Ketogenic diet regulates Uch-L1(C) to improve cerebral energy metabolism and cognitive function in Alzheimer's disease mice

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ABSTRACT

The ketogenic diet (KD), a high-fat, low-carbohydrate diet, can effectively regulate energy metabolism in the brain. The regulation of cerebral energy metabolism in patients with Alzheimer's disease (AD) has attracted the attention of researchers. Recent studies have shown that ubiquitin carboxyl terminal hydrolase L1 (Uch-L1) deficiency leads to neurodegeneration by increasing energy demand and endoplasmic reticulum stress. However, the effect of Uch-L1 on AD remains to be explored. This study first combined Uch-L1 with cerebral energy metabolism to explore its role in long-term KD in AD. We found that AD mice with long-term KD showed better spatial recognition and working memory. KD promoted Uch-L1(C) and Mfn2 expression by inhibiting oxidative stress in the hippocampus of mice, improved mitochondrial function, increased ATP content, and significantly reduced neuronal apoptosis. In conclusion, KD can increase Uch-L1(C) and Mfn2 expression in the brain, and improve cerebral energy metabolism and cognitive function in AD mice.

Key words: ketogenic diet; Alzheimer's disease; oxidative stress; Uch-L1; cognition; energy metabolism.

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Introduction

Alzheimer's disease (AD) is a common group of progressive and fatal neurodegenerative diseases in older population characterized by the accumulation of amyloid β-protein in the form of extracellular plaques and intracellular neurofibrillary tangles.[1] In addition, it was found that there were obvious energy metabolism in the hippocampus, occipital visual area, posterior cingulate cortex and other brain areas of AD patients by means of fluorodeoxyglucose positron emission tomography (FDG-PET).[2] The regulation of cerebral energy metabolism in AD patients has attracted the attention of researchers.

In aging neurodegenerative diseases, brain glucose metabolism is depleted in a progressive, region-specific and disease-specific manner at the beginning of the disease, which is the most typical pathological feature of AD.[3] Mitochondria damage in neurons leads to energy metabolism disturbance, which is a mark of neurodegenerative diseases including AD.[4] Studies have shown that mitochondria are essential for the brain to provide sustained and effective energy supply, so maintaining mitochondrial homeostasis can promote the formation and progression of AD.[5]

Ketogenic diet (KD), a high-fat, low-carbohydrate diet (carbohydrate content within 5%), provides ketone bodies instead of glucose as energy substrates for human in cases of meeting nutritional requirements. KD can induce and sustain a state of chronic ketosis, so that the body mainly uses ketone bodies instead of glucose to supply energy and alleviate the impact of impaired glucose metabolism.[6] Pavón *et al*.[7] concluded that ketone body metabolites increase the score of the AD Assessment Scale, and KD-induced ketosis may alleviate the damage in AD or improve cognition.[7,8] It has been suggested that ketogenic diet provides energy in the form of ketone bodies and fatty acids rather than glucose, and plays a protective role in brain neurons in neurodegenerative diseases, nerve damage and epilepsy by regulating oxidative stress, neuroinflammation, insulin receptor sensitivity and synaptic plasticity.[9-11] The latest study found that KD can lead to ketone metabolism to improve mitochondrial function, reduce the regulator that mediates apoptosis, oxidative stress and inflammation, and increase available energy.[12,13] Thus, it shows the potential to maintain homeostasis of neural networks and improve brain functioning.

Ubiquitin carboxyl terminal hydrolase L1 (Uch-L1) is a deubiquitinase that is highly expressed in neurons (accounting for 1-5% of the total neuroproteins).[14] Previous studies have shown that Uch-L1(C) functions as a key soluble de-ubiquitinase in the cytoplasm, playing a role in maintaining neuronal homeostasis and alleviating oxidative damage. However, the membrane-bound Uch-L1(M) lacks these protective functions and is more associated with the pathology of Parkinson's disease. Therefore, Uch-L1 is gradually becoming a diagnostic and therapeutic target for neurodegenerative diseases and nerve injuries.[15] In an animal experiment, researchers found that inhibition of Uch-L1 activity led to memory and environmental regulation dysfunction in mice.[16] After protein profiling of the frontal cortex from AD donors, Chen J, etc. had found that the level of Uch-L1in the cytoplasm of patients with AD was significantly decreased, indicating the important intervention effect of Uch-L1 on AD.[17] Follow-up studies have shown that Uch-L1 deficiency leads to accelerate protein renewal in the early stage, which leads to neurodegeneration by increasing energy demand and endoplasmic reticulum stress. Recent clinical cohort studies further support this, demonstrating that variations in serum Uch-L1 levels correlate significantly with cognitive decline severity in AD patients, reinforcing its viability as a reliable diagnostic and prognostic tool.[18] However, the function and molecular mechanism are still in the preliminary exploration stages.

Butterfield DA asserts that the changes of oxidation and nitrosation of Uch-L1 are the primary targets to the mechanism of neuronal death in AD and its early mild cognitive impairment (MCI).[19] After oxidation and nitrosation modification, Uch-L1 function is destroyed, thus reducing the expression level of Uch-L1 in AD brain. As oxidative stress can affect the morphology and function of Uch-L1 *in vivo*, the cysteine residue 152 (Cys^{152}) of Uch-L1, as an antioxidant, binds to H_2O_2 to maintain its protein spatial conformational stability and deubiquitinase activity under a certain intensity of oxidative stress.[20] If the oxidative stress is too strong, the Cys^{152} of Uch-L1 can transmit -NO, and finally transfer -NO to mitochondrial dynamic related proteins (Drp1) to cause its excessive activation, leading to mitochondrial breakage.[21] It should be noted that the C-terminal of Uch-L1 plays a key role in protein stability. As is well known, Uch-L1 exists in neurons in two forms: one is soluble in the cytoplasm (Uch-L1 (C)) and the other is bound to the cell membrane after farnesylation of UCH-L1 at Cys220 (Uch-L1 (M)).[22] The level of Uch-L1 (C) decreased in the brain of patients with AD, while Uch-L1 (M) is not only unable to stabilize proteins, but also related to the occurrence and development of Parkinson's disease (PD) (α-synuclein dysfunction).[23] This suggests that Uch-L1 (C) is the effective form of Uch-L1 to play key role of deubiquitinase.

Gegg *et al*. found that the mitochondrial fusion protein (Mitofusin-2, Mfn2) connects ubiquitin monomer at site K48 that could be removed by Uch-L1.[24] In addition, previous studies have shown that the knockdown of the corresponding Uch-L1 homolog will reduce the level of Mfn2, indicating that Mfn2 is directly affected by Uch-L1[25], thus confirming that Uch-L1 can act as a deubiquitinase for Mfn2.

Since ketogenic diet can improve oxidative stress in the body, and oxidative stress does play a significant role in the morphology and function of Uch-L1 (C) *in vivo*, Uch-l1 (C) regulates mitochondrial morphology and function by regulating mitochondrial Mfn2, which is one of the feasible pathways to regulate neuronal energy metabolism. Therefore, we hypothesized that Uch-L1 (C) is also involved in the pathway of increasing nervous system energy metabolism by ketogenic diet in AD. In order to test our hypothesis, we analyzed the cognitive behavioral performance and oxidative stress status of brain tissue, mitochondrial morphology and function, Uch-L1 (C) and Mfn2 related protein expression of transgenic mice in AD model after long-term KD intervention.

Materials and Methods

Animals

The experimental animals used included 6-week-old male APP/PS1 transgenic mice and wild-type (WT) C57BL/6 control mice matched with their genetic background, which were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. In this model, the mice are based on the C57BL/6 background that is consistent with the wild-type control, and the Alzheimer's disease-related phenotypes (such as Ab lipid deposition and cognitive impairment) typically begin to manifest at 6 months of age. To reduce the effects of hormonal and sex differences on the results, we only used male mice in this study. Mice were housed in the Laboratory Animal Center of North Sichuan Medical College according to a protocol approved by the Animal Care and Use Committee of North Sichuan Medical College, with a light/dark cycle of 12/12 h and free access to water. After one week of adap-

tation, the amount of feed required by random diet of mice per day was measured, and the mice were divided into groups and treated with diet intervention after one week: mice in WT group and APP/PS1 transgenic mice in standard diet group (APP con) received standard diet (SD) every day, while APP/PS1 transgenic mice in KD group (APP KD) received ketogenic diet with the same calories as normal diet every day. The ketogenic diet feed was provided by the Experimental Animal Center of the North Sichuan Medical College. The diet consists of approximately 75% fat, 15% protein, and 5% carbohydrates by weight. The dietary intervention lasted for 40 weeks. Behavioral trials including Y-Maze test and Morris Water Maze test (MWM) were conducted after the dietary intervention. Besides, the mice in APP KD group continued to be fed ketogenic diet after 40 weeks. The mice were then euthanized by injection of sodium pentobarbital, and the brains were harvested.

Y-maze test

The Y-maze alternating test was performed to evaluate the working memory performance. As earlier described,[26] the Y-maze device consists of three arms (one starting arm and two target arms) of 30 × 10 × 20 cm connected by an intersection. At the beginning of the test, each mouse was placed at the end of a center-facing maze arm and was allowed to explore the maze for 10 minutes. The entry of arms was recorded by the observers who did not know the experimental group in front of the camera screen. If the mice did not enter the new arm within 2 min or entered the number of arms less than 12 times during the 10-min exploration, they were excluded from the analysis. The number of mice that were excluded due to insufficient exploration of the arm area (n=3 for the WT group, and n=6 for the APP con group). Successful alternation is defined as entering a new arm continuously before returning to the two previously visited arm, and the maximum number of alternations is equal to the total number of arm entries - 2. Percentage of alternation = number of alternations / (total number of arm entries - 2).

Morris water maze test

The Morris water maze test was performed to evaluate spatial memory performance. As previously mentioned,[27] several references with different black patterns were painted on the walls of the water maze, and the water was dyed white with a non-toxic dye to facilitate the camera system to identify black mice. The opaque platform with a diameter of 10 cm was located 1 cm below the surface of the water. The entire experiment lasted for six days. During the first five training days, mice were placed in the maze and swam freely until they found a hidden platform for up to 60 s, and the mice completed four trails a day with an interval of at least 20 min between the two training sessions so that the mice can have a full rest. If the mice fail to reach the platform within 60 s, they will be gently guided to the platform and stay there for 15 s. The latency and average swimming speed of finding the platform were recorded. On the 6th day, the free exploration experiment was carried out with an interval of at least 5 h. The platform was removed from the pool and the mice searched the platform for 180 s. The number and time of passing through the platform area and the time spent in the platform and contralateral quadrant was recorded and analyzed by using the computer video imaging analysis system (Guangzhou enclave).

Weight, blood glucose and blood ketone

The mice were weighed on a scale and then their weights were read. Mice were fixed with a small animal holder, the tails were fully exposed, and the distal tail was repeatedly wiped with 75% alcohol cotton balls and left to dry. The scissors cut the tail tip 1mm and saw blood flow from the broken end. One drop of whole blood was added to blood glucose or blood ketone strip, and blood glucose and blood β-hydroxybutyrate were tested by blood glucose / blood ketone detector. After blood collection, the tail was disinfected with 75% alcohol cotton balls, and the dry cotton ball compressed the broken end to stop bleeding.

Western blotting

Proteins were extracted from the hippocampus and cortex stored in the refrigerator at -80°C and separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the proteins were transferred to nitrocellulose membrane. After successful transfer, the TBST buffer was gently shaken and washed for 3 times, and the nitrocellulose membrane was sealed with TBST buffer containing 5% skimmed milk powder for 1 h. After sealing, rinsed and incubated with primary antibody (1:1000) at 4°C overnight, and then incubated them with secondary antibody (1:1000) at 37°C for 1 h. Finally, the bands were exposed after washing with TBST buffer for 3 times. Using β-actin as the control band (1:1000), the value used for statistical analysis is expressed as the ratio of the gray value of each protein band to its upper sample. The related primary antibodies used in this study are listed in Table 1.

Transmission electron microscopy

The hippocampus CA1 was immediately placed in 2.5% glutaraldehyde electron microscope fixed solution (TED PELLA) diluted by PBS and fixed at room temperature for 1 h. The hippocampus area (particles whose side length was smaller than 2 mm) was dehydrated and embedded, and the images were performed on transmission electron microscope (TEM) (Hitachi, HT7700). The morphology of mitochondria in the image was mea-

Table 1. The related primary antibodies used in this study.

Name	Manufacturer	Identification	Dilution
Uch-L1	R&D	MAB60072	1:200 (IF) 1:1000 (WB)
Mfn2	Abcam	ab124773	1:200 (IF) 1:500 (WB)
8-OHdG	Abcam	ab183393	1:200
4-HNE	Stressmarq	SMC-511D	1:200 (IF) 1:1000 (WB)
β-actin	Beyotime	AF5003	1:5000
DAPI	Servicebio	G1012	1:20000

sured by using ImageJ software. First, trace both the outer mitochondrial membrane and the ER membrane. Next, to obtain ER-mitochondria interface length, draw a line with the length of the contact site, and then use ROI Manager to measure the length. To obtain ER-mitochondria contact distance, draw a line between the two organelles and measure it.[28] All the statistical analyses were conducted on the pyramidal neurons in the CA1 region of the hippocampus.

ATP assay

ATP assay kit (FF2000; Promega, Madison, WI, USA) was used to analyze the concentration of ATP in mouse brain. First, the frozen hippocampal and cortical tissues were crushed with a grinder by adding boiled double distilled water at 1:10 by weight, in which 0.5% TCA was added as a lytic. After 10 min of boiling water bath, the supernatant was centrifuged (3500 r/min, 10 min), and then added with Tris buffer to adjust the PH value to an appropriate value (PH7.75). Finally, after adding 100 µL sample or standard solution with proper solubility to the opaque white 96-well plate, the fluorescence reaction was carried out immediately and detect the fluorescence intensity by spectrophotometer (SpectraMax Paradigm; Molecular Devices, Cardiff, UK).

Immunostaining

In order to evaluate the morphology and apoptosis of nerve cells, the paraffin sections of mouse brain (coronal section) were dewaxed and repaired with citrate repair solution (ZLI-9065; Beijing Zhongshan Jinqiao, Beijing, China), and then sealed with goat serum for 20 min. Then, incubated with primary antibody as described in Table 1, at 4°C overnight, then incubated with the secondary antibody (AP-9001/9002; Beijing Zhongshan Jinqiao,) at 37°C for 30 min, and finally developed color with concentrated DAB kit (K135925C; Beijing Zhongshan Jinqiao). TdT-mediated dUTP Nick-End Labeling (TUNEL) (49330900; Roche, Basel, Switzerland) was used to detect apoptosis of nerve cells. OlyVIA software was used for image acquisition and processing, and ImageJ software was used to quantify nuclear co-localization labeling and quantitative analysis of apoptotic cells. The related primary antibodies used in this study are listed in Table 1.

Statistical analysis

Statistical analysis was performed by Statistic Package for Social Science (SPSS) 23 statistical software (IBM, Armonk, NY, USA). Before the intervention, the mice were divided into three groups by digital random method, data normality was assessed using the Shapiro-Wilk test and the differences between different groups were analyzed by one-way analysis of variance followed by Tukey's *post-hoc* test. The results were represented by mean ± SEM, and $p<0.05$ indicated that the difference was statistically significant.

Results

Effects of a 40-week ketogenic diet intervention on body weight, blood glucose, and blood ketone levels in APP/PS1 mice

To verify how ketogenic diet changed the glucose metabolism and the ketone body metabolism in APP/PS1 mice, we observed the body weight, blood glucose and blood ketone of APP/PS1 mice after intervention of ketogenic diet for 40 weeks. As shown in

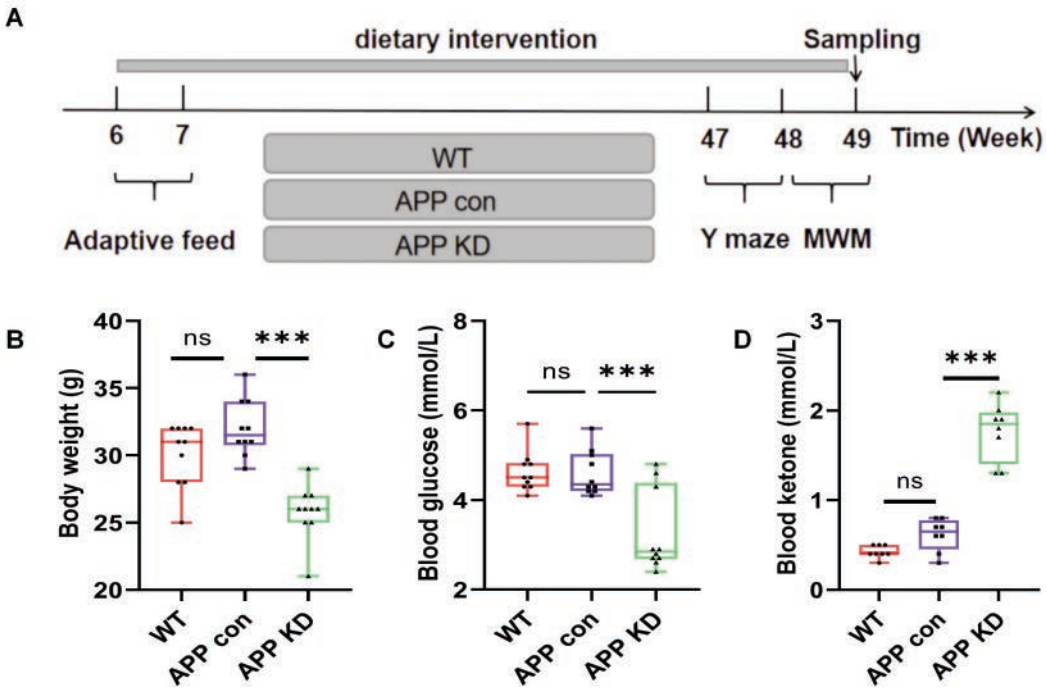

Figure 1. Experimental schematic of dietary intervention and behavioral tests in mice (**A**). Body weight (**B**), blood glucose (**C**) and blood ketone (**D**) value in mice after 40 weeks of dietary intervention. The "box" depicts the median and the 25th and 75th quartiles and the "whisker" shows the 5th nd 95th percentile. n=10, ***$p<0.001$.

Figure 1 A,B, after 40 weeks of diet intervention, the body weight and blood glucose of APP KD group were significantly lower than those of WT and APP con group, while the blood ketone was on the contrary. But there was no significant difference between WT and APP con groups. The results showed that long-term KD could decrease the glucose metabolism and increase the ketone body metabolism in APP/PS1 mice, and significantly reduce the body weight of mice.

Ketogenic diet improves spatial memory and working memory in APP/PS1 mice

To investigate the effect of ketogenic diet intervention on cognitive function in AD mice, the Y-maze test was performed to evaluate the working memory and MWM test was used to detect the spatial memory of AD mice. As can be seen from the MWM (Figure 2 A-G), Compared with WT, AD mice need more time to find the hidden platform in the water, and it is more difficult to locate the hidden platform accurately in free exploration mode. Compared with the APP con, the latent period of searching for the platform of APP KD was significantly shorter, and it was more accurate to locate the hidden platform in free exploration. In order to exclude the influence of motor ability on the results, we performed a statistical analysis of the average speed and total distance traveled during the exploration phase. We found no statistical difference in average speed between the three groups, and the total distance travelled of APP group was significantly greater than that of WT and APP KD group.

Similarly, the APP KD group in the Y-maze showed better working memory than its control group (Figure 2H). These results suggest that long-term ketogenic diet intervention can improve the

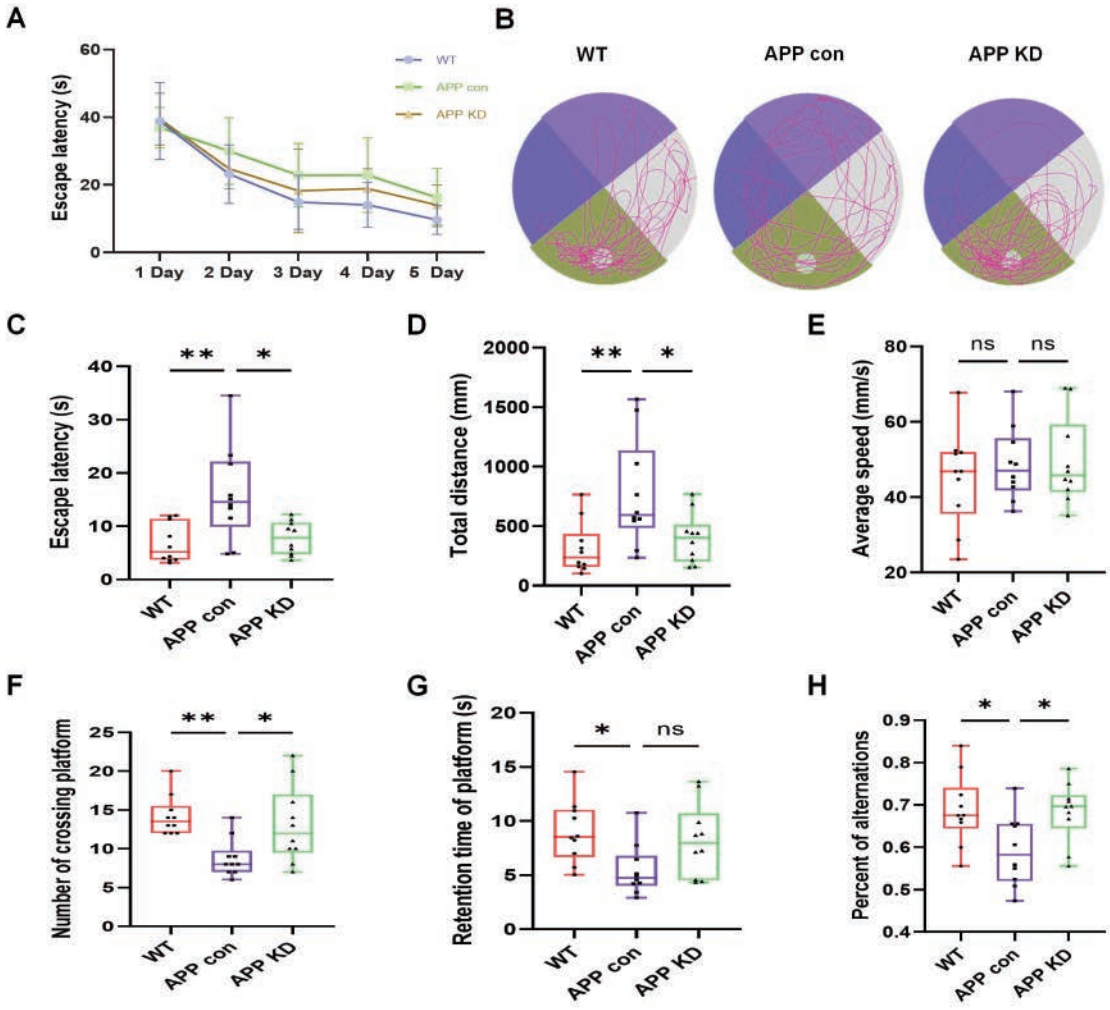

Figure 2. Behavioral data of mice. **A-G**) Behavioral data of mice in Morris Water Maze. Average latency of searching platform every day during training (**A**). Movement trajectory diagram of mice during the exploration phase of water maze (**B**). The escape latency of searching platform during the exploration phase (**C**). Total distance travelled during the exploration phase (**D**). Average swimming speed during the exploration phase (**E**). Times of crossing the platform area during the exploration phase (**F**). Retention time of platform area during the exploration phase (**G**). Percentage of alternations in the Y maze test (**H**). The "box" depicts the median and the 25th and 75th quartiles and the "whisker" shows the 5th and 95th percentile. n=10, *p<0.05, **p<0.01.

cognitive functions such as spatial memory and working memory in AD mice.

Ketogenic diet reduces oxidative stress in the brain of APP/PS1 mice

Based on the enhancement of oxidative stress in AD,[29] in order to study the level of oxidative stress in the brain of mice, we analyzed the expression of 8-OHdG and 4HNE in temporal cortex and hippocampus. Through immunohistochemical fluorescence staining, we found that compared with WT, 8-OHdG and 4HNE of APP con significantly increased in temporal cortex and hippocampus. Compared with APP con, 8-OHdG and 4HNE of APP KD significantly decreased in temporal cortex and hippocampus (Figure 3 A-F). In addition, we also performed Western blot (WB) of 4HNE, which confirmed the immunofluorescence results (Figure 4 A-B,E-F). These results suggest that oxidative stress in the brain of AD mice is significantly higher than that of WT mice, and long-term KD intervention can improve the oxidative stress in the brain of AD mice.

Ketogenic diet increases the expression of Uch-L1(C) and Mfn2 in APP/PS1 mice

Excessive oxidative stress can destroy the function of UCH-L1(C), thereby reducing the expression level of UCH-L1(C) in AD brain. And the expression of Mfn2 is regulated by Uch-L1(C).[25] In order to verify whether KD increases the expression of Uch-L1(C) and Mfn2 in mice, we analyzed the expression of Uch-L1(C) and Mfn2 in temporal cortex and hippocampus by WB. Compared with WT group, the expression level of Uch-L1(C) and Mfn2 in hippocampus in APP con group decreased significantly. In addition,

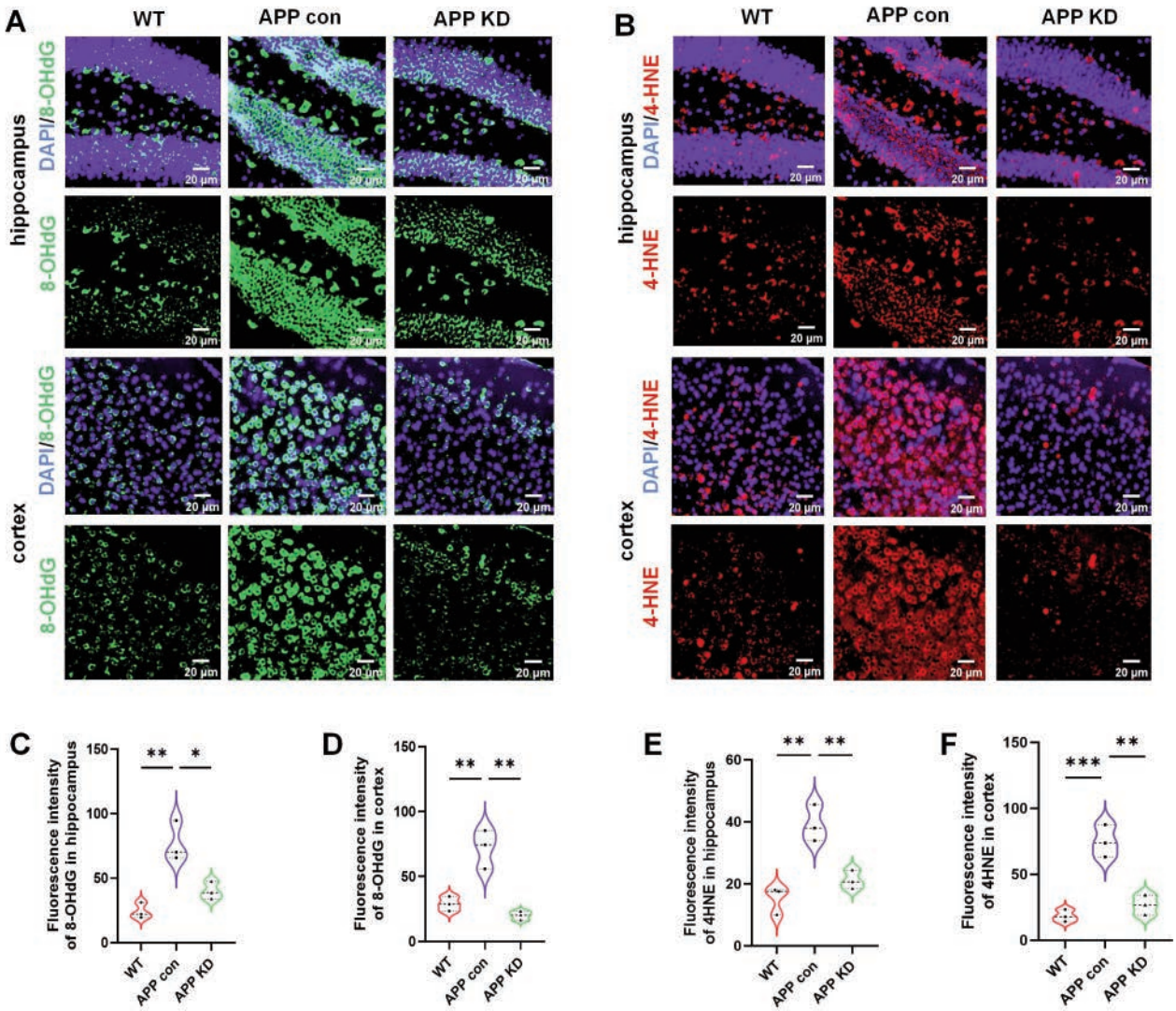

Figure 3. Detection of oxidative stress in the hippocampus and cortex of mice brain. **A,B**) Expression of 8-OHdG and 4-HNE in hippocampus and temporal cortex in different groups examined by immunofluorescence images (n=3). **C,D**) Analysis of fluorescence intensity of 8-OHdG in hippocampus and temporal cortex respectively. **E,F**) Analysis of fluorescence intensity of 4HNE in hippocampus and temporal cortex, respectively. n=3, *p<0.05, **p<0.01, ***p<0.001.

the expression level of Uch-L1(C) and Mfn2 in hippocampus in APP KD group was significantly higher than that in APP con group. Unfortunately, there was no significant difference in temporal cortex among the three groups (Figure 4 C-D,G-J). Subsequently, the expression of Uch-L1(C) and Mfn2 in different brain regions was also analyzed by immunofluorescence staining whose result was consistent with WB (Figure 5 A-F). There was also no significant difference in temporal cortex among the three groups. Besides, we found there existed common labeling of Uch-L1(C) and Mfn2. These results suggest that long-term KD intervention can increase the expression of Uch-L1(C) and Mfn2 in hippocampus in AD mice, and there is interaction between Uch-L1(C) and Mfn2.

Ketogenic diet improves endoplasmic reticulum-mitochondrial cross-linking in APP/PS1 mice

To examine the effect of long-term KD intervention on mitochondrial morphology and function, the mitochondria of hippocampal neurons of three groups of mice were observed by transmission electron microscope (TEM). First, the mitochondria in hippocampal neurons of APP con group were longer than WT group and APP KD group. Given the important role of endoplasmic reticulum (ER)-mitochondria contact in mitochondrial function, we analyzed the ER-mitochondrial contact. Compared with WT group, the distance between ER and mitochondria in hippocampal neurons of APP con group was farther, and the degree of cross-linking was lower (Figure 6 A,C), which was significantly improved in APP KD group. And then we used the ER–mitochondria contact coefficient (ERMICC) to objectively quantify ER-mitochondria cross-linking (Figure 6 B,D).[30] The larger the ERMICC value, the better the ER-mitochondria cross-linking. Since the effective tether length between ER-mitochondrial connection is within 30 nm,[31] we only included the distance within 30nm to calculate the ERMICC. The results show that long-term KD intervention can improve the ER-mitochondrial contact in AD.

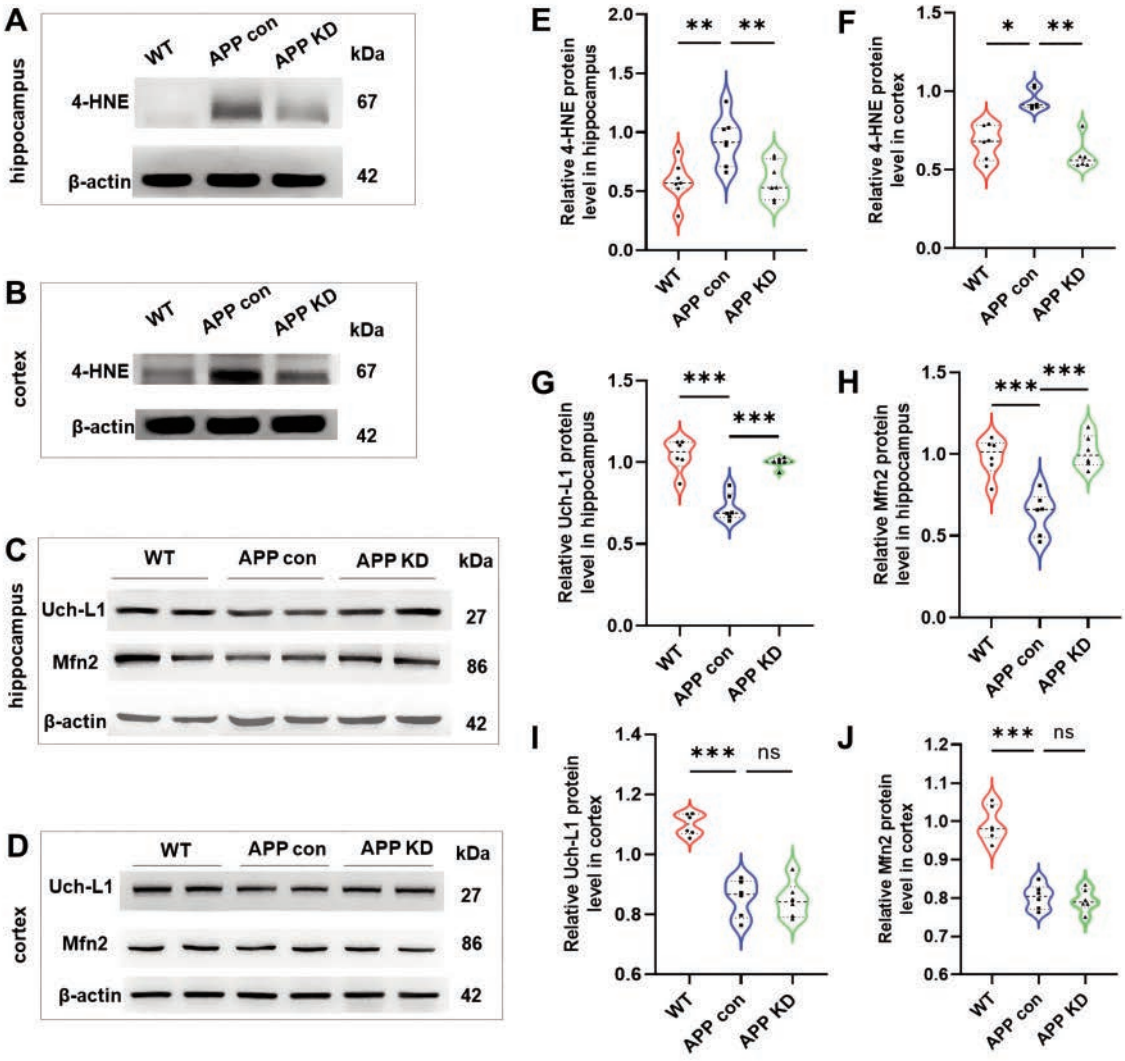

Figure 4. Expression of 4HNE, Uch-L1(C) and Mfn2 in hippocampus and cortex in APP/PS1 mice by Western blotting. Results of Western blotting (**A-D**) and gray value analysis (**E-J**) of 4HNE, Uch-L1(C) and Mfn2, respectively (n=5/group). n=3, *p<0.05, **p<0.01, ***p<0.001.

Ketogenic diet increases ATP energy supply and reduces neuronal apoptosis in the brain of APP/PS1 mice

Brain energy is mainly supplied continuously by ATP, most produced by oxidative phosphorylation in mitochondria, supplemented by anaerobic glycolysis in the cytoplasm.[3] In order to detect the cerebral energy metabolism of mice, we detected the ATP values of cortex and hippocampus of three groups of mice by ELISA (Figure 7 D,E).[32] As expected, the ATP values in the cerebral cortex and hippocampus of mice in the AD con group were significantly lower than WT group, while the brain ATP values in the AD KD group were significantly increased.

To analyze the apoptosis of cerebral neurons in the three groups of mice, we performed fluorescent TUNEL detection on the paraffin sections of the three groups of mouse brain (Figure 7 A-C). Compared with the WT group, there were significantly more apoptotic cells in the cerebral cortex and hippocampus in the APP con group, but fewer apoptotic cells in the APP KD group, which is consistent with the ATP test. These indicate that long-term ketogenic diet intervention can increase cerebral energy metabolism and reduce neuronal apoptosis in AD mice.

Discussion

With the acceleration of the global population aging process, the situation of AD has been not optimistic.[33,34] Although there has been a little progress in conquering AD worldwide, the way to cure it has not been found so far. Therefore, it is urgent to find effective ways to prevent or slow down the process of AD.

In this study, we tested spatial learning ability through Morris water maze and working memory through Y maze. Brownlow *et*

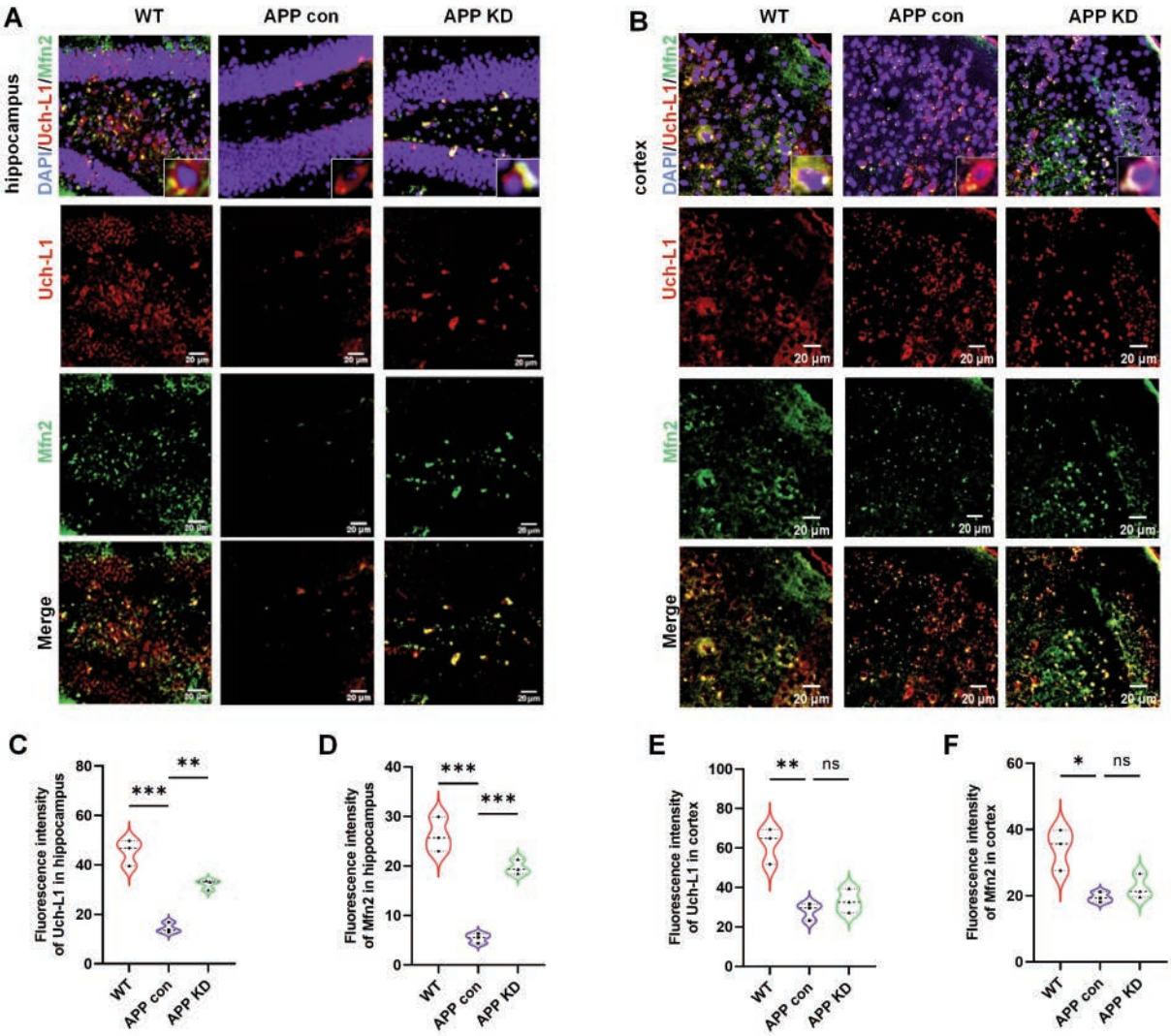

Figure 5. Expression of Uch-L1(C) and Mfn2 in hippocampus and cortex in APP/PS1 mice by immunofluorescence. **A,B**) Expression of Uch-L1(C) and Mfn2 in hippocampus and temporal cortex in different groups examined by immunofluorescence images (n=3). **C-F**) Corresponding data analysis of immunofluorescence analysis. n=3, *p<0.05, **p<0.01, ***p<0.001.

al. found that 4-month KD intervention may enhance motor performance but not cognition in APP/PS1 and Tg4510 mice by accelerating rotarod, open field test, Y-maze and radial arm water maze.[35] Xu *et al*. studied that four months of ketogenic diet could improve spatial learning and working memory in 5XFAD mice by Barns maze and T maze.[36] Next, it may be meaningful to conduct KD intervention in other AD animal models such as 3xTg-AD and Tg2576 mice with more behavioral experiments. In our previous study, it was shown that the ketogenic diet reduces the deposition of Ab.[37] Although previous studies have explored the beneficial effect of KD on AD, for instance, ketogenesis can increase available energy,[1,3] enhance mitochondrial function,[12] prevent amyloid protein from entering nerve cells,[38] regulate intestinal microorganism,[39] and so on, the specific molecular mechanism of enhancing energy metabolism is still blank. In this study, we considered the direct relationship and molecular mechanism between oxidative stress and energy metabolism for the first time.

The result about weight in this study shows that long-term KD intervention can control the weight of aged mice within a stable and healthy range.[40] Studies have shown that obesity and excess weight may be associated with the incidence and progression of AD. The ketogenic diet can reduce body weight, which may reduce inflammatory response and improve insulin sensitivity, thus may have a positive impact on the pathological process of AD.[41] From the results about blood glucose and blood ketones, we can know that after long-term KD intervention, the metabolic pathway in AD mice has been transformed from glucose metabolism to KB metabolism.[42] KB metabolism reduces the production of ROS by increasing mitochondrial uncoupling proteins.[43] The ketogenic diet improves glucose metabolism in the brain by lowering blood glucose levels and reducing insulin secretion. In addition, reducing glucose intake can suppress the inflammatory response and reduce amyloid deposition, and improve cognitive function of AD. However, maintaining moderate blood glucose levels is essential for brain function. In some cases, too low a blood glucose level can lead to cognitive decline and nerve damage. Although long-term ketogenic diet has shown neuroprotective effects, its impact on liver function, electrolyte balance, and intestinal microbial community dysbiosis still requires careful monitoring in future preclinical and clinical studies. Meanwhile, when it comes to the effects of a ketogenic diet on AD gradually, it is necessary to balance blood ketone and blood glucose levels to ensure adequate energy

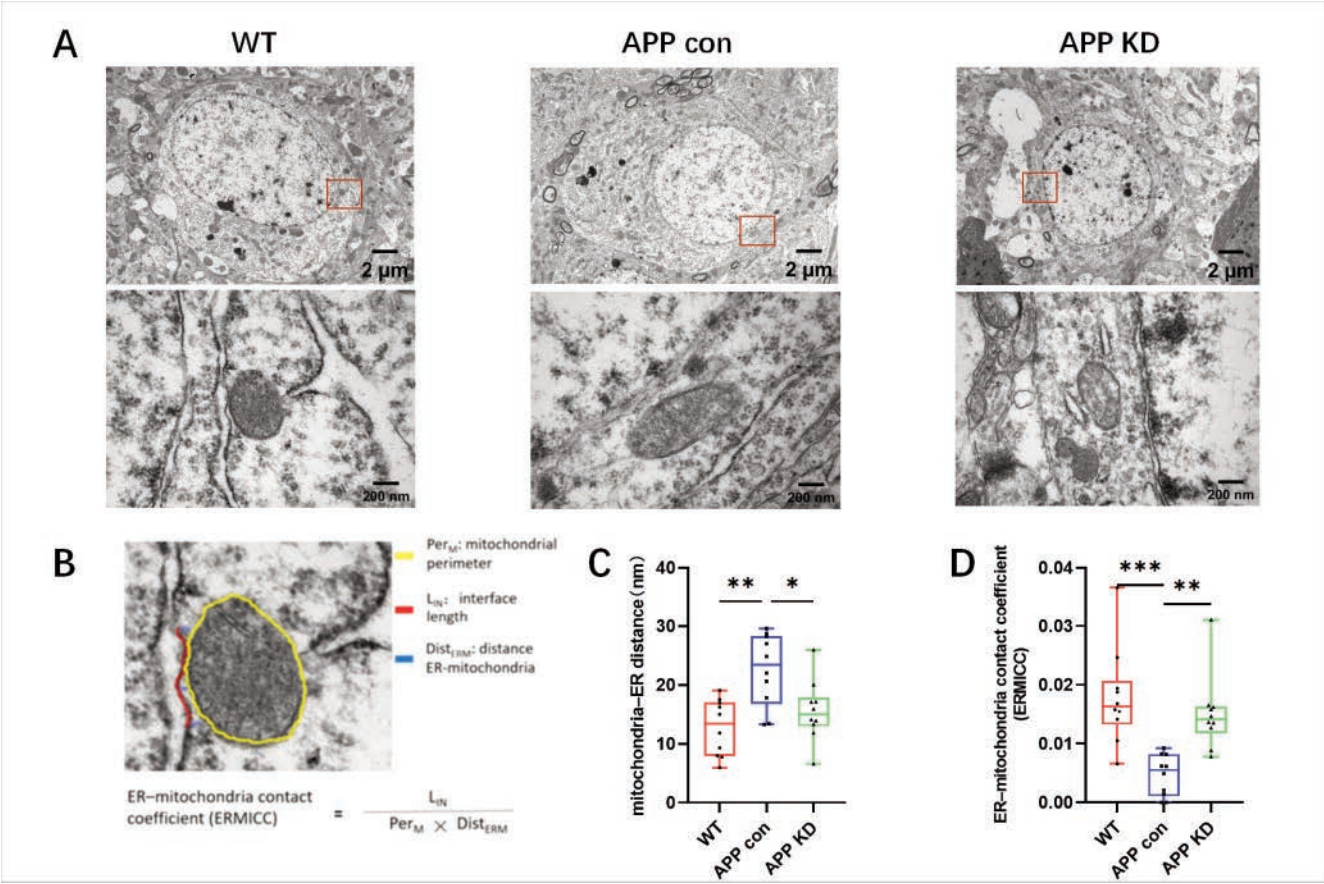

Figure 6. Ketogenic diet increases endoplasmic reticulum-mitochondrial cross-linking in APP/PS1 mice. **A**) Transmission electron microscopy images of hippocampal neurons mitochondria of the three groups (scale bars: 2 μm) and enlarged view of hippocampal neurons mitochondria of the three groups (scale bars: 200 nm). **B**) Description of the ER- mitochondria contact index (ERMICC); scale bars: 200 nm. **C**) Mean ± SEM of mitochondria-ER distance calculated in ten independent experiments as in panel A. **D**) Mean ± SEM of ERMICC calculated from ten independent experiments performed as in panel A. The "box" depicts the median and the 25th and 75th quartiles and the "whisker" shows the 5th and 95th percentile. All the statistical analyses were conducted on the pyramidal neurons in the CA1 region of the hippocampus. n=3, *p<0.05, **p<0.01, ***p<0.001.

supply in brain.[44,45] Therefore, we found two oxidative stress markers (8-OHdG and 4HNE) decreased after long-term KD intervention, confirming that the KB metabolism caused by long-term KD intervention can improve brain oxidative stress in AD mice. Besides, the expression of Uch-L1 (C) was decreased in the APP con group with enhanced oxidative stress, while increased in the APP KD group with attenuated oxidative stress. Excessive oxidative stress will also lead to changes in S-glutathionylation of mitochondrial Mfn2. Ubiquitination of Mfn2 by Parkin (an E3 ubiquitin ligase) or the S-glutathione is closely related to mitochondrial division and cellular oxidative stress.[46-48] Therefore, we found that the expression of Mfn2 and Uch-L1 were both increased in the APP KD group with reduced oxidative stress. The immunofluorescence confirmed that Uch-L1(C) and Mfn2 were co-labeled, proving that there is an interaction between them. We speculate that the possible effects of Uch-L1 (C) on Mfn2 are as follows: i) Cys152 of Uch-L1(C) acts as an antioxidant binding to H_2O_2 to protect Mfn2 from S-glutathione and maintain THE activity of Mfn2 under oxidative stress; ii) Uch-L1(C) blocks the degradation of Mfn2 by the ubiquitin-proteasome system by deubiquitinating the K48 site of Mfn2. Our future work may further validate the effect of Uch-L1 (C) on Mfn2.

ER-mitochondria cross-linking is an important part controlling mitochondrial Ca^{2+} uptake, autophagosome formation and apoptosis, closely related to mitochondrial function.[49-51] Mfn2 is a recognized GTPase protein that regulates the ER-mitochondria cross-linking.[51-53] And it has also been proved to be the physical chain of ER-mitochondria cross-linking.[30] The result of transmission electron microscopy and WB confirmed that Uch-L1(C) and Mfn2 could regulate endoplasmic reticulum-mitochondrial cross-linking. Long-term KD intervention can improve ER- mitochondrial cross-linking by increasing the expression of Uch-L1(C) and Mfn2, further improving mitochondrial function. Mitochondria are the main source of ATP. When the mitochondria function improves, the

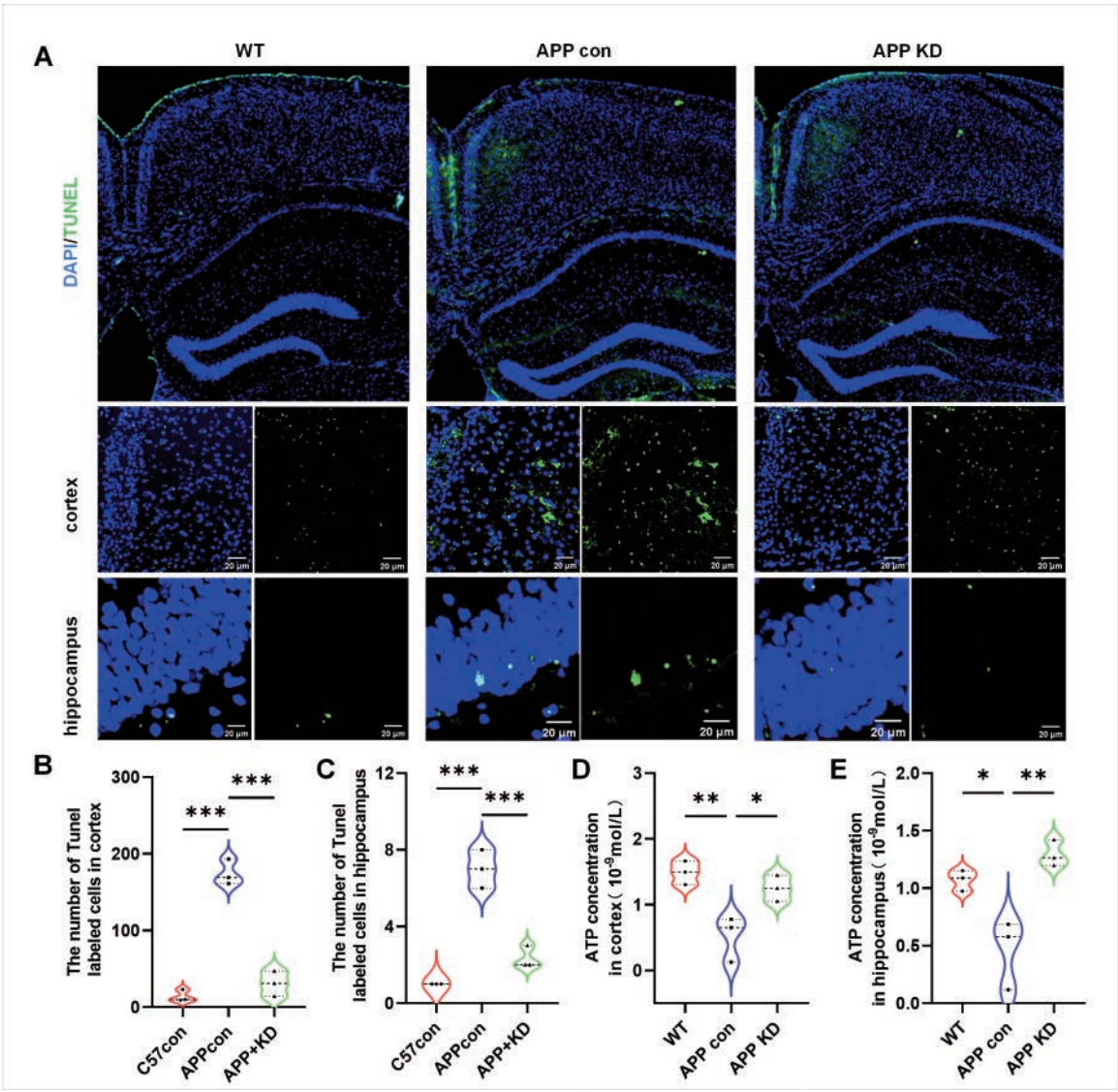

Figure 7. ogenic diet increases ATP energy supply and reduces neuronal apoptosis in mouse brain. **A**) Fluorescent TUNEL staining of brain neurons of mice in the three groups. **B,C**) Fluorescence intensity in cortex and hippocampus in three groups analyzed by ImageJ software. **D,E**) Analysis of actual detected ATP content in cortex and hippocampus (detected by ATP kit Promega, FF200). n=3, *p<0.05, **p<0.01, ***p<0.001.

energy produced increases.[54,55] The result of ATP assay confirmed that long-term ketogenic diet intervention can enhance energy metabolism in the brain of AD mice. And we found that the number of apoptotic neurons in APP KD group decreased in TUNEL staining, so we confirmed that restoring brain energy can reduce neuronal apoptosis in neurodegenerative diseases.[3]

Our experimental results affirmed the previous conjecture: long-term KD intervention increased the expression of Mfn2 along with Uch-L1(C) by reducing oxidative stress *in vivo*. Specifically, the metabolic mode of KD may reduce the production of reactive oxygen species (ROS) by upregulating endogenous antioxidant enzymes and by enhancing mitochondrial uncoupling, thereby alleviating oxidative stress. Therefore, the improvement of mitochondria morphology and function to produce more energy could reduce the neuronal apoptosis due to energy crisis and then improve the cognitive function in AD. After searching for Uch-L1 gene from AlzData and AlzCode, some high-throughput histological databases about AD, we found that compared with the control group, the expression level of each brain region in the AD group was lower. Besides, in our previous clinical studies, we found that serum Uch-L1 was related to various cognitive scores, which proved its significance in nervous system.[56] And in clinical trials, serum Uch-L1 has been proved to be useful in diagnosing intracranial injury and evaluating immediate and delayed memory performance after injury,[57,58] which is promising to become a new biomarker of AD.

There are already studies of KD and AD. Similarly to other studies, we explored the regulation of KD on cognitive function in AD mice. However, this is the first study to link cerebral energy metabolism with Uch-L1 in the mechanism of KD improving AD, which fills the blank of molecular mechanism in KD improving AD energy metabolism. Of course, this exciting study still has some limitations. First, animal model used in this study is not entirely suitable for human. Although the genetically modified AD model used in this study simulates human familial AD in many aspects, sporadic AD (ADs) in human is not in this category.[59] Therefore, we are considering using sporadic AD (such as 3xTg-AD mice) in future studies to verify the general applicability of the research results. This study exclusively employed male mice to avoid hormonal fluctuations; however, given the critical role of sex differences in AD pathology, this is a major limitation that must be addressed in subsequent research. Then, the experimental group lacks the group that knock out the Uch-L1 gene, which makes us lack strong verification of the key role of Uch-L1 in KD improving AD. But the results of this experiment show that the deficiency of Uch-L1 is an important factor in the energy metabolism crisis in AD, so we will continue our work in the future. In terms of cell apoptosis detection, our TUNEL assay can indicate DNA degradation, but it may produce non-specific markers. In future research, we must use precise markers (such as cleaved caspase-3, BAX and Bcl-2) to accurately verify the regulatory role of specific apoptotic pathways to avoid drawing unverified conclusions. Finally, the diet used is semi-synthetic diet we purchased, which may have slightly different effect on those that consume natural foods directly in clinic. However, despite the limitations of this study, the results can also provide a valuable reference for the study of KD in the prevention or delay of human AD.

To sum up, this study shows that KD can regulate the expression of Uch-L1(C) in AD mice and subsequently improve cerebral energy metabolism and cognitive function. Importantly, this is the first study to identify Uch-L1(C) as a key molecular mediator linking ketogenic diet to mitochondrial function and neural energy homeostasis, providing a concise mechanistic explanation for how KD alleviates neurodegenerative changes. By demonstrating that KD restores Uch-L1(C)–dependent Mfn2 stability, enhances ER–mitochondria coupling, and increases ATP supply, our work fills a critical gap in understanding the metabolic mechanism of KD in AD and highlights Uch-L1(C) as both a potential therapeutic target and biomarker of energy-related neural dysfunction. However, we noted that successful clinical translation will face challenges, particularly the need to ensure adequate blood-brain barrier (BBB) penetration of targeted therapeutics and the necessity of minimizing potential off-target effects of systemic Uch-L1 modulators. In the future, pharmacological modulation of Uch-L1(C) may offer a promising strategy for treating neurodegenerative diseases driven by impaired mitochondrial and metabolic regulation.

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