Cerebralcare Granule® attenuates cognitive impairment in rats continuously overexpressing microRNA-30e

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Abstract. Previous studies have demonstrated that dysregulation of micro (mi)RNAs is associated with the etiology of various neuropsychiatric disorders, including depression and schizophrenia. Cerebralcare Granule® (CG) is a Chinese herbal medicine, which has been reported to have an ameliorative effect on brain injury by attenuating blood-brain barrier disruption and improving hippocampal neural function. The present study aimed to evaluate the cognitive behavior of rats continuously overexpressing miRNA-30e (lenti-miRNA-30e), prior to and following the administration of CG. In addition, the mechanisms underlying the ameliorative effects of CG were investigated. The cognitive ability of the rats was assessed using an open-field test and a Morris water maze spatial reference/working memory test. A terminal deoxynucleotidyl transferase dUTP nick end labeling assay was used to detect neuronal apoptosis in the dentate gyrus of the hippocampus. Immunohistochemical analysis and western blotting were conducted to detect the expression levels of B-cell lymphoma 2 (BCL-2) and ubiquitin-conjugating enzyme 9 (UBC9), in order to examine neuronal apoptosis. The lenti-miRNA-30e rats exhibited increased signs of anxiety, depression, hyperactivity and schizophrenia, which resulted in a severe impairment in cognitive ability. Furthermore, in the dentate gyrus of these rats, the expression levels of BCL-2 and UBC9 were reduced and apoptosis was increased. The administration of CG alleviated cognitive impairment, enhanced the expression levels of BCL-2 and UBC9, and reduced apoptosis in the dentate gyrus in the lenti-miRNA-30e rats. No significant differences were detected in behavioral indicators between the lenti-miRNA-30e rats treated with CG and the normal controls. These findings suggested that CG exerts a potent therapeutic effect, conferred by its ability to enhance the expression levels of BCL-2 and UBC9, which inhibits the apoptotic process in neuronal cells. Therefore, CG may be considered a potential therapeutic strategy for the treatment of cognitive impairment in mental disorders.

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Introduction

Gene-environment interactions have been demonstrated to contribute to the etiology of various mental disorders (1,2). Although the genetic risk factors for different mental disorders have previously been considered to be distinct, studies have demonstrated that five major mental disorders: Autism spectrum disorder, attention deficit-hyperactivity disorder, bipolar disorder, major depressive disorder and schizophrenia, share markedly overlapping genetic roots (3). Therefore, the identification of candidate genes associated with mental disorders has increased in prominence in modern psychiatric investigations.

MicroRNAs (miRNAs) are short RNA molecules, which negatively regulate the stability and translation of mRNA targets at the post-transcriptional level. miRNAs regulate target mRNA expression by binding to the 3'-untranslated region through base pairing, resulting in target mRNA cleavage or translational inhibition (4-6). miRNAs are regarded as master regulators of gene expression, in that a single miRNA may regulate several hundred genes, and collectively miRNAs may regulate as much as two thirds of the transcriptome (7). Notably, miRNAs regulate mRNA translation locally in the axosomal and synaptodendritic compartments, thereby contributing to the dynamic spatial organization of axonal and dendritic structures and their functions, and consequently, synaptic and neural plasticity (8). Increasing evidence has suggested that the dysregulation of miRNAs is associated with the etiopathology of various neuropsychiatric disorders (9-13). In particular, miRNAs have been found to be associated with

intellectual disabilities, including cognitive impairment and cognitive retardation (14).

Although the etiopathology of mental disorders remains unclear, disruptions across whole cellular networks and the dysregulation of multiple signaling pathways, particularly those contributing to synaptic plasticity, likely contribute to pathogenesis. These disruptions are likely lead to aberrant information processing in the circuits that regulate mood, cognition and neurovegetative function, including sleep, appetite and energy (15,16). As miRNAs are able to suppress the translation of hundreds of target mRNAs, they are well-positioned to target numerous cellular processes; therefore, therapeutic strategies capable of affecting changes in cellular plasticity to restore synaptic function and neuronal connectivity hold significant potential (13). Increasing evidence has implicated miR-30e in a number of diseases, including heart failure, neoplasms, lymphoma, melanoma, mesothelioma, aortic aneurysm, glioma, obesity, periodontal diseases, lung diseases and, most notably, schizophrenia (17-19). miR-30e regulates its target gene, ubiquitin-conjugating enzyme 9 (UBC9), in order to inhibit cell growth in carcinoma (20,21). In addition, miR-30e has been identified as an immediate target activated by the β-catenin/transcription factor 4 complex during intestinal cell differentiation (22). Through bioinformatic analysis, our previous study demonstrated that miR-30e may target a functional network, comprising 10 interrelated genes, to regulate their levels of expression in schizophrenia (19). Downregulation in the expression of miR-30e has been demonstrated to promote neuronal survival in long-lived calorie-restricted mice, suggesting that it may regulate neuronal death (23). Our previous investigations revealed that the expression of miR-30e is increased in the peripheral blood leukocytes of patients with schizophrenia (19), which was consistent with the findings of other previous studies that observed abnormal expression levels of miR-30e in the brain and peripheral blood leukocytes of subjects with schizophrenia (24-26). Rege *et al* (27) demonstrated that miR-30e is upregulated in the frontal cortex and striatum of certain neurodegenerative disorders, including Huntington's disease. Furthermore, Banigan *et al* (28) revealed that exosomal miR-30e-5p is significantly differentially expressed in patients with schizophrenia and bipolar disorder.

Cerebralcare Granule® (CG; Tasly Pharmaceutical Co., Ltd., Tianjin, China) is a Chinese herbal medicine compound, which is used for the treatment of cerebrovascular diseases. Compounds identified in CG include rhynchophylline, genistein, ursolic acid, 2-alpha-hydroxyursolic acid, naphthopyrones, alaternin, ferulic acid, ligustrazine, L-tetrahydropalmatine, peoniflorin, rehmannioside and methyleugenol, among which a substantial proportion exhibit antioxidant properties (29). Wang *et al* (30) identified six active components in rat plasma following oral administration of CG: Protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, rosmarinic acid and paeoniflorin, using liquid chromatography-tandem mass spectrometry and pharmacokinetics. CG inhibits the production of superoxide in the cerebral venular endothelium and reduces albumin leakage across venules, as well as attenuating bilateral common carotid artery occlusion-elicited cerebral microcirculatory disturbance, hippocampal neuron injury and blood-brain barrier disruption. In addition, CG protects the brain from edema following ischemia and reperfusion injury (29,30-33), suggesting that it may alleviate cognitive impairment induced by chronic cerebral hypoperfusion.

The present study aimed to investigate the effects of CG on behavioral impairment in rats induced to continuously overexpress miR-30e. Furthermore, the present study aimed to investigate the molecular and cellular mechanisms underlying the therapeutic effects of CG.

Materials and methods

Recombinant lentiviral vector production and verification. Lentiviral vectors are useful for *in vivo* investigations of the central nervous system (CNS), as they are capable of maintaining expression for the life of an animal, when injected into the brain (34-36). In addition, as lentiviral vectors are replication-deficient and do not leave the site of injection, they stably and safely deliver the target gene into the CNS (37). In the present study, vectors containing the target nucleotide sequence were constructed. Rat miR-30e cDNA was amplified by polymerase chain reaction (PCR) from the miR-30e-pLVX-IRES-Zs Green1 vector (Shanghai SBO Medical Biotechnology Co., Ltd., Shanghai, China) using a Biosafer 9703 (Shenzhen Safer Science and Technology Co., Ltd., Shenzhen, China). The following oligonucleotide primers (Invitrogen Life Technologies, Carlsbad, CA, USA) were used for the PCR isolation of miR-30e: Forward 5'-CAACAGAAGGCTCGAGCTGTTGGAGAAGTGGGCATC-3' and reverse 5'-ATTCTGATCAGGATCCCTCCAAACGAAGAGAGACAGTC-3', which carried restriction sites for *Bam*HI and *Xho*I, respectively. The PCR cycling conditions were as follows: 98˚C for 3 min, followed by 30 cycles at 98˚C for 10 sec, 55˚C for 15 sec and 72˚C for 30 sec, and a final extension at 72˚C for 10 min. The products were subsequently stored at 4˚C. The virus was generated by transient co-transfection of the expression plasmid (10 μg), the pseudotyping construct PMD2 G (10 μg; Shanghai SBO Medical Biotechnology Co., Ltd.) and the packaging construct psPAX2 (10 μg; Shanghai SBO Medical Biotechnology Co., Ltd.) in a 75-mm plate containing 90% confluent 293T cells (ATCC, Manassas, VA, USA), as previously described [Naldini *et al* (36); Rattiner *et al* (38) and Heldt *et al* (39)]. Following transfection for 12 h at 37˚C, the medium was discarded and 10% Dulbecco's modified Eagle's medium was added (Invitrogen Life Technologies). The medium was collected 48 and 72 h post-transfection, cleared of debris by low-speed centrifugation at 4,378 x g for 30 min at 4˚C, and filtered through 0.45-μm filters. High-titer stocks were prepared by initial ultracentrifugation for 1 h at 296,000 x g at 4˚C, and a secondary centrifugation at 96,360 x g for 30 min at 4˚C. The viral pellets were resuspended in 1% bovine serum albumin in phosphate-buffered saline and stored at -80˚C. Green fluorescent protein (GFP)-positive cells (pLVX-IRES-ZsGreen1 is able to express GFP) were visualized using fluorescence microscopy (Leica Microsystems Canada Inc., Richmond Hill, ON, Canada). The quantity of p24 Gag conical virion capsid was measured using the QuickTiter™ Lentivirus Titer kit (Cell Biolabs, San Diego, CA, USA). Lentiviral particle (LP) titers were assayed based on the manufacturer's control titer of 1 ng p24 = 1.25×10^7 LPs. The yield ranged between 1 and

25 μg/ml p24, corresponding to values between $1.25x10^{10}$ and $0.3x10^{12}$ LPs/ml.

Animals and stereotaxic surgery. The present study was approved by the ethics committee of the Laboratory Animal Facility Biomedical Analysis Center, Tsinghua University (Beijing, China; 100084; 2012-LiuPZ- mir30e). Male Sprague-Dawley (SD) rats (age, 4 weeks; average body weight, 100±10 g) were bred at the Laboratory Animal Facility of the Biomedical Analysis Center of Tsinghua University (Beijing, China). The rats were housed (four animals per cage) with access to food and water *ad libitum*, under a controlled 12-h/12-h light-dark cycle (lights on at 7:00AM) at 22±2˚C and 55±5% humidity. All experimental protocols (permit no. 2012-LiuPZ-mir30e) were approved by the Tsinghua University Laboratory Animal Administration Committee, were performed according to the Tsinghua University Guidelines for Animal Experimentation, and conformed to the Guide for the Care and Use of Laboratory Animals (40) published by Tsinghua University.

The rats were intraperitoneally anesthetized with 10% chloral hydrate (2 mg/kg) and placed in a stereotaxic apparatus (ST-51600; David Kopf Instruments, Tujunga, CA, USA). A 1 μl aliquot of the lentivirus was injected into the hippocampal dentate gyrus (DG) at a rate of 0.2 μl/min (UltramicropumpII; World Precision Instruments, Inc., Sarasota, FL, USA). The injection coordinates relative to the bregma were AP, -3.0; ML, ±2.6 and DV, -3.0 [Paxinos and Franklin (41)] and were based on previously described coordinates. The needle remained in place for an additional 5 min and was then slowly withdrawn. The rats were allowed to recover for 14 days, in order to allow the virus to infect cells at the injection site and begin producing miR-30e or GFP (used as an exogenous marker). The lentivirus expressing GFP was used to control for surgery-induced hippocampal damage, which may lead to abnormal behavior. Theoretically, the lentivirus results in expression of miR-30e or GFP for the remainder of the animal's life.

A total of 40 male SD rats (4-week-old; average body weight, 100±10 g) were randomly divided into five groups, with eight rats in each group: i) Control group; ii) lenti-GFP group; iii) lenti-miR-30e group; iv) lenti-miR-30e plus CG (100 mg/kg, 14 days) group; and v) lenti-miR-30e plus fluoxetine (10 mg/kg, 14 days) group (Fig. 1). The optimal concentration of CG for treatment of the rats was determined in preliminary experiment, and was equivalent to a 6-7% concentration in humans.

Drug administration. The CG used in the present study was produced by Tasly Pharmaceutical Co., Ltd. A 50 mg/ml stock of CG (cat. no. 110306) solution was prepared using sterile water. Appropriate quantities of CG were ground with a pestle and mortar to remove lumps, and the resulting power was mixed with sterile water for injection (50 mg/ml) once a day for 14 days.

Chronic administration was performed by dissolving fluoxetine hydrochloride (10 ml/kg; Sigma-Aldrich, St. Louis, MO, USA) in deionized water and dividing the solution into 14 equal volumes (1 mg/ml), which were used for daily treatment over a 14-day period. The control rats received injections of deionized water.

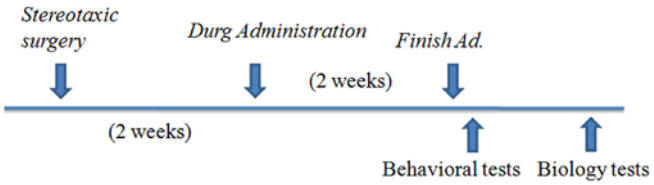

Figure 1. Schematic of the experimental protocol. Ad., administration.

Behavioral testing open field test. The open field test assesses hyperactivity through locomotion and anxious behavior. The open field box used in the present study consisted of a square black box (60x60x25 cm) consisting of plexiglass with an outlined center area. The center area (30x30 cm) was demarcated with Tartan 1710 vinyl electrical tape. Each rat was placed in the box for 10 min. The overall activity of the rat in the box was measured using a videotracker (Noldus version 8.0; ZS Dichuang, Beijing, China), and the duration and distance travelled in the center area of the maze were measured. This paradigm was based on the premise that rodents naturally prefer to be close to a protective wall, rather than being exposed to danger in the central area.

Water maze test. A modified version of the water maze task originally developed by Morris (42) was used in the present study to assess spatial learning and memory. The maze consisted of a circular tank (180 cm in diameter) filled with water at room temperature, which was made opaque by the addition of non-toxic white paint. Extra-maze distal cues were positioned on the walls around the tank. A 15-cm-wide circular platform was fixed to the bottom of the tank and submerged 2 cm below the water surface, in order to remain invisible to the animals.

In the version of the test assessing working memory, the animals were subjected to two trials per day over six consecutive days. In each trial, the rats were released from a different position and had 60 sec to locate the platform and climb onto it. The platform position was changed daily, but remained constant throughout a given day. The rats were allowed an inter-trial interval of 15 sec, during which they remained on the platform. The latency in locating the platform was recorded.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Four weeks following treatment, four animals/group were intraperitoneally injected with 10% chloral hydrate (Tianjin Kermel Chemical Reagent Co., Ltd., Tianjin, China) at 4 ml/kg. The chest was sectioned and opened following anesthesia. A fast injection of normal saline was given through a catheter placed in the left ventricle; at the same time, the right auricle was sectioned and opened. When the liver turned white from bleeding, 4% paraformaldehyde (Sinopharm Chemical Reagent Co., Ltd., Beijing, China) was infused until rigor mortis occurred. The brain was removed and fixed in a solution containing 30% sucrose and 4% paraformaldehyde. Once the brain sunk to the bottom of the solution, 10 μm cryostat sections were prepared using a freezing microtome (CM 1850; Leica Microsystems Inc., Buffalo Grove, IL, USA). Apoptotic cells were identified using a TUNEL assay (*In Situ* Cell Death Detection kit; TMR red; Roche Diagnostics,

Basel, Switzerland), according to the manufacturer's instructions. For nuclear counterstaining, the sections were dyed with DAPI (1:10,000) in PBS with Tween 20 (PBS-T) for 10 min, and then briefly rinsed with PBS-T and PBS. The quantification of TUNEL-positive cells on images of the hippocampal DG sections were performed manually. A minimum of four sections (10 μm apart) were quantified per animal.

Western blotting. The brains were removed from the cranium, the hippocampus was rapidly dissected four weeks following treatment, frozen in liquid nitrogen, and stored at -80˚C prior to further analysis. Extracts for western blot analysis were prepared by homogenizing the tissues in ice-cold extraction buffer (Cancer Type, Beijing, China) consisting of 75 mM β-glycerophosphate, 20 mM MOPS, 15 mM EGTA, 2 mM EDTA, 1 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin (pH 7.2) and then sonicated on ice. Insoluble material was removed by centrifugation at 12,000 x g at 4˚C for 30 min. Protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Total protein (10 μg) was separated by 10% SDS-PAGE (Cancer Type) and transferred onto nitrocellulose membranes (Sartorius AG, Goettingen, Germany). Subsequently, the membranes were incubated with anti-B-cell lymphoma 2 (BCL-2; 1:1,000; mouse monoclonal; cat. no. 15071; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-UBC9 (1:1,000; rabbit monoclonal; cat. no. 47861; Cell Signaling Technology, Inc.), and anti-GAPDH (1:1,000; mouse monoclonal; cat. no. 14433; Cancer Type) primary antibodies at 2-8˚C overnight in 5% non-fat dried milk. The membranes were then incubated with secondary antibodies, including goat anti-mouse for Bcl-2 and GAPDH (cat. no. ZF-0512) and goat anti-rabbit for UBC9 (cat. no. ZF-0511) (1:5,000; Cancer Type) for 2 h at room temperature. The blots were visualized using enhanced chemiluminescence detection, following which images were captured and protein expression levels were quantified using ImageJ software (1.38e/Java 1.5.0_09; National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry. Following removal and fixing of the brains of the rats, 10 μm cryostat sections were prepared and immunohistochemistry was performed using BCL-2 and UBC9 antibodies, according to the manufacturer's instructions. The primary antibody was replaced with normal serum in negative controls.

For quantitative immunohistochemistry, three rats were selected from each group, and one in every four samples was selected from a continuous series of the prepared hippocampal tissue sections, which were processed immunohistochemically. In total, 15 sections were observed under a x20 objective (Leica DM3000; Leica Microsystem, Wetzlar, Germany), and positively stained cells were randomly selected from each section. ImageJ software (National Institute of Health) was used to determine the mean cytoplasmic grey scale and to assess the staining intensity.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Data between two groups were compared using Student's t-test. Results were analyzed using repeated measures analysis of variance (ANOVA), followed by one-way ANOVA and a post-hoc Fisher's protected least significant difference (PLSD) test. The number of TUNEL-positive cells were analyzed using one-way ANOVA, followed by a post-hoc Fisher's PLSD test. Statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Open field test. The lenti-miR-30e rats exhibited a high degree of cognitive impairment, as well as signs of anxiety, hyperactivity, depression and schizophrenia. Administration of fluoxetine significantly improved the cognitive abilities of the experimental rats. Notably, administration of CG exerted a more marked ameliorating effect on cognitive abilities, compared with fluoxetine. Furthermore, the CG-treated rats exhibited marginally improved cognitive performance, compared with the control group. A significant difference in cognitive performance was observed between the miR-30e group and the control group ($P<0.01$), and a significant difference was also observed between the CG group and the miR-30e group ($P<0.01$). There was a difference in performance between the CG group and the fluoxetine group, however, it was not significant ($P>0.05$). No significant difference was observed between the CG group and the control group ($P>0.05$; Fig. 2A).

Morris water maze test. The lenti-miR-30e rats exhibited increased signs of working memory impairment. The group treated with CG exhibited no significant difference in behavior, compared with the control group, whereas the rats in the CG group exhibited a marginal improvement in performance, compared with the rats in the fluoxetine group. These results suggested that CG robustly alleviated cognitive impairment in rats exhibiting miR-30e overexpression. Significant differences were observed between the miR-30e group and the control group ($P<0.01$), and between the CG group and the miR-30e group ($P<0.01$). No significant difference was observed between the CG group and the control or fluoxetine groups ($P>0.05$; Fig. 2B).

TUNEL assay. In the lenti-miR-30e rats, the overexpression of miR-30e induced apoptosis of the neuronal cells in the DG of the hippocampus, whereas treatment with CG significantly inhibited the apoptotic process. Notably, CG exhibited similar anti-apoptotic effects to those of fluoxetine (Fig. 3).

Protein expression levels of BCL-2 and UBC9. The protein expression levels of BCL-2 and UBC9 were significantly reduced in the lenti-miR-30e rats. Following treatment with either fluoxetine or CG, the protein expression levels of BCL-2 and UBC9 returned to normal in the hippocampus, and were not significantly different from those in the control group. There was a significant difference in the protein expression levels between the CG group and the miR-30e group, with higher expression levels in the former ($P<0.01$). However, no significant differences were observed between the CG group and the control or fluoxetine groups ($P>0.05$; Fig. 4A).

BCL-2 and UBC9 immunohistochemical analysis. The immunohistochemistry results obtained in the present study were

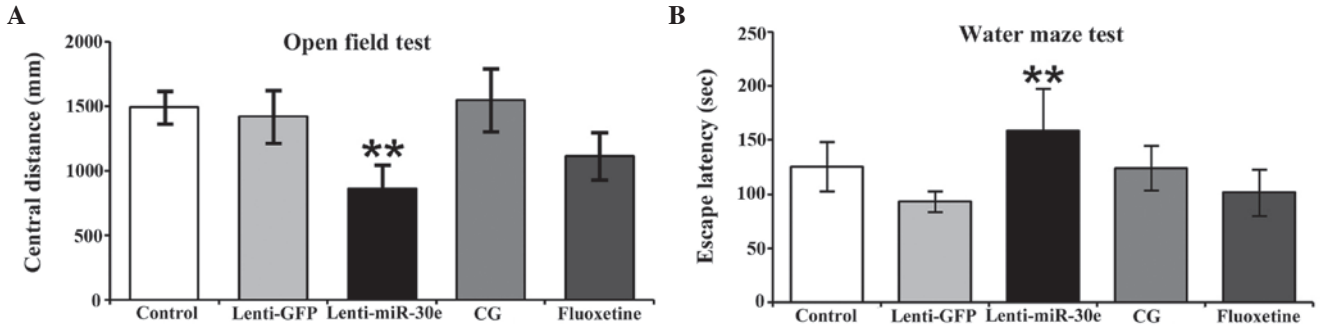

Figure 2. Effects of chronic administration (14 days) of CG, measured using an (A) open field test and (B) water maze test. (A) In the open field test, untreated lenti-microRNA (miR)-30e rats exhibited decreased central distance values, compared with the rats treated with CG. The central distance values of the rats administered with CG for 2 weeks were no different to the controls. (B) In the water maze test, untreated lenti-mir-30e rats exhibited increased escape latency values, compared with the rats treated with CG. The escape latency values of the rats administered CG for 2 weeks were no different to the controls. Data are presented as the mean ± standard error of the mean. **P<0.01, vs. control group. CG, Cerebralcare Granule®; GFP, green fluorescent protein.

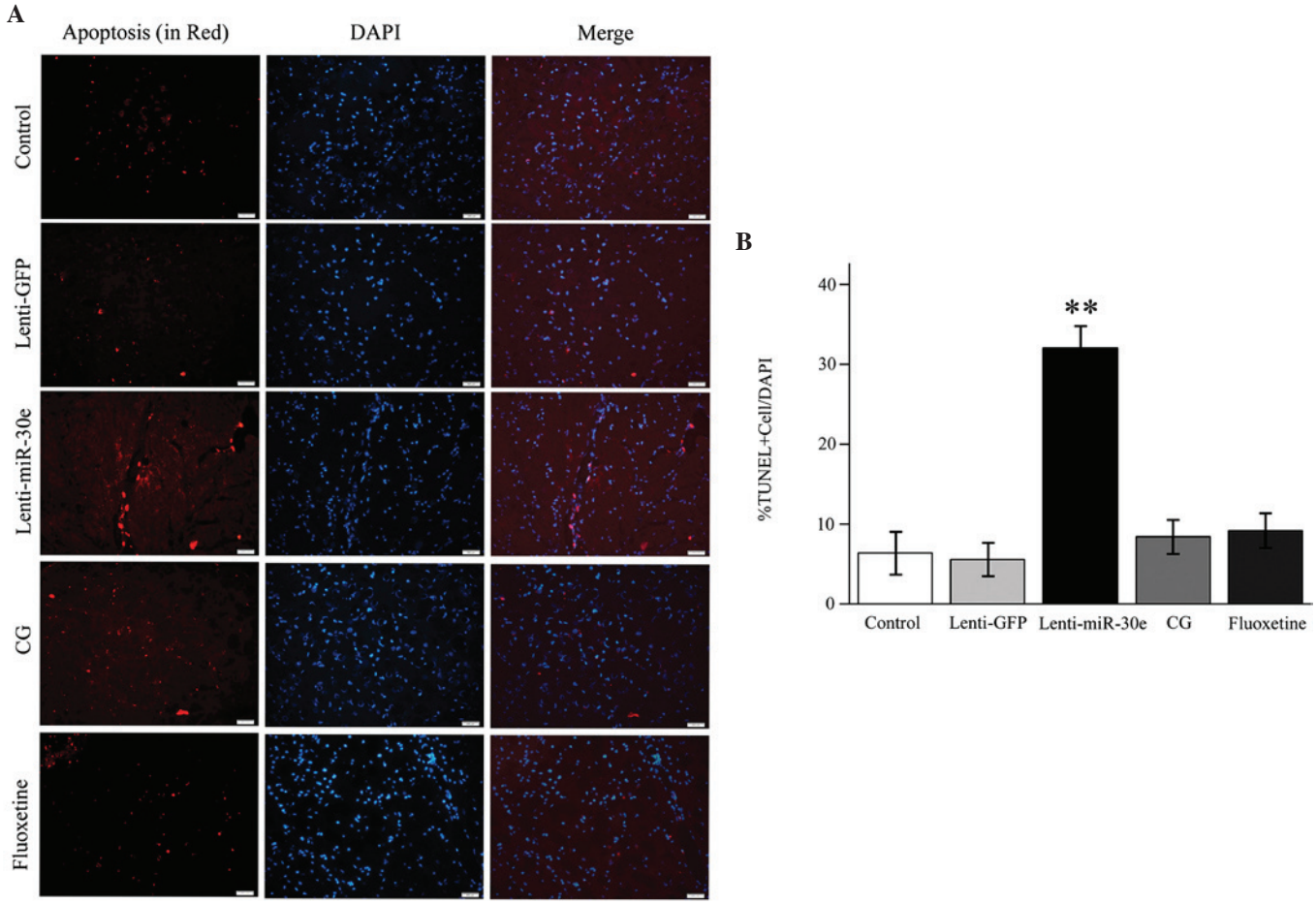

Figure 3. CG inhibits miR-30e-induced apoptosis of neuronal cells. (A) TUNEL-positive cells (red) co-localized with 4',6-diamidino-2-phenylindole (DAPI; blue)-stained nuclei (scale bar=200 mm). (B) No difference in TUNEL-positive cells were identified between the rats treated with CG and the fluoxetine control. Data are presented as the mean ± standard error of the mean. **P<0.01, vs. control. CG, Cerebralcare Granule®; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

consistent with the results of the western blot analysis. The protein expression levels of BCL-2 and UBC9 were significantly reduced in the hippocampus of the lenti-miR-30e rats. Following treatment with either fluoxetine or CG, the protein expression levels of BCL-2 and UBC9 in the hippocampus of the lenti-miR-30e rats returned to normal, and were not significantly different from those in the control group. There was a significant difference between the CG group and the miR-30e group, with higher expression levels in the former (P<0.01). However, there was no significant difference between the CG group and the control or fluoxetine groups (P>0.05; Fig. 4B).

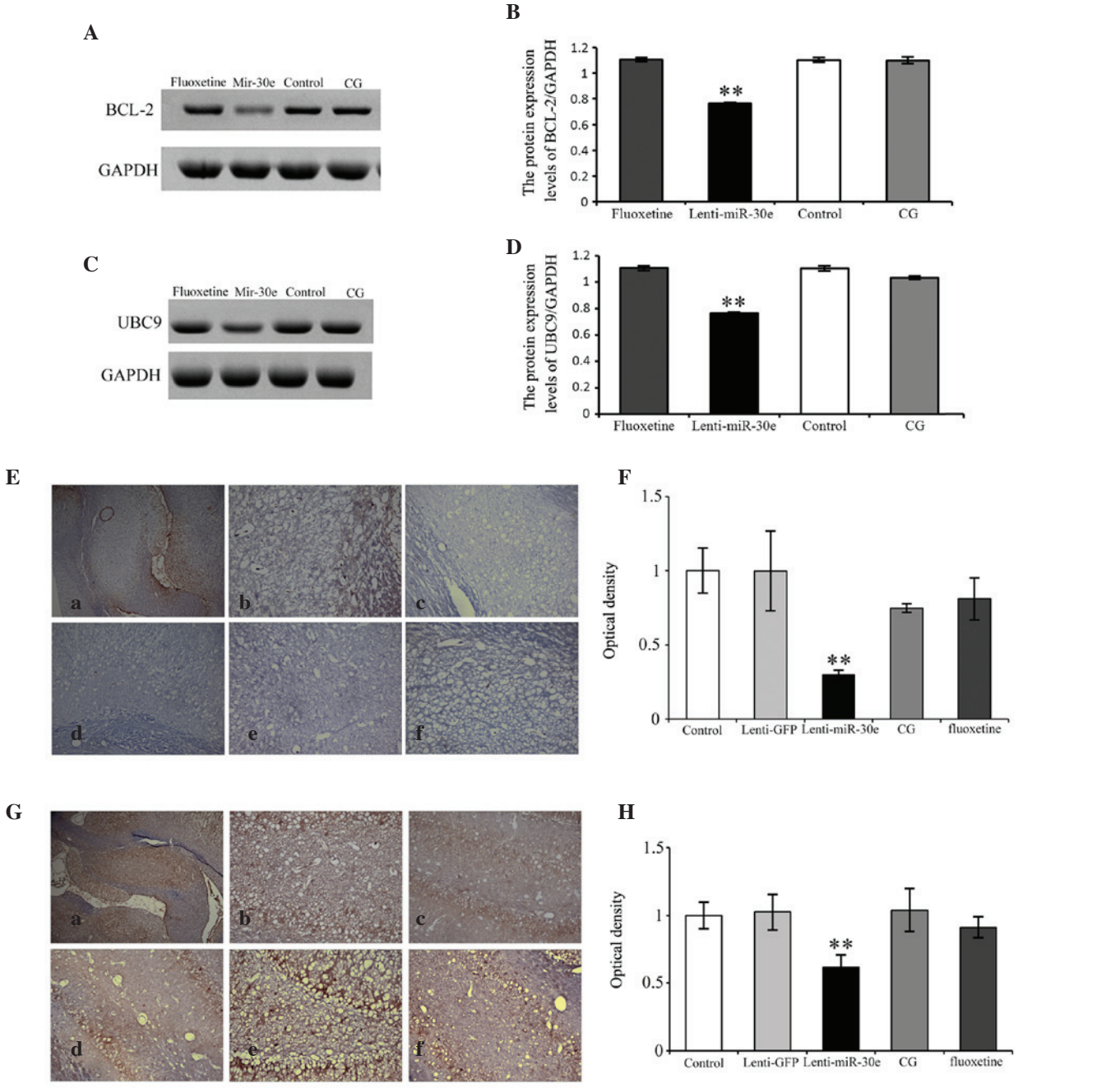

Figure 4. Western blotting to detect the protein expression levels of (A and B) BCL-2) and (C and D) UBC9 in the hippocampus of lenti-miR-30e rats. The protein expression levels were decreased in the lenti-miR-30e rats, compared with the controls, however there was no difference between the CG and control groups. Following staining with (E and F) BCL-2 and (G and H) UBC9, few positively-stained hippocampal neurons were identified in the lenti-miR-30e rats; however, numerous positively-stained hippocampal neurons were observed in the CG and control groups, and staining was dark. (a) Hippocampus image (original magnification, x4); (b) normal control group (original magnification, x20); (c) lenti-GFP group (original magnification, x20); (d) lenti-miR-30e group (original magnification, x20); (e) lenti-miR-30e+fluoxetine (10 mg/kg, 14 days) group (original magnification, x20); and (f) lenti-miR-30e+CG (100 mg/kg, 14 days) group (original magnification, x20). Scale bar=50 mm. Data are presented as the mean ± standard error of the mean. **P<0.01, vs. control. CG, Cerebralcare Granule®; BCL-2, B-cell lymphoma-2; UBC9, ubiquitin-conjugating enzyme 9; GFP, green fluorescent protein.

Discussion

Mental disorders are disabling diseases, which frequently afflict individuals throughout their entire life. In addition, the pathogenetic mechanisms remain to be fully elucidated and current clinical therapies are only partially effective.

The present study aimed to determine whether miR-30e is important in the initiation and progression of neuropsychiatric disorders. The results demonstrated that the overexpression of miR-30e induced behavioral abnormalities and cognitive impairment in the rat model, resulting in signs of anxiety, hyperactivity and schizophrenia. The present study also aimed to assess the clinical efficacy of CG on mental disorders, and to assess the degree to which it attenuates cognitive disabilities in the rat model overexpressing miR-30e. Furthermore, the molecular mechanisms underlying the therapeutic effects of

CG were evaluated. The overall aim of these investigations was to identify novel therapeutic strategies for the treatment of mental disorders.

Genome-wide association studies of schizophrenia (43) have suggested that only a very few single-nucleotide polymorphisms (SNPs) are located in exons, and that the majority are detected in introns, suggesting that gene regulatory mechanisms may be key in the pathogenesis of schizophrenia. miRNAs represent the predominant gene regulatory factor in multicellular genomes, and are important in a wide range of biological processes, including developmental timing, growth control and differentiation (44,45). Previous studies (46,47) have suggested that there are abnormalities in miRNA expression levels in patients with schizophrenia. For example, abnormal expression levels of DGCR8 exhibits marked correlation with the pathogenesis of DiGeorge's syndrome, and the protein product of this gene is closely associated with miRNA processing. Notably, approximately one in four patients with DiGeorge's syndrome develop schizophrenia (48). Autopsy investigations have reported that the expression of miRNAs in certain parts of the brain in patients with schizophrenia is significantly different from that in healthy controls (26,49,50). Studies in molecular genetics have also suggested that genetic alterations in miRNAs are associated with the onset of schizophrenia (51,52). Juhila *et al* (53) investigated miRNA expression in various brain regions, including the prefrontal cortex, hippocampus and hypothalamus, and identified inverse correlations between miRNA and mRNA pathways. In this case, as expression of a miRNA increased, expression of its target mRNA decreased.

Previous studies have reported that miR-30e has a relatively lower level of expression in the adult hippocampus, compared with other tissues, including the immune system, even lower levels of expression in the adult midbrain and frontal cortex, and almost no expression in the cerebellum. miRNAs have been found to possess differential expression regulation in various brain regions. For example, acute stress increases the expression levels of let-7a, miR-9 and miR-26a/b in the mouse frontal cortex, but not in the hippocampus (54). Previous studies have demonstrated that the depletion of miRNAs in the cerebral cortex and hippocampus, via genetic inactivation of Dicer following the onset of forebrain neurogenesis, profoundly impairs the morphological and proliferative characteristics of neural stem and progenitor cells. The cytoarchitecture and self-renewal potential of radial glial (RG) cells located within the cerebral cortex and the hippocampus are profoundly altered, thereby causing a significant disruption of the normal development of the dorsal sub-ventricular zone and the DG. This effect has been attributed to the high-temperature requirement A serine peptidase 1 (HtrA1) gene product, whose overexpression in the developing forebrain mimics certain features of the Dicer phenotype (55). miR-30e was identified as a post-transcriptional negative regulator of HtrA1 by binding to its 3'-untranslated region, and *in vivo* overexpression of miR-30e in the Dicer forebrain rescues RG proliferation defects (56). This suggests that miR-30e may inhibit neuronal cell proliferation and promote neuronal apoptosis.

In the present study, a novel rat model of mental illness was constructed using targeted miRNA gene transfection. The lenti-miR-30e rats, which exhibited miR-30e overexpression in the hippocampus, exhibited features of mental disease, including anxiety, hyperactivity and signs of schizophrenic behavior. The hippocampus was selected for the site of miR-30e injection, as it is closely associated with schizophrenia, depression and cognitive impairment. In 2010, our previous study demonstrated that SNPs in the miR-30e precursor are associated with schizophrenia (19) and depression (57), and are a clinical indicator of cognitive ability and P300 latency (57). Therefore, the hippocampus was considered an ideal target for investigating the role of miR-30e in schizophrenia. In addition, the expression levels of miR-30e in the hippocampus of patients with schizophrenia and depression remained to be elucidated, particularly as living brain tissues cannot be acquired from patients with mental illness. Furthermore, autopsy is not reliable, as patients are subject to the effects of long-term medication prior to mortality. The present study hypothesized that miR-30e levels are elevated in pathological conditions, including schizophrenia and depression, and a rat model overexpressing miR-30e in the hippocampus.

The present study aimed to examine the effects of miR-30e overexpression on the cognitive ability of rats, which may provide insight into the role of miR-30e in the pathogenesis and development of schizophrenia. The present study also aimed to examine the therapeutic effect of CG on cognitive impairment in these rats, and determine how it compares to other medicine intervention in its therapeutic efficacy.

The lenti-miR-30e rats exhibited increased signs of anxiety, hyperactivity and schizophrenia, resulting in a severe impairment in cognitive abilities, confirmed using open field and water maze tests. Treatment of the rat model with CG attenuated the cognitive impairment to control levels. The present study also demonstrated that miR-30e overexpression lead to increased neuronal apoptosis and decreased protein expression levels of BCL-2 and UBC9, and that treatment with CG increased the expression levels of these proteins, thereby inhibiting the apoptosis of neuronal cells. CG had a therapeutic effect similar to fluoxetine. Therefore, CG may have similar therapeutic effects to dopamine, serotonin and/or fluoxetine in the treatment of neuropsychiatric disorders. Furthermore, the natural ingredients in the herbal medicine offer potential in examining the pharmaceutical properties of herbal medicines as alternatives in the treatment of mental illness, although their toxicity requires careful characterization in the future.

The mechanisms underlying the ameliorative effect of CG on mental disorders and cognitive impairment may be associated with its ability to inhibit the apoptosis of neuronal cells, which may, in part, be attributed to its antioxidant properties. BCL-2 is an inhibitor of apoptosis. The present study detected a significant reduction in the protein expression levels of BCL-2 in the hippocampus of the lenti-miR-30e rats, providing evidence that miR-30e promoted the apoptotic process in the neuronal cells. These findings are consistent with previous reports on the regulatory role of miR-30e on the expression of BCL-2. The protein expression of BCL-2 protein in the hippocampus of rats in the CG group was markedly enhanced, compared with that in the lenti-miR-30e group, resulting in levels similar to those in the control group.

UBC9 is a downstream target of miR-30e. The present study demonstrated that, in the various groups, the expression of UBC9 paralleled that of BCL-2, suggesting that miR-30e

directly regulated BCL-2 and UBC9, or that it directly regulated UBC9 and indirectly regulated BCL-2, in order to affect neuronal apoptosis.

In conclusion, the lenti-miR-30e rats, induced to overexpress miR-30e in the DG of the hippocampus, exhibited signs of cognitive impairment and anxious behavior. CG effectively alleviated these symptoms, with a therapeutic effect similar to that of fluoxetine. In addition, miR-30e overexpression induced neuronal apoptosis, as revealed by a significant reduction in the protein expression levels of BCL-2 and UBC9, whereas treatment with CG markedly enhanced the expression levels of these proteins, resulting in the inhibition of neuronal apoptosis.

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