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Maternal Deprivation Enhances Contextual Fear Memory via Epigenetically Programming Second-Hit Stress-Induced Reelin Expression in Adult Rats

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Abstract

Background: Early-life stress increases the risk for posttraumatic stress disorder. However, the epigenetic mechanism of early-life stress-induced susceptibility to posttraumatic stress disorder in adulthood remains unclear.
Methods: Rat pups were exposed to maternal deprivation during postnatal days 1 to 14 for 3 hours daily and treated with the DNA methyltransferase inhibitor zebularine, L-methionine, or vehicle 7 days before contextual fear conditioning, which was used as a second stress and to mimic the reexperiencing symptom of posttraumatic stress disorder in adulthood. Long-term potentiation, dendritic spine density, DNA methyltransferase mRNA, Reelin gene methylation, and Reelin protein expression in the hippocampal CA1 were measured.
Results: Maternal deprivation enhanced contextual fear memory in adulthood. Meanwhile, maternal deprivation decreased DNA methyltransferase mRNA and Reelin gene methylation in the hippocampal CA1 on postnatal days 22 and 90. Reelin protein expression was increased in the hippocampal CA1 following contextual fear conditioning in adulthood. Furthermore, compared with rats that experienced maternal deprivation alone, rats also exposed to contextual fear conditioning showed an enhanced induction of hippocampal long-term potentiation and increased dendritic spine density in the hippocampal CA1 following contextual fear conditioning in adulthood. Zebularine pretreatment led to an enhancement of contextual fear memory, hypomethylation of the Reelin gene, and increased Reelin protein expression in adult rats, while L-methionine had the opposite effects.
Conclusions: Maternal deprivation can epigenetically program second-hit stress-induced Reelin expression and enhance the susceptibility to contextual fear memory in adulthood. These findings provide a new framework for understanding the cumulative stress hypothesis.

Keywords: early life stress, susceptibility, posttraumatic stress disorder, Reelin, methylation

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Significance Statement

Early-life stress (ELS) increases the risk of posttraumatic stress disorder (PTSD) in adulthood. However, few studies have been conducted that explore the epigenetic mechanism underlying ELS-generated programming effects on a second stressor in adulthood to account for PTSD susceptibility. In the present study, we provide evidence that ELS epigenetically programs Reelin gene expression and synaptic plasticity induced by a second stress and enhances the vulnerability to contextual fear memory in adulthood. By revealing the lifelong impact of ELS, these findings help to clarify the mechanism by which adverse childhood experiences lead to the development of PTSD when individuals are coping with second or multiple stressors experienced in adulthood and provide a new framework for understanding the cumulative stress hypothesis.

Introduction

Posttraumatic stress disorder (PTSD) is a common stress-related psychiatric disorder that may occur in individuals after exposure to some severe life-threatening traumatic events. Previous studies have shown that 20% of people who experience traumatic events develop PTSD, whereas some people recover from the traumatic experiences (Block and Liberzon, 2016), suggesting that the vulnerability to PTSD differs within the population. Clinical and animal studies have indicated that early-life stress (ELS) results in enhanced susceptibility to PTSD following a second trauma exposure in adulthood (Widom, 1999).

As with studies of PTSD susceptibility in the context of ELS, abundant evidence has indicated that early adversities alter the brain both structurally and functionally during development (Cowan et al., 2016). However, recent studies have proposed that neuropsychiatric diseases not only are affected by ELS but also result from 2 (or more) stressors experienced over the lifespan based on the cumulative or diathesis stress hypothesis (Nederhof and Schmidt, 2012; Mc Elroy and Hevey, 2014). As stated, this classical theory hypothesizes that ELS elevates the risk for individuals to develop stress-related diseases in response to second or multiple stressors experienced during later life by programming effects on the nervous system (Lesse et al., 2017; Peña et al., 2017). However, the exact molecular mechanisms underlying ELS-generated vulnerability to adulthood second stressors are still unclear.

Epigenetics has been increasingly recognized as an important factor in various psychiatric disorders (Ju et al., 2016). One well-studied form of epigenetic processes is DNA methylation, a covalent modification of DNA catalyzed by DNA methyltransferases (DNMTs) (Goll and Bestor, 2005). ELS has been well established to potentially cause DNA methylation alterations in several neurotransmitter genes (Alelu-Paz et al., 2015). In addition, a previous study suggested that maternal care alters DNA methylation and has long-term effects on gene expression following a second stress in adulthood (Weaver et al., 2004). Interestingly, our previous works and other studies have suggested that ELS alters DNA methylation and Reelin gene transcription in the hippocampus (Qin et al., 2011; Gross et al., 2012; Zhang et al., 2013).

Reelin is a large extracellular matrix glycoprotein that is involved in the etiopathology of several psychiatric disorders (Fatemi et al., 2000). Recently, a series of studies showed that Reelin is also crucial for synaptic plasticity, the formation of fear memory, and long-term potentiation (LTP) in the postnatal hippocampus (Weeber et al., 2002; Qiu et al., 2006; Levenson et al., 2008). Numerous sites of the Reelin gene appear to be susceptible to methylation, potentially leading to inhibition of Reelin gene expression, and are associated with memory formation and cognition (Levenson et al., 2008). The hippocampus is involved in fear memory formation and has been highlighted to play a key role in the etiology of PTSD (Alvarez et al., 2008; Acheson et al., 2012; Maren et al., 2013). The several different subfields of the hippocampus might play distinct roles in contextual fear conditioning (CFC). For example, the CA1 area has been shown to be involved in the rapid acquisition of CFC and the retrieval of contextual memory after a long time (i.e., 24 hours) as well as the consolidation process, which is specifically important for long-term contextual fear memory (Lee and Kesner, 2004; Daumas et al., 2005; Ji and Maren, 2008; Langston et al., 2010). In contrast, the dentate gyrus and CA3 areas have primarily been associated with the rapid acquisition and retrieval of contextual memory (Lee and Kesner, 2004; Daumas et al., 2005; Langston et al., 2010). Meanwhile, the CA1 area was found to be an important output structure of the hippocampal network (Lee and Kesner, 2004). Previous studies generally focused on the effects of Reelin gene methylation on resilience to PTSD after a single ELS at a young age but ignored the influence of a second stress in adulthood (Qin et al., 2011; Palacios-García et al., 2015; Ju et al., 2016). Therefore, we hypothesized that Reelin gene methylation in the adult hippocampal CA1 might be altered by ELS and involved in PTSD susceptibility after a second stress in adulthood.

The aim of this study was to clarify whether Reelin promoter methylation in the adult hippocampal CA1 induced by ELS is involved in the development of PTSD vulnerability after a second stress in adulthood. Maternal deprivation (MD), one of the most common animal models for inducing ELS (Qin et al., 2011), was used as a potential influencing factor of PTSD, while the CFC paradigm mimicked the second stress and reexperiencing symptom of PTSD in adulthood in this study.

Methods

Animals

Primiparous pregnant Sprague-Dawley rats were housed individually in standard polypropylene cages with wood-chip bedding and standard rat chow and water available ad libitum. The rats (see Figure 1 for experimental setup) were kept in a temperature-controlled room (23°C ± 1°C) on a 12-hour-light/-dark cycle (lights on at 8:00 AM). All procedures (see Figure 1 for experimental setup) were approved by the Institutional Animal Care and Use Committee of the Zhongshan School of Medicine, Sun Yat-sen University, and complied with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

MD Procedure (First Stress)

The birth day was designated as postnatal day 0 (PND 0). The MD procedure was conducted as previously described (Barreau et al., 2004). After PND 1, each pup was randomly assigned to either the MD group or mother-reared control (MRC) group. During MD (PND 1–14), all pups were removed from their home cages and

placed into an individual clean cage where the ambient temperature was controlled at 28°C ± 1°C. Then, the new cages were moved to another room for 3 hours daily (from 9:00 AM to 12:00 PM). The rat pups of the MRC group were not removed from the home cage until weaning. All rat pups were weaned on PND 22, at which time 6 same-sex rats were housed in each cage until PND 90. Only male pups were used for the experiments. Rats were weighed on PND 22 and 90.

CFC Procedure (Second Stress)

CFC was conducted on PND 90 and served as the second stressor. All rats were allowed to habituate for 5 minutes to the conditioning chamber on day 1. On day 2, the animals were placed in the conditioning chamber (conditioned stimulus) and received 3 footshocks (0.6 mA, 2 seconds) at 1-minute intervals after a 2-minute acclimation period in the chamber. All rats were retained for another 1 minute in the chamber after the last footshook. After conditioning, rats were placed in their home cages and returned to the rearing room. Twenty-four hours after fear conditioning, all the conditioned rats were returned to the chamber for 5 minutes to test for long-term contextual fear.

Cannula Implantation

For stereotaxic surgery, rats were anesthetized with 2% pentobarbital sodium. Anesthetized rats were secured on a stereotaxic frame (68001; RWD Life Science, Shenzhen, China). Cannulas (62001; RWD Life Science) were bilaterally implanted in the CA1 region of the dorsal hippocampus (AP: -3.8 mm relative to bregma; ML: ±2.5 mm; DV: -3.5 mm from skull) (Miller and Sweatt, 2007). Rats were housed individually for 7 days for postoperative recovery before drug infusion. Accurate cannula placement was confirmed by postmortem histological analysis.

Drugs

Zebularine (Sigma) was dissolved in 10% DMSO and diluted to a concentration of 600 ng/mL in 0.9% sterile saline (Roth et al., 2009). L-methionine (Sigma) was dissolved and diluted to a concentration of 100 μg/mL in 0.9% sterile saline (Weaver et al., 2005).

Intra-CA1 Infusion

After 7 days of postoperative recovery as described above, MRC rats received a single infusion every day for 7 consecutive days before CFC. A total 2 μL of each drug was bilaterally infused at a rate of 0.5 μL/min for 2 minutes. Infusion cannulas were left in place for 3 minutes after the infusion to allow for diffusion of the drug.

Isolation of the CA1 Area

On PND 22, PND 90, and after completion of CFC, rats were killed and brains were rapidly removed. The CA1 was dissected from the other hippocampal subfields under a dissecting microscope, immediately frozen in liquid nitrogen, and stored at -80°C (Miller and Sweatt, 2007).

Real-Time Reverse Transcription Quantitative PCR

RNA extraction was conducted on CA1 tissue from rats that were killed on PND 22. RNA was extracted using Trizol (Invitrogen) and further purified with $CHCl_3$. The concentrations of the RNA samples were determined spectrophotometrically, and reverse transcription (RT)-PCR was performed with commercially available reagents (AORT-0060, GeneCopoeia) following the manufacturer's protocol. DNMT mRNA levels were measured by quantitative PCR (qPCR) using commercially available super SYBR Green PCR Mix (QP002, GeneCopoeia) and primers for DNMT1, DNMT3A, DNMT3B, and β-actin (RQP051580, RQP044961, RQP044963, RQP051050, GeneCopoeia). All primers were designed to span exon boundaries, ensuring amplification of only mRNA. Relative gene expression comparisons were carried out using β-actin as an invariant endogenous control. qPCRs and comparative Ct method were conducted according to previous protocols (Livak and Schmittgen, 2001; Pfaffl, 2001; Miller and Sweatt, 2007).

DNA Methylation Assay

DNA was extracted from the CA1 of the brain tissue from rats killed on PND 22 and 90. Genomic DNA was extracted and purified by using the standardized phenol-chloroform extraction method. Purified DNA was processed for bisulfite modification enabling high cytosine conversion rates of >99% (EpTect Bisulfite Kit, QIAGEN). The DNA methylation of the Reelin gene was determined using quantitative real-time PCR. The unmethylated Reelin DNA was detected using the following primers: forward (5'-TGTTAAATTTTTGTAGTATTGGGGATGT-3') and reverse (5'-TCCTTAAAATAATCCAACAACACACC-3'). Methylated Reelin DNA was detected using the following primers: forward (5'-GGTGTTAAATTTTTGTAGTATTGGGGAC-3') and reverse (5'-TCCTTAAAATAAT CCAACAACACGC-3') (Miller and Sweatt, 2007). qPCRs and methylation index (%) equation were conducted according to previous protocols (Miller and Sweatt, 2007; Toda et al., 2014).

Western Blotting

Total protein extracted from the CA1 area of rat brain tissue was subjected to SDS-PAGE on 8% separating gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% nonfat milk and then separately incubated with a 1:500 anti-Reelin monoclonal antibody (MAB5364, Millipore) and 1:1000 anti-β-actin antibody at 4°C overnight. Then the membranes were washed and incubated with a corresponding secondary antibody for 2 hours at room temperature. Reelin blots were normalized to actin blots for each individual lane. Optical densities of the individual bands were visualized using ImageJ for Windows.

Golgi-Cox Impregnation

Animals were anesthetized with 2% pentobarbital sodium and transcardially infused with 0.9% saline solution. Brains were rapidly removed. The whole-brain impregnation procedure was conducted using a kit (GMS80020.2. vA, Genmed Scientifics Inc.) as described in the manufacturer's protocol, and the brain was then sectioned at a thickness of 180 μm on a vibratome (VT 1000S; Leica). The sections were stained, dehydrated, and coverslipped with mounting medium. The dendritic spines in the CA1 of the hippocampus were analyzed by counting the tertiary apical dendrites using a 100× oil microscope (BX51). Spine density was normalized as counted spines per 10 μm of dendrite length. Image acquisition and quantitative analysis method were conducted according to the previous protocols (Li et al., 2004; Chen et al., 2018).

Electrophysiological Recordings

Adult male animals were anesthetized with pentobarbital sodium and decapitated on PND 90 or after CFC. Brains were immediately removed and placed in oxygenated (95% O_2/5% CO_2) ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 120 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 2.0 $CaCl_2$, 2.0 $MgSO_4$, 26 $NaHCO_3$, and 10 glucose. Coronal hippocampal slices (360 μm) were prepared using a vibratome (VT 1000S, Leica). After the tissue was sectioned, hippocampal slices were allowed to recover in oxygenated ACSF at 30°C ±1°C for at least 90 minutes. The slices were gently placed into a recording chamber that was superfused (a flow rate of 4–5 mL/min) with oxygenated ACSF and maintained at 30°C. Field excitatory synaptic potentials (fEPSPs) were evoked with a 2-concentric bipolar stimulating electrode and recorded through glass pipettes (3–8 M, filled with ACSF) using Axon Multiclamp 1500A (Molecular Devices). A stimulating electrode evoked fEPSPs from the Schaffer collateral pathway at 0.05 Hz, and fEPSP responses at half of the maximum response were recorded in the stratum radiatum of the CA1. LTP was induced with high-frequency stimulation (HFS; 100 pulses at 100 Hz, 4 trains with 20-second intertrain intervals), and the average slope of 6 recording sweeps (across 2 minutes) was plotted. The magnitude of LTP was measured by the average slope of the recordings over the last 30 minutes (90 sweeps). Each group consisted of 4 to 5 slices obtained from 4 rats.

Statistical Analysis

SPSS Statistics version 19 was used for statistical analysis. Numerical data were analyzed by an unpaired *t* test, multiple *t* tests, ANOVA for repeated measures, 1-way ANOVA, or 2-way ANOVA followed by posthoc LSD test or posthoc Tukey test. In all cases, the significance level was set at $P<.05$. All data are shown as the means ± SEM.

Results

Contextual Fear Memory Enhancement in Adulthood Was Induced by MD

To determine whether MD affected the formation of fear memory in adulthood, a CFC paradigm, which is related to the functions of the hippocampal CA1, was performed. We found no significant difference in freezing level between the MRC+CFC and MD+CFC groups during fear conditioning training ($F_{(1, 84)}=0.02$, $P=.88$) (Figure 2A), but the rats in the MD+CFC group showed a significantly higher level of freezing than those in the MRC+CFC group 24 hours posttraining ($t_{(15)}=3.00$, $P<.05$) (Figure 2B), suggesting that MD enhanced contextual fear memory.

Locomotor activity, anxiety-like behavior in a novel environment, and nociception were examined to ascertain whether CFC was influenced by baseline behaviors and perception of a footshock, which might be affected by MD. We found no significant difference between MRC and MD rat pups in body weight ($t_{(20)}=0.98$, $P=.34$) (supplementary Figure 1A) or forelimb grasp reflex on PND 90 ($t_{(10)}=0.64$, $P=.54$) (supplementary Figure 1B). In the open field test, the MD and MRC rats showed no significant differences in total distance ($t_{(10)}=0.22$, $P=.83$) (supplementary Figure 1C) or percentage of the distance in the center zone ($t_{(10)}=0.04$, $P=.98$) (supplementary Figure 1D). Furthermore, in the elevated plus maze, no significant effect of MD was found on the percent of entries in the open arm ($t_{(16)}=1.13$, $P=.28$) or closed arm ($t_{(16)}=0.81$, $P=.43$) (supplementary Figure 1E) or the percent of time spent in open arm ($t_{(16)}=0.67$, $P=.51$) or closed arm ($t_{(16)}=0.46$, $P=.65$) (supplementary Figure 1F). Finally, there was no significant difference found in the withdrawal thresholds between groups ($F_{(1, 32)}=0.05$, $P=.83$) (supplementary Figure 1G). These results suggested that MD treatment had no obvious effect on somatic development, locomotion, habituation, thigmotaxis, anxiety-like behavior, or nociception in adulthood.

Hypomethylation of the Reelin Gene in the Hippocampal CA1 in Adulthood Was Induced by MD

Our previous works have shown that ELS alters DNA methylation of the Reelin gene in the hippocampus on PND 22 (Qin et al., 2011). Therefore, we first investigated whether the expression of 3 DNMT isoforms, which catalyze DNA methylation (Levenson et al., 2008), in the CA1 area were altered by MD on PND 22. MD rats showed lower DNMT1, DNMT3A, and DNMT3B mRNA expression in the CA1 area than MRC rats (DNMT1: $t_{(6)}=5.82$, $P<.05$; DNMT3A: $t_{(6)}=2.54$, $P<.05$; DNMT3B: $t_{(6)}=2.93$, $P<.05$) (Figure 3B).

To evaluate whether the MD-induced hypomethylation of the Reelin gene was a long-lasting epigenetic mark in adulthood, its expression in the CA1 was examined on PND 22 and PND 90. We found that MD rats showed an obvious reduction in Reelin gene methylation on PND 22 ($t_{(10)}=2.44$, $P<.05$) (Figure 3C) and PND 90 ($t_{(10)}=5.01$, $P<.001$) (Figure 3D) compared with MRC rats. As the epigenetic changes are potentially associated with gene expression, we further examined whether the decreased Reelin gene methylation affected gene expression following CFC. We found a significantly greater level of Reelin protein in the MD+CFC group than in the MRC+CFC group 24 hours posttraining ($t_{(8)}=2.80$, $P<.05$) (Figure 3E). These results showed that MD promoted the hypomethylation of the Reelin gene, which subsequently upregulated the expression of Reelin protein in the hippocampal CA1 area following CFC in adulthood.

Hypomethylation and Hypermethylation of the Reelin Gene Showed Opposite Effects on Contextual Fear Memory in Adulthood

To determine whether Reelin gene DNA methylation was critically involved in contextual fear memory formation in

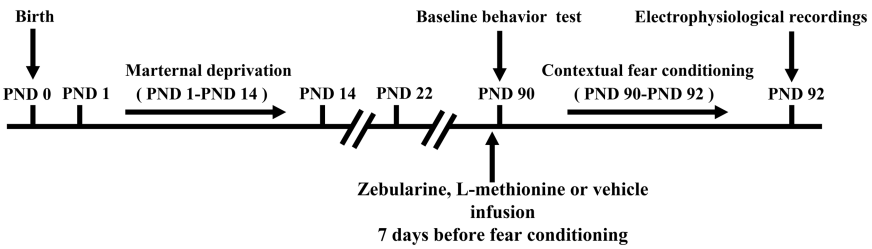

Figure 1. Schematic representation of a timeline for the experimental methods.

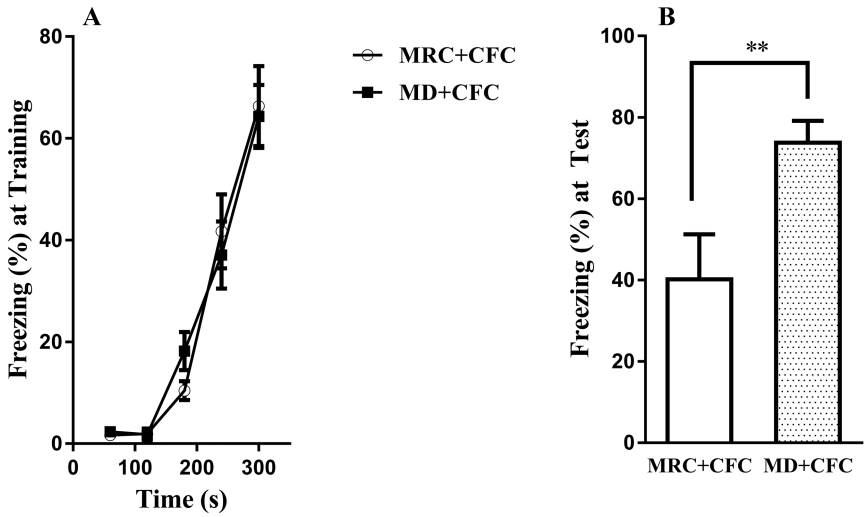

Figure 2. Effects of maternal deprivation (MD) on contextual fear memory in adulthood. (A) The MD+contextual fear conditioning (CFC) and mother-reared control (MRC)+CFC groups acquired similar levels of freezing during fear conditioning training (n = 7–10 per group). (B) A higher freezing level was exhibited in the MD+CFC group in the test performed 24 hours posttraining (n = 7–10 per group). Data presented as the mean ± SEM. $^{**}P < .01$ compared with the MRC+CFC group.

adulthood, we examined the effects of Reelin DNA methylation on contextual fear memory and Reelin protein expression in the hippocampal CA1 area following CFC through performing intra-CA1 microinjections of zebularine or L-methionine in adult MRC rats. Zebularine is a DNA methyltransferase inhibitor that has been validated to cause DNA hypomethylation (Lubin et al., 2008). L-methionine, a methyl group donor for DNA methylation, has been proposed to cause DNA hypermethylation (Detich et al., 2003). The effects of drug treatment on Reelin DNA methylation were evident in our results ($F_{(2, 13)} = 17.69$, $P < .001$) (Figure 4B) and were in agreement with previous studies (Tremolizzo et al., 2002; Miller and Sweatt, 2007). Compared with the vehicle group, the zebularine group showed a significant reduction in Reelin gene methylation (LSD-$t_{(13)} = 2.21$, $P < .05$) (Figure 4B), while the L-methionine group revealed the opposite effect (LSD-$t_{(13)} = 3.83$, $P < .05$) (Figure 4B).

The expression of Reelin protein influenced by Reelin gene hypomethylation or hypermethylation in adulthood showed remarkable changes 24 h post training ($F_{(2, 8)} = 31.26$, $P < .001$) (Figure 4C). We found a significantly higher level of Reelin protein expression in the zebularine group (LSD-$t_{(8)} = 4.54$, $P < .05$) (Figure 4C) and an evidently lower level in the L-methionine group (LSD-$t_{(8)} = 3.64$, $P < .05$) (Figure 4C) compared to that in vehicle controls. These results suggested that hypomethylation and hypermethylation of the Reelin gene caused opposite alterations in Reelin gene expression in the hippocampal CA1.

The effects of DNA hypomethylation or hypermethylation on contextual fear memory were also evaluated by intra-CA1 microinjections of zebularine or L-methionine 7 days before training. Significant effects of drug treatments were observed on contextual fear memory 24 h posttraining ($F_{(2, 17)} = 16.11$, $P < .05$) (Figure 4D). Compared with the vehicle group, the zebularine group showed a significantly higher level of freezing (LSD-$t_{(17)} = 2.82$, $P < .05$) (Figure 4D), while the L-methionine group exhibited a significantly lower freezing level (LSD-$t_{(17)} = 2.63$, $P < .05$) (Figure 4D). Thus, our results confirmed that modifications of Reelin gene methylation play a crucial role in the formation of contextual fear memory.

Hippocampal LTP Induction was Facilitated by the Second Stress Compared with That in Adult Rats Who Experienced MD Alone

LTP is considered to be associated with learning and memory in the hippocampus (Bliss and Collingridge, 1993) at the cellular level, and lasting synaptic plasticity facilitates memory consolidation. To determine whether the fear memory-augmenting effect of MD was correlated with enhanced synaptic plasticity following a second stress, LTP in the hippocampal CA1 was measured in rats exposed to MD alone and in those exposed to MD+CFC. Two-way ANOVA revealed a significant interaction between MD and the second stress ($F_{(1,280)} = 9.64$, $P < .05$). Compared with slices from the MRC group, slices from the MD group exhibited a greater induction of LTP (MRC: 123.18 ± 3.14%, MD: 170.22 ± 2.59%, Tukey-$t_{(280)} = 12.64$, $P < .001$) (Figure 5B). CFC further facilitated hippocampal LTP in MD rats but showed no significant effects on LTP in MRC rats (MRC+CFC: 129.20 ± 1.70%, MD+CFC: 198.68 ± 5.81%, Tukey-$t_{(280)} = 19.86$, $P < .001$; MD: 170.22 ± 2.59%, MD+CFC: 198.68 ± 5.81%, Tukey-$t_{(280)} = 7.82$, $P < .001$; MRC: 123.18 ± 3.14%, MRC+CFC:129.20 ± 1.70%, Tukey-$t_{(280)} = 1.68$, $P = .63$) (Figure 5B). There was no significant difference in input-output curves or basal synaptic transmission at a series of stimulation intensities across groups ($F_{(3,567)} = 0.12$, $P = .946$, n = 4–5 slices from 4 rats for each group) (Figure 5C). These results suggested that MD facilitated LTP induction in the hippocampal CA1 following CFC in adulthood.

Dendritic Spine Density in the Hippocampal CA1 Was Reversed by the Second Stress in Adulthood

Furthermore, we examined whether the MD exposure-induced enhancement of fear memory cooccurred with structural synaptic plasticity following CFC through examining changes in dendritic spines pre- vs post-second-hit stress. Two-way ANOVA revealed a significant interaction between MD and the second stress ($F_{(1,60)} = 38.58$, $P < .001$). Furthermore, CFC reversed the reduction in dendritic spine density in the hippocampal CA1 caused by MD (MRC: 5.17 ± 0.23, MD: 3.72 ± 0.20, Tukey-$t_{(60)} = 5.39$, $P < .01$;

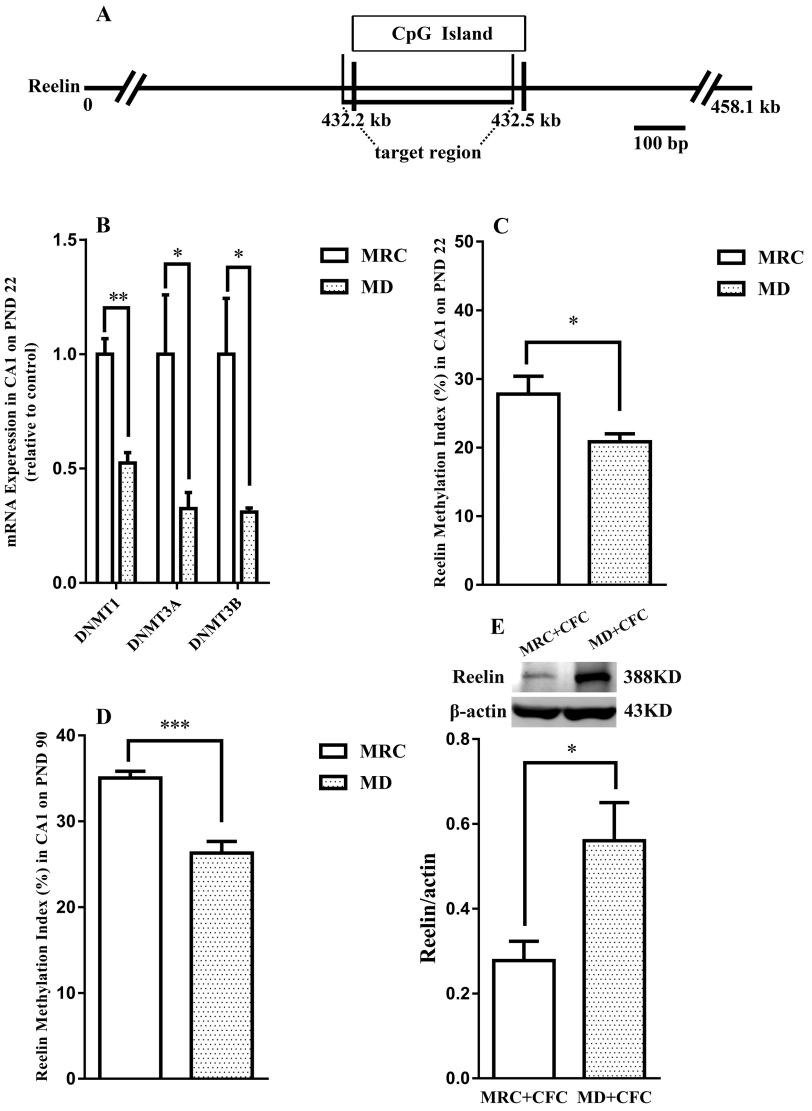

Figure 3. Effects of maternal deprivation (MD) on Reelin gene methylation in the hippocampal CA1. (A) Schematic representation of the location of the methylation changes. Primer sets, as described in Experimental Procedures, were designed to amplify the target region within a CpG island located in the Reelin gene. All base pair (bp) annotations are relative to the start of the promoter. The scale bar represents 100 bp (Miller and Sweatt, 2007). (B) DNA methyltransferase (DNMT)1, DNMT3A, and DNMT3B mRNA expression in the CA1 on postnatal day (PND) 22 was downregulated by MD (n = 4 per group). MD decreased methylation of the Reelin gene on PND 22 (C) (n = 6 per group) and PND 90 (D) (n = 5–7 per group). (E) Protein expression of Reelin was increased by MD following contextual fear conditioning (CFC) (n = 5 per group). Data presented as the mean ± SEM. *P < .05, **P < .01, ***P < .001 compared with the mother-reared control (MRC) group or MRC+CFC group.

MRC+CFC: 8.37 ± 0.38, MD+CFC: 11.11 ± 0.71, Tukey-$t_{(60)}$ = 6.96, P < .001) (Figure 6B). The results showed that the dendritic spine density in the hippocampal CA1 was reversed by the second stress in adulthood.

Discussion

The major finding of the present study was that MD, a stressor applied in the early postnatal period, promoted the lifelong hypomethylation of the Reelin gene in the hippocampal CA1, which subsequently led to the upregulation of Reelin protein expression and the facilitation of LTP in the hippocampal CA1 area, further strengthening fear memory induced by a second stress in adulthood and potentially increasing the susceptibility of individuals to PTSD.

ELS has been demonstrated to increase the risk of developing PTSD in adulthood (Widom, 1999). The present study showed that MD did not affect normal baseline behavior or sensitivity to nociception that could affect CFC (Sultan et al., 2012). MD has the potential to alter the anxiety levels of rats, but there was no significant difference in baseline anxiety level among the groups in the present study. Meanwhile, the freezing level of the MD+CFC group was higher than that of the MRC+CFC group 24 hours posttraining. Several lines of evidence also support the observed MD-enhanced fear memory in adulthood (Oomen et al., 2010; Diehl et al., 2014; Toda et al., 2014). Our previous studies and other independent studies also reported altered Reelin mRNA and protein levels in the total hippocampus of ELS-exposed male rats at PND 90 (Gross et al., 2012; Zhang et al., 2013). Therefore, our present and previous study showed that destabilization of Reelin expression might disturb the response to a second stress in adulthood and alter the susceptibility to fear memory in MD rats. In contrast to our results, our previous study and some independent studies found that ELS hampered

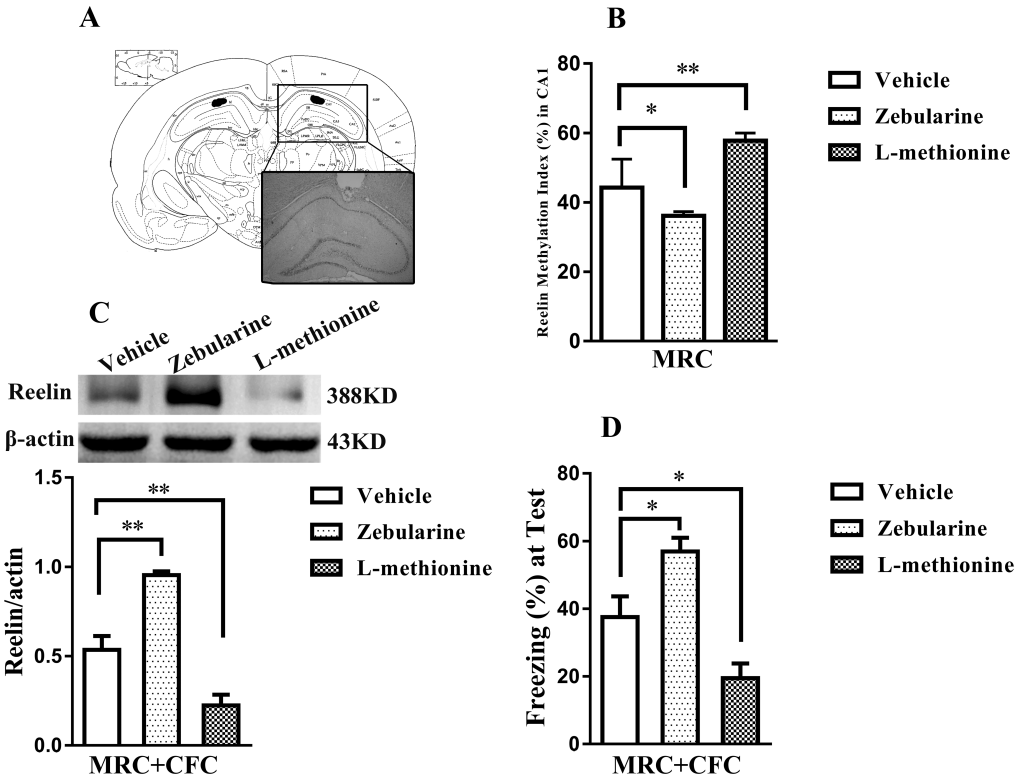

Figure 4. Effects of Reelin gene hypomethylation or hypermethylation in the hippocampal CA1 on contextual fear memory. (A) Representative microphotograph depicting the injection site in the hippocampal CA1 (4.16 mm posterior to bregma). (B) The zebularine group showed a significant reduction in Reelin gene methylation, and the L-methionine group exhibited the opposite effects (n = 5–6 per group). (C) The zebularine group showed higher Reelin protein expression than the vehicle group, whereas the L-methionine group showed the opposite effect (n = 3–4 per group). (D) The zebularine group displayed a higher level of freezing, and the L-methionine group showed a lower level of freezing at 24 hours posttraining (n = 6–7 per group). Data presented as the mean ± SEM. *P < .05, **P < .01 compared with the vehicle group.

contextual fear memory and exerted anxiety-like behavior (Chocyk et al., 2014; Sun et al., 2014; Rana et al., 2015). However, we speculate that these discrepancies are dependent on different experimental conditions such as animal strain, sex, genetic background (Pryce and Feldon, 2003; Sun et al., 2014; Rana et al., 2015), experimental model, timing of ELS, and second stress procedure, which may cause differences in the stressful environment and the effects on the development of the brain (Lehmann et al., 1999; Meerlo et al., 1999; Chocyk et al., 2014; Pierce et al., 2014; Sun et al., 2014; Banqueri et al., 2017).

Gene-environment interactions, such as those occurring with ELS exposure, often lead to epigenetic changes in genes associated with the stress response and synaptic plasticity (Roth et al., 2009; Jawahar et al., 2015). Interestingly, in the current study, we found that MD decreased the expression of 3 DNMT subtypes and Reelin gene methylation in the CA1 area of the hippocampus on PND 22. Our results were in agreement with a previous study that reported that DNMT1 was the primary methyltransferase for the Reelin promoter (Noh et al., 2005). In addition, DNMT3A and DNMT3B may also work together to affect the methylation of the Reelin gene. Our previous study suggested that MD results in upregulation of Reelin gene methylation in the total hippocampus on postnatal day 22 (Qin et al., 2011), which seems to be inconsistent with the results of our present study. In fact, the alteration of Reelin gene methylation may differ across the subfields of the hippocampus in MD rats. Meanwhile, Reelin gene methylation level also changes dynamically from infancy to adulthood. An additional intriguing implication of the current study is that there might be a DNA demethylase regulated by MD in the hippocampal CA1. No specific DNA demethylase has been identified so far; however, current research suggests that there might be at least 2 candidates. Detich et al. have reported that overexpression of MBD2 in cell culture induced demethylation at a number of sites within the promoter region of 2 reporter genes (Detich et al., 2002). In addition, a recent study found that knockdown of Gadd45b, a regulator of active DNA demethylation, reduced expression of Reelin (Kigar et al., 2015). Our study implied the existence of a signaling cascade controlling the demethylation of the Reelin gene induced by MD and suggested that the demethylase might be identifiable in the hippocampal CA1 region. Future studies are needed to investigate the upstream molecules that may regulate DNA demethylase and DNMT activities (Levenson et al., 2004; Chwang et al., 2006). One upstream candidate molecule is extracellular signal-regulated kinase, an upstream kinase of a signaling cascade resulting in 2 chromatin modifications, phosphorylation and histone acetylation, which play an integral role in contextual fear memory (Levenson et al., 2004; Miller and Sweatt, 2007). Thus, DNMTs or demethylases might be a target for extracellular signal-regulated kinase as a means of translating general signals during MD. Furthermore, the MD-induced Reelin gene hypomethylation not only was found in early life but also had profound effects in adulthood. The relationship between the downregulation of Reelin gene methylation in adulthood and increased contextual fear memory was causative. Hypomethylation of the Reelin gene and increased Reelin protein levels caused by zebularine pretreatment in adult rats resulted in enhanced contextual fear memory, while L-methionine had the opposite effect. Furthermore, numerous studies have also suggested that contextual fear memory could be affected by the level of Reelin

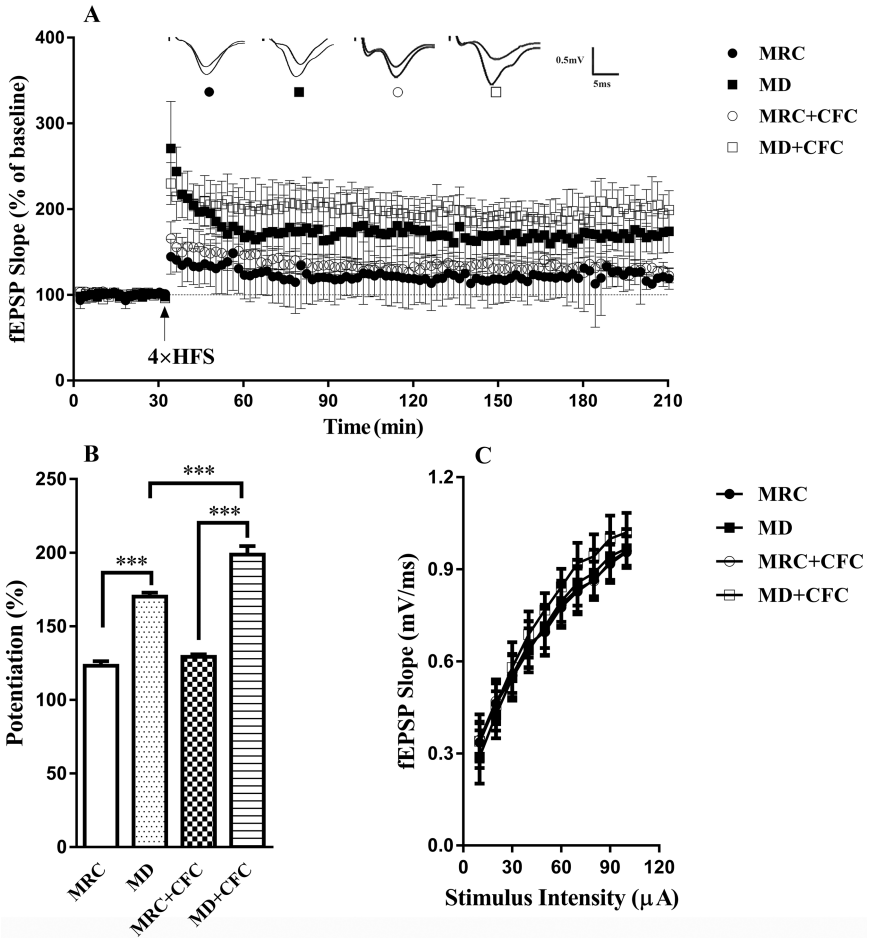

Figure 5. Effects of maternal deprivation (MD) on hippocampal long-term potentiation (LTP) after a second stress. (A) The field excitatory synaptic potential (fEPSP) slope was plotted from mother-reared control (MRC) slices (close circles), MD slices (close square), MRC+CFC slices (open circles), and MD+CFC slices (open square). Arrow indicates 4× high-frequency stimulation (HFS) (100 Hz, 1 second with 20-second intervals). (B) Histogram shows the average percentage of potentiation (150–180 min after HFS vs baseline). LTP was more easily induced in slices obtained from the MD group. Furthermore, CFC further facilitated the hippocampal LTP of MD rats but had no effect on MRC rats (n = 4–5 slices from 4 rats for each group). (C) MD, CFC, and MD+CFC slices did not show a shift in the input-output (I-O) curve (n = 4–5 slices from 4 rats for each group). Data presented as the mean ± SEM. ***$P < .001$ compared with the MRC, MD, or MRC+CFC group.

protein in the hippocampus (Weeber et al., 2002; Qiu et al., 2006; Reichelt et al., 2015). However, a previous study reported that infusion of zebularine immediately after CFC impaired fear memory (Miller and Sweatt, 2007). The observed inconsistencies might be due to the differences in drug dose, the time of injection, and the CFC procedures. Furthermore, zebularine infusion at different times of fear conditioning has been shown to have different effects on fear memory (Miller and Sweatt, 2007). Consistent with our findings, another study found that zebularine increased brain-derived neurotrophic factor mRNA expression and enhanced fear memory (Lubin et al., 2008). Together, our results provide compelling evidence that MD promoted the persistent hypomethylation of the Reelin gene, which was associated with downregulated DNMT expression, and finally facilitated the formation of fear memory induced by a second stress in adulthood. Furthermore, a previous study found that Reelin mRNA expression and the density of Reelin-synthesizing cells in the hippocampus during adulthood were not altered by MD alone (Gross et al., 2012), which suggested that MD-induced hypomethylation of the Reelin gene might be insufficient to alter Reelin expression. However, MD might prime a "stress-like" state in the hippocampal CA1 area, which could be activated by a second stress in adulthood, leading to upregulation of Reelin protein expression and elevated sensitivity to the second stress. Together, we concluded that MD rats might be less capable of dealing with a second stress in adulthood, predisposing them to the development of enhanced contextual fear memory.

LTP is the most widely used model to study learning and memory underlying neuronal plasticity in the hippocampus (Bliss and Collingridge, 1993). Dendritic spines are principal loci of activity-dependent synaptic rearrangements and often correlate with the functional changes associated with synaptic plasticity (Briner et al., 2011; Penzes et al., 2011). We found that LTP was more easily induced in MD rat slices on PND 90, in accordance with the results of previous studies (Zhang et al., 2014; Derks et al., 2016). Our study also showed that the second stress could further facilitate the induction of hippocampal LTP in rats that experienced MD, while the second stress had no effect on LTP in the MRC group. These results are in line with a previous study showing that CFC did not affect hippocampal fEPSP LTP (Abrari et al., 2009; Simoes et al., 2016) but do not mean that there was no behavioral effect on hippocampal potentiation. Some studies have indicated that different behavior interventions induce different forms of hippocampal potentiation (e.g., population spike and long-term depression) (Abrari et al., 2009; Aarse et al., 2016). Taken together, our

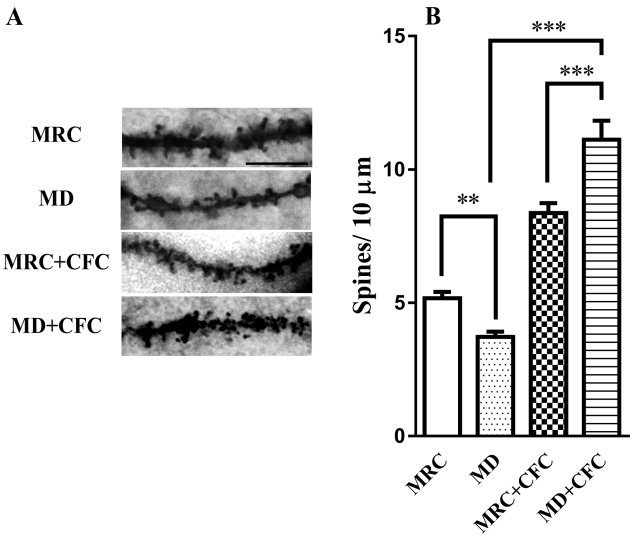

Figure 6. Effects of maternal deprivation (MD) on dendritic spines density in the hippocampal CA1 following the second stress. (A) Representative photomicrographs (×100) of tertiary apical dendrites of hippocampal CA1 pyramidal neurons. Scale bar: 5 μm. (B) The average spine density per oblique dendritic length of hippocampal CA1 pyramidal neurons was reversed in the MD+ contextual fear conditioning (CFC) group (n = 7–19 slices from 4 rats for each group). Data presented as the mean ± SEM. **P < .01, ***P < .001 compared with the MRC, MD, or MRC+CFC group.

results further suggested that MD might enhance contextual fear memory via hippocampal fEPSP LTP facilitated by a second stress. In addition, the change in dendritic spine density in the hippocampal CA1 was reversed by the second stress in adulthood. In line with our study, previous studies have shown that hippocampal spine formation is attenuated by ELS alone (Monroy et al., 2010; Ohta et al., 2017), while an acute stressful event could greatly increase the presence of dendritic spines (Dalla et al., 2009). These observations suggest that the reversal of the change in dendritic spine density might result from an MD-induced enhanced vulnerability to a second stress in adulthood. The Reelin protein, the expression of which was upregulated by MD-induced hypomethylation of the gene following the second stress, and the relevant receptor might be involved, as they have been shown to affect neuroplasticity and neuronal structure in the hippocampal CA1 (Stranahan et al., 2013). Reelin increased Disabled-1 and cAMP-response element binding protein activation and enhanced dendritic spine density in the hippocampal CA1 in the context of improved CA1 LTP (Rogers et al., 2011). Two major forms of N-methyl-D-aspartate receptors (NR) 2 subunits in the hippocampus, NR2A and NR2B, were developmentally regulated, subjected to activity- as well as behavior-induced changes, and determined the polarity of synaptic plasticity under certain circumstances (Levenson et al., 2008). Reelin was required for the normal developmental switch from NR2B to NR2A in the hippocampus (Sinagra et al., 2005). NR2A has been proposed to be more likely to favor the induction of LTP and control the direction of LTP than NR2B (Iafrati et al., 2014). Moreover, both NR2A and NR2B could be tyrosine phosphorylated in response to synaptic activity by Reelin (Beffert et al., 2005). Thus, the activation of Disabled-1 and cAMP-response element binding protein, the alteration of NMDAR activity by changes in subunit composition, the modulation of NMDAR conductivity via phosphorylation by Reelin, which is regulated by the MD-induced alteration of Reelin gene methylation following a second stress, might impact LTP induction and contextual fear memory in adulthood.

The major limitation of our study was that we only investigated one synaptic plasticity-related gene regulated by DNA methylation. Whether other genes were involved in the MD-induced enhanced fear memory remains unclear, and the roles of other genes and epigenetic programming should be investigated in the future.

In conclusion, our results suggest that MD promoted the lifelong hypomethylation of the Reelin gene in the hippocampal CA1, which subsequently upregulated the expression of the Reelin protein and further facilitated the induction of LTP, finally strengthening the vulnerability to fear memory following a second stress in adulthood. Importantly, these findings could be explained within the framework of the cumulative stress model, which is based on the hypothesis that individual genes interact with environmental factors (Nederhof and Schmidt, 2012; Daskalakis et al., 2013; Mc Elroy and Hevey, 2014). A great number of studies have revealed that ELS results in significant epigenetic changes of genes in or out of the hypothalamic–pituitary–adrenal axis such as glucocorticoid receptor, arginine vasopressin, and brain-derived neurotrophic factor (Gray et al., 2014; Jawahar et al., 2015). Recent studies have suggested that compared with a combination of different stressors, a single stressor results in different gene expression activation patterns, further indicating that a history of stress indeed permanently alters stress susceptibility and gene expression patterns in the hippocampus following a novel stressor, which might be regulated by epigenetic variation-induced programming effects (Lesse et al., 2017). Altogether, our findings provide further evidence for long-term epigenetic alterations induced by ELS that alter the susceptibility for contextual fear memory and the mechanism for the programming effects of ELS on gene expression in response to a second stressor in later life.

Supplementary Material

Supplementary data are available at *International Journal of Neuropsychopharmacology* online.

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Statement of Interest

None.

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