Chronic Sucralose or L-Glucose Ingestion does not Suppress Food Intake

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The overall metabolic impact of distorting dietary sweetness through consumption of non-nutritive sweeteners (NNS) is the focus of much debate (Fowler, 2016; Swithers, 2013). We have previously demonstrated that chronically feeding flies a nutritional diet spiked with the NNS sucralose (termed exposure phase; **Figure S1A**) can promote subsequent food intake when sucralose is removed (post-exposure phase; **Figure S1A**), and we provide the first molecular mechanism for how NNS can regulate appetite (Wang et al., 2016). Recently, Park *et al* (2017) reported that the hyperphagic effect arises as a result of sucralose suppressing food intake during the exposure phase, leading to increasing intake in the post-exposure phase due to food deprivation. Park *et al.* conclude that the added sweetness of the sucralose or other NNS to a diet rich in carbohydrate tricks an animal into eating fewer calories than they need. While this simple mechanism was also our original hypothesis, it was not supported by our data. We only observed a slight and temporal (~24 h) decrease in food intake during exposure to sucralose in the context of a nutritional diet, with no significant chronic suppression of food intake as shown by Park *et al*. As such our data support that the increase in food intake after sucralose exposure reflects physiological changes induced by sucralose *per se*, rather than by creating a caloric deficit during sucralose exposure.

Park *et al.* (2017) make the general claim that "Sucralose Suppresses Food Intake". To directly test this, we offered flies a low carbohydrate diet (5% yeast extract solution) sweetened with sucralose. We show that adding sucralose to this low carbohydrate diet promotes an immediate (within 24 h) and dose-dependent increase in food intake compared with unsweetened control (**Figure S1B**). These data are in accordance with multiple independent studies (Dus et al., 2011; Gordesky-Gold et al., 2008; Keene et al., 2010) that all show sucralose generally promotes food intake under a variety of acute experimental conditions.

In the original chronic sucralose consumption assay (Wang et al., 2016), we provided animals with a calorically sufficient control diet (5.4 % sucrose, and 3.6 % yeast) +/- additional sweetness from sucralose (Exposure phase), then after 6 days we removed sucralose and tested food intake over the next 24 h (Post-exposure phase). In this setup we found exposure to sucralose led to increased food intake post-exposure. Although Park *et al.* did not test post-exposure food intake in their study, however, they report that sucralose ingestion suppresses food intake during the exposure phase, presumably leading to

undernourished animals that consume more food in the post-exposure period. To further clarify this issue, we quantified food intake during the exposure phase with control or sucralose-spiked food. Using a high (2.5%) dose of sucralose, we observed a slight (11%) but reproducible decrease in food intake within the first 24 h; however this response was transient and no longer observed at day 6 (**Figure S1C**). Further, we performed additional experiments with a lower (0.5%) sucralose dose and found that at this dose, sucralose (when added to the caloric sufficient diet) did not suppress food intake during 1 or 6 days of exposure phase (**Figure S1C**). Importantly, in accordance with Wang *et al.* (2016), both 0.5% and 2.5% sucralose-sweetened food promoted a significant increase in food intake during the post-exposure phase (**Figure S1D**). These results are consistent with an independent study showing that in conditions of nutritional deprivation, flies rely primarily on dietary caloric content to ensure they meet energy requirements (Dus et al., 2011). More generally, we and others (Carvalho et al., 2005; Dethier, 1976; Dus et al., 2013; Lee et al., 2008; Stafford et al., 2012; Wang et al., 2016) have found that *Drosophila* show robust nutritional homeostatic responses to dietary manipulations- either sweetness, energy content of food or macronutrient composition - and over time will adapt feeding behaviour to ensure appropriate nutrient intakes. Taken together our data show that sucralose did not suppress food intake throughout the exposure phase, and flies exposed to sucralose were not underfed as suggested by Park *et al.* Importantly, however, after chronic sucralose ingestion, post-exposure flies consumed more naturally sweetened food and this response was a direct effect of ingesting sucralose and not due to a cumulative caloric debt.

In our original study, we confirmed these effects of NNS on food intake using a second NNS, L-glucose (Wang et al., 2016), a non-metabolizable steroisomer of D-glucose. Park *et al.* provide data suggesting that L-glucose ingestion also suppresses appetite over 24 h, but do not address the effects of long-term exposure or the effect on post-exposure phase. To test the chronic responses to L-glucose sweetened food, we performed additional experiments as in Wang *et al.*(2016), quantifying food intake both during and after L-glucose pre-treatment. Again in contrast to Park *et al.*, we found no anorexigenic effect of L-glucose in the exposure phase (**Figure S1E**); however, consistent with our previous report, L-glucose promoted a significant increase in food intake post-exposure (**Figure S1F**). Thus, we provide further evidence that chronic ingestion of artificially sweetened food does not result in a reduction in caloric intake during exposure, but as we originally concluded (Wang et al., 2016), does promote subsequent increased food intake in the post-exposure phase.

Regulation of energy homeostasis is complex, and to understand the overall mechanisms involved, multiple independent variables must be measured and integrated. Park *et al.* (2017) base their conclusions that sucralose promotes an energy deficit solely on the measurement of food intake. Further evidence that this did not occur in our previous study comes from the fact that, after the sucralose exposure phase, we observed no changes in triglyceride content, body weight, resting hemolymph sugar, or glycogen levels, and continued exposure to sucralose did not alter lifespan (Wang et al., 2016). Sucralose-containing food does, however, consistently promote sustained hyperactivity (32% increase daily activity over 6 days) (Wang et al., 2016). If sucralose consuming animals do in fact decrease daily food intake by up to ~5-10% as reported by Park *et al.*, thereby creating a chronic caloric debt in the context of sustained hyperactivity, it is unclear how this could occur without affecting energy stores or other measured metabolic parameters or lifespan.

Whether the observed hyperactivity itself is somehow responsible for subsequent hyperphagia, potentially via an increased central excitatory state (Dethier, 1976) further enhanced by gustatory sensitization to sucrose (Wang et al., 2016), remains to be investigated. Regardless, the data (including those from Park *et al.*, 2017) as a whole support the conclusion that during the exposure phase, the NNS sucralose and L-glucose are not creating a sustained caloric debt, and the resulting increase in food intake post exposure is consistent with a higher order recalibration of the value of sweetness verses energy content of food. Moreover, the discrepancy between Park *et al.* (2017) and Wang *et al.* (2016) highlights that measuring food intake alone is not sufficient to understand how dietary or genetic interventions impact *Drosophila* physiology. Instead a multi-parameter comprehensive investigation of whole animal physiology constitutes the gold standard for investigations into genetic and environmental regulators of energy homeostasis.

Park *et al.* (2017) make a second point concerning the role of neuropeptide F (NPF), the fly ortholog of the orexigenic peptide NPY, in regulating food intake in the fly. They present data where silencing NPF-producing neurons with tetanus toxin increased baseline food intake independent of sucralose, and suggest that in Wang *et al.* (2016), baseline changes in food intake may have been misconstrued as components of a sucralose pro-appetitive response. In Wang *et al.* we included baseline food intake values for all RNAi and control animals used to construct the sucralose response pathway (Wang *et al.* Table S1) and reported no significant difference in baseline food intake in the absence of sucralose. Thus, the suggestion that altered baseline food intake explains our described sucralose response pathway was ruled out in our original study. Secondly, Park *et al.* show that blocking the NPF-system promotes food intake, suggesting NPF is a negative regulator of appetite; in contrast the NPF/NPY system is known to promote feeding in a variety of experimental conditions (Krashes et al., 2009; Loh et al., 2015; Wang et al., 2013; Wu et al., 2003; Wu et al., 2005a; Wu et al., 2005b). In our studies however, silencing NPF-producing neurons did not affect baseline food intake (**Figure S1G**), despite being essential for increased food intake after sucralose pre-treatment. We observed the same results with a second independent NPF-Gal4 (**Figure S1H**) and ruled out background effects because NPF-Gal4 6X backcross to *w1118* still showed no effect on food intake (not shown). To further investigate the role of NPF in baseline regulation of food intake we targeted NPF pan-neuronally using RNAi and observed a significant decrease in baseline food intake (**Figure S1I**). A recent study (Eriksson et al., 2017) also showed that blocking the NPF system suppresses baseline food intake. We therefore conclude that the weight of evidence is that disrupting the NPF system does not promote food intake.

In assessing the discrepancies between Park *et al.* (2017) and our work, we have noted issues with experimental design or replication and measurement precision that could potentially help explain the differences between these studies. Firstly, Park *et al*. did not test post-exposure food intake or evaluate other metabolic parameters, so results are not all directly comparable. Secondly, Park *et al*. state they use active yeast in their solid food assays, whereas we used inactivated yeast, which could, *inter alia*, potentially affect the use of radiolabels as a measure of intake. Thirdly, a systematic assessment of fly food intake measurements by these authors concluded that both CAFÉ and radiolabelling assays have thresholds for meaningful data between ~10-20% change in food intake (Deshpande et al., 2014). In both Wang *et al.* and this current study, all significant data are in this meaningful

range, whereas Park *et al.* report extensive variation in the sucralose suppression effects, mostly below the meaningful threshold (e.g. **Figure S1B**, **S1E, Figure S1F**, **S1G** Park *et al.*). Regardless of this threshold, for studies involving ~5-10% change in feeding, power calculations would require ~60 replicates for CAFÉ assay and ~30 replicates for radiolabelled experiments to yield definitive results (Deshpande et al., 2014). By these calculations, much of the Park *et al.* study was underpowered, both for CAFÉ results (13 replicates; **Figure S1C** Park *et al.*), and for radiolabelled experiments (20 replicates; **Figure S1D**, authors state only 3 replicates, n = 18 for day 6, **S1F-G** Park *et al.***)**. In our studies, we have optimised reproducibility extensively. First, in Wang *et al*. we were studying an effect size ≥20%. Second, we perform each experiment on at least three separate occasions with 7 replicates per day, ≥21 replicates total based on ≥105 animals from independent experimental set ups. Thus, while both Wang *et al.* and the data we present here are well powered and informative, much of the data in Park *et al.* is inadequately powered and with an effect size below the range for meaningful interpretation in these assays (Deshpande et al., 2014).

Another complication with the Park *et al.* study is that the radiolabelled assay is only accurate if both internal and excreted label is evaluated. For solid food, ~10% of the ingested radiolabel is excreted without absorption (Deshpande et al., 2014) and when the effect size being investigated is 5-10%, a 10% error rate based on differential label excretion could confound data interpretation, especially when these diets differ significantly in composition. Moreover, it is not clear what fraction, if any, of the ingested radiolabel is absorbed by the microbiome. Since sucralose can be toxic to the microbiome (Suez et al., 2014), this could further confuse interpretation of the results. Importantly, there are multiple studies showing that NNS such as sucralose can increase GI tract secretion, promote serotonin which drives peristaltic activity, alter gut permeability, and inhibit passive nutrient transport in the gut (Spencer et al., 2016). We observed a strong laxative effect in mice (unpublished). If sucralose alters gut motility or nutrient absorption in the fly, these effects could mistakenly be interpreted as an apparent reduction in food intake. As such, until these issues are experimentally addressed, we argue that only direct measurements of food intake be used for studies involving sucralose or other NNS.

While much has been done to establish the acute safety of ingesting NNS such as sucralose, the impact of chronically distorting the perceived energy value of food is unclear. While originally considered benign, there is emerging evidence from multiple groups (Fowler, 2016) that sucralose or other NNS may have unanticipated consequences on animal or human physiology. To fully understand the impact of NNS on overall health will require carefully controlled, adequately powered systematic investigation of NNS effects on multiple metabolic parameters and across numerous experimental systems.

SUPPLEMENTAL INFORMATION

Supplemental information including Supplemental Experimental Procedures and one figure can be found with this letter online at http://

ACKNOWLEDGEMENTS

We thank all members of the CPC for helpful discussions and excellent advice and technical support. We also thank the VDRC and Bloomington stock centres for providing an essential service to the fly community. This work was supported in part through NHMRC project grants APP1026310, APP1029672, APP1028887, APP1046090, APP1042416, APP1086851. G.G.N. is supported by an NHMRC career development fellowship II CDF1111940.

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Figure S1. **Sucralose and the conserved NPF/NPY system promote food intake.** (A) Experimental set up for Wang *et al*. vs. Park *et al*. In Park et al. food intake was measured during 1 or 6 days exposure to sucralose or L-Glu-laced food. In Wang et al. animals were pre-treated with sucralose or L-Glu for 6 days and then tested for subsequent food intake on naturally sweetened food. (B) Sucralose promoted consumption of a high protein (5% yeast extract) diet (C) Feeding was not suppressed on day 6 during sucralose treatment, $n \geq 21$. (D) Sucralose promoted feeding after 6 days sucralose exposure. (E) L-glucose did not suppress feeding on day 1 and day 6 during L-glucose treatment. (F) L-glucose promoted feeding after 6 days L-glucose exposure. (G) Block of NPF neuron synaptic transmission does not affect feeding. (H) Pan-neuronal *NPF* RNAi neuron reduced feeding. All data represented as mean ± S.E.M., unpaired t-test or One-way ANOVA Dunnett's multiple comparisons test were used appropriate. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$, n.s., not significant. $n \geq 21$ replicates, 5 animals per replicate, 7 replicates per experiment and 3 independent experiments for all feeding data.

Fly Strains

Fly stocks were maintained on standard diet and were raised in 25°C incubator with a 12/12 light/dark cycle. w^{1118} is from Hugo Bellen. *UAS-TeTxLC.TNT* (*UAS-TNT*, #28838) and *UAS-TeTxLC.IMP TNT* (*UAS-iTNT*, (#28841) (Sweeney et al., 1995), *UAS-NPF-IR (#27237)* were obtained from the Bloomington Stock Center. *NPF-Gal4* (Wu et al., 2003*)* is from Ping Shen. *UAS-Dicer 2* (Neely et al., 2010) is from VDRC, *nSyb-Gal4 (III)* is from Partrik Verstreken. *Gr64f-Gal4* (Weiss et al., 2011) is from Alex Keene.

Diet Conditioning

For exposure phase 3 to 7 day old male flies were fed with control diet +/- sucralose (Sigma, #69293) or L-glucose (Sigma # G5500-5G) 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 (0.5% and 2.5%). L-glucose diet was made from the control diet plus l-glucose (0.45%). Post-exposure food intake was determined after 6 days of sucralose preconditioning.

Feeding Assay

Food intake was measured by CAFÉ assay, which was modified from previous studies (Deshpande et al., 2014; Ja et al., 2007). Five flies were housed in an empty vial with wet kimwipes and liquid food was supplied to flies in 5µl of capillaries. In the acute sucralose feeding, flies were fed with 5% yeast extract (Merck #103753) plus sucralose. During sucralose treatment (exposure phase), flies were fed with 5.4 % sucrose, 3.6 % soluble yeast (MPB, #02103304) plus 0.5% or 2.5% sucralose. During L-glucose treatment (exposure phase), flies were fed with 5.4 % sucrose, 3.6 % soluble yeast plus 0.45% l-glucose. After exposure, control food used for assessing food intake after conditioning was 5% yeast extract and 10% sucrose. For measurement of normal food intake, flies were fed with 5% yeast extract and 10% sucrose. In all cases, food intake was measured over 24 hours. 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 hours after start of food loading.

Statistical Analysis: Data are represented as means ± SEM. Statistical tests were performed use unpaired t test, One-way ANOVA with Dunnett's multiple comparisons test. All statistical analysis was performed using GraphPad Prism 7.0.

Supplemental References

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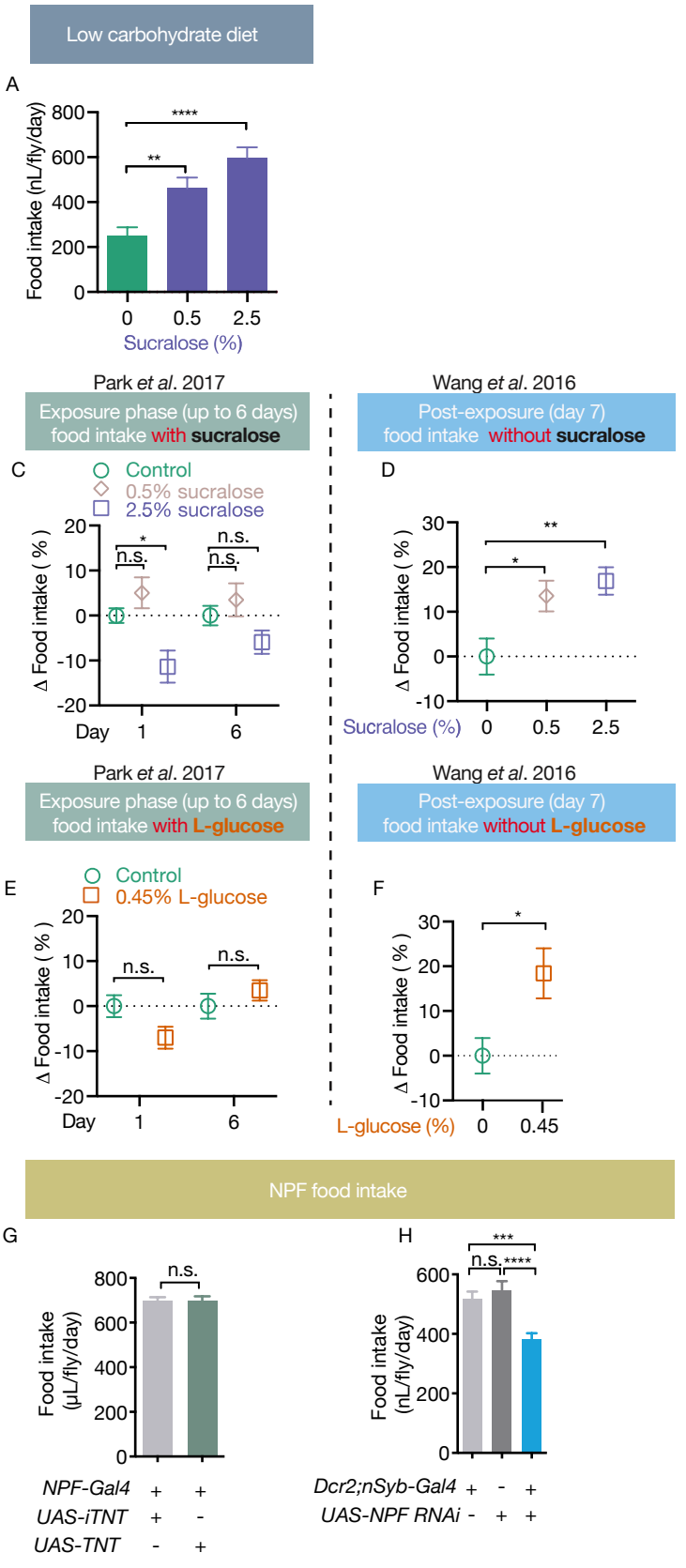
Low carbohydrate diet
A
Food intake (nL/fly/day)
800
600
400
200
0
****
**
0
0.5
2.5
Sucralose (%)
Park et al. 2017
Exposure phase (up to 6 days)
food intake with sucralose
Wang et al. 2016
Post-exposure (day 7)
food intake without sucralose
C
Control
0.5% sucralose
2.5% sucralose
Δ Food intake (%)
20
10
0
-10
-20
*
n.s.
n.s.
n.s.
Day
1
6
D
Δ Food intake (%)
30
20
10
0
-10
**
*
Sucralose (%)
0
0.5
2.5
Park et al. 2017
Exposure phase (up to 6 days)
food intake with L-glucose
Wang et al. 2016
Post-exposure (day 7)
food intake without L-glucose
E
Control
0.45% L-glucose
Δ Food intake (%)
20
10
0
-10
-20
n.s.
n.s.
Day
1
6
F
Δ Food intake (%)
30
20
10
0
-10
*
L-glucose (%)
0
0.45
NPF food intake
G
Food intake (μL/fly/day)
800
600
400
200
0
n.s.
NPF-Gal4 + +
UAS-iTNT + -
UAS-TNT - +
H
Food intake (nL/fly/day)
600
400
200
0
***
n.s.
****
Dcr2;nSyb-Gal4 + - +
UAS-NPF RNAi - + +