Melatonin prevents diabetes-associated cognitive dysfunction from microglia-mediated neuroinflammation by activating autophagy via TLR4/Akt/mTOR pathway

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Abstract
Cognitive dysfunction often occurs in diabetes mellitus patients. This study aimed to investigate the efficacy of melatonin (MLT) in improving diabetes-associated cognitive decline and the underlying mechanism involved. Type 2 diabetic mice and palmitic acid (PA)-stimulated BV-2 cells were treated by MLT, and the potential mechanisms among MLT, cognition, and autophagy were explored. The results showed that type 2 diabetic mice showed obvious learning and memory impairments in the Morris water maze test compared with normal controls, which could be ameliorated by MLT treatment. Meanwhile, MLT administration significantly improved neuroinflammation and regulated microglial apoptosis. Furthermore, autophagy inhibitor 3-methyladenine (3-MA) increased the microglial inflammation and apoptosis, indicating that the treatment effect of MLT was mediated by autophagy. Lastly, MLT treatment significantly decreased the levels of toll-like receptors 4 (TLR4), phosphorylated-protein kinase B (Akt), and phosphorylated-mechanistic target of rapamycin (mTOR), indicating that blocking TLR4/Akt/mTOR pathway might be an underlying basis for the anti-inflammatory and anti-apoptosis effects of MLT. Collectively, our study suggested that MLT could improve learning and memory...
1 | INTRODUCTION

Diabetes is a serious global public health problem, and its worldwide prevalence is increasing yearly. It is one of the most common metabolic diseases; it can extensively damage a variety of organs and tissues, including the heart, kidney, retina, and central nervous system (CNS), and cause serious complications. Cognitive impairment is a common but severely underestimated complication of diabetes. Impaired cognitive function in diabetes is manifested by many aspects, including learning and memory, executive ability, attention, and emotion. Compared with nondiabetics, patients with diabetes experience faster cognitive decline. Given the prevalence of diabetes, the cognitive dysfunction related to diabetes is expected to exacerbate substantially and poses challenges to public health. However, the current understanding of diabetes-related cognitive dysfunction is insufficient, and the clinical treatment effect of this complication is poor. Therefore, deciphering the mechanism and finding new strategies to delay or reduce the occurrence of cognitive dysfunction are crucial.

Neuroinflammation is closely related to cognitive dysfunction. Microglia are immune cells in the brain that act as important regulators of neuroinflammation. Activated microglia can secrete multiple proinflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and reactive oxygen species (ROS), which have toxic effects on neurons and further activate microglia, thereby aggravating neuroinflammation. Neuroinflammation caused by microglial overactivation is the main feature of neuropathology. Thus, microglial activation must be properly and strictly regulated to maintain normal physiological homeostasis.

A growing body of evidence has demonstrated that autophagy can regulate microglial activation. Autophagy is a lysosomal-dependent cellular catabolism mechanism that can clear protein aggregates and damaged organelles to maintain cell homeostasis. In cellular and animal models, autophagy has been shown to contribute to different aspects of neuronal and microglial physiology, including axonal homeostasis, synaptic pruning, and neurogenesis. Inhibiting inflammation and preventing apoptosis are the two major functions of autophagy. Microglial autophagy is related to microglial activation and contributes to the development of neurodegenerative diseases. Han et al examined the role of autophagy in activated BV-2 cells and found that the activation of autophagy inhibits the expression of IL-6 and the death of lipopolysaccharide (LPS)-stimulated microglial cells. Therefore, autophagy may regulate inflammation and apoptosis by regulating the activation of microglia.

Melatonin (MLT) is the main secretory product of the pineal gland. It acts as a regulator of the circadian rhythm. It also acts on energy expenditure, glucose metabolism, inflammatory factor secretion regulation, and anti-apoptosis. The peak concentration of MLT is around 10 pg/mL (43 pmol/L) in the blood and 3 pg/mL in the saliva; however, patients with type 2 diabetes have decreased serum MLT level and disordered circadian rhythm. Previous studies indicated that the doses and concentrations of MLT required for a beneficial effect are much higher than the physiological dose and concentration. Lo et al indicated that MLT at the dose of 50 mg/kg could decrease the level of blood glucose, whereas MLT at the dose of 10 mg/kg could not decrease the level of blood glucose obviously. Some studies showed that MLT at the dose of 10 mg/kg could improve cognition significantly. In order to avoid the influence of blood glucose on cognitive function, MLT at the dose of 10 mg/kg was chosen in our experiments. Exogenous MLT can penetrate the blood–brain barrier and play a role in different brain regions. Moreover, a recent study demonstrated that MLT can alleviate memory and cognitive impairment. MLT has been shown to inhibit microglial activation and reduce proinflammatory cytokine levels in many experimental models, including Alzheimer’s disease (AD). Notably, the administration of MLT significantly suppresses the expression of toll-like receptor 4 (TLR4). However, the therapeutic effects and mechanism of MLT in diabetes-associated cognitive decline remains unknown.

Therefore, in the present study, we analyzed the potential mechanism of MLT in diabetes-associated cognitive decline. For this purpose, we investigated whether MLT could improve learning and memory in type 2 diabetic mice by activating autophagy via the TLR4/Akt/mTOR pathway, thereby inhibiting neuroinflammation and microglial apoptosis.

2 | MATERIALS AND METHODS

In this study, 8-week-old male C57BL/6J mice were used. The mice were randomly divided into four groups (n = 10 per group): control, MLT (10 mg/kg/day, M813985; Merck), type 2 diabetes, and type 2 diabetes plus MLT (10 mg/kg/day). All mice were maintained on a 12-hour light: 12-hour dark cycle. The type 2 diabetic mice model was induced by injection of streptozocin (STZ) (100 mg/kg body weight, M0659; Sigma-Aldrich) to achieve a blood glucose concentration of at least 20 mmol/L 7 days post-injection. Control mice were injected with the same volume of saline. After 7 days of diabetes induction, mice were randomly divided into four groups (n = 10 per group): control, MLT (10 mg/kg/day), type 2 diabetes, and type 2 diabetes plus MLT (10 mg/kg/day). All mice were maintained on a 12-hour light: 12-hour dark cycle.
S0130; Sigma-Aldrich) dissolved in a 50-mM citric acid buffer and fed with a high-fat diet (HFD, 60% calories from fat) for 16 weeks, whereas the control mice were injected with the citric acid buffer and fed with a normal chow diet (NCD, 10% calories from fat). The type 2 diabetic mice model was considered successful if there were two consecutive fasting glucose levels over 16.7 mmol/L. MLT was injected intraperitoneally once a day for 1 month. Behavioral tests were performed 1 month later. Body weight and blood glucose were monitored weekly.

All experimental procedures performed in this study followed the ethical guidelines for animal studies and were approved by the Institutional Animal Care and Qilu Hospital of Shandong University, China.

2.1 | Intraperitoneal glucose tolerance test and intraperitoneal insulin tolerance test

For intraperitoneal glucose tolerance test (IPGTT), mice were fasted for 16 hours and given an intraperitoneal injection of glucose (2 g/kg, Sigma, G7021). For intraperitoneal insulin tolerance test (IPITT), mice were fasted for 6 hours and given an intraperitoneal injection of insulin (0.75 U/kg, Wanbang Pharmaceutical, Jiangsu, China). Blood was collected from the tail vein, and glucose concentrations were measured at 0, 15, 30, 60, 90, 120, and 180 minutes using a handheld glucose meter (Roche, Grenzach-Wyhlen, Germany).

2.2 | Morris water maze (MWM) test

Cognitive performance was evaluated by the MWM and was performed between 9:00 and 17:00. The MWM was conducted in a circular pool (120 cm diameter; 50 cm of depth) filled with water maintained at 22 ± 1°C and divided into four quadrants. The visible platform test was performed on the first day, in which the black circular platform (12 cm diameter) was 1 cm above the water surface. The hidden platform test was performed for the next 5 days, and mice were given four trials (60 seconds per trial) per day with four different positions while the platform was 1 cm below the water surface and located at a fixed quadrant center. If the mice failed to find the platform within the permitted time, the trial was terminated, and the mice were gently guided to the platform and were allowed to stay for 15 seconds. For all training trials, time and distance to reach the platform (escape latency and path length) were recorded, respectively. The probe trial was conducted on Day 7 to evaluate memory consolidation, during which the platform was removed from the maze, and the animals were allowed to swim freely for 60 seconds. The number of original platform crossings and time spent in each quadrant was recorded.

2.3 | Tissue collection

After finishing the behavioral tests, the mice were anesthetized for euthanasia. The brain of each mouse was harvested by decapitation. The left hemibrain was immediately fixed in 4% paraformaldehyde, and the hippocampus of the right hemibrain was collected for further biochemical analysis.

2.4 | Cell culture and treatments

BV-2 microglial cell line was widely used in neuroinflammation research. BV-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/high-glucose medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and penicillin (100 U/mL)-streptomycin (100 μg/mL) at 37°C with 5% CO₂. BV-2 cells were incubated with 0.4 mM of palmitic acid (PA, Sigma-Aldrich, USA) for 24 hours to induce metabolic stress and were cotreated with MLT (100 nM). To further test the effects of MLT on PA-induced changes, cells were pretreated with 3-methyladenine (3-MA, 5 mM, Sigma-Aldrich, USA), TLR4 inhibitor TAK-242 (10 μM, Selleck, USA), Akt inhibitor MK-2206 (10 μM, Selleck, USA), and mTOR inhibitor Rapamycin (100 nM, Selleck, USA) for 1 hour.

2.5 | Cell viability

BV-2 cells incubated in 96-well plates were treated as indicated and cell viability was assessed by Cell Counting Kit-8 (CCK-8, DoJinDo, Japan) at 24 and 48 hours according to the manufacturer's instructions. The absorbance was detected by a microplate reader at a test wavelength of 450 nm.

2.6 | Western blot

BV-2 cells or hippocampi were lysed in RIPA buffer (Beyotime, China). After running on 10% or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (EpiZyme, China), proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA), which were blocked with 5% milk at room temperature for 1 hour. Transferred membranes were incubated with antibodies against TLR4 (sc-293072; Santa, USA), MAP1LC3B (L8918; Sigma, USA), GFAP (16825-1-AP; Proteintech, China), NF-κB p65 (3034; CST, USA), p-NF-κB p65 (Ser536)(3033; CST, USA), IκBα (4814; CST, USA), p-IκBα (Ser32)(2859; CST, USA), TNF-α (BIOSS: bs-2081R), IL-1β (bs-0812R; BIOSS, China), IL-6 (66146-1-Ig; Proteintech, China), Cleaved-caspase3
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(9661; CST, USA), Caspase3 (9662; CST, USA), Bax (2772S; CST, USA), Atg7 (133528; Abcam, USA), Atg5 (108327; Abcam, USA), p62 (91526; Abcam, USA), p-Akt (Ser473)(4060S; CST, USA), Akt (60203-2-Ig; proteintech, China), p-mTOR (Ser2448)(2971S; CST, USA), and mTOR (2983S; CST, USA). After incubation with horseradish-peroxidase-labeled secondary antibodies, protein bands were visualized by Image Lab software (BioRad, USA). Protein-band intensities were measured via ImageJ and were normalized to β-actin.

2.7 | RNA extraction and qRT-PCR

RNA extraction, cDNA synthesis, and qRT-PCR were performed as previously described.20 β-actin was used as a reference gene. Primer sequences are as follows: Mouse β-actin (F: 5’-AGCCATGTACGTAGCCATCCA-3’; R: 5’-TCTCCC GGAGTCCCATCAAATG-3’); Mouse TNF-α (F: 5’-ATCTTCTCAAAAAATCTCAGTGAAC-3’; R: 5’-GAGGGAGT AGACAAGGTCACAACC-3’); Mouse IL-1β (F: 5’-AGG CACAGGTTATTTGTC-3’; R: 5’-GCCCCATCTCTGTGTA CTC-3’); Mouse IL-6 (F:5’-GCTACCAAACTGGATATAACTGGA-3’; R:5’-CCAGGTAGCTATGGTACTCCAGA A-3’).

2.8 | Immunofluorescence

Hippocampus sections were used for immunofluorescent staining for GFAP (16825-1-AP; Proteintech, China), Iba1(17198; CST, USA), TLR4(sc-293072; Santa, USA), and MAP1LC3B (L8918; Sigma, USA). Sections were incubated with 5% bovine serum albumin (BSA) for 1 hour at room temperature and then incubated overnight with the anti-GFAP, anti-Iba1, anti-TLR4, and anti-MAP1LC3B antibodies. On the following day, the sections were washed and subsequently incubated with secondary antibodies for 1 hour in the dark. The nucleus was stained with 4, 6 diamidino-2-phenylindole (DAPI) for 5 minutes.

BV-2 cells were cultured on 24-well plates and treated according to their respective experiments. Cells were washed with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 20 minutes, and then washed with PBS again for three times. Cells were blocked in 5% BSA with 0.1% Triton X-100 for 1 hour and then incubated with Iba1 (17198; CST, USA), TLR4 (sc-293072; Santa, USA), and MAP1LC3B (L8918; Sigma, USA) at 4°C overnight. The next day, cells were stained with the secondary antibody for 1 hour at room temperature in the dark. The nucleus was stained with DAPI at room temperature for 5 minutes. The tissue sections and cells were imaged under a fluorescence microscope (BX61, Olympus, Japan), and images intensity was measured in Image pro plus.

2.9 | Hematoxylin and eosin (HE) staining

Four percent paraformaldehyde-fixed brain tissues from each group were subjected to HE staining. All tissue samples were treated with the reagent HE, after which these samples were evaluated for pathological changes by an optical microscope (BX61, Olympus, Japan).

2.10 | Nissl staining

Four percent paraformaldehyde-fixed brain tissues from each group were subjected to Nissl staining. The sections were deparaffinized, treated with Nissl staining solution (G1434; Solarbio, China), washed, differentiated in 95% alcohol, dehydrated, cleared, and mounted. The morphological changes were observed and photographed under the microscope (BX61, Olympus, Japan).

2.11 | Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay

TUNEL assays of hippocampus sections and BV-2 cells were performed using the TUNEL Apoptosis Detection Kit (KGA702, KeyGEN BioTECH, China) and detected according to the manufacturer’s instructions. Briefly, BV-2 cells were fixed with 4% paraformaldehyde for 20 minutes. A 50-μL reaction mixture containing 45-μL equilibration buffer, 4-μL TdT enzyme, and 1-μL Biotin-11-dUTP was then added to each sample for 60-minute incubation at 37°C. Then, the sections and cells were washed with PBS three times and incubated with Streptavidin-TRITC for 30 minutes at 37°C, and finally counterstained with DAPI for 5 minutes. The tissue sections and cells were imaged under a fluorescence microscope (BX61, Olympus, Japan).

2.12 | Statistical analysis

Three independent experiments were performed, and results were expressed as the mean ± SEM. Statistical comparisons were made by paired Student’s t-test or one-way analysis of variance (ANOVA) in GraphPad Prism 8 software (San Diego, CA, USA). P < .05 was considered statistically significant.
3 | RESULTS

3.1 | MLT improved glucose homeostasis in type 2 diabetic mice

Type 2 diabetic mice were injected intraperitoneally with MLT once a day for 1 month (Figure 1A). As shown in Figure 1B, blood glucose levels were increased significantly after STZ injection in mice; however, MLT treatment failed to decrease blood glucose levels of type 2 diabetic mice significantly. Meanwhile, body weights of mice in four groups were measured. The results showed that the body weight gain of type 2 diabetic mice was significantly lower than that of blank controls, which could be reversed by MLT treatment (Figure 1C). Compared with type 2 diabetic mice, glucose homeostasis of MLT-treated type 2 diabetic mice was significantly improved. IPGTT showed significant and persistent glucose intolerance in type 2 diabetic mice, which could be

|        | HFD diet | STZ(100mg/kg i.p.) | MLT(10mg/kg i.p.) | IPGTT | MWM | probe trial | Sample collection |
|--------|----------|-------------------|-------------------|-------|-----|-------------|-------------------|
|        | 16 weeks | Day 1-7           | Day 15-44         | Day 45-58 | Day 59-64 | Day 65       | Day 66            |

FIGURE 1  Melatonin promote glucose homeostasis in type 2 diabetic mice. Mice were randomly divided into four groups: control, MLT (10 mg/kg/day), type 2 diabetes, type 2 diabetes plus melatonin (MLT) (10 mg/kg/day). A. The schedule of the present study, (B) blood glucose, (C) body weight levels of each group were monitored once a week. D, E, Intraperitoneal glucose tolerance test (IPGTT) and area under the curve (AUC). F, G, Intraperitoneal insulin tolerance test (IPITT) and AUC. Data are expressed as mean ± SEM (n = 10). *P < .05; **P < .01; ***P < .001 versus control; *P < .05, **P < .01; ***P < .001 versus type 2 diabetes.
improved in MLT-treated type 2 diabetic mice (Figure 1D). IPITT showed markedly reduced insulin sensitivity in type 2 diabetic mice, whereas the response to insulin increased after MLT treatment (Figure 1F). The areas under the curve (AUC) of IPGTT and IPITT of MLT-treated type 2 diabetic mice were lower than that of type 2 diabetic mice (Figure 1E,G).

### 3.2 MLT ameliorated learning and memory deficits in type 2 diabetic mice

MWM test was performed to prove the effects of MLT on hippocampus-dependent learning and memory function in type 2 diabetic mice. As shown in Figure 2A,B, there were no significant differences in the swimming speed and escape latency in the visible platform experiment, which proved that the four groups of mice had no obvious movement or spatial vision disorders. During the hidden platform test, compared with blank controls, type 2 diabetic mice showed higher escape latency on Days 1, 3, and 4 (Figure 2C) and longer path length on Day 4 (Figure 2D), whereas the escape latency and path length decreased notably after MLT treatment. Additionally, a probe trial was conducted to assess memory retention. A notable difference in swimming traces was represented among four groups (Figure 2E). The type 2 diabetic mice mainly swam on the edge of the pool and had a reduced platform crossing numbers compared with the controls. MLT-treated type 2 diabetic mice showed an increased trend in crossing the platform, although there was no significant difference (Figure 2F). Moreover, in the target quadrant, the swimming time (Figure 2G) and swimming length (Figure 2H) of MLT-treated type 2 diabetic mice were longer than those of type 2 diabetic mice. These results demonstrated that MLT treatment ameliorated cognitive deficits in learning and memory function in type 2 diabetic mice.

### 3.3 MLT prevented apoptosis in hippocampus

The neuronal morphology and neuronal density were detected by HE staining and Nissl staining. It has been confirmed that the neurons of hippocampal CA1 region in type 2 diabetic mice exhibited pathological features, such as irregular arrangement, widened intercellular space, reduced cell volume, and nuclear condensation (Figure 3A). The neuronal density of type 2 diabetic mice was decreased significantly (Figure 3B). Our data showed that MLT treatment could reverse the pathological features of cells and increase the density of neurons in the hippocampal CA1 region in type 2 diabetic mice.

The TUNEL assay showed that the number of TUNEL-positive microglia in type 2 diabetic mice was increased dramatically compared with blank controls. MLT treatment significantly reduced the number of apoptosis cells, which indicated that MLT could prevent microglial apoptosis induced by type 2 diabetes (Figure 3C). Additionally, we examined the protein levels of cleaved-caspase3 and Bax. The results revealed that the expressions of cleaved-caspase3 and Bax were obviously increased in type 2 diabetic mice, compared with blank controls. The mechanism of MLT with the function of reducing apoptosis may be caused by inhibiting the expressions of cleaved-caspase3 and Bax, which are well-recognized indicators of apoptosis (Figure 3D). These results suggested that MLT prevented cell death in the hippocampus induced by type 2 diabetes.

### 3.4 MLT suppressed microglial activation and inhibited proinflammatory cytokine expression in hippocampus by NF-κB signaling pathway

To explore the effect of MLT on neuroinflammation, the expressions of Iba1, GFAP, TNF-α, IL-1β, IL-6, p-NF-κB p65, and p-IκBα in the hippocampus were detected. Ionized calcium binding adapter molecule 1 (Iba1), which is widely used as a marker of microglia, and its level is related to the activation of microglia. Glial fibrillary acidic protein (GFAP), which is a special marker of astrocyte, and its level is related to the activation of astrocyte. The immunofluorescence assay and western blot showed increased expression of GFAP in the hippocampus of type 2 diabetic mice, whereas MLT treatment reduced the expression of GFAP (Figure S7A,B). On the contrary, the immunofluorescence assay showed a stronger presence of Iba1-positive microglia in the hippocampus of type 2 diabetic mice, and MLT treatment reduced the number of such microglia (Figure 4A). Therefore, we focused on microglia to study the effect of MLT on type 2 diabetic neuroinflammation. Compared with blank controls, the protein and mRNA levels of TNF-α, IL-1β, and IL-6 were upregulated in type 2 diabetic mice, and MLT treatment reversed these changes (Figure 4B,C). Western blot showed that the levels of p-NF-κB p65 and p-IκBα were increased in the hippocampus of type 2 diabetic mice, which could be reversed by MLT treatment (Figure 4D). To sum up, the results showed that MLT might inhibit neuroinflammation in the hippocampus of type 2 diabetes by NF-κB signaling pathway.

### 3.5 MLT prevented the apoptosis and inflammation of BV-2 cells induced by PA-stimulation

BV-2 microglial cell line was widely used in the research of neuroinflammation. The CCK8 assay was performed to
determine the concentration of PA and MLT. As a result, we chose a 0.4-mM PA and 100-nM MLT for future experiments (Figure S1A,B).

The TUNEL assay showed an increase in the number of TUNEL-positive apoptotic BV-2 cells when cocultured with PA. MLT treatment significantly reduced the number
8 of 18 of apoptotic cells (Figure 5A). Additionally, western blot revealed that the expressions of cleaved-caspase3 and Bax increased obviously in PA-stimulated BV-2 cells. However, the expressions of cleaved-caspase3 and Bax were reduced after MLT treatment (Figure 5B).

The immunofluorescence assay indicated that PA stimulated microglial activation and increased the number of Iba1-positive BV-2 cells. After MLT treatment, Iba1 levels showed a prominent fall (Figure 5C). PA stimulation increased the protein and mRNA expressions of TNF-α, IL-1β,
and IL-6 in the BV-2 cells significantly. MLT treatment significantly prevented the upregulation of these proinflammatory cytokines induced by PA (Figure 5D,E). Moreover, MLT treatment resulted in a decrease in the expressions of p-NF-κB p65 and P-IκBα of PA stimulated BV-2 cells (Figure 5F). Collectively, these findings suggested that MLT could exert anti-apoptosis and anti-inflammation effects in PA stimulated BV2 cells.

3.6 | MLT could improve the process of autophagy

Autophagy in hippocampal microglia and BV-2 cells was detected by western blot and immunofluorescence. Autophagosomal markers (Atg5, Atg7, and MAP1LC3B) mediate the formation of the autophagosome. A degradation marker (p62) mediates the degradation of target proteins and the binding of damaged organelles to the autophagosome. Our results showed that the levels of MAP1LC3B-II, Atg7, and Atg5 were decreased in type 2 diabetic mice, indicating the formation of autophagosomes was impaired. Besides, the level of p62 was increased (Figure 6A). These results indicated that autophagy was suppressed in type 2 diabetic mice.

In order to verify whether MLT could improve the process of autophagy, BV-2 cells were treated with MLT in the presence of PA. The results showed that MLT treatment increased the expressions of MAP1LC3B-II, Atg7, and Atg5 and decreased the level of p62 (Figure 6C). Besides, immunofluorescence assay showed that MLT markedly increased the percentage of MAP1LC3B-positive microglia in hippocampus or BV-2 cells (Figure 6B,D), implying that MLT could improve the process of autophagy.
Melatonin prevented the apoptosis and inflammation of BV-2 cells induced by PA-stimulation. BV-2 cells were treated with melatonin (MLT) (100 nM) in the presence of palmitic acid (PA) (0.4 mM) for 24 hours. A, Immunofluorescence images of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) (red)/4,6 diamidino-2-phenylindole (DAPI) (blue) colocalization in BV-2 cells. B, Expressions of Bax and cleaved-caspase3 were detected by western blot. C, Immunofluorescence images of ionized calcium binding adapter molecule 1 (Iba1) (green)/4,6 diamidino-2-phenylindole (DAPI) (blue) colocalization in BV-2 cells. D, E, The production of tumor necrosis factors-α (TNF-α), interleukin-1β (IL-1β), and IL-6 were measured by western blot and qRT-PCR. F, Protein levels of p-nuclear factor kappa B (NF-κB p65), NF-κB p65, p-recombinant inhibitory subunit of NF kappa B α (IκBα) and IκBα were measured by western blot. Data are expressed as mean ± SEM. Scale bar = 50 µm. *P < .05; **P < .01; ***P < .001 versus control group; #P < .05; ##P < .01; ###P < .001 versus PA group.
FIGURE 6 Melatonin-activated autophagy in hippocampus and BV-2 cells. A, Expressions of autophagy-related genes (Atg7), Atg5, p62, and microtubule-associated protein 1 light chain 3 beta (MAP1LC3B) in the hippocampus were detected by western blot. B, Immunofluorescence images of Iba1 (red)/MAP1LC3B (green)/4,6 diamidino-2-phenylindole (DAPI) (blue) colocalization in the hippocampus. C, Expressions of Atg7, Atg5, p62, and MAP1LC3B in the BV-2 cells were detected by western blot. D, Immunofluorescence images of MAP1LC3B (green)/DAPI (blue) colocalization in the BV-2 cells. Data are expressed as mean ± SEM (n = 5). Scale bar = 50 µm. *P < .05; **P < .01; ***P < .001 versus control group; #P < .05; ##P < .01; ###P < .001 versus type 2 diabetes or PA group.
3.7 | MLT inhibited apoptosis and neuroinflammation by promoting autophagy

To confirm whether MLT could improve microglial apoptosis and inflammation by regulating autophagy, BV-2 cells were treated with autophagy inhibitor 3-MA in the presence of PA. Western blot analysis demonstrated that MLT increased the expressions of Atg5, Atg7, and MAP1LC3B-II and decreased the expression of p62 in PA-stimulated BV-2 cells, whereas the expression of above proteins in BV-2 cells remained unchanged in response to MLT in the culture medium containing PA and 3-MA (Figure 7A). TUNEL assay revealed that the apoptosis of BV-2 cells was significantly enhanced after 3-MA treatment (Figure 7B), which was characterized by upregulation of the expressions of cleaved-caspase3 and Bax (Figure 7C).

Moreover, immunofluorescence assay showed that there were fewer Iba1-positive BV-2 cells after MLT treatment, whereas the number of such BV-2 cells was increased when pretreated with 3-MA (Figure 7D). When cotreated with 3-MA, MLT-induced inhibitive expressions of TNF-α, IL-1β, and IL-6 were markedly reversed (Figure 7E,F). Additionally, the levels of p-NF-κB p65 and p-IκBα were elevated after 3-MA cotreatment (Figure 7G). These results indicated that autophagy is necessary for MLT to resist apoptosis and inflammation in BV-2 cells.

3.8 | MLT promotes autophagy by inhibiting TLR4/Akt/mTOR pathway

TLR4 inhibitor TAK-242, Akt inhibitor MK-2206, and mTOR inhibitor Rapamycin were used to observe whether MLT could activate autophagy through TLR4/Akt/mTOR pathway. Western blot showed that MLT reduced the levels of TLR4, p-Akt, and p-mTOR in the hippocampus of type 2 diabetic mice, and similar results were found in BV-2 cells when cotreated with PA and MLT (Figure 8A,B). Moreover, immunofluorescence assay also showed that MLT significantly reduced the number of TLR4-positive microglial cells (Figure 8C). Pretreatment with TAK-242, MK-2206, and Rapamycin showed that PA-induced activation of TLR4, Akt, and mTOR was inhibited, accompanied by an increase in the expressions of Atg5, Atg7, and MAP1LC3B-II and a decrease in the expression of p62 (Figures 8D and 8E). These results proved that TLR4/Akt/mTOR pathway played a vital role in the regulation of autophagy.

Besides, TUNEL assay showed that TAK-242, MK-2206, and Rapamycin could reverse the apoptosis induced by PA (Figures 8E and 8F). The protein levels of cleaved-caspase3 and Bax were recovered as well (Figures 8F and 8G). Immunofluorescence assay showed that the number of Iba1-positive BV-2 cells was decreased after TAK-242, MK-2206, or Rapamycin treatment (Figures 8G and S6D). When cotreated with TAK-242, MK-2206, and Rapamycin, the production of TNF-α, IL-1β, and IL-6 (Figures 8H and S6E,F) and the levels of p-NF-κB p65, p-IκBα (Figures 8J and S6G) were significantly reduced. These results indicated that MLT promoted autophagy by inhibiting the TLR4/Akt/mTOR pathway.

4 | DISCUSSION

In this study, we revealed the role of MLT in the cognitive impairment of type 2 diabetic mice and explored the potential mechanism of this role by using the mouse microglial cell line BV-2. Our results suggested that diabetes-associated cognitive decline was related to neuroinflammation, as well as the hippocampal neuronal and microglial apoptosis induced by microglial activation, which was possibly due to the suppression of autophagy. Notably, MLT may promote autophagy by inhibiting the TLR4/Akt/mTOR pathway, which plays a protective role in microglial inflammation and apoptosis (Figure 9).

Herein, we observed that diabetes-associated cognitive decline was prevented by MLT treatment. Consistent with previous studies, MLT treatment could improve cognitive decline in AD rats. A growing body of evidence suggests that diabetes is associated with cognitive decline, and the level of hyperglycemia and the duration of diabetes are linked with cognitive decline. Previous studies have reported that oxidative stress, energy metabolism disorders, and neuroinflammation may be the underlying pathogenesis of diabetes-associated cognitive decline. Some studies have indicated that MLT has a regulatory effect on oxidative stress and energy metabolism. Meanwhile, MLT can ameliorate brain insulin resistance induced by neuroinflammation. Overall, these indicate that MLT may have the potential of preventing the cognitive impairments induced by diabetes.

PA is widely used to induce lipotoxicity. However, there are few researches on PA and microglia. Moreover, the effect and the underlying molecular mechanism of MLT in PA-induced lipotoxicity are poorly understood. Exposure to lipotoxicity activates the caspase pathway, thus leads to cell death and neurodegeneration. The apoptosis of neurons and microglia can lead to cognitive dysfunction. PA can induce cell death through a variety of mechanisms, including oxidative stress, ER stress, and autophagy. Our experiments showed that type 2 diabetes and PA could induce the activation of caspase3-dependent apoptosis and that MLT treatment could reverse the changes caused by type 2 diabetes and PA.

In the present study, the microenvironment of diabetes and PA stimulation increased the expression of the
FIGURE 7  Melatonin ameliorated apoptosis and neuroinflammation by promoting autophagy. BV-2 cells were precultured in the presence of 3-methyladenine (3-MA) (5 mM) for 1 hour and stimulated with palmitic acid (PA) for 24 hours. A, Expressions of autophagy-related genes (Atg7), Atg5, p62, and microtubule-associated protein 1 light chain 3 beta (MAP1LC3B) in the BV-2 cells were detected by western blot. B, Immunofluorescence images of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) (red)/4,6 diamidino-2-phenylindole (DAPI) (blue) colocalization in BV-2 cells. C, Expressions of Bax and cleaved-caspase3 were detected by western blot. D, Immunofluorescence images of ionized calcium binding adapter molecule 1 (Iba1) (green)/4,6 diamidino-2-phenylindole (DAPI) (blue) colocalization in BV-2 cells. E, F, The production of proinflammatory cytokines tumor necrosis factors-α (TNF-α), interleukin-1β (IL-1β), and IL-6 were detected by western blot and qRT-PCR. G, Protein levels of p-nuclear factor kappa B (NF-κB p65), NF-κB p65, p-recombinant inhibitory subunit of NF kappa B α (IkBα) and IkBα were measured by western blot. Data are expressed as mean ± SEM. Scale bar = 50µm. *P < .05; **P < .01; ***P < .001 versus control group; †P < .05; ††P < .01; †††P < .001 versus PA group.
microglial marker, Iba1. Previous studies showed that the increased expression of Iba1 corresponds to the microglial activation in neuroinflammatory diseases. Importantly, in this study, MLT treatment prevented the activation of microglia and improved neuroinflammation by inhibiting proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and NF-κB signaling pathway in the hippocampus of type 2 diabetic mice and PA stimulated BV-2 cells. Activated microglia can release proinflammatory mediators, thus damage the neuronal survival. Hippocampal inflammation is characterized by an increase in TNF-α, IL-1β, and IL-6. IL-1β can inhibit synaptic enhancement, long-term potentiation,
and glutamate release in the hippocampus.\textsuperscript{30,31} TNF-\(\alpha\) can inhibit the division of hippocampal progenitor cells, thereby impairing neurogenesis, leading to memory and learning defects.\textsuperscript{32} I\(\kappa\)B\(\alpha\) binds to the p65-p50 heterodimer and is degraded when an exogenous substance stimulates cells. This process translocates NF-\(\kappa\)B to the nucleus and activates the expression of specific genes, such as TNF-\(\alpha\), IL-1\(\beta\), and IL-6. In the presence of acute and chronic infections, activated microglia activates the NF-\(\kappa\)B pathway and mediates neuroinflammation.\textsuperscript{33} Studies have reported that MLT can inhibit neuroinflammation by changing the polarization state of microglia. Our results demonstrated that MLT exerted an anti-neuroinflammation effect.

Autophagy is an important physiological process that influences cellular homeostasis. Importantly, autophagy deregulation possibly affects the clearance of dysfunctional mitochondria; ER parts; and protein aggregates, which are the main cause of neurodegenerative diseases. Researches on the role of autophagy in the brain in cognition have mainly focused on neurons; however, in recent years, an increasing number of researchers have begun to pay growing attention to the effect of autophagy on microglia.\textsuperscript{34}
Autophagy can regulate inflammation by adjusting the activation of inflammasomes and controlling the polarization of microglia. Moreover, autophagy is a type of programmed cell death, also called type II programmed death. The interplay between autophagy and apoptosis is extensive. Some evidence indicates that autophagy plays important roles in the regulation of apoptosis, including mitochondrial dysfunction, p53, and interactions between BECN-1 and Bcl-2 family members.

There are few studies on autophagy in MLT and cognition. Recent studies have reported that MLT treatment exerts beneficial effects via the induction of autophagy in animal models of diabetic cardiomyopathy, and some carcinomas, demonstrated that autophagy activation appears to be one of the important mechanisms for MLT in the treatment of diseases. Therefore, the role between MLT and autophagy in cognition process is worth studying. We observed that PA stimulation significantly reduced the expressions of Atg5, Atg7, and MAP1LC3B-II and increased the level of autophagy substrate p62, and MLT exerted a comparable effect on autophagy induction. Autophagy includes a series of interrelated steps, including initiation, nucleation, elongation, fusion, and degradation. This complete dynamic process is also called autophagy flux. Atg5, Atg7, and MAP1LC3B mediate the formation of the autophagosome, and p62 mediates the binding of damaged organelles to the autophagosome.

The abnormal levels of Atgs proteins represent autophagy defects. 3-MA is used to block autophagy by inhibiting autophagy substrate p62, and MLT exerted a comparable effect on autophagy induction. Autophagy includes a series of interrelated steps, including initiation, nucleation, elongation, fusion, and degradation. This complete dynamic process is also called autophagy flux. Atg5, Atg7, and MAP1LC3B mediate the formation of the autophagosome, and p62 mediates the binding of damaged organelles to the autophagosome.

Next, we discussed the possible mechanisms through which MLT modulated autophagy in microglia. TLR4 is mainly expressed in microglia and is stimulated by appropriate ligands. Some studies have demonstrated that MLT could directly act on TLR4 and mediates the downstream signaling pathway. Xia et al suggested that MLT could modulate TLR4-mediated inflammatory genes in LPS-stimulated RAW264.7 cells. Wei et al found that MLT ameliorates hyperglycemia-induced renal inflammation by inhibiting the activation of TLR4. All these indicate that MLT could exert an effect directly through the TLR4 pathway. At the same time, Lee et al found that autophagic flux is significantly suppressed by TLR4 activation in microglia. The stimulation of the TLR4 extracellular domain sequentially triggers the Akt/mTOR signaling pathway, which acts as a vital autophagy regulatory pathway. Therefore, we speculated that MLT might improve microglial autophagy through the TLR4/Akt/mTOR pathway. Our data showed that the protein expression levels of TLR4, p-Akt, and p-mTOR were increased in type 2 diabetes mice and PA-stimulated BV-2 cells, and MLT treatment reversed these changes. We next utilized TAK-242, MK-2206, and Rapamycin to further validate the role of TLR4 signaling. The inhibition of TLR4, Akt, and mTOR exerted a comparable effect on the inhibition of the TLR4/Akt/mTOR signaling pathway in PA-stimulated BV-2 cells. This result suggested that MLT exerts its anti-inflammatory and anti-apoptosis effects by directly acting on TLR4.

However, some limitations of our work should be mentioned. In the present study, we did not investigate the effect of MLT in female mice. Growing evidence indicated that there are gender differences in diabetes and cognitive function. Therefore, the effect of MLT on gender-dependent differences in diabetes-associated cognitive decline should be further confirmed. Furthermore, the MWM test was employed to study the cognitive function, and we would perform an additional cognitive test to support the findings in future studies.

This study provides a new basis for the beneficial effects of MLT in diabetes-associated cognitive dysfunction, and provides a novel strategy for delaying the progress of cognitive dysfunction through autophagy.

ACKNOWLEDGEMENTS
This work was supported by grants from the National Natural Science Foundation of China (82070852, 81873650, and 82070799), the Natural Science Foundation of Shandong Province (ZR2020MH105), and Fundamental research funds of Shandong University (2018JC015). The supplementary experiments were supported by three additional authors (Yilin Wang, Jinmin Ren, and Peng Lin).

CONFLICT OF INTEREST
The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS
Y. Cui performed the experiments, analyzed the data, and wrote the manuscript. M. Yang, Y. Wang, J. Ren, and P. Lin participated in the research and data collection. C. Cui, Q. He, H. Hu, and J. Song helped with the sample collection. Y. Sun and K. Wang supervised the overall study design.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Cui Y, Yang M, Wang Y, et al. Melatonin prevents diabetes-associated cognitive dysfunction from microglia-mediated neuroinflammation by activating autophagy via TLR4/Akt/mTOR pathway. The FASEB Journal. 2021;35:e21485. https://doi.org/10.1096/fj.202002247RR