A dual-targeted multifunctional nanoformulation for potential prevention and therapy of Alzheimer’s disease

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Graphical abstract

Public summary
- Dual-targeted PTCN that were assembled from traditional biomaterials can traverse the blood-brain barrier, target Aβ aggregates, exert antioxidant effects and ameliorate other pathological processes
- The adjustable PTCN dosing strategy designed according to the OS level of AD stages can improve cognitive decline and hippocampal atrophy of APP/PS1 mice in both preventive and therapeutic trials
- PTCN has broad prospects for the early prevention, mild remission and late treatment of AD
A dual-targeted multifunctional nanoformulation for potential prevention and therapy of Alzheimer’s disease

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Antioxidation and adjustable treatment strategies are critical for the effective treatment of Alzheimer’s disease (AD). Here, we design a dual-targeted Prussian blue nanoformulation (PTCN) that can cross the blood-brain barrier and target amyloid beta aggregates further exert antioxidant effects. An adjustable gradient dosing strategy with PTCN is used for the first time to design the preventive and therapeutic trials based on the severity of oxidative stress at different AD stages. The results show that PTCN could effectively ameliorate AD-related pathological processes, improve the cognitive decline, and rescue hippocampal atrophy of APP/PS1 mice in both preventive and therapeutic trials. Altogether, PTCN provided here is a successful combination of three traditional biomaterials with good biosafety, which has broad prospects for the early prevention, mild remission, and late treatment of AD, and is expected to be developed into personalized therapeutic drugs and healthcare products for clinical AD in the future.

Keywords: Alzheimer’s disease; dual-targeted nanoformulation; adjustable dosing strategy; antioxidation; prevention and therapy

INTRODUCTION

Alzheimer’s disease (AD) is a common neurodegenerative disease characterized by cognitive decline. However, most clinical drugs end in failure in AD treatment. Oxidative stress (OS), which refers mainly to oxidative damage induced by excessive accumulation of reactive oxygen species (ROS), plays a significant role in AD.¹,² The brain is highly susceptible to oxidative attacks,³,⁴ and the body’s own antioxidant system normally defends against oxidative attacks to ensure low ROS levels. Under pathological conditions, endogenous antioxidants are extremely vulnerable to damage and interference, which weakens antioxidant functions in the brain. The produced ROS accumulate continuously and cannot be effectively eliminated before resulting in irreversible OS, which interacts with other pathogenic mechanisms during AD and eventually leads to cognitive impairments.⁵–⁷ Therefore, development of exogenous antioxidants that can combat OS is very important for AD treatment.

Many developed exogenous molecular antioxidants exhibit poor performance in cognitive function improvement for several potential reasons.⁸–¹⁰ Firstly, the blood-brain barrier (BBB) blocks access to the brain.¹⁰¹¹ Secondly, some antioxidants that entered the brain diffuse and cannot effectively concentrate in the pathological areas of AD, resulting in insufficient working concentration.¹² Nanoantioxidants can target lesions through surface modification and have been developed to treat diseases associated with OS, including AD.¹³,¹⁴ Notably, Prussian blue (PB)-associated nanoparticles (NPs) have been reported to be effective in removing ROS,¹⁵,¹⁶ which have been generally considered to be biosafe because the major component, PB, has been approved by the U.S. Food and Drug Administration (FDA).¹⁷ In addition, due to the simple preparation process and good reproducibility, PB NPs have considerable potential as exogenous antioxidants for use in AD treatment.

Designing a flexible and adjustable treatment program based on the occurrence and development of AD remains a major challenge. Clinical AD is usually divided into three stages (early stage, mild; middle stage, moderate; and late stage, severe).¹⁸ Currently, most research focuses on the middle and late stages of AD, lacking intervention strategies for the early stage or even the whole disease process. OS has been proposed to be one of the causes of AD and worsens with disease progression,¹⁹–²¹ while other pathological molecules have also been found to exhibit different changes in different stages.²²–²⁴ Therefore, flexible and adjustable antioxidant treatment based on the different degrees of OS in different AD stages would help alleviate other pathologies and enable personalized treatment of AD.

Herein, a dual-targeted PB@PEG-TF/CR NPs (PTCN) was assembled from traditional biomaterials and administrated using an antioxidation-guided adjustable dosing strategy for potential prevention and therapy of AD. The PB NPs in PTCN can exert antioxidant effects to scavenge ROS, and surface modification with transferrin (TF) and Congo red (CR) enables to traverse the BBB and target amyloid beta (Aβ) aggregates, respectively.²⁵–²⁷ The designed antioxidation-guided adjustable dosing strategy with PTCN can effectively attenuate OS in the hippocampus, thereby ameliorating other pathological processes and ultimately improving the cognitive functions and rescuing hippocampal atrophy for both preventive and therapeutic trials of APP/PS1 mice. Altogether, this work reveals the potential of PTCN to be developed as a promising drug and healthcare product for clinical personalized treatment of AD, realizing the early prevention, mild remission, and late treatment of AD.

RESULTS AND DISCUSSION

Synthesis and characterization of PTCN

PTCN was synthesized by simultaneous surface modification of carboxylated PEGylated PB NPs (PB@PEG NPs) with TF and CR (Figure S1). Transmission electron microscopy (TEM) images showed that PTCN exhibited a clear spherical shape and good uniformity (Figure 1A). The hydrodynamic diameter and zeta potential of PTCN (198 ± 18.27 nm, –15.97 ± 0.28 mV) (Figure S2) were larger than those of PB@PEG NPs (88.79 ± 4.50 nm, –35.83 ± 0.23 mV) (Figure S3). Moreover, PTCN exhibited a TF characteristic peak at 270 nm and CR characteristic peaks at both 350 and 500 nm in UV-vis-NIR spectra (Figure 1B). These results indicated that PB@PEG NPs were successfully modified with TF and CR. Notably, the hydrodynamic diameter of PTCN showed no significant changes for 14 days, indicating its excellent stability for further application (Figure S4).

Next, we verified two important functions of PTCN, including crossing of the BBB and targeting of Aβ aggregates in the brain. TF modification has been widely demonstrated to help NPs cross the BBB via TF receptor-mediated endocytosis.²⁶ To demonstrate TF-mediated BBB crossing function of PTCN, a compact monolayer of mouse brain endothelial cells (bEnd.3)
was implanted into transwell filters. Transendothelial electrical resistance (TEER) values during the experiments were all above 180 Ω·cm², indicating the membrane integrity (Figure 1C). After 12 h incubation of NPs, 7.2% of PTCN penetrated through the bEnd.3 monolayers, which was obviously higher than 0.8% of PB@PEG NPs (Figure 1D). After excess free TF was pre-incubated to block TF receptor-mediated endocytosis, the penetration efficiency of PTCN was reduced to 3.5%, indicating that TF receptor-mediated endocytosis played a key role in the penetration process of PTCN. Then, on the basis of removing the endogenous Fe content in the untreated control group, the efficiency of PTCN entering the brain was evaluated by quantitative analysis of the increased Fe content in the brain. The results showed that the brain utilization of PTCN was approximately 5.9% at 3 h post-injection, reached the peak at 12 h post-injection, and retained 24 h after injection, while that of NPs without TF modification was almost zero (Figure 1E). In addition to brain utilization, the mass fraction of Fe in the brain was calculated; the results showed that PTCN increased the brain average Fe content by 90% 3 h after injection, which was significantly higher than the control group (Figure 1E). Furthermore, the presence of PTCN in hippocampal tissues of APP/PS1 mice at 3 h post-injection was further confirmed by biological TEM. Unmodified PB@PEG NPs were found in blood vessels but not in the hippocampus, demonstrating that PB@PEG NPs without TF modification cannot pass through the BBB into the brain (Figure S5). As expected,
PTCN was observed not only in blood vessels but also in the hippocampus. In particular, we not only observed the presence of PTCN on the luminal side of endothelial cells but also the efflux of PTCN on the abluminal side of the BBB, intuitively showing that PTCN can penetrate to the hippocampus from the blood vessels. These results strongly demonstrate that TF-modified PTCN can effectively cross the BBB, enter the brain, and enrich in the hippocampus of mice.

What’s more, it has been reported that CR can be specifically bound to Aβ aggregates by unique hydrogen bonds and that the resulting CR-Aβ complex greatly enhances the red fluorescence of CR, which is typically weak. To verify this specific binding of PTCN with Aβ aggregates, the fluorescence intensities of PTCN and CR combined with Aβ aggregates were measured and compared with those of free CR and Aβ aggregates alone. After binding with Aβ aggregates, both PTCN and CR showed significantly heightened fluorescence intensities, and the fluorescence intensity of PTCN combined with 100 μM Aβ aggregates was approximately 1.5 times stronger than that of PTCN alone (Figure S6). Under an inverted fluorescence microscope, only these complexes were clearly observed and had greatly enhanced red fluorescence (Figure S7). Furthermore, the intravital fluorescence of the PTCN-Aβ complex was also evaluated by an in vivo imaging system. The fluorescence enhancement of the PTCN-Aβ complex was not significantly less than that of the CR-Aβ complex (Figures 1F and 1G). Then, in vivo fluorescence imaging was performed on APP/PS1 mice with high levels of Aβ aggregates in the brain, and demonstrated that only PTCN-treated APP/PS1 mice showed strong fluorescence signals in the brain region, but not in other organs (Figures 1H and 1I), indicating that fluorescence was only visible when the CR on PTCN bound to Aβ in the brain. All these results confirm that PTCN can pass through the BBB and specifically combine with Aβ aggregates.

**ROS-scavenging ability of PTCN**

The ROS-scavenging ability of PTCN is essential for its effective antioxidant activity, as well as in the detection of the BBB. Therefore, we investigated the ability of PTCN to act like multiple enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), as well as its hydroxyl radical scavenging activity (HRS) activity.25,28,29 These activities are key to removing ROS. First, SOD-like activity was evaluated via assessment of O2•− scavenging. Excessive O2•− was generated by the xanthine/xanthine oxidase system and detected by formazan formation using a WST-8 assay kit. The O2•− inhibition rate gradually increased with PTCN concentration (Figure 1J), indicating good SOD-like activity of PTCN. Second, O2•− can be dismutated spontaneously or catalyzed by SOD to form H2O2, which is another important ROS. H2O2 scavenging was used to evaluate CAT-like activity and POD-like activity. CAT-like activity was investigated via assessment of H2O2 decomposition to produce O2. After PTCN was added into 30% H2O2 solution, the amount of bubbles increased with PTCN concentration, while PTCN alone did not exhibit bubbles (Figure S8). Quantitative analysis of dissolved oxygen showed that PTCN catalyzed production of O2 from H2O2 in a concentration- and time-dependent manner (Figure 1K). Furthermore, POD-like activity was investigated by catalyzing the reaction of H2O2 with the natural substrate 3,5,3',5'-tetramethylbenzidine (TMB). PTCN treatment resulted in the characteristic absorbance of the products of H2O2-mediated TMB oxidation at 650 nm, and the effect increased with the PTCN concentration (Figure 1L). These results confirmed the good CAT-like activity and POD-like activity of PTCN. Finally, HRS activity was evaluated via assessment of scavenging of -OH, which is considered to be an initiator of lipid peroxidation. A TiO2/UV system was chosen to generate -OH, which was trapped by 5,5-dimethyl-pyroline-N-oxide (DMPO) and detected by electron spin resonance. The signal intensity of DMPO/ -OH was progressively reduced with increasing PTCN concentrations, suggesting a direct scavenging effect of PTCN on -OH (Figure S9). These results reveal that all the enzyme-like activities of PTCN exhibit concentration dependence and that PTCN is an effective antioxidant with ROS-scavenging activity.

It is generally believed that iron-based NPs can react with H2O2 to cause Fenton reaction and produce -OH, thus mediating biotoxicity.30 Therefore, the assessment of whether PTCN can undergo Fenton reaction was crucial for the correct evaluation of ROS-scavenging activity and the potential biosafety of PTCN. As shown in Figure S10, the FeSO4 and H2O2 system has been considered a classic Fenton reaction model, and the produced -OH could specifically react with salicylic acid to form 2,3-dihydroxybenzoic acid, exhibiting an obvious UV absorption peak at 510 nm.31 However, there was no absorption peak at 510 nm representing -OH generation observed after replacing FeSO4 with PTCN. Our results demonstrated that PTCN had no Fenton-like activity, which was similar to previous studies.32,33 Furthermore, considering that the chemical stability of PTCN in acidic solutions was not negligible for the efficiency of antioxidant activity and TF-mediated BBB crossing, we further assessed the possible iron release from PTCN as well as the detachment of targeting groups in the lysosomal mimic fluid. Acetate buffer, pH 3.6, was used to mimic lysosomal-like acidic condition, and PTCN was incubated with this buffer for 48 h, and then the suspension was centrifuged to obtain precipitate and supernatant. After replacing FeSO4 in the Fenton reaction model with the supernatant and precipitate of PTCN, no absorption peak at 510 nm was observed, and the characteristic absorption peak of PB at 700 nm was still observed in the precipitation (Figure S10), demonstrating that the structure of PB as the core component of PTCN was not affected and that no iron was released from the PTCN in the lysosomal mimic fluid. In addition, the characteristic peaks of TF, CR, and PB were still retained in the precipitate, but not found in the supernatant (Figure S11), indicating that targeting groups were not detached from PTCN in the lysosomal mimic fluid.

**Antioxidant and neuroprotective effects of PTCN in PC12 cells**

To test whether ROS scavenging by PTCN could provide neuroprotection, we evaluated the effect of PTCN on the viability of PC12 cells under H2O2 oxidative shock.35 Cytotoxicity tests were first carried out on PC12. Among the components of PTCN, PB and TF have been approved by the FDA, and CR shows very low cytotoxicity (Figure S12). Cell viability after treatment with PTCN, at all studied concentrations, exceeded 90%, indicating PTCN’s low cytotoxicity (Figure S13). Then, H2O2 was used for oxidative shock to construct a cell model of OS. Oxidative shock significantly reduced cell viability, with greater effects at higher H2O2 concentrations (Figure 2A). To assess the therapeutic effects of PTCN on cells under oxidative shock, cells were first treated with H2O2 and then with PTCN. Cell viability was obviously lower in H2O2-treated cells than in untreated control cells, and the reduction was exacerbated with increasing H2O2 concentrations (Figure 2B). PTCN posttreatment greatly rescued the cell viability, resulting in even higher viability in the posttreatment group than in the control group (Figure 2B), which may have been attributable to the oxygen produced through H2O2 decomposition. To assess the preventive effects of PTCN on oxidative shock in cells, cells were treated with PTCN and then with H2O2. PTCN pretreatment also greatly enhanced cell viability, as the viability was greater in pretreated cells subjected to oxidative shock than in the cells subjected to oxidative shock alone, and the effect increased with increasing PTCN concentration (Figure 2C). These results indicate that PTCN posttreatment and pretreatment can effectively rescue cells and prevent cell death caused by H2O2 oxidative shock, respectively.

Then, the effects of PTCN posttreatment and pretreatment on cellular ROS levels and apoptosis under H2O2 oxidative shock were further studied. DCFH-DA staining was performed to measure cellular ROS levels. Unlike the control treatment and PTCN alone, H2O2 treatment caused excessive ROS production in cells and resulted in shrunken cells and decreased numbers of synapses (Figures 2D and 2E). PTCN posttreatment and pretreatment restored the morphology of cells and synapses, while reducing the elevations in ROS levels caused by H2O2 oxidative shock (Figures 2D and S14). These results were further demonstrated by flow cytometric quantitative fluorescence analysis (Figures 2E and S15). Dual staining with Calcein-AM and propidium iodide was performed to mark live and dead cells. The results showed that cells were destroyed after H2O2 oxidative shock, while PTCN posttreatment and pretreatment prevented the cells from being damaged (Figure S16). Moreover, flow cytometric quantitative apoptosis analysis demonstrated that...
PTCN posttreatment significantly improved the survival rate from 54.6% (under H$_2$O$_2$ oxidative shock) to 83.4%; similarly, PTCN pretreatment increased the survival rate to 84.6% (Figure 2F). These results suggest that PTCN provides excellent neuroprotection against oxidative shock for both preventive and therapeutic treatments.

Behavioral and hippocampal volume assessment for evaluation of the efficacy of PTCN for AD prevention and therapy in vivo

Before in vivo treatments, the biosafety of PTCN was demonstrated by hematoxylin and eosin staining (Figure S17) and routine blood analysis (Table S1). Next, we quantitatively analyzed Fe content in the blood of APP/PS1 mice after intravenous injection of PTCN using ICP-MS. As shown in Figure S18, we observed high blood Fe content at 0.5 h post-injection and a decrease of only 33% at 48 h post-injection, suggesting that PTCN composed mainly of Fe might have longer blood circulation. On the basis of the brain utilization and blood Fe content of PTCN, we designed a gradient dosing strategy for the preventive and therapeutic trials with APP/PS1 mice according to the severity of OS in different AD stages (Figures 3A and S19). For the preventive trial, APP/PS1 mice at the age of 10 weeks were treated with a low dose of PTCN until 16 weeks and then with a medium dose until 22 weeks, while,
Figure 3. Behavioral and hippocampal volume evaluations for the preventive and therapeutic trials of PTCN in vivo (A) Time schedules for the preventive and therapeutic trials. (B) Representative swimming paths of mice in the preventive trial. (C–E) MWM results of the preventive trial: (C) Escape latency. (D) Swimming time spent in the targeted quadrant. (E) Number of times the mice cross over the platform site. In addition, we adjusted the legend of panels G–I as follows: (G–I) MWM results of the therapeutic trial: (G) Escape latency. (H) Swimming time spent in the targeted quadrant. (I) Number of times the mice cross over the platform site. (F) Representative swimming paths of mice in the therapeutic trial. (J and K) (J) T1-weighted MRI of the mice brain and (K) corresponding analysis of hippocampal volume. WT, C57BL/6 mice; AD, APP/PS1 mice; PTCN, PTCN-treated APP/PS1 mice. For all graphs, the data are presented as the mean ± SD and statistical significance was calculated by two-tailed t test; *p < 0.05, **p < 0.01, ***p < 0.001.
for the therapeutic trial, APP/PS1 mice at the age of 25 weeks were treated with a high dose until 31 weeks.

Cognitive behaviors were assessed with a Morris water maze (MWM) test (Figures 3B and 3F). To exclude the possible difference in sensorimotor functions between each group, the swimming speed of each group was first calculated and no significant difference was found among the three groups in both preventive and therapeutic trials (Figure S20). In both the preventive and therapeutic trials, the escape latency of PTCN-treated APP/PS1 mice was significantly shorter than that of untreated APP/PS1 mice (Figures 3C and 3F). Similarly, PTCN treatment significantly increased the number of crossings of APP/PS1 mice over the platform in both the preventive and therapeutic trials (Figures 3E and 3I). Then, we evaluated hippocampal volume changes by magnetic resonance imaging (MRI) since hippocampal atrophy is a recognized biological marker of AD. In both the preventive and therapeutic trials, there were no differences in hippocampal volumes between PTCN-treated APP/PS1 mice and WT mice, while the volumes in untreated APP/PS1 mice were much smaller than those in WT mice (Figures 3J, 3K, and S21). Overall, these results suggest that, as AD progresses, cognitive impairment gradually worsens, and the impairment is accompanied by pronounced hippocampal atrophy; however, PTCN treatment more effectively alleviated these results suggest that, as AD progresses, cognitive impairment gradually worsens, and the impairment is accompanied by pronounced hippocampal atrophy; however, PTCN treatment more effectively alleviated cognitive impairments and hippocampal atrophy.

Ascertainment of the pathological mechanisms targeted by PTCN antioxidant treatment

To further explore the pathological mechanisms targeted by PTCN antioxidant treatment, pathological changes in the mouse hippocampus were detected monthly during both preventive (Figure 4A) and therapeutic trials (Figure 5A) by western blot analysis. First, lipid peroxidation and DNA oxidative damage represented by 4-hydroxynonenal (4-HNE) and 8-hydroxyguanosine (8-OHG), respectively, were analyzed to evaluate the alleviation of OS by PTCN. The 4-HNE assay results showed that 4-HNE appeared at 10 weeks in APP/PS1 mice and that the levels gradually increased (Figure 4A); however, the levels were slightly reduced after PTCN treatment in the therapeutic trial (Figure 4B). Notably, the levels of 4-HNE were higher in the older APP/PS1 mice than in the younger mice (Figure 5A) and were significantly reduced by PTCN treatment in the therapeutic trial (Figure 5B). The 8-OHG results showed that, during the preventive trial, a difference in 8-OHG level between APP/PS1 mice and WT mice appeared at 10 weeks, but the difference was gradually eliminated by PTCN treatment (Figures 4A and 4B). During the therapeutic trial, the 8-OHG level in APP/PS1 mice was quite high and was also greatly reduced by PTCN treatment to reach the same level as that in WT mice (Figures 5A and 5B). These results indicated that lipid peroxidation and DNA oxidative damage occurred at the beginning of the AD process and were gradually aggravated. PTCN treatment more effectively alleviated DNA oxidative damage than lipid peroxidation in the early stage of AD, while it alleviated both in the late stage of AD.

Next, we investigated other AD-related pathological changes that have been reported to interact with OS, including Aβ aggregates, mitochondrial dysfunction, inflammation, synaptic damage, and neuronal apoptosis. PTCN treatment slightly but nonsignificantly reduced Aβ aggregates (Figures 4A, 4B, 5A, and 5B) in APP/PS1 mice. Given the apparent improvements in cognitive behavior and hippocampal volume, Aβ is very likely not the primary cause of cognitive impairments in AD, which is consistent with the fact that most clinical drugs for Aβ have failed. Obvious increases in dynamin-related protein 1 (DRP1) levels and decreases in mitofusin 2 and cytochrome c oxidase subunit IV levels were observed from 10 to 32 weeks in the hippocampus of APP/PS1 mice, indicating mitochondrial dysfunction (Figures 4A and 5A). PTCN treatment regulated the levels of mitochondrial function markers to close to normal levels, except for DRP1 (Figures 4C and 5C). The continuous activation of astrocytes and microglia as well as the increased levels of inflammatory cytokines in APP/PS1 mice indicated severe inflammation (Figures 4A and 5A). Notably, PTCN treatment markedly inhibited microglial activation and reduced inflammatory cytokine levels in the preventive trial but only slightly inhibited microglial activation and
Conclusion

In summary, PTCN, which consists of dual-targeted PTCN, was constructed for potential prevention and therapy of AD. Compared with the current nanotechnology to treat AD, the designed strategy in this study is superior in the following three aspects. Firstly, this work provides a simple and easy assembly of three traditional biomaterials. PB is a clinical therapeutic drug approved by the FDA, TF is a transporter of iron in human plasma that mediates BBB crossing, and CR is a common clinical diagnostic reagent for amyloidosis, all of which are widely used biomaterials and have good biosafety for in vivo application. These three components are connected with chemical bonds to form multifunctional PTCN, revealing a new combination of traditional biomaterials. Our results validated the function of the above three components and demonstrated that PTCN with excellent biosafety could largely accumulate in AD lesions and exert substantial antioxidant effects. Secondly, this work first proposes an antioxidant-guided gradient dosing strategy that can be implemented throughout the course of AD from prevention to therapy. At present, the application of nanoantioxidant in AD treatment has gradually attracted attention, but most studies focus on the late treatment of severe cognitive impairment, and there is still a lack of research on early prevention and mild remission. In this work, the doses of PTCN can be adjusted according to the OS levels at different stages of the AD process, and the results showed that this strategy could effectively inhibit OS, ameliorate other pathological processes to varying degrees, and ultimately rescue cognitive impairment and hippocampal atrophy of APP/PS1 mice in both preventive and therapeutic trials. Finally, PTCN has great potential to be developed as a therapeutic drug and healthcare product for AD. As mentioned above, PTCN is an integration of three traditional biomaterials with a simple preparation method and high repeatability, and both in vitro and in vivo results have confirmed good biosafety of PTCN, which is conducive to future industrialization and clinical application. More importantly, PTCN can be applied to different stages of AD through the above antioxidation-guided gradient dosing strategy. We even hope that PTCN can be developed as a healthcare product similar to vitamins, which can be taken in a healthy state to prevent future occurrences. In addition to AD, the roles of OS in the occurrence and development of other diseases have received increasing attention, and PTCN may have considerable potential as a broad antioxidant for the treatment of various other OS diseases.

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AUTHOR CONTRIBUTIONS

Y.D., J.C., and D.Z. conceived and designed the experiments. D.Z. conducted most experiments. Y.T. helped with experiments in cells and MWM test. X.S. performed the detection of hippocampal volume and contributed analysis tools in MRI. C.Z. helped with flow cytometer analysis. Y.D. and D.Z. analyzed the data and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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