Spearmint extract Neumentix downregulates amyloid-β accumulation by promoting phagocytosis in APP23 mice

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Abbreviations used: 4-HNE, 4-hydroxynonenal; 8-OHdG, 8-hydroxydeoxyguanosine; AD, Alzheimer's disease; AKT, protein kinase B; Aβ, amyloid-β; ANOVA, analysis of variance; BSA, bovine serum albumin; CA1, cornu ammonis 1; CA3, cornu ammonis 3; CD11c, integrin αX; CTX, cortex; CNS, cerebral nervous system; DAB, diaminobenzidine; DAM, disease-associated microglia cell; DG, dentate gyrus; EGCG, epigallocatechin-3-gallate; HI, hippocampus; Iba-1, ionized calcium binding adapter protein 1; LPL, lipoprotein lipase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RA, rosmarinic acid; ROS, reactive oxygen species; SD, standard deviation; TH, thalamus; Trem2, triggering receptor expressed on myeloid cells 2; TNF-α, tumor necrosis factor-α

Keywords: Alzheimer's disease; amyloid-beta; inflammation; Neumentix; phagocytosis; survival rate

Abstract

In recent years, many researchers have focused on natural compounds that can effectively delay symptoms of Alzheimer's disease (AD). The spearmint extract Neumentix, which is rich in phenolic compounds, has been shown to reduce inflammatory responses and oxidative stress in mice. However, the effect of Neumentix on AD has not been thoroughly studied. In this study, APP23 transgenic female and male mice were administered Neumentix orally from 4 to 18 months of age at a dosage of 2.65 g/kg/day (containing 0.41 g/kg/day of rosmarinic acid). The impact was evaluated by behavioral tests and histological analyses and compared with APP23 mice to which Neumentix was not administered. The results showed that Neumentix administration increased the survival rate of APP23 mice and effectively reduced Aβ accumulation by enhancing its phagocytosis by microglial cells. These findings suggest that Neumentix is a potential natural nutritional treatment for improving the progression of AD.

Introduction

Alzheimer's disease (AD) is the primary cause of dementia. It is characterized by senile plaques and neurofibrillary tangles due to the accumulation of amyloid-beta (Aβ) and phosphorylated tau, respectively, in the brain, especially in the medial temporal lobe and neocortical structures[1]. However, several aspects of how AD progresses remain unknown. Currently, curative therapies are lacking, so treatment strategies mainly focus on slowing down symptoms and managing behavioral change[2]. Thus, much research is focused on finding potentially modifiable risk factors, such as diets and blood pressure, which can play a role in AD development.

Polyphenols are considered beneficial to the inhibition of AD development through several biological mechanisms[3]. As one example, rosmarinic acid (RA) can strongly inhibit Aβ aggregation, alleviating synaptic toxicity in vitro and in vivo[4]. Neumentix, a spearmint extract rich in phenolic compounds (approximately 30%, including salvianolic,

caffeoylquinic, and hydroxyphenyl propanoic acids), also contains RA (approximately 15%)[5]. We previously reported that Neumentix, when used as a dietary supplement, inhibited inflammation and oxidative stress in an ischemic stroke mouse model[6, 7]. We wanted to determine whether Neumentix, a natural phenolic-rich product, had any beneficial effects on AD, or whether it could improve AD symptoms. In this study, we thus focused on the effect of Neumentix on an AD mouse model and evaluated the mechanism underlying the action of Neumentix on AD pathology.

Materials and methods

Animals

All experimental procedures were conducted using a protocol approved by the Animal Committee of the Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University (OKU-2022589). APP23 mice overexpress the human APP751 isoform, which contains the Swedish double mutation (KM670/671NL) under the control of the 6.7 kbp murine Thy1 promoter[8]. APP23 male and female mice were

maintained as heterozygotes by mating APP23 male mice with C57BL/6J female mice. All mice, which were purchased from Japan SLC Inc. (Shizuoka, Japan), were maintained in a 12/12 h light-dark cycle room under constant humidity and a temperature of around 23 ℃.

Neumentix

The Neumentix used in this study was extracted from dry spearmint (*Mentha spicata* L.) using a method involving a solid phase microextraction (SPME) method. As noted in a previous paper, Neumentix contains RA and other RA derivatives (such as sageinic acid), which together account for about 88% of the total detected phenolics. This is followed by salvianolic acids, which make up 5.6% of the total phenolics, and caffeoylquinic acids, which constitute 1.2% of the total phenolics. Hydroxycinnamic acids, including caffeic acid (an ester of caffeic and tartaric acids), represent about 1.1% of the total phenolics. Other detected phenolic groups, such as flavonols, flavanones, flavones, hydroxybenzoic acids, and hydroxycinnamic acids, collectively make up approximately 1% of the total phenolic compounds.[5]

Experimental groups

Mice were randomly separated into two groups in this study: the APP23 group (males: n = 10; females: n = 10), and the APP23+Neumentix group (males: n = 10; females: n = 10). However, at the time of sacrifice, the number of surviving mice was lower in both the APP23 group (males: n = 5; females: n = 4) and the APP23+Neumentix group (males: n = 9; females: n = 8). There are two main reasons for the death of animals, the first being that APP23 transgenic mice became ill after aging and died of epilepsy, while the other mice died from dieting during the behavioral test. These processes were reported in detail in the animal experiment plan, and there were no unethical steps.

Drug treatment

Mice in the APP23 group received no special treatment whereas for the APP23+Neumentix group, Neumentix (Kemin Japan, Tokyo, Japan) was dissolved in tap water and administered orally to mice that were between 4 and 18 months old. Regarding the dosage, the target administered dose of Neumentix was 6.67g/kg/day (containing 1 g/kg/day of RA) based on

previous findings[9, 10]. To prepare the Neumentix solution, we conducted a preliminary study with 14 mice, confirming the average body weight (25.37 g) and daily Neumentix intake volume (8.81 mL) of the mice (Supplementary Fig. 1a). Based on these findings, the dosing concentration was prepared according to the mice's body weight and the expected daily Neumentix intake volume of 8.81 mL. The required dose for the mice was calculated by multiplying the dosage by the average body weight of the mice in each group, with the average body weight being measured every two weeks, and the result was divided by 8.81 mL to obtain the concentration of the solution. For example, for a 25 g mouse, the required dose of Neumentix was 166.75 mg/day, and dividing by 8.81 mL resulted in a concentration of 18.93 mg/mL. However, after administration, daily monitoring over one month revealed that the actual water intake of the mice averaged only 3.50 mL/day (Supplementary Fig. 1b). This reduction in water intake resulted in an actual RA dose of 0.41 g/kg/day, corresponding to a Neumentix dose of 2.65 g/kg/day. Recognizing that the actual water intake was insufficient to achieve the

target dose, we attempted to adjust the dosing concentration. However, this led to a further decline in water intake, potentially compromising the health of the mice. To ensure their well-being, we maintained the original dosing concentration throughout the study.

Behavioral analysis

An 8-arm radial maze test was used to evaluate behavioral memory (mainly working memory), as described in previous literature[11, 12]. In brief, in this test, mice were deprived of food as part of a schedule designed to maintain ≤15% body weight deficiency but had free access to water. Mice were allowed to adapt to the maze once a day for 7 consecutive days before the test. For each test trial, each mouse was allowed to make arm choices until either all four pellets (F0071-J50; Bio Serv, Flemington, NJ, USA) had been eaten, or until 5 min had elapsed. Re-entry into the baited arms previously visited was scored as a working memory error. The radial maze task was performed separately when mice were 6, 11, and 18 months old.

Tissue preparation

When mice were 18 months old, they were deeply anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg). Intracardiac blood was collected for serum analysis, then transcardially perfused with 20 mL of ice-cold phosphate-buffered saline (PBS) followed by 20 mL of ice-cold 4% paraformaldehyde (PFA) in 0.10 mol/L phosphate buffer. Brains were removed and post-fixed in 4% PFA for 24 h at 4 ℃. Floating coronal sections (50 μm in thickness) were sliced with a vibrating blade microtome (LEICA VT1000S; Leica, Germany).

Quantitative analysis of antioxidative activity and oxidative stress

To evaluate antioxidant capacity and reactive oxygen species (ROS) in serum, OXY-adsorbent (OX191207; Wismerll, Tokyo, Japan) and d-ROMs tests (DI-003b; Wismerll) were assayed with a spectrophotometer according to the manufacturer's instructions.

Histochemistry and immunohistochemical staining

For Nissl staining, three sections between -1.34 mm and -2.30 mm adjacent to the bregma were laid onto a microscope glass slide and dried for two days, immersed in 1% cresyl violet for 5 min at room temperature,

dehydrated in graded alcohol, and overlaid with a glass slide.

For immunohistochemical staining, brain sections were immersed in 0.6% periodic acid to block intrinsic peroxidase and treated with a mixture of 5% bovine serum albumin in 50 mM PBS and 0.1% Triton X-100 for 3 h to block any non-specific antibody binding, then incubated at 4 ℃ overnight with the primary antibody. We used the following primary antibodies: rabbit anti-human amyloid β (Aβ) (1-40) antibody (1:500, 18580; Immuno-Biological Laboratories, Gunma, Japan), rabbit anti-human Aβ (1-42) antibody (1:500, 18582, Immuno-Biological Laboratories), mouse anti-4-hydroxynonenal (4-HNE) antibody (1:50, MHN-020P; JaICA, Shizuoka, Japan), mouse anti-8-hydroxydeoxyguanosine (8-OHdG) antibody (1:20, MOG-020P; JaICA), and tumor necrosis factor-alpha (TNF-α) (1:100, AF-410-NA; goat, R&D systems, Minneapolis, MN, USA). The sections were washed in PBS and biotinylated secondary antibodies corresponding to the respective hosts (1:500; Vector Laboratories, Newark, CA, USA) for 2 h at room temperature. Sections were then treated with

avidin-biotin-peroxidase complex solution (1:100, PK-6104; Vector Laboratories) for 30 min and incubated with diaminobenzidine (DAB) standard dissolved in PBS. The treated sections were laid on a glass slide, dried for 2 days, then visualized under a light microscope (BX-51; Olympus, Tokyo, Japan).

Double immunofluorescent histochemistry

For immunofluorescent histochemistry, after incubation in 5% bovine serum albumin (BSA) in PBS with 0.1% TritonX-100 at room temperature for 3 h, sections were incubated at 4 ℃ overnight with one of several primary antibodies: rabbit anti-ionized calcium binding adapter protein 1 (Iba-1) antibody (1:1000, 019-19741; Wako, Osaka, Japan), mouse anti-triggering receptor expressed on myeloid cells 2 (Trem2) antibody (1:200, AF1729; R&D systems), mouse anti-lipoprotein lipase (LPL) antibody (1:100, ab21356; Abcam), and Armenian hamster anti-integrin αX (CD11c) antibody (1:150, 14-0114-82; Thermo Fisher Scientific, Waltham, MA, USA). Following washes with PBS, sections were incubated with fluorescent secondary antibodies. The antibodies

against Iba-1, Trem2, and LPL were detected with a suitable secondary antibody conjugated with Alexa Fluor (Invitrogen, Carlsbad, CA, USA). The antibodies against CD11c were detected with a suitable secondary antibody conjugated with CyTM3 (Jackson ImmunoResearch Inc., West Grove, PA, USA). Fluorescent signals were visualized with a confocal microscope (LSM-780; Zeiss, Jena, Germany).

Semi-quantitative analysis

For semi-quantitative analysis of Aβ accumulation, 4-HNE, 8-OHdG, and TNF-α, three sections between -1.34 mm and -2.30 mm adjacent to the bregma were used following immunofluorescent staining. For each section, eight areas on the cerebral cortex (CTX), hippocampus (HI), and thalamus (TH) on both the left and right sides were captured by the light microscope.

For semi-quantitative analysis of Trem2/Iba-1, LPL/Iba-1, and CD11c/Iba-1, two sections between -1.34 mm and -2.30 mm adjacent to the bregma were used following immunohistochemical staining. For each section, six areas on the CTX and HI on both the left and right sides were

randomly captured by the confocal microscope.

The number of positive cells in each position was counted using image processing software (ImageJ, version 2.1.0; NIH, Bethesda, MD, USA). For DAB staining, positive cells were identified by applying thresholds, and the average number of positive cells per 0.14 mm^2 was calculated. Signal intensity was analyzed by measuring mean gray values and integrated densities after thresholding. For fluorescence staining, the Hue channel (threshold 205–225) was isolated to detect yellow regions, and signal intensity was quantified.

Statistical analysis

Statistical software (Prism 9 for macOS version 9.1.1; Graphpad Software, LLC, Boston, MA, USA) was used to analyze the data of this experiment. Power analyses with G*Power (G*Power 3.1.9.6; NRW, Germany) determined sample sizes of 17 mice per group were necessary with a two-sided 5% significance level and 80% power. Survival rate was analyzed by the log-rank (Mantel–Cox) test. The serial 8-arm radial maze test results were analysis by repeated measure two-way analysis of

variance (ANOVA). The remaining results are expressed as the mean ± standard deviation (SD). A one-way ANOVA, followed by Bonferroni's multiple comparison test, was performed to verify differences in immunohistochemical expression. The results of the normality test, analytical methods, post-hoc analysis, and p-values have been tabulated in Supplementary Fig. 1b. In all statistical analyses, statistical significance was considered at $p < 0.05$.

During data analysis, we removed all data from mice that died during the experiment, such as their behavioral test data. Other than that, all remaining data were included in the results.

Results

Survival rate and behavioral test

We measured mouse weight every four weeks during treatment and recorded survival rate. The survival rate at 18 months of the APP23+Neumentix group was 90% (n = 17), which was significantly higher ($p = 0.0152$; log-rank test; Fig. 1a) than that of the APP23 group

(53%, n = 9).

Across separate two-way repeated measures ANOVA analyses, the 8-arm radial maze tests showed there were no consistent significant effects for the APP23 and APP23+Neumentix groups in mice that were 6, 11, and 18 months old (Fig. 1b).

Neuronal loss

Compared with the APP23 group, Nissl staining showed no significant change in the number of neurons in the APP23+Neumentix group for 18 months old mice in the cornu ammonis 1 (CA1), cornu ammonis 3 (CA3), and dentate gyrus (DG) (Fig. 2).

Aβ accumulation

DAB staining of Aβ40 and Aβ42 indicates the accumulation of Aβ plaque in the brains of 18 months old APP23 mice. Compared with the APP23 group, the APP23+Neumentix group showed no significant difference in Aβ40 intensity in CA1, DG, and TH (Fig. 3a, c). However, Aβ40 decreased significantly in CTX in the APP23+Neumentix group compared with the APP23 group ($p = 0.0454$; one-way ANOVA test with

Bonferroni's correction; Fig. 3a, c).

Also in 18 months old mice, compared with the APP23 group, Aβ42 immunohistochemistry showed no significant difference between CA1 and TH in the APP23+Neumentix group (Fig. 4b, d). However, Aβ42 was significantly attenuated in CTX and DG in the APP23+Neumentix group relative to the APP23 group ($p < 0.0001$ and $p = 0.0044$, respectively; one-way ANOVA test with Bonferroni's correction; Fig. 4b, d).

Oxidative stress in the brains of APP23 mice

In 18 months old mice, compared with the APP23 group, Neumentix did not decrease oxidative stress, as evaluated by d-ROMs ($p = 0.98$; Mann–Whitney test; Fig. 4a, b). Similar to the d-ROMs result, the OXY-adsorbent test showed no significant difference between the two groups (Fig. 4a, b).

The pixel intensity of 4-HNE showed no significant differences between the two groups in the CTX, CA1, CA3, DG, and TH (Fig. 4c, e). There were no significant differences in the number of 8-OHdG-positive cells between the two groups in the CTX and other regions (CA1, CA3,

DG, and TH) (Fig. 4d, f). Since they displayed fewer 8-OHdG-positive cells, CA1, CA3, DG, and TH were collectively analyzed.

Neuroinflammation in the brains of APP23 mice

There was no significant difference in integrated intensity in the immunohistochemistry of TNF-α between the two groups in the CTX, CA1, CA3, TH, and DG (Fig. 5).

Trem2 and microglia cells in the brains of APP23 mice

To detect the activity of Trem2 in microglia, double immunofluorescence analysis using Trem2 and Iba-1 antibodies was performed. Double immunofluorescence in the APP23 and APP23+Neumentix groups showed about 85% Trem2 colocalization with Iba-1-positive microglia cells around the Aβ plaque.

There was no significant difference in the number of double-positive Trem2/Iba-1 cells between the two groups in the CTX and HI (Fig. 6a, b). The integrated intensity of the Iba-1 signal showed no significant difference between the two groups in the CTX and HI (Fig. 6a, c).

DAM cells and microglia cells in the brains of APP23 mice

LPL and macrophage marker CD11c have been recommended as markers of DAM cells[13]. Double immunofluorescence in the APP23 and APP23+Neumentix groups showed that about 43.5% LPL and 27.9% CD11c colocalized with Iba-1-positive microglia cells around the Aβ plaque.

There was no significant difference in the number of LPL/Iba-1 double-positive cells between the two groups in the CTX and HI (Fig. 7a, b).

There was no significant difference in the number of CD11c/Iba-1 double-positive cells in HI between the two groups (Fig. 8a, b). However, the number of CD11c/Iba-1 double-positive cells showed a significant increase in the APP23+Neumentix group in the CTX compared with the APP23 group ($p = 0.0082$; one-way ANOVA test with Bonferroni's correction; Fig. 8a, b). We analyzed the integrated intensity of Iba-1 in the DAM-positive area. The results showed no significant difference between the two groups in the LPL expression area in the CTX and HI (Fig. 7a, c). The integrated intensity of Iba-1 increased significantly in the CD11c

expression area between the CTX and HI of the APP23+Neumentix group compared with the same regions of the APP23 group ($p = 0.0077$ and $p = 0.0007$, respectively; one-way ANOVA test with Bonferroni's correction; Fig. 8a, c).

Discussion

The results of the present study show that oral administration of phenolic-rich Neumentix significantly increased the survival rate of mice (Fig. 1), attenuated Aβ accumulation (Fig. 3), and increased the number of activated microglia cells expressing CD11c at the late stage of AD (Fig. 8).

This is the first time to report that Neumentix significantly increased the survival rate of AD mice compared with the vehicle group at 18 months (Fig. 1). Neumentix, a phenolic-rich spearmint extract developed as a dietary and food supplement, has been proven safe in both human and animal studies[14]. In addition to its potential disease-modifying effects in AD, the survival benefit observed in our study may reflect a broader

improvement in systemic health. Neumentix has demonstrated antioxidative and anti-inflammatory effects in ischemic stroke models, and was also reported to increase body weight in aged mice, suggesting a supportive role in maintaining overall physiological function[6, 7]. These findings raise the possibility that Neumentix enhances systemic resilience, potentially reducing mortality through general health promotion. Although wild-type controls were not included in our study, future research is warranted to determine whether this survival effect is disease-specific or indicative of a broader physiological benefit. Similar systemic effects have been reported for other polyphenols such as resveratrol and epigallocatechin-3-gallate (EGCG)[15-17]. This survival benefit may be partially attributable to the observed reduction in Aβ accumulation (Fig. 3)[8, 18]. Previous studies reported that Neumentix supported cognitive performance, including overall working memory[19, 20]. However, this type of effect was not shown in our results. We suspect that mortality selection bias (Fig. 1a) might be one reason why no significant differences were observed in the behavioral test (Fig. 1b) and neuronal

loss test (Fig. 2). In addition, overtraining during the behavioral test may have minimized behavioral variability and masked subtle group differences.

Microglia, the principal innate immune cells, act as the first line of defense against immune responses in the cerebral nervous system (CNS), and can be activated in response to certain environmental stimuli[21]. In AD, a specialized subset of microglia are termed disease-associated microglia cells (DAMs). These cells, typically marked by high expression of genes such as *CD11c* and *LPL*, are characterized by a high expression of phagocytosis and lipid metabolism[13, 22, 23] (Supplementary Fig. 2). In our study, we observed that the number of CD11c/Iba-1 double-positive cells increased significantly in the APP23+Neumentix group (Fig. 8), suggesting activation of a DAM-like phenotype in response to treatment. In previous studies, $CD11c^{+}$ microglia have been associated with improved Aβ clearance, phagocytosis that decreases Aβ aggregation, reduced plaque toxicity, and enhanced tissue remodeling[24-26]. These cells may help to counteract chronic neuroinflammation and contribute to

maintaining local immune homeostasis in the aging AD brain.

Trem2, a negative regulator of inflammation, can inhibit the secretion of pro-inflammatory cytokines, and enhances the phagocytic function of microglia[26-28]. Trem2 reduces neuroinflammation in AD by regulating microglia polarization from M1 towards the M2 phenotype and plays an important role in the activation of DAMs[29, 30]. DAM activation is a mechanistically coupled temporal event that must first be initiated in a Trem2-independent pathway, followed by activation of the Trem2-dependent program[13]. Trem2 plays a central role in lipid metabolism and phagocytosis, and its loss impairs the microglial response to amyloid plaques[31, 32]. In our study, however, we did not observe significant changes in the number of $Trem^+$ microglia following Neumentix treatment (Fig. 6), despite a clear increase in $CD11c^+$ cells. This discrepancy suggests that the DAM-like microglial response may have been triggered via a Trem2-independent mechanism. One possible explanation is that Neumentix, as a polyphenol-rich compound, may activate alternative immune-modulatory pathways that bypass

Trem2-dependent transcriptional programming. This result highlights the complexity and heterogeneity of microglial responses in AD and suggests that Neumentix may exert its effects through alternative immunomodulatory mechanisms, rather than classical Trem2-driven signaling.

For instance, polyphenols such as resveratrol have been shown to modulate microglial polarization and phagocytosis through SIRT1 activation and suppression of NF-κB signaling[17, 33]. More broadly, polyphenols—including RA, resveratrol, and EGCG—have been shown to activate conserved stress-response mechanisms, notably the Sirtuin 1(SIRT1) and NRF2 pathways[16, 33, 34]. SIRT1 modulates inflammation, promotes mitochondrial function, and enhances cellular resilience, while NRF2 signaling increases antioxidant capacity by upregulating cytoprotective genes such as *HO-1* and *SOD2*, thereby mitigating oxidative and nitrosative stress commonly associated with AD[16, 34-36]. The present study was only able to reveal the effect of Neumentix, a mixture of polyphenols, so further investigation is needed to determine whether

this effect is due to any one polyphenol or a combination of factors.

In addition, the NRF2-regulated vitagene network, which includes cytoprotective genes such as HO-1, thioredoxin, and Hsp70, plays an important role in long-term cellular adaptation to oxidative and inflammatory stress. Calabrese et al. emphasized its therapeutic potential in neurodegenerative diseases[36, 37]. Although we did not directly assess SIRT1 or NRF2 activation in this study, the polyphenol-rich composition of Neumentix suggests it may engage multiple protective pathways simultaneously. Such multi-targeted strategies may offer greater efficacy in multifactorial diseases like AD than single-compound interventions.

In summary, this study is the first to report that Neumentix increases the survival rate of aging APP23 mice, potentially by enhancing microglial phagocytosis and reducing Aβ aggregation. These findings suggest that Neumentix, a polyphenol-rich spearmint extract, may serve as a promising natural nutritional intervention to mitigate AD pathology or slow disease progression.

Declaration of Competing Interests

Neumentix was kindly provided by Kemin Foods (Des Moines, IA, USA). Kemin Foods was not involved in the study conception, collection, interpretation, or analysis of data, or writing the manuscript. The authors declare no other conflicts of interest.

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Authors' contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization: KA, TY and XRH; Methodology: RSOE, XRH; Investigation: YF, XRH, YTB; Formal Analysis: XRH; Resources: TY; Data curation: HMS; Writing - Original Draft: XRH; Writing -

Review & Editing: HI, RM and TY; Visualization: XRH; Supervision, TY; Funding Acquisition, TY.

Figure legends.

Fig. 1. Neurobehavioral analysis of APP23 mice sacrificed at 18 months. (a) Survival rate of the APP23 and APP23+Neumentix groups was 52.9% (n = 9) and 89.5% (n = 17), respectively. (b) The 8-arm radial maze test indicated no significant differences in errors between the APP23 and APP23+Neumentix groups at 6, 11, and 18 months (*$p < 0.05$).

Fig. 2. Neuronal loss was evaluated by Nissl staining in the hippocampal CA1, CA3, and DG. (a, b) Analysis of pixel intensity showed no significant differences between the APP23 and APP23+Neumentix groups. Scale bar: 50 μm.

Fig. 3. Aβ accumulation by immunohistochemical staining in the CTX, CA1, DG, and TH. (a, c) Aβ40 pixel intensity decreased significantly in

CTX in the APP23+Neumentix group compared with the APP23 group. (b, d) Aβ42 pixel intensity decreased significantly in the CTX and DG in the APP23+Neumentix group compared with the APP23 group (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$). Scale bars: 50 μm.

Fig. 4. Quantitative analysis of antioxidative activity and oxidative stress. (a) d-ROMs test and (b) OXY-adsorbent test showed no significant differences between the two groups. DAB staining of (c, e) 4-HNE and (d, f) 8-OHdG showed no significant differences between the two groups in the CTX, CA1, CA3, DG, or TH. Scale bars: 50 μm.

Fig. 5. Quantitative analysis of an inflammatory marker TNF-α in the CTX, CA1, CA3, DG, and TH. (a, b) DAB staining of TNF-α showed no significant differences between the two groups in the CTX, CA1, CA3, DG, or TH. Scale bars: 50 μm.

Fig. 6. Double immunofluorescence staining of Trem2/Iba-1. (a) Double

immunofluorescence staining shows around 85% Trem2 colocalization with Iba-1-positive microglia cells around the Aβ plaque. (b) The number of double-positive Trem2/Iba-1 cells and (c) the integrated intensity of the Iba-1 signal showed no significant differences between the two groups in the CTX and HI. Arrows indicate Trem2/Iba-1 double-positive signals. Scale bars: 50 μm.

Fig. 7. Double immunofluorescence staining of LPL/Iba-1. (a) Double immunofluorescence staining shows 43.5% LPL colocalization with Iba-1-positive microglia cells around the Aβ plaque. (b) The number of double-positive LPL/Iba-1 cells and (c) the integrated intensity of the Iba-1 signal showed no significant differences between the two groups in the CTX and HI. Arrows indicate LPL/Iba-1 double-positive signals. Scale bar: 50 μm.

Fig. 8. Double immunofluorescence staining of CD11c/Iba-1. (a) Double immunofluorescence staining shows 27.9% CD11c colocalization with

Iba-1-positive microglia cells around the Aβ plaque. (b) The number of double-positive CD11c/Iba-1 cells showed a significant increase in the APP23+Neumentix group in the CTX, and (c) the integrated intensity of the Iba-1 signal showed a significantly increased in the area of CD11c expression between the CTX and HI of the APP23+Neumentix group. Arrows indicate Cd11c/Iba-1 double-positive signals (**p < 0.01, ***p < 0.001, versus APP23 group). Scale bar: 50 μm.

Supplementary Fig. 1 Results of the pilot study and formal experiment on water intake and body weight. (a) The daily water intake of APP23 transgenic mice during the one-week pilot study. The first panel shows the detailed daily intake volume of Neumentix (n = 6) and tap water (n = 6) groups for each mouse. The second panel summarizes the average daily water intake for each group. The third panel displays the average body weight of all mice during the same period. (b) The daily water intake of the APP23+Neumentix group (n = 20) during the 30-day formal experiment. Each point represents the average water intake of all mice in

the group for a given day. (b) The table displays the results of the normality test, the analytical methods, and the p-values.

Supplementary Fig. 2. Schematic diagram of the putative mechanism of Neumentix action in microglia. Neumentix activates resting microglia to active microglia by stimulating Trem2. Activated microglia are mainly of three types: M1, a pro-inflammatory phenotype; M2, an anti-inflammatory phenotype, and DAMs, characterized by high expression of phagocytosis and lipid metabolism. Trem2 regulates microglial polarization from M1 towards the M2 phenotype, reducing neuroinflammation factor TNF-α expression. Trem2-dependent transition from resting microglia to the DAMs phenotype induces improved phagocytosis and decreased Aβ aggregation in AD pathology. Created with BioRender.com.

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