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# Allicin alleviated learning and memory deficits caused by lead exposure at developmental stage

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ABSTRACT

*Aims:* It is a promising approach to search the therapeutic strategies for treating lead (Pb) toxicity. Allicin, a natural compound extracted from *Allium sativum* (garlic), has been reported to have many beneficially biological properties. In this study, we investigated the protective effects of allicin on learning and memory function of rats exposed by lead acetate at developmental stage.

*Materials and methods:* Rats received lead acetate for inducing toxicity, and gavaged with allicin to ameliorate this toxicity. Morris water maze test was performed to determine learning and memory function. Superoxide dismutase (SOD), glutathione (GSH) and methane dicarboxylic aldehyde (MDA) was measured to determine oxidative stress. Immunofluorescence was carried out to analyze GFAP-positive cells. The protein expression of ERK, p-ERK, EGFR and p-EGFR were detected using western blot.

*Key findings:* We found that allicin ameliorated lead acetate-caused learning and memory deficits by promoting hippocampus astrocyte differentiation, which mainly through EGFR/ERK signaling. Moreover, allicin attenuated the increased ROS level by regulating the oxidative defense system.

*Significance:* These results suggest that allicin is a potent agent able to ameliorate lead acetate-induced learning and memory deficits during early development, and may thus be useful for defeating lead acetate toxicity.

## 1. Introduction

Lead (Pb) is one of the most common environmental heavy metal toxicants. Human organisms are highly vulnerable to lead toxicity, especially in relation to learning and memory function deficits of the central nervous system [1]. Lead-induced learning and memory deficits are amplified during developmental stage because of greater lead absorbance [2]. The mechanisms responsible for lead toxicity involve disorganized oxidative balance leading to overproduction of reactive oxygen species (ROS) [3]. Physiological ROS perform numerous important functions by activating various cellular responses, such as gene transcription, cell proliferation, apoptosis, and other signaling pathways [4]. Thus, mechanistic elucidation on lead toxicity has allowed us to explore potential medical strategies for lead-induced functional deficits treatment. Recently, antioxidation has been shown to be a critically effective avenue for defending against lead toxicity, including ascorbic acid [5], quercetin [6], curcumin [7], and garlic [8], etc.

Allicin, a bioactive component extracted from *Allium sativum* (garlic), has a variety of beneficial effects [9], including antibiotic, antioxidant, antithrombotic, and antiatherogenic effects [10,11]. These findings were supported by a recent study, which found that 2-acrylic acid, an unstable metabolite of allicin, spontaneously antagonized free

*Abbreviations:* SOD, superoxide dismutase; GSH, glutathione; MDA, methane dicarboxylic aldehyde; Gpx, glutathione peroxidase; EGFR/ERK, epidermal growth factor receptor/extracellular signal-regulated kinase

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radicals, partly contributing to these beneficial effects [12]. Allicin has also been reported to reduce lead levels in adult mice, including in the peripheral blood, bone, and liver [13]. However, the effects and underlying mechanisms of allicin on lead-induced learning and memory dysfunction are not fully understood. In the present study, we investigated the protective effects of allicin against lead toxicity in terms of learning and memory function in developmentally lead acetate exposed rats, and also investigated the potential regulatory mechanisms involved in this process.

## 2. Materials and methods

### 2.1. Animals and drug preparation

Lead acetate [$(CH_3COO)_2Pb{\cdot}3H_2O$, PbAc] was obtained from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in tap water at a concentration of 0.2%. Allicin was purchased from Shanghai Mengry Bio-Technology Co., Ltd. (Shanghai, China), and dissolved in 0.9% saline at a concentration of 2 mg/ml as a stock solution. The body weight of each rat was ascertained prior to each administration for adjusting the amount of allicin. The dosage of lead acetate was determined based on previous studies [14]. Sexually mature Wistar rats (240 ± 20 g, 6–8 weeks) were provided by SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All animal experiments were performed in accordance with the National Institutes of Health guidelines. The animals were kept in germ-free conditions at a constant room temperature of 22 °C with free access to standard food and tap water. Female Wistar rats were mated with sexually mature males (2 female: 1 male). After confirmation of pregnancy (by the presence of a vaginal plug), the females were transferred into new individual cages for further study.

### 2.2. Experimental design

Twenty pregnant female rats were randomly divided into four groups of five each. The lead acetate exposed dams were received tap water containing 0.2% lead acetate for 63 days from the day of conception to the day of weaning (postnatal day 21). After weaning of the respective pups, three pups were randomly selected per litter regardless of sexuality, and given the same amount of tap water twice a day for 21 days via intragastrical gavage for next study. The control dams were received tap water only throughout the whole gestation and lactation period, the respective pups were selected according the principles mentioned above, and continuously given tap water twice a day for 21 days via intragastrical gavage. The Pb/allicin group dams were received tap water containing 0.2% lead acetate from the day of conception and throughout gestation and lactation, until the day of weaning. After weaning of the respective pups, one pup was randomly selected per litter regardless of sexuality, and given 30 mg/kg allicin twice a day for 21 days via intragastrical gavage. The control/allicin group dams were received tap water without lead acetate from the day of conception and throughout gestation and lactation until the day of weaning. After weaning of the respective pups, one pup were randomly selected per litter regardless of sexuality, and given 30 mg/kg allicin twice a day for 21 days via intragastrical gavage. The experimental groups are shown in Fig. 1a.

### 2.3. Lead concentration determination

To determine the lead concentrations in the peripheral blood and hippocampus tissue, blood samples were collected from postorbital vein, and analyzed by inductively-coupled plasma mass spectroscopy (VG Elemental Ltd., UK). Hippocampus samples were collected, digested with nitric and perchloric acids and analyzed by inductively-coupled plasma mass spectroscopy (VG Elemental Ltd., UK). All tests were performed in triplicate.

### 2.4. Morris water maze test

At the end of allicin treatment, learning and memory function was evaluated by Morris water maze test. Briefly, the Morris water maze test was performed using an apparatus consisting of a 120 cm diameter round pool, a 10 cm diameter platform, and a computer-controlled video tracking system (MobileDatum, Shanghai, China). To evaluate the spatial learning ability, the platform was placed 1 cm above the water in one of the four quadrants of the round pool in a bright surrounding. Animals with normal eyesight would escape the water and find the platform within a short time. The escape latency of each test was recorded using the computer-controlled video tracking system. Each subject was tested in four quadrants at the actual time in a day, and performed for 5 consecutive days. In the space exploration test, the platform was hidden blew the water, rats were released from the actual quadrant and allowed to swim freely in the pool for 60 s. The time spent in the target quadrant containing the hidden platform was recorded by the computer-controlled video tracking system. There was an interval of at least 15 min between two consecutive tests, and a different release quadrant was used in each of four tests in a day, for 5 consecutive days.

### 2.5. Oxidative stress assay

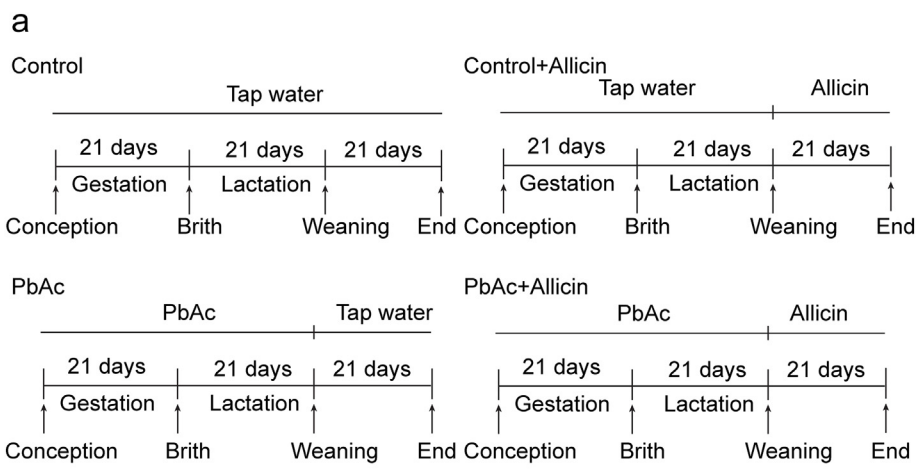
To determine the levels of oxidative stress in hippocampus, cerebral cortex and liver, superoxide dismutase (SOD) and glutathione (GSH) content, and the lipid peroxidation product methane dicarboxylic aldehyde (MDA) were detected. For SOD detection, the activities of SOD were measured by the 19,160 SOD determination kit (Sigma Aldrich, MO, USA), according to the manufacture's protocol. Briefly, 20 μl protein samples were added with 200 μl WST Working Solution, 20 μl of Dilution Buffer, and 20 μl Enzyme Working Solution, and incubated at 37 °C for 20 min. The absorbance of each sample was recorded by a spectrophotometer at 450 nm. GSH content was measured using a Total GSH Assay Kit (Beyotime, Hangzhou, China), according to the manufacturer's protocol. Briefly, 10 μl protein samples tissue were added to 50 μl 0.16 mg/ml NADPH and incubated at 25 °C for 5 min. The absorbance of each sample was then recorded at 412 nm using a spectrophotometer. MDA content was measured using a Lipid Peroxidation MDA Assay Kit (Beyotime), according to the manufacturer's protocol. During oxidative stress, MDA combined with thiobarbituric acid (TBA) to create a TBA-MDA adduct that was detected spectrophotometrically at 532 nm.

### 2.6. Immunofluorescence

Rat pups were anesthetized with 2% pentobarbital sodium the day after the end of the experiment. Hippocampus tissue was collected and fixed in 2% paraformaldehyde in PBS (0.1 M, pH 7.4) at 4 °C overnight, and stored in the liquid nitrogen. For section preparation, hippocampus was coronally cut by using microtome at a thickness of 30 μm. For immunofluorescence analysis on the GFAP-positive cells in hippocampus dentate gyrus (DG) region, sections were washed with cold PBS and incubated with fluorescein isothiocyanate-conjugated anti-glial fibrillary acidic protein (GFAP) antibody (eBioscience Inc., San Diego, CA, USA) for 2 h at room temperature. DAPI (Invitrogen) was used to identify cell nuclei. Images were acquired under a fluorescence microscope (Olympus, Japan). The number of GFAP-positive cell was counted using Image-Pro Plus software. More than 3 areas per section were randomly selected and calculated to evaluate the GFAP-positive cells.

### 2.7. Western blotting assay

Briefly, hippocampus tissue was collected and homogenized for protein separation. Proteins were determined using a BCA Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equivalent amounts of



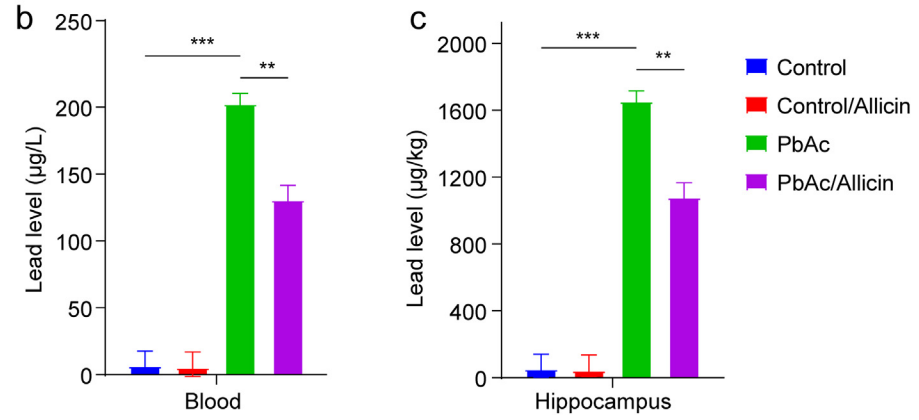




**Fig. 1.** Schematic diagram of the experimental procedures and determination of lead level.
Chronological schedule of experimental set-up for pregnant rats administered tap water (control), lead acetate (PbAc), allicin (control/allicin), and lead acetate plus allicin (PbAc/allicin), respectively. (b,c) The levels of lead in peripheral blood and hippocampus tissues of different group rats. $^{**}p < 0.01$, $^{***}p < 0.001$.

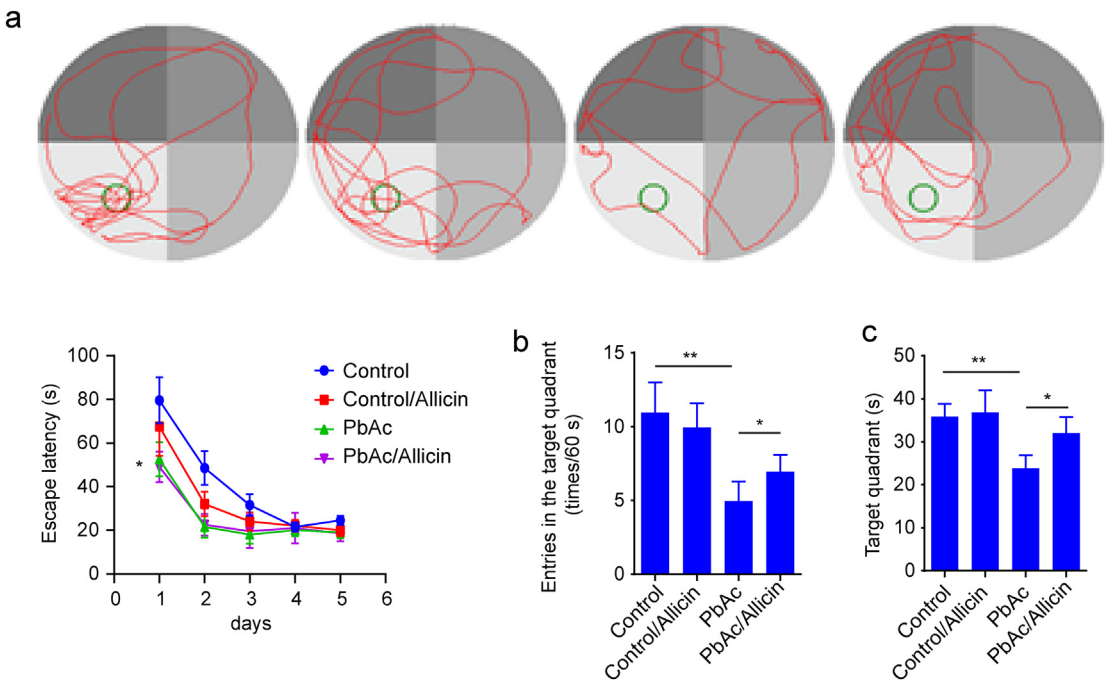



**Fig. 2.** Allicin alleviated learning and memory deficits caused by developmental lead exposure.
(a) The movement track and mean escape latencies of rat pups from day 1 to day 5 after exposure were examined by the Morris maze test. (b) Bar graph of entries into the target quadrant. (c) Duration in the target quadrant. $^{*}p < 0.05$, $^{**}p < 0.01$.

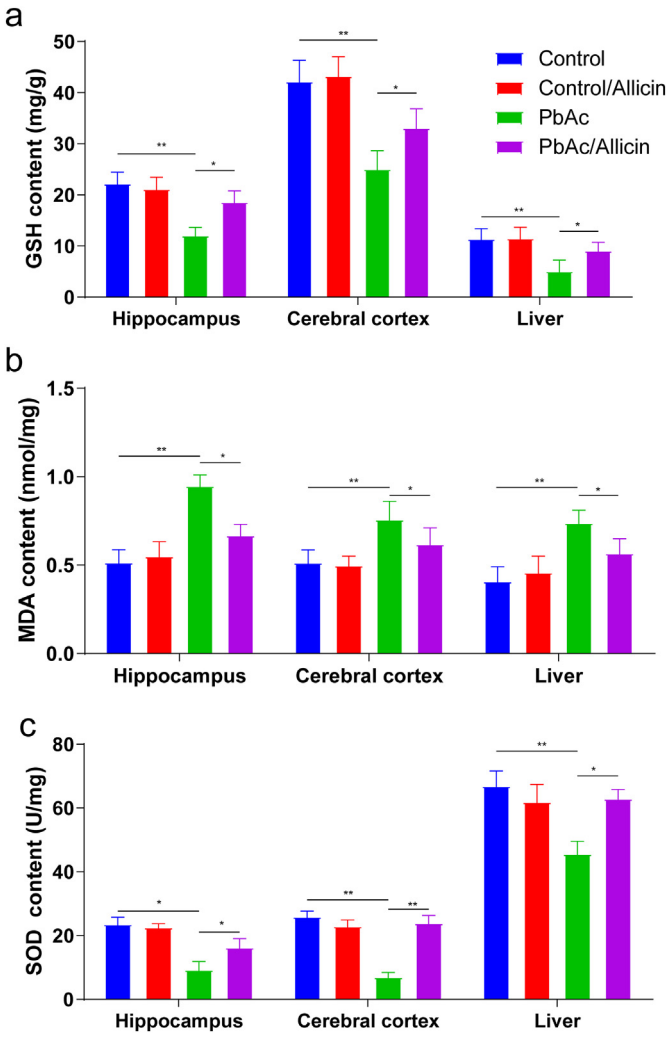



**Fig. 3.** Allicin reversed lead acetate caused oxidative stress.
Representative bar graph of glutathione (GSH) activity (a), methane dicarboxylic aldehyde (MDA) content (b), and superoxide dismutase (SOD) activity (c) in the hippocampus, liver, and cerebral cortex, respectively. $^{**}p < 0.01$, $^{***}p < 0.001$.

proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated overnight with primary mouse anti-phosphorylated extracellular signal-regulated kinase (ERK) antibody, mouse anti-GFAP antibody (Abcam, USA), and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then rinsed and incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG; 1:10000; Bio-Rad). Chemiluminescence detection was performed using an ECL kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Densitometric quantification was performed using Image-Pro Plus (version 6.0) software (Media Cybernetics, Inc., Rockville, USA).

### 2.8. Statistical analysis

All statistical analyses were performed using SPSS.18 software. All data were expressed as mean ± standard deviation. Comparisons between groups were analyzed by one-way analysis of variance followed by Student-Newman-Keuls multiple comparison test. Statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Allicin decreased lead levels in peripheral blood and hippocampus

It is more vulnerable to lead toxicity at developmental stage. To assess the direct effect of allicin on lead toxicity in developmental rats, we firstly detected the lead levels in peripheral blood (PB) and hippocampus tissues. As is shown in Fig. 1b and c, lead exposure caused a significantly increased lead level in peripheral blood and hippocampus tissues compared with the control group. Also, treatment with allicin led to a reduced lead level in PB and hippocampus in developmentally lead acetate-exposed rats. Interestingly, allicin treatment alone had limited effects on control group rats excluding the potential adverse effects of allicin itself. These results indicate that allicin significantly reduced lead levels in the peripheral blood and hippocampus in lead acetate-exposed rats at developmental stage.

### 3.2. Allicin alleviated learning and memory deficits caused by developmental lead exposure

It is noteworthy that lead accumulated in hippocampus tissue in lead-acetate exposed rat. Next, we investigated the effects of allicin and lead acetate on learning and memory function in rats. As shown in Fig. 2a, lead acetate exposure led to a significant impairment in spatial learning ability; while allicin treatment led to a significantly reversion of spatial learning ability to the similar levels of the control rats. Treatment with allicin alone showed similar spatial learning ability to that of the control rats. These results indicate that allicin ameliorated the lead-induced spatial learning ability impairment in developmentally lead acetate-exposed rats.

In the spatial probe trail, the frequency of entry into the target quadrant and duration in the target quadrant was analyzed to evaluate learning and memory function. As illustrated in Fig. 2b and c, lead acetate exposed rats had a significant impairment in learning and memory function, which is confirmed by the decreased frequency of entry into the target quadrant and duration in the target quadrant. Allicin treatment lead to an increased frequency of entry into the target quadrant and duration in the target quadrant compared with the Pb group. Moreover, allicin treatment alone had no significant effect on learning and memory function. These results indicate that allicin effectively ameliorated lead-induced learning and memory deficits in developmentally lead acetate- exposed rats.

### 3.3. Allicin reversed lead acetate caused oxidative stress condition

It reported that allicin has antioxidant effects in multiple biological process [15]. Now, we investigated whether allicin ameliorated lead-induced learning and memory deficits by modulating redox balance. To explore the effects of allicin on oxidative defense system, the content of SOD and GSH in hippocampus tissues was detected. As illustrated in Fig. 3, SOD and GSH content was significantly lower in Pb group compared with control group, but allicin treatment led to a significant increase in SOD and GSH content to the similar level of control group. There were no significant differences in SOD and GSH content between the control and allicin groups. We also detected the changes of MDA content, a product of oxidative stress, in hippocampus and showed that the MDA contents were significantly higher in Pb group compared with the control group. It has been significantly ameliorated by allicin treatment. To confirm the oxidative defense role of allicin in vivo, we detected the biomarkers of SOD, GSH, and MDA in liver and cerebral cortex tissues. In line with our expectation, the tendency of changes in SOD, GSH, and MDA in liver and cerebral cortex tissues is consistent with that in hippocampus (Fig. 3). These results indicate that allicin attenuated lead acetate-induced oxidative stress damage by regulating oxidative defense system.

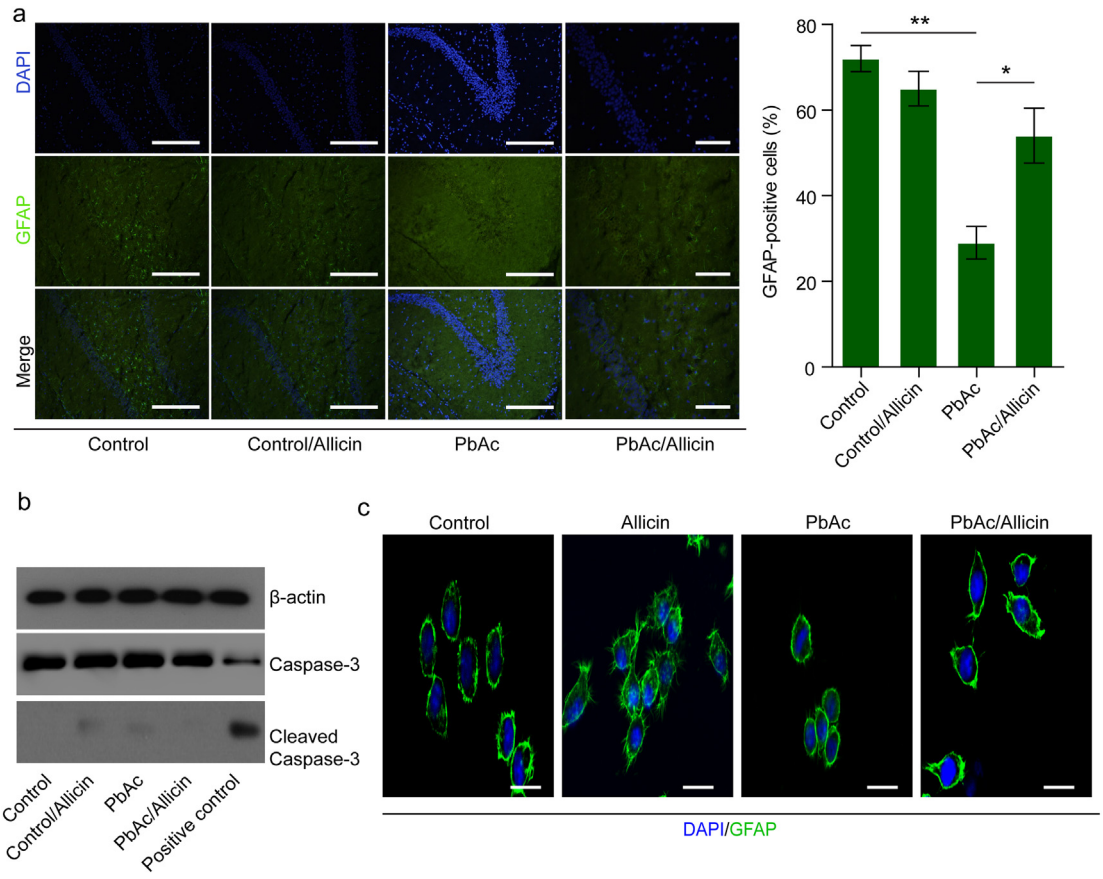



**Fig. 4.** Allicin reversed lead acetate-caused defects of hippocampal astrocyte differentiation at development stage.
(a) The quantification and representative distribution of DAPI and GFAP-positive cells determined by immunofluorescence staining; Pictures are shown on a dark back ground of DAPI (up panels) and GFAP-positive cells (middle panels) or merged (down panels) or as overlap with the phase contrast picture; Scale bar = 20 μm. (b) The expressions of active Caspase-3 in hippocampus tissues of different group rats. (c) The representative distribution of DAPI and GFAP-positive cells (astrocytes differentiated from NS-5 cells) determined by immunofluorescence staining; Scale bar = 10 μm. $*p < 0.05$, $**p < 0.01$.

### *3.4. Allicin reverses lead acetate caused defects of hippocampal astrocyte differentiation*

To further explore the mechanisms of lead acetate induced learning and memory deficits, we analyzed the changes of various type cells in hippocampus zone. It showed that no significant difference has been found in neuron number in hippocampal zone between these groups. Whereas, astrocyte numbers were significantly decreased in lead acetate exposed rats compared with the control. Moreover, there was no difference in astrocyte numbers between the control and control/allicin groups (Fig. 4a). Next, we investigated whether astrocyte loss is contributed by ROS-induced apoptosis. We found that there is no remarkable apoptosis occurred in astrocyte in the PbAc group, as showed by the inactivation of Caspase-3 protein (Fig. 4b). To confirm whether astrocyte differentiation was blunted by lead exposure, neural stem cell (NSC) line NS-5 was used to induce astrocyte differentiation in the presence or absence of lead acetate and allicin in vitro. We found that astrocyte numbers (astrocyte marker GFAP-positive cells) were significantly decreased in the presence of lead acetate compared with the control. Allicin treatment significantly increased the astrocyte numbers compared with the lead acetate group. Moreover, there was no difference in astrocyte numbers between the control and allicin groups (Fig. 4c). These data indicated that exposure to lead acetate did not induced astrocyte apoptosis, but possibly impaired the process of astrocyte differentiation.

### *3.5. EGFR/ERK signaling is involved in mediating astrocyte differentiation*

It reported that EGFR/ERK signaling modulated astrocyte differentiation [16]. We next investigated the expressions of phosphorylated EGFR (p-EGFR) and ERK (p-ERK) protein in hippocampus tissues. It showed that the expressions of p-EGFR and p-ERK were significantly decreased in the PbAc group than that in control group, but significantly attenuated by allicin treatment (Fig. 5a). Next, we investigated the expressions of phosphorylated EGFR (p-EGFR) and ERK (p-ERK) protein in NS-5 cells exposed by lead acetate in the presence or absence of allicin. We found that the expressions of p-EGFR and p-ERK were significantly decreased after PbAc treatment. And allicin treatment significantly reversed PbAc-induced decreased p-EGFR and p-ERK expression (Fig. 5b). To confirm it, the ERK inhibitor LY3214996 was used to treat NS-5 cell followed by PbAc/Allicin treatment. Compared with the PbAc/Allicin group, LY3214996 significantly reduced the expressions of p-EGFR and p-ERK to a level similar to PbAc group suggesting abrogating the ameliorating effects of allicin on PbAc-activated EGFR/ERK signaling in NS-5 cells (Fig. 5b). These results suggest that EGFR/ERK signaling is contributed to regulate astrocyte differentiation. The signaling is inhibited by lead acetate exposure at developmental stages which could be ameliorated by allicin treatment.

## 4. Discussion

Lead has toxic effects in a wide variety of organs. It can impair the nervous, hematopoietic, renal, cardiovascular, and reproductive systems as a result of swallowing, inhalation, or skin absorption.

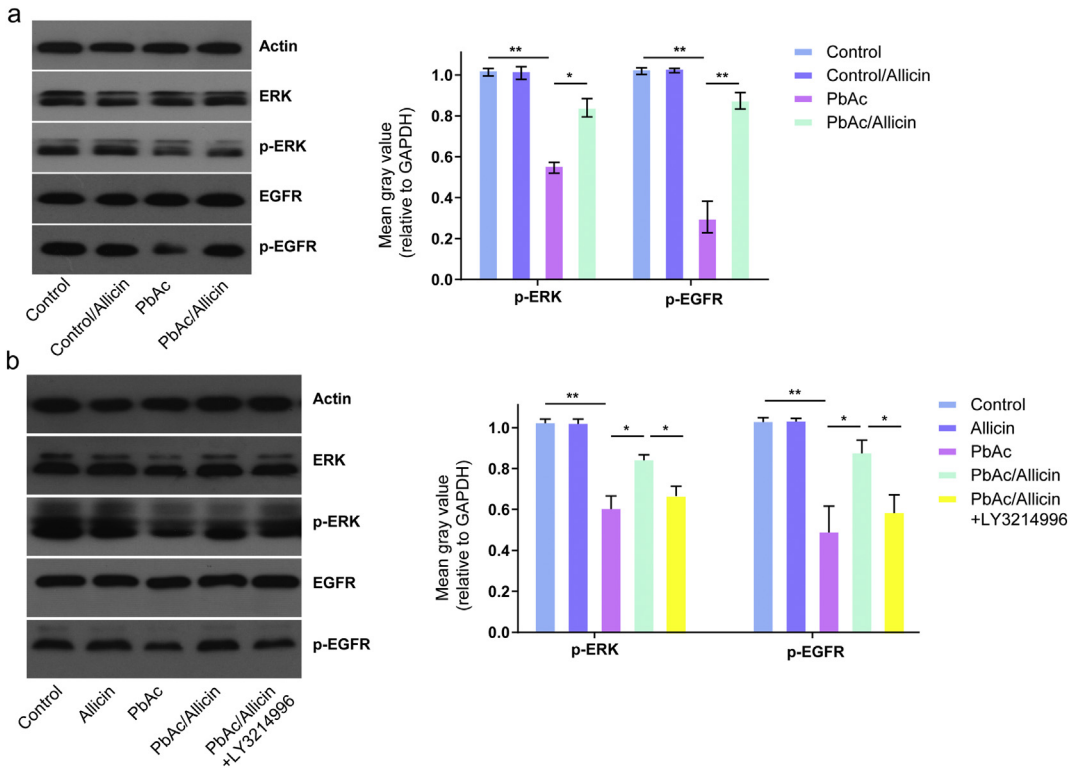



**Fig. 5.** EGFR/ERK signaling is involved in mediating astrocyte differentiation.
(a, b) Grayscale images of phosphorylated EGFR (p-EGFR) protein and phosphorylated ERK (p-ERK) protein and the total proteins of hippocampus tissue (a) from different groups' subjects (left panel) or NS-5 cells (b) by western blotting. Representative bar graph of protein expression quantification normalized to GAPDH (right panel); the experiment was repeated three times. *$p < 0.05$, **$p < 0.01$.

Compared with other organ systems, the central nervous system is particularly susceptible to lead toxicity, especially during its development [14,17], partly because of the weak blood-brain barrier, leading to higher absorbance of lead in the brain [18]. Lead poisoning in the central nervous system leads to disruption of learning and memory [6], as confirmed by the results of the current study. Hippocampal astrocyte in the central nervous system play an important role in nerve conduction and in the learning and memory activities responsible for learning and memory function, and are considered to be a potential target of lead intoxication. It reported that allicin effectively reduced the lead load in mice [13]. The present results demonstrated that astrocyte development is impaired by lead exposure. The restoration of learning and memory function consistent with an increase in astrocyte number after allicin treatment demonstrated the potential of allicin to protect astrocyte development from lead intoxication at developmental stage.

Oxidative stress has been reported as a major mechanism of lead toxicity. The redox balance is regulated by several endogenous enzymes including SOD, GSH, and Gpx (glutathione peroxidase). Disruption of the redox reaction may result in oxidative stress damage and activation of a variety of cellular process [19]. In current study, we found that the content of SOD and GSH, two critical components of the intracellular oxidant defense system, was significantly increased by allicin treatment. These results suggest that regulation of the redox balance is the defining mechanism responsible for the ameliorating effects of allicin on astrocyte development. The ERK pathway is a major signaling cassette of MAPK signaling, and has been reported to perform a number of important signaling functions, including regulating synaptic plasticity through long-term potentiation in hippocampal neurons responsible for learning and memory [20]. Consistent with previous study [16], in the current study, allicin affected activation of EGRF/ERK suggesting that EGFR/ERK signaling was involved in regulating the ameliorating effects of allicin on astrocyte differentiation.

In conclusion, allicin significantly ameliorated lead-induced learning and memory deficits in rats. Allicin maintained physiological levels of redox balance through regulation of the oxidative defense system, and ameliorated lead-induced impairments in hippocampus astrocyte differentiation through EGFR/ERK signaling.

## Authors' contribution

Shizhong Cai and Lina Zhao designed the study and drafted the manuscript. Jun Liu performed the experiments. Xiaoyan Shi analyzed the data. Shousen Hu and Shizhong Cai contributed to the quality control of the manuscript.

## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

## Acknowledgments

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