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ORIGINAL ARTICLE

Melatonin ameliorates cognitive deficits through improving mitophagy in a mouse model of Alzheimer's disease

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Abstract

While melatonin is known to have protective effects in mitochondria-related diseases, aging, and neurodegenerative disorders, there is poor understanding of the effects of melatonin treatment on mitophagy in Alzheimer's disease (AD). We used proteomic analysis to investigate the effects and underlying molecular mechanisms of oral melatonin treatment on mitophagy in the hippocampus of 4-month-old wild-type mice versus age-matched 5 × FAD mice, an animal model of AD. 5 × FAD mice showed disordered mitophagy and mitochondrial dysfunction as revealed by increased mtDNA, mitochondrial marker proteins and MDA production, decreased electron transport chain proteins and ATP levels, and co-localization of Lamp1 and Tomm20. Melatonin treatment reversed the abnormal expression of proteins in the signaling pathway of lysosomes, pathologic phagocytosis of microglia, and mitochondrial energy metabolism. Moreover, melatonin restored mitophagy by improving mitophagosome–lysosome fusion via Mcoln1,

Abbreviations: AD, Alzheimer's disease; ATG5, Autophagy protein 5; ATG7, Autophagy protein 7; CQ, Chloroquine;; CTSD, Cathepsin D; DE, Differentially Expressed;; ETC, Electron transport chain; Hk2, Hexokinase-2; Lamp1, Lysosome-associated membrane glycoprotein 1; LC3b, Microtubule-associated proteins light chain 3B; Mcoln1, Mucolipin-1; MDA, Malondialdehyde; p62, Sequestosome-1.

Chongyang Chen and Chao Yang contributed equally to this work.

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and thus, ameliorated mitochondrial functions, attenuated Aβ pathology, and improved cognition. Concurrent treatment with chloroquine and melatonin blocked the positive behavioral and biochemical effects of administration with melatonin alone. Taken in concert, these results suggest that melatonin reduces AD-related deficits in mitophagy such that the drug should be considered as a therapeutic candidate for the treatment of AD.

KEYWORDS
Alzheimer's disease, melatonin, mitochondrial function, mitophagy, proteomic

1 | INTRODUCTION

The mitochondrion is crucial for energy production, synthesis of key metabolites, regulation of apoptosis, and generation of endogenous reactive oxygen species (ROS), among other functions.[1] Neurons are particularly sensitive to changes in mitochondrial function because synaptic transmission, axonal and dendritic transport, and ion channel activity are extremely energy dependent.[2,3] Mitochondrial dysfunction is an early pathological event in mouse models of Alzheimer's disease (AD). Mitochondrial respiration and pyruvate dehydrogenase activity decreased in the brain of 3×Tg-AD mice as early as 3 months old.[4] Likewise, in the brains of AD patients, mitochondrial dysfunction evidenced by a decrease in cytochrome oxidase (COX) activity, an increase in free radical generation, and a reduction in energy metabolism occur prior to senile plaque formation.[4–7]

Mitophagy is a form of cellular autophagy that selectively removes defective mitochondria. Impairment of mitophagy resulting from cellular energy deficits and oxidative damage is proposed to participate in the etiology of AD.[8] Abnormal mitophagy is evidenced by autophagic accumulation of mitochondria in the soma of vulnerable neurons in AD patient brains.[9] The AD-related proteins Aβ and hyperphosphylated Tau (p-tau) interact with other mitochondrial proteins (Drp1, VDAC, CypD, and ABAD) to cause excessive fragmentation of mitochondria, disordered mitochondrial dynamics, and impairment of mitophagy, ultimately leading to synaptic plasticity and cognitive deficits.[10–13] We also found that IST1, a positive modulator for the formation of ESCRT complex that is required for autophagosome–lysosome fusion, decreased the protein level by overexpressing wild-type human tau, ultimately leading to autophagosome accumulation and cognitive impairment.[14] Accumulated wild-type human tau proteins also translocated into the mitochondria in a dose-dependent manner to disrupt mitochondrial dynamics and cause deficient mitophagy.[15,16] These findings suggest that maintaining mitochondrial homeostasis by optimizing mitophagy is a potential therapeutic strategy for AD.

As a hormone secreted by the pineal gland, melatonin (N-acetyl-5-methoxytryptamine) has protective effects in mitochondria-related diseases, aging, and neurodegenerative diseases.[17,18] Previous studies have found that melatonin reduced AD-like pathology by restoring autophagy flux[19] and promoting mitophagy to alleviate myocardial ischemia-reperfusion injury.[20] Melatonin also improved cognitive ability and reduced Aβ deposition in an AD mouse model.[21,22] However, there is no clear understanding of whether melatonin affects AD through mitophagy and the underlying mechanisms involved therewith.

The present study used 5 × FAD mice as a murine model of AD to examine the effects of daily oral melatonin treatment for a 1-month period. We found that melatonin restored mitophagy by promoting fusion of mitophagosomes with lysosomes and inhibiting Aβ formation, with resultant improvement in cognitive function.

2 | MATERIAL AND METHODS

2.1 | Regents and antibodies

Melatonin (stated purity≥99.47%, catalog number: HY-B0075), memantine (stated purity≥98%, catalog number: HY-B0365A), and chloroquine (CQ) (stated purity≥99.50%, catalog number: HY-17589A) were purchased from the company of MedChemExpress (MCE, Monmouth Junction, NJ, USA). Information on antibodies and dilution ratios used in this study has been shown in Table S1.

2.2 | Animal treatment

5 × FAD (B6. Cg-Tg(APPSwFlLon,PSEN1*M146L*L286V)6799V) mice and control mice (WT) were obtained from the Jackson Laboratory (Maine, USA). The brains

of 5 × FAD mice develop cortical amyloid deposition at 2 months of age and develop cognitive impairment by the age of 4 months,[23] the age selected for this study. The animals were housed in standard laboratory cages under 12-h light/12-h dark cycle. Memantine, a noncompetitive NMDA receptor antagonist approved for AD treatment by the U.S. FDA, was administrated as a positive control drug.

Four-month-old WT and 5 × FAD mice were treated orally by gavage with 10 mg/kg melatonin or combined with chloroquine (50 mg/kg, intraperitoneal injection), or 10 mg/kg memantine, or the same volume of corresponding vehicle (1% dimethyl sulfoxide) daily for 1 month. The chosen dose of melatonin and administration time were based on previous studies.[24–28] The behavioral tests were beginning after 1 month melatonin administration, and performed at 13:00 pm to 18:00 pm. All animal experiments followed the "Policies on the Use of Animals and Humans in Neuroscience Research" revised and approved by the Society for Neuroscience (USA) in 1995. The Ethics Committee of the Shenzhen Center for Disease Control and Prevention reviewed and approved this study.

2.3 | Behavioral tests

2.3.1 | Y maze

The Y maze was used to assess the spatial reference memory of mice.[29] Animals were first acclimated for 1 h in the behavioral testing room. The arms of the maze were labeled as start arm, novel arm, and other arm. Before each mouse training and testing, the floor and arms were cleaned with 70% ethanol and wiped with dry paper, and at least 5 min were left to allow ethanol evaporation. With the novel arm closed off with a divider, mice were placed individually facing the center into one of the open arms to explore for 15 min. One hour after this period of exploration, the test animal was again placed into the distal part of the same arm that used in the previous training period, facing toward the center of the maze for 5-min undisturbed exploration with the divider removed. The time of an arm entry was recorded when the mouse had all four paws inside the arm. Time in the novel arm time percentage was expressed as novel arm exploration time/total exploration time.

2.3.2 | Object recognition test

The mice were placed individually in an empty plastic box (length × width × height: 47.5 cm × 35 cm × 20 cm) and allowed to adapt to the environment for 5 min on day 1. On the second day, the mice were placed into the same plastic box but with two same objects which were placed on opposite sides of the test area, and allowed to explore freely for 5 min. One hour after the exploration, one of the two objects was replaced with a new object of the same material and size but different in shape. Mice were then individually placed into the box for free exploration for 5 min. The time was recorded when the mouse's nose was in contact with the object or directed at the object within ≤2 cm. And the time the mouse spent on standing, sitting, or leaning on the object was excluded in the calculation. Before each mouse training and testing, the arena and objects were cleaned with 70% ethanol and wiped with dry paper; at least 5 min were left to allow ethanol evaporation. Results were expressed as a preferential index percentage calculated as the new object (exploration time / new object exploration time + old object exploration time) × 100%.

2.3.3 | Morris water maze

The Morris water maze (MWM) test was performed to assess the learning and spatial memory of mice.[30] The circular pool was filled with water containing dissolved milk powder and divided into four quadrants of equal area. A platform was hidden in the third quadrant at a position 1.2 cm beneath the surface of the water. Animals were individually started from one of the middles of the four quadrants facing the wall of the pool to train to find the platform for five consecutive days, four trials per day with a 30 s interval. A stationary array of cues outside the pool tub was used for mice to establish spatial orientation. For the training test, mice were allowed to find the platform within a period of 60 s and stayed on the platform for another 30 s. If the animal failed to find the platform within 60 s, we recorded the time as 60 s. Then, these mice were guided onto the platform and allowed to stay for 30 s. The probe trial was performed 24 h after the training test. The platform was removed and mice were individually placed into the quadrant opposed the location of original platform. The time for the animal to arrive at the site of previous platform placement, the number of times the platform site was crossed, the swimming speed, and swimming distance were automatically recorded by a video camera.

2.4 | Proteomic analysis

The method of proteomic was referenced from previous research.[31–33] Briefly, the protein extracted from hippocampal tissue was digested with trypsin. After digestion, the peptide was labeled with tandem mass tags

(TMTs), dried, and redissolved in 100 μl 0.1% FA for peptide fraction. A total of 45 fractions was collected from high-performance liquid chromatography (HPLC), dried, and redissolved 20 μl 0.1% FA for liquid chromatography (LC)–mass spectrometry (MS)/MS analysis. Acquired data were searched against the database of UniProt-Mus musculus with Proteome Discoverer 2.1 software. The differential expression (DE) of protein in any two groups was set at $p < 0.05$.

2.5 | Assessment of relative mitochondrial DNA copy number

The relative mitochondrial DNA (mtDNA) copy number was measured according to a method descried in recently published research.[34] The DNA of hippocampal tissue was extracted with a DNA Extraction Kit (Tian gen, DP304-03, China). The relative mtDNA copy number was measured by qPCR. The primers of nuclear control: (F) TTGAGACTGTGATTGGCAATGCCT and (R) CCAGAAATGCTGGGCGTACT; and the primers of mitochondrialDNA:(F)GCCAGCCTGACCCATAGCCATAAT and (R) GCCGGCTGCGTATTCTACGTTA.

2.6 | Statistical analysis

Data were expressed as mean ± standard error (S.E.M). Statistical analyses were performed using two-way repeated measures ANOVA tests or one-way ANOVA followed by Tukey's multiple-comparison tests with the GraphPad Prism 7. A p value <.05 was taken as significant.

3 | RESULTS

3.1 | Melatonin ameliorated cognitive defects of 5 × FAD mice

To explore whether melatonin treatment affected diurnal pattern of behavioral activity of mice, the open field test was used. As shown in Figure S1, the total traveled distance and crossed line had no significant difference in all treatment groups over 24 h. 5 × FAD mice were treated with melatonin or memantine for 1 month, after which cognitive performance was detected. Compared with WT mice, untreated 5 × FAD animals showed significantly less time in the Y-maze novel arm and a lower NOR preferential index. Treatment with melatonin reversed these trends, while memantine only reversed the NOR preferential index trend (Figure 1A, B). In the training stage of the MWM test, the 5 × FAD control mice spent more time to find the platform on training day 5 compared with WT mice, while melatonin treatment significantly decreased the escape latency time on the 3rd and 5th days, and memantine treatment also significantly decreased the latency time on day 5 (Figure 1C).

For the test stage of the MWM test, the 5 × FAD controls showed a significantly increased escape latency compared with WT controls, decreased numbers of platform crossings, reduced distance traveled in the correct quadrant, and time spent in the correct quadrant. Melatonin treatment reversed all these behavioral indices while memantine also reversed the above proxies but had no significant effect on the traveled distance in the correct quadrant (Figure 1D–H). There was no significant difference in swimming speed (Figure 1I) or total swimming distance (Figure 1J), which excluded motor deficits. Unexpectedly, melatonin had no effects on the behavior of WT mice (Figure 1). Taken together, these data showed that melatonin attenuated cognitive deficits of 5 × FAD mice.

3.2 | Melatonin treatment reversed autophagy dysfunction in the hippocampus of 5 × FAD mice

To explore the possible mechanism by which melatonin treatment positively affected cognitive function in 5 × FAD mice, the hippocampus was lysed for proteomic analysis. Heatmap analysis showed the differentially expressed (DE) proteins of the four treatment groups of animals: melatonin treatment obviously reversed the protein expression pattern of 5 × FAD mice (Figure 2A), while showed few proteins changed in WT mice (Figure S2A–D and Table S2). Cluster analysis found three reversed protein expression modules (Figure S3A). By gene ontology analysis, Clusters 1 and 3 showed proteins with increased expression in 5 × FAD control mice compared with untreated WT mice, while treatment with melatonin reversed such expression. These proteins showed enriched biological processes including: protein autophosphorylation, mitophagy in response to mitochondrial depolarization, phagocytosis, engulfment, and innate immune response, among others. Cluster 2 showed expression of proteins downregulated in 5 × FAD control mice and increased in animals treated with melatonin, with associated biological processes including: protein transport, reactive oxygen species metabolic process, mitochondrial electron transport, ubiquinol to cytochrome c, and so on (Figure S3B). ClueGO analysis further found that proteins in Cluster 1- and 3-enriched pathways mainly involved the lysosome, microglia pathogen phagocytosis pathway, and phagosome, while proteins in Cluster 2 reflected mainly

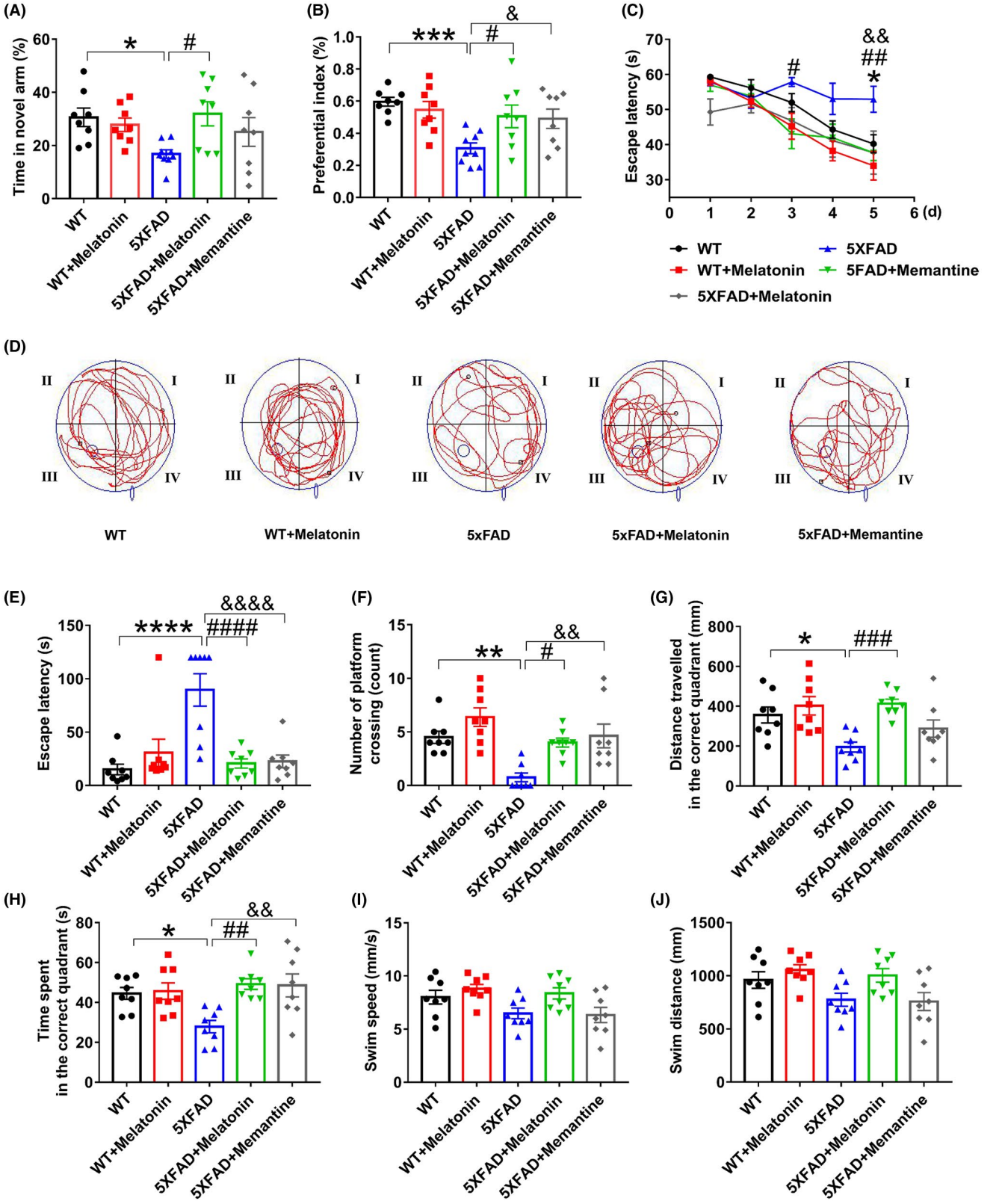

FIGURE 1 Melatonin improved cognitive behavior of 5 × FAD mice. After melatonin or memantine treatment for 1 month of 4-month-old 5 × FAD mice, behavior was assessed. (A) Time spent in the novel arm of the Y maze; (B) the preferential index in the NOR test; (C) the escape latency of mice spent during the training stage of MWM test; (D) representative movement trajectories of mice during the test stage of MWM; (E–J) the escape latency (E); number of platform crossings (F); distance traveled in the correct quadrant (G); time spent in the correct quadrant (H); swimming speed (I); and swimming distance (J) during the test stage of MWM. Data were shown as Mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ versus WT vehicle group. #$p < .05$, ##$p < .01$, ###$p < .001$, ####$p < .0001$, &$p < .05$, &&$p < .01$, &&&&$p < .0001$ versus 5 × FAD vehicle-treated (control) group. $N = 8$ for each group

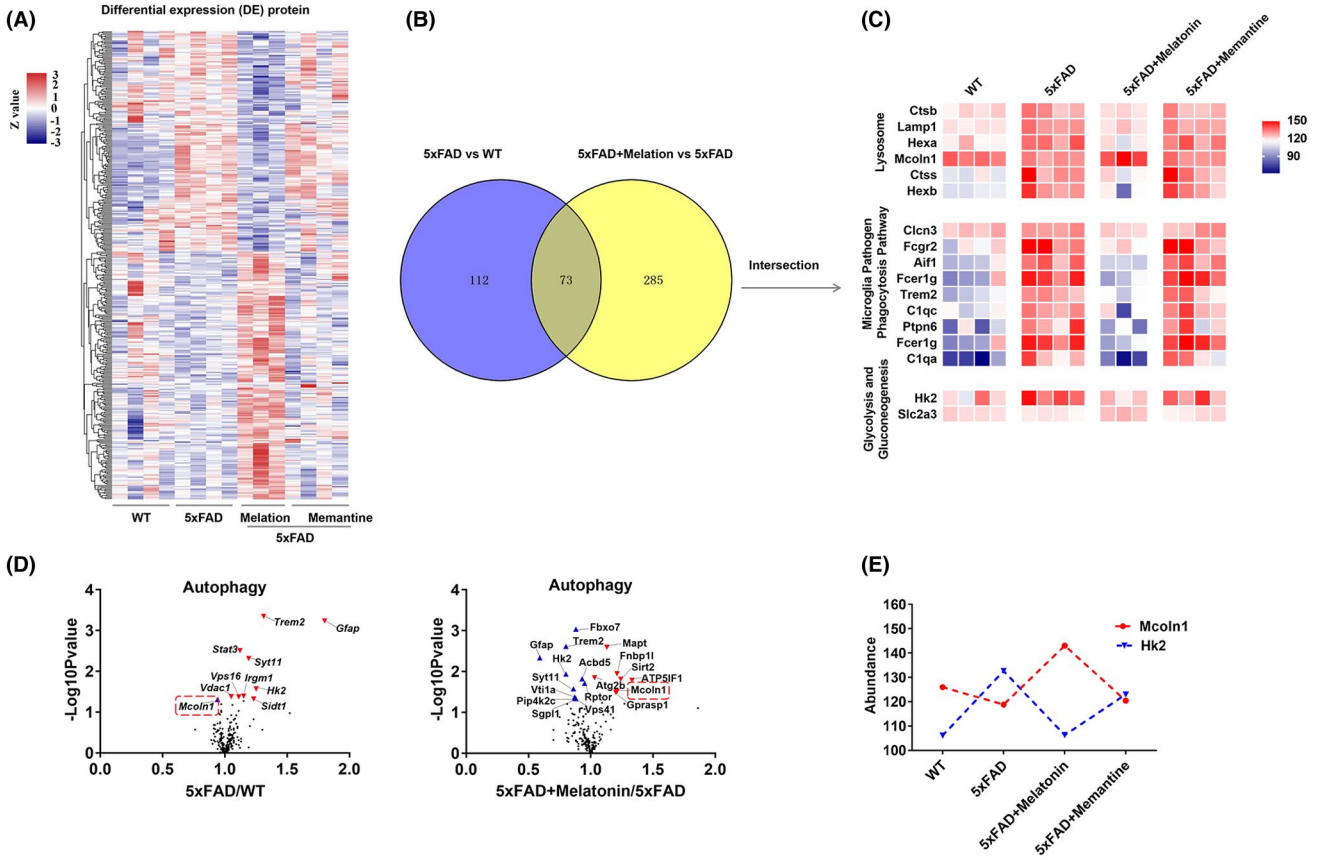

FIGURE 2 Melatonin treatment displayed proteomic changes in the hippocampus of 5 × FAD mice. (A) The heatmap of differentially expressed proteins ($p < .05$) for WT mice, 5 × FAD treated with vehicle (5 × FAD), 5 × FAD treated with melatonin (5 × FAD + melatonin), and 5 × FAD treated with memantine (5 × FAD + memantine). The Z value of protein abundance was plotted in a red-blue color scale, with red and blue indicating increased and decreased protein expression, respectively. (B) The Venn diagram to find proteins shared between 5 × FAD mice versus WT mice and 5 × FAD mice + melatonin versus 5 × FAD mice. (C) The heatmap of pathways enriched from the shared proteins are shown in (B) with the red color representing increased expression and blue color reduced expression. (D) Volcano plots of WT mice or 5 × FAD + melatonin versus 5 × FAD mice for autophagy-relative proteins: the X-axis represented the ratio between two compared groups, and the Y-axis is the -log10-transformed *p*-value. The red color represented increased expression and the blue color represented reduced expression. The red frame indicated Mcoln1, which is involved in the regulation of mitophagosome and lysosome fusion. (E) Profile plot of protein expression of Hk2 and Mcoln1; the dotted line connects the average abundance of the protein in the different animal groups

enriched pathways in ribosome, oxidative phosphorylation, and electron transport chain (Figure S3C). Taken together, these data suggested that melatonin treatment had positive effects on lysosome, auto/mitophagy, microglia activation, and mitochondrial energy metabolism of 5 × FAD mice.

By Venny analysis, there was a total of 185 DE proteins between WT and 5 × FAD control mice, 358 DE proteins between 5 × FAD control and melatonin-treated 5 × FAD mice (Table S2), and 73 DE proteins were shared between WT versus 5 × FAD control mice and 5 × FAD control versus melatonin-treated 5 × FAD mice (Figure 2B). These shared DE proteins were mainly focused on lysosomes, the microglia pathogen phagocytosis pathway, glycolysis, and gluconeogenesis (Figure 2C). Hippocampal expression of lysosome proteins (Lamp1, cathepsin b (Ctsb), cathepsin s (Ctss), beta-hexosaminidase subunit alpha (Hexa), and beta-hexosaminidase subunit beta (Hexb)) was increased in 5 × FAD mice and deceased in melatonin-treated 5 × FAD mice, both relative to expression in WT controls. Mucolipin-1 (Mcoln1) was the only protein that showed reduced expression in 5 × FAD control mice relative to WT animals and increased expression in melatonin-treated 5 × FAD mice. There was increased expression of Lamp1, which functions in the maintenance of lysosomal structural integrity, while Ctsb, Ctss, Hexa, and Hexb are intra-lysosomal hydrolases. This change indicated there was an accumulation of lysosomes in 5 × FAD mice that was attenuated by treatment with melatonin.

Since there is overlap in proteins used for autophagy and lysosomal function, hippocampal autophagy-related proteins were analyzed and searched for in the

collaborative DE proteins. A volcano plot showing protein expression in WT versus 5 × FAD mice revealed increased expression in the latter of nine proteins, including Gfap, Trem2, Stat3, Syt11, Irgm1, Vps16, Sidt1, Hexokinase-2 (Hk2), Vdac1, and decreased expression of Mcoln1. Treatment with melatonin reversed the expression of Trem2, Gfap, Syt11, HK2, and Mcoln1 expression. Among them, Gfap is a marker of astrocyte; Trem2 is involved in microglial phagocytosis; Syt11 is a member of the synaptotagmin protein family; and Hk2 is a protein in the mitochondrion outer membrane. Mcoln1 plays a role in phagosome–lysosome fusion.[35] The melatonin induced increased expression of proteins (Fnbp1l, Sirt2, ATP5IF1, Atg2b, and Mcoln1) involved in the process of mitophagy; they positively regulate endosome to lysosome transport or lysosome-mediated degradation. Expression of Mcoln1 and Hk2 in the different animal groups was shown in Figure 2E. Mcoln1 was the only protein that showed reduced hippocampal expression in 5 × FAD mice and the expression was reversed with systemic melatonin treatment. Expression of HK2 and VDAC1 was also increased in 5 × FAD mice, which indicated accumulation of hippocampal mitochondria.

3.3 | Melatonin ameliorated mitophagy deficits

The results of bioinformatics analysis of protein expression were consistent with the hypothesis that melatonin might ameliorate autophagy/mitophagy defects in the hippocampus of 5 × FAD mice by promoting auto/mitophagosome and lysosome fusion. To confirm the bioinformatics results, by Western blotting, p62 and LC3b levels were significantly increased compared with WT mice, while melatonin treatment significantly decreased p62 and LC3b levels. ATG7 and ATG5 (the upstream protein of autophagy pathway that involves in the maturation of LC3b) showed no significant difference (Figure 3A–E); these data indicated that the initiation of autophagy was normal but that auto/mitophagosomes accumulated in the hippocampus of 5 × FAD mice, while melatonin administration promoted auto/mitophagosome degradation.

To confirm further the ameliorating effect of melatonin administration on deficient mitophagy in the 5 × FAD hippocampus, we detected the mitochondrial DNA (mtDNA) relative copy number and mitochondrial markers Hk2, pyruvate dehydrogenase (PDH), cytochrome c (Cytc), Tomm20, and Tomm40. Compared with WT mice, mtDNA significantly increased in 5 × FAD control mice, while melatonin treatment significantly decreased mtDNA (Figure 3F). Melatonin administration also reversed the increased protein level of Hk2, PDH, Cytc, Tomm20, and Tomm40 in 5 × FAD mice (Figure 3G–L). However, treatment with memantine had no effect on the level of mtDNA or mitochondrial protein marker. In addition, in WT mice, oral treatment with melatonin had no effect on expression levels of the above-noted proteins (Figure S4).

Compared with WT mice, the co-localized yellow puncta of Lc3 and Tomm40 increased, and the co-localized puncta of Lamp1 and Tomm20 decreased, in the hippocampal CA3 region of 5 × FAD mice. Melatonin treatment decreased the co-localized puncta of Lc3 and Tomm40 and increased the co-localized puncta of Lamp1 and Tomm20 (Figure 4E, F). These results suggested that melatonin attenuated mitochondrial accumulation via increased mitophagy in 5 × FAD mice. By proteomic analysis, melatonin was found to remodulate the expression of proteins involved in lysosome (Figure 2C). Western blotting further validated the proteomic profiling results. Compared with WT mice, Mcoln1 showed a reduced expression with lysosomal markers (Lamp1 and CTSD) had increased expression in the 5 × FAD control, while melatonin treatment reversed these protein patterns (Figure 4A–D). Melatonin had no effect on the expression of Mcoln1 and lysosomal markers in WT mice (Figure S5). These data suggest that melatonin ameliorated mitophagy deficits by promoting the fusion of autophagosome/mitophagosome and lysosome in 5 × FAD mice.

3.4 | Melatonin improved mitochondrial function in the hippocampus of 5 × FAD mice

The accumulated mitochondria were eliminated by melatonin treatment in 5 × FAD mice, which implied that mitochondrial dysfunction might also be ameliorated by melatonin. To test this hypothesis, the expression of ETC proteins (Ndufs1 (complex Ⅰ), SDHB (complex Ⅱ), UQCRFS1 (complex Ⅲ), and Cox5b (complex Ⅳ)) was analyzed by Western blotting. In 5 × FAD mice, melatonin treatment significantly reversed the reduced hippocampal levels of Ndufs1, SDHB, and UQCRFS1, increased the ATP level, and decreased MDA production (Figure 5A–G), while such treatment had no effect on the levels of the above-noted mitochondrial-associated proteins in WT mice (Figure S6). All the DE mitochondrial proteins were searched for their cellular component in mitochondria. As shown in Figure S7, the decreased 5 × FAD hippocampal expression levels of some proteins involved in mitochondrial ribosomal protein and mitochondrial electron transport chain (ETC) were reversed by melatonin treatment. Taken together, these data suggested that melatonin treatment improved mitochondrial energy supply, which was decreased in the hippocampus of 5 × FAD mice versus WT controls.

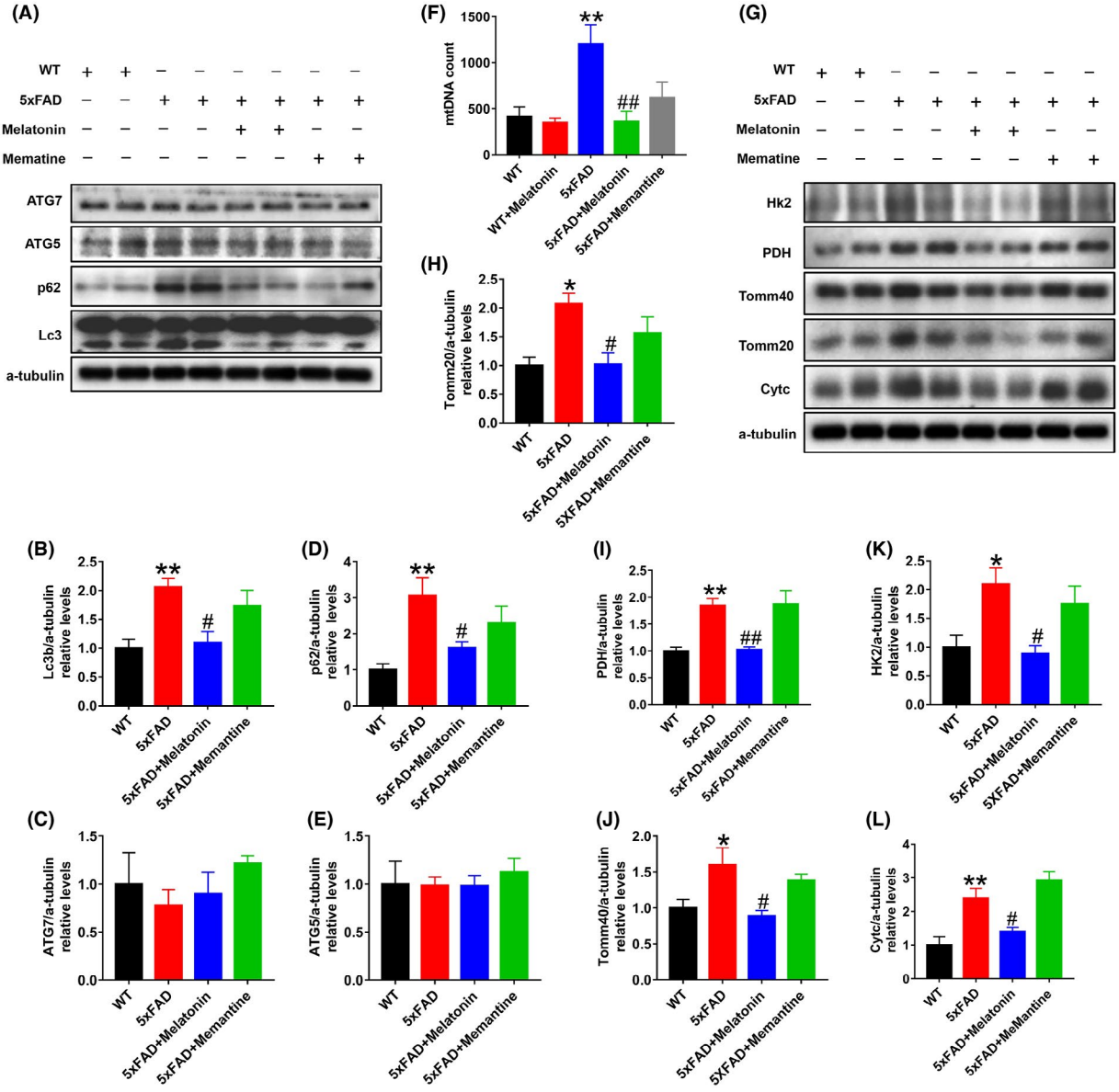

FIGURE 3 Melatonin ameliorated the accumulation of mitochondria in 5 × FAD mice. (A–E) The expression level of autophagy-related proteins (ATG7, ATG5, p62, and LC3b) detected by Western blotting and quantitative analysis. (F) The relative mtDNA copy number was detected by qPCR. (G–L) The expression level of proteins of the mitochondrial outer membrane (Hk2, Tomm40, and Tomm20), mitochondrial inner membrane (Cytc), and mitochondrial matrix (PDH) was detected by Western blotting and quantitative analysis. Data were expressed as Mean ± SEM. *$p < .05$, **$p < .01$ versus WT vehicle group. #$p < .05$, ##$p < .01$ versus 5 × FAD vehicle group. $N = 4$ for each group

3.5 | Melatonin attenuated Aβ deposition in the hippocampus of 5 × FAD mice

We also detected the hippocampal Aβ level. Compared with WT mice, Aβ plaques were obviously increased in the hippocampus of 5 × FAD control mice, and melatonin treatment significantly decreased the number of Aβ plaques (Figure 6A, B). Moreover, dot blots also showed a significantly decreased level of 6E10 after melatonin treatment (Figure 6C, D).

3.6 | Chloroquine (CQ) attenuated the improvement effects of melatonin on the cognitive ability

Chloroquine suppresses fusion of the auto/mitophagosome and lysosome. We used CQ treatment of 5 × FAD mice to confirm melatonin-induced amelioration effects via modulation of autophagy/mitophagy. CQ treatment significantly reversed the positive effects of melatonin on behavioral measures as indicated by the significantly decreased time mice spent in the novel arm of the Y maze

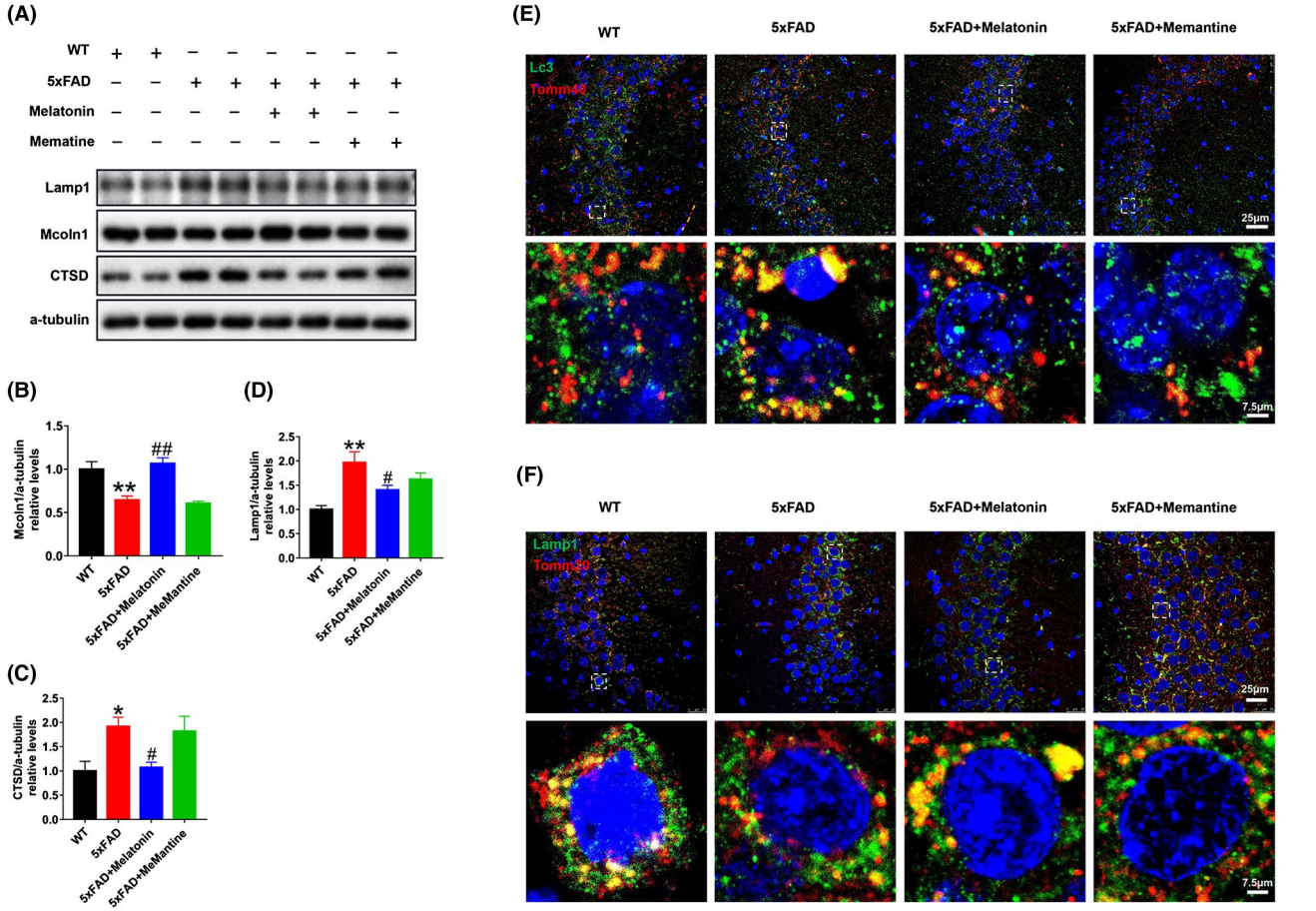

FIGURE 4 Melatonin promoted the fusion of mitophagosome and lysosome in 5 × FAD mice. (A–D) The expression of Mconl1, Lamp1, or CTSD was detected by Western blotting and quantitative analysis. (E, F) The co-immunofluorescence staining of Lc3 and Tomm40 (E), and co-staining of Lamp1 and Tomm20 (F) in the hippocampus CA3. Data were expressed as Mean ± SEM. *$p < .05$, **$p < .01$ versus WT vehicle group. #$p < .05$, ##$p < .01$, ###$p < .001$ versus 5 × FAD vehicle group. $N = 4$ for each group

and preferential index of NOR (Figure 7A, B). By WMW test, animals treated with CQ plus melatonin showed significantly increased escape latency on the 3rd, 4th, and 5th days of the training stage (Figure 7C), and a significantly increased escape latency, decreased number platform crossings, and distance traveled in the targeted quadrant compared with melatonin-treated 5 × FAD mice in the test stage, while the swimming speed and total distance had no significant difference (Figure 7D, E). Taken together, the behavioral tests suggested that CQ reversed the improved cognition of 5 × FAD mice induced by melatonin.

3.7 | Chloroquine (CQ) disturbed melatonin-induced mitophagy, and improved mitochondrial function and pathology of Aβ deposition in 5 × FAD mice

Western blotting showed that p62 and Lc3b levels were significantly increased in melatonin + CQ-treated 5 × FAD mice compared with melatonin-treated 5 × FAD mice, while the expression levels of ATG5 and ATG7 showed no significant difference (Figure 8A–E). The accumulation of mitochondria after CQ administration was proved by a significantly increased level of mtDNA (Figure 8F). CQ treatment also significantly reversed the decreased expression of mitochondrial markers in the hippocampus of 5 × FAD mice induced by melatonin (Figure 8G–L).

CQ treatment also significantly decreased the ETC proteins (Ndufs1, SDHB) and the hippocampal ATP level, and increased MDA production, thereby reversing melatonin-induced improved mitochondrial function (Figure 9A–E). Immunohistochemistry and dot blots showed that Aβ plaques number and the 6E10 level were significantly increased in the hippocampus of CQ + melatonin-treated 5 × FAD mice (Figure 10).

4 | DISCUSSION

In this study, by proteomic and biochemical analysis, we found that DE proteins enriched in the molecular pathway

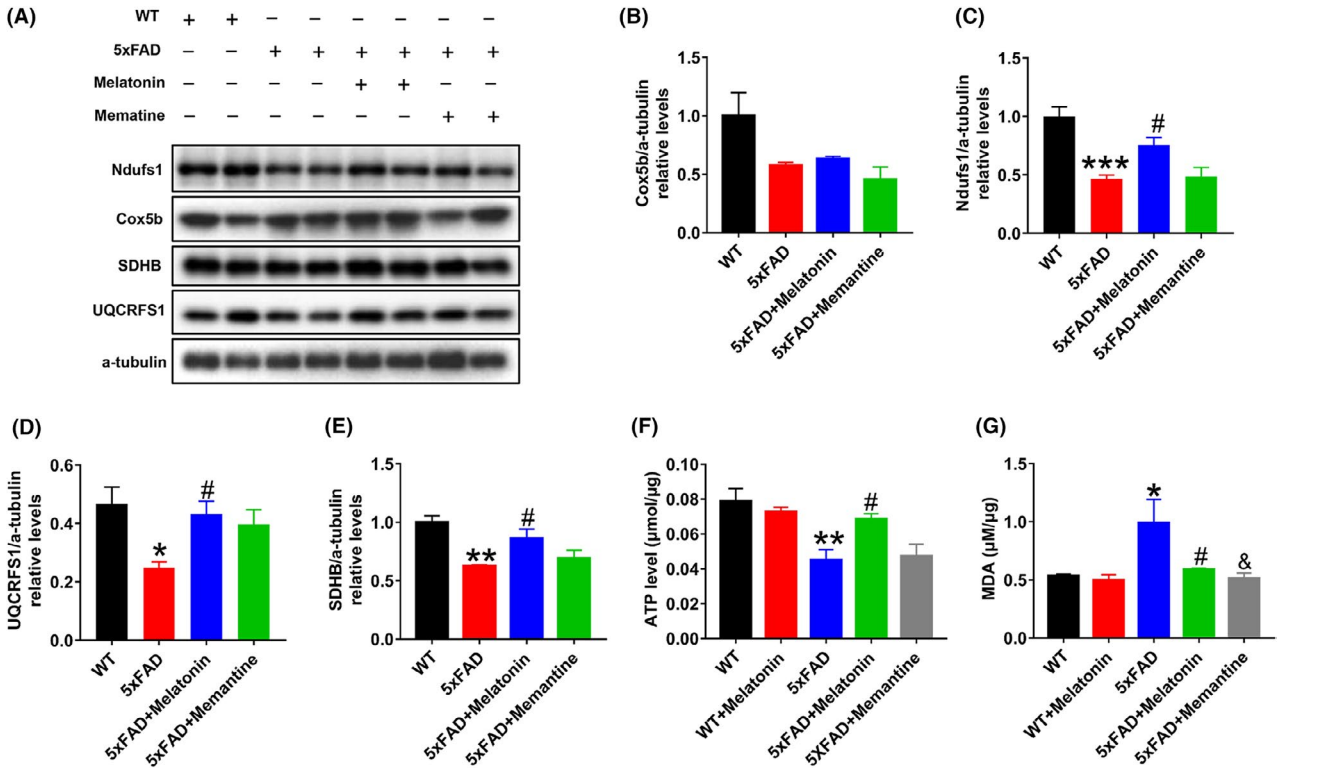

FIGURE 5 Melatonin improved mitochondrial function in 5 × FAD mice. (A–E) The expression level of electron transport chain proteins (Ndufs1, SDHB, Cox5b, and UQCRFS1) detected by Western blotting and quantitative analysis. (F, G) Melatonin treatment increased the ATP level and decreased MDA production in the hippocampus of 5 × FAD mice. Data were shown as Mean ± SEM. *$p < .05$, **$p < .01$ versus WT vehicle group. #$p < .05$, &$p < .05$ versus 5 × FAD vehicle group. $N = 4$ for each group

of lysosomes, the microglial pathogen phagocytosis pathway, glycolysis and gluconeogenesis, and the accumulation of mitochondria and lysosomes in the hippocampus of 5 × FAD due to an autophagosome and lysosome fusion disorder. Melatonin treatment restored mitophagy by promoting autophagosome and lysosome fusion, and improved mitochondrial function, as indicated by a significantly increased expression of ETC protein, enhanced ATP level, and decreased MDA production, which were consistent with previous reports that melatonin restored mitochondrial functions in the brain of several animal models.[24,36,37] Long-term melatonin administration protected mitochondria from aging.[24] Dragicevic et al. also reported that melatonin treatment decreased mitochondrial Aβ levels accompanied by a near complete restoration of mitochondrial respiratory rates, membrane potential, and ATP levels in isolated mitochondria from the hippocampus, cortex, or striatum in AD mice. They also demonstrated direct melatonin receptor involvement in the mitochondrial effects of melatonin in vitro.[37] In addition, hippocampal Aβ pathology and associated cognitive decline in 5 × FAD mice were also attenuated by melatonin treatment. Many studies also reported that melatonin treatment decreased Aβ levels and improved cognitive performance of AD mice.[38,39] For example, melatonin's cognitive benefits involved its anti-Aβ aggregation, anti-inflammation, etc.[39] By genetic deletion of MTNRs, melatonin's positive effects on behaviors of $A\beta PP^{swe}$/PSEN1dE9 mouse model of AD were considered to be receptor dependent in the case of spatial learning and memory such as the Morris water maze and Barnes maze, but receptor independent in the case of nonspatial cognitive performance such as the object recognition test.[38] The action of melatonin was mediated not only by MTNR but also by the brain serotoninergic system. Systemic administration of melatonin (30–120 mg/kg, i.p.) caused a decrease in hypothalamic serotonin (5-HT) release in rats, and potentiated the 5-HT_{1A} receptor activation in the hypothalamus.[40] Although the same or higher dose melatonin than 10 mg/kg had shown protective effects in neurodegenerative diseases,[19,25,41,42] as serotonin receptors are associated with emotional and cognitive deficits,[43] the side effects of serotonin receptors in cognition and mitochondrial functions improved by melatonin should be investigated in the future study.

The hippocampus of 5 × FAD mice showed increased expression of lysosomal marker proteins (Lamp1, CTSD, Hexb, Hexa, etc.) and of mitochondrial proteins (Hk2,

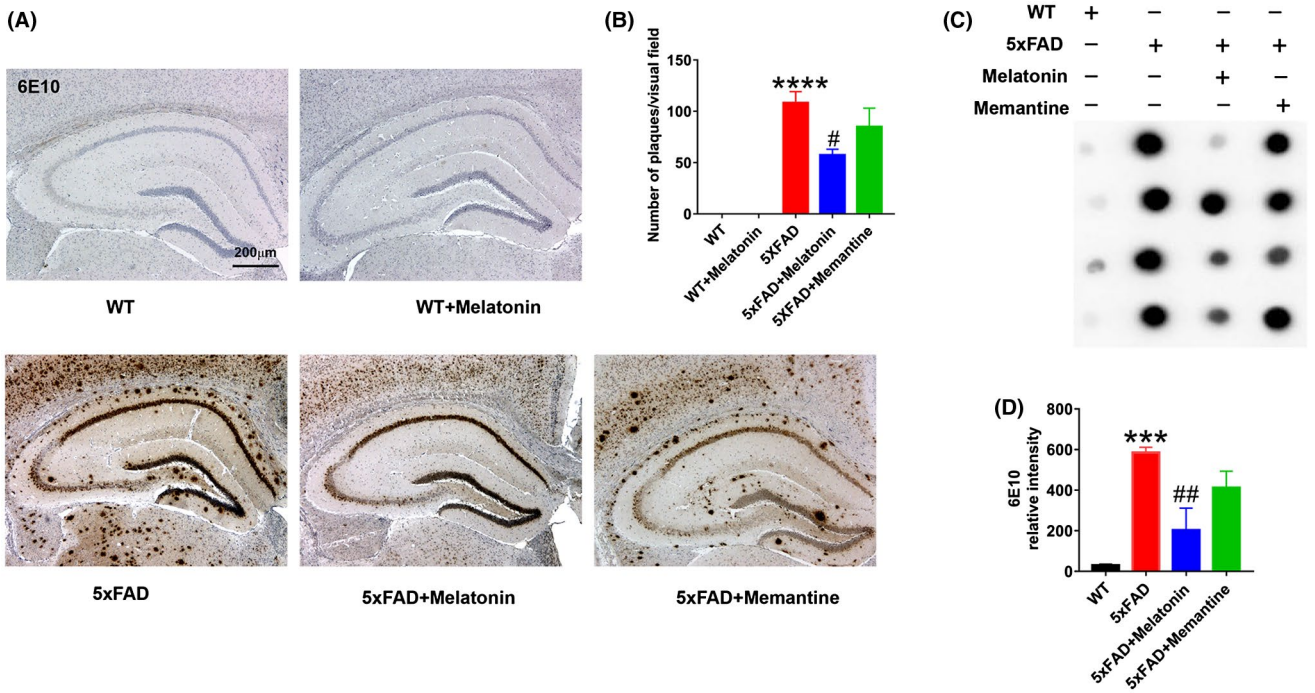

FIGURE 6 Melatonin treatment attenuated Aβ pathology in 5 × FAD mice. (A, B) Immunohistochemical staining of 6E10 in the hippocampus of 5 × FAD mice (A), and amyloid plaque quantification (B). (C, D) The dot blots were used to detect Aβ level and quantitative analysis. Data were shown as Mean ± SEM. ***$p < .001$, ****$p < .0001$ versus WT vehicle group. #$p < .05$, ##$p < .01$ versus 5 × FAD vehicle group. $N = 4$ for each group

VDAC1, etc.), which indicated the accumulation of lysosomes and mitochondria in 5 × FAD mice. By volcano plot analysis, most DE proteins were involved in mitophagy and the autophagy–lysosome pathway. LC3b and p62 levels increased, while ATG5 and ATG7 levels were unchanged, which suggested that the autophagy/mitophagy pathway was impaired but that initiation of auto/mitophagy was normal. Greater co-localization of autophagosomes and mitochondria and less co-localization of mitochondria and lysosomes in 5 × FAD mice further confirmed that the fusion of auto/mitophagosomes and lysosomes was disordered. This finding was consistent with previous researches, which reported autophagy–lysosomal fusion dysfunction in the brains of AD patients and mice models thereof.[44,45]

As it is selectively taken up by mitochondria, melatonin is thought to have major therapeutic potential for neurodegenerative diseases. Melatonin attenuated cognitive deficits of AD models with several different mechanisms, such as: decreasing Aβ aggregation; reduction in tau hyperphosphorylation; regulation of neuroinflammation and calcium homeostasis; and promotion of neurogenesis, among others.[46] In the present study, a 1-month course of oral melatonin remodulated the protein expression of lysosome and mitochondria in 5 × FAD mice, up-regulated autophagy-associated proteins, and induced less co-localization of autophagosome and mitochondria and more co-localization of mitochondria and lysosomes. Together, these observations suggest that, in 4- to 5-month-old 5 × FAD mice, melatonin ameliorated mitophagy deficits by promoting the fusion of auto/mitophagosome and lysosomes. Mcoln1, which is involved in many lysosome-dependent cellular events, including the fusion and trafficking of discarded organelles and autophagy, was the only protein that showed reduced expression in the hippocampus of 5 × FAD mice and increased expression in melatonin-treated 5 × FAD mice, thereby suggesting this protein has an important role in promoting mitophagy by melatonin. CQ, an inhibitor of the fusion of auto/mitophagosome and lysosome (A/M + L), reversed the ameliorated accumulation of mitochondria and lysosome plus the improved mitochondrial function and cognition, indicating that melatonin indeed arrested the normal downhill course of 5 × FAD mouse brain function by promoting organelle (A/M + L) fusion.

The AD brain has impaired neuronal mitochondrial function and reduced activity of mitochondrial proteins involved in TCA cycle.[47] Mitochondrial proteomic analysis revealed that melatonin treatment reversed the reduced expression of proteins enriched in the electron transport chain and in glycolysis and gluconeogenesis; concurrently, ATP production increased while the MDA level decreased. Additionally, in the 5 × FAD mouse brain, there was increased expression

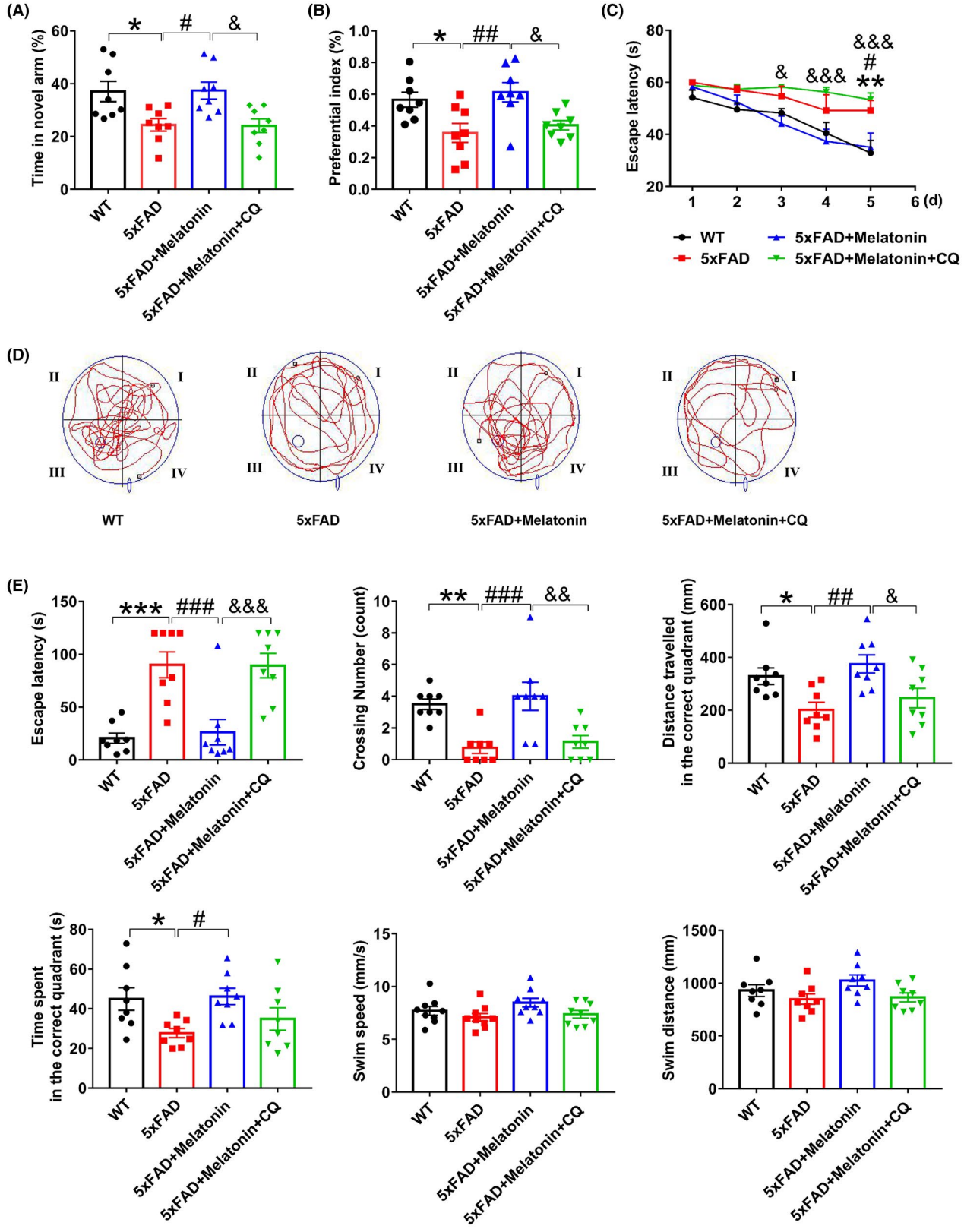

FIGURE 7 Chloroquine reversed the cognitive improvement of 5 × FAD mice induced by melatonin. Behavioral measures of 4-month-old 5 × FAD mice treated with chloroquine (CQ) and melatonin for 1 month. (A) Time spent in the novel arm of the Y maze; (B) the preferential index of the NOR test; (C) the escape latency during the training stage of the MWM test; (D) representative movement trajectories of mice during the test stage of the MWM test; (E) the escape latency, number of platform crossings, distance traveled in the correct quadrant, time spent in the correct quadrant, swim speed, and swim distance during the test stage. Data were expressed as Mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$ versus WT vehicle group. #$p < .05$, ##$p < .01$, ###$p < .001$ versus 5 × FAD vehicle group. &$p < .05$, &&$p < .01$, &&&$p < .001$ versus melatonin-treated 5 × FAD group. $N = 8$ for each group

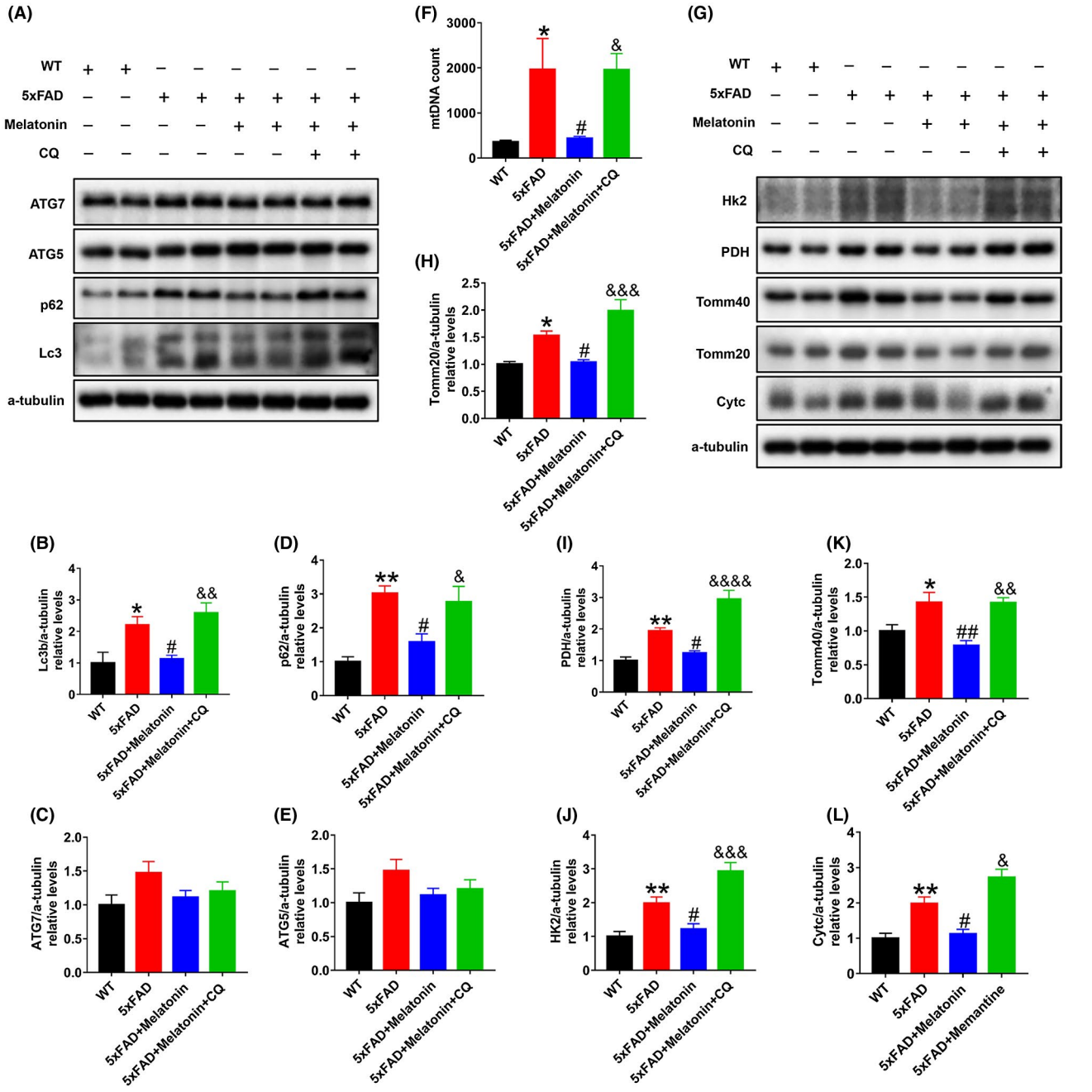

FIGURE 8 Chloroquine reversed the removal of accumulated mitochondria induced by melatonin. (A–E) The expression level of autophagy-related proteins (ATG7, ATG5, p62, and LC3b) detected by Western blotting and quantitative analysis. (F) The relative mtDNA copy number detected by qPCR. (G–L) The expression level of proteins of the mitochondrial outer membrane (Hk2, Tomm40, and Tomm20), mitochondrial inner membrane (Cytc), or mitochondrial matrix (PDH) detected by Western blotting and quantitative analysis. Data were shown as Mean ± SEM. *$p < .05$, **$p < .01$ versus WT vehicle group. #$p < .05$, ##$p < .01$ versus 5 × FAD vehicle group. &$p < .05$, &&$p < .01$, &&&$p < .001$, &&&&$p < .0001$ versus melatonin-treated 5 × FAD group. $N = 4$ for each group

of proteins involved in the microglia pathogen phagocytosis pathway that might arise from dysfunctional lysosomes and defective autophagy induced by Aβ accumulation.

Memantine, a noncompetitive NMDA receptor antagonist, also improved cognitive impairments of 5 × FAD mice in the absence of any evidence of attenuated auto/mitophagy deficits or clearance of Aβ deposition. Melatonin also had no influence on auto/mitophagy and mitochondrial function in the hippocampus of WT mice. Further research is needed to understand these observations.

The melatonin substitution may very well shift the phase of the mice circadian activity profile, and thus,

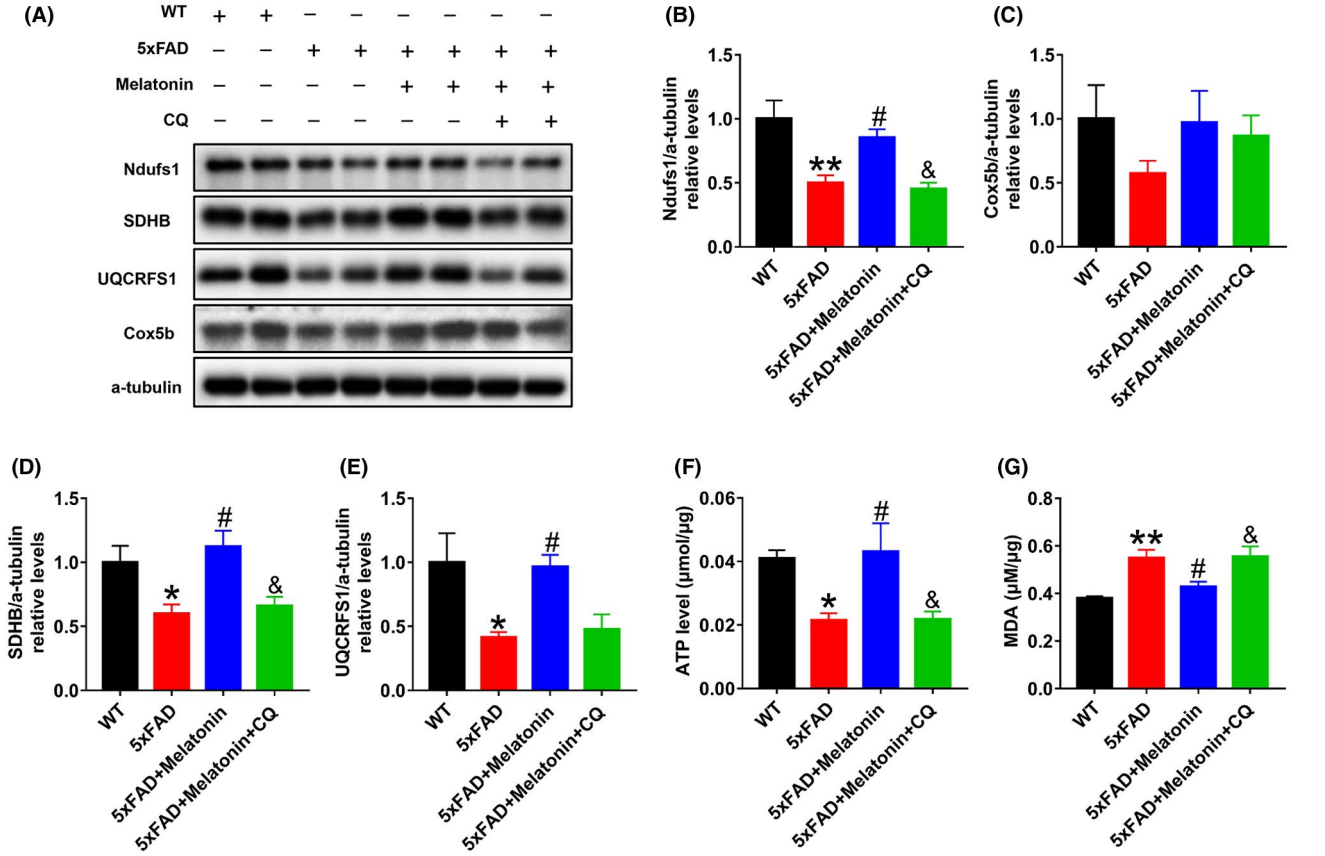

FIGURE 9 Chloroquine suppressed the improvement of mitochondrial function by melatonin. (A–E) The expression level of Ndufs1, SDHB, Cox5b, and UQCRFS1 detected by Western blotting and quantitative analysis. (F, G) ATP level and MDA production. Data were shown as Mean ± SEM. *$p < .05$, **$p < .01$ versus WT vehicle group. #$p < .05$ versus 5 × FAD vehicle group. &$p < .05$ versus melatonin-treated 5 × FAD group. $N = 4$ for each group

change protein expression profiles. Many studies in mice suggest that the circadian system regulates thousands of protein-coding genes,[48,49] and up to half of these proteins are regulated in an organ-specific manner.[48] Disordered circadian rhythm caused changes in human plasma proteomics.[50] Melatonin treatment was reported to regulate genes controlling the cell cycle, cell/organism defense, protein transport, and also increase the expression of some mitochondrial genes.[51] In the present study, Melatonin treatment did not alter diurnal pattern of behavioral activity detected by open field test. There are different results about the activity pattern of animals by exogenous administration of melatonin. Five-month-old mice were treated with melatonin for 10 months, and displayed an increase in locomotor activity.[52] However, other study demonstrated that chronic melatonin treatment (0.5 mg per day for 3.5~4 months) had no effect on the free-running circadian period or entrainment capacity in AD mice and wild-type control mice.[39] Andrea Corrales also reported that chronic melatonin treatment does not cause noncognitive behavioral (spontaneous activity) side effects in Ts65Dn mice.[53] Acute and chronic dosing of ≤5 mg melatonin produced mild, transient sedative effects; nevertheless, doses of ≥10 mg/kg did not cause sustained sedative effects.[54] The reasons for these different conclusions about the activity pattern of animals induced by melatonin treatment may be due to species, animal's age, drug dose, duration of administration, etc. We also searched all the DE proteins against the database of Uniprot related to circadian rhythm and locomotor activity. Melatonin treatment induced few proteins changes in the hippocampus of WT mice, which did not contain circadian rhythm and locomotor activity-related proteins. However, among the total 358 DE proteins, only five proteins (Kdm5a, Ntrk2, Ube3a, Thrap3, and Mapk10) were enriched in circadian rhythm biological process (Figure S2, Table S2), and only Mapk10 was involved in locomotor rhythm,[55] in the 5 × FAD control versus melatonin-5 × FAD mice. In fact, it was quite difficult to distinguish the change in protein expressions induced by the change in circadian activity alone, as the activity change also was induced by melatonin substitution. And Mapk10 also

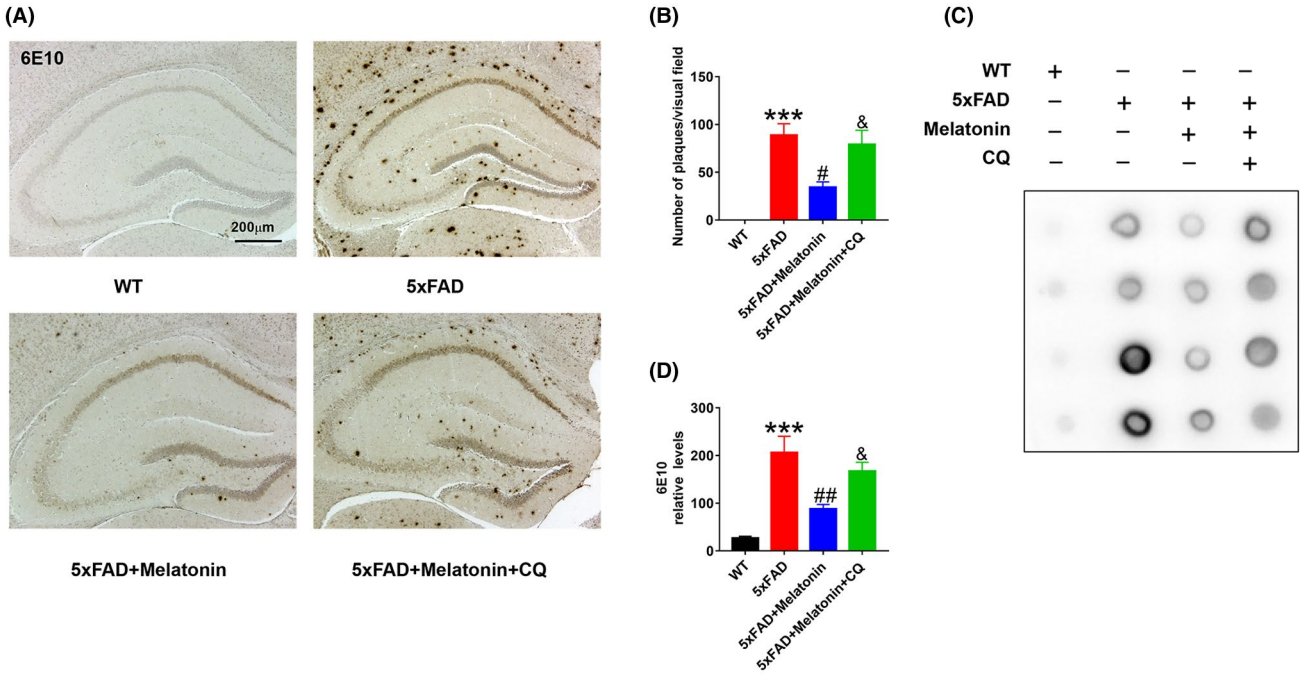

FIGURE 10 Chloroquine reversed Aβ pathology in the hippocampus of melatonin-treated 5 × FAD mice. (A, B) Immunohistochemical staining of 6E10 in the hippocampus of melatonin-treated 5 × FAD mice after CQ treatment (A), and the number of plaques quantified (B). (C, D) The dot blots were used to detect the Aβ level of the hippocampus of CQ treated with melatonin-treated 5 × FAD mice. Data were expressed as Mean ± SEM. ***$p < .001$ versus WT vehicle group. #$p < .05$, ##$p < .01$ versus 5 × FAD vehicle group. &$p < .05$ versus melatonin-treated 5 × FAD group. $N = 4$ for each group.

involved in many other biological processes including intracellular signal transduction, protein phosphorylation, neurite growth in spiral ganglion neurons, and so on.[55,56] All these suggested that the proteomic profiles were mainly induced by melatonin.

In summary, we report for the first time that melatonin attenuated mitophagy deficits by promoting the fusion of mitophagosomes and lysosomes in the hippocampus of 5 × FAD mice. Melatonin reduced cognitive impairments and ameliorated mitochondrial dysfunction and decreased Aβ deposition in the brain of this reliable mouse model of AD (Figure S8).

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CONFLICT OF INTEREST

All authors claimed no conflicts of interest.

AUTHOR CONTRIBUTIONS

CYC drafted the manuscript and performed the experiments. CY analyzed the data. JW, XH, HTY, and SML helped with behavioral test. SPL, ZJZ, JJL, XFY, and GPL designed the study and analyzed the data. XFY and GPL revised the manuscript.

ETHICAL APPROVAL

All the animal experiments and manipulation were supported by the Ethics Committee of the Shenzhen Center for Disease Control and Prevention.

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SUPPORTING INFORMATION

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