S-adenosylmethionine Decarboxylase 1 Participates in $PM_{2.5}$ Exposure Induced Neuronal Apoptosis via Mitochondria-Mediated Pathway

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Research

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Abstract

Background: Fine particle (Particulate matter 2.5, $PM_{2.5}$), as the primary ambient pollutant, is considered harmful to some neurodegenerative diseases, while the specific biochemical mechanism underlying is still unrevealed. Neuronal apoptosis is believed the crucial event in neurodegenerative pathogenesis, but evidence supporting neuronal apoptosis as $PM_{2.5}$ induced neuronal injury is insufficient. S-adenosylmethionine decarboxylase 1 (AMD1) and its related spermidine synthesis have been shown to participate in cellular apoptosis, but its role in $PM_{2.5}$ exposure induced neuronal apoptosis was rarely reported. To better understand contribution of AMD1 activity and spermidine in $PM_{2.5}$ exposure induced neuronal apoptosis, may provide novel therapeutic and preventive targets for air pollution associated neurodegenerative diseases.

Methods: In the current work, sixteen C57BL/6 male mice were randomly divided into ambient $PM_{2.5}$ chamber or filtered air chamber, and the mouse model of whole-body ambient $PM_{2.5}$ chronic exposure was established. Behavioral and cognitive ability, together with corresponding biomedical index were recorded and tested to evaluated neurotoxicity by $PM_{2.5}$ exposure in mice. In parallel, PC12 cells and primary hippocampal neurons were applied for $PM_{2.5}$ treatment to explore the possible cellular and molecular mechanism which may be critically involved in the process. AMD1 activity and cellular spermidine content were modulated by pharmacological approach to examine their participation in $PM_{2.5}$ triggered neuronal apoptosis, followed by better examination of typical index for mitochondrial membrane potential and mitochondrial-mediated apoptosis pathway signaling.

Results: Chronic ambient $PM_{2.5}$ exposure attenuated spatial learning and memory ability, and triggered neuronal apoptosis together with increased expression of apoptosis-related Bax/Bcl-2 and cleaved caspase-3. $PM_{2.5}$ exposure impaired AMD1 expression and spermidine synthesis. AMD1 inhibition could mimick $PM_{2.5}$ exposure induced neuronal apoptosis. Spermidine supplementation rescued against neurotoxicity and inhibited $PM_{2.5}$ induced apoptosis, in which mitochondrial pathway signaling.

Conclusions: Chronic real-time exposure to ambient $PM_{2.5}$ led to the reduced the ability of spatial learning and memory in mice. Neuronal apoptosis was the key event in the process of neurodegenerative development induced by $PM_{2.5}$ exposure. AMD1 and spermidine participated in neuronal apoptosis induced by $PM_{2.5}$ exposure, which was at least partially dependent on mitochondria mediated pathway.

Background

With the rapid development of industry in the 21st century, the threat of air pollution to human health has attracted wide attention in recent years. The Global Burden of Disease (GBD) pointed out that air pollution is one of the main causes of the largest burden of diseases, especially in low- and middle-income countries [1]. Fine particle (Particulate matter 2.5, $PM_{2.5}$) is considered as the primary pollutant in air pollution. The surface of $PM_{2.5}$ is coated with polycyclic aromatic hydrocarbons (PAHs) and volatile

organic compounds (VOCs), leading to adverse effects on human health [2]. In recent years, more and more epidemiological studies have suggested that $PM_{2.5}$ exposure is an important risk factor for neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [3, 4]. Previously, we have reported that chronic $PM_{2.5}$ exposure led to cognitive decline in mice [5]. However, we found that neuroinflammation may only occur later than cognitive impairment in mice, the event earlier than behavior phenotype induced by $PM_{2.5}$ exposure and the underlying molecular mechanism remain unclear.

Exposure to $PM_{2.5}$ not only causes excessive neuroinflammatory responses in glial cells [6], but also induces synaptic damage and neuronal apoptosis [7]. Neuronal apoptosis is considered a crucial event in the development of neurodegenerative diseases, which is closely related to the pathologic degree of such diseases [8]. Additionally, Chen found that $PM_{2.5}$ exposure could induce neuronal apoptosis and synaptic injuries in a season-dependent manner, accompanied by changes in apoptosis-related proteins *in vitro* [9], although little was known about the mechanism by which $PM_{2.5}$ triggered apoptosis. Therefore, we speculate that neuronal apoptosis may be critical event in the pathogenesis of cognitive dysfunction induced by $PM_{2.5}$ exposure in mice.

Cell apoptosis is regulated by many intracellular signal pathways and specific kinases, including many polyamines. S-adenosylmethionine decarboxylase 1 (AMD1) as one of the rate-limiting enzymes for polyamine synthesis in the body [10], may have chance to participate in cellular apoptotic process. AMD1 decarboxylated S-adenosylmethionine, which provide aminopropyl for putrescine conversion to spermidine and spermine. AMD1 and related polyamines have been reported to contribute to cellular apoptosis via p53-Mdm2-Akt signaling in neuroblastoma [11]. Besides, spermidine, whose metabolism is closely related to AMD1, could efficiently attenuate neuronal apoptosis via restoration of cellular autophagy *in vitro* [12], and also improve cognitive function and alleviate neurodegeneration in mice [13]. Apparently, little is known about whether AMD1 or spermidine participates in $PM_{2.5}$ exposure-induced neurological dysfunction. To further explore the possible contribution of AMD1 and related polyamines in neuronal apoptosis induced by $PM_{2.5}$ exposure, may help us to better interpret air pollution associated neuronal impairment.

In the current work, the mouse model of whole-body ambient $PM_{2.5}$ chronic exposure was established. Behavioral and cognitive ability, together with corresponding biomedical index were recorded and tested to evaluated neurotoxicity by $PM_{2.5}$ exposure in mice. In parallel, PC12 cells and primary hippocampal neurons were applied for $PM_{2.5}$ treatment to explore the possible cellular and molecular mechanism that may be critically involved in the process. AMD1 activity and spermidine were modulated by pharmacological approach to examine their participation in $PM_{2.5}$ triggered neuronal apoptosis. To better understand the possible role of AMD1 activity and spermidine in $PM_{2.5}$ induced neuronal injury, may provide promising therapeutic and preventive targets for air pollution associated neurodegenerative diseases.

Results

$PM_{2.5}$ exposure attenuated spatial learning and memory ability in mice

Sixteen 8-week-old male mice were exposed to $PM_{2.5}$ for 24 weeks via a whole-body exposure manner. Prior to assignment to exposure protocols, there was no statistically significant difference of body weight in mice at baseline between the filtered air (FA) group and PM group. During the exposure period, the body weight of mice in both groups increased steadily (Fig. 1A). After 24 weeks of $PM_{2.5}$ exposure, we found that the weight of the mice in the PM group increased significantly compared to the FA group (p<0.05) (Fig. 1A). However, there is no significant difference in brain weight between FA and PM mice (p>0.05) (SFig. 1).

In previous study, the average $PM_{2.5}$ concentration in the exposure chamber was calculated as 40.79 μg/m^3. The main inorganic component of $PM_{2.5}$ is sulfur, while benzo(b)fluoranthene has the highest proportion in PAHs [5]. Given that learning and memory deficits are the main features of cognitive dysfunction, morris water maze (MWM) test was applied to assess whether $PM_{2.5}$ exposure would impair spatial learning and memory. In the probe trial, mice in the PM group exhibited a longer latency time to reach the hidden platform than mice in FA group, and the number of platform crossings and the time spent in the target quadrant were decreased (Fig. 1B). The results that differences in all indicators were statistically significant (p<0.05) indicated that chronic real-time $PM_{2.5}$ exposure could lead to reduced learning and memory function in mice.

$PM_{2.5}$ exposure induced neuronal apoptosis

In order to understand the molecular mechanism for cognitive dysfunction, neuronal apoptosis in the hippocampus was determined by TdT-mediated dUTP Nick-End Labeling (TUNEL) assays in mice. Results showed that the average number of apoptotic cells in hippocampus CA1 area of mice from PM group were significantly higher than those from FA group (Fig. 2A). The quantitative results showed that the average apoptosis rate in hippocampus of mice from FA group was 9.6%, which was 29.4% from PM group (Fig. 2A). Due to the difference of apoptosis level, we then detected the expression of apoptosis related proteins in brain tissues. Immunoblotting results showed an increase in expression of Bax and cleaved caspase-3 following $PM_{2.5}$ exposure. Meanwhile the expression of Bcl-2 was down-regulated by $PM_{2.5}$ exposure (Fig. 2B). Subsequently, Bax/Bcl-2 ratios in mice from PM group was calculated as 2.19-fold as the control, while cleaved caspase-3 was 2.68-fold as the control (Fig. 2B).

To further verify the neurotoxicity of $PM_{2.5}$ *in vitro*, PC12 cells and primary hippocampal neurons were treated with $PM_{2.5}$ at different concentrations for 24 hr. The viability of PC12 decreased in a concentration-dependent manner (Fig. 3A), and the significant difference (p<0.05) was first shown in 100 μg/mL group. As the exposure concentration increased, $PM_{2.5}$ caused a significant reduction in Bcl-2

and an increase in Bax and cleaved caspase-3 (Fig. 3B). Meanwhile, similar results were obtained in primary hippocampal neurons (Fig. 3A, C). Taken together, $PM_{2.5}$ exposure could surly induce neuronal apoptosis in animal and cell models.

$PM_{2.5}$ exposure impaired AMD1 expression

To explore differences in gene-level expression, mRNA transcription levels were screened with transcriptome sequencing analysis. After the preliminary screening of the difference in gene expression, the top 20 genes with statistically significant differences were selected to draw a heat map (Fig. 4A). And then qPCR was applied for further verification of AMD1 transcription level in brain tissue. Results showed that the relative transcription level of AMD1 from brain in PM group was significantly reduced (Fig. 4A). Furthermore, $PM_{2.5}$ induced down-regulation of AMD1 protein expression was observed in both brain tissue sample and PC12 cells (Fig. 4B-C). Given that 100 μg/mL $PM_{2.5}$ treatment led to the obvious reduction in cell viability and AMD1 expression, and was the comparatively lower concentration to trigger the apoptotic reactions in PC12 cells, 100 μg/mL of $PM_{2.5}$ exposure was selected as the dose for further *in vitro* experiment.

AMD1 inhibition mimicked $PM_{2.5}$ exposure induced neuronal apoptosis

Considering the obvious low expression level of AMD1 was resulted from $PM_{2.5}$ exposure both *in vivo* and *in vitro*, pharmacological approach was then applied to manipulate AMD1 activity to further investigate its role in cellular apoptosis. SAM486A is an inhibitor of AMD1, which can effectively limit the synthesis of polyamines [14]. We found that AMD1 inhibition could mimic $PM_{2.5}$ exposure induced cellular apoptosis in PC12 cells. Immunoblotting results also showed the expression of Bax and cleaved caspase-3 could be significantly upregulated by SAM486A (10 μmol/L) treatment ($p<0.01$), which was similar with the $PM_{2.5}$ exposure (Fig. 5). The current evidence implied that AMD1 may participate in $PM_{2.5}$ exposure induced neuronal apoptosis.

Spermidine rescued against neurotoxicity and apoptosis induced by $PM_{2.5}$

Regarding to the obvious reduction of AMD1 expression level by $PM_{2.5}$ exposure, the production of spermidine, one of the most relevant polyamines metabolized by AMD1, was tested by high performance liquid chromatography (HPLC) in PC12 cells. The results showed that both $PM_{2.5}$ exposure and SAM486A treatment could lead to a reduced cellular spermidine production (Fig. 6A).

After that, exogenous spermidine supplementation was then tested to explore its possible protective effect against $PM_{2.5}$ induced neurotoxicity. Increasing concentrations of spermidine treatment did not impact on cell viability in PC12 cells ($p>0.05$), until the concentration of 30 μmol/L (SFig. 3). And then pretreatment with spermidine at 10, 20, and 30 μmol/L for 24 hr improved the cell survival following

100 μg/mL $PM_{2.5}$ exposure in PC12 cells (Fig. 6B). 10 μmol/L spermidine pretreatment was applied for following assays. Subsequently, Annexin V-FITC apoptosis staining and flow cytometry were performed to further evaluate the apoptotic level of PC12 cells in each group. Results showed the apoptotic rates of PM and SAM486A group were 18.7% and 16.8% respectively, significantly higher than the control. On contrary, pretreatment with spermidine effectively attenuate the apoptotic rates by $PM_{2.5}$ exposure, which was 9.6% and 9.9%, respectively (Fig. 6C).

Spermidine inhibited $PM_{2.5}$ induced apoptosis via mitochondrial pathway

Mitochondrion is the main organelle that produces ATP and promotes cell energy conversion. It is also responsible for regulating cell growth and cell cycle. Moreover, mitochondrial apoptosis pathway is one of the main pathways of cell apoptosis [15]. Thus, we next put effort to explore the changes in mitochondrial membrane potential and apoptosis-related proteins expression by $PM_{2.5}$ exposure. Results of mitochondrial membrane potential evaluated by flow cytometry (Fig. 7A) showed that the ratios of depolarization of mitochondrial membrane potential were 3.6%, 11.7%, 10.8% for the groups of control, $PM_{2.5}$ and SAM486A respectively, which demonstrated that $PM_{2.5}$ or SAM486A treatment induced mitochondrial damage in PC12 cells. However, pretreatment with spermidine significantly alleviated depolarization of mitochondrial membrane potential ($p<0.05$).

Meanwhile, the expression levels mitochondrial apoptosis related proteins, Bax, cytochrome C were up-regulated by $PM_{2.5}$ treatment and SAM486A treatment, along with down-regulation of Bcl-2, followed by higher expression levels of downstream cleaved caspase-9, cleaved caspase-3 (Fig. 7B). Spermidine pretreatment could dramatically reduce Bax/Bcl-2 ratio, expression levels of cytochrome C, cleaved caspase-9, and cleaved caspase-3. Taken together, these results indicated that spermidine supplementation could attenuate $PM_{2.5}$ induced mitochondrial apoptosis in PC12 cells.

Discussion

Air pollution is an important environmental risk factor that influences people's health. WHO indicated that 92% of the world's population were breathing air which does not meet minimum standards [16]. Earlier studies demonstrated that the exposure of $PM_{2.5}$ could lead to increased incidence and mortality from cardiovascular and respiratory diseases [17, 18]. Moreover, epidemiological studies have shown that exposure to $PM_{2.5}$ may be related to obesity and type 2 diabetes [19, 20]. Children who are chronically exposed to $PM_{2.5}$ above the standards would suffer from obesity and cognitive deficits [21]. Recently, we have found that chronic $PM_{2.5}$ exposure for 9 months could led to neuroinflammatory and cognitive decline in mice [5]. However, $PM_{2.5}$ exposure induced neuroinflammatory reactions in hippocampus and cortex could be found only after 12-month exposure, which occurred after cognitive impairment, implying that neuroinflammtion may be the results but did not trigger the neuronal dysfunction [5]. The underlying molecular mechanism of early events in brain induced by $PM_{2.5}$ exposure remains unclear. In the current

study, neuronal apoptosis were observed earlier by 24-week $PM_{2.5}$ exposure, in parallel with aggravated cognitive function in mice. Besides, we also found AMD1, as one of most important enzymes responsible for polyamine synthesis, together with its product spermidine, participated in $PM_{2.5}$ exposure triggered neuronal apoptosis.

During whole-body exposure, the concentration and composition of $PM_{2.5}$ were analyzed, as it has been reported before [5]. As we all know, $PM_{2.5}$ is an important pollutant composed of a complex mixture of organic and inorganic components. Previous evidence showed that inorganic elements such as sulfur, potassium, chlorine, calcium were the major composition of $PM_{2.5}$ and the PAHs contained benzo(b)fluoranthene were detected in $PM_{2.5}$ as well, which has carcinogenic and neurotoxic properties [22]. In the current study, mice were exposed to ambient $PM_{2.5}$ in a whole-body manner for 24 weeks. In our expectation, in MWM test, mice from PM group manifested longer latency time to reach the hidden platform and reduced frequency and time spent in the target quadrant (Fig. 1B). Therefore, these results again demonstrated that $PM_{2.5}$ was complex and harmful mixture which could do harm to central nervous system.

We then put efforts on to explore the molecular mechanism triggering the neuronal dysfunction. Though neuroinflammation is the common pathway of neurological diseases [23], much evidence has shown neuronal apoptosis as important event in the pathologic process of cognitive dysfunction [24, 25]. Concerning to about $PM_{2.5}$ exposure induced neuronal apoptosis, evidence from mouse model of gestational $PM_{2.5}$ exposure showed neuronal apoptosis in hippocampus of mice offspring [24]. *In vitro* data also showed that $PM_{2.5}$ exposure season-dependently induced neuronal apoptosis and synaptic injuries [9]. From our results, TUNEL positive cells were obviously increased in CA1 area of the hippocampus from mice exposed to $PM_{2.5}$ for 24 weeks, indicating elevated neuronal apoptosis rate by $PM_{2.5}$ exposure (Fig. 2A). Besides, we found the upregulated levels of pro-apoptotic protein Bax and cleaved caspase-3, and the downregulated level of anti-apoptotic protein Bcl-2 from both *in vivo* and *in vitro* experiments (Fig. 2B, Fig. 3B, C), which was consistent with evidence reported before. Therefore, in the next step, we will focus on exploring the specific molecular mechanism of $PM_{2.5}$ inducing neuronal apoptosis.

In order to explore novel target that may contribute to $PM_{2.5}$ induced neuronal apoptosis, total RNA from brain tissues homogenate was screened for transcriptome sequencing analysis. The results showed that genes with upregulated transcription levels included Pgrmc1, Tceal6, Syt11, Pea15a, AMD1 *et al*, while with downregulated transcription levels included Tubb2b, mt-Nd5, Tubb4b, Shisa9 *et al*. By literature research, AMD1, as one of the speed-limiting enzymes in polyamine metabolism in eukaryotic cells [26], became one of our interests. The reduced relative transcriptional level and protein expression level of AMD1 by $PM_{2.5}$ exposure in mice were further verified by qPCR and immunoblotting (Fig. 4A-B), which was confirmed as dose-dependent *in vitro* (Fig. 4C). AMD1 silencing or inhibition was reported to regulate apoptosis in neuroblastoma [11], but little evidence was provided from neurons. By our results, we found

that reduced cell viability and increased neuronal apoptosis could be triggered not only by $PM_{2.5}$ exposure, but also by AMD1 inhibitor in PC12 cells and primary neurons (Fig. 5), demonstrating the possible participation of AMD1 in $PM_{2.5}$ induced neuronal dysfunction.

AMD1 is believed to play an important role in cell growth and proliferation, which may be dependent on metabolic process it participates in. AMD1 decarboxylated S-adenosylmethionine, which provide aminopropyl for putrescine conversion to spermidine and spermine [27]. Spermidine, one of natural polyamines, is not only a major inducer of growth and proliferation in eukaryotic cell, but also has significant mitigative and protective effects against the development of neurodegenerative diseases and impaired cognitive ability [28]. Numerous studies have showed that spermidine treatment could attenuate oxidative stress, neuroinflammation and possess promising neuroprotective effect against degenerative changes [29, 30]. Reduced level of spermidine was reported concomitant with declining memory abilities in aging fruit flies [31]. Another study showed that spermidine promoted cell liability in retinal ganglion neuronal cells [32]. By our work, intracellular concentration of spermidine was evaluated by HPLC, and data showed that spermidine concentration in PM group was significantly reduced (Fig. 6A). By contrast, exogenous spermidine supplementation could efficiently rescue the impaired cell viability, increased apoptotic rate and corresponding apoptosis related protein expression triggered by $PM_{2.5}$ treatment (Fig. 6B-C). Piled up all the results, we may speculate that AMD1 participate in $PM_{2.5}$ induced neuronal apoptosis, while its product spermidine was the key target.

As is known to all, mitochondria are one of the most important organelle involved in apoptosis [33, 34]. Numerous studies have shown that mitochondrial dysfunction may eventually lead to apoptosis [35, 36]. Besides, spermidine was proven to ameliorate neuronal aging by improving mitochondrial function *in vitro* [37]. Based on these evidence, mitochondrial membrane potential was examined in PC12 cells by JC-1 staining, showing that exogenous application of spermidine could rescue $PM_{2.5}$ treatment induced the mitochondrial membrane potential depolarization (Fig. 7A). Mitochondrial membrane potential depolarization may then switch on the intrinsic apoptosis pathway. Once activated, mitochondrial intermembrane space proteins, notably cytochrome C, are released into the cytosol whereupon they activate caspases. Executioner caspase, such as caspase 3, effectively kills the cell within minutes by cleavage of hundreds of different substrates in parallel [38–40]. By our results, the expression levels of mitochondrial function closely related protein Bax/Bcl-2, cytochrome C, cleaved caspase-9 and cleaved caspase-3 were up-regulated by $PM_{2.5}$ treatment, which could be recovered by spermidine pretreatment (Fig. 7B). Therefore, our results suggested that $PM_{2.5}$ aggravated PC12 cells via activating mitochondrial-mediated apoptosis pathway, which could be alleviated by exogenous spermidine. All these data suggested that AMD1 and its product spermidine played crucial role in $PM_{2.5}$ induced neuronal injury.

Conclusions

In summary, the present study demonstrated that chronic ambient $PM_{2.5}$ exposure led to cognitive impairment in mice, and neuronal apoptosis was one of the crucial events. $PM_{2.5}$ reduced cell viability,

lead to mitochondrial damage and finally neuronal apoptosis both *in vivo* and *in vitro*. AMD1 and related spermidine synthesis participated in $PM_{2.5}$ induced neuronal apoptosis, at least partially dependent on the mitochondrial-mediated apoptosis pathway. The distinct molecular mechanism contributed to $PM_{2.5}$ neuronal apoptosis and corresponding participation of AMD1/spermidine were summarized in Fig. 8. The current work raised up AMD1 as the novel clue and possible promising therapeutic target for $PM_{2.5}$-induced neurodegenerative disease.

Methods

Animal treatment and behavior test

Sixteen 8-week-old C57BL/6 male mice were purchased from Shanghai Laboratory Animal Co., Ltd. (SLAC, China). All mice were fed the respective diet (Xietong Organism, China) and water *ad libitum*, and maintained on a 12 hr light/dark cycle at 25 ℃. The mice were randomly assigned to ambient $PM_{2.5}$ chamber or FA chamber in Ambient $PM_{2.5}$ real-time Exposure System (APES) (Jukang, China) for 24 weeks via a whole-body exposure manner. All animal procedures are conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals in China, and approved by the Experimental Animal Ethics Committee of Hangzhou Normal University. APES is a whole-body aerosol exposure device which can carry out research on animal models to simulate personal long-term real-time exposure to ambient $PM_{2.5}$. It is configured with two humidified, temperature-controlled chambers. The PM chamber is directly connected to the outside air and the cyclone separator is used as a substitute for a $PM_{2.5}$ filter. The FA chamber equipped with high efficiency particulate air (HEPA) filter can remove all the $PM_{2.5}$ from the air. All mice in the chambers were weighed every week throughout the exposure period.

The ability of spatial learning and memory in mice were evaluated by MWM following a previous protocol [41]. Briefly, at the end of $PM_{2.5}$ exposure, mice received a consecutive 4-day maze training with 4 trials per day to search for the hidden platform prior to test. On the test day (Day 6), the probe trial was conducted by removing the platform and each mice was given 120 s to seek the platform. The latency and frequency to platform and duration in the target quadrant were recorded.

Brain slice preparation and TUNEL test

After the MWM test, mice under pentobarbital anesthesia (80 mg/kg body weight) were sacrificed by cervical dislocation, and perfused with saline via the left ventricle. Then brain tissues were carefully excised from the body and weighed. Hemisphere brain from each mice was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into a thickness of 4 μm. TUNEL staining was used for neural apoptosis detection and performed according to the manufacturer's instructions for the TUNEL assay kit (Roche, USA). The prepared brain tissues were dehydrated with gradient alcohol after being embedded using conventional paraffin. After permeabilization, the brain slice was labeled with fluorescein-dUTP and peroxidase-labeled anti-fluorescein antibody and visualized using DAB chromogen. Finally the sections were sealed with neutral gum and was observed under optical microscope. The

percentage of apoptotic cells was determined and calculated by counting 200 cells in 5 fields in each experiment by 3 pathologist independently.

Transcriptome RNA-seq analysis and qPCR

Hemisected brains were homogenized in Tris-buffered saline (TBS), sieved with 100 μm cell strainer (Falcon, USA) for transcriptome RNA-seq analysis. The brain tissues homogenate was clarified by centrifugation at 17500 g for 30 min. Total RNA from brain tissues homogenate was extracted using TRIzol reagent (Invitrogen, USA) and purified by poly-T oligo-attached magnetic beads. The extracted mRNA was randomly interrupted into short fragments by Fragmentation Buffer. Then the cleaved RNA fragments were reverse-transcribed to create the final complementary DNA (cDNA) library and the sequencing was performed on an Illumina Hiseq 4000 (LC Science, USA) following the vendor's recommended protocol.

Total RNA samples from brain tissues homogenate and cell was isolated by using RNAiso plus reagent (Sangon Biotech, China) according to the manufacture's protocol. The extracted RNA was reverse transcribed into cDNA using a reverse transcription kit (Takara, Japan). The quantification of gene expression was determined by qPCR followed by SYBR Green PCR Master Mix (Cwbiotech, China) standard protocol. The relative expression levels were determined using $2^{-\Delta\Delta Ct}$ method relative to β-actin. The sequences of primers are list in Table 1.

Table 1
Primers used in qPCR test

Primer		Sequence (5' to 3')
AMD1	Forward	CCGAGTAATCAGTCAGCC
	Reverse	ATCGAGTAGCCACAAGGA
β-actin	Forward	CTGTCCCTGTATGCCTCTG
	Reverse	ATGTCACGCACGATTTCC

$PM_{2.5}$ preparation, cell treatment and viability determination

$PM_{2.5}$ samples were collected on Teflon filter membranes with pore size of 2 μm (Pall Life Sciences, USA), which was mounted on the air samplers connected with exposure chambers in Hangzhou, Zhejiang Province, China. The sampling flow rate was set at 5 L/min for consecutively 48 hr. The membranes were then weighed at a room with constant air temperature and humidity using ultramicro electronic balance (Mettler Toledo, Schweiz) before and after sampling to calculate the concentrations of $PM_{2.5}$. $PM_{2.5}$ extract was prepared from Teflon filter membranes by using a soxhlet extractor with 300 mL acetone for 24 hr [42]. The extracts were then dried by rotary evaporation and diluted to 100 mg/mL stock solution with dimethylsulfoxide (DMSO). Finally, the samples were stored at -20 ℃ till use.

PC12 cells were cultured in DMEM (BBI Life Sciences, China) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA), 1% penicillin-streptomycin (BBI Life Sciences, China), and maintained in an incubator at 37 °C with 5% CO_2. Primary hippocampal neurons were prepared from the brains of C57BL/6 at postnatal day 1 as previously described [43] and briefly described in supplementary materials.

Cell Counting Kit-8 (CCK-8) was usually chosen to detect cell viability after cells were exposed to toxicant [17]. PC12 cells and primary hippocampal neurons were seeded at 1×10^4 cells/well in a 96-well plate for 24 hr. After pretreatment with or without spermidine for further 24 hr, the cells were exposed to $PM_{2.5}$ for another 24 hr. At the end of exposure, the medium was discarded and 10 μL/well CCK-8 (Biosharp, China) was added and incubated at 37 °C for 2 hr. The absorbance at 450 nm was detected with a microplate reader (Molecular Devices, USA).

Immunoblotting analysis

Total protein from brain tissues homogenate or cells was extracted with RIPA lysis buffer (Solarbio, China) containing protease and phosphatase inhibitors (Sangon Biotech, China), and quantified according to our previous study [41]. In brief, 40 μg of total protein was electrophoresed on SDS-PAGE and transferred onto a PVDF membrane (Merck, Germany), and blocked with 5% bovine serum albumin (BSA) at room temperature for 1 hr. The membrane was incubated with primary antibodies β-actin, Bax, Bcl-2 (1:2000; Abcam, UK), AMD1, cytochrome C (1:1000; Proteintech, USA), cleaved caspase-9, cleaved caspase-3 (1:1000; Cell Signaling Technology, USA) overnight at 4 °C. After 3 washes with TBST, the membrane was incubated with HRP-conjugated secondary antibodies for 1 hr and visualized with ECL reagents (Millipore, USA). The density of each band was analyzed with Quantity One software (Bio-Rad, USA) and normalized by β-actin.

Cellular spermidine detection by HPLC

The cellular spermidine content was measured using HPLC method. In brief, PC12 cells were treated with $PM_{2.5}$ (100 μg/mL) for 24 hr. Then PC12 cells were harvested, counted, and lysed by repeated freeze-thaw at -80 °C and 43 °C for 3 times. The cell lysates were benzoylated and filtered through 0.22 μm filter into an amber glass HPLC vial prior to analysis. HPLC analysis was performed according to the following procedures. Derivative spermidine was separated on a Venusil XBP C18(L) column (250 mm×4.6 mm, 5 μm) held at room temperature. The column was eluted with a 62% acetonitrile (phase A) and 38% water (phase B) at the flow rate of 1 mL/min. The detection wavelength was 254 nm.

Flow cytometry

The cell apoptotic rate was determined by Annexin V-FITC apoptosis detection kit (Beyotime Biotechnology, China). At least 1×10^4 cells were collected in each simple and the fluorescent intensity was determined by flow cytometry (Beckman Coulter, USA). The excitation wavelength was 488 nm, and emission wavelengths were 530 nm for FITC and 630 nm for PI, respectively.

The mitochondrial membrane potential was measured by JC-1 test kit (Beyotime Biotechnology, China). Briefly, cells were treated according to the manufacturer's protocol. Subsequently, samples were added to flow cytometer for detection. The excitation wavelength was 488 nm, and emission wavelengths were 525 nm for FL1 and 585 nm for FL2, respectively.

Statistical analysis

All the assays were repeated more three times independently, and data was expressed as the Mean±SEM. SPSS 20.0 software was conducted to do the statistical analysis. Two tailed unpaired Student's *t*-test and one-way ANOVA were used to detect the average difference between the treatment group and the control group. Differences were considered significant when $p<0.05$.

Abbreviations

AD: Alzheimer's disease; AMD1: S-adenosylmethionine decarboxylase 1; APES: Ambient $PM_{2.5}$ real-time Exposure System; BSA: Bovine serum albumin; DMSO: Dimethylsulfoxide; FA: Filtered air; FBS: Fetal bovine serum; GBD: Global Burden of Disease; HEPA: High efficiency particulate air; HPLC: High performance liquid chromatography; MWM: Morris water maze; PAHs: Polycyclic aromatic hydrocarbons; PD: Parkinson's disease; $PM_{2.5}$: Particulate matter 2.5; VOCs: Volatile organic compounds; TBS: Tris-buffered saline; TUNEL: TdT-mediated dUTP Nick-End Labeling

Declarations

Acknowledgements

Not applicable.

Authors' contributions

H.W and Y.H designed the study. X.Z performed the animal exposure, cognitive evaluation, immunoblotting analysis, and apoptosis detection. Y.S performed cell exposure, spermidine detection. X.J helped with data collection and analysis. X.Z drafted the manuscript. H.W supervised the entire project and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

All animal procedures are conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Experimental Animal Ethics Committee of Hangzhou Normal University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

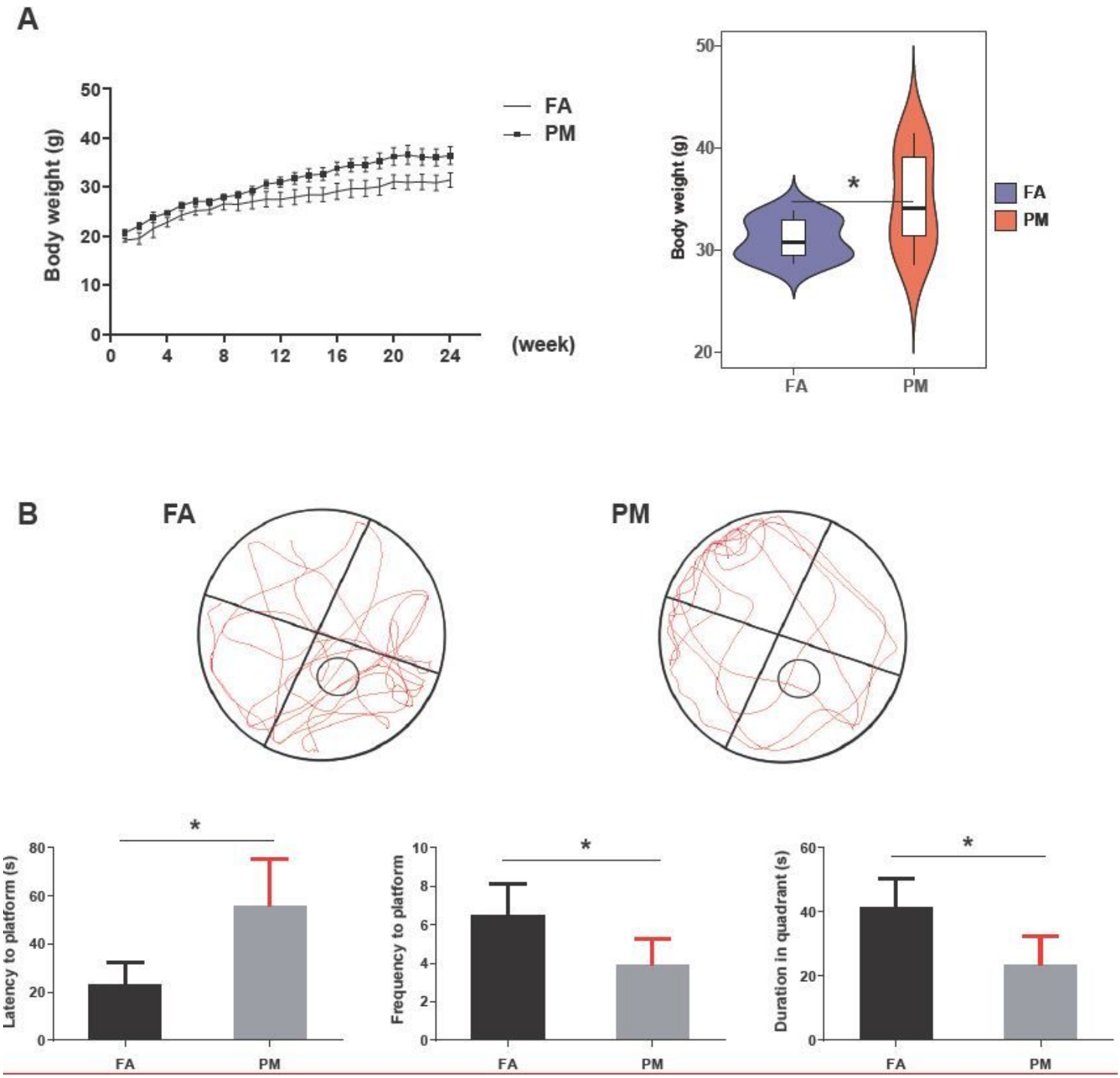

Figure 1

PM2.5 exposure impaired spatial learning and memory function in mice Sixteen 8-week-old male mice were exposed to PM2.5 for 24 weeks via whole body exposure. A) Body weights of mice were recorded once a week. Body weights of mice during 24-week PM2.5 exposure and body weights of mice before sacrifice were shown in the chart. B) Spatial learning and memory function were tested by MWM in mice. Swimming tracking routs of mice in MWM test, latency to platform, frequency to platform, and duration in quadrant were recorded (n=8, *p<0.05).

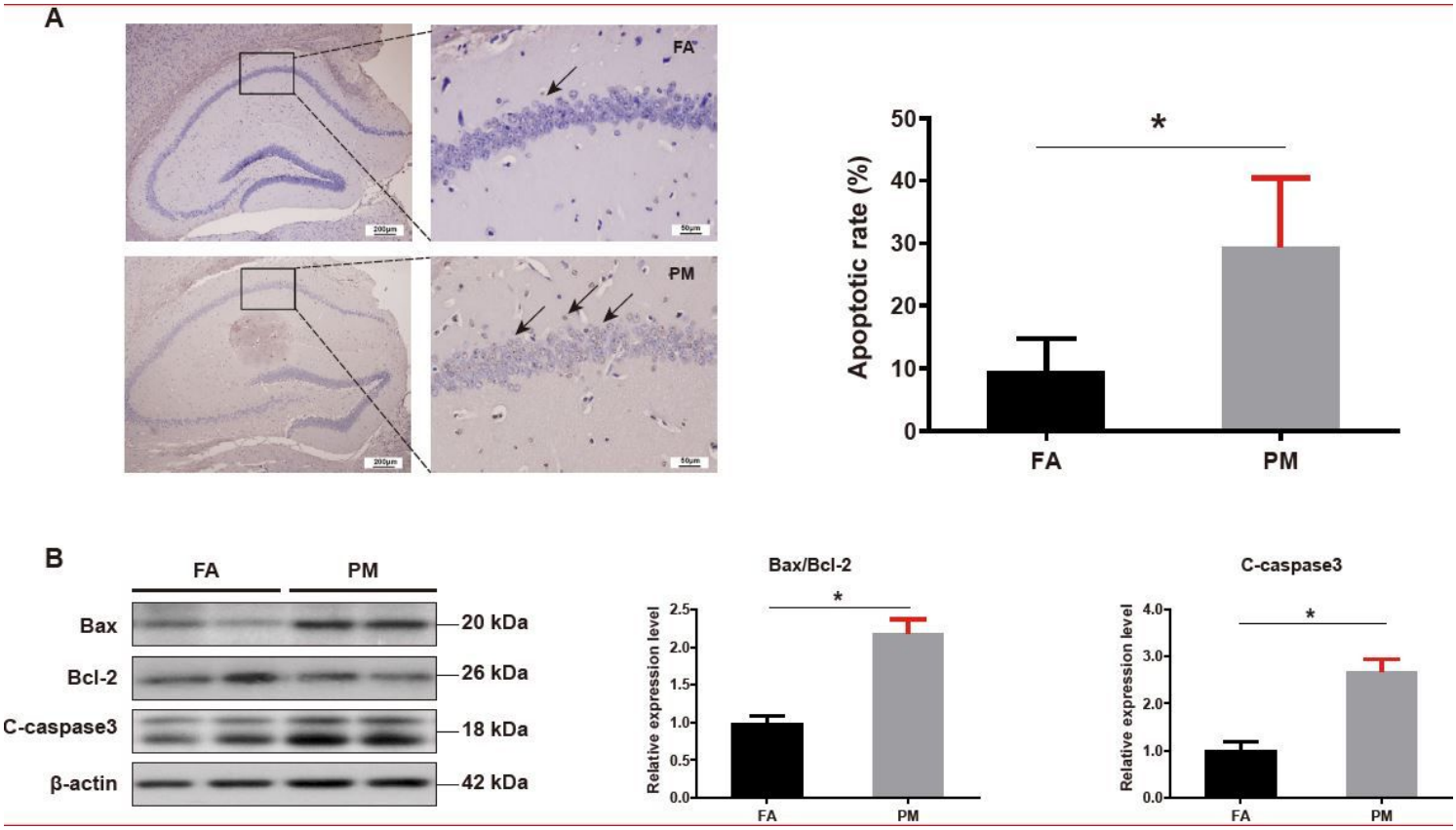

Figure 2

PM2.5 exposure induced neuronal apoptosis in mice A) Neuronal apoptosis was evaluated by TUNEL assay. 5 non-overlapping pictures were randomly selected from the brain slice. TUNEL positive cells rate/index was defined as apoptotic positive cells number/total cellular number. TUNEL positive apoptosis cells and apoptosis rate in CA1 area of the hippocampus were shown in the chart. B) Hemisphere of brain tissue were homogenated and lysed andapoptosis related protein was tested by immunoblotting. And intensity quantification was performed by Quantity one and normalized to β-actin (n=8, scale bar=50 μm, *p<0.05).

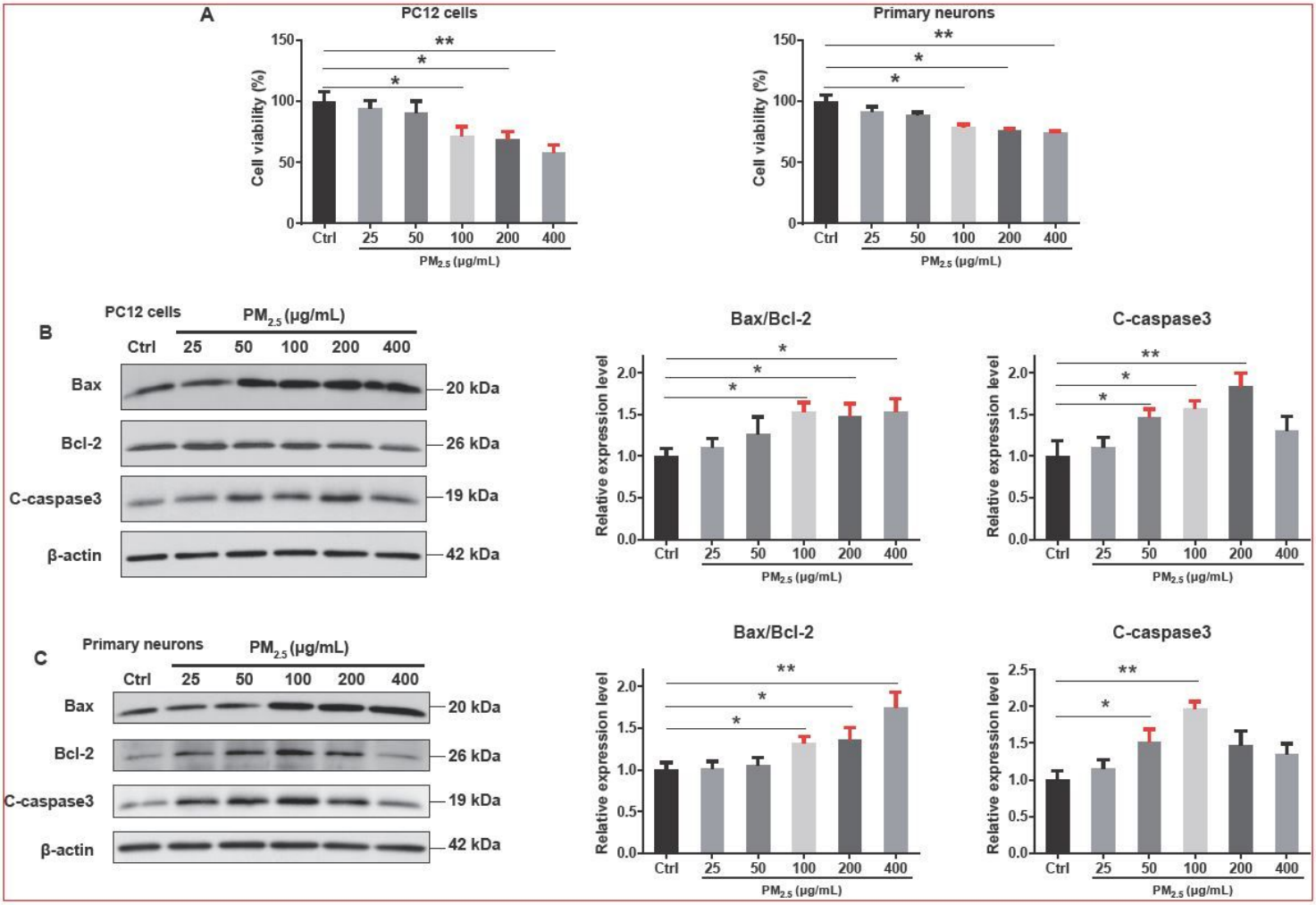

Figure 3

PM2.5 treatment accelerated apoptosis in PC12 cells and primary hippocampal neurons Cells were seeded at 1×104 cells/well in a 96-well plate for 24 hr, serum-free medium dissolved PM2.5 was added for another 24 hr, cell viability was evaluated by CCK8 assay. A) Cell viability measurement of PC12 cells and primary hippocampal neurons, respectively. After exposure to PM2.5 for 24 hr, cells were collected and regarded as a protein sample, apoptosis-related proteins were detected by immunoblotting and quantification was performed by Quantity one. B) Represented bands for Bax, Bcl-2, cleaved caspase-3 in PC12 cells treated by different dose of PM2.5 and associated quantifications. C) Represented bands for Bax, Bcl-2, cleaved caspase-3 in primary neurons treated by different dose of PM2.5 and associated quantifications (n=3, *p<0.05, **p<0.01).

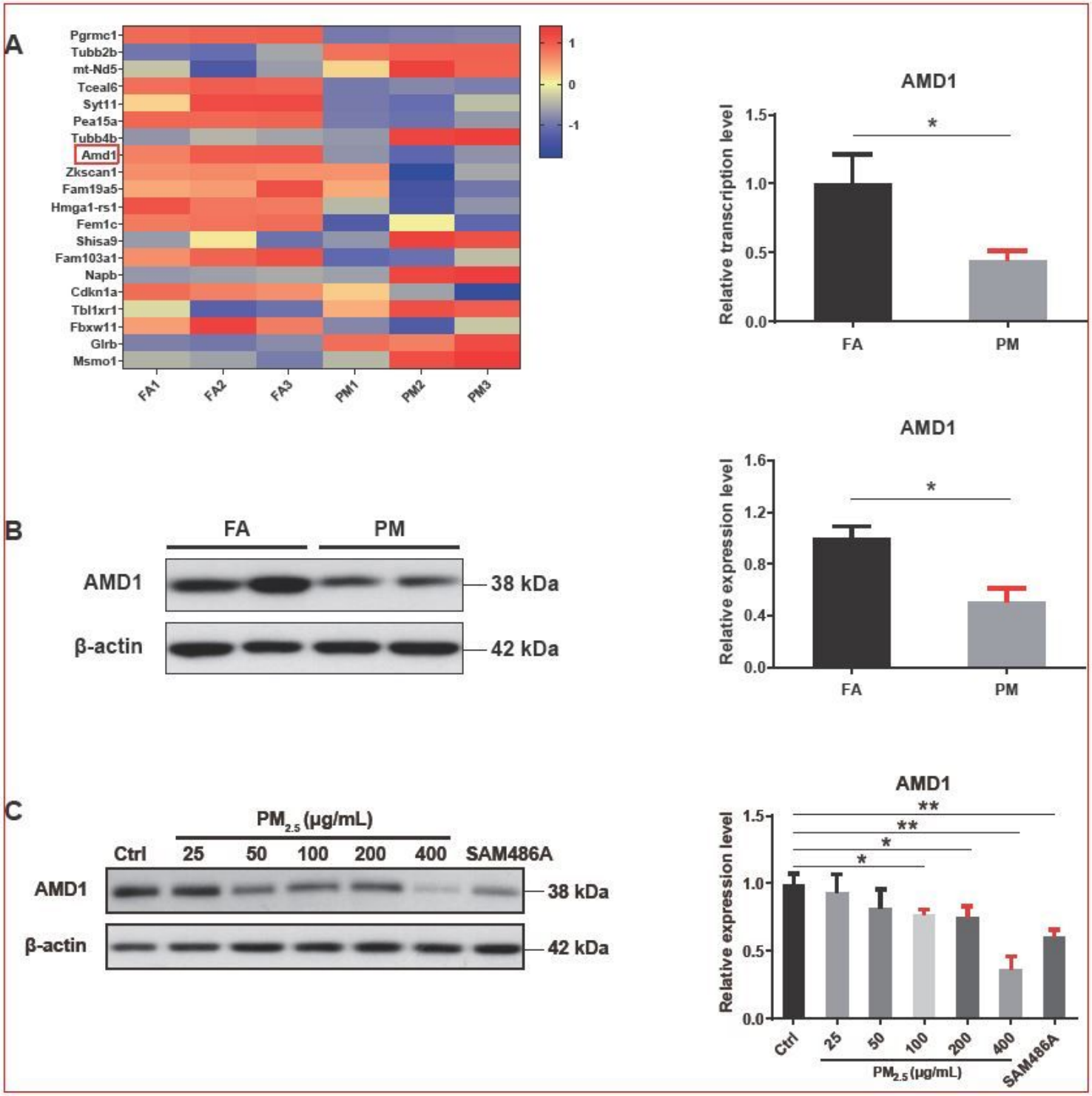

Figure 4

PM2.5 exposure impacted on of AMD1 expression in vivo and in vitro A) Heat-map analysis to differential gene-level expression in brain tissues. The names of different samples are indicated at the bottom of the heatmaps. “FA” represents filtered air group and “PM” represents PM2.5 treated group. Color bar at the right represents the color scale reflecting gene-level expression differences. The transcriptional level of AMD1 was detected by qPCR (n=8, *p<0.05). B) AMD1 expression level and intensity quantification in brain tissues exposed to PM2.5 were evaluated by immunoblotting (n=8, *p<0.05). PC12 cells were

treated with different dose of PM2.5 or AMD1 inhibitor for 24 hr, then cells were collected and regarded as a protein sample. C) AMD1 expression level and intensity quantification from PC12 cells treated with different concentration of PM2.5 and AMD1 inhibitor (SAM486A 10 μmol/L) (n=3, *p<0.05, **p<0.01).

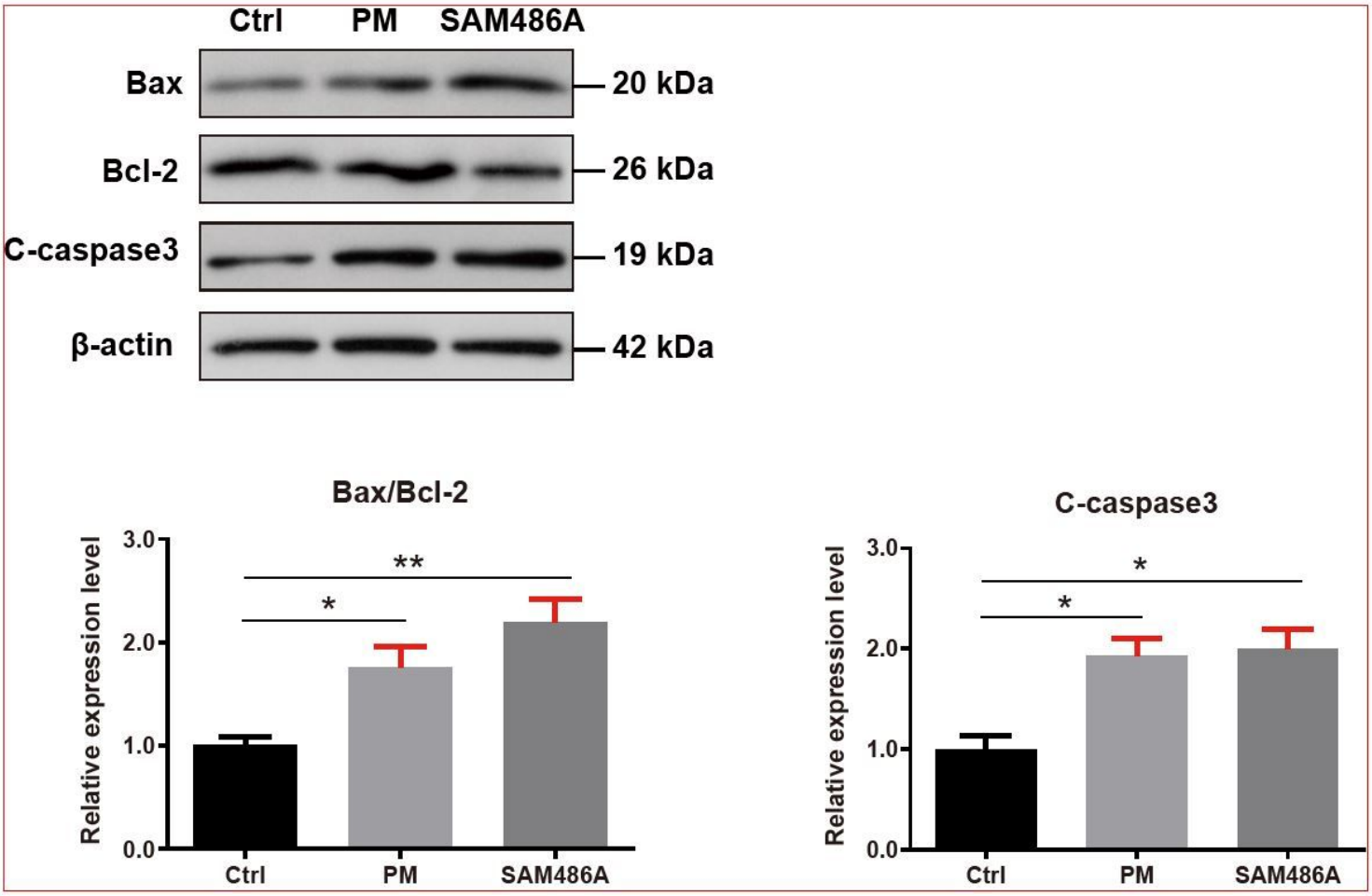

Figure 5

AMD1 inhibition mimicked PM2.5 treatment induced neuronal apoptosis PC12 cells were treated with 100 μg/mL PM2.5 or AMD1 inhibitor (SAM486A 10 μmol/L) for 24 hr, then cells were collected and lysed for detection. Relative protein expression and intensity quantification of Bax/Bcl-2 ratios and cleaved caspase-3 in PC12 cells were listed (n=3, *p<0.05, **p<0.01).

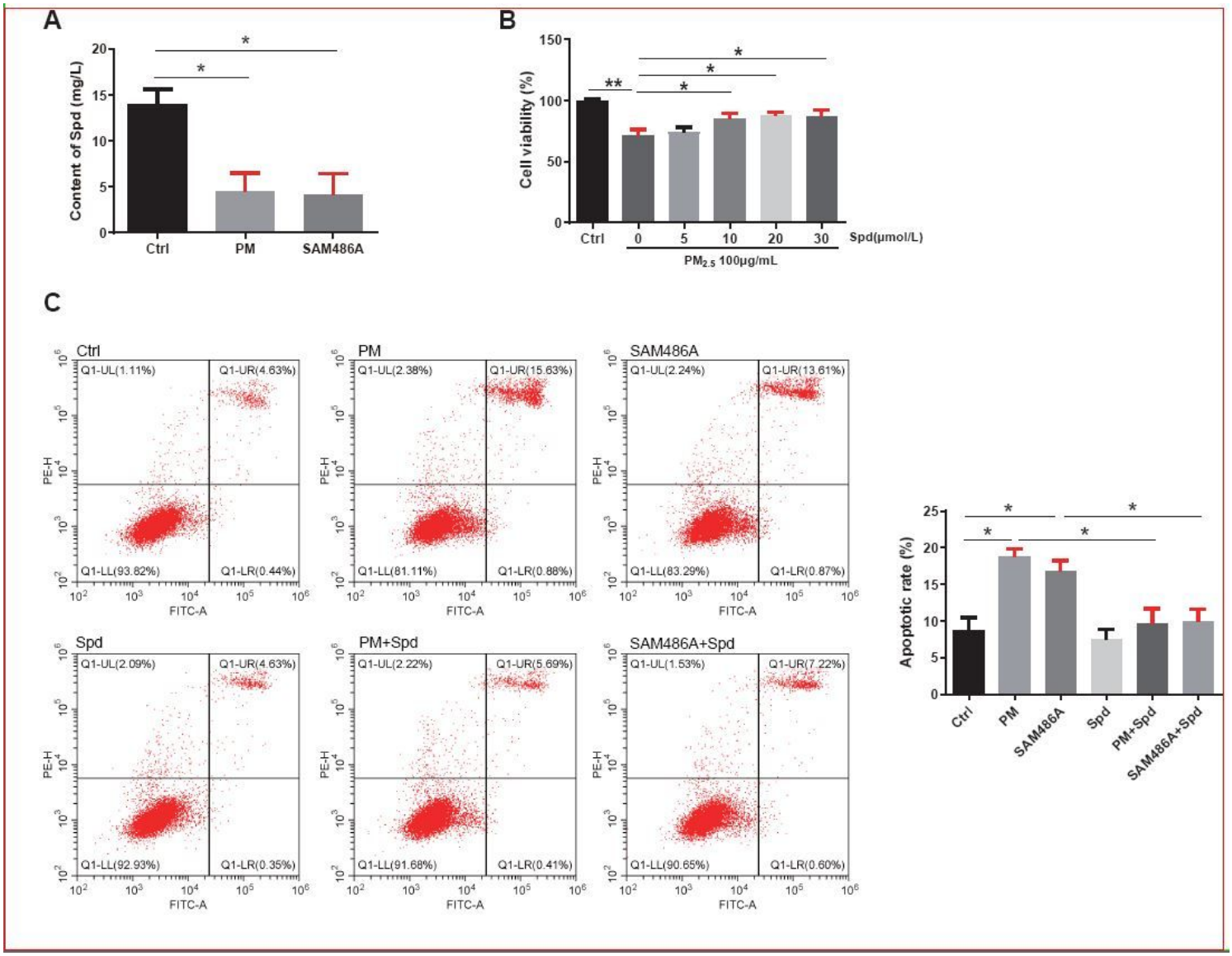

Figure 6

Spermidine protected cells against neurotoxicity and apoptosis induced by PM2.5 PC12 cells were pretreated with different dose of spermidine (Spd) for 24 hr, then the medium was added with 100 μg/mL PM2.5 for another 24 hr. A) Cellular Spd concentration was detected by HPLC. B) Cell viability was evaluated by CCK8 assay. C) PC12 cells treated with PM2.5 and Spd were double stained by PI and FITC-labeled Annexin V and quantification of cell apoptosis was performed by flow cytometer. The represented flow cytometer figure and its quantification (n=3, *p<0.05, **p<0.01).

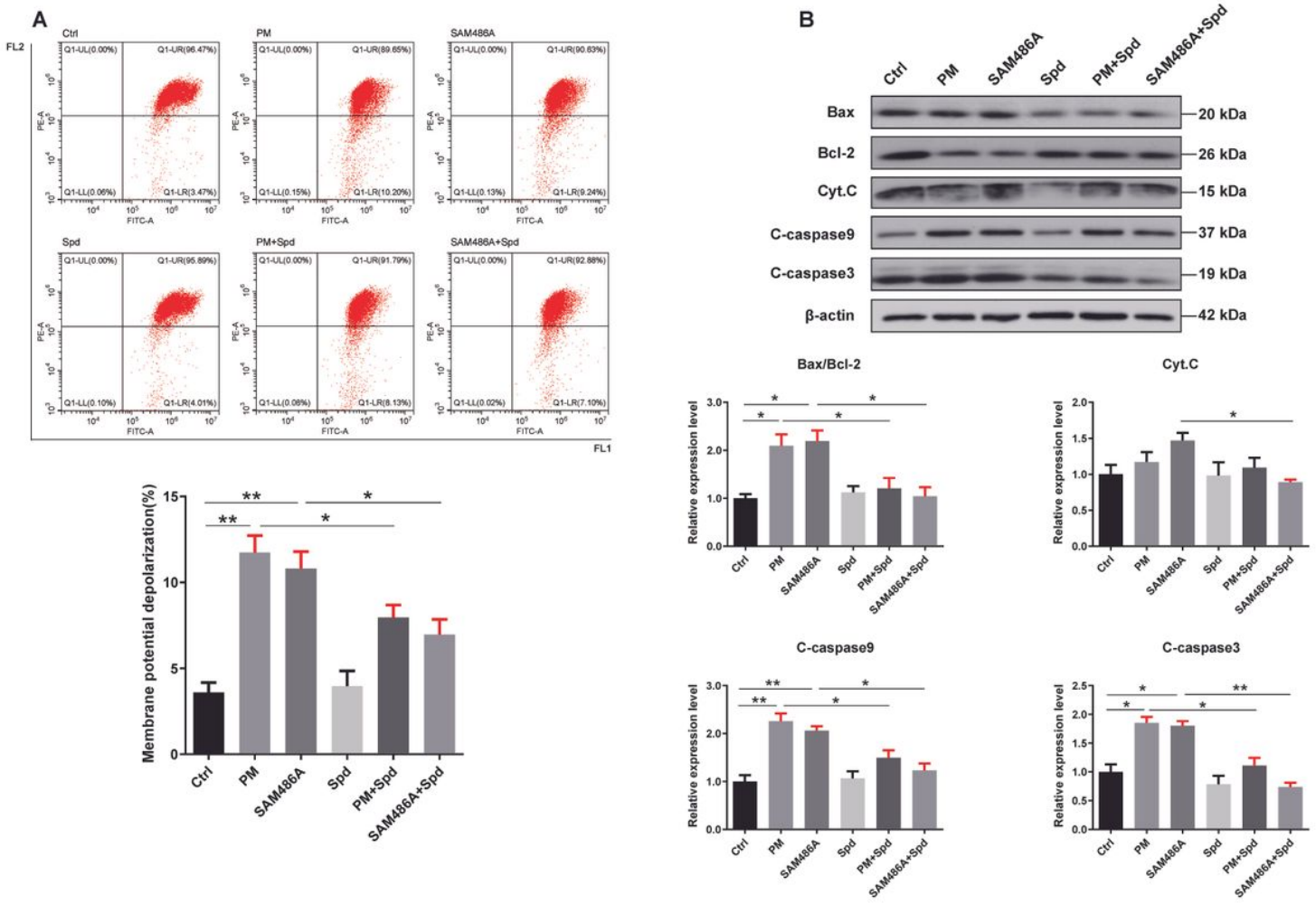

Figure 7

Spermidine supplementation rescued PM2.5 induced neuronal apoptosis via the mitochondrial pathway PC12 cells were pretreated with 10 μmol/L Spd for 24 hr, then the medium was added with 100 μg/mL PM2.5 or AMD1 inhibitor for another 24 hr. A) At the end of exposure, PC12 cells were stained by JC-1 assay and mitochandrial membrane potential were evaluated by flow cytometry. The representative flow cytometry and its quantification. B) PC12 cells were treated with Spd, PM2.5 or AMD1 inhibitor for 24 hr, then cells were collected and regarded as protein samples. Mitochondrial mediated apoptosis pathway related proteins Bax, Bcl-2, cytochrome C, cleaved caspase-9, cleaved caspase-3 were examined by immunoblotting. Quantification were performed by Quantity one normalized to β-actin (n=3, *p<0.05, **p<0.01).

Figure 8

Scheme for proposed molecular mechanism by which PM2.5 aggravates neuronal apoptosis in vitro and in vivo and participation of AMD1 related metabolism

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SFig3.pdf
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- SFig1.pdf
- SupplementaryMaterials.docx