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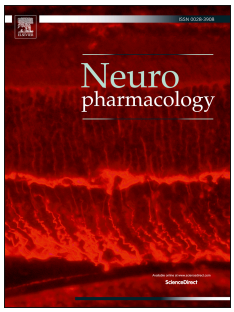

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Cuiqin Fan[1], Xiuzhi Zhu[1], Qiqi Song[1], Peng Wang[1], Zhuxi Liu[1], Shu Yan Yu[1, 2] *

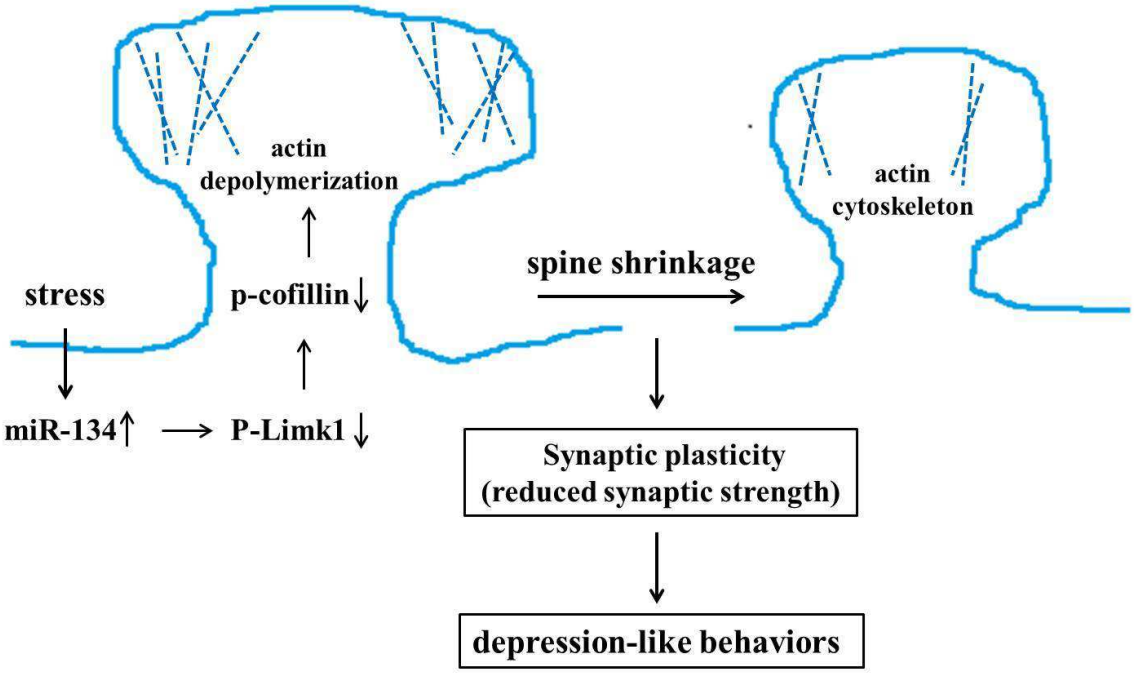
1. Department of Physiology, Shandong University, School of Medicine, Wenhuaxilu Road, Jinan, Shandong Province, 250012, PR China;

2. Shandong Provincial Key Laboratory of Mental Disorders, School of Medicine, Wenhuaxilu Road, Jinan, Shandong Province, 250012, PR China;

* Corresponding author: Shu Yan Yu,

E-mail address: shuyanyu@sdu.edu.cn

Tel: +86-0531-88383902; fax: +86-0531-88382502



The hypothesis of molecular mechanisms underlying the modulatory effects of miR-134 on neuroplasticity and depression-like behaviors.

MiR-134 modulates chronic stress-induced structural plasticity and depression-like behaviors via downregulation of Limk1/cofilin signaling in rats

Cuiqin Fan[1], Xiuzhi Zhu[1], Qiqi Song[1], Peng Wang[1], Zhuxi Liu[1], Shu Yan Yu[1, 2] *

1. Department of Physiology, Shandong University, School of Medicine, Wenhuaxilu Road, Jinan, Shandong Province, 250012, PR China;

2. Shandong Provincial Key Laboratory of Mental Disorders, School of Medicine, Wenhuaxilu Road, Jinan, Shandong Province, 250012, PR China;

* Corresponding author: Shu Yan Yu,

E-mail address: shuyanyu@sdu.edu.cn

Tel: +86-0531-88383902; fax: +86-0531-88382502

Abstract

Increasing evidence has suggested that depression is a neuropsychiatric condition associated with neuroplasticity within specific brain regions. However, the mechanisms by which neuroplasticity exerts its effects in depression remain largely uncharacterized. In the present study we show that chronic stress effectively induces depression-like behaviors in rats, an effect which was associated with structural changes in dendritic spines and synapse abnormalities within neurons of the ventromedial prefrontal cortex (vmPFC). Moreover, unpredictable chronic mild stress (UCMS) exposure significantly increased the expression of miR-134 within the vmPFC, an effect which was paralleled with a decrease in the levels of expression and phosphorylation of the synapse-associated proteins, LIM-domain kinase 1 (Limk1) and cofilin. An intracerebral infusion of the adenovirus associated virus (AAV)-miR-134-sponge into the vmPFC of stressed rats, which blocks mir-134 function, significantly ameliorated neuronal structural abnormalities, biochemical changes and depression-like behaviors. Chronic administration of ginsenoside Rg1 (40 mg/kg, 5 weeks), a potential neuroprotective agent extracted from ginseng, significantly ameliorated the behavioral and biochemical changes induced by UCMS exposure. These results suggest that miR-134-mediated dysregulation of structural plasticity may be related to the display of depression-like behaviors in stressed rats. The neuroprotective effects of ginsenoside Rg1, which produces an antidepressant like effect in this model of depression, appears to result from modulation of the miR-134 signaling pathway within the vmPFC.

Key words: Structural plasticity; miR-134; Ventromedial prefrontal cortex; Depression; Ginsenoside Rg1

1. Introduction

Depression, which is associated with notable alterations in neural activities within specific brain circuits, is considered a major neuropsychiatric disorder in contemporary society (Vaidya and Duman 2001; Mayberg 2003). Recently, the ventromedial prefrontal cortex (vmPFC) has emerged as the important brain region in the pathogenesis of depression (Holmes and Wellman 2009; McLaughlin et al. 2009; Radley et al. 2006). Although the functional changes within the vmPFC as related to depression have yet to be fully elucidated, the reciprocal pathways that exist between the vmPFC and other critical structures associated with depression suggest some potential relationships. In specific, the connections involving the vmPFC with that of the hippocampus and amygdala, insinuate its involvement in deficits of executive functions such as memory, cognition and reward-related processes, as well as emotional dysregulation among other stress-related behaviors in rodents. In further support for a significant role of the vmPFC in depression are results obtained from neuroimaging studies which revealed that reductions of vmPFC activity were associated with depression (Takahashi et al. 2004). Moreover, alterations in the structure and function of dendrites were also found within the vmPFC in response to chronic stress in animal models (Cook et al. 2004; Goldwater et al. 2009). These

findings demonstrate that modulations in the plasticity of vmPFC neurons are associated with modifications in brain circuity related to depression. However, the molecular mechanisms underlying these neuronal plasticity processes within the vmPFC and how depression-inducing stressors leads to these changes have yet to be investigated.

In the mammalian nervous system, microRNAs have been suggested to regulate the translation of messenger RNAs, and thereby play critical roles in neuronal development, differentiation and plasticity (Iacoangeli et al. 2010; Presutti et al. 2006). For example, miR-124, which was found to be preferentially expressed in neurons, represents an important regulator for the temporal progression of adult neurogenesis (Cheng et al. 2009), while miR-132 appears to be critical for the formation and plasticity of neuronal connections (Pathania et al. 2012). It has been hypothesized that miRNAs can repress the translation of synaptically localized mRNAs until these neurons are exposed to the appropriate extracellular stimuli, which will then result in the formation, maturation and/or plasticity of the synapse (Bredy et al. 2011; Higuchi et al. 2016). MiR-134, a brain-specific microRNA which is mainly localized to the synapto-dendritic compartment of neurons, has been suggested to negatively regulate dendritic spine development and plasticity (Schratt et al. 2006). Findings from recent studies have also demonstrated that miR-134 negatively regulates hippocampal synaptic plasticity to result in cognitive deficits by inhibiting mRNAs of plasticity-associated proteins such as Lim-domain-containing protein kinase1 (Limk1) (Liu et al. 2017). Limk1 is considered to function as a positive regulator of actin

filament dynamics via its capacity to inhibit cofilin activity, the key actin depolymerizing factor located in postsynaptic sites. The phosphorylation of Limk1, which subsequently phosphorylates and inhibits the activity of cofilin, prevents the cleavage of filamentous actin, thereby stabilizing the actin cytoskeleton and spine sizes (Bamburg 1999; Gunning et al. 2015; Sarmiere and Bamburg 2002). It has been reported that a Limk1 deficiency leads to abnormalities in dendritic spine structure and synaptic transmission efficiency in Limk1 knockout mice, effects similar to that found with miR-134 overexpression (Meng et al. 2002). Moreover, studies in rats have demonstrated that stress produces a long-term inhibition of Limk1 mRNA translation, an effect which is critical for changes in synaptic plasticity. Interestingly, both of these alterations are ameliorated with antidepressant treatment (Nava et al. 2017). Thus, when subjected to stress, the miR-134-mediated reductions in Limk1 phosphorylation levels may be responsible for the de-phosphorylation of cofilin, thereby resulting in depolymerization of actin filaments in post-synaptic sites to induce morphological changes in dendritic spines. However, whether such mechanisms are involved in the dysregulation of neuronal structural plasticity within the vmPFC as induced by chronic stress to produce depression-like behaviors are unknown.

Recently, traditional herbal medicines have become novel pharmacological tools for use in the treatment of various neurological disorders. The benefits of these agents reside in their neuroprotective effects and high safety margins (Nemeroff 2007; Van der Watt et al. 2008). Ginsenoside Rg1, the active principle ingredient of the herb

Panax Ginseng, is now being investigated as a valuable neuroprotective agent in the treatment of Alzheimer's disease and other neurodegenerative disorders (Zhang et al. 2012; Song et al. 2013). It has also been reported that ginsenoside Rg1 shows significant neuroprotective effects in a variety of cognitive and memory deficit animal models (Wang et al. 2014; Zhu et al. 2014). In addition, findings from recent studies have revealed that ginsenoside Rg1 could also function as an antidepressant through its ability to reverse the decreases in hippocampal BDNF protein levels in mice subjected to chronic stress (Jiang et al. 2012). Studies within our laboratory have shown that chronic treatment of ginsenoside Rg1 significantly ameliorated depression-like behaviors in rats, possibly via activating the CREB-BDNF signaling pathway, a crucial regulator in protecting and sustaining the normal structure and function of neurons (Zhu et al. 2016; Liu et al. 2016). Recent evidence has implicated that ginsenoside Rg1 promotes the neural differentiation of mouse adipose-derived stem cells via upregulation of miRNA-124 expression (Dong et al. 2017), as well as by promoting angiogenesis, possibly through the modulation of miRNA-214 or miRNA-23a expression (Chan et al. 2009; Kwok et al. 2015). Therefore, these results suggest that the neuroprotective effects of ginsenoside Rg1 might serve as the basis to explain its antidepressant effects. However, detailed characterization of the neuronal mechanisms underlying neuronal structural and functional plasticity in depression and thus the antidepressant-like effects of ginsenoside Rg1is remains to be explored.

Therefore, in the present study, we first investigated whether treatment with

ginsenoside Rg1 could prevent the neuronal structural changes and depression-like behaviors induced by UCMS exposure in rats. To explore some of the underlying mechanisms of the neuroprotective and antidepressant-like effects of ginsenoside Rg1, the regulatory roles of miR-134 upon the structural plasticity-related proteins, Limk1 and cofilin, were examined within the vmPFC, a crucial brain region involved in the pathogenesis of depression in the UCMS animal model.

2. Materials and Methods

2.1. Animals

Male Wistar rats (160-180g) were obtained from the Shandong University Animal Centre. All procedures were approved by the Shandong University Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Animals were housed under standard laboratory conditions and allowed to adapt to these conditions for 7–8 days prior to use in the experiments. Behavioral tests were performed in the dark phase of the light/dark cycle. All efforts were made to minimize the pain and numbers of the animals used in the experiments.

2.2. Drugs and treatment

Ginsenoside Rg1 (Winherb Medical S & T Development Co. Ltd., Shanghai, China) was dissolved in physiological saline (NaCl, 0.9%) at a concentration of 10 mg/ml. Animals were randomly allocated to one of the following four groups with N=12/group: (a) control (non-stressed group), (b) UCMS, (c) ginsenoside Rg1 treatment (40 mg/kg, administered daily) followed by UCMS, (d) vehicle treatment (physiological saline 10 ml/kg, administered daily) followed by UCMS. Dose, route and administration schedules of ginsenoside Rg1 used in the present experiment were based upon previous studies (Zhu et al. 2016; Liu et al. 2016; Xie et al. 2015). Ginsenoside Rg1 or saline were administered intraperitoneally (i.p.) at 60 min prior to UCMS exposure. All drugs were administered daily for 5 weeks.

The UCMS procedure was performed according to previously studies (Mao et al. 2009). Animals in the control (non-stressed) group were housed in groups of four per cage in a separate room from that of the stressed groups. Animals in the stressed-groups were housed individually and subjected to a variable sequence of mild and unpredictable stressors. Stressors applied over the 5-week course of the UCMS procedure included 24h food deprivation followed by 24h water deprivation, 45° cage tilt (24h), shaking 2 h, 5 min cold swimming (at 4°C), 2 h physical restraint, wet bedding (24h), and overnight illumination. Rats were subjected to one of above stressors each day as administered in a pseudo-random order (Figure 1A).

2.3. Stereotaxic injection of the AAV virus

We constructed the HBAAV2/8-U6-miR134-CAG-GFP virus (AAV-miR-134, Hanbio biotechology, Shanghai, China) to overexpress miR-134 in the vmPFC and the HBAAV2/8-U6-miR134-sponge-CAG-GFP virus (AAV-miR-134-sponge, Hanbio biotechology, Shanghai, China) to block miR-134 in the vmPFC. In a separate series of experiments, animals were allocated to one of the following five groups with N=12/group: (a) naive (non-stressed and non-injected group), (b) naive + AAV-control (GFP-Cre construct), (c) naive+AAV-miR-134 (d) stressed, (d) stressed + AAV-control (GFP-Cre construct) and (e) stressed + AAV-miR-134-sponge. For viral treatments, rats were anesthetized with 10% chloral hydrate (4ml/kg of body weight; i.p.) and placed in a stereotaxic frame. Rats were then infused bilaterally with 1-1.5ul of purified and concentrated AAV virus (~10^{12} infection units per ml) into the vmPFC (coordinates from bregma: +3.24mm; medial/lateral: ±0.5mm; dorsal/ventral: -5.0mm, for Wistar rats at age 8 weeks) using a slow infusion rate of 100-150nl/min. Behavioral experiments or biochemical assays were performed at a minimum of 10 days after infusion into the vmPFC. Infusion sites were assessed at the end of the behavioral tests and only data from rats with infusions confined to the vmPFC were included in the analyses. An infection rate within the vmPFC region was calculated for each rat. Counting of infected cells was conducted in serial 2.64 um anterior to posterior sections. The total number of neurons was averaged from counting NeuN signals in serial sections of three control rats. Infection rate = number of infected cells/number of total neurons.

2.4. Behavioral assays

All behavioral tests were conducted during the dark period of the light/dark cycle (1900-2400 h) after the 5-week regime of UCMS exposure.

Sucrose preference test. The sucrose preference test was performed as described previously with minor modifications (Mao et al. 2009). Briefly, animals were initially placed individually in the test cages and two bottles of 1% sucrose solution were placed in the cage for the first 24 h. Then one bottle was replaced with tap water for the second 24 h period. The animals were then food and water deprived for 24 h and exposed to two bottles for a 3 h period, with one containing 100 ml of sucrose solution (1%, w/v) and the other 100 ml of tap water. The bottle position was alternated every hour. Total consumption of the sucrose solution and tap water were measured and the sucrose preference, which provides the index of anhedonia, was defined as sucrose consumption / [water consumption + sucrose consumption] ×100% during the 3 h test.

Forced swim test. At 24 h following the sucrose preference test, the forced swim test was performed as previously described (Porsolt et al. 1997; Duman et al. 2007). Animals were placed individually in a cylinder of water (height: 80 cm, diameter: 30 cm) at 25 °C for 15 min of forced swimming (training session). The depth of water was set to prevent the rats from contacting the bottom of the tank or escape. Twenty-four hours after this training session, animals were again individually placed in the cylinder for a 5-min period (test session). The immobility time, which provides

an index of despair, another core symptom of depression, was defined as floating with only limited movements necessary to maintain their head above the water.

Open field test. At 24 h after the forced swim test, animals were tested for spontaneous exploratory activity as described previously (Walsh and Cummins 1976). Animals were individually placed in the center of a large square box (100 x 100 x 40 cm) and were permitted unrestricted exploration of the arena for a 5-min session. The number of locomotor (segments crossed with the four limbs) and exploratory (number of rearings, consisting of standing on their hind limbs) activities were recorded and subsequently analyzed.

2.5. Golgi Staining

At 24 h following completion of behavioral tests Golgi staining was performed to assess changes in neuronal dendrites and dendritic spines within vmPFC neurons. A Golgi-Cox impregnation based FD Rapid GolgiStain™ Kit (PK401, FD Neuro-Technologies, MD21041, USA) was employed to stain neurons and glia according to the manufacturer's instructions. Rats were anaesthetized with sodium pentobarbital (150 mg/kg, i.p.) and the brains were rapidly removed, quickly rinsed with distilled water to remove blood from the surface, then immersed in the impregnation solution (A/B=1:1, total 15 ml/rat) in a 50ml plastic tube and covered with aluminum foil according to the manufacture's protocol. Brains were then sectioned serially into 100 µm coronal sections. Each section was transferred to a

gelatin-coated slide and was dried at room temperature in the dark for up to 3 days, at which time staining and washing procedures were performed according to the manufacture's protocol. The sections were then cleaned in xylene and cover-slipped with Rhamsan gum for light microscopic observation. Pyramidal neurons of the vmPFC, which were defined by the presence of a basilar dendritic tree and a clearly defined single apical dendrite, were chosen for morphological analysis. For each group, at least 3 to 5 dendritic segments of apical dendrites per neuron were randomly selected, and 5 pyramidal neurons were analyzed per animal. The number of spines per dendritic segment was counted by Image-Pro plus software. Four rats were included per group.

2.6. Electron microscopy analysis

Transmission electron microscopy (TEM) was performed at 24 h after completion of behavioral tests as described previously (Zhang et al. 2014). Rats were anaesthetized with sodium pentobarbital (150 mg/kg, i.p.) and the vmPFC (1mm×1mm×1mm) was carefully dissected, placed in 2.5% glutaraldehyde at 4°C for 4 h and then fixed with 1% osmium tetroxide for 1 h. After graded ethanol dehydration, the tissues were infiltrated with a mixture of one-half propylene oxide overnight and then embedded in resin. The tissues were then cut into serial ultrathin sections (70 nm thick) and stained with 4% uranyl acetate for 20 min and with 0.5% lead citrate for 5 min. The ultrastructure of vmPFC neurons were then observed with use of transmission

electron microscopy (Philips Tecnai 20 U-Twin, Holland). In the present study, at least 30 micrographs from each rat were randomly selected for analysis. Analysis of synaptic density was performed with use of Image J analysis software (NIH, Scion Corporation, Frederick, MD).

2.7. Real-time quantitative PCR

Total RNA was isolated from vmPFC samples and 2μg of total RNA was used as a template to generate cDNA by reverse transcription using the All-in-One^{TM} miRNA First-Strand cDNA Synthesis Kit（QP018, GeneCopoeia Inc.，USA). Real-time PCR was performed with use of the Bio-rad IQ5 Real Time PCR System (Bio-Rad, USA). The PCR conditions consisted of: 95°C × 10min, followed by 40 cycles of 95°C × 10s, 55°C × 20s and 72℃×15s. The relative fold change in expression of miR-134 was determined using the ΔΔCT method of the Bio-Rad IQ5 Software (Bio-Rad, USA).

2.8. Western blot analysis

At 24 h following completion of behavioral testing, rats were anaesthetized with sodium pentobarbital (150 mg/kg, i.p.), decapitated and the vmPFC was carefully dissected while the brain was maintained on ice. Thirty μg of proteins were loaded per lane, electrophoretically separated on 12% SDS-PAGE gels and transferred onto the PVDF membrane. Membranes were then incubated with their appropriate primary

antibodies overnight at 4° C for western blot analysis. The antibodies used were polyclonal rabbit anti-Limk1 (1:1000, CST-3842S, Cell Signaling Technology, Beverly, MA, USA), anti-cofilin (1:1000, CST-5175S, Cell Signaling Technology, Beverly, MA, USA) and anti-phospho-cofilin (1:200, CST-3313S, Cell Signaling Technology, Beverly, MA, USA), anti-βactin (1:8000) (SC-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the secondary antibody was horseradish peroxidase-conjugated antibody (1:5000, SC-2030, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were detected using an enhanced chemiluminescence detection kit (GE Healthcare, Buckinghamshire, UK) and protein band densities were quantified using Image-J software (NIH, Scion Corporation, Frederick, MD). Final data were expressed as a percent difference from that of the control group. In this study, the vmPFC of each rat (eight rats per group) was analyzed separately.

2.9. Statistics

All statistical procedures were performed using the SPSS version 13.0. Data were expressed as mean ± SEM and represented values generated from at least four individual animals. A one-way analysis of variance (ANOVA) was used and multiple post-hoc comparisons were performed using the Newman-Keuls test. Differences with a $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. Ginsenoside Rg1 treatment ameliorated depression-like behaviors caused by UCMS exposure

Results from the sucrose preference test revealed that there was an overall statistically significant difference among the four groups for the percent of sucrose consumption [$F_{(3, 44)} = 17.68$, $P < 0.05$] (Figure 1B). Post-hoc analysis indicated that the percent of sucrose consumption in UCMS rats was significantly reduced as compared to the non-stressed control rats. In contrast, 5-weeks of pretreatment with ginsenoside Rg1 significantly increased the consumption of sucrose solution in UCMS-exposed rats. No significant differences in sucrose consumption were obtained between vehicle-treated UCMS rats and UCMS rats ($p > 0.05$). The increased amounts of sucrose consumption observed within ginsenoside Rg1 treated UCMS-exposed rats suggest a potential antidepressant-like effect of ginsenoside Rg1.

Another test often used to screen for compounds with antidepressant-like effects is the forced swim test. As presented in Figure 1C, an overall statistically significant difference was obtained among these groups with regard to immobility times [$F_{(3, 44)} = 18.72$, $P < 0.05$] and swimming times [$F_{(3, 44)} = 15.18$, $P < 0.05$] in this behavioral test. Post-hoc analysis revealed that the UCMS procedure significantly increased immobility times and decreased swimming times in rats, while daily pretreatment with ginsenoside Rg1 significantly prevented these behavioral changes in UCMS rats. This ability for ginsenoside Rg1 to decrease immobility times as well as to increase

swimming times in the forced swim test provide further evidence for its possible antidepressant-like effects in this animal model of depression.

The effects of ginsenoside Rg1 treatments on locomotor and exploratory activities of rats were examined in the open field test. An overall statistically significant difference was obtained for both the number of crossings [$F_{(3, 44)} = 12.76$, $p < 0.05$] and rearings [$F_{(3, 44)} = 10.17$, $p < 0.05$] (Figure 1D). Post-hoc analysis revealed that horizontal locomotor activities (crossings defined as segments crossed with all four paws) and vertical exploratory activities (number of rearings defined as standing on their hind paws) in rats were significantly reduced after 5-weeks of UCMS exposure ($p < 0.05$), while pretreatment with ginsenoside Rg1 increased both of these activities in UCMS-exposed rats. Overall, the results obtained from these three behavioral assays demonstrate that UCMS serves as an effective model for inducing depression in rats and that ginsenoside Rg1 treatment alleviates depression-like behavioral responses.

3.2. Ginsenoside Rg1 treatment ameliorated structural changes in vmPFC neurons induced by UCMS exposure

Examination of the vmPFC was performed to assess possible involvement of changes in neuronal plasticity as related to the anti-depressant effects induced by ginsenoside Rg1. Results obtained from the Golgi Staining assay showed that stress induced morphological alterations in dendritic spines, the main anatomical substrate for

synapse formation. An overall statistically significant difference was obtained for the density of dendritic spines among these groups [$F_{(3, 12)} = 16.81$, $P < 0.05$] (Figure 2A). Chronic UCMS exposure significantly decreased the density of dendritic spines within vmPFC regions as compared with that of the non-stressed control group. In contrast, long term pretreatment with ginsenoside Rg1 significantly prevented this UCMS-induced decrease in dendritic spine density. To further corroborate these Golgi Staining results, a transmission electron microscopy evaluation of vmPFC neuronal ultrastructure was also performed. Similar to that observed with Golgi Staining, there was an overall statistically significant difference in vmPFC synaptic number density among these groups [$F_{(3, 12)} = 17.29$, $P < 0.05$]. As shown in Figure 2B, after 5 weeks of UCMS exposure, synapse numbers in the vmPFC were significantly reduced, while chronic pretreatment with ginsenoside Rg1 ameliorated these ultrastructural abnormalities, as indicated by significant increases in synapse numbers within the vmPFC of UCMS-exposed rats receiving ginsenoside Rg1. No significant differences were observed between vehicle-treated UCMS-exposed rats and UCMS-exposed rats with regard to dendritic spine and synapse numbers within the vmPFC ($p > 0.05$). Accordingly, these results demonstrate that depression is accompanied with a disruption in structural plasticity within vmPFC neurons and that the amelioration of this structural plasticity suggests a potential neuroprotective effect of ginsenoside Rg1which may then be related to its antidepressant-like effects.

3.3. UCMS induced overexpression of miR-134 and dysregulation of

plasticity-associated signaling was ameliorated by ginsenoside Rg1 treatment

As miR-134 is a potential modulator of synaptic plasticity and we found that ginsenoside Rg1 ameliorated changes in structural plasticity within vmPFC neurons, we next examined whether ginsenoside Rg1 would regulate miR-134 expression within the vmPFC of UCMS-exposed rats. Analysis of miR-134 expression levels within the vmPFC revealed that an overall statistically significant difference was obtained among these groups [$F_{(3, 20)} = 19.17$, $P < 0.05$] (Fig 3A). Specifically, post-hoc analysis showed that chronic UCMS exposure significantly increased miR-134 expression within the vmPFC as compared with that observed in non-stressed rats, while chronic pre-administration of ginsenoside Rg1 significantly decreased vmPFC expression of miR-134 in UCMS-exposed rats. There were no significant differences between vehicle-treated UCMS-exposed rats and UCMS-exposed rats in miR-134 expression within the vmPFC ($p > 0.05$). These results suggest that the miR-134 pathway may participate in the antidepressant effects of ginsenoside Rg1 as assessed in these UCMS-exposed rats.

MiR-134 is mainly located in synaptic sites on dendrites and is involved in regulating dendritic spine morphology (Schratt et al. 2006). To further explore whether chronic stress would exert effects on synaptic-associated proteins, we investigated the activity of Limk1 pathways within the vmPFC of the four treatment groups. Limk1 is believed to be the major neuronal cofilin kinase (George et al. 2015). Expression levels of Limk1 within the vmPFC were significantly different among the

four groups [$F_{(3, 20)} = 15.17$, $P < 0.05$] (Figure 3B). Post-hoc analysis revealed that 5-weeks of UCMS exposure significantly reduced the expression of Limk1 protein in the vmPFC, while these protein levels were significantly increased in UCMS rats receiving a chronic pre-administration of ginsenoside Rg1.

Statistical analysis of cofilin activity within the vmPFC also showed an overall significant difference among the four groups with regard to phosphorylation levels of cofilin [$F_{(3, 20)} = 16.39$, $P < 0.05$] (Figure 3C). Post-hoc analysis indicated that cofilin phosphorylation levels in the vmPFC were significantly reduced in the UCMS-exposed groups, while these levels remained significantly increased in UCMS rats receiving ginsenoside Rg1 pretreatment. No significant differences between vehicle-treated UCMS-exposed and UCMS-exposed groups were observed in the activity of these two synaptic-related proteins within the vmPFC ($p > 0.05$). These results provide evidence indicating the possible involvement of the Limk1/cofilin signaling pathway within the vmPFC as contributing to the antidepressant-like effects of ginsenoside Rg1 in this UCMS animal model.

3.4. MiR-134 modulates neuronal structural plasticity and depression-like behaviors through the plasticity-associated cascade of Limk1/cofilin signaling

To continue with these investigations, the AAV-miR-134 virus was infused bilaterally into the vmPFC of non-stressed rats to overexpress the miR-134, and thus examine whether UCMS-induced dysregulation of neuronal plasticity and depression-like

behaviors may be mediated by the vmPFC miR-134 signaling pathway (Figure 4A). Behavioral testing of depression was then conducted at 10-days post-infusion (Figure 4B). An overall statistically significant difference was obtained for these behaviors related to depression [sucrose preference test: $F_{(2, 33)} = 16.87$, $P < 0.05$; forced swim test: $F_{(2, 33)} = 14.52$, $P < 0.05$; open field test: $F_{(2, 33)} = 11.79$, $P < 0.05$] (Figure 4C). Post-hoc analysis revealed that an overexpression of miR-134 within the vmPFC of unstressed rats produced significant increases in immobility times in the forced swim test and resulted in anhedonia in the sucrose preference test as compared with rats receiving a control injection (green fluorescent protein (GFP)-Cre construct). These results suggest that the overexpression of miR-134 is required to produce depression-like behaviors. In addition, we found that miR-134 overexpression also produced an overall statistically significant decrease in the expression of Limk1 [$F_{(2, 33)} = 17.25$, $P < 0.05$] and the phosphorylation of cofilin [$F_{(2, 33)} = 15.93$, $P < 0.05$] (Figure 4D), as well as in the density of dendritic spines [$F_{(2, 15)} = 18.26$, $P < 0.05$] and synapses [$F_{(2, 15)} = 17.03$, $P < 0.05$] (Figure 4E).

To determine whether a down-regulation of miR-134 or blockade of miR-134 function within the vmPFC reversed the display of these depression phenotypes, we expressed a small interference RNA sponge sequence form of miR-134 in the AAV virus (AAV-miR-134-sponge) (Figure 5A) and targeted its expression to the vmPFC of stressed rats by bilateral stereotaxic infusion into this site to block miR-134 function (Figure 5B). The results showed that in stressed rats infused with the AAV-miR-134-sponge to block miR-134 function within the vmPFC, significant

reductions were observed in depression-like behavioral responses [sucrose preference test: $F_{(3, 44)} = 15.28$, $P < 0.05$; forced swim test: $F_{(3, 44)} = 12.18$, $P < 0.05$; open field test: $F_{(3, 44)} = 14.23$, $P < 0.05$] (Figure 5C), neuronal structural plasticity [spine: $F_{(3, 20)} = 17.21$, $P < 0.05$; synapse: $F_{(3, 20)} = 15.93$, $P < 0.05$] (Figure 5D) and biochemical markers associated with UCMS-induced depression [Limk1: $F_{(3, 20)} = 14.27$, $P < 0.05$; p-cofilin: $F_{(3, 20)} = 17.61$, $P < 0.05$] (Figure 5E). Taken together, these results indicate that Limk1/cofilin may be a critical downstream molecular target for miR-134 in mediating neuronal structural plasticity and behavioral responses related to depression.

4. Discussion

Although emerging evidence has demonstrated that depression is associated with structural and functional neuroplasticity in specific brain areas, the underlying molecular mechanisms have yet to be clarified. This gap in our understanding seriously hinders the development of effective therapeutic tools for the treatment of depression. In the present study we demonstrate that: 1) chronic stress produces depression-like behaviors in rats, 2) ginsenoside Rg1 exerts antidepressant-like effects in this animal model of depression, 3) the amelioration of these depression-like behavioral responses are paralleled with neuronal structural changes within the vmPFC and 4) chronic treatment with ginsenoside Rg1 also significantly ameliorated neuronal and biochemical alterations caused by chronic stress, including those

associated with specific effects on multiple molecular signals that are crucial for neuroplasticity, such as miR-134, Limk1 and cofilin. Taken together, these findings indicate that the dysregulation of structural plasticity might be involved in the promotion of depression-like behaviors, while ginsenoside Rg1 exhibits antidepressant-like effects via its neuroprotective ability by modulating the plasticity-associated changes within the vmPFC that result from depression.

In the present study, the display of depression-like behaviors in UCMS exposed rats was associated with dramatic changes in neuronal structures within the vmPFC, a key region proposed to be involved with depression. For example, results from the Golgi staining assay showed that 5 weeks of UCMS exposure significantly decreased the density of dendritic spines within vmPFC neurons. Dendritic spines provide the possible contact sites between neurons to achieve synaptic transmission (Caroni et al. 2012; Kasai et al. 2010). The results of our ultrastructural assessment, as achieved using electron microscopy, showed that accompanying this reduction in vmPFC spine density within these UCMS rats were ultrastructural neuronal changes, consisting mainly of decreases in synapse number and post synaptic density (PSD) thickness. Such morphological, and presumably resultant functional, alterations in dendritic spines are observed in a number of neurological and neurodegenerative disorders in human subjects as well as in animal models of these conditions (Blundell et al. 2010; Dorostkar et al. 2015; Lee et al. 2016; Zhang et al. 2016). Findings from a recent study have demonstrated that stress-induced apical dendrite atrophy of pyramidal neurons within the vmPFC correlated with reduced excitatory postsynaptic currents to

serotonin application (Liu and Aghajanian, 2008). Taken together, these latter findings along with our current results suggest that structural changes in neurons, localized to specific brain regions, may be involved in the pathophysiology of depression. In specific, our current results show that the antidepressant-like effects of ginsenoside Rg1 observed upon behavioral responses in this UCMS-induced depression model were accompanied with a significant amelioration of neuronal structural changes within the vmPFC. A number of neuroprotective effects of ginsenoside Rg1 have been shown upon some neurodegenerative diseases (Zhang et al. 2012; Song et al. 2013). Here, we now show that this neuroprotective function of ginsenoside Rg1 extends to modulating vmPFC spine and synapse density within an animal model of depression, suggesting that this ability to regulate structural plasticity of neurons within this site might prove to be a novel tool for use in the treatment of depression.

In recent years, mounting evidence has been presented demonstrating that miRNAs play an important role in the pathophysiology of stress-related disorders (Im and Kenny 2012; Dwivedi 2014; Geaghan and Cairns 2015; Issler and Chen 2015; Kocerha et al. 2015). The effects of external stressors and antidepressant drugs can alter the expression levels of miRNAs in the prefrontal cortex, amygdala, nucleus accumbens, locus coeruleus, and other brain regions associated with mood regulation (Baudry et al. 2010; Uchida et al. 2010; Dias et al. 2014; Issler et al. 2014). One such miRNA, miR-134 is thought to regulate a distinct set of target genes such as Limk1 and CREB mRNA translation locally within dendrites, thus controling dendritic spine morphology and the development or plasticity of synapses (Schratt et

al. 2006; Gao et al. 2010; Roberto et al. 2014). To examine one potential molecular signaling pathway underlying the dysregulation of structural plasticity in vmPFC neurons induced by chronic stress, we measured the expression of the dendritically localized miR-134. The UCMS exposure used in the present study lead to a significant increase in the expression of miR-134 levels within the vmPFC, while long-term pretreatment with ginsenoside Rg1 significantly ameliorated this miR-134 overexpression. As an approach to corroborate these findings, we infused miR-134-expressing viruses into the vmPFC of unstressed wide type rats to overexpress miR-134 and examined the resultant effects on neuronal structure and animal behaviors. The results of this experiment showed that overexpression of miR-134 within the vmPFC of unstressed rats produced a significant dysregulation in structural plasticity within these vmPFC neurons along with the display of depression-like behaviors. In contrast, down-regulation of miR-134 expression or blocking of miR-134 functions within the vmPFC of stressed rats significantly reversed these depression phenotypes in these UCMS-exposed rats. In this way, the present study revealed a crucial role for the miR-134 pathway in regulating dendritic and synaptic plasticity in response to UCMS exposure. Moreover, these findings raise the question as to the nature of downstream molecular targets of miR-134 in mediating the vmPFC neuroplasticity dysregulation in depression. This signal is considered an important factor in regulating the expression of the synaptic proteins, Limk1 and cofilin, which can then control dendritic spine density (Schratt et al. 2006). Actin filaments represent essential cytoskeleton components of synapses whose

rearrangement by actin-binding proteins is essential for regulating synaptic plasticity (Lei et al. 2016). Cofilin is a critical regulator which cooperates with many other actin-binding proteins to remodel the actin cytoskeleton and thus produce the responses required for modulating the shape and volume of dendritic spines (Zhou et al. 2004; Bamburg et al. 2016). LIM kinases are considered to be the major neuronal cofilin kinase (George et al. 2015). Inactivation of Limk1 inhibits cofilin phosphorylation and thus increases cofilin dimerization activity in dendritic spines (Soosairajah et al. 2005). Therefore, activation of cofilin may lead to a loss in dendritic complexity and spine density which can then affect synapse function (Liu et al. 2016). The present results indicate that overexpression of miR-134 within the vmPFC decreased the expression of Limk1, an effect which was paralleled with a dephosphorylation of cofilin. These findings suggest that activated cofilin possess the capacity to sever actin filaments, thereby decreasing spine size and density. Taken together, these results suggest the hypothesis that chronic stress-induced overexpression of miR-134 regulates the expression and/or activity of actin-binding proteins. As a result, these alterations have the potential of modulating neuronal structures within specific brain regions to promote the development of neuronal dysfunctions.

The next important issue to address was that of the mechanisms through which stress stimuli and antidepressants lead to bidirectional changes in miR-134 levels in the vmPFC region. Previous studies have implicated that changes in miR-134 were related to synaptic plasticity and cognitive deficits (Liu et al. 2017). Thus, together

with the results in this present study, it is tempting to speculate that miR-134 levels could be modulated by chronic stress exposure. With the onset of depression, prolonged stress stimuli may result in an up-regulation of miR-134 levels. Such an effect can produce a down-regulation of Limk1/cofilin expression and phosphorylation, which would then eventually lead to an alteration in neuronal structures within the vmPFC. Conversely, antidepressant suppression of miR-134 may up-regulate the activity of the Limk1 pathway, which can ameliorate this dysregulation of structural plasticity. Results from previous studies have demonstrated that chronic stress impairs rodent executive functions mediated by the vmPFC, such as working memory (Mizoguchi et al. 2000), behavioral flexibility (Cerqueira et al. 2007) and decision making (Dias-Ferreira et al. 2009). It has also been reported that local pharmacological inactivation of vmPFC neurons significantly delayed the collection of earned sucrose pellets, suggesting that the vmPFC signaling pathway facilitates activities related to reward (Burgos-Robles et al. 2013). Hence, we suggest that the structural plasticity observed, as indicated by changes in the densities of synaptic spines, may change vmPFC neuronal activity. Such effects have repercussions in the formation of perception-action processes, and thus to eventually drive maladaptive behavioral changes toward emotionally disorder states. In support of this idea are the findings of the present study which demonstrate that ginsenoside Rg1 ameliorated both spine and synapse density decreases imposed by miR-134 overexpression. As miR-134 can regulate synaptic plasticity and depression-like behaviors via a Limk1-mediated mechanism and we showed that ginsenoside Rg1

could downregulate miR-134, we next examined whether ginsenoside Rg1 could regulate the Limk1/cofilin pathway. The results of this experiment revealed that a daily ginsenoside Rg1 regimen of 40 mg/kg significantly decreased the expressions of both miR-134 and Limk1 as well as cofilin phosphorylation within the vmPFC. As miR-134 knockdown has been shown to regulate synaptic plasticity and thus improve memory deficits in mice (Gao et al. 2010), it seems likely that this miR-134/Limk1 pathway may also participate in the ameliorative effects of ginsenoside Rg1 on structural plasticity and depression-like behaviors in our UCMS rats. Taken together, these results provide strong evidence in support of the hypothesis that the antidepressant mechanisms of ginsenoside Rg1 may result from its neuroprotective effects by regulating structural plasticity within localized neuronal sites. However, detailed molecular mechanisms regarding the means through which stress may regulate miR-134 expression await further investigation.

It should be pointed out that the present study mainly investigated the modulation effects of miR-134 on the UCMS-induced behavioral and neurobiological changes in male rats. However, whether this mechanism was applied equally to female rats have not been fully identified on account of the female rats usually undergo the effects of estrous cycle. Previous studies have been reported that estrogen influenced neurotransmitter systems and mood regulatory systems in women via interacting with life stress (Newhouse and Albert 2015). Therefore, the potential molecular mechanism of the sex hormone effects on depression would be achieved further investigation in the future studies.

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In conclusion, we identified miR-134 as being a key molecular determinant of vmPFC neuroplasticity and behavioral depression in an animal model of depression. In specific, we report that chronic exposure to stress induced an overexpression of miR-134 within the vmPFC and a subsequent resultant dysregulation in the structural plasticity via the Limk1/cofilin pathway in these neurons. Moreover, each of these effects, as well as the behavioral depression resulting from chronic stress exposure, was ameliorated by ginsenoside Rg1 treatment. Therefore, the present study not only clarifies some of the potential mechanisms underlying chronic stress-associated alterations in neuroplasticity, but also provides support for the potential use of ginsenoside Rg1 treatment strategies against stress-related mental disorders.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure legends:

Figure 1. Effects of ginsenoside Rg1 treatment on depression-like behaviors induced by UCMS exposure. (A) Experimental design: schematic figure of the treatment protocol. (B) Effects of ginsenoside Rg1 on the percent of sucrose consumption. Ginsenoside Rg1 (40 mg/kg) increased the consumption of sucrose solution in UCMS-exposed rats in the sucrose preference test. (C) Effects of ginsenoside Rg1 on immobility times of rats in the forced swim test. Ginsenoside Rg1 decreased immobility times and increased swimming times in UCMS-treated rats in this test. (D) Effects of ginsenoside Rg1 on locomotor activities in the open-field test. Each column represents the number of horizontal (white, crossings) or vertical (gray, rearing) exploratory activities as measured in a 5-min period. All values are presented as means ± SEM (N=12). * $P < 0.05$ as compared with the control group (non-stressed). $^{\#}P < 0.05$ as compared to the UCMS-exposed group. (G-Rg1: Ginsenoside Rg1)

Figure 2. Ginsenoside Rg1 prevented the decreases in dendritic spine and synapse density within the vmPFC neurons as induced by UCMS exposure. (A) Representative images of Golgi staining from vmPFC regions. A view of a dendritic segment is shown in the top of the picture. Scale bar is 10μm. Bar graph illustrating the mean spine density of individual dendrites within the vmPFC region of rats. At least 12 dendrites from neurons of 4 rats per group were analyzed. (B) Electron micrograph of vmPFC neuronal ultrastructure. Arrows indicate spine synapses. Scale bar is 0.5 μm. Bar graph illustrating the mean synapse density of individual neurons

within the vmPFC region. At least 30 micrographs from neurons of 4 rats per group were analyzed. Error bars show SEM (N=4). * $P < 0.05$ as compared with the control group (non-stressed). $^{\#}P < 0.05$ as compared to the UCMS-exposed group. (G-Rg1: Ginsenoside Rg1)

Figure 3. Ginsenoside Rg1ameliorated the dysregulation of miR-134/Limk1/cofilin activity within the vmPFC as induced by UCMS exposure. (A) Ginsenoside Rg1 reduced miR-134 overexpression within the vmPFC as induced by UCMS exposure. Band 1: control group; Band 2: UCMS group; Band 3: ginsenoside Rg1 (40 mg/kg) + UCMS group; Band 4: vehicle (0.9% physiological saline, 10ml/kg) + UCMS group. Relative quantity of miR-134 expression versus U6 expression in each group are presented as the means ± SEM (N=12). (B) Ginsenoside Rg1 increased Limk1 protein expression in the vmPFC induced by UCMS exposure. Band 1: control group; Band 2: UCMS group; Band 3: ginsenoside Rg1 (40 mg/kg) + UCMS group; Band 4: vehicle (0.9% physiological saline, 10ml/kg) + UCMS group. Normalized intensity bands of Limk1 are presented as the means ± SEM (N=12). (C) Ginsenoside Rg1 increased cofilin phosphorylation levels within the vmPFC as induced by UCMS exposure. Band 1: control group; Band 2: UCMS group; Band 3: ginsenoside Rg1 (40 mg/kg) + UCMS group; Band 4: vehicle (0.9% physiological saline, 10ml/kg) + UCMS group. Phosphorylated-cofilin levels were normalized to their corresponding total cofilin levels and expressed as the means ± SEM (N=12). *$P < 0.05$ as compared with the control group (non-stressed). $^{\#}P < 0.05$ as compared to the UCMS-exposed group.

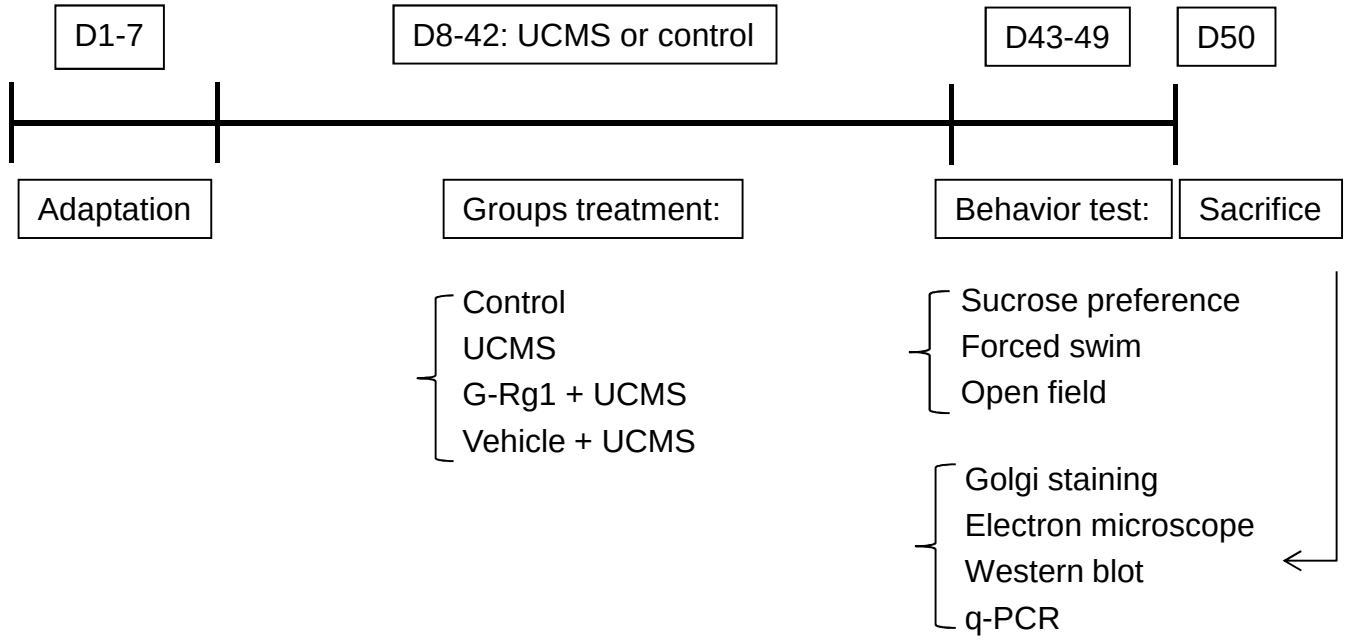
(G-Rg1: Ginsenoside Rg1)

Figure 4. Overexpression of miR-134 within the vmPFC leads to depression-like phenotypes in normal rats. (A) Top: Schematics of AAV vectors engineered to overexpress a control construct or miR-134. ITR, inverted terminal repeats; CAG, ZsGreen promoter; U6, miR-134 promoter. Bottom: Experimental paradigm for behavioral testing of rats infected by the virus. (B) Illustration of viral infusion of AAV-miR-134 into the vmPFC. PrL, prelimbic cortex; IL, infralimbic cortex. Scale bar is 100μm. (C) Behavioral effects of expressing viral AAV-miR-134 in the vmPFC. (D) Dendritic spine and synapse density in vmPFC neurons infected with AAV-miR-134. (E) Limk1 and cofilin expression levels in vmPFC infected with AAV-miR-134. Spine and synapse analysis: N= 4. For all the other analyses, N=12 in all groups. * $P < 0.05$ as compared with the non-infused control group. All values are presented as the means ± SEM.

Figure 5. Blocking of miR-134 within the vmPFC reduced the display of depression-like phenotypes of stressed rats. In these experiments, prior to AAV-miR-134-sponge infusion, rats were exposed to a 5-week period of UCMS to induce a depression-like phenotype. (A) Top: Schematics of AAV vectors engineered to overexpress a control construct or miR-134-sponge. ITR, inverted terminal repeats; CAG, ZsGreen promoter; U6, miR-134-sponge promoter. Bottom: Experimental paradigm for behavioral testing of rats infected by the virus. (B) Illustration of viral

infusion of AAV-miR-134-sponge into the vmPFC. Scale bar is 100µm. (C) Behavioral effects of expressing AAV-miR-134-sponge within the vmPFC of stressed rats. (D) Dendritic spine and synapse density in vvmPFC neurons infected with the AAV-miR-134-sponge. (E) Limk1 and cofilin expression levels in the vmPFC infected with the AAV-miR-134-sponge. Spine and synapse analysis: N= 4. For all the other analyses, N=12 in all groups. * $P < 0.05$ as compared with the non-infused stressed group. All values are presented as the means ± SEM. WT, wide type; S, stressed.

A



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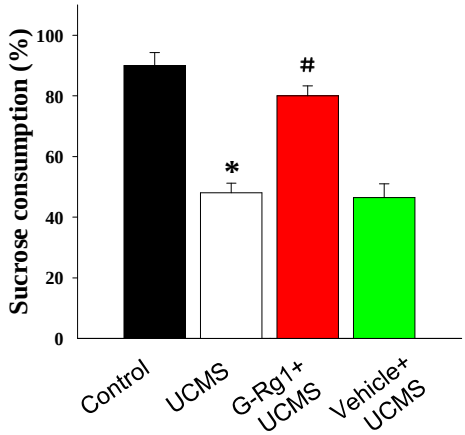

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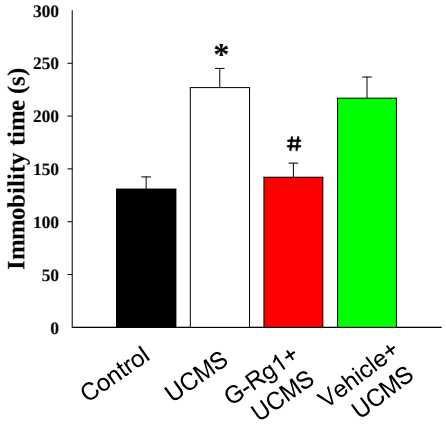

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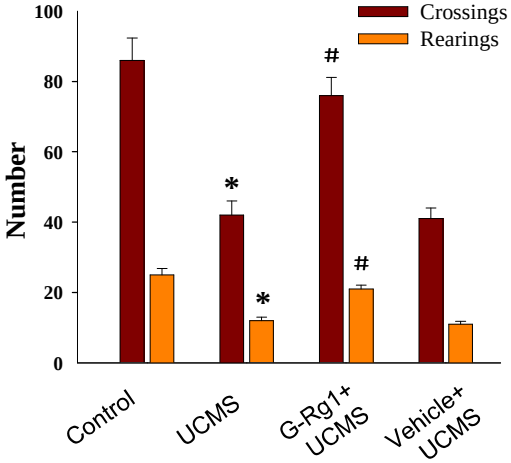

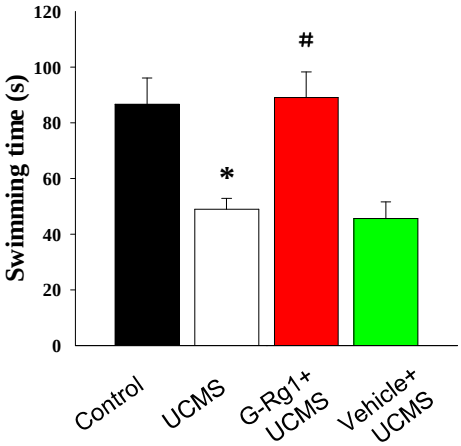

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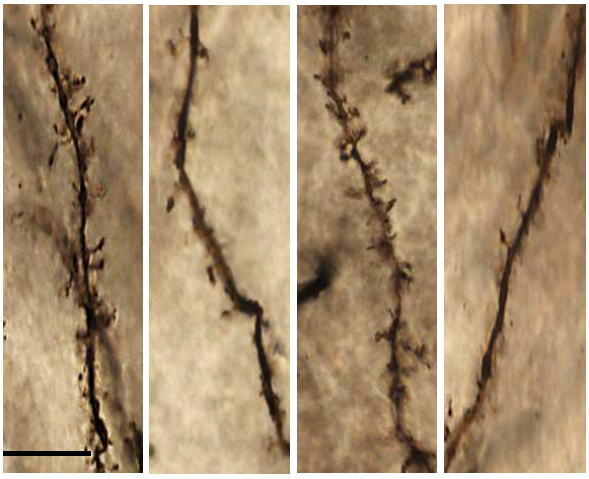
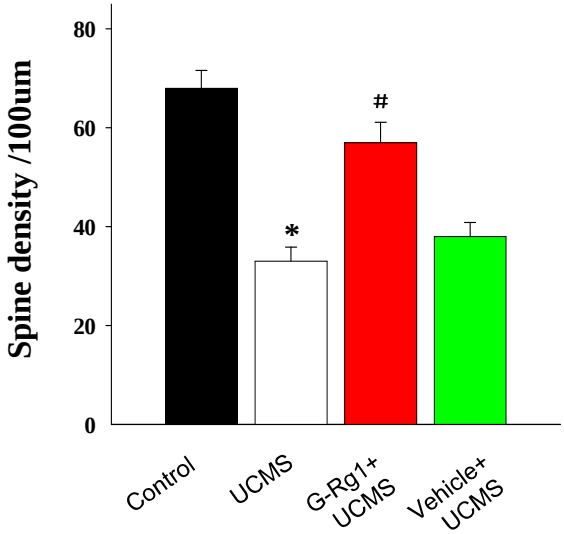
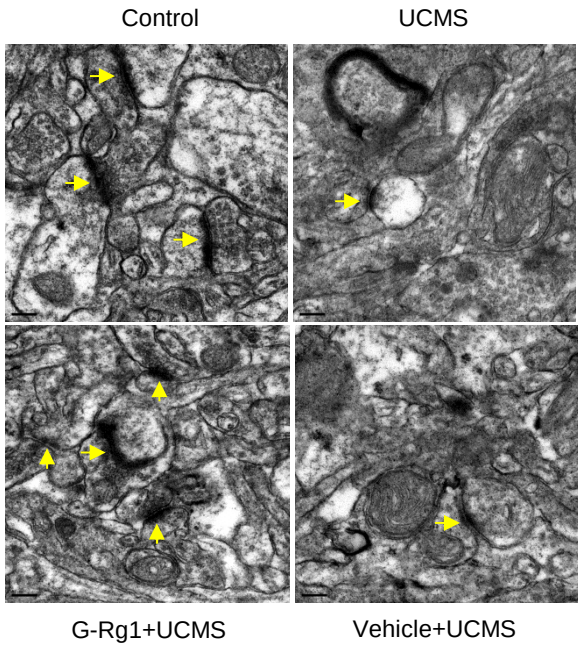
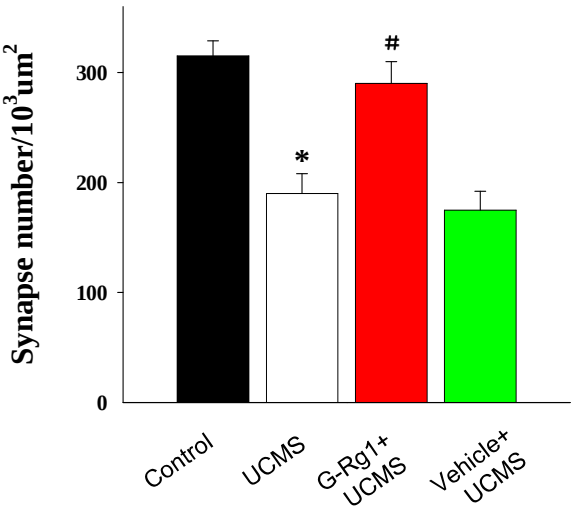
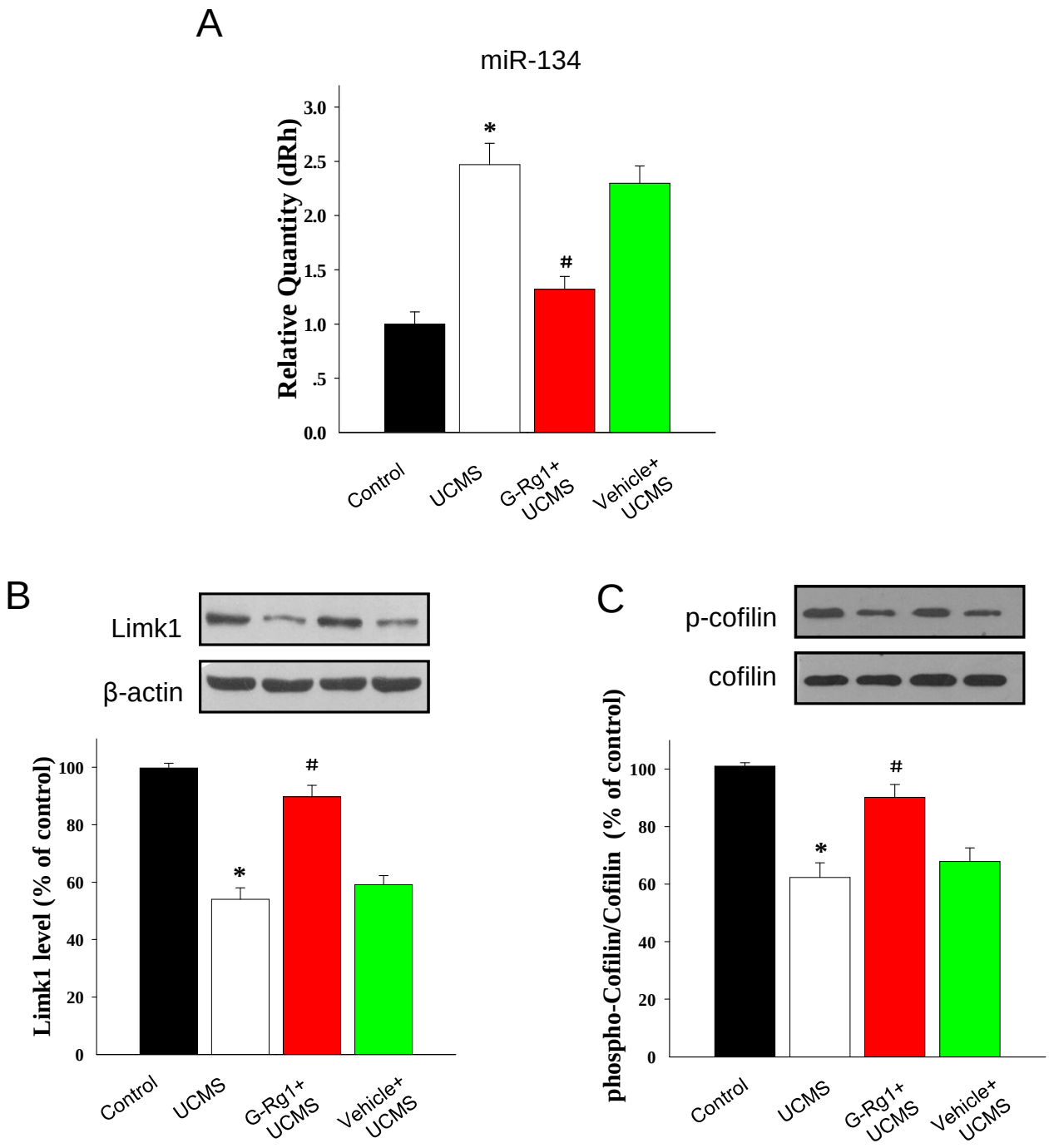

Control
UCMS
G-Rg1+ UCMS
Vehicle+ UCMS

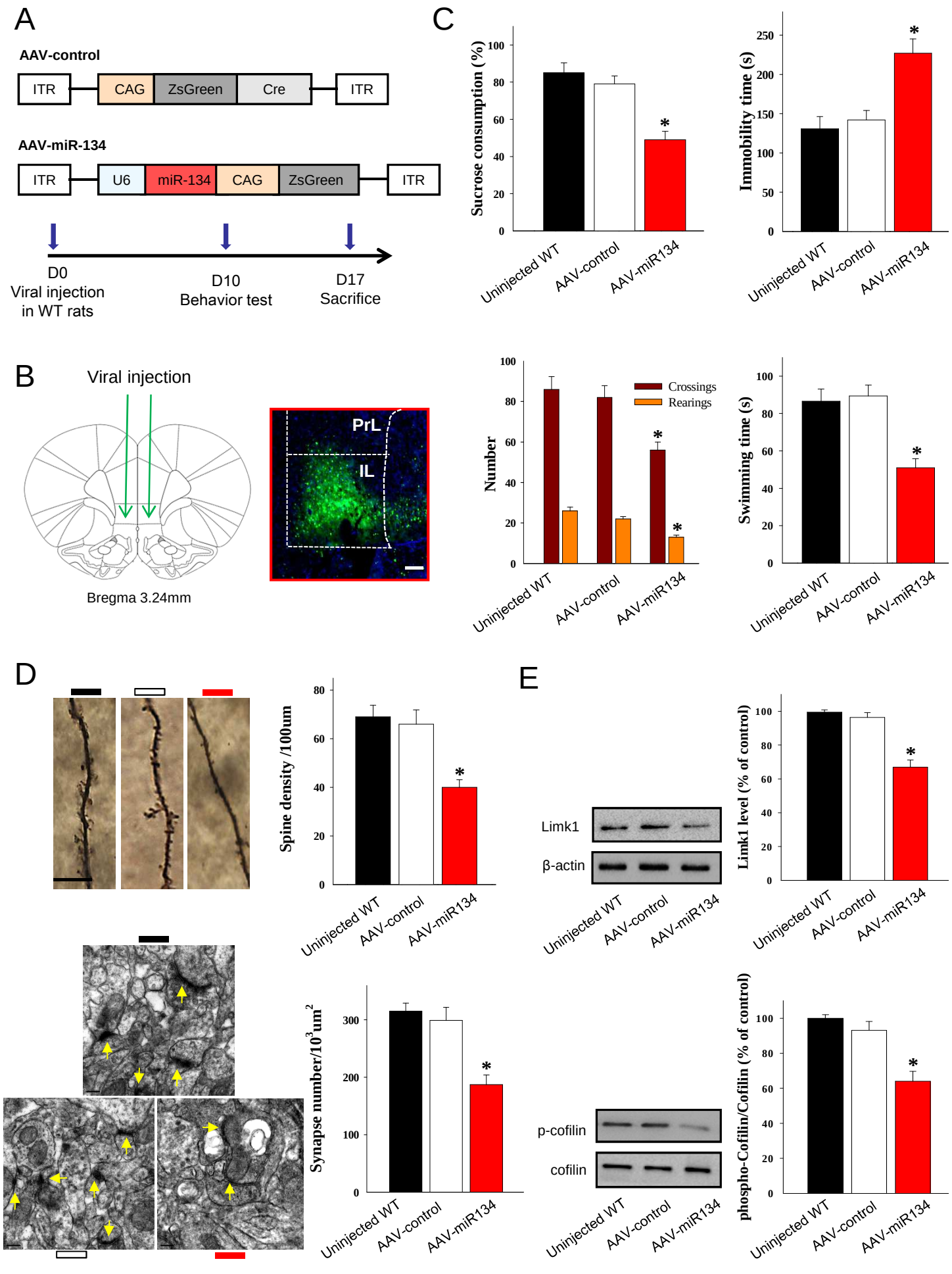

Spine density /100um
80
60
40
20
0
*
#
Control
UCMS
G-Rg1+ UCMS
Vehicle+ UCMS

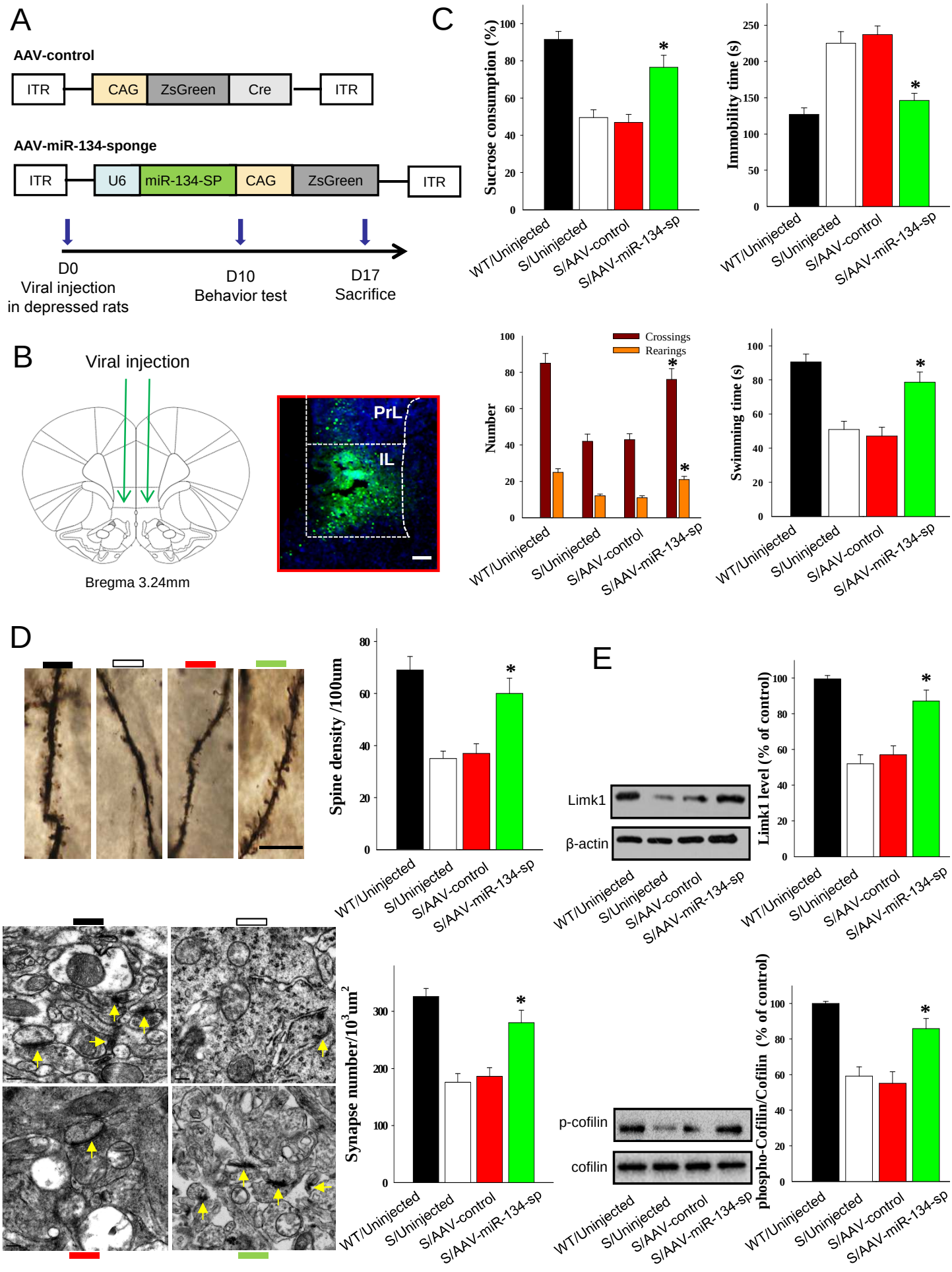
B


Control
UCMS
G-Rg1+UCMS
Vehicle+UCMS


Synapse number/10^3um^2
300
200
100
0
*
#
Control
UCMS
G-Rg1+ UCMS
Vehicle+ UCMS


A
miR-134
Relative Quantity (dRh)
3.0
2.5
2.0
1.5
1.0
.5
0.0
*
#
Control
UCMS
G-Rg1+ UCMS
Vehicle+ UCMS
B
Limk1
β-actin
Limk1 level (% of control)
100
80
60
40
20
0
*
#
Control
UCMS
G-Rg1+ UCMS
Vehicle+ UCMS
C
p-cofilin
cofilin
phospho-Cofilin/Cofilin (% of control)
100
80
60
40
20
0
*
#
Control
UCMS
G-Rg1+ UCMS
Vehicle+ UCMS





Research Highlights

1. UCMS induced depression-like behavior accompanied with neuronal structural changes in mPFC.
2. UCMS induced miR-134 overexpression and Limk1/cofilin downregulation in mPFC.
3. MiR-134 modulates the structural neuroplasticity via Limk1/cofiling signaling pathway in mPFC.
4. Ginsenoside Rg1 ameliorated behavioral and neuronal structural changes induced by UCMS.
5. Ginsenoside Rg1 ameliorated the dysregulation of miR-134-Limk1/cofilin signaling induced by UCMS.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.