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Research Report

Intrathecal 5-azacytidine inhibits global DNA methylation and methyl- CpG-binding protein 2 expression and alleviates neuropathic pain in rats following chronic constriction injury

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ABSTRACT

The pathogenesis of neuropathic pain remains largely unknown. Epigenetic mechanisms may play a major role in regulating expression of pro- or antinociceptive genes. DNA methylation is a major epigenetic mechanism in vertebrates, and methyl- CpG-binding protein 2 (MeCP2) is directly involved in methylation-mediated gene silencing. To determine how changes in global DNA methylation and MeCP2 expression occur following chronic constriction injury (CCI) and how repression of DNA methylation affects these changes and attenuates neuropathic pain, we used intrathecal 5-azacytidine, a DNA methyltransferase inhibitor, in CCI rats. Rats received 0.9% saline or 5-azacytidine (10 $\mu mol \cdot d^{-1}$) via spinal injection once daily from day 3 to day 14 after CCI surgery. Global DNA methylation and MeCP2 expression increased in the spinal cord in CCI rats on day 14 after CCI surgery. Mechanical allodynia and thermal hyperalgesia induced by CCI were attenuated by intrathecal 5-azacytidine from day 5 to day 14 after CCI surgery. The increases in global DNA methylation and MeCP2 expression in the spinal cord in CCI rats were also significantly inhibited by intrathecal 5-azacytidine. These results demonstrate that increased global DNA methylation and MeCP2 expression in the spinal cord after nerve damage may play an important role in neuropathic pain. 5-azacytidine shows potential for treating neuropathic pain.

1. Introduction

Chronic neuropathic pain is caused by primary or secondary damage or dysfunction of the nervous system. Neuropathic pain is characterized by hyperalgesia, allodynia, and spontaneous pain (Cruccu et al., 2004; Moalem and Tracey, 2006). The pathogenesis of neuropathic pain is complex, and many factors remain unknown. Recent evidence suggests that expression levels of pain-associated genes in sensory neurons, immune cells, and glial cells are highly involved in the generation and maintenance of neuropathic pain (Scholz and Woolf, 2007). Epigenetic mechanisms that induce heritable changes in gene expression without changing the DNA sequence can regulate the transcription and expression of pro- or antinociceptive genes (Doehring et al., 2011). DNA methylation is one of the most characterized epigenetic mechanisms in vertebrates and is a major contributor to the stability of gene expression (Jaenisch and Bird, 2003). Methyl-CpG-binding protein 2 (MeCP2) was the first

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methyl-CpG binding protein to be cloned, which binds methyl-CpG and inhibits the transcription of DNA methylation-dependent genes (Lewis et al., 1992). MeCP2 is expressed predominantly in neurons and nearly absent in glial cells in the mature mammalian central nervous system (CNS) (Akbarian et al., 2001; Kishi and Macklis, 2004; Shahbazian et al., 2002). In the adult spinal cord, MeCP2 is expressed in all dorsal horn neurons (Geranton et al., 2007). X-linked MeCP2 gene mutation or rearrangement is the main cause of Rett syndrome (Amir et al., 1999). A recent study of patients with MeCP2 mutations suffering from Rett syndrome revealed an abnormal response and sensitivity to pain (Downs et al., 2010). Reports also suggest that MeCP2 may play an important role in regulating gene expression in inflammatory pain (Geranton et al., 2007, 2008). Therefore, we hypothesized that DNA methylation and MeCP2 may be involved in the development and maintenance of neuropathic pain. In this study, we examined global DNA methylation and MeCP2 expression in the spinal cord in rats following chronic constriction injury (CCI) surgery. We further examined the effects of intrathecal administration of the demethylation reagent 5-azacytidine (5-AZA), the most extensively studied DNA methyltransferase (DNMT) inhibitor (Oki and Issa, 2006), on global DNA methylation, MeCP2 expression, and neuropathic pain in CCI rats.

2. Results

2.1. *Changes in behavior and neuropathic pain measurements*

CCI rats suffering from neuropathic pain showed various signs of protecting postures and movement disorders such as foot and toe closing, dorsiflexion, eversion, and a marked limp. Sham-operated rats did not show any such signs. The ipsilateral mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) showed similar changes in CCI rats (Fig. 1). After catheterization and before CCI surgery, there was no significant difference in MWT or TWL among the rats ($P>0.05$). On the third day after CCI surgery, the MWT and TWL in CCI rats decreased and were obviously lower than before the operation ($P<0.05$), indicating that mechanical allodynia and thermal hyperalgesia had become apparent in CCI rats. The MWT and TWL were reduced in all vehicle-treated CCI rats from day 3 to day 14 after CCI surgery. Mechanical allodynia and thermal hyperalgesia were consistently attenuated in 5-AZA-treated CCI rats from day 5 to day 14 after CCI surgery.

2.2. *Changes in the level of global DNA methylation*

The level of global DNA methylation in the lumbar spinal cord in all rats 14 days following CCI surgery was significantly different among the groups (Fig. 2; $P<0.05$). Global DNA methylation levels increased in vehicle-treated CCI rats compared with sham-operated rats ($P<0.05$). 5-AZA-treated CCI rats showed lower global DNA methylation levels compared to vehicle-treated CCI rats ($P<0.05$). Thus, upregulation of global DNA methylation by CCI is inhibited by 5-AZA, a DNMT blocker.

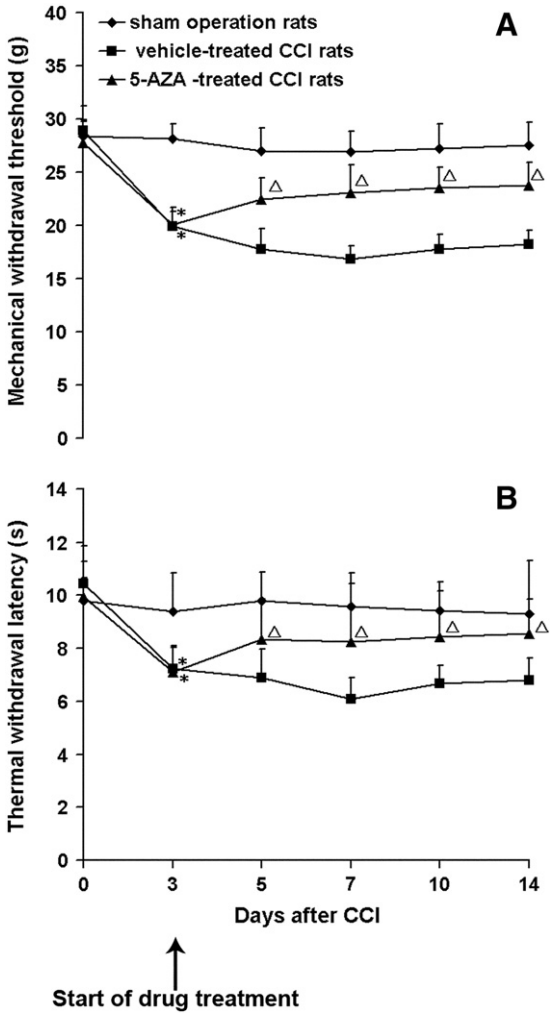

Fig. 1 – The mean (±S.E.M.) data of ipsilateral mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) in all rats. A: Change in mechanical allodynia in all rats over time following CCI. B: Change in thermal hyperalgesia in all rats over time following CCI. Comparison of MWT and TWL in rats with different treatments: * vs. before operation, $P<0.05$; △ vs. vehicle-treated CCI rats, $P<0.05$.

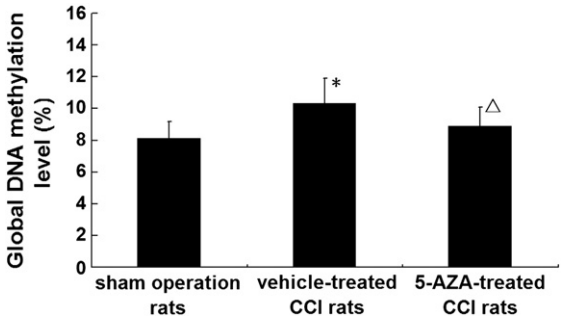

Fig. 2 – Global DNA methylation profiles. The mean (±S.E.M.) global DNA methylation levels in the lumbar spinal cord in all rats 14 days following CCI. Comparison of global DNA methylation levels in rats with different treatments: * vs. sham-operated rats, $P<0.05$; △ vs. vehicle-treated CCI rats, $P<0.05$.

2.3. Changes in MeCP2 expression

2.3.1. Reverse transcription PCR (RT-PCR) analysis

RT-PCR analysis showed clear expression of MeCP2 and β-actin mRNA in the lumbar spinal cord in all rats 14 days following CCI, and the results were significantly different among the groups (Figs. 3A and B; $P<0.05$). Sham-operated rats showed the lowest MeCP2 mRNA expression level, and a significant increase was seen in vehicle-treated CCI rats ($P<0.05$ vs. sham control). 5-AZA-treated CCI rats showed only a moderate increase in MeCP2 mRNA expression, which was lower than vehicle-treated CCI rats ($P<0.05$), indicating that 5-AZA reduced the upregulation of MeCP2 mediated by CCI.

2.3.2. Western blot analysis

Western blot results were consistent with those from RT-PCR (Figs. 3C and D). We observed clear expression of nuclear MeCP2 protein and β-actin in the lumbar spinal cord in all rats 14 days following CCI, and significant differences were observed among the groups ($P<0.05$). Nuclear MeCP2 expression was significantly increased in vehicle-treated CCI rats ($P<0.05$ vs. sham control); 5-AZA-treated CCI rats showed a moderate increase that was lower than that in vehicle-treated CCI rats ($P<0.05$).

2.3.3. Immunocytochemistry analysis

Immunostaining results were also consistent with findings from RT-PCR and western blotting. Abundant MeCP2 labeling was seen in the dorsal horn of the lumbar spinal cord in all rats 14 days following CCI surgery (Fig. 4). Vehicle-treated CCI rats had the most MeCP2-positive cells (Fig. 4B), 5-AZA-treated CCI rats had intermediate levels (Fig. 4C), and sham-operated rats had the least MeCP2 labeling (Fig. 4A).

3. Discussion

Transcription and expression of pain-associated genes, on which DNA methylation may exert a major effect, are highly relevant to the genesis and development of neuropathic pain. Elucidating the role of DNA methylation in neuropathic pain should facilitate development of new therapies that are urgently needed because conventional painkillers such as opioids and non-steroidal anti-inflammatory drugs have poor efficacy in neuropathic pain (Dworkin et al., 2003).

In this study, MeCP2 was strongly expressed in the lumbar spinal cord in rats, which is in agreement with Geranton's report (Geranton et al., 2007). Increased global DNA methylation, MeCP2 mRNA level, and nuclear MeCP2 protein localization were clearly detected in the lumbar spinal cord 14 days after CCI surgery, accompanied by mechanical allodynia and thermal hyperalgesia in CCI rats. The increases in global DNA methylation and MeCP2 expression in CCI rats indicates an important role for both in neuropathic pain, and the increases in global DNA methylation and MeCP2 expression may be involved in downregulating the expression of some DNA methylation-dependent antinociceptive genes in the lumbar spinal cord in CCI rats.

DNA methylation is catalyzed by DNMTs, which transfer a methyl group from S-adenosyl-L-methionine to cytosine bases in DNA (Guz et al., 2010). 5-AZA is one of the most intensively studied DNMT inhibitors, and shows potential to reduce DNA methylation and reactivate silenced genes when cells are synthesizing DNA (Holliday and Ho, 2002), such as 5-AZA could restore expression of tropomyosin-related kinase A (TrkA), TrKB and TrKC in liver cell lines (Jin et al., 2011). The spinal cord is made up of two classes of cells, neurons and glial cells. For a long time, it was considered that mature neurons had no

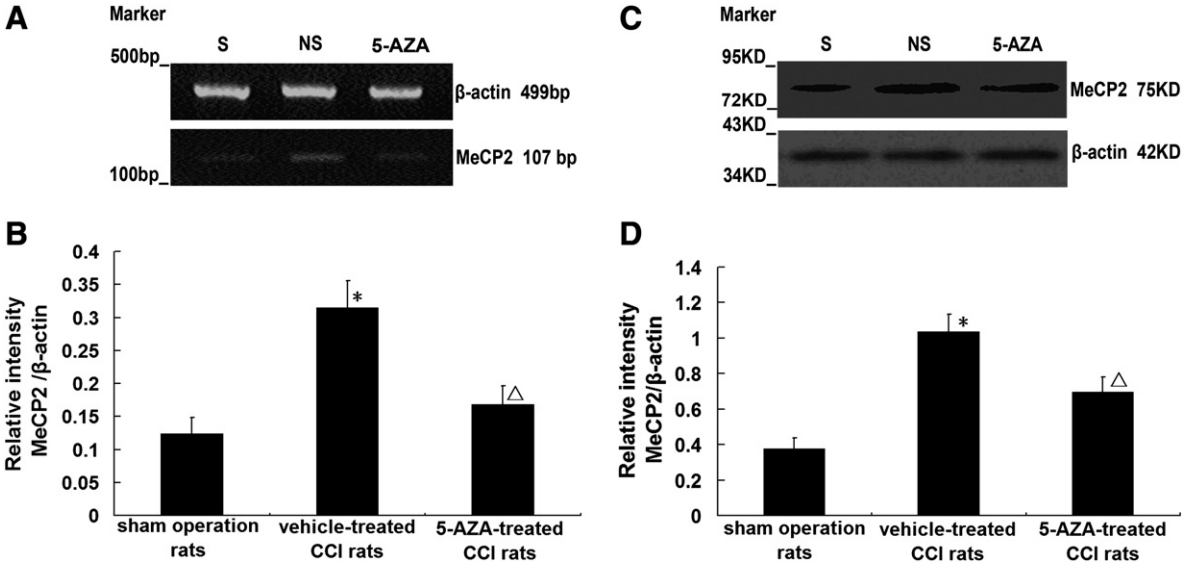

Fig. 3 – RT-PCR and western blot analysis. A: Expression of mRNAs for MeCP2 (107 bp) in the lumbar spinal cord in all rats 14 days following CCI. The β-actin fragment (499 bp) was used as an internal control. B: The mean (±S.E.M.) of integral optical density (IOD) of MeCP2/β-actin in all rats. C: Expression of nuclear MeCP2 (75 kDa) in the lumbar spinal cord in all rats 14 days following CCI. β-actin (42 kDa) was used as an internal control. D: The mean (±S.E.M.) of IOD of MeCP2/β-actin in all rats. Comparison of the IOD of MeCP2/β-actin in rats with different treatments: * vs. sham-operated rats, $P<0.05$; △ vs. vehicle-treated CCI rats, $P<0.05$. S: sham-operated rats; NS: vehicle-treated CCI rats; 5-AZA: 5-AZA-treated CCI rats.

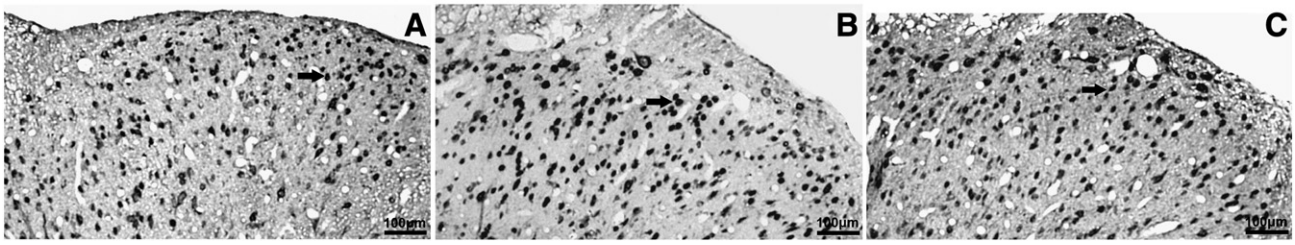

Fig. 4 – Representative images showing immunoreactivity for MeCP2 in the lumbar spinal cord in rats 14 days following CCI. Representative MeCP2-positive cells in the dorsal horn were pointed by arrows A: The lumbar spinal cord in sham-operated rats with basal MeCP2 expression. B: The lumbar spinal cord in vehicle-treated CCI rats with highly increased MeCP2 expression. C: The lumbar spinal cord in 5-AZA-treated CCI rats with moderately increased MeCP2 expression.

potential to synthesize DNA and neurogenesis was non-existent in the adult mammalian CNS. Recent studies have shown that DNA synthesis and neurogenesis occur in the adult mammalian CNS under pathological conditions such as injury (Bauer and Patterson, 2005; Kuan et al., 2004; Vessal et al., 2007; Ziv and Schwartz, 2008), and even under normal physiological conditions (Halim et al., 2004; Korr and Schultze, 1989; Shechter et al., 2007). Cell proliferation and DNA synthesis had been examined in the glia cells in the adult mammalian CNS after injury (Oishi et al., 1998; Scholz and Woolf, 2007). So, DNA synthesis may occur in the neurons and glia cells in the spinal dorsal horn after nerve damage in CCI rats. In our current study, mechanical allodynia and thermal hyperalgesia induced by CCI were markedly attenuated by intrathecal 5-AZA treatment from day 5 to day 14 in CCI rats. Intrathecal 5-AZA treatment may alleviate neuropathic pain by upregulating the expression of some DNA methylation-dependent antinociceptive genes in the lumbar spinal cord in CCI rats. Similarly, Stephanopoulos reported that 5-AZA significantly enhances the rate of recovery from herpes simplex virus in latent herpes simplex virus type 2-infected dorsal root ganglia and spinal cords (Stephanopoulos et al., 1988). Reduced global DNA methylation and MeCP2 expression as the result of 5-AZA treatment may contribute to this effect. Determining the exact mechanisms of how 5-AZA leads to a decrease in MeCP2 expression is beyond the scope of our current experiment. One hypothesis is that less MeCP2 may be needed to bind methyl-CpG, followed by decreased methyl-CpG in nuclei. Therefore, 5-AZA may indirectly inhibit nuclear MeCP2 expression by inducing hypomethylation in CCI rats. Thus, DNA methylation may be a significant mechanism in neuropathic pain. 5-AZA has been approved by the U. S. Food and Drug Administration for chemotherapy against myelodysplastic syndrome and some other malignant diseases (Ghoshal and Bai, 2007; Oki and Issa, 2006). It may also be a potential drug for treating neuropathic pain. However, this study had some limits and only focused on changes on 14 days post CCI. There will be further complete to expand this observation of neuropathic pain and DNA methylation to longer term CCI and other models such as Chung's model.

In summary, our results suggest that DNA methylation and MeCP2 may play an important role in neuropathic pain. Inhibition of DNMTs with drugs such as 5-AZA may be a new therapeutic approach for neuropathic pain and may provide some hints for further studies of the involvement of DNA methylation in neuropathic pain.

4. Experimental procedures

4.1. *Animals*

This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Animal Care Committee of Central South University in China approved all the procedures. Twenty-seven male Sprague–Dawley rats weighing 250–280 g were provided by the animal experiment center of Xiangya Medicine School, Central South University.

4.2. *Surgeries*

Under deep anesthesia induced by intraperitoneal injection of 10% chloral hydrate (300–350 mg/kg), a chronic lumbar intrathecal catheter was implanted in the subarachnoid space in all rats as described (Xu et al., 2009). After 5 days of recovery, rats were randomly divided into the sham-operated group (n=9) and two CCI groups (vehicle-treated and 5-AZA-treated groups, n=9 each). Under deep anesthesia, a modified CCI to the sciatic nerve was performed on the left side (Bennett and Xie, 1988). In the sham operation, the nerve was exposed but not ligated.

4.3. *Pharmacological treatments*

Four different doses (100 nmol·d^{-1}, 1 μmol·d^{-1}, 10 μmol·d^{-1}, 100 μmol·d^{-1}) of intrathecal 5-AZA were used in our pilot study according to previous reports (Aizawa et al., 2009; Sciandrello et al., 2003; Stephanopoulos et al., 1988), and we found that 10 μM intrathecal 5-AZA was the most effective dose that did not produce significant side effects on the rats' motor functions. Sham-operated rats received vehicle (0.9% saline), and CCI rats received either vehicle (0.9% saline) or 5-AZA (10 μmol·d^{-1}, Sigma, USA) intrathecally once a day from days 3 to 14 after CCI surgery.

4.4. *Neuropathic pain measurements*

Ipsilateral mechanical allodynia and thermal hyperalgesia in all rats were monitored by a 2390 Electronic von-Frey Anesthesiometer (IITC Life Science, USA) and a Hargreaves Tes7370 (Ugo Basile, Italy), respectively (Wang et al., 2010). The MWT and TWL of each rat were measured before the CCI, and on days 3,

5, 7, 10, and 14 after CCI surgery. All behavioral testing was done by observers unaware of the animal treatments.

4.5. *Sample preparation*

On day 14 after CCI, six rats from each group were chosen randomly to observe global DNA methylation with ELISA, MeCP2 mRNA expression with RT-PCR, and nuclear MeCP2 protein localization with western blot analysis. The rats were killed under deep anesthesia, and their lumbar spinal cords were immediately dissected. The remaining three rats in each group were used for immunostaining of MeCP2 expression. Under deep anesthesia, 200 ml normal saline followed by 400 ml 4% paraformaldehyde was perfused into the rats through their ascending aorta. The rats were then killed, and their lumbar spinal cords were dissected.

4.6. *Global DNA methylation measurements*

To measure global DNA methylation, genomic DNA was extracted with the Cwbio TissueGen DNA kit (Cwbio, China). Global DNA methylation was measured using an ELISA method with the Methylamp™ Global DNA Methylation Quantification kit (Epigentek Group Inc, USA) (Luo et al., 2008).

4.7. *RT-PCR analysis*

For RT-PCR analysis, the MeCP2 primer sequences were: forward 5′-CGCGAAAGCTTAAACAGAGGA-3′, and reverse 5′-TGCAAT CAATTCTACTTTAGAGCGA-3′. β-actin primer sequences were: forward 5′-TGAGCTGCGTGTGGCCCCTGAG-3′, and reverse 5′-GGGGCATCGGAACCGCTCATTG-3′. Total RNA was extracted using Trizol Reagent (Invitrogen, USA). cDNA templates were synthesized by reverse transcription and stored at −20 °C. PCR conditions for MeCP2 amplification were 1 cycle of predenaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; 1 cycle of extension at 72 °C for 2 min, and termination at 4 °C. Positive bands were analyzed with Quantity One software (Bio-Rad, USA) to measure optical density. MeCP2 expression levels were normalized to β-actin levels.

4.8. *Western blot analysis*

For western blot analysis, nuclear proteins were extracted from tissues with the Keygen Nucleoprotein and cytoplasmic protein extraction kit (Keygen Biotech, China). Each nuclear protein sample (10 μg) was separated on a 12% polyacrylamide gel and electrotransferred to a nitrocellulose membrane in the Mini Trans-Blot electrophoresis Transfer Cell (Bio-Rad, USA). Membranes were blocked in 5% skim milk powder at 37 °C for 2 h. Membranes were incubated with anti-rabbit MeCP2 (molecular weight 75 kDa, 1:400, Proteintech Group Inc., USA) and anti-mouse β-actin (molecular weight 42 kDa, 1:1000, ProMab, USA) at room temperature on a shaker for 2 h and then at 4 °C overnight, followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:40,000) and goat anti-mouse IgG conjugated to horseradish peroxidase (1:50,000) at room temperature on a shaker for 1 h. The immunocomplexes were visualized by chemiluminescence using an ECL kit (Pierce, USA). Positive bands were analyzed with Quantity One software (Bio-Rad). Nuclear MeCP2 expression levels were normalized to β-actin levels (Sharma et al., 2008; Tikoo et al., 2009).

4.9. *Immunocytochemistry analysis*

For immunocytochemistry, tissues were fixed with 4% paraformaldehyde for 6 h, dehydrated, embedded in paraffin, sectioned at 5 μm thickness, and stored at 4 °C. After dewaxing, the sections were treated with 0.01 M citrate buffer at 95 °C for 20 min for antigen retrieval. Then, the sections were treated with 3% H_2O_2 for 10 min, blocked with 10% goat serum at 37 °C for 20 min, incubated with rabbit polyclonal anti-MeCP2 (1:50, Proteintech Group Inc.) at 4 °C overnight and then at 37 °C for 1 h. The sections were then incubated with biotinylated anti-rabbit IgG at 37 °C for 20 min, and then with horseradish peroxidase–conjugated streptavidin at 37 °C for 20 min. Finally, sections were incubated with 0.05% diaminobenzidine and 0.01% H_2O_2 for 1–2 min for development of color. Between each incubation, sections were washed with 0.01 M phosphate-buffered saline three times for 5 min. Negative controls lacked the primary antibody. Cells with brown-yellow granules were considered positive.

4.10. *Statistical analysis*

Data reflect the mean ± S.E.M. ANOVA was used to compare values obtained for MWT, TWL, global DNA methylation, and MeCP2 expression, using the SNK-q test, and the LSD test was used to compare groups. A P value of <0.05 was considered significant.

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