Early life stress increases stress vulnerability through BDNF gene epigenetic changes in the rat hippocampus

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Early life stress (ELS) exerts long-lasting epigenetic influences on the brain and makes an individual susceptible to later depression. It is poorly understood whether ELS and subsequent adult chronic stress modulate epigenetic mechanisms. We examined the epigenetic mechanisms of the BDNF gene in the hippocampus, which may underlie stress vulnerability to postnatal maternal separation (MS) and adult restraint stress (RS). Rat pups were separated from their dams (3 h/day from P1–P21). When the pups reached adulthood (8 weeks old), we introduced RS (2 h/day for 3 weeks) followed by escitalopram treatment. We showed that both the MS and RS groups expressed reduced levels of total and exon IV BDNF mRNA. Furthermore, RS potentiated MS-induced decreases in these expression levels. Similarly, both the MS and RS groups showed decreased levels of acetylated histone H3 and H4 at BDNF promoter IV, and RS exacerbated MS-induced decreases of H3 and H4 acetylation. Both the MS and RS groups had increased MeCP2 levels at BDNF promoter IV, as well as increased HDAC5 mRNA, and the combination of MS and RS exerted a greater effect on these parameters than did RS alone. In the forced swimming test, the immobility time of the MS + RS group was significantly higher than that of the RS group. Additionally, chronic escitalopram treatment recovered these alterations. Our results suggest that postnatal MS and subsequent adult RS modulate epigenetic changes in the BDNF gene, and that these changes may be related to behavioral phenotype. These epigenetic mechanisms are involved in escitalopram action. © 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Early adverse experiences in humans augment the risk for developing major depression in adulthood (Heim and Nemeroff, 2001; Kendler et al., 2002). Exposure to early life stress (ELS) in rodent models induces long-lasting effects on neural and behavioral functions (Lee et al., 2007; Nishi et al., 2013). These effects may increase vulnerability to subsequent stress exposure in adulthood.

Recent studies indicated that epigenetic mechanisms, the process by which environmental factors change gene expression, may be involved (Zannas and West, 2014).

Epigenetic mechanisms are most commonly regulated by posttranslational modifications of histones and DNA methylation, which can promote or repress gene transcription (Chahrour et al., 2008; Gräff and Mansuy, 2008). In general, acetylation of histones H3 and H4 loosens the interaction between DNA and histones and allows the transcriptional machinery access to the promoters of particular genes. Thus, hyperacetylation at the promoters increases transcriptional activity, whereas hypoacetylation decreases activity (Grunstein, 1997). The methylation of CpG islands at the promoter leads to stable silencing of gene expression (Moore et al., 2013). The gene silencing effect of DNA methylation is mediated by a family of methyl-CpG binding proteins, such as methyl CpG binding protein 2.
(MeCP2). MeCP2 binds to methylated CpG sites at the promoter and can block transcription factor binding through its interaction with a histone deacetylase (HDAC)/Sin3 complex, thereby silencing gene expression (Jones et al., 1998; Moore et al., 2013). On the other hand, one study found MeCP2 functioned as both an activator and a repressor on gene expression in the hypothalamus (Chahrour et al., 2008).

Evidence suggests that chromatin remodeling at the BDNF gene is involved in depression and stress-related models as well as in antidepressant drug action (Boule et al., 2012; Fuchikami et al., 2010; Sun et al., 2013). It was initially thought that the rodent BDNF gene formed four 5′ noncoding exons (Timmusk et al., 1993). However, it was confirmed that the rodent BDNF gene consists of eight 5′ noncoding exons and one 3′ protein-coding exon, each of which can be alternatively spliced to a common coding exon to generate nine different transcripts (Aid et al., 2007). Therefore, due to this newly described exon arrangement, we defined the previously labeled exons III and IV as exons IV and VI in the present study. Each alternatively spliced mRNA transcript has a distinct promoter region, which is differentially activated in response to various types of neuronal stimulation.

BDNF exon III (i.e., exon IV in the present study) is the major activity-dependent exon, and promoter III (i.e., promoter IV in the present study), upstream of exon III, is the best characterized BDNF promoter (Shieh et al., 1998; Tao et al., 1998). Cyclic AMP response element (CRE) binding protein (CREB) is one of the transcription factors that can modulate BDNF exon IV transcription (Zheng et al., 2012). CREB activates BDNF transcription by binding to CRE within BDNF promoter III (i.e., promoter IV in the present study) (Shieh et al., 1998; Shieh and Ghosh, 1999). MeCP2 reportedly selectively binds to CRE site of the BDNF promoter III (i.e., exon IV in the present study) (Chen et al., 2003). In rat cortical neurons, membrane depolarization significantly increased BDNF exon III (i.e., exon IV in the present study) transcription, accompanied by dissociation of the MeCP2−CREB complex from BDNF promoter III (i.e., promoter IV in the present study) (Chen et al., 2003; Martinowich et al., 2003). Conflicting result has been shown that in the hypothalamus of mice overexpressing MeCP2, MeCP2 activates CREB, and BDNF is upregulated (Chahrour et al., 2008). Thus, we focused on the BDNF promoter IV mediates by CREB. Numerous animal studies have shown that ELS can leave persistent epigenetic marks, which can affect neural and behavioral functioning throughout adulthood (Lutz and Turecki, 2014). We investigated whether ELS and subsequent adult stress affected stress vulnerability through epigenetic mechanisms that regulate BDNF expression. For this, we used two kinds of stress models: (1) maternal separation (MS) during early postnatal life and (2) chronic restraint stress (RS) during adulthood. MS has been widely used to characterize the long-term effects of early life adversity on subsequent behavior in adulthood (Pryce and Feldon, 2003). In our previous studies, chronic RS decreased BDNF expression in the adult rat hippocampus (Park et al., 2006, 2009). Therefore, we used RS, a well-established model of adult stress. First, we examined the levels of total and exon IV BDNF mRNA in the rat hippocampus. Second, the acetylated levels of histones H3 and H4 and the level of MeCP2 at BDNF gene promoter IV were investigated. Third, we examined the histone deacetylase 5 (HDAC5) mRNA level. Fourth, we determined whether depression-like behavioral changes were induced in maternally separated adult (MS group), restrained (RS group), and maternally separated, restrained rat (MS + RS group) rats. Furthermore, we investigated whether chronic escitalopram treatment affected the observed alterations in these groups.

2. Experimental procedures

2.1. Animals

All experiments involving animals were approved by the Committee for Animal Experimentation and the Institutional Animal Laboratory Review Board of Inje Medical College (approval no. 2012–019). Timed-pregnant Sprague–Dawley (SD) female rats (Orient Bio, GyeongGi-Do, South Korea) arrived at the animal facility when they were 15 days pregnant. All animals were maintained under standard laboratory conditions (21 ± 1°C, 12/12 h light/dark cycle, food and water ad libitum). Pregnant females were individually housed with sawdust until delivery.

2.2. Experimental design

There were eight experimental groups (male rats; Fig. 1). The first group (control rats; control group) consisted of non-separated, non-restrained rats that received vehicle (1 mL/kg). The second group (control + escitalopram group) consisted of non-separated, non-restrained rats that received escitalopram (10 mg/kg). The third group (maternally separated adult rats; MS group) consisted of maternally separated, non-restrained rats that received vehicle. The fourth group (MS + escitalopram group) consisted of maternally separated, non-restrained rats that received escitalopram. The fifth group (restrained rats; RS group) consisted of non-separated, restrained rats that received vehicle. The sixth group (RS + escitalopram group) consisted of non-separated, restrained rats that received escitalopram. The seventh group (maternally separated, restrained rats; MS + RS group) consisted of maternally separated, restrained rats that received vehicle. The eighth group (MS + RS + escitalopram group) consisted of maternally separated, restrained rats that received escitalopram. Escitalopram oxalate (obtained as a generous gift from Sandoz, UK) was mixed fresh daily, dissolved in vehicle (0.7% glacial acetic acid in 0.9% saline), and intraperitoneally (i.p.) administered to the animals. The 10–mg/kg escitalopram dose was selected based on a report showing that this dose exerted antidepressant-like effects in rats in which depression was induced by chronic mild stress (Eren et al., 2007). This dose also reportedly ameliorated hippocampal neurochemical alterations associated with maternal separation in rats (Hui et al., 2010).

2.3. Maternal separation (MS)

The MS procedure, previously described in detail by Lippmann et al. (2007), was used with slight modifications. MS was administered to 50% of the total pups in each of the MS, MS + escitalopram, MS + RS, and MS + RS + escitalopram groups; the rest pups were assigned to the control, control + escitalopram, RS, and RS + escitalopram groups. Pups were separated from their dam for 3 h daily from postnatal day (P)1 to P21. During separation, the litters were placed in a small container filled with nesting material. To minimize any body temperature loss during deprivation, these containers were placed in an incubator maintained at 30–32°C. At the end of each daily separation period, the pups were returned to their home cage and reunited with their dam. Litters were housed with animals of the same rearing groups until P35, at which time they were separated by sex and were randomly housed three per cage. Only male rats were used. Non-separated animals were left undisturbed until P35, except during cage cleaning.

2.4. Restraint stress (RS)

RS was performed as reported in Park et al. (2006, 2009). When
all animals reached adulthood (8 weeks old), the RS, RS + escitalopram, MS + RS, and MS + RS + escitalopram groups were individually subjected to RS (2 h per day) for three weeks by placing them into a specially designed plastic restraint tube (dimensions: 20 cm high, 7 cm diameter). Beginning on RS day 1, escitalopram or vehicle was administered 1 h before daily stress for 3 weeks.

2.5. Forced swimming test (FST)

The FST, developed by Porsolt et al. (1978), was performed 24 h after the last RS with minor modifications, as described in our previous report (Seo et al., 2012). Briefly, rats forced to swim in a restricted space adopt a typical immobile posture after an initial period of vigorous attempts to escape. The observed immobility reflects a state of despair. We used transparent plastic containers (40 cm height × 18 cm diameter) filled with water (23–25°C) deep enough that the rats could not touch the bottom. Fresh water was used for each rat. Rats were placed into the tank and left there for 7 min. Each rat’s behavior was videotaped for later scoring. For 5 min (i.e., from 2 min to 7 min of the test) the immobility time was measured by a trained experimenter blinded to the groups. Because little immobility was observed during the first 2 min, only that occurring during the last 5 min was taken into account (Porsolt et al., 1978).

2.6. Measurement of mRNA levels by quantitative real-time polymerase chain reaction (qRT-PCR)

Tissue from the hippocampus of rats was snap-frozen in liquid nitrogen and then stored at −80°C. RNA was extracted from the hippocampus using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The procedure was carried out at room temperature. The RNA concentration of each sample was determined using a NanoDrop™ ND-1000 UV−Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the RNA was subsequently stored at −20°C until further use. RNA isolated from the hippocampus (1 μg) was employed for single-strand cDNA synthesis using an amfiRivertII™ cDNA Synthesis Master Mix (GenDepot, Baker, TX, USA) according to the manufacturer’s instructions. Following optimization of qRT-PCR, cDNA synthesized from 100 ng RNA template per reaction was used. To measure the amplification of the different samples, a master mix consisting of iQ™SYBR®GreenSupermix (Bio-Rad, Hercules, CA, USA) and forward and reverse primers were added. Duplicate samples were amplified in 96-well plates using an iCycler™ iQ 170–8740 System (Bio-Rad, Marnes-la-Coquette, France) programmed to execute the following parameters: 95°C for 3 min, followed by 45 cycles of heating at 95°C for 35 s, annealing at 55°C for 35 s, and extension at 72°C for 35 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Sequences for the primers were as follows: Total BDNF: forward 5’-CCAACGAAGAAAACCTATAAG-3’; reverse 5’-ATTTCCCCTTCTCTT-3’; HDAC5: forward 5’-TTCTTCAACTCCGTAGCC-3’; reverse 5’-TCCCATTCTGCTAGCC-3’; BDNF exon IV: forward 5’-AGATCAAATGGAGCTTCTCA-3’; reverse 5’-ATACTTGTGCTTCTC-3’; HDAC5: forward 5’-TCTTTCACTCAGGCC-3’; reverse 5’-TCCCTTCATTCTTAC-3’; GAPDH: forward 5’-ATTCCTCCTCAGTCA-3’; reverse 5’-GAGATCCACACGATACTAT-3’. ΔCt, representing the difference between GAPDH and the target gene Ct, was calculated using the formula ΔCt = Ct target gene − Ct.
and the fold difference was then quantified using the 2^−ΔΔCt method. The final value was expressed as a value relative to the control group.

2.7. Chromatin immunoprecipitation (ChIP) assays

For ChIP analysis, we used a Simple ChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer’s instructions. Briefly, cross-linked cell lysates were sheared by sonication to generate chromatin fragments of approximately 200–500 bp on average. Chromatin was then subjected to immunoprecipitation (IP) using antibodies specific to acetyl-histone H3 (K9 + K14) (06–599; Millipore, Billerica, MA, USA) and H4 (K5 + K8 + K12 + K16) (06–866; Millipore, Billerica, MA, USA) or MeCP2 (ab2828; Abcam, Cambridge, UK) at 4 °C overnight. Protein-DNA-antibody complexes were precipitated with Protein G Magnetic Beads and then subjected to a series of washes. The precipitated protein–DNA complexes were eluted from the antibody with elution buffer. We reversed the cross-links of all the samples by adding NaCl and proteinase K for 2 h at 65 °C. Protein-free DNA was extracted in phenol/chloroform (Amresco, Solon, OH, USA) and precipitated in ethanol (Merck, Hunterdon, NJ, USA). The purified DNA was subjected to PCR amplification using real-time PCR. Each sample was run in duplicate with 2 μL of DNA elute per reaction. A master mix consisting of iQ™ SYBR® Green Supermix and forward and reverse primers were added to each well and the program executed the following parameters: 95 °C for 3 min, followed by 45 cycles of heating at 95 °C for 35 s, annealing at 55 °C for 35 s, and extension at 72 °C for 35 s. The following primers were used to selectively amplify the portion of BDNF promoter IV: forward 5′-CACGCTGCCTTGACG-3′. The ChIP results were normalized to the input, and the fold difference was then quantified using the 2^−ΔΔCt method. The final value was expressed as a value relative to the control group.

2.8. Statistical analysis

For the FST, the Kruskal–Wallis test was used to detect significant differences in the immobility time among groups, followed by the Mann–Whitney U test. Changes in gene expression, histone acetylation, and MeCP2 levels were analyzed using a one-way analysis of variance (ANOVA). Scheffe’s test was used for post hoc comparisons. To determine the individual and interactive effects of MS and RS on these levels, a two-way ANOVA was performed in the control, MS, RS, and MS + RS groups. P-values ≤0.05 were considered statistically significant.

3. Results

3.1. Effects on hippocampal BDNF mRNA levels

The Two-way ANOVA revealed a significant interaction between MS and RS on the levels of total and exon IV BDNF mRNA (for total BDNF: interaction, F1,44 = 5.212, p = 0.027, Fig. 2A; for exon IV BDNF: interaction F1,44 = 4.418, p = 0.041, Fig. 2B). Significant individual effects of MS and RS on these mRNA levels were also found (for total BDNF: MS: F1,44 = 54.164, p < 0.001; RS: F1,44 = 201.970, p < 0.001, Fig. 2A; for exon IV BDNF: MS: F1,44 = 84.753, p < 0.001; RS: F1,44 = 227.647, p < 0.001, Fig. 2B). Post hoc analysis revealed that the MS, RS, and MS + RS groups had significantly decreased levels of total and exon IV BDNF mRNA compared with the control group (for total BDNF: MS vs. control: 0.79 ± 0.06 vs. 1.00 ± 0.03, p = 0.032; RS vs. control: 0.58 ± 0.06 vs. 1.00 ± 0.03, p < 0.001; MS + RS vs. control: 0.37 ± 0.09 vs. 1.00 ± 0.03, p < 0.001, Fig. 2A; for exon IV BDNF: MS vs. control: 0.78 ± 0.06 vs. 1.00 ± 0.04, p = 0.010; RS vs. control: 0.63 ± 0.04 vs. 1.00 ± 0.04, p < 0.001; MS + RS vs. control: 0.42 ± 0.04 vs. 1.00 ± 0.04, p < 0.001, Fig. 2B). Moreover, these mRNA levels were clearly lower in the MS + RS group than that in the RS group (for total BDNF: 0.37 ± 0.09 vs. 0.58 ± 0.06, p < 0.001, Fig. 2A; for exon IV BDNF: 0.42 ± 0.04 vs. 0.63 ± 0.06, p < 0.001, Fig. 2B), suggesting that RS potentiated the MS-induced decreases of these levels.

There was no significant difference in the effects of total and exon IV BDNF mRNA in the control and the control + escitalopram groups. Escitalopram administration significantly increased the levels of total and exon IV BDNF mRNA in MS, RS, and MS + RS-exposed rats (post hoc: for total BDNF: MS + escitalopram vs. MS:
Having observed a significant reduction in the levels of total and exon IV BDNF mRNA in MS, RS, and MS + RS-exposed rats, we next examined the levels of acetylated histones H3 and H4 and MeCP2 at promoter IV of the BDNF gene in the hippocampus of rats. MS, RS, and their interaction had significant effects on the levels of acetylated histones H3 and H4 (for H3: MS: F(3,44) = 102.359, p < 0.0001; RS: F(3,44) = 164.079, p < 0.0001; interaction: F(3,44) = 10.213, p = 0.003, Fig. 3A; for H4: MS: F(3,44) = 121.483, p < 0.001; RS: F(3,44) = 229.767, p < 0.001; interaction: F(3,44) = 7.821, p = 0.008, Fig. 3B). The levels of acetylated histones H3 and H4 in the MS, RS, and MS + RS groups were significantly reduced as compared with those of the control group (post hoc for H3: MS vs. control: 0.75 ± 0.05 vs. 1.00 ± 0.05, p = 0.011; RS vs. control: 0.67 ± 0.06 vs. 1.00 ± 0.05, p < 0.001; MS + RS vs. control: 0.39 ± 0.07 vs. 1.00 ± 0.05, p < 0.001, Fig. 3A; for H4: MS vs. control: 0.78 ± 0.05 vs. 1.00 ± 0.04, p = 0.002; RS vs. control: 0.68 ± 0.04 vs. 1.00 ± 0.04, p < 0.001; MS + RS vs. control: 0.45 ± 0.05 vs. 1.00 ± 0.04, p < 0.001, Fig. 3B). Specifically, the histone H3 and H4 levels in the MS + RS group were significantly lower than those in the RS group (post hoc for H3: 0.39 ± 0.07 vs. 0.67 ± 0.06, p < 0.001, Fig. 3A; for H4: 0.45 ± 0.05 vs. 0.68 ± 0.04, p < 0.001, Fig. 3B), suggesting that RS exacerbates the MS-induced decreases in H3 and H4 acetylation.

There was no significant difference in the effects of histone H3 and H4 acetylation on the control and the control + escitalopram groups. Chronic escitalopram treatment significantly increased histone H3 and H4 acetylation in the MS, RS and MS + RS-exposed rats (post hoc for H3: MS + escitalopram vs. MS: 1.11 ± 0.08 vs. 0.75 ± 0.05, p < 0.001; RS + escitalopram vs. RS: 1.00 ± 0.06 vs. 0.67 ± 0.06, p < 0.001; MS + RS + escitalopram vs. MS + RS: 0.77 ± 0.06 vs. 0.39 ± 0.07, p < 0.001, Fig. 3A; for H4: MS + escitalopram vs. MS: 1.16 ± 0.07 vs. 0.78 ± 0.05, p < 0.001; RS + escitalopram vs. RS: 0.97 ± 0.06 vs. 0.68 ± 0.04, p < 0.001; MS + RS + escitalopram vs. MS + RS: 0.86 ± 0.05 vs. 0.45 ± 0.05, p < 0.001, Fig. 3B). An additional statistical analysis revealed that the level of histone H3 acetylation in the MS + RS + escitalopram group, but not the MS + escitalopram group and the RS + escitalopram group, was significantly different from that of the control group (post hoc, p = 0.029, Fig. 3A), indicating that effect of drug in the MS + RS group is not bringing up levels of the control group.

MeCP2 can modulate BDNF gene expression by suppressing the transcription of promoter IV (Chen et al., 2003; Martinowich et al., 2003). Fig. 3C shows the MeCP2 level at BDNF promoter IV. The Two-way ANOVA indicated that the individual effects of MS and RS were statistically significant, whereas the interaction between the two was not significant (MS: F(3,44) = 61.605, p < 0.001; RS: F(3,44) = 89.862, p < 0.001; interaction: F(3,44) = 0.003, p = 0.957, Fig. 3C). The Post hoc analysis indicated that the MeCP2 level at promoter IV was significantly increased in the MS, RS, and MS + RS groups compared with that in the control group (MS vs. control: 1.23 ± 0.03 vs. 1.00 ± 0.02, p = 0.009; RS vs. control: 1.28 ± 0.04 vs. 1.00 ± 0.02, p < 0.001; MS + RS vs. control: 1.58 ± 0.06 vs. 1.00 ± 0.02, p < 0.001). Additionally, the MeCP2 level in the MS + RS group was significantly higher than that in the RS group (1.58 ± 0.06 vs. 1.28 ± 0.04, p = 0.007), indicating that the combination of MS and RS exerted a greater effect than did RS alone.

There was no significant difference in the effects of MeCP2 binding at promoter IV in the control and the control + escitalopram groups. Chronic escitalopram significantly decreased the MeCP2 level in MS, RS, and MS + RS-exposed rats (post hoc: MS + escitalopram vs. MS: 0.90 ± 0.06 vs. 1.23 ± 0.03, p < 0.001; RS + escitalopram vs. RS: 0.96 ± 0.05 vs. 1.28 ± 0.04, p < 0.001; MS + RS + escitalopram vs. MS + RS: 1.07 ± 0.06 vs. 1.58 ± 0.06, p < 0.001, Fig. 3C).

3.2. Effects on BDNF promoter IV epigenetic state in the hippocampus

High HDAC expression causes histone hypoacetylation, and HDAC inhibitors increase histone acetylation (Covington et al., 2011; Schroeder et al., 2007). Thus, we examined whether HDAC5 expression was changed in these groups. There was no significant relationship between the MS × RS interaction on HDAC5 mRNA level, but the individual effects of MS and RS were significant (MS: F(3,44) = 53.896, p < 0.001; RS: F(3,44) = 137.366, p < 0.001; interaction: F(3,44) = 0.475, p = 0.495, Fig. 4). Significantly increased HDAC5 mRNA was found in the hippocampus of the MS, RS, and MS + RS groups (post hoc: MS vs. control: 1.24 ± 0.04 vs. 1.00 ± 0.04, p = 0.007; RS vs. control: 1.40 ± 0.03 vs. 1.00 ± 0.04, p < 0.001; MS × RS vs. control: 1.67 ± 0.04 vs. 1.00 ± 0.04, p < 0.001; Fig. 4). Furthermore, the HDAC5 level in the MS + RS group was significantly higher than that in the RS group (post hoc: 1.67 ± 0.04 vs. 1.40 ± 0.03, p = 0.005), indicating that the combination of MS and RS exerted a greater effect than did RS alone.

Escitalopram administration did not affect HDAC5 expression in control rats, whereas it significantly reduced HDAC5 expression in MS, RS, and MS + RS-exposed rats (post hoc: MS + escitalopram vs. MS: 1.00 ± 0.04 vs. 1.24 ± 0.04, p = 0.007; RS + escitalopram vs. RS: 1.07 ± 0.07 vs. 1.40 ± 0.03, p < 0.001; MS + RS + escitalopram vs. MS + RS: 1.06 ± 0.06 vs. 1.67 ± 0.04, p < 0.001, Fig. 4).

3.4. Behavioral changes in adult rats during the FST

Behavioral changes of the eight groups observed in the FST are presented in Fig. 5. The immobility time of the MS and RS groups was not statistically different from that of the control group, although there was a trend toward an increased immobility time (MS vs. control: 119.2 ± 27.1 vs. 81.3 ± 11.5 s; RS vs. control: 118.2 ± 21.2 vs. 81.3 ± 11.5 s). However, the immobility time was significantly increased in the MS + RS group compared with the control group (191.0 ± 21.0 s vs. 81.3 ± 11.5 s, p < 0.027). Specifically, the MS + RS group exhibited significantly longer immobility time than did the RS group (191.0 ± 21.0 s vs. 118.2 ± 21.0 s, p = 0.028).

The control + escitalopram, RS + escitalopram, and MS + RS + escitalopram groups had significantly reduced immobility time compared with matched groups that did not receive escitalopram (control + escitalopram vs. control: 19.5 ± 8.9 vs. 81.3 ± 11.5 s, p = 0.021; RS + escitalopram vs. RS: 56.9 ± 17.8 vs. 118.2 ± 21.0 s, p = 0.07; MS + RS + escitalopram vs. MS + RS: 108.4 ± 12.1 vs. 191.0 ± 21.0 s, p = 0.018). No significant difference was observed between the MS group and the MS + escitalopram group, although the results from the MS + escitalopram group showed a trend toward reduced immobility time compared with
the MS group (55.9 ± 15.9 vs. 119.2 ± 27.1 s).

4. Discussion

Our main findings were that postnatal maternal separation and subsequent adult chronic restraint stress (MS + RS group) modulated the epigenetic mechanisms underlying the regulation of BDNF exon IV in the hippocampus, and this modification may be related to augmented depression-like behavior. Both the MS and RS groups also showed (1) decreased total and exon IV BDNF mRNA expression, (2) decreased acetylation of H3 and H4 at BDNF promoter IV, (3) increased MeCP2 levels at promoter IV, and (4) increased HDAC5 mRNA expression. We further, showed that chronic escitalopram treatment affected epigenetic BDNF gene modifications in MS, RS, and MS + RS groups.

4.1. Regulation of total and exon IV BDNF mRNA in the hippocampus of control, MS, RS, and MS + RS rats

In the present study, the MS group, which was subjected to 3 h MS during the first 3 weeks of postnatal life, showed reduced levels of total and exon IV BDNF mRNA in the adult hippocampus. This is consistent with previous reports showing that (1) a single 24-h MS on P9 resulted in persistently reduced BDNF protein expression in the rat hippocampus (Roceri et al., 2002), (2) repeated 3-h MS (from P2 to P14) caused a significant reduction in mature BDNF protein in the rat hippocampus (de Lima et al., 2011), and (3) 3-h MS (from P1 to P14) decreased the BDNF protein level in the rat hippocampus (Lippmann et al., 2007). These findings suggest that MS results in BDNF expression changes that persist for several weeks and may thereby affect long-term functioning of the adult hippocampus.

BDNF regulates neurogenesis in the brain. Specifically, MS rats have decreased cell proliferation and immature neuron production in the dentate gyrus of the hippocampus (Kikusui et al., 2009; Mirescu et al., 2004). Reduced BDNF levels and neurogenesis in the hippocampus cause functional alterations, including impaired learning and memory (Lafenetre et al., 2011; Monteggia et al., 2004). Indeed, MS rats had significant deficits in hippocampal learning and memory in the Morris water maze task (Lippmann et al., 2007). Furthermore, a recent study reported that MS (3 h daily from P2 to P14) induced deficits in hippocampal-dependent memory tasks that persisted throughout adulthood, and escitalopram treatment improved these deficits (Couto et al., 2012).

We found that the RS group had decreased expression of total and exon IV BDNF. Decreased BDNF has been reported in many animal studies that used the RS model (Park et al., 2006, 2009, 2011; de Lima et al., 2011; Lippmann et al., 2007). MS rats also showed decreased BDNF expression in the hippocampus compared to the control group (Fig. 3). These findings suggest that MS and RS treatments have distinct effects on BDNF expression, with MS resulting in more pronounced decreases compared to RS.

4.2. Regulation of acetylated histones H3 and H4 and methyl CpG binding protein 2 (MeCP2) at BDNF promoter IV

Chromatin immunoprecipitation (ChIP) assays were performed to measure the levels of acetylated H3 and H4 and MeCP2 at BDNF promoter IV in the hippocampus using specific antibodies. These levels were quantified by RT-PCR. PCR products (102 bp) were detected using 3% agarose gel electrophoresis. This experiment was repeated twice. Quantitative analysis was normalized to the input DNA. Results are expressed as a value relative to the control group using the 2^(-△△CT) method. CON, non-separated, non-restrained rats; MS, maternally separated adult rats with or without escitalopram; RS, non-separated, restrained rats with or without escitalopram; MS + RS, maternally separated, restrained rats with or without escitalopram. Data are mean ± S.E.M. "p < 0.05 vs. control group; "p < 0.01 vs. control group; "p < 0.01 vs. RS group; "p < 0.01 vs. drug-untreated matched group.
In particular, acute RS reportedly decreased total BDNF mRNA, with significantly reduced levels of exon I and IV BDNF mRNA in the rat hippocampus (Fuchikami et al., 2009). MeHg exposure caused a decrease in BDNF mRNA, an increase in DNA methylation and histone H3K27me3, and a decrease in H3 acetylation at promoter IV. In particular, reduced BDNF mRNA and H3 (K9 + K14) acetylation was reversed by chronic fluoxetine treatment. Interestingly, it was reported that epigenetic alterations associated with ELS were dependent on age, showing that young adult rats (2 months) subjected to MS (daily 3 h from P2 to P14) decreased repressive histone methylation at BDNF promoter IV, and this was associated with enhanced BDNF expression, whereas opposing changes were observed in middle (15 months)-aged MS rats (Suri et al., 2013). This study also showed that chronic antidepressant treatment with amitriptyline attenuated the repressive epigenetic modification at BDNF promoter IV and the decreased exon IV expression in middle-aged MS rats (Suri et al., 2013). More recently, it was reported that adult offspring of pregnant mice subjected to prenatal stress, such as predator odor exposure and restraint, decreased BDNF expression and increased DNA methylation in BDNF exon IV (Boersma et al., 2014; Dong et al., 2015; St-Cyr and McGowan, 2015). These studies indicate that ELS affects BDNF expression in adulthood through epigenetic changes.

Acetylation of the lysine (K) residues neutralizes the basic charge of the N-terminal tails, thus loosening chromatin and allowing binding of the transcription factor to the promoters of particular gene. K 9, 14, 18, 23, 27 and 36 on histone H3 were acetylated, and K5, 8, 12 and 16 on histone H4 were acetylated (He and Lehming, 2003). Among these sites, acetylation at K9 and K14 of histone H3, as well as acetylation at multiple K sites of histone H4, are common modifications enhanced at transcriptionally active genes (Turner, 2002). In the present study, we used antibodies that blocked chronic antidepressant drug treatment (Nibuya et al., 1995). In the present study, chronic escitalopram treatment reversed the decreased levels of total and exon IV BDNF mRNA in the hippocampus of MS, RS, and MS + RS-treated rats; but not in control rats. Overall, these results suggest that changes in BDNF transcripts may be involved in the beneficial effects of escitalopram treatment in counteracting the stress-induced decrease in BDNF expression.

### 4.2. Regulation of histone modification at BDNF promoter IV of control, MS, RS, and MS + RS rats

In the present study, chronic RS significantly decreased acetylated histone H3 and H4 at promoter IV of the BDNF gene, and this was reversed by chronic escitalopram treatment. Several studies showed interactions between stressors and antidepressant drugs with involvement of BDNF gene histone modification in the hippocampus. Chronic social stress reduced the transcripts, accompanied by a significant increase in the levels of repressive histone methylation (H3K27me2) at BDNF promoters; chronic imipramine treatment reversed these changes (Tsankova et al., 2006). Acute RS impacted the levels of transcripts and H3 acetylation (Fuchikami et al., 2009). Chronic-exposure to variable stress reduced H3 (K9) and H4 (K12) acetylation in CA1, CA3, and the dentate gyrus of the hippocampus (Ferland and Schrader, 2011). Chronic RS (6 h daily for 21 days) increased the H3K4me3 level and reduced the H3K9me3 level in the dentate gyrus of the hippocampus, and fluoxetine treatment during stress reversed the decrease in H3K9me3 level (Hunter et al., 2009). Therefore, histone modification of the BDNF gene in the hippocampus is likely to play a critical role in responses to a stressful environment or in antidepressant drug treatment.

Hypocetylation of histone H3 and H4 at BDNF promoter IV was also observed in the MS group, and was reversed by escitalopram treatment. Similar to our observations under stress, perinatal exposure to methyl mercury (MeHg) induced epigenetic suppression of BDNF expression in the hippocampus (Onishchenko et al., 2008). MeHg exposure caused a decrease in BDNF mRNA, an increase in DNA methylation and histone H3K27me3, and a decrease in H3 acetylation at promoter IV. In particular, reduced BDNF mRNA and H3 (K9 + K14) acetylation was reversed by chronic fluoxetine treatment. Interestingly, it was reported that epigenetic alterations associated with ELS were dependent on age, showing that young adult rats (2 months) subjected to MS (daily 3 h from P2 to P14) decreased repressive histone methylation at BDNF promoter IV, and this was associated with enhanced BDNF expression, whereas opposing changes were observed in middle (15 months)-aged MS rats (Suri et al., 2013). This study also showed that chronic antidepressant treatment with amitriptyline attenuated the repressive epigenetic modification at BDNF promoter IV and the decreased exon IV expression in middle-aged MS rats (Suri et al., 2013). More recently, it was reported that adult offspring of pregnant mice subjected to prenatal stress, such as predator odor exposure and restraint, decreased BDNF expression and increased DNA methylation in BDNF exon IV (Boersma et al., 2014; Dong et al., 2015; St-Cyr and McGowan, 2015). These studies indicate that ELS affects BDNF expression in adulthood through epigenetic changes.

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detected histone H4 acetylated at all sites (K5, 8, 12 and 16) and histone H3 acetylated at K9 and K14, while another group (Ferland and Schrader, 2011) used antibodies to detect one specific site.

While the present study investigated changes in whole hippocampal tissues, several studies have examined epigenetic changes in specific subregions of the hippocampus: the dentate gyrus, CA1 or CA3. One study found that chronic RS for 21 days induced an increase in the H3K4me3 levels and a reduction in the H3K9me3 levels in the dentate gyrus, whereas acute RS had no effect on H3K4me3, but increased H3K9me3 levels in the dentate gyrus and in CA1 (Hunter et al., 2009). Another study reported that psychosocial stress via predator exposure produced the most robust hypermethylation of BDNF exon IV in the CA1 subregion (Roth et al., 2011). It appears that specific patterns of chromatin remodeling are produced regionally within the hippocampus in response to various stresses.

The present study also found that the levels of histone H3 and H4 acetylation at BDNF promoter IV were much lower in the M5 + RS group than in the RS group, suggesting that RS potentiated the M5-induced decrease of these levels. These epigenetic changes were similar to the patterns of the BDNF mRNA levels. However, we cannot explain whether downregulation of BDNF expression by MS and RS was directly due to decreased acetylation of histone H3 and H4, because we did not specifically test whether histone acetylation levels were directly responsible for the altered mRNA levels.

4.3. Regulation of MeCP2 at BDNF promoter IV in control, MS, and MS + RS rats

DNA methylation at BDNF promoter IV is involved in BDNF gene silencing through the transcriptional repressor MeCP2 (Chen et al., 2003; Martinowich et al., 2003). Once bound to methylated DNA, MeCP2 participates in chromatin remodeling by recruiting HDAC binding via the corepressor, mSin3A (Martinowich et al., 2003). On the other hand, activity-dependent MeCP2 phosphorylation is dissociated from the BDNF promoter, enabling BDNF transcription (Chen et al., 2003; Martinowich et al., 2003).

A few studies have reported evidence indicating that ELS in rats or mice regulates MeCP2 levels. One study showed that ELS caused MeCP2 phosphorylation and subsequent relief of MeCP2 binding at the arginine vasopressin promoter in the paraventricular nucleus of the hypothalamus (Murgatroyd et al., 2009). These changes were associated with decreased CpG methylation of this promoter and increased AVP expression. According to studies measuring MeCP2 mRNA or immunoreactivity, exposure to caregiver maltreatment resulted in significantly decreased MeCP2 mRNA in the prefrontal cortex (Blaze and Roth, 2013), and maternally separated adult rats had reduced MeCP2 cell counts in the nucleus accumbens (Lewis et al., 2013). Another study also showed that stressors and antidepressant drugs can act via MeCP2 to regulate stress responses (Hutchinson et al., 2012). A previous study used MeCP2 S421A knock-in (KI) mice in which Ser421 was changed to a non-phosphorylatable Ala residue. These MeCP2 KI mice had increased immobility in the FST compared with wild-type (WT) mice. Furthermore, chronic imipramine administration improved social interaction in the MeCP2 WT mice after chronic social defeat stress, whereas imipramine administration did not affect social interaction in MeCP2 KI mice. These findings suggest that MeCP2 KI mice have enhanced sensitivity to stressors, and MeCP2 phosphorylation at Ser421 is required for chronic imipramine to affect depression-like behavior after chronic social defeat stress (Hutchinson et al., 2012).

Based on the studies described above, changes in the MeCP2 level may differ according to the experimental methods employed (i.e., specific promoter binding, phosphorylation, protein, mRNA, or cell counting). In the present study, we detected MeCP2 binding at promoter IV of the BDNF gene. MS increased MeCP2 occupancy at this promoter, and RS increased MeCP2 induction with or without MS.

Our observation that chronic escitalopram treatment reduced MeCP2 occupancy in BDNF promoter IV of stress-exposed rats indicates that MeCP2-dependent transcriptional regulation may be involved in a novel mechanism underlying antidepressant drug action. Interestingly, only chronic, but not acute, imipramine treatment (28 d) produced a significant increase in MeCP2 phosphorylation in the lateral habenula, an important brain region involved in modulating behavioral responses to pain, stress, and anxiety (Hutchinson et al., 2012). MeCP2 immunoreactivity was increased in the dorsal caudate–putamen, frontal cortex, and hippocampal dentate gyrus of rats repeatedly treated with fluoxetine (10 d), and both MeCP2 mRNA isoforms, MeCP2_e1 and MeCP2_e2, were increased by long-term, but not short-term, fluoxetine treatment in these brain regions (Cassel et al., 2006). These studies suggest that treatment with some antidepressant drugs alters the epigenetic factor MeCP2 in the adult rat brain.

4.4. Regulation of HDAC5 in the hippocampus of control, MS, and MS + RS rats

It is generally believed that histone hypoacetylation caused by high HDAC expression hinders gene transcription, and HDAC inhibitors increase histone acetylation. There is increasing evidence that HDAC inhibition is involved in antidepressant-like activity (Covington et al., 2011; Schroeder et al., 2007). Mechanistic evidence for a role of HDAC5, a class II HDAC, in histone acetylation was reported in both rodent and human studies of depression. The hippocampal mRNA level of HDAC5 was downregulated in chronically defeated mice that received chronic imipramine; moreover, HDAC5 overexpression in the hippocampus blocked imipramine’s action to reverse depression-like behavior due to chronic defeat stress (Tsankova et al., 2006). In the peripheral leukocytes of depressive patients, the HDAC5 mRNA level was significantly higher in drug-free patients than in controls, and this elevation was downregulated to the control level after 8 weeks of paroxetine treatment (Iga et al., 2007). Another study found that chronic unpredictable stress produced a significant decrease in H3 and H4 acetylation, in addition to strongly increasing HDAC5 expression, in the rat hippocampus; administration of sodium valproate, a HDAC inhibitor, prevented high HDAC5 expression and H4 hypoacetylation caused by chronic unpredictable stress, and relieved depression-like behavior in rats (Liu et al., 2014). A more recent study investigated the regulation of the expression of the different classes (from class I to class IV) of HDACs in the prefrontal cortex by chronic defeat stress and imipramine treatment (Erburu et al., 2015). Specifically, this study identified that HDAC5 was regulated by stress and the antidepressant drug (Erburu et al., 2015). Taken together, expression of HDAC5 seems to have a role in mediating stress or antidepressant-induced changes in histone acetylation. Thus, we chose to focus our investigation specifically on HDAC5.

However, a limitation of the present study was that the levels of HDAC5 protein were not examined. Additional studies that include Western blot analysis are needed to strengthen the findings of the present work. In addition, the functional mechanism of HDAC5 inhibition should be investigated in future studies. Since no selective compound towards HDAC5 exist yet, the specific class II HDAC inhibitor is necessary to study the possible functional implications of HDAC5 inhibition.

The abovementioned studies are consistent with our present study showing that HDAC5 mRNA expression in the hippocampus was increased by chronic RS and that this elevation was reversed by
chronic escitalopram treatment. The present study also found that MS significantly increased HDAC5 mRNA expression in adulthood, and RS increased this level with or without MS. However, other studies examining chronic social defeat stress and chronic variable mild stress found reduced HDAC5 expression in the nucleus accumbens and amygdala, respectively (Renthal et al., 2007; Sterrenburg et al., 2011). These findings suggest that HDAC5 expression differentially contributes to stress vulnerability in different brain regions. Regulation of HDAC expression in ELS was only examined in one study showing that HDAC1 mRNA was reduced in the medial prefrontal cortex of rats subjected to caregiver maltreatment (Blaze and Roth, 2013). Thus, the role of HDAC classes and brain regions according to stress response needs further investigation.

4.5. Behavioral changes in control, MS, RS, and MS + RS rats

Our behavioral results showed that the FST immobility time was increased in the MS + RS group, indicating more depression-like behavior compared with the RS group. Previous studies indicated reducing immobility (Shirayama et al., 2002; Deltheil et al., 2008). Particularly, mice lacking expression of hippocampal BDNF through promoter IV (BDNF-KIV mice) exhibited depression-like behavior (increased immobility) in a tail suspension test (Jha et al., 2011). BDNF infusions into the hippocampus produced antidepressant-like behavior in FST, reducing immobility (Shirayama et al., 2002; Deltheil et al., 2008).

Based on our findings, the effect seen in the MS + RS group may be caused by stronger epigenetic modifications of the BDNF gene compared with the RS group.

The FST is a well-established screening paradigm for estimating depression-like behavior and antidepressant drug activity (Cryan et al., 2002). In adult rats that have experienced a stressful situation, antidepressant drugs can normalize depression-like behavior. Our results showed that escitalopram treatment for 21 days exerted antidepressant-like effects under stressful conditions (RS + escitalopram or MS + RS + escitalopram groups). Escitalopram treatment in the MS group slightly decreased immobility time, although this was not statistically significant (MS + escitalopram group). This is consistent with a recent study showing that escitalopram significantly improved latency to despair in the MS rats (3 h per day between P2 and P14) but did not change their immobility time. Our results also showed that the immobility time was decreased in escitalopram-treated control rats. This escitalopram effect on FST was also observed in another study showing that systemic escitalopram treatment (0.1–10 mg/kg, i.p.) was effective in producing significant FST antidepressant-like effects without affecting locomotor activity in normal mice (Zomkowski et al., 2010).

Taken together, these findings illustrate the important antidepressant effect of escitalopram. However, escitalopram treatment did not affect the levels of BDNF transcripts or epigenetic regulators in the control group. Thus, the alterations in the BDNF gene observed in this study cannot be the sole mechanism of antidepressant action on behavioral phenotype.

In conclusion, our results provide the first in vivo evidence showing that ELS and subsequent adult stress affect the epigenetic modification of the BDNF gene in the hippocampus, although there are numerous other studies in this field, and these effects may be related to the behavioral phenotype associated with the FST. Furthermore, these epigenetic changes are not permanent, but can be controlled by chronic escitalopram treatment. However, epigenetic changes induced by chronic escitalopram treatment need to be confirmed in other stress-related models and humans before any firm conclusion can be drawn regarding the action of escitalopram.

Conflicts of interest

The authors declare no competing financial interests.

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