ORIGINAL ARTICLE

Long-term treatment with 3,4-methylenedioxymethamphetamine caused retina damage in C57BL/6 mice

Tratamento a longo prazo com 3,4-metilenedioximetanfetamina causando dano na retina em camundongos C57BL/6

Xiangqin Liu[1], Hong Ye[2]

1. Department of Ophthalmology, Beijing Royal Integrative Medicine Hospital, Beijing 102200, People's Republic of China.
2. Department of Ophthalmology, Beijing Changping Hospital of Integrated Chinese and Western Medicine, Beijing 102208, People's Republic of China.

ABSTRACT | Purpose: As a class of psychostimulant drugs, amphetamines are widely abused for their stimulant, euphoric, and hallucinogenic properties. Many of these effects result from acute increases in dopamine and serotonin neurotransmission. Following the onset of these effects, 3,4 methylenedioxymethamphetamine produces persistent damage to dopamine and serotonin nerve terminals, resulting in long-lasting neurotoxicity. The purpose of this investigation was to assess the effects of treatment with low dose of methylenedioxymethamphetamine on retinal function of C57BL/6 mice and its underlying mechanisms. **Methods:** C57BL/6 mice were divided randomly into two groups (n=10): one group was treated with phosphate buffered saline by intraperitoneal injection daily; the other group was treated with 1 mg/kg methylenedioxymethamphetamine by intraperitoneal injection daily for three months. Electroretinography was used to test retinal function every month. H&E staining and terminal deoxynucleotidyl transferase assay were used to evaluate the retinal morphology and histology. Enzyme-linked immunosorbent assay assays were used to measure markers of oxidative stress and inflammatory factors. Gene and protein expression was detected by real-time PCR and western blot. **Results:** Three-month treatment with methylenedioxymethamphetamine induced significant retinal dysfunction via photoreceptor cell apoptosis by oxidative stress and inflammatory responses. **Conclusions:** These results suggest that long-term treatment with methylenedioxymethamphetamine increases inflammatory responses in photoreceptor cells resulting in retinal dysfunction in C57BL/6 mice. Thus, this investigation provides preclinical rationale for the retina damage caused by the methylenedioxymethamphetamine abuse.

Keywords: 3,4-methylenedioxymethamphetamine; Oxidative stress; Inflammatory response; Retina/injuries; Mice

RESUMO | Objetivos: Como uma classe de drogas psicoestimulantes, as anfetaminas são amplamente usadas por suas propriedades estimulantes, eufóricas e alucinógenas. Muitos desses efeitos resultam de aumentos agudos na neurotransmissão da dopamina e da serotonina. Após o início desses efeitos, a 3,4-metilenedioximetanfetamina produz danos persistentes nos terminais nervosos de dopamina e serotonina, resultando em neurotoxicidade duradoura. O objetivo desta investigação foi avaliar os efeitos do tratamento baixa dose de metilenedioximetanfetamina na função da retina em camundongos C57BL/6 e seus mecanismos subjacentes. **Métodos:** Camundongos C57BL/6 foram divididos aleatoriamente em dois grupos (n=10): um grupo foi tratado com solução salina tamponada de fosfato por injeção intraperitoneal diária; o outro grupo foi tratado com 1 mg/kg de metilenedioximetanfetamina por injeção intraperitoneal diária durante 3 meses. Eletroretinografia foi utilizada para testar a função da retina a cada mês. A coloração H&E e análise com deoxinucleotidil terminal transferase foram utilizados para avaliar a morfologia e histologia da retina. Testes de imunoabsorção enzimática foram utilizados para medir marcadores de estresse oxidativo e fatores inflamatórios. A expressão de genes e proteínas foi detectada por PCR em tempo real e western blot. **Resultados:** O tratamento de três meses com metilenedioximetanfetamina induziu disfunção de retina significativa por apoptose de células fotorreceptoras por estresse oxidativo e resposta inflamatória. **Conclusões:** Estes resultados sugerem que o tratamento a longo prazo com metilenedioximetanfetamina aumenta as respostas inflamatórias em células fotorreceptoras, resultando em disfunção de retina em camundongos C57BL/6. Assim, a investigação foence uma justificação pré-clínica para os danos na retina causados pelo abuso de metilenedioximetanfetamina.

INTRODUCTION

3,4-methylenedioxymethamphetamine (MDMA), commonly known as ecstasy, is a ring-substituted amphetamine derivative that has become increasingly popular in North America and Europe and is linked with nightlife settings and particularly techno dance music[1]. MDMA abuse is a growing concern around the world due primarily to its ability to produce significant short term feelings of euphoria. MDMA easily crosses the blood-brain barrier with wide-ranging effects on the central nervous system (CNS). Although much progress has been made in understanding the acute effects as they promote the abuse liability of MDMA, the long-term consequences of their abuse are rapidly emerging and include evidence of neuropsychiatric disorders and brain damage[2,3]. However, the toxic effects of MDMA on the retina have not been elucidated completely. Studies showed that chronic exposure to MDMA resulted in irreversible damage on photoreceptor cells, causing retinal degeneration[4,5].

MDMA has also been associated with the degeneration of 5-HT nerve terminals and resulted in an imbalance between the release and reuptake of dopamine (DA), leading to neurological abnormalities[6,7]. Acute increases in cytoplasmic and extracellular DA contribute to oxidative damage of axon terminals following MDMA exposure. Although the underlying mechanisms have not been fully understood yet, several factors were proposed to justify the toxicity caused by MDMA, including the production of reactive nitrogen species resulting from increased nitric oxide synthase activity and reactive oxygen species (ROS) which lead to a subsequent induction of oxidative stress, compromising mitochondrial function, and activating the apoptotic pathway[8-10].

Exposure to a high dose of MDMA results in direct toxicity in animal models, which may be related to lipid peroxidation processes and oxidative stress[11]. However, little is known about the effects of systemic treatment with low dose of MDMA on adult mouse retinas. MDMA has been shown to trigger inflammatory responses in areas where DA and 5-HT terminals are damaged. Given the potentially damaging effects of inflammation within the CNS, these processes have been suggested to play a role in the damage to monoamine nerve terminals. Neuroinflammation has been implicated as an important mechanism associated with some neuropsychological impairments[12]. The ability of activated microglia to promote neuronal damage during MDMA exposure has not been elucidated fully. Nevertheless, cytokines produced during microglial activation are known to enhance glutamate neurotransmission, and, thus, promote excitotoxicity[13,14]. In addition, MDMA has been shown to induce an inflammatory response causing photoreceptor cell damage *in vitro*[15]. The purpose of this study was to investigate whether long-term treatment with MDMA affects the retinas of C57BL/6 mice, providing preclinical rationale for the retina damage caused by the MDMA abuse.

METHODS

Mouse treatment

C57BL/6 mice were purchased from Charles River Laboratory (Beijing, China). The mice were housed in standard cages with food and water available *ad libitum* in a temperature-controlled environment under a 12-h light and 12-h dark cycle. All the experimental procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Anhui Medical University. MDMA was purchased from the National Institute for Food and Drug Control (Beijing, China) with a purity of more than 98%. Mice were treated with PBS (vehicle, n=10) or MDMA (1 mg/kg, n=10) by intraperitoneal injection daily.

Electroretinography

After overnight dark adaptation, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg) under dim red illumination to perform the ERG test using the UTAS-E3000 Electrophysiology System (LKC Technologies, Gaithersburg, USA)[16]. The pupils were dilated with eye drops (2.5% phenylephrine HCl, 1% cyclopentolate HCl, 1% mydriacyl) and the corneal surface was anesthetized with 0.5% proparacaine HCl. Reference and ground electrodes were attached to the mouth and placed in the neck-back region subcutaneously. In the light-adapted session, the flash luminance ranged from -0.8 to 1.9 log cd s/m². In the dark-adapted session, the flash luminance ranged from -2.4 to 2.1 log cd s/m². The ERG A-wave was derived from the cones and rods of the outer photoreceptors and reflects the hyperpolarization of the photoreceptors due to the closure of sodium ion channels in the outer-segment membrane. The amplitude of the A-wave was measured from the baseline to

the trough of the A-wave. The ERG B-wave was derived from the inner retina, predominantly Muller and bipolar cells. The amplitude of the B-wave was measured from the trough of the A-wave to the peak of the B-wave.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

After a sacrifice by inhaling CO_2, mouse eyes were enucleated and fixed with 4% paraformaldehyde for 4 h, and, then, dehydrated by a graded sucrose solution. Eyes were embedded in Optimal Cutting Temperature compound. 10 μm thick frozen sections were then cut sagittally passing through the optic disc. Frozen sections were incubated with the TUNEL reaction mixture for 60 min at 37°C and then rinsed with PBS. Sections were sealed with VECTASHIELD mounting medium containing 4',6-diamidino-2-phenylindole and visualized under the fluorescence microscope[17]. ImageJ software (Version 1.48v, National Institutes of Health) was used to count the TUNEL-positive cells.

Hematoxylin and eosin staining

Ten μm thick frozen sections were stained with hematoxylin and eosin. An Olympus BX60 microscope (Olympus, Shinjuku, Japan) was used to take photographs. The thickness of the outer nuclear layer (ONL) was measured at 200 μm from the edge of the optic disc using ImageJ 1.48v software (National Institutes of Health).

Measurement of superoxide dismutase (SOD) activity, GSH, and MDA content

After a sacrifice by inhaling CO_2, retinas were separated and lysed to determine the SOD activity, glutathione (GSH), and malondialdehyde (MDA) levels by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instruction.

Measurement of inflammatory factors

Nitric oxide (NO) was determined by Griess assay[18]. TNFα, IL-1β, IL-15, and IL-18 levels were detected by ELISA kits according to the manufacturer's instruction.

Measurement of cytochrome-c

After the animals were sacrificed via CO_2 inhalation, retinas were separated and lysed to measure the cytochrome-c levels by the assay kit (R&D systems, Minneapolis, MN, USA). The optical density was measured at 490 nm.

Measurement of caspase activity

After animal sacrifice via CO_2 inhalation, retinas were separated and lysed to measure the caspase-3/caspase-9 activities by the assay kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instruction.

Quantitative real-time PCR

After animal sacrifice via CO_2 inhalation, the total RNA was extracted from retinas using TRIzol reagent and quantified. The cDNA reverse-transcription was performed using 0.5 μg RNA with SuperScript master mix. Quantitative PCR was run on StepOne systems (Thermo Fisher) with SYBR green Supermix (Thermo Fisher), using comparative C_t value method to detect the expression level of the targeted genes. The mRNA levels were normalized to the housekeeping gene β-*actin*. The primer sequences are: for *Sod*, forward: gcggtcgtgtaaacctcaat; reverse: ccagagcctcgtggtacttc, NCBI reference: NM_013671.3; for *Catalase*, forward: ttgacagagagcggattcct; reverse: agctgagcctgactctccag, NCBI reference: NM_009804.2; for *Il-1*β, forward: tgaaatgccacctttgaca; reverse: tgtcctcatcctggaaggtc, NCBI reference: NM_008361.4; for *Il-15*, forward: cattttgggctgtgtcagtg; reverse: gcaattccaggagaaagcag, GenBank: U14332.1; for *Il-18*, forward: gaggaaatggatccacctga; reverse: acaaaccctcccacctaac, NCBI reference: NM_008360.2; for *Tnf*α, forward: agcccccagtctgtatcctt; reverse: gaggcaacctgaccactctc, NCBI reference: NM_013693.3; for β-*actin*, forward: gctacagcttcaccaccaca; reverse: aaggaaggctggaaaagagc, GenBank: BC138614.1.

Western blot

After animal sacrifice via CO_2 inhalation, the retinas were separated and homogenized with RIPA lysis buffer. The lysates were centrifuged at 12,000 rpm at 4°C for 20 minutes to remove the insoluble material. The supernatant was used for protein quantification with the Bradford method. After boiling for 5 min, samples with equal protein (40 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 4%-12% gel. Then, protein was transferred to polyvinylidene fluoride membranes. After blocking with 1% BSA for 1 h, the membranes were washed three times and incubated with primary antibodies at 4°C (anti-cleaved Caspase 3 (Asp175) Rabbit mAb #9664, 1:1000; anti-Caspase 3 rabbit pAb ab13847, 1:500; anti-Caspase 9 rabbit pAb ab52298, 1:500; anti-cleaved Caspase 9 (Asp353) Rabbit mAb #9509, 1:1000; anti-Bcl-2

rabbit pAb ab59348, 1:500; anti-Bax rabbit mAb ab32503, 1:2000; anti-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 rabbit pAb ab55831, 1:500; anti-NADPH oxidase 2 rabbit pAb ab31092, 1:500; anti-NADPH oxidase 3 rabbit pAb ab82708, 1:1000; anti-NADPH oxidase 4 rabbit pAb ab154244, 1:1000; anti-NF-κB P65 rabbit mAb ab207297, 1:1000; anti-NF-κB p-P65 (phosphor S536) rabbit pAb ab86299, 1:2000; anti-β-actin rabbit pAb ab8227, 1:3000) overnight. After washing, membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit

IgG H&L HRP, ab6721, 1:3000) for 1 h. Protein bands were detected by the ECL system (Pierce, IL, USA).

Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analyzed with the SAS 9.1 software (SAS Institute, USA). The ERG data were analyzed by two-way repeated ANOVA. The power analysis was conducted by the F-test of one-way ANOVA, where numbers were considered as outcome and groups as the factor. The experimental group and the control group were compa-
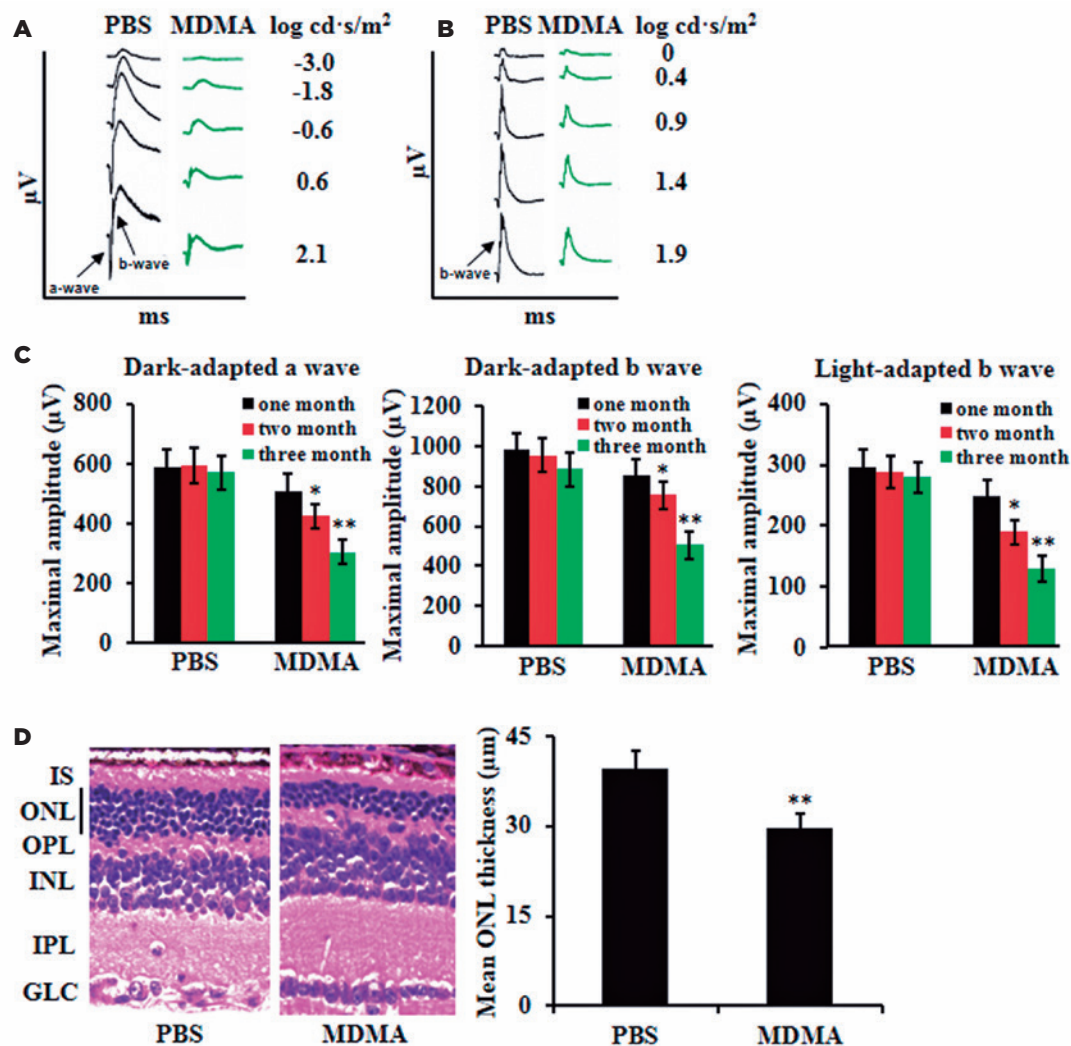
Data are expressed as mean ± SD. *$p<0.05$, **$p<0.01$ vs. PBS group.
Figure 1. Three-month treatment with MDMA caused retinal dysfunction and damage. ERG response was obtained from C57BL/6 mice treated with PBS (n=10) or MDMA (n=10), shown by typical dark-adapted ERG waveforms (A) and typical light-adapted ERG waveforms (B). MDMA treatment decreased the intensity-response of dark-adapted ERG A-wave, dark-adapted ERG B-wave, and light-adapted ERG B-wave (C). Representative images (20×) of retina cross--sections with H&E staining from C57BL/6 mice treated with PBS or MDMA were presented (D). The thickness of ONL significantly decreased after three-month MDMA treatment.

red by Dunnett's *t*-test. A value of p less than 0.05 was considered statistically significant.

RESULTS

Long-term treatment with MDMA caused retinal dysfunction and damage

ERG measures the electrical activity generated by neural and non-neural retinal cells in response to a light stimulus, and is the widely accepted method to eva-luate the outer retinal functions and potential disease progression. Treating mice with MDMA for one month had modest effects on ERG. However, after 3-month treatment with MDMA, the amplitude dramatically decreased, suggesting MDMA caused retinal dysfunction. The thickness of ONL significantly decreased after treatment with MDMA for three months, indica-ting MDMA treatment causing photoreceptor cell loss (p<0.01, Figure 1).
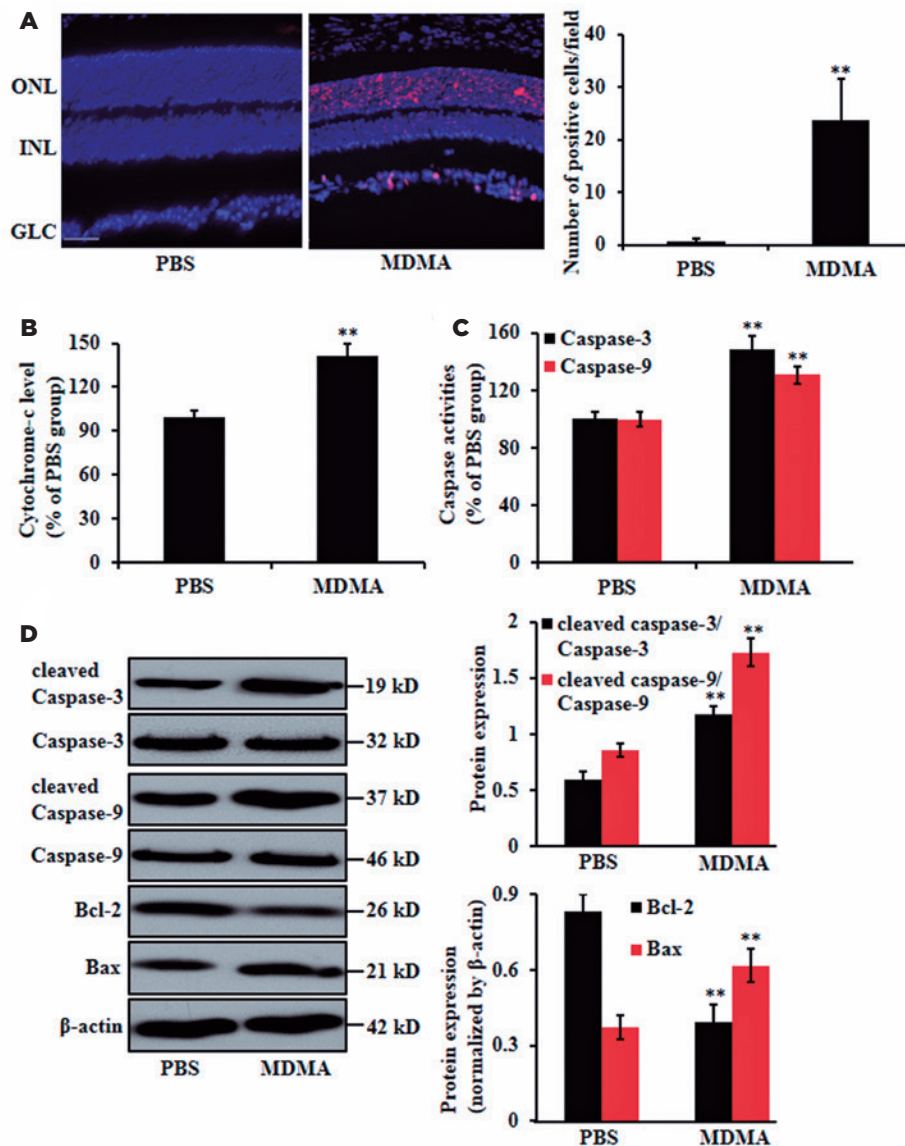
Data are expressed as mean ± SD. ****p<0.01 vs. PBS group.
Figure 2. Three-month treatment with MDMA caused apoptosis of photoreceptor cells. The apoptotic cells (TUNEL-positive) were indicated by the red spots (20×) (A). Three-month treatment with MDMA significantly increased DNA damage (a), cytochrome-c release (B) and caspase activities (C). Three-month treatment with MDMA also decreased expression of the anti-apoptotic protein, while increased pro-apoptotic protein expression (D).

Long-term treatment with MDMA induced apoptosis of photoreceptor cells

MDMA treatment significantly increased the TUNEL-positive cells in retinas of C57BL/6 mice after three months. Cytochrome-c and caspase activities also increased significantly (p<0.01). Expression of anti-apoptotic protein decreased, while pro-apoptotic protein expression significantly increased after MDMA treatment (Figure 2).

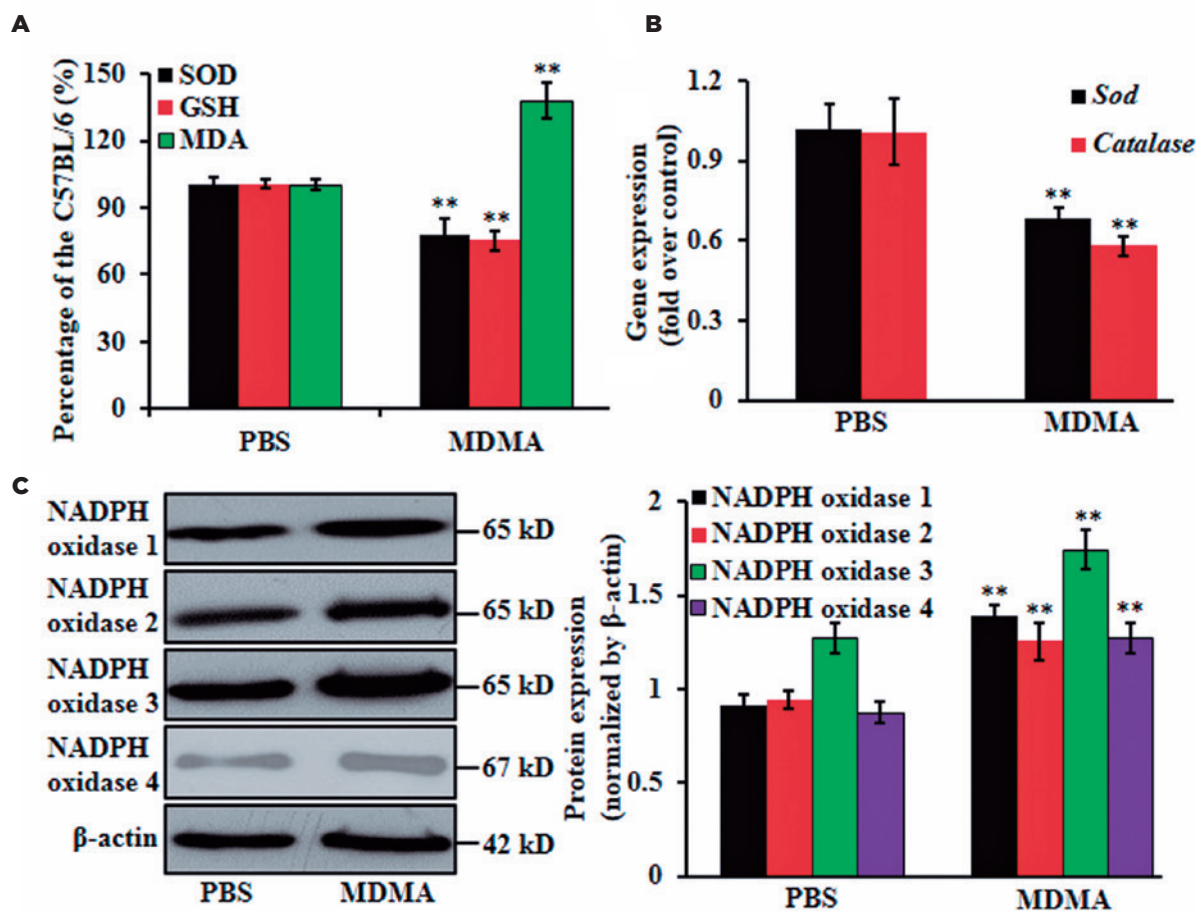## Long-term treatment with MDMA increased the oxidative stress in C57BL/6 mouse retinas

Several intracellular oxidative and anti-oxidative agents were measured in retina lysates with ELISA assay, qRT-PCR and western blot. A decrease in the SOD, GSH, and catalase was found in retina lysate. Consistently, an increase in MDA and NADPH oxidases was observed after three-month MDMA treatment, relative to the PBS control (Figure 3).

Long-term treatment with MDMA induced the inflammatory response

Production of NO and IL-1β, IL-15, IL-18, as well as TNFα in the retinas significantly increased after three-month MDMA treatment. Gene expression of *Il-1*β, *Il-15*, *Il-18*, and *TNFα* also significantly increased after MDMA treatment. MDMA treatment increased protein expression of p-P65 in the C57BL/6 mouse retinas (p<0.01, Figure 4).

DISCUSSION

MDMA is administered systemically and may exert pharmacological effects on the function of both brain and peripheral organs. Many investigations only consider how these effects originate within neurons, making it essential to explore the non-neuronal effects of MDMA due to the potential to significantly contribute to the excitotoxicity after exposure to MDMA. Moreover, the

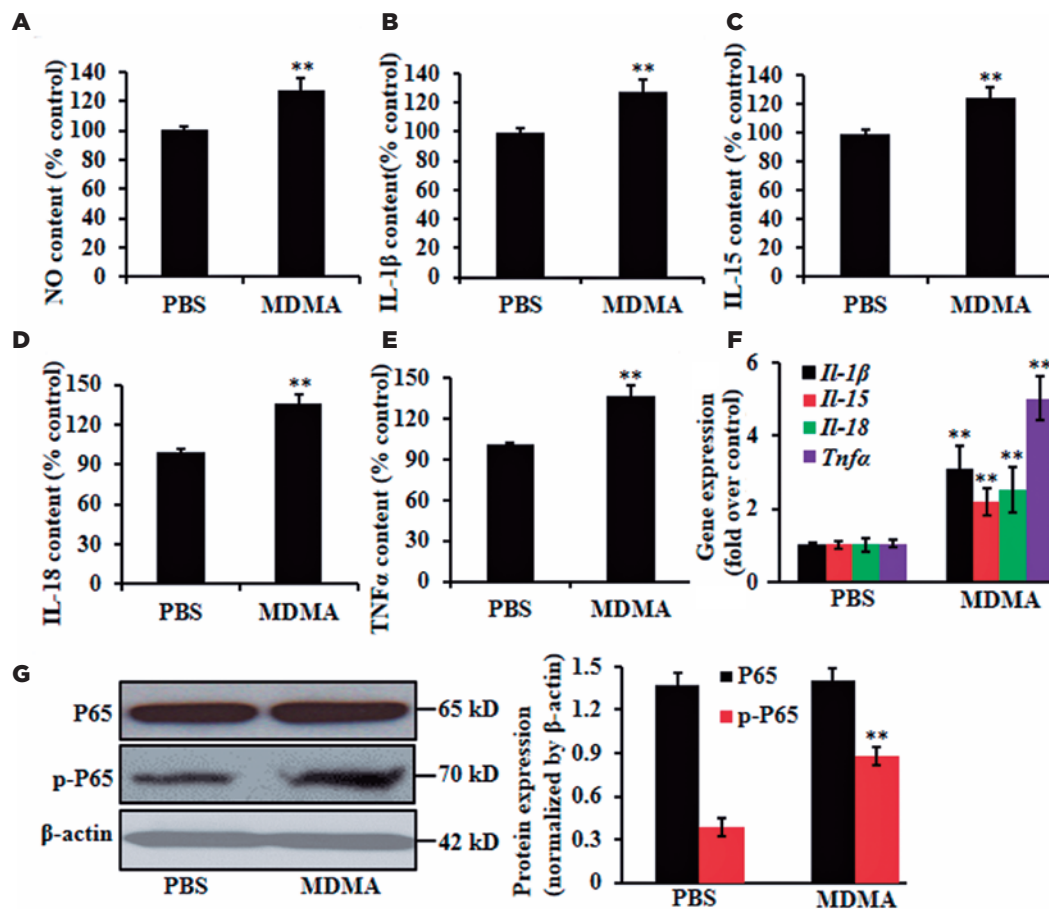Data are expressed as mean ± SD. **p<0.01 vs. PBS group.

Figure 3. Three-month treatment with MDMA increased oxidative stress in C57BL/6 mouse retinas. Retinas were harvested to measure the oxidative stress: SOD activity, GSH level and MDA content (A); gene expression of *Sod* and *Catalase* (B) and oxidative stress-related protein expression (C).

elucidation of these non-neuronal mediators of toxicity could reveal novel targets for the treatment of the complex neurotoxicity produced by MDMA[19]. There is far less known about the effects of MDMA abuse on retinas and even little is known about the MDMA caused chronic toxicity on retinas[20,21]. The purpose of this investigation was to assess the effects of long-term treatment with MDMA on mouse retinas and its underlying mechanisms resulting in dysfunction.

MDMA induced toxicity involves an excessive release of dopamine, which initiates the production of oxygen-based free radicals to induce apoptosis[22]. Repeated treatment with MDMA in rats resulted in an imbalance of oxidative/anti-oxidative system in retinas and the compromised antioxidant defense impaired the normal function of the visual system[4]. Oxidative stress affects a variety of biological macromolecules, and is considered to be a fundamental mechanism by which MDMA exerts its toxic

effects[23,24]. An excessive increase of free radicals can cause damage to the lipids, a process known as lipid peroxidation (LPO). LPO significantly alters the physiological properties of membrane lipid bilayers, causing serious and irreversible cellular dysfunctions[25,26].

Moreover, it is well-established that a variety of secondary products (such as malondialdehyde, hexanal, and 4-hydroxynonenal) result from LPO, some of which can also exert adverse biological effects[27]. Another production of free radicals in the retina results from the activity of NADPH oxidases, which generates superoxide extracellularly as a defense against bacteria, and contributes to angiogenesis in the retinal pigment epithelium and phagocytosis[28-30]. SOD and GSH are intracellular antioxidants alleviating oxidative damage caused by ROS. Repeated treatment with MDMA caused a progressive increase of hydroxyl radicals in the brain of mice, which is significantly greater than that observed

Data are expressed as mean ± SD. **$p<0.01$ vs. PBS group.
Figure 4. Three-month treatment with MDMA induced inflammatory response in retinas of C57BL/6 mice. Pro-inflammatory factors were measured by: NO (A); IL-1β (B); IL-15 (**cC**; IL-18 (D); TNFα (E). MDMA treatment increased gene expression of pro-inflammatory factors (F) and the protein expression of p-P65 (G).

following a single dose of MDMA[31]. However, rats were treated with MDMA at 5 mg/kg for one week[4], which was far from the real situation of chronic toxicity. In this study, mice were treated with MDMA up for three months to explore the potential mechanisms. The results indicated the decrease of SOD activity and GSH depletion, together with markedly increase of MDA and protein expression of NADPH oxidases in retinas of mice.

Moreover, results from the present investigation demonstrate that apoptotic cells in retinas significantly increased after treatment with MDMA. While it is well known that ecstasy causes apoptosis in the liver and brain[32] these results show that apoptosis is induced in the retinas after treatment with MDMA for three months. Additionally, treatment with MDMA also increased the release of cytochrome-c, and activities of caspase-3/caspase-9. Expression of anti-apoptotic protein decreased, and pro-apoptotic protein expression increased.

Inflammatory cytokines are important mediators involved in the progression of a variety of systemic diseases[33]. These cytokines stimulate oxidant production with subsequent peroxidative damage to macromolecules, resulting in intracellular toxic events by increasing the mitochondrial membrane permeability, cytochrome-c release, activating caspase-related apoptotic proteins and finally leading to cell death[34]. NO possesses cytotoxic properties and exerts damaging effects on host tissues as a mediator of inflammatory responses. In addition, NO is a potent neurotransmitter at the neuron synapses and contributes to the regulation of apoptosis[35,36]. NF-κB, a transcription factor involved in many inflammatory and immune pathways, has been considered as a prototypical pro-inflammatory factor[37]. In the present investigation, MDMA treatment upregulated gene expression of IL-1β, IL-15, IL-18, and TNFα, and increased their levels in the retinas through NF-κB. These results are consistent with an *in vitro* study in which MDMA caused photoreceptor cell damage through inducing an inflammatory response[15].

To summarize, long-term treatment with MDMA caused retina damage in C57BL/6 mice via the promotion of an inflammatory response and increase in oxidative stress. Chemical intervention, such as antioxidant, could eliminate ROS. Therefore, reducing the chronic inflammatory response and inhibiting oxidative stress may be an effective strategy to prevent the pathological progression caused by the MDMA abuse.

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