Maternal high-fat diet induces hypothalamic metabolic stress responses and autophagy in the offspring

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Short title: maternal obesity & hypothalamic stress responses

Key words: Maternal obesity, hypothalamus, ER stress, autophagy, mitophagy

Word count: 6212

Abstract

Maternal obesity has been shown to increase the risk of obesity and related disorders in the offspring. The molecular responses to metabolic stress in the hypothalamus, the central region of appetite regulation, may underline this mechanism. To test this hypothesis, female Sprague-Dawley rats (8 weeks old) were fed a high-fat diet (HFD) for 6 weeks prior to mating and throughout gestation and lactation. From postnatal day 4, half of the pups in each litter were treated with 4-phenyl butyrate (PBA), a chemical chaperone of protein folding. At weaning, offspring from the HFD-fed dams (MHF) showed significantly increased body weight and glucose intolerance, adiposity and plasma triglyceride level. Hypothalamic mRNA levels of both orexigenic neuropeptide Y (NPY) and anorexigenic pro-opiomelanocortin (POMC) were significantly upregulated. mRNA expression of unfolded protein response markers spliced X-box binding protein (sXBP)1, protein levels of autophagy-related genes Atg5 and Atg7, as well as mitophagy marker Parkin, were significantly upregulated; while Protein kinase B/Mammalian target of rapamycin (Akt/mTOR) signalling was suppressed in the MHF offspring. PBA administration in the offspring significantly reduced their body weight, fat deposition, and normalised their hypothalamic levels of NPY, POMC, Akt, mTOR and mitophagy markers, which were associated with improved sXBP1 and Microtubule-associated protein light chain 3 (LC3)-II/LC3-I ratio. These results suggest that cellular metabolic stress responses are likely to contribute to the transgenerational effects of maternal obesity on appetite and energy homeostasis.

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), ultimately resulting in approximately 2.8 million deaths every year worldwide (WHO 2015). 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.* 2008a, Nivoit *et al.* 2009, Chen *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 have shown that offspring exposed to maternal and postnatal high-fat diet (HFD) exhibited increased density of orexigenic peptide-expressing neurons in the hypothalamus (Chang *et al.* 2008). Additionally, hypothalamic mRNA expression of orexigenic neuropeptides overreact to fasting compared with control offspring (Férézou-Viala *et al.* 2007, Page *et al.* 2009, Chen *et al.* 2008). These results suggest that dysregulation of hypothalamic

homeostatic circuitry is a key factor leading to hyperphagia and adiposity by maternal HFD consumption.

The endoplasmic reticulum (ER) is a Ca^{2+}-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 means of *in vitro* overexpression of spliced X-box binding protein 1 (sXBP1, a fundamental transcription factor of chaperones for protein folding), or administration of 4-phenyl butyrate (PBA, a FDA approved chemical chaperone) in *ob/ob* and 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 ubiquination-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. Autophagy-related gene/protein (Atg) 7 is one of the most important proteins in this process, where it acts as an E1-like

activating enzyme to initiate two main branches: one for autophagosome formation via Atg12-Atg5 complex and the other for autophagosome maturation by lipidation of Microtubule-associated protein light chain 3 (LC3). The fusion of autophagosome and lysosome for degradation is then facilitated by the binding of sequestome 1 (p62) to LC3 (Pankiv *et al.* 2007). 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, Lim *et al.* 2014, Meng & Cai 2011). ARC specific inhibition of such pathways was able to attenuate obesity related phenotypes (Benzler *et al.* 2015). As such, we hypothesised that central ER stress and autophagy are important factors implicated in the transgenerational effects of maternal obesity.

Materials and methods

1. 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). Randomly selected 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 4-16, half of the female pups from each dam were randomly selected and

118 treated with 4-phenylbutyrate (PBA, 250mg/kg/day, s.c, Scandinavian Formulas, USA); and the
119 other half with vehicle (vegetable oil). The dose was determined according to (Özcan *et al.*
120 2006, Nogueira *et al.* 2011). This yielded four experimental groups, Chow-fed dam's offspring
121 receiving vehicle (MChow-VEH), Chow-fed dam's offspring receiving PBA (MChow-PBA),
122 HFD-fed dam's offspring receiving vehicle (MHF-VEH), and HFD-fed dam's offspring
123 receiving PBA (MHF-PBA). At weaning (P20), all pups were sacrificed under fasting. Blood
124 was collected via cardiac puncture after anaesthesia (Pentothal®, 0.1mg/g, i.p., Abbott
125 Australasia Pty Ltd, NSW, Australia). Retroperitoneal fat, gonadal fat, mesenteric fat, and liver
126 were weighed. The total fat was reported as the sum of these 3 fat pads. The whole
127 hypothalamus was dissected, snap frozen, and stored at -80°C for later analysis. Insulin was
128 measured in the plasma using an ELISA kit (Millipore, MA, USA). All animals were assigned a
129 cull ID and all the samples were analysed in a blind manner. The data were only grouped
130 according to the cull IDs when the statistical analysis was performed.
131
132 The Homeostatic model assessment and insulin resistance (HOMA-IR) was calculated using
133 fasting insulin and glucose as previously described (Chen et al. 2009). Power calculation was
134 used to determined the sample size. The primary comparison of interest is the difference in
135 mean fat mass between 2 groups; i. female offspring from the control dams, and ii. offspring
136 from the HFD-fed dams. Based on our previous studies, the body of control offspring was
137 30.18g with a standard deviation 1.02 at weaning, while the body of offspring from HFD-fed
138 dam was 39.35g with a standard deviation of 4.51. Using a t-test at a 2-sided 0.05 level, 8 mice
139 per group will provide 73% power, and 12 mice per group will provide 88% power. Therefore a
140 minumn sample size of 8 was determined for each group, and minimum one pup was randomly
141 selected from each litter for each experimental group to ensure genetic variation.
142
143 *2. Intraperitoneal glucose tolerance test (IPGTT)*

At P19, the animals were weighed and fasted for 5 hours prior to IPGTT (Chen et al. 2009), then a glucose solution (50%) was injected (2g/kg, ip). 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, Nutley, USA). The area under the curve (AUC) was calculated for each animal. The rats were excluded if the blood glucose level failed to double at 15 minutes post glucose injection.

3. Quantitative real time PCR (qRT-PCR)

Total RNA was isolated from all hypothalamus using Tri Reagent (Sigma Aldrich, VIC, Australia) 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, WI, USA). Pre-optimised TaqMan primers (Applied Biosystems, CA, USA, sequence of the probes in Table 1) were used for qRT-PCR (Eppendorf Realplex 2, Eppendorf AG, Hamburg, Germany). To determine appetite control, orexigenic neuropeptides NPY and AgRP, anorexigenic neuropeptide POMC, NPY Y1 receptor (NPY-1R) and melanocortin-4 receptor (MC4R), single minded gene (Sim)1, and suppressor of cytokine signalling (SOCS)3 were measured in hypothalamic RNA extracts. To assess ER stress, immunoglobin-binding protein (BiP), unspliced and spliced X-box binding protein 1 (XBP1 and sXBP1), and C/EBP homologous protein (CHOP) were examined for mRNA expression. The probes for target genes other than sXBP-1 were labelled with FAM and the housekeeping gene 18s were labelled with VIC. sXBP1 (f: CTG AGT CCG AAT CAG GTG CAG; r: ATC CAT GGG AAG ATG TTC TGG) was amplified with SYBR Green probes. Gene expression was quantified in a single multiplexing reaction, where target genes were standardized to 18s rRNA. The average of the control group was arbitrarily assigned as a calibrator against which all other samples were expressed as fold difference.

4. 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, Hilden, Germany), followed by centrifugation at 1,500 g for 5 min to pellet down the nucleus and cell debris. The supernatant was collected and centrifuged again at 10,000 g for 15 min to isolate cytosolic and mitochondrial fractions. Alternatively, the tissues were homogenised in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, MA, USA) 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, Amersham, UK), 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), Mammalian target of rapamycin (mTOR), phosphorylated mTOR (pmTOR) (1:2000), and Parkin (Prk8, 1:500) are from Cell Signalling (MA, USA). p62, PTEN-induced putative kinase (PINK)1 and Dynamin-related protein (Drp)1 (1:2000) are from Novus Biologicals (CO, USA). Mitochondrial oxidative phosphorylation (OXPHOS) complex Rodent Western Blotting Antibody Cocktail (1:2000) is from Abcam (VIC, Australia). For housekeeping proteins, COX IV (1:4000, Novus Biologicals, CO, USA) was used to determine mitochondrial proteins; while α-Tub (1:10000, Sigma-Aldrich, MO, USA) 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, MA, USA) to the membrane and exposed for an appropriate duration using ImageQuant LAS 4000 (Fujifilm, Tokyo, Japan). ImageJ

(National Institutes of Health, USA) was used for densitometry. Randomly selected samples were used for the reprentative blots.

5. Statistical analysis

The results are expressed as mean ± SEM. Data of IPGTT were analysed by ANOVA with repeat measures followed by Turkey post hoc tests. The other data sets were analysed by Two-way ANOVA, followed by Turkey 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

Body weight, adiposity, food intake and glucose intolerance in the offspring were increased by MHF and reduced by PBA

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 2). 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 3). 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 3. Fasting BGL and plasma triglyceride (TG) levels were significantly increased in offspring of HFD-fed dams ($P < 0.01$, maternal effect, Table 3), in consistence with their impaired glucose clearance during IPGTT ($P < 0.05$, maternal effect, Figure 1A, B). Plasma insulin levels and HOMA-IR indexes were also significantly higher in MHF offspring, reflecting insulin resistance (Table 1, $P < 0.05$).

Overall, PBA treatment during the suckling period significantly reduced body weight ($P < 0.01$, PBA effect, Table 3). 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 3), 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, Figure 1B).

Upregulated NPY and POMC expression in MHF offspring was normalised by PBA

Consistent with previous studies, rat offspring born to HFD-fed dams showed increased hypothalamic mRNA expression of orexigenic peptide NPY ($P < 0.01$, Figure 2A). However, no significant difference in the mRNA expression of its receptor NPY1R and its orexigenic partner AgRP was detected (Figue 2B, 2C). Regarding anorexigenic signalling, POMC mRNA was significantly upregulated ($P < 0.01$, Figure 2D), whereas the expression of its receptor MC4R was not significantly changed (Figure 2, Figure 2D). Interestingly, the administration of PBA in MHF neonates nearly normalised the hypothalamic mRNA levels of both NPY and POMC (conditional t test $P < 0.05$, MHF-PBA vs MHF-VEH, Figure 2A,B). The treatment caused no change in the AgRP and Sim1 mRNA expression levels (Figure 2C, F).

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

To determine the effects of maternal obesity on offspring hypothalamic ER stress, we examined several downstream markers of UPR, including BiP, XBP1, sXBP1 and CHOP. The first three are positive regulators of protein stability, and CHOP is known as a pro-apoptic marker associated with severe ER stress (Schönthal 2012). Our results showed that hypothalamic mRNA expression of XBP1, sXBP1 and CHOP were significantly upregulated in the offspring of the HFD-fed dams at weaning ($P < 0.05$ and $P < 0.001$, respectively, Figure 3A). The protein levels of sXBP1 showed a non-significant trend of increase while CHOP protein levels are not different among the groups (Figure 3B).

PBA administration significantly increased hypothalamic mRNA expression of BiP, XBP1 and sXBP1 independent of maternal diet ($P < 0.05$, Figure 3A). The combination of maternal HFD and PBA treatment in the offspring led to a further upregulation of hypothalamic sXBP1 mRNA expression in the offspring ($P < 0.05$, Figure 3A). By contrast, the protein level of sXBP1 was normalised to the level of MChow-VEH offspring (Figure 3B). Hypothalamic CHOP protein level was unchanged by PBA treatment (Figure 3B).

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 (Figure 4A,B). LC3-I protein level was significantly reduced due to maternal HFD consumption ($P < 0.05$ maternal effect, Figure 4C). LC3-II/LC3-I ratio and p62 level were not significantly changed (Figure 4D). PBA administration in the MHF offspring did not change the hypothalamic levels of any Atg proteins examined, but significantly reduced LC3-I and increased the LC3-II/LC3-I ratio ($P < 0.05$, Figure 4C, 4E). In the MChow-PBA offspring, only p62 protein level was significantly reduced ($P < 0.05$, Figure 4F), while other autophagy

markers including Atg7 and LC3-II showed a trend of increase ($P = 0.12$ and $P = 0.14$ respectively, Figure 4A, 4D).

Regulation of hypothalamic Akt/mTOR signalling in offspringby MHF and PBA

Akt and mTOR are essential nutrient sensors which stimulate protein synthesis and cellular growth, with Akt lying upstream of mTOR. It has been shown that during ER stress, Akt/mTOR signalling is downregulated, mediating the upregulation of autophagy (Qin *et al.* 2010). Indeed, out study deninstrates that in association with elevated expression of the ER stress markers, hypothalamic protein expression of both Akt and mTOR was significantly reduced in the offspring of MHF dams ($p < 0.05$, Figure 5A and $p < 0.01$, Figure 5C respectively). In addition, the level of phosphorylated mTOR (pmTOR) was also downregulated by maternal HFD consumption ($p < 0.05$, Figure 5D), suggesting reduced activaty of the signialling pathway. PBA administration in the MHF offspring normalised hypothalamic Akt, pAkt and mTOR levels but did not significantly reverse the level of pmTOR (Figure 5). By contrast, in the MChow offspring, PBA significantly reduced the levels of pmTOR ($P < 0.001$, Figure 5D).

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 (Figure 6A) and Prk8 (Figure 6B) in the offspring. PBA administration, on the other hand, significantly reversed such maternal effect ($P < 0.05$, Figure 6A and $P < 0.01$, Figure 6B respectively). Additionally, mitochondrial fission marker Drp1 protein expression was also significantly reduced by PBA administration in the offspring of HFD-fed dams ($P < 0.01$, Figure 6C).

Regulation of mitochondrial OXPHOS complexes by MHF and PBA

The OXPHOS complexes catalyse electron transport chain for the production of ATP. Consistent with the increase in mitophagy markers in MHF offspring, the levels of mitochondrial OXPHOS complex III (Figure 7C) and V (Figure 7E) on mitochondrial membrane were also suppressed ($P < 0.05$, maternal effect), while no change was observed in the other complexes (Figure 7A, B, D). PBA administration did not improve the hypothalamic levels of these complexes in MHF offspring (Figure 7), although it increased the level of OXPHOS complex I in the MChow offspring (Figure 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 unfolded protein response (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, hypothalamic levels of the orexigenic peptide NPY were increased in the MHF offspring, in line with increased milk intake observed in such offspring in previous studies (Bergen *et al.* 1999, Chen & Morris 2009). Interestingly, the anorexigenic peptide POMC was also upregulated, which is opposite to what we have previously demonstrated in male offspring (Bergen et al. 1999, Chen & Morris 2009). Female offspring tend to have lower fat mass compared to male offspring due to the effect of maternal obesity (Samuelsson *et al.* 2008b).

Since POMC suppresses feeding, the distinct regulation of its hypothalamic expression between the two genders may be a contributing factor for such gender difference in adiposity. Although the dysregulation of NPY and POMC is generally linked to impaired leptin signalling in obese animals (Friedman & Halaas 1998), our previous study indicated that during the suckling period, central leptin sensitivty was not affected by HFD-consumption in rat dams (Chan et al. 2015). Supporting this finding, the current study found no significant change in hypothalamic leptin receptor (lep-R) expression in the MHF offspring (Supplemetary data S1). Additionally, lep-R expression was upregulated by PBA administration in control animals, in line with a previous study (Ozcan et al. 2009). This effect was diminished by maternal HFD consumption (Supplemetary data S1), suggesting the impairment of hypothalamic PBA-mediated actions by maternal HFD consumption.

Together with the alteration in appetite and metabolic markers in the MHF offspring, hypothalamic ER stress markers sXBP1 and CHOP were upregulated. sXBP1 is a positive transcriptional factor of ER chaperones known to assist in protein folding (Hetz & Mollereau 2014). The elevation of hypothalamic sXBP1 at both transcriptional and translational levels in the female offspring is likely to reflect an adaptive response to increased ER protein load due to maternal obesity. In addition, sXBP1 can also bind to CHOP promoter to improve its transcription, which explains why CHOP mRNA expression was concomitantly increased. Although increased CHOP abundance is considered detrimental to neuronal survival due to its induction of apoptosis (Oyadomari & Mori 2003), the protein has also been demonstrated to be essential in preventing hypoxic injuries (Halterman *et al.* 2010). Despite increased mRNA expression, CHOP protein level was unchanged, suggesting that the level of hypothalamic ER stress induced by maternal HFD consumption in the offspring was not severe. Collectively, maternal HFD-consumption can induce ER stress in the offspring hypothalamus, which may play a role in the development of childhood obesity (Melo *et al.* 2014).

Autophagy machinery closely interacts with UPR to rescue cells from the accumulation of non-functional misfolded proteins and metabolic stress (Senft & Ronai 2015). As such, it is expected to observe an increase in autophagy components in the setting of increased ER stress . Indeed, Atg7 and Atg12-Atg5 complex, two major constituents of the autophagosome, were significantly increased in offspring of obese dams. As LC3-I/II and p62 expression remained normal, autophagosome degradation was likely to be unaffected. Similar to UPR, enhanced autophagy activity is likely to be an adaption against the accumulation of cellular misfolded/damaged proteins in the hypothalamus due to maternal obesity. In addition, hypothalamic autophagy has also been linked to increased food intake (Kaushik *et al.* 2011), which aligns with the increased NPY mRNA expression in the MHF offspring.

The Akt/mTOR signalling pathway stimulates protein synthesis and inhibits protein degradation. In this study, it was downregulated in the MHF offspring. Together with the elevation of UPR and autophagy markers, such change suggests possible interactions among these pathways to prevent misfolding protein accumulation in the hypothalamic cells of the MHF offspring. Indeed, it has been shown that upon ER stress and UPR activation, mTOR signalling is inhibited, leading to the initiation of autophagy (Qin et al. 2010). Given the established role of hypothalamic mTOR as a nutrient sensor (Cota *et al.* 2006), its correlation with UPR and autophagy markers further supports the implication of the latter two in hypothalamic regulation of energy homeostasis.

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 (Figure 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). To our knowledge, this is the first study to show a link between hypothalamic autophagy/mitophagy and obesity-related phenotypes in the offspring due to maternal obesity.

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 can not 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.

PBA administration increased hypothalamic mRNA expression of BiP and sXBP1. As both BiP and sXBP1 are positive regulator of protein stability, their increase can assist protein folding and relieve ER stress (Bertolotti *et al.* 2000). Despite the increase in mRNA level, the protein level of sXBP1 in the MHF-PBA offspring was comparable to baseline. This is most probably due to the fact that sXBP-1 protein has a short life (Calfon *et al.* 2002) and its abundance has a negative effect on the regulation of sXBP-1 mRNA stability (Majumder *et al.* 2012). Hence, in early stress responses, sXBP1 protein synthesis is repressed at baseline so that sXBP1 mRNA molecules can accumulate. In the later phase, the relief of such repression results in a boost of sXBP1 protein level as part of the stress response program (Majumder et al. 2012). In this study, the mismatch between sXBP1 mRNA and protein levels in the MHF-PBA offspring may therefore imply early UPR.

Regarding autophagy, PBA significantly increased the LC3-II/LC3-I ratio, suggesting an overall increase in the turnover rate of misfolded proteins and impaired cellular organelles. However, the lack of concomitant elevation in hypothalamic expression of Atg7 and Atg12-Atg5 complex implies that autophagosome formation rate may not be altered. As ER stress is one of the main drivers of autophagy (Yorimitsu et al. 2006), the subtle change of LC3-II/LC3-I ratio in the MHF-PBA group could be due to the effect of PBA which was shown ER stress in the offspring.

In contrast to the moderate elevation of 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 homeostatsis (Zorzano & Claret 2015).

Conclusion

Maternal obesity altered metabolic homeostasis in the offspring inducing weight gain, adiposity, glucose tolerance and insulin sensitivity. These changes were not only associated with 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.

Funding

Long Nguyen was supported by Sydney Medical School's ECR PhD Scholarship and Amgen research scholarship. This study and Dr Yi Tan were supported by a Gift Grant Awarded to Dr Hui Chen from Perpetual Ramaciotti Foundation, Australia.

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Figure legends:

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 ± SEM (n = 8-10). Results in A were analysed by one-way ANOVA with repeat measures, followed by Turkey 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.

Figure 2. Hypothalamic mRNA expression of appetite regulators in the offspring at weaning. Orexigenic regulators (A, B, C) and anorexigenic regulators (D, E, F). Results are expressed as means ± SEM (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) , †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. NPY (Neuropeptide Y), NPY1R (Y1 receptor); POMC (Pro-opiomelanocortin); MC4R (melanocortin-4 receptor); AgRP (Agouti-related peptide); Sim1 (Single-minded homolog 1)

Figure 3. Hypothalamic mRNA and protein levels of ER markers in the offspring at weaning. A. mRNA expression (n = 8 – 12). B. Protein expression (n = 5). Results are expressed as means ± SEM 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. BiP (immunoglobin binding protein), XBP1 (X-box binding protein 1), sXBP1 (spliced XBP1) and CHOP (C/EBP homologous protein).

Figure 4. Hypothalamic protein levels of autophagy markers in the offspring at weaning. Results are expressed as means ± SEM (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), p62 (Sequestosome 1).

Figure 5. Hypothalamic Akt/mTOR signalling in the offspring at weaning, Results are expressed as means ± SEM (n = 4) and analysed by Two-way ANOVA followed by Turkey (if P-interaction < 0.05) or conditional t-test; c (P<0.05, interaction between MHF and PBA); *P<0.05, **P<0.01, ***P<0.001 (vs MChow-VEH), ††P<0.01 (vs MHF-VEH). Akt (Protein kinase B), mTOR (mechanistic target of rapamycin), pAkt (phosphorylated Akt), pmTOR (phosphorylated mTOR).

Figure 6. Hypothalamic protein levels of mitophagy markers in the offspring at weaning. Results are expressed as means ± SEM (n = 4 - 6) and analysed by Two-way ANOVA followed by Turkey (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).

Figure 7. Hypothalamic protein expression of mitochondrial oxidative phosphorylation (OXPHOS) complex I-V in the offspring at weaning. Results are expressed as means ± SEM (n = 4 - 6) and analysed by Two-way ANOVA followed by Turkey (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.

Tables

Table 1. TaqMan probe sequence (Applied Biosystem, 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	GCAGAGGTGCTAGATCCAC AGAACC	Rn01431703_g1
NPY	NM_012614.1	GCCCGCCCGCCATGATGCTA GGTAA	Rn00561681_m1
POMC	NM_139326.2	AAGCAACCTGCTGGCTTGC ATCCGG	Rn00595020_m1
Y1 receptor	NM_001113357.1, Z11504.1	TTCATATGCTACTTCAAGAT ATACG	Rn01402912_g1
MC4R	NM_013099.2	AGCAGAAGCCTGATTCCAC TGTTTA	Rn01491866_s1
Sim 1	XM_228329.4, NM_001107641.1	CCTGGACTCCAGGGTAGCA GAGCTG	Rn01440876_g1
XBP-1	NM_001004210.1, BC079450.1	CCTCTTCAGATTCTGAGTCT GATAT	Rn01752572_g1
BiP	NM_013083.1, M14050.1, BC062017.1	AACAATCAAGGTCTACGAA GGTGAA	Rn00565250_m1
CHOP	NM_001109986.1, NM_024134.2, XM_006241444.2, XM_006241445.2	AGGAAACGAAGAGGAAGA ATCAAAA	Rn00492098_g1

Table 2. Body weight, food intake and organ mass of dams fed with Chow or HFD

	Chow-fed dam (n=9)	HFD dam (n=9)
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 *
Epididymal fat (g)	5.92 ± 0.61	6.23 ± 0.34
Epididymal 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 ± SEM. Data were analysed by student t-test. *P < 0.05

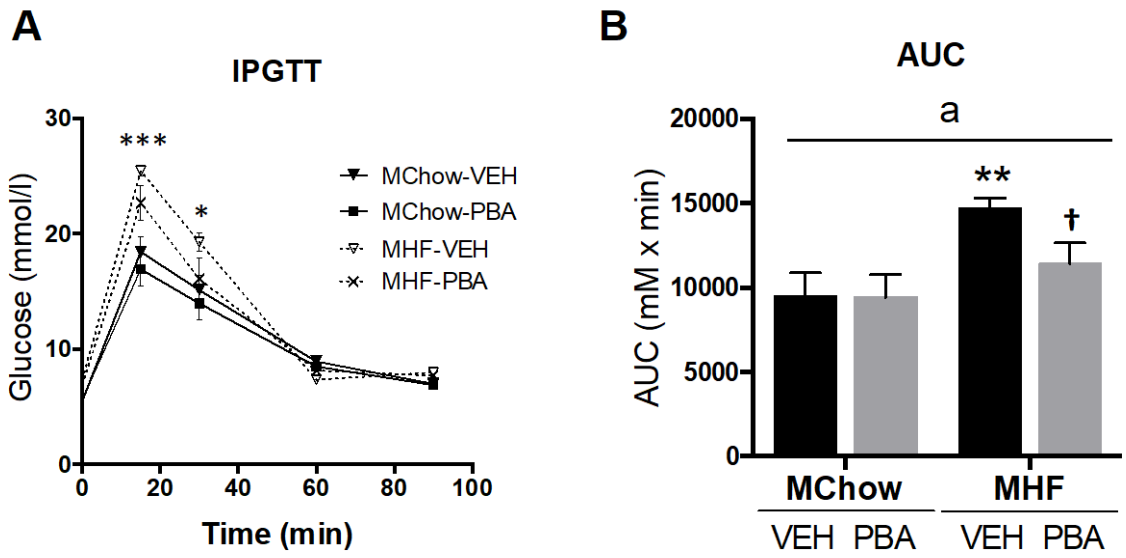
Table 3. Effects of maternal HFD and PBA treatment on offspring body weight, organ mass, blood glucose, insulin and triglyceride levels at weaning.

	MChow-VEH	**MChow-PBA**	**MHF-VEH**	**MHF-PBA**	**P value**		
	n = 8	n = 10	n = 10	n = 12	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 ± SEM. Data were analysed by Two-way ANOVA followed by Turkey post hoc test or conditional t-test.

a (maternal effect; $P < 0.05$), b (PBA effect; $P < 0.05$), c (interaction 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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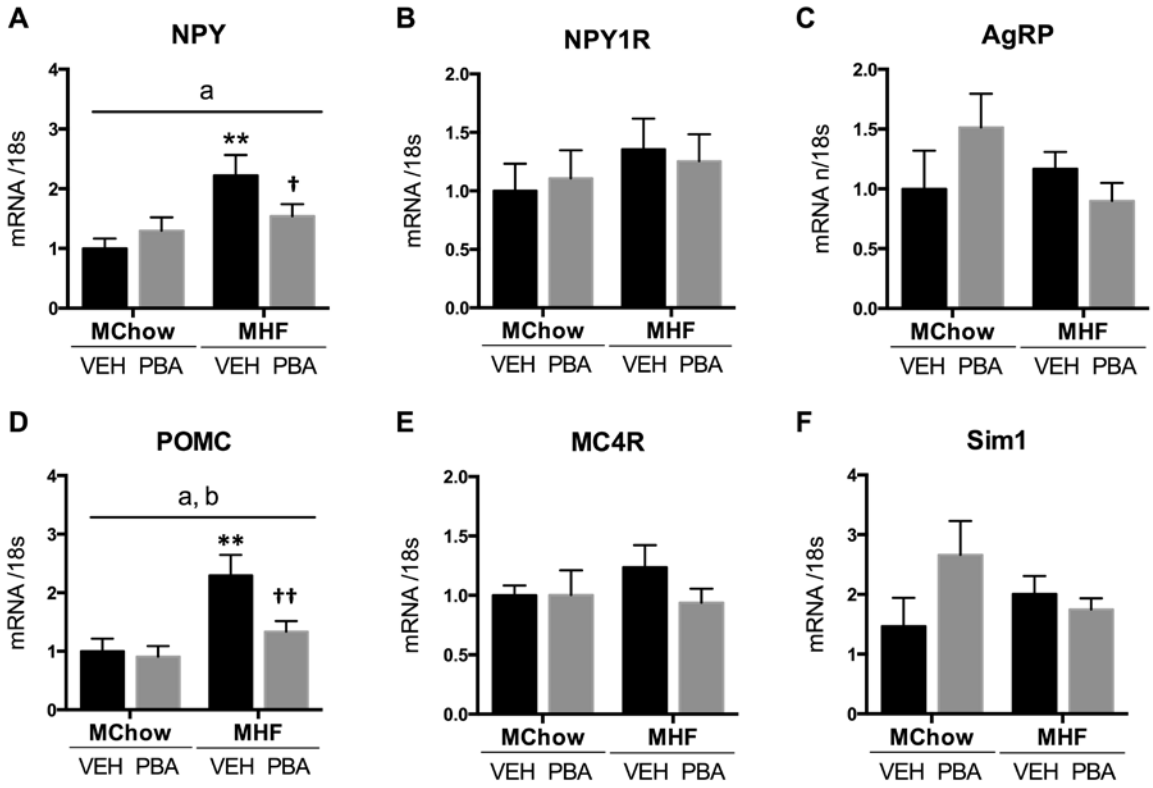
VEH PBA
VEH PBA

Figure 2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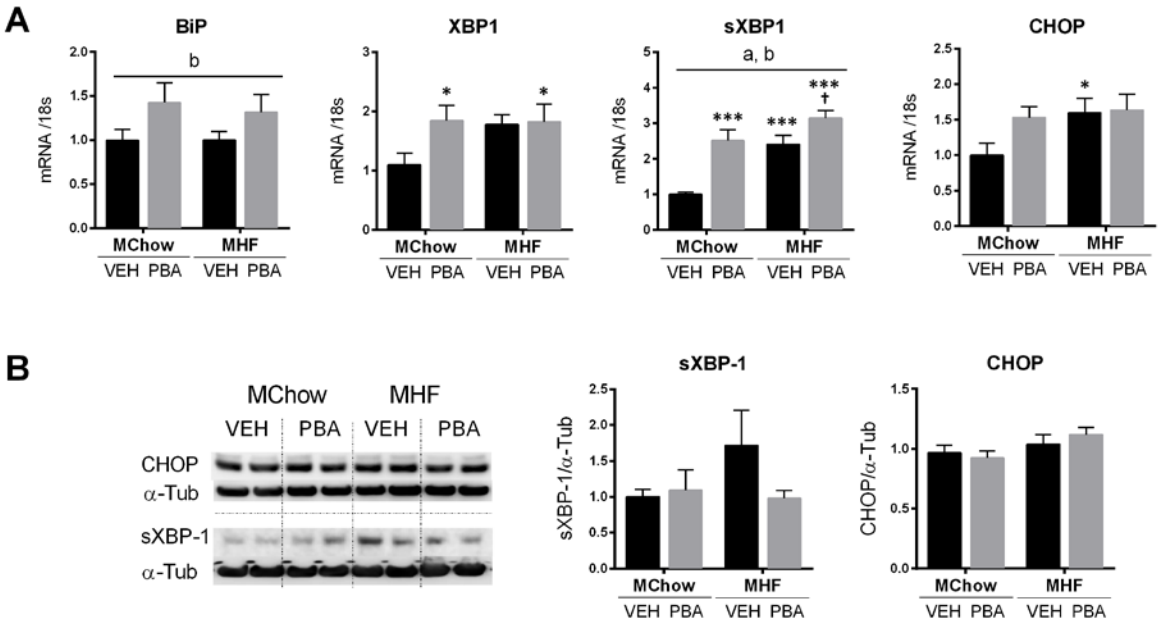
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Figure 4

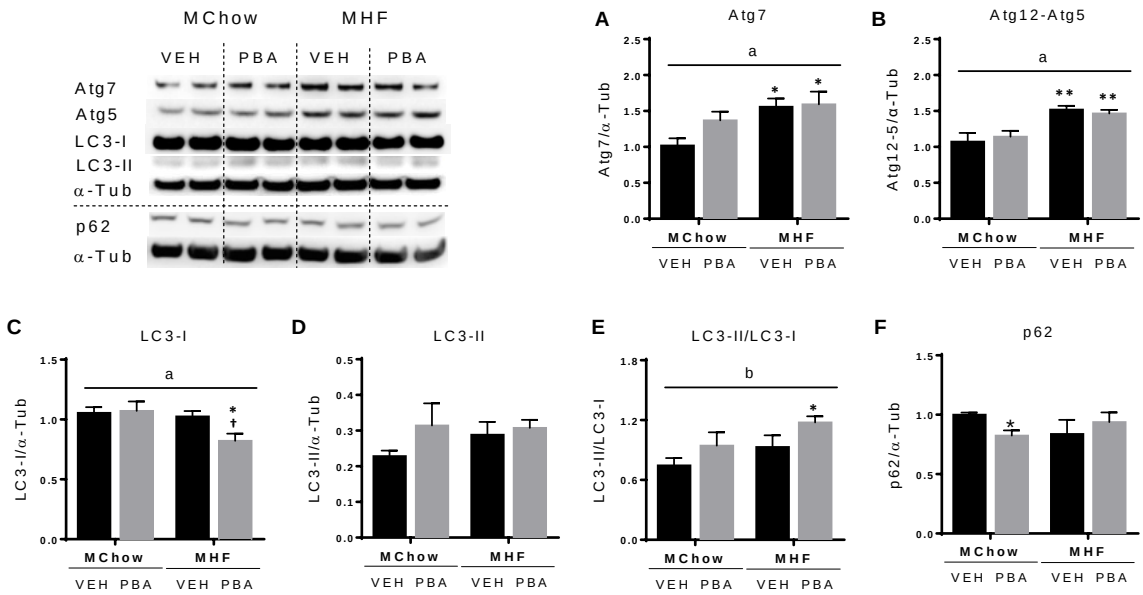
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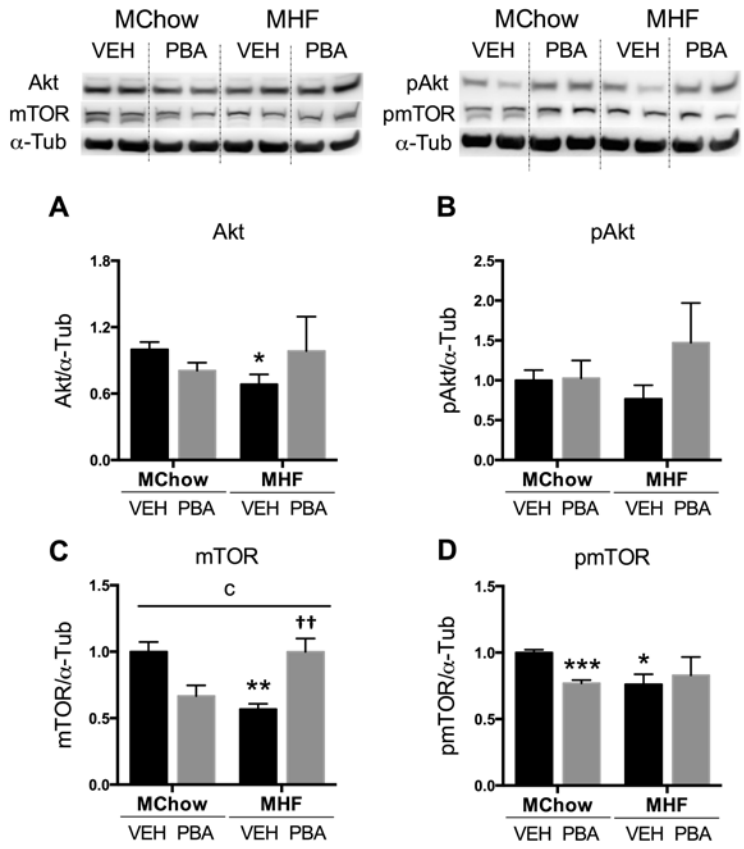
Figure 5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Akt/α-Tub
1.8
1.2
0.6
0.0
*
MChow
MHF
VEH PBA VEH PBA
B
pAkt
pAkt/α-Tub
2.5
2.0
1.5
1.0
0.5
0.0
MChow
MHF
VEH PBA VEH PBA
C
mTOR
c
††
**
mTOR/α-Tub
1.5
1.0
0.5
0.0
MChow
MHF
VEH PBA VEH PBA
D
pmTOR
pmTOR/α-Tub
1.5
1.0
0.5
0.0
***
*
MChow
MHF
VEH PBA VEH PBA

Figure 6

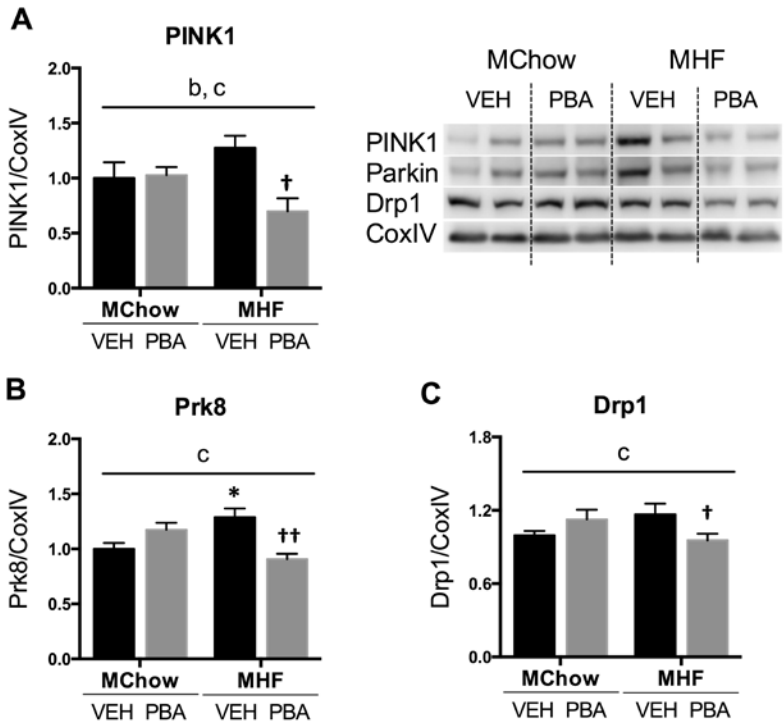
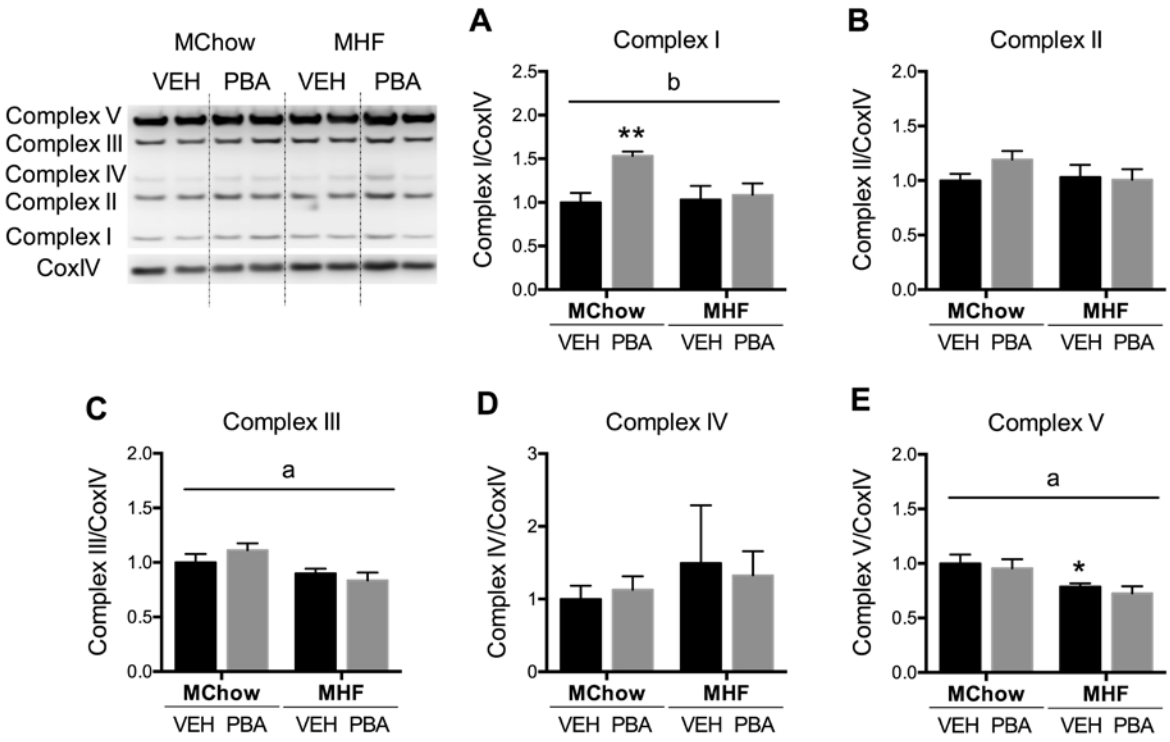

A
PINK1
b, c
PINK1/CoxIV
2.0
1.5
1.0
0.5
0.0
†
MChow
MHF
VEH PBA VEH PBA
MChow
MHF
VEH
PBA
VEH
PBA
PINK1
Parkin
Drp1
CoxIV
B
Prk8
c
Prk8/CoxIV
*
††
C
Drp1
c
Drp1/CoxIV
1.8
1.2
0.6
†

Figure 7


MChow
MHF
VEH
PBA
VEH
PBA
Complex V
Complex III
Complex IV
Complex II
Complex I
CoxIV
A
Complex I
b
**
Complex I/CoxIV
B
Complex II
Complex II/CoxIV
C
Complex III
a
Complex III/CoxIV
D
Complex IV
Complex IV/CoxIV
E
Complex V
a
*
Complex V/CoxIV
MChow
MHF
VEH PBA VEH PBA