Abbreviations

AGRP: Agouti-related protein; ATGL: adipocyte triglyceride lipase; ARC: arcuate nucleus; AZGP1: α(2)-zinc-glycoprotein 1; CPT-1: carnitine palmitoyltransferase-1; Ex-4: exendin-4; FASN: fatty acid synthase;FOXO1: Forkhead box protein O 1; GLP-1: glucagon-like peptide-1; HFD: high-fat diet; IUGR: intrauterine growth restriction; MC4R: melanocortin 4 receptor; NPY: neuropeptide Y ; NPY Y1R: Neuropeptide Y1 receptor; Ob-Rb: active lepton receptor; P: postnatal day; POMC: pro-opiomelanocortin; PGC1α: peroxisome proliferator activated receptor gamma coactivator 1α; PPARγ: peroxisome proliferator-activated receptor γ; SREBP: sterol regulatory element binding protein.; SOCS: suppressor of cytokine-signaling; STAT: signal transducer and activator of transcription.

Short term exendin-4 treatment reduces markers of metabolic disorders in female offspring of obese rat dams

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Abbreviations

AGRP: Agouti-related protein; ATGL: adipocyte triglyceride lipase; ARC: arcuate nucleus; AZGP1: α(2)-zinc-glycoprotein 1; CPT-1: carnitine palmitoyltransferase-1; Ex-4: exendin-4; FASN: fatty acid synthase;FOXO1: Forkhead box protein O 1; GLP-1: glucagon-like peptide-1; HFD: high-fat diet; IUGR: intrauterine growth restriction; MC4R: melanocortin 4 receptor; NPY: neuropeptide Y ; NPY Y1R: Neuropeptide Y1 receptor; Ob-Rb: active lepton receptor; P: postnatal day; POMC: pro-opiomelanocortin; PGC1α: peroxisome proliferator activated receptor gamma coactivator 1α; PPARγ: peroxisome proliferator-activated receptor γ; SREBP: sterol regulatory element binding protein.; SOCS: suppressor of cytokine-signaling; STAT: signal transducer and activator of transcription.

Abstract

Objectives: Maternal obesity imposes significant health risks in the offspring including diabetes and dyslipidemia. We previously showed that the hypoglycaemic agent exendin-4 (Ex-4) administered from weaning can reverse the maternal impact of 'transmitted disorders' in such offspring. However daily injection for six-weeks was required and the beneficial effect lapsed upon drug withdrawal. This study aimed to investigate whether short term Ex-4 treatment during suckling in a rodent model can reverse transmitted metabolic disorders due to maternal obesity.

Methods: Maternal obesity was induced in female Sprague Dawley rats by high-fat diet feeding for 6 weeks, throughout gestation and lactation. Female offspring were treated with Ex-4 (5μg/kg/day) between postnatal day (P) 4 and 14. Female offspring were harvested at weaning (P20). Lipid and glucose metabolic markers were measured in the liver and fat. Appetite regulators were measured in the plasma and hypothalamus.

Results: Maternal obesity significantly increased body weight, fat mass, and liver weight in the offspring. There was an associated inhibition of peroxisomal proliferator activated receptor gamma coactivator 1α (PGC1α), increased fatty acid synthase (FASN) expression in the liver, and reduced adipocyte triglyceride lipase (ATGL) expression. It also increased the plasma gut hormone ghrelin and reduced glucagon-like peptide-1. Ex-4 treatment partially reversed the maternal impact on adiposity and impaired lipid metabolism in the offspring, with increased liver PGC1α and inhibition of FASN mRNA expression. Ex-4 treatment also increased the expression of a novel fat depletion gene a2-zinc-glycoprotein 1 in the fat tissue.

Conclusion: Short term Ex-4 treatment during the suckling period significantly improved the metabolic profile in the offspring from the obese mothers at weaning. Long-term studies are needed to follow such offspring to adulthood to examine the sustained effects of Ex-4 in preventing the development of metabolic disease.

Key words: adiposity, lipid metabolism, leptin sensitivity, PGC-1α, AZGP1

Introduction

The global obesity epidemic is associated with a dramatic rise in the rate of childhood obesity. Epidemiological and animal studies have established that maternal obesity plays a significant role in the increased incidence of childhood obesity and the future risk of obesity and obesity related chronic diseases, including diabetes and cardiovascular disorders (Bayol *et al.*, 2005; Samuelsson *et al.*, 2007; Chen & Morris, 2009; Bayol *et al.*, 2010).

The hypothalamic neuronal systems that regulate appetite and energy homeostasis are not mature in newborn rats, with development continuing until weaning (Pinto *et al.*, 2004; Bouret & Simerly, 2006). Specifically between postnatal days (P) 4–14, there is a marked increase in serum levels of the hormone leptin (called the 'leptin surge'). This is believed to support the post-natal development and maturation of hypothalamic neuronal projections between nuclei involved in the regulation of energy homeostasis (Ahima *et al.*, 1998; Bouret *et al.*, 2004). Leptin binds to its active form of the receptor Ob-Rb, followed by a phosphorylation of its downstream signal transducer and activator of transcription (STAT)3 leading to nuclear gene transcription. Any homeostatic disturbances during the 'leptin surge' period can alter the neural development resulting in metabolic disorders (e.g., obesity, hyperlipidemia), as observed in leptin-deficient *ob/ob* mice or pups of obese dams with an abnormally high level of blood leptin (Pinto *et al.*, 2004; Yura *et al.*, 2005; Bouret & Simerly, 2006; Kirk *et al.*, 2009). We have shown that maternal obesity prior to gestation changes the hypothalamic appetite stimulator neuropeptide Y (NPY) expression (Chen *et al.*, 2008), and impairs NPY production in response to both hypo- and hyperglycaemia (Chen & Morris, 2009; Chen *et al.*, 2014a). Dysregulation of NPY production is closely linked to hyperphagia during the suckling period and obesity at weaning in such offspring (Chen & Morris, 2009). Additional nutrient influx during the suckling period exacerbates metabolic disorders (Chen *et al.*, 2008; Chen *et al.*, 2009), which can subsequently impact on the second generation (Rajia *et al.*, 2010). The replenishment of blood leptin in *ob/ob* mice during the time of the leptin surge induces synaptic reorganization and may reverse their abnormal phenotype (Pinto *et al.*, 2004). This suggests that an intervention during the suckling period, especially during the leptin surge period, may reduce obesity and metabolic derangements in the offspring. The obese phenotype in pups from obese rats has been believed to be partially due to the composition of breast milk (Gorski *et al.*, 2006) as the birth weight of pups from lean and obese dams are similar. However, nutritional approaches that use breast milk from lean rats have been shown to be ineffective in changing the obese phenotype in offspring of obese mothers (Gorski *et al.*, 2006).

We have previously used the hypoglycaemic drug exendin-4 (Ex-4), a glucagon-like peptide-1 (GLP-1) receptor analogue, to treat offspring of obese dams for 6 weeks starting at weaning and have shown a reversal of the maternal impact on the propensity of the offspring to high-fat diet (HFD)-induced obesity (Chen *et al.*, 2014b). However, such treatment needs to be administered daily and requires 6 weeks to exert significant effects to normalize glucose homeostasis. In a previous study, Ex-4 was injected daily into neonate rats with intrauterine growth restriction (IUGR) from P0-6 (Stoffers *et al.*, 2003). Such short term Ex-4 treatment in early life permanently reversed the diabetic phenotype in the IUGR rats, via promoting the development of the pancreas, in particular insulin generating β-cells (Stoffers *et al.*, 2003). Since maternal obesity causes insulin resistance at a very young age lasting until adulthood (Chen *et al.*, 2008; Chen *et al.*, 2009), we propose that short-term Ex-4 treatment during the early postnatal period may improve the metabolic profile in such offspring.

We have recently demonstrated this in post-weaning rats (Chen *et al.*, 2014b), but through mechanisms that are independent of pancreatic function. Since early postnatal neural development plays a critical role in determining the future risk of lipid and glucose metabolic disorders, we hypothesized that short-term Ex-4 treatment during P4-14 (leptin surge period) could improve the metabolic profile at weaning in the offspring of obese mothers. In this study we treated female rat pups with daily Ex-4 between P4 and P14, and measured body weight, adiposity, blood lipid levels and hypothalamic expression of regulators for energy homeostasis. In addition, we also measured mRNA expression of lipid and glucose metabolic regulators, such as adipose triglyceride lipase (ATGL), lipid oxidative regulator carnitine palmitoyltransferase-1 (CPT-1), peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α) and its downstream signaling pathway, as well as the novel 'fat depletion gene' α(2)-zinc-glycoprotein 1 (AZGP1), in the fat and the liver.

Animal and methods

1. Modeling maternal obesity and treatment

The study was approved by the Animal Care and Ethics Committees of University of New South Wales and University of Technology Sydney. Maternal obesity was modeled as previously described (Chen *et al.*, 2012; Chen *et al.*, 2014b). Briefly, female Sprague Dawley breeders (8 weeks, Animal Resource Centre Pty. Ltd, WA, Australia) were housed under the standard conditions (Chen *et al.*, 2012; Chen *et al.*, 2014b). Maternal obesity was induced by consuming a pellet high-fat diet (HFD, 20kJ/g, 43.5% calorie as fat, SF03-020, Specialty Feeds, WA, Australia, n=8) for 6 weeks (Chen *et al.*, 2014b), while the control rats were fed standard chow (11kJ/g, 14%

calorie as fat, Gordon's specialty Stockfeeds, New South Wales, Australia, n=8). Then females were mated with lean male rats (8 weeks) from the same source. Litter size was adjusted to 10/litter (sex ~1:1). The same control or HFD diet was continued in the dams until the pups reached P20.

From P4-14, 2-3 female pups from every litter were injected with saline daily to act as the control group (CS: chow offspring + saline, HS: HFD offspring + saline), while the other females were injected with Ex-4 (5μg/kg/day i.p., Auspep, VIC, Australia. CE: chow offspring + Ex-4, HE: HFD offspring + Ex-4).

2. Leptin sensitivity test

In a sub-cohort of offspring, leptin (15 μg/g, i.p., Sigma-Aldrich Pty Ltd, NSW, Australia) was injected into P10 pups (n=3-4, 1 per litter), with saline-injected into littermates to act as the control group as previously described (Kirk *et al.*, 2009; Glavas *et al.*, 2010). The whole hypothalamus was then harvested 45 min post-injection for the measurement of the protein levels of the downstream signaling of the leptin receptor, including STAT3 and its phosphorylated form p-STAT3 by western blotting. Blood glucose level was measured using a glucose meter (Accu-Chek®, Roche, Nutley, NJ) in saline treated pups before harvesting the brain.

3. Sample collection

At weaning (P20), female offspring were deeply anesthetised with sodium thiopental (Pentothal®, 0.1mg/g, i.p, Abbott Australasia, NSW, Australia), immediately after being taken away from their mothers. Body weight and naso-annual length were measured prior to the blood collection by cardiac puncture, and blood glucose levels were measured using a glucose meter (Accu-Chek®, Roche, Nutley, NJ). Serum was immediately separated by centrifugation at 4ºC (12,000g, 8 minutes) and stored at -20°C in DNAse and RNAse free Eppendorf tubes for later measurement of hormones (ghrelin, GLP-1 and leptin) and lipids (triglyceride and non-esterified fatty acid). Pups were then killed by decapitation and the liver and fat pads (retroperitoneal, mesenteric fat and gonadal fat) were weighed. The hypothalamus was micro-dissected into regions containing arcuate nucleus (ARC) and paraventricular nucleus (PVN) as previously described (Chen *et al.*, 2009). The brain, liver and retroperitoneal fat were then snap-frozen for mRNA measurement.

4. Plasma lipid and hormone assays

Liver lysis (n=8) was homogenized using chloroform:methanol mixture (2:1), and air-evaporated. Absolute ethanol (250μl) was used to dissolve the sample, which was used for triglyceride assay

(Chen *et al.*, 2014b). Plasma or liver extracts, and glycerol standards (Sigma-Aldrich, Saint Louis, MO, USA) were incubated with triglyceride reagent (Roche Diagnostics, NJ, USA) to measure triglyceride concentration as previously described (Chen *et al.*, 2014b). Plasma non-esterified free fatty acids (NEFA) were measured using a NEFA kit according to the manufacture's instruction (WAKO, Osaka, Japan) (Chen *et al.*, 2014b). Insulin levels were measured using an enzyme-linked immunosorbent assay (Crystal Chem, Downers Grove, IL, USA). Non-fasting plasma ghrelin, GLP-1, and leptin levels were measured using Bio-Plex Pro™ Assays kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Bio-Plex system® and Bio-Plex Manager™ (Chen *et al.*, 2014b).

5. Quantitative real-time PCR

Tissue RNA (n=6-8) was extracted using TRIzol reagent (Sigma-Aldrich Pty Ltd, NSW, Australia), and cDNA synthesized as previously described (Chen *et al.*, 2014b). Pre-optimised TaqMan probe/primers were used to measure the specific mRNA expression in the hypothalamus, liver and fat using real-time PCR (Eppendorf Realplex 2, Eppendorf AG, Hamburg, Germany). In the retroperitoneal fat, mRNA expression of the essential fatty acid and glucose metabolic markers, including PGC-1α and its downstream gluconeogenesis marker Forkhead box protein O (FOXO)1, fatty acid oxidative and lipid synthesis markers (peroxisome proliferator-activated receptor gamma (PPAR-γ), sterol regulatory element binding protein (SREBP)1c, CPT-1, and fatty acid synthase (FASN)) were measured in liver. CPT-1, lipase ATGL, and the 'fat depletion gene' AZGP1 were measured. Appetite regulators, including orexigenic NPY and Agouti-related protein (AGRP) and anorexigenic proopiomelanocortin (POMC), as well as active leptin receptor (OB-Rb) were measured in the ARC; while their downstream receptor neuropeptide Y1 receptor (NPY Y1R) and melanocortin 4 receptor (MC4R) and suppressor of cytokine signaling-3 (SOCS3) were measured in the PVN and ARC respectively. The probe sequences for all the target genes provided by the manufacturer are listed in Table 1. Target genes were labeled with FAM and housekeeping genes 18s were labeled with VIC. The average of the CS group was used as a calibrator against which all other samples were expressed as fold difference.

Table 1: TaqMan probe sequence (Life Technology, Foster City, USA) used for real time-PCR.

Gene name	NCBI gene references	FAM-labeled Probes (5'→ 3')	Assay ID
AGRP	XM_574228.2,AF206017.1	GCAGAGGTGCTAGATCCACAGAACC	Rn01431703_g1
ATGL	XM_341960.3	GCCTGCCTGGGCGAAGCGGGTGCCA	Rn01479969_m1
AZGP1	NM_012826.1,D21058.1,X75309.1,BC091166.1,BC105822.1	TGGACCGAACAGATCCTCCCACTGT	Rn00563201_m1
CPT-1α	NM_031559.1,L07736.1,U88294.1,BC072522.1	CCAGGAGAGTGCCAGGAGGTCATAG	Rn00580702_m1
FASN	NM_017332.1,M76767.1,J03514.1,X13415.1,X62888.1	AACCTGTCCCAGGTGTGTGATGGGA	Rn01645297_g1
FOXO1	NM_001191846	AAGAGTTAGTGAGCAGGCTACATTT	Rn01494868_m1
Ob-Rb	NM_012596.1	TTAATTTCCAAAAGCCTGAAACATT	Rn01433205_m1
MC4R	NM_013099.2	AGCAGAAGCCTGATTCCACTGTTTA	Rn01491866_s1
NPY	NM_012614.1	GCCCGCCCGCCATGATGCTAGGTAA	Rn00561681_m1
PPARγ	NM_013124.2,AB019561.1,AF156666.1,Y12882.2	AGCACTTCACAAGAAATTACCATGG	Rn00440940_m1
PGC1 α	NM_031347.1,AB025784.1,AY237127.1	TCTGGAACTGCAGGCCTAACTCCTC	Rn00580241_m1
POMC	NM_139326.2	AAGCAACCTGCTGGCTTGCATCCGG	Rn00595020_m1
SOCS3	NM_053565.1	ACCCCCGGAGCACGCAGCCAGTGCC	Rn00585674_s1
SREBP1c	XM_213329.5,AF286469.2,AF286470.2	GGGCACTGAGGCAAAGCTGAATAAA	Rn01495769_m1
Y1 receptor	NM_001113357.1,Z11504.1	TTCATATGCTACTTCAAGATATACG	Rn01402912_g1

6. Western blotting

Proteins were isolated from the hypothalamus and quantified as previously described (Chen *et al.*, 2011). Proteins were separated with Novex® system (Life Tech, CA, USA) and transferred to polyvinylidene difluoride membranes using a transBlot semi-dry Transfer cell (Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight at 4°C with the primary antibodies (STAT3 (1:500), p-STAT3 (1:500), or housekeeping β-actin (1:1000), Cell Signaling Technology, Inc. Beverly, MA, USA), followed by secondary antibody (donkey anti-rabbit-HRP (1:10,000) for STAT3, goat anti-mouse, (1:5000) for p-STAT3 and β-actin), Santa Cruz Biotechnology, Dallas, TX, USA) for 1h at room temperature. Then the membranes were incubated with Stable Peroxide Solution and Enhancer Solution (Thermo Scientific, Waltham, MA, USA). Protein expression was detected by Universal Hood II and quantified using Quantity One software (Bio-Rad).

7. Statistical Methods

All the results in the Table and text are expressed as mean ± SEM. Those in the figures are presented as mean + SEM. Normality was tested and if the data were not normally distributed, they were log transformed prior to statistical analysis. The differences between the groups were analysed using a two-way ANOVA. If there was a significant interaction between the maternal and Ex-4 effects, *post hoc* Bonferonni tests were performed. $P<0.05$ was considered significant (Statistica 8.0, StatSoft Inc., Tulsa, OK, USA).

Results

1. Effect of maternal obesity on the offspring

1.1 Anthropometric characteristics

There was no significant difference in birth weights between the offspring of the control and obese dams (7.07 ± 0.17g vs 6.91 ± 0.16g in offspring of control and obese dams, respectively). Maternal obesity led to significantly increased body weight, liver and fat mass of the offspring at P20 ($P<0.05$, maternal effect, HS & HE vs CS & HE. Table 2). Body length, BMI, body and liver weight, as well as the percentages of liver, gonadal fat and mesenteric fat mass were significantly increased by maternal obesity ($P<0.05$, Table 2). Maternal obesity also increased liver triglyceride concentration ($P<0.05$, maternal effect, HS & HE vs CS & HE. Table 2), and plasma levels of lipids, including triglycerides and NEFA, but NEFA was only higher in saline-treated offspring ($P<0.05$, maternal effect, HS & HE vs CS & HE. Table 2). Plasma insulin level was also increased by maternal obesity ($P<0.05$, maternal effect, HS & HE vs CS & HE. Table 2). Plasma gut-derived GLP-1 levels were significantly lower, while total ghrelin levels were higher in the offspring of

obese dams (P<0.05, maternal effect, HS & HE vs CS & HE. Table 2). The level of the plasma hormone leptin was more than tripled in the offspring of obese dams compared to those from lean dams (P<0.05, HS vs CS. Table 2). However, blood glucose level at p10 and P20, and glucagon levels were not changed by maternal obesity (Table 2).

Table. 2 Effects of maternal obesity and Ex-4 treatment on the body parameters at P20

Parameters	**CS (n=15)**	**CE (n=17)**	**HS (n=11)**	**HE (n=13)**
Body weight (g) *†δ	59.3 ± 0.5	38.8 ± 0.7†	72.8 ± 2.9*	65.1 ± 2.4*†
Body length (cm) *†δ	12.45 ± 0.09	10.93 ± 0.11†	13.22 ± 0.17*	12.81 ± 0.16
BMI (kg/m^2) *†δ	3.81 ±0.05	3.26 ±0.08 †	4.09 ± 0.07 *	3.90 ± 0.07
Liver (g) *†δ	2.80 ± 0.04	1.54 ± 0.04†	3.54 ± 0.17*	3.05 ± 0.15*†
Liver (%) *†δ	4.72 ± 0.05	3.98 ± 0.04†	4.86 ± 0.07	4.60 ± 0.09*
Retroperitoneal fat (g) *†	69.1 ± 3.2	46.2 ± 3.0	296 ± 24	241 ± 23
Retroperitoneal fat (%) *	0.12 ± 0.01	0.11 ± 0.01	0.40 ± 0.02	0.35 ± 0.03
Gonadal fat (g) *†	95.5 ± 4.9	69.1 ± 19.9	478 ± 42	364 ± 37
Gonadal fat (%)*†δ	0.17 ± 0.01	0.17 ± 0.06	0.69 ± 0.04*	0.54 ± 0.04*†
Mesenteric fat (g) *†	585 ± 20	381 ± 12	893 ± 44	724 ± 40
Mesenteric fat (%) *†δ	0.99 ± 0.03	0.95 ± 0.02	1.26 ± 0.02*	1.10 ± 0.02*†
Liver triglyceride concentration (mmol/g) *†δ	10.2 ± 0.9	12.2 ± 1.9	42.9 ± 2.6*	30.9 ± 2.3*†
Blood and plasma markers				
Glucose at P10 (mM)	6.20 ± 0.22	6.32 ± 0.38	6.72 ± 0.43	6.50 ± 0.33
Glucose at P20 (mM)	7.93 ± 0.13	8.38 ± 0.16	8.25 ± 0.41	8.19 ± 0.17
Insulin at P20 (ng/ml)*	0.76 ± 0.07	0.94 ± 0.20	1.91 ± 0.45	1.01 ± 0.20
Triglyceride at P20 (mM) *	0.21 ± 0.03	0.24 ± 0.05	1.29 ± 0.24	1.30 ± 0.14
NEFA at P20 (mM) *δ	0.89 ± 0.03	0.93 ± 0.06	1.35 ± 0.11*	1.06 ± 0.06†
GLP-1 at P20 (pg/ml)	191 ± 9	182 ± 12	130 ± 15*	150 ± 9*
Ghrelin at P20 (pg/ml) *	168 ± 28	236 ± 65	265 ± 44	410 ± 80
Glucagon at P20 (pg/ml)	268 ± 17	360 ± 25	291 ± 68	268 ± 39
Leptin at P20 (ng/ml) *†	2.86 ± 0.30	2.06 ± 0.10	9.60 ± 0.88	6.89 ± 0.82

Results are expressed as mean ± S.E.M. *P<0.05, overall maternal effect; † P<0.05 overall Exendin-4 effect. δ P<0.05, interactions between maternal and Exendin-4 effect.* and † next to the numbers indicate P< 0.05 in the *post hoc* test.

1.2 Liver mRNA expression

In pups from the obese mothers the liver insulin sensing marker PPARγ mRNA expression was reduced by ~50% although it did not research statistical significance (Fig 1a). Maternal obesity reduced some glucose and lipid metabolic markers, including liver PGC1a and fat ATGL mRNA expression (P<0.05, maternal effect, HS & HE vs CS & HE. Fig 1d, h); while it also significantly increased mRNA expression of some lipid metabolic markers, including liver SREBP1c, FASN and fat CPT-1α expression in P20 pups (P<0.05, maternal effect, HS & HE vs CS & HE. Fig 1b, c, g). Maternal obesity did not affect liver CPT-1α (Fig 1e) and FOXO1 (Fig 1f) mRNA in offspring.

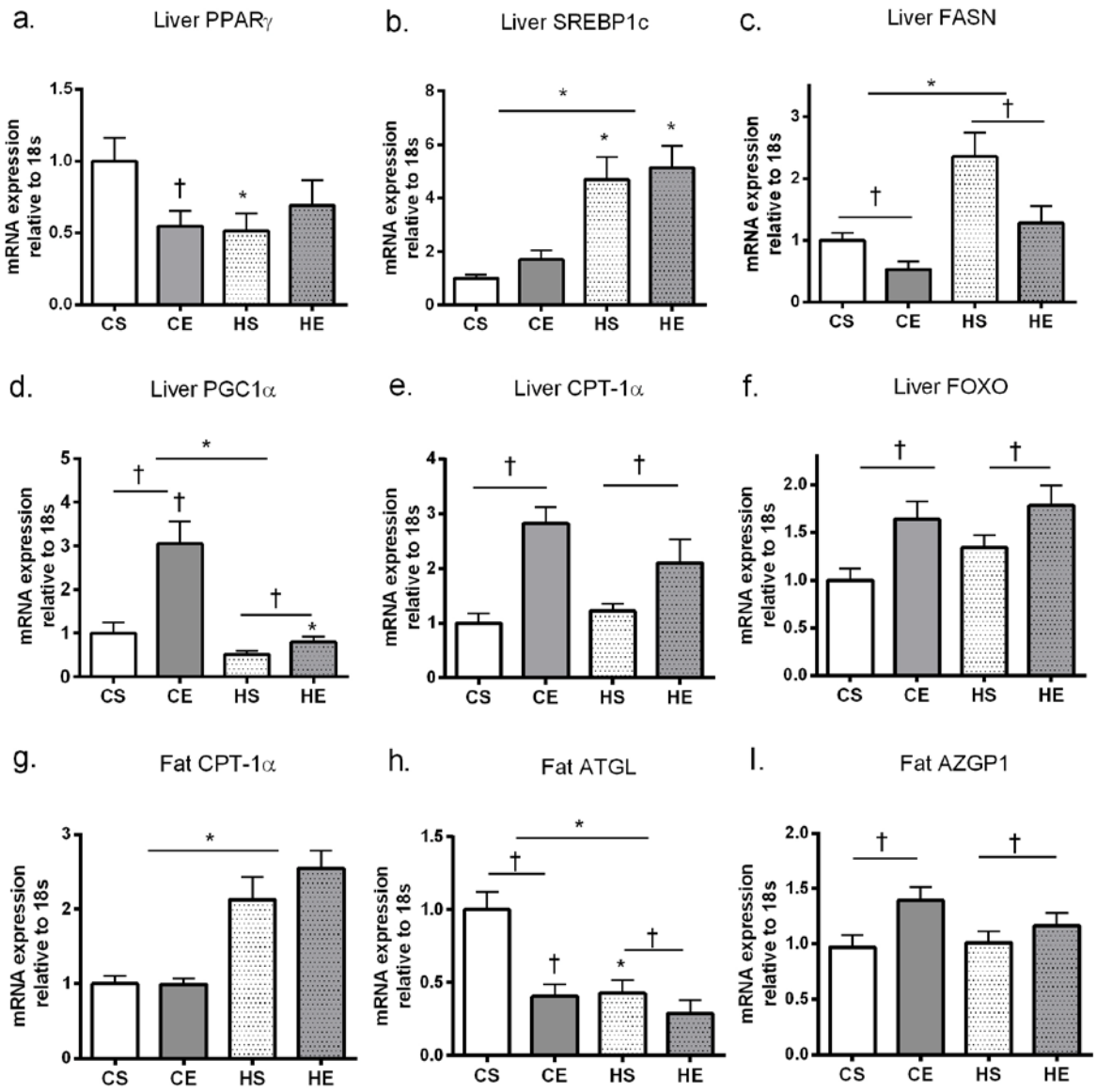

Figure 1. Fat metabolic markers in the liver (a-f) and retroperitoneal fat (g-i). Data are expressed as mean + SEM. *P<0.05, overall maternal effect; †P<0.05, overall Ex-4 effect. * and † appear directly on top of the bars indicate P< 0.05 in the post hoc test.

1.3 Hypothalamic mRNA and protein expression

In the hypothalamus, maternal obesity reduced mRNA expression of appetite-stimulating NPY, its receptor NPY Y1R (P<0.05, maternal effect, Fig 2b), and AGRP, as well as appetite-inhibiting MC4R in the offspring (P<0.05, maternal effect, Fig 2a, b, c, e). ARC appetite-inhibiting POMC was reduced by 35% in the saline-treated pups only. However this did not reach statistical significance (Fig 2d).

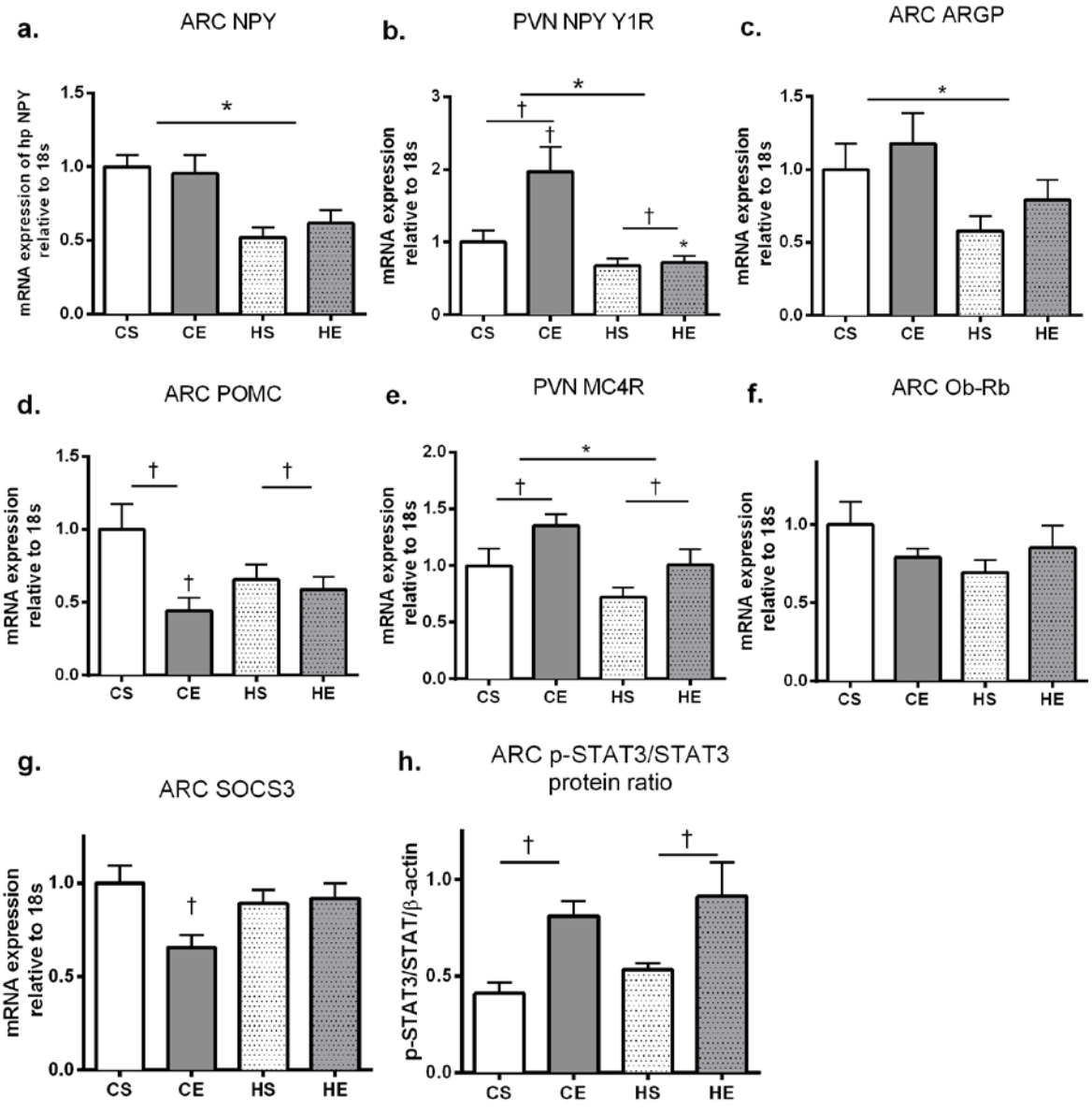

Figure 2. mRNA expression appetite regulators in the hypothalamus at 20 days (a-g, n = 6-8) and protein ratio between p-STAT3 and STAT3 in the ARC at P10 (h, n = 3). Data are expressed as mean + SEM. *P<0.05, overall maternal effect; † P<0.05 overall Ex-4 effect. * and † appear directly on top of the bars indicate P< 0.05 in the post hoc test.

The ARC leptin receptor Ob-Rb expression was not affected by maternal obesity (Fig 2f), neither was its downstream signaling p-STAT3/STAT3 ratio at P10 in saline injected rats. However, exogenous leptin injection failed to increase hypothalamic p-STAT3/STAT3 ratio or protein levels (data not shown) after 45 minutes.

2. Effect of Ex-4 treatment on offspring

2.1 Anthropometric characteristics

Ex-4 treatment significantly reduced body length and BMI, especially in offspring of chow-fed mothers by 12% and 14% respectively ($P<0.05$, Ex-4 effect, CE & HE vs CS & HS; interaction effect between maternal obesity and Ex-4 on body length and BMI. *Post hoc* $P<0.05$, CS vs HS and CS vs CE, for both body length and BMI. Table 2). It also reduced body and organ weights (liver and fat pads) in P20 offspring regardless of the maternal group ($P<0.05$, Ex-4 effect, CE & HE vs CS & HS; interaction effect between maternal obesity and Ex-4 on body and liver weights. *Post hoc* $P<0.05$, CS vs HS, and CE vs HE, CS vs CE, HS vs HE, for both body and liver weights. Table 2). Ex-4 also reduced the percentage of liver ($P<0.05$, Ex-4 effects, CE & HE vs CS & HS; interaction effect between maternal and Ex-4. *post hoc* $P<0.05$, CE vs HE, CS vs CE), gonadal and mesenteric fat ($P<0.05$, Ex-4 effects, CE & HE vs CS & HS; interaction effect between maternal and Ex-4, *post hoc* $P<0.05$, CS vs HS, CE vs HE, HS vs HE, Table 2). Liver triglyceride concentration was significantly lower in Ex-4 treated pups ($P<0.05$, Ex-4 effect, CE & HE vs CS & HS; interaction effect between maternal and Ex-4. *Post hoc* $P< 0.05$, CS vs HS, CE vs HE, HS vs HE, Table 2). Blood NEFA levels were reduced by Ex-4 in offspring of obese mothers in particular ($P<0.05$, Ex-4 effect, CE & HE vs CS & HS; interaction between maternal and Ex-4. *Post hoc* $P< 0.05$, HE vs HS, Table 2). Plasma leptin levels were also reduced by ~28% in Ex-4 treated offspring from both lean and obese mothers ($P<0.05$, Ex-4 effect, CE & HE vs CS & HS. Table 2). However, neither blood glucose, insulin, and triglycerides levels nor other metabolic hormones were significantly affected by Ex-4 treatment, although the insulin level in the HE group was nearly halved compared with the untreated littermates in the HS group (Table 2).

2.2 Liver mRNA expression

Liver PCG1α, CPT-1, and FOXO1 mRNA expression were upregulated by Ex-4 treatment ($P<0.05$, Ex-4 effect, CE & HE vs CS & HS. Fig 1d, e, f); whilst liver FASN and fat ATGL mRNA expression were decreased by Ex-4 ($P<0.05$, Ex-4 effect, CE & HE vs CS & HS. Fig 1c, h). There was a significant interaction effect between maternal obesity and Ex-4 on liver PGC1α (*post hoc*

P<0.05, CS vs CE, CE *vs* HE, Fig 1d) and fat ATGL (*post hoc* P<0.05, CS vs HS, CS vs CE, Fig 1h) mRNA expression, with *post hoc* testing indicated that Ex-4 effects were only significant in offspring of chow-fed mothers (P<0.05). Ex-4 also upregulated mRNA expression of AZGP1 (P<0.05, Ex-4 effect, CE & HE vs CS & HS. Fig 1i). Although liver PPARγ mRNA expression was reduced by nearly 50% in both CE and HS groups compared with the CS group, there was no statistical significance (P<0.05, interaction effect between maternal obesity and Ex-4. Fig 1a).

2.3 Hypothalamic mRNA expression

In the hypothalamus, Ex-4 treatment increased the appetite-stimulating PVN NPY Y1R and the appetite-inhibiting MC4R mRNA expression, as well as the hypothalamic leptin receptor downstream signaling p-STAT3/STAT3 ratio at P10 (P<0.05, Fig 2b, e, h). Ex-4 treatment significantly reduced ARC POMC expression in the hypothalamus (P<0.05, Ex-4 effect, CE & HE vs CS & HS; Fig 2b, d). An interaction effect between maternal obesity and Ex-4 was observed in PVN NPY Y1R (*post hoc* P<0.05, CE vs HE, CS vs CE, Fig 2b), and ARC POMC and SOCS3 mRNA expression (*post hoc*, P<0.05 CS vs CE, Fig 2d, g).

Discussion

In this study, we found that Ex-4 treatment during the suckling period led to significant reductions in body weight, liver mass, and fat mass of the female offspring from obese mothers. BMI and the percentage of liver weight were nearly normalized to the levels of the control group (CS). Ex-4 improved markers of lipid metabolism in the liver and fat, which may have contributed to reduced fat mass and the improved lipid profile. Ex-4 increased hypothalamic leptin sensitivity during the neural developmental period in the suckling pups, leading to upregulated anorexigenic receptor expression.

Paternal obesity may result in epigenetic modification in the offspring. However, recent human studies demonstrated that maternal obesity has a much stronger influence than paternal obesity not only on the phenotype and metabolic profile of immediate offspring (Casas *et al.*, 2013; Patro *et al.*, 2013), but can also predispose the second generation to metabolic disorders (Rajia *et al.*, 2010; King *et al.*, 2013; Shasa *et al.*, 2014). As we plan to study the second generation into the future, we have focused on first generation of female offspring in this study. In both human and animal studies maternal obesity has been shown to be strongly associated with early childhood obesity and metabolic disorders in both (Danielzik *et al.*, 2002; Wu & Suzuki, 2006; Gibson *et al.*, 2007; Chen *et al.*, 2008; Chen *et al.*, 2009). Since the BMI in childhood correlates with adult obesity (Danielzik

et al., 2002; Wu & Suzuki, 2006; Gibson *et al.*, 2007), early intervention to ameliorate an obesity phenotype in childhood could lead to reduced chronic disease in adulthood. In this study, maternal obesity significantly increased the body weight and adiposity in weaning offspring, as well as their blood insulin and lipids levels, which is consistent with our previous observations in a similar model (Chen *et al.*, 2008). These alterations were partially reversed by Ex-4 treatment during the critical postnatal neural developmental period. However, Ex-4 restricts the growth of the offspring of the lean mothers, reflected in a smaller body length and BMI, compared with the un-treated litter mates. The disproportionate reduced liver weight in those offspring may limit the total lipid storage capacity. In addition, as liver is also an important organ for glucose and lipid metabolism, in the offspring from the lean mothers the reduced size together with downregulated PPARγ (insulin sensing marker) and slight increase in insulin level (23%) suggests that early Ex-4 treatment may impair their glucose homeostasis which requires further long-term investigation. This growth restrictive effect is also consistent with the observations by Stoffers and colleagues after a six-day treatment from birth (Stoffers *et al.*, 2003). This effect could be due to the appetite suppression effect of Ex-4 (Bojanowska & Nowak, 2007; Barrera *et al.*, 2009; Dalvi *et al.*, 2012). It has been shown that the brain response to GLP-1 can be blunted by HFD consumption (Nogueiras *et al.*, 2009). This could also happen in the offspring from the obese mothers as maternal obesity increases milk intake (Chen & Morris, 2009), and it can be speculated that the breast milk from the obese mothers may be richer, potentially recapitulating the effect of HFD (Gorski *et al.*, 2006). In addition, the effect of Ex-4 in the offspring of the obese dams was not as pronounced as in our previous study focusing on post-weaning rats, where Ex-4 treated offspring did not develop HFD-induced obesity and related metabolic disorders (Chen *et al.*, 2014b). There are two reasons that may account for this difference. First, the Ex-4 dose in this study was 1/3 that of our previous study (15μg/kg/day), considering the young age and much smaller size of the rats. Second, immature homeostatic circuitry, especially in the developing hypothalamus can respond to Ex-4 differently from the fully developed circuitry in older rats after weaning. Indeed, the glucose lowering effect of Ex-4 was not significant in the weaning rats in this study, and neither blood glucose nor plasma glucagon was changed, suggesting a differential effect of Ex-4 in immature suckling rats. However, the lowered insulin level in the HE group may suggest some benefit of insulin action in maintaining normal blood glucose level.

Hepatic biosynthesis and secretion of glucose and lipids plays a critical role in controlling blood glucose and lipid levels, and dysregulation of both are significant risk factors for both type 2 diabetes and cardiovascular disease (Ginsberg, 2002; D'Adamo *et al.*, 2010). Hyperlipidemia, liver

steatosis, and glucose intolerance co-exist in the offspring of obese mothers from weaning to adulthood (Elahi *et al.*, 2009; Rajia *et al.*, 2010). PPARγ is a ligand-activated transcription factor which is responsible for insulin sensitivity. As such, PPARγ activators such as rosiglitazone are still used as oral hypoglycemic drugs in patients with type 2 diabetes in some countries. Under fasting conditions, PPARγ expression can be increased by its upstream regulator PGC-1α to increase fatty acid oxidation to support energy supply (Puigserver *et al.*, 1999; Wallberg *et al.*, 2003). PGC-1α plays a key role in substrate (glucose/fatty acid) metabolism and regulating mitochondrial function (Finck & Kelly, 2006). In this study, reduced liver PGC-1α and a trend to a reduction in PPARγ expression in the offspring of the obese mothers, which may contribute to the development of insulin resistance (Oben *et al.*, 2010) and result in glucose intolerance (Chen *et al.*, 2008) as we have reported previously. Reduced abdominal fat mass due to Ex-4 treatment in the HE offspring may improve liver insulin sensitivity (Finelli & Tarantino, 2012). In addition, previously it has been shown that Ex-4 treatment or GLP-1 receptor antagonist can improve liver insulin sensitivity in both *in vitro* and *in vivo* studies (Aviv *et al.*, 2009; Gupta *et al.*, 2010; Lee *et al.*, 2012). However, without the measurement of insulin receptor signaling cascade and insulin sensing hormone adiponectin, it cannot be concluded here whether liver insulin sensitivity is affected at this age (Finelli & Tarantino, 2013). PGC-1α activity may also increase hepatic gluconeogenesis to raise blood glucose level (Finck & Kelly, 2006), via its downstream signaling FOXO1 (Puigserver *et al.*, 2003) which was however not affected by maternal obesity. FOXO1 is normally increased by starvation to maintain blood glucose level (Zhou *et al.*, 2011). In this study, FOXO1 as well as PGC-1α were increased by Ex-4. However blood glucose levels were not affected. As the protein level was not measurement, we were unable to confirm the functional relevance of upregulated FOXO1.

The expression of several genes in the liver was changed by maternal obesity, which may be link to increased hepatic triglyceride concentration in such offspring here. Increased PGC-1α expression was shown to improve mitochondrial lipid oxidative metabolism, and inhibit the expression of lipogenic genes to prevent the development of hepatic steatosis (Leone *et al.*, 2005; Finck & Kelly, 2006). Long-term obesity has been shown to reduce PGC-1α mRNA expression and is associated with decreased mitochondrial lipid oxidation and increased local triglycerides accumulation resulting in hepatic steatosis (Petersen *et al.*, 2003; Leone *et al.*, 2005). Here, liver PGC-1α expression was downregulated by maternal obesity, which may be closely linked to the phenotype of liver steatosis (Bruce *et al.*, 2009; Bayol *et al.*, 2010; Oben *et al.*, 2010; Caruso *et al.*, 2011; Chen *et al.*, 2014b). CPT-1α, a key downstream target of PGC-1α in lipid metabolism (Finck & Kelly,

2006; Liang & Ward, 2006), functions as a rate limiting step to transport fatty acid into the mitochondria to support ATP synthesis (Louet *et al.*, 2002). In this study, at weaning liver CPT-1α mRNA level was unchanged. Normally during increased lipid influx (eg. consuming high-lipid diet) without an increase in lipid transportation into the mitochondria, the excess dietary fat is redirected for *de novo* lipid synthesis leading to liver steatosis, which is observed in offspring of the obese mothers reflected by increased liver TG concentration in this study. Excessive dietary fat influx can also increase liver SREBP1c mRNA expression and further FASN activation to induce lipogenesis, resulting in hepatic ectopic lipid accumulation (Aragno *et al.*, 2009). In this study, SREBP1c and FASN was synchronically upregulated with PGC-1α, maybe linked to hyperlipidemia and increased liver triglyceride concentration observed in the obese offspring here. However, without the measurement of hepatic inflammation markers and hormones, such as IL-6, TNF-α, and adiponectin, the increase of which is one of the hallmarks hepatic steatosis (Tarantino *et al.*, 2010; Finelli & Tarantino, 2013), we are unable to conclude whether such offspring developed hepatic steatosis.

Ex-4 reduced blood NEFA level in offspring from obese mothers. This may be related to downregulated FASN, and upregulated PCG-1α and its downstream CPT-1α by Ex-4. In general, such changes have the potential to increase lipid oxidation, reduce ectopic lipid synthesis and accumulation in the liver. High expression of FASN signaling is closely linked to the development of fatty liver disease in humans (Zou *et al.*, 2007). However, the upstream regulator of FASN, SREBP1c expression was unchanged, suggesting that Ex-4 may act via another unknown pathway to suppress FASN expression. A previous study showed that the upregulation PCG-1α mRNA can prevent mice from developing HFD-induced liver mitochondrial dysfunction, which is a major contributor to the development of fatty liver disease (Lagouge *et al.*, 2006; Zou *et al.*, 2014). Ex-4 is able to ameliorate the impact of maternal obesity on PGC-1α expression.

Adipose tissue is a metabolically active organ in addition to the major role it plays in energy storage. Fat CPT-1α mRNA was increased in the offspring of obese mothers which can be a response to increased fat influx into the adipocyte. However, a concurrent reduction in fat ATGL mRNA expression may suggest a reduced lipolysis, which can be linked to increased adiposity. Here we report a novel finding of the effect of Ex-4 on fat AZGP1 mRNA expression. AZGP1 has been shown to stimulate lipolysis leading to fat loss in humans and mice, in conditions such as cigarette smoking and cancer cachexia (Vanni *et al.*, 2009; Haugen *et al.*, 2011; Cabassi & Tedeschi, 2013). AZGP1polymorphism on the other hand was shown to contribute to the onset of type 2 diabetes in mice (Gohda *et al.*, 2003), while recombinant human AZGP was shown to

attenuate the symptoms related to type 2 diabetes in *ob/ob* mouse (Russell & Tisdale, 2010). In this study, Ex-4 significantly increased AZGP1 expression, but more so in the offspring from the lean dams, in the face of significantly smaller fat mass. This may be a new mechanism by which Ex-4 causes fat loss. However, downregulated AZGP1 in the offspring of the obese mothers may be counteracted by downregulated ATGL expression by Ex-4 to prevent fat depletion.

Maternal obesity changed both blood and brain appetite regulators. The changes in gut-derived hormones ghrelin and GLP-1 may directly contribute to their observed hyperphagic phenotype previously reported (Chen & Morris, 2009), which were not significantly affect by Ex-4 in the present study. There are two major molecular forms of ghrelin, acylated ghrelin and des-acyl ghrelin. Recently it has been suggested that des-acyl ghrelin counteracts the orexigenic effects of acylated ghrelin (Stevanovic *et al.*, 2014). Ghrelin levels usually increase in response to fasting. However, somewhat paradoxically exogenous ghrelin induces more energy intake in obese individuals compared with lean subjects (Ariyasu *et al.*, 2001; Druce *et al.*, 2005; Cummings, 2006). Although total fasting ghrelin levels are not different between obese and lean humans, the acylated ghrelin levels were lower in human obese individuals (Tschop *et al.*, 2001; Druce *et al.*, 2005). Controversially, Briggs and colleagues showed that exogenous ghrelin failed to induce orexigenic effects in diet-induced obese rats (Briggs *et al.*, 2010). The limitation of our ghrelin result is that our method did not preserve the acylated ghrelin and measured both ghrelin isoforms, so the result reflects total ghrelin levels. In addition, ghrelin was also measured in the non-fasting state of the rats. Therefore, we cannot conclude whether increased total ghrelin in offspring by maternal obesity in this study is linked to their hyperphagic phenotype (Chen & Morris, 2009), nor whether Ex-4 treatment can cause hyperphagia after weaning due to increased total plasma ghrelin level. Additional experiments to measure acylated ghrelin in adult offspring will conducted in future studies. Both anorexigenic and orexigenic neurotransmitters were downregulated by maternal obesity potentially due to reduced neural activity as we have shown previously (Chen *et al.*, 2014a), which may exert a balanced effect on feeding regulation. However, reduced anorexigenic MC4R and unchanged Y1R mRNA may be associated with hyperphagia as observed in our previous study (Chen & Morris, 2009). At P10, leptin resistance was not evident as suggested by the activation levels of p-STAT3/STAT3. Leptin resistance can develop in response to increased milk availability resulting from litter size reduction as early as P16 in mice (Glavas *et al.*, 2010), when the leptin surge is finished. In this study, leptin sensitivity was measured at P10 when the major function of leptin is to support neural development. However, this did not normalize the appetite regulator expression in offspring obese mother at weaning, suggesting that other growth factors may be

involved in neural development during the leptin surge. The observed lack of increase in STAT3 phosphorylation in response to exogenous leptin is also surprising and this might have been affected by the already elevated circulating leptin levels at P10 as previously reported (Kirk *et al.*, 2009). Therefore, the dose of leptin used previously in the literature may not be sufficient to over-ride the effects of endogenously high leptin levels.

It was interesting that Ex-4 increased leptin signaling in the hypothalamus at P10. In the HE group, although Ex-4 had no effect on SOCS3 (the classical inhibitor of STAT3), STAT3 activity appeared to be increased. Therefore, a different upstream pathway may contribute to increased leptin signaling activity in the obese offspring, which requires further investigation. In the short term, Ex-4 treated offspring from lean mothers had increased both orexigenic and anorexigenic receptor expression, but reduced anorexigenic POMC expression; whereas offspring from obese mothers had an increase in anorexigenic MC4R expression. It seems that the former may support hyperphagia, while the latter may reduce food intake. This suggests that such an intervention using Ex-4 is only applicable in offspring of obese mothers, but not as prevention in those of lean mothers. Although it is well-known that a child's BMI correlates with his/her own BMI in adulthood, long term follow up studies are needed, especially with additional environmental enrichment, such as HFD consumption, which is beyond the scope of this study. In addition to the short-term nature of the study, there are several other limitations of this paper that need to be addressed in future studies. Firstly, male offspring need to be examined although their direct impact to the second generation is not as potent as the females. Secondly, cross generational studies are of great interest to follow up such interventions. Thirdly, protein levels and functional studies are necessary to validate these findings.

In summary, Ex-4 treatment during the early postnatal period showed metabolic benefit in the offspring of obese mothers in association with diverse effects on neurohormonal regulation of metabolism; whereas it caused growth restriction in those from the lean mothers. Hence Ex-4 should be explored in the offspring of obese mothers to limit future metabolic complications.

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References

Ahima, R.S., Prabakaran, D. & Flier, J.S. (1998) Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. *J Clin Invest*, **101**, 1020-1027.

Aragno, M., Tomasinelli, C.E., Vercellinatto, I., Catalano, M.G., Collino, M., Fantozzi, R., Danni, O. & Boccuzzi, G. (2009) SREBP-1c in nonalcoholic fatty liver disease induced by Western-type high-fat diet plus fructose in rats. *Free Radic Biol Med*, **47**, 1067-1074.

Ariyasu, H., Takaya, K., Tagami, T., Ogawa, Y., Hosoda, K., Akamizu, T., Suda, M., Koh, T., Natsui, K., Toyooka, S., Shirakami, G., Usui, T., Shimatsu, A., Doi, K., Hosoda, H., Kojima, M., Kangawa, K. & Nakao, K. (2001) Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab*, **86**, 4753-4758.

Aviv, V., Meivar-Levy, I., Rachmut, I.H., Rubinek, T., Mor, E. & Ferber, S. (2009) Exendin-4 promotes liver cell proliferation and enhances the PDX-1-induced liver to pancreas transdifferentiation process. *J Biol Chem*, **284**, 33509-33520.

Barrera, J.G., D'Alessio, D.A., Drucker, D.J., Woods, S.C. & Seeley, R.J. (2009) Differences in the Central Anorectic Effects of Glucagon-Like Peptide-1 and Exendin-4 in Rats. *Diabetes*, **58**, 2820-2827.

Bayol, S.A., Simbi, B.H., Fowkes, R.C. & Stickland, N.C. (2010) A Maternal "Junk Food" Diet in Pregnancy and Lactation Promotes Nonalcoholic Fatty Liver Disease in Rat Offspring. *Endocrinology*, en.2009-1192.

Bayol, S.A., Simbi, B.H. & Stickland, N.C. (2005) A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. *J Physiol*, **567**, 951-961.

Bojanowska, E. & Nowak, A. (2007) Interactions between leptin and exendin-4, a glucagon-like peptide-1 agonist, in the regulation of food intake in the rat. *J Physiol Pharmacol*, **58**, 349-360.

Bouret, S.G., Draper, S.J. & Simerly, R.B. (2004) Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science*, **304**, 108-110.

Bouret, S.G. & Simerly, R.B. (2006) Developmental programming of hypothalamic feeding circuits. *Clin Genet* **70**, 295-301.

Briggs, D.I., Enriori, P.J., Lemus, M.B., Cowley, M.A. & Andrews, Z.B. (2010) Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. *Endocrinology*, **151**, 4745-4755.

Bruce, K.D., Cagampang, F.R., Argenton, M., Zhang, J., Ethirajan, P.L., Burdge, G.C., Bateman, A.C., Clough, G.F., Poston, L., Hanson, M.A., McConnell, J.M. & Byrne, C.D. (2009) Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology*, **50**, 1796-1808.

Cabassi, A. & Tedeschi, S. (2013) Zinc-α2-glycoprotein as a marker of fat catabolism in humans. *Curr Opin Clin Nutr Metab Care*, **16**, 267-271.

Caruso, V., Chen, H. & Morris, M.J. (2011) Early hypothalamic FTO overexpression in response to maternal obesity--potential contribution to postweaning hyperphagia. *PLoS One*, **6**, e25261.

Casas, M., Chatzi, L., Carsin, A.-E., Amiano, P., Guxens, M., Kogevinas, M., Koutra, K., Lertxundi, N., Murcia, M., Rebagliato, M., Riaño, I., Rodríguez-Bernal, C.L., Roumeliotaki, T., Sunyer, J., Mendez, M. & Vrijheid, M. (2013) Maternal pre-pregnancy overweight and obesity, and child neuropsychological development: two Southern European birth cohort studies. *Int J Epidemiol*, **42**, 506-517.

Chen, H., Iglesias, M.A., Caruso, V. & Morris, M.J. (2011) Maternal cigarette smoke exposure contributes to glucose intolerance and decreased brain insulin action in mice offspring independent of maternal diet. *PLoS One*, **6**, e27260.

Chen, H. & Morris, M.J. (2009) Differential responses of orexigenic neuropeptides to fasting in offspring of obese mothers. *Obesity*, **17**, 1356-1362.

Chen, H., Simar, D., Lambert, K., Mercier, J. & Morris, M.J. (2008) Intrauterine and postnatal overnutrition differentially impact appetite regulators and fuel metabolism. *Endocrinology*, **149**, 5348-5356.

Chen, H., Simar, D. & Morris, M.J. (2009) Hypothalamic neuroendocrine circuitry is programmed by maternal obesity: interaction with postnatal nutritional environment. *PLoS ONE*, e6259.

Chen, H., Simar, D. & Morris, M.J. (2014a) Maternal obesity impairs brain glucose metabolism and neural response to hyperglycemia in male rat offspring. *J Neurochem*, **129**, 297-303.

Chen, H., Simar, D., Pegg, K., Saad, S., Palmer, C. & Morris, M. (2014b) Exendin-4 is effective against metabolic disorders induced by intrauterine and postnatal overnutrition in rodents. *Diabetologia*, **57**, 614-622.

Chen, H., Simar, D., Ting, J.H.Y., Erkelens, J.R.S. & Morris, M.J. (2012) Leucine Improves Glucose and Lipid Status in Offspring from Obese Dams, Dependent on Diet Type, but not Caloric Intake. *J Neuroendocrinol* **24**, 1356-1364.

Cummings, D.E. (2006) Ghrelin and the short- and long-term regulation of appetite and body weight. *Physiol Behav*, **89**, 71-84.

D'Adamo, E., Cali, A.M.G., Weiss, R., Santoro, N., Pierpont, B., Northrup, V. & Caprio, S. (2010) Central Role of Fatty Liver in the Pathogenesis of Insulin Resistance in Obese Adolescents. *Diabetes Care*, **33**, 1817-1822.

Dalvi, P.S., Nazarians-Armavil, A., Purser, M.J. & Belsham, D.D. (2012) Glucagon-Like Peptide-1 Receptor Agonist, Exendin-4, Regulates Feeding-Associated Neuropeptides in Hypothalamic Neurons in Vivo and in Vitro. *Endocrinology*, **153**, 2208-2222.

Danielzik, S., Langnase, K., Mast, M., Spethmann, C. & Muller, M.J. (2002) Impact of parental BMI on the manifestation of overweight 5-7 year old children. . *Eur J Nutr*, **41**, 132-138.

Druce, M.R., Wren, A.M., Park, A.J., Milton, J.E., Patterson, M., Frost, G., Ghatei, M.A., Small, C. & Bloom, S.R. (2005) Ghrelin increases food intake in obese as well as lean subjects. *Int J Obes*, **29**, 1130-1136.

Elahi, M.M., Cagampang, F.R., Mukhtar, D., Anthony, F.W., Ohri, S.K. & Hanson, M.A. (2009) Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice. *Br J Nutr*, **102**, 514-9.

Finck, B.N. & Kelly, D.P. (2006) PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest*, **116**, 615-622.

Finelli, C. & Tarantino, G. (2012) Is visceral fat reduction necessary to favour metabolic changes in the liver? *J Gastrointestin Liver Dis*, **21**, 205-8.

Finelli, C. & Tarantino, G. (2013) What is the role of adiponectin in obesity related non-alcoholic fatty liver disease? *World J Gastroenterol*, **19**, 802-812.

Gibson, L.Y., Byrne, S.M., Davis, E.A., Blair, E., Jacoby, P. & Zubrick, S.R. (2007) The role of family and maternal factors in childhood obesity. *Med J Aust*, **186**, 591-595.

Ginsberg, H.N. (2002) New perspectives on atherogenesis: role of abnormal triglyceride-rich lipoprotein metabolism. *Circulation*, **106**, 2137-2142.

Glavas, M.M., Kirigiti, M.A., Xiao, X.Q., Enriori, P.J., Fisher, S.K., Evans, A.E., Grayson, B.E., Cowley, M.A., Smith, M.S. & Grove, K.L. (2010) Early Overnutrition Results in Early-Onset Arcuate Leptin Resistance and Increased Sensitivity to High-Fat Diet. *Endocrinology*, **151**, 1598-1610.

Gohda, T., Makita, Y., Shike, T., Tanimoto, M., Funabiki, K., Horikoshi, S. & Tomino, Y. (2003) Identification of epistatic interaction involved in obesity using the KK/Ta mouse as a Type 2 diabetes model: is Zn-alpha2 glycoprotein-1 a candidate gene for obesity? *Diabetes*, **52**, 2175-2181.

Gorski, J.N., Dunn-Meynell, A.A., Hartman, T.G. & Levin, B.E. (2006) Postnatal environment overrides genetic and prenatal factors influencing offspring obesity and insulin resistance. *Am J Physiol Regul Integr Comp Physiol*, **291**, R768-778.

Gupta, N.A., Mells, J., Dunham, R.M., Grakoui, A., Handy, J., Saxena, N.K. & Anania, F.A. (2010) Glucagon-like Peptide-1 Receptor (GLP-1R) is present on human hepatocytes and has a direct role in decreasing hepatic steatosis in vitro by modulating elements of the insulin signaling pathway. *Hepatology*, **51**, 1584-1592.

Haugen, F., Labori, K.J., Noreng, H.J., Buanes, T., Iversen, P.O. & Drevon, C.A. (2011) Altered expression of genes in adipose tissues associated with reduced fat mass in patients with pancreatic cancer. *Arch Physiol Biochem*, **117**, 78-87.

King, V., Dakin, R.S., Liu, L., Hadoke, P.W., Walker, B.R., Seckl, J.R., Norman, J.E. & Drake, A.J. (2013) Maternal Obesity Has Little Effect on the Immediate Offspring but Impacts on the Next Generation. *Endocrinology*, **154**, 2514-2524.

Kirk, S.L., Samuelsson, A.M., Argenton, M., Dhonye, H., Kalamatianos, T., Poston, L., Taylor, P.D. & Coen, C.W. (2009) Maternal obesity induced by diet in rats permanently influences central processes regulating food intake in offspring. *PLoS One*, **4**, e5870.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., Geny, B., Laakso, M., Puigserver, P. & Auwerx, J. (2006) Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell*, **127**, 1109-1122.

Lee, J., Hong, S.W., Chae, S.W., Kim, D.H., Choi, J.H., Bae, J.C., Park, S.E., Rhee, E.J., Park, C.Y., Oh, K.W., Park, S.W., Kim, S.W. & Lee, W.Y. (2012) Exendin-4 improves steatohepatitis by increasing Sirt1 expression in high-fat diet-induced obese C57BL/6J mice. *PLoS One*, **7**, e31394.

Leone, T.C., Lehman, J.J., Finck, B.N., Schaeffer, P.J., Wende, A.R., Boudina, S., Courtois, M., Wozniak, D.F., Sambandam, N., Bernal-Mizrachi, C., Chen, Z., Holloszy, J.O., Medeiros, D.M., Schmidt, R.E., Saffitz, J.E., Abel, E.D., Semenkovich, C.F. & Kelly, D.P. (2005) PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biology*, **3**, e101.

Liang, H. & Ward, W.F. (2006) PGC-1alpha: a key regulator of energy metabolism. *Adv Physiol Educ*, **30**, 145-151.

Louet, J.-F., Hayhurst, G., Gonzalez, F.J., Girard, J. & Decaux, J.-F. (2002) The Coactivator PGC-1 Is Involved in the Regulation of the Liver Carnitine Palmitoyltransferase I Gene Expression by cAMP in Combination with HNF4α and cAMP-response Element-binding Protein (CREB). *J Biol Chem*, **277**, 37991-38000.

Nogueiras, R., Perez-Tilve, D., Veyrat-Durebex, C., Morgan, D.A., Varela, L., Haynes, W.G., Patterson, J.T., Disse, E., Pfluger, P.T., Lopez, M., Woods, S.C., DiMarchi, R., Dieguez, C., Rahmouni, K., Rohner-Jeanrenaud, F. & Tschop, M.H. (2009) Direct control of peripheral lipid deposition by CNS GLP-1 receptor signaling is mediated by the sympathetic nervous system and blunted in diet-induced obesity. *J Neurosci*, **29**, 5916-5925.

Oben, J.A., Mouralidarane, A., Samuelsson, A.M., Matthews, P.J., Morgan, M.L., McKee, C., Soeda, J., Fernandez-Twinn, D.S., Martin-Gronert, M.S., Ozanne, S.E., Sigala, B., Novelli, M., Poston, L. & Taylor, P.D. (2010) Maternal obesity during pregnancy and lactation programs the development of offspring non-alcoholic fatty liver disease in mice. *J Hepatol*, **52**, 913-920.

Patro, B., Liber, A., Zalewski, B., Poston, L., Szajewska, H. & Koletzko, B. (2013) Maternal and Paternal Body Mass Index and Offspring Obesity: A Systematic Review. *Ann Nutr Metab*, **63**, 32-41.

Petersen, K.F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D.L., DiPietro, L., Cline, G.W. & Shulman, G.I. (2003) Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science*, **300**, 1140-1142.

Pinto, S., Roseberry, A.G., Liu, H., Diano, S., Shanabrough, M., Cai, X., Friedman, J.M. & Horvath, T.L. (2004) Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science*, **304**, 110-115.

Puigserver, P., Adelmant, G., Wu, Z., Fan, M., Xu, J., O'Malley, B. & Spiegelman, B.M. (1999) Activation of PPARgamma coactivator-1 through transcription factor docking. *Science*, **286**, 1368-1371.

Puigserver, P., Rhee, J., Donovan, J., Walkey, C.J., Yoon, J.C., Oriente, F., Kitamura, Y., Altomonte, J., Dong, H., Accili, D. & Spiegelman, B.M. (2003) Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature*, **423**, 550-555.

Rajia, S., Chen, H. & Morris, M.J. (2010) Maternal overnutrition impacts offspring adiposity and brain appetite markers-modulation by postweaning diet. *J Neuroendocrinol*, **22**, 905-914.

Russell, S.T. & Tisdale, M.J. (2010) Antidiabetic Properties of Zinc-α2-Glycoprotein in ob/ob Mice. *Endocrinology*, **151**, 948-957.

Samuelsson, A.-M., Matthews, P.A., Argenton, M., Christie, M.R., McConnell, J.M., Jansen, E.H.J.M., Piersma, A.H., Ozanne, S.E., Twinn, D.F., Remacle, C., Rowlerson, A., Poston, L. & Taylor, P.D. (2007) Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance. A novel murine model of developmental programming. *Hypertension*, **51**, 383-392.

Shasa, D.R., Odhiambo, J.F., Long, N.M., Tuersunjiang, N., Nathanielsz, P.W. & Ford, S.P. (2015) Multi-generational impact of maternal overnutrition/obesity in the sheep on the neonatal leptin surge in granddaughters. *Int J Obes*. **39**, 695-701.

Stevanovic, D.M., Grefhorst, A., Themmen, A.P., Popovic, V., Holstege, J., Haasdijk, E., Trajkovic, V., van der Lely, A.J. & Delhanty, P.J. (2014) Unacylated ghrelin suppresses ghrelin-induced neuronal activity in the hypothalamus and brainstem of male rats. *PLoS One*, **9**, e98180.

Stoffers, D.A., Desai, B.M., DeLeon, D.D. & Simmons, R.A. (2003) Neonatal Exendin-4 Prevents the Development of Diabetes in the Intrauterine Growth Retarded Rat. *Diabetes*, **52**, 734-740.

Tarantino, G., Savastano, S. & Colao, A. (2010) Hepatic steatosis, low-grade chronic inflammation and hormone/growth factor/adipokine imbalance. *World J Gastroenterol*, **16**, 4773-4783.

Tschop, M., Weyer, C., Tataranni, P.A., Devanarayan, V., Ravussin, E. & Heiman, M.L. (2001) Circulating ghrelin levels are decreased in human obesity. *Diabetes*, **50**, 707-709.

Vanni, H., Kazeros, A., Wang, R., Harvey, B.-G., Ferris, B., De, B.P., Carolan, B.J., Hübner, R.-H., O'Connor, T.P. & Crystal, R.G. (2009) CIgarette smoking induces overexpression of a fat-depleting gene azgp1 in the human. *Chest*, **135**, 1197-1208.

Wallberg, A.E., Yamamura, S., Malik, S., Spiegelman, B.M. & Roeder, R.G. (2003) Coordination of p300-mediated chromatin remodeling and TRAP/mediator function through coactivator PGC-1alpha. *Mol cell*, **12**, 1137-1149.

Wu, Q. & Suzuki, M. (2006) Parental obesity and overweight affect the body-fat accumulation in the offspring: the possible effect of a high-fat diet through epigenetic inheritance. *Obes Rev*, **7**, 201-208.

Yura, S., Itoh, H., Sagawa, N., Yamamoto, H., Masuzaki, H., Nakao, K., Kawamura, M., Takemura, M., Kakui, K., Ogawa, Y. & Fujii, S. (2005) Role of premature leptin surge in obesity resulting from intrauterine undernutrition. *Cell Metab*, **1**, 371-378.

Zhou, Y., Lee, J., Reno, C.M., Sun, C., Park, S.W., Chung, J., Lee, J., Fisher, S.J., White, M.F., Biddinger, S.B. & Ozcan, U. (2011) Regulation of glucose homeostasis through a XBP-1-FoxO1 interaction. *Nat Med*, **17**, 356-365.

Zou, C., Ma, J., Wang, X., Guo, L., Zhu, Z., Stoops, J., Eaker, A.E., Johnson, C.J., Strom, S., Michalopoulos, G.K., DeFrances, M.C. & Zarnegar, R. (2007) Lack of Fas antagonism by Met in human fatty liver disease. *Nat Med*, **13**, 1078-1085.

Zou, X., Yan, C., Shi, Y., Cao, K., Xu, J., Wang, X., Chen, C., Luo, C., Li, Y., Gao, J., Pang, W., Zhao, J., Zhao, F., Li, H., Zheng, A., Sun, W., Long, J., Szeto, I.M., Zhao, Y., Dong, Z., Zhang, P., Wang, J., Lu, W., Zhang, Y., Liu, J. & Feng, Z. (2014) Mitochondrial dysfunction in obesity-associated nonalcoholic fatty liver disease: the protective effects of pomegranate with its active component punicalagin. *Antioxid Redox Signal*, **21,** 1557-70.