Cardiometabolic and reproductive benefits of early dietary energy restriction and voluntary exercise in an obese PCOS-prone rodent model

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Abstract

Polycystic ovary syndrome (PCOS) is one of the most common endocrine-metabolic disorders in women of reproductive age characterized by ovulatory dysfunction, hyperandrogenism and cardiometabolic risk. The overweight-obese PCOS phenotype appears to have exacerbated reproductive dysfunction and cardiometabolic risk. In overweight-obese adult women with PCOS, exercise and energy restricted diets have shown limited and inconsistent effects on both cardiometabolic indices and reproductive outcomes. We hypothesized that an early lifestyle intervention involving exercise and dietary energy restriction to prevent or reduce the propensity for adiposity would modulate reproductive indices and cardiometabolic risk in an obese PCOS-prone rodent model. Weanling obese PCOS-prone and Lean-Control JCR:LA-cp rodents were given a chow diet *ad libitum* or an energy-restricted diet combined with or without voluntary exercise (4 h/day) for 8 weeks. Dietary energy restriction and exercise lowered total body weight gain and body fat mass by 30% compared to free-fed sedentary or exercising obese PCOS-prone animals ($P<0.01$). Energy restriction induced an increase in exercise intensity compared to free-feeding plus exercise conditions. Energy restriction and exercise decreased fasting plasma triglycerides and apoB48 concentrations in obese PCOS-prone animals compared to free-fed and exercise or sedentary groups. The energy restriction and exercise combination in obese PCOS-prone animals significantly increased plasma sex-hormone binding globulin, hypothalamic cocaine-and amphetamine-regulated transcript (CART) and Kisspeptin mRNA expression to levels of the Lean-Control group, and this was further associated with improvements in estrous cyclicity. The combination of exercise and dietary energy restriction when initiated in early life exerts beneficial effects on cardiometabolic and reproductive indices in an obese PCOS-prone rodent model, and this may be associated with normalization of the hypothalamic neuropeptides, Kisspeptin and CART.

Key Words
- PCOS
- energy restriction
- cardiometabolic risk
- endocrine
- estrous cyclicity
- hypothalamic neuropeptides
- CART
- kisspeptin

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Introduction

More than 60% of adolescents and women with polycystic ovary syndrome (PCOS) are overweight or obese, and this adiposity is associated with exacerbation of cardiometabolic risk and reproductive-endocrine dysfunction (Bekx *et al*. 2010, Hart *et al*. 2011, Lim *et al*. 2013). The manifestation of PCOS in overweight-obese adolescents is likely to originate in prepuberty (Diamanti-Kandarakis *et al*. 2007), predisposing individuals to increased cardiometabolic risk, including dyslipidemia and impaired glucose tolerance, leading to early development of type II diabetes and cardiovascular disease (Alexander *et al*. 2009, Fulghesu *et al*. 2010, Sathyapalan & Atkin 2012, Dantas *et al*. 2013). Insulin resistance (IR) is strongly implicated in the etiology of PCOS (Baillargeon & Nestler 2006, Diamanti-Kandarakis & Dunaif 2012) and hyperinsulinemia has been shown to stimulate androgen production in ovarian thecal cells and to reduce hepatic sex-hormone binding globulin (SHBG) synthesis (Barbieri *et al*. 1984, Nestler *et al*. 1991). PCOS patients have a high incidence of IR and this has been shown to be independent of body weight; however, IR is more common and exacerbated in obese PCOS patients (Marcondes *et al*. 2007, Hart *et al*. 2011, Mehrabian *et al*. 2011). Lifestyle interventions focusing on diet and exercise are considered first-line treatments to target reductions in total body weight in the overweight-obese PCOS phenotype (Moran *et al*. 2011, 2013*a*,*b*). Several studies implementing dietary caloric restriction in overweight-obese women with PCOS have shown some improvements in hyperinsulinemia, hyperlipidemia, menstrual cyclicity and fertility (Thomson *et al*. 2008, Hamayeli Mehrabani *et al*. 2010, Altieri *et al*. 2013). Exercise training (lasting 10 min or more per week) may improve cardiometabolic risk factors and reproductive indices in women with PCOS (Giallauria *et al*. 2007, Moran *et al*. 2013*b*). These improvements appear to be independent of reductions in total body weight and adiposity (Sprung *et al*. 2013). Despite evidence that energy-restricted diets and exercise may assist in the effective management of PCOS and cardiometabolic risk, limited research has been conducted on the mechanistic pathways regulated by these interventions in PCOS. Multiple metabolic signals link energy metabolism and reproductive function via activation of neurons located in different hypothalamic nuclei, such as the arcuate, paraventricular, lateral and ventromedial nuclei. The arcuate nucleus (ARC) has been the most studied due to its close proximity to the peripheral blood stream and high responsiveness to peripheral cues that regulate energy balance and reproduction (Myers 2008, Quennell *et al*. 2011). Hypothalamic nuclei expressing the leptin receptor produce different neuropeptides (proopiomelanocortin (POMC), neuropeptide Y (NPY), cocaine-and amphetamine-regulated transcript (CART), Kisspeptin) and peptides such as mammalian target of rapamycin complex 1 (mTORC1). These neuropeptides and peptides have been found to mediate the effects of leptin receptor signaling involved in feeding behaviour, reproduction and fertility (Israel *et al*. 2012, Cravo *et al*. 2013, Diane *et al*. 2013*a*). Of note, the expression of Kisspeptin, mTORC1 and CART in the hypothalamus is downregulated in leptin deficiency, leptin receptor dysfunction and diet-induced leptin resistant rodent models of obesity and infertility (Quennell *et al*. 2011). Indeed, leptin treatment has been shown to increase mTORC1, in turn stimulating the expression of CART and Kisspeptin (Altarejos *et al*. 2008). However, limited data is available on these ARC hypothalamic neuropeptides and peptides in PCOS and how they may mediate effects on feeding behaviour and reproductive outcomes, particularly in response to dietary energy-restriction and exercise in the obese PCOS phenotype.

In overweight-obese adult women with PCOS, exercise and energy-restricted diets have shown limited and inconsistent effects on improving cardiometabolic risk and hormonal and reproductive outcomes (Thomson *et al*. 2008, Moran *et al*. 2011, 2013*b*). This has been proposed to be associated with an inherent resistance to metabolic shifts in metabolism due to abnormalities in the hypothalamic–pituitary–ovarian axis in overweight-obese PCOS patients (Pasquali 2012, Altieri *et al*. 2013). It has been proposed that early intervention in prepuberty, adolescence and young adulthood in overweight-obese individuals at risk or in those that have already developed PCOS may be more effective for long-term improvement in reproductive and cardiometabolic risk factors (Neal *et al*. 2009, Ojaniemi *et al*. 2010). According to the developmental origins of adult disease hypothesis, interventions in childhood or young adulthood compared to adulthood may be more successful in reprogramming metabolic pathways and predisposition to develop chronic disease (Barker 1998). Consistently, studies in rodents have shown that early implementation of energy-restriction diets (at weaning to postnatal day 45) reduces long-term body weight gain and adiposity, even in the presence of genetic proneness to obesity compared to late interventions (postnatal days 45–70) (Schroeder *et al*. 2010). Therefore, it may be suggested

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that early lifestyle interventions to prevent or reduce the propensity for adiposity may mitigate the onset of obesity-associated PCOS phenotypes and cardiometabolic risk. We have previously shown that obese-prone rodents are resistant to engage in voluntary exercise interventions; however, severe dietary energy restriction does induce exercise-food seeking behaviour and promotes cardiometabolic improvements in obese-prone weaning and prepubertal animals (Diane *et al.* 2012, 2013*b*). The aim of this study was to determine the effect of early intervention of an energy-restricted diet combined with exercise on the development of adiposity, cardiometabolic risk and reproductive dysfunction and the relationship to expression of hypothalamic neuropeptides and peptides in an established rodent model prone to develop obesity and a PCOS-like phenotype.

Materials and methods

Animal model

We obtained 24 female JCR:LA-cp rats (18 obese PCOS-prone and six lean control) at weaning, 21 days of age, from our established breeding colony at the University of Alberta (Shi *et al.* 2009, Vine *et al.* 2014). The obese PCOS-prone JCR:LA-cp/cp rats have an autosomal recessive cp gene mutation (a Tyr763Stop mutation for the leptin receptor) that manifests in an absence of functional leptin receptors (Takaya *et al.* 1996). The rats that are homozygous for the cp trait (cp/cp) spontaneously develop PCOS-like characteristics similar to the overweight-obese PCOS phenotype observed in adolescents and women (Shi *et al.* 2009, Shi & Vine 2012, Leblanc *et al.* 2014, Vine *et al.* 2014). The heterozygous cp trait (+/cp) or wildtype (+/+) are Lean-Control animals, and these are both metabolically and physiologically indistinguishable (Shi *et al.* 2009). The autosomal recessive cp gene expression also excludes the possibility of including obese-non-PCOS Control animals in the experimental design (Shi *et al,* 2009, Shi & Vine 2012, Vine *et al.* 2014). At 6 weeks of age the obese PCOS-prone rodents display hyperphagia and increased dietary energy intake, increased adiposity and early cardiometabolic risk compared to Lean-Control animals (Shi *et al.* 2009, Vine *et al.* 2014). At 12 weeks of age, the cp/cp genotype animals spontaneously develop hyperandrogenism, acyclicity and ovarian pathology inclusive of a decreased number of corpora lutea, atretic and cystic-type follicles (Shi *et al.* 2009). In addition, the animals are obese, dyslipidemic and insulin resistant (Shi *et al.* 2009, Vine *et al.* 2014). Rats were housed individually in clear polycarbonate cages (47 cm×27 cm×20 cm) with sterile wood chip bedding in 22±2 °C and a humidity-controlled environment and maintained on a 12 h light:12 h darkness cycle (lights off 0700–1900 h). The care and use of animals were in accordance with Guidelines of the Canadian Council of Animal Care and subject to prior review and approval by the Animal Care and Use Committee: Health Sciences of the University of Alberta.

Exercise apparatus and diet

Wahmann running wheels (1.1 m circumference) with metal side cages (25 cm×15.5 cm×12.5 cm) were used and computerized revolutions/min were recorded as previously described (Diane *et al.* 2012). Animals were fed standard laboratory chow (LabDie 5010 Rodent Diet, PMI Nutrition Intl., Inc., St Paul, MN, USA; energy=3.42 kcal/g). An electronic scale (Sartoris, Goettingen, Germany) was used to measure food and body weight to the nearest gram. Rats were given a fixed amount of food (50 g) at the beginning of the feeding period and the amount of chow remaining after was subtracted from this fixed amount to provide a measure of food intake (g). Subsequently, food intake was converted to calories (food (g)×3.42 kcal/g), yielding a measure of daily energy intake.

Exercise and energy-restriction diet protocol

Weanling obese PCOS-prone rats (cp/cp) aged 3 weeks were randomly assigned to free-feeding non-exercise or sedentary (FF-Sedentary) or free feeding and exercise (FF-Exercise) and dietary energy restriction and exercise (ER-Exercise) groups (n=6 rats per group). The Lean-Control rats were assigned to free feeding and non-exercise or sedentary conditions (Lean-Control) group. In previous studies we have used an energy restriction and non-exercise or sedentary (ER-Sedentary) group (Diane *et al.* 2013*b*), and for the purposes of this manuscript, the data was not included. Food-energy restriction diets were based on daily pair feeding of the obese/PCOS-prone rats to the daily mean amount of food consumed by the Lean-Control group. Animals in the exercise intervention were placed in the wheel running apparatus for 4 h/day with free access to water but no food was provided at this time. This exercise was voluntary in nature compared to forced treadmill exercise to reflect free living conditions observed in the clinical setting and to determine the effect of voluntary exercise in relationship to energy restriction and free-feeding conditions in this rodent model. The FF-Sedentary

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or Lean-Control groups were also placed in the wheel running apparatus for 4 h/day but the wheels were locked. Previous data from our group has shown that dietary early energy caloric restriction plus sedentary behaviour without exercise significantly improves cardiometabolic parameters in young animals in this rodent model (Russell *et al.* 2008, Diane *et al.* 2013*a*). Therefore, in this study a dietary ER-Sedentary group was not included, as the aim was to compare the effect of exercise under free feeding and dietary energy restriction conditions.

Food intake and wheel running distance were recorded daily, and body weight was measured three times a week. Fat and lean body mass were measured weekly using nuclear magnetic resonance (Minispec LF90 Body Composition Analyzer, Bruker, Ontario, Canada). Five weeks following the intervention (60-day-old rats), the estrous cyclicity was measured daily for 21 consecutive days. After 8 weeks of feeding and exercise intervention, a standardized meal tolerance test (MTT) was performed. Briefly, animals were fasted overnight and given a 5.0 g chow meal pellet, and blood was taken using an established tail-snip procedure (Vine *et al.* 2007).

Estrous cyclicity and ovarian morphology assessment

Obese PCOS-prone JCR-LA:cp/cp adult rats have been demonstrated to have abnormal cyclicity or acyclicity as compared to their lean-prone counterparts (Shi *et al.* 2009). Vaginal cytology was examined every day for 21 days (days 60–81 of the intervention and at the same time of day 1300–1500 h). Vaginal smears were collected by introduction and immediate extraction of a small amount of sterilized saline (0.9%) with a micropipette as previously described (Shi *et al.* 2009, McLean *et al.* 2012). The estrous cycle of the rat is 4–5 days and the different stages (metestrous, diestrous, proestrous or estrous) of the estrous cycle were assessed based on appearance and abundance of cells in each vaginal smear sample using microscopy. Metestrous was characterized by the presence of leukocytes, cornified and nucleated epithelial cells; diestrous as predominance of leukocytes and nucleated epithelial cells; proestrous as primarily nucleated epithelial cells; and estrous as an abundance of anucleated cornified cells (Shi *et al.* 2009, McLean *et al.* 2012, Witchel *et al.* 2013).

Ovaries were longitudinally and serially sectioned (4 μm) using three consecutive sections from the mid-ovary and stained with hematoxylin and eosin. All sections were scanned and histologically analysed using an inverted microscope (Zeiss Axio Observer A1, Zeiss Canada Axio, Toronto, Ontario, Canada) by two persons blinded to the identification of ovary. Large pre-ovulatory or cystic follicles may be observed in consecutive slides, therefore transitioning between consecutive slides allows a large follicle to be tracked and therefore not counted twice. The identification of types of ovarian follicles – primary, secondary, tertiary and pre-ovulatory and corpus luteum or albicans – was assessed and quantified, as previously described (Shi *et al.* 2009).

Post-mortem and tissue preparation

After 8 weeks of intervention, rats were fasted overnight (16 h) and anaesthetized with Isoflurane, and blood was taken by cardiac puncture and plasma prepared by centrifugation (3900 ***g***, 10 min). Plasma samples were stored at −80 °C until analysis. The rats were immediately perfused intracardially with ice-cold isotonic saline, and the brain excised and immediately frozen at −80 °C before sectioning, as described below. The ovaries were also excised and stored in 4% paraformaldehyde.

Brain micro-dissection and hypothalamic ARC gene expression

Cryostat (Model tissue Tek II, Miles, Cyrostat, Oshawa, Ontario, Canada) at an operating temperature of −20 °C was used for coronal sections from frozen rat brains. The hypothalamic ARC region was targeted to take punches using Stereotaxic Coordinates (Paxinos & Watson 2005). Punches were stored at −80 °C until further RNA extraction processing. NPY, cocaine- and amphetamine-regulated transcript (CART), melanocortin 3 receptor (Mc3r), Kisspeptin, mTORC1 and GnRH mRNA levels in the ARC were assessed by qPCR method. Total RNA was isolated from frozen ARC tissues using TRIzol (Invitrogen) as described in the manufacturer's protocol and reversed transcribed into cDNA (MMLV reverse transcriptase; Applied Biosystems). Target gene copy number was quantified using the comparative cycle threshold (*C*t) and normalized to the housekeeping gene, Cyclophilin. Data was expressed as a ratio in mRNA expression relative to housekeeping gene control ($2^{-\Delta Ct}$). All assays were performed in duplicate.

Plasma biochemical analyses

Total plasma cholesterol and triglyceride (TG) concentrations were determined enzymatically with kits (Wako, Richmond, VA, USA). Plasma glucose was determined using a glucose oxidase method (Diagnostic Chemical Ltd, Charlottetown, Price Edward Island, Canada) and insulin was assessed by ELISA for rodents (Mercodia,

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Uppsala, Sweden). Total testosterone and SHBG levels were assessed by ELISA for rats (CUSABIO Biotech, Hubei, China). The free androgen index (FAI) was calculated (FAI = 100; total testosterone/SHBG) (Azziz *et al*. 2009). Apolipoproteins (apo) B48 (a marker of intestinal chylomicron (CM)) and B100 (a marker of hepatic VLDL and LDL) were quantified in fasting plasma using a western blotting procedure, SDS–PAGE combined with ECL analysis, as described previously (Vine *et al*. 2007).

Statistical analysis

Results are presented as mean ± S.E.M. Food intake, body weight, body composition and wheel turns (exercise) were analysed by two-way ANOVAs for repeated measures with four experimental groups (or three obese/PCOS-prone groups) as the between subjects factor and days (or weeks) and the within subjects (repeated measures). One-way ANOVAs were used to compare the four experimental groups including the Lean-Control or to compare only the three PCOS-prone groups on dependent measures, followed by *post-hoc* Bonferroni tests for pairwise comparison of means. Pearson correlations were calculated to determine the direction and strength of the relationship among the different physiological and behavioural parameters using only the PCOS-prone rats. All tests and comparisons with $P<0.05$ were considered statistically significant using GraphPad Prism Software version 5.0a (Graphpad Prism, San Diego, CA, USA) and IBM SPSS Statistics Software, version 21.

Results

Body weight, dietary energy intake, exercise and body composition

Body weight (g) and energy intake (kcal/week) in the intervention period is shown in Fig. 1. In obese PCOS-prone rats, the FF-Exercise and FF-Sedentary groups had 1.6-fold increase in body weight gain compared to the Lean-Control group ($P<0.001$, Fig. 1A). In obese PCOS-prone rats, the combination of dietary energy restriction and exercise lowered the total body weight by 33.2% compared to the obese PCOS-prone FF rats in either the sedentary or exercising groups ($P<0.0001$). The ER-Exercise group had a final body weight comparable to the Lean-Control group. Exercise without dietary energy restriction had no significant effect on body weight in obese PCOS-prone rats (Fig. 1A).

Energy intake over the intervention period is shown in Fig. 1B. As expected the PCOS FF-Sedentary consumed significantly more calories than the Lean-Control group ($P<0.001$), indicating a genotype effect. The FF-Exercise group had significantly higher total caloric intake compared to the ER-Exercise group ($P<0.001$), indicating exercise did not have an independent effect to lower energy intake in animals under free-feeding conditions (Fig. 1B).

The percentages of fat mass and lean body mass over the course of the exercise and dietary intervention are shown in Fig. 1. After 8 weeks, obese PCOS-prone rats with free access to food (both FF-Exercise and FF-Sedentary) had a 30% higher fat mass and a 40% lower lean body mass compared to Lean-Control animals ($P<0.001$). Exercise appeared to have no significant effect on body composition in free-fed rats (Fig. 1C and D), where the ER-Exercise rats showed reduced fat mass (16%) and greater lean mass (55%) compared to FF-Sedentary rats ($P<0.01$, Fig. 1C and D). However, the total percentage of fat mass and percentage lean mass in the ER-Exercise group remained significantly higher and lower, respectively, compared to Lean-Control rats at the end of the intervention ($P<0.01$). These results reflect the inherent genetic proneness of this leptin-receptor deficient model to develop increased adiposity (Shi *et al*. 2009).

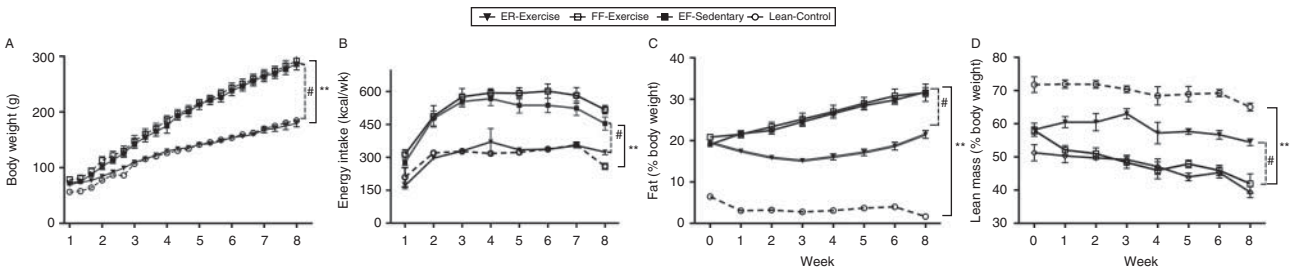

Figure 1
Body weight gain (A), energy intake (B), percent fat mass (C) and percent lean body mass (D) in the JCR:LA-cp obese PCOS-prone rodent model following dietary energy restriction and exercise intervention ($n=6$ rats/group). Data are shown as mean ± S.E.M., $^{**}P<0.01$ Lean-Control compared to FF-Sedentary; $^{\#}P<0.05$ FF-Sedentary compared to ER-Exercise. FF, free feeding; ER, energy restricted.

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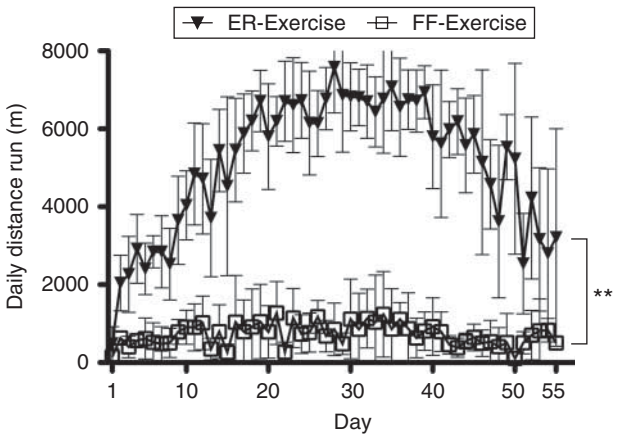

Figure 2
Daily running rate over time in obese PCOS-prone animals following dietary energy restriction and exercise intervention ($n=6$ rats/group). Data are shown as mean ± S.E.M., $^{**}P<0.01$ FF-Exercise compared to ER-Exercise. FF, free feeding; ER, energy restricted.

ER-Exercise rats showed substantially greater wheel running (daily distance) compared to the FF-Exercise animals (Fig. 2, $P<0.01$), consistent with free-feeding conditions impeding motivation or capacity for exercising in this rodent model (Diane *et al.* 2012). The results suggest that energy restriction and exercise initially increased voluntary exercise in response to food restriction, and then exercise intensity was lowered following prolonged food restriction (days 30–40, quadratic trend, $P<0.01$). The ER-Exercise animals also maintained a lower body weight compared to FF-Exercise animals. Pearson's correlation revealed that the total distance run (exercise) by animals was negatively correlated with total caloric intake, body weight gain and fat mass and positively correlated with lean body mass (Table 1). These findings demonstrate that a combination of reduced dietary caloric intake and increased exercise intensity in obese PCOS-prone animals lowered total body weight gain.

Cardiometabolic risk factors

Free-fed obese PCOS-prone groups had significantly higher fasting plasma insulin, glucose, TGs, apoB100 and apoB48 concentrations compared to Lean-Control animals ($P<0.01$), while HDL-cholesterol was significantly lower in all obese PCOS-prone groups ($P<0.01$, Table 2). Notably, the ER-Exercise group showed significantly reduced plasma TG and apoB48 concentrations compared to the FF-Sedentary group ($P<0.01$).

The postprandial glucose response following a MTT, total area under the curve (AUC), was similar between the four experimental groups (Fig. 3A and Table 2). Plasma postprandial insulin response (AUC) was 50% lower in ER-Exercise animals compared to the FF-Sedentary and FF-Exercise groups (Fig. 3B and Table 2). Moreover, the combination of exercise and energy restriction significantly reduced the insulin-to-glucose ratio to a level comparable to the Lean-Control, while voluntary exercise under free-feeding conditions (FF-Exercise) showed no effect on the insulin-to-glucose ratio (Fig. 3C). Plasma leptin concentrations were 20% lower in the FF-Exercise group and 30% lower in the ER-Exercise group compared to FF-Sedentary rats, although this did not reach significance (Table 2).

Endocrine, estrous cyclicity and ovarian morphology parameters

Serum total testosterone concentrations were not significantly altered by exercise or dietary energy restriction in

Table 1 Pearce correlations between physiological and biochemical parameters in the JCR:LA-cp obese PCOS-prone rodent model following dietary and exercise intervention

Parameters	BWG (g)	Total intake (kcal)	Exercise (m)	Fat mass (%)	Lean mass (%)	Insulin (ng/ml)	Total cholesterol (mg/dl)	TG (mg/dl)	ApoB48 (mg/dl)	SHBG (ng/ml)	CART (mRNA)
Intake (kcal)	0.88[a]	–									
Exercise (m)	−0.90[a]	−0.86[a]	–								
Fat mass (%)	0.90[a]	0.87[a]	−0.82[a]	–							
Lean mass (%)	−0.88[a]	−0.81[a]	0.77[a]	−0.78[a]	–						
Insulin (ng/ml)	0.27	0.45	−0.08	0.32	−0.26	–					
SHBG (ng/ml)	−0.53[b]	−0.47[b]	0.54[b]	−0.58[b]	0.44[b]	−0.15	−0.22	−0.50[b]	−0.50[b]	–	
CART (mRNA)	−0.67[a]	−0.60[b]	0.66[b]	−0.72[a]	0.65[a]	−0.04	−0.05	−0.37	−0.39	0.57[b]	–
Kiss (mRNA)	−0.68[a]	−0.52[b]	0.66[a]	−0.66[a]	0.66[a]	0.32	−0.06	−0.35	−0.45	0.55[b]	0.74[a]

BWG, body weight gain; TG, triglycerides, ApoB48, apolipoprotein B 48; SHBG, sex hormone binding globulin; NPY, neuropeptide Y, CART, Cocaine and amphetamine regulated transcript; Kiss, kisspeptin.
[a]Correlation is significant at the 0.01 level.
[b]Correlation is significant at the 0.05 level.

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Table 2 Body weight gain, biochemical parameters and mTORC1 mRNA expression in the JCR:LA-cp obese PCOS-prone rodent model following dietary restriction and exercise intervention

Parameters	Lean-Control ($n=6$)	FF-Sedentary ($n=6$)	FF-Exercise ($n=6$)	ER-Exercise ($n=6$)
Body weight gain (g)	114.65±2.66^a	212.90±10.73^b	219.73±7.90^b	117.06±6.11^a
Fasting glucose (mg/dl)	131.6±9.8^a	179.2±6.1^b	184.5±20.0^b	152.2±6.9^b
Glucose AUC	6447±582.2^a	7329±601.7^a	6710±978.4^a	5709±734.3^a
Fasting insulin (ng/ml)	0.39±0.11^a	4.24±1.20^b	6.11±1.47^b	4.20±1.43^b
Insulin AUC	22.71±2.84^a	552.9±130.2^b	513.4±118.1^b	214.5±36.03^b
HOMA-IR	3.40±0.94^a	46.30±10.86^b	67.98±16.65^b	39.18±13.32^b
Leptin (ng/ml)	20.36±11.87^a	69.50±7.51^b	53.78±18.55^b	46.39±8.44^b
TG (mg/dl)	29.42±6.04^a	798.1±180.0^b	560.9±93.77^b	207.1±21.00^c
Total cholesterol (mg/dl)	71.20±4.03^a	130.0±24.87^b	107.7±9.35^b	79.29±13.29^b
HDL (mg/dl)	33.27±2.13^a	16.97±1.99^b	23.13±2.83^b	23.23±2.76^b
ApoB48 (μg/ml)	55.63±3.23^a	513.9±69.47^b	386.6±51.76^b	273.4±32.24^c
ApoB100 (μg/ml)	457.5±24.46^a	1831.0±198.5^b	1620.0±357.6^b	2559.0±273.1^c
mTORC1 mRNA	5.55±0.34^a	4.19±0.88^a	5.33±0.62^a	6.93±1.17^a

Values are mean±S.E.M. The superscripts a, b and c are used to denote statistical differences among groups. Within the same row, means with different superscripts (corresponding to the results from follow-up tests using one-way ANOVAs with *post-hoc* comparisons) are significantly different ($P<0.05$). mTORC1, mammalian target of rapamycin complex 1.

obese PCOS-prone rats, and there was no difference in total testosterone between obese PCOS-prone and Lean-Control animals, as shown in Table 3. Plasma SHBG concentrations were significantly lower in FF-Sedentary rats and appeared to be normalized in ER-Exercise rats compared to Lean-Control, indicating that exercise and food restriction in combination may improve SHBG levels. As shown in Table 1, serum SHBG concentrations were positively correlated with lean body mass ($r=0.44$, $P<0.05$) and inversely correlated with total body weight gain ($r=-0.53$, $P<0.05$) and fat mass ($r=-058$, $P<0.05$). In addition, serum SHBG levels were negatively correlated with both plasma TGs ($r=-050$, $P<0.05$) and apoB48 concentrations ($r=-0.50$, $P<0.05$; Table 1).

Feeding regime and exercise altered the estrous cyclicity of obese PCOS-prone rats. FF-Sedentary rats had a lower total number of cycles, and the ER-Exercise group showed a normalization of estrous cyclicity compared to Lean-Controls (Fig. 4). The total number of cycles in the FF-Exercise group did not significantly differ from either the Lean-Control or ER-Exercise groups. Also no differences were observed in the number of primary, secondary, tertiary follicles or pre-ovulatory follicles between obese PCOS-prone groups and the Lean-Control group (Table 3). No corpus luteum (a marker of ovulation) was observed in the FF-Sedentary group, while corpus lutea were observed in the ovaries of the FF-Exercise and ER-Exercise groups (Table 3). Interestingly, lipid droplets were observed in 60% of the ovaries in FF-Sedentary animals, consistent with our previous report (Shi *et al.* 2009), and lipid droplets were absent in the ER-Exercise group (data not shown).

Hypothalamic ARC neuropeptide and peptide expression

ARC neuropeptide expression levels after an 8-week period of intervention are shown in Figs 5 and 6. NPY mRNA expression was ~25% greater in all obese PCOS-prone animals compared to Lean-Controls ($P<0.05$; Fig. 5A), consistent with the leptin-receptor deficiency in this animal model (Shi *et al.* 2009, Diane *et al.* 2013*a*,*b*). Exercise and energy restriction had no significant effect on NPY mRNA expression. FF-Sedentary rats displayed a 50% lower mRNA expression of Kisspeptin compared to Lean-Controls ($P<0.05$; Fig. 5B). Notably, the ER-Exercise group had increased Kisspeptin mRNA similar to the levels of the Lean-Control group. The mTORC1 mRNA expression levels were not different between the experimental groups (Table 2). As expected, the FF-Sedentary group had lower CART and POMC) mRNA expression compared to Lean-Controls (Fig. 6A and C), while the neuropeptide receptor expression for Mc3r and Mc4r did not differ between these groups (Fig. 6B and D). Dietary energy restriction and exercise had no effect on POMC and Mc4r mRNA expression but significantly increased CART and Mc3r mRNA expression in the obese PCOS-prone rats ($P<0.05$; Fig. 6).

Serum SHBG concentrations but not total testosterone were positively correlated with both CART and Kisspeptin (Table 1). In addition, SHBG, CART and Kisspeptin mRNA expression were negatively correlated with body weight, caloric intake and percent fat mass, and positively correlated with lean body mass and exercise (total running, m), consistent with the interrelationship of these pathways with dietary intake and energy metabolism (Table 1).

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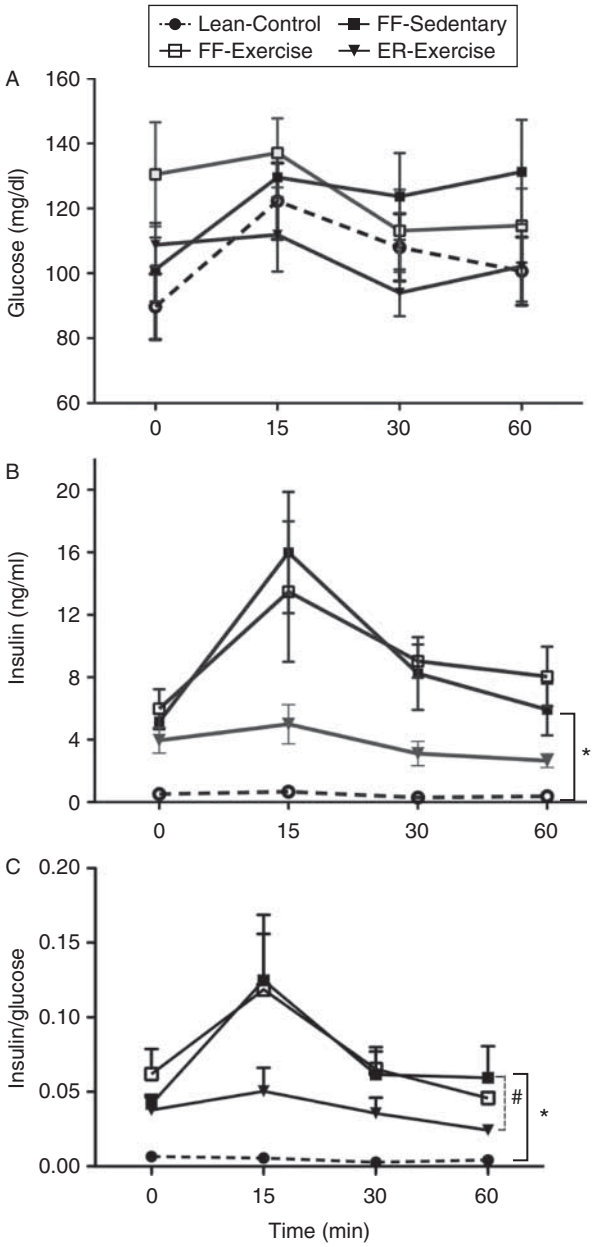

Figure 3
The postprandial response in plasma glucose (A), insulin (B) and insulin/glucose ratio (C) following a meal tolerance test the JCR:LA-cp obese PCOS-prone rodent model following dietary and exercise intervention ($n=6$ rats/group). Data are shown as mean ± S.E.M. $^{*}P<0.05$ Lean-Control compared to FF-Sedentary; $^{\#}P<0.05$ FF-Sedentary compared to ER-Exercise.

Discussion

The results of this study have shown that a combination of a dietary energy restriction and exercise intervention at an early developmental age, post-weaning, can favourably modulate the development of adiposity, cardiometabolic risk and reproductive function in this obese PCOS-prone rodent model. Dietary energy restriction and exercise intervention were further associated with normalization of the ARC mRNA expression of CART, Mc3r and Kisspeptin in this rodent model. These early interventions suggest a potential resetting of the hypothalamic regulation of feeding, energy metabolism and reproductive-gonadal axis. In terms of adiposity, early dietary energy restriction or normalizing energy intake similar to Lean-Control animals, combined with voluntary exercise, reduced total body weight gain in the obese PCOS-prone genotype. These results are consistent with our previous observations in the male obese and female obese PCOS-prone rodent model in which dietary energy restriction alone and/or with voluntary exercise (wheel running) reduced body weight gain and adiposity in young rather than older animals (Diane *et al.* 2012, 2013*a*,*b*). An ER-Sedentary group was not included in this study based on our previous findings that dietary energy restriction alone and a combination of exercise and dietary energy restriction reduce body weight to the same extent (Diane *et al.* 2013*b*). Interestingly, despite the decrease in body weight gain and percentage of fat mass following exercise and dietary energy restriction, the body composition of the obese PCOS-prone animals remained predisposed toward adiposity compared to Lean-Controls. Our results are consistent with Schroeder *et al.* (2010), showing that early caloric restriction at weaning, without exercise, in genetically predisposed Otsuka Long-Evans Tokushima Fatty (OLETF) rats can reduce long-term body weight gain and adiposity. Our results also highlight that the presence of an inherent leptin-receptor defect is persistent, and despite reduced caloric intake, animals remain predisposed to develop adiposity. Collectively, these results may highlight the importance of early dietary energy restriction and exercise intervention in those genetically prone or with familial history of obese proneness. Indeed, the JCR:LA-cp obese PCOS-prone animal model represents a 'thrifty gene' phenotype in which animals accumulate adipose stores despite reduced dietary energy intake (Diane *et al.* 2012, 2013*a*). Leptin receptor ablation or dysfunction has been associated with reduced thermogenesis (Simonds *et al.* 2012). Decreased thermogenesis as a consequence of leptin-receptor deficiency in the ER-Exercise group may explain the persistent adiposity observed despite reduced energy intake similar to that of lean-free-fed control animals.

Another significant finding of this study is that dietary energy restriction promoted a sevenfold increase in the rate of exercise activity or distance run by ER-Exercise

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Table 3 Endocrine profile and ovarian morphology assessment in the JCR:LA-cp obese PCOS-prone rodent model of following dietary restriction and exercise intervention

Parameters	Lean-Control ($n=6$)	FF-Sedentary ($n=6$)	FF-Exercise ($n=6$)	ER-Exercise ($n=6$)
Total testosterone (ng/ml)	0.37 ± 0.04^{a}	0.27 ± 0.06^{a}	0.29 ± 0.03^{a}	0.39 ± 0.06^{a}
SHBG (ng/ml)	468.00 ± 19.30^{a}	417.70 ± 7.21^{b}	450.50 ± 7.56^{b}	472.80 ± 8.63^{c}
FAI	0.079 ± 0.008^{a}	0.065 ± 0.006^{a}	0.064 ± 0.006^{a}	0.084 ± 0.012^{a}
Primary follicles	2.57 ± 0.87^{a}	0.67 ± 0.39^{a}	0.50 ± 0.21^{a}	1.40 ± 0.42^{a}
Secondary follicles	1.86 ± 0.60^{a}	2.50 ± 0.48^{a}	1.17 ± 0.29^{a}	2.40 ± 0.66^{a}
Tertiary follicles	3.57 ± 1.11^{a}	4.67 ± 1.57^{a}	5.67 ± 1.64^{a}	3.20 ± 0.66^{a}
Pre-ovulatory	1.00 ± 0.38^{a}	1.33 ± 0.32^{a}	1.83 ± 1.01^{a}	2.00 ± 0.72^{a}
Atretic follicles	3.14 ± 1.10^{a}	6.33 ± 1.53^{a}	6.33 ± 1.93^{a}	3.40 ± 0.96^{a}
Corpus luteum	0.86 ± 0.60	0.00	0.50 ± 0.31	0.60 ± 0.54
Corpus albicans	2.00 ± 0.49^{a}	4.83 ± 1.37^{a}	2.00 ± 0.53^{a}	4.20 ± 1.24^{a}

Values are mean$\pm$S.E.M. The superscripts a, b and c are used to denote statistical differences among groups. Within the same row, means with different superscripts (corresponding to the results from follow-up tests using one-way ANOVA with *post-hoc* comparisons) are significantly different ($P<0.05$).

animals compared to the FF-Exercise animals. We have observed similar findings in the male genotype of this rodent model (Diane *et al.* 2012) in which increased exercise activity under energy restriction conditions appears to promote behaviours associated with food foraging and food-related travel (Mistlberger *et al.* 2006). Furthermore, the total distance run by animals was found to be negatively correlated with total food intake in ER-Exercise animals, suggesting the increase in exercise activity stimulated an increase in food-seeking travel behaviour, consistent with previous observations (De Rijke *et al.* 2005, Diane *et al.* 2012).

Cardiometabolic risk parameters in the obese PCOS-prone animals were shown to improve with both exercise and dietary energy restriction. Plasma TG, cholesterol and apoB48 concentrations were lowered and HDL cholesterol was increased in the ER-Exercise group. These parameters tended to improve in the FF-Exercise group (TG (−30%), apoB48 (−28%) and apoB100 (−11.5%)) but did not reach statistical significance compared to the FF-Sedentary group, indicating that a low exercise level in free-feeding obese PCOS animals may potentiate improvements in blood lipids if intervened for a longer duration. These findings are consistent with clinical reports that show low to moderate physical activity improves dyslipidemia in women with PCOS in the absence of changes in body weight and body composition (Moran *et al.* 2013*a*,*b*, Sprung *et al.* 2013) The obese PCOS-prone animals exhibit severe IR (Shi *et al.* 2009). ER-Exercise tended to reduce (although not significantly) fasting plasma insulin (22%) and total insulin secretion (50%) in response to a meal challenge compared to FF-Sedentary animals. This modest improvement in insulin sensitivity may be mediated by the effects of exercise on insulin signaling in skeletal muscle (Bradley *et al.* 2008). Furthermore this animal model, like clinical PCOS phenotypes, is resistant to dietary and/or exercise improvements in metabolism, thus an 8-week intervention may not be adequate to facilitate marked shifts in insulin-glucose metabolism (Pasquali *et al.* 2011, Altieri *et al.* 2013).

In addition to beneficial effects on body composition, blood lipids and insulin metabolism, exercise combined with energy restriction positively impacted reproductive indices in these young obese PCOS-prone animals. The exercise-dietary energy restriction intervention commenced when animals were 3 weeks old (at weaning) and ended when animals were 11 weeks of age (before adulthood), suggesting that early intervention when compared to late intervention (Diane *et al.* 2013*b*) is more effective in terms of reproductive improvements in this obese PCOS-prone model. Also, compared to previous studies from our group and others, the findings from this current study showed that the combination of energy restriction and voluntary exercise mediates beneficial effects on metabolic and reproductive outcomes in this obese PCOS-prone rodent model compared to dietary energy restriction alone (Bronson 1987, Russell *et al.* 2008, Diane *et al.* 2013*a*,*b*). Our findings that plasma total testosterone, FAI and ovarian follicular morphology were not statistically different between experimental groups are a reflection of the use of younger animals in this PCOS-prone model, as these animals do not display the full PCOS phenotype until adulthood (>12 weeks) (Shi *et al.* 2009). However, SHBG concentrations were lower, and altered cyclicity was observed in these young obese PCOS-prone animals compared to Lean-Controls. Indeed, in these young pre-adulthood animals, dietary energy restriction and exercise improved estrous cyclicity and SHBG

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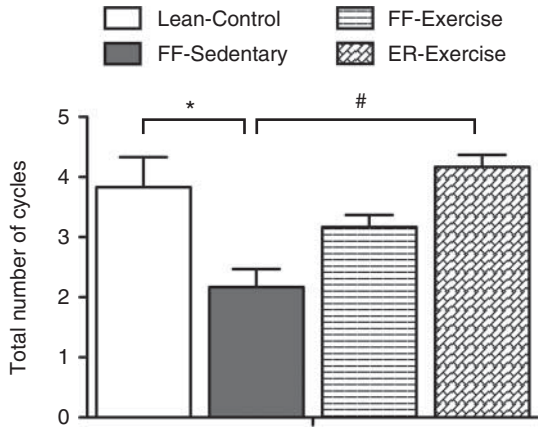

Figure 4
Estrous cyclicity in the JCR:LA-cp obese PCOS-prone rodent model following dietary and exercise intervention ($n=6$ rats/group). Data are shown as mean ± S.E.M. $^{*}P<0.05$ Lean-Control compared to FF-Sedentary; $^{\#}P<0.05$ FF-Sedentary compared to ER-Exercise. FF, free feeding; ER, energy restricted. Estrous cyclicity was assessed by vaginal cytology for each rat for 21 consecutive days at the end of the intervention, days 60–81.

concentrations. Although not measured in this study, improvements in cardiometabolic and ovarian function following dietary energy restriction and exercise may also be related to favourable effects on inflammatory pathways in conditions of obesity, IR and PCOS (Giallauria *et al.* 2008, Ojeda-Ojeda *et al.* 2013, Gower & Goss 2015).

Despite the potential benefits of combining dietary energy restriction and exercise on reproductive function in obese PCOS phenotypes, the central molecular mechanisms have remained unclear. The hypothalamic regulation of feeding behaviour and reproductive function are intimately linked with leptin metabolism and the secretion of neuropeptides (Okamura *et al.* 2013). The ARC neuropeptides CART and Kisspeptin are secreted in response to leptin-receptor signaling to modulate satiety and reproductive function (Israel *et al.* 2012, Cravo *et al.* 2013). Previous reports have revealed that decreases in hypothalamic Kisspeptin and CART mRNA expression are associated with reproductive dysfunction (Witchel *et al.* 2013). Indeed, obese PCOS-prone rats (FF-Sedentary) were shown to have reproductive impairment associated with reduced CART, Kisspeptin and mTORC1 mRNA expression. We also observed a positive correlation between plasma SHBG levels and both CART and Kisspeptin mRNA expression in the ARC of the obese PCOS-prone animals. A reduction in the mRNA expression of these genes is thought to reflect reduced activation of CART and KISS1 genes to signal secretion of these neuropeptides. This appears to be also associated with impaired leptin-receptor signaling in obese PCOS-prone animals, particularly the FF-Sedentary group. Consistently, total body weight gain and percent fat mass were negatively correlated with Kisspeptin and CART mRNA expression in the obese PCOS-prone rats. Interestingly, these neuropeptides increased to a level similar to that of Lean-Control animals in response to exercise and dietary energy restriction, which possibly suggests an increased activation of the associated neurons in the ARC. CART, Kisspeptin and mTORC1 expressions were not affected under free feeding and low exercise conditions in obese PCOS-prone animals, suggesting that caloric restriction together with high-level physical activity upregulated these neuropeptides (CART and Kisspeptin) and peptide (mTORC1) in the ER-Exercise group. The Kisspeptin mRNA level has also been shown to be reduced following severe food restriction in rodent models (Castellano *et al.* 2005); however, our obese PCOS-prone model has an

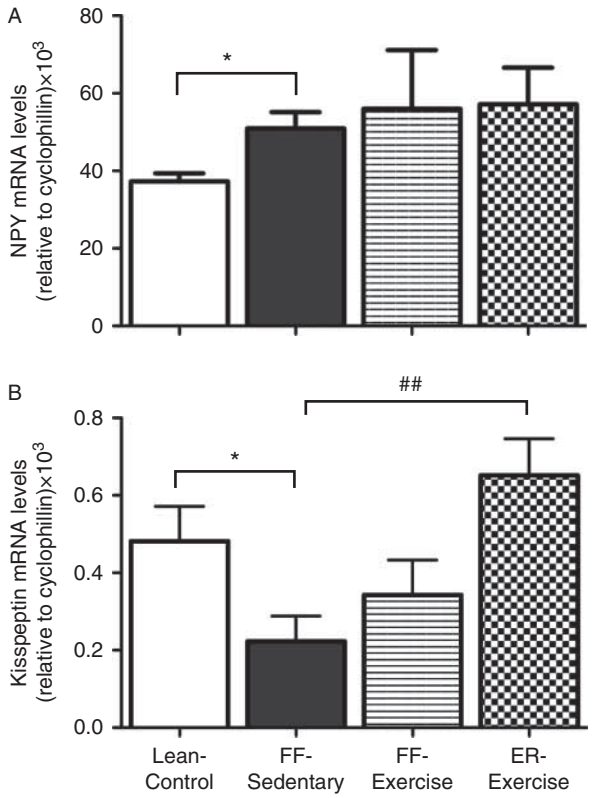

Figure 5
Hypothalamic arcuate nucleus mRNA expression of neuropeptides (A) NPY and (B) Kisspeptin in the JCR:LA-cp obese PCOS-prone rodent model following dietary and exercise intervention ($n=6$ rats/group). Data are shown as mean ± S.E.M., $^{*}P<0.05$ indicates a genotype effect (Lean-Control compared to FF-Sedentary), $^{\#\#}P<0.05$ FF-Sedentary vs ER-Exercise. Target gene expression was normalized to a reference gene (cycophilin). NPY, neuropeptide Y; Kiss, kisspeptin.

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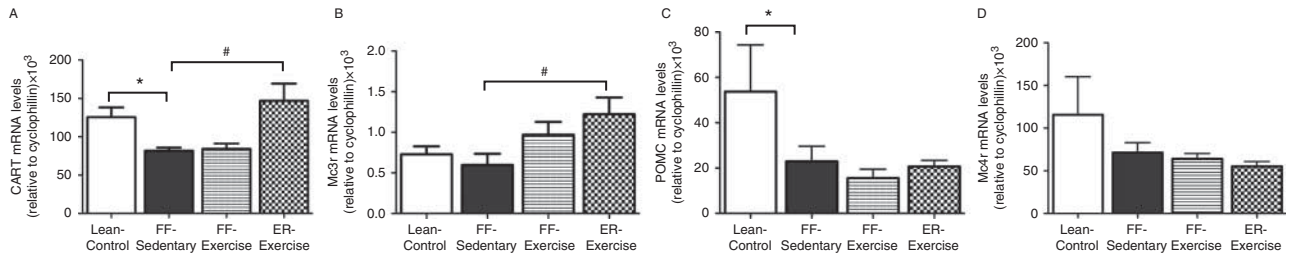

Figure 6

Hypothalamic arcuate nucleus mRNA expression of neuropeptides (A) CART, (B) Mc3r, (C) POMC and (D) Mc4r in the JCR:LA-cp obese PCOS-prone rodent model following dietary and exercise intervention ($n=6$ rats/group). Data are shown as mean ± S.E.M., $^{*}P<0.05$ indicates a genotype effect (Lean-Control compared to FF-Sedentary), $^{\#}P<0.05$ FF-Sedentary vs ER-Exercise. Target gene expression was normalized to a reference gene (cyclophilin). CART, Cocaine and amphetamine regulated transcript; Mc3r, melanocortin receptor 3; Mc4r, melanocortin receptor 4; POMC, proopiomelanocortin. FF, free feeding; ER, energy restricted.

inherent leptin-receptor defect that may cause a part inactivation of ARC neurons and consequently reduced Kisspeptin mRNA expression. In this study we implemented food restriction by pair feeding, which stimulated exercise activity and reduced percentage body fat mass, rather than severe starvation conditions used in other studies (Castellano *et al.* 2005). The conditions used may be synergistically acting to increase the secretion of CART and Kisspeptin to improve reproductive indices (Fuqua & Rogol 2013). Moreover, our results show that Mc3r but not Mc4r mRNA was increased in the ER-Exercise group and this was associated with improved estrous cyclicity. This finding is consistent with the role of the α-MSH pathway in reproduction through Mc3r, where mice lacking Mc3r display subfertility (Begriche *et al.* 2011). Furthermore, dietary energy restriction mediates leptin-mediated onset of puberty and this is reversed by the use of a Mc3r antagonist (Roa 2013). One of the limitations of this study is that we were unable to end each animal on the same day of the estrous cycle due to the variability in normal cycles and the presence of acyclicity or arrested cycles in obese PCOS-prone animals, and this may have impacted the hypothalamic neuropeptide mRNA expression. Future studies to characterize hypothalamic neuropeptide mRNA expression at different stages of the estrous cycle using a semi-quantitative method (*in situ* hybridization) in the ARC would be useful in this obese PCOS-prone animal model but were beyond the scope of the present study.

The findings from this study demonstrate that early intervention at weaning in predisposed obese PCOS-prone animals can modulate hypothalamic regulatory pathways to improve adiposity, cardiometabolic and reproductive indices. The use of a voluntary wheel running protocol (which occurs under non-stressful conditions) as opposed to enforced treadmill running was used in this study, reflecting a measure of 'self-motivation' to exercise and therefore may best mimic voluntary exercise in the clinical setting (Uysal *et al.* 2014). To our knowledge, studies using treadmill-enforced running have not been undertaken in weaning animals that are PCOS prone. However, these studies would be warranted to determine if there are additional beneficial effects of exercise in combination with dietary energy restriction in PCOS-prone animal models, as observed in our previous studies (Diane *et al.* 2013*b*). In adulthood (>12 weeks of age), when animals have developed obesity and a PCOS-like phenotype, the use of enforced treadmill exercise may be more effective to improve cardiometabolic and reproductive indices. Similarly, in the clinical setting strong encouragement of high-intensity exercise may provide additional health benefits to dietary caloric restriction (Altieri *et al.* 2013, Moran *et al.* 2013*a*,*b*). The findings of this current study may further translate to lifestage-epigenetic modulation of the hypothalamic–pituitary–gonadal axis via dietary energy restriction and exercise programs to target young adolescent girls diagnosed with PCOS. Furthermore, in prepubertal or adolescents predisposed to the development of PCOS associated with obesity, early intervention to address dietary energy intake and exercise habits may prevent the development and onset of PCOS in these individuals. This in turn may prevent long-term cardiometabolic risk and adverse reproductive and fertility outcomes associated with PCOS (Alexander *et al.* 2009, Sathyapalan & Atkin 2012).

In conclusion, our finding shows for the first time that lifestyle interventions that combine dietary energy restriction and regular exercise initiated early in life appear to be an efficacious strategy to prevent or ameliorate the adverse cardiometabolic and reproductive outcomes associated

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with a predisposition to develop obesity and PCOS. The molecular mechanism underlying these effects appears to be mediated through the modulation of the hypothalamic neuropeptides Kisspeptin, CART and Mc3r. The translation of these findings to the clinical setting suggest early intervention with dietary energy restriction and exercise in young adolescents with PCOS or in prepubertal children at risk of PCOS may ameliorate the development of aberrant reproductive-endocrine and cardiometabolic outcomes via modulation of neuropeptides in the hypothalamic–pituitary–gonadal axis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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