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Chronic mild stress induced anxiety-like behaviors can Be attenuated by inhibition of NOX2-derived oxidative stress

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ABSTRACT

Chronic stress-induced anxiety disorder is a highly-prevalent, modern social disease in which oxidative stress plays an important role. It is necessary to determine the underlying mechanisms governing this disorder to establish an effective treatment target for anxiety disorders. In this study, we examined the behavioral changes in mice subjected to chronic mild stress (CMS). We found that CMS exposure leads to anxiety-like phenotypes and increased levels of oxidative stress in the ventral hippocampus of mice. Furthermore, CMS increased the excitatory synaptic transmission of pyramidal cells in the ventral CA1 (vCA1). Administration of 4-hydroxy-3-methoxy-acetophenone (apocynin), an inhibitor of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, clearly ameliorated the changes induced by CMS exposure. In addition, our results of behavioral tests and analyses of reactive oxygen species (ROS) using NOX2-deficient mice indicate that CMS-induced enhanced oxidative stress level is primarily caused by the increased expression of NOX2. NOX2-derived oxidative stress can serve as a target for anxiety therapy led by chronic stress.

1. Introduction

Anxiety disorder is a highly prevalent mental disorder characterized by a pathological state of high arousal and elevated vigilance in the absence of immediate threats (Zhu et al., 2014; Parihar et al., 2011; Calhoun and Tye, 2015). Recent studies have identified stress, particularly chronic stress, as an important inducer of anxiety (Zhu et al., 2014; McKim et al., 2018; Zhang et al., 2018). Chronic stress is acknowledged as one of the predominant mental health issues currently affecting society (Shepard and Coutellier, 2018). Exposure to chronic stress purportedly leads to a series of detrimental effects on the human body, especially the brain, which ultimately recognizes threatening stimuli and then modulates defensive behaviors (McEwen, 2007). Prolonged stressful stimuli can be harmful to the brain, inducing psychiatric diseases like depression and anxiety disorders in both humans and rodent models. (Zhu et al., 2014; Parihar et al., 2011; McEwen, 2007; Cohen et al., 2007; Gross and Pinhasov, 2016). Early studies led to the identification of key loci controlling anxiety, mainly through activity in the prefrontal cortex (PFC), the basolateral amygdala (BLA), and the ventral hippocampus (vHPC) (Zhu et al., 2014; Calhoun and Tye, 2015; Zhang et al., 2018; Liang et al., 2016). Among these, vHPC-circuits were recently shown to play an important role in the process of anxiety by exciting neurons that drive anxiety-related behavior (Jimenez et al., 2018).

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Oxidative stress describes a state of imbalance between pro-oxidants and antioxidants that caused by excessive reactive oxygen species (ROS) which cause damage to nucleic acids, cell membranes, and proteins (Lichtenberg and Pinchuk, 2015; Fedoce et al., 2018; Kim et al., 2015). The brain consumes 20% of the body's total oxygen and is thus especially vulnerable to oxidative damage (Fedoce et al., 2018; Kim et al., 2015), which is known to be a key factor in the development and progression of neurodegenerative diseases (Kim et al., 2015) and chronic stress-induced mental illnesses such as schizophrenia, depression, and anxiety (Steullet et al., 2017; Hovatta et al., 2005; Matchkov et al., 2015; Trumbull et al., 2012; Black et al., 2017; Hernandes et al., 2010; Smaga et al., 2015). The nicotinamide adenine dinucleotide

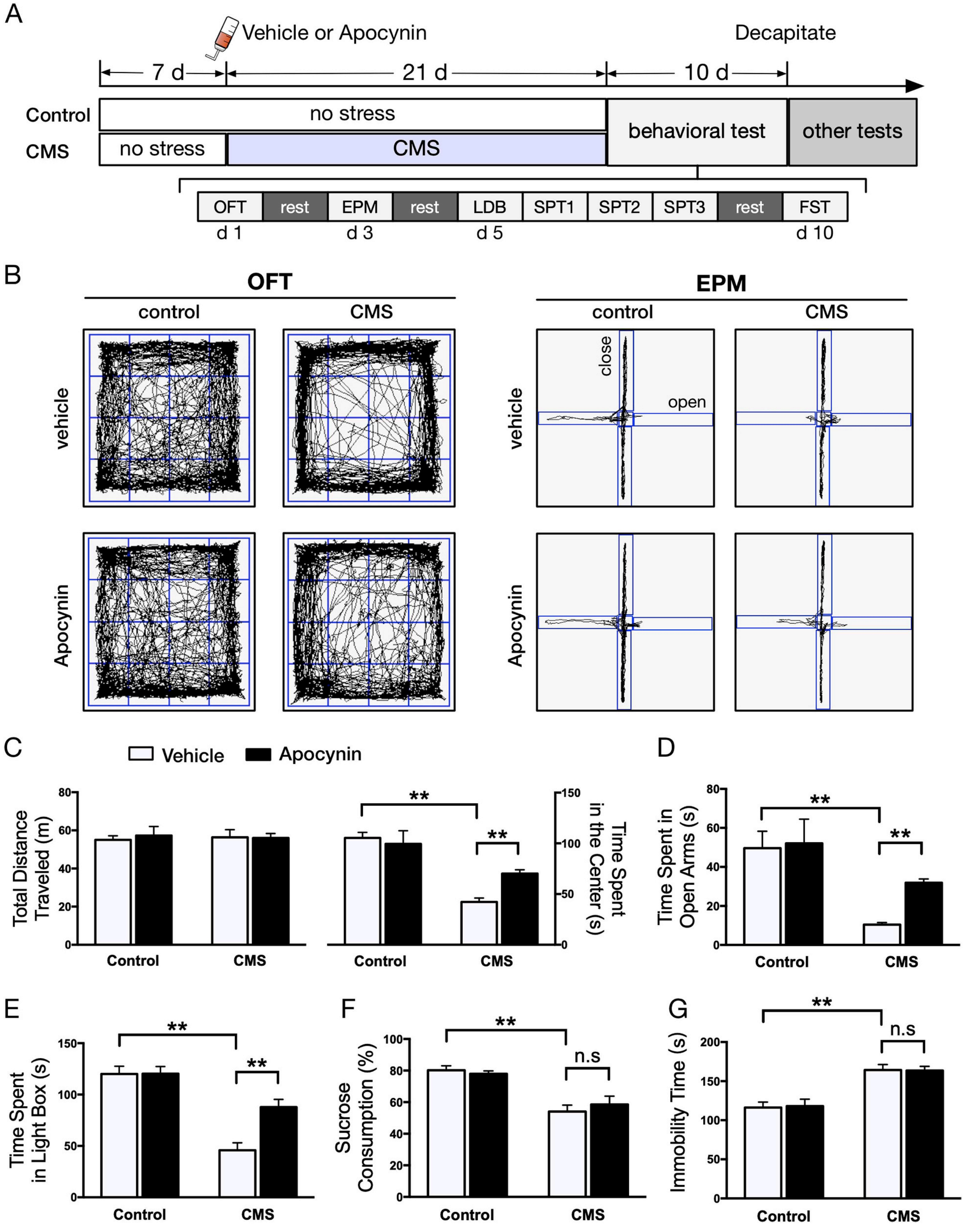

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Fig. 1. Apocynin ameliorates CMS-induced anxiety-like behaviors, but not depression-like behaviors. (A) The experimental schedule of CMS, apocynin administration, and behavioral tests.(B) Representative traces of the movement of mice during an open field box test or elevated plus maze test.(C) Apocynin increased the time spent in the center area, counteracting the effect of CMS. Left, total distance travelled. Apocynin × CMS two-way ANOVA: Interaction: $F(1,36) = 0.145$, $p = 0.706$; main effect of apocynin: $F(1,36) = 0.083$, $p = 0.775$; main effect of CMS, $F(1,36) = 0.000$, $p = 0.997$. Right, time spent in center area in the open field box. Apocynin × CMS two-way ANOVA: Interaction: $F(1,36) = 5.715$, $p = 0.022$; main effect of apocynin: $F(1,36) = 2.409$, $p = 0.129$; main effect of CMS, $F(1,36) = 42.1$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$.(D–E) Apocynin increased the time spent in the open arms and in the light box, counteracting CMS in the elevated plus maze and light/dark box test, respectively. For time in open arms, Apocynin × CMS two-way ANOVA: Interaction: $F(1,36) = 2.243$, $p = 0.143$; main effect of apocynin: $F(1,36) = 2.431$, $p = 0.128$; main effect of CMS, $F(1,36) = 16.63$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$. For time in the light box, Apocynin × CMS two-way ANOVA: Interaction: $F(1,36) = 8.108$, $p = 0.007$; main effect of apocynin: $F(1,36) = 8.281$, $p = 0.007$; main effect of CMS, $F(1,36) = 52.96$, $p < 0.001$, Tukey post hoc test, $^{**}p < 0.01$. (F–G) Apocynin had no effect on CMS-induced reduction in sucrose consumption or increased time of immobility in the forced swimming test. For sucrose consumption, Apocynin × CMS two-way ANOVA: Interaction: $F(1,36) = 0.797$, $p = 0.378$; main effect of apocynin: $F(1,36) = 0.098$, $p = 0.756$; main effect of CMS, $F(1,36) = 36.49$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$. For immobility time, Apocynin × CMS two-way ANOVA: Interaction: $F(1,36) = 0.031$, $p = 0.860$; main effect of apocynin: $F(1,36) = 0.009$, $p = 0.939$; main effect of CMS, $F(1,36) = 41.99$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$, no significant (n.s) $p > 0.05$. Data represent means ± SEM for each group (n = 9–11 mice per group).

phosphate (NADPH) oxidases (NOX) are an important source of ROS (Kim et al., 2015; Bedard and Krause, 2007), which are involved in a variety of psychiatric or neurodegenerative disorders (Bedard and Krause, 2007; Sorce and Krause, 2009). Different types of pharmacologic tools like iodonium (DPI), 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), and 4-hydroxy-3-methoxy-acetophenone (apocynin) have been applied to block NOX enzymes activity (Bedard and Krause, 2007; Altenhofer et al., 2015). Among them, apocynin is one of the most frequently used NOX inhibitor (Altenhofer et al., 2015; Stefanska and Pawliczak, 2008) and can penetrate the intact blood-brain barrier (Wang et al., 2008), we therefore applied apocynin in our following study.

NOX2, one of the NOX enzyme subunits, is widely expressed across various regions of the human and rodent brain (Sorce and Krause, 2009; Infanger et al., 2006; Schiavone et al., 2017), and has been reported as a major source of ROS in the central nervous system (CNS) (Bedard and Krause, 2007). Several studies suggest that NOX2 is responsible for the majority of ROS production in different types of rodent model of psychiatric disorders, including post-traumatic stress disorder (PTSD) induced by single prolonged stress (Liu et al., 2016) and schizophrenia induced by social isolation exposure (Schiavone et al., 2009). Furthermore, apocynin administration effectively ameliorated the behavioral deficits in these rodent models (Liu et al., 2016; Schiavone et al., 2009). Generally, oxidative stress has been considered as an important factor in the development of anxiety (Fedoce et al., 2018; Rammal et al., 2008). However, the source of ROS and how oxidative stress leads to anxiety behaviors still remain unclear.

Here, we applied pharmacologic tools and NOX2 knockout (KO) mice to investigate CMS-induced changes in NOX2-derived oxidative stress, glutamatergic synaptic transmission in vCA1 and neurobehavioral features. This study revealed the role that NOX2-derived oxidative stress playing in CMS-induced disorders of neurotransmission and anxiety behaviors, which provided potential pathogenic mechanisms and therapeutic strategies for anxiety disorders.

2. Materials and methods

2.1. Animals

A total number of 48 male C57/BL6 mice, 16 male NOX2-deficient mice (B6.129S-Cybbtm1Din/J, stock number: 002365, Jackson Laboratory) and 16 male littermate control mice were used in this study. Mice were housed at controlled temperature (20–22 °C) with a 12-h light/dark cycle and had free access to food and water. The experimental procedure has been approved by the Animals Care and Use Committee of Guangzhou University of Chinese Medicine.

2.2. CMS schedule and apocynin treatment

After one week of habituation, mice (8–9 weeks old) were subjected to 3 weeks of CMS procedure which designed as previously described (Ducottet et al., 2003). During the process, animals were randomly exposed to various types of stressors including restraint in a small tube, forced warm water bath, water/food deprivation, housing in wet sawdust, and reversed day/night cycle, etc. During CMS progress, all mice were group-housed and half of the control and CMS mice received apocynin administration (5 mg/kg/day, Abam-ab120615, dissolved in drinking water) (Schiavone et al., 2012). Based on our measurements, the daily water intake for each mouse (body weight: 23.48 ± 1.43 g) in all experimental groups was 8.11 ± 0.51 ml.

2.3. Behavioral tests

For all behavioral tests, mice were brought to the testing room 1 h before each test and were kept in the same room throughout the tests. The sequence of behavioral tests was designed as shown in Figs. 1A and 5A. All the tests were performed in apparatuses with a video analysis system from Shanghai Jiliang Software Technology Co., Ltd (Shanghai, China).

The open field test (OFT) was performed as we previously described (Xia et al., 2014). Briefly, mice were placed in a random order into a 45 × 45 × 45 cm open-field arena which located in a sound proof box. Mice were initially placed in the box through the entrance and then allowed to move freely in the arena for 30 min. The total distance travelled (mm) and the time they spent in the center area (s) in 30 min were measured with the video analysis system.

The elevated plus maze (EPM) was assessed as described previously (Walf and Frye, 2007). Briefly, the EPM apparatus consists of two open arms (30 × 5 cm), two enclosed arms (30 × 5 × 15 cm), and a central platform (5 × 5 cm) located at the intersection of the four arms. At the start of the test, each mouse was placed in the central platform facing one of the two opened arms and allowed to explore the maze for 5 min. Time spent in the open arms was measured with the video analysis system.

The light-dark box (LDB) test was performed as described previously (Zhu et al., 2014). Briefly, the LDB apparatus has two chambers: one dark chamber and one brightly illuminated chamber. The apparatus was enclosed in a sound-attenuating box to mask noise. Mice were allowed to move freely between the two chambers and the time that mice spent in each division was recorded for 5 min.

The sucrose preference test (SPT) was performed using a two-bottle choice procedure as described previously (Parihar et al., 2011). Briefly, mice were presented with two drinking bottles: one contained 1% sucrose and the other contained water. On day −2, mice were individually housed and habituated to 1% (w/v) sucrose for 24 h. On day −1, mice were deprived of water with free access to food for 16 h. On the test day, mice were housed singly and given access to two preweighed bottles, one containing water and the other containing 2% (w/v) sucrose. These two bottles were weighed again after 24 h of consumption. The preference for sucrose over water was measured by sucrose/(sucrose + water) × 100%.

The forced swim tests (FST) procedure was assessed as described previously (Sellmeijer et al., 2018). In brief, mice were placed into a transparent plastic cylinder (10 cm diameter, 30 cm height) which was filled with 20-cm-deep water (24 ± 2 °C). The forced swimming lasted for 6 min, and the immobility time, which defined as mice were absence of all motions except those required to keep their head above water, in the last 4 min were manually recorded.

2.4. Electrophysiology

After behavioral tests, electrophysiology experiments were completed within 12 days. Acute hippocampal slices were prepared as we described in a previous study (Yin et al., 2013). Briefly, mice were anesthetized with 1% sodium pentobarbital (i.p, 100 mg/kg, Sigma) and perfused transcardially for 1 min with 4 °C modified artificial cerebrospinal fluid (ACSF) containing (in mM): 2.5 KCl, 1 $NaH_2PO_4-2H_2O$, 2.5 $MgSO_4$, 1.3 $CaCl_2-2H_2O$, 26 $NaHCO_3$, 220 sucrose, and 10 D-glucose bubbled with a mixture of 5%CO_2 and 95%O_2. Mice were then decapitated and brains were quickly removed and chilled in ice-cold ACSF for 1 additional min. Transverse hippocampal slices (300 μm) were prepared using a Vibroslice (VT 1200S; Leica, GER) in ice-cold ACSF. All slices were allowed to recover for 30 min at 32–34 °C and 1 h at room temperature (about 24 °C) in ACSF as follows (in mM): 126 NaCl, 3 KCl, 1.2 $NaH_2PO_4-2H_2O$, 1 $MgSO_4$, 2 $CaCl_2-2H_2O$, 26 $NaHCO_3$, and 10 D-glucose bubbled with a mixture of 5% CO_2 and 95% O_2.

To measure the excitatory/inhibitory balance of pyramidal cells, electrophysiology experiments were performed as described previously (Zhou et al., 2009). Whole-cell voltage clamp recordings were performed in hippocampal vCA1 pyramidal neurons using an upright microscope equipped with a 40 × water-immersion lens (Nikon FN1, Tokyo, Japan) and an infrared-sensitive CCD camera (Hamatsu C11440−42U, Bridgewater, USA). Patch electrodes (3–5 MΩ) were filled with a solution containing the following (in mM): 125 $CsCH_3SO_3$, 5 CsCl, 10 HEPES, 0.2 EGTA, 1 $MgCl_2$, 4 Mg-ATP, 0.3 Na-GTP, 10 phosphocreatine, 5 Qx314, pH 7.30, and 280 mOsm. To measure spontaneous excitatory current (sEPSC) recordings, voltage clamp recordings were performed at −60 mV and spontaneous inhibitory postsynaptic current (sIPSC) recordings were measured at +10 mV; all recordings were made with a Multiclamp 700B amplifier and 1550B digitizer (Molecular Devices, CA, USA) and analyzed with a Clampfit 10.7 (Molecular Devices, CA, USA). Data were collected when series resistance fluctuated within 20% of initial values (20–30 MΩ), filtered at 1 kHz, and sampled at 10 kHz.

2.5. Measurement of oxidative stress

As described previously, ROS was assessed by dihydroethidium (DHE) imaging (Qin et al., 2017) and the concentration of malondialdehyde (MDA) (An et al., 2018). In brief, DHE (Sigma, USA) was injected intraperitoneally (2 mg/kg) 1 h before the mice were anesthetized. Then the brain was taken out and sliced into 10 μm sections with a cryostat microtome (Microm HM525 NX, Thermo Scientific, China). The DHE fluorescence in brain sections was observed using a confocal microscope (Nikon A1, Tokyo, Japan). The intensity of the fluorescence was quantified on 4–6 sections per mouse using NIH ImageJ software and the averaged intensity of fluorescence was expressed as a percentage (mean of control group was set as 100%). The MDA test kit was conducted according to the instructions of the MDA test Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), tissues were processed with corresponding reagents. Then, the samples were heated at 95 °C for 40 min and centrifuged at 4000 rpm from additional 10 min at 4 °C. The OD values were measured using an ultra-violet spectrophotometer (TechcompUV1000, Shanghai, China) at 532 nm.

2.6. Immunofluorescence

The procedure of immunofluorescence was performed as we previously described (Tao et al., 2013). In brief, mice were deeply anesthetized with 1% sodium pentobarbital and transcardially perfused with normal saline and 4% paraformaldehyde (in PBS; PH = 7.4). Then, brains were immediately removed and post fixed in 4% paraformaldehyde for 24 h and separately dehydrated in 15% and 30% sucrose at 4 °C. Brains were embedded in optimum cutting compound and cut into 40 μm sections. The sections were blocked with 3% BSA (containing 0.25% Triton X-100 and 10% goat serum) for 1 h at room temperature. The sections were then exposed to monoclonal mouse antibody against NOX2 (1:100, BD) and rabbit antibody against Iba-1 (1:200, Abcam) as primary antibody and incubated at room temperature for 14 h. We then exposed the sections to Abcam-488 goat anti-rabbit (1:2000) and Abcam-594 goat anti-mouse (1:1000) secondary antibody, incubated for 1 h at 37 °C. Then, 4′,6′-Diamidino-2-phenylindole (DAPI) counterstaining was performed to label cell nuclei. The fluorescent images were taken using a confocal microscope (Nikon Eclipse Ti, Japan). The total number of NOX2 positive (NOX2+) cells in vCA1 (4–6 sections per mouse) were manually counted for each double-immunolabeled z-stack.

2.7. Real-time quantitative PCR

Real-time quantitative PCR was performed as we previously described (Yao et al., 2017). In brief, total RNA from the ventral hippocampus was isolated using E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, Inc., Norcross, GA). Total RNA was reversed transcribed with the PrimeScript™RT reagent Kit (Takara, Japan) to generate cDNA. Gene expression was determined by quantitative PCR with SYBR Green Dye Gene Expression Assays, which was performed on an ABI7500 system (Applied Biosystems, Carlsbad, CA, USA). The primers were synthesized by Shanghai Sangon Biological Engineering Technology Company (Shanghai, China). Primer sequences were as follows: NOX2 (forward: 5′- CTCC TTGG GTCA GCAC TGGC; reverse: 5′- CTCC TTGG GTCA GCAC TGGC) and β-actin (forward: 5′- CTCC TTGG GTCA GCAC TGGC; reverse: 5′- CTCC TTGG GTCA GCAC TGGC). The cycle threshold, determined as the initial increase in fluorescence above background, was determined for each sample. β-actin was used as internal control for normalization.

2.8. Statistics

All the tests were performed by experimenters who were blinded to the genotype and grouping of the animals. Data were analyzed using SPSS 19.0. All results were expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA. Comparisons between two groups were analyzed using Tukey post hoc tests. For all tests, a probability of $P < 0.05$ was considered to statistically significant.

3. Results

3.1. Apocynin attenuates CMS-induced anxiety-like but not depressive-like behaviors

To test the impact of chronic stress on animal behavioral deficits, we performed a series of behavioral tests in adult C57/BL6 mice with CMS exposure. In our study, mice subjected to CMS showed decreased time in the central field (OFT), open arms (EPM) and light box (LDB), compared to control mice in each respective test (Fig. 1B–E). Compared to vehicle groups, apocynin administration significantly ameliorated the decreased time in the central field (Fig. 1C), open arms (Fig. 1D), and light box (Fig. 1E). We also found that CMS decreased the consumption of sucrose in the SPT (Fig. 1F) and increased immobility time during FST (Fig. 1G). Unlike the effects found in tests of anxiety-like

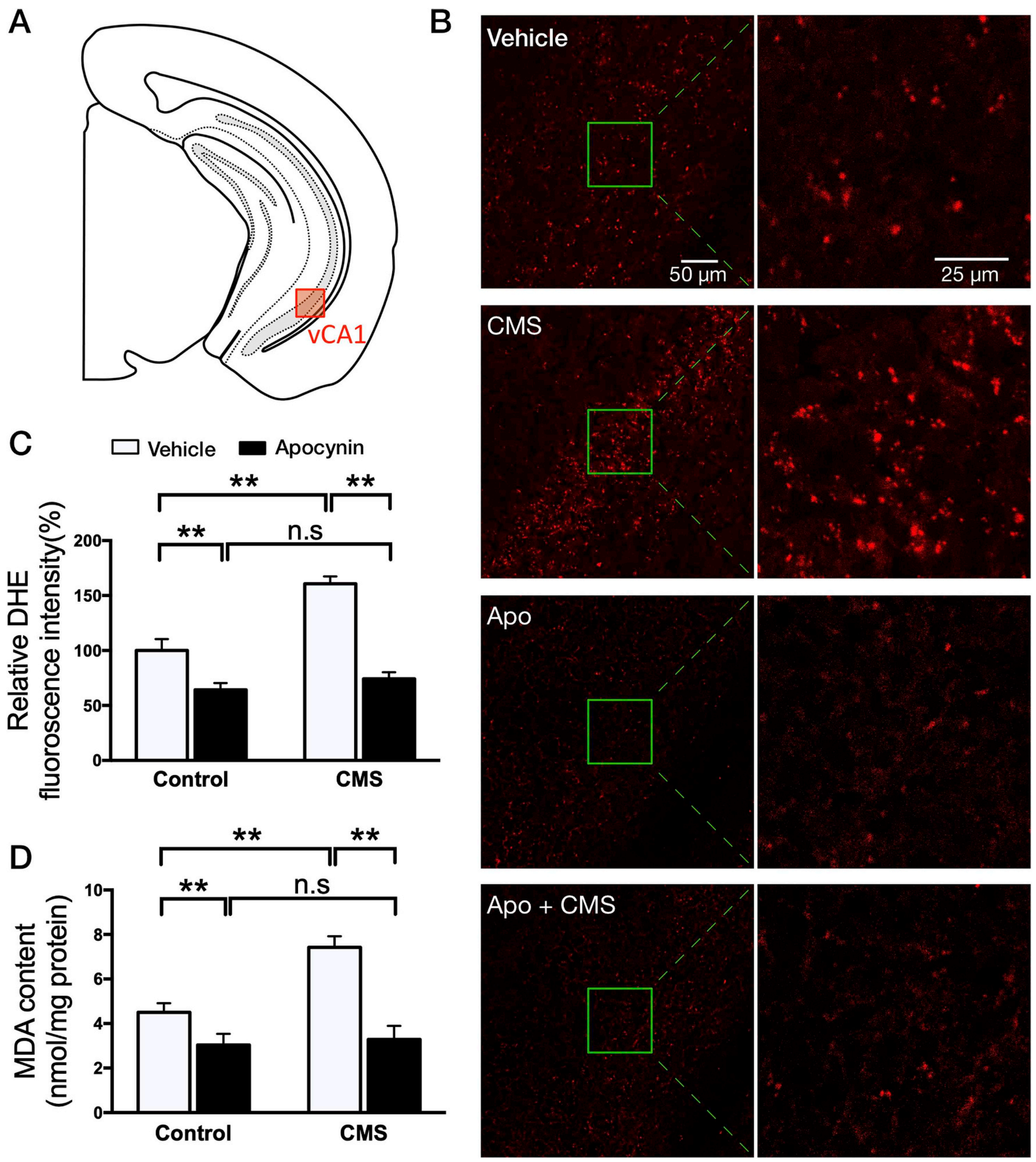

Fig. 2. CMS leads to increased levels of oxidative stress in vCA1 of mice. (A, B) Dihydroethidium (DHE) staining showing ROS levels in the vCA1 region of mice. (C) Fluorescence intensity of DHE in the vCA1 region of mice. Apocynin × CMS two-way ANOVA: Interaction: $F(1,8) = 3.006$, $p = 0.121$; main effect of apocynin: $F(1,8) = 49.7$, $p < 0.001$; main effect of CMS, $F(1,8) = 9.845$, $p = 0.014$, Tukey post hoc test,$^{**}p < 0.01$, no significant (n.s) $p > 0.05$. ($n = 3$ mice per group). (D) The MDA content in vHPC of mice. Apocynin × CMS two-way ANOVA: Interaction: $F(1,11) = 6.609$, $p = 0.260$; main effect of apocynin: $F(1,11) = 29.02$, $p < 0.001$; main effect of CMS, $F(1,11) = 9.335$, $p = 0.109$, Tukey post hoc test,$^{**}p < 0.01$, no significant (n.s) $p > 0.05$. ($n = 3$–4 mice per group). Data represent means ± SEM for each group. $^{*}p < 0.05$, $^{**}p < 0.01$.

behaviors, apocynin administration did not affect the decreased sucrose consumption (Fig. 1F) or increased immobility time in the swimming box (Fig. 1G). We monitored the weekly body weight of mice and there was no significant difference between all groups before and during CMS period (Fig S1).

3.2. CMS leads to oxidative stress in ventral CA1 (vCA1)

To further determine the level of oxidative stress, we tested the levels of oxidative stress in the vCA1 of mice. As measured by DHE staining, mice subjected to CMS displayed a significant increase of ROS levels in vCA1 compared to control mice, while apocynin administration prevented the increase of ROS levels in the vCA1 of mice exposed to CMS (Fig. 2B and C). Similarly, CMS also augmented the expression levels of MDA in the vHPC compared to that of control mice (Fig. 2D). Apocynin administration significantly lowered the level of MDA in both control and CMS-exposed mice compared to vehicle administrated mice (Fig. 2D). As a control, we investigated the level of oxidative stress in the medial prefrontal-cortex (mPFC), another brain area involved in the process of anxiety (Padilla-Coreano et al., 2016). We found that CMS causes no significant changes in the density of DHE imaging and the level of lipid peroxides in the mPFC (Fig S2).

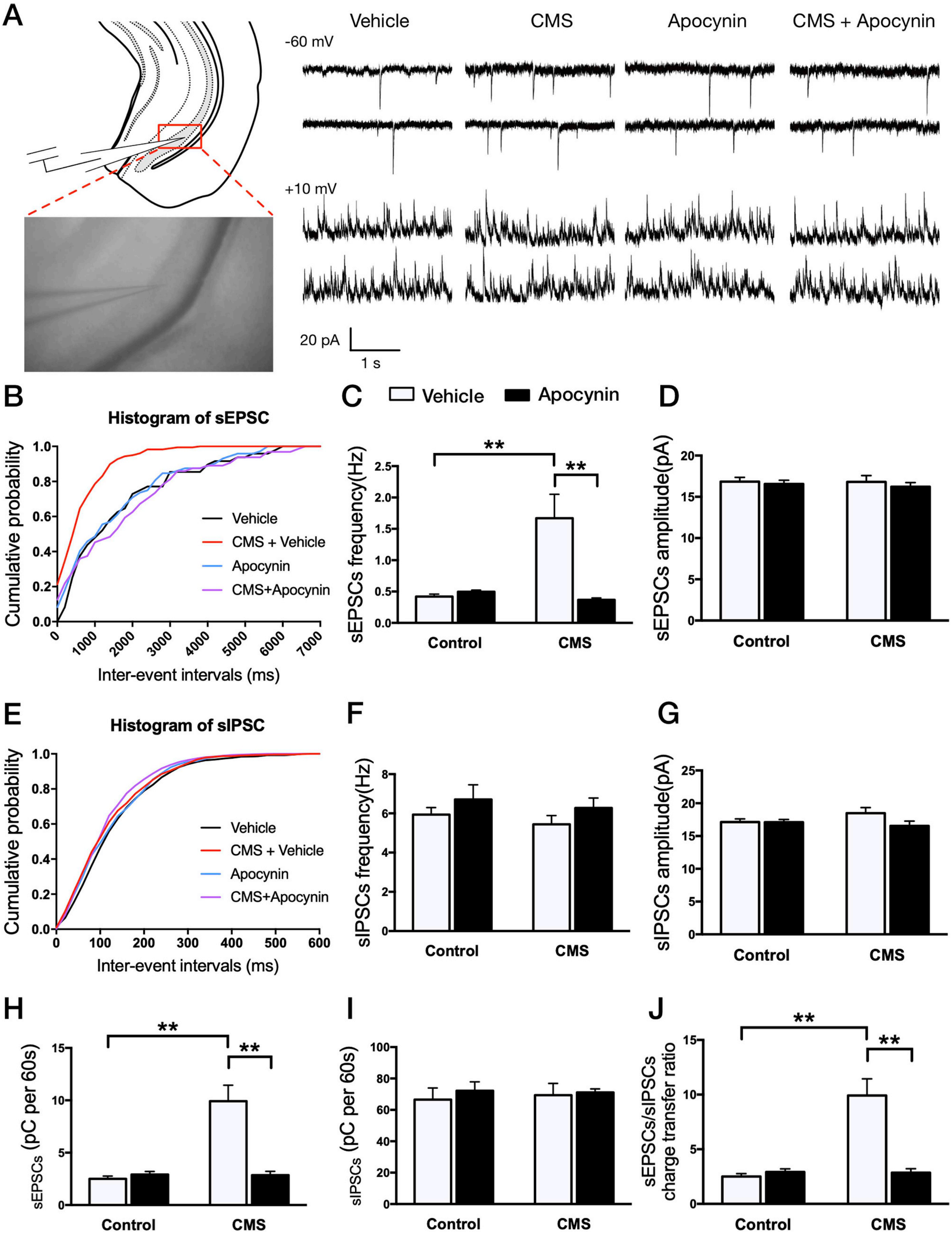

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Fig. 3. CMS leads to an increased excitatory synaptic transmission in vCA1 pyramidal cells which can be ameliorated by apocynin. (A) Representative traces showing spontaneous EPSC (sEPSC, upper) and IPSC (sIPSC, lower) recorded in the same cell at −60 mV (upper) and +10 mV (lower). (B) Cumulative probability plots of sEPSC inter-event intervals. (C) frequency of sEPSC. Apocynin × CMS two-way ANOVA: Interaction: F(1,76) = 11.68, p = 0.001; main effect of apocynin: F(1,76) = 9.183, p = 0.003; main effect of CMS, F(1,76) = 7.669, p = 0.007, Tukey post hoc test,**p < 0.01. (D) amplitude of sEPSC. Apocynin × CMS two-way ANOVA: Interaction: F(1,76) = 0.065, p = 0.799; main effect of apocynin: F(1,76) = 0.580, p = 0.449; main effect of CMS, F(1,76) = 0.097, p = 0.756. (E) Cumulative probability plots of sIPSC inter-event intervals. (F) frequency of sIPSC. Apocynin × CMS two-way ANOVA: Interaction: F(1,76) = 0.003, p = 0.958; main effect of apocynin: F(1,76) = 2.19, p = 0.143; main effect of CMS, F(1,76) = 0.738, p = 0.393.(G) amplitude of sIPSC. Apocynin × CMS two-way ANOVA: Interaction: F(1,76) = 2.211, p = 0.141; main effect of apocynin: F(1,76) = 2.331, p = 0.131; main effect of CMS, F(1,76) = 0.382, p = 0.538.(H) and Quantification of sEPSC. Apocynin × CMS two-way ANOVA: Interaction: F(1,76) = 19.89, p < 0.001; main effect of apocynin: F(1,76) = 15.7, p < 0.001; main effect of CMS, F(1,76) = 19.19, p < 0.001, Tukey post hoc test,**p < 0.01.(I) Quantification of sIPSC. Apocynin × CMS two-way ANOVA: Interaction: F(1,76) = 0.101, p = 0.751; main effect of apocynin: F(1,76) = 0.354, p = 0.554; main effect of CMS, F(1,76) = 0.019, p = 0.890. (J) sEPSC/sIPSC charge transfer ratios. Apocynin × CMS two-way ANOVA: Interaction: F(1,76) = 19.89, p < 0.001; main effect of apocynin: F(1,76) = 15.7, p < 0.001; main effect of CMS, F(1,76) = 19.19, p < 0.001, Tukey post hoc test,**p < 0.01. Data represent means ± SEM for each group (n = 19–21 cells, 3–4 mice per group).

3.3. Apocynin prevents the increase of excitatory synaptic transmissions caused by CMS in vCA1 pyramidal cells

To detect whether CMS-induced oxidative stress impacts on the vCA1 pyramidal cells, the sEPSCs and sIPSCs of vCA1 pyramidal cells were recorded alternately on a cell-by-cell basis. These data demonstrated that mice exposed to CMS exhibited an increased frequency but not amplitude of sEPSC (Fig. 3A–D), compared to control mice. Apocynin administration reversed the increased sEPSC frequency caused by CMS (Fig. 3A–C). Unlike the results of sEPSC, neither CMS nor apocynin administration affected the frequency or amplitude of sIPSC (Fig. 3A, E-G). To further determine the balance of excitatory and inhibitory synaptic currents of the pyramidal cells in vCA1, the sEPSC/sIPSC charge transfer ratio was analyzed. Consistent with the changes in sEPSC frequency, CMS resulted in a significant increase of the sEPSC/sIPSC charge transfer ratio, while apocynin treatment reversed this change (Fig. 3H–J).

3.4. NOX2 expression in vCA1 is elevated by CMS

To investigate whether the level of NOX2 is altered in mice exposed to CMS, we measured the expression of NOX2 in the vHPC of mice. We found that CMS exposure increased *Nox2* mRNA levels in the vHPC of control mice, while apocynin administration significantly suppressed *Nox2* mRNA transcription in both control and CMS-exposed mice compared to mice with vehicle administration (Fig. 4A). It has been previously demonstrated that NOX2 is mainly expressed in microglia of rodent brains (Bedard and Krause, 2007; Schiavone et al., 2009). Similarly, we found that CMS significantly increased the number of NOX2+ cells, among which 92% are co-labeled with Iba-1 (Fig. 4B–D). However, in both control and CMS-exposed mice, apocynin administration reduced the number of these cells in vCA1 (Fig. 4B–D). This observation is consistent with data showing increased *Nox2* mRNA levels.

3.5. NOX2 deletion reduces CMS-induced oxidative stress in vCA1

To further confirm whether NOX2 is involved in the increase in oxidative stress caused by chronic stress, we examined the effects of NOX2 deletion on CMS-elevated ROS levels and lipid peroxidation (Fig. 5A). As shown in Fig. 5B, *Nox2* mRNA levels and immunoreactivity were largely undetected in NOX2-deficient (NOX2−/−) mice compared to their control littermates (WT). WT mice exposed to CMS displayed significant enrichment of ROS by DHE staining in vCA1 (Fig. 5C). We observed significantly reduced ROS levels in NOX2−/− vCA1 compared to WT mice with and without CMS stimuli (Fig. 5C and D). Consistent with DHE staining, the elevation of lipid-peroxide by CMS was significantly curtailed in NOX2−/− mice (Fig. 5E).

3.6. NOX2 deletion attenuates CMS-induced anxiety-like behaviors

We next assessed a set of behaviors in NOX2-deficient mice with and without CMS exposure. In comparison with WT mice, NOX2−/− mice had no significant differences in total distance and time spent in the central field of the OFT box, nor in the time spent in open arms of the EPM and light box of LDB (Fig. 6A–D). However, CMS-decreased time spent in the central field of the OFT box, open arms of EPM and light box of LDB were significantly elevated in NOX2−/− mice compared to WT mice (Fig. 6A–D). We also found that NOX2−/− mice exhibited a similar immobility time in sucrose consumption (Fig. 6E) and FST (Fig. 6F) compared with WT mice.

4. Discussion

In our study, we found that 1) CMS increased the levels of oxidative stress and NOX2 expression in vCA1, 2) CMS altered the E/I balance of pyramidal cells in vCA1 and consequently aggravated anxiety-like behaviors, and 3) both apocynin administration and NOX2 deletion attenuated the above deficits.

CMS is one of the most often used animal model for the study of stress-related disorders (Lin et al., 2015). In our CMS model, mice exhibited both anxiety- and depressive-like behaviors, which confirmed previous conclusions that CMS leads to anxiety and depression (Parihar et al., 2011; Shepard and Coutellier, 2018; Gross and Pinhasov, 2016; Ducottet et al., 2003). Meanwhile, our results showed that no significant changes in the body weight between the CMS and control group (Fig S1). This is consistent with some other studies using the same animal model (Grippo et al., 2006; Dandekar et al., 2018). However, some studies have shown that CMS decreased the body weight (Shang et al., 2017; Xu et al., 2016). The reasons for this difference remain unclear, which may be related to variation of the CMS protocol applied in each study (Grippo et al., 2006; Dandekar et al., 2018; Shang et al., 2017; Xu et al., 2016; Sacharczuk et al., 2010) or the intensity of the stressors (Marti et al., 1994). Our results indicate that all the interpretations in this study are not secondary to body weight change.

We found that the attenuation of oxidative stress by apocynin administration can prevent CMS-induced anxiety, which potentially improves the efficacy of interventive measures for mental illnesses caused by chronic stress. In support of our conclusion, other studies have reported that anxiety-like behaviors induced by exposing to L-Buthionine-sulfoximine (BSO) and hypoxia can be alleviated by apocynin treatment (Masood et al., 2008; Yuan et al., 2015). However, CMS-induced depression-like behaviors were not reversed by apocynin treatment. Although one study found that the inhibition of NADPH oxidase with apocynin provided beneficial effects to counter depression caused by chronic stress (Seo et al., 2012), this was probably due to differences in the chronic stress protocol (CMS vs restraint stress), apocynin dosage (5 mg/kg vs 15 mg/kg), and route of administration (oral administration vs intraperitoneal injection). In various animal models, the therapeutic potential dose range of apocynin is 2.5–12 mg/kg (Kimura et al., 2005; Sonta et al., 2004). In addition, several studies have shown

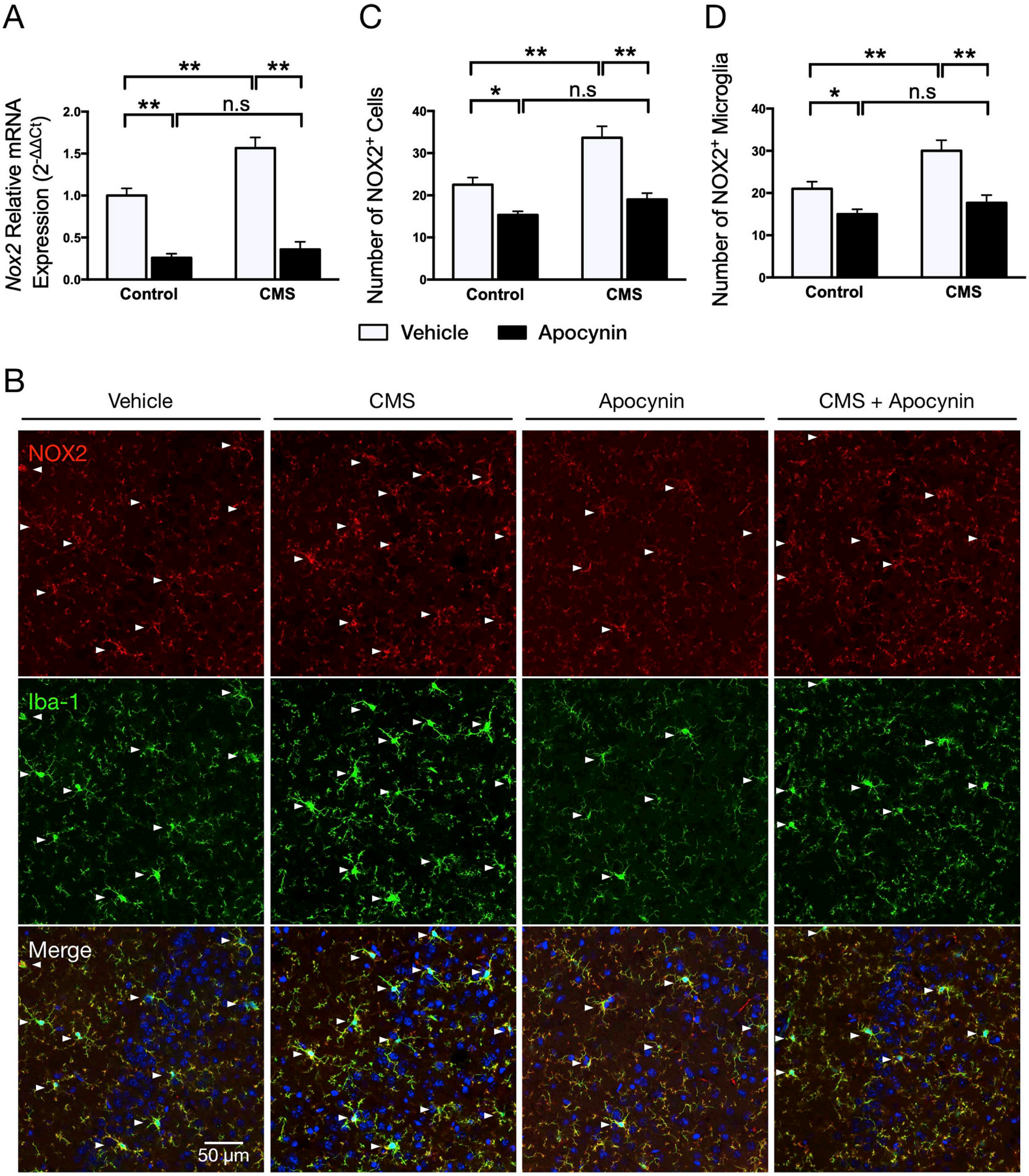

Fig. 4. CMS leads to increased expression of NOX2 and microglia in vHPC. (A) *Nox2* mRNA expression in vHPC of mice. Apocynin × CMS two-way ANOVA: Interaction: $F(1,8) = 6.31$, $p = 0.036$; main effect of apocynin: $F(1,8) = 110$, $p < 0.001$; main effect of CMS, $F(1,8) = 12.76$, $p = 0.007$, Tukey post hoc test,$^{**}p < 0.01$, no significant (n.s) $p > 0.05$. ($n = 3$ mice per group). (B) Immunofluorescence of NOX2 (red) and microglia (green, labeled by Iba-1) in vCA1 of mice (white arrowheads indicate NOX2, Iba-1 co-labeled cells).(C) Amount of NOX2+ cells in vCA1 of mice. Apocynin × CMS two-way ANOVA: Interaction: $F(1,9) = 4.119$, $p = 0.073$; main effect of apocynin: $F(1,9) = 34.9$, $p < 0.001$; main effect of CMS, $F(1,9) = 16.11$, $p = 0.003$, Tukey post hoc test,$^{**}p < 0.01$, no significant (n.s) $p > 0.05$. ($n = 3$–4 mice per group). (D) Amount of NOX2+ microglia in vCA1 of mice. Apocynin × CMS two-way ANOVA: Interaction: $F(1,9) = 2.869$, $p = 0.125$; main effect of apocynin: $F(1,9) = 24.04$, $p < 0.001$; main effect of CMS, $F(1,9) = 9.735$, $p = 0.012$, Tukey post hoc test,$^{**}p < 0.01$, no significant (n.s) $p > 0.05$. ($n = 3$–4 mice per group). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that, except NOX2, apocynin exerts an inhibitory effect on NOX4, NOX5, and anti-inflammation (Altenhofer et al., 2015; Aldieri et al., 2008), which may likely cause some significant consequences under high-dose administration. It was also reported that high doses of apocynin may also lead to side-effects like suppression of the phosphorylation of Akt (Kucera et al., 2016). In addition to these effects,

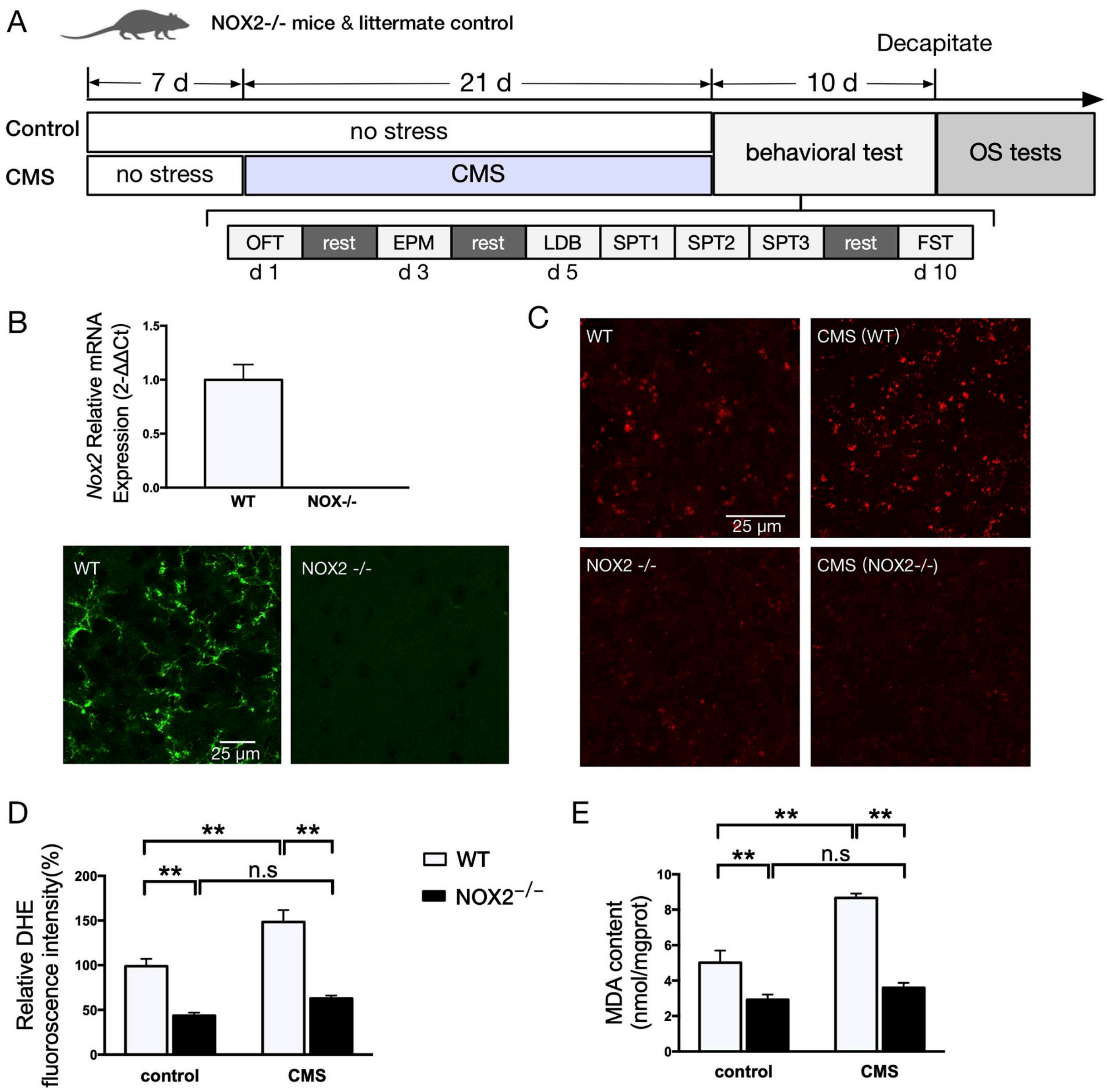

Fig. 5. NOX2 deletion ameliorates the increased level of oxidative stress in vHPC or vCA1. (A) Schematic of the procedure used in the present study, which started 7 weeks after birth of mice (OS tests: oxidative stress tests). (B) Typical image of genetic identification of NOX2−/− mice via RT-qPCR and immunofluorescence (white arrowheads indicate NOX2+ cells). (C,D) DHE staining of NOX2−/− mice and their wild-type littermate. Genotype × CMS two-way ANOVA: Interaction: $F(1,12) = 3.472$, $p = 0.087$; main effect of Genotype: $F(1,12) = 74.25$, $p < 0.001$; main effect of CMS, $F(1,12) = 17.69$, $p = 0.001$, Tukey post hoc test,$^{**}p < 0.01$, no significant (n.s) $p > 0.05$. ($n = 4$ mice per group). (E) The MDA content in ventral hippocampus of mice. Genotype × CMS two-way ANOVA: Interaction: $F(1,12) = 12.88$, $p = 0.004$; main effect of Genotype: $F(1,12) = 74.45$, $p < 0.001$; main effect of CMS, $F(1,12) = 27.14$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$, no significant (n.s) $p > 0.05$. ($n = 4$ mice per group). Data represent means ± SEM for each group. $^{*}p < 0.05$, $^{**}p < 0.01$.

daily intraperitoneal injection of apocynin may also exert, to some extent, stressful stimuli to mice.

The vHPC is an important brain region which involved in the development of anxiety (Calhoon and Tye, 2015; Padilla-Coreano et al., 2016). Furthermore, a recent study indicated that the activation of vCA1 pyramidal cells directly controls the anxious response (McEwen, 2007).In our study, mice exposed to CMS in this study exhibited significant enhancement of oxidative stress level in vCA1, suggesting that vCA1 oxidative stress may take part in the process of CMS-induced anxiety. Whereas, we found the level of ROS and lipid peroxidation in the mPFC were not significantly increased by CMS, suggesting that oxidative stress levels are different in various brain regions. Similarly, several studies have reported that, in animal models of social isolation and ischemic reperfusion, the level of oxidative stress was varied in different brain regions or time points (Schiavone et al., 2009; Candelario-Jalil et al., 2001). In addition, one postmortem study found that superoxide dismutase (SOD), an important antioxidant in the balance of oxidative stress, was highly expressed in the PFC, but not in the hippocampus, of depression patients (Michel et al., 2007). These findings suggest that, in various neuro-disorders, there is a bias of oxidative stress in different brain areas.

One previous study has provided evidence that 20 min of restraint stress increases the neuronal release of excitatory amino acids in the hippocampus and prefrontal cortex, though the mechanism is unknown (Moghaddam, 1993). Another study reported that chronic stress, in this case 4 or 7 weeks of social isolation, up-regulated the levels of oxidative stress and glutamate (Schiavone et al., 2012). Consistent with these findings, our results showed that CMS increased oxidative levels and

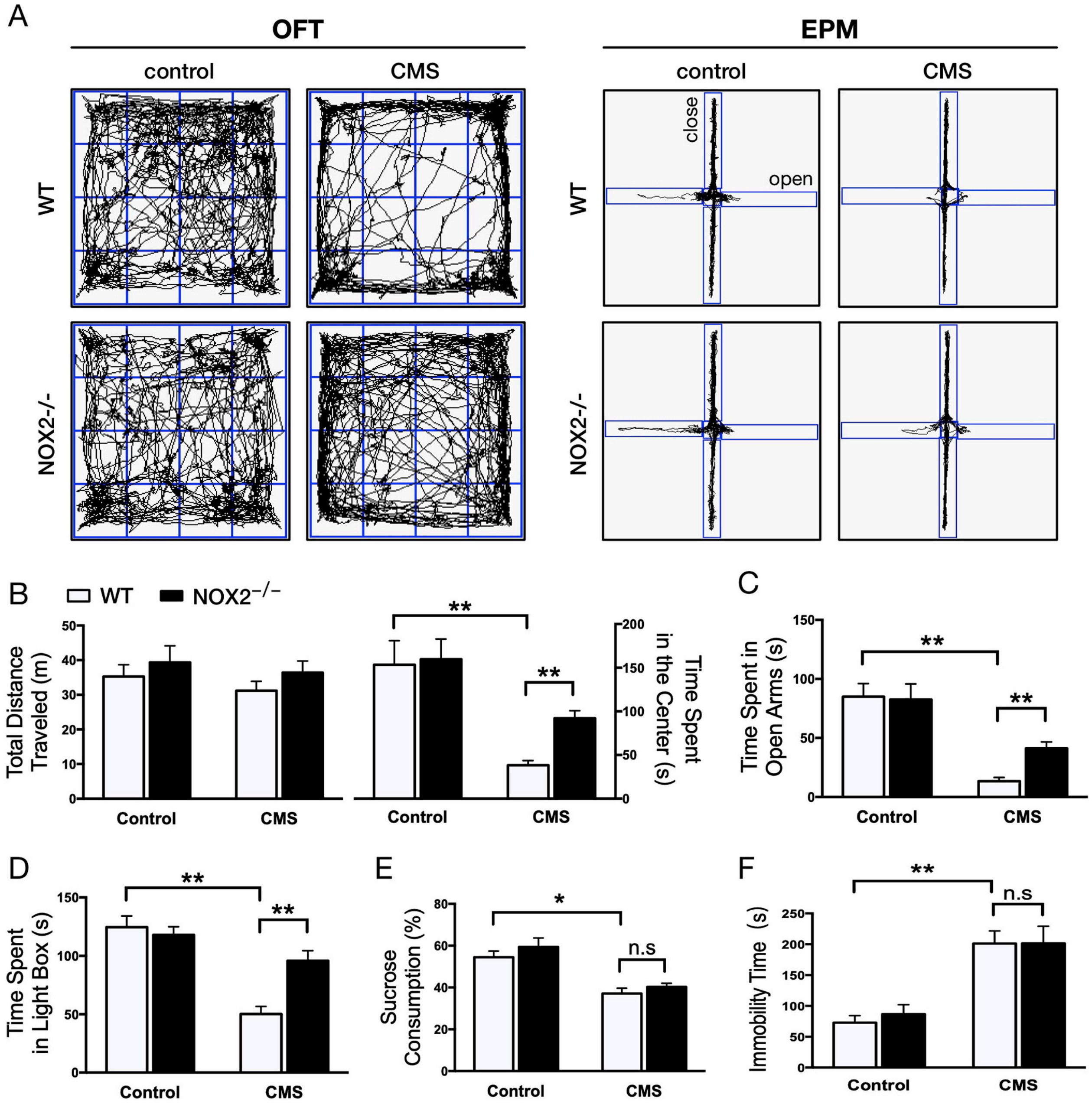

Fig. 6. NOX2 deletion ameliorates CMS-induced anxiety-like behaviors, but not depression-like behaviors. (A) Representative traces of the movement of mice during an open field box test and elevated plus maze test. (B) Left, total distance travelled. Genotype × CMS two-way ANOVA: Interaction: $F(1,28) = 0.001$, $p = 0.971$; main effect of Genotype: $F(1,28) = 1.159$, $p = 0.291$; main effect of CMS, $F(1,28) = 1.316$, $p = 0.261$. Right, time spent in center area in the open field box. Genotype × CMS two-way ANOVA: Interaction: $F(1,28) = 1.554$, $p = 0.223$; main effect of Genotype: $F(1,28) = 2.512$, $p = 0.124$; main effect of CMS, $F(1,28) = 23.52$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$. (C) Time spent in open arms of the elevated plus maze test. Genotype × CMS two-way ANOVA: Interaction: $F(1,28) = 2.785$, $p = 0.106$; main effect of Genotype: $F(1,28) = 1.988$, $p = 0.170$; main effect of CMS, $F(1,28) = 38.09$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$. (D) Time spent in the light box in light/dark box test. Genotype × CMS two-way ANOVA: Interaction: $F(1,28) = 10.85$, $p = 0.003$; main effect of Genotype: $F(1,28) = 7.034$, $p = 0.013$; main effect of CMS, $F(1,28) = 34.21$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$. (E) Sucrose consumption in the sucrose preference. Genotype × CMS two-way ANOVA: Interaction: $F(1,28) = 0.091$, $p = 0.765$; main effect of Genotype: $F(1,28) = 1.706$, $p = 0.202$; main effect of CMS, $F(1,28) = 35.34$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$. no significant (n.s) $p > 0.05$. (F) Time of immobility (or floating) in forced swimming test. Genotype × CMS two-way ANOVA: Interaction: $F(1,28) = 0.341$, $p = 0.564$; main effect of Genotype: $F(1,28) = 0.016$, $p = 0.902$; main effect of CMS, $F(1,28) = 41.21$, $p < 0.001$, Tukey post hoc test,$^{**}p < 0.01$. no significant (n.s) $p > 0.05$. Data represent means ± SEM for each group ($n = 8$ mice per group). $^{*}p < 0.05$, $^{**}p < 0.01$.

sEPSCs frequency in vCA1 pyramidal cells (Fig. 3). Furthermore, inhibition of oxidative stress reversed these abnormalities caused by CMS (Figs. 2 and 3), indicating that oxidative stress is at least one of the key factors promoting efficacy of excitatory synaptic transmission. Consistent with this conclusion, one study indicated that oxidative stress in the PFC increased the release of glutamate transmission (Sorce et al., 2010). Another report demonstrated that chronic stress may promote the maturity of dendritic spines involved in anxiety-related neural circuits (Zhang et al., 2018). In addition, anxiety cells enriched in vCA1 can be activated by an anxiogenic environment, thus increase avoidance to open arms in the EPM and central field in the open-field box (Jimenez et al., 2018). Considering our results, we propose that

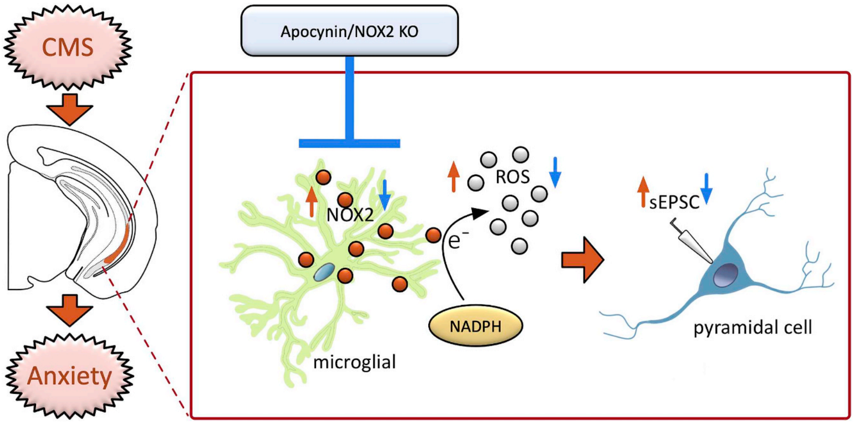

Fig. 7. CMS exposure increases the expression of NOX2 on microglia and subsequently up-regulates the level of ROS. The ROS-induced oxidative stress ultimately contributed to the enhancement of pyramidal cells in vCA1 and subsequently led to psychiatric anxiety.

oxidative stress is possibly the route between the activation of these pyramidal cells and a stressful external environment.

Previous studies have demonstrated that the antioxidant effects of apocynin work mainly through inhibition of NADPH oxidase (NOX) (Altenhofer et al., 2015; Jaquet et al., 2009). In the present study, we found that the expression of *Nox2* mRNA is up-regulated in vHPC of mice subjected to CMS and further examination showed that increased expression of NOX2 was observable in microglia of vCA1 (Fig. 4). Our study revealed that NOX2 in microglia may be the main contributor to chronic stress-induced anxiety. Consistent with our results, several previous studies demonstrated that NOX2, which is highly expressed in microglia, generates a prodigious amount of ROS that potentially influence neurological disease progression via producing neurotoxicity (Bedard and Krause, 2007; Belarbi et al., 2017; Gao et al., 2003). In addition, other studies have shown that the level of NOX2-derived oxidative stress were enhanced in rats exposed to social isolation or a pro-inflammatory environment led by advanced age (Schiavone et al., 2009; von Leden et al., 2017). One study reported that the proliferation of cultured microglia can be blocked by apocynin treatment (Mander et al., 2006). Consistent with these studies, our results showed that the number of NOX2+ microglia decreased by apocynin administration with or without CMS exposure (Fig. 4D). These results suggest that increased number of NOX2+ microglia induced by CMS may contribute to the enhancement of NOX2 level in vCA1, and subsequently the level of oxidative stress.

Although apocynin is one of the most frequently used NOX2 inhibitors, it has been demonstrated to show other unspecific effects (Altenhofer et al., 2015; Aldieri et al., 2008; Kucera et al., 2016). In light of that possibility, we included chronic stress experiments with NOX2−/− mice that were also used in our previous work (Yokota et al., 2011). We found that NOX2 deletion completely blocked CMS-induced elevation of oxidative stress levels in the vCA1 of mice, indicating that NOX2 is the primary source of ROS in chronic stress-induced oxidative stress in vCA1. Moreover, consistent with test results for mice with apocynin administration (Fig. 1), NOX2 deletion attenuated CMS-induced anxiety-, but not depression-like behaviors (Fig. 6). However, these preventive effects of NOX2 deletion or apocynin administration only partially reversed anxiety-like behaviors of CMS mice. These findings suggest that there are multiple mechanisms, in addition to oxidative stress pathways, involved in anxiety disorders led by chronic stress. For example, other studies found that increased CAPON-nNOS coupling and decreased neurogenesis in the hippocampus may contribute to the modulation of anxiety-related behaviors induced by CMS (Zhu et al., 2014; Parihar et al., 2011).

Consistent with previous studies that demonstrated the importance of NOX2 in another psychiatric disorder like PTSD (Liu et al., 2016), our results indicated that NOX2 may play a key role in chronic-stress-induced anxiety. Thus, blockade of NOX2-derived oxidative stress may provide a strategy to prevent anxiety and other psychiatric disorders induced by stress, which is a prevalent risk factor in modern society. One previous study reported that the activity of vCA1 pyramidal cells is important to anxiety (Jimenez et al., 2018), and our results further suggest that E/I balance of pyramidal cells may link oxidative stress and behavioral features underlying the etiology of psychiatric disorders, which can be a potential therapeutic target. However, it still needs to be further investigated that how microglial NOX2 regulates the neurotransmission of vCA1 pyramidal cells.

In conclusion, this study demonstrated that CMS causes increased level of NOX2 expression in microglia and, consequently, an upregulated level of oxidative stress. Additionally, the efficacy of excitatory synaptic transmission in vCA1 pyramidal cells is impacted by oxidative stress and thereby leads to anxiety-like behaviors (Fig. 7).

Conflicts of interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2019.04.008.

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