Neuronal E93 regulates metabolic homeostasis

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Abstract

We report a novel function of E93, a transcriptional factor essential for metamorphosis, in the metabolic and circadian regulation of *Drosophila melanogaster*. When *E93* is specifically knocked down in neurons, flies exhibit a systemic dysregulation of metabolic homeostasis. They become hyperphagic and obese with increased energy stores and disrupted circadian rhythms. A screen of Gal4 lines targeting subsets of neurons and endocrine cells identified neurons producing GABA and myoinhibitory peptide (MIP) as the main sites of E93 action. Inhibition of GAL4 by a GAL80 repressor specific to MIP neurons partially but significantly reverses the neuronal *E93* knockdown phenotype. Knockdown of the ecdysone receptor, which acts upstream of *E93*, specifically in MIP neurons partly phenocopies the MIP neuron-specific knockdown of *E93*. Based on these results we suggest that *E93*, essential for metamorphosis, is a key switch for the metabolic transition acting in the neurons.

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 *Actias luna* belongs, do not have a feeding organ, and survive and reproduce solely by 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 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 depending on the females' mating status, suggesting their continuous roles in balancing metabolic states after metamorphosis.

E93 (*Eip93F*: Ecdysone-induced protein 93F), is one of the master regulators of metamorphosis and has been intensively studied due to its crucial role in the MEKRE93 pathway which many pesticides target[9,10]. E93 belongs to the pipsqueak transcription factor family[11,12] 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)[13,14]. Conserved from *C. elegans* to mammals[15], it regulates neurite pruning in *C. elegans*[14], metamorphosis in insects including *Drosophila*[13], and is associated with body size variations and lipid metabolism in mammals[16,17]. In *Drosophila*, E93 plays an essential role during metamorphosis as a switch to suppress expression of larval genes and promote expression of adult genes[18]. 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[19]. Consistent with this role as a switch, E93 is

also required for termination of neurogenesis in mushroom body neuroblasts, presumably to help the neurons adapt their adult fate[20]. *E93* expression is maintained at low levels until larva L3 puffstage, the beginning of metamorphosis, when expression is induced by ecdysone (20E)[21]. *E93* expression persists in adults mostly in neurons, including subsets of antennal sense organ and olfactory neurons[22], suggesting its roles beyond metamorphosis. Despite its essential roles in this developmental program tightly linked to metabolic transition, the mechanisms of E93 action are largely unknown. Moreover, the role of E93 action in metabolism controlled by the nervous system has not been studied.

We report that reduced expression of *E93* in neurons results in hyperphagia and various metabolic and physiological abnormalities. Knockdown increases attraction to food, as well as food intake, body weight, and energy stores with defective circadian rhythm. This failure of metabolic homeostasis is largely due to E93's action in two populations of neurons: GABA-ergic and myoinhibitory peptidergic (MIP) neurons, both of which have been implicated in controlling food intake, metabolism, circadian rhythm, and sleep[23-29]. MIP neuron-specific knockdown of the ecdysone receptor (EcR), the receptor of ecdysone (20E) which acts upstream of *E93* in metamorphosis, partially phenocopies the MIP neuron-specific knockdown of *E93* in the obese phenotype. Additionally, restoring *E93* only in MIP neurons partially reverses the metabolic abnormalities observed in neuron-specific knockdown of *E93*. Together, our study reveals a novel neuron-specific role of E93 in controlling metabolic homeostasis and circadian rhythm via a steroid hormonal signal.

Materials and Methods

***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) and vGat RNAi (#41958). 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.

Quantification of triglycerides (TAG). Triglycerides and protein were quantified as described by Tennessen *et al*[30] 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 three minutes 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 minutes. Absorbance levels were read at 540 nm.

Quantification of protein. Protein level was quantified using the BCA assay kit (ThermoFisher Scientific, Waltham, MA; product no. 23228) and used to normalize 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 × number of samples + 20) ×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 minutes and absorbance values were read at 562 nm of wavelength.

Quantification of glycogen. Glycogen quantification was performed as described by Tennessen *et al.*[30] 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 minutes and then centrifuged at 14,000 rpm for three minutes 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 µl of PBS in a separate 1.5 ml tube. 100 µl of amyloglucosidase solution was added and samples were incubated while shaking at 55°C for one hour. For quantification of glucose, 20 µl of remaining supernatant was added to a 96 well plate in duplicates and were diluted in 60 µl PBS. Similarly, 20 µl of incubated glycogen sample was added to the plate in duplicates and 100 µl 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 minutes and 10 µl of 12 N sulfuric acid were added before absorbance was measured at 540 nm[30].

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

Quantification of body weight. A group of five 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 5-10 groups / genotype / sex.

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.

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 µl pipette tip with the head exposed. 1 µl 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 five minutes.

Blue dye feeding assay. Two-day old flies of 7-18 were fasted for 24 hours 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 µl 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 minutes if previously fasted, and 10 minutes 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 µl of water, then centrifuged at 14,000 rpm for 5 minutes. To measure absorbance, 10 µl of supernatant was transferred to a 96 well plate and diluted in 40 µl of water. Absorbance was measured at 630 nm.

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

RNA isolation and MIP qPCR. RNA was isolated using the Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA) following manufacture instructions. 10 heads were used per replicate. 250 ng of head RNA was converted to cDNA using Superscript IV cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA). qPCR was performed using TaqMan probes: *Mip*, Dm02367867_s1 and normalized to *alphaTub* Dm02361072_s1 (Thermo Fisher Scientific). Fold change was calculated using the delta-delta CT ($\Delta\Delta CT$) method. The experiment was repeated twice.

Starvation assay. Vials were prepared with 0.7% agar. Age-matched males and 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.

***Drosophila* Activity Monitor (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. An infra-red beam breaking horizontal locomotor activity was monitored from individual fly under a 12 h light and 12 h dark or in constant darkness for 3 days. Because some of nSyb>E93 flies become unhealthy after being kept in the tube more than 5 days, we didn't 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 of beam breaks / 6 min from 16-73 flies of 1-5 cohorts of each genotype and sex were used to generate actograms (ClockLab, Actimetrics, Wilmette, IL). Files died during 3 days of recording were excluded from the group average actogram. Double plotted group averaged actograms were generated from 1- 3 cohorts in each genotype and sex with 30 min bin and scaled plot (0-120 counts / 30 min).

Statistics

We examined whether all experimental groups were normally distributed using Sturges' class number and a symmetric distribution. Most 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. Two-way ANOVA was used for Figure 1A and Figure 4B.

Ethics statement

No animal work in this study requires specific regulations.

Data availability

Source data are provided with this paper.

Results

Neural specific knockdown of E93 increases body weight and energy stores.

When we knocked down the expression of *E93* pan-neuronally using an nSyb-GAL4 driver and a UAS-E93 RNAi (from here, nSyb>E93$_{RNAi}$), the flies became obese; their body weight was increased compared to the three UAS, GAL4, and genetic background control lines (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$_{RNAi}$) or nSyb>mCherry$_{RNAi}$ as a control depending on the experiment. nSyb>mCherry$_{RNAi}$, nSyb>E93$_{RNAi}$ and none>E93 $_{RNAi}$ lines were outcrossed to w Berlin for five generations to equalize the background.

The body weight of nSyb>E93$_{RNAi}$ flies gradually increased each day after eclosion (Figures 1B and 1C). Three days after eclosion, both male and female nSyb>E93$_{RNAi}$ flies were visibly obese (Figure 1D). When we measured abdomen width, both knockdown males and females had increased abdomen size (Figure S1A). Female knockdowns had significantly enlarged abdomens beginning one day after eclosion compared to controls, but both nSyb>E93$_{RNAi}$ and controls retained similar numbers of mature eggs (14.4 ± 5.3, n=18, vs 14.9 ± 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 triglycerides (TAG), the main storage form of lipid, and glycogen, we found that males had more TAG whereas females had more glycogen than controls (Figures 1E and 1F). These results suggest that neuronal E93 regulates energy stores and that the energy stores may be regulated in a sexually dimorphic manner.

Neural specific knockdown of E93 increases food intake.

Next, we asked whether the increased body weight and energy stores in nSyb>E93$_{RNAi}$ flies were due to an increase in food intake. When nSyb>E93$_{RNAi}$ flies were introduced to a vial with a pad containing sucrose solution (150 mM) and blue dye, a higher percentage of nSyb>E93$_{RNAi}$ flies gathered to the pad than controls (Figure 2A). Consistent with this observation, nSyb>E93$_{RNAi}$ flies showed increased feeding frequency measured by the percentage of total fed flies with blue abdomens (Figure 2B). Furthermore, the majority of nSyb>E93$_{RNAi}$ 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$_{RNAi}$ flies was fully extended at a higher frequency than the control, suggesting they are feeding more frequently than the control (Figures 2C and 2D).

The Capillary Feeding (CAFE) assay is commonly used to assess food intake and relies on the ability of flies to reach a suspended food source and hang upside down long enough to feed until satiated[31]. As nSyb>E93$_{RNAi}$ 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 λ_{630} (see Methods)[32]. Virgin male and female nSyb>E93$_{RNAi}$ flies fasted for 24 h had increased food intake compared to controls (Figure 2F).

We used the proboscis extension reflex (PER) assay to further examine the observed hyperphagia of nSyb>E93$_{RNAi}$ flies in detail. Flies were individually trapped in a pipet tip and presented with 1 μl of sucrose solution containing blue dye under the proboscis[27]. The duration of proboscis extension and the volume of consumed food were measured over 5 min by subtracting the remaining volume from the 1 μl 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$_{RNAi}$ flies and controls. All nSyb>E93$_{RNAi}$ flies maintained extended proboscises for the entire 5 min (100%, 5 out of 5) and 80% (4 out of 5) of flies finished 1 μl 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 μl (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 to increased food intake when nutritional demand is high[33]. The average diameter of the midgut of nSyb>E93$_{RNAi}$ females was increased compared to controls, suggesting an increase in food intake (Figures S1B and 1C). In males increased food intake is often associated with increased defecation rate[34]. nSyb>E93$_{RNAi}$ males show an increased defecation rate than controls suggesting their increased food intake (Figure S1D). nSyb>E93$_{RNAi}$ flies survived starvation longer than controls (Figures 2G and 2H), 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 metabolism.

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

To determine the sites of E93 action, we screened 17 GAL4 lines limiting expression of the E93 RNAi construct in 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$_{RNAi}$ 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 remaining ten drivers were selected based on their known roles in feeding and metabolism, and include cells producing MIP (myoinhibitory peptide)[27], Akh (Adipokinetic hormone)[35], Eth (Ecdysone triggering hormone)[36], Phm (ecdysone producing cells of the prothoracic glands)[37], Tk (Tachykinin)[38,39], Npf (neuropeptide F)[40], Fmrfa (FMRFamid neuropeptide), Dilp2 (Insulin-like peptide), Lk (Leucokinin), and SIFaR (SIFamide receptor)[41]. To control for the effect of different GAL4s, we used UAS-mCherry$_{RNAi}$ as a control for each driver. UAS-mCherry$_{RNAi}$ (none>mCherry$_{RNAi}$) flies had comparable metabolic parameters, including body weight and starvation response, to other controls (Figures 1A, 2G and 2H).

Among classical neurotransmitter-producing neurons, *E93* knockdown in GABA-ergic neurons increased glycogen levels. For neuropeptidergic/endocrine cells, knocking down *E93* in MIP neurons increased the level of glycogen, while knocking down *E93* in the rest did not (Figure 3A). Interestingly *E93* knockdown in serotonergic neurons (driven by Trh-GAL4) reduced glycogen levels, suggesting a differential role of E93 depending on neuron types (Figure 3A).

Specific GABA-ergic interneurons are known to play a critical role in restraining feeding[23]. Indeed, body weight and feeding frequency were greater in both male and female vGAT>E93$_{RNAi}$ flies compared to controls (Figures 3B-3D). In addition, a higher percentage of vGAT>E93$_{RNAi}$ males and females extended their proboscises than controls (93% of vGAT>E93$_{RNAi}$ (N=57) vs. 2% of vGAT>mCherry$_{RNAi}$ (N=46)). The abdomen size of the vGAT>E93$_{RNAi}$ females was increased without an increase of the number of retained eggs (Figures S2A and S2B). 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. vGAT>E93$_{RNAi}$ 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 results indicate that GABA-ergic neurons are one of the main sites of E93 action in controlling feeding and metabolism.

Next, we examined flies with *E93* knocked down in MIP neurons. MIP/allatostatin-B homologs are present in almost all animals[42], and serve versatile roles related to metabolism such as feeding and metamorphosis[43]. 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[27-29]. Both males and females of MIP>E93$_{RNAi}$ are obese and feed more (Figures 3E-3G), indicating MIP neurons are also where E93 acts. The MIP>E93$_{RNAi}$ females exhibit similar phenotypes to the reported phenotypes of the MIP mutant females[27] and an increased abdomen size without changing in the number of retained mature eggs (Figures S2C and S2D)

Because MIP>E93$_{RNAi}$ flies show similar phenotypes to those of MIP mutants and because E93 is a transcription factor, we next asked whether MIP transcription level is reduced in nSyb>E93$_{RNAi}$ flies. qPCR from head samples of nSyb>E93$_{RNAi}$ female flies showed reduced MIP transcription, suggesting that E93 controls food intake and metabolism partly via MIP signaling (Figure 4A).

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$_{RNAi}$ flies. Repression of GAL4 by GAL80 prevents RNAi of *E93* so that 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$_{RNAi}$ flies (Figures 4B and 4C), confirming that MIP neurons are one of the sites of E93 action.

Steroid nuclear receptor controls metabolism in MIP neurons.

During the process of metamorphosis, ecdysone binds to the ecdysone receptor (EcR) to induce expression of *E93*[12]. To delineate the signaling pathway of E93, we examined whether EcR also acts upstream of E93 and mediates the metabolic phenotypes observed in adults. Ecdysone and EcR regulate many essential functions not only during metamorphosis but also in adult flies; ecdysone is secreted mainly in the fat body in males and ovaries in females, and controls reproduction and metabolism[10,44,45]. Because knocking down EcR expression pan-neuronally or specifically in GABA-ergic neurons (nSyb>EcR$_{RNAi}$ or vGAT>EcR$_{RNAi}$) result in lethality, we could not examine the EcR functions in the two types of neurons in feeding and metabolism. However, using MIP-GAL4 and UAS-EcR$_{RNAi}$ lines, we found that MIP-specific knockdown of EcR (MIP>EcR$_{RNAi}$) phenocopied MIP>E93$_{RNAi}$. MIP>EcR$_{RNAi}$ flies showed increased body weight and visibly obese (Figures 4D and 4E), suggesting that E93 functions downstream of EcR in MIP neurons and regulates metabolism. However, we found the feeding frequency of MIP>EcR$_{RNAi}$ was not different from the control (Figure 4E female) or slightly reduced (Figure 4E male). Although we do not know the reason for the discrepancy between increased body weight without increased food intake in MIP>EcR$_{RNAi}$, we think it could be due to a different activity pattern of MIP>EcR$_{RNAi}$ (see below) which might change their feeding timing and pattern. It is also possible that because unlike E93, EcR plays a significant role from the birth, knocking down EcR in MIP neurons throughout development might cause certain adaptations or defects some of which might override the feeding phenotype we observed in adult MIP>E93$_{RNAi}$. Nonetheless, similar to nSyb>E93$_{RNAi}$ females, MIP>E93$_{RNAi}$ females had increased abdomen size without changes in the number of retained mature eggs (Figures S3A and S3B).

Because GABA is important for controlling food intake[23,24] and is secreted from some MIP neurons[46], we examined whether GABA produced in MIP neurons contributes to the nSyb>E93$_{RNAi}$ phenotypes. However, no differences in body weight were observed when GABA production in MIP neurons was reduced by RNAi of the vesicular transporter of GABA (vGAT), suggesting that GABA produced by MIP neurons might not contribute to the obesity phenotype of MIP>E93$_{RNAi}$ flies (Figures S3C and S3D).

Neuronal knockdown of E93 alters locomotor activity and disrupts circadian rhythm.

Metabolic abnormality is often associated with abnormal locomotive activity[47,48]. In mice, *Clock* mutant is obese[49] and high-fat diet disrupts the activity and feeding rhythms[50,51], indicating the reciprocal interaction between biological rhythm and metabolism is conserved in many animals. Studies in *Drosophila* also reveal evolutionarily conserved mechanisms of circadian rhythm controlled by metabolism[52-55]. Therefore, we monitored locomotor activity rhythms of flies with E93 knockdown. When we measured the daytime and nighttime activity of nSyb>E93$_{RNAi}$ 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 (Figure 5A). Similar reduction in activity was observed in MIP>E93$_{RNAi}$ flies, but the reduced activity was less pronounced in MIP>EcR$_{RNAi}$ flies and no obvious reduction in activity was observed in vGAT>E93$_{RNAi}$ flies (Figures 5C, 5D). Interestingly, nighttime activity of vGAT>E93$_{RNAi}$ 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[26,56]. Therefore, the increased nighttime activity observed in vGAT>E93$_{RNAi}$ suggests E93 could play a role in neuronal GABA activity in general to control food intake and promote sleep.

Drosophila melanogaster expresses a bimodal activity pattern under the light and dark cycle with anticipation of lights-on (dawn) or lights-off (dusk) by gradually increasing the activity before lights-on or lights-off. Each anticipation is controlled by different subsets of circadian pacemaker neurons[57-59]. While all the control flies exhibit the anticipatory activities controlled by circadian pacemakers, the anticipations are absent in nSyb>E93 $_{RNAi}$ flies (Figure 5A), suggesting that autonomous circadian clocks in those flies are disrupted. In fact, nSyb>E93 $_{RNAi}$ flies exhibit nearly arrhythmic activity pattern in constant darkness (Figure 6A). The majority of individual flies exhibit arrhythmic behavior and only a few flies exhibit low amplitude of rhythms (1 out of 16 knockdown males, 2 out of 15 knockdown females, Figure S4A). Similar to nSyb>E93 $_{RNAi}$ flies, anticipations for lights-on and off in vGAT>E93$_{RNAi}$, MIP>E93$_{RNAi}$, MIP>EcR$_{RNAi}$ flies were also absent (Figure 5B-D) suggesting that their circadian rhythms are also disrupted. vGAT>E93$_{RNAi}$, MIP>E93$_{RNAi}$, MIP>EcR$_{RNAi}$ flies exhibited dampened circadian rhythms under constant darkness as the

amplitude of the rhythm is much lower than that of the control (Figure 6B-D). Male vGAT>E93$_{RNAi}$ flies exhibited disrupted circadian rhythm (Figure 6B-D). Based on our observations, we suggest that circadian rhythms in vGAT>E93$_{RNAi}$, MIP>E93$_{RNAi}$, MIP>EcR$_{RNAi}$ flies are disrupted, but less severely than that of nSyb>E93 $_{RNAi}$ flies under constant darkness. The overall activity in knockdown flies in constant darkness was reduced, similar to their activity in light and dark conditions.

Together, our results suggest that E93 in the two subsets of neurons, MIP and vGAT, play an essential role in the regulation of metabolism, locomotive activity, and circadian rhythm.

Discussion

In this study, we report a new role of E93, a conserved adult specifier[13], in regulating feeding, metabolism, and biological rhythm. Neuron-specific knockdown of E93 causes multiple behavioral and physiological abnormalities such as incessant feeding, increased energy stores, and disrupted circadian rhythm. From screening of 17 different GAL4 drivers, we identified that E93 function in metabolism and circadian rhythm is primarily through its action in GABA-ergic and MIP neurons, both of which have been implicated in regulation of feeding, metabolism, and sleep[23,25,27,29,56]. Furthermore, we found that, in MIP neurons, E93 partly acts downstream of the ecdysone receptor, EcR. Although both males and females with neuron-specific knockdown of *E93* have increased energy stores, males favored storing excess energy as TAG whereas females stored energy in the form of glycogen, suggesting a sexual dimorphism in how energy is stored. Since glycogen is the major resource accumulated in eggs, there might be an evolutionary advantage for females to store excess energy as glycogen rather than lipid. Increased frequency of feeding and resulting increased food intake is most likely the main cause of obesity in flies with neuron-specific knockdown of *E93.*

Neuro-specific knockdown of *E93* also reduces activity and disrupts circadian rhythm. This is most pronounced when the flies are exposed to constant darkness (Figure 6). Similar circadian disruption was observed in flies with GABA-specific or MIP-specific knockdown of *E93*, supporting E93's role in circadian rhythm related to metabolism. All nSyb>E93$_{RNAi}$, vGAT>E93$_{RNAi}$, MIP>E93$_{RNAi}$ and MIP>EcR$_{RNAi}$ flies failed to show lights-on and off anticipations but exhibited either arrhythmicity or low amplitude of rhythms under constant darkness. It is possible that reduced activity itself causes the circadian phenotype. For instance, the knockdown flies might stay on the side of the DAM tube with food and spend a significantly longer time feeding than the control, resulting in a decrease in the number of beam crosses. We didn't observe any reduction of activity in vGAT>E93$_{RNAi}$ flies, yet they showed disrupted circadian rhythm; males exhibited arrhythmicity in constant darkness similar to nSyb>E93$_{RNAi}$, while females exhibited very low amplitude of rhythm in constant darkness. Therefore, disrupted circadian rhythms in vGAT>E93$_{RNAi}$ flies is independent from reduced activity. This suggests that as least some part of disrupted circadian rhythm of nSyb>E93$_{RNAi}$ is more likely associated with their metabolic abnormalities.

The mammalian homolog of E93 is LCOR (Ligand-dependent Corepressor) and has been shown to interact with nuclear receptors critical for metabolism[60-63]. This suggests that E93 might regulate circadian rhythm directly by interacting with the key nuclear receptors required for the metabolic clock[64,65]. Indeed, the fly orthologs of the key nuclear receptors, E75 (ROR in mammals) and DHR51 (REV-ERB in mammals) have conserved roles in regulating circadian rhythm[66,67].

Although, the detailed neuro-molecular mechanisms by which E93 regulates these pleiotropic metabolic phenotypes require further investigation, we suggest that E93 is the key switch in shaping the adult brain to confer the fittest behavior, metabolism, and physiology in adults. 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 Figure 7. Neuron-specific knockdown of *E93* could disrupt the function of several neuron types including GABA and MIP neurons. Our identification of those two groups as the major sites of action of E93 supports their roles as regulators of adult metabolism and physiology.

Recent study shows that several of the genes enriched in larvae are upregulated during metamorphosis in the E93 deletion mutant[18]. Interestingly, a lot of them are implicated in neural fate determination (e.g. *sqz*, *ko*), metabolism (e.g. *sdr*) and circadian rhythm (e.g. *clk*), supporting the potential role of E93 in transitioning to adult metabolism and physiology. One way by which E93 could control the transition from the larva brain to the adult brain is to establish the adult neuronal circuitry by controlling neurite pruning as its *C. elegans* ortholog does[14]. In fact, the nervous system undergoes dramatic rewiring often via steroid hormones during adolescence in many animals to switch the focus from growth to reproduction[68,69]. *Drosophila* also undergoes intensive remodeling of the nervous system during metamorphosis when E93 plays its essential roles[70].

It is equally possible that E93 continues to function in adult neurons in those two populations to maintain the function of adult neurons. In fact, a few genes that play essential roles during development are repurposed to play roles in adults. For example, bursicon is essential for metamorphosis and also plays a role in energy homeostasis in adults[71]. That ecdysone, the upstream hormone of E93, continues to function in adults to control metabolism depending on reproduction state suggests that E93 could continue to function downstream of the 20E steroid signal to control metabolic homeostasis in adults.

Together, our results provide an insight into how a transcription factor acting in specific sets of neurons in the nervous system mediates steroid hormone signaling to shape and regulate adult metabolism and physiology.

Author contribution

JE, SL and Y-JY planed and supervised the study. CY, SW, SY, Y-JY devised ideas, performed experiments, and analyzed the data. SY and AR provided technical help, CY, SY and Y-JY wrote the manuscript with inputs from SW, SL and JE.

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Figure legends

Figure 1: Neuronal specific knockdown of E93 increases body weight and energy stores

A: The body weight (3-day old) of males and females of nSyb>E93$_{RNAi}$ is increased compared to all controls of RNAi only (none>E93$_{RNAi}$ and none>mCherry$_{RNAi}$) and GAL-4 driver (nSyb>mCherry$_{RNAi}$). Individual data was plotted with the mean ± S.E.M. *** $p < 0.001$ by two-way ANOVA.

B-C: The time course of body weight measurements in males (B) and females (C) shows there is slight (male) or no (female) difference in initial body weight compared to controls (none>E93$_{RNAi}$), but the weight gradually increases in nSyb>E93$_{RNAi}$. Errors are S.E.M. *** $p < 0.001$ by Student *t* test.

D: The representative images of males (D) and females (E) of control (nSyb>mCherry$_{RNAi}$: white eyes) and nSyb>E93$_{RNAi}$ (orange eyes). Scale bar = 1 mm.

E: The level of triglyceride (TAG) is increased in nSyb>E93$_{RNAi}$ males but not in nSyb>E93$_{RNAi}$ females when compared to controls (nSyb>mCherry$_{RNAi}$).

F: The level of glycogen is increased in nSyb>E93$_{RNAi}$ females but not in nSyb>E93$_{RNAi}$ males when compared to controls (nSyb>mCherry$_{RNAi}$).

E-F: Individual data was plotted with the mean ± S.E.M. * $p < 0.05$, ** $p < 0.005$, ns = not significant by Student *t* test.

Figure 2: Neuronal specific knockdown of E93 increases feeding frequency, food intake and starvation survival

A-B: nSyb>E93$_{RNAi}$ flies are more attracted to food (**A**) and show a higher feeding frequency than the control (nSyb>mCherry$_{RNAi}$) (**B**). Individual data was plotted with the mean ± S.E.M.

C: nSyb>E93$_{RNAi}$ flies constantly feed as indirectly measured by % extended proboscis. N=144-187 males, 83-100 females

D: The images of proboscises in each state of closed, half-open and fully open. Scale bar = 100 μm

E: The % of unexpanded wing of nSyb>E93$_{RNAi}$ flies. N= 82 males, 113 females

F: nSyb>E93$_{RNAi}$ flies increase food intake. After 10 min of feeding, the flies were homogenized, and the food intake was measured by absorbance of the blue dye of 630 λ. Control is none>E93$_{RNAi}$.
Individual data was plotted with the mean ± S.E.M.

G-H: Both males (G) and females (H) of nSyb>E93$_{RNAi}$ survive starvation better than the all three controls.

** $p < 0.005$, *** $p < 0.001$, ns = not significant by Student *t* test.

Figure 3: The metabolic changes in neuro-specific knockdown of E93 are mediated by E93 function in GABA and MIP neurons

A: Glycogen levels after knockdown of E93 in 17 subsets of neurons in females indicates MIP-producing and GABA-ergic neurons most significantly contribute to the increase of glycogen. **nSyb**: pan-neuronal, **MIP**: Myoinhibitory, **Akh**: Adipokinetic, **vGlut**: Glutamatergic, **ChAT**: Cholinergic, **vGAT**: GABAergic, **Tdc**: Octopaminergic/ tyraminergic, **OK107**: mushroom body, **Trh:** Serotonergic, **LK:** Leucokinin-producing, **Phm:** ecdysone producing cells of the prothoracic glands, **Eth:** Ecdysis triggering hormone-producing, **NPF:** Neuropeptide F-producing, **Dilp2:** Insulin-like peptide-producing, **FMRFa:** FMRFamide peptide-producing, **TK:** Tachykinin-producing, **Ple:** Dopaminergic, **SiFa:** SIFamide-producing. The red-colored bars indicate a significant increase in glycogen level and the blue bar indicates significant decrease. Errors are S.E.M. Control is each Gal4 driver with $mCherry_{RNAi}$.

B-D: vGAT>$E93_{RNAi}$ phenocopies nSyb>$E93_{RNAi}$ in increased body weight (B), obese appearance (C) and high feeding frequency (D). Control is vGAT>$mCherry_{RNAi}$. Individual data was plotted with the mean ± S.E.M.

C: Scale bar = 1 mm.

E-G: MIP>$E93_{RNAi}$ phenocopies nSyb>$E93_{RNAi}$ in increased body weight (E), obese appearance (F) and high feeding frequency (G). Control is MIP>$mCherry_{RNAi}$. Individual data was plotted with the mean ± S.E.M.

* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ by Student *t* test.

Figure 4: E93 acts in MIP neurons downstream of ecdysone receptor.

A: The level of MIP mRNA level is reduced in nSyb>E93$_{RNAi}$ compared to controls (none>E93$_{RNAi}$). Each data point represents 10 female heads. The graph is a representative of two independent experiments.

B-C: MIP>GAL80 partially but significantly reverses the increased body weight phenotype of nSyb>E93$_{RNAi}$. Individual data was plotted with the mean ± S.E.M. ** $p < 0.005$, *** $p < 0.001$ by Two-way ANOVA. C: Scale bar = 1 mm.

D-F: MIP>EcR$_{RNAi}$ phenocopies MIP>E93$_{RNAi}$ in increased body weight (D) and obese appearance (E), but not in feeding frequency (F). Control is MIP>mCherry$_{RNAi}$. Individual data was plotted with the mean ± S.E.M. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, ns = not significant by Student *t* test.

Figure 5: Knockdown of E93 attenuates circadian controlled morning and evening anticipation.

Beam braking activity was recorded every 6 min from individual fly with DAM assay system under 12 h light and 12 h dark condition. Group average double plotted actograms were generated with 30 min bin 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 in each sex (males (♂): top, females (♀): bottom). White background indicates light and gray background indicates dark.

A: Knockdown of E93 expression pan-neuronally using nSyb>GAL4 driver. nSyb>mCherry$_{RNAi}$ males (N=27, 2 cohorts), nSyb>mCherry$_{RNAi}$ females (N=29, 2 cohorts), nSyb>E93$_{RNAi}$ males (N=31, 2 cohorts), nSyb>E93$_{RNAi}$ females (N=31, 2 cohorts)

B: Knock down of E93 expression in GABA-ergic neurons. vGAT>mCherry$_{RNAi}$ males (N=39, 3 cohorts), vGAT>mCherry$_{RNAi}$ females (N=73, 5 cohorts), vGAT>E93$_{RNAi}$ males (N=45, 3 cohorts), vGAT>E93$_{RNAi}$ females (N=64, 5 cohorts)

C: Knock down of E93 expression in MIP neurons. MIP> mCherry$_{RNAi}$ males (N=31, 2 cohorts), MIP> mCherry$_{RNAi}$ females (N=32 2 cohorts), MIP> E93$_{RNAi}$ males (N=32, 2 cohorts), MIP> E93$_{RNAi}$ females (N=32, 2 cohorts)

D: Knock down of EcR expression in MIP neurons. MIP> mCherry$_{RNAi}$ males (N=32, 2 cohorts), MIP> mCherry$_{RNAi}$ females (N=28, 2 cohorts), MIP>EcR$_{RNAi}$ males (N=30, 2 cohorts), MIP>EcR$_{RNAi}$ females (N=32, 2 cohorts)

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 hour light and 12 hour dark condition were placed in DAM during 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: Knockdown of E93 expression pan-neuronally using nSyb>GAL4 driver. nSyb>mCherry$_{RNAi}$ males (N=16, 1 cohort), nSyb>mCherry$_{RNAi}$ females (N=15, 1 cohort), nSyb>E93$_{RNAi}$ males (N=16, 1 cohort), nSyb>E93$_{RNAi}$ females (N=15, 1 cohort)

B: Knock down of E93 expression in GABA-ergic neurons. vGAT>mCherry$_{RNAi}$ males (N=26, 2 cohorts), vGAT>mCherry$_{RNAi}$ females (N=39, 3 cohorts), vGAT>E93$_{RNAi}$ males (N=31, 2 cohorts), vGAT>E93$_{RNAi}$ females (N=44, 3 cohorts)

C: Knock down of E93 expression in MIP neurons. MIP> mCherry$_{RNAi}$ males (N=31, 2 cohorts), MIP> mCherry$_{RNAi}$ females (N=31 2 cohorts), MIP> E93$_{RNAi}$ males (N=32, 2 cohorts), MIP> E93$_{RNAi}$ females (N=31, 2 cohorts)

D: Knock down of EcR expression in MIP neurons. MIP> mCherry$_{RNAi}$ males (N=31, 2 cohorts), MIP> mCherry$_{RNAi}$ females (N=31, 2 cohorts), MIP>EcR$_{RNAi}$ males (N=30, 2 cohorts), MIP>EcR$_{RNAi}$ females (N=32, 2 cohorts)

Other conditions are the same with actograms in Figure 5.

Figure 7: Model

E93 conveys a steroid signal to shape the nervous system fitting to adult metabolism and physiology.

Figure 1

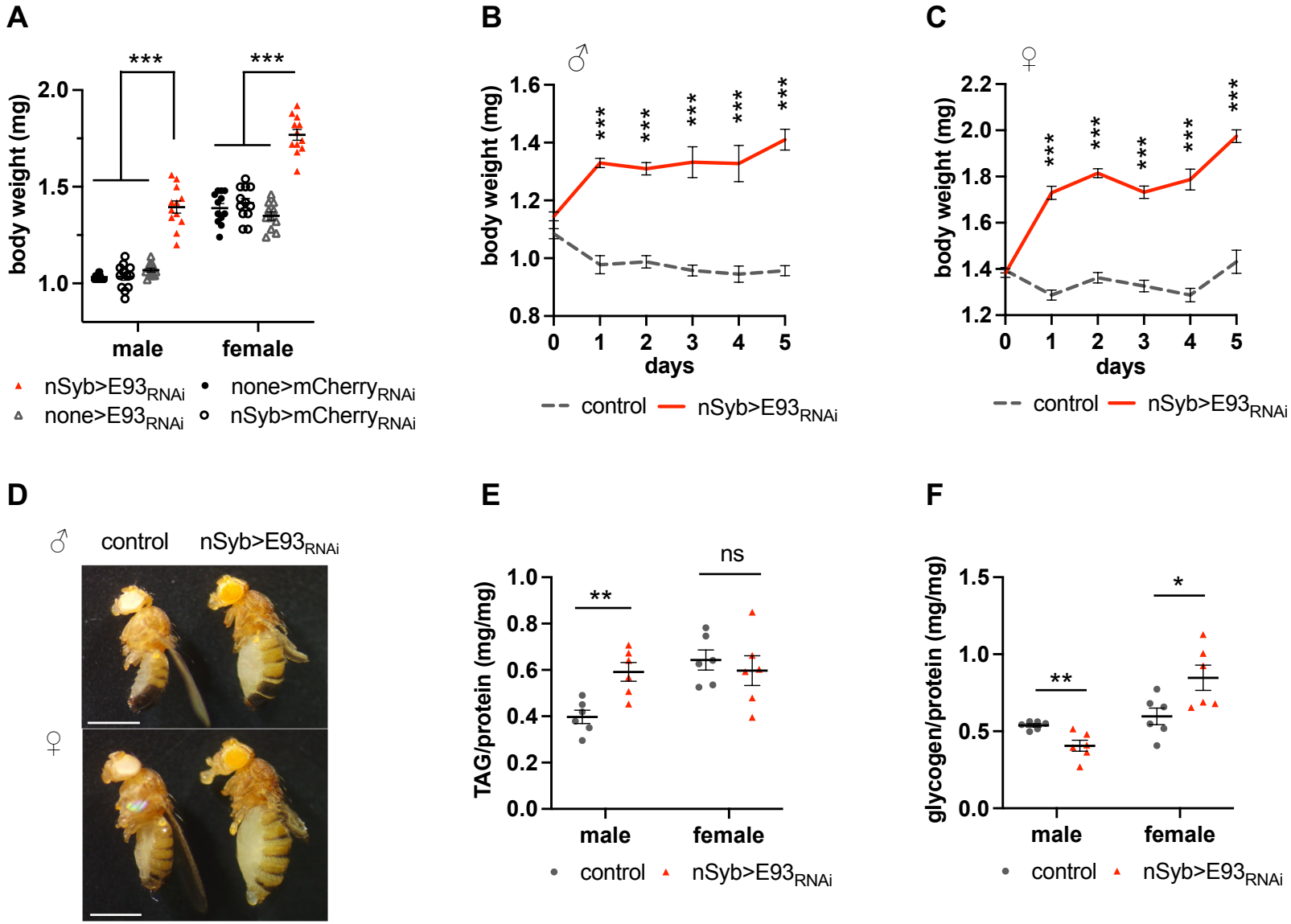

A
***
***
body weight (mg)
2.0
1.5
1.0
male
female
nSyb>E93RNAi
none>E93RNAi
none>mCherryRNAi
nSyb>mCherryRNAi
B
♂
body weight (mg)
1.6
1.4
1.2
1.0
0.8
0
1
2
3
4
5
days
control
nSyb>E93RNAi
C
♀
body weight (mg)
2.2
2.0
1.8
1.6
1.4
1.2
days
control
nSyb>E93RNAi
D
♂
control
nSyb>E93RNAi
♀
E
ns
**
TAG/protein (mg/mg)
1.0
0.8
0.6
0.4
0.2
0.0
male
female
control
nSyb>E93RNAi
F
*
**
glycogen/protein (mg/mg)
1.5
1.0
0.5
0.0
male
female
control
nSyb>E93RNAi

Figure 2

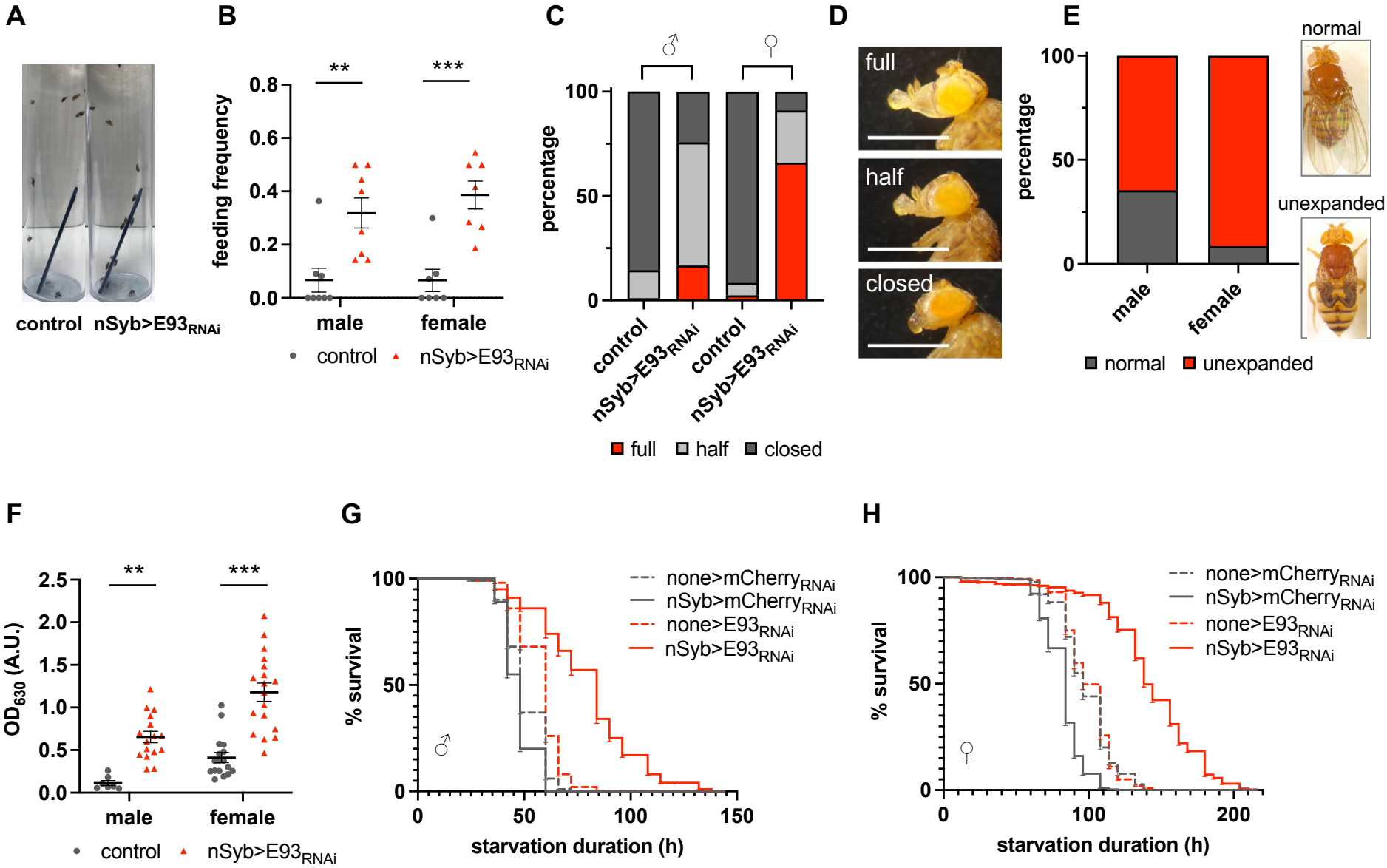

A
control
nSyb>E93RNAi
B
feeding frequency
0.8
0.6
0.4
0.2
0.0
**
***
male
female
control
nSyb>E93RNAi
C
♂
♀
percentage
100
50
0
control
nSyb>E93RNAi
control
nSyb>E93RNAi
full
half
closed
D
full
half
closed
E
normal
unexpanded
percentage
100
50
0
male
female
normal
unexpanded
F
OD630 (A.U.)
2.5
2.0
1.5
1.0
0.5
0.0
**
***
male
female
control
nSyb>E93RNAi
G
% survival
100
50
0
♂
0
50
100
150
starvation duration (h)
none>mCherryRNAi
nSyb>mCherryRNAi
none>E93RNAi
nSyb>E93RNAi
H
% survival
100
50
0
♀
0
100
200
starvation duration (h)
none>mCherryRNAi
nSyb>mCherryRNAi
none>E93RNAi
nSyb>E93RNAi

Figure 3

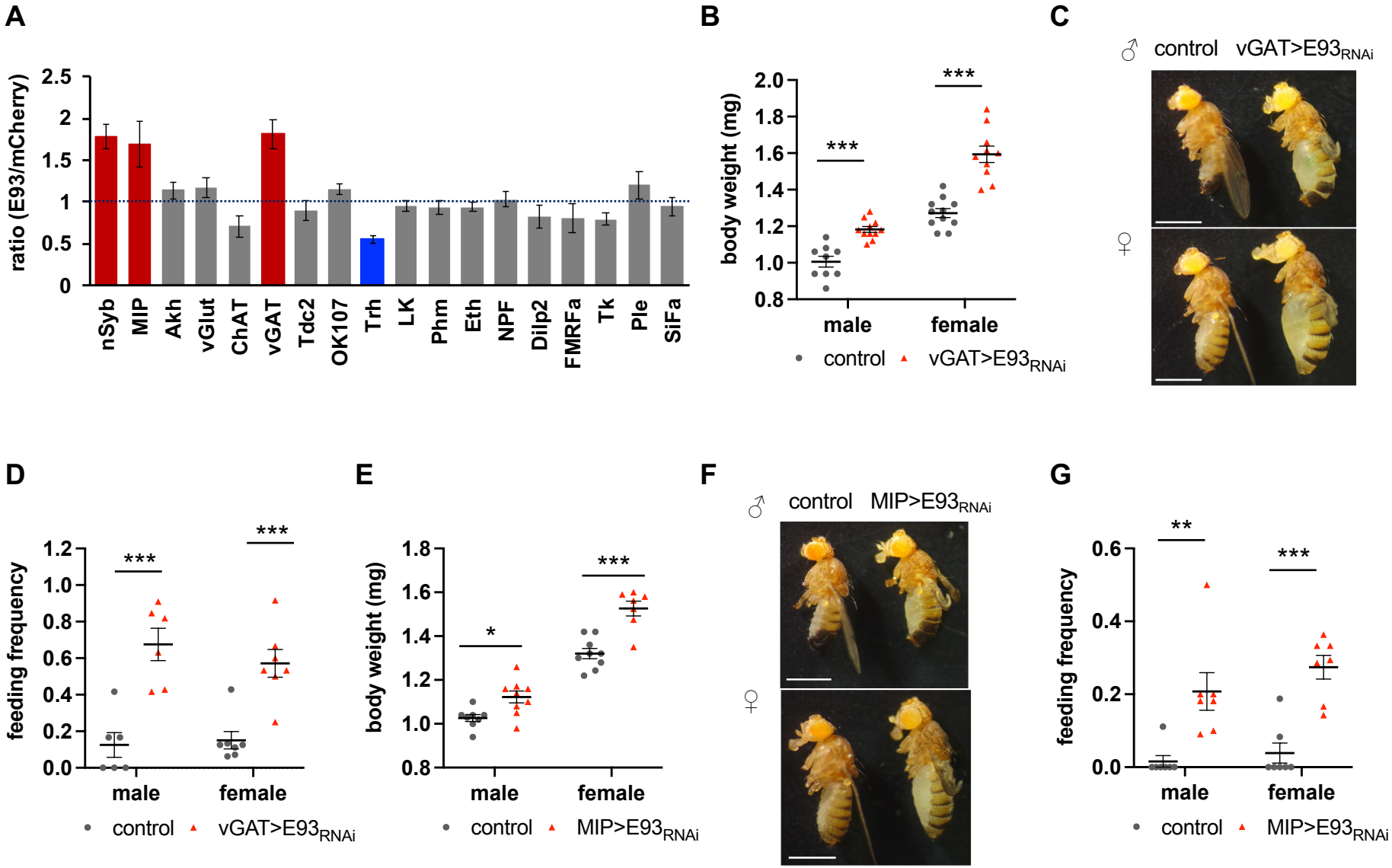

A
ratio (E93/mCherry)
2.5
2
1.5
1
0.5
0
nSyb
MIP
Akh
vGlut
ChAT
vGAT
Tdc2
OK107
Trh
LK
Phm
Eth
NPF
Dilp2
FMRFa
Tk
Ple
SiFa
B
body weight (mg)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
***
***
male
female
control
vGAT>E93RNAi
C
♂ control vGAT>E93RNAi
♀
D
feeding frequency
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
male
female
control
vGAT>E93RNAi
E
body weight (mg)
1.8
1.6
1.4
1.2
1.0
0.8
*
***
male
female
control
MIP>E93RNAi
F
♂ control MIP>E93RNAi
♀
G
feeding frequency
0.6
0.4
0.2
0.0
**
***
male
female
control
MIP>E93RNAi

Figure 4

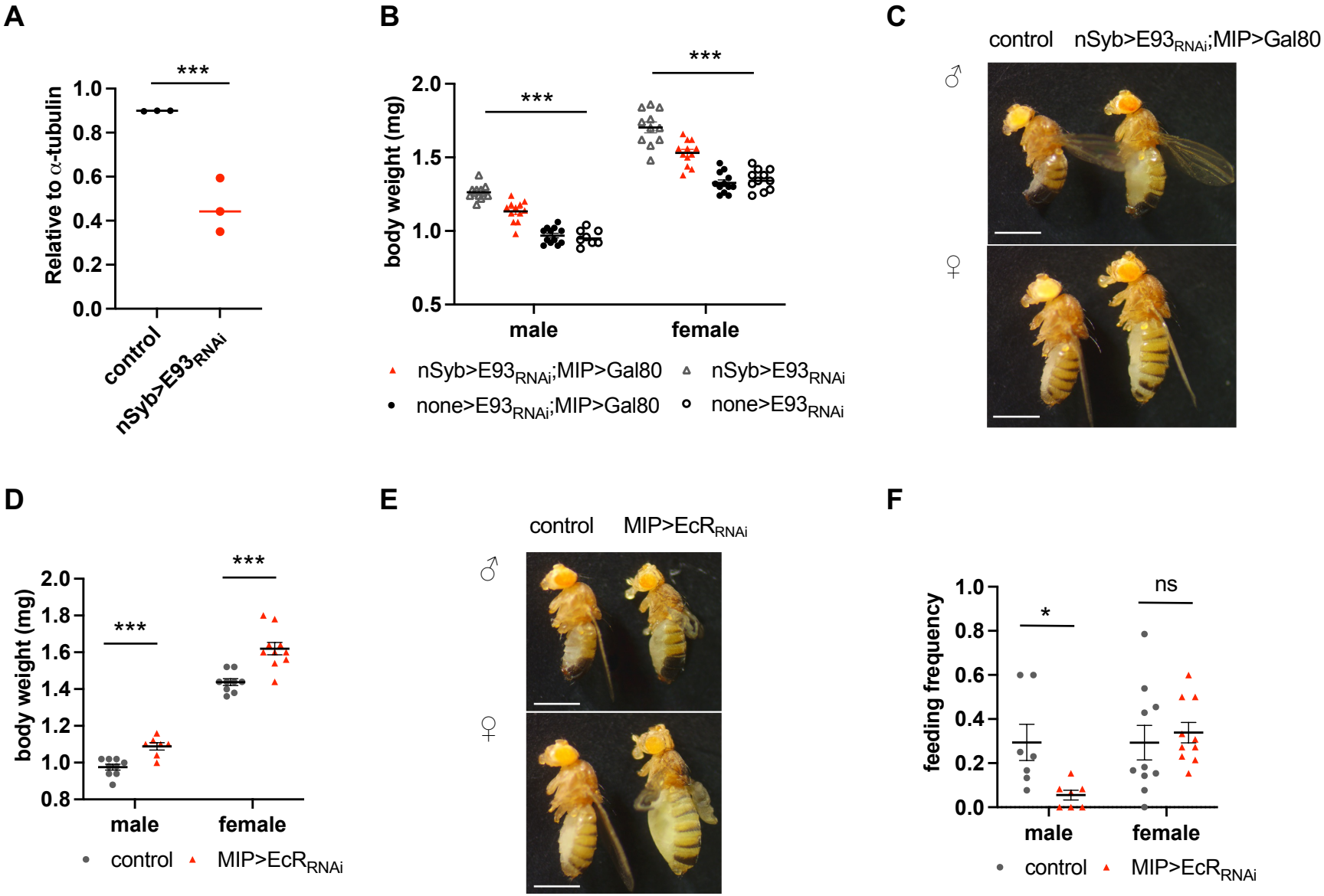

A
Relative to α-tubulin
1.0
0.8
0.6
0.4
0.2
0.0
***
control
nSyb>E93RNAi
B
body weight (mg)
2.0
1.5
1.0
0.5
***
***
male
female
nSyb>E93RNAi;MIP>Gal80
nSyb>E93RNAi
none>E93RNAi;MIP>Gal80
none>E93RNAi
C
control
nSyb>E93RNAi;MIP>Gal80
♂
♀
D
body weight (mg)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
***
***
male
female
control
MIP>EcRRNAi
E
control
MIP>EcRRNAi
♂
♀
F
feeding frequency
1.0
0.8
0.6
0.4
0.2
0.0
*
ns
male
female
control
MIP>EcRRNAi

Figure 5

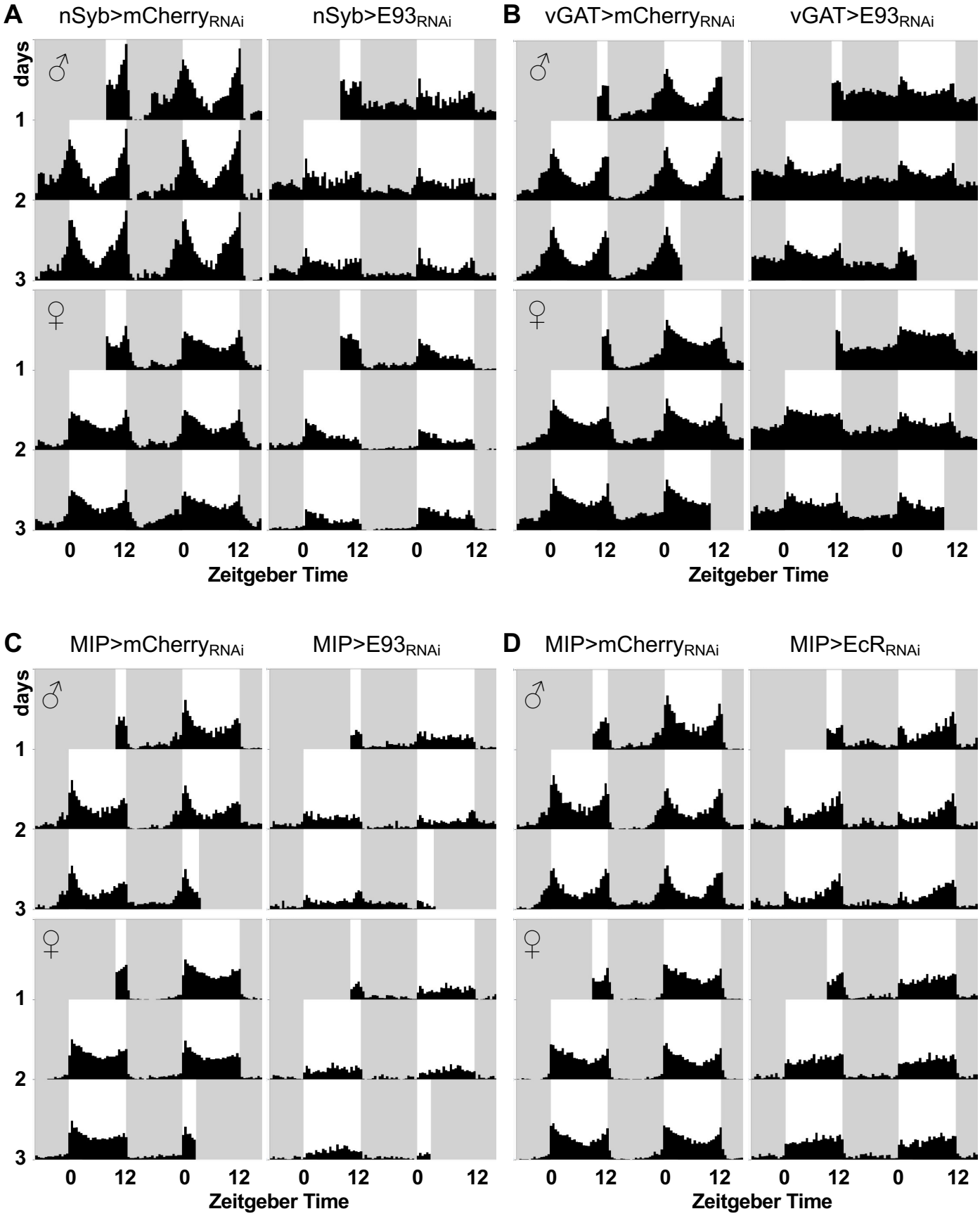

A
nSyb>mCherry$_{RNAi}$
nSyb>E93$_{RNAi}$
B
vGAT>mCherry$_{RNAi}$
vGAT>E93$_{RNAi}$
C
MIP>mCherry$_{RNAi}$
MIP>E93$_{RNAi}$
D
MIP>mCherry$_{RNAi}$
MIP>EcR$_{RNAi}$
days
1
2
3
0
12
Zeitgeber Time

Figure 6

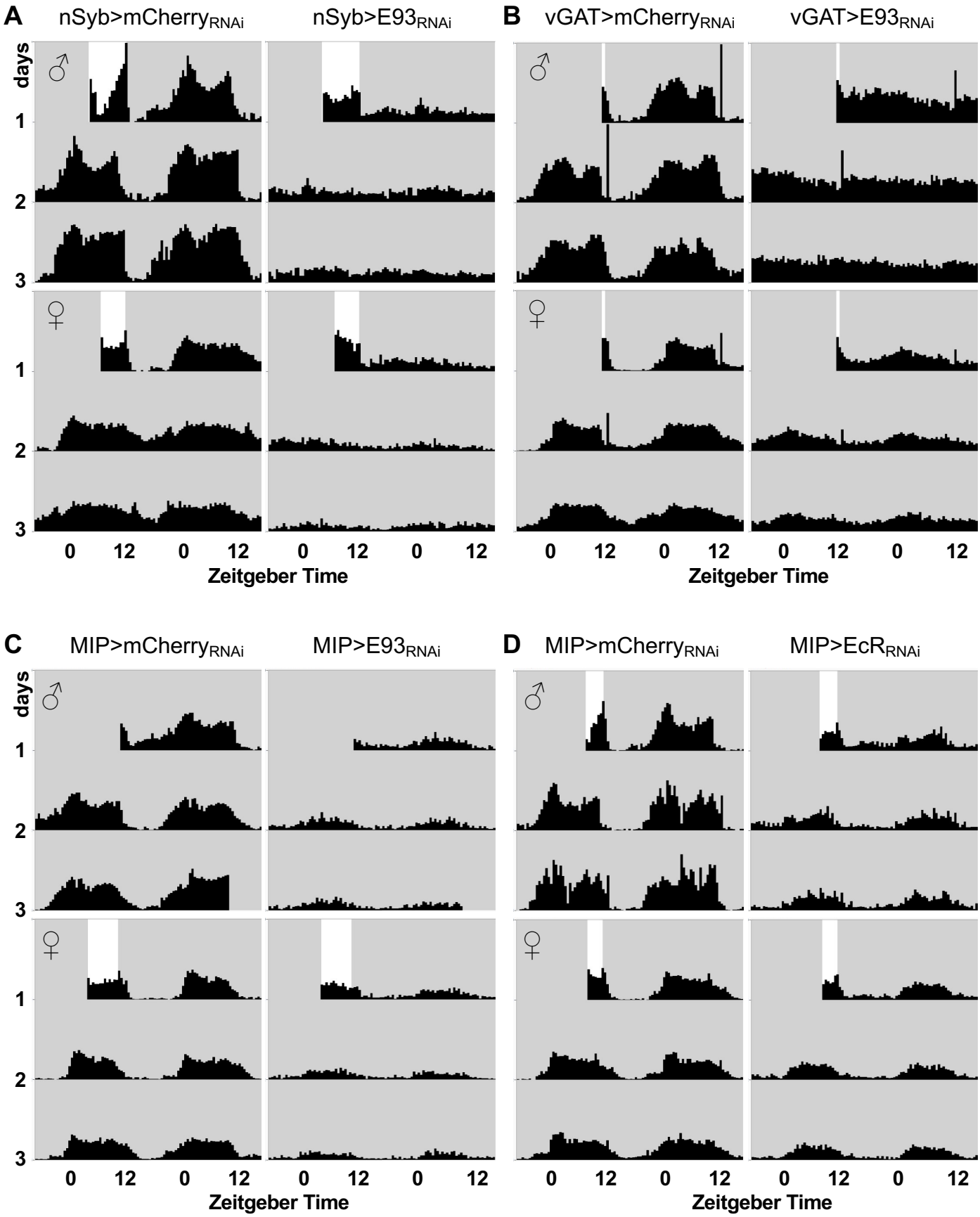
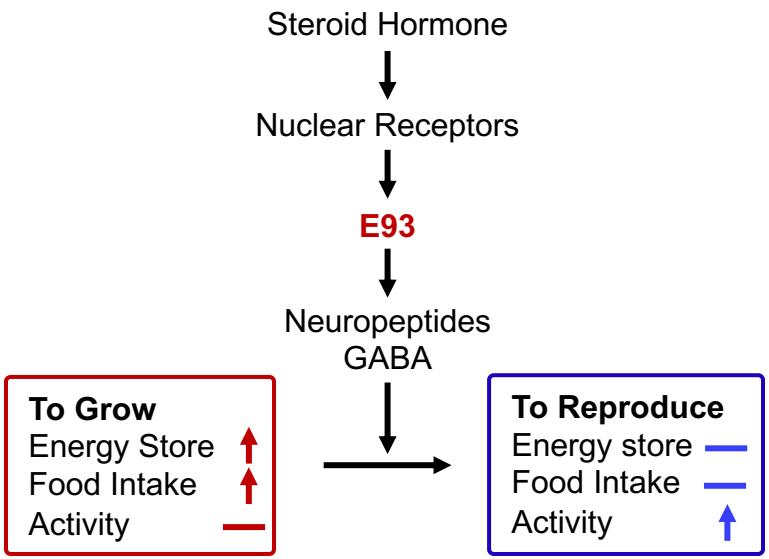

A
nSyb>mCherry$_{RNAi}$
nSyb>E93$_{RNAi}$
B
vGAT>mCherry$_{RNAi}$
vGAT>E93$_{RNAi}$
C
MIP>mCherry$_{RNAi}$
MIP>E93$_{RNAi}$
D
MIP>mCherry$_{RNAi}$
MIP>EcR$_{RNAi}$
days
♂
♀
1
2
3
0
12
Zeitgeber Time

Figure 7


Steroid Hormone
Nuclear Receptors
E93
Neuropeptides
GABA
To Grow
Energy Store
Food Intake
Activity
To Reproduce
Energy store
Food Intake
Activity