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Research paper

# Metformin attenuates diabetes-induced tau hyperphosphorylation in vitro and in vivo by enhancing autophagic clearance

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ABSTRACT

Diabetes mellitus (DM) can increase the risk of Alzheimer's disease (AD) in patients. However, no effective approaches are available to prevent its progression and development. Recently, autophagy dysfunction was identified to be involved in the pathogenesis of neurodegenerative diseases. This study was designed to investigate the effect of metformin on hyperphosphorylated tau proteins in diabetic encephalopathy (DE) by regulating autophagy clearance. db/db mice were randomly divided into four groups, db/+ mice were used as control group. Twelve-week old male db/db mice received consecutive intraperitoneal injection of 200 mg/kg/d metformin or (and) 10 mg/kg/d chloroquine for eight weeks. Morris water maze (MWM) tests were performed to test cognitive functions before the mice were euthanized. Metformin attenuated cognitive impairment in db/db mice, reduced hyperphosphorylated tau proteins, restored the impaired autophagy in diabetic mice, all of which were reversed by inhibiting of autophagy activity. In high glucose-cultured HT22 cells, metformin increased autophagy in a dose-dependent manner. Besides, metformin enhanced autophagy activity in an AMPK dependent manner. These data show that metformin may reduce tauopathy and improve cognitive impairment in db/db mice by modulating autophagy through the AMPK dependent pathway. These findings highlight metformin as a new therapeutic strategy for the treatment of DE.

## 1. Introduction

Epidemiological evidence indicates that diabetes mellitus (DM) is associated with a higher risk of cognitive impairment, dementia and Alzheimer's disease (AD) (Leibson et al., 1997), DM-induced cognitive impairment is called diabetic encephalopathy (DE). Two major pathological hallmarks of AD are extracellular amyloid plaques formed by β-amyloid peptides (Aβs) and intracellular neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau protein. Interestingly, in diabetic patients, cerebrospinal fluid (CSF) levels of total tau and phosphorylated-tau (pTau) are significantly increased, but is not associated with $A\beta_{42}$ level, which indicates that tau and pTau instead of Aβ plaques are more likely to be pathological hallmarks for diabetes associated dementia (Moran et al., 2015). A recent study reported that leptin resistance-induced diabetes accelerates the development of tauopathy, and may ultimately lead to cognitive impairment (Platt et al., 2016). And other studies have shown that obvious phosphorylation of tau protein in the brain starts as early as 6–8 weeks of age in db/db (BKS.Cg-Dock7m+/+Lepr$^{db}$/J) mice and in the streptozotocin (STZ)-treated mice (Kim et al., 2009; Planel et al., 2007). Tau is a microtubule-associated protein, abnormally hyperphosphorylated tau forms NFTs which is closely related to cognitive impairment (Ballatore et al., 2007). Hence, targeting hyperphosphorylated tau protein has been considered as one of the promising approaches to diabetes associated dementia.

Metformin is one of the first-line treatments for glycemic control in type 2 diabetes mellitus (T2D) patients. Interestingly, aside from blood glucose control, metformin also has beneficial effects for various central nervous system (CNS) disorders, including ischemic brain disease (Jiang et al., 2014a), Parkinson's disease (Perez-Revuelta et al., 2014), and Huntington's disease (Vazquez-Manrique et al., 2016), etc. Recent clinical trials report that long term use of metformin in T2D patients significantly lower the incidence of dementia (Ng et al., 2014; Hsu et al., 2011), and animal studies have also shown a protective effect of metformin in cognitive impairment in different animal models of diabetes (Pintana et al., 2012; Oliveira et al., 2016; Li et al., 2012). To date, the underlying mechanisms of neuroprotective effect of metformin still remain poorly understood.

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Macroautophagy (hereafter called autophagy) is a highly conserved catabolic process that degrades the aggregate-prone proteins and damaged cytoplasmic organelles within cells. Autophagy dysfunction in neurons has been reported to contribute to various neurodegenerative diseases, including AD (Kiriyama and Nochi, 2015; Komatsu et al., 2006). Down regulated autophagy in neurons leads to accumulation of misfolded proteins, and then aggravates the deposition of Aβ and formation of NFTs in AD brain (Li et al., 2016). Furthermore, studies have reported that pharmacological induction of autophagy could be a promising therapeutic strategy in neurodegenerative diseases (Jiang et al., 2014b; Jang et al., 2016; Friedman et al., 2015). T2D is also associated with down-regulation of autophagy (Kanamori et al., 2015; Wilson et al., 2014), which could be the pathogenesis of diabetes associated dementia.

To better understand the potential mechanisms of metformin on diabetes associated dementia, high glucose-treated mouse HT22 cells and db/db mice were used as in vitro and in vivo models of diabetes. We demonstrated that metformin treatment enhances autophagic clearance of hyperphosphorylated tau in high glucose-incubated HT22 cells and in brain of db/db mice through the activation of the AMP-activated protein kinase (AMPK), ultimately attenuates cognitive impairment in db/db mice.

## 2. Materials and methods

### 2.1. Reagents

Metformin and chloroquine (CQ) were purchased from Sigma (Sigma, St. Louis, MO, USA). 3-methyladenine (3-MA) and compound C (CC) were purchased from Selleckchem (Selleck, Westlake Village, CA, USA). In vitro studies, 3-MA and CC were dissolved in DMSO and adjusted to the final concentration with culture medium.

### 2.2. Cell culture and treatments

Mouse hippocampal neuron cells HT22 were a generous gift from prof. Wei-Lin Jin (Institute of Bio-Nano-Science and Engineering, Department of Instrument Science and Engineering, School of Electronic Information and Electrical Engineering, and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China). The cells were cultured in standard Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and incubated at 37 °C with 5% $CO_2$. The glucose concentration of 25 mM in DMEM was considered the normal glucose (NG), additional 25 mM glucose was added into DMEM (50 mM) and considered the high glucose (HG). Cells were incubated with either NG or HG medium for 24 h, and then were treated with vehicle (DMSO) or metformin (3.2 mM) in the presence or absence of 3-MA (10 mM) or compound C (10 μM) for another 24 h.

### 2.3. Transfection of cells with Beclin 1 siRNA

Specific small interfering RNA (siRNA) targeting Beclin 1 (Sense: 5′-CAGUUUGGCACAAUCAAUAUUTT-3′; Anti-sense: 5′-AAUAUUGAU UGUGCCAAACUGTT-3′) and control siRNA (Sense:5′-UUCUCCGAACG UGUCACGUTT-3′; Anti-sense: 5′-ACGUGACACGUUCGGAGAATT-3′), which should not knock down any known proteins, were purchased from Gene Pharma, Inc. (Gene Pharma, Shanghai). The above-mentioned siRNA molecules were transfected into the cells using Lipofectamine 2000 (Invitrogen, USA). 48 h after transfection, cells were exposed to various treatments as specifically indicated.

### 2.4. Animals and drug treatments

Six-week old male db/db (BKS.Cg-Dock7m+/+Leprdb/Nju) mice and their age-, sex-matched wild-type db/+ control mice were purchased from Model Animal Research Center of Nanjing University. All animals were housed five per cage in a temperature-controlled room (22 ± 2 °C) with a 12 h light/dark cycle (lights on 8 a.m.-8 p.m.), and given free access to food and water. All experimental procedures were carried out during the light phase. The animal protocol was approved by the Animal Care and Use Committee of The First Affiliated Hospital of Chongqing Medical University. All animal experiments followed Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies complied with the ARRIVE guidelines.

The mice (n = 60) were randomly selected and allocated to five groups: (Leibson et al., 1997) db/+ group (control group, n = 12); (Moran et al., 2015) db/db group (DM group, n = 12); (Platt et al., 2016) DM + metformin treatment group (Met group, n = 12); (Kim et al., 2009) DM + metformin + CQ treatment group (Met + CQ group, n = 12); DM + CQ treatment group (CQ group, n = 12). CQ is one of the most widely used autophagy inhibitors in vivo which can inhibit the fusion of autophagosomes with lysosomes. Drug treatment was started when mice were 12 weeks old. Metformin (200 mg/kg/d) and CQ (10 mg/kg/d) were dissolved in saline. Mice were injected intraperitoneally with vehicle (control group, DM group), metformin (Met group), metformin + CQ (Met + CQ group) and CQ (CQ group) every day for 8 consecutive weeks, respectively. Their body weights were measured every one week and fasting blood glucose levels were measured every one month.

### 2.5. Morris water maze

Cognitive performance was evaluated by the Morris water maze (MWM) during the last 6 days of experimental period. The mice (n = 10 per group) were given 4 training trials per day for 5 consecutive days. For each trial, the mouse was placed in the pool (facing pool wall) at one of the selected quadrants. The duration of each trial lasted until the mouse found the platform or until a maximum of 60 s. If the mouse failed to find the platform within 60 s, it was guided to the platform by technician for 15 s. Once a mouse mounted the platform, it was allowed to remain there for 5 s. Acquisition was measured as escape latency to reach the platform. Twenty-four hours after the last trial, mice were subjected to a probe trial in which the platform was removed and they were allowed to swim freely for 60 s. The frequency of an individual mouse passing the platform area and the time the animal spent in the target quadrant were recorded as a measure of spatial memory.

After probe trials, all mice performed a visible platform test to detect possible deficits in visual acuity and motor ability. The platform was fixed in a new quadrant 1 cm above the water level, and the latency to reach the platform was recorded.

### 2.6. Brain tissue preparation

Following behavioral testing, all mice were deeply anesthetized with 1% sodium pentobarbital (40 mg/kg) at 21 weeks of age. For western blotting, hippocampi were dissected out quickly and stored in liquid nitrogen. For immunohistochemistry (IHC) and hematoxylin & eosin (HE) staining, rats were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were removed and fixed in 4% PFA at 4 °C.

### 2.7. Western blotting

Cells and hippocampal tissues were homogenized in RIPA buffer (Beyotime Inc.) supplemented with a protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Protein concentrations were determined by using BCA Protein Assay Kit (Beyotime Inc.). Equal amount of protein from different samples were loaded onto 8–10% SDS polyacrylamide gels and separated by electrophoresis at 100 V for 120 min. The proteins were transferred onto PVDF

**Table 1**
Body weight (g) of mice in the control, db/db, Met, Met + CQ and CQ groups.

| Group | Control | db/db | Met | Met + CQ | CQ |
|---|---|---|---|---|---|
| Week 8 | 19.67 ± 0.37 | 38.30 ± 0.88[a] | 37.55 ± 1.04[a] | 38.55 ± 0.52[a] | 36.95 ± 0.48[a] |
| Week 9 | 22.73 ± 0.45 | 47.93 ± 1.13[a] | 45.57 ± 1.38[a] | 47.12 ± 0.92[a] | 45.22 ± 0.37[a] |
| Week 10 | 23.17 ± 0.27 | 51.35 ± 1.11[a] | 49.31 ± 1.35[a] | 50.90 ± 1.00[a] | 48.87 ± 0.44[a] |
| Week 11 | 23.97 ± 0.27 | 53.55 ± 1.24[a] | 51.77 ± 1.63[a] | 52.72 ± 1.05[a] | 51.35 ± 0.50[a] |
| Week 12[1] | 24.68 ± 0.33 | 53.62 ± 1.26[a] | 53.25 ± 1.33[a] | 53.65 ± 1.12[a] | 53.03 ± 0.45[a] |
| Week 13 | 24.57 ± 0.29 | 54.85 ± 1.58[a] | 54.53 ± 1.18[a] | 55.95 ± 1.16[a] | 54.87 ± 0.64[a] |
| Week 14 | 24.50 ± 0.21 | 57.00 ± 1.54[a] | 55.12 ± 1.29[a] | 57.55 ± 1.28[a] | 55.28 ± 0.69[a] |
| Week 15 | 25.47 ± 0.19 | 58.15 ± 1.41[a] | 55.05 ± 1.43[a] | 58.57 ± 1.38[a] | 56.15 ± 0.60[a] |
| Week 16 | 26.35 ± 0.25 | 59.68 ± 1.10[a] | 56.53 ± 1.20[a] | 59.42 ± 1.39[a] | 57.38 ± 0.68[a] |
| Week 17 | 26.17 ± 0.24 | 59.85 ± 1.09[a] | 55.68 ± 1.24[a] | 58.03 ± 1.40[a] | 57.91 ± 0.82[a] |
| Week 18 | 25.85 ± 0.36 | 59.77 ± 0.97[a] | 55.63 ± 1.14[a] | 57.80 ± 1.40[a] | 57.65 ± 0.92[a] |
| Week 19 | 26.40 ± 0.36 | 58.85 ± 1.53[a] | 54.80 ± 1.20[a] | 57.73 ± 1.34[a] | 57.15 ± 0.78[a] |
| Week 20[2] | 25.85 ± 0.29 | 58.80 ± 1.39[a] | 54.13 ± 1.02[a] | 56.97 ± 1.60[a] | 57.18 ± 0.87[a] |

[1] Body weight of all mice before the treatment of metformin, metformin + chloroquine, chloroquine or vehicle.
[2] Body weight of all mice after the treatment of different drugs.
[a] $P < 0.01$ versus the control group. There were 10–12 animals in each experimental group at each time point.

**Table 2**
Blood glucose level (mmol/L) of mice in the control, db/db, Met, Met + CQ and CQ groups.

| Group | Control | db/db | Met | Met + CQ | CQ |
|---|---|---|---|---|---|
| Week 12[1] | 6.23 ± 0.15 | 27.37 ± 1.04[a] | 27.22 ± 0.90[a] | 26.15 ± 1.35[a] | 26.50 ± 0.45[a] |
| Week 16 | 6.15 ± 0.19 | 29.20 ± 1.20[a] | 26.37 ± 0.72[a] | 27.73 ± 0.92[a] | 28.42 ± 0.39[a] |
| Week 20[2] | 6.16 ± 0.17 | 30.48 ± 0.51[a] | 27.15 ± 1.13[a,b] | 29.32 ± 1.05[a] | 30.25 ± 0.71[a] |

[1] Blood glucose levels before drug administration.
[2] Eight weeks after *i.p.* administration of metformin, metformin + chloroquine, chloroquine or vehicle.
[a] $P < 0.01$ versus the control group.
[b] $P < 0.05$ versus the db/db group. There were 10–12 animals in each experimental group at each time point.

membranes (Millipore), and then blocked with 5% nonfat milk or 5% bovine serum albumin (BSA) for 1 h at room temperature. The membranes were incubated with different primary antibodies against p-Tau (Ser396) (1:1000, Cell Signaling Technology, CST), p-Tau (Ser404) (1:1000, Abcam), AT8 (Ser202/Thr 205) (1:1000, Pierce Biotechnology), Tau5 (1:1000, Abcam), P62 (1:1000, CST), Beclin 1 (1:1000, CST), LC3B (1:1000, Sigma), AMPK (1:1000, CST), p-AMPK (1:1000, CST), ACC (1:1000, CST), p-ACC (1:1000, CST), β-actin (1:1000, zhongshan Inc.) at 4 °Covernight. The next day, membranes were washed three times in TBST and incubated with a horseradish peroxidase conjugated anti-mouse (1:5000, zhongshan Inc.) or anti-rabbit (1:5000, Cell Signaling Technology) antibody for 1 h at 37 °C. The membranes were washed for another three times in TBST, protein bands were detected with enhanced chemiluminescence substrate (Thermo Scientific) by exposing to X-ray film (Vilber Lourmat, France). The band intensity were quantified by using Bio-1D software (Vilber Lourmat, France).

### 2.8. Histological examination

Brains were immersed in 4% PFA for 24 h after they were dissected out, then they were embedded in paraffin and tissue sections of 4 μm were obtained. Samples were prepared and stained with hematoxylin and eosin for morphological observation.

### 2.9. Immunohistochemistry

Brains were immersed in 4% PFA for 24 h after they were dissected out, then they were embedded in paraffin and tissue sections of 4–6 μm were obtained for IHC analysis. For heat-induced epitope retrieval, deparaffinized and hydrated sections were incubated in 1 mM citrate buffer (pH 6.0) at 95 °C for 30 min using a microwave oven. Sections were then incubated with 3% $H_2O_2$ for 20 min to block endogenous peroxidase. Nonspecific binding was blocked with 10% goat serum for 30 min at 37 °C. The sections were incubated with the primary antibodies against (rabbit monoclonal anti-Tau ($Ser^{404}$), 1:100, Abcam; mouse monoclonal anti-AT8 ($Ser^{202}$/Thr $^{205}$), 1:400, Thermo Fisher Scientific Inc) overnight at 4 °C. After washing with PBS, sections were treated with biotinylated secondary antibody (Zhongshan Inc.) for 30 min at 37 °C. Finally, immunoreactivity was detected using diaminobenzidine, followed by counterstaining with hematoxylin, then dehydrated and mounted on the slides. All the sections were examined blindly with a microscope at 400× magnification.

### 2.10. Transmission electron microscope

After specific treatment, cells were collected and fixed with a solution containing 3% glutaraldehyde + 2% paraformaldehyde in 0.1 M PBS. After fixation, all samples were then processed for transmission electron microscope (TEM) analysis in the School of Life Science at our university. The ultrathin sections were then viewed with a TEM (Hitachi-7500, Japan) by another technician who was blinded to the experiment. From each section, ten fields of images were captured randomly at a magnification of 20,000×.

### 2.11. Statistical analysis

All statistical analysis was carried out using SPSS 22.0. After confirming a normal distribution with the Shapiro-Wilk test and equal variances of the data, acquisition data from the MWM tests were analyzed using the two-way repeated measures ANOVA, and data from different treatment groups at each measurement time were compared using a multivariate ANOVA. A one-way ANOVA followed by Turkey's post hoc tests was used to analyze the other data obtained in this experiment. All data are presented as the mean ± SEM. $P < 0.05$ was considered to be statistically significant.

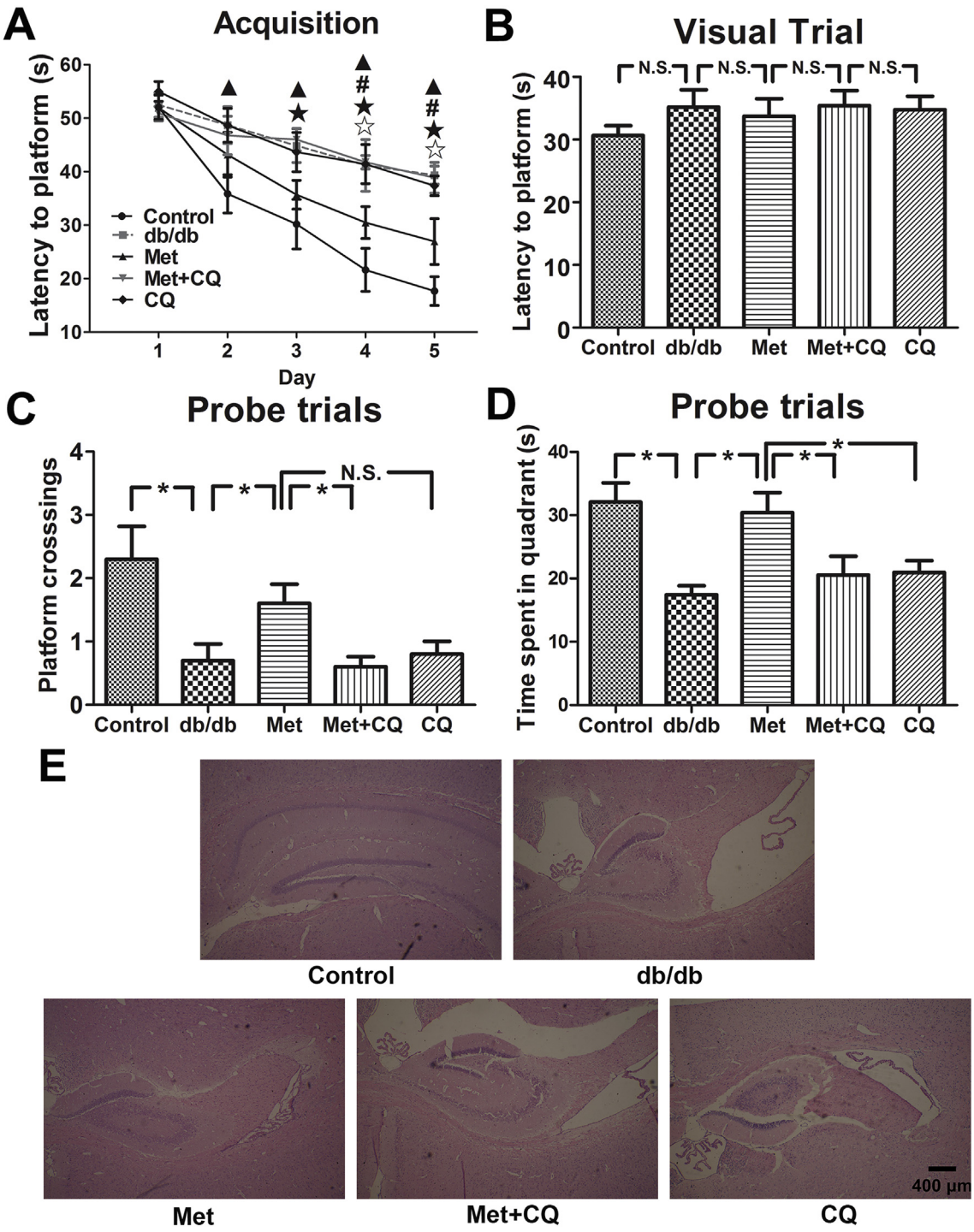


**Fig. 1.** Effect of metformin treatment on spatial cognitive impairment and hippocampal structure abnormalities in db/db mice. **A** The latency to find the hidden platform during the 5 days of acquisition. Data were analyzed using a two-way ANOVA of repeated measures followed by Turkey's post hoc tests. ▲$P < 0.05$ represent the db/db group versus the control group. ★$P < 0.05$ represent the Met group versus the db/db group. ☆represent the Met + CQ versus the Met group. #$P < 0.05$ represent the CQ group versus the Met group. **B** The latency to find the visible platform during the visual trial. **C** The number of platform crossings during the probe trial. **D** Time spent in the target quadrant during the probe trial. Data were analyzed using a one-way ANOVA followed by Turkey's post hoc tests (B, C, D). *$P < 0.05$, N.S., not significant. Results are expressed as the mean ± SEM; n = 10 animals/group. **E** Representative photos of HE staining in mice hippocampal regions. Magnification ×100. Scale bars represent 400 μm. n = 3 animals/group.

## 3. Results

### 3.1. Body weight and blood glucose level in all groups of mice

As shown in Table 1, the average body weight of the db/db, Met, Met + CQ and CQ groups was significantly heavier than the control group ($P < 0.01$) during the experimental period. Eight weeks treatment of Met, Met + CQ, CQ or vehicle did not show any significant effect on body weight in db/db mice. Blood glucose level was measured on week 12, week 16 and week 20, as shown in Table 2, the blood glucose level of the db/db, Met, Met + CQ and CQ groups was significantly higher than the control group ($P < 0.01$) during the

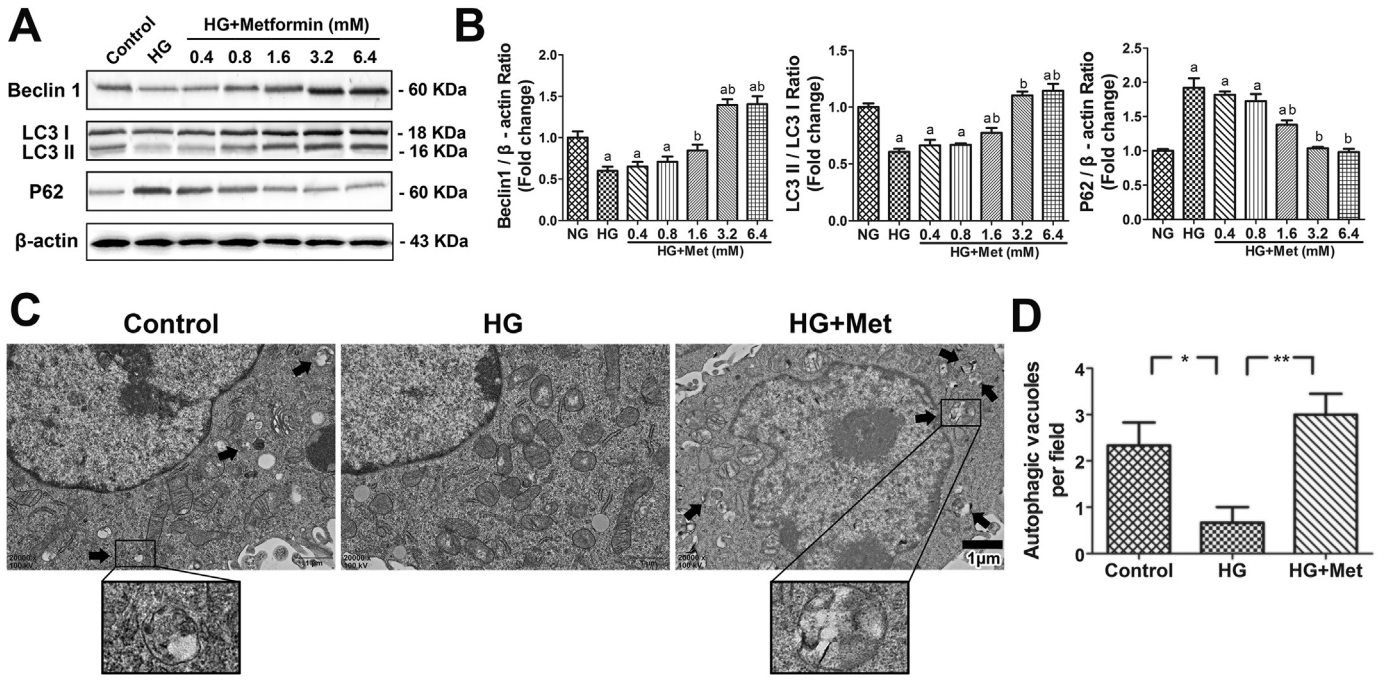


**Fig. 2.** Metformin induces autophagy in high glucose-cultured HT22 cells. **A** Western blot analyses of autophagic indicators in HT22 cells treated with different concentrations of metformin (0.4, 0.8, 1.6, 3.2 and 6.4 mM), β-actin was used as the loading control. (n = 6) **B** Quantitative analysis of Beclin1/β-actin, LC3 II/LC3 I and P62/β-actin levels. Values for the control group were arbitrarily set to a unit of 1. **a** represents $P < 0.01$ versus control group, **b** represents $P < 0.01$ versus HG group. **C** Representative transmission electron micrographs from different groups of HT22 cells. Metformin concentration is 3.2 mM. The arrowhead indicates an autophagic vacuole and the box below shows an amplified figure. Magnification ×20,000. Scale bars represent 1 μm. **D** Quantification of autophagic vacuole counted from six randomly selected fields, $^{**}P < 0.01$, $^{*}P < 0.05$. (n = 6).

experimental period. There was no significant difference between the blood glucose level of the db/db group and the Met group at week 12, week 16. However, a significant difference of blood glucose levels between the db/db group (30.48 ± 0.51 mmol/L) and the Met group (27.15 ± 1.13 mmol/L) was observed at week 20 ($P < 0.05$). No significant difference was observed regarding blood glucose level among the db/db, Met + CQ and CQ groups.

### 3.2. Metformin ameliorates spatial cognitive impairment and hippocampal structure abnormalities in db/db mice

We tested the effect of metformin on DM-induced cognitive deficit using the MWM. The spatial learning abilities of mice were evaluated with 5 days of hidden platform tasks. As shown in Fig. 1A, compared with the control group, the db/db group showed increased escape latencies during the hidden platform acquisition from 2nd to 5th days ($P < 0.05$), while the mice treated with metformin exhibited better performances than db/db mice from 3rd to 5th days ($P < 0.05$). Of note, from the 4th day, the protective effect of metformin was significantly reversed by co-administration of CQ in the Met + CQ group ($P < 0.05$), and single injection of CQ did not show any cognition ameliorating effect.

Twenty-four hours after the last training test, probe tests were conducted without the hidden platform, which evaluated the memory of the trained mice. As shown in Fig. 1C and D, the db/db mice showed less platform crossing time (0.7 ± 0.26 versus 2.3 ± 0.52, $P < 0.05$) and spent less time in the target quadrant (17.43 ± 1.42 s versus 32.10 ± 3.01 s, $P < 0.05$) than the control group. However, mice treated with metformin showed more platform crossing time (1.6 ± 0.31 versus 0.7 ± 0.26, $P < 0.05$) and stayed longer time in the target quadrant (30.42 ± 3.18 s versus 17.43 ± 1.42 s, $P < 0.05$) than db/db mice. Of note, these protective effects were significantly reversed by co-administration of CQ as compared to the Met group (0.6 ± 0.16 versus 1.6 ± 0.31, 20.55 ± 2.98 s versus 30.42 ± 3.18 s, respectively, $P < 0.05$). Single administration of CQ did not show any cognition ameliorating effect on diabetic mice. These results indicated that metformin treatment could attenuate cognitive impairment in db/db mice, and these neuroprotective effects of metformin could be reversed by CQ. In the visual trial, there were no significant differences in latency to the visible platform among the five groups (Fig. 1B), excluding the possibility that these results were affected by the animal's visual ability.

We assessed hippocampal structure changes in all groups of mice with histological examination. As shown in Fig. 1E, HE staining revealed a significant reduction of hippocampal volume in db/db mice compared with that in the control group. Metformin treatment attenuated the reduction of hippocampal volume in db/db mice. Met + CQ treatment did not obviously reverse the protective effect of metformin on hippocampal volume compared with the Met group. Single administration of CQ had no effect on the structure of hippocampus.

### 3.3. Metformin promotes autophagy in the hippocampi of db/db mice and in high glucose-cultured HT22 cells

To investigate whether metformin triggered the autophagic machinery and induced autophagosome formation in db/db mice and in high glucose-cultured HT22 cells, we detected the formation of autophagosomes and measured the protein expression of Beclin 1, LC3B II/I and P62. As demonstrated in Fig. 4A and B, western blot analysis revealed a significant reduction of LC3B II/I ratio and Beclin 1 expression, and a significant increase of P62 levels in db/db group compared with the control group, indicating the impaired formation of autophagosomes in the hippocampi of db/db mice. Besides, compared with the db/db group, treatment with metformin increased the protein expression of Beclin 1 and LC3B II/I ratio, and decreased the protein level of P62.

In HT22 cells, compared with the control group, high glucose incubation significantly decreased LC3B II/I ratio and Beclin 1

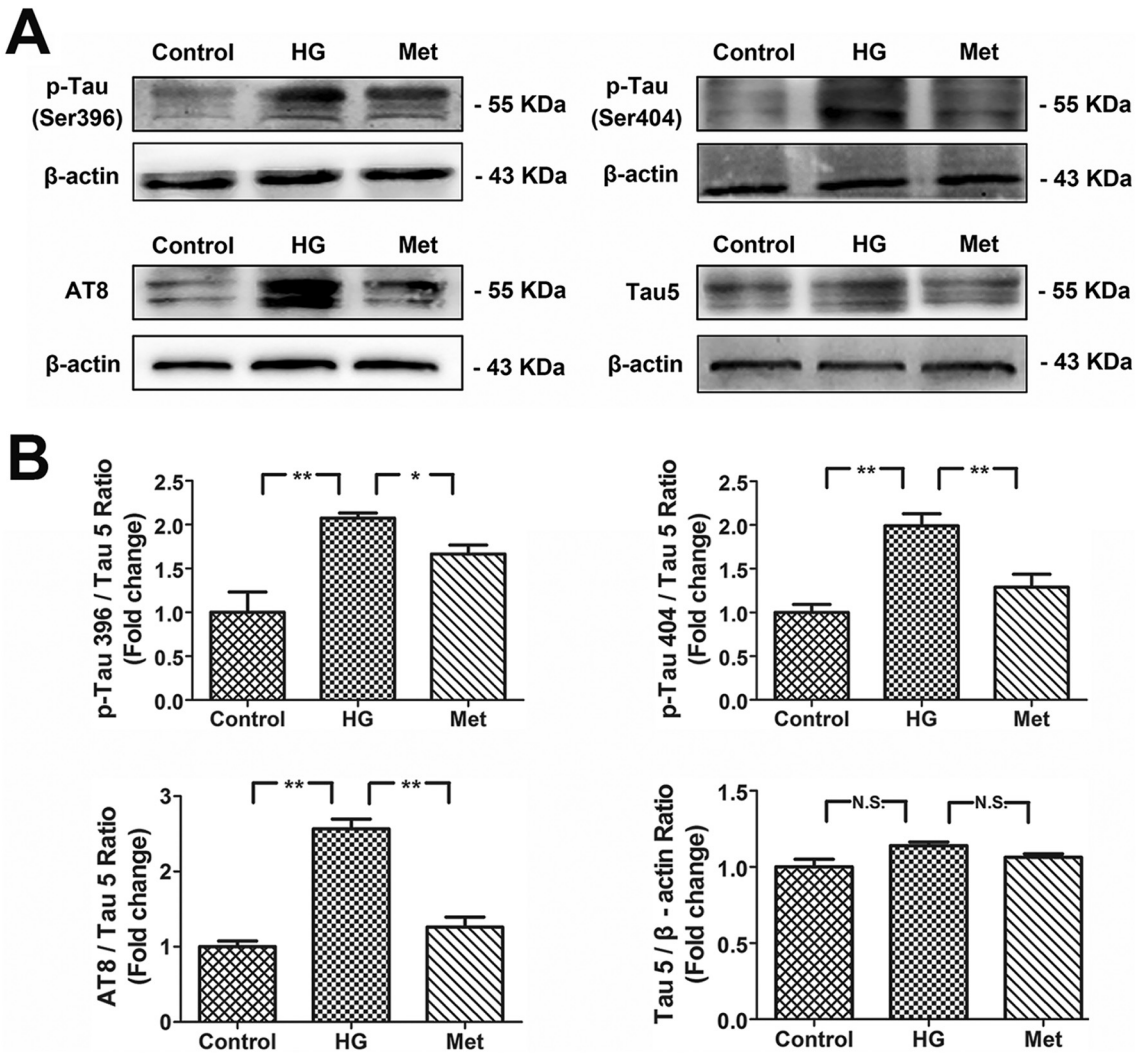


**Fig. 3.** Effect of metformin on phosphorylated tau proteins expression in high glucose-cultured HT22 cells. **A** p-Tau (Ser396), p-Tau (Ser404), AT8 (Ser202/Thr205), and Tau5 were measured via western blotting in HT22 cells. β-actin was used as the loading control. (n = 6) **B** Quantification of different Tau protein levels in control, HG and metformin groups of HT22 cells. Values for the control group were arbitrarily set to a unit of 1. $^{**}P < 0.01$, $^{*}P < 0.05$, N.S., not significant.

expression, increased P62 level. While incubation with metformin at the concentrations of 1.6, 3.2 and 6.4 mM for 24 h increased the protein levels of Beclin 1 and LC3B II/I, and decreased the protein levels of P62 in a dose-dependent manner (Fig. 2A and B), indicating that metformin activated autophagy in HT22 cells. The autophagosome and autophagolysosome, collectively referred to as autophagic vacuoles (AVs), are considered as the characteristic components of autophagy. Furthermore, we observed the structures of AVs in different groups of HT22 cells using a TEM. As presented in Fig. 2C and D, high glucose incubation significantly reduced AVs in HT22 cells, while treatment with metformin at 3.2 mM significantly increased the structure of AVs in HT22 cells. These data indicated that metformin treatment induces the formation of autophagy both in vivo and in vitro.

### *3.4. Metformin reduces hyperphosphorylated tau in the hippocampi of db/db mice and in high glucose-cultured HT22 cells*

As autophagy plays an important role in degradation of aggregated/misfolded proteins, we further test whether metformin could enhance the degradation of DE associated hyperphosphorylated tau proteins in the hippocampi of db/db mice and in high glucose-cultured HT22 cells. As shown in Fig. 4A and B, increased tau proteins phosphorylated at Ser396, Ser404 and Ser202/Thr205 (AT8) in the hippocampi of db/db mice were detected compared with that in the control group ($P < 0.01$) via western blot analysis: the ratios of p-Tau (Ser396)/total tau, p-Tau (Ser404)/total tau and AT8 (Ser202/Thr205)/total tau in db/db mice were increased by 2.45-, 4.54- and 4.76-fold, respectively. Metformin treatment significantly decreased the expression of p-Tau (Ser396) ($P < 0.01$), p-Tau (Ser404) ($P < 0.01$) and AT8 (Ser202/Thr205) ($P < 0.01$). Total tau protein levels showed no significant differences among the groups.

Similar results were obtained in the in vitro study. HT22 cells were incubated with either NG or HG medium for 24 h, and then the control group and the HG group were treated with vehicle and the Met group was treated with metformin for another 24 h. As shown in Fig. 3A and B, western blot analysis revealed that high glucose incubation significantly increased the expression of tau proteins phosphorylated at Ser396, Ser404 and Ser202/Thr205 (AT8) by 2.07-, 1.99- and 2.57-fold, respectively, in the HT22 cells compared with that in the control group ($P < 0.01$). Metformin treatment significantly decreased the expression of p-Tau (Ser396) ($P < 0.05$), p-Tau (Ser404) ($P < 0.01$) and AT8 (Ser202/Thr205) ($P < 0.01$). Total tau protein levels showed no significant differences among the three groups.

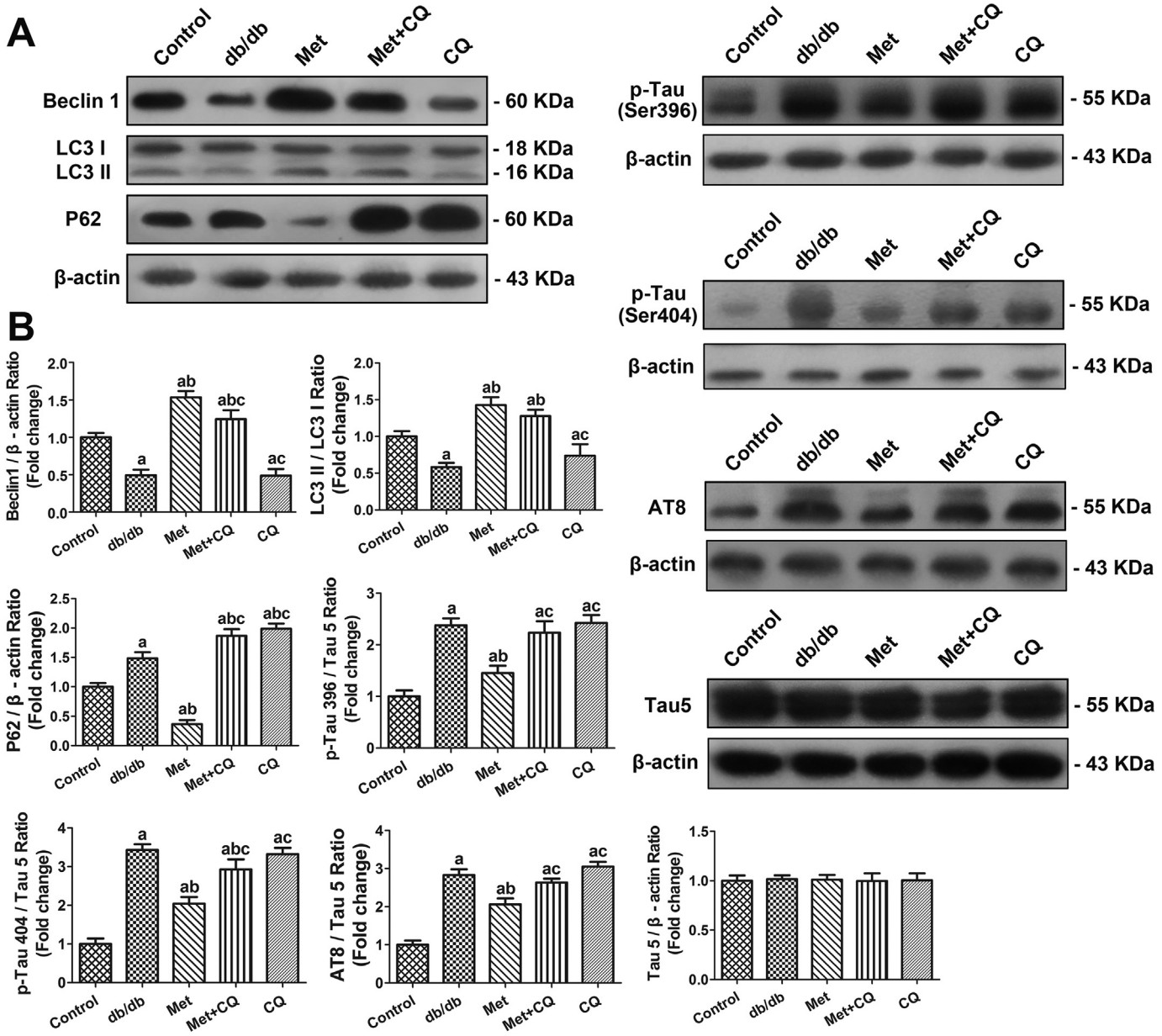


**Fig. 4.** Autophagic markers and phosphorylated tau protein expression in the hippocampi of mice in different groups. **A** Beclin1, LC3I, LC3II, P62, p-Tau (Ser396), p-Tau (Ser404), AT8 (Ser202/Thr205) and Tau5 were measured via western blotting. β-actin was used as the loading control. **B** Quantification of different protein levels in mouse hippocampi. Values for the control group were arbitrarily set to a unit of 1. **a** represents $P < 0.01$ versus control group, **b** represents $P < 0.01$ versus db/db group, **c** represents $P < 0.01$ versus Met group. n = 6 animals/group.

### *3.5. Metformin-induced reduction of hyperphosphorylated tau in vivo and in vitro mediated through autophagy induction*

To further determine whether the neuroprotective effects of metformin on tau clearance in hippocampi in db/db mice were mediated through the activation of autophagy, mice were injected with metformin and/or CQ. As shown in Fig. 4A and B, co-treatment with metformin and CQ significantly increased the protein levels of Beclin 1 and LC3B II/I ratio compared with db/db group. However, P62 expression was significantly increased in Met + CQ group compared with db/db or Met group, indicating the blockade of the autophagy flux. Meanwhile, there was no significant difference in the protein levels of Beclin 1 and LC3B II/I ratio between CQ and db/db group, indicating that CQ had no effects on induction of autophagy. However, P62 levels were significantly increased in CQ group, suggesting that the autophagy flux was blocked at the late stage by CQ. We have found that metformin treatment significantly reduced the hyperphosphorylated tau protein at Ser396, Ser404 and Ser202/Thr205 (AT8) in the hippocampi of db/db mice. However, co-treatment with CQ reversed these effects. Besides, phosphorylated tau protein levels in CQ group had no obvious change compared with db/db group, indicating that metformin enhances tau protein clearance mainly through autophagic activation. We further confirmed these findings via immunohistochemistry staining for hyperphosphorylated tau protein at Ser404 and Ser202/Thr205 (AT8), as demonstrated in Fig. 5. Consistent with the western blotting, these results were also confirmed by immunohistochemical analyses both in hippocampus and in cortex.

Consistent results were observed in high glucose-incubated HT22 mouse hippocampal neuron cells. HT22 cells were pretreated with vehicle or 3-MA (10 mM) for 3 h followed by exposure to high glucose DMEM in the absence or presence of metformin (3.2 mM) for another 24 h. 3-MA was widely used as autophagy inhibitor (Seglen and

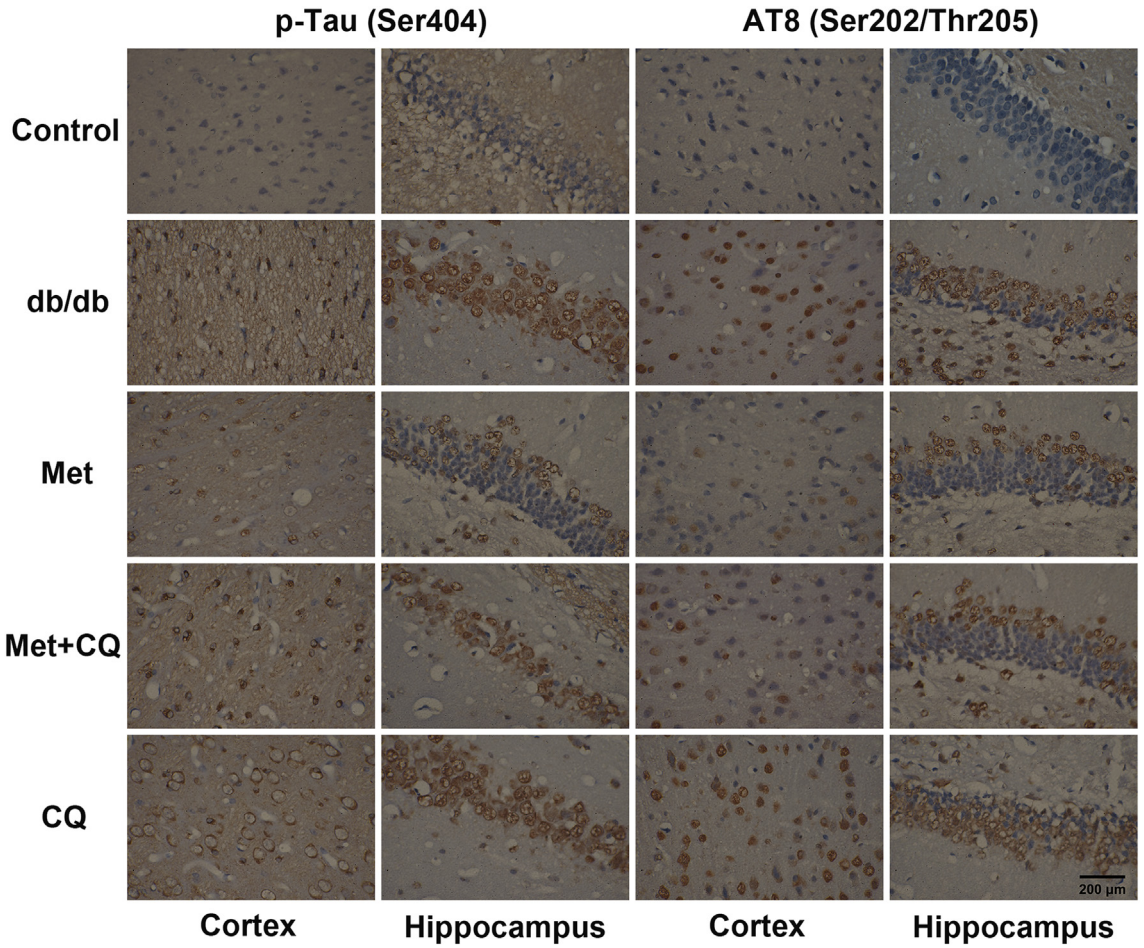


**Fig. 5.** The IHC staining of p-Tau (Ser404) and AT8 in the brain cortex and hippocampus of db/db mice among different groups. Magnification ×400. Scale bars represent 200 μm. n = 3 animals/group.

Gordon, 1982). As shown in Fig. 6A and B, western blot analysis showed that metformin-induced increase of LC3B II/I ratio, Beclin 1 protein levels were significantly reduced when HT22 cells were pretreated with 3-MA. Meanwhile, the effects of metformin attenuated high glucose-induced tau hyperphosphorylation at Ser396, Ser404 and Ser202/Thr205 (AT8) were reversed by pretreatment with 3-MA, indicating that autophagy induction is required for metformin in neuron tau clearance. Total tau levels did not show any difference among the groups.

Beclin 1 is an activator of autophagy necessary for vesicle nucleation. To further consolidate our findings that autophagy induction was involved in the metformin-induced reduction of hyperphosphorylated tau in neurons, we suppressed the expression of autophagy-related gene Beclin 1 in HT22 cells by Beclin 1 siRNA transfection. As shown in Fig. 7A and B, compared with the control siRNA group, Beclin 1 siRNA group significantly suppressed the Beclin 1 expression, and the role of metformin in autophagy induction was blocked accordingly. Moreover, we found that the role of metformin in diminishing high glucose-induced increase of hyperphosphorylated tau was also blocked when Beclin 1 gene expression was knocked down accompanied by the inhibition of autophagy.

### *3.6. Metformin promotes autophagy in an AMPK-dependent manner in HT22 cells*

Previous studies indicate that metformin exerts its therapeutic effects by activating AMPK (Jiang et al., 2014a; Duan et al., 2017; Xie et al., 2011). To determine whether metformin induces autophagy via AMPK activation in tau clearance in neurons, both metformin and Compound C (CC) were utilized in HT22 cells. As shown in Fig. 8A and B, western blot analysis revealed that metformin treatment significantly increased the phosphorylation levels of AMPK at Thr172 and acetyl-CoA carboxylase (ACC) at Ser79, because ACC is a substrate for AMPK, the determination of ACC phosphorylation also served as an indicator of AMPK activity, indicating that metformin treatment increased the AMPK activity. Meanwhile, metformin also significantly increase the protein levels of Beclin 1 and LC3B II/I ratio, decreased the P62 levels. CC is a widely used AMPK inhibitor, our results revealed that CC markedly prevented metformin-induced activation of p-AMPK and p-ACC, accompanied by the decrease of Beclin 1 expression and LC3B II/I ratio, indicating that inhibition of AMPK caused autophagy inhibition accordingly. Additionally, as presented in Fig. 8C and D, TEM showed a significant reduction of the structure of AVs in HT22 cells by CC treatment compared with HG + Met group. These results suggested that metformin promoted autophagy in HT22 cells mainly through AMPK activation.

## 4. Discussion

Metformin is the most widely prescribed drug for patients with T2D and other metabolic disorders, while the effects of metformin on DE remains unclear. Recently, the accumulation and aggregation of hyperphosphorylated tau protein in DE has aroused considerable attention. Impaired of tau degradation and clearance has been considered to be involved in the pathogenesis of DE. Here, our study shows that metformin treatment significantly enhances autophagic clearance of

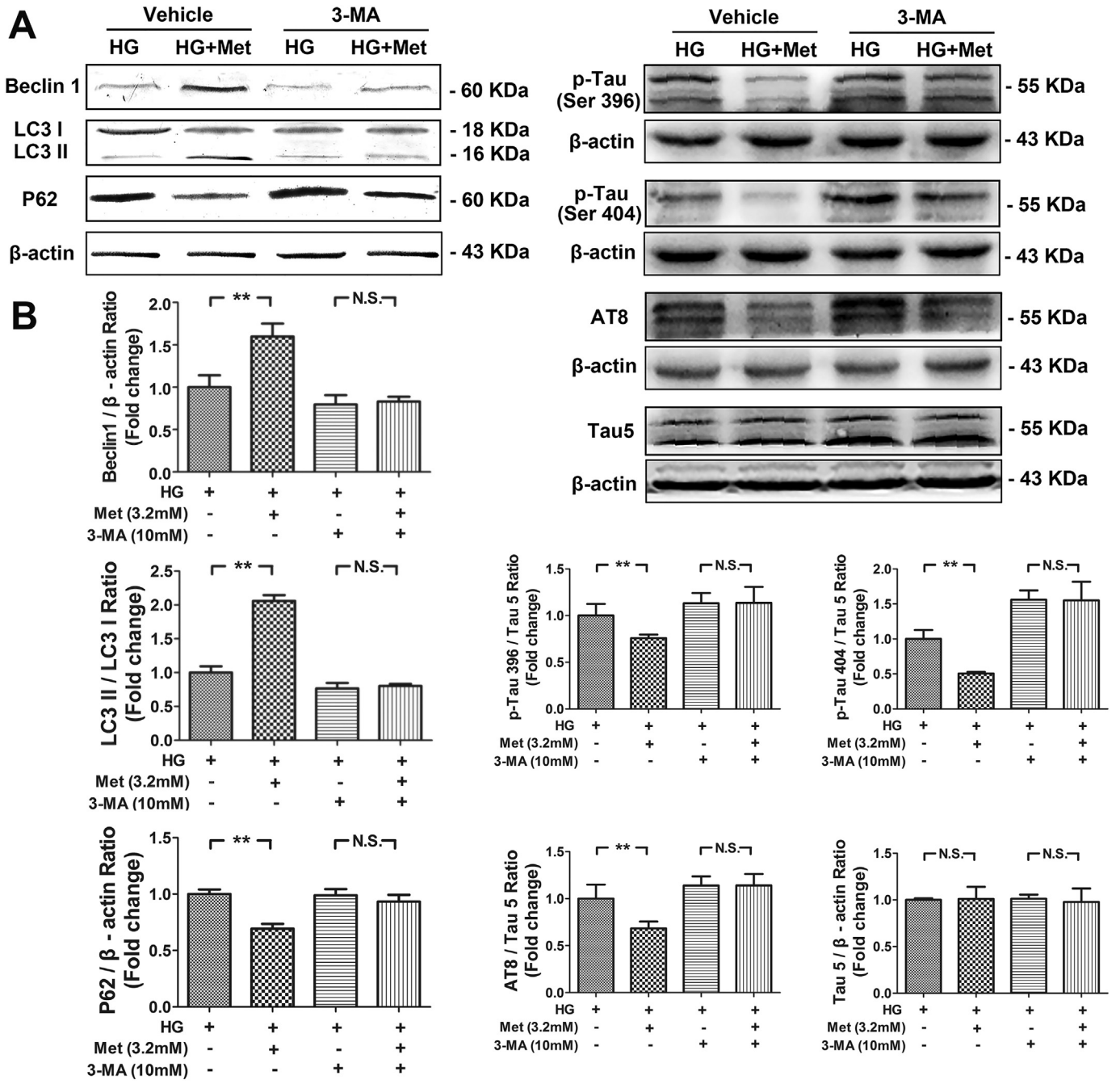


**Fig. 6.** Effect of metformin treatment on phosphorylated tau protein expression in HT22 cells after inhibition of autophagic flux by co-treatment with 3-MA. **A** Beclin1, LC3I, LC3II, P62, p-Tau (Ser396), p-Tau (Ser404), AT8 (Ser202/Thr205) and Tau5 were measured via western blotting. β-actin was used as the loading control. (n = 6) **B** Quantification of different protein levels. Values for the control group were arbitrarily set to a unit of 1. $^{**}P < 0.01$, N.S., not significant.

hyperphosphorylated tau proteins in both the hippocampi of db/db mice and high glucose-induced HT22 cells, thus attenuates cognitive impairments in db/db mice. Furthermore, we find that metformin prevents the development of DE by restoring the autophagy in the hippocampi and HT22 cells through the modulation of AMPK activity.

Since an increasing proportion of AD individuals are also diabetic, diabetes has been regarded as an independent risk factor for AD. As the safest first-line therapy for T2D, the neuroprotective effect of metformin has been widely discussed in patients with metabolic disorder. Previous studies have shown that metformin reduces the progression of cognitive impairment in different animal models (Pintana et al., 2012; Oliveira et al., 2016; Wang et al., 2012; Chen et al., 2016; DiTacchio et al., 2015). Consistent with our present result, Chen et al. (2016) reported that six weeks treatment of metformin improved learning and memory in db/db mice reflected by improved performance on MWM and Y maze tasks. In addition, it revealed that metformin treatment attenuated the cognitive impairment in db/db mice by restoring the RAGE-mediated influx of Aβ across the blood brain barrier (BBB). Li J et al. (Li et al., 2012) demonstrated that a long-time administration of metformin, apart from its hypoglycemic effect, attenuated the increased hyperphosphorylated tau protein and reduction of synaptophysin in the hippocampi of db/db mice. Furthermore, the authors explored the main tau protein kinases, such as GSK-3β, cyclin-dependent kinase-5 (CDK-5), c-jun N-terminal kinase (JNK), and the catalytic subunit of PP2A. The results showed that the expression of GSK-3β and CDK5 were not different between the db/db and db/+ mice, but db/db mice had an

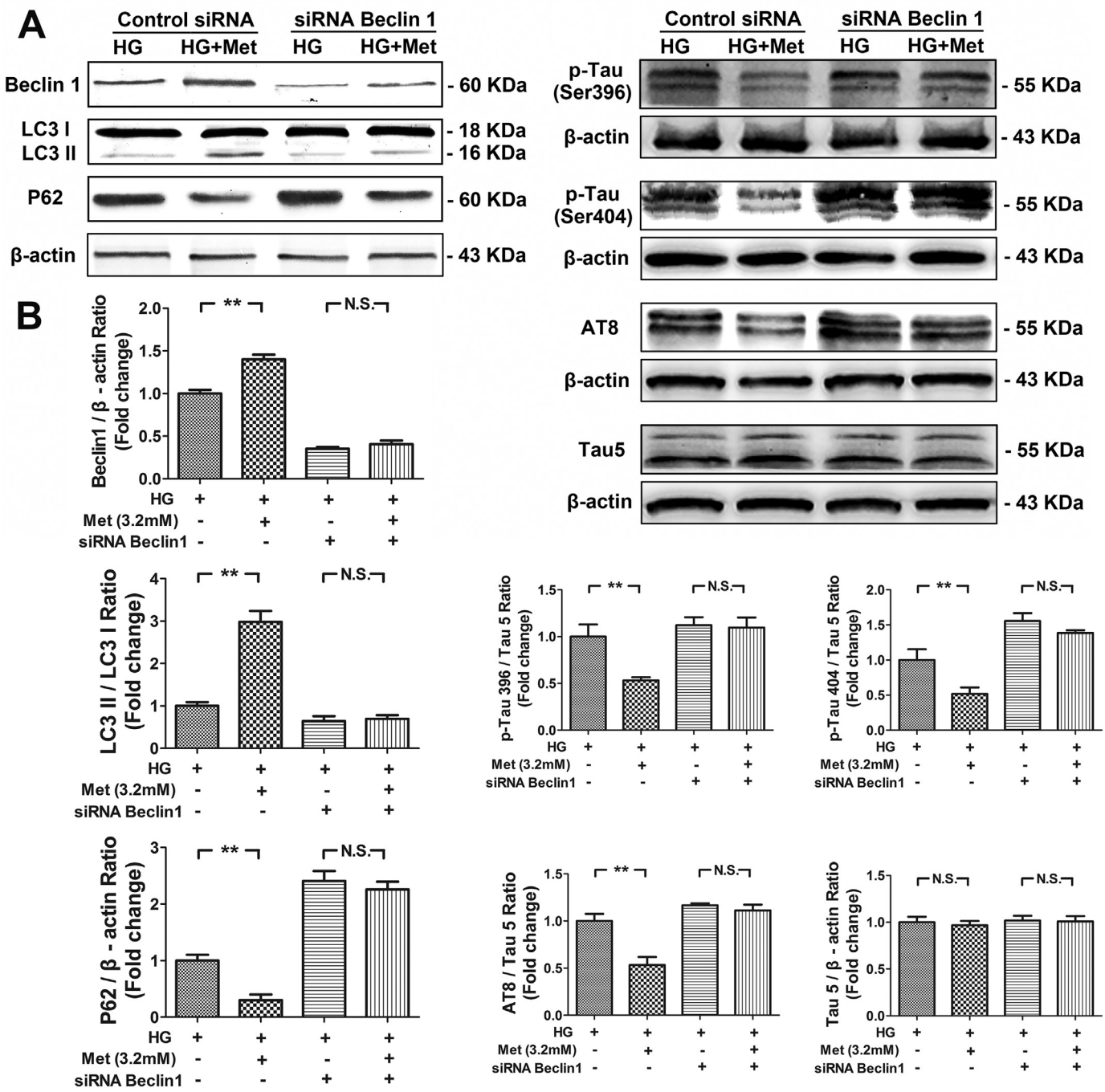


**Fig. 7.** Effect of metformin treatment on phosphorylated tau protein expression in HT22 cells after inhibition of autophagic flux by transfection with Beclin 1 siRNA. **A** Beclin1, LC3I, LC3II, P62, p-Tau (Ser396), p-Tau (Ser404), AT8 (Ser202/Thr205) and Tau5 were measured via western blotting. β-actin was used as the loading control. (n = 6) **B** Quantification of different protein levels. Values for the control group were arbitrarily set to a unit of 1. $^{**}P < 0.01$, N.S., not significant.

increase in JNK activity and an decrease in PP2A activity in the hippocampi, while treatment with metformin only decreased the activity of JNK and did not attenuate the decrease of PP2A expression in the db/db mice. Then the authors speculated that the JNK is involved in tau phosphorylation in hippocampus and that attenuation of JNK activation may be a mechanism for metformin to decrease tau phosphorylation in db/db mice, but they did not go further to verify it. In the current study, we demonstrated that chronic treatment with metformin significantly decreases the expression of pTau protein phosphorylated at Ser396, Ser404 and Ser202/Thr205 in neurons both in vivo and in vitro. More importantly, it improved the learning and memory in db/db mice by improving performance in MWM. We further discussed the possible mechanisms on neuroprotective effects of metformin through modulating the activity of autophagy.

Autophagy maintains a balance between the synthesis, degradation, and subsequent recycling of cellular components. It plays an important role on maintaining normal cellular and organismal physiology, both over increased and decreased autophagy have been associated with disease (Thorburn, 2017). Interestingly, recent studies reported that metformin treatment exerted beneficial effects via induction of autophagy in animal models of diabetic cardiomyopathy (Xie et al., 2011), hepatic steatosis (Song et al., 2015), spinal cord injury (Zhang et al., 2017), lymphoma (Shi et al., 2012) and other carcinomas (Tomic et al., 2011; Feng et al., 2014; Tsai et al., 2017). All of these studies

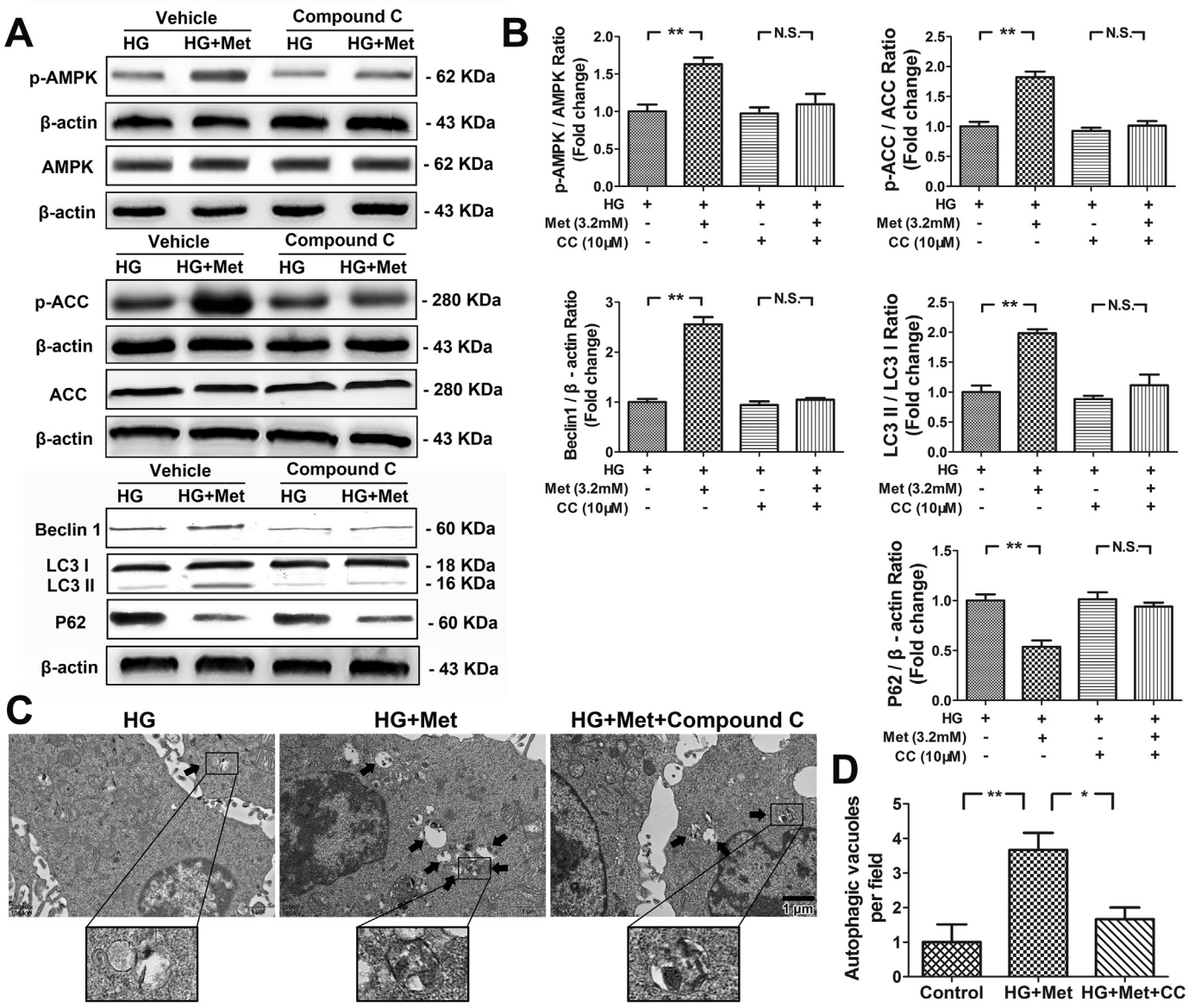


**Fig. 8.** Effect of metformin treatment on autophagy activity in high glucose-induced HT22 cells after inhibition of AMPK activation. **A** p-AMPK,AMPK,p-ACC,ACC,Beclin1,LC3I,LC3II and P62 were measured via western blotting. β-actin was used as the loading control. (n = 6) **B** Quantification of different protein levels. Values for the control group were arbitrarily set to a unit of 1. **C** Representative transmission electron micrographs from different groups of HT22 cells. Metformin concentration is 3.2 mM, Compound C concentration is 10 μM. The arrowhead indicates an autophagic vacuole and the box below shows an amplified figure. Magnification ×20,000. Scale bars represent 1 μm. **D** Quantification of autophagic vacuole counted from six randomly selected fields. $^{**}P < 0.01$, $^{*}P < 0.05$, N.S., not significant. (n = 6).

demonstrated that activation of autophagy seems to be one of the important mechanisms for metformin in the treatment of diseases. In the last decade, there has been a growing body of evidence indicating that autophagy plays an important role in neuronal aging and health. Autophagy is involved in the degradation and removal of aggregated proteins which directly cause the neurodegenerative diseases. Thus, a strong link between autophagy and neurodegenerative diseases has been established. Today, our knowledge is increasing regarding the role of autophagy and its mechanisms in the pathogenesis of various neurodegenerative diseases such as AD (Li et al., 2017), Parkinson's disease (Sanchez-Perez et al., 2012), Huntington's disease (Ravikumar et al., 2004; Walter et al., 2016) and Amyotrophic lateral sclerosis (Chen et al., 2012). It should be noted that metformin also exerts a neuroprotective effect via modulating autophagy activity. Jiang T and his group found that acute metformin preconditioning confers neuroprotection against subsequent cerebral ischaemia via inducing autophagy (Jiang et al., 2014a). Sarkaki et al. (2015) reported that metformin improves anxiety-like behaviors through AMPK-dependent regulation of autophagy following transient forebrain ischemia. On consideration of the above evidence, in the current study, we first discuss the possible protective effect of metformin in DE through modulating of autophagy. In line with these studies, we found that treatment with metformin restored the impaired neuron autophagy both in vivo and in vitro. Besides, inhibiting autophagy with CQ in db/db mice, abolished the protective effect of metformin on hippocampi injury, and increased both the protein levels of LC3B-II/I and hyperphosphorylated tau. In HT22 cells, co-treatment with 3-MA or Beclin 1 siRNA decreased the protein expression of LC3B-II/I and the number of autophagic vacuoles, but significantly increased the expression of phosphorylated tau proteins. These data provide some evidences that diabetes-inhibited neuronal autophagy may play an important role in the pathogenesis of DE, while metformin may reduce hyperphosphorylated tau proteins against

DE by enhancing autophagy clearance. Furthermore, we discussed the possible mechanisms through which signaling metformin modulates autophagy in neurons. AMPK is a critical energy sensor in all types of cells and tissues, including neurons (Liu and Chern, 2015), which can be activated in response to an increase in the intracellular AMP/ATP ratio. Emerging evidences have demonstrated that AMPK is one of the key modulators in autophagy activity (Zhang et al., 2016; Mihaylova and Shaw, 2011). As a well-known AMPK activator, metformin has been revealed to induce autophagy through an AMPK-dependent manner in high glucose-induced HT22 cells. In HT22 cells, inactivation of AMPK signaling by CC abolished the autophagy induction effects of metformin. Thus, we speculate that metformin modulates neuronal autophagy activity through an AMPK dependent manner.

However, the effects of metformin on the prevention of dementia in patients with T2D still remain controversial. Studies by Moore et al. (2013) conducted in Australia and by Kuan et al. (2017) conducted in Taiwan demonstrated that chronic use of metformin is associated with an increased risk of cognitive impairment in patients with T2D. While most human studies showed that metformin use can reduce the risk of dementia in patients with T2D (Ng et al., 2014; Hsu et al., 2011; Cheng et al., 2014; Orkaby et al., 2017). Recently, a meta-analysis conducted by Areosa Sastre A et al. revealed that no specific treatment or treatment strategy for T2D can prevent or delay cognitive impairment (Areosa Sastre et al., 2017). All these studies have its own limitations. Different study designs, dosage of metformin, follow-up time, race, etc., which may bias the interpretation of results. Thus, large-scale randomized controlled trials are warranted.

In conclusion, the current study provides the evidence that metformin treatment reduces hyperphosphorylated tau proteins through enhancing autophagic clearance both in high glucose-induced HT22 cells and in brain of db/db mice, rescues spatial learning and memory impairment in db/db mice, which is a valuable, standard and widely used murine spontaneous T2D model. Furthermore, metformin modulates autophagic activity through an AMPK dependent signaling pathway. Our results suggest that metformin may provide potential therapeutic strategy for preventing cognitive impairment in T2D.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Acknowledgements

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