The down-regulation of PP2Ac demethylation attenuates learning and memory impairment in Manganism

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Research

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Abstract

Background: Protein phosphatase 2A (PP2A) is considered a potential therapeutic target for neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), and catalytic unit methylation of PP2A that relies on methionine and its intermediate S-adenosylmethionine (SAM) regulates most of protein phosphorylation. Manganese overload caused methionine decrease and tau phosphorylation in brain. However, the relationship between PP2Ac and manganese poisoning is not fully understood. In present study, we concentrate on the ameliorative effect of PP2Ac demethylation on cognitive impairment induced by manganese.

Methods: Adult male Sprague-Dawley rats (n = 80; 200-250 g) were divided into Subacute (4W) and chronic (16W) exposure groups. Each exposure group consisted of four treatment groups: control (i.p., saline), Mn, methionine, and methionine plus Mn treatment group. N2a cells with different manganese exposure concentrations were cultured with or without methionine, furthermore, ABL127 is used in follow-up studies. Morris water maze, Alamar Blue assay, flow cytometry, Western blot, liquid chromatography, 2,7-dichlorofluorescein diacetate assay were performed to assess spatial memory, cell viability and apoptosis levels，phosphorylated tau and PP2Ac pathway protein expression, SAM content in brain tissue and cells, oixidative stress related markers.

Results: Manganese-exposed rats had elevated levels of phosphorylated Tau and PP2Ac demethylation accompanied by abnormal rise of PPME1 expression in cortex and hippocampus as well as restricted SAM in hippocampus and spatial memory function. Up-regulation of PP2Ac demethylation caused by SAM increase after methionine treatment or PPME1 inhibition after ABL127 treatment significantly reduced p-tau, apoptosis and ROS levels, and increased cell viability and GSH levels. In addition, methionine treatment in vivo significantly increased the number of crossings and reduced the escape latency.

Conclusion: Methionine and ABL127 treatment can prevent manganese-induced tau hyperphosphorylation and oxidative stress injury, thus improve spatial memory. This improvement is mediated by down-regulation of PP2Ac demethylation. This study confirms that PP2Ac demethylation plays an important role in manganese neurotoxicity and provides a potentially powerful therapeutic strategy for such neurodegenerative diseases.

Background

Although manganese serves as an essential required trace metal in mammalian animals, over exposure to Mn from occupational sets and ambient environment is a critical public health concerns. Epidemiological studies have disclosed that chronic Mn exposure through underwater caused cognitive and memory impairment in children [1–3]. Other investigations have indicated that elevated Mn exposure is likely a risk factor of neurodegenerative disease, including Alzheimer’s disease(AD), Parkinson’s disease (PD) [4, 5]. Moreover, Mn level is significantly higher in the brain of patients with Alzheimer disease (AD)

[6]. Animal models also suggested that Mn can cause cognitive deficit and memory impairment [7]. Hyperphosporylated tau, a topical trait of tau pathgy was found in cell model overload to manganese [8]. However, the molecular mechanism of cognitive and memory impairment induced by manganese exposure has not been fully understood yet.

Tau, namely as microtubule-associated protein, is enriched in neurons where it or low level tau phosphorylation form function as maintaining microtubule stability. Hyperphosphorylation tau is vulnerable to disconnect from microtubule and abnormally aggregates to form neurofibrillary tangles (NFTs) which is the characteristic of tau pathology including Parkinson's disease and Alzheimer disease (AD). More than 40 serine and threonine residues have been reported so far. Hyperphosporylated tau is regulated by the balance between the activity of protein kinases and phosphoprotein phosphatases [9]. Multiple protein kinases were reported to be involved in tau hyperphosphorylation induced by Mn exposure. However, few results were elucidated how phosphoprotein phosphatases govern tau phosphorylation in neurotoxicity induced by Mn.

Compared with numerous of protein kinases, there are only 2 identified phosphoprotein phosphphatases contribution to removal phosphoryl groups, including PP1 and PP2 [10]. PP2A is the major member of a family of phosphoprotein phosphatases which is preferentially expressed in the brain. PP2A is found in heterotrimeric holoenzymes consisting of a highly conversed catalytic subunit C, a scaffold-like A subunit and a family of B regulatory subunit. The activity of PP2A is involved in complex process including protein-protein interaction and posttranslational modification [11, 12]. Specifically, the methylation of catalytic subunit of PP2A (PP2Ac) is crucial for PP2A activity. PP2Ac specific leucine carboxyl methyltransferase (LCMT-1) and PP2Ac specific methylesterase (PME-1) are reversible removal carboxyl group at leucine-309 residue, thus modulates the methylation of catalytic subunit [13]. Researches have indicatd that hypomethylated PP2A counted for the taupaththies in AD patients, quarrel with methylating enzyme LCMT-1 reduction and demethylating enzyme PME-1 promotion [14, 15]. Methionine was found to decreased in olfactory bulb of mouse treated with Mn [16]. As a major precursor of methyl group, whether methionine impacts the methylation of PP2Ac hasn't been investigated yet.

Herein, we postulate that up-regulation of PP2Ac demethylation caused by high level Mn resulted to tau hyperhosphorylation and cell impairment, down-regulation could therefore revised this pathogenesis. We firstly established two animal models by subacute and chronic Mn treatment (15 mg kg day^{-1},4w and 16w) in rats to explore the roles of regulation PP2Ac demethylation on spatial learning and memory function deficit by using the Morris water maze. Moreover, we investigated the effect and mechanism of methionine supplementation on manganese poisoning in rats and N2a cytotoxicity. We determined the level of tau and its hyperphosphorylation forms, the methylation status of PP2Ac as well as the expression of 2 reversible enzymes which contribute to govern PP2Ac methylation, LCMT-1 and PME-1 in animals brain tissues and in N2a cells. Furthermore, a specific inhibitor of PPME-1, ABL127 is applied to investigate the impact of down-regulation of PP2Ac demethylation on Mn neurotoxicity in vitro [17]. The present study attempts to address the molecular mechanism of tau aberrant phosphorylation underlying Mn neurotoxicity.

Results

Methionine supplement improves the Mn-induced learning and memory deficit of rats

To determine the impact of Mn and the protective effect of methionine on cognitive function in subacute and chronic experiments, the MWM test was used in this study. In the training phase, both subacute and chronic experiments showed that Mn administration resulted in a significant lower spatial learning ability compared to the control, while methionine treatment significantly improved this cognitive function, and especially in the last two days of 16W (days 4 and 5, $p < 0.005$), the difference in latency between Mn rats and control or methionine treatment group was more significant than that of 4W experiment (Fig. 1a). In the spatial probe trial at 24 hr, rats of chronic Mn administration showed decreased probe times and swimming time in the target quadrant compared with the control rats ($p < 0.01$), with no significant changes in swimming speed, and 16W rats exposed to Mn treated with methionine showed a remarkable reversion in the low level of memory (Fig. 1b, Fig. 1c, Fig. 1d). These results suggest that methionine could protects the Mn-induced deficit in spatial learning and memory abilities, and this positive effect is more obvious in the 16-week treatment experiment.

Mn concentration in whole blood

To verify whether methionine exerts a protective effect through reducing blood manganese levels in Mn-induced neurotoxicity in rats, we measured the manganese content of whole blood by ICP-MS. After four weeks of treatment, we found that the blood manganese level in the Mn group increased significantly compared with the control group (all $p < 0.0001$; Fig. 2a), and with the prolongation of exposure time, the blood manganese level of the 16-week Mn-treated group continued to rise, with the average value is close to twice that of the 4-week treatment group. However, the blood Mn levels did not decrease significantly after methionine supplementation, suggesting that methionine attenuates Mn-induced cognitive dysfunction not by lowering blood manganese levels.

Cell viability and apoptosis treated $MnCl_2{\cdot}4H_2O$ with or without methionine

N2a cells treated $MnCl_2{\cdot}H_2O$ were of round shape, axons disappearance and detached from the surface. The morphology profile was worse with higher Mn exposure. Lower growth density of N2a cells was observed in methionine-free medium, but reversed after methionine supplement.

$MnCl_2{\cdot}4H_2O$ induced cell toxicity decrease in a dose-dependent manner in N2a cells, with the medium inhibition concentration (IC50) as 1.6 μmol/L ($p < 0.05$; Fig. S1-2). Sub-lethal concentrations $MnCl_2{\cdot}4H_2O$ (0.5 mM and 1.0 mM) were chosen to use in the subsequent measurements. Compared with the cells cultured in methionine-free medium, those treated with various levels methionine had higher cell viability

($p < 0.05$), but no dose-dependent manner ($p > 0.05$) and with peak value at 10 mg/L, at which methionine can promote cell proliferation, shown as Fig. S3-4. Thus, 10 mg/L methionine will be utilized in the following tests. According to the results, Fig. S5-6 showed that methionine (10 mg/L) significantly reversed decline in cell viability induced by $MnCl_2 \cdot 4H_2O$ to increase the fluorescence value of N2a cells($p < 0.05$). The cell fluorescence value of methionine group was higher than methionine lack of group in the same concentration of manganese injury, and the difference was statistically significant ($p < 0.05$, Fig. 3a-b). Next, we used flow cytometry to find out that Mn significantly up-regulated N2a cells apoptosis in a dosed-dependent manners without methionine($p < 0.05$). Methionine supplementary reversed apoptosis caused by Mn（$p < 0.05$）. Without Mn treatment, no difference of apoptosis was found in cells treated with or without methionine (Fig. 3c).

Supplementation of methionine promots a decrease in the level of tau hyperphosphorylation induced by Mn

In the present study, we used $MnCl_2 \cdot 4H_2O$ to establish tau hyperphosphorylation rat model of subacute and chronic manganese poisoning, and in behavioral tests, we observed significant changes in ability of spatial learning and memory in rats. we measured the level of tau hyperphosphorylation by western blotting assay. Notably, we found that tau hyperphosphorylation at S396 was more obvious than that at S404 and S199, all typical phosphorylation sites involved in PD and AD. Analysis results exhibited that the expression of hyperphosphorylated tau at S396 was increased in the cortex and hippocampus of manganese model rats (4W: all $p < 0.05$, 16W: all $p < 0.005$), while methionine (200 mg/kg) markedly decreased the level of tau phosphorylation at this site induced by Mn (4W: all $p < 0.05$, 16W: all $p < 0.01$) (Fig. 4a). In vitro, with the increase of manganese concentration, the relative expression of tau-total protein did not decrease significantly, and the difference was not statistically significant ($p > 0.05$). However, in the tau phosphorylation of 199 and 396 sites increased with the increase of manganese dose, and the difference was statistically significant ($p < 0.05$). The relative expression of PP2Ac protein was not statistically significant ($p > 0.05$). The relative expression density of demethylation PP2Ac and LCMT-1 protein increased with the increase of Mn concentration, and the difference was statistically significant ($p < 0.05$) (Fig. 4b).

Methionine contributes to reverse Mn-induced imbalance of PP2Ac methylation/demethylation to attenuate tau hyperphosphorylation in specific regions of the brain and in cells

The methylation of PP2Ac is regulated by leucinecarboxyl methyltransferase-1 (LCMT-1) and protein phosphatase methylesterase-1 (PME-1), and the former promotes methylation of PP2Ac while the latter catalyzes demethylation [18, 19].We continued to explore the causes of tau phosphorylation in our model rats by Western blotting. As illustrated in Fig. 5a, compared with the control group, the expression level of

LCMT-1 was significantly decreased and these changes are found in both the cortex and the hippocampus. Furthermore, the results displayed that Mn induced a remarkable increase in PME-1 in the hippocampus and cortex of rats. Of note, in the same brain region, with the extension of Mn exposure time, the difference in PME-1 expression between the model group and the control group became more pronounced, but this effect was not shown in LCMT-1 expression. As shown in Fig. 5b, the up-regulation of PME-1 is accompanied by a significant increase in the level of demethylation of PP2Ac in both two brain regions, except in the 4-week cortex. In summary, we can see from this part that Mn destroys the balance of PP2Ac methylation/demethylation, i.e. increases PP2Ac demethylation levels and reduces PP2Ac methylation levels, and the cause of this change may be related to the expression of Mn regulation LCMT-1/PME-1. Then we look at the results of methionine treatment, after supplementing exogenous methionine, the decrease of LCMT-1 and the increase of PME-1 in hippocampus and cortex of rats in Mn group were also restored to the normal level. Furthermore, the protein expression of demethylated PP2Ac was significantly lower than that of Mn group after methionine supplementation. Most importantly, methionine was extremely effective in reversing the adverse outcomes at the protein level induced by Mn, even with prolonged exposure, in both subacute and chronic manganese exposures. In vitro, under the same concentration of manganese injury, the relative expression density of total-Tau protein of methionine group was no significant change compared with the lack of methionine group, and the difference was not statistically significant ($p > 0.05$). The relative expression density of tau protein in 199 sites of methionine group was lower than methionine lack group, and the difference was statistically significant ($p < 0.05$); But, the relative expression of tau 396 site protein of methionine group was high compared with the lack of methionine group in manganese dose of 0 and 500 μmol/L, and then the difference was statistically significant ($p < 0.05$).The relative expression density of demethylation PP2Ac protein in methionine group was lower than methionine lack group, and there were statistically significant ($p < 0.05$), while the LCMT-1 protein relative expression density value present opposite trends, and Mn = 500 the difference is statistically significant ($p < 0.05$) (Fig. 5c).

Methionine treatment restores Mn-induced SAM reduction in hippocampus of rats and cultured cells

We know that SAM is the universal methyl donor throughout the body which is derived from methionine. Significantly, the activity of LCMT-1, the sole PP2Ac methyltransferase, is dependent on SAM supply [20, 21]. So we were interested in whether the Mn-induced methylation/demethylation imbalance of PP2Ac is related to the breakdown of one-carbon metabolic cycle homeostasis caused by SAM reduction. To confirm the above conjecture, we measured intracerebral tissue SAM and SAH levels using Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS). We found that the results of 16 week hippocampus are most representative, as shown in Fig. 6a, the concentration of SAM in brain tissue in Mn group was significantly lower than that in normal group ($p < 0.05$). On the other hand, normal rats did not increase ratios of SAM to SAH in the hippocampus after methionine supplementation. But surprisingly, we found that the SAM level in brain in the Mn plus methionine group increased significantly compared with the Mn model group ($p < 0.05$). In Vitro, Mn caused methionine metabolism abnormal in methionine-free medium, disruption of the balance of its metabolites SAM and SAH. The levels of SAM in

N2a treated by 500 μM and 1000 μM Mn was significantly decrease as well as the ratio of SAM/SAH ($p < 0.05$). However, SAH was obviously enhanced in 1000 μM Mn treatment ($p < 0.05$). 10 mg/L methionine remarkably restored SAM level and SAM/SAH ratio in N2a treated by 1000 μM Mn ($p < 0.05$), with SAH levels decreasing trend (Fig. 6b-d).

ABL127 reduces Mn-induced tau hyperphosphorylation by reversing excessive demethylation of PP2Ac in Vitro

In order to further verify that PME-1 is a potential target of manganese induced abnormal phosphorylation toxicity of tau, ABL127 was applied to the study of neurodegenerative pathogenesis for the first time. From the results of Western blot analysis, as shown in Figure. 7a, the addition of ABL127 effectively inhibited the expression of PME-1 in N2a cells induced by Mn (500 and 1000 μmol/L), thereby significantly reducing level of PP2Ac demethylation ($p < 0.05$). In addition, it was surprising that the upregulation of PP2Ac methylation after ABL127 treatment was also very significant in the control group and the two Mn treatment groups ($p < 0.05$), while the change in LCMT-1 was not so significant, its expression increased only at 1000 μmol/L Mn concentration ($p < 0.05$). At the same time, we found a significant decrease in Mn-induced tau phosphorylation at the S199 and S396 sites after ABL127 treatment ($p < 0.05$, Figure. 7b). These results revealed that ABL127 reversed PP2Ac over-demethylation to decreased the abnormal phosphorylation of tau in vitro.

Methionine and ABL127 exterminates reactive oxygen species in N2a cell exposured to $MnCl_2{\cdot}4H_2O$

With the increase of manganese dose, the fluorescence intensity value both were decreased in Mn, methione plus Mn and ABL127 plus Mn group, and the difference was statistically significant ($p < 0.05$). At the same time, the fluorescence values of methione plus Mn and ABL127 plus Mn group were lower than Mn group, and differences were statistically significant in Mn = 1000 μM ($p < 0.05$); The microscope fluorescence brightness of three group cells were consistent with the trend of fluorescence intensity change. In addition, the GSH value of Mn and methione plus Mn group presented decreased trend with the increasing of Mn concentration, but ABL127 plus Mn group was increasing trend. The GSH value of methione plus Mn and ABL127 plus Mn group were high than Mn group, and difference were statistically significant at 500 and 1000 μM ($p < 0.05$) (Fig. 8a-d).Above results revealed methione and ABL127 could exterminate reactive oxygen species from Mn inducing.

Discussion

Overexposure to Mn is considered as a risk factor of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, characterizing as motor and cognitive performance [22]. Methionine, one of sulfur-contained essential amino acids, functions as a precursor for protein synthesis, methyl donator. No effective intervention has been applied to treat neurodegenerative impairment relative to Mn exposure. In

the present study, we evaluated the molecular basic and potential therapeutic of regulation PP2Ac demethylation on Mn-induced neurotoxicity in vivo and vitro. We found methionine supplementary ameliorates the cognitive deficits in rats and N2a cells cytotoxicity induced by Mn. The protection of methionine, which scavenges ROS and donates methyl contributes to down regulate PP2Ac demethylation, thus decrease tau hyperphosphorylation. Furthermore, ABL127 administration reverses Mn cytotoxicity with down-regulation PP2Ac methylation and tau hyperphosphorylation accumulation. To our knowledge, our study firstly hightlights that PP2Ac is likely a prospective therapeutic target in Mn neurotoxicity.

Accumulating evidences demonstrated that Mn overexposure induced school-age students cognitive deficit as well as cells lesion [23, 24]. Methionine level was declined in animal brain after manganese treated [16]. Thus, methionine is involved in neurotoxicity induced by Mn. In present study, methionine improved learning and memory impairment in animal and protected N2a cells from apoptosis caused by Mn in vivo. However, methionine protecting cytotoxicity induced by Mn has not been fully elucidated. Considering methionine and its sequence metabolisms, it is not surprising that methionine exerts oxidative defense, protein structure and cellular regulation. Our investigation indicated that PP2Ac are likely a prospective therapeutic target for neurotoxicity induced by Mn over-exposure, whose methylation is critical for regulating tau hyerphosphorylation. It seemed that oxidative stress instead of one-carbon cycle governed the methylation of PP2Ac in N2a exposure to Mn. Both methionine and ABL127 reverted demethylation of PP2Ac through their antioxidant properties (Fig. 9). Though methionine plays a crucial role in cell metabolism, no dose-response relationship was detected in cell viability after supplemented different level methionine, with optimal protective level at 10 mg/L,which is similar to the level in mediate. Methionine has also been demonstrated to protect cecal tonsils cellar apoptosis, the conflict evidence from animal and clinical trials showed that excessive methionine intake is likely contribute to Alzheimer's-like neurodegeneration [25]. The narrow adequate level of methionine in cellular was maintained by its metabolism process, which ubiquitously occurs in mammalian. In its metabolism procedure, a methyl was generated then transferred into DNA, protein methylation modification and methionine was recycled. The intermediate productions of methionine metabolism like SAM, Hcys and SAH have been demonstrated to involve in various diseases [26].

As a hallmark of tau pathology, tau hyperphosporylation can caused abnormally neurofibrillary tangle contributable to destruction of the neuronal cytoskeleton and axonal transport [27]. Many neurotoxins can induce tau hyperphosphorylation, including aluminum, MPTP, BMAA, etc [28–32]. More than 30 tau phosphorylation sites were tested so far in neuronal diseases, with limited sites phosphorylation related to neurodegenerative diseases [9]. The present study indicated that in our animal or cell model, the manganese exposure level we set caused tested sites at Ser199, Ser202 sites, Ser396 and Ser404 phosphorylation, with Ser199, Ser396 more sentivity. Other literatures have discovered that other heavy metals, including cadmium, lead caused tau hyperphosphorylation, but showing different phosphorylated sites [33–36]. Even exposed to Mn, the sites of phosphorylation of tau were not consistent in different models. Methionine supplement attenuates Tau phosphorylation, which is consistent with some literatures. Conflicting evidences was reported by Tapia and his colleagues [25]. The balance of tau

phosphorylation is supervised by numerous protein kinases and phosphatases. Various protein kinases are related to neurodegenerative diseases, including casein kinase, calcium calmodulin-dependent kinase and glycogen synthase kinase-3β (GSK-3β) ,which specifically was identified to get involved in tau phosphorylation in PC12 cells exposed to Mn [8]. How Mn stimulated tau phosphorylation has not been fully understood yet. Nonetheless, the impact of phosphatases on tau hyperphosphorylation induced by Mn has not been fully elucidated yet.

PP2A, a major member of phosphoprotein phosphatases, its posttranslational modifications are responsible for dephosphorization in tau in neurodegenerative diseases. PP2A is composed of three subunits; those are a scaffolding unit (A), a regulatory unit (B) and a catalytic unit(C). Specifically, the methylation status of catalytic unit is crucial for PP2A activity. It have been demonstrated that the methylation of catalytic unit enhanced PP2A activity and decreased tau hyper phosphorylation. Conversely, demethylation of PP2Ac is contributed to dephosphorylate of hyperphosphorylated tau in AD brain [10, 37–40]. In present study, Mn caused demethylated PP2Ac increasing, paralleled with methylated PP2Ac decrease, which implicated inactivation of PP2A (Fig. 9). However, other investigation proved that lower level Mn^{2+} activated PP2A [41]. Of note, the Mn concentration applied in Zhang et al. was 0.1-1% percent of ours.

Both one-carbon-cycle and oxidative were assumed to regulate PP2Ac methylation modification. Methionine and its intermediate, S-adenosylmethionine (SAM) were demonstrated to offer a methyl to promote PP2Ac methylation, thus dephosphorylate tau [42]. It is known little about the impact of Mn on methionine metabolism. We found methionine supplement consistently increase SAM and decreased SAH level whatever with or without manganese, suggesting that decreases in methionine cycle activity was associated with tau hyperphosphorylation caused by Mn. Moreover, previous study indicated that oxidative stress can promptly and continuously demethylation PP2Ac, independent of other signal pathway [43]. It is well accepted that oxidative stress can directly interact with cysteine residue then impact spatial structure and biological effects of protein. Sequence analysis pointed out that a pairs of conserved cysteine residues C266/269 close to the active site of PP2Ac [44], which is likely a potential target of oxidative stress. This hypothesis was proved that disulfide bond reducing agent seized the increasing ROS, restoring PP2A activity as well [45]. Our present results demonstrated that Mn exposure enhanced ROS and declined GSH, which might partly explain the mechanism of PP2Ac demethylation after Mn exposure (Fig. 9).

The reversible methylation and demethylation of Leu309 at carboxyl end of PP2Ac is catalyzed by leucine carboxyl methyltransferase-1 (LCMT-1) and protein phosphatase methylesterase-1 (PME-1) [46]. The levels of LCMT-1 and methylated PP2Ac are decreased, accordant with hyperphosphorylation of tau in AD brains [47]. Deregulation of PP2Ac methylation also disrupts the interaction between PP2A and tau, and alters tau distribution [48]. These previous studies have indicated that methylation of PP2Ac is essential for it to dephosphorylate tau. It has been discovered that Mn, being required for PP2A phosphatase activity, is evicted while the interaction between PME-1 and PP2A [13], but how does Mn

disrupt the level of LCMT-1 and PME-1 hasn't been explained yet. There was evidence show that ROS can cover the active site of PP2A to LCMT-1, thus deregulate PP2A activity [43, 49].

Conclusion

In conclusions, we found that PP2Ac demethylation mediated tau abnormal phosphorylation thus contributed to cognitive impairment caused by Mn exposure. Functioning as an exogenous methyl donor and antioxidant, methionine supplement can promote learning and memory impairment induced by manganese. Methionine cycle disruption and ROS generation were associated with a decreased cell viability and apoptosis in cultured cells exposed to manganese. Down-regulation of PP2Ac demethylation through methionine supplement and ABL127 can decrease tau hyperphosphorylation. Of note, ABL127, a specific inhibitor of PME-1 restored was firstly introduced to Mn poisoning and worked as antioxidant as well. Our results suggested that down-regulation of PP2Ac demethylation through methionine supplement and ABL127 can ameliorate cell apoptosis and reduced tau hyperphos-phorylation, thus improve cognitive capacity.

Material And Methods

Chemicals

MEM, 1640 medium and methionine lack of culture medium were purchased from Sigma company. Fetal bovine serum from Gibco company, 0.25% trypsin - EDTA digestive juices and 100 x penicillin and streptomycin mixture were from Solarbio company, the Resazurin sodium salt-powder, methionine and Manganese chloride ($MnCl_2 \cdot 4H_2O$) powder, ABL127 were purchased from Sigma company. Anti-de-methylated PP2Ac, anti-LCMT1, anti-PME1, anti-total PP2Ac were from Santa Cruz Biotechnology. Anti-tau (phosphoS199, S396, S199, S202), anti-tau antibody (tau-5), were purchased from Abcam company. cyclophilin B was purchased from Cell Signaling company, mouse and rabbit monoclonal were obtained from ZSGB-BIO company.

Animals, treatment and samples collection

All animal studies were approved by the ethical committee for animal experiment of the Guangxi Medical University (GXMU-2014-036). and in accordance with the international standards of guidelines for the Care and Use of Laboratory Animal in animal experiments. This study strived to minimize the number and the suffering of experimental animals. 80 male Sprague-Dawley Rats (200 ± 10 g weight, SPF grade) were provided by the experimental animal center of Guangxi Medical University (LicenseNo:SCXKGui2009-0002). Rats were randomly assigned to 8 groups (4 treatment groups lasted 4 weeks and 16 weeks respectively, 10 rats/group): control, Mn, methionine, and methionine plus Mn group. Mn-administrated rats were injected intraperitoneally by $MnCl_2 \cdot 4H_2O$ (15 mg/kg, in saline). In the methionine treatment group, methionine (200 mg/kg, in saline) were treated. The rats in the taurine plus Mn group were injected

intraperitoneally for 15 mg $MnCl_2 \cdot 4H_2O$/kg and 200 mg methionine/kg. Control animals received saline (0.9% NaCl) injection. The injection was given every day, respectively, lasting for 4 weeks and 16 weeks. The volume of administration was 5 mL/kg. After behavioral testing, blood samples were collected from the abdominal aorta and collected in heparin-treated blood collection tubes. The brains were quickly removed and placed on a plastic petri dish with cold saline, the hippocampus and cortex was dissected. All samples were stored at -80℃.

Cell culture and treatment

Mouse neuroblastoma cell line (N2a) was obtained from Shanghai Institutes for Biological Sciences, CAS, China. N2a cells were grown in normal MEM medium containing 10% fetal bovine serum and 1% antibiotics (100Unit/mL) in CO_2 (5%) incubator at 37 °C. After 70%~ 80% confluences, N2a cells were cultured in MEM without methionine for Mn toxicity (ranging from 0 to 10000uM) to pick out two sub-lethal concentrations for the consequent experiments. Meanwhile, methionine (0, 10, 15 and 20 mg/L) were added to methionine-free MEM to figure out the appropriate concentration used for consequent tests. The same tragedy was applied to choose the suitable level of ABL127, a specific inhibitor of protein phosphatase methylesterase-1 (PPME-1), which usually demethylases PP2A catalytic unit. N2a cells were exposed to specific concentration of methionine or ABL127 combined with two sub-lethal levels of $MnCl_2 4H_2O$ (0 ~ 10000uM) as the following test. Morphology of N2a cells was observed after 24 h by using an inverted microscope (DMi8; Leica, Germany).

Morris Water Maze test

The Morris water maze test (MWM) was used to determine changes in spatial learning and memory ability 24 hours after last injection. The experimental device was a cylindrical pool of 160 cm in diameter and 60 cm in height, which was divided into four quadrants I, II, III and IV. A black circular platform with a diameter of 12 cm was placed in the III quadrant and was 2 cm below the surface of the water. Keep the objects around the water maze fixed, and the water temperature was stable at 23 ± 2 °C. A camera was suspended 2 m above the maze and a computer equipped with the Morris Water Maze analysis software (Huaibei Zhenghua biological equipment Co, China) was used to record the swimming track in the water maze. All the test was started at 8:00 am of six continuing days. In the first five days, each rat entered maze from four different quadrants and trained four times a day, with a training interval of 30 s. When the rats did not find the platform within 60 s, the tester guided it to the platform and recorded the escape latency of 60 s. At the 6th day, the platform was removed and each mouse was put into maze at a fixed quadrant, and the time (s) spent in target quadrant, probe time were used to evaluate the level of animal spatial memory.

Mn concentration in blood

At the end of the behavioral study, whole blood samples were collected from rats after decapitation in each group. The samples were prepared by digestion with nitric acid (65%) in microwave digestion

apparatus (MARS6; CEM, Matthews, NC, USA). The level of Mn was determined by inductively coupled plasma massspectrometry (ICP-MS, X Series 2; Thermo Scientific, Waltham, MA, USA) (6 samples per treatment group).

Cell viability determined by Alamar Blue assay

N2a cells were seeded in 96-well plate at a density of 2×10^4 cells per well, six replicate wells each treatment and triplicates. Briefly, after 24 h treatment, 1% Alamar Blue (Sigma, USA) was added to each well for another 4 h incubation. Then, optical density of cells was detected on a plate reader at a wavelength of 570 nm and 600 nm (EL-800, Micro-Tek instruments). The cell viability was presented by the amount of reduced Alamar Blue calculated by the formula as [AR570 = A570-(A600XRo),Ro = AO570/AO600].The value was expressed was the percentage of the control.

Detection of apoptosis by flow cytometry

N2a cell apoptosis level was quantified by flow cytometry. The N2a cells were washed three times with pre-chilled PBS at 4 degrees Celsius for three minutes. The cells were digested with trypsin without EDTA. The cells in suspension were centrifuged at 1000 rcf for 5 minutes. Discard the supernatant, cells were washed with PBS, and then centrifuge at 1000 rcf for 5 minutes. Next we Resuspend the pellet in 100 µL of Binding Buffer, 5 µL of Annexin V-FITC, and 5 µL of propidium iodide. The sample was incubated for 15 minutes at room temperature in the dark and 400 µl was added for loading buffer. The samples were detected by flow cytometry (CytoFLEX; Beckman Coulter, USA) within 1 h. Each sample was analyzed in triplicates.

Western blot analysis

The whole protein was extracted from tissue or cells by lysis buffer (P0013, Beyotime, China) and the protein concentration was detected by Pierce® (BCA) protein assay kit (TaKaRa, Japan) according to the vendor’s protocol. Sample containing the same amount of protein was loaded on to a SDS-polyacrylamide gel. After separation, the proteins were transferred to a PVDF membrane followed by blocking of unspecifific

binding sites and treatment with primary antibodies. The profile of primary and secondary antibodies tested in this study was shown as Table. 1. Protein expressions were detected by chemiluminescence assay and quantitated by imaging analysis system (FL1000, Thermo Fisher, USA). The relative fold of target protein towards to reference protein in treatment group was normalized to that in untretment.

Table 1. Antibodies Used in the Study

Antibodies	Epitopes	Dilution	References and Sources
PP2A-C α/β antibody (1D6)	PP2A C submit	1/2000	Santa Cruz (USA)
demethylated-PP2A-C antibody (4B7)	demethylated-PP2A-C submit	1/2000	Santa Cruz (USA)
methyl-PP2A-C α/β antibody (2A10)	methyl-PP2A-C submit	1/500	Santa Cruz (USA)
Anti-LCMT1 antibody(4A4)	LCMT-1	1/1000	Santa Cruz (USA)
Anti-PME1 antibody(B12)	PME-1	1/1000	Santa Cruz (USA)
Anti-Tau antibody	Tau (Ser 396)	1/2000	Abcam (Cambridge, UK)
Anti-Tau antibody	Tau (Ser 199)	1/2000	Abcam (Cambridge, UK)
Anti-Tau antibody	Tau (Ser 202)	1/4000	Abcam (Cambridge, UK)
Anti-Tau antibody	Tau (Ser 404)	1/2000	Abcam (Cambridge, UK)
Anti-Tau antibody	Tau-5	1/2000	Abcam (Cambridge, UK)
α-Tubulin antibody	α-Tubulin	1/2000	Beyotime (Shanghai, China)
Cyclophilin B antibody	Cyclophilin B	1/2000	Beyotime (Shanghai, China)

Quantification of SAM and SAH in brain tissue and cells

SAM and SAH were determined by ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS). SAM and SAH in brain tissues and cultured cells were extracted. Brieflfly, counted cells were collected in 1.5-ml Eppendorf tubes containing 200 μl of initial mobile phase (5.0 mmol/L ammonium acetate + 0.4% acetic acid + 2% methanol) and repeatedly frozen and thawed at -80 °C for three times. Frozen tissues at − 80 °C were removed and placed in 1.5-ml Eppendorf tubes containing 200 μl of extraction buffer, and the tissues were fully ground by using a hand-held homogenizer, followed by sonication at 40 W and 60% duty cycle for about 40 s, and then, all samples were clarified by centrifugation at 10 000 g for 10 min at 4℃. The supernatant was taken and made up to 1 ml, passed through an organic phase filter (0.22 μm, 13 mm), and tested within 24 hours after bottling. The levels of SAM and SAH were assayed using the UPLC system (CORTES, Waters Corp, USA) interfaced with a mass spectrometer (QTRAP 4500, AB SCIEX, USA). Brieflfly, the samples were separated on a CORTES UPLC-C18 + column and subjected to multiple reaction monitoring (MRM) scanning in electrospray ion source positive ion mode (ESI+). The column temperature was maintained at 40℃ and

the flow rate was 0.35 mL/min, and the gradient elution process is as follows: buffer A: 0.4% (v/v) acetic acid aqueous solution containing 10 mmol/L ammonium acetate; buffer B: methanol. T = 0 min, 0%B; T = 1 min, 2%B; T = 3 min, 75%B; T = 4 min, 2%B; T = 5 min, 2%B. The ESI source voltages were 5.5 kV with a capillary temperature of 400 °C; CUR: 206 kPa; Collision Gas(CAD): Medium; GS1: 384 kPa; GS2: 384 kPa. The injection volume was 2 μl and triplicate injections were performed for each sample. The area under each peak was quantified using software and the accuracy was re-examined. The results were quantified by external standard method.

Measurement of oxidative stress index in virto

The level of reactive oxygen species (ROS) in cells induced by above treatment after 24 hours was tested by 2,7-dichlorofluorescein diacetate (DCFH-DA) assay (Beyotime, China). Briefly, DCFH-DA (10 μmol/L) was added and incubated with cell at 37 °C for 30 min then washed twice with PBS.Subsequently, the fluorescence intensity was read by nulti-function microplate reader (excitation wavelength of 488 nm and the emission wavelength of 525 nm). GSH content of cells was detected by GSH and GSSG Assay Kit (Beyotime, China), the specific method was based on the previous research [50]. Briefly, total glutathione (GSSG plus GSH) was determined spectrophotometrically with DTNB and NADPH at 412 nm. GSSG was measured in the same way in the presence of 2-vinylpyridine, the amount of GSH was calculated by subtracting the amount of GSSG from the amount of total glutathione.

Data analysis

Results are presented as means ± SEM as independt experiments. Statistical graph was performed using GraphPad Prism 6 statistical software (GraphPad Software). The one-way ANOVA was used to determine the differences among groups. The difference was considered as significane while $p < 0.05$.

Abbreviations

ROS: Reactive oxygen species; MWM: Morris water maze; AD: Alzheimer’s disease; PD: Parkinson's disease; i.p: Intraperitoneal

Declarations

Acknowledgements

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Authors’ Contributions

Cailing Lu and Xiyi Li conceptualized and designed the study. Bin Wu, Haiqing Cai，Shen Tang performed the experiment, drafted the initial manuscript, and approved the final manuscript as submitted. Deqiang

Xiao processed and analyzed data. Xinhang Wang, Yilu Xu, Qianqian Shi, Ling Meng and Lancheng Wei coordinated the project. Yunfeng Zou and Xiaobo Yang supervised experiments and revised manuscript.

Funding

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Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal studies were approved by the ethical committee for animal experiment of the Guangxi Medical University (GXMU-2014-036). and in accordance with the international standards of guidelines for the Care and Use of Laboratory Animal in animal experiments. This study strived to minimize the number and the suffering of experimental animals.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Additional File

Figure S1. Morphology images of N2a cell after exposure to Mn. **Figure S2.** Cell viability of N2a cells after exposure to Mn at different concentration. **Figure S3.** Morpho-logy images of N2a cell after treated in growth medium with methionine(10~20mg/L). **Figure S4** Cell viability of N2a cells after treated in growth medium with methionine at different concentration.

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Figures

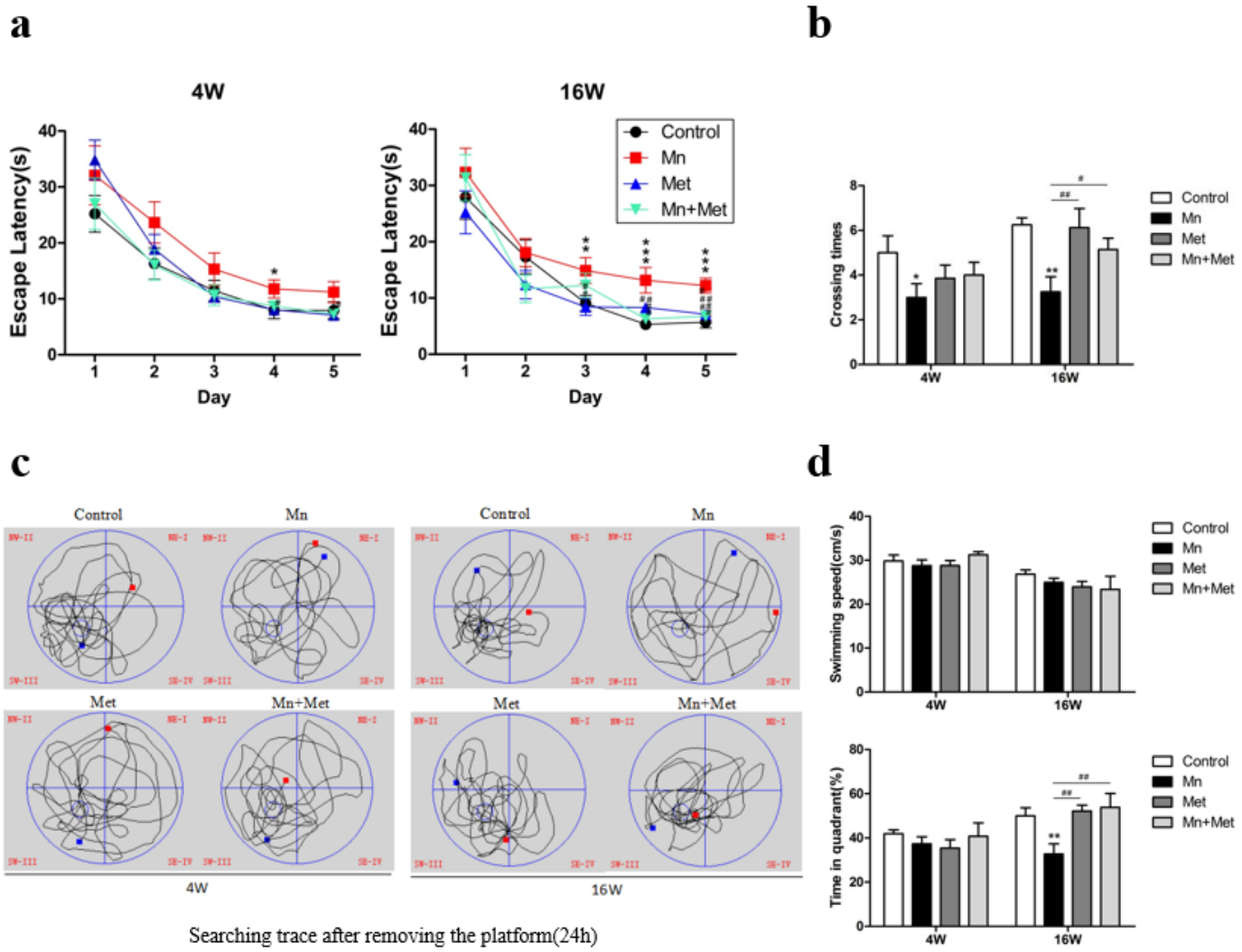

Figure 1

Evaluation of spatial learning and memory in rat. a The latency to reach the hidden platform. b The representative searching trace after removing the platform at 24 hr in the probe trial after training. c The times to cross the platform is shown. d Up: swimming speed after removing the platform in the probe trial. Down: the percentage of time spent in target quadrant is shown. All data are expressed as means ± S.D. (n = 8). *p < 0.05 and **p < 0.01 vs the control group; # p < 0.05 and ##p < 0.01 vs Mn group.

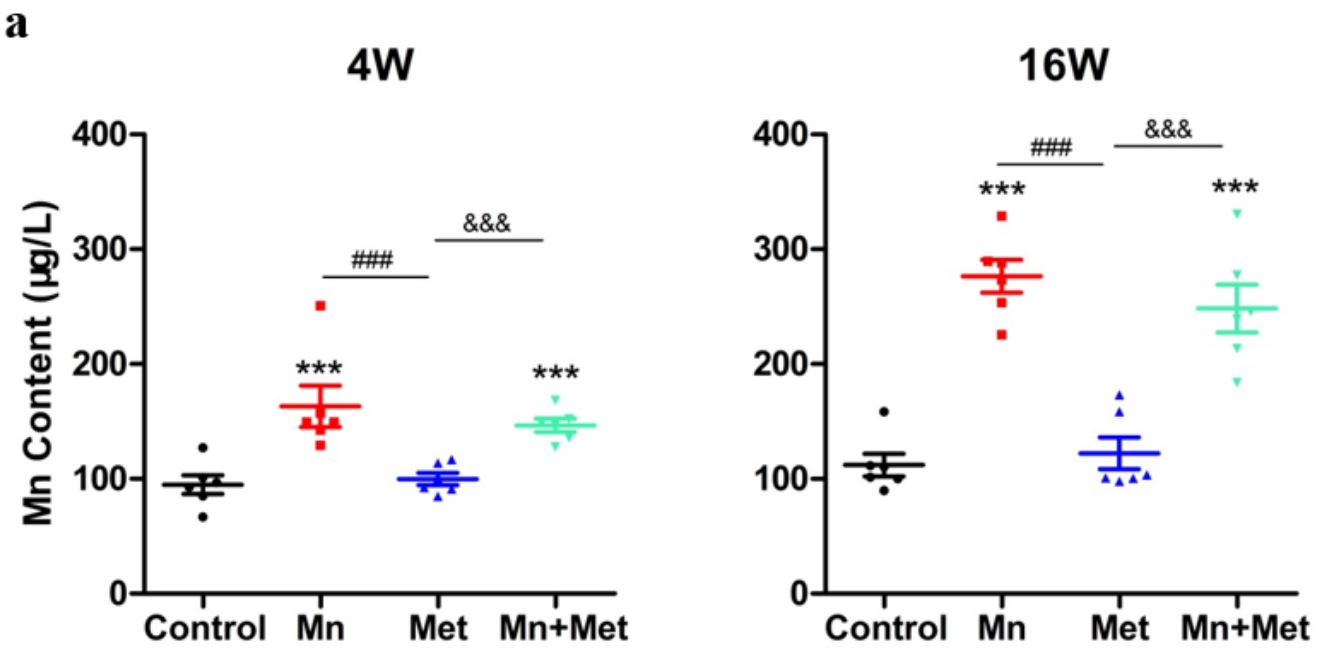

Figure 2

Determination of manganese content in blood. a The whole blood manganese level of rats in different treatment groups. All data are expressed as means ± S.D. (n = 6). ***p < 0.005 vs the control group; ###p < 0.005 vs Mn group; &&&p < 0.005 vs methionine group.

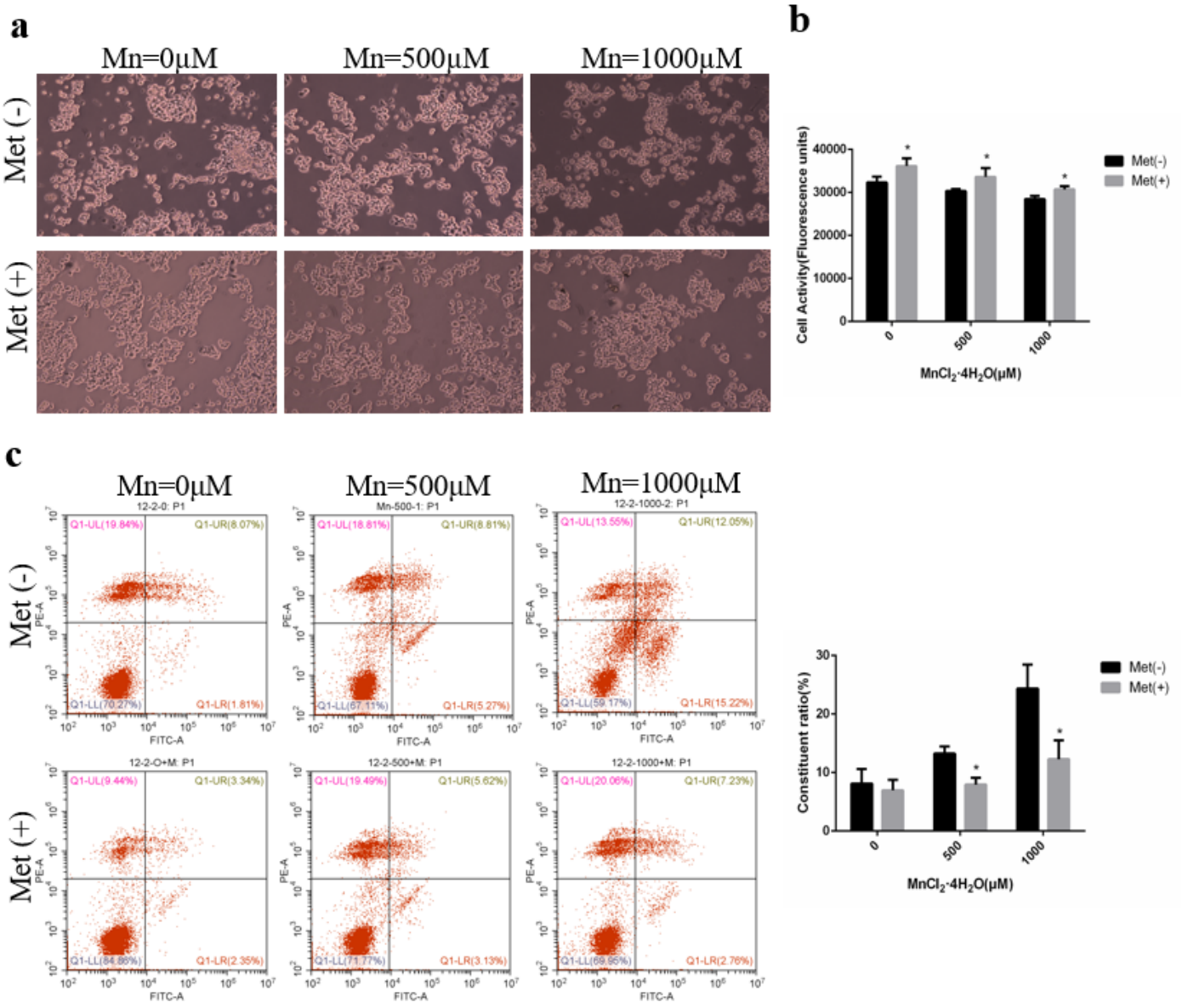

Figure 3

The role of methionine in Mn induced N2a cytotoxicity. a Morphology images of N2a cell after incubated in different concentration Mn and methionine medium. b Cell viability of N2a cells after incubated in Mn and met medium. c Protective effect of methionine against Mn-induced apoptosis in N2a cells. Methionine deficiency was used as the control group, the data shown represent the mean ± SD, three independent trials(n = 3). *p < 0.05 vs the the control group.

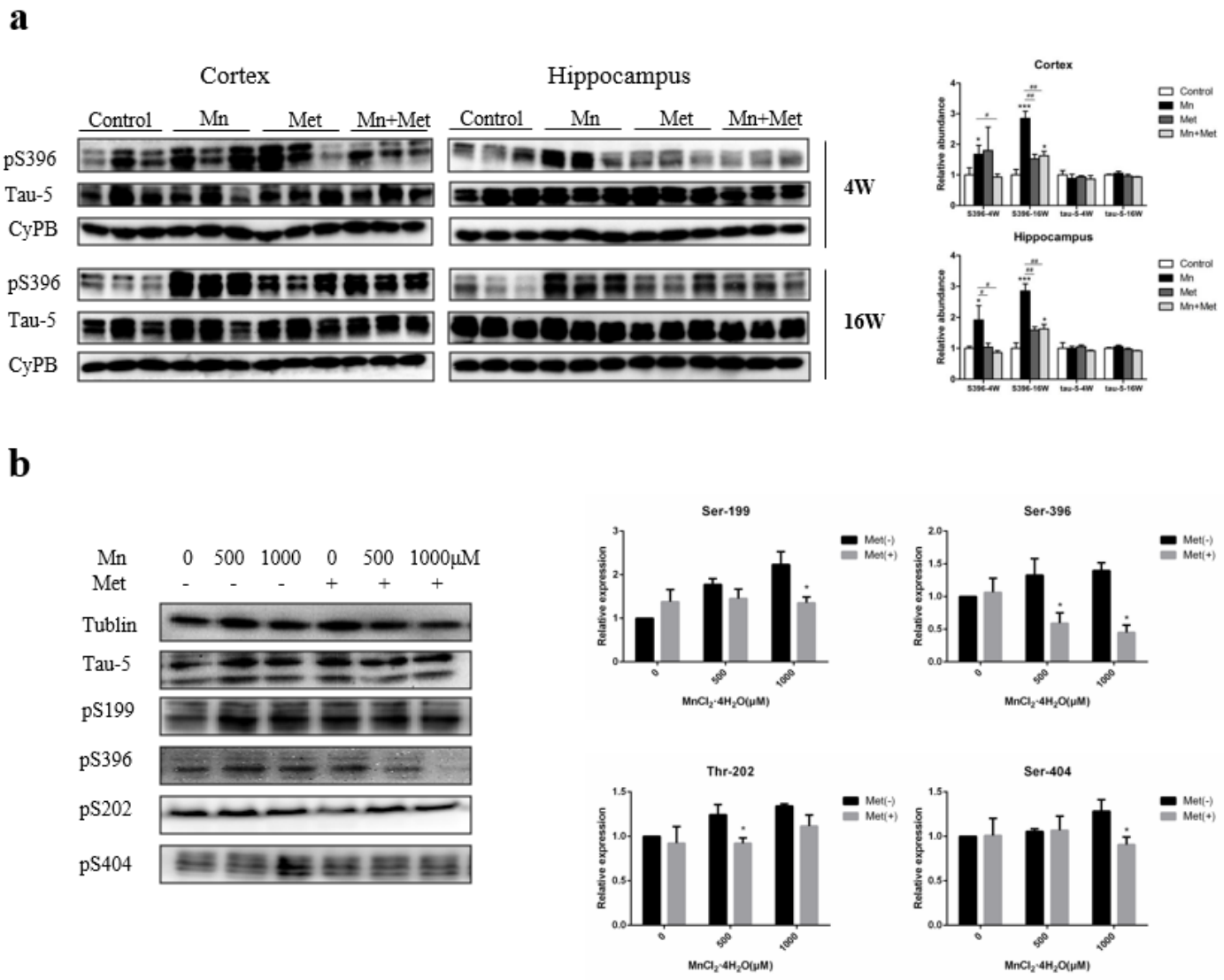

Figure 4

Regional alterations in p-Tau levels in vivo and vitro. a Methionine promoted dephosphorylation of tau in rat models of subacute and chronic manganese exposure. b Methionine promotes the dephosphorylation of different phosphorylation sites of tau in N2a cells. All data are presented as mean ± S.D. from each group (n = 6). Methionine deficiency was used as the control group. *p < 0.05 and ***p < 0.005 vs the control group; # p < 0.05 and ##p < 0.01 vs Mn group.

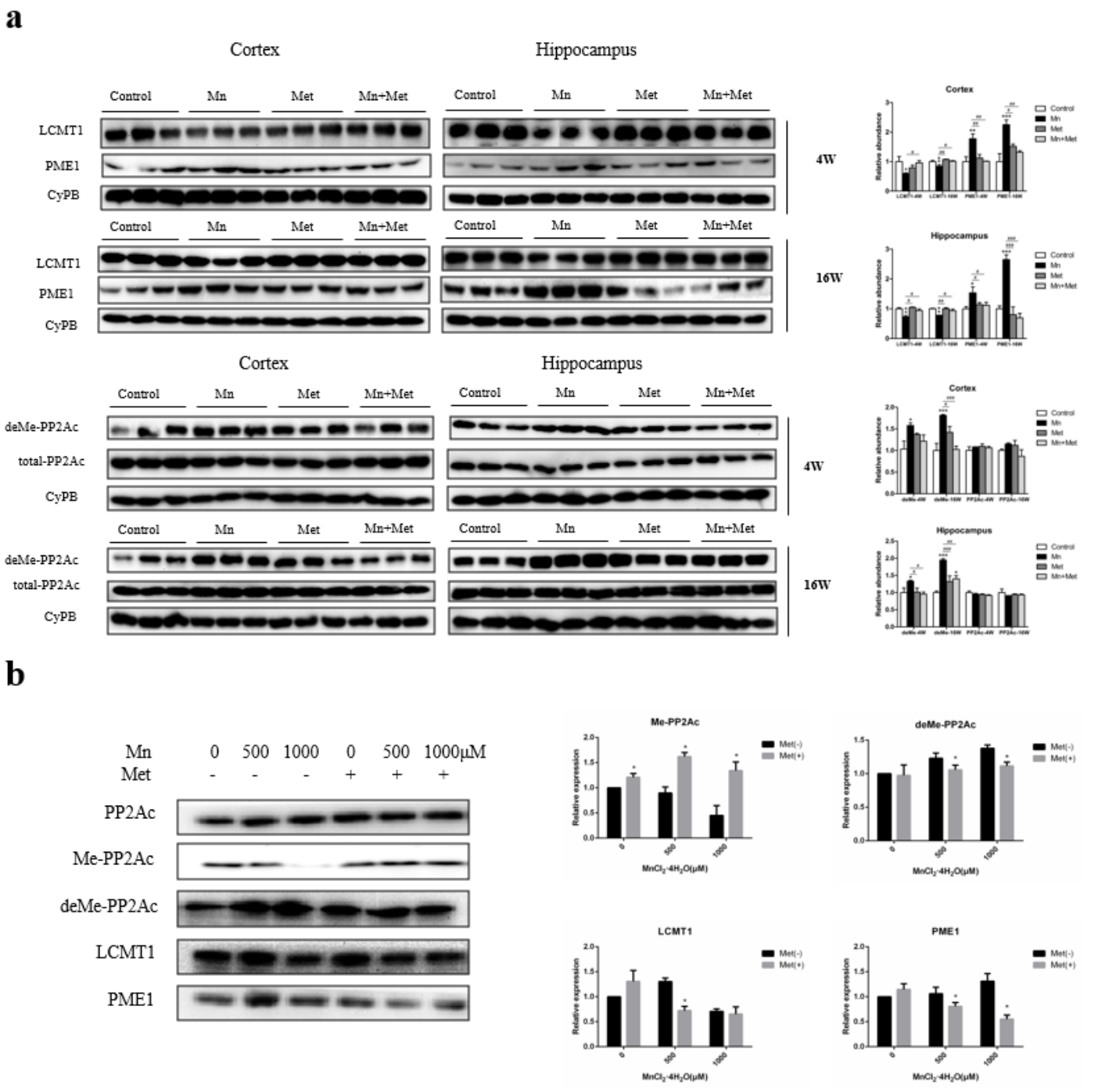

Figure 5

Expression of PP2Ac methylation / demethylation and phosphorylation related proteins in tissues and cells. a Methionine down regulated the protein level of PP2Ac demethylation in cortex and hippocampus of Mn rats. b The protective effect of methionine on Mn induced imbalance of methylation / demethylation of PP2Ac and hyperphosphorylation of PP2Ac in N2a cells. Methionine deficiency was used as the control group. All data are presented as mean ± S.D, from each group (n = 6). *p < 0.05, **p < 0.01 and ***p < 0.005 vs the control group; # p < 0.05, ##p < 0.01 and ###p < 0.005 vs Mn group.

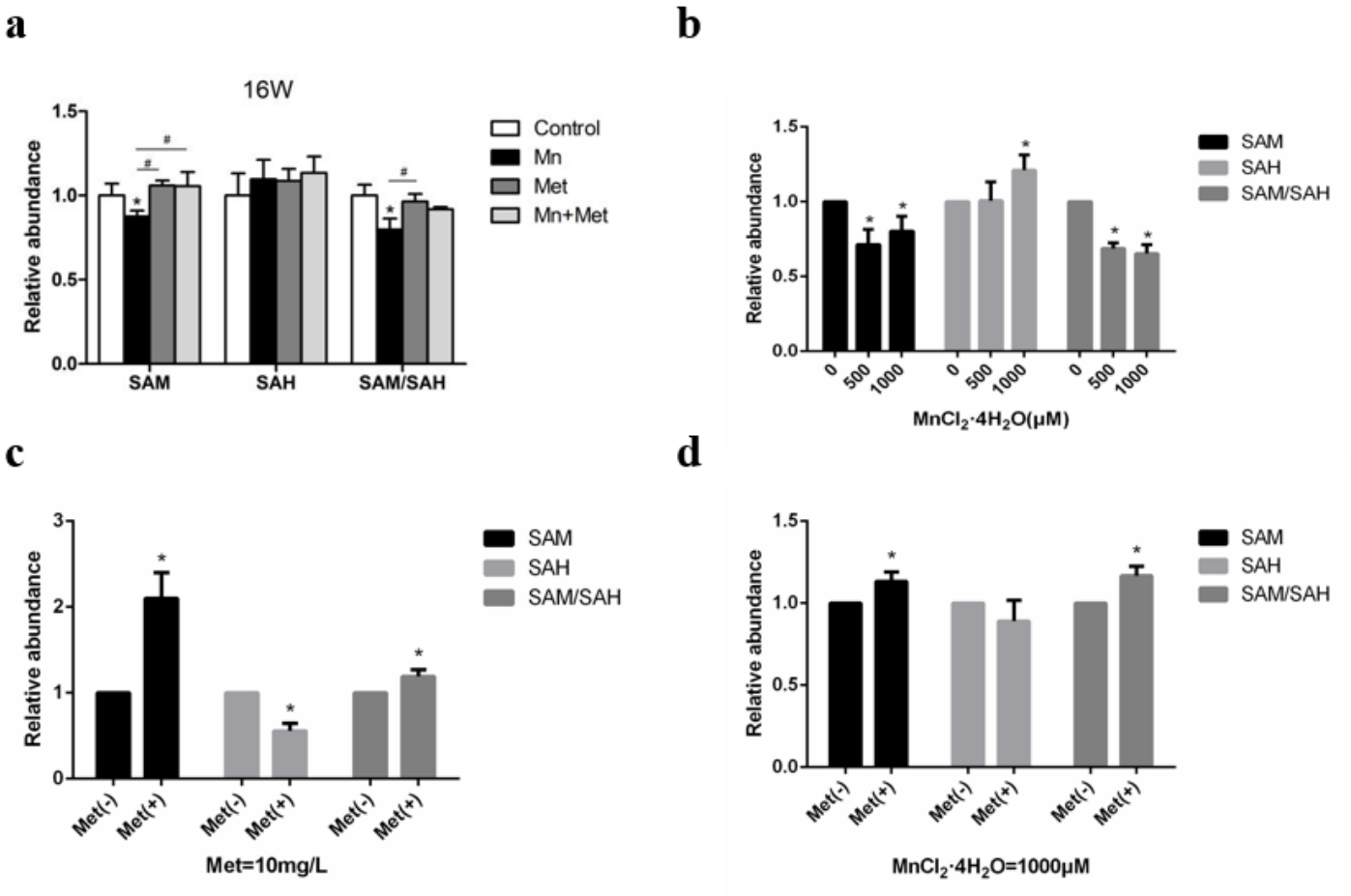

Figure 6

Changes of Sam and SAH contents in hippocampus and cells. a The contents of SAM and SAH in the hippocampus of 16-weeks rats. b The contents of SAM, SAH and SAM/SAH of N2a cells after exposure to Mn. c The contents of SAM, SAH and SAM/SAH of N2a cells after treated in methionine. d The contents of SAM, SAH and SAM/SAH of N2a cells after treated in methionine and Mn. Methionine deficiency was used as the control group. All data are presented as mean ± S.D. from each group (n = 6). *p < 0.05 vs the control group; #p < 0.05 vs Mn group.

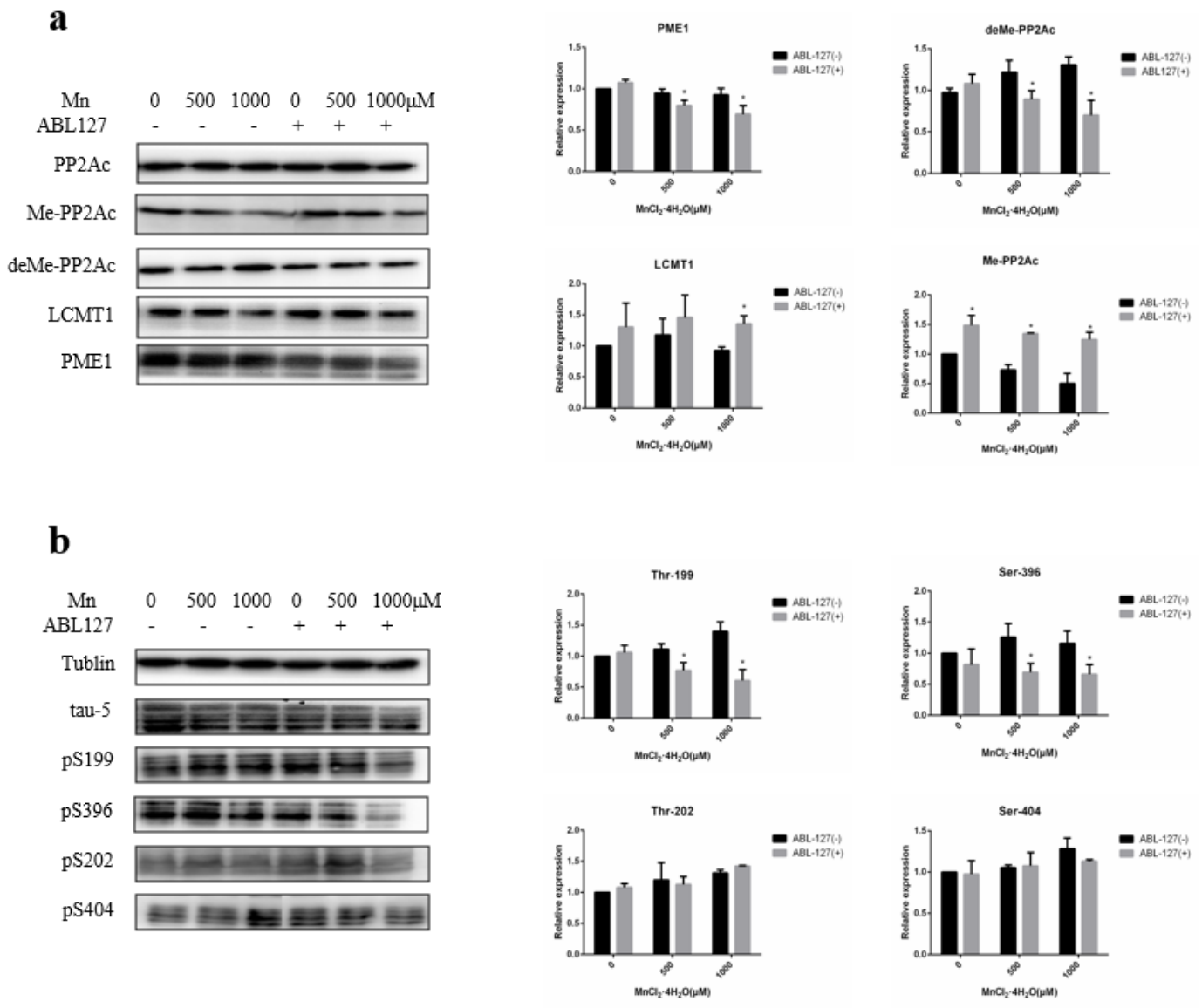

Figure 7

Effect of ABL127 on tau dephosphorylation and PP2Ac demethylation of N2a cells. a ABL127 inhibited Mn-induced PME1 and demethylation of PP2Ac in N2a cells. b ABL127 inhibited Mn-induced dephosphorylation of tau in N2a cells. All data are presented as mean ± S.D. from each group (n = 6). *p < 0.05 vs the ABL127 deficiency group.

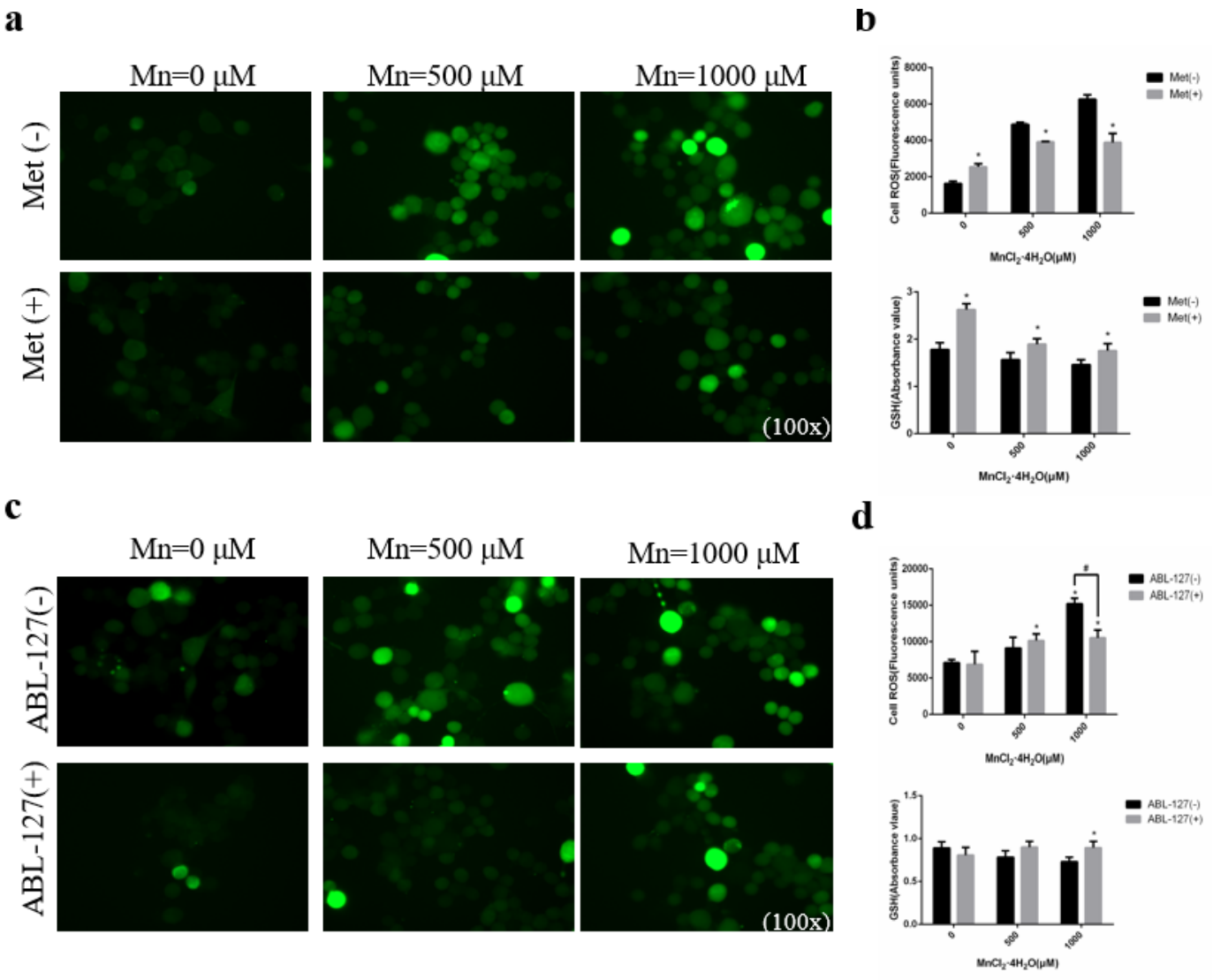

Figure 8

ABL127 and methionine increaed GSH and decreased Mn-induced ROS. a Fluorescence image of N2a cell after treated in Mn and methionine. b Methionine decreaed Mn-induced ROS level in N2a cells by increaing GSH. c Fluorescence image of N2a cell after treated in Mn and ABL127. d ABL127 decreaed Mn-induced ROS level in N2a cells by increaing GSH. All data are presented as mean ± S.D, three independent trials(n=3). *p < 0.05 vs the ABL127 deficiency group or methionine deficiency group.

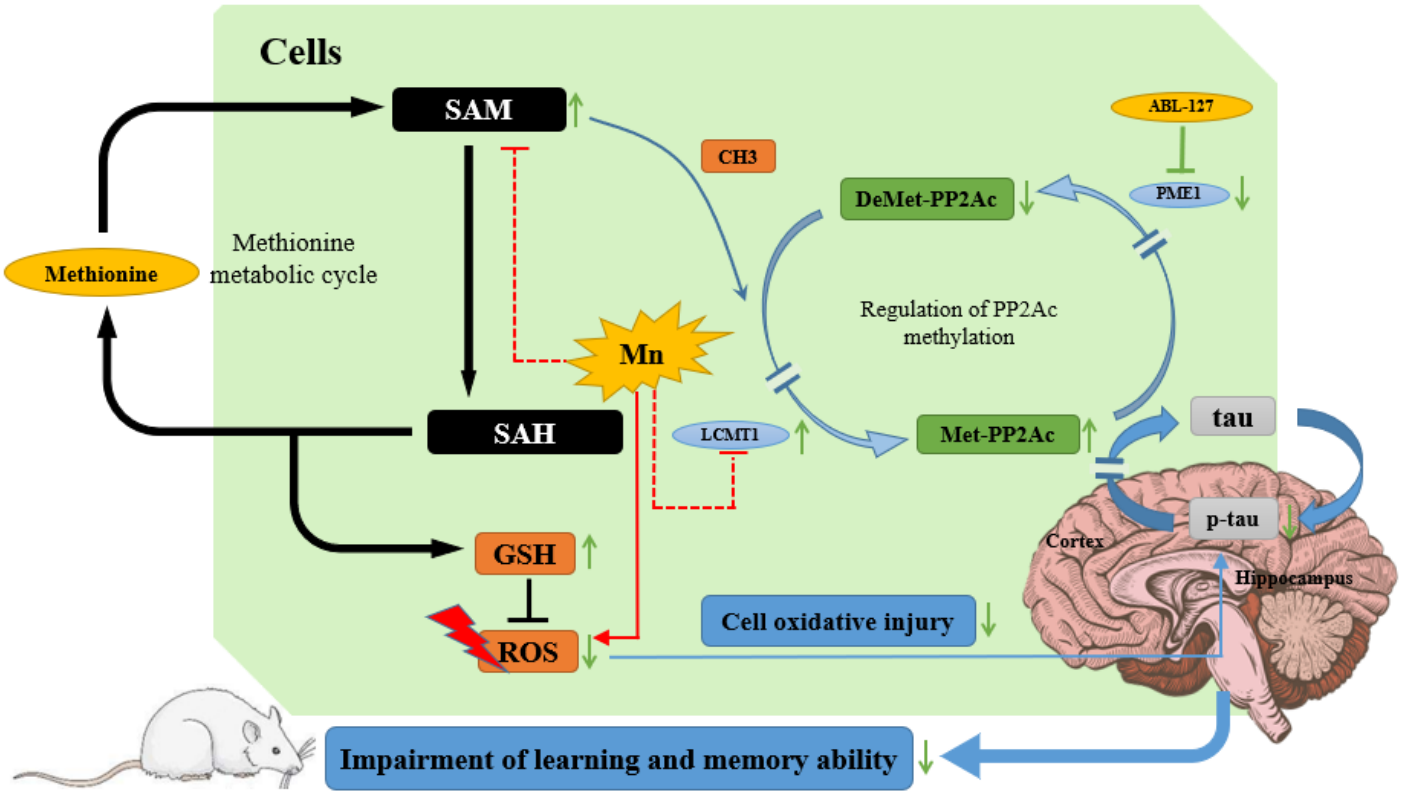

Figure 9

Schematic diagram of methionine and ABL127 protecting manganese-induced learning and memory impairment. Tau hyperphosphorylation is controlled by PP2A inactivatione which is regulated by PP2Ac demethylation. Methionine induced an increase in SAM levels, down-regulated the expression of PME1, and up-regulated the expression of LCMT1, thereby reducing the abnormal increase in PP2Ac demethylation induced by manganese. ABL127 down-regulates PP2Ac demethylation by specifically inhibiting PME-1. The down-regulation of PP2Ac demethylation prevents the accumulation of phosphorylated tau, and increases the GSH, a downstream product of the methionine cycle, thereby reducing the Mn-induced ROS level, eventually reducing oxidative stress damage in rats and improving learning and memory abilities.

Supplementary Files

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

- Additionalfile1.pptx