Combination therapy with octyl gallate and ferulic acid improves cognition and neurodegeneration in a transgenic mouse model of Alzheimer’s disease

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To date, there is no effective Alzheimer’s disease (AD)-modifying therapy. Nonetheless, combination therapy holds promise, and nutraceuticals (natural dietary compounds with therapeutic properties) and their synthetic derivatives are well-tolerated candidates. We tested whether combination therapy with octyl gallate (OG) and ferulic acid (FA) improves cognition and mitigates AD-like pathology in the presenilin-amyloid octyl gallate (OG) and ferulic acid (FA) improves cognition and neurodegeneration in a transgenic mouse model of cerebral amyloidosis. One-year-old mice with established β-amyloid plaques received daily doses of OG and FA alone or in combination for 3 months. PSAPP mice receiving combination therapy had statistically significant improved cognitive function versus OG or FA single treatment on some (but not all) measures. We also observed additional statistically significant reductions in brain parenchymal and cerebral vascular β-amyloid deposits as well as brain amyloid β-protein abundance in OG- plus FA-treated versus singly-treated PSAPP mice. These effects coincided with enhanced nonamyloidogenic amyloid β-protein precursor (APP) cleavage, increased α-secretase activity, and β-secretase inhibition. We detected elevated expression of nonamyloidogenic soluble APP-α and the α-secretase candidate, a disintegrin and metalloproteinase domain-containing protein 10. Correspondingly, amyloidogenic β-carboxy-terminal APP fragment and β-site APP-cleaving enzyme 1 expression levels were reduced. In parallel, the ratio of β- to α-carboxy-terminal APP fragment was decreased. OG and FA combination therapy strikingly attenuated neuroinflammation, oxidative stress, and synaptotoxicity. Co-treatment afforded additional statistically significant benefits on some, but not all, of these outcome measures. Taken together, these data provide preclinical proof-of-concept for AD combination therapy.

Dementia has become a worldwide public health concern. The World Alzheimer Report warns that 46.8 million people worldwide are living with this debilitating illness in 2015, with an estimated increase to 131.5 million by 2050. Dementia has a titanic economic impact, with an 818 billion dollar total estimated worldwide cost that will eclipse a trillion dollars by 2018 and increase to 2 trillion dollars by 2030. Around the world, there were 9.9 million new cases of dementia in 2015, which is one new case every 3 s (1).

Alzheimer’s disease (AD)3 is the most common form of age-related dementia, and it is earmarked by impaired episodic memory, ultimately leading to progressive cognitive and behavioral disturbance. Extracellular deposition of amyloid β-protein (Aβ) in the brain parenchyma and cerebral vessels is a pathognomonic feature of the disease (2), and the “amyloid cascade hypothesis” purports that cerebral Aβ deposition drives AD pathogenesis (3).

In the amyloidogenic pathway, Aβ is generated via sequential endoproteolytic cleavage of amyloid β-protein precursor (APP) by β- and γ-secretases. These cleavage events create an

3 The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β-protein; APP, amyloid β-protein precursor; CTF, carboxyl-terminal fragment; sAPP, soluble APP; PS, presenilin; OG, octyl gallate; FA, ferulic acid; ADAM10, a disintegrin and metalloproteinase domain-containing protein 10; ANOVA, analysis of variance; RAPP, radial arm water maze; RSC, retrosplenial cortex; EC, entorhinal cortex; H, hippocampus; ROI, regions of interest; CAA, cerebral amyloid angiopathy; BACE1, β-site APP-cleaving enzyme 1; pADAM10, precursor ADAM10; mADAM10, mature (active) ADAM10; QPCR, real-time polymerase chain reaction; SOD1, superoxide dismutase 1; GPx1, glutathione peroxidase 1; GAFP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; IR, immunoreactivity; LD50, lethal dose for 50%; APPsw, “Swedish” APPswe; M11472, Mid-Career Award in Aging Research M11472 (to T. T.), and generous startup funds from the Zilkha Neurogenetic Institute of the Keck School of Medicine at the University of Southern California (to T. T.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Aβ-containing β-carboxyl-terminal fragment (CTF) (C99), an amino-terminal soluble APP-β (sAPP-β), and a smaller γ-CTF (C57) (4–7). Conversely in the nonamyloidogenic pathway, α-secretase cleavage of APP precludes Aβ formation and produces amino-terminal soluble APP-α (sAPP-α), α-CTF (C83), and p3 peptide (5, 8). Once generated, Aβ enters into dynamic equilibrium between soluble and deposited forms (9). APP molecules are homeostatically processed by nonamyloidogenic α-secretase cleavage of APP; therefore, constitutive Aβ generation is quantitatively minor. In autosomal dominant AD, a subset of mutations in the APP or the presenilin (PS) genes drive the amyloidogenic pathway, whereas other APP mutations impact Aβ assembly without affecting production of Aβ peptides.

Acetylcholinesterase inhibitors and N-methyl d-aspartate antagonists are current standard-of-care for treating AD-related symptoms with limited efficacy and no disease modification. In addition, although anti-psychotic drugs are still prescribed at advanced disease stages to treat behavioral and psychological symptoms of dementia, associated adverse effects (e.g. arousal disturbance, ileus, tardive dyskinesia, malignant syndrome, rhabdomyosis, etc.) often worsen activities of daily living and quality of life.

Clinical trials are presently underway based on the amyloid cascade hypothesis, and we await results from those studies. Unfortunately, drugs designed to block Aβ production have, so far, produced unwanted side effects. Nonetheless, secretase modulation remains an important AD therapeutic approach. In this regard, naturally occurring dietary compounds, so-called “nutraceuticals” can be used to modulate APP processing with very little (if any) side effects. We screened a class of nutraceuticals and their synthetic derivatives, and we identified two promising phenolic compounds: octyl gallate (OG) (10) and ferulic acid (FA) (11).

OG (octyl 3,4,5-trihydroxybenzoate, C15H16O4) is a phenolic compound that is permitted for use as an anti-oxidant and preservative in foods (e.g. oil, margarine, lard, and peanut butter) and cosmetics. OG is an alkyl gallate, which is synthetically derived from gallic acid. Its multifunctional biological properties include anti-fungal and anti-oxidant activities (12–14). We previously reported that OG promotes nonamyloidogenic processing of both human wild-type (WT) and “Swedish” mutant forms of APP via estrogen receptor-mediated a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) activation. Moreover, intracerebroventricular injection of OG into Tg2576 mice decreased cerebral Aβ abundance and increased sAPP-α expression (10).

FA (trans-4-hydroxy-3-methoxycinnamic acid, C10H10O4) is a phenolic compound that is copiously present in seed plants and leaves (e.g. rice, wheat, and oat), vegetables (e.g. tomato and carrot), and fruits (e.g. pineapple and orange). FA is generated by the metabolism of phenylalanine and tyrosine, and it is found both in free form and covalently linked to lignin and other biopolymers. The compound has both anti-inflammatory and anti-oxidant properties (15), and our previous report showed that oral FA treatment for 6 months remedies behavioral impairment, reduces amyloidogenic APP metabolism by modulating β-secretase, and mitigates AD-like pathology in the PSAPP transgenic mouse model of cerebral amyloidosis (11). Both OG and FA have low molecular weights, are cell-permeable, and are highly bioavailable. OG is metabolized in the intestine to gallic acid and octanol, and finally to acetate and butyrate via sequential enzymatic action. Gallic acid (3,4,5-trihydroxybenzoic acid) is a smaller phenolic compound that is a hydrolysable tannin constituent of many plants (13, 14). Tannins (e.g. gallic acid) have anti-oxidant and free radical scavenging, anti-viral/bacterial, anti-carcinogenic, anti-mutagenic, and anti-inflammatory properties (16). Gallic acid is rapidly absorbed in the intestine over a period of ~60 min in rodents (17), equating to 76 min in humans (18), and it crosses the blood-brain barrier in rodents (19). FA is absorbed in free form through stomach mucosa and is then transported into the hepatic portal vein where it is metabolized in the liver. Interestingly, FA can be recovered in rat plasma 5 min after oral administration. At this point, the ratio of free FA to total FA in plasma is considerably elevated but rapidly gives rise to conjugated FA. Both free and conjugated FA are reportedly distributed via the systemic circulation into tissues (15, 20).

Given similar anti-oxidant and anti-inflammatory properties of OG and FA as well as complementary modes of action on APP processing, we hypothesized that combination therapy with OG plus FA would lower cerebral Aβ pathology and improve cognitive function. To test this, we orally administered these agents alone or in combination (all at 30 mg/kg) daily for 3 months (commencing at 12 months of age) to the PSAPP-accelerated mouse model of cerebral amyloidosis, and we examined behavioral impairment, AD-like pathology, and APP processing at 15 months. For some (but not all) measures, results showed additional benefit afforded by combination therapy.

Results

Combination therapy with OG plus FA reverses behavioral deficits

When cerebral and vascular β-amloid deposits are already present in the PSAPP mouse model of cerebral amyloidosis (at 12 months of age), we orally administered (via gavage) vehicle, OG, FA, or OG plus FA (all at 30 mg/kg) once daily for 3 months to PSAPP or WT littermate mice. Prior to randomized group assignment, we assessed baseline cognitive status in all mice at 12 months of age. In this study, we only included cognitively impaired PSAPP mice (four treatments with n = 8 per group; equal numbers of four males and four females) and cognitively normal WT littermates (same group sizes and sex distribution as PSAPP mice).

At the end of treatment (15 months of age), we conducted novel object recognition testing for episodic memory, and all eight groups had comparable recognition indices (49.3–52.0%) in the training phase of the test (Fig. 1A, left). By contrast, in the retention phase, one-way analysis of variance (ANOVA) followed by post hoc testing demonstrated statistically significant differences between PSAPP-V mice and the other seven mouse groups (Fig. 1A, right; *p < 0.05 for PSAPP-V versus all other groups). PSAPP mice treated with OG plus FA or OG/FA alone had significantly increased novel object exploration frequency by 59.8–70.0% versus PSAPP-V mice (50.6%). Moreover, combined treatment with OG plus FA additionally improved episodic
Figure 1. Combined OG plus FA treatment reverses PSAPP transgene-associated behavioral impairment. Data were obtained from PSAPP mice treated with vehicle (PSAPP-V, n = 8), OG (PSAPP-OG, n = 8), FA (PSAPP-FA, n = 8), or OG plus FA (PSAPP-OGFA, n = 8) and also wild-type mice treated with vehicle (WT-V, n = 8), OG (WT-OG, n = 8), FA (WT-FA, n = 8), or OG plus FA (WT-OGFA, n = 8) for 3 months beginning at 12 months of age. All mice were behaviorally tested at 15 months. A, recognition index (%) in the novel object recognition test is shown from training (left) and retention test phases (right). B, Y-maze test data are represented as total arm entries (left) and spontaneous alternation (right). C, 2-day radial arm water maze test data are shown for errors (left) and escape latency (right). *, p < 0.05 for PSAPP-V versus the other treated mice; †, p < 0.05 for PSAPP-OGFA versus PSAPP-OG or PSAPP-FA mice.

memory versus OG or FA single treatment (Fig. 1A, right; †, p < 0.05, PSAPP-OGFA versus PSAPP-OG or PSAPP-FA mice). Combination treatment completely remediated episodic memory impairment, because there was no significant difference (p > 0.05) versus any of the WT mouse groups (Fig. 1A, right).

In the Y-maze test for exploratory activity and spatial working memory, one-way ANOVA followed by post hoc comparison demonstrated statistically significant differences for total arm entries between PSAPP-V mice and the other seven mouse groups (Fig. 1B, left; *, p < 0.05). This behavioral phenotype has been reported in mouse models of cerebral amyloidosis (11, 21–25) and may reflect disinhibition resulting from cortical and/or hippocampal injury (26). Of note, PSAPP transgene-associated hyperactivity was completely mitigated in PSAPP-OGFA mice (Fig. 1B, left; †, p < 0.05, PSAPP-OGFA versus PSAPP-OG or PSAPP-FA mice), which did not significantly differ from any of the WT mouse groups (p > 0.05). Mice tend to spontaneously alternate arm entries in the Y-maze, such that they visit the three arms in sequence more frequently than would occur by chance (50%, see dashed line in Fig. 1B, right); this phenotype is generally interpreted as a measure of spatial working memory. PSAPP-V mice had fewer tendencies to alternate, thus demonstrating impaired spatial working memory. One-way ANOVA followed by post hoc testing revealed statistically significant differences on Y-maze spontaneous alternation between PSAPP-V mice and the other seven mouse groups (Fig. 1B, right; *, p < 0.05). Importantly, PSAPP-OGFA mice significantly increased alternation behavior compared with OG or FA singly-treated mice (Fig. 1B, right; †, p < 0.05, PSAPP-OGFA versus PSAPP-OG or PSAPP-FA mice), and did not significantly differ from any of the WT mouse groups (p > 0.05); demonstrating completely remediated spatial working memory.

Finally, we evaluated mice in the radial arm water maze (RAWM) test for hippocampus-dependent spatial reference learning and memory. On day 1, overall ANOVA showed main effects of block (p < 0.001 for both errors and escape latency), genotype (p < 0.01 for errors and p = 0.001 for escape latency), and treatment (p < 0.05 for escape latency). Repeated-measures ANOVA followed by post hoc comparison did not show statistically significant differences between PSAPP-V mice and the other seven mouse groups, whereas PSAPP-V mice tended toward more errors and greater escape latencies to reach the visible or hidden platform locations than the other seven groups. On day 2, overall ANOVA demonstrated main effects of block (p < 0.001 for both errors and escape latency), genotype (p < 0.01 for errors and p < 0.001 for escape latency), and treatment (p < 0.001 for both errors and escape latency).
Repeated-measures ANOVA followed by post hoc evaluation showed statistically significant differences between PSAPP-V mice and the other seven mouse groups (Fig. 1C; *p < 0.05 for both errors and escape latency). PSAPP-V mice had increased errors and longer latencies to reach the visible or hidden platform locations than the other seven mouse groups; importantly however, PSAPP-OGFA, PSAPP-OG, and PSAPP-FA mice completed the task with significantly less errors and shorter latencies, and their behavior did not significantly differ from the four WT mouse groups (p > 0.05). This demonstrates that 3-month single or double treatment with OG and FA completely reverses mutant human APP transgene-associated spatial reference learning and memory deficits. In addition, there were no significant between-group differences (p > 0.05) on swim speed, nor did we observe thigmotaxis (an index of anxiety) in any of the mice tested. For all behavioral tests, multiple ANOVA models were used with sex as a categorical covariate, but we did not observe significant main effects or interactive terms with sex (p > 0.05). To be certain, we also stratified data by sex, and a similar pattern of results as above was evident in both males and females (data not shown).

Cerebral amyloid pathology is mitigated in PSAPP-OGFA mice

At 15 months of age, PSAPP-V mouse brains had severe cerebral β-amyloid load (mean of 7–8%) throughout the retrosplenial cortex (RSC), entorhinal cortex (EC), and hippocampus (H) regions of interest (ROI). By contrast, PSAPP-OG, PSAPP-FA, and PSAPP-OGFA mice had 29–59% plaque reduction across all three brain regions. Greatest reductions were observed in the EC (Figs. 2 and 3, A−C; **p < 0.01; ***p < 0.001). Importantly, β-amyloid burden was further significantly reduced in H and EC PSAPP-OGFA brain areas (28–33% additional reduction versus OG or FA single treatment) (Fig. 3, B and C; †, p < 0.05). To determine whether treatment prevented versus reversed kinetics of cerebral amyloid accumulation, eight untreated PSAPP mice at 12 months of age (when dosing started) were included in the analysis and had cerebral β-amyloid burden averaging from 4 to 5% (Fig. 3, A−C). Intriguingly, in RSC, H, and EC regions, 3-month treatment with OG plus FA trended toward reversing cerebral amyloid pathology compared with untreated baseline (at 12 months of age, Fig. 3, A−C). This effect was sex-independent (data not shown).

To further elucidate effects of treatment on cerebral amyloid pathology, we conducted morphometric analysis of β-amyloid deposits. In the entire RSC, EC, and H regions, Aβ(1−24) monoclonal antibody 4G8-stained β-amyloid deposits were assigned to one of three mutually exclusive groupings based on maximum diameter as follows: small (<25 μm), medium (between 25 and 50 μm), or large (>50 μm). PSAPP-OGFA mice had the greatest magnitude of statistically significant reductions in all three subcategories: 24−45% for small, 34−49% for medium, and 39−65% for large plaques (Fig. 2 and 3, D−F; *, p < 0.05; **, p < 0.01; ***, p < 0.001). Of note, doubly-treated PSAPP mice showed additional statistically significant reductions in large plaques in H and EC regions compared with OG or FA singly-treated mice (39−43% additional reduction; Fig. 3F; †, p < 0.05). Stratification by sex demonstrated the same pattern of results in both males and females (data not shown).

We confirmed histological observations by biochemical analysis of Aβ species in brain homogenates using sandwich enzyme-linked immunosorbent assay (ELISA). In the TBS-soluble fraction, all three groups of treated PSAPP mice had statistically significant reductions in Aβ(1−40) (from 45 to 70%) and Aβ(1−42) (from 42 to 65%) abundance compared with PSAPP-V controls (Fig. 4A*; p < 0.05). Aβ(1−40) levels were markedly and significantly decreased by combined treatment (43–46% further decrease versus OG or FA alone) (Fig. 4A; ††, p < 0.01). A similar pattern of results emerged when considering the detergent-soluble fraction, where the three PSAPP treatment groups had statistically significant reductions in both Aβ(1−40) (from 42 to 61%) and Aβ(1−42) (from 26 to 37%) (Fig. 4B; *, p < 0.05; **, p < 0.01). Moreover, the guanidine HCl-soluble fraction, which most closely mirrors cerebral β-amyloid deposits, revealed statistically significant reductions for each PSAPP treatment group for Aβ(1−40) (from 25 to 51%) and Aβ(1−42) (from 30 to 58%) (Fig. 4C; **, p < 0.01). Here again, PSAPP-OGFA mice had the deepest reductions in both Aβ(1−40) and Aβ(1−42) levels (30–40% further reduction versus OG or FA single treatment) (Fig. 4C; †, p < 0.05).

We moved on to assess cerebral amyloid angiopathy (CAA). At 15 months of age, PSAPP-V mice had numerous cerebral vascular β-amyloid deposits in the walls of penetrating arteries at the pial surface in RSC and EC regions and in small arteries at the hippocampal fissure and brachium of the superior colliculus (Fig. 5A). Mean numbers of CAA deposits showed sta-
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**Figure 3. Cerebral β-amyloid deposits are mitigated by combination of OG plus FA treatment.** Data were obtained from PSAPP mice treated with vehicle (PSAPP-V, n = 8), OG (PSAPP-OG, n = 8), FA (PSAPP-FA, n = 8), or OG plus FA (PSAPP-OGFA, n = 8) for 3 months commencing at 12 months of age (mouse age at sacrifice = 15 months) for A–F and from 12-month-old untreated PSAPP mice (PSAPP-12M, n = 8) for A–C. A–C, quantitative image analyses for β-amyloid burden using an amino-terminal β-amyloid monoclonal antibody (4G8) are shown. D–F, morphometric analyses of cerebral parenchymal β-amyloid plaque size are shown. A–F, brain region is indicated on the x axis. Statistical comparisons for A–F are within each brain region, between groups. *, p < 0.05; **, p < 0.01; †††, p < 0.001 for PSAPP-V versus the other treated mice; †, p < 0.05 for PSAPP-OGFA versus PSAPP-OG or PSAPP-FA mice.

**Statistically significant reductions in PSAPP-OG (20–36%), PSAPP-FA (17–35%), and PSAPP-OGFA (37–41%) versus PSAPP-V mice in all three brain regions examined (Fig. 5, B–D; *, p < 0.05), and combination treatment produced a significant decrease in the H region (22–24% further decrease versus OG or FA single treatment) (Fig. 5C; †, p < 0.05).**

**Modulation of APP metabolism in PSAPP-OGFA mouse brains**

To rule out the possibility that treatment reduced expression of transgene-derived APP or PS1, we examined holoprotein expression by Western blot in brain homogenates from each PSAPP treatment group. Results showed comparable band intensities, confirming that neither single nor double treatment altered APP (Fig. 6A) or PS1 (data not shown) expression.

We moved on to determine whether OG and/or FA treatment modulated amyloidogenic APP metabolism by examining APP metabolites, including nonamyloidogenic sAPP-α and α-CTF/C83 as well as amyloidogenic β-CTF/C99, phospho-β-CTF/P-C99, and monomeric/oligomeric Aβ species. Densitometry confirmed that nonamyloidogenic APP metabolism to sAPP-α was significantly increased in brain homogenates from PSAPP-OG and PSAPP-OGFA mice (Fig. 6, A and B; **, p < 0.01 for PSAPP-OG and PSAPP-OGFA versus PSAPP-V or PSAPP-FA mice), which was further supported by significantly decreased ratios of C99 to C83 in brain homogenates from these treatment groups (Fig. 6, A and C; ***, p < 0.001). In complementary fashion, densitometry confirmed that amyloidogenic APP metabolism to C99 and P-C99 was significantly decreased in brain homogenates from the three groups of treated PSAPP mice (Fig. 6, A and C; ***, p < 0.001). Importantly, brain homogenates from PSAPP-OGFA mice showed significant reductions in P-C99 and C-99 protein compared with PSAPP-OG or PSAPP-FA mice (Fig. 6C; †††, p < 0.001). In parallel, the 4-kDa monomeric Aβ band was attenuated in the three PSAPP treatment groups (Fig. 6A), and there was reduced abundance of Aβ species between 25 and 75 kDa (putative Aβ oligomers, Fig. 6A). These effects were verified by sandwich ELISA for (N) 82E1 Aβ oligomers (Fig. 6D; *, p < 0.05 for PSAPP-V versus PSAPP-OG, PSAPP-FA, or PSAPP-OGFA mice).

Given the biochemical evidence for enhanced nonamyloidogenic and reduced amyloidogenic APP metabolism after treatment, we wanted to explore how these compounds impacted α/β-secretase activity. We began by examining ADAM10 (α-secretase candidate) and β-site APP-cleaving enzyme 1 (BACE1, β-secretase) protein abundance in brain homogenates from each group of treated PSAPP mice. Both precursor...
ADAM10 (pADAM10, 90-kDa band) and mature (active) ADAM10 (mADAM10, 68-kDa band) were more highly expressed in brain homogenates from PSAPP-OG and PSAPP-OGFA mice, and densitometry reached statistical significance (Fig. 7, A and B; ***, *p*/< 0.001 for PSAPP-OG and PSAPP-OGFA mice versus PSAPP-V or PSAPP-FA mice). Analysis of BACE1 disclosed decreased protein expression in brain homogenates from treated versus PSAPP-V mice, which was confirmed by densitometry (Fig. 7, A and C; ***, *p*/< 0.001). Moreover, combined treatment with OG plus FA significantly reduced BACE1 protein expression compared with OG alone (Fig. 7, A and C; ††, *p*/< 0.01). When taken together, these data demonstrate that OG and FA together enhance ADAM10 and inhibit BACE1, beneficially shifting toward non-amyloidogenic APP metabolism.

**Combination therapy stabilizes neuroinflammation, oxidative stress, and synaptotoxicity**

To determine whether treatment impacted neuroinflammatory processes and oxidative stress in PSAPP mice, we examined β-amyloid deposit-associated astrocytosis and microgliosis and quantified brain mRNA expression of the proinflammatory innate immune cytokines: tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by real-time polymerase chain reaction (QPCR). We also examined two cardinal oxidative stress markers: superoxide dismutase 1 (SOD1) and glutathione peroxidase 1 (GPx1). PSAPP-V mice had exacerbated β-amyloid plaque-associated reactive astrocytosis and microgliosis, as evidenced by increased expression of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba1) in glial somata and processes. Numerous minute GFAP-positive granules, which were likely within astrocytic processes, were dispersed between neurons. In addition, GFAP expression was strongly detected in dystrophic neurites associated with β-amyloid deposits. It is noteworthy that PSAPP-V mice had astroglial and microglial hyperplasia and hypertrophy in and around β-amyloid deposits, which were strongly immunoreactive in all three brain regions examined (Fig. 8). Reactive astrocytes and microglia were significantly attenuated in PSAPP-OG, PSAPP-FA, and PSAPP-OGFA mice compared with PSAPP-V mice (Fig. 9, A–F; **, *p*/< 0.01; ***, *p*/< 0.001). Astrocytosis and microgliosis were additionally reduced in PSAPP-OGFA mice in H and EC regions (16–21% further reduction for astrocytosis and 18–21% for microgliosis versus OG or FA alone treatment) (Fig. 9, B, C, E, and F; †, *p*/< 0.05; ††, *p*/< 0.01). In support, TNF-α and IL-1β brain mRNAs
were significantly decreased in PSAPP-OG, PSAPP-FA, and PSAPP-OGFA mice compared with PSAPP-V animals (Fig. 9G; *, p < 0.05). Of interest, OG- plus FA-treated PSAPP mice had significantly reduced proinflammatory cytokines compared with OG or FA alone (Fig. 9G; †, p < 0.05). Proinflammatory cytokines were completely blocked by combination therapy and did not significantly differ from any of the WT control groups (p > 0.05).

A similar pattern of results was observed for expression of the key oxidative stress markers: SOD1 and GPx1. Both were elevated in PSAPP-V mice, and all three treatment groups had significant reductions (Fig. 9H; *, p < 0.05 for PSAPP-V versus PSAPP-OG, PSAPP-FA, or PSAPP-OGFA mice). Of import, OG- plus FA-treated PSAPP mice showed significantly reduced GPx1 mRNA expression compared with OG or FA alone (Fig. 9H; †, p < 0.05). Similar to proinflammatory cytokines, combined treatment demonstrated complete reductions compared with OG or FA single treatment that did not significantly differ from the three WT mouse groups (p > 0.05). These effects were sex-independent (data not shown). Collectively, combined treatment with OG and FA mitigated neuroinflammation and oxidative stress in PSAPP mouse brains.

Finally, to investigate whether single or double OG and/or FA treatment impacted synaptotoxicity in PSAPP mice, we examined synaptophysin immunoreactivity (IR) in the hippocampal CA1 and EC regions. Synaptophysin IR was significantly increased in both brain areas from PSAPP-OG, PSAPP-FA, and PSAPP-OGFA mice compared with PSAPP-V animals (Fig. 10, A–C; ***, p < 0.001). Synaptophysin IR was strikingly enhanced in PSAPP-OGFA mice in the EC region (Fig. 10, A and C; †, p < 0.05).

**Discussion**

In this report, we show that combination therapy with OG (10) plus FA (11) for 3 months in the aged PSAPP transgenic mouse model of cerebral amyloidosis restores cognitive func-
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Figure 6. Combination therapy with OG plus FA promotes nonamyloidogenic and inhibits amyloidogenic APP processing. A, Western blots are shown for holo-APP, sAPP-α, and amyloidogenic APP cleavage fragments, including Aβ monomer and oligomers as well as phospho-C99 (P-C99), C99, and C83. Actin is included as a loading control, and densitometry data are shown below each lane. Densitometry analysis is shown for ratios of sAPP-α to APP (B) and for ratios of C-99 or P-C99 to actin or ratios of C99 to C83 (C). D, abundance of (N) 82E1 Aβ oligomers in the 2% SDS-soluble brain homogenate fraction (measured by sandwich ELISA) are shown. Data were obtained from PSAPP mice treated with vehicle (PSAPP-V, n = 8), OG (PSAPP-OG, n = 8), FA (PSAPP-FA, n = 8), or OG plus FA (PSAPP-OGFA, n = 8) for 3 months starting at 12 months of age. B, **, p < 0.01 for PSAPP-OGFA and PSAPP-OG mice versus PSAPP-V and PSAPP-FA mice; C and D, *, p < 0.05; **, p < 0.01; ***, p < 0.001 for PSAPP-V versus PSAPP-OGFA, PSAPP-OG, and PSAPP-FA mice; †††, p < 0.001 for PSAPP-OGFA versus PSAPP-OG or PSAPP-FA mice.

Although one interpretation is that OG and FA exert effect(s) at the level of the brain, it is important to consider that OG is likely more brain-permeable than FA, and either or both compounds may act indirectly at the level of the periphery (15, 20).

The connection between cognitive improvement and cerebral Aβ/β-amyloid pathology deserves consideration. There are numerous reports showing mitigation of transgene-associated behavioral impairment associated with reduced cerebral amyloidosis (11, 23–25, 27–30). Complementarily, our pathological results show that brain parenchymal and cerebral vascular β-amyloid deposits as well as Aβ levels are ameliorated by each treatment, with OG plus FA producing the strongest effects (see Figs. 2–5). This tight association between reduced cerebral Aβ/β-amyloid pathology and cognitive benefit in PSAPP mice is remarkable.

Nonetheless, other reports decouple β-amyloidosis from behavioral impairment (31, 32). In part, this discordancy has led some to propose that oligomeric Aβ is the primary neurotoxic species (33–35). In support, there is a strong correlation between soluble Aβ oligomers and cognitive disturbance in
Figure 7. OG plus FA treatment activates ADAM10 and inhibits BACE1. A, Western blots are for ADAM10 (α-secretase candidate) and BACE1 (β-secretase). Actin is included as a loading control, and densitometry data are shown below each lane. Densitometry analysis is shown for B, and ratios of precursor ADAM10 (pADAM10) or mature ADAM10 (mADAM10; two isoforms are shown) to actin, or C, ratios of BACE1 to actin. Data were obtained from PSAPP mice treated with vehicle (PSAPP-V, n = 8), OG (PSAPP-OG, n = 8), FA (PSAPP-FA, n = 8), or OG plus FA (PSAPP-OGFA, n = 8) for 3 months starting at 12 months of age. B, ***, p < 0.001 for PSAPP-OGFA and PSAPP-OG versus PSAPP-V and PSAPP-FA mice; C, ***, p < 0.001 for PSAPP-V versus PSAPP-OGFA, PSAPP-OG, or PSAPP-FA mice; ††, p < 0.01 for PSAPP-OGFA versus PSAPP-OG mice.

mouse models of cerebral amyloidosis (28, 32). In this report, Western blots showed decreased abundance of Aβ species between 25 and 75 kDa (putative oligomers) in PSAPP brains after treatment, which was confirmed by significantly decreased levels of (N) 82E1 Aβ oligomers by sandwich ELISA (see Fig. 6). This may be due to inhibiting amyloidogenesis and/or enhancing nonamyloidogenic APP processing.

The action of nonamyloidogenic α-secretase on APP produces sAPP-α and C83, whereas the amyloidogenic pathway (via the action of β-secretase) produces C99 and sAPP-β. In this regard, the Western blot analyses reported here show that sAPP-α protein expression is significantly increased in PSAPP brain homogenates treated with OG alone or in combination with FA, whereas C99 and P-C99 are correspondingly reduced. Additionally, the ratio of C99 to C83 is attenuated. Somewhat surprisingly, even OG single-treatment reduces amyloidogenic and increases nonamyloidogenic APP metabolism (see Fig. 6).

In parallel, the combined treatment significantly increases both pADAM10 and mADAM10 expression in PSAPP brains. By contrast, BACE1 protein abundance is significantly decreased even in OG singly-treated brains. Interestingly, combination therapy further decreases BACE1 protein expression (see Fig. 7). These data raise a question as to why both C99 and BACE1 proteins are reduced by OG treatment alone, as we and our colleagues initially reported that OG promotes nonamyloidogenic processing of APP through estrogen receptor-mediated ADAM10 maturation (10). However, we also showed that sAPP-α can indirectly modulate β-secretase activity via a negative feedback loop (36), and this is in line with previous reports showing that sAPP-α can autoregulate APP processing (37–39). Other beneficial properties of sAPP-α include neurotrophism and neuroprotection (40, 41) and even enhanced long-term potentiation (42).

Amyloidogenic APP metabolism via β-secretase cleavage is thought to be a rate-limiting step for Aβ production (4–6, 43), and both α- and β-secretases compete for APP substrate (44). When considering this, we hypothesize that combined enhancement of α-secretase and inhibition of β-secretase activity would shift APP metabolism toward nonamyloidogenic α-secretase cleavage; and this is consistent with observations in this report.

Although we previously published that OG or FA treatment promotes nonamyloidogenic APP processing in cerebral amyloidosis model mice, it is important to recognize that dual treatment can have pleiotropic effects in vivo. In terms of combined OG/FA therapy, some of the most robust effects that we reported are on behavioral improvement and mitigated neuroinflammation and oxidative stress. Others have shown that the OG metabolite, gallic acid, has anti-oxidant, free radical scavenging, and anti-inflammatory properties (16). Furthermore, gallic acid inhibits amyloid fibril formation (45) and Aβ oligomerization (46), and it suppresses microglial-mediated neuroinflammation in response to β-amyloid (47). With regard to FA, the compound inhibits β-amyloid aggregation (48) and reduces Aβ/β-amyloid neurotoxicity (49). A derivative of FA (FA ethyl ester) has been shown to protect neurons against Aβ(1–42)-induced oxidative stress and neurotoxicity via induction of heme oxygenase-1 and heat shock protein 72 in parallel with down-regulation of inducible nitric-oxide synthase (50). Fur-
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Figure 8. Combination therapy attenuates reactive astrocytosis and microgliosis in PSAPP mice. Representative images were obtained from PSAPP mice treated with vehicle (PSAPP-V), OG (PSAPP-OG), FA (PSAPP-FA), or OG plus FA (PSAPP-OGFA) for 3 months starting at 12 months of age (mouse age at sacrifice = 15 months). Immunohistochemistry for glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba1) reveals β-amyloid deposit-associated astrocytosis (GFAP, left panels) and microgliosis (Iba1, right panels). Brain regions shown include the following: RSC (top), H (middle), and EC (bottom). Scale bars, 50 μm.

Thus, long-term FA treatment lowers microglial activation induced by intracerebroventricular Aβ(1–42) injection (51).

Anti-inflammatory properties shared by OG and FA are particularly relevant, as neuroinflammation is now recognized to be a prime contributor to AD evolution (52–54). In this report, we demonstrate that combined OG plus FA treatment attenuates plaque-associated microgliosis and astrocytosis and decreases proinflammatory cytokine expression to baseline levels (see Figs. 8 and 9). One possibility is that dual treatment has anti-inflammatory effects that are Aβ/β-amyloid-independent. Of course, because gliosis and cerebral β-amyloid deposition are spatiotemporally linked in human AD and in rodent models of the disease (11, 23–25, 55–59), addressing this hypothesis in vivo would be complex.

Oxidative stress also contributes to AD pathogenesis (60, 61), and both OG and FA have anti-oxidant and free radical scavenging properties (14, 62). Importantly, our data show that combined treatment reduces cerebral expression of two key oxidative stress markers (SOD1 and GPx1) to baseline levels (see Fig. 9). In this regard, OG can donate three electrons from its benzene ring (i.e. 3-, 4-, 5-hydroxyl) to readily form a resonance-stabilized phenoxy radical. In addition, the compound’s tertiary structure (i.e. the FA carboxylic acid group with adjacent unsaturated C–C double bond) can stabilize free radicals via resonance or by preventing free radical membrane attack. Like gallic acid, the carboxylic acid group can protect against lipid peroxidation (14, 16). FA has an intriguing chemical structure that imbues it with free radical scavenging properties. The presence of electron-donating sites on the benzene ring (i.e. 3-methoxy and, more importantly, 4-hydroxyl) can readily form a resonance-stabilized phenoxy radical. In addition, the compound’s tertiary structure (i.e. the FA carboxylic acid group with adjacent unsaturated C–C double bond) can stabilize free radicals via resonance or by preventing free radical membrane attack. Like gallic acid, the carboxylic acid group can protect against lipid peroxidation by acting as a lipid anchor (62). Assessment of OG/FA combined effects on lipid peroxidation and protein oxidation (including formation of carbonyls) is warranted to definitively establish synergism between the two compounds at the level of oxidative stress.

Synaptic loss is characteristic of AD pathology, and the role of Aβ in synapse loss and dysfunction is well-documented. Synaptic plasticity or change in synaptic strength is widely considered to be the cellular foundation for learning and memory (63–65). As dysregulation of plasticity precedes synaptic loss, reduced synaptic abundance is a reliable predictor of disease progression and cognitive decline (63). Synaptophysin is found in synaptic vesicles and is the most widely used marker to determine synaptic density (66). To assess whether beneficial effects of OG and/or FA on behavioral outcome(s) were accompanied by increased synaptic density, we quantified synaptophysin in hippocampal CA1 and EC regions of PSAPP mice. Synaptophysin IR was significantly increased in each PSAPP treatment group, with the greatest benefit after combined treatment (see Fig. 10). One possibility is that dual treatment restored mossy fiber innervation, preserving synaptic integrity of Schaffer collateral projections to the hippocampal CA1 region.

Despite these beneficial effects of co-treatment, unwanted side effects must be taken into account for any drug, natural or synthetic. It is important to note that we did not detect any evidence of adverse events (changes in behavior, body weight, food intake, or mortality) in any of the mice. We also did not observe pathological features in any major organs upon post-mortem examination. Lack of adverse events dovetails with OG having a highly acute oral lethal dose for 50% (LD50) of rats of 2,710 mg/kg in males and 2,330 mg/kg in females (14). Similarly, acute oral LD50 for FA is as high as 2,370 mg/kg in mice (11). The doses that we administered to PSAPP mice (30 mg/kg/day) are orders of magnitude lower. The tolerable daily intake in humans can be extrapolated from rodent LD50 threshold data (67). Assuming that the default uncertainty factor accounting for interspecies variation is 10 (68), the tolerable human daily intake for OG equates to 16.26 g in males and 13.98 g in females; FA is 14.22 g (both assume 60 kg human weight). These values are well above the doses administered in this study, and such favorable profiles reinforce the notion that combined OG/FA treatment is safe. However, it should be noted that ADAM10 and BACE1 have a multitude of substrates and modulators that could be indirectly affected by altered secretase activity, possibly leading to long-term unwanted side effects. This would need to be properly investigated in humans.

In conclusion, we provide evidence that oral treatment with OG plus FA to aged PSAPP mice reverses behavioral impair-
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Figure 9. Neuroinflammation and oxidative stress are attenuated by combination therapy with OG plus FA. Data for A–F were obtained from PSAPP mice treated with vehicle (PSAPP-V, n = 8), OG (PSAPP-OG, n = 8), FA (PSAPP-FA, n = 8), or OG plus FA (PSAPP-OGFA, n = 8) for 3 months commencing at 12 months of age (mouse age at sacrifice = 15 months). Data for G and H additionally included wild-type mice treated in parallel with vehicle (WT-V, n = 8), OG (WT-OG, n = 8), FA (WT-FA, n = 8), or OG plus FA (WT-OGFA, n = 8). Quantitative image analysis for astrocytosis (A–C) or microgliosis (D–F) burden is shown. Each brain region is indicated on the x axis, and statistical comparisons for A–F are within each brain region, between groups. **, p < 0.01; ††, p < 0.01 for PSAPP-V versus the other treated mice; †, p < 0.05; ††, p < 0.01 for PSAPP-OGFA versus PSAPP-OG or PSAPP-FA mice. QPCR for tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β) proinflammatory cytokines (G) or key oxidative stress markers superoxide dismutase 1 (SOD1) or glutathione peroxidase 1 (GPx1) (H). Data are expressed as relative fold over WT-V mice. *, p < 0.05 for PSAPP-V versus the other treated mice; †, p < 0.05 for PSAPP-OGFA versus PSAPP-OG or PSAPP-FA mice.

Experimental procedures

Ethics statement

All experiments were performed in accordance with the guidelines of the National Institutes of Health, and all animal studies were approved by the Saitama Medical University Institutional Animal Care and Use Committee. Animals were humanely cared for during all experiments, and all efforts were made to minimize suffering.
Mice

Male B6.Cg-Tg(APPsw, PSEN1dE9)85Dbm/Mmjax mice (bearing “Swedish” APP<sub>K595N/M596L</sub> (<em(APP</em>swe) and <em>PS1</em> exon 9-deleted human transgenes) on the congenic C57BL/6J background (designated PSAPP mice) (69) and female C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). For this study, male PSAPP mice on the congenic C57BL/6J background were bred with female C57BL/6J mice to yield PSAPP and WT offspring, so all experimental PSAPP and WT littermates used in this study are genetically comparable.

OG and FA were obtained from Sigma and resuspended in distilled water containing dimethyl sulfoxide at a final concentration of 0.2%. PSAPP mice received four treatments (<em>n</em> = 8 per condition; four males and four females) as follows: OG (PSAPP-OG); FA (PSAPP-FA); OG plus FA (PSAPP-OGFA); or vehicle (distilled water containing dimethyl sulfoxide at a final concentration of 0.2%; PSAPP-V). Additionally, WT littermates received the same four treatments (<em>n</em> = 8 per group; four males and four females) as follows: WT-OG; WT-FA; WT-OGFA mice; or vehicle (WT-V). In parallel, to determine whether each treatment prevented versus reversed kinetics of cerebral amyloid accumulation, eight untreated PSAPP mice at 12 months of age (PSAPP-12M mice, four males and four females) were included for analysis of <em>β</em>-amyloid pathology. After baseline cognitive determination just prior to dosing (at 12 months of age), animals were gavaged with OG, FA, or OG plus FA (all at 30 mg/kg), or vehicle once daily for 3 months. Mice were housed in a specific pathogen-free barrier facility under a 12/12-h light/dark cycle, with <em>ad libitum</em> access to food and water.

Behavioral analyses

To assess novel object recognition memory, animals were habituated in a cage for 4 h, and then two objects of different shapes were concurrently provided for 10 min. The number of times that the animal explored the familiar object (defined as number of instances where an animal directed its nose 2 cm or less from the object) that was later replaced by a novel object were counted for the initial 5 min of exposure (training phase). To test memory retention on the following day, one of the familiar objects was replaced with a different shaped novel object, and then explorations were recorded for 5 min (retention test). The recognition index, taken as surrogate measure of episodic memory, is reported as frequency (%) of explorations of the novel versus familiar objects.

Figure 10. Combined treatment attenuates synaptotoxicity in PSAPP mice. Data for A–C were obtained from PSAPP mice treated with vehicle (PSAPP-V, <em>n</em> = 8), OG (PSAPP-OG, <em>n</em> = 8), FA (PSAPP-FA, <em>n</em> = 8), or OG plus FA (PSAPP-OGFA, <em>n</em> = 8) for 3 months commencing at 12 months of age (mouse age at sacrifice = 15 months). A, immunohistochemistry using a carboxyl-terminal synaptophysin monoclonal antibody. Brain regions shown include CA1 (left) and EC (right). Scale bars, 25 μm. B and C, quantitative image analysis for synaptophysin immunoreactivity (IR) is shown. Brain region is indicated on the x-axis, and statistical comparisons are within brain region, between groups. ***, <em>p</em> < 0.001 for PSAPP-V versus the other treated mice; †, <em>p</em> < 0.05 for PSAPP-OGFA versus PSAPP-OG or PSAPP-FA mice.
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entries and total number of entries were counted over a period of 8 min, beginning when the animal first entered the central area. Percent alternation was defined as entries into sequentially different arms on consecutive occasions using the following formula: % alternation = number of alternations/(number of total arm entries – 2) × 100%

To assess spatial reference learning and memory, the RAWM test was conducted over 2 days and consisted of triangular wedges in a circular pool (80 cm diameter) configured to form swim lanes that enclose a central open space. Mice naïve to the test were placed in the pool and allowed to search for the platform for 60 s. The animal was dropped into a random start arm and allowed to swim until it located and climbed onto the platform (goal) over a period of 60 s. Latency to locate the platform and errors were recorded. Each mouse was given a total of 15 trials. On day 1, the goal alternated between visible and hidden as the trials proceeded for each animal; on day 2, the goal was always hidden. All data were organized as individual blocks of three trials each. The goal arms remained in the same location for both days, whereas the start arm was randomly altered. All trials were done at the same time of day (±1 h), during the animals’ light phase. To evade interference with behavioral testing, each treatment was carried out 1 h after concluding behavioral testing (25).

Brain tissue preparation

At 15 months of age, animals were anesthetized with sodium pentobarbital (50 mg/kg) and euthanized. Brains were isolated, quartered, and processed according to our published methods (23–25). Briefly, left anterior hemispheres were weighed and snap-frozen at −80 °C for Western blot analysis. Right anterior hemispheres were weighed and immersed in RNA stabilization solution (RNeAlater®, Applied Biosystems, Foster City, CA) and then snap-frozen at −80 °C for QPCR analysis. Left posterior hemispheres were immersed in 4% paraformaldehyde fixative at 4 °C overnight and routinely processed in paraffin. Right posterior hemispheres were weighed and snap-frozen at −80 °C for sandwich ELISA.

Immunohistochemistry

Five coronal paraffin sections (per set) were cut with a 100-μm interval and 5-μm thickness spanning bregma −2.92 to −3.64 mm (70). Three sets of five sections were prepared for analyses of Aβ deposits/β-amyloid plaques, astrocytosis, and microgliosis. An additional set of five sections was used for analysis of synaptophysin IR. Primary antibodies included biotinylated anti-Aβ(1–40) and Aβ(1–42) monoclonal (4G8; Covance Research Products, Emeryville, CA), anti-GFAP polyclonal (Dako, Carpinteria, CA), carboxyl-terminal anti-Iba1 polyclonal (WAKO, Osaka, Japan), and carboxyl-terminal anti-synaptophysin monoclonal (DAK-SYNAP; Dako) antibodies. Immunohistochemistry was performed using a Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) coupled with the dianinobenzidine reaction, except that the biotinylated secondary antibody step was omitted for the biotinylated Aβ(17–24) monoclonal antibody.

Image analysis

Images were acquired and quantified using SimplePCI software (Hamamatsu Photonics, Shizuoka, Japan). Images of five 5-μm sections through each anatomic ROI (i.e. RSC, EC, and H) were captured based on anatomical criteria (70), and a threshold optical density that discriminated staining from background was set. Selection bias was controlled for by analyzing each ROI in its entirety. For Aβ, GFAP, and Iba1 burden analyses, data are reported as the percentage of positive pixels captured divided by the full area captured. We used mouse monoclonal Aβ(17–24) (4G8) antibody, which recognizes amino acids 18–22 (VFFAE), for Aβ burden analysis. Although it should be noted that this antibody can cross-react with APP under certain conditions, it is widely used for conventional Aβ plaque burden analyses (see Fig. 2).

For β-amyloid plaque morphometric analysis, diameters (maximum lengths) of β-amyloid plaques were measured, and three mutually exclusive plaque size categories (<25, 25–50, or >50 μm) were blindly tabulated. For quantitative analysis of CAA, numbers of Aβ antibody-positive cerebral vessels were blindly counted in each ROI. To evaluate synaptophysin IR, images of five 5-μm sections through hippocampal CA1 and EC were captured based on anatomical criteria (70) and converted to gray scale. The average optical density of positive signals from each image was quantified as a relative number from zero (white) to 255 (black) and expressed as mean intensity of synaptophysin IR.

ELISA

Brains were homogenized using TissueLyser LT (Qiagen, Valencia, CA) in Tris-buffered saline (TBS: 25 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing protease inhibitor cocktail (Sigma) and centrifuged at 18,800 × g for 60 min at 4 °C, and supernatants were collected (representing the TBS-soluble fraction). Resulting pellets were treated with 2% SDS in H2O with protease inhibitors and homogenized using TissueLyser LT. Homogenates were then centrifuged at 18,800 × g for 60 min at 4 °C, and supernatants were collected (representing the 2% SDS-soluble fraction). The remaining pellets were treated with 5 mM guanidine HCl and dissolved by occasional mixing on ice for 30 min and centrifuged at 18,800 × g for 60 min at 4 °C, and supernatants were then collected; this is taken as the guanidine HCl-soluble fraction.

Aβ(1–40) and Aβ(1–42) species were separately quantified in each sample in duplicate by sandwich ELISA (IBL, Gunma, Japan). Putative Aβ oligomers were quantified in the 2% SDS-soluble fraction in duplicate by Human Amyloidβ Oligomers (82E1-specific) Assay Kit (IBL). All samples fell within the linear range of the standard curve.

Western blotting

We lysed brain homogenates in TBS solution containing protease inhibitor cocktail (Sigma) followed by TNE buffer (10 mM Tris-HCl, 1% Nonidet P-40, 1 mM EDTA, and 150 mM NaCl), and aliquots corresponding to 10 μg of total protein were electrophoretically separated using 10 or 15% Tris glycine gels based on target protein molecular weights. Electrophoresed proteins were transferred to polyvinylidene difluoride mem-
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Data are presented as the mean ± 1 S.E. A hierarchical analysis strategy was used in which the first step was an overall ANOVA (repeated measures ANOVA was used for behavioral data) to assess significance of the main effects and interactive terms. If significant, post hoc testing was done with Tukey’s HSD or Dunnett’s T3 methods, and appropriate p values are reported based on adjustment according to Levene’s test for equality of the variance. In instances of multiple mean comparisons, one-way ANOVA was used, followed by post hoc comparison of the means using Bonferroni’s or Dunnett’s T3 methods (where appropriateness was determined by Levene’s test for equality of the variance). The α levels were set at 0.05 for analyses. Analyses were performed using SPSS, release 22.0 (IBM, Armonk, NY).

Author contributions—T. M. and T. T. designed the study, analyzed the data, and wrote the paper. T. M., N. K., T. S., and M. M. performed the experiments. J. T. provided technical assistance and contributed new analytical reagents/tools. All authors reviewed the results and approved the final version of the manuscript.

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作者贡献—T. M.和T. T.设计了研究，分析了数据，并撰写了论文。T. M.，N. K.，T. S.，和M. M.执行了实验。J. T.提供了技术支持，并贡献了新的分析试剂/工具。所有作者都审阅了结果并同意了手稿的最终版本。

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