Emodin Rescued Hyperhomocysteinemia-Induced Dementia and Alzheimer’s Disease-Like Features in Rats

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Abstract

Background: Hyperhomocysteinemia is an independent risk factor for dementia, including Alzheimer’s disease. Lowering homocysteine levels with folic acid treatment with or without vitamin B12 has shown few clinical benefits on cognition.

Methods: To verify the effect of emodin, a naturally active compound from *Rheum officinale*, on hyperhomocysteinemia-induced dementia, rats were treated with homocysteine injection (HCY, 400 μg/kg/d, 2 weeks) via vena caudalis. Afterwards, HCY rats with cognitive deficits were administered intragastric emodin at different concentrations for 2 weeks: 0 (HCY-E0), 20 (HCY-E20), 40 (HCY-E40), and 80 mg/kg/d (HCY-E80).

Results: β-Amyloid overproduction, tau hyperphosphorylation, and losses of neuron and synaptic proteins were detected in the hippocampi of HCY-E0 rats with cognitive deficits. HCY-E40 and HCY-E80 rats had better behavioral performance. Although it did not reduce the plasma homocysteine level, emodin (especially 80 mg/kg/d) reduced the levels of β-amyloid and tau phosphorylation, decreased the levels of β-site amyloid precursor protein-cleaving enzyme 1, and improved the activity of protein phosphatase 2A. In the hippocampi of HCY-E40 and HCY-E80 rats, the neuron numbers, levels of synaptic proteins, and phosphorylation of the cAMP responsive element-binding protein at Ser133 were increased. In addition, depressed microglial activation and reduced levels of 5-lipoxygenase, interleukin-6, and tumor necrosis factor α were also observed. Lastly, hyperhomocysteinemia-induced microangiopathic alterations, oxidative stress, and elevated DNA methyltransferases 1 and 3β were rescued by emodin.

Conclusions: Emodin represents a novel potential candidate agent for hyperhomocysteinemia-induced dementia and Alzheimer’s disease-like features.

Keywords: homocysteine, emodin, dementia, Aβ, tau
Significance Statement
Hyperhomocysteinemia (HHcy) is an independent risk factor for dementia, including Alzheimer’s disease (AD). In this study, we tested the effect of emodin, a natural active compound from *Rheum officinale*, on HHcy-induced dementia. Emodin rescued HHcy-induced dementia by counteracting the AD-like features including β-amyloid (Aβ) overproduction, tau hyperphosphorylation, neuron loss, synaptic damage, cerebral microvascular damage and inflammation, and alleviating the oxidative stress and methylation impairments in the brains of rats. Our results suggest that emodin represents a novel potential candidate agent for HHcy-induced cognitive deficits.

Introduction
Homocysteine (Hcy) is a crucial intermediate in the metabolism of methionine (Met) to cysteine (Cys) (Prudova et al., 2006). Three pathways are involved in Hcy metabolism: remethylation, transsulfuration, and cyclization (Finkelstein, 1998; Selhub, 1999; Troen, 2005; Kumar et al., 2017). Hcy can be remethylated to Met by methionine synthase enzymes such as 5-methyltetrahydrofolate-homocysteine methyltransferase (remethylation), or it can be metabolized to Cys by cystathionine β-synthase (CBS) and with the help of the cofactors folate, vitamin B12 (Vit B12), and Vit B6 (transsulfuration). Additionally, Hcy can undergo cyclization to form homocysteine thiolactone (cyclization). Elevated Hcy, or hyperhomocysteinemia (HHcy), is mainly due to genetic defects in the Hcy metabolism enzymes such as CBS and methylenetetrahydrofolate reductase or deficiencies of certain cofactors in Hcy metabolism including folate, Vit B6, Vit B12, and choline (Troen et al., 2008a, 2008b; Kumar et al., 2017; 2018). Additionally, several other factors such as pregnancy and lactation are reported to contribute to HHcy (Desouza et al., 2002; Cotter et al., 2003).

HHcy is a risk for dementia, including Alzheimer’s disease (AD) and vascular dementia (Obeid et al., 2007; Sharma et al., 2015). Clinical studies concluded that Hcy is associated with the degree of cognitive impairment and acts as a biomarker for the progression of dementia (Kitzlerová et al., 2014). Previously, we reported the correlation between higher plasma Hcy levels with cognition deficits and AD-like features including β-amyloid (Aβ) overproduction and tau hyperphosphorylation in the hippocampi of HHcy rats (Zhang et al., 2008; 2009).

Supplements of Vit B12 and folate are regarded as effective Hcy-lowering interventions (Malouf et al., 2003; de Koning et al., 2016). However, studies from the trials (Fioravanti et al., 1998; Clarke et al., 2003; Sommer et al., 2003) reported that folate with or without Vit B12 did not improve any measures of cognition or mood for healthy, cognitively impaired or demented people or without Vit B12 did not improve any measures of cognition or mood for healthy, cognitively impaired or demented people. The injections were performed with an insulin syringe (29 gauge, 0.33 × 12.7 mm) and filtered before injection. Emodin was obtained from Shanghai Base Industry (Shanghai, China) and dissolved in 0.5% carboxymethylcellulose sodium, which was from Xilong Chemical (Guangzhou, China). The sandwich enzyme-linked immunosorbent assay (ELISA) kit for Hcy was purchased from Mosak Biotecnology (Wuhan, China), and the ELISA kits for Aβ40, Aβ42, malondialdehyde (MDA), superoxide dismutase 1 (SOD1), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) were from Elabscience Biotecnology (Wuhan, China). The antibodies used are listed in Table 1.

Animals and Treatments
Sprague-Dawley rats (2 months old, 280 ± 10 g) were obtained from the Disease Control and Prevention Center of Hubei Province (Wuhan, China). All rats received food and water ad libitum in a standard animal house, with a 12-hour-light/dark cycle with the lights on from 7:00 AM to 7:00 PM, relative humidity (55 ± 15%), and a constant room temperature (22 ± 2°C). All experiments in this study were performed according to the Policies on the Use of Animals and Humans in Neuroscience Research revised and approved by the Society for Neuroscience in 1995.

Sixty-five male rats were randomly divided into 2 groups: 52 rats were administered 2 weeks of Hcy injections (400 μg/kg/d, via vena caudalis, HCY rats) as previously reported (Zhang et al., 2008; 2009), and 13 rats were administered NS injections (CON rats) in the same volume as the Hcy treatments (Figure 1A). The injections were performed with an insulin syringe (29 gauge, 0.33 × 12.7 mm) at 9:00 AM every day. After the final injection, the animals completed the open field test (OFT) and novel object recognition test (NORT) to assess their locomotor and cognitive activities. In the preliminary experiments, we observed that intragastric administrations of 100 and 120 mg/kg/d emodin induced obvious weight loss and lipid stop in adult SD male rats. Therefore, HCY rats were randomly divided into 4 groups (HCY-E0, HCY-E20, HCY-E40, and HCY-E80, n = 13/group) to receive intragastric administrations of emodin at different concentrations (e.g., 0, 20, 40, and 80 mg/kg/d). These administrations were performed from 9:00 AM to 12:00 PM every day. HCY-E0 rats were administered a vehicle (0.5% carboxymethylcellulose sodium). Two weeks later, all rats novel object recognition and spatial cognition tests than HCY-E0 rats. Further, emodin treatment improved AD-like features including Aβ overproduction, tau hyperphosphorylation, neuron loss, synaptic impairments, cerebral microvascular damage, and inflammation, and it alleviated oxidative stress and methylation impairments in the brains of rats. Our results suggest that emodin represents a novel potential candidate agent for HHcy-induced dementia.

Materials and Methods
Hcy from Sigma (St. Louis, MO) was dissolved in normal saline (NS) (400 μg/mL) and filtered before injection. Emodin was obtained from Shanghai Base Industry (Shanghai, China) and dissolved in 0.5% carboxymethylcellulose sodium, which was from Xilong Chemical (Guangzhou, China). The sandwich enzyme-linked immunosorbent assay (ELISA) kit for Hcy was purchased from Mosak Biotecnology (Wuhan, China), and the ELISA kits for Aβ40, Aβ42, malondialdehyde (MDA), superoxide dismutase 1 (SOD1), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) were from Elabscience Biotecnology (Wuhan, China). The antibodies used are listed in Table 1.

Drugs and Antibodies
HCY-E0 rats. Further, emodin treatment improved AD-like features including Aβ overproduction, tau hyperphosphorylation, neuron loss, synaptic impairments, cerebral microvascular damage, and inflammation, and it alleviated oxidative stress and methylation impairments in the brains of rats. Our results suggest that emodin represents a novel potential candidate agent for HHcy-induced dementia.
completed the NORT and Morris water maze test (MWMT). All behavioral tests were performed during the light period.

**OFT**

Before tests, rats were placed in the specialized testing room for 1 hour of habituation. The floor of the chamber was divided into 25 equal squares with lines, and the arena was cleaned with 75% alcohol after every test. The rat was placed in the center of an open-field arena for 5 minutes, and its behaviors were recorded by an overhanging camera. The zone crossing times were used to assess the general locomotor activities of rats.

**NORT**

NORT is widely used to assess recognition memory in rodents (Broadbent et al., 2010). On the 1st day (acquisition trial), 2 identical objects were placed in opposite corners of the testing chamber, and the rat was placed in the chamber to explore the objects for 10 minutes. The behaviors were recorded with an overhanging camera. On the 2nd day (retention trial), a distinctly different novel object and one of the original objects in the same location were placed in the chamber. Each rat was allowed to explore the objects for 5 minutes. For the acquisition trial, the recognition index (RI) was calculated as the percentage of time spent exploring one object of the total exploring time. The RI in the retention trial was calculated as the percentage of time spent exploring the novel object over the whole exploring time.

**MWMT**

The spatial cognition abilities of rats were evaluated by MWMT (Morris, 1984). For learning, rats were trained in the water maze to find a hidden platform for 5 consecutive days (4 trials/d). In each trial, the test ended when the animals climbed onto the platform. If the rats failed to find the hidden platform within 60 seconds, they were manually guided to the platform and remained there for 30 seconds. The rats were allowed 1 day of rest before the memory test, in which the platform was removed. The escape latencies, the numbers of platform crossings, and the swimming speed were monitored by a computerized tracking system.

**Western Blotting and ELISA**

Rats were anesthetized with isoflurane and killed. The hippocampi were homogenized at pH 7.6, 8% SDS, 40% glycerol, and 40 mM dithiothreitol boiled for 10 minutes and then centrifuged at 12 000 × g for 10 minutes before the memory test, in which the platform was removed. The escape latencies, the numbers of platform crossings, and the swimming speed were monitored by a computerized tracking system.

### Table 1. Antibodies Used in This Study

| Antibody   | Epitopes       | mAb/pAb | Dilution | Source                          |
|------------|----------------|---------|----------|---------------------------------|
| DM1A       | α-tubulin      | mAb     | 1:2000 for WB | Sigma (St. Louis, MO)          |
| β-actin    | Total β actin  | mAb     | 1:1000 for WB | Abcam (Cambridge, UK)          |
| pCREB      | p-CREB at Ser133 | pAb       | 1:1000 for WB | Cell Signaling (Danvers, MA) |
| CREB       | Total CREB     | pAb     | 1:1000 for WB | Cell Signaling (Danvers, MA) |
| DNMT1      | Total DNMT1    | pAb     | 1:400 for WB | Boster Biological Technology (Wuhan, China) |
| DNMT3α     | Total DNMT3α   | mAb     | 1:200 for WB | Boster Biological Technology (Wuhan, China) |
| DNMT3β     | Total DNMT3β   | mAb     | 1:200 for WB | Boster Biological Technology (Wuhan, China) |
| GluR1      | Total GluR1    | pAb     | 1:1000 for WB | Millipore (Billerica, MA) |
| NR1        | Total NR1      | pAb     | 1:1000 for WB | Chemicon                        |
| NR2B       | Total NR2B     | pAb     | 1:1000 for WB | Abcam (Cambridge, UK)          |
| Synaptophysin | SYP 245–258  | pAb     | 1:1000 for WB | Abcam (Cambridge, UK)          |
| Synapsin1  | Synapsin1 C-term | pAb       | 1:1000 for WB | Abcam (Cambridge, UK)          |
| NF-κB p65  | p-NF-κB p65 at Ser536 | pAb       | 1:1000 for WB | Cell Signaling (Danvers, MA) |
| Iba1       | A marker of microglia | pAb     | 1:200 for IHC | Wako (Osaka, Japan)            |
| RECA-1     | Rat endothelial cell surface antigen | mAb | 1:50 for IHC | AbD Serotec (Oxford, UK) |
| BACE1      | BACE C-15      | pAb     | 1:500 for WB | Santa Cruz                     |
| Tau1       | np-tau at Ser-198/199/202 | mAb | 1:1000 for WB | Chemicon (Temecula, CA) |
| Tau5       | Total tau      | mAb     | 1:500 for WB | Millipore (Billerica, MA) |
| pT321      | p-tau at Thr231 | pAb     | 1:1000 for WB | Biosource (Camarillo, CA) |
| pS396      | p-tau at Ser396 | pAb     | 1:1000 for WB | Biosource (Camarillo, CA) |
| pS214      | p-tau at Ser214 | pAb     | 1:500 for WB | Biosource (Camarillo, CA) |
| 5-LO       | Total 5 Lipoxigenase | mAb | 1:1000 for WB | Abcam (Cambridge, UK)          |
| PP2Ac      | PP-2A catalytic subunit | pAb | 1:1000 for WB | Biosource (Camarillo, CA) |
| p-PP2Ac    | p-PP2A at Tyr307 | pAb     | 1:1000 for WB | Santa Cruz                     |
| DM-PP2Ac   | Demethylated PP2A at Leu309 | pAb       | 1:1000 for WB | Abcam (Cambridge, UK)          |

Abbreviations: IHC, immunohistochemistry; mAb, monoclonal antibody; np-, non-phosphorylated; p-, phosphorylated; pAb, polyclonal antibody; WB, western blotting.

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visualized and quantitatively analyzed by an Odyssey infrared imager system (Li-Cor Bioscience).

The hippocampal levels of A\(\beta\)\(_{40}\), A\(\beta\)\(_{42}\), IL-6, TNF-\(\alpha\), MDA, and SOD1 and plasma levels of Hcy, IL-6, and TNF-\(\alpha\) were assayed by ELISA. The hippocampi were homogenized in phosphate-buffered saline (PBS) (0.01 M) on ice and centrifuged for 5 minutes at 5000 \(\times\) g to obtain the supernatant. Blood samples were drawn from a heart puncture and promptly centrifuged at 2000 \(\times\) g for 15 minutes at 4°C. The blood or hippocampal supernatants were incubated in the ELISA microplate for 90 minutes at 37°C. After adding the stop solution, the optical densities were measured by a BioTek Synergy 2 microplate reader (Winooski, VT).

**Brain Slice Preparation and Immunohistochemical, Nissl, and HE Stainings**

Rats were anaesthetized and sacrificed with an over-dose of isoflurane and immediately perfused with 100 mL of NS followed by 400 mL of 4% paraformaldehyde solution. To visualize the cerebral vasculature, fluorescein isothiocyanate (FITC) was used as reported (Miyata and Morita, 2011). Briefly, the rats received intracardiac perfusion with FITC-containing NS at pH 7.0 followed by paraformaldehyde fixative at pH 8.0. The brains were dissected and post-fixed in perfusate overnight at 4°C. After being dehydrated twice in 30% sucrose in PBS, brains were cut into coronal slices (24 \(\mu\)m) with a freezing microtome (Leica). The brain slices were collected consecutively in PBS.

For immunohistochemistry staining, free-floating slices were blocked with 0.3% H\(_2\)O\(_2\) in absolute methanol (30 minutes) and nonspecific sites were blocked with bovine serum albumin (30 minutes). The slices were then incubated overnight at 4°C with primary antibodies (see Table 1). After washing with PBS, the slices were subsequently incubated with biotin-labelled secondary antibodies for 1 hour at 37°C. The immunoreaction was detected using hors eradish peroxidase-labelled antibodies and visualized with the diaminobenzidine tetrachloride system.

Figure 1. Cognitive deficits in homocysteine-injected rats were rescued by emodin. The experimental workflow is shown in (A). Sixty-five male rats were randomly divided into 2 groups, 52 rats were administered 2 weeks of homocysteine (Hcy) injections (400 \(\mu\)g/kg/d, via the vena caudalis, HCY rats, n = 52), and 13 rats were administered normal saline (NS) injections (control [CON] rats, n = 13) in the same volume as the Hcy treatment. The injections were performed with an insulin syringe (29 gauge, 0.33 \(\times\) 12.7 mm) at 9:00 AM every day. After the final injection, the animals completed the open field test (OFT) and novel object recognition test (NORT) to assess their activities of locomotion and cognition. The numbers of zone crossings in OFT (B) and the recognition indexes (RI) during the acquisition trial and retention trial in NORT (C) of HCY and CON rats were recorded. Then, HCY rats were randomly divided into 4 groups (HCY-E0, HCY-E20, HCY-E40, and HCY-E80, n = 13/group) to be administered intragastric emodin at different concentrations (e.g., 0, 20, 40, and 80 mg/kg/d, respectively). HCY-E0 rats received a vehicle control (0.5% carboxymethyl-cellulose sodium). Two weeks later, the RI during the acquisition trial and retention trial in NORT (D), the escape latencies on the 5th day in the learning phase (E), and the times crossing the platform in the memory tests in Morris water maze (F) of the rats were recorded. The plasma Hcy levels of the rats were detected by ELISA (n = 4) (G). Data were expressed as the means \(\pm\) SEM. *P < .05, **P < .01 vs the data of same rats in acquisition trial. $P < .05$, $$P < .01$ vs HCY-E0 (HCY). $P < .05$, $P < .01$ vs CON. $P < .05$ vs HCY-E0.
each primary antibody, 3 to 5 consecutive slices from each brain were used. For Nissl staining, the slices were washed twice for 5 minutes in 0.01 M PBS and incubated in 0.1% toluidine blue staining solution for 10 minutes at room temperature. Then they were rinsed in distilled water, soaked in 95% ethanol for 10 minutes, and dehydrated in 100% ethanol. After dehydration, the slices were cleared in xylene. For HE staining, the slices were stained with haematoxylin and eosin after being immersed in distilled water for 3 minutes. Then they were dehydrated through increasing concentrations of ethanol and xylene. All slices were covered with neutralized resin. The images were acquired with the same parameters by a microscope (Olympus SV120, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL). The data were expressed as the means ± SEM. The 1-way ANOVA procedure followed by Tukey’s multiple comparisons test was used to determine the differences between groups. For the 2 group comparisons, the data were analysed statistically using the t test. The level of significance was defined as P<.05.

Results

Cognitive Deficits of HCY Rats Were Rescued by Emodin

HCY and CON rats had equivalent zone crossing in OFT (t63 = 0.2703, P = .7878, independent t test; Figure 1A–B) and equal RI levels in the acquisition trial of NORT (t63 = 0.2193, P = .8271, independent t test; Figure 1C). In the retention trial of NORT, the CON rats showed a higher RI (0.64 ± 0.03) than the HCY rats (0.54 ± 0.02) (t63 = 2.295, P = .0251, independent t test; Figure 1C). After 2 weeks of emodin treatments, the rats were subjected to the behavior tests again (Figure 1A). In NORT, RIs of the HCY-E0 (0.69 ± 0.07) and HCY-E80 (0.76 ± 0.06) rats in the retention trial were significantly increased compared with the acquisition trial (0.56 ± 0.05 and 0.58 ± 0.05, respectively) (t63 = 2.086, P = .0478, paired t test, HCY-E40; t63 = 2.233, P = .0351, paired t test, HCY-E80; Figure 1D), showing that HCY-E0 and HCY-E80 rats had more interest in the novel object as CON rats. On the 5th day of learning in MMWT, the HCY-E0 rats had the longest latency (17.0 ± 5.2 seconds) to find the submerged platform [F(4, 25) = 5.307, P = .001; Figure 1E], while the latency in the HCY-E80 rats was 7.5 ± 1.7 seconds and in CON rats was 5.5 ± 1.0 seconds. In the memory testing, both the CON (4.7 ± 0.45) and HCY-E80 rats (4.5 ± 0.6) had more crossing times than the HCY-E0 rats (3.1 ± 0.55) [F(4, 25) = 4.114, P = .0052; Figure 1F]. The moving speeds of rats in OFT, NORT, and MMWT showed no difference. All these data suggested that emodin treatment (40, 80 mg/kg/d) significantly improved the cognitive deficits of HHcy rats. The plasma Hcy levels were detected after behavior tests. HCY-E0 rats had higher Hcy levels (10.1 ± 0.8 μM) than CON rats (6.1 ± 1.1 μM), while the Hcy levels in HCY-E40 (8.1 ± 0.4 μM) and HCY-E80 (8.9 ± 0.6 μM) rats decreased slightly [F(4, 25) = 5.384, P = .0068; Figure 1G].

Emodin Increased Hippocampal Neurons and Synapse-Related Proteins

Nissl staining was used to show the neuron numbers in the hippocampi (Figure 2A). Decreased neurons were observed in the CA3 region of the HCY-E0 rats (approximately 86.5% of the CON rats) [F(4, 25) = 7.406, P = .0004; Figure 2B], but this difference was not observed in the CA1, CA2, and dentate gyrus regions. The neuron numbers of the CA3 region in the HCY-E40 and HCY-E80 rats were increased compared with the HCY-E0 rats (Figure 2B). CAMP responsive element-binding protein (CREB) is a nuclear transcription factor essential for memory formation and retention, and its activation primarily depends on the phosphorylation at Ser133 (pCREB) (Shaywitz and Greenberg, 1999). The hippocampal pCREB level in the HCY-E0 rats was only 26.3% of the CON rats. Both the HCY-E40 (49.7% of CON) and HCY-E80 (81.7% of CON) rats had higher pCREB levels than the HCY-E0 rats [F(4, 25) = 41.54, P < .0001; Figure 2C–D].

Synapses propagate electrical or chemical signals between neurons. Here, the hippocampal presynaptic proteins (synapsin 1 and synaptophysin) and postsynaptic proteins (glutamate receptor 1 (Glur1), N-methyl D-aspartate receptor (NR) subtype 1 (NR1) and NR2B) were detected (Figure 2E–G). The HCY-E0 rats had significantly decreased Glur1 (58.5%, F(4, 25) = 3.037, P = .036), NR1 (44.8%, F(4, 25) = 7.77, P = .0003), synaptophysin (48.1%, F(4, 25) = 6.422, P = .0011), and synapsin 1 (48%, F(4, 25) = 13.02, P < .0001) levels, while NR2B levels were unchanged. Compared with the CON rats, HCY-E0 rats had increases of 107.2% in NR1, 67.3% in synaptophysin, and 125.9% in synapsin 1 levels, and HCY-E80 rats had increases of 138.4% in Glur1, 108.2% in NR1, 50.2% in NR2B, 44.2% in synaptophysin, and 256.3% in synapsin 1 levels (Figure 2E–G). These data suggested that emodin may attenuate HHCy-induced loss of neurons and synaptic proteins.

Emodin Eliminated Hippocampal Aβ Overproduction and Tau Hyperphosphorylation

By ELISA, higher levels of hippocampal Aβ40 (4496 ± 248 pg/g tissue) and Aβ42 (689.5 ± 35 pg/g tissue) were found in the HCY-E0 rats compared with the CON rats (Aβ40, 3439 ± 190; Aβ42, 689.5 ± 35 pg/g tissue) [F(4, 25) = 6.751 for Aβ40, P = .0008; F(4, 25) = 4.618 for Aβ42, P = .0063; Figure 3A–B]. Decreased Aβ40 levels in the HCY-E20, HCY-E40, and HCY-E80 rats and decreased Aβ42 in the HCY-E80 rats (621.9 ± 82 pg/g tissue) (Figure 3A–B) were observed. Significantly decreased β-site amyloid precursor protein-cleaving enzyme 1 (BACE1), the rate-limiting enzyme for Aβ production, was found in the HCY-E40 (147.3% of CON) and HCY-E80 rats (139.3% of CON) [F(4, 25) = 8.714, P = .0002] (Figure 3C–D).

In the HCY-E0 rats, the levels of hippocampal tau phosphorylated at Thr231, Ser396, and Tau1, respectively, and the level of tau unphosphorylated at Ser198/199/202 (Tau1) was significantly decreased (52.3% of CON) [F(4, 25) = 21.31 for Tau1, P < .0001; F(4, 25) = 9.788 for pT231, P < .0001; F(4, 25) = 31.92 for pS396, P < .0001; F(4, 25) = 13.41 for pS214, P < .0001; Figure 3E–F]. In the HCY-E20, HCY-E40, and HCY-E80 rats, the levels of tau phosphorylation at Thr231, Ser396, Ser214, and Ser198/199/202 were significantly decreased. The immunohistochemical staining with Tau1 on brain slices confirmed the results [F(4, 25) = 17.89, P < .0001; Figure 4E–F]. Total tau showed no differences (Tau5, Figure 3E–F).

Protein phosphatase 2A (PP2A) is involved in HHCy-induced Aβ overproduction and tau hyperphosphorylation (Zhang et al., 2008, 2009). In the HCY-E0 rats, significantly increased levels of Leu309-demethylation (DM-PP2Ac, 157.1% of CON) and Tyr307-phosphorylation (p-PP2Ac, 133.6% of CON) of the PP2A catalytic subunit (PP2Ac) [F(4, 25) = 0.1303 for PP2Ac, P = .9699; F(4, 25) = 4.978 for p-PP2Ac, P = .0043; F(4, 25) = 6.849 for DM-PP2Ac, P = .0007; Figure 3G–H] were observed, indicating the inhibition of PP2A.
In the HCY-E40 and HCY-E80 rats, the levels of DM-PP2Ac and p-PP2Ac were obviously reduced. Analysis of total PP2Ac levels showed no differences (Figure 3G–H). We also measured the activation of glycogen synthase kinase 3β, a key kinase activated in AD processing, but no alterations were observed (data not shown).
Oxidative stress and DNA methylation impairments are included in HHcy-induced damages. Here, hippocampal levels of MDA \( [F(4, 15) = 3.568, P = .0309] \) were significantly increased, and SOD1 levels \( [F (4, 15) = 4.339, P = .0157] \) were decreased in HCY-E0 rats compared with the CON rats (Figure 4A–B). The increased MDA and decreased SOD1 levels were attenuated in the HCY-E40 and HCY-E80 rats (Figure 4A–B).

The hippocampal levels of DNA methyltransferases (DNMTs) were detected, and DNMT1 and DNMT3β, but not DNMT3α, were increased in the HCY-E0 rats. The levels of DNMT1 and DNMT3β in the HCY-E40 and HCY-E80 rats were significantly lower than in the HCY-E0 rats \( [F_{(4, 25)} = 21.37 \text{ for } \text{DNMT1}, P < .0001; F_{(4, 25)} = 14.02 \text{ for } \text{DNMT3β}, P < .0001; \text{Figure 4C–D}] \).

**Emodin Attenuated Damage of Cerebral Microvessels in HCY Rats**

Cerebral vessels were visualized by the immunohistochemical staining of RECA-1 (an endothelial marker, Figure 5A–C), fluorescence of FITC (Figure 5D–E), and HE staining (Figure 5F). No differences were observed in the microvessel densities in the hippocampi and primary somatosensory cortex (S1Cx) of these 5 groups of rats (data not shown). However, in the RECA-1-imaged
vascular networks, we observed decreased lumen diameters of microarterioles by an intermittent dilatation manner both in the S1Cx (Figure 5B) and hippocampi (Figure 5C) of the HCY-E0 rats. Comparatively, the cerebral microvessels in the HCY-E80 rats presented normal morphological features similar to the CON rats (Figure 5B–C). Extravascular leakage of microvessels was assayed by FITC perfusion, which is a method of visualizing vasculature and extravascular leakage (Miyata and Morita, 2011). In the hippocampi and corpus calosum of the HCY-E0 rats, the green fluorescence of FITC was observed outside of the microvessels (Figure 5D). And in some hippocampal microvessels in the HCY-E0 rats, the bunchy exfoliated endothelial cells were clogged the vessels (Figure 5E, angiemphraxis). By HE staining, hyalinosis, increased perivascular space and splitting

Figure 4. Emodin reversed hippocampal oxidative stress and upregulation of DNA methylation methyltransferases. Hippocampal malondialdehyde (MDA) (A) and superoxide dismutase 1 (SOD1) (B) were detected by ELISA (ng/g tissue) (n = 4). Levels of DNA methyltransferases (DNMTs), e.g., DNMT1, DNMT3α and DNMT3β (C–D), were measured by western blotting and quantitatively analyzed (n = 6). Hippocampi with Tau1-based immunohistochemical staining were shown (E), and the densities of Tau1 positive cells were quantitatively analyzed (F) (bar = 200 μm, n = 6). Data were expressed as the means ± SEM. *P < .05, **P < .01, ***P < .001 vs CON. #P < .05, ##P < .01, ###P < .001 vs HCY-E0.
of the walls (double-barrel formation) were also detected in the hippocampi of the HCY-E0 rats (Figure 5F). However, extravascular leakage of FITC, angiemphraxis, hyalinosis, increased perivascular space, and splitting of the walls were not observed (Figure 5D–F) in the brains of the HCY-E40 and HCY-E80 rats.

**Microglia Activation in the Hippocampi of HCY Rats Was Inhibited by Emodin**

Increased solidity (the ratio between the positive area and the convex area) represents the activation of microglia (Soltys et al., 2001). By Iba1 (a microglial marker)-based immunohistochemical staining (Figure 6A), HCY-E0 rats had higher microglial solidities in both the hippocampi (0.26 ± 0.01) and S1Cx (0.24 ± 0.01), while the solidities in the HCY-E40 (hippocampi, 0.18 ± 0.01; S1Cx, 0.16 ± 0.01) and HCY-E80 rats (hippocampi, 0.17 ± 0.02; S1Cx, 0.15 ± 0.01) were lower [F (4, 70) = 20.34 in the S1Cx, P < .001; F (4, 70) = 28.21 in hippocampi, P < .001; Figure 6B–C].

The HCY-E0 rats had higher levels of IL-6 (4028 ± 55.7 pg/g tissue) and TNF-α (2609 ± 88.5 pg/g tissue) than the CON rats in hippocampi (IL-6, 3646 ± 50.3 pg/g tissue; TNF-α, 2085 ± 121.4 pg/g tissue), while both the HCY-E40 and HCY-E80 rats had lower levels of IL-6 and TNF-α. The plasma levels of IL-6 and TNF-α showed no difference (data not shown). The hippocampal nuclear factor-κB p65 (NF-κB p65) was increased in the HCY-E0 rats (190.9% of CON rats), but not in the HCY-E40 and HCY-E80 rats [F (4, 25) = 16.36, P < .0001; Figure 6F–G]. Further, 5-lipoxygenase (5-LO) levels in the HCY-E0 rats increased to approximately 220% of CON rats [F (4, 25) = 5.68, P = .0021; Figure 6F, H], and this increase was confirmed by immunohistochemical staining (Figure 6I). The HCY-E40 and HCY-E80 rats had significantly lower 5-LO levels (Figure 6F, H, I). All these data suggested that HHcy-induced inflammation could be rescued by emodin treatment.

**Discussion**

Although there are reports of cognitive function improvements after high-dose Vit B therapy in individuals with cognition decline and elevated plasma Hcy levels (Nilsson et al., 2001; Durga et al., 2007; de Jager et al., 2012), 3 randomized controlled trials found no effects of vitamin supplementation on cognitive tests (Aisen et al., 2008; Kwok et al., 2011; Köbe et al., 2016). Several other trials in elders with vascular disease (Stott et al., 2005) and individuals with mild cognitive impairment (van Uffelen et al., 2008) and dementia (Sommer et al., 2003) also showed that Vit B treatment did not improve cognition despite lowering Hcy. Furthermore, some meta-analyses suggest that folate and Vit B supplementation do not provide any significant advantage in slowing down or preventing the progression of cognitive decline (Ford and Almeida, 2012; van der Zwaluw et al., 2014; Smith et al., 2015; Zhang et al., 2017). Therefore, reducing Hcy levels is not sufficient to improve HHcy-induced cognitive impairments. Emodin is a bioactive anthraquinone present in
some prescriptions of traditional Chinese medicine with cerebral protections. Here, although plasma Hcy levels did not decrease, the emodin-treated HHcy rats had better behavioral performances. And in the hippocampi of emodin-treated HHcy rats, the neuron loss in the CA3 region, the decreased synapse-related proteins including synaptophysin, synapsin 1 and NR1, and the reduced phosphorylated CREB at Ser133 were remedied by emodin.

Cerebral hypoperfusion is a risk factor for cognitive impairments (Park et al., 2014; Garcia-Bonilla et al., 2015; Faraco et al., 2016). HHcy was reported to impair cerebrovascular endothelial function and interfere with nitric oxide-mediated cerebral blood flow (Woo et al., 1997; Bang et al., 2016). In the CBS-knockout mouse (an HHcy model), HHcy was shown to increase brain permeability (Tyagi et al., 2012). A study on a mouse model produced by feeding with food containing excess methionine and deficits in folate, Vit B6, and Vit B12 reported that HHcy was associated with increased microhemorrhages in the brain (Sudduth et al., 2013). Hcy level was regarded as the marker of endothelial function not only in macroangiopathy but also clinically in cerebral microangiopathy (Bang et al., 2016). And HHcy was claimed more closely associated with small vessel disease than large vessel disease (Feng et al., 2013). In this study, HHcy induced several microangiopathic alterations including intermittent increased lumen diameter, extravascular leakage, hyalinosis, increased perivascular space, and splitting of the walls, all of which contribute to cerebral hypoperfusion (Kalaria, 2016). Emodin significantly ameliorated HHcy-induced...
microangiopathic alterations, suggesting its role in improving cerebral perfusion. Besides cerebral hypoperfusion, HHcy induces Aβ accumulation, tau hyperphosphorylation, and cognition impairments in AD models and normal animals (Fuso et al., 2008; Troen et al., 2008a, 2008b; Zhang et al., 2009; Fuso et al., 2012; Nicolaia et al., 2010; Chai et al., 2013; Li et al., 2014). We reported that HHcy enhanced the phosphorylation of amyloid precursor protein at Thr688 by inhibiting PP2A and thus increased Aβ production in vivo (Zhang et al., 2009). BACE1 is the rate-limiting enzyme for Aβ production. Levels of BACE1 and Aβ were reported upregulated in Vit B deficiency mice with HHcy (Fuso et al., 2008). Here, the increased BACE1 was also detected in the hippocampi of HCY-E0 rats, suggesting its potential role in HHcy-induced Aβ overproduction. The density of neurofibrillary tangles containing hyperphosphorylated tau, which could be dephosphorylated by PP2A, correlates with dementia of AD (Tian and Wang, 2002). HHcy was reported to increase tau phosphorylation by inhibiting PP2A in vivo (Zhang et al., 2008). Previous studies showed that the anthraquinone family (including emodin) inhibited tau aggregation in vitro (Pickhardt et al., 2005). In this research, emodin opposed HHcy-induced inhibition of PP2A in vivo and thus attenuated HHcy-induced tau hyperphosphorylation and Aβ overproduction.

PP2A, a serine/threonine protein phosphatase, exists predominantly as heterotrimers containing a 65-kDa subunit A (PP2A-A) acting as a scaffold for the association of a 36-kDa PP2A-B, one of a variety of regulatory subunits (PP2A-C), and PP2A-C. The highly conserved carboxyl-terminal sequence of PP2A-C has the methylesterase (PME) and methyltransferase, promotes the methylation at Leu309, which is specifically catalyzed by demethylases. PME and methyltransferase, promotes the demethylation of PP2A in vitro and thus attenuated HHcy-induced tau hyperphosphorylation and Aβ overproduction. The reduction of DNMT1 in heterozygous null mice conferred protection against stroke (Endres et al., 2000), and reduction of DNMT3α decreased the apoptosis of NSC34 cells (Chen et al., 2013). Thus, emodin might exert different effects on DNMTs in different pathological conditions.

Inflammation is a potential mediator of cognitive deficits in AD (Serpente et al., 2014). Microglia with different morphological types are key players of cerebral inflammation, and this microglia engaged in inflammation is a major source of reactive oxygen species (Soltyss et al., 2001; Block and Hong, 2007). Activated microglia release a series of proinflammatory mediators including cytokines, complement components, and free radicals, all of which potentially contribute to oxidative stress and eventually trigger neuronal dysfunction and death, synapse loss, and cognitive impairments (Chinnici et al., 2007; Hong et al., 2016). 5-LO, an inflammatory enzyme widely expressed in the brain, plays a key role in brain damage and increases with aging (Bessis et al., 2007). Pharmacologic inhibition of 5-LO improves memory, rescues neuroinflammation and synaptic dysfunction, and ameliorates tau pathology in a transgenic model of tautopathy (Chu et al., 2012). NF-κB is a master regulator of inflammation and also a sensor of oxidative stress. Activating NF-κB induces some cytotoxic factors that exacerbate inflammation and oxidative stress and promote apoptosis (Fahl, 1999), suggesting its prominent strategic position in oxidative stress and inflammation. In this study, HHcy-induced cerebral inflammation—characterized by activated microglia, increased levels of 5-LO, NF-κB p65, TNF-α, and IL-6 and activated oxidative stress—was rescued by emodin. However, the plasma TNF-α and IL-6 levels were not affected by HHcy, indicating the possibility that cerebral inflammation may occur very early after HHcy treatment.

Here, HHcy rats developed obvious cognitive deficits with several AD-like features in the brain, including Aβ overproduction, tau hyperphosphorylation, loss of neurons and synapses, inflammation, and abnormal microangiopathic alterations. Emodin effectively improved the cognitive deficits and rescued the AD-like features induced by HHcy, although it did not reduce the plasma Hcy level. Thus, emodin represents a novel potential candidate agent for HHcy-induced cognitive deficits.
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Statement of Interest
None.

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