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REGULAR ARTICLE

The antidepressant effect of musk in an animal model of depression: a histopathological study

Nasra Naeim Ayuob[1,2] · **Soad Shaker Ali**[1] · **Mansour Suliaman**[3] · **Manal Galal Abd El Wahab**[4,5] · **Samra Mansour Ahmed**[6]

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Abstract Depression is a significant public health concern all over the world, especially in modern communities. This study aims to assess the efficacy of musk in alleviating the behavioral, biochemical and histopathological changes induced by chronic unpredictable mild stress (CUMS) in an animal model of depression and to explore the underlying mechanism of this effect. Male Swiss albino mice were divided into four groups ($n = 10$): control, CUMS, CUMS+fluoxetine and CUMS+musk. At the end of the experiment, behavioral tests were administered and serum corticosterone and testosterone levels were assessed. Surface markers, proteins and gene expressions of brain-derived neurotropic factor (BDNF) and glucocorticoid receptors (GRs) in the hippocampus were assessed. The immunoexpression of glial fibrillary acidic protein, Ki67 and caspase-3 was also assessed. Data were analyzed using the Statistical Package for the Social Sciences and a P value of less than 0.05 was considered significant. Musk alleviated the behavioral changes caused by CUMS and reduced elevated corticosterone levels. It reduced CUMS-induced neuronal atrophy in the CA3 and dentate gyrus of the hippocampus and restored astrocytes. Musk reduced the neuro- and glial apoptosis observed in stressed mice in a manner comparable to that of fluoxetine. Musk induced these effects through up-regulating both BDNF and GR gene and protein expressions. Musk has an antidepressant-like effect in an animal model of depression, so it is advisable to assess its efficacy in people continually exposed to stressors.

Keywords Musk · Depression · Hippocampus · Neurogenesis · Apoptosis · BDNF-GR

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Introduction

Depression is a significant public-health concern all over the world, especially in modern communities. Both low- and high-income populations can be affected by depressive disorders. Major depressive episodes are reported more frequently in high-income countries as compared to low-income countries (28 vs. 20 %) (Bromet et al. 2011). Conventional interventions for treating depression, including pharmacologic and psychologic treatments, have remained the recommended approach for the past 30 years (Schmidt et al. 2008). Although psychological therapies can help, affected individuals still require pharmacological medications such as tricyclic antidepressants, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors and others (Nemeroff 2007; Rodgers et al. 2012). Around the globe, complementary and alternative medicine (CAM) is increasingly used for the treatment of many medical disorders as a result of the increasing number of people and the diseases affecting modern communities (Seifi et al. 2014; Ji et al. 2014).

Aromatherapy, which means using the essential and aromatic oils of natural fruits and flowers, is considered a common approach in CAM. It is claimed to benefit patients mentally, psychologically, spiritually and socially, although these are not very quantitatively measurable. With respect to safety, it is reported that aromatherapy is relatively free of adverse effects as compared with conventional drugs (Schmidt et al. 2008).

Musk is a powerful odoriferous material obtained from the dried secretions of a gland of the male musk deer. The gland is located under the abdomen, near the pubis. It is used as a fragrance and fixative in perfumes. It has been used in Chinese medicine for thousands of years in treating stroke, coma, neurasthenia and convulsions (Khan and Abourashed 2010). Musk has also been reported to decrease cortisol levels in males and, therefore, musk odor has been supposed to attenuate stress (Fukui et al. 2007). Its antidepressant effect is investigated in this study.

Imbalance between forebrain systems involved in processing and responding to affective information has been suggested to result in depression. This can arise either from the action of high levels of stress, causing damage to the hippocampus, or from the combination of low levels of stress with a variety of factors that confer vulnerability to depression, which may be present from an early age, or may represent 'scars' left by previous depressive episodes (Willner et al. 2013).

Although many animal models of depression were found on reviewing the literature, for this study, chronic unpredictable mild stress (CUMS) was chosen because it is a valid and reliable model in rodents and has proven etiological, face and predictive validity. CUMS induces behavioral, biochemical and cellular changes consistent with those described in patients suffering from major depression. Because of this, many previous studies have utilized it in investigating the antidepressant effect of the drugs, as well as their mechanism of action (Vollmayr and Henn 2003; Song and Leonard 2005). Therefore, this study aims to assess the efficacy of musk in alleviating the behavioral, biochemical and histopathological changes induced by CUMS in an animal model of depression and to explore the mechanism underlying this effect.

Materials and methods

Animals

Male Swiss albino mice weighing 30–40 g were purchased from the animal house at the King Fahed Medical Research Center (KFMRC) of King Abdulaziz University (KAU), Jeddah, Saudi Arabia. They were housed in groups of 10 stainless steel cages (40 × 30 × 15) with sawdust bedding renewed daily. They were maintained in a 12-h light–dark cycle (lights on: 0700–1900 hours), with a room temperature thermostatically maintained at 27 ± 1 °C, under hygienic conditions. Water and food was available ad libitum. The mice were kept under these conditions to acclimatize for 2 weeks before exposure to the CUMS procedure. The experiment was performed according to the guidelines for animal care set by KFMRC, which were in compliance with the National Institute of Health Guide (NIH publication No. 80–23, revised 1996).

Drugs and chemicals

Musk (*Moschus moschiferus*) was purchased from the local market in Jeddah, Saudi Arabia. The constituents of the musk were analyzed via gas chromatography, coupled with mass spectrometry (GC-MS; Agilent, Columbia, USA) with a DB-5 ms column (30 m × 0.25 mm × 0.25 μm). The composition of the musk is shown in Table 1. The musk was diluted in propylene glycol to obtain concentrations of 1.0 % (v/v) just prior to the experiments (Fukayama et al. 1999). The administration of musk and amyl acetate was performed through inhalation in an odor-isolated chamber (32 cm × 24 cm × 32 cm), as described by Chioca et al. (2013). Inhalation was performed once per day immediately after the CUMS procedure and lasted for 15 min.

Fluoxetine hydrochloride was obtained from Dar Al Dawa Pharmaceuticals (Jordan). It was dissolved in 0.03 % sodium carboxymethyl cellulose and was given once a day at a dose of

Table 1 Chemical composition of the musk obtained by GC-MS

Compound	Retention time (min)	Percentage
Steroids		13.801
Androstan-3-one semicarbazone	33.062	9.345
Spirostan-23-ol #; Spirostan-23-ol	32.164	4.456
Essential oils		12.445
Alpha-Cedrol	23.858	0.102
Other essential oils		2.343
Organic compound		41.515
5-Ethyl-5-[(E)-styryl] barbituric acid (sedative)	27.303	0.355
Other organic compounds		29.259
Alchlols and phenols		2.216
1,1′-Oxybis-2-Propanol,	13.408	1.217
2-(2-Hydroxypropoxy)-1-propanol	13.804	0.799
3,3′-Oxybis-2-Butanol,	14.235	0.158
Anise alcohol	17.599	0.028
2,4-Di-tert-butylphenol (antioxidants)	21.766	0. 600
2-Fluoro-6-nitrophenol	24.447	0.008
Esters		9.423
Pyridine		8.233
Terpenes		0.091
Pyrans		5.126
Polcyclic musk		5.154
Others		1.996

20 mg/kg through intragastric gavage according to Li et al. (2014). Amyl acetate (5 %; Sigma, St. Louis, MO, USA) was administrated via inhalation to the positive control group because it has no effect on anxiety, as shown in previous studies (Kilpatrick and Cahill 2003; Pavesi et al. 2011).

Experimental procedure

The mice were randomly divided into four groups (10 mice each): control, CUMS (exposed to the CUMS for 4 weeks plus amyl acetate during the last 2 weeks of the experiment), CUMS+ Fluoxetine (FLU) (exposed to the CUMS for 4 weeks plus FLU for the last 2 weeks) and CUMS+musk (exposed to the CUMS for 4 weeks plus musk for the last 2 weeks). The mice were subjected to CUMS, which was first used by Willner (1997) and modified for mice by Ducottet and Belzung (2004). The mice were exposed to different types of stressors at different time points during the day for 4 weeks to prevent habituation and to provide an unpredictable feature to the stressors. Stressors included social stress, achieved by: placing mice in the soiled cages of other mice; inverting the light/dark cycle; placing mice in cages with wet sawdust; placing mice in tilt cages at 30°; restraining the mice and water stress, which was achieved by placing mice in an empty cage with 1 cm of water at the bottom (Fig. 1a). After 2 weeks of exposure to CUMS, mice were treated with amyl acetate, FLU and musk daily and this was continued throughout the following 2 weeks, along with the CUMS. At the end of the experiment, behavior tests were performed between 0800 and 1130 hours in a dimly lit room as described by Mineur et al. (2006) starting with the elevated plus maze test, open field test and finally the forced swimming test on days 29, 30 and 31, respectively, with a 24-h pause between the tests. On day 32, blood samples were collected in the morning, then the mice were sacrificed via decapitation (Fig. 1b).

Assessment of behavior

Forced swim test (FST) This was performed as described by Doron et al. (2014). Each mouse was placed in a glassy cylindrical container (height 20 cm, diameter 14 cm) with 15 cm of water at 25 ± 2 °C. The mouse was videotaped for 6 min using behavior software (Noldus Information Technology, EthoVision XT®) and the total time spent immobile during the 6 min was measured by a technician blind to the experiment groups; it is presented in seconds. Immobility was defined as the cessation of limb movement, except for the minor movement necessary to keep the mouse afloat.

Elevated plus-maze (EPM) This was carried out according to Carobrez and Bertoglio (2005). The maze was elevated 40 cm above the floor. Each mouse was placed in the center of the EPM and its behavior was videotaped for 5 min using behavior software (Noldus Information Technology) and is presented in seconds. The numbers of closed-arm entries and the time spent inside the open arm were measured.

Open field test (OFT) This was performed as previously described by Mineur et al. (2006). The mice were individually placed in the center of a dimly illuminated observation cage (109 cm × 49 cm × 49 cm). The animals were observed for 25 min directly and continuously by an observer. The number of rearings (standing upright on hind legs while the forepaws are free) was registered manually. These vertical movement scores reflect exploratory behaviors. Locomotor activity (distance traveled in 25 min) was quantified using the video tracking system (Columbus Instruments, OH, USA).

Assessment of serum corticosterone and testosterone levels

At the end of the experiment, the mice were anaesthetized using ether and blood samples were obtained in the morning from the retrorbital venous plexus. Blood was placed into EDTA-coated tubes. Then, the blood was centrifuged for 10 min and the collected serum samples were kept at −80 °C until the corticosterone (ALPCO Diagnostics, Orangeburg, NY, USA) and testosterone (Siemens Healthcare Diagnostics, USA) levels were assessed using ELISA kits according to the manufacturers' instructions.

Assessment of hippocampal GR and BDNF protein expression levels

Immediately after the decapitation of the mouse, the brain was dissected on an ice-plate and the entire hippocampus was isolated according to Paxinos and Watson's atlas (1998). Tissue punches were homogenized in cold extraction buffer (Tris-buffered saline, pH 8.0, with 1 % NP-40, 10 % glycerol, 5 mM sodium metavanadate, 10 mM PMSF, 100 μg/ml aprotinin and 10 μg/ml leupeptin). Homogenates were acidified with 0.1 MHCl (pH 3.0), incubated at room temperature (22–24 °C) for 15 min and neutralized (pH 7.6) with 0.1 M NaOH. The homogenates were then microfuged at 7000*g* for 10 min. BDNF and GR protein levels were assessed using sandwich enzyme-linked immunosorbent assay (ELISA) according to Baker-Herman et al. (2004).

Assessment of hippocampal GR and BDNF gene expression levels

Total RNAs were isolated from 30–60 mg of hippocampus using an EZ RNA Clean Up Plus DNase Kit (EZ BioResearch, St Louis, MO, USA). RNA concentrations were measured using a NanoDrop Spectrophotometer (Jenway, UK). Reverse transcriptions (RTs) were performed using oligo-dT primers (Bioneer, Daejeon, Republic of Korea) in a 20-μL reaction including 5 μL RNA. The cDNAs obtained were amplified by using PCR Master Mix (Bioneer) with primers designed by

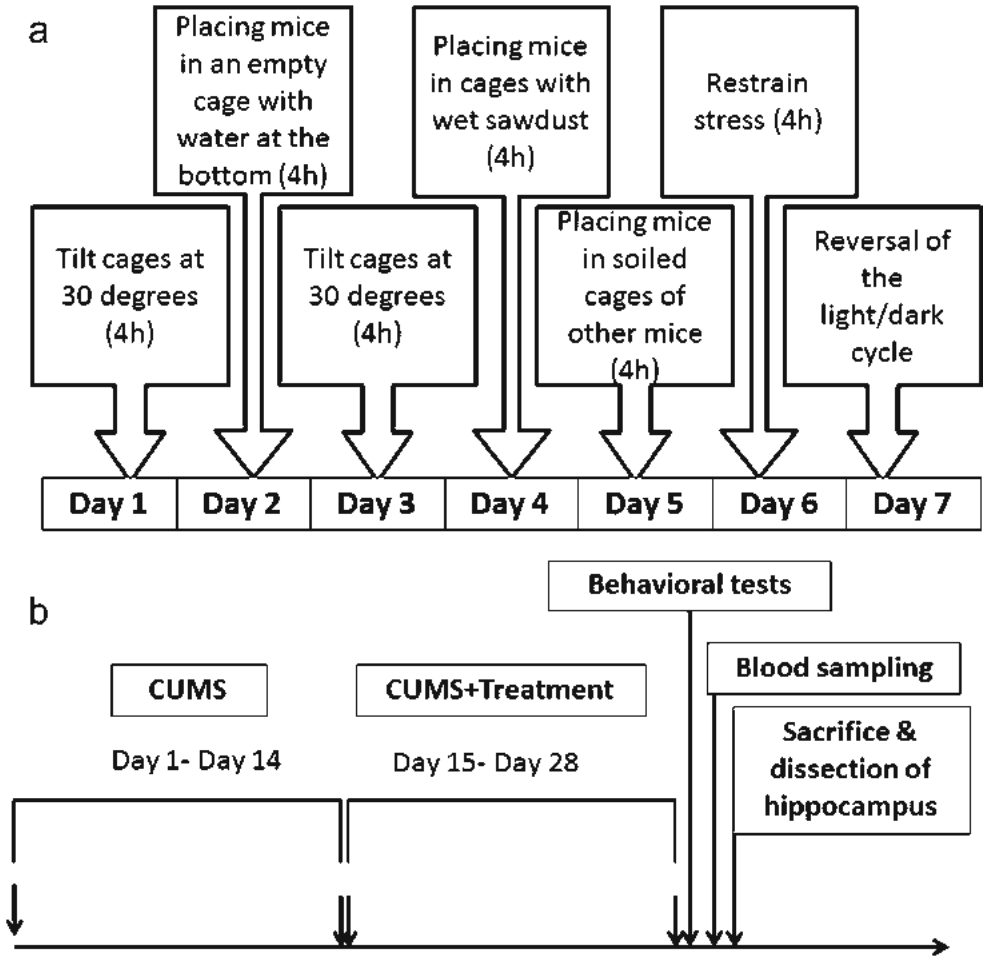

Fig. 1 **a** Experimental design. In this experiment, mice ($n = 10$ in each group) were exposed to either chronic unpredictable mild stress (*CUMS*) daily from day 1 to day 28 or left unexposed to stress all throughout. On day 15 to day 28, the mice were exposed daily to the control treatment (amyl acetate), fluoxetine, or musk along with the CUMS. On days 29, 30 and 31, the behavioral tested are sequentially done for all groups. On day 32, a blood sample was obtained, then the mice were sacrificed and the brain was dissected to obtain the hippocampus. **b** The stressor to which the mice were exposed during the CUMS procedure for 1 week. It was repeated for 4 weeks. To prevent habituation and to provide an unpredictable feature to the stressors, the stressors were administered at different time points during the day (Doron et al. 2014)

Metabion International (Semmelweisser, Germany) as follows: GR (forward 5′-AGCTCCCCCTGGTAGAGAC −3′; reverse 5′-GGTGAAGACGCAGAAACCTT-3′), BDNF (forward 5′-TATTTCATACTTCGGTTGC-3′; reverse 5′-TGTC AGCCAGTGATGTCG-3′) and β-actin (forward 5′-TCTG GCACCACA CCTTCTA-3′; reverse 5′-AGGC ATACAGGGACAGCAC-3′). PCR amplification was performed in a thermocycler (manufactured by Labnet International). The amplified fragments were analyzed via gel electrophoresis using a DNA ladder in order to assess the size of the amplicon products. The images were obtained using a gel documentation system (manufactured by Ultra-Violet Products). The size of the amplicons was determined using software available with the gel documentation system. The expression patterns of the GR gene and BDNF gene in the hippocampus were examined through RT-PCR using SYBR Green qPCR Master mix containing ROX as a reference dye (Biotool, Houston, TX, USA). All amplified fragments were achieved in three independent replicates; in addition, the results were normalized to β-actin as a reference gene using the comparative Ct method.

Histopathological assessment

Animals were sacrificed via cervical decapitation to avoid any effects on the part of anesthetic agents on brain histochemistry. The skull was opened and the brain was dissected on an iced plate. Then, it was cut into two halves in the sagittal plane. The right half was fixed in 10 % neutral buffered formalin overnight and then processed to obtain paraffin blocks. Serial paraffin sections were cut into slices 3–4 μm in thickness and stained with hematoxylin and eosin (H&E) for histopathological examination (Bancroft and Gamble 2008). Immunohistochemical studies were carried out using the peroxidase-labeled streptavidin-biotin technique according to Makhlouf et al. (2014). The paraffin sections were deparaffinized and then rehydrated. They were boiled in a

microwave for 20 min in 0.01 M sodium citrate buffer (pH: 6) in order to retrieve the antigen. Three percent H2O2 in methanol was used for 5 min at room temperature to block endogenous peroxidase activity, followed by washing twice in phosphate-buffered saline (PBS). The slides were incubated overnight at 4 °C and anti-GFAP (Dako Cytomation, USA) was performed for 1 h with a 1:1000 dilution for the demonstration of astrocytes. For the demonstrate of apoptosis, anti-caspase-3 (Santa Cruz Biotechnology, USA) was used at a dilution of 1:1000 for 1 h. Anti-Ki-67 (rabbit polyclonal Ig G produced by Abcam, Cambridge, UK) was used at a dilution 1:100 for the demonstration of cell proliferation. Rabbit anti-GR antibody and anti-BDNF (Santa Cruz Biotechnology) were used with dilutions of 1:1000 and 1:400, respectively, overnight at room temperature and subsequently exposed to biotinylated goat anti-rabbit IgG and streptavidin peroxidase complex (1:200 dilution; Vector Laboratories) at room temperature. After washing, the slides were incubated with the avidin-biotin-peroxidase complexes (Dako, USA) for 10 min, covered with DAB and incubated for 10 min and then counterstained with haematoxylin, dehydrated, cleared and mounted.

Morphometric and statistical analysis

A light microscope (Olympus BX-61, Los Angeles, CA, USA) connected to a digital camera was used for examination and photographing. Both the thickness and surface area of the pyramidal cell layer in the CA3 area, as well as the granular cell layer in the DG areas, were measured using Image ProPlus Software v.6.0 (Media Cybernetics, Silver Spring, MD, USA). Ten sections from each mouse were examined. In each section, five non-overlapping fields were assessed and the mean for each of the ten mice was calculated. In addition, the number of GFAP-positive cells in CA3 was counted in five high-power fields (×400 magnification) in each of the ten mice according to the method of Makhlouf et al. (2014). The number of caspase-3, Ki67-positive cells was assessed by the cubic millimeter using the same software. The relative optical density (ROD) of BDNF and GR immunoexpression was assessed as described by Chen et al. (2015).

The data were analyzed using the Statistical Package for the Social Sciences (SPSS, v.16). Data are presented in the form of means and standard deviations. For the non-parametric data, Kruskal–Wallis analysis of variance (ANOVA), followed by a post hoc test (based on Dunnett's C procedure), were used to analyze each pair of groups to avoid a multiple-comparison effect. For the parametric data, the various groups were compared using ANOVA (F test), followed by a Bonferroni post hoc test. Significance was considered to be indicated at a p value of less than 0.05.

Results

Effect of musk on serum corticosterone level

On the biochemical level, CUMS significantly increased ($p < 0.001$) the basal serum corticosterone level as compared with the control. The administration of FLU and musk significantly ($p < 0.001$) reduced the basal serum corticosterone level as compared with the CUMS group (Fig. 2a).

Effect of musk on serum testosterone level

CUMS significantly increased ($p < 0.001$) the serum testosterone level as compared with the control while the administration of musk significantly ($p = 0.01$) increased its level as compared with the CUMS group (Fig. 2b).

Effect of musk on forced swimming test (FST)

Depressive-like behavior was increased in the CUMS mice, as evidenced by the significant increase in immobility time on the FST (control: 302.16 ± 7.12; CUMS: 357.24 ± 35.26; $p < 0.001$). Exposure to FLU and musk along with the CUMS reduced this depressive-like behavior, as evidenced by the significant decrease in immobility time in comparison to CUMS alone (CUMS: 357.24 ± 35.26; CUMS+FLU: 328.11 ± 11.64; $p = 0.008$; CUMS+M: 327.49 ± 8.44; $p = 0.006$) (Fig. 2c).

Effect of musk on elevated plus maze test (EPM)

Anxiety-like behavior was found to be increased in mice exposed to CUMS because the mice exhibited a significant decrease (control: 27.40 ± 3.51; CUMS: 11.77 ± 0.93; $p < 0.001$) in the time spent in the open arms of the EPM when compared with the control mice. The administration of FLU and musk resulted in the reduction of anxiety-like behavior, as evidenced by the increase of the time spent in the open arms in comparison to the CUMS mice (CUMS: 11.77 ± 0.93; CUMS+FLU: 16.76 ± 2.53; $p = 0.001$; CUMS+M: 19.29 ± 3.2; $p < 0.001$) (Fig. 2d, e).

Effect of musk on open field test (OFT)

As shown in Fig. 2f, g, mice exposed to CUMS showed an increase in spontaneous locomotor activity on the OFT because they travelled significantly more than the control (control: 3616.3 ± 238.2; CUMS: 4572.8 ± 270.3; $p < 0.001$). Exposure to FLU and musk along with CUMS significantly reduced this increased activity in comparison with the CUMS group (CUMS: 4572.8 ± 270.3; CUMS+FLU: 2617.8 ± 650.6; $p < 0.001$; CUMS+M: 3011.2 ± 102.9; $p < 0.001$).

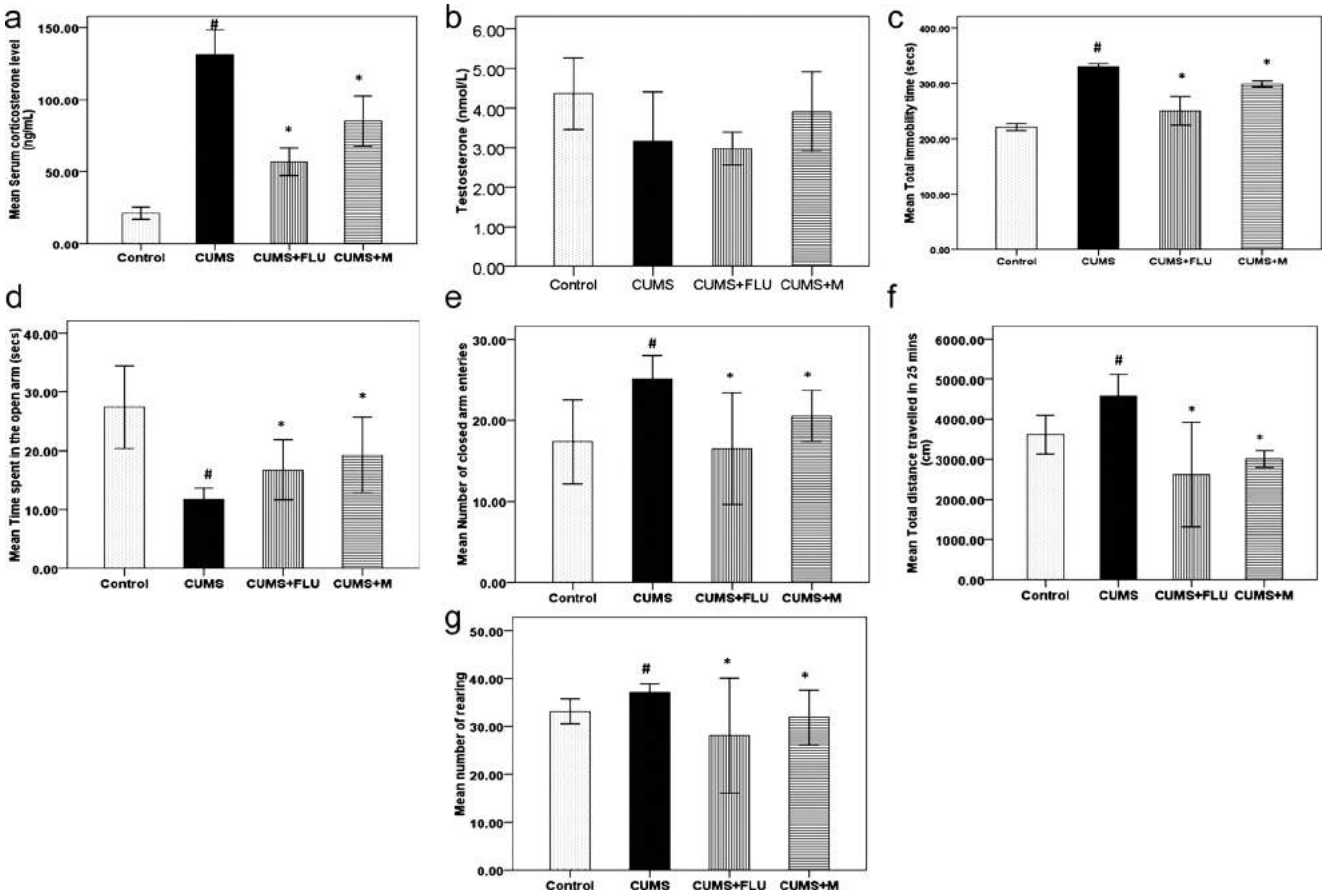

Fig. 2 Effect of musk on the serum corticosterone level (**a**), serum testosterone level (**b**), the immobility time of the FST (**c**), the time spent in the open arm (**d**), the number of closed arm entries (**e**) of the EPM test, the distance travelled in 25 min (**f**) and number of rearing (**g**) of the OFT. Data are expressed as mean ± SD ($n = 10$). #p significance versus control, *p significance versus CUMS. *EPM* elevated plus maze, *OFT* open field test, *CUMS* chronic unpredictable mild stress, *FLU* fluoxetine, *M* musk

CUMS also induced a significant increase in the number of rearings as compared to the control (control: 32.29 ± 1.29; CUMS: 37.1 ± 0.85; $p = 0.02$), while FLU and musk significantly reduced rearings in comparison to the CUMS group (CUMS: 37.1 ± 0.85; CUMS+FLU: 28.05 ± 6.02; $p < 0.001$; CUMS+M: 31.84 ± 2.8; $p = 0.007$).

Effect of musk on the histologic structure of the hippocampus

The hippocampus is formed by the C-shaped cornu amonis, or CA and the interlocking V-shaped dentate gyrus, or DG. The cornu amonis consists in three areas: CA1, CA2 and CA3. CA3 and the DG were specifically examined in this study because they have been reported to be affected in depression. The control CA3 consisted of polymorphic, pyramidal and the molecular cell layers. The pyramidal layer contains crowded pyramidal cells with large vesicular nuclei, while many of those cells in mice exposed to CUMS appeared to be smaller with dark cytoplasm and small condensed nuclei. On the other hand, mice exposed to CUMS along with FLU and musk showed fewer of these small dark cells and the majority of cells appeared to be normal (Fig. 3). A significant increase in both the thickness and surface area of the pyramidal cell layer was observed following exposure to FLU and musk (Table 2).

The control DG was composed of molecular, granular cell and pleomorphic layers. The granular cell layer contained polyhedral cells with vesicular nuclei. In mice exposed to CUMS, many of those cells appeared to be smaller with dark cytoplasm and small condensed nuclei (apoptotic). These dark cells were less frequently observed in mice exposed to CUMS along with FLU and musk (Fig. 4). Significant increases in both the thickness and surface area of the granular cell layer were observed following exposure to FLU and musk (Table 2).

Effect of musk on the immunoexpression of GFAP

The immunoexpression of GFAP in the hippocampal CA3 and DG was assessed (Figs. 5, 6). It was observed that with CUMS, GFAP immunoexpression was obviously decreased ($p = 0.003$, $p < 0.001$) in the CA3 and DG as compared to the

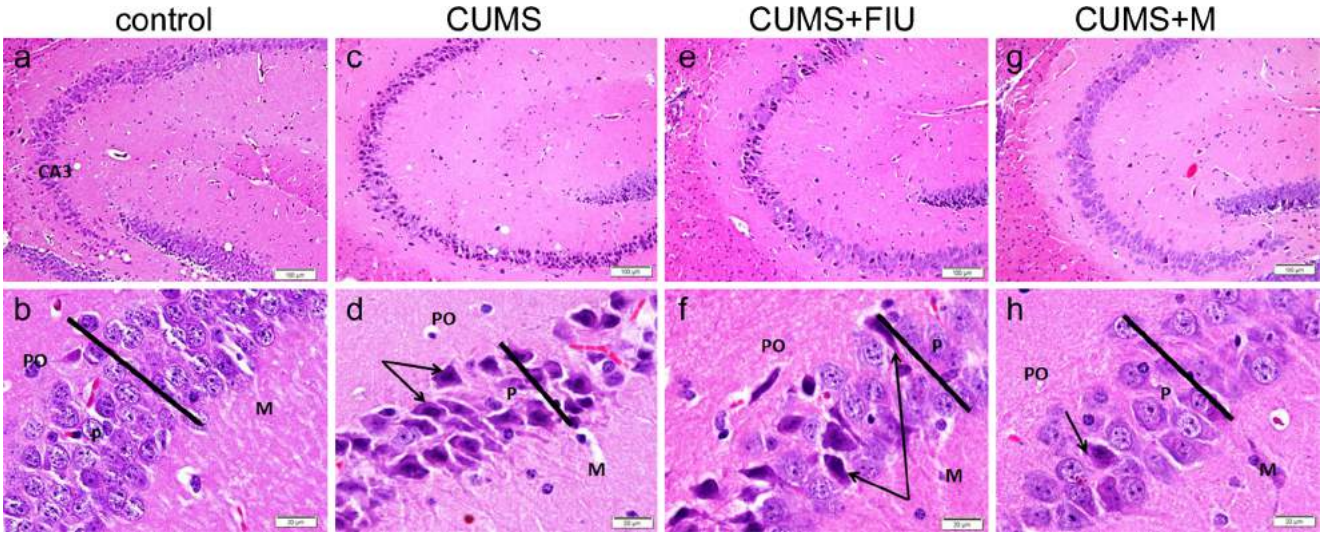

Fig. 3 The CA3 region of the hippocampus of the *control* (**a**, **b**), *CUMS* (**c**, **d**), *CUMS+FLU* (**e**, **f**) and *CUMS+M* (**g**, **h**) group show three layers; the polymorphic (*PO*), the pyramidal (*P*) and the molecular (*M*). Note the reduction in the pyramidal layer thickness indicated by the *black line*. H&E staining. (**a**, **c**, **e**, **g** ×200; **b**, **d**, **f**, **h** ×1000). *CUMS* chronic unpredictable mild stress, *FlU* fluoxetine, *M* musk

control and that it was increased after fluoxetine administration ($p = 0.04$, $p = 0.05$). Musk significantly increased ($p = 0.01$, $p = 0.03$) GFAP expression in the CA3 and DG as compared to the CUMS group (Fig. 7a).

Effect of musk on the immunoexpression of caspase

Figure 7b summarizes the anti-apoptotic effect of musk in the CUMS model. FLU ($p < 0.001$) and musk ($p < 0.001$) significantly decreased the number of caspase-positive cells as compared to the CUMS group in both the CA3 and DG.

Effect of musk on the immunoexpression of Ki67

The effect of musk on DG neurogenesis is summarized in Fig. 7c. FLU ($p = 0.003$) and musk ($p < 0.001$) significantly increased the number of proliferating Ki-67-positive cells in the subgranular layer of DG as compared to the CUMS group.

Effect of musk on immunoexpression of BDNF

BDNF immunoexpression was obviously decreased ($p = 0.001$, $p = 0.01$) in the hippocampal CA3 and DG of the CUMS group as compared to the control. It was significantly increased following fluoxetine ($p < 0.001$) and musk ($p < 0.001$) administration as compared to the CUMS group in both areas (Fig. 7d).

Effect of musk on immunoexpression of GR

A similar trend was observed in GR immunoexpression. It was significantly decreased ($p = 0.001$, $p = 0.02$) in the CA3

Table 2 Effect of Musk on morphometric measurement of the hippocampus

Parameter	Control ($n = 10$)	CUMS ($n = 10$)	CUMS+FlU ($n = 10$)	CUMS+M ($n = 10$)
Thickness of CA3 pyramidal cell layer (μm)	77.7 ± 5.9	49.04 ± 4.7 $p < 0.001$	64.11 ± 6.1 p1 < 0.001	68.7 ± 7.6 p1 < 0.001
Surface area of CA3 pyramidal cell layer ($\times 10^3 \mu m^2$)	53.37 ± 4.15	42.20 ± 5.14 $p = 0.002$	49.22 ± 4.27 p1 = 0.004	52.36 ± 5.75 p1 = 0.006
Thickness of DG granular cell layer (μm)	94.34 ± 11.2	74.22 ± 20 $P = 0.02$	89.54 ± 10 p1 = 0.04	90.5 ± 11.3 p1 = 0.03
Surface area of DG granular cell layer ($\times 10^3 \mu m^2$)	143.2 ± 14.2	86.12 ± 12.33 $p < 0.001$	112.44 ± 28.2 p1 = 0.02	129.2 ± 16 p10.001

Data are expressed as mean ± SD

p significance versus group control; *p1*: significance versus group CUMS; significance is considered at $p < 0.05$

HPF High power field, *CUMS* chronic unpredictable mild stress; *FlU* fluoxetine; *M* musk

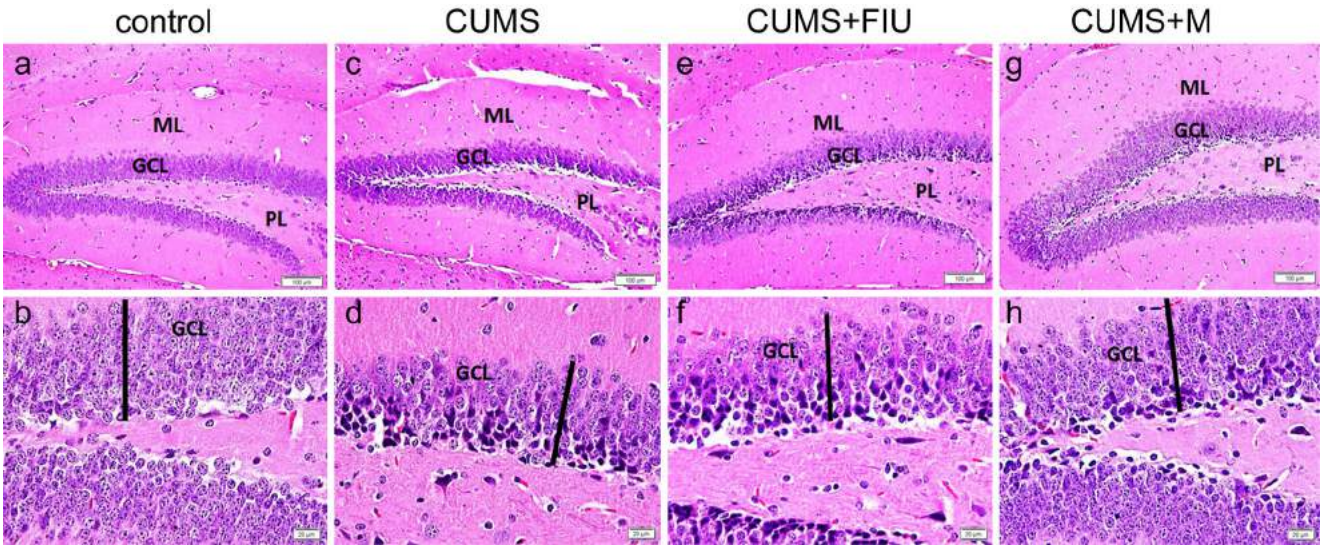

Fig. 4 The dentate gyrus of the hippocampus of the control (**a**, **b**), CUMS (**c**, **d**), CUMS+FLU (**e**, **f**) and CUMS+M (**g**, **h**) groups show molecular (ML), granular cell (GCL) and pleomorphic layers (PL). Note the reduction in the granular cell layer thickness indicated by the *black line*. H&E staining. (**a**, **c**, **e**, **g** ×200; **b**, **d**, **f**, **h** ×600). *CUMS* chronic unpredictable mild stress; *FlU* fluoxetine; *M* musk

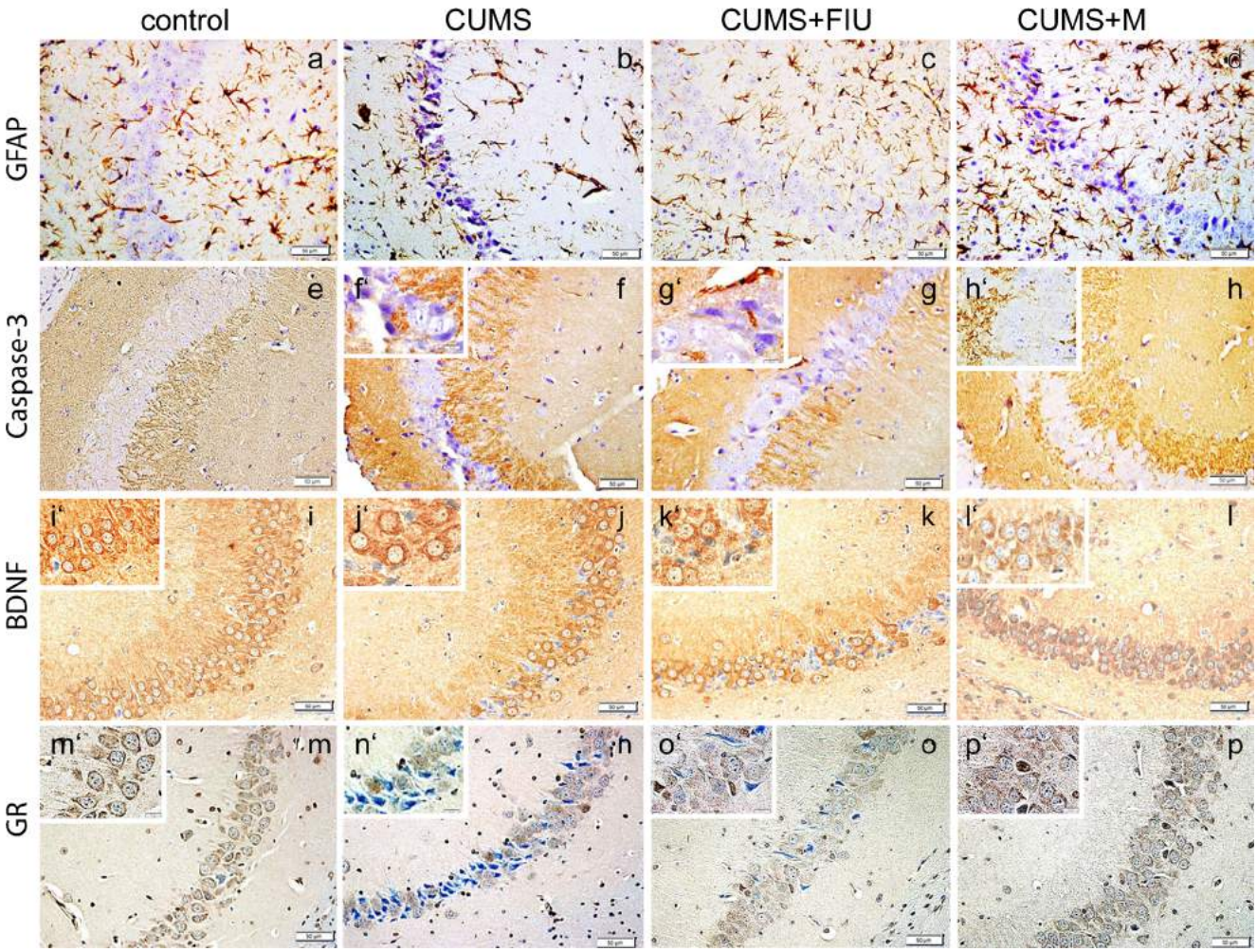

Fig. 5 Immunoexpression of GFAP (**a**–**d**), caspase (**e**–**h**), BDNF (**i**–**l**) and GR (**m**–**p**) in the CA3 region of the hippocampus in the studied group (×400, *insets* ×1000). *CUMS* chronic unpredictable mild stress; *FlU* fluoxetine; *M* musk

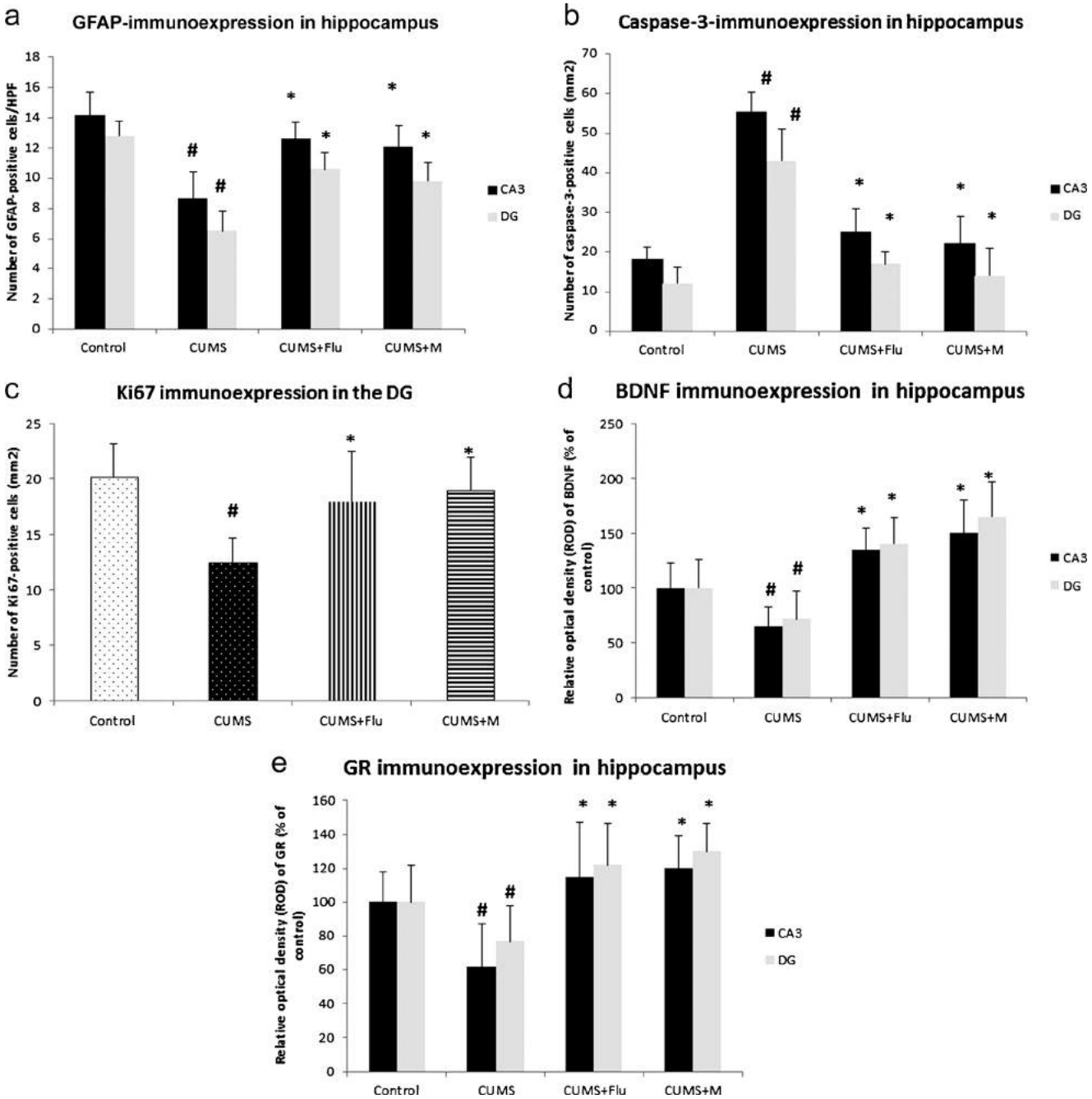

Fig. 6 Immunoexpression of GFAP (**a**–**d**), caspase-3 (**e**–**h**), Ki67 (**i**–**l**), BDNF (**m**–**p**) and GR (**q**–**t**) in the dentate gyrus of the hippocampus of the studied groups (**a**–**l** ×600, **m**–**t** ×400, *insets* ×1000). *CUMS* chronic unpredictable mild stress; *FlU* fluoxetine; *M* musk

and DG of the CUMS group. FLU ($p < 0.001$) and musk ($p < 0.001$) administration could significantly increase GR immunoexpression as compared to the CUMS group in both areas (Fig. 7e).

Effect of musk on BDNF gene and protein expression level

Quantitative RT-PCR showed that BDNF mRNA expression levels were significantly down-regulated ($p = 0.03$) in the hippocampi of the stress group as compared with the control group. FLU ($p < 0.001$) and musk ($p = 0.01$) significantly up-regulated this expression (Fig. 8a). Similar observations were noticed regarding BDNF protein expression because CUMS significantly down-regulated ($p < 0.001$) BDNF protein level expression as compared with the control group. Administering FLU and musk significantly up-regulated ($p < 0.001$, $p = 0.02$) this expression (Fig. 8b).

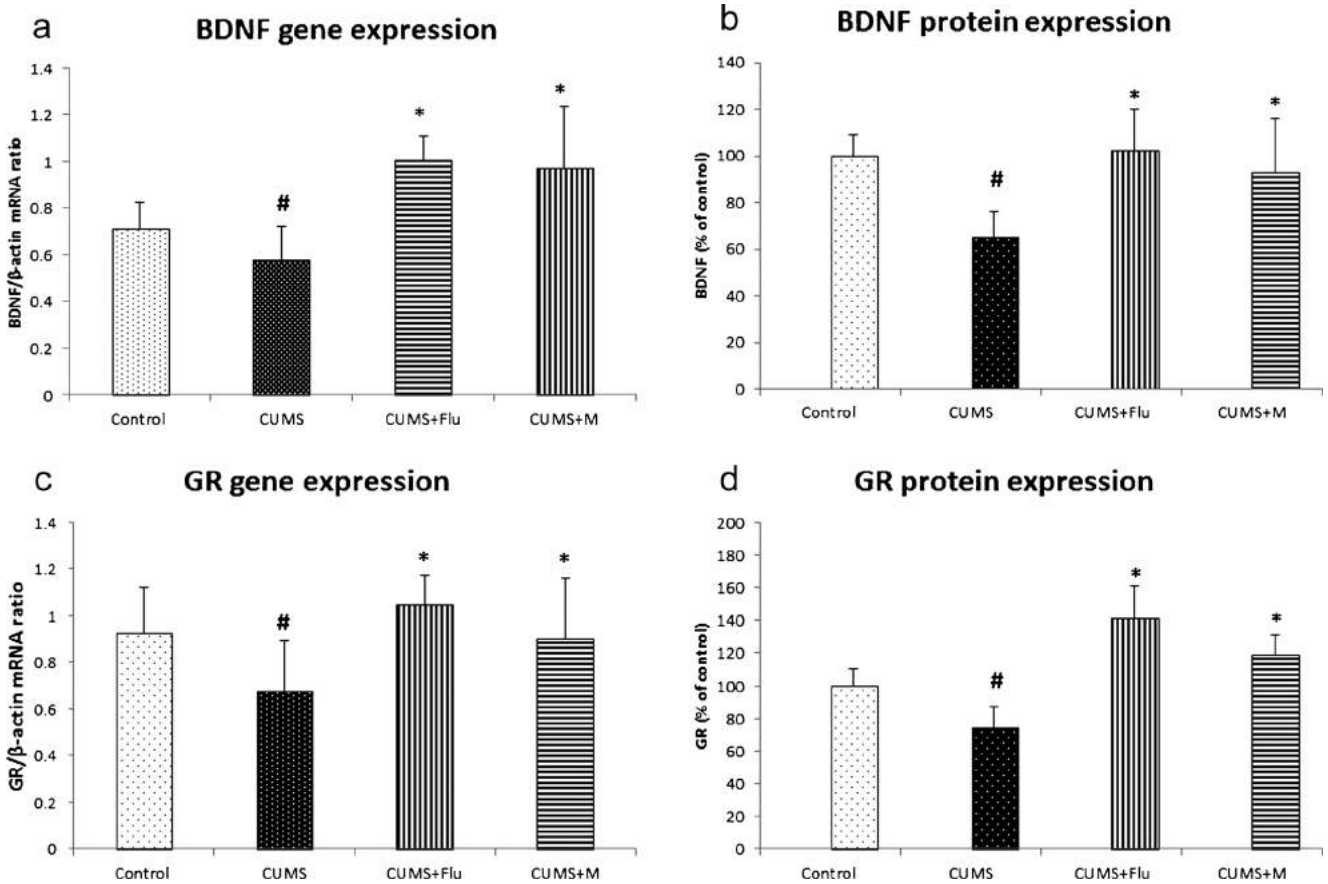

Fig. 7 Quantitative assessment of immunoexpression of GFAP (**a**), caspase-3 (**b**), Ki67 (**c**), BDNF (**d**) and GR (**e**) in the hippocampus of the studied groups. Data of GFAP, caspase-3, Ki67 expression are expressed as mean ± SD and that of BDNF and GR is expressed as mean percent of control value ± SD. #p *s*ignificance versus control, **p* significance versus CUMS. *CUMS* chronic unpredictable mild stress; *FLU* fluoxetine; *M* musk. (ANOVA followed by a Bonferroni post hoc test)

Effect of musk on GR gene and protein expression levels

The one-way ANOVA analysis of the data, followed by the LSD test of the quantitative RT-PCR, showed that GR mRNA expression levels were significantly down-regulated ($p = 0.01$) in the hippocampi of the stress group as compared with the control group. FLU ($p < 0.001$) and musk ($p = 0.03$) significantly prevented this down-regulation induced by chronic stress (Fig. 8c). A similar trend was observed in GR protein expression. Exposure to CUMS significantly down-regulated ($p < 0.001$) the GR protein level as compared with the control and administrating FLU and musk significantly up-regulated ($p < 0.001$, $p < 0.001$) this expression (Fig. 8d).

Discussion

This study hypothesized that musk inhalation may create anxiolytic and antidepressant effects. To test this hypothesis, mice were exposed to musk inhalation after the induction of depression through exposure to CUMS. The mechanism of the effect induced by musk was also explored. In this study, mice exposed to CUMS displayed depressive status, as evidenced by the prolongation of immobility in the FST, as well as the time spent in the open arms in the EPM and this was confirmed by increased spontaneous locomotor activity. The main finding of this study is that musk can alleviate the behavioral changes caused by the CUMS process and reduce the elevated corticosterone level associated with CUMS. Musk can reduce CUMS-induced neuronal atrophy in the hippocampus and restore astrocytes. In addition, musk reduced the neuro- and glial apoptosis observed in the hippocampi of CUMS mice. Musk alleviated the CUMS-induced corticosterone level elevation and degenerative cellular changes in a manner comparable to that of fluoxetine, the selective serotonin reuptake inhibitor (SSRI) antidepressant that was used as a positive control for pharmacological validation. The antidepressant effect of musk is induced through the up-regulation of both the gene and protein expression of BDNF and GR and this was also true for fluoxetine.

Depression symptoms are frequently reported to be associated with disturbed glucocorticoid secretion in patients with depression, as well as in many animal models of depression,

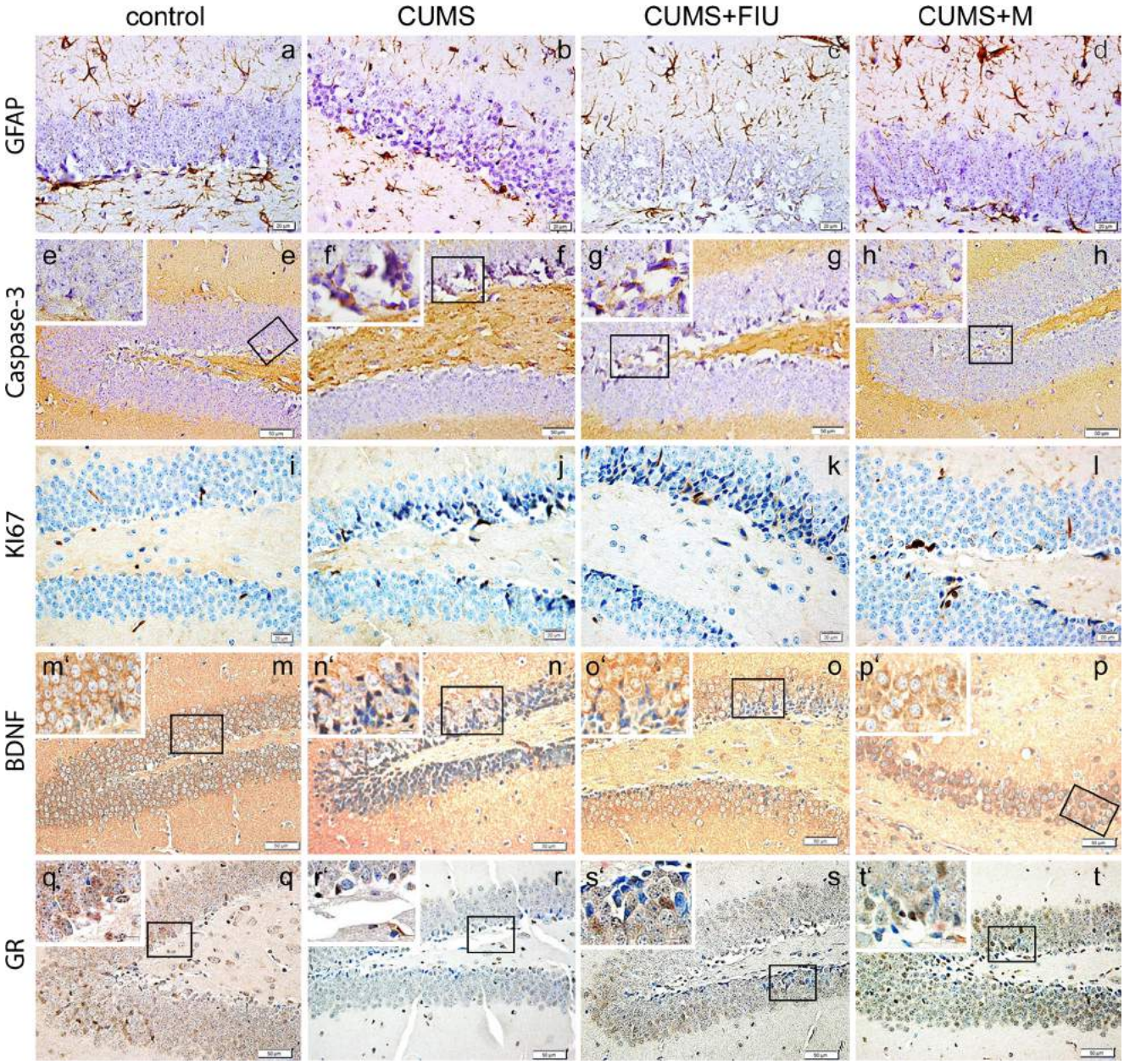

Fig. 8 BDNF mRNA (**a**) and GRm RNA (**c**) expression levels in the hippocampus of the studied groups assessed by quantitative RT-PCR. Data are expressed as mean ± SD ($n = 10$). BDNF protein (**b**) and GR protein (**d**) expression levels in the hippocampus assessed by ELISA and expressed as percent of control value ± SD. #*p* significance versus control, **p* significance versus CUMS. *CUMS* chronic unpredictable mild stress; *FLU* fluoxetine; *M* musk (ANOVA followed by a Bonferroni post hoc test)

so dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis was investigated through the assessment of the serum corticosterone level (Zunszain et al. 2011). In this study, CUMS resulted in a significant increase in serum corticosterone, which indicated hyperactivity in the HPA axis. Many previous studies have reported similar findings (Grippo et al. 2005; Mizuki et al. 2014). However, other studies reported no changes in the corticosterone levels in some depressive subtypes and attributed this finding to the difference in the biological correlates of these subtypes (Keller et al. 2006; Lamers et al. 2013). Fluoxetine and musk significantly reduced the corticosterone level in the present study. Liu et al. (2014) also reported a reduced corticosterone level after fluoxetine administration in stressed mice. Fukui et al. (2007) reported that musk can decrease cortisol levels in males.

Because testosterone levels have been reported to greatly affect behavior mood and personality (Mazur and Booth 1998), they were assessed in this study. It was observed that CUMS decreased testosterone levels in mice and administration of FLU further decreased them, although this was of no statistical significance. This was in accordance with the findings of Sakr et al. (2015) during their study on CUMS in rats.

Excessive glucocorticoid (corticosterone in rodents) exposure, the hallmark of stress, leads to decreased testosterone in circulation through suppressed androgen synthesis and reductions in the numbers of Leydig cells as a result of apoptosis (Hardy et al. 2005). Bataineh and Daradka (2007) reported that long-term intake of FLU caused a decrease in testosterone and attributed that to the action of FLU on Leydig cells or the effect of ROS on testicular steroidogenesis. The administration of musk significantly increased testosterone levels as compared with the CUMS group and this might be attributed to the reduction of corticosterone levels induced by musk.

To assess the number and integrity of astrocytes in the hippocampus, a selective immune-marker, GFAP, was used (Webster et al. 2001). The number of the GFAP-positive astrocytes was markedly reduced in the CA3 area and DG of the CUMS mice. This finding is consistent with that of Li et al. (2013) in their study of stressed rats, Webster et al.'s (2001) study on patients with mood disorders and Bowley et al.'s (2002) post-mortem studies of tissues from patients with depression. Also, Banasr and Duman (2008) suggested that reduced GFAP expression in astrocytes is a contributing factor in developing depression symptoms. Fluoxetine, as an antidepressant, was reported to prevent the reduction of GFAP expression, as well as glial atrophy (Liu et al. 2010) and this was evident in this study. Musk has induced the same effect as FLU. Regarding the apoptotic effect of chronic stress, Liu et al. (2010) reported an increase in TUNEL-positive hippocampal neurons, an indication of cell death. In addition, a recent study by Yu et al. (2014) demonstrated that CUMS induced an increase in bax and caspase-3, as well as a decrease in bcl-2 expression, in the hippocampus and this is consistent with the present study regarding caspase-3. The administration of FLU, along with CUMS, resulted in a slight reduction of caspase-3 expression, while the administration of musk markedly reduced it. This anti-apoptotic effect on the part of antidepressants has been previously reported in many studies (Manji and Duman 2001; Lucassen et al. 2004).

In this study, it was observed that exposure to CUMS was associated with a decreased number of proliferating neurons in the SGZ of the DG, as demonstrated by Ki67 immunostaining. This was in agreement with previous studies (Alonso et al. 2004). Chronic but not acute antidepressant treatment was reported to increase SGZ proliferation and prevent the down-regulation of neurogenesis caused by chronic mild stress in mice (Alonso et al. 2004; Malberg et al. 2000). This new cell birth is necessary for the behavioral actions of these agents in selected rodent models (Banasr et al. 2011). In this study, this finding was supported regarding fluoxetine, as well as musk. Increased apoptosis of neurons or glial cells and reduced neurogenesis in the SGZ of the dentate gyrus seem to be behind the reduction in hippocampal volume reported by Sahay et al. (2007) and the reduction in thickness of the CA3 pyramidal and granular cell layer of the DG observed in this study.

The BDNF is the brain neurotrophic/growth factor that has been extensively studied in stress and depression research (Castren et al. 2007; Monteggia 2007). BDNF is modulated by many internal and external factors. It might be differently involved in particular processes in depression and may be more important for anti-depressive therapy than for pathophysiological courses (Chourbaji et al. 2011). Chronic stress, as well as the heterozygous deletion of BDNF in mice, resulted in the atrophy of neurons in the hippocampus and PFC (Egan et al. 2003; Duman 2004). Regarding GR, hippocampal neurons can be damaged by high levels of glucocorticoid because they are specifically rich in GR (Szymánska et al. 2009). Repeated stress and glucocorticoid administration were reported to result in the atrophy of pyramidal CA3 neurons (Warner-Schmidt and Duman 2006). Because of that, both BDNF and GR were selected to be sensitive indicators of the antidepressant effect of musk. In this study, both BDNF and GR immuno-, gene and protein expressions were up-regulated after the administration of fluoxetine and musk. The increased level of circulating glucocorticoids in response to chronic stress seems to result primarily in the activation of GR, which then translocates to the nucleus of the cell, where it triggers changes in gene expression, with subsequent long-lasting effects on the structure and functioning of the cells (Warner-Schmidt and Duman 2006). In 2005, the first experimental evidence that compromised GR function concurrently evokes a BDNF dysregulation and a predisposition to depressive behavior was established by Ridder et al. (2005). They reported a down-regulation of BDNF protein content in the hippocampus of GR+/− mice (GR-heterozygous mutant mice with a 50 % GR gene dose reduction), which is in agreement with the neurotrophin hypothesis of depression. Sustained stress, along with the subsequent release of pro-inflammatory cytokines, leads to chronic neuroinflammation (Kim et al. 2016). They added that the elevated pro-inflammatory cytokine levels and hippocampal glucocorticoid receptors' (GRs') functional resistance are among the most widely investigated factors in the pathophysiology of depression (Kim et al. 2016). Because musk was reported by Wang et al. (2003) and Lin et al. (2004) to be used in treating inflammation in Chinese medicinal remedies due to its proved anti-inflammatory effect, this effect could be one of the possible mechanisms explaining the antidepressant-like effect of musk. In this study, it was observed that steroids account for up to 14 % of musk constituents and this is consistent with what was previously reported (Oh et al. 2002). Thus, another suggested mechanism of action on the part of musk is through GRs, which are reported to be abundant in the hippocampus (McEwen and Magarinos 2001).

Limitations of the study: This study did not confirm whether the anti-inflammation activities of musk participated in the anti-depressive effect in CUMS mice. In addition, the

connection between GR and BDNF expressions in the stress procedure was not further clarified. Further studies are needed to detect and explore the detailed mechanism underlying the anti-depressive effects of musk.

In conclusion, this study indicates that the inhalation of musk has an antidepressant effect on CUMS-induced depression in an animal model. The behavioral changes and elevated serum glucocorticoid levels induced by CUMS were significantly improved as compared with the control group. Neuronal and glial apoptosis were reduced, while neurogenesis was increased after musk inhalation as compared with the control group. GR and BDNF hippocampal expressions, as detected by RT-PCR, ELISA and immunohistochemical methods, were significantly up-regulated as compared with the model group.

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