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Impact of prenatal stress on long term body weight is dependent on timing and maternal sensitivity

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Abstract

Stress experienced during pregnancy increases the risk for altered birth weights. Recent studies have revealed a link between abnormal birth weights and a future predisposition toward developing overweight or obesity. To determine the gestational time window when stress exposure produces the greatest impact on offspring body weight regulation, we have examined the birth weights and long-term body weight changes in offspring exposed to chronic variable stress (CVS) early, mid-, or late in gestation. As it is likely that the influences of prenatal stress on development stem from a complex interaction between both environmental and genetic factors, our study has included comparisons with offspring born to stress-sensitive (corticotropin-releasing factor receptor-2 deficient) mice. Stress experienced late in pregnancy significantly elevated offspring birth weights in wild type mice compared to unstressed controls. However, this weight difference diminished postnatally. In contrast, stress experienced mid- to late in pregnancy produced significant and long-term effects on body weight in offspring from stress-sensitive dams, were the male offspring were 15% heavier as adults. Adult offspring plasma glucose and leptin levels were also dependent on the timing of stress exposure, indicating that alterations in energy homeostasis may be influencing long-term body weight. Results from these studies support our hypothesis that the ultimate effect of prenatal stress on offspring long-term outcome is dependent on the timing of exposure and maternal sensitivity.

Keywords: Pregnancy; Development; Obesity; Overweight

1. Introduction

It is well established that stress experienced during gestation can significantly alter birth weight [1–3]. Abnormal birth weights are strongly linked to later development of metabolic disorders including obesity and diabetes [3–7]. A recent study following the development of 14,000 infants reported that with each kilogram above the average birth weight, the odds for developing obesity increased by nearly 50% [5]. Surprisingly, children born with abnormally low birth weights are also at an increased risk for later overweight and obesity [4,8]. The rapid weight gain, or 'catch-up' growth, that frequently follows low birth weight may alter metabolism or feeding behaviors, predisposing an individual to increased weight gain [9]. As we are currently facing an obesity epidemic in this country, with the fastest growing population children aged 6–11, elucidating those factors that may predispose an individual to increased body weight and obesity is critical.

Animal models allow administration of stress in a controlled environment, avoiding confounding social factors inevitably present in human retrospective studies. Such research has illustrated the profound and deleterious impact of prenatal stress on the developing offspring. However, a heavy reliance on repeated restraint stress administered in a predictable manner to the dam late in pregnancy has placed a limitation on our understanding of the mechanism and timing whereby the effects of stress are elicited [10–12]. As the effect of a physical restraint stress on plasma corticosterone is progressively attenuated with repeated exposure, a variable stress paradigm may be preferable to a repeated design [13–15]. Furthermore, as organ and tissue development, including the brain, occurs over the course of gestation, it follows that the impact of stress would depend on the specific point during development at which stress was experienced.

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However, the temporal specificity of prenatal stress influences on offspring body weight regulation is not currently known.

The recent development of a genetic mouse model of increased stress sensitivity permits an investigation of how specific genetic differences in maternal susceptibility may affect offspring development during periods of stress. The corticotropin-releasing factor receptor-2 deficient (CRFR2 KO) mouse is a useful model of heightened stress sensitivity, as these mice display significantly elevated stress hormone levels following restraint [16], a decreased rate of recovery following stress exposure [17], elevated anxiety-like behaviors [16,18], and increased maladaptive responses in learned helplessness studies [19]. In addition, these mice have elevated central CRF expression, a likely contributor to their increased stress responsivity in the absence of CRFR2 [16,17,20]. Thus, these mice provide a useful genetic model with which the possible influence of an increased maternal vulnerability to stress can be examined as it relates to exaggerated offspring outcomes following prenatal stress exposure. While a global CRFR2 deficiency may pose unanticipated caveats on pregnancy outcome, our studies have included non-stressed control groups for both genotypes for comparison of such effects.

These studies have utilized a chronic variable stress (CVS) administered to dams with normal (WT) or elevated stress sensitivity (KO) during early, mid-, or late pregnancy and determined body weight and energy homeostasis alterations in the offspring to assess the developmental window of vulnerability when a perturbation in the uterine environment may lead to birth and long-term body weight alterations.

2. Materials and methods

2.1. Animals

Wild type (WT) and stress-sensitive CRFR2-deficient (KO) mice on a mixed C57Bl/6:129 background were generated as previously described [16]. Dams of both genotypes were siblings from heterozygous x heterozygous crosses randomly distributed across treatment groups. Mice were housed under a 12-h light/dark cycle (lights on at 7:00 am) with ambient temperature of 22 °C, and relative humidity of 42%. Food (Purina Rodent Chow; 28.1% protein, 59.8% carbohydrate, 12.1% fat) and water was provided throughout the study *ad libitum*. All studies were done according to experimental protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

2.2. Maternal stress response

In order to confirm that the increased sensitive neuroendocrine stress response of KO mice remained exaggerated throughout all stages of pregnancy, corticosterone levels of WT ($n=5$) and KO ($n=4$) dams were compared following a 15-min stress exposure. While measuring pregnant dam stress levels following CVS would be complex, as most of the stressors are long in duration, we compared the stress response between the 2 genotypes following an acute CVS stressor during each of the 3 trimesters. On gestation day 6, 1000 h, dams were exposed to a 15-min restraint in a 50-mL conical tube. On gestation day 12, 0900 h, dams were placed in a cage with a cotton swab soaked in fox odor (1:5000 2,4,5-Trimethylthiazole; Acros Organics) for 15 min. On gestation day 18, 1100 h, 12 marbles of similar shape and color were placed in the home cage of the pregnant dam for 15 min. To investigate stress response and recovery, tail blood was collected immediately prior to the stress start (0 min), immediately following the stress exposure (15 min), and 15 and 45 min following end of stress (30 and 60 min respectively). The blood collection procedure lasted less than 1 min. Samples were immediately centrifuged and stored at −80 °C until the assay was conducted. Corticosterone levels were determined by radioimmune assay (ICN Biomedicals).

2.3. Breeding

Wild type and KO virgin female mice were mated at 6–8 weeks of age. Three females were housed with one male of the same genotype. Homozygous matings were conducted in these studies such that all offspring would be of a known genotype, providing a consistent, controlled uterine environment within groups, and to limit the animal numbers utilized. At the start of the light cycle, each morning following mating, females were examined for the presence of a vaginal copulation plug. Presence of a copulation plug denoted day 1 of gestation. The female was individually housed, given a cotton nestlet, and food pellets were kept on cage floor to allow food intake measurement throughout pregnancy.

2.4. Variable stress paradigm

WILD type ($n=18$) and KO females ($n=17$) were randomly assigned to treatment groups to receive stress during one of the three trimesters, or to a control non-stressed group. Pregnant mice assigned to the stress groups experienced a different daily stressor on each of seven days during early (days 1–7), mid- (days 8–14), or late (days 15–21) gestation. These variable stressors included: 36 h of constant light, 1 h of fox odor during light cycle, novel object (marbles) exposure overnight, 5 min restraint stress during light cycle, novel noise (White Noise/Nature Sound-Sleep Machine®, Brookstone) overnight, multiple cage changes during the light cycle, and saturated bedding (700 mL, 23 °C water) overnight. These mild stressors were selected for not inducing pain or directly influencing maternal food intake or weight gain.

2.5. Prepartum measurements

Dams were briefly removed from the home cage daily (0800–0900 h) and weighed. Food pellets were also weighed to measure 24-h food intake. Nesting material was introduced in the cage once per week. 24 h following nestlet placement, nest quality was scored by an investigator blind to genotype and treatment group using the following scale: (1) nest material unmodified; (2) partially modified material on flat nest; (3) shredded material forming shallow walls; (4) nest with well formed walls; (5) nest in the shape of a cocoon with roof [21].

2.6. Postpartum measurements

During postpartum weeks, nesting behavior was measured as time the dam spent on the nest with the pups. During postnatal week 3 (PN 25–PN 27) pups were ear-tagged to provide a method of permanent identification.

2.7. Offspring measurements

2.7.1. Litter size

Each cage was inspected for the presence of a litter at 0800 h. If a litter was present (postnatal day 1), pups were quickly counted. Litters were culled to 8 pups and litters containing fewer than 6 pups were excluded from analysis.

2.7.2. Body weight

On postnatal day 1, litters were weighed (0800 h). As the study was designed to examine prenatal stress, postnatal manipulations were avoided as much as possible. To minimize handling [22], pups were weighed as a group weekly during cage changes (PN 8, PN 15, PN 21, and PN 26). Total weights were averaged for the number of pups per litter. Offspring were weighed individually at postnatal week 10 and postnatal week 16.

2.7.3. Corticosterone

At 16 weeks of age, corticosterone levels of adult offspring were compared to evaluate the HPA axis response to stress. All endocrine measures were obtained from the same set of animals. Blood was taken by tail bleed following a 5-min restraint stress at 0900 h. Our previous work has shown no genotypic differences in corticosterone levels following a 5-min restraint stress [16]. Plasma samples were immediately centrifuged and stored at −80 °C until the assay was conducted. The corticosterone assay (ICN Biomedicals) used 3 μl of plasma.

2.7.4. Glucose

Adult offspring fasted glucose levels were obtained (0900 h) following an overnight (12-h) fast when offspring were 18–20 weeks of age. Glucose was measured from tail blood using the Lifescan One Touch glucometer (Johnson and Johnson, Milpitas, CA). As glucose levels are highly dependent on environmental stressors, care was taken to ensure an undisturbed environment prior to sampling.

2.7.5. Leptin

To compare basal levels of circulating leptin between offspring groups, a blood sample was collected immediately following decapitation (postnatal week 19–21), centrifuged, and stored at −80 °C until assay was performed. The leptin assay (LINCO Research) used 100 μl of plasma. The sensitivity of the assay was 0.5 ng/ml. The intraassay coefficient of variation was less than 6%.

2.8. Data analysis

Effects of genotype and time of blood collection on maternal corticosterone levels were evaluated by a two-way ANOVA. Effects of stress treatment and genotype on maternal food intake, body weight, and litter growth were determined by a two-way ANOVA. Sex ratio was tested against the expected value of 0.5, by two-way ANOVA for prenatal stress treatment, and maternal genotype [23,24] followed by a Chi-square test. Litter weight is normalized by pup number and compared among treatments. The mean obtained from same-sex littermates is defined as a single measurement. These data are analyzed by a three-way ANOVA for stress treatment, maternal genotype, and sex. ANOVAs were corrected for repeated measures where necessary. Significance was set at $P=0.05$. Bonferroni multiple comparisons test determined post hoc significance. All statistical analysis was performed using StatView SE+ (Abacus Concepts, Berkeley, CA).

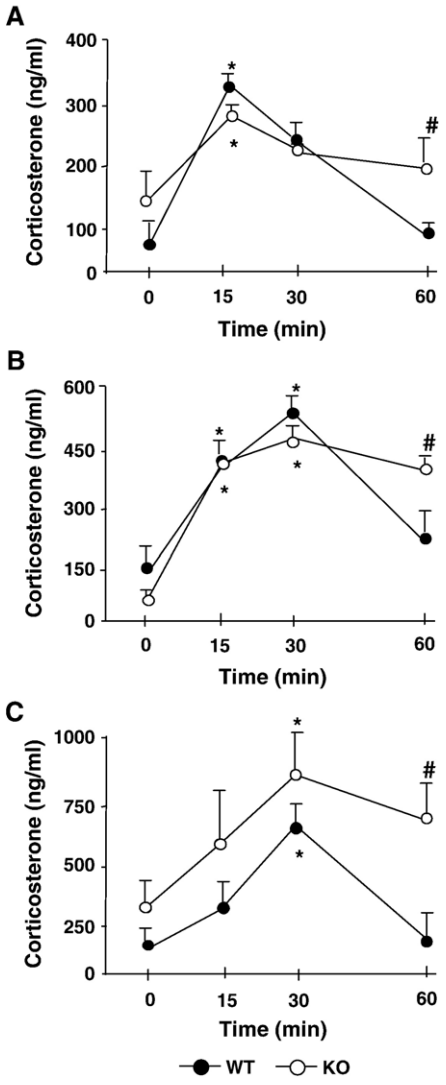

Fig. 1. Stress-sensitive phenotype of CRFR2-deficient (KO) mice is present during pregnancy. Following a 15-min stress exposure, KO mice show a prolonged stress recovery time compared to wild type (WT) dams throughout pregnancy as measured on day 6 (A), 12 (B) and 18 (C) of gestation. Novel, acute stressors were used for each of the 3 points: (A) restraint stress, (B) fox odor, and (C) marble exposure. Corticosterone levels were measured at baseline (0), immediately following the 15-min stress (15), 15-min following the end of stress (30), and 45-min following the end of stress (60). (*, $P<0.05$ as compared to time 0; #, $P<0.05$, as compared to WT at identical time point). Data are mean±SEM.

3. Results

3.1. Verification of CRFR2-KO maternal HPA axis

To confirm that the heightened stress-sensitivity of CRFR2-deficient mice persists throughout gestation, corticosterone levels of pregnant WT and KO dams were compared following a 15-min stress. Supporting our previous findings [16], baseline levels of corticosterone did not significantly differ between genotypes ($F_{(1/22)}$=0.32, P>0.05). On pregnancy day 6, there was a significant interaction between genotype and stress time point ($F_{(3/26)}$=4.24, P<0.05, Fig. 1). Following 45 min of recovery, WT corticosterone levels returned to baseline, while KO corticosterone levels remained significantly elevated compared to WT levels (Fig. 1A). On gestation day 12, there were main effects for genotype ($F_{(1/26)}$= 5.28, P<0.05) and time of blood collection ($F_{(3/29)}$=28.74, P< 0.001, Fig. 1B). After 45 min of recovery, WT corticosterone levels return to near-baseline while KO corticosterone levels are significantly elevated compared to WT levels (Fig. 1B). On gestation day 18, there was a main effect of genotype ($F_{(1/23)}$=4.20, P<0.05, Fig. 1C). Again, following 45 min of recovery, WT corticosterone levels return to near-baseline, while KO corticosterone levels remain significantly higher compared to WT levels (Fig. 1C). These results verified that CRFR2-deficient mice provide a valid model of increased stress-sensitivity during pregnancy.

3.2. Maternal measurements

As maternal undernutrition can exert effects on offspring outcome [25], dam body weights and food intake were measured daily during pregnancy. Body weights and food intake did not differ between genotypes ($F_{(1/32)}$=1.12, P>0.05) or stress treatment groups ($F_{(3/34)}$=0.90, P>0.05, Table 1). Gestation duration was not significantly different between genotypes ($F_{(1/32)}$=2.01, P>0.05) or stress treatment groups ($F_{(3/34)}$=1.69, P>0.05). As maternal behavior has been shown to influence offspring development [26], we compared pre-partum maternal behavior between genotypes by investigating quality of nest building during pregnancy. No significant differences were found between genotypes ($F_{(1/34)}$=0.50, P>0.05) or stress treatment ($F_{(3/34)}$= 0.30, P>0.05, Table 2). Nesting time of the dam spent with her pups was recorded as a measure of post-partum maternal care. Time that the dam spent on the nest with pups did not significantly differ between genotype ($F_{(1/34)}$=0.20, P>0.05) or stress treatments ($F_{(3/34)}$=0.40, P>0.05, Table 2).

Table 1
Pregnant dam food intake and body weight

Maternal group	Conception weight (g)	Final dam body weight (g)	Total food intake (g)
WTC	20.9±2.0	37.0±0.6	100.1±9.1
WT 1	21.5±0.8	37.2±0.5	115.2±5.7
WT 2	21.6±1.0	34.9±1.9	103.3±8.7
WT 3	21.3±0.9	35.9±0.7	108.3±5.5
KOC	22.8±1.0	38.3±0.3	113.2±7.6
KO 1	22.5±0.7	37.2±0.6	106.0±5.3
KO 2	21.8±0.9	37.6±0.7	109.3±6.7
KO 3	23.4±0.6	37.4±1.7	107.4±3.6

Food intake (g) and body weight (g) of wild type (WT) and CRFR2-deficient (KO) dams exposed to early (1), mid- (2), late (3) gestational stress or unstressed, control dams (C). Values are mean±SEM.

Table 2
Quality of nest (scale 1-5)

Maternal group	Nest quality	Time w/ offspring on nest		
		Week 1	Week 2	Week 3
WTC	4.5±0.3	9.7±0.3	6.5±0.3	1.5±0.4
WT 1	4.4±0.4	9.0±0.3	6.8±0.3	2.0±0.2
WT 2	4.6±0.2	9.3±0.5	7.0±0.5	2.0±0.5
WT 3	4.4±0.3	9.0±0.4	6.3±0.4	1.8±0.5
KOC	4.2±0.2	9.4±0.2	7.0±0.2	1.3±0.6
KO 1	4.4±0.6	9.3±0.5	7.0±0.5	2.0±0.7
KO 2	4.5±0.7	9.3±0.3	7.8±0.3	2.0±1.0
KO 3	4.6±0.4	9.0±0.3	6.7±0.3	1.5±0.4

Mean time (min) dam spent on nest with pups during each of the three nursing weeks prior to weaning during ten min observation period. Value are mean± SEM.

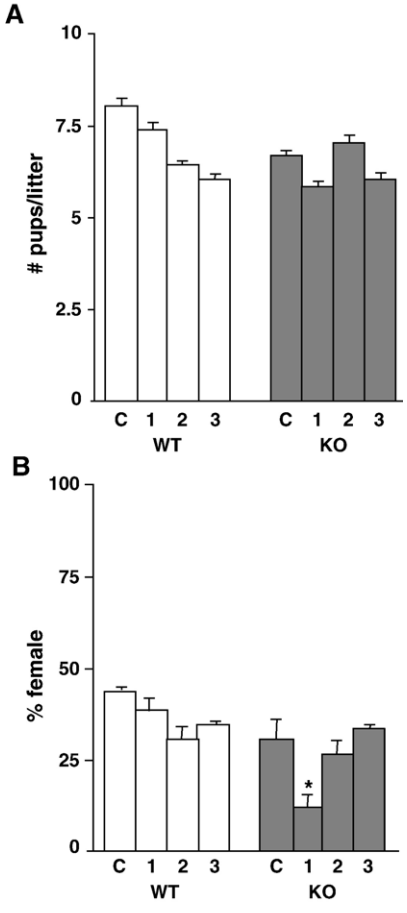

Fig. 2. Number of female offspring from early stress exposed CRFR2-deficient (KO) dams is decreased. (A) There was no significant difference in average litter size between treatment groups of WT and KO litters (P>0.05). (B) The percent female for WT and KO litters. KO dams stressed during early (1) gestation gave birth to litters that contained a significantly lower ratio of female:male pups than unstressed KO dams (*, P<0.05). Data are mean±SEM. Offspring were born to unstressed, control dams (C), or dams exposed to early (1), mid- (2), or late (3) gestational stress.

Table 3A
Offspring litter size average weight (g), and number of male and female offspring born to wild type (WT) dams

Maternal group	Litter size	# male pups	# female pups	Average pup weight (g)
WT control	10[a]	5	5	1.33
	7	4	3	1.36
	7	3	4	1.34
WT early stress	7	3	4	1.44
	7	3	4	1.31
	7	1	6	1.19
	9[a]	5	4	1.21
	6	3	3	1.37
WT mid-stress	9[a]	5	4	1.08
	8	3	5	1.04
	6	3	3	1.37
	6	2	4	1.39
	5[b]	3	2	1.19
WT late stress	8	4	4	1.28
	7	4	3	1.50
	6	3	3	1.70
	6	3	3	1.64
	5[b]	2	3	1.62

[a] If litter contained >8 pups, it was culled to include 8 pups.
[b] If litter contained <6 pups, these offspring were eliminated from all analyses.

3.3. Sex ratio of litters

On postnatal day one, pups were counted and sexed to determine the influence of genotype and stress treatment on litter size and offspring sex ratios. Litter size did not significantly differ between genotype ($F_{(1/37)}=0.10$, $P>0.05$) or stress treatment groups ($F_{(3/37)}=0.90$, $P>0.05$, Fig. 2, Table 3A and B). The overall sex ratio of total pups differed two-fold depending on maternal genotype and timing of prenatal stress treatment ($F_{(3/37)}=5.30$, $P<0.01$, Fig. 2, Table 3A and B). Although there was no effect of stress treatment on WT litter sex ratios, the ratio of female:male pups born to stress-sensitive KO litters was significantly reduced ($\chi^2=3.9$, $P<0.05$).

Table 3B
Offspring litter size average weight (g), and number of male and female offspring born to CRFR2-deficient (KO) dams

Maternal group	Litter size	# male pups	# female pups	Average pup weight (g)
KO control	10[a]	5	5	1.32
	8	4	4	1.36
	8	4	4	1.34
	2[b]	1	1	1.67
	2[b]	1	1	1.85
KO early stress	8	4	4	1.22
	7	5	2	1.41
	6	3	3	1.43
	6	5	1	1.36
	5[b]	3	2	1.37
KO mid-stress	8	4	4	1.08
	7	4	3	1.04
	7	5	2	1.37
	6	4	2	1.39
KO late stress	9[a]	5	4	1.22
	6	3	3	1.38
	6	3	3	1.70

[a] If litter contained >8 pups, it was culled to include 8 pups.
[b] If litter contained <6 pups, these offspring were eliminated from all analyses.

3.4. Offspring birth weights and early growth

To assess the impact of maternal stress sensitivity and prenatal stress treatment on body weight regulation, offspring birth weight and growth was monitored during nursing. At birth, a main effect of stress treatment is evident ($F_{(3/35)}=3.80$, $P<0.05$). Wild type offspring stressed during late gestation weighed significantly more than unstressed WT offspring at birth (Fig. 3). At postnatal day 26, an interaction between maternal genotype and stress treatment is present ($F_{(3/35)}=4.80$, $P<0.05$). KO litters stressed during early gestation weighed significantly more than unstressed KO litters (Fig. 3). At PN 8 and PN 15 body weights did not significantly differ between groups.

3.5. Offspring body weights as adults

Offspring were weighed as adults to determine the possible long-term body weight changes. At postnatal week 10, there was a significant interaction between stress treatment, maternal genotype, and sex ($F_{(3/35)}=6.30$, $P<0.01$) with main effects for stress treatment ($F_{(3/35)}=5.73$, $P<0.005$, genotype ($F_{(1/37)}=70.15$, $P<0.0001$) and sex ($F_{(3/37)}=144.70$, $P<0.001$). The body weights of prenatally stressed WT males and females did not differ from controls of the same sex, at postnatal week 10 (Fig. 4A and B). However, significant treatment differences were found in male and female offspring born to KO dams. Prenatally stressed KO male offspring weighed significantly more than unstressed KO male controls; this effect was exaggerated in males stressed during mid- and late gestation (Fig. 4A). KO female offspring stressed during early and late, but not mid gestation, weighed significantly more than WT females exposed to the same stress treatment (Fig. 4B). By postnatal week 16, stress treatment and maternal genotype continued to significantly affect offspring body weight ($F_{(1/35)}=4.80$, $P<0.0001$). Specifically, KO males

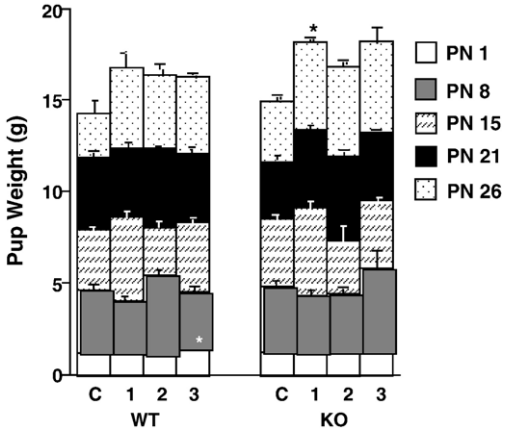

Fig. 3. Maternal stress sensitivity and timing of prenatal stress exposure alter early litter growth prior to weaning. At birth, (PN1), offspring from WT dams stressed during late (3) gestation weighed significantly more than offspring from unstressed (C) WT dams (*, $P<0.05$). At PN26, offspring born to KO dams exposed to early (1) gestational stress weighed significantly more than offspring born to unstressed KO dams (*, $P<0.05$). Data are mean±SEM.

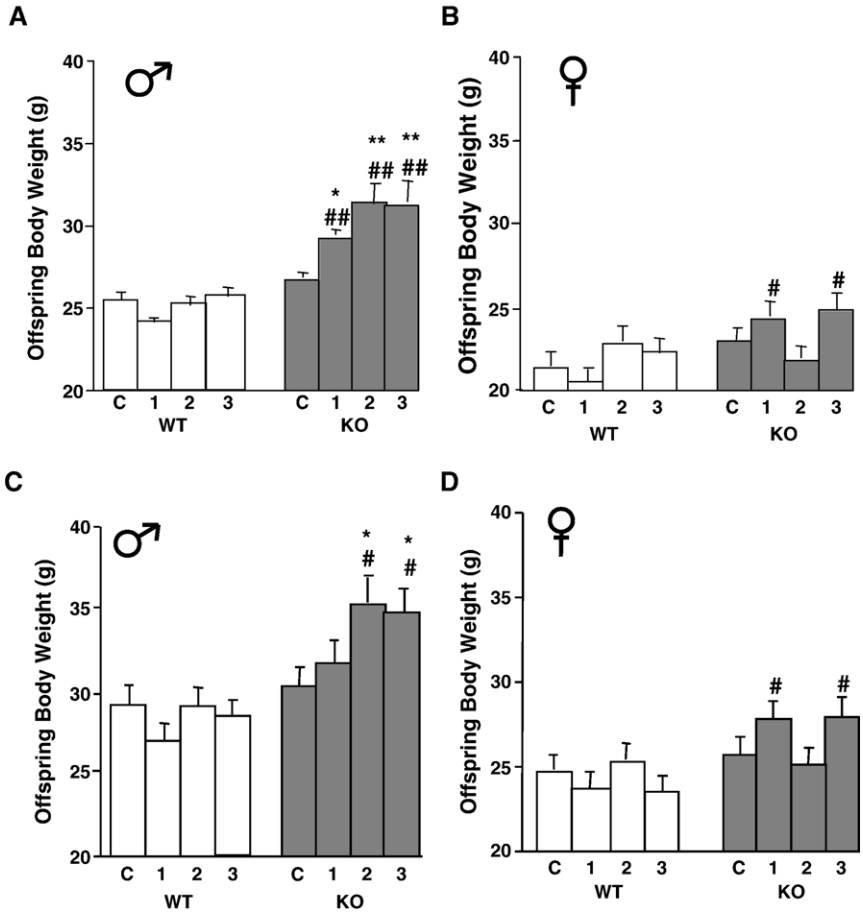

Fig. 4. Timing of prenatal stress exposure and offspring sex influence long-term adult body weight. Adult offspring body weight at postnatal week 10 (A and B) was significantly elevated for males from stressed KO dams compared to unstressed KO males (*, $P<0.01$; **, $P<0.001$) and WT males of the same stress group (##, $P<0.001$). Female offspring from early (1) and late (3) stressed KO dams weighed significantly more than females from WT dams of the same stress group (#, $P<0.01$). Offspring body weight at postnatal week 16 (C and D) was significantly elevated for males from KO dams exposed to mid- (2) and late (3) gestation stress compared to males from unstressed KO dams (*, $P<0.01$) and WT males exposed to the same stress treatment (#, $P<0.01$). Female offspring body weight from early (1) and late (3) stressed KO dams remained significantly higher compared to WT females of the same stress group (#, $P<0.01$). Data are mean ± SEM.

exposed to mid- and late gestation stress remained significantly heavier than unstressed KO males (Fig. 4C), while KO female offspring prenatally stressed early and late, but not mid, gestation weighed significantly more than WT females exposed to the same stress treatment (Fig. 4B and D).

3.6. Glucose

To determine potential metabolic consequences of prenatal stress, fasted plasma glucose levels were examined. Stress treatment ($F_{(1/35)}=6.80$, $P<0.005$) and maternal genotype ($F_{(1/35)}=5.90$, $P<0.05$) affected glucose levels. Female WT offspring exposed to prenatal stress had significantly lower fasted glucose levels than their unstressed controls (Fig. 5A and B). In contrast, glucose levels of WT males and KO offspring were not significantly influenced by prenatal stress (Fig. 5A and B).

3.7. Leptin

To characterize the impact of prenatal stress and maternal stress sensitivity on circulating leptin, basal plasma leptin levels were measured at the time of sacrifice. Significant interactions between stress treatment and sex ($F_{(3/26)}=3.26$, $P<0.05$), genotype and sex ($F_{(3/26)}=6.42$, $P<0.05$) affected leptin levels for males and females. Leptin levels did not differ between offspring born to WT dams (Fig. 5C and D). KO females stressed in late gestation had significantly higher leptin compared to WT females stressed in late gestation (Fig. 5D).

3.8. Corticosterone

To determine if prenatal stress exposure altered the neuroendocrine stress response of offspring, we measured corticosterone following five minutes of restraint. There is a significant interaction between sex, stress treatment, and genotype ($F_{(3/31)}=4.6$, $P<0.05$, Fig. 5E and F). Female offspring overall showed a greater corticosterone response than males, independent of stress treatment and genotype (Fig. 5F). The timing of prenatal stress exposure determined plasma corticosterone levels following stress in WT and KO males (Fig. 5E). Wild type males stressed during mid-gestation displayed higher corticosterone levels following restraint stress compared to unstressed WT males, while KO males stressed during

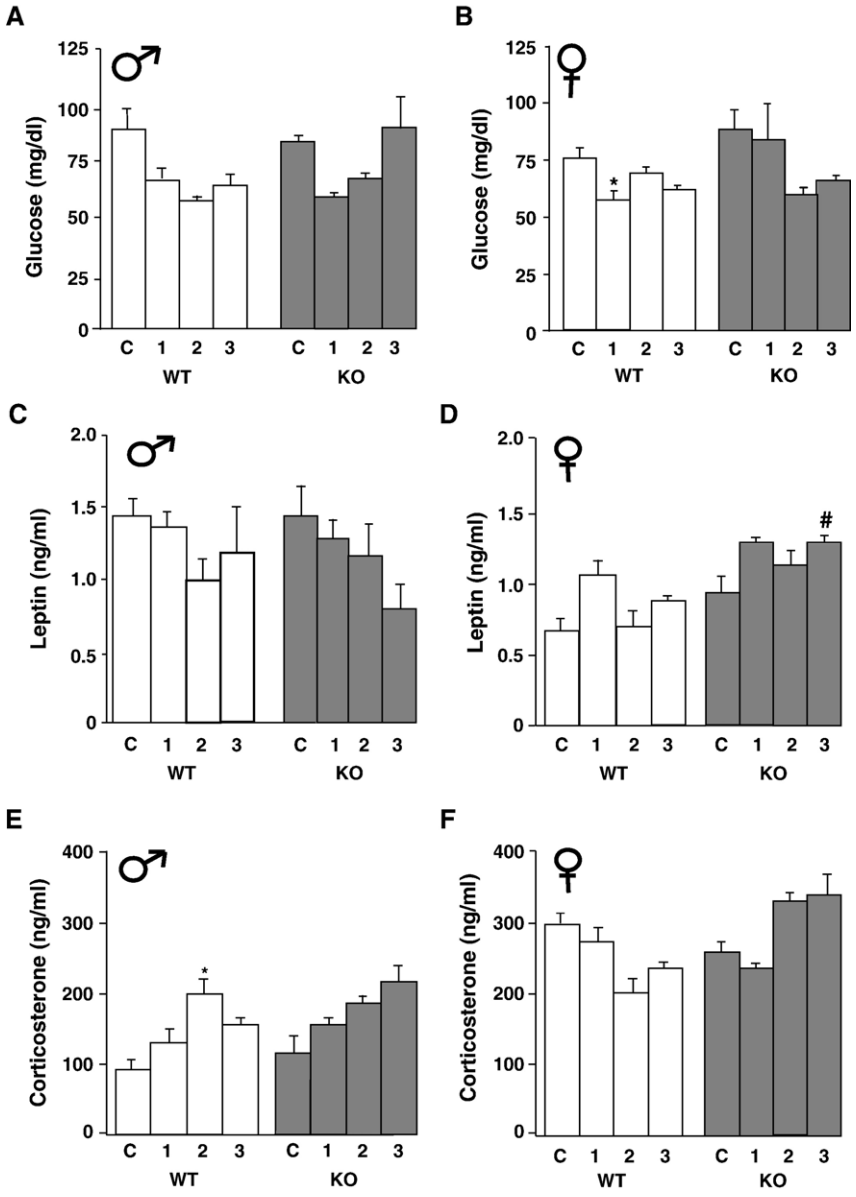

Fig. 5. Hormone levels important in energy homeostasis are altered by prenatal stress exposure. Fasted glucose levels (A and B) were decreased for WT female offspring exposed to early (1) gestational stress compared to females from unstressed (C) WT dams (*, $P<0.05$). No differences in fasted glucose levels were seen for males. Basal plasma leptin (C and D) was unchanged in male offspring. Leptin levels for KO females exposed to stress were significantly higher than WT females of the same stress group (#, $P<0.05$). Corticosterone levels (E and F) in response to a 5-min restraint were elevated in WT males stressed during mid- (2) gestation compared to unstressed (C) WT males (*, $P<0.05$). Female corticosterone levels are not significantly altered by stress treatment or maternal genotype. Data are mean± SEM.

late gestation appeared to exhibit higher corticosterone levels than unstressed KO males.

4. Discussion

Given the difficulties and limited success of permanent weight loss, studies elucidating environmental and genetic risk factors that may predispose an individual to increased body weight are needed. It is well established that perturbations in the uterine environment during development can permanently alter neuroendocrine function in offspring, resulting in abnormal metabolism and body weight alterations [3,12,27]. Our aim in these studies was to examine factors that may heighten the risk of long-term body weight increases following prenatal stress. Using a genetic model of increased stress sensitivity and CVS, we investigated the dependence of offspring body weight on maternal stress sensitivity and time of prenatal stress exposure.

In order to confirm that the increased sensitive neuroendocrine stress response of KO mice remained exaggerated throughout all stages of pregnancy, corticosterone levels of WT and KO dams were compared following a 15-min stress exposure. Our results showed that the KO dams continue to exhibit a prolonged recovery following stress exposure throughout pregnancy. Plasma corticosterone of WT dams returned to near-baseline 1 h following stress exposure. In contrast, KO dams display a prolonged

stress recovery during all three trimesters, and an increased magnitude during late pregnancy. Both genotypes showed an expected progression in magnitude of basal and stress-induced corticosterone production across pregnancy [28].

Utilizing this genetic model of increased stress sensitivity, we examined the time-specific effects of prenatal stress on offspring outcome. An initial unexpected finding in these studies was a decrease in female offspring from litters exposed to prenatal stress. Offspring from non-stressed dams of both genotypes showed an expected Mendelian 50:50 offspring sex ratio. In contrast to earlier studies that have reported decreased male births following prenatal challenges (including dam heat and malnutrition exposure) [29], our findings revealed that CVS early in pregnancy significantly reduced the number of female offspring born to stress-sensitive KO dams. These results may be explained by the difference in timing and nature of stressors, as well as the increased stress sensitivity of KO dams, suggesting an elevated vulnerability of females to prolonged glucocorticoid exposure during implantation and early gestation.

Numerous studies have reported *low* birth weights following maternal stress or synthetic glucocorticoid exposure [12,30,31]. Surprisingly, we found that WT offspring exposed to stress late in gestation weighed *more* than unstressed WT control offspring. As our study used CVS, while previous reports have typically utilized a repeated stress, these results may suggest a correlation between the 'predictability' of the stress experienced and the ultimate impact on offspring body weights [14,15,32]. Further, chronic restraint stress late in gestation has been shown to reduce maternal food intake and body weight gain [33–36]. As maternal undernutrition can produce fetal intrauterine growth retardation [25], utilizing a stress model that does not directly affect maternal food intake may be preferable when evaluating the effects of stress on offspring body weight outcome distinct from maternal nutritional parameters. In our study using CVS, dam body weight gain and food intake did not differ between treatment groups or genotypes throughout pregnancy.

Prior to weaning, prenatally stressed offspring from both genotypes appeared heavier than unstressed controls, supporting recent findings indicating the prenatal environment may influence the likelihood that offspring will develop overweight and obesity [37]. This effect on body weights was exaggerated in offspring from KO dams, as first trimester stressed KO offspring were significantly heavier by postnatal day 26. Given that pups were weighed as a litter to minimize handling and maternal separation time, the increased body weight in this group may be a reflection of the increased percentage of males in these litters. However, male and female body weights remained elevated at 10 weeks.

In adulthood, the effects of prenatal stress on body weight were no longer evident in WT offspring, but remained significant in KO mice. Increased birth weight of prenatally stressed WT males had normalized by postnatal week 10. In contrast, adult KO males exposed to prenatal stress were 15–20% heavier than unstressed control offspring. This sustained impact of prenatal stress on offspring from stress-sensitive KO dams supports our hypothesis that maternal stress-sensitivity can be a critical determinant as to the magnitude and longevity of the offspring phenotype. While the effect of prenatal stress on body weight was not specific to time of stress exposure, the magnitude of increased body weight was greater for offspring exposed to mid- and late gestational stress. Furthermore, at postnatal week 16, only those KO males exposed to mid- and late gestational stress remained significantly heavier than unstressed controls. As observed in males, long-term body weight changes were only evident in females born to stress-sensitive KO dams. At both 10 and 16 weeks, early and late prenatally stressed KO females weighed more than KO controls and significantly more than WT females of the same stress treatment groups.

To investigate potential mechanisms by which prenatal stress alters offspring long-term body weight, we examined several indices of energy homeostasis, including plasma leptin and fasted glucose levels. Leptin levels were not elevated in KO males exposed to stress late in gestation despite their elevated body weights, suggesting that their increased weight may result from increased lean body mass. The increase in female KO body weight correlated with higher leptin levels, suggesting prenatal stress may have specifically altered long-term adiposity of stress-sensitive females. As there are distinct sex differences in energy storage and in obesity susceptibility, delineating the influence of stress during development on these parameters may provide insight into the possible underlying contributions of stress [38]. Future studies will more specifically examine body composition of prenatally stressed offspring.

With a few group exceptions, prenatal stress for both genotypes appeared to reduce fasting glucose levels independent of stress exposure timing. Effects of prenatal stress on fasted glucose levels were most pronounced in WT female offspring stressed during early gestation. Similar to our findings on body weights, these results are in opposing direction to those previously shown where stress late in pregnancy produces glucose intolerance and hyperglycemia in aged animals [12]. As the predominant stress utilized in such studies is that of repeated restraint late in pregnancy, differences in offspring outcome may be directly related to distinctions in methodology. In a repeated restraint model, the prolonged exposure (45 min to 3 h) of the largely pregnant animal in a plastic tube under bright lights with no access to food or water is likely to produce a physiologic stress effect due to an inability to properly thermoregulate, increasing the dam's body temperature [11,12,34,39]. Studies in pregnant rats exposed to brief heat during late pregnancy showed significant changes in fetal hypothalamic glucose utilization and uptake at birth, supporting a profound effect of maternal body temperature on offspring brain function that may produce long-term alterations in key hypothalamic neurocircuitry [40]. To avoid these potential confounds, we selected variable stressors that did not directly influence maternal nutrient intake, body weight gain, induce pain, or alter core body temperature. Therefore, results from our model may be distinct from those using a chronic restraint stress and may be more indicative of the effects of prenatal stress resulting from repeated activation of the maternal neuroendocrine HPA axis. Further studies are needed to determine if this model may be more akin to the stressors experienced in human pregnancy.

It is well established that rodent and primate offspring exposed to prenatal stress or glucocorticoids display exaggerated stress reactivity as adults [41–44]. However, research delineating the contribution of the timing of prenatal stress exposure to this

phenotype has not been determined. Our study found that WT male adult offspring exposed to mid-gestation stress showed significantly elevated corticosterone levels in response to an acute mild stress. Knockout males appeared to have an exaggerated stress response most pronounced in offspring stressed late in gestation. Possibly due to a ceiling effect, where females have an overall greater magnitude stress response than males, prenatal stress treatment did not further augment corticosterone levels in females of either genotype [11,45,46]. As these results were limited to a single time point following an acute stress exposure, future studies will more specifically examine stress response curves and recovery in these offspring.

As it has been well established that postnatal manipulations (neonatal handling, maternal deprivation, and modified maternal behaviors) during early development can permanently modify the phenotype of adult offspring [47–51], we controlled for possible postnatal maternal influences by measuring prepartum nest quality and postpartum nesting prior to weaning. We detected no differences in these broad indices of pre- and postpartum maternal behaviors in our study. Dams of both genotypes spent similar time on the nest with pups prior to weaning. Previous work from our laboratory has shown that cross-fostering KO offspring to WT dams reduced the heightened stress-responsive behaviors specifically of male KO offspring. However, this was not the case with the reverse cross. WT offspring cross-fostered to KO dams showed no change in their stress responsivity [52]. Further, in our previous examination of postpartum maternal behaviors, we found no genotypic differences in a test for pup retrieval where dams from both genotypes retrieved their pups to the nest and spent equal time on the nest following retrieval [52]. While a complete analysis of maternal licking and grooming behaviors has not been conducted, these studies support the absence of profound maternal care deficits in our KO mice. Future studies will utilize cross-fostering to dissect the possible independent influences of prenatal versus postnatal environment on offspring outcome.

Results from out study illustrate how CVS exposure produces alterations in long-term body weight and energy homeostasis regulation. As the prolonged effects on body weight were most evident in offspring from stress-sensitive KO dams, these results suggest that the influence of prenatal stress may be exaggerated when the prenatal environment is more susceptible to external stimuli. While previous studies have illustrated the profound effects of mild prenatal stress on multiple postnatal parameters, the results presented here demonstrate that the specific outcomes are likely dependent on the timing of the stress exposure during gestation. Mechanistic studies can now begin to elucidate how stress experienced during these specific developmental windows produces long-term alterations in offspring body weight.

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