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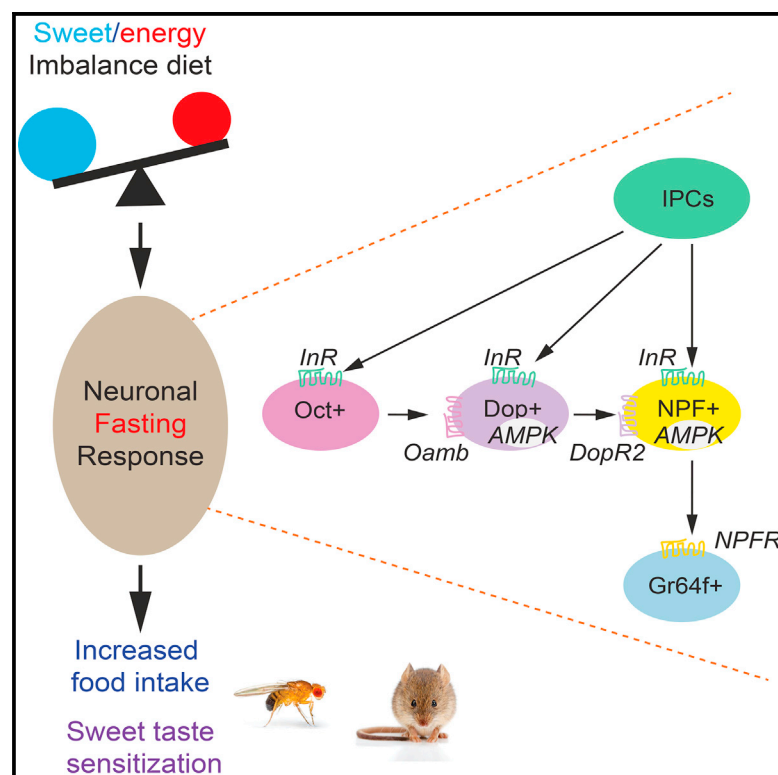
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# Cell Metabolism

## Sucralose Promotes Food Intake through NPY and a Neuronal Fasting Response

### Graphical Abstract



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### In Brief

Wang et al. show that chronic consumption of the synthetic sweetener sucralose causes increased food intake in the fruit fly. This effect involves a neuronal starvation pathway, and a similar effect was observed in mice. These findings suggest that disrupting the sweet/energy balance of food may have unanticipated consequences.

### Highlights

- Chronic sucralose diet triggers increased food intake
- Dietary sucralose creates a sweet/energy imbalance
- Sweet/energy imbalance activates a conserved neuronal starvation response
- Sucralose effect on feeding is conserved from flies to mammals

### Accession Numbers

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# Sucralose Promotes Food Intake through NPY and a Neuronal Fasting Response

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## SUMMARY

Non-nutritive sweeteners like sucralose are consumed by billions of people. While animal and human studies have demonstrated a link between synthetic sweetener consumption and metabolic dysregulation, the mechanisms responsible remain unknown. Here we use a diet supplemented with sucralose to investigate the long-term effects of sweet/energy imbalance. In flies, chronic sweet/energy imbalance promoted hyperactivity, insomnia, glucose intolerance, enhanced sweet taste perception, and a sustained increase in food and calories consumed, effects that are reversed upon sucralose removal. Mechanistically, this response was mapped to the ancient insulin, catecholamine, and NPF/NPY systems and the energy sensor AMPK, which together comprise a novel neuronal starvation response pathway. Interestingly, chronic sweet/energy imbalance promoted increased food intake in mammals as well, and this also occurs through an NPY-dependent mechanism. Together, our data show that chronic consumption of a sweet/energy imbalanced diet triggers a conserved neuronal fasting response and increases the motivation to eat.

## INTRODUCTION

Synthetic or non-nutritive sweeteners (NNSs) can be found in thousands of products and are consumed by billions of people annually. Despite widespread usage, the impact of a synthetically sweetened diet on metabolic health remains unclear and controversial. Experiments using animal models support a role for NNSs in metabolic dysregulation; however, the molecular mechanisms involved are unclear. For example, rats given a saccharin solution showed increased food intake compared to animals given water, and this increase occurred even when the sweetener was removed from the diet (Tordoff and Friedman, 1989a, 1989b, 1989c, 1989d). Beyond food intake, numerous studies have shown that animals consuming synthetic sweet-

eners exhibit weight gain (Feijó et al., 2013; Swithers and Davidson, 2008; Swithers et al., 2010, 2013), accumulation of body fat (Swithers et al., 2010, 2013), or impaired glucose homeostasis (Suez et al., 2014; Swithers et al., 2012) or exhibit weaker caloric compensation (Swithers et al., 2010).

The majority of observational studies addressing NNS consumption show an association with metabolic dysregulation. An early study reported a significant correlation between NNS consumption and weight gain in an ~80 000 participants study (Stellman and Garfinkel, 1988). Other independent studies confirmed these associations, with synthetically sweetened beverage consumption being associated with a much higher incidence of metabolic syndrome (odds ratio ~1.93) when compared to non-users (Fowler et al., 2008; Lutsey et al., 2008), and NNS consumption has been identified as a significant risk factor for metabolic disease in children (Blum et al., 2005), middle-aged adults (Dhingra et al., 2007), and the elderly (Fowler et al., 2015). One study showed that NNS consumers exhibit reduced weight gain (Schulze et al., 2004); however, these participants showed increased risk for developing diabetes in an 8-year follow-up. Furthermore, human intervention studies have also shown that ingestion of NNS could enhance appetite (Blundell and Hill, 1986; Rogers and Blundell, 1989), promote hunger (Tordoff and Alleva, 1990), and increase food consumption (Lavin et al., 1997; Rogers and Blundell, 1989; Tordoff and Friedman, 1989a), resulting in impaired glucose tolerance (Pepino et al., 2013; Suez et al., 2014). However, other studies have reported no major effect or weight loss as a result of consuming NNSs (De La Hunty et al., 2006; de Ruyter et al., 2012; Raben et al., 2002), and the overall impact of NNS on metabolic health remains controversial.

In order to more definitively determine the impact of NNSs on energy homeostasis, and potential mechanisms involved in such effects, we used the fruit fly *Drosophila melanogaster*. Fruit flies possess systems that assess both sweetness and caloric content of food (Burke and Waddell, 2011; Dus et al., 2011, 2013, 2015; Fujita and Tanimura, 2011; Gordesky-Gold et al., 2008; Stafford et al., 2012) and the insulin and gustatory reward pathways are conserved from invertebrates to mammals (Burke et al., 2012). Moreover, the available genetic tools (Dietzel et al., 2007), conserved metabolic pathways (Pospisilik et al., 2010) and controlled behavioral assays (Dethier, 1976; Ja et al., 2007)

make the fruit fly an ideal system to dissect the impact of synthetic sweetness on energy homeostasis.

## RESULTS

### Synthetically Sweetened Food Has a Broad Impact on Energy Homeostasis

Of the commercially available NNSs, fruit flies find sucralose the most palatable (Gordesky-Gold et al., 2008). Sucralose is highly stable and thus is often used to sweeten food that requires baking or products with a longer shelf life. To investigate if consumption of a sucralose-sweetened diet impacts fly energy homeostasis, we fed flies either a standard control diet (sucrose and yeast) or a control diet plus the synthetic sweetener sucralose for various times. Animals were then removed from the sucralose diet and food (sucrose and yeast) intake was determined over the next 24 hr using the capillary feeding (CAFE) assay. Animals consuming the sweetened diet for 1–4 days did not differ from controls; however, flies exposed to a diet laced with sucralose for prolonged ( $\geq 5$  days) periods exhibited a robust increase in subsequent food intake (Figure 1A) and calories consumed (Figure 1B); this effect was observed in both male (Figures 1A and 1B) and female (not shown) animals. Interestingly, the appetite-stimulating effect was reversible, and food intake returned to control levels within 3 days of sucralose being removed from the diet (Figure 1A). The proboscis extension response (PER) assay can also be used to evaluate taste sensitivity and the overall motivation to consume food (Dethier, 1976). Interestingly, compared to control-treated flies, animals fed the sucralose diet for 6 days showed a marked increase in sucrose sensitivity by PER (Figure 1C), but removing sucralose from the diet abolished this sensitization after 3 days (Figure 1D). One report (Suez et al., 2014) recently showed that in mice, saccharin could trigger metabolic abnormalities through actions on the host microbiome. We tested if a similar mechanism contributed to the appetite-stimulating effect of sucralose observed in our system. However, after prolonged consumption of a sucralose-sweetened diet, flies continued to exhibit a robust appetite-stimulating response to sucralose pretreatment in both tetracycline-treated (Figure S1A) or germ-free (Figure S1B) flies, indicating that in *Drosophila* this effect is independent of the microbiome. In addition to sucralose, flies find L-glucose, a sweet-tasting non-caloric enantiomer of D-glucose, palatable. Importantly, 6 days ingestion of a diet containing L-glucose also had a sustained appetite-stimulating effect (Figure 1E).

Sustained sucralose ingestion did not cause a significant change in body weight (Figure S1C), triglycerides (Figure S1D), glycogen (Figure S1E), or resting hemolymph glucose (Figure S1F). However, sucralose-treated flies did show impaired glucose tolerance in an oral glucose tolerance test (Figure 1F). Furthermore, flies became hyperactive after 4 days of sucralose-sweetened food (Figure 1G) but not after L-glucose-sweetened food (Figure S1G), and sucralose-treated animals also exhibited sleep fragmentation (Figure 1H) and reduced total sleep (Figure 1I). Elevated energy expenditure caused by increased activity and altered sleep behavior may explain why these animals do not significantly expand energy stores despite increased caloric intake. Of interest, similar effects (i.e., altered sleep behavior and insomnia) have been reported in human sub-

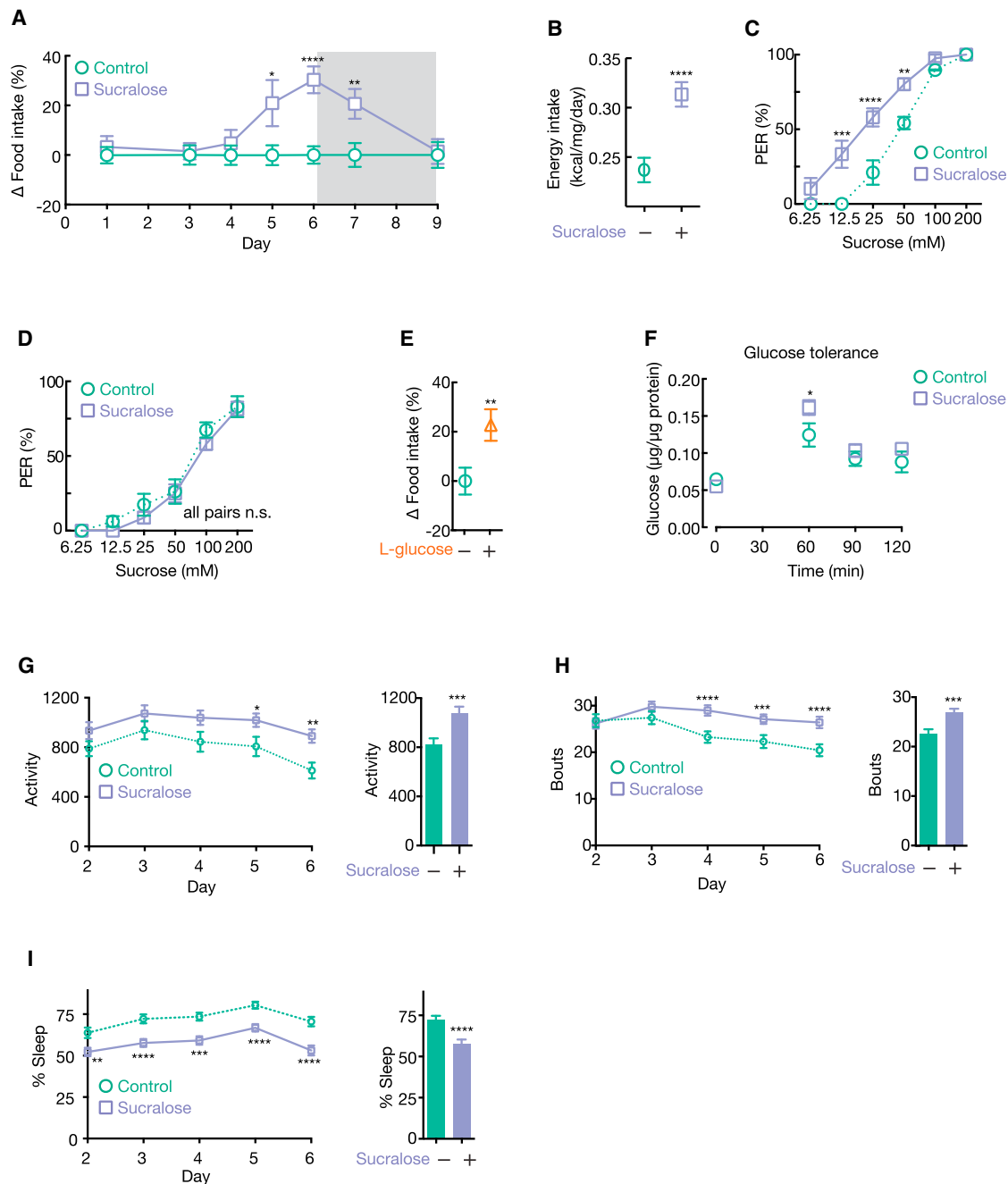
jects ingesting NNSs (Roberts, 1988). These data show that, in flies, prolonged ingestion of sucralose-sweetened diet triggers broad physiological changes similar to effects reported in rodents and human studies.

### Sucralose Ingestion Increases Appetite through Sweet Taste Receptor Gr64a

Sucralose may increase appetite directly via taste-dependent or indirectly via taste-independent mechanisms (Burke and Wadell, 2011; Dus et al., 2011; Fujita and Tanimura, 2011). To test this, we manipulated sweet taste neurons genetically using the *Gr64f-Gal4* driver line (Klapoetke et al., 2014). Synaptic silencing of sweet taste neurons with tetanus toxin (*Gr64f-Gal4* > *UAS-TNT*), but not an inactive toxin (*UAS-ITNT*), blocked the sucralose effect (Figure 2A), demonstrating these neurons are required to promote increased appetite. Importantly, prolonged activation of sweet taste neurons was also sufficient to drive a lasting increase in food intake, since 6 days of thermogenetic preconditioning (Hamada et al., 2008) of these cells mimicked sucralose pretreatment and enhanced appetite for 24 hr after temperature stimulation ended (Figure 2B). In flies, many sweet tastants require the sweet taste receptors *Gr5a* or *Gr64a* (Dahanukar et al., 2007). We found that sucralose requires *Gr64a* to mediate increased food intake (Figure 2C; see Table S1A for basal food intake) and PER sensitization to sucrose (Figure 2D; see Table S1B for  $S_{50}$  values). Thus, prolonged ingestion of a sweetened diet containing sucralose acts specifically through the sweet receptor *Gr64a*, and importantly, sustained thermogenetic activation of sweet taste neurons is sufficient to produce a lasting increase in food intake even in the absence of the sucralose-sweetened diet.

### The Fly Insulin System Is Critical for the Sucralose Effect on Appetite

To understand how sucralose promotes changes in food intake, we used mRNA sequencing. Flies were collected at day 0, or after 6 days on a sucralose-sweetened diet; mRNA was extracted from fly heads; and RNA seq was performed. 175 transcripts showed differential regulation in sucralose-fed versus control flies, with 30 transcripts upregulated and 145 downregulated (Figure 3A; Table S2). The 30 upregulated transcripts included the fly insulin receptor *InR* (Figure 3B), and upregulation of the *InR* starting 4 days after sucralose diet was confirmed by qPCR (Figure 3C), suggesting an involvement of the fly insulin system in sucralose-induced feeding changes. To directly test a role for neuronal *InR*, we used tissue-specific transgenic RNAi (Dietzl et al., 2007). While the pan-neuronal driver line *nSyb-Gal4*, or the *UAS-InR* RNAi lines crossed to *w<sup>1118</sup>* control lines, exhibited robust increases in food intake following 6 days on the sucralose diet, when the *InR* was knocked down constitutively (Figure 3D; *nSyb-Gal4* > *UAS-InR* RNAi) or inducibly (Figure 3E; *nSyb-Gal4* > *UAS-InR* RNAi *UAS-Gal80<sup>ts</sup>*), these flies no longer showed an increase in appetite or PER response (Figure 3F), and these findings were confirmed with a second RNAi hairpin (not shown). The output from insulin-producing cells was also critical for the appetite-stimulating effect of sucralose pre-conditioning, since blocking output with tetanus toxin (*Dilp2-Gal4* > *UAS-TNT*) but not inactive control toxin (*Dilp2-Gal4* > *UAS-ITNT*) abolished the sucralose effect (Figure 3G),



**Figure 1. Consumption of a Synthetically Sweetened Diet Has Broad Impact on Energy Homeostasis**

(A) A sucralose-sweetened diet caused a reversible increase in food intake. Animals were fed a control diet  $\pm$  sucralose for 1 to 6 days, and then food intake was measured over 24 hr by CAFE assay. The gray shading depicts animals fed sucralose for 6 days and then switched to control diet for 1–3 days. Unless otherwise indicated, 6 days pretreatment was used for all subsequent experiments;  $n \geq 20$  replicates (five animals per replicate for all feeding experiments).

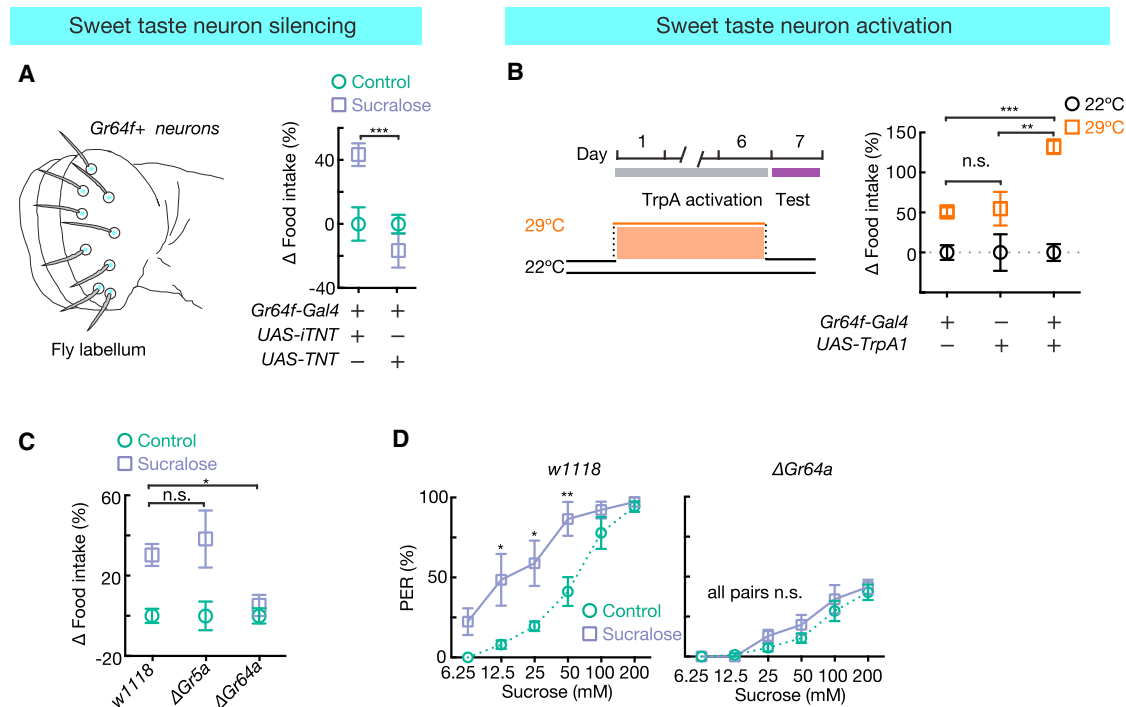
(B) Animals fed with sucralose diet exhibited increased caloric consumption;  $n \geq 18$ .

(C and D) (C) A sweetened diet caused an enhanced proboscis extension response (PER); (D) this effect was reversed after 3 days' removal of sucralose from the diet;  $n \geq 3$  replicates (10–13 animals per replicate for all PER experiments).

(E) Pretreatment with a diet containing the NNS L-glucose also caused increased feeding;  $n \geq 20$ .

(F) Prolonged sucralose-sweetened diet caused glucose intolerance ( $n \geq 8$  replicates).

(G–I) Sucralose diet caused (G) hyperactivity, (H) increased sleep fragmentation, and (I) decreased sleep time; a representative experiment ( $n = 32$  animals) is shown. All animals tested were *WT* (*w<sup>1118</sup>*) flies. Data represented as mean  $\pm$  SEM; unpaired t test was used for all analysis, except for PER where two-way ANOVA with Sidak's multiple comparisons test was used. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ . n.s., not significant. Also see Figure S1.



**Figure 2. A Sucralose-Sweetened Diet Promotes Hunger through the Sweet Taste Receptor Gr64a**

(A) Blocking synaptic output from Gr64f+ sweet taste neurons prevented increased appetite in response to sweetened food;  $n \geq 10$ .  
 (B) TrpA1 activation in Gr64f+ neurons mimicked the sucralose effect on food intake. Scheme of TrpA1 activation, flies were raised at 22°C and then taste neurons expressing TrpA1 were activated at 29°C for 6 days. Food intake was then measured at 22°C between day 6 and 7 by CAFE assay;  $n \geq 7$ .  
 (C)  $\Delta$ Gr64a mutant animals did not increase food intake in response to a sucralose diet;  $n \geq 12$ .  
 (D)  $\Delta$ Gr64a mutant animals did not exhibit enhanced sucrose sensitivity after a sucralose diet;  $n \geq 5$ . Data represented as mean  $\pm$  SEM. One-way ANOVA with Turkey's multiple comparisons test was used for all analysis except PER, where two-way ANOVA with Sidak's multiple comparisons test was used. \* $p < 0.05$  and \*\* $p < 0.01$ . n.s., not significant. Also see Table S1.

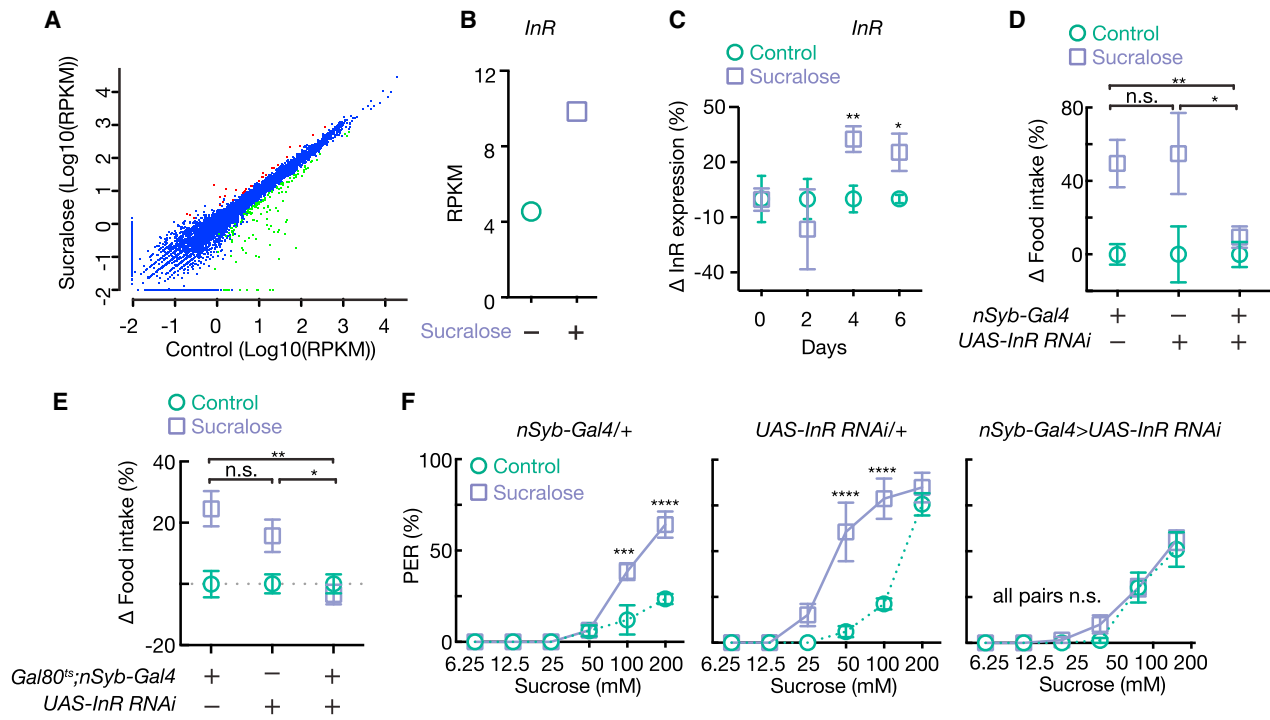
and inducible silencing of synaptic output was also sufficient to block the sucralose response (Figure 3H). Importantly, inducible thermogenetic activation of insulin-producing cells using TrpA1 (*Dilp2-Gal4 > UAS-TrpA1*) mimicked sucralose treatment and was sufficient to promote a lasting increase in food intake (Figure 3I), and this is consistent with human studies where endogenously or exogenously increasing insulin levels also promotes food intake (Rodin et al., 1985; Ryan et al., 2008). Taken together, sucralose-sweetened food triggers the fly insulin system (Figure 3J) to promote appetite, and thermogenetic activation of this system in the absence of sucralose is sufficient to mediate a lasting increase in food intake.

### Sucralose Promotes Hunger through a Pathway Involving Octopamine and Dopamine

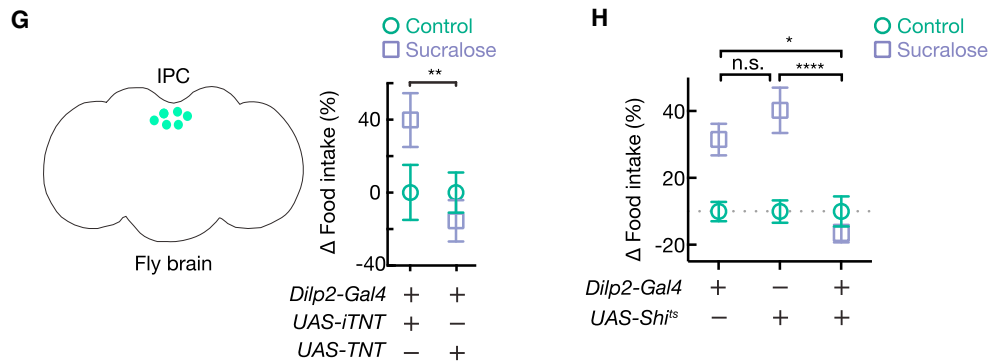
Since sucralose works through a taste-dependent mechanism, and food consumption can be rewarding, we reasoned sucralose might also act through a reward pathway. The gustatory reward pathway is an ancient system conserved from invertebrates like *Drosophila* through to mammals (Burke et al., 2012). The biogenic amine octopamine, which is related to norepinephrine, is a major neurotransmitter in the insect reward pathway (Hammer, 1993; Schwaerzel et al., 2003) and can promote gustatory reward in flies (Burke et al., 2012; Kim et al., 2013). Octopamine is synthesized from tyrosine, and this requires tyrosine

decarboxylase (TDC) and tyramine  $\beta$ -hydroxylase (Tbh). Tbh mutant (*Tbh<sup>M18</sup>*) flies (Burke et al., 2012), which cannot produce octopamine, were indeed resistant to the effect of persistent sucralose ingestion (Figure 4A). Importantly, when we blocked synaptic output in octopamine-producing cells (*Tdc2-Gal4 > UAS-TNT*), animals were completely resistant to the appetite-stimulating effect of a sweetened diet, and in this case, prolonged sucralose ingestion had an appetite-suppressing effect (Figure 4B). Moreover, we found octopamine-producing neurons require the fly insulin receptor to promote increased food intake (Figure 4C) and PER sensitization (Figure S2A) in response to sucralose. Thermogenetic activation of octopamine-producing neurons (*Tdc2-Gal4 > UAS-TrpA1*) could not mimic sucralose pretreatment (Figure S2B). Thus, the octopamine system is required downstream of insulin signaling to enhance appetite in response to a sucralose-sweetened diet.

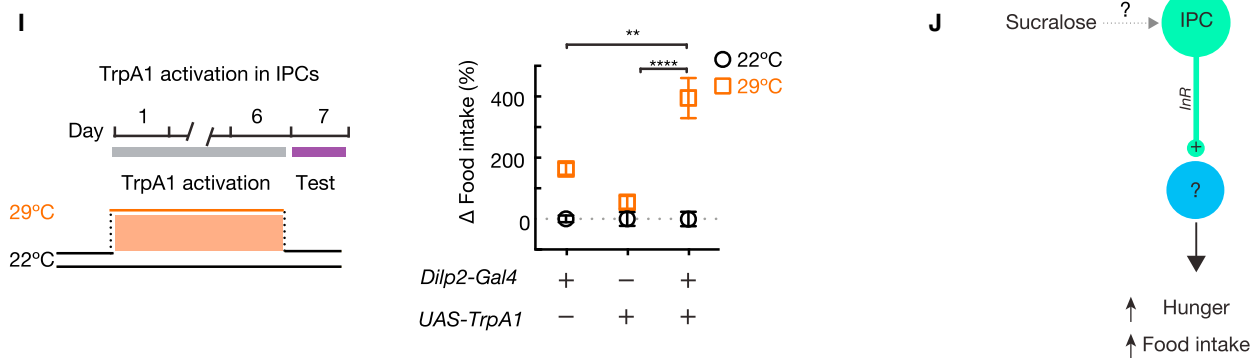
Next using pan-neuronal RNAi, we tested all fly octopamine receptors and found only *Oamb* (Figures 4D and S2C) was required for increased appetite. This was confirmed using a second RNAi hairpin (data not shown), as well as using an *Oamb* mutant line (*oamb<sup>584</sup>*), which no longer increased food intake (Figure S2D) or showed enhanced PER response to sucrose following the synthetically sweetened diet (Figure 4E). Octopaminergic neurons have been shown to interact directly with dopaminergic neurons to promote gustatory reward (Burke



### IPC silencing



### IPC activation



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et al., 2012; Huetteroth et al., 2015). Moreover, dopamine has been shown to regulate taste behavior (Marella et al., 2012) and constitutes a major gustatory reward pathway both in flies (Burke et al., 2012) and mammals (de Araujo et al., 2008). In flies, dopaminergic neurons can be manipulated genetically using *Gal4* driven by the *tyrosine hydroxylase* promoter (*TH-Gal4*) (Friggi-Grelin et al., 2003). To assess if dopamine is involved in the appetite-stimulating effect of sucralose, we blocked synaptic output from dopamine neurons (*TH-Gal4>UAS-TNT*), which caused a complete resistance to sucralose pretreatment (Figure 4F). Interestingly, however, thermogenetic activation of dopamine-producing cells (*TH-Gal4 > UAS-TrpA1*) was not sufficient to mimic the appetite-stimulating effects of sucralose (Figure S2E). Moreover, when *InR* or *Oamb* was knocked down in dopaminergic neurons, these flies were resistant to the proappetitive (Figures 4G and 4H) and PER-sensitizing (Figures S2F and S2G) effects of sucralose ingestion. To determine the responsible dopamine receptor, we tested all known dopamine receptors (pan-neuronal *nSyb-Gal4 > UAS-DopR* RNAi) and identified *DopR2* as being required for the appetite-stimulating effects of sweetened food (Figures 4I and S2H). This was confirmed using a second hairpin (data not shown) and a *DopR2* hypomorphic mutant line (*DopR2<sup>MB05108</sup>*) (Liu et al., 2012b), both of which were resistant to the sucralose effect on food intake (Figure S2I) and PER receptivity (Figure 4J). Of note, a second dopamine receptor, *DopEcR*, was also required for sucralose to increase appetite (Figures 4I and S2I) and PER response (Figure 4J). *DopEcR* has previously been implicated in regulating PER in response to starvation (Inagaki et al., 2012). Furthermore, to identify if there is bi-directional signaling between dopamine and insulin or octopamine-producing neurons, we knocked down *DopR2* using *Dilp* or *Tdc2-Gal4*; however, this had no effect on food intake (Figure S2J). Of note, *TH-Gal4* does not target the PAM cluster of dopaminergic neurons that have been implicated previously in gustatory appetitive memory, suggesting alternative mechanism of action in this system (Liu et al., 2012a). Overall, the dopamine system is necessary for sucralose to increase appetite, and this response requires insulin (*InR*) and octopamine (*Oamb*) receptors in dopamine-producing neurons (Figure 4K).

To identify the components of this response downstream of dopamine, we crossed *UAS-DopR2* to candidate neuronal *Gal4* drivers. The sucralose response did not require *DopR2* expression in sweet taste neurons (*Gr64f-Gal4 > DopR2* RNAi; Figure S3A). However, when we knocked down *DopR2* in NPF-

producing neurons, the sucralose effects on food intake (Figure 5A) and PER sensitization (Figure 5B; RNAi control in Figure S3B) were completely blocked. Of note, by comparing *S<sub>50</sub>* values, *NPF-Gal4 > DopR2* RNAi animals exhibit a significant baseline sucrose sensitization that could explain the observed loss of sucralose response (Figure S3C); however, basal food intake was unaffected in these animals (data not shown) and thus the observed loss of sucralose effect on food intake is dependent on *DopR2* expression in NPF+ neurons. Neuropeptide F (NPF), the fly ortholog of the potent mammalian appetite-stimulating neurotransmitter neuropeptide Y (NPY) (Loh et al., 2015), has been shown to directly interact with dopaminergic neurons to regulate feeding behavior in flies (Krashes et al., 2009; Wang et al., 2013; Wu et al., 2005). NPF was indeed involved in the sucralose response, as output from both NPF-producing (Figure 5C) and *NPFR*-expressing (Figure S3D) neurons were critical for the appetite-stimulating activity of sweetened food. Moreover, pan-neuronal knockdown of the *NPFR* also conferred resistance to sucralose effects on food intake (Figure 5D) and PER sensitization (Figure S3E; confirmed with a second hairpin, not shown). Of note, *NPFR* expression was not required in insulin-producing cells (Figure S3F), but was required in dopamine-producing cells (Figure 5E), indicating a bi-directional interaction between these two reward pathways. Further, NPF-producing neurons did not require *Oamb* (not shown) but did require the fly insulin receptor for sucralose to cause increased appetite (Figure 5F) and PER sensitization (Figure S3G). Together, these data demonstrate that insulin acts upstream of NPF-producing cells to promote food intake in response to sucralose.

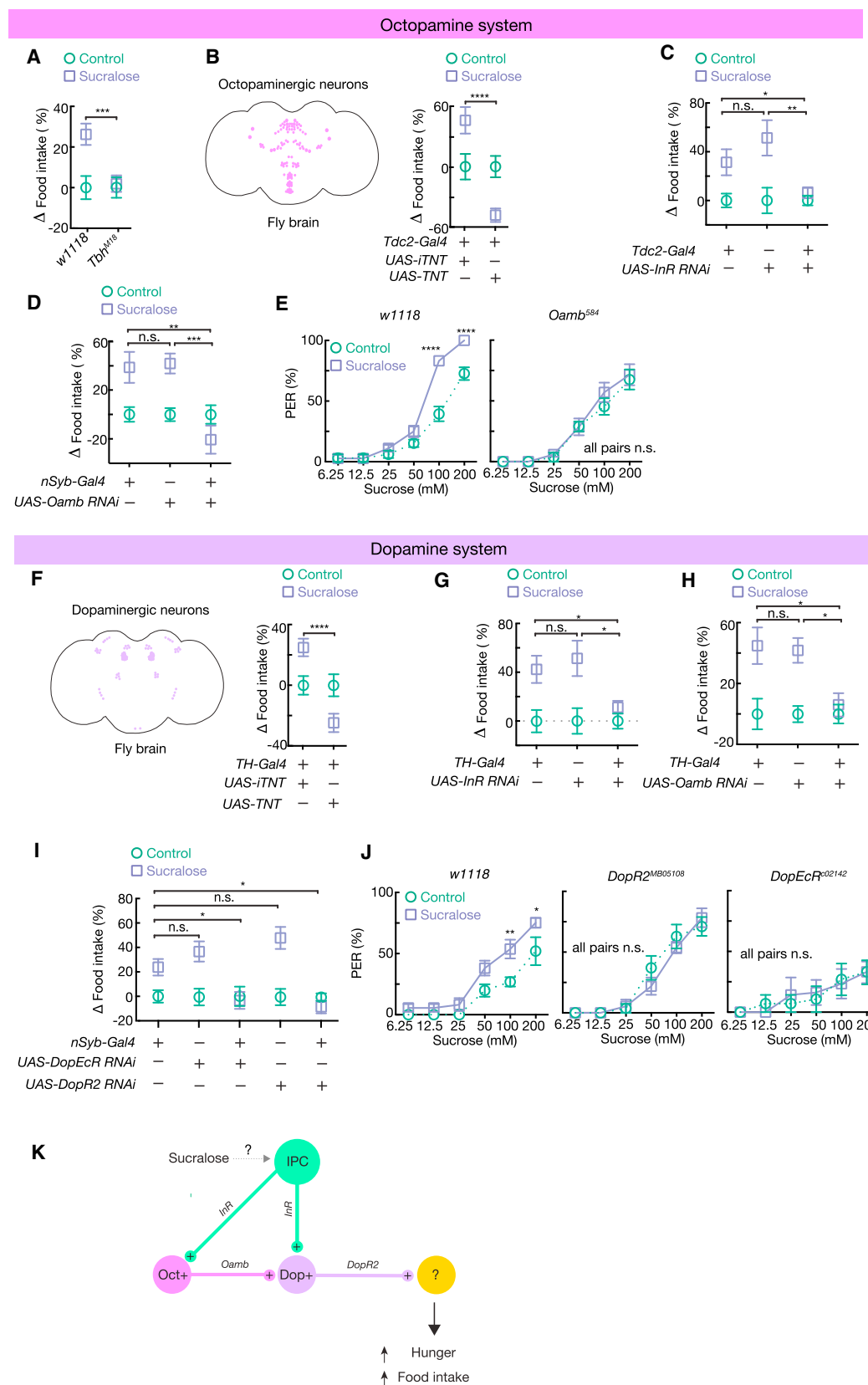
### Sucralose Causes Sweet Taste Sensitization through NPFR in Taste Neurons

Given the role for *NPFR* in modulating food preference (Wu et al., 2005), and that sweet taste neurons are involved in the sucralose response (Figure 2), we hypothesized that *NPFR* was acting directly on sweet taste neurons. Consistent with this idea, knockdown of *NPFR* in sweet taste neurons nullified the sucralose-mediated increased food intake (Figure 5G) and PER sensitization (Figure 5H). We next tested if sucralose was directly altering sweet taste perception using electrophysiological recordings from single taste sensilla on the fly labellum (Figure 5I). Flies were fed control food  $\pm$  sucralose for 6 days, and then sucrose responses were recorded. Compared to control food, flies on the sucralose-sweetened diet exhibited a strong (50%)

### Figure 3. Sucralose-Sweetened Food Promotes Hunger via the Insulin System

- (A) Differentially expressed genes in response to 6 days on a sucralose-sweetened diet.
- (B) Expression level (RPKM) of the fly insulin receptor (*InR*) before and after sucralose treatment.
- (C) qPCR confirmed *InR* expression was upregulated after sucralose diets; *n* = 3 replicates (30–40 animals per replicates).
- (D) Pan-neuronal knockdown of *InR* blocked increased food intake (*n*  $\geq$  15).
- (E) Inducible pan-neuronal knockdown of *InR* using *Gal80<sup>ts</sup>* blocked increased food intake, flies raised at 18°C and tested at 29°C; *n*  $\geq$  21.
- (F) Pan-neuronal knockdown of *InR* blocked increased PER sensitivity (*n*  $\geq$  3).
- (G) Blocking secretion from insulin-producing cells (IPCs) suppressed the appetite-stimulating effect of the sweetened diet; *n*  $\geq$  14.
- (H) Inducible silencing of IPCs output using *UAS-shirbire<sup>ts</sup>* blocks the appetite-stimulating effect of a sucralose-sweetened diet, flies raised at 25°C and tested at 29°C; *n*  $\geq$  10.
- (I) 6 days of thermogenetic activation of IPCs caused a lasting increase in food intake; *n*  $\geq$  7.
- (J) Schematic of how sucralose promotes hunger and food intake through the fly insulin system. Data represented as mean  $\pm$  SEM; unpaired t test was used for single comparison, one-way ANOVA with Turkey's multiple comparisons test was used for multiple comparisons, and two-way ANOVA with Sidak's multiple comparisons test was used for PER assay. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, and \*\*\*\**p* < 0.0001. n.s., not significant. Also see Table S2.





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sensitization to sucrose (Figure 5J). Importantly, when *NPFR* was knocked down in *Gr64f*+ sweet taste neurons, these animals showed a complete loss of sweet taste sensitization in response to sucralose (Figure 5J). Thus, a sucralose-sweetened diet alters the intensity of natural sugar through NPF acting directly on sweet taste neurons (Figure 5K).

### Consumption of Sucralose Promotes Hunger through Activation of Neuronal AMPK

We next addressed if the effect of a sucralose diet is due to the sweetness itself or a result of the imbalance between the sweetness versus actual caloric content of the ingested food. If this response was due to enhanced dietary sweetness alone, a similar effect should be observed using a nutritive sweetener. Importantly, while pretreatment with the sucralose-sweetened diet promoted increased food intake, 6 day pretreatment with 2× or 3× the sucrose content of control food caused a significant reduction in food intake relative to 1× sucrose control food (Figure 6A). To address the dietary imbalance between perceived and actual caloric content, we used sorbitol, a tasteless source of sugar calories for flies (Burke and Waddell, 2011; Fujita and Tanimura, 2011). While 6-day treatment of the synthetically sweetened diet again promoted increased food intake, the additional inclusion of nutritive but tasteless sorbitol to this sweetened diet nullified the appetite-stimulating effect, whereas additional sorbitol without sucralose had no effect (Figure 6B). Since rebalancing sweetness and caloric content blocked the sucralose effect, we hypothesized that sucralose is likely working through an energy-sensing intermediate. To this end, we investigated a role for the key cellular energy sensor AMP-activated protein kinase (AMPK). Following 6 days of ingesting sucralose-containing food, we saw significant activation of AMPK in the brain (Figure 6C), and when we knocked down AMPK constitutively (Figure 6D, and second hairpin, not shown) or inducibly (Figure 6E), or expressed kinase-dead AMPK using pan-neuronal *nSyb-Gal4* (Figure S4A), we found that the effect of sucralose pretreatment on both food intake (Figures 6D and 6E) and PER (Figure 6F) was abolished. Furthermore, functional mapping of this response revealed that AMPK was dispensable in insulin-like peptide-producing neurons, octopaminergic neurons, and sweet taste neurons (Figures S4B and S4C). Importantly, however, AMPK expression in both dopaminergic and NPF-producing neurons was essential for increased appetite (Figures 6G and S4D) and PER sensitivity (Figures 6H, 6I, and S4E) after sucralose. Thus, long-term ingestion of sucralose-sweetened food activates

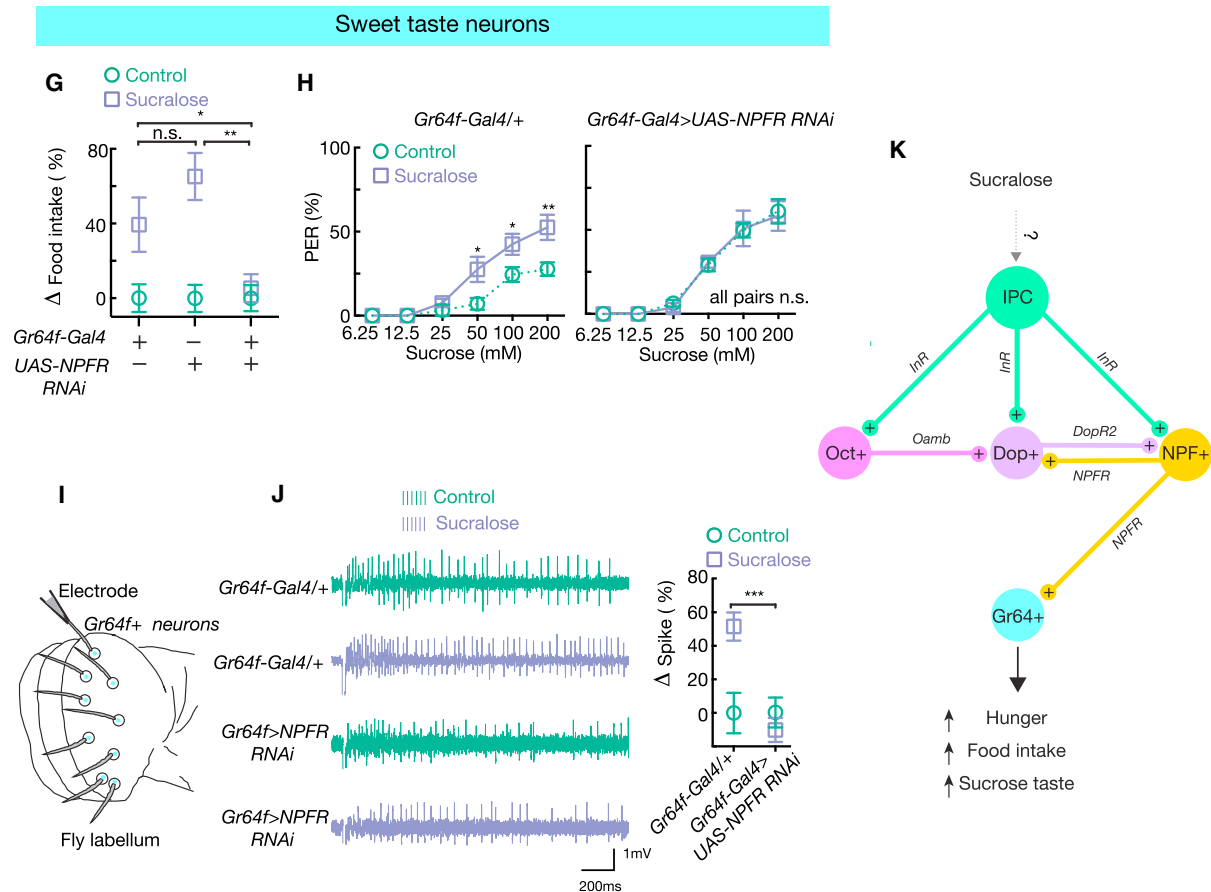
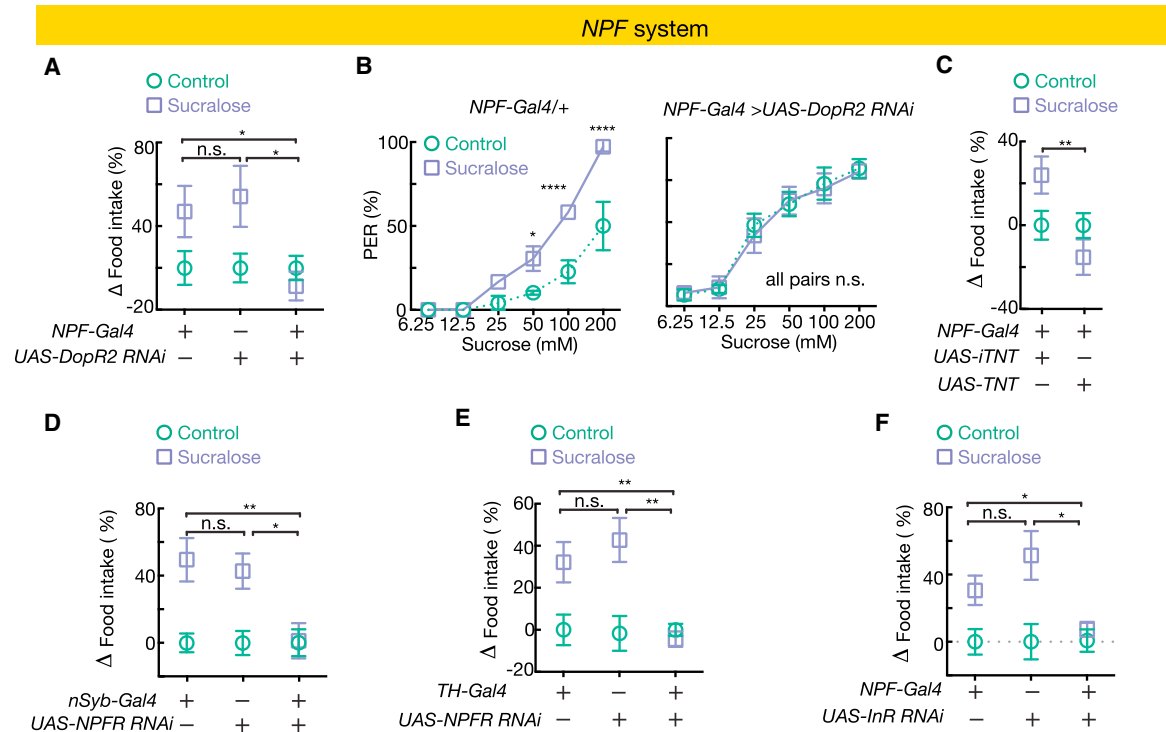
neuronal AMPK, which acts within dopamine and NPF-producing cells to promote hunger. Importantly, these data define a novel neural pathway that integrates taste, hunger, reward, and nutritional content (Figure 6J) to directly alter the taste of food and promote an increased motivation to eat.

### Consuming Synthetically Sweetened Food Mimics Neuronal Fasting

Our results show that sucralose pretreatment triggers an increase in appetite and sucrose receptivity and promotes hyperactivity, insomnia, and sleep fragmentation, behaviors that are consistent with a mild starvation or fasting state (Keene et al., 2010). Moreover, previous work has implicated NPF (Inagaki et al., 2014) and the dopamine receptor *DopEcR* (Inagaki et al., 2012) in sweet taste sensitization after a 6 hr fast, the same molecular machinery we identified as critical regulators of the sucralose response. To directly test if the sucralose effect is working through a neuronal fasting response, we assessed the motivation to feed (PER) following 6 hr of fasting in animals that showed resistance to sucralose pretreatment. Wild-type flies exhibited a robust PER sensitization following fasting (Figure 7A), and as previously described (Inagaki et al., 2012), this response was completely absent in *DopEcR* mutants (Figure 7B; see Table S1C for all *S<sub>50</sub>* values). As with sustained sucralose ingestion, this response was also blocked when synaptic output was blocked from octopamine-, dopamine-, or NPF-producing neurons (Figures S5A–S5C). Fasting-induced PER sensitization also required neuronal *InR* (Figures 7C and S5D) expressed in octopamine- (Figure 7D; Figure S5D), dopamine- (Figures 7E and S5D), and NPF-producing (Figures 7F and S5D) neurons. Moreover, this response required *Oamb* expression in dopamine-producing neurons (Figures 7G and S5D), *DopR2* expression in NPF-producing neurons (Figures 7H and S5D), and *NPFR* expression in dopaminergic (Figures 7I and S5D) and sweet taste neurons (Figures 7J and S5D). Of note, by comparing *S<sub>50</sub>* values, *NPF-Gal4* > *DopR2* RNAi still showed some residual sensitization in response to fasting (2-fold compared to fed animals; Table S1C), suggesting a partial requirement for NPF-expressed *DopR2* in the fasting response. Fasting-induced PER sensitization was also dependent on neuronal AMPK (Figure S5E), and as with sucralose responses, this function was mapped to dopamine- (Figures 7K and S5D) and NPF-producing (Figures 7L and S5D) neurons. Thus, the molecular pathway promoting appetite in response to a synthetically sweetened diet also modulates hunger in response to fasting.

### Figure 4. Sucralose-Sweetened Food Promotes Hunger via Octopamine and Dopamine Pathways

- (A) Octopamine-deficient *Tbh* mutant flies did not increase food intake after consuming a sweetened diet; *n* ≥ 19.  
 (B) Blocking synaptic output from octopaminergic neurons impaired the appetite-stimulating effect of a sweetened diet; *n* ≥ 14.  
 (C) Knockdown of *InR* in octopaminergic neurons suppressed increase in food intake; *n* ≥ 15.  
 (D) Pan-neuronal knockdown of *Oamb* blocked increased food intake in response to sucralose diet; *n* ≥ 13.  
 (E) *Oamb* mutants fail to show PER sensitization to sucrose after a sweetened diet; *n* = 6.  
 (F) Blocking synaptic output from dopaminergic neurons suppressed increased food intake after a sweetened diet; *n* ≥ 14.  
 (G) Increased feeding was impaired when *InR* was knocked down in dopaminergic neurons; *n* ≥ 14.  
 (H) Knockdown of *Oamb* in dopaminergic neurons prevented increased food intake after a sweetened diet; *n* ≥ 12.  
 (I) Increased feeding in response to a sweetened diet was impaired in flies with pan-neuronal knockdown of *DopR2* and *DopEcR*; *n* ≥ 15.  
 (J) Increased PER was also impaired in *DopR2* and *DopEcR* mutants; *n* ≥ 6.  
 (K) Sucralose promotes hunger and food intake via insulin, octopamine, and dopamine system. Data represented as mean ± SEM. Two-way ANOVA with Sidak's multiple comparisons test was used for all PER data, and unpaired t test or one-way ANOVA with Turkey's multiple comparisons test was used for food intake data as appropriate. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, and \*\*\*\**p* < 0.0001. n.s., not significant. See also Figure S2 and Table S1.



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In addition, we tested if fasting also promotes direct sensitization of sweet taste to sugar (Figure 7M). We confirmed previous findings that 6 hr fasting does not induce taste sensitization to a 100 mM sucrose solution (Figure S5F) (Inagaki et al., 2012). However, at sucrose concentrations of 20 mM, we found a robust sucrose sensitization in response to fasting when we recorded single taste sensilla, and this is in line with similar observations in the locust (Simpson et al., 1991). Importantly, this effect required *NPFR* expression in sweet taste neurons (Figure 7N). Thus, in flies, synthetically sweetened food or fasting activates a previously unknown neuronal fasting response pathway (Figure 7O) that integrates the internal nutritional state and gustatory cues to regulate food palatability and the motivation to eat. Finally, we tested if sucralose ingestion also increased food intake in mammals. Wild-type mice that orally consumed sucralose jelly once per day showed a significant increase in food intake following the 7<sup>th</sup> jelly treatment, while vehicle control-treated mice showed no change (Figure 7P). Importantly, the increased food intake following prolonged sucralose ingestion was completely absent in *NPY* knockout (KO) mice (Figure 7Q). Together, prolonged ingestion of a synthetically sweetened diet activates a conserved neuronal response pathway involving NPF/NPY, and these factors are critical for sucralose to promote increased food intake.

## DISCUSSION

While sweetness of an ingested substance often correlates with nutritional value, this is not the case when food contains synthetic sweeteners such as sucralose. Numerous studies have reported an appetite-stimulating effect of consuming synthetic sweeteners in both humans and rodents; however, our understanding of this effect has been hampered due to lack of a molecular mechanism. Here we show that sustained sucralose ingestion activates a conserved neural fasting response. This response integrates pathways that govern feeding, gustatory reward, and energy sensing that together modify how sweet food is perceived. In conditions of fasting, or when the sensory sweetness of food does not match the caloric content for a sustained period, a compensatory response is activated that alters taste sensitivity and feeding behavior accordingly.

Previous work has suggested that synthetic sweeteners act through the microbiome (Suez et al., 2014) or by reducing the validity of “sweetness” to predict caloric content (Swithers et al., 2010). We found no role for the microbiome in our system. This may be a result of the lower overall diversity in commensal gut

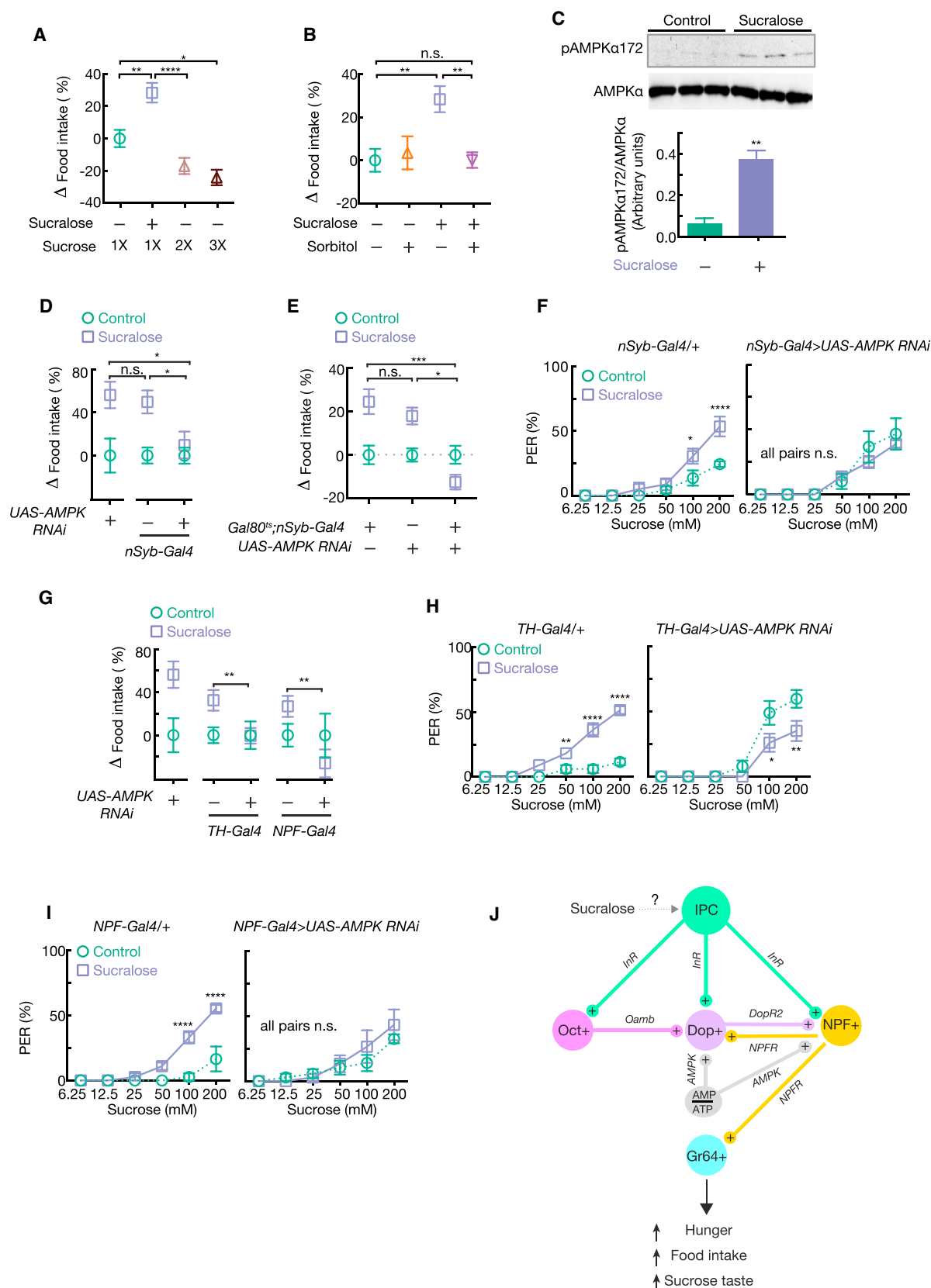
microflora in the fly compared to mammals (Wong et al., 2011). Instead, our data support a mechanism where an imbalance of sweetness versus caloric content of a diet simulates a fasting state and triggers a sensory and behavioral response that increases caloric consumption. These data are compatible with a mechanism where the validity of “sweetness” as a means of predicting nutritive value is flexible and is recalibrated by ingesting a synthetically sweetened diet.

The discrepancy between dietary sweetness and energy is detected at multiple levels. In the fly, insulin signaling was integrated at the systems level, with insulin acting upstream of octopamine, dopamine, and NPF in this response. Interestingly, we found insulin required for the orexigenic effect of sucralose, and chronic activation of insulin-producing cells was sufficient to mimic this effect. In mammals, the increase of circulating insulin postprandial under normal conditions is associated with the state of satiety, and the lack of neuronal insulin signaling has been shown to increase food intake and obesity in rodents (Brüning et al., 2000). Administration of exogenous insulin directly into the cerebroventricular system of lean rodents inhibits NPY/AgRP neurons while simultaneously stimulating POMC neurons (Benoit et al., 2002; Sato et al., 2005), leading to a positive energy balance. However, specific deletion of insulin receptors from POMC or *Agrp* neurons (Köner et al., 2007) did not reproduce the hyperphagic obese phenotype observed in the global neuronal insulin receptor KO. This suggests a more complex regulatory network of insulin action in the mouse brain, though the phenotype of rodents lacking the insulin receptor specifically in NPY+ neurons remains to be seen. In addition, a role for insulin in controlling the rewarding properties of feeding has also been suggested, with altered insulin signaling in the ventral striatum potentially leading to inaccurate valuation of nutritive foods, leading to overconsumption or the selection of foods that don’t accurately meet the body’s current physiological needs (Woods et al., 2016).

In line with our results in the fly, exogenous administration as well as excessive and prolonged release of insulin (like under conditions of type 2 diabetes) paradoxically gives rise to the sensation of hunger, which in the early phase occurs on the background of normal glycemia. Importantly, insulin therapy can cause weight gain, and this is most likely occurring through increased energy intake (Ryan et al., 2008). Moreover, a role for insulin in promoting increased food intake is also consistent with human data, where dysregulation of the insulin system through clamping could increase hunger, food intake, sucrose sweetness intensity, and the overall perceived pleasantness of sugar taste (Rodin et al., 1985). Similarly, we found the insulin system

### Figure 5. Sucralose-Sweetened Food Acts through NPF System to Alter Sweet Taste Perception

- (A and B) Knockdown of *DopR2* in *NPF*+ neurons blocked the effect of a sweetened diet on (A) food intake ( $n \geq 13$ ) and (B) PER ( $n \geq 3$ ).  
 (C) Synaptic output from *NPF*+ is required for increased food intake after sucralose diet;  $n \geq 14$ .  
 (D) Pan-neuronal knockdown of *NPFR* blocked increased feeding in response to the sweetened diet;  $n \geq 14$ .  
 (E) Knockdown of *NPFR* in dopaminergic neurons blocked increased feeding;  $n \geq 18$ .  
 (F) Knockdown of *InR* in *NPF*+ neurons blocked increased feeding;  $n \geq 14$ .  
 (G and H) Knockdown of *NPFR* in *Gr64f*+ sweet taste neurons blocked increased (G) feeding ( $n \geq 18$ ) and (H) PER ( $n \geq 3$ ).  
 (I) Scheme of electrophysiological recording of sweet taste neurons.  
 (J) Prolonged (6-day) sucralose ingestion leads to sucrose sensitization in taste neurons. Control animals showed 50% increase in sucrose spikes after 6 days on a sucralose diet, while knockdown of *NPFR* in *Gr64f*+ sweet taste neurons blocked this effect;  $n = 9$ –14 animals.  
 (K) Sucralose acts through the insulin, octopamine, dopamine and NPF systems to promote hunger, food intake, and sweet taste sensitization. Data represented as mean  $\pm$  SEM. Two-way ANOVA with Sidak’s multiple comparisons test was used for all PER data, and unpaired t test or one-way ANOVA with Turkey’s multiple comparisons test was used for food intake data, as appropriate. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\*\* $p < 0.0001$ . n.s., not significant. See also Figure S3 and Table S1.



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was upregulated following a sucralose diet, while glucose tolerance was reduced, and an identical response has been observed in rats fed a saccharin diet (Swithers et al., 2012) or humans that have consumed saccharin (Suez et al., 2014) or sucralose (Pepino et al., 2013).

Prolonged ingestion of a sucralose-sweetened diet triggered an activation of neuronal AMPK, and we found, for both fasting and sucralose responses, AMPK was essential within dopamine- and NPF-producing cells of the gustatory reward system. These effects are similar to data from the mammalian system, where activation of neuronal AMPK in the hypothalamus has also been shown to increase appetite and regulate NPY expression in response to starvation (Minokoshi et al., 2004). Mammalian NPY is a potent regulator of food intake, especially during food deprivation, and is a critical mediator of insulin's control on whole body energy homeostasis (Loh et al., 2015). Through our systematic dissection of the sucralose response in flies, we identified the conserved NPF/NPY system as a critical downstream component of the sucralose response and confirmed a conserved role for NPY in promoting food intake in response to sucralose-sweetened food.

Despite inclusion in thousands of products, and consumption by billions of people, the molecular effects of ingesting synthetically sweetened food are not well understood. Moreover, there is conflicting evidence from both human and animal studies as to whether or not synthetic sweeteners interact with overall physiology or regulation of energy homeostasis. Our results show that prolonged consumption of a sucralose-sweetened diet promotes hunger and changes how animals perceive nutritive sugar. This involves layered neuronal regulation through conserved metabolic regulatory pathways that we report are also novel components of a neuronal response to fasting. Importantly, in mice, NPY was also critical to mediate increased food intake following chronic ingestion of sucralose-sweetened food, and a similar mechanism may also mediate these effects in humans.

## EXPERIMENTAL PROCEDURES

Detailed experimental procedures are provided in the [Supplemental Information](#).

### Fly Strains

Fly stocks were maintained on standard diet and were raised in 25°C incubator with a 12/12 light/dark cycle. See detailed fly stock information in [Supplemental Experimental Procedures](#).

### Diet Conditioning

3- to 7-day-old male flies were fed with control diet  $\pm$  sucralose (Sigma, #69293) for indicated time. The control diet was made from 1% agar, 5.4% sucrose, and 3.6% yeast. Sucralose diet was made from the control diet plus sucralose (2.5%). Unless otherwise stated, conditioning with sucralose diet occurred for 6 days. In the experiments involving conditioning with sucralose diet then rest on control diet, flies were fed with sucralose diet for 6 days and then were transferred to control diet for 1 or 3 days. For the excessive nutritive sweetness experiment, flies were fed control diet plus additional 5.4% (2 $\times$ ) or 10.8% (3 $\times$ ) sucrose for 6 days. For the sorbitol rescue experiment, flies were fed with control diet plus sorbitol (1%) or sucralose diet plus sorbitol (1%) for 6 days. Flies were transferred into fresh vials every 2 to 3 days.

### Feeding Assay

Food intake was measured by CAFE assay, which was modified from previous studies (Ja et al., 2007). In all cases, food intake was measured over 24 hr. Empty vials were used for evaporation controls. All food intake experiments were set up at Zeitgeber time 6–8, and food intake was recorded exactly 24 hr after start of food loading.

### PER Assay

For PER responses after sucralose-sweetened diet, flies were pretreated with control or sucralose-sweetened diet for 6 days, and then PER assay was performed as described (Masek and Keene, 2013).

### Electrophysical Recording

Tip recordings were performed on flies that were pretreated with or without sucralose diet for 6 days (Figure 5) or after 6 hr of fasting (Figure 7). Three to five L-type labellar bristles were recorded on each fly.

### RNA Sequencing and Bioinformatics Analysis

Fifty fly heads were collected at day 0 or day 6 (6 days sucralose treatment). RNA was extracted using Trizol (Life Technologies, #10296010) and then sequenced by Illumina HiSeqTM 2000. Gene expression level was calculated using RPKM (Reads Per kb per Million reads). Transcripts with Log2 ratio > 1, FDR < 0.001 were considered differentially regulated.

### Western Blot

Fly heads were collected and homogenized in PBS with Roche protease inhibitors cocktail. Western blot was performed according to a standard protocol. Rabbit-anti-AMPK $\alpha$  (Cell signaling, #2532), Rabbit-anti-pAMPK $\alpha$ 172 (Cell signaling, #2535). Secondary antibodies were used at a dilution of 1 in 10,000 (BioRad). Qualification was performed using Image J software.

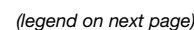
### Mouse Food Intake

All animals experiments were conducted in accordance with relevant guidelines and regulations. 10-week-old female wild-type (C57BL/6) and *NPY*<sup>−/−</sup> mice (on a C57BL/6 background) were fed with a piece of jelly (vehicle group) or jelly containing 7.5 mg of sucralose (sucralose group) daily for 7 days. Food intake was determined using Promethion metabolic cages (PromethionTM Line, Sable Systems International, NV USA).

## Figure 6. Neuronal AMPK Is Activated by a Sucralose-Sweetened Diet and Is Required for the Sustained Effects on Food Intake and PER Response

- (A) Flies fed excessive nutritive sweetness (sucrose) for 6 days exhibit decreased food intake;  $n \geq 20$ .  
 (B) Addition of the tasteless but nutritive sugar sorbitol to the sucralose diet was sufficient to suppress the sucralose-mediated increased feeding response;  $n \geq 18$ .  
 (C) 6 days of sucralose-sweetened diet caused increased phosphorylated AMPK.  
 (D) Pan-neuronal AMPK knockdown abolished changes in feeding;  $n \geq 15$ .  
 (E) Inducible pan-neuronal knockdown of AMPK blocked the sucralose-mediated increased food intake; flies raised at 18°C and tested at 29°C;  $n \geq 10$ .  
 (F) Pan-neuronal AMPK knockdown abolished changes in PER;  $n \geq 3$ .  
 (G) Knockdown of AMPK in dopaminergic neurons or NPF+ neurons blocked increased feeding from a sweetened diet;  $n \geq 13$ .  
 (H and I) Knockdown of AMPK in (H) dopaminergic or (I) NPF+ neurons blocked increased PER after a sucralose diet;  $n \geq 3$ .  
 (J) A working model of how prolonged ingestion of a synthetically sweetened, calorically sufficient diet alters taste perception and feeding behavior. Sucralose triggers a response through the insulin system that requires octopamine-, dopamine-, and NPF-producing neurons to alter sweet taste perception and promote hunger. Moreover, this system involves the cell-autonomous energy sensor AMPK acting within *Dop*+ and *NPF*+ neurons. Data represented as mean  $\pm$  SEM. Two-way ANOVA with Sidak's multiple comparisons test was used for all PER data, one-way ANOVA with Turkey's multiple comparisons test was used for food intake data, and unpaired t test for western blot data. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ . n.s., not significant. See also Figure S4 and Table S1.





## Statistical Analysis

Data are represented as means  $\pm$  SEM. Statistical tests were performed using unpaired t test, one-way ANOVA with Tukey's multiple comparisons test, two-way ANOVA with Sidak's multiple comparisons test, or repeated-measures ANOVA, as appropriate. All statistical analysis was performed using GraphPad Prism 6.0.

## ACCESSION NUMBERS

The RNA-sequencing data in this article have been deposited in the NCBI GEO and are accessible through GEO: GSE73179.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.cmet.2016.06.010>.

## AUTHOR CONTRIBUTIONS

Q.-P.W. designed and performed the majority of the experiments with help from L.J.O., J.C., Y.Q., T.M.K., N.B., D.T.B., M.T.L., and Y.P. Y.Q.L. performed electrophysiological recordings. L.Z. performed mouse studies. Y.A.W., T.A.C., A.C.N.W., and S.J.S. supported the metabolic and lifespan experiments, and G.G.N. conceived of and coordinated the project and wrote manuscript with help from Q.-P.W., H.H., A.R.C., and J.M.P.

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## Figure 7. Prolonged Sucralose Ingestion Alters Food Intake and Sweet Taste Perception through a Novel Fasting Response Pathway

- (A) 6 hr fasting caused enhanced PER to sucrose in wild-type (*w<sup>1118</sup>*) flies;  $n \geq 6$ .  
 (B) Enhanced PER was impaired in *DopEcR* mutant flies;  $n \geq 6$ .  
 (C–F) Knockdown of *InR* (C) pan-neuronally or in (D) octopaminergic, (E) dopaminergic, or (F) *NPF*<sup>+</sup> neurons blocked fasting induced PER sensitization;  $n \geq 3$ .  
 (G) Knockdown of *Oamb* in dopaminergic neurons prevented enhanced PER;  $n \geq 3$ .  
 (H) Knockdown of *DopR2* in *NPF* neurons prevented increased PER;  $n \geq 3$ .  
 (I and J) Knockdown of *NPFR* in (I) dopaminergic neurons and (J) *Gr64*<sup>+</sup> neurons abolished increased PER;  $n \geq 3$ .  
 (K and L) Fasting-induced increased PER was blocked when AMPK knockdown was driven in (K) dopaminergic or (L) *NPF*<sup>+</sup> neurons;  $n \geq 3$ .  
 (M) Scheme of electrophysiological recording of sweet taste neurons.  
 (N) Fasting leads to sucrose sensitization in taste neurons. Fasted animals show increased spikes in response to 20 mM sucrose, while *NPFR* knockdown in *Gr64*<sup>+</sup> sweet taste neurons blocked this effect;  $n = 9$ –14 animals.  
 (O) The sucralose response pathway is part of a novel fasting response.  
 (P) Food intake increased following prolonged sucralose ingestion in WT mice.  
 (Q) Increased food intake following prolonged sucralose jelly intake was abolished in *NPY* KO mice. Data represented as mean  $\pm$  SEM. Two-way ANOVA with Sidak's multiple comparisons test was used for all PER data, repeated-measure ANOVA was for mouse data, and unpaired t test for electrophysiology data.  
 \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ . n.s., not significant. See also Figure S5 and Table S1.

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