A multicomponent screen for feeding behaviour and nutritional status in *Drosophila* to interrogate mammalian appetite-related genes

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**ABSTRACT**

**Objective:** More than 300 genetic variants have been robustly associated with measures of human adiposity. Highly penetrant mutations causing human obesity do so largely by disrupting satiety pathways in the brain and increasing food intake. Most of the common obesity-predisposing variants are in, or near, genes expressed highly in the brain, but little is known of their function. Exploring the biology of these genes at scale in human obesity do so largely by disrupting satiety pathways in the brain and increasing food intake. Most of the common obesity-predisposing genes that influence energy balance in flies to those of 10 control genes. We then used this screen to explore the effects of disrupted expression of genes either a) implicated in energy homeostasis through human genome-wide association studies (GWAS) or b) expressed and nutritionally responsive in specific populations of hypothalamic neurons with a known role in feeding/fasting.

**Methods:** We validated a screen for feeding behaviour in *Drosophila* by comparing results after disrupting the expression of centrally expressed genes. We then used this screen to explore the effects of disrupted expression of genes either a) implicated in energy homeostasis through human genome-wide association studies (GWAS) or b) expressed and nutritionally responsive in specific populations of hypothalamic neurons with a known role in feeding/fasting.

**Results:** Using data from the validation study to classify responses, we studied 53 *Drosophila* orthologues of genes implicated by human GWAS in body mass index and found that 15 significantly influenced feeding behaviour or energy homeostasis in the *Drosophila* screen. We then studied 50 *Drosophila* homologues of 47 murine genes reciprocally nutritionally regulated in POMC and agouti-related peptide neurons. Seven of these 50 genes were found by our screen to influence feeding behaviour in flies.

**Conclusion:** We demonstrated the utility of *Drosophila* as a tractable model organism in a high-throughput genetic screen for food intake phenotypes. This simple, cost-efficient strategy is ideal for high-throughput interrogation of genes implicated in feeding behaviour and obesity in mammals and will facilitate the process of reaching a functional understanding of obesity pathogenesis.

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**Keywords** Appetite; Food intake; GWAS; Transcriptomics; *Drosophila*; Obesity

1. **INTRODUCTION**

Obesity is arguably the most serious public health threat of the 21st century [1] because of its association with comorbidities such as type 2 diabetes, cardiovascular disease, hypertension, and certain cancers [2]. Modern lifestyles have been the driver of the increase in obesity, and the variation in individuals’ response to this ‘obesogenic’ environment is large [3]. Underlying this variable response is a powerful genetic element: twin and adoption studies have revealed the heritability of fat mass to be between 40% and 70% [4,5].

Over the past 20 years, genetic and ‘omics’ approaches have been used to characterise the molecular and physiological mechanisms of food intake control. For instance, studies of human and mouse genetics have uncovered circuits within the brain that play a central role in modulating mammalian appetitive behaviour [6,7]. The best characterised of these circuits is the hypothalamic leptin-melanocortin signalling pathway, genetic disruption of which causes the majority of monogenic severe obesity disorders in mice and humans [8–11]. In addition, genome-wide association studies (GWAS) have identified more than 300 human genetic loci associated with variations in body
mass index (BMI) [12,13]. The genes closest to these loci, including many components of the melanocortin pathway, are primarily expressed in the central nervous system (CNS) [13,14]. Where their function is known, many of these genes influence food intake [6–11,15–19].

In addition to genetic approaches, transcriptomic analyses of discrete neuronal populations are playing an increasingly important role in illuminating novel genes and pathways that may play a role in appetite control [20,21]. At least 2 populations of neurons sense peripheral nutritional signals and play a central role in the melanocortin pathway: pro-opiomelanocortin (POMC) neurons decrease food intake when activated, and agouti-related peptide (AgRP) neurons increase food intake [22,23]. Some transcripts are reciprocally regulated in these 2 populations [21], but the function of these transcripts remains mostly unknown.

One reason for the disappointing rate of translating the genetic signals into insightful biological knowledge is that investigations of candidate genes have been mainly addressed in complex model organisms such as mice [24–32]. Given the significant resources required to generate and phenotype each murine model, they are not ideal for studying the effects of disruption of gene function at scale. Thus, a high-throughput model is necessary to ‘pre-screen’ genes for a potential role in feeding behaviour, before committing the resources necessary for further research in mammalian models. The fruit fly Drosophila melanogaster is a key model organism for research in developmental biology, cell biology, and neurobiology; has recently been demonstrated as an excellent model for dissecting metabolic homeostasis and nutrient-sensing pathways; and has a recently been demonstrated as an excellent model for dissecting metabolic homeostasis and nutrient-sensing pathways; and has a

2. MATERIALS AND METHODS

2.1. Fly husbandry

All flies were raised on a normal diet (ND: 1.25% agar, 10.5% dextrose, 9% maize, 2.6% yeast, 3.5% tapioca in water). Some assays required the use of food with added dye (ND + 1% Fast Green FCF dye [Sigma]) or a high-fat diet (HFD: ND + 20% coconut oil). Experimental flies were maintained at 25 °C and 60%–70% humidity in a 14-hour light: 10-hour dark cycle, except for flies on the HFD, which were housed at 20 °C in the dark. Unless otherwise noted, we used male flies aged between 5 and 10 days for the experiments, 15 flies per sample, and 5 biological replicates per genotype per assay.

Drosophila orthologues of human genes of interest were identified by using ENSEMBL [37] or NCBI BLAST searches of publicly available protein sequences [38,39]. Flies stocks (listed in Supplementary Table 1) were acquired from the Vienna Drosophila Resource Centre (VDRC) and the Bloomington Drosophila Stock Centre. For RNAi stocks, preference was given to GD lines to avoid potential complications from tip tap gene expression [40]. Two GAL4 lines were used: elav-GAL4/CyO, expressed in neurons [41], and act5C-GAL4/CyO, constitutively expressed throughout the body. UAS-RNAi lines were crossed with Gal4 lines (elav-Gal4 or act-Gal4), and controls were created by crossing each Gal4 line with control lines for GD, KK, and KKtip tap. Experimental flies were compared with the relevant background control (Supplementary Table 1). Three of the GD lines (ADCY3/Ac3, LRRN6C/Fili, and NEGR1/AnmA) were supplied as compound X stocks from VDRC and were rebacked by using Flm7 before phenotyping.

To standardise the effects of the parental environment on offspring fitness, all UAS-RNAi stocks were stored in bottles at approximately constant density. Flies for phenotyping were generated by crossing virgin female UAS-RNAi flies to male GAL4 flies and collecting the male offspring. To standardise the effects of parental age on offspring fitness, crosses were set up by using 1–5-day-old flies. Briefly, for each cross, on day 1, 5 UAS-RNAi/female virgin were placed in a vial of ND at 25 °C with 2 elav-Gal4 or 5 act-Gal4 males. On day 4, these parents were removed. On day 14, offspring were transferred to a new vial and allowed to mate because mating alters gene expression and metabolic parameters [42]. On day 15, the females were removed. On day 16 at 2 pm, male flies were placed on starvation media for 3 h to synchronise their metabolism before being returned to the appropriate diet for the experiment. Because Drosophila activity and feeding behaviours are affected by age and circadian rhythms [43], all assays were performed within a fixed time window: the capillary feeder (CAF) assay was performed at 2 pm; the fasting-induced overfeeding assay, at 9 am; and sample collection for wet mass and triglyceride analysis, at 10 am.

2.2. PCR-based diagnostic assay to determine appropriate background line for the ‘KK’ collections

Genomic DNA was isolated by crushing flies in ‘squishing buffer’ (10 mM Tris, pH 8.2, 1 mM EDTA, 25 mM NaCl, 200 µg/ml protease K), followed by a 30-minute incubation at 37 °C, after which the proteinase K was inactivated by heating at 95 °C for 5 min. We used a PCR-based diagnostic assay to determine the KK insertion site, as described in Green et al. [40]. Briefly, occupancy of the transgene at the annotated insertion site reported by the VDRC, 40D, was determined in a multiplex PCR. The following primers and PCR using these primers yield a ~450-bp product in the case of an insertion, or a ~1050-bp product in the case of an empty insertion site, at 40D:

40D Genomic_F 5′- GCCCACTGTGAGCCTCAAC -3′
pKC26_R 5′- TGTTAAAAGACGGCCAGT -3′
pC43_R 5′- TCCTGCTGGCAAGATGTCAC -3′

For detecting the non-annotated insertion, 30B (thus we used tip tap as control), the pKC26_R and pC43_R primers were multiplexed with the following primer:

30B_Genomic_F 5′- GCTGGCCAACGTCAAC -3′
Solutions were centrifuged (16,100 rcf, 4°C, 30 min) using a FastPrep-24 homogeniser (MP Biomedicals) for 60 s at 6 m/s. Homogenates were heat-inactivated (5 min, 70°C). PCR using these primers results in a ~600-bp product in the case of an insertion, and a ~1200-bp product in the case of an empty insertion site, at 30°C. PCR was performed with the following programme: 95°C 120 s initial denaturation, 30 cycles (95°C 15 s denaturation, 50°C 15 s annealing, 72°C 45 s extension), and a final 72°C 120s extension.

2.3. CAFE assay
The CAFE assay is an accurate method of measuring food intake in Drosophila [44] for which the liquid food in calibrated capillary glass tubes (5 μl, WVR International) is the only food available to the study flies. The CAFE assay was performed using a custom-made acrylic cap with 4 holes that hold 200 μl pipette tips; of the 4 holes, 2 act as air holes and 2 hold the glass capillaries (WVR 53432-706) in place. CAFE chambers were made from standard fly vials (25 x 95 mm) with 3 ml of 1% agar that serves as a water source and maintains internal chamber humidity (Supplementary Figure 1). Eight flies were placed into each vial. Two capillary tubes were filled with liquid food (5% sucrose and 5% yeast extract) via capillary action. The top of the meniscus was marked, and the filled capillaries were inserted into the chamber through the lid and left at 25°C. After an initial 48 h of habituation in the CAFE setup, the movement of the meniscus was measured over 24 h and evaporation (measured by a vial containing no flies) was subtracted to yield the volume of food consumed in 24 h.

2.4. Fasting-induced overfeeding assay
This protocol was modified from Williams et al., 2014 [45] and is particularly useful for examining satiety signals. Fifteen male flies were fasted for 24 h in vials with 1% agar. On the day of the experiment, these flies were transferred to ND and allowed to feed. After 20 min, the flies were then transferred to vials containing ND with 1% Fast Green dye for 15 min (Supplementary Figure 1). The number of flies with visible dye in their abdomen (mid-gut and/or crop) were counted under a dissecting microscope and scored as a percentage of the total number in the vial.

2.5. Wet mass
Groups of 15 Drosophila were frozen on dry ice and weighed by using a microbalance (Sartorius) rounded to 2 decimal places in milligrams.

2.6. Triglyceride assays
For triglyceride analysis, frozen Drosophila were placed in FastPrep tubes containing Lysis Beads and Matrix D (MP Biomedicals) and 350 μl cold PBST (PBS + 0.05% Tween-20) and then homogenised by using a FastPrep-24 homogeniser (MP Biomedicals) for 60 s at 6 m/s. Solutions were centrifuged (16,1000 rcf, 4°C, 3 min) to pellet debris, and 300 μl of supernatant pipetted into a fresh Eppendorf on ice. Homogenates were heat-inactivated (5 min, 70°C). Triglyceride levels were analysed by using enzymatic assays by the Cambridge Core Biochemical Assay Laboratory. Triglyceride amount was normalised to the number of Drosophila.

2.7. Phenotyping of larvae
Cages were set up containing 60 virgin female and 30 male flies on apple juice agar plates with fresh yeast paste and left for 3 days at 25°C. On the day of collection, a new plate with fresh yeast paste was provided every 30 min for 1 h to clear old embryos from the oviducts of female flies. Embryos were collected on a fresh plate for 3 h and then left for 72 h to develop into 3rd instar larvae. Larvae were either fed liquid yeast or fasted (provided with distilled water instead) for 2 h. For the assay, yeast paste was placed in the centre of an apple juice agar plate, and 20 larvae were placed on the inside rim. The number of larvae in/out of the food was counted after 20 min.

2.8. Scoring algorithm
The scoring system 'sums up' data for all 3 assays performed in adult flies to provide a quantitative measure of the overall phenotype. The scoring sums the p values for each assay and then subtracts from 1 so that more significant results yield higher scores. We used at least 5 sets of biological replicates for each assays and 15 flies per assay (except for the CAFE assay, which had 8 flies per assay).

2.9. Statistical analysis
Each assay was repeated at least 5 times by using independent crosses (biological replicates). Mean and standard error of the mean were calculated from all biological replicates of each experiment and analysed by using GraphPad Prism. Data from all assays were analysed using unpaired homoscedastic Student t tests. ANOVA with appropriate post hoc analysis for multiple comparisons was employed where appropriate.

3. RESULTS

3.1. Selection of assays for the high-throughput screen
To create a Drosophila-based functional screen focused on appetitive behaviour, we selected 3 assays: body mass and 2 measures of energy intake (the CAFE assay and the fasting-induced feeding assay). To validate these assays, we tested each for their ability to differentiate wild-type flies on a ND from those either fasted for 24 h or placed on an HFD for 5 days (Figure 1). The CAFE assay measures the amount of liquid food (sucrose plus yeast extract) that flies consume from a capillary tube by tracking the distance moved by the meniscus over a given time. As expected, fasted flies consumed significantly more than the controls (144%; p = 0.0012) did, and flies on an HFD consumed less (70%; p = 0.0538; Figure 1A). The fasting-induced feeding assay measures food intake after fasting. Flies were fasted for 24 h and then exposed to normal food for 20 min before being transferred onto food coloured with dye for 15 min, which is then visible through their translucent abdomen. In the HFD group, the number of flies with green stomachs decreased to 29% of that of controls (Figure 1B, p = 0.036). Fasted flies, as expected, had less wet mass (80%, p < 0.0001) than flies fed ad libitum, and a HFD had no measurable effect on mass (Figure 1C). Therefore, the 3 assays detected physiologically meaningful responses of wild-type flies to nutritional perturbation.

3.2. Validation of the high-throughput screen using 'control' genes
Next, we tested our screen on 4 genes that play roles in Drosophila energy homeostasis (Table 1a). Because most genes linked to BMI are enriched for expression or function in the CNS [13], we took advantage of the UAS2-GAL4 system [46] to knock down the expression of each gene specifically in neurons by using the neuronal driver elav-GAL4 [41]. Results from the neuronal knockdown were compared with background-matched control lines (KK-elavGAL4, GD-elavGAL4, or KK_copine-elavGAL4 as appropriate; Supplementary Table 1). As one example, neuronal knockdown of upd1, a Drosophila orthologue of leptin [47], led to flies ingesting 1.5x more in the CAFE assay than in controls (p = 0.0149; Figure 1D and 2.4x more flies ingesting dyed food in the fasting-induced feeding assay (p = 0.0013; Figure 1E).
To take advantage of the multiple assays, we used a simple algorithm to produce an integrated score that reflects the overall phenotype resulting from neuronal knockdown of each gene. The score was calculated by taking the sum of the p values from the 3 assays for each gene and subtracting it from 1. Thus, the lower the p value, the more significant the results for a given gene, and the closer the score would be to 1. Using this method produced an integrated score of 0.99 for upd1 (Figure 1G). The scores for the other positive controls were 0.80 for DSK; 0.97 for Ttap2; and 0.70 for the twz (the Drosophila orthologues of CCK [48], Ttap-2, and KCTD15, respectively [45]).

In addition, we tested 10 negative control genes linked to diseases other than obesity (Table 1b). When the scores for all of the controls were plotted, the flies with neuronal knockdown of positive control genes had a significantly higher average score than the negative controls (p = 0.0006; Figure 1H). There was overlap between the scores for peripheral negative and positive controls, but on the basis of its ability to distinguish these 2 groups, we set a score of 0.80 as a threshold for the selection of genes of interest, with consideration given to genes with a score ≥0.70.

### Table 1 — Positive and negative control genes

| Human Gene | Drosophila gene | % Identity | Note          |
|------------|----------------|------------|---------------|
| leptin (47) | upd1           | 6          | Functional Orthologue |
| CCK (48)   | DSK            | 14         | Functional Orthologue |
| TFAP2B (45)| Ttap2          | 40         |               |
| KCTD15 (45)| twz            | 36         |               |

| Disease | Body weight association | Human gene | Drosophila gene | % Identity |
|---------|-------------------------|------------|----------------|------------|
| Schizophrenia | Obesity (65) | FZD3 (71) | fz | 42 |
| ALS     | Weight loss (63) | UNC13 (72) | unc13 | 56 |
| ALS     | Weight loss (63) | UNC13 (72) | unc13-4a | 56 |
| Alzheimer’s | Weight loss (66) | PSEN1 (70) | psn | 51 |

### 3.3. Screening of candidate genes from GWAS for BMI

Having validated our assays, we used them to screen candidate genes associated with BMI by GWAS. We focussed on 36 genetic loci identified by Speliotes et al. [49] and Lu et al. [50]. We studied the closest gene to each of the 36 BMI-associated SNPs (Supplementary Table 2), as well as additional genes within 500 kb of 7 of the SNPs, for a total of 53 human genes (Supplementary Tables 2 and 3). Forty-two of the human genes (81%) had at least 1 Drosophila orthologue and some had more than 1, giving a total of 56 Drosophila genes (Figure 2). UAS-RNAi lines were readily available for 53 of these genes. Of the 56 Drosophila genes selected for our screen, 55 are expressed in the Drosophila brain [51–53], suggesting conservation of expression.

CG10820/MITCH2 is not neuronally expressed but is expressed in the fat body and should therefore be unaffected by neuronal RNAi. Ubiquitous RNAi knockdowns (using the act5C-GAL4 driver) of 29 of the 53 lines tested (55%) were not viable as adult flies (Figure 3). This was much higher than the Drosophila genome-wide figure of 25% [36], suggesting that the BMI-associated GWAS genes are enriched for those that are essential for life.

In contrast with the ubiquitous knockdowns, neuron-specific knockdown (using the elav-GAL4 driver) of 29 of the 53 lines tested (55%) were not viable as adult flies (Figure 3). Several of these genes have murine orthologues with known roles in energy homeostasis (ATXN2L, PCSK1, NEGR1, MTIF3, SEC16B, and TFAP2B), increasing our confidence that our screen can reveal biologically relevant candidates. Other genes (GNPDA2, NRXN3, C2112020 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

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GABRG1, LRRN6C, KLF9, and SULT1A2) are relatively unexplored in relation to energy homeostasis, marking them prime candidates for further study. At 7 of the GWAS loci, multiple human genes were studied in our screen (Supplementary Table 3). At 3 of these loci, the nearest gene to the SNP resulted in the highest score of all the other genes in the vicinity: GNPDA2 (0.96), DNAJC27 (0.72), and QPCTL (0.77). At 3 other loci, the highest score was observed by neuronal knockdown of ‘non-nearest’ genes: ATXN2L (0.99), COL4A3BP (0.89), and APC1 (0.74). In addition, at the rs16951275 locus, neither the nearest gene (LBXCOR1) nor the nearby gene (MAP2K5) had a score >0.70, perhaps implicating other genes farther away than 500 kb. Baranski and colleagues [54] performed a screen of triglyceride levels in Drosophila, in which BMI GWAS candidate genes were concurrently knocked down in the CNS and fat body (using the cg-GAL4 driver). Sixteen Drosophila genes were studied in their screen and ours (Supplementary Table 4). In their screen, 5 of these genes showed an increase in triglycerides when knocked down. In our screen, 3 of these, namely NRXN3, SEC16B, and COL4A3BP, had scores >0.80 (Figure 3; Supplementary Table 4). Notably, Atx2 (corresponding to the human gene ATXN2L) showed no phenotype in the Baranski et al. triglyceride screen but achieved the highest score of 0.99 in our screen. However, in our hands, the knockdown of Atx2 resulted in flies that ate less, and mice without Abxn2 show a phenotype of adult-onset obesity [55]. Thus, although perturbation of its expression resulted in opposite feeding phenotypes in flies and mice, Atx2 and Abxn2 play a role in energy balance [56]. Notably, when Baranski and colleagues knocked down RPL27A in the CNS and fat body, the result was a lethal phenotype. In our hands, neuron-specific manipulation of RPL27A expression resulted in a score of 0.88, implicating RPL27A in the control of food intake.

3.4. Follow-up studies in larvae and adult knockout lines

Next, we followed up the results of the neuronal RNAi experiments by studying Drosophila third-instar larvae with genetic knockout of the same genes [57] as an orthogonal biological replication. Knockout lines were available for 38 of the 56 genes tested, of which 15 were homozygous viable. We used an assay that measured feeding behaviour after a fast. Larvae were either fed on liquid yeast or fasted (provided with distilled water) for 2 h. Twenty larvae from each group were then transferred to the inside rim of a plate containing apple juice agar with yeast paste placed at its centre, and the numbers of larvae inside and outside the food were counted after 20 min. This assay was validated by its ability to distinguish fed from fasted third-instar wild-type larvae:

![Figure 2: Selection of BMI GWAS genes.](image-url)
Figure 3: Neuronal (elav-GAL4) knockdown of BMI GWAS genes.
Figure 4: Selection of genes reciprocally regulated in murine POMC and AgRP neurons in response to fasting.

56% of fed larvae were found in the food versus 73% of fasted larvae (p = 0.017; Supplementary Figure 2). Of the 15 viable lines, 5 (PCSK1, NRXN3, NEGR1, OPCTL, and GIPR) did not display this anticipated increase in the number of larvae found in food after a fast (Supplementary Figure 2). PCSK1, NRXN3, and NEGR1 have been highlighted by the aforementioned neuronal RNAi studies, with composite phenotype scores >0.80 (Figure 3). We also studied the adult PCSK1, NRXN3, and NEGR1 knockout lines, as well as TFAP2B, which was also highlighted by the neuronal RNAi studies with a phenotype score of 0.97 (Figure 3). We examined dry mass, wet mass, triglyceride levels, and survival during starvation in these 4 lines and found that PCSK1 showed a consistent ‘obesity’ phenotype (Supplementary Figure 3), in accord with the involvement of PCSK in human monogenic obesity [18]. In human and rodent data, mutations affecting expression of PCSK1 result in an increase in body weight and food intake [24,28,30].

### 3.5. Screening of genes reciprocally regulated in mammalian POMC and AgRP neurons

Next, we assessed candidate genes emerging from transcriptomic analyses of neurons with key roles in the control of mammalian appetitive behaviour. Henry et al. [21] reported the expression levels of 35,266 mouse genes in POMC and AgRP neurons. We used a workflow to select a list of candidate transcripts with potential relevance to feeding, from this dataset (Figure 4). Of the 1,038 and 3,554 genes whose expression significantly changes (>1.5×; p < 0.05) in POMC and AgRP neurons, respectively, upon fasting, 192 responded in a reciprocal manner in the 2 neuron types. Using ENSEMBL, we identified Drosophila orthologues for 58% of these mouse genes, including some with multiple orthologues, and 2 mouse genes with the same fly orthologue. Overall, this resulted in a list of 157 Drosophila genes that corresponded to 112 murine genes. To maximise the likelihood of generating relevant biological insights, we selected orthologues of 61 mouse genes with amino-acid identity >30% and expressed in either larval or adult Drosophila CNS [51] (Figure 4). We again identified Drosophila UAS-RNAi lines for these genes, and after excluding 2 genes with no available UAS-RNAi line, and 6 for which only lines with multiple off-target effects were available, we screened the remainder for the effects of neuronal knockdown. In total, we assayed 11 ‘orexigenic’ genes (decreased expression in POMC neurons and increased expression in AgRP neurons during a fast) and 39 ‘anorexigenic’ genes (increased expression in POMC and decreased expression in AgRP neurons during fasting; Figure 5; Supplementary Table 5).

We also measured triglyceride levels in these lines and demonstrated that the values correlated well with wet mass (Pearson r = 0.6; p < 0.0001; Supplementary Figure 4). When we then used the triglyceride levels instead of wet mass in our screen, the ranking of the overall selected candidate gene list remained similar to either measurement (Supplementary Table 6). Because it is cheaper and quicker to measure mass than triglyceride levels, we suggest that wet mass is a suitable proxy for triglyceride levels in the context of a high-throughput screen.

The results of the screen, with genes ranked by score, are presented in Figure 5. The phenotype scores had a larger range (0.23–0.98) than the GWAS genes did. Seven of the ‘anorexigenic’ genes (Nrd2, Cacnb4, Dtna, Pofut1, Htr1a, Lrch2, and Tub) and none of the candidate ‘orexigenic’ gene had a score >0.80. For 2 of the genes with a score >0.80 (Htr1a, Tub) and 3 of the genes with a score between 0.70 and 0.80 (RPS6ka5, Nedd4l, and Ruyt2), evidence is available from mouse and/or human studies for roles in energy homeostasis.

### 4. DISCUSSION

With the rapid proliferation of gene lists emerging from large-scale ‘omics’ approaches, the aim of this study was to develop an in vivo high-throughput screen of feeding behaviour and energy balance phenotypes by using Drosophila as a model. We identified key characteristics that would make such a screen effective for prioritising long lists of gene candidates for more detailed and resource-intensive follow-up studies: examines multiple phenotypes and is economical in time and cost and simple to perform. Together, these factors allow the screen to be deployed at scale. We screened approximately 60 different Drosophila lines in 5 weeks, at a cost of less than £50 per gene, resulting in 30% of human genes screened being highlighted as warranting further study.

Prior Drosophila screens have focused on single metabolic measures in either adult flies [54,58–60] or larvae [61]. By contrast, we pulled together 3 assays that measured food intake and body weight, all of which fulfilled the requirements of low cost, speed, and hence, scalability. Using multiple assays and generating an integrated score offers 2 major advantages over single-assay screens. First, the screen is less likely to produce false positive results. Second, the sensitivity of the screen is increased, highlighting genes that do not show a strong phenotype in any single assay but that have subtle changes in multiple assays. Notably, this approach could possibly miss genes that affect single parameters. For example, SBK1/CG4943 and SLC39A8/Zip71B might be involved in the starvation-induced feeding regulation, as
Figure 5: RNAi screen of murine “anorexigenic” and “orexigenic” genes.

Summary of phenotypes, ranked by overall score, of flies with neuronal RNAi knockdown (using elav-GAL4) of the murine anorexigenic genes (a) and orexigenic genes (b). The top hit genes are those with score ≥ 0.80. The viability of flies when ubiquitous knockdown of the gene (using actin-GAL4) was performed are shown in the far right column.
suggested from their significant effect on the fasting-induced overeating assay, but not on normal food intake and body weight (Figure 3). While validating the screen, we initially grappled with selecting suitable negative controls. The problem is that many human diseases are associated with changes in body weight [62–68], as either a cause or a consequence [69], making it difficult to differentiate these genes from those that primarily influence feeding behaviour. Our solution was to use 2 categories of genes—those associated with neuronal diseases [70–72] and those associated with peripheral diseases [73–77] and our combined assay successfully discriminated between the negative and positive controls (Figure 1H) with significantly different average scores between the 2 groups. However, because a higher score for the unc13-4a/UNC13 is associated with amyotrophic lateral sclerosis (a neurological condition commonly associated with weight loss and dysphagia), we set a high scoring threshold for a gene to be considered for further study (0.80). Nevertheless, because the principal aim of this screen was to prioritise candidate genes for follow-up, avoiding false positive results was important; hence, we used a high threshold.

After validating the screen, we successfully demonstrated its utility for studying genes associated with BMI through GWAS [14,49], as well as genes reciprocally regulated by fasting in AgRP and POMC neurons that play a key role in the central melanocortin pathway [21]. Several genes highlighted by our screen (PCS1K1, ATXN2, NEGR1, MTF3, and SEC16B) have been shown to play a role in energy homeostasis in mouse models [24,27,28,30,32], demonstrating that the screen was effective and produced biologically relevant results. Critically, we also identified genes relatively unexplored in relation to energy homeostasis, marking them as prime candidates for further study. These included GNPDA2, NRXN3, GABRG1, LRRN6C, COL4A3BP, and RPL27A. NRXN3 (Neurexin 3), for example, is a neuronal cell surface protein involved in cell recognition and cell adhesion [78], GABRG1 (GABA(A) Receptor Subunit Gamma-1) belongs to the ligand-gated ion channel family and is predominantly expressed in the brain reward circuitry and may be implicated in addiction [79], and GNPDA2 is an enzyme that catalyses the deamination of glucosamine-6-phosphate and is critical for lipid and glucose metabolism in human adipose-derived mesenchymal stem cells [80]. Some genes, including Nr1d2 and Cacnb4, showed a Drosophila phenotype of significantly increased feeding but decreased mass, suggesting increased metabolic demands. We are unable to explain these apparently discordant phenotypes without detailed follow-up studies, but Nr1d2 has been demonstrated to be important to the mammalian circadian rhythm and might play a role in energy homeostasis [81].

A problem with GWAS is that the vast majority of SNPs associated with disease are located in non-coding regions, making the identification of the ‘causative’ gene(s) driving the phenotype challenging. Therefore, we used our high-throughput screen to assay multiple genes at 7 of the BMI loci, to observe if this provided insights into the potential causative gene. Of the 24 human genes studied in these 7 loci, 5 were scored as ‘positive hits’ by our screen: GNPDA2 and GABRG1 (near rs10938397), COL4A3BP (near rs21112347), ATXN2L, and SULT1A2 (near rs7359397). ATXN2L, SULT1A2, and COL4A3BP are not the genes closest to their respective SNPs. This result is comparable to those reported by Baranski, in which knocking down the nearest genes did not necessarily provide the largest phenotype.

The advantages of multiple assays over a single assay were highlighted when we compared our results to those of Baranski and colleagues, who also studied BMI-associated GWAS hits by knocking them down in Drosophila [54]. Sixteen genes were included in both studies, 5 of which were identified by Baranski and colleagues to have increased triglycerides storage when knocked down in the brain and fat body. Three of these 5 genes, NRXN3, SEC16B, and COL4A3BP, were positively identified by our screen, with an integrated score >0.80, providing independent validation of the utility of our approach. Crucially, ATXN2L, which when knocked down did not result in increased triglyceride storage, was positive when using our screen, because of its consistent anorexigenic effect on food intake. Abn2 plays a role in the metabolism of branched-chain and other amino acids, metabolism of fatty acids, and in the citric acid cycle [56], with mice lacking Abn2 demonstrating sensitivity to diet-induced obesity [55]. Although triglyceride levels are an important readout of nutrient status, body mass was a useful proxy for them in a high-throughput screen (Supplementary Figure 4 & Supplementary Table 6).

Another unique element of our study was the use of a neuron-specific RNAi. BMI-associated genes identified by GWAS show enriched expression in the CNS [13], and 55 of the 56 genes that we studied were expressed in the Drosophila CNS. When knocked down ubiquitously, 55% of the lines were lethal. This is a higher proportion of lethality than for knockdown of fasting-related genes (38%) and the Drosophila genome-wide figure of 25% [36], suggesting that the GWAS-identified genes are essential for life. Even the Baranski study, which perturbed gene expression in both the brain and fat body, yielded 13% lethality. By contrast, all our neuron-specific knockdown lines were viable. Our more tissue-targeted approach therefore allowed us to screen more genes and obtain more phenotypic information.

In our quest for speed and scalability in this screen, we compromised on our selection of control lines: instead of testing UAS-RNAi lines as controls, we used the relevant GAL4 line. However, the use of multiple UAS-RNAi lines from each collection should limit the extent of line-to-line variation, and other Drosophila screens for GWAS genes have applied a similar approach (e.g. Baranski et al. [54] and Pende et al. [59]; thus, there is precedent. The use of multiple background controls, as well as additional confirmation of the findings using full knockout lines (as we did in some of our positive hits here), would be essential before taking a candidate gene further into a mammalian model.

One limitation of working with Drosophila is its distant relation to humans. However, energy homeostasis and feeding are fundamental processes for all organisms and much of the circuitry and pathways are conserved between species. Notably, nearly 80% of the genes we studied here had a Drosophila orthologue. Even for genes with no obvious Drosophila orthologue, the expression of the human gene in question can sometimes transfer the function of the encoded protein, at least in part, to the fly [82].

In conclusion, we have demonstrated that the use of Drosophila for screening feeding behaviour phenotypes is effective in moving from large lists obtained from whole genome or transcriptomic approaches, to more selective lists of relevant genes which can then be studied in ‘lower throughput’ and more time-consuming mammalian models. Furthermore, the detrimental effects observed in many of the ubiquitous versus neuron-specific Drosophila knockdowns suggests that at least for a subset of candidate genes, neuron-specific models may also be necessary to explore genotype—phenotype relationships relevant to energy balance in higher organisms.

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AUTHOR CONTRIBUTION

Chalmers J. Methodology, Investigation, Formal analysis, Visualisation, Project administration, Funding acquisition. Tung YCL: Conceptualisation, Methodology, Validation, Supervision, Fundin acquisition, Writing—Original draft preparation and editing. Liu C. Methodology, Resources, Supervision. O’Kane CJ. Methodology, Supervision, Writing—Reviewing and Editing. O’Rahilly: Conceptualisation, Writing—Reviewing and Editing, Funding acquisition. Yeo GSH: Conceptualisation, Supervision, Writing—Reviewing and Editing, Funding acquisition.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.101127.

REFERENCES

[1] Finkelstein, E.A., Trogdon, J.G., Brown, D.S., Allaire, B.T., Dellea, P.S., Kannal-Bahl, S.J., 2008. The lifetime medical cost burden of overweight and obesity: implications for obesity prevention. Obesity (Silver Spring, Md 16(8):1843–1848.
[2] Must, A., Spadano, J., Coakley, E.H., Field, A.E., Colditz, G., Dietz, W.H., 1999. The disease burden associated with overweight and obesity. Journal of the American Medical Association 282(16):1523–1529.
[3] Maes, H.H., Neale, M.C., Eaves, L.J., 1997. Genetic and environmental factors in relative body weight and human adiposity. Behavior Genetics 27(4):325–351.
[4] Stunkard, A.J., Foch, T.T., Hrubec, Z., 1986. A twin study of human obesity. Journal of the American Medical Association 256(1):51–54.
[5] Stunkard, A.J., Harris, J.R., Pedersen, N.L., McLean, G.E., 1990. The body-mass index of twins who have been reared apart. New England Journal of Medicine 322(21):1483–1487.
[6] Barsh, G.S., Farooqi, I.S., O’Rahilly, S., 2000. Genetics of body-weight regulation. Nature 404(6778):644–651.
[7] Morton, G.J., Cummings, D.E., Baskin, D.G., Barsh, G.S., Schwartz, M.W., 2006. Central nervous system control of food intake and body weight. Nature 443(7109):289–295.
[8] Boston, B.A., Blaydon, K.M., Varnerin, J., Cone, R.D., 1997. Independent and additive effects of central POMC and leptin pathways on murine obesity. Science (New York, N.Y.) 278(5343):1641–1644.
[9] Clement, K., Vaisse, C., Lahou, N., Cabrol, S., Pelloux, V., Cassuto, D., et al., 1998. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 392(6674):398–401.
[10] Montague, C.T., Farooqi, I.S., Whitehead, J.P., Soos, M.A., Rau, H., Wareham, N.J., et al., 1997. Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 387(6636):903–908.
[11] Yeo, G.S., Farooqi, I.S., Aminian, S., Haisall, D.J., Stanhope, R.G., O’Rahilly, S., 1998. A frameshift mutation in MCHR4 associated with dominantly inherited human obesity. Nature genetics 20(2):111–112.
[12] Akijama, M., Okada, Y., Kanai, M., Takahashi, A., Momozawa, Y., Ikeda, M., et al., 2017. Genome-wide association study identifies 112 new loci for body mass index in the Japanese population. Nature Genetics 49(10):1458–1467.
[13] Locke, A.E., Kahali, B., Berndt, S.I., Justice, A.E., Pers, T.H., Day, F.R., et al., 2015. Genetic studies of body mass index yield new insights for obesity biology. Nature 518(7538):197–206.
[14] Lu, Y., Loos, R.J., 2013. Obesity genomics: assessing the transferability of susceptibility loci across diverse populations. Genome Medicine 5(6):55.
[15] Benzinou, M., Creemers, J.W., Choquet, H., Lobbens, S., Dina, C., Durand, E., et al., 2008. Common nonsynonymous variants in PCSK1 confer risk of obesity. Nature Genetics 40(8):943–945.
[16] Borman, A.D., Pearce, L.R., Mackay, D.S., Nagel-Wolfrum, K., Davidson, A.E., Henderson, R., et al., 2014. A homozygous mutation in the TUB gene associated with retinal dystrophy and obesity. Human Mutation 35(3):289–293.
[17] Doche, M.E., Bochukova, E.G., Su, H.W., Pearce, L.R., Keogh, J.M., Henning, E., et al., 2012. Human SH2B1 mutations are associated with maladaptive behaviors and obesity. Journal of Clinical Investigation 122(12): 4732–4736.
[18] Jackson, R.S., Creemers, J.W., Ohagi, S., Raffin-Sanson, M.L., Sanders, L., Montague, C.T., et al., 1997. Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. Nature Genetics 16(3):303–306.
[19] Yeo, G.S., Connie Hung, C.C., Rochford, J., Keogh, J., Gray, J., Sivaramakrishnan, S., et al., 2004. A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. Nature Neuroscience 7(11):1187–1189.
[20] Campbell, J.N., Macosko, E.Z., Fenselau, H., Pers, T.H., Lyubetskaya, A., Tenen, D., et al., 2017. A molecular census of arcuate hypothalamus and median eminence cell types. Nature Neuroscience 20(3):484–496.
[21] Henry, F.E., Sugino, K., Tozer, A., Branco, T., Sternson, S.M., 2015. Cell type-specific transcriptomics of hypothalamic energy-sensing neuron responses to weight-loss. eLife 4.
[22] Aponte, Y., Atasoy, D., Sternson, S.M., 2011. AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nature Neuroscience 14(4):351–355.
[23] Krashes, M.J., Koda, S., Ye, C., Rogan, S.C., Adams, A.C., Cusher, D.S., et al., 2011. Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. Journal of Clinical Investigation 121(4):1424–1428.
[24] Dickinson, M.E., Flenniken, A.M., Ji, X., Teboul, L., Wong, M.D., White, J.K., et al., 2016. High-throughput discovery of novel developmental phenotypes. Nature 537(7621):508–514.
[25] Larder, R., Sim, M.F.M., Gulati, P., Antrobus, R., Tung, Y.C.L., Rimmington, D., et al., 2017. Obesity-associated gene TMEM18 has a role in the central control of appetite and body weight regulation. Proceedings of the National Academy of Sciences of the United States of America 114(35):9421–9426.
[26] Rathjen, T., Yan, X., Kronenwo, N.L., Ku, M.C., Song, K., Ferrarese, L., et al., 2017. Regulation of body weight and energy homeostasis by neuronal cell adhesion molecule 1. Nature Neuroscience 20(8):1096–1103.
[27] Speakman, J.R., 2013. Functional analysis of seven genes linked to body mass index and adiposity by genome-wide association studies: a review. Human Heredity 75(2–4):57–79.
White, H., Pieper, C., Schmader, K., Fillenbaum, G., 1996. Weight change in Alzheimer’s disease. Journal of the American Geriatrics Society 44(3):265–272.

Zhang, A., Silverberg, J.I., 2015. Association of atopic dermatitis with being overweight and obese: a systematic review and metaanalysis. Journal of the American Academy of Dermatology 72(4):606–616 e4.

Gao, X., Schwarzschild, M.A., Wang, H., Ascherio, A., 2009. Obesity and restless legs syndrome in men and women. Neurology 72(14):1255–1261.

Vercruysse, P., Vieau, D., Blum, D., Petersën, Å., Dupuis, L., 2018. Hypothalamic alterations in neurodegenerative diseases and their relation to abnormal energy metabolism. Frontiers in Molecular Neuroscience 11(2).

Kelleher 3rd, R.J., Shen, J., 2017. Presenilin-1 mutations and Alzheimer’s disease. Proceedings of the National Academy of Sciences of the United States of America 114(4):629–631.

Pantavou, K.G., Braliou, G.G., Kontou, P.I., Dimou, N.L., Bagos, P.G., 2016. A meta-analysis of FZD3 gene polymorphisms and their association with schizophrenia. Psychiatric Genetics 26(6):272–280.

van Rheenen, W., Shatunov, A., Dekker, A.M., McLaughlin, R.L., Diekstra, F.P., Pulit, S.L., et al., 2016. Genome-wide association analyses identify new risk variants and the genetic architecture of amyotrophic lateral sclerosis. Nature Genetics 48(9):1043–1048.

Ferreira, M.A., Vonk, J.M., Baurecht, H., Marenholz, I., Tian, C., Hoffman, J.D., et al., 2017. Shared genetic origin of asthma, hay fever and eczema elucidates allergic disease biology. Nature Genetics 49(12):1752–1757.

Johnson, E.O., Hancock, D.B., Gaddis, N.C., Levy, J.L., Page, G., Novak, S.P., et al., 2015. Novel genetic locus implicated for HIV-1 acquisition with putative regulatory links to HIV replication and infectivity: a genome-wide association study. PloS One 10(3):e0118149.

Paternoster, L., Standl, M., Waage, J., Sauretch, H., Hotze, M., Strachan, D.P., et al., 2015. Multi-ancestry genome-wide association study of 21,000 cases and 95,000 controls identifies new risk loci for atopic dermatitis. Nature Genetics 47(12):1449–1456.

Salminen, A.V., Lam, D.D., Winkelmann, J., 2019. Role of MEIS1 in restless legs syndrome: from GWAS to functional studies in mice. Advances in Pharmacology 84:175–184.

Zheng, H.F., Forgetta, V., Hsu, Y.H., Estrada, K., Rosello-Diez, A., Leo, P.J., et al., 2015. Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture. Nature 526(7571), 112–117.

Kasem, E., Kunihara, T., Tabuchi, K., 2018. Neurexins and neuropsychiatric disorders. Neuroscience Research 127:53–60.

Enoch, M.A., 2008. The role of GABA(A) receptors in the development of alcoholism. Pharmacology Biochemistry and Behavior 90(1):95–104.

Wu, L., Ma, F., Zhao, X., Zhang, M.X., Wu, J., Mi, J., 2019. GNPDA2 gene affects adipogenesis and alters the transcriptome profile of human adipose-derived mesenchymal stem cells. International journal of endocrinology 2019:9145452.

Dankel, S.N., Degerud, E.M., Borkowski, K., Fjære, E., Midtbo, L.K., Haugen, C., et al., 2014. Weight cycling promotes fat gain and altered clock gene expression in adipose tissue in C57BL/6J mice. American Journal of Physiology - Endocrinology And Metabolism 306(2):E210–E224.

St Johnston, D., 2002. The art and design of genetic screens: Drosophila melanogaster. Nature Reviews. Genetics 3(3):176–188.