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GM6 Attenuates Alzheimer's Disease Pathology in APP Mice

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Abstract

Alzheimer's disease (AD) results in the deposition of amyloid β (Aβ) peptide into amyloid fibrils and tau into neurofibrillary tangles. Regardless of whether or not these entities are a cause or consequence of the disease process, preventing their accumulation or accelerating their clearance may slow the rate of AD onset. Motoneuronotrophic factor (MNTF) is an endogenous neurotrophin that is specific for the human nervous system, and some of the observed effects of MNTF include motoneuron differentiation, maintenance, survival, and reinnervation of target muscles and organs. GM6 is a six-amino-acid component of MNTF that appears to replicate its activity spectrum. In this study, we investigated the effect of GM6 in a mouse model of AD before the development of amyloid plaques and determined how this treatment affected the accumulation of Aβ peptide and related pathologic changes (e.g., inflammation, nerve growth factor (NGF) expression, cathepsin B, and memory impairment). Application of GM6 over a 4-month period in young APP/ΔPS1 double-transgenic mice resulted in attenuation in Aβ peptide levels, reduction of inflammation and amyloid load, increased cathepsin B expression, and improved spatial orientation. In addition, treatment with GM6 increased brain NGF levels and tempered memory impairment by ~ 50% at the highest dose. These data suggest that GM6 may modulate disease-determining pathways at an early stage to slow the histological and clinical progression of AD.

Keywords Alzheimer's · Aβ peptide · Behavior · Cathepsin B · Nerve growth factor

Introduction

Alzheimer's disease (AD) affects more than 35 million people worldwide and is known as the most common form of dementia; the number of persons afflicted with dementia is also expected to grow further with the increase in the aging population worldwide [1]. The frequency of diagnosis of AD increases to 1 in 3 after the age of 85 and results in death 3–9 years after the onset of symptoms [2]. AD is associated with specific clinical features such as cognitive impairment and neuropsychiatric disturbances [3, 4]. AD is characterized by the aggregation of amyloid-β (Aβ) peptide into neuritic plaques and hyperphosphorylated tau protein accumulating into neurofibrillary tangles (NFTs). Aβ is generated from the sequential cleavage of the amyloid precursor protein (APP) resulting in the production of $Aβ_{40}$ and $Aβ_{42}$ peptides, which give rise to aggregated fibrils and plaques [5, 6]. Aβ is known to be neurotoxic and is thought to play a major role in the pathogenesis of AD; it accumulates as a result of either decreased turnover and/or increased production [7, 8].

About 25 years ago, motoneuronotrophic factor 1 (MNTF1) was isolated from rat muscle tissue [9]. This factor was capable of supporting in vitro growth and/or regeneration of anterior horn motoneurons and spinal cells of rat lumbar spinal cord. Over the years, a number of studies have established that MNTF was efficacious in various rat nerve systems [9–12]. In a hemi-sectioned rat spinal cord injury

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model, MNTF reduced inflammation, limited degeneration, and enhanced regeneration of the grafted nerves [12]. In addition, in wobbler mice, an ALS model, one dose of 35 μg/kg MNTF at the age of 6 weeks slowed the progression of the disease phenotype.

Within the MNTF protein is a six-amino-acid peptide (Phe-Ser-Arg-Tyr-Ala-Arg) referred to as MNTF6mer or GM6 [13]. Studies have shown that GM6 had similar activity as the parent MNTF molecule [14, 15]. Studies using the synthesized GM6 demonstrated trophic effects in a transected femoral nerve rat model. In addition, in a zebrafish bioassay, GM6 was shown to protect the animals from L-2-hydroxyglutaric acid (LGA)–induced CNS oxidative stress and apoptosis and attenuate midbrain apoptotic cell death by 85%. Finally, we have shown that GM6 was able to penetrate the blood-brain barrier and at both 1 and 5 mg/kg showed a significant protection from infarct damage and improved neurological outcomes in a mouse middle cerebral artery occlusion (MCAo) model [16]. These studies suggest that GM6 has significant protective effects in the CNS.

Based on the impact of GM6 on inflammation and oxidative stress, we decided to test the efficacy of GM6 in a mouse model of AD. Using the APP/PS1 mouse model previously established, mice were injected daily with the GM6 intravenously and examined for the impact on neurological outcomes, biochemical changes, and amyloid pathology. We report that the administration of GM6 to transgenic mice expressing familial AD (FAD) mutant AβPP and presenilin 1 (PS1) cDNAs (APP-PS1) with GM6 for 4 months significantly decreased amyloid plaque burden and levels of Aβ peptide and increased brain NGF compared to APP-PS1 control mice. These data suggest that GM6 might be beneficial in the attenuation of AD pathology in man.

Materials and Methods

Animals

A total of 60 3-month-old male transgenic mice were used for the studies. The mice expressed the mutant form of human presenilin-1 (DeltaE9) and the mutant form of the chimeric mouse/human amyloid precursor protein (APP695) [17, 18]. The mouse prion protein promoter directed the expression of both transgenes. The DeltaE9 mutation of the human presenilin-1 gene is a deletion of exon 9 and corresponds to a form associated with early-onset AD. The APP695 gene harbors the K595N/M596L (Swedish) AD-causing mutations. The coding sequence of mouse Aβ peptide domain was humanized by replacing the three amino acids that differ between the two species with the human residues. These APP/PS1-Tg mice start developing amyloid plaques at ~ 3 to 4 months of age. These mice were on a C3H/HeJ*C57BL/6J background. Ten 7-month-old wild-type (C3H/HeJ*C57BL/6) mice were used to obtain baseline levels of the different variables. Mice were given free access to food and water before the experiment. Animals were 12 weeks of age at the start of the experiment and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). All animals were randomized to the various groups. All studies were approved that the Institutional Animal Care and Use Committee at the University of South Florida and the Veterans Affairs Medical Center. This study adhered to the Guide for the Care and Use of Laboratory Animals developed by the Office of Laboratory Animal Welfare.

Treatment with GM6

Animals were randomly assigned to a vehicle group ($n = 20$) or treatment groups ($n = 20$) treated with daily intravenous injection of a 6-mer active component of MNTF (GM6) at a dose of 1 or 5 mg/kg. The formulation of GM6 (CS Bio Co., Menlo Park, CA, Catalog# CS1507, GMP013, lot C811) was performed by reconstituting GM6 with normal saline solution that was stored at 4 °C. Vehicle control received phosphate-buffered saline (PBS) solution. The IV injections via tail vein were given. Four months after the treatment, animals were transcardially perfused under deep anesthesia with 1× PBS and their brains removed for further analysis. The right brain hemispheres were immersion-fixed in 4% paraformaldehyde for 24 h, followed by immersion in 4% paraformaldehyde containing 30% sucrose for 2 to 3 days. After fixation, the right brain hemispheres were frozen in OCT medium and sectioned with a cryostat to obtain 30-μm frozen sections for immunohistochemical analysis. The left brain hemispheres were frozen as quickly as possible and used to quantitate the levels of Aβ peptide ($Aβ_{1–40}$ and $Aβ_{1–42}$), inflammatory markers, NGF, and cathepsin B.

Immunohistochemistry Staining

Cryosections of the right brain hemispheres were washed three times (5 min/wash) with Tris-buffered saline (TBS) (pH 7.4) buffer, followed by washing one time with 0.1% Triton X-100-TBS buffer for 5 min. Sections were then incubated in 3% H_2O_2 and TBS buffer for 30 min at room temperature to eliminate endogenous peroxidase activity. After 1 h of blocking with 5.0% serum (horse or goat), the sections were incubated overnight with primary antibodies. Primary antibodies and dilutions were as follows: Aβ peptide, mouse anti-human Aβ peptide antibody (1:500 dilution, 10D5; Elan Pharmaceuticals, San Francisco, CA); CD68-positive microglia, anti-mouse CD68 antibody (1:400 dilution, KP1; Abcam, Cambridge, MA); GFAP-positive astrocytes (1:200 dilution, 2E1; BD Biosciences, San Jose, CA). The next day, sections were

washed three times (5 min/wash) with 0.1% Triton X-100 and TBS buffer to remove excess primary antibody. Thereafter, primary antibodies were detected using horseradish peroxidase-conjugated mouse or rat IgG Vectastain ABC kit and DAB/substrate reagents (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Image analysis in each section was determined with a computer-assisted image analysis system, consisting of a Power Macintosh computer (Apple Inc., Cupertino, CA) equipped with a QuickCapture frame grabber card, Hitachi CCD camera (Hitachi Kokusai Electric Inc., Tokyo, Japan) mounted on an Olympus microscope (Olympus, Tokyo, Japan), and camera stand. Images were captured and a total number of plaques or cells was determined over sections using National Institutes of Health (NIH) Image Analysis Software (v. 1.55; NIH, Bethesda, MD) conducted by a single operator blinded to treatment status for analyses of all measurements.

Aβ Peptide, sAPPα, and CTFβ Analysis

Left brain hemispheres were weighted and homogenized with 4 vol of PBS buffer (125 mg/ml) containing complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Half the volume of the homogenates was mixed with 8.2 mol/L guanidine-HCl (pH 7.4) to a final concentration of 5 mol/L for 4 h at room temperature. Guanidine extracts were then diluted 1:50 in BSAT-DPBS buffer (Dulbecco's phosphate-buffered saline with 5% bovine serum albumin, 0.03% Tween 20, and 1× protease inhibitor cocktail), mixed, and spun at 16,000×*g* for 20 min at 4 °C. The supernatants were used to measure Aβ peptide levels by human $Aβ_{1–40}$ and $Aβ_{1–42}$ ELISA kits (Biosource International, Camarillo, CA). The remainder of the brain homogenates was subsequently centrifuged at 12,000×*g* for 20 min at 4 °C. Content of NGF in brain tissue was measured using the Chemikine NGF ELISA kit (Millipore) according to the manufacturer's directions.

CTFβ and sAβPPα Analyses

Western blot assays measured CTFβ and sAβPPα as previously described with the same amounts of protein in each lane [19]. CTFβ was determined in the pellet fraction from the brain extract (antibody 8717, Sigma). In addition, sAβPPα was assessed in the supernatant fraction from the brain extract (antibody 6E10, Signet Laboratories). Relative amounts of CTFβ and sAβPPα were measured by densitometry and results were expressed as percentage of the mean CTFβ and sAβPPα compared to the control group. As control, β-actin Western blots (anti-β-actin from Cell Signaling Technology) monitored equal loading of the same amounts of samples (20 μg protein) in gel lanes.

ELISA Analysis

For quantitative analysis of cytokines, an ELISA was used to measure the levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), or transforming growth factor-β (TGF-β) in brain tissue [20]. Cytokines were extracted from mouse brains as follows: frozen hemibrains were placed in tissue homogenization buffer containing protease inhibitor cocktail (Sigma, St Louis, MO, USA) 1:1000 dilution immediately before use and homogenized using polytron. Tissue sample suspensions were distributed in aliquots and snap frozen in liquid nitrogen for later measurements. Invitrogen ELISA kits were then used, according to manufacturer directions (Carlsbad, CA, USA).

The Morris Water Maze Test

A circular water tank, made from aluminum (diameter, 100 cm; height, 40 cm) was filled to a depth of 25 cm with water (23 °C) and rendered opaque by the addition of a small amount of nontoxic white powder. Four positions around the edge of the tank were arbitrarily designated north (N), south (S), east (E), and west (W), to provide four alternative start positions and define the division of the tank into four quadrants: NE, SE, SW, and NW. A square white Perspex escape platform (10 × 10 × 2 cm) was submerged 1.0 cm below the water surface and placed at the midpoint of NE quadrant. The platform was invisible to the mice. Four visible cues to assist the mice for spatial analysis were placed outside the wall of the pool. A video camera, connected to SMART video tracking system (San Diego Instruments, San Diego, CA), was fixed 1.6 m above the center of the swim tank, and all swimming trials were recorded for further analysis. Four months after the start of injections, each APP/ΔPS1-Tg mouse with and without GM6 was trained for 4 consecutive days to reach the platform from the four different starting points. Starting points were randomized every day during the 4-day trials. Each mouse was placed gently in the pool facing the tank wall. Every mouse was allowed to swim and reach the platform within 60 s (spatial learning). If the mouse reached the platform, it was allowed to stay on the platform for another 30 s. If the mouse failed to reach the platform, it was gently placed on the platform for 30 s. Before the next trial, each mouse was allowed to rest for 10 min. On the day after the last training session, the platform was removed, and each mouse was allowed to search for the platform within 60 s (memory retention). To compare the spatial learning and memory retention between the mice with and without GM6, the escape latency and distance taken by the mice to reach the platform were

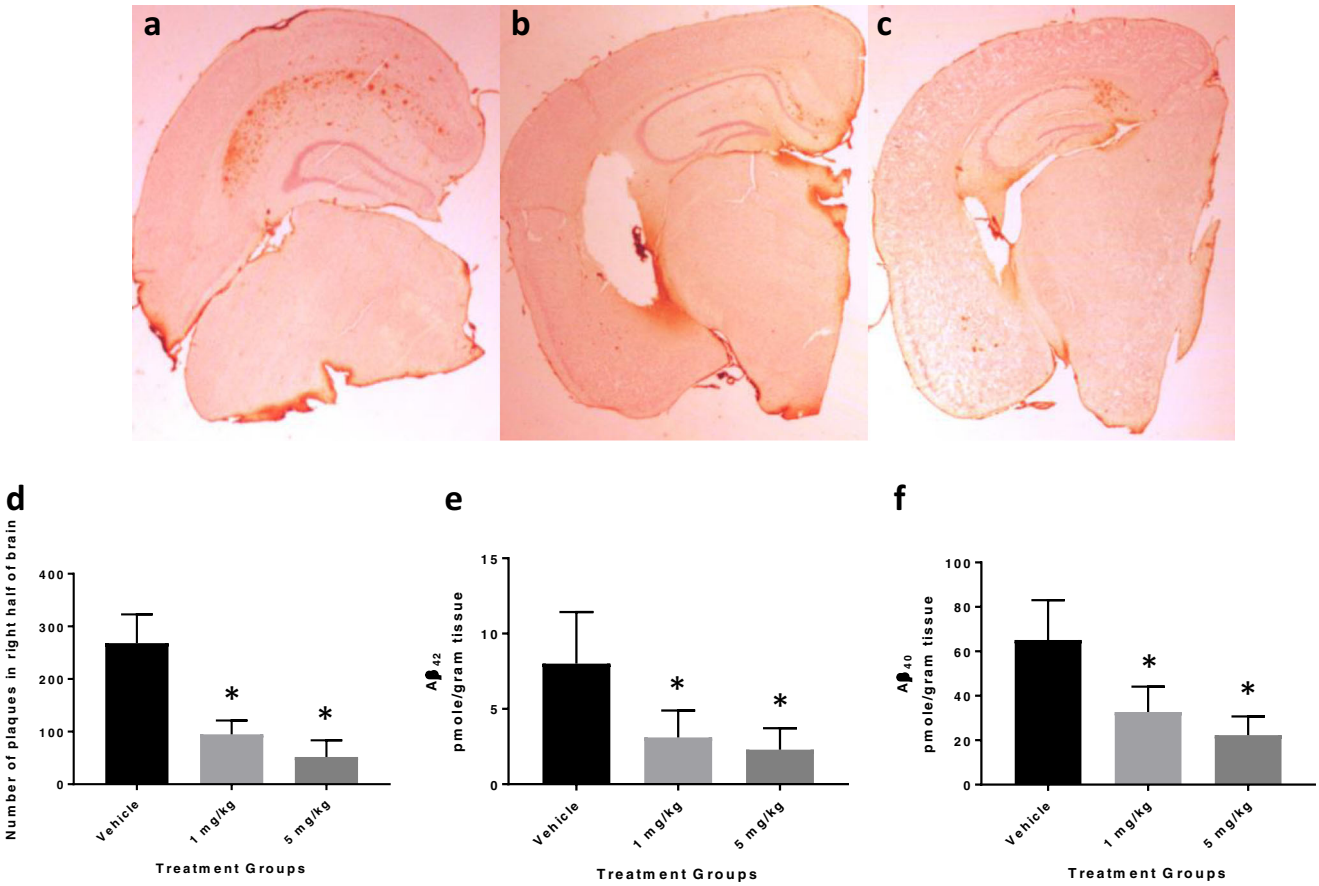

Fig. 1 Level of amyloid load after 4 months of GM6 treatment in APP/ΔPS1-Tg mouse brains. Right brain hemisphere sections obtained from control mice (**a**), GM6 treated with 1 mg/kg GM6 (**b**), or GM6 treated with 5 mg/kg GM6 (**c**) were immunostained with mouse anti-human Aβ peptide (clone 10D5) antibody to detect amyloid plaques. The number of amyloid plaques in the brain sections (10 sections per mouse) from each set of control mice or mice treated with GM6 were counted and averaged (**d**). The left brain hemisphere was examined for guanidine-extractable $A\beta_{1–40}$ (**e**) and $A\beta_{1–42}$ peptides (**f**). Vehicle: 7-month-old APP/ΔPS1-Tg mice that were not treated with GM6 were used as controls (*$P < 0.001$, $n = 20$ per group)

measured on the fourth day before removing the platform. In addition, the percent time that each mouse spent in the NE quadrant and in the outer annular was measured on the last day in absence of the platform.

Statistical Analysis

Results were expressed as mean ± SEM. The multiple comparison of amyloid plaque numbers, the concentration of Aβ peptide ($A\beta_{1–40}$ and $A\beta_{1–42}$), and the inflammatory markers were performed by one-way analysis of variance followed by Tukey's multiple comparison tests. A two-way analysis of variance was used to evaluate the differences in a number of CD68-positive microglia and GFAP-positive astrocytes in the hippocampus and cortex areas. Statistical analysis of the Morris water maze test was performed using a two-way analysis of variance. In all comparisons, a difference with a P value less than 0.05 was considered statistically significant.

Results

Impact of GM6 on Amyloid Pathology in the APP/PS-1 Mice

The APP/PS1 mice used in this study began to exhibit an increased level of Aβ peptide and deposition of amyloid plaques in the brain at about 3 months of age. The increase of amyloid load in the brain was associated with both GFAP-positive astrocytes and CD-68-positive microglia in the cortex area and the hippocampus region [21]. Therefore, 3-month-old APP/PS1 mice exhibit early stages of AD-like pathology and were selected for testing GM6 treatment. In addition, these mice show a dramatic increase in amyloid load and memory impairment at 6 to 8 months of age [21].

Three-month-old APP/PS1 mice were treated with GM6 at 1 mg/kg or 5 mg/kg daily (i.v.) for 4 months to allow for adequate pathological and behavioral changes to occur. Age-matched, vehicle-treated transgenic mice were also used in these experiments. After 4 months or treatment, all mice were

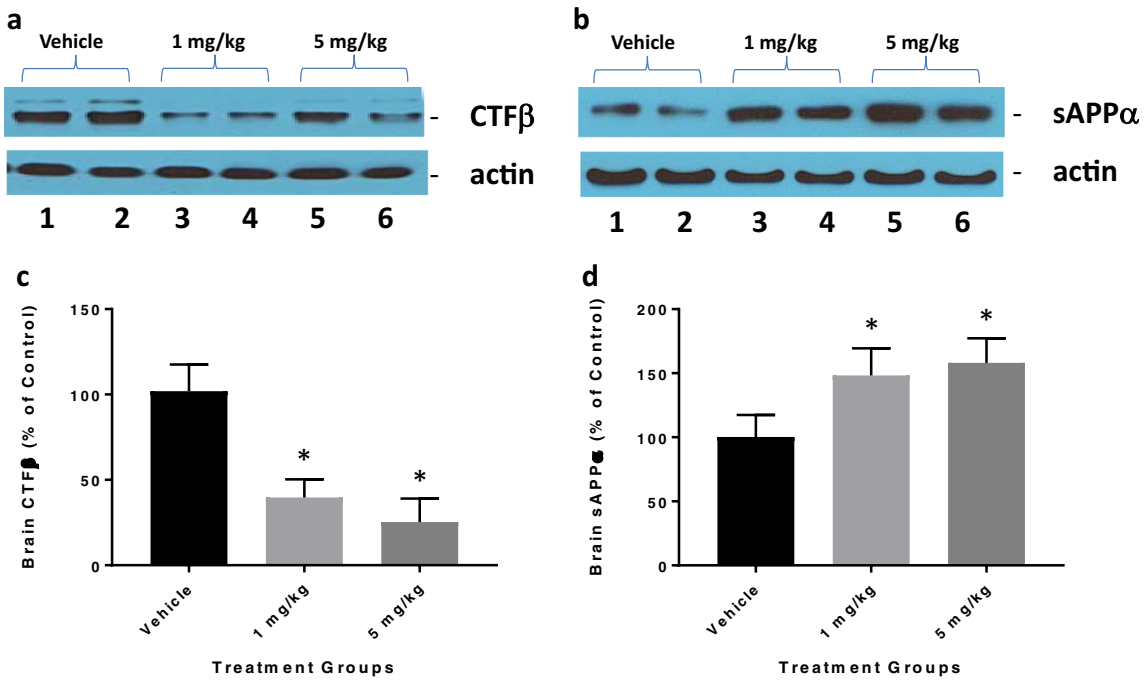

Fig. 2 GM6 lowers and increases brain CTFβ and sAβPPα, respectively. **a** GM6 caused a reduction in brain CTFβ. CTFβ is a proteolytic product of β-secretase cleavage of AβPP and thus, these data are also consistent with GM6 acting through the inhibition of β-secretase activity. **b** In contrast, GM6 mediated an increase in brain sAβPPα levels. **c**, **d** Graphical representation of Western blots in **a** and **b**, respectively. These data are consistent with GM6 mediating the inhibition of β-secretase activity because sAβPPα is derived from AβPPα-secretase cleavage, which competes with β-secretase cleavage of AβPP, and thus, the inhibition of β-secretase activity provides more AβPP for β-secretase to produce more sAβPPα (*$P < 0.001$; $n = 20$)

subjected to behavioral analysis (see the "Attenuation of Neurological Deficits in APP/PS-1 Mice during GM6 Treatment" section) and sacrificed, and sections of the right brain hemisphere were subjected to immunohistochemistry. To evaluate the effect of GM6 on the Aβ peptide levels in the brain, we measured the levels of $Aβ_{1–40}$ and $Aβ_{1–42}$ peptides and the number of amyloid plaques. Immunostaining of 10 random cryostat sections (each spaced by at least five unused sections to prevent double counts) of each right brain hemisphere was performed on APP/PS1 mice (Fig. 1). As seen in Fig. 1, a through c, the number of amyloid plaques was attenuated 4 months after the GM6 treatment. Treatment with GM6 at 1 mg/kg or 5 mg/kg correlated with a 65% or 81% decrease in the number of amyloid plaques (vehicle, 268 ± 54.8; GM6 (1 mg/kg), 95 ± 26.4; GM6 (5 mg/kg), 51.8 ± 31.8) (Fig. 1d). In addition, the levels of Aβ peptide ($Aβ_{1–40}$ and $Aβ_{1–42}$) in the brain (left hemisphere) were analyzed by ELISA (Fig. 1e, f). The guanidine-extractable $Aβ_{1–40}$ and $Aβ_{1–42}$ peptides in the brain were decreased by 50% and 66% and 61% and 71% in the brain, at the 1 and 5 mg/kg dosing.

To help understand the mechanisms for the increase in Aβ peptide, we examined the impact of GM6 on the cleavage products CTFβ and sAPPα. GM6 animals demonstrated a reduction in brain CTFβ (Fig. 2a, c). The CTFβ levels were significantly lower (60% and 75%) than in the vehicle animals. These data are also consistent with GM6 regulating a reduction in β-secretase activity as CTFβ is the cleavage product of β-secretase [22]. Mice treated with GM6 showed an increase in brain sAPPα (Fig. 2b, d). The sAPPα levels were significantly higher in the GM6 animals (148% and 158%) than the vehicle-treated animals. Again, these data support the hypothesis that GM6 is facilitating a reduction in β-secretase activity because sAβPPα is a cleavage product of α-secretase cleavage, which competes with β-secretase activity, and the inhibition of β-secretase activity affords more APP for α-secretase to generate more sAPPα.

Attenuation of Neurological Deficits in APP/PS-1 Mice during GM6 Treatment

APP/PS1 mice exhibit significant amyloid plaque deposition at 6 to 8 months of age. Also, they show signs of memory impairment as it has been reported [21]. Four months after the GM6 treatment started, mice were examined for spatial learning and memory retention in the Morris water maze test [23]. On the fourth day of training and before removing the hidden platform, the vehicle- and GM6-treated groups were tested for the spatial learning ability (escape latency and total distance traveled by mice to reach the platform in the presence of distal visual cues outside the swimming pool). The group of mice treated

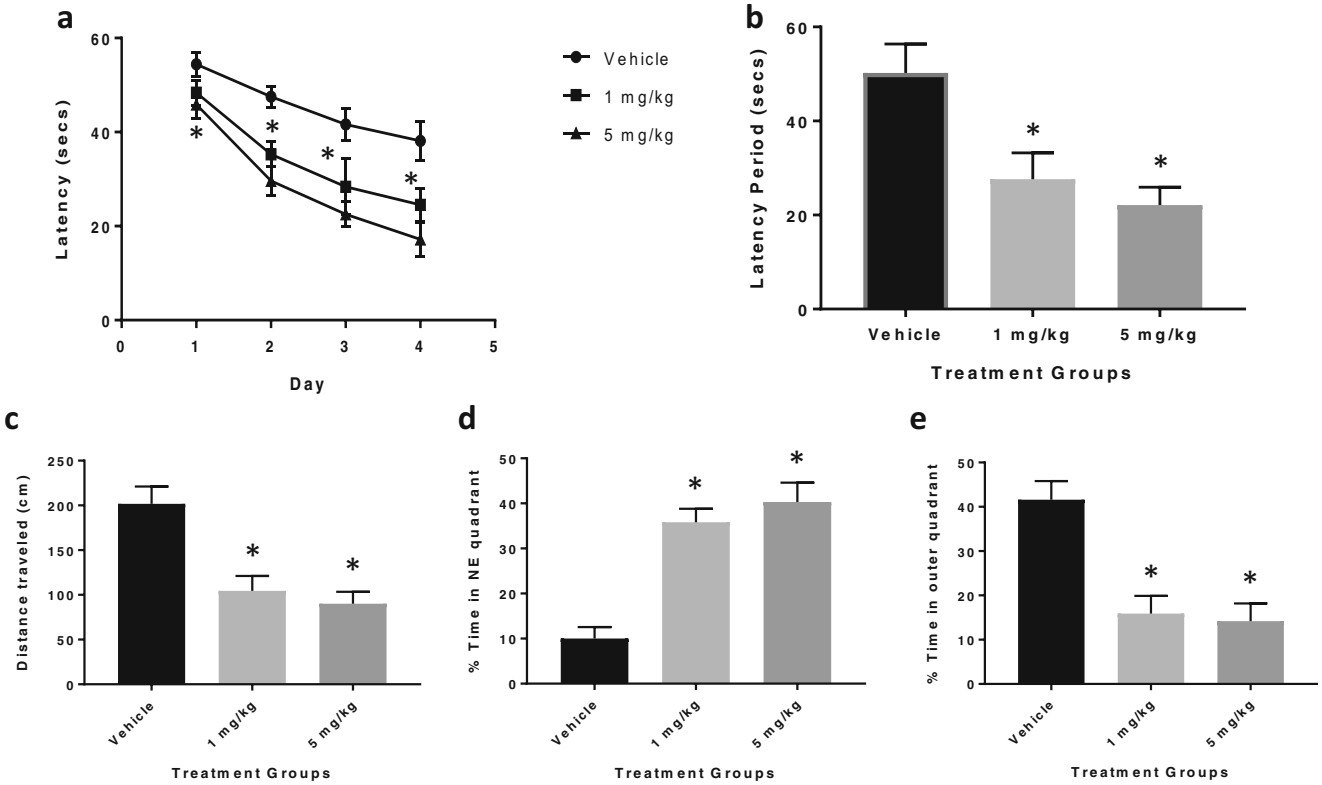

Fig. 3 Behavioral analysis of mice treated with GM6 in the Morris water maze. **a** APP/PS-1 vehicle and 1 mg/kg and 5 mg/kg GM6 were examined for memory acquisition. APP/PS-1 mice were trained in the Morris water maze test on each of 4 consecutive days to learn the location of a submerged, invisible platform in a pool of water. The time that it took the mice to swim to the platform was recorded each day, measured as the latency period (seconds), with shorter latency times indicating better memory acquisition. Latency (s) is shown as $x \pm$ s.e.m. (*Statistically significant, $P < 0.05$, $n = 20$ per group). Memory deficits of APP/PS-1 mice were assessed 2 days after completion of the training in the Morris water maze test by measuring the latency period (**b**) and distance traveled (**c**) for animals to swim to the submerged, invisible platform. The shorter latency periods and shorter distances traveled indicate improved memory. Vehicle APP/PS-1, APP/PS-1 (1 mg/kg), and APP/PS-1 (5 mg/kg) mice had mean latency periods of 49, 26, and 21 s, respectively (**b**), and mean distances traveled at 201, 109, and 95 cm, respectively (**c**). Values are expressed as $x \pm$ s.e.m., and $n = 10$ per group. *Statistically significant ($P < 0.001$). The percent time each animal swam in the quadrant from which the platform had been removed (northeast (NE) quadrant (**d**) and the percent time an animal swam in the annulus of the pool were recorded (**e**)). Greater memory retention is reflected in a higher percent time in the NE quadrant and lower percent time in the annulus. APP/PS-1 (vehicle), APP/PS-1 (1 mg/kg), and APP/PS-1 (5 mg/kg) mice had percent times in the quadrant of 10%, 36%, and 41% (**d**) and in the annulus of 42%, 17%, and 15% (**e**). Values are expressed as the mean ± s.e.m., and $n = 20$ per group. *Statistically significant with $P < 0.001$

with GM6 showed a significantly enhanced spatial learning ability compared to the vehicle-treated mice (Fig. 3a, b). The latency period and distance traveled were 27.6 ± 5.62 s and 104.4 ± 16.8 cm (1 mg/kg) and 22.1 ± 3.84 s and 90.1 ± 13.3 cm (5 mg/kg) for the mice treated with GM6. For the vehicle mice, the latency period and distance were 50.2 ± 6.20 s and 202.7 ± 19.35 cm, respectively. In the spatial probe (memory retention) trial, in the absence of a platform, the GM6-treated mice showed significantly less cognitive impairment (better memory retention) compared to vehicle mice (Fig. 3d, e). The percentage time points spent in the NE quadrant by GM6-treated mice were 35.8 ± 3.01 (1 mg/kg) and 40.3 ± 4.30 (5 mg/kg) s compared to 10.0 ± 2.58 s spent by vehicle mice. The percentage time points spent by the groups of mice in the outer annular area were 15.9 ± 3.99 (1 mg/kg) and 14.2 ± 3.99 (5 mg/kg) s and 41.6 ± 4.20 s.

Reduced Inflammation in APP/PS-1 Mice with GM6

To determine whether there were any measurable effects on the brain of treated APP/PS1 mice as a result of amyloid load attenuation, we performed immunohistochemical staining on the brain sections obtained from vehicle- and GM6-treated mice. The density of CD68-positive microglial cells was reduced in mice treated with the GM6 (Fig. 4b, c) compared to vehicle mice (Fig. 4a). A quantitative analysis of CD68-positive microglia revealed that the number of cells was reduced by 65% and 70% in the brain (Fig. 4d) (165.2 ± 36.81 and 139.1 ± 61.94 versus 466.2 ± 60.44). Similarly, the density of GFAP-positive astrocytes was reduced in the APP mouse brains (Fig. 4f, g) compared to vehicle-treated APP mouse brains (Fig. 4e). The number of GFAP-positive astrocytes was reduced by 63% and 71% (332.3 ± 102.9 and 264.1 ± 80.36 versus 901.3 ± 76.55) (Fig. 4h).

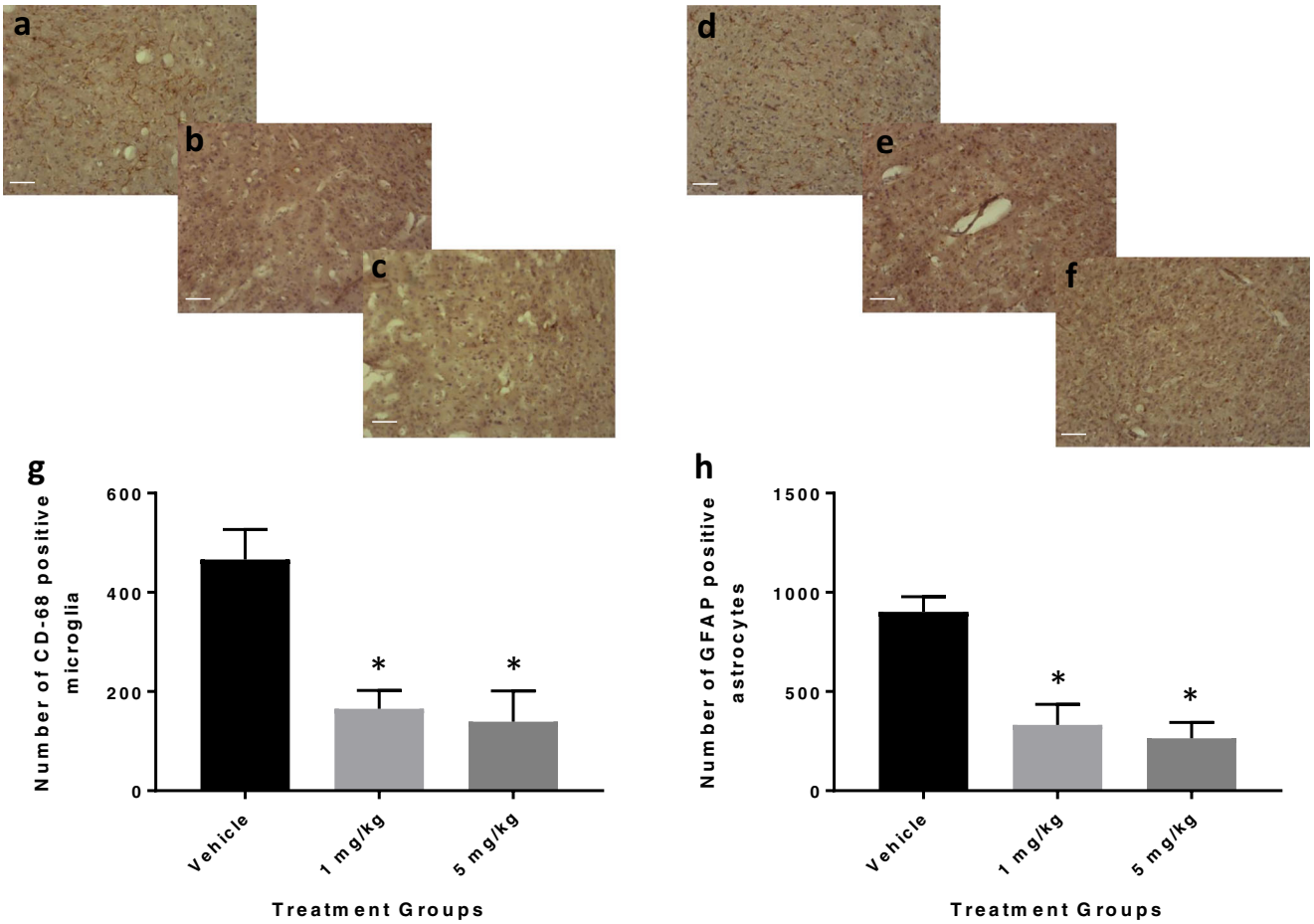

Fig. 4 Quantitative analysis of microglia and astrocytes in the brain of APP/PS1 mice after 4 months of GM6 treatment. Brain sections from APP/PS1 control mice (**a**) or mice treated with GM6 1 mg/kg (**b**) or 5 mg/kg (**c**) were stained with anti-mouse CD68 antibody to detect the microglial cells. Five sections of each mouse were counted and averaged for the number of CD68-positive microglia in the brain (*$P < 0.001$, $n = 20$). Brain sections from APP/PS1 control mice (**e**) or mice treated with GM6 1 mg/kg (**f**) or 5 mg/kg (**g**) were immunostained with anti-mouse GFAP antibody to detect the activated astrocytes. Five sections of each mouse were counted and averaged for the number of GFAP-activated astrocytes in the brain (*P 0.001; $n = 20$). **d**, **h** Quantification of data from the section for CD-68 and GFAP, respectively. Imaged with × 20 objective. Scale bar represents 20 μm

To determine the impact of the GM6 on neuroinflammation in the APP/PS-1 mouse brain, mouse brains were examined for the expression of inflammatory markers. We determined the levels of the cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and transforming growth factor-β (TGF-β) at the end of the experiment (Fig. 5). As seen in the figure, the GM6 significantly reduced TNF-α, IL-1β, and TGF-β levels. The GM6 at 1 mg/kg and 5 mg/kg showed

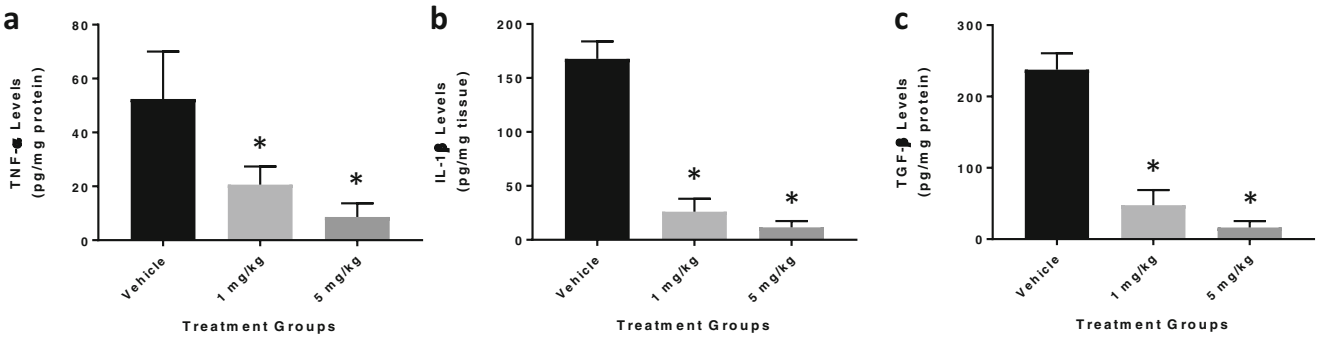

Fig. 5 Reduced inflammatory markers in the brain of APP/PS-1 mice. Mice were treated with vehicle (vehicle) or GM6 for 4 months at 1 mg/kg or 5 mg/kg. Quantitative analysis of TNF-α (**a**), IL-1β (**b**), and TGF-β (**c**) in the APP/PS-1 brains was determined by enzyme-linked immunosorbent assay (ELISA). $n = 20$ per group. *$P < 0.01$ (compared with vehicle)

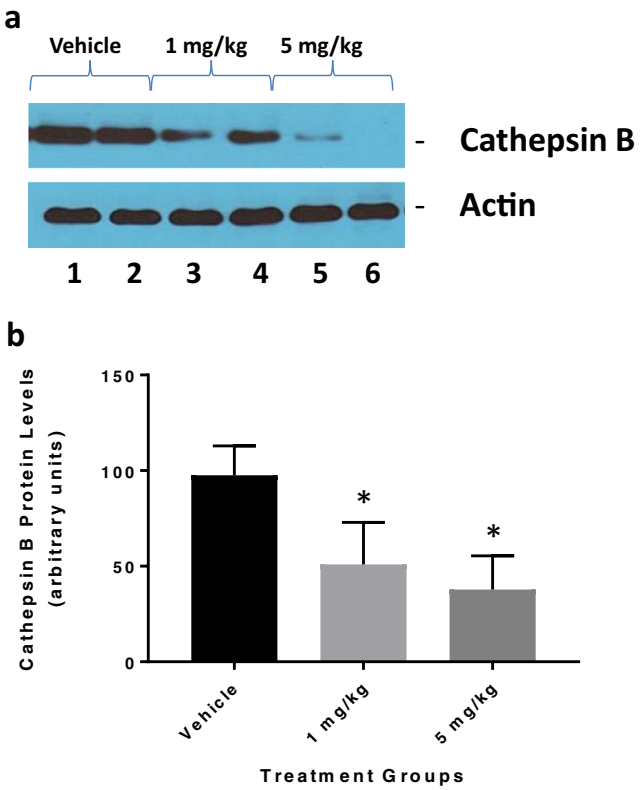

Fig. 6 The effect of GM6 on cathepsin B activity. **a** Brain cathepsin B protein levels were determined at the end of the experiment. Western blot analysis of the cathepsin B levels in the brains of vehicle-treated and 1-mg/kg and 5-mg/kg GM6–treated mice. **b** Quantitative analysis of cathepsin B protein levels of the mice in **a**. The results are expressed as mean ± SD ($n = 20$; *$P < 0.001$ compared to the vehicle group)

an effect reducing the above cytokine levels by 61% and 84% % (TNF-α), 84% and 93% (IL-1β), and 80% and 93% (TGF-β).

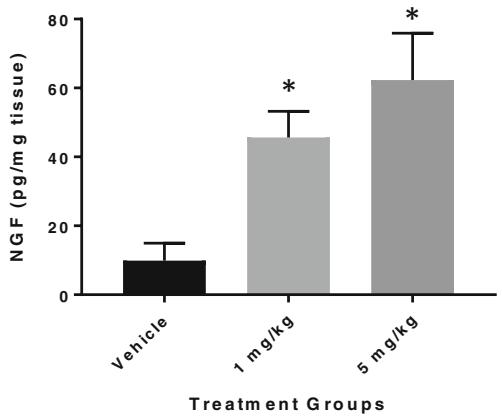

Fig. 7 Concentration of NGF in the brain of APP/PS-1 mice. Bar graphs show the concentration of NGF in picograms (pg) per milligram (mg) of brain tissue from APP/PS-1 mice treated with GM6 (1 or 5 mg/kg) versus APP/PS-1 mice untreated. The difference was statistically significant (*$P < 0.001$; $n = 20$ per group)

Reduced Cathepsin B Expression in GM6-Treated APP/PS-1 Mice

We have shown that cathepsin B is elevated in APP mice and that it contributes to Aβ peptide generation and inflammation [24]. Studies have shown that increased cathepsin B protein and activity can lead to inflammatory mediators such as IL-1β [24]. To understand the role of GM6 and the association with the increase in inflammation (Fig. 6), we established the impact on cathepsin B protein levels. GM6 decreased cathepsin B levels (49% (1 mg/kg) and 62% (5 mg/kg)) in the brain suggesting that reduction in inflammation occurring with treatments was partially related to the inhibition of cathepsin B activity.

Increased Expression of NGF in GM6-Treated Mice

To provide a better understanding as to the role of GM6 in AD pathogenesis, we examined the levels of the trophic factor NGF in brain tissue extracts from the APP/PS-1 mice treated with vehicle or GM6. We observed that the APP/PS-1 GM6–treated mice had significantly higher concentrations of NGF in the brain than APP/PS-1 vehicle–treated mice (45.6 ± 7.66 (1 mg/kg) and 62.3 ± 13.61 (5 mg/kg) vs. 9.90 ± 5.09 pg/mg) (Fig. 7). We found that the GM6 translated to a four- to sixfold increase of NGF content in the brain and that the increase was highly significant ($P < 0.001$).

Discussion

Alzheimer's disease (AD) is a devastating neurodegenerative disorder characterized primarily by progressive memory loss [25]. As the disease advances, more severe symptoms manifest, including disorientation, depression, anxiety, insomnia, confusion, and paranoia [26]. In the final stages of AD memory, cognitive and behavioral changes become more pronounced, and eventually, patients can lose the ability to speak, walk, or even swallow [27]. AD is incurable, represents 60–80% of dementia cases, and is the sixth leading cause of death in the USA [28, 29]. Unfortunately, current treatments for AD do not stop the progressive loss of neurons, but only improve cognition by elevating the decreased acetylcholine levels seen in AD or by limiting glutamate-induced excitotoxicity [30]. Other pharmacological therapies can only manage the coexisting symptoms of AD such as depression and insomnia [31]. Given these lamentable circumstances, new drugs that can stop the progression of AD are badly needed.

AD has been defined histopathologically by the accumulation of extracellular plaques composed of the amyloid β protein (Aβ) and intracellular neurofibrillary tangles of the hyperphosphorylated tau protein [32]. The precise role that these aspects of the disease play in neuronal cell death is still

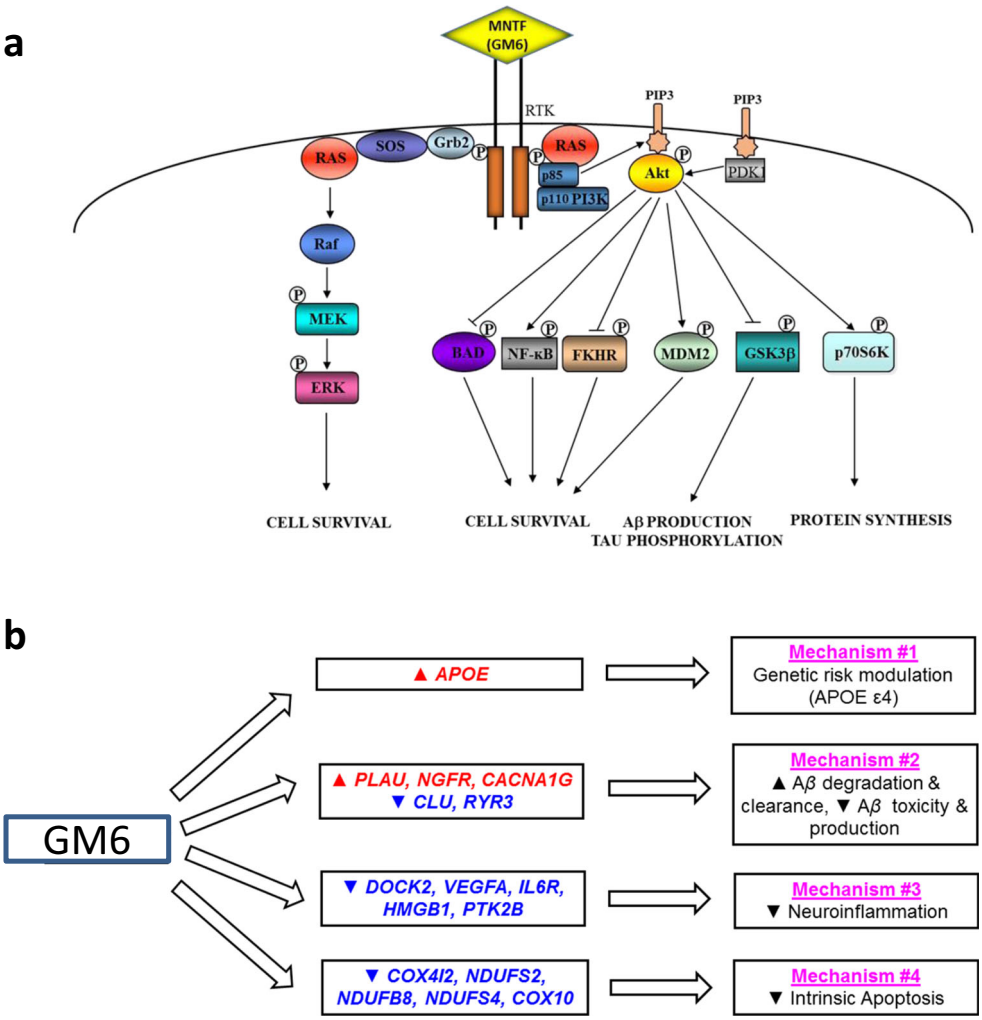

Fig. 8 Mechanistic insight into the mode of action of GM6. The figure helps to elucidate the actions of GM6 in the brain. **a** Intracellular signaling pathways mediated by GM6 (MNTF) in glial and neuronal cells. **b** Overall impact of GM6 on gene expression and global conduits that regulate genetic risk, pathogenic mechanisms, neuroinflammation, and cell death

not known, but the data that has accrued over the decades underscore a number of critical mechanisms. The Aβ pathway hypothesis of AD states that this peptide disrupts synaptic function and can lead to neuronal loss directly [33], or indirectly by activation of glial cells [34]. Neurofibrillary tangle formation occurs following Aβ deposition [35], and the imbalance between the production and removal of the amyloid peptide from brain tissue leads to toxic aggregation and formation of senile plaques. This process stimulates hyperphosphorylation of tau, which is involved in the stabilization of cytoskeletal microtubules, resulting in destabilization of the cytoskeleton and degeneration of nerve cells [36].

MNTF is a trophic factor that has been shown in preclinical models to protect the brain from neurodegenerative disorders and may stimulate regeneration of neurons following injury [10, 16, 37]. Several published experiments have implicated MNTF as an important component associated with the differentiation of embryonic stem (ES) cells into motor neurons [38, 39]. Due to our previous studies and the results indicated above, we decided to test the efficacy of GM6 (the six-amino-acid analog of MNTF) in an animal model of AD [16, 24]. In this study, we demonstrated that i.v. administration of GM6 was capable of attenuating the increase in Aβ peptide levels and amyloid plaque deposition in the APP/PS1 mouse model. Intravenous administration of GM6 at 1 and 5 mg/kg daily dosage revealed a dose-dependent effect in the brain that manifested in a decrease in plaque formation, improved behavioral features, and reduced inflammatory conditions. These results may be due to replication of the endogenous MNTF neurotrophic effects by GM6 [40]. MNTF, and specifically GM6, might function as master regulators of a number of different pathways that can alter Aβ production and inflammation and mediate pathways to enhance recovery and repair [16, 40]. As indicated in the present study, the reduced levels of Aβ might be due to the inhibitory effect on β-secretase and/or an increase in α-secretase activities [24]. In addition, GM6 appears to regulate cathepsin B expression which functions as not only a regulator of the inflammasome, but also as a β-secretase and generating Aβ peptide. We expect that these effects of GM6 involve a number of different pathways rather than limited activation or inhibition of any one pathway individually [41, 42].

Here, we show that animals injected with GM6 exhibited a reduction in inflammation. We found that in the AD mouse models, inflammation seems to play a key role in the disease process [24]. Studies have shown that cytokines (TNF-α, IL-1, IL-2, etc.) and inflammatory mediators (CD-68 and GFAP) can contribute to the pathogenesis [24]. In addition, attenuation of inflammation can limit the development of the pathology [43]. We have shown that both CD-68 and GFAP are elevated following in both the preclinical and clinical states. Modulation of inflammation has been an approach to treat AD; however, this strategy has not proven to be successful [44]. In our hands, the modulation of inflammation via the inflammasome might provide an alternative and more efficacious approach to treating the disease [45]. This may be achieved through the modulation of cathepsin B and cleavage of APP to Aβ as we have shown with GM6. However, whether this is a direct effect on the enzymes or mediated via upstream pathways that facilitate the expression of the genes/proteins needs to be further developed.

Based on the data provided here and in other published and unpublished information, we are beginning to understand the mechanisms associated with MNTF (GM6) activity [9–16, 40, 41, 43, 46]. Figure 8 attempts to address the cellular signaling and overall impact of GM6 in AD. Figure 8a illustrates that GM6 functions through receptor tyrosine kinases (possibly the insulin receptor) and through the activation of p85/p110 and the generation of PIP3 activates Akt. This activation mediates a number of intracellular signaling pathways that help to mediate cell survival downregulate Aβ production and tau phosphorylation (through GSK3β), and reduce inflammation. These processes culminate in the impact of GM6 on gene expression and global conduits that regulate genetic risk, pathogenic mechanisms, neuroinflammation, and cell death (Fig. 8b). Further studies are needed to tease out these potential mechanisms and provide optimal targets for GM6 intervention.

Our data suggest that GM6 (subcomponent of MNTF) can attenuate disease symptoms in an AD mouse model with corresponding improvements in histological and biochemical features. This may reflect replication of MNTF neurotrophic activity as a consequence of GM6 treatment. These studies establish a framework for clinical trials to clarify effects of GM6 in AD and other neurodegenerative disorders.

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Author Contribution Conceived and designed the experiments: JY, ST, CK, DK, and MSK. Performed the experiments: JY, HZ, ST, WM, and MSK. Performed data analysis: CK and MSK. Contributed reagents/materials/analysis tools: DK. Wrote and contributed to the writing of the manuscript: CK, DK, and MSK. All authors discussed the results and edited the manuscript.

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Compliance with Ethical Standards

This research was approved by the IACUC of the University of South Florida, following the guidelines for the Care and Use of Laboratory Animals.

Competing Interest Dorothy Ko is an employee of Genervon and has interest in the company.

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