Lasting Epigenetic Influence of Early-Life Adversity on the *BDNF* Gene

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Background: Childhood maltreatment and early trauma leave lasting imprints on neural mechanisms of cognition and emotion. With a rat model of infant maltreatment by a caregiver, we investigated whether early-life adversity leaves lasting epigenetic marks at the brain-derived neurotrophic factor (*BDNF*) gene in the central nervous system.

Methods: During the first postnatal week, we exposed infant rats to stressed caretakers that predominately displayed abusive behaviors. We then assessed DNA methylation patterns and gene expression throughout the life span as well as DNA methylation patterns in the next generation of infants.

Results: Early maltreatment produced persisting changes in methylation of *BDNF* DNA that caused altered *BDNF* gene expression in the adult prefrontal cortex. Furthermore, we observed altered *BDNF* DNA methylation in offspring of females that had previously experienced the maltreatment regimen.

Conclusions: These results highlight an epigenetic molecular mechanism potentially underlying lifelong and transgenerational perpetuation of changes in gene expression and behavior incited by early abuse and neglect.

Key Words: *BDNF*, child abuse, DNA methylation, epigenetics, hippocampus, prefrontal cortex, *Reelin*, transgenerational inheritance

Childhood abuse and neglect compromise neural structure and function, rendering an individual susceptible to later cognitive deficits (1–3) and psychiatric illnesses, including schizophrenia, major depression, and bipolar disorder (1–4). Clinical and experimental studies indicate that the prefrontal cortex (PFC) and hippocampus might play a pivotal role in the cognitive deficits and aberrant emotional behaviors originating from early-life adversity (2,4–7). Thus, it has been hypothesized that the stress-induced changes in behavior are attributable to changes in neural plasticity in these areas (5,8). Indeed, key mediators of neural plasticity in the PFC and hippocampus, such as brain-derived neurotrophic factor (BDNF) protein levels, expression of N-methyl-D-aspartate receptor subunits, and measures of synaptic long-term potentiation, are strongly affected by early adverse experiences (5,9–12).

Epigenetic modulation of gene transcription, a newly proposed substrate for regulating gene expression changes underlying neural plasticity, might likewise be affected by early-life adversity. Epigenetic modifications are most commonly regulated by either direct methylation of DNA or by posttranslational modifications of histones, both of which can either promote or repress gene transcription (13–16). In the central nervous system (CNS), DNA methylation has historically been viewed as a static process after neural development and cell differentiation; however, recent data continue to highlight the dynamic role of DNA methylation in gene regulation (17–20). Furthermore, there is growing evidence for the role of epigenetic modifications in support of adult cognition and emotional health. For example, recent work has provided support for epigenetic marking in neural and behavioral plasticity (14,21–28). With a mouse model of depression, it has been demonstrated that changes in *BDNF* gene expression after chronic adult stress are attributable to epigenetic modification of the *BDNF* gene in the hippocampus (29). Epigenetic modulation of gene transcription has also been implicated in the long-term impact of positive caregiver experiences on adult rat stress responses and maternal behavior (30). Specifically, adult patterns of DNA methylation of the glucocorticoid receptor gene in the hippocampus, which plays a pivotal role in mediating stress responses, are directly associated with the quality of maternal care received in infancy (30). Additionally, epigenetic dysregulation within the human PFC and hippocampus continues to gain support as a likely factor in the etiology of mental illness (31–32). For example, S-adenosyl methionine (a methyl donor), DNA methyltransferases, and methylation of the *Reelin* promoter are increased in schizophrenia and bipolar disorder patients (33–34).

Thus, increased susceptibility to cognitive impairments and psychiatric illnesses in adults with a history of childhood maltreatment might reflect a lasting imprint of early maltreatment on epigenetic mechanisms regulating gene expression. Indeed seminal work supporting this hypothesis demonstrates that there is increased methylation of the human ribosomal RNA (*rRNA*) gene promoter that is correlated with reduced rRNA gene expression in the hippocampus of suicide victims with a history of childhood maltreatment (35). In this study we used a rat model of infant maltreatment to assess the possibility of a lasting impact of early-life adversity on DNA methylation, as a potential transcription-regulating mechanism mediating gene expression in the PFC and hippocampus.

Methods and Materials

Subjects

We used male and female pups (Long-Evans) obtained from our breeding colony. Mothers were housed in polypropylene cages with wood shavings and kept in a temperature- (20°C) and light-controlled (12-hour light/dark cycle, lights on at 7:00 AM) environment with food and water continually available. Day of parturition was termed 0 days of age, and litters were culled to 5–6 males and 5–6 females on postnatal (PN) day 1. All behavioral manipulations and observations were performed dur-

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Received September 3, 2008; revised November 24, 2008; accepted November 25, 2008.

0006-3223/09/$36.00
doi:10.1016/j.biopsych.2008.11.028

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ing the light cycle. The University of Alabama at Birmingham Animal Care and Use Committee approved all procedures. For complete detail on methodologies, please see Supplement 1.

Maltreatment Regimen

With an adaptation of a method previously reported (36,37), rat neonates were exposed to either a stressed-abusive mother (maltreatment) or positive caregiving mother (cross-fostered care) 30 min daily during the first postnatal week (PN1–7). Additional littermates served as normal maternal care control subjects by remaining in the home cage.

DNA Methylation and Gene Expression Assays

Tissue was collected from the PFC and hippocampus in PN8, PN30, and PN90 male and female rats exposed to the maltreatment regimen. Methylation status was assessed via methylation specific real-time polymerase chain reaction (PCR) (MSP) or direct bisulfite DNA sequencing PCR (BSP) on bisulfite-modified DNA (Chemicon, Temecula, California, or Qiagen, Valencia, California) or via methylated DNA immunoprecipitation with an antibody against 5-methylcytosine (Epigentek, Brooklyn, New York). Primers were designed to target CpG sites within the promoter region of *BDNF* exon IV, downstream of a promoter region within *BDNF* exon IX, and both the promoter region and an intragenic region of *Reelin*. Real-time one-step reverse transcriptase PCR was performed with primers designed for *BDNF* exon IV and total *BDNF* messenger RNA (mRNA) (exon IX) and Taqman probes (Applied Biosystems, Foster City, California) for *Reelin* mRNA. Primer sets and the locations they amplify are listed in Supplement 2.

Drug Treatment

A stainless steel guide cannula was implanted in the left lateral ventricle of male and female adults. After the recovery period, animals were given a single daily infusion (2 μL volume, infusion rate of 1 μL/min) of zebularine (600 ng/μL in 10% dimethylsulfoxide [DMSO]) or vehicle (10% DMSO in saline) over a 7-day period. Brains were removed 24 hours after the last drug infusion to assess DNA methylation and gene expression.

Maternal Behavior in Adults Exposed to Maltreatment Paradigm

Adult females that had been exposed to maltreatment in infancy or their littermate control subjects were mated. Beginning 3 days prepartum and through 7 days postpartum, maternal behavior within the home cage was recorded. In a second set of adults that had been exposed to the maltreatment paradigm, within 12 hours of birth, the dam was removed from the cage, litters were culled to 10 pups, and 4 pups/litter were cross-fostered. On PN8, both native and cross-fostered pups (males and females) were rapidly decapitated, and the PFC and hippocampus were isolated and stored at −80°C until processing for measurement of DNA methylation.

Statistical Analysis

The relative fold change between maltreatment and control samples for MSP and gene expression assays was assessed by the comparative Ct method (38,39). Differences in methylation and mRNA levels were analyzed by two-tailed one-sample *t* tests and two-tailed unpaired *t* tests when appropriate (male and female data were collapsed after no differences were found between the genders). Differences in BSP data were analyzed by analysis of variance tests (two-way and three-way where appropriate) and either Bonferroni or Fisher least significant difference post hoc tests as indicated. Behavioral data were analyzed with two-tailed paired or unpaired *t* tests when appropriate. For all analyses, significance was set at $p \leq .05$.

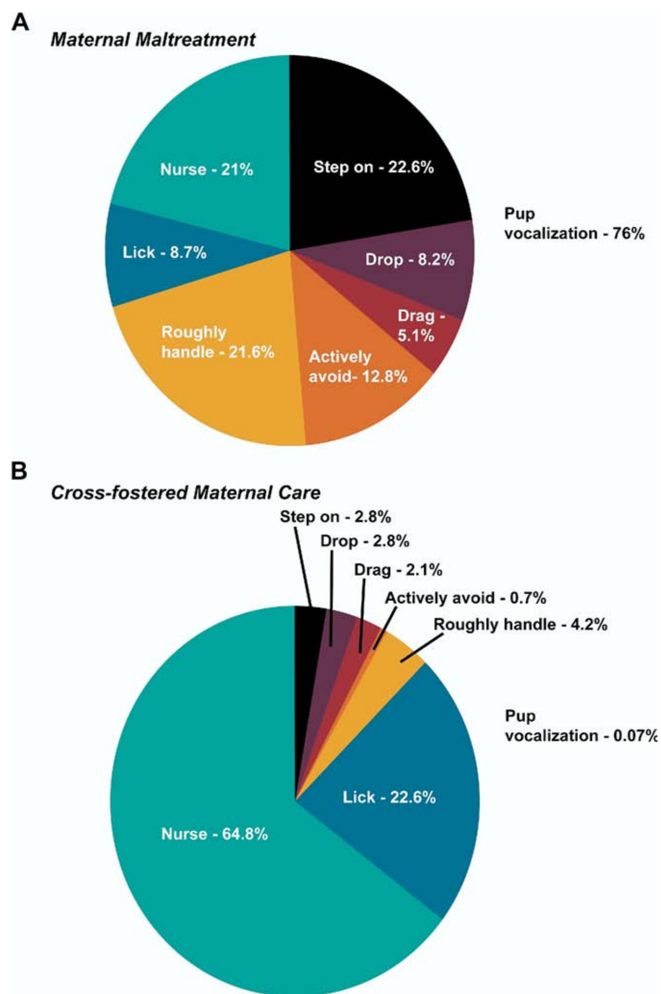

Figure 1. Infants experienced an adverse caregiving environment. **(A)** Qualitative assessment of the percent occurrence of pup-directed behaviors in the maltreatment condition indicates that pups experienced predominately abusive behaviors, which resulted in considerable audible pup vocalization. **(B)** In sharp contrast, pups experienced significant amounts of normal maternal care behaviors in the cross-fostered maternal care condition. Statistical analyses of the maternal behaviors (abusive vs. normal care) are provided in Supplement 3.

Results

To model early maltreatment, we exposed infant male and female rats to a stressed, "abusive" mother 30 min daily during the first postnatal week (maltreatment condition). Mothers were stressed by providing limited nesting resources in an unfamiliar environment. Littermate control subjects were exposed to a non-stressed, positive caregiving mother (cross-fostered care condition; mothers were provided with abundant nesting resources in a familiar environment). As illustrated in Figure 1A, neonate pups within the maltreatment condition received significant amounts of abusive maternal behaviors, thus subjecting them to the experience of an adverse caregiving environment. During the maltreatment regimen, pups were frequently stepped on, dropped during transport, dragged, actively rejected, and roughly handled. Additionally, pups were often neglected. In contrast, neonates in the cross-

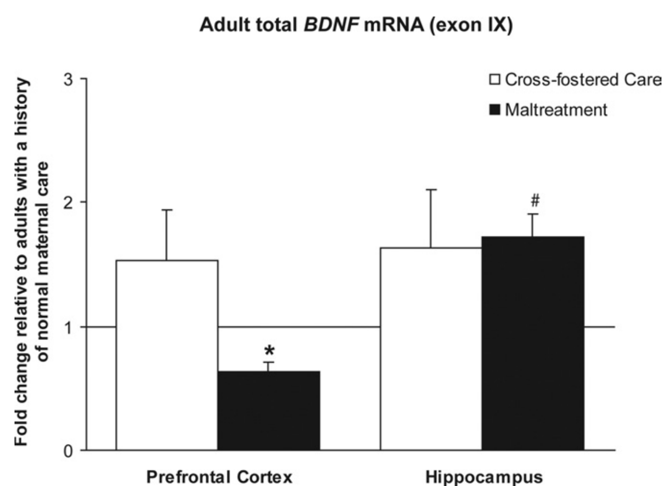

Figure 2. Maltreatment during infancy decreases brain-derived neurotrophic factor (*BDNF*) gene expression in the adult prefrontal cortex (PFC). Adult males and females exposed to maltreatment had less total *BDNF* messenger RNA (mRNA) (exon IX) in the PFC than control subjects. Adults were derived from five mothers; $n = 7$/group; *p values significant versus both normal maternal care [$t(6) = 5.17$, $p = .0021$, one-sample t test] and cross-fostered control subjects [$t(12) = 2.19$, $p = .0495$, Student two-tailed t test]. Maltreatment also increased levels of *BDNF* total mRNA in the hippocampus [$t(8) = 4.10$, #$p = .0034$, one-sample t test]; however, maltreated-adults did not differ from cross-foster care control subjects; $n = 7–9$/group. Error bars represent SEM.

fostered care condition rarely experienced behaviors classified as abusive and received significant amounts of normal and positive caregiving behaviors (retrieval to the nest, licking, nursing), thus experiencing a positive caregiving environment (Figure 1B).

Maltreatment During Infancy Decreases *BDNF* Gene Expression in the Adult PFC

It is well-documented that both prenatal and postnatal adverse experiences yield a reduction in *BDNF* mRNA expression and BDNF protein levels that persists into adulthood (5,10,11,40,41). Importantly, aberrant *BDNF* gene expression continues to be implicated in the onset of several mental illnesses subsequent to early-life adversity (42–44). However, the mechanism by which lasting alterations in *BDNF* gene expression might be triggered is unclear. Thus, we sought to determine whether early maltreatment might trigger lasting changes in *BDNF* DNA methylation as a transcription-regulating mechanism for *BDNF* gene expression changes.

We first assessed *BDNF* total mRNA levels (exon IX) within the PFC and hippocampus of adult male and female rats that were exposed to the maltreatment paradigm as neonates. Figure 2 demonstrates that in the PFC adult rats exposed to maltreatment during infancy had significant suppression of *BDNF* mRNA relative to both cross-fostered and normal maternal care control subjects. Although maltreatment produced a significant increase in mRNA levels in the hippocampus, maltreated-adults did not differ from cross-foster care control subjects (Figure 2). This suggests that the increase in the hippocampus was not exclusive to the experience of maltreatment but also reflective of other variables, such as exposure to new caretakers, experience in a novel environment, and/or removal from the biological mother and home cage.

Maltreatment During Infancy Elicits a Lasting Increase in *BDNF* DNA Methylation in the PFC

Because we found deficits in *BDNF* gene expression within the adult PFC specific to maltreatment, we next wanted to identify whether these patterns were correlated with lasting changes in *BDNF* DNA methylation, because global or site-specific methylation of CpG sites near and within regulatory regions of genes is often associated with transcriptional inactivity and gene suppression (13–16).

The *BDNF* gene contains nine 5′ non-coding exons (I-IXA), each linked to a unique promoter that differentially splices to the common 3′ coding exon IX (45,46). The activity of each non-coding promoter region dictates differential expression of *BDNF* exon-specific transcripts, providing tissue-specific and activity-dependent regulation of the *BDNF* gene across development and in adulthood (41,45–47). To assess *BDNF* DNA methylation, we evaluated the region of exon IV (formerly rat exon III) encompassing the transcription start site and cyclic adenosine monophosphate (cAMP) response element (Supplement 4), because epigenetic regulation of this particular region continues to gain support for its pivotal role in neural activity-dependent *BDNF* gene expression (22,29,45,48). We also evaluated a region of the common coding exon IX (formerly rat exon V) that encompasses a large number of CpG sites and is located downstream of a transcription start site for that exon (Supplement 4). Expression of exon IV- and IX-containing mRNA transcripts gradually increases during postnatal development, particularly within the cortex and hippocampus (47,49,50). Importantly, dynamic methylation of exon IV has been suggested to be a likely mechanism mediating *BDNF* gene expression during development and thus susceptible to environmental insults (49).

We first used MSP to screen methylation of exon IV and IX DNA in the PFC of male and female infants (PN 8), adolescents (PN30), and adults (PN90) that had been exposed to maltreatment or normal maternal care during the first postnatal week. Our MSP results demonstrate that early experiences trigger changes in *BDNF* DNA methylation within the PFC that persist into adulthood (Figure 3). Specifically, at each age examined, maltreatment produced a significant increase in methylated *BDNF* exon IX DNA and a corresponding decrease in unmethylated *BDNF* exon IX DNA in comparison with both cross-fostered and normal maternal care control subjects. Furthermore, maltreatment-induced changes in methylation of exon IV were detected in adults. Thus, adults with a history of maltreatment showed significant methylation at both exons IV and IX DNA regions. We confirmed our MSP results for the adult PFC via two approaches. First, we used direct BSP to examine site-specific methylation of 12 CpG dinucleotides within the same region of exon IV screened by MSP. The results showed significant increases in methylation across the region in adults with a history of maltreatment (Figure 4). We likewise examined site-specific methylation of 11 CpG dinucleotides within the same region of exon IX screened by MSP. The results showed an increase in methylation only at site 11 with maltreatment (Supplement 5). Second, we used a methylated DNA immunoprecipitation assay (meDIP), which confirmed the increased presence of methylated *BDNF* DNA in our adult animals that had experienced the maltreatment regimen (Supplement 6). Overall, our data indicate that there were maltreatment-induced changes in methylation of *BDNF* DNA, and these changes were maintained through adolescence and into adulthood, even though the exposure to the abusive mothers ended at PN7.

To determine whether such experiences produced similar

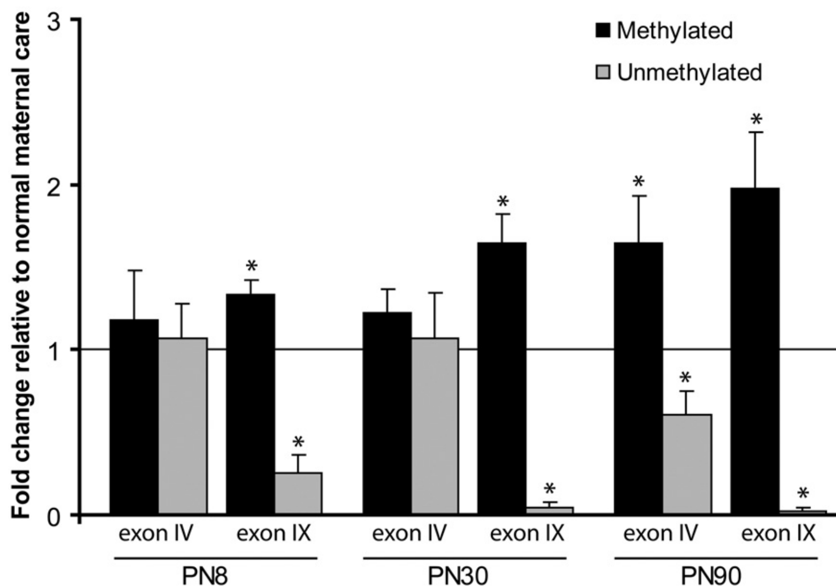

Figure 3. Maltreatment during infancy elicits methylation of *BDNF* DNA in the PFC. Methylation specific real-time polymerase chain reaction indicates that maltreatment results in methylation of *BDNF* DNA that persists into adulthood. For clarity of presentation, only the data generated in the abusive condition are illustrated. Experimental subjects (males and females) were derived from 13 mothers. $n = 4–9$/group; *p values significant versus both normal maternal care and cross-fostered care control subjects ($p < .05$). Error bars represent SEM. Other abbreviations as in Figure 2.

changes in other genes, we also examined the methylation status of *Reelin* DNA within the PFC. *Reelin* is another gene associated with neural plasticity and emotional health, and epigenetic regulation of the *Reelin* gene has been implicated in cognition and mental health (26,33,51). Although very little is known of how postnatal experiences affect *Reelin* gene regulation, what is known is that *Reelin* mRNA levels are higher in adults that as infants experienced abundant positive maternal behaviors (52). The effect of an adverse caregiving environment on transcriptional molecular mechanisms supporting *Reelin* gene expression is unknown. We first measured *Reelin* mRNA levels in the adult PFC and found no difference between adults with a history of maltreatment and those who had normal infancy (Panel A in Supplement 7). With MSP, we failed to detect changes in methylation of DNA within the examined region of the *Reelin* promoter (Panels B and C in Supplement 7). However, it is worth noting that we observed transient changes in *Reelin* DNA methylation in the cross-fostered care condition that might reflect environmental enrichment or learning of some sort (data not shown). This is in agreement with recent data that demonstrate the beneficial effect of environmental enrichment on chromatin remodeling (23).

Maltreatment-Induced Deficits in *BDNF* Gene Expression in the Adult PFC Are Rescued by Treatment with a DNA Methylation Inhibitor

Our studies support the hypothesis that experience-induced changes in adult *BDNF* gene expression could be due to changes in *BDNF* DNA methylation. To assess a causal relationship between maltreatment-induced changes in *BDNF* DNA methylation and *BDNF* mRNA expression in the adult PFC, we chronically infused zebularine, a DNA methylation inhibitor, in male and female adults from our maltreatment

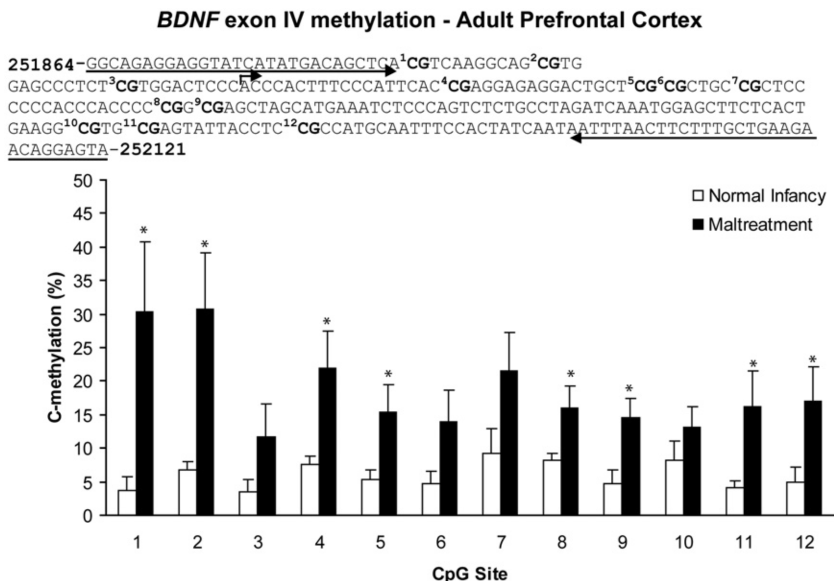

Figure 4. Methylation analysis of individual CpG dinucleotides (*BDNF* exons IV) from the PFC of adults with a history of maltreatment or normal maternal care. Top panel: Location of 12 CpG sites relative to the transcription initiation site (bent arrow) of exon IV. Note that this region of exon IV contains a cyclic adenosine monophosphate (cAMP) response element site (TCACGTCA) for transcription factor cAMP response element binding protein, which encompasses CpG site 1. Sequencing primer pair positions are indicated by the left and right arrows, and primer sequences can be found in Supplement 2. Bottom panel: Bisulfite DNA sequencing analysis indicates that maltreatment increases methylation of all CpG sites within the examined region of exon IV DNA in the adult PFC [two-way analysis of variance (ANOVA) with Bonferroni post hoc tests; significant effect of infant condition $F = 46.62$, $p < .0001$]. $n = 7–8$/group; *p values significant versus control subjects ($p \leq .05$). Error bars represent SEM. Male and female adults were derived from seven mothers. Abbreviations as in Figure 2.

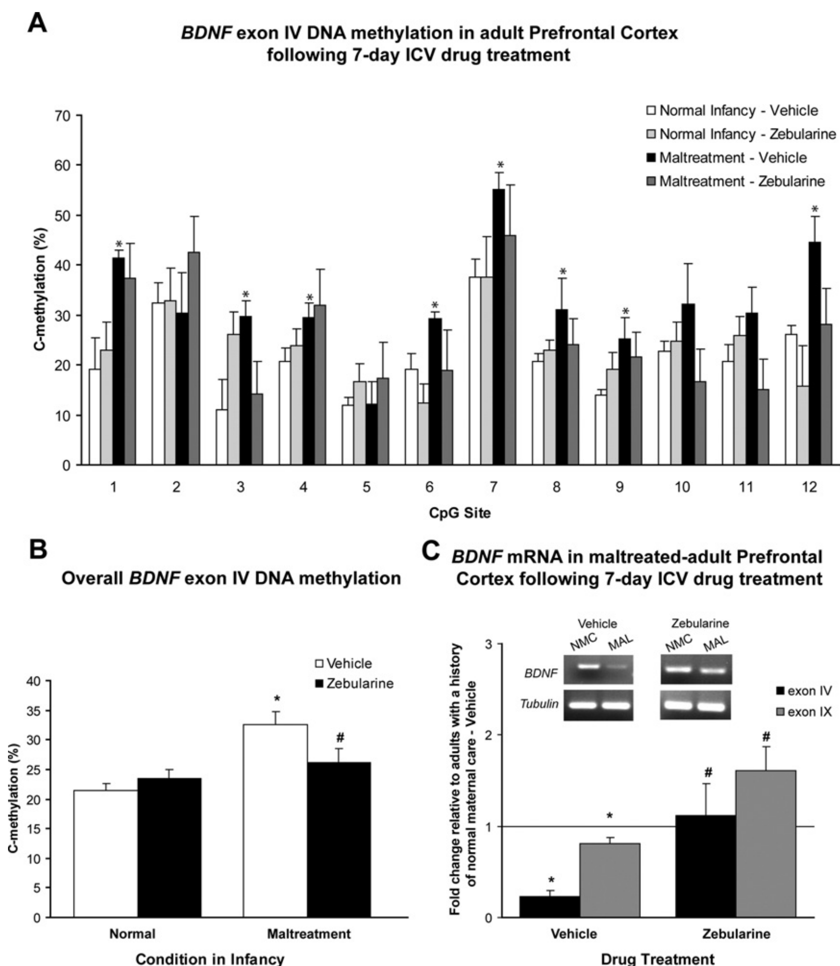

Figure 5. Infusions of a DNA methylation inhibitor in the adult rescues maltreatment-induced deficits in *BDNF* gene expression in the PFC. **(A)** The effects of zebularine treatment on methylation of *BDNF* exon IV DNA (n = 3–5/group; male and female adults were derived from three mothers). Maltreated animals infused with vehicle demonstrated significantly higher levels of methylation than control subjects, whereas zebularine treatment lowered levels of methylation in maltreated-animals [three-way ANOVA with Bonferroni post hoc tests; significant effects of infant condition $F = 18.44$, $p < .0001$, CpG region $F = 7.69$, $p < .0001$, and infant condition × drug interaction $F = 6.73$, $p = .0103$]. *p values significant versus adults with a normal infancy infused with vehicle ($p \leq .05$). **(B)** Average percentage of methylation across the examined CpG sites of exon IV in subjects from panel **A** [two-way ANOVA with Bonferroni post hoc tests; significant effect of infant condition $F = 13.54$, $p = .0003$ and infant × drug interaction $F = 4.94$, $p = .0273$]. *p value significant versus adults with normal infancy ($p < .0001$); #p value significant versus maltreated-animals infused with vehicle ($p = .05$). Maltreated-animals that received zebularine were not significantly different from normal control subjects. **(C)** The effects of zebularine treatment on mRNA (n = 4–6/group). Animals with a history of maltreatment infused with vehicle demonstrated a significant decrease in both exon IV *BDNF* mRNA and total *BDNF* mRNA (exon IX). *p values significant versus adults with a normal infancy infused with vehicle [exon IV $t(5) = 10.94$, $p = .0001$; exon IX $t(5) = 2.82$, $p = .0373$, one-sample t tests] or zebularine [exon IV $t(8) = 2.19$, $p = .06$; exon IX $t(9) = 2.29$, $p = .0474$, Student two-tailed t test]. Zebularine treatment rescued the maltreatment-induced reduction in *BDNF* mRNA expression [exon IV $t(8) = 3.05$, #$p = .0159$; exon IX $t(10) = 2.91$, #$p = .0156$, Student two-tailed t test], and animals did not differ from normal maternal care control subjects. Agarose gel images represent total *BDNF* gene expression (IX). For all panels, error bars represent SEM. ICV, intracerebroventricular.

regimen and then assessed *BDNF* exon IV DNA methylation, because exon IV is the best-characterized target of epigenetic regulation of *BDNF* gene expression (22,29,45,48). We also assessed *BDNF* exon IV mRNA levels as well as total *BDNF* mRNA levels (exon IX) after the drug treatment regimen.

The results indicate that zebularine treatment for 7 days was sufficient to decrease methylation of *BDNF* exon IV DNA and rescue both *BDNF* exon IV mRNA and total mRNA levels in adults with a history of maltreatment (Figure 5). We note that there were differences in baseline methylation between vehicle-treated groups in Figure 5A and animals in Figure 4. However, it is reasonable to ascribe these differences to any of several variables not present in the earlier experiment, including surgery for cannulation, daily handling of animals for drug infusions, and the 7-day infusion regimen. The precise mechanism of how zebularine is able to rescue aberrant methylation and gene expression in the adult CNS is not understood, but it might do so by inhibiting DNA methyltransferases through its incorporation as a cytosine analog (53) or by actively demethylating DNA in non-dividing cells through a replication-independent event, such as DNA repair (19,54–56). Nevertheless, we show that aberrant *BDNF* gene expression patterns induced by early experiences are reversible in the adult PFC by pharmacological manipulation of DNA methylation, and at least one of the *BDNF* promoters responsible for this increase in gene expression is associated with exon IV.

BDNF DNA Methylation Patterns in the PFC Incited by Maltreatment Are Perpetuated to the Next Generation

Experiences in the nest provide a learning environment that serves to program the quality of maternal behavior that will be displayed toward the next generation. For example, infant rats reared without a mother display deficits in maternal behavior toward their own offspring, and the amount of licking rat neonates receive from the mother correlate with the amount of licking they will display toward their own offspring (57–59). In addition, rhesus macaques that were abused in infancy display maladaptive behaviors toward their own offspring (60,61). One brain area that might play a pivotal role in the programming of maternal behavior is the PFC (58,62–64). Given our findings of persisting maltreatment-induced changes in *BDNF* DNA methylation and gene expression in the PFC, we hypothesized that adult females would display atypical maternal behavior toward their own offspring. As illustrated in Figure 6, females with a history of maltreatment (maltreated-females) displayed significant amounts of abusive behaviors toward their offspring, and these interactions resulted in audible pup vocalization. In the realm of normal maternal care behaviors, maltreated-females also frequently displayed low-posture nursing positions. Thus, from our behavioral assessment we found that atypical maternal behavior correlated with the aberrant *BDNF* DNA methylation and gene expression patterns that we had observed.

Because our maltreated-females showed higher abusive behaviors toward their own offspring, we next sought to determine

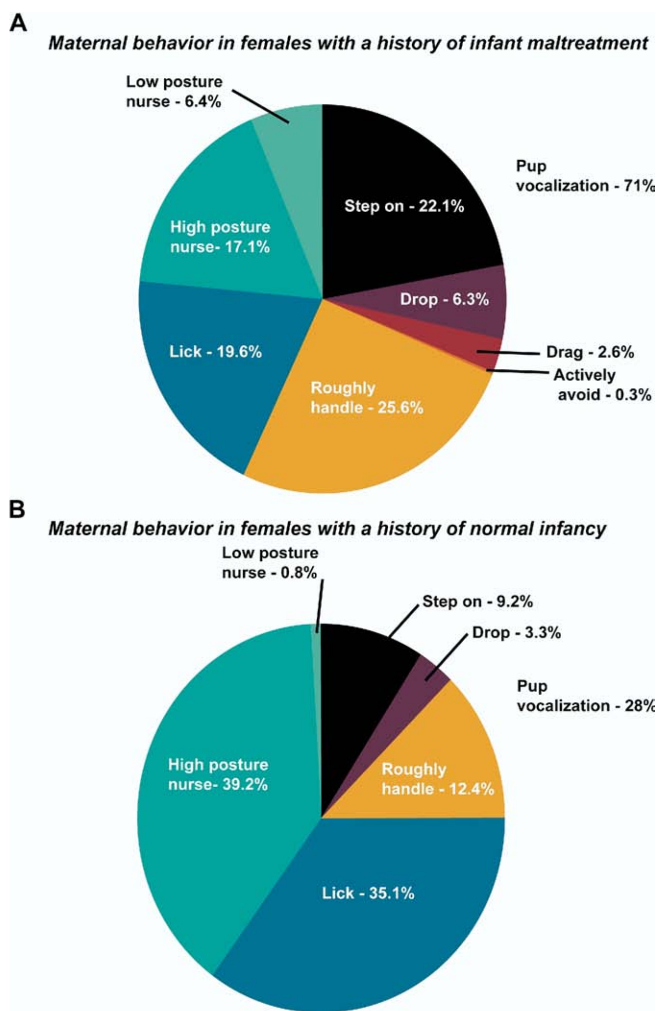

Figure 6. Females with a history of maltreatment display aberrant maternal behavior toward their own offspring. **(A)** Qualitative assessment of the percent occurrence of pup-directed behaviors in females with a history of infant maltreatment indicates that females display considerable abusive behaviors toward their first offspring. **(B)** In sharp contrast, females with a history of normal maternal care display predominately normal maternal care behaviors toward their first offspring. $n = 4$–5/group, derived from three mothers. Statistical analysis of the maternal behaviors (abusive vs. normal care) is provided in Supplement 8.

whether their offspring would also exhibit changes in methylation of *BDNF* DNA. Indeed, PN8 offspring (both males and females) derived from maltreated-females had significantly greater methylated *BDNF* DNA in their PFC and hippocampus than offspring derived from normal-treated females (Figure 7, Supplements 9 and 10). Sequencing data indicated a site-specific increase in exon IV DNA methylation within the PFC of offspring born to females with a history of maltreatment (Figure 7A). Although MSP failed to detect any changes in DNA methylation within the hippocampus (Panel A in Supplement 9), sequencing data revealed significant increases in methylation at several CpG sites within the examined region of exon IV DNA (Figures 7B and 7C). Furthermore, sequencing also revealed site-specific increases in methylation of exon IX DNA in the hippocampus (Supplement 10). Overall, these data indicate that there is increased DNA methylation in the next generation.

To evaluate whether these changes were directly attributable to the postnatal environment (i.e., the maternal behavior received), we cross-fostered offspring derived from maltreated-females to normal-treated females for the duration of the first postnatal week and then assessed methylation of *BDNF* DNA at PN8. Sequencing data again indicated site-specific effects in methylation of exon IV DNA within the infant PFC, with cross-fostering producing an increase in methylation at CpG site 1 yet a decrease at site 12 (Figure 7A). Likewise, there were site-specific effects in methylation of exon IX DNA in the hippocampus (Panel A in Supplement 10). Finally, whereas there was an overall significant decrease in methylation of exon IV DNA within the hippocampus, cross-fostered offspring still had higher levels of methylation in comparison with normal control subjects (Figures 7B and 7C; Panel B in Supplement 9). Thus surprisingly, we failed to observe a complete rescue of the altered methylation patterns with cross-fostering. These data suggest that the perpetuation of maltreatment-induced DNA methylation patterns is not simply a product of the postnatal experience.

We also cross-fostered offspring derived from normal-treated females to maltreated-females for the duration of the first postnatal week and then assessed *BDNF* DNA methylation at PN8. Although sequencing did detect significant increases at both CpG site 12 of exon IV in the PFC (Figure 7A) and CpG site 5 of exon IX in the hippocampus (Panel A in Supplement 10), overall methylation levels did not significantly change (Figures 7B and 7C; Panel C in Supplement 9, Supplement 10). These data again suggest that the perpetuation of methylation in this generation reflects a variable other than postnatal experience.

Because it is well-documented that stress experienced during gestation produces brain and behavioral alterations in offspring that are comparable to those produced by postnatal adversity (65,66), we wanted to document whether there were differences in prepartum behaviors between maltreated- and normal-treated females that might be indicative of stress and thus could influence fetal development. Our prepartum behavioral observations indicate that females with history of maltreatment displayed significantly more anxiety-related behaviors (excessive self-grooming and rearing) during this period (Figure 8). Although these are not direct measures of stress or the physiology of the prenatal environment, it is worthwhile to speculate that in addition to postnatal maltreatment pups born to females with a history of abuse were potentially subjected to prenatal stress. Together, data suggest that the transgenerational perpetuation of maltreatment-induced changes in CNS DNA methylation is likely attributable to an interplay of both prenatal and postnatal experiences, and the hormonal environment in utero.

Discussion

Here we use a rodent model of infant maltreatment from a caregiver to explore the hypothesis that epigenetic modulation of gene transcription underlies the perpetuation of changes in gene expression incited by early-life adversity. First, we show that infant maltreatment results in methylation of *BDNF* DNA through the lifespan to adulthood that dovetails reduced *BDNF* gene expression in the adult PFC. Second, we demonstrate that the altered epigenetic marks and gene expression in the adult can be rescued with chronic treatment of a DNA methylation inhibitor. Finally, we explore the perpetuation of maternal behavior and DNA methylation across a generation. We demonstrate that not only do rodents that have experienced abuse grow up and mistreat their own offspring but that their offspring also have significant DNA methylation. To summarize, our results demonstrate a surpris-

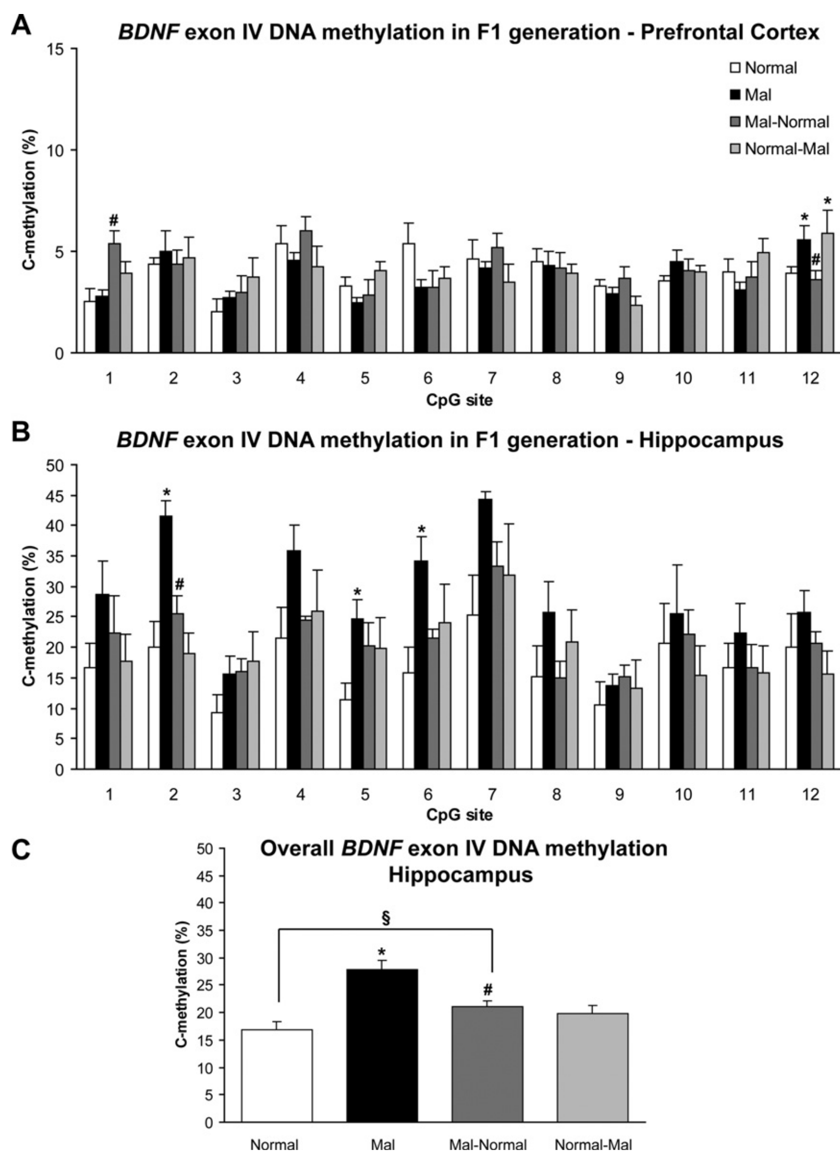

Figure 7. Direct bisulfite sequencing confirms that DNA methylation patterns incited by maltreatment are perpetuated to the next generation and that cross-fostering fails to completely rescue DNA methylation patterns. **(A)** Sequencing reveals site-specific effects of mother history on methylation of exon IV DNA in offspring's PFC [two-way ANOVA with Fisher post hoc tests; significant effect of CpG site $F = 4.02$, $p < .0001$ and marginal effect of CpG site $\times$ mother history interaction $F = 1.45$, $p = .06$]. At CpG site 12, offspring born to females with a history of maltreatment (Mal) have significant methylation in comparison with offspring from females with a history of normal infancy (*$p = .05$). Cross-fostering mal-offspring to a female with a history of normal infancy (Mal-Normal) evokes an increase in methylation at CpG site 1 (#p value significant vs. mal and normal, $p < .01$), whereas it reverses methylation at site 12 (#p value significant vs. mal, $p = .023$). Cross-fostering normal offspring to a female with a history of maltreatment (Normal-Mal) is sufficient to increase methylation at site 12 (*p value significant vs. normal, $p = .05$) **(B)** Sequencing detected significant methylation of exon IV DNA in the hippocampus of mal offspring [two-way ANOVA with Fisher post hoc tests; significant effect of CpG site $F = 5.21$, $p < .0001$ and mother history $F = 10.29$, $p < .0001$]. *p values significant versus normal, $p < .05$. Cross-fostering (Mal-Normal) significantly reversed methylation at site 2 (#p value significant vs. mal offspring, $p = .04$). **(C)** Average percentage of methylation across the examined CpG sites of exon IV in subjects from panel **B** [one-way ANOVA with Fisher post hoc tests; $F = 8.59$, $p < .0001$]. *p value significant versus normal, $p < .0001$. Although cross-fostering (Mal-Normal) was able to reduce methylation (#p value significant vs. mal, $p = .004$), methylation in these animals was still significantly higher than normal control subjects ($p = .034$). Cross-fostering normal offspring to maltreated-females did not induce significant methylation. In all panels, n = 3–8/group, derived from seven mothers; error bars represent SEM.

ing robustness to the perpetuation of changes in *BDNF* DNA methylation both within the individual across its lifespan and in passing that altered methylation from one generation to the next.

It is clear that early social experiences and experience-related changes in neural correlates of cognition and emotion play a pivotal role in transgenerational transmission of phenotype and particularly of maternal behavior. Recent support for the existence of transgenerational inheritance in mammals suggests that the epigenetic status of particular genes in the previous generation influences the next generation (57,67–72). Indeed, transmission of positive aspects of maternal behavior as well as adult stress responses seems attributable to the methylation status of the promoter of the glucocorticoid receptor gene in the hippocampus of the mother as well as the methylation status of the promoter of the estrogen receptor α in the medial preoptic area (57,67,68,72). With the results presented here, we now provide the first evidence of perpetuation of maltreatment-induced changes in DNA methylation across a generation. The impact of these inherited DNA methylation changes on gene expression and behavior in this generation is currently unknown and thus a focus of ongoing studies. Nevertheless, data highlight the possibility that epigenetic changes contribute to the cycle of maltreatment through generations as well as the perpetuation of nurturing behavior that has been previously proposed.

One striking finding from our studies was the inability of cross-fostering to completely rescue CNS DNA methylation changes that a pup acquired as a result of being born to a mother that had herself experienced neonatal maltreatment. If the changes in DNA methylation were entirely attributable to postnatal treatment and experience, one would expect cross-fostering to a normal mother to completely rescue the altered methylation, which it did not. This finding suggests that certain epigenetic marks or at minimum a predisposition to form these marks might be inherited through mechanisms involving the prenatal milieu. The importance of the quality of the prenatal environment is underscored by several studies. For example, human infants of mothers with high levels of depression and anxiety during the third trimester have increased methylation of the glucocorticoid receptor gene promoter in cord blood cells (73). Exposure to cocaine during the second and third trimesters

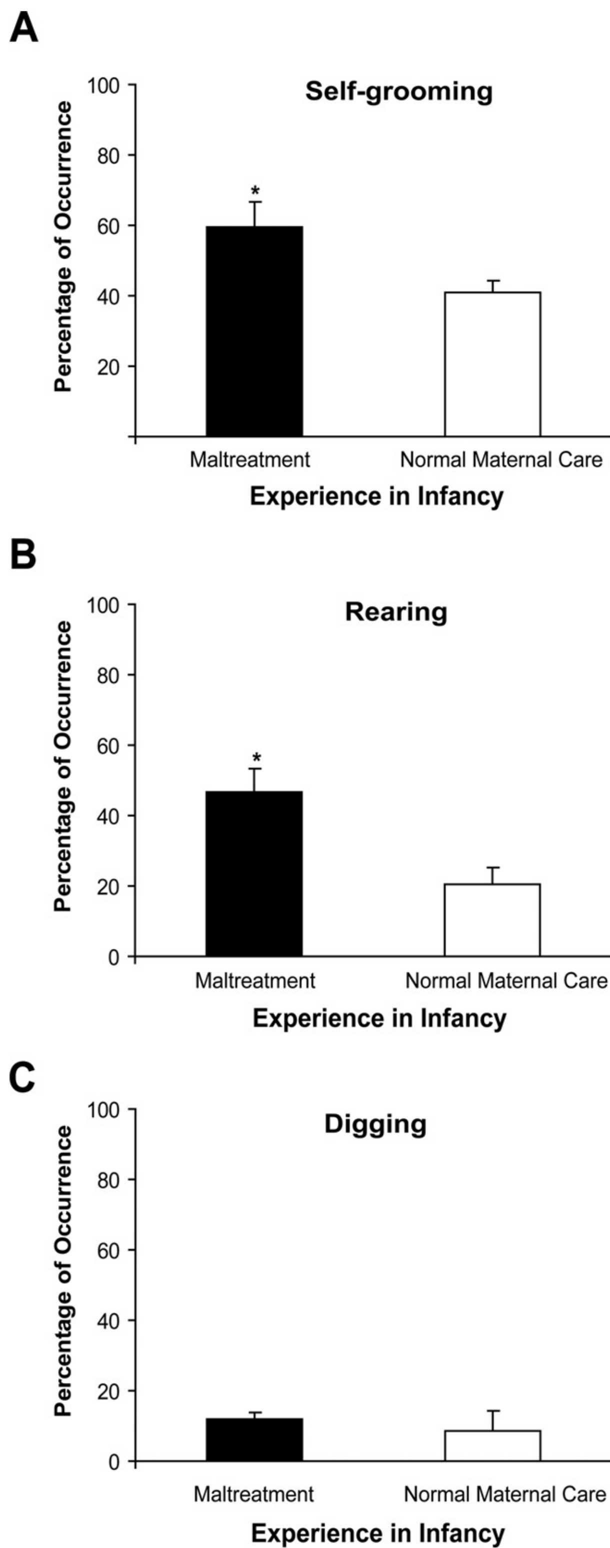

Figure 8. Females with a history of maltreatment display more anxiety-related behaviors during the prepartum period. Assessment of behaviors 3 days before giving birth indicates that females with a history of maltreatment had increased **(A)** self-grooming and **(B)** rearing behaviors than females with a normal infancy [$n = 4–5$/group; self-grooming $t(7) = 2.44$, $p = .0448$; rearing $t(6) = 3.12$, $p = .0205$; Student two-tailed t tests]. **(C)** Digging behaviors were not affected by prior experience. Error bars represent SEM.

of gestation is sufficient to induce global changes in DNA methylation in the infant hippocampus as well as changes that later emerge in the adolescent (74), and prenatal stress is sufficient to alter site-specific methylation of stress-related genes in the adult hypothalamus and amygdala (75). Still it remains to be determined what types of cells are responsible for the observed DNA methylation changes in these studies as well as in our own study.

As epigenetic mechanisms continue to be linked with neuronal plasticity and psychiatric illnesses, manipulating chromatin structure continues to gain support as a viable avenue for therapeutic intervention to restore cognitive and emotional health. This raises the intriguing speculation that such interventions, such as early exposure to complex environments (enrichment), handling, or treatment with DNA demethylases or histone deacetylase inhibitors, might prove useful as therapeutic strategies for reversing persisting effects of early-life adversity.

We thank Drs. Y. Li, T.O. Tollefsbol, M. Han, and the UAB Genomics Core Facility for their assistance in bisulfite sequencing. We thank Drs. B.R. Korf, E. Klann, J.H. Meador-Woodruff, and R.M. Sullivan for their comments and critical reading of this manuscript; and F. Hester, E. Ellis, J. Glover, and J. Tate for their technical assistance. This work was funded by Grants MH57014, NARSAD, NS048811, and the Evelyn McKnight Brain Research Foundation. The authors report no biomedical financial interests or potential conflicts of interest.

Supplementary material cited in this article is available online.

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Supplement 1 – Additional Methods

Early Experience Paradigm. On PN 1 – 7 infant rats within a litter were exposed to one of 3 conditions: 1) maltreatment; 2) cross-fostered care; and 3) normal maternal care. Maltreatment and cross-fostered care occurred outside of the home cage and within chambers that consisted of a 45.5 x 30.5 x 45 (l x w x h) cm opaque Plexiglas boxes. Chambers were lit with a red light and covered with Privacy Mirror Film to ensure behavior was not disturbed by experimenter observations (Gila, CPFilms Inc., Martinsville, VA).

Maltreatment. Neonates were exposed to maltreatment from non-biological mothers using an adaptation of a method previously reported (36-37). Limited clean aspen shavings and a 5 min habituation period prior to receiving neonates served as effective stressors in eliciting maternal maltreatment. An experimental session lasted 30 min, during which maternal and pup behaviors were recorded in 5 min intervals. Abusive maternal behaviors observed were: (1) stepping: the mother steps or jumps on the pup; (2) dropping: the mother drops a pup during retrieval or transport; (3) dragging: the mother drags a pup across the chamber; (4) pushing away/actively avoiding: the mother runs from a pup's approaches or pushes a pup away from her, crushing the infant onto the floor; and (5) rough handling: the mother aggressively grooms a pup or transports a pup by a limb. These behaviors typically elicit audible pup vocalization, which were recorded by both the observer and a video camera. Less frequently, positive maternal behaviors were observed, and included pup grooming, anogenital licking, and nursing. These behaviors do not typically elicit pup vocalization. Following

training, pups were placed in a 30 °C incubator for 15 min, and then returned to the biological mother.

Cross-fostered care and normal maternal care controls. For comparative purposes, littermates were exposed to non-biological mothers who were non-abusive, thus representing exposure to a normal and positive caregiving environment (cross-fostered maternal care). Copious shavings on the floor (2 cm layer) and a 1 hour habituation time before receiving pups were effective in evoking normal maternal behaviors. Additional littermates served as normal maternal care controls, and were only away from the home cage long enough for marking and weighing by the experimenter.

Gene Expression. RNA was extracted from the prefrontal cortex or hippocampus using Trizol (Invitrogen). For *BDNF* mRNA, real-time one-step RT-PCR was performed using iScript one-step Supermix (Bio-Rad) and primers designed for *BDNF* and *tubulin* or *ribosomal 18S* mRNA as listed in Supplement 2. For *Reelin* mRNA, real-time one-step RT-PCR was performed using commercially available reagents (QuantiTect Probe RT-PCR, Qiagen) and Taqman probes for Reelin and tubulin, beta 2B (Applied Biosystems). All primer and probes were designed to span exon boundaries, ensuring amplification of only mRNA. Equal amounts of RNA were analyzed in triplicate and normalized to *tubulin* or *18S*. Ct values were chosen within the linear range, and the comparative Ct method was used to calculate differences in gene expression between samples (38-39).

Methyl-specific quantitative real time PCR. On PN8, PN30, or PN90 brains were removed and the prefrontal cortex was isolated and stored at -80 °C. DNA was isolated and purified using a Wizard genomic DNA purification kit (Promega), and subsequently subjected to bisulfite modification (Chemicon or Qiagen). Methyl-specific primers were designed using Methprimer software (www.urogene.org/methprimer), and CpG islands for *BDNF* exon IV and exon IX and *Reelin* DNA were confirmed with CpG Island Searcher software (www.cpgislands.com). Quantitative real-time PCR was used to determine the DNA methylation status of *BDNF* and *Reelin* using either a Chromo4 or iQ5 iCycler real-time PCR detection system (Bio-Rad). PCR reactions were performed in a total volume of 20 μl, consisting of 2 μl of bisulfite modified DNA, 10 μl of iQ SYBR Green Supermix (Bio-Rad), 1 μl of primer and adjusted to final volume with DEPC-treated water. Primer sequences are listed in Supplement 2. Product specificity was verified by both a melting curve analysis and electrophoresis on a 2% agarose gel. All samples were assayed in triplicates, from at least 2 independent bisulfite treatments. Samples were normalized to either *tubulin, beta 4* or *ribosomal 18S*. Ct values were chosen within the linear range, and the comparative Ct method was used to calculate differences in methylation between samples (38-39).

Bisulfite sequencing. Bisulfite-treated samples (as described above) were amplified by primers that amplify the same region of exon IV or exon IX DNA, but independent of methylation status. The PCR products for both regions were then purified using a gel extraction kit (Qiagen), and sequenced using the reverse primer at the University of Alabama at Birmingham Genomics Core Facility of the Heflin Center for Human

Genetics (http://www.heflingenetics.uab.edu). The percent methylation of each CpG site within the region amplified was determined by the ratio between peaks values of G and A (G/[G+A]), and these levels on the electropherogram were determined using Chromas software.

Methylated DNA Immunoprecipitation. Tissue (~400 mg) from the prefrontal cortex was homogenized in buffer (10 mM KCL, 20 mM HEPES, 1 mM $MgCl_2$, 1 mM dithiothreitol, supplemented with protease inhibitors) with a Dounce homogenizer. Samples were then incubated on ice for 5 min and centrifuged at 1500 rpm for 10 min at 4 °C. The centrifuged pellet was resuspended in 0.5 ml 1 x TE with protease inhibitor. Genomic DNA was extracted by phenol and choloroform, and then precipitated with ethanol (containing 75 mM NaAcetate), and then resuspended in TE buffer (20 μg/ml). Genomic DNA was sheared by sonication (Branson Sonifier 250, 1.5 power, constant duty cycle). Immunoprecipitations (on 4 μg of DNA) were carried out at 4 °C overnight with 10 μg of monoclonal antibody against 5-methylcytosine (Epigentek), or with no antibody, or an equivalent amount of normal IgG (anti-mouse; Santa Cruz). A portion of the sonicated DNA was left untreated to serve as input control. Immune complexes were collected with protein A beads and sequentially washed 2 times with low salt buffer (20 mM Tris, pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), high salt buffer (20 mM Tris, pH 8.1, 0.1% SDS, 1% Triton X-100, 500 mM NaCl, 2 mM EDTA), LiCl immune complex buffer (0.25 M LiCl, 10 mM Tris, pH 8.1, 1% deoxycholic acid, 1% NP-40, 1 mM EDTA), and TE buffer. After proteinase K digestion (100 μg; 2 h at 37°C), DNA was extracted by phenol/chloroform/isoamyl alcohol and then ethanol-

precipitated. Immunoprecipitated DNA was subjected to quantitative real-time PCR using primers listed in Supplement 2. The cumulative fluorescence for each amplicon was normalized to input amplification.

Surgery and Adult Drug Treatment. Animals were anesthetized and placed in a stereotaxic apparatus. A stainless steel guide cannula (22 gauge, 8 mm length, Plastic One Inc., Roanoke, VA) was implanted in the left lateral ventricle (1.5 mm posterior to bregma, 2.0mm lateral to the midline, 3.0 mm ventral). Animals were allowed to recover for 5 days, after which, a single infusion was delivered daily for 7 consecutive days. On each day, animals were removed from the home cage and briefly held while an infusion cannula (28 gauge) attached to PE20 tubing was lowered into the guide. Over the course of 2 min, animals received a 2 µl volume (infusion rate of 1µl/min) of zebularine (600 ng/µl in 10% DMSO) or vehicle (10% DMSO in saline) while freely moving about the home cage. Following the infusion, a 1 min period was allowed to elapse before removing the infusion cannula.

Maternal Behavior in Adults Exposed to Early Experience Paradigm. Adult females that had been exposed to maltreatment in infancy or their littermate controls were mated and allowed to give birth. Beginning 3 days prepartum, the behavior of each dam within the home cage was recorded in 5 min intervals during a 30 min observation period between 0900 and 1700 hr. Behaviors recorded include: self-grooming, rearing (vertical movements, indicative of exploration and increased vigilance), and digging. Within 12 hours of birth, the dam was removed from the cage, and litters were culled to 10 pups.

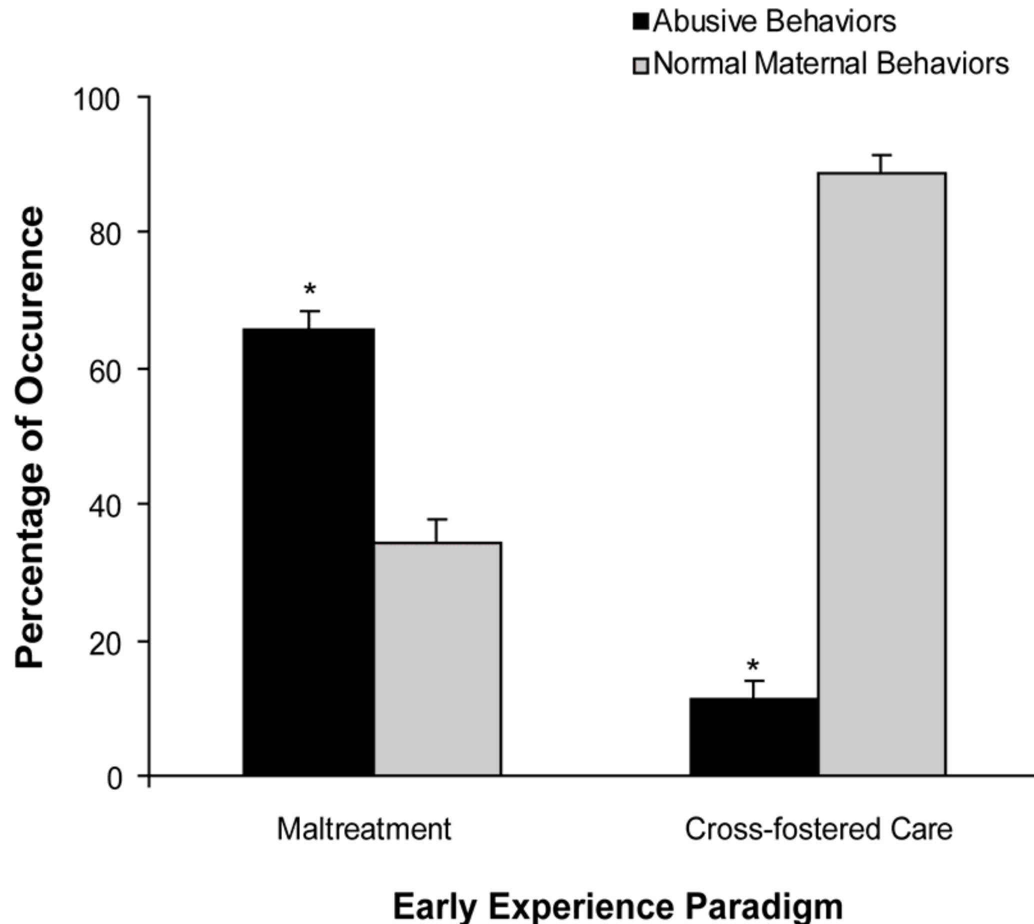
The entire procedure took less than 15 min, after which the dam was returned. Subsequent, the behavior of each dam was observed for 7 days postpartum. On each test day immediately before commencing the behavioral observation period, pups were removed from the nest, marked for identification and weighed, and then returned to the home cage opposite of the nest. During the postnatal observation periods, the following behaviors were recorded: pup retrieval and transport, pup licking and grooming, nursing position (mother lays over the pups or mother lays on either her back or her side while the pups nurse), any of the aforementioned maltreatment behaviors displayed in the maltreatment paradigm, and pup vocalization. Both prepartum and postnatal behaviors were analyzed as the percentage of behavior over the course of the observation period.

Supplement 2

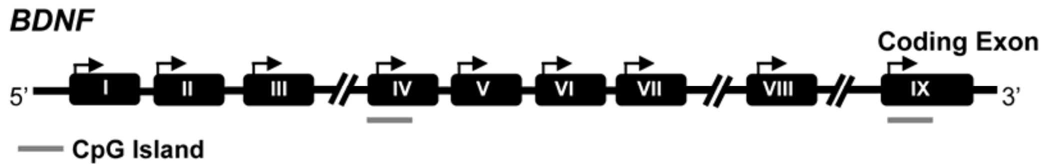
Primers used in DNA methylation and gene expression assays

Target Gene	Primer Sequence (5' – 3')
One-Step RT-PCR	
BDNF total mRNA (exon IX)	GAGAAGAGTGATGACCATCCT
	TCACGTGCTCAAAAGTGTCAG
BDNF exon IV mRNA	TGCGAGTATTACCTCCGCCAT
	TCACGTGCTCAAAAGTGTCAG
Tubulin mRNA	AGCAACATGAATGACCTGGTG
	GCTTTCCCTAACCTGCTTGG
Ribosomal 18S mRNA	CGGCTACCACATCCAAGGAA
	GCTGGAATTACCGCGGCT
Methylation specific Real-time PCR	
BDNF exon IV Methylated	GGTAGAGGAGGTATTATATGATAGTTTACG
NW_047673.1: 251864-251983	TAAATAAAAAAAACGACAACGCGAA
BDNF exon IV Unmethylated	AGGTAGAGGAGGTATTATATGATAGTTTAT
NW_047673.1: 251863-251983	TAAATAAAAAAAACAACAACACAAA
BDNF exon IX Methylated	GTGAATGGGTTTAGGGTAGGTTCG
NW_047673.1: 280779-280928	CGAAAAATATACAAATCCGCGTC
BDNF exon IX Unmethylated	GTGAATGGGTTTAGGGTAGGTTTGA
NW_047673.1: 280779-280931	ACCCAAAAAATATACAAATCCACATC
Reelin Methylated	GTCGAGGGGCGTCGTATGTATAC
NW_047687.1: 2408952-2409069	GAAATTACTTTAAACCGCGAAAACGA

Target Gene	Primer Sequence (5' – 3')
Reelin Unmethylated	GGTTGAGGGGTGTTGTATGTATATGG
NW_047687.1: 2408951-2409070	CAAAATTACTTTAAACCACAAAAACAAA
Tubulin, beta4	GGAGAGTAATATGAATGATTTGGTG
	CATCTCAACTTTCCCTAACCTACTTAA
Ribosomal 18S	TTTGTCGTTTTTTGTTTTGTTACGA
	CTAAATTCGATTCCCAACTCCG
Bisulfite DNA Sequencing	
BDNF exon IV	GGTAGAGGAGGTATTATATGATAGTTTA
NW_047673.1: 251864-252121	TACTCCTATTCTTCAACAAAAAAATTAAAT
BDNF exon IX	GTGAATGGGTTTAGGGTAGGTT
NW_047673.1: 280780-280981	CCAACAAAAAAAACAAAAAAAACTC
Methylated DNA IP Real-time PCR	
BDNF exon IX - Set 1	GACAAGGCAACTTGGCCTAC
NW_047673.1: 280727-280883	CGAACCTTCTGGTCCTCATC
BDNF exon IX - Set 2	CCTTCGAGAGGTCTGACGAC
NW_047673.1: 280760-280877	AGTCCGCGTCCTTATGGTTT



Supplement 3
Infants experienced an adverse caregiving environment. Assessment of the behaviors collapsed indicates that mothers displayed predominately abusive behaviors within the maltreatment condition, thus modeling an adverse caregiving environment. In sharp contrast, mothers in the cross-fostered care condition were rarely abusive and displayed copious amounts of normal and positive behaviors toward neonates. n=15-20/group; *$p<0.0001$, Student's two-tailed *t* tests. Error bars represent SEM.



Exon IV MSP primer locations

```
2518464-GGCAGAGGAGGTATCATATGACAGCTCACGTCAAGGCAGCGTG
GAGCCCTCTCGTGGACTCCCACCCACTTTCCCATTCACCGAGGAGAGGACTGCTCGCGCTGCCGC
TCCCCCCACCCACCCCCGGCGAGCTAGCATGAAATCTCCCAGTCTCTGCCTAGATCAAATGGAGC
TTCTCACTGAAGGCGTGCGAGTATTACCTCCGCCATGCAATTTCCACTATCAATAATTTAACTTC
TTTGCTGAAGAACAGGAGTA-252121
```

Exon IX MSP primer locations

```
              TSS
280143-CCCTCTAGCCACTACTGTTTCCACCACACATTCCTACCAAGGACTGTGCTGCTGACTTG
AAAGGAAGATTACTATTCCACTTGCAGTTGTTGCTTAGTAACATGTGTGATTGTGTTTCTGGTGACGGCG
TGAACAGAGATCATTAAAAATTAAACCCCCAGAGCTGCTAAAGTGGGAGGAAGTGGGAGCGGGGAAGCCA
CAGTGCCGTCACTTGCTTAGAAACCGTTTACTCTCAGGCTTACATGTAAATCTTGAGGAAAACACTGCAT
GTCTCTGGTTTATATTAAGCTACATACAGCACGGTAACTGACATTGATTTGTGTCCCCTGCAGGTGGAGT
GGATCAGTAAGACATTAAAAAAAAAAGAAAAAGAAAAAAAGAAAGAAAAAAAGGTCTGAAATTACAAGCAG
ATGGGCCACATGGTGTCCCCAAGAAAGTAAGGTCTAACCTGTTCTGTGTCTGTCTCTGCTTCCTTCCCAC
 *
AGTTCCACCAGGTGAGAAGAGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATACTTCGGTTGCAT
GAAGGCTGCGCCCATGAAAGAAGCAAACGTCCACGGACAAGGCAACTTGGCCTACCCAGCTGTGCGGACC
CATGGGACTCTGGAGAGCGTGAATGGGCCCAGGGCAGGTTCGAGAGGTCTGACGACGACGTCCCTGGCTG
ACACTTTTGAGCACGTGATCGAAGAGCTGCTGGATGAGGACCAGAAGGTTCGGCCCAACGAAGAAAACCA
TAAGGACGCGGACTTGTACACTTCCCGGGTGATGCTCAGCAGTC-280945
```

Supplement 4

Schematic of the *BDNF* gene, with positions of CpG islands (in grey) relative to the transcription start site (TSS, indicated by the bent arrow) of exons IV and IX. Methylated primer pair positions for each exon are indicated by the left and right arrows, and primer sequences can be found in Supplement 2.

BDNF exon IX methylation - Adult Prefrontal Cortex

```
280143-CCCTCTAGCCACTACTGTTTCCACCACACATTCCTACCAAGGACTGTGCTGCTG
ACTTGAAAGGAAGATTACTATTCCACTTGCAGTTGTTGCTTAGTAACATGTGTGATTGTGTTTCTGGTGAC
GGCGTGAACAGAGATCATTAAAAATTAAACCCCCAGAGCTGCTAAAGTGGGAGGAAGTGGGAGCGGGGAAG
CCACAGTGCCGTCACTTGCTTAGAAACCGTTTACTCTCAGGCTTACATGTAAATCTTGAGGAAAACACTGC
ATGTCTCTGGTTTATATTAAGCTACATACAGCACGGTAACTGACATTGATTTGTGTCCCCTGCAGGTGGAG
TGGATCAGTAAGACATTAAAAAAAAAAGAAAAAGAAAAAAAAGAAAGAAAAAAAGGTCTGAAATTACAAGCAG
ATGGGCCACATGGTGTCCCCAAGAAAGTAAGGTCTAACCTGTTCTGTGTCTGTCTCTGCTTCCTTCCCACA
 *
GTTCCACCAGGTGAGAAGAGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATACTTCGGTTGCATGA
AGGCTGCGCCCATGAAAGAAGCAAACGTCCACGGACAAGGCAACTTGGCCTACCCAGCTGTGCGGACCCAT
GGGACTCTGGAGAGCGTGAATGGGCCCAGGGCAGGTT¹CGAGAGGTCTGA²CGA³CGA⁴CGTCCCTGGCTGAC
ACTTTTGAGCA⁵CGTGAT⁶CGAAGAGCTGCTGGATGAGGACCAGAAGGTT⁷CGGCCCAA⁸CGAAGAAAACCAT
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CTTTCTGCTGG-280981
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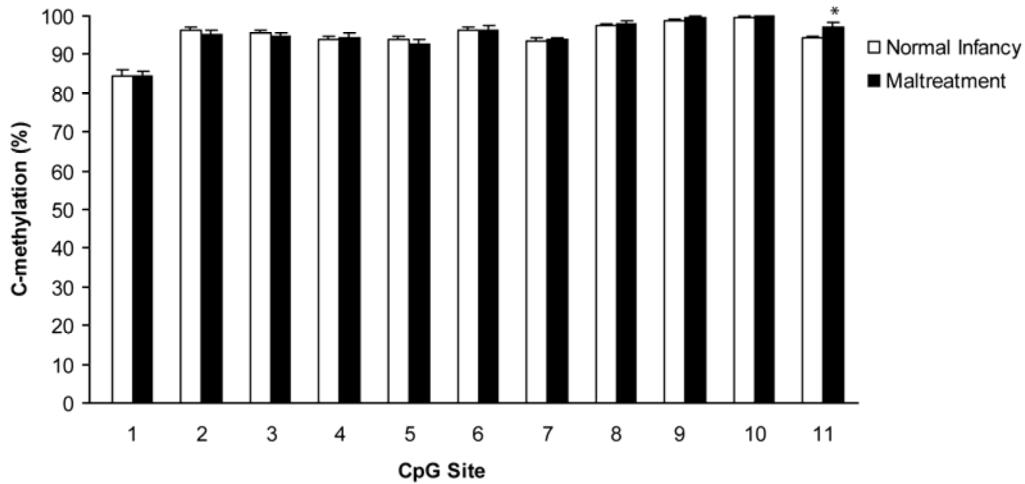
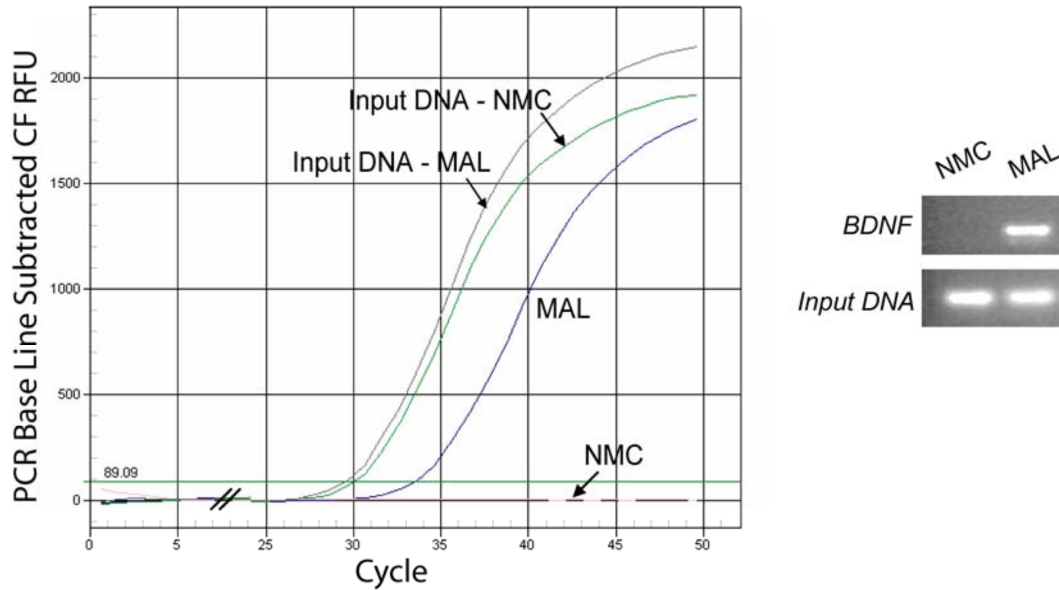


Supplement 5

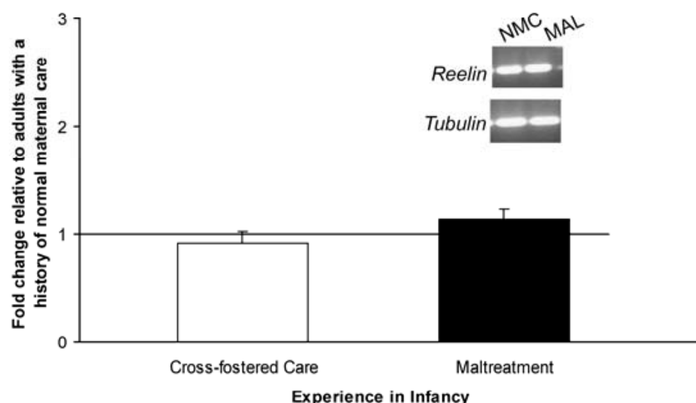
Methylation analysis of individual CpG dinucleotides of *BDNF* exon IX from the PFC of adults with a history of maltreatment or normal maternal care. Top panel - Location of 11 CpG sites within the common coding exon (IX) relative to two transcription initiation sites (bent arrows) in promoter IXA. *major splice site; sequencing primer pair positions are indicated by the left and right arrows, and primer sequences can be found in Supplement 2. Lower panel - Bisulfite DNA sequencing analysis confirms that maltreatment during infancy results in site-specific methylation of exon IX DNA in the adult prefrontal cortex (n=9-11/group; two-way ANOVA with Bonferroni's post hoc tests), with a significant increase at CpG site 11 (p=0.0161). Error bars represent SEM.

Methylated *BDNF* exon IX DNA Immunoprecipitation - Adult Prefrontal Cortex

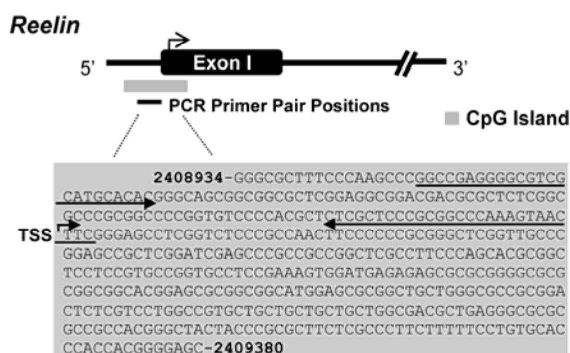


Supplement 6
Additional confirmation of persisting methylated *BDNF* DNA in the prefrontal cortex of adults with a history of maltreatment. (A) Representative quantitative real-time PCR plot and 2% agarose gel electrophoresis of the methylated DNA immunoprecipitation assay (meDIP), confirming the presence of methylated *BDNF* DNA in the adult prefrontal cortex of maltreated-animals. n=4/group; MAL=maltreatment; NMC=normal maternal care.

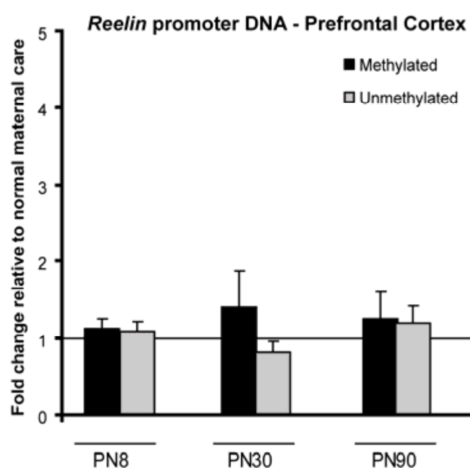
A



B

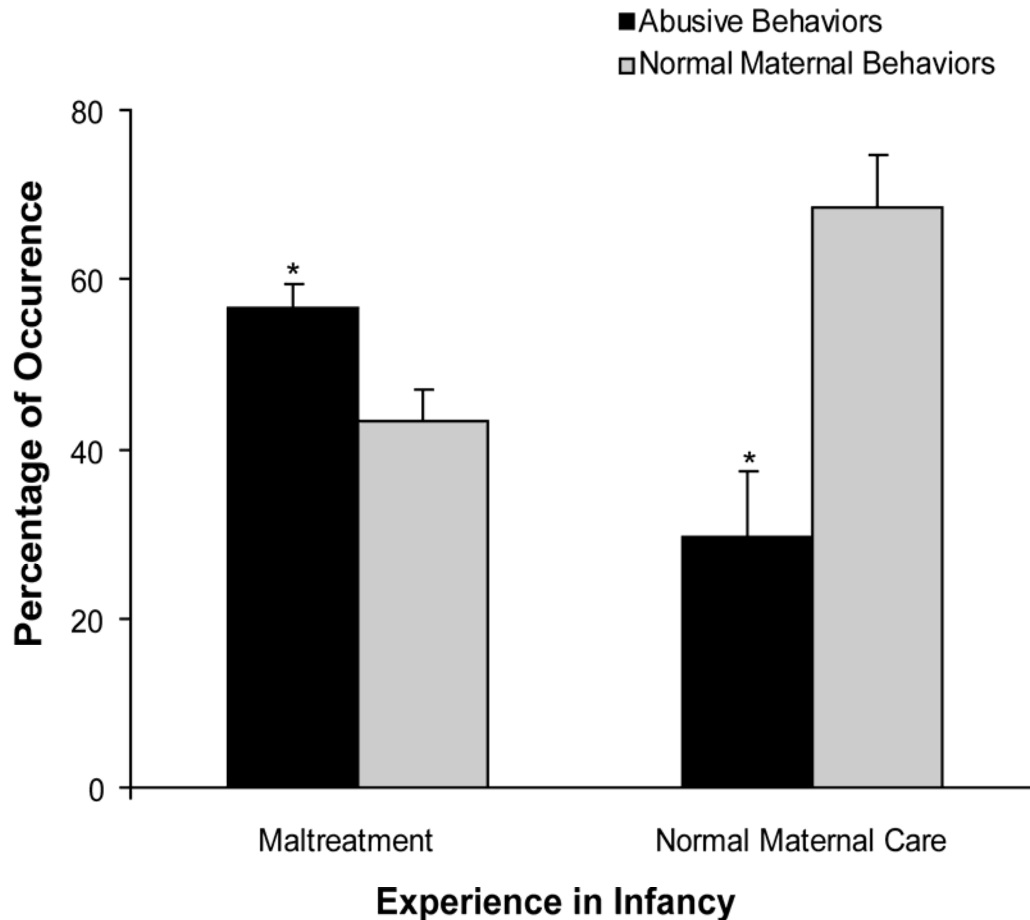


C



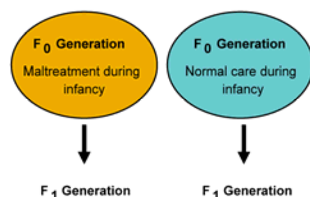
Supplement 7

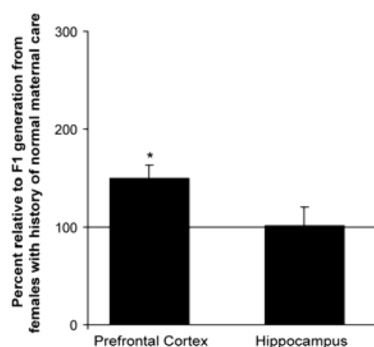
Exposure to an adverse caregiving environment had no effect on *Reelin* gene expression or methylation of *Reelin* DNA. (A) Maltreatment or cross-fostered care during infancy had no detectable effect on *Reelin* mRNA levels in the adult prefrontal cortex (n=8-10/group). Male and female adults were derived from 5 mothers. (B) Schematic of the large CpG island (in grey) relative to the transcription start site (TSS) of the *Reelin* gene (note that the entire CpG island is not represented, but only the portion relevant to the primer target region). Methylated primer pair positions are indicated by the left and right arrows, and primer sequences can be found in Supplement 2. (C) Exposure to the maltreatment condition had no observable effect on methylation of *Reelin* DNA within the prefrontal cortex. We also observed no changes in methylation of a CpG island located within an intragenic region of the *Reelin* gene (at location 2841591-2841823; data not shown). Male and female subjects were derived from 13 mothers; n=4-8/group. For panels A and C, error bars represent SEM. PN=postnatal day.



Supplement 8
Females with a history of maltreatment display deficits in maternal behavior toward their own offspring. Assessment of the behaviors collapsed indicates that mothers with a history of maltreatment display significant amounts of abusive behaviors toward their first offspring. In sharp contrast, mothers with a normal infancy display predominately normal care behaviors toward their first offspring. n=4-5/group; *$p<0.01$, Student's two-tailed *t* tests. Error bars represent SEM.

A





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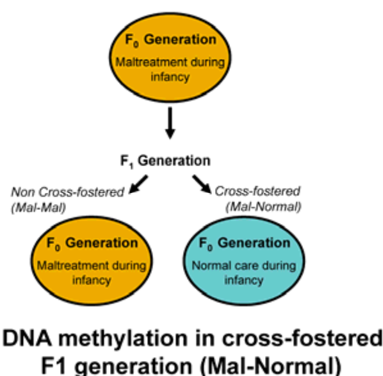
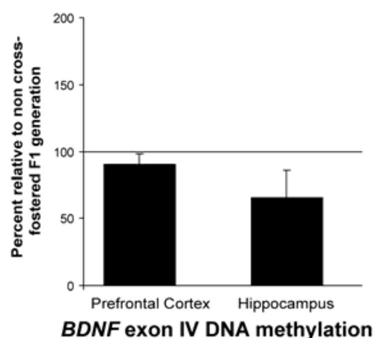
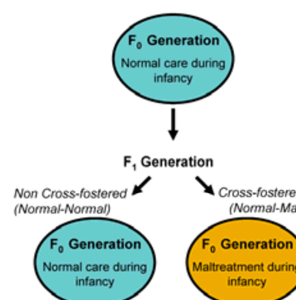
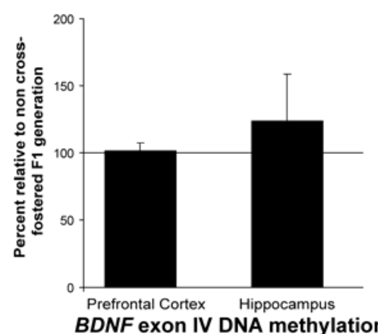




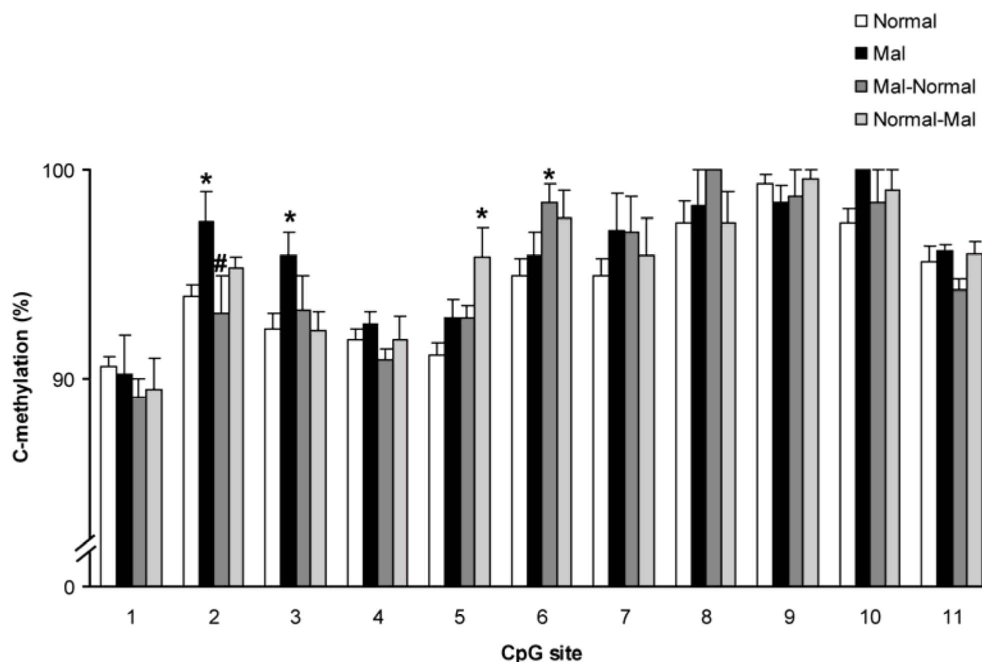
C

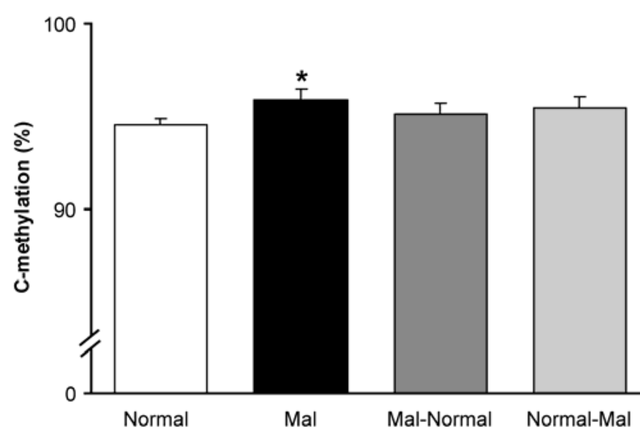




Supplement 9

BDNF DNA methylation patterns incited by maltreatment are perpetuated to the next generation. (A) Schematic of mother history of the F_1 generation. F_1 offspring (male and female) of females that had been exposed to maltreatment during infancy had significantly higher levels of methylated *BDNF* exon IV in their prefrontal cortex, as detected by methylation specific real-time PCR. n=6-8/group, derived from 7 mothers; *p-values significant versus pups born to females with no history of maltreatment (prefrontal cortex IV t_6=3.45, p=0.0136; one-sample *t* test). (B) Schematic of the cross-fostering of pups born to females with a history of maltreatment to females with a normal infancy. Cross-fostering of F_1 offspring yielded no detectable changes in methylation, although a strong trend towards a decrease in the hippocampus (methylation specific real-time PCR). n=4-8/group. (C) Schematic of the cross-fostering of pups born to females with a history of normal infancy to females with a history of maltreatment. Cross-fostering of F_1 offspring yielded no detectable changes in methylation (methylation specific real-time PCR). For all panels, error bars represent SEM.





Supplement 10
Offspring born to females with a history of maltreatment show an increase in exon IX DNA methylation in their hippocampus. (A) Bisulfite DNA sequencing indicates that offspring from females with a history of maltreatment have increased methylation at specific CpG sites within the examined region of exon IX DNA (two-way ANOVA with Fisher's post hoc tests; significant effect of CpG site $F=31.75$, $p<0.0001$ and condition $F=4.25$, $p=0.0063$). n=3-8/group; *p-values significant versus normal offspring ($p<0.05$), #p-value significant versus mal offspring ($p=0.0164$). (B) Average *BDNF* exon IX DNA methylation in subjects from panel A. *p-value significant versus normal offspring ($p=0.05$). Error bars represent SEM. Mal=maltreatment.