Acta Pharmacologica Sinica

www.nature.com/aps

ARTICLE

# Cinnamaldehyde prevents intergenerational effect of paternal depression in mice via regulating GR/miR-190b/BDNF pathway

Zhi-ying Gao[1], Tian-yu Chen[1], Ting-ting Yu[1], Li-ping Zhang[1], Si-jie Zhao[1], Xiao-yang Gu[1], Ying Pan[1,2] and Ling-dong Kong[1,2]

Paternal stress exposure-induced high corticosterone (CORT) levels may contribute to depression in offspring. Clinical studies disclose the association of depressive symptoms in fathers with their adolescent offspring. However, there is limited information regarding the intervention for intergenerational inheritance of depression. In this study we evaluated the intervention of cinnamaldehyde, a major constituent of Chinese herb cinnamon bark, for intergenerational inheritance of depression in CORT- and CMS-induced mouse models of depression. Depressive-like behaviors were induced in male mice by injection of CORT (20 mg·kg$^{-1}$·d$^{-1}$, sc) for 6 weeks or by chronic mild stress (CMS) for 6 weeks. We showed that co-administration of cinnamaldehyde (10, 20, or 40 mg·kg$^{-1}$·d$^{-1}$, ig) for 6 weeks in F0 males prevented the depressive-like phenotypes of F1 male offspring. In addition, co-administration of cinnamaldehyde (20 mg·kg$^{-1}$·d$^{-1}$, ig) for 4 weeks significantly ameliorated depressive-like behaviors of chronic variable stress (CVS)-stimulated F1 offspring born to CMS mice. Notably, cinnamaldehyde had no reproductive toxicity, while positive drug fluoxetine showed remarkable reproductive toxicity. We revealed that CMS and CORT significantly reduced testis glucocorticoid receptor (GR) expression, and increased testis and sperm miR-190b expression in F0 depressive-like models. Moreover, pre-miR-190b expression was upregulated in testis of F0 males. The amount of GR on miR-190b promoter regions was decreased in testis of CORT-stimulated F0 males. Cinnamaldehyde administration reversed CORT-induced GR reduction in testis, miR-190b upregulation in testis and sperm, pre-miR-190b upregulation in testis, and the amount of GR on miR-190b promoter regions of F0 males. In miR-190b-transfected Neuro 2a (N2a) cells, we demonstrated that miR-190b might directly bind to the 3′-UTR of brain-derived neurotrophic factor (BDNF). In the hippocampus of F1 males of CORT- or CMS-induced depressive-like models, increased miR-190b expression was accompanied by reduced BDNF and GR, which were ameliorated by cinnamaldehyde. In conclusion, cinnamaldehyde is a potential intervening agent for intergenerational inheritance of depression, probably by regulating GR/miR-190b/BDNF pathway.

**Keywords:** depression; intergenerational inheritance; cinnamaldehyde; fluoxetine; corticosterone; GR

*Acta Pharmacologica Sinica* (2022) 43:1955–1969; https://doi.org/10.1038/s41401-021-00831-0

## INTRODUCTION

Depression is a prevalent and devastating psychiatric disorder and causes considerable mental pain and social-economic burden to patients [1]. The current consensus is that the genetic make-up of an individual contributes to depression risk and heritability, and environmental factors, particularly lifetime exposure to chronic stress, dramatically increase the risk of depression in an individual [2]. Recent studies have been paying increased attention to environmental factors experienced by parents that can affect their offspring across multiple generations [3]. To date, the association between depressive symptoms in fathers and their adolescent offspring has been observed in clinical cohort studies [4, 5]. Early interventions to paternal depression may reduce the prevalence of adolescent depression [4].

Methylation alteration of glucocorticoid receptor (GR) gene exon 1F promoter induced by posttraumatic stress disorder, is related to offspring's depression [6]. Exposue of adult males to synthetic glucocorticoids could affect DNA methylation in sperm of filial generation (F0) male mice and GR expression in hippocampus and kidney of first filial generation (F1) offspring, indicating that adults' stress-induced high level of endogenous glucocorticoids may lead to transcriptional and DNA methylation changes in nuclear steroid receptors in hippocampus of male offspring [7]. F1 offspring born to F0 males of chronic mild stress (CMS) exposure are susceptible to depression-like symptoms [8]. The expression levels of microRNA-190 (miR-190b), miR-144, and miR-98 in sperm are upregulated in corticosterone (CORT)-induced depression of mice [9]. In particular, miR-190b is an attractive regulation candidate of the synaptic plasticity in amygdala, which may be involved in the development or the maintenance of impulse phenotype [10]. Fluoxetine, a selective serotonin reuptake inhibitor, is one of the first-line antidepressants in clinical practice. Fluoxetine in F1 male rats of maternal separation can reverse anxiety-like behaviors in second filial generation 2 (F2) offspring [11] and attenuate pre-reproductive stress-induced alteration in maternal oocyte miRNA expression

[1]School of Life Sciences, Nanjing University, Nanjing 210023, China and [2]Institute of Chinese Medicine, Nanjing University, Nanjing 210023, China
Correspondence: Ying Pan (pany@nju.edu.cn) or Ling-dong Kong (kongld@nju.edu.cn)

Received: 26 July 2021 Accepted: 23 November 2021
Published online: 4 January 2022

and offspring phenotypes, while the treatment with fluoxetine in pregnant mice increases pup mortality [12]. Thus, it is an urgent need to explore safe and effective antidepressants to intervene the intergenerational inheritance of depression.

*Cinnamomum cassia* (*C. cassia*) has been used as a traditional Chinese medicine to improve depression, sexual function, and others [13–16]. Cinnamaldehyde (3-phenylprop-2-enal, Fig. 1a) is a kind of aldehydes extracted from *C. cassia*. Cinnamaldehyde is reported to have neuroprotective, anti-inflammatory, analgesic, and other pharmacological effects [17–22]. It alleviates chronic unexpected stress-induced depression-like behaviors in middle-aged rats [23]. However, it is unknown whether cinnamaldehyde intervenes the intergenerational inheritance of depressive phenotypes.

In the present study, we evaluated the intervention of cinnamaldehyde for intergenerational inheritance of depression in CORT- and CMS-induced mouse models of depression, respectively. We also assessed the male reproductive toxicity of cinnamaldehyde and fluoxetine. As the functional endpoint of the hypothalamic-pituitary-adrenal (HPA) axis, GR-mediated negative feedback is dysfunctional in depression and other psychiatric disorders [24]. Brain-derived neurotrophic factor (BDNF) expression in hippocampus of mice is increased by ketamine, its deletion in hippocampus attenuates antidepressant responses [25, 26]. Furthermore, BDNF-tropomysin-related kinase B (TrkB) signaling pathway as the upstream molecular effector of GR is responsible for the enhancement of fear memory induced by restraint stress [27]. To define the intervention mechanism of cinnamaldehyde, we focused on the regulation of GR to miR-190b in testis and sperm of F0 CORT-induced mouse model of depression. Furthermore, we predicted and validated the downstream target of miR-190b in hippocampus of F1 CORT- and CMS-induced mouse models of depression.

## MATERIALS AND METHODS

### Mice

C57BL/6 mice from Charles River (SCXK 2016-0003, Beijing, China) was maintained on a 12 h light/dark cycle with *ad libitum* access to normal food and water. All animal procedures were conducted in accordance with the guidelines established by the Institutional Animal Care Committee of Nanjing University. All behavioral tests occurred during the animals' light cycle (8:00–20:00). Experimenters were blinded to animal group, and the order of testing was counterbalanced during behavioral experiments.

### Depression models, treatments and breeding scheme

*Subcutaneous injection of CORT*. C57BL/6 male mice aged 7 weeks old were habituated to animal facilities for 1 week before the experiment. A schematic timeline and behavioral paradigm were shown in Fig. 1b. Mice subcutaneously injected with 20 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ CORT (containing 0.1% DMSO and 0.1% Tween-80) were randomized into five subgroups, receiving saline (vehicle), 10, 20, or 40 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ cinnamaldehyde (≥99%, Sigma-Aldrich, St. Louis, MO, USA) or $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ fluoxetine (≥99%, Aladdin, Shanghai, China) via daily intragastric gavage (10 mL/kg) for 6 weeks. Both cinnamaldehyde and fluoxetine were suspended in water by ultrasound, respectively.

Cinnamaldehyde is reported to alleviate chronic unexpected stress-induced depressive-like behaviors in male Sprague-Dawley rats at 22.5, 45, and 90 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ for 3 weeks [23]. It has antihyperglycemic and antihyperlipidemic actions in C57BLKS/J *db/db* mice at 20 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ for 4 weeks [28], inhibits inflammation and brain damage in a mouse model of permanent cerebral ischemia at 25, 50, and 75 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ for 3 d [29], reduces fructose-induced cardiac inflammation and fibrosis at 20, 40, and 80 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ for 5 weeks [30]. Fluoxetine is reported to reverse stress-induced behavioral changes at 15 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ in unpredictable chronic mild stress-stimulated mice [31]. Accordingly, based on these reports, the doses of 10, 20, and 40 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ cinnamaldehyde, as well as 15 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ fluoxetine were used by gavage once a day for 6 weeks in this study. During the 5th–6th week of treatment, body weight, sucrose preference test (SPT), open field test, and forced swim test (FST) were measured. After the behavioral tests, F0 male mice of CORT-induced model of depression and F0 male controls were mated with 8-week-old C57BL/6 female mice for 5 days to breed F1 offspring. After 5 days, males were removed and females were single housed until they littered down. On postnatal week 3, offspring were weaned and divided into new standard-housing boxes. Every box contained 3–5 mice of the same sex and same paternal treatment with *ad libitum* access to food and water. SPT, open field test, and FST were carried out when F1 offspring of CORT-induced model of depression and controls were 8 weeks of age.

*CMS*. C57BL/6 male mice aged 7 weeks old were habituated to animal facilities for 1 week before the experiment. CMS animals were housed separately and subjected to standard CMS protocols consisting of three stressors per day for 6 weeks before behavior testing [32]. Mice in this group experienced two stressors during the day and a different stressor during the night. Fully validated and approved standard stressors were randomly selected from the following stressors, which were unpredictable to the subjects: food deprivation for 12–16 h; water deprivation for 12–16 h; damp bedding (200 mL water poured into sawdust bedding) for 12–16 h; white noise (https://simplynoise.com/) for 1–16 h; individual housing (separating cage-mates into single-housing cages) for 1–16 h; cage tilt at a 45° angle for 1 to 16 h; strobe light illumination for 1 to 16 h; crowded housing (4–5 mice in a 10 cm × 13 cm × 13 cm plastic box with air holes) for 1–3 h; dark cycle (continuous darkness) for 24–36 h; and light cycle (continuous illumination) for 24–36 h. During the 5th–6th week of stress, body weight, SPT, open field test and FST were measured. After the behavioral test, F0 male mice of CMS-induced model of depression and F0 male controls were mated with 8-week-old C57BL/6 female mice for 5 d to breed F1 offspring. After 5 d, males were removed and females were single housed until they littered down. On postnatal week 3, offspring were weaned and divided into new standard-housing boxes. Every box contained 3–5 mice of the same sex and same paternal treatment with *ad libitum* access to food and water. SPT, open field test, and FST were carried out when F1 offspring of CMS-induced model of depression and controls were 8 weeks of age.

*Chronic variable stress (CVS)*. To investigate whether cinnamaldehyde could ameliorate the susceptibility of F1 offspring born to CMS mice in response to stress, we conducted a CVS experiment for 4 weeks according to previous study [33]. CVS was randomly assigned every day, and the order of seven kinds of stress was different every week. F1 males born to CMS/control F0 males were exposed to fox odor (1:5000; W332518, Sigma-Aldrich, St. Louis, MO, USA), constant light, presence of a novel object, multiple cage changes, saturated bedding overnight, novel 100 dB white noise (Sleep Machine; Brookstone, Merrimack, NH, USA), and restrained in a 50 mL conical tube. According to the dose of cinnamaldehyde and fluoxetine in CORT-induced mouse models of depression, CVS-exposed mice were intragastrically administered 20 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ cinnamaldehyde, 15 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ fluoxetine or an equivalent volume of saline as a control for 4 weeks in a volume of 10 mL/kg body weight. After the behavioral test, F1 male mice of CMS-induced model of depression and F1 male controls were mated with 8-week-old C57BL/6 female mice for 5 d to breed F2 offspring. The vaginal plug was checked every morning. After 5 d, males were removed and females were single housed until they littered down.

Behavioral tests

*SPT*. C57BL/6 mice of F0 males and F1 offspring of CORT- and CMS-induced model of depression were housed in a single cage and given two bottles of 1% sucrose solution for 24 h to adapt to the concentration of sucrose solution. Afterward, one of the bottles of sucrose solution was replaced with drinking water and given for 24 h, during which the position was changed at 12 h to prevent location habituation. After 24 h of water and food shortage, the bottles of 1% sucrose solution and drinking water were given to mice for 24 h weighed separately, and the positions were changed at 12 h. The percent of sucrose preference was calculated as the amount (g) of sucrose solution consumed over the total amount (g) of water and sucrose consumed [34].

*Open field test*. C57BL/6 mice of F0 males and F1 offspring of CORT- and CMS-induced models of depression were measured in an open field arena (50 cm × 50 cm × 40 cm) during a 10 min test under red lighting. A video tracking system (Xinruan, Shanghai, China) measured locomotor activity, as well as the time spent in the center and periphery of the test arena as an index of anxiety.

*Forced swim test (FST)*. The FST was performed to assess behavioral despair of C57BL/6 mice of F0 males and F1 offspring of CORT- and CMS-induced models of depression. Swim training was performed the day before the experiment, and the mice were placed in 15–20 cm deep water at 24 ± 1 °C for 15 min. On the day of the experiment, the mice were placed in water at a depth of 15–20 cm at 24 ± 1 °C for 5 min, and a video-tracking system (Xinruan, Shanghai, China) was used for video recording and software statistics.

Sperm count and motility measurement

The cauda epididymis of F0 male mice of CORT-induced model of depression and F1 offspring of CMS-induced model of depression were minced in normal saline at 37 °C and incubated for 5 min at 37 °C to release the sperm. The sperm mixture was dropped on a glass slide in a wet preparation ~20 μm deep. After the sample was settled on the slide (within 1 min), the slide was observed with a phase-contrast microscope at 400 magnification. Approximately 200 spermatozoa were evaluated for each specimen to calculate the percentage of the active type, inactive type, and completely immobile type. The sperm count was quantified in a hemocytometer under a light microscope. Sperm morphology was assessed with a light microscopic following Wright-Giemsa staining.

Histological examination

The testes or epididymis of F0 male mice of CORT-induced model of depression and F1 offspring of CMS-induced model of depression was removed, fixed in 4% formalin for 24 h, dehydrated in graded ethanol, embedded in paraffin, and sectioned. The sections were stained with hematoxylin-eosin staining (H&E) and observed under a light microscope (Leica DM500, Wetzlal, Germany). Six microscopic fields of every section of the testes were randomly selected (×400 magnification) and different germ cells (spermatogonia, spermatocytes, and round spermatids) were counted.

CORT assay

Blood samples of animals were collected through the posterior orbital venous plexus between 09:00 and 12:00 under anesthesia by intraperitoneal injection of 50 mg/kg sodium pentobarbital. These samples were allowed to clot for 2 h at room temperature and centrifuged at 3000 × *g* for 10 min at 4 °C, after which serum was collected and stored at −80 °C. CORT in serum was quantified by enzyme-linked immunosorbent assay (Parameter Corticosterone Assay KGE009, R&D Systems, Minneapolis, MN, USA).

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from hippocampus (F0 male mice of CORT-induced model of depression and F1 offspring of CORT- and CMS-induced models of depression), testes, epididymis, and sperm (F0 male mice of CORT- and CMS-induced model of depression) after behavioral tests using TRIzol reagent (15596018, Invitrogen, Carlsbad, CA, USA). To quantify GR, BDNF, and GAPDH mRNA expression, oligo (dT)18 primers (3805, TaKaRa, Dalian, China) were used to reverse transcribe total RNA into cDNA. The reaction mixture was incubated at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. Then, RT-qPCR was performed using SYBR Green (31000, Biotium, Bay Area, California, USA) and specific primers for GR, BDNF, and GAPDH. To quantify precursors of miR-190b (pre-miR-190b), miR-190b, miR-144, and miR-98 expression, PCR primers were synthesized by GENERAL BIOL (Anhui, China). The primer sequences were listed in Supplementary Table S1. The real-time PCR cycles consisted of a pre-denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 30 s. The fold-change in GR and BDNF expression was normalized to GAPDH expression. The fold-change in pre-miR-190b, miR-190b, miR-144, and miR-98 expression was normalized to U6 expression, respectively. The relative expression levels of target genes were determined by the Ct ($2^{-\Delta\Delta Ct}$) method.

Western blot analysis

RIPA lysis buffer freshly mixed with a protease inhibitor cocktail was used to isolate proteins from hippocampus (F0 male mice of CORT-induced model of depression and F1 offspring of CORT- and CMS-induced models of depression), testes, epididymis, and sperm (F0 male mice of CORT- and CMS-induced model of depression) after behavioral tests. Proteins were separated by SDS-PAGE with 12.5% gels. Blots were incubated with primary antibodies, including anti-GR antibody (1:1000; 12041, Cell Signaling Technology, Danvers, MA, USA), anti-BDNF antibody (1:2000; ab108319, Abcam, Cambridge, UK), and anti-β-actin antibody (1:1000; sc-47778, Santa Cruz, Dallas, TX, USA), overnight at 4 °C followed by the incubation of secondary antibodies. Immunoreactive bands were visualized via chemiluminescence and quantified using ImageJ software (version 1.42q, NIH, Bethesda, MD, USA).

Immunohistochemical staining of testes

The paraffin testes sections of F0 male mice of CORT-induced model of depression after behavioral tests were deparaffinized, rehydrated, treated with 3% $H_2O_2$ to block endogenous peroxidase activity, and washed in $ddH_2O$. Antigen retrieval was performed using citric acid. The sections were incubated with blocking solution, processed using an AB blocking kit, and incubated overnight with anti-GR antibody (1:400; 12041, Cell Signaling Technology, Danvers, MA, USA). The sections were then incubated with mouse biotin, processed with the ABC kit (Servicebio Technology, Wuhan, China), and stained with DAB and hematoxylin. The sections were dehydrated, cleared in xylene and cover slipped. The sections were viewed using a microscope under ×20 magnification to monitor the color of the nucleus: a cell was considered positive if the nucleus was stained brown. The integral optical density was determined automatically by software (Image-pro plus 6.0, Media Cybernetics, MD, USA).

Chromatin immunoprecipitation (ChIP) assay of testes

The ChIP assay of testes of F0 male mice of CORT-induced model of depression was performed using a commercial kit (9005, Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. An antibody against GR (1:50; 12041, Cell Signaling Technology, Danvers, MA, USA) was used to immunoprecipitate GR-chromatin complexes. Anti-IgG served as a negative control. The ChIP products were assessed by real-time PCR. The primers for amplification are listed in Supplementary

Table S1. The real-time PCR cycles consisted of a pre-denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s.

Luciferase reporter assay of Neuro 2a (N2a) cells

N2a cells was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The wild-type (WT) BDNF-3′-UTR vector was constructed by inserting the amplified 3′-UTR of BDNF into a luciferase reporter vector. The sites that interact with the miR-190b seed sequence were mutated from ACATATC to TGTATAG to create a mutant BDNF 3′-UTR, which was inserted into a luciferase reporter plasmid to construct the BDNF-3′-UTR vector. Luciferase reporter plasmid, β-galactosidase (β-gal) expression plasmid, and the miR-190b mimic, inhibitor or scrambled negative control RNA were co-transfected into N2a cells using Lipofectamine 2000, with the β-gal plasmid serving as a transfection control. After 24 h, the cells were analyzed for luciferase activity on a Modulus Luminometer using luciferase assay kit (E1500, Promega, Madison, WI, USA).

Statistical analysis

The results are presented as the mean ± SD. Analyses with more than two groups and one variable were performed using one-way ANOVA with Bonferroni *post hoc* test or Fisher's LSD *post hoc* test. Analyses with more than two variables were performed using two-way ANOVA with Bonferroni *post hoc* test or Fisher's LSD *post hoc* test. Analyses with two groups were performed using an unpaired, two-tailed Student's *t* test. Observed differences were considered statistically significant at $P < 0.05$. Statistical analysis was performed using GraphPad Prism software 7 (GraphPad Software, San Diego, CA, USA).

## RESULTS

Cinnamaldehyde exerts antidepressant-like effects in F0 males of CORT-induced mouse model of depression

Cinnamaldehyde and fluoxetine decreased serum CORT levels in F0 males of CORT-induced depression-like model (Fig. 1c). There was no remarkable difference in body weight between F0 control males (F0-Ctl) and F0 males of CORT-induced depression-like model treated with vehicle (F0-CORT) (Fig. 1d). Compared with F0-Ctl, F0-CORT showed sucrose consumption reduction in SPT (Fig. 1e), the decreased time in the center and similar total travel distance in an open field test (Fig. 1f), and the increased immobility time in FST (Fig. 1g), which were attenuated by cinnamaldehyde in F0 males of CORT-induced depression-like model (F0-CA). These data indicated cinnamaldehyde effectively decreased the anhedonia, anxiety and behavioral despair in F0 males of CORT-induced depression-like model. However, fluoxetine in F0 males of CORT-induced depression-like model (F0-FLX) only ameliorated behavioral despair (Fig. 1g), without prominent effect on anhedonia and anxiety (Fig. 1e, f).

We next tested the expression of GR and BDNF in hippocampus, and observed a prominent downregulation of GR and BDNF mRNA (Fig. 1h, i) and protein levels (Fig. 1j) in hippocampus of F0-CORT, which were substantially restored by cinnamaldehyde and fluoxetine (Fig. 1h-j).

Fluoxetine but not cinnamaldehyde substantially damaged fertility in F0-CORT

To investigate the effect of cinnamaldehyde and fluoxetine on the intergenerational inheritance of depression traits in F0-CORT, our experimental strategies to breed F1 offspring were shown in Fig. 2a. Consistent with one previous study [9], CORT did not affect male fertility compared with F0-Ctl (Fig. 2b–i). F0-CA also had no effect on the percentage of successful mating and pregnancy, number of F1 pups, weight ratio of testis and epididymis, sperm count, sperm motility as well as morphology of testis, epididymis, and sperm compared with F0-CORT (Fig. 2b–i). However, fluoxetine substantially reduced the percentage of successful mating between F0-CORT and naive females (Fig. 2b). Strikingly, the percentage of successful pregnancy and number of F1 pups were zero in mating between F0-FLX and naive females (Fig. 2c, d). Moreover, weight ratios of testis and epididymis, sperm count, and sperm motility were markedly decreased in F0-CORT (Fig. 2e–h). Histological examination further showed that the number of spermatocytes and round spermatids in the seminiferous tubules as well as sperm filling capacity of the epididymis was remarkably reduced in F0-FLX (Fig. 2i). There was no observed difference in sperm morphology between F0-CORT and F0-FLX (Fig. 2i).

Cinnamaldehyde prevents paternal transmission of CORT-induced depressive phenotype to F1 male offspring

In F1 male offspring, differences in body weight, sucrose consumption in SPT, time spent in the center of an open field, and locomotor activity were not observed between F1 males born to F0-CORT (F1-CORT males) and F1 males born to F0-Ctl (F1-Ctl males) (Fig. 3a–c). Under baseline condition, F1-CORT males showed more time of immobility in the FST than F1-Ctl males (Fig. 3d). Moreover, there was higher serum CORT levels in F1-CORT males than in F1-Ctl males (Fig. 3e). F1 males born to F0-CA (F1-CA males) had less time of immobility (Fig. 3d) and lower serum CORT levels (Fig. 3e) than F1-CORT males, indicating that cinnamaldehyde prevented intergenerational inheritance of behavioral despair of F0-CORT.

In F1 female offspring, only body weight was higher in F1 females born to F0-CORT (F1-CORT females) than in F1 females born to F0 control (F1-Ctl females), but there was no difference in body weight among F1 females born to F0-CA (F1-CA females) and F0-CORT females (Supplementary Fig. S1a). However, prominent differences in anhedonia, anxiety, locomotion, and behavioral despair among F1 females of F1-CORT, F1-CA and F1-Ctl were not observed (Supplementary Fig. S1b–d). Serum CORT levels in females of F1-CORT and F1-CA were similar to F1-Ctl females (Supplementary Fig. S1e). These data indicated that the intergenerational effect of CORT exposure was different between males and females. Paternal CORT exposure-induced depressive phenotype was not transmitted to F1-CORT females, and F1-CA females also did not have depressive phenotype.

Cinnamaldehyde treats depression after CVS in F1 males born to a paternal CMS-induced depression model

To reproduce stress-induced intergenerational impact, we adapted a CMS mouse model of depression [32] and followed mouse breeding protocol to produce F1 offspring as previously described [3]. As expected [31, 32], CMS-stimulated F0 males (F0-CMS) showed body weight loss, anhedonia, anxiety, and behavioral despair with the HPA axis over-activation compared with F0-Ctl (Supplementary Fig. S2a–e). However, these remarkable body weight change, anhedonia, anxiety, or behavioral despair were not observed in F1 offspring born to F0-CMS (F1-CMS) under baseline condition (Supplementary Fig. S3a–d). F1 offspring born to F0 males of CMS-induced depression model is susceptible to depression-like symptoms by exposure to a slight CVS [8].

To investigate the therapeutic effect of cinnamaldehyde and fluoxetine on the inheritance of depression, cinnamaldehyde and fluoxetine were administered to F1 males exposed to CVS (the scheme as Fig. 4a). After exposure to CVS, body weight of F1 males born to F0-Ctl and F0-CMS was still similar, and the treatment of cinnamaldehyde and fluoxetine did not affect body weight of F1 males born to F0-Ctl and F0-CMS (Fig. 4b). Although F1 males born to F0-Ctl (F1-Ctl) had mild anxiety after CVS, F1-CMS animals were more susceptible to anhedonia, anxiety, and behavioral despair than F1-Ctl (Fig. 4c–e). Moreover, F1-CMS animals had higher serum CORT levels than F1-Ctl by CVS (Fig. 4f). Cinnamaldehyde

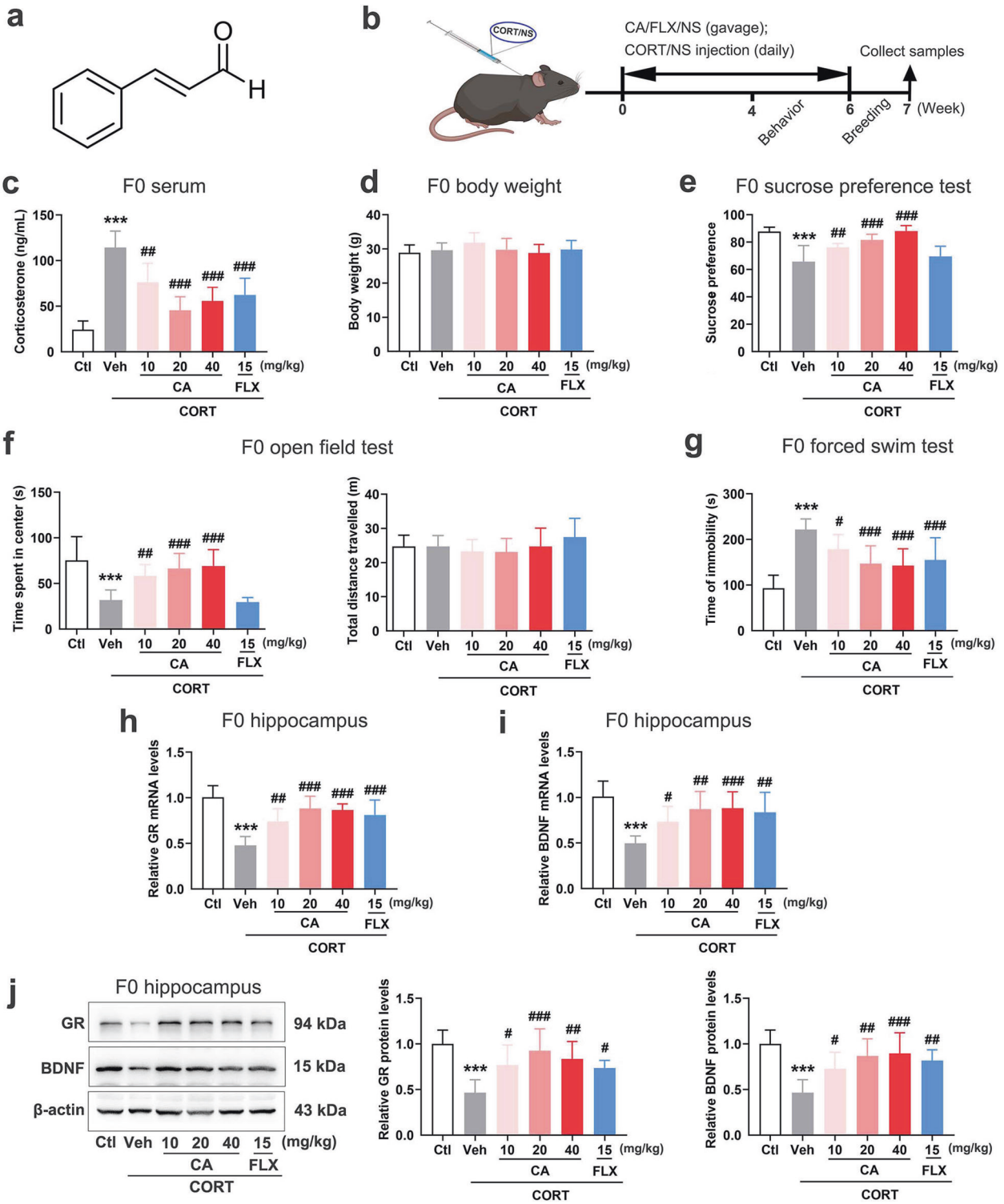


**Fig. 1** Effects of cinnamaldehyde (CA) on depression-like behaviors and physiology in CORT-stimulated F0 males. **a** Chemical structure of CA. **b** The timeline of CA/fluoxetine treatment, behavioral testing and breeding in F0 CORT-stimulated mice. Created in BioRender.com. **c** Serum corticosterone levels ($n = 6$). **d** Body weight ($n = 10$). **e** Percentage preference for 1% sucrose solution in a two-bottle choice test ($n = 10$). **f** Open field center (left) exploration time and (right) total distance traveled ($n = 10$). **g** Immobility time in FST ($n = 10$). **h**, **i** RT-qPCR analysis of GR and BDNF mRNA levels in hippocampus ($n = 5$–6). **j** Western blot analysis of GR and BDNF protein levels in hippocampus ($n = 5$). Ctl, Control; Veh, Vehicle; CA, Cinnamaldehyde; FLX, Fluoxetine. Data are expressed as mean ± SD, $^{***}P < 0.001$ vs F0-Control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs F0-CORT vehicle group.

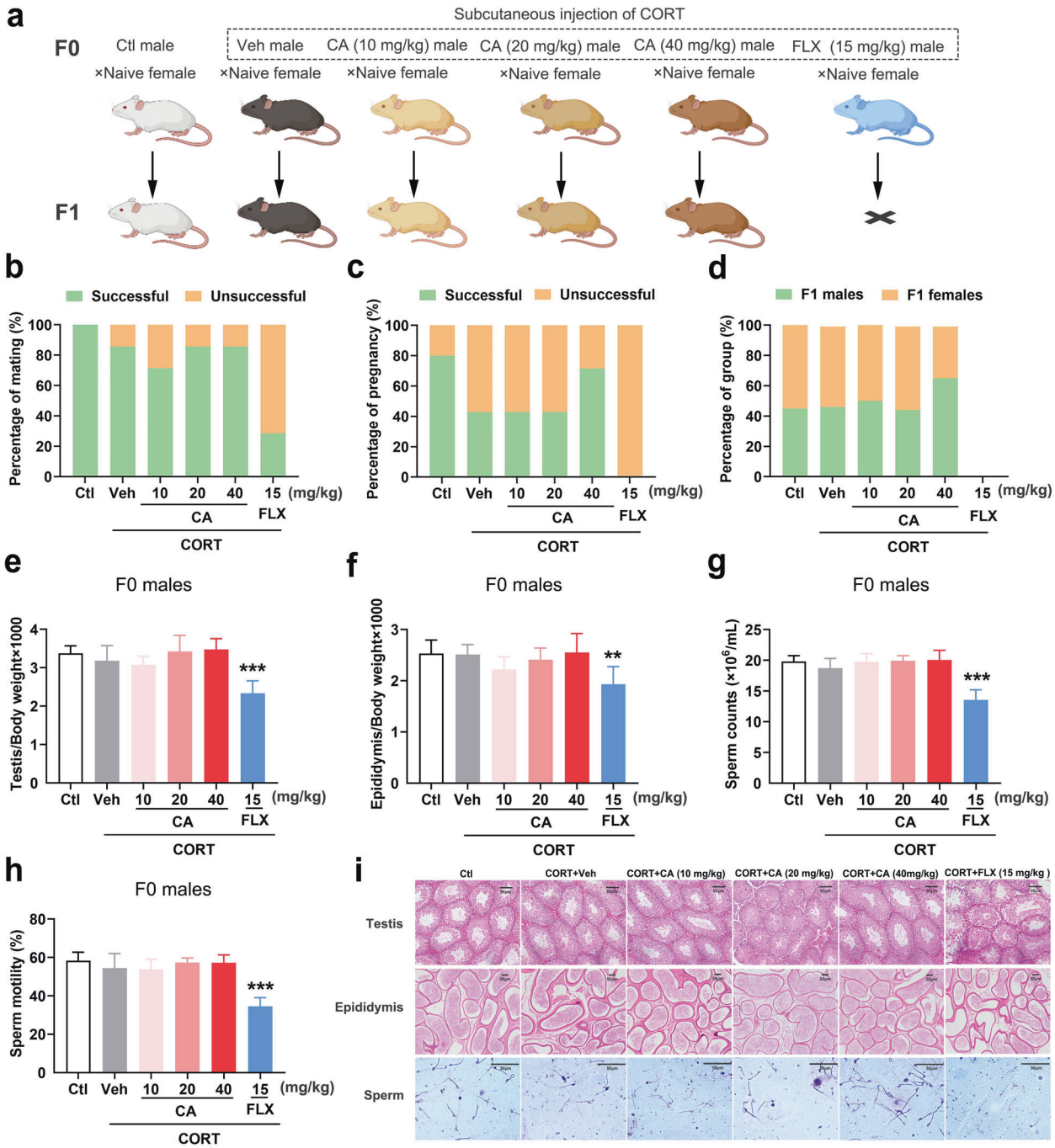


**Fig. 2** Effects of cinnamaldehyde (CA) on male reproductive functions in CORT-stimulated F0 males. **a** Weaning strategies for CORT-stimulated F0 males. Created in BioRender.com. **b** The percentage of successful mating between F0-CORT and naive females. **c** The percentage of successful pregnancy. **d** The percentage of sex of F1 Pups. **e** Ratio of testis and body weight (testis/body weight ×1000) was examined ($n = 7$). **f** Ratio of epididymis and body weight (epididymis/body weight ×1000) was examined ($n = 7$). **g**, **h** Sperm counts (**g**) and motility (**h**) were examined from cauda epididymis in each mouse ($n = 7$). **i** Morphological examination in the testis, epididymis and sperm of CORT-stimulated F0 males ($n = 5$). The sections of testis and epididymis were stained with H&E, the sperm morphology of smear was stained with Wright-Giemsa staining. Scale bars, 50 μm. Ctl, Control; Veh, Vehicle; CA, Cinnamaldehyde; FLX, Fluoxetine. Data are expressed as mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$ vs F0-CORT vehicle group.

markedly alleviated CVS-induced anxiety and elevation of serum CORT in F1-Ctl (Fig. 4d and f), as well as ameliorated CVS-induced anhedonia, anxiety, behavioral despair, and elevation of serum CORT in F1-CMS (Fig. 4c–f). Fluoxetine substantially decreased CVS-induced elevation of serum CORT in F1-Ctl (Fig. 4f), and attenuated CVS-induced anxiety, behavioral despair and elevation of serum CORT in F1-CMS (Fig. 4d–f), without difference in anhedonia of F1-CMS and anxiety of F1-Ctl (Fig. 4c, d).

### Fluoxetine but not cinnamaldehyde substantially affects fertility in F1 males of CVS

To validate the effect of cinnamaldehyde and fluoxetine for 4 weeks on fertility, F1-CMS/F1-Ctl after CVS and drug treatment were mated with naive females to breed F2 offspring. Compared with F1 males of control, CVS did not affect the percentage of successful mating and pregnancy, number of F2 pups, the weight ratio of testis and epididymis, sperm count, sperm motility as well

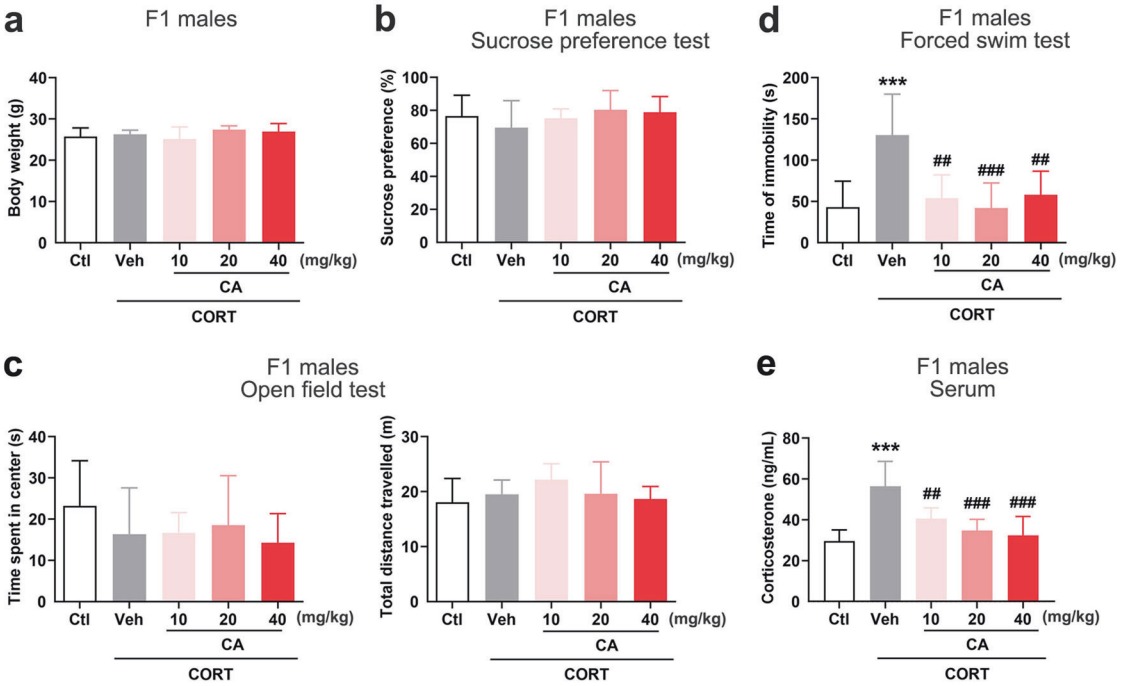


**Fig. 3** Cinnamaldehyde (CA) prevents paternal transmission of CORT-induced depression-like behaviors to F1 male offspring. **a** Body weight ($n = 6–10$). **b** Percentage preference for 1% sucrose solution in a two-bottle choice test ($n = 6–10$). **c** Open field center (left) exploration time and (right) total distance traveled ($n = 6–10$). **d** Immobility time in FST ($n = 5–9$). **e** Serum corticosterone levels ($n = 6$). Ctl, Control; Veh, Vehicle; CA, Cinnamaldehyde. Data are expressed as mean ± SD, $^{***}P < 0.001$ vs F1-Ctl group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs F1-CORT vehicle group.

as morphology of testis, epididymis, and sperm (Fig. 5a–j). Compared with F1 males of CVS with vehicle, cinnamaldehyde had no effect on the percentage of successful mating and pregnancy, number of F2 pups, the weight ratio of testis and epididymis, sperm count, sperm motility as well as morphology of testis, epididymis, and sperm in F1 males of CVS (Fig. 5a–j). Consistent with above observations (Fig. 2b–i), fluoxetine substantially reduced the percentage of successful mating, pregnancy and number of F2 pups in mating between F1 males and naive females (Fig. 5a–c). Moreover, fluoxetine markedly decreased weight ratio of testis and epididymis, sperm count, and sperm motility in F1 males of CVS (Fig. 5d–g). Similarly, histological examination further showed that fluoxetine obviously decreased the number of spermatocytes and round spermatids in the seminiferous tubules and sperm filling capacity of the epididymis in F1 males (Fig. 5h, i), however, fluoxetine did not impact the morphology of sperm (Fig. 5j).

### Cinnamaldehyde reverses CMS/CORT-induced downregulation of GR in testis of F0 males

GR expression in hippocampus of F0-CORT was substantially less than F0-Ctl (Fig. 1h, j). These data raised the possibility that enhanced HPA axis activity may induce GR dysregulation in male reproductive system, possibly contributing to the intergenerational inheritance of depression. Therefore, we examined GR expression in testis and epididymis of F0-CMS. The decrease in GR expression was testis-specific, as there was less GR expression in F0-CMS than in F0-Ctl, but there was no difference in GR expression in caput epididymis and cauda epididymis of F0-CMS compared with F0-Ctl (Fig. 6a, b). Low GR expression levels were also observed in testis of F0-CORT, which were substantially increased by cinnamaldehyde and fluoxetine (Fig. 6c–e). These results indicated that CMS/CORT might induce GR-dependent epigenetic changes in testis, which might be transmitted to zygotes via sperm.

### Cinnamaldehyde suppresses miR-190b expression in testis and sperm of F0-CORT by restoring GR downregulation in testis of F0-CORT

Next, we examined miR-190b, miR-144 and miR-98 expression levels in testis and sperm of CORT- and CMS-induced mouse models of depression, which were increased in sperm by CORT as reported previously [9]. RT-qPCR analysis revealed high miR-190b expression in both testis and sperm of F0-CMS versus F0-Ctl, respectively (Fig. 7a, b). However, miR-144 and miR-98 expression levels were not changed in testis and sperm of F0-CMS versus F0-Ctl (Fig. 7a, b). Moreover, F0-CMS had more pre-miR-190b expression in testis than F0-Ctl, but there was no difference in pre-miR-190b expression in sperm of F0-CMS compared with F0-Ctl (Fig. 7c). These observations indicated that the increase in pre-miR-190b expression was testis-specific, and miR-190b upregulation in testis may contribute to the upregulation of miR-190b in sperm of F0-CMS. Similarly, miR-190b expression levels in both testis and sperm (Fig. 7d, e) and pre-miR-190b expression in testis (Fig. 7f) of F0-CORT were increased compared with F0-Ctl. Cinnamaldehyde and fluoxetine blocked upregulation of miR-190b expression in both testis and sperm of F0-CORT (Fig. 7d, e), and of pre-miR-190b expression in testis of F0-CORT (Fig. 7f).

As GR expression was decreased in testis of F0-CMS and F0-CORT (Fig. 6a–e), we next hypothesized that the downregulation of transcription factor GR would enhance the transcription of miR-190b in testis. To test this hypothesis, the potential promoter region (2 kb upstream of the transcriptional start site) of miR-190b was analyzed using JASPAR [35] and LASAGNA-Search 2.0 [36]. The results showed that GR could potentially target the promoter region of miR-190b (Fig. 7g). ChIP showed that GR was localized on miR-190b promoter regions in testis, and F0-CORT had less amount of GR on miR-190b promoter regions in testis than F0-Ctl (Fig. 7h), indicating that GR may negatively regulate the transcription of miR-190b via specific GR-binding motifs in the promoter region of miR-190b in testis. Cinnamaldehyde and fluoxetine increased the amount of GR on miR-190b promoter

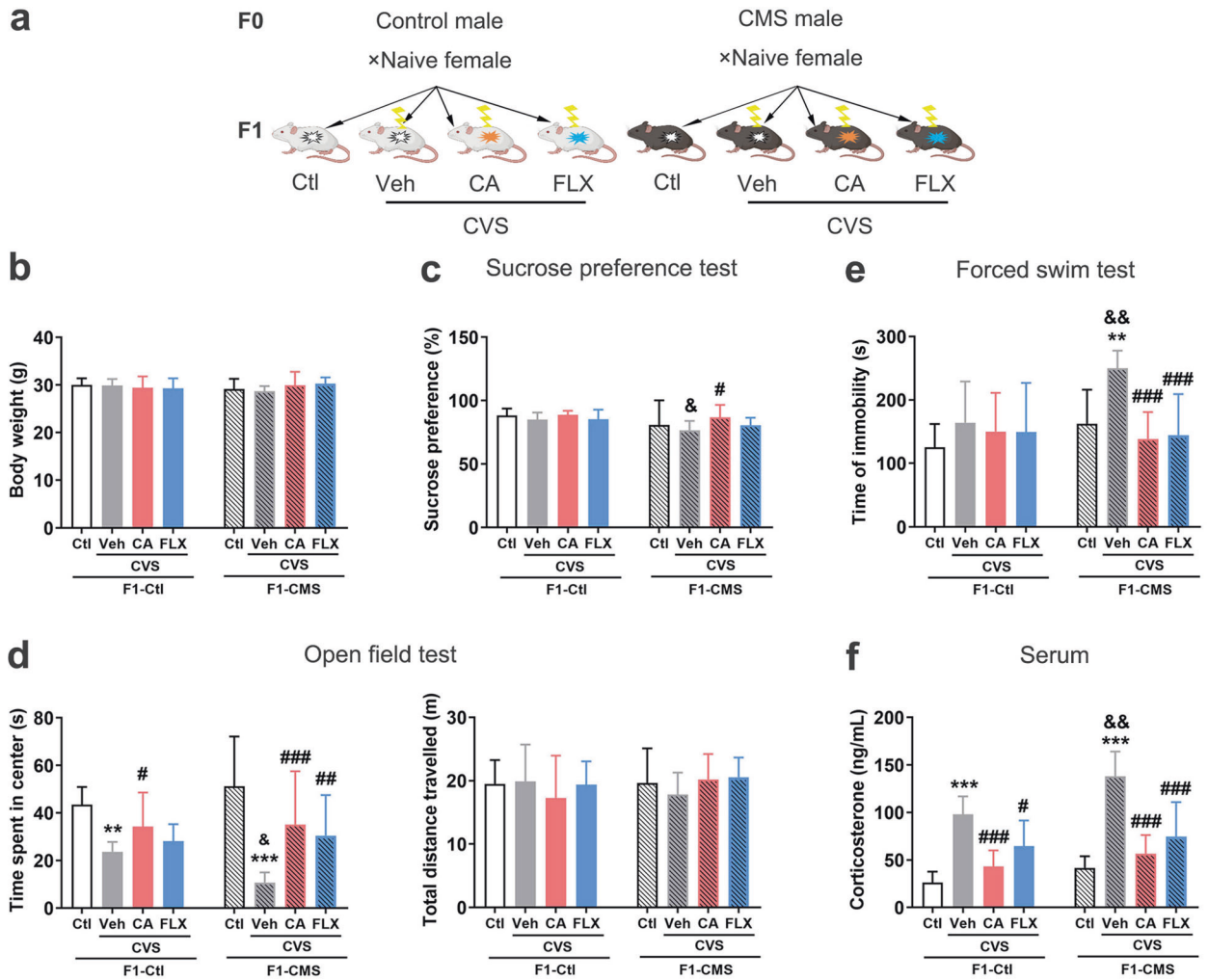


**Fig. 4** Cinnamaldehyde (CA) ameliorates depression-like behaviors of CVS-stimulated F1 offspring born to F0 males of CMS-induced depression model. **a** The timeline of CA/fluoxetine treatment and behavioral testing in F1 offspring born to F0-CMS/Control. White star: vehicle; orange star: CA; blue star: Fluoxetine. Created in BioRender.com. **b** Body weight ($n = 10$). **c** Percentage preference for 1% sucrose solution in a two-bottle choice test ($n = 10$). **d** Open field center (left) exploration time and (right) total distance traveled ($n = 10$). **e** Immobility time in FST ($n = 10$). **f** Serum corticosterone levels ($n = 6$). Ctl, Control; Veh, Vehicle; CA, Cinnamaldehyde; FLX, Fluoxetine. Data are expressed as mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs CVS vehicle groups; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs F1-Ctl groups, respectively.

regions in testis of F0 mice of CORT-induced model of depression (Fig. 7h), possibly showing the suppression of miR-190b transcription by upregulating GR expression in testis.

Cinnamaldehyde upregulates GR, downregulates miR-190b, and increases BDNF expression in hippocampus of F1-CORT and F1-CMS

To investigate the target gene of miR-190b on modulating depression of intergenerational inheritance in males, a combination of bioinformatics software TargetScan [37] and RNAhybrid [38] was used to predict candidate genes. Among these candidate genes, BDNF was also one of them, and the minimum free energy hybridization of the binding site between BDNF and miR-190b was −20.7 kcal/mol (Fig. 8a). To verify the direct binding of miR-190b to the target gene BDNF, a firefly luciferase reporter plasmid containing wild-type or mutant fragment of BDNF 3′-UTR across the miR-190b binding sites were constructed. The wild-type or mutant plasmid was transfected into N2a cells along with miR-190b mimic, miR-190b inhibitor or scrambled negative control RNA, respectively. As shown in Fig. 8b, miR-190b mimic reduced the luciferase activity compared with control mimic, whereas miR-190b inhibitor increased the luciferase activity compared with control inhibitor. However, the luciferase activity of mutant reporter plasmid was no longer influenced by miR-190b mimic or inhibitor (Fig. 8b). These data indicated that miR-190b might directly bind to the 3′-UTR of BDNF.

Subsequently, F1-CORT males had more miR-190b expression in hippocampus than F1-Ctl males, whereas F1 males born to cinnamaldehyde had less miR-190b expression in hippocampus than F1-CORT males (Fig. 8c). As anticipated, F1-CORT males exhibited GR and BDNF downregulation in hippocampus compared with F1-Ctl males (Fig. 8d, e). F1-CA males had higher GR and BDNF expression in hippocampus than F1-CORT males (Fig. 8d, e). Either baseline condition or exposure to CVS, miR-190b expression levels were higher in hippocampus of F1-CMS than that in F1-Ctl, respectively (Fig. 8f). Additionally, the downregulation of GR and BDNF in hippocampus of F1-CMS was observed (Fig. 8g, h). Cinnamaldehyde in F0 males of CORT-induced depression-like mode prevented downregulation of GR, upregulation of miR-190b, and downregulation of BDNF in hippocampus of F1-CORT (Fig. 8c–e). Cinnamaldehyde and fluoxetine in male F1-CMS were observed to upregulate GR,

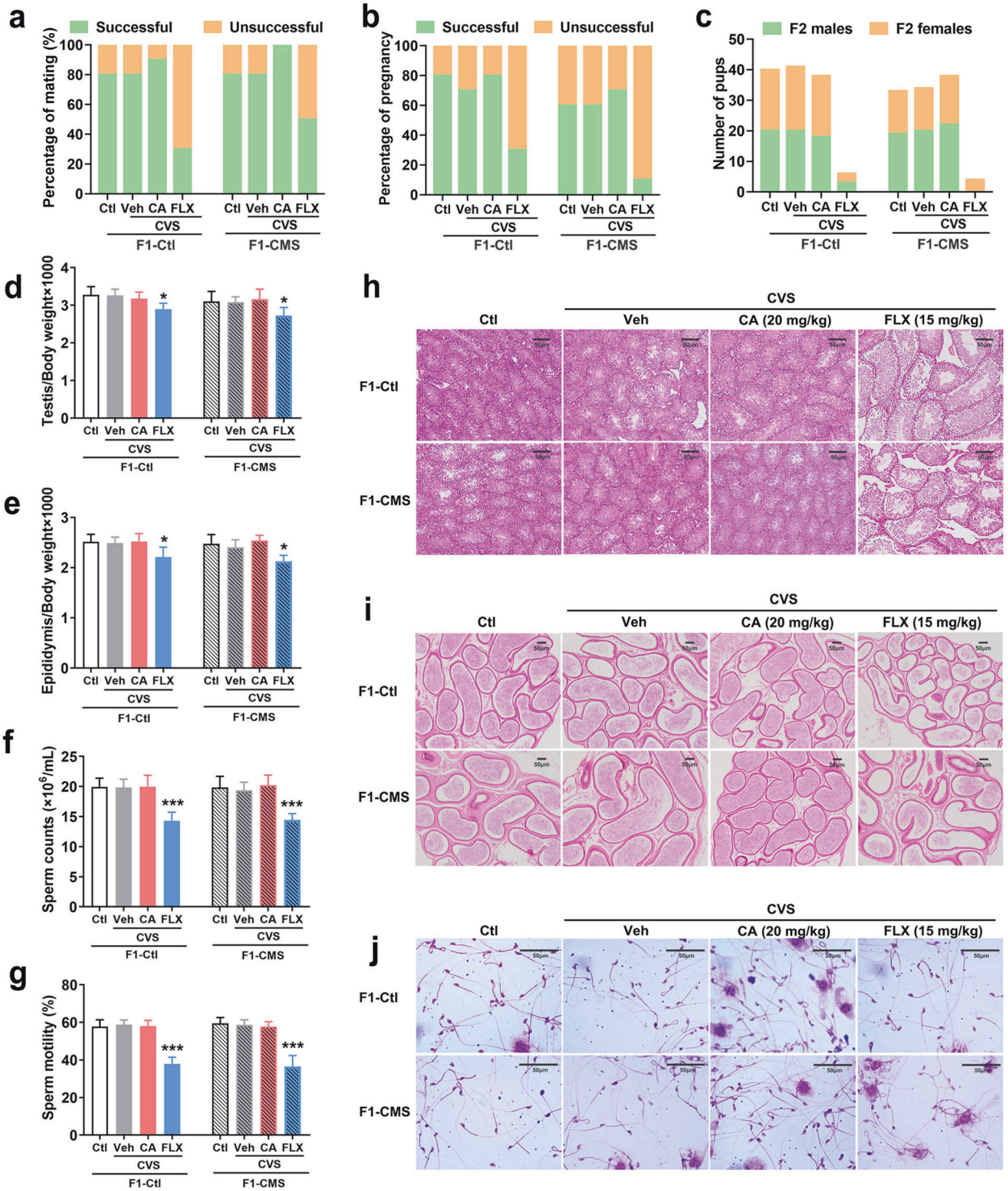


**Fig. 5** Effects of cinnamaldehyde (CA) on male reproductive functions in CVS-stimulated F1 offspring born to F0 males of CMS-induced depression model. **a** The percentage of successful mating between F1-males and naive females. **b** The percentage of successful pregnancy. **c** Number of F2 Pups. **d** Ratio of testis and body weight (testis/body weight ×1000) was examined ($n = 6$). **e** Ratio of epididymis and body weight (epididymis/body weight ×1000) was examined ($n = 6$). Sperm counts (**f**) and motility (**g**) were examined from cauda epididymis in each mouse ($n = 6$). Morphological examination in testis (**h**), epididymis (**i**) and sperm (**j**) of F1 males ($n = 5$). The sections of testis and epididymis were stained with H&E, the sperm morphology of smear was stained with Wright-Giemsa staining. Scale bars, 50 μm. Ctl, Control; Veh, Vehicle; CA, Cinnamaldehyde; FLX, Fluoxetine. Data are expressed as mean ± SD, $^{*}P < 0.05$, $^{***}P < 0.001$ vs CVS vehicle group.

downregulate miR-190b and increase BDNF expression in hippocampus of male F1-CMS (Fig. 8f–h).

Taken together, these results indicated that miR-190b post-transcriptionally suppressed BDNF expression in hippocampus of F1-CORT and F1-CMS. BDNF-TrkB signaling pathway was the upstream molecular effector of GR [27]. The inhibition of the molecular cascade reaction of BDNF-related signaling pathway might result in GR downregulation in hippocampus of males of F1-

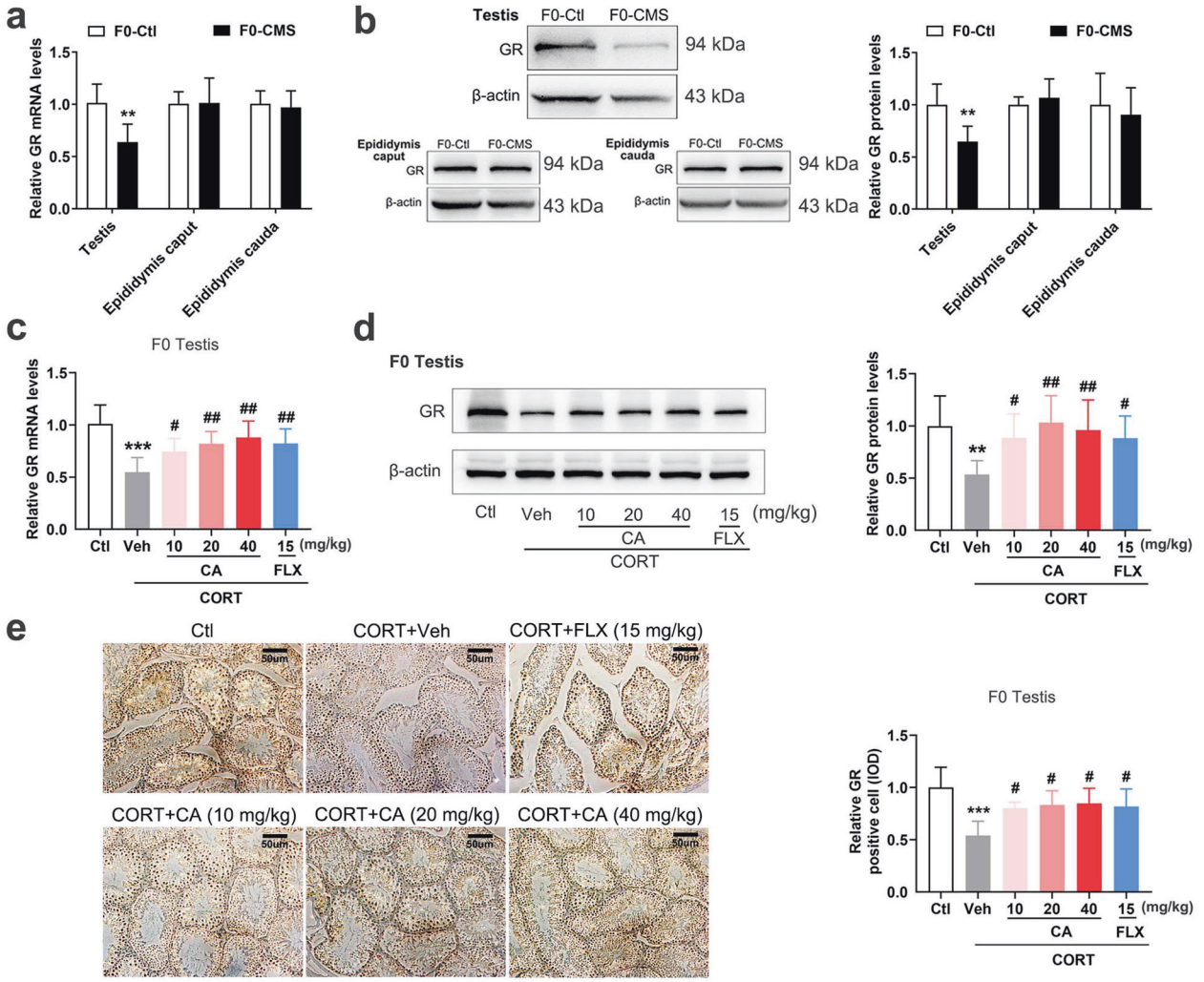


**Fig. 6** CMS/CORT exposure markedly downregulates GR expression in testis of F0 males, which are attenuated by cinnamaldehyde (CA). Analysis of GR mRNA (**a**) and protein (**b**) levels in testis, caput epididymis and cauda epididymis of F0-CMS versus F0-Control ($n = 6$). Analysis of GR mRNA (**c**) and protein (**d**) levels in testis of F0-CORT ($n = 5$). **e** Immunohistochemical staining for GR in testis of F0-CORT ($n = 5$). Scale bars, 50 μm. Ctl, Control; Veh, Vehicle; CA, Cinnamaldehyde; FLX, Fluoxetine. Data are expressed as mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$ vs F0-Control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs F0-CORT vehicle group.

CORT and F1-CMS. Cinnamaldehyde may intervene intergenerational inheritance of depression in males by modulating GR/miR-190b/BDNF pathway.

## DISCUSSION

Evidence has shown that paternal exposure to stress and elevated CORT can mediate depression-like traits in offspring [2, 9]. Our study demonstrated that cinnamaldehyde could intervene depression in F1 males born to CORT- and CMS-induced mouse models of depression. Previously, there is limited information regarding the intervention for intergenerational inheritance of depression with safe and effective drugs. In addition, the existing antidepressants often have treatment limitations, especially reproductive toxicity [39–45], further highlighting these unmet needs [1]. Despite cinnamaldehyde had an obvious antidepressant effect in mouse model of depression, which was supported by both our study and another previous report [23], it remains unknown whether cinnamaldehyde can intervene the intergenerational inheritance of depression. Notably, our study found that cinnamaldehyde had no male reproductive toxicity, while the classical antidepressant fluoxetine had obvious male reproductive toxicity and even resulted in complete infertility. Additionally, fluoxetine treatment failed to substantially improve anxiety-like behavior of CORT-disturbed mice, which might be due to the substantial time lag and low rate of efficacy of fluoxetine.

There are some important points that deserve more in-depth discussion. First, several typical depression models have been applied in the literature. Stress-related models are were the earliest established models. Considering the uncontrollability of stressors and passive/active coping responses of rats, HPA axis activation-related models are then established based on the fact that glucocorticoid homeostasis is disrupted by excessive activity of HPA axis in depression [46]. Therefore, these models can be divided into two categories: stress-related models (including CMS, chronic social defeat stress, and unpredictable maternal separation combined with unpredictable maternal stress) and HPA axis-related models (including CORT-induced model and mice models of mutations in HPA axis [2, 8, 47–49]. In order to comprehensively evaluate the role of cinnamaldehyde in depression, we selected one model from each of the two category models (CMS and CORT models) in our study. Additionally, we used CORT-induced mouse model of depression by subcutaneous injection of CORT, so as to avoid the individual differences caused by oral CORT. Second, cinnamic acid is the first metabolite of cinnamaldehyde. A part of cinnamaldehyde was is metabolized into cinnamic acid in

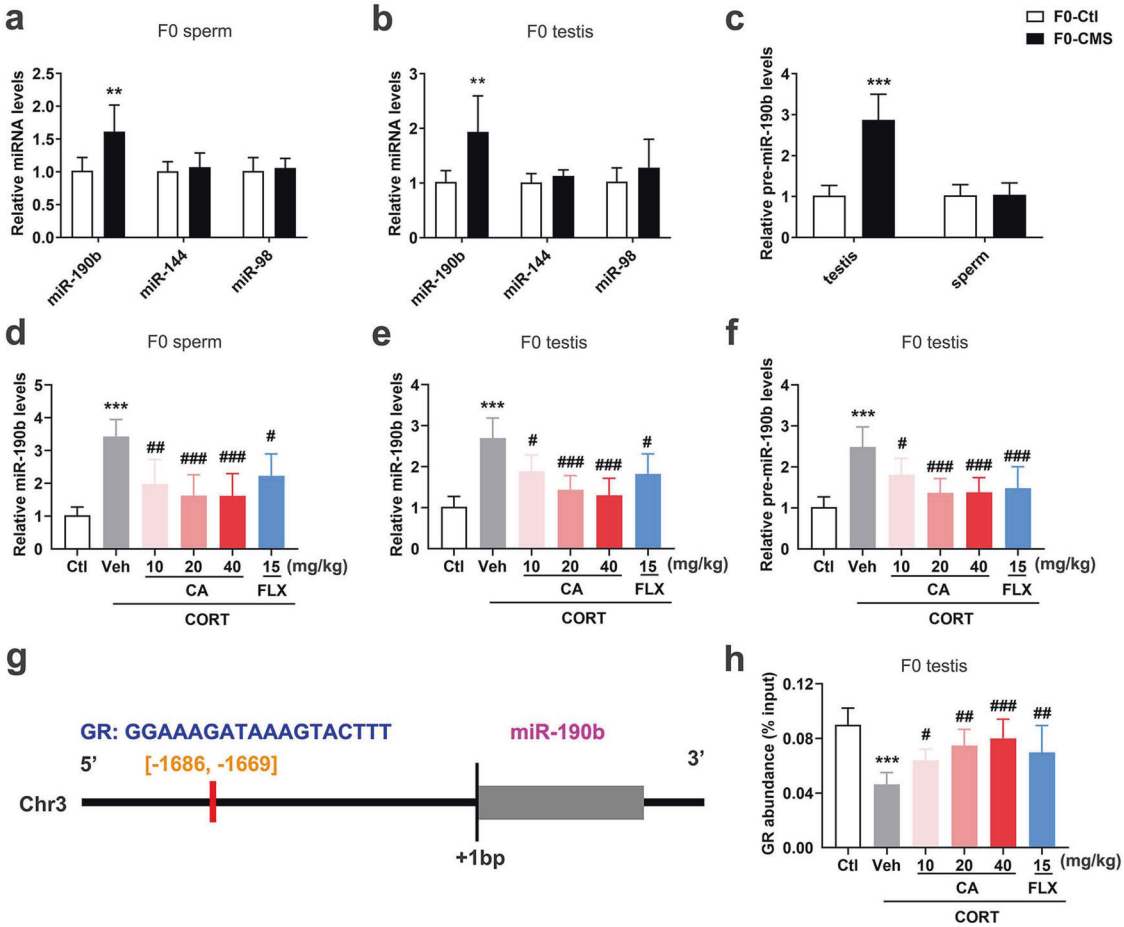


**Fig. 7** CMS/CORT exposure upregulated miR-190b transcription by suppressing GR in testis of F0 males, which is ameliorated by cinnamaldehyde (CA). RT-qPCR analysis of miR-190b, miR-144 and miR-98 in sperm (**a**) and testis (**b**) of F0-CMS ($n = 6$). **c** RT-qPCR analysis of pre-miR-190b in testis and sperm of F0-CMS ($n = 6$). RT-qPCR analysis the effect of cinnamaldehyde on miR-190b expression in sperm (**d**) and testis (**e**) of CORT-stimulated F0 males ($n = 5$). **f** RT-qPCR analysis the effect of cinnamaldehyde on pre-miR-190b expression in testis of CORT-stimulated F0 males ($n = 5$). **g** Potential targeting of GR at the promoter region of miR-190b. **h** ChIP assay was used to analyze the effect of cinnamaldehyde on the abundance of GR at miR-190b promoter in testis of CORT-stimulated F0 males. Ctl, Control; Veh, Vehicle; CA, Cinnamaldehyde; FLX, Fluoxetine. Data are expressed as mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$ vs F1-Ctl group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs F1-CORT vehicle group.

stomach and small intestine and almost completely metabolized into cinnamic acid in the liver before it is absorbed into blood in rats [50]. Although there are evidence that cinnamic acid protects bone marrow, spleen and liver of Swiss albino mice from the toxic effects of cyclophosphamide [51], ameliorates nonalcoholic fatty liver disease of fatty liver disease mice [52] and decreases $H_2O_2$-induced DNA damage in human peripheral blood lymphocytes [53], there is lack of direct evidence to identify its influence on testis. Nevertheless, indirect evidences suggest that cinnamic acid may have no obvious testis toxicity. Because *C. cassia* and cinnamon bark oil, both contain cinnamaldehyde, have been used to treat male sexual disorder and prevent $CCl_4$-induced impairments of testis, epididymis, and sperm quality in Wistar rats [54]. Pure cinnamaldehyde also improves erectile function of human and rat corpus cavernosum [55]. No obvious reproductive toxicity of cinnamaldehyde on male is reported by these studies. However, more evidence shows that cinnamaldehyde has some influences on female reproductivity [56, 57]. To our knowledge, there are three studies about the reproductive toxicity of cinnamaldehyde to female in literature [56–58], while the results are inconsistent. In one study, female rats receive oral cinnamaldehyde before pregnancy [58], and oral cinnamaldehyde reduces mortality and improves the glucose/insulin levels of infants born to rat model of gestational diabetes [58]. Moreover, the hyperphagia, glucose intolerance, and lipid metabolism symptoms of fatty-sucrose diet/streptozotocin-induced rat model of gestational diabetes are also improved [58]. The other two studies gave cinnamaldehyde by subcutaneous injection and gavage to pregnant rats, respectively [56, 57]. One study observes that cinnamaldehyde has a dose-dependent anti-5-azacytidine-induced digital malformations in the fetus of pregnant rats [56]. Interestingly, cinnamaldehyde shows a time-dependent fetal mortality increasing effect [56]. The other study reports found that cinnamaldehyde increases the incidence of poor cranial ossification in the fetus of pregnant rats [57].

Accumulating evidence indicates that various environmental factors experienced by males induce epigenetic changes in sperm, which may lead to a disease cycle and disease risk in their offspring [3, 59, 60]. Spermatogenesis occurs in testis, and further maturation occurs in epididymis. Thus, we examined GR expression in testis and epididymis. Our results of mouse models of depression showed that CMS and CORT exposure markedly downregulated GR expression in testis of F0 males. However, GR expression in epididymis of F0 males was not influenced by exposure to CMS and CORT. These results indicated that the downregulation of GR expression in testis of F0 CMS and F0-CORT was tissue-specific, which may be the basis of the difference of CORT response [61–63].

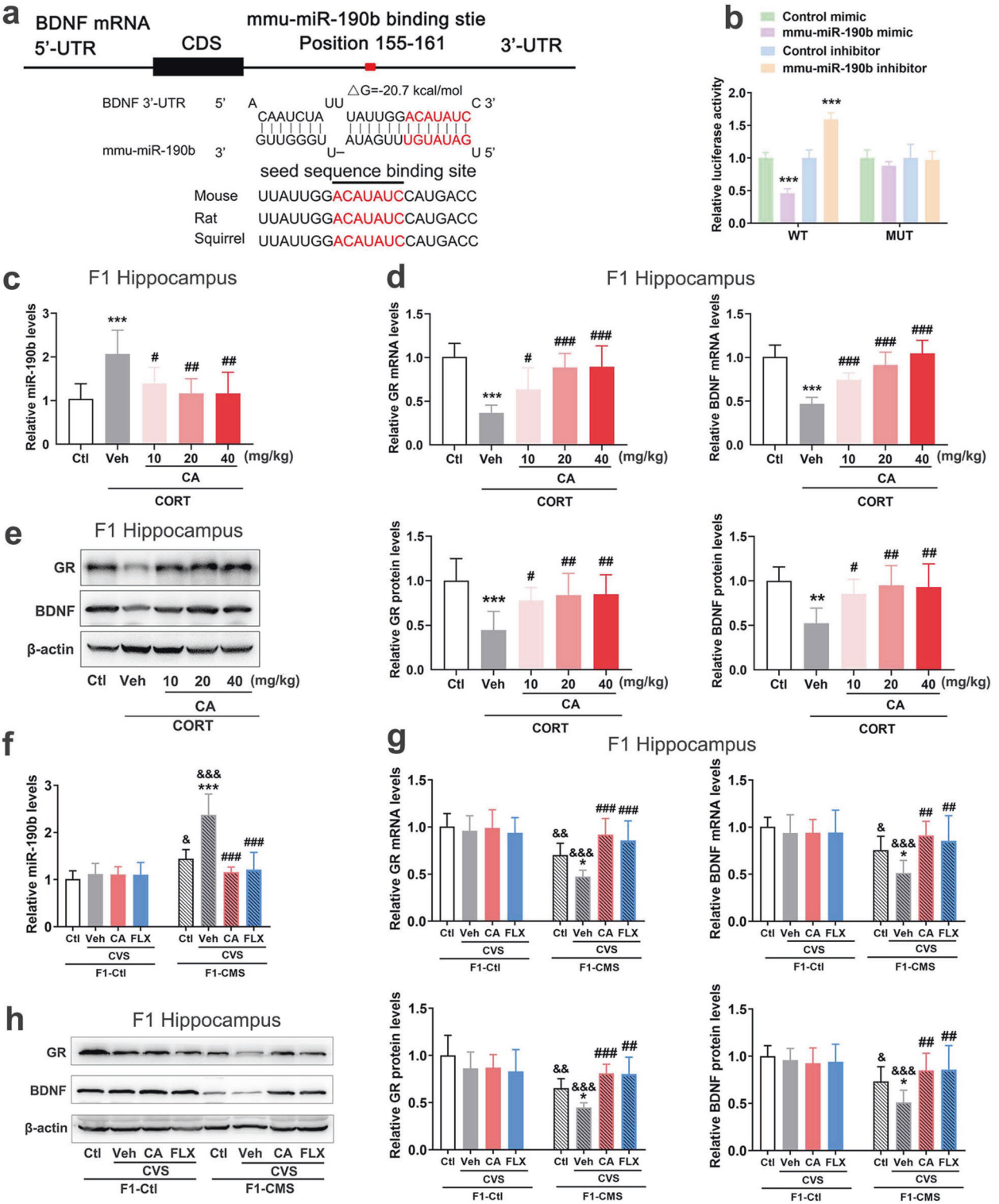


**Fig. 8** The effects of cinnamaldehyde (CA) on GR, miR-190b and BDNF expression in hippocampus of F1-CORT and F1-CMS. **a** Schematic descriptions of the hypothetical duplexes formed by miR-190b with the 3′-UTR of BDNF. **b** Relative luciferase activities in N2a cells treated with a miR-190b mimic or inhibitor ($n = 3$). **c** RT-qPCR analysis of miR-190b in hippocampus of F1-CORT ($n = 5$–6). **d** RT-qPCR analysis of GR and BDNF mRNA levels in hippocampus of F1-CORT ($n = 6$–8). **e** Western blot analysis of GR and BDNF protein levels in hippocampus of F1-CORT ($n = 5$). **f** RT-qPCR analysis of miR-190b in hippocampus of F1-CMS ($n = 5$). **g** RT-qPCR analysis of GR and BDNF mRNA levels in hippocampus of F1-CMS ($n = 5$). **h** Western blot analysis of GR and BDNF protein levels in hippocampus of F1-CMS ($n = 5$). Ctl, Control; Veh, Vehicle; CA, Cinnamaldehyde; FLX, Fluoxetine. Data are expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs F1-CORT/F1-CMS vehicle group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ vs F1-Ctl groups, respectively.

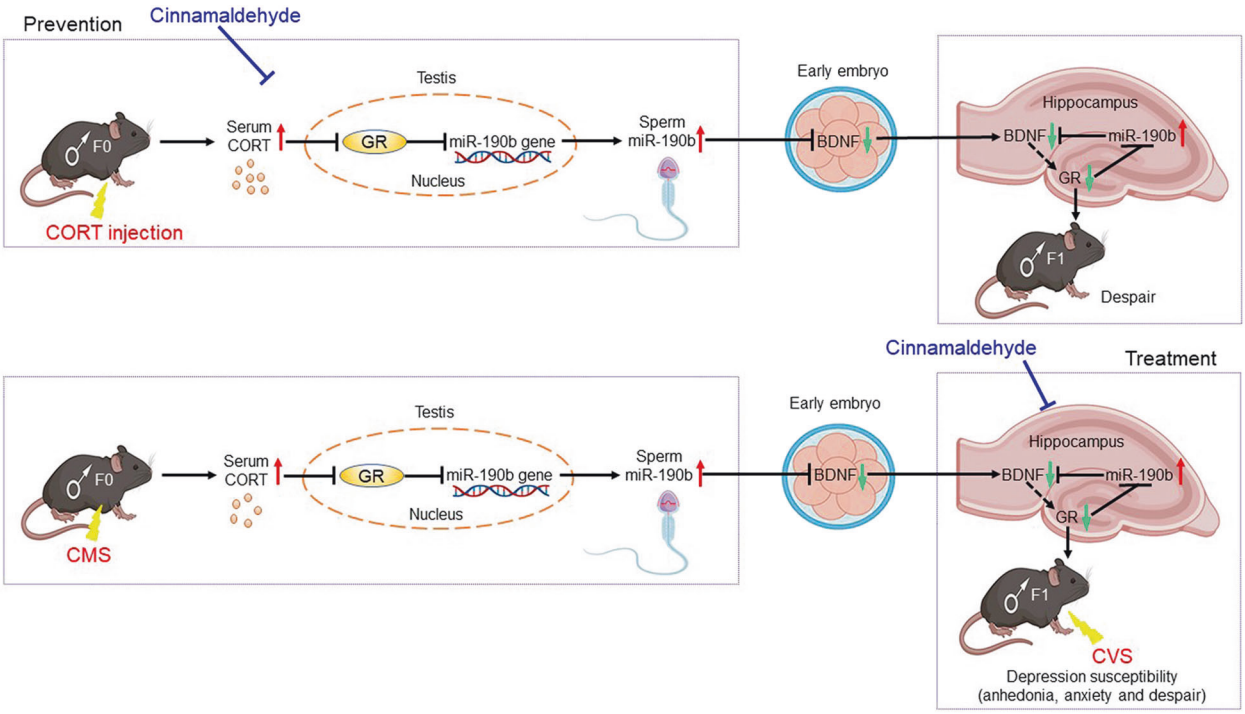


**Fig. 9** Schematic representation of cinnamaldehyde for the intervention of intergenerational effect of paternal depressive phenotypes possibly through GR/miR-190b/BDNF axis. Created in BioRender.com.

Mature sperm in mammals has abundant small non-coding RNAs (sncRNAs) [64, 65]. Recent studies in *Caenorhabditis elegans* [66, 67] and mice [2, 68, 69] have shown that sncRNAs, specifically miRNAs, can mediate non-Mendelian inheritance of traits or phenotypes acquired throughout life. Recently, miR-190b, miR-144, and miR-98 expression levels were altered by CORT exposure in mouse sperm, and altered miRNAs in sperm of CORT-stimulated F0 males may affect gene expression patterns in offspring embryos [9]. In CMS- and CORT-disturbed males, miR-190b expression was found to be upregulated in testis and sperm, while pre-miR-190b expression was upregulated only in testis, indicating that miR-190b was transcriptionally upregulated in testis possibly contributing to miR-190b upregulation in sperm. However, we did not observe a prominent change in the expression levels of miR-144 and miR-98 in sperm of F0-CMS.

To determine the regulation of GR on miR-190b, we used bioinformatics predictions and ChIP assay and validated that GR was upstream transcription factor of miR-190b by directly binding to its promoter region. Our results showed that paternal CORT exposure decreased the amount of GR on miR-190b promoter regions in testis of F0 mice. A recent study shows that spermatogenesis-related miRNAs are mainly expressed in the early stages of meiosis [70]. Moreover, spermatogenesis-related miRNAs have a conserved testicular-specific high expression pattern in different mammals [70]. Therefore, paternal CMS/CORT exposure downregulated GR expression and upregulated transcription of miR-190b in testis of F0 mice, which may lead to miR-190b upregulation in sperm of F0-CMS/F0-CORT. In testicular germ cells, miRNAs control mRNA translation by compartmentalization based on 3′UTR length [71]. Sperm miRNAs can be transferred into zygotes during fertilization [72]. Moreover, small RNAs are critical regulators of gene expression in early embryonic development [72]. These results raise the possibility that stress induces GR-dependent miR-190b increase in testis, which may be a primary mechanism of intergenerational inheritance of depression.

Using bioinformatic algorithms, we predicted that miR-190b was a potential miRNA targeting BDNF and confirmed that miR-190b binds directly to the 3′-UTR of BDNF mRNA. BDNF is required for activity-dependent formation and maintenance of synaptic plasticity [73]. In males of F1-CORT and F1-CMS, the expression levels of BDNF and GR were decreased, and the expression of miR-190b was increased in hippocampus. These results suggest that miR-190b post-translationally regulates BDNF expression in hippocampus of males of F1-CORT and F1-CMS. The BDNF-TrkB signaling pathway was the upstream molecular effector of GR [27]. The inhibition of the molecular cascade reaction of BDNF-related signaling pathway may result in the downregulation of GR in hippocampus of males of F1-CORT and F1-CMS. F1-CA males had higher BDNF and GR expression and lower miR-190b in hippocampus than in F1-CORT males. Moreover, cinnamaldehyde substantially ameliorated CVS-induced BDNF and GR downregulation and miR-190b upregulation in hippocampus of F1-CMS males.

Our results showed that CORT exposure induced serum CORT levels elevation, anhedonia, anxiety, and behavioral despair in F0 males (Fig. 1c, e–g), but F1-CORT males only showed behavioral despair and elevated serum CORT levels (Fig. 3d, e), which may result from the different miRNA expression profiles in sperm between F0-CORT and F0-Ctl. The discrepancy of sperm miRNA expression between generations in different depression models has also been verified by other studies [2, 8, 9]. These findings cross-validate that sperm miRNAs were crucial to intergenerational inheritance of depression. Different miRNAs expression profiles and functions in sperm may lead to different trait.

The clinical therapy of depression includes blocking the reuptake and breakdown of monoamines and increasing synaptic plasticity by chronic administration of typical antidepressants [26, 74]. However, typical antidepressants have notably a substantial time lag, low rates of efficacy and side effects, especially reproductive toxicity [39–45]. The therapeutic limitations of typical antidepressants highlight the major unmet needs of depression treatment [1]. The safety outcomes of cinnamaldehyde were supported by the emerging preclinical evidence [75]. Our study suggested that the administration of cinnamaldehyde is an excellent choice for depression, not only therapy for

depression but also prevention of intergenerational inheritance of depression. Further studies should be conducted to confirm the clinical efficacy of cinnamaldehyde in the intervention of intergenerational inheritance of depression, such as multicenter large-scale trials.

In summary, we demonstrate the efficacy of cinnamaldehyde as a potential intervention for intergenerational inheritance of depression in F1 males of CORT- and CMS-induced mouse models of depression. Compared with fluoxetine, cinnamaldehyde may not have male reproductive toxicity. This study identified an essential GR/miR-190b regulatory axis in testis of F0 males of CORT- and CMS-induced mouse models of depression and highlighted the vital role of miR-190b in suppressing BDNF expression in hippocampus of F1 males of CORT- and CMS-induced mouse models of depression. These changes in testis were ameliorated by cinnamaldehyde (the working model is summarized in Fig. 9). Future studies are warranted to explore GR-dependent epigenetic changes that contribute to the transmission of acquired traits in mammals, which should be considered in the development of novel antidepressants.

## ACKNOWLEDGEMENTS

This work was supported by the Fundamental Research Funds for the Central Universities.

## AUTHOR CONTRIBUTIONS

LDK and YP supervised and conceptualized the study. LDK, YP, and ZYG prepared the manuscript. ZYG, TYC, TTY, LPZ, SJZ, and XYG performed the experiments. All authors have read and approved the final manuscript.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41401-021-00831-0.

**Competing interests:** The authors declare no competing interests.

## REFERENCES

1. Duman RS, Aghajanian GK, Sanacora G, Krystal JH. Synaptic plasticity and depression: new insights from stress and rapid-acting antidepressants. Nat Med. 2016;22:238–49.
2. Gapp K, Jawaid A, Sarkies P, Bohacek J, Pelczar P, Prados J, et al. Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. Nat Neurosci. 2014;17:667–9.
3. Bohacek J, Mansuy IM. A guide to designing germline-dependent epigenetic inheritance experiments in mammals. Nat Methods. 2017;14:243–9.
4. Lewis G, Neary M, Polek E, Flouri E, Lewis G. The association between paternal and adolescent depressive symptoms: evidence from two population-based cohorts. Lancet Psychiatry. 2017;4:920–6.
5. Stein A, Pearson RM, Goodman SH, Rapa E, Rahman A, McCallum M, et al. Effects of perinatal mental disorders on the fetus and child. Lancet. 2014;384:1800–19.
6. Yehuda R, Daskalakis NP, Lehrner A, Desarnaud F, Bader HN, Makotkine I, et al. Influences of maternal and paternal PTSD on epigenetic regulation of the glucocorticoid receptor gene in Holocaust survivor offspring. Am J Psychiatry. 2014;171:872–80.
7. Petropoulos S, Matthews SG, Szyf M. Adult glucocorticoid exposure leads to transcriptional and DNA methylation changes in nuclear steroid receptors in the hippocampus and kidney of mouse male offspring. Biol Reprod. 2014;90:43.
8. Wang Y, Chen ZP, Hu H, Lei J, Zhou Z, Yao B, et al. Sperm microRNAs confer depression susceptibility to offspring. Sci Adv. 2021;7:eabd7605.
9. Short AK, Fennell KA, Perreau VM, Fox A, O'Bryan MK, Kim JH, et al. Elevated paternal glucocorticoid exposure alters the small noncoding RNA profile in sperm and modifies anxiety and depressive phenotypes in the offspring. Transl Psychiatry. 2016;6:e837.
10. Pietrzykowski AZ, Spijker S. Impulsivity and comorbid traits: a multi-step approach for finding putative responsible microRNAs in the amygdala. Front Neurosci. 2014;8:389.
11. Xiong GJ, Yang Y, Cao J, Mao RR, Xu L. Fluoxetine treatment reverses the intergenerational impact of maternal separation on fear and anxiety behaviors. Neuropharmacology. 2015;92:1–7.
12. Zaidan H, Galiani D, Gaisler-Salomon I. Pre-reproductive stress in adolescent female rats alters oocyte microRNA expression and offspring phenotypes: pharmacological interventions and putative mechanisms. Transl Psychiatry. 2021;11:113.
13. Zada W, Zeeshan S, Bhatti HA, Mahmood W, Rauf K, Abbas G. Cinnamomum cassia: an implication of serotonin reuptake inhibition in animal models of depression. Nat Prod Res. 2016;30:1212–4.
14. Goswami SK, Inamdar MN, Jamwal R, Dethe S. Effect of Cinnamomum cassia methanol extract and sildenafil on arginase and sexual function of young male Wistar rats. J Sex Med. 2014;11:1475–83.
15. Blevins SM, Leyva MJ, Brown J, Wright J, Scofield RH, Aston CE. Effect of cinnamon on glucose and lipid levels in non insulin-dependent type 2 diabetes. Diabetes Care. 2007;30:2236–7.
16. Iqbal S, Jabeen F, Peng C, Ijaz MU, Chaudhry AS. Cinnamomum cassia ameliorates Ni-NPs-induced liver and kidney damage in male Sprague Dawley rats. Hum Exp Toxicol. 2020;39:1565–81.
17. Bae WY, Choi JS, Jeong JW. The neuroprotective effects of cinnamic aldehyde in an MPTP mouse model of Parkinson's disease. Int J Mol Sci. 2018;19:551. https://doi.org/10.3390/ijms19020551.
18. Lv C, Yuan X, Zeng HW, Liu RH, Zhang WD. Protective effect of cinnamaldehyde against glutamate-induced oxidative stress and apoptosis in PC12 cells. Eur J Pharmacol. 2017;815:487–94.
19. Zhang L, Zhang Z, Fu Y, Yang P, Qin Z, Chen Y, et al. Trans-cinnamaldehyde improves memory impairment by blocking microglial activation through the destabilization of iNOS mRNA in mice challenged with lipopolysaccharide. Neuropharmacology. 2016;110:503–18.
20. Mateen S, Shahzad S, Ahmad S, Naeem SS, Khalid S, Akhtar K, et al. Cinnamaldehyde and eugenol attenuates collagen-induced arthritis via reduction of free radicals and pro-inflammatory cytokines. Phytomedicine. 2019;53:70–8.
21. Chen BJ, Fu CS, Li GH, Wang XN, Lou HX, Ren DM, et al. Cinnamaldehyde analogues as potential therapeutic agents. Mini Rev Med Chem. 2017;17:33–43.
22. Buglak NE, Jiang W, Bahnson ESM. Cinnamic aldehyde inhibits vascular smooth muscle cell proliferation and neointimal hyperplasia in Zucker Diabetic Fatty rats. Redox Biol. 2018;19:166–78.
23. Yao Y, Huang HY, Yang YX, Guo JY. Cinnamic aldehyde treatment alleviates chronic unexpected stress-induced depressive-like behaviors via targeting cyclooxygenase-2 in mid-aged rats. J Ethnopharmacol. 2015;162:97–103.
24. Arloth J, Bogdan R, Weber P, Frishman G, Menke A, Wagner KV, et al. Genetic differences in the immediate transcriptome response to stress predict risk-related brain function and psychiatric disorders. Neuron. 2015;86:1189–202.
25. Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng PF, et al. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. Nature. 2011;475:91–5.
26. Duman RS, Aghajanian GK. Synaptic dysfunction in depression: potential therapeutic targets. Science. 2012;338:68–72.
27. Revest JM, Le Roux A, Roullot-Lacarriere V, Kaouane N, Vallee M, Kasanetz F, et al. BDNF-TrkB signaling through Erk1/2 MAPK phosphorylation mediates the enhancement of fear memory induced by glucocorticoids. Mol Psychiatry. 2014;19:1001–9.
28. Li J, Liu T, Wang L, Guo X, Xu T, Wu L, et al. Antihyperglycemic and antihyperlipidemic action of cinnamaldehyde in C57BLKS/J db/db mice. J Tradit Chin Med. 2012;32:446–52.
29. Zhao J, Zhang X, Dong L, Wen Y, Zheng X, Zhang C, et al. Cinnamaldehyde inhibits inflammation and brain damage in a mouse model of permanent cerebral ischaemia. Br J Pharmacol. 2015;172:5009–23.
30. Kang LL, Zhang DM, Ma CH, Zhang JH, Jia KK, Liu JH, et al. Cinnamaldehyde and allopurinol reduce fructose-induced cardiac inflammation and fibrosis by attenuating CD36-mediated TLR4/6-IRAK4/1 signaling to suppress NLRP3 inflammasome activation. Sci Rep. 2016;6:27460.
31. Apazoglou K, Farley S, Gorgievski V, Belzeaux R, Lopez JP, Grenier J, et al. Antidepressive effects of targeting ELK-1 signal transduction. Nat Med. 2018;24:591–7.
32. Tye KM, Mirzabekov JJ, Warden MR, Ferenczi EA, Tsai HC, Finkelstein J, et al. Dopamine neurons modulate neural encoding and expression of depression-related behaviour. Nature. 2013;493:537–41.
33. Jasarevic E, Howard CD, Morrison K, Misic A, Weinkopff T, Scott P, et al. The maternal vaginal microbiome partially mediates the effects of prenatal stress on offspring gut and hypothalamus. Nat Neurosci. 2018;21:1061–71.
34. Pena CJ, Kronman HG, Walker DM, Cates HM, Bagot RC, Purushothaman I, et al. Early life stress confers lifelong stress susceptibility in mice via ventral tegmental area OTX2. Science. 2017;356:1185–8.
35. Mathelier A, Fornes O, Arenillas DJ, Chen CY, Denay G, Lee J, et al. JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles. Nucleic Acids Res. 2016;44:D110–5.
36. Lee C, Huang CH. LASAGNA-Search 2.0: integrated transcription factor binding site search and visualization in a browser. Bioinformatics. 2014;30:1923–5.

37. Sun W, Li J, Zhou L, Han J, Liu R, Zhang H, et al. The c-Myc/miR-27b-3p/ATG10 regulatory axis regulates chemoresistance in colorectal cancer. Theranostics. 2020;10:1981–96.
38. Gao Z, Zhou L, Hua S, Wu H, Luo L, Li L, et al. miR-24-3p promotes colon cancer progression by targeting ING1. Signal Transduct Target Ther. 2020;5:171.
39. Alzahrani HA. Sister chromatid exchanges and sperm abnormalities produced by antidepressant drug fluoxetine in mouse treated in vivo. Eur Rev Med Pharmacol Sci. 2012;16:2154–61.
40. Ayala ME, Gonzáles A, Olivarez RM, Aragón-Martínez A. Fluoxetine treatment of prepubertal male rats uniformly diminishes sex hormone levels and, in a subpopulation of animals, negatively affects sperm quality. Reprod Fertil Dev. 2018;30:1329–41.
41. Bataineh HN, Daradka T. Effects of long-term use of fluoxetine on fertility parameters in adult male rats. Neuro Endocrinol Lett. 2007;28:321–5.
42. Bezerra MS, Martins ABM, Trajano FMG, Pontes THA, Gomes L, Gavioli EC, et al. Fluoxetine and sertraline effects on rat distal cauda epididymis contraction, sperm count and sperm transit time trough epididymis. Eur J Pharmacol. 2019;865:172774.
43. Karimipour M, Ahmadi A, Zirak Javanmard M, Jafari A, Mohebi M, Hosseinalipour E. The effects of exposure to fluoxetine during lactation on testicular tissue and sperm parameters in mice offspring. Vet Res Forum. 2020;11:35–42.
44. Roboon J, Nudmamud-Thanoi S, Thanos S. Recovery effect of pre-germinated brown rice on the alteration of sperm quality, testicular structure and androgen receptor expression in rat model of depression. Andrologia. 2017;49:1–10.
45. Sakr HF, Abbas AM, Elsamanoudy AZ, Ghoneim FM. Effect of fluoxetine and resveratrol on testicular functions and oxidative stress in a rat model of chronic mild stress-induced depression. J Physiol Pharmacol. 2015;66:515–27.
46. Cryan JF, Holmes A. The ascent of mouse: advances in modelling human depression and anxiety. Nat Rev Drug Discov. 2005;4:775–90.
47. Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, et al. Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell. 2007;131:391–404.
48. Gourley SL, Kiraly DD, Howell JL, Olausson P, Taylor JR. Acute hippocampal brain-derived neurotrophic factor restores motivational and forced swim performance after corticosterone. Biol Psychiatry. 2008;64:884–90.
49. Muller MB, Holsboer F. Mice with mutations in the HPA-system as models for symptoms of depression. Biol Psychiatry. 2006;59:1104–15.
50. Chen Y, Ma Y, Ma W. Pharmacokinetics and bioavailability of cinnamic acid after oral administration of Ramulus Cinnamomi in rats. Eur J Drug Metab Pharmacokinet. 2009;34:51–6.
51. Patra K, Bose S, Sarkar S, Rakshit J, Jana S, Mukherjee A, et al. Amelioration of cyclophosphamide induced myelosuppression and oxidative stress by cinnamic acid. Chem Biol Interact. 2012;195:231–9.
52. Wu Y, Wang M, Yang T, Qin L, Hu Y, Zhao D, et al. Cinnamic acid ameliorates nonalcoholic fatty liver disease by suppressing hepatic lipogenesis and promoting fatty acid oxidation. Evid Based Complement Altern Med. 2021;2021:9561613.
53. Taner G, Ozkan Vardar D, Aydin S, Aytac Z, Basaran A, Basaran N. Use of in vitro assays to assess the potential cytotoxic, genotoxic and antigenotoxic effects of vanillic and cinnamic acid. Drug Chem Toxicol. 2017;40:183–90.
54. Yuce A, Turk G, Ceribasi S, Guvenc M, Ciftci M, Sonmez M, et al. Effectiveness of cinnamon (Cinnamomum zeylanicum) bark oil in the prevention of carbon tetrachloride-induced damages on the male reproductive system. Andrologia. 2014;46:263–72.
55. Onder A, Yilmaz-Oral D, Jerkovic I, Akdemir AO, Gur S. Evaluation of relaxant responses properties of cinnamon essential oil and its major component, cinnamaldehyde on human and rat corpus cavernosum. Int Braz J Urol. 2019;45:1033–42.
56. Kurishita A, Ihara T. Inhibitory effects of cobalt chloride and cinnamaldehyde on 5-azacytidine-induced digital malformations in rats. Teratology. 1990;41:161–6.
57. Mantovani A, Stazi AV, Macri C, Ricciardi C, Piccioni A, Badellino E. Pre-natal (segment II) toxicity study of cinnamic aldehyde in the Sprague-Dawley rat. Food Chem Toxicol. 1989;27:781–6.
58. Hosni AA, Abdel-Moneim AA, Abdel-Reheim ES, Mohamed SM, Helmy H. Cinnamaldehyde potentially attenuates gestational hyperglycemia in rats through modulation of PPARgamma, proinflammatory cytokines and oxidative stress. Biomed Pharmacother. 2017;88:52–60.
59. Pang TYC, Short AK, Bredy TW, Hannan AJ. Transgenerational paternal transmission of acquired traits: stress-induced modification of the sperm regulatory transcriptome and offspring phenotypes. Curr Opin Behav Sci. 2017;14:140–7.
60. Klengel T, Dias BG, Ressler KJ. Models of intergenerational and transgenerational transmission of risk for psychopathology in mice. Neuropsychopharmacology. 2016;41:219–31.
61. John S, Sabo PJ, Thurman RE, Sung MH, Biddie SC, Johnson TA, et al. Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. Nat Genet. 2011;43:264–8.
62. Vockley CM, D'Ippolito AM, McDowell IC, Majoros WH, Safi A, Song L, et al. Direct GR binding sites potentiate clusters of TF binding across the human genome. Cell. 2016;166:1269–81 e19.
63. Gertz J, Savic D, Varley KE, Partridge EC, Safi A, Jain P, et al. Distinct properties of cell-type-specific and shared transcription factor binding sites. Mol Cell. 2013;52:25–36.
64. Wu PH, Fu Y, Cecchini K, Ozata DM, Arif A, Yu T, et al. The evolutionarily conserved piRNA-producing locus pi6 is required for male mouse fertility. Nat Genet. 2020;52:728–39.
65. Sharma U, Conine CC, Shea JM, Boskovic A, Derr AG, Bing XY, et al. Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. Science. 2016;351:391–6.
66. Burton NO, Burkhart KB, Kennedy S. Nuclear RNAi maintains heritable gene silencing in Caenorhabditis elegans. Proc Natl Acad Sci USA. 2011;108:19683–8.
67. Gu SG, Pak J, Guang S, Maniar JM, Kennedy S, Fire A. Amplification of siRNA in Caenorhabditis elegans generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. Nat Genet. 2012;44:157–64.
68. Liu WM, Pang RT, Chiu PC, Wong BP, Lao K, Lee KF, et al. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. Proc Natl Acad Sci USA. 2012;109:490–4.
69. Chen Q, Yan M, Cao Z, Li X, Zhang Y, Shi J, et al. Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. Science. 2016;351:397–400.
70. Zhang F, Zhang Y, Lv X, Xu B, Zhang H, Yan J, et al. Evolution of an X-Linked miRNA family predominantly expressed in mammalian male germ cells. Mol Biol Evol. 2019;36:663–78.
71. Zhang Y, Tang C, Yu T, Zhang R, Zheng H, Yan W. MicroRNAs control mRNA fate by compartmentalization based on 3′ UTR length in male germ cells. Genome Biol. 2017;18:105.
72. Yang Q, Lin J, Liu M, Li R, Tian B, Zhang X, et al. Highly sensitive sequencing reveals dynamic modifications and activities of small RNAs in mouse oocytes and early embryos. Sci Adv. 2016;2:e1501482.
73. Koo JW, Chaudhury D, Han MH, Nestler EJ. Role of mesolimbic brain-derived neurotrophic factor in depression. Biol Psychiatry. 2019;86:738–48.
74. Castren E, Hen R. Neuronal plasticity and antidepressant actions. Trends Neurosci. 2013;36:259–67.
75. Zhao H, Yuan J, Yang Q, Xie Y, Cao W, Wang S. Cinnamaldehyde in a novel intravenous submicrometer emulsion: pharmacokinetics, tissue distribution, antitumor efficacy, and toxicity. J Agric Food Chem. 2015;63:6386–92.