

Octopamine integrates the status of internal energy supply into the formation of food-related memories

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Abstract Summary

The brain regulates food intake in response to internal energy demands and food availability. However, can internal energy storage influence the type of memory that is formed? We show that the duration of starvation determines whether *Drosophila melanogaster* forms appetitive short-term or longer-lasting intermediate memories. The internal glycogen storage in the muscles and adipose tissue influences how intensely sucrose associated information is stored. Insulin-like signaling in octopaminergic reward neurons integrates internal energy storage into memory formation. Octopamine, in turn, suppresses the formation of long-term memory. Octopamine is not required for short-term memory, because octopamine-deficient mutants can form appetitive short-term memory for sucrose and to other nutrients depending on the internal energy status. The reduced positive reinforcing effect of sucrose at high internal glycogen levels combined with the increased stability of food-related memories due to prolonged periods of starvation could lead to increased food intake.

eLife assessment

This **important** study dissects the role of octopamine in the interplay between internal energy homeostasis, food intake and food-related memories. The **solid** experimental evidence will shed additional light on previously published work and should be of interest to the growing community of biologists interested in how internal state shapes behavior, including decision making processes, learning and memory.

Introduction

To ensure survival, the internal energy status of an animal needs to be adjusted to the energy expenditure of the organism and the availability of external food. Increased storage of energy correlates with increased food intake in the past. Dysregulation of food intake can lead to diseases such as obesity and diabetes. Sucrose is a carbohydrate enriched in Western diets, and the breakdown product of sucrose - glucose - can be stored in the organism as glycogen, primarily in

the liver and muscles. Elevated glycogen levels are a hallmark of glycogen storage diseases that are accompanied by defects in the liver, muscles and brain (Ellingwood and Cheng, 2018 [↗](#)). Similar to vertebrates, the fruit fly *Drosophila melanogaster* uses glucose as its primary energy source and stores glycogen primarily in the muscles—the main site of energy expenditure—and the fat body—the equivalent of the vertebrate liver (Galikova and Klepsatel, 2023 [↗](#); Wigglesworth, 1949 [↗](#)). As in vertebrates, glycogen levels are also found in the brain (Yamada et al., 2018 [↗](#)).

In *Drosophila*, the monoamine octopamine - functionally related to noradrenalin in vertebrates - is involved in the regulation of energy homeostasis. In tyramine- β -hydroxylase (*T β h*) mutants lacking the neurotransmitter octopamine, the glucose and trehalose concentrations in the hemolymph change less upon starvation, and the life span is extended (Damrau et al., 2017 [↗](#); Li et al., 2016 [↗](#)). *T β h* mutants show an increased threshold to respond to sucrose with the extension of their proboscis when sucrose is offered to their tarsi – a structure that contains gustatory receptor neurons. The reduced responsiveness to sucrose correlates with a reduced sucrose intake in *T β h* mutants (Li et al., 2016 [↗](#); Scheiner et al., 2014 [↗](#)). Octopaminergic neurons potentiate the response of sugar-sensing gustatory receptor neurons in satiated flies, suggesting that octopaminergic neurons regulate feeding behavior by changing the sensitivity of taste receptor neurons (Youn et al., 2018 [↗](#)). The reduced responsiveness to sucrose is also thought to be responsible for defects observed in habituation in *T β h* mutants (Scheiner et al., 2014 [↗](#)). In addition to their changes in simple forms of neuronal plasticity, *T β h* mutants fail to form a positive association with a food reward in a classical olfactory conditioning paradigm. Flies quickly learn to associate an odorant with sucrose, and *T β h* mutants do not show this positive association directly after learning (Schwaerzel et al., 2003 [↗](#)). However, when a longer time after training has elapsed, memory appears (Das et al., 2014 [↗](#)). The release of octopamine mediates the reinforcing effect of sweet taste in short-term memory and the anesthesia-resistant form of long-term memory (Burke et al., 2012 [↗](#); Huetteroth et al., 2015 [↗](#); Wu et al., 2013 [↗](#)). The question arises how the internal energy supply is integrated into the formation of food related memories and feeding behavior in *T β h* mutants.

The peptide hormone insulin regulates glucose homeostasis at the cellular level (Saltiel and Kahn, 2001 [↗](#)). Insulin and its *Drosophila* counterpart, insulin-like peptides, perform their functions through well-conserved signal transduction cascades (Chatterjee and Perrimon, 2021 [↗](#); Inoue et al., 2018 [↗](#)). In vertebrates, in addition to its function in fat tissue and muscles, the insulin receptor is broadly expressed in the brain and regulates neuronal plasticity (Nakai et al., 2022 [↗](#)). For example, in rats, reduced insulin receptor function in the hypothalamus results in loss of long-term potentiation and impaired spatial memory (Grillo et al., 2015 [↗](#)). In *Drosophila*, the insulin receptor substrate Chico is required for the development of mushroom bodies, a brain region required for learning and memory, and loss of Chico results in learning defects (Naganos et al., 2012 [↗](#)). Disrupting the function of the insulin receptor in the mushroom bodies also results in learning defects, whereas the insulin receptor and Chico are required in the ellipsoid body for protein biosynthesis-dependent long-term memory (Chambers et al., 2015 [↗](#)). While there is evidence that insulin receptor signaling regulates cellular physiology, it is not clear how the internal energy supply of the animal influences the formation of food-related memories.

To analyze the relationship between energy status, the evaluation of a food reward and the formation of memory and food intake, we used the olfactory associative learning and memory paradigm. Using nutrients as a positive reward allows for investigating how the internal energy status influences the evaluation of the reinforcer and in turn learning and memory. As a step to understand the interconnection between reward evaluation, nutrient intake and the formation of food-related memories, we analyzed the function of octopamine in the regulation of internal energy homeostasis, learning and memory and food intake.

Results

Starvation increases learning and memory performance when using a food-related reward (Colomb et al., 2009). However, how the internal energy status is integrated into the evaluation of the reward and the stability of food-related memories and whether there is a correlation between the perceptions of the food reward, food-related memories and food intake are not clear.

The duration of hunger influences the strength and stability of memories

To investigate whether changes in internal energy storage influence food-related memories, we used the olfactory associative learning and memory paradigm using sucrose as a food-related reinforcer (Schwaerzel et al., 2003). In the paradigm, flies learn to associate an odorant with sucrose and are later tested on whether they remember the association. A prerequisite for this association to be made is that the animals are hungry. During the training, starved flies were exposed for 2 min to the unconditioned odorant, 4-methylcyclohexanol (MCH), followed by a 2 min exposure to a second odorant, 3-octanol (3-OCT), that was paired with the reward of 2 M sucrose. After the training, flies were given a choice between the two odorants (Figure 1A). Normally, flies prefer the rewarded odorant already 2 min after the training. To avoid a bias towards one odorant, in a reciprocal experiment, the first odorant was rewarded. The performance index presents the average positive association between the reward and both odorants and describes whether information is learnt and remembered. To ensure that observed differences in learning and memory were not due to changes in odorant perception, odorant evaluation or sucrose sensitivity, different fly populations of the same genotypes were tested for their odorant acuity, odorant and sucrose preference (Table S1). The flies of the different genotypes sensed the odorants and evaluated them as similar salient in comparison. This is important to avoid a bias where flies have to choose between the two odorants after training. They also sensed sucrose. We next determined whether differences in sucrose preference influence sucrose intake during training. Therefore, we measured the intake of food-colored sucrose of starved flies in the behavioral set up. After 2 min, we evaluated whether there was dyed food in the abdomen of the fly (Table S1). Flies of both genotypes fed sucrose within 2 min and there was no difference between w^{1118} and $T\beta h^{nM18}$ flies.

To alter the internal energy status, flies were starved either for 16 h or for 40 h before the training (Figure 1C). We used 3- to 5-day-old male flies to minimize differences in body weight and to control for differences in food preferences. We included $T\beta h^{nM18}$ mutants lacking octopamine in the experiments, since they showed defects in energy metabolism and sucrose reward learning (Li et al., 2016; Schwaerzel et al., 2003). Flies that were starved for 16 h established a positive association between the odorant and the reward. They remembered this association for 2 min, but not for 6 h or 24 h. Consistent with previous results, learning and memory performance was significantly improved by prolonged starvation (Colomb et al., 2009). The memory was also remembered for longer, since it was still detectable 6 h after training. The $T\beta h^{nM18}$ mutants starved for 16 h did not show 2 min memory but emerged memory 6 h later that lasted up to 24 h (Das et al., 2014; Schwaerzel et al., 2003). In contrast, $T\beta h^{nM18}$ mutants remembered the rewarded odorant after only 2 min following starvation extended to 40 h. The memory was still detectable after 6 h and 24 h (Figure 1B). We repeated similar experiments using a lower concentration of 0.15 M sucrose as reinforcer (Figure 1C). In contrast to the experiments using 2 M sucrose as a reward, 16 h starved $T\beta h^{nM18}$ mutants form a negative association with 0.15 M sucrose directly after the training, that turns into a positive association when the flies were starved longer 40 h. Again, prolonged starvation significantly increased learning and memory performance in controls and $T\beta h^{nM18}$ mutants. Thus, the length of starvation and the reinforcer strength influence appetitive memory strength.

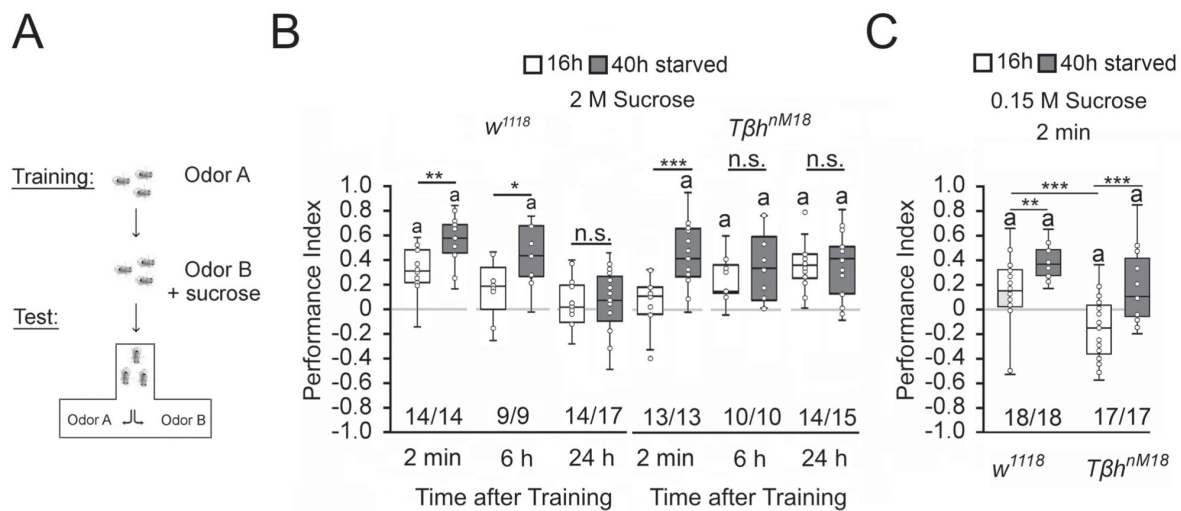


Figure 1.

Starvation influences the strength of memory

A, Appetitive olfactory learning and memory paradigm. **B**, Appetitive 2 min STM, 6 h and 24 h memory of *w¹¹¹⁸* or *Tβh^{NM18}* that were starved either for 16 h (white bars) or 40 h (dark gray bars) before the training. 2 M sucrose was used as reward. **C**, 0.15 M sucrose was used as reinforcer. Prolonged starvation increases memory performance. Prolonged starvation from 16 h to 40 h leads to the formation of STM in *Tβh^{NM18}*. Independent of the duration of starvation, 6 h after training, memory appeared in the mutants. **C**, *Tβh^{NM18}* mutants starved for 16 h developed an aversive STM memory to 0.15 M sucrose. After 40 h of starvation, the mutants developed an appetitive STM. Numbers below box plots indicate pairs of reciprocally trained independent groups of male flies. The letter "a" marks a significant difference from random choice as determined by a one-sample sign test ($P < 0.05$). The Student's t tests were used to determine differences between two groups. For differences between more than two groups one-way ANOVA with Tukey's post hoc HSD test was used. ($P^* < 0.05$; $P^{**} < 0.01$; $P^{***} < 0.001$).

Next, we investigated how starvation affects the stability of memory. Protein synthesis-dependent long-term memory is still labile immediately after training and can be blocked by 4°C cold-shock anesthesia immediately after training. After consolidation, long-term memory is cold shock resistant and insensitive to a cold shock 1 h before the test (Krashes and Waddell, 2008). To investigate how hunger influences the stability of food-related memories, we administered cold shock anesthesia immediately after the training and 1 h before the test and analyzed the effect on memory formation in differently starved control and *Tβh^{nM18}* flies (Figure 2A). We reduced the time for the memory test after training from 6 h to 3 h to be able to detect memory in 16 h starved flies. The memory was completely abolished by a cold shock directly after training or shortly before the test. Prolonging the starvation period resulted in memory that was sensitive to cold-shock directly after training but not shortly before the test. In contrast, 16 h starved *Tβh^{nM18}* mutants developed memory that was sensitive to cold shock directly after training and cold shock insensitive shortly before the test. The results together with the observed memory 24 h later of *Tβh^{nM18}* after the same training (Figure 1B) support that *Tβh^{nM18}* mutant develop long-term memory. Longer periods of starvation in *Tβh^{nM18}* mutants resulted in anesthesia-resistant long-term memory.

To further confirm that the observed memory in 16 h starved *Tβh^{nM18}* is indeed long-term memory, we wanted selectively inhibit long-term memory in *Tβh^{nM18}* mutants and analyze their food-related memory (Figure 2B). We analyzed memory performance 6 h after training, as control flies show no memory at this time point (Figure 1B, Figure 2B). The R15A04-Gal4 driver targets dopaminergic neurons of the PAM cluster, which are specifically required for appetitive long-term memory but not short-term memory (Yamagata et al., 2015). Blocking the function of these dopaminergic neurons immediately after training using a temperature-sensitive *shibire* transgene (UAS-shi^{ts}) and a 30-min heat pulse of 31°C resulted in loss of memory in *Tβh^{nM18}* mutants (Figure 2B). Control flies carrying one copy of the Gal4 transgene showed no memory. Without heat shock, the *Tβh^{nM18}* mutants carrying the UAS-shi^{ts}, the R15A04-Gal4 or both transgenes developed memory (Figure S1). Thus, although they do not show memory immediately after training, *Tβh^{nM18}* mutants that were briefly starved develop long-term memory.

These results, together with the course of memory loss, show that lightly hungry flies form a memory that lasts up to 3 h and is sensitive to anesthesia (Figure 2D). Since no memory can be observed 24 h later, this indicates that it is an anesthesia-sensitive intermediate memory (ITM). Prolonged starvation results in anesthesia-resistant ITM (Figure 2D). In contrast, mildly starved *Tβh^{nM18}* mutants form a long-term memory that is sensitive to anesthesia-sensitive and detectable 24 h after the training. Prolonged starvation in *Tβh^{nM18}* mutants leads to anesthesia-resistant long-term memory (Figure 2D).

Thus, the longer the starvation lasts, the more stable the memory becomes. Depending on the duration of starvation after the same training phase, the animals first form an STM memory, then anesthesia-sensitive or insensitive intermediate memory. Mildly starved *Tβh^{nM18}* mutants do not form a short-term memory, but a long-term memory and after prolonged starvation, short-term and anesthesia resistant memory.

Octopamine is a negative regulator of memory

Since the *Tβh^{nM18}* mutants lack the neurotransmitter octopamine and form LTM, we next investigated whether octopamine normally suppresses LTM. To do so, we first blocked the function of octopamine receptors in controls immediately after the training by feeding the octopamine antagonist epinastin for 1 h and analyzed memory 5 h later (Figure 3A). If the octopamine receptor function is required for long-term memory, prolonged memory should also occur in control flies that normally show no memory after 16 hours of starvation. This was the case. Consistent with the idea that octopamine is a negative regulator of long-term memory, feeding octopamine to *Tβh^{nM18}* mutants immediately after training blocked long-term memory (Figure

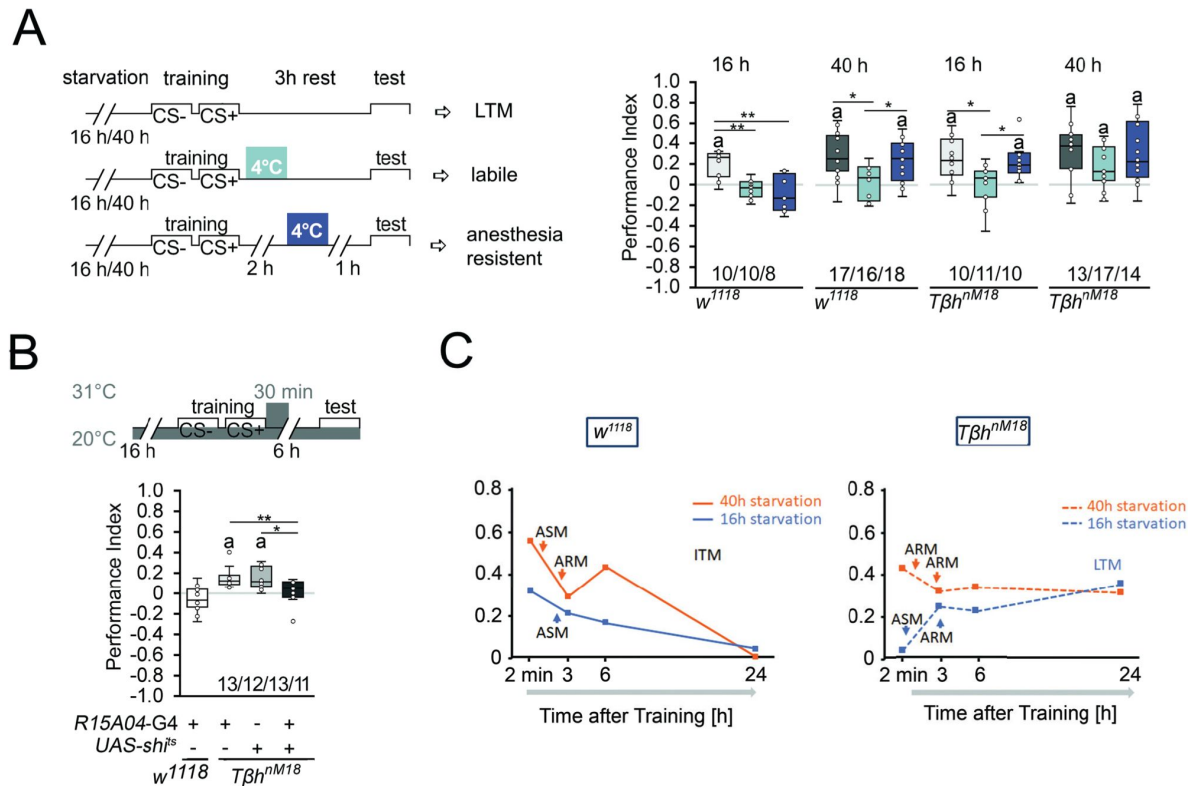


Figure 2.

Starvation influences the type of memory

A, Appetitive STM training with 2 M sucrose and cold shock directly or 2 h after training. Mildly starved control flies exhibit an appetitive memory sensitive to cold shock 3 h after training. Severely starved control flies develop a memory that is initially sensitive to cold shock, but becomes insensitive after 2 h. This phenotype is shared with mildly starved $T\beta h^{nM18}$ mutants. Prolonged starvation in the mutants shifts memory to cold shock insensitive memory. **B**, To block neuronal activity and the formation of LTM, a 30-min heat shift was applied immediately after training to flies expressing a temperature-sensitive *shibire* transgene under the control of the R1504-Gal4 driver. The block results in $T\beta h^{nM18}$ mutants losing LTM. Numbers below box plots indicate pairs of reciprocally trained independent groups of male flies. The letter “a” marks a significant difference from random choice as determined by a one-sample sign test ($P < 0.05$). One-way ANOVA with Tukey’s post hoc HSD test or for data in **A**, and Kruskal-Wallis followed by post-hoc Dunn’s test and Bonferroni correction was used to determine differences in **B**. ($P^* < 0.05$; $P^{**} < 0.01$). **C**, Model summarizing memory performance of control and mutant flies, that were either starved for 16 h (blue line) or 40 h (orange line). The dots present the average of the data presented in **Figure 1** and **Figure 2**. Anesthesia sensitive memory (ASM), anesthesia resistant memory (ARM), intermediate memory (ITM) and long-term memory (LTM).

3A). To analyze whether octopamine is also able to block STM, we fed octopamine prior to training to control flies (Figure 3B). A short pulse of octopamine before the training inhibits STM.

We were wondering whether octopamine could reduce the appetite in starved animals and thereby reducing the value of the food reward. To investigate this, we fed starved flies with a short octopamine pulse and assayed their sucrose intake after 3 h (Figure S2). Flies fed with octopamine showed a significant reduction of sucrose intake, but not flies fed with a similar amount of tyramine or a 10-fold increase in tyramine concentration. Therefore, it is possible that increased octopamine content influences how food is evaluated. This is consistent with a function of octopamine as a signal for food or high internal energy levels. Taken together, octopamine is a negative regulator of appetitive long-term memory and STM (Figure 3C).

Starvation influences sucrose consumption preference

Starvation reduces the internal energy storage. The reduction could lead to a reassessment of the external food cues and increased food consumption to restore the energy supplies. First, we investigated how starvation reduces the glycogen storage in whole animals (Figure 4A). In controls and *Tβh^{nM18}* mutants, starvation reduces the glycogen level. However, non-starved *Tβh^{nM18}* mutant males had significantly higher glycogen levels at baseline. After 40 h of starvation, the glycogen levels of *Tβh^{nM18}* mutants were still higher than those of controls that were starved for the amount of time. Adult *Tβh^{nM18}* mutants have a reduced sucrose intake (Li et al., 2016; Scheiner et al., 2014). The assessment of external food sources might be reflected in the choice of food. To examine whether hunger influences the evaluation of an external food stimulus, we starved flies and determined the preference for consuming sucrose over protein-enriched food using the capillary feeder assay (Figure 4B). Non-starved flies reared on standard fly food preferred sucrose over protein-enriched food, but *Tβh^{nM18}* mutants to a significantly lesser extent than controls. 18 h of starvation resulted in a decreased sucrose preference in controls and *Tβh^{nM18}* mutants, but to a significantly lower level in *Tβh^{nM18}* mutants. Extending the starvation period to 40 h resulted in a similar sucrose consumption in control and mutant flies.

To further investigate whether *Tβh^{nM18}* mutants integrate their internal glycogen storage into their feeding preference, we deprived male flies of sucrose- or protein-enriched food by feeding them 5% sucrose, 5% yeast or standard fly food for 3 days. The food preference was analyzed after deprivation (Figure 4C). As controls, we included mated females in our analysis as they have different nutritional requirements due to their mating status (Ribeiro and Dickson, 2010; Vargas et al., 2010). Male flies fed standard food for 3 days strongly preferred sucrose over protein-enriched food, with *Tβh^{nM18}* mutants showing a significant reduction. Yeast-deprived and sucrose-deprived *Tβh^{nM18}* mutant showed a significantly reduced preference for sucrose. In mated females, no differences were observed in food preference between controls and the *Tβh^{nM18}* mutant. In conclusion, the reduced preference for sucrose consumption correlates in *Tβh^{nM18}* with increased glycogen levels. Furthermore, *Tβh^{nM18}* mutants can sense the reduction of specific internal energy stores and change their food preferences accordingly.

Internal glycogen storage influences sucrose-related memories

Since memory performance increases when the internal energy supply is reduced by starvation, we wanted to investigate whether the internal energy supply influences memory performance. In *Drosophila*, glycogen is mainly found in the fat bodies - the major energy storage organ - and the muscles, a major site of energy expenditure (Wigglesworth, 1949). Glycogen synthase and glycogen phosphorylase control glycogen content in the body (Figure 5A). Knockdown of glycogen synthase using *Glyc^{HMS01279}-RNAi* efficiently reduced glycogen levels in larvae, while knockdown of glycogen phosphorylase using *Glyc^{HMS00032}-RNAi* efficiently increased glycogen levels (Yamada et al., 2018). We altered glycogen levels in the muscles using the *mef2*-Gal4 driver (Ranganayakulu et al., 1998) and/or the fat bodies using the FB-Gal4 driver (Gronke et al.,

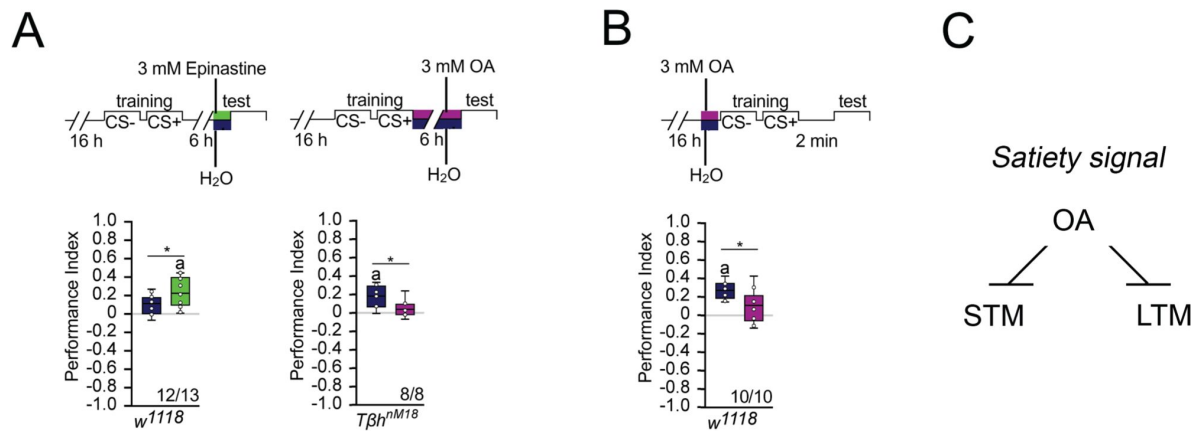


Figure 3.

Octopamine suppresses memory

A, Feeding 3 mM of the octopamine receptor antagonist epinastine for 1 h after training resulted in memory 6 h later in w^{1118} flies. Feeding 3 mM octopamine for 6 h after training suppresses LTM in $T\beta h^{nm18}$. **B**, A 3 mM octopamine feeding pulse 30 min before training inhibits STM in $T\beta h^{nm18}$ mutants. Controls were water-fed. **A, B**, Flies were starved for 16 h and 2 M sucrose was used as reinforcer. The letter “a” marks a significant difference from random choice as determined by a one-sample sign test ($P < 0.05$). Student’s t tests were used to determine differences between two groups. ($P < 0.05$). Numbers below box plots indicate pairs of reciprocally trained independent groups of male flies. **C**, Model for memory suppression.

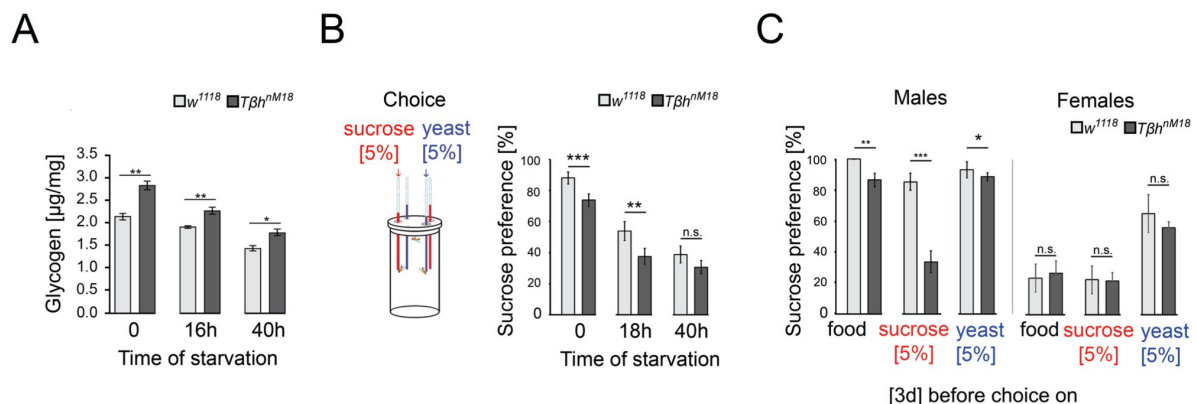


Figure 4.

Elevated glycogen levels correlate with reduced sucrose preference

A, Analysis of whole-body glycogen levels in w^{1118} and $T\beta h^{nm18}$ flies. In $T\beta h^{nm18}$ flies, glycogen content is significantly higher than in w^{1118} flies under similar starvation conditions. $N = 3$ groups of 5 male flies. **B**, Flies were starved 18 h or 40 h before food intake was measured for 24 h. Flies chose between 5% sucrose and 5% yeast. The preference was determined. All flies showed a significant preference for sucrose consumption. Starvation reduced the preference. $T\beta h^{nm18}$ showed a significantly reduced preference for sucrose in comparison to control flies, but not after 40 h starvation. $N = 20 - 26$ groups of eight flies. **C**, Feeding male flies for 3 days on standard fly food (food), 5% sucrose or 5% yeast resulted in control flies preferring to consume sucrose. Male $T\beta h^{nm18}$ mutants fed normal food, sucrose and yeast showed a significant reduction in sucrose preference. Female flies of controls and mutants did not differ in their preferences. $N = 14 - 28$ groups of eight flies. To determine differences between two groups, the Mann-Whitney U test was used. $P^* < 0.05$; $P^{**} < 0.01$, $P^{***} < 0.001$.

2003) and analyzed the effect of altered glycogen levels on short-term memory (**Figure 5**). We used PAS staining to confirm the down- or up-regulation of glycogen in larval muscles or fat bodies, respectively (**Figure 5**) (Yamada et al., 2018). In addition, we quantified the glycogen levels in the bodies of adult flies (Figure S3). As we could not always detect differences in glycogen content in whole flies, we also analyzed the glycogen level in the muscle-rich thorax or fat body-rich abdomen (Figure S3). The knockdown of glycogen phosphorylase increased glycogen levels and the knockdown of glycogen synthase reduced glycogen levels.

Increasing glycogen levels in the muscles had no effect on the short-term memory of the 16 h starved flies, but decreasing glycogen levels significantly improved memory performance (**Figure 5B**). Increasing or decreasing glycogen levels in the fat bodies had no effect on memory performance (**Figure 5C**). When the muscles and fat bodies glycogen levels were significantly increased, flies showed a reduced memory to odors paired with sucrose. An increase in memory performance was observed when glycogen levels were significantly reduced in both tissues (**Figure 5D**). Recently, it has been shown that energy metabolism in mushroom bodies is important for the formation of LTM (Placais et al., 2017). To analyze the function of the mushroom bodies, the expression of *GlyS^{HMS01279}-RNAi* under the control of the Mef2-Gal4 driver was repressed in the mushroom bodies using the mb247-Gal80 driver (Krashes et al., 2007). The memory was still increased (Figure S4). Conversely, the reduction of *GlyS^{HMS01279}-RNAi* using the mb247-Gal4 driver targeting the mushroom bodies (Zars et al., 2000) did not alter short-term memory (Figure S4). Thus, low levels of glycogen in the muscles after starvation positively influence appetitive short-term memory, whereas high levels of glycogen in the muscles and fat body decrease reduce short-term memory.

Internal glycogen levels reduce sucrose-related memories in *Tβh* mutants

The increased glycogen levels in *Tβh^{nM18}* mutants could be responsible for the reduced STM. To determine whether the reduction in glycogen levels in the muscles or fat bodies restores STM, we expressed *GlyS^{HMS01279}-RNAi* under the control of the *mef2*-Gal4 or *FB*-Gal4 driver in *Tβh^{nM18}* mutants and analyzed STM (**Figure 6A**). Neither the reduction in the muscles nor the reduction in fat bodies of *Tβh^{nM18}* mutants improved STM. Only when glycogen was reduced in both tissues did the *Tβh^{nM18}* mutants show improved STM compared to controls. Thus, *Tβh^{nM18}* mutant flies can form appetitive STM similar to controls when the energy storage is sufficiently reduced. Next, we analyzed whether male *Tβh^{nM18}* mutants can form STM to other nutrients than carbohydrates by using a protein-enriched diet in the form of 5% yeast as a positive reinforcer (**Figure 6B**). To determine whether there is a difference in the evaluation of protein as a food source between male flies and *Tβh^{nM18}* mutants, we measured yeast intake in non-starved and starved flies (Figure S5). Non-starved *Tβh^{nM18}* mutant males have a significantly higher protein intake than controls. However, after 16 h of starvation, the protein intake was comparable to that of control flies. When 5% yeast was used as a food reward, male *Tβh^{nM18}* mutants showed comparable STM levels controls (**Figure 6B**).

To further analyze the influence of the internal energy status on memory performance, we took advantage of the observation that female flies have different nutritional requirements depending on their mating status (Ribeiro and Dickson, 2010; Vargas et al., 2010). Virgin females showed a higher consumption preference for sucrose than mated females when given the choice between yeast and sucrose (**Figure 4C**), demonstrating a difference in internal energy demands and suggesting a difference in valence for different diets. We tested virgin and mated female flies of controls and *Tβh^{nM18}* mutants for STM with 2 M sucrose as reinforcer (**Figure 6C**). Virgin females remembered the odorant paired with sucrose significantly better than mated females. This was also true for *Tβh^{nM18}* mutant females. Thus, the internal energy status influences how the reward is evaluated in learning and memory.

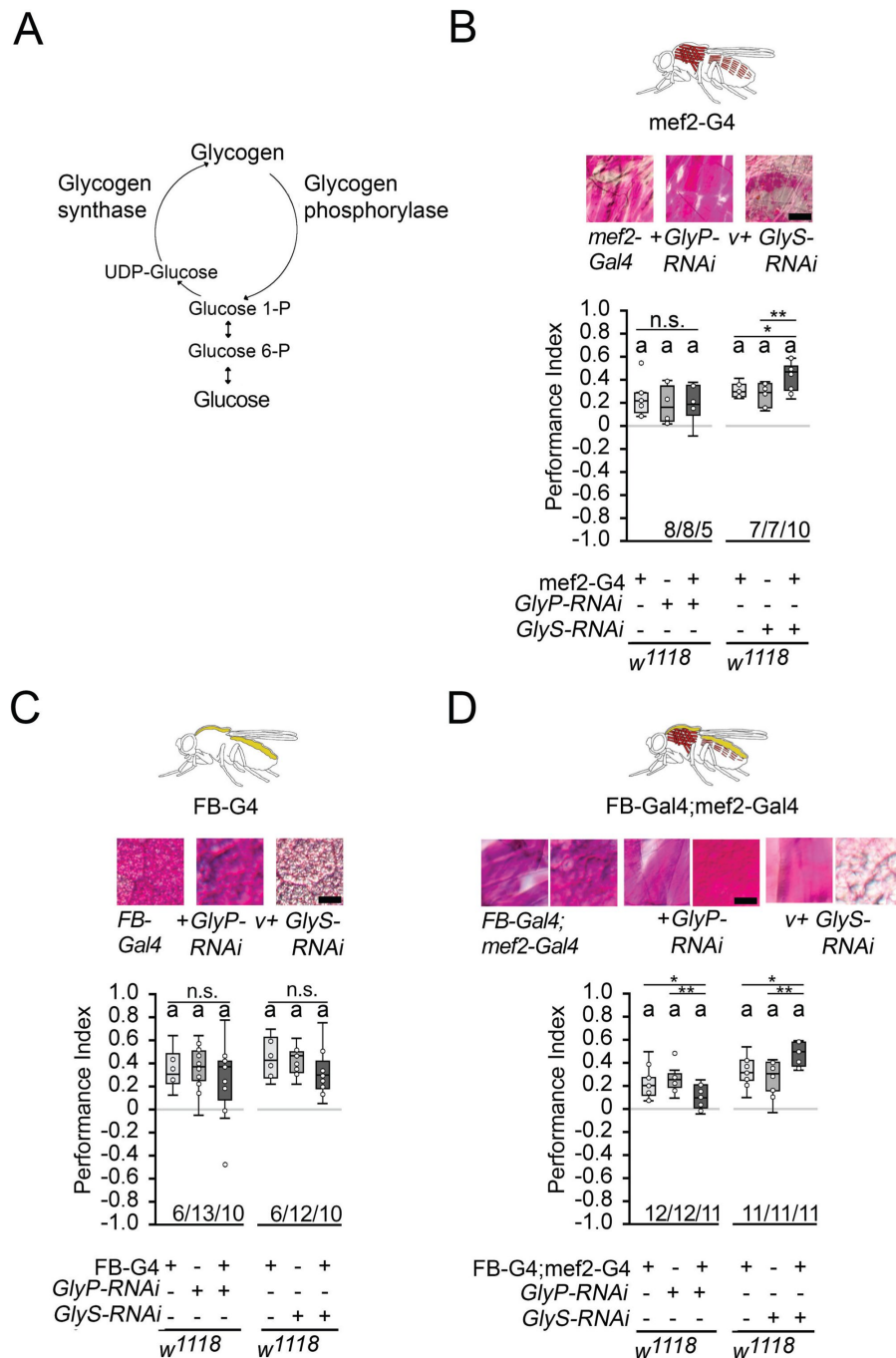
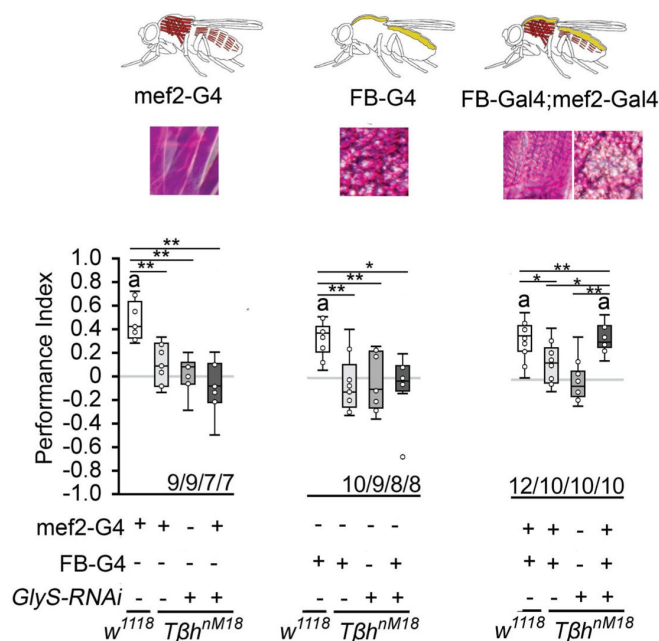


Figure 5.

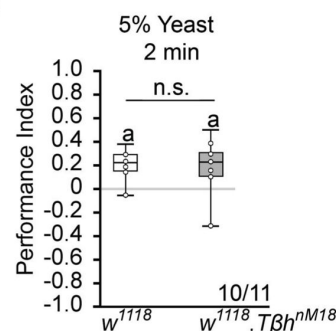
Carbohydrate storage influences appetitive STM

A, Schemata of glycogen synthesis. The expression of *GlyP-RNAi* reduced glycogen phosphorylase and increased glycogen levels, whereas *GlyS-RNAi* reduced glycogen synthase and decreased glycogen levels in target tissues (Figure S3). **B - D**, PAS was used to visualize glycogen levels in larval muscle or fat bodies. **B**, Increases in glycogen in the muscles have no effect on STM, whereas reduced muscle glycogen increases appetitive STM. **C**, Increased or decreased glycogen levels in the fat bodies did not interfere with STM. **D**, A combined increase in glycogen in muscles and fat bodies reduced STM, and a decrease in glycogen increased STM. Flies were starved for 16 h and 2 M sucrose was used as reinforcer. Numbers below box plots indicate one pair of reciprocally trained independent fly groups. The letter "a" marks a significant difference from random choice as determined by a one-sample sign test ($P < 0.05$). The one-way ANOVA with post hoc Tukey's HSD was used to determine differences between three groups ($P^* < 0.05$; $P^{**} < 0.01$).

A



B



C

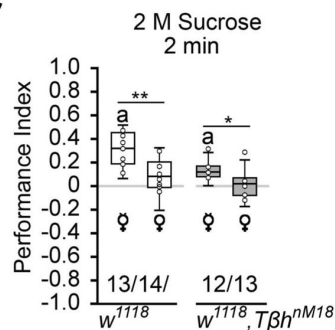


Figure 6.

Reducing glycogen in $T\beta h^{NM18}$ improves appetitive STM

A, Decreasing glycogen concentration using UAS-*GlyS^{RNAi}* in the muscles or fat bodies in $T\beta h^{NM18}$ mutants did not improve STM, but decreasing glycogen in both tissues improved STM to control levels. Flies were starved for 16 h and 2 M sucrose was used as reinforcer. **B**, 16 h starved w^{1118} and $T\beta h^{NM18}$ flies formed similar levels of appetitive STM when 5% yeast was used as a reinforcer. **C**, 16 h starved virgin females of w^{1118} and $T\beta h^{NM18}$ displayed STM, whereas mated females of both genotypes did not. Differences from random choice were determined using a one-sample sign test and marked with the letter "a" ($P < 0.05$). Differences between two groups were determined using Student's t tests, and differences among four groups were determined with one-way ANOVA with Tukey's HSD post hoc test. $P^* < 0.05$; $P^{**} < 0.01$. Numbers below box plots indicate one pair of reciprocally trained independent fly groups.

Insulin-like signaling in octopaminergic neurons regulates STM

How is the internal energy status integrated into the reward system? Insulin-like signaling regulates glycogen levels in invertebrates and vertebrates (Semaniuk et al., 2021 [↗](#)). Loss of the insulin receptor results in more circulating sugar but not increased glycogen levels (Shingleton et al., 2005 [↗](#)). Thus, the insulin receptor might be a good candidate that links the internal energy level to reinforcing neurons. First, we analyzed whether the insulin receptor is expressed in octopaminergic reward neurons in the brain (**Figure 7A** [↗](#)). To detect the expression of an activated insulin receptor, we used an insulin antibody that recognizes the phosphorylated form of the insulin receptor (InR). This region is highly conserved between humans and flies. First, we tested whether the antibody indeed recognized the activated insulin receptor. Therefore, we overexpressed the activated insulin receptor using the UAS-InR.A1325D transgene under the control of the *dTdc2*-Gal4 driver (Figure S6). The InR.A1325D protein variant mimics the human V938D protein variant, which is constitutively active (Longo et al., 1992 [↗](#)) and the *dTdc2*-Gal4 driver targets octopaminergic reward neurons (Busch et al., 2009 [↗](#)). The expression of activated InR resulted in increased immunoreactivity (Figure S6). The InR antibody therefore detects the activated InR. In the brain, activated InR is broadly expressed in a punctate manner and more specifically in the soma of *dTdc2*-Gal4-targeted neurons (**Figure 7A** [↗](#)). To uncouple energy sensing via the insulin receptor in reward neurons, we expressed UAS-InR.K1409A under the control of the *dTdc2*-Gal4 driver. The transgene encodes a dominant negative variant of the InR (InR^{DN}) and disrupts with InR function (Wu et al., 2005 [↗](#)). In animals starved for 16 h, the expression of InR^{DN} under the control of the *dTdc2*-Gal4 driver did not alter STM, but uncoupling of InR-dependent energy sensing in reward neurons in *Tβh^{nM18}* restored STM to normal levels (**Figure 7B** [↗](#) and **C** [↗](#)).

To investigate whether the improved STM also affects the emerging LTM of the *Tβh^{nM18}* mutants, we performed cold shock experiments in *Tβh^{nM18}* mutants in which InR^{DN} was expressed in octopaminergic reward neurons (**Figure 7D** [↗](#)). The 3 h memory in controls is cold shock sensitive, but the memory in *Tβh^{nM18}* mutants is cold shock insensitive, supporting the idea that the mutants formed anesthesia-resistant memory. Given that octopamine is a negative regulator of memory and that it is still absent in *Tβh^{nM18}* mutants with blocked insulin signaling on octopaminergic reward neurons, it is not surprising that ARM is still observed in the mutants.

The results can be described with the following model (**Figure 7E** [↗](#)). Insulin receptor signaling on reward neurons integrated the internal energy status into memory formation. When energy levels are high enough due to increased glycogen levels or food intake, the insulin pathway suppresses the formation of STM. In return, the system releases octopamine to suppress the formation of food-related LTM. In mildly starved *Tβh^{nM18}* mutants, the integration of the internal energy status into the reward neurons is still intact. In addition, the level of the internal energy supply combined with the loss of octopamine is still high enough to support the formation of protein biosynthesis-dependent LTM. In *Tβh^{nM18}* mutants, two functionally different memory traces are formed after training, an insulin receptor-sensitive appetitive short-term memory and a long-term memory. In starved control animals, the glycogen levels are more severely depleted and octopamine is still present. This supports the formation of STM and an intermediate form of memory (ITM).

Increased starvation results in overconsumption in *Tβh^{nM18}* mutants

To analyze whether the increased glycogen levels and the reduced sucrose reward of *Tβh^{nM18}* mutants correlate with food consumption, the energy requirements in flies under different starvation conditions were analyzed by measuring food intake (**Figure 8** [↗](#)). Control flies starved for shorter or longer periods consumed similar amounts of 5% sucrose. In contrast, *Tβh^{nM18}*

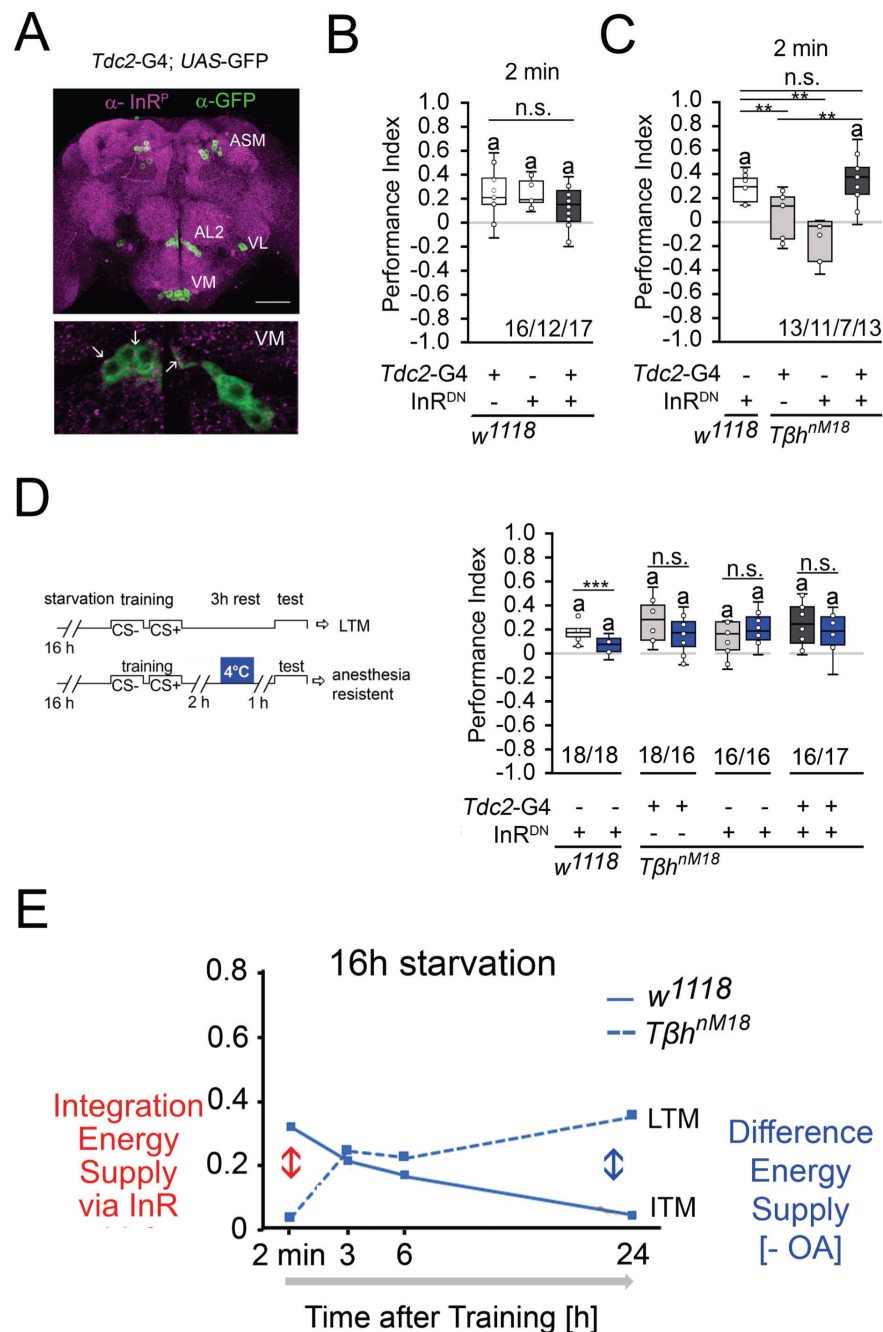


Figure 7.

Insulin signaling in reward neurons regulates STM

A, The activated form of the InR is expressed in punctuate manner throughout the brain (in magenta) and is also detected in octopaminergic reward neurons visualized by using the UAS-mCD8::GFP transgene under the control of the *Tdc2-Gal4* driver (in green). **B**, Blocking InR signaling in *Tdc2-Gal4*-targeted octopaminergic neurons does not change appetitive STM in 16 h starved flies using 2 M as reinforcer. **C**, Blocking InR signaling in *Tdc2-Gal4*-targeted octopaminergic neurons in *Tβh^{NM18}* mutants restored STM to control levels. **D**, A cold shock did not disrupt emerging memory in *Tβh^{NM18}* mutants with blocked InR under the control of the *Tdc2-Gal4* driver. Student's *t* test was used to determine differences between two groups, and one-way ANOVA with Tukey's post hoc HSD test to determine differences between three or more groups. n.s. is not significant; $P^* < 0.05$; $P^{**} < 0.01$, $P^{***} < 0.001$. The letter "a" marks a significant difference from random choice as determined by a one-sample sign test ($P < 0.05$). **E**, Model of memory regulation in *Tβh^{NM18}* mutants. Long-term memory (LTM), intermediate-term memory (ITM).

mutants starved for 16 h consumed significantly less sucrose than controls. Longer-starved mutants consumed approximately 34% more sucrose (**Figure 8B**). After longer starvation, the glycogen levels in *Tβh^{nM18}* mutants were still higher than those in controls (**Figure 4A**). Therefore, they overconsumed sucrose. The overconsumption was independent of the diet, as they showed similar overconsumption when fed with a solution containing 5% yeast and 5% sucrose (**Figure 8B**). To investigate whether the internal energy status is also integrated into the feeding behavior by the octopaminergic reward neurons, we blocked insulin signaling in reward neurons in 16 h-starved *Tβh^{nM18}* mutants and analyzed sucrose consumption (**Figure 8C**). The reduced sucrose consumption of *Tβh^{nM18}* mutants was significantly improved compared to controls. Thus, the octopaminergic reward system integrates via insulin receptor signaling the internal energy level into the regulation of food consumption. The mechanism is still intact in *Tβh^{nM18}* mutants.

Discussion

Depending on the duration of starvation, flies develop STM and an anesthesia-sensitive intermediate type of a food-related memory. The extending the hunger period, memory formation shifts to an anesthesia-resistant intermediate memory. Presentation of the reward leads to the release of octopamine and this the suppression of LTM. The shifting of different memories phases in response to different energy supplies has been also described for memories that are formed of aversive non-food-related memories. Depending on the reduction of the internal energy supply, flies initially form protein-dependent aversive olfactory LTM and later ARM, a protein synthesis-independent form of memory (Wu et al., 2005). In times of energy shortage, the organism exchanges the costly protein biosynthesis-dependent LTM for the “less costly” protein biosynthesis-independent ARM (Mery and Kaweck, 2005). The effect of memory transition can also be observed at the level of appetitive LTM. In *Tβh^{nM18}* mutants, mild starvation along with elevated glycogen levels leads to the formation of LTM and prolonged starvation leads to the formation of anesthesia-resistant LTM. Furthermore, *Tβh^{nM18}* mutants form only anesthesia-sensitive aversive memory (Wu et al., 2013). In contrast, *Tβh^{nM18}* mutants form cold shock-resistant LTM. Therefore, *Tβh^{nM18}* mutants are not defective in appetitive anesthesia-resistant types of memories rather the internal energy status defines how quickly anesthesia-resistant appetitive memories forms.

Octopamine gates memory formation

What function does octopamine play in memory formation? Octopamine is a negative regulator of LTM, as feeding octopamine receptor antagonists to control flies results in memory, and emerging memory in *Tβh^{nM18}* mutants is suppressed by octopamine after conditioning. Octopamine acts in the formation of LTM upstream of dopaminergic neurons, as blocking neurotransmitter release of dopaminergic LTM-mediating neurons immediately after conditioning blocks memory formation in *Tβh^{nM18}* mutants. The results are consistent with the model describing that dopamine release is not required during the pairing of the conditioned stimulus and the reward rather than as gain control after the training (Adel and Griffith, 2021). The inhibitory function of octopamine controls the gain.

During the acquisition of appetitive STM, octopamine acts upstream of dopaminergic neurons M (Burke et al., 2012; Liu et al., 2012). However, at first glance, the function of octopamine appears to differ in STM and LTM. Loss of octopamine in *Tβh^{nM18}* mutants results in loss of STM, indicating that octopamine is required as a positive regulator for appetitive STM (Schwaerzel et al., 2003). The loss of STM in *Tβh^{nM18}* mutants has been attributed to the loss of labile STM which forms in response to the sweetness of sucrose, but not due to defects in stable LTM, which forms in response to the caloric value of sugar (Burke et al., 2012; Fujita and Tanimura, 2011). Sweetness is sensed by taste receptor that change their sensitivity during hunger (Inagaki et al., 2012; Marella et al., 2012) and thereby influencing the response to the external reinforcer.

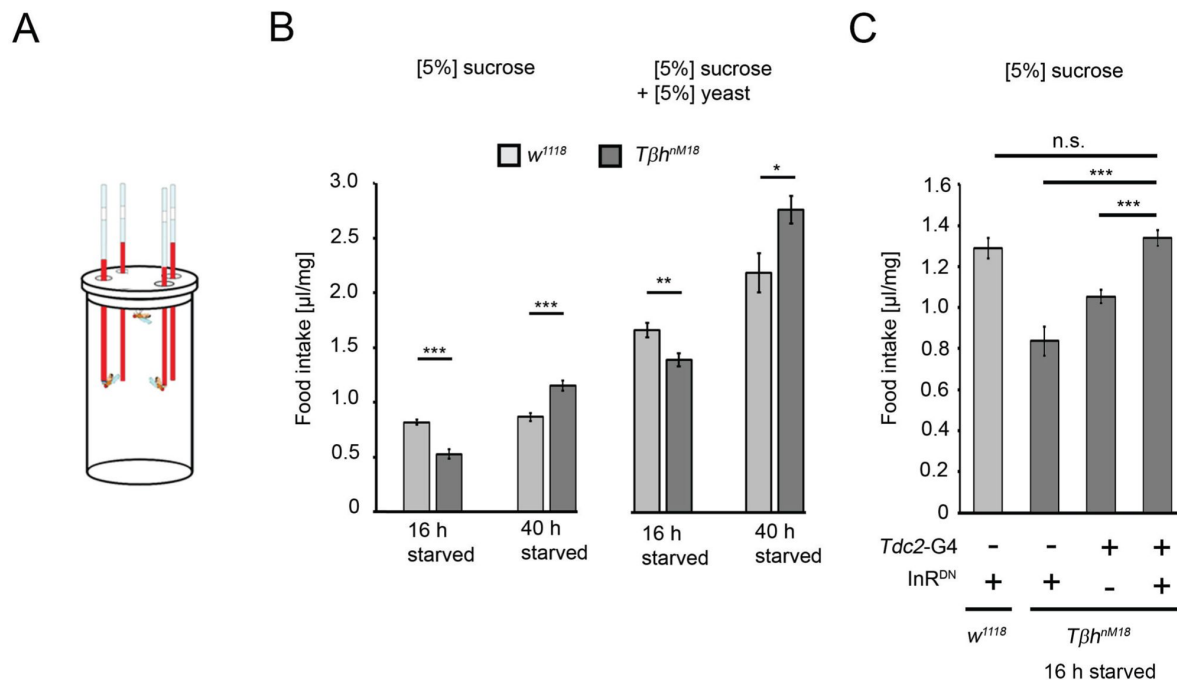


Figure 8.

Prolonged starvation results in rebound sucrose intake in hyperglycemic *Tβh^{NM18}* mutants

A, Capillary feeder assay used to determine food intake. **B**, Flies were starved 16 h or 40 h before 24 h food intake was measured. After 16 h of starvation, *Tβh^{NM18}* mutants significantly consumed less 5% sucrose or 5% sucrose with 5% yeast. After 40 h of starvation, *Tβh^{NM18}* mutants significantly consumed more sucrose and sucrose with yeast. N = 20 - 26 groups of eight flies. **C**, Blocking InR signaling in *Tdc2-Gal4*-targeted octopaminergic neurons in *Tβh^{NM18}* mutants significantly increased 5% sucrose consumption. N = 20 - 28 groups of eight flies. To determine differences between two groups, Student's t test was used, and to determine differences between three or more groups, one-way ANOVA with post hoc Tukey's HSD was used. n.s. = non-significant; *P** < 0.05; *P*** < 0.01, *P**** < 0.001.

The responsiveness could be regulated by the octopaminergic system, as octopaminergic neurons regulate the sucrose sensitivity of gustatory neurons in *Drosophila* (Youn et al., 2018 [↗](#)). However, the reduced sensitivity of gustatory receptors cannot explain why octopamine-deficient mutants do not form STM. The *Tβh^{nM18}* mutants form aversive memory to lower concentrations of sucrose and the differences in sucrose preference of *Tβh^{nM18}* do not translate to differences in food intake during training.

The internal energy status influences how the external source of sucrose is evaluated rather than octopamine being directly involved in the acquisition of appetitive STM. Consistently, *Tβh^{nM18}* mutants can exhibit STM after prolonged periods of starvation. In mildly starved *Tβh^{nM18}* flies that do not show STM, uncoupling of energy sensing in reward neurons by blocking insulin receptor function leads to STM. Furthermore, *Tβh^{nM18}* mutant females with different energy requirements form appetitive STM, and a short pulse of octopamine before conditioning can even block STM. Thus, octopamine is not required in the acquisition of appetitive STM but rather suppresses LTM when enough energy is available. The function of octopamine as a suppressor of different memory types allows for selecting the memory type depending the internal and external information. This gating function in memory formation can also be observed in other behaviors. For example, octopamine regulates the decision to approach or turn away from a food source (Classen and Scholz, 2018 [↗](#)). Taken together, the octopaminergic system integrates internal energy demands and the evaluation of external food supplies.

The internal energy level of the animal influences memory formation

At the cellular level, insulin receptor signaling regulates energy metabolism (Chatterjee and Perrimon, 2021 [↗](#)). The broad expression pattern of the insulin receptor in the *Drosophila* brain (Figure 7 [↗](#)) indicates that every cell in the brain needs to regulate its own energy homeostasis. However, the fly's entire energy resources and the evaluation of the external food supply must also be integrated. The reward system evaluates the external food supply. In mice, insulin receptor signaling in dopaminergic neurons mediates food reward (Konner et al., 2011 [↗](#)). Consistently, insulin receptor function in octopaminergic reward neurons regulates the rewarding properties of sucrose in appetitive STM memory (Figure 6A [↗](#)) and during food intake (Figure 7C [↗](#)).

The systemic metabolic rate is regulated by fat bodies. For example, after food intake, the fat body secretes Unpaired 2 (Upd2), which in turn regulates the secretion of *Drosophila* insulin-like peptides via GABAergic neurons (Rajan and Perrimon, 2012 [↗](#)). We show that the reduction of glycogen in the fat bodies is not sufficient to change appetitive STM, but reduction in the muscles or both tissues is. The results support a feedback mechanism between the assessment of glycogen levels in the muscles and the brain. In addition, the muscles and the fat bodies communicate about the energy content of both structures, and this information is signaled back to the brain. Such long-range signals from the muscles exist. Skeletal muscles are secretory organs (Pedersen and Febbraio, 2012 [↗](#)). For example, the muscle-secreted Amyrel amylase reduces the age-related accumulation of polyubiquitinated proteins in the brain (Rai et al., 2021 [↗](#)). Regardless of how the glycogen levels of both tissues are communicated to the brain, they influence how the reinforcer is evaluated. The feedback between the energy levels in both structures is still intact in octopamine-deficient mutants, as *Tβh^{nM18}* mutants still form STM when the energy storage is sufficiently reduced.

Are food-related memories and internal glycogen levels predictive of food intake?

The feeding behavior in hungry animals is regulated by various neural systems, including networks that receive and process sensory information and networks that assign reward properties to food (Berridge, 2009 [↗](#)). Blocking insulin receptor function in dopaminergic neurons

results in increased food intake and weight gain in mice (Konner et al., 2011). Similarly, blocking insulin receptor function in octopaminergic reward neurons in flies with increased glycogen levels and reduced food intake increases food intake. Thus, the octopaminergic system assigns reward properties to food. However, blocking insulin receptor signaling on reward neurons does not result in extensive overconsumption, supporting that the regulation of the food quantity is still intact and is not due to insulin receptor resistance in reward neurons. Increased levels of internal energy, e.g., glycogen, reduced the reward properties of food, resulting in a decreased positive association of food-related memories (Figure 5D; Figure 7A) and decreased food consumption (Figure 8). This is intended to prevent increased food intake when there is excess food. Insulin resistance could contribute to weight gain, because in addition to high internal levels of glycogen, the flies show normal food intake. However, the overconsumption only becomes visible after a prolonged period of hunger, which correlates with the emerging stable appetitive long-term memory. This suggests that the stability of appetitive memory could lead to the re-evaluation of food and could trigger the overconsumption of food.

In summary, the octopaminergic neurotransmitter system integrates several aspects that influence the regulation of food memories. Octopamine modulates the sensory perception of the conditioned stimulus and the sensory perception of the reward. Here, we show that the evaluation of food reward in the context of the energy storage is integrated by the octopaminergic system and influences the stability of food-related memories. The function of octopamine as a negative regulator of various forms of memories allows for selective regulation of food related behaviors such as food intake, and the loss of this regulation could also promote dysregulation of food intake. The close relationship between the octopaminergic neurotransmitter and the noradrenergic neurotransmitter system suggests that the integrator function may be conserved.

Material and methods

Drosophila melanogaster

Flies were raised on an ethanol-free standard cornmeal-molasses food at 25°C and 60% relative humidity on a 12 h/12 h day-night cycle. The following lines were used: *w¹¹¹⁸*; *w¹¹¹⁸, Tbh^{TM18}* (Monastirioti et al., 1996); *UAS-GlyS^{HMS01279}-RNAi* (BDSC #34930); *UAS-GlyP^{HMS00032}-RNAi* (BDSC #33634); *w¹¹¹⁸, UAS-shi^{ts}* (Kitamoto, 2001); *w¹¹¹⁸, P{UAS-InR. K1409A}* (BDSC#8253); *w¹¹¹⁸, FB-Gal4* (a generous gift from the Partridge Lab); *mef2-Gal4* (Ranganayakulu et al., 1998); *R15A04-Gal4* (BDSC #48671); *Tdc2-Gal4* (Cole et al., 2005). All lines were backcrossed to *w¹¹¹⁸* (Scholz Lab) for at least five generations to isogenize the genetic background. For behavioral experiments, three- to five-day-old male flies were used, if not otherwise indicated. Male flies of the F1 generation carrying one copy of the transgene were used as controls for behavioral experiments. Animal studies using *Drosophila melanogaster* were conducted in agreement with the regulations of the DFG and the Land North Rhine-Westphalia. Other ethics approval and informed consent statement are not applicable for research using *Drosophila*.

Olfactory learning and memory

Associative olfactory learning and memory was trained and tested with a modified version of the Tully and Quinn olfactory conditioning apparatus (Schwaerzel et al., 2003; Tully and Quinn, 1985). Approximately 70 one- to two-day-old male flies were collected with CO₂ anesthesia and were kept for 2 days at 25°C to recover from CO₂ sedation. Briefly, three- to five-day-old male flies were starved for 16 h or 40 h in vials with water-soaked filter paper at the bottom. Flies were transferred to training tubes and exposed to the first odorant for 2 min, either 3-octanol (3-OCT diluted 1:80 in paraffin oil) or 4-methylcyclohexanol (MCH diluted 1:100 in paraffin oil). After that, they were transferred to a second tube and exposed to the second odorant in the presence of filter paper soaked with either 0.15 M sucrose, 2 M sucrose or 5% yeast. To analyze the abilities of the flies to learn and remember the odorant paired with the reward, 2 min, 3 h, 6 h or 24 h after

training, flies were given the choice between odorant 1 (CS+) and odorant 2 (CS-) for 2 min. The performance index (PI) was calculated as $PI = (\#(CS+) + \#(CS-)) / (\text{total } \# \text{ flies})$, where CS+ indicates the odorant associated with the appetitive reinforcer and CS- indicates the odorant that was not associated with the reinforcer. To exclude non-associative effects, each “n” consists of a reciprocally trained, independent group of naïve flies. For pharmacological experiments, flies were fed water, 3 mM OA or 2 M sucrose before training or with 3 mM OA or 3 mM epinastine between training and testing. The assay was performed at RT with 60% relative humidity. For cold shock experiments, flies in vials were placed in ice-cold water for 2 min.

Odorant acuity and odorant balance were tested with naïve flies that were not used later for learning and memory experiments. For testing, the flies were placed into the Tully Quinn setup. They chose between the odorant- or the solvent paraffin oil-containing sides or between both odorants for 2 min. The sucrose and yeast preference was determined by analyzing whether they preferred the side with a sucrose or yeast-soaked filter paper over a water-soaked filter paper. To determine sucrose intake, flies were fed with a food-safe colored sucrose solution and food intake was analyzed by the color in the abdomen (Table S1).

Food intake

To analyze the consumption of nutrients in flies, the capillary feeder (CaFe) assay was performed (Diegelmann et al., 2017 [DOI](#); (Ja et al., 2007 [DOI](#))). Briefly, eight three- to five-day-old male or female flies that were either non-starved or starved for 18 h or 40 h had access to 4 capillaries filled with either 5% sucrose or 5% yeast or a mixture of both for 24 h at 25°C and 60% humidity. The solution was colored with red food dye. During starvation, flies were kept in vials with wet filter paper at 25°C and 60% humidity. The amount of consumed solution was determined using an electronic caliper. To account for evaporation, the average evaporation of three CAFE setups without flies was measured and used to normalize the average food intake per fly. To normalize food intake to body weight, at least 5 times 100 male flies per genotype and condition were weighed, and the average body weight of a single fly was determined. The total consumption per µg fly was calculated by dividing the total consumption per fly by the mean weight of a fly. N indicates the number of tested groups.

Glycogen content

Whole body glycogen levels were determined with the Glucose (HK) Assay Kit (Sigma Aldrich, #GAHK20-1KT) according to the protocol of (Tennessen et al., 2014 [DOI](#)). A group of five male flies that were either sated or starved for 16 h or 40 h were homogenized in 100 µl ice-cold 1x PBS. To reduce enzymatic degradation, proteins and enzymes were heat inactivated at 70°C for 10 min. The supernatant was removed and diluted 1:3 with 1x PBS. Twenty microliters of the samples were either added to 20 µl of 1x PBS or PBS/Amyloglucosidase mix and incubated at 37°C for 60 min. Then, 100 µl of HK-reagent was added to the sample or glucose standard and incubated for 15 min at RT. Absorbance was measured at 340 nm. The glycogen content was calculated by subtracting the total glucose concentration from 1x PBS-treated samples from the total glucose of amyloglucosidase-treated samples.

Periodic acid staining

To visualize glycogen levels in the fat bodies and muscle tissue of larvae and adult *Drosophila*, PAS staining was performed after Yamada et al., 2018 [DOI](#) (Yamada et al., 2018 [DOI](#)) with a slight modification. The samples were fixed in 3.5% formaldehyde for 20 min and washed two times for 5 min with 1% BSA/PBS. Periodic acid solution was added for 5 min, followed by two washes of 5 min with 1% BSA/PBS. Schiff's Reagent was added for 5 min, followed by two washes of 5 min with 1% BSA/PBS. Tissue was stored in 50% glycerol.

Immunohistochemistry

For immunohistochemistry, antibodies raised in rabbits against the activated insulin-like receptor (cell signaling technology #3021) were used at a dilution of 1:50 in 5% normal goat serum in PBS with 0.1% Triton and incubated for two days. The brains were washed with PBS with 0.3% Triton.

Quantification and statistical analysis

Food intake was displayed as the mean $\pm$ s.e.m. For learning and memory experiments, the data were displayed as boxplot $\pm$ minimum ($Q1 - 1.5 * IQR$) and maximum ($Q1 + 1.5 * IQR$). We used the Shapiro-Wilk test (significance level $P < 0.05$) followed by a QQ-Plot chart analysis to determine whether the data were normal distributed. For normal distributed data, we used the Student's t test to compare differences between two groups and the one-way ANOVA with Tukey's post hoc HSD test for differences between more than two groups. For nonparametric data, we used the Mann-Whitney U test for differences between two groups and for more than two groups the Kruskal-Wallis test with post hoc Duenn analysis and Bonferroni correction. The nonparametric one-sample sign test was used to analyze whether behavior was not based on random choice and differed from zero ($P < 0.05$). The statistical data analysis was performed using statskingdom (<https://www.statskingdom.com>). Boxplots were generated with Microsoft Excel 2016 and GIMP 2.10.12.

Data availability

All data related to figures are included in the supplement Table S2.

Acknowledgements

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Author contributions

H.S. initiated the project. M.B., H.S. and K.D. designed the experiments. M.B., K.A., K. D., M.F. and T.E.K. performed the experiments and, together with H.S., analyzed the data. M.B, M.F. and K.A. performed learning and memory tests. K.D. characterized insulin receptor expression. M.B. and T.E.K. performed food consumption assays. M.B. wrote a draft of the manuscript, and H.S. wrote the manuscript.

Declaration of interests

The authors declare no competing interests.

Supplemental information titles and legends

Supplemental information includes 6 figures and two tables and can be found with this article online.

Figure S1 Controls for heat shift experiment in Figure 2B [↗](#)

To control for memory performance without a heat-shift, flies expressing a temperature-sensitive *shibire* transgene under the control of the R1504-Gal4 driver were trained and tested at the permissive temperature and compared to the respective controls. The tested groups showed memory 6 h after the training and did not differ from each other. Flies were starved for 16 h and 2 M sucrose was used as reinforcer. The behavior differed from random choice as determined using a one-sample sign test and marked with the letter “a” ($P < 0.05$). The one-way ANOVA with post hoc Tukey’s HSD was used to analyze difference between groups. n.s = no significant differences.

Figure S2. Octopamine but not tyramine suppresses food intake (related to Figure 3 [↗](#))

The w^{1118} flies were starved for 17 h followed by feeding 1 h octopamine [10 mg/ml], tyramine [10 or 100 mg/ml] or water. The intake of 5% sucrose after 3 h was determined using the CAFÉ assay. Student’s t tests were used to determine differences between two groups and one-way ANOVA with Tukey’s post hoc HSD test between three groups. ($P^{***} < 0.001$; n.s. = non-significant). N = 13 – 20 groups of 20 male flies.

Figure S3. Glycogen level in adult flies with reduced GlyP and GlyS (related to Figure 5 [↗](#))

The relative glycogen content in flies with altered GlyP and GlyS in muscles, fat bodies or both. The expression of *GlyP-RNAi* and *GlyS-RNAi* under the control of *mef2-Gal4* did not significantly change the relative glycogen level in whole animals, but in the thorax. The expression of *GlyP-RNAi* under the control of *FB-Gal4* increased significantly glycogen levels in whole animals, and the expression of *GlyS-RNAi* decreased glycogen in the abdomen. When both drivers are combined, the expression of *GlyP-RNAi* increases glycogen in whole animals and the expression of *GlyS-RNAi* decreases the glycogen levels. Glycogen was measured in 3 groups of 5 male flies and normalized to the protein levels and to the glycogen levels of flies of the Gal4 driver. ANOVA with post hoc Tukey’s HSD was used to determine differences between three groups. ($P^* < 0.05$; $P^{**} < 0.01$, $P^{***} < 0.001$).

Figure S4. The glycogen level in the mushroom bodies does not influence appetitive STM (related to Figure 5 [↗](#))

A, The expression of *GlyS-RNAi* under control of the *mef2-Gal4*; *mb247-Gal4* drivers increased STM. **B**, The expression of *GlyS-RNAi* under control of the *mb247-Gal4* driver did not change STM. Flies were starved for 16 h and 2 M sucrose was used as reinforcer. One-way ANOVA with Tukey’s HSD post hoc test was used to determine differences between the groups. ($P^* < 0.05$; $P^{**} < 0.01$; n.s. = non-significant). The letter “a” marks a significant difference from random choice as determined by a one-sample sign test ($P < 0.05$). Numbers below box plots indicate one pair of reciprocally trained independent fly groups. **C**, Sensory acuity tests for the genotypes used.

Figure S5. Starvation influences yeast consumption of *Tβh^{nm18}* flies (related to Figure 6 [↗](#))

Non-starved *Tβh^{nm18}* male flies consumed significantly more yeast within 24 h. Starved *Tβh^{nm18}* mutants consumed similar amounts of yeast. N = 26 groups of eight flies. To determine differences between both groups, Student’s t test was used. $***P < 0.001$; n.s. = non-significant difference.

Figure S6. The antibody against phosphorylated InR recognizes activated InR (related to Figure 7 [↗](#))

A, B, In the antennal lobes (AL) of the male adult brain, the immunoreactivity recognized by the anti-InR^P antibody is shown in magenta, and GFP expression is shown in green. The Gal4 expression of the *Tdc2-Gal4* driver targeting octopaminergic neurons is visualized using the UAS-

mCD8::GFP transgene. **B**, The expression of the constitutively active insulin receptor results in increased immunoreactivity detected by the anti-InR^P antibody.

Table S1. Sensory acuity

Odorant acuity and odorant balance were tested in independent fly groups of each genotype used in the behavioral experiments. For testing, flies were placed in the Tully Quinn setup. To analyze odorant preference and balance, they were allowed to choose between the odorant- or paraffin oil-containing sides or between both odorants for 2 min. Preference for sucrose and yeast was determined by analyzing whether flies preferred the side with a sucrose- or yeast-soaked filter paper over a water-soaked filter paper. Sucrose intake was analyzed by examining whether flies absorbed colored sucrose solution into the abdomen.

Table S2. Data related to Figures

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Reviewer #1 (Public Review):

The present study conducted by Berger et al. delves into the extent to which memory formation relies on available energy reserves. While aversive memory formation has been extensively studied in this context, the investigation into appetitive memory formation has been comparatively sparse. It has long been recognized that flies can only form appetitive memory under conditions of starvation. However, the authors of this study go beyond this understanding by revealing that not only the duration of starvation matters, but it also dictates the type of memory formed, whether short- or long-term memory. The authors illustrate that internal glycogen stores play a crucial role in this process, facilitated by insulin-like signaling in octopaminergic reward neurons, which integrates internal energy reserves into memory formation. Consequently, the authors propose that octopamine serves as a negative regulator of various forms of memory, shedding light on the enduring question of the octopaminergic neuronal system's involvement in appetitive memory formation, which has been overshadowed by the focus on the dopaminergic system in recent years. Additionally, the findings contribute to the ongoing debate concerning the role of insulin receptors, whether they function within neurons themselves or in glial cells. Moreover, the authors not only convincingly demonstrate that octopamine negatively regulates appetitive memory formation, but they also propose a mechanism whereby the insulin receptor in octopaminergic neurons senses the internal energy status and subsequently modulates the activity of these neurons. The experiments are meticulously designed, employing a variety of behavioral assays, genetic tools for manipulating neuronal activity, and state-of-the-art

imaging techniques. The conclusions are well supported by the data and carefully performed controlled experiments, yielding high-quality data.

<https://doi.org/10.7554/eLife.88247.2.sa2>

Reviewer #2 (Public Review):

How organism physiological state modulates establishment and perdurance of memories is a timely question that the authors aimed at addressing by studying the interplay between energy homeostasis and food-related conditioning in *Drosophila*. Specifically, they studied how starvation modulates the establishment of short-term vs long-term memories and clarified the role of the monoamine Octopamine in food-related conditioning, showing that it is not per se involved in formation of appetitive short-term memories but rather gates memory formation by suppressing LTM when energy levels are high. This work clarifies previously described phenotypes and provides insight about interconnections between energy levels, feeding and formation of short-term and long-term food-related memories.

Strengths

- Previous studies have documented the impact of Octopamine on different aspects of food-related behaviors (regulation of energy homeostasis, feeding, sugar sensing, appetitive memory...), but we currently lack a clear understanding of how these different functions are interconnected. The authors have used a variety of experimental approaches to systematically test the impact of internal energy levels in establishment of different forms of appetitive memory and the role of Octopamine in this process.

- The authors have used a range of approaches, performed carefully controlled experiments and produced high quality data.

<https://doi.org/10.7554/eLife.88247.2.sa1>

Reviewer #3 (Public Review):

In this manuscript, Berger et al. study how internal energy storage influence learning and memory. Since in *Drosophila melanogaster*, octopamine (OA) is involved in the regulation of energy homeostasis they focus on the roles of OA. To do so they use the tyramine- β -hydroxylase (Tbh) mutant that is lacking the neurotransmitter OA and study short term memory (STM), long-term memory (LTM) and anesthesia-resistant memory (ARM). They show that the duration of starvation affects the magnitude of both short- and long-term memory. In addition, they show that OA has a suppressive effect on learning and memory. In terms of energy storage, they show that internal glycogen storage influences how long sucrose is remembered, and high glycogen suppresses memory. Finally, they show that insulin-like signaling in octopaminergic neurons, which is also related to internal energy storage, suppresses learning and memory.

The revised version of the manuscript is greatly improved, and I thank the authors for taking the comment seriously. This is an important study that extends our knowledge on OA activity in learning and memory and the effects the metabolic state has on learning and memory. The authors nicely use the genetic tools available in flies to try and unravel the complex circuitry of metabolic state level, OA activity and learning and memory. The overall take-home message of the manuscript is clear and supported by the data presented.

<https://doi.org/10.7554/eLife.88247.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

The present study by Berger et al. analyzes to what extent memory formation is dependent on available energy reserves. This has been dealt with extensively in the case of aversive memory formation, but only very sparsely in the case of appetitive memory formation. It has long been known that an appetitive memory in flies can only be formed by starvation. However, the authors here additionally show that not only the duration of starvation plays a role, but also determines which form of memory (short- or long-term memory) is formed. The authors demonstrated that internal glycogen stores play a role in this process and that this is achieved through insulin-like signaling in octopaminergic reward neurons that integrates internal energy stores into memory formation. Here, the authors suggest that octopamine plays a role as a negative regulator of different forms of memory.

The study sheds light on an old question, to what extent the octopaminergic neuronal system plays a role in the formation of appetitive memory, since in recent years only the dopaminergic system has been in focus. Furthermore, the data are an interesting contribution to the ongoing debate whether insulin receptors play a role in neurons themselves or in glial cells. The experiments are very well designed and the authors used a variety of behavioural experiments, genetic tools to manipulate neuronal activity and state-of-the-art imaging techniques. In addition, they not only clearly demonstrated that octopamine is a negative regulator of appetitive memory formation, but also proposed a mechanism by which the insulin receptor in octopaminergic neurons senses the internal energy status and then controls the activity of those neurons. The conclusions are mostly supported by the data, but some aspects related to the experimental design, some explanations and literature references need more clarification and revision.

(1) Usually, long-term memory (LTM) is tested 24 hours after training. Here, the authors usually refer to LTM as a memory that is tested 6 hours after training. The addition of a control experiment to show that LTM that the authors observe here lasts longer would increase the power of this study immensely.

We thank the reviewer for this comment, as it helped greatly to clarify the matter.

We measured memory of control and mutant flies 24 h after the training and included the data into the manuscript (Figure 1B and summarized in a model in Figure 2C). We show that control flies develop an intermediate type of memory, that is depending on the length of starvation either anesthesia-sensitive or resistant. Mutants lacking octopamine develop either anesthesia-sensitive or resistant long-term memory.

(2) The authors define here another consolidated memory component as ARM, when they applied a cold-shock 2 hours after training. However, some publications showed that LTM is formed after only one training cycle (Krashes et al 2008, Tempel et al 1983). This makes it difficult to determine, whether appetitive ARM can be formed. Furthermore, one study showed that appetitive ARM is absent after massed training (Colomb et al 2009). Therefore, the conclusion could be also, that different starvation protocols, would lead to different stabilities of LTM. Therefore, additional experiments could help to clarify this opposing explanation. From these results, it can then be concluded either that different stable forms of LTM are formed depending on the starvation state, or that two differently consolidated memory phases (LTM, ARM) are formed, as has already been shown for aversive memory. This is also important for other statements in the manuscript, and

therefore the authors should address this. For example, the findings about the insulin receptor (is it two opposing memories or different stabilities of LTM).

The flies indeed develop different types of memory depending on the length of starvation and the internal energy supply.

Reviewer #2 (Public Review):

How organism physiological state modulates establishment and perdurance of memories is a timely question that the authors aimed at addressing by studying the interplay between energy homeostasis and food-related conditioning in Drosophila. Specifically, they studied how starvation modulates the establishment of short-term vs long-term memories and clarified the role of the monoamine Octopamine in food-related conditioning, showing that it is not per se involved in formation of appetitive short-term memories but rather gates memory formation by suppressing LTM when energy levels are high. This work clarifies previously described phenotypes and provides insight about interconnections between energy levels, feeding and formation of short-term and long-term food-related memories. In the absence of population-specific manipulation of octopamine signaling, it however does not reach a circuit-level understanding of how these different processes are integrated.

Strengths

- *Previous studies have documented the impact of Octopamine on different aspects of food-related behaviors (regulation of energy homeostasis, feeding, sugar sensing, appetitive memory...), but we currently lack a clear understanding of how these different functions are interconnected. The authors have used a variety of experimental approaches to systematically test the impact of internal energy levels in establishment of appetitive memory and the role of Octopamine in this process.*
- *The authors have used a range of approaches, performed carefully controlled experiments and produced high quality data.*

Weaknesses

(1) In the tbh mutant flies, Tyramine -to- Octopamine conversion is inhibited, resulting not only in a lack of Octopamine, but also in elevated levels of Tyramine. If and how elevated levels of Tyramine contributes to the described phenotypes is unclear. In the current version of the manuscript, only one set of experiments (Figure 2) has been performed using Octopamine agonist. This is particularly important in light of recent published data showing that starvation modifies Tyramine levels. (2) Octopamine (and its precursor Tyramine) have been implicated in numerous processes, complicating the analysis of the phenotypes resulting from a general inhibition of tbh.

We thank the reviewer for raising these points. The observed memory defects of the Tbh mutants can be solely explained by loss of octopamine. We included models into the manuscript to illustrate this (Figure 2 C and Figure 7E).

To address whether the elevated levels of tyramine observed in Tbh mutants interfere with food consumption, we analyzed the effect of increased levels of tyramine and octopamine on food consumption. We included the data (Figure S2). An increase in tyramine levels did not result in a change in food intake, rather the increase in octopamine levels reduced food intake. Our data show that the reduction of food intake observed in starved Tbh mutants is due to the increased internal energy supply.

(3) The manuscript explores various aspects of the impact of energy levels on food-related behaviors and the underlying sensing and effector mechanism, both in wild-type and *tbh* mutants, making it difficult to follow the flow of the results.

We included models illustrating the results to clarify the content of the manuscript.

Reviewer #3 (Public Review):

*In this manuscript, Berger et al. study how internal energy storage influence learning and memory. Since in *Drosophila melanogaster*, octopamine (OA) is involved in the regulation of energy homeostasis they focus on the roles of OA. To do so they use the tyramine- β -hydroxylase (*Tbh*) mutant that is lacking the neurotransmitter OA and study short term memory (STM), long-term memory (LTM) and anesthesia-resistant memory (ARM). They show that the duration of starvation affects the magnitude of both short- and long-term memory. In addition, they show that OA has a suppressive effect on learning and memory. In terms of energy storage, they show that internal glycogen storage influences how long sucrose is remembered and high glycogen suppresses memory. Finally, they show that insulin-like signaling in octopaminergic neurons, which is also related to internal energy storage, suppresses learning and memory.*

This is an important study that extends our knowledge on OA activity in learning and memory and the effects the metabolic state has on learning and memory. The authors nicely use the genetic tools available in flies to try and unravel the complex circuitry of metabolic state level, OA activity and learning and memory.

Nevertheless, I do have some comments that I think require attention:

(1) The authors use RNAi to reduce the level of glycogen synthase or glycogen phosphorylase. These manipulations are expected to affect the level of glycogen. Using specific drivers the authors attempt to manipulate glycogen level at the muscles and fat bodies and examine how this affects learning and memory. The conclusions of the authors arise solely from the manipulation intended (i.e. the genetics). However, the authors also directly measured glycogen levels at these organs and those do not follow the manipulation intended, i.e. the RNAi had very limited effect on the glycogen level. Nevertheless, these results are ignored.

We agreed with the reviewer and repeated the experiments. While we could not detect differences in whole animals, we detected differences in tissues enriched for muscles or fat, e.g. thorax or abdomen. We added the data.

*(2) The authors claim in the summary that OA is not required for STM. However, according to one experiment OA is required for STM as *Tbh* mutants cannot form STM. In another experiment OA is suppressive to STM as *wt* flies fed with OA cannot form STM. Therefore, it is very difficult to appreciate the actual role of OA on STM.*

During mild starvation, the internal energy supply is greater in *Tbh* mutants than in control flies. This information is integrated into the reward system via insulin receptor signaling. Therefore, the association between the odorant and sucrose is not meaningful to the mutants and no STM is formed. At the same time there is no release of octopamine and therefore no repression of LTM. In starved animals, octopamine suppresses food intake (we added the data). This is consistent with a function of Octopamine as a signal for the presence of food. Depending on when the signal comes, this might suppress the formation of STM or LTM.

(3) The authors use t-test and ANOVA for most of the statistics, however, they did not perform normality tests. While I am quite sure that most datasets will pass normality test, nevertheless, this is required.

Thanks for pointing this out. We have included a description in the “Materials and Methods” section that explains how we tested the data for normal distribution. We corrected the figure legends accordingly.

“We used the Shapiro-Wilk test (significance level $P < 0.05$) followed by a QQ-Plot chart analysis to determine whether the data were normal distributed. “

(4) While it is logical to assume that OA neurons are upstream to R15A04 DA neurons, I am not sure this really arises from the experiment that is presented here. It is well established that without activity in R15A04 DA neurons there is no LTM. Since OA acts to decrease LTM, can one really conclude anything about the location of OA effect when there is no learning?

Normally control flies did not form memory 6 h after training, only Tbh mutants. We wanted to investigate what kind of memory develops in Tbh mutants. During the experiments of the manuscript, we kept the training procedure constant.

(5) It is unclear how expression of a dominant negative form of insulin receptor (InR) in OA neurons can rescue the lack of OA due to the Tbh mutation. If OA neurons cannot release anything to the presumably downstream DA neurons, how can changing their internal signaling has any effect?

The expression of the dominant negative form of the insulin receptor signals no food or low energy levels and activation of the insulin receptor that there is enough food. The reward is a source of food, but the energy content is not high enough to fill the energy stores. The insulin receptor activation can activate at least three different signaling cascades, one of which might regulate octopamine release.

While I stressed some comments that need to be addressed, the overall take-home message of the manuscript is supported and the authors do show that the metabolic state of the animal affects learning and memory. I do think though, that some more caution is required for some of the conclusions.

We added additional data to address the points raised.

We addressed all points raised by the reviewers, clarified the content or added more data.

Reviewer #1 (Recommendations For The Authors):

(1) Throughout the manuscript, the full stop of a sentence is always placed before the references.

We fixed this.

(2) I find the English in the manuscript not yet sufficient for publication. I suggest that the authors carefully revise the manuscript. I think if the sentences are structured a little more clearly, this paper has enormous potential to be read by your broad community.

Recommendations for the authors:

We addressed all points raised by the reviewers, clarified the content or added more data.

Reviewer #1 (Recommendations For The Authors):

(1) Throughout the manuscript, the full stop of a sentence is always placed before the references.

We fixed this.

(2) I find the English in the manuscript not yet sufficient for publication. I suggest that the authors carefully revise the manuscript. I think if the sentences are structured a little more clearly, this paper has enormous potential to be read by your broad community.

We agree and revised the manuscript. We hope the manuscript is now clearer.

(3) Sentences l114 to l117 are misleading. The authors imply that they tested the same flies for changes in odor perception or sucrose sensitivity. I assume that the authors meant that they analyzed different groups of animals.

We clarified the sentence as follows:

“To ensure that the observed differences in learning and memory were not due to changes in odorant perception, odorant evaluation or sucrose sensitivity, different fly populations of the same genotypes were tested for their odorant acuity, odorant preference and their sucrose responsiveness (Table S1).”

(4) In the title as well as in the abstract the influence of octopamine on appetitive memory formation is described in more detail, this is also the main focus of this study. However, in the introduction, the influence of the insulin receptor on memory formation is discussed first. Personally, I would describe this later in the manuscript, ideally in the results section. At this point in the manuscript, this leads to an interruption in the flow of reading.

Thanks for the suggestion. We changed the order in the introduction.

*(5) The authors could consider, since they only used *Drosophila melanogaster*, changing "*Drosophila melanogaster*" to "*Drosophila*" throughout the manuscript.*

We modified the text accordingly.

(6) All evaluations and statistical tests are state of the art. However, I have one comment. For each statistical test, a correction should be made depending on the number of tests. However, I could not determine whether this was also done for the parametric or non-parametric one-sample t-test. From the results and the methods section, I would guess not. Here I would recommend a Bonferroni correction or even better a Sidak-Holm correction. Furthermore, the authors could also go into more detail about which non-parametric one-sample t-test they used.

We described the statistic used in more detail in the material and method section.

“We used the Shapiro-Wilk test (significance level $P < 0.05$) followed by a QQ-Plot chart analysis to determine whether the data were normal distributed. For normal distributed

data, we used the Student's t test to compare differences between two groups and the one-way ANOVA with Tukey's post hoc HSD test for differences between more than two groups. For nonparametric data, we used the Mann-Whitney U test for differences between two groups and for more than two groups the Kruskal-Wallis test with post hoc Dunn analysis and Bonferroni correction. The nonparametric one-sample sign test was used to analyze whether behavior was not based on random choice and differed from zero ($P < 0.5$). The statistical data analysis was performed using statskingdom (<https://www.statskingdom.com>)."

(7) In nearly all figure legends the sentence "The letter "a" marks a significant difference from random choice as determined by a one-sample sign test ($P^ < 0.05$; $P < 0.01$)" occur. This is correctly indexed in the figures. However, I do not understand here what then $P < 0.05$; $P^* < 0.01$ means. The significance level should be described here. I would strongly recommend the authors to make the definition clearer.*

We corrected this in the figure legends (see also above).

(8) In Fig. 1B the labelling is a bit confusing. I interpret the two right groups as the mutants for octopamine, but there is still w[1118] in front.

We modified the Figure 1B.

Reviewer #2 (Recommendations For The Authors):

Suggestions

(1) Assessing the contribution of Tyramine in the observed phenotypes (for example by reducing the levels of Tyramine or its specific receptor) would help understand the contribution of Tyramine in the observed phenotypes.

See comments above.

We thank the reviewer for raising these points. The observed memory defects of the Tbh mutants can be solely explained by loss of octopamine. We included models into the manuscript to illustrate this (Figure 2 C and Figure 7E).

To address whether the elevated levels of tyramine observed in Tbh mutants interfere with food consumption, we analyzed the effect of increased levels of tyramine and octopamine on food consumption. We included the data (Figure S2). An increase in tyramine levels did not result in a change in food intake, rather the increased octopamine levels reduced food intake. Our data show that the reduction of food intake observed in starved Tbh mutants is due to the increased internal energy supply.

(2) Cell-specific inhibition of octopamine receptors should thus be performed to precisely interpret the observed phenotypes and dissect how interconnected the different phenotypes are, which is the object of this publication.

We observed that the time point and duration of octopamine application changes the behavioral output. The behavior analyzed depends on pulses of octopamine and differences of the internal energy status. A cell-specific inhibition via RNAi knock down of octopamine receptors might not clarify the issue.

(3) Defining of streamline and progressively integrating the different observations into a unifying model would improve the clarity and flow of the manuscript.

We included models explaining the observed results (Figure 2C and Figure 7E).

Minor comments

Line 129: Figure 1B should be mentioned, not 2B.

Figure 1 legend: E should be replaced by C (after A,B).

Figure S5: what are the arrows pointing to? Why are the Inr foci visible in A not seen in B? It should be mCD8-GFP and not mCD on top of the images.

We fixed this.

Reviewer #3 (Recommendations For The Authors):

Major:

(1) Can one really conclude from Figure 2A that OA acts on R15A04 DA neurons? It is well established that without activity in these DA neurons there is no LTM. Since OA acts to decrease learning, how one can conclude anything about the location of OA effect when there is no learning? With STM the situation was opposite, OA supported learning and this was abolished when DA neurons were silenced. I think some supporting experiment are required, i.e. how OA affects DA neurons activity or, alternatively, tone down a bit the writing.

Normally control flies did not form memory 6 h after training, only Tbh mutants. We wanted to investigate what kind of memory develops in Tbh mutants. During the experiments of the manuscript, we kept the training procedure constant. The inhibition of dopaminergic neurons blocks the memory of Tbh mutants. Taken together the duration of the memory, the cold-shock experiments and the inhibition of the dopaminergic neurons, Tbh develops LTM after training. This training does not evoke memory in controls.

The loss of STM in mildly starved Tbh mutants depends on the integration of the high internal energy levels via InR signaling. Reducing the internal energy levels further by extension of starvation result in STM supporting that OA is not directly involved in the formation of STM.

(2) Figure 4 requires some clarifications. In Supplementary Figure S2 the authors show that they could not manipulate glycogen levels in muscles. However, in Figure 4B they show that "Increasing glycogen levels in the muscles did not change short-term memory in 16 h starved flies, but the reduction in glycogen significantly improved memory strength (Figure 4B)" (lines 231-233). How can this be reconciled?

While we could not detect differences in whole animals, we detected differences in glycogen content in body parts enriched with muscles or fat, e.g. thorax or abdomen when using UAS-GlyP-RNAi or UAS-GlyS-RNAi under the control of the respective Gal4 drivers.

We added the data.

Likewise, the authors write that "Increasing or decreasing glycogen levels in the fat bodies had no effect on memory performance (Figure 4C)" Line (233-234). However, in Figure S2 they show that they can only increase glycogen levels but not decrease them.

As explained above the conclusion of Figure 4 "Thus, low levels of glycogen in the muscles upon starvation positively influence appetitive short-term memory, while high levels of glycogen in the muscles and fat body reduce short-term memory" lines 245-246, is not supported by the direct measurements of glycogen presented in Figure S2.

We added the data showing that the reduction or increase can be measured when analyzing the specific body parts enriched in muscles tissue or fat tissue.

(3) In cases where mutant flies do not display learning, a control should be done to see if they ate the sugar (with dye). Especially since the genetic manipulation affects metabolism.

We analyzed how much sucrose the animals consumed in the behavioral test. Tbh and controls fed and there was no difference in feeding behavior between the mutants and the controls.

“We next determined whether differences in preferences influence sucrose intake during training. Therefore, we measured the sucrose intake of starved flies in the behavioral set up. We used a food-colored sucrose solution and evaluated the presence of food in the abdomen of the fly after two 2 min (Table S1). Flies fed sucrose within 2 min and there was no difference between w1118 and TβhnM18 flies. “

(4) The use of t-test requires the data to be normally distributed. If I am not mistaken this was not demonstrated for any of the datasets used. I did a quick check on one of the datasets provided in the excel sheet and it is normally distributed. Therefore, please add normality test for all data sets. If some do not pass normality, please use a suitable non-parametric test.

We added normality test to all data sets and used non-parametric tests for non-normal distributed data. We clarify this in the material and method section and the figure legends.

(5) The authors show that OA suppresses also STM. This result is in contradiction to previous published results. This by itself is not a problem. However, this result also seems to me in contradiction to the authors own results. According to Figure 1B, OA is required for STM as it absence in the tbh mutant results in loss of STM. According to Figure 2C, OA is reducing STM as wt flies fed with OA just prior to learning do not form STM. This appears in other places in the manuscript as well.

In addition, in the text lines 178-180, the authors write "A short pulse of octopamine before the training inhibits the STM. Thus, octopamine is a negative regulator of appetitive dopaminergic neuron-dependent long-term memory and can block STM." But in the summary they write "Octopamine is not required for short-term memory, since octopamine deficient mutants form appetitive short-term memory to sucrose and to other nutrients depending on the internal energy status." So, the take-home message regarding OA and STM is unclear.

The authors need to better clarify this point.

We clarified these points. See comments above. The loss of memory in Tbh mutants is not due to loss of octopamine, but increased energy levels that changes the reward properties of sucrose.

(6) The manuscript is very difficult to follow. The authors constantly change between 16 and 40 hours starvation, short term memory, 3 hour memory and 6 hour memory. I think it would have been better to have a more focused manuscript. However, if this is not possible, I recommend preparing a diagram with the different neurons or signaling pathways (i.e. insulin) and how they affect each other. Also, perhaps add to each figure a panel describing exactly the experimental conditions. I think also simplifying the text and adding more conclusions throughout the results section will help the readers to follow.

Finally, I think that it would help understanding the conclusions if the authors can add a diagram of the flow that they think occurs. For example, the authors show that glycogen suppresses learning as its reduction increases learning. They also show that InR activity receptor suppresses learning as its KD also increases learning. If I am not mistaken the link between the two is not straight forward (but I may be wrong here). A diagram of the flow would be very helpful.

We prepared diagrams summarizing and explaining the results.

Minor

(1) I may not have understood correctly as I am not sure that I found Table S1.

Also, there was no legend for Table S1.

Nevertheless, if I understood correctly, the authors write that "Before the experiments, flies were tested to determine whether they perceived the odorants, preferred one odorant over other and responded to the reward similarly to ensure that the observed differences in behavior were not due to changes in odorant perception or sucrose sensitivity (Table S1)." However, according to the Table that I found it seems that following 40h starvation wt flies show preference to OCT whereas this does not occur for the mutant. Also, it seems that at 16h the mutant has a much higher preference to the odors than after 40h. This is a bit odd. I am also not sure what the balance value refers to. Finally, the mutant shows really low 2M sucrose preference after 40h. In general, this set of experiments requires a bit more explanation.

I think it is better to show these experiments using graphs and add this to the supplementary figures.

We clarified the experiments in the result section as follows and added an explanation to the material and method section. We tested the odorant acuity and sucrose preference for all genotypes used in the manuscript and added the data to the Table S1.

"The flies of the different genotypes sensed the odorants and evaluated them as similar salient in comparison. This is important to avoid a bias in the situation where flies have to choose between the two odorants after training. They also sensed sucrose. We next determined whether differences in preferences influence sucrose intake during training. Therefore, we measured the sucrose intake of starved flies in the behavioral set up. We used a food-colored sucrose solution and evaluated the presence of food in the abdomen of the fly after two 2 min (Table S1). Flies fed sucrose within 2 min and there was no difference between w1118 and TβhnM18 flies."

(2) Line 129 should be Figure 1B

Is corrected.

(3) Line 133, Figure 1C, how can one explain the negative reinforcement? I can understand no reinforcement, but negative?

The effect of glucose might be doses dependent. 0.15 M sucrose is a much closer to a realistic concentration found in fruits than 2 M sucrose and might therefore elicit aversion. When animals are starved enough they might find any food source attractive, even when the concentrations of sucrose is unrealistic.

(4) Figure 1, why are the graphs different between panel B and C?

Is corrected.

(5) In Figure S1, are the *TβhnM18* groups differ significantly from zero? I think they are, so better to state this somewhere. If not, the claims in lines 134-135 are not supported by the data.

We added the significance and added the data to Figure 1.

Figure S1 legend: there is no A panel. Also "below box blots" should be box plots.

Thanks for pointing that out. We corrected it.

(6) It is not clear what is the duration of starvation used in Figure 2A. I assume that 16h and sucrose 2M used were used, but I would state that explicitly.

We added the information to the figure legends.

(7) Figure 2A is missing a control of flies with both the driver and UAS shibirets at the permissive temperature.

We added the controls to the supplement (Figure S1).

(8) It seems to me that Figure 3B, in which the author state that "Only after 40 h of starvation did *TβhnM18* mutants show a similar preference to control sucrose consumption" (line 198) is somewhat in contradiction to Table S1 in which I see Sucrose preference for wt 0.36 and for *tbh* 0.17. I think this comment arise because I did not understand Table S1 correctly, so please better explain.

We rewrote this section.

(9) In Figure 3C, consider not using *std* as this stands for standard deviation and may be confusing.

We now use the term "food" instead of "std" and explained in the legend that food means standard fly food.

We fixed this.

(10) Please check the Supplementary Figures. I think Figures S2 and S3 are switched.

We fixed this.

(11) There is a mistake in Figure S3A. The right column should have another "+" sign.

Thanks, we fixed this.

(12) I am somewhat puzzled by Figures 4 and 5. If I understand correctly figure 4B w1118 *mef2-G4* is exactly the same experiment as Figure 5A w1118 *mef2-G4* and yet in Figure 4B performance index is 0.2 and in Figure 5A about 0.4. According to other comparisons it seems to me that these will be significantly different and yet it is the same experiment.

They are two independent experiments done at different times. The controls were independently repeated.

| (13) Line 273 should be Figure 5C.

Is corrected.

| (14) I don't think this is a correct sentence "Virgin females remembered sucrose significantly better than mated females." Line 274.

Reads now:

"Virgin females remembered the odorant paired with sucrose significantly better than mated females."

| (15) Line 340 there is no Figure 1E

Is fixed (1 C)

| (16) The data excel file is difficult to follow. In Figure 2 there are references to Figure 5. The graphs are pointing to other files. Text is not always in English. It is not clear what W stands for. I recommend making it more accessible.

We corrected the data excel files.

| (17) The manuscript is difficult to follow. I recommend preparing a diagram with the different neurons or signaling pathways (i.e. insulin) and how they affect each other.

We improved the data presentation by

- a) adding a model showing the kinetics of memory formation in controls and mutants (Figure 2C)
- b) a model explaining how the internal state is integrated into the formation of memory (Figure 7D).