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Grandmaternal high-fat diet primed anxiety-like behaviour in the second-generation female offspring

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ABSTRACT

The health consequences of maternal obesity during pregnancy are disturbing as they may contribute to mental disorders in subsequent generations.

We examine the influence of suboptimal grandmaternal diet on potential metabolic and mental health outcome of grand-progenies with a high-fat diet (HFD) manipulation in adulthood in a rat HFD model.

Grandmaternal exposure to HFD exacerbated granddaughter's anxiety-like phenotype.

Grandmaternal exposure to HFD led to upregulated corticotropin-releasing hormone receptor 2 mRNA expression involved in the stress axis in the male F_2 offspring. Thus, we demonstrate that suboptimal grandmaternal diet prior to and during pregnancy and lactation may persist across subsequent generations. These findings have important implications for understanding both individual rates of metabolic and mental health problems and the clinical impact of current global trends towards comorbidity of obesity and depression and anxiety.

In conclusion, the effect of grandmaternal HFD consumption during pregnancy on stress axis function and mental disorders may be transmitted to future generations.

1. Introduction

An increasing amount of evidence suggest that pre-gestational and early postnatal events such as maternal malnutrition can increase later-life susceptibilities to disease in offspring [1]. In fact, obesity, metabolic syndrome and type 2 diabetes mellitus (T2DM) are strongly comorbid with major depressive disorder (MDD) and anxiety [2,3]. Furthermore, it has been shown that fat mass is not only a result of energy intake and expenditure, but that genetics and environment are strong contributing factors [4–6], although the underlying mechanisms are still unknown.

A growing body of literature indicate that developmental exposure to high-fat diet (HFD) leads to adverse health outcomes later in life. Maternal Westernized diet (herein referred to as HFD consumption) before and during gestation has been associated with an increased prevalence of offspring metabolic diseases, reduced insulin sensitivity and diabetes in a variety of pre-clinical studies [7–12]. More so, maternal HFD has been shown to alter emotional behaviour in rodents and primates in the first generation (F_1) offspring [12–15].

Furthermore, a recent study linked maternal inflammation related to HFD exacerbated anxiety-like traits in the second generation (F_2) offspring as assessed in the rodent elevated plus maze (EPM) [16]. In fact, recent data regarding maternal diet in mice suggest that the metabolic phenotype of the F_1 can be passed on to the F_2 [17] and may even be passed on to the F_3 generation [18]. Thus, it is important to elucidate the mechanisms of intergenerational inheritance of metabolism and mental health to understand how the population-wide epidemics occur, and lastly, how treatments can be initiated.

The hypothalamic-pituitary-adrenal (HPA) axis is exceptionally vulnerable to fetal programming by prenatal metabolic stress [19]. A previous study demonstrated that prenatal stress induced anxiety-like behaviour in F_2 male offspring associated with increased CRH mRNA expression [20]. Up until now, it has been unknown how the HPA axis function and related behavioural effect of a maternal obesogenic diet, as a stressor, can be transmitted to the F_2 offspring and whether gender-determined differences are inherited in an intergenerational manner.

In the present study, we exposed F_0 female rats to a HFD prenatally

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and during lactation. F_1 animals were therefore exposed during development *in utero* and early postnatal life, whereas F_2 animals were potentially exposed as germ cells of the F_1. The outcome from these F_2 offspring are termed intergenerational.

The study hypothesized that the consequences of F_0 exposure to HFD would result in intergenerational inheritance of potentially pathogenic traits, such as glucose intolerance, increased body weight and occurrence of MDD and anxiety in the F_2 offspring, which might be mediated through an altered developmental programming of the HPA axis function.

2. Materials and methods

2.1. Animals

Seven weeks old male (n = 10) and females (n = 10) Sprague Dawley rats were obtained from Taconic Bioscience A/S (Ry, Denmark). Rats were housed in same-sex pairs (Cage 1291H Eurostandard Type III H, 425 × 266 × 185, Techniplast, Buguggiate, Italy, including a tunnel shelter, nesting material, pine bedding and a wooden stick), maintained on a 12 h light-dark cycle (lights on from 7:00 AM). The animal welfare committee appointed by the Danish Ministry of Justice granted ethical permission for the studies. All experimental procedures complied with the EU Directive 2010/63/EU and the Danish Experimentation Act (LBK 1306 from 23/11/2007 with 2011 amendments). The protocol was approved by the Danish Animal Experimentation Committee (j.no 2012-15-2934-00254).

2.2. Diets

Rats were challenged with a HFD (D12492, Research Diets, Inc., New Brunswick, NJ, USA) comprised of 60% kcal fat (mainly lard) and refined carbohydrates (sucrose and maltodextrin) with a total kcal/g 5.24. The control diet (CON) (D12450 J, Research Diets, Inc., New Brunswick, NJ, USA) consisted of 10% kcal fat (soybean oil and lard) and more complex carbohydrates including corn-starch, total kcal/g 3.85. Food (HFD or CON) and water was available *ad libitum*. The composition of the dietary regimens can be seen in Table 1. Food consumption and weight gain was monitored by weighing food and rats once a week. Weekly caloric intake per rat was considered as [rat weight gain / total cage weight gain × total cage caloric intake].

2.3. Experimental design

To investigate whether obesogenic grandmaternal HFD (F_0) would alter metabolism and behaviour in the grand-offspring (second-generation/F_2), sexually mature female rats (F_0, 8 weeks old), were randomly allocated to control diet (CON, n = 10) or HFD (n = 10) (Fig. 1). F_0 received their respective diets for 8 weeks prior to breeding to ensure a female weight gain prior to gestation. Female F_0 were bred with a mature F_0 CON male to produce F_1 offspring of the CON and HFD lineage.

Two breeding combinations were used to produce F_2: the F_2 CON lineage (produced from F_1 CON females crossed with F_1 CON males, n = 10), and F_2 HFD lineage through the maternal line (F_1 HFD females crossed with F_1 CON males, n = 10).

F_1 and F_2 offspring were weaned at postnatal day (PND) 21 and had *ad libitum* CON diet access. To examine postnatal environmental effects, adult male and female F_2 offspring (14 weeks old), were randomly allocated into one of four groups. Thus, F_2 offspring were challenged with a HFD for 14 weeks in order to investigate whether they would develop a more distinct phenotype. This generated the following experimental groups CON-CON, CON-HFD, HFD-CON HFD-HFD, indicating the F_0/F_2 diets, respectively.

Table 1
Main nutrient composition of diets used. Fat in the CON was mainly soybean oil, whereas fat in the HFD consisted of mostly lard.

	CON (D12450 J)		HFD (D12492)	
	gm	kcal	gm	kcal
Macronutrients (% energy)				
Protein	19.2	20	26	20
Carbohydrate	67.3	70	26	20
Fat	4.2	10	35	60
Total		100		100
Energy Density (kcal/gm)	3.85		5.24	
Micronutrients sources (kcal)				
Protein, Casein, Lactic, 30 Mesh	200	800	200	800
Protein, Cysteine L	3	12	3	12
Corn starch	506.2	2024.8	0	0
Maltodextrin	125	500	125	500
Sucrose	68.8	275	68.8	275
Soybean oil	25	225	25	225
Lard	20	180	245	2205
Total	1055.05	4057	773.85	4057

2.4. Behavioural assessment

Each behavioural test session was separated by 7 days from the previous test to minimize cross- interference between tests and stress for the animals. The order of each of the four F_2 experimental groups were pseudo-randomized to eliminate potential interactions due to circadian rhythm and kept through all tests. Animals were allowed to habituate for 1 h in a neighbouring room, prior to testing and all apparatus and objects were cleaned with 70% ethanol solution and allowed to air dry to neutralize odorants between sessions. Furthermore, all animals were returned to their home cages between trials and after each session. All tests were carried out between 9 a.m. and 3 p.m.

2.4.1. Elevated plus-maze

To assess anxiety-like behaviour, the EPM was utilised [21,22]. The plus-shaped apparatus (elevated 80 cm above the ground) was constructed of two open arms (50 × 10 cm) and two closed arms, with opaque, black plastic walls on the sides and at the end of the two arms (20 cm) [23]. Light intensity on the open arms was 60 lx and the other two enclosed arms had limited lighting (10 lx). Rats were placed in the centre of the maze facing the closed arm opposite from the experimenter and allowed to freely explore the maze for 5 min. Rats were recorded by a camera in the ceiling and videos were manually scored by a blinded experimenter. Entry was defined as at least two front paws placed on the arms.

As an index of anxiety-like behaviour, the percentage of time spent on the open arms was calculated as follows – [time spent on open arms / time spent on all arms × 100%]. Furthermore, the percentage of entries onto open arms was similarly calculated and reported.

2.4.2. Light-dark box test

The light-dark box test (LDB) was included as an additional test for anxiety-like behaviour. This test is also based on an approach-avoidance conflict between exploration of novel environments and avoidance of brightly lit, open spaces [24]. The test was conducted in an apparatus consisting of two identical chambers (40 × 40 × 42 cm), separated by a wall with a hole (20 × 20 cm), allowing the animal to move across the two chambers [25,26]. An animal was placed in the light box (200 lx) in the centre of the box and allowed to move freely and video recorded from the ceiling for 5 min. Number of entries into the light chamber, latency to re-enter the light box and time spent in each chamber were recorded.

2.4.3. Open field test

Gross locomotor activity was determined in an open field test (OFT) in an arena with opaque black background (100 × 100 cm^2, height

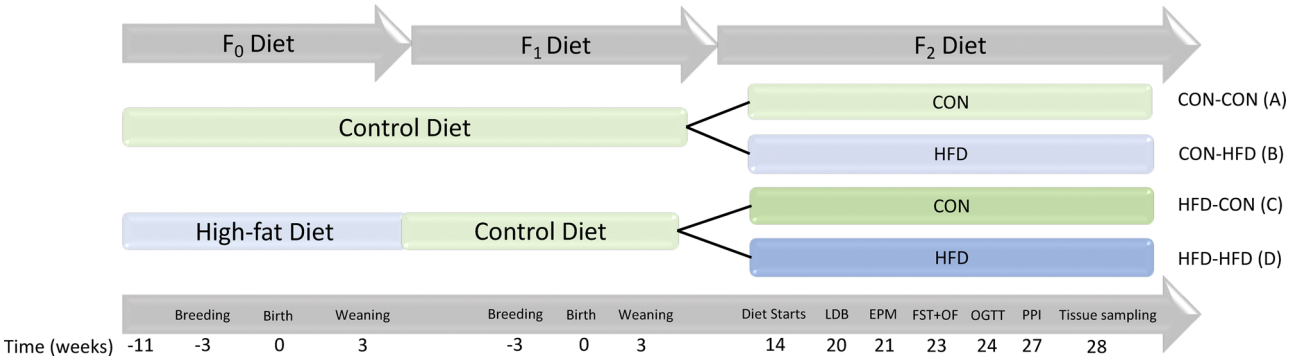

Fig. 1. Experimental design. F_0 diet was initiated 8 prior to breeding. After weaning all F_1 offspring were fed CON diet. F_2 offspring were challenged with HFD (B and D) at age 14 weeks or kept on a standard diet (A and C).

50 cm), according to previously described (Kim 2011). The rat was placed in the centre of the arena and could freely explore the arena for 5 min. Trials were recorded by a video camera located above the arena and distance travelled was automatically tracked using Ethovision (version 11, Noldus Information Technology, Wacheningen, Netherlands).

2.4.4. Forced swim test

To evaluate behavioural despair, an index of depression-like behaviour, animals were exposed to the modified forced swim test (FST) [27]. Briefly, each rat was subjected to a swim session twice, 24 h apart. Each animal was placed in a transparent, water-filled (25 °C), plastic, cylindrical tank (H: 54 cm, φ: 24 cm, water depth: 40 cm) and allowed to swim for 15 min during the pre-test (day 1) and 7 min on the test trial (day 2). All swim sessions were video-recorded from the side, by a camera positioned in front of the cylinders. Immobility was scored if this was the most dominant behaviour within 5 s intervals as described elsewhere [28,29] by an observer blind to study groups.

2.4.5. Prepulse inhibition of the acoustic startle response

Patients with neuropsychiatric diseases or schizophrenia often suffer from a dysfunctional sensorimotor gating mechanism [30–32]. This was evaluated by the prepulse inhibition (PPI) of an acoustic startle response, using the startle response system from SR-Lab™ SDI (San Diego Instruments (Europe) Ltd., Birmingham, UK). Background, white noise (70 dB) was provided through the entire session by a 3.5 tweeter (model BT2, MG electronics, Hauppauge, NY, USA) 14 cm over the animal chamber (Plexiglas tube φ 8.8 cm males and φ 6.5 females). Testing began with a 5 min habituation period followed by three blocks lasting 20 min. The first block consisted of five startle- elicited stimuli (120 dB, 40 ms). The second block involved five different types of trials; either the startle stimuli or the startle preceded by a prepulse (20 ms) (PPI) at 72, 74, 78 and 86 dB, separated from the startle with 40 ms. All five stimuli were presented in pseudo-random order (5 × 7). The last block consisted of five startle stimuli. The V max from all five trials in block 2 was used to generate an average V constituting of the independent variable for analysis. Percentage of PPI was calculated as decrease in mean V max: % PPI [100% × (PPI / startle alone) – 100].

2.5. Oral glucose tolerance test

To investigate the glucose response, an oral glucose tolerance test (OGTT) was performed after a 10 h fasting period. Blood glucose was measured in duplicates using OneTouch® Vita blood glucose metre (Lifescan Inc., Cilag GmbH, Switzerland) from blood obtained by a small incision at the tip of the tail. All plasma glucose levels were measured in duplicates at 30, 60 and 120 min after oral gavage of glucose (2.5 g kg^{-1}).

2.6. Tissue sampling and preparation

All offspring were euthanized by decapitation (PND ≥ 150) and heart, liver, and epididymal and gonadal fat tissue were dissected and weighed immediately. The brain was removed and hippocampus dissected and rapidly snap frozen on powdered dry ice and stored at −80 °C until further analysis.

2.7. Hippocampal RNA extraction

Tissue was homogenised using the bead beating technology by Precellys equipment (Bertin Technologies, Villeurbanne, France) for 2 × 15 s (5000 rpm) in lysis buffer (Applied Biosystems, Foster City, CA, USA) and one 2.8 mm stainless steel bead. Total RNA was isolated using the ABI PRISM 6100 Nucleic acid Prepstation (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' protocol. 11 mg homogenized tissue was loaded per well. RNA concentration and purity was assessed using Nanodrop spectrophotometer (Thermo Fisher Sci, Massachusetts, USA), and RNA was stored at −80 °C prior to use.

2.8. cDNA synthesis

Total RNA was reversely transcribed using random primers and Superscript IV Reverse Transcriptase (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol. The RNA concentration from the Nanodrop spectrophotometer was utilized to ensure the same amount of RNA was present in each sample. The input RNA concentration per reaction was 16 ng/μL. The cDNA samples were stored undiluted at −80 °C until quantitative real-time polymerase chain reaction (real-time qPCR) analysis.

2.9. Real-time qPCR

We performed real-time qPCR on individual samples in 96-well PCR plates using the Mx3005 P (Stratagene, La Jolla, CA, USA) and SYBR Green reaction containing 5 μl SYBR Green mastermix (Sigma-Aldrich, St. Louis, MO, USA), 0.5 μM primer pair, 1.5 μl DEPC water and 3 μL diluted DNA (10 μL total volume) as previously reported [33]. Prior to use, cDNA samples were diluted 1:9 with diethylpyrocarbonate (DEPC). The thermal profile for the PCR was 3 min at 95 °C to activate the hot-start iTaqDNA polymerase, followed by 40 cycles of: 10 s denaturation at 95 °C, 30 s annealing at 60 °C and 60 s extension at 72 °C. Finally, the PCR product was exposed to heat-denaturing by increasing the temperature from 60 °C to 95 °C to generate a melting curve. Each plate included a fivefold standard curve run in duplicate. We investigated five genes encoding for proteins associated with HPA axis regulation (glucocorticoid receptor (*Gr*), mineralocorticoid receptor (*Mr*), 11beta-hydroxysteroid dehydrogenase type 1 (*11β-hsd1*), corticotrophin-

releasing hormone receptor 1 and 2 (*Crh-r1*, *Crh-r2*)), one gene related to structural plasticity (brain derived neurotrophic factor (*Bdnf*)) and eight potential references genes (*Actb, CycA, Gapdh, Hmbs, Hprt, Rpl13 A, Ywhaz, 18 s rRNA*). Characteristics of the primers are given in Table S1. The individual sample genes were normalized to the geometric mean of the two most stable reference genes (*ActB/Hprt*) detected by Normfinder software (http://moma.dk/norm-software; [34]).

2.10. Statistical analyses

All analyses were performed using Stata 14 (StataCorp LP, Texas, USA) and SPSS statistics (IBM® SPSS statistics, version 2). Normality was assessed by Shapiro Wilk test and QQ plot, and homoscedacity was confirmed using Levene's test. In case of non-normality or heteroscedasticity, data were log-transformed.

Area under the curve (AUC) was calculated by summation of trapezoids between the different time points for blood glucose levels during OGTT, body weight and %PPI.

Blood glucose levels and body weight were analysed using four-way ANOVA with the independent variables (gender × F_0 diet × F_2 diet × time). In case of significant effect of time, separate three-way ANOVAs were performed at each time point.

We performed three-way ANOVA (gender × F_0 diet × F_2 diet) for OFT, LDB, EPM, FST, for each time point during the glucose measure, body weight and real-time qPCR. If no three-way and no two-way interactions were present we reported main effects.

If there was a two-way interaction in the three-way ANOVA, we performed analysis of simple main effects at each level of the two independent variables that had interactions.

If we had a three-way interaction, we performed an analysis for simple two-way interactions at each level of gender using the error term from the three-way ANOVA as described by [35].

If we had a simple two-way interaction in this analysis, we performed an analysis for simple-simple main effects at each level of the two independent variables that had interactions still using the error term and degrees of freedom from the three-way ANOVA, otherwise simple main effects were reported. Multiple comparisons were corrected using Bonferroni's method.

False discovery rate correction by Benjamini and Hochberg, [36] was applied to the real-time qPCR data with Q = 0.05 by ranking the significant p-values and evaluating the likelihood of false positives. All results are presented as means ± SEM, and the statistical significance level is set at $\alpha = 0.05$. Group sizes were chosen based on a power calculation for FST ($1\text{-}\beta = 0.8$ and $\alpha = 0.05$).

3. Results

3.1. Effects of F_0 and F_2 diet on F_2 adult offspring body weight

There was a significant interaction between gender, F_2 diet, F_0 diet and time for F_2 offspring body weight (gender × F_2 diet × F_0 diet × time: $F_{(3,\ 226)} = 5.37$, $p < 0.001$, Fig. 2A). *Post hoc* comparisons revealed that both male and female F_2 offspring gained weight with F_2 HFD (male: F_2 diet: $F_{(1,\ 70)} = 52.78$, $p < 0.001$; female: $F_{(1,\ 70)} = 28.64$, $p < 0.001$). In the three-way ANOVA of AUC body weight, there was a significant gender and F_2 diet interaction (gender × F_2 diet: $F_{(1,\ 70)} = 5.36$, $p = 0.024$). Simple effect analysis showed that F_2 diet significantly increased AUC body weight for male F_2 offspring (F_2 diet: $F_{(1,\ 70)} = 52.39$, $p < 0.001$, Fig. 2B) and for female F_2 offspring (F_2 diet: $F_{(1,\ 70)} = 15.71$, $p < 0.001$, Fig. 2B).

F_2 HFD significantly increased total epididymal fat tissue in F_2 offspring (F_2 diet: $F_{(1,\ 70)} = 183.3$, $p < 0.001$, Table 2). Specifically, total epididymal and gonadal fat tissue was increased in CON-HFD compared to HFD-HFD F_2 offspring ($F_{(1,\ 70)} = 8.8$, $p = 0.004$, Table 2).

3.2. No effects of F_0 or F_2 HFD on F_2 offspring locomotor activity in adulthood

Neither F_0 nor F_2 diet ($p < 0.05$) had impact on locomotor activity (Fig. 3A).

3.3. Effects of F_0 and F_2 diet on F_2 adult offspring anxiety-like behaviour in the LDB

In the LDB, there was a significant gender, F_0 diet and F_2 diet interaction on time spent in the light box (gender × F_0 diet × F_2 diet: $F_{(1,\ 64)} = 7.13$, $p = 0.01$). For male F_2 offspring there was no effect of F_0 and F_2 diet on time spent in the light box (F_0 diet × F_2 diet: $F_{(1,\ 64)} = 0.78$, $p = 0.38$). For female F_2 offspring there was a significant simple F_0 and F_2 diet interaction (F_0 diet × F_2 diet: $F_{(1,\ 64)} = 8.89$, $p = 0.004$, Fig. 3B). *Post hoc* Bonferroni's comparisons test revealed that female HFD-HFD spent less time in the light box than HFD-CON ($F_{(1,\ 64)} = 9.37$, $p = 0.003$, Fig. 3B).

3.4. Effects of F_0 and F_2 diet on F_2 adult offspring risk-taking behaviour in the EPM

In the EPM, there was a significant gender and F_0 diet interaction in the three-way ANOVA (gender × F_0 diet: $F_{(1,\ 68)} = 9.03$, $p = 0.004$, Fig. 3C). Simple main effect analysis revealed that female offspring from F_0 HFD spent less time in the open arms than female offspring from F_0 CON (F_0 diet: $F_{(1,\ 68)} = 8.19$, $p = 0.006$, Fig. 3C). In addition, there was a significant gender and F_2 diet interaction (gender × F_2 diet: $F_{(1,\ 68)} = 4.23$, $p = 0.044$, Fig. 3C). Simple main effect analysis revealed that F_2 HFD male offspring spent less time in the open arms compared to F_2 CON male offspring (F_2 diet: $F_{(1,\ 64)} = 9.37$, $p = 0.003$, Fig. 3C).

3.5. Effects of F_0 and F_2 diet on F_2 adult offspring depression-like behaviour in the FST

In the FST, there was a significant gender × F_0 diet × F_2 diet interaction within time spent immobile (gender × F_0 diet × F_2 diet: $F_{(1,\ 70)} = 4.43$, $p = 0.039$; Fig. 3D). There was a significant simple F_0 diet and F_2 diet interaction in male F_2 offspring (F_0 diet × F_2 diet: $F_{(1,\ 70)} = 4.1$, $p = 0.047$) but not for female F_2 offspring (F_0 diet × F_2 diet: $F_{(1,\ 70)} = 0.9$, $p = 0.34$). *Post hoc* Bonferroni's multiple comparisons analysis revealed that F_2 diet significantly increased percentage of time spent immobile in F_0 HFD male F_2 offspring ($F_{(1,\ 70)} = 11.92$, $p = 0.001$; Fig. 3D) and in F_0 CON male F_2 offspring ($F_{(1,\ 70)} = 38.27$, $p < 0.001$; Fig. 3D). Similarly, in female F_2 offspring, F_2 HFD significantly exacerbated percentage immobility in F_0 HFD female F_2 offspring ($F_{(1,\ 70)} = 11.58$, $p = 0.001$; Fig. 3D) and in F_0 CON female F_2 offspring ($F_{(1,\ 70)} = 3.93$, $p = 0.05$; Fig. 3D).

3.6. Effects of F_0 and F_2 diet on F_2 adult offspring prepulse inhibition

In the four-way ANOVA, %PPI increased with increasing pre-pulse intensity (dB) in the repeated measurements analysis ($F_{(3,\ 210)} = 79.8$, $p < 0.001$), indicating successful PPI setup. There was a significant main effect of gender ($F_{(1,\ 70)} = 6.3$, $p < 0.01$), but F_0 diet, F_2 diet and gender interactions were not significantly different ($p > 0.05$, Fig. S1).

3.7. Effect of F_0 HFD on F_2 offspring during an oral glucose tolerance test

To determine the metabolic effects of early prenatal diet exposure, we analysed the glucose levels from male and female F_2 offspring from CON and HFD grandmothers. There was a significant gender, F_0 diet, F_2 diet and time interaction during the OGTT (gender × F_0 diet × F_2 diet × time: $F_{(2147)} = 3.1$, $p = 0.045$, Fig. 4A). We therefore performed three-way ANOVAs (gender × F_0 diet × F_2 diet) at each time point. At

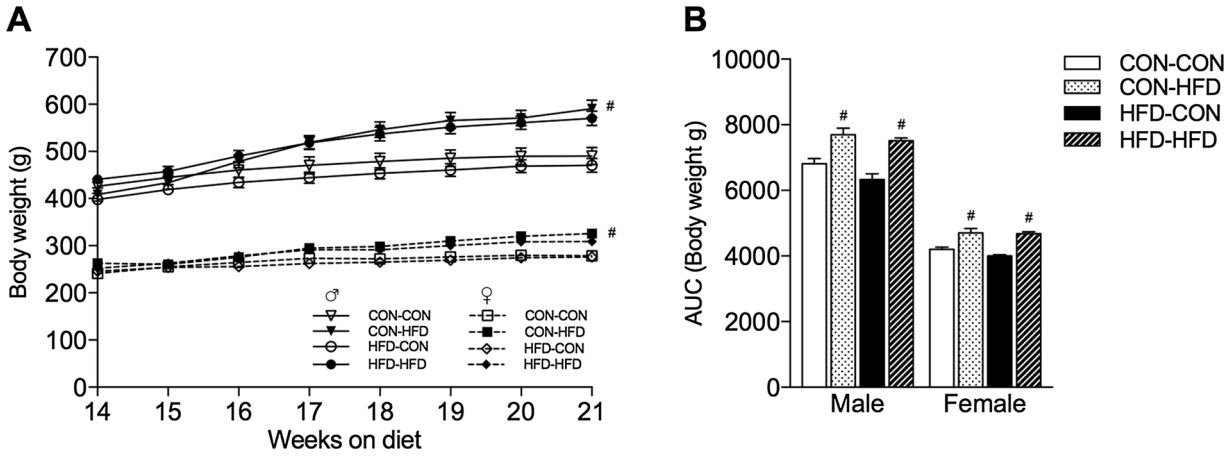

Fig. 2. Body weight increased with adult postnatal HFD challenge in male and female F_2 offspring (A) AUC body weight (B). Results are expressed as mean ± SEM. Hashtag indicate significant main effect of F_2 diet. $^{\#}$ $p < 0.001$, effect of F_2 HFD, n = 10 per group. CON, control diet; HFD, high-fat diet. Intact line indicates male F_2 offspring, line with gaps indicates female F_2 offspring. CON-CON: control diet (F_0) – control diet (F_2); HFD-CON, high-fat diet (F_0) – control diet (F_2); HFD-HFD, high-fat diet (F_0) – high-fat diet (F_2); CON-HFD, control-diet (F_0) – high-fat diet (F_2).

time points 60 min (gender × F_0 diet × F_2 diet: $F_{(1,\ 70)} = 38.99$, $p < 0.001$) and 120 min (gender × F_0 diet × F_2 diet: $F_{(1,\ 70)} = 104.46$, $p < 0.001$) F_2 HFD significantly increased the blood glucose levels, irrespective of other factors.

At time 0 min (fasting glucose), there was a significant interaction between gender, F_0 diet and F_2 diet (gender × F_0 diet × F_2 diet: $F_{(1,\ 70)} = 16.19$, $p < 0.001$). There was a significant simple F_0 diet and F_2 diet interaction for male offspring (F_0 diet × F_2 diet: $F_{(1,\ 70)} = 8.83$, $p = 0.004$) and for female F_2 offspring (F_0 diet × F_2 diet: $F_{(1,\ 70)} = 7.39$, $p = 0.008$). Simple main effects showed that in male F_2 offspring from CON grandmothers, F_2 HFD significantly increased glucose levels (F_2 diet: $F_{(1,\ 70)} = 10.15$, $p = 0.002$) and from HFD grandmothers (F_2 diet: $F_{(1,\ 70)} = 56.78$, $p < 0.001$). In female F_2 offspring from CON grandmothers, F_2 HFD significantly increased glucose levels (F_2 diet: $F_{(1,\ 70)} = 26.06$, $p < 0.001$), but not in female F_2 offspring from HFD grandmothers (F_2 diet: $F_{(1,\ 70)} = 1.81$, $p = 0.18$).

Finally, at 30 min, there was a significant gender, F_0 diet and F_2 diet interaction in the glucose levels (gender × F_0 diet × F_2 diet: $F_{(1,\ 70)} = 4.47$, $p = 0.04$). There was a trend for a significant simple F_0 diet and F_2 diet interaction for female F_2 offspring (F_0 diet × F_2 diet: $F_{(1,\ 70)} = 3.59$, $p = 0.06$), but not for male F_2 offspring (F_0 diet × F_2 diet: $F_{(1,\ 70)} = 1.19$, $p = 0.27$). Simple main effects showed that in male F_2 offspring from CON grandmothers, F_2 HFD significantly increased glucose levels (F_2 diet: $F_{(1,\ 70)} = 65.75$, $p < 0.001$) and from HFD grandmothers (F_2 diet: $F_{(1,\ 70)} = 11.73$, $p = 0.003$). In female F_2 offspring from HFD grandmothers, F_2 HFD significantly increased glucose levels (F_2 diet: $F_{(1,\ 70)} = 12.69$, $p = 0.002$), but not in female F_2 offspring from CON grandmothers (F_2 diet: $F_{(1,\ 70)} = 0.86$, $p = 0.36$).

We also performed a three-way ANOVA on the AUC of glucose levels at the OGTT. Here we found increased AUC with F_2 HFD (F_2 diet: $F_{(1,\ 70)} = 40.21$, $p < 0.001$, Fig. 4B).

3.8. Effects of F_0 HFD on F_2 Offspring Hippocampal Neuroendocrine receptor expression, synaptic plasticity and neuronal growth at the mRNA levels

To determine underlying mechanisms driving the sex-specific effects of early prenatal diet exposure, we analysed the hippocampus from male and female F_2 offspring from CON and HFD grandmothers.

In the hippocampus, there was no significant effect of gender, F_0 diet and F_2 diet on *Crh-r1* mRNA levels ($F_{(1,\ 70)} = 0.27$, $p = 0.6$). Nevertheless, *Crh-r2* mRNA levels showed a significant gender × F_0 diet interaction in the tree-way ANOVA (gender × F_0 diet: $F_{(1,\ 70)} = 4.97$, $p = 0.029$). In fact, within male F_2 offspring, grandmaternal HFD significantly increased the hippocampal *Crh-r2* mRNA levels ($F_{(1,\ 70)} = 6.33$, $p = 0.01$; Fig. 5A). However, in female F_2 offspring, no significant difference was found ($F_{(1,\ 70)} = 0.23$, $p = 0.61$; Fig. 5A).

There was a significant gender × F_0 diet × F_2 diet interaction within *Mr* mRNA levels (gender × F_0 diet × F_2 diet: $F_{(1,\ 70)} = 5.81$, $p = 0.026$; Fig. 5B). We then performed simple two-way ANOVAs (F_0 diet × F_2 diet) for each gender. There was a significant simple F_0 diet and F_2 diet interaction for female F_2 offspring (F_0 diet × F_2 diet: $F_{(1,\ 70)} = 4.59$, $p = 0.04$), but not for male F_2 offspring (F_0 diet × F_2 diet: $F_{(1,\ 70)} = 1.11$, $p = 0.3$). In female F_2 offspring from HFD

Table 2
Effect of F_0 and F_2 diet on body weight, fat mass, liver weight and heart weight. Data were analysed by three-way ANOVA. Results are expressed as mean ± SEM, n = 9 – 10. $^{+}$ $p < 0.001$, effect of gender; $^{\alpha}$ $p < 0.01$, compared to HFD-HFD; $^{\&}$ $p < 0.01$ $^{\#}$ $p < 0.001$, effect of F_2 diet.

	Male				Female			
	CON-CON	CON-HFD	HFD-CON	HFD-HFD	CON-CON	CON-HFD	HFD-CON	HFD-HFD
Body weight, start (g)$^{+}$	426.1 ± 45.7	408.5 ± 44.5	398.2 ± 29.9	432.7 ± 27.9	241 ± 12.3	263 ± 27.8	246.1 ± 17.8	252.4 ± 13.7
Body weight, end (g)$^{+}$	525.7 ± 40.1	690.1 ± 24.5$^{\#}$	490.7 ± 37.4	614.6 ± 24.7$^{\#}$	295.9 ± 16.3	335.3 ± 14.1$^{\#}$	289.5 ± 15.0	347.9 ± 24.3$^{\#}$
Epididymal/gonadal fat tissue								
Weight (g)$^{+}$	8 ± 1.6	21.4 ± 4.7$^{\#}$	6.9 ± 1.6	16.1 ± 4.4$^{\#}$	6.1 ± 1.9	14.2 ± 3.7$^{\#}$	5.7 ± 1.6	13.3 ± 3.5$^{\#}$
Weight (% of BW)$^{+}$	1.5 ± 0.2	3.1 ± 0.4$^{\#,\alpha}$	1.4 ± 0.2	2.6 ± 0.6$^{\#}$	2.1 ± 0.6	4.1 ± 0.8$^{\#,\alpha}$	1.9 ± 0.5	3.8 ± 0.8$^{\#}$
Liver								
Weight (g)$^{+}$	16.1 ± 2.6	17.7 ± 2.3	16.6 ± 2.03	16.1 ± 1.9	9.7 ± 1.2	10.1 ± 2.0	9.3 ± 1.2	9.3 ± 0.8
Weight (% of BW)	3.1 ± 0.3	2.53 ± 0.2$^{\#}$	3.3 ± 0.21	2.6 ± 0.2$^{\#}$	3.3 ± 0.5	2.8 ± 0.3$^{\#}$	3.1 ± 0.3	2.7 ± 0.2$^{\#}$
Heart								
Weight (g)$^{+}$	1.66 ± 0.18	1.84 ± 0.18$^{\alpha}$	1.52 ± 0.13	1.67 ± 0.18	1.12 ± 0.13	1.25 ± 0.13$^{\alpha}$	1.06 ± 0.1	1.09 ± 0.13
Weight (% of BW)$^{+}$	0.31 ± 0.02	0.27 ± 0.02$^{\#}$	0.30 ± 0.02	0.26 ± 0.02$^{\#}$	0.37 ± 0.03	0.35 ± 0.04$^{\&}$	0.36 ± 0.02	0.32 ± 0.04$^{\&}$

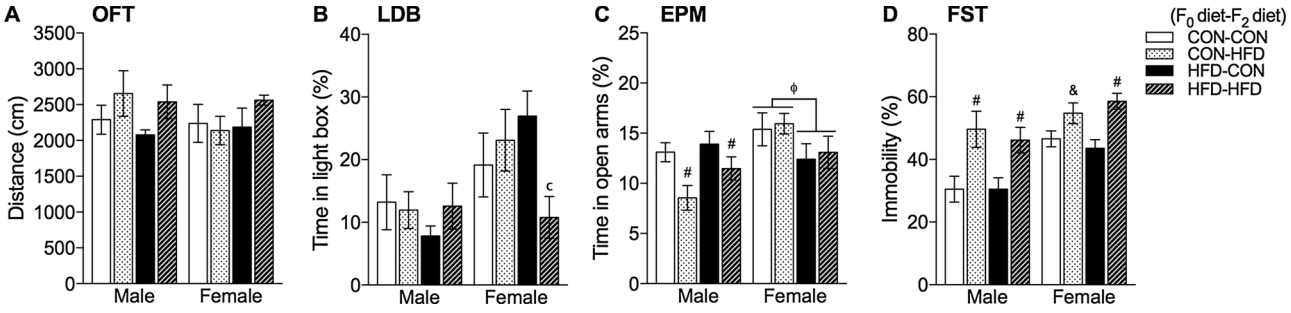

Fig. 3. Behaviour was altered differently in male and female F_2 offspring with F_0 and F_2 diet. In the OFT there were no changes (A). In the LDB, HFD-HFD female F_2 offspring showed anxiety-like behaviour (B). In the EPM female F_2 offspring showed an anxiety-like behaviour with F_0 HFD and male F_2 offspring showed anxiety-like behaviour with F_2 HFD (C). Immobility was increased in F_2 offspring with F_2 HFD in the FST (D). Data are presented as mean ± SEM, n = 8–10 per group. $^{\&}$ $p < 0.05$, compared to F_2 CON; ^{c} $p < 0.01$, compared to HFD-CON; $^{\#}$ $p < 0.01$, compared to F_2 CON; $^{\varphi}$ $p < 0.01$, compared to F_0 CON. CON, control diet; HFD, high-fat diet. CON-CON: control diet (F_0) – control diet (F_2); HFD-CON, high-fat diet (F_0) – control diet (F_2); HFD-HFD, high-fat diet (F_0) – high-fat diet (F_2); CON-HFD, control-diet (F_0) – high-fat diet (F_2).

grandmothers, there was a simple main effect of F_2 diet (F_2 diet: $F_{(1,\ 70)} = 10.55$, $p = 0.004$). *Post hoc* Bonferroni's comparisons test revealed significantly higher *Mr* mRNA level in HFD-CON compared to CON-CON ($F_{(1,\ 70)} = 4.65$, $p = 0.034$, Fig. 5B) and to HFD-HFD ($F_{(1,\ 70)} = 11.67$, $p < 0.001$, Fig. 5B), respectively.

The three-way ANOVA showed a significant main effect of gender within *Gr* mRNA levels as such that female F_2 offspring transcript levels were higher than male F_2 offspring's (gender: $F_{(1,\ 70)} = 5.78$, $p = 0.019$; Fig. 5C).

In the three-way ANOVA there was a significant gender × F_2 diet interaction within *11β-hsd1* mRNA levels (gender × F_2 diet: $F_{(1,\ 70)} = 4.14$, $p = 0.046$). Simple main effect analysis showed that male F_2 offspring challenged with F_2 HFD had lower levels compared to female F_2 offspring fed F_2 HFD (F_2 diet: $F_{(1,\ 70)} = 7.91$, $p = 0.006$; Fig. 5D).

The three-way ANOVA showed a significant gender × F_2 diet interaction on *Bdnf* mRNA levels (gender × F_2 diet: $F_{(1,\ 70)} = 4.85$, $p = 0.031$). In male F_2 offspring, F_2 HFD did not alter *Bdnf* mRNA levels compared to F_2 CON male F_2 offspring (F_2 diet: $F_{(1,\ 70)} = 0.14$, $p = 0.71$). Within female F_2 offspring, F_2 HFD increased the *Bdnf* mRNA levels compared to F_2 CON female F_2 offspring (F_2 diet: $F_{(1,\ 70)} = 7.47$, $p = 0.008$; Fig. 5E).

Furthermore, there was a significant gender × F_0 diet interaction (gender × F_0 diet: $F_{(1,\ 70)} = 11.28$, $p = 0.001$). Male F_2 offspring from F_0 CON had higher *Bdnf* mRNA levels than female F_2 offspring from F_0 CON (F_0 diet: $F_{(1,\ 70)} = 6.42$, $p = 0.01$; Fig. 5E). Female F_2 offspring from F_0 HFD had increased *Bdnf* mRNA levels compared to male F_2 offspring from F_0 HFD (F_0 diet: $F_{(1,\ 70)} = 4.9$, $p = 0.03$; Fig. 5E). In addition, female F_2 offspring from F_0 HFD had higher transcript levels than female F_0 CON (F_0 diet: $F_{(1,\ 70)} = 9.37$, $p = 0.003$; Fig. 5E).

4. Discussion

We describe for the first time, sex-dependent intergenerational heritability of grandmaternal HFD via the maternal line on the anxiety-like behaviour and HPA axis response in rats.

The main findings of the present study are that grandmaternal obesogenic diet indicates a predisposition to an increased anxiety-like behaviour in adult female F_2 offspring in the EPM. In the LDB, HFD in adult female F_2 offspring exacerbates a predisposed latent trait inherited from the F_0 generation to an increased anxiogenic phenotype in the female F_2 generation. Additionally, F_2 HFD induced anxiogenic behaviour in male F_2 offspring, independent of F_0 diet in EPM, but no effects were found for male F_2 offspring in the LDB. In our study, it could indicate that male offspring are more resilient to early life stressors. The underlying mechanism programming the effect of grandmaternal diet exposure remains unclear.

Although early life maternal diet generally has demonstrated anxiety in the F_1 generation across many animal models of early life stressors [13,15,37,38], task variances are occasionally observed [39]. There may be differences in performance in these tasks as the LDB correlates with more responses to novelty than with exploration [40]

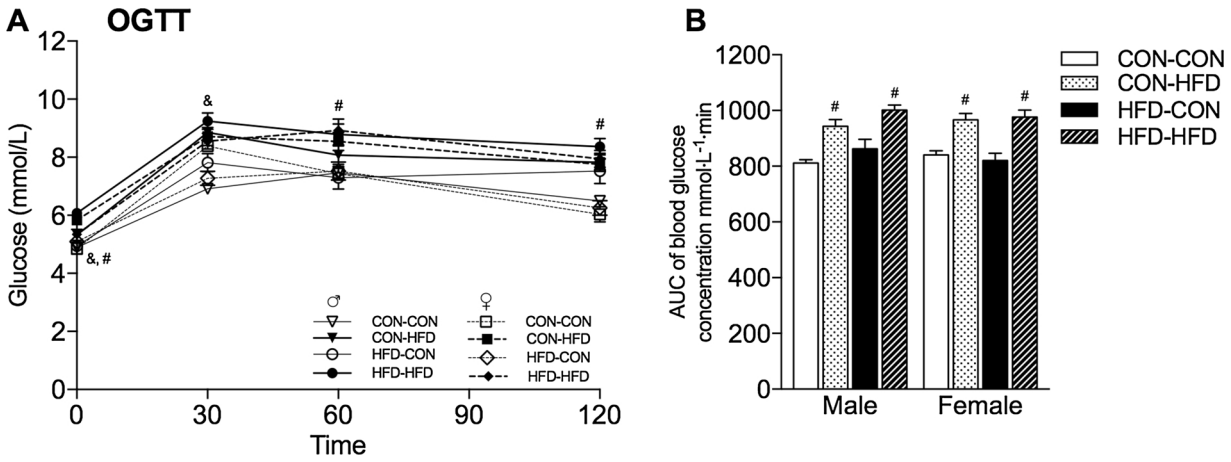

Fig. 4. F_2 HFD, but not F_0 HFD, impairs blood glucose homeostasis. Plasma glucose levels were increased in male and female F_2 offspring with F_2 HFD, but not F_0 HFD (A). The AUC in male and female F_2 offspring (B). Data are presented as mean ± SEM, n = 10 per group. $^{\&}$ $p < 0.05$, $^{\#}$ $p < 0.01$, compared to F_2 CON diet. Intact line indicates male F_2 offspring, line with gaps indicates female F_2 offspring CON-CON: control diet (F_0) – control diet (F_2); HFD-CON, high-fat diet (F_0) – control diet (F_2); HFD-HFD, high-fat diet (F_0) – high-fat diet (F_2); CON-HFD, control-diet (F_0) – high-fat diet (F_2).

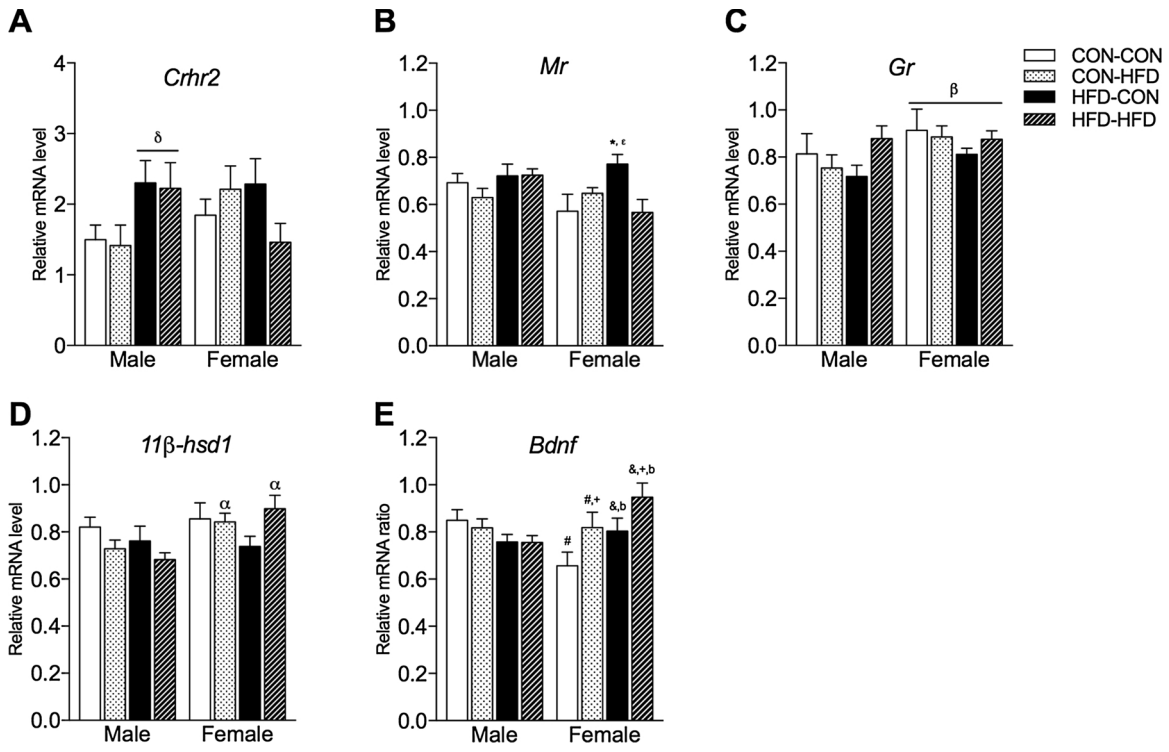

Fig. 5. Second-generation male and female offspring hippocampal mRNA levels. *Crhr2* mRNA levels were increased in F_2 male offspring with F_0 diet (A). *Mr* mRNA levels were higher in HFD-CON compared to CON-CON and HFD-HFD, respectively (B). *Gr* mRNA levels were higher in male F_2 offspring (C). *11β-hsd1* mRNA levels were higher in F_2 HFD exposed female F_2 offspring compared to HFD exposed male F_2 offspring (D). *Bdnf* mRNA levels were higher in F_0 HFD female F_2 offspring than F_0 HFD male F_2 offspring. In addition, *Bdnf* mRNA levels were higher in F_0 HFD female F_2 offspring than F_0 CON female F_2 offspring. *Bdnf* mRNA levels were higher in F_2 HFD exposed female F_2 offspring than F_2 CON female F_2 offspring (E). Data were analysed by three-way ANOVA. CON: control diet, HFD: high-fat diet. Data are expressed as mean ± SEM, n = 9 – 10. $^{\delta}p < 0.01$, simple main effect of F_0 HFD within male F_2 offspring; $^{*}p < 0.05$, compared to CON-CON; $^{\varepsilon}p < 0.01$, compared to HFD-HFD; $^{\beta}p < 0.05$ compared to male F_2 offspring; $^{\alpha}p < 0.01$, compared to F_2 HFD male F_2 offspring; $^{\#}p < 0.01$, compared to F_0 CON male F_2 offspring; $^{\&}p < 0.05$ compared to F_0 HFD male F_2 offspring; $^{b}p < 0.01$, compared to F_0 CON female F_2 offspring; $^{+}p < 0.01$, compared to F_2 CON female F_2 offspring. CON-CON: control diet (F_0) – control diet (F_2); HFD-CON, high-fat diet (F_0) – control diet (F_2); HFD-HFD, high-fat diet (F_0) – high-fat diet (F_2); CON-HFD, control-diet (F_0) – high-fat diet (F_2).

and the EPM correlates with increased novelty-seeking behaviour and risk-assessment [22]. Differences in anxiety-like behaviour detected in the EPM and LDB may be due to differences in dietary habits which have been connected not only to disruption in energy balance but also to mood and anxiety disorders [41].

In the FST, F_2 HFD exposed offspring displayed depression-like behaviour, independently of F_0 diet and sex. This suggests, that depression-like behaviour in the FST in not being passed on in an intergenerational manner, at least with the HFD used in our study. We did not detect any changes in general locomotor activity in the OFT induced by diet, thus confirming that changes in the immobility in the FST cannot be ascribed to changes in general activity.

Sensorimotor gating, being impaired in many schizophrenic patients and in animal model of psychosis [42,43], can be measured by the PPI. Although we could not detect any difference in the PPI with F_0 HFD, the inheritance of such trait has been demonstrated in a study administering methylazoxymethanol acetate, which induced schizophrenic-like phenotype in adolescent rats and was inherited to the F_2 and the F_3 generation, with a significant correlation of the hippocampal parvalbumin expression and the neuronal activity of dopamine in the F_2 rats [44]. These studies may be valuable to examine the aspects of gene and environment interactions.

Our data demonstrate a heritable aspect on the molecular level as we found that the effect of grandmaternal HFD exposure altered hippocampal genes involved in the HPA axis response in the F_2 offspring, which were clearly sexually dimorphic. As CRH-R2 previously has been shown to correlate to anxiety and depression [45], we measured hippocampal *Crh-r2* mRNA levels in F_2 offspring. Among the adult male F_2 offspring, grandmaternal HFD exposure resulted in increased levels of *Crh-r2* mRNA levels, with no difference in female F_2 offspring. Animal studies suggest that *Crhr2* mRNA expression and functioning differs in response to stressor early in life, as such that expression is both up and down regulated [46]. In our study, the increased *Crh-r2* mRNA level in male F_2 offspring by grandmaternal HFD exposure could attribute to an altered HPA axis function, however, future studies are needed to underpin the inherited trait of the HPA axis.

MR and GR play a role in the neuroendocrine stress axis feedback regulation and difference between GR and MR involves resilience and adaption to stress [47]. We found that the hippocampal *Mr* mRNA gene expression was higher in female F_2 offspring from grandmothers exposed to CON diet than in female F_2 offspring from grandmothers exposed to HFD, suggesting that adult F_2 diet had a programming effect on latent trait expression of the MR affinity for corticosteroids [48]. This finding suggest that grandmaternal diet exposure could be important to MR function in the HPA axis response to maternal HFD.

In our study, we demonstrated a sex-dependent difference in the neuroendocrine stress axis function of *Gr* mRNA level in response to grandmaternal diet exposure and adult diet manipulation, providing additional indication of sex-dependent programming in the mechanism of phenotype manifestation initiating at early life development. This finding may be in line with a previous study demonstrating an inherited elevated level of the GR target receptor phosphoenolpyruvate carboxykinase (PEPCK) in the liver in the F_2 generation male offspring, following maternal dexamethasone administration during gestation [49].

Surprisingly, we found that in female F_2 offspring, hippocampal *11β-hsd1* mRNA expression was increased with F_2 HFD compared to

male F_2 HFD, independently of grandmaternal diet. In the brain, 11β-HSD1 acts as a reductase, and may amplify glucocorticoid action under certain circumstances [50]. Inhibition of 11β-HSD1 reversed spatial memory impairments and reduced fear memories [51,52], indicating a potential target for treatment option for certain subgroups suffering from anxiety. Our finding suggests that consumption of a highly palatable food modulates the regulation of hippocampal *11β-hsd1* mRNA in female F_2 offspring and could play a role in the observed behaviour.

Interestingly, we found a sex-dependent effect in the BDNF expression with HFD in adulthood leading to upregulation of *Bdnf* mRNA expression levels in female F_2 offspring. This result may be in line with a previous study that demonstrated that maternal HFD increased BDNF and induced a depression-like phenotype in offspring [53]. Increased HFD has been demonstrated to improve stress and ameliorate anxiety and depression-like phenotype in rats [54,55]. Thus, although conflicting results, this study indicates, that grandmaternal HFD consumption can alter behaviour via altered BDNF, which may play a role in the observed behavioural phenotypes in the EPM in the adult progeny.

As expected, offspring receiving a F_2 HFD had increased body weight, visceral adipose tissue, liver weight, and heart weight, suggesting a dysmetabolic physiological condition. However, F_2 offspring exposed to F_2 HFD had elevated levels of visceral adipose tissue by F_0 CON diet compared to F_0 HFD. We evaluated the dynamics of glucose response by an oral glucose tolerance test. Overall, F_2 HFD increased glucose metabolism in male and female F_2 offspring with effects of F_0 diet at 30 and 60 min. Thus, these data may suggest that F_0 diet exposure may play a role in programming adult physiology of weight gain and metabolic dysfunction in the F_2 generation, inherited through the maternal lineage. An increased HPA axis activity is an important link between early life and adult metabolic syndrome and T2DM [56,57]. Although the mechanism for developmental programming is not fully clear, alterations in the trajectories of neuroendocrine and metabolic pathways during early developmental share common features.

Limitations of the current study include inability to confirm whether the observed phenotypes are germ-line modifications or re-transmission via development in the programmed brain. Future studies should include a thorough understanding of the specific molecular mechanisms by which inherited material is altered by the diet and inherited transgenerationally. In addition, due to total amount of hippocampal tissue, it was not possible to execute protein, DNA methylation analysis and other confirming studies. However, for better understanding the mechanism by which grandmaternal diet could lead to such impairment in anxiety, future studies should include larger sample and include the gene and environment interaction model.

The hippocampus was chosen as it is involved in anxiety and affective disorders, however other regions as for example amygdala or prefrontal cortex could have been investigated as these regions are implicated in anxiety and memory. Moreover, the hippocampus was not divided into ventral and dorsal which could have further reduced variability in the measured outcome. Our finding that developmental programming has effects on female offspring following a grandmaternal nutritional stimulus has important implications, not only for improved understanding of how the brain is influenced by the early life environment, but also how programming effects could persist or be repeated in future generations. Furthermore, the lack of published multigenerational studies on diet induced animal models transgenerational effect on behaviour, will require future work on the third generation to address the mechanisms of inheritance from the maternal lineage.

In summary, our findings demonstrate that female offspring exposed to grandmaternal HFD could be more sensitive than male offspring and when exposed to a F_2 diet, and that grandmaternal diet could trigger changes in MR activity, but not in GR, which might describe the extent of diverse results in the subtypes of anxiety. The finding that grandmaternal environment leads to altered behaviour in females, but not in males is of interest in the increased prevalence of anxiety and depression in women than men [58].

This suggests the possibility that the mechanism of action of behavioural development may differ between the sexes and that the HPA axis responsivity may be important in male and female development of mental disorders. However, future studies should include larger samples to confirm this to develop early intervention options, such as pharmacological and lifestyle strategies.

Author contributions

All experiments were conducted by Gudrun Winther. First draft of this manuscript was conducted by Gudrun Winther and all authors assisted in the further interpretation of data, editing and final approval of the manuscript.

Disclosure

Gregers Wegener declares having received lecture/consultancy fees from H. Lundbeck A/S, Servier SA, Astra Zeneca AB, Eli Lilly A/S, Sun Pharma Pty Ltd, and Pfizer Inc., Shire A/S, HB Pharma A/S, Arla Foods A.m.b.A., Alkermes Inc, and Mundipharma International Ltd., and research funding from the Danish Medical Research Council, Aarhus University Research Foundation (AU-IDEAS initiative (eMOOD)), the Novo Nordisk Foundation, the Lundbeck Foundation, and EU Horizon 2020 (ExEDE). All other authors report no potential conflicts of interest. All other authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbr.2018.10.017.

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