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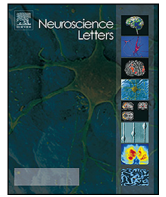

Research article

Long-term effects of maternal separation coupled with social isolation on reward seeking and changes in dopamine D1 receptor expression in the nucleus accumbens via DNA methylation in mice

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HIGHLIGHTS

- Early life stress by MS reduced reward seeking in adult female mice.
- The expression of dopamine D1 receptor in the NAc was decreased in adult MS females.
- The promoter region of the D1 receptor was hypermethylated by MS experience in female mice.

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ABSTRACT

Early-life stress has long-lasting effects on the stress response, emotions, and behavior throughout an individual's life. Clinical reports have demonstrated that child abuse victims exhibit impairments in reward-associated behavior; yet, the mechanism for this effect remains unclear. Maternal separation (MS) or MS coupled with social isolation (SI) (MS + SI) is widely used as a model for early-life stress in rodent studies. We employed mice subjected to MS + SI to clarify the long-term effect of early-life stress on reward-seeking involving palatable foods by a conditioned place-preference (CPP) paradigm. Prior MS + SI experience decreased exploration time in a chocolate-paired compartment in adult female mice, but not in male mice. We then focused on the mesolimbic dopamine pathway associated with reward-seeking behavior and measured both mRNA and protein levels of tyrosine hydroxylase (TH) in the ventral tegmental area (VTA) and dopamine D1 and D2 receptors in the nucleus accumbens (NAc). MS + SI female mice had significantly lower D1 receptor mRNA and protein levels than controls, whereas the expression of TH and the D2 receptor was similar in the 2 groups. All mRNA and protein levels were unchanged in MS + SI male mice. When attempting to elucidate the mechanism underlying downregulation of the D1 receptor in the NAc of MS + SI females, we found hypermethylation of the *Drd1a* promoter region. These results suggest that early-life stress affects reward-seeking behavior in female mice, which may be associated with the downregulation of D1 receptor in the NAc via epigenetic modification of its promoter region.

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1. Introduction

Early-life stress has long-lasting effects on an individual's stress response, emotions, and behavior throughout life, and increases the risk for various psychiatric diseases, including depression and posttraumatic stress disorder [1]. Recently, clinical report have indicated that child abuse victims exhibit reduced sensitivity to reward and show decreased activation of the striatum and thalamus during reward processing [2]. Maternal separation (MS) or

Abbreviations: CpG, cytosine-phospho-guanine dinucleotide; CPP, conditioned place preference; DNMT, DNA methyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MS, maternal separation; MS + SI, maternal separation coupled with social isolation; NAc, nucleus accumbens; PND, postnatal day; SI, social isolation; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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MS coupled with social isolation (SI) (MS + SI) is an animal model for studying the mechanisms underlying the effects of early-life stress on the development of psychiatric disorders. Animal studies related to early-life stress have revealed that an abnormal maternal environment, including MS, can impair innate reward-seeking behavior [3–5]. The mesolimbic dopamine system is a key brain circuit to regulate reward seeking, which comprises dopaminergic neurons that project from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) [6]. The principal cells of the NAc are medium spiny neurons that express $G_{s\alpha}$-coupled dopamine D1 receptors and $G_{i\alpha}$-coupled dopamine D2 receptors. Although some reports have demonstrated that early-life stress decreased dopamine receptor expression [7,8], the molecular mechanisms underlying receptor regulation remain unclear.

Epigenetic modifications, such as DNA methylation of promoters and histone modification regulate gene expression and have been implicated in the pathologies of several psychological disorders [9]. DNA methylation involves methyltransferase-catalyzed direct covalent addition of a methyl group to the fifth position of cytosine residues within cytosine-phospho-guanine dinucleotide (CpG); at gene promoters, this typically represses gene transcription. Importantly, MS and MS + SI is known to increase the expression of DNA methyltransferases (DNMTs) in the NAc of infant and adult rats [10–12]. Furthermore, different rearing environments during the first postnatal week in rodents modify epigenetic regulation of the glucocorticoid receptor gene [13]. Thus, MS + SI is likely to alter expression of several genes at epigenome level.

In this study, we hypothesized that early-life stress has long-lasting effects on reward-seeking behavior by altering the mesolimbic reward system. We assessed this using the MS + SI paradigm and examined reward-seeking behavior by a conditioned place preference (CPP) test, using chocolate as reward. To clarify whether MS + SI affects the mesolimbic dopamine system, we examined both mRNA and protein levels of tyrosine hydroxylase (TH) in the VTA and dopamine D1 and D2 receptors in the NAc. Furthermore, we evaluated DNA methylation of the D1 receptor promoter region in the NAc to elucidate the mechanism underlying expression of abnormal reward-seeking behavior induced by MS + SI.

2. Materials and methods

2.1. Animals

The numbers of animals used for each experiment are shown in Fig. 1. C57BL/6N female mice at day 14 of pregnancy were purchased from Japan SLC Inc. (Hamamatsu, Japan). Pregnant mice were randomly assigned to control (n = 18) or MS + SI (n = 18) groups and individually housed on a 12-h light/dark cycle (lights on at 08.00 h), in a temperature-controlled facility (23 °C) with 55% relative humidity. Standard laboratory chow and water were given *ad libitum*. The date of birth was designated as postnatal day (PND) 0. All animal protocols were approved by the Animal Care Committee of Nara Medical University and were performed in accordance with the policies established in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Maternal separation coupled with social isolation

The experimental schedule of MS + SI is shown in Fig. 1A and the procedure was performed as described previously [14]. In brief, pups in the MS + SI group were subjected to MS + IS for 3 h (09.30 – 12.30) daily from PND 1–14. During MS + SI sessions, each pup was isolated in a separate cup on a heating pad maintained at 32 °C, separated from the dams. Pups in the control group were left undisturbed with the dam until weaning, except for cage-cleaning once a week. All pups were weaned on PND 21 and housed in same-sex groups of 3 – 5 mice. After weaning, body weight and food consumption were measured at the time of weekly cage-cleaning.

2.3. CPP test

The CPP schedule including the pre-test, conditioning phase, and test and the numbers of animals used are shown in Fig. 1B. The CPP test was performed during the light phase (13.00 – 18.00) with mice (3 – 4 months of age) in a place-conditioning apparatus that consisted of 2 equal-sized compartments (20 × 20 × 25 cm) separated by a sliding door (O'Hara & Co., Ltd, Tokyo, Japan). The compartments had differently colored walls (black or white) and distinct floor textures (bar or grid). Mice were transferred to this room 1 h prior to the start of testing each day. CPP comprised 3 phases on consecutive days: pre-conditioning (2 days), conditioning (12 days), and testing (1 day). During the pre-conditioning phase, the sliding door was open and mice were allowed free access to both compartments for 15 min to determine the baseline box-preference of each mouse. Mice showing a biased baseline preference >80% or a difference in length of stay > 200 s during the pre-conditioning phase were omitted. During the conditioning phase, mice were confined to 1 compartment per day, with compartments alternated daily, by closing the sliding door for 30 min. Confinement to the preferred box was accompanied by access to standard laboratory chow, while confinement to the non-preferred box was accompanied by access to milk chocolate (Meiji Co., Ltd., Tokyo, Japan). On the testing day, the sliding door was opened, and mice were again allowed free access to both compartments for 15 min. Preference scores were expressed as the change in the time (s) spent in the chocolate-paired compartment before and after conditioning.

2.4. Food-choice test

A food-choice test was performed to evaluate the preference for chocolate under conditions in which mice were not required to seek reward (chocolate), by presenting chocolate and standard laboratory chow simultaneously to mice. The test was performed for 6 days continuously (see Fig. 1C), using 3 – 4-month-old female mice naïve to chocolate. Mice were individually transferred to a new cage daily, and given milk chocolate and standard laboratory chow simultaneously for 30 min. Mice were then returned to their home cage and the amounts of the foods consumed were measured. Food-choice ratios were calculated as the amount of chocolate consumed relative to the total amount of food consumed.

2.5. Tissue collection

Mice (11-week-old) were anesthetized and decapitated, and the brains were harvested for real-time polymerase chain reaction (PCR), western blot, and DNA methylation analysis. Brain slices were cut using a linear slicer (Pro.7; DOSAKA EM Co., Ltd., Kyoto, Japan) based on a mouse brain atlas [15], and the 2 slices that included the VTA (Fig. S1A-B, from bregma −3.16 mm, 350-μm thickness) and NAc (Fig. S1C-D, from bregma +1.70 mm, 600-μm thickness) were mounted on a rubber pad. The bilateral VTA was collected using a 21-gauge needle with a rounded tip. The bilateral NAc was collected using a puncher with 1.5-mm diameter after removing the anterior commissure using a 23-gauge needle with a rounded tip. Collected samples were stored at −80 °C until use.

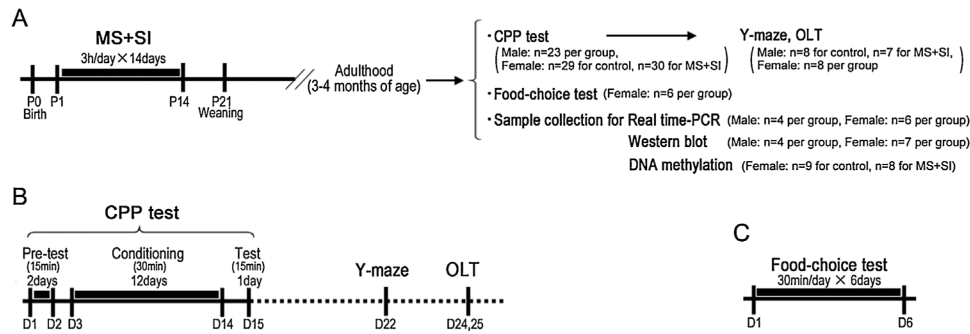

Fig. 1. Summary of experimental design and sample size in each experiment. (A) An illustration for MS + SI paradigm (3 h/day, P1-14) and overview of experimental design. (B) Schedule for CPP test consisting of two times of pre-test (2days), conditioning phase (12days), and test (1 day), followed by Y-maze (1 day) and OLT (2 day). (C) Food choice test were performed for continuous 6 days. P = postnatal day, D = day, MS + SI = maternal separation coupled with social isolation, CPP test = conditioned place preference test, OLT = object location test.

2.6. Real-time PCR

Total RNA was isolated using the Pure Link® RNA Mini Kit (Ambion/Life Technologies, Grand Island, NY, USA) and all RNA samples were determined to have A260/280 values > 1.8 (Nanodrop, ThermoFisherScientific, Waltham, USA). Reverse transcription was performed using the Super Script® VILO™ Master Mix (Invitrogen, Waltham, USA). Gene expression was evaluated using TaqMan Gene Expression Assays (Applied Biosystems, Saint Aubin, France) for *Th* (tyrosine hydroxylase, Mm00447557_m1), *Drd1a* (dopamine D1 receptor, Mm02620146_s1), *Drd2* (dopamine D2 receptor, Mm00438545_m1,) and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase, Mm99999915_g1). Real-time PCR was performed using the Step One Plus™ system (Applied Biosystems). Gene expression was quantified using the 2-$\Delta\Delta$Ct (threshold cycle) method. Ct was defined as the number of cycles required for fluorescent signal intensity to surpass the threshold. ΔCt values were obtained by subtracting the *Gapdh* Ct value from the target gene Ct value, for normalizing to housekeeping gene levels. To calculate between-group differences, the mean ΔCt value of the control group was used to calculate the $\Delta\Delta$Ct of each gene (2-$\Delta\Delta$Ct). Data were expressed as fold-change compared to the control group.

2.7. Western blot

Protein samples were extracted from the NAc using cell lysis buffer (Cell Signaling Technology, Danvers, USA) supplemented with a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). After determining protein concentration, equal amounts of protein (20 μg) were loaded in each lane of a 10% sodium dodecylsulfate-polyacrylamide gel, resolved, and transferred onto a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare, Little Chalfont, UK). Then, the membrane was blocked with 7.5% skim milk and incubated with rabbit anti-D1 receptor (1:8000, Abcam, Cambridge, UK), rabbit anti-D2 receptor (1:5000, Millipore, Darmstadt, Germany), and mouse anti-GAPDH (1:10000, Abcam) overnight at 4 °C, washed, and incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies. Finally, membranes were incubated with Super Signal™ West Dura Extended Duration Substrate (ThermoFisher Scientific) and visualized and analyzed using the Fusion system (Vilber Lourmat, Eberhardzell, Germany).

2.8. Bisulfite sequencing

Genomic DNA was isolated from NAc tissue samples using the NucleoSpin Tissue Kit (Takara Bio Inc., Shiga, Japan) and treated with sodium bisulfite to converted unmethylated cytosine to uracil (leaving 5-methylcytosine unchanged), using the EZ Methylation Kit (Zymo Research Corp., Orange, CA, USA). Bisulfite-treated DNA was then subjected to strand-specific PCR with primers targeting bisulfite-converted CpG islands neighboring the transcription start site of *Drd1a* (GenBank accession no. NM_010076; forward primer: 5′-GTA GAA GGG GAT AGG GAA AAG T-3′; reverse primer: 5′-AAA TCA AAA CTA ACC CTA CCC A-3′). PCR products were separated by electrophoresis on a 1% agarose gel, and specific bands of DNA (431 bp) were extracted and purified. Purified DNA fragments were cloned and transformed (TOPO TA Cloning Kit with the pCR2.1-TOPO Vector and DH5α-T1, Invitrogen). To verify DNA insertion into plasmid constructs, we performed colony PCR with M13 Forward [−20] primer: 5′-GTA AAA CGA CGG CCA G-3′ and M13 Reverse primer: 5′-CAG GAA ACA GCT ATG AC-3′. Only PCR products containing inserted DNA (10 positive DNA fragments per animal) were collected and sequenced (Takara Bio Inc.).

2.9. Statistical analysis

Statistical analyses were performed using JMP8 software (SAS Institute Japan, Tokyo, Japan). Except for DNA methylation analysis, all data were first assessed for normal distribution with the Shapiro – Wilk test, followed by either Student's *t*-tests for parametric or Mann – Whitney *U* tests for non-parametric data. Fisher's exact tests were used for DNA methylation analysis. The level of significance was set at $p < 0.05$. Sample size calculation was not performed and the size of each experiment was determined according to published literature using the same experiments [4,13,16].

3. Results

3.1. The effect of MS + SI on CPP for palatable foods

There were no significant differences in body weight or daily food consumption between MS + SI and control mice of both sexes (Tables S1,S2). During the conditioning phase of CPP, there were no significant differences in consumption of standard laboratory chow and chocolate between control and MS + SI mice of either sex (Fig. 2A for males and Fig. 2C for females). Among males, MS + SI mice showed comparable preference for the chocolate-paired compartment as the control mice (Fig. 2B). In contrast, female MS + SI mice showed significant lower preference for the chocolate-paired compartment than did control female mice (Fig. 2D). Comparing the preference score between male and female in control group, the female score was significantly higher than that of male (n = 23 for male, n = 29 for female; $p < 0.01$). To assess whether the lower CPP score observed in MS + SI females arises from decreased preference for chocolate, the food-choice test was performed under condi-

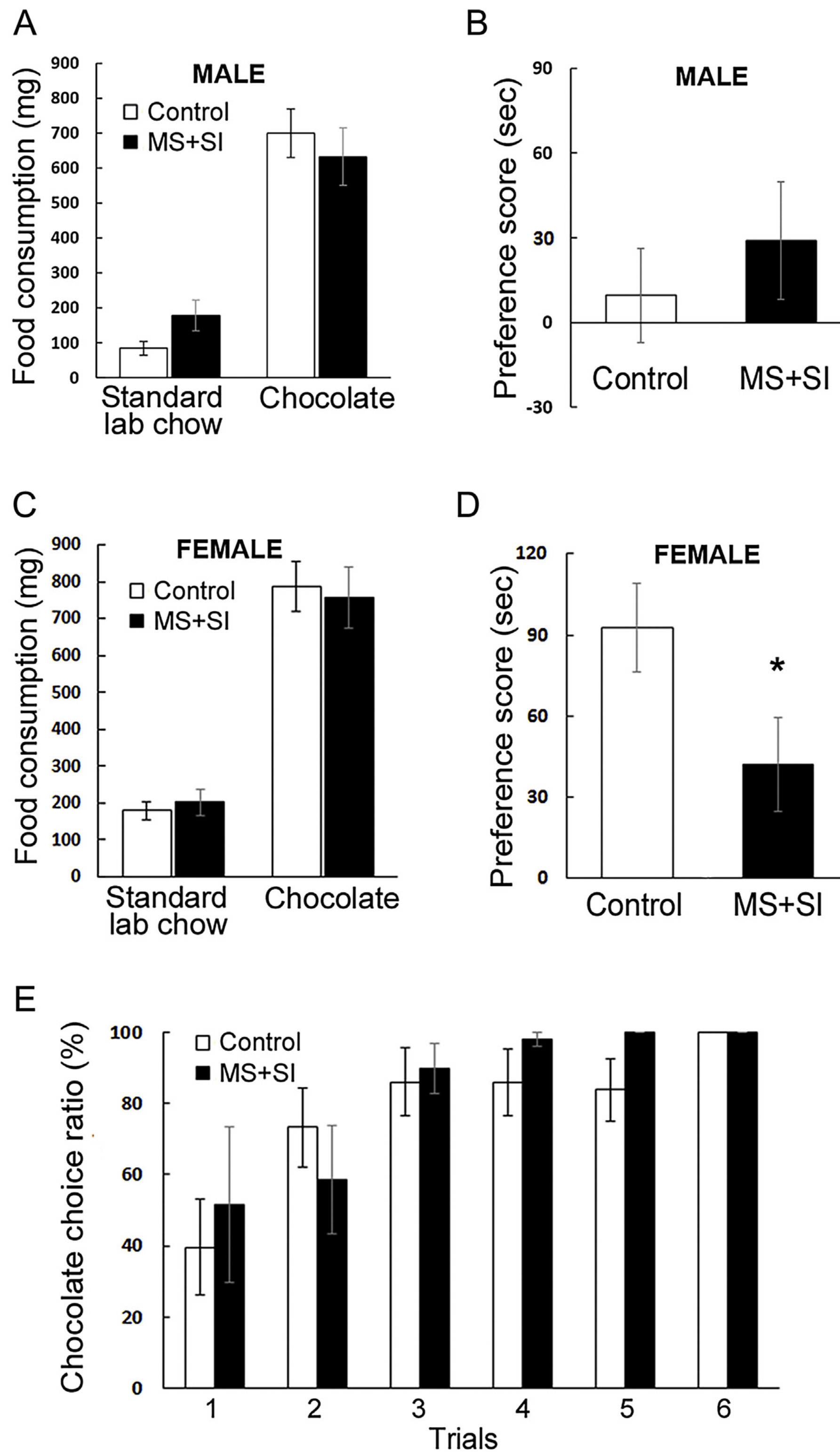

Fig. 2. MS + SI reduces CPP to palatable food in adult female mice. The consumption of standard laboratory chow and chocolate during the CPP conditioning phase in males (A, n = 23 per group, $p > 0.05$) and females (C, n = 29 for control, n = 30 for MS + SI, $p > 0.05$). Chocolate-induced CPP in adult males (B, n = 23 per group, $p = 0.47$) and females (D, n = 29 for control, n = 30 for MS + SI, $p = 0.037$). Preference scores are expressed as the mean ± SEM (s) time spent in the chocolate-paired chamber on the test day minus the time spent in the same chamber during the pretest phase. (E) Choice rates for chocolate in food choice test trials. Data points were calculated as chocolate consumption relative to total food consumption for the 30-min test period (n = 6 per group, $p > 0.05$). * indicates $p < 0.05$ versus control.

tions that did not require mice to seek chocolate. Both control and MS + SI female mice showed a gradual increase in chocolate intake over the initial 3 days, and there were no significant differences in the chocolate choice ratio between control and MS + SI groups at any time point (Fig. 2E). Moreover, although the CPP paradigm is widely used to evaluate preference for reward, the results can be affected by changes in spatial recognition memory [17]. To examine whether the females' lower CPP score was attributable to impaired

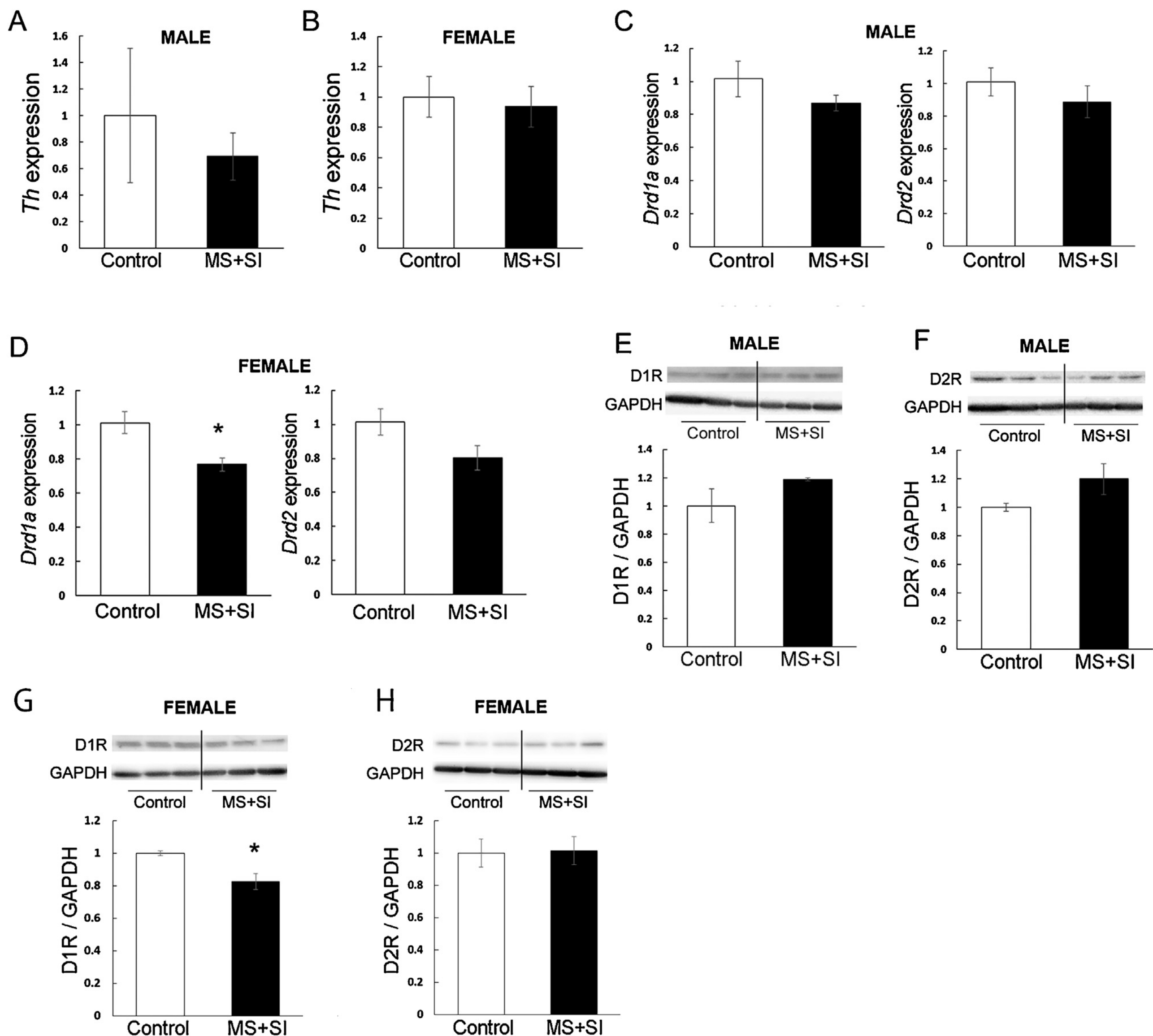

Fig. 3. Effect of MS + SI on mesolimbic dopamine pathway. Real-time PCR analysis of TH mRNA in the VTA of control and MS + SI mice in males (A, n = 4 per group, $p = 0.89$) females (B, n = 6 for control, n = 5 for MS + SI, $p = 0.75$). (C) Real-time PCR analysis of mRNA expression for *Drd1a* and *Drd2*in the NAc of males (n = 4 per group, *Drd1a*, $p = 0.26$; *Drd2*, $p = 0.38$). (D) Real-time PCR analysis of mRNA expression for *Drd1a* and *Drd2*in the NAc of females (n = 4 per group, *Drd1a*, $p = 0.009$; *Drd2*, $p = 0.07$). Western blot analysis of NAc D1 and D2 receptor protein expression in males (E-F, n = 4 per group, D1, $p = 0.16$; D2, $p = 0.13$) and females (G-H, n = 7 per group D1, $p = 0.01$; D2, $p = 0.91$), respectively. All data are presented as the mean ± SEM. * indicates $p < 0.05$ versus control. GAPDH was used as a loading control in real-time PCR and western blot analyses. Data was expressed as fold change versus control group.

spatial recognition memory, we also performed Y-maze and object location tests. Both tests showed no significant changes in spatial recognition memory and locomotor activity in MS + SI females (Fig. S2D-F). Male mice also showed normal behavior in these tests (Fig. S2A-C). These results suggested that MS + SI reduced chocolate-seeking behavior only in female mice; this reduction was not caused by a reduced chocolate preference or impaired spatial recognition memory.

3.2. Effects of MS + SI on mesolimbic dopamine pathway

TH is the rate-limiting enzyme for dopamine synthesis in neurons of the mesolimbic pathway. We quantified TH mRNA expression in the VTA of adult mice to evaluate differences in dopaminergic function. There were no significant differences in TH mRNA levels between MS + SI and control mice of either sex (Fig. 3A,B). The expression of *Drd1a* and *Drd2* mRNA in the NAc were also investigated; there were no significant differences in the expression of these genes at mRNA (Fig. 3C) or in the protein levels of D1 (Fig. 3E) and D2 receptors in male mice (Fig. 3F). However, MS + SI females exhibited reduced *Drd1a* mRNA expression relative to control mice, while there was no significant difference in *Drd2* mRNA expression between these 2 groups (Fig. 3D). Moreover, D1 receptor protein levels were also significantly decreased in MS + SI female mice (Fig. 3G), while those of the D2 receptor was not changed (Fig. 3H). These findings indicated that D1 receptor expression in the NAc was reduced by MS + SI exposure in female mice.

3.3. DNA methylation at the D1 receptor promoter region in the NAc

We next examined DNA methylation patterns across CpG islands of *Drd1a* in the NAc. Overall, CpG methylation of *Drd1a* in

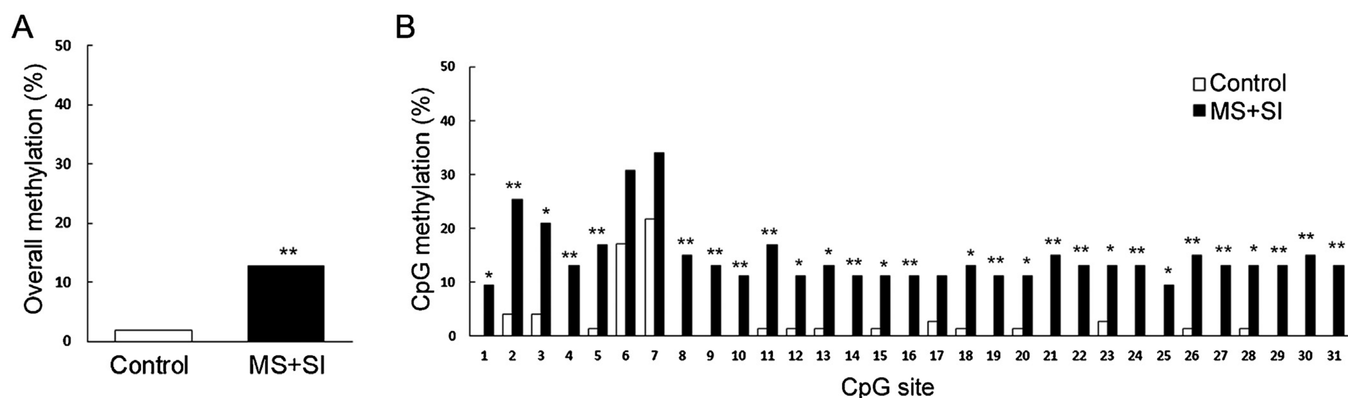

Fig. 4. Methylation patterns across *Drd1a* CpG islands in female MS + SI mice. CpG methylation profiles of the *Drd1a* CpG islands in the NAc using bisulfate sequencing. (A) Overall methylation is presented as the percentage of methylated sites (n = 9 for control, n = 8 for MS + SI, $p < 0.0001$). (B) Individual CpG methylation statuses are presented as the percentage of methylated sites (n = 9 for control, n = 8 for MS + SI, 31 CpG sites). * indicates $p < 0.05$ versus control and ** indicates $p < 0.01$ versus control, Fisher's exact test.

MS + SI female mice was significantly increased compared with that in control mice (Fig. 4A, control: 1.9%, MS + SI: 12.7%, $p < 0.0001$). A detailed analysis revealed that 29 of 31 CpG sites within *Drd1a* were more frequently methylated in MS + SI mice than in control mice (Fig. 4B, CpG sites 2, 4, 5, 8 – 11, 14, 16, 19, 21, 22, 24, 26, 27, and 29–31, $p < 0.01$; CpG sites 1, 3, 12, 13, 15, 18, 20, 23, 25, and 28, $p < 0.05$).

4. Discussion

The developing brain is sensitive to environmental influences, and early life experiences during heightened periods of brain plasticity can affect lifelong structural and functional aspects of the brain and behavior [1,18]. The present study demonstrated that MS + SI reduced chocolate-seeking behavior in female mice, but had no effect on such behavior in male mice. Similar sex differences in the effect of early-life stress on reward-seeking behavior have been reported previously using a different MS paradigm in which pups are separated from the dam for 6 h daily from PND 5 – 20 [19], cross-fostering stress [5], and prenatal restraint stress reduced food-induced reward-associated behavior in female mice [4]. However, it should be noted that CPP scores measured using chocolate as a reward were innately higher in females than in males, as also found previously [20]. Such innate sex differences in chocolate-induced CPP seem to be relevant to both sex hormones and the levels of D1 receptor expression, because previous studies have suggested that chocolate-induced CPP is reduced in ovariectomized females [4] and D1 receptor expression in the NAc is innately higher in females than in males [20,21]. Regarding the effect of MS + SI on sex hormones, a previous study had reported that MS + SI from PND 2–14 did not affect sex hormone levels in adulthood [22]. Although sex hormone levels in MS + SI females should be investigated in future, current evidence suggests that the reduction of CPP scores in MS + SI females is associated with downregulation of D1 receptor expression in the NAc, rather than with changes in sex hormones.

The neural connection between the VTA and NAc is known to be reciprocal. The NAc is connected to the VTA via 2 different (direct and indirect) pathways. In the direct pathway, D1 receptor-expressing NAc neurons directly project to the VTA, while in the indirect pathway, D2 receptor-expressing neurons of the NAc project to ventral pallidum neurons that innervate the VTA [23]. Previous studies implied that the direct pathway plays a critical role in reward-associated behaviors and D1 receptor-expressing neurons respond to reward stimuli, while D2 receptor-expressing neurons in the NAc mainly respond to aversive stimuli [24,25]. Our results showed that MS + SI reduced the expression of D1 receptor without affecting the D2 receptor in female mice. This change in the D1 and D2 receptor expression pattern is thought to be consistent with the behavioral results shown by CPP in our study.

Various MS paradigms are now used in studies of early-life stress, and differences in the experimental conditions sometimes have different consequences. For example, MS + SI, but not MS, attenuated stress response induced by novel environment exposure in juvenile period [26]. However, MS + SI and MS likely have similar effects on reward-seeking behavior, as shown by a previous study reporting that MS without SI also reduced reward-seeking behavior [19]. Furthermore, differences in the separation duration can also yield different results; for example, a brief separation of 15 min daily during PND 1–21 increased D1 receptor expression in the NAc [8], while MS for 6 h daily for the first 3 weeks reduced the D1 receptor expression. Together, these findings suggest that D1 receptor expression level in the NAc is susceptible to early-life stress and can be either upregulated or downregulated depending on MS conditions.

Animal studies have improved our knowledge of gene-environment interactions and elucidated the pathways that program animal brain and behavior in response to early life experiences [27]. Epigenetic mechanisms involving DNA methylation are major candidates for the integration of intrinsic and environmental signals in the genome [28]. The present study revealed that MS + SI increases DNA methylation at the promoter of the *Drd1a* in the NAc in adult females. Importantly, previous studies have suggested that MS caused increased DNMT levels in infant rats, which persist into adulthood [10–12]. In addition, glucocorticoids also act to alter DNA methylation [29,30]. Our previous study showed that MS + SI markedly increased plasma corticosterone levels during separation [14] and also heightened the basal corticosterone level in adulthood [31]. Thus, corticosterone increases during MS + SI and/or in adulthood might be related to DNA methylation of the *Drd1a* via the upregulation of DNMTs.

5. Conclusion

The present study indicated that MS + SI reduced chocolate-induced CPP and decreased D1 receptor expression in the NAc in adult female mice. Furthermore, hypermethylation of the *Drd1a* promoter region was raised as a possible mechanism for downregulation of D1 receptor expression in the NAc of female mice. These results suggest that early-life stress has long-lasting effects on the mesolimbic dopamine system via epigenetic modification, which could lead to behavioral changes in reward-seeking.

Conflicts of interest

The authors declare no competing financial or personal interests.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2017.01.025.

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