Emodin Prevented Depression in Chronic Unpredicted Mild Stress Exposed Rats by Targeting miR139-5p/5-Lipoxygenase

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Research

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Abstract

Background: Compelling evidences have highlighted the role of inflammation as a possible mechanism linking stress to depression. Anti-inflammatory treatment might block the occurrence of stress-induced depression. Emodin, a natural active compound extracted from *rhubarb*, has the biological activity of anti-inflammation. Here, the effect of emodin on the depressed young male rats and the underlying mechanism were studied.

Methods: The proteomics analysis of hippocampi, which were taken from depression (Dep) rats, depression resistant (Res) rats and control (Ctrl) rats after 7 weeks' chronic unpredicted mild stress (CUMS), were performed. We also screened out depression susceptible (DeS) rats and stress insensitive (Ins) rats at the end of 5th week in CUMS. While exposed to CUMS, DeS rats received daily emodin (Emo, 80mg/kg) or vehicle treatment (Veh) for 2 weeks.

Results: Inflammation was suggested by the proteomics analysis of hippocampi taken from Dep rats, Res rats and Ctrl rats. Emodin obviously improved the depression behaviors and a series of pathological changes in hippocampus, such as hippocampal neuron and spine loss, microglia activation, increased interleukin-1b (IL-1b) and tumor necrosis factor- α (TNF- α), and the activation of 5-lipoxygenase (5-LO). Furtherly, we demonstrated that emodin inhibited its excess inflammatory responses possibly by targeting miR139-5p/5-LO and modulating the downstream glycogen synthase kinase 3 β (GSK3 β) and nuclear factor erythroid 2-related factor 2 (Nrf2).

Conclusions: These results established a key role of miR139-5p/5-LO in the development of depression and provided an important evidence that emodin may be a candidate agent for the treatment of depression.

Introduction

Depression is a widespread chronic illness with a wide array of symptoms affecting personal somatic, cognitive, affective, and social processes. It is characterized by low mood, sadness, insomnia, lack of interests in study, work and life, and so on. As a leading cause of the global burden, the main treatments of depression are drug and psychological interventions[1, 2]. While effective, one third of people accepting drug intervention did not respond to these antidepressants, and others did not experience complete remission, or they suffered from relapse [3, 4].

Although both stress and neuroinflammation are the predisposing factors in the development of depression[5, 6], the more important role of inflammation has been highlighted by compelling clinical and preclinical evidences as a possible mechanism linking stress to depression[7]. Clinic and rodents studies have shown that exposure to repeated psychosocial and environmental could cause considerable immunological alterations, including accumulation of pro-inflammatory cytokines and decrease of anti-inflammatory cytokines in the blood and brain[8-10]. As the brain-resident macrophages, microglia account for about 10% of the total glial population in brain and are capable of changing morphology

rapidly from a ramified surveillant state to an ameboid like activated state in response to stresses[11]. Depending on the cytokines secreted, activated microglia can be categorized into two broad phenotypes, pro-inflammatory (M1) and anti-inflammatory (M2). M1 microglia release pro-inflammatory cytokines including interleukin (IL)-1, IL-6, tumor necrosis factor- α (TNF- α) and nitric oxide (NO). M2 microglia express anti-inflammatory cytokines, such as IL-4 and IL-10. Acute psychological stressors in human have been identified continuously increase the circulating inflammatory factors[12]. In a Trier social stress test (TSST), the elevated levels of pro-inflammatory cytokines IL-6 and TNF- α were observed in healthy controls[13]. Chronic unpredicted mild stress (CUMS), an experimental method of stress whereby the subject is exposed to mild and constantly changing stress stimuli[14], has been shown cause hippocampal microglial activation detected by positron emission tomography (PET), immunofluorescence staining, and Western blotting[15, 16]. Activation of NOD-like receptor protein 3 (NLRP3) inflammasome and upregulation of pro-inflammatory cytokines IL-1 β , IL-6, and IL-18 were observed in the hippocampus of depressed rats after stress [15, 16].

Thus, anti-inflammation therapy might act as antidepressants in stress triggered depression. Some clinical trials have indicated better antidepressant effects for anti-inflammatory drugs, especially nonsteroidal anti-inflammatory drugs (NSAIDs) and cytokine-inhibitors. However, some reported side effects have also raised controversy about whether NSAIDs can be used safely[17]. Emodin, a natural active compound extracted from an herb *rhubarb*, has the biological activity of anti-inflammation[18, 19]. Here, we observed the effect of emodin on the depression-like behaviors of young male rats after CUMS exposure.

Depression-like behaviors were identified according to sucrose preference test (SPT), force swimming test (FST) and open field test (OFT). Inflammation in the development of depression was suggested by the proteomics analysis of hippocampi, which were taken from depression (Dep) rats, depression resistant (Res) rats and control (Ctrl) rats after 7 weeks' CUMS. To observe the effect of emodin, we screened out depression susceptible (DeS) rats and stress insensitive (Ins) rats after 5 weeks' CUMS. DeS rats received daily emodin (Emo, 80mg/kg) or the same volume of Tween-80 (Veh) treatment. 2 weeks later, Emo rats had their depression-like behaviors obviously improved. And a series of pathological changes in hippocampus, such as hippocampal neuron and spine loss, microglia activation, increased IL-1 β and TNF- α , and the activation of 5-lipoxygenase (5-LO) have been reversed by emodin. Furtherly, we demonstrated that emodin inhibited the excess inflammatory responses possibly by targeting miR139-5p/5-LO, and further inhibited glycogen synthase kinase 3 (GSK3 β) and activated nuclear factor erythroid 2-related factor 2 (Nrf2). These results established a key role of miR139-5p/5-LO in the development of depression and provided an important evidence that emodin may be a candidate agent for the treatment of depression.

Materials And Methods

Antibodies and drugs

The primary antibodies used in this study are listed in Table 1. Emodin was obtained from Shanghai Base Industry (Shanghai, China) and dissolved in Tween-80, which was from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Anti-rabbit or anti-mouse IgG conjugated to IRDye@ (800CW) (1:10,000) was from Lincoln (USA). Toluidine blue and dimethyl sulfoxide (DMSO) were from Sigma (St. Louis, MO, USA). Diaminobenzidine (DAB) tetrachloride system was from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China). The miR139-5p inhibitor was from RiboBio Co., Ltd. (Guangzhou, China). 3'-UTR of 5-*LO* was amplified with the following primers: forward 5'-CGGGGTCTACAGTGCACGT-3', reverse 5'-CTCAACTGGTGTCTGGAGTC-3'.

Cell culture and transfection

N2a cells were cultured with 45% DMEMhigh glucose medium and 45% Opti-MEM® I Reduced Serum Medium and supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin (all from Hyclone) at 37°C in the presence of 5% CO₂. Transfection was performed with neofect (Neofect biological Technology) when cells were cultured to 70%~80% confluence in six-well plates. 48 hours after transfection, cells were collected and lysed for further research. HEK293 cells were cultured in high-glucose DMEM added with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all from Hyclone). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. HEK293 transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA).

Animals

8-week-old male Sprague-Dawley rats were supplied by Experimental Animal Central of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All efforts were made to minimize animal suffering and to reduce the number of rats used and all experimental procedures in this research have been approved by the Animal Care and Use Committee of Huazhong University of Science and Technology.

In the first part of this research, 45 rats were evaluated depressive status by SPT, FST and OFT. Then, 15 rats were randomly chosen as Ctrl rats, and 30 rats were daily exposed for 7 weeks' CUMS as reported[20, 21]. After 7 weeks of CUMS exposure, we obtained 13 Res rats and 14 Dep rats. In the second part, 30 rats were randomly chosen as Ctrl rats, and 64 rats were daily exposed for 7 weeks' CUMS. At the end of the 5th week in CUMS, 28 rats showed stress insensitive (Ins rats) and 30 rats showed depression susceptible (DeS rats). Therefore, we used 15 Ctrl and 15 DeS rats accepted 14 days' Emo treatment (Ctrl+Emo, DeS+Emo, intragastric administration, 80mg/kg) or the same volume of Tween-80 (Veh, 15 Ctrl+Veh and 15 DeS+Veh). The dosage of emodin was referenced from previous studies[19]. Rats were housed 5 per cage in temperature (26 ± 2 °C) controlled rooms with standard rodent chow and water available ad libitum, keeping on a standard 12 hours light or dark cycle with the light on from 7:00 a.m. to 7:00 p.m. All behavioral tests were performed during the light cycle in a dedicated sound-proof behavioral facility by experimenters blind to treatment information. Rats were brought to the procedure room 1 hour before the

start of behavioral training and remained in the same room through the test. At all times, the sound was masked with 60-65 Db white noises.

CUMS

The procedure of CUMS was performed as we previously reported[20, 21]. Briefly, all stress-exposed rats were subjected to three or four following stressors each day, such as water or food deprivation for 24 hours, empty water bottles for 2 hours, cold room (4 °C) for 2 hours, hot room (45 °C) for 15 minutes, cage tilt for 16 hours, continuous overnight lighting for 12 hours, soiled cage (200 ml of water spilled onto the bedding) for 12 hours, grouped housing in one cage (4-5 per cage) for 12 hours, strobe lighting (200 flashes/minute) for 4 hours, intermittent white noise (85 dB) for 6 hours. The procedure was repeated for 7 weeks. The Ctrl rats were left undisturbed throughout with the exception of general handling (i.e., regular cage cleaning, water or food deprivation for SPT, and measuring body weight). Every week, SPT and weight weighing were performed.

Sucrose preference test (SPT)

SPT consists of 7 days of training phase, 24 hours of food and water deprivation phase, and 1 hour of testing phase. As previously, we trained the rat to consume 1.5% water sucrose solution for 1 hour (9:30 am-10:30 am) every day to adapt to novelty in the training phase. Then, the rat was deprived of food and water for 24 hours. In testing phase, rats were allowed free access to two pre-weighed bottles (containing water or 1.5% sucrose solution) for 1 hour. The sucrose preference was calculated as a percentage sucrose consumption $\times 100 / (\text{water consumption} + \text{sucrose consumption})$.

Open field test (OFT)

The test was performed in a bare square box with 100 cm of length, 100 cm of width and 40 cm of height. As previously described[20], each rat were placed in the center of a black floor with 25 equal squares (20 cm $\times$ 20 cm square) including 16 peripheral squares and 9 central squares. The activity of rats was recorded by an overhanging camera linking to a computer over a 5 minutes' period. The number of total squares a rat crossing in the arena defined as the number of zones crossing was analyzed as measures of locomotor activity. The rearings were taken as measures of anxiety. The box was cleaned with 75% alcohol between tests.

Forced swimming test (FST)

Next, rats were tested in a transparent plexiglas cylinders (20 cm of diameter, 50 cm of deepth, filled with 23-25 °C water to a depth of 25 cm) as previously[21]. As previously described [21], rats were individually forced to swim for 5 minutes. Each session was videotaped for analysis and the water was changed between sessions. The duration of immobility in 5 minutes was measured. The floating vertically in the water and making only those movements necessary to maintain the head above the surface of the water for living were both considered as immobility. The immobility time was used for assessing feeling of hopelessness in rats.

Proteomic analysis

The hippocampal proteomic analysis was conducted as previously described[20-21]. Briefly, the hippocampus proteins were extracted, digested and labeled by iTRAQ-6plex reagents in accordance with the manufacturer's protocol. After labeled, the peptides were fractionated by high pH reverse-phase HPLC. The resulting fractions were dissolved, loaded onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific), and then separated using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific). The peptides were accepted to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. In order to identify the proteins, we analyzed the resulting MS/MS data using Mascot search engine (v.2.3.0) and searched against Uniprot_rat database (32,983 sequences). As reportedly, we defined observed proteins with iTRAQ ratios of >1.2 and <0.83 coupled with $p < 0.05$ as differentially expressed proteins (DEPs). Interaction network of DEPs were made by STRING 11.0.

Morphological techniques on brain slices

Rats were anesthetized with isoflurane and transcardially perfused with 100 milliliters normal saline and then perfused with 400 milliliters 4% paraformaldehyde solution. The brain sample was removed from the skull carefully to avoid damaging or pressing. For Nissl staining, immunohistochemical staining and immunofluorescence staining, brain sample was post-fixed in 4% paraformaldehyde solution for another 24 hours at 4 °C. In the dehydration, the sample was subjected to 20% and 30% sucrose gradient dehydration for twice until completely sunken. All brains were sliced into 30 µm coronal sections with a freezing microtome (Kryostat 1720, Leitz, Wetzlar, Germany). The sections were consecutively collected and stored in 50% glycerinum in PBS at -20 °C.

Nissl staining

The sections were washed with PBS for 2 minutes ´ 3 times, pasted on the slides and air-dried for 2 hours. Then they were immersed into Nissl dye liquor for several minutes according to the color changing, followed by decoloration in 75% alcohol, 95% alcohol twice for several minutes each. Subsequently, use the absolute ethyl alcohol for dehydration for 5 minutes ´ 3 times and the dimethylbenzene for transparency for 10 minutes twice. The sections were sealed and dried in a fume hood. The images were obtained by an optical microscope (Nikon 90i, Tokyo, Japan).

Immunohistochemical and immunofluorescence staining

After being washed with PBS for 5 minutes ´ 3 times and treated by PBS containing 0.3% H₂O₂ and 0.5% Triton X-100 for 30 minutes at room temperature, the sections were pre-incubated with 3% normal goat serum and incubated in the primary antibodies (Table 1). 24 hours later, they were incubated for 1 hour with the secondary antibodies (Table 1) for 1 hour at 37°C after being washed with PBS. For immunohistochemical staining, immunoreaction was developed using Histostain TM-SP kits (ZSGB-Bio, Beijing, China) and visualized with diaminobenzidine (brown). The images were obtained by an optical

microscope (Nikon 90i, Tokyo, Japan). For immunofluorescence stained sections, the images were observed using a laser scanning confocal microscope (Zeiss LSM 710, Germany).

Golgi staining

After the brain was removed from the skull, golgi staining was developed using the FD Rapid GolgiStain kit (FD Neurotechnologies, Baltimore, MD) according to the manufacturer's protocol. The samples were cut into horizontal sections of 100 μm thicknesses using a vibratome (Leica, Nussloch, Germany; S100, TPI) and mounted on the gelatin-coated slide. The sections were dehydrated in successive alcohol and transparency in xylene, and then the slide was sealed. The sections were observed and imaged by using an ordinary optical microscope (Nikon 90i, Tokyo, Japan). The number of dendritic spines on hippocampus pyramidal neurons was counted in Image-Pro Plus 6.0 software (Media Cybernetics, Inc. USA).

Western blotting

The rats were anaesthetized with isoflurane and sacrificed. The hippocampi were quickly dissected out of brain and frozen at $-80\text{ }^{\circ}\text{C}$. For Western blotting analysis of whole cell components, the sample of hippocampus was homogenized in cold buffer solution containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 0.5 mM Na_3VO_4 , 1 mM EDTA, 1 mM benzamidine, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/ml leupeptin, 5 mg/ml aprotinin and 2 mg/ml pepstatin. Lysates were mixed with 4 $\times$ extracting buffer and protein concentrations were determined using a BCA protein assay kit (Rockford, IL, USA). All the sample solutions were stored at $-80\text{ }^{\circ}\text{C}$ for use. For Western blotting analysis of nuclear and cytoplasmic fractions[22], proteins were extracted with the kit from KeyGen Biotech (NanJing KeyGen Biotech Co.,Ltd.).

Before sample-loading, a final concentration of 10% β -mercaptoethanol and 0.05% bromophenol blue were added. The proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and probed with primary antibodies (Table 1). Secondary antibodies were anti-rabbit or anti-mouse IgG conjugated to IRDye@ (800 CW; 1:10,000). The intensities of immunoblotting strips were automatically recognized by the Odyssey system (Li-Cor Bioscience, Lincoln, Nebraska, USA). All intensities of strips were normalized by the average intensity of DM1A. Then take the average value of Ctrl group as 1, and calculate the relative intensity of each strip.

Quantitative real-time PCR

Total RNA was extracted from hippocampal using TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. $\text{RNA}_{260/280}$ was measured spectrophotometrically for determining the concentration and purity. To synthesise cDNA from miRNA, we used M-MLV Reverse Transcriptase cDNA Synthesis Kit (Invitrogen, Carlsbad, California, USA) according to the supplier's recommendations. Quantitative PCR amplification was performed using CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) and SYBR Green Premix Ex TaqTM (TaKaRa, Kyogo, Japan).

The reaction conditions for PCR is an initial denaturation 3 minutes at 95 °C, followed by 45 cycles 10 seconds at 95 °C, 30 seconds at 60 °C, and extension 30 seconds at 72 °C. The primers for qPCR analysis were designed and synthesized. The data was quantified using the $\Delta\Delta C_t$ -method.

Luciferase assays for identifying miR-target interactions

Normal and mutated 3'-UTR sequences of *5-LO* were subcloned into the psiCHECK-2 reporter plasmid (Qijing Biotechnology Co., Ltd., Wuhan, China) as previously described[23]. HEK293T cells were transfected with psiCHECK-2 plasmid containing the 3'-UTR and the overexpressing vector for a specific miRNA. 24 hours after transfection, cells were lysed and luciferase reporter activities were assayed as previously described[23].

Enzyme-linked immuno sorbent assay (ELISA)

The hippocampal levels of IL-1b, TNF- α and leukotriene B4 (LTB4) were assayed by ELISA according to the protocols of ELISA kits (Elabscience Biotechnology Co., Ltd, Wuhan, China). A microplate reader (Biotek, Winooski, VT, USA) set to 450 nm was used to determine the optical density (OD) value. A standard curve was created by plotting the mean OD value for each standard. The sample concentration was determined from the standard curve.

Predictions of microRNA targeting

The miR-targeting predictions were performed by 3 different web-based algorithms, Targetscan software (<http://www.targetscan.org>), miRbase (<http://www.mirbase.org/>), and miRDB (<http://www.mirdb.org/miRDB/>).

Statistical analysis

The data were analyzed using SPSS 12.0 (SPSS Inc., Chicago, Illinois, USA) and the statistical graphs were produced by GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Data were expressed as means $\pm$ SEM. Differences among groups were tested with the one-way analysis of variance (ANOVA) procedure. The level of significance was set at $p < 0.05$.

Results

Inflammation is the prominent differential signal in hippocampi of Dep, Res and Ctrl rats

Before CUMS exposure, all the rats (n=45) have identical rate of preference to sucrose (about 85%) in SPT, equivalent immobility time (about 79.3 seconds) in FST, and equivalent number of zone crossing (about 154) and rearing times (about 27) in OFT. A depression (Dep) rat was manifested as having more than 30% decrease in sucrose water intake in SPT and a 50% increase in the immobility time in FST. A depression resistant (Res) rat was manifested as having a greater sucrose water intake than the lower endpoint of the 95% confidence interval of that of control rats in SPT and a shorter immobility time than

the upper endpoint of the 95% confidence interval of that of control rats in FST. In this research, 13 Res rats and 14 Dep rats were obtained from 30 rats accepted CUMS exposure. The Dep rats showed a robust decrease (45.1%) in sucrose intake, whereas Res rats displayed a sucrose preference (83.2%) similar to that of unstressed Ctrl rats (Fig. 1b). In FST, the Dep rats had a much longer immobility time (220 ± 4.4 seconds) than Res rats (83.3 ± 5.8 seconds) and Ctrl rats (78.1 ± 5.3 seconds) (Fig. 1c). In OFT, the number of zone crossing and rearing times of Dep rats were less than those of Res rats and Ctrl rats (Fig. 1d, e). Additionally, we found both Dep (354.1 ± 6.4 g) and Res (353.5 ± 9.5 g) rats had lighter body weights than Ctrl rats (471.1 ± 5.8 g) (Fig. 1f).

In the proteomics data with 3645 quantified proteins, 262 DEPs were obtained, including 40 up-regulated and 53 down-regulated DEPs in Dep/Ctrl, 75 up-regulated and 34 down-regulated DEPs in Dep/Res, and 40 up-regulated and 101 down-regulated DEPs in Res/Ctrl (fig. 1g). Of the 20 down-regulated proteins in Dep/Ctrl, 7 proteins were down-regulated in Dep/Res and 13 down-regulated proteins were down-regulated in Res/Ctrl (fig. 1h). 18 up-regulated proteins in Dep/Ctrl and Dep/Res and 4 up-regulated proteins in Dep/Ctrl and Res/Ctrl were shown in fig. 1i. 22 up-regulated proteins in Dep/Res were down-regulated in Res/Ctrl (fig. 1j), and 6 down-regulated proteins in Dep/Res were up-regulated in Res/Ctrl (fig. 1k). 9 differential proteins were common in Dep/Ctrl, Dep/Res and Res/Ctrl (fig. 1l).

The protein–protein interaction (PPI) network analysis suggested inflammation was the characteristic signal in hippocampus of Dep rats (Fig.1m, n). Fibrinogen (Fg) is the acute phase protein and a marker of inflammation[24]. Increased Fg was reported contribute to nuclear factor- κ B (NF- κ B) activation[24]. Up-regulated Fga, Fgb and Fgg levels in whole blood samples were reported in depressed patients[25]. a2-HS glycoprotein (Ahsg, also known as fetuin-A), an endogenous ligand for [toll-like receptor-4](#) (TLR4), is reported involved in lipid-induced inflammation by activating NF- κ B[26, 27]. Elevated serum Ahsg was reported in male patients with major depressive disorder (MDD)[28]. Increased Fga, Fgb, Fgg and Ahsg were shown in Dep/Ctrl and Dep/Res, but not Res/Ctrl (Fig.1o). Additionally, increased vimentin (Vim)[29], collagen type I α 1 chain (Col1a1)[30], annexin A1 (Anxa1)[31] and Anxa2[32] indicated the elevated inflammatory responses to CUMS and increased the permeability of blood-brain barrier (BBB) in hippocampus[29] (Fig.1o). Glutathione S-transferase A4 (GSTA4) is a key enzyme for removal of toxic lipid peroxidation products[33]. Decreased GSTA4 was shown in Dep/Ctrl (Fig.1o), but not Res/Ctrl, which may contribute to a depression phenotype.

Emodin ameliorates depression-like behaviors in depression-susceptible rats

5-LO (ALOX5) has more connections with DEPs in Dep/Ctrl and Dep/Res as shown in Figure 1m and n. Emodin acts as an inhibitor of 5-LO[34]. Then, we studied whether emodin could ameliorate the depression-like behaviors in rats. As the depression rate was 46.67% in rats accepted 7 weeks' CUMS, we selected out depression-susceptible (DeS) rats after 5 weeks' CUMS, which would accept 2 weeks' emodin treatment. A DeS rat should have a more than 20% decrease in sucrose water intake in SPT (Fig. 2b). A stress insensitive (Ins) rats should have a greater sucrose water intake than the lower endpoint of the 95% confidence interval of that of control rats in SPT (Fig. 2b).

In this research, 30 DeS rats and 30 Ctrl rats received daily emodin (Emo, 80mg/kg) or the same volume of Tween-80 (Veh) for 2 weeks (Fig. 2a). Meanwhile, DeS rats were exposed to CUMS continuously. 2 weeks later, DeS+Veh rats (body weight 355.7 ± 5.0 g) presented depressive behaviors, e.g. a lower sucrose preference (44.1%) in SPT (Fig. 2c) and an obvious longer immobility time (210.2 ± 3.4 seconds) in FST (Fig. 2d). However, DeS+Emo rats had a higher percent of sucrose preference (78.5%) in SPT (Fig. 2c) and a much shorter immobility time (78.5 ± 5.0 seconds) in FST (Fig. 2d), much more crossing zones and rearing times in OFT (Fig. 2e, f), and an increased body weight (435.6 ± 2.9 g, Fig. 2g) than DeS+Veh rats. Emodin treatment had no effects on the emotional behaviors and body weight of Ctrl rats.

Studies in humans and animals have confirmed the reduced volume of the hippocampus in depressed brain, which was characterized by loss of neurons and loss of synapses [35, 36]. In this research, by Nissl staining we found DeS+Veh rats had much less neurons in hippocampal CA1, CA3 and DG regions (Fig. 2h-k), whereas no obvious hippocampal neuron loss was observed in DeS+Emo rats (Fig. 2h-k). By Golgi staining, DeS+Veh rats showed the significant decreased density of dendritic spines and number of mushroom-type spines in CA1 (Fig. 2l-n). DeS+Emo rats had more spines, especially mushroom-type spines, than DeS+Veh rats (Fig. 2l-n). Similar alterations of dendritic spines in CA3 and DG were observed (data not shown). Emodin treatment had no effects on the hippocampal neuron number and spine density of Ctrl rats.

All these data suggested that emodin improved the depressive behaviors and ameliorated the losses of hippocampal neuron and dendritic spine in depressed brain.

Depression associated microglia activation were inhibited by emodin

Microglial cells, the brain-resident macrophages, are associated with inflammation in depression [37, 38]. By Iba1 (a microglial marker)-based immunohistochemical staining (Fig. 3a), the obvious increased densities of microglia in hippocampal CA1, CA3 and DG regions of DeS+Veh rats were found. The densities of microglia in DeS+Emo rats were significantly decreased compared with DeS+Veh rats (Fig. 3a-d). Increased solidity (the ratio between the positive area and the convex area) represents the activation of microglia[39-41]. In this research, we divided the solidity value of the microglia into 3 grades, e.g. <0.25 (ramified), 0.25-0.31 (hypertrofied) and >0.31 (bushy, also termed as amoeboid), and the higher solidity value indicates higher activation of microglia[39]. As shown in Figure 3e and f, 69% microglia in Ctrl+Veh rats, 67.1% in Ctrl+Emo rats and 56.6% in DeS+Emo rats had their solidity value below 0.25. Only 16.4% microglia in DeS+Veh rats had their solidity value below 0.25. DeS+Veh rats had the most microglia (48.4%) with the highest solidity value, greater than 0.31, indicating their activation. By ELISA, DeS+Veh rats had the obvious increased levels of IL-1 β and TNF- α in their hippocampi (Fig. 3g, h). There was no difference in the proportion of microglia solidity value and levels of IL-1 β and TNF- α between DeS+Emo and Ctrl rats. All these data suggested that the depression associated microglia activation was inhibited by emodin.

Emodin inhibited the activation of 5-LO by upregulating miR-139-5p

5-LO, an important pro-inflammatory enzyme widely expressed in brain, initiates leukotrienes (LTs) synthesis from arachidonic acid. DeS+Veh rats showed significantly elevated levels of 5-LO and LTB₄ (a major metabolic product of 5-LO activation) (Fig. 4a-c). However, DeS+Emo rats showed significantly lower levels of 5-LO and LTB₄ compared with DeS+Veh (Fig. 4a-c). Upon stimulation, 5-LO could be translated to nuclear and perinuclear membranes, and this translocation is regarded as a determinant of its LTB₄ synthetic capacity[42]. Therefore, the homogenate of hippocampus was divided into cytoplasmic and nuclear fractions. It was found that DeS+Veh rats, but not DeS+Emo rats, had the significantly higher nuclear 5-LO level than those in Ctrl+Veh and Ctrl+ Emo rats (Fig. 4e, f). By immunofluorescence staining and fluorescence intensity distribution analysis (FIDA) in neurons of CA1 (Fig. 4h-j), CA3 and DG (data not shown), more 5-LO was observed in the nucleus in DeS+Veh rats, whereas it was mainly in the cytoplasm in DeS+Emo (Fig. 4h-j) and Ctrl rats (data not shown).

5-LO inhibition might exhibit anti-inflammation capacity by down-regulating NF- κ B[43, 44]. NF- κ B, consisting of p50 and p65, resides in the cytoplasm of resting cells. In response to stimulation, NF- κ B p65 is activated by phosphorylation and translocates to the nucleus, followed by binding to specific DNA sequences in target genes involved in inflammation [43, 45] and apoptosis [46]. In this research, increased NF- κ B p65 was found in hippocampus of DeS+Veh rats (Fig. 4a, d), and this increasing was mainly concentrated in the nuclear fraction (Fig. 4e, g). Emodin treatment significantly inhibited the increasing nuclear NF- κ B p65 (Fig. 4e, g).

miR-139-5p, a neuron enriched miRNA in brain, plays critical roles in regulating neuronal differentiation, maturation, and survival in rodents and humans[47]. By Targetscan software (<http://www.targetscan.org>), miRbase (<http://www.mirbase.org/>), and miRDB (<http://www.mirdb.org/miRDB/>), miR139-5p was suggested bind to a 7-bp sequence in the 5-*LO* 3'-UTR. This binding site is localized at the 5' end of the 3'-UTR (51-57 bp from the 5' end). In this research, hippocampal miR139-5p was significantly decreased in DeS+Veh rats (Fig. 5a), whereas no obvious decreasing of miR139-5p was shown in DeS+Emo rats (Fig. 5a). To verify the posttranscriptional regulation of 5-LO by miR139-5p, we constructed the wild-type 3'-UTR of 5-*LO* (5-LO Wt), which contains the binding site to miR139-5p, and a mutated version (5-LO Mut) to the luciferase reporter vector, which were cotransfected into HEK293 cells with miR139-5p mimic or a negative control (miR-mimic NC) (Fig. 5b). Overexpression of miR139-5p suppressed the expression of the luciferase reporter gene with the 5-*LO* 3'-UTR, whereas reporter expression was not suppressed when we mutated the miR139-5p recognition site located on the 5-*LO* 3' UTR (Fig. 5c). Moreover, the miR139-5p inhibitor elevated 5-LO protein level in N2a cells (Fig. 5d, e). Thus, all these data showed miR139-5p inhibited 5-LO expression via an interaction with the 5-*LO* 3'-UTR, and emodin might inhibit the activation of 5-LO partially by upregulating miR139-5p.

Emodin inhibited hippocampal GSK3 β activation

Previous research suggested stress leads to GSK3 β activation depending on 5-LO[48]. In this research, the total levels of hippocampal GSK3 β were equal in all groups, while its phosphorylation level at Ser9 (p-GSK3 β , inactive form) was significantly decreased in DeS+Veh rats (Fig. 6a, b). Furthermore, the

decreasing of phosphorylated GSK3 β at Ser9 was observed in both cytoplasmic and nuclear fractions of DeS+Veh rats (Fig. 6c, d). In hippocampi of DeS+Emo rats, the phosphorylation levels of GSK3 β at Ser9 in both cytoplasmic and nuclear fractions were higher than those in DeS+Veh rats, indicating the inhibition of emodin on GSK3 β activation (Fig. 6c, d). Furtherly, we detected the levels of Nrf2, which is a transcriptional activator of antioxidant genes. GSK3 β could indirectly phosphorylate Nrf2 (at Tyr568) by activating src-related kinase Fyn [49, 50] or directly phosphorylate Nrf2 (at Ser335 and Ser338), resulting in nuclear export and cytoplasmic degradation of Nrf2[51]. We observed decreased nuclear Nrf2 and increased cytoplasmic Nrf2 in hippocampi of DeS+Veh rats, indicating the increased the nuclear export of Nrf2 (Fig. 6e, f). In hippocampi of DeS+Emo rats, the nuclear export of Nrf2 was not observed (Fig. 6e, f). The phosphorylation of Nrf2 at Ser40 is important for its transcriptional property. In DeS+Veh rats, decreased phosphorylation of Nrf2 at Ser40 was in the nuclear fraction (Fig. 6e, f). DeS+Emo rats had much more nuclear Ser40 phosphorylated Nrf2 than the other three groups of rats. The alterations of phosphorylated Nrf2 at Ser40 were confirmed by its immunohistochemical and immunofluorescence staining in brain slices (Fig. 6g, h). All these data suggested that emodin might inhibit GSK3 β activation and act as an Nrf2 activator.

Discussion

In this research, emodin, a natural compound with anti-5-LO activity, blocked the occurrence of stress-induced depression. The significantly up-regulated hippocampal DEPs, e.g. Fga, Fgb, Fgg, Ahsg, Col1a1 and Anxa2, suggested hippocampal inflammation in the development of depression, which was confirmed by the activated microglia and the increased IL1b, TNFa and NF- κ B p65 in hippocampi of depressed rats. Increased hippocampal 5-LO and its major metabolic product LTB4 suggested the activation of 5-LO. Emodin obviously improved the depression behaviors and a series of pathological changes in hippocampus, such as hippocampal neuron and spine loss, microglia activation, increased IL-1b and TNF-a, and the activation of 5-LO and GSK3 β . Furtherly, we demonstrated that emodin blocked 5-LO related inflammatory by targeting miR139-5p.

Stress could induce a series of serious mental disorders including major depressive disorder (MDD), anxiety, mania, and bipolar disorder. However, some individuals exposed to the similar levels of stress maintain normal psychological and physical functioning and escape serious mental illness, who are considered to be resilient. In this research, rats showing a graded response to stress after CUMS exposure, were divided into stress-susceptible group and stressed-resilient as reported[52, 53]. Previous studies have reported that the different pathophysiological features of the two groups [52, 54]. In this research, hippocampal inflammation in the development of depression was suggested by the proteomics data. And anti-inflammatory was effective in blocking the stress-induced depression.

There is growing evidence indicating that stress alters miRNAs in the brain, and alterations of miRNAs contribute to depression[55, 56]. Nevertheless, the involvement of miRNA-mediated gene dysregulation in chronic stress-induced excessive neuroinflammation and depression-like behaviors remains largely unexplored. Based on our data and several miR-screening studies, we found that miR139-5p, a noncoding

RNA involved in the inflammatory responses, was markedly suppressed in the hippocampus of depressed rats. miR139-5p had been demonstrated to attenuate brain damage through regulating neuronal apoptosis and mediating behavioral responses to chronic stress in rats[57, 58]. We focused on downstream target genes of miR139-5p contributing to depression-like behaviors and inflammation. The miR-targeting predictions were performed by 3 different web-based algorithms, Targetscan software, miRbase, and miRDB, and then, 5-LO was screened out. By experiments *in vitro*, we confirmed the regulation of miR139-5p on the expression of 5-LO.

Emodin was reported to inhibit lipopolysaccharide (LPS) induced microglial activation, as well as protect synaptic transmission of hippocampal neurons from glutamate excitotoxicity[59, 60]. 5-LO plays a key role in the formation of LTs and appears to be a key part of the pathophysiology of inflammation[61]. Our findings are consistent with previous studies where it was found that pharmacological inhibition 5-LO ameliorates depression-like behaviors or cognitive impairment[62, 63]. In our study, CUMS induced the activation of 5-LO in DeS+Veh rats. Although the elevated 5-LO was not restored to a normal level in DeS+Emo rats, the emodin-treated DeS rats had a normal activity of 5-LO. Previous research suggested that stress led to an elevation of 5-LO activity due to the level and translocation of 5-LO[64, 65]. By further study, the 5-LO in the nucleus was found to significantly increase in DeS+Veh rats, while emodin treated DeS rats had an evaluated 5-LO in the cytoplasm other than in the nucleus. We also found decreased miR139-5p along with increased 5-LO in DeS+Veh rats and a normal miR139-5p level rescued by emodin. However, the emodin treated DeS rats had a lower level of 5-LO not a normal level compared with DeS+Veh rats, but had a normal activity of 5-LO, which indicated that emodin not only decreased 5-LO level by acting on miR139-5p, but also decreased 5-LO activity by inhibiting its nuclear translocation. The involved mechanisms need further investigations.

5-LO has been revealed to be associated with a stress-induced elevation of GSK3 β kinase activity[66]. GSK3 β activation was reported in brains of AD and depression, and GSK3 β inhibition was a therapeutic target both of AD and depression[67, 68]. Further, GSK3 β could indirectly phosphorylate Nrf2 (at Tyr568) by activating src-related kinase Fyn[49, 50] or directly phosphorylate Nrf2 (at Ser335 and Ser338), resulting in nuclear export and cytoplasmic degradation of Nrf2[51]. In this study, GSK3 β activation was shown in hippocampi of DeS+Veh rats by decreased phosphorylation levels of GSK3 β at Ser9. The decreased nuclear Nrf2 and increased cytoplasmic Nrf2 in hippocampi of DeS+Veh rats were observed, indicating the increased the nuclear export of Nrf2.

It was reported that ablation of Nrf2 triggers depression-like behaviors related to an increased inflammation and Nrf2 agonist afforded antidepressant-like effects in an animal inflammatory model of depression[69]. Nrf2, a transcription factor, belongs to the basic leucine zipper transcription factor family and plays a central role in regulating inflammation and regulating production of antioxidants and antioxidant enzymes[70, 71]. Under basal conditions, Keap-1 maintains Nrf2 in the cytoplasm by forming Keap1-Nrf2 complex, and then Nrf2 is ubiquitinated and subsequently degraded by the 26S proteasome[72]. When a moderate stimulus appears, Nrf2 is more phosphorylated at Ser40 and transferred to the nucleus, and then play a protective role[73, 74]. In DeS+Veh rats, decreased

phosphorylation of nuclear Nrf2 at Ser40 was observed. DeS+Emo rats had much more nuclear Ser40 phosphorylated Nrf2 than the other three groups of rats. Meanwhile, the nuclear export of Nrf2 was not observed in DeS+Emo rats.

Conclusions

Taken together, this study demonstrated emodin blocked the occurrence of stress-induced depression by inhibiting 5-LO related inflammatory *via* upregulating miR139-5p. These results established a key role of miR139-5p/5-LO in the development of stress-induced depression and provided an important evidence that emodin may be a candidate agent for the treatment of depression.

Abbreviations

Dep: Depression; Res: Depression resistant; Ctrl: Control; CUMS: Chronic unpredicted mild stress; DeS: Depression susceptible; Ins: Stress insensitive; Emo: Emodin; Veh: Vehicle; IL: Interleukin; TNF- α : Tumor necrosis factor- α ; 5-LO: 5-lipoxygenase; GSK3 β : Glycogen synthase kinase 3 β ; Nrf2: Nuclear factor erythroid 2-related factor 2; NF- κ B: Nuclear factor- κ B; TSST: Trier social stress test; NO: Nitric oxide; PET: Positron emission tomography; NLRP3: NOD-like receptor protein 3; NSAIDs: Nonsteroidal anti-inflammatory drugs; SPT: Sucrose preference test; FST: Force swimming test; OFT: Open field test; PMSF: Phenylmethylsulfonyl fluoride; LTB4: Leukotriene B4; OD: Optical density; PPI: Protein-protein interaction; MDD: Major depressive disorder; Fg: Fibrinogen; Ahsg: α 2-HS glycoprotein; Vim: Vimentin; TLR4: [Toll-like receptor-4](#); Col1a1: Collagen type I α 1 chain; Anxa1: Annexin A1; BBB: Blood-brain barrier; GSTA4: Glutathione S-transferase A4; LPS: Lipopolysaccharide.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Zhang Teng and Yang Can conceived and directed the study, interpreted the results, and wrote the manuscript. Zhang Teng and Tian Qing designed and performed most of the experiments and analyzed most of the results. Chu Jiang, Ning Linna, Zeng Peng, Wang Xiaoming, Shi Yan, and Qin Baojian contributed to the experiments. Qu Na and Zhang Qi provided supervision. Zhang Teng, Ning Linna, Qu Na, and Tian Qing provided funding for experiments. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets and materials used and/or analyzed during current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Care and Use Committee of Huazhong University of Science and Technology.

Consent for publication

Not applicable.

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table

Table 1 Antibodies used in this study

Antibody	Epitopes	mAb/pAb	WB	IHC or IF	Source
DM1A	alpha-tubulin	mAb	1:2000 for WB		Abcam
Calba1	ionized calcium binding adapter molecule 1	pAb		1:200	Wako
anti-GSK3β	total GSK3β	pAb	1:1000 for WB		Cell Signaling
anti-GSK3β	p-GSK3β at Ser9	pAb	1:1000 for WB		Cell Signaling
anti-Nrf2	total Nrf2	pAb	1:500 for WB		Abcam
anti-Nrf2	p-Nrf2 at Ser40	pAb	1:1000 for WB	1:100	Abcam
anti-5-LO	total 5-Lipoxygenase	mAb	1:1000 for WB	1:100	Abcam
anti-NF-κB p65	total NF-κB p65	mAb	1:500 for WB		Cell Signaling

p, phosphorylated; mAb, monoclonal antibody; pAb, polyclonal antibody;WB, western blotting; IHC, immunohistochemistry; IF, Immunofluorescence.

Figures

Figure 1

Inflammation, the prominent differential signal in hippocampi of depression, depression resistant and control rats. (a) Schematic illustration of the first part of this research, e.g. proteomics research. 45 rats were evaluated depressive status by sucrose preference test (SPT), forced swimming test (FST) and open field test (OFT). Then, 15 rats were randomly chosen as control (Ctrl) rats, and 30 rats were daily exposed to chronic unpredicted mild stress (CUMS). After 7 weeks' CUMS exposure, depression resistant (Res) rats

and 14 depression (Dep) rats were obtained by SPT, SPT and OFT. The hippocampi were extracted and investigated by isobaric tags for relative and absolute quantitation (iTRAQ) (n=3/group). The sucrose preference rates in SPT (b), immobility time in FST (c), numbers of zone crossing (d) and rearing times (e) in OFT, and the body weights of rats (f) were recorded. 259 differential expressed proteins (DEPs) were obtained, e.g. 93 DEPs in Dep/Ctrl, 109 DEPs in Dep/Res, and 141 DEPs in Res/Ctrl (g). In 20 down-regulated DEPs of Dep/Ctrl, 7 were down-regulated in Dep/Res and 13 were down-regulated in Res/Ctrl (h). 18 up-regulated proteins in both Dep/Ctrl and Dep/Res and 4 up-regulated proteins in both Dep/Ctrl and Res/Ctrl were shown (i). 22 up-regulated DEPs in Dep/Res were down-regulated (j) and 6 down-regulated DEPs in Dep/Res were up-regulated (k) in Res/Ctrl. 9 DEPs were common in Dep/Ctrl, Dep/Res and Res/Ctrl (l). By STRING 11.0 (<https://string-db.org/>), protein-protein interaction (PPI) networks construction for DEPs in Dep/Ctrl rats (m) and Dep/Res rats (n) were qualified. The thickness of edges was decided by the combined score. The DEPs related to inflammatory responses were listed with a ratio (o). Data were analyzed by one-way ANOVA and expressed as the mean $\pm$ SEM, ** p <0.01 Res or Dep versus Ctrl. ## p <0.01 Dep versus Res.

Figure 2

Emodin ameliorated depression-like behaviors in depressive tendency rats. Schematic illustration of the second part of this research (a). After evaluated by SPT, OFT and FST, 64 rats were exposed to CUMS for 5 weeks, and the other 30 rats were left unstressed as control (Ctrl). By the percentage of sucrose water preference in SPT performed on 36th day (b), 30 depression-susceptible (DeS) rats and 28 stress insensitive (Ins) rats were obtained. Then, DeS and Ctrl rats daily received emodin (Emo) (80mg/kg) or

the same volume of Tween-80 (Veh) by intragastric administration (n=15/per group) from 37th to 50th day. Simultaneously, DeS rats (DeS+Emo rats and DeS+Veh rats) were also exposed to CUMS. The sucrose preference rates in SPT on 51st day (c), immobility time in FST on 52nd day (d), numbers of zone crossing (e) and rearing times (f) in OFT on 53rd day, and the body weights on 51st day (g) were recorded. Hippocampal neurons were shown by Nissl staining (h, left scale bar=200 μ m, right scale bar=20 μ m) and quantified in CA1 (i), CA3 (j) and DG (k) regions (n=3 brains/group). The dendrites of the CA1 neurons were shown by Golgi staining (l, scale bar=1 μ m). Quantification of the density of dendritic spine (m) and mushroom-type spine (n) were calculated by Image-Pro Plus 6.0 software (n=21 dendrites from 3 brains/group). Data were analyzed by one-way ANOVA and presented as mean $\pm$ S.E.M., ** p<0.01 DeS+Veh versus Ctrl+Veh, ## p<0.01 DeS+Emo versus DeS+Veh.

Figure 3

Depression associated microglia activation were inhibited by emodin. By immunohistochemistry staining with Iba1 (a marker of microglia), the hippocampal microglia were shown (a) (left scale bar=200 μ m, right scale bar=20 μ m), and their densities in CA1 (b), CA3 (c) and DG (d) regions were calculated (n=3 brains/group). The solidity value analysis was used to evaluate the activation of microglia. Higher solidity value indicates higher activity of microglia. We divided the solidity value of the microglia into 3

grades (<0.25, 0.25–0.31, >0.31), and the percentages of microglia with different grades in different groups were shown in (e) and (f) (n=3 brains/group). By ELISA, the hippocampal levels of interleukin-1b (IL-1b, g) and tumor necrosis factor-a (TNF-a, h) were assessed (n=3 brains/group). Data were analyzed by one-way ANOVA and presented as mean ± S.E.M., ** p<0.01 DeS+Veh versus Ctrl+Veh, ## p<0.01 DeS+Emo versus DeS+Veh.

Figure 4

Emodin inhibited the activation of 5-lipoxygenase (5-LO) and nuclear factor-κB (NF-κB). The levels of 5-LO and NF-κB p65 (activated form of NF-κB) in hippocampus were tested by Western blotting (a) and quantitative analysis (b, d) (n=6 brains/group). By ELISA, the hippocampal levels of leukotriene B4 (LTB₄) were assessed (n=3 brains/group). The levels of 5-LO and NF-κB p65 in the nuclear and cytoplasmic fractions were detected by Western blotting (e) and quantitative analysis (f, g) (n=6 brains/group). By

immunofluorescence staining (h), the significant co-localization between 5-LO and DAPI (blue, marking nucleus) was shown in the hippocampal CA1 of DeS+Veh rats, and 5-LO was transferred from the nucleus to the cytoplasm by emodin (scale bar=20 μ m, n=3 brains/group). (i, j) Fluorescence signal intensities of 5-LO (green) and DAPI (blue) were measured by line tool. Data were analyzed by one-way ANOVA and presented as mean $\pm$ S.E.M., ** p<0.01 DeS+Veh versus Ctrl+Veh, $\Delta\Delta$ p<0.01 DeS+Emo versus Ctrl+Emo, # p<0.05, ## p<0.01 DeS+Emo versus DeS+Veh.

Figure 5

Emodin decreased 5-LO level by upregulating miR-139-5p. Hippocampal levels of miR139-5p were analyzed by quantitative real-time PCR (a, n=3 brains/group). ** p<0.01 DeS+Veh versus Ctrl+Veh, ## p<0.01 DeS+Emo versus DeS+Veh. The binding site for miR139-5p seed sequence in the ALOX5 3'-UTR

(b). Luciferase reporter assay results demonstrated that miR139-5p targets ALOX5 3'-UTR (c). The level of 5-LO in N2a cells treated with miR139-5p inhibitor was tested by Western blotting (d) and quantitatively analyzed (e). ** $p < 0.01$ A-miR139-5p versus Veh. Data were analyzed by one-way ANOVA and presented as mean $\pm$ S.E.M..

Figure 6

Emodin inhibited hippocampal GSK3 β activation. Levels of hippocampal total GSK3 β (t-GSK3 β) and phosphorylated GSK3 β (p-GSK3 β , Ser9) were shown by Western blotting (a) and quantitatively analyzed (b) (n=6 brains/group). Meanwhile, the levels of t-GSK3 β and p-GSK3 β in the nuclear and cytoplasmic fractions were tested by Western blotting (c) and quantitatively analyzed (d) (n=6 brains/group). The levels of t-Nrf2 and p-Nrf2 in the nuclear and cytoplasmic fractions were tested by Western blotting (e) and quantitatively analyzed (f) (n=6 brains/group). Data were analyzed by one-way ANOVA and presented as mean $\pm$ S.E.M., ** p<0.01 DeS+Veh versus Ctrl+Veh, $\Delta\Delta$ p<0.01 DeS+Emo versus Ctrl+Emo, ## p<0.01 DeS+Emo versus DeS+Veh. By immunohistochemistry staining (g), phosphorylated Nrf2 atSer40 (p-Nrf2) positive hippocampal neurons were shown (left scale bar=200 μ m, right scale bar=20 μ m, n=3 brains/group). By double-label immunofluorescence staining (h), in DeS+Emo rats most p-Nrf2 (red) positive cells were NeuN (a marker of neuron, green) positive, and all p-Nrf2 co-localized with DAPI (blue, marking nucleus) (scale bar=20 μ m, n=3 brains/group).