Steady-state energy balance in animal models of obesity and weight loss

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Abstract

Objective: We wanted to exam the steady-state energy balance by using high fat diet-induced obese (DIO) rats and mice as models for positive energy balance, and gastric bypassed (GB) rats and gene knockout of muscarinic acetylcholine M3 receptor (M3KO) mice as models for negative energy balance. ***Methods:*** 132 rats and mice were used. Energy balance was measured by a comprehensive laboratory animal monitoring system. Gene expression was analysed by *in situ* hybridization in M3KO mice. ***Results:*** DIO rats reached the plateau of body weight 28 weeks after starting high-fat diet (25% heavier than controls), whereas DIO mice reached the plateau after 6 weeks (23% heavier than controls). At the plateau, DIO rats had higher calorie intake during light phase but not during dark phase, while mice had the same calorie intake per day as controls. DIO rats and mice had lower energy expenditure (EE) and respiratory exchange ratio (RER) than controls. GB-rats reached the plateau (15% weight loss) 2 weeks after surgery and had the same calorie intake as sham-operated controls. EE, but not RER, was higher in GB rats than controls during dark phase. The lean M3KO mice (25% lighter than wild-type (WT) mice at the plateau between 6-15 months of age) had the same calorie intake but higher EE, RER and hypothalamic mRNA expression of NPY, AgRP and leptin receptor than WT mice. ***Conclusion***: When body weight gain or loss reached a plateau, the steady-state energy balance was mainly maintained by EE and/or RER rather than calorie intake.

Keywords

Eating behaviour, High-fat diet, Gastric bypass, Muscarinic acetylcholine M3 receptor knockout, Mice, Rats

Introduction

The number of overweight individuals is increasing, leading to a global obesity epidemic [1, 2]. This is of concern because obesity is a major contributor in the development of type 2 diabetes, cardiovascular diseases, certain forms of cancer, overall reduced quality of life and premature mortality [3, 4]. Currently, weight loss treatments include diet control, physical training, drug therapies, and bariatric surgery [5]. Weight loss resulting from either dieting or exercise remains usually for a shorter period of time, whereas weight loss after bariatric surgery (such as sleeve gastrectomy or gastric bypass) has much longer effect [6, 7]. With intensive lifestyle interventions, a majority of obese participants in clinical trials lose 7% to 10% of their initial weight at 1 year, but longer-term weight maintenance is difficult [8, 9]. The development of anti-obesity drugs to target energy balance by acting on either appetite control or energy expenditure (EE) is yet without great success [9, 10, 11, 12]. For example, the pharmacological interventions reduce body weight only by 3-9% of initial weight on average at 1 year and the proportion of patients achieving clinically meaningful (>5%) weight loss ranges from 37% to 70% depending on different drugs and/or dosages [9]. In order for current lifestyle interventions, drug therapies and bariatric surgeries to be maximally effective for body weight control in individual patients as well as in a patient population, better understanding of the energy balance is essential [13, 14].

The concept of energy balance for regulating body weight is simple in principle. When energy output equals input, i.e., energy homeostasis, the body is at steady state energy balance. A new set point for steady state balance occurs when energy intake (EI) exceeds EE over a given period of time and weight is gained, or when EE exceeds EI and weight is lost [15]. In this study, we defined the dynamic phase of energy balance as weight gain or loss of more than 5% for at least one week, and steady state as less than 5% change in body weight for at least one week. From the energy balance point of view, it has not been understood why it is easier to control the body weight during the dynamic phase of energy balance than to reverse it under the steady state condition [16]. In fact, it is not uncommon that many obese patients seek weight-loss treatments when they are under the steady state in terms of body weight. Thus, a better understanding of steady-state energy balance may help us to better understand why it is difficult to perturb a shift in EE particularly to achieve a long-term body weight loss by either dieting or exercise after obesity is established.

Previously, we and others have shown that dynamic development of obesity is associated with increased calorie per meal [17, 18], and that there are compensatory changes in hypothalamic gene expression of AgRP and NPY in rodents fed high-fat diet or low-energy

dense diet for a prolonged period of time, which is indicative of defence against changes in body weight [19, 20, 21, 22]. Gastric bypass-induced weight loss is mainly associated with increasing EE rather than reducing EI [23, 24, 25, 26, 27], which is in line with the hypothalamic gene expression of AgRP, NPY and POMC [28]. However, the initial report on M3KO mice showed a reduced food intake with increased expression of hypothalamic AgRP mRNA and reduced POMC mRNA [29], which is inconsistent with homeostatic mechanism of food intake regulation [30].

In the present study, we wanted to explore the steady-state energy balance to defend a change in body weight in response to either positive or negative energy balance. Thus, we used high-fat-diet fed rats and mice as models for positive energy balance displaying an obese phenotype [31], and gastric bypassed (GB) rats and gene knockout of muscarinic acetylcholine M3 receptor (M3KO) mice as models for negative energy balance [23, 32].

Materials and Methods

Experimental design

Four sets of experiments were conducted in rodent models, comprising two sets examining obesity development in rats and mice, and another two sets examining weight loss in rats and mice.

All animals were housed in groups of three or four together in individually ventilated cages (1500 cm^2 in size) on wood chip bedding with a 12 h light/dark cycle, room temperature of 21-22ºC and 50-60% relative humidity. The standard housing conditions were specific pathogen free and in agreement with FELASA (Federation of European Laboratory Animal Science Association) recommendations. Throughout the experiment, all animals had free access to tap water and food regardless of being in metabolic or Makrolon cages. The high fat diet (D12492) was purchased from Research Diets Inc. (New Brunswick, NJ, USA), and the normal chow (RM1 811004) was purchased from Scanbur BK AS (Sweden). Animal experiments were performed according to the guidelines for the design and statistical analysis of experiments using laboratory animals after being approved by the Norwegian National Animal Research Authority (Forsøksdyrutvalget, FDU).

Diet-induced obese rats: Four weeks old male Sprague-Dawley rats were purchased from Taconic (Ejby, Denmark). The rats were divided into two groups: diet-induced obese (DIO) (n=12) and controls (n=8). To allow the DIO rats to adapt to high-fat diet, they were put on a mixture of 50% high fat diet and 50% normal chow for two weeks before being fed

high fat diet only (60% fat) [17]. The DIO and control rats received high fat diet or normal chow, respectively, throughout the study. Body weight was measured every week from arrival until euthanization. Food intake, eating behaviour, metabolic parameters and body composition were measured when the body weight-plateau was reached (35 weeks of age). To follow the 3R principle, this group of rats were also used in a separated study entitled "Time-restricted feeding during weekdays against obesity: a study using high-fat diet-induced obese rat models" (Olsen MK, Choi MH, Kulseng B, Zhao C-M, Chen D. unpublished data).

Diet-induced obese mice: Five weeks old male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were divided into two groups: DIO (n=10) and controls (n=10). To allow the DIO mice to adapt to the high-fat diet, they were fed a mixture of 50% high fat diet and 50% normal chow diet for two weeks before being fed high fat diet only (60% fat) for the remainder of the study. The control mice were fed normal chow for the entire study. Body weight was measured every week from arrival until euthanization. Food intake, eating behaviour, metabolic parameters and body composition was measured when the body weight-plateau was reached (11 weeks of age).

Gastric bypassed rats: 48 weeks old male Sprague-Dawley rats were purchased from Taconic (Ejby, Denmark) and were divided into two groups: GB (n=14) and sham operation (n=6). The GB was performed according to previous experiments [25] and the rats were fed normal-chow throughout the whole study. Food intake, eating behaviour and metabolic parameters were measured when the body weight-plateau had been reached (3 weeks post-surgery). To follow the 3R principle, the data from this group of rats were taken from our previous study [23] and re-analysed and re-used in the present study.

M_3 receptor knockout (M3KO) mice: M3KO mice were generated [33] and backcrossed 14 generations onto C57BL/6J background at Prof. Takeuchi's laboratory (Kyoto Pharmaceutical University, Japan) and imported to Norway by Prof. D. Chen for this study. The M3KO mice were further bred through sibling matings for 2 generations. Age-matched wild-type (WT) mice on the same background (C57BL/6J) were purchased from Taconic (Ejby, Denmark). All the mice including M3KO and WT mice were fed normal chow in-house together during the study period. Body weight was measured at 2, 6, 11 and 15 months of age. At 2 months of age there were 7 mice in each group, 3:4 male:female ratio in M3KO group and 4:3 male:female ratio in WT group. At 6, 11 and 15 months of age there were 8 mice in each group, with male:female ratio of 4:4 ratio in the WT group, and 4:4, 5:3 and 6:2 in the M3KO group, respectively. Brain tissue from 15 months old mice was collected for *in situ* hybridization.

148 ***Measurements of energy balance by CLAMS***

149 The parameters for energy balance were measured by a Comprehensive Laboratory
150 Animal Monitoring System.

151 The animals were acclimatized to the Comprehensive Laboratory Animal Monitoring
152 System (CLAMS; Columbus Instruments International, Columbus, OH USA) for 24 h before
153 data collection. At data collection the animals were kept in CLAMS for 48 h and data from
154 the last 24 h were used for analysis. Before CLAMS the animals were habituated to their
155 normal food as powder for three days, as the food in CLAMS is in powder form. The animals
156 were placed in CLAMS when the body weight had reached a plateau. This is defined by less
157 than 5% weight loss or gain over a period of 7 days or more. The DIO rats and mice were
158 placed in CLAMS at 35 and 11 weeks of age, respectively, together with their respective
159 control groups. GB and control rats were placed in CLAMS 3 weeks post-surgery, while
160 M3KO and WT mice were 6, 11 and 15 months old when placed in CLAMS.

161 Animals were placed in CLAMS with free access to standard powder food (RM1
162 801002, Scanbur BK AS, Sweden) or to high fat diet, and tap water. This system is composed
163 of a four-chamber open circuit indirect calorimeter designed for continuous monitoring of
164 individual animals. Eating behaviour and metabolic parameters were recorded automatically.
165 High-resolution feeding data was generated by monitoring all feeder balances every 0.5
166 seconds. The end of an eating event (meal) was determined when the balances were stable for
167 more than 10 seconds and a minimum of 0.05 g of food were eaten. An air sample was
168 withdrawn every 5 min. The EE (kcal/h) was calculated according to this equation:
169 $(3.815+1.232\ \mathrm{RER}) \times \mathrm{VO_2}$, where RER (respiratory exchange ratio) was the volume of CO_2
170 produced per volume of O_2 consumed. VO_2 was the volume of O_2 consumed per hour per
171 kilogram of mass of the animal. Parameters that were obtained during light phase (7 am–7
172 pm) and dark phase (7 pm–7 am) for each individual animal included number of meals, meal
173 size, meal duration, accumulated food intake, intermeal interval, rate of eating, satiety ratio,
174 drinking activity, EE and ambulatory activity. The intermeal interval was defined as the
175 interval in minutes between two meals. Rate of eating was calculated by dividing the average
176 meal size by the average duration of a meal, and satiety ratio, an index of the non-eating time
177 produced by each gram of food consumed, was calculated by dividing the average intermeal
178 interval by the average meal size.
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Measurement of body composition

Body composition was determined for DIO rats and mice, and respective control groups, using dual energy X-ray absorptiometry (DXA) with small animal software (Hologic QDR 4500A, Hologic Inc., Bedford, MA, USA). Total bone area (cm^2), total fat mass (g) and total fat-free mass (g) were measured, and total mass (g), lean percentage (%) and fat percentage (%) were calculated. DXA was performed under anaesthesia (isoflurane).

In situ hybridization

Brain samples were taken at euthanization and snap-frozen in isopentane on dry ice before being stored at -80°C wrapped in aluminium foil. The frozen brains were cut (14 μm) in the region spanning the hypothalamus between Bregma –0.10 to –2.54 mm according to the Mouse Brain Atlas of Franklin & Paxinos 1997 and sections were mounted onto poly-L-lysine-coated slides. Primers for the amplification of AgRP spanned were based on Genbank sequence U89484 to amplify the sequence between bases 113-341 (forward primer 5'-TGTTCCCAGAGTTCCCAGGTC-3', reverse primer 5'-GCATTGAAGAAGCGGCAGTAGCAC-3'). Primers for the amplification of POMC were based on Genbank sequence J00162 to amplify the sequence between bases 263-665 (forward primer 5'-GGGCAAGCGCTCCTACTCCAT-3', reverse primer 5'-GCCCTTCTTGTRSRCGTTCTTGA-3'). The DNA sequence for NPY was a full-length cloned rat NPY gene sequence. Primers for the amplification of Ob-Rb (leptin receptor) were based on Genbank sequence U49107 to amplify the sequence between bases 5'-GTGTGAGCATCTCTCCTGGAG-3' (+2829 to +2849) and 5'-ACCACACCAGACCCTGAAAG-3' (+3362 to +3343)(see MCH information). Integrated optical density (IOD) was obtained by reference to the 14C microscale. Values were averaged for each animal.

Statistical analysis

The results are expressed as means ± SEM. Statistical comparisons were performed using two-sided independent *t*-test between the groups, and paired *t*-test was used for comparisons within groups. If assumption of normal distribution was not met, Mann-Whitney U test (denoted as U test when applied) were used. A p-value of <0.05 was considered statistically significant. Shapiro-Wilk test was used to test for normal distribution and Levine's test for homogeneity was used to test for equal variances between the groups and p-values were reported accordingly. A power of >0.80 was set as minimal to ensure sufficient

sample sizes and were calculated using G*Power version 3.1 and a randomization table was used to assign the animals to different groups. The data analysis was performed in SPSS version 20.0.

Results

Diet-induced obese rats

The increase of body weight reached the plateau 28 weeks after starting high-fat diet feeding (33 weeks of age). DIO rats were 25% heavier than age-matched and normal diet (ND) controls at the plateau (p=0.001) (Fig. 1a, Supplementary Table S1).

At 35 weeks of age, the rats were placed in CLAMS, and we found calorie intake was higher during dark phase than light phase in both ND (p<0.001) and DIO (p<0.001) rats indicating a good sensitivity of CLAMS. Calorie intake was slightly higher in DIO rats than ND rats during light phase (p=0.039), but not significantly different during the dark phase (p=0.083) (Fig. 1b). EE was higher during dark phase than light phase in ND rats (p<0.001). However, there were no differences between ND and DIO rats during light phase (p=0.917), but there was a trend for lower EE in DIO rats during dark phase (p=0.068) (Fig. 1c). RER was higher during dark phase than light phase in control rats (p<0.001). In comparison with ND rats, DIO rats had a lower RER during both light- and dark-phase (p=0.008 and <0.001, respectively)(Fig. 1d). Body composition analysis showed that total body mass (p<0.001) and surface area (p<0.001), fat mass (p<0.001, U test) and fat percentage (p<0.001) were higher, whereas the lean percentage (p<0.001) (but not lean mass (p=0.657, U test)) was lower in DIO than ND rats (Fig. S1).

Diet-induced obese mice

The increase of body weight reached the plateau 6 weeks after starting high-fat diet feeding (11 weeks of age). DIO mice were 23% heavier than age-matched and normal diet controls at the plateau (p<0.001) (Fig. 2a, Supplementary Table S2).

At the plateau of body weight, the mice were placed in CLAMS. We found that calorie intake was higher during dark phase than light phase particularly in ND mice (p<0.001). There was no difference in calorie intake between DIO and ND mice neither during light phase (p=0.326) nor during dark phase (p=0.943) (Fig. 2b). EE was higher during dark phase than light phase in both ND (p<0.001) and DIO (p<0.001) mice, suggesting higher active EE. There was no difference in the EE during light phase (p=0.274), but DIO had lower EE than

ND during dark phase (p=0.043) (Fig. 2c). RER was higher during dark phase than light phase in both ND (p=0.001) and DIO (p=0.008) mice. In comparison with ND mice, DIO mice had a lower RER only during dark phase (p=0.003) (Fig. 2d). Body composition analysis showed that total body mass (p<0.001) and surface (p=0.004), and fat mass (p<0.001, U test) and percentage (p<0.001) were higher, whereas the lean mass (p=0.020) and percentage (p<0.001) were lower in DIO than ND mice (Fig. S2).

Gastric bypassed rats

GB-induced body weight loss reached the plateau, i.e., 15% weight loss, 2 weeks after surgery (p<0.001)(Fig. 3a, Supplementary Table S3), and 3 weeks post-surgery, the rats were placed in CLAMS. Calorie intake was higher during dark phase than light phase in both sham-operated and GB rats (p=0.001 and 0.003, respectively). There was no difference in calorie intake between the two groups neither during light phase nor during dark phase (p=0.289 and 0.870, respectively) (Fig. 3b). EE was higher in GB rats than sham-operated rats during dark phase (p=0.033) (Fig. 3c). RER was higher during dark phase than light phase in both sham- and GB-operated group (p=0.001 and 0.008, respectively). There was no difference in RER between groups either during dark- or light-phase (p=0.389 and 0.516, respectively) (Fig. 3d).

M3KO mice

At the birth, the body weight did not differ between M3KO and WT mice [29]. At 2 months of age there was a trend for lower body weight in M3KO than WT mice (p=0.065). The mice was placed in CLAMS at 6, 11 and 15 months of age, and M3KO mice weighed significantly less than WT mice at all time-points (p<0.001, 0.001 and 0.001, respectively). There was no significant difference in body weight between 6, 11, and 15 months of age (6 vs. 11: p=0.599, 6 vs. 15: p=0.514 and 11 vs 15: p=0.292)(Fig. 4a). Both M3KO and WT mice had a continuous growth throughout the lifespan. (Supplementary Tables S4-6). Calorie intake was higher during dark phase than light phase in WT mice (p=0.002, 0.092 and 0.081, respectively) and more so in M3KO mice (p=0.022, 0.004 and <0.001, respectively). There were no differences in calorie intake between WT and M3KO mice at all time points examined, except light phase at 6 months where calorie intake was lower in M3KO compared to WT, (p=0.010) and dark phase at 15 months of age where calorie intake was unexpectedly higher in M3KO than WT mice (p=0.034) (Fig. 4b). Both EE and RER were higher during light- as well as dark-phase in M3KO than in WT mice at the time points measured (Light:

EE: $p<0.001$, =0.005 and =0.005, respectively. RER: $p<0.001$, =0.028 and <0.001, respectively) (Dark: EE: $p<0.001$, <0.001 and =0.003, respectively; RER: $p<0.001$, 0.001 and 0.001, respectively) (Fig.4c, d).

In comparison with age-matched WT mice, the M3KO mice had higher mRNA expression of NPY, AgRP and leptin receptor in the arcuate nucleus in the hypothalamus (p=0.001, <0.001 and =0.003, respectively) (Fig. 5). Pro-opiomelanocortin (POMC) in the ARC and melanin-concentrating hormone (MCH) in ventromedial hypothalamus were unchanged in terms of mRNA expression (p=0.356 and 0.200, respectively) (Fig. 5).

Discussion

The results of the present study using 4 different animal models have demonstrated a common phenotype, i.e. steady-state energy balance at the plateau of body weight development. EE was measured during light- and dark-phases separately to indicate basal and active metabolic rates, respectively. Interestingly, the basal metabolic rate was unchanged in both obese rats and mice compared to normal chow-fed controls, but the active metabolic rate seemed to be reduced significantly in mice and non-significantly in rats (probably due to the sample size). The results also showed a higher RER in dark phase than light phase during the steady-state energy balance, indicating a higher oxidative capacity of the muscle for the provision of energy in the dark phase [34]. Furthermore, the obese rats had a low RER during both light-and dark- phases, suggesting a lower muscle's oxidative capacity.

These results may help us to explain what we often see in clinic, i.e., the large variability in individual responses to weight loss interventions in patients could be because the differences in the time frame over which a steady-state energy balance establishes between individuals. This is in line with a recent study showing that the longer it took to reach a steady state following GB surgery, the greater the weight loss was. In addition, the majority of patients undergoing GB surgery did not lose significant weight after reaching steady state [35].

Previously it has been reported that NPY and AgRP mediate complementary functions of hyperphagia and reduce EE in leptin receptor deficient mice [36]. In addition, it has been demonstrated that there are compensatory changes in hypothalamus, particularly NPY and AgRP gene expression, in normal rats and mice fed either with high-fat diet or low-energy dense diet for long period, indicative of a compensation to defend the body weight changes

[19, 20, 21, 22]. Previously, we and others have shown that GB-induced weight loss in rats was not due to decreased calorie intake, but due to an increase in EE [23, 24, 25, 26, 27], which is in line with the hypothalamic gene expression of NPY, AgRP, or POMC [28]. Therefore, we did not analyse the hypothalamic gene expression in obese rats, obese mice and GB rats in the present study.

However, we further analysed the M3KO mouse model with respect to both energy balance and the hypothalamic gene expression. This was because in the initial publication [29] reported that M3KO mice had reduced food intake with increased expression of hypothalamic AgRP mRNA and decreased expression of POMC mRNA, which appeared to be inconsistent with the homeostatic mechanism of food intake regulation associated with the regulatory neuropeptides encoded by these genes[30]. In contrast to this previous study we found M3KO mice did not have significantly overall reduced calorie intake as measured using a more precise method (the state-of-the-art CLAMS) than one used in the study by Yamada et al. [29]. Furthermore, we found that reduced calorie intake during the light phase and increased calorie intake during the dark phase were accompanied by increase EE during both light phase and dark phases throughout all stages of growth and maturity. Additionally, in contrast to this previous study, we found that RER was increased in M3KO mice, implicating use of carbohydrates as the predominant fuel for cellular respiration. This may also suggest high rates of *de novo* lipogenesis in these mice as carbohydrates are partially oxidized and directed towards fatty acid synthesis. This would have a considerable effect on the fat mass of these mice and is highly relevant to their steady state. Similar to EE, RER was higher in both the light and dark phases. Taken together with the findings of equal body weights at birth and during the initial post-natal development, we suggest that the lean phenotype of the M3KO mice is not caused by prenatal factors or environment, but develops as the mice grow and is maintained as the mice achieve a new steady-state of energy balance. Since the absence of M3 receptor has beneficial effects, which protect mice against obesity, some studies with new classes of M3-receptor selective antagonists have been initiated but still in its preliminary phases, and the lack of specific antagonists or agonists for M3 receptors in CNS, make it impossible to validate this anti-obesity target at present [37]. In addition, there are several other possible pharmacological treatments for obesity targeting EE, such as thyroid hormone receptor β agonists (GC-1 and GC-24) and NADPH:quinone reductase 1 (NQO1) activators (MB12066) [38, 39]. Some of these compounds have shown promising results as obesity treatment in animal studies, with MB12066 having completed Phase I clinical trial [40, 41, 42].

The results of the present study suggest that when a body weight plateau is reached following either weight gain or loss, a new steady-state energy homeostasis occurs which is mainly due to alterations in EE and RER. The decreased EE in obese animals and increased EE in lean animal models suggest that interventions for reduction in body weight or for continuation of weight loss should target EE.

Disclosure of interest

No conflicts of interest, financial or otherwise, are declared by the authors

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Author contributions

MKO, HJ, NC, PB, KT, BK, DC, and C-MZ conceived and designed the experiments. MKO, HJ, NC, PB, DC and C-MZ performed the experiments and analysed the results. KT provided animal models. MKO, HJ, NC, PB, KT, BK, DC and C-MZ wrote the manuscript. All authors reviewed the manuscript.

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Figures Captions

Figure 1 | (a) Body weight of high-fat-diet (HFD) induced obese (DIO) (n=12) rats relative to normal diet (ND) (n=8) rats from 5 to 35 weeks of age. Grey area represents body weight plateau (less than 5% increase or decrease in BW over last couple of weeks). CLAMS was performed at 35 weeks of age for both DIO (n=11) and ND (n=7) rats. (b) Calorie intake (g/100g body weight) during light phase and dark phase of DIO and ND rats. (c) EE (kcal/h/100g body weight) during light phase and dark phase of DIO and ND rats. (d) RER during light phase and dark phase of DIO and ND rats. White background indicates light phase and grey background indicates dark phase. Data is expressed as mean ± SEM. *, **, ***: $p<0.05$, 0.01, 0.001 between DIO and ND. Independent t-test was used between groups and paired *t*-test within groups (light vs. dark phase).

Figure 2 | (a) Body weight of high-fat-diet (HFD) induced obese (DIO) (n=10) mice relative to normal diet (ND) (n=10) mice from 5 to 12 weeks of age. Grey area represents body weight plateau (less than 5% increase or decrease in BW over last couple of weeks). CLAMS was performed at 11 weeks of age for both DIO (n=10) and ND (n=10) mice. (b) Calorie intake (g/100g body weight) during light phase and dark phase of DIO and ND mice. (c) EE (kcal/h/100g body weight) during light phase and dark phase of DIO and ND mice. (d) RER during light phase and dark phase of DIO and ND mice. White background indicates light phase and grey background indicates dark phase. Data is expressed as mean ± SEM. **, ***: $p< 0.01$, 0.001 between DIO and ND. Independent *t*-test was used between groups and paired *t*-test within groups (light vs. dark phase).

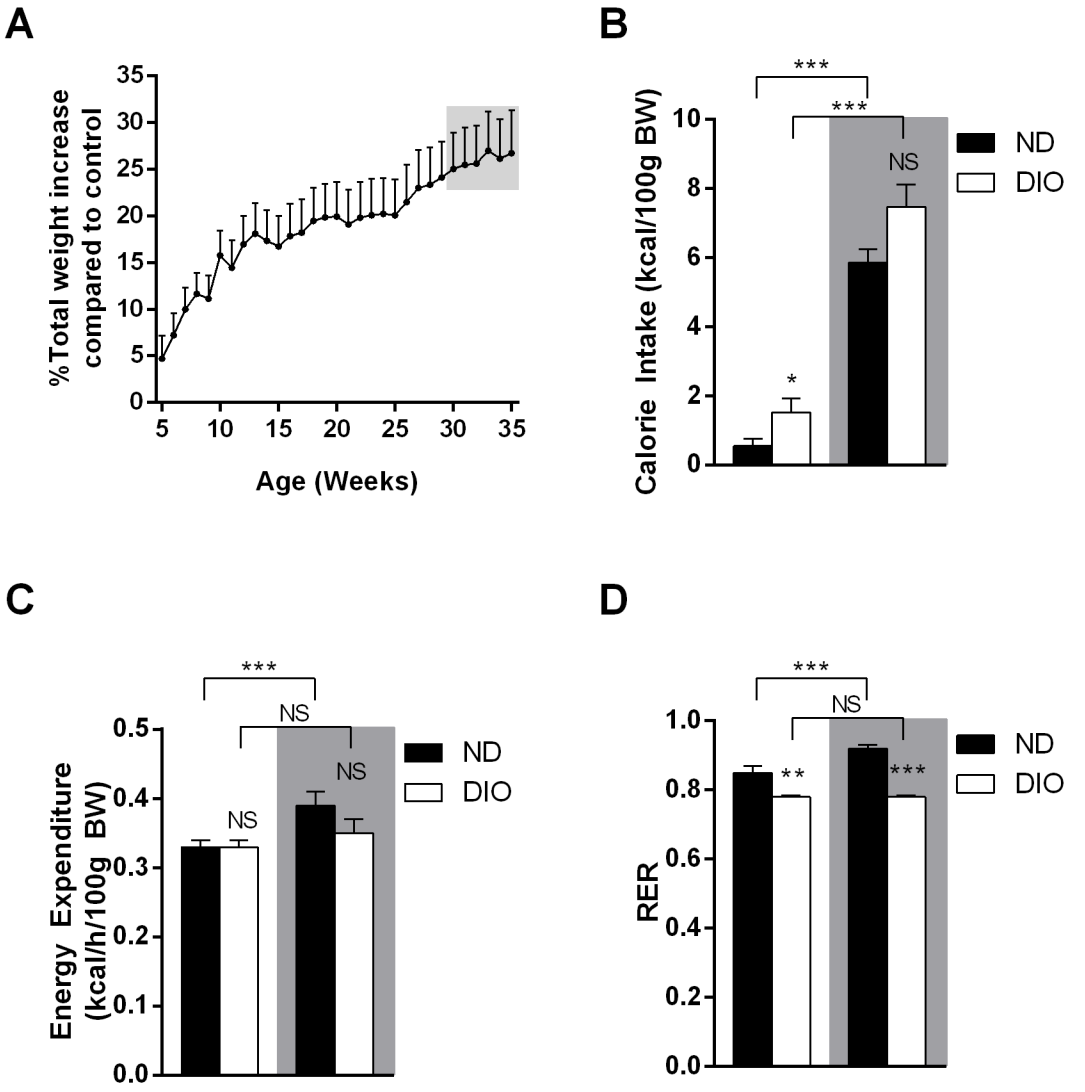
Figure 3 | (a) Body weight of GB-operated (n=14) rats relative to sham-operated (n=6) rats up to 5 weeks post-surgery. Grey area represents body weight plateau (less than 5% increase or decrease in BW over last couple of weeks). CLAMS was performed at 3 weeks after surgery for both GB (n=8) and sham-operated (n=6) rats. (b) Calorie intake (g/100g body weight) during light phase and dark phase of GB and sham rats. (c) EE (kcal/h/100g body weight) during light phase and dark phase of GB and sham rats. (d) RER during light phase and dark phase of GB and sham rats. White background indicates light phase and grey background indicates dark phase. Data is expressed as mean ± SEM. *, ** $p<0.05$, 0.01 between GB and sham. Independent *t*-test was used between groups and paired *t*-test within groups (light vs. dark phase).

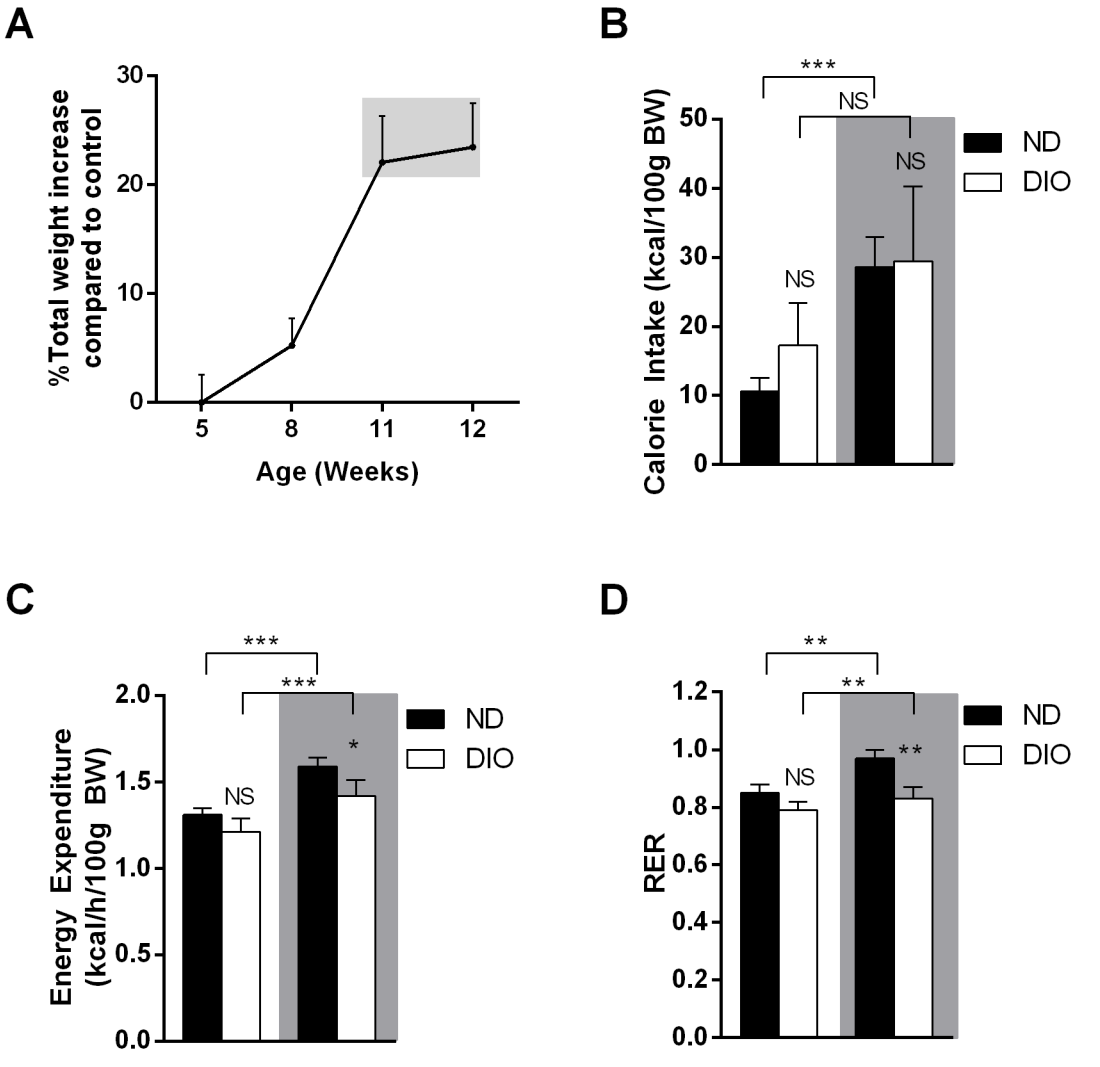
Figure 4 | (a) Body weight of M3KO mice relative to wild-type (WT) (n=7) at 2 (n=8), 6 (n=8), 11 (n=8) and 15 (n=8) months of age. Grey area represents body weight plateau (less than 5% increase or decrease in BW over last couple of weeks). CLAMS was performed at 6, 11 and 15 months of age for both M3KO (n=8) and WT (n=8) mice. (b) Calorie intake (g/100g body weight) during light phase and dark phase of WT and M3KO mice at 6, 11 and 15 months of age. (c) EE during light phase and dark phase of WT and M3KO mice at 6, 11 and 15 months of age. (d) RER during light phase and dark phase of WT and M3KO mice at 6, 11 and 15 months of age.

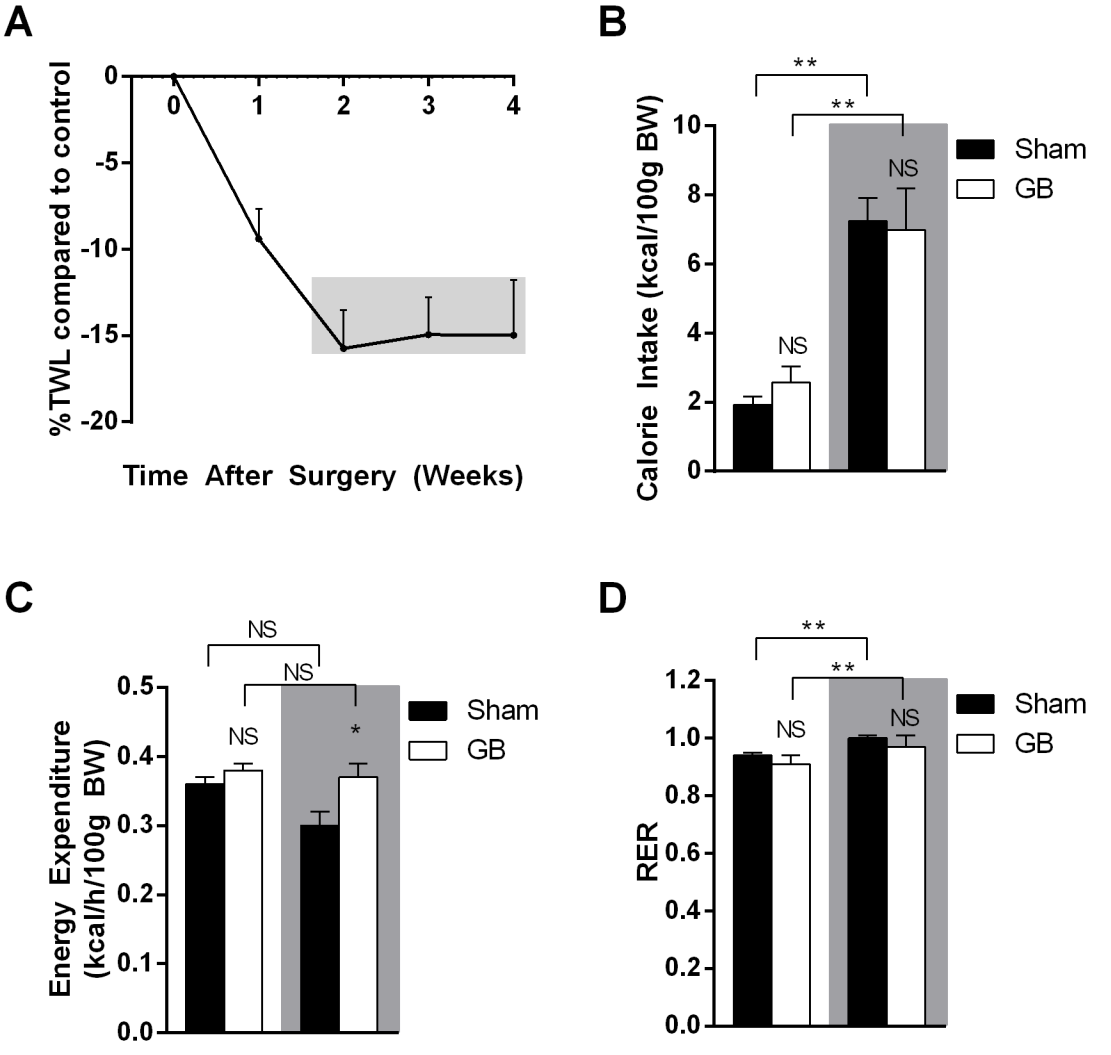
Figure 5 | mRNA expression (integrated optical density, %IOD) of AgRP, NPY, POMC and leptin receptor (Lepr) in arcuate nucleus and of MCH in ventromedial hypothalamus of WT (n=8) and M3KO (n=8) mice at 15 months of age. Data is expressed as mean ± SEM. *, **, ***: p<0.05, 0.01, 0.001 between M3KO and WT. Independent *t*-test was used between groups.

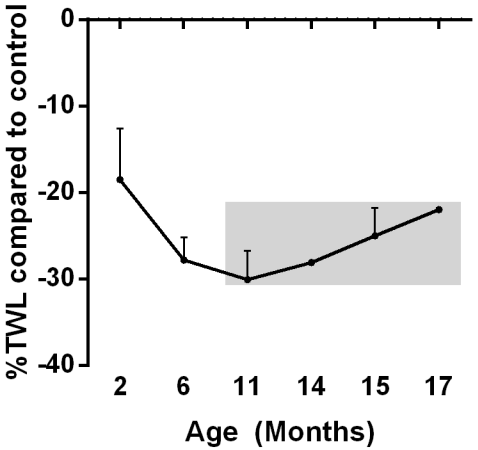
Figure S1 | Body composition of DIO (n=11) and normal diet (ND) (n=8) rats at 35 weeks of age. (a) Total mass (g), (b) Fat mass (g), (c) Fat (%), (d) Surface area of the rats (cm^2), (e) Lean mass (g) and (f) Lean (%). Data is expressed as mean ± SEM. **, ***: p< 0.01, 0.001 between DIO and ND. Independent *t*-test was used for all comparisons except for fat and lean mass where U test was used.

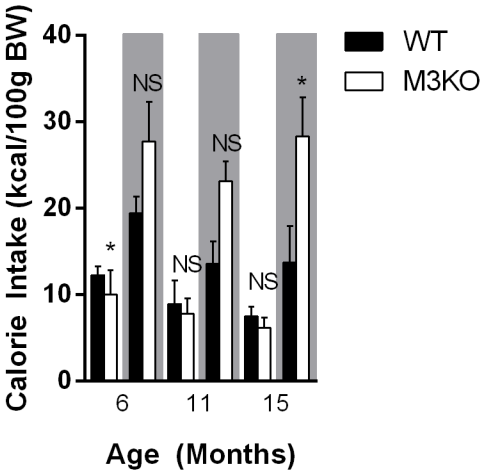
Figure S2 | Body composition of DIO (n=10) mice and normal diet (ND) (n=10) mice at 11 weeks of age. (a) Total mass (g), (b) Fat mass (g), (c) Fat (%), (d) Surface area of the rats (cm^2), (e) Lean mass (g) and (f) Lean (%). Data is expressed as mean ± SEM. *, **, ***: p< 0.05, 0.01, 0.001 between DIO and ND. Independent *t*-test was used for all comparisons except for fat mass where U test was used.

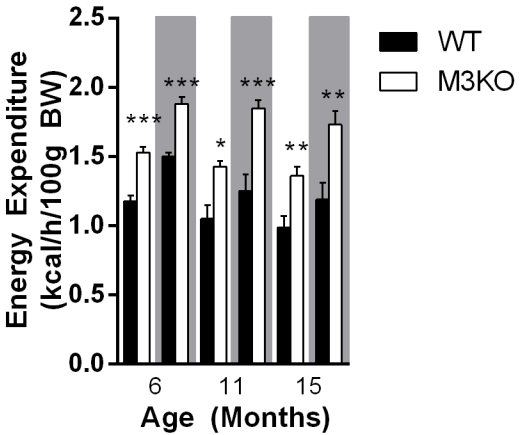

A
% Total weight increase compared to control
Age (Weeks)
B
Calorie Intake (kcal/100g BW)
ND
DIO
NS
C
Energy Expenditure (kcal/h/100g BW)
D
RER

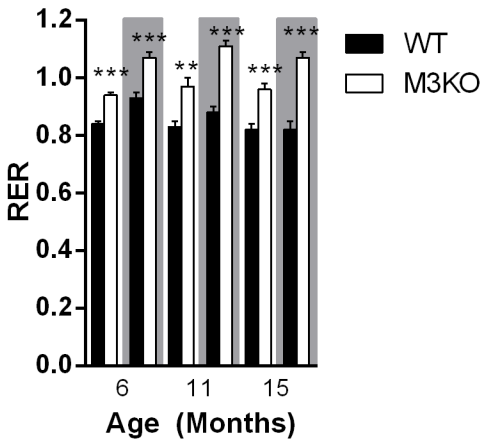

A
% Total weight increase compared to control
30
20
10
0
5
8
11
12
Age (Weeks)
B
Calorie Intake (kcal/100g BW)
***
NS
NS
NS
50
40
30
20
10
0
ND
DIO
C
Energy Expenditure (kcal/h/100g BW)
***
***
*
NS
2.0
1.5
1.0
0.5
0.0
ND
DIO
D
RER
**
**
**
NS
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ND
DIO

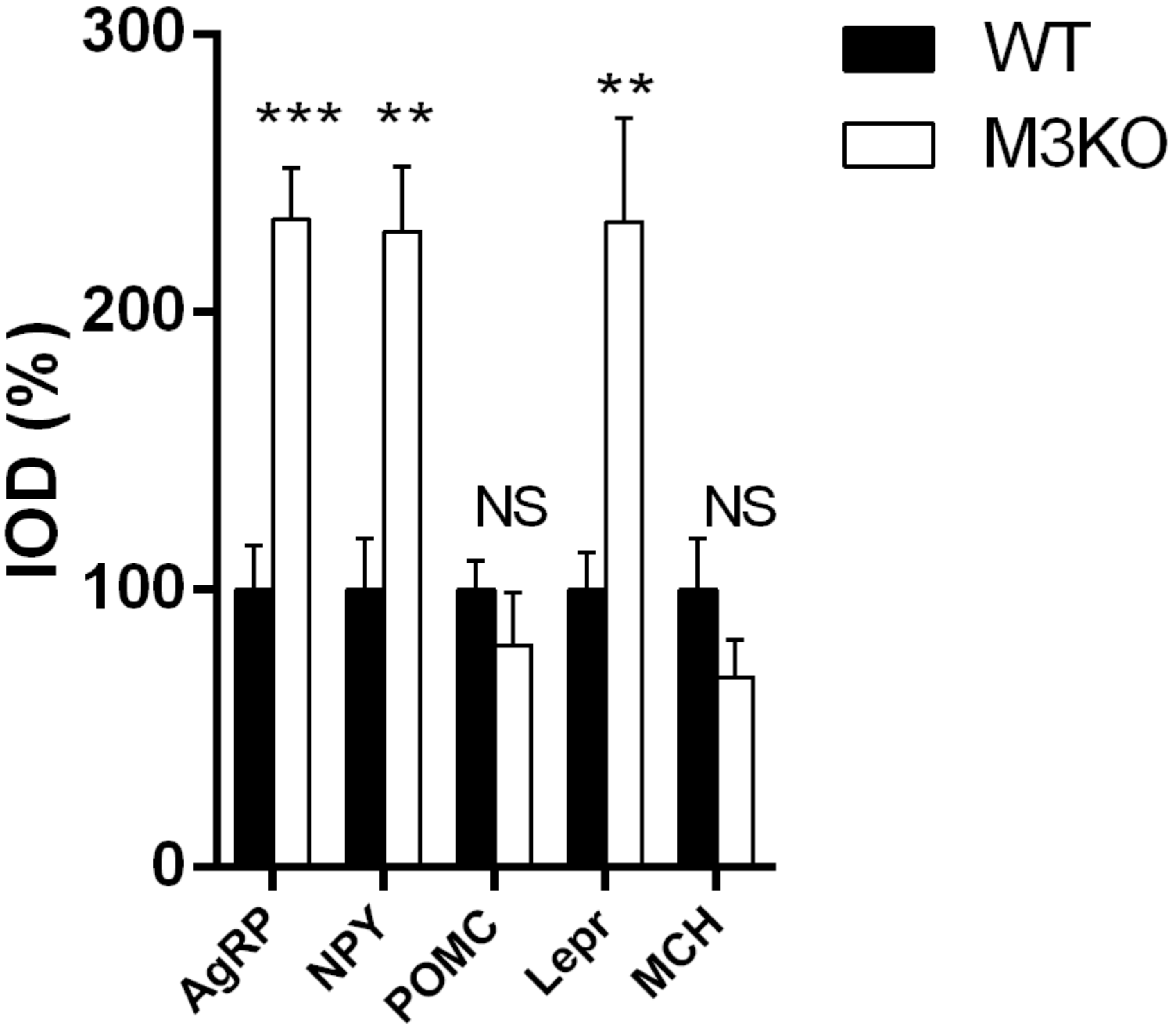

A
0
-5
-10
-15
-20
%TWL compared to control
0
1
2
3
4
Time After Surgery (Weeks)
B
**
**
NS
NS
Sham
GB
Calorie Intake (kcal/100g BW)
10
8
6
4
2
0
C
NS
NS
NS
*
Sham
GB
Energy Expenditure (kcal/h/100g BW)
0.5
0.4
0.3
0.2
0.1
0.0
D
**
**
NS
NS
Sham
GB
RER
1.2
1.0
0.8
0.6
0.4
0.2
0.0

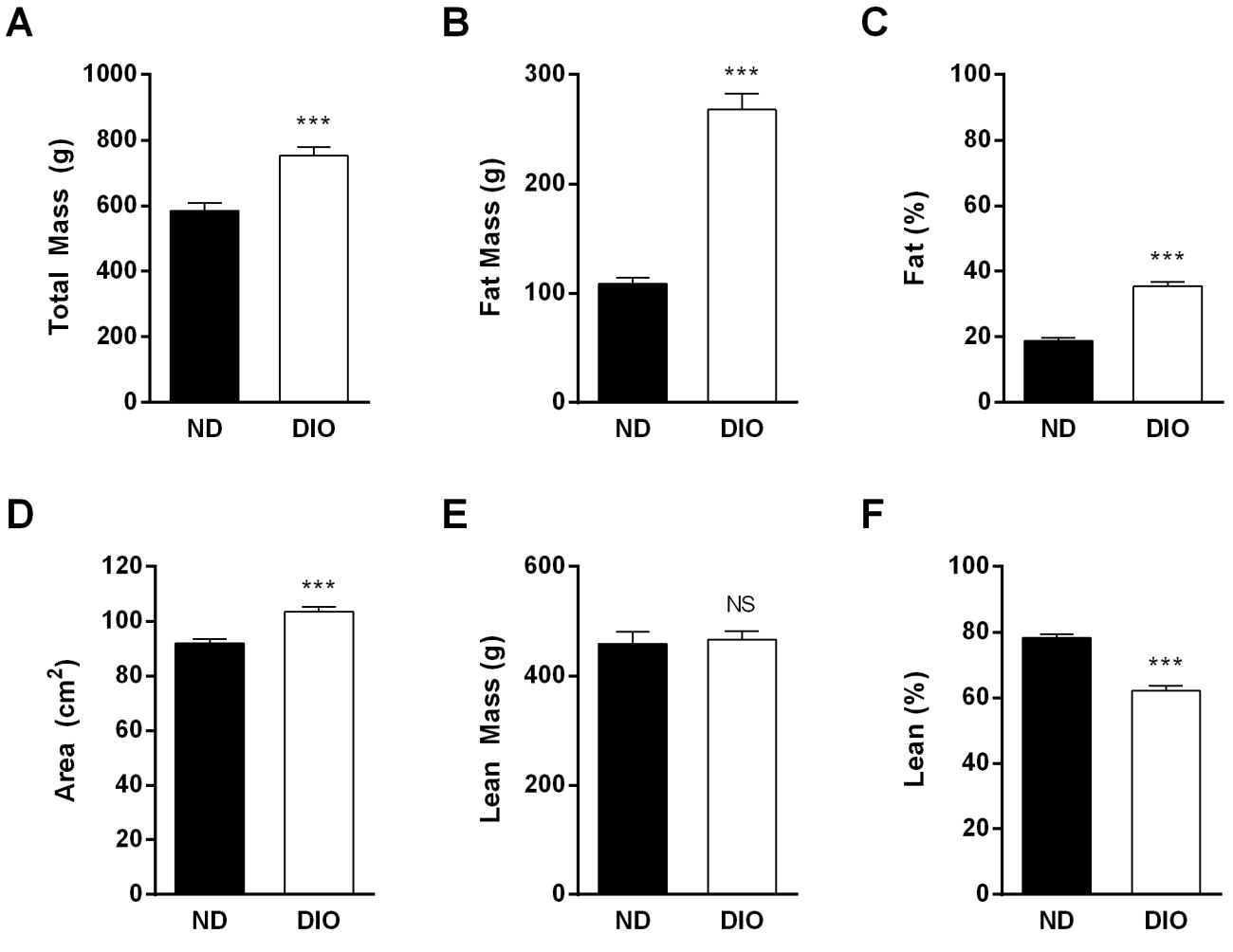

%TWL compared to control
0
-10
-20
-30
-40
2
6
11
14
15
17
Age (Months)


Calorie Intake (kcal/100g BW)
40
30
20
10
0
*
NS
NS
NS
*
6
11
15
Age (Months)
WT
M3KO


Energy Expenditure (kcal/h/100g BW)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
*
***
**
**
6
11
15
Age (Months)
WT
M3KO


RER
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
**
***
***
***
6
11
15
Age (Months)
WT
M3KO


300
200
100
0
IOD (%)
***
**
**
NS
NS
AgRP
NPY
POMC
Lepr
MCH
WT
M3KO


A
Total Mass (g)
1000
800
600
400
200
0
***
ND
DIO
B
Fat Mass (g)
300
200
100
0
***
ND
DIO
C
Fat (%)
100
80
60
40
20
0
***
ND
DIO
D
Area (cm2)
120
100
80
60
40
20
0
***
ND
DIO
E
Lean Mass (g)
600
400
200
0
NS
ND
DIO
F
Lean (%)
100
80
60
40
20
0
***
ND
DIO

Supplementary Table S1 | Energy balance in normal diet (ND) and diet-induced obese (DIO) rats at 35 weeks of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U –test were used if normality assumptions were not met (denoted U).

	Parameters	**ND (n=7)**	**DIO (n=11)**	***p*-value (two-tailed)**
24 Hours	Food Intake (g)	15.05±1.05	12.84±0.97	0.155
	Food Intake (g/100g body weight)	2.49±0.18	1.73±0.13	0.004[u]
	Calories intake (kcal)	38.67±2.71	67.3±5.07	0.001
	Calories intake (kcal/100g body weight)	6.41±0.45	9.05±0.69	0.008[u]
	Number of meals	64.29±7.61	24.64±1.92	0.002
	Meal size (g/meal)	0.25±0.03	0.54±0.05	0.000
	Meal size (kcal/meal)	0.64±0.07	2.85±0.27	0.000
	Meal duration (min)	82.22±5.1	33.72±1.85	0.000
	Meal duration (min/meal)	1.39±0.16	1.44±0.12	0.771
	Intermeal interval (min)	22.84±3.08	58.31±4.73	0.000
	Satiety ratio (min/g)	91.27±6.15	115.6±14.95	0.246[u]
	Rate of eating (g/min)	0.19±0.02	0.38±0.02	0.000
	Water intake (mL)	14.86±0.65	11.56±0.77	0.009
	Water intake (ml/100g body weight)	2.47±0.14	1.54±0.1	0.000
	Water intake during one interval (ml/time)	0.96±0.14	0.96±0.11	0.988
	Energy expenditure (kcal/h)	2.19±0.13	2.53±0.12	0.084
	Energy expenditure (kcal/h/100g body weight)	0.36±0.01	0.34±0.01	0.219
	Energy expenditure (kcal/h/cm2 body surface)	0.004±0	0.004±0	0.970
	RER	0.89±0.01	0.78±0.01	0.000
	VO2	733.04±25.46	702.51±25.88	0.438
	VCO2	653.15±29.62	549.67±21.47	0.011
	Ambulatory activity	8059.8±225.77	5736±257.01	0.104[u]

	Parameters	**ND (n=7)**	**DIO (n=11)**	***p*-value (two-tailed)**
Light Phase	Food Intake (g)	1.26±0.49	2.23±0.52	0.328[u]
	Food Intake (g/100g body weight)	0.21±0.08	0.3±0.07	0.423
	Calories intake (kcal)	3.23±1.27	11.67±2.75	0.035[u]
	Calories intake (kcal/100g body weight)	0.55±0.2	1.52±0.38	0.039
	Number of meals	6.57±1.85	5.09±0.96	0.445
	Meal size (g/meal)	0.17±0.04	0.39±0.07	0.033
	Meal size (kcal/meal)	0.45±0.09	2.04±0.36	0.001
	Meal duration (min)	6.08±1.93	6.1±1.52	0.996
	Meal duration (min/meal)	0.89±0.13	1.08±0.12	0.322
	Intermeal interval (min)	141.98±40.4	155.39±27.7	0.780
	Satiety ratio (min/g)	1265.18±653.95	3650.66±3232.5	0.285[u]
	Rate of eating (g/min)	0.19±0.02	0.36±0.04	0.004
	Water intake (mL)	0.15±0.08	0.9±0.28	0.044[u]
	Water intake (ml/100g body weight)	0.03±0.01	0.11±0.03	0.085[u]
	Water intake during one interval (ml/time)	0.07±0.04	0.4±0.11	0.027[u]
	Energy expenditure (kcal/h)	1.99±0.11	2.45±0.1	0.009
	Energy expenditure (kcal/h/100g body weight)	0.33±0.01	0.33±0.01	0.917
	Energy expenditure (kcal/h/cm2 body surface)	0.003±0	0.003±0	0.044[u]
	RER	0.85±0.02	0.78±0.01	0.008
	VO2	673.28±19.97	681.08±21.43	0.807
	VCO2	572.11±26.3	531.86±18.05	0.210
	Ambulatory activity	1486.4±94.14	1516.89±88.74	0.596[u]

	Parameters	**ND (n=7)**	**DIO (n=11)**	***p*-value (two-tailed)**
Dark Phase	Food Intake (g)	13.79±0.95	10.62±0.9	0.033
	Food Intake (g/100g body weight)	2.28±0.15	1.43±0.12	0.000
	Calories intake (kcal)	35.44±2.45	55.63±4.72	0.002
	Calories intake (kcal/100g body weight)	5.86±0.4	7.47±0.64	0.083
	Number of meals	57.71±6.75	19.55±2.23	0.001
	Meal size (g/meal)	0.26±0.03	0.6±0.07	0.000
	Meal size (kcal/meal)	0.66±0.07	3.14±0.35	0.000
	Meal duration (min)	76.13±4.81	27.62±1.5	0.000
	Meal duration (min/meal)	1.43±0.17	1.6±0.18	0.536
	Intermeal interval (min)	12.04±1.66	38.94±5.23	0.000[u]
	Satiety ratio (min/g)	47.09±3.15	67.18±7.04	0.020[u]
	Rate of eating (g/min)	0.19±0.02	0.38±0.02	0.000
	Water intake (mL)	14.71±0.61	10.67±0.62	0.001[u]
	Water intake (ml/100g body weight)	2.44±0.13	1.43±0.09	0.000
	Water intake during one interval (ml/time)	1.05±0.16	1.08±0.13	0.897
	Energy expenditure (kcal/h)	2.39±0.16	2.61±0.14	0.345
	Energy expenditure (kcal/h/100g body weight)	0.39±0.02	0.35±0.02	0.068
	Energy expenditure (kcal/h/cm2 body surface)	0.004±0	0.004±0	0.429
	RER	0.92±0.01	0.78±0.01	0.000
	VO2	792.8±32.26	723.93±32.88	0.177
	VCO2	734.19±34.67	567.49±26.79	0.001
	Ambulatory activity	6573.4±296.45	4219.11±267.14	0.104[u]

Supplementary Table S2 | Energy balance in normal diet (ND) and diet-induced obese (DIO) mice at 11 weeks of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U –test were used if normality assumptions were not met (denoted U).

	Parameters	**ND (n=10)**	**DIO (n=10)**	***p*-value (two-tailed)**
24 Hours	Food Intake (g)	4.22±0.61	3.07±1.13	0.063[u]
	Food Intake (g/100g body weight)	15.25±2.27	8.91±3.18	0.035[u]
	Calories intake (kcal)	10.84±1.56	16.07±5.91	0.353[u]
	Calories intake (kcal/100g body weight)	39.19±5.83	46.69±16.67	0.280[u]
	Number of meals	85.3±7.71	61.7±8.45	0.054
	Meal size (g/meal)	0.05±0	0.04±0.012	0.105[u]
	Meal size (kcal/meal)	0.13±0.01	0.11±0.03	0.105[u]
	Meal duration (min)	105.27±11.07	32.23±5.66	0.000
	Meal duration (min/meal)	1.24±0.07	0.51±0.044	0.000
	Intermeal interval (min)	16.54±1.4	27.7±4.78	0.048
	Satiety ratio (min/g)	361.8±42.88	970.82±208.87	0.017
	Rate of eating (g/min)	0.04±0.01	0.09±0.022	0.029[u]
	Water intake (mL)	2.21±0.05	1.68±0.18	0.018
	Water intake (ml/100g body weight)	7.99±0.26	5.15±0.72	0.002
	Water intake during one interval (ml/time)	0.05±0	0.07±0.021	0.280[u]
	Energy expenditure (kcal/h)	0.4±0.01	0.44±0.026	0.023[u]
	Energy expenditure (kcal/h/100g body weight)	1.45±0.04	1.31±0.085	0.165[u]
	Energy expenditure (kcal/h/cm2 body surface)	0±0	0.05±0	0.796[u]
	RER	0.91±0.03	0.81±0.031	0.016
	VO2	2941.72±89.33	2718.82±170.02	0.529[u]
	VCO2	2702.743±92.59	2210.76±182.3	0.027
	Ambulatory activity	11262.8±2522.14	10998.4±1933.35	0.436[u]

	Parameters	**ND (n=10)**	**DIO (n=10)**	***p*-value (two-tailed)**
Light Phase	Food Intake (g)	1.14±0.2	1.15±0.42	0.165[u]
	Food Intake (g/100g body weight)	4.12±0.75	3.29±1.18	0.075[u]
	Calories intake (kcal)	2.94±0.52	6±2.21	0.684[u]
	Calories intake (kcal/100g body weight)	10.59±1.93	17.25±6.17	1.000[u]
	Number of meals	28.1±3.03	17.4±2.32	0.012
	Meal size (g/meal)	0.04±0	0.06±0.02	0.739[u]
	Meal size (kcal/meal)	0.1±0.01	0.15±0.05	0.739[u]
	Meal duration (min)	31.15±2.92	6.87±1.37	0.000[u]
	Meal duration (min/meal)	1.15±0.1	0.38±0.04	0.000
	Intermeal interval (min)	25.83±2.49	43.87±4.7	0.005
	Satiety ratio (min/g)	685.11±73.44	1420.31±335.79	0.059
	Rate of eating (g/min)	0.04±0.01	0.19±0.08	0.001[u]
	Water intake (mL)	0.67±0.04	0.62±0.11	0.692
	Water intake (ml/100g body weight)	2.41±0.15	1.93±0.39	0.264
	Water intake during one interval (ml/time)	0.05±0	0.05±0.01	0.436[u]
	Energy expenditure (kcal/h)	0.36±0.01	0.41±0.02	0.140
	Energy expenditure (kcal/h/100g body weight)	1.31±0.04	1.21±0.08	0.274
	Energy expenditure (kcal/h/cm2 body surface)	0±0	0±0	0.684[u]
	RER	0.85±0.03	0.79±0.03	0.190[u]
	VO2	2701.94±87.25	2521.09±161.52	0.338
	VCO2	2303.14±96.38	1994.48±161.82	0.105[u]
	Ambulatory activity	2780.08±659.06	3022.8±570.81	0.785

	Parameters	**ND (n=10)**	**DIO (n=10)**	***p*-value (two-tailed)**
Dark Phase	Food Intake (g)	3.07±0.45	1.92±0.72	0.035[u]
	Food Intake (g/100g body weight)	11.13±1.68	5.62±2.06	0.007[u]
	Calories intake (kcal)	7.9±1.16	10.07±3.8	0.315[u]
	Calories intake (kcal/100g body weight)	28.59±4.31	29.44±10.78	0.280[u]
	Number of meals	57.2±5.44	44.3±6.67	0.151
	Meal size (g/meal)	0.05±0.01	0.04±0.01	0.052[u]
	Meal size (kcal/meal)	0.14±0.01	0.1±0.03	0.052[u]
	Meal duration (min)	74.12±10.46	25.36±5	0.001
	Meal duration (min/meal)	1.28±0.1	0.55±0.05	0.000
	Intermeal interval (min)	12.24±1.45	20.42±4.43	0.247[u]
	Satiety ratio (min/g)	253.23±42.66	741.89±156.87	0.013
	Rate of eating (g/min)	0.05±0.01	0.07±0.02	0.165[u]
	Water intake (mL)	1.54±0.05	1.06±0.08	0.000[u]
	Water intake (ml/100g body weight)	5.58±0.24	3.23±0.36	0.000[u]
	Water intake during one interval (ml/time)	0.06±0	0.08±0.03	0.190[u]
	Energy expenditure (kcal/h)	0.44±0.02	0.48±0.03	0.075[u]
	Energy expenditure (kcal/h/100g body weight)	1.59±0.05	1.42±0.09	0.043[u]
	Energy expenditure (kcal/h/cm2 body surface)	0.01±0	0.01±0	0.796[u]
	RER	0.97±0.03	0.83±0.04	0.003
	VO2	3181.38±94.72	2916.54±186.14	0.579[u]
	VCO2	3102.16±108.17	2427.04±207.7	0.010
	Ambulatory activity	8482±1911.33	7975.6±1433.72	0436[u]

Supplementary Table S3 | Energy balance in sham- and gastric bypass (GB)-operated rats 3 weeks post-surgery. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U –test were used if normality assumptions were not met (denoted U).

	Parameters	**Sham (n=6)**	**GB (n=8)**	***p*-value (two-tailed)**
24 Hours	Food Intake (g)	20.51±1.17	18.63±3.09	0.852[u]
	Food Intake (g/100g body weight)	3.57±0.24	3.72±0.6	0.345[u]
	Calories intake (kcal)	52.72±3	47.88±7.94	0.852[u]
	Calories intake (kcal/100g body weight)	9.18±0.63	9.56±1.53	0.345[u]
	Number of meals	44.83±4.48	44±6.95	0.928
	Meal size (g/meal)	0.48±0.04	0.39±0.06	0.323
	Meal size (kcal/meal)	1.22±0.11	1±0.16	0.323
	Meal duration (min)	58.43±7.37	65.15±11.08	0.648
	Meal duration (min/meal)	1.3±0.1	1.41±0.11	0.505
	Intermeal interval (min)	31.64±3.15	84.44±56.47	0.755[u]
	Satiety ratio (min/g)	66.85±3.71	914.52±847.79	0.852[u]
	Rate of eating (g/min)	0.37±0.03	0.28±0.05	0.184
	Water intake (mL)	20.22±1.83	17.85±2.38	0.472
	Water intake (ml/100g body weight)	3.5±0.31	3.61±0.49	0.865
	Water intake during one interval (ml/time)	2.09±0.2	1.32±0.17	0.013
	Energy expenditure (kcal/h)	1.92±0.07	1.86±0.05	0.852[u]
	Energy expenditure (kcal/h/100g body weight)	0.33±0.01	0.38±0.01	0.003
	Energy expenditure (kcal/h/cm2 body surface)	0.003±0	0.003±0	0.055
	RER	0.97±0.01	0.94±0.04	0.852[u]
	VO2	663.32±16.09	755.31±15.33	0.005[u]
	VCO2	638.31±17.4	707.19±27.26	0.020[u]
	Ambulatory activity	6423.33±375.13	6186±623.55	0.770

	Parameters	**Sham (n=6)**	**GB (n=8)**	***p*-value (two-tailed)**
Light Phase	Food Intake (g)	4.35±0.54	4.99±0.91	0.593
	Food Intake (g/100g body weight)	0.75±0.09	1±0.18	0.289
	Calories intake (kcal)	11.18±1.39	12.82±2.35	0.593
	Calories intake (kcal/100g body weight)	1.93±0.23	2.57±0.46	0.289
	Number of meals	12.67±1.8	16.13±3.04	0.389
	Meal size (g/meal)	0.37±0.07	0.29±0.03	0.260
	Meal size (kcal/meal)	0.96±0.17	0.74±0.09	0.260
	Meal duration (min)	12.3±2.07	15.76±3.29	0.429
	Meal duration (min/meal)	1.02±0.19	0.9±0.1	0.552
	Intermeal interval (min)	57.05±8.2	80.3±40.2	0.414[u]
	Satiety ratio (min/g)	168.48±31.65	674.16±546.43	0.573[u]
	Rate of eating (g/min)	0.38±0.04	0.32±0.02	0.172
	Water intake (mL)	0.36±0.26	1.67±0.81	0.081[u]
	Water intake (ml/100g body weight)	0.06±0.04	0.34±0.16	0.008[u]
	Water intake during one interval (ml/time)	0.21±0.12	0.6±0.23	0.491[u]
	Energy expenditure (kcal/h)	2.08±0.03	1.9±0.06	0.029
	Energy expenditure (kcal/h/100g body weight)	0.36±0.01	0.38±0.01	0.126
	Energy expenditure (kcal/h/cm2 body surface)	0.003±0	0.003±0	0.824
	RER	0.94±0.01	0.91±0.03	0.389
	VO2	726.68±15.56	776.37±20.45	0.094
	VCO2	682.5±12.08	705.98±29.9	0.531
	Ambulatory activity	1636.17±103.11	1591.63±86.9	0.746

	Parameters	**Sham (n=6)**	**GB (n=8)**	***p*-value (two-tailed)**
Dark Phase	Food Intake (g)	16.16±1.3	13.64±2.44	0.426
	Food Intake (g/100g body weight)	2.82±0.26	2.72±0.47	0.870
	Calories intake (kcal)	41.54±3.34	35.06±6.28	0.426
	Calories intake (kcal/100g body weight)	7.24±0.67	6.99±1.2	0.870
	Number of meals	32.17±4.17	27.88±4.64	0.521
	Meal size (g/meal)	0.53±0.04	0.45±0.08	0.464
	Meal size (kcal/meal)	1.35±0.11	1.16±0.2	0.464
	Meal duration (min)	46.13±6.52	49.39±8.17	0.772
	Meal duration (min/meal)	1.43±0.06	1.77±0.16	0.086
	Intermeal interval (min)	22.2±3.06	63.6±42.28	0.852[u]
	Satiety ratio (min/g)	41.7±3.46	787.62±742.99	0.662[u]
	Rate of eating (g/min)	0.37±0.03	0.27±0.06	0.043[u]
	Water intake (mL)	19.86±1.77	16.18±1.86	0.108[u]
	Water intake (ml/100g body weight)	3.44±0.31	3.27±0.39	0.750
	Water intake during one interval (ml/time)	2.43±0.32	1.49±0.2	0.013[u]
	Energy expenditure (kcal/h)	1.75±0.14	1.82±0.1	0.414[u]
	Energy expenditure (kcal/h/100g body weight)	0.3±0.02	0.37±0.02	0.033
	Energy expenditure (kcal/h/cm2 body surface)	0.003±0	0.003±0	0.113
	RER	1±0.01	0.97±0.04	0.516
	VO2	600.02±36.7	734.01±37.61	0.029
	VCO2	594.19±37.54	708.2±40.76	0.070
	Ambulatory activity	4787.17±284.06	4594.38±555.18	0.785

Supplementary Table S4 | Energy balance in wild-type (WT) and M3KO mice at 6 months of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U – test were used if normality assumptions were not met (denoted U).

	Parameters	WT (n=8)	M3KO (n=8)	*p*-value (two-tailed)
24 Hours	Food Intake (g)	4±0.24	3.49±0.51	0.380
	Food Intake (g/100g body weight)	12.33±0.8	14.69±1.79	0.249
	Calories intake (kcal)	10.28±0.62	8.97±1.31	0.380
	Calories intake (kcal/100g body weight)	31.68±2.06	37.75±4.61	0.249
	Number of meals	80.25±8.76	24±6.17	0.000
	Meal size (g/meal)	0.05±0.01	0.19±0.04	0.005
	Meal size (kcal/meal)	0.14±0.02	0.5±0.09	0.005
	Meal duration (min)	69.29±11.55	23.51±4.71	0.005
	Meal duration (min/meal)	0.86±0.09	1.5±0.51	0.130[u]
	Intermeal interval (min)	18.28±1.92	86.49±18.76	0.008
	Satiety ratio (min/g)	346.19±19.64	430.65±51.52	0.160
	Rate of eating (g/min)	0.07±0.01	0.18±0.03	0.002
	Water intake (mL)	2.61±0.2	3.46±0.68	0.267
	Water intake (ml/100g body weight)	8.03±0.63	14.94±2.9	0.050
	Water intake during one interval (ml/time)	0.07±0.01	0.06±0.01	0.539
	Energy expenditure (kcal/h)	0.44±0.01	0.4±0.01	0.028
	Energy expenditure (kcal/h/100g body weight)	1.34±0.03	1.71±0.04	0.000
	Energy expenditure (kcal/h/cm2 body surface)	0.005±0	0.005±0	0.000
	RER	0.89±0.01	1±0.01	0.000
	VO2	2742±67.57	3370.29±96.59	0.000
	VCO2	2440±57.88	3417.59±84.93	0.000
	Ambulatory activity	14323.75±3489.96	16439.88±4172.16	0.703

	Parameters	WT (n=8)	M3KO (n=8)	*p*-value (two-tailed)
Light Phase	Food Intake (g)	1.55±0.11	0.93±0.27	0.005[u]
	Food Intake (g/100g body weight)	4.78±0.37	3.9±1.08	0.010[u]
	Calories intake (kcal)	3.98±0.28	2.38±0.71	0.005[u]
	Calories intake (kcal/100g body weight)	12.28±0.96	10.02±2.79	0.010[u]
	Number of meals	28.25±4.14	7.25±2.96	0.001
	Meal size (g/meal)	0.06±0.01	0.31±0.1	0.039
	Meal size (kcal/meal)	0.16±0.02	0.8±0.26	0.039
	Meal duration (min)	17.94±3.24	6.48±2.07	0.010
	Meal duration (min/meal)	0.65±0.09	2.57±1.4	0.878[u]
	Intermeal interval (min)	27.23±3.64	196.09±51.27	0.013
	Satiety ratio (min/g)	448.53±25.91	717.32±100.33	0.032
	Rate of eating (g/min)	0.11±0.03	0.35±0.12	0.084
	Water intake (mL)	0.65±0.09	0.62±0.1	0.859
	Water intake (ml/100g body weight)	2.01±0.31	2.71±0.43	0.212
	Water intake during one interval (ml/time)	0.06±0.01	0.05±0.01	0.431
	Energy expenditure (kcal/h)	0.38±0.01	0.36±0.01	0.045
	Energy expenditure (kcal/h/100g body weight)	1.18±0.04	1.53±0.04	0.000
	Energy expenditure (kcal/h/cm2 body surface)	0.004±0	0.005±0	0.000
	RER	0.84±0.01	0.94±0.01	0.000
	VO2	2457.6±77.21	3073.19±99.3	0.000
	VCO2	2059.68±72.3	2903.66±61	0.000
	Ambulatory activity	3964.83±401.03	4951.83±617.33	0.210

	Parameters	WT (n=8)	M3KO (n=8)	*p*-value (two-tailed)
Dark Phase	Food Intake (g)	2.45±0.25	2.56±0.49	0.843
	Food Intake (g/100g body weight)	7.55±0.76	10.79±1.78	0.117
	Calories intake (kcal)	6.3±0.63	6.59±1.27	0.843
	Calories intake (kcal/100g body weight)	19.4±1.96	27.73±4.58	0.117
	Number of meals	52±5.69	16.75±3.44	0.000
	Meal size (g/meal)	0.05±0.01	0.18±0.03	0.003
	Meal size (kcal/meal)	0.13±0.02	0.47±0.08	0.003
	Meal duration (min)	51.35±8.6	17.03±3.25	0.005
	Meal duration (min/meal)	0.99±0.12	1.39±0.45	0.402
	Intermeal interval (min)	13.75±1.54	52.23±9.71	0.005
	Satiety ratio (min/g)	287.66±30.5	303.25±40.23	0.762
	Rate of eating (g/min)	0.06±0.01	0.18±0.03	0.005
	Water intake (mL)	1.96±0.17	2.84±0.6	0.198
	Water intake (ml/100g body weight)	6.02±0.52	12.23±2.53	0.044
	Water intake during one interval (ml/time)	0.07±0.01	0.07±0.01	0.582
	Energy expenditure (kcal/h)	0.49±0.01	0.44±0.02	0.034
	Energy expenditure (kcal/h/100g body weight)	1.5±0.03	1.88±0.05	0.000
	Energy expenditure (kcal/h/cm2 body surface)	0.005±0	0.006±0	0.002
	RER	0.93±0.02	1.07±0.02	0.000
	VO2	3026.41±63.52	3667.4±102.96	0.000
	VCO2	2820.32±65.79	3931.53±129.81	0.000
	Ambulatory activity	15133.5±1779.52	16968±2483.48	0.562

Supplementary Table S5 | Energy balance in wild-type (WT) and M3KO mice at 11 months of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U – test were used if normality assumptions were not met (denoted U).

	Parameters	WT (n=8)	M3KO (n=8)	*p*-value (two-tailed)
24 Hours	Food Intake (g)	3.04±0.56	3.1±0.25	0.003[u]
	Food Intake (g/100g body weight)	8.78±1.99	12.03±0.73	0.000[u]
	Calories intake (kcal)	7.82±1.43	7.97±0.64	0.003[u]
	Calories intake (kcal/100g body weight)	22.56±5.12	30.91±1.87	0.000[u]
	Number of meals	72.63±9.47	46.38±16.07	0.181
	Meal size (g/meal)	0.04±0.01	0.32±0.18	0.328[u]
	Meal size (kcal/meal)	0.11±0.02	0.83±0.47	0.328[u]
	Meal duration (min)	38.1±3.65	36.63±13.44	0.918
	Meal duration (min/meal)	0.61±0.1	1.09±0.34	0.442[u]
	Intermeal interval (min)	21.91±3.23	112.61±56.13	0.505[u]
	Satiety ratio (min/g)	529.99±62.39	438.87±46.99	0.263
	Rate of eating (g/min)	0.08±0.01	0.4±0.22	0.574[u]
	Water intake (mL)	2.28±0.27	3.74±0.41	0.010
	Water intake (ml/100g body weight)	6.48±1	14.62±1.71	0.001
	Water intake during one interval (ml/time)	0.06±0.01	0.06±0	0.515
	Energy expenditure (kcal/h)	0.42±0.04	0.42±0.02	0.970
	Energy expenditure (kcal/h/100g body weight)	1.15±0.11	1.64±0.03	0.003
	Energy expenditure (kcal/h/cm2 body surface)	0.004±0	0.005±0	0.023
	RER	0.85±0.02	1.04±0.02	0.000[u]
	VO2	2363.17±228.09	3199.54±61.23	0.008
	VCO2	2021.22±198.3	3357.79±66.95	0.000
	Ambulatory activity	17856.83±976.98	17046.83±2190.65	0.743

	Parameters	WT (n=8)	M3KO (n=8)	*p*-value (two-tailed)
Light Phase	Food Intake (g)	1.19±0.31	0.77±0.17	0.263
	Food Intake (g/100g body weight)	3.48±1.06	3.02±0.7	0.723
	Calories intake (kcal)	3.06±0.81	1.99±0.45	0.263
	Calories intake (kcal/100g body weight)	8.95±2.72	7.77±1.81	0.723
	Number of meals	26.5±5.95	22.25±7.98	0.676
	Meal size (g/meal)	0.05±0.01	0.18±0.09	0.199
	Meal size (kcal/meal)	0.13±0.02	0.45±0.23	0.199
	Meal duration (min)	12.07±1.84	14.7±7.58	0.028[u]
	Meal duration (min/meal)	0.64±0.13	0.73±0.25	0.753
	Intermeal interval (min)	39.93±10.26	151.88±61.13	0.028[u]
	Satiety ratio (min/g)	774.76±132	1399.58±662.91	0.007[u]
	Rate of eating (g/min)	0.1±0.03	0.34±0.17	0.219
	Water intake (mL)	0.76±0.14	0.78±0.2	0.950
	Water intake (ml/100g body weight)	2.14±0.42	2.97±0.72	0.340
	Water intake during one interval (ml/time)	0.05±0.01	0.05±0.01	0.717
	Energy expenditure (kcal/h)	0.38±0.03	0.37±0.02	0.737
	Energy expenditure (kcal/h/100g body weight)	1.05±0.1	1.43±0.04	0.005
	Energy expenditure (kcal/h/cm2 body surface)	0.004±0	0.005±0	0.328[u]
	RER	0.83±0.02	0.97±0.03	0.028[u]
	VO2	2167.93±221.84	2841.45±79.9	0.013
	VCO2	1794.32±182.51	2755.23±105.23	0.000
	Ambulatory activity	3857.33±425.65	4110.5±656.7	0.753

	Parameters	WT (n=8)	M3KO (n=8)	*p*-value (two-tailed)
Dark Phase	Food Intake (g)	1.85±0.27	2.33±0.24	0.645[u]
	Food Intake (g/100g body weight)	5.3±0.99	9±0.88	0.442[u]
	Calories intake (kcal)	4.75±0.69	5.98±0.63	0.645[u]
	Calories intake (kcal/100g body weight)	13.61±2.54	23.14±2.26	0.442[u]
	Number of meals	46.13±5.85	24.13±8.36	0.049
	Meal size (g/meal)	0.05±0.01	0.52±0.36	0.083[u]
	Meal size (kcal/meal)	0.12±0.02	1.33±0.92	0.083[u]
	Meal duration (min)	26.03±3.47	21.93±6.37	0.581
	Meal duration (min/meal)	0.65±0.12	1.33±0.45	0.328[u]
	Intermeal interval (min)	16.26±1.8	82.67±40.89	0.195[u]
	Satiety ratio (min/g)	424.69±65.51	298.51±48.29	0.143
	Rate of eating (g/min)	0.07±0.01	0.49±0.29	0.328[u]
	Water intake (mL)	1.52±0.17	2.97±0.42	0.007
	Water intake (ml/100g body weight)	4.34±0.67	11.65±1.9	0.028[u]
	Water intake during one interval (ml/time)	0.06±0	0.06±0	0.366
	Energy expenditure (kcal/h)	0.46±0.05	0.48±0.03	0.745
	Energy expenditure (kcal/h/100g body weight)	1.25±0.12	1.85±0.06	0.000[u]
	Energy expenditure (kcal/h/cm2 body surface)	0.005±0	0.006±0	0.009
	RER	0.88±0.02	1.11±0.02	0.000[u]
	VO2	2558.41±238.86	3557.62±107.81	0.000[u]
	VCO2	2248.11±216.3	3960.36±137.2	0.000
	Ambulatory activity	13999.5±762.25	12936.33±1838.05	0.610

Supplementary Table S6 | Energy balance in wild-type (WT) and M3KO mice at 15 months of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U – test were used if normality assumptions were not met (denoted U).

	Parameters	WT (n=8)	M3KO (n=8)	*p*-value (two-tailed)
24 Hours	Food Intake (g)	2.98±0.51	3.73±0.5	0.304
	Food Intake (g/100g body weight)	8.25±1.85	13.41±2.17	0.092
	Calories intake (kcal)	7.65±1.3	9.59±1.28	0.304
	Calories intake (kcal/100g body weight)	21.2±4.75	34.46±5.57	0.092
	Number of meals	93±21.96	42.88±13.88	0.959[u]
	Meal size (g/meal)	0.04±0.01	0.22±0.1	0.015 [u]
	Meal size (kcal/meal)	0.1±0.02	0.57±0.26	0.015[u]
	Meal duration (min)	53.23±12.81	58.31±20.9	0.015[u]
	Meal duration (min/meal)	0.76±0.2	2.48±0.88	0.092
	Intermeal interval (min)	20.42±4.11	82.2±32.6	0.234[u]
	Satiety ratio (min/g)	611.73±149.57	413.72±82.69	0.021[u]
	Rate of eating (g/min)	0.07±0.02	0.11±0.03	1.000[u]
	Water intake (mL)	2.37±0.38	4.67±0.94	0.040
	Water intake (ml/100g body weight)	6.49±1.35	16.83±3.84	0.000[u]
	Water intake during one interval (ml/time)	0.08±0.02	0.08±0.02	0.905
	Energy expenditure (kcal/h)	0.42±0.04	0.44±0.02	0.679
	Energy expenditure (kcal/h/100g body weight)	1.09±0.1	1.54±0.08	0.003
	Energy expenditure (kcal/h/cm2 body surface)	0.004±0	0.005±0	0.000[u]
	RER	0.82±0.03	1.01±0.02	0.001[u]
	VO2	2263.56±213.94	3041.98±161.91	0.012
	VCO2	1837.37±157.2	3114.74±180.56	0.000
	Ambulatory activity	18779±3267.54	14149.83±3576.57	0.362

	Parameters	WT (n=8)	M3KO (n=8)	*p*-value (two-tailed)
Light Phase	Food Intake (g)	1.09±0.15	0.66±0.12	0.043
	Food Intake (g/100g body weight)	2.9±0.45	2.39±0.46	0.440
	Calories intake (kcal)	2.8±0.39	1.7±0.31	0.043
	Calories intake (kcal/100g body weight)	7.47±1.16	6.15±1.19	0.440
	Number of meals	35.75±10.68	19.38±6.89	0.219
	Meal size (g/meal)	0.04±0.01	0.12±0.05	0.180
	Meal size (kcal/meal)	0.11±0.02	0.3±0.13	0.180
	Meal duration (min)	15.11±5	16.64±6.38	0.021[u]
	Meal duration (min/meal)	0.74±0.23	2.33±1.02	0.166
	Intermeal interval (min)	30.78±7.92	118.35±45.17	0.095
	Satiety ratio (min/g)	733.59±130.19	1281.43±340.48	0.050[u]
	Rate of eating (g/min)	0.15±0.05	0.07±0.01	0.328[u]
	Water intake (mL)	0.82±0.16	0.78±0.28	0.908
	Water intake (ml/100g body weight)	2.24±0.51	2.83±1.06	0.626
	Water intake during one interval (ml/time)	0.08±0.02	0.05±0.01	0.279[u]
	Energy expenditure (kcal/h)	0.38±0.04	0.39±0.02	0.946
	Energy expenditure (kcal/h/100g body weight)	0.99±0.08	1.36±0.07	0.005
	Energy expenditure (kcal/h/cm2 body surface)	0.004±0	0.005±0	0.034
	RER	0.82±0.02	0.96±0.02	0.000
	VO2	2056±180.32	2716.36±141.6	0.012
	VCO2	1664.28±131.51	2608.84±144.57	0.000
	Ambulatory activity	4753±486.8	4258±1153.65	0.701

	Parameters	WT (n=8)	M3KO (n=8)	*p*-value (two-tailed)
Dark Phase	Food Intake (g)	1.88±0.46	3.07±0.4	0.072
	Food Intake (g/100g body weight)	5.34±1.64	11.02±1.77	0.034
	Calories intake (kcal)	4.84±1.18	7.89±1.03	0.072
	Calories intake (kcal/100g body weight)	13.74±4.22	28.32±4.55	0.034
	Number of meals	57.25±11.66	23.5±8.4	0.034
	Meal size (g/meal)	0.03±0.01	0.32±0.15	0.001[u]
	Meal size (kcal/meal)	0.09±0.02	0.83±0.39	0.001[u]
	Meal duration (min)	38.12±9.03	41.68±15.35	0.845
	Meal duration (min/meal)	0.79±0.19	2.62±0.8	0.057
	Intermeal interval (min)	15.16±2.79	69.65±27.16	0.105[u]
	Satiety ratio (min/g)	586.3±170.24	231.18±42.9	0.002[u]
	Rate of eating (g/min)	0.06±0.02	0.14±0.03	0.105[u]
	Water intake (mL)	1.56±0.28	3.89±0.72	0.014
	Water intake (ml/100g body weight)	4.24±0.95	14±2.99	0.028[u]
	Water intake during one interval (ml/time)	0.08±0.02	0.09±0.02	0.955
	Energy expenditure (kcal/h)	0.46±0.04	0.49±0.02	0.500
	Energy expenditure (kcal/h/100g body weight)	1.19±0.12	1.73±0.1	0.003
	Energy expenditure (kcal/h/cm2 body surface)	0.005±0	0.006±0	0.015
	RER	0.82±0.03	1.07±0.02	0.000[u]
	VO2	2471.12±250.41	3367.61±185.81	0.012
	VCO2	2010.47±185	3620.64±222.35	0.000
	Ambulatory activity	14026±2998.34	9891.83±2435.21	0.310