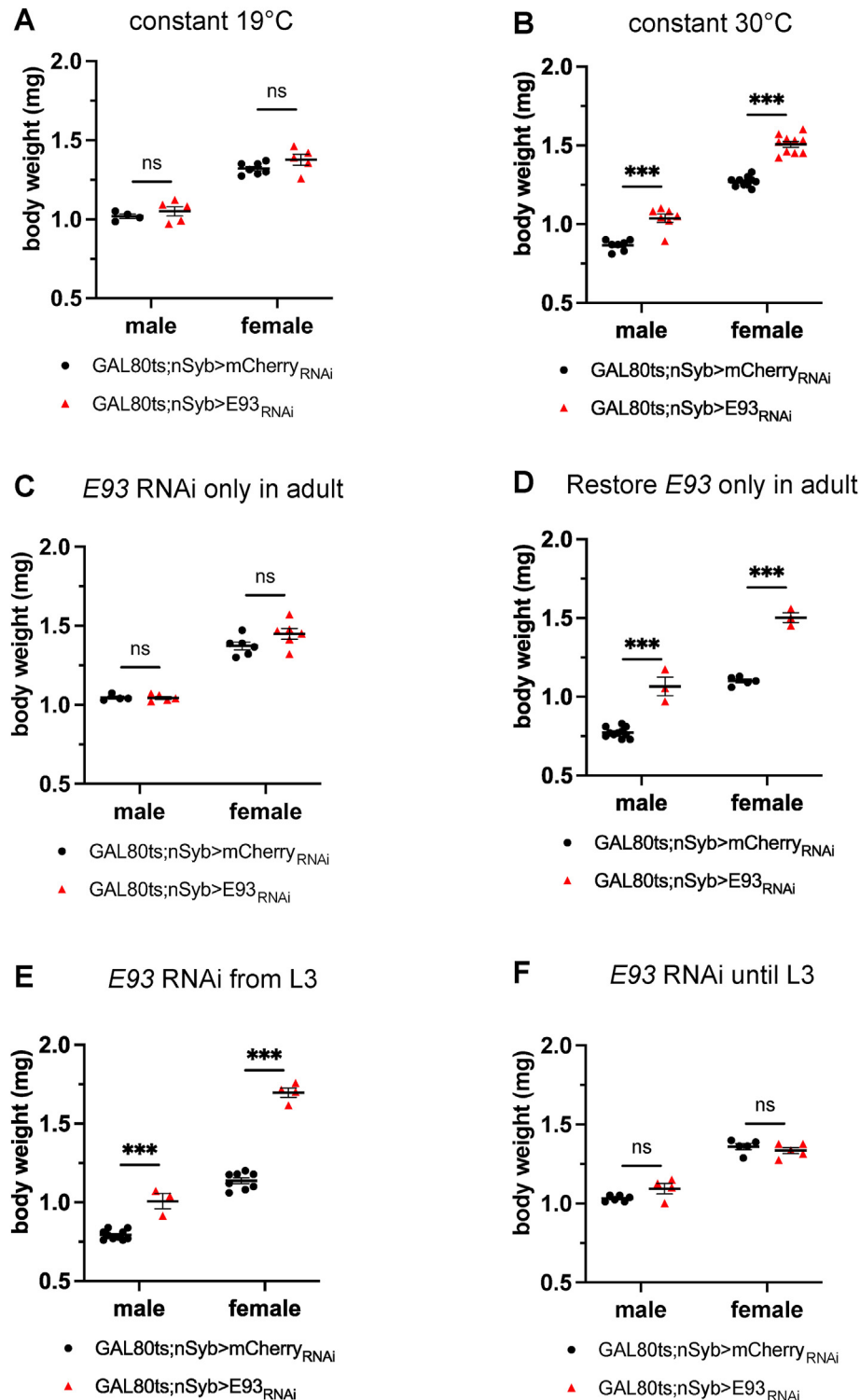
Because *nSyb>E93<sup>RNAi</sup>* flies show increased attraction to food (Figure 2A), We next tested whether the lack of courtship behaviors in *nSyb>E93<sup>RNAi</sup>* males was due to a preference for food over mates. When we performed the mating assay in the absence of food, however, *nSyb>E93<sup>RNAi</sup>* males still did not show courtship behavior (Figure 8D, Supplementary Video 5). Instead, they usually rested in the corner, suggesting that the lack of courtship behavior in *nSyb>E93<sup>RNAi</sup>* males is not due to a preference for food over a mate.

The lack of courtship behavior resulted in failure of reproduction. When a female and male pair was placed in a vial with food for 24 h, the control pairs produced progeny, as did the *nSyb>E93<sup>RNAi</sup>* female with a control male. However, *nSyb>E93<sup>RNAi</sup>* males with a control or a *nSyb>E93<sup>RNAi</sup>* female failed to produce progeny (Figure S7A). Because wings play a significant role in courtship behavior and because *nSyb>E93<sup>RNAi</sup>* flies do not have functional wings, we tested whether the infertility of *nSyb>E93<sup>RNAi</sup>* males was due to their lack of functional wings. When we removed wings of control males, however, the de-winged controls produced progeny, indicating that in our setup, the wings are dispensable for mating (Figure S7B). Both *vGAT>E93<sup>RNAi</sup>* males and *MIP>E93<sup>RNAi</sup>* males fail to exhibit mating behavior regardless of food (Figure 8B–C, E–F, Supplementary Videos 6–9), suggesting these two neuron populations could also be the main action sites of *E93* to regulate reproductive activity.

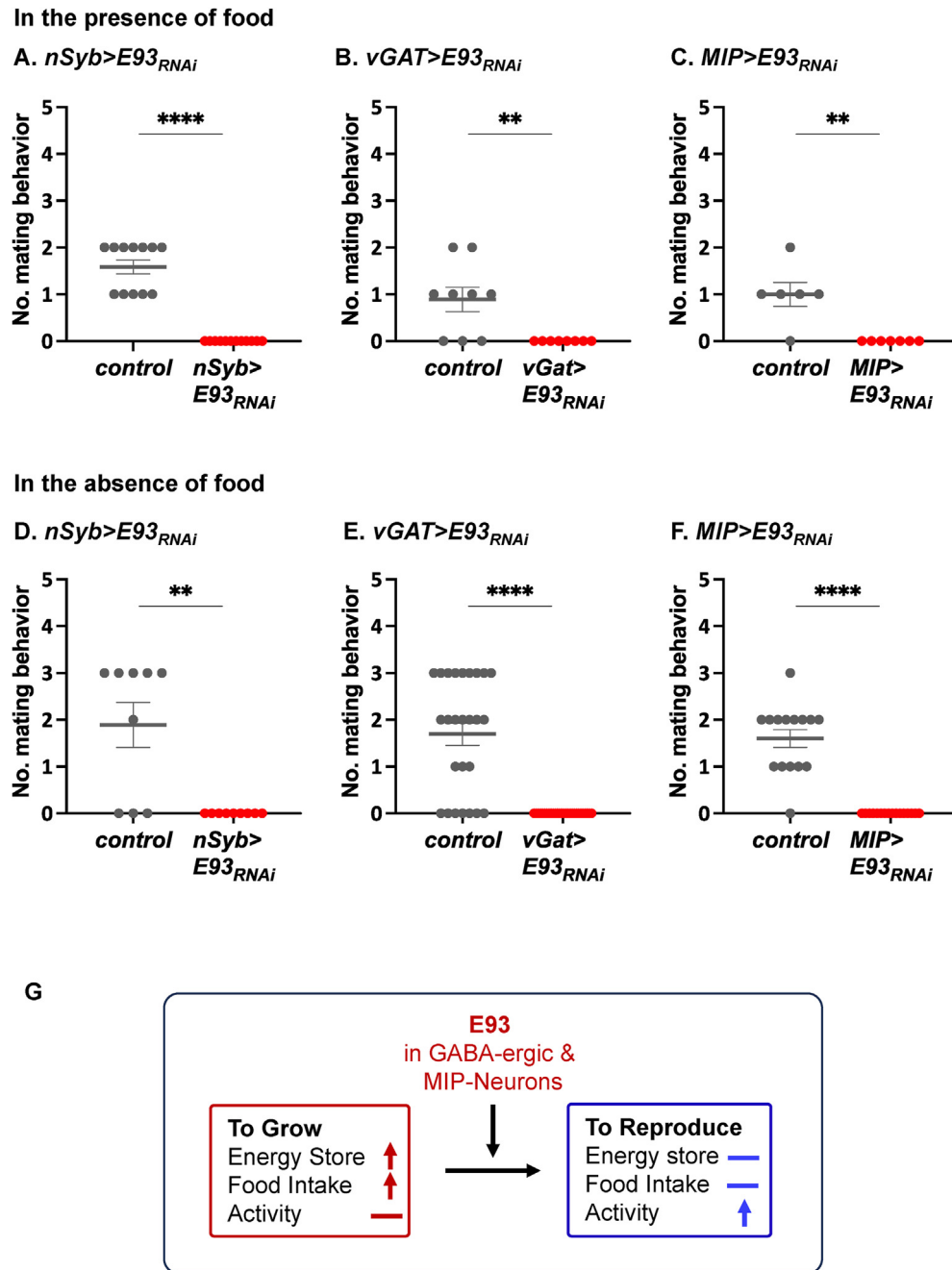
During mating, a female fly evaluates the male partner via several pre-mating responses such as flapping, escaping, and kicking, before she eventually accepts the male [75,76]. Although *nSyb>E93<sup>RNAi</sup>* females could reproduce (Figure S7A), it is possible that their receptivity is reduced. We tested the receptivity indirectly by a modified fertility assay, where a pair of flies was given only 3 h for mating. We reason that if the receptivity of *nSyb>E93<sup>RNAi</sup>* females were reduced, the mating rate would be also reduced. In two independent experiments where 20 or 24 females of *nSyb>mCherry<sup>RNAi</sup>* or *nSyb>E93<sup>RNAi</sup>* were each individually paired with a *nSyb>mCherry<sup>RNAi</sup>* male for 3 h, 15 and 20 *nSyb>mCherry<sup>RNAi</sup>* females produced progeny (15/20, 20/24, an average of 79% mating rate). However, only 2 *nSyb>E93<sup>RNAi</sup>* females produced progeny (2/20, 2/24, an average of 10% mating rate, Figure S7C). This result suggests that reduction of neuronal *E93* expression reduces reproductive activity both in males and females.

## 4. DISCUSSION

In this study, we report *E93* as a new monogenic factor of metabolic syndrome, identified from a targeted screen of 70 selected genes orthologous to mammalian genes enriched in VMH. Neuron-specific knockdown of *E93* causes a systemic failure of metabolic



**Figure 7: E93 function is critical during larval-to-adult transition.** **A:** The body weights are not different between *tubP-GAL80ts; nSybGAL4>E93<sub>RNAi</sub>* flies (shown as GAL80ts;nSyb>E93<sub>RNAi</sub>) and *tubP-GAL80ts; nSybGAL4>mCherry<sub>RNAi</sub>* (shown as GAL80ts;nSyb>mCherry<sub>RNAi</sub>) flies when both were raised at constant 19 °C, a permissive temperature, throughout life. **B:** *tubP-GAL80ts; nSybGAL4>E93<sub>RNAi</sub>* flies are heavier than *tubP-GAL80ts; nSybGAL4>mCherry<sub>RNAi</sub>* flies when both were raised at constant 30 °C, a non-permissive temperature, throughout life. **C:** The body weights are not different between *tubP-GAL80ts; nSybGAL4>E93<sub>RNAi</sub>* flies and *tubP-GAL80ts; nSybGAL4>mCherry<sub>RNAi</sub>* flies when both were raised at 19 °C and then moved to 30 °C immediately after eclosion. **D:** *tubP-GAL80ts; nSybGAL4>E93<sub>RNAi</sub>* flies are heavier than *tubP-GAL80ts; nSybGAL4>mCherry<sub>RNAi</sub>* flies when both were raised at 30 °C and then moved to 19 °C immediately after eclosion. **E:** *tubP-GAL80ts; nSybGAL4>E93<sub>RNAi</sub>* flies are heavier than *tubP-GAL80ts; nSybGAL4>mCherry<sub>RNAi</sub>* flies when both were raised at 19 °C and then moved to 30 °C at the L3 stage. **F:** The body weights are not different between *tubP-GAL80ts; nSybGAL4>E93<sub>RNAi</sub>* flies and *tubP-GAL80ts; nSybGAL4>mCherry<sub>RNAi</sub>* flies when both were raised at 30 °C and then moved to 19 °C at the L3 stage. **A–F:** Each data point represents one group of 7–10 flies. Flies were 3–8 days old. The bars indicate the mean  $\pm$  S.E.M. ns: not significant, \*\*\* $p < 0.001$  by Student *t*-test.



**Figure 8: Reduced *E93* expression in neurons reduces reproductive behavior.** **A–C:** The number of exhibition of mating behavior of males in the presence of food (see the [Methods](#) and [Supplementary videos](#)). **A:** *nSyb>mCherry<sup>RNAi</sup>* males (control) and *nSyb>E93<sup>RNAi</sup>*, N = 12 for both. 3–5 days old. **B:** *vGAT>mCherry<sup>RNAi</sup>* males (control) N = 9, and *vGAT>E93<sup>RNAi</sup>* males N = 8. 2–3 days old. **C:** *MIP>mCherry<sup>RNAi</sup>* males (control) and *MIP>E93<sup>RNAi</sup>*, N = 7 for both. 3 days old. Each data point represents a single male's behavior. **D–F:** The number of exhibition of mating behavior in the absence of food. **D:** *nSyb>mCherry<sup>RNAi</sup>* (control) and *nSyb>E93<sup>RNAi</sup>*, N = 9 for both. 2–3 days old. **E:** *vGAT>mCherry<sup>RNAi</sup>* (control) N = 26, and *vGAT>E93<sup>RNAi</sup>*, N = 28. 3–5 days old. **F:** *MIP>mCherry<sup>RNAi</sup>* (control) and *MIP>E93<sup>RNAi</sup>*, N = 16 for both. 3 days old. The bars indicate the mean  $\pm$  S.E.M. \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$  by Student *t*-test. **G: Model** A larva and an adult have distinct goals of growth and reproduction, respectively. This difference in goals directs the behavior and activity of each form to maximize fitness. Larvae's main activity is feeding to increase the mass, whereas the adults' is coordinated behavior such as circadian rhythmicity, optimum fitness, and energy stores for mating success. Our study suggests that *E93* in GABA-ergic neurons and MIP-producing neurons acts as a switch critical for this transition.

homeostasis, leading to phenotypes such as hyperphagia, increased energy stores, and impaired exercise endurance. Neuronal *E93* is also required for circadian rhythmicity, and for mating behavior for males, indicating *E93* is critical for the animals to exhibit adult behaviors. Screening of 17 different GAL4 drivers revealed that *E93* function in metabolism, adult physiology and behavior is primarily via its action in

GABA-ergic and MIP-producing neurons, both of which have been implicated in regulation of feeding, metabolism, and sleep [22,24,26,28,70]. Consistent with its known role in metamorphosis, *E93* action during the 3rd instar (L3) and metamorphosis is the critical developmental time window for its normal body weight. *E93* partly interacts with the ecdysone receptor (*Ecr*) in MIP neurons, linking

steroid signal to a peptidergic signal. Increased frequency of feeding and the resulting increased food intake most likely underlies the obesity in flies with neuron-specific knockdowns of *E93*.

Neuron-specific knockdown of *E93* also exhibits multiple physiological defects such as impaired exercise endurance, reduced climbing speed, disrupts circadian rhythm, and reduced reproduction activity. Similar defects in exercise endurance, climbing speed, circadian rhythm, and reproduction activity were observed in flies with GABA-specific or MIP-specific knockdowns of *E93*, supporting the idea that these neurons are the main sites of *E93* action. Disruption of circadian rhythm is most pronounced when the flies are exposed to constant darkness (Figure 6). All *nSyb*>*E93*<sup>RNAi</sup>, *vGAT*>*E93*<sup>RNAi</sup>, *MIP*>*E93*<sup>RNAi</sup>, and *MIP*>*Ecr*<sup>RNAi</sup> flies failed to show lights-on and off anticipatory behavior but exhibited either arrhythmicity or low amplitude rhythms under constant darkness. It is possible that reduced activity itself underlies the circadian phenotype. For instance, in a DAM assay, flies that stay near the food and spend a significantly longer time feeding than the controls would show decreased numbers of beam crosses. However, *vGAT*>*E93*<sup>RNAi</sup> flies showed disrupted circadian rhythms, yet their activity levels were comparable to controls, suggesting that the arrhythmicity in males or the low amplitudes in females in constant darkness could be independent of overall activity. Taken together, our data from *vGAT*>*E93*<sup>RNAi</sup> and *nSyb*>*E93*<sup>RNAi</sup> flies suggest that at least some part of disrupted circadian rhythm of *nSyb*>*E93*<sup>RNAi</sup> is likely associated with their global metabolic abnormalities rather than overall reduction of activity.

A mammalian homolog of *E93* is *LCoR* (Ligand-dependent Corepressor) and has been shown to directly interact with nuclear receptors critical for metabolism [77–80]. This suggests that *E93* might regulate circadian rhythm by interacting with the key nuclear receptors required for the metabolic clock [81,82]. Indeed, the fly orthologs of the key nuclear receptors, *E75* (Retinoic acid-related Orphan Receptors in mammals) and *DHR51* (Drosophila Hormone Receptor 51, REV-ERB in mammals) have conserved roles in regulating circadian rhythm [83,84]. Although, the detailed neuro-molecular mechanisms by which *E93* regulates these pleiotropic metabolic phenotypes require further investigation, based on its role in body weight regulation from the temperature sensitive experiment, we suggest that *E93* is the key switch in shaping the adult brain to confer the adult metabolism. As a transcription factor, *E93* could directly control gene expression in coordination with specific nuclear receptors and switch the neuron's profile from larva to adult, as suggested in the model (Figure 8G). Our result that obesity can be induced by reducing neuronal *E93* expression only during L3 and metamorphosis also supports this hypothesis. Indeed, a recent study shows that several larva-enriched genes are abnormally upregulated during metamorphosis in the *E93* deletion mutant [17]. Interestingly, many of these genes are also implicated in neural fate determination (e.g. *squeeze*, *ko*), metabolism (e.g. *secreted decoy of Inr*), and circadian rhythm (e.g. *clock*), supporting a role for *E93* in the transition from larval to adult metabolism and physiology. One way by which *E93* could control the transition from the larval brain to the adult brain is to establish the adult neuronal circuitry by controlling neurite pruning as its *C. elegans* ortholog does [13]. In fact, the nervous system undergoes dramatic rewiring via steroid hormones during adolescence in many animals to switch the focus from growth to reproduction [85,86]. *Drosophila* also undergoes intensive remodeling of the nervous system during metamorphosis a stage in which *E93* plays its essential roles [87].

Together, our results provide insights into how a transcription factor acting in specific sets of neurons in the nervous system shapes and regulates adult metabolism, physiology, and behavior.

## FUNDING

This work was supported by the National Institutes of Health (NIH) through R01DK100659 (JE), P30DK127984 (JE), R01NS114527 (SY), R01AA019526 (AR), R01AA026818 (AR), R01AG059683 (RW), 2T32HL120822 (TC), and by the National Science Foundation (NSF) through IOS-1931115 (SY).

## ETHICS STATEMENT

No animal work in this study requires specific regulations.

## CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

**Cecilia Yip:** Writing — review & editing, Writing — original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Steven C. Wyler:** Writing — review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Katrina Liang:** Investigation. **Shin Yamazaki:** Writing — review & editing, Writing — original draft, Validation, Software, Funding acquisition, Formal analysis. **Tyler Cobb:** Investigation, Funding acquisition, Formal analysis. **Maryam Safdar:** Writing — original draft, Investigation, Formal analysis. **Aarav Metai:** Investigation, Formal analysis. **Warda Merchant:** Methodology, Investigation. **Robert Wessells:** Writing — review & editing, Writing — original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Adrian Rothenfluh:** Writing — review & editing, Supervision, Funding acquisition. **Syann Lee:** Writing — review & editing, Supervision, Conceptualization. **Joel Elmquist:** Supervision, Funding acquisition, Conceptualization. **Young-Jai You:** Writing — review & editing, Writing — original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization.

## ACKNOWLEDGEMENT

We thank Drs. Kim J, Fujikawa T, and Sieber M, and the Sieber lab members for invaluable discussions, Drs. Smith D, Buszczak M, and Kramer H, Ms. Chelsea Limboy for technical help. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. We used FlyBase to obtain information on multiple genes including *E93*. We thank Korea Drosophila Resource Center, and Dr. Kim Y (GIST) for fly strains.

## DECLARATION OF COMPETING INTEREST

The authors declare that there are no conflicts of interest.

## DATA AVAILABILITY

No data was used for the research described in the article.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2024.101939>.

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