Differential Methylation of Genes in the Medial Prefrontal Cortex of Developing and Adult Rats Following Exposure to Maltreatment or Nurturing Care During Infancy

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Key Words

Epigenetics · DNA methylation · Early-life stress · Maternal care · Medial prefrontal cortex · *bdnf* gene · *reelin* gene

Abstract

Quality of maternal care in infancy is an important contributing factor in the development of behavior and psychopathology. One way maternal care could affect behavioral trajectories is through environmentally induced epigenetic alterations within brain regions known to play prominent roles in cognition, emotion regulation, and stress responsivity. Whereas such research has largely focused on the hippocampus or hypothalamus, the prefrontal cortex (PFC) has only just begun to receive attention. The current study was designed to determine whether exposure to maltreatment or nurturing care is associated with differential methylation of candidate gene loci (*bdnf* and *reelin*) within the medial PFC (mPFC) of developing and adult rats. Using a within-litter design, infant male and female rats were exposed to an adverse or nurturing caregiving environment outside their home cage for 30 min per day during the first postnatal week. Additional littermates remained with their biological caregiver within the home cage during the manipulations. We observed that infant rats subjected to caregiver maltreatment emitted more audible and ultrasonic vocalizations than littermates subjected to nurturing care either within or outside of the home cage. While we found no maltreatment-induced

changes in *bdnf* DNA methylation present in infancy, sex-specific alterations were present in the mPFC of adolescents and adults that had been exposed to maltreatment. Furthermore, while maltreated females showed differences in *reelin* DNA methylation that were transient, males exposed to maltreatment and both males and females exposed to nurturing care outside the home cage showed differences in *reelin* methylation that emerged by adulthood. Our results demonstrate the ability of infant-caregiver interactions to epigenetically mark genes known to play a prominent role in cognition and psychiatric disorders within the mPFC. Furthermore, our data indicate the remarkable complexity of alterations that can occur, with both transient and later emerging DNA methylation differences that could shape developmental trajectories and underlie gender differences in outcomes.

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Introduction

Clinical research and animal models have emphasized the importance of early-life caregiving environments in affecting behavioral outcomes and mental health throughout the lifetime and even transgenerationally. For example, clinical studies have shown that maltreatment early in childhood can cause a lifelong reduction in cognitive performance, alter social development, negatively impact stress physiology and neurobiological function, and in-

crease the risk for mood and stress-related disorders [1–4]. Both rodent and nonhuman primate models of aberrant maternal care or infant separation have likewise demonstrated alterations in behavioral outcomes, brain structure, and function [5–8]. Furthermore, animal models have been instrumental in helping us understand that not only are these neurobiological changes long lasting, but that they too can be passed on to future generations via nongenetic transmission [9–11].

Current theories advocate that both genetic makeup and early-life environmental factors play a significant role in the development of normal behavior and psychopathology [12, 13]. A challenge has been untangling the relationship between nature and nurture, and recently we have begun to see empirical evidence for a mechanism that allows this. Epigenetic mechanisms provide a way to integrate an organism's genes with experience through various environmentally induced dynamic and stable modifications to DNA and its associated proteins that can alter gene expression. The mechanisms by which epigenetics can affect gene expression are varied and include DNA methylation and demethylation, which involve the addition or removal (respectively) of methyl groups from cytosines typically in and around gene promoters [14].

Studies in rodents have suggested a causal relationship between the quality of the caregiving environment and DNA methylation patterns within the brain. For example, a landmark paper from Michael Meaney's laboratory utilized natural variations in maternal caregiving, in particular levels of licking/grooming and arched-back nursing, to demonstrate that experience with low levels of nurturing care result in offspring that have significantly more methylation of the *glucocorticoid receptor* gene promoter in the hippocampus [15]. The same phenomenon has since been found in humans with a history of childhood abuse [16, 17]. Subsequent work using the same rodent model [18–21] or paradigms where infants were separated from the caregiver [10, 22] has shown changes in other gene loci within the hippocampus, and in additional brain regions, including nuclei of the hypothalamic-pituitary-adrenal axis.

Though a handful of studies have demonstrated a relationship between the epigenetic regulation of genes within the prefrontal cortex (PFC) and memory capacity in adult animals [23–25], the relationship between the quality of infant-caregiver experiences and the epigenetic regulation of genes within the PFC has only begun to receive attention [11, 26–29], though it is a brain region known to be particularly susceptible to the damaging effects of early-life adversity [30–32]. Previous work in our laboratory has shown that maltreatment of infant rats by

caregivers during the first 7 days of life produces increased methylation of the brain-derived neurotrophic factor (*bdnf*) gene in the PFC (as a whole), an effect also associated with decreased *bdnf* mRNA levels in adulthood [11]. The PFC consists of numerous distinct regions, and recent work has now begun to show an association between early-life adversity and abnormal function of the medial PFC (mPFC) [33, 34]. The biological basis of how exposure to maltreatment early in life could contribute to long-term effects on mPFC structure and function is not known and could involve epigenetic mechanisms. To our knowledge, no studies have investigated a link between DNA methylation correlates within the mPFC and exposure to caregiver maltreatment.

Here, we used our rodent model of caregiver maltreatment and a candidate gene approach to investigate this link. The *bdnf* gene codes for a protein that is essential in development and synaptic plasticity and has been linked to several psychiatric disorders [35, 36]. *Reelin* is another gene that codes for a protein crucial for brain development and synaptic plasticity, is a gene implicated in psychiatric disorders [37–39], and a gene whose methylation patterns too can be altered by environmental factors [25, 40]. We also examined whether there were any sex-specific alterations and incorporated a lifespan approach to follow the developmental trajectory of methylation patterns.

Methods

Subjects

Male and female outbred Long-Evans rats were obtained from Harlan and housed in our breeding colony. The animals were housed in polypropylene cages with plentiful wood shavings in a temperature- and light-controlled colony room (12-hour light/dark cycle with lights on at 6.00 a.m.) with ad libitum access to food and water. All behavioral manipulations were performed during the light cycle. All females had experience in raising at least one litter prior to the beginning of the experiment so that no first-time mothers were ever used. Postnatal day (PN) 0 was designated as the day of pup birth, and on PN1, litters were culled to 5–6 males and 5–6 females. Twenty-two litters were used to generate PN8, PN30, and PN90 cohorts for tissue collection. The University of Delaware Animal Care and Use Committee approved all procedures.

Caregiving Manipulations

Using a method previously reported [11] and adapted from other studies [41–43], infant rats were divided into three equal groups on PN1 using a within-litter design. For 30 min a day beginning on PN1 and ending on PN7, up to 4 pups (2 males and 2 females) from a litter were exposed to a stressed dam outside the home cage (maltreatment condition). The maltreatment caregiver was a lactating female who was placed in a novel environment with inadequate nesting material (only 100 ml of wood shavings). Up to

4 additional pups (2 males and 2 females) from the same litter were exposed to a nonstressed dam outside of the home cage (cross-foster care condition). The cross-foster caregiver was another lactating female who was given at least 1 h to habituate to the exposure chamber and provided ample wood shavings (an approx. 2-cm layer across the chamber floor) for nesting material. Chamber temperatures were maintained between 24 and 29°C. The remaining pups from the litter (up to 2 males and 2 females) were only marked for identification and weighed, and returned to the biological mother in the home cage for a control group. After the 30-min session, experimental pups were removed from the test chamber and placed back into the home cage with the biological mother. Stimulus dams (maltreatment and cross-foster) were also reunited with their biological pups immediately after each exposure session. Except for weekly cage changes, pups remained undisturbed until PN8 (if brains were removed in infancy) or PN21–23 when they were housed in same-sex pairs through adolescence and into adulthood.

Nurturing and adverse-caregiving behaviors were scored for all 3 conditions via live observations and/or video recordings, and both 40-kHz ultrasonic (Batbox III D, NHBS Ltd., UK) and audible vocalizations emitted by the infant rats during each exposure session were scored from digital recordings. For caregiver behavior observations, nurturing and adverse-caregiving behaviors were tallied in 5-min time bends and averaged across the 7 exposure days. Ultrasonic vocalizations (only recorded 40 kHz across our 3 groups) were scored by simply tallying whether a vocalization, regardless of its duration, occurred or did not occur within each minute time bend during a 30-min session. Vocalizations were then averaged across the 7 exposure days and sessions (as we did for caregiving behaviors). Audible vocalizations were scored and summarized in the same manner.

DNA Methylation and Gene Expression Assays

Animals were sacrificed at baseline conditions, at PN8 (24 h after the last caregiving manipulation), PN30, or PN90. Brains were removed, sliced using a 1-mm brain matrix, flash-frozen on untreated slides with 2-methylbutane, and placed in a –80°C freezer until later processing. The mPFC (consisting of bilateral prelimbic and infralimbic tissue) was dissected on dry ice using stereotaxic coordinates and DNA/RNA were simultaneously extracted (Qiagen Inc., Valencia, Calif., USA). Quantification and assessment of nucleic acid quality from samples were determined using spectrophotometry (NanoDrop 2000). Methylation status was later assessed via methylation-specific real-time PCR (MSP on a Bio-Rad CFX96 system) on bisulfite-modified DNA (Qiagen Inc.). Primer sets as previously published [11, 44] targeted methylated and unmethylated CG dinucleotides in DNA associated with *bdnf* exons I and IV (encompassing each transcription start sites), the promoter region of *reelin*, or *tubulin* (as a reference gene). Reverse transcription was performed using a cDNA synthesis kit (Qiagen) on RNA, and cDNA was amplified by real-time PCR (Bio-Rad CFX96) with Taqman probes (Applied Biosystems) to target *bdnf* (exon IX), *reelin*, or *tubulin* (for a reference gene) mRNA. All reactions for each gene in the MSP and gene expression assays were run in triplicate. Product specificity was determined by melt curve analysis (MSP only) and gel electrophoresis.

Statistical Analyses

Caregiver and pup behaviors were analyzed using 1-way or 2-way ANOVAs, Bonferroni's and unpaired post hoc tests where appropriate. We used the comparative C_t method to obtain the

relative fold change of experimental (maltreatment or cross-foster) versus control (normal care) groups for MSP and gene expression assays [45]. For our MSP data, the methylation index was calculated by dividing the fold change value for the methylated primer set by the fold change value for the unmethylated primer as previously described [25]. Outliers with values 2 standard deviations from the mean were identified and removed from analyses (*bdnf* I MSP $n = 1$ for PN8, $n = 2$ for PN90; *reelin* MSP $n = 2$ for PN8, $n = 3$ for PN30, $n = 2$ for PN90; *bdnf* gene expression $n = 1$). Differences in methylation and mRNA levels were analyzed by 1-sample t tests (for comparison with normal care controls), and 2-way ANOVAs followed by post hoc tests (Bonferroni's or unpaired) where appropriate. Differences were considered to be statistically significant for $p < 0.05$, and nonsignificant trends at $p < 0.1$ are also reported.

Results

Caregiver Behaviors

To manipulate the quality of early-life caregiving experiences, infant male and female rats were exposed to maltreatment or nurturing care either outside of (cross-foster care) or within (normal care) the home cage. Caregiving conditions differed greatly across our treatment groups, as there was a main effect of caregiving behavior ($F_{1,98} = 245.8$, $p < 0.001$) and a behavior $\times$ infant condition interaction ($F_{2,98} = 173.7$, $p < 0.001$). Figure 1a shows that infants within both the normal and cross-foster care groups experienced high levels of nurturing care and low levels of adversity, which did not differ between the two conditions ($p > 0.05$). In contrast, infants within the maltreatment group experienced significant adversity and less nurturing care ($p < 0.001$). The types and percentages of infant-directed behaviors within the normal and cross-foster care conditions are illustrated in figure 1b and c, and consisted primarily of licking, grooming, nesting, and nursing. These infant-directed behaviors were less prevalent within the maltreatment condition, and instead dams predominately stepped on, dropped during transport, dragged while nipple attached, actively avoided, and roughly handled infants (fig. 1d). Analyses for each type of infant-directed behavior between the 3 conditions revealed no differences in levels of individual behaviors between the normal and cross-foster care conditions but significant differences when compared to the maltreatment condition (table 1).

Infant Responses to Caregiving Conditions

To determine infant responses to our caregiving environments, we measured audible and ultrasonic (40-kHz) vocalizations emitted during each exposure session across

Fig. 1. Differences in caregiving behaviors across treatment groups. To manipulate the early-life caregiving environment, infant male and female rats experienced nurturing care within the home cage (normal care), were exposed to nurturing care outside the home cage (cross-foster), or were exposed to caregiver maltreatment outside the homecage. **a** Behavioral observations show that there were significant differences in the amount of nurturing and ad-

verse caregiving behaviors displayed by dams across the three treatment groups. Assessment of individual behaviors experienced by infants across the three conditions revealed high levels of infant licking, grooming, and nursing in the normal (**b**) and cross-foster (**c**) care conditions but aberrant caregiving behaviors in the maltreatment (**d**) condition. $n = 18$ – 22 dams/group; error bars are SEM.

Table 1. Analyses of individual infant-directed behaviors across the three conditions

	One-way ANOVAs			p values for Bonferroni's tests		
	F	d.f.	p value	CFC vs. NMC	MAL vs. CFC	MAL vs. NMC
Step on	21.39	51	<0.001	>0.05	<0.001	<0.001
Drop	14.99	51	<0.001	>0.05	<0.001	<0.001
Drag	2.29	51	0.112	n.a.	n.a.	n.a.
Actively avoid	36.61	51	<0.001	>0.05	<0.001	<0.001
Roughly handle	27.04	51	<0.001	>0.05	<0.001	<0.001
Lick/groom	14.94	51	<0.001	>0.05	<0.001	<0.01
Crouch/nurse	19.92	51	<0.001	>0.05	<0.001	<0.001

CFC = Cross-foster care; NMC = normal care; MAL = maltreatment.

Fig. 2. Pup responses to caregiving environments. Infants in the maltreatment condition emitted significantly more audible (a) and ultrasonic (b) vocalizations compared to infants in the normal and cross-foster care conditions. * $p < 0.01$, ** $p < 0.001$; error bars represent SEM.

the 3 conditions (fig. 2). 40-kHz ultrasonic vocalizations have been previously classified as a distress call emitted by rat pups [46]. Both audible ($F_{2,40} = 7.29$, $p < 0.01$) and ultrasonic vocalizations ($F_{2,20} = 110.5$, $p < 0.001$) differed significantly across our treatment groups. While there was no difference in audible or ultrasonic vocalizations emitted between infants in the normal and cross-foster care conditions ($p > 0.05$), infants in the maltreatment condition emitted significantly more audible ($p < 0.05$ vs. normal and $p < 0.01$ vs. cross-foster care) and ultrasonic ($p < 0.001$ vs. normal and cross-foster care) vocalizations.

Methylation Patterns across Development

We used a lifespan approach and measured DNA methylation after our caregiving manipulations of two genes essential for development and plasticity, *bdnf* and

reelin. We first used MSP to characterize methylation of DNA associated with *bdnf* exons I and IV in infant (PN8), adolescent (PN30), and adult (PN90) animals (fig. 3). Though the *bdnf* gene can produce numerous *bdnf* transcripts due to its large number of exons and promoters [47], we chose to focus on DNA associated with exon I and IV, since environmentally driven changes in methylation are known to occur at these specific loci on the gene [11, 23, 44, 48, 49]. In our youngest cohort, 24 h after the last manipulation, the only difference in methylation levels detected was in females exposed to nurturing care outside their home cage, who showed an increase in methylation of DNA associated with exon IV ($t_9 = 3.17$, $p < 0.05$ vs. normal care controls, fig. 3a). A 2-way ANOVA for each *bdnf* exon across our infant groups revealed no significant main effect of infant condition, sex, or an infant $\times$ sex interaction (all p values > 0.1).

In our adolescent cohort we detected differences in methylation specific to our maltreatment group (fig. 3b). Specifically, we found increased methylation of DNA associated with *bdnf* exon I in maltreated males ($t_9 = 3.29$, $p < 0.01$ vs. normal care controls), but decreased methylation of DNA associated with exon IV in maltreated females ($t_9 = 6.65$, $p < 0.001$ vs. normal care controls). A 2-way ANOVA for each *bdnf* exon across our adolescent groups revealed no significant main effect of infant condition (all p values > 0.01), a marginally significant effect of sex at exon I ($F_{1,35} = 3.37$, $p = 0.07$), and no significant infant condition $\times$ sex interactions (all p values > 0.01).

As illustrated in figure 3c, we likewise detected methylation differences in methylation levels in maltreated adults. Both PN90 males ($t_7 = 4.167$, $p < 0.01$ vs. normal care controls) and females ($t_4 = 3.03$, $p < 0.05$ vs. normal care controls) who had been exposed to maltreatment during infancy showed decreased methylation of DNA associated with exon I. Additionally, maltreated females had increased methylation of DNA associated with exon IV ($t_5 = 2.689$, $p < 0.05$ vs. normal care controls). A 2-way ANOVA for *bdnf* exon I methylation across our adult groups showed no main effect of infant condition, sex, or an infant condition $\times$ sex interaction (all p values > 0.1). A 2-way ANOVA for *bdnf* exon IV methylation showed no main effect of infant condition or sex on methylation patterns (p values > 0.1), but revealed a significant infant condition $\times$ sex interaction ($F_{1,23} = 7.77$, $p = 0.01$). Methylation patterns of maltreated females were significantly higher than those of maltreated males ($t_{13} = 2.71$, $p < 0.05$) and cross-foster females ($t_{10} = 2.85$, $p < 0.05$).

We also used MSP to characterize methylation of DNA associated with the *reelin* promoter in infant, adolescent,

Fig. 3. Lifespan *bdnf* DNA methylation. Transient and emerging DNA methylation (MI) differences for *bdnf* exons I and IV in infant (a), adolescent (b), and adult (c) animals. Infant and adolescent cohorts, n = 10/group; adult cohort, n = 6–10/group; ^a p < 0.05, ^b p < 0.01, ^c p < 0.001 versus normal care controls; ^d p < 0.05 between sexes; ^e p < 0.05 between groups; error bars represent SEM.

and adult animals (fig. 4). In our infant cohort, maltreated females showed decreased methylation ($t_8 = 3.49$, $p < 0.01$ vs. normal care controls). A 2-way ANOVA revealed a significant main effect of infant condition in our PN8 cohort ($F_{1,33} = 6.37$, $p < 0.05$), but no main effect of sex

or an infant condition $\times$ sex interaction (p values > 0.1). Maltreated females showed marginally lower levels of methylation than cross-foster care females ($t = 1.945$, $p = 0.07$). In our adolescent cohort, there were no significant differences in *reelin* methylation compared to normal

Fig. 4. Lifespan *reelin* DNA methylation. Methylation (MI) of DNA associated with the *reelin* promoter varies by condition, age, and sex. $n = 6\text{--}10/\text{group}$; ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ versus normal care controls; ^d $p < 0.001$ between sexes; error bars represent SEM.

maternal care controls, and a 2-way ANOVA showed no effect of infant condition, sex, or an infant condition $\times$ sex interaction (all p values >0.1).

Adult *reelin* methylation patterns were also affected by infant experiences. Males exposed to nurturing care ($t_4 = 3.70$, $p < 0.05$ vs. normal care controls) or maltreatment ($t_7 = 2.18$, $p = 0.06$ vs. normal care controls) outside the home cage showed increased methylation. Females who had been exposed to nurturing care outside the home cage showed decreased methylation ($t_5 = 8.90$, $p < 0.001$ vs. normal care controls). There was no main effect of infant condition ($p > 0.05$) but a significant effect of sex ($F_{1,21} = 20.09$, $p < 0.001$) and infant condition $\times$ sex interaction ($F_{1,21} = 4.94$, $p < 0.05$) were found for adult *reelin* methylation patterns. Cross-foster females differed significantly from both cross-foster males ($t_9 = 5.37$, $p < 0.001$) and maltreated females ($t_{10} = 2.14$, $p = 0.06$) in their methylation levels.

Gene Expression in Adulthood

The last question we asked was whether DNA methylation changes present in adults coincided with alterations in gene expression at baseline conditions. For the *bdnf* gene we assessed this by measuring all *bdnf* mRNA transcripts (exon IX-containing) in the tissue samples (fig. 5a). One-sample t tests comparing maltreated or cross-foster care groups to normal care controls revealed no changes in basal *bdnf* mRNA levels (all p values >0.1). A 2-way ANOVA also revealed no significant differences in *bdnf* levels across groups based upon infant condition, sex, or an infant condition $\times$ sex interaction (all p values >0.1). At the *reelin* gene locus (fig. 5b), however, there was a trending decrease in gene expression in both males exposed to maltreatment ($t_8 = 2.07$, $p = 0.07$ vs. normal care controls)

Fig. 5. Adult gene expression. Baseline measures of adult *bdnf* (a) and *reelin* (b) mRNA levels. $n = 6\text{--}10/\text{group}$; ^a $p < 0.05$, ^b $p = 0.07$ (trend) versus normal care controls; error bars represent SEM.

and cross-foster care ($t_5 = 2.22$, $p = 0.07$ vs. normal care controls). Additionally, there was a significant decrease in *reelin* gene expression in maltreated females ($t_5 = 2.71$, $p < 0.05$ vs. normal care controls). *Reelin* levels across groups did not differ by infant condition or sex nor was there an interaction (all p values >0.1).

Discussion

The present study was designed to investigate a link between DNA methylation within the mPFC and caregiving conditions in infancy. We did this for both males and females across development after using a model where we are able to assign infants within the same litter to different, reoccurring treatments. We exposed infant rats to adversity outside of the home cage, providing a unique means of studying infant-caregiver dynamics without the confounding metabolic consequences of maternal milk or warmth deprivation. An additional strength of our model is the cross-foster and home cage control groups, which allow us to distinguish effects produced by exposure to another caregiver/caregiving environment and removal from the home cage/biological mother from those produced by caregiver maltreatment.

To produce maltreatment behaviors towards infant rats, we restricted a lactating female's nesting material while she was in a novel environment and observed high levels of aversive caregiving behaviors towards pups. These data replicate those from our previous reports [11, 42] and complement those of others demonstrating the ability of resource deprivation (within the home cage) to produce aberrant caregiving behaviors [41, 43]. We now also significantly extend our prior work here by providing the additional characterization of caregiver behavior within our normal care group (something we had not done previously) and infant vocalization behavior from the three treatment groups. We observed that both lactating females in a familiar environment with adequate nesting material (cross-foster care condition) and the biological caregivers within their home cage (normal care condition) exhibited similar high levels of nurturing care. We also observed that infants responded differentially to our three caregiving conditions, with high amounts of audible and ultrasonic distress vocalizations emitted within our adverse caregiving environment but not within the environments where pups experienced nurturing care (our normal and cross-foster groups). Ultrasonic vocalizations serve to elicit maternal behavior and are normally emitted when infants are separated from the mother or experience extreme physical stress [46, 50–52]. Since one of the physical stressors that has been established as a potentiator of infant ultrasonic vocalizations is cold exposure, we recorded infant body temperatures at the end of each 30-min exposure period for each of our conditions. We found similar temperatures across our conditions (data not shown), suggesting that a decrease in body temperature was not a leading factor underlying the high levels of ultrasonic vocalizations specific to the maltreatment condition.

Biochemical findings show that exposure to the different caregiving conditions produced a complex array of DNA methylation differences that vary between developmental time points, sexes, treatment groups, and gene locus. In regard to the *bdnf* gene, throughout development females experiencing maltreatment or nurturing care outside the home cage were observed to have more methylation changes at exon IV, while males had more methylation changes at exon I. For exon I, data show that experience-induced changes in methylation were not present in infancy but were in both adolescence and adulthood. For exon IV, while cross-fostered-females showed differences in methylation of DNA 24 h after the last manipulation but not later, maltreated females did not show differences in methylation at this locus until adolescence and adulthood. Strikingly, the directional nature of the differences in methylation at either *bdnf* locus for both maltreated males and females changed between adolescence and adulthood. While PN30 maltreated males were observed to have increased methylation associated with exon I, PN90 males instead had decreased methylation. While PN30 maltreated females were observed to have decreased methylation associated with exon IV, PN90 maltreated females had increased methylation. Our findings of differential epigenetic marking of the *bdnf* gene are consistent with other reports, where environmental stimuli and conditions have been shown to evoke a complex pattern of DNA methylation changes that vary across *bdnf* gene loci [11, 44, 53, 54]. In addition to our previous report of stress-induced changes at exons IV and IX in the PFC as a whole [11], we now demonstrate that these changes occur locally at the level of the mPFC, show that they also occur at exon I, and furthermore, show that there are divergent trajectories in regard to exon I and IV methylation differences between sexes.

Though our previous work showed no changes in *reelin* DNA methylation in the PFC as a whole in animals with a history of maltreatment or nurturing caregiving [11], our results here provide evidence of changes occurring at the level of the mPFC. Similar to our *bdnf* results, differences in *reelin* methylation likewise varied between developmental time points, sexes, and treatment conditions. While infant females experiencing maltreatment showed a decrease in methylation relative to normal care controls, their methylation levels after infancy were no longer different from controls. This is in contrast to males that experienced maltreatment, who showed no difference in methylation in infancy but instead appeared to show higher methylation levels (a trend) with maturation. In the cross-foster care condition, adult

males showed increased methylation whereas females showed significantly less than controls. The similar high methylation levels in adult males exposed to maltreatment or nurturing care outside the home cage suggest experience-induced changes reflecting commonalities between the conditions, such as exposure to a novel environment, exposure to another caretaker, and removal from the home cage and biological mother.

Although several lines of work have shown the ability of postnatal experiences to cause immediate changes in DNA methylation that persist into adulthood [11, 15, 18], our data here show that the majority of group differences at the gene loci we examined within the mPFC do not appear immediately after behavioral manipulations, but emerge over the course of development. A similar observation of late-emerging effects has been reported for the *AVP* gene in the PVN of male mice subjected to early-life stress, which was attributed to reduced MeCP2 binding (a methyl-binding domain protein that binds to methylated DNA) that was already present early in development and presumed to confer later changes in methylation [22]. Whether a similar phenomenon has occurred in our animals is not known. In addition, our data show clear sex differences that were a function of infant experience. These observations are consistent with a growing number of reports showing sex-specific differences in basal expression of chromatin-regulating enzymes, experience-induced changes in DNA methylation, and differential sensitivity to subsequent hormonal modulation [55–58]. It is likely that the basis of our sex-specific patterns reflects a number of factors, including differential stimulation of males and females by dams in the caregiving environments we created in addition to within the home cage. For example, maternal behavior is known to differ by sex of the pup, with males receiving more anogenital licking than females [59, 60].

As one way to explore the functional relevance of our mPFC DNA methylation changes, we characterized basal group differences in gene expression in our adult cohort. We did not detect any significant differences in total (exon IX) levels of *bdnf* mRNA in animals at rest. Although no baseline differences were found, it is possible that DNA methylation alterations were manifest in changes in specific *bdnf* transcript levels (i.e. exon I- or exon IV-containing mRNA transcripts), which we did not examine here. Our previous work has shown downregulation of exon-IV-containing *bdnf* transcripts in the PFC of adults who had experienced maltreatment [11].

At the *reelin* gene locus, we observed changes in mRNA levels in all groups that also showed changes in methyla-

tion of DNA associated with the *reelin* promoter. A handful of studies have demonstrated changes in hippocampal *reelin* gene expression following exposure to high levels of nurturing care or infant separation from the mother [19, 32, 61]. A study by Cassidy et al. [62] demonstrated the capacity of early-life stress in the form of social isolation (soon following weaning) to alter mPFC *reelin* protein expression and mPFC-mediated behaviors. In parallel to the developmental emergence of prepulse inhibition deficits, they found *reelin* deficits in isolated animals. Similar to our results, they found that *reelin* alterations emerged with development, as there were no group differences early in development but later, with increased protein expression at PN60 while decreased expression at PN80 in isolated rats. Group differences in methylation of the *reelin* promoter could be a driving factor in their observations, but this was not investigated. Though the capacity of prenatal stress to affect mPFC *reelin* methylation and later behavior outcomes has been established [63], to our knowledge, our study is the first to establish a link between infant-caregiver experiences and altered *reelin* gene methylation and expression within the mPFC.

In addition to causing changes in basal mRNA levels, DNA methylation alterations would likely be relevant in terms of activity-evoked regulation of these genes. For example, the ability of an adult animal to learn and form a memory has been linked with activity-evoked demethylation of *reelin* within the PFC [24] and demethylation/methylation of *bdnf* loci within the hippocampus [44, 49]. Both *reelin* and *bdnf* have recently been shown to undergo demethylation in response to the induction of long-term potentiation in the mPFC [25]. If epigenetic regulation of genes plays an active role in regulating an animal's ability to respond to its environment and experiences later in life, then altered methylation patterns of genes induced by experiences early in development have the potential to subsequently affect behavior.

Rodent models and clinical research continue to show a strong relationship between early-life stress and later behavioral abnormalities, including deficits in cognitive function, stress vulnerability, and an increase in depressive- and anxiety-like behaviors. Though studies are beginning to highlight a key role for mPFC dysfunction in aspects of these behaviors, the molecular mechanisms underlying this link are unclear. Investigations into epigenetic mechanisms in the mPFC (and the PFC in general) due to early-life stress have been few [27–29], and have focused on measurements made at one time point (for example, only in young adulthood). A lifespan approach may be necessary to fully understand the link between an

experience and outcome, as epigenetic patterns are known to naturally change over the course of the lifespan in the 'healthy' human cortex [64]. Here we provide a critical first step toward providing an understanding of DNA methylation differences at gene loci during lifespan development in an animal model with clinical relevance. Further work will be necessary to establish an epigenetic link with subsequent phenotypic outcomes, both at the cellular (mPFC neuronal dendritic length and spine density for example) and behavioral levels. Regardless of underlying mechanisms for the later-emerging and sex-specific observations we report here, our study contributes to the emerging insight of an epigenetic basis for the long-lasting and even transgenerational effects of early-life ad-

versity. Considering the remarkable complexity of alterations that can occur, more longitudinal studies that incorporate both sexes are warranted to reveal etiological information regarding stress-associated and sex-specific behavioral trajectories.

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References

- Cicchetti D, Toth SL: Child maltreatment. *Annu Rev Clin Psychol* 2005;1:409–438.
- Bremner JD, Elzinga B, Schmahl C, Vermetten E: Structural and functional plasticity of the human brain in posttraumatic stress disorder. *Prog Brain Res* 2007;167:171–186.
- Heim C, Nemeroff CB: The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biol Psychiatry* 2001;49:1023–1039.
- Neigh GN, Gillespie CF, Nemeroff CB: The neurobiological toll of child abuse and neglect. *Trauma Violence Abuse* 2009;10:389–410.
- Szyf M, McGowan P, Meaney MJ: The social environment and the epigenome. *Environ Mol Mutagen* 2008;49:46–60.
- Howell BR, Sanchez MM: Understanding behavioral effects of early life stress using the re-active scope and allostatic load models. *Dev Psychopathol* 2011;23:1001–1016.
- Ivy AS, Rex CS, Chen Y, Dubé C, Maras PM, Grigoriadis DE, Gall CM, Lynch G, Baram TZ: Hippocampal dysfunction and cognitive impairments provoked by chronic early-life stress involve excessive activation of CRH receptors. *J Neurosci* 2010;30:13005–13015.
- Landers MS, Sullivan RM: The development and neurobiology of infant attachment and fear. *Dev Neurosci* 2012;34:101–114.
- Maestripieri D, Lindell SG, Higley JD: Intergenerational transmission of maternal behavior in rhesus macaques and its underlying mechanisms. *Dev Psychobiol* 2007;49:165–171.
- Franklin TB, Russig H, Weiss IC, Gräff J, Linder N, Michalon A, Vizi S, Mansuy IM: Epigenetic transmission of the impact of early stress across generations. *Biol Psychiatry* 2010;68:408–415.
- Roth TL, Lubin FD, Funk AJ, Sweatt JD: Lasting epigenetic influence of early-life adversity on the *bdnf* gene. *Biol Psychiatry* 2009;65:760–769.
- Bagot RC, Meaney MJ: Epigenetics and the biological basis of gene × environment interactions. *J Am Acad Child Adolesc Psychiatry* 2010;49:752–771.
- Murgatroyd C, Spengler D: Epigenetics of early child development. *Front Psychiatry* 2011;2:16.
- Moore LD, Le T, Fan G: DNA methylation and its basic function. *Neuropsychopharmacology* 2013;38:23–38.
- Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ: Epigenetic programming by maternal behavior. *Nat Neurosci* 2004;7:847–854.
- McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M, Turecki G, Meaney MJ: Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 2009;12:342–348.
- Labonte B, Yerko V, Gross J, Mechawar N, Meaney MJ, Szyf M, Turecki G: Differential glucocorticoid receptor exon 1b, 1c, and 1h expression and methylation in suicide completers with a history of childhood abuse. *Biol Psychiatry* 2012;72:41–48.
- Champagne FA, Weaver IC, Diorio J, Dymov S, Szyf M, Meaney MJ: Maternal care associated with methylation of the estrogen receptor- α 1b promoter and estrogen receptor- α expression in the medial preoptic area of female offspring. *Endocrinology* 2006;147:2909–2915.
- Weaver ICG, Meaney MJ, Szyf M: Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. *Proc Natl Acad Sci USA* 2006;103:3480–3485.
- Bagot RC, Zhang T-Y, Wen X, Nguyen TTT, Nguyen H-B, Diorio J, Wong TP, Meaney MJ: Variations in postnatal maternal care and the epigenetic regulation of metabotropic glutamate receptor 1 expression and hippocampal function in the rat. *Proc Natl Acad Sci USA* 2012;109:17200–17207.
- Zhang T-Y, Hellstrom IC, Bagot RC, Wen X, Diorio J, Meaney MJ: Maternal care and DNA methylation of a glutamic acid decarboxylase 1 promoter in rat hippocampus. *J Neurosci* 2010;30:13130–13137.
- Murgatroyd C, Patchev AV, Wu Y, Micale V, Bockmuhl Y, Fischer D, Holsboer F, Wotjak CT, Almeida OF, Spengler D: Dynamic DNA methylation programs persistent adverse effects of early-life stress. *Nat Neurosci* 2009;12:1559–1566.
- Bredy TW, Wu H, Crego C, Zellhoefer J, Sun YE, Barad M: Histone modifications around individual *bdnf* gene promoters in prefrontal cortex are associated with extinction of conditioned fear. *Learn Mem* 2007;14:268–276.
- Miller CA, Gavin CF, White JA, Parrish RR, Honasoge A, Yancey CR, Rivera IM, Rubio MD, Rumbaugh G, Sweatt JD: Cortical DNA methylation maintains remote memory. *Nat Neurosci* 2010;13:664–666.
- Sui L, Wang Y, Ju LH, Chen M: Epigenetic regulation of reelin and brain-derived neurotrophic factor genes in long-term potentiation in rat medial prefrontal cortex. *Neurobiol Learn Mem* 2012;97:425–440.
- Provençal N, Suderman MJ, Guillemin C, Massart R, Ruggiero A, Wang D, Bennett AJ, Pierre PJ, Friedman DP, Côté SM, Hallett M, Tremblay RE, Suomi SJ, Szyf M: The signature of maternal rearing in the methylome in rhesus macaque prefrontal cortex and T cells. *J Neurosci* 2012;32:15626–15642.
- Mychasiuk R, Gibb R, Kolb B: Prenatal stress produces sexually dimorphic and regionally specific changes in gene expression in hippocampus and frontal cortex of developing rat offspring. *Dev Neurosci* 2011;33:531–538.

- 28 Mychasiuk R, Schmold N, Ilnytsky S, Kovalchuk O, Kolb B, Gibb R: Prenatal bystander stress alters brain, behavior, and the epigenome of developing rat offspring. *Dev Neurosci* 2011;33:159–169.
- 29 Uchida S, Hara K, Kobayashi A, Funato H, Hobara T, Otsuki K, Yamagata H, McEwen BS, Watanabe Y: Early life stress enhances behavioral vulnerability to stress through the activation of *rest4*-mediated gene transcription in the medial prefrontal cortex of rodents. *J Neurosci* 2010;30:15007–15018.
- 30 De Bellis MD, Keshavan MS, Shifflett H, Iyengar S, Beers SR, Hall J, Moritz G: Brain structures in pediatric maltreatment-related posttraumatic stress disorder: a sociodemographically matched study. *Biol Psychiatry* 2002;52:1066–1078.
- 31 Hanson JL, Chung MK, Avants BB, Shirtcliff EA, Gee JC, Davidson RJ, Pollak SD: Early stress is associated with alterations in the orbitofrontal cortex: a tensor-based morphometry investigation of brain structure and behavioral risk. *J Neurosci* 2010;30:7466–7472.
- 32 Qin L, Tu W, Sun X, Zhang J, Chen Y, Zhao H: Retardation of neurobehavioral development and *reelin* down-regulation regulated by further DNA methylation in the hippocampus of the rat pups are associated with maternal deprivation. *Behav Brain Res* 2011;217:142–147.
- 33 Bluhm RL, Williamson PC, Osuch EA, Frewen PA, Stevens TK, Boksman K, Neufeld RWJ, Théberge J, Lanius RA: Alterations in default network connectivity in posttraumatic stress disorder related to early-life trauma. *J Psychiatry Neurosci* 2009;34:187–194.
- 34 Philip NS, Sweet LH, Tyrka AR, Price LH, Bloom RF, Carpenter LL: Decreased default network connectivity is associated with early life stress in medication-free healthy adults. *Eur Neuropsychopharmacol* 2013;23:24–32.
- 35 Calabrese F, Molteni R, Racagni G, Riva MA: Neuronal plasticity: a link between stress and mood disorders. *Psychoneuroendocrinology* 2009;34:S208–S216.
- 36 Autry AE, Monteggia LM: Brain-derived neurotrophic factor and neuropsychiatric disorders. *Pharmacol Rev* 2012;64:238–258.
- 37 Grayson DR, Guidotti A: The dynamics of DNA methylation in schizophrenia and related psychiatric disorders. *Neuropsychopharmacology* 2013;38:138–166.
- 38 Roth TL, Lubin FD, Sodhi M, Kleinman JE: Epigenetic mechanisms in schizophrenia. *Biochim Biophys Acta* 2009;1790:869–877.
- 39 Tissir F, Goffinet AM: Reelin and brain development. *Nat Rev Neurosci* 2003;4:496–505.
- 40 Martínez L, Jiménez V, García-Sepúlveda C, Ceballos F, Delgado JM, Niño-Moreno P, Doniz L, Saavedra-Alanís V, Castillo CG, Santoyo ME, González-Amaro R, Jiménez-Capdeville ME: Impact of early developmental arsenic exposure on promotor CPG-island methylation of genes involved in neuronal plasticity. *Neurochem Int* 2011;58:574–581.
- 41 Ivy AS, Brunson KL, Sandman C, Baram TZ: Dysfunctional nurturing behavior in rat dams with limited access to nesting material: a clinically relevant model for early-life stress. *Neuroscience* 2008;154:1132–1142.
- 42 Roth TL, Sullivan RM: Memory of early maltreatment: neonatal behavioral and neural correlates of maternal maltreatment within the context of classical conditioning. *Biol Psychiatry* 2005;57:823–831.
- 43 Raineke C, Moriceau S, Sullivan RM: Developing a neurobehavioral animal model of infant attachment to an abusive caregiver. *Biol Psychiatry* 2010;67:1137–1145.
- 44 Lubin FD, Roth TL, Sweatt JD: Epigenetic regulation of *bdnf* gene transcription in the consolidation of fear memory. *J Neurosci* 2008;28:10576–10586.
- 45 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 2001;25:402–408.
- 46 Portfors C: Types and functions of ultrasonic vocalizations in laboratory rats and mice. *J Am Assoc Lab Anim Sci* 2007;46:28–34.
- 47 Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T: Mouse and rat *bdnf* gene structure and expression revisited. *J Neurosci Res* 2007;85:525–535.
- 48 Roth TL, Zoladz PR, Sweatt JD, Diamond DM: Epigenetic modification of hippocampal *bdnf* DNA in adult rats in an animal model of post-traumatic stress disorder. *J Psychiatr Res* 2011;45:919–926.
- 49 Mizuno K, Dempster E, Mill J, Giese KP: Long-lasting regulation of hippocampal *bdnf* gene transcription after contextual fear conditioning. *Genes Brain Behav* 2012;11:651–659.
- 50 Wöhr M, Schwarting RKW: Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat. *Behav Neurosci* 2008;122:310–330.
- 51 Hofer MA: Multiple regulators of ultrasonic vocalization in the infant rat. *Psychoneuroendocrinology* 1996;21:203–217.
- 52 Blumberg MS, Alberts JR: On the significance of similarities between ultrasonic vocalizations of infant and adult rats. *Neurosci Biobehav Rev* 1991;15:383–390.
- 53 Fuchikami M, Morinobu S, Segawa M, Okamoto Y, Yamawaki S, Ozaki N, Inoue T, Kusumi I, Koyama T, Tsuchiyama K, Terao T: DNA methylation profiles of the brain-derived neurotrophic factor (*bdnf*) gene as a potent diagnostic biomarker in major depression. *PLoS One* 2011;6:e23881.
- 54 Smith AK, Conneely KN, Kilaru V, Mercer KB, Weiss TE, Bradley B, Tang Y, Gillespie CF, Cubells JF, Ressler KJ: Differential immune system DNA methylation and cytokine regulation in post-traumatic stress disorder. *Am J Med Genet B Neuropsychiatr Genet* 2011;156:700–708.
- 55 Jessen HM, Auger AP: Sex differences in epigenetic mechanisms may underlie risk and resilience for mental health disorders. *Epigenetics* 2011;6:857–861.
- 56 Nugent B, McCarthy M: Epigenetic underpinnings of developmental sex differences in the brain. *Neuroendocrinology* 2011;93:150–158.
- 57 McCarthy MM, Arnold AP, Ball GF, Blaustein JD, De Vries GJ: Sex differences in the brain: the not so inconvenient truth. *J Neurosci* 2012;32:2241–2247.
- 58 McCarthy MM, Auger AP, Bale TL, De Vries GJ, Dunn GA, Forger NG, Murray EK, Nugent BM, Schwarz JM, Wilson ME: The epigenetics of sex differences in the brain. *J Neurosci* 2009;29:12815–12823.
- 59 Hao Y, Huang W, Nielsen DA, Kosten TA: Litter gender composition and sex affect maternal behavior and DNA methylation levels of the *oprm1* gene in rat offspring. *Front Psychiatry* 2011;2:21.
- 60 Moore CL, Wong L, Daum MC, Leclair OU: Mother-infant interactions in two strains of rats: implications for dissociating mechanism and function of a maternal pattern. *Dev Psychobiol* 1997;30:301–312.
- 61 Gross CM, Flubacher A, Tinnes S, Heyer A, Scheller M, Herpfer I, Berger M, Frotscher M, Lieb K, Haas CA: Early life stress stimulates hippocampal *reelin* gene expression in a sex-specific manner: evidence for corticosterone-mediated action. *Hippocampus* 2012;22:409–420.
- 62 Cassidy AW, Mulvany SK, Pangalos MN, Murphy KJ, Regan CM: Developmental emergence of reelin deficits in the prefrontal cortex of Wistar rats reared in social isolation. *Neuroscience* 2010;166:377–385.
- 63 Matrisciano F, Tueting P, Dalal I, Kadriu B, Grayson DR, Davis JM, Nicoletti F, Guidotti A: Epigenetic modifications of GABAergic interneurons are associated with the schizophrenia-like phenotype induced by prenatal stress in mice. *Neuropharmacology* 2013;68:184–194.
- 64 Numata S, Ye T, Hyde Thomas M, Guitart-Navarro X, Tao R, Wininger M, Colantuoni C, Weinberger DR, Kleinman JE, Lipska BK: DNA methylation signatures in development and aging of the human prefrontal cortex. *Am J Hum Genet* 2012;90:260–272.