BHBA treatment improves cognitive function by targeting pleiotropic mechanisms in transgenic mouse model of Alzheimer's disease

Yancheng Wu1,2 | Yuhong Gong2 | Yongxin Luan3 | Yang Li4 | Juxiong Liu2 |
Zitong Yue5 | Boyu Yuan2 | Jingxuan Sun2 | Changxin Xie2 | Lijuan Li4,6 |
Junli Zhen4 | Xinxin Jin2 | Yan Zheng4 | Xiaomin Wang4 | Liwei Xie7 | Wei Wang1,2,4

1Innovative Institute of Animal Healthy Breeding, Key Laboratory of Waterfowl Healthy Breeding of Guangdong Province, College of Animal Sciences and Technology, Zhongkai University of Agriculture and Engineering, Guangzhou, P.R. China
2College of Veterinary Medicine, Jilin University, Changchun, P.R. China
3Department of Neurosurgery, First Hospital of Jilin University, Changchun, P.R. China
4Department of Neurobiology, Key Laboratory for Neurodegenerative Disorders of the Ministry of Education, Capital Medical University, Beijing, P.R. China
5Changchun Jida Middle School Experimental School, Changchun, P.R. China
6The Second Hospital of Hebei Medical University, Shijiazhuang, P.R. China
7State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial

Abstract
Accumulation of amyloid β (Aβ) peptide, inflammation, and oxidative stress contribute to Alzheimer's disease (AD) and trigger complex pathogenesis. The ketone body β-hydroxybutyrate (BHBA) is an endogenous metabolic intermediate that protects against stroke and neurodegenerative diseases, but the underlying mechanisms are unclear. The present study aims to elucidate the protective effects of BHBA in the early stage of AD model and investigate the underlying molecular mechanisms. Three-and-half-month-old double-transgenic mice (5XFAD) overexpressing β-amyloid precursor protein (APP) and presenilin-1 (PS1) were used as the AD model. The 5XFAD mice received 1.5 mmol/kg/d BHBA subcutaneously for 28 days. Morris water maze test, nest construction, and passive avoidance experiments were performed to assess the therapeutic effects on AD prevention in vivo, and brain pathology of 5XFAD mice including amyloid plaque deposition and microglia activation were assessed. Gene expression profiles in the cortexes of 5XFAD- and BHBA-treated 5XFAD mice were performed with high-throughput sequencing and bioinformatic analysis. Mouse HT22 cells were treated with 2 mM BHBA to explore its in vitro protective effects of BHBA on hippocampal neurons against Aβ oligomer toxicity, ATP production, ROS generation, and mitochondrial aerobic respiratory function. APP, BACE1, and nephrilysin (NEP) expression levels were evaluated in HT22 cells following treatment with

Abbreviations: 5XFAD, β-amyloid precursor protein and presenilin-1 double-transgenic mice; AcAc, Acetoacetate; ACE, angiotensin-converting enzyme; AD, Alzheimer's disease; APP, β-amyloid precursor protein; ATP, Adenosine Triphosphate; Aβ, Amyloid β; BACE1, β-secretase 1; BHBA, β-hydroxybutyrate; CNS, central nervous system; DAB, diaminobenzidine; FBS, fetal bovine serum; GPR109A, G protein-coupled receptor 109A; IL-1β, interleukin-1β; IL-6, interleukin-6, KBs, Ketones bodies; KD, ketogenic diet; NEP, nephrilysin; NFTs, neurofibrillary tangles; OCR, oxygen consumption rate; PAGE, polyacrylamide gel electrophoresis; PFC, pre-frontal cortex; PS1, presenilin-1; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; WT, wild type.

Yancheng Wu, Yuhong Gong, Yongxin Luan, and Yang Li contributed equally to this work.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2019 The Authors. The FASEB Journal published by Wiley Periodicals, Inc. on behalf of Federation of American Societies for Experimental Biology
1 | BACKGROUND

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by progressive impairment in memory, language, judgment, and decision making. AD is considered an aging-related disease with complicated etiology and may be related to a variety of genetic and environmental factors. The extracellular senile plaques (SP) and intracellular neurofibrillary tangles (NFTs), especially in the cortex and hippocampus are the pathological hallmarks of AD. Amyloid β (Aβ) peptides, which are mainly composed of 40-42 amino acids is considered as an trigger during amyloid cascade and SP formation in AD. Therefore, inhibition of Aβ production is considered to be a potential therapeutic strategy for AD treatment. However, current therapeutic agents targeting Aβ fail to prevent or reverse disease development in clinical AD patients. Besides Aβ toxicity, neuroinflammation and oxidative stress are also involved in the progression the of AD. Thus, other more effective treatments with the therapeutic potential to restrict the AD process from multiple aspects are urgently needed.

The ketogenic diet has been shown to improve the cognitive ability in drug-resistant epilepsy patients and animal models and is often used in the treatment of the disorder. Ketone bodies (KBs) consist of acetoacetate (AcAc), beta-hydroxybutyrate (also called 3-HB, BHBA), and acetone. Interestingly, BHBA treatment exerted antidepressant-like effects in a depression rat model and attenuated stress-induced increases in levels of IL-1β in the hippocampus. It could also protect dopaminergic neurons against inflammatory challenges both in vivo and in vitro in Parkinson’s disease models. The neuroprotective effect of BHBA has been shown to be mediated by hydroxycarboxylic acid receptor 2 (HCA2), also called

BHBA by measuring the presence or absence of G protein-coupled receptor 109A (GPR109A). BHBA improved cognitive function of 5XFAD mice in Morris water maze test, nesting construction and passive avoidance experiments, and attenuated Aβ accumulation and microglia overactivation in the brain. BHBA also enhanced mitochondrial respiratory function of hippocampal neurons and protected it from Aβ toxicity. The enzymes, APP and NEP were regulated by BHBA via G-protein-coupled receptor 109A (GPR109A). Furthermore, RNA sequencing revealed that BHBA-regulated genes mainly annotated in aging, immune system, nervous system, and neurodegenerative diseases. Our data suggested that BHBA confers protection against the AD-like pathological events in the AD mouse model by targeting multiple aspects of AD and it may become a promising candidate for the prevention and treatment of AD.
GPR109A, which modulates monocyte/macrophage functions and redirect these cells to a neuroprotective pathway. This evidence suggests that BHBA may exert the protective role in the brain through multiple pharmacological mechanisms. Nevertheless, the opposite results reported by Milene et al showed that ketogenic diet (KD)-enhanced motor performance but did not rescue memory deficits in a 5-month-old AD mouse model indicating that the late intervention was not effective after the onset of neurodegenerative disease. However, it has not yet been fully elucidated whether BHBA has preventive, therapeutic effects in early stages of AD models. The mechanisms underlying the protective effects of BHBA on AD development also remain largely unknown.

In this study, 5XFAD mice were used as the AD model. Morris water maze test, nest construction, and passive avoidance experiment were performed to evaluate the therapeutic effects of BHBA on AD prevention in vivo. Also, HT22 cells were treated as hippocampal neuron model to explore the protective effects of BHBA in vitro. We found that BHBA treatment improved the spatial learning ability of AD transgenic mice. BHBA treatment attenuated the production and accumulation of Aβ as well as microglia activation in the hippocampus and cortex. BHBA also exerted anti-inflammatory and antioxidative effects and decreased the expression of inflammatory cytokines in the brain of 5XFAD mice. Furthermore, we found that BHBA protected HT22 cells from Aβ toxicity and improved mitochondrial function. Importantly, we identified that processing of amyloidogenic β-amyloid precursor protein (APP) was inhibited by treatment with BHBA, both in vivo and in vitro. We also demonstrated BHBA increased both the transcription and the protein levels of neprilysin (NEP), a key enzyme degrading Aβ in a GPR109A-dependent manner. Thus, our study suggests that BHBA has the potential as a novel preventive or therapeutic candidate by targeting multiple aspects of the AD.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All animal experiments in this study have been approved by the Jilin University Institutional Animal Care and Use Committee (Permit number: 201704005).

2.2 | Animals and β-hydroxybutyrate treatment

AD mouse model-5XFAD (number 006554, from Jackson Laboratory), GPR109A mice were a generous gift from Dr Martin Sager of Zentrale Einrichtung für Tierforschung und Tierschutzaufgaben der Heinrich-Heine Universität Düsseldorf, Germany. Wild-type (WT) littermates (The female parent of the first generation was provided by the Yisi Experimental Animal Center, Changchun, China.) were raised in the Experimental Animal Center of Jilin University and served as controls. Mice were housed in a controlled environment of 22-25°C, 50% humidity, and a 12-hour light-dark cycle with free access to standard chow and water. All animal experiments were approved by the Jilin University Institutional Animal Care and Use Committee. We used 3.5-month-old female mice for the experiments. Mice in the BHBA-treated group (1.5 mmol/kg/d, H6501, Sigma-Aldrich) received subcutaneous administration (0.25 μL/h) using the Alzet mini-osmotic pump (DURECT Corp, Cupertino, California, CA, USA). There were six to eight mice in each group. After 21 days, the administration was terminated, and the subsequent experiment was started.

2.3 | Morris water maze

The Morris water maze test (Morris, 1984) was used to examine the learning and memory abilities of different subgroups of mice. The mice undergoing the visual platform training phase were first placed into the water from the opposite side of the visual platform and the elapse latency was recorded. Mice that could not find the platform within 60 seconds were guided to the visible platform. The elapse latency was recorded as 60 seconds and the mice remained for 30 seconds to adapt them to the experimental environment. The spatial reference memory test phase could be further divided into training period and exploration period. The mouse did not find the platform within 60 seconds, it was led to the hidden platform, the elapse latency was 60 seconds, and the mouse had to stay for 30 seconds. In addition, the path through which the mouse passed was observed. The exploration period was: 24 hours after the last training, following which the platform was removed from the pool. All mice were tested for 60 seconds, and the time they first crossed the hidden platform and the swimming path were recorded and analyzed.

2.4 | Nest building

On the second day after the completion of the water maze experiment, the mice were subjected to nesting experiments. At 7 o’clock in the afternoon, an unscented paper towel of uniform size (thickness of about 0.5 mm, length × width: 13.8 × 10.8 cm) was placed in the cage 1 hour before the start of the night rhythm. Several images were taken 15 minutes before the active period to assess nesting behavior. Scoring standard (5 points total) consisted of (a) paper towels intact (100%); (b) paper towels remain largely intact (> 90% complete); (c) paper towels are mostly chopped (<90%) but there
is no recognizable nest position (flat if there is a nest); (d) the paper towel is torn, there are obvious nests (>90%, the paper towels are arranged in a certain direction, the nest is flat); (e) nearly perfect nest (the nest is constructed higher than the mouse, and the circumference can completely surround the mouse).

2.5 Passive avoidance test

The passive avoidance experiment was used as a method of behavioral assessment to measure the associative memory retention of mice. For this purpose, the experimental device consisted of two identical bright and dark boxes, with stainless steel tubes evenly distributed at the bottom, and two boxes connected by arched small doors. The mouse could be freely shuttled through the small door, and the door was opened and closed by software. First, a single mouse was placed in a bright box to familiarize with the environment. When the mouse entered the black box from the bright box, the small door was closed, and the corresponding elapsed latency was recorded. If the mouse did not spontaneously enter the dark box, it was excluded. After the mouse entered the dark box, it was given a foot shock (0.7 mA) for 2 seconds and 30 seconds after the end of the shock, the mouse was returned to the cage, and the bottom of the box was cleaned with 10% alcohol to remove the smell following which the next mouse was carried out. The retention test was performed 24 hours after the training period. The test procedure was similar except that the foot shock was omitted and the elapsed latency into the dark box was recorded. If the mouse did not enter the black box within 600 seconds, the incubation period was recorded as 600 seconds, and the test was ended.

2.6 Immunohistochemistry

After completion of the behavioral experiments, mice (three in each group) were anesthetized by intraperitoneal administration of sodium pentobarbital (45 mg/kg), perfused intracardially with 0.9% sodium solution, and decapitated. Subsequently, the brain was fixed in a 4% paraformaldehyde solution for 24 hours, dehydrated in 20% and 30% sucrose solutions for 48 hours, and 30-μm sections were taken along the coronal plane with a cryostat. After pretreatment, sections were blocked with 0.5% goat serum for 1 hour at room temperature and the excess serum was blotted with filter paper without washing. Next, the sections were incubated with the mouse monoclonal anti-6E10 (1:1000; SIG-39300, Covance) and rabbit polyclonal anti-Iba1 (1:200, Proteintech, Chicago, IL, USA) for 12 hours at 4°C. The sections were washed and incubated for 10 minutes at room temperature with biotin-labeled secondary antibodies (Biopol, Eching, Germany) at a dose of 50 μL/slice. The avidin-peroxidase solution was incubated for 10 minutes at room temperature with a 50 μL/slice dose washed with PBS for 5 minutes, stained with DAB (IBL, Germany), washed, and dehydrated.

2.7 Aβ-peptide ELISA and western blotting

Beta-amyloid ELISA kits 1-40 and 1-42 (Biolegend, 842301 and 842401) were used. Cortex and hippocampus were lysed in TBS (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 1% Triton-X100, and protease inhibitor cocktail (Roche, 11 697 498 001) and centrifuged at 100 000 g. Aβ-peptide levels were quantified according to the manufacturer’s instructions.

Total proteins were extracted from HT22 cells or tissues following the procedure we described previously. Subsequently, western blotting was performed according to the standard protocol. Antibody information is as follows: specific antibodies including-Tubulin (1:4000), APP (1:5000; A8717, Sigma-Aldrich), NEP (1:1000; ab79423, Abcam), GPR109A (1:100); Goat anti-rabbit IgG-HRP (1:3000; Santa Cruz Biotechnology); and goat anti-mouse IgG-HRP antibody (1:3000; Santa Cruz Biotechnology).

2.8 RNA interference, real-time QPCR, and RNA-Seq Analysis

Lentiviral interference vector of G protein-coupled receptor 109A (GPR109A) was synthesized by Shanghai Sangon Biological Engineering Technology. The infection method was followed according to the manufacturer’s instructions. In brief, HT22 cells, grown overnight in six-well plates (2 × 10^5 cells/well), were infected with HCV (MOI 20) and incubated for 24 hours. Successful lentivirus infection was observed by monitoring GFP-positive cells under a fluorescence microscope and were used for the subsequent experiments.

RNA extraction, reverse transcription, and RT-PCR were performed following our previously published procedure. The primer sequences were listed in Table 1.

Clean reads were obtained by filtering out more than 20% of low-quality reads and reads with adapters and unknown bases (more than 5%). Subsequently, the clean reads were assembled into Unigenes, following functional annotation, SSR detection, and calculating the Unigene expression levels and SNPs of each sample. Finally, differential expressed genes (DEGs) between samples were identified and clustering analysis and functional annotation were performed. Internal software SOAPnuke was used to filter reads and Hierarchical
Indexing for Spliced Alignment of Transcripts (HISAT) was used for the mapping step. Clean reads were mapped to the reference using Bowtie, and then the gene expression level was calculated with RSEM. DEGs were detected with DEGseq based on the Poisson distribution and performed as described by Wang et al.

2.9 Treatment of HT22 cells

Aβ25-35 (synthesized by Shanghai Sangon Biological Engineering Technology & Services Co) was diluted in distilled water at a concentration of 16 mmol/L and was maintained at 37°C for 7 days to pre-age the peptide. HT22 cells were divided into four groups: the control (NT) group, the BHBA (2 mM) group, the Aβ25-35 (20 μmol/L) treatment group, and the Aβ25-35 (20 μmol/L) with BHBA (2 mM) pretreatment group. Cells were starved for 4 hours and then pretreated with BHBA for 2 hours before Aβ25-35 was added. Expression of the intracellular amyloid precursor protein and neprilysin were detected after 24 hours. Each experiment was repeated 3 times.

2.10 Effect of BHBA on the viability, ROS production, and oxidative respiration rate in HT22 cells

HT22 cells were centrifuged and seeded in 96-well plates at a density of 2 × 10^4 cells/mL. Two hundred microliters of PBS were added and the cells were incubated at 37°C. After 24 hours, the complete medium was removed and 200-μL prewarmed DMEM basal medium was added for cell starvation. Subsequently, BHBA was added after 4 hours to pretreat the cells, and after 2 hours, Aβ25-35 was added for stimulation. There were five replicates for each test group. After 24 hours and 48 hours of treatment, the culture medium was discarded and 100 μL of 10 × diluted CCK8 solution was added. Finally, the absorbance at 450-nm wavelength was detected after 1-4 hours.

HT22 cells were seeded in a 96-well plate at a liquid volume of 2 × 10^4 cells/well, and each treatment group was set to five repeats. After culture 24 hours, replace with DMEM incomplete medium to starved cell for 4 hours. Then, 2 mM BHBA was added for pre-protection for 2 hours, and DCFH-DA was diluted with non-complete medium at a ratio of 1:2000, and cultured for 40 minutes. The cells were treated with Aβ25-35 at a dose of 20 μM/well for 24 hours. The detection wavelength is: 488 nm excitation wavelength and 525-nm emission wavelength.

After the end of the treatment, the mitochondrial oxygen consumption rate (OCR) of HT22 was determined by XF24 Extracellular Flux Analyzer (Seahorse Biosciences). The experimental platform, reagents, and instruments involved in determination of OCR were provided by the company (Liaoning Baihao Biotech Co., Ltd., China) and processed in strict accordance with our experimental requirements.

2.11 Statistical analysis

The results are expressed as the mean ± SD (or mean ± SEM). Statistical analysis was performed using Student's t test, or one-way ANOVA followed by Tukey’s post hoc test using...
impairment in 5XFAD mice

improvement in nesting scores (Figure 1G), demonstrating

treated with BHBA, on the other hand, showed a significant

PBS did not destruct or even chew the paper tissue indicat-

mice immediately exhibited chewing and tearing behavior to-

BHBA or PBS and WT mice are shown in Figure 1F. WT

mice and 5XFAD mice treated with

behavior, we explored nest construction with fragrant-free

in the original platform containing quadrants were observed

Nevertheless, no differences in total exploration time spent

that BHBA not only prevented the learning ability deteriora-

deficits of 5XFAD mice (Figure 1B). On the contrary, the

mice to find the hidden platform was shorter than that of the control mice treated with PBS in the training

session. Remarkably, the behavioral impairment could be

BHBA treatment as there was no significant dif-

BHBA-treated 5XFAD mice and WT con-

mice. Additionally, there was no statistically significant dif-

BHBA and WT mice.

In passive avoidance experiments, 5XFAD mice showed

long-term behavioral deficits characterized by shorter la-

the ALZET Osmotic pump into 3.5-month old 5XFAD mice

for 4 weeks. Negative controls received phosphate buff-

the ALZET Osmotic pump into 3.5-month old 5XFAD mice

the overexpression of inflammatory cytokines in 5XFAD

mice than WT mice (Figure S2). BHBA treatment impeded

GPR109A mRNA was also higher in the brain of 5XFAD

cytokines including IL-1β, TNF-α, and IL-6 were increased

occupied by Iba1-positive microglia in the brain was remark-

mice cortex and hippocampus (Figure 3C-H). Taken together,

mice cortex and hippocampus. As expected, BHBA significantly

homogenates. Real-time PCR was employed to evaluate

mRNA expression of the inflammatory cytokines in the
cortex and hippocampus. As expected, BHBA significantly

decreased the number of senile plaques in the hippocam-
pus compared to those in the saline-treated 5XFAD mice

(Figure 2A-C); the average area occupied by the plaques

in the BHBA-treated 5XFAD mice was reduced both in the
cortex and hippocampus (Figure 2D,E). Accordingly, the soluble Aβ40 and the soluble or insoluble Aβ42 in the
brain homogenate were decreased under BHBA treatment

(Figure 2N-P). These results confirmed that BHBA treat-
ment attenuated the Aβ burden and inhibited Aβ deposition

mice. Additionally, there was no statistically significant dif-

between 5XFAD mice treated with BHBA and WT mice.

To further examine the effects of BHBA on 5XFAD mice

mice pathology, mice were sacrificed and decapitated after

behavioral tests. Diaminobenzidine (DAB) staining method

was used to assess the distribution and morphology of am-

loid plaques and microglia in brains sectioned along the
coronal plane. Human Aβ42 and Aβ40 levels were assessed

using sandwich ELISA in the cortex and hippocampus

homogenates. Real-time PCR was employed to evaluate

mRNA expression of the inflammatory cytokines in the
cortex and hippocampus. As expected, BHBA significantly

decreased the number of senile plaques in the hippocam-
pus compared to those in the saline-treated 5XFAD mice

(Figure 2A-C); the average area occupied by the plaques

in the BHBA-treated 5XFAD mice was reduced both in the
cortex and hippocampus (Figure 2D,E). Accordingly, the soluble Aβ40 and the soluble or insoluble Aβ42 in the
brain homogenate were decreased under BHBA treatment

(Figure 2N-P). These results confirmed that BHBA treat-
ment attenuated the Aβ burden and inhibited Aβ deposition

mice. Additionally, there was no statistically significant dif-

between 5XFAD mice treated with BHBA and WT mice.

In passive avoidance experiments, 5XFAD mice showed

long-term behavioral deficits characterized by shorter la-

the bright compartment, and a higher frequency of entering into

the dark compartment compared with WT mice. BHBA treat-
ment group showed a longer latency to cross over into the

dark chamber and longer retention time in the bright com-

partments when compared to their PBS-exposed littermates,

indicating better task retention by BHBA treatment (Figure

1H-J). These experiments proved that BHBA prevented cog-

nitive dysfunction in AD mice.

3.2 | BHBA attenuates Aβ accumulation and
neuroinflammation in the brain

To further examine the effects of BHBA on 5XFAD mice

mice pathology, mice were sacrificed and decapitated after

behavioral tests. Diaminobenzidine (DAB) staining method

was used to assess the distribution and morphology of am-

loid plaques and microglia in brains sectioned along the
coronal plane. Human Aβ42 and Aβ40 levels were assessed

using sandwich ELISA in the cortex and hippocampus

homogenates. Real-time PCR was employed to evaluate

mRNA expression of the inflammatory cytokines in the
cortex and hippocampus. As expected, BHBA significantly

decreased the number of senile plaques in the hippocam-
pus compared to those in the saline-treated 5XFAD mice

(Figure 2A-C); the average area occupied by the plaques

in the BHBA-treated 5XFAD mice was reduced both in the
cortex and hippocampus (Figure 2D,E). Accordingly, the soluble Aβ40 and the soluble or insoluble Aβ42 in the
brain homogenate were decreased under BHBA treatment

(Figure 2N-P). These results confirmed that BHBA treat-
ment attenuated the Aβ burden and inhibited Aβ deposition

mice. Additionally, there was no statistically significant dif-

between 5XFAD mice treated with BHBA and WT mice.

In passive avoidance experiments, 5XFAD mice showed

long-term behavioral deficits characterized by shorter la-

the bright compartment, and a higher frequency of entering into

the dark compartment compared with WT mice. BHBA treat-
ment group showed a longer latency to cross over into the

dark chamber and longer retention time in the bright com-

partments when compared to their PBS-exposed littermates,

indicating better task retention by BHBA treatment (Figure

1H-J). These experiments proved that BHBA prevented cog-

nitive dysfunction in AD mice.

To further examine the effects of BHBA on 5XFAD mice

mice pathology, mice were sacrificed and decapitated after

behavioral tests. Diaminobenzidine (DAB) staining method

was used to assess the distribution and morphology of am-

loid plaques and microglia in brains sectioned along the
coronal plane. Human Aβ42 and Aβ40 levels were assessed

using sandwich ELISA in the cortex and hippocampus

homogenates. Real-time PCR was employed to evaluate

mRNA expression of the inflammatory cytokines in the
cortex and hippocampus. As expected, BHBA significantly

decreased the number of senile plaques in the hippocam-
pus compared to those in the saline-treated 5XFAD mice

(Figure 2A-C); the average area occupied by the plaques

in the BHBA-treated 5XFAD mice was reduced both in the
cortex and hippocampus (Figure 2D,E). Accordingly, the soluble Aβ40 and the soluble or insoluble Aβ42 in the
brain homogenate were decreased under BHBA treatment

(Figure 2N-P). These results confirmed that BHBA treat-
ment attenuated the Aβ burden and inhibited Aβ deposition

mice. Additionally, there was no statistically significant dif-

between 5XFAD mice treated with BHBA and WT mice.

In passive avoidance experiments, 5XFAD mice showed

long-term behavioral deficits characterized by shorter la-

the bright compartment, and a higher frequency of entering into

the dark compartment compared with WT mice. BHBA treat-
ment group showed a longer latency to cross over into the

dark chamber and longer retention time in the bright com-

partments when compared to their PBS-exposed littermates,

indicating better task retention by BHBA treatment (Figure

1H-J). These experiments proved that BHBA prevented cog-

nitive dysfunction in AD mice.

To further examine the effects of BHBA on 5XFAD mice

mice pathology, mice were sacrificed and decapitated after

behavioral tests. Diaminobenzidine (DAB) staining method

was used to assess the distribution and morphology of am-

loid plaques and microglia in brains sectioned along the
coronal plane. Human Aβ42 and Aβ40 levels were assessed

using sandwich ELISA in the cortex and hippocampus

homogenates. Real-time PCR was employed to evaluate

mRNA expression of the inflammatory cytokines in the
cortex and hippocampus. As expected, BHBA significantly

decreased the number of senile plaques in the hippocam-
pus compared to those in the saline-treated 5XFAD mice

(Figure 2A-C); the average area occupied by the plaques

in the BHBA-treated 5XFAD mice was reduced both in the
cortex and hippocampus (Figure 2D,E). Accordingly, the soluble Aβ40 and the soluble or insoluble Aβ42 in the
brain homogenate were decreased under BHBA treatment

(Figure 2N-P). These results confirmed that BHBA treat-
ment attenuated the Aβ burden and inhibited Aβ deposition

mice. Additionally, there was no statistically significant dif-

between 5XFAD mice treated with BHBA and WT mice.

In passive avoidance experiments, 5XFAD mice showed

long-term behavioral deficits characterized by shorter la-

the bright compartment, and a higher frequency of entering into

the dark compartment compared with WT mice. BHBA treat-
ment group showed a longer latency to cross over into the

dark chamber and longer retention time in the bright com-

partments when compared to their PBS-exposed littermates,

indicating better task retention by BHBA treatment (Figure

1H-J). These experiments proved that BHBA prevented cog-

nitive dysfunction in AD mice.
FIGURE 1  BHBA ameliorates cognitive impairment in 5XFAD mice. A, Experimental procedure. (B-E) Results of Morris water maze test. B, The escape latency of saline-treated WT mice (WT, n = 9), saline-treated 5XFAD mice (AD, n = 7) and BHBA-treated 5XFAD mice (AD + BHBA, n = 7) to find the hidden platform was recorded on every training day (means ± SEM, ANOVA, Tukey's test, *P < .05). C-E, One day after finishing the acquisition task, a probe trial was performed to evaluate spatial memory. The escape strategy adopted by three groups of mice was detected by using a camera (C). Time required for the first crossing over the original platform site (D) and the platform crossing time (E) were used to determine the memory retention of the three groups of mice (means ± SEM, ANOVA, Tukey's test, *P < .05). F-G, Examples of nesting from three groups. G, Number of animals receiving each score (means ± SEM, ANOVA, Tukey’s test, *P < .05). H-J, Results of passive avoidance experiment. Latency to dark compartment (H), entry dark compartment times (I) and retention times in bright compartment (J) revealed the effect of BHBA treatment on memory loss in passive avoidance task paradigm of the three groups mice (means ± SEM, ANOVA, Tukey’s test, *P < .05, **P < .01).
BHBA attenuates Aβ toxicity and maintains mitochondrial respiratory function in hippocampal neurons

5XFAD mice recapitulate major features of AD pathology due to Aβ accumulation in the brain and allow exploration of the toxicity of Aβ on neurons and the protective effects of BHBA against Aβ injury. For in vitro assays, we used HT22 cells as the hippocampal cell model. As is shown in Figure 4, compared with no treatment controls, incubation with 20 μM Aβ oligomer for 24 hours significantly decreased the cell viability and ATP production in HT22 cells accompanied by higher intracellular ROS accumulation. However, pretreatment with 2 mM BHBA remarkably reversed the decline of cell viability and ATP production, and decreased ROS production induced by Aβ (Figure 4B-D). These observations indicated that BHBA, as an alternative energy supply, could maintain cell viability and reduce oxidative stress injury caused by the Aβ toxicity. We also examined the mitochondrial aerobic respiratory function following treatment with BHBA under Aβ challenge. Figure 4E shows that Aβ treatment reduced maximal respiration and respiratory capacity in terms of the oxygen consumption rate (OCR) in HT22 cell and BHBA impeded the decrease in OCR induced by Aβ in maximal respiration and rescued the respiratory capacity (Figure 4F). These results indicated that BHBA improved mitochondrial respiratory function and increased ATP production in hippocampal neurons which contributed to the recovery of neurons from Aβ toxicity and ROS injury.
3.4 | BHBA suppresses APP expression and increases NEP level in GPR109A-dependent manner

To exclude the possibility that altered APP levels caused the decreased Aβ seen in BHBA-treated mice, real-time PCR and western blotting were carried out to detect the expression of APP. As Figure 5 shows, high levels of APP mRNA and protein expression were detected in the hippocampus and cortex of brains from 5XFAD mice compared with WT mice. It is of note that BHBA treatment partially decreased the APP expression both in the cortex and hippocampus in vivo (Figure 5A,C,E-G). To demonstrate the hypothesis that the decrease in Aβ burden in BHBA treated 5XFAD mice brain was mediated by decreased Aβ production or increased Aβ clearance, we next assessed the expression of BACE1, ACE, and NEP. We found that BHBA affected BACE1 protein levels neither in the cortex nor in the hippocampus of 5XFAD mice (Figure S3). However, NEP, a degradation enzyme for Aβ, was significantly upregulated by BHBA in the cortex and hippocampus of 5XFAD mice (Figure 5B,D,H-J). Similar results were observed for the expression of ACE, which is also a peptidase that cleaves Aβ1-42 into Aβ1-40 and other less amyloidogenic peptides (Figure S4). Interestingly, in the absence of...
FIGURE 4  BHBA enhances mitochondrial respiratory function and attenuates Aβ toxicity on hippocampal neurons in HT22 cells. A. Effect of Aβ25-35 on HT22 cell viability. Cells were incubated with 0, 20, 40, 60, 80, or 100 μM Aβ25-35 for 24 or 48 hours (n = 5, means ± SD, Student’s t test, ***P < .001). B. Effect of 2 mM BHBA pretreatment on cell viability challenged by 20 μM Aβ 25-35 for 24 hours; cell viability was analyzed by the CCK-8 assay (n = 5, means ± SD, Student’s t test, *P < .05). C. Intracellular ATP levels were measured after the last treatment with different drug formulations (n = 3, means ± SD, Student’s t test, **P < .01). D. Cells were incubated with DCFH2-DA and ROS production was observed using fluorescence microscopy. Protective effect of BHBA on Aβ25-35-stimulated HT22 cells by decreasing the intracellular ROS production was validated (n = 3, means ± SD, Student’s t test, **P < .01). E,F. Analysis of mitochondrial OCR in Aβ25-35-treated HT22 cells. E. OCR was recorded in cultures treated with Aβ25-35 in the presence or absence of 2 mM BHBA. F. Relative basal and maximal OCR, spare respiratory capacity, and ATP production were calculated (n = 3, means ± SD, Student’s t test, ns, no significant difference, *P < .05, **P < .01)
GPR109A, BHBA failed to regulate APP and NEP expression in vivo in the brains of 5XFAD. To further investigate the mechanism underlying the regulation of APP and NEP expression, APPSWE 293 and HT22 cells were treated with 2 mM BHBA for 24 hours. As shown in Figure 6, BHBA reduced the mRNA level of APP in HT22 cells (Figure 6A). In contrast, the mRNA level of NEP was upregulated by BHBA treatment in both APPSWE 293 (Figure S5) and HT22 cell lines. We also used shRNA lentivirus for GPR109A or PTX to knock down (Figure 6D) or block GPR109A in HT22 cells which is a Gi-coupled receptor and is believed to mediate the effects of BHBA and niacin. As predicted, PTX (Figure 6B,C) and shRNA (Figure 6E-I) impeded the effects of BHBA on APP and NEP regulation at both mRNA and protein levels. These results revealed that BHBA inhibited APP expression and upregulated NEP expression in a GPR109A-dependent manner.

3.5 | BHBA transcriptionally regulates global gene expression in the brain

For comprehensive profiling of BHBA-mediated transcriptional regulation in the brain, total RNA was isolated from cortices of WT, 5XFAD, and BHBA-treated 5XFAD mice and high-throughput sequencing was performed followed by bioinformatic analysis. Among the three groups, 17,450 common genes and 231, 270, and 174 distinct genes were annotated in WT, 5XFAD, and 5XFAD + BHBA groups, respectively (Figure 7A). Differentially expressed genes were further compared among groups showing up- or down-regulation of 665 and 369 genes, respectively, between WT and 5XFAD, while between 5XFAD and 5XFAD + BHBA groups, 693 and 363 genes were up- or downregulated, respectively (Figure 7B). The expression level in the cortex of
5XFAD + BHBA mice was similar to that in WT mice (Figure 7C-D). For differentially expressed genes between 5XFAD and 5XFAD + BHBA groups, gene ontology analysis was performed which demonstrated that upon BHBA treatment, most differentially expressed genes were associated with the immune system, nervous system, and neurodegenerative diseases. Thus, this systematic investigation of BHBA-mediated transcriptional regulation led to the identification of differentially expressed genes associated with the immune and nervous systems.

4 | DISCUSSION

Emerging data suggested that, due to its neuroprotective effects, the ketogenic diet (KD) could be applied for purposes
other than the treatment of epileptic patients. For example, KDs can enhance cognitive function in experimental animals and AD patients. Ester et al found that a ketogenic diet with Triheptanoin supplementation for three months reduced the memory deficit of AD transgenic mice. Similarly, Yin and colleagues reported the protective mechanism of ketones in...
AD mouse model including blockage of amyloid-β 42 entry into neurons, reduced oxidative stress, and improved synaptic plasticity.25 Very low carbohydrate consumption could improve memory function in older adults at an increased risk for Alzheimer’s disease as shown by Krikorian and co-workers26; this effect may be associated with ketosis, reduced inflammation, and enhanced energy metabolism contributing to improved neurocognitive function. Another study reported that, compared with placebo, administration of AC-1202 in AD patients resulted in elevated serum ketone bodies and significant differences in AD Assessment Scale-Cognitive subscale (ADAS-Cog scores).27 All these findings indicated that ketogenic diet supplements could be considered a potential therapeutic strategy in the early stages of AD. The primary circulating ketone is 3-hydroxybutyrate or BHBA, which has been demonstrated to show an anti-inflammatory effect in various diseases28,29 contributing to its neuroprotective function. Another study reported that administration of ketone diet resulted in elevated plasma BHB and improvement in memory function in older sporadic AD patient treated with ketone monoester extract.30 In this study, we observed the protective effect of BHBA treatment on 5XFAD mice leading to the improvement of cognitive functions.

The 5XFAD mouse co-expressing five familial AD mutations has been widely used as an AD model for many years. 5XFAD mice show deficits in learning and memory at 4.5 month31 and exhibit significant neuron loss at 9 month.32 Accordingly, we chose 3.5-month-old 5XFAD mice to examine the preventive effect of BHBA treatment on AD-like phenotypes. Peripheral administration of 1.5 mM BHBA greatly improved learning ability and memory, as well as significantly reduced amyloid burden in the asymptomatic mouse model of AD. Consistent with our findings, Yoshihiro et al reported that administration of ketone diet resulted in a higher β-hydroxybutyrate concentration, superior cognitive performance, and suppression of Aβ accumulation and pTau pathology in 3xTgAD mice.33 Takehiko and colleagues demonstrated that depressive- and anxiety-like behaviors were attenuated by BHB administration in a rodent chronic unpredictable stress model.34 In another example, a 63-year-old sporadic AD patient treated with ketone monoester exhibited elevated plasma BHB and improvement in memory retrieval.35 Together with these findings, our results not only highlight the importance of BHBA, a component of ketone bodies, in improving cognitive function in AD transgenic mice, but also suggests BHBA as a potential therapeutic strategy for AD prevention.

Nesting behavior is important for the survival of animal. Several brain regions including the caudate putamen, hippocampus, septum, and medial preoptic area in hypothalamus are implicated in nesting behavior. Overexpression of the mutant APP gene has been shown to dramatically disrupt nest building in mice.36 These observations highlighted the potential usefulness of nesting score in studies of mouse model neurodegenerative diseases as a pathological index or indicator of cognitive dysfunction during AD.

Neuro-inflammation has been shown to play a critical role in the development of neurodegenerative diseases. Microglia, the resident innate immune cells in the CNS, play a major role in the inflammatory process. On the one hand, these cells were found to be highly concentrated around the SP.36-38 On the other hand, the microglia had been shown to play an important role in synaptic formation and plasticity during brain development.39,40 Synaptic elimination by microglia may also contribute to the synaptic loss observed in the AD brain.41,42 Furthermore, activated microglia express pro-inflammatory enzymes (iNOS and COX-2) and cytokines (TNF-α, IL-1β, and IL-6),43 which may mediate neuronal degeneration.44,45 Using mass spectrometry with bioinformatic analysis, Antonio et al found activation of JAK/STAT, p38 MAPK, and interleukin pathways in microglial cells of 5XFAD mice, indicating that innate immune alterations are elicited in microglial cells before plaque deposition in the AD mouse model.46 Therefore, inhibition of inflammation in the nervous system, especially prior to microglia activation and inflammatory cytokine production may be a potential strategy for the treatment of neurodegenerative diseases.47,48

We found increased microglia accumulation and expression of inflammatory cytokines in the cortex or the hippocampus in 5XFAD mice compared with WT mice, while BHBA treatment significantly attenuated neuro-inflammation. We had previously demonstrated that BHBA exerted neuroprotective effects on dopaminergic neurons by inhibiting microglial activation, both in vitro and in vivo, in the LPS-induced PD model, and this inhibitory effect was mediated by GPR109A signaling pathway.14,15 We also observed elevated GPR109A mRNA level in 5XFAD mice cortex and hippocampus compared with their WT controls suggesting that upregulated GPR109A in the brain serves as a negative feedback and limits excessive neuro-inflammation. Thus, inhibition of microglial activation and inflammatory cytokine production may be one of the protective mechanisms of BHBA for improving cognitive performance of AD mice.

Growing evidence showed that microvascular endothelial dysfunction precedes, often by decades, the cognitive decline associated with AD. Although the relevance between endothelial dysfunction and AD pathogenesis need to be fully clarified, there is already evidence that BHBA may protect the vascular endothelium.49 We found that BHBA upregulates claudin-5 and occludin expression in BMEM cells and increases the integrity of the blood-brain barrier model in vitro (data not shown). Further study will focus on the therapeutic properties of ketone bodies with an emphasis on their effects on the cerebral vascular endothelium during the onset of AD.

The brain is one of the most energy-demanding organs in the body. Any disruption in the energy supply leads to AD as well as various other abnormalities. AD is characterized by the release of amyloid beta peptides in the form of monomers/oligomers which may cause mitochondrial dysfunction.
and oxidative stress, and consequently lead to neuroinflammation, neurotoxicity, and synaptic loss. About two decades back Yoshihiro et al had reported that D-β-hydroxybutyrate protected hippocampal neurons in Alzheimer's cell model induced by Aβ1-42 peptides; they hypothesized that ketone bodies not only increased mitochondrial acetyl CoA but also reduced the free mitochondrial NAD couple and oxidized the mitochondrial coenzyme Q couple ultimately decreasing the major source of mitochondrial ROS.

In this study, we used Aβ oligomers to induce oxidative stress and mitochondrial dysfunction in HT22 cells. Our results confirmed that the preservation of ATP levels in combination with reduced ROS production accounted for the protective action of BHBA on hippocampal neurons. Consistent with our findings, when Yukitoshi and coworkers assessed the effects of D-βHB on the preservation of neuronal integrity in hippocampal slices at different developmental stages, they observed that D-βHB was not only able to substitute for glucose as an energy substrate but also preserved neuronal integrity and stability. Similarly, in another study in an in vivo non-coma hypoglycemia model, D-βHB could substitute for glucose and remarkably prevented ROS generation and cell death in affected cortical areas. Also, 3-HB was reported to change neuronal bioenergetics by increasing mitochondrial respiration that resulted in increased BDNF expression. We had previously shown that Aβ oligomers decreased mitochondrial respiration spare capacity in HT22 cells. Also similar to other neurodegenerative disease profiles, iPSC-derived neural cells and fibroblasts from PD patients showed a lower OCR compared to controls after FCCP treatment. BHBA pretreatment attenuated the decrease of spare capacity induced by Aβ, indicating that mitochondrial respiration was maintained by BHBA. These findings suggest that the enhancement of metabolic activity in combination with its antioxidant role contributes to the effective neuron protective function of BHBA in neurodegenerative diseases.

Aβ42 is believed to play a critical role in the pathological context of AD leading to the ultimate neuronal loss. The association between intraneuronal Aβ42, Caspase-3 activation, and neuronal death has been shown in the 5XFAD mouse model. Aβ is generated from the sequential cleavage of APP by β-secretase (BACE1) and γ-secretase on the cell surface, where BACE-1 limits the proteolysis in Aβ production. NEP is the major extracellular enzyme which prevents deposition of Aβ by its cleavage. NEP mRNA and the protein are downregulated in AD brain, suggesting it is an initiating factor in AD.

Although BHBA treatment did not influence BACE1 expression in this study, we have demonstrated both in vivo and in vitro that BHBA effectively inhibited the expression of APP and promoted the expression of NEP, suggesting that BHBA treatment not only reduced the source of Aβ formation but also accelerated its degradation. Most agents that exclusively target amyloid-related alterations in AD have been unsuccessful so far. This is likely due to ineffective late interventions and insufficient knowledge of molecular targets. Significantly, in the current study, we confirmed that GPR109A was essential in mediating the inhibitory action of BHBA on Aβ formation and deposition in both 5XFAD GPR109A−/− hybrid mice and a GPR109A knockdown cell line. Combined with our previous studies, our current findings not only underscore the biological function of GPR109A but also highlight the potential of GPR109A as a novel target in neurodegenerative diseases.

FIGURE 8 Mechanisms of BHBA on improving cognitive function in the 5XFAD mouse model
Human disease genes including AD are enriched for polymorphisms that affect gene expression. By analyzing RNA-Seq data sets, age-associated transcriptomic differences between two transgenic mouse models, Tg2576 and TgCRND8 were evaluated.\(^\text{61}\) Region-specific enrichment of populations of mRNAs in the mouse hippocampus and prefrontal cortex (PFC) as well as characteristic lncRNA expression signatures in subregions of the brain and specific neuronal populations have been reported.\(^\text{62}\) Natalie and colleagues reported transcriptomic analysis for different regions of the AD brain using RNA-Seq.\(^\text{63}\) Using Gene Ontology term enrichment analysis, they revealed overrepresentation of genes associated with the cytological structure and synapse function of the neurons in AD brain samples. In our study, GO analysis showed that the differentially expressed genes (DEGs) between 5XFAD and BHBA-treated 5XFAD mice were involved in many processes including environmental information processing, human diseases, metabolism, cellular processes, and organismal system. These DEGs might play important roles in the development, physiology and pathology of the brain. It is noteworthy that the genes mainly annotated in aging, immune system, nervous system, and neurodegenerative diseases were similar between WT and BHBA-treated 5XFAD mice compared with 5XFAD mice indicating that abnormal aberrations in the gene expression might contribute to the occurrence and progression of AD. Our findings provide a foundation for further research into the molecular mechanisms of BHBA in regulating brain functions.

Figure 8 summarizes the underlying mechanisms of BHBA involved in improving cognitive function by targeting multiple aspects in the AD brain model. Our findings not only explained the protective effect of BHBA on AD process, but also furnished evidence that GPR109A activation, mitochondrial respiratory function maintenance, and microglia inhibition likely constitute valid therapeutic targets in neurodegenerative diseases.

**ACKNOWLEDGMENTS**
We are grateful to Dr Martin Sager (Zentrale Einrichtung für Tierforschung und Tierschutzaufgaben der Heinrich-Heine Universität Düsseldorf, Germany) for providing the GPR109a\(^{-/-}\) mice (whole body KO). We thank Key Laboratory for Neurodegenerative Disorders of the Ministry of Education of China in Capital Medical University for providing behavioral test facilities.

**CONFLICT OF INTEREST**
The author declares that they have no conflict of interests.

**AUTHOR CONTRIBUTIONS**
Designed research: W. Wang, X. Wang, Y. Zheng; Conducted research: W. Wang, Y. Wu, Y. Luan, Y. Gong, Y. Li, Z. Yue, J. Sun, C. Xie, B. Yuan, X. Jin; Behavioral experiment: Y. Li, L. Li, J. Zhen, Z. Yue; Immunohistochemical and ELISA: Y. Wu, Y. Li, L. Li, J. Zhen; Mitochondrial function related experiments: Y. Wu, Y. Gong, J. Liu, B. Yuan, J. Sun, C. Xie, X. Jin; Molecular mechanism including RT-PCR and western blot: Y. Wu, Y. Gong, J. Liu, B. Yuan, J. Sun, C. Xie, X. Jin; Analyzed data: W. Wang, L. Xie, Y. Wu, Y. Zheng; Wrote paper (W. Wang, Y. Wu, Y. Zheng; Have primary responsibility for final content: W. Wang.

**REFERENCES**
1. Reitz C, Brayne C, Mayeux R. Epidemiology of Alzheimer disease. *Nat Rev Neurol* 2011;7(3):137-152.
2. RI N, Ce E. Alzheimer’s disease and Parkinson’s disease. *N Engl J Med* 2003;348(14):1356-1364.
3. Goedert M, Spillantini MG. A century of Alzheimer’s disease. *Science*. 2006;314(5800):777-781.
4. Braak H, Braak E. Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol*. 1991;82(4):239-259.
5. Selkoe DJ. Physiological production of the beta-amyloid protein and the mechanism of Alzheimer’s disease. *Trends Neurosci*. 1993;16(10):403-409.
6. Mucke L, Selkoe DJ. Neurotoxicity of amyloid beta-protein: synaptic and network dysfunction. *Cold Spring Harb Perspect Med*. 2012;2(7):a006338.
7. Mangialasche F, Solomon A, Winblad B, Mecocci P, Kivipelto M. Alzheimer’s disease: clinical trials and drug development. *Lancet Neurol*. 2010;9(7):702-716.
8. McGeer PL, McGeer EG. The amyloid cascade-inflammatory hypothesis of Alzheimer disease: implications for therapy. *Acta Neuropathol*. 2013;126(4):479-497.
9. Ballard C, Gauthier S, Corbett A, Brayne C, Aarsland D, Jones E. Alzheimer’s disease. *Lancet*. 2011;377(9770):1019-1031.
10. Nei M, Ngo L, Sirven JI, Sperling MR. Ketogenic diet in adolescents and adults with epilepsy. *Seizure Eur J Epilept*. 2014;23(6):439-442.
11. Cross H. Epilepsy: behavioural, psychological, and ketogenic diet treatments. *Bmj Clin Evid*. 2015;2015:1214.
12. Augustin K, Khabbush A, Williams S, et al. Mechanisms of action for the medium-chain triglyceride ketogenic diet in neurological and metabolic disorders. *Lancet Neurol*. 2018;17(1):84-93.
13. Yamanashi T, Iwata M, Kamiya N, et al. Beta-hydroxybutyrate, an endogenic NLRP3 inflammasome inhibitor, attenuates stress-induced behavioral and inflammatory responses. *Sci Rep*. 2017;7(1):7677.
14. Fu SP, Wang JF, Xue WJ, et al. Anti-inflammatory effects of BHBA in both in vivo and in vitro Parkinson’s disease models are mediated by GPR109A-dependent mechanisms. *J Neuroinflammation*. 2015;12(1):1-14.
15. Fu SP, Li SN, Wang JF, et al. BHBA suppresses LPS-induced inflammation in BV-2 cells by inhibiting NF-kB activation. *Mediators Inflamm*. 2014;2014:983401.
16. Rahman M, Muhammad S, Khan MA, et al. The β-hydroxybutyrate receptor HCA2 activates a neuroprotective subset of macrophages. *Nat Commun*. 2014;5:3944.
17. Brownlow ML, Benner L, D’Agostino D, Gordon MN, Morgan D. Ketogenic diet improves motor performance but not cognition in two mouse models of Alzheimer’s pathology. *PLoS ONE*. 2013;8(9):e75713.
18. Feng W, Wu Y, Chen G, et al. Sodium butyrate attenuates diarrhea in weaned piglets and promotes tight junction protein expression in colon in a GPR109A-dependent manner. *Cell Physiol Biochem.* 2018;47(4):1617-1629.

19. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods.* 2015;12(4):357-360.

20. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* 2012;9(4):357-359.

21. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics.* 2011;12(1):323.

22. Wang L, Feng Z, Wang X, Wang X, Zhang X. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics.* 2010;26(1):136-138.

23. Kossoff EH, Rho JM. Ketogenic diets: evidence for short- and long-term efficacy. *Neurotherapeutics.* 2009;6(2):406-414.

24. Aso E, Semakova J, Joda L, et al. Triheptanoin supplementation to ketogenic diet curbs cognitive impairment in APP/PS1 mice used as a model of familial Alzheimer's disease. *Curr Alzheimer Res.* 2013;10(3):290-297.

25. Yin JX, Maalouf M, Han P, et al. Ketones block amyloid entry and improve cognition in an Alzheimer's model. *Neurobiol Aging.* 2016;39:25-37.

26. Krikorian R, Shidler MD, Dangelo K, Couch SC, Benoit SC, Clegg RL. A new way to produce hyperketonemia: use of ketone ester in a case of Alzheimer's disease. *Alzheimers Dement.* 2015;11(1):99-103.

27. Henderson ST, Vogel JL, Barr LJ, Garvin F, Jones JJ, Costantini LC. Study of the ketogenic agent AC-1202 in mild to moderate Alzheimer's disease: a randomized, double-blind, placebo-controlled, multicenter trial. *Nutr Metab.* 2009;10(6):31.

28. Goldberg EL, Asher JL, Molony RD, et al. β-Hydroxybutyrate de-activates neutrophil NLRP3 inflammasome to relieve gut flares. *Cell Rep.* 2017;18(9):2077-2087.

29. Nakamura K, Tonouchi H, Sasayama A, Ashida K. A ketogenic formula prevents tumor progression and cancer cachexia by attenuating systemic inflammation in colon 26 tumor-bearing mice. *Nutrients.* 2018;10(2):E206.

30. Lim S, Chesser AS, Grima JC, et al. D-β-Hydroxybutyrate is protective in mouse models of huntington's disease. *PLoS ONE.* 2011;6(9):e24620.

31. Wang Q, Xiao B, Cui S, et al. Triptolide treatment reduces accumulation and Caspase-3 activation. *Neurobiol Aging.* 2011;32(10):1539-1556.

32. Koshio K, Takeshima T, Mori N, Nakashima K, Clarke K, Clegg RL. Study of the ketogenic agent AC-1202 in mild to moderate Alzheimer's disease. *J Cereb Blood Flow Metab.* 2015;35(5):851.

33. Itagaki S, Mcgeer PL, Akiyama H, Zhu S, Selkoe D. Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. *J Neuroimmunol.* 1989;24(3):173-182.

34. Orr ME, Kamphuis W, Dooves S, et al. Reactive glia show increased immunoproteasome activity in Alzheimer's disease. *Brain.* 2013;136(Pt 5):1415-1431.

35. Boza-Serrano A, Yang Y, Paulus A, Deierborg T. Innate immune neurons. *Nat Methods.* 2016;13(5):769-781.
54. Cooper O, Seo H, Andrabi S, et al. Familial Parkinson's disease iPSCs show cellular deficits in mitochondrial responses that can be pharmacologically rescued. Sci Transl Med. 2012;4(141):141ra90.
55. Ambrosi G, Ghezzi C, Sepe S, et al. Bioenergetic and proteolytic defects in fibroblasts from patients with sporadic Parkinson's disease. Biochem Biophys Acta. 2014;1842(9):1385.
56. Vassar R, Bennett BD, Babu-Khan S, et al. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science. 1999;286(5440):735-741.
57. El-Amouri SS, Zhu H, Yu J, Marr R, Verma IM, Kindy MS. Neprilysin: an enzyme candidate to slow the progression of Alzheimer's disease. Am J Pathol. 2008;172(5):1342-1354.
58. Yasojima K, McGee EG, McGeer PL. Relationship between beta amyloid peptide generating molecules and neprilysin in Alzheimer disease and normal brain. Brain Res. 2001;919(1):115-121.
59. Iwata N, Higuchi M, Saido TC. Metabolism of amyloid-beta peptide and Alzheimer's disease. Pharmacol Ther. 2005;108(2):129-148.
60. Corbett A, Pickett J, Burns A, et al. Drug repositioning for Alzheimer's disease. Nat Rev Drug Discovery. 2012;11(11):833-846.
61. Rothman SM, Tanis KQ, Gandhi P, et al. Human Alzheimer's disease gene expression signatures and immune profile in APP mouse models: a discrete transcriptomic view of Abeta plaque pathology. J Neuroinflammation. 2018;15(1):256.

62. Kadakkuzha BM, Liu XA, McCrate J, et al. Transcriptome analyses of adult mouse brain reveal enrichment of lncRNAs in specific brain regions and neuronal populations. Front Cell Neurosci. 2015;9:63.
63. Twine NA, Janitz K, Wilkins MR, Janitz M. Whole transcriptome sequencing reveals gene expression and splicing differences in brain regions affected by Alzheimer's disease. PLoS ONE. 2011;6(1):e16266.

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

How to cite this article: Wu Y, Gong Y, Luan Y, et al. BHBA treatment improves cognitive function by targeting pleiotropic mechanisms in transgenic mouse model of Alzheimer’s disease. The FASEB Journal. 2020;34:1412–1429. https://doi.org/10.1096/fj.201901984R