Effects of Co-exposure to Lead and Manganese on Learning and Memory Deficits

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Abstract

Background: Lead and manganese are common neurotoxins. However, individuals are subject to co-exposures in real life, and it is therefore important to study these metals in combination.

Methods: Weaning Sprague-Dawley rats were given ad libitum access to drinking water solutions containing lead (100 ppm), manganese (2.5 mg/mL) or a mixture, and each treatment has its own minocycline (50 mg/Kg.d) supplement group.

Results: The results showed a significant difference in spatial memory and the induction levels of hippocampal long-term potentiation (LTP) in all exposure groups when compared with controls. The combined exposure group exhibited the most pronounced effect when compared with each of the single metal exposure groups. Microglia displayed activation at day 3 after exposure alone or in combination, while astrocytes showed activation at day 5, accompanied by decreased expression levels of glutamate/aspartate transporter (GLAST), glutamate transporter-1 (GLT-1), and glutamine synthetase (GS). Furthermore, the levels of the glutamate in the synaptic cleft increased significantly. When microglial activation was inhibited by minocycline, the activation of astrocytes, and the expression of GLAST, GLT-1, and GS were both reversed. In addition, upon minocycline treatment, the hippocampal LTP impairment and the cognitive injury were significantly alleviated in each of the exposure groups.

Conclusions: These results suggest that combined exposure to lead and manganese can cause greater effects on cognition and synaptic plasticity when compared to single metal exposure groups. And the reason may involve in microglia abnormal activation leading to an excessive regulation of astrocytes, resulting in glutamate reuptake dysfunction in astrocytes and lead to perturbed cognition and synaptic plasticity.

Introduction

Human exposure to neurotoxic metals is a global public health problem [1]. Metals such as lead (Pb) and manganese (Mn) are of particular concern due to the long-lasting and possibly irreversible nature of their effects [2, 3]. Exposure to these neurotoxic metals can occur via contaminated air, food, water, or in hazardous occupations. While in the developed world, in large measure, exposures to neurotoxic metals have decreased in recent decades, the developing world still experiences high levels of metal pollution and exposure [4, 5]. The source of this contamination is largely derived from anthropogenic sources, such as the combustion of leaded gasoline [6, 7], unregulated industrial emissions [8, 9], mining [10], metal smelting [11] and unregulated electronic waste recycling [12, 13].

Lead exposure in the developing period may result in cognitive deficits in children [14]. These effects are long-lasting and persist into adulthood even after Pb exposure has been reduced or eliminated altogether [15]. It is believed that Pb targets learning and memory processes by several means. For example, lead inhibits the expression and ontogeny of *N*-methyl-D-aspartate receptor (NMDAR) subunit [16], impairs neurotransmission [17, 18] in hippocampus and consequently disrupts synaptic development [19],

excitotoxicity [20, 21] and synaptic plasticity [22], eventually inducing hippocampal learning and memory impairment.

Unlike Lead, Manganese is an essential element of the human diet and it serves many cellular functions. Nonetheless, elevated Mn levels can also result in cognitive deficits as well as synaptic plasticity impairment [23]. In addition, elevated intracellular manganese concentrations result in its accumulation in mitochondria, where it disrupts adenosine triphosphate (ATP) synthesis by inhibiting the F1/F0 ATP synthase or complex 1 (NADH dehydrogenase) of the mitochondrial respiration chain [24, 25]. Reduction in intracellular ATP levels has been shown to alter glutamate recycling, resulting in neuronal excitotoxicity [1].

Optimal glutamate removal in synaptic cleft is considered to play a critical role in synaptic transmission. Astrocytes function to maintain neuronal homeostasis in the brain, occupying strategically important positions around the synapse [26], and express glutamate transporters and glutamine synthetase (GS) activity. The concentration of glutamate in the synaptic cleft is influenced by the rate of its release and diffusion and regulated predominantly by glutamate transporters. Extracellularly released glutamate is rapidly taken up into astrocytes. Five distinct high-affinity glutamate transporters have been cloned [27]. Two of them, glutamate/aspartate transporters GLAST/EAAT1 and GLT-1/EAAT2 (rat/human, respectively) are astrocytic and play a crucial role in glutamate clearance in the adult brain [28].

In addition, several studies have confirmed that microglia is more sensitive to cerebral microenvironment changes *vs*. the astrocyte. Furthermore, microglia can influence the functions of reactive astrocytes, ranging from neuroprotection to neurotoxicity [29].

Taken together, this study was designed to evaluate whether lead and manganese co-exposures affect glutamate-glutamine cycle-related proteins expression by interfering with glutamate uptake and catabolism, and whether these effects are due to the activation of microglia and their abnormal regulation of astrocytes.

Materials And Methods

Materials

Analytical pure lead acetate and manganese chloride were purchased from SIGMA (USA). Anti-OX42, Anti-GFAP, Anti-GLAST, Anti-GLT-1 and Anti-GS antibodies were purchased from Millipore (Millipore, USA). Minocycline (MC) was obtained from Sigma (USA). All other reagents were purchased from Sigma (USA). The inverted microscope and the fluorescence microscope were purchased from Olympus (Tokyo, Japan). The Patch clamp system was bought from Axon (USA). The enzyme linked immunosorbent spectrophotometer was obtained from Shimadzu (Nakagyo-ku, Kyoto, Japan).

Animals and treatments

All procedures involving animals were carried out in strict accordance with the international standards of animal care guidelines and were approved by the local Care of Experimental Animals Committee. Male Sprague-Dawley rats (SD rats) were obtained from the Animal Experiment Center of the Fourth Military Medical University. The animals were maintained in a 12/12 light/dark cycle and a temperature-controlled room, with food and water available ad libitum. The rats were assigned to four or eight groups (20 in each group): control group, Pb treated group, Mn treated group, Pb and Mn treated group, minocycline treated group, Pb and minocycline treated group, Mn and minocycline treated group, Pb and Mn and minocycline treated group. The animals were fed with lab chow pellets (obtained from the Animal Experiment Center of Fourth Military Medical University). On the third day after arrival (aged 24 days), the animals were exposed to Pb and/or Mn *via* drinking water. Lead acetate and/or manganese chloride (SIGMA, USA) were dissolved in distilled water (Pb: 0 or 100 ppm, Mn: 0 or 2.5 mg/ml). Pb and Mn concentrations in distilled water and blood lead and manganese level were verified using electrothermal atomization atomic absorption spectroscopy (AAS). Rats were exposed to Pb and Mn from 24 to 80 days of age. Minocycline was also supplemented via drinking water (50 mg/Kg.d). The approximate minocycline consumption was calculated by calculating the water consumption of each rat in each group. Water consumption was monitored every two days and individual body weight was measured weekly during the experimental period. A food restriction schedule was established one week before the beginning of the appetitive Y-maze task. The animals were weighed throughout the experiment to ensure that they did not lose more than 15% of their initial weight [30].

Determination of Pb and Mn concentrations in blood

At the end of the study, six animals from each group were decapitated. Blood samples were collected into heparinized syringes and used to determine blood Pb using AAS.

Morris water maze test

Morris water maze (MWM) studies were performed the day after the 8-week exposure period to assess spatial learning. The MWM consisted of a circular water tank (120 cm diameter, 50 cm height) that was partially filled with water (25 °C, dyed white with edible pigment). The pool was divided into four equal quadrants labeled 1-2-3-4 in the middle position of each pool wall. A white escape platform (10 cm in diameter) was hidden 2 cm below the surface of the water in a fixed location in one of the four quadrants (defined as the target quadrant) of the pool. The platform remained in the same place during the entire experiment. The maze was surrounded with a curtain and located in a quiet test room, surrounded by fixed visual cues (e.g. the label on each quadrant's pool wall, rack, etc.), which were visible from within the pool and could be used by the rats for spatial orientation. The movement of the animals was recorded by a TV camera located over the center of the pool and was connected to a personal computer. Before the training started, rats were allowed to swim freely in the pool for 60 s without the platform and were then put on the platform for 30 s in order to let them be familiar with the experimental condition. Rats were given four trials (once from each starting position) per session for 4 d, with each trial having a ceiling of 60 s and a trial interval of approximately 30 s. After climbing onto the platform, the animal remained there for 30 s before the commencement of the next trial. The recording was automatically terminated as

escape latency when the animal found the target. The time required to reach the platform is defined as the escape latency. When a tested rat could not escape to the platform within 60 s, it was placed on the platform and allowed to remain there for the same amount of time and their escape latency was recorded as 60 s. The mean latency of finding the invisible platform was measured for individual animals on each day. The day after the acquisition phase, a probe test was conducted by removing the platform. Rats were allowed to swim freely in the pool for 60 s. The time spent in the target quadrant, which had previously contained the hidden platform, was recorded. Results were calculated as the percent of time spent in the target quadrant.

Y maze test

The spatial learning and memory were also tested with a Y maze [31]. The maze has three arms of equal size (50 cm long, 15 cm wide and 25 cm height) and was located in a laboratory with prominent distal extra-maze cues, and was elevated 80 cm above the ground. The movement of the animals was recorded by a TV camera located over the center of the maze and was connected to a personal computer. The arm where the rats were placed at the beginning of each trial was considered the start arm. The other arms, had food cups located at the ends and they were considered the choice arms. Peanuts were used as food pellets and were put in one cup. Another cup was painted with peanut oil to ensure both cups have a similar odor. The experiment consisted of placing the rats at the start arm, permitting them to move to the correct arm holding the peanuts. Pre-training was carried out for 10 min to familiarize the rats with the maze. To assess the cognitive behavior, each rat had 3 trials (10 min, 24 h and 48 h after pre-training) and each trial was repeated 10 times. In each trial, rats were allowed to explore the maze for 2 min. A correct trial was designated when the rat's hind legs crossed the boundary line between the central district and the correct arms. If the rat made an incorrect response, it was allowed to move to the empty food cup at the end of the incorrect arm and was removed after 5 s. From the day prior to the experiment and throughout the whole experimental period, the rats were maintained in the experimental room.

Long-term potentiation recording in hippocampal slices

1. Slice preparation

Coronal brain slices (300 μm) from 6 to 8 week-old SD rats, containing the hippocampus, were prepared using standard methods [32]. Slices were transferred to a submerged recovery chamber containing oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 4.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 10 mM glucose) at room temperature for at least 1 h.

2. Whole-cell recordings

Experiments were performed in a recording chamber on the stage of an Axioskop 2 FS microscope with infrared differential interference contrast (DIC) optics for visualizing whole-cell patch-clamp recordings. Excitatory postsynaptic currents (EPSCs) were recorded from pyramidal neurons in the CA1 region using an Axon 200B amplifier (Axon Instruments, CA, USA), and stimulations were delivered using a bipolar

tungsten stimulating electrode which was placed on Schaffer collateral-commissural fibers in the CA3 stratum radiatum. Alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) receptor-mediated EPSCs were induced by repetitive stimulations at 0.02 Hz and neurons were voltage clamped at -70 mV. After obtaining stable EPSCs for at least 10 min, LTP was induced by 80 pulses at 2 Hz paired with postsynaptic depolarization at + 30 mV (we called pairing training). The recording pipettes (3–5 MΩ) were filled with solution containing (mM) 145 K-gluconate, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, and 0.1 Na_3-GTP (adjusted to pH 7.2 with KOH). Picrotoxin (100 μM) was always present to block gamma-aminobutyric acid (GABA) A receptor-mediated inhibitory synaptic currents. Access resistance was 15–30 MΩ and monitored throughout the experiment. Data were discarded if access resistance changed more than 15% during an experiment. Results are expressed as means ± SEM. Statistical comparisons were performed using the Student's t test.

Immunocytochemistry

Hippocampal microglial and astrocyte cells were detected with anti-OX42 and anti-GFAP antibody, respectively. Briefly, slices form cryopreserved tissues were followed by blocking for 1 h with phosphate buffered saline (PBS) containing 0.4% Triton X-100, 2% bovine serum albumin (BSA) and 3% normal goat serum. After blocking, slices were incubated with primary antibody overnight at 4 °C. Slices were then washed with PBS and incubated for 1 h with the secondary antibodies (antimouse-Rhodamine, Jackson ImmunoResearch, West Grove, PA, USA) at room temperature and next rinsed with PBS buffer. The number of activated microglia was determined by counting the number of OX42-immunoreactive cells in DG zone of hippocampus. Briefly, each image results from one group were divided equally into 16 lattices, and then only calculate the activated cell number (based on morphological characteristics of the cell). The total number of microglial cells was detected and calculated by Image pro plus (IPP) software (only OX42 and Hoechst double-labeling cells were calculated). The number of activated astrocytes was determined by counting the number of GFAP-immunoreactive cells in DG zone of hippocampus as well. Cells were examined and recorded blindly under an Olympus BX51 fluorescent microscope equipped with DP-BSW software (Olympus, Japan).

Western blot analysis

Protein samples from the hippocampus of the different groups were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by immersing the membranes in 5% bovine serum albumin in PBS at room temperature, followed by incubation with primary antibodies. Subsequently, the membranes were incubated with appropriate secondary antibodies (Santa Cruz, USA). All secondary antibodies were horseradish peroxidase conjugated. ECL Western Blotting Substrate (Pierce, USA) was used to detect the immunoreactive signals with an ECL-based Fluorchem® FC2 image system (Alpha Innotech, USA). Primary antibodies included GLAST, GLT-1 and GS antibody (Millipore, USA). All western blot analyses were performed in triplicate. The FluorChem FC2 software was used to analyze the gray value of the protein expression in each group.

Glutamate assay in the cerebrospinal fluid

The cerebrospinal fluid (CSF) was collected on day 81 of age, for glutamate measure according to Almeida et al., 2017 [33]. Briefly, rats were anesthetized with inhaled isoflurane 3% (May & Baker, Dagenham, Essex, UK) in oxygen and positioned for fixation in the stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). The liquor (100–150 μL) was collected by direct puncture of the cisterna magna with a syringe (27 gauge × 0.5 in. in length). All samples were centrifuged at 10,000 g for 4 min in a tube to obtain clear supernatants. After centrifugation, the samples were immediately frozen in liquid nitrogen and stored at − 80 °C until analysis. Glutamate concentration was determined in the CSF by high-performance liquid chromatography (HPLC) [34]. Samples were analyzed in a Shimadzu Instruments liquid chromatography (50 μl loop valve injection, injection volume: 40 μl) with a reverse phase column (Supelcosil LC-18, 250 mm × 4.6 mm × 5 μm, Supelco) and fluorescent detection after pre-column derivatization with 100 μl o-phtalaldehyde (OPA) (5.4 mg OPA in 1 ml 0.2 M sodium borate pH 9.5) plus 25.5 μl 4% mercaptoethanol. The mobile phase flowed at a rate of 1.4 ml/min and column temperature was 24 °C. Buffer composition was A: 0.04 mol/l sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 80% of methanol; B: 0.01 mol/l sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 20% of methanol. The gradient profile was modified according to the content of buffer B in the mobile phase: 100% at 0.10 min, 90% at 15 min, 48% at 10 min, 100% at 60 min. Absorbance was read at 360 nm and 455 nm (excitation and emission, respectively). The analysis was performed on samples of 20 μl and concentration was expressed in micromoles. A known glutamate standard samples were used for calibration. Glutamate was identified by its retention time and quantified by its chromatographic peak area.

Statistical analysis

Data were analyzed using SPSS 16. The results were presented as the mean ± SD, and for all of the statistical analyses, a value of $P < 0.05$ was considered significant for n = 6–20, where n corresponds to the number of samples. The MWM data (escape latency) were analyzed by a repeated measure analysis of various (ANOVA) with “group” as the between-subjects variable (four levels: Con, Pb, Mn, and Pb + Mn. Or eight levels: Con, Pb, Mn, Pb + Mn, Con + minocycline, Pb + minocycline, Mn + minocycline, and Pb + Mn + minocycline) and “day” as the within subjects variable (seven levels: 1, 2, 3, 4, 5, 6, 7 days) with a general liner model. The other data were analyzed with LSD t-test unless otherwise specified.

Results

Blood lead and manganese level, body weight following exposure

Exposure to Pb (100 ppm) in drinking water resulted in a 4.82-fold increase in blood Pb (BPb) compared to control rats ($P < 0.05$) (Fig. S1B) [35, 36]. Exposure to Mn in drinking water under the current dosing regimen (2.5 mg/ml) resulted in a 1.76-fold increase in blood Mn (BMn) compared to control rats ($P < 0.05$) (Fig. S1C) [37]. Co-exposure to Pb and Mn in drinking water resulted in a 4.25-fold ($P < 0.05$ *vs.* Con. Fig. S1B) and 2.35-fold ($P < 0.05$ *vs.* Con. Fig. S1C) increase in BPb and BMn, respectively, compared to

control rats. There were no significant differences between the four groups ($P > 0.05$) in body weights (Fig. S1A).

Effect of Pb and Mn exposure on spatial memory

We compared the effects of single metal and combined exposure on spatial learning and memory in the Morris water maze (Con: n = 9, Pb: n = 8, Mn: n = 9, Pb + Mn: n = 9). Although the latencies to reach the submerged platform decreased gradually both in the control group and exposure groups during the 7 days of training, the mean latency was significantly prolonged in the single metal and combined exposure groups compared to the control group (Fig. 1A) ($P < 0.05$ Pb, Mn and Pb + Mn *vs.* Con). Compared to the single metal exposure groups, combined exposures caused a significantly prolonged mean latency to reach the submerged platform ($P < 0.05$ Pb + Mn *vs.* Con, Pb and Mn).

On the probe trial, with the platform removed, exposure to Pb and/or Mn also impaired memory retrieval as indicated by a reduction in time spent in the target quadrant (Control, 57.72 ± 4.99%; Pb, 43.69 ± 5.19%; Mn, 42.73 ± 2.44%; Pb + Mn, 35.26 ± 5.21%) where the hidden platform was placed during the training session (Fig. 1B) ($P < 0.05$ Pb, Mn and Pb + Mn *vs.* Con). In addition, co-exposure to Pb and Mn led to a significant decrease in the amount of time spent in the target quadrant compared to single metal exposure groups (Fig. 1B) ($P < 0.05$ Pb + Mn *vs.* Pb, $P < 0.05$ Pb + Mn *vs.* Mn). For the swimming speed, no significant differences were noted between the four experimental groups ($P > 0.05$, Fig. 1C).

Single metal and combined exposures caused significant reduction in performance in the Y maze (Fig. 1D), both at the early and late test points ($P < 0.01$ Pb, Mn, Pb + Mn *vs.* Con. 30 min, 24 h and 48 h; Fig. 1D). Furthermore, co-exposure to Pb and Mn resulted in a significantly decreased ratio of correct responses in the Y-maze compared to the single metal exposure groups ($P < 0.05$ Pb + Mn *vs.* Pb, $P < 0.05$ Pb + Mn *vs.* Mn. 30 min, 24 h and 48 h).

Effects of Pb and Mn exposure on hippocampal long-term potentiation in rats

LTP represents an important mechanism underlying hippocampal learning and memory [38–40]. Thus, it's important to investigate the effect of single metal and combined exposures on hippocampal LTP. We used the traditional LTP induction paradigm to trigger LTP in hippocampal slices [41]. The results showed that pairing training induced a significant LTP of synaptic responses in slices from control rats (163.19 ± 10.92% of baseline, n = 10 slices/6 rats, t-test; $P < 0.01$ compared with baseline responses, Fig. 2A). In contrast, synaptic potentiation was attenuated in slices from Pb- and Mn-exposed rats (Pb: 114.35 ± 13.24%, n = 11 slices/6 rats, t-test; $P < 0.05$ compared with baseline, $P < 0.05$ *vs.* control group, Fig. 2B. Mn: 131.66 ± 9.45%, n = 10 slices/6 rats, t-test; $P < 0.05$ compared with baseline, $P < 0.05$ *vs.* control group, Fig. 2C). Furthermore, co-exposure to both Pb and Mn resulted in more pronounced attenuation of synaptic potentiation compared to each of the single metal exposure groups (Pb + Mn: 105.59 ± 15.24%, n = 12 slices/6 rats, t-test; $P < 0.05$ compared with baseline, $P < 0.05$ *vs.* Pb group, $P < 0.05$ *vs.* Mn group, Fig. 2D).

Effects of Pb and Mn single and combined exposure on microglia and astrocyte activation in rats

Our previous studies have demonstrated that exposure to lead and manganese alone can cause microglial activation. To investigate whether co-exposure to lead and manganese could induce a significantly higher activation ratio, we assessed the number of activated microglia in hippocampal slices. Results showed that on day 3 of exposure, microglia in slices derived from control rats were predominantly in the resting state (Fig. 3A Con), while microglia in the exposed rats were predominantly in an activated state ($P < 0.05$ Pb, Mn and Pb + Mn *vs.* Con. Figure 3A Pb, Mn and Pb + Mn). Furthermore, the combined exposure group exhibited the most pronounced activation ratio when compared with each of the single metal exposure groups ($P < 0.05$ Pb + Mn *vs.* Pb, $P < 0.05$ Pb + Mn *vs.* Mn. Figure 3).

Since existing evidence suggests that both lead and manganese exposure may contribute to neurotransmitter dysfunction, we also examined the activity of astrocytes. Unlike microglia, no activation of astrocytes was detected on day 3 after exposures (Fig. 3B) ($P > 0.05$ Pb, Mn and Pb + Mn *vs.* control group). Nonetheless, we detected astrocytic activation on day 5 after exposure. The results corroborated that in the exposure groups astrocytes expressed significantly higher levels of GFAP ($P < 0.05$ Pb, Mn and Pb + Mn *vs.* control. Figure 3D) *vs.* controls. Notably, the combined exposure group exhibited significantly higher GFPA expression level compared to each of the single metal exposure groups ($P < 0.05$ Pb + Mn *vs.* Pb, $P < 0.05$ Pb + Mn *vs.* Mn. Figure 3D).

Effects of Pb and Mn exposure on glutamate-glutamine cycling-related protein expression

The metabolism of the excitatory neurotransmitter glutamate in the brain is primarily performed in astrocytes. The proteins involved in glutamate-glutamine cycling in astrocytes include glutamate transporter GLAST, GLT-1 and GS. Our results showed that after exposure, the expression of GLAST, GLT-1 and GS were decreased significantly in each of the exposure groups compared with the control group ($P < 0.05$) (Fig. 4A, B). Furthermore, the decreased levels were most pronounced and significant in the combined exposure group ($P < 0.05$ Pb + Mn *vs.* Pb, $P < 0.05$ Pb + Mn *vs.* Mn. Figure 4A, B).

Effects of Pb and Mn single or combined exposure on glutamate concentration

We compared the effects of Pb and Mn exposure on glutamate concentration in cerebrospinal fluid (Con: n = 6, exposure groups: n = 6). The result shows that the glutamate levels in exposure group were significantly higher than in controls ($P < 0.05$ Pb, Mn and Pb + Mn *vs.* control. Figure 4D). Furthermore, glutamate levels in CSF were significantly higher in the combined exposure group than in the single metal exposure groups ($P < 0.05$ Pb + Mn *vs.* Pb, $P < 0.05$ Pb + Mn *vs.* Mn. Figure 4D).

Minocycline treatment alleviates Pb and Mn exposure-induced hippocampal microglia activation

Minocycline, a tetracycline antibiotic, has anti-inflammatory properties and is used experimentally in the treatment of several central nervous system (CNS) disorders. Minocycline has been shown to inhibit microglial activation and to protect the CNS from inflammatory conditions [42–44]. In order to establish whether the activation of microglia triggered a series of reactions as mentioned above, rats were treated with minocycline (50 mg/kg per day in dd water; Sigma, USA) via drinking water during the exposure. Minocycline strongly inhibited microglial activation ($P < 0.05$ Pb + MC *vs.* Pb, $P < 0.05$ Mn + MC *vs.* Mn, $P < 0.05$ Pb + Mn + MC *vs.* Pb + Mn. Figure 5A). The results also showed no significant differences in microglial activation between control and minocycline treated offspring ($P > 0.05$ Con + MC *vs.* Con. Figure 5A).

Inhibition of microglia activation reversed exposure induced astrocyte activation, glutamate-glutamine cycling-related protein expression and glutamate concentration

After minocycline inhibited microglial activation, we also detected the activation of astrocyte and its glutamate-glutamine cycling-related protein expression. The results showed that the activation of astrocyte were also significantly inhibited ($P < 0.05$ Pb + MC *vs.* Pb, $P < 0.05$ Mn + MC *vs.* Mn, $P < 0.05$ Pb + Mn + MC *vs.* Pb + Mn. Figure 5B), meanwhile, enhanced the GLAST, GLT-1 and GS expression($P < 0.05$) (Fig. 6A-D). The results also showed that the accumulation of glutamate in synaptic cleft was also significantly alleviated ($P < 0.05$) (Fig. 6E, F).

Minocycline treatment alleviates exposure induced impairments of spatial memory and hippocampal long-term potentiation

We confirmed that minocycline decreased the exposure induced hippocampal microglial activation. In turn, it also alleviates the activation of astrocytes and restored its glutamate-glutamine cycling-related protein expression. However, does this lead to the prevention of spatial memory impairment?

The effects of minocycline on spatial learning and memory were tested in the Morris water maze. The results showed that minocycline had no effect in control rats (Escape latency: $P > 0.05$ Con *vs*. Con + MC). The mean latency was significantly shortened in the exposure plus minocycline group relative to the exposure group ($P < 0.05$ Pb + MC *vs*. Pb, $P < 0.05$ Mn + MC *vs*. Mn, $P < 0.05$ Pb + Mn + MC *vs*. Pb + Mn. Figure 7A-E). However, the recovery level in the combined exposure plus minocycline treatment group was significantly attenuated *vs*. the single metal exposure plus minocycline group ($P < 0.05$ Pb + Mn + MC *vs*. Pb + MC, $P < 0.05$ Pb + Mn + MC *vs*. Mn + MC. Figure 7E).

On the probe trial, with the platform removed, minocycline also alleviated the impairment in memory retrieval as indicated by greater percent of time which was spent in the target quadrant where the hidden platform was placed during the training session ($P < 0.05$ Pb + MC *vs*. Pb, $P < 0.05$ Mn + MC *vs*. Mn, $P < 0.05$ Pb + Mn + MC *vs*. Pb + Mn. Figure 7F). Similarly, the percentage of time spent in the target quadrant was not as long as in the combined exposure plus minocycline group compared with single metal

exposure plus minocycline group ($P < 0.05$ Pb + Mn + MC *vs.* Pb + MC, $P < 0.05$ Pb + Mn + MC *vs.* Mn + MC. Figure 7F). For the swimming speed, no significant differences were found between each group ($P > 0.05$, Fig. 7G).

After confirming that minocycline alleviates lead and manganese single and co-exposure induced spatial memory impairment, we determined if this effect was paralleled by rescued hippocampal LTP. The study on hippocampal LTP showed that the induction level of LTP were partly but significantly restored in exposure plus minocycline group (Pb + MC: 155.58 ± 17.74%, n = 10 slices/6 rats, t-test; $P < 0.05$ *vs.* baseline. Mn + MC: 145.54 ± 8.93%, n = 11 slices/6 rats, t-test; $P < 0.05$ *vs.* baseline. Pb + Mn + MC: 142.19 ± 11.42%, n = 9 slices/6 rats, t-test; $P < 0.05$ *vs.* baseline. Figure 8B-D) relative to the exposure group (Pb: 104.16 ± 17.99%, n = 10 slices/6 rats, t-test; $P < 0.05$ compared with baseline, $P < 0.05$ compared with Pb + MC group. Mn: 122.59 ± 8.24%, n = 10 slices/6 rats, t-test; $P < 0.05$ compared with baseline, $P < 0.05$ compared with Mn + MC group. Pb + Mn: 108.63 ± 10.79%, n = 11 slices/6 rats, t-test; $P < 0.05$ compared with baseline, $P < 0.05$ compared with Pb + Mn + MC group. Figure 8B-D). The results also showed that there was no significant difference between control rats (145.54 ± 14.84%, n = 10 slices/6 rats, t-test; $P < 0.05$ compared with baseline, Fig. 8A) and minocycline treated rats (146.59 ± 13.90%, n = 11 slices/6 rats, t-test; $P < 0.05$ compared with baseline, $P > 0.05$ *vs.* control group, Fig. 8A). This suggested that minocycline may partly prevent lead and manganese single and co-exposure induced LTP impairment.

Discussion

This study evaluated the effects of Pb and Mn alone or their co-exposure on cognition and synaptic plasticity. Metal exposure over a period of 8-weeks induced spatial memory and LTP impairment in rats. Furthermore, cognition and synaptic plasticity impairment induced by co-exposures was more pronounced than in each of the single metal exposure groups. Furthermore, this study shows for the first time that co-exposure to Pb and Mn caused microglial activation and this activation likely was the reason for astrocytic activation and its glutamate-glutamine cycle related proteins aberrant expression. These interferences may culminate in altered glutamine-glutamine cycling and ensuing cognitive impairment. In support of these findings, we corroborated these changes in glutamine-glutamine cycling as well as learning and memory impairment could be reversed by microglial activation specific inhibitors.

Heavy metal pollution remains a global public health problem. Metals, such as Pb and Mn are of particular concern due to potential to inflict irreversible neurotoxic effects. Both Pb and Mn can cause cognition deficits [45, 46], each exhibiting distinct modes of action, nevertheless, both disrupting synaptic transmission [47–50]. Since the banning of leaded gasoline in China (July 1, 2000), a general decrease in blood Pb concentrations in the Chinese population had been noted. Mean Pb blood levels have decreased from 92.9 μg/L in 2004 to 34.9 μg/L in 2014 in urban environments [51]. However, there remains a significant problem with metal contamination from mining in developing countries, which results in elevated metal levels in water and air [52, 53]. Furthermore, electronic waste recycling has gradually become a major source of metal contamination in developing countries. Electronic waste, such as used or broken computers, mobile phones and other electronic devices, exported from developed countries for

disposal in developing countries, where few regulations are in place regarding safe disposal [54]. Unfortunately, unsafe methods are used for the extraction of the precious metals, resulting in contamination of the local environment of highly toxic metals level [12, 53]. Accordingly, it is prudent to investigate whether co-exposure to both Mn and Pb may aggravate their individual effects on cognition.

To date, only a few studies have focused on the combined effects of Pb and Mn. One epidemiological study [55] has shown that adolescents (11–14-year-old) with average BPb levels of 1.71 μg/dL, show a reduction of about 2.4 IQ points with a two-fold increase in BPb. Mn exposure (average blood manganese, 11.1 μg/L) has not associated with cognitive and behavioral outcomes, nor was there any interaction with Pb. These findings demonstrate that low levels of Pb exposure have a significant negative impact on cognitive function in adolescent children. Mn may not cause cognitive effects at these low exposure levels. Another epidemiological study [56] showed that in adolescents (8–11-years-old) with average BPb and BMn level of 1.73 μg/dL and 14.3 μg/L, respectively, both Pb and Mn showed significant linear relationship with full-scale IQ and verbal IQ. Full-scale IQ and verbal IQ of the children with blood Mn > 14 μg /L showed significant association with Pb.

The effects of co-exposures to Pb and Mn have been rarely addressed in animal models. One earlier study [57] established an adult rats co-exposure model with manganese chloride (3 mg Mn^{2+}/ml water) through drinking water in conjunction with intraperitoneal lead acetate injection (5.0, 8.0 and 12.0 mg Pb^{2+}/kg daily) for a period of 14 days. Results from this study suggest that combined exposure to Mn and Pb is associated with changes in animal behavioral patterns and levels of biogenic amines in the brain. However, it must be acknowledged that the intraperitoneal exposure route fails to recapitulate real-life human exposures to metals. As inhalation of polluted air and ingestion of contaminated water or food are the main routes of exposure to Pb and Mn, our study recapitulates real-life exposure scenarios.

The present research aimed to focus on cognition disturbances in infants and young children during critical age windows for neurodevelopment. Accordingly, we chose an exposure period from weaning to postnatal day (PND) 80. In a related study [37], pregnant rats were dosed *ad libitum* with drinking water solutions containing Pb (10 μg/mL), Mn (2 mg/mL) or a mixture of the two, from the beginning of gestation, through lactation and weaning. On PND 24, mixture-exposed male pups exhibited a better performance in learning and memory, assessed with the MWM, compared to animals with single metal exposures. This effect was absent between PNDs 56 and 60. Meanwhile our results suggest that the performance in learning and memory were significantly reduced in the single metal and combined exposure groups compared to the control group after exposure for 8 weeks (PND 80). While the previous studies have suggested that exposure to neurotoxic metal such as Pb and Mn during neurodevelopmental period may cause an irreversible cognitive impairment [56]. Our data are consistent with this thesis.

Both Pb and Mn alter cognition by affecting neurotransmission [17, 18]. In turn, impairment in neurotransmission may affect synaptic plasticity [19] in the hippocampus, and induce learning and memory deficit. Pb effects on hippocampal synaptic plasticity have been widely addressed [36]. However,

the effect of Mn on hippocampal synaptic plasticity injury has yet to be reported. On the other hand, the co-exposure of Pb and Mn on synaptic plasticity has not been previously addressed. Accordingly, we evaluated the single metal and combined exposure to Pb and Mn on hippocampal synaptic plasticity impairment. Our data suggest that not only lead but also manganese may reduce the hippocampal long-term potentiation induction level. Moreover, co-exposure to lead and manganese caused more pronounced inhibitory effects on the hippocampal long-term potentiation. These findings are novel and its underlying mechanisms need further study.

Since abnormal levels of LTP induction were detected, suggesting impaired neurotransmission, and given previous literature reports [58], we determined the levels of the excitatory neurotransmitter glutamate in the hippocampus. The result shows that the level of glutamate was abnormally high after single or combined exposure to lead and manganese. Accordingly, co-exposure to lead and manganese caused more severe accumulation of glutamate in hippocampus.

It has been widely confirmed that astrocytes are capable of intake excess glutamate in the synaptic cleft and produce glutamine [59]. Glutamine is released from astrocytes and uptake by neurons as a precursor of glutamate. The synthesis of glutamine is the key part of the glutamate-glutamine cycle. The synthesis reaction is catalyzed by GS, which is almost exclusively present in astrocytes, but is rare in oligodendrocytes and absent from neurons, so it is the signature enzyme of astrocytes [60]. In addition, it has been confirmed that although both neurons and astrocytes express glutamate transporters, the uptake of glutamate by astrocytes is still the most important means to maintain stable extracellular glutamate concentration. When the expression of astrocytes GLAST, GLT-1 transporter or neuron essential amino acid transporter were selectively inhibited by antisense nucleic acid technology, extracellular glutamate concentrations increased [61]. In this study, we also detected key proteins expression of astrocyte GLAST, GLT-1 and GS, and the results showed that both lead and manganese can lead to a significant decrease in their expression. However, the combined exposure to lead and manganese lead a more significant reduction.

Along with the glutamate-glutamine cycle-related proteins abnormal expression, our results also found that the expression level of GFAP in astrocytes and OX42 in microglia was significantly increased on day 5 after single or co-exposure to lead and manganese, suggesting that astrocytes and microglia were in an activated state.

Microglia-astrocyte crosstalk has recently been at the forefront of glial research. Emerging evidence illustrates that microglia determine the functions of reactive astrocytes, ranging from neuroprotective to neurotoxic [29]. However, the effect of lead and manganese exposure on microglia and astrocytes activation has not been reported. To test if the activation of astrocytes were affect by microglia, we used a classic microglial activation inhibitor, minocycline. After significantly inhibiting the activation of microglia, we found that the activation of astrocytes was also significantly reversed. Meanwhile, the impaired expression of glutamate-glutamine cycle-related proteins in astrocytes was also significantly alleviated. To further confirm the regulatory effect of microglia on astrocytes, we also measured the

cognitive function and the level of LTP induction in all animals. The results showed that after the inhibition of microglial activation and the subsequent reversal of astrocyte activation, the reduction of the induction level of LTP was significantly reversed in both single metal and combined exposure animals, and the cognitive impairment in the single metal and combined exposure groups was also significantly alleviated.

In summary, both Pb and Mn can induce learning and memory deficit and this deficit may mainly cause through hippocampal synaptic plasticity impairment. In addition, the present study also suggests the possibility that there may be an additive interaction between Pb and Mn on cognition and synaptic plasticity. The first trigger step of underlying cascade may mainly due to the activation of microglia during exposure. Thus, the activated microglia may play a regulatory role on astrocyte activation and its glutamate-glutamine cycle-related proteins abnormal expression. Eventually, these abnormally expressed proteins contribute to the accumulation of glutamate in the synaptic cleft and induce a series of subsequent damaging effects.

Conclusion

We suggest that microglial activation might be a novel target for the prevention of heavy metals co-exposure on learning and memory deficits. Astrocyte activation and its glutamate-glutamine cycle-related proteins abnormal expression was shown to be involved in microglial activation induced cognition and synaptic plasticity. However, further in vivo and in vitro experiments are necessary to better comprehend the mechanistic effects of microglial activation on astrocyte activation and the accumulation of glutamate in the synaptic cleft. These findings suggest that blocking microglial activation should be explored as a potential therapeutic strategy for infants who suffered from multiple heavy metals exposure.

Abbreviations

AAS
Atomization atomic absorption spectroscopy; AMPA:Alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid; ANOVA:Analysis of various; ATP:Adenosine triphosphate; BMn:Blood Mn; BPb:Blood Pb; BSA:bovine serum albumin; CNS:Central nervous system; CSF:Cerebrospinal fluid; DIC:differential interference contrast; EPSCs:Excitatory postsynaptic currents; GLAST:Glutamate/aspartate transporter; GLT-1:Glutamate transporter-1; GS:Glutamine synthetase; HPLC:High-performance liquid chromatography; IPP:Image pro plus; LTP:Long-term potentiation; MC:Minocycline; Mn:Manganese; MWM:Morris water maze; NMDAR:*N*-methyl-D-aspartate receptor; OPA:O-phtalaldehyde; Pb:Lead; PBS:phosphate buffered saline; PND:Postnatal day; SD-rats:Sprague-Dawley rats;

Declarations

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AUTHORS' CONTRIBUTIONS

RC and ML conceived and supervised the project. RG , ML and RC drafted the manuscript. TW and XD revised the manuscript. RG, TW, XD and KD were responsible for behavioral experiments. JL and FZ were responsible for western blot. GZ, XS were responsible for morphological experiment. JX, BL, BC and JW worked on the data management and statistical analyses. RG and TW worked on electrophysiological experiment. MA worked on revision of language. All authors critically reviewed the manuscript. All authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

All data are provided in the manuscript and in the additional files.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Committee of the Fourth Military Medical University.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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Figures

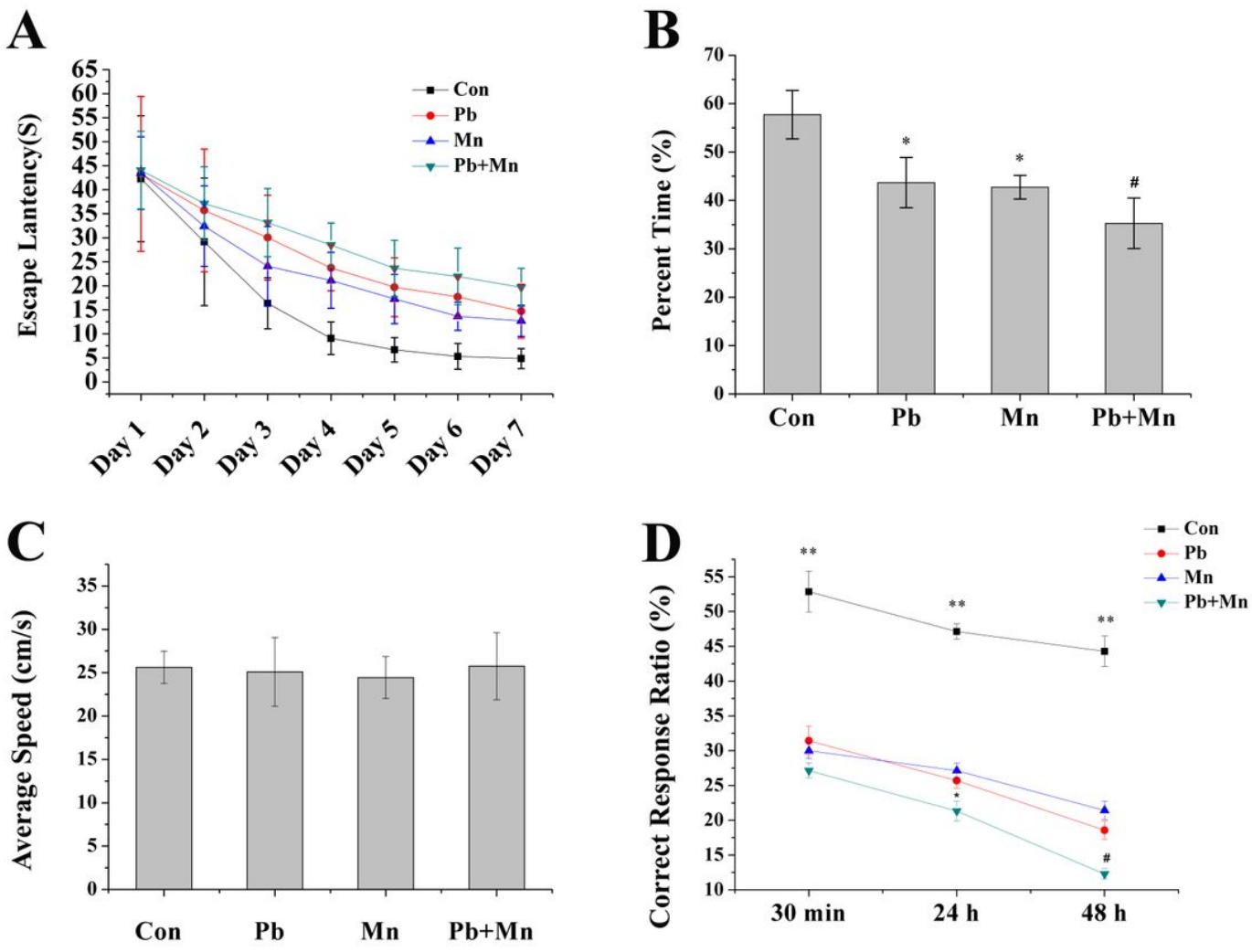

Figure 1

Effects of single metal and combined exposure on spatial learning and memory in the Morris water maze and Y maze. (A) Single metal and combined exposure rats require a longer time to escape to the hidden platform. Combined exposure caused a more prolonged mean latency to reach the hidden platform. $P < 0.05$ Pb+Mn vs. Con, Pb and Mn. (B) Single metal and combined exposure induced memory retrieval impairment as indicated by reduced time to searching in the target quadrant. Co-exposure to Pb and Mn led to a significant decrease in the amount of time spent in the target quadrant compared to single metal exposure groups *$P < 0.05$ vs. Con, #$P < 0.05$ vs. Pb and Mn. (C) There was no significant difference between the four groups in the swim speed. (D) The ratio of single metal and combined exposure to find the right arm was significantly lower than in the control rats. Combined exposure rats exhibit more significant memory lost compared to single metal exposure rats. **$P < 0.01$ vs. Pb, Mn and Pb+Mn. *$P < 0.05$ vs. Pb and Mn (24 h). #$P < 0.05$ vs. Pb and Mn (48 h). (MWM: Con: n = 9, Pb: n = 8, Mn: n = 9, Pb + Mn: n = 9. Y Maze: n = 6 for each group).

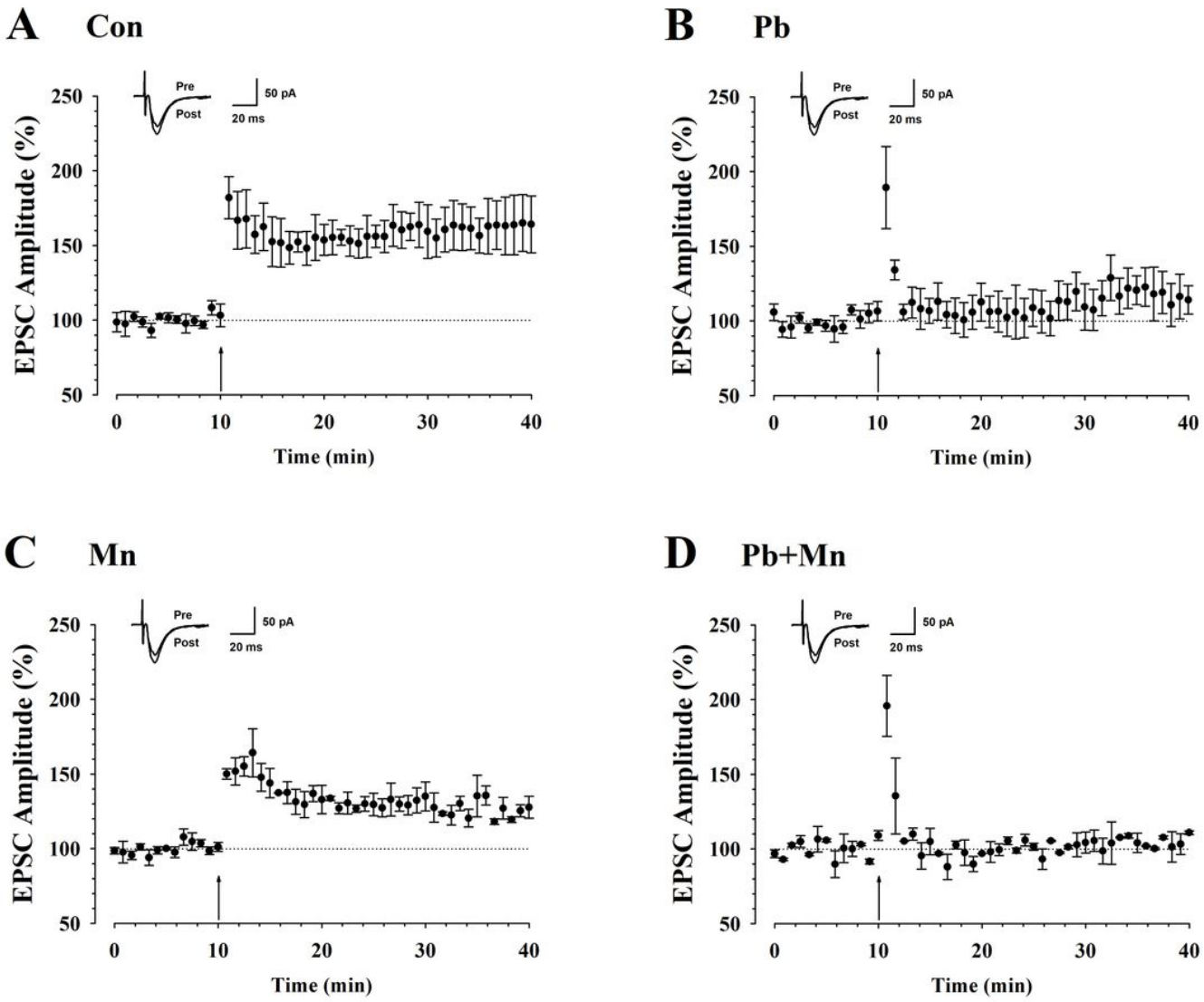

Figure 2

Effects of single metal and combined exposure on hippocampal long-term potentiation in rats. (A) LTP was induced in hippocampal pyramidal neurons in control rats (163.19 ± 10.92% of baseline, n = 10 slices/6 rats, t-test; P < 0.01 compared with baseline responses). (B) LTP was lost in hippocampal pyramidal neurons in Pb treated rats (Pb: 114.35±13.24%, n = 11 slices/6 rats, t-test; P < 0.05 compared with baseline, P < 0.05 vs. control group). (C) LTP was lost in hippocampal pyramidal neurons in Mn treated rats (Mn: 131.66±9.45%, n = 10 slices/6 rats, t-test; P < 0.05 compared with baseline, P < 0.05 vs. control group). (D) co-exposure to both lead and manganese resulted in more pronounced attenuation of synaptic potentiation compared to each of the single metal exposure groups (Pb+Mn: 105.59±15.24%, n = 12 slices/6 rats, t-test; P < 0.05 compared with baseline, P < 0.05 vs. Pb group, P < 0.05 vs. Mn group).

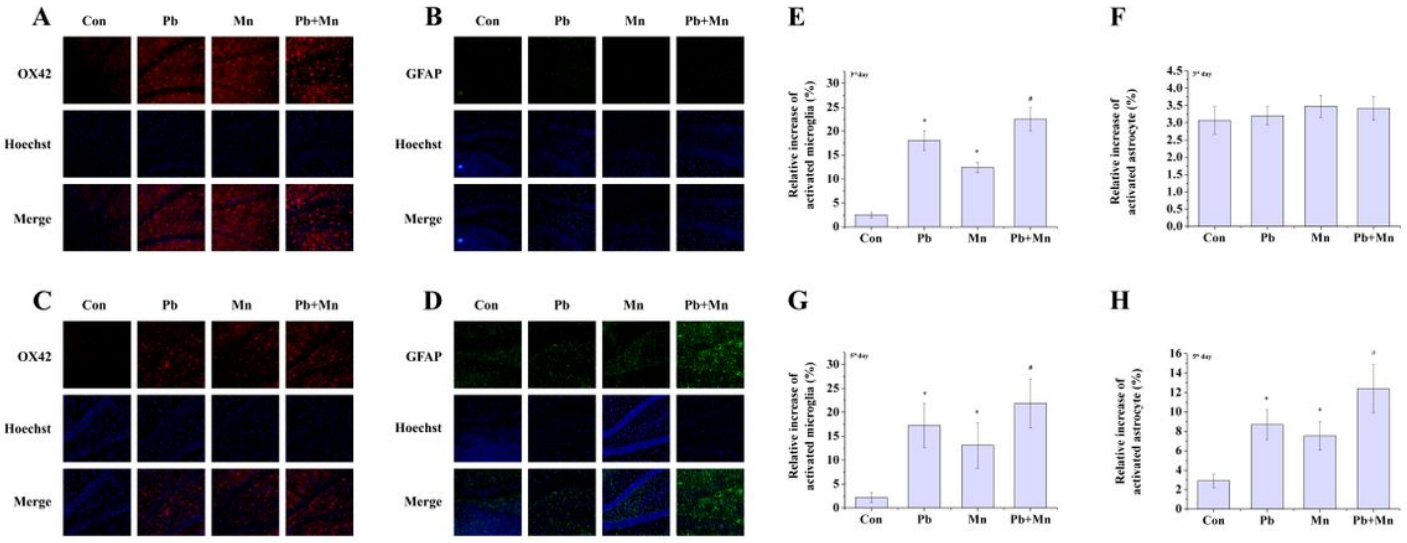

Figure 3

Effects of lead and manganese and combined exposure on microglial and astrocytic activation. Microglial activation was detected by immunocytochemistry (OX42) at 3rd day of exposure, while astrocytes were in a resting state (A: microglia; B: astrocyte). Astrocytes activation was detected by immunocytochemistry (GFAP) at 5th day of exposure (C: microglia; D: astrocyte). (E-H) The results were quantified. Results (A-D) are expressed as the mean ± S.D. of average activated cell rate in random fields (n = 20). *P < 0.05 vs. control. #P < 0.05 vs. Pb and Mn. A and B: 3rd day of exposure; C and D: 5th day of exposure.

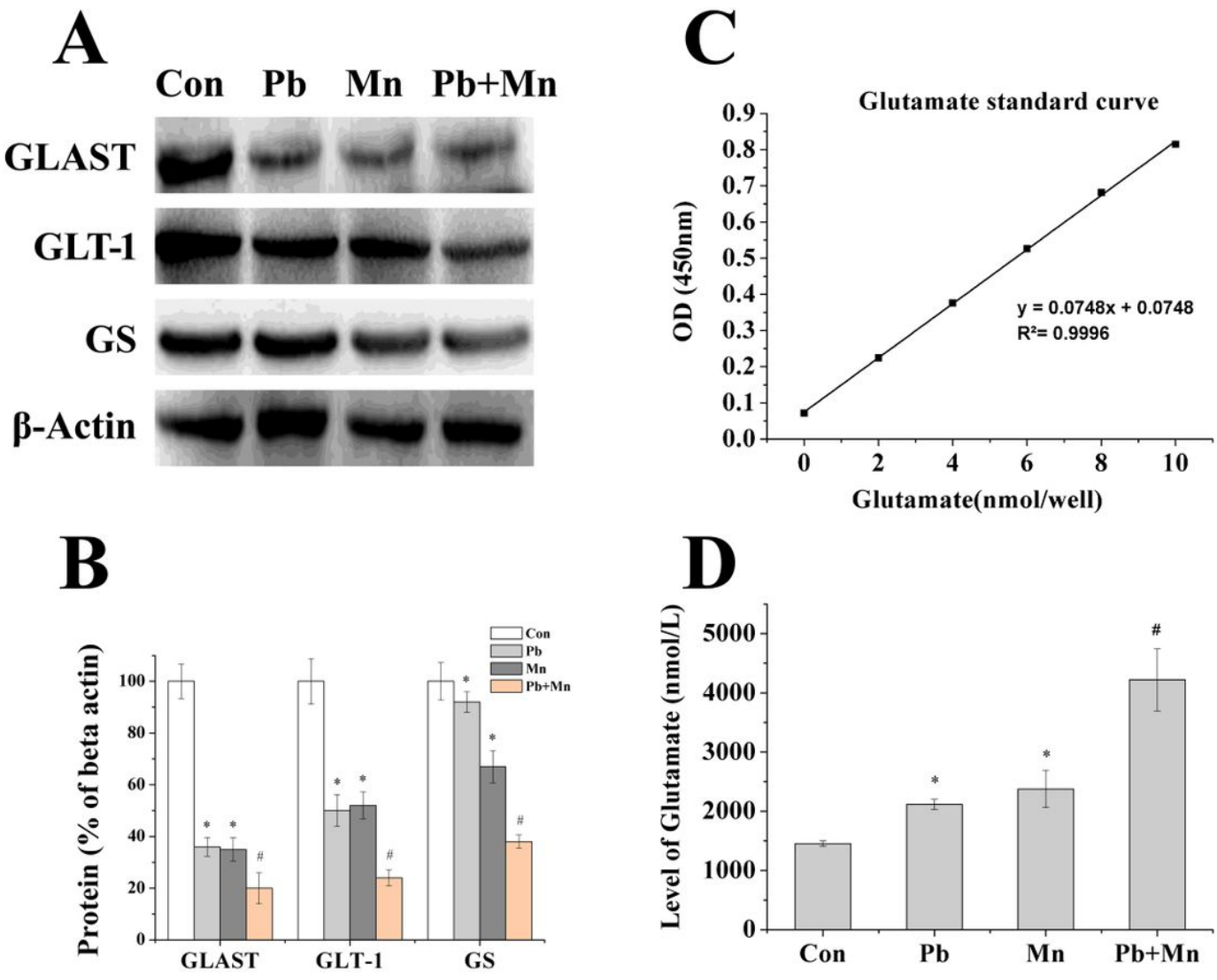

Figure 4

Effects of Pb and Mn exposure on glutamate-glutamine cycle-related protein expression and glutamate recycling. (A, B) Single metal exposure significantly inhibits glutamate-glutamine cycling-related protein expression compared to controls (GLAST, GLT-1 and GS: *P < 0.05 vs. Con, n = 6); co-exposure to both lead and manganese resulted in more pronounced attenuation of protein expression compared to each of the single metal exposure groups (GLAST, GLT-1 and GS: #P < 0.05 vs. Pb and Mn, n = 6). (C, D) Single metal exposure significantly inhibit glutamate reuptake compared to controls (*P < 0.05 vs. Con, n = 6); co-exposure to both lead and manganese resulted in more pronounced accumulation of glutamate compared to each of the single metal exposure groups (#P < 0.05 vs. Pb and Mn, n = 6).

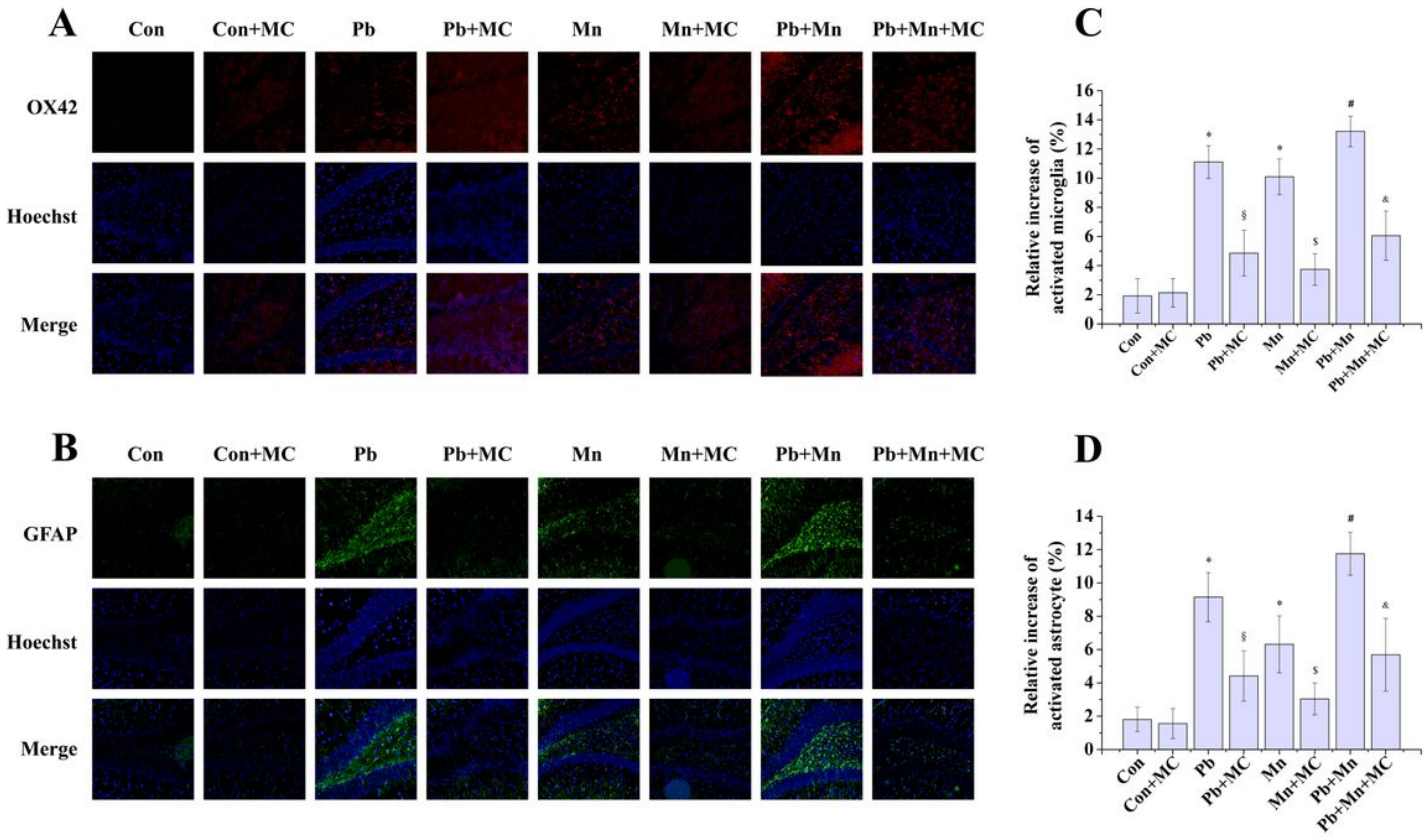

Figure 5

Minocycline blocks the activation of microglia and astrocyte induced by lead and manganese single and combined exposure. Microglia activation was detected by OX42 antibody. (C) The results were quantified. Results (A) are expressed as the mean ± S.D. of average activated cell rate in random fields (n = 20). *P < 0.05 vs control groups. #P < 0.05 vs Pb and Mn groups. §P < 0.05 vs Pb groups. $P < 0.05 vs Mn groups. &P < 0.05 vs Pb+Mn groups. Astrocytes activation was detected by GFAP antibody. (D) The results were quantified. Results (B) are expressed as the mean ± S.D. of average activated cell rate in random fields (n = 20). *P < 0.05 vs control groups. #P < 0.05 vs Pb and Mn groups. §P < 0.05 vs Pb groups. $P < 0.05 vs Mn groups. &P < 0.05 vs Pb+Mn groups. MC means minocycline.

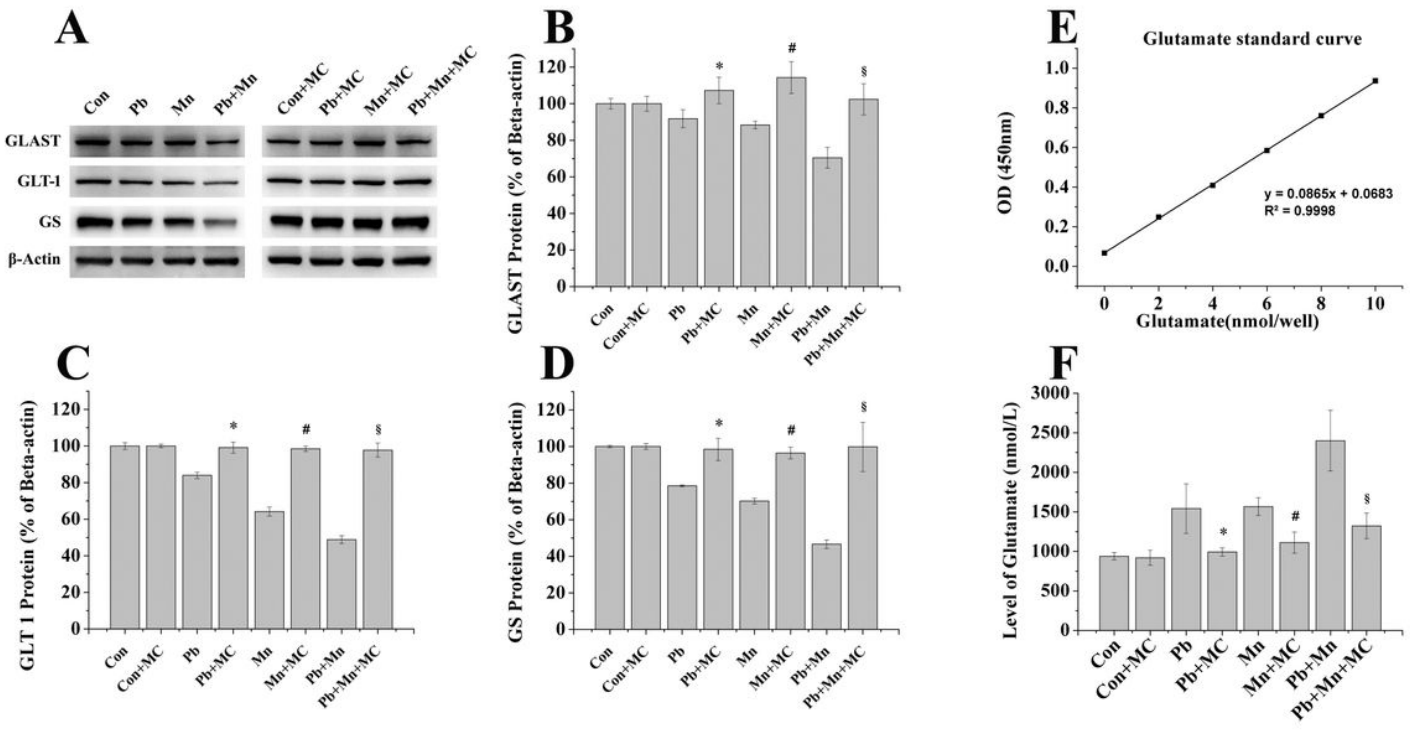

Figure 6

Minocycline alleviates glutamate-glutamine cycling-related protein impairments and glutamate accumulation. (A-D) Minocycline treatment significantly reversed the inhibition of lead and manganese single and combine exposure on GLAST, GLT-1 and GS expression (B-D: *P < 0.05 vs. Pb. #P < 0.05 vs. Mn. §P < 0.05 vs. Pb+Mn. n = 6). (E, F) Minocycline treatment significantly alleviates the accumulation of glutamate induced by lead and manganese single and combined exposure. (*P < 0.05 vs. Pb. #P < 0.05 vs. Mn. §P < 0.05 vs. Pb+Mn. n = 6).

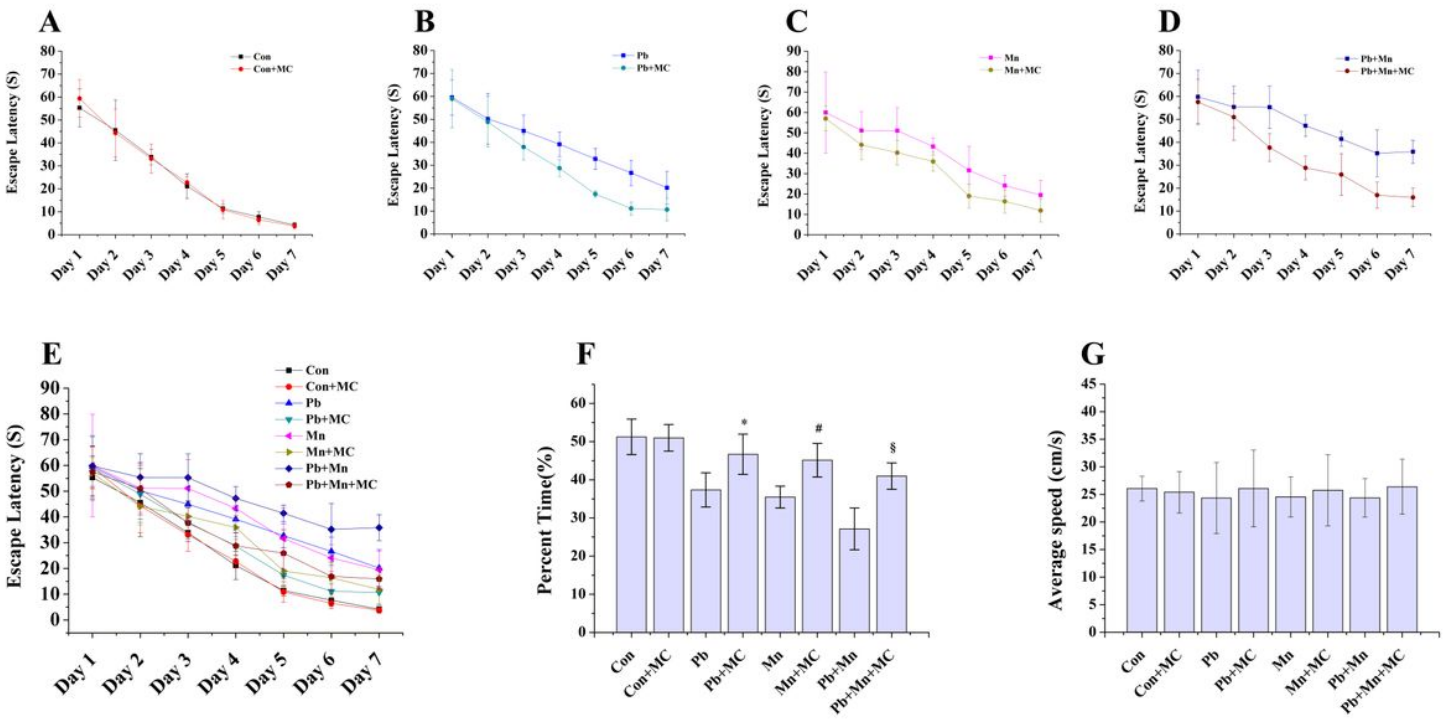

Figure 7

Minocycline alleviates spatial memory impairment induced by lead and manganese single and combine exposure. (A-E) Minocycline treatment reduced the escape latency time than lead and manganese single or combine exposure rats ($P < 0.05$). (F) Minocycline treatment enhanced the search time in target quadrant than lead and manganese single or combine exposure group (*$P < 0.05$ vs. Pb. #$P < 0.05$ vs. Mn. §$P < 0.05$ vs. Pb+Mn. n = 6). (G) There was no significant difference among each group for the swim speed ($P > 0.05$).

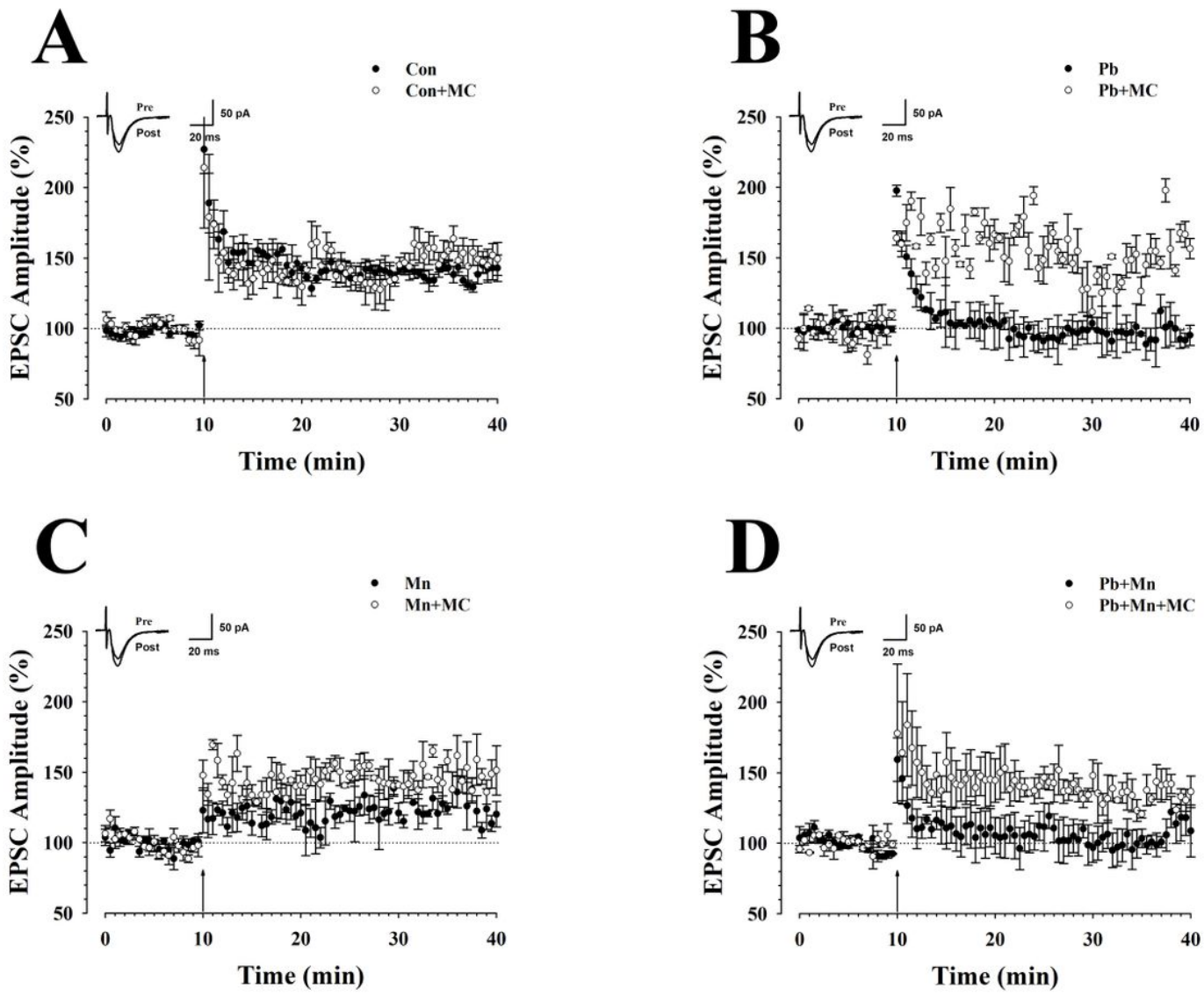

Figure 8

Minocycline prevents the lead and manganese single and combine exposure induced hippocampal long-term potentiation impairment. (A) LTP was induced in hippocampal pyramidal neurons in control and control plus minocycline treated rats (Con: 145.54 ± 14.84%, n = 10 slices/6 rats, t-test; P < 0.05 vs. baseline. Con+MC: 146.59 ± 13.90%, n = 11 slices/6 rats, t-test; P < 0.05 vs. baseline, P > 0.05 vs. control group). (B-D) LTP was significantly reversed in hippocampal pyramidal neurons in exposure plus minocycline treatment rats. (Pb: 104.16 ± 17.99%, n = 10 slices/6 rats, t-test; P < 0.05 vs. baseline. Pb+MC: 155.58 ± 17.74%, n = 10 slices/6 rats, t-test; P < 0.05 vs. baseline. P < 0.05 Pb vs. Pb+MC. Mn: 122.59 ± 8.24%, n = 10 slices/6 rats, t-test; P < 0.05 vs. baseline. Mn+MC: 145.54 ± 8.93%, n = 11 slices/6 rats, t-test; P < 0.05 vs. baseline. P < 0.05 Mn vs. Mn+MC. Pb+Mn: 108.63 ± 10.79%, n = 11 slices/6 rats, t-test; P < 0.05 vs. baseline. Pb+Mn+MC: 142.19 ± 11.42%, n = 9 slices/6 rats, t-test; P < 0.05 vs. baseline. P

< 0.05 Pb+Mn vs. Pb+Mn+MC). Pairing training is indicated by an arrow. The dashed line indicates the mean basal synaptic responses.

Supplementary Files

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

- figs1BWandBPbandBMn.tif