Neuromodulatory circuit effects on *Drosophila* feeding behaviour and metabolism

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Abstract

All animals have evolved to adapt their behaviour and internal metabolism to a changing external environment in order to maintain homeostasis. Both metabolism and feeding behaviour are coordinated by hormone activity and neuromodulation, and a number of the implicated neuromodulatory systems are homologous between mammals and the important neurogenetic model *Drosophila melanogaster*. We hypothesized that silencing broad neuromodulatory systems would elicit systematic, cohesive changes in feeding, behavioural activity and metabolism. To test our hypothesis, we employed transgenic drivers that allowed us to inhibit large cellular sets of the dopaminergic, serotonergic, octopaminergic, tyraminergic and neuropeptide F systems. The resulting groups of genetically manipulated fly stocks were then assessed for changes in their overt behavioural response and metabolism by monitoring eleven parameters: activity, climbing ability, individual feeding, group feeding, food discovery, respiration (fed/starved), lipid content (fed/starved) and whole-body weight (fed/starved). Our data indicated that individual neuromodulatory systems can have dissociated effects on feeding behaviour, motor activity and metabolism. These results refute our original hypothesis, and instead suggest that neuromodulatory systems in *D. melanogaster* exert specialised functions across and within neurotransmitter cell types.

Introduction

The brain is responsible for monitoring internal metabolic and nutritional status and inducing corresponding changes in behaviour, food intake, and metabolism to maintain energy homeostasis (Loftus et al. 2000; Leibel, Rosenbaum, and Hirsch 1995; Obici et al. 2002; Harris-Warrick and Marder 1991; Pfaff, Kieffer, and Swanson 2008). Many of the neuromodulators involved in this process are evolutionarily conserved between mammals and invertebrates, and exhibit homologous biosynthesis pathways and similar neuromodulatory functions (Baker and Thummel 2007; Melcher, Bader, and Pankratz 2007). Studying these neuromodulators in mammals is complicated by the immense complexity of the brain and the expense of genetic experiments. *Drosophila melanogaster* (a vinegar fly) is one of the predominant model systems used to dissect the role of genetic pathways involved in mediating animal behaviour, and the neuromodulatory regulation of feeding, foraging and metabolism is becoming a popular topic of *Drosophila* research. The regulatory pathways that modify metabolic homeostasis are conserved between flies and mammals (Melcher, Bader, and Pankratz 2007). Neurogenetic tools currently available for use in *Drosophila* allow temporally-resolved manipulation of specific neurons, which permits an analysis of their role in energy homeostasis, feeding-associated behaviours and metabolism (Owusu-Ansah and Perrimon 2014; Kaun, Devineni, and Heberlein 2012; Pandey and Nichols 2011). Such temporal control also eliminates the possibility of incurring unwanted developmental effects or compensatory mechanisms as a result of neuronal inhibition.

The majority of known neuromodulators are thought to have an effect on feeding behaviour in the fly. Serotonin regulates larval food intake and stomatogastric responses (Neckameyer 2010; Shimada-Niwa and Niwa 2014; Schoofs et al. 2014); a decreased level of neuronal serotonin increases appetite during the larval stages of development (Neckameyer 2010; Gasque et al. 2013). A similar role is seen for octopamine, where obesity-linked *Drosophila* homologs Transcription factor AP-2 and Tiwaz regulate octopamine signalling, which in turn exerts positive and negative effects on feeding, including a negative feedback loop that controls feeding frequency (Williams et al. 2014). In rodents, neuropeptide Y (NPY) modulates feeding behaviour (Leibowitz and Alexander 1991), and the *Drosophila* homolog, neuropeptide F (NPF) is thought to respond to feeding-associated signals to help regulate feeding (Shen and Cai 2001; Lee et al. 2004). Dopamine signalling is required for normal food intake in flies and locomotor activity (T. Riemensperger et al. 2011)(T. Riemensperger et al. 2011; Q. Y. Zhou and Palmiter 1995).

In the present study we examined the effects of silencing various neuromodulatory systems in *D. melanogaster* on appetitive control, food driven behaviours, metabolism and locomotion. We hypothesized that lesions in these neuromodulatory systems would have coordinated effects on behaviour, feeding and metabolism; for example, that a lesion that increased behavioural activity would also concomitantly increase metabolism and feeding. To test this hypothesis, we used five transgenic Gal4 drivers (using the enhancers of

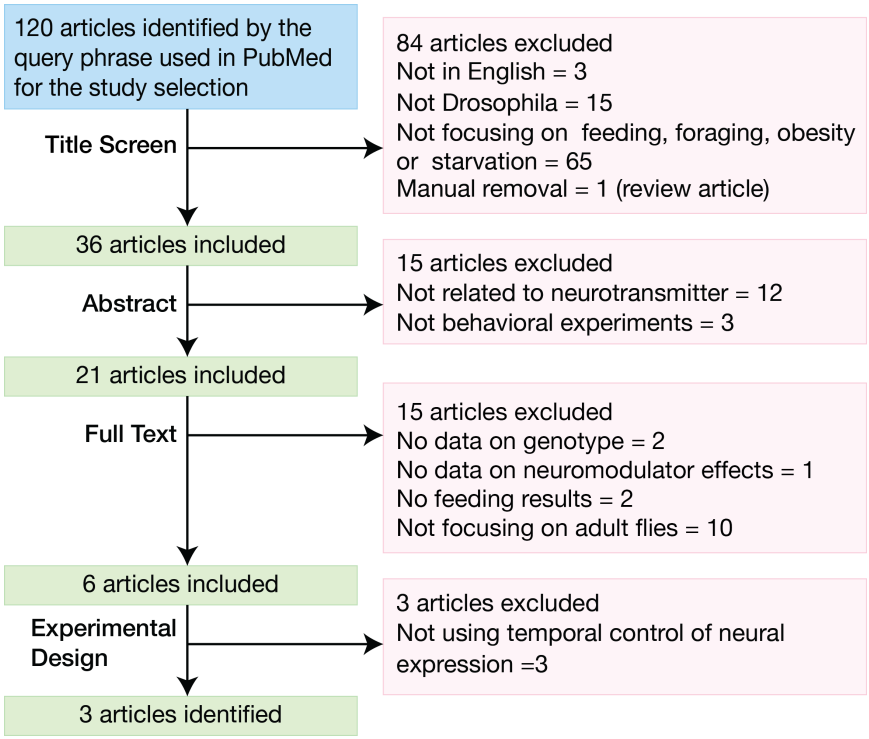

Figure 1. A systematic literature review of the effects of neuromodulatory neurotransmission on feeding behaviours in *Drosophila*.
PubMed was interrogated using the search expression [(*Drosophila* or fruitfly or "vinegar fly" or "fruit fly") AND (feeding or obesity or foraging or starvation) AND (NPF or "neuropeptide F" or octopamine or serotonin or dopamine or tyramine)], which yielded 120 articles. Four successive screens were then used to review the resulting literature, whereby a title screen was followed by three exclusions of increasing detail: abstract, full text and experimental design. A total of 117 article were excluded by the selection criteria and only three articles were identified for comparison with our study.

the genes *pale/Tyrosine hydroxylase* (*TH*), *Dopa decarboxylase* (*Ddc*), *Tyrosine decarboxylase 2* (Tdc2), *Tryptophan hydroxylase* (*Trh*) and *neuropeptide F* (*NPF*)) to examine the circuit function of five neuromodulators (dopamine, octopamine, tyramine, serotonin, and neuropeptide F) in 11 different assays. Interestingly, we found that feeding phenotypes were not necessarily associated with metabolic or behavioural changes. These results indicate dissociated neuromodulator function, eroding confidence in the hypothesis, and instead suggest that the normal coordination of feeding, behaviour and metabolism requires the organized action of several modulatory systems.

Results

Systematic literature review of neuromodulators and food intake in *Drosophila*

A total of 120 articles were identified in the initial search of PubMed; applying the selection criteria reduced this number to three articles (Figure 1). The three identified articles contained five different experiments that utilized two different methods for assessing food intake: the CAFE assay and the proboscis extension reflex (PER) test. *Drosophila* feeding behaviour was characterized using these two assays after either silencing or activating dopaminergic, serotonergic, NPF-ergic and octopaminergic (or tyraminergic) neurons (Figure 2) (Marella, Mann, and Scott 2012; Williams et al. 2014; Inagaki et al. 2012). The relative paucity of data relating adult *Drosophila* neuromodulator function to feeding and metabolism led us to conduct new experiments on this topic.

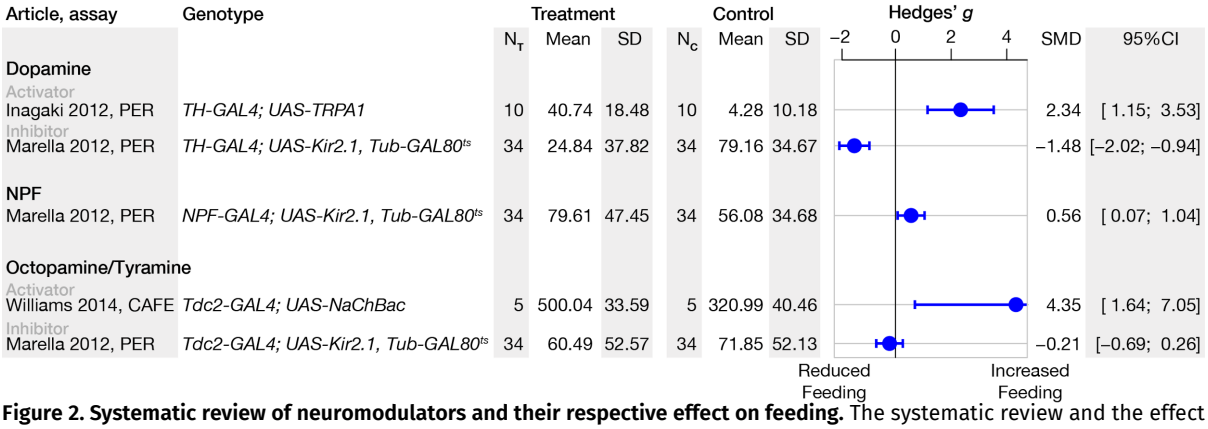

Figure 2. Systematic review of neuromodulators and their respective effect on feeding. The systematic review and the effect size is illustrated as a forest plot of standardized effect sizes (Hedges' *g*). The forest plot is grouped by neuromodulator and sub-grouped by activator and inhibitor. Error bars indicate the 95% confidence intervals (95%CI) of the standardized mean difference (SMD). Control and treatment samples sizes are given in the columns listed as N_C and N_T respectively. Abbreviations: *TH*, tyrosine hydroxylase; *Ddc*, dopa-decarboxylase; *Tdc2*, tyrosine decarboxylase 2; *Trh*, tryptophan hydroxylase; *NPF*, neuropeptide F precursor; CAFE, capillary feeding; PER, proboscis extension reflex; SD, standard deviation.

Silencing of *TH-Gal4* neurons reduces activity

We used *Gal4* drivers in combination with the *Tub-Gal80^{ts}* conditional repressor transgene (McGuire, Mao, and Davis 2004) to drive expression of Kir2.1, an inward-rectifying potassium channel that silences neuronal activity (Baines et al. 1999). Following warm-treatment induction of Kir2.1 expression, motor coordination and activity were compared with two sets of control flies: a driverless line (*UAS-Kir2.1; Tub-Gal80^{ts}*/+) subjected to heat, or flies maintained at 21°C that had intact Gal80^{ts} repression of *Kir2.1* expression but were otherwise genetically identical. Inhibiting electrical activity in *TH-Gal4* dopaminergic cells had a profound effect on the activity index, defined as the proportion of time a fly spent moving. The activity index of induced

TH>Kir2.1 flies was reduced by −70% compared to uninduced isogenic animals, Δactivity = −0.25 [95CI −0.19, −0.32], g = −1.2, P = 2.2 × 10^{-13} (Figure 3A). Inhibiting the *Ddc-Gal4* cells, which include both dopaminergic and serotonergic neurons, reduced activity by −35%. Flies with silenced *Tdc2-Gal4* cells, which include both octopaminergic and tyraminergic neurons, exhibited a +48% increase in activity. Silencing the *Trh-Gal4* cells, which includes most of the serotonergic neurons, had only a negligible effect on activity.

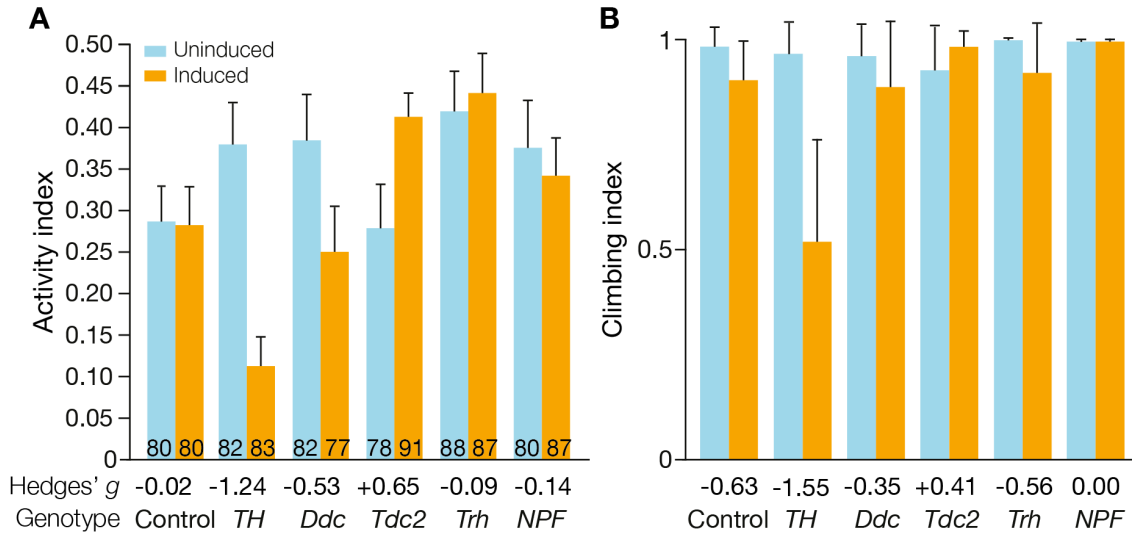

Figure 3. Temporal inactivation of TH-Gal4 dopaminergic neurons disrupts motor function.
Activity index and climbing index for the progeny of *TH-Gal4*, *Ddc-Gal4*, *Tdc2-Gal4*, *Trh-Gal4* and *NPF-Gal4 flies crossed with Kir2.1; Tub-Gal80^{ts}*, and a responder control line (progeny of *UAS-Kir2.1; Tub-Gal80^{ts}* crossed with wild type CS flies). Controls were maintained at 22°C only (blue bars) whereas Kir2.1-induced flies were raised at 22°C before being transferred to 31°C for 24 h prior to the assay (orange bars) to elicit Kir2.1 expression. The control flies carried the *UAS-Kir2.1; Tub-Gal80^{ts}/+* construct but not the *Gal4* drivers.
A. An activity index was calculated for each fly, and represented the proportion of time that the fly spent moving. *TH-Gal4>UAS-Kir2.1; Tub-Gal80^{ts}/+* flies exhibited reduced activity: Δactivity = −0.25 [95CI −0.19, −0.32], g = −1.24, P = 2.2 × 10^{-13}, N_{flies} = 73, 75. Induced *Ddc>Kir2.1* flies also exhibited reduced activity but to a lesser extent compared to the *TH>Kir2.1* flies: −0.10 [95CI −0.01, −0.18], g = −0.53, P = 0.025. Conversely, neural inhibition with *Tdc2>Kir2.1* resulted in increased activity: +0.67 [95CI +0.19, +0.06], g = +0.65, P = 2.35 × 10^{-5}, N_{flies} = 78, 91.
B. The climbing ability of *TH>Kir2.1* flies was impaired compared to controls: Δclimbing = −0.45 [95CI −0.21, −0.70], g = −1.55, P = 0.002, N_{tubes} = 10, 10. No differences were observed in *Ddc>Kir2.1* flies. All error bars represent 95% CI. The numbers indicated above each bar denote the effect size (g) for the individual driver lines. The climbing index was repeated 10 times with at least five male flies, and no more than 20 flies in each vial. Abbreviations: *TH*, tyrosine hydroxylase; *Ddc*, dopa-decarboxylase; *Tdc2*, tyrosine decarboxylase 2; *Trh*, tryptophan hydroxylase; *NPF*, neuropeptide F precursor.

Inhibition of TH cells impairs climbing ability

We next assessed climbing ability to determine whether the observed reduction in activity in the transgenic dopamine lines was associated with functional motor deficits (Bartholomew et al. 2015). Flies expressing Kir2.1 in the *TH-Gal4* cells exhibited dramatically worse climbing ability compared to control flies, Δclimbing index = −0.45 [95CI −0.2, −0.70], g = −1.55, P = 0.002. No notable deficit was seen in *Ddc>Kir2.1* flies, but it should be considered as above that this driver also includes some dopaminergic cells (Li et al. 2000). All other induced *Gal4>Kir2.1* lines exhibited only negligible differences in climbing index (Figure 3B).

Silencing *Trh* neurons increased food intake

Neuromodulators are known to affect foraging and feeding behaviours in vertebrates and invertebrates (Shen and Cai 2001; T. Zhang, Branch, and Shen 2013; Gasque et al. 2013; Williams et al. 2014; Vucetic and Reyes 2010). Thus, we measured cumulative food intake in the five driver lines over a period of 6 h in a group of flies using a CAFE assay. Control *UAS-Kir2.1; Tub-Gal80^{ts}/+* flies drank +40% more liquid food after 31°C warm treatment, which represented the expected change in food intake as a result of warm treatment alone without Kir2.1 induction (Figure 4). Despite this large change in motor activity in response to temperature, *TH>Kir2.1* displayed only a minor change in food intake (Figure 4A). Interestingly, *Trh>Kir2.1* was the only line that displayed a substantial increase (51% over 6 h) in consumption after Kir2.1 induction (Figure 4).

Silencing *Ddc*, *Tdc2* and *NPF* cells reduced food intake

Data obtained from CAFE assays indicated that the silencing of neuronal cells in several driver lines produced decreases in feeding behaviour in flies that had been starved for 24 h. However, in all cases, the food intake of the uninduced flies was higher than that of the driverless controls: uninduced, Gal80^{ts}-repressed *Ddc>Kir2.1*, *Tdc2>Kir2.1* and *NPF>Kir2.1* flies had +105%, +185% and +166% increased food consumption, relative to *UAS-Kir2.1; Tub-Gal80^{ts}/+* controls, respectively (Figure 4A). As the only shared genetic dif-

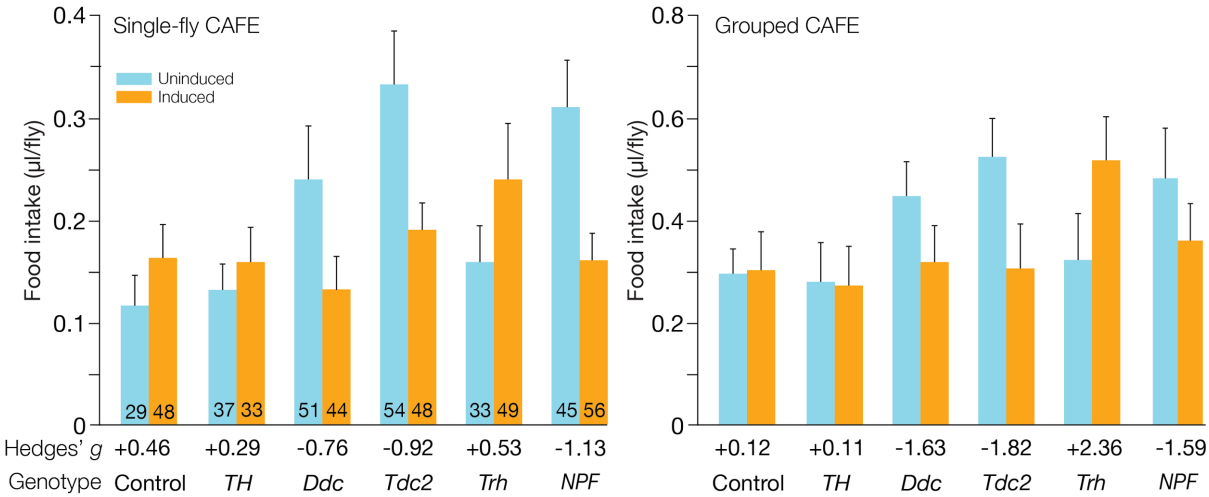

Figure 4. Silencing neuromodulatory circuits has effects on food intake.

A. The total food intake over 6 h was measured by capillary feeding (CAFE) assay. Control *UAS-Kir2.1; Tub-Gal80ts/+* flies drank a little more after 31°C warm treatment: Δfood intake = +0.05 µl [95CI +0.09, +0.004], g = +0.46 P = 0.036, N_{flies} = 29, 48. *Ddc-Gal4* flies deficient in dopamine exhibited a reduction in food intake: Δfood intake = −0.11 µl [95CI −0.05, −0.16], g = −0.76, P = 0.0003, n = 51, 44. Inhibition of Tdc2-Gal4 neurons also caused a substantial decrease in cumulative food consumption: Δfood intake = −0.14 µl [95CI −0.10, −0.18], g = −0.92, $P = 3.8 \times 10^{-5}$, n = 54, 48. Conversely, inhibition of Trh-Gal4 cells increased food consumption: Δfood intake = +0.08 µl [95CI +0.14, +0.02], g = +0.53, P = 0.02, n = 33, 49. *NPF-Gal4* flies had a reduced food intake: Δfood intake = −0.15 µl [95CI = −0.09, −0.20], g = −1.125, $P = 1.8 \times 10^{-7}$, n = 45, 56).

B. The results of the individual CAFE assay were confirmed with conventional CAFE: groups of *Ddc-Gal4* flies had a reduced food intake with Δfood intake = −0.16 µl [95CI −0.062, −0,27], g = −1.63, P = 0.02; *Tdc2-Gal4* Δfood intake = −0.22 µl [95CI −0.15, −0.294] g = −1.82, P = 0.01; *Trh-Gal4* Δfood intake = +0.19 µl [95CI +0.27, +0.1], g = +2.36, P = 0.003; *NPF-Gal4* Δfood intake = −0.12 µl [95CI −0.04, −0.2] g = −1.59, P = 0.02. The data represent the means with their 95% CI. A total of 10 males were used for each replicate and the assay was repeated at least five times for each genotype. The control data are depicted in blue, and the experimental (induced) data in orange. The numbers above each column denote the effect sizes for the individual driver lines. The numbers at the base of each column denote the sample size (N). Uninduced *D. melanogaster* lines exhibited increased food intake relative to the control line: *Ddc-Gal4* Δfood intake = +0.15 µl [95CI +20, +0.09]; Tdc2-Gal4 Δfood intake = +0.23 µl [95CI +0.29, +0.18]; NPF-Gal4 Δfood intake = +0.19 µl [95CI +025, +0.12]. Abbreviations: TH, tyrosine hydroxylase; Ddc, dopa-decarboxylase; Tdc2, tyrosine decarboxylase 2; Trh, thyrotropin-releasing hormone; NPF, neuropeptide F precursor.

ference between the uninduced experimental flies and the uninduced driverless control is the presence of a driver, the increased intake could be due to partially incomplete Gal80^{ts} repression at 22°C (Marella, Mann, and Scott 2012; Masek and Keene 2013; Pool et al. 2014; Jeong et al. 2016). Relative to the elevated baseline of uninduced *Ddc>Kir2.1* controls, inhibition of *Ddc* cells reduced their 6 h food intake by −0.11 µl [95CI −0.05, −0.16], g = -0.76, P = 0.0003 (Figure 4A). Blockade of neural activity in *Tdc2-Gal4* cells also reduced food intake by −0.14 µl [95CI −0.10, −0.18], g = −0.92, $P = 4.41 \times 10^{-10}$; similarly, silencing NPF-ergic neurotransmission decreased food intake by −0.15 µl [95CI = −0.09, −0.20], g = -1.13, $P = 1.8 \times 10^{-7}$ (Figure 4A). These results indicate that these neuromodulatory systems normally function to promote feeding.

A group feeding assay confirmed two types of feeding changes

The unusual changes in feeding behaviour in the uninduced flies in the single-fly CAFE assay, and large changes in feeding in the induced flies, raised the question as to whether these data were due to isolation of the flies versus group behaviour or might be sporadic results. As such, we replicated all the experiments in a group CAFE assay (Ja et al. 2007). In the group CAFE experiment, the overall effects of genotype and induction were very similar to the original, individual-level data. The increases in the baseline feeding level were also replicated, but were less marked than in the single-fly assay: uninduced *Ddc>Kir2.1*, *Tdc2>Kir2.1, Trh>Kir2.1* and *NPF>Kir2.1* flies demonstrated a +51%, +77% and +63% increase in feeding, respectively, compared to the uninduced controls (Figure 4B). This experiment also reproduced the effect of decreased feeding after Kir2.1 induction in these lines, eliciting −29%, −42%, and −25% reductions, respectively (Figure 4B).

Neuromodulators influence foraging behaviour

To further assess the role of neuromodulators in feeding behaviour we assessed the effects of neural silencing on a food-discovery task (Navawongse et al. 2016). Liquid food was provided to hungry flies over six sessions and the ability to approach the transient food source was measured. In each epoch, flies had 100 sec to find the food outlet alcove. Analyzing the number of successful alcove entries revealed that most lines exhibited a similar behaviour to their uninduced sibling controls. Only induced *TH>Kir2.1* animals entered the food alcove markedly less than uninduced controls, with a mean of 1.01/6 possible entries compared to 3.45/6: Δentries = −2.44 [95CI −2.02, −2.80], g = −1.44, $P = 8.86 \times 10^{-26}$ (Figure 5A). Induced *TH>Kir2.1* flies also travelled a shorter distance before entering the alcove: Δdistance = −40.2 mm [95CI −8.29, −66.66], g = +0.63, $P = 2.11 \times 10^{-6}$ (Figure 5D). Neither the time to alcove entry nor the path efficiency were affected in any of the lines, although *Tdc2>Kir2.1* induction resulted in a small reduction in the number of alcove entries, from 3.2/6 to 2.41/6: Δentries = −0.8 [95CI −0.19, −1.32], g = −0.44, P = 0.007. These data suggest that neuromodulator silencing has a minimal effect on food-discovery in *D. melanogaster*, with the exception of dopaminergic neuron silencing, as observed in the induced *TH>Kir2.1* flies that performed poorly. This effect is likely due to the generally impaired motor function of these flies.

Silencing aminergic circuits affects oxygen consumption during different nutritional paradigms

Neuromodulators are involved in metabolic homeostasis and energy regulation in both mammals and *Drosophila* (Loftus et al. 2000; Leibel, Rosenbaum,

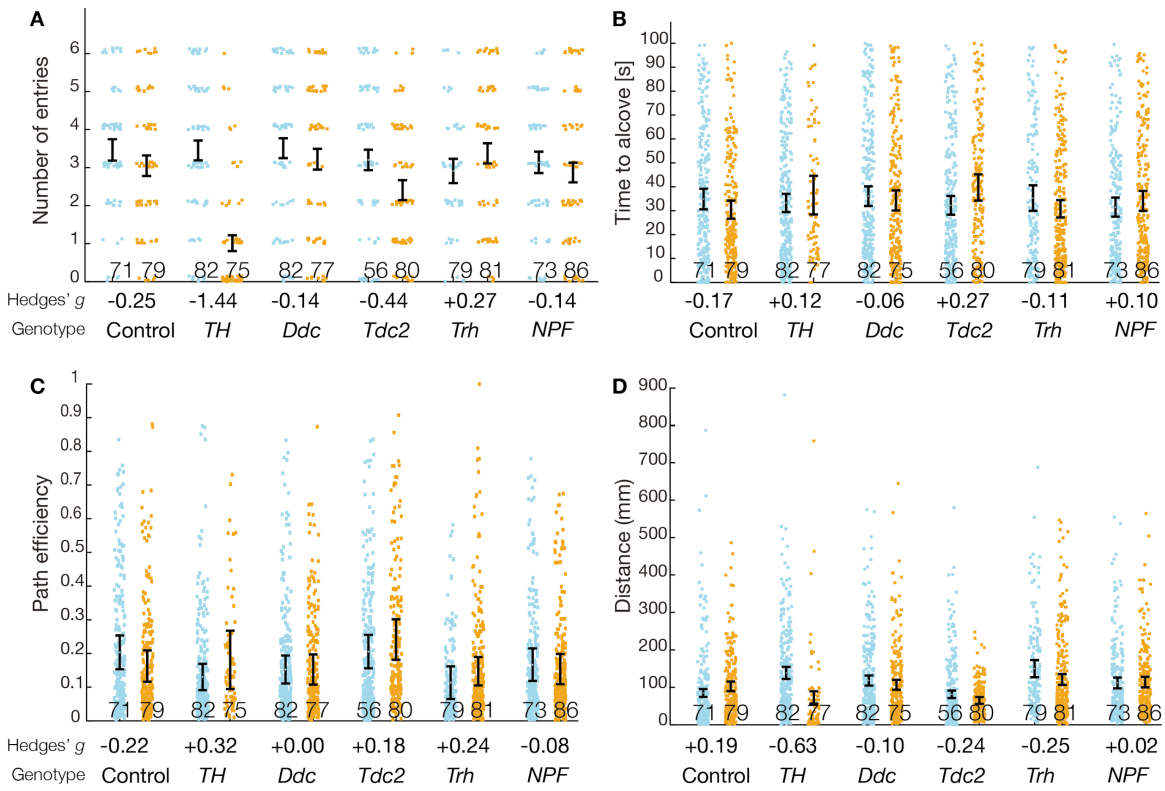

Figure 5. Effect of dopaminergic neuron inhibition on feeding behaviour in TH>Kir2.1 flies.
A. Mean number of entries to the feeding alcove. Induced *TH>Kir2.1* flies had a reduced number of entries relative to controls: Δentries = −2.44 [95CI −2.02, −2.80], g = −1.44, P = 8.86 × 10^{-26}, n = 82, 75.
B. Silencing neuromodulators did not affect the time latency to enter the alcove.
C. No differences were seen in the path efficiency between induced and uninduced animals.
D. Induced *TH>Kir2.1* flies did not travel as far to the alcove as control flies: Δdistance = −65 mm [-33.4, −90.7], g = −0.63, P = 1.93 × 10^{-5}, n = 82, 75. Control flies showed no effect on distance travelled to the alcove. Flies with inhibited neurons (induced) are depicted in orange and control flies (uninduced) are depicted in blue. Dots represent the distances travelled in all epochs in which a fly successfully entered the feeding alcove. Error bars represent the 95% confidence intervals. Numbers above the columns denote the effect sizes. Numbers at the base of each scatter plot denote the sample size (N). Abbreviations: Ctrl, control; TH, tyrosine hydroxylase; Ddc, dopa-decarboxylase; Tdc2, tyrosine decarboxylase 2; Trh, thyrotropin-releasing hormone; NPF, neuropeptide F precursor.

and Hirsch 1995; Obici et al. 2002; Harris-Warrick and Marder 1991; Pfaff, Kieffer, and Swanson 2008). We asked, therefore, whether manipulation of neuromodulatory circuits might affect respiration in a nutritional status-dependent manner (L. Zhang et al. 2015). Flies were either allowed to feed *ad libitum* or were starved for 24 h prior to warm-induced Kir2.1 expression. Warm treatment at 31°C caused a moderate decrease in the respiration rate of fed, control flies: ΔVO_2 = −0.81 µl/fly/h [95CI −0.19, −1.4], g = −0.59, P = 0.014, n = 51, 27. Interestingly, in the starved state, silencing cells with either dopaminergic driver (*TH-Gal4* or *Ddc-Gal4*) resulted in decreased respiration and reduced oxygen consumption: induced *TH>Kir2.1* ΔVO_2 = −1.63 µl/fly/h ([95CI −0.36, −2.64], g = −0.80, P = 0.046); induced *Ddc>Kir2.1* ΔVO_2 = −1.32 µl/fly/h ([95CI −0.78, −1.83], g = −0.94 P = 6.59 × 10^{-6}) (Figure 6B). Consistent with their increase in food intake, induced *Trh-Gal4* flies also showed an increase in metabolism after starvation: *Trh>Kir2.1* ΔVO_2 = +3.44 µl/fly/h [95CI +4.7, +2.4], g = +1.14, P = 1.2 × 10^{-11} (Figure 6B).

Silencing neuromodulator cells produced no major overnight changes in body weight or fat content.

We next assessed whether neuromodulator disruptions caused any changes in body weight by measuring the mass and lipid content of fed and starved flies, before and after Kir2.1 induction (Figure 6C). Differences in the lipid levels

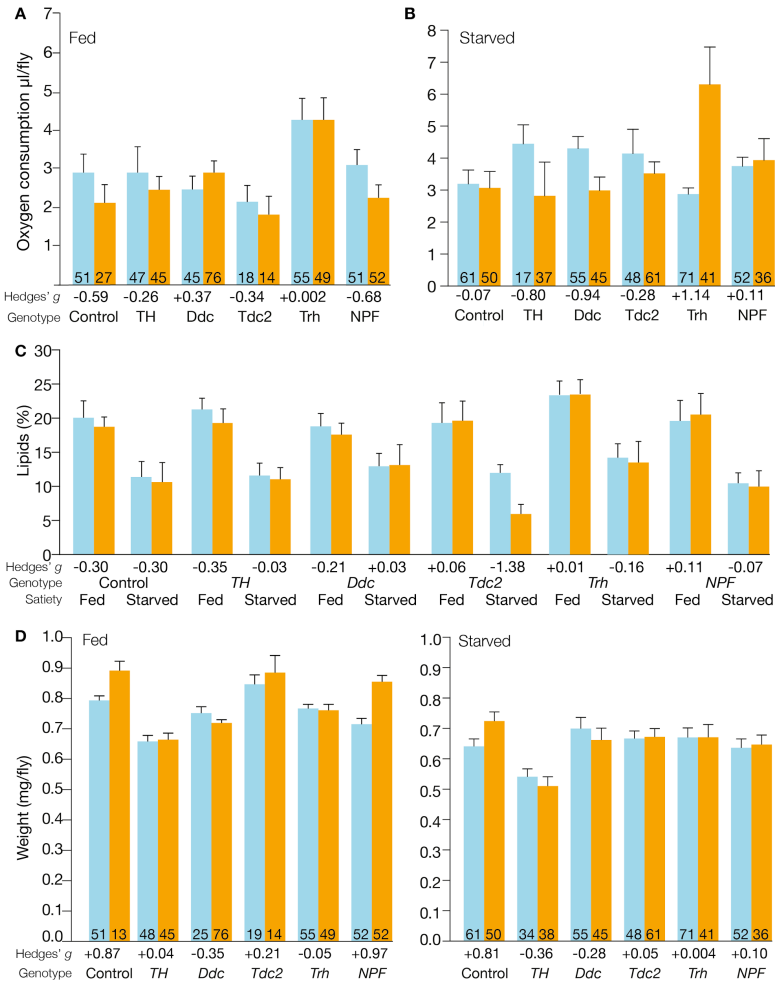

Figure 6. Manipulation of dopaminergic, serotonergic and NPF-ergic activity during different dietary paradigms alters metabolism.

A. Respirometry measurements of neuromodulator driver lines with induced Kir2.1 or *UAS-Kir2.1; Tub-Gal80^{ts}/+* flies (Ctrl). The progeny were assayed in the uninduced state or after overnight warm induction to elicit Kir2.1 expression, and after *ad libitum* feeding. Several lines underwent modest changes in oxygen consumption (VO_2). Inhibition of NPF moderately decreased respiration: ΔVO_2 = −0.85 µl/fly/h [95CI −0.34, −1.32],g = −0.6835, P = 0.008, n = 51, 52).

B. Respiration rate after the flies were wet starved for 24 h. Several lines underwent substantial changes in consumption: *TH>Kir2.1*, ΔVO_2 = −1.63 µl/fly/h less oxygen ([95CI −.35, −2.64], g = −0.6, P = 0.045, n = 17, 37) ; *Ddc>Kir2.1*, ΔVO_2 = −1.32 µl/fly/h less oxygen ([95CI −0.78, −1.83, g = −0.96, P = 6.59 × 10^{-6}, n = 55, 45); *Trh>Kir2.1*, ΔVO_2 +3.44 µl/fly/h [95CI +4.7, +2.4], g = +1.14, P = 1.2 × 10^{-11}, n = 71, 41.

C. Whole-body lipid levels were determined in fed flies (0 h) and flies starved for 24 h. All lines underwent substantial changes in lipid levels during starvation, but these were comparable to control flies, with the exception of *Tdc2>Kir2.1*, which displayed a −50% loss of fat after 24 h starvation when compared to its uninduced control: Δlipid = −6.08% [95CI −4.32, −7.7], g = −1.38, P = 0.0003. Five males were used for each replicate, and the assay was repeated at least five times for each genotype.

D. Whole-body weight for controls or respective genotypes in 5–7 day-old flies. No marked changes in weight were observed in any of the lines 24 h after induction, in either fed or starved animals. Flies with inhibited neurons (induced) are depicted in orange and control flies (uninduced) are depicted in blue . Data represent the means with their 95% CI; numbers at the base of each column denote the sample size (n) and the numbers at the top denote the respective effect size. Abbreviations: Ctrl, control; TH, tyrosine hydroxylase; Ddc, dopa-decarboxylase; Tdc2, tyrosine decarboxylase 2; Trh, thyrotropin-releasing hormone; NPF, neuropeptide F precursor.

were not observed in the majority of the experimental lines, as both uninduced and induced flies displayed similar lipid levels in both the fed and 24 h-starved states (Figure 6). The exception was observed in the induced *Tdc2-Kir2.1* flies, which had reduced lipid levels compared to uninduced controls after 24 h starvation: Δlipid = −6.08%, [95CI −4.32, −7.7], g = −1.38, P = 0.0003 (Figure 6C). When compared with the warm-treated wild type control flies (*UAS-Kir2.1; Tub-Gal80^{ts}*/+) the Δlipid of the *Tdc-Kir2.1* flies was −4.76% ([95CI −3.24, −6,92], g = −2.65, P = 0.002) after 24 h of starvation (Figure 6C). Interestingly, none of the flies showed any substantial changes in overall wet weight under any of the experimental conditions (Figure 5D).

Table 1. Summary of results from the behavioural and metabolic assays.
Standardized effect sizes (Hedges' g) are shown for all assays conducted on neuromodulator-silenced flies. Hedges' g represents the difference (in standard deviations) between uninduced control flies and warm-treated flies with de-repressed Kir2.1 expression. Effect sizes are listed for all 14 metrics from 11 assays conducted on five different neuromodulator driver lines. Moderate (g > 0.50) and larger effect sizes are coloured in red for decreases and green for increases. Silencing *TH-Gal4* cells produced coordinated decreases in motor-related phenotypes (activity, climbing, alcove entries, distance travelled). The other four lines had reproducible effects on post-starvation food intake, but these were not accompanied by consistent behavioural or metabolic phenotypes.

Assay	*TH-Gal4*	*Ddc-Gal4*	*Tdc2-Gal4*	*Trh-Gal4*	*NPF-Gal4*
Activity	-1.24	-0.53	+0.65	-0.09	-0.14
Climbing	-1.55	-0.35	+0.41	-0.56	-0.00
Food intake - Single	+0.29	-0.76	-0.90	+0.53	-1.13
Food intake - Group	+0.11	-1.63	-1.82	+2.36	-1.59
Alcove entries	-1.44	-0.14	-0.44	+0.27	-0.14
Time to alcove	+0.12	-0.06	+0.27	-0.11	+0.10
Path efficiency	+0.32	+0.00	+0.18	+0.24	-0.08
Distance travelled	-0.55	-0.10	-0.24	-0.25	+0.02
VO2 - fed	-0.26	+0.37	-0.34	+0.00	-0.68
VO2 - starved	-0.80	-0.94	-0.28	+1.14	+0.11
Lipid - fed	-0.35	-0.21	+0.06	+0.01	+0.11
Lipid - starved	-0.03	+0.03	-1.38	-0.16	-0.07
Weight - fed	+0.04	-0.35	+0.21	-0.05	+0.97
Weight - starved	-0.36	-0.28	+0.05	+0.00	+0.10

Discussion

Dopaminergic effects on motor function and feeding

Previous studies have shown that activating *TH-Gal4* cells in transgenic flies promotes the PER (Inagaki et al. 2012), whereas inhibiting these cells reduces the PER (Marella, Mann, and Scott 2012), suggesting that dopaminergic activity modulates feeding drive (Figure 2). However, our data indicate that inhibiting *TH-Gal4* cells has only a trivial effect on post-starvation food intake (Figure 4). Rather, silencing the *TH-Gal4* cells resulted in substantial effects on overall activity, climbing, food discovery and respiration—outcomes that are consistent with a disruption in motor function (Table 1). From these data we conclude that all the phenotypes we observed in this transgenic line are due to this generalized motor deficit. These findings confirm previous work that has shown that loss of either dopaminergic neurons or the dopamine synthetic enzyme, tyrosine hydroxylase, results in motor impairments (Thomas Riemensperger et al. 2013; T. Riemensperger et al. 2011; Shaltiel-Karyo et al. 2012; Islam et al. 2012; Coulom and Birman 2004). Si-

lencing the dopaminergic cells in the *Ddc-Gal4* driver did not result in motor deficits; rather, inhibiting the *Ddc-Gal4* cells (which include both dopaminergic or serotonergic neurons) robustly and specifically suppressed food intake and reduced respiration. While *Ddc-Gal4* targets some serotonergic cells, silencing cells using the nearly comprehensive *Trh-Gal4* serotonergic driver, had the opposite effect: induced *Trh>Kir2.1* flies increased their food intake and consumed more oxygen (Table 1). These distinct results suggest that *Ddc-Gal4* contains a subset of dopaminergic cells (or maybe serotoninergic cells) that normally act to promote feeding. One candidate group of feeding-promoting cells is the paired antero-medial cells, which are known to promote appetitive memory (Burke et al. 2012; Liu et al. 2012) and are numerous in *Ddc-Gal4* cells but sparse in *TH-Gal4* cells.

Reduced feeding in animals with silenced *Tdc2-Gal4* cells

Tyrosine decarboxylase is required for the synthesis of both octopamine and tyramine (Roeder 2005). Octopamine is known to have roles in a range of behaviours (Crocker and Sehgal 2008; Crocker et al. 2010; C. Zhou and Rao 2008), including appetitive learning (Burke et al. 2012), and starvation-induced hyperactivity (Yang et al. 2015); less is known about the role of tyramine (Roeder 2005). Previous work found that activating the *Tdc2-Gal4* cells produces a dramatic increase in capillary feeding, while silencing these cells causes only a minor reduction in PER (Williams et al. 2014; Marella, Mann, and Scott 2012). In our experiments, silencing *Tdc2-Gal4* cells caused a substantial decrease in food intake, consistent with the earlier evidence that *Tdc2-Gal4* cells function to promote feeding. Additionally, inhibition of the *Tdc2-Gal4* cells was found to increase lipid depletion during starvation (Figure 6C), but it is unlikely that this response directly relates to the food intake phenotype as both parameters were measured after 24 h starvation.

Are feeding, activity and metabolism dissociable?

We originally proposed that perturbed neuromodulatory function would have concomitant behavioural and metabolic phenotypes. Both the individual and group assays of post-starvation feeding found that silencing the *Trh-Gal4* cells led to an increase in food intake (Table 1). These animals also displayed an increased respiration rate, but this effect was not accompanied by an increase in activity. Silencing *NPF-Gal4* cells produced decreases in post-starvation feeding, but there were no concomitant metabolic or behavioural changes. Even though silencing *Th-Gal4* cells had coordinated effects on activity and metabolism, these effects did not translate into reduced feeding after starvation. These isolated phenotypes suggest that incapacitating one neuromodulatory system may have specific, dissociated effects on one or several aspects of feeding, activity and/or metabolism. These findings erode confidence in our original hypothesis of coordinated neuromodulation of behaviour and metabolism, and implies that these physiological functions are regulated by distinct, separable neuromodulatory subsystems. The present study is only the fourth to specifically examine the effects of neuromodulatory circuit-silencing on feeding in adult *Drosophila* (Marella, Mann, and Scott 2012; Inagaki et al. 2012; Williams et al. 2014). Recently developed, specific drivers, such as split-Gal4 lines targeting dopaminergic cells (Aso et al. 2014), will allow for new analyses of subsets of the neuromodulatory systems examined here. Such future studies will help determine the extent to which multi-phenotypic effects are due to pleiotropy of single neuromodulator cell types, and which effects are due to dissociable functions within the overall system.

Methods and materials

Fly stocks and maintenance

The five Gal4 driver lines used were *TH-Gal4* (Friggi-Grelin et al. 2003), *Ddc-Gal4* (Li et al. 2000), *Tdc2-Gal4* (Cole et al. 2005), *NPF-Gal4* (Wu et al. 2003), *Trh-Gal4* (Alekseyenko, Lee, and Kravitz 2010). Driver lines were outcrossed with the Canton-Special (CS) wild type strain for five generations. Driver lines and CS were then crossed with *UAS-Kir2.1; Tub-Gal80^{ts}* flies to produce the male F1 flies used in all behavioural experiments. The CS flies crossed with *UAS-Kir2.1; Tub-Gal80^{ts}* were used as a negative control for each assay and are hereafter referred to as controls. Flies (5–8 days old) were maintained at 22°C, in 60-70% relative humidity, under 12:12 hour artificial light-dark cycles before the experiment day. Neuronal silencing mediated by over-expression of the potassium Kir2.1 channel was induced in flies carrying a Gal4 driver transgene and *UAS-Kir2.1; Tub-Gal80^{ts}* by incubation at 31°C for 24 h followed by 22°C for 24 h, 1 day prior to the start of the experiment. If starvation was required, flies were wet starved (deprived of food but not water) for 24 h before the start of the experiment; these flies were maintained in a vial containing a strip of filter paper soaked in deionized water.

Systematic review - database search

A systematic literature search was conducted as previously described (Yildizoglu et al. 2015). The search expression [(*Drosophila* or fruitfly or "vinegar fly" or "fruit fly") AND (feeding or obesity or foraging or starvation) AND (NPF or "neuropeptide F" or octopamine or serotonin or dopamine or tyramine)] was used to query PubMed in September 2016, which resulted in 120 records (Figure 1). All the bibliographic information, including Title and Abstract, was exported to a Microsoft Excel spreadsheet, and the titles, abstracts and/or full texts were interrogated. The spreadsheet was used to record the results of the full text screen, which included a detailed screen of experimental design.

Systematic review - study selection

The literature selection process was designed to identify experiments that examined the involvement of neuromodulation on feeding in *Drosophila* by using various feeding assays. Our analysis focused on experiments that aimed to understand the role of neuromodulators on feeding by using inducible neuronal activation or deactivation in genetically modified flies. The 120 records yielded from the PubMed search were screened in four stages based on title review, abstract reading, full text scan and a detailed review of experimental design, to systematically exclude studies that were not relevant (Supplementary Table 1). After systematic review, 117 studies were eliminated (Figure 1), and data were collected from the remaining three studies, as detailed below.

Systematic review - data extraction

The following data were collected from the included studies: authors, year of publication, figure and panel numbers, genotype, mean number of flies in the control and experimental groups and the corresponding standard errors of the mean and sample sizes of each group, age and gender of the flies, type of food used during feeding experiments, and the number of hours of starvation prior to starting the experiment. Numeric data were digitized from graphics with the Measuring Tool in Adobe Acrobat Pro.

Small-animal Nutritional Access Control (SNAC) food discovery assay

A microfluidic feeding assay was performed as previously described (Navawongse et al. 2016). Briefly, each microfluidic SNAC chip contained a 20 × 22 mm arena with a feeding alcove connected to a microfluidic channel that is designed to deliver 70 nl of liquid food via an actuator pump. Eight chips were run simultaneously. Experiments were conducted inside an incubator maintained at 22°C. The flies were given 10 min to habituate to the chip/incubator environment prior to the start of the experiment. A combined stimulus of blue light with an 85 dB, 300 Hz sound was administered at the onset of the 70 nl liquid food reward (Navawongse et al. 2016). The liquid food (5% sucrose 10% red food dye in deionized water) was retracted automatically when the fly was detected inside the feeding alcove or a timeout of 100 sec was reached. Six foraging-feeding epochs were imposed in succession with a 120 sec inter-epoch interval. The following parameters were recorded with custom LabView code as previously described: the number of entries into the feeding alcove, time to feed, path efficiency, and the distance travelled during the time the food was presented (Navawongse et al. 2016). Task performance was calculated by dividing the number of epochs with successful alcove entries by the total number of epochs. We defined "time to feed" as the latency to enter the feeding alcove, and "path efficiency" as the distance of the most direct path to the feeding alcove divided by the actual distance travelled by the fly in each feeding epoch.

Climbing assay

Male flies were separated from the F1 progeny and maintained in a freshly prepared food vial 24 h before the start of the experiment. No more than 20 flies were housed within the same vial. Five male flies were put inside a 50 ml disposable polystyrene serological pipet (Fisher Scientific, Waltham, USA) that was cut to 50 mm in length. The top and bottom of the tube were sealed with parafilm that was punctured with three small holes to provide ventilation. The tube was then placed flat on a surface for 1 h at 22°C while the flies acclimatised. At the beginning of the experiment the tube was tapped 2–3 times to encourage the flies to the bottom of the tube. The time for each fly to reach the top marked point of the tube was then recorded. Any flies that could not reach the top mark within 60 sec were denoted as failing to climb.

CO_2 metabolism assay

Male flies were transferred to a food vial 24 h before the start of the experiment. In the starvation group, flies were maintained inside a vial with only water soaked in the filter paper. All flies were maintained at 22°C under a 12:12 hour light-dark cycle. The respiration chamber consisted of a 1 ml syringe (Becton Dickinson, USA) and a glass microcapillary tube (53432-728, VWR, USA) glued to the 16-gauge needle (Becton Dickinson, USA). A thin layer of absorbent and non-absorbent cotton filled the top of syringe. A fly was anesthetized by cooling and loaded into the syringe. The syringe plunger was inserted to close the chamber, leaving a 1–1.5 cm space for the fly to move. A total of 15 µl potassium hydroxide (KOH, Sigma, USA) was loaded on the top of the syringe and the glass microcapillary tube was assembled onto the syringe. The gap between the syringe and holder were wrapped with parafilm. The respiration chambers were incubated under water inside the 22°C incubator (Sanyo, Japan) for 10 min. A small volume of 30% sucrose solution containing 10% food colour dye filled the top of the glass capillary to isolate the respiration chamber from the outside environment. The position of the coloured solution was measured 15 min after it was filled and its final

position was determined after 1 h. The CO_2 metabolic volume consumption during a 1 h period was obtained from the increase in volume within the glass capillary.

Body weight and lipid measurements

Male flies were starved for 1 h under non-dehydrating conditions to limit the amount of food-derived lipids present in the gut. Each group of flies were then weighed to 0.1 mg accuracy using an analytical balance (Sartorious, Singapore). For lipid measurements, groups of five males were weighed to obtain their wet mass and then dehydrated at 65°C for 24 h and subsequently weighed to obtain their dry mass. Lipid extraction was performed by placing intact, dehydrated flies in a glass vial containing 9 ml diethyl ether for 24 h at room temperature under gentle agitation. After incubation the diethyl ether was removed and the flies were air dried for an additional 24 h at room temperature. The weight of the flies was then remeasured to obtain lean dry mass. The total lipid content of the flies was considered to be the difference between the dry mass and lean dry mass.

Capillary feeding (CAFE) assays

Male flies were anesthetized by cooling and placed in chambers for the CAFE assay, where capillaries delivered liquid food (5% sucrose 10% red colour food dye in deionized water) to the fly. The experiment was conducted within an incubator that was maintained at 22°C (Ja et al. 2007). The level of the fluid was noted at the beginning of the experiment and 6 h later. A control experiment, whereby no flies were housed inside the chamber, was also conducted in order to calculate the loss of fluid due to evaporation. The difference in the reduction of fluid level between the experimental and control chambers was assigned as the volume partaken by an individual fly. We used a group assay in which 10 flies were assayed in a vial with a single capillary as previously described (Ja et al. 2007), as well as a single-fly assay, in which each capillary was accessible by a single fly kept in a 12 mm × 12 mm × 2 mm chamber cut from acrylic.

Data analysis

Data were analyzed with custom scripts written in LabView, Matlab and Python, and visualized with GraphPad. The data were analyzed with estimation methods to calculate mean differences and Hedges' g where appropriate (Claridge-Chang and Assam 2016). Standardized effect sizes are referred to as 'trivial' or 'negligible' ($g < 0.2$), 'small' ($0.2 < g < 0.5$), 'moderate' ($0.5 < g < 0.8$) or 'large' ($g > 0.8$) (Cumming 2012). Both significance testing and power calculations were avoided per recommended practice (Altman et al. 2000; Cumming 2012), though Student's t statistic was used to calculate P values for *pro forma* reporting. To indicate estimate precision, 95% confidence intervals (95CI) were calculated using bootstrap methods and reported in text and/or as error bars (Cumming 2012).

Author Contributions

Conceptualization: RN, ZPW and ACC; *Methodology*: RN, MR, DC and AE; *Software*: JCS and RN; *Investigation*: AE (group CAFE, respiration, lipid, weight, genetics, systematic review), MR (activity, climbing, individual CAFE, SNAC, genetics, systematic review), and YLT (systematic review); *Resources*: RN, JCS, DC, ZPW (instrumentation); *Data Analysis*: RN (SNAC, single CAFE, respiration), MR (SNAC, single CAFE), AE (group CAFE, lipid, weight), YLT (systematic review); *Writing – Original Draft*: MR, AE; *Writing – Revision*: AE and ACC; *Visualization*: MR, AE, RN and ACC; *Supervision*: ZPW and ACC; *Project Administration*: ACC; *Funding Acquisition*: ZPW and ACC.

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