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RESEARCH PAPER

Antidepressant-like effects of ginsenoside Rg1 are due to activation of the BDNF signalling pathway and neurogenesis in the hippocampus

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BACKGROUND AND PURPOSE

Ginsenoside Rg1 (Rg1) is one of the major bioactive ingredients of *Panax ginseng* with little toxicity and has been shown to have neuroprotective effects. In this study, we investigated the antidepressant-like effect of Rg1 in models of depression in mice.

EXPERIMENTAL APPROACH

The effects of Rg1 were assessed in the forced swimming test (FST) and tail suspension test (TST) in mice. Rg1 was also investigated in the chronic mild stress (CMS) mouse model of depression with imipramine as the positive control. Changes in hippocampal neurogenesis and spine density, the brain-derived neurotrophic factor (BDNF) signalling pathway, and serum corticosterone level after chronic stress and Rg1 treatment were then investigated. The tryptophan hydroxylase inhibitor and the tyrosine kinase B inhibitor were also used to explore the antidepressive mechanisms of Rg1.

KEY RESULTS

Ginsenoside Rg1 exhibited antidepressant-like activity in the FST and TST in mice without affecting locomotor activity. It was also effective in the CMS model of depression. Furthermore, Rg1 up-regulated the BDNF signalling pathway in the hippocampus and down-regulated serum corticosterone level during the CMS procedure. In addition, Rg1 was able to reverse the decrease in dendritic spine density and hippocampal neurogenesis caused by CMS. However, Rg1 had no discernable effect on the monoaminergic system.

CONCLUSIONS AND IMPLICATIONS

Our results provide the first evidence that Rg1 has antidepressant activity via activation of the BDNF signalling pathway and up-regulation of hippocampal neurogenesis.

Abbreviations

BDNF, brain-derived neurotrophic factor; CMS, chronic mild stress; CREB, cAMP response element-binding protein; DCX, doublecortin; DG, dentate gyrus; FST, forced swimming test; GCL, granule cell layer; HPA, hypothalamic-pituitary-adrenocortical; PCPA, *p*-chlorophenylalanine methyl ester; Rg1, ginsenoside Rg1; SGZ, subgranular zone; SSRIs, selective serotonin reuptake inhibitors; TrkB, tyrosine kinase B; TST, tail suspension test

Introduction

Depression is a group of syndromes characterized by notable and persistent mood disorders. It is one of the leading causes of total disability and constitutes a serious economic burden (Kessler *et al.*, 1994). Despite decades of clinical use, the molecular and cellular mechanisms underlying the long-term, therapeutic actions of antidepressants remain poorly understood and, in recent years, the research has focused on the development of non-monoamine-based antidepressants (Berton and Nestler, 2006). At present, most antidepressants used clinically are synthetic compounds, including the selective 5-HT reuptake inhibitors (SSRIs). Although current therapy improves depression, the symptoms fail to resolve completely in as many as half of the cases. Remission rates are even worse for those who have failed initial medication trials (McGrath *et al.*, 2006). Thus, antidepressants with higher efficacy and fewer side effects are needed.

Traditionally, dysfunction of the 5-hydroxytryptaminergic system is considered to be the cause of depression and SSRIs are the most widely used antidepressants (Berton and Nestler, 2006). Recently, a leading hypothesis of depression suggests that neurotrophic factors and adult neurogenesis play critical roles in mediating the behavioural responses to antidepressants (Duman *et al.*, 1997; Krishnan and Nestler, 2008). The brain-derived neurotrophic factor (BDNF) is regulated by antidepressants and exerts an antidepressant-like effect in short-term behavioural models of depression (Shirayama *et al.*, 2002). Moreover, the time course of maturation of newly generated neurons in the dentate gyrus (DG) is generally consistent with the delayed onset of therapeutic effects of current antidepressants (Sahay and Hen, 2007). These studies indicate that stimulation of the BDNF signalling pathway and hippocampal neurogenesis could provide a novel approach to the treatment of depression.

Ginsenoside Rg1 (Rg1; Figure 1A), one of the most abundant and active ingredients of *Panax ginseng*, has already been shown to have neuroprotective effects with little toxicity. A wide range of neurotrophic and neuroprotective effects of Rg1 have been reported, including protective effects against ischaemia/reperfusion and Alzheimer's disease (Zhang *et al.*, 2008; Wang and Du, 2009; Shi *et al.*, 2010), promotion of progenitor cell proliferation (Shen and Zhang, 2004; Shi *et al.*, 2009), and improvement of learning and memory (Shi *et al.*, 2009). Recent surveys indicate that ginseng is one of the most commonly used natural products by adults in the United States, with usage rates ranging up to 30% of the population (Harnack *et al.*, 2001; Kaufman *et al.*, 2002; Barnes *et al.*, 2004). Preliminary reports indicate that Rg1 is a potent neuroprotective agent that promotes neurotrophin expression and neurogenesis (Shen and Zhang, 2007; Lu *et al.*, 2010). Taken together, these results indicate that Rg1 may produce antidepressant-like effects.

In the present study, we first assessed the antidepressant effects of the pure ingredient of *ginseng* – Rg1, using various models of depression, including the forced swimming test (FST), tail suspension test (TST) and chronic mild stress (CMS). Furthermore, the mechanisms for these antidepressant effects were explored.

Methods

Animals

All animal care and experimental procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). Adult male C57BL/6J mice (8–10 weeks old) were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. Before use, mice were housed (5 per cage) under standard conditions (12 h light/dark cycle; lights on from 7:00 a.m. to 7:00 p.m.; 23 ± 1°C ambient temperature; 55 ± 10% relative humidity) for 1 week with free access to food and water. Each experimental group consisted of 20 mice and behavioural experiments were carried out during the light phase. A total of 827 mice were used in these experiments.

Treatment schedules

The repeated drug treatment of CMS animals was performed once daily at 11:00 a.m.–12:00 p.m. during the last 14 days. The doses chosen were based on the behavioural results and previous reports (Xu *et al.*, 2009; Shi *et al.*, 2010). All these compounds were administered i.p. in a volume of 10 $mL \cdot kg^{-1}$. Control animals were given the corresponding vehicle, also in the same volume.

Forced swimming test

The FST was carried out in mice according to our previous and other reports with slight modification (Porsolt *et al.*, 1977; Yan *et al.*, 2010; Xiong *et al.*, 2011). Briefly, 30 min after injection, mice were individually placed into a glass cylinder (25 cm in height, 10 cm in diameter) filled with 10 cm high water (25 ± 1°C). The water was exchanged after each trial. All animals were forced to swim for 6 min, and the immobility time during the final 4 min interval of the test was recorded. Immobility time was defined as the time spent by the mouse floating in the water without struggling, and making only those movements necessary to keep its head above the water. The observers were unaware of the treatment of mice.

Tail suspension test

The total duration of immobility induced by tail suspension was measured according to the methods described previously (Steru *et al.*, 1985; Sarkisyan *et al.*, 2010). Briefly, 30 min after injection, mice were suspended 50 cm above the floor for 6 min by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time during the final 4 min interval of each test was recorded. Mice were considered immobile only when they hung passively and were completely motionless, and any mice that did climb their tails were removed from the experimental analysis. The observers were unaware of the treatment of the mice.

Open field test

Locomotor activity was studied using an open-field test, and the open-field apparatus was similar to those described pre-

viously (Liang *et al.*, 2008; Xu *et al.*, 2008; Covington *et al.*, 2009; Muller *et al.*, 2009; Cui *et al.*, 2011). The mice were placed individually in the dark in a wooden box (100 × 100 × 40 cm) with the floor divided into 25 (5 × 5) squares. The apparatus was illuminated with a red bulb (50 W) on the ceiling. The animals were placed in the central sector 30 min after injection and the total number of squares entered was recorded for 5 min under dim light conditions. The open field arena was thoroughly cleaned between each test. The observers were unaware of the treatment of the mice.

CMS procedure

The CMS was adopted as we have designed with slight modifications (Willner *et al.*, 1992; Xiong *et al.*, 2011). During this experiment, the mice in the control group were left undisturbed in the home cages in a separate room with the exception of general handing (e.g. regular cage cleaning) that matched the CMS groups, whereas the mice in other groups were housed in individual cages (one cage per mouse) and exposed to CMS. Mice were subjected once a day for 8 weeks to one of the following stressors such as food or water deprivation (23 h), damp sawdust, cold water swimming (4°C for 4 min), tail suspension (5 min), inversion of light/dark cycle, cage tilting (45°C) and placement in an empty cage. To prevent habituation and provide an unpredictable feature to the stressors, all the stressors were randomly scheduled over a 1 week period and repeated throughout the 8 week experiment.

Sucrose preference test

The sucrose preference test was conducted over a 48 h period using a two-bottle test, one with 1% sucrose solution and the other with water (Pothion *et al.*, 2004). All mice were acclimatized for 3 consecutive days to two-bottle choice conditions before 2 additional days of choice test. To prevent potential location preference of drinking, the position of the bottles was changed after 24 h. The mice were deprived of food and water for 24 h prior to the test. On each test, bottles were pre-weighed and the position of the bottles was interchanged. One hour later, the amount of sucrose solution or water consumed was determined by weighing the bottles again, and the preference for the sucrose solution was calculated as the percentage of sucrose solution ingested relative to the total amount of liquid consumed. Sucrose preference was assessed in individually housed, stressed and unstressed mice.

Measurement of plasma corticosterone level

The mice were killed by cervical dislocation, 30 min after the last stressor and drug exposure. Blood samples were collected between 10:00 a.m. and 11:00 a.m. and kept on ice, then centrifuged at 3000× *g* at 4°C for 15 min (Solich *et al.*, 2008). The resulting plasma was kept at –80°C until analysis. The corticosterone levels were measured using a commercially available RIA kit (ICN Biomedicals, Costa Mesa, CA, USA).

RT-PCR

This experiment was undertaken as before with slight modifications (Wang *et al.*, 2009). The animals were killed 24 h after the last stressor and drug exposure. Bilateral hippocampi were rapidly dissected and total RNA was isolated from them with the use of Trizol reagent (Invitrogen, San Diego, CA, USA). Then, total RNA (1 μg) was reverse transcribed, and the resulting cDNA (1 μL) was used to detect the transcripts. The primers used for BDNF (Invitrogen) were: forward 5′-GAC AAG GCA ACTTGG CCT AC-3′, reverse 5′-CCT GTC ACA CAC GCT CAGCTC-3′, product size: 356 bp; and GAPDH: forward 5′-ACA TTGTTG CCATCA ACG AC-3′, reverse 5′-ACG CCA GTA GAC TCC ACG AC-3′, product size: 216 bp. An amplified reaction was performed for a single 5 min initial denaturation at 94°C followed by 30 cycles (BDNF) or 25 cycles (GAPDH) under the conditions: 94°C (40 s), 55°C (GAPDH, 30 s) or 53°C (BDNF, 30 s), and 72°C (40 s) and final extension at 72°C for 10 min. The PCR products were separated on 1.5% agarose gels and stained with GoldView (Promega, Madison, WI, USA). The BDNF PCR product was normalized to that of the GAPDH PCR product in each sample.

Western blotting analysis

The experiment was conducted as we have described with slight modifications (Wang and Du, 2009; Wang *et al.*, 2009; Wu *et al.*, 2011). The animals were killed 24 h after the last stressor and drug exposure. Bilateral hippocampi were rapidly dissected and homogenized in lysis buffer for 30 min. The homogenate was centrifuged and supernatants were collected. Total protein was estimated by Coomassie blue protein-binding assay (Jiancheng Institute of Biological Engineering, Nanjing, China). Then, the samples were mixed with SDS sample buffer and boiled for 5 min. Equal amounts of protein samples (30 μg) were separated by 10% SDS/PAGE gel electrophoresis and then transferred to nitrocellulose membranes. Transferred membranes were incubated overnight at 4°C with primary antibodies to ERK1/2 (1:1000), phospho-ERK1/2 (pERK1/2; 1:1000; Santa Cruz, CA, USA); cAMP response element-binding protein (CREB; 1:500), phospho-CREB-ser133 (pCREB; 1:500; Cell Signaling, MA, USA); doublecortin (DCX; 1:500; Cell Signaling), BDNF (1:500; Epitomics, CA, USA) or β-actin (1:1000; Santa Cruz, CA, USA). The antigen-antibody complexes were visualized with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:2000; Santa Cruz) by using enhanced chemiluminescence (Pierce, Rockford, IL, USA). The optical density of the bands was determined using Optiquant software (Packard Instruments BV, Groningen, Netherlands).

Immunohistochemical studies

This was performed as described with some modification (Huang *et al.*, 2010). The mice were deeply anaesthetized with pentobarbital sodium and perfused transcardially with 4% paraformaldehyde in 0.01 M phosphate buffer 24 h after the last session. The brains were removed and postfixed for 24 h, then dehydrated with 30% sucrose solution. After that, coronal brain sections of hippocampus were cut at 25 μm with a freezing microtome (CM1900; Leica Microsystems, Wetzlar, Germany) and collected serially.

For double fluorescence staining, the sections were sequentially treated with 0.3% Triton X-100 in 0.01 M PBS for 30 min and 3% BSA in 0.01 M PBS for 30 min. They were

then incubated with diluted rabbit anti-DCX antibody (1:200; Santa Cruz) and mouse anti-NeuN antibody (1:100; Chemicon International, CA, USA) overnight at 4°C. The sections were subsequently exposed to fluorescein isothiocyanate-labelled horse anti-rabbit IgG (1:50; Pierce) and rhodamine-labelled goat anti-mouse IgG (1:50; Pierce) for 1 h. They were then washed in 0.01 M PBS and mounted on slides following dehydration, and coverslipped.

All quantitative histological evaluations were performed in a uniform fashion by an observer who was unaware of the experimental group. Examination of DCX-positive (DCX+) cells was confined to the DG, especially in the granule cell layer (GCL), including the subgranular zone (SGZ) of the hippocampus that was defined as a two-cell body-wide zone along the border between the GCL and the hilus. Quantifications of DCX+ cells were respectively conducted from 1-in-12 series of hippocampal sections spaced at 300 μm and spanning the rostrocaudal extent of the DG bilaterally. Every DCX+ cell within the GCL and SGZ was counted.

All immunostained sections were analysed using a laser confocal microscope (FV500; Olympus, Tokyo, Japan) equipped with a motorized X-Y sensitive stage and a video camera connected to a computerized image analysis system (ExploraNova, La Rochelle, France). The plotting of labelled cells was conducted bilaterally using Mercator v.2 software (ExploraNova) that allowed the measurement of the DG corresponding surface (GCL + SGZ). The reference volume was determined as the sum of the traced areas multiplied by the distance between sampled sections (300 μm). The density of DCX+ cells was then calculated by dividing the number of DCX+ cells by the GCL sectional volume. Numbers of DCX+ cells were normalized as density per volume unit (mm^3).

Golgi silver staining

The mice were deeply anaesthetized with pentobarbital sodium (60 $mg \cdot kg^{-1}$, i.p.) and perfused intranscardially with 0.9% saline solution 24 h after the last session. The brains were removed and stained by the modified Golgi-Cox method (Gibb and Kolb, 1998; Flores *et al.*, 2005; Alquicer *et al.*, 2008), and then stored for 14 days in Golgi-Cox solution, followed by 3 days in 30% sucrose solution. Coronal sections of 50 μm thickness of regions to be studied were obtained using a vibratome (Camden Instrument, MA752, Leicester, UK). Sections were then treated with ammonium hydroxide for 30 min, followed by 30 min in Kodak Film Fixer (Eastman Kodak Company, Rochester, NY, USA) and finally rinsed with distilled water, dehydrated and mounted with resinous medium. Golgi-impregnated pyramidal neurons in the CA3 subfield of hippocampus were studied. For details on staining protocol and dendritic spine density analyses, see Supplementary materials and methods.

Data analysis

All analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) and data which are presented as means ± SEM. Differences between mean values were evaluated using one-way ANOVA or two-way ANOVA, as appropriate. For all one-way ANOVA analyses, *post hoc* tests were performed using the least significant difference test. For all two-way ANOVA analyses, Bonferroni *post hoc* tests were used to assess isolated comparisons. $P < 0.05$ was considered statistically significant.

Materials

Rg1 (purity >90%; molecular weight: 801.01), imipramine hydrochloride and *p*-chlorophenylalanine methyl ester (PCPA) were purchased from Sigma (St. Louis, MO, USA) and dissolved in 0.9% saline. K252a was purchased from Alomone Laboratories (Jerusalem, Israel) and dissolved in 0.1% DMSO. Receptor nomenclature follows Alexander *et al.*, (2011).

Results

Antidepressant-like effects of Rg1 in the FST and TST of mice

FST and TST in mice are the most widely used behavioural assays for detecting potential antidepressant-like activity and have a high predictive validity for antidepressant activity (Cryan and Holmes, 2005; Cryan and Slattery, 2007). Thus, the possible antidepressant effects of Rg1 were first examined in the FST. Rg1 (2.5, 5, 10, 20 $mg \cdot kg^{-1}$) was given i.p. and imipramine (15 $mg \cdot kg^{-1}$, i.p.) was included as the positive control. The results showed that a single injection of Rg1 produced a strong antidepressant effect in the FST (Figure 1B). The data were subjected to a one-way ANOVA with drug treatment as the factor and revealed a significant main effect of drug treatment [$F(5, 54) = 44.956$, $P < 0.01$]. Subsequent *post hoc* analysis indicated that increasing doses of Rg1 from 2.5 to 20 $mg \cdot kg^{-1}$ decreased the immobility time in the FST in a dose-dependent manner, as immobility time was significantly reduced at the higher doses of 10 and 20 $mg \cdot kg^{-1}$ of Rg1 ($n = 10$, $P < 0.01$ vs. control). Imipramine also produced a significant reduction of immobility time ($n = 10$, $P < 0.01$ vs. control), an effect that has been consistently described (de Felipe *et al.*, 1989).

The dose–response studies were also performed to assess the antidepressant-like effects of Rg1 in the TST (Figure 1C). Data from this test revealed a significant main effect of drug treatment [$F(5, 54) = 99.869$, $P < 0.01$]. Rg1 (2.5–20 $mg \cdot kg^{-1}$, i.p.) treatment produced a dose-dependent reduction in the duration of immobility in the TST as well as FST. *Post hoc* analysis indicated that Rg1 at doses from 2.5 to 20 $mg \cdot kg^{-1}$ significantly reduced the immobility time compared with saline-treated animals ($n = 10$, $P < 0.01$ vs. control), and imipramine also decreased immobility time as expected ($n = 10$, $P < 0.01$ vs. control).

To exclude the possibility that the reduced immobility in these tests might be due to an increase in spontaneous activity (Bourin *et al.*, 2001), naive mice treated as in the above schedule were exposed to the open-field apparatus for 5 min. The results showed that there was no difference in the number of squares an animal crossed between areas of the centre and periphery in all groups (Figure 1D), and ANOVA demonstrated no effect for Rg1 treatment [$F(5, 54) = 2.197$, $P = 0.065$]. Acute Rg1 treatment had no effect on locomotor activity, indicating that the reduction of immobility observed in the two tests after Rg1 treatment was not due to locomotor hyperactivity.

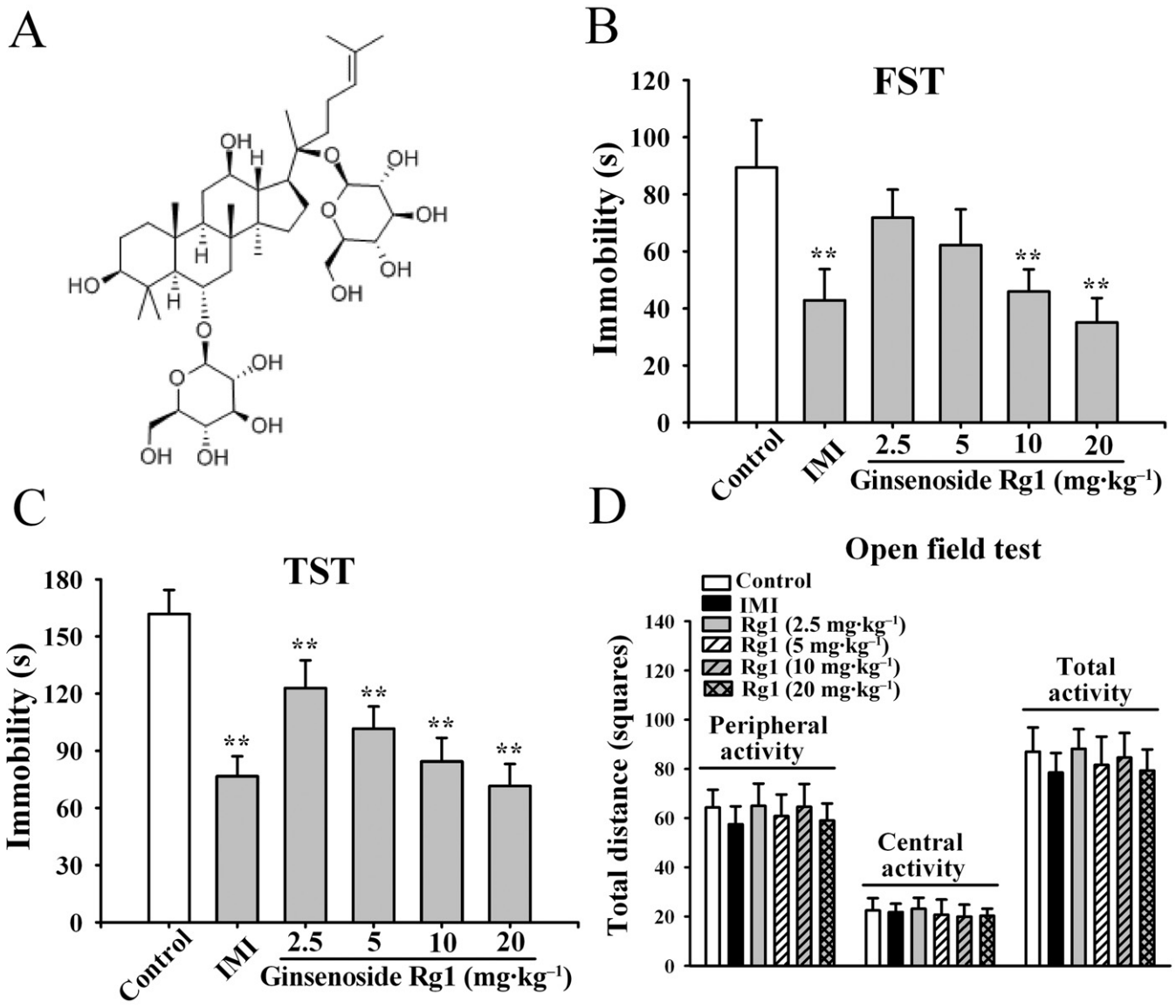

Figure 1

Rg1 produces antidepressant-like effect in the FST and TST. The mice were injected with a single dose of saline (control), imipramine (IMI, 15 mg·kg^{-1}) or Rg1 (2.5, 5, 10, 20 mg·kg^{-1}). The behavioural tests were conducted 30 min after the injection. (A) The chemical structure of Ginsenoside Rg1. (B) Rg1 decreased the immobility time in the FST in a dose-dependent manner. (C) Rg1 decreased the immobility time in the TST in a dose-dependent manner. (D) Rg1 had no effect on the spontaneous locomotor activity in the open-field test. The data are expressed as means ± SEM (n = 10). **P < 0.01, significantly different from control; one-way ANOVA followed by *post hoc* LSD test.

Chronic Rg1 treatment reverses the CMS-induced depressive symptoms of mice

To further characterize the antidepressant effects of Rg1, we employed CMS, which is currently regarded as one of the most predictive animal models of depression (Willner, 1984; Forbes *et al.*, 1996; Chourbaji *et al.*, 2005). In the present study, we examined the effects of Rg1 on the sucrose intake and weight gain as indices of stress-induced responses. As shown in Figure 2A, CMS induced a 58 ± 4.5% decrease in sucrose consumption in the mice compared with control (n = 10, P < 0.01). This effect was significantly reversed by chronic systemic administration of Rg1, and two-way ANOVA indicated a significant interaction [F(5, 58) = 38.714, P < 0.01] with significant effects for CMS [F(1, 58) = 101.981, P < 0.01] and drug [F(5, 58) = 40.384, P < 0.01]. *Post hoc* analysis showed that the sucrose consumption was restored by 5, 10 and 20 mg·kg^{-1} Rg1. Further study revealed that the sucrose consumption was increased by 101 ± 17% and 121 ± 21% with administration of 10 and 20 mg·kg^{-1} Rg1 (n = 10, P < 0.01 vs. CMS), respectively, similar to that in the imipramine-treated group (n = 10, P < 0.01 vs. CMS). These results suggest that Rg1 may increase hedonic states in mice. Figure 2B illustrates the effects of CMS and Rg1 on weight gain; two-way

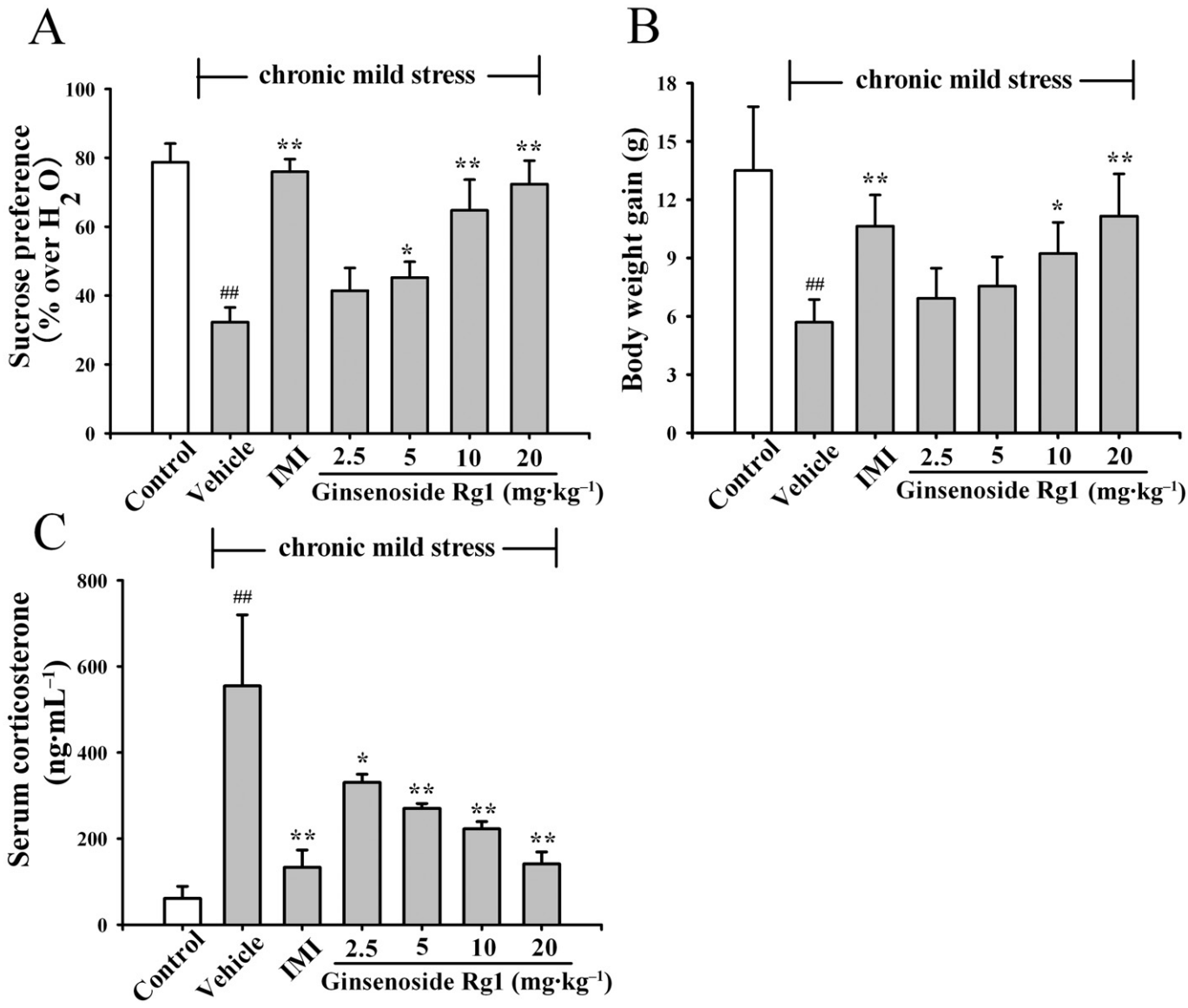

Figure 2

Rg1 reverses depressive-like behaviour in the CMS paradigm. Mice were exposed to CMS for 8 weeks and received a daily injection (i.p.) of saline (vehicle), imipramine (IMI; 15 mg·kg^{-1}) or Rg1 (2.5, 5, 10, 20 mg·kg^{-1}) during the last 2 weeks. Behavioural tests were then conducted. (A) Chronic Rg1 treatment produced robust antidepressant effects in the sucrose preference test. CMS + Rg1 mice displayed higher sucrose preference than CMS + vehicle mice. (B) Antidepressant effects of Rg1 on body weight of CMS-exposed mice. CMS + Rg1 mice displayed higher weight gain than CMS + vehicle mice. (C) Rg1 treatment significantly reduced CMS-induced elevation of serum corticosterone levels. Data are expressed as means ± SEM (n = 10). $^{\#\#}P < 0.01$, significantly different from control; $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from CMS + vehicle group; two-way ANOVA followed by *post hoc* Bonferroni's test.

ANOVA reported a significant interaction [F(5, 58) = 20.555, $P < 0.01$] with significant effects for CMS [F(1, 58) = 86.847, $P < 0.01$] and drug [F(5, 58) = 12.521, $P < 0.01$]. At 8 weeks after CMS, the weight gain in CMS-treated mice was less than that of control mice, which was consistent with previous reports (Ghiglieri *et al.*, 1997). By contrast, Rg1 administration brought the weight gain in CMS-treated mice to control levels within 14 days of treatment (10 mg·kg^{-1}: n = 10, $P <$ 0.05 vs. CMS; 20 mg·kg^{-1}: n = 10, $P < 0.01$ vs. CMS), which was similar to that of imipramine (n = 10, $P < 0.01$ vs. CMS). These findings suggest that Rg1 corrects the weight loss caused by CMS. Further behavioural experiments revealed that treatment with Rg1 for 14 days had no significant effects on sucrose preference [Supporting Information Figure S1A; F(5, 54) = 3.136, P = 0.078] and weight gain [Supporting Information Figure S1B; F(5, 54) = 2.894, P = 0.071] in naive mice, indicating that the effect of Rg1 was limited to stressed animals.

One major neuroendocrine response to stress is the secretion of corticosterone via activation of the hypothalamic-

pituitary-adrenocortical (HPA) axis (Carroll *et al.*, 1976; Stokes, 1995). Therefore, we assessed the effects of Rg1 on serum corticosterone levels in CMS mice. As shown in Figure 2C, the level of plasma corticosterone in CMS-treated mice was significantly higher than that of unstressed control ($n = 10$, $P < 0.01$). Two-way ANOVA revealed that the effects of drug treatment [$F(5, 58) = 20.007$, $P < 0.01$] and CMS [$F(1, 58) = 103.468$, $P < 0.01$] were significant, as was the interaction between the two [$F(5, 58) = 22.849$, $P < 0.01$]. Repeated treatment with Rg1 (2.5, 5, 10, 20 mg·kg^{-1}) for 14 days significantly reduced CMS-induced elevation of serum corticosterone levels compared with CMS group ($n = 10$, $P < 0.01$). Imipramine also markedly prevented CMS-induced changes in serum corticosterone levels ($n = 10$, $P < 0.01$ vs. CMS). This result indicates that Rg1 can reverse the impaired feedback regulation of the HPA axis induced by CMS.

Rg1 reverses the CMS-induced decrease in neurogenesis and dendritic spine density

One notable stress-induced hippocampal neuroadaptation is inhibition of cell proliferation in the DG region (Lagace *et al.*, 2010). Hippocampal neurogenesis has also been shown to be required for antidepressant-like behaviour (Santarelli *et al.*, 2003). In this study, neurogenesis was studied by DCX immunohistochemistry in the DG region of hippocampus. DCX is a microtubule-associated protein that serves as a marker of neurogenesis by virtue of transient expression in newly formed neurons between the timing of their birth and final maturation (Brown *et al.*, 2003). The immunohistochemical staining in Figure 3A showed the DCX+ cells in the DG region of mice. Because ANOVA revealed a significant interaction [$F(3, 17) = 11.884$, $P < 0.01$] with significant effects for CMS [$F(1, 17) = 28.348$, $P < 0.01$] and drug treatment [$F(3, 17) = 13.249$, $P < 0.01$], a main effect of antidepressant treatment on the number of DCX+ cells in the DG region was found (Figure 3B). It was shown that CMS resulted in a 60 ± 1.6% reduction in the number of DCX+ cells compared with that in control mice ($n = 5$, $P < 0.01$), which was in agreement with previous report (Yap *et al.*, 2006). The decreased number of DCX+ cells in CMS group was reversed by Rg1 treatment, especially at dose of 20 mg·kg^{-1} ($n = 5$, $P < 0.01$ vs. CMS). Imipramine also increased the number of DCX+ cells compared with stressed mice ($n = 5$, $P < 0.01$). Correspondingly, the results of Western blotting showed a significant decrease in DCX protein level in the stressed animals. However, Rg1 and imipramine counteracted this deficiency induced by CMS (Figure 3C).

Chronic stress produces atrophy and dendritic arborization of CA3 pyramidal neurons (Magarinos *et al.*, 2011). The spine density of the CA3 subfield of hippocampus was measured using a Golgi-Cox stain. Distal dendrites in this region are shown in Figure 3D. Two-way ANOVA for the analysis of the dendritic spine density produced a significant interaction [$F(3, 22) = 12.093$, $P < 0.01$] with significant effects for CMS [$F(1, 22) = 33.208$, $P < 0.01$] and drug [$F(3, 22) = 13.391$, $P < 0.01$]. A *post hoc* test revealed that the dendritic spine density was markedly decreased in CMS-treated mice compared with the control group ($n = 6$, $P < 0.01$; Figure 3E), and Rg1 reversed the reduction of spine density ($n = 6$, $P < 0.01$ vs. CMS), which was similar to that of imipramine ($n = 6$, $P < 0.01$ vs. CMS). These results indicate that the CMS-induced decrease in neurogenesis and dendritic spine density were rescued by Rg1 treatment.

Chronic Rg1 treatment restores the CMS-induced decrease in the BDNF signalling pathway in the hippocampus

To investigate the possible mechanisms underlying Rg1-induced neurogenic and antidepressant effects, we first examined the expression of BDNF, which is important for neuronal survival and neurogenesis in the brain and plays a prominent role in current theories of depression (Dranovsky and Hen, 2006; Sahay and Hen, 2007). Therefore, we measured BDNF mRNA levels in the hippocampus following CMS. The BDNF mRNA expression level was expressed as a ratio of the expression of GAPDH. Data are summarized in Figure 4A. Two-way ANOVA indicated a significant interaction [$F(5, 23) = 8.137$, $P < 0.01$] with significant effects for CMS [$F(1, 23) = 26.908$, $P < 0.01$] and drug treatment [$F(5, 23) = 9.355$, $P < 0.01$]. Consistent with the findings from Duman and Monteggia (2006), the average BDNF mRNA level was decreased in the hippocampus of mice exposed to CMS compared with unstressed control mice ($n = 5$, $P < 0.01$). A 14 day treatment with Rg1 increased the BDNF mRNA level by 50 ± 2.5% at the dose of 2.5 mg·kg^{-1} ($n = 5$, $P < 0.05$ vs. CMS), and induced a 170 ± 20% increase at 20 mg·kg^{-1} ($n = 5$, $P < 0.01$ vs. CMS), similar to that of 15 mg·kg^{-1} imipramine ($n = 5$, $P < 0.01$ vs. CMS). We then determined if such changes were paralleled by modification of BDNF protein levels. As shown in Figure 4B, there was a main effect of both CMS [$F(1, 23) = 12.525$, $P < 0.01$] and drug administration [$F(5, 23) = 6.199$, $P < 0.01$], and a significant interaction between the two [$F(5, 23) = 5.477$, $P < 0.01$]. While CMS decreased hippocampal BDNF protein levels ($n = 5$, $P < 0.01$ vs. control), Rg1 treatment significantly increased its expression at the dose of 20 mg·kg^{-1} ($n = 5$, $P < 0.01$ vs. CMS), in line with the up-regulation of BDNF mRNA levels. Thus, Rg1 treatment prevented the ability of stress to lower BDNF in the hippocampus.

The ERK MAP kinase pathway is a key downstream signalling pathway of BDNF and ERK1/2 phosphorylation has been proposed as an intracellular signalling mechanism mediating antidepressant efficacy in depressed humans and animal models of depression (Gourley *et al.*, 2008). We examined the expression of ERK1/2 and pERK1/2 (the active form of ERK1/2) among the treatment groups in the hippocampus. Two-way ANOVA revealed a significant interaction [$F(5, 23) = 3.851$, $P < 0.05$] with significant effects for CMS [$F(1, 23) = 6.397$, $P < 0.05$] and drug [$F(5, 23) = 3.368$, $P < 0.05$]. Compared with the CMS group, imipramine restored the hippocampal pERK1/2 expression to the control level ($n = 5$, $P < 0.01$; Figure 4C). Similarly, chronic treatment with Rg1 also increased pERK1/2 in the hippocampus of CMS mice, especially at the dose of 20 mg·kg^{-1} ($n = 5$, $P < 0.01$ vs. CMS). By contrast, ERK1/2 levels remained unchanged among all treatment groups.

The CREB in the hippocampus is a critical mediator of neural plasticity and the transcription factor for BDNF. It has also been implicated in the long-term actions of antidepressants (Lane-Ladd *et al.*, 1997; Milner *et al.*, 1998; Silva *et al.*,

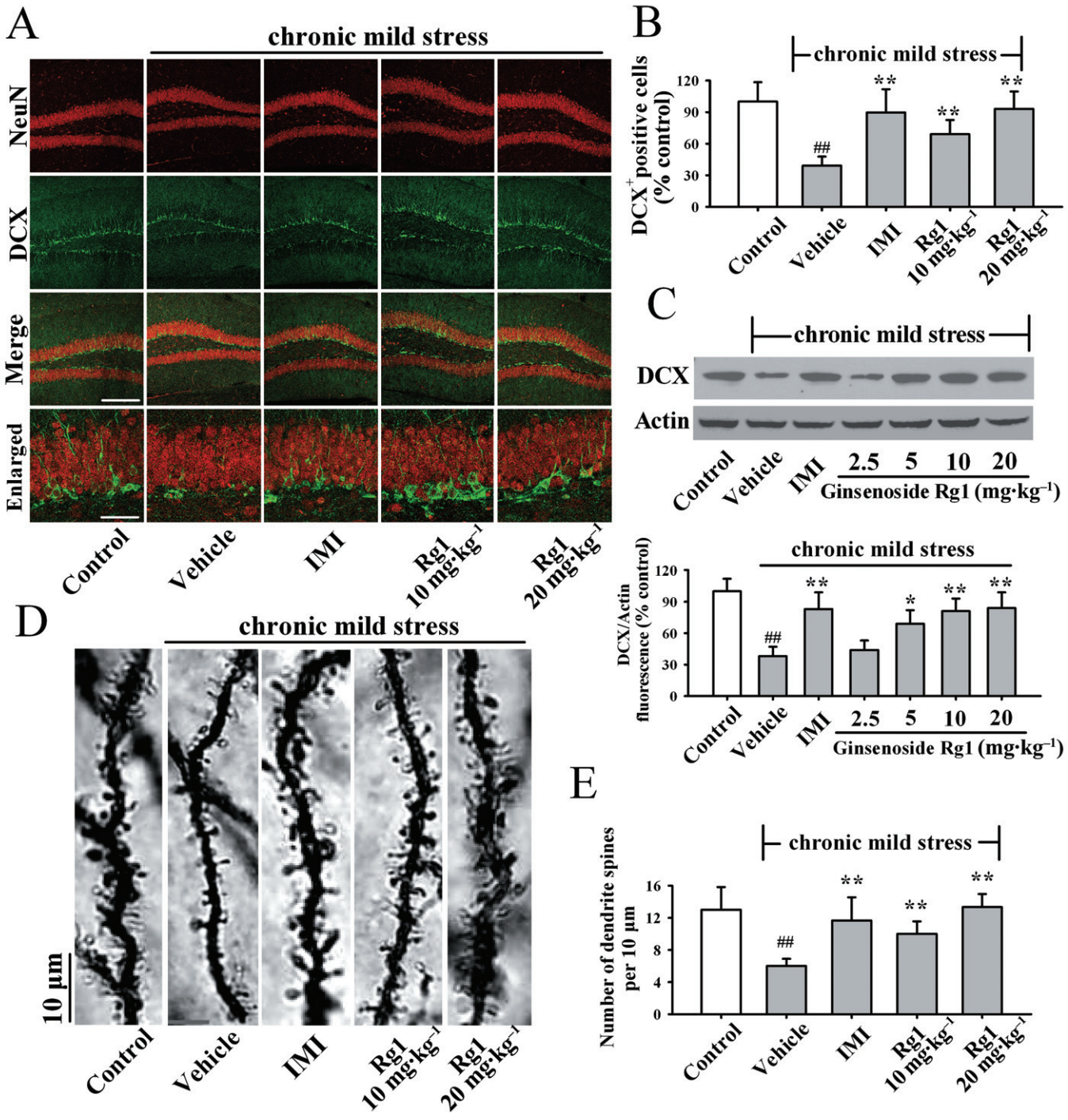

Figure 3

Rg1 reverses the change in hippocampal spine density and the decreased proliferation of hippocampal progenitor cells induced by CMS. (A) Representative confocal microscopic images showed co-localization (yellow) of NeuN (red) with DCX (green) in the DG. The scale bar is 200 μm for representative images and 25 μm for enlarged images respectively. (B) Density statistics (two-way ANOVA followed by *post hoc* Bonferroni's test) indicated that the decrease in the number of DCX positive cells induced by CMS was significantly reversed by Rg1 administration for 14 days ($n = 5$). (C) Representative Western blotting of DCX coincided with immunohistochemical changes. β-actin blotting was used to ensure equal loading. The summary data were shown in the lower histogram, revealing that CMS-induced decrease in the protein level of DCX was restored by Rg1 ($n = 5$). (D) Representative photomicrograph of a Golgi-Cox stained pyramidal neuron of CA3 hippocampus from animals of each group. Scale bar = 10 μm. (E) Summary data showed that the decrease in the spine density induced by CMS was completely reversed by Rg1 ($n = 6$). Data are expressed as means ± SEM. $^{\#\#}P < 0.01$, significantly different from control; $^{**}P < 0.01$, significantly different from CMS + vehicle group; two-way ANOVA followed by *post hoc* Bonferroni's test. IMI, imipramine.

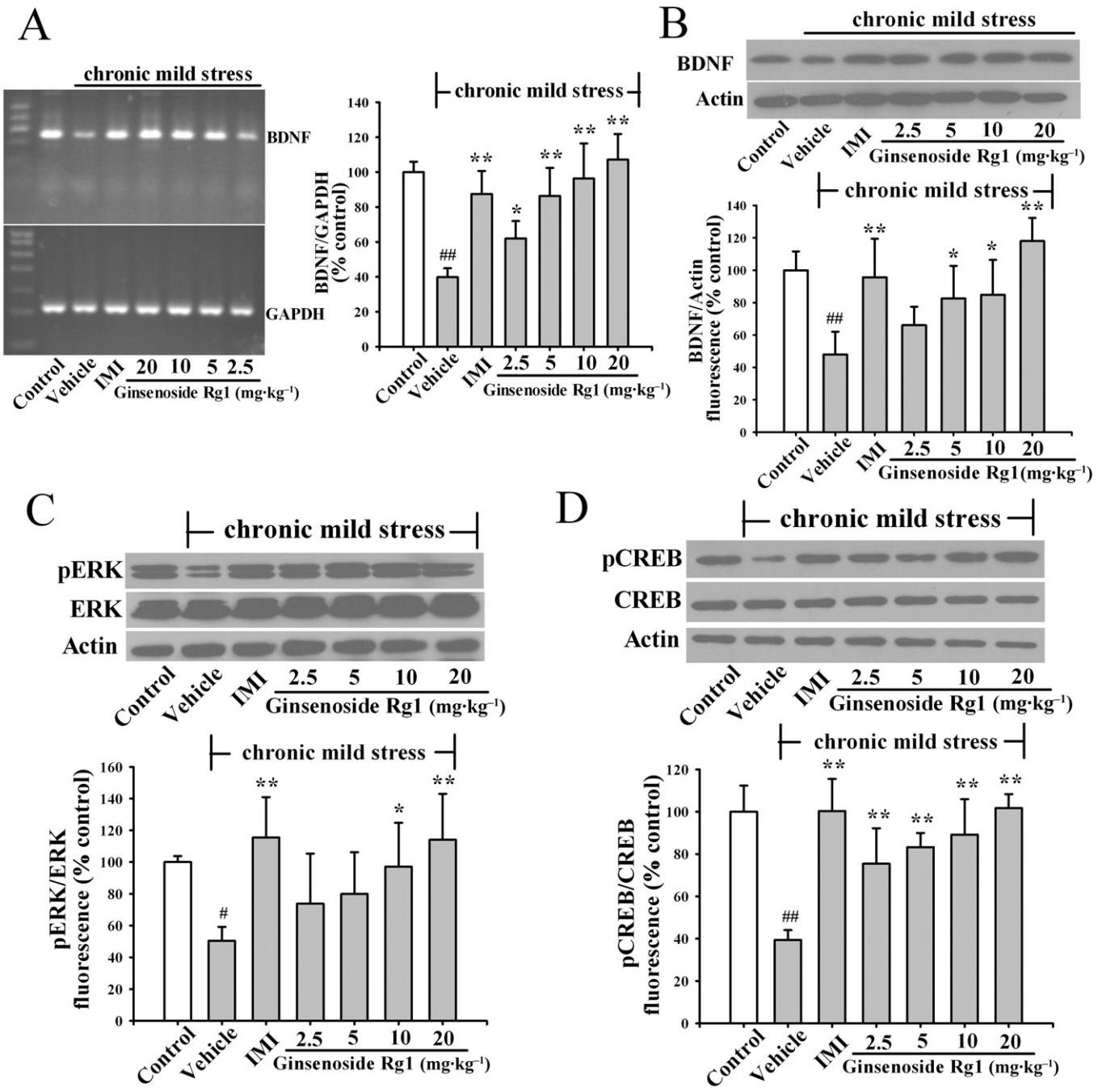

Figure 4

Chronic Rg1 treatment increases BDNF signalling in the hippocampus of stressed animals. (A) RT-PCR results showed that chronic treatment with Rg1 for 14 days reversed the decrease in BDNF mRNA in the hippocampus induced by CMS procedures. (B) Western blotting showed that Rg1 treatment reversed the CMS-induced reduction of hippocampal BDNF protein. (C) Rg1 reversed the decreased hippocampal pERK1/2 expression caused by CMS. (D) In a depression model, Rg1 restored the CMS-induced inhibition of hippocampus CREB phosphorylation. Data are expressed as means ± SEM ($n = 5$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from control; $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from CMS + vehicle group; two-way ANOVA followed by *post hoc* Bonferroni's test. IMI, imipramine.

1998). Enhancing hippocampal pCREB (the active form of CREB) has been proposed as a common antidepressant mechanism (Li *et al.*, 2009). In this study, pCREB level was significantly decreased in the hippocampus of CMS mice ($n = 5$, $P < 0.01$ vs. control; Figure 4D), and this was reversed by chronic treatment with imipramine ($n = 5$, $P < 0.01$ vs. CMS). Also, Rg1 (2.5–20 mg·kg^{-1}) increased pCREB level in the hippocampus of CMS mice in a dose-dependent manner, espe-

cially at the dose of 20 mg·kg^{-1} (n = 5, P < 0.01 vs. CMS). Two-factor ANOVA indicated significant treatment effects of CMS [F(1, 23) = 46.549, P < 0.01] and drug [F(5, 23) = 13.522, P < 0.01], as was the interaction between the two [F(5, 23) = 12.153, P < 0.01]. However, the level of total CREB protein was not altered. Thus, Rg1 induced antidepressant-like effects in parallel to the effects on the level of pERK1/2 and pCREB, suggesting that Rg1-induced changes in the BDNF signalling pathway in hippocampus are involved in the mediation of the behavioural effects of Rg1.

BDNF is necessary for the antidepressant effects of Rg1

Several lines of evidence support an important role of BDNF in the response to antidepressants (Nestler *et al.*, 2002; Shirayama *et al.*, 2002; Adachi *et al.*, 2008). To further determine whether BDNF-TrkB signalling is necessary for the effects of Rg1, K252a, a potent pharmacological inhibitor of the BDNF receptor TrkB was used (Tapley *et al.*, 1992; Yan *et al.*, 2010). Mice were first treated with K252a (25 μg·kg^{-1}, i.p., daily) for 3 days, then with Rg1 (20 mg·kg^{-1}) and followed by FST. It was found that K252a pretreatment dramatically prevented the Rg1-induced decrease in the duration of immobility in the FST (Figure 5A), and ANOVA showed a main effect for K252a [F(1, 36) = 4.256, P < 0.05] and for Rg1 [F(1, 36) = 7.270, P < 0.05] with significant interaction [F(1, 36) = 10.231, P < 0.01]. Furthermore, CMS-treated mice were co-injected with Rg1 (20 mg·kg^{-1}) and K252a (25 μg·kg^{-1}) for 14 days and behavioural tests were performed 24 h after the last injection. As shown in Figure 5B, co-treatment Rg1 with K252a inhibited the sucrose preference in the CMS-treated mice, and two-factor ANOVA reported a significant interaction [F(1, 26) = 44.659, P < 0.01] with significant effects for K252a [F(1, 26) = 68.452, P < 0.01] and Rg1 [F(1, 26) = 47.603, P < 0.01]. These results indicate that K252a antagonizes the antidepressant effects of Rg1.

SSRIs are the most widely used antidepressants, therefore we wanted to test whether the behavioural effects of Rg1 depended on 5-HT signalling. We depleted 5-HT with the tryptophan hydroxylase inhibitor PCPA. Although PCPA does not increase depression-like behaviour, it blocks the antidepressant effects of 5-HT reuptake inhibitors in the FST and in other animal models of depression (Coryell *et al.*, 2009). We reasoned that if 5-HT was necessary for the antidepressant effect of Rg1, then Rg1 should be sensitive to 5-HT depletion. Mice were given intraperitoneal injections of PCPA (300 mg·kg^{-1}, daily, 3 days) or vehicle control (saline, daily, 3 days) following injection of Rg1 (20 mg·kg^{-1}), and then antidepressant activity was assessed. The results showed that PCPA had no effect on the immobility time in the FST (Figure 5C), and two-way ANOVA revealed a significant effect of Rg1 [F(1, 36) = 127.414, P < 0.01] but no effects of PCPA [F(1, 36) = 0.523, P = 0.474]. To further test this result, CMS-treated mice were co-injected with Rg1 (20 mg·kg^{-1}) and PCPA for 14 days, and then sucrose preference was examined. Two-factor ANOVA showed a significant effect for Rg1 [F(1, 26) = 39.418, P < 0.01] and no effect for PCPA [F(1, 26) = 0.883, P = 0.335]. As shown in Figure 5D, PCPA had no effect on the sucrose consumption of Rg1. These results suggest that Rg1 produces antidepressant effects through mechanisms different from fluoxetine.

Next, we examined whether K252a blocked the effect of Rg1 on hippocampal neurogenesis, spine density and the ERK/CREB signalling pathway. There was a significant difference between control and CMS groups. More importantly, K252a also blocked the effect of Rg1 on neurogenesis [Rg1, F(1, 11) = 56.567, P < 0.01; K252a, F(1, 11) = 31.896, P < 0.01; interaction, F(1, 11) = 29.952, P < 0.01; Figure 6A and B], spine density [Rg1, F(1, 14) = 35.735, P < 0.01; K252a, F(1, 14) = 28.235, P < 0.01; interaction, F(1, 14) = 21.471, P < 0.01; Figure 6C and D], BDNF [Rg1, F(1, 11) = 21.409, P < 0.01; K252a, F(1, 11) = 33.728, P < 0.01; interaction, F(1, 11) = 18.052, P < 0.01; Figure 7A], pERK [Rg1, F(1, 11) = 18.101, P < 0.01; K252a, F(1, 11) = 40.225, P < 0.01; interaction, F(1, 11) = 21.686, P < 0.01; Figure 7B] and pCREB [Rg1, F(1, 11) = 9.558, P < 0.01; K252a, F(1, 11) = 16.919, P < 0.01; interaction, F(1, 11) = 8.514, P < 0.01; Figure 7B] level in the hippocampus. Together, these results suggest that the BDNF signalling pathway is necessary for the neurogenic and antidepressant effects of Rg1.

Discussion

In the present study, we have demonstrated for the first time that Rg1 produced robust antidepressant effects in rodent models of depression, which was similar to imipramine, and mediated by the BDNF signalling pathway, and not the 5-hydroxytryptaminergic system.

Panax ginseng C.A. Meyer (Araliaceae family) is a popular 'tonic' herb in Chinese traditional medicine. The biological actions of ginseng are complex and include some effects that can be related to affective and anxiety disorders, such as modulation of the HPA axis (Kim *et al.*, 2003) and the monoaminergic system (Fugh-Berman and Cott, 1999). In the context of novel theories related to depression, the active ingredients of ginseng were demonstrated to have neuroprotective effects and to increase neuronal survival. *In vitro*, ginsenoside treatment increased survival and promoted neuronal plasticity and neurogenesis in dopaminergic cells (Radad *et al.*, 2004). *In vivo*, ginsenoside was shown to reduce hypoxic brain injury in rats (Park *et al.*, 2004; Ji *et al.*, 2005), and protect against toxic interventions in Parkinson's disease (Van Kampen *et al.*, 2003). Previous studies have demonstrated the antidepressant effects of total ginseng saponin (Dang *et al.*, 2009), which contains several ingredients. However, it is important to determine the active ingredient which improved the depression-like behaviour. Rg1 shares a similar molecular structure to that of ginsenoside Rb3, which possesses antidepressant properties (Cui *et al.*, 2011), and has been reported to increase BDNF expression following focal cerebral ischaemia (Shen and Zhang, 2003). Our study provides direct evidence that the active ingredient Rg1 displays significant antidepressant activity.

Acute injection of Rg1 at doses of 10 and 20 mg·kg^{-1} in mice produced a significant reduction of immobility time in the FST and TST, while Rg1 treatment did not result in any significant changes in locomotor activity even at the higher dose (20 mg·kg^{-1}), indicating that this compound was well tolerated and the reduction of immobility was not due to locomotor abnormality. Furthermore, chronic daily injections of Rg1 for 14 days robustly ameliorated the behavioural

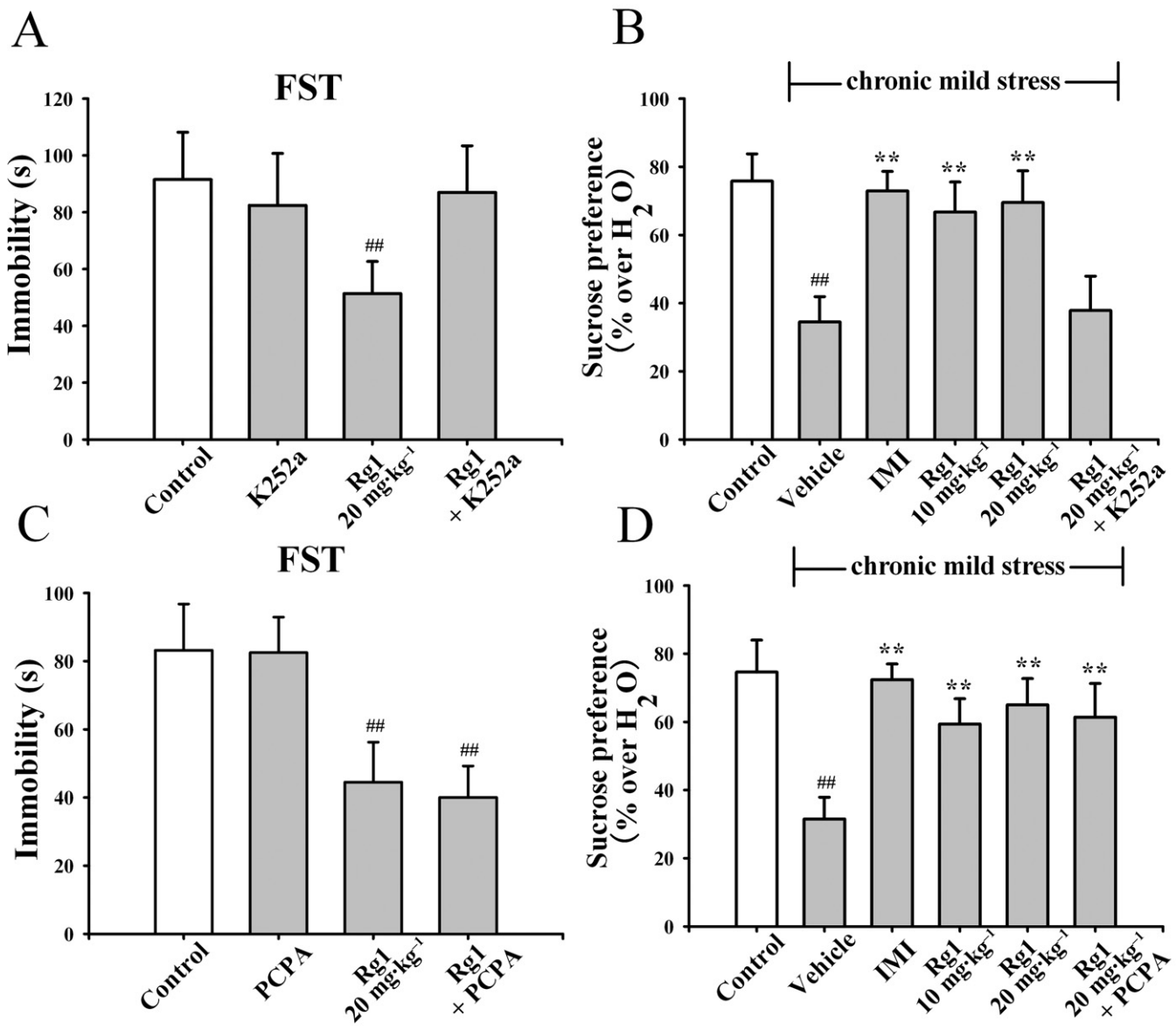

Figure 5

Blockade of BDNF-TrkB signalling by K252a, not depletion of 5-HT by PCPA, abolishes the antidepressant actions of Rg1. (A) Rg1 + K252a mice displayed higher duration of immobility than Rg1-treated mice in the FST. (B) In CMS model, Rg1 + K252a mice displayed lower sucrose preference than Rg1-treated mice. (C) PCPA injection had no effect on the antidepressant effects of Rg1 in the FST. (D) PCPA injection had no effect on the antidepressant effects of Rg1 in the CMS. Results are expressed as means ± SEM ($n = 10$). $^{\#\#}P < 0.01$, significantly different from control; $^{**}P < 0.01$, significantly different from CMS + vehicle group; two-way ANOVA followed by *post hoc* Bonferroni's test. IMI, imipramine.

deficits of CMS-treated mice to the basal level of non-stressed control mice. More importantly, the antidepressant effects of Rg1 at the dose of 20 mg·kg^{-1} were similar to that of imipramine, suggesting that Rg1 may be developed as a novel antidepressant.

Chronic stress produces atrophy and dendritic arborization of CA3 pyramidal neurons as well as stress-induced inhibition of hippocampal neurogenesis. This dendritic remodelling may be related to the prolonged activation of the HPA axis and the resulting elevation of excitatory amino acids and corticosteroid activation during stress (Sapolsky, 1996; Lupien *et al.*, 1999), and can be reversed by antidepressant treatment (Watanabe *et al.*, 1992). In this study, the CMS-induced elevation of plasma corticosterone level was completely reversed by chronic Rg1 administration, like the classical antidepressants. Rg1 administration also reversed the change in spine density of pyramidal neurons and the decreased neurogenesis caused by CMS. More interestingly, our results also demonstrated that Rg1 up-regulated the expression of BDNF mRNA in the hippocampus of stressed mice. BDNF is known to play an important role in adult neurogenesis and the activity of antidepressants in depressed

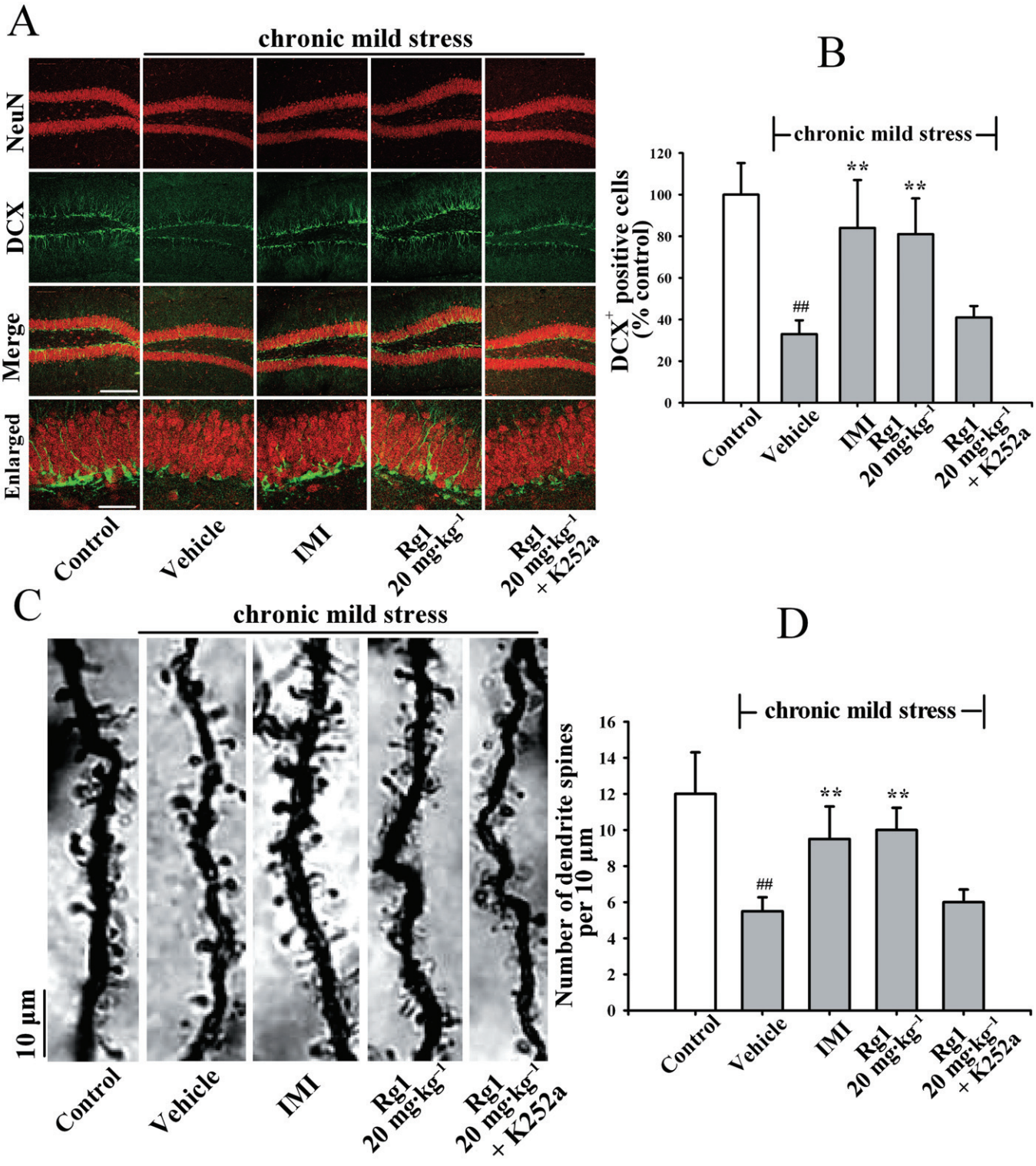

Figure 6

K252a blocks the neurogenic effect of Rg1. (A) Representative confocal microscopic images showed co-localization (yellow) of NeuN (red) with DCX (green) in the DG region. The scale bar is 200 μm for representative images and 25 μm for enlarged images respectively (B) Density statistics indicated that the enhancement of cell proliferation induced by Rg1 administration was blocked by co-injection with K252a (n = 5). (C) Representative photomicrograph of a Golgi-Cox stained pyramidal neuron of CA3 area of hippocampus from animals of each group. Scale bar = 10 μm. (D) Summary data showed that the enhancement of spine density induced by Rg1 administration was blocked by co-injection with K252a (n = 6). Results are expressed as means ± SEM. $^{\#\#}P < 0.01$, significantly different from control; $^{**}P < 0.01$, significantly different from CMS + vehicle group; two-way ANOVA followed by *post hoc* Bonferroni's test. IMI, imipramine.

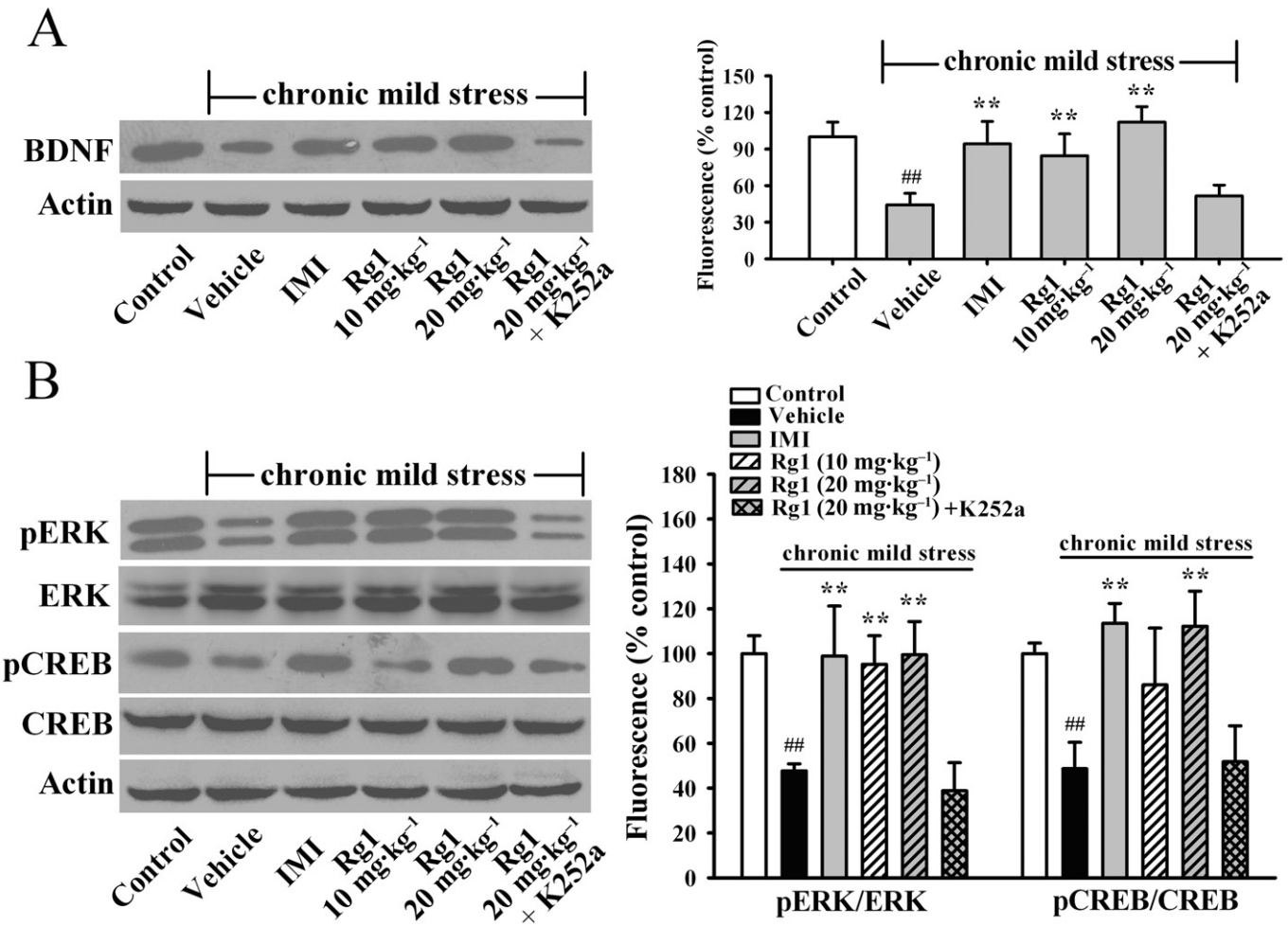

Figure 7

K252a treatment prevents the effect of Rg1 on the BDNF signalling cascade in the hippocampus. (A) Western blotting results showed that the enhancement of BDNF in the hippocampus induced by Rg1 administration was blocked by K252a. (B) Western blotting results showed that the Rg1-induced enhancement of hippocampal pERK1/2 and pCREB was also blocked by K252a administration. Results are expressed as means ± SEM ($n = 5$). $^{\#\#}P < 0.01$, significantly different from control; $^{**}P < 0.01$, significantly different from CMS + vehicle group; two-way ANOVA followed by *post hoc* Bonferroni's test. IMI, imipramine.

patients and in animal models of depression (Smith *et al.*, 1995; Shirayama *et al.*, 2002; Newton and Duman, 2004). Various stress procedures decrease BDNF levels in the frontal cortex and hippocampus, whereas chronic treatment with almost all classes of antidepressants increases BDNF in those regions. Given that both bipolar disorder and major depressive disorder patients have a reduced hippocampal expression of BDNF and other neurotrophic factors (Knable *et al.*, 2004; Lee and Kim, 2009), increasing BDNF expression might be a common pathway for antidepressants to exert therapeutic actions. In this study, Rg1 reversed the CMS-induced decrease in the level of hippocampal BDNF in parallel to the up-regulation of progenitor cell proliferation.

We then examined the changes in ERK activity because BDNF is an upstream regulator of the ERK cascade and ERK 1/2 phosphorylation has also been proposed as an intracellular signalling mechanism mediating antidepressant efficacy in depressed humans and animal models of depression. The activity of ERK, as evaluated by the anti-phospho-ERK specific antibody, was much higher in Rg1-treated mice than that in stressed mice. Finally, an effect of Rg1 on CREB activity, which is involved in BDNF signalling pathway, was also observed. Increasing evidence shows that the level of pCREB is reduced in the temporal cortex (presumably including the hippocampus) in depressed patients (Dowlatshahi *et al.*, 1998), and major classes of antidepressants increase the level and/or function of pCREB in several brain regions including the hippocampus (Thome *et al.*, 2000; Tardito *et al.*, 2009). In this study, we found that chronic treatment of Rg1 up-regulated level of phosphorylated CREB in the hippocampus of stressed animals, to the basal level of saline-treated mice. In addition, the antidepressant effects as well as enhancement of cell proliferation induced by Rg1 administration were also blocked by co-treatment with K252a, a potent inhibitor of the BDNF receptor TrkB, suggesting that

the BDNF-TrkB signalling pathway is essential in the antidepressant and neurogenic effects of Rg1.

In addition, monoaminergic systems, especially the 5-hydroxytryptaminergic system, contribute to the pathophysiology of mental depression (Elhwuegi, 2004), and most antidepressants exert their action by elevating synaptic monoamine concentrations. However, we found that depleting 5-HT by PCPA did nothing to lessen the antidepressant action of Rg1, indicating that the molecular mechanisms of Rg1 were distinct from the conventional antidepressants.

In summary, the present study shows that Rg1 exhibited antidepressant-like properties in animal models of depression, which appeared to be mediated through up-regulation of the hippocampal BDNF-TrkB signalling pathway. This newly discovered effect of Rg1 provides a new insight to understand the pharmacological effects of Rg1, a further insight into the possible therapeutic use of Rg1 for treating major depression, and more importantly, sheds light on the development of new antidepressants with higher efficacy and fewer side effects.

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Rg1 has no significant effects on sucrose preference and weight gain in naïve mice. The mice received daily injection of saline (control), imipramine (IMI, 15 mg·kg^{-1}) and Rg1 (2.5, 5, 10, 20 mg·kg^{-1}) for 14 consecutive days. The behavioural tests were conducted 24 h after the last injection. (A) The sucrose consumption in Rg1-treated mice was similar to that in vehicle-treated mice. (B) The body weight gain in Rg1-treated mice was similar to that in vehicle-treated mice. The data are expressed as means ± SEM (n = 10); one-way ANOVA followed by *post hoc* LSD test.

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