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Ambient PM2.5 caused depressive-like responses through Nrf2/NLRP3 signaling pathway modulating inflammation

Chen Chu[a], Haiya Zhang[b], Shijie Cui[c], Bin Han[a], Lixiao Zhou[a], Ning Zhang[a], Xuan Su[a], Yujie Niu[b,c], Wen Chen[d], Rui Chen[e], Rong Zhang[a,c,*], Yuxin Zheng[f,**]

[a] Department of Toxicology, Hebei Medical University, Shijiazhuang, 050017, PR China
[b] Deportment Occupational Health and Environmental Health, Hebei Medical University, Shijiazhuang, 050017, PR China
[c] Hebei Province Key Laboratory of Environment and Human Health, Shijiazhuang, 050017, PR China
[d] Department of Toxicology, School of Public Health, Sun Yat-sen University, Guangzhou, 510080, PR China
[e] Key Laboratory of Environmental Medicine Engineering, Ministry of Education, School of Public Health, Southeast University, Dingjiaqiao 87, Nanjing, 210009, PR China
[f] Department of Toxicology, Public Health College, Qingdao University, 266000, Qingdao, PR China

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ABSTRACT

PM2.5 pollution has been associated with numerous adverse effects including cardiovascular, respiratory and metabolic diseases as well as emotional disorders. However, the potential mechanism has not known clearly. Twenty-four rats were divided into 3 groups and exposed to various airs: filtered air (FA), unfiltered air (UA) and concentrated PM2.5 air (CA), respectively. Thirty wild type (WT) and 30 Nrf2 knockout (KO) mice were divided into 2 groups and exposed to FA and UA, respectively. The changes of neurobehavioral function, neurotransmitter secretion, toxic elements deposition, oxidative stress and the inflammation in prefrontal cortex were investigated during 9–12 weeks with/without PM2.5 exposure. Results showed that CA rats and KO-UA mice emerged obviously depressive-like responses. Li, Be, Al, Cr, Co, Ni, Se, Cd, Ba, Ti and Pb could deposit in the prefrontal cortex of rats after PM2.5 exposure. The neurotransmitters were significantly disorder in prefrontal cortex of CA rats. The NLRP3 signaling pathway was more activated in $Nrf2^{-/-}$ than WT mice after PM2.5 exposure for 9 weeks. Nrf2/ NLRP3 signaling pathway modulating the inflammation might play an important role in the depression induced by ambient PM2.5.

1. Introduction

Ambient particulate matter 2.5 (PM2.5) poses a significant risk to public health worldwide and the PM2.5 pollution has been associated with numerous adverse health effects [1]. World Health Organization (WHO) reports that in 2012 around 7 million people died - one in eight of total global deaths - as result of air pollution exposure. According to the WHO global burden of disease (GBD) study, ambient PM2.5 was responsible for 3.2 million premature deaths worldwide annually, and 1.2 million premature deaths in China alone [2]. These data suggest that PM2.5 is now one of the world's largest environmental health risks. Therefore, the WHO ambient air quality guidelines suggest an annual mean PM2.5 concentration limit of 10 μg/m^3 and 25 μg/m^3 for the 24-hourly mean to prevent the adverse effects of PM2.5 on public health [3].

Recent study had reported adverse associations between air pollution exposures and the nervous system [4]. Increases in PM2.5 exposure was associated with depression, emotional symptoms and suicide attempts or elevated anxiety [5,6]. Consistent with these findings, time-series studies examining acute associations had reported increased depression-related hospital admissions with increasing pollution levels [7–9]. A significant association between PM2.5 and hospital admission for mental and behavioral disorders was found in Shijiazhuang, as one of the most polluted cities in China [10]. There was association between chronic exposures to PM2.5 and onset of depression among cohorts in the US [11,12].

However, the current insufficient epidemiological evidences are hard to explain the mechanism which would hold back the treatment and prevention of psychological diseases and mental disorders induced by PM2.5 exposure. PM2.5 has complex constituents including heavy

* Corresponding author at: Dept. Toxicology, Hebei Medical University, 361 Zhongshan east Rd., Shijiazhuang, Hebei 050017, PR China; Dept. Toxicology, Public health College, Qingdao University, 266000, Qingdao, PR China.
** Corresponding author at: Dept. Toxicology, Public health College, Qingdao University, 266000, Qingdao, PR China.
E-mail addresses: rongzhang@hebmu.edu.cn (R. Zhang), yxzheng@qdu.edu.cn (Y. Zheng).

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metals and PAHs [13], which towards to result in depressive responses and increased hippocampal pro-inflammatory cytokines [14]. The nucleotide-binding domain and leucinerich repeat protein 3 (NLRP3) inflammasome is reported as a central mediator by which psychological and physical stressors could contribute to the development of depression [15].

In the literature, NLRP3 had been associated with oxidative stress [16]. After phagocytosing exogenous particles, macrophages could generate a large amount of reactive oxygen species (ROS) [17], which in turn was known to play an important role in depression [18,19]. Excessive ROS could bind to the inflammasome assembly and finally activated NLRP3 inflammasome [17]. PM2.5 extracts could induce inflammatory responses through NLRP3 inflammasome pathway activating then mediate ROS generation and trigger the following oxidative stress [20].

Nuclear factor E2-related factor-2 (Nrf2) is an essential transcription factor that mediates cellular antioxidant responses. PM2.5 exposure significantly increased the expressions of Nrf2 and Nrf2-regulated antioxidant genes then performed anti-oxidative stress effect [21]. Nrf2-mediated defenses could be activated by PM2.5-induced ROS and many functions as signaling molecules [22]. Though there were evidences of the oxidative stress and inflammation response to depression induced by PM2.5 [23,24], the interaction mechanism of oxidative stress and inflammation regulated by Nrf2 has not been known clearly. Nrf2 signal pathway took responsibility to PM2.5-caused hypothalamus inflammation [25] and its critical proinflammatory effect was mediated by NLRP3inflammasome activation. The NLRP3 inflammasome contains a C-terminal LRR, a central NACHT domain, and an N-terminal pyrin domain (PYD). NLRP3 binds to the adaptor protein, apoptosis speck-like protein, containing a CARD domain (ASC) which in turn recruits and activates Caspase-1. Biochemical analysis revealed that Nrf2 appeared in the ASC-enriched cytosolic compartment after NLRP3 inflammasome activation [26]. Therefore, the Nrf2/ NLRP3 signal regulatory oxidative stress and inflammation interaction mechanism was hypothesized to explain the depressive-like responses induced by sub-chronic PM2.5 exposure.

Here, sub-chronic PM2.5 exposure animal models were established to explore the depression and it's perhaps mechanism. The evaluation of trace or toxic elements deposition in brain partly explained the direct reason of depressive-like responses in rats. Nrf2 knockout mice were used to study the regulating role of Nrf2/ NLRP3 signaling pathway modulating inflammation on the depression induced by ambient PM2.5. The present study suggested toxic elements deposited in prefrontal cortex after PM2.5 exposure. And PM2.5 exposure triggered Nrf2/ NLRP3 signaling pathway mediating inflammation, then resulting in depressive-like responses.

2. Materials and methods

2.1. Whole-body ambient inhalational protocol

Male, pathogen-free Sprague-Dawley rats at 6 weeks of age were purchased from the Animal Experimental Center of Hebei Medical University and acclimated for a week before the commencement of inhalation. The humidity was 50% and the temperature was 25–26 °C in the cages with a 12 h light/dark cycle. Twenty-four rats were divided randomly into 3 groups and exposed to filtered air (FA), unfiltered air (UA) and concentrated PM2.5 air (CA) by real time ambient particulate matter exposed chambers, respectively. The FA, UA and CA rats were exposed to clean air in the chamber with HEPA-filters, atmosphere in the chamber without HEPA-filters and concentrated PM2.5 atmosphere, respectively. The exposure time was 6 h per day, 6 days per week from May 1st, 2017 to July 24th, 2017 at a total of 12 weeks. $Nrf2^{-/-}$ (KO) mice were used to explain the effects of Nrf2 signaling pathway on the depression induced by ambient PM2.5. Thirty male wild type (WT) and 30 male $Nrf2^{-/-}$ (KO) mice at 6-week age were divided randomly into two groups, respectively. WT-FA and KO-FA mice were exposed to clean air whereas WT-UA and KO-UA mice were exposed to unfiltered air, respectively. The mice were exposed for 6 h per day, 6 days per week from May 1st, 2017 to July 3th, 2017 at a total of 9 weeks. Animals were housed in chamber with HEPA-filters after exposure. Mice and rats began a series of behavioral testing at 8th and 11th week and then executed (Fig. S1A). The present protocol was approved by the Committee of the Ethics Animal Experiments of Hebei Medical University (IACUC-Hebmu-20170116) and carried out under the institutional guidelines for ethical animal use.

2.2. The exposure equipment and parameters monitor

The concentrated PM2.5 was generated using a PM2.5 concentration enrichment system (VACES) with a concentration monitor (HRH-7886, Beijing Huironghe Technology Co., Ltd. Beijing, China, Fig. S1B), which located at Shijiazhuang, China. The surroundings of exposure equipment is near a major traffic arterial (Black line in Fig. S1C), 2.4 km north of the local National Air Pollutant Surveillance (NAPS) monitoring stations (Point A), and 1.1 km north of a pharmaceutical factory (Point B) as well as 2.1 km north of a coal-fired power plants (Point C).

The meteorological condition inside the chambers was closely monitored to keep a relatively constant temperature (20–25 °C), humidity (40–60%), ventilation frequency (18–20/h) and noise (30–35 dB). The concentrations of ambient PM were monitored using an Aerosol Detector DUSTTRAKTM II-Model 8530 (TSI Instrument, Shoreview, MN) and the spectrum of particle size was analyzed by an Aerodynamic Particle Sizer (APS) Spectrometer 3321 (TSI Instrument, Shoreview, MN). During the exposure periods, we collected the PM2.5 on the Teflon filter by High-Volume Air Samples (The Thermo Scientific, Franklin, MA [27] and then PM2.5 was extracted. Briefly, the filters were cut into small pieces and taken into a 50 mL centrifuge tube. After an ultrasonic processing in ice cold water for 30 min, then shaking for 20 min on a shaker and repeating the processes three times, the extracts were passed sequentially through a sterile 40 μm nylon filter (Corning Life Sciences, Corning, NY) and dried by lyophilization [28]. The PM 2.5 extraction powder was stored in −20 °C until used. The levels of NO_2, SO_2 and O_3 in chambers were monitored by the gas detector GasAlertMicro 5 PID (BW Canada) during the exposure to compare the differences among the different chambers.

2.3. Behavioral tests

2.3.1. Open field test (OFT)

OFT detects hyperactivity by movement and anxious behavior. A 60 cm × 60 cm × 25 cm square black box was used to rat and a 30 cm × 30 cm × 25 cm square white box was used to mice. Each rat or mouse was placed in the box and observed for five minutes. The amount of traveling time and distance in the center area of the maze were recorded [29].

2.3.2. Novelty suppressed feeding test (NSF)

An open-field arena (60 cm × 60 cm × 25 cm) with several pellets of food placed on a piece of white paper (10 × 10 cm) in the center was used to detect the exploratory behavior. The rats were fasting for 24 h before the test. Next day, the rat was individually placed in a corner of the cage. The latency that the rats approached to foods and began to eat was recorded. The observed maximum time was for 10 min [29].

2.3.3. Sucrose preference test (SPT)

SPT was used to evaluate the anhedonia of animals. Each rat was isolated in one cage with two bottles. One bottle is containing water and another is containing 3% sucrose solution. The rats could choose the two bottles freely. The intakes from each bottle were checked daily for total 3 days. The bottles were shifted daily throughout the test to avoid potential side preferences [30]. The sucrose preference of rat in

different treatment group was calculated as a percentage of the amount of sucrose solution ingested to the total liquid intake.

2.3.4. Mouse tail suspension test (TST)

According to a previous publication, the mice were suspended 50 cm above the floor with adhesive tape placed 1 cm from the tip of the tail. The trace of mice was recorded by videotaped and immobility time within 6 min was measured. Except of those required for respiration, the absence of any limb or body movements was defined as immobility when the mouse hung passively and completely motionless. The mice were separated to avoid visual and acoustic associations from each other during the test [31].

2.4. The contents of elements in PM2.5 extracted samples and the prefrontal cortex were measured by ICP-MS

The elements analysis was detected by inductively coupled plasma-mass spectrometry (ICP-MS) (Tianrui Co. Jiangsu, China). According to the literature with a minor modification, 1 mL 30% H_2O_2 was added to about 100 mg PM2.5 extraction samples and tissues, respectively, then digested by 5 mL concentrated HNO_3 in a closed vessel microwave assisted reaction system (MARS 6, CEM Corporation, MWJ, NC). Finally, 1 ml mixture was diluted to 2 mL with 2% HNO_3 for ICP-MS analysis [32] (Supplementary files).

2.5. Determination of neurotransmitter, GSH and GSSG levels in prefrontal cortex by HPLC

Neurotransmitter levels, including norepinephrine (NE), 5-hydroxyindoleacetic acid (5-HIAA), 5-hydroxytryptamine (5-HT), dopamine (DA), levodopa (L-DOPA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by high performance liquid chromatography (HPLC, LC-20AT, Shimadzu Corporation, Japan) with a fluorescence detector (RF-10AXL, Shimadzu Corporation, Japan). Briefly, the prefrontal cortex was homogenized in 2.4 mL 0.04 mol/L of $HClO_4$ and then centrifuged at 10,000 × g for 20 min at 4 ℃. 1 mL supernatant was neutralized along with 10 μL working solution and 10 μL water and then centrifuged at 300 × g for 10 min. The supernatants were transferred quantitatively to another tube and evaporated to dryness under a slight stream of nitrogen at 35℃. Finally, the dried extract was dissolved by 100 μL water and 2 μL aliquot injected into HPLC system for analysis [33].

The levels of GSH and GSSG in prefrontal cortex were determined by HPLC-CL detection system which consisted of the HPLC and a post-column CL detection system (Shimadzu Corporation, Japan) [34]. 0.5 mg wet tissue was added to 10 μl ice-cold extraction solutions and then homogenized on ice. After centrifuged at 20,000 rpm at 4 ℃ for 15 min, supernatants were diluted with mobile phase (containing 1 mM EDTA) in the ratio of 1:1 (v/v) and then centrifuged for 2 min to analyze immediately [35].

2.6. Histopathology, immunohistochemical staining (IHC) and Immunofluorescence

The brain was fixed in 10% formalin and then embedded in paraffin. Thereafter, the paraffin-embedded tissue samples were cut into 5 μm serial sections and stained with hematoxylin and eosin according to our previous study with a small modification [36].

For IHC and Immunofluorescence, after subjected to antigen retrieval, the slices were incubated overnight at 4 ℃ with antibodies: Ionized calcium bindingadaptor molecule-1 (IBA-1, 1:100, Bioworld, Nanjing, China), glial fibrillary acidic protein (GFAP), (1:100, BD Biosciences Pharmingen, San Diego, CA), brain derived neurotrophic factor (BDNF, 1:100, Abcam Inc. Cambridge, UK), respectively. For IHC, slides were treated with an anti-rabbit secondary antibody (ZSGB-Biology, Beijing, China). Avidin-conjugated HRP with diaminobenzidine (DAB) was used as a substrate (ZSGB-Biology) and hematoxylin counterstaining was followed. The slices were observed and taken pictures with a light microscope (Olympus Ltd., Japan). For Immunofluorescence, the slides were washed by PBS/0.1% Tween 20, then incubated with the secondary antibodies: Cy3 coupled IgG (DPC Biermann, Bad Nauheim, Germany) or Alexa 488 coupled IgG (Sigma chemical, Hazelwood, MO). The images were obtained under a Leica TCS SL confocal system (Leica Microsystem, Wetzlar, Germany) [36,37].

2.7. Western blot

The prefrontal cortex of brain was isolated and homogenized in lysis buffer (Sigma chemical, Hazelwood, MO). After the homogenates were centrifuged at 14,000 rpm for 5 min, the supernatants were harvested. Proteins were separated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride membrane (Bio-Rad Laboratories Inc., Hercules, CA), probed with the indicated antibodies and detected with enhanced chemiluminescence.

2.8. Statistical analysis

The results were expressed as means ± standard deviation (SD). The data were statistically analyzed by IBM SPSS statistics 21.0 software. The comparison between the FA, UA and CA was analyzed using a one-way ANOVA followed by Least-significant difference and Student-Newman-Keuls tests. Before ANOVA analysis, the homogeneity and normality were checked. Independent-sample *t*-test was used to compare the difference between WT-FA, KO-FA and WT-UA, KO-UA groups, respectively. *P*-value < 0.05 was considered statistically significant. Orgin 2017 software (Orginlab, Inc., Northampton, MA) was used for graph plotting.

3. Results

3.1. PM2.5 concentration and physical characteristics

For exposure week 1–12, the average PM2.5 concentrations were 0, 46.37 ± 27.66, and 305.58 ± 254.22 μg/m^3 in the FA, UA and CA chambers, respectively. For exposure week 1–9, the average PM2.5 concentrations were 0.0 and 48.25 ± 24.36 μg/m^3 in the FA and UA exposure chambers, respectively. The averages of NO_2, SO_2 and O_3 levels were not significant different among the FA, UA and CA chambers (Table S1).

3.2. The depressive behavior of rats induced by PM2.5

The representative traces of rats' movements by OFT were showed in Fig. 1A. The total distances moved by UA rats and CA rats were significantly decreased compared to FA rats throughout the course of the experiment ($P < 0.05$). CA rats showed a reduction in total distance moved compared to the UA group ($P < 0.05$). The retention time and distance within the center were decreased trend in UA rats compared to FA rats, but the differences were no statistically significances ($P > 0.05$). In CA rats, both of retention time and distance were significant decreased compared to FA rats ($P < 0.05$).

The results of SPT suggested that the percentages of sucrose preference in UA rats were slightly declined ($P > 0.05$), further more a decreased significantly of sucrose preference was found in CA rats ($P < 0.05$ Fig. 1B), compared to the FA. Neither CA nor UA rats' total water intake showed significant differences compared to that of FA rats. ($P > 0.05$).

The results of NSF indicated that the latency to feeding in the UA group were significantly increase compared to FA ($P < 0.05$ Fig. 1C) but the food consumption was not significantly different ($P > 0.05$). Both of the feeding latency and food consumption decline of CA rats were significantly different from those of FA and UA rats ($P < 0.05$).

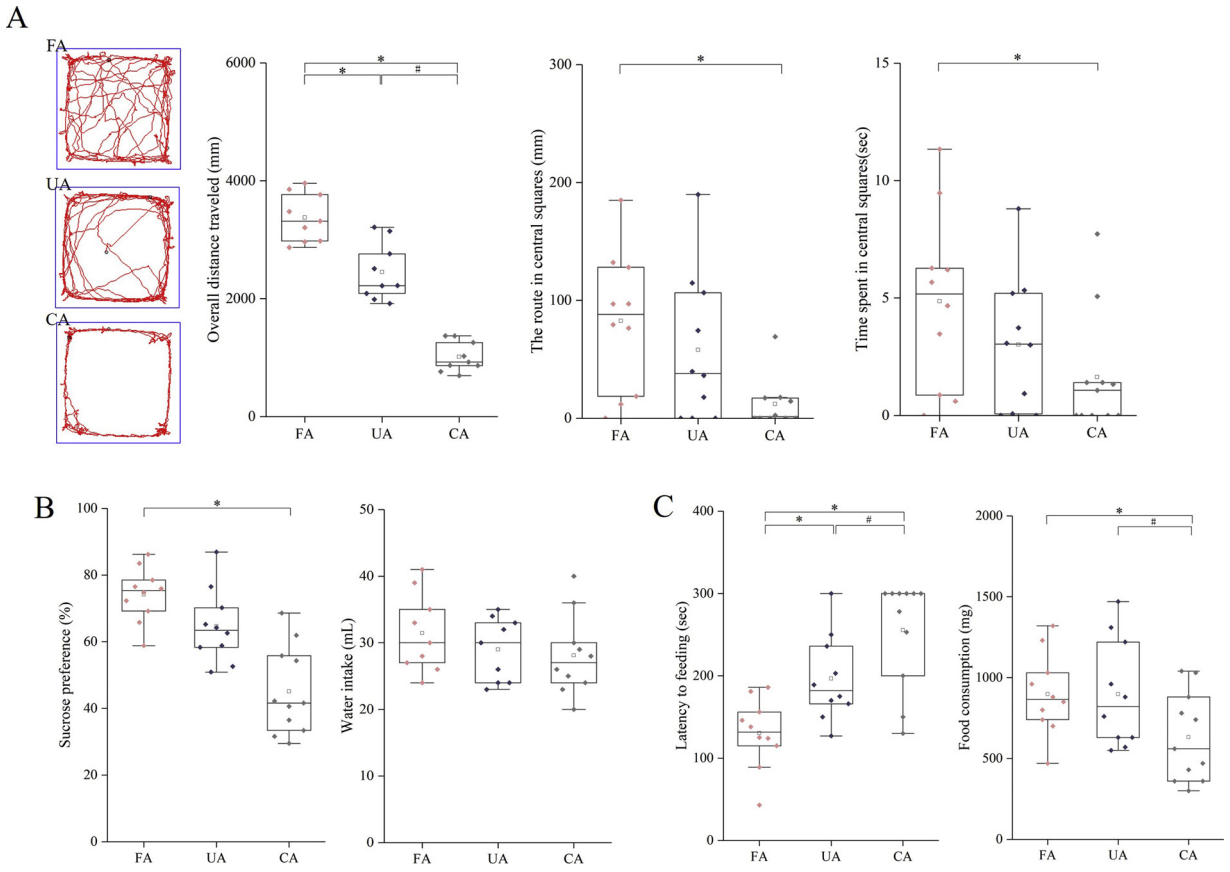

Fig. 1. Changes of behavior after PM2.5 exposure in rats. A, The represented motion trail of OFT, the total distance, route in central squares and time in central squares. B, Sucrose preference of SPT and total water intake. C, Latency to feeding of NSF and Food consumption. OFT, Open field test; SPT, Sucrose preference test; NSF, Novelty-suppressed feeding. N = 8, * $P <$ 0.05 compared with the FA group. # $P < 0.05$ compared with the UA group.

3.3. Toxic elements accumulated in prefrontal cortex of rats after PM2.5 exposure

In UA rats, the contents of Cr, Co and Ti were significantly increased compared to FA ($P < 0.05$). The contents of Li, Be, Al, Cr, Co, Ni, Se, Cd, Ba, Ti and Pb in CA rats were significantly increased, compared to FA ($P < 0.05$) (Table 1). These data indicated the toxic elements could deposit in prefrontal cortex after PM2.5 exposure.

Table 1
The levels of elements in prefrontal cortex of rats (Mean ± SD).

Element	FA	UA	CA
^{7} Li (ug/L) Lithium	8.96 ± 0.46	10.92 ± 0.48*	11.34 ± 0.45*
^{9} Be (ug/L) Beryllium	0.024 ± 0.003	0.028 ± 0.005	0.033 ± 0.003*
^{27} Al (ug/L) Aluminum	185.27 ± 5.94	321.52 ± 60.99*	408.82 ± 114.78*
^{52} Cr (ug/L) Chromium	4.44 ± 0.42	5.23 ± 0.57	6.52 ± 0.99*,#
^{55} Mn (ug/L) Manganese	22.17 ± 2.93	23.57 ± 2.56	23.37 ± 4.26
^{59} Co (ug/L) Cobalt	0.224 ± 0.011	0.237 ± 0.006	0.35 ± 0.05*,#
^{60} Ni (ug/L) Nickel	1.81 ± 0.48	5.43 ± 0.41*	6.66 ± 2.57*
^{63} Cu (ug/L) Copper	77.47 ± 2.42	70.59 ± 12.77	86.13 ± 13.66
^{75} As (ug/L) Arsenic	5.89 ± 1.12	5.51 ± 1.08	7.21 ± 2.74
^{78} Se (ug/L) Selenium	5.67 ± 0.42	6.62 ± 1.50	7.71 ± 0.92*
^{111} Cd (ug/L) Cadmium	0.036 ± 0.001	0.047 ± 0.002*	0.050 ± 0.012*
^{137} Ba (ug/L) Barium	4.18 ± 0.20	5.47 ± 1.35*	5.76 ± 0.49*
^{205} Ti (ug/L) Titanium	0.013 ± 0.001	0.014 ± 0.003	0.018 ± 0.004*,#
^{208} Pb (ug/L) Lead	3.20 ± 1.47	4.53 ± 1.59	5.18 ± 0.95*

* $P <$ 0.05 compared with the FA group.
# $P < 0.05$ compared with the UA group.

3.4. The histopathological changes, neurotrophic factors levels and neurotransmitter secretion in prefrontal cortex of rats

The histopathological changes of prefrontal cortex were shown in Fig. 2A. Normal neuronal structures were defined as those with slightly oval shaped nuclei and evenly distributed nuclear chromatin without shrinkage or edema. UA rats exhibited slightly swollen or shrunken neuros with the nuclear edge. Apparently, the histopathology changes of neuros in CA rats were severe and were characterized by necrotic neurons and pycnotic or condensed nuclei in the apoptotic bodies.

The results of immunofluorescence determination showed the GFAP and BDNF expression in prefrontal cortex of rats (Fig. 2B). The GFAP was 1.36-fold increase in UA and 2.03-fold in CA rats compared to FA, respectively ($P < 0.05$). The BDNF was declined 7% in UA and 57.5% in CA rats compared to FA rats, respectively ($P < 0.05$).

Changes of NE levels in rats' brain were shown in Fig. 2C. NE levels in the prefrontal cortex of rats after PM2.5 exposure were significantly decreased compared to FA ($P < 0.05$). The L-DOPA levels significantly increased and DA and DOPAC levels dose dependently decreased both in UA and CA rats compared to FA ($P < 0.05$,). Dose dependently decreases of 5-HT and increases of 5-HIAA were observed in the prefrontal cortex both of UA and CA compared to FA ($P < 0.05$).

With regard to neurotransmitter turnover, the index of neuronal activity was calculated as a ratio of metabolite to transmitter. There were significant increases of 5-HIAA/5-HT ratios in the prefrontal cortex both of UA and CA rats compared to FA ($P < 0.05$). Slightly increases of DOPAC/DA ratios were found in the prefrontal cortex of UA rats ($P > 0.05$) whereas significant increases in CA rats compared to FA ($P < 0.05$).

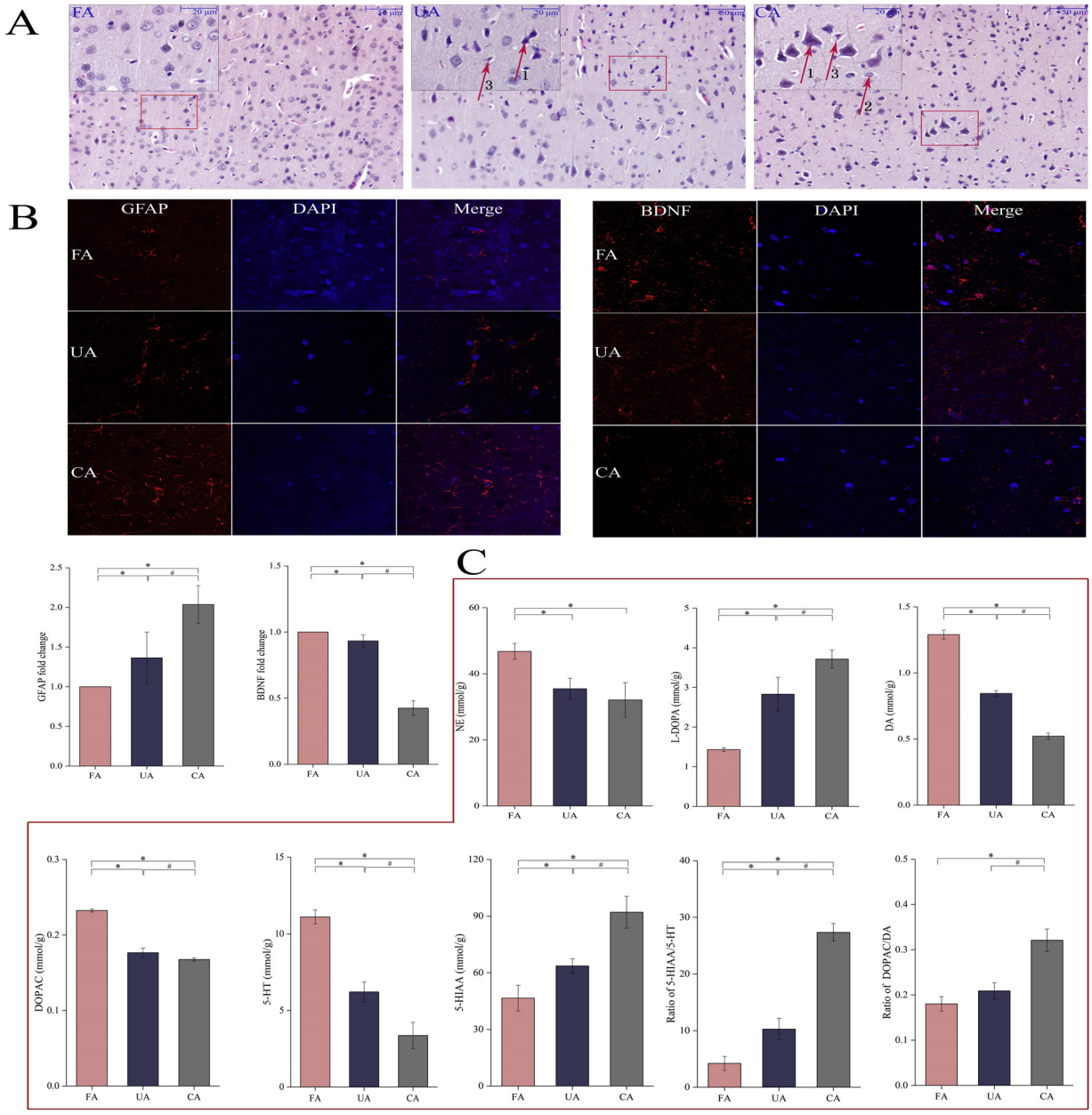

Fig. 2. The changes of histopathology and the expression of GFAP and BDNF as well as the levels of neurotransmitters in prefrontal cortex of rats after PM2.5 exposure for 12 weeks. A, Representative histopathology of the prefrontal cortex in rats by HE staining (100×) and inset a higher magnification of the tissue (400×) in the top left corner. The arrows indicate that 1, swollen neuros, 2, necrotic neuros and 3, shrunken neuros. B, The representative images of GFAP-positive astrocytes and BDNF-positive cells in the prefrontal cortex by confocal microscopy and the fold changes of GFAP and BDNF expression. C, The levels of neurotransmitters in prefrontal cortex by HPLC. GFAP, Glial fibrillary acidic protein; BDNF, Brain derived neurotrophic factor; HPLC, High performance liquid chromatography; NE, Norepinephrine; L-DOPA, Levodopa; DA, Dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid. N = 8, * $P < 0.05$ compared with the FA group. $^{\#}$ $P < 0.05$ compared with the UA group.

3.5. The inflammation cytokines and NLRP3 inflammasome-related protein expression in prefrontal cortex of rats

The represented bands of inflammatory cytokines expression suggested that bands were gradually dark in FA, UA and CA rats (Fig. 3A). Compared to FA rats, IL-6 levels in UA rats were slightly increased ($P > 0.05$), whereas significant increases occurred in CA rats ($P < 0.05$). UA rats appeared 1.17 and 1.34-fold increases of IL-8 and IL-17 levels compared to FA rats ($P < 0.05$). Significant up-regulation of IL-8, IL-17 levels was also observed in CA rats ($P < 0.05$). The statistical data showed the IL-8 and IL-17 levels increased 1.39 and 1.44-fold compared to FA rats.

The represented bands of inflammasome protein expression suggested that bands were gradually dark in FA, UA and CA rats (Fig. 3B). Compared to FA rats, the NLRP3, ASC and pro-Caspase-1 expression in UA rats were slightly increased ($P > 0.05$) whereas 2.65, 1.16, 1.47-fold increases of these markers (NLRP3, ASC and pro-Caspase-1 expression) were observed in CA rats, respectively. The Caspase-1 and IL-1β expression in UA and CA rats showed significant increases compared to FA rats, respectively ($P < 0.05$). The Caspase-1 was 1.76-fold increase in UA and 2.44-fold in CA rats compared to FA, respectively. The IL-1β was 2.32- and 2.62-fold up-regulated in UA and CA rats compared to FA, respectively.

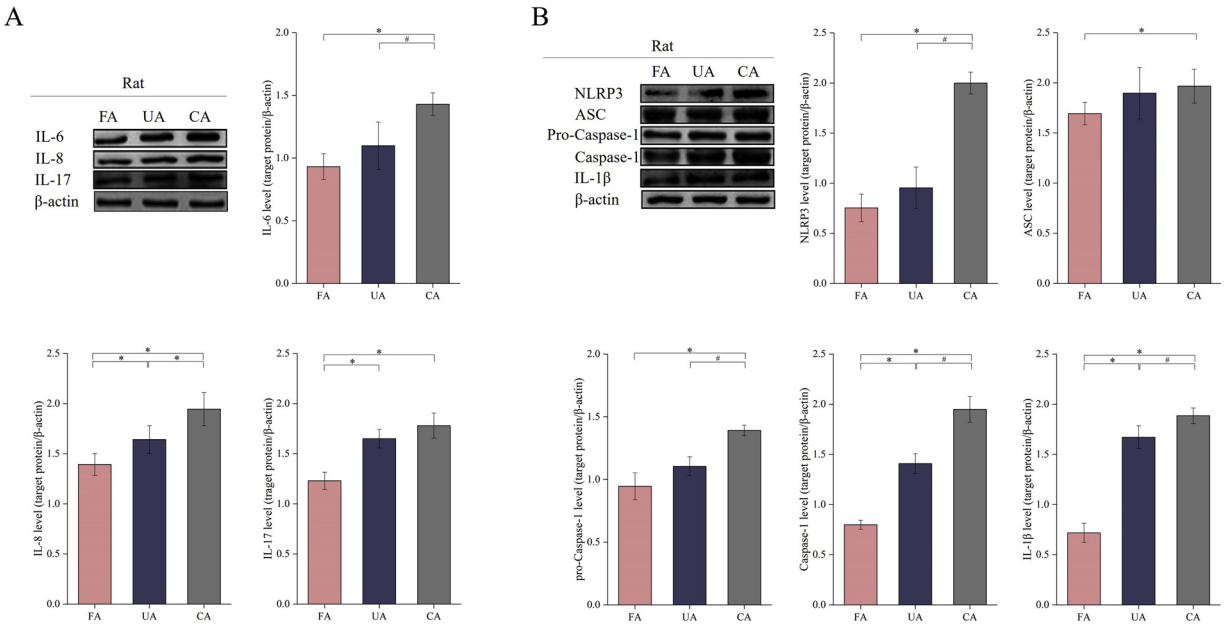

Fig. 3. Changes of inflammatory cytokines and NLRP3 signal pathways in prefrontal cortex of rats exposed to PM2.5 for 12 weeks. A, The represented protein bands and fold changes of IL-6, IL-8 and IL-17 levels. B, The represented protein bands and fold changes of NLRP3, ASC, pro-Caspase-1, Caspase-1, IL-1β protein levels. NLRP3, Nod-like receptor protein 3; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-17, Interleukin-17; ASC, Apoptosis associated speck like protein; IL-1β, Inflammatory cytokines interleukin-1β. N = 5, * $P < 0.05$ compared with the FA group. # $P < 0.05$ compared with the UA group.

3.6. The levels of oxidative stress and Nrf2-related protein in prefrontal cortex of rats

Significant decrease of GSH level was observed in UA and CA rats, compared to the FA rats ($P < 0.05$). The data showed that GSH contents declined 52.1% and 73.4% in UA and CA rats compared to FA, respectively. The GSSG contents increased slightly in UA rats ($P > 0.05$) but significantly increased in CA rats compared to FA rats ($P < 0.05$ Fig. 4A). Here we found significantly drops of GSH/GSSG ratio both in UA and CA rats dependent on the increases of PM2.5 concentration ($P < 0.05$).

The represented bands of Nrf2 signal related protein expression suggested that bands were gradually dark in FA, UA and CA rats (Fig. 4B). Compared to FA rats, the expression of those proteins in UA and CA rats were significant increase ($P < 0.05$). In comparsion with FA rats, the Nrf2 increased 1.33- and 1.84-fold in UA and CA rats, respectively. The NQO1 had 1.75- and 4.10-fold up-regulation in UA and CA rats, respectively. The γ-GCS had 2.05- and 2.40-fold up-regulation in UA and CA rats compared to FA, respectively.

3.7. The effects of Nrf2 deficiency on behavioral changes, the histopathological changes of the brain and neurotransmitter secretion

In the initial characterization, we focused on the analysis of spontaneous exploratory locomotor behavior evaluated by the OFT (Fig. 5A). The time spent in central squares and the length of movement by WT and Nrf2$^{-/-}$ mice had no difference in UA group, compared to the FA group ($P > 0.05$). The distance within center in WT−UA mice had no significant differences compared to WT−FA mice ($P > 0.05$) whereas significant less of distance within the center were observed in KO−UA mice compared to KO−FA mice ($P < 0.05$).

To further assess depression-like phenotype, we performed the tail suspension test (TST). Neither of immobility time nor latency in WT-UA mice exhibited significant difference compared to that of WT-FA mice ($P > 0.05$). In contrast, KO-UA mice exhibited a significant increase in immobility time ($P < 0.05$) but not the latency of immobility compared to KO-FA mice ($P > 0.05$ Fig. 5B).

From the histopathological images of cortex in mice (Fig. 5C), we did not find obvious pathological changed in WT-UA mice whereas there were cells with slightly swollen or shrunken as well as the unclear edge in KO-UA mice after PM2.5 exposure for 9 weeks.

Compared to FA mice, the NE and 5-HIAA levels of WT-UA and KO-UA mice decreased slightly but not significantly, respectively ($P > 0.05$, Fig. 5D). The 5-HT levels in KO-UA mice significantly decreased compared to KO-FA mice ($P < 0.05$) whereas the WT-UA mice were not significantly different compared to WT-FA ($P > 0.05$). The DA levels in KO-UA mice significantly decreased compared to KO-FA mice ($P < 0.05$) whereas DOPAC levels were slightly increased ($P > 0.05$). The L-DOPA levels in WT-UA and KO-UA mice significantly increased compared to WT-FA and KO-FA mice, respectively ($P < 0.05$).

The 5-HIAA/5-HT ratio of WT-UA mice increased 1.23-fold compared to WT-FA mice ($P > 0.05$) whereas the ratio of KO-FA mice increased 4.32-fold compared to KO-UA mice ($P < 0.05$). Compared to FA mice, the WT-UA mice increased 1.45-fold ($P > 0.05$) whereas in the meanwhile the KO-UA mice increased 2.15-fold, respectively ($P < 0.05$).

3.8. The effects of Nrf2 deficiency on inflammatory activated in prefrontal cortex

There were 1.06-fold increases of Iba-1 expression in WT-UA mice ($P > 0.05$) and 1.34-fold increases in KO-UA mice compared to FA mice ($P < 0.05$), respectively (Fig. 6A).

The IL-8 and IL-17 levels were shown in Fig. 6B. Significant up-regulation of IL-8 levels were observed in KO-UA mice compared to KO-FA mice ($P < 0.05$) but not in WT-FA mice ($P > 0.05$). Compared to FA mice, the IL-17 levels in WT-UA mice were slightly increased ($P > 0.05$) whereas significantly increased IL-17 was detected in KO-UA mice ($P < 0.05$). Compared to FA mice, the expression of NLRP3, Caspase-1 and IL-1β were significantly higher in UA mice, both of WT and KO mice ($P < 0.05$ Fig. 6C). There was 2.30-, 1.38-, 1.60-fold increases in WT-UA mice and 2.02-, 1.84-, 1.92-fold increases in KO-UA mice compared to FA mice, respectively. The expression of ASC was not significantly higher in WT-UA mice, compared to WT-FA mice ($P > 0.05$) whereas significantly higher in KO-UA mice compared to

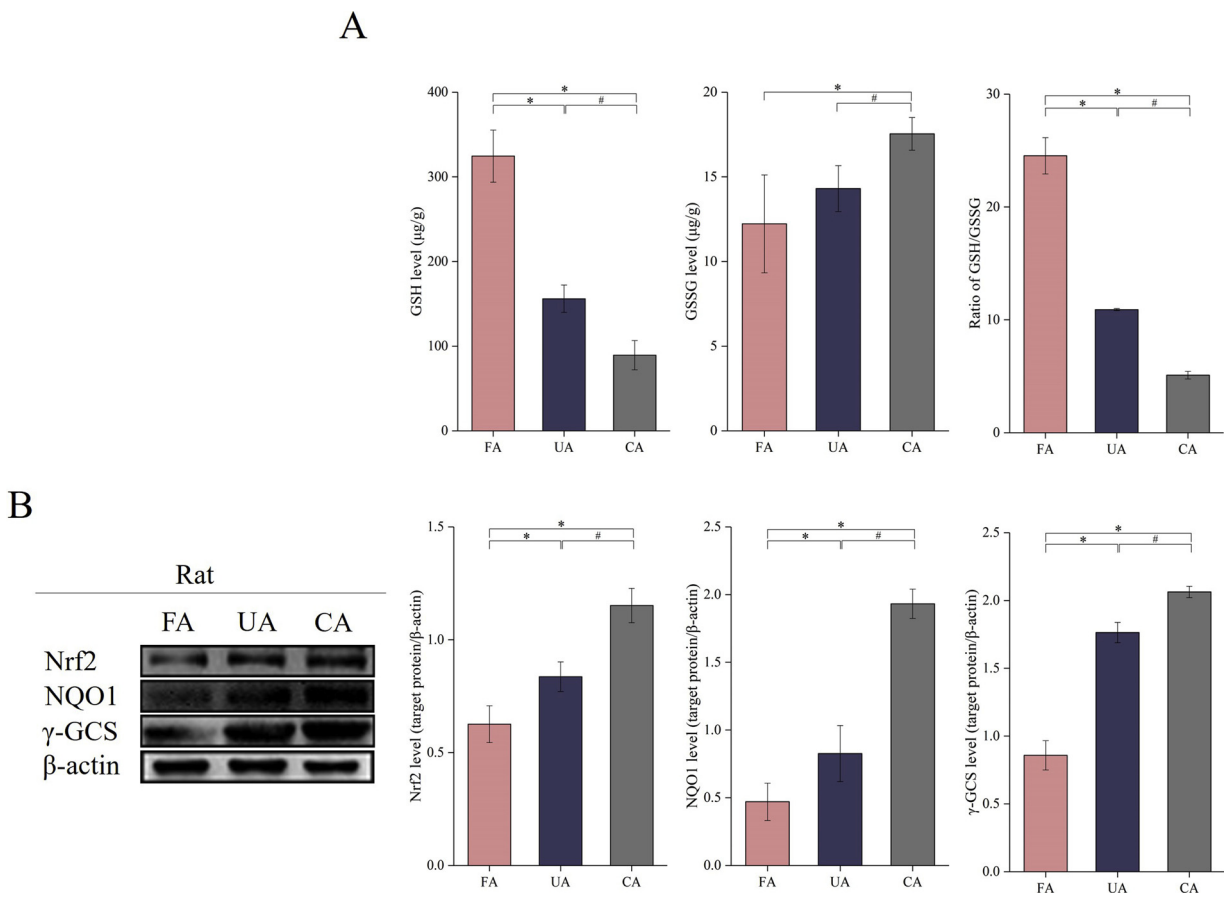

Fig. 4. Changes of oxidative stress levels and Nrf2 signal pathways in prefrontal cortex of rats exposed to PM2.5 for 12 weeks. A, The changes of oxidative stress levels in prefrontal cortex by HPLC. B, The represented protein bands and the fold changes of Nrf2, NQO1 and γ-GCS protein levels. Nrf2, Nuclear factor-like 2; GSH, Glutathione; GSSG, Oxidized glutathione; NQO1, NAD(P)H: quinone oxidoreductase 1; γ-GCS, γ glutamylcysteine synthetase. N = 5, * $P < 0.05$ compared with the FA group. # $P < 0.05$ compared with the UA group.

KO-FA mice ($P < 0.05$).

4. Discussion

According to the WHO ambient air quality guidelines, the suggested PM2.5 concentration limit is 25 μg/m^3 for the 24-hourly [2], and it is 75 μg/m^3 in China [38]. As well, there were some countries setting at 150 μg/m^3 which were much higher than the WHO standards. [39]. In the present study, after rats exposed to 305 μg/m^3 PM 2.5 for 12 w, the significant depressive symptoms were observed. Even though 46 μg/m^3 in this study excesses the limit of WHO (25 μg/m^3), it is far less than the concentration limit of China (75 μg/m^3), and the slightly depressive symptoms were appeared under this concentration. Recent years, there were very serious air pollution occurred in Shijiazhuang, China. The annual mean concentration of PM2.5 was 87.2, 98.8 and 86 μg/m^3 in 2015–2017, respectively. The mean concentration of PM2.5 reached to 200 μg/m^3 in January 2017.

Air pollution has been associated with depression and emotional disorders. In previous studies, increases in PM10, PM2.5, NO_2, CO, SO_2, O_3 could elevate the depression in the elder [2,40] and emotional symptoms and suicide attempts in younger [5]. In the present study, we monitored the NO_2, CO, SO_2, O_3 concentration in the FA, UA and CA chambers and there were no significant differences during the exposure (Table S1). In the UA chamber, more than 90% particles were less than 2.5 μm whereas more than 99% particles were less than 2.5 μm in CA chamber (Tables S2–3). Therefore, the damages of rats after exposure we observed in the present study indicated the effects of PM2.5 but not PM10, NO_2, CO, SO_2, O_3.

In a previous study, it is reported positive association of PM2.5 long-term exposure with phobic anxiety among a cohort of nurses in the US [41]. However, there was no significant association between exposure to PM2.5 and mental risk in a middle aged and older women cohort study [42]. In animal models, after 94.38 mg/m^3 PM2.5 exposure for 3 weeks, both of OFT and Porsolt forced swim test revealed there was increased anxiety and despair behaviors compared with the control [43]. However, another study did find there had negative relationship between PM exposure and anxiety-like responses in mice [44].The conflicting findings might be attributed to the different subject size, different PM2.5 concentration, greater geographical coverage, and different exposure term. In the present study, both of anxiety and despair behaviors were found in rats dose dependently, suggested that the concentration of PM2.5 was partly contribute to the depressive-like response. Taken the gender into consideration, depression seemed disproportionately affect women [45]. In Indian, cooking with biomass would increase the risk of depression in women [46]and in the US, depression was related with both ozone and PM2.5 exposures in a women cohort [42]. Although there were cohorts' studies found there was relationship between air pollution and depression in human, both men and women [47], there were rare researches in man cohort solely. In animal study, male mice might also be sensitive to PM2.5 [48]. In the present study, male rats were used to study the relationship between the air pollution and depression. We found serious depressive-like symptoms in rats after high concentration PM2.5 exposure. Therefore, we have to pay attention to the depressive-like disorders in man who exposure to high particulate matter pollution levels in the future.

The pathogenesis of depression has been implicated in the 5-HT

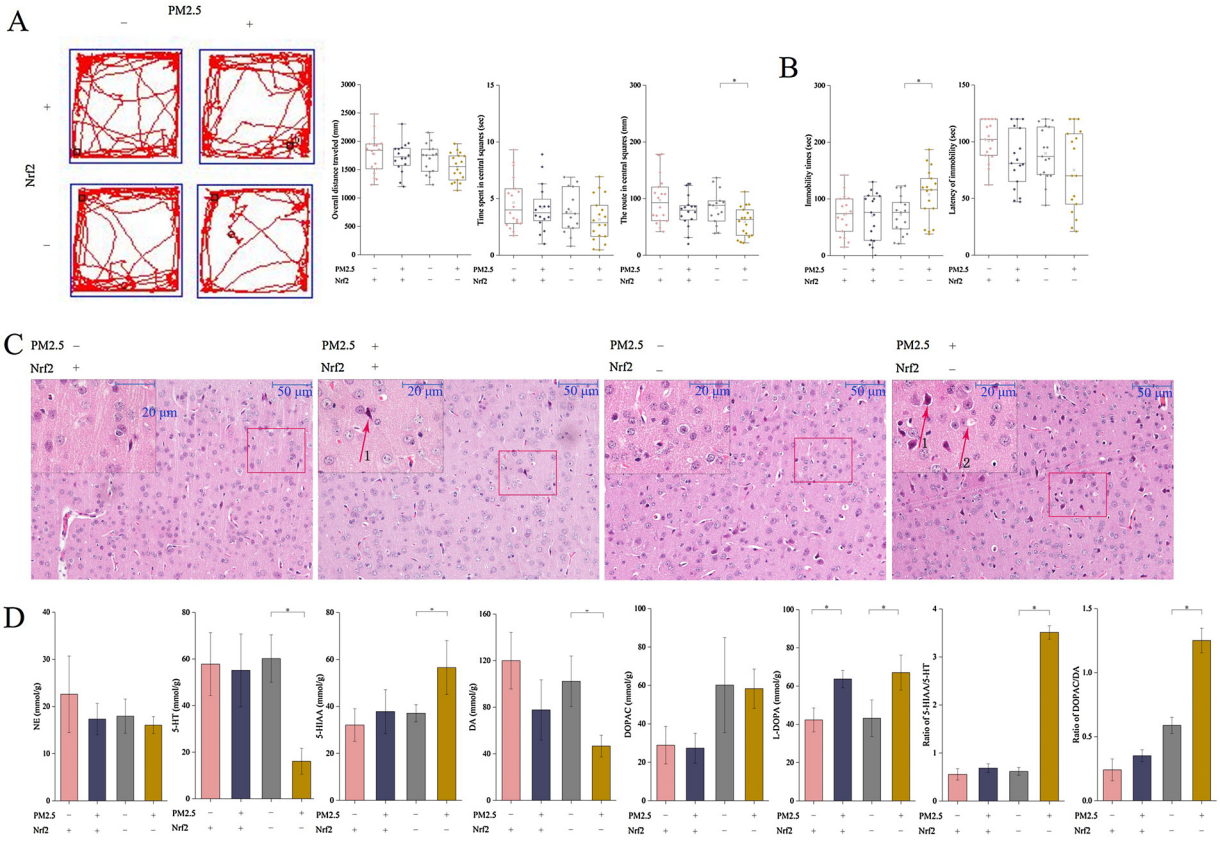

Fig. 5. The effects of Nrf2 deficiency on behavioral changes, the histopathological changes of the brain and neurotransmitter secretion after PM2.5 exposure. A, The represented motion trail of OFT, the total distance, time spent in central squares and the route in central squares (N = 10). B, The immobility times and immobility latency of TST (N = 10). C, Representative histopathology of the prefrontal cortex in mice by HE staining (100×) and inset a higher magnification of the tissue (400×) in the top left corner. The arrows indicate that 1, swollen neuros, and 2, shrunken neuros. D, The levels of neurotransmitters in prefrontal cortex by HPLC. (N = 5). TST, Tail Suspension Test. * $P < 0.05$ compared with the FA group, respectively.

system. There was a strong positive correlation between 5-HIAA and the severity of symptoms. A 4-fold increase of 5-HIAA in patients was diagnosed with panic disorder compared to healthy subjects [49]. The enhancement of 5-HIAA led to dampening of the activities of NE and DA neurons, which related with the depressive symptoms. The functional connectivity between the 5-HT, 5-HIAA, NE and DA systems could be used to understand the pathogenesis of depression [50]. In the present study, we found 5-HIAA/5-HT had 2.1- and 6.8-fold increases in UA and CA rats compared with FA rats, respectively. Both of NE and DA levels in prefrontal cortex of UF and CA rats were significantly decreased compared with the FA rats. Our data implicated the 5-HIAA/5-HT ratio elevation as well the 5-HT, NE and DA levels decreases might partly explain the pathogenesis of depression induced by PM2.5 exposure.

As we know, PM2.5 has a heterogeneous composition and is known to contain or be associated with metals, polycyclic aromatic hydrocarbons (PAHs) and other trace elements [51,52]. It has recently been reported that PM2.5 exposure leads to deposition of metals within numerous tissues including brain [53]. In the present study, the toxic elements including heavy metals including Be, Al, Cr, Mn, Co, Ni, Cu, As, Se, Cd, Ba, Ti and Pb were found in atmosphere during the exposure time (Table S4) and most of them could deposit in prefrontal cortex of rats after PM2.5 exposure (Table 1). It has long been known that heavy metals have neurotoxic effects on development [54] and growing evidence suggests that metals exposure may also result in neuroinflammation [55]. The neurotoxicity induced by PM2.5 in this study partly attribute to the toxic elements deposition in the prefrontal cortex of rats.

Jones and Thomsen [56] suggested that increases of proinflammatory cytokines resulted in depressive behaviors in animals. In brain, glial cells could up regulate the proinflammatory cytokines releases and participate the activation of inflammation process in hypothalamus [57]. We did observe increased expression of GFAP in the prefrontal cortex of UA and CA rats (Fig. 3B). GFAP is known to be upregulated in activated astrocytes suggesting that PM2.5 exposure promotes a proinflammatory- like response [58]. Macrophages released pro-inflammatory cytokines, IL-1β, which was implicated in the depression when phagocytosing pollution particles [59,60]. In addition, IL-1β could produce signal amplification cascades involving multiple inflammatory factors, thus aggravating inflammatory responses [61]. In a meta-analysis, Hiles et al. [62] found that IL-6 increased in depressed patients as compared with non-depression. In the present study, we found there were significantly increased in IL-1β, IL-6, IL-8 and IL-17 levels (Fig. 4A) in prefrontal cortex of UA and CA rats, which indicated the inflammation was activated by PM2.5 and then induced the depression.

Activation of NLRP3 inflammasome signaling is a pivotal mediator of IL-1β function, could contribute to depression [63]. Activation of NLRP3 is a multi-step process consisting of initial priming that upregulates NLRP3 or pro-IL-1β expression levels, followed by activation signals leading to oligomerization and assembly of the inflammasome. After PM2.5 exposure for 12 weeks, the NLRP3 inflammasome signal pathway including NLRP3, ASC, pro-caspase-1 and caspase-1 protein was activated and inflammation was induced in brain of rats.

Oxidative stress had been strongly associated with NLRP3 sensing, and antioxidants attenuate caspase-1 activation [64,65]. Air pollution exposures had also been found to increase systemic oxidative stress [66,67], which in turn was known to play an important role in depression [18,19]. In the present study, we found the GSH levels were

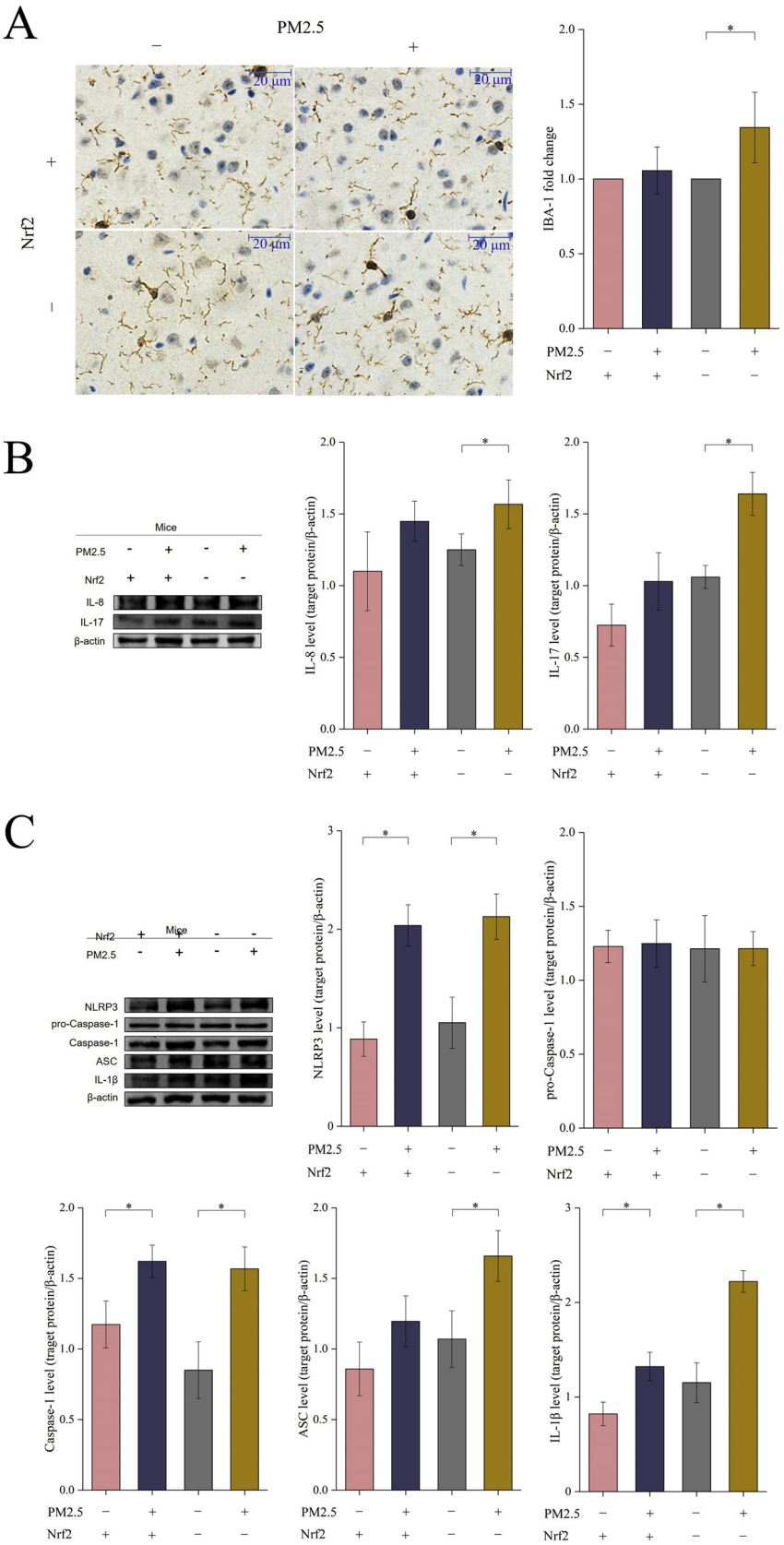

Fig. 6. The effects of Nrf2 deficiency on activation of NLRP3 signal pathways in mice after PM2.5 exposure for 9 weeks. A, Representative images of Iba-1 positive cells in the prefrontal cortex by immunohistochemical staining and the fold changes of Iba-1 levels. B, The represented protein bands and fold changes of IL-8 and IL-17 levels. C, The represented protein bands and fold changes of NLRP3, ASC, pro-Caspase-1, Caspase-1, IL-1β protein. Abbreviations: Iba-1, Ionized calcium bindingadaptor molecule-1. N = 5, $^{*}P < 0.05$ compared with the FA group, respectively.

significantly decreased while GSSG levels were significantly increased in prefrontal cortex of UA and CA rats. The ratio of GSH/GSSG was decreased significantly and dose dependently indicated that the oxidative damages in brain of rats after PM2.5 exposure.

The enhanced oxidative stress after PM2.5 exposure could activate Nrf2 pathway in astrocytes in vitro [25]. In the present study, the Nrf2 and its downstream proteins including NQO1 and γ-GCS levels were significantly and dose-dependently increased in prefrontal cortex of UA and CA rats. Nrf2 gene deficiency may delay and decrease antioxidant process. The lower expression of SOD, GSH-Px activities and higher expression of MDA levels in Nrf2$^{-/-}$ mice were exhibited, suggesting Nrf2 deficiency strengthen oxidative stress caused by PM2.5 administration (Fig. S2). In a word, PM2.5 exposure could activate Nrf2 pathway so that enhance anti-oxidative stress and the Nrf2 deficiency could strength the oxidative stress.

Furthermore, we confirmed that Nrf2$^{-/-}$ did significantly up regulate pro-inflammatory cytokines expression and NLRP3 inflammatsome activities, compared with WT mice. Nrf2 also had critical proinflammatory effect mediated by inflammasome activation [26]. In prefrontal cortex of mice, Iba-1 expression significantly increased in Nrf2$^{-/-}$ mice whereas WT mice had no difference. As is common knowledge, microglia is the main source of pro-inflammatory cytokines in the brain [68]. The activation of microglia may have detrimental effects on neurons by expressing and synthesizing pro-inflammatory cytokines such as IL-1β, which induces neuroinflammation [69,70]. Therefore, Nrf2 deficiency reduce anti-oxidative stress effect, confered animal more inflammatory response caused by oxidants in previous study [57] and in the present study. In Nrf2$^{-/-}$ mice, there exhibited serious depression induced by PM2.5 compared with WT mice. And the ratio of 5-HIAA/5-HT had significantly increased while DA had significantly decreased in Nrf2$^{-/-}$ mice after PM2.5 exposure but not in WT mice. Our data indicated that the Nrf2 deficiency would aggravate the depression induced by PM2.5 through neurotransmitters disorder.

5. Conclusion

Our study demonstrated the depressive-like responses were caused by ambient PM2.5 dose-dependently in rats and mice, which might partly attribute to the disorders of neurotransmitters as well as the deposition of toxic elements from contaminated air, including Be, Al, Cr, Co, Ni, Se, Cd, Ba, Ti and Pb. After PM2.5 exposure for 9 weeks in mice and 12 weeks in rats, the significant inflammatory changes and oxidative stress had been observed. Though both of oxidative stress and inflammation could contribute to depression, our data suggested that Nrf2, usually regulates oxidative response, could regulate inflammation through NLRP3 inflammasome signal pathway after PM2.5 exposure. Nrf2 deficiency activating NLRP3 inflammasome might have a contributory role in depressive-like response induced by PM2.5. Due to the complex nature of airborne particulate matter, the direct and indirect mechanisms may act synergistically leading to depressive-like responses, further study on mechanisms and signal pathways involved in depression induced by particulate matter is required in order to work towards preventing depression provoked by polluted air.

Ethical standards

The animal use protocol has been reviewed and approved by the Laboratory Animal Ethical and Welfare Committee Hebei Medical University, Shijiazhuang, China. Approval No. is IACUC-Hebmu-20170116.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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