



Maternal high fat diet programs hypothalamic-pituitary-adrenal function in adult rat offspring

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ABSTRACT

Maternal environmental factors such as diet have profound effects on offspring development and later health. The hypothalamic-pituitary-adrenal (HPA) axis is an important stress neuroendocrine system that is subject to programming by early life challenges. The present study was further to investigate whether maternal high fat diet (HFD) exposure during rat pregnancy and lactation can alter the HPA axis activity in adult male offspring. We observed that maternal HFD consumption exerted long-term effects on the basal activity of the HPA axis in adult offspring, with increased mean plasma corticosterone levels that result from elevated steroid pulse frequency and pulse amplitude. More importantly, maternal HFD offspring displayed enhanced corticosterone responses to restraint (1 h) and lipopolysaccharide (25 µg/kg, iv) but not insulin-induced hypoglycemia (0.3U/kg, iv) stress, suggesting a stressor-specific effect of maternal diet on the hyperresponsiveness of the HPA axis to stress. Additionally, maternal HFD exposure markedly attenuated the habituation of HPA responses to repeated restraint stress. These findings demonstrate that perinatal HFD exposure has a potent and long-lasting influence on development of neuroendocrine regulatory mechanisms. Maternal HFD consumption significantly increased basal corticotropin-releasing factor (CRF) mRNA expression in the paraventricular nucleus; nevertheless, similar increments in CRF mRNA levels following restraint were observed between maternal HFD offspring and control rats. Furthermore, the medial and central nuclei of amygdala played a pivotal role in maternal HFD-induced sensitization of the HPA response to psychological and systemic stress, respectively, suggesting that different neural pathways may mediate maternal HFD-induced HPA hyperresponsivity to different types of stressors. Taken together, the long-term effects of maternal HFD challenge on the central regulation of the HPA axis, therefore, expose the adult offspring to greater HPA function throughout lifespan, in stressor-specific and region-specific manner.

1. Introduction

Epidemiological and animal studies suggest that, besides the parental heredity and adult lifestyle, the specific environmental factors that a developing offspring experiences in early life may also have a tremendous impact on the development and health problems throughout the life (Barker, 2004). Early life challenges such as mother-infant separation and neonatal immune challenge has been reported to increase adult susceptibility to obesity, insulin resistance, hypertension and cardiovascular dysfunction (Gluckman and Hanson, 2004; Craft et al., 2006; Warner and Ozanne, 2010; Liang et al., 2011). Therefore,

although developmental plasticity in physiological systems is an important mechanism by which organisms can adapt their responses to fit environmental demands for immediate survival, such alterations may cause long-lasting changes in the responses to environmental challenges and alter predisposition to health problems in later life.

Maternal diet and metabolic status are important factors which influence the intrauterine and early postnatal environment that offspring experiences in early life. Nowadays, the most common maternal dietary imbalance is over intake of dietary fat in many countries. There is accumulating evidence supporting that perinatal overnutrition or high fat diet (HFD) consumption may result in developmental and health

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problems, such as metabolic syndrome, hypertension, cardiovascular remodeling and cerebrovascular dysfunction (Khan et al., 2004; Alfaradhi and Ozanne, 2011; Lin et al., 2017). Moreover, maternal HFD exposure also confers offspring susceptibility to behavioral disorders and mental problems, including anxiety, depression, cognitive deficit, impairments in social behavior and reward-based behaviors (Lin et al., 2015).

As the core mediator of the neuroendocrine stress response, the hypothalamic-pituitary-adrenal (HPA) axis is subject to programming by early life challenges. Shanks and colleagues reported that animals exposed to neonatal endotoxin are more stress responsive as adults, exhibiting increased adrenocorticotropic hormone (ACTH) and corticosterone responses to restraint stress compared with saline-treated controls (Shanks et al., 2000). Moreover, prenatal restraint exposure in rats also induces long-lasting neuroendocrine alternations including disruption of circadian rhythms and impaired feedback mechanisms of the HPA axis in offspring (Maccari and Morley-Fletcher, 2007). Animal studies also suggested that maternal diet and metabolic status continuously affect the HPA axis activity throughout the lifespan. Nevertheless, the results of a limited number of previous studies of developmental HFD exposure on HPA axis activity have been mixed. Trotter and coworkers reported that increased fat intake during lactation decrease corticosterone responsiveness to ether vapors in developing rat pups, but enhance ACTH stress response in 35-day-old offspring (Trotter et al., 1998). However, rats fed a HFD starting mid-pregnancy have male offspring with increased basal corticosterone levels at post-natal day 10 (D'Asti et al., 2010). More recently, Abuaish and colleagues reported that perinatal HFD induces early activation of endocrine stress responsivity and anxiety-like behavior during the stress hyporesponsive period (Abuaish et al., 2018). Whereas as for adult offspring, Sasaki et al. reported that rat dams consuming a HFD during pregnancy and lactation had offspring with decreased basal corticosterone levels, but a heightened response to restraint stress (Sasaki et al., 2013). Whilst in another study, adult offspring of HFD-fed mother demonstrate elevated circulating levels of corticosterone (Walker et al., 2008). Our previous study also reported that maternal HFD exposure significantly increases HPA profile response to acute restraint stress, but does not influence the mean basal plasma corticosterone level in adult male offspring (Lin et al., 2015). The effect of maternal HFD exposure on the activity of the HPA axis in adult offspring have been unclear due to these conflicting findings.

The paraventricular nucleus (PVN) of the hypothalamus directly modulates the HPA axis activity, whilst the limbic amygdaloid complex has been implicated in many aspects of the neuroendocrine responses during stress (Dunn and Whitener, 1986). Boitard and colleagues reported that, adolescent, but not adult, HFD exposure leads to enhanced amygdala function (Boitard et al., 2015, 2016). Moreover, perinatal HFD alters the expression of the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in the limbic areas including amygdala in both adolescent and adult offspring, which are known to regulate circulating corticosterone levels during basal and stress conditions (Sasaki et al., 2013, 2014). Furthermore, in accordance with the notion that different types of stressors activate distinct neuronal pathways (Dayas et al., 2001; Pacak and Palkovits, 2001), numerous studies have implicated that the effect of the amygdala on the HPA axis is stressor- and region-specific (Herman et al., 2005). Out of a number of subdivisions of the amygdala, its medial (MeA) and central nuclei (CeA) play key roles in psychogenic and systemic stress-induced activation of the HPA axis activity, respectively. Therefore, it is worth to explore whether dietary environment during development also program the amygdala relevant for the regulation of the HPA function in stressor- and region-specific manner.

In the current study, we hypothesized that maternal HFD exposure would program the HPA axis activity in adult offspring under non-stressed and stressful conditions. Moreover, we examined whether maternal HFD program the HPA response in a stressor-specific manner.

To test the hypothesis, the neuroendocrine response to different types of stress paradigms, including psychological (restraint), systemic (lipopolysaccharide, LPS) and metabolic (insulin-induced hypoglycemia, IIH) stress, were assessed in adult male offspring of fat-fed dams during pregnancy and lactation. Additionally, repeated psychological stress-induced habituation of HPA axis activity in maternal HFD offspring was also investigated. Moreover, we investigated the corticotropin-releasing factor (CRF) mRNA expression in the PVN of maternal HFD offspring under stress-free and stressed conditions. The further goal of the present study was to evaluate the involvement of amygdala in maternal diet-induced alternation of HPA responses to stress. In details, we explored whether neurotoxic lesions specific to the MeA attenuate maternal HFD-induced HPA hypersensitivity to psychological stress and compare the response with commonly used systemic stressor. A differential response to these two stress paradigms in CeA-lesioned maternal HFD animals was also explored.

2. Materials and methods

2.1. Animals and surgical procedures

Female Sprague–Dawley rats (120–140 days), obtained from Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China), were housed under a 12 h:12 h light-dark cycle (lights on at 0600 h) at 22 ± 2 °C temperature and provided with food and water ad libitum. The dam rats were fed with either a standard control diet [5.3% fat (corn oil), 21.2% protein, 57.4% carbohydrate, 4.6% fiber; Medicience Ltd., JiangSu, China] or HFD (25.7% fat, 19.5% protein, 41.3% carbohydrate, 3.5% fiber; estimated fats: palmitic acid 4.5%, stearic acid 1.99%, palmitoleic acid 0.12%, oleic acid 6.86%, linoleic acid 2.58%, α -linolenic acid 0.25%, arachidonic acid 0.19%; Medicience Ltd.), for 10 days before mating and throughout pregnancy and lactation. All animals were bred in house, and litters were culled evenly to 8 pups on the day of parturition (day 0), to standardize competition for food and maternal attention. Because the present study focused on the effect of maternal diet on the HPA axis in male offspring, we included 6 males and 2 females in each litter (except 2 of the control and 3 HFD-fed litters in which only 5 males were delivered). All litters were weaned on day 21 and housed 3 animals per cage. All offspring were fed a control diet until 15–16 weeks of age, thereafter, to obtain control offspring fed with control diet (C/C) and offspring from fat-fed dams fed with control diet (HFD/C), and were split across experimental groups. Animal weight was recorded weekly post-weaning. Experiments were performed according to the Regulations of the Chinese Council on Animal Care, approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University and reported following the ARRIVE guidelines. All surgical procedures were carried out under ketamine anesthesia (100 mg/kg i.p.; Pharmacia and Upjohn Ltd, Crawley, UK) and xylazine (10 mg/kg i.p.; Bayer, Leverkusen, Germany). The rats were fitted with cardiac catheters via the jugular veins, for collection of blood samples without interruption in the experiment (Lin et al., 2011).

2.2. Basal corticosterone determination

On the morning of experimentation, the adult offspring rats were attached via the cardiac catheter to a computer-controlled automated blood sampling system, which allows for the intermittent withdrawal of small blood samples for corticosterone measurement without disturbing the animals. Basal secretion patterns of corticosterone in adult offspring rats were assessed over 24 h (Shanks et al., 2000). Small blood samples (15 μ l) were taken every 10 min over 24 h starting at 0800 (lights on 0600) and frozen at -20°C for later assay to determine corticosterone levels. The patterns of basal corticosterone secretion were analyzed for 6 h into each of the light and dark cycles and over the 24 h of blood sampling.

2.3. HPA profile response to differential stressors

On the morning of experimentation, the rats were attached to the automated blood sampling system, and patterns of corticosterone response to stress in adult offspring rats were assessed over 4 h. Blood samples were collected every 10 min, starting 10 min before the onset of stress. The stressors were performed between 0800 and 0900, avoiding the elevation of basal corticosterone levels and peak HPA responses to stress associated with the dark phase of the cycle. For psychological stressor, animals were placed in restraint devices for 60 min (Lin et al., 2011). In the repeated restraint stress, the same experimental regimen was repeated 4 times at 5-day intervals, but with much less sampling time points. For the immunological stressor, animals were injected with a bolus of LPS (25 µg/kg in 0.3 ml saline, Sigma-Aldrich) via the second iv catheter. For the metabolic stress, the rats were fasted overnight but were provided with water ad libitum throughout. After controlled blood sampling insulin (0.3 IU/kg in 0.3 ml of saline) was administered through the second iv catheter in rats. Blood glucose was monitored during the experiment to determine the hypoglycemia (Lin et al., 2011).

2.4. The involvement of the amygdala sub-nuclei in maternal HFD-induced changes in HPA responsiveness to stress

To evaluate the involvement of amygdala in maternal HFD-induced modulation of the HPA axis response to stress, offspring rats received bilateral MeA or CeA excitotoxic lesions via bilateral injections of 0.4 µl of ibotenic acid (10 µg/µl in sterile 0.1 M sodium phosphate buffer, pH 7.4; Sigma-Aldrich Ltd, Poole, UK) using a 25 gauge 1 µl Hamilton injection syringe, following the co-ordinates: 3.3 mm lateral, 2.6 mm posterior to Bregma and 8.7 mm below the surface of the dura for the MeA; 4.1 mm lateral, 2.5 mm posterior to Bregma and 7.7 mm below the surface of the dura for the CeA (Paxinos and Watson, 1986), at 15 weeks of age. Sham lesions were carried out using the same procedure but with sterile artificial cerebrospinal fluid (aCSF). After a 10-day recovery period, the rats were implanted with jugular cannulae. Thereafter, CeA-lesioned rats and MeA-lesioned offspring were exposed to restraint or LPS injection, and corticosterone response were assessed.

2.5. Corticosterone radioimmunoassay

Total corticosterone concentrations in plasma were measured directly in heparinized plasma through radioimmunoassay, by using a citrate buffer at pH 3.0 to denature the binding globulin (1 µl of plasma fraction diluted to 100 µl with buffer, antiserum and ¹²⁵I-labeled corticosterone (North Bio, BeiJing, China) with a specific activity of 4–5 mCi/µg (1 mCi = 37 MBq). The sensitivity of the assay was 0.075 ng/ml. The intraassay variation was 6.4% and the interassay variation was 8.0%.

2.6. In situ hybridization

A separate group of rats were enrolled for the determination of CRF mRNA in the PVN under nonstressed and stressful conditions. In situ hybridization was performed as previously reported (Bowe et al., 2008). Coronal sections (15 µm) were cut through the area of the PVN, using identifiable anatomical landmarks according to a rat brain atlas (Paxinos and Watson, 1986), mounted on gelatin-coated slides, and stored at –80 °C. Before hybridization, sections were thawed and allowed to dry at room temperature for 10 min, and then fixed in 4% paraformaldehyde for 5 min. Following three phosphate-buffered saline washes, sections were acetylated for 10 min in 0.1 M triethylamine in 0.9% saline containing 0.025% acetic anhydride. Sections were then dehydrated, and delipidated in chloroform for 5 min. Sections were rehydrated in a series of ethanol baths and left to dry.

For determination of CRF mRNA, the probe used was a 48-mer

cDNA oligoprobe complementary to part of the exonic portion the CRF mRNA coding sequence. Probes were labelled at the 3'-end with ³⁵S-dATP using terminal deoxyribonucleotidyl transferase (Boehringer, Mannheim) following the manufacture's instructions. All sections were hybridized in the same hybridization reaction. In brief, sections were hybridized with a labelled probe (1 × 10⁶ cpm/section) using a hybridization buffer containing 50% deionized formamide, 0.5 mg/ml sheared salmon sperm DNA, 0.25 mg/ml yeast tRNA, 10 mM DTT and 100 mg/ml dextran overnight at 37 °C. Hybridized sections were placed in X-ray cassettes and then apposed to autoradiographic film (Kodak Biomax MS¹, Sigma-Aldrich) for 10 days, together with ¹⁴C-labelled standards of known radioactivity (Amersham International plc) to compensate for the nonlinear response of the film to radioactivity. The relative optical density of the autoradiographic images of CRF mRNA was measured by computer-assisted densitometry (Scion Image, NIH). For each rat, an average integrated optical density was obtained, consisting of at least four anatomically matched brain slices (every fourth sections). CRF mRNA levels are expressed as integrated density, and were calculated by comparing the optical density due to silver grain over the PVN to that of the background, using the analysis package Scion Image.

2.7. Quantitative reverse transcriptase-polymerase chain reaction

Expression of GR and MR mRNA in the amygdala was determined by real-time quantitative RT-PCR. Rats were decapitated, and amygdala was collected and stored at –80 °C until RNA extraction. The amygdala was homogenized in 1 ml Trizol reagent (Sigma-Aldrich) and total RNA was isolated. The primers were designed and synthesized by Shengggong Biotech Company (Shanghai, China) according to the serial number from Genbank: GR: (sense) 5'-ACTGCAGGAGTCTACAAGACAC-3', (antisense) 5'-TGTCTGGAAGCAGTAGGTAAGGA-3'; MR: (sense) 5'-GGCAGTGTCAAAGTCTTCTT-3', (antisense) 5'-GACAGTCTTTTCGC CGAATC-3'; 28S rRNA: (sense) 5'-TTGAAAATCCGGGGGAGAG-3', (antisense) 5'-ACATTGTTCCAACATGCCAG-3'. The LightCycler™ (Roche Biochemicals, Lewes, UK) was used for real-time quantitative analysis of GR mRNA expression. The cDNA was synthesized using reverse transcriptase (Sigma-Aldrich) after the RNA quality was verified by a spectrometer. The GR cycling conditions consisted of an initial single cycle of 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 60 s at 72 °C. The MR cycling conditions consisted of an initial single cycle of 4 min at 94 °C, followed by 36 cycles of 45 s at 94 °C, 40 s at 60 °C, and 50 s at 72 °C. The 28S rRNA reaction conditions were 10 min at 95 °C for one cycle, then 15 s at 95 °C, 10 s at 54 °C and 5 s at 72 °C for 28 cycles. Preliminary experiments were done to prepare the PCR products used to generate standard curves in real-time PCR. GR and MR mRNA was quantified, against a standard curve of samples containing known GR and MR PCR product concentrations, using the LightCycler™ program. The 28S rRNA was quantified as a reference gene against a separate standard curve of samples containing known concentrations of 28S rRNA product. PCR product for GR and MR mRNA was sequenced and analysed using an ABI Stepone system (Applied Biosystems Inc., Foster City, CA, USA). The Values of receptor mRNA were expressed as a ratio of mRNAs for the receptor and 28S rRNA.

2.8. Western blot analysis

GR and MR protein levels were determined by immunoblotting. The collected amygdala tissue were homogenized in RIPA lysis buffer. Total protein content in the sample was determined by the Bradford method (Thermo Scientific, IL, USA). The extracts were separated on 10% SDS-PAGE gel and transferred to a nitrocellulose membrane in Tris-glycine transfer buffer supplemented with 20% methanol. The immunoblots were blocked for 1 h in 5% bovine serum albumin diluted in 0.2 M Tris base, 1.4 M NaCl, 0.1% Tween 20, and 0.02% NaN₃. The membrane

were then incubated overnight with primary antibodies for GR and MR (Santa Cruz Biotechnology, Dallas, USA). The specificity of the bands was confirmed by using increasing concentrations of competing peptide for each antibody. The protein bands were visualized with ECL detection reagent and quantified by optical densitometry and ImageJ software. The densitometry values represented the pixel intensity, and were normalized to GAPDH to correct for loading.

2.9. Statistical analysis

All quantitative data were represented as the mean \pm SEM. The number of corticosterone pulses and mean pulse amplitudes were determined by using algorithm ULTRA as previously described (Van Cauter, 1988). Two intraassay coefficients of variation of the assay were used as the reference threshold for the pulse detection, and pulse amplitude was defined as the difference between the nadir and peak value. For comparison of in situ hybridization data, four to five sections encompassing the PVN were assessed in each animal, and the mean optical density compared between groups. The statistical significance of pulses numbers and amplitude, CRF mRNA level and mRNA and protein of MR/GR were evaluated by a one-way ANOVA followed by Dunnett's *t*-test. The corticosterone levels were assessed by ANOVA followed by Dunnett's *t*-test to assess statistically significant differences within the same strain at different time points, as well as between the different strains at the same time point. A repeated measures ANOVA with a Bonferroni post-test was used to compare the corticosterone responses between groups. For all experiments, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Basal corticosterone secretion

Representative examples of basal corticosterone secretion from

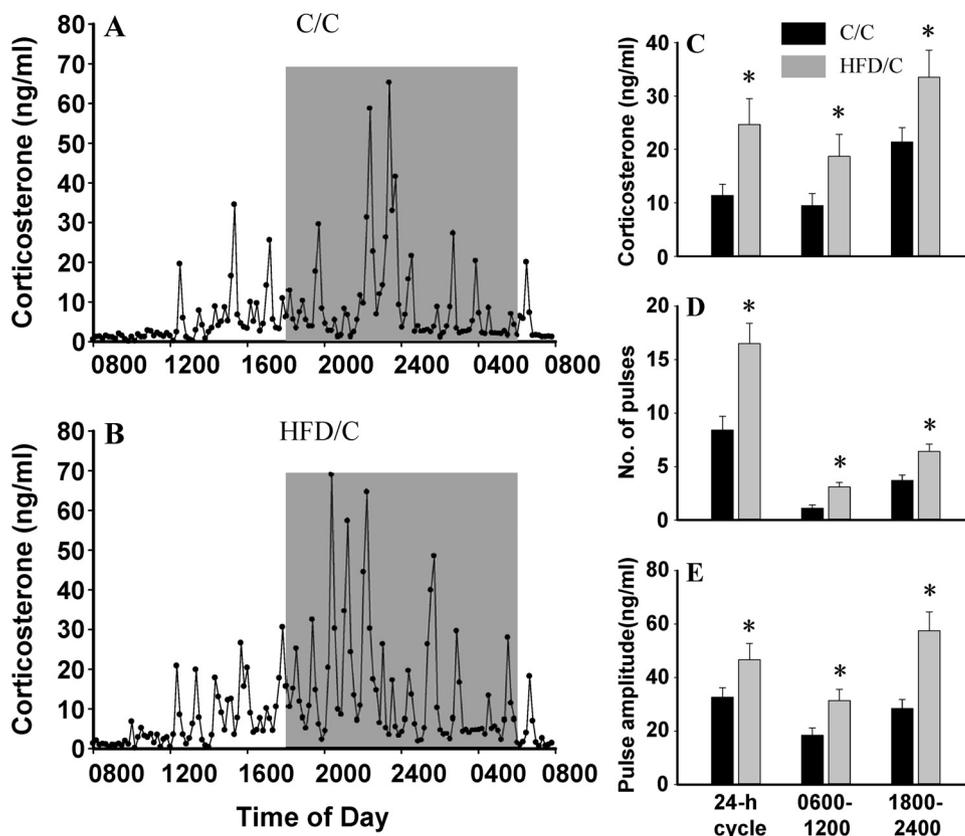


Fig. 1. Representative examples of plasma corticosterone levels collected every 10 min for 24 h in adult male offspring of dams fed with normal diet (A) or high fat diet (B) throughout pregnancy and lactation. It was observed that both groups displayed diurnal pattern of steroid secretion, with high levels in the dark phase of the light cycle (1800–0600) and a nadir early during the light phase. Basal secretion of corticosterone was analyzed over 24 h cycle, during the first 6 h of the light phase (nadir, 0600–1200) and the first 6 h of the dark phase (acrophase, 1800–2400). Summary analysis (C–E) revealed that HFD/C animals exhibited greater corticosterone levels, more pulses of steroid secretion and greater pulse amplitudes. * $P < 0.05$ versus C/C group. Data are expressed as means \pm SEM. C/C ($n = 11$) and HFD/C ($n = 12$).

groups are shown in Fig. 1 (A) and (B). Both C/C and HFD/C animals displayed a pulsatile rhythm of corticosterone secretion with a circadian variation in pulse amplitude. Summary analysis showed that, although a nadir in the morning (approximately 0700 h) was observed in both groups, peak corticosterone levels in HFD/C animals were 'shifted' 2 h toward the light phase of the light/dark cycle (Fig. 1). Moreover, compared with C/C animals, HFD/C rats displayed elevated basal levels of corticosterone in both light and dark phases of the diurnal cycle, resulting from both an increased number of pulses and an increased pulse amplitude (Fig. 1 C–D). These data demonstrate that fat-rich diet exposure in the early life exerts long-term effects on the mechanisms generating basal pulsatile corticosterone secretion in adult offspring.

3.2. Corticosterone responses to different stressors

3.2.1. Corticosterone responses to restraint

During the psychological restraint stress experiment, the levels of corticosterone were markedly increased following restraint in both HFD/C and C/C rats ($P < 0.05$ versus basal). Repeated ANOVA revealed that HFD/C and C/C rats did not differ in their corticosterone responses during the period of 1 h restraint procedure ($F_{(1, 15)} = 0.9$, $P > 0.05$); however, the steroid levels in C/C rats reached peak levels 20 min before the end of restraint, whereas the corticosterone levels kept raising in HFD/C animal beyond 10 min after the end of stress (Fig. 2A). Moreover, compared with C/C animals, HFD/C rats displayed higher levels of plasma corticosterone during the postrestraint period ($F_{(1, 15)} = 14.5$, $P < 0.01$, repeated measures ANOVA). Additionally, corticosterone levels returned to baseline within 3 h after stress exposure in C/C rats, whereas HFD/C animals maintained elevated steroid levels beyond 3 h after stress. These data indicate possible differences in the dynamics of the corticosterone response to acute restraint or differences in the ability of the HPA axis to respond to acute psychological signals.

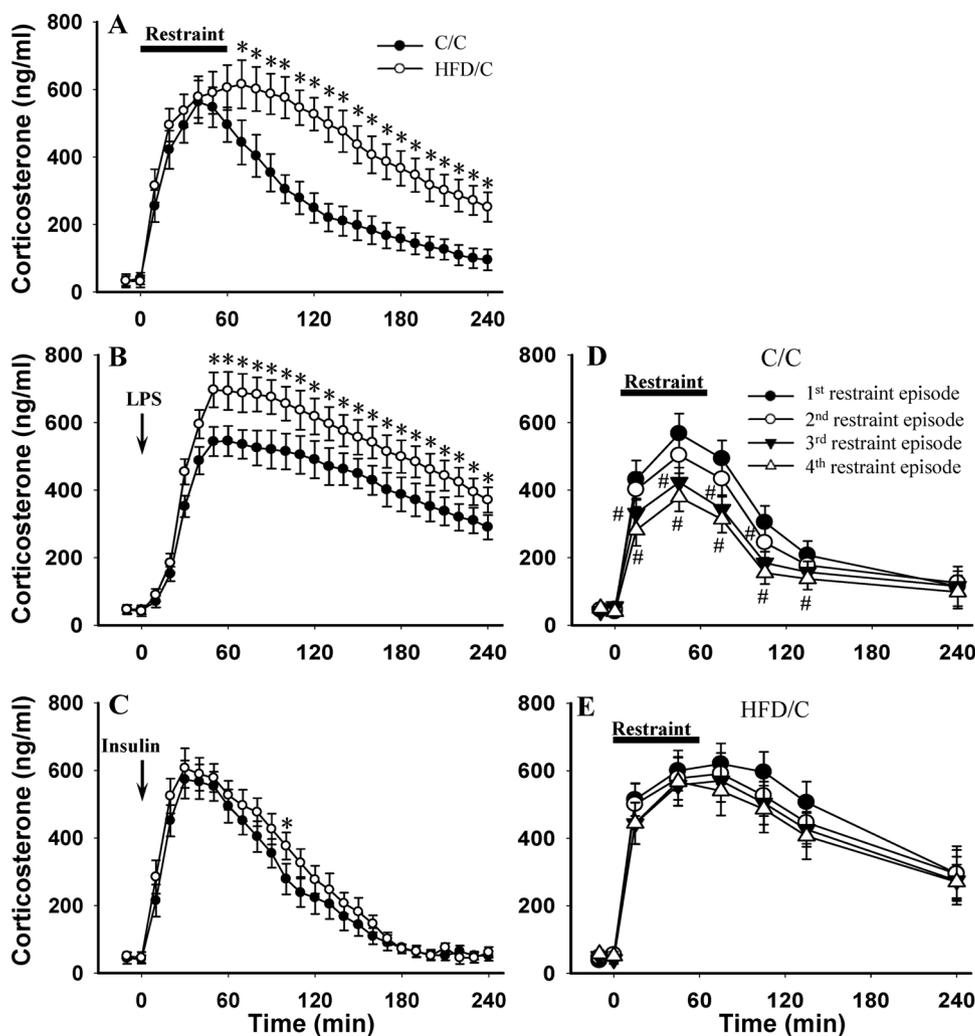


Fig. 2. Mean (\pm SEM) plasma corticosterone levels in response to different stressors and repeated restraint stress. (A–C) Maternal high fat diet exposure exaggerated the corticosterone responses to the restraint (A) and LPS (B), but not insulin-induced hypoglycemia (C). (D) showing repeated restraint stress resulted in an adaptation of the corticosterone responses to the third and fourth episode in C/C offspring. (E) No significant differences in corticosterone level among the repeated restraint episodes were observed in HFD/C offspring. * $P < 0.05$ versus C/C group. # $P < 0.05$ compared with the first corticosterone response to restraint. Restraint : C/C ($n = 8$) and HFD/C ($n = 9$). LPS: C/C ($n = 7$) and HFD/C ($n = 9$). IIH: C/C ($n = 8$) and HFD/C ($n = 8$). Repeated restraint stress: C/C ($n = 7$) and HFD/C ($n = 8$).

3.2.2. Corticosterone responses to LPS

C/C rats and HFD/C animals were also given an acute inflammatory challenge with an i.v. bolus injection of LPS. We observed that plasma corticosterone levels were increased in both groups of animals after the LPS exposure, reaching the peak corticosterone level within 1 h after the injection, but to a greater extent in HFD/C rats (Fig. 2B; $F_{(1, 14)} = 5.1$, $P < 0.05$, repeated measures ANOVA). Moreover, both groups maintained elevated plasma corticosterone levels beyond 4 h after the injection of LPS.

3.2.3. Corticosterone responses to IIH

For the metabolic stress, animals received an acute metabolic attack with an i.v. injection of insulin after fasting. Surprisingly, no significant difference in speed or extent of the responsiveness to IIH was observed between C/C and HFD/C rats (Fig. 2C; $F_{(1, 14)} = 0.9$, $P > 0.05$, repeated measures ANOVA). Both C/C rats and HFD/C rats responded to metabolic challenge with a marked increase in plasma corticosterone concentrations, and the peak levels of corticosterone in response to the stimulus did not differ between the two groups. Whereafter, both groups displayed a rapid decline in the corticosterone levels, and the steroid levels returned to baseline within 3 h after stress exposure.

3.3. HPA responses to repeated restraint stress

During the repeated restraint exposure, the levels of corticosterone were all significantly ($P < 0.05$ versus basal) increased in response to the stimuli in both groups, but with variable extent and speed of

responsiveness. Repetitive exposure to restraint stress resulted in habituation of the HPA axis as shown by a significant attenuation of the corticosterone response to restraint in C/C offspring, especially in the third and fourth episodes (Fig. 2D; $F_{(3, 12)} = 11.5$, $P < 0.05$, repeated measures ANOVA). In contrast, no significant differences in corticosterone level among the restraint episodes were observed in the HFD/C offspring (Fig. 2E; $F_{(3, 14)} = 1.1$, $P > 0.05$, repeated measures ANOVA).

3.4. CRF mRNA expression in the PVN

Fig. 3 shows darkfield images of in situ hybridization for CRF mRNA in the PVN in animals under stress-free, acute restraint and repeated restraint stress conditions. Basal level of CRF mRNA expression in the PVN was very low in C/C rats (Fig. 3A), although CRF hybridization signals can be clearly seen in parvocellular neurones of the hypothalamic PVN. Maternal HFD exposure significantly increased basal CRF mRNA expression in the PVN (Fig. 3A, D and G; $F_{(1, 13)} = 6.8$, $P < 0.05$, one-way ANOVA). Moreover, acute restraint stress resulted in a marked increase in CRF mRNA levels in both HFD/C and C/C rats. Surprisingly, similar increments in PVN CRF mRNA expression were observed between the two groups in response to restraint stress at 120 min, the only time point examined (Fig. 3B, E and G; $F_{(1, 15)} = 0.8$, $P > 0.05$, one-way ANOVA). After repetitive exposure of restraint stress, C/C rats displayed a significant decline in the levels of CRF mRNA, compared with HFD/C offspring (Fig. 3C, F and G; $F_{(1, 14)} = 6.4$, $P < 0.05$, one-way ANOVA). Nevertheless, there is no significant

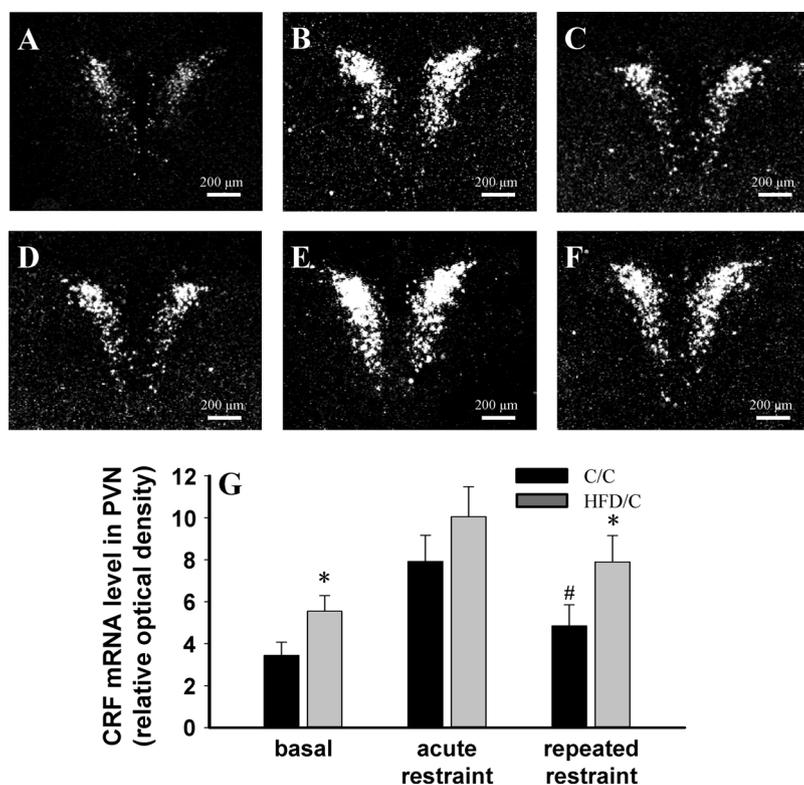


Fig. 3. Examples depicting corticotropin-releasing factor (CRF) mRNA expression in the paraventricular nucleus (PVN) following restraint stress in groups. (A) C/C (0 min, $n = 7$); (B) C/C exposed to restraint (120 min, $n = 8$); (C) C/C exposed to fourth restraint (120 min, $n = 8$); (D) HFD/C (0 min, $n = 8$); (E) HFD/C exposed to restraint (120 min, $n = 9$); (F) C/C exposed to fourth restraint (120 min, $n = 8$); (G) Summary analysis revealed that HFD/C rats displayed significantly increased basal CRF mRNA level in the PVN, nevertheless, C/C and HFD/C offspring exhibited similar increments in CRF mRNA expression following acute restraint stress. Moreover, there is no significant difference in CRF mRNA level between the first and fourth restraint exposure in HFD/C offspring. The mRNA was measured as optical density arbitrary units relative to standard values from autoradiograph images. In situ hybridizations were performed with oligonucleotide probes corresponding to rat CRF mRNA coding sequences. * $P < 0.05$ versus C/C rats with same treatment; # $P < 0.05$, versus the acute restraint in the same group.

difference in CRF mRNA expression in response to the first or fourth restraint stress in HFD/C offspring (Fig. 3E, F and G; $F_{(1, 15)} = 1.0$, $P > 0.05$, one-way ANOVA).

3.5. MeA and CeA lesion verification

The location and extent of the neurotoxic lesions were confirmed by microscopic histological inspection, using Nissl staining. The presence of extensive neuronal loss, gliosis infiltration, and injector trajectory within the MeA or CeA (Fig. 4 E and F) were used as parameters to determine the existence of successful lesions (Lin et al., 2011). Fig. 4 A and B show the variation in MeA and CeA lesion extents, respectively. Animals were excluded from the groups if unsuccessful lesion, unilateral lesion or damage extending outside of the MeA or CeA was observed. All aCSF treated rats sustained no damage, especially no neuronal loss, to the sub-nuclei of the amygdala, but the injector trajectory within the MeA or CeA were observed (Fig. 4 C and D).

3.6. The MeA or CeA lesions

Restraint stress caused an immediate elevation of plasma corticosterone level in both intact MeA and MeA-lesioned rats. In the intact MeA animals, plasma corticosterone levels were increased after the restraint exposure in both C/C and HFD/C group, but to a greater extent in HFD/C rats. Bilateral MeA damage significantly decreased the corticosterone responses to the restraint, in not only C/C rats but also HFD/C offspring. More importantly, no significant differences were observed in plasma corticosterone levels responses to the restraint between MeA-lesioned C/C and HFD/C rats (Fig. 5A; $F_{(1, 13)} = 1.3$, $P > 0.05$, repeated measures ANOVA), indicating that bilateral MeA damage markedly attenuated maternal HFD-induced HPA hypersensitivity to restraint stress. In contrast, bilateral CeA damages had no effect on plasma corticosterone levels responses to restraint in rats. These data suggest that the MeA but not CeA may play an important role in maternal HFD-induced programming of HPA hyperresponsiveness to restraint stress.

On the other hand, bilateral lesion of the CeA markedly blocked LPS stress-induced elevation of corticosterone levels in both C/C and HFD/C rats. Unlike the marked difference between the corticosterone levels of CeA intact C/C and HFD/C rats response to the systemic stress, there was no significant difference between the plasma corticosterone concentration of CeA-lesioned C/C and C/HFD rats following the injection of LPS (Fig. 5D; $F_{(1, 13)} = 1.2$, $P > 0.05$, repeated measures ANOVA). In contrast, bilateral MeA damages had no effect on LPS stress-induced elevation of corticosterone levels in both C/C and HFD/C groups. These data suggest that the CeA but not MeA may play a potent role in the maternal HFD-induced hypersensitivity of the HPA response to LPS stress.

3.7. Protein and mRNA expression of GR and MR in the amygdala

In order to determine whether maternal HFD exposure alter the GR and MR density in the amygdala, the mRNA and protein levels for GR and MR were measured by RT-PCR and immunoblotting, respectively (Fig. 6). Compared with C/C animals, HFD/C offspring showed increased basal GR mRNA ($F_{(1, 14)} = 13.8$, $P < 0.01$, one-way ANOVA) and protein ($F_{(1, 14)} = 5.4$, $P < 0.05$, one-way ANOVA) in the amygdala. Nevertheless, no significant differences in basal MR mRNA ($F_{(1, 14)} = 0.9$, $P > 0.05$, one-way ANOVA) and protein ($F_{(1, 14)} = 1.2$, $P > 0.05$, one-way ANOVA) level were observed between the C/C and HFD/C offspring.

4. Discussion

Nutritional status or diet intake during critical periods of early life has important effects on development and later health of the offspring. The present study provided the further evidence revealing the long-lasting influence of maternal diet exposure on the development of the HPA axis in adult offspring. We observed that maternal HFD consumption resulted in long-term changes in basal activity of the HPA axis in offspring, with increased corticosterone pulse frequency and pulse amplitude. More importantly, we demonstrated that maternal HFD

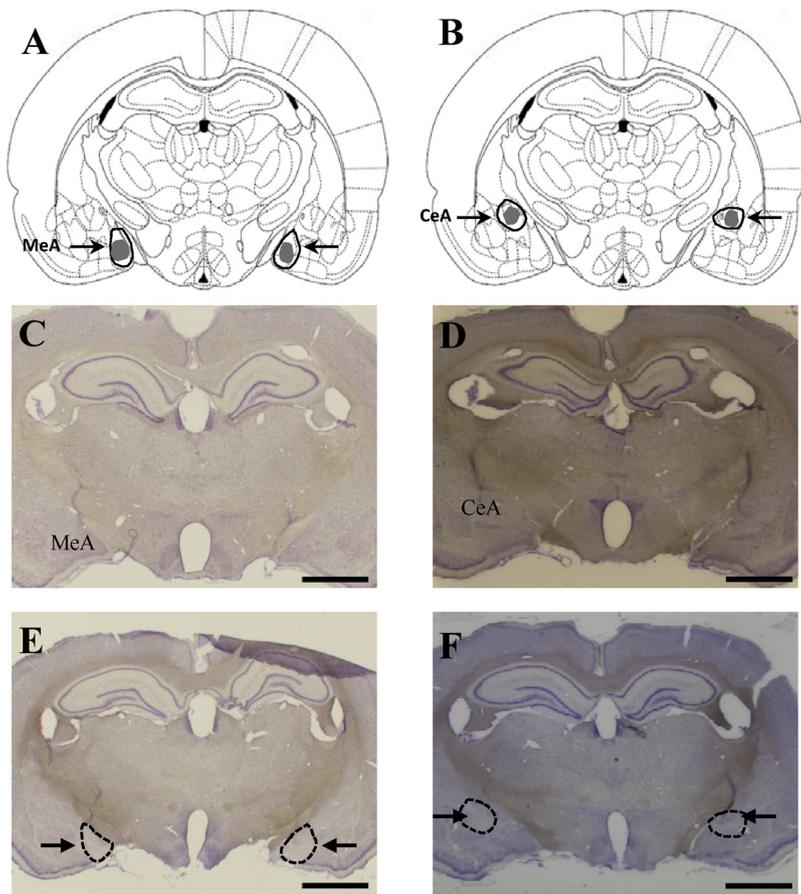


Fig. 4. Schematic diagrams and representative examples illustrating bilateral lesions of the medial nucleus (MeA) or central nucleus (CeA) of the amygdala. A and B show the variation in extent of the bilateral MeA and CeA lesions targeted by ibotenate acid injections, respectively; the grey areas highlight the smallest extent of the lesions, whilst the black bold line denotes the largest extent of the lesions. C and D, intact MeA and CeA, respectively, with injector trajectory within the sub-nucleus. E and F, lesioned MeA and CeA, respectively, showed by Nissl staining and outlined by the broken line. Bars, 500 μm .

exposure sensitize the offspring HPA responses to stress in a stressor-specific manner, supported by the evidence showing that adult maternal HFD offspring displayed exaggerated corticosterone responses to

restraint and LPS, but not insulin-induced hypoglycemia. Furthermore, neurotoxic lesions of the MeA markedly attenuated the enhanced corticosterone responses to restraint but not LPS in maternal HFD

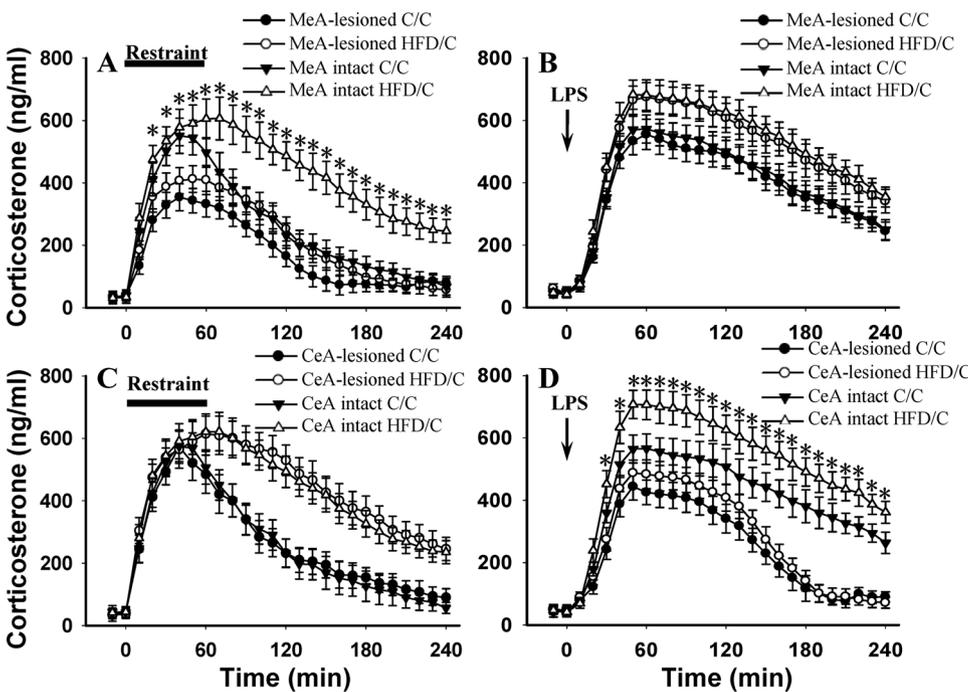


Fig. 5. The effect of selective lesion of the amygdala sub-nucleus on the corticosterone responses to the restraint or LPS stress in offspring. A and B, rats with intact (sham lesions) MeA and MeA lesions, treated with restraint and LPS, respectively. C and D, rats with intact CeA and CeA lesions, treated with restraint and LPS, respectively. A and C showing no significant differences were observed in plasma corticosterone levels responses to the restraint between MeA-lesioned C/C and C/HFD rats, indicating that the MeA but not CeA may play an important role in maternal HFD-induced programming of HPA hyperresponsiveness to restraint stress. B and D showing the CeA but not MeA play a potent role in the maternal HFD-induced hypersensitivity of the HPA response to LPS stress. * $P < 0.05$ versus lesion group at the same time point in HFD/C animals. Results are shown as means \pm SEM. Restraint: MeA-lesioned C/C (n = 7), MeA intact C/C (n = 7), MeA-lesioned HFD/C (n = 8), MeA intact HFD/C (n = 8). LPS: MeA-lesioned C/C (n = 6), MeA intact C/C (n = 7), MeA-lesioned HFD/C (n = 8), MeA intact HFD/C (n = 8). Restraint: CeA-lesioned C/C (n = 6), CeA intact C/C (n = 7), CeA-lesioned HFD/C (n = 8), CeA intact HFD/C (n = 8). LPS: CeA-lesioned C/C (n = 7), CeA intact C/C (n = 7), CeA-lesioned HFD/C (n = 8), CeA intact HFD/C (n = 8).

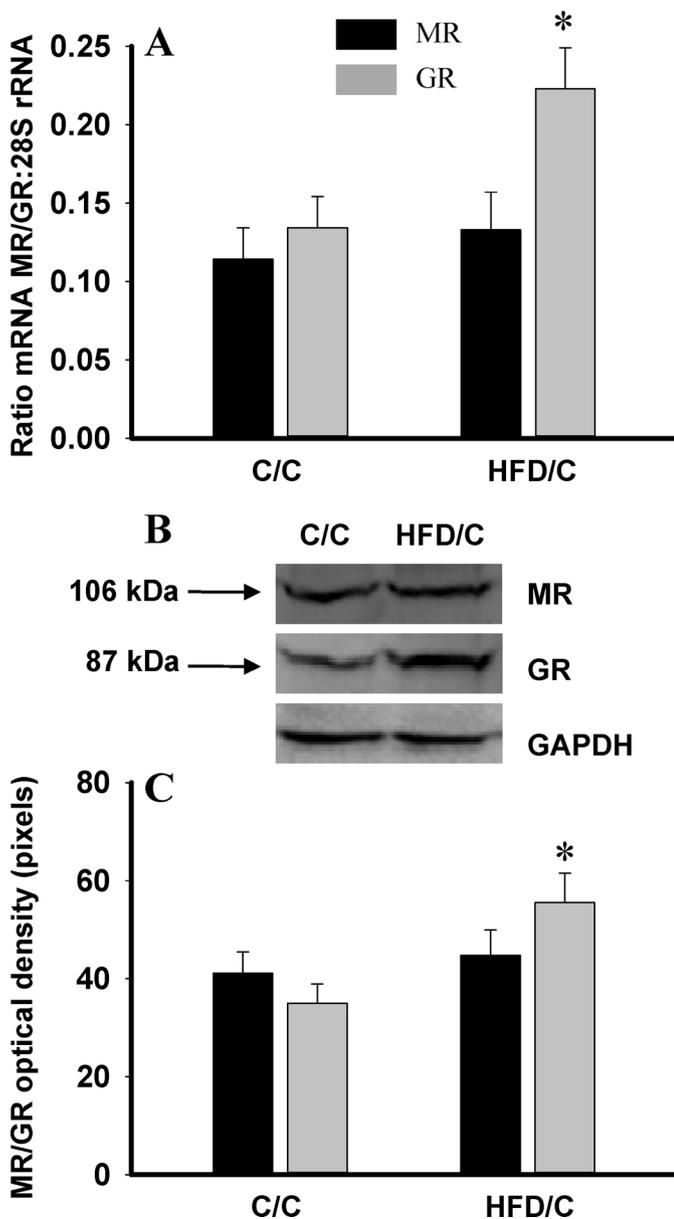


Fig. 6. Basal glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) expression in the amygdala. The analyses indicated maternal HFD consumption increased baseline GR mRNA and protein but not MR mRNA and protein in offspring. (B) Representative immunoblot showing GR and MR protein levels. The mRNA values are expressed as a ratio of receptor mRNA and 28S rRNA. Densitometry values reported are normalized to GAPDH levels to account for differences in loading. * $P < 0.05$ versus C/C group. Results are shown as mean \pm SEM. C/C ($n = 8$) and HFD/C ($n = 8$).

offspring, especially that there was no significant difference in plasma corticosterone levels responses to the restraint between MeA-lesioned C/C and C/HFD rats. In contrast, maternal HFD-induced HPA hyperresponsiveness to LPS but not restraint was blocked by the CeA lesions. These evidence indicated that the amygdala may play a region-specific role in maternal HFD-induced HPA axis hyperresponsiveness to stress, suggesting that different neural pathways mediate maternal diet-induced programming of HPA responses to different types of stressors. The results are consistent with other data showing that a fat-rich diet during rat pregnancy and suckling confers susceptibility to mental health and behavioral disorders in offspring, such as depression, anxiety, impairments in social behavior and cognitive deficit (Bilbo and Tsang, 2010; Raygada et al., 1998; Sullivan et al., 2014, 2015).

Moreover, maternal HFD exposure also induces hypertension, metabolic syndrome, cardiovascular dysfunction and cerebrovascular remodeling in adult offspring (Gluckman and Hanson, 2004; Craft et al., 2006; Liang et al., 2011; Warner and Ozanne, 2010). Taken together, although maternal diet results in developmental plasticity in physiological systems which may prove beneficial for immediate survival, they may cause a permanent alteration in physiological responses to environmental challenges and alter predisposition to disease later in life. It is worth noting that, we did not observe any differences in offspring body weight (Lin et al., 2017), or maternal behavior between the two treatment conditions, with dams of HFD-fed or control diet-fed litters showing similar number of periods away from the nest and time spent off the nest (Lin YS, unpublished observation), suggesting that the remodeling of the HPA axis programmed by maternal HFD exposure is unlikely to be due to altered growth rate or maternal contact.

The importance of early-life challenges in modulating the HPA axis activity in adult offspring has been demonstrated in several animal models, such as neonatal endotoxin or restraint exposure (Shanks et al., 2000; Maccari and Morley-Fletcher, 2007). Moreover, fat-rich diet feeding in the early life also affects the basal HPA axis activity and the responsiveness to stress in offspring animals. Nevertheless, several studies reported that rat dams consuming HFD in the early life have offspring with increased, decreased or unchanged basal corticosterone levels (D'Asti et al., 2010; Sasaki et al., 2013; Lin et al., 2015). Maternal HFD exposure has been demonstrated to increase or decrease the HPA profile response to stress (Trottier et al., 1998; Sasaki et al., 2013; Lin et al., 2015). Additionally, increased or decreased anxiety behavior is also observed in offspring perinatally exposed to HFD in different animal studies (Bilbo and Tsang, 2010; Sasaki et al., 2014). The results of these studies of developmental fat-rich diet exposure on basal and stress-challenged levels of corticosterone have been mixed. Differences in procedural details including species and ages of animals, fat sources, durations of diet manipulation and corticosterone measurement time points may account in part for these discrepancies. Nonetheless the studies presented above, regardless of whether they demonstrate an stimulatory or inhibitory role for maternal diet on HPA axis activity, focus on changes in mean circulating levels of corticosterone. The present study demonstrates that perinatal HFD exposure programs the adult offspring HPA activity in both stress-free and stressed environment. Under nonstressed condition, we observed that maternal HFD offspring displays a pulsatile pattern of corticosterone secretion with a circadian variation in pulse amplitude, serving to prepare the organism for potential homeostatic challenge (Jankord and Herman, 2008). Maternal HFD consumption upregulates the basal activity of the HPA axis in adult offspring, as reflected in increased diurnal corticosterone concentrations, resulting from increased corticosterone pulse frequency and pulse amplitude. Thus, maternal HFD offspring might be exposed to greater corticosterone levels throughout their lifespan and this may program predisposition to pathologies that have a significant HPA regulatory component, such as behavior disorders. Moreover, maternal HFD exposure also sensitizes the corticosterone responses to stressful stimuli, including psychological and systemic stressors. Surprisingly, maternal HFD consumption does not affect the corticosterone responses to insulin-induced hypoglycemia. These result suggest that maternal dietary fat may affect the responsivity of HPA to stress in offspring in an stressor-specific manner. To the best of our knowledge, the present study provides the first evidence revealing that perinatal fat-rich diet intake differentially modulates the neuroendocrine stress reactivity to types of stressors in adult offspring. This suggests that a centrally mediated mechanism programmed by the maternal HFD exposure can selectively modulate the response to different incoming signals. Taken together, it appears that the long-term effects of neonatal metabolic challenge on the central regulation of the HPA axis, therefore, expose an animal to greater basal and stress-induced steroid signals throughout its lifespan. Consistent with other study (Bellisario et al., 2014), maternal HFD-induced changes of the neuroendocrine profile overlap with

those caused by prenatal stress, such as restraint (Louvar et al., 2009), neonatal immunological challenge (Shanks et al., 2000) and maternal care (Meaney, 2001; Brunton, 2010). The overlapping consequences of maternal HFD and stress exposure in neuroendocrine response raise the possibility that perinatal HFD may initially be perceived as a stressor or mimic stress exposure, as both interfere with the neuroendocrine development, altering the HPA axis activity in later life (Bellisario et al., 2015).

Repeated stress would result in an adaptation or desensitization of the HPA axis to the same stressor whereby the corticosterone response to subsequent homotypic stress is reduced and may even be abolished, especially some mild stress such as restraint stress, water immersion and foot shock (Ma and Lightman, 1998). Adaptation of HPA axis to repeated stress is an important mechanism that the organisms can adapt their physiological responses to meet environmental demands and prevent over-responsivity of neuroendocrine axis to familiar stimuli with low threat level. It is now clear that the development of habituation depends upon not only the duration of each repeated restraint period, but also interval and number of restraint episodes (Ma and Lightman, 1998; Pitman et al., 1988). The present study observed that four times restraint at 5-days intervals result in significant adaptation of corticosterone responses to the stimulus in control rats. Nevertheless, maternal HFD offspring still displays a prominent corticosterone profile response following repetitive stress exposure, with similar degree of responsiveness to the homotypic stressor. Thus, adaptive responses to repeated stress are attenuated by maternal HFD exposure and this may result in overreaction of the HPA axis to repeated environmental challenges and thereafter increase the susceptibility to the disease in offspring. The poor habituation to repetitive stress may be partially related to their hyperresponsiveness of the HPA axis to acute restraint stress. Moreover, high levels of CRF mRNA in the PVN following repetitive restraint observed in the present study may also contribute to maternal HFD-induced poor adaptation of the corticosterone responses to the repeated restraint stress, while CRF expression of normal animals fail to respond to repeated restraint stress which is in accordance with previous studies (Aguilera, 1994; Kusek et al., 2017). On the other hand, exposure of chronically stressed rats to a heterotypic stressor induces greater and more rapid increases in plasma ACTH and corticosterone responses (Ma and Lightman, 1998), but what degree of HPA responses to heterotypic stressor happens to maternal HFD offspring still need to be explored.

The increased basal activity of the HPA axis and aggravated corticosterone response to stress reflect changes in the regulatory components of the neuroendocrine axis. CRF produced by the hypothalamic PVN drive the HPA axis by stimulating the pituitary release of ACTH and ultimately the adrenocortical release of corticosterone. As the principal regulator of the HPA axis, CRF has also been shown to be programmed by early-life events wherein both mRNA and peptide levels within the PVN and median eminence, respectively, are decreased by neonatal handling, yet increased by endotoxin treatment (Shanks et al., 1995, 2000). In the current study, maternal HFD consumption increases basal CRF mRNA levels in the PVN, which may partially cause the increase of basal plasma corticosterone levels. Nevertheless, although maternal HFD exposure upregulates corticosterone responses to acute restraint stress, it induces similar increments in the expression of PVN CRF mRNA. Additionally, maternal HFD offspring shows high levels of CRF expression in the PVN after repetitive stress. These results further exemplify that the fat-rich diet exposure in the early life induces complex changes in regulatory components of the HPA axis. The amygdaloid complex play an important role in modulating the response to stress through direct projections to PVN of the hypothalamus. In accordance with the notion that different types of stressors activate distinct neuronal pathways, it is now the understanding that the effect of the amygdala on the HPA axis is stressor-and region-specific (Herman et al., 2005). The MeA and CeA play key roles in psychogenic and systemic stress-induced activation of the HPA axis activity,

respectively. The current study showed that bilateral lesions of the MeA markedly attenuates psychogenic (restraint) but not immunological (LPS) stress-induced activation of the HPA axis in animals. More importantly, the selective neurotoxic lesion of the MeA removes the difference in corticosterone levels responses to restraint stress between the animals exposed to different diets in early life. The lesions of the CeA lessen LPS but not restraint stress-induced activation of corticosterone secretion in maternal HFD rat, supporting the stressor selective nature of the CeA in the development of the HPA axis induced by maternal HFD consumption. It is further supported by the evidence showing that the significant difference in corticosterone levels responses to LPS stress was abrogated by the neurotoxic lesion of the CeA. Therefore, the MeA and CeA play an important role in maternal HFD-induced programming of the neuronal pathway specifically for psychogenic and systemic stressor respectively, suggesting that the amygdala may play a stressor specific role in maternal HFD-induced sensitization of the HPA axis to stress. It is worth noting that other limbic brain structures such as the hippocampus and the bed nucleus of the stria terminalis may also play an important role in maternal HFD-induced programming of the HPA axis, and more studies need to be conducted to characterize the neural circuitry by which maternal HFD consumption influences the HPA axis. In the amygdala, MR is a high-affinity receptor that is normally bound to corticosterone in basal conditions, determining the threshold of the stress response, whereas GR is involved with the extent of the stress response. We observed that maternal HFD exposure increases GR expression but not MR expression in the amygdala of offspring. Therefore, increased GR levels in the amygdala may partially mediate the hyperresponsiveness of HPA axis to stress as a result of maternal HFD exposure. Consistently, Sasaki and colleagues reported evidence of increased GR in amygdala and a heightened response to stress in maternal HFD offspring (Sasaki et al., 2013, 2014). However, they also demonstrated that maternal HFD exposure increased MR levels in the amygdala, which may lead to decreased basal levels of corticosterone. Nevertheless, all these studies focused on the changes of GR and MR expression in the whole amygdala, rather than in the subdivisions of the complex. Since the receptors are differentially distributed in the nuclei such as the MeA and CeA, it is worth to explore the changes of GR and MR expression in more precise brain structures. Therefore, more work will be required to identify the changes of the amygdala induced by maternal fat-rich diet exposure.

It needs to be mentioned that, the present study only explored the influence of maternal HFD exposure on the HPA axis in male offspring, and sex differences should be taken into account. Brunton and Russell demonstrated that prenatal social stress programs anxiety behaviour and HPA axis responses to stress in adult male, but not female offspring (Brunton and Russell, 2010). Moreover, the effects can also be transmitted to future generations in sex-dependent manner (Grundwald and Brunton, 2015). Consistently, it has been reported that stress experience early in pregnancy may contribute to sex-specific neurodevelopmental disorders in male offspring (Mueller and Bale, 2008). However, as for the maternal HFD consumption, Khan and colleagues reported the sex-linked hypertension in offspring of lard-fed pregnant rats (Khan et al., 2003), supported by the evidence showing that blood pressure was raised in female but not male offspring. Therefore, it remains to be established whether maternal HFD consumption also exerts sex-specific effects on the HPA axis activity.

5. Conclusion

Overall, the present experiments support the hypothesis that a fat-rich diet during pregnancy and lactation fundamentally alters the activity of the HPA axis in adult offspring, under both stress-free and stressful conditions. These maternal HFD-induced remodeling of the HPA axis would ultimately not only affect the adult responsiveness to the stressful challenge, but also give rise to an offspring phenotype predisposed to the development of behavior disorders and other health

problems in adult. The present study demonstrated, for the first time, maternal diet affects the HPA response in a stressor-specific manner, with alternations of the neuroendocrine response to psychological and systemic but not metabolic stressors. Moreover, the MeA and CeA play an important role in the hyperresponsiveness of the HPA axis to psychological and systemic stress in maternal HFD offspring, respectively, suggesting that maternal HFD exposure may selectively modulate the HPA response to different incoming signals through differential neural pathways. Identification of the mechanisms and pathways that produce long-term vulnerability in response to perinatal environmental factors will facilitate development of clinical intervention and prevention strategies to reduce the incidence and severity of disease. Therefore, more studies need to be conducted to characterize the pathways and mechanisms by which maternal HFD consumption influences the HPA axis activity.

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Authors' contributions

LCC, LYS and SB did experimental design and study conceptualization. NXT and WXY did animal operation, tissue collection, RIA protocols, In situ hybridization protocols and molecular work. ZWL conducted the statistical analyses. LYS supervised the research. NXT, and WXY drafted and revised the manuscript, and all authors approved the manuscript.

Conflict of interest

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Declaration of interest

The authors report no conflicts of interest.

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