

# PERINATAL HIGH FAT DIET ALTERS GLUCOCORTICOID SIGNALING AND ANXIETY BEHAVIOR IN ADULTHOOD

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**Abstract**—Maternal obesity carries significant health risks for offspring that manifest later in life, including metabolic syndrome, cardiovascular disease and affective disorders. Programming of the hypothalamic–pituitary–adrenal (HPA) axis during development mediates both metabolic homeostasis and the response to psychosocial stress in offspring. A diet high in fat alters maternal systemic corticosterone levels, but effects in offspring on limbic brain areas regulating the HPA axis and anxiety behavior are poorly understood. In addition to their role in the response to psychosocial stress, corticosteroid receptors form part of the glucocorticoid signaling pathway comprising downstream inflammatory processes. Increased systemic inflammation is a hallmark of high-fat diet exposure, though altered expression of these genes in limbic brain areas has not been examined. We studied the influence of high-fat diet exposure during pre-weaning development in rats on gene expression in the amygdala and hippocampus by quantitative real-time polymerase chain reaction (PCR), anxiety behavior in the Open field, elevated plus maze and light–dark transition tasks, and corticosterone levels in response to stress by radioimmunoassay. As adults, offspring exposed to perinatal high-fat diet show increased expression of corticosterone receptors in the amygdala and altered pro-inflammatory and anti-inflammatory expression in the hippocampus and amygdala in genes known to be regulated by the glucocorticoid receptor. These changes were associated with increased anxiety behavior, decreased basal corticosterone levels and a slower return to baseline levels following a stress challenge. The data indicate that the dietary environment during development programs glucocorticoid

signaling pathways in limbic areas relevant for the regulation of HPA function and anxiety behavior. © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** glucocorticoid receptor, stress, anxiety behavior, maternal, obesity, gene expression programming.

## INTRODUCTION

Obesity is increasingly common during pregnancy, exposing the developing child to significant health risks (King, 2006). The overconsumption of saturated fat is most closely linked with deleterious health effects on offspring development (Bersamin et al., 2008). Exposure to a diet high in fat during development is a well known risk factor for coronary heart disease, type 2 diabetes and the metabolic syndrome in adulthood (Marx, 2002; Boksa, 2004; Kahn et al., 2006; Van Gaal et al., 2006; Peleg-Raibstein et al., 2012). In addition, obesity increases the risk of behavioral disorders associated with anxiety in humans, effects that are particularly pronounced among females (Desai et al., 2009; Rofey et al., 2009).

Animal models of diet-induced obesity have been important tools for understanding the influence of overnutrition on metabolic development (Bouret, 2009). For example, maternal diets high in saturated fats and refined sugar compared to standard chow impact gene expression in brain reward systems and alter offspring food preferences for fat-rich foods (Ong and Muhlhausler, 2011). Few studies to date have examined the influence of perinatal high-fat diet on the development of neural systems mediating anxiety behavior. The results of studies of the role of perinatal high-fat diet on both basal and stress-challenged levels of circulating corticosterone in adult offspring have been unclear due to conflicting findings (Walker et al., 2008; Auvinen et al., 2011). Non-human primates developmentally exposed to a high-fat diet show increased fear responses in the presence of novelty (Sullivan et al., 2010) and in rodents high-fat diet exposure during development appears to increase anxiety behavior in the Open field and Elevated plus maze tasks (Bilbo and Tsang, 2010; Peleg-Raibstein et al., 2012). To date, the impact of high-fat diet exposure during perinatal development on brain-region specific changes associated with increased anxiety behavior remains poorly understood.

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*Abbreviations:* CD11b, cluster of differentiation molecule 11B; CHD, chow diet; CORT, corticosterone; EPM, elevated plus maze task; GR, glucocorticoid receptor; HFD, high-fat diet; HPA, hypothalamic–pituitary–adrenal; I $\kappa$ B $\alpha$ , I-kappa-B-alpha; IL-1Ra, interleukin-1 Receptor antagonist; IL-6, interleukin-6; LD, light–dark transition task; LPS, lipopolysaccharide; MKP-1, mitogen-activated protein kinase phosphatase-1; MR, mineralocorticoid receptor; NF $\kappa$ B, nuclear factor kappa beta; OF, Open field task; PD, postnatal day; TLR4, Toll-like receptor 4.

Many studies in animal models have demonstrated that environmental factors during perinatal development have a long-term impact on the function of the hypothalamic–pituitary–adrenal (HPA) axis, a primary mediator of the endocrine response to stress. For example, maternal stress during pregnancy or maternal care in early postnatal life have long-term effects on anxiety behavior in offspring and the expression of genes in the brain critical for the function of the HPA axis (Meaney, 2001; Welberg and Seckl, 2001; McGowan et al., 2008; Brunton, 2010). These factors are known to alter HPA function in offspring in part via changes in the expression of mineralocorticoid and glucocorticoid receptors in limbic brain areas, including the amygdala and hippocampus, that regulate basal and stress-activated levels of corticosterone in circulation (Welberg and Seckl, 2001; Brunton, 2010). There is also increasing recognition of the role of glucocorticoid receptors in the regulation of downstream inflammatory processes as a result of altered HPA function (Sorrells et al., 2009).

Obesity has long been known to induce markers of inflammation in the body (Bray et al., 2002). Recent data in animal models suggest that high-fat diet exposure changes glial activation of inflammatory processes in the hypothalamus and in the hippocampus (Bilbo and Tsang, 2010; Peleg-Raibstein et al., 2012). In the hypothalamus, pro-inflammatory cytokines including NF $\kappa$ B, IL-6 and other downstream signaling molecules including I $\kappa$ B represent the largest class of genes with altered expression as a function of chronic high-fat diet consumption (De Souza et al., 2005; Velloso et al., 2008; Thaler and Schwartz, 2010). However, to our knowledge, the influence of developmental exposure to high-fat diet on the expression of these inflammatory genes in the amygdala, a brain area long known to be critically involved in anxiety behavior (Davis, 1992), has not been studied.

Here, we examine the impact of perinatal high-fat diet exposure on corticosteroid receptor and downstream pro- and anti-inflammatory gene expression in limbic brain areas important for the response to psychosocial stress and anxiety behavior in adulthood, two components of the adaptive response system mediated by the HPA axis.

## EXPERIMENTAL PROCEDURES

### Animals

Adult male and female Long Evans rats (7 week) used were obtained from Charles River Canada (St. Constant, QC), housed in same-sex pairs and maintained on a 12:12-h light–dark cycle (lights on 7:00 am–7:00 pm) with *ad libitum* access to food and water. Experimental protocols were approved by the Local Animal Care Committee at the University of Toronto, Scarborough, and were in accordance with the guidelines of the Canadian Council on Animal Care.

### Diets

Female breeders were placed on one of two diets: a high-fat diet (HFD,  $n = 8$ ) or a house chow diet (CHD,  $n = 8$ ). The 5.24-kcal/g high-fat diet was obtained from Research Diets, Inc. (New Brunswick, NJ: cat. no. D12492), and contained (by kcal): 20% protein, 60% fat, 20% carbohydrate. The 3.02-kcal/g house chow

diet was obtained from Purina Lab Diets (St. Louis, MO: cat. no. 5001) and contained 28.5% protein, 13.5% fat, and 58% carbohydrate. A comparison between similar formulations of high-fat diet and house chow diet has been used to examine diet-induced obesity in several previous studies (El-Haschimi et al., 2000; De Souza et al., 2005; Dunn and Bale, 2009; Tamashiro et al., 2009; Purcell et al., 2011). Females remained on the diet for 4 weeks prior to mating and throughout pregnancy and lactation. Upon weaning at Postnatal day (PD) 21, offspring were maintained on house chow diet throughout adulthood.

### Subjects and general procedures

Female breeders were individually housed after mating and weighed daily until the birth of their pups. There were no significant differences in litter size or sex ratio among the diet groups. Except for weighing during weekly cage changes, the offspring remained undisturbed until PD21 when they were housed in same-sex pairs throughout adulthood (PD90). A subset of male and female adult offspring (1–2/sex/L) were used in behavioral assays (HFD-F  $n = 14$ , CHD-F  $n = 12$ , HFD-M  $n = 14$ , CHD-M  $n = 11$ ) and brains for a subset of six animals per sex and per diet group were collected at PND110 ( $\pm 10$ ) for gene expression analysis. Bodyweights in adulthood were taken at sacrifice shortly after completion of the behavioral experiments. A separate subset of adult animals were used for blood collection and corticosterone radioimmunoassays (HFD-F  $n = 10$ , CHD-F  $n = 10$ , HFD-M  $n = 10$ , CHD-M  $n = 11$ ). All testing, blood collection, and animal sacrifices occurred at the mid-point of the light phase of the circadian cycle (11–3 pm) to control for possible circadian effects on behavior, hormone levels and gene expression.

### Open field

The Open field (OF) consisted of an opaque square arena (40.3  $\times$  40.3 cm) placed in a dimly lit room (33.7 lux). The number of entries into the center of the arena (20.15  $\times$  20.15 cm) as well as the distance traveled were recorded using ANY-maze software over the course of a 15-min trial. The order of testing was pseudo-randomized between diet groups and sexes. After each test, the maze was cleaned using a 70% ethanol solution and allowed to air dry to remove or homogenize odorants.

### Elevated plus maze

The Elevated plus maze (EPM) contained two open and two closed arms (45  $\times$  10 cm) and a center platform (10  $\times$  12 cm) elevated 80 cm above the floor. The maze was placed in a dimly lit room (33.7 lux). The number of visits to the open arm as well as the closed arm and the distance traveled in both arms were recorded using ANY-maze software over a 5-min trial. In addition, we noticed that many animals also exhibited ‘stretch-attend’ postures when exploring the arms of the maze such that only their head and shoulders fully entered a given zone, a behavioral measure known to be sensitive to the effects of anxiolytic drugs (Bailey and Crawley, 2009). Head entries in each arm were therefore defined in ANY-maze software as the presence of 60% of the animal in a given arm. After each test, the maze was cleaned using a 70% ethanol solution and allowed to air dry.

### Light–dark transition

The light–dark (LD) transition box consisted of two Plexiglas chambers of equal size (30  $\times$  30 cm): one black (dark) and one white (light). The boxes were placed in a dimly lit room with a

single light bulb centered over the light portion of the box. Each trial was 5 min. The wall separating the two chambers contained a small opening (12 × 12 cm) to allow passage between the chambers. The number of visits to the light chamber and the distance traveled in the light chamber were recorded using ANY-maze software. After each test, the chambers were cleaned using a 70% ethanol solution and allowed to air dry.

### Blood collection and serum corticosterone (CORT) radioimmunoassay

Rats were handled (2 min/day) for five consecutive days prior to testing. On the day of testing, rats were habituated to the procedure room for 2 h and then hand-restrained with a loosely fitting towel and blood was immediately withdrawn from a small nick in the tail into a heparinized tube and placed on ice. Rats were then placed into Plexiglas restrainers (6.4 cm diameter × 21.6 cm length; Plas-Labs) and after 20 min a second sample of blood was withdrawn while rats were in the restrainer. Rats were then returned to their home-cage without their conspecific pair and left undisturbed for 70 min in the same room. At the end of 70 min, a third sample of blood was withdrawn.

Blood was kept on ice for at least 30 min before being centrifuged at 4 °C, 4000 rpm for 20 min. Serum was then extracted and stored at –80 °C. Levels of serum corticosterone were determined using commercially available radioimmunoassay kits with 125I-labeled anti-corticosterone antibody (MP Biomedicals Inc., CA., USA: sensitivity 7.7 ng/ml and the intra-assay coefficient of variation was 7.1%). Five outliers with CORT values  $\pm 2$  STDEV from the mean were identified and removed from analysis (HFD-F  $n = 1$ , CHD-F  $n = 1$ , HFD-M  $n = 2$ , CHD-M  $n = 1$ ).

### Tissue preparation

Animals were sacrificed by CO<sub>2</sub> inhalation followed by decapitation. The entire hippocampus and amygdala were rapidly dissected from adult (PD110 ± 10) HFD and CHD offspring for gene expression analysis using stereotaxic coordinates (Paxinos and Watson, 1997), flash-frozen and stored at –80 °C. RNA extraction (RNeasy plus, Qiagen) and quantification/quality assessment (Nanodrop ND-2000C spectrophotometer, Thermo Scientific) were performed according to the manufacturers' protocol.

### Gene expression analysis by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression patterns of seven transcripts were quantified and analysis were performed by StepOne Plus real-time PCR using Fast SYBR Green PCR master mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and by relative normalization against the expression of an additional five housekeeping genes (Gapdh, Actb, Ubc, Ywhaz, and rRNA). The genes showing the least variance between high-fat diet and in house chow groups were selected for the analysis (Gapdh, Actb, and Ywhaz). A standard curve was generated from 11 serial dilutions of a mixture of cDNA from offspring, and gene expression was quantified relative to a geometric mean of the three housekeeping genes. All reactions for all genes were performed in triplicate.

### Primer specifications

The primers used in this study were purchased or designed using sequence information from GenBank at the National Center for Biotechnology Information (NCBI; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) as

follows: GR: NR3C1 RT2 quantitative-PCR (qPCR) Primer Assay (PPR52805B, SA biosciences, Qiagen, Valencia, CA, USA), NFkB: RT2 qPCR Primer Assay (PPR42746A, SA biosciences, Qiagen, Valencia, CA, USA), IL-6: F: 5'-GGC AAA TTT CCT GGT TAT ATC C-3' and R: 5'-AGA AAA GAG TTG TGC AAT GGC A-3', MR: F: 5'-GGC AGC TGC AAA GTC TTC TT-3' and R: 5'-GAC AGT TCT TTC GCC GAA TC-3', UBC: F: 5'-CAC CAA GAA GGT CAA ACA GGA A-3'; GAPDH: F: 5'-ACA TCA AAT GGG GTG ATG CT-3' and R: 5'-GTG GTT CAC ACC CAT CAC AA-3', ActinB: F: 5'-TTT GAG ACC TTC AAC ACC CC-3' and R: 5'-ATA GCT CTT CTC CAG GGA GG-3', 18S rRNA: F: 5'-ATG GTA GTC GCC GTG CCT A-3' and R: 5'-CTG CTG CCT TCC TTG GAT G-3', Ywhaz: F: 5'-TTG AGC AGA AGA CGG AAG GT-3' and R: 5'-GAA GCA TTG GGG ATC AAG AA-3', IL-1Ra: F: 5'-CTG GGT ACT TAC AAG GAC CAA ATA CC-3' and R: TGG ATG CCC AAG AAC ACA TTC CGA-3', MKP-1: F: 5'-GCT CCA CTC AAG TCT TCT TCC TCC AA-3', IkBa: F: CAG GAT TCT GCA GGT CCA CT-3' and R: TGG AGC ACT TGG TGA CTT TG-3'.

### Statistical analyses

Statistical analyses were carried out using Statview (SAS institute, Cary NC). Data for maternal and pup weights were analyzed by 2 × 2 diet by sex mix-model repeated measures analysis of variance (ANOVA). Data for caloric intake between HFD and CHD groups were analyzed using a student-t test. Behavioral data for OF, EPM and LD were analyzed by 2 × 2 factorial ANOVA followed by post hoc tests for identified effects of high-fat diet using a Bonferroni correction for multiple comparisons. Mixed-model repeated measures ANOVA were used to examine diet, sex and changes in the CORT response over time with stress challenge using Bonferroni post hoc tests for planned comparisons. For gene expression analysis, 2 × 2 factorial ANOVA examining the influence of diet and sex were used, followed by Bonferroni tests for post hoc comparisons. Effects considered statistically significant at  $P \leq 0.05$  and non-significant trends at  $P \leq 0.10$  are reported.

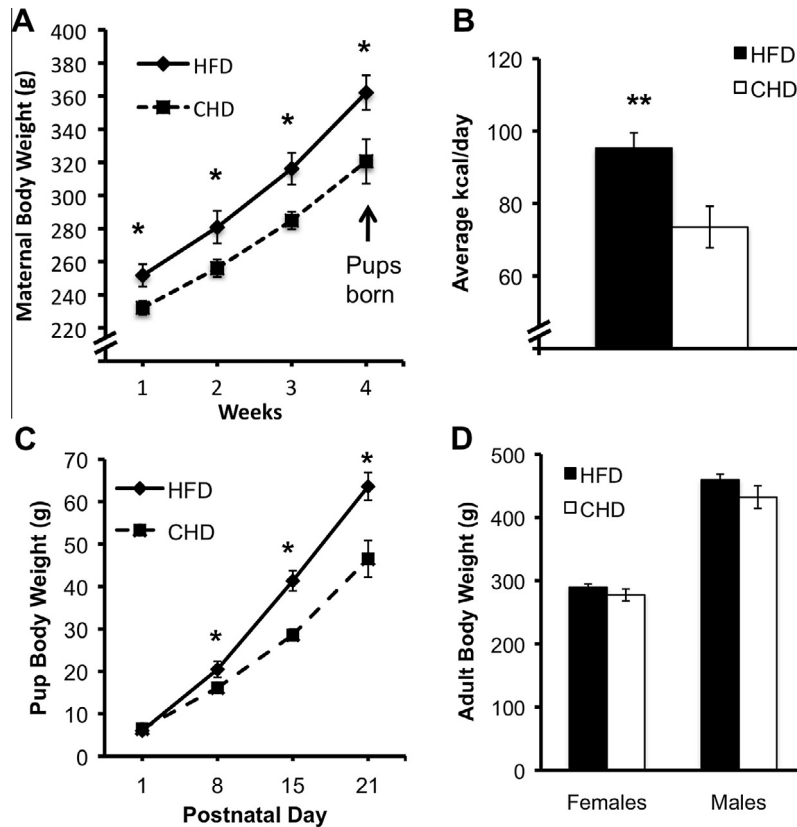
## RESULTS

### Maternal body weight and caloric intake

To ensure adequate exposure to high-fat diet, dams were given *ad libitum* access to high-fat diet or control chow diet for 4 weeks prior to pregnancy and throughout gestation and lactation. Dams consuming high-fat diet gained significantly more weight than chow-fed dams [ $F_{(1,56)} = 22.97$ ,  $P < 0.01$ ; Fig. 1A]. In addition, the high fat-fed dams' average caloric intake during the last week prior to birth was significantly higher than that of chow-fed dams [ $t_{(1,14)} = 3.05$ ,  $P < 0.01$ ; Fig. 1B].

### Offspring body weight

We next determined the influence of maternal high-fat diet on offspring weight gain until weaning at PD21 and in adulthood (PD90). High-fat diet-exposed offspring gaining significantly more weight than chow-exposed offspring throughout the pre-weaning period [ $F_{(1,15)} = 15.58$ ,  $P < 0.01$ ; Fig. 1C]. The rate of weight gain among high-fat diet-exposed offspring was also significantly higher than for the chow-exposed offspring [day by diet interaction,  $F_{(3,15)} = 130.72$ ,  $P < 0.01$ ]. Upon weaning, both the group developmentally exposed to high-fat diet and the group exposed to chow were maintained on chow diet until adulthood. In adulthood,



**Fig. 1.** High-fat diet alters maternal and pre-weaning offspring body weight. (A) Maternal body weights during gestation, (B) maternal daily caloric intake during the last week prior to pup birth, (C) pre-weaning pup weights and (D) adult offspring body weights (PD90). HFD = high-fat diet, CHD = chow diet. \*\* $P < 0.01$ , \* $P < 0.05$  main effect of diet.

male offspring were significantly heavier than female offspring overall [ $F_{(1,86)} = 237.29$ ,  $P < 0.01$ ; Fig. 1D] and there were no significant differences in offspring weight between high-fat diet- and chow-exposed males or females.

### Open field

In the OF, entries into or time spent in the center of the field and, conversely, thigmotactic behaviors are typically used as measures of anxiety behavior. To index potential differences in thigmotactic behavior, we examined the proportion of time spent in the center of the OF relative to time spent in both the center and area proximal to the walls. Offspring exposed to maternal high-fat diet spent significantly less time in the center of the OF as a proportion of total time [ $F_{(1,48)} = 15.73$ ,  $P = 0.01$ ]. This decrease in the relative time spent in the center portion of the OF was more pronounced among males [diet by sex interaction,  $F_{(1,48)} = 3.88$ ,  $P = 0.05$ ; Fig. 2A]. There were no differences between diet groups or sexes in the mean speed, time spent mobile or number of entries into the center portion of the OF.

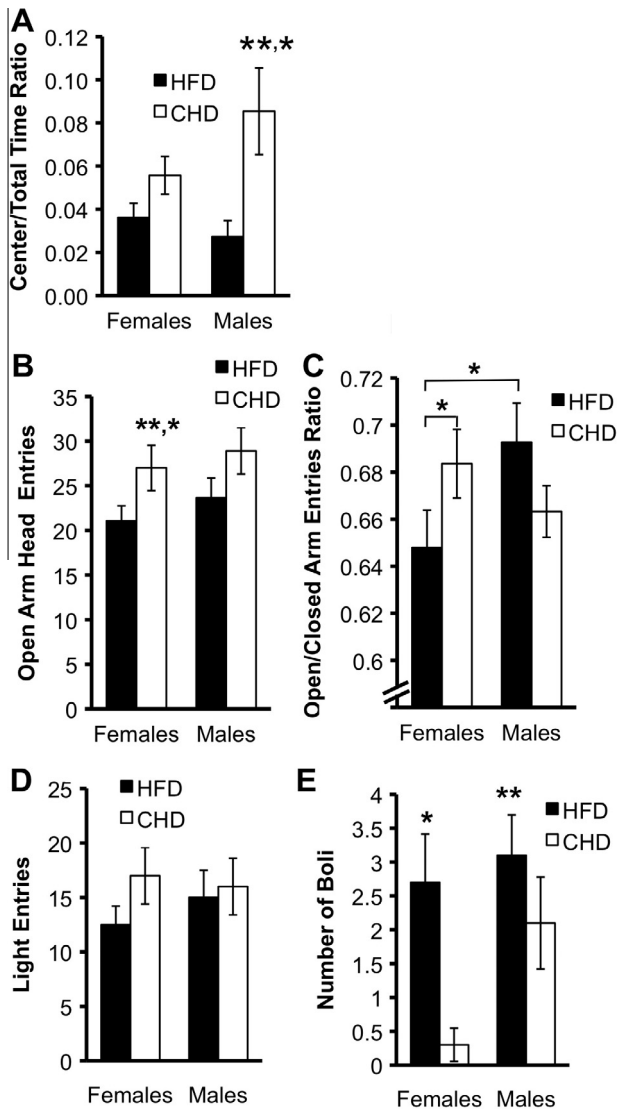
### Elevated plus maze

Maternal high-fat diet-exposed offspring generally showed a decreased preference for the open arms of

the EPM, suggesting increased anxiety behavior. Maternal high-fat diet-exposed offspring displayed fewer head entries into the open arms compared to chow-exposed offspring, defined as behavioral postures in which 60% of the body of the animal entered the arm [ $F_{(1,47)} = 6.14$ ,  $P = 0.01$ ; Fig. 2B]. High-fat diet-exposed female offspring also made significantly fewer entries into the open arms relative to the closed arms of the EPM compared to chow-exposed females or high-fat diet exposed males [diet by sex interaction,  $F_{(1,47)} = 4.68$ ,  $P = 0.03$ ; Fig. 2C]. There were no differences between diet groups or sexes in the distance traveled, mean speed, time spent mobile or the time spent in the open versus closed arms of the EPM.

### Light–dark transition

There was no significant influence of maternal high-fat diet or sex on the number of entries into the lighted portion of the LD box [ $P$ 's  $> 0.1$ ; Fig. 2E]. However, the number of fecal boli was also recorded at the end of each trial, as an increased number of boli is sometimes used as a measure of anxiety in the light–dark transition task (e.g. Ennaceur et al., 2006). Adult offspring exposed to maternal high-fat diet showed a significantly greater number of boli compared to chow-exposed offspring [ $F_{(1,47)} = 10.92$ ,  $P = 0.01$ ] and there was a marginal but non-significant influence of sex on the



**Fig. 2.** Perinatal high-fat diet exposure increases anxiety behavior in adulthood. (A) Open field (B, C) elevated plus maze (D, E) light–dark transition box. HFD = high fat diet; CHD = chow diet. \*\* $P < 0.01$ , main effect of diet; \* $P < 0.05$  post hoc comparison within diet or sex.

number of boli observed, with chow-exposed female offspring showing fewer boli than chow-exposed males [ $F_{(1,47)} = 2.71$ ,  $P = 0.10$ ; Fig. 2E]. There were no differences between diet groups or sexes in the distance traveled, speed, time spent mobile or time spent in the lighted portion of the LD box.

#### Basal and stress-induced serum corticosterone

CORT levels were assessed just prior to restraint stress challenge (0 min), at the end of the stress (20 min) and after 70 min to examine return to baseline levels. Repeated-measures ANOVA showed that CORT levels varied across the time-points sampled, with offspring in both diet conditions showing a significant increase between 0 and 20 min followed by a significant decrease between the 20- and 70-min time-point [ $F_{(2,74)} = 125.39$ ,  $P < 0.01$ ; Fig. 3A]. Overall, females showed higher levels

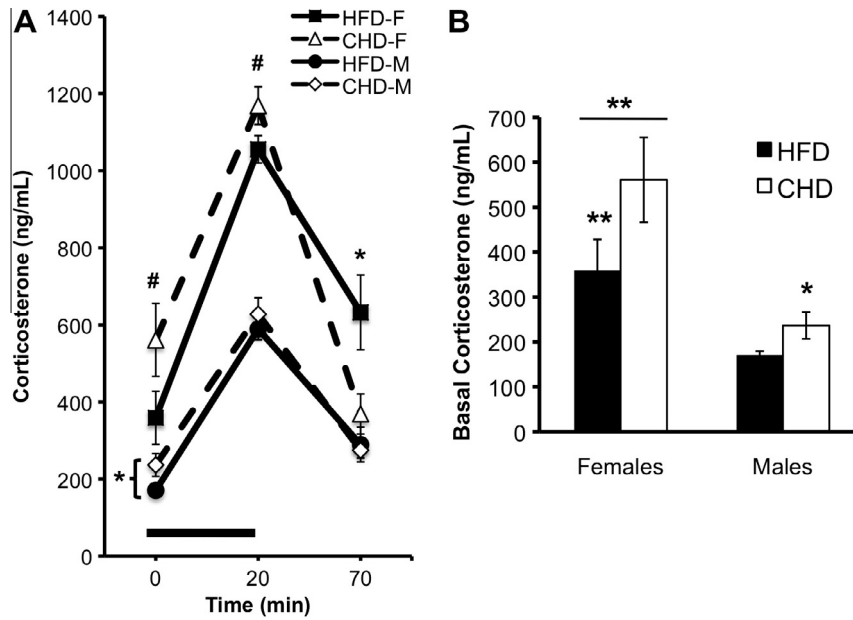
of CORT compared to males at each time-point [ $F_{(1,37)} = 97.99$ ,  $P < 0.01$ ]. The influence of maternal diet exposure varied over time, as indicated by significant interactions between sample time and diet, sample time and sex and sample time by diet by sex [all  $F$ 's  $> 3.99$ ,  $P$ 's  $< 0.03$ ]. Basal levels of CORT were significantly lower overall in males compared to females [ $F_{(1,37)} = 18.64$ ,  $P < 0.01$ ] and were significantly lower among offspring exposed to maternal high fat diet [ $F_{(1,37)} = 5.08$ ,  $P < 0.05$ ; Fig. 3B]. Within each sex, CORT levels tended to be lower for maternal high-fat diet exposed female offspring relative to chow-exposed females ( $P = 0.10$ ). CORT levels were significantly lower in maternal high-fat diet-exposed males compared to chow-exposed males ( $P = 0.05$ ). Among females, high-fat diet-exposed offspring also showed a trend for lower levels of CORT after 20 min of stress challenge ( $P = 0.07$ ), and high-fat diet-exposed female offspring had significantly higher levels of CORT at 70 min ( $P = 0.02$ ) compared to chow-exposed female offspring, demonstrating a slower return to baseline levels (Fig. 3A). Among males, CORT levels in high fat exposed offspring remained significantly elevated 70 min after stress challenge relative to the 0 min baseline ( $P = 0.01$ ), while levels in chow-exposed offspring were no different from baseline after 70 min ( $P = 0.30$ ), suggesting a slower return to baseline CORT levels in high fat exposed males.

#### Corticosterone receptor gene expression

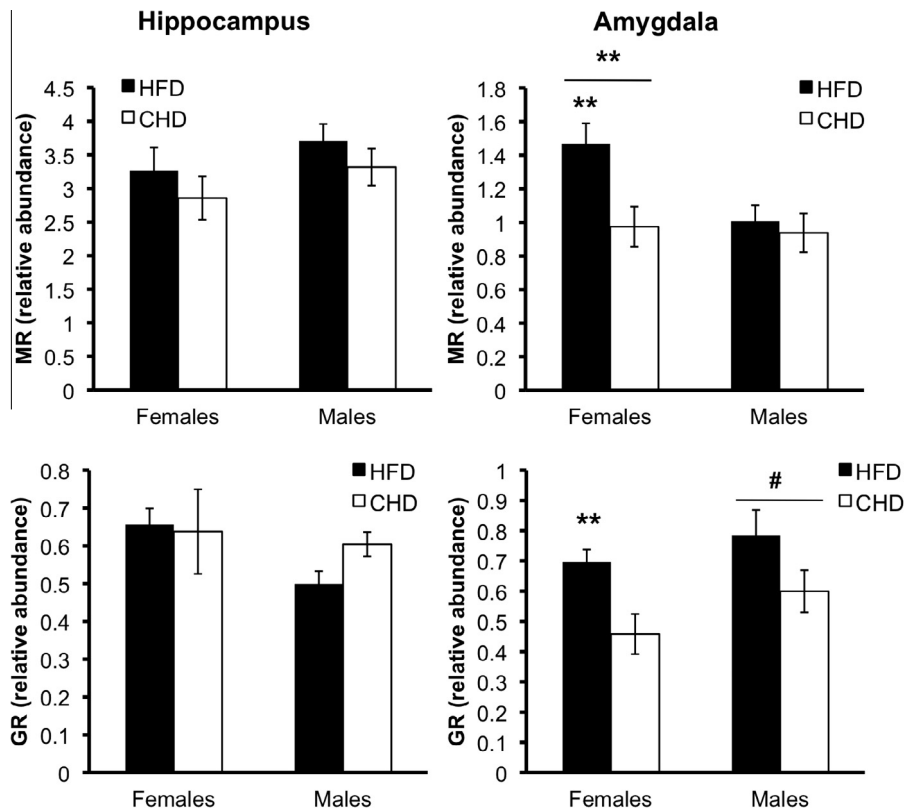
We examined relative abundance of transcript for the two major corticosteroid receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) in the amygdala and hippocampus. MR transcript abundance in the amygdala was significantly higher overall among high-fat diet exposed offspring relative to chow-exposed offspring [ $F_{(1,20)} = 8.29$ ,  $P < 0.01$ ; Fig. 4] and among females compared to males [ $F_{(1,20)} = 6.54$ ,  $P = 0.01$ ], with maternal high-fat diet-exposed female offspring showing greater expression than chow-exposed female offspring ( $P < 0.01$ ) or high-fat diet-exposed male offspring [diet by sex interaction:  $F_{(1,20)} = 4.76$ ,  $P = 0.04$ ]. For GR, high-fat diet-exposed offspring showed higher abundance of transcript overall in the amygdala compared to chow-exposed offspring [ $F_{(1,20)} = 9.90$ ,  $P < 0.01$ ] and males tended to show higher levels than females [ $F_{(1,20)} = 2.87$ ,  $P = 0.10$ ]. Post hoc comparisons revealed that high fat-exposed females showed significantly more GR than chow-exposed females ( $P = 0.01$ ). In the hippocampus, expression levels of MR and GR did not differ significantly between diet groups or sexes.

#### Pro-inflammatory gene expression

We examined transcript abundance of nuclear factor kappa beta (NFkB), interleukin-6 (IL-6) and cluster of differentiation molecule 11B (CD11b), markers of innate immune activation previously associated with perinatal high-fat diet exposure in the hypothalamus and hippocampus (Bilbo and Tsang, 2010). In the amygdala, high-fat diet-exposed offspring showed increased



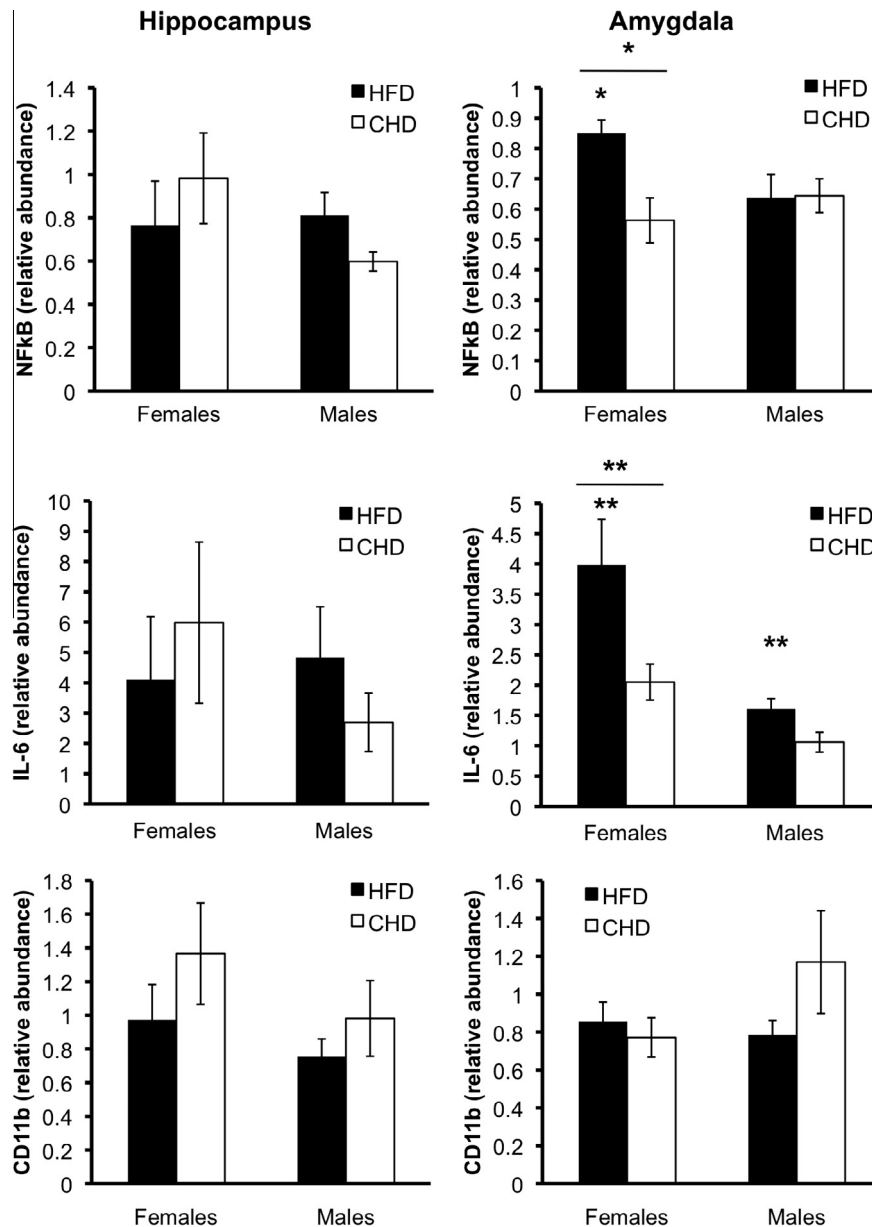
**Fig. 3.** Perinatal high-fat diet exposure alters HPA in adulthood. (A) Stress-challenged levels of CORT. Black line shows duration of restraint stressor. Differences are shown between and within each sex. (B) Basal levels of CORT. HFD = high fat diet; CHD = chow diet. Bar,  $**P < 0.05$ , main effect of sex;  $**P < 0.01$  main effect of diet;  $*P < 0.05$ ,  $\#P < 0.10$  post hoc comparison within sex.



**Fig. 4.** Perinatal high-fat diet exposure increases corticosteroid receptor expression in the amygdala in adulthood. HFD = high fat diet; CHD = chow diet, Bars,  $**P < 0.01$ ,  $\#P < 0.10$  main effect of sex;  $**P < 0.01$  main effect of diet and post hoc comparison within sex.

transcript abundance of NF $\kappa$ B relative to chow-exposed offspring [ $F_{(1,20)} = 4.73$ ,  $P = 0.04$ ; Fig. 5] and maternal high-fat diet-exposed female offspring had higher levels of NF $\kappa$ B transcript compared to maternal high-fat diet-

exposed male offspring or chow-exposed females [diet by sex interaction:  $F_{(1,20)} = 5.27$ ,  $P = 0.03$ ]. High-fat diet-exposed offspring also showed increased abundance of IL-6 [ $F_{(1,20)} = 8.84$ ,  $P < 0.01$ ], with



**Fig. 5.** Perinatal high-fat diet exposure increases pro-inflammatory gene expression in the amygdala in adulthood. HFD = high fat diet; CHD = chow diet. Bars, \*\* $P < 0.01$ , \* $P < 0.05$  main effect of sex; \*\* $P < 0.01$ , \* $P < 0.05$  main effect of diet; \*\* $P < 0.01$ , \* $P < 0.05$  post hoc comparison within diet and sex.

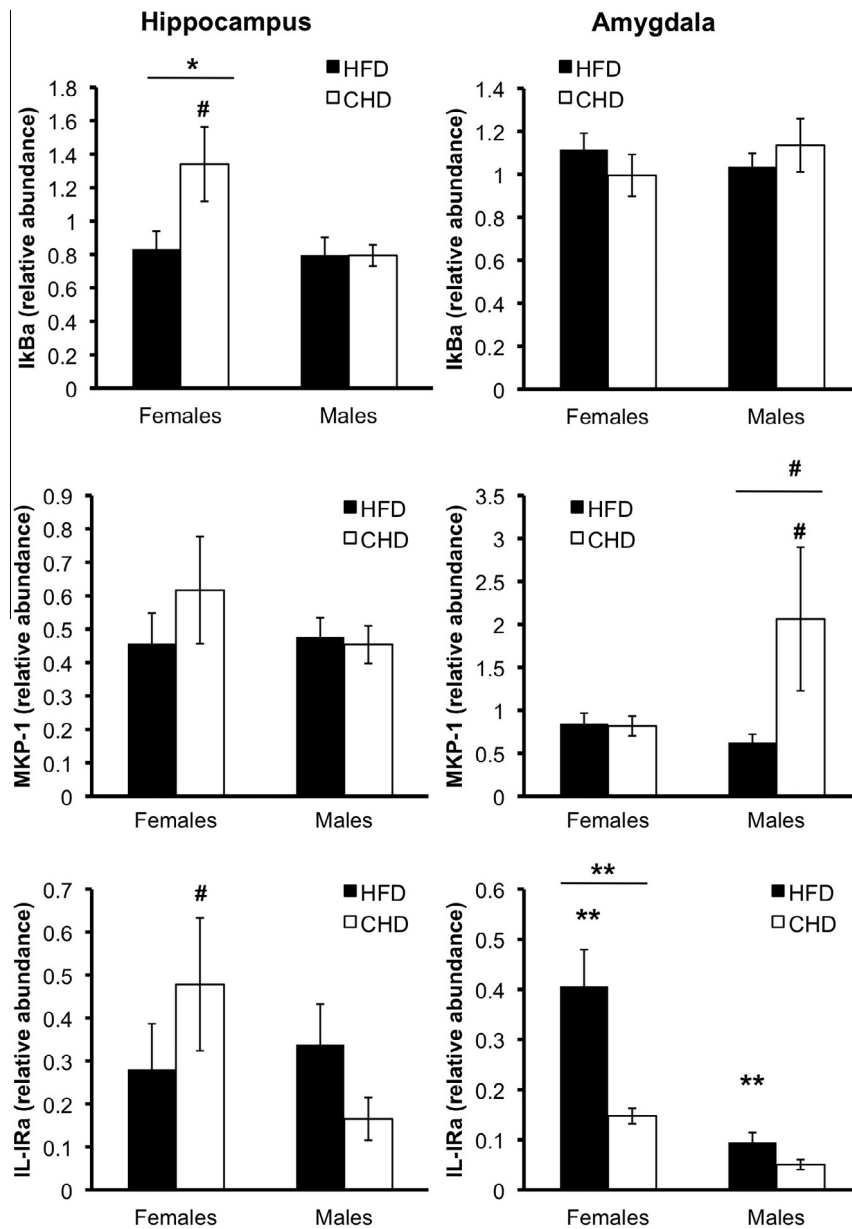
females showing higher levels overall than males [ $F_{(1,20)} = 16.23$ ,  $P < 0.01$ ; Fig. 5]. In the hippocampus, there were no significant differences between diet groups or sexes in NFκB or IL-6 expression. There were also no statistically significant differences between diet groups or between sexes in the expression of CD11b in the amygdala or hippocampus.

#### Anti-inflammatory gene expression

Next, we determined the impact of maternal high-fat diet exposure on expression levels of negative regulators of inflammatory response. Pro-inflammatory stimuli by mitogen activation is known to alter the expression of the signaling molecules I-kappa-B-alpha (IκBa),

mitogen-activated protein kinase phosphatase-1 (MKP-1) and interleukin-1 receptor antagonist (IL-1Ra) via changes in GR, NFκB and IL-6 signaling (Sorrells et al., 2009), but their regulation in the context of the inflammatory response to high-fat diet is unknown.

In the amygdala, high-fat diet-exposed offspring showed significantly elevated abundance of IL-1Ra [ $F_{(1,20)} = 15.38$ ,  $P < 0.01$ ; Fig. 6], and females showed higher overall levels compared to males [ $F_{(1,20)} = 28.04$ ,  $P < 0.01$ ] an effect that was significantly greater among high-fat diet-exposed females [diet by sex interaction:  $F_{(1,20)} = 6.95$ ,  $P = 0.01$ ]. High-fat diet-exposed offspring tended to show reduced abundance of MKP-1 transcript [ $F_{(1,20)} = 2.69$ ,  $P = 0.11$ ], with chow-exposed male offspring showing a trend for higher levels of MKP-1



**Fig. 6.** Perinatal high-fat diet exposure alters anti-inflammatory gene expression in the hippocampus and amygdala in adulthood. HFD = high fat diet; CHD = chow diet. Bars, \*\* $P < 0.01$ , # $P < 0.10$  = main effect of sex; \*\* $P < 0.01$ , \* $P < 0.05$ , # $P < 0.10$  main effect of diet and post hoc comparison within diet and sex.

transcript compared to high fat diet-exposed male offspring or female offspring [diet by sex interaction:  $F_{(1,20)} = 2.91$ ,  $P = 0.10$ ]. There were no differences between diet groups or between sexes in the expression of IkBa in the amygdala.

In the hippocampus, females showed significantly higher levels of IkBa expression than males [ $F_{(1,20)} = 4.45$ ,  $P = 0.04$ ], there was a trend for lower levels of expression among high fat exposed offspring [ $F_{(1,20)} = 3.37$ ,  $P = 0.08$ ], with chow-exposed female offspring tending to show higher levels of expression compared to either high-fat diet-exposed female or male offspring [diet by sex interaction:  $F_{(1,20)} = 3.43$ ,  $P = 0.07$ ]. Planned comparisons showed that chow-exposed female offspring had significantly higher levels

of IkBa ( $P = 0.04$ ) compared to chow-exposed male offspring. For IL-1Ra, there was a trend among chow-exposed female offspring for increased expression of IL-1Ra relative to high-fat diet-exposed female and chow-exposed male offspring [diet by sex interaction:  $F_{(1,20)} = 2.96$ ,  $P = 0.10$ ]. Expression levels of MKP-1 did not differ between diet groups or sexes in the hippocampus.

## DISCUSSION

To our knowledge, this is the first study to examine changes in glucocorticoid signaling in response to perinatal high-fat diet exposure in limbic areas known to regulate HPA function and anxiety behavior. The results



indicate that altered expression of corticosteroid receptors and inflammatory genes in the brain of adult offspring as a function of high-fat diet exposure during the perinatal period alone may contribute to a heightened endocrine response to stress and increased anxiety behavior in adulthood.

The maternal phenotype of high fat diet dams during gestation and lactation consisted of increased body weight and caloric intake. This phenotype was transmitted to offspring prior to weaning, who were heavier than chow-exposed offspring beginning at postnatal day 8. However, there was no difference in body weight between maternal high-fat diet-exposed and chow-exposed offspring in adulthood when anxiety and physiological measures were taken, suggesting that the observed phenotype was associated with perinatal high fat diet exposure rather than current obesity.

At present we do not know whether differences in the maternal care received may have contributed to the difference between our high-fat and chow-fed groups. To our knowledge, the only two recent studies that have examined this question arrive at different conclusions. One study found that dams consuming high-fat diet from mating through lactation displayed increased maternal nursing (i.e. arched-back and passive nursing postures) but no change in licking and grooming behavior during the first week postpartum compared to chow-fed controls (Purcell et al., 2011). Another study found that dams consuming high-fat diet from mating through lactation exhibited decreased maternal licking and grooming but no change in nursing behavior between postnatal days 3 and 8 (Connor et al., 2012). These disparate results indicate that more study is needed to elucidate the role of maternal behavior in offspring phenotype in the context of maternal high-fat diet.

A potential limitation in our study is that, in addition to relatively high levels of fat (60% versus 13.5%), our dietary manipulation also consisted of somewhat higher levels of refined sugars (7% sucrose) compared to the chow diet control (3.7% sucrose). Although previous studies have used this model of diet-induced obesity compared to control chow diet to examine the developmental impact of overnutrition (El-Haschimi et al., 2000; De Souza et al., 2005; Dunn and Bale, 2009; Tamashiro et al., 2009; Purcell et al., 2011), the present data do not rule out the possibility that increased exposure to refined sugar during development contributed to the observed effects. There is an obvious tradeoff among levels of fats, carbohydrates and proteins among diets used to generate obese phenotypes. Importantly, however, whereas levels of protein were higher in the chow diet control in this study (28% versus 20%), we did not observe a decreased body weight at birth among the high-fat diet exposed offspring at birth, indicative of insufficient levels of protein. Thus, these data indicate that our model of diet-induced obesity likely did not involve protein restriction during development. Future studies are needed to examine the relative influence of fats and carbohydrates on the observed phenotype.

In this study, we used a variety of measures to assess anxiety behavior. Two previous studies in rats (Bilbo and Tsang, 2010) and mice (Peleg-Raibstein et al., 2012) found that offspring exposed to maternal high-fat diet show increased anxiety in the elevated plus maze. Our data extend the link between perinatal high-fat diet exposure and anxiety behavior in males and females in adulthood; perinatally exposed animals showed evidence of increased anxiety in light–dark transition task as well as a relative avoidance of the open areas on both the Open field and Elevated plus maze. Importantly, these differences in the Open field and Elevated plus maze were observed in the absence of effects on locomotor activity, suggesting that differences between diet groups likely reflected an anxiety-like phenotype rather than differences related to metabolic or motivational effects.

A heightened HPA response to stress challenge is typically associated with anxiety behavior, as basal corticosterone levels set the threshold for activation by psychosocial stress (Lupien et al., 2009). The results of a limited number of previous studies of developmental levels of corticosterone have been mixed (Walker et al., 2008; Shalev et al., 2010; Auvinen et al., 2011). Differences in procedural details including duration of dietary manipulation and the time of day of corticosterone sampling may account in part for these discrepancies (Walker et al., 2008; Auvinen et al., 2011). In this study, both males and females showed a similar corticosterone response to high-fat diet exposure. We found that basal corticosterone was attenuated in adult males in response to perinatal high-fat diet exposure, and adult females showed a trend toward lower basal corticosterone levels. High-fat diet-exposed animals also showed a heightened response to restraint stress and a slower return to baseline corticosterone levels, particularly among females, indicating less efficient corticosterone feedback. These data are consistent with evidence of heightened HPA response to stress challenge among females compared to males in both rodents (Weinstock, 2007) and humans (Kudielka and Kirschbaum, 2005), and suggest that perinatal high-fat diet exposure may exacerbate HPA reactivity to stress among females.

The hippocampus and amygdala play key roles in modulating the response to stress through direct projections to the paraventricular nucleus of the hypothalamus. In turn, the hypothalamus directs the endocrine response via adrenocorticotropin releasing hormone from the pituitary, leading to the release of corticosterone from the adrenal cortex and exerting feedback on limbic brain areas via corticosteroid receptors. Mineralocorticoid receptor is a high-affinity receptor that is normally bound to corticosterone in basal conditions, determining the threshold of the stress response. In the hippocampus, increased corticosterone as a result of psychosocial stress activates the lower affinity glucocorticoid receptor, inhibiting the further release of corticosterone, whereas in the amygdala glucocorticoid receptor activation enhances the HPA

response (Joels et al., 2008). In this manner, the limbic system has been proposed to refine the adaptive response to stress (Groeneweg et al., 2011). We found evidence of increased mineralocorticoid receptor levels in the amygdala in response to maternal high fat diet exposure. Increased mineralocorticoid receptor in amygdala has been shown in male Zucker diabetic fatty rats, a model of diet-induced obesity (Johren et al., 2007). Our data indicate that increased mineralocorticoid receptor levels in amygdala may lead to decreased basal levels of corticosterone as a result of maternal high-fat diet exposure. We also found evidence of increased glucocorticoid receptor in amygdala and a heightened response to stress in offspring exposed to maternal high fat diet. These data support a number of previous observations that increased glucocorticoid receptor in the amygdala enhances the corticosterone-mediated response to stress (Joels et al., 2008).

Glucocorticoids have well known anti-inflammatory activities in the body, and have been recently shown to have both inflammatory and anti-inflammatory roles in the brain (Sorrells et al., 2009). Increased glucocorticoid receptor expression under conditions of chronic stress or altered levels of glucocorticoids generally enhance central inflammatory responses. Indeed, under conditions of chronic stress, glucocorticoid receptor activation is required for inflammatory responses as a result of increased NFkB (Sorrells et al., 2009). Altered inflammatory gene expression in the hypothalamus is a well known consequence of diet-induced obesity, and diet-induced obesity is associated with increased IL-6 expression in the hypothalamus (De Souza et al., 2005) and in the cortex (White et al., 2009) in a diet-dose responsive manner. That is, increased IL-6 transcript is only observed with prolonged high-fat diet exposure. In this study, we found that transcript abundance of the major pro-inflammatory genes NFkB and IL-6 was also increased in the amygdala among animals exposed to high-fat diet only during perinatal life. We did not find a change in IL-6 in the hippocampus, supporting previous work (Bilbo and Tsang, 2010). The present data showing parallel increases in glucocorticoid receptor, NFkB and IL-6 suggest that, in addition to changes induced by high-fat diet consumption, changes in the regulation of these genes occur with high-fat diet exposure during development, independent of current consumption. We did not detect an influence of high-fat diet on IkB $\alpha$  expression, a known negative regulator of NFkB (Munhoz et al., 2010), suggesting a disruption in the negative inhibition of NFkB normally mediated by IkB $\alpha$ . Among the other negative regulators of inflammation examined in this study and known to be activated by increased glucocorticoid receptor, we found a high-fat diet-dependent increase in the expression of IL-1Ra, an inhibitor of IL-1, and a trend for decreased expression of MKP-1 particularly among females. A balance between inflammatory responses is a critical mechanism to maintain an adequate response to pathological challenges such as in disease and infection (Spulber et al., 2009). In peripheral monocytes, analysis

by microarray has shown that synthetic glucocorticoid increases the expression of a number of both pro- and anti-inflammatory genes (Galon et al., 2002). Seen in this context, our data indicate that the central homeostatic equilibrium between inflammatory and anti-inflammatory responses may be disrupted by high-fat diet exposure during development.

A number of previous studies of the interaction between glucocorticoids, glucocorticoid receptors and inflammatory processes have examined the effects of mitogen challenge by lipopolysaccharide (LPS) on glucocorticoid signaling. LPS activates signal transduction through the Toll-like receptor 4 (TLR4) pathway, leading to increased NFkB and MAP kinase activation. Studies of the effect of stress and sustained alterations in glucocorticoids have demonstrated that glucocorticoid receptors modulate the ability of LPS to direct a pro-inflammatory response in part by modulating the activity of MKP-1, IkB $\alpha$ , IL-1Ra and subunits of NFkB (Madrigal et al., 2001; Sorrells et al., 2009; Frank et al., 2010, 2012). These data indicate that the TLR4 inflammatory pathway is sensitive to altered glucocorticoid signaling. High-fat diet exposure is known to induce inflammation through the TLR4 pathway in the hypothalamus, which leads to endoplasmic reticulum stress, the expression of inflammatory cytokines and eventually apoptosis of neurons, contributing to the dysregulation of energy homeostasis (Zhang et al., 2008; Milanski et al., 2009; Moraes et al., 2009). The effects of glucocorticoids on signaling via this pathway may, to some extent, be brain-region (Munhoz et al., 2010) and cell-type specific (Sorrells et al., 2009). Therefore, some degree of caution is warranted in extrapolating such findings to limbic brain areas. However, our data implicate glucocorticoid modulation of inflammatory signaling in limbic areas in anxiety behavior as a function of high-fat diet exposure.

Anxiety behavior has been linked to changes in inflammatory gene expression in several studies (Dantzer et al., 2008). In turn, mitogen-activated inflammation in limbic areas of the brain, including amygdala and the hippocampus, is linked to anxiety behavior, indicating a direct effect of inflammation on anxiety (Rodgers et al., 2012). Future studies are needed to identify interactions among key regulators of glucocorticoid signaling such as glucocorticoid receptor, NFkB and other downstream inflammatory signaling molecules and their contribution to anxiety behavior.

Female rats showed the largest changes in gene expression as a function of the maternal diet in this study. Few studies have examined sex differences in gene expression programming by diet. However, similar results have been reported in studies of maternal high fat diet on transcriptional regulation during placental development. Maternal high-fat diet has a more pronounced influence on gene expression in female placenta in mice, with a greater number of genes showing increased expression in females compared to males (Mao et al., 2010). A recent study using a genome-wide transcriptomic approach reported a

selective increase in the expression of a number of genes involved in the inflammatory responses in females compared to males (Gaborly et al., 2012). These data may indicate a sexually dimorphic developmental trajectory of immune signaling through adaptations at the fetal-maternal interface. In humans, incidences of anxiety disorders as well as obesity are much more common in females (Desai et al., 2009; Rofey et al., 2009). There is also evidence that females may be more sensitive to the effects of alterations in immune function on anxiety behavior (Bouret, 2009; Schwarz and Bilbo, 2012). Our data to date are consistent with the hypothesis that the response to perinatal high-fat diet observed in this study may arise as a function of both increased HPA reactivity and increased inflammatory gene expression, particularly among females.

A clear majority of adults in developed countries are now either overweight or obese (68% in the US (Flegal et al., 2010; Sullivan et al., 2010)), a condition linked to important health risks. When experienced during development, a high-fat diet may have long-term impacts on mental disorders associated with anxiety. It will be important to elucidate changes in signaling pathways in critical brain areas in offspring so that targeted interventions may be effectively aimed at mitigating deleterious effects of overnutrition on developmental programming.

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