Involvement of Microglia Activation in the Lead Induced Long-Term Potentiation Impairment

Ming-Chao Liu1,3, Xin-Qin Liu1,3, Wen Wang2, Xue-Feng Shen1, Hong-Lei Che1, Yan-Yan Guo2, Ming-Gao Zhao3, Jing-Yuan Chen1*, Wen-Jing Luo1*

1 Department of Occupational & Environmental Health and the Ministry of Education Key Lab of Hazard Assessment and Control in Special Operational Environment, School of Public Health, Fourth Military Medical University, Xi’an, China, 2 Department of Anatomy, Histology and Embryology, K.K. Leung Brain Research Centre, Fourth Military Medical University, Xi’an, China, 3 Department of pharmacology, Fourth Military Medical University, Xi’an, China

Abstract

Exposure of Lead (Pb), a known neurotoxicant, can impair spatial learning and memory probably via impairing the hippocampal long-term potentiation (LTP) as well as hippocampal neuronal injury. Activation of hippocampal microglia also impairs spatial learning and memory. Thus, we raised the hypothesis that activation of microglia is involved in the Pb exposure induced hippocampal LTP impairment and neuronal injury. To test this hypothesis and clarify its underlying mechanisms, we investigated the Pb-exposure on the microglia activation, cytokine release, hippocampal LTP level as well as neuronal injury in in vivo or in vitro model. The changes of these parameters were also observed after pretreatment with minocycline, a microglia activation inhibitor. Long-term low dose Pb exposure (100 ppm for 8 weeks) caused significant reduction of LTP in acute slice preparations, meanwhile, such treatment also significantly increased hippocampal microglia activation as well as neuronal injury. In vitro Pb-exposure also induced significantly increase of microglia activation, up-regulate the release of cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and inducible nitric oxide synthase (iNOS) in microglia culture alone as well as neuronal injury in the co-culture with hippocampal neurons. Inhibiting the microglia activation with minocycline significantly reversed the above-mentioned Pb-exposure induced changes. Our results showed that Pb can cause microglia activation, which can up-regulate the level of IL-1β, TNF-α and iNOS, these proinflammatory factors may cause hippocampal neuronal injury as well as LTP deficits.

Citation: Liu M-C, Liu X-Q, Wang W, Shen X-F, Che H-L, et al. (2012) Involvement of Microglia Activation in the Lead Induced Long-Term Potentiation Impairment. PLoS ONE 7(8): e43924. doi:10.1371/journal.pone.0043924

Copyright: © 2012 Liu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by: National Basic Research Program of China (973 Program, #2012CB525002); Grants from National Natural Science Foundation of China: #30830087, URL: http://159.226.244.22/portal/Proj_List.asp; #81001233, URL: http://159.226.244.22/portal/Proj_List.asp; #30901176, URL: http://159.226.244.22/portal/Proj_List.asp; Program for New Century Excellent Talents in University, URL: http://news.fmumu.edu.cn/nr.jsp?urltype=004&newsid=776376&btreeid=1004; Science and Technology Innovation Projects of Shaanxi Province (#2011KTCL03-19); Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: luowenj@fmmu.edu.cn (W-JL); jy_chen@fmmu.edu.cn (J-YC)

† These authors contributed equally to this work.

Introduction

Lead (Pb) is known to cause irreversible neurological disturbances [1]. Environmental low-level Pb caused neurotoxic impairment is a severe hazard worldwide [2,3]. Extensive studies have shown the effect of low-level Pb exposure on growth, development and cognitive function [4,5]. These extensive studies revealed that Pb exposure at low doses is extremely dangerous and can cause learning and memory impairments [6]. The hippocampus is a brain center for learning and memory [7]. LTP of the hippocampal excitatory synaptic transmission is thought to be a pattern of manifestation for synaptic plasticity [8] which is believed to be the underlying mechanisms for hippocampus-dependent learning and memory [9].

On the other hand, the contribution of microglia to the hippocampus dependent learning and memory becomes the research focus recently. Microglia activation can impair the learning and memory via released interleukin (IL)-1 [10]. Inhibiting the microglia activation can rescue the learning and memory deficits in a murine model of human immunodeficiency virus (HIV) type 1 encephalitis [11]. Meanwhile, the microglia activation is suggested to be involved in some form of LTP [12] and such effect may be mediated by the adenosine receptor P2X7 located on the microglia [13]. Although one previous study suggested that the microglia released IL-8 is involved in the inhibition of hippocampal LTP [14], whether and how microglia contributes to hippocampal LTP remains unclear.

Our pilot study revealed that low-level Pb-exposure induced hippocampus-dependent learning and memory deficits as well as activation of microglia. Microglia may contribute to the hippocampal LTP which is recognized as one mechanism for learning and memory. Thus, we raised the hypothesis that activation of microglia is involved in the Pb exposure induced hippocampal LTP impairment, neuronal injury and learning and memory deficits. To test this hypothesis and clarify its underlying mechanisms, we investigated the Pb-exposure on the microglia activation, cytokine release, hippocampal LTP level as well as neuronal injury in adult Sprague-Dawley rats (SD-rats) or primary
hippocampal and microglial cell cultures. The involvement of microglia activation in the Pb induced LTP impairment was also confirmed by inhibiting microglia activation with minocycline, a microglia inhibitor.

Materials and Methods

Materials

Cell culture ingredients were purchased from Invitrogen (Grand Island, NY, USA). D-Hanks solution was bought from GibCO (Grand Island, NY, USA). Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and poly-L-lysine were obtained from SIGMA (USA). Transwell co-culture plates and other cell culture equipment were bought from Costar (Corning, NY, USA). SD rats were obtained from the Animal Experiment Center of the Fourth Military Medical University. Analytical pure lead acetate was purchased from SIGMA (USA). Minocycline was bought from SIGMA (USA). Polyclonal mouse anti-OX42 antibody, and polyclonal mouse anti-NeuN antibody were from CHEMICON (Hampshire, UK). The fluorescence secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). TNF-α and IL-1β Enzyme-linked immunosorbent (ELISA) kits were purchased from eBioscience (San Diego, CA, USA). Lactate dehydrogenase (LDH)-cytotoxicity assay kits were purchased from Biovision (Biovision Inc, USA). All other reagents were purchased from SIGMA (USA). The inverted microscope, the fluorescence microscope and the Laser confocal microscope were bought from OLYMPUS (Tokyo, Japan). 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 (guide for the care and use of laboratory animals) and were approved by the institutional animal care and use committee of Fourth Military Medical University (Permit Number:12001). Male 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 groups (20 for each group): control group, minocycline treated group, Pb treated group, Pb 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 22–24 days), the animals were exposed to Pb via drinking water. Lead acetate (SIGMA, USA) was dissolved in distilled water with a concentration of either 0 or 100 ppm [15]. Pb concentrations were verified using electrothermal atomization atomic absorption spectroscopy (AAS). Rats were exposed to Pb from 24 to 80 days of age. Minocycline (120 mg/kg per day in 5% sucrose; SIGMA, USA) was delivered via oral gavage [16] in Pb expose 0 or 100 ppm rats. Water consumption was monitored every two days and individual body weight was measured weekly during the experimental period.

Primary cultures and cell stimulation

1. Hippocampal neuronal cultures. Rat primary hippocampal cultures were prepared following a previously described protocol [17,18,19] with some modifications. Briefly, hippocampal tissues were dissected from postnatal day 1 SD rats and dissociated mechanically without using enzymes. Cells were seeded in 12-well (6×10^7/ml) culture plates pre-coated with poly-L-lysine (20 μg/ ml) and maintained in 0.5 ml/well of Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (Ham) (1:1) (DMEM/F12) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1 g/L glucose, 2 mmol L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cultures were replenished with 0.5 ml/well fresh medium 3 days later and were used for treatment 3 days later.

2. Microglia cultures. Microglia cells were derived from postnatal day 1 SD rat brains. Cerebro-cortices were isolated and trypsinized. Cortices were dissociated mechanically and plated into tissue culture T-75 flasks in DMEM/F12 with L-glutamine (Invitrogen, Carlsbad, CA, USA) containing 15% heat-inactivated FCS and 20 μg/L GM-CSF. After 24 h all media and tissue were removed and replaced with fresh media. After 7 days, one half of the media was replaced and cells were maintained in a mixed glial culture until day 14. At 14 days in vitro, microglia were removed from the mixed glial culture via a rotating shaker at 60 rpm for 20 h, the supernatant was collected and seeded in 12-well culture plates and cultured as above.

3. Cell stimulation. For the microglia activation assay, the purified microglia were seeded in 12 well culture plates, cultured in 0.5 ml/well of DMEM/F12 containing 10% FCS, 2 mmol/L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were treated with 50 μmol/L Pb for 48 h.

For the neuroprotective effects assay, the hippocampal neurons were seeded and cultured in the bottom of Transwell plate, the purified microglia were seeded on the semipermeable membrane of Transwell insert, then put the Transwell insert into a normal 12 well culture plate, cultured and treated with 50 μmol/L Pb and 45 μmol/L minocycline for 48 h. The inserts were removed from the normal 12 well culture plate and immediately co-cultured with hippocampal neuronal cultures for 48 h in Transwell plates.

For cytotoxicity detection, the culture medium of each group were removed for LDH release assay using a LDH-cytotoxicity assay kit (Biovision Inc, USA) according to the manufacturer’s protocol. The relative absorbance of all samples was measured at 490 nm. The measurement was repeated three times at a 5-s interval and the numbers of each group were calculated with the following formula: Cytotoxicity = (A test sample-A blank control) / (A positive control - A blank control) × 100%.

Determination of Pb concentrations in blood and hippocampal samples

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. Brain tissues were dissected and the hippocampus was quickly dissected on ice and used to determine Pb using an electrothermal atomization AAS.

Long-term potentiation (LTP) recording in hippocampal slices

1. Slice preparation. Coronal brain slices (300 μm) from a 6 to 8 week-old SD rat, containing the hippocampus, were prepared using standard methods [20]. Slices were transferred to a submerged recovery chamber containing oxygenated (95% O2 and 5% CO2) artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 4.4 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 25 mM NaHCO3, 1 mM Na2HPO4, 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-

Figure 1. Abolishment of hippocampus synaptic potentiation in 100 ppm Pb treated rats. (A) LTP was induced in hippocampal pyramidal neurons in control rats $(n = 13$ slices/6 rats, t-test; $P<0.001$ compared with baseline). (B) LTP was lost in hippocampal pyramidal neurons in Pb treated rats $(n = 8$ slices/6 rats, t-test; $P>0.05$ compared with baseline). Pairing training is indicated by an arrow. The dashed line indicates the mean basal synaptic responses.
doi:10.1371/journal.pone.0043924.g001

Figure 2. Effects of hippocampal neuronal injury and microglia activation induced by Pb in the hippocampus. Microglia activation was detected by immunocytochemistry (OX42). (A) control; (B) 100 ppm Pb for 8 weeks. Activation of microglia was evaluated by counting the number of activated microglial cells (B blue arrow); White arrows (A) indicate resting microglia. (C) The results were quantified and are expressed as the mean ± S.D. of average activated cell rate in random fields $(n = 20)$. * $P<0.05$ compared with control groups. Scale bar indicates 50 μm. Hippocampal neuronal injury was detected with in situ TUNEL (green fluorescence). (K) The apoptotic neurons in the Pb group were significantly higher than the control group. (L) The results were quantified and are expressed as the mean ± S.D. of apoptotic neurons in DG zone of hippocampus $(n = 8)$. * $P<0.05$ compared with control groups. Scale bar indicates 50 μm.
doi:10.1371/journal.pone.0043924.g002
Lead Induced Long-Term Potentiation Impairments

A: Lead induced long-term potentiation impairments

B: Lead induced long-term potentiation impairments

C: The rate of activation (%) of Con and Pb

D: The average fluorescence intensity

E: The average fluorescence intensity

F: The rate of cell apoptosis (%)

G: Level of TNF-alpha (ng/ml)

H: Level of IL-1 Beta (ng/ml)

I: The rate of cell apoptosis (%)

J: NeuN

K: Pb-DS

L: Con-CC

M: Pb-CC

N: TUNEL

O: Pb-DS

P: Con-CC

Q: Pb-CC

R: Merge

S: Pb-DS

T: Con-CC
Figure 3. Pb activated microglia, enhanced TNF-α and IL-1β secretes, iNOS expression, and induces hippocampal neuronal injury.

Purified microglia cells were seeded in 12-well culture plates. Cells were treated with vehicle (control) (A) or Pb (50 μmol) (B) for the indicated times. Microglia activation was detected by immunocytochemistry (OX42). (A) control; (B) 50 μmol Pb for 48 h. (C) The results were quantified. Results (A and B) are expressed as the mean ± S.D. of average activated cell rate in random fields (n = 20). **P<0.001 compared with control groups. Scale bar indicates 50 μm. iNOS was detected by immunofluorescence staining. Activation of iNOS was evaluated by measure the average fluorescence of iNOS-immunoreactive cells (D, E). (F) The results were quantified and are expressed as the mean ± S.D. of average fluorescence of iNOS-immunoreactive cells in random fields (n = 20). ** P<0.001 compared with control groups. Scale bar indicates 50 μm. TNF-α (G) and IL-1β (H) were determined by ELISA. Purified microglia were cultured with vehicle (control) or Pb (50 μmol) for 48 h for the TNF-α and IL-1β assay, respectively. ** P<0.001 compared with control groups. The purified microglia were co-cultured with hippocampal neurons for 48 h after culturing with vehicle (control) (L, P, T) or Pb (50 μmol) (M, Q, U) for 48 h. Apoptosis of hippocampal neurons was detected in situ TUNEL (green fluorescence). Degeneration of hippocampal neurons was evaluated by counting the number of apoptotic neurons. As compared with the co-culture method, hippocampal neurons were also treated with vehicle (control) (J, N, R) or Pb (50 μmol) (K, O, S) directly for 48 h. (I) The results were quantified and are expressed as the mean ± SD of apoptotic neurons percentage in random fields (n = 20). ** P<0.001 compared with control groups. Scale bar indicates 50 μm. CC in figure means co-culture method; DS in figure means direct stimulation method.

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-glucuronate, 5 NaCl, 1 MgCl2, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, and 0.1 Na3-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

Neurons were detected with anti-NeuN antibody and microglial cells were detected with anti-OX42 antibody. Briefly, cells from primary culture were fixed with 3.7% paraformaldehyde for 30 min and cells from primary culture or slices form cryopreserved tissue 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, cells or slices were incubated with primary antibody overnight at 4°C. Cells or slices were then washed with PBS and incubated for 1 h with the secondary antibodies (anti-rabbit/or goat-FITC and antimouse-Rhodamine, Jackson ImmunoResearch, West Grove, PA, USA) at room temperature and then rinsing with PBS buffer. In in vivo experiments, neurons were stained with anti-NeuN antibody and then the apoptotic neurons were labeled in situ using the TUNEL. The rate of cell apoptosis was counted apoptotic cells and total neurons from 8–9 random fields of a coverslip and calculates the rate of cell apoptosis. In in vitro experiments, neurons were stained with anti-NeuN antibody and then the apoptotic cells were labeled in situ using the TUNEL. The number of apoptotic cells was counted in DG zone of hippocampus. Layers were determined according to anatomical criteria [21]. The rates of activated microglia were evaluated by counting the number of activated microglial cells from OX42-immunoreactive cells in DG zone of hippocampus or from 8–9 random fields of a coverslip. Expression of iNOS was evaluated by measure the average fluorescence of iNOS-immunoreactive cells. Cells were examined and recorded under an OLYMPUS BX51 fluorescent microscope equipped with DP-BSW software (OLYMPUS, Japan).

Quantitation of secreted TNF-α and IL-1β

TNF-α and IL-1β in the culture supernatant were detected by an ELISA kit (eBIOSCIENCE). Briefly, the 96 well culture plates were coated with coating buffer at 4°C overnight, the plates were rinsed five times with wash buffer, and the plates were blocked with 1× Assay Diluent and incubated for 1 h at room temperature. The standard preparation was diluted with Assay Diluent and 100 μl of the standard preparation was added to the wells, at the same time, 100 μl of the sample was added to the wells, the plate was covered and incubated for 2 h at room temperature. The plate was then rinsed as before and 100 μl of the detecting antibody was added to the wells and incubated for 1 h at room temperature, then rinsed as before. 100 μl of Avidin-HRP was added to each well and incubated for 30 min at room temperature, the plate was rinsed 7 times, 100 μl of base solution was added to each well and incubated for 15 min at room temperature, then 50 μl of stop solution was added to each well and the plate was read at 450 nm.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) except specified and analyzed with one-way analysis of variance (ANOVA) followed by a least significant-difference test for multiple comparisons where appropriate. A P value less than 0.05 was considered as statistically significant.

Results

Blood and hippocampal Pb level, body weight and water consumption following Pb exposure

Exposure to Pb in drinking water under the current dose regimen (100 ppm) resulted in a 4.5-fold increase in blood Pb (BPb) as compared to control rats (P<0.05) (Fig. S1). Similar to changes in BPb, Pb exposure resulted in 1.26-fold increases in Pb concentrations among hippocampus compared to control rats (P<0.05) (Fig. S2). Results also showed there are no significant differences between control and Pb groups (P>0.05) in body weight (Fig. S3) and water consumption (Fig. S4). This result demonstrates that 100 ppm Pb in drinking water didn’t significantly affect the diet and growth of rats.

Pb treatment reduced hippocampal LTP levels

Previous reports have shown that Pb triggers a series of neuronal injuries and causes learning and memory deficits [22,23,24]. LTP is one important mechanism underlying hippocampal learning and memory [7]. Thus, it’s important to investigate the effect of Pb on hippocampal LTP. To determine how Pb affects hippocampal LTP, we used an in vivo approach using a Pb poisoning model in the present study. Using ex vivo slice preparations derived from the CA1 region of the rat hippocampus, we first assessed the impact of Pb on synaptic plasticity. We used the typical LTP induction paradigm to trigger LTP in hippocampal slices [25]. The results show that pairing training induced a significant, long-lasting potentiation of synaptic responses in slices.
Figure 4. Minocycline blocks the activation of microglia; attenuates Pb-induced secreted of TNF-α, IL-1β and expression of iNOS; and protects co-cultured hippocampal neurons. Purified microglia were treated with vehicle (control) (A), Pb (50 μmol) (B), or Pb (50 μmol)+minocycline (45 μmol) (C) for 48 h, and were co-cultured with hippocampal neurons for another 48 h. (A, B, C) Microglia activation
was detected by OX-42 antibody. (D) The results were quantified. Results (A, B and C) are expressed as the mean ± S.D. of average activated cell rate in random fields (n = 20). **P<0.001 compared with control groups. *P<0.01 compared with Pb-treated groups. iNOS was detected by anti-iNOS antibody (E, F, G, red fluorescence). Activation of iNOS was evaluated by the average fluorescence of iNOS-immunoreactive cells. (H) The results were quantified. Results (E, F and G) are expressed as the mean ± S.D. of average fluorescence of iNOS-immunoreactive cell number in random fields (n = 20). **P<0.001 compared with control groups. *P<0.01 compared with Pb-treated groups. TNF-α (l) and IL-1β (J) were determined by ELISA. Purified microglia were treated with vehicle (control) or Pb (50 μmol), or Pb (50 μmol)+minocycline (45 μmol) for 48 h for the TNF-α and IL-1β assay, respectively. **P<0.001 compared with control groups. *P<0.01 compared with Pb-treated groups. Apoptotic hippocampal neurons were detected and compared using TUNEL (green fluorescence) (L–T). Degeneration of hippocampal neurons was evaluated by counting the number of apoptotic neurons. (K) The results were quantified and are expressed as the mean ± S.D. of apoptotic neurons percentage in random fields (n = 20). **P<0.001 compared with control groups. *P<0.01 compared with Pb-treated groups. All scale bar indicates 50 μm.

doi:10.1371/journal.pone.0043924.g004

from control rats (mean 129.6±7.5% of baseline, n = 13 slices/6 rats, t-test; P<0.001) compared with baseline responses, Fig. 1A). In contrast, synaptic potentiation was absent in slices from Pb treated rats (38.6±7.1%, n = 8 slices/6 rats, t-test; P>0.05 compared with baseline, Fig. 1B). These results show that Pb reduced the induction of LTP in the hippocampus of adult rats.

**In vivo** Pb exposure activates microglia and induces hippocampal neuronal injury

Recent studies have demonstrated that administration of lipopolysaccharide (LPS) can activate microglia and impair the level of learning and memory [26]. In this study, we subjected weaning rats to Pb. Analysis of the activation level of microglia from the hippocampus of weaning rats treated with Pb by immunocytochemistry, revealed microglia activation as evidenced by the key morphological changes including the thickening and retraction of branches, the increasing of cell body (33.69±3.48%, n = 20) (Fig. 2B and C) from the resting state compared with the control group (12.37±1.09%, n = 20) (Fig. 2A and C).

Studies have shown that treatment with Pb can cause hippocampal neuronal injury. In this study we also confirmed that Pb induced obvious neuronal apoptosis (31±4, n = 8) (Fig. 2H-K and L) in the hippocampus of weaning rats treated with Pb compared with the control group (3±1, n = 8, P<0.05 VS Pb group) (Fig. 2D-G and L).

**Figure 5. Effect of minocycline on LDH-release after Pb exposure.** Purified microglia were treated with vehicle (control), Pb (50 μmol) or Pb (50 μmol)+minocycline (45 μmol) for 48 h, and were co-cultured with hippocampal neurons for another 48 h. LDH-release was measured from co-culture supernatant by LDH-cytotoxicity assay kit. The mean value of LDH-release of the positive control group (0.1% triton X-100) was considered as 100%. n = 6. Mean ± S.D. *P<0.01 compared with Pb-treated groups.

doi:10.1371/journal.pone.0043924.g005

Pb pretreatment induced **in vitro** microglia activation

To investigate if Pb induced microglia activation first and then followed with neuronal injury, we established an **in vitro** neuron-microglia co-culture model. In order to determine whether Pb exposure can activate microglia, we treated microglial cultures with 50 μmol/L Pb for 48 h based on MTT assay (Fig. S5). Immunocytochemistry showed that stimulated microglia were present as mononuclear macrophages (91.21±4.56%, n = 20) (Fig. 3B), and cells in the control group were mostly in the resting state (17.11±0.86%, n = 20) (Fig. 3A), which strongly indicated that Pb stimulation caused microglial activation (P<0.001 VS control) (Fig. 3C).

**Activated microglia cause hippocampal neuronal apoptosis in vitro**

After treatment with Pb, microglial cells were co-cultured with hippocampal neurons. The effect of activated-microglia on hippocampal neuronal apoptosis in cultures was determined by TUNEL assay. As shown in Fig. 3, pretreatment of microglia with Pb significantly increased the number of apoptotic neurons from the hippocampal primary culture (Con: 3.11±0.16%, n = 20, Fig. 3 I, M, Q, U; Pb: 68.13±3.41%, n = 20, Fig. 3 I, L, P, T). In that group that Pb was applied directly to isolated neurons, the number of apoptotic neurons was not different from that of the control group (2.13±0.11%, n = 20) (Fig. 3 I, J, N and R) and the Pb-treated group (2.01±0.11%, n = 20) (Fig. 3 I, K, O and S). These results indicated that activated microglia caused hippocampal neuronal death. These findings raise the possibility that hippocampal neurones may be easily damaged by microglias which were activated with Pb.

**Inflammatory factors were up-regulated after stimulation with Pb in vitro**

It has previously been reported that activated microglia secrete many different inflammatory factors [27,28]. We found that after treatment with Pb, the expression of iNOS was at a significantly high level (71.08±3.55, n = 20) (Fig. 3 E and F) compared with control group (2.13±0.11, n = 20) (Fig. 3 D and F). We also found that TNF-α (Con: 13.18±0.54%, n = 6; Pb: 53.88±2.71%, n = 6) (Fig. 3 G) and IL-1β (Con: 16.04±0.63%, n = 6; Pb: 71.30±3.68%, n = 6) (Fig. 3 H) were induced after exposure to Pb.

**In vitro** pretreatment with minocycline inhibits Pb induced upregulations of iNOS, TNF-α and IL-1β

Minocycline, a tetracycline antibiotic, has profound anti-inflammatory properties and is used experimentally for the treatment of many central nervous system (CNS) disorders. Minocycline has been shown to inhibit microglia activation, and to protect the CNS under inflammatory conditions [29,30,31]. In order to establish whether secretions from activated microglia caused hippocampal neuronal death, cultures were treated with 50 μM Pb and 45 μM minocycline. The results showed that...
minocycline strongly inhibited microglia activation (Con: 13.34±0.67%, n = 20; Pb: 90.13±4.51%, n = 20; Minocycline: 27.67±1.38%, n = 20) (Fig. 4 A–D), meanwhile, decreased iNOS (Con: 4.46±0.22, n = 20; Pb: 66.10±3.30, n = 20; Minocycline: 9.32±0.47, n = 20) (Fig. 4 E–H), TNF-α (Con: 15.22±0.76%, n = 6; Pb: 73.76±3.37%, n = 6; Minocycline: 23.04±1.10%, n = 6) (Fig. 4 I) and IL-1β (Con: 21.12±1.23%, n = 6; Pb: 84.20±3.70%, n = 6; Minocycline: 28.39±1.36%, n = 6) (Fig. 4 J) expression.

In vitro pretreatment with minocycline prevents hippocampal neuronal injury

We confirmed that minocycline inhibited microglia activation and the expression of their inflammatory factors. However, does this inhibition lead to the prevention of hippocampal neuronal death? Minocycline pretreated microglia were co-cultured with hippocampal neurons, and number of apoptotic neurons were significantly less (4.11±0.21%, n = 20) (Fig. 4 K, N, Q and T) than that of cells pretreated with 50 μM Pb alone (73.15±3.66%, n = 20) (Fig. 4 K, M, P and S). On the other hand, we also confirmed that after Pb treatment, the average value of LDH was similar to that of the positive control group treated with 0.1% Triton X-100 (88.17±12.92%, n = 6, P>0.05 VS positive control) (Fig. 5). When minocycline was added to the cultures during Pb exposure, the average value of LDH greatly declined (21.21±1.63%, n = 6) (Fig. 5) as compared with Pb-treated groups (P<0.01). These suggested that minocycline could prevent Pb-induced hippocampal neuronal injury.

Minocycline pretreatment reversed Pb-induced inhibition of hippocampal LTP

We confirmed that pretreatment with minocycline partly reversed Pb-induced activation of microglia and hippocampal neuronal injury in vitro. The study of the LTP in hippocampus in vivo also showed that, after minocycline administration, the induction of LTP (121.2±4.8%, n=9 slices/6 rats, t-test; P<0.05 compared with baseline, Fig. 6D) were partly reversed compared with Pb treated rats (95.4±5.3%, n=10 slices/6 rats, t-test; P>0.05 compared with baseline, Fig. 6C). The results also showed that there is no significant different between control rats (135.1±4.1% of baseline, n=11 slices/6 rats, t-test; P>0.001 compared with baseline, Fig. 6A) and minocycline treated rats (130.7±6.7% of baseline, n=8 slices/6 rats, t-test; P>0.001 compared with baseline, Fig. 6B). This suggested that minocycline could partly prevent Pb-induced LTP impairment.

Figure 6. Minocycline reversed the induction of LTP in hippocampus after 100 ppm Pb treated. (A) LTP was induced in hippocampal pyramidal neurons in control rats (n = 11 slices/6 rats, t-test; P<0.001 compared with baseline). (B) LTP was induced in hippocampal pyramidal neurons in minocycline treated rats (n = 8 slices/6 rats, t-test; P>0.05 compared with baseline). (C) LTP was lost in hippocampal pyramidal neurons in Pb treated rats (n = 10 slices/6 rats, t-test; P<0.001 compared with baseline). (D) LTP was partly reversed in hippocampal pyramidal neurons in minocycline treated rats during Pb exposure (n = 9 slices/6 rats, t-test; P>0.05 compared with baseline). Pairing training is indicated by an arrow. The dashed line indicates the mean basal synaptic responses.

doi:10.1371/journal.pone.0043924.g006
Discussion

In the present study, we revealed that low-level Pb exposure can activate microglia and increase the release of some cytokines such as IL-1β and TNF-α and up-regulate the expression of iNOS, meanwhile, such changes were accompanied with hippocampal neuronal injury and hippocampal LTP impairment. Inhibiting Pb-induced microglia activation with minocycline also reversed Pb enhanced cytokine release as well as hippocampal neuronal injury and LTP impairment. This might be the underlying mechanisms that Pb impairs the hippocampus dependent learning and memory.

Pb toxicity on neurons but not glia cells is commonly taken into consideration when investigating the Pb-induced LTP and learning and memory impairments [32,33]. However, microglial cells play very important roles in varied physiological or pathophysiological functions [34,35]. They are the only nucleus phagocytes within CNS which are very important to the CNS immune responses [36]. The most remarkable feature of microglia is their extreme sensitivity to external environmental stimuli [36]. Moreover, activated microglia can release many cytokines, including TNF-α, IL-1β and interferon gamma (IFN-γ), and they also up-regulate the expression of iNOS. These factors are believed to be the main causes of neurotoxicity [27]. Treatment with some neurotoxic substance such as LPS impairs the rat learning and memory [26], and induces the microglia activation [37]. Pb is also a neurotoxic substance, therefore, it is natural to speculate that microglia may play an important role in the Pb-exposure induced learning and memory decline. In consistent with our hypothesis, we did detect Pb-induced microglia activation accompanied with the long-term potentiation impairment. Moreover, within our expectation, all these changes were also accompanied with hippocampal neuronal injury (by in situ TUNEL and LDH release assay).

Pb-exposure can impair the hippocampal LTP and such impairment is suggested to be mediated by the alteration of glutamate-N-methyl-D-aspartate (NMDA) receptor system [30]. These findings fell into the conventional neuron-centered theory. We moved forward and revealed the substantial involvement of microglia in the Pb-induced hippocampal LTP impairment. Furthermore, such effects of microglia might be mediated by the released cytokines. But it is not elucidated whether these effects are direct or indirect. By using microglia and hippocampal neurons co-culture model, we offered the evidence that microglia affects the neuronal functions. By treating rats with minocycline we evidence that microglia impair the hippocampal LTP. In other word, microglia affects hippocampal LTP and learning and memory indirectly. However, the possibility of direct effects can not be ruled out, as other studies demonstrated such evidence [39,40].

In this study, we treated microglia with 50 μmol Pb based on our MTT assay results. Although this dose is well beyond USA CDC stated safe limit, we reported these data to demonstrate that in an in vitro experiment, this dose Pb exposure results in robust activation of microglia and cytokines release, which may contribute to the primary cultured hippocampal neuronal injury.

In summary, our current study suggests that Pb neurotoxicity may be mediated by microglia activation, which induces high-level expression of many factors. Either alone or in combination, these inflammatory factors may cause hippocampal neuronal injury. This neurotoxic damage may affect the induce level of LTP, thus leading to learning and memory deficits.

Supporting Information

Figure S1  SD-rat blood leads level. SD-rat were treated with 0 (control) or 100, 200, 300 ppm lead acetate for 8 weeks from drink water. Data are expressed as mean ± SD (n = 6). (TIF)

Figure S2  SD-rat hippocampus lead level after treated with lead acetate for eight weeks. SD-rat were treated with 0 (control) or 100, 200, 300 ppm lead acetate for 8 weeks from drink water. Data are expressed as mean ± SD (n = 6). (TIF)

Figure S3  SD-rat body weight. SD-rat were treated with 0 (control) or 100, 200, 300 ppm lead acetate for 8 weeks. Data are expressed as mean ± SD (n = 20). (TIF)

Figure S4  SD-rat water consumption after treated with lead acetate. SD-rat were treated with 0 (control) or 100, 200, 300 ppm lead acetate for 8 weeks. Data are expressed as mean ± SD (n = 40). (TIF)

Figure S5  Effect of lead on primary cultured microglia by MTT assay. Primary cultured microglia were treated with 0, 25, 50, 100, 200, 300, 400, 500, 600 μmol lead acetate for 0, 3, 6, 9, 12, 24 and 48 hours. All data are expressed as mean ± SD (n = 6 well). (TIF)

Author Contributions

Conceived and designed the experiments: W-JL, J-YC M-CL. Performed the experiments: M-CL X-QL Y-YG M-GZ. Analyzed the data: M-CL H-LC. Contributed reagents/materials/analysis tools: WW X-FS. Wrote the paper: M-CL WW.

References

1. Bellinger DC (2004) Lead. Pediatrics 113: 1016–22.
2. Finkestein Y, Markowitz ME, Rosen JF (1998) Low-level lead-induced neurotoxicity in children: an update on central nervous system effects. Brain Res Brain Res Rev 27: 168–76.
3. Rosen JF (1995) Adverse health effects of lead at low exposure levels: trends in the management of childhood lead poisoning. Toxicology 97: 11–7.
4. Koller K, Brown T, Spurgeon A, Levy L (2004) Recent developments in low-level lead exposure and intellectual impairment in children. Environ Health Perspect 112: 987–94.
5. Needelman HL, McFarland C, Ness RB, Feuble SG, Tobin MJ (2002) Bone lead levels in adjudicated delinquents. A case control study. Neurotoxicol Teratol 24: 711–7.
6. Al-Saleh I, Nester M, Mashhour A, Moncari L, Shinwari N, et al. (2009) Prenatal and postnatal lead exposure and early cognitive development: longitudinal study in Saudi Arabia. J Environ Pathol Toxicol Oncol 28: 283–302.
7. Bird CM, Burgess N (2006) The hippocampus and memory: insights from spatial processing. Nat Rev Neurosci 9: 162–94.
8. Blis TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361: 31–9.
9. Morris RG, Frey U (1997) Hippocampal synaptic plasticity: role in spatial learning or the automatic recording of attended experience? Philos Trans R Soc Lond B Biol Sci 352: 1489–503.
10. Tanaka S, Kondo H, Kanda K, Ashino T, Nakamachi T, et al. (2011) Involvement of interleukin-1 in lipopolysaccharide-induced microglial activation and learning and memory deficits. J Neurosci Res 89 (4): 506–14.
11. Kebleh JP, Dou H, Geudelina HE, Xiong H (2009) 4-Aminopyridine improves spatial memory in a murine model of HIV-1 encephalitis. J Neuroimmune Pharmacol 4:317–27.
12. Zhou JJ, Yang T, Wei X, Liu Y, Xin WJ, et al. (2011) Brain-derived neurotrophic factor contributes to spinal long-term potentiation and mechanical hypersensitivity by activation of spinal microglia in rat. Brain Behav Immun 25:322–34.
13. Chu YX, Zhang Y, Zhang YQ, Zhao QZ (2010) Involvement of microglial P2X7 receptors and downstream signaling pathways in long-term potentiation of spinal nociceptive responses. Brain Behav Immun 24:1176–89.
14. Xiong H, Boyle J, Winkelhauer M, Gorantla S, Zheng J, et al. (2003) Inhibition of long-term potentiation by interleukin-8: implications for human immunodeficiency virus-1-associated dementia. J Neurosci Res 71:600–7.

15. Reza B, Ali N, Azhdar H, Alireza A, Ali K (2008) Effects of low-level lead exposure on blood pressure and function of the rat isolated heart. Indian J Pharmacol 40: 69–72.

16. Zhao F, Cai TJ, Liu MC, Zheng G, Luo WJ, et al. (2009) Manganese induces dopaminergic neurodegeneration via microglial activation in a rat model of manganism. Toxicol Sci 107: 156–64.

17. Brewer GJ (1997) Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods 71: 143–55.

18. Potter SM, DeMarce TB (2001) A new approach to neural cell culture for long-term studies. J Neurosci Methods 110: 17–24.

19. Yu AC, Sun CX, Li Q, Liu HD, Wang CR, et al. (2005) Identification of a mouse synaptic glycoprotein gene in cultured neurons. Neurochem Res 30: 1209–94.

20. Wei F, Wang GD, Kerchner GA, Kim SJ, Xu HM, et al. (2001) Genetic enhancement of inflammatory pain by forebrain NR2B overexpression. Nat Neurosci 4: 164–9.

21. Paxinos G, Franklin K (2001) The mouse brain in stereotaxic coordinates. San Diego: Academic Press.

22. Brown S, Dragann N, Vogel WH (1971) Effects of lead acetate on learning and memory in rats. Arch Environ Health 22: 370–2.

23. Song B, Han CC, Sun X, Peng L, Niu YJ (2006) Effects of prenatal exposure to low level lead on learning and memory of rats' offspring. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 24: 399–402.

24. Yang Y, Ma Y, Ni L, Zhao S, Li L, et al. (2003) Lead exposure through gestation-only caused long-term learning and memory deficits in young adult offspring. Exp Neurol 184: 489–95.

25. Zhao M G, Toyoda H, Lee YS, Wu LJ, Ko SW, et al. (2005) Roles of NMDA NR2B subtype receptor in preferential long-term potentiation and contextual fear memory. Neuron 45: 403–15.

26. Tanaka S, Ide M, Shibutani T, Ohtaki H, Numazawa S, et al. (2006) Lipopolysaccharide-induced microglial activation induces learning and memory deficits without neuronal cell death in rats. J Neurosci Res 83: 537–66.

27. Frigo DE, Vigil KA, Struckhoff AP, Elliott S, Beckman BS, et al. (2005) Xenohiotic-induced TNF-alpha expression and apoptosis through the p38 MAPK signaling pathway. Toxicol Lett 155: 227–38.

28. Liu MC, Cai TJ, Zhao F, Zheng G, Wang Q, et al. (2009) Effect of microglia activation on dopaminergic neuronal injury induced by manganese, and its possible mechanism. Neurotox Res 16: 42–9.

29. Arvin KL, Han BH, Du Y, Lin SZ, Paul SM, et al. (2002) Minocycline markedly protects the neonatal brain against hypoxic-ischemic injury. Ann Neurol 52: 54–61.

30. Tomas-Camardiel M, Riee I, Herrera AJ, de Pablos RM, Cano J, et al. (2004) Minocycline reduces the lipopolysaccharide-induced inflammatory reaction, peroxynitrite-mediated nitration of proteins, disruption of the blood-brain barrier, and damage in the nigral dopaminergic system. Neurobiol Dis 16: 190–201.

31. Yrjanheikki J, Tikka T, Kriinmae R, Goldsteins G, Chan PH, et al. (1999) A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci USA 96: 13496–90.

32. Yin ST, Tang ML, Su L, Chen L, Hu P, et al. (2006) Effects of Epigallocatechin-3-gallate on lead-induced oxidative damage. Toxicology 249: 45–54.

33. She JQ, Wang M, Zhu DM, Tang M, Chen JT, et al. (2008) Monosialoanglioside (GM1) prevents lead-induced neurotoxicity on long-term potentiation, SOD activity, MDA levels, and intracellular calcium levels of hippocampi in rats. Naunyn Schmiedebergs Arch Pharmacol 379: 517–24.

34. Schlegelmilch T, Herke K, Peri F (2011) Microglia in the developing brain: from immunity to behaviour. Curr Opin Neurobiol 21: 5–10.

35. Nakajima K, Kobayashi S (2001) Microglia activation and their significance in the central nervous system. J Biochem 130: 169–75.

36. van Rossum D, Hanisch UK (2004) Microglia. Metab Brain Dis 19: 393–411.

37. Espinosa-Oliva AM, de Pablos RM, Villarain RF, Arguelles S, Venero JL, et al. (2009) Stress is critical for LPS-induced activation of microglia and damage in the rat hippocampus. Neurobiol Aging 32: 85–102.

38. Gilbert ME, Lasley SM (2007) Developmental lead (Pb) exposure reduces the ability of the NMDA antagonist MK-801 to suppress long-term potentiation (LTP) in the rat dentate gyrus, in vivo. Neurotoxicol Teratol 29: 305–9.

39. Cao XJ, Cao J, Chen TT, Chen WH, Ruan DY (2010) Protective effects of omega-3 fish oil on lead-induced impairment of long-term potentiation in rat dentate gyrus in vivo. Sheng Li Xue Bao 62: 225–30.

40. Cao XJ, Wang M, Chen WH, Zhu DM, She JQ, et al. (2009) Effects of chronic administration of melatonin on spatial learning ability and long-term potentiation in lead-exposed and control rats. Biomed Environ Sci 22: 70–5.