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INTENSITY MATTERS: BRAIN, BEHAVIOUR AND THE EPIGENOME OF PRENATALLY STRESSED RATS

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Abstract—There is a general consensus that prenatal stress alters offspring brain development, however, the details are often inconsistent. Hypothesising that variation to the level of stress would produce different maternal experiences; this study was designed to examine offspring outcomes following a single prenatal stress paradigm at two different intensities. Pregnant Long Evans rats received mild, high, or no-stress from gestational days 12–16. Offspring underwent early behavioural testing and global methylation patterns were analysed from brain tissue of the frontal cortex and hippocampus. The two different prenatal stress intensities produced significantly different and often, opposite effects in the developing brain. Mild prenatal stress decreased brain weight in both males and females, whereas extreme stress increased female brain weight. Mild prenatal stress slowed development of sensorimotor abilities and decreased locomotion, whereas high prenatal stress also slowed development of sensorimotor learning but increased locomotion. Finally, mild prenatal stress increased global DNA methylation levels in the frontal cortex and hippocampus whereas high prenatal stress was associated with a dramatic decrease. The data from this study provide evidence to support a dose-dependent effect of prenatal stress on multiple aspects of brain development, potentially contributing to long-term outcomes. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: epigenetics, prenatal stress, brain development, behaviour.

There is an abundance of evidence illustrating the negative consequences associated with stress early in life (McCormick et al., 1995; Lemaire et al., 2000; Huizink et al., 2003; Patin et al., 2004; Lee et al., 2007). But there is also evidence suggesting that mild stress may be beneficial. For example, exposure to low doses of corticotrophin-releasing factor improves memory retention in rats (Wang et al., 1998) and studies with monkeys have demonstrated that mild early life stress actually serves to inoculate monkeys to be resilient to stress later on (Katz et al., 2009).

Less is known about the effects of stress during gestation, although any effects are likely to be mediated by glucocorticoid exposure (Meaney et al., 2007). Although glucocorticoids are necessary for normal brain growth and maturation (Meyer, 1983), suppression or amplification of glucocorticoid expression is detrimental at this developmental time. The extent of fetal exposure to these stress-related hormones is dependent on maternal production (White et al., 1997). Although there has been extensive research conducted on the effects of prenatal stress (Gue et al., 2004; Murmu et al., 2006; Maccari and Morely-Fletcher, 2007; Zuena et al., 2008) very few studies have examined outcomes for the same prenatal stress experience at different intensities (Patin et al., 2002). Furthermore, to our knowledge, no experiments have examined the effects of prenatal stress intensity on the genome, and most importantly—epigenome, at a global level.

The current study was designed to investigate the effects of a single prenatal stress paradigm, at two different intensities, on offspring brain, behaviour and global epigenetic DNA methylation levels. Pregnant dams received mild, high, or no stress from gestational days 12–16 and offspring were tested behaviourally before the brains were harvested at weaning for epigenetic analysis.

EXPERIMENTAL PROCEDURES

Subjects and stressing procedures

All experiments were carried out in accordance with the Canadian Council of Animal Care and approved by the University of Lethbridge Animal Care Committee. Eighteen pregnant Long-Evans rats were paired with a female Long-Evans rat and housed in shoe-box cages (36 females in total). All animals were maintained on 12:12 h light:dark cycle in a temperature controlled breeding room (21 °C) and were given access to food and water *ad libitum*. The female rats were in-bred in the facility for at least five generations. Two different doses of prenatal stress were given to a subset of the pregnant dams. Both prenatal stress paradigms involved placing the pregnant dam on an elevated Plexiglas® platform (1 m tall, 21×21 cm^2) twice daily on gestational days 12–16 (G12–G16) (Wong et al., 2007). The mild prenatal stress treatment was characterised by 10-min sessions on the elevated platform (n=5), while the high prenatal stress treatment entailed 30-min sessions (n=4). Stressing occurred at 9:00 AM and 3:00 PM. Control dams (n=9) were left undisturbed. Once the pups were born, each of the mothers and their litters were housed individually.

Behavioural methods

Negative geotaxis. Pups were individually placed facing downward on Plexiglas® board set to a 40° angle. Pups were filmed for 60 s. If the pup slid off the board, they were replaced in the downward position. Pups were scored for the amount of time they spent facing in an upward direction. A pup was considered to be in an upward position when its head crossed the horizontal

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plane. Pups were tested on postnatal day 9 (P9) and P10. At this time point, pups eyes were still closed.

Open field. Pups were individually placed in the centre of a transparent Plexiglas® box measuring 16×20×30 cm^3. The base of the box was divided into roughly 130 squares measuring 2×2 cm^2. Pups were filmed for 60 s and scored for the total number of novel squares their front paws entered. Two analysts scored each video and the average score for each pup was used. Pups were tested on P10–P13 and P15.

Methylation procedure

At approximately 21 days of age, pups were subjected to isoflurane inhalation, weighed and quickly decapitated. The brains were then removed from the skull (cutting the optic nerves ahead of the optic chiasm and removing the olfactory bulbs), and weighed. The frontal cortex and hippocampi of each pup was removed, immersed in RNAlater RNA Stabilisation Reagent (Qiagen; Valencia, CA, USA), immediately flash frozen on dry ice and stored at −80 °C.

To determine the extent of global methylation, we used a well established radiolabeled [^{3}H]-dCTP HpaII/MspI-based cytosine extension assay that measures the proportion of CpG islands that lost methyl groups on both strands of DNA (Pogribny et al., 1999). While the restriction enzyme HpaII cleaves CpG sequences when internal cytosine residues are unmethylated on both strands, its isoschizomer MspI cleaves CpG sites in DNA regardless of CpG methylation status. The absolute percent of double-stranded unmethylated CpG sites can be calculated by relating the data of HpaII and MspI digests (Pogribny et al., 2004; Koturbash et al., 2007). As the vast majority of the frequently occurring HpaII tetranucleotide recognition sequences are constitutively methylated *in vivo*, an increase in cleavage at these sites is an indicator of genome-wide hypomethylation (Pogribny et al., 2004; Koturbash et al., 2007).

In brief, 1 μg of genomic DNA was digested with the methylation-sensitive HpaII restriction endonuclease (New England Biolabs, Beverly, MA, USA). A second DNA aliquot (1 μg) was digested with methylation-insensitive isoschizomer MspI (New England Biolabs, Beverly, MA, USA). Another aliquot (1 μg) of undigested DNA served as background control. The single nucleotide extension reaction was performed in a 25 μl reaction mixture containing 1.0 μg DNA, 1× PCR buffer II, 1.0 mM $MgCl_2$, 0.25 U AmpliTaq DNA polymerase and 0.1 μl of [^{3}H]dCTP (57.4 Ci/mmol), and incubated at 56 °C for 1 h. Samples were applied to DE-81 ion-exchange filters and washed three times with 0.5 M Na-phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for scintillation counting. The [^{3}H]dCTP incorporation into DNA was expressed as mean disintegrations per min (dpm) per μg of DNA after subtraction of the dpm incorporation in undigested samples (background). Two technical repeats of each experiment were conducted to ensure consistency of the data. The absolute percent of double stranded unmethylated CCGG sites was calculated as described (Pogribny et al., 2004; Koturbash et al., 2007).

Statistical analysis

All statistical analysis was carried out using SPSS 16.0 for Mac. Analysis was conducted to ensure results could not be attributed to a specific litter. Two-way ANOVA's with Stress Level and Sex as factors were run to compare the prenatal stress groups and the control group. Significant results are reported as significant differences between either prenatal stress group and the no-stress control group.

RESULTS

Effects of stress on maternal behaviour

Maternal behaviour exhibited dose dependent alterations associated with the two prenatal stress paradigms. Observation revealed a loss of fur in dams exposed to the high prenatal stress paradigm that was not evident in dams exposed to mild prenatal stress. High prenatal stress dams were also more aggressive towards animal care technicians who did not know their group affiliation, whereas mild prenatal stress dams were timid. Furthermore, when compared to control dams, mild prenatal stress was associated with a significant increase in the amount of weight gained during gestation and high prenatal stress was associated with a significant decrease (Weight gain during gestation=weight at gestational day 21—weight at gestational day 0, measured daily; (grams): control, 116.3±10.7; Mild Prenatal Stress, 135.0±13.6; High Prenatal Stress, 74.1±15.2; $F(2,16)=4.535$, $P=0.030$). Stress protocol did not have an effect on duration of pregnancy or litter composition.

Brain weight

Mild prenatal stress animals had decreased brain weights when compared to control animals. Similarly, high prenatal stress males had decreased brain weights, whereas females exhibited an increase in brain weight when compared to controls (Fig. 1). A two-way ANOVA with stress level and sex as factors showed a main effect of stress level, $F(2,221)=6.919$, $P=0.001$ and sex, $F(1,221)=14.855$, $P<0.0001$, but not the interaction $F(2,221)=2.632$, $P=0.074$. Given that brain and body weight fluctuate, it is possible for increased brain weight to be a result of increased body weight and vice versa. When brain weights were standardised against body weight results were the same as demonstrated above (data not shown).

Body weight

Both male and female offspring from the mild prenatal stress group demonstrated a decrease in body weight when compared to controls. Female offspring from the high prenatal stress group exhibited an increase in body weight, and males

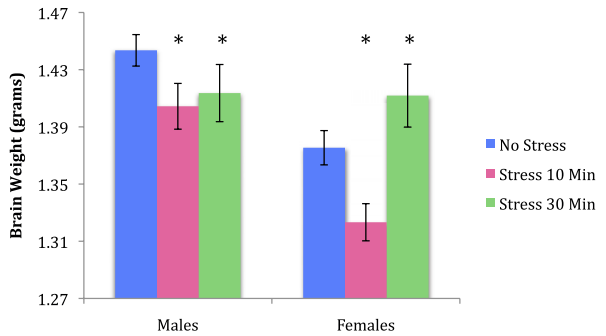

Fig. 1. Average brain weight of offspring at time of sacrifice (P21) (* $P<0.001$). * Represent significant differences between stressed offspring and control offspring. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

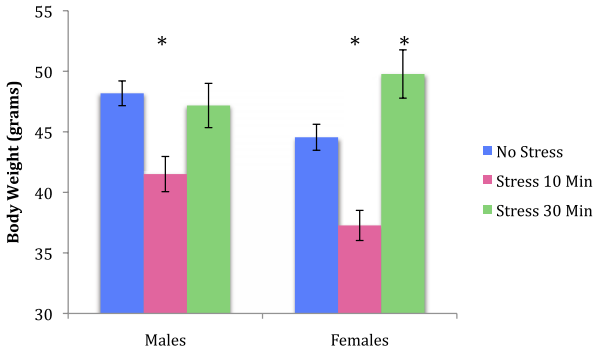

Fig. 2. Average body weight of offspring at time of sacrifice (P21) (* $P<0.001$). * Represent significant differences between stressed offspring and control offspring. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

from this group were not different than controls (Fig. 2). A two-way ANOVA with stress level and sex as factors showed a main effect of stress level, $F(2,221)=21.757$, $P<0.0001$, but not of sex, $F(1,221)=2.110$, $P=0.148$. The interaction was not significant, $F(2,221)=2.436$, $P<0.090$.

Negative geotaxis

Both male and female offspring from the mild prenatal stress group demonstrated deficits on the negative geotaxis task on postnatal day 9 and 10 when compared to controls. Male and female offspring from the high prenatal stress group show no deficits on postnatal day 9. However, they both fail to demonstrate any improvement on postnatal day 10 and therefore show deficits when compared to controls (Figs. 3 and 4). For negative geotaxis scores on postnatal day 9, a two-way ANOVA with stress level and sex as factors showed a main effect of stress level, $F(2,181)=19.390$, $P<0.0001$, but not of sex, $F(1,181)=0.910$, $P=0.341$. The interaction was not significant, $F(2,181)=1.980$, $P=0.141$. Similarly, for scores on postnatal day 10, a two-way ANOVA with stress level and sex as factors showed a main effect of stress level, $F(2,181)=17.355$, $P<0.0001$, but not of

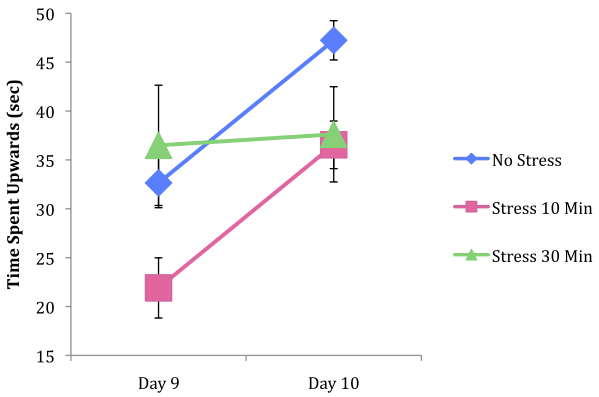

Fig. 3. Amount of time male offspring spent in the upward direction during the negative geotaxis task on P9 and P10. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

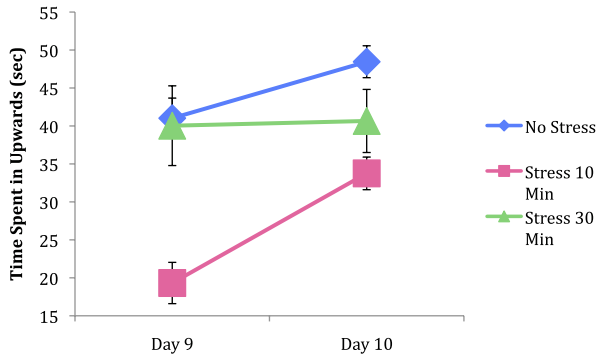

Fig. 4. Amount of time female offspring spent in the upward direction during the negative geotaxis task on P9 and P10. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

sex, $F(1,181)=0.037$, $P=0.848$. The interaction was not significant, $F(2,181)=0.565$, $P=0.569$.

Open field

When total number of novel fields entered is summed across all 5 days; activity level of both male and female mild prenatal stress offspring decreases while, activity level for male offspring of high prenatal stress increases when compared to controls (Fig. 5). A two-way ANOVA with stress level and sex as factors shows a main effect of stress level, $F(2,138)=13.482$, $P<0.0001$, and sex, $F(1,138)=5.214$, $P=0.024$. The interaction was not significant $F(2,138)=2.090$, $P=0.128$.

Global DNA methylation

Frontal cortex. Both male and female offspring in the high prenatal stress group showed a decrease in global DNA methylation when compared to offspring in the control group. Males of the mild prenatal stress group demonstrated an increase in global DNA methylation whereas female offspring in this group was not different from the control group (Fig. 6). A two-way ANOVA with stress level and sex as factors demonstrated a main effect of stress level, $F(2,56)=38.773$, $P<0.0001$ but not

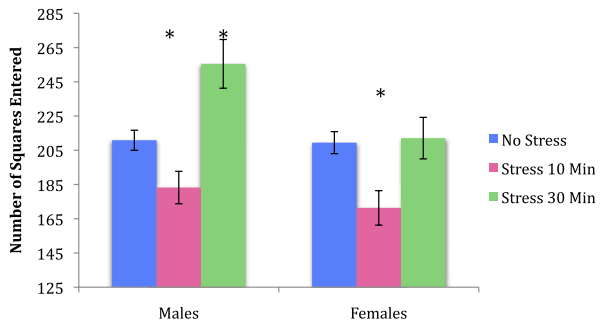

Fig. 5. Total number of novel fields offspring entered on postnatal days 10–13 and 15 (* $P<0.001$). * Represent significant differences between stressed offspring and control offspring. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

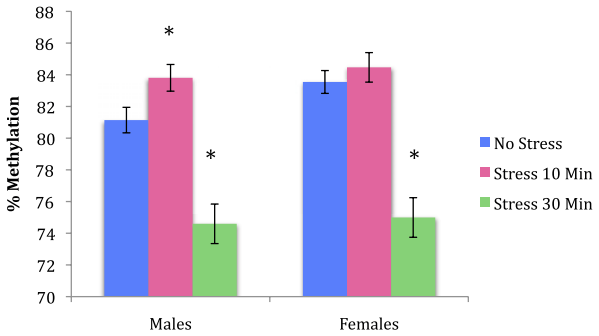

Fig. 6. Levels of global DNA methylation shown as percentage of methylated CpG sites in frontal cortex of offspring at postnatal day 21 (* $P<0.0001$). * Represent significant differences between stressed offspring and control offspring. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

of sex, $F(1,56)=2.045$, $P=0.159$. The interaction was not significant, $F(2,56)=0.765$, $P=0.470$.

Hippocampus. All offspring from the mild prenatal stress group demonstrated an increase in global DNA methylation of the hippocampus whereas offspring from the high prenatal stress group demonstrated a decrease in global DNA methylation when compared to offspring of the control group (Fig. 7). A two-way ANOVA with stress level and sex as factors exhibited a main effect of stress level, $F(2,55)=133.153$, $P<0.0001$ but not of sex, $F(1,55)=0.225$, $P=0.637$. The stress level by sex interaction was not significant, $F(2,55)=0.161$, $P=0.852$.

DISCUSSION

There is an abundance of research illustrating the important effects early stress has on brain and behavioural development (e.g. (Meaney et al., 1996, 2007; Anda et al., 2006; Danese et al., 2009). The present study clearly demonstrates that not only does prenatal stress affect brain and behavioural development; the effects of prenatal stress differ with intensity.

Effects of prenatal stress on brain weight

Prenatal stress had different effects on offspring brain weight depending on the dose. Mild prenatal stress decreased brain weight in both males and female offspring, whereas high prenatal stress increased brain weight in females but decreased brain weight in males. This is contradictory to results reported from other laboratories where no change in offspring brain weight was found (Van Den Hove et al., 2006). Similarly, mild prenatal stress decreased body weight in both sexes, whereas high prenatal stress resulted in an increase in all offspring body weight. The mild prenatal stress results are consistent with other research demonstrating a decrease in body weight of male offspring at birth (Patin et al., 2002; Gue et al., 2004; Lesage et al., 2004), adolescence (Van Waes et al., 2006) and adulthood (Vallee et al., 1996). The identified offspring brain and body weight changes are likely not due to maternal weight changes during pregnancy as dams exposed to mild stress gained more weight than controls and dams exposed to high stress gained less weight.

Effects of prenatal stress on behavioural development

Although the skills required to complete the negative geotaxis task are not fully understood, the task is often used as a milestone for early sensorimotor development in rats (Alberts et al., 2004; Patin et al., 2004). The current results suggest that prenatal stress alters sensorimotor development. Offspring exposed to high prenatal stress appear to show no deficits in the task on P9 but they fail to demonstrate any learning or improvements and thus are significantly impaired when compared to controls on P10. In contrast offspring of the mild prenatal stress group learned the task in a similar fashion as controls, but their starting performance was significantly impaired on P9 and were therefore unable to reach performance levels of controls by P10. It may be the case that high prenatal stress blocks sensorimotor learning, whereas mild prenatal stress has no impact on sensorimotor learning but does changes the starting point of sensorimotor capability.

The effect of prenatal stress on locomotor activity appears to be dependent upon the level of prenatal stress and the age at which the offspring are studied. In this study mild prenatal stress was associated with a decrease in locomotor activity for both males and females when compared to controls. This study is the first to look at locomotion at such an early time point (P10–P15) but the results are similar to Patin and colleagues (2004) who found that there was a decrease in spontaneous locomotor activity at 1 and 2 months in male offspring exposed to repeated prenatal stress. In contrast, high prenatal stress in this study was associated with an increase in locomotor activity in male offspring but no change in female offspring. Gue and colleagues (2004) also found an increase in locomotor activity in male and female offspring, however they failed to mention the age at which testing occurred. Our research indicates that there may be a reason for the conflicting results seen in previous studies examining locomotor ac-

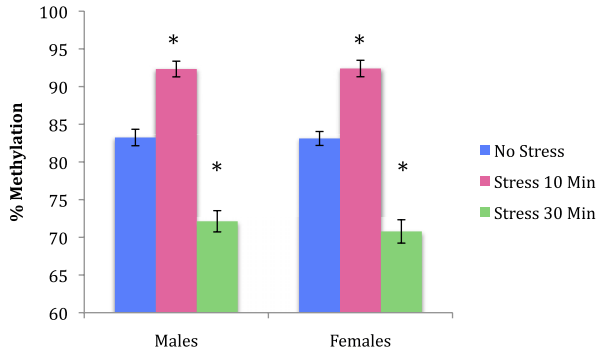

Fig. 7. Levels of global DNA methylation shown as percentage of methylated CpG sites in hippocampus of offspring at postnatal day 21 (* $P<0.0001$). * Represent significant differences between stressed offspring and control offspring. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

tivity and prenatal stress as altering the intensity of prenatal stress appears to alter the locomotor response of male and female offspring.

Prenatal stress alters gene methylation

Cytosine DNA methylation is a well-studied key epigenetic phenomenon that regulates important cellular processes, such as differentiation, proliferation and apoptosis during normal development (Jaenisch and Bird, 2003; Baylin and Ohm, 2006; Bird, 2007), giving rise to cell- and tissue-specific gene expression patterns (Illingworth et al., 2008). DNA methylation is crucially important for the processes of X-chromosome inactivation, genomic imprinting and silencing of foreign DNA. It acts as a repressor of gene expression and helps to maintain genome stability (Bird, 2007). Altered DNA methylation levels may in turn lead to aberrant gene expression and overall genome destabilisation. Therefore, the most surprising, and by far the most interesting finding arising from our experiment, are the dramatic DNA methylation changes observed in the frontal cortex and hippocampus of the stressed offspring. Mild prenatal stress was related to a significant increase in global DNA methylation in the hippocampus of both male and female offspring and the frontal cortex of male offspring. Contrary to this, both female and male offspring exposed to high prenatal stress showed a significant reduction in global DNA methylation levels in the hippocampus and the frontal cortex. It is likely that these epigenetic changes are the consequence of the maternal stress response. The precise mechanisms of DNA methylation changes and their cellular and organismal repercussions need to be further delineated. Yet we suggest that some of the altered behavioural changes may in turn be associated with altered DNA methylation and gene expression levels. The precise gene-specific nature of the aforementioned changes needs to be further established.

The hypothalamic-pituitary-adrenal (HPA) axis is a well-studied mediator of the relationship between maternal experiences and fetal development (Meaney et al., 1985; McCormick et al., 1995; Matthews, 2002). Lipophilic steroids like glucocorticoids easily cross the placenta (White et al., 1997) and glucocorticoid receptors are expressed in most fetal tissues early in embryonic development (Meaney et al., 1985). Although the placenta and the fetus have developed mechanisms to protect the fetus from maternal cortisol, this mechanism can only compensate to a certain threshold (White et al., 1997). Based on the evidence from this study one may speculate that a small increase in maternal stress hormone release (Wong et al., 2007) may silence the genome by increasing global DNA methylation whereas a much greater increase in maternal stress hormone release (Wong et al., 2007) may activate the genome by decreasing global DNA methylation. Yet, the exact nature of the genes affected by maternal stress hormones still needs to be established.

Limitations and future directions

A potential limitation of this study is the inability to exclude the behavioural testing as a contributor to the changes in global methylation. It is possible that the behavioural testing differentially affected DNA methylation patterns in stressed versus control rats. However significant modification to global methylation, as seen here, is unlikely to occur in response to such a brief, noninvasive postnatal experience. The second limitation to our study is the lack of plasma or serum corticosterone concentrations for pregnant dams in the different prenatal stress groups. These were not taken during the course of this experiment, as the act of drawing blood is an additional stressor and may have confounded our results. Had we anticipated the nonlinear relationship between stress level and offspring outcome we may have emphasised the importance of this test. Similarly, future studies would include more gradations of prenatal stress intensity, possibly at 5, 15, 20, 25, 35 and 40-min intervals. This would help explain if our results exist as part of an inverted U theory of stress and performance, in which some levels of stress act as inoculations where as others are detrimental. The current findings suggest that future studies on the effects of prenatal stress should be mindful of the details of the stress paradigm. Although we varied the intensity of stress in the current study, we held duration (G12–G16) and timing (9:00 AM and 3:00 PM) of stress constant. It seems likely that duration and timing will be important variables as well and will require systematic study.

CONCLUSION

The current experiments carried out in this study provide initial evidence to support a relationship between intensity of prenatal stress and effect on offspring. Although we did not investigate the entire prenatal stress-offspring continuum, the data suggest that increasing prenatal stress intensity results in very different outcomes for offspring. Thus, prenatal stress changed brain and body weights, sensorimotor learning, locomotor activity and global methylation patterns differentially based upon the intensity. These experiments also provide insight into the relationship between long-term health risks and early adverse experiences; a relationship that is thought to depend on epigenetic patterning (Meaney et al., 2007; Darnaudery and Maccari, 2008; Lupien et al., 2009). This study demonstrated that the same experience, varied in magnitude, produces drastically different epigenetic changes. The net effect of comparing mild to high prenatal stress is a global methylation swing of ~20% in the hippocampus and ~10% in the frontal cortex. Although both prenatal stress paradigms result in DNA methylation patterning that differs from controls, the most remarkable divergence in methylation occurs between the two prenatal stress groups.

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