

## Neuropeptide Y mediates the short-term hypometabolic effect of estrogen deficiency in mice

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### ABSTRACT

**Background:** Estrogen deficiency increases body weight or total and central adiposity and decreases energy expenditure. Hypothalamic neuropeptide Y (NPY) expression is altered by estrogen deficiency in rodents, but the long-term consequences on energy homeostasis are unknown.

**Objective:** To investigate the role of NPY in the changes in energy expenditure and physical activity, as well as the associated changes in body weight and composition in response to short-term and long-term estrogen deficiency.

**Design:** Sham and ovariectomy (OVX) operations were performed at 8 weeks of age in wild-type (WT) and NPY<sup>-/-</sup> mice. Energy expenditure, physical activity, body composition and weight, as well as food intake were measured at 10–18 days (short-term) and 46–54 days (long-term) after OVX.

**Results:** OVX influences energy homeostasis differently at early compared with later time-points. At the early but not the late time point, OVX in WT mice reduced oxygen consumption and energy expenditure and tended to reduce resting metabolic rate. Interestingly, these effects of short-term estrogen deficiency were ablated by NPY deletion, with NPY<sup>-/-</sup> mice exhibiting significant increases in energy expenditure and resting metabolic rate. In addition to these hypermetabolic effects, OVX NPY<sup>-/-</sup> mice exhibited significantly lower body weight and whole-body fat mass relative to OVX WT controls at the short-term but not the long-term time point. Food intake and physical activity were unaltered by OVX, but NPY<sup>-/-</sup> mice exhibited significant reductions in these parameters relative to WT.

**Conclusion:** The effects of estrogen deficiency to reduce energy metabolism are transient, and NPY is critical to this effect as well as the early OVX-induced obesity.

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### INTRODUCTION

Menopause is associated with weight gain and increased adiposity (particularly visceral adiposity).<sup>bib11, bib22, bib33</sup> Given the contribution of excess weight and central obesity to pathologies, such as insulin resistance, diabetes and cardiovascular disease,<sup>bib44, bib55, bib66</sup> identifying the mechanisms contributing to menopause-induced weight gain could lead to improved means of attenuating

these pathologies. Ovariectomy (OVX) in animals, a model of menopause, induces increases in body weight and total adiposity,<sup>bib77, bib88, bib99, bib1010</sup> consistent with effects of menopause in women.<sup>bib11, bib22, bib33</sup> Importantly, lack of estrogens in humans is also associated with reductions in lean body mass and muscle strength,<sup>bib1111, bib1212</sup> perhaps explaining why menopause is consistently associated with increases in total and/or visceral fat,<sup>bib77, bib88, bib99, bib1010, bib1313</sup> but not always with increased body weight.<sup>bib1414, bib1515</sup> Administration of exogenous estrogens to OVX animals or estrogen-deficient women reduces body weight gain, prevents abdominal or visceral fat gain, and reduces loss of fat-free mass and muscle strength,<sup>bib1010, bib1616, bib1717</sup> demonstrating the pivotal role of estrogens in regulating body weight and composition.

Alterations in food intake are unlikely to have a long-term role in sustaining the positive energy balance associated with OVX or menopause. In rodents, food intake was increased at 2 and 3 weeks following OVX,<sup>bib77, bib1818</sup> however, this OVX-induced hyperphagia appears transient, with food intake normalized within 5–7 weeks post surgery.<sup>bib77, bib1818</sup> A longitudinal study following women transitioning through menopause shows no difference in energy intake at 2 years after the complete cessation of menses compared with that measured before menopause, despite increased body weight.<sup>bib1919</sup> In fact, 3–4 years before the onset of menopause, dietary energy, protein, carbohydrate and fiber intake were significantly higher than corresponding intakes measured at the onset of menopause.<sup>bib1919</sup> Thus, if energy intake is not consistently elevated when ovarian estrogens are lacking, reductions in energy expenditure may contribute to the associated weight and fat gain.

Women transitioning through menopause display marked decreases in energy expenditure and spontaneous physical activity.<sup>bib1919</sup> However, it cannot be determined whether these changes were permanent or transient because of the limited follow-up period of that study (2 years). Animals also show a drop in energy expenditure and physical activity in response to OVX.<sup>bib2020</sup> However, these changes were measured at 22 weeks after surgery, thus it is not known whether these responses to estrogen deficiency are stronger in the early period after estrogen deficiency, as is suggested by the transient increase in food intake in OVX rodents.<sup>bib77, bib1818</sup> Identifying the time course and mechanisms with which these decreases in energy expenditure and physical activity occur during estrogen deficiency could allow optimization of timing of intervention programs aimed at attenuating menopause-induced weight or fat gain.

The hypothalamus is likely to have a critical role in the estrogen-mediated regulation of energy expenditure and physical activity via actions through estrogen receptors (ER $\alpha$  and ER $\beta$ ).<sup>bib2121, bib2222, bib2323, bib2424, bib2525</sup> One of the major regulators of energy expenditure and physical activity in the central nervous system—and a target for the effects of estrogen deficiency—is neuropeptide Y (NPY), a potent orexigenic peptide primarily expressed in the hypothalamic arcuate nucleus. Centrally-administered NPY acutely stimulates hyperphagia, and repeated doses increase body weight and adiposity.<sup>bib2626</sup> In addition, NPY can reduce energy expenditure and physical activity.<sup>bib2727</sup> Marked increases in hypothalamic *NPY* gene expression have been described in post-mortem samples from post-menopausal women (59–86 years of age) when compared with that of young women (21–39 years of age).<sup>bib2828</sup> In rodents, there is also a consistent increase in hypothalamic NPY expression in response to OVX, but this appears to be transient as it is only observed at 2 and 3 weeks but not at 4–7 weeks following OVX.<sup>bib77, bib2929, bib3030</sup> The transient increase in NPY expression in response to estrogen deficiency may contribute to the transient hyperphagia and may also initiate the reduction in energy expenditure and physical activity evident following estrogen deficiency. However, there is no direct evidence for the role of NPY in mediating hyperphagia and the reductions in energy expenditure and physical activity in response to estrogen deficiency. In order to determine the role of NPY in the changes in energy expenditure and

physical activity, as well as the associated changes in body weight and composition with estrogen deficiency, we compared the effects of OVX on these parameters in wild-type (WT) and NPY knockout (NPY<sup>-/-</sup>) mice. Moreover, to determine whether the decreases in energy expenditure and physical activity that have been observed with estrogen deficiency may be more marked in the early than in the later stages of estrogen deficiency, we investigated the effect of estrogen deficiency on these parameters at both short- and long-term time points after OVX, when food intake<sup>bib77, bib1818</sup> and hypothalamic NPY expression are known to be different.<sup>bib77, bib2929, bib3030</sup>

## MATERIALS AND METHODS

### Animals

All research and animal care procedures were approved by the Garvan Institute/St Vincent's Hospital Animal Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Mice were housed under conditions of controlled temperature (22°C) and illumination (12-h light cycle). All mice were fed a normal chow diet (2.6 kcal g<sup>-1</sup>; Gordon's Specialty Stock Feeds, Yanderra, New South Wales Australia). Food and water were available *ad libitum* unless otherwise stated. Details of generation of the germline NPY knockout mice (NPY<sup>-/-</sup>) were published previously.<sup>bib3131</sup> OVX and sham operations were performed on female WT and NPY<sup>-/-</sup> mice at 8 weeks of age as described previously.<sup>bib2020</sup> Uterine weights were measured at the end of the study to confirm the success of OVX procedures as described previously.<sup>bib1010</sup>

### Food intake

Food intake was measured at 8–14 and 45–48 days post surgery. These time points are referred to as 'short-term' and 'long-term' effects of surgery. Food intake for each mouse was determined as the average of measurements taken over four consecutive days, with actual food intake (as opposed to food spillage) being determined as described previously.<sup>bib3232</sup>

### Indirect calorimetry

Energy expenditure was determined with indirect calorimetry (Oxymax Series; Columbus Instruments, Columbus, OH, USA) as described previously.<sup>bib3232</sup> Measurements were performed at 14–18 and 50–54 days post surgery ('short-term' and 'long-term' effects of surgery). Pre-weighed mice were housed individually in specially built plexiglass cages (20.1 × 10.1 × 12.7 cm<sup>3</sup>). They were left to acclimatize to the cages for 24h before 72h recordings commenced. Temperature was maintained at 22°C with airflow of 0.6 l min<sup>-1</sup>. Oxygen consumption (VO<sub>2</sub>) and carbon dioxide production (VCO<sub>2</sub>) were measured every 27 min. The respiratory exchange ratio (RER) was calculated as the quotient of VCO<sub>2</sub>/VO<sub>2</sub>, with 100% carbohydrate oxidation resulting in an RER of 1, and 100% fat oxidation resulting in an RER of 0.7.<sup>bib3333, bib3434</sup> Energy expenditure (kcal heat produced) was calculated as calorific value (CV) × VO<sub>2</sub>, where CV is 3.815 + 1.232 × RER.<sup>bib3535</sup> Data for the 72-h monitoring period was averaged for 1-h intervals for energy expenditure and RER. Actual food intake was determined during indirect calorimetry over four consecutive days, taking into account food spillage.

### Measurement of physical activity and resting metabolic rate

Ambulatory activity of individually housed mice was evaluated at the same time as measurement of energy expenditure via indirect calorimetry, using an OPTO-M3 sensor system (Columbus Instruments, Columbus, OH, USA). Consecutive adjacent photo-beam breaks were counted as ambulatory counts. Cumulative ambulatory counts of X and Y directions were recorded every minute and summed for 1-h intervals as previously described.<sup>bib3232</sup> In order to elucidate the

contribution of possible changes in physical activity to changes in energy expenditure, resting metabolic rate was estimated using correlation analysis between physical activity and energy expenditure as previously described.<sup>bib3636</sup>

#### **Analysis of body composition**

Immediately upon completion of indirect calorimetry and physical activity measurements, animals were anesthetized and then scanned using dual-energy X-ray absorptiometry (Lunar PIXImus; GE Healthcare, Madison, WI, USA) to determine whole-body fat and lean mass. The head was excluded from analyses of body composition.

#### **Tissue collection**

At 8 weeks (56 days) following surgery, animals were culled between 1200 and 1700, h by cervical dislocation followed by decapitation. White adipose tissue depots (right inguinal, right parametrial, right retroperitoneal and mesenteric) and the interscapular brown adipose tissue were removed and weighed. Total adipose tissue mass was estimated as the sum of the mass of these white adipose tissue depots. Brown adipose tissue was frozen then stored at  $-80^{\circ}\text{C}$  for subsequent analysis.

#### **Western blotting**

Brown adipose tissue samples were homogenized in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostic, Mannheim, Germany). Following centrifugation, supernatants were collected and protein concentrations were measured spectrophotometrically. Fifteen microgram protein were resolved by SDS-PAGE and immunoblotted with antibodies against uncoupling protein-1 (UCP-1) (Alpha Diagnostic International, San Antonio, TX, USA) and peroxisome proliferator activated receptor  $\gamma$  coactivator 1  $\alpha$  (PGC1 $\alpha$ ) (Calbiochem, Merck Pty Ltd, Kilsyth, Victoria, Australia). Immunolabelled bands were quantified by densitometry.

#### **Serum estrogen and corticosterone analyses**

Blood samples were collected from the tail immediately after indirect calorimetry at both time points. Serum estradiol levels were measured at the long-term time point using an enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI, USA). Serum corticosterone levels were determined at both time points using a radioimmunoassay kit (MP Biomedicals, Solon, OH, USA).

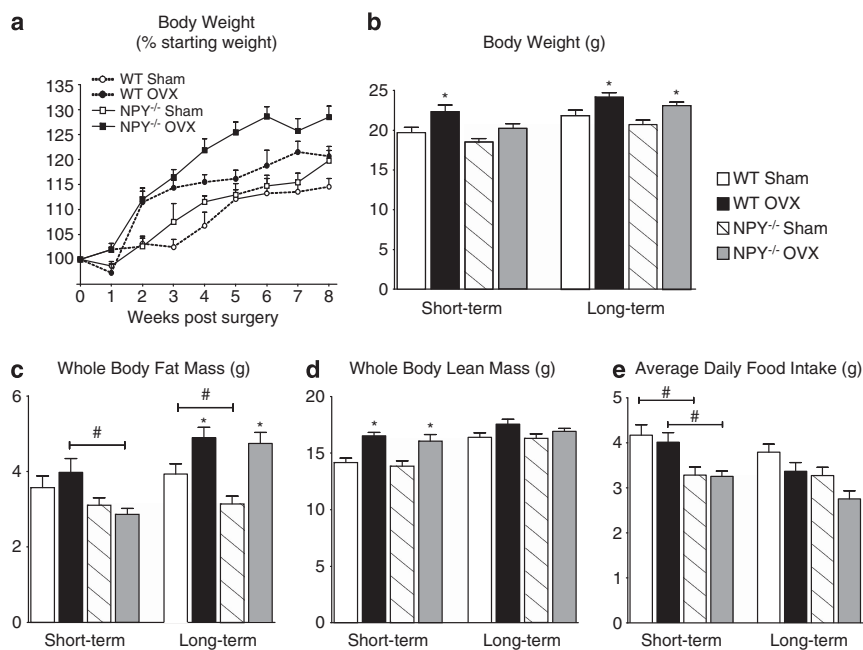
#### **Statistical analyses**

All data are expressed as means $\pm$ s.e. of the mean (s.e.m.). Differences among groups were assessed by two-way analysis of variance (ANOVA) with Bonferroni *post-hoc* tests when appropriate. Differences in the overall time course between knockout and WT mice with respect to  $\text{VO}_2$ , physical activity and RER over the continuous 72-h period were averaged for the whole 72-h period, as well as for the light and dark periods, were assessed by two-way ANOVA with repeated measures. Comparisons of energy expenditure (kcal per h) and resting metabolic rate (kcal per h) were carried out by analysis of covariance with lean mass as a covariate. Adjusted means of energy expenditure or resting metabolic rate at a common lean mass were generated by analysis of covariance and presented. When significant interactions between genotype and surgery were observed, energy expenditure and resting metabolic rate data were split for genotype or surgery and analyzed by analysis of covariance. Statistical analyses were performed with SPSS version 18.0 (SPSS, Chicago, IL, USA). Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### Effect of NPY deficiency on OVX-induced obesity

To investigate the interaction between the NPY and estrogen pathways, we first examined body weight, body composition and food intake. Our model of OVX was found to be effective in all animals, as demonstrated by decreased uterine weight in WT and NPY<sup>-/-</sup> OVX animals relative to sham controls (WT: 1.4±0.3 versus 10.2±1.4mg; NPY<sup>-/-</sup>: 1.5±0.1 versus 10.1±1.3mg, 8–11 mice per group, *P*<0.0001 OVX versus sham). In addition, serum estrogen was reduced to undetectable levels by OVX in both genotypes, and levels in sham controls were not significantly different between genotypes (25.8±2.1 versus 29.6±3.7pgml<sup>-1</sup> in WT and NPY<sup>-/-</sup> mice, respectively, 6–8 mice per group, ns). Before surgery, all groups showed similar body weights (19.1±0.5g in WT sham; 19.0±0.6g in WT OVX; 18.2±0.5g in NPY<sup>-/-</sup> sham; 18.4±0.4g in NPY<sup>-/-</sup> OVX, 9–11 mice per group, ns). There was no significant difference between sham WT and NPY<sup>-/-</sup> mice with respect to body weight changes in the 8 weeks after surgery (fig1Figures 1a and b). At the short-term time point following OVX, WT but not NPY<sup>-/-</sup> mice exhibited increased body weight when compared with sham controls of the same genotype (fig1Figure 1b). However, at the long-term period of estrogen deficiency, OVX induced an increase in body weight in both WT and NPY<sup>-/-</sup> mice (fig1Figure 1b).



**Figure 1.** NPY deficiency does not prevent OVX-induced obesity. (a, b) OVX increases weight gain and body weight in both WT and NPY knockout mice relative to sham control mice. (c) Dual-energy X-ray absorptiometry (DXA) scans at 18 and 54 days post surgery, referred to as 'short-term' and 'long-term' time-points, respectively, reveal that OVX-induced increases in whole-body fat mass in both genotypes occur only after long-term estrogen deficiency, with NPY<sup>-/-</sup> mice exhibiting a reduction in fat mass after short-term estrogen deficiency. Dual-energy X-ray absorptiometry (DXA) scans (d) show that short-term estrogen deficiency increases whole-body lean mass in WT and NPY<sup>-/-</sup> mice. (e) Average daily food intake measured over four consecutive days demonstrates that OVX had no effect on food intake in WT or NPY<sup>-/-</sup> mice at either the short-term or long-term time points, with decreased average daily food intake in NPY<sup>-/-</sup> versus WT mice at the short-term time point. Data are means ± s.e.m. of 8–11 mice per group. \**P*<0.05 for comparisons within genotype. #*P*<0.05 for comparisons within surgery type.

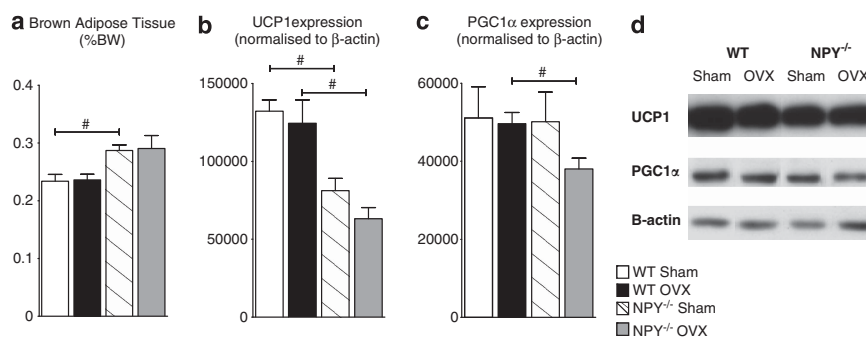
Dual-energy X-ray absorptiometry at both the early and late time points revealed that significant OVX-induced increases in fat mass in both genotypes were not observed until the long-term time point (fig1Figure 1c), which was also demonstrated by the weight of individual white adipose tissue depots dissected at the long-term point (Supplemental Figure 1). In fact, there was a significant reduction in whole-body fat mass at the short-term time point in OVX NPY<sup>-/-</sup>



versus OVX WT mice (fig1Figure 1c), suggesting that NPY may indeed have a role in promoting fat accretion in the initial 18 days following estrogen deficiency in mice. Interestingly, at the short-term, but not the long-term time point, OVX induced a significant increase in whole-body lean mass in WT and NPY<sup>-/-</sup> relative to sham animals of the same genotype, with no significant difference between genotypes (fig1Figure 1d).

There was no significant effect of OVX on food intake in WT or NPY<sup>-/-</sup> mice at either time point (fig1Figure 1e). However, NPY<sup>-/-</sup> mice exhibited significantly decreased average daily food intake compared with WT mice at the short-term time point (fig1Figure 1e). This indicates that the increases in body weight and fat mass seen in OVX mice of either genotype occur in the absence of any increase in food intake at these time points.

Brown adipose tissue is an important regulator of energy balance in rodents.<sup>bib3737</sup> We showed that NPY deficiency *per se* significantly increased brown adipose tissue weight, with no significant effect of OVX in mice of either genotype (fig2Figure 2a). There was no significant effect of OVX on UCP-1 or PGC1 $\alpha$  expression in WT or NPY<sup>-/-</sup> mice (fig2Figures 2b–d), however, NPY deficiency significantly decreased brown adipose tissue UCP-1 expression in both sham and OVX animals (fig2Figures 2b–d). Interestingly, OVX decreased PGC1 $\alpha$  expression in NPY<sup>-/-</sup> mice compared with sham-operated knockouts, and NPY<sup>-/-</sup> OVX mice exhibited significantly reduced PGC1 $\alpha$  expression relative to WT OVX mice (fig2Figures 2c and d). These data suggest that NPY is critical for the regulation of UCP-1 and PGC1 $\alpha$ , particularly under conditions of estrogen deficiency.

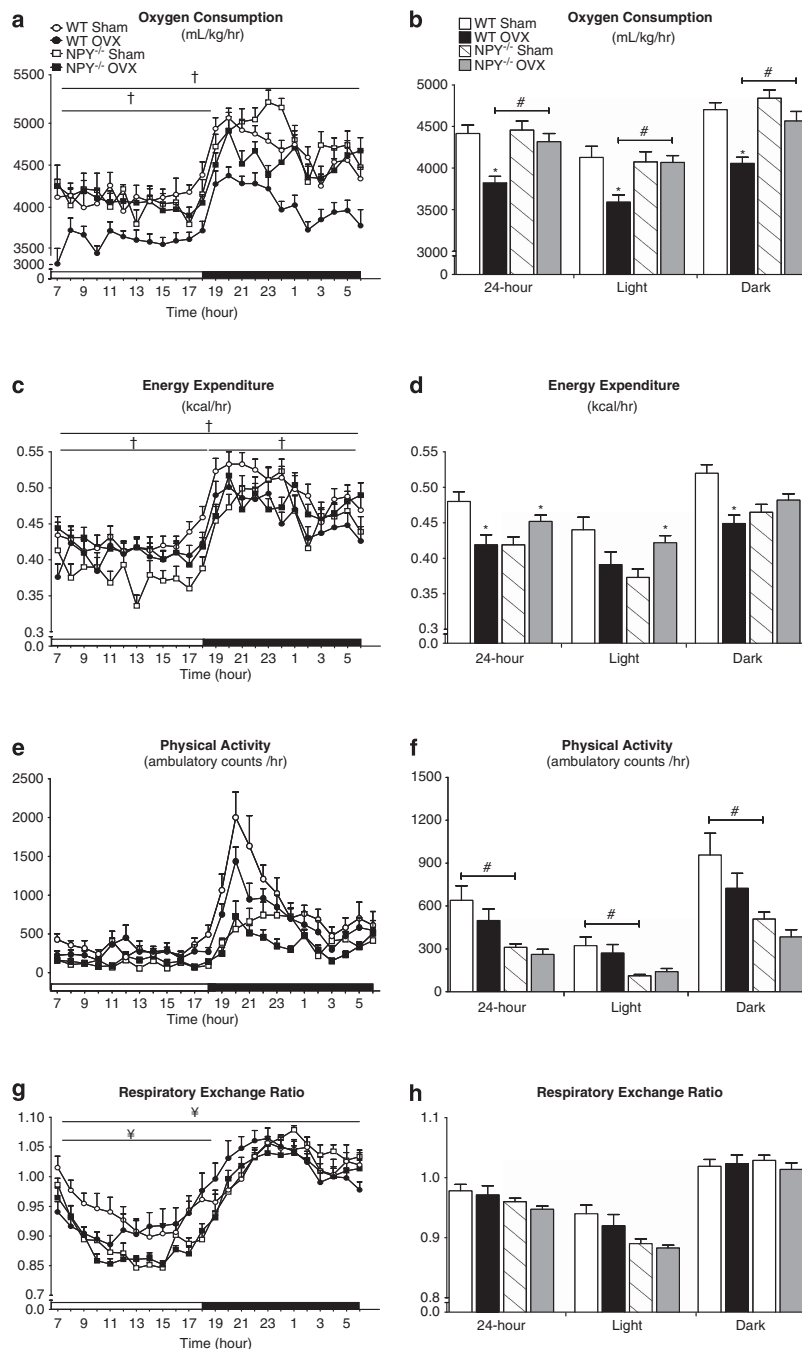


**Figure 2.** NPY deletion decreases markers of thermogenesis in estrogen deficiency. (a) Dissected interscapular brown adipose tissue weight as a percent of body weight is increased in NPY<sup>-/-</sup> relative to WT mice, with no significant effect of OVX relative to sham operation in either genotype. (b) Quantification of western blot analysis demonstrates that NPY deficiency decreases brown adipose tissue uncoupling protein-1 (UCP-1) protein levels relative to corresponding WT values, with no significant effect of OVX versus sham operation in either genotype. (c) PGC1 $\alpha$  protein levels, as determined by quantification of western blots, are significantly lower in NPY<sup>-/-</sup> OVX versus WT OVX mice. (d) Representative western blot showing protein levels of UCP-1 and PGC1 $\alpha$  in sham-operated and OVX WT and NPY<sup>-/-</sup> mice. Data are means  $\pm$  s.e.m. of six mice per group. \* $P < 0.05$  for comparisons within genotype. # $P < 0.05$  for comparisons within surgery type.

### NPY deficiency prevents the short-term reduction in oxygen consumption and energy expenditure induced by estrogen deficiency

At the short-term time point, OVX WT mice exhibited significant reductions in oxygen consumption and energy expenditure compared with sham-operated controls, during the light and/or the dark phase (fig3Figures 3a–d). In contrast, this OVX-induced reduction in oxygen consumption was not seen in NPY<sup>-/-</sup> mice (fig3Figures 3a and b). Indeed, the oxygen consumption of NPY<sup>-/-</sup> OVX mice was not significantly different from sham-operated WT mice, and was significantly greater than that of WT OVX mice (fig3Figures 3a and b). Furthermore, energy expenditure of sham-operated NPY<sup>-/-</sup> mice was significantly lower than sham WT mice, as determined by repeated measures ANOVA (fig3Figure 3c). Unlike the OVX-induced drop in energy expenditure seen in WT mice, OVX in NPY<sup>-/-</sup> mice induced significant increases in 24 h and light phase energy expenditure (fig3Figures 3c and d). There were no significant differences in food intake among groups during these measurements of energy metabolism after short-term

estrogen deficiency (tbl1Table 1), demonstrating that the observed differences in oxygen consumption and energy expenditure were independent of differences in food intake. Thus, the effect of OVX to reduce oxygen consumption and energy expenditure during the initial phase of estrogen deficiency is dependent upon NPY.



**Figure 3.** NPY deficiency prevents the reductions in oxygen consumption and energy expenditure in response to acute estrogen deficiency. (a, b) At 14–18 days after surgery, ovariectomized (OVX) WT but not germline NPY knockout (NPY<sup>-/-</sup>) mice exhibited significant reductions in 24 h, light and dark phase oxygen consumption compared with sham-operated control mice. (c, d) OVX decreased 24 h and dark phase energy expenditure in WT mice, whereas NPY<sup>-/-</sup> mice showed OVX-induced increases in energy expenditure. (e, f) OVX has no significant effect on physical activity; however, NPY deficiency reduced physical activity compared with WT. (g, h) RER was decreased with NPY deficiency in the total 24-h period and in the light phase, with no significant effect of OVX. Energy expenditure was adjusted for lean mass by analysis of covariance (ANCOVA). Adjusted energy expenditure was presented at a common lean mass of 15.22 g. Data are means ± s.e.m. of 8–11 mice per group, measured at 14–18 days post surgery. \**P* < 0.05 for comparisons within genotype. #*P* < 0.05 for comparisons within surgery type. †*P* < 0.05 for the interaction effect between surgery and genotype and ‡*P* < 0.05 for the effect of genotype by repeated measures ANOVA.

OVX had no significant effect on physical activity at the short-term time point in WT or NPY<sup>-/-</sup> mice; however, NPY<sup>-/-</sup> mice exhibited significantly lower

activity levels than WT (fig3Figures 3e and f). In addition, there was a significant effect of genotype on RER over the 24-h period and during the light phase, with both sham-operated and OVX NPY<sup>-/-</sup> mice displaying a reduced RER compared with WT mice, determined by repeated measures ANOVA (fig3Figures 3g and h). Hence, NPY<sup>-/-</sup> mice may preferentially use fat as a fuel source compared with WT mice.

### Long-term estrogen deficiency has no effect on energy metabolism in WT or NPY<sup>-/-</sup> mice

The significant decreases in oxygen consumption and energy expenditure observed in WT mice at the short-term time point following OVX were no longer apparent at the long-term time point (fig4Figures 4a-d). Moreover, there was no significant effect of NPY deficiency *per se* on oxygen consumption or energy expenditure at the long-term time point after surgery (fig4Figures 4a-d). However, consistent with the effects seen at the short-time point, there was a trend to an increase in energy expenditure following OVX in NPY<sup>-/-</sup> mice, reaching statistical significance during the light period (fig4Figure 4d).

| <b>Table 1.</b> Average daily food intake in wild-type (WT) and NPY knockout (NPY <sup>-/-</sup> ) mice during indirect calorimetry is not affected by ovariectomy (OVX) |            |            |                    |            |
|--|------------|------------|--------------------|------------|
|  | WT         |            | NPY <sup>-/-</sup> |            |
|  | Sham       | OVX        | Sham               | OVX        |
| Short-term   | 4.40 ± 0.2 | 4.51 ± 0.3 | 3.72 ± 0.5         | 4.06 ± 0.1 |
| Long-term  | 4.83 ± 0.2 | 4.06 ± 0.1 | 3.86 ± 0.1         | 3.85 ± 0.5 |

Abbreviation: NPY, neuropeptide Y. Daily food intake (g) was measured during indirect calorimetry at 14–18 days ('short-term') and 50–54 days ('long-term') after OVX or sham operation. Data are means ± s.e.m. of 8–11 mice per group. There were no significant differences between groups.

(Figure 5a). This finding indicates that these differences in oxygen consumption or energy expenditure are driven, at least to some extent, by changes in resting metabolic processes.

Food intake measured during indirect calorimetry at the long-term time point showed no differences among groups (tbl1Table 1), consistent with the long-term food intake data shown in fig1Figure 1e.

Physical activity was unchanged by OVX in WT or NPY<sup>-/-</sup> mice, albeit NPY<sup>-/-</sup> mice exhibited significantly reduced activity levels relative to WT counterparts at the long-term time point (fig4Figures 4e and f), consistent with the data collected at the short-term time point (fig3Figures 3e and f). Also consistent with data collected at the short-time point, there was a significant effect of genotype on RER during the light phase as determined by repeated measures ANOVA (fig4Figure 4g), with no effect of OVX (fig4Figures 4g and h), suggesting that NPY deficiency increases fat oxidation. Together, the data in fig3Figures 3 and fig44 suggest that the effects of OVX on oxygen consumption and energy expenditure are transient, and that NPY is critical to these short-term effects of estrogen deficiency.

### Resting metabolic rate increases with simultaneous estrogen and NPY deficiency

Our observation of changes in oxygen consumption and energy expenditure in the absence of corresponding changes in physical activity (fig3Figures 3a-f and



fig44a-f) suggest that the differences may be because of differences in resting metabolic rate, the largest component of daily energy expenditure.<sup>bib3838</sup> OVX in WT mice produced a transient trend to decreased resting metabolic rate that was seen at the short- but not at the long-term time point (fig5Figure 5a). Importantly, this reduction was not observed in OVX NPY<sup>-/-</sup> mice (fig5Figure 5a). In fact, resting metabolic rate was significantly increased by OVX in NPY<sup>-/-</sup> mice relative to intact NPY<sup>-/-</sup> controls or sham-operated WT mice at both the short-term and the long-term time points of estrogen deficiency (fig5Figure 5a). This finding indicates that these differences in oxygen consumption or energy expenditure are driven, at least to some extent, by changes in resting metabolic processes.

### **Serum corticosterone levels reduces with simultaneous estrogen and NPY deficiency**

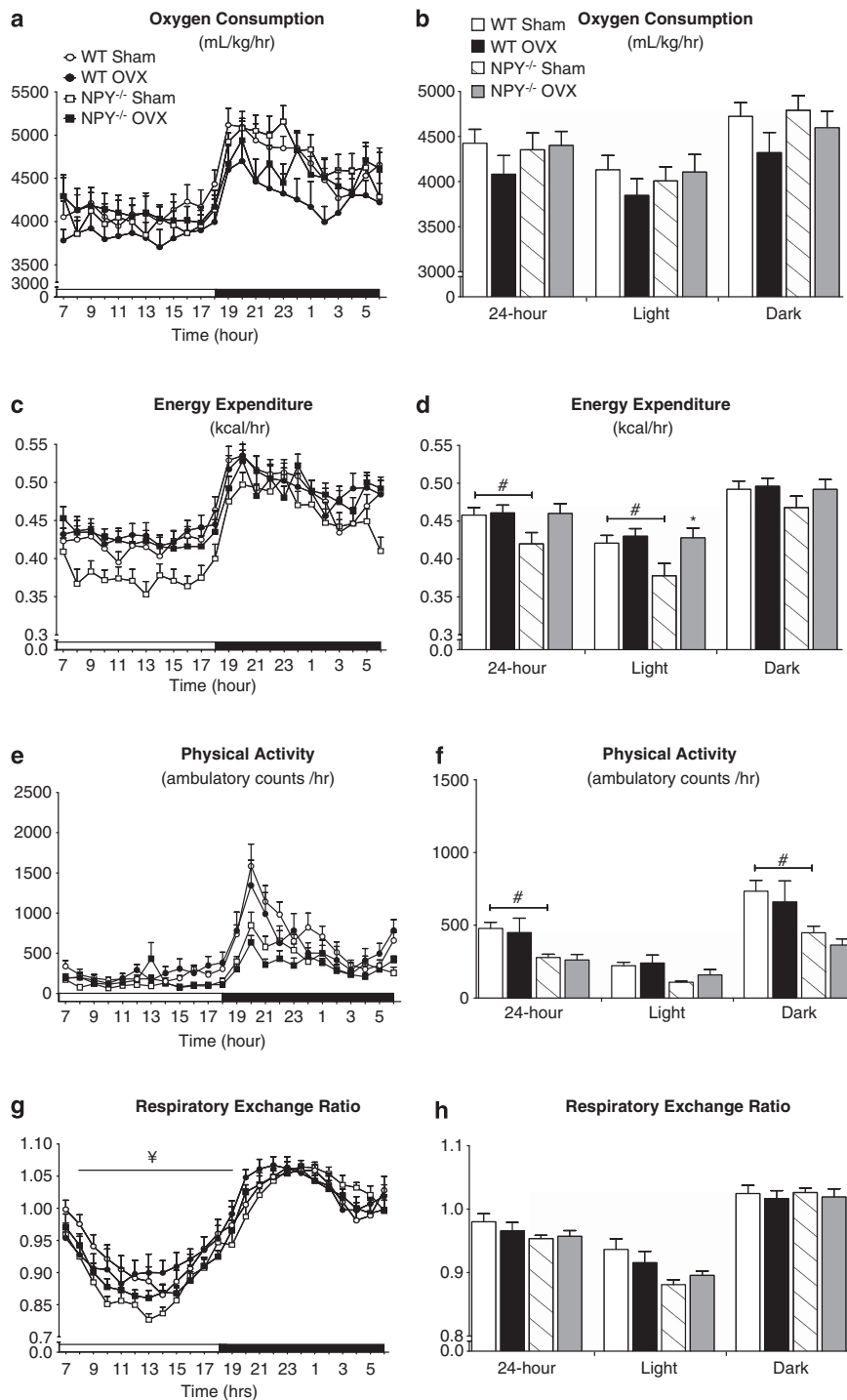
Having seen strong reductions in physical activity in NPY<sup>-/-</sup> compared with WT mice, and knowing that the anxious phenotype of NPY<sup>-/-</sup> mice could contribute to reduced physical activity,<sup>bib3131</sup> serum corticosterone levels were measured. However, OVX NPY<sup>-/-</sup> mice exhibited marked reductions in serum corticosterone levels relative to OVX WT mice, significantly so after short-term estrogen deficiency (fig5Figure 5b).

## **DISCUSSION**

This study reveals that the decreases in oxygen consumption and energy expenditure occurring in response to estrogen deficiency are transient, as these changes were apparent only at 2 weeks, but not at 7 weeks after OVX in mice. NPY is a critical mediator of the short-term decreases in oxygen consumption and energy expenditure induced by estrogen deficiency, because NPY<sup>-/-</sup> mice did not exhibit any such metabolic depression after OVX. This finding extends previous observations that hypothalamic NPY expression is increased in post-menopausal women<sup>bib2828</sup> and transiently increased by OVX in rodents,<sup>bib77, bib2929, bib3030</sup> by demonstrating the functional significance of NPY in mediating metabolic effects of estrogen deficiency. Importantly, NPY ablation led to a significant decrease in whole-body fat mass relative to WT mice at 2 weeks after OVX, suggesting that NPY contributes not only to OVX-induced metabolic depression but also to OVX-induced fat accretion.

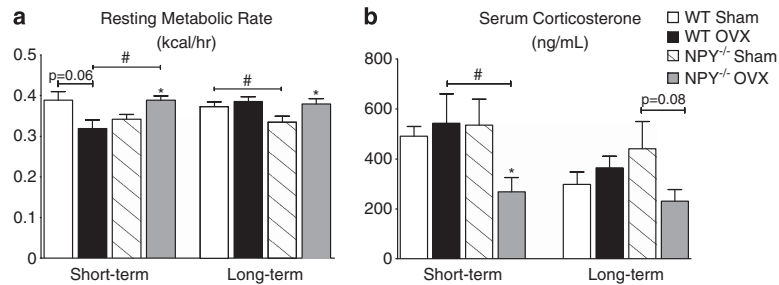
Increases in sympathetic nervous activity and thyroid function with NPY ablation may have contributed to the lack of hypometabolic effects of short-term estrogen deficiency in NPY<sup>-/-</sup> mice. It is known that estrogen deficiency in humans<sup>bib3939</sup> and rodents increases sympathetic activity,<sup>bib4040</sup> as indicated by increased circulating and hypothalamic catecholamine levels. This change might be expected to increase metabolic rate following OVX, as increases in sympathetic activity have been shown to increase metabolic rate in rodents.<sup>bib3939</sup> However, the transient OVX-induced increase in hypothalamic NPY expression in WT animals<sup>bib77, bib2929, bib3030</sup> could have opposed any such hypermetabolic effect, as NPY inhibits sympathetic nervous output as well as thyroid function, with both changes depressing metabolic rate.<sup>bib2727, bib4141, bib4242</sup> In NPY-deficient animals no such OVX-induced increase in hypothalamic NPY expression could occur, nor any consequent metabolic depression, resulting in neither reduction in oxygen consumption nor change in metabolic rate relative to non-OVX WT animals.

Despite abolishing the short-term hypometabolic effect of estrogen deficiency and inducing a transient decrease in whole-body fat mass, NPY ablation did not block the increases in body weight and fat mass seen in response to long-term estrogen deficiency. Adaptations in NPY<sup>-/-</sup> mice may have overcompensated for the lack of NPY, thereby promoting obesity in estrogen deficiency. However, previous studies in NPY<sup>-/-</sup> mice have not revealed any changes in hypothalamic immunoreactivity of possible candidate compensators, namely agouti-related



**Figure 4.** Long-term estrogen deficiency has no effect on energy metabolism in WT or germline NPY knockout (NPY<sup>-/-</sup>) mice. **(a, b)** At 50–54 days after surgery, there was no significant difference between ovariectomized (OVX) and sham-operated WT and NPY<sup>-/-</sup> mice with respect to oxygen consumption. **(c, d)** OVX had no significant effect on energy expenditure in WT mice when measured at 50–54 days post surgery. NPY deficiency in sham-operated mice significantly decreased energy expenditure relative to WT control values, while OVX normalized this difference. **(e, f)** Long-term (50–54 days) estrogen deficiency in OVX WT or NPY<sup>-/-</sup> mice has no significant effect on physical activity, but NPY<sup>-/-</sup> mice showed reduced physical activity compared with WT values. **(g, h)** RER is decreased with NPY deficiency in the light phase. Energy expenditure was adjusted for lean mass by analysis of covariance (ANCOVA). Adjusted energy expenditure was presented at a common lean mass of 16.76 g. Data are means ± s.e.m. of 8–11 mice per group, measured at 50–54 days post-surgery. \**P* < 0.05 for comparisons within genotype. #*P* < 0.05 for comparisons within surgery type. †*P* < 0.05 for the interaction effect between surgery and genotype and ‡*P* < 0.05 for the effect of genotype by repeated measures ANOVA.

protein, pro-opiomelanocortin and cocaine-amphetamine-regulated transcript.<sup>bib4343, bib4444</sup> An alternate explanation for the eventual OVX-induced obesity in NPY<sup>-/-</sup> mice is our finding that these animals were hypermetabolic and hypophagic relative to WT mice. Indeed, rodents placed on calorie restricted diets exhibit increased body fat and decreased lean mass during weight regain because of preferential replenishment of fat stores.<sup>bib4545, bib4646</sup> These changes may be due to adaptive responses to energy deficiency, which have been shown to favor fat accretion at the expense of lean tissues and bone.<sup>bib2727</sup> In keeping with this, NPY<sup>-/-</sup> mice showed no compensatory gain in lean mass as a consequence of the increase in energy deposition as fat and the associated increase in weight.



**Figure 5.** OVX increases resting metabolic rate in NPY deficiency and simultaneously reduces serum corticosterone levels. (a) OVX in WT mice produces a transient decrease in resting metabolic rate at 14–18 days ('short-term') but not at 50–54 days ('long-term') after surgery relative to sham-operated WT mice. In contrast, resting metabolic rate was significantly increased after OVX in NPY<sup>-/-</sup> mice after both short-term and long-term estrogen deficiency. Resting metabolic rate was adjusted for lean mass by analysis of covariance (ANCOVA). Adjusted resting metabolic rate was presented at a common lean mass of 15.34 g during the short-term time point and 16.76 g at the long-term time point. (b) OVX decreased serum corticosterone levels in NPY<sup>-/-</sup> mice after both short-term and long-term estrogen deficiency, with no effect in WT mice. Data are means  $\pm$  s.e.m. of 8–11 mice per group; \* $P < 0.05$  for comparisons within genotype. # $P < 0.05$  for comparisons within surgery type.

Previous studies in humans<sup>bib1919</sup> and in rodents<sup>bib2020, bib4747, bib4848</sup> have shown that estrogen deficiency leads to a decrease in physical activity in association with the decrease in energy expenditure.<sup>bib1919, bib2020, bib4747, bib4848</sup> However, it was not known from those studies whether the associated reduction in energy expenditure was due entirely to this reduction in physical activity, or whether reduced basal metabolic rate may have also contributed. Our study has clearly shown that OVX-induced reductions in oxygen consumption and energy expenditure can occur in the absence of significant reductions in activity, suggesting that estrogen deficiency also reduces basal metabolic processes. Indeed, OVX reduced resting metabolic rate by 18% in WT mice. Further evidence for independent regulation of physical activity and resting metabolic rate is our observation that physical activity was significantly reduced in OVX NPY<sup>-/-</sup> mice despite significantly increased energy expenditure and resting metabolic rate relative to corresponding values in OVX WT animals.

The reduced oxygen consumption and energy expenditure in response to estrogen deficiency raises the possibility that the decrease in energy expenditure observed in women transitioning through menopause<sup>bib1919</sup> may also be transient. If so, then targeting intensive weight management programs to the early period of estrogen deficiency may lead to better prevention of menopause-induced obesity. Circumstantial evidence for this comes from a randomized controlled trial in overweight post-menopausal women aged 50 to 75 years.<sup>bib4949</sup> A 12-month exercise intervention program resulted in significant losses of body weight (1.0%), intra-abdominal fat (8.6 g cm<sup>-2</sup>) and subcutaneous abdominal fat (28.8 g cm<sup>-2</sup>) compared with women randomized to the non-exercise control arm.<sup>bib4949</sup> Furthermore, when the results were stratified by age, women aged 50–59 years exhibited significantly reduced body weight, total body fat and subcutaneous body fat when compared with women aged 70–75 years, in which no significant reductions in these parameters were observed.<sup>bib4949</sup> Taken together, weight

management interventions targeted to the short-term rather than the long-term phase after estrogen deficiency may have greater potential to reduce body weight and fat mass, albeit human studies would be required to investigate this. In summary, our findings demonstrate that the effect of estrogen deficiency to reduce energy expenditure is transient, and that NPY is an important mediator of this effect, possibly via known inhibitory effects of NPY on the sympathetic nervous system and on thyroid function. Despite this effect, long-term NPY deficiency in our NPY<sup>-/-</sup> mice did not prevent OVX-induced weight and fat gain. The relevance of these findings to changes in fat mass in women around the time of menopause remains to be evaluated.

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