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Research paper

Role of NF-κB in lead exposure-induced activation of astrocytes based on bioinformatics analysis of hippocampal proteomics

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ABSTRACT

Lead (Pb), as a heavy metal, is used in batteries, ceramics, paint, pipes, certain ceramics, e-waste recycling, etc. Chronic Pb exposure can result in the inflammation of the central nervous system, as well as neurobehavioral changes. Both glial cells and neurons are involved in central nervous injury following Pb exposure. However, significant cellular events and their key regulators following Pb exposure remain to be elucidated. In this study, rats were randomly exposed to 250 or 500 mg/L PbAc for 9 weeks. Hippocampal proteomics was performed using isobaric tags for relative absolute quantification. Bioinformatics analysis was used to identify 301 and 267 differentially expressed proteins—which were involved in biological processes, including glial cell activation, neural nucleus development, and mRNA processing—in the low and high Pb exposure groups, respectively. Gene Set Enrichment Analysis showed that astrocyte activation was identified as a significant cellular event occurring in the low- or high-dose Pb exposure group. Subsequently, in vivo and in vitro models of Pb exposure were established to confirm astrocyte activation. As a result, glial fibrillary acidic protein expression in astrocytes was much higher in the Pb exposure group. Moreover, the mRNA expression of neurotoxic reactive astrocyte genes was much higher than that of the control group. The analysis of transcription factors indicated that NF-κB was screened as the top transcription factor, which might regulate astrocyte activation following Pb exposure in the rat hippocampus. The data also showed that the inhibition of NF-κB transcription suppressed astrocyte activation following Pb exposure. Overall, astrocyte activation was one of the significant cellular events following Pb exposure in the rat hippocampus, which was regulated by the NF-κB transcription factor, suggesting that inhibiting astrocyte activation may be a potential target for the prevention of Pb neurotoxicity.

1. Introduction

Lead (Pb) is a heavy metal used in industrial production and found in daily life environments [1]. Chronic environmental Pb exposure can impair the central nervous system (CNS) and cardiovascular system [2,3]. Studies have demonstrated that Pb can accumulate in the hippocampus, leading to cognitive impairment and depression [4]. It can also trigger neurotoxicity via various cellular events. Long-term Pb exposure can trigger oxidative stress, leading to neuronal apoptosis [5]. Moreover, Pb exposure exacerbates neuroinflammation via microglial activation in the developing mouse brain [6]. However, significant cellular events following Pb exposure remain to be elucidated.

Recent studies have shown that Pb can impair glial cell function in the CNS [5,7]. Glial cells, including astrocytes, microglia, and oligodendrocytes, supply energy to neurons, maintain the blood–brain barrier, and support synaptic and immune response [8–10]. Dysfunctional

Abbreviations: Pb, Lead; CNS, central nervous system; GFAP, glial fibrillary acidic protein; GSEA, Gene Set Enrichment Analysis; TGF, transforming growth factor; NOR, Novel Object Recognition; MWM, Morris water maze; GO, gene ontology; DEPs, differentially expressed proteins; PDTC, pyrrolidinedithiocarbamic acid; ELISA, enzyme-linked immunosorbent assay.

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Table 1
The primer sequences used for real-time PCR.

Gene	Forward primer	Reverse primer	Annealing temperature
Lcn2	5′-CACAGTCGGGAGCCCTAAAG-3′	5′-TTCCCTCAGACACATGGTGC-3′	55 °C
Steap4	5′-CACTATCGGCCCCAGAACAG-3′	5′-AGCAACCGAGTTCTGTCGAG-3′	55 °C
S100a10	5′-GGGGGCAATGTTGCTCAATG-3′	5′-TGAGGACCTTGGGTCTCTGT-3′	57 °C
Cd14	5′-GGTTTGCAGGCGACAAAGAC-3′	5′-ATTCCTCAAGTGACCCCGTG-3′	60 °C
Gfap	5′-AAGAACTTGGACCAGGACGC-3′	5′-CTCTGCTTTTCGCTTGAGCC-3′	60 °C
Amigo2	5′-AGGGCCATAGGGAAGCTTGA-3′	5′-AGCCATCCACTGGGTAAAGG-3′	56 °C
Tgm1	5′-TCGCACTCAATACGAGGCAG-3′	5′-CATTTGCCGCTCTAGGGACT-3′	60 °C
Cd109	5′-ACTGAAGCCTTTCTCGGAGC-3′	5′-TGAAAGCGCTGGACCAATCT-3′	57 °C
Cxcl10	5′-TCGAGAAGGTGTTGGCAGTC-3′	5′-ATGGCCTGACTCTCGTGTTG-3′	55 °C
β-actin	5′-GTTGGTTGGAGCAAACATCCC-3′	5′-TTAGGAGTGGGGGTGGCTTT-3′	55–60 °C

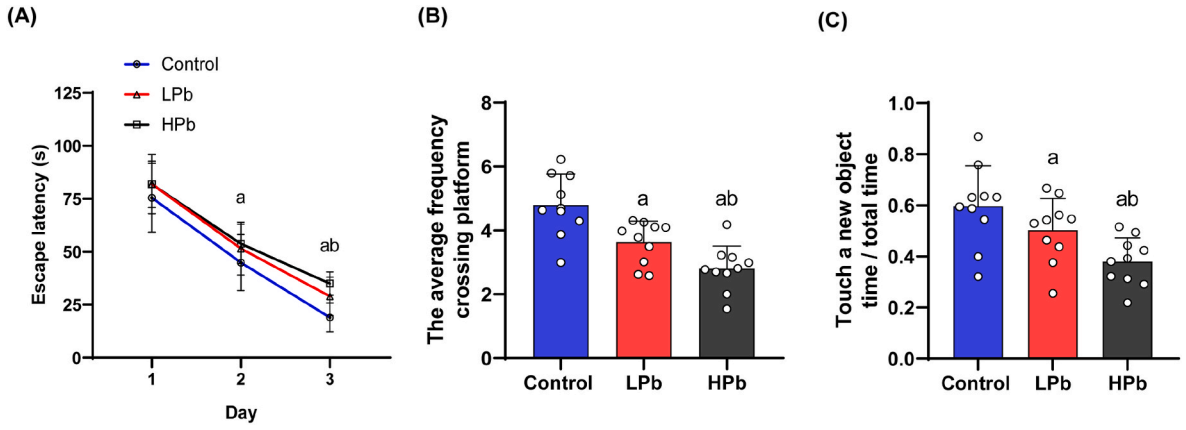

Fig. 1. Pb exposure induced learning memory deficits and decreases in exploring ability. A, B: Changes of directional navigation and space exploration ability in LPb and HPb groups after Pb exposure in MWM (n = 10); C: Effect of Pb exposure on new object recognition time in LPb and HPb groups (n = 10); $^{a}P < 0.05$ vs Control; $^{b}P < 0.05$ vs LPb.

astrocytes or microglia aggravated Aβ accumulation and impaired the learning memory ability of 5xFAD mice [11,12]. Pb exposure can also induce the homeostatic state of microglia or astrocytes in the activated state [13]. Previous studies have shown that Pb promotes hippocampal microglial activation via the TLR4-MyD88-NF-κB signaling pathway, aggravating CNS damage in mice [14]. Pb also promotes astrocyte activation and enhances glial fibrillary acidic protein (GFAP) expression in the hippocampus [15]. However, the significant changes in glial cells following Pb exposure remain unclear.

Mass spectrometry-based proteomics is an effective method for identifying different proteins under the pathophysiological conditions of cells or tissues [16]. Bioinformatic approaches have been widely used to determine underlying molecular mechanisms, which form the basis of proteomics analysis [17–19]. Gene Set Enrichment Analysis (GSEA) is a powerful analytical method for identifying significant cellular events [20,21]. GSEA has been used to determine that the metabolic disorders of sphingolipids and glutathione were vital processes in Parkinson's disease [22]. In addition, microglial transforming growth factor (TGF)-β signaling has been found to be a therapeutic target for depression through GSEA [23]. In this study, we established a Pb-exposed rat model to identify the differentially expressed proteins in the hippocampus. GSEA was performed to explore significant cellular changes. Transcription factor analysis was used to explore the mechanisms underlying significant cellular changes. Our findings may provide new insights into the mechanisms underlying Pb neurotoxicity.

2. Methods

2.1. Animals and treatment

A total of 30 SPF male Fisher 344 rats, aged 6–8 weeks (277.72 ± 18.28 g), were purchased from Beijing Huafukang Biotechnology Co., Ltd., and randomly divided into the control, Low Pb (LPb) (drinking water with 250 mg/L PbAc), or High Pb (HPb) (drinking water with 500 mg/L PbAc) groups for 9 weeks [24,25]. The rats were housed (n = 2/cage) in a room with a fully controlled environment—at constant temperature and humidity on a 12:12 h light/dark cycle—with free access to food and water. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the North China University of Science and Technology Animal Ethics Committee (No. 2020-SY-001).

2.2. Cell culture and treatment

Astrocyte cell-line MA-c cells were cultured in Dulbecco's modified Eagle's medium, which was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C with 5% CO_2 in the incubator. For each experiment using MA-c cells, the plating density was 1×10^4 cell well^{-1} in 96-well plates and 5×10^5 cell well^{-1} in 6-well plates. After reaching 80% confluence, MA-c cells were randomized to receive one of the following treatments: 5, 10, and 20 μM PbAc, pyrrolidine dithiocarbamic acid (PDTC) (dissolved in 1 × PBS, 10 μM), or PDTC+20 μM PbAc (pretreated PDTC for 1 h) for 24 h.

2.3. Morris water maze (MWM) and novel object recognition (NOR) test

After nine weeks of Pb exposure, rat spatial memory performance was evaluated using an MWM (180 cm diameter, 45 cm high) test. Briefly, the rats were placed in the maze and allowed to search for the platform within 120 s for three days. On the fourth day, the platform was removed, the rats were placed in the water away from the previous platform, and the frequency of crossing the platform was recorded within 120 s.

NOR was used to detect the memory ability of the rats. During the

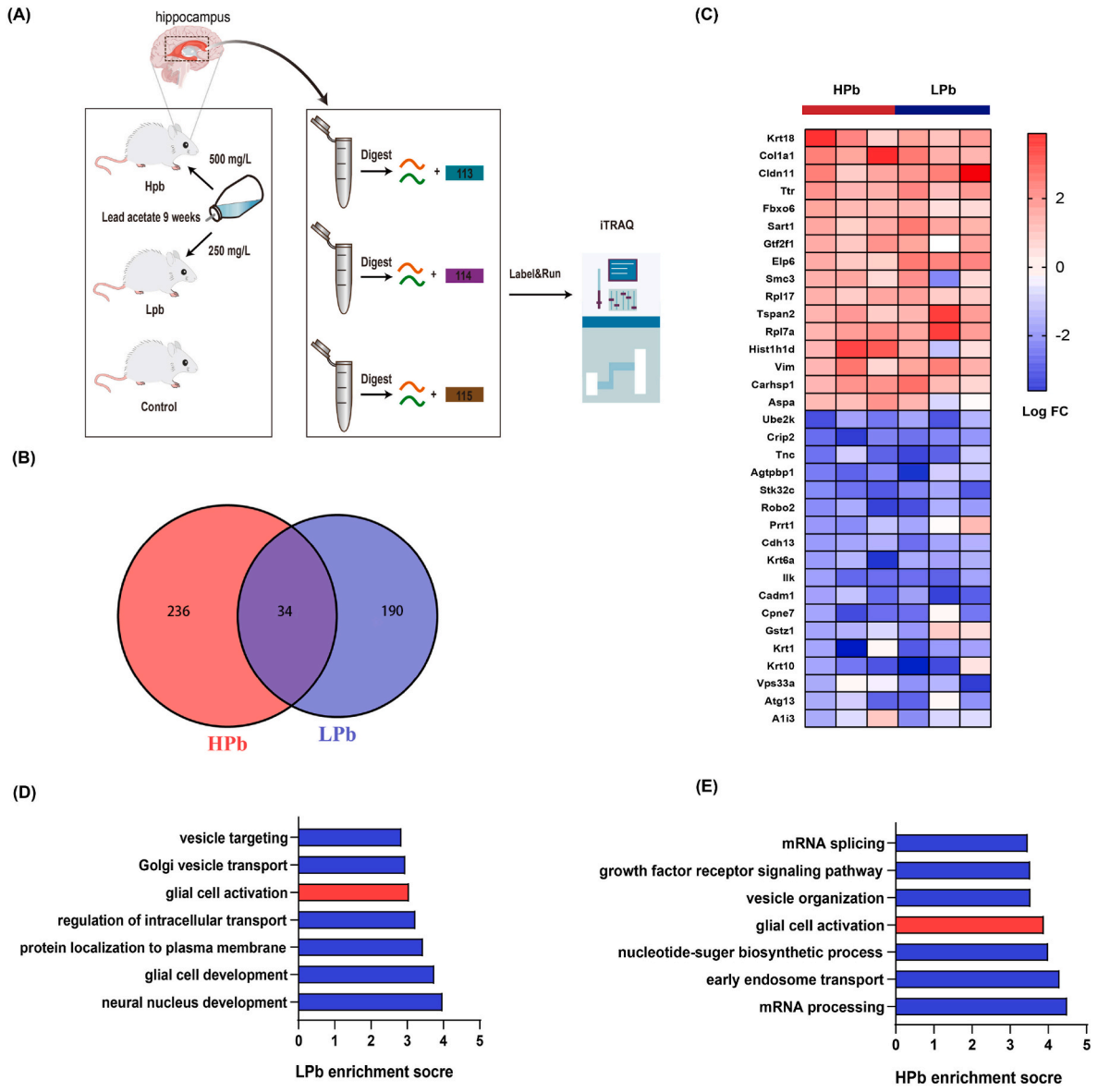

Fig. 2. DEPs were involved in glial cell activation in the rat hippocampus following Pb exposure. A: Flow chart of proteomics detection in rats. SPF male Fisher 344 rats were divided into control, LPb, and HPb groups. After 9 weeks of Pb acetate exposure, the hippocampus was extracted from the rats. Proteomic sequencing was performed on the digested tissue by trypsin using iTRAQ; B: The Venn plot of DEPs in the LPb and HPb groups; C: The heatmap of DEPs in the LPb and HPb groups; D, E: The top 7 GO terms for DEPs in the LPb and HPb groups.

adaptation period, the rats were allowed to exercise for 10 min in the experimental apparatus. In the training session, two identical objects were placed on both the right and left sides of the arena. The rats were placed in the device with their backs facing the object, and the time spent exploring each object was recorded. During the testing session, one of the objects was changed to a new object with different shapes, sizes, and colors, and the recognition time of the old or new objects was recorded within 3 min.

2.4. Hippocampal protein sequencing

The hippocampus was carefully isolated from the brains of individual rats for protein sequencing (n = 3 for each group). Proteomic detection was conducted based on the workflow developed by Huada Biotechnology Limited Company (Beijing, China). Briefly, rat hippocampal tissue was dissected and homogenized by ultrasonication for 30 s on ice. Tissue proteins were released by protein lysis (Solarbio, China). Soluble proteins were obtained by centrifugation at 12,000 rpm for 15 min at 4 °C. Protein concentration was determined using a bicinchoninic acid protein assay kit (Solarbio, China). Subsequently, 100 μg of protein was isolated from each sample for protein enzymolysis and isobaric tags for relative absolute quantification (iTRAQ) labeling. A Triple TOF 5600 (SCIEX, USA) was used to quantify the relative protein levels.

2.5. Bioinformatics analysis of proteomics

2.5.1. Differential protein screening and enrichment analysis in LPb and HPb

Hippocampal proteins were analyzed using isobaric tags for relative and absolute quantification (iTRAQ). An FC of $\geq$1.2 or FC $\leq$ 0.8 and P-value $<$0.05 were set as the thresholds for screening differentially expressed proteins. Furthermore, we performed gene ontology (GO) functional analysis to identify the significant biological processes in the LPb and HPb groups. GO functional analysis was performed using the R package ClusterProfiler [26]. A corrected P value of 0.05 was used to identify enriched GO terms. The top seven items filtered by protein

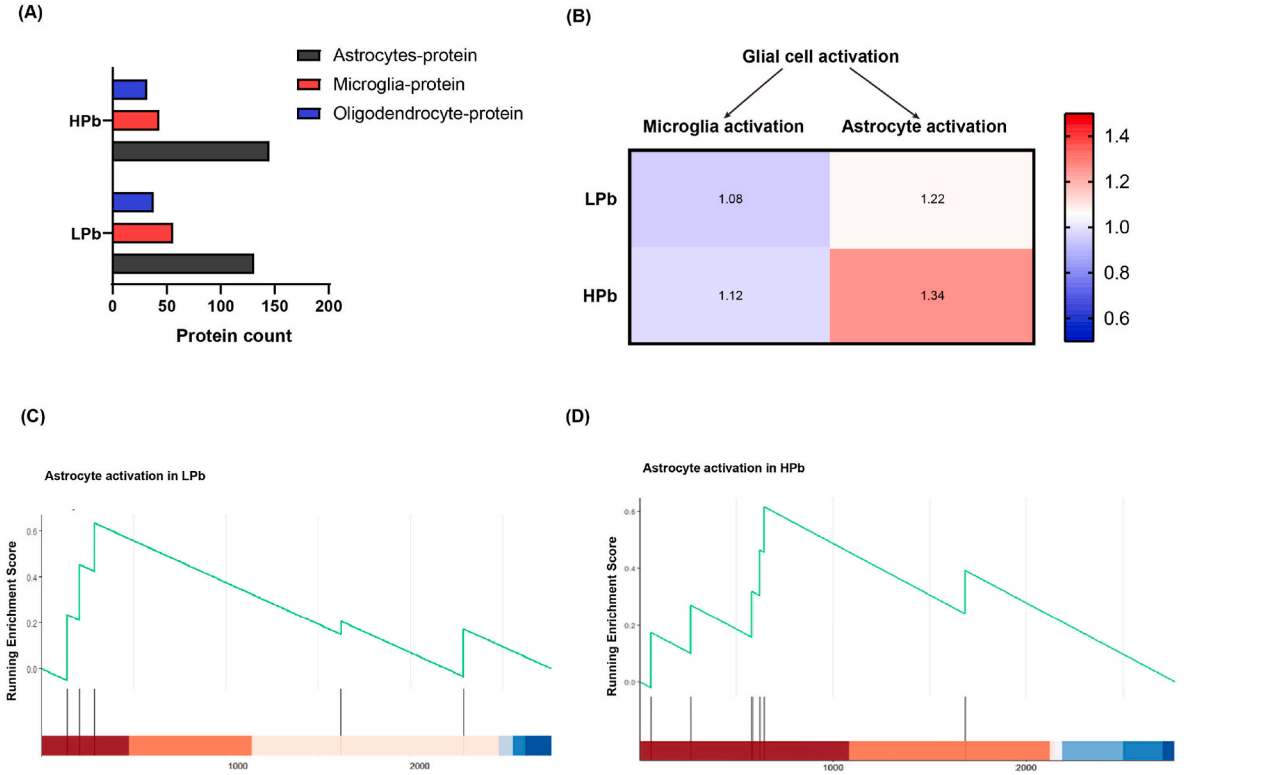

Fig. 3. Astrocyte activation was identified as one of the significant cellular events in the rat hippocampus following Pb exposure. A: Cell-type analysis of all proteins in the LPb and HPb groups; B: The glial cell-related functional modules following Pb exposure; C, D: The functional modules of astrocyte activation in the LPb and HPb groups.

count and fold enrichment are shown in the figures.

2.5.2. Identification of the proteins counts in different glial cells of the rat hippocampus following Pb exposure

Protein marker sets of astrocytes, microglia, and oligodendrocytes were obtained from the GenBank database. The keywords of search in Genecard (https://www.genecards.org/) included "astrocyte," "microglia," and "oligodendrocyte." Furthermore, a total of 1000 proteins were selected as protein markers, which had the highest correlation for each cell in the dataset to match the total proteins in LPb or HPb.

2.5.3. Identification of the significant changes in the cellular events in LPb and HPb

GSEA was used to identify significant changes in cellular events. The data were used to perform GSEA analysis as follows: (1) The subseries terms of glial cell activation include astrocyte and microglial activation; (2) NF-κB transcription factor activity, proteins involved in astrocyte activation, and microglial activation; NF-κB transcription factor activity files were obtained from the MSigDB website database; and (3) Protein marker sets of astrocytes in the LPb and HPb; GSEA was performed using the R package ClusterProfiler [26]. The parameters were set as follows: minGSSize = 3, maxGSSize = 10, p-value cutoff = 0.05, and = BP. The standard for differential biological process identification was set at $p < 0.05$, NES < -1, or >1.

2.5.4. Prediction of potential transcription factors of astrocyte activation

The TRRUST database (https://www.grnpedia.org/trrust/) and analytical tools were used to identify transcription factors related to astrocyte activation. Based on the overlapping genes and q value, the -top 15 transcription factors were selected, and t in Cytoscape was applied to calculate the center node of the network. The score of the center node was based on the MNN algorithm in CytoHubba (analysis APP of Cytoscape).

2.6. Cell viability assay

Cell viability was measured by using CCK 8 kit (Solarbio, China). MA-c cells were seeded in 96-well plates. When the MA-c cells cultures reached 80% density, they were treated with different dose of PbAc for 24 h, the cells were incubated with CCK-8 solution. Absorbance (A) values at 450 nm were measured on an automated reader (Bio Tek, USA).

2.7. Immunofluorescence assays

The MA-c cells were fixed with 4% paraformaldehyde for 20 min. After permeabilization with Triton × 100, the cells were blocked with a 3% blocking solution for 1.5 h. The cells were incubated with primary antibodies against NF-κB (1:100) and GFAP (1:100) at 4 °C overnight. Nuclei were stained with Hoechst 33342. Images were obtained using an Olympus FV3000 confocal microscope (Olympus, Tokyo, Japan).

2.8. Enzyme-linked immunosorbent assay (ELISA)

The contents of IL6, IL-1β, and TNF-α in the rat hippocampus and MA-c cell supernatant with different treatments were measured using commercially available ELISA kits (Solarbio, China). Briefly, the supernatant of MA-c cells and the hippocampal tissues of rat in different groups were collected separately. Hippocampal tissues were dissected and homogenized by ultrasonication for 30s on ice. Tissue homogenate solution and supernatant of MA-c cells were centrifuged at 5000×*g* for 10 min at 4 °C. The solution of tissue homogenate and the supernatant of MA-c cells were collected and diluted (supernatant of MA-c cells was undiluted) for measurement. Then, all operations were carried out in strict accordance with the operating instructions of the kit. The detection range for the IL6, IL-1β, TNF- α kits were 18.75–1000, 7.8–500, 7.8–500 pg/ml(mg) respectively.

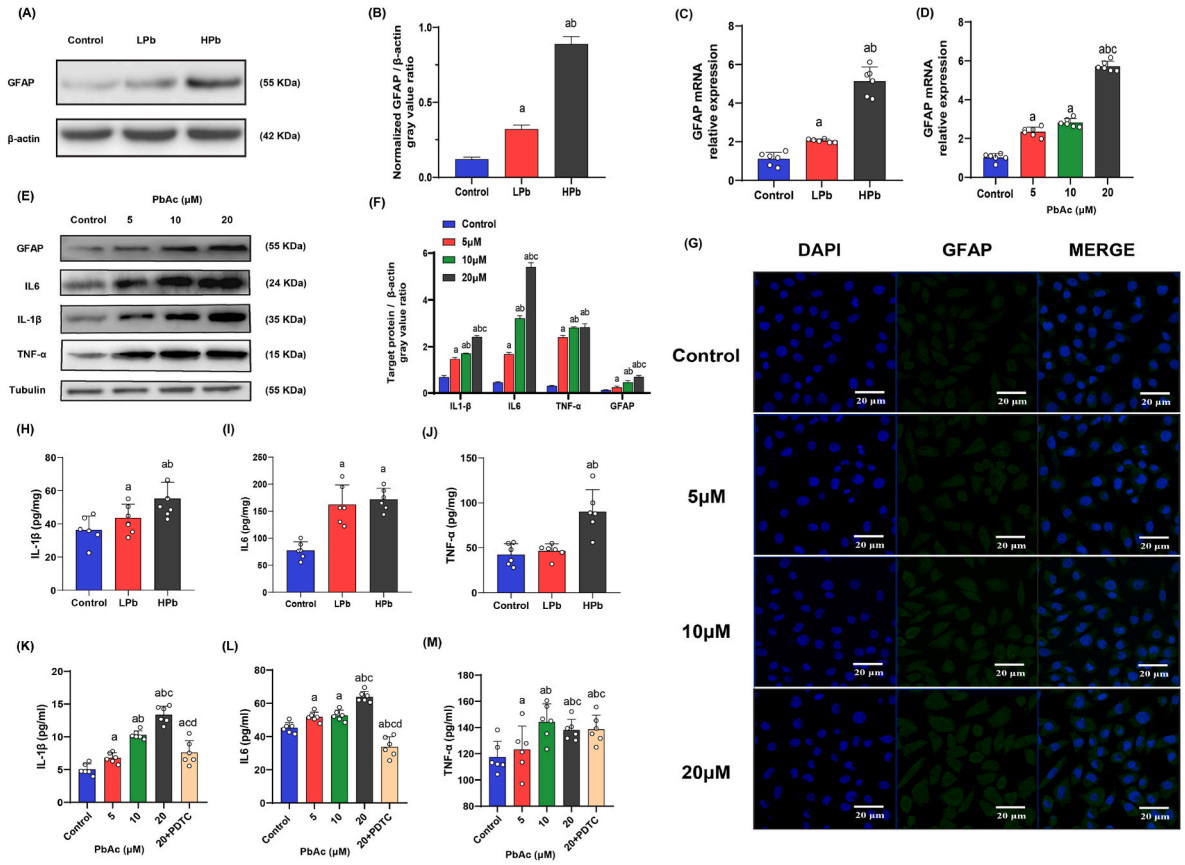

Fig. 4. Pb exposure induced the activation of astrocytes in vivo and in vitro. A, B: The protein expression of GFAP in the rat hippocampus following Pb exposure (n = 3); C, D: the GFAP mRNA expression in vivo and in vitro (n = 6); E: The protein expression of IL6, IL-1β, TNF-α, GFAP, and tubulin in astrocyte-treated Pb acetate with different concentrations (n = 3); G: Effect of Pb exposure on GFAP expression in MA-c cells (immunofluorescence, × 400, n = 3); H–J: The TNF-α, IL6, and IL-1β protein concentrations in the rat hippocampus following Pb exposure (n = 6); K–M: TNF-α, IL6, and IL-1β expression in MA-c cells supernatant treated with PbAc in different dosages (n = 6). The MA-c cell line was treated with PbAc for 24 h or pretreated with PDTC for 1 h; $^{a}P < 0.05$ vs Control; $^{b}P < 0.05$ vs LPb in A-D; $^{b}P < 0.05$ vs 5, $^{c}P < 0.05$ vs 10, $^{d}P < 0.05$ vs 20 in F-M.

2.9. Western blotting

Protein concentration was determined using a BCA protein assay kit. (Solarbio, China). The protein was separated by 12% SDS-PAGE gel electrophoresis and followed by transferal to polyvinylidene fluoride membrane. The membranes were blocked in 5% skim milk (in TBST) and then followed by incubation with anti–NF–κB (1:1000), anti-Tubulin (1:2000), IL6 (1:1000), IL-1β (1:500), TNF-α (1:500), GFAP (1:1000) (Proteintech, China) antibodies overnight at 4 °C. After washing with TBST, blots were reacted with secondary HRP-linked anti-rabbit (1:3000) (Proteintech, China) at room temperature for 2 h.

2.10. Real-time PCR

Total RNA was extracted using TRIZOL, and RNA was converted into complementary DNA (cDNA) using a cDNA Reverse Transcription Kit (Roche, Switzerland). β-Actin was chosen as the reference gene (housekeeping). Amplification was performed over 40 cycles. All primer sequences and annealing temperatures are listed in Table 1:

2.11. Statistical analysis

All statistical analyses were performed using the IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA). Normal distributions of data are expressed as mean ± SD. One-way ANOVA followed by Tukey's post hoc test was used for group analyses, and Student's t-test was used for statistical analyses between two groups.

3. Results

3.1. Pb exposure impaired the learning memory and exploring ability

To investigate whether Pb exposure affects learning memory and exploring ability, the MWM and NOR tests were performed. The results showed that the escape latency of rats in the LPb or HPb groups was increased compared to that of the control group (Fig. 1 A, B). Meanwhile, the average frequency of crossing platforms in the LPb and HPb groups was reduced by 23% and 45%, respectively, compared with that of the control group. In addition, the NOR results also showed that the ratio of new object touching time in the LPb and HPb groups was much lower than that of the control group (Fig. 1C). These results demonstrated that Pb exposure could impair learning memory and exploratory activity of rats.

3.2. Glial cell activation was identified as an essential biological process in rat hippocampus following Pb exposure

iTRAQ was performed to detect changes in protein levels in the rat hippocampus following Pb exposure (Fig. 2A). A total of 2888 proteins were identified in the LPb group, among which 216 were upregulated and eight were downregulated in comparison to control. Meanwhile, 2890 proteins were identified in the HPb group, among which 263 proteins were upregulated and seven proteins were downregulated (Supplemental Fig. S1 A, B). The shared differentially expressed proteins (DEPs) in the LPb and HPb groups included Krt18, Col1a1, Cldn11, Ttr,

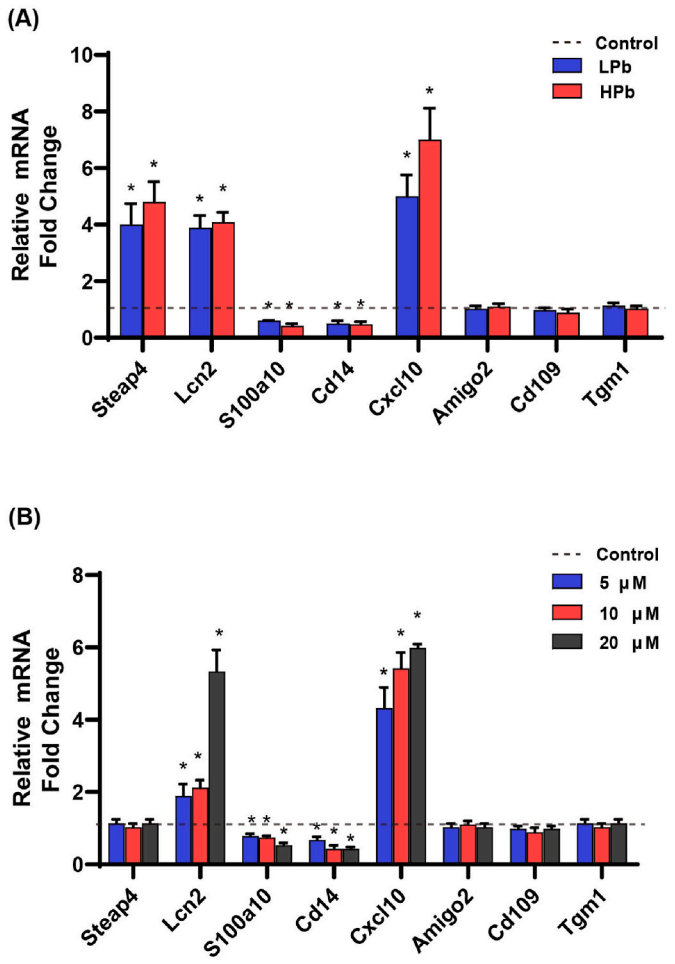

Fig. 5. Pb exposure affects the expression of neurotoxic and neuroprotective reactive astrocyte genes in vivo and in vitro (n = 6); A: The neurotoxic and neuroprotective astrocyte gene expression in LPb and HPb; B: The neurotoxic and neuroprotective astrocyte gene expression in MA-c cells treated with PbAc in different dosages (n = 6); *$P < 0.05$ vs Control.

Fbxo6, Sart1, Gtf2f1, Elp6, Smc3, Rpl17, Tspan2, Rpl7a, Hist1h1d, Vim, Carhsp1, Aspa, Ube2k, Crip2, Tnc, Agtpbp1, Stk32c, Robo2, Prrt1, Cdh13, Krt6a, Ilk, Cadm1, Cpne7, Gstz1, App, Grn, Vps33a, Atg13, and A1i (Fig. 2 B, C). The upregulated and downregulated proteins were analyzed using GO enrichment and KEGG analyses. The results showed that the top five biological processes in the LPb group were glial cell activation, neural nucleus development, glial cell development, protein localization to the plasma membrane, and regulation of intracellular transport. The top five biological processes in the HPb group were early endosome transport, mRNA processing, glial cell activation, vesicle organization, and nucleotide-sugar biosynthetic process. Interestingly, glial cell activation was upregulated in both LPb and HPb groups (Fig. 2 D, E). KEGG analysis results showed that the DEPs were enriched in the VEGF signaling pathway in the LPb group and AMPK signaling pathway in the HPb group (Supplemental Fig. S1 C). These results indicated that glial cell activation is an essential biological process in the rat hippocampus following Pb exposure.

3.3. Astrocyte activation was screened as significant cellular event in rat hippocampus following Pb exposure

The identified proteins (2888 proteins in the LPb and 2890 proteins in the HPb groups) were matched to a known protein database in terms of microglia, astrocytes, and oligodendrocytes. The results showed that altered proteins of astrocytes accounted for the largest proportion, and the number of astrocyte-changed proteins was 151 and 138 in the HPb and LPb groups, respectively (Fig. 3 A). Thus, astrocyte and microglial activation were involved in glial cell activation. GSEA analysis was performed to identify astrocyte and microglial activation in astrocyte-related proteins and microglia-related proteins. Notably, astrocyte activation was the top module, which was 1.22- fold in the LPb group and 1.34- fold in the HPb group compared with the control group (Fig. 3B–D). In addition, the GSEA results also showed that altered protein expression in Pb exposed astrocytes was accompanied by upregulated IL6, IL-1β, and TNF-α secretion (Supplemental Fig. S2 A, B).

3.4. Pb exposure did induce the astrocytes activation in vitro and in vivo

It is well known that GFAP upregulation serves as a marker for astrocyte activation. The results showed that GFAP expression was markedly higher in the rat hippocampus following Pb exposure, which was 1.9- fold in the LPb group and 4.1- fold in the HPb group compared with the control group (Fig. 4 A, B). Furthermore, MA-c cells, an astrocyte cell line, were treated with PbAc at dosages of 5, 10, and 20 μM to investigate the changes in GFAP expression. The results showed that GFAP protein and mRNA expression increased in the 5 μM, 10 μM, and 20 μM groups in a concentration-dependent manner (Fig. 4D–F) (Supplemental Fig. S3). In addition, the immunofluorescence staining results also showed that GFAP expression increased with increasing PbAc concentration, which further supported astrocyte activation (Fig. 4 G). Another characteristic feature of astrocyte activation is an increase in the levels of inflammation factors. TNF-α, IL6, and IL-1β protein expression was detected in the rat hippocampus and MA-c cells after Pb exposure. The results showed that IL6 was highly expressed in the LPb and HPb groups, which was 2.1- and 2.3- fold higher than that in the control group (Fig. 4 I). TNF-α and IL-1β protein expression in the hippocampus of the HPb groups was 1.7-fold and 1.5-fold higher than that in the control group (Fig. 4 H, J). Meanwhile, TNF-α, IL6, and IL-1β protein expression in MA-c cells was significantly increased in the 5 μM, 10 μM, and 20 μM groups compared with the control group (Fig. 4 E, F). TNF-α, IL6, and IL-1β expression in MA-c cell supernatants following Pb treatment also significantly increased. (Fig. 4K–M). Collectively, our results suggest that Pb exposure induces astrocyte activation both in vivo and in vitro.

CNS injuries can elicit at two types of "reactive" astrocytes with strikingly different properties, one type being neurotoxic and the other neuroprotective. We detected neurotoxic astrocyte genes, including Lcn2, Steap4, Cxcl10, and Amigo, and neuroprotective astrocyte genes, including S100a10, Cd14, Tgm1, and Cd109, in the rat hippocampus and MA-c cells following Pb exposure. The mRNA expressions of Lcn2, Steap4, and Cxcl10 were significantly higher in the LPb and HPb groups than in the control group. In addition, S100a10 and Cd14 mRNA expression levels were lower in the LPb and HPb groups (Fig. 5 A). There were no significant differences in Amigo2, Cd109, or Tgm1 mRNA expression. Moreover, the mRNA expression of Lcn2 and Cxcl10 was increased and the mRNA expression of Cd14 was decreased in MA-c cells treated with different concentrations of PbAc (Fig. 5 B). There were no significant differences in Steap4, Amigo2, Cd109, or Tgm1 mRNA expression.

3.5. NF-κB transcription factor contributes to astrocyte activation following Pb exposure

Transcription factor identification was performed using the online tool TRRUST to screen for transcription factors involved in astrocyte activation. Based on the analysis results, the top five transcriptional regulators related to astrocyte activation were NF-κB, Stat1, Jun, Rela, and AHR. Additionally, a regulatory network for astrocyte activation-associated transcription factors was constructed. Interestingly, the NF-κB transcription regulator was identified as the central node of the network (Fig. 6 A). Meanwhile, GSEA analysis results also showed that

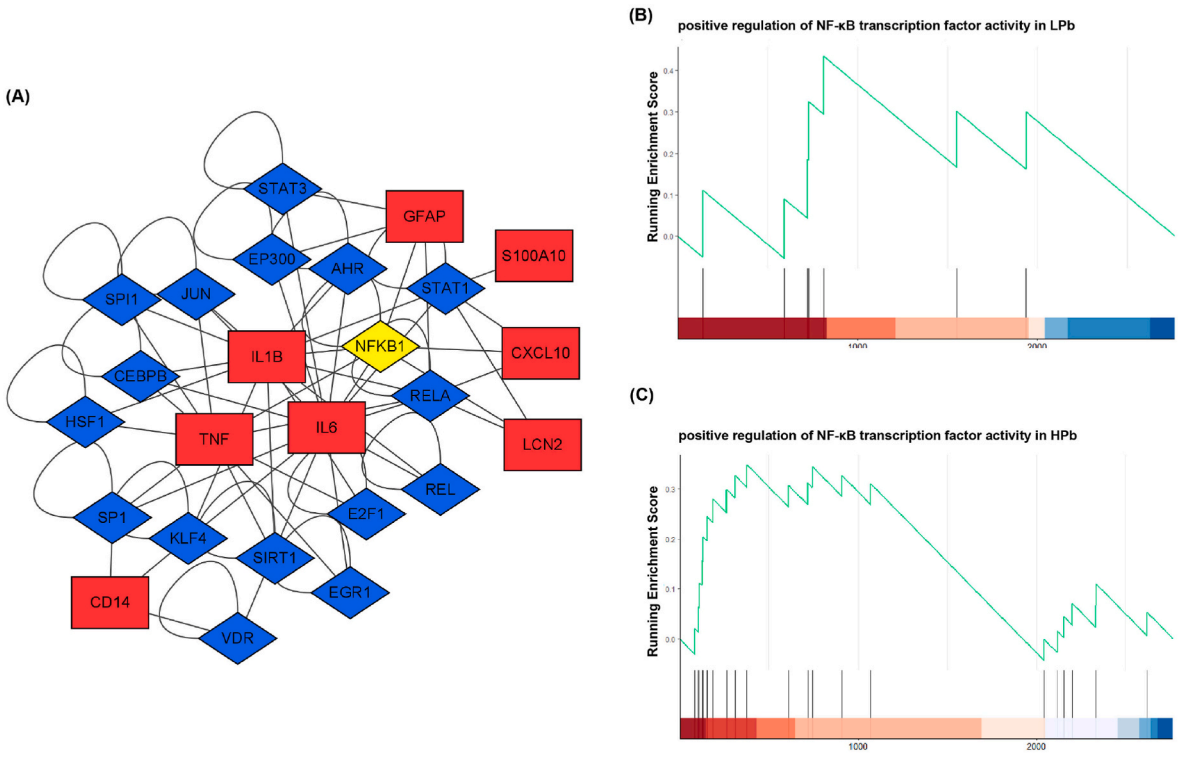

Fig. 6. Transcriptional factors regulate prediction of neurotoxic astrocyte gene expression and inflammatory cytokines following Pb exposure. A: Transcription factor regulatory network involved in neurotoxic reactive astrocyte gene expression and inflammatory cytokines; B, C: The NF-κB transcription factor activity module for GSEA following Pb exposure in the LPb and HPb groups.

NF-κB transcription regulator activity was upregulated in the Pb exposure group (Fig. 6 B, C). Therefore, we hypothesized that astrocyte activation is regulated by the NF-κB transcription factor following Pb exposure.

To verify this hypothesis, NF-κB protein expression was measured in MA-c cells. The results showed that NF-κB protein expression in the 20 μM Pb acetate groups was 3.25-fold higher than that in the control group (Fig. 7 A, B). To confirm that NF-κB regulates astrocyte activation, PDTC was used to inhibit NF-κB activation. GFAP expression in the 20 μM + PDTC group was decreased by 88% (Fig. 7E–G). IL6 and IL-1β protein expression in the supernatant of MA-c astrocytes in the PDTC +20 μM group was lower than that in the 20 μM group (Fig. 4K–M). In addition, Cxcl10 and Lcn2 mRNA expression decreased by 80% and 62.5%, respectively, compared with that in the 20 μM Pb group (Fig. 7E–G). No significant differences were observed in terms of S100a10 mRNA expression (Supplemental Fig. S4). These results indicate that the NF-κB transcription factor regulates the activation of astrocytes following Pb exposure.

4. Discussion

Several studies have shown that Pb can accumulate in the hippocampus or cortex, which results in cellular dysfunction [27–29]. Proteomic and bioinformatics analyses can facilitate the understanding of the main changes in cellular events following Pb exposure [30,31]. Therefore, we compared and analyzed the protein profiles of the rat hippocampus from the Pb-exposed and control groups. The results showed that the DEPs in the Pb exposure group were different from those in the control group, and that they were involved in glial activation. Further analysis showed that astrocyte activation was the most altered cellular event in the rat hippocampus. Furthermore, the NF-κB transcription factor was found to be involved in astrocyte activation following Pb exposure. The above results revealed that astrocyte activation, which is regulated by the NF-κB transcription factor, is the top cellular event in the rat hippocampus following Pb exposure.

GSEA is one of the most widely used approaches for interpreting transcriptomic and other omics data [32]. Rather than focusing on highly regulated single genes, GSEA focuses on gene sets that tend to be more reproducible and interpretable [33]. GSEA is also thought to be an effective method that can provide a comprehensive analysis of biological processes at the cellular level [34,35]. In the present study, GSEA analysis showed that the most important functional module was astrocyte activation, which is considered to be one of the significant cellular events affected by Pb exposure. This method was also used in the study of primary hepatocellular carcinoma, which revealed that DNA replication, spliceosome, cell cycle, and cellular senescence are essential biological processes in patients with primary hepatocellular carcinoma [36]. In Alzheimer's disease, GSEA analysis identified that synaptic signaling, oxidative phosphorylation, and immune response are key biological processes and are intimately involved in Alzheimer's disease progression, which suggests that GSEA analysis serves as a powerful tool for exploring one of the top cellular events [37].

Astrocytes are the most abundant glial cells in the CNS and play a series of indispensable roles in maintaining normal CNS health and function [9,38,39]. The cell type analysis results showed that astrocytes exhibited the greatest change in protein numbers compared to other glial cells, which suggested that astrocytes were more sensitive to Pb toxicity in glial cells. The same changing trend was also observed in the proteomics of human hippocampal subfield CA1 from AD patients [40]. This also illustrates that hippocampal astrocytes are more vulnerable to nerve injuries. Some studies have found that reduced astrocyte activation can ameliorate learning and memory in LPS-induced rat model and 5xFAD mice [41,42]. Our data showed that GFAP expression was much higher in the rat hippocampus and MA-c cells after Pb exposure. In addition, TNF-α, IL-1β, and IL-6 levels increased in the rat hippocampus and MA-c cell supernatant, suggesting that astrocyte activation is involved in neuroinflammation following Pb exposure. This may be one of the mechanisms underlying behavioral impairment in rats following

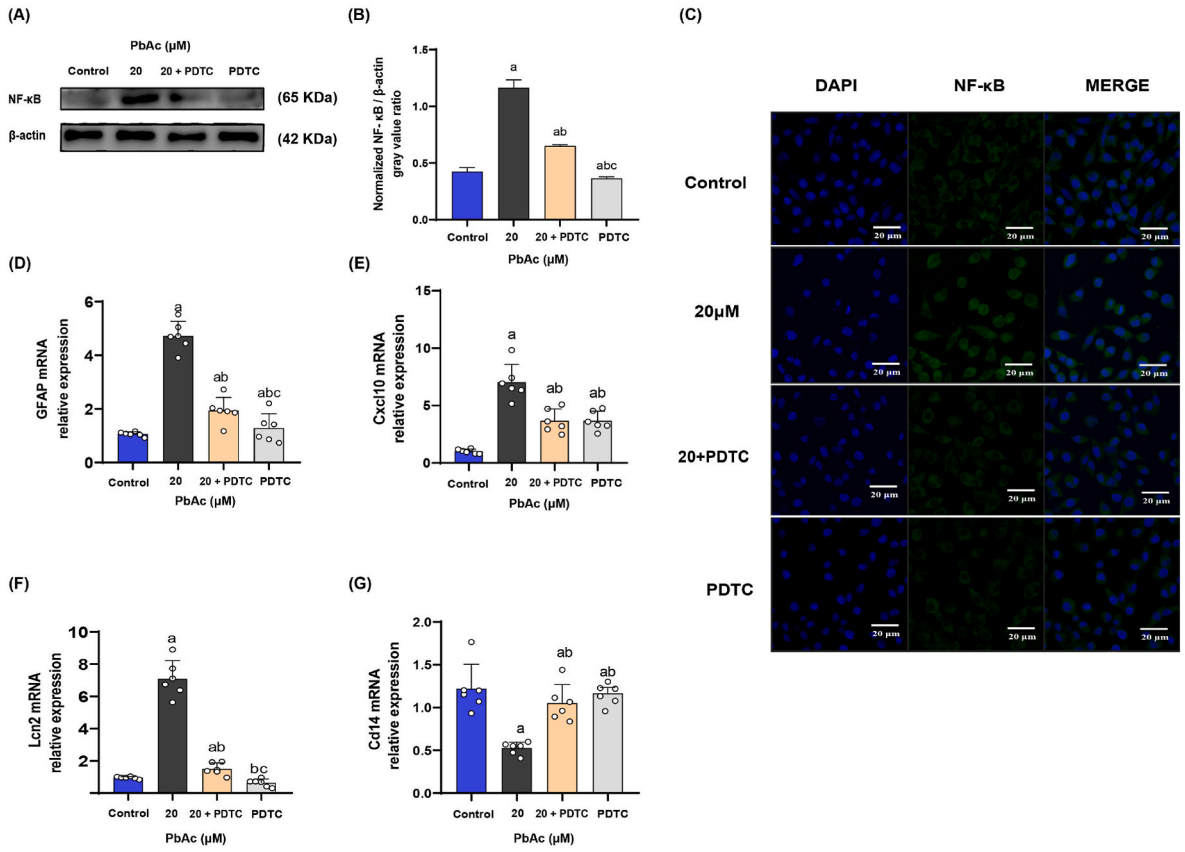

Fig. 7. The NF-κB transcriptional factor contributes to activation of astrocytes following Pb exposure. A, B: The protein expression of NF-κB in MA-c cells (n = 3); C: The NF-κB protein expression in MA-c cells in the control, 20 μM PbAc, 20 μM PbAc + PDTC, and PDTC groups (immunofluorescence, × 400); D–G: Real-time PCR results of neurotoxic and neuroprotective astrocyte gene expression in MA-c cells treated with PbAc or PDTC (n = 6). The MA-c cell lines were treated with PbAc for 24 h or pretreated with PDTC for 1 h $^{a}p < 0.05$ vs Control; $^{b}p < 0.05$ vs 20; $^{c}p < 0.05$ vs 20 + PDTC.

Pb exposure. Astrocytes can be transferred to reactive astrocytes in response to CNS impairment [43]. Studies have shown that reactive astrocytes have two distinct phenotypes, termed neurotoxic (A1) and neuroprotective (A2) [44,45]. The present study found that A1-specific astrocyte genes Lcn2, Steap4, and Cxcl10 were increased, and A2-specific astrocyte genes S100a10 and Cd14 were reduced following Pb exposure, suggesting that Pb can induce astrocyte activation and alter astrocyte neurotoxic and neuroprotective gene expression. Similar A1 and A2-specific astrocyte genes were also observed in the hippocampus of mice subjected to chronic mild stress for six weeks [46]. In summary, our findings indicated that astrocytes were activated in vivo and in vitro following Pb exposure.

Transcription factors regulate gene transcription and cellular processes [47]. In this study, transcription factor analysis identified several transcription factors that regulate astrocyte activation. Among these, NF-κB transcription factors are considered the most important. NF-κB can activate corresponding cell biological processes, including proliferation and inflammation [48]. A previous study showed that Aβ-activated astrocytes secrete inflammatory cytokines via NF-κB transcription factor activity [49]. Laquinimod downregulates proinflammatory cytokines in astrocytes by reducing NF-κB transportation factors [50]. Our results showed that the NF-κB transcription factor is activated in astrocytes following Pb exposure and can regulate astrocyte activation. In addition, inhibition of NF-κB transcription factor activation resulted in a decrease in Lcn2, Steap4, and Cxcl10 mRNA expression. However, inhibiting the NF-κB transcription factor did not affect A2-specific astrocyte gene expression in MA-c cells following Pb exposure. Thus, it is reasonable to speculate that the NF-κB transcription factor can mainly regulate astrocyte activation and inflammatory factor expression, which exacerbates the inflammatory response following Pb exposure. Studies have shown that astrocytes exposed to manganese release inflammatory mediators in an NF-κB-dependent manner, leading to neuronal death [51]. Another study showed that NF-κB mediates antimony-induced astrocyte activation [52], suggesting that the NF-κB transcription factor is involved in astrocyte activation, which is a common mechanism underlying heavy metal exposure.

5. Conclusion

In summary, our findings demonstrated that astrocyte activation is a significant cellular event in the rat hippocampus following Pb exposure. NF-κB transcription factors can regulate astrocyte activation following Pb exposure. In addition, proteomics and bioinformatics were more effective in revealing the potential cellular mechanisms following Pb exposure. Furthermore, our findings provide insights and perspectives for the investigation of Pb-induced learning memory impairments.

Credit authorship contribution statement

Fan Shi: Methodology, Investigation, Formal analysis, Writing -original draft. Weixuan Wang: Methodology, Investigation, Software. Han Hao: Data curation, Formal analysis. Liansheng Zhang: Conceptualization, Supervision. Gang liu: Conceptualization, Supervision. Jierui Wang: Conceptualization, Supervision. Yanshu Zhang: Project administration, Conceptualization, Writing-review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2022.110310.

References

[1] Z. Sarlak, H. Hosseini, F. Garavand, R. Mohammadi, M. Rouhi, The occurrence of lead in animal source foods in Iran in the 2010s decade: a systematic review, Biol. Trace Elem. Res. 200 (2002) 1917–1936, https://doi.org/10.1007/s12011-021-02787-y.
[2] P. Su, D. Wang, Z. Cao, J. Chen, J. Zhang, The role of NLRP3 in lead-induced neuroinflammation and possible underlying mechanism, Environ. Pollut. 287 (2021), 117520, https://doi.org/10.1016/j.envpol.2021.117520.
[3] J.G. Dorea, Environmental exposure to low-level lead (Pb) co-occurring with other neurotoxicants in early life and neurodevelopment of children, Environ. Res. 177 (2019), 108641, https://doi.org/10.1016/j.envres.2019.108641.
[4] A. Hernandez-Coro A, B.E. Sanchez-Hernandez, S. Montes, J.C. Martínez-Lazcano, E. González-Guevara, F. Pérez-Severiano, Alterations in gene expression due to chronic lead exposure induce behavioral changes, Neurosci. Biobehav. Rev. 126 (2021) 361–367, https://doi.org/10.1016/j.neubiorev.2021.03.031.
[5] I. Baranowska-Bosiacka, A. Falkowska, I. Gutowska I, A. Kolasa-Wołosiuk, M. Tarnowski, K. Chibowska, et al., Glycogen metabolism in brain and neurons - astrocytes metabolic cooperation can be altered by pre- and neonatal lead (Pb) exposure, Toxicology 390 (2017) 146–158, https://doi.org/10.1016/j.tox.2017.09.007.
[6] Q. Han, W. Zhang, J. Guo, Q. Zhu, H. Chen, Y. Xia, et al., Mitochondrion: a sensitive target for Pb exposure, J. Toxicol. Sci. 46 (2021) 345–358, https://doi.org/10.2131/jts.46.345.
[7] K. Chibowska, I. Baranowska-Bosiacka, A. Falkowska, I. Gutowska, M. Goschorska, D. Chlubek, Effect of Lead (Pb) on inflammatory processes in the brain, Int. J. Mol. Sci. 17 (2016) 2140, https://doi.org/10.3390/ijms17122140.
[8] K.M. Tate, J.M. Munson, Assessing drug response in engineered brain microenvironments, Brain Res. Bull. 150 (2019) 21–34, https://doi.org/10.1016/j.brainresbull.2019.04.027.
[9] I.H. Hernández, M. Villa-González, G. Martín, M. Soto, M.J. Pérez-Álvarez, Glial cells as therapeutic approaches in brain ischemia-reperfusion injury, Cells 10 (2021) 1639, https://doi.org/10.3390/cells10071639.
[10] A. Rai, S.K. Maurya, P. Khare, A. Srivastava, S. Bandyopadhyay, Characterization of developmental neurotoxicity of As, Cd, and Pb mixture: synergistic action of metal mixture in glial and neuronal functions, Toxicol. Sci. 118 (2010) 586–601, https://doi.org/10.1093/toxsci/kfq266.
[11] D. Kaur, V. Sharma, R. Deshmukh, Activation of microglia and astrocytes: a roadway to neuroinflammation and Alzheimer's disease, Inflammopharmacology 27 (2019) 663–677, https://doi.org/10.1007/s10787-019-00580-x.
[12] A. Gomez-Arboledas, J.C. Davila, E. Sanchez-Mejias, V. Navarro, C. Nuñez-Diaz, R. Sanchez-Varo, et al., Phagocytic clearance of presynaptic dystrophies by reactive astrocytes in Alzheimer's disease, Glia 66 (2018) 637–653, https://doi.org/10.1002/glia.23270.
[13] L. Wu, S. Li, C. Li, B. He, L. Lv, J. Wang, et al., The role of regulatory T cells on the activation of astrocytes in the brain of high-fat diet mice following lead exposure, Chem. Biol. Interact. 351 (2022), 109740, https://doi.org/10.1016/j.cbi.2021.109740.
[14] J.T. Liu, B.Y. Chen, J.Q. Zhang, F. Kuang, L.-W. Chen, Lead exposure induced microgliosis and astrogliosis in hippocampus of young mice potentially by triggering TLR4-MyD88-NFkappaB signaling cascades, Toxicol. Lett. 239 (2015) 97–107, https://doi.org/10.1016/j.toxlet.2015.09.015.
[15] U. Wilhelmsson, A. Pozo-Rodrigalvarez, M. Kalm, Y. de Pablo, Å. Widestrand, M. Pekna, et al., The role of GFAP and vimentin in learning and memory, Biol. Chem. 400 (2019) 1147–1156, https://doi.org/10.1515/hsz-2019-0199.
[16] M. Wang, S. Yang, J. Cai, et al., Proteomic analysis using iTRAQ technology reveals the toxic effects of zearalenone on the leydig cells of rats, Food Chem. Toxicol. 141 (2020), 111405, https://doi.org/10.1016/j.fct.2020.111405.
[17] W.W.B. Goh, Y.H. Lee, M. Chung, L. Wong, How advancement in biological network analysis methods empowers proteomics, Proteomics 12 (2012) 550–563, https://doi.org/10.1002/pmic.201100321.
[18] S. Virakul, P. Somparn, T. Pisitkun, P.J. van der Spek, V.A.S.H. Dalm, D. Paridaens, et al., Integrative Analysis of Proteomics and DNA methylation in orbital fibroblasts from Graves' ophthalmopathy, Front Endocrinol (Lausanne) 11 (2020), 619989.
[19] L. Zhang, Y. Dong, Q. Wang, C. Du, W. Xiong, X. Li, et al., iTRAQ-based proteomics analysis and network integration for kernel tissue development in maize, Int. J. Mol. Sci. 18 (2017) 1840, 10.3390%2Fijms18091840.
[20] A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, et al., Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15545–15550, https://doi.org/10.1073/pnas.0506580102.
[21] P. Kong, G. Huang, W. Liu, Identification of protein complexes and functional modules in E. coli PPI networks, BMC Microbiol. 20 (2020) 243, https://doi.org/10.1186/s12866-020-01904-6.
[22] J. Zhang, R. Chen, F. Shi, P. Yang, K. Sun, X. Yang, et al., Genome-wide data mining to construct a competing endogenous RNA network and reveal the pivotal therapeutic targets of Parkinson's disease, J. Cell Mol. Med. 25 (2020) 5912–5923, https://doi.org/10.1111/jcmm.16190.
[23] F. Caraci, S. Spampinato, M.A. Sortino, P. Bosco, G. Battaglia, V. Bruno, et al., Dysfunction of TGF-β1 signaling in Alzheimer's disease: perspectives for neuroprotection, Cell Tissue Res. 347 (2012) 291–301, https://doi.org/10.1007/s00441-011-1230-6.
[24] L. Shvachiy, V. Geraldes, A. Amaro-Leal, I. Rocha, Persistent effects on cardiorespiratory and nervous systems induced by long-term lead exposure: results from a longitudinal study, Neurotox. Res. 37 (2020) 857–870, https://doi.org/10.1007/s12640-020-00162-8.
[25] S. Li, C. Yang, X. Yi, R. Wei, M. Aschner, Y. Jang, et al., Effects of sub-chronic lead exposure on essential element levels in mice, Biol. Trace Elem. Res. (2022), https://doi.org/10.1007/s12011-022-03137-2.
[26] T. Wu, E. Hu, S. Xu, et al., clusterProfiler 4.0: a universal enrichment tool for interpreting omics data, Innovation (Camb). 2 (2021), 100141, https://doi.org/10.1016/j.xinn.2021.100141.
[27] N. Li, S. Cao, Z. Yu, et al., Perinatal lead exposure alters calsyntenin-2 and calsyntenin-3 expression in the hippocampus and causes learning deficits in mice post-weaning, Biol. Trace Elem. Res. 199 (2021) 1414–1424, https://doi.org/10.1007/s12011-020-02241-5.
[28] K.M. Bakulski, J.F. Dou, R.C. Thompson, et al., Single-cell analysis of the gene expression effects of developmental lead (pb) exposure on the mouse hippocampus, Toxicol. Sci. 176 (2020) 396–409, https://doi.org/10.1093/toxsci/kfaa069.
[29] T. Wang, R.L. Guan, M.C. Liu, et al., Lead exposure impairs hippocampus related learning and memory by altering synaptic plasticity and morphology during juvenile period, Mol. Neurobiol. 53 (2016) 3740–3752, https://doi.org/10.1007/s12035-015-9312-1.
[30] M. Rahmatbakhsh, A. Gagarinova, M. Babu, Bioinformatic analysis of temporal and spatial proteome alternations during infections, Front. Genet. 12 (2021), 667936, 10.3389%2Ffgene.2021.667936.
[31] J.W. Shin, J.H. Lee, H. Kim, et al., Bioinformatic analysis of proteomic data for iron, inflammation, and hypoxic pathways in restless legs syndrome, Sleep Med. 75 (2020) 448–455, https://doi.org/10.1016/j.sleep.2020.09.002.
[32] C. Weidner, M. Steinfath, E. Opitz, et al., Defining the optimal animal model for translational research using gene set enrichment analysis, EMBO Mol. Med. 8 (2016) 831–838, https://doi.org/10.15252/emmm.201506025.
[33] A.K. Sommer, A. Hermawan, B. Ljepoja, et al., A proteomic analysis of chemoresistance development via sequential treatment with doxorubicin reveals novel players in MCF7 breast cancer cells, Int. J. Mol. Med. 42 (2018) 1987–1997, https://doi.org/10.3892/ijmm.2018.3781.
[34] N.T. Doncheva, J.H. Morris, J. Gorodkin, et al., Cytoscape StringApp: network analysis and visualization of proteomics data, J. Proteome Res. 18 (2019) 623–632, https://doi.org/10.1021/acs.jproteome.8b00702.
[35] X. Wu, M.A. Hasan, J.Y. Chen, Pathway and network analysis in proteomics, J. Theor. Biol. 362 (2014) 44–52, https://doi.org/10.1016/j.jtbi.2014.05.031.
[36] Q. Zhang, Z. Xiao, S. Sun, et al., Integrated proteomics and bioinformatics to identify potential prognostic biomarkers in hepatocellular carcinoma, Cancer Manag. Res. 13 (2021) 2307–2317, https://doi.org/10.2147/cmar.s291811.
[37] Z. Liu, H. Li, S. Pan, Discovery and validation of key biomarkers based on immune infiltrates in Alzheimer's disease, Front. Genet. 12 (2021), 658323, 10.3389%2Ffgene.2021.658323.
[38] M. Santello, N. Toni, A. Volterra, Astrocyte function from information processing to cognition and cognitive impairment, Nat. Neurosci. 22 (2019) 154–166, https://doi.org/10.1038/s41593-018-0325-8.
[39] H. Wood, Evidence for altered astrocyte function in Duchenne muscular dystrophy, Nat. Rev. Neurol. 18 (2022) 1, https://doi.org/10.1038/s41582-021-00601-w.
[40] Y. Gao, J. Liu, J. Wang, et al., Proteomic analysis of human hippocampal subfields provides new insights into the pathogenesis of Alzheimer's disease and the role of glial cells, Brain Pathol. 32 (2022), e13047, https://doi.org/10.1111/bpa.13047.
[41] M. Choi, S.M. Lee, D. Kim, et al., Disruption of the astrocyte-neuron interaction is responsible for the impairments in learning and memory in 5XFAD mice: an Alzheimer's disease animal model[J], Mol. Brain 14 (1) (2021) 111, https://doi.org/10.1016/j.biopha.2018.08.136.
[42] F. Zhang, J.G. Zhang, W. Yang, et al., 6-Gingerol attenuates LPS-induced neuroinflammation and cognitive impairment partially via suppressing astrocyte overactivation, Biomed. Pharmacother. 107 (2018) 1523–1529, https://doi.org/10.1016/j.biopha.2018.08.136.
[43] L.E. Clarke, S.A. Liddelow, C. Chakraborty, et al., Normal aging induces A1-like astrocyte reactivity, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E1896–E1905, https://doi.org/10.1073/pnas.1800165115.

[44] S.A. Liddelow, K.A. Guttenplan, L.E. Clarke, et al., Neurotoxic reactive astrocytes are induced by activated microglia, Nature 541 (2017) 481–487, https://doi.org/10.1016/j.celrep.2020.107776.
[45] K.A. Guttenplan, B.K. Stafford, R.N. El-Danaf, et al., Neurotoxic reactive astrocytes drive neuronal death after retinal injury[J], Cell Rep. 31 (12) (2020), 107776.
[46] Y. Fang, X. Ding, Y. Zhang, et al., Fluoxetine inhibited the activation of A1 reactive astrocyte in a mouse model of major depressive disorder through astrocytic 5-HT2BR/beta-arrestin2 pathway, J. Neuroinflammation 19 (2020) 23, https://doi.org/10.1186/s12974-022-02389-y.
[47] A. Leung, C. Trac, H. Kato, et al., LTRs activated by Epstein-Barr virus-induced transformation of B cells alter the transcriptome, Genome Res. 28 (2018) 1791–1798, 10.1101%2Fgr.233585.117.
[48] C.M. Simplicio-Revoredo, P.R. de Oliveira, M.M. de Almeida, et al., Expression of Nrf2 and NF-kappaB transcription factors in breast cancer and breast fibroadenoma: insights for a new therapeutic approach, Oncotarget 11 (2020) 1629–1636, https://doi.org/10.18632/oncotarget.27574.
[49] N.O. Alami, C. Schurr, H.F. Olde, et al., NF-kappaB activation in astrocytes drives a stage-specific beneficial neuroimmunological response in ALS, EMBO J. 37 (2018), https://doi.org/10.15252/embj.201798697.
[50] W. Bruck, R. Pfortner, T. Pham, et al., Reduced astrocytic NF-kappaB activation by laquinimod protects from cuprizone-induced demyelination, Acta Neuropathol. 124 (2012) 411–424, https://doi.org/10.1007/s00401-012-1009-1.
[51] X. Liu, J.A. Buffington, R.B. Tjalkens, NF-kappaB-dependent production of nitric oxide by astrocytes mediates apoptosis in differentiated PC12 neurons following exposure to manganese and cytokines, Brain Res Mol Brain Res 141 (2005) 39–47, https://doi.org/10.1016/j.molbrainres.2005.07.017.
[52] T. Zhang, Y.D. Zheng, M. Jiao, et al., Nuclear factor-kappaB signaling mediates antimony-induced astrocyte activation, Biomed. Environ. Sci. 34 (2021) 29–39, https://doi.org/10.3967/bes2021.005.