Effects of caffeic acid on epigenetics in the brain of rats with chronic unpredictable mild stress

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Abstract. The present study hypothesized that caffeic acid (3,4-dihydroxycinnamic acid; CaA) may exert antidepressant-like effects in rats with chronic unpredictable mild stress via epigenetic mechanisms, such as DNA methylation and hydroxymethylation. The chronic unpredictable mild stress (CUMS) model was used to analyze the effects of CaA on behavioral phenotypes, and to evaluate the distribution of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) in the hippocampus and prefrontal cortex using immunohistochemistry and immunofluorescence. mRNA levels of the genes encoding brain-derived neurotropic factor (BDNF) and catechol-O-methyltransferase (COMT), and key enzymes regulating DNA methylation [DNA methyltransferase (DNMT)1 and DNMT3A] and hydroxymethylation [Ten-eleven translocation (TET)1-3] were examined using quantitative (q)PCR. Furthermore, enrichment of 5mC and 5hmC at the promotor regions of the *Bdnf* and *Comt* genes was quantified using chromatin immunoprecipitation-qPCR. Behavioral data showed that CaA exerted a slight antidepressant-like effect. *Bdnf* and *Comt* genes showed differential expression patterns due to CUMS. CaA intervention induced different *Dnmt1*/*Dnmt3a* and *Tet1*/*Tet2* mRNA levels in the hippocampus and prefrontal cortex, respectively. CaA regulated the ratio of 5mC/5hmC at the promotor region of the *Bdnf* and *Comt* genes and therefore influenced gene expression, which may be a valuable therapeutic option for major depressive disorder (MDD). In conclusion, there were epigenetic changes in the hippocampus and prefrontal cortex in CUMS rats, and CaA may function as a modulator of DNA methylation to regulate gene transcription, thus providing a mechanistic basis for the use of this phytochemical agent in the treatment of MDD.

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Introduction

Caffeic acid (3,4-dihydroxycinnamic acid; CaA) is a natural hydroxycinnamic acid that is found in coffee, argan oil and barley grain (1). Studies have shown that CaA has anticancer, anti-oxidant and anti-inflammatory properties and can be activated both *in vitro* and *in vivo* (2-5). In addition, as a dietary catechol, CaA can function as an inhibitor of DNA methylation through the increased formation of S-adenosyl-L-homocysteine via catechol-O-methyltransferase (COMT)-mediated O-methylation (6). COMT has multiple functions in neurological, estrogen-associated and methylation metabolic pathways, such as catecholamine, estradiol and estrone metabolism (7,8). Expression of the *COMT* gene primarily determines dopaminergic levels in the prefrontal cortex. Therefore, variations in COMT activity and expression may be involved in the pathogenesis of various psychiatric and neurological diseases, including schizophrenia and depression (9,10). A large amount of research has focused on a common, single nucleotide polymorphism of the *COMT* gene, Val158Met, which results in high activity of the COMT enzyme, contributing to the pathophysiology of major depressive disorder (MDD) (11). Previous studies have suggested that methylation of the *COMT* gene impacts its subsequent expression (12,13). Our previous study showed that phytochemically-stimulated *COMT* expression reversed the estrogen-induced inhibition of *COMT* via epigenetic mechanisms (14).

A recent study indicated that aberrations in epigenetic modification, particularly DNA methylation of 5-methylcytosine (5mC) in regions that are differentially methylated, are associated with depression (15). DNA methylation is initiated and maintained by DNA methyltransferases (DNMTs), which would be expected to decrease gene expression. Recent studies have shown that 5mC can be oxidized by members of the ten-eleven translocation (TET) protein family to form 5-hydroxymethylcytosine (5hmC), which could mediate DNA hydromethylation and increase gene expression (16,17). These epigenetic enzymes, including methyl CpG binding proteins, DNMTs and TETs, can bind to DNA and thus regulate gene expression. It has been reported that alteration

in the expression of neurogenesis-associated genes via methylation regulation is associated with depression in a rat model (18).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors. Previous research has shown that expression of *Bdnf* was decreased in psychiatric disorders, such as depression (19), ultimately leading to atrophy of the hippocampus. *Bdnf* transcription is controlled by eight promotors, leading to different mRNAs containing one of the eight untranslated 5'-exons (I to VIII) and the 3' encoding exon (IX) (20). The promotor IV region contains a cAMP responsive element as the key regulatory component (21), the expression of which is a good indicator of total *Bdnf* expression in the brain (22). The *Bdnf* promotor IV is also epigenetically regulated, through both DNA methylation and hydroxymethylation (23).

In the present study, it was hypothesized that CaA could influence gene-specific methylation status in the brain of a rat model with chronic unpredictable mild stress (CUMS), potentially mediated by the epigenetic effects of CaA on the transcription of *Dnmt* and/or *Tet* genes. Therefore, the aim of the present study was to investigate antidepressant-like activity via the epigenetic mechanisms of CaA focusing on the hippocampus and prefrontal cortex using a well-established model of CUMS (24,25).

Materials and methods

Animals. A total of 40 male Wistar rats (age, 4 weeks; mean weight ± SD, 100±10 g), were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The size of cages was 460x300x160 mm and animal designs were set up as previously described (26) (Table SI; Appendices S1 and S2). Briefly, after 2 weeks of acclimatization the rats were first trained over a period of 3 weeks to consume a 1% sucrose solution. Then, the rats were randomly and equally divided into four groups: A control group, a CUMS model group, a model group treated with the antidepressant paroxetine (Sigma-Aldrich; Merck KGaA), and a model group treated with CaA (Sigma-Aldrich; Merck KGaA). Paroxetine and CaA were administered intraperitoneally at doses of 10and 50 mg/kg for at 9:00 a.m. every day for 4 weeks, respectively. Rats in the control (no treatment) and treated groups were exposed to a series of CUMS and behavioral tests, including the sucrose preference test (SPT) and the forced swimming test (FST) (26). During these tests, food consumption and body weight were evaluated as an indicator of depression as previously described (26) (data not shown). The present study was approved by The Nanjing Medical University Institutional Animal Care and Use Committee (Nanjing, China).

Sample collection and preparation. After 4 weeks, 300 mg/kg 10% chloral hydrate solution per rat was injected intraperitoneally. No rats exhibited signs of peritonitis following administration of the anesthetic, and rats were euthanized by dislocation of the cervical vertebrae. The heart was exposed and heartbeat was assessed to confirm death. No mortality occurred outside of planned euthanasia. The hippocampus and the prefrontal cortex were dissected from the brain of sacrificed animals by a trained expert technician on ice, fixed with 4% formaldehyde overnight at room temperature, and were subsequently paraffin-embedded, or frozen with liquid nitrogen and stored at -80˚C for subsequent experiments.

Immunofluorescence (IF) and immunohistochemistry (IHC) analyses. After paraffin embedding, 3-μm thick sections were obtained, dewaxed and washed in water. Then, sections were immersed in xylene and dehydrated in graded concentrations of absolute ethanol (95, 85, 75 and 50%) at room temperature. For IHC (5mC) and IF (5hmC), sections were blocked for 30 min at room temperature with 5% BSA (Beyotime Institute of Biotechnology) and incubated overnight at 4˚C with anti-5mc (1:200; cat. no. 28692; Cell Signaling Technology, Inc.) and anti-5hmC (1:200; cat. no. 39770; Active Motif, Inc.) antibodies, respectively. Afterwards, sections were washed three times with PBS and incubated for a further 1 h at room temperature with horseradish peroxidase-labeled goat anti-rabbit antibody (1:1,000; cat. no. GB23303; Wuhan Servicebio Technology Co., Ltd.) in blocking buffer (Beyotime Institute of Biotechnology). For IHC, nuclei were stained with Harris hematoxylin for 2 min at room temperature. Staining intensity for IHC was digitalized with Panoramic SCAN (3DHISTECH Ltd.) (light microscopy mode). Each immunohistochemically stained slide was scanned with a 20x objective and images were captured at x10 magnification. For further evaluation of IHC imaging, 5hmC level was quantified by integrated optical density (IOD) of positive brown staining using Image-Pro Plus version 6.0 software (Media Cybernetics, Inc.).

For IF, an Alexa-488 labeled secondary antibody (1:300; cat. no. GB25301; Wuhan Servicebio Technology Co., Ltd.) in blocking buffer (Beyotime Institute of Biotechnology) was used for 2 h at 4˚C, and nuclei were stained with DAPI at room temperature for 1 h. Images for IF were captured using a Zeiss LSM 700B confocal microscope and the magnification was x400. The proportion (%) of 5hmC-positive foci was then quantified and calculated by the positive (green) fluorescent intensity normalized by positive staining area using Image-Pro Plus version 6.0 software (Media Cybernetics, Inc.). All IF experiments were performed in at least three independent biological replicates.

Quantification of mRNA levels of epigenetic modulators and specific genes using reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted using the FastPure Cell/Tissue Total RNA Isolation kit (Vazyme Biotech Co., Ltd.) and reverse transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.) following the manufacturer's instructions. mRNA levels of Dnmt1, Dnmt3a, Tet1-3, methyl CpG binding protein 2 (Mecp2), Bdnf and Comt genes were quantified using SYBR Green-based qPCR using 2X SYBR Green PCR mix (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. Primer sequences are listed in Table I (15,19,26). All amplifications were performed in triplicate using a LightCycler® 96 (Roche Diagnostics) according to the manufacturer's instructions with the following cycle parameters: 95˚C for 600 sec, followed by 45 repeats of 95˚C for 15 sec and 60˚C for 60 sec. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression level of the transcripts normalized to levels of *GAPDH* (27).

Specific promotor methylation and hydroxymethylation analysis. Chromatin immunoprecipitation was performed using the chromatin immunoprecipitation (ChIP)-IT Express kit (Active Motif, Inc.) according to the manufacturer's instructions. A total of 20-30 mg tissues were fixed with

Table I. Primers for quantitative PCR.

Gene	Primer sequence, 5'→3'
Tet1	
Forward	CCGGTCGCCAAGTGGGTGAT
Reverse	GGTCCACACGCTCACGAACCA
Tet2	
Forward	TACCGTACAGCCACCCAAAC
Reverse	CGT GACTGGAACTGCTCACT
Tet3	
Forward	GGACTTCTGTGCCCACGCCC
Reverse	TCAGGGTGCAGACCACAGTGC
Dnmt1	
Forward	GGCCAGCCCCATGAAACGCT
Reverse	GGGGCGTCCAGGTTGCTTCC
Dnmt3a	
Forward	TCCAACATGAGCCGCTTGGCG
Reverse	GGTGGCGGATGACTGGCACG
Mecp2	
Forward	CTTGACCTCAATGCTGACGGT
Reverse	GGGTAGAAAGCCTGGGAGTGT
Bdnf P4	
Forward	GCTGCCTTGATGTTTACTTTGA
Reverse	GCAACCGAAGTATGAAATAACC
Total *Bdnf*	
Forward	GGCCCAACGAAGAAAACCAT
Reverse	AGCATCACCCGGGAAGTGT
Comt	
Forward	TCCACAACCTGATCATGGGT
Reverse	ACATCGTACTTCTTCTTCAGCTGG
GAPDH	
Forward	TCGGTGTGAACGGATTTGGCCG
Reverse	CCGTTGAACTTGCCGTGGGT

Tet, Ten-eleven translocation; *Dnmt*, DNA methyltransferase; *Mecp*, methyl CpG binding protein; brain-derived neurotropic factor; *Bdnf*, brain-derived neurotropic factor; P4, promotor IV region; *Comt*, catechol-O-methyltransferase.

1% formaldehyde at room temperature for 15 min and lysed with lysis buffer in ChIP-IT Express kit (Active Motif, Inc.) to release chromatin. Chromatin was then enzymatically sheared to obtain chromatin, 100-500 base pairs (bp) in length, using an Enzymatic Shearing kit (Active Motif, Inc.). Approximately 50 ng sheared chromatin was then immunoprecipitated with antibodies against 5mc (1:200; cat. no. 61479) and 5hmc (1:200; cat. no. 39769) (both from Active Motif, Inc.) overnight at 4˚C. Immunoglobulin G was used as a mock control. DNA released from the reverse crosslink was purified (Universal DNA Purification kit; cat. no. DP214-02; Tiangen Biotech Co., Ltd.) according to the manufacturer's instructions. Native chromatin was used as an initial input sample. The level of DNA bound to 5mC and 5hmC antibodies was quantified using SYBR-green based qPCR. Primers were designed to amplify regions of

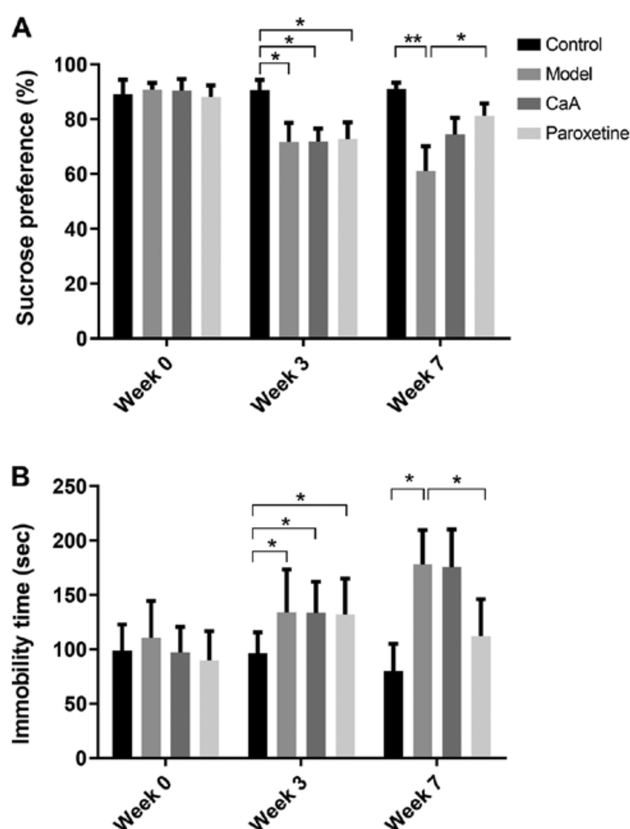

Figure 1. Results of behavioral tests for (A) sucrose preference test and (B) forced swim test for the control, model, CaA and paroxetine treatment groups. *P<0.05, **P<0.01. CaA, caffeic acid.

Bdnf and *Comt* promotor as follows: ChIP-*Bdnf*, forward: 5'-TTGTGGCATGGTTCTCAACC-3' and reverse: 5'-TAGAT CTCTGAGAAGAGGTA-3'; and ChIP-*Comt*, forward: 5'-TTT GGAGCAGGAGTAGACC-3' and reverse: 5'-TTTTAACACG CGCGGGACG-3'. PCR conditions were programmed as follows: 95˚C for 20 sec, followed by 40 cycles 95˚C for 3 sec and 60˚C for 30 sec. The levels of bound DNA sequences were then calculated using the percent input method ($2^{-[Cq\ (ChIP)\ -\ Ct\ (Input)]\ x\ 100}$) (14) by calculating the qPCR signal relative to the input sample.

Statistical analysis. All data are presented as the mean ± standard deviation. For group comparisons, Kruskal-Wallis tests were used followed by Dunn's post hoc test. All data were analyzed and plotted with GraphPad Prism version 6.0 (GraphPad Software). P<0.05 was considered to indicate a statistically significant difference.

Results

CaA can reverse changes in the behavior test induced by the CUMS procedure. The SP of each rat in the control group decreased in the first three CUMS procedures. Over the next 4 weeks of the CUMS procedure, the SP of the model group was continually decreased compared with that of the control group. Furthermore, SP was reversed by CaA and paroxetine treatment. During week 3, the model group, CaA and paroxetine groups were significantly decreased compared with the control (P<0.05). However, only paroxetine treatment showed

A Hippocampus

Control Model

CaA Paroxetine

B Prefrontal cortex

Control Model

CaA Paroxetine

Figure 2. Immunohistochemistry staining of 5-methylcytosine in the (A) hippocampus and (B) prefrontal cortex of the control, model, CaA and paroxetine treatment groups. Scale bar, 200 μm. CaA, caffeic acid.

statistical significance when compared with the model group during week 7 (P<0.05; Fig. 1A). Immobility time in the FST (26) was clearly increased in the model group during the CUMS procedure. In the model group, CaA and paroxetine treatment were significantly increased compared with the control during week 3. However, paroxetine treatment caused a significant decrease in immobility time compared with the model group and CaA treatment had no significant effect on FST during week 7 (P<0.05; Fig. 1B).

Comparison of global 5mC and 5hmC in the hippocampus and prefrontal cortex. The levels of global 5mC (IHC) and global 5hmC (IF) in the hippocampus and prefrontal cortex were detected, respectively. Immunostaining of 5mC was localized

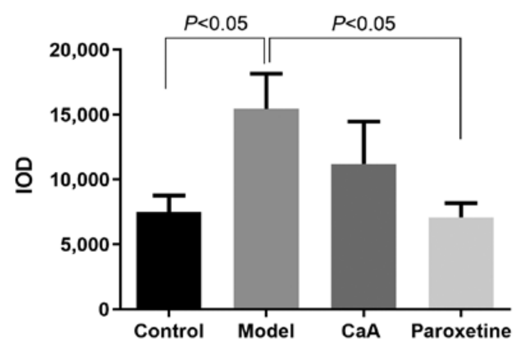

Figure 3. IOD for immunohistochemistry image analysis in the hippocampus. The same density of dark brown was selected from all images as standards using Image-Pro Plus 6.0 software. IOD, integrated optical density; CaA, caffeic acid.

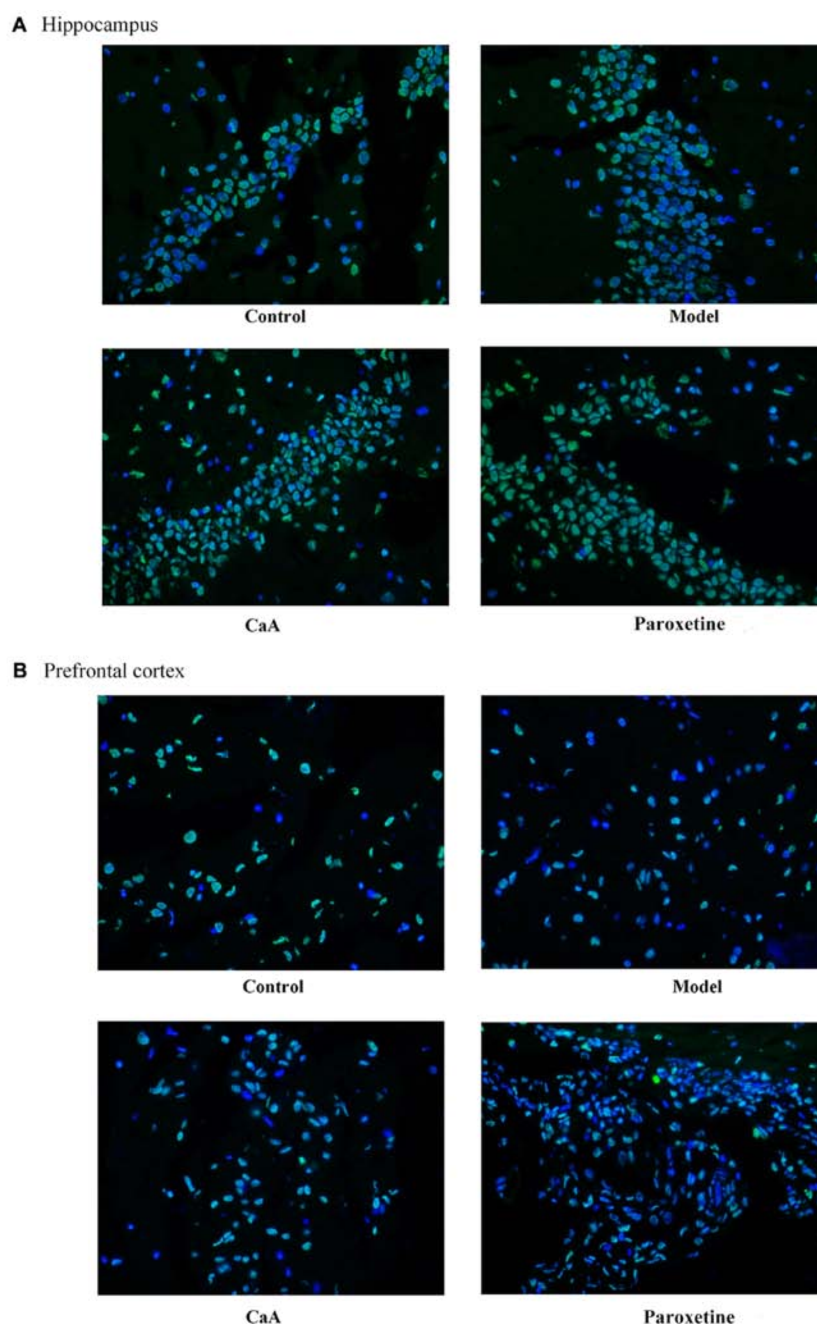

Figure 4. Global DNA hydroxymethylation levels in the (A) hippocampus and (B) prefrontal cortex of the control, model, CaA and paroxetine treatment groups. Global nuclear (blue) 5hmC levels (green) were examined using immunofluorescent staining with a 5hmC antibody. Magnification, x400. 5hmC, 5-hydroxymethylcytosine; CaA, caffeic acid.

in the nuclei of the cells, and was visualized as brown-colored staining (Fig. 2). As presented in Fig. 3, there was a significant difference in IOD value of 5mC between the model and control groups, and also between the paroxetine group and the model group ($P<0.01$ and $P<0.001$, respectively). Although the difference was not statistically significant, 5mC level was slightly lower in the CaA-treated group compared with the model group. However, in the prefrontal cortex, there was no significant difference in 5mC level among groups. Levels of 5hmC were confirmed by IF. The levels of 5hmC in the hippocampus were slightly increased in the CaA-treated group when compared with the model group. Notably, the levels of 5hmC increased in the paroxetine-treated group compared with the

Table II. Expression levels of 5hmC in the hippocampus in the control, model, CaA and paroxetine-treated groups analyzed using immunofluorescent staining.

Group	Mean ± SD	P-value
Control	0.0804±0.002	0.18
Model	0.0664±0.009	0.01[a]
CaA	0.0823±0.002	0.12
Paroxetine	0.0936±0.011	0.01

[a]$P<0.05$ vs. paroxetine. 5hmC, 5-hydroxymethylcytosine; CaA, caffeic acid.

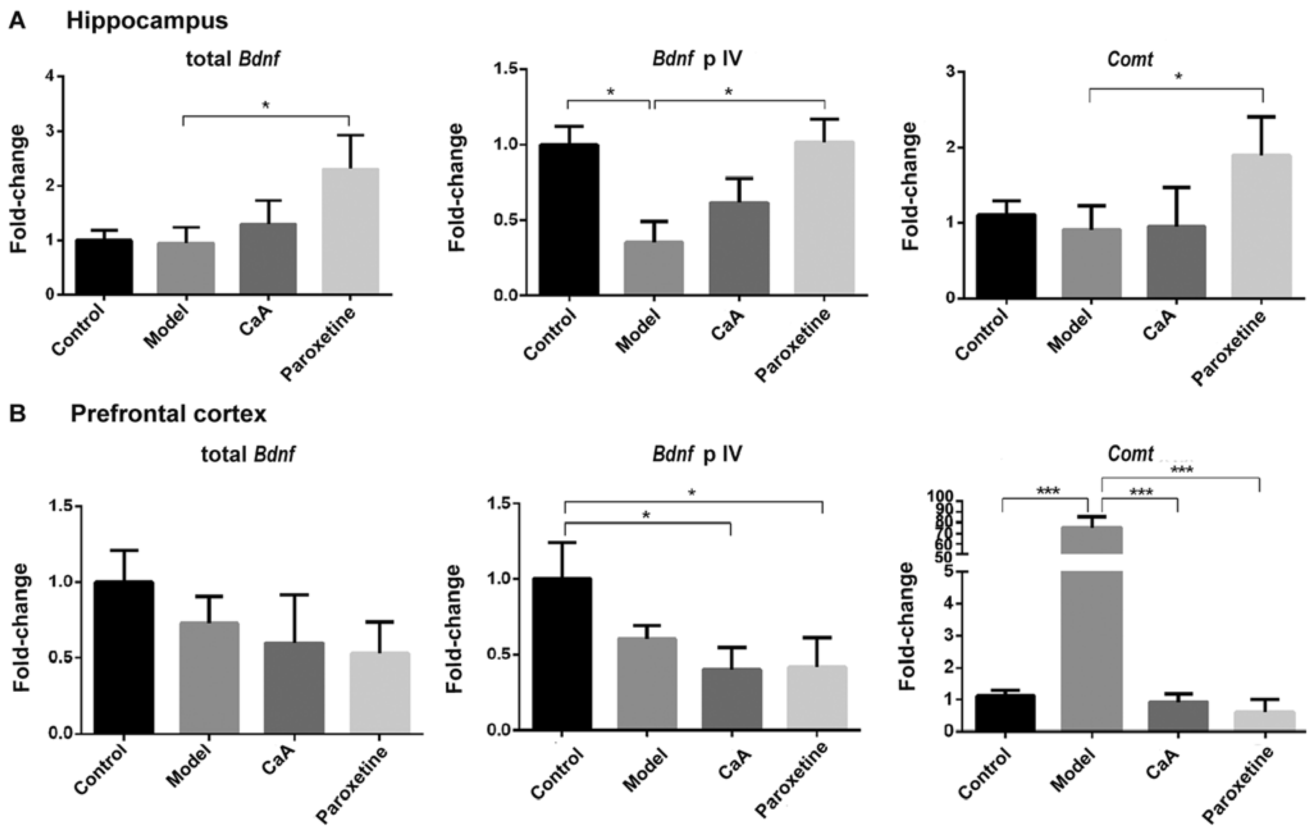

Figure 5. Gene expression levels of total *Bdnf*, *Bdnf* p IV and *Comt* in the (A) hippocampus and (B) prefrontal cortex of the control, model, CaA and paroxetine treatment groups. The mRNA levels were measured by reverse transcription-quantitative PCR. The experiment was repeated twice. Quantitative analyses were normalized to *GAPDH*. Results are expressed as a value relative to the control group using the $2^{-\Delta\Delta Cq}$ method. *P<0.05, ***P<0.001. *Bdnf*, brain-derived neurotropic factor; *Comt*, catechol-O-methyltransferase; p, promotor; CaA, caffeic acid.

model group in the hippocampus (Fig.4A; Table II). However, there was no difference in 5hmC levels in the prefrontal cortex when compared between all groups (Fig. 4B).

Effects of CaA on the expression of Bdnf and Comt. The present study assessed whether *Bdnf* and *Comt* expression could be affected by CaA and paroxetine, and whether these were associated with changes in the regulation of DNA methylation. In the hippocampus, levels of *Bdnf* promotor IV were decreased in the model group compared with the control group (P<0.05), and CaA caused a non-significant increase in *Bdnf* promotor IV levels. However, paroxetine treatment significantly reversed the inhibitory effects of CUMS. Total *Bdnf* and *Comt* levels were increased in the paroxetine-treated group compared with the model group (both P<0.05), although there was no significant difference between the model group and the CaA-treated group. In the prefrontal cortex, there was no change in total *Bdnf* expression levels, but a significant difference was observed in the expression levels of the *Bdnf* promotor IV in CaA and paroxetine treatment groups vs. control. Notably, levels of *Comt* mRNA in the model group were significantly higher when compared with those in the control group (P<0.001); however, both CaA and paroxetine treatment significantly decreased *Comt* mRNA levels compared with the model group (both P<0.001; Fig.5).

CaA influences the expression of methylated and hydroxymethylated genes. *Dnmt1*, *Dnmt3a* and *Mecp2* serve important roles in the regulation of methylation, while the *Tet1-3* family is involved in the regulation of hydroxymethylation (28,29). Previous studies have demonstrated that aberrant epigenetic modification, such as DNA methylation and hydroxymethylation, is associated with psychiatric disorders, including depression (30,31). In the present study, epigenetic-mediated gene expression was detected in the hippocampus and prefrontal cortex of rats. In the hippocampus, *Dnmt1* expression was increased in the model group when compared with the control group. CaA and paroxetine treatment blocked this increase but without statistical significance. *Tet1* expression was decreased in the model group (P<0.05) and paroxetine treatment could reverse this change. MeCP2 belongs to the family of methyl-CpG-binding domain proteins (MBDs), which bind to methylated DNA (32). In the present study, the expression of *Mecp2* did not show any difference among groups. (Fig. 6A). There were no changes in the expression levels of *Dnmt1, Dnmt3a* or *Tet1-3* genes in the prefrontal cortex; however, *Mecp2* levels were increased in the model, CaA and paroxetine-treated groups when compared with those in the control group (all P<0.05; Fig. 6B).

5mC and 5hmC enrichment in the promotor of Bdnf and Comt. In order to improve the current understanding of how changes in *Bdnf* and *Comt* gene expression may be associated with methylation levels, the present study predicted 5'-CpG island(s) in the promotor regions of both the *Comt* and *Bdnf* genes using online

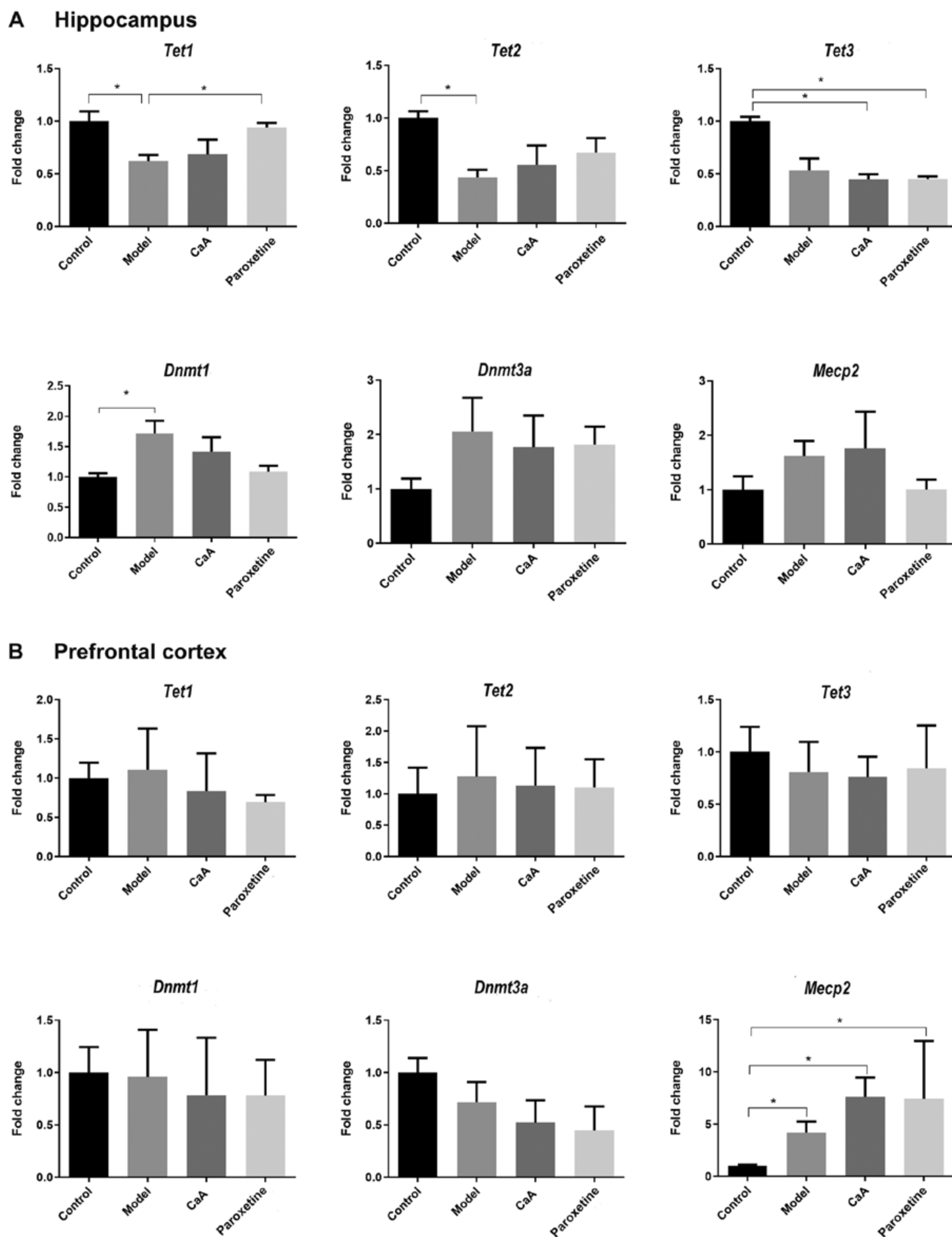

Figure 6. *Tet1-3*, *Dnmt1, Dnmt3a* and *Mecp2* mRNA levels in the (A) hippocampus and (B) prefrontal cortex of the control, model, CaA and paroxetine treatment groups. *P<0.05. *Tet*, Ten-eleven translocation; *Dnmt*, DNA methyltransferase; *Mecp*, methyl CpG binding protein; CaA, caffeic acid.

software (urogene.org/cgi-bin/methprimer/methprimer.cgi) (33) (Fig. S1). According to the position of the predicted 5'-CGI(s), the quantities of 5mC and 5hmC in these regions was determined using ChIP-qPCR. As presented in Fig. 7A, in the hippocampus, levels of 5mC were increased in the *Bdnf* and *Comt* promotors in the model group compared with the control. However, no changes were observed in the CaA and paroxetine-treated group. The expression levels of 5mC were significantly decreased in the *Comt* promotor of the prefrontal cortex model compared with those in the control group (P<0.05; Fig. 7B). Neither of the two promotors showed any changes with respect to 5hmC levels. There was an increase of 5mC/5hmC in the *Bdnf* and *Comt* gene promotors in the model group compared with the control group in the hippocampus. CaA treatment may adjust the balance of 5mC/5hmC in the hippocampus. There was a decrease of 5mC/5hmC in the *Comt*

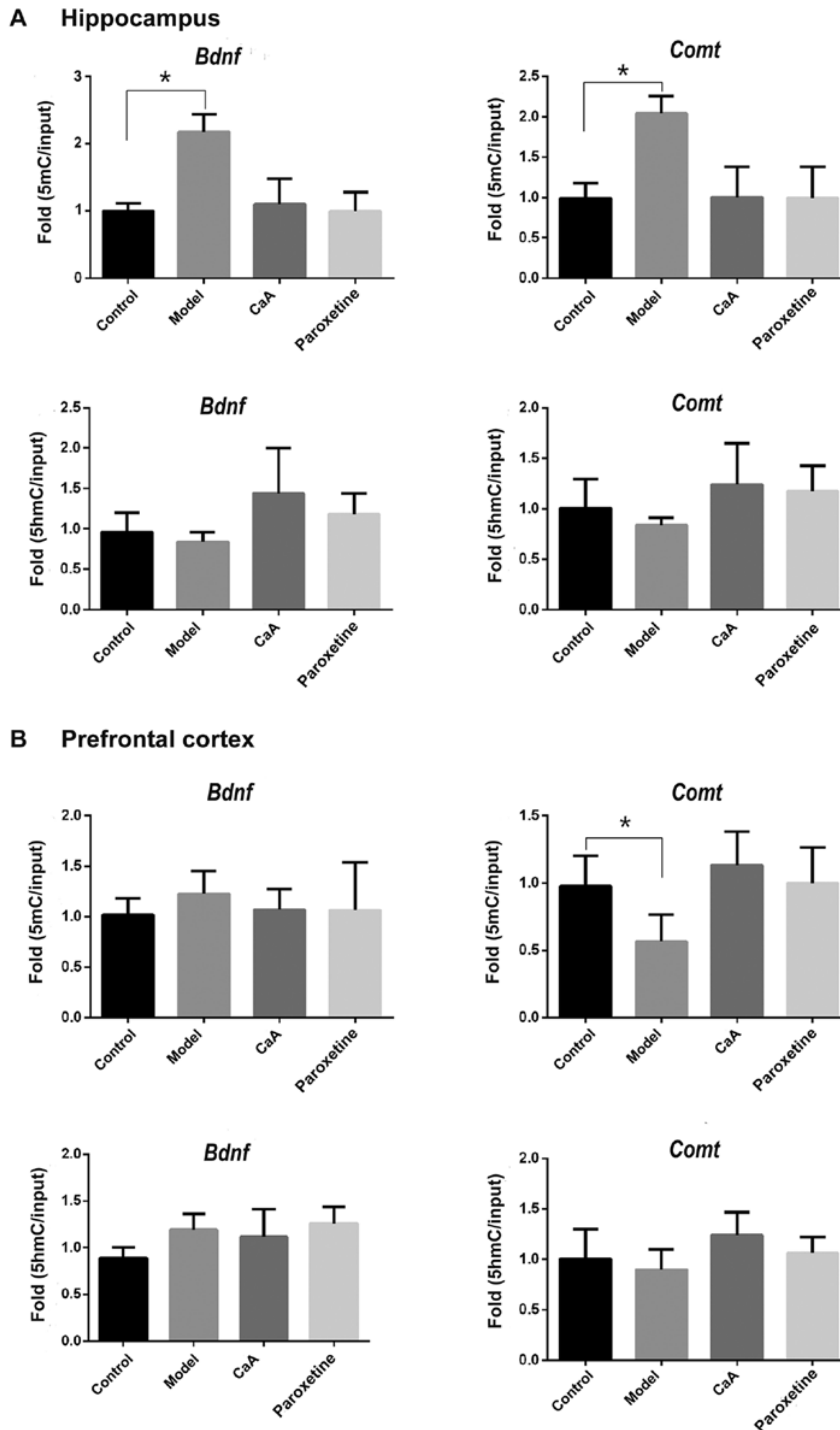

Figure 7. Enrichment of 5mC and 5hmC was measured at the *Bdnf* and *Comt* promotor regions in the (A) hippocampus and (B) prefrontal cortex. Data were normalized by input and then compared with the control. *P<0.05. *Bdnf*, brain-derived neurotrophic factor; *Comt*, catechol-O-methyltransferase; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; CaA, caffeic acid.

gene promotor in the model group vs. the control group in the prefrontal cortex, which may be restored by CaA.

Discussion

The results of the present study may provide novel insight into genetic in depression, and demonstrated that epigenetic changes for this illness are likely to involve multiple genes. Extensive studies have indicated that epigenetic modifications, such as histone acetylation and DNA methylation, serve a crucial role in the modification of DNA during neuronal gene expression and memory formation (34,35). In addition, levels of 5hmC are most abundant in the brain, which is controlled by the activation of gene transcription (36,37). In the present

study, it was hypothesized that the antidepressant-like properties of CaA may partially involve changes at the DNA methylation and hydroxymethylation levels.

In the present study, the CUMS model was selected for investigation, which is regarded as one of the most valid and reliable animal models (24,25). Behavioral data showed that CaA exerted a slight antidepressant-like effect. In the analysis of global methylation status, no robust evidence of widespread methylation differences was observed between the control and CaA groups. However, non-significant trends towards lower DNA methylation levels in the CaA group were observed. Furthermore, in the hippocampus, *Dnmt1* and *Dnmt3a* mRNA levels in the model group were increased compared with those in the control, and CaA treatment may lower this trend but this was not significant. *Tet1* and *Tet2* mRNA levels were decreased in the model group compared with those in the control, and this was reversed in the CaA group. The expression levels of *Dnmt1*/*Dnmt3a* and *Tet1*/*Tet2* were associated with the levels of global DNA methylation and hydroxymethylation, respectively, which were consistent with the results of the 5mC IHC and 5hmC IF expression analysis in the hippocampus. A previous epigenetic study has indicated potential associations between epigenetic patterns and abnormalities in the hippocampus, which is vulnerable to the pathogenesis of MDD (38). No obvious changes in global 5mC and 5hmC levels were observed in the prefrontal cortex following CaA treatment. The present study revealed that *Bdnf* and *Comt* genes had differential expression patterns in the hippocampus and prefrontal cortex, respectively. Specifically, the model group showed decreased *Bdnf* mRNA levels, which were restored to within the normal range in the hippocampus following CaA and paroxetine treatment. These results are consistent with previous human patient and animal model studies demonstrating that BDNF deficiency, particularly those lacking promotor IV-driven *Bdnf* transcription, underlies depressive states, and that antidepressant treatments restore this expression in the hippocampus (39,40). Preclinical studies have established that hippocampal BDNF expression serves a critical role in the etiology of depression; however, the exact underlying molecular mechanisms of this role remain unclear. Notably, the complex regulation of *Bdnf* gene transcription may provide the opportunity to clarify this uncertainty. Keller *et al* (40) first reported that lower *Bdnf* expression is associated with increased DNA methylation of *Bdnf* promotor IV. Therefore, the present study analyzed the levels of 5mC and 5hmC in the promotor region of the *Bdnf* gene following CaA treatment in rats. It was demonstrated that increased *Dnmt1* levels in the model group were associated with increased levels of 5mC in the *Bdnf* gene promotor. There was a decrease in 5hmC levels in the model group compared with the control group; however, this was not statistically significant. When these findings were expressed as a ratio of 5mC/5hmC, the data showed a statistically significant increase in the *Bdnf* promotor. These data suggested a shift in the equilibrium towards methylation in the hippocampus following CUMS, which may have been driven by the increased quantity of steady state 5mC in the promotor regions. CaA treatment may restore the expression of *Bdnf* by adjusting the balance of 5mC/5hmC in the hippocampus. Notably, there was a marked increase in *Comt* gene expression in the prefrontal cortex in the model group compared with the control group, and CaA reversed this. There was also a decrease of 5mC/5hmC, which was shifted towards hydroxymethylation in the prefrontal cortex. These findings indicate that the expression of *Comt* gene can be regulated by DNA methylation and hydroxymethylation in the brain. *COMT* is the primary regulator of dopamine clearance in extra-striatal regions of the brain, including the prefrontal cortex (13,41). Elevated levels of COMT may accelerate the metabolism of dopamine and result in decreased dopamine levels, which may in turn influence brain function, potentially leading to depression. Of note, CaA treatment resulted in differential DNA methylation regulation of *Bdnf* and *Comt* genes in the hippocampus and the prefrontal cortex, respectively. During antidepressant treatment, epigenetic mechanisms may exert a dynamic and tissue-specific regulation of gene transcription (42). Therefore, studies investigating epigenetic mechanisms have improved the current understanding of the molecular basis for antidepressant treatment in MDD.

In the present study, the effects of CaA on MDD had, in part, the same trend as the antidepressant, paroxetine. It was revealed that there were epigenetic changes in the genes (*Bdnf* and *Comt*) associated with depression, which were reversible with CaA treatment. It has previously been demonstrated that CaA could enhance the expression of microRNA (miR)-148a by decreasing *Dnmt1* mRNA levels (43). There is also a support that CaA may act as a histone deacetylase inhibitor for breast cancer treatment (44). Thus, CaA, as a phytochemical and a dietary supplement, may be a comprehensive epigenetic modulator, and may have potential as an antidepressant. The results of the present study are promising and these trends require further validation in studies with larger sample size. However, it should be noted that the present study did not detect the effects of every epigenetic-associated enzyme on gene transcription, such as DNMTs/MBDs mediating DNA methylation and histone deacetylases/histone acetyltransferases. It is hypothesized that CaA may recruit the binding of the aforementioned enzymes to specific CpG sites of the gene promotor. These putative bulky enzymatic complexes may inhibit the binding of transcription co-activators at the promotor leading to decreased gene expression, and *vice versa*. Future mechanistic studies should aim to elucidate the mechanisms of CaA as a direct or indirect epigenetic modulator. Further experiments should also investigate the effects of different CaA dosages and administration methods.

In conclusion, the present results demonstrated that there were epigenetic changes in the hippocampus and prefrontal cortex in rats with CUMS. CaA may function as a modulator of DNA methylation to regulate *Bdnf* and *Comt* gene transcription. Although a limitation of the present study was a lack of statistical power, the trends observed provide a mechanistic basis for the use of this phytochemical agent in the treatment of depression.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QW and LL conceived and designed the experiments. JH, SC, DW and LW performed the experiments. QW, ZZ and SC analyzed data. QW wrote the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Nanjing Medical University Institutional Animal Care and Use Committee (Nanjing, China) (approval no. NJMU2015/81473020).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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