Maternal high-fat diet induces metabolic stress response disorders in offspring hypothalamus

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Abstract

Maternal obesity has been shown to increase the risk of obesity and related disorders in the offspring, which has been partially attributed to changes of appetite regulators in the offspring hypothalamus. On the other hand, endoplasmic reticulum (ER) stress and autophagy have been implicated in hypothalamic neuropeptide dysregulation, thus may also play important roles in such transgenerational effect. In this study, we show that offspring born to high-fat diet-fed dams showed significantly increased body weight and glucose intolerance, adiposity and plasma triglyceride level at weaning. Hypothalamic mRNA level of the orexigenic neuropeptide Y (NPY) was increased, while the levels of the anorexigenic pro-opiomelanocortin (POMC), NPY1 receptor (NPY1R) and melanocortin-4 receptor (MC4R) were significantly downregulated. In association, the expression of unfolded protein response (UPR) markers including glucose-regulated protein (GRP)94 and endoplasmic reticulum DNA J domain-containing protein (Erdj)4 was reduced. By contrast, protein levels of autophagy-related genes Atg5 and Atg7, as well as mitophagy marker Parkin, were slightly increased. The administration of 4-phenyl butyrate (PBA), a chemical chaperone of protein folding and UPR activator, in the offspring from postnatal day 4 significantly reduced their body weight, fat deposition, which were in association with increased activating transcription factor (ATF)4, immunoglobulin-binding protein (BiP) and Erdj4 mRNA as well as reduced Parkin, PTEN-induced putative kinase (PINK)1 and dynamin-related protein (Drp)1 protein expression levels. These results suggest that hypothalamic ER stress and mitophagy are among the regulatory factors of offspring metabolic changes due to maternal obesity.

Key Words

- ▶ maternal obesity
- ▶ hypothalamus
- ▶ ER stress
- ▶ autophagy
- ▶ mitophagy

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Introduction

Obesity is a metabolic disorder characterised by a long-lasting positive energy balance, conservatively affecting 600 million people worldwide (WHO 2015). It is a critical factor leading to the development of various comorbidities such as hypertension and type 2 diabetes mellitus (Guh *et al.* 2009). Multiple factors can contribute

to the development of obesity, among which is the transgenerational effects of maternal obesity. It has been shown in animal models and humans that offspring born to obese mothers tend to have higher risk of obesity and related complications (O'Reilly & Reynolds 2013). Such effect has been partially attributed to dysregulated appetite

and energy metabolism in the offspring (Samuelsson *et al.* 2008, Chen *et al.* 2009, Nivoit *et al.* 2009).

The hypothalamus is the central regulator of appetite and energy homeostasis. It consists of neurons in the arcuate nucleus (ARC) that react to metabolic hormones such as ghrelin, insulin and leptin to orchestrate feeding behaviours and energy expenditure (Yeo & Heisler 2012). In individuals with increased adiposity, the level of leptin secreted by adipose tissues into circulation is increased (Shah & Braverman 2012). The binding of leptin to leptin receptors on hypothalamic neurons leads to the downregulation of orexigenic neuropeptides Agouti-related peptide (AgRP) and Neuropeptide Y (NPY), as well as upregulation of anorexigenic pro-opiomelanocortin (POMC). As a result, hyperphagia is limited. Studies in rat models of maternal obesity showed that offspring exposed to maternal and postnatal high-fat diet (HFD) exhibit increased density of orexigenic peptide-expressing neurons in the hypothalamus (Chang *et al.* 2008). Additionally, hypothalamic mRNA expression of orexigenic neuropeptides overreacts to fasting compared with control offspring (Férézou-Viala *et al.* 2007, Chen *et al.* 2008, Page *et al.* 2009). These results suggest that dysregulation of hypothalamic homeostatic circuitry is a key factor leading to hyperphagia and adiposity by maternal HFD consumption.

The ER is a Ca²⁺-rich intracellular membrane network that is required for protein synthesis, folding and post-translational modifications, as well as lipogenesis. During pathophysiological conditions including obesity, the metabolic stress induced by excess glucose and lipid influx can cause an imbalance between ER workload and capacity, leading to the accumulation of misfolded proteins in the ER lumen (Verfaillie *et al.* 2010). This condition, namely ER stress, subsequently triggers a series of adaptive responses known as unfolded protein response (UPR) to limit protein translation, improve protein-folding capacity, as well as activating autophagy machinery for disposal of misfolded molecules (Yorimitsu *et al.* 2006). Interestingly, hypothalamic ER stress has been suggested to result in leptin resistance, upregulation of NPY and AgRP and disrupted post-translation of POMC-derived peptide in diet-induced obese mice (Ozcan *et al.* 2009, Çakir *et al.* 2013). Moreover, improving ER function by administration of 4-phenyl butyrate (PBA, a FDA-approved chemical chaperone) in dietary obese mice was able to rescue leptin sensitivity to reduce the level of adiposity (Ozcan *et al.* 2009).

Acting in concert with UPR, autophagy is also an adaptive response to metabolic stress (Senft & Ronai 2015),

which can be classified into macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy, the most-studied type of autophagy, is composed of a series of ubiquitination-like reactions to create a vesicle-like structure for engulfment and bulk degradation of misfolded proteins and impaired organelles. When the target organelle is mitochondrion, the process is specifically termed mitophagy. Hypothalamic autophagy, in particular, has been linked to leptin sensitivity and appetite stimulation following the elevation of fatty acids in the blood stream (Quan *et al.* 2012). A hypothalamic autophagy defect has been shown to mediate leptin resistance and hyperphagia in animal model of diet-induced obesity via the IκB kinase β (IKKβ)/NF-κB pathway (Meng & Cai 2011), which is activated following ER stress/UPR elevation (Zhang *et al.* 2008, Meng & Cai 2011, Lim *et al.* 2014). As such, we hypothesised that hypothalamic ER stress and autophagy/mitophagy regulation are important factors implicated in the transgenerational effects of maternal obesity.

Materials and methods

Animals

The study was approved by the Animal Care and Ethics Committee of the University of Technology Sydney (ACEC# 2009-350), and followed the 'Australian code of practice for the care and use of animals for scientific purposes' (NHM&RC, Australia). Female Sprague–Dawley rats (8 weeks) were fed HFD (20 kJ/g, 43.5% calorie as fat, Specialty Feed, WA, Australia) or standard rodent chow (11 kJ/g, 14% calorie as fat, Gordon's Speciality Stockfeeds, NSW, Australia) for 6 weeks before mating, throughout gestation and lactation (Chen *et al.* 2014). On postnatal day (P) 1, litter size was adjusted to 10 pups/litter (sex ratio 1:1). As the impact of maternal obesity on metabolic disorders in offspring has been shown to be more prominent in females (Bayol *et al.* 2008), only female pups were selected for this study. From postnatal day 4 to day 16, half of the female pups from each dam were treated with 4-phenylbutyrate (PBA, 250 mg/kg/day, s.c., Scandinavian Formulas, USA); and the other half with vehicle (vegetable oil, s.c.). The dose was determined according to the previous studies (Özcan *et al.* 2006, Nogueira *et al.* 2011). This yielded four experimental groups, Chow-fed dam's offspring receiving vehicle (MChow-VEH), Chow-fed dam's offspring receiving PBA (MChow-PBA), HFD-fed dam's offspring receiving vehicle (MHF-VEH), and HFD-fed dam's offspring receiving PBA

(MHF-PBA). At weaning (P20), all pups were killed under fasting. Blood was collected via cardiac puncture after anaesthesia (Pentothal, 0.1 mg/g, i.p., Abbott Australasia Pty Ltd). Retroperitoneal fat, gonadal fat, mesenteric fat and liver were weighed. The total fat was reported as the sum of these 3 fat pads. The whole hypothalamus was dissected, snap frozen and stored at -80°C for later analysis. Insulin was measured in the plasma using an ELISA kit (Millipore). The homeostatic model assessment and insulin resistance (HOMA-IR) was calculated using fasting insulin and glucose as previously described (Chen *et al.* 2009).

Intraperitoneal glucose tolerance test (IPGTT)

At P19, the animals were weighed and fasted for 5 h prior to IPGTT (Chen *et al.* 2009), then a glucose solution (50%) was injected (2 g/kg, i.p.). Tail blood glucose level was recorded prior to glucose injection at 15, 30, 60 and 90 min post injection using a glucometer (Accu-Chek glucose meter; Roche Diagnostics). The area under the curve (AUC) was calculated for each animal.

Quantitative real time PCR (qRT-PCR)

Total RNA was isolated from the hypothalamus using Tri Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The purified total RNA was used as a template to generate first-strand cDNA using M-MLV Reverse Transcriptase, RNase H-, Point Mutant Kit (Promega). SYBR Green probes were used for qRT-PCR. To determine appetite control, orexigenic neuropeptides NPY (f: GGC TGT GTG GAC TGA CCC T, r: GAT GTA GTG TCG CAG AGC GG), anorexigenic neuropeptide POMC (f: GAG ATT CTG CTA CAG TCG CTC, r: TTG ATG ATG GCG TTC TTG AA), NPY Y1 receptor (NPY-1R, f: TGA TCT CCA CCT GCG TCA AC, r: ATG GCT ATG GTC TCG TAG TC) and melanocortin-4 receptor (MC4R, f: GAC GGA GGA TGC TAT GAG, r: GCA GGT TCT TGT TCT TGG C) were measured in hypothalamic RNA extracts. To assess ER stress, immunoglobulin-binding protein (BiP), glucose-regulated protein (GRP)94 (f: GAT GGT CTG GAA ACA TGG AG, r: GCC TTG GTG TCG GGT AGA AG), endoplasmic reticulum DNA J domain-containing protein (Erdj)4 (f: CCG AAC AGG ACG AAG GTT G, r: ACT GAC TGT GGA GTT GCC AT), activating transcription factor (ATF)4 (f: GGT TTC TGT CTT CCA CTC CA, r: TAT AAG CAG CAG AGT CAG GC), spliced X-box binding protein 1 (sXBP1, f: CTG AGT CCG AAT

CAG GTG CAG; r: ATC CAT GGG AAG ATG TTC TGG) and C/EBP homologous protein (CHOP, f: CCA CCA CAC CTG AAA GCA GA, r: AGG CGA AAG GCA GAG ACT CA) were examined for mRNA expression. β -Actin was used as the housekeeping gene (f: CGC CAC CAG TTC GCC ATG GA, r: TAC AGC CCG GGG AGC ATC GT). BiP mRNA was measured using Taqman probe (Rn00565250_m1). The average of the control group was arbitrarily assigned as a calibrator against which all other samples were expressed as fold difference.

Western blot

Frozen hypothalamus tissues were homogenised in HEPES buffer (20 mM, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) using TissueRuptor (Qiagen), followed by centrifugation at 1500g for 5 min to pellet down the nucleus and cell debris. The supernatant was collected and centrifuged again at 10,000g for 15 min to isolate cytosolic and mitochondrial fractions. Alternatively, the tissues were homogenised in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) to extract total protein. Protein concentrations were determined and stored at -80°C for further analysis. Proteins were electrophoresed and electroblotted onto the Hybond nitrocellulose membrane (Amersham Pharmacia Biotech), which was then incubated with a primary antibody at 4°C overnight. Antibodies against Atg12–Atg5 complex, Atg7, LC3, protein kinase B (Akt), phosphorylated Akt (pAkt, Thr308), mammalian target of rapamycin (mTOR), phosphorylated mTOR (pmTOR, Ser2448) (1:2000), heat shock protein (Hsp) 90 (1:1000) and Parkin (Prk8, 1:500) are from Cell Signalling. p62, PTEN-induced putative kinase (PINK)1 and dynamin-related protein (Drp)1 (1:2000) are from Novus Biologicals. ER degradation-enhancing alpha-mannosidase-like (EDE) 1 antibody (1:1000) is from Biovision. Mitochondrial oxidative phosphorylation (OXPHOS) complex (I–V) Antibody Cocktail (1:2000) is from Abcam. For housekeeping proteins, COX IV (1:4000, Novus Biologicals) was used to determine mitochondrial proteins; while α -Tub (1:10,000, Sigma-Aldrich) was used to determine proteins in the cytosolic and total protein extracts. All primary antibodies were derived from rabbit except Prk8 and α -Tub antibodies, which are from mouse. Then the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (goat anti-mouse for α -Tub, otherwise goat anti-rabbit). The immunoblots were developed by adding the Luminata Western HRP Substrates (Millipore) to the membrane and

Table 1 Body weight, food intake and organ mass of dams fed with Chow or HFD.

	Chow-fed dam (n=17)	HFD dam (n=17)
Body weight prior to diet (g)	179±2.28	183±2.44
Body weight at mating (g)	250±4.66	287±5.67*
Body weight at weaning (g)	320±15.7	362±10.4*
Food intake (kJ/rat/day)	208±15.56	281±23.37*
Retroperitoneal fat (g)	5.62±1.17	11.9±1.35*
Retroperitoneal fat (%)	1.71±0.27	3.31±0.38*
Paragonadal fat (g)	5.92±0.61	6.23±0.34
Paragonadal fat (%)	1.92±0.16	1.73±0.12
Mesenteric fat (g)	4.44±0.19	5.50±0.53*
Mesenteric fat (%)	1.39±0.05	1.52±0.14
Liver (g)	10.5±0.60	15.1±0.63*
Liver (%)	3.29±0.09	4.19±0.15*

Results are expressed as means±S.E.M. Data were analysed by student's *t*-test.

**P*<0.05.

exposed for an appropriate duration using ImageQuant LAS 4000 (Fujifilm, Tokyo, Japan). ImageJ (National Institutes of Health) was used for densitometry.

Statistical analysis

The results are expressed as mean±S.E.M. Data of IPGTT were analysed by ANOVA with repeat measures followed by Tukey's *post hoc* tests. The other data sets were analysed

by two-way ANOVA, followed by Tukey's *post hoc* tests if there were significant interactions between the maternal and PBA effects. If there was no significant interaction, conditional *t*-test was performed between the treated and non-treated litter mates within the same maternal group. *P*<0.05 was considered significant.

Results

Anthropometric and metabolic characteristics

At mating, HFD-fed dams had significantly greater body weight, fat mass, liver mass and food intake compared to chow-fed dams, and their adiposity persisted until the pups weaned (*P*<0.05, unpaired *t*-test, Table 1). At weaning, the offspring of the HFD-fed dams showed significantly greater body weight than those born to chow-fed dams (*P*<0.01, maternal effect, Table 2). The net and relative mass of the adipose tissues sampled (retroperitoneal, gonadal and mesenteric) as well as liver was significantly greater in the offspring of HFD-fed dams compared to that of the chow-fed dams (*P*<0.01, maternal effect, Table 2). Fasting BGL and plasma triglyceride (TG) levels were significantly increased in offspring of HFD-fed dams (*P*<0.01, maternal effect, Table 2), in consistent with their impaired glucose clearance during IPGTT (*P*<0.05, maternal effect, Fig. 1A and B). Plasma insulin levels and HOMA-IR indexes were also significantly higher in MHF offspring, reflecting insulin resistance (Table 1, *P*<0.05).

Table 2 Effects of maternal HFD and PBA treatment on the anthropometric and metabolic characteristics of offspring at weaning.

	MChow-VEH (n=8)	MChow-PBA (n=10)	MHF-VEH (n=10)	MHF-PBA (n=12)	P value		
					Maternal	PBA	Interaction
Body weight (g)	50.69±1.83	45.57±1.45	61.87±2.38***	57.75±1.00†	<0.001 ^a	<0.01 ^b	0.919
Fasting BGL (mM)	5.69±0.23	5.67±0.18	7.51±0.14***	6.85±0.17	<0.001 ^a	0.070	0.089
Plasma insulin	2.59±1.10	2.94±1.15	10.48±2.98**	5.62±2.27	<0.01 ^a	0.227	0.166
HOMA-IR	0.94±0.42	1.04±0.41	4.44±1.56*	1.96±0.44	<0.05 ^a	0.150	0.182
Plasma triglyceride	0.23±0.02	0.27±0.03	0.60±0.08***	0.72±0.06	<0.001 ^a	0.118	0.466
Total fat (g)	0.53±0.03	0.42±0.04	1.09±0.09***	0.99±0.05††	<0.001 ^a	<0.01 ^b	0.312
Total fat (%)	1.05±0.06	0.93±0.05	1.83±0.07***	1.61±0.07†	<0.001 ^a	0.016	0.465
Retroperitoneal fat (g)	0.04±0.01	0.03±0.00	0.26±0.03***	0.18±0.02†	<0.001 ^a	0.020 ^b	0.046 ^c
Retroperitoneal fat (%)	0.07±0.01	0.07±0.01	0.41±0.03***	0.31±0.03††	<0.001 ^a	0.038 ^b	0.058
Epididymal fat (g)	0.04±0.01	0.03±0.00	0.23±0.04***	0.15±0.01††	<0.001 ^a	0.020 ^b	0.101
Epididymal fat (%)	0.08±0.02	0.06±0.01	0.37±0.05***	0.27±0.02††	<0.001 ^a	0.023 ^b	0.129
Mesenteric fat (g)	0.45±0.02	0.37±0.03	0.65±0.05***	0.60±0.03	<0.001 ^a	0.068	0.684
Mesenteric fat (%)	0.89±0.05	0.80±0.05	1.05±0.06	1.04±0.05	<0.001 ^a	0.376	0.455
Liver (g)	2.16±0.09	1.90±0.08	2.90±0.19***	2.62±0.06	<0.001 ^a	0.026 ^b	0.924
Liver (%)	4.26±0.14	4.16±0.07	4.65±0.16	4.54±0.08	0.003 ^a	0.363	0.965

Results are expressed as means±S.E.M. Data were analysed by two-way ANOVA followed by Tukey's *post hoc* test or conditional *t*-test.

^aMaternal effect; *P*<0.05, ^bPBA effect; *P*<0.05, ^cinteraction between MHF and PBA; *P*<0.05, **P*<0.05, ***P*<0.01, ****P*<0.001 (vs MChow-VEH), †*P*<0.05 (vs VEH controls), ††*P*<0.01 (vs VEH controls).

BGL, blood glucose level.

Figure 1

Glucose tolerance test in offspring at weaning. (A) BGL at different time points post glucose injection. (B) Area under the curve (AUC) of (A). Results are expressed as means $\pm$ s.e.m. ($n=8-10$). Results in (A) were analysed by one-way ANOVA with repeat measures, followed by Tukey's *post hoc* $P<0.01$, $***P<0.001$ (MHF-VEH vs MChow-VEH). Results in (B) were analysed by two-way ANOVA followed by conditional *t*-test. ^a($P<0.05$, overall maternal effect); ^b($P<0.05$, overall PBA effect); ^c($P<0.05$, interaction between MHF and PBA); * $P<0.05$ (vs MChow-VEH), † $P<0.05$ (vs MHF-VEH). MChow-VEH, offspring of Chow-fed dams, treated with vehicles; MChow-PBA, offspring of Chow-fed dams, treated with PBA; MHF-VEH, offspring of HFD-fed dams, treated with vehicles; MHF-PBA, offspring of HFD-fed dams, treated with PBA.

Overall, PBA treatment during the suckling period significantly reduced body weight ($P<0.01$, PBA effect, Table 2). Similarly, the net and percentage of fat mass (total, retroperitoneal and gonadal) in PBA-treated rats was also significantly smaller compared to that of the VEH-treated littermates ($P<0.05$, PBA effect, Table 2), suggesting adiposity was reduced by PBA regardless of the maternal diet. Liver weight was significantly decreased by PBA (PBA effect, $P<0.05$). The percentage reduction in organ weight by PBA was more pronounced among the offspring of the HFD-fed dams than those from chow-fed dams. Additionally, there was a significant interaction between maternal HFD and PBA treatment in reducing retroperitoneal fat (*post hoc* $P<0.01$ MHF-VEH vs MChow-VEH, $P<0.05$ MHF-PBA vs MHF-VEH). The AUC value in MHF-PBA group was 22% lower than MHF-VEH group and nearly normalised to MChow-VEH level (conditional *t*-test $P<0.05$ MHF-PBA vs MHF-VEH, Fig. 1B).

Dysregulated NPY and POMC signalling in MHF offspring hypothalamus

Consistent with previous studies, rat offspring born to HFD-fed dams showed increased hypothalamic mRNA expression of orexigenic peptide NPY ($P<0.01$, Fig. 2A) and reduced mRNA expression of anorexigenic peptide POMC ($P<0.05$, Fig. 2C). Their respective receptors NPY1R and MC4R were also significantly decreased in MHF offspring ($P<0.001$ and $P<0.05$, respectively, Fig. 2B

Figure 2

Hypothalamic mRNA expression of appetite regulators in the offspring at weaning. Orexigenic regulators (A, B) and anorexigenic regulators (C, D). Results are expressed as means $\pm$ s.e.m. ($n=8-12$), and analysed by two-way ANOVA, followed by conditional *t*-test; ^a($P<0.05$, overall maternal effect); ^b($P<0.05$, overall PBA effect); * $P<0.05$, $***P<0.01$ (vs MChow-VEH). MChow-VEH, offspring of Chow-fed dams, treated with vehicles; MChow-PBA, offspring of Chow-fed dams, treated with PBA; MHF-VEH, offspring of HFD-fed dams, treated with vehicles; MHF-PBA, offspring of HFD-fed dams, treated with PBA. NPY (Neuropeptide Y), NPY1R (Y1 receptor), POMC (Pro-opiomelanocortin) and MC4R (melanocortin-4 receptor).

and D). The administration of PBA in MHF neonates had no significant effect on NPY, NPY1R and POMC mRNA expression levels (Fig. 2A, B and C) but normalised the level of MC4R (Fig. 2D).

Regulation of hypothalamic Akt/mTOR signalling in offspring by MHF and PBA

Central mTOR signalling has been shown to regulate food intake (Cota *et al.* 2006). Herein, we show that the protein expression of mTOR and its active form phosphorylated mTOR (pmTOR) were significantly decreased in the offspring hypothalamus due to maternal HFD consumption ($P<0.01$ and $P<0.05$, respectively, Fig. 3C and D). Akt, lying upstream of mTOR, was also suppressed ($P<0.05$, Fig. 3A). PBA administration increased the level of Akt, pAkt and mTOR in MHF offspring (Fig. 3A, B and C) but had minor effect on pmTOR expression (Fig. 3D). In opposition, PBA administration had negative effects on mTOR and pmTOR expression in MChow offspring.

Figure 3

Hypothalamic Akt/mTOR signalling in the offspring at weaning. Results are expressed as means $\pm$ S.E.M. ($n=4$) and analysed by two-way ANOVA followed by Tukey's (if P -interaction <0.05) or conditional t -test; $\dagger$ ($P < 0.05$, interaction between MHF and PBA); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (vs MChow-VEH), $\dagger\dagger P < 0.01$ (vs MHF-VEH). Akt (protein kinase B), mTOR (mechanistic target of rapamycin), pAkt (phosphorylated Akt) and pmTOR (phosphorylated mTOR).

The ratio between the phosphorylated and total Akt or mTOR was not significantly different among the groups.

Selective regulation of hypothalamic markers of unfolded protein response in the offspring by MHF and PBA

It has been shown that during ER stress, Akt/mTOR signalling is downregulated, mediating the upregulation of autophagy (Qin *et al.* 2010). First, to determine the effects of maternal obesity on offspring hypothalamic ER stress, we examined several downstream markers of UPR, including BiP, Erdj4, Grp94, ATF4, sXBP1 and CHOP. Our results show that Erdj4 and Grp94 mRNA levels were significantly reduced in the offspring of the HFD-fed dams ($P < 0.001$, respectively, Fig. 4B and C), while ATF4 was significantly increased ($P < 0.05$, maternal HFD effect, Fig. 4D). The mRNA expression of sXBP1 was slightly increased (non-significantly), and CHOP was not changed (Fig. 4E and F). These are consistent with the protein expression reflected by the immunoblot results (Fig. 4G and H). The protein expression of Hsp90 was unchanged by maternal HFD consumption (Fig. 4I); whereas EDEM1 was significantly suppressed ($P < 0.05$, Fig. 4K).

PBA administration significantly increased hypothalamic mRNA expression of BiP independent

of maternal diets ($P < 0.05$, PBA effect, Fig. 4A). The expression of ATF4 was also increased but not significantly ($P = 0.06$, PBA effect, Fig. 4D). In addition, MHF offspring treated with PBA have a significantly higher level of Erdj4 compared to those treated with vehicle control ($P < 0.05$, Fig. 4B). Both PCR and Western blot results indicate the same trend of normalisation of sXBP1 in MHF-PBA offspring (Fig. 4E and G). No effect of PBA on CHOP mRNA or protein expression was detected (Fig. 4F and H). PBA administration significantly increased the level of Hsp90 and reduced the level of EDEM1 in the MChow offspring ($P < 0.05$, Fig. 4I and K). Such effect was not evidence in the MHF offspring.

Regulation of hypothalamic autophagy markers in the offspring by MHF and PBA

Hypothalamic protein levels of Atg7 and Atg12-Atg5 complex were significantly increased in offspring of HFD-fed dams ($P < 0.05$ and $P < 0.01$, respectively) compared to those of chow-fed dams (Fig. 5A and B). LC3-I protein level was significantly reduced due to maternal HFD consumption ($P < 0.05$ maternal effect, Fig. 5C). LC3-II/LC3-I ratio and p62 level were not significantly changed (Fig. 5D). PBA administration in the MHF offspring did not change the hypothalamic levels of any Atg proteins

Figure 4

Hypothalamic mRNA and protein levels of ER markers in the offspring at weaning. (A) mRNA expression ($n=6$). (B) Protein expression ($n=4-6$). Results are expressed as means $\pm$ S.E.M. and analysed by two-way ANOVA followed by conditional t -test; $a(P<0.05$, overall maternal effect); $b(P<0.05$, overall PBA effect); $*P<0.05$, $***P<0.001$ (vs MChow-VEH), $\dagger P<0.05$ (vs MHF-VEH). MChow-VEH, offspring of Chow-fed dams, treated with vehicles; MChow-PBA, offspring of Chow-fed dams, treated with PBA; MHF-VEH, offspring of HFD-fed dams, treated with vehicles; MHF-PBA, offspring of HFD-fed dams, treated with PBA. BiP (immunoglobulin-binding protein), sXBP1 (spliced X-box binding protein 1) and CHOP (C/EBP homologous protein).

examined, but significantly reduced LC3-I and increased LC3-II as well as the LC3-II/LC3-I ratio ($P<0.05$, Figs 4E and 5C). In the MChow-PBA offspring, only p62 protein level was significantly reduced ($P<0.05$, Fig. 5F), while other autophagy markers including Atg7 and LC3-II showed a trend of increase ($P=0.12$ and $P=0.14$, respectively, Figs 4D and 5A).

Regulation of hypothalamic mitophagy by MHF and PBA

Mitophagy has important roles in maintaining mitochondrial structure and function. Our result indicated that overall, maternal HFD consumption increased the hypothalamic protein expression of mitophagy markers PINK1 (Fig. 6A) and Prk8 (Fig. 6B) in the offspring. PBA administration, on the other hand, significantly reversed such maternal effect ($P<0.05$, Fig. 6A and $P<0.01$, Fig. 6B,

respectively). Additionally, mitochondrial fission marker Drp1 protein expression was also significantly reduced by PBA administration in the offspring of HFD-fed dams ($P<0.01$, Fig. 6C).

Regulation of mitochondrial OXPHOS complexes by MHF and PBA

The OXPHOS complexes are essential components of the mitochondria, which constantly drive electron transport chain for ATP production. Herein, we show that the levels of mitochondrial OXPHOS complex III (Fig. 7C) and V (Fig. 7E) were suppressed ($P<0.05$, maternal HFD effect). No change was observed in the other complexes (Fig. 7A, B and D). PBA administration did not improve the hypothalamic levels of these complexes in MHF offspring (Fig. 7), although it

Figure 5

Hypothalamic protein levels of autophagy markers in the offspring at weaning. Results are expressed as means $\pm$ S.E.M. ($n=4-6$) and analysed by two-way ANOVA followed by conditional *t*-test; ^a($P<0.05$, overall maternal effect); ^b($P<0.05$, overall PBA effect); * $P<0.05$, *** $P<0.001$ (vs MChow-VEH), [†] $P<0.05$ (vs MHF-VEH). MChow-VEH, offspring of Chow-fed dams, treated with vehicles; MChow-PBA, offspring of Chow-fed dams, treated with PBA; MHF-VEH, offspring of HFD-fed dams, treated with vehicles; MHF-PBA, offspring of HFD-fed dams, treated with PBA. Atg (Autophagy-related gene), LC3 (microtubule-associated protein 1A/1B-light chain 3) and p62 (sequestosome 1).

Figure 6

Hypothalamic protein levels of mitophagy markers in the offspring at weaning. Results are expressed as means $\pm$ S.E.M. ($n=4-6$) and analysed by two-way ANOVA followed by Tukey's (if *P*-interaction <0.05) or conditional *t*-test; ^b($P<0.05$, overall PBA effect); ^c($P<0.05$, interaction between MHF and PBA); * $P<0.05$ (vs MChow-VEH), [†] $P<0.05$, ^{††} $P<0.01$ (vs MHF-VEH). MChow-VEH, offspring of Chow-fed dams, treated with vehicles; MChow-PBA, offspring of Chow-fed dams, treated with PBA; MHF-VEH, offspring of HFD-fed dams, treated with vehicles; MHF-PBA, offspring of HFD-fed dams, treated with PBA. Prk8 (Parkin), PINK1 (PTEN-induced putative kinase 1), Drp1 (dynamin-related protein 1).

increased the level of OXPHOS complex I in the MChow offspring (Fig. 7A).

Discussion

Maternal obesity due to long-term HFD consumption is associated with an increased risk of obesity in the offspring, partially attributed to fetal programming of metabolic and appetite control by the hypothalamus (Chen *et al.* 2009, Nivoit *et al.* 2009). The current study showed that these programming effects are associated with multiple metabolic stress responses in the hypothalamus of the offspring, including UPR, autophagy and mitophagy. In addition, early administration of PBA, a chemical chaperone shown to relieve ER stress, can attenuate these adverse effects induced by maternal obesity in female offspring.

Consistent with previous studies (Chen *et al.* 2008, Chan *et al.* 2015), the offspring from obese dams gained more weight and fat at weaning, reflecting the effects of maternal HFD consumption on nutrient influx especially lipid influx during gestation and lactation (Zhu *et al.* 2010). In association, the hypothalamic level of the orexigenic peptide NPY was increased in the MHF offspring, while the level of the anorexigenic peptide

Figure 7

Hypothalamic protein expression of mitochondrial oxidative phosphorylation (OXPHOS) complex I–V in the offspring at weaning. Results are expressed as means $\pm$ S.E.M. ($n=4-6$) and analysed by two-way ANOVA followed by Tukey's (if P -interaction <0.05) or conditional t -test; ^b($P<0.05$, overall PBA effect); ^c($P<0.05$, interaction between MHF and PBA); * $P<0.05$ (vs MChow-VEH), [†] $P<0.05$, ^{††} $P<0.01$ (vs MHF-VEH). MChow-VEH, offspring of Chow-fed dams, treated with vehicles; MChow-PBA, offspring of Chow-fed dams, treated with PBA; MHF-VEH, offspring of HFD-fed dams, treated with vehicles; MHF-PBA, offspring of HFD-fed dams, treated with PBA.

POMC was downregulated, reflecting an imbalance in appetite regulation that favours food intake (Bergen *et al.* 1999, Chen & Morris 2009). Inconsistent with the reduced POMC level, the reduction of MC4R is another indicator of anorexigenic signalling suppression. On the other hand, the downregulation of NPY1R appears to conflict with the increased NPY level, which may reflect an automatic feedback regulation of the orexigenic signalling within the offspring hypothalamus.

Together with the alteration in appetite and metabolic markers in the MHF offspring, several ER stress/UPR markers were also affected. Erdj4 and Grp94 were markedly reduced, while ATF4 was slightly upregulated. Upon accumulation of misfolded proteins, BiP molecules detach from the three ER transmembrane proteins including PRKR-like ER kinase (PERK), inositol-requiring protein 1 α (IRE1 α) and activating transcription factor 6 α (ATF6 α), leading to three pathways of UPR induction. Grp94 is an endoplasmic reticulum-specific isoform of heat shock protein (Hsp)90 (Marzec *et al.* 2012), which stabilizes the interactions of BiP with the cytoplasmic domains of the three main UPR inducing factors, leading to suppression of ER stress and UPR (Marcu *et al.* 2002). As such, its depletion in MHF offspring hypothalamus is a clear indicator of ER stress. Erdj4 is a BiP cochaperone that has been suggested to be involved in endoplasmic reticulum-associated degradation (ERAD) of protein (Lai *et al.* 2012). The hypothalamic reduction of this protein in MHF offspring hypothalamus suggests reduced ERAD activity and hence higher risk of misfolded protein accumulation. ATF4 is an important transcriptional factor of multiple UPR components including XBP1, Erdj4 and Grp94, thus the increased mRNA level of this marker is

likely to reflect a compensatory response to the reduced protein manufacturing capacity due to maternal HFD consumption. BiP and sXBP1 are also positive regulators of ER chaperones (Hetz & Mollereau 2014). While the expression of BiP remained unchanged, the level of hypothalamic sXBP1 appears to be slightly increased by maternal HFD consumption, supporting the implication of ER stress and UPR activation. Similar observation of sXBP1 upregulation has been reported in the male offspring (Melo *et al.* 2014). On the other hand, GRP94 was upregulated in males (Melo *et al.* 2014), which is opposite to the change in the females in this study. The level of CHOP, a pro-apoptotic marker, was normal in the MHF female offspring, suggesting that the apoptotic branch of the ER stress responses had not yet been activated. As expected from an UPR activator, PBA significantly increased the levels of chaperone BiP, Hsp90 and Erdj4 in the offspring, suggesting improved chaperone availability, protein-folding capacity, hence reduced ER stress. Interestingly, it also reduced the level of EDEM1, an ERAD marker, in the MChow offspring. This result may indicate that PBA does not only improve protein folding and stability but also attenuates their degradation through modulating ERAD activity. The association of these changes with improved metabolic indexes in the offspring suggests a role of hypothalamic UPR in the transgenerational effects of maternal diets on the offspring.

mTOR is an essential nutrient sensor for feeding regulation in the hypothalamus. Its expression and activity have been shown to be suppressed following 48 h of fasting and recovered upon refeeding (Cota *et al.* 2006). Moreover, central-specific activation of the signalling pathway by ι -leucine led to the suppression of food

intake (Cota *et al.* 2006). In this study, the hypothalamic expression of both phosphorylated and total Akt/mTOR was suppressed by maternal HFD consumption, while the ratio between the two was not significantly changed. This suggests that the signalling was downregulated in total expression and activity, whereas the specific activity (phosphorylation state per amount of expression) was rather unaffected. In this study, PBA as an UPR activator significantly reduced the hypothalamic levels of mTOR and pmTOR in MChow offspring, which can be expected as UPR activation has been shown to inhibit mTOR signalling (Qin *et al.* 2010). By contrast, when administered in MHF offspring, it actually relieved the suppression of Akt and mTOR induced by maternal HFD consumption. Similar findings have been found in adipose tissue of obese mice treated with PBA, which was linked to improved insulin sensitivity (Guo *et al.* 2017). These results suggest that Akt/mTOR signalling is likely to be implicated in increased weight gain and lipid storage in the MHF offspring at weaning. Neonatal PBA administration can partially rescue the signalling, thereby contributing to the attenuation of such effects.

The autophagy machinery closely interacts with UPR to rescue cells from the accumulation of non-functional misfolded proteins and metabolic stress (Senft & Ronai 2015). On the other hand, it is also negatively regulated by mTOR signalling (Jung *et al.* 2010). In the study, autophagy markers were selectively upregulated by maternal HFD consumption with significant changes in Atg5 and Atg7 but no change in LC3 and p62. Our results are in line with a previous study in male offspring which reported that hypothalamic LC3 and p62 levels in the offspring of the HFD dams were similar to those in the control offspring, although they were impaired at birth (Reginato *et al.* 2016). The selective changes in autophagy markers are likely to reflect a compensation to the suppression of UPR and ERAD markers in the offspring hypothalamus. PBA administration did not change the levels of Atg proteins but improved the LC3-I conversion to LC3-II, suggesting an increase in autophagosomal degradation of misfolded proteins. As mTOR is a well-known negative regulator of autophagy, offspring hypothalamic autophagy can also be regulated through the suppression of mTOR signalling. In addition, hypothalamic autophagy has also been linked to increased food intake (Kaushik *et al.* 2011), which aligns with the increased NPY and reduced POMC mRNA expression in the MHF offspring.

Mitophagy is the autophagy mechanism specific for the disposal of dysfunctional/depolarised mitochondria. During this process, PINK1 and Prk8 are recruited to

the outer membrane of the impaired mitochondrion, leading to ubiquitination, autophagosomal engulfment and degradation. Dysfunctional mitochondria undergo mitochondrial fission (Youle & Van Der Bliek 2012), which is mediated by a number of fission factors including Drp1. Similar to autophagy, hypothalamic levels of mitophagy markers PINK1 and Prk8 were also moderately upregulated in the offspring of obese dams, suggesting that more hypothalamic mitochondria were damaged and hence became more susceptible to autophagosomal engulfment. Indeed, hypothalamic mitochondrial OXPHOS complexes III and V were found to be reduced in the MHF offspring, suggesting functional impairment (Fig. 7). This is well supported by a previous study in *ob/ob* mice where reduced OXPHOS complex III and V was associated with impaired mitochondrial respiration rate (Boudina *et al.* 2005). These changes in mitophagy and mitochondrial complexes might in turn disturb energy metabolism, leading to the dysregulation of metabolic markers. Supporting this hypothesis, Prk8 knockout mice exhibited resistance to weight gain and improved insulin sensitivity with reduced hepatic fat uptake and adipocyte differentiation, although without significant change in the intake of HFD (Kim & Sack 2012). In contrast to autophagy markers, mitophagy markers (PINK1, Prk8 and Drp1) were significantly attenuated by PBA administration in the offspring of obese dams. This may imply that mitochondrial homeostasis, together with UPR and autophagy, form a network in hypothalamic regulation of energy homeostasis (Zorzano & Claret 2015).

In this study, PBA treatment during the suckling period significantly improved metabolic phenotypes in the offspring of obese dams, including improved fat and glucose metabolism. Normalised appetite regulator expression and neuronal responses to metabolic stress by PBA may be a key mechanism, although the peripheral actions of PBA cannot be excluded (Kawasaki *et al.* 2012). As PBA is also a histone deacetylase inhibitor, it has the potential to modify the neonatal metabolism through epigenetic regulation. Although a recent study suggested that the primary acting of PBA in neuronal ER stress is as a chemical chaperone rather than histone deacetylase inhibitor (Mimori *et al.* 2013), the possibility of its implication in intergenerational epigenetic modifications by maternal obesity still needs to be examined in future studies.

Although the association between maternal HFD and hypothalamic ER stress in the offspring has been partially investigated by Melo and coworkers (Melo *et al.* 2014), the study was conducted on male offspring only,

which is the commonly chosen gender for investigation on metabolic regulation; whereas females have not been well studied in the literature. The novelty of our study is in part because we studied female mice which may also transmit the phenotype to the next generation. The importance of using female offspring was based on a recent publication from our group (Chan *et al.* 2015). That said, further studies involving both sexes are worthwhile to specifically address the possible sex-dependence in the regulation of hypothalamic UPR/autophagy/mitophagy in response to maternal HFD consumption, which may underline the distinct risk of obesity between males and females (Power & Schulkin 2008). More importantly, we explored the relevance of appetite-regulating factors with UPR and other stress responses including autophagy and mitophagy. We showed 4-PBA as a therapeutic candidate capable of attenuating these abnormalities. Our results shed some light on the re-purposing of 4-PBA in the prevention of metabolic disorders caused by maternal obesity. To our knowledge, these aspects have not been published in the setting of maternal HFD consumption.

Conclusion

Maternal obesity altered metabolic homeostasis in the offspring inducing weight gain, adiposity, glucose tolerance and insulin sensitivity. These changes were associated with not only alteration in appetite regulators but also markers of metabolic stress response mechanisms such as UPR, autophagy and mitophagy in the hypothalamus, which could represent some of the earliest metabolic changes that mediate the development of obesity later in life. Administration of PBA from postnatal day 4 to day 16, a critical developmental period for hypothalamic neural projection in neonates, is likely to have positive effects on hypothalamic metabolic stress response and its regulation of energy homeostasis, reflected by improved metabolic outcomes in the offspring. Whether such treatment can have long-term impacts on the health outcome of such offspring requires further investigation.

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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