Original Article
https://doi.org/10.9758/cpn.2022.20.2.373
Clinical Psychopharmacology and Neuroscience 2022;20(2):373-388
pISSN 1738-1088 / eISSN 2093-4327

Risperidone Induced DNA Methylation Changes in Dopamine Receptor and Stathmin Genes in Mice Exposed to Social Defeat Stress

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Objective: Understanding complex epigenetic mechanisms is necessary to fully elucidate the effects of antipsychotic drug. This study investigated DNA methylation and mRNA expression levels of dopamine D2 and D1 receptor (Drd2 and Drd1, respectively), nuclear receptor subfamily 3, group C, member 1 (Nr3c1) and stathmin 1 (Stmn1) in brain regions of mice exposed to social defeat stress (SDS) and effects of risperidone on altered methylation and mRNA expression levels induced by SDS.
Methods: Following SDS for 10 days, risperidone (0.2 mg/kg) or vehicle was administered to adult mice for 7 days. Brain tissues from the prefrontal cortex (PFC), hippocampus (HIP) and amygdala (AMY) were processed to measure methylation and mRNA levels of Drd2, Drd1, Nr3c1 and Stmn1 using pyrosequencing and real time-polymerase chain reaction.
Results: We found altered methylation status of Nr3c1 and Stmn1 in the HIP and AMY of mice exposed to SDS. These changes were reversed by risperidone treatment. In addition, different methylation patterns of Drd2 and Drd1 in the PFC and AMY between defeated and control mice were identified with risperidone treatment.
Conclusion: These findings suggest that risperidone can cause epigenetic changes in Drd2, Drd1, Nr3c1 and Stmn1 in defeated mice. These changes could be epigenetic mechanisms underlying antipsychotic efficacy.

KEY WORDS: Social defeat stress; Risperidone; DNA methylation; Drd2; Nr3c1; Stmn1.

INTRODUCTION

Evidence pertaining to the specific effects of antipsychotics on epigenetics comes mostly from DNA methylation studies. Animal methylation studies measured changes of DNA methylation levels induced by antipsychotics at specific CpG sites of candidate gene promoters or methylome-wide CpG sites [1-3]. The genes of interest were related to the GABAergic system [4,5], glycine receptor subunit alpha-1 [6] and the cadherin gene family [7]. Most of the studies compared epigenetic effects between antipsychotics and vehicle [1-3,7,8], although a few looked at the reversal/ameliorating effect of antipsychotics on the altered methylation induced by stress [5] and methionine treatment [4]. Clozapine has consistently shown a demethylating effect, whereas other agents such as haloperidol and olanzapine showed mixed results [9]. Risperidone (RIS) is the first second-generation antipsychotic specifically designed as a combined dopamine D2 receptor (Drd2) and serotonin (5-hydroxytryptamine)2A receptor antagonist, and has superior efficacy to first-generation antipsychotics (quetiapine, aripiprazole and ziprasidone) [10]. To date, only one study has measured the effect of risperidone on DNA methylation, in 84 neurotransmitter genes in rat [6].

Social defeat stress (SDS), which is induced in the resident/intruder paradigm, causes a variety of molecular, physiological, and behavioral changes (for a review, see [11]). Because of its ethologically salient characteristics, it

Received: May 20, 2021 / **Revised:** July 11, 2021
Accepted: July 12, 2021
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is a good model for investigating the etiology of stress-related disorders in humans and has been widely used as an animal model of depression, anxiety disorders and psychosis [12-14]. Especially, this model could be useful to investigate impact of environmental factors associated with schizophrenia given that social defeat results in deficits in prepulse inhibition [15], an enhanced mesocorticolimbic dopamine response [16,17], increased phasic activity in ventral tegmental area dopaminergic neurons [18], reductions in striatal dopamine transporter binding [19], and behavioral and neuronal cross-sensitization to amphetamine [20]. These evidences prompted us to hypothesize that antipsychotics may prevent dopamine-related changes induced by SDS. Several studies measured the impact of SDS on DNA methylation using target genes [21-24] or the genome-wide approach [25,26]. However, to the best of our knowledge, no study has identified methylation changes induced by antipsychotics in rodents exposed to SDS. The target genes in the present study were Drd2, dopamine D1 receptor (Drd1), nuclear receptor subfamily 3, group C, member 1 (Nr3c1) and stathmin 1 (Stmn1). The Drd2 and Drd1 are closely associated with action mechanisms of antipsychotics and pathophysiology of schizophrenia [27,28]. The regulation of Nr3c1, which is a glucocorticoid receptor (GR) gene, is important for adaptation to stress [29]. Stmn1 produces a protein critical for microtubule (MT) polymerization, and is also involved in fear processing in both mice [30] and humans [31]. Given that antipsychotics have anti-stress properties [32] and reduce fear and anxiety [33], we hypothesized that altered methylation induced by SDS could be attenuated by administration of antipsychotics. The aims of present study were to investigate the DNA methylation and mRNA expression levels of Drd2, Drd1, Nr3c1 and Stmn1 in brain regions of mice exposed to SDS, and the effects of risperidone on the changes of methylation and mRNA expression levels induced by SDS.

METHODS

Animals

All experiments were conducted using young adult male C57BL/6J mice and old male CD1 (ICR) mice (Orient Company, Seongnam, Korea) aged 6 and 15 weeks, respectively, and weighing 18−22 and 40−44 g, respectively. The C57BL/6J mice were group-housed while the CD1 mice were single-housed. All procedures were conducted in strict accordance with the guidelines for animal experiments of the Institutional Animal Care and Use Committee (IACUC) of Jeonbuk National University and the National Institutes of Health (NIH) principles for the Care and Use of Laboratory Animals based on the 3Rs (replacement, refinement, and reduction). The project was reviewed and approved by the IACUC (cuh-IACUC-151027-32) of Jeonbuk National University Medical School (Care and Animals 1986).

Study Design

Following the 1-week habituation period, C57BL/6J mice were subjected to chronic social defeat for 10 consecutive days. The defeated mice were categorized into susceptible (SUS) and unsusceptible (UNS) groups based on their performance in the social avoidance test. After 1 week of risperidone administration (0.2 mg/kg, intraperitoneal [i.p.]), mice were sacrificed and brain tissues were obtained for the molecular studies (Fig. 1).

Social Defeat Stress

The mice were exposed to SDS via the resident-intruder paradigm. Specifically, C57BL/6J mice (n = 50) experienced 10 days of SDS via confrontations with an aggressive and larger CD-1 mouse that was approximately 16 weeks old. All male CD1 mice were screened for aggressiveness by measuring the latency to attack a naive C57BL/6J mouse. Only CD1 mice that attacked in less than 60 seconds in at least two consecutive 180 seconds screening sessions (among three sessions) were used.

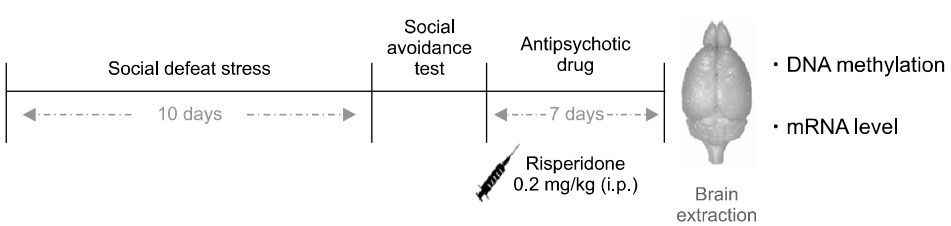

Fig. 1. Timeline of the experimental procedure.

The C57BL/6J mice were introduced into the home cage of the unfamiliar CD1 aggressor mouse and allowed to interact for 10 minutes. After 10 minutes of full interaction, the defeated mouse was separated from the aggressive resident by inserting a perforated Plexiglas divider into the cage, which also allowed for sensory contact for the rest of the day. On the subsequent day, the C57BL/6J mouse was exposed to a new resident CD1 aggressor mouse to prevent habituation. The social defeat procedure lasted for 10 consecutive days. As a control (CON) group, C57BL/6J mice (n = 20) were placed into equivalent cages with members of the same strain, which were changed daily.

Social Avoidance Test

Following completion of the social defeat procedure, the social avoidance test was performed on day 11 of the study, to categorize the mice into UNS and SUS groups. Each defeated mouse was placed into an interaction box (42 × 42 cm) that consisted of a wire mesh cage (10 × 4.5 cm) located at one end and an interaction zone (8-cm wide) surrounding the cage. The test comprised two sessions, separated by a 1-minute interval. In the first session, no CD1 mouse was present in the wire mesh cage and the movement of the defeated animal was tracked for 2.5 minutes. In the second session, a novel CD1 mouse was introduced into the wire mesh cage and the same defeated animal from the first session was placed into the box and tracked for a further 2.5 minutes. The total time spent by the experimental mouse in the 8-cm-wide corridor surrounding the wire mesh cage (interaction zone) was calculated automatically using SMART software (Panlab, Barcelona, Spain) and a social interaction (SI) ratio was derived as follows: 100 × (interaction time with target mouse present) / (interaction time with no target mouse present). Based on previous studies [34], a SI ratio of 100 was used as the cut-off value, such that scores $<$ 100 were defined as "SUS" and scores $\geq$ 100 as "UNS". As UNS and SUS mice could be powerful tools for studying the mechanisms underlying individual differences in stress resiliency and susceptibility [34], two groups were all used in the experiment.

Drug Administration

Male C57BL/6J mice were assigned to six groups (n = 7 per group): CON-Vehicle (VEH), UNS-VEH, SUS-VEH, CON-Drug (DRG), UNS-DRG and SUS-DRG. RIS dissolved in 0.1% tartaric acid (0.2 mg/kg) or VEH was given once daily for 7 days. The dose of risperidone was chosen based on the previous study on DNA methylation [8]. All of the solutions were administered via i.p. injection in a volume of 10 ml/kg.

Brain Tissue Collection

After drug administration, the mice were euthanized via cervical dislocation. The prefrontal cortex (PFC), hippocampus (HIP) and amygdala (AMY) were dissected using micro-spatulas on an ice plate. The tissues (15−18 mg of the PFC, 4−5 mg of the AMY, and 18−22 mg of the HIP) were quickly cryopreserved in liquid nitrogen and stored at −80°C until assay.

DNA Methylation Analysis

DNA extraction and bisulfite treatment

Genomic DNA was isolated from samples of the PFC, HIP, and AMY using DNase Blood & Tissue Kits (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Subsequently, bisulfite conversion of 500 ng of genomic DNA was achieved using the EpiTect bisulfite kit (QIAGEN) according to the manufacturer's instructions.

Bisulfite pyrosequencing

DNA methylation was measured by pyrosequencing the polymerase chain reaction (PCR) products. Primers were designed against the putative promoter and first intron region of the Drd2 (Fig. 2), Drd1 (Fig. 3) and Stmn1 (Fig. 4) genes, which were assumed to be located between positions −1 kb and +500 bp of the transcription start site (TSS). For Nr3c1 (Fig. 5), a primer was designed to span CpG sites in the promoter region (exon 17) of the GR [35], which has been extensively studied with regard to stress. Several regions were initially designed using PyroMark Assay Design 2.0 software (QIAGEN): for Drd2 and Stmn1, we had five and eight regions, respectively. Afterwards, regions that had more transcription factor binding sites were selected; regions 1 (CpG1 and 2) and 4 (CpG3−7) for Drd2, and regions 5 (CpG1−7) and 6 (CpG8−11) for Stmn1, using JASPAR (http://jaspar.genereg.net/). (Fig. 2−5). Details about the PCR primers and sequencing primer are shown in Supplementary Table 1 (available online).

Next, 40 ng of bisulfite-treated DNA was amplified in a

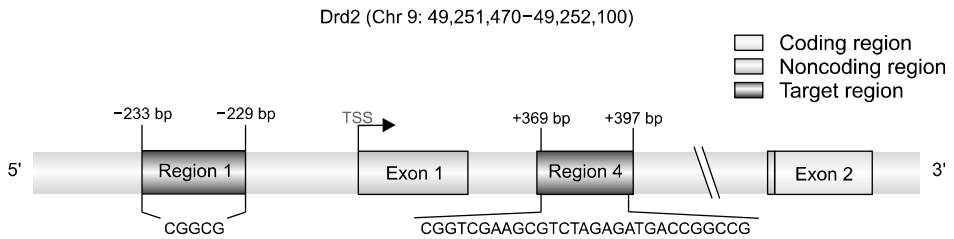

Fig. 2. Schematic representation of the mouse Drd2 gene, including the CpG island that extends from the promoter "region 1" (CpG 1 and 2) into the first intron "region 4" (CpG 3 to CpG 7).
CpG, cytosine-phosphate-guanine; TSS, transcription start site; Drd2, dopamine receptor D2.

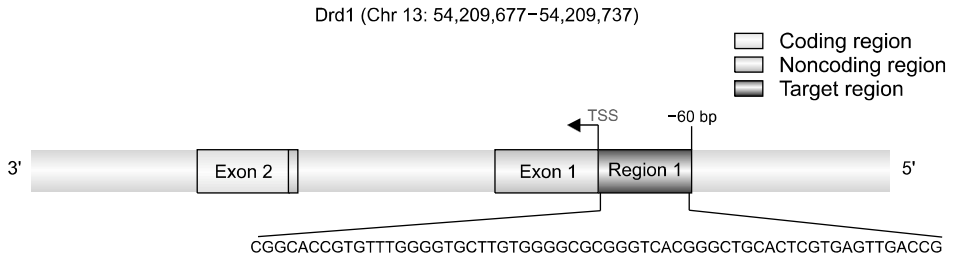

Fig. 3. Schematic representation of the mouse Drd1 gene, including the CpG island at the promoter "region 1" (CpG 1 to CpG 7).
CpG, cytosine-phosphate-guanine; TSS, transcription start site; Drd1, dopamine receptor D1.

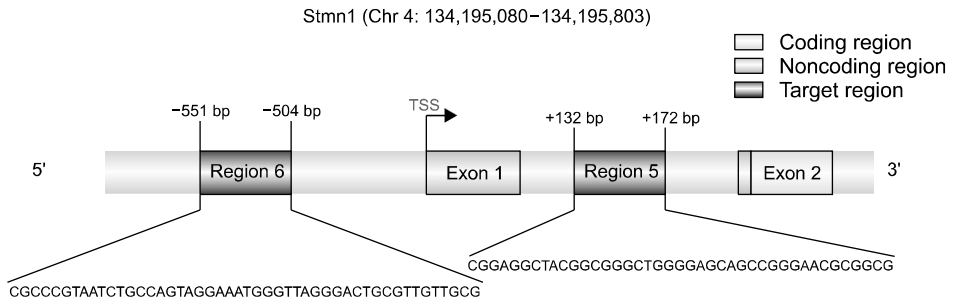

Fig. 4. Schematic representation of the mouse Stmn1 gene, including the CpG island that extends from the promoter "region 6" (CpG 8 to CpG 11) into the first intron "region 5" (CpG 1 to CpG 7).
CpG, cytosine-phosphate-guanine; TSS, transcription start site; Stmn1, stathmin 1.

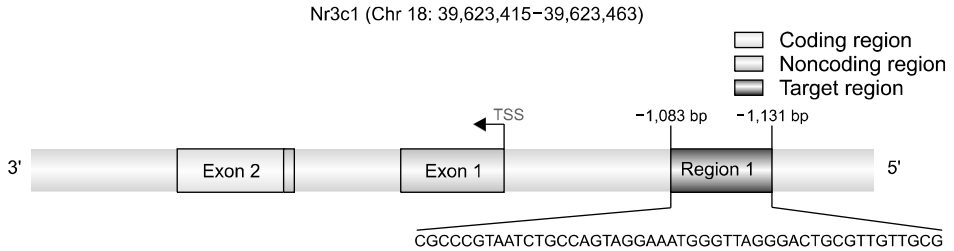

Fig. 5. Schematic representation of the mouse Nr3c1 gene, including the CpG island at the promoter "region 1" (CpG 1 to CpG 8).
CpG, cytosine-phosphate-guanine; TSS, transcription start site; Nr3c1, nuclear receptor subfamily 3 group c member 1.

25-µl reaction volume using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Either the forward or reverse primer was biotinylated to convert the PCR product to single-stranded DNA templates, or a sequencing primer that annealed to the single-stranded DNA template was then added [36]. The pyrosequencing reactions were performed in a PyroMark Q48 Autoprep system (QIAGEN) and quantification of the CpG methyl-

ation level (percentage of the relative light unit [RLU] of the C peak [methylated cytosine]/RLU of C peak + T peak [unmethylated cytosine]) was performed with PyroMark Q48 Autoprep 2.4.2 software (QIAGEN). When the peak value of a base exceeded 20 RLU, the pyrosequencing results were considered to be reliable.

Pyrogram results were reanalyzed if they did not meet the following criteria: in the overlapped histogram of the expected and actual results, the magnitude of the RLU difference of any mismatched peaks among all samples was $>$ 20 RLU; the background peak was inconsistent among the samples and the RLU of a background peak was $>$ 7% of the mean RLU of a single peak; the analysis for a certain CpG site failed due to the preceding polybases ($\geq$ three identical bases) and the peak heights among all samples for that CpG site were inconsistent; and any peak showed a double-peaked structure.

Real-time Polymerase Chain Reaction

Total RNA from the PFC, HIP, and AMY was extracted using Isol-RNA Lysis reagent according to the manufacturer's protocol (5 PRIME, Gaithersburg, MD, USA) and RNA quantitation was carried out using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). We followed standard RT-PCR procedures. RT-PCR was performed in triplicate using the following sequences of the primer: D2L (F): AACTGTACCCACCCTGAGGA, D2L (R): GTTGCTATGTAGACCGTG; D2S (F): CACCACTCAAGG ATGCTGCCC, D2S (R): GTTGCTATGTAGACCGTG; Drd1 (F): CCAGACCACCACAGGTAAT, Drd1 (R): CCACAG AAGGGCACCATA; Stmn1 (F): GTTCGACATGGCATCT TCTGAT, Stmn1 (R): CTCAAAAGCCTGGCCTGAA; Nr3c1 (F): AGCTCCCCCTGGTAGAGAC, Nr3c1 (R): GGTGAA GACGCAGAAACCTTG; β-actin (F): CTGACAGACTAC CTCATGAAGATCC, β-actin (R): AGTCTAGAGCAACATAGC ACAG. Relative mRNA expression for the UNS or SUS group was determined using the $2^{-\Delta\Delta Ct}$ value of each group (subtracting ΔΔCt of CON from ΔΔCt of the UNS or SUS group).

Table 1. Effects of risperidone on DNA methylation of Drd2 gene in the three brain regions of mice exposed to social defeat stress

Region	CpG sites	Vehicle			p value	Drug			p value
		CON (n = 6)	UNS (n = 6)	SUS (n = 6)		CON (n = 6)	UNS (n = 6)	SUS (n = 6)	
PFC	CpG1	12.23 ± 0.59	12.97 ± 0.68	13.11 ± 0.67	0.476	12.14 ± 0.58	12.46 ± 0.38	13.62 ± 1.18	0.484
	CpG2	10.33 ± 0.77	9.72 ± 0.29	10.20 ± 1.00	0.587	9.20 ± 0.81	9.52 ± 0.53	10.03 ± 1.17	0.834
	CpG3	4.13 ± 0.38	4.58 ± 0.34	4.06 ± 0.21	0.359	4.05 ± 0.36	4.71 ± 0.20	3.64 ± 0.40	0.090
	CpG4	5.11 ± 0.38	5.35 ± 0.35	4.53 ± 0.21	0.267	4.56 ± 0.45	5.49 ± 0.24	4.66 ± 0.39	0.164
	CpG5	3.49 ± 0.34	3.78 ± 0.25	2.93 ± 0.26	0.090	3.36 ± 0.24	3.83 ± 0.18	2.63 ± 0.20*	0.046
	CpG6	6.29 ± 0.54	7.65 ± 0.36†	5.08 ± 0.56	0.019	6.37 ± 0.57	6.16 ± 0.11	6.42 ± 0.51	0.738
	CpG7	7.01 ± 0.49	8.20 ± 0.44†	6.34 ± 0.38	0.029	6.65 ± 0.69	7.17 ± 0.44	6.87 ± 0.50	0.523
	Mean	6.94 ± 0.30	7.46 ± 0.21	6.61 ± 0.35	0.140	6.62 ± 0.35	6.91 ± 0.17	6.84 ± 0.43	0.777
HIP	CpG1	12.91 ± 1.43	13.04 ± 1.18	12.98 ± 1.74	0.854	11.00 ± 0.37	11.58 ± 0.87	10.59 ± 1.02	0.548
	CpG2	9.89 ± 0.92	9.43 ± 1.50	10.93 ± 1.22	0.567	8.63 ± 0.78	9.32 ± 1.17	9.74 ± 0.62	0.519
	CpG3	4.21 ± 0.31	3.82 ± 0.26	4.65 ± 0.46	0.399	4.47 ± 0.37	4.40 ± 0.49	4.04 ± 0.22	0.834
	CpG4	4.01 ± 0.26	5.63 ± 0.53	4.23 ± 0.25	0.069	4.56 ± 0.57	4.80 ± 0.35	4.50 ± 0.67	0.580
	CpG5	2.59 ± 0.16	3.29 ± 0.37	3.31 ± 0.47	0.390	3.14 ± 0.19	3.15 ± 0.33	2.99 ± 0.24	0.834
	CpG6	5.64 ± 0.29	6.08 ± 0.67	5.76 ± 0.34	0.927	5.81 ± 0.53	5.81 ± 0.53	5.87 ± 0.64	0.977
	CpG7	6.54 ± 0.54	5.98 ± 0.84	6.11 ± 0.69	0.911	6.82 ± 0.43	5.45 ± 0.63	7.43 ± 0.46	0.071
	Mean	6.54 ± 0.31	6.75 ± 0.44	6.85 ± 0.59	0.949	6.35 ± 0.33	6.36 ± 0.43	6.45 ± 0.25	0.983
AMY	CpG1	14.00 ± 1.36	11.83 ± 0.78	12.59 ± 0.56	0.372	12.92 ± 1.18	14.68 ± 0.47†	12.01 ± 0.47	0.034
	CpG2	11.62 ± 1.59	10.86 ± 1.07	10.10 ± 0.59	0.864	12.34 ± 0.96	11.86 ± 0.80	9.17 ± 0.81*	0.039
	CpG3	4.13 ± 0.31	4.18 ± 0.33	4.51 ± 0.22	0.421	3.90 ± 0.24	4.22 ± 0.44	4.52 ± 0.30	0.331
	CpG4	5.08 ± 0.45	5.56 ± 0.79	5.01 ± 0.09	0.630	4.92 ± 0.35	4.37 ± 0.31	5.18 ± 0.19	0.185
	CpG5	3.96 ± 0.61	3.48 ± 0.40	3.64 ± 0.13	0.738	2.77 ± 0.14	2.83 ± 0.15†	3.89 ± 0.28*	0.008
	CpG6	6.19 ± 0.35	5.82 ± 0.44	6.58 ± 0.36	0.359	5.77 ± 0.46	6.08 ± 0.54	6.55 ± 0.26	0.372
	CpG7	6.27 ± 0.40	7.16 ± 0.66	7.17 ± 0.27	0.191	7.77 ± 0.66	7.79 ± 0.75	7.70 ± 0.55	0.983
	Mean	7.32 ± 0.38	6.98 ± 0.45	7.09 ± 0.18	0.641	7.20 ± 0.42	7.40 ± 0.22	7.00 ± 0.35	0.587

Data were expressed in mean ± standard error of the mean.
AMY, amygdala; CON, control; HIP, hippocampus; PFC, prefrontal cortex; SUS, susceptible; UNS, unsusceptible; Drd2, dopamine receptor D2.
*$p < 0.05$ vs. control group; †$p < 0.05$ vs. susceptible group by Bonferroni *post-hoc* test.

Statistical Analysis

Our main goal was to investigate the effects of SDS in normal mice and risperidone in defeated mice. For both DNA methylation and mRNA data, the Kruskal−Wallis test was employed with the Bonferroni *post-hoc* test, because of the non-normal data distribution. In cases without significant *post-hoc* results, the false discovery rate was calculated. Methylation data were analyzed by two-way analysis of variance (ANOVA) with group (CON, UNS, and SUS) and treatment (vehicle and drug) as the main effects and methylation percentage as the dependent variable. If an interaction or main effect was significant, appropriate pairwise comparisons were performed using Tukey's honest significant difference test. All results are presented as mean ± standard error of the mean. Statistical significance was defined as $p \leq 0.05$. Graphs were drawn using GraphPad Prism software (version 9.1; GraphPad Software Inc., San Diego, CA, USA).

RESULTS

During the social defeat procedure, all CD1 mice attacked and defeated the intruder C57BL/6J mice, of which all showed signs of subordination. Five mice were later found dead. The remaining defeated mice (n = 45) were subjected to the social avoidance test. Following this procedure, 55.7% of mice were classified as SUS (n = 24) and 44.3% as UNS (n = 21). For the results of the two-way ANOVA, see Supplementary Tables 2−6 (available online).

Effects of Social Defeat Stress on Methylation Levels

For Drd2, significant differences were found in CpG 6 ($p = 0.019$) and CpG 7 ($p = 0.029$) of the PFC among the three groups (Table 1, Fig. 6). Post hoc analyses revealed significantly increased methylation levels in CpG6 ($p = 0.015$) and CpG7 ($p = 0.024$) in the UNS group compared to the SUS group. For Drd1, no significant differences

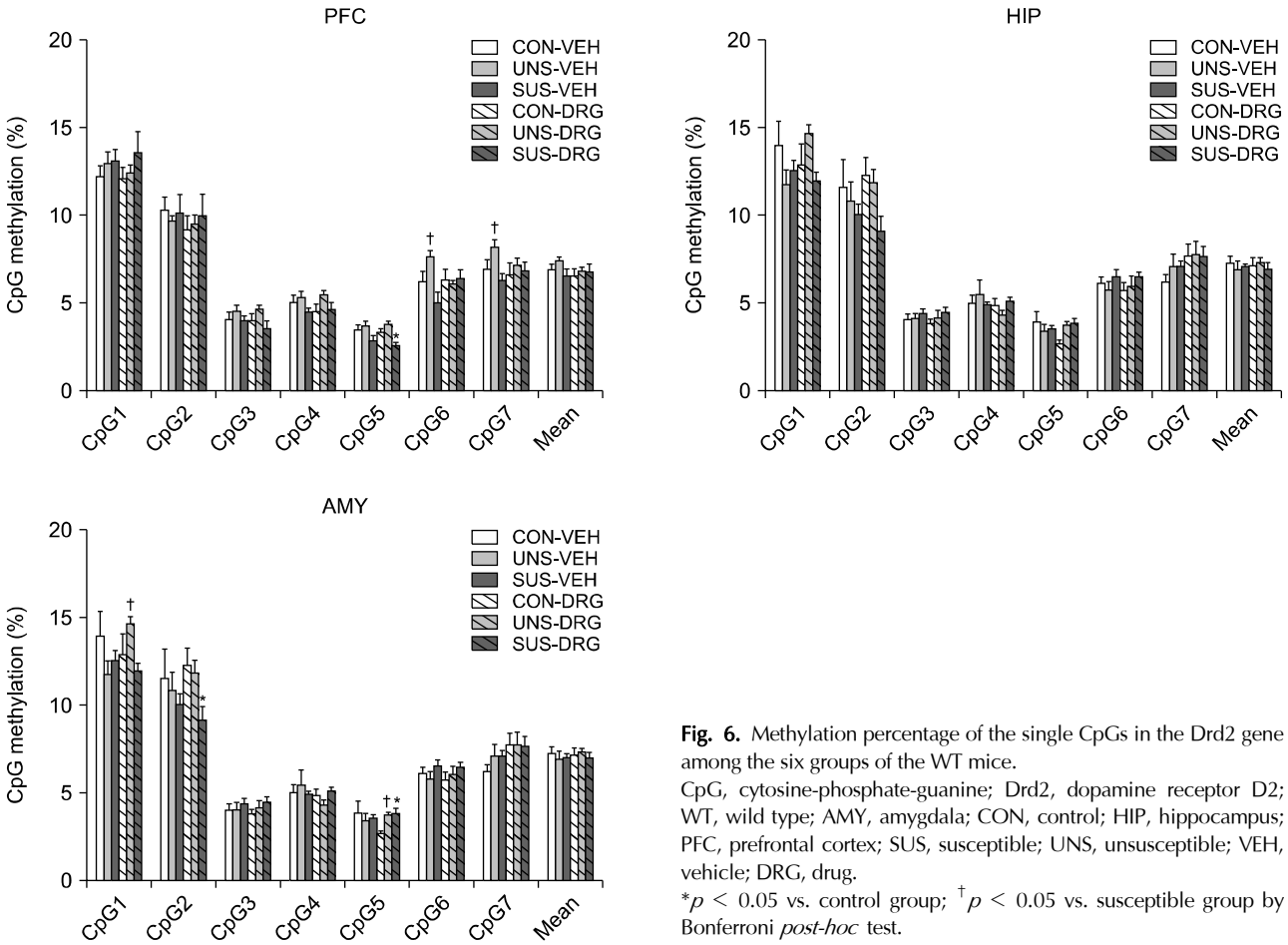

Fig. 6. Methylation percentage of the single CpGs in the Drd2 gene among the six groups of the WT mice.
CpG, cytosine-phosphate-guanine; Drd2, dopamine receptor D2; WT, wild type; AMY, amygdala; CON, control; HIP, hippocampus; PFC, prefrontal cortex; SUS, susceptible; UNS, unsusceptible; VEH, vehicle; DRG, drug.
*$p < 0.05$ vs. control group; †$p < 0.05$ vs. susceptible group by Bonferroni *post-hoc* test.

Table 2. Effects of risperidone on DNA methylation of Drd1 gene in the three brain regions of mice exposed to social defeat stress

Region	CpG sites	Vehicle			p value	Drug			p value
		CON (n = 6)	UNS (n = 6)	SUS (n = 6)		CON (n = 6)	UNS (n = 6)	SUS (n = 6)	
PFC	CpG1	1.99 ± 0.19	1.55 ± 0.13	1.91 ± 0.16	0.125	2.17 ± 0.40	1.74 ± 0.14	2.58 ± 0.50	0.241
	CpG2	2.03 ± 0.16	1.83 ± 0.13	2.05 ± 0.19	0.641	2.12 ± 0.16	1.80 ± 0.08	2.03 ± 0.29	0.330
	CpG3	1.05 ± 0.24	1.16 ± 0.07	1.30 ± 0.13	0.519	1.00 ± 0.06	1.17 ± 0.11	1.52 ± 0.31	0.120
	CpG4	1.08 ± 0.10	1.04 ± 0.12	1.16 ± 0.05	0.828	1.13 ± 0.09	0.99 ± 0.12	1.41 ± 0.27	0.421
	CpG5	1.08 ± 0.04	0.97 ± 0.15	1.01 ± 0.25	0.612	1.08 ± 0.20	1.17 ± 0.12	1.71 ± 0.38	0.235
	CpG6	1.96 ± 0.21	2.05 ± 0.11	1.94 ± 0.09	0.806	2.22 ± 0.11	1.88 ± 0.10	2.72 ± 0.49	0.082
	CpG7	2.22 ± 0.21	2.10 ± 0.12	1.94 ± 0.12	0.559	1.97 ± 0.09	1.93 ± 0.12 ‡	2.98 ± 0.40 †	0.041
	Mean	1.63 ± 0.12	1.52 ± 0.07	1.61 ± 0.08	0.805	1.67 ± 0.11	1.52 ± 0.05	2.13 ± 0.36	0.312
HIP	CpG1	2.10 ± 0.19	2.35 ± 0.22	1.95 ± 0.14	0.348	1.99 ± 0.11	2.12 ± 0.14	1.75 ± 0.05	0.079
	CpG2	1.98 ± 0.15	2.17 ± 0.29	1.78 ± 0.15	0.621	1.81 ± 0.10	1.70 ± 0.12	1.81 ± 0.11	0.854
	CpG3	1.45 ± 0.21	1.48 ± 0.20	1.24 ± 0.10	0.574	1.22 ± 0.15	1.30 ± 0.07	1.23 ± 0.17	0.834
	CpG4	1.19 ± 0.11	1.46 ± 0.26	0.98 ± 0.04	0.284	1.12 ± 0.14	0.96 ± 0.05	1.18 ± 0.06	0.164
	CpG5	1.14 ± 0.14	1.38 ± 0.22	1.32 ± 0.09	0.548	1.27 ± 0.10	1.96 ± 0.20	0.90 ± 0.18	0.144
	CpG6	2.23 ± 0.22	2.39 ± 0.29	2.01 ± 0.25	0.503	2.01 ± 0.06	1.76 ± 0.11	1.86 ± 0.15	0.441
	CpG7	2.26 ± 0.39	2.22 ± 0.28	1.87 ± 0.13	0.806	1.81 ± 0.15	2.02 ± 0.24	1.98 ± 0.12	0.738
	Mean	1.76 ± 0.17	1.92 ± 0.23	1.59 ± 0.05	0.778	1.60 ± 0.07	1.54 ± 0.06	1.53 ± 0.04	0.630
AMY	CpG1	1.78 ± 0.14	2.10 ± 0.19	1.86 ± 0.14	0.703	1.77 ± 0.22	2.39 ± 0.26	1.59 ± 0.16	0.038
	CpG2	1.91 ± 0.24	1.82 ± 0.13	1.83 ± 0.12	0.949	1.60 ± 0.07	1.71 ± 0.02	1.22 ± 0.26	0.055
	CpG3	1.08 ± 0.10	1.04 ± 0.12	1.16 ± 0.05	0.417	1.13 ± 0.09	0.99 ± 0.12	1.41 ± 0.27	0.390
	CpG4	0.80 ± 0.18	1.11 ± 0.04	1.07 ± 0.11	0.244	0.95 ± 0.07	1.04 ± 0.10	1.00 ± 0.03	0.666
	CpG5	1.26 ± 0.37	1.19 ± 0.13	1.13 ± 0.15	0.931	0.98 ± 0.09	1.40 ± 0.23	1.12 ± 0.12	0.399
	CpG6	2.14 ± 0.24	2.30 ± 0.34	2.00 ± 0.11	0.830	1.79 ± 0.10	2.17 ± 0.12	2.08 ± 0.20	0.099
	CpG7	1.94 ± 0.17	1.90 ± 0.17	1.80 ± 0.11	0.860	1.66 ± 0.09	2.15 ± 0.14*	2.03 ± 0.20	0.042
	Mean	1.60 ± 0.15	1.62 ± 0.10	1.57 ± 0.09	0.911	1.41 ± 0.05	1.75 ± 0.11*	1.46 ± 0.03	0.013

Data were expressed in mean ± standard error of the mean.
AMY, amygdala; CON, control; HIP, hippocampus; PFC, prefrontal cortex; SUS, susceptible; UNS, unsusceptible; Drd1, dopamine receptor D1.
*$p < 0.05$ vs. control group by Bonferroni *post-hoc* test. †$p < 0.05$ vs. control group; ‡$p < 0.05$ vs. susceptible group by false discovery rate *post-hoc* test.

were detected (Table 2, Fig. 7).

For Nr3c1, significant differences were found in CpG4 ($p = 0.035$) of the PFC and CpG2 ($p = 0.027$) and CpG3 ($p = 0.018$) of the HIP among the three groups (Table 3, Fig. 8). Post hoc analyses revealed significantly decreased methylation levels in CpG4 of the PFC ($p = 0.044$) in the UNS group compared to the SUS group, and in CpG2 ($p = 0.031$) and CpG3 ($p = 0.017$) of the HIP in the UNS group compared to the CON and SUS groups, respectively.

For Stmn1, a significant difference was found in CpG9 of the PFC ($p = 0.029$) among the three groups (Table 4, Fig. 9). Post hoc analyses revealed significantly increased methylation levels in CpG9 ($p = 0.039$) of the UNS group compared to the CON group. In the HIP region, significant differences were found in CpG9 ($p = 0.014$), CpG10 ($p = 0.042$) and CpG11 ($p = 0.003$) among the three groups. Post hoc analyses revealed significantly reduced methylation levels in CpG9 ($p = 0.024$) and CpG10 ($p = 0.45$) of the UNS group compared to the CON group, and in CpG11 of the UNS ($p = 0.007$) and SUS ($p = 0.015$) groups compared to the CON group. In the AMY region, significant differences were found in CpG1 ($p = 0.002$), CpG7 ($p = 0.018$), CpG8 ($p = 0.009$) and mean ($p = 0.040$) among the three groups. *Post-hoc* tests revealed significantly decreased methylation levels in CpG1 ($p = 0.001$), CpG7 ($p = 0.048$) and CpG8 ($p = 0.019$) of the SUS group compared to the CON group, and a trend toward decreased mean methylation levels in the SUS ($p = 0.052$) and UNS ($p = 0.052$) groups compared to the CON group.

Effects of Risperidone on Methylation Levels

For Drd2, significant differences were found in CpG5 of the PFC ($p = 0.046$), and CpG1 ($p = 0.034$), CpG2 ($p = 0.039$) and CpG5 ($p = 0.008$) of the AMY among the three groups (Table 1, Fig. 6). *Post-hoc* analyses revealed a significantly decreased methylation level in CpG5 of the PFC ($p = 0.045$) and CpG2 ($p = 0.039$) of the AMY in the SUS

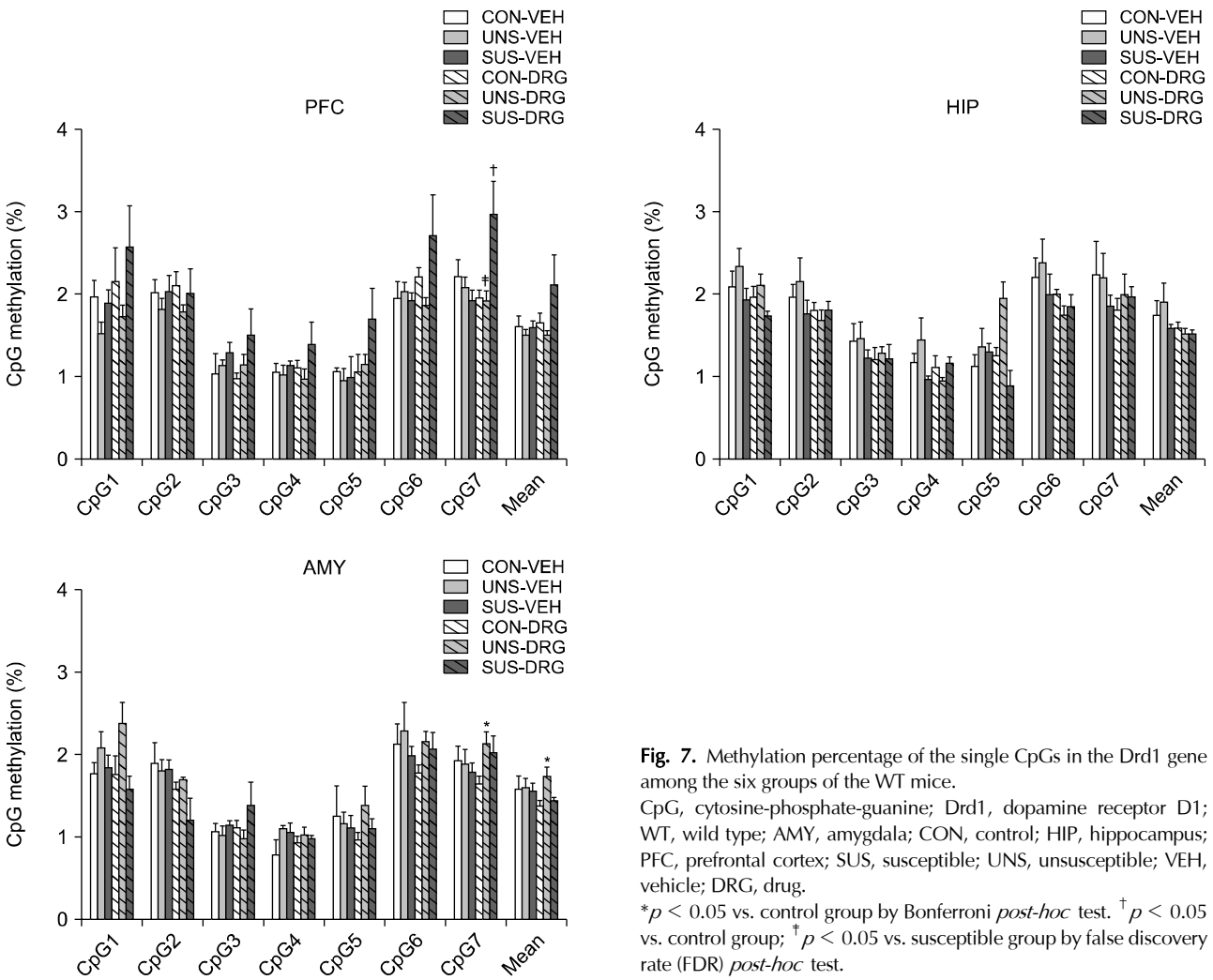

Fig. 7. Methylation percentage of the single CpGs in the Drd1 gene among the six groups of the WT mice.
CpG, cytosine-phosphate-guanine; Drd1, dopamine receptor D1; WT, wild type; AMY, amygdala; CON, control; HIP, hippocampus; PFC, prefrontal cortex; SUS, susceptible; UNS, unsusceptible; VEH, vehicle; DRG, drug.
$^{*}p < 0.05$ vs. control group by Bonferroni *post-hoc* test. $^{\dagger}p < 0.05$ vs. control group; $^{\ddagger}p < 0.05$ vs. susceptible group by false discovery rate (FDR) *post-hoc* test.

group compared to CON group, and significantly increased methylation level in CpG5 ($p = 0.015$) of the AMY in the SUS group compared to the CON group. In addition, the UNS group showed significantly increased methylation levels in CpG1 ($p = 0.028$), and decreased methylation levels in CpG5 ($p = 0.033$), of the AMY compared to the SUS group. For the Drd1 gene, significant differences were found in the methylation levels in CpG7 ($p = 0.041$) of the PFC, and CpG1 ($p = 0.038$), CpG7 ($p = 0.042$) and mean ($p = 0.013$) for the AMY among the three groups (Table 2, Fig. 7). *Post-hoc* analyses revealed significantly increased methylation levels in CpG7 ($p = 0.045$), and mean ($p = 0.035$), for the AMY in the UNS group compared to the CON group.

For Nr3c1, a significant difference was found in the methylation level in CpG1 of the PFC among the three groups ($p = 0.020$) (Table 3, Fig. 8). *Post-hoc* analysis revealed significantly decreased methylation levels ($p = 0.019$) in the SUS compared to CON group.

For Stmn1, significant differences were found in the methylation levels in CpG1 ($p = 0.015$) of the PFC, CpG8 ($p = 0.049$) and CpG9 ($p = 0.008$) of the HIP, and CpG1 ($p = 0.039$), CpG9 ($p = 0.018$) and CpG11 ($p = 0.035$) of the AMY among the three groups (Table 4, Fig. 9). *Post-hoc* analyses revealed significantly increased methylation levels in CpG1 of the PFC and AMY ($p = 0.033$ and $p = 0.039$, respectively) in the UNS group compared to the SUS group, and decreased methylation levels in CpG9 ($p = 0.009$) of the HIP in the UNS group compared to SUS group. In addition, the SUS group showed a significantly decreased methylation level in CpG9 ($p = 0.015$) of the AMY compared to the CON group.

Table 3. Effects of risperidone on DNA methylation of Nr3c1 gene in the three brain regions of mice exposed to social defeat stress

Region	CpG sites	Vehicle			p value	Drug			p value
		CON (n = 6)	UNS (n = 6)	SUS (n = 6)		CON (n = 6)	UNS (n = 6)	SUS (n = 6)	
PFC	CpG1	2.05 ± 0.17	2.14 ± 0.16	1.82 ± 0.14	0.331	2.46 ± 0.39	1.84 ± 0.13	1.74 ± 0.04*	0.020
	CpG2	1.86 ± 0.26	1.84 ± 0.09	1.66 ± 0.11	0.493	1.92 ± 0.15	1.86 ± 0.20	1.92 ± 0.44	0.526
	CpG3	2.20 ± 0.20	2.05 ± 0.11	2.01 ± 0.14	0.977	2.06 ± 0.16	2.21 ± 0.13	1.93 ± 0.09	0.281
	CpG4	1.01 ± 0.08	0.96 ± 0.05 †	1.27 ± 0.13	0.035	1.26 ± 0.11	1.09 ± 0.10	1.01 ± 0.05	0.586
	CpG5	1.89 ± 0.12	1.90 ± 0.05	1.65 ± 0.08	0.097	1.91 ± 0.15	1.91 ± 0.12	1.82 ± 0.09	0.911
	CpG6	1.60 ± 0.15	1.59 ± 0.07	1.70 ± 0.14	0.777	1.51 ± 0.12	1.58 ± 0.09	1.42 ± 0.05	0.248
	CpG7	1.37 ± 0.10	1.31 ± 0.06	1.40 ± 0.15	0.850	1.21 ± 0.17	1.12 ± 0.08	1.32 ± 0.13	0.494
	CpG8	0.99 ± 0.10	1.04 ± 0.13	1.13 ± 0.14	0.806	1.31 ± 0.29	1.16 ± 0.14	1.24 ± 0.13	0.850
	Mean	1.62 ± 0.12	1.60 ± 0.04	1.58 ± 0.11	0.548	1.68 ± 0.17	1.60 ± 0.05	1.55 ± 0.06	0.778
HIP	CpG1	2.18 ± 0.20	1.76 ± 0.25	1.67 ± 0.24	0.359	1.72 ± 0.10	1.56 ± 0.10	1.59 ± 0.11	0.373
	CpG2	1.81 ± 0.12	1.28 ± 0.12*	1.85 ± 0.28	0.027	1.78 ± 0.13	1.38 ± 0.10	1.53 ± 0.16	0.142
	CpG3	1.92 ± 0.15	1.50 ± 0.10 †	2.20 ± 0.16	0.018	2.11 ± 0.07	1.84 ± 0.13	1.95 ± 0.11	0.224
	CpG4	1.15 ± 0.09	1.08 ± 0.10	1.07 ± 0.11	0.581	1.13 ± 0.12	0.96 ± 0.08	0.94 ± 0.05	0.321
	CpG5	1.90 ± 0.09	1.36 ± 0.13	1.75 ± 0.19	0.057	1.72 ± 0.13	1.51 ± 0.15	1.65 ± 0.14	0.495
	CpG6	1.83 ± 0.06	1.50 ± 0.13	1.66 ± 0.20	0.082	1.49 ± 0.11	1.24 ± 0.08	1.62 ± 0.14	0.070
	CpG7	1.28 ± 0.11	1.34 ± 0.10	1.14 ± 0.08	0.357	1.03 ± 0.03	1.04 ± 0.07	1.22 ± 0.14	0.675
	CpG8	1.19 ± 0.18	0.90 ± 0.21	1.09 ± 0.11	0.806	1.06 ± 0.07	0.21 ± 0.13	1.01 ± 0.04	0.535
	Mean	1.66 ± 0.06	1.34 ± 0.08	1.55 ± 0.11	0.052	1.50 ± 0.05	1.34 ± 0.04	1.44 ± 0.05	0.101
AMY	CpG1	1.60 ± 0.21	1.79 ± 0.13	1.81 ± 0.08	0.526	1.63 ± 0.21	1.89 ± 0.16	1.87 ± 0.14	0.630
	CpG2	1.52 ± 0.20	1.37 ± 0.11	1.48 ± 0.12	0.548	1.64 ± 0.12	1.48 ± 0.11	1.61 ± 0.10	0.530
	CpG3	1.82 ± 0.15	1.68 ± 0.21	2.11 ± 0.08	0.143	1.66 ± 0.07	1.76 ± 0.16	2.01 ± 0.10	0.140
	CpG4	1.15 ± 0.20	1.27 ± 0.10	1.02 ± 0.06	0.204	0.98 ± 0.08	1.21 ± 0.09	1.04 ± 0.06	0.149
	CpG5	1.58 ± 0.11	1.73 ± 0.16	1.56 ± 0.09	0.528	1.46 ± 0.08	1.53 ± 0.21	1.68 ± 0.06	0.309
	CpG6	1.58 ± 0.11	1.73 ± 0.16	1.56 ± 0.09	0.532	1.46 ± 0.08	1.53 ± 0.21	1.68 ± 0.06	0.327
	CpG7	1.47 ± 0.12	1.46 ± 0.04	1.31 ± 0.08	0.269	1.18 ± 0.07	1.31 ± 0.10	1.17 ± 0.04	0.557
	CpG8	1.12 ± 0.20	1.57 ± 0.20	1.02 ± 0.12	0.096	1.14 ± 0.05	1.10 ± 0.12	1.16 ± 0.12	0.926
	Mean	1.49 ± 0.15	1.55 ± 0.08	1.52 ± 0.06	0.452	1.41 ± 0.06	1.50 ± 0.07	1.56 ± 0.06	0.202

Data were expressed in mean ± standard error of the mean.
AMY, amygdala; CON, control; HIP, hippocampus; PFC, prefrontal cortex; SUS, susceptible; UNS, unsusceptible; Nr3c1, nuclear receptor subfamily 3 group c member 1.
*$p < 0.05$ vs. control group; †$p < 0.05$ vs. susceptible group by Bonferroni *post-hoc* test.

Effects of Social Defeat Stress and Risperidone on mRNA Expression Levels

There were significant differences in D2L mRNA expression levels in the HIP ($p = 0.013$) and in AMY ($p = 0.038$) among the three groups after SDS. *Post-hoc* analyses revealed significantly decreased mRNA expression in the HIP ($p = 0.003$), and increased levels in the AMY ($p = 0.014$), in the SUS group compared to the CON group (Table 5).

There were significant differences in D2S mRNA expression levels in the PFC ($p = 0.046$) and HIP ($p = 0.018$), Drd1 mRNA expression levels in the HIP ($p = 0.016$), and Stmn1 mRNA expression levels in the HIP ($p = 0.034$) among the three groups after risperidone treatment. *Post-hoc* analyses revealed significantly decreased levels of D2S ($p = 0.047$), Drd1 ($p = 0.030$) and Stmn1 ($p = 0.029$) mRNA expression in the HIP in the UNS compared to SUS and CON groups.

DISCUSSION

Epigenetics has been shown to be involved in the pharmacological effects of antipsychotics, as well as the pathophysiology of psychiatric illness. The majority of previous studies assessed the effects of antipsychotics on methylation levels in specific neurotransmitter-associated candidate genes, or at the genome-wide level. However, there is a paucity of evidence of the effects of antipsychotics on the methylation changes identified in various animal models of stress. We measured DNA methylation and mRNA levels of target genes in the brains of mice exposed to SDS, and the influence of risperidone on those changes.

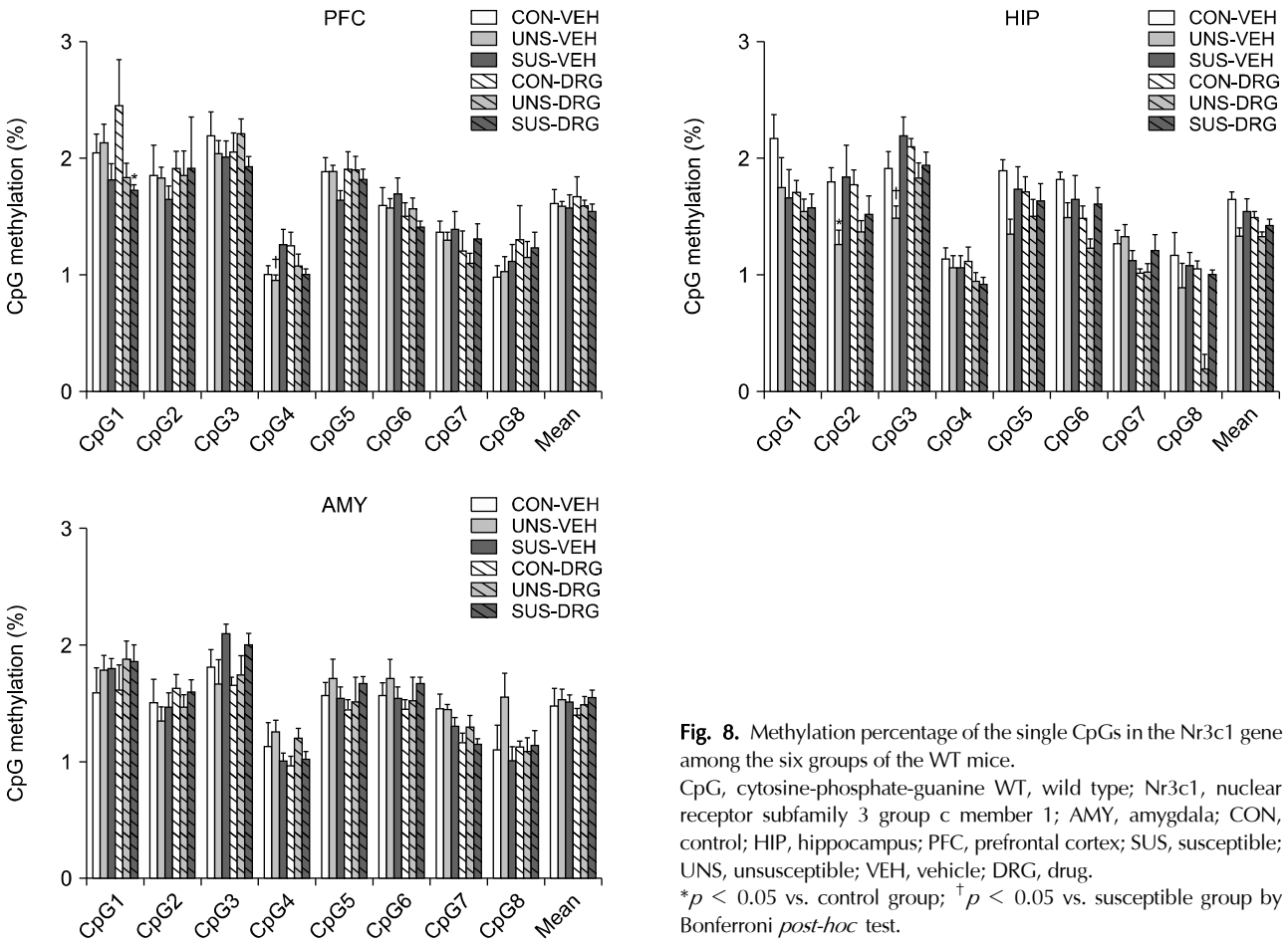

Fig. 8. Methylation percentage of the single CpGs in the Nr3c1 gene among the six groups of the WT mice.
CpG, cytosine-phosphate-guanine WT, wild type; Nr3c1, nuclear receptor subfamily 3 group c member 1; AMY, amygdala; CON, control; HIP, hippocampus; PFC, prefrontal cortex; SUS, susceptible; UNS, unsusceptible; VEH, vehicle; DRG, drug.
$^{*}p < 0.05$ vs. control group; $^{\dagger}p < 0.05$ vs. susceptible group by Bonferroni *post-hoc* test.

Effects of Social Defeat Stress on Methylation Levels

We observed no significant differences in the methylation levels of Drd2 or Drd1 between the UNS and CON, or SUS and CON, groups, although there was a significant difference in the PFC between the UNS and SUS groups. These findings replicate our previous study [37]. In addition, these results are partially in line with the lack of significant differences in Drd2 and Drd1 expression between defeated and CON mice [38]. Collectively, these findings suggest that SDS does not affect the dopaminergic system at both methylation and protein levels. This view is partially supported by two genome-wide methylation studies in which no methylation changes were found in relation to Drd2 or Drd1 [25,26].

For the Nr3c1 gene, significantly decreased methylation was seen only in the HIP of the UNS group compared to the CON and SUS groups. This suggests that resilience to stress is associated with demethylation of Nr3c1 exon 1 in the HIP. Similarly, it was reported that resilience to SDS coincided with demethylation of corticotrophin-releasing factor promoter in mice [22]. In this study, for the Stmn1 gene, altered methylation was observed in the PFC and HIP of the UNS group in comparison to the CON group. Given the role of Stmn1 in regulating MT polymerization and the fear response [30,31], these findings suggest that the methylation changes of Stmn1 might have protected against SDS. More importantly, significantly decreased methylation at the three CpG sites, and decreasing trend in methylation of mean, were observed in the AMY of the SUS group compared to the CON group. Assuming that decreased methylation of Stmn1 enhances its expression levels, and that this change is detrimental for axonal growth [39], these findings suggest that decreased methylation of Stmn1 in the AMY could be an epigenetic marker of vulnerability to social stress.

Effects of Risperidone on Methylation Levels

For the Drd2 gene, we observed significantly decreased

Table 4. Effects of risperidone on DNA methylation of Stmn1 gene in the three brain regions of mice exposed to social defeat stress

Region	CpG sites	Vehicle			p value	Drug			p value
		CON (n = 6)	UNS (n = 6)	SUS (n = 6)		CON (n = 6)	UNS (n = 6)	SUS (n = 6)	
PFC	CpG1	3.23 ± 0.11	3.54 ± 0.28	2.88 ± 0.21	0.160	2.58 ± 0.25	3.61 ± 0.19*,‡	2.56 ± 0.30	0.015
	CpG2	2.51 ± 0.29	2.38 ± 0.17	1.97 ± 0.16	0.278	2.23 ± 0.08	2.57 ± 0.39	2.04 ± 0.26	0.401
	CpG3	2.02 ± 0.09	1.87 ± 0.19	1.74 ± 0.09	0.191	1.63 ± 0.06	2.03 ± 0.30	1.79 ± 0.13	0.353
	CpG4	2.74 ± 0.16	2.34 ± 0.09	0.39 ± 0.17	0.153	2.44 ± 0.09	2.69 ± 0.42	2.53 ± 0.26	0.899
	CpG5	2.34 ± 0.19	2.33 ± 0.13	2.01 ± 0.16	0.347	2.09 ± 0.10	2.35 ± 0.49	1.95 ± 0.26	0.778
	CpG6	1.98 ± 0.18	1.90 ± 0.19	1.97 ± 0.19	0.823	1.96 ± 0.10	2.02 ± 0.41	1.72 ± 0.17	0.470
	CpG7	1.88 ± 0.16	1.85 ± 0.19	1.62 ± 0.19	0.476	1.63 ± 0.12	1.65 ± 0.38	1.55 ± 0.12	0.417
	CpG8	2.19 ± 0.13	2.47 ± 0.21	2.30 ± 0.29	0.499	2.16 ± 0.13	2.54 ± 0.06	2.18 ± 0.23	0.107
	CpG9	2.67 ± 0.19	3.50 ± 0.18*	3.39 ± 0.33	0.029	3.38 ± 0.23	3.58 ± 0.31	3.02 ± 0.24	0.504
	CpG10	1.55 ± 0.15	1.70 ± 0.10	1.92 ± 0.24	0.278	1.67 ± 0.10	2.01 ± 0.20	1.82 ± 0.31	0.331
	CpG11	1.64 ± 0.08	1.79 ± 0.11	1.70 ± 0.12	0.567	1.62 ± 0.09	1.96 ± 0.13	1.84 ± 0.19	0.202
	Mean	2.25 ± 0.11	2.33 ± 0.06	2.17 ± 0.16	0.224	2.13 ± 0.07	2.45 ± 0.21	2.09 ± 0.18	0.473
HIP	CpG1	2.79 ± 0.25	3.44 ± 0.53	3.55 ± 0.47	0.359	3.05 ± 0.27	3.32 ± 1.12	3.86 ± 0.49	0.281
	CpG2	2.40 ± 0.34	2.37 ± 0.26	2.46 ± 0.19	0.927	2.47 ± 0.17	2.56 ± 1.00	2.68 ± 0.36	0.173
	CpG3	2.11 ± 0.35	2.62 ± 0.43	1.89 ± 0.15	0.644	2.10 ± 0.23	2.38 ± 0.68	2.10 ± 0.21	0.884
	CpG4	2.77 ± 0.38	3.08 ± 0.45	3.08 ± 0.45	0.796	2.50 ± 0.24	2.93 ± 0.61	3.27 ± 0.44	0.323
	CpG5	2.13 ± 0.25	3.12 ± 0.41	2.56 ± 0.51	0.281	2.05 ± 0.22	2.88 ± 0.57	2.84 ± 0.42	0.338
	CpG6	2.19 ± 0.28	2.37 ± 0.30	2.31 ± 0.20	0.806	2.19 ± 0.33	2.24 ± 0.57	2.28 ± 0.49	0.973
	CpG7	2.03 ± 0.29	2.14 ± 0.38	1.92 ± 0.23	0.983	1.77 ± 0.20	2.00 ± 0.43	2.20 ± 0.33	0.700
	CpG8	2.18 ± 0.17	1.85 ± 0.15	1.75 ± 0.16	0.234	2.78 ± 0.33	1.73 ± 0.14	3.52 ± 0.88	0.049
	CpG9	2.72 ± 0.23	2.03 ± 0.12*	2.59 ± 0.13	0.014	3.27 ± 0.28	2.34 ± 0.10‡	4.22 ± 0.74	0.008
	CpG10	1.61 ± 0.15	1.06 ± 0.07*	1.17 ± 0.13	0.042	2.37 ± 0.28	1.32 ± 0.24	2.09 ± 0.33	0.060
	CpG11	1.79 ± 0.07	1.19 ± 0.06*	1.21 ± 0.09*	0.003	2.34 ± 0.17	1.33 ± 0.22	1.95 ± 0.48	0.088
	Mean	2.25 ± 0.19	2.30 ± 0.23	2.23 ± 0.19	0.977	2.44 ± 0.16	2.28 ± 0.43	2.82 ± 0.24	0.135
AMY	CpG1	4.08 ± 0.53	2.61 ± 0.08	1.86 ± 0.13†	0.002	2.52 ± 0.43	3.17 ± 0.19‡	1.91 ± 0.19	0.039
	CpG2	2.57 ± 0.39	1.85 ± 0.15	1.87 ± 0.18	0.489	2.15 ± 0.21	2.56 ± 0.30	1.86 ± 0.19	0.121
	CpG3	2.09 ± 0.39	1.59 ± 0.12	1.70 ± 0.14	0.757	1.81 ± 0.24	2.19 ± 0.14	1.61 ± 0.11	0.061
	CpG4	2.93 ± 0.46	2.38 ± 0.16	2.56 ± 0.30	0.796	2.66 ± 0.27	2.90 ± 0.29	2.39 ± 0.11	0.347
	CpG5	2.63 ± 0.42	2.21 ± 0.17	1.86 ± 0.16	0.421	2.35 ± 0.25	2.64 ± 0.28	1.92 ± 0.13	0.090
	CpG6	2.41 ± 0.37	1.84 ± 0.18	1.70 ± 0.12	0.323	1.91 ± 0.22	2.39 ± 0.39	1.64 ± 0.16	0.327
	CpG7	2.31 ± 0.32	1.40 ± 0.08	1.34 ± 0.09*	0.018	1.90 ± 0.36	2.01 ± 0.33	1.48 ± 0.15	0.587
	CpG8	4.20 ± 0.91	1.82 ± 0.13	1.69 ± 0.18†	0.009	3.27 ± 0.84	1.90 ± 0.15	1.52 ± 0.12	0.124
	CpG9	4.13 ± 0.57	2.82 ± 0.30	2.65 ± 0.14	0.069	3.56 ± 0.62	2.43 ± 0.18	1.87 ± 0.17*	0.018
	CpG10	1.93 ± 0.30	1.22 ± 0.12	1.37 ± 0.16	0.149	2.29 ± 0.62	1.39 ± 0.19	1.22 ± 0.13	0.430
	CpG11	2.18 ± 0.31	1.28 ± 0.14	1.57 ± 0.16	0.075	2.39 ± 0.73	1.11 ± 0.03§,‖	1.45 ± 0.13	0.035
	Mean	2.86 ± 0.41	1.91 ± 0.04	1.83 ± 0.12	0.040	2.44 ± 0.34	2.24 ± 0.15	1.71 ± 0.09	0.064

Data were expressed in mean ± standard error of the mean.
AMY, amygdala; CON, control; HIP, hippocampus; PFC, prefrontal cortex; SUS, susceptible; UNS, unsusceptible; Stmn1, stathmin 1.
*$p < 0.05$, †$p < 0.01$ vs. control group; ‡$p < 0.05$ vs. susceptible group by Bonferroni *post-hoc* test. §$p < 0.05$ vs. control group; ‖$p < 0.05$ vs. susceptible group by false discovery rate *post-hoc* test.

methylation levels in the PFC and AMY (CpG2 site) of the SUS compared to CON group. Assuming that decreased methylation of Drd2 enhances its expression levels, our findings are consistent with previous studies showing that various antipsychotics, including risperidone, upregulated mRNA levels of Drd2 in the brains of rats [40-42]. However, it should be noted that the methylation level at CpG5 of Drd2 in the AMY of the SUS group was significantly higher compared to the CON group, which is not compatible with the above interpretation. It may be that combined effects of CpG2 and CpG5 sites increase the mRNA expression of Drd2. For the Drd1 gene, we observed increased methylation levels in the PFC of the SUS group, and in the AMY of the UNS group, compared to CON group. The increased methylation levels may result in decreased expression of Drd1, in turn decreasing the activation of adenylyl cyclase. Given the opposing roles of Drd2 and Drd1 in adenylyl cyclase and cyclic AMP [43], our findings on the methylation levels of Drd2 and Drd1 suggest decreased activation of adenylyl cyclase.

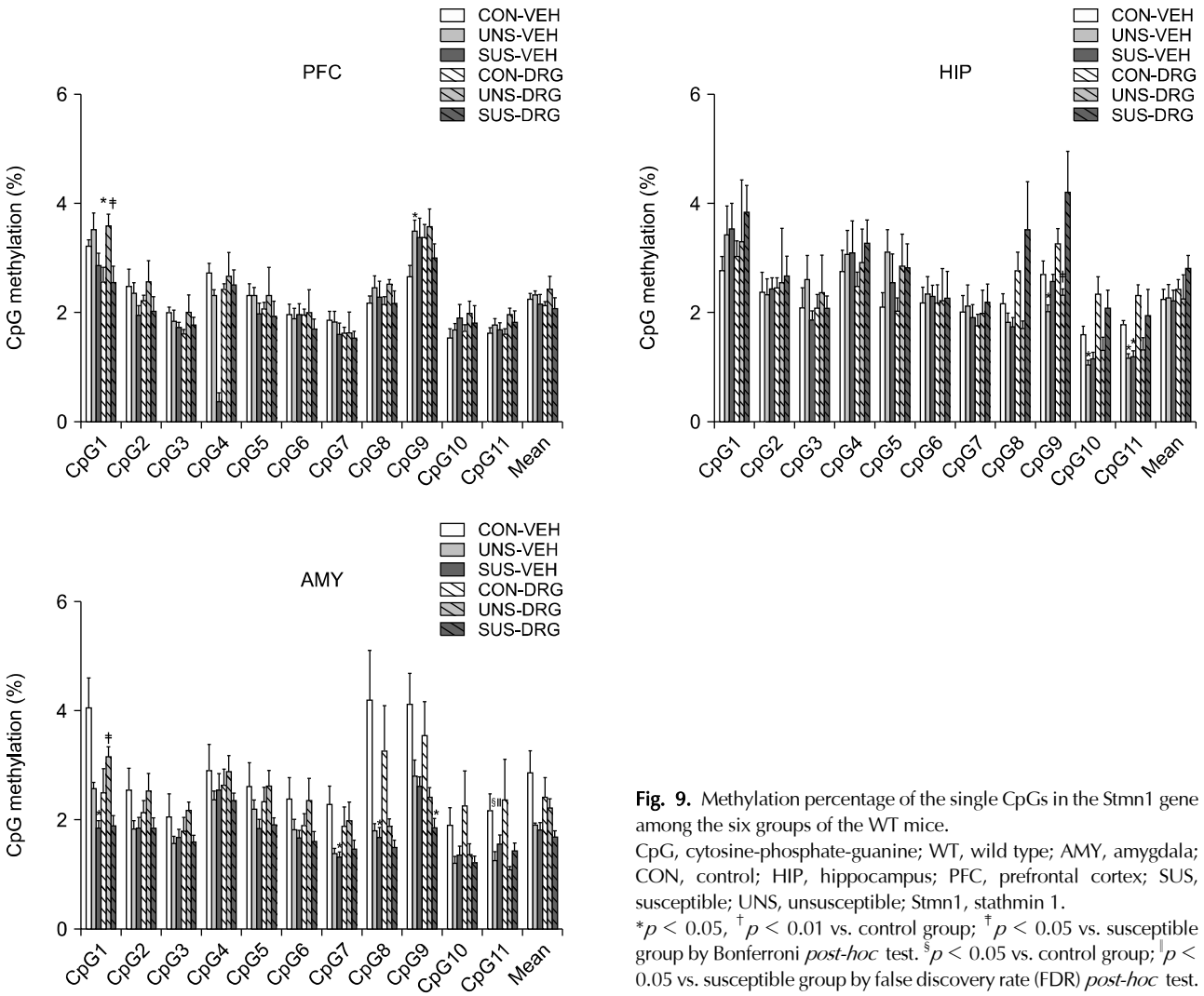

Fig. 9. Methylation percentage of the single CpGs in the Stmn1 gene among the six groups of the WT mice.
CpG, cytosine-phosphate-guanine; WT, wild type; AMY, amygdala; CON, control; HIP, hippocampus; PFC, prefrontal cortex; SUS, susceptible; UNS, unsusceptible; Stmn1, stathmin 1.
*$p < 0.05$, †$p < 0.01$ vs. control group; ‡$p < 0.05$ vs. susceptible group by Bonferroni *post-hoc* test. §$p < 0.05$ vs. control group; ‖$p < 0.05$ vs. susceptible group by false discovery rate (FDR) *post-hoc* test.

Thus, it seems that Drd1 is involved in a compensatory mechanisms to offset the stimulation of adenylyl cyclase induced by the Drd2-blocking action of risperidone.

Regarding the Nr3c1 gene, altered methylation in the HIP of the UNS group relative to the CON group induced by SDS was abolished by administering risperidone. This finding can be interpreted in two ways: if the abolishment reflects attenuation of resilience, it could be harmful; but if the abolishment reflects attenuation of the stress response, it could be beneficial. Evidence for the latter comes from a study showing that GR expression in the HIP of rats was increased by chronic mild stress (CMS), which was normalized by antidepressant treatment [44]. On the other hand, risperidone treatment significantly decreased the methylation level in the PFC of the SUS group relative to the CON group. Assuming that this decreased level leads to increased expression of GRs in SUS mice and subsequent activation of functions involving cortisol, such as hypothalamic–pituitary–adrenal (HPA) axis self-regulation and anti-inflammatory actions, this finding may reflect a therapeutic mechanism, i.e., attenuation of susceptibility. Although the HPA axis is not represent a direct target of antipsychotics, several drugs have been reported to modulate the stress response. For instance, atypical antipsychotics such as clozapine, risperidone and aripiprazole target multiple stress-related metabolic pathways [45], and lurasidone treatment can prevent the increase of GR membrane levels that follow CMS exposure, as well as restore the transcription of GR-responsive genes [46].

For the Stmn1 gene, interestingly, the greater methylation changes in the HIP and AMY of the SUS group rela-

Table 5. Effects of risperidone on mRNA expression levels of target genes in the PFC, HIP, and AMY regions of mice exposed to social defeat stress

Protein	Region	Vehicle			p value	Drug			p value
		CON (n = 5–7)	UNS (n = 5–7)	SUS (n = 5–7)		CON (n = 5–7)	UNS (n = 5–7)	SUS (n = 5–7)	
D2L	PFC	1.00 ± 0.00	4.37 ± 4.07	2.51 ± 0.79	0.217	1.00 ± 0.00	0.78 ± 0.58	50.90 ± 42.41	0.072
	HIP	1.00 ± 0.00	9.10 ± 8.91	0.21 ± 0.07*	0.013	1.00 ± 0.00	50.85 ± 40.02	2.13 ± 0.52	0.419
	AMY	1.00 ± 0.00	3.47 ± 0.81	11.99 ± 6.00*	0.038	1.00 ± 0.00	1.30 ± 0.56	2.12 ± 0.71	0.323
D2S	PFC	1.00 ± 0.00	2.17 ± 1.76	0.96 ± 0.56	0.072	1.00 ± 0.00	6.45 ± 5.21	7.02 ± 4.33	0.046
	HIP	1.00 ± 0.00	2.63 ± 1.90	2.50 ± 0.58	0.269	1.00 ± 0.00	0.27 ± 0.15†	4.05 ± 2.03	0.018
	AMY	1.00 ± 0.00	1.11 ± 0.37	1.04 ± 0.28	0.935	1.00 ± 0.00	1.56 ± 0.47	1.35 ± 0.40	0.859
Drd1	PFC	1.00 ± 0.00	1.89 ± 0.65	0.92 ± 0.17	0.076	1.00 ± 0.00	1.97 ± 0.99	2.66 ± 1.33	0.359
	HIP	1.00 ± 0.00	1.88 ± 1.36	4.13 ± 0.96	0.072	1.00 ± 0.00	0.27 ± 0.11*	2.43 ± 1.45	0.016
	AMY	1.00 ± 0.00	0.76 ± 0.21	0.83 ± 0.35	0.118	1.00 ± 0.00	2.58 ± 0.87	2.88 ± 1.20	0.104
Nr3c1	PFC	1.00 ± 0.00	1.15 ± 0.23	0.90 ± 0.09	0.426	1.00 ± 0.00	0.78 ± 0.10	1.01 ± 0.33	0.228
	HIP	1.00 ± 0.00	0.85 ± 0.16	0.97 ± 0.08	0.861	1.00 ± 0.00	0.79 ± 0.17	0.97 ± 0.17	0.111
	AMY	1.00 ± 0.00	1.74 ± 0.35	2.19 ± 0.68	0.100	1.00 ± 0.00	0.87 ± 0.19	1.07 ± 0.19	0.476
Stmn1	PFC	1.00 ± 0.00	1.20 ± 0.12	1.24 ± 0.18	0.184	1.00 ± 0.00	1.01 ± 0.12	1.25 ± 0.15	0.186
	HIP	1.00 ± 0.00	1.07 ± 0.30	1.65 ± 0.19	0.097	1.00 ± 0.00	0.65 ± 0.25*	0.91 ± 0.16	0.034
	AMY	1.00 ± 0.00	0.90 ± 0.10	1.60 ± 0.22	0.365	1.00 ± 0.00	0.63 ± 0.07	0.92 ± 0.13	0.098

Data were expressed in mean ± standard error of the mean.
AMY, amygdala; CON, control; HIP, hippocampus; PFC, prefrontal cortex; SUS, susceptible; UNS, unsusceptible; Drd1, dopamine receptor D1; Nr3c1, nuclear receptor subfamily 3 group c member 1; Stmn1, stathmin 1.
*$p < 0.05$ vs. control group; †$p < 0.05$ vs. susceptible group by Bonferroni *post-hoc* test.

tive to the CON group were abolished by risperidone treatment. It may be inferred that detrimental effects in SUS mice of MT dysfunction were attenuated. However, significantly decreased methylation at a different CpG site, CpG9, in the AMY of the SUS group relative to the CON group, remained. It may be that the dosage of risperidone in the present study (0.2 mg/kg) was not enough to prevent harmful effects of SDS. On the other hand, in the PFC, we observed significantly increased methylation in the UNS group compared to the CON group. Considering the role of Stmn1 as a MT-destabilizing factor [47], this finding suggests that risperidone treatment may exert a MT-stabilizing effect by decreasing Stmn1 expression levels in the UNS mice. In humans, fear and anxiety, as well as cognitive and affective processing, were shown to be associated with Stmn1 polymorphisms [31,48]. There is increasing evidence of correlations between the Stmn1 gene and a broad range of neuropsychiatric disorders, including schizophrenia [49] and post-traumatic disorder [50,51]. A greater understanding of the precise mechanisms underlying changes in the DNA methylation of Stmn1 induced by antipsychotics would be invaluable for developing novel agents.

Effects of Social Defeat Stress and Risperidone on mRNA Expression Levels

SDS decreased mRNA expression of D2L in the HIP of the SUS group compared to the CON group. Given that enhanced mesocorticolimbic dopamine response [16] and increased phasic activity of ventral tegmental area dopamine neurons [18] were reported in animals exposed to SDS, this finding seems to reflect a compensatory response to the overstimulation of D2L caused by increased dopamine release. On the other hand, the finding of increased expression of D2L in the AMY of the SUS group compared to the CON group conflicts with previous studies variously reporting decreased [52,53] and increased [54] expression of Drd2 in the AMY of defeated mice. Nevertheless, considering the role of AMY in vigilance and danger detection, and its higher concentration of Drd2 [55], this result may reflect vulnerability in SUS mice. With risperidone treatment, altered expression of D2L in the HIP and AMY of our SUS group disappeared. Although increased expression of Drd2 in the mesolimbic region is general reported in association with antipsychotic treatment [56,42], this finding should be interpreted in terms of how antipsychotic treatment altered mRNA expression of D2L in defeated mice. In other words, it may signify that antipsychotic treatment attenuates or abolishes altered mRNA expression of D2L in SUS

mice. In addition, decreased expression levels of Drd1 and Stmn1 in the HIP of the UNS versus CON group were seen. Assuming that decreased expression of Stmn1 may contribute to MT stabilization and neuronal plasticity [57], this finding suggests that risperidone treatment exerts a beneficial effect in the HIP of UNS mice. Down-regulation of Stmn1 by clozapine and risperidone was also reported [58]. Given proteomic evidence for up-regulation of Stmn1 in schizophrenia [39], the decreased mRNA expression of Stmn1 in response to risperidone observed in this study may have clinical relevance.

The present study had several limitations that should be mentioned. First, only a single dose of risperidone was administered, which is not sufficient to fully elucidate the effects of this agent. Second, our results on DNA methylation do not match those on mRNA expression levels. Although an inverse correlation between DNA methylation of the first intron and gene expression was seen consistently across tissues and species [59], this issue is controversial considering that the relationship between DNA methylation and mRNA and protein levels is not straightforward [60,61]. Further studies measuring target proteins using Western blot are required to validate the present findings. Third, genome-wide methylation studies with antipsychotics are scarce. This should be addressed in future using microarray or methylation sequencing covering a wider range of CpG sites. In summary, the present study found that 10 days of SDS altered the methylation status of Nr3c1 and Stmn1 in the HIP and AMY of mice, where these changes were reversed by risperidone treatment. In addition, different methylation patterns of Drd2 and Drd1 in the PFC and AMY of the SUS and UNS groups compared to the CON group were identified following risperidone treatment. These findings suggest that risperidone can cause epigenetic changes in Drd2, Drd1, Nr3c1 and Stmn1, where such changes could underlie the therapeutic effects of antipsychotics.

■ Funding

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2020R1A2C2009413).

■ Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

■ Author Contributions

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