Oleocanthal Enhances Amyloid-β Clearance from the Brains of TgSwDI Mice and in Vitro across a Human Blood-Brain Barrier Model

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ABSTRACT: Numerous clinical and preclinical studies have suggested several health-promoting effects for the dietary consumption of extra-virgin olive oil (EVOO) that could protect and decrease the risk of developing Alzheimer’s disease (AD). Moreover, recent studies have linked this protective effect to oleocanthal, a phenolic secoiridoid component of EVOO. This protective effect of oleocanthal against AD has been related to its ability to prevent amyloid-β (Aβ) and tau aggregation in vitro, and enhance Aβ clearance from the brains of wild type mice in vivo; however, its effect in a mouse model of AD is not known. In the current study, we investigated the effect of oleocanthal on pathological hallmarks of AD in TgSwDI, an animal model of AD. Mice treatment for 4 weeks with oleocanthal significantly decreased amyloid load in the hippocampal parenchyma and microvessels. This reduction was associated with enhanced cerebral clearance of Aβ across the blood-brain barrier (BBB). Further mechanistic studies demonstrated oleocanthal to increase the expression of important amyloid clearance proteins at the BBB including P-glycoprotein and LRP1, and to activate the ApoE-dependent amyloid clearance pathway in the mice brains. The anti-inflammatory effect of oleocanthal in the brains of these mice was also obvious where it was able to reduce astrocytes activation and IL-1β levels. Finally, we could recapitulate the observed protective effect of oleocanthal in an in vitro human-based model, which could argue against species difference in response to oleocanthal. In conclusion, findings from in vivo and in vitro studies provide further support for the protective effect of oleocanthal against the progression of AD.

KEYWORDS: Alzheimer’s diseases, amyloid-β, blood-brain barrier, clearance, oleocanthal

Mediterranean diet is considered one of the most important healthy habits of the Mediterranean population. Emerging evidence from clinical studies has correlated Mediterranean diets to the low risk of several noncommunicable diseases such as cardiovascular disease and certain types of cancers.1–3 Moreover, a very recent study reported that nutrition intervention with Mediterranean diet to significantly improve the participants’ cognitive performance.4 Mediterranean diet contains several elements that were evaluated for their health promoting effect.5,6 Extra-virgin olive oil (EVOO) is one of the characteristic elements of Mediterranean diet that has been extensively studied for its potential health promoting activities.5,7 Several clinical studies have shown the enrichment of Mediterranean diet with EVOO to improve cognitive performance and slow the progression of memory impairment.3,4,8,9 EVOO is defined as a high quality olive oil that is obtained from the first pressing of olive fruit by mechanical means (European Commission, 2003). EVOO has long been recognized for its extraordinary fat content, which is composed of two fractions, the glycerol (∼95%) and nonglycerol (∼5%) fractions.10 About 75% of fat content of EVOO (in its glycerol fraction) is in the form of oleic acid (a monounsaturated, omega-9 fatty acid) that was previously reported to improve cardiovascular functions such as reduction in blood cholesterol and blood pressure.11 The nonglycerol fraction contains phenolic compounds that account for EVOO resistance to oxidative rancidity.12 Most of EVOO phenolic compounds are antioxidant molecules that are able to counter the toxic effects of oxygen metabolism such as free radical formation, thus protecting cells against oxidative damage.13,14 The total nonglycerol content of EVOO is about 500 mg/kg and includes over 30 chemical substances belonging to different classes, such as alcohols, sterols, hydrocarbons, and volatile compounds.5 The most abundant phenolic compounds in EVOO are tyrosol, hydroxytyrosol, and other complex ester secoiridoids, which share the hydroxytyrosol or tyrosol alcohol moiety. Among EVOO phenolics, S(−)-oleocanthal, a dialdehydic form of (−)-deacetoxyligstroside glycoside, is a
naturally occurring phenolic secoiridoid that has related chemical structure to the secoiridoids ligstroside and oleuropein aglycones, which are common in EVOO. Oleocanthal is responsible for the bitter and pungent taste of EVOO, and has anti-inflammatory and antioxidant properties similar to the nonsteroidal anti-inflammatory drug ibuprofen.\textsuperscript{15}

Several animal and in vitro studies have shown that oleocanthal and phenolic compounds of EVOO possess important neuroprotective activities against Alzheimer’s disease (AD).\textsuperscript{16–20} In vitro studies with oleocanthal demonstrated its effect on the key mediators of AD pathogenesis, amyloid-\(\beta\) (A\(\beta\)) and hyperphosphorylated tau proteins,\textsuperscript{16,21,22} which contribute significantly to neurodegeneration and memory loss.\textsuperscript{23} In these studies, oleocanthal prevented the aggregation of hyperphosphorylated tau by locking tau into the naturally unfolded state,\textsuperscript{21} and altered the oligomerization state of soluble A\(\beta\)\textsubscript{12} oligomers that protected neurons from their synaptopathological effect.\textsuperscript{22} In addition, we recently showed the ability of oleocanthal to enhance cerebrovascular clearance of exogenous A\(\beta\) across the blood-brain barrier (BBB) of wild type mice brains.\textsuperscript{16}

While previous in vitro studies and our in vivo study in wild type mice provided insights on the mechanisms of oleocanthal neuroprotective activity, none of these studies tested the reported beneficial effects of oleocanthal in an AD model. Therefore, in the current study, we examined the effect of oleocanthal on A\(\beta\) load in the brain parenchyma of a mouse model of AD, namely, TgSwDI mice, and on A\(\beta\) deposit on brain microvessels. In addition, the effect of oleocanthal on cerebral clearance and production of A\(\beta\), tau hyperphosphorylation and its anti-inflammatory effect on astrocytes and brain inflammatory cytokines release were investigated. To test whether the observed effects in the mouse model could be extended to humans, we studied the effect of oleocanthal on A\(\beta\) clearance and production using a cell-based in vitro model.

\section*{RESULTS AND DISCUSSION}

Previous clinical and preclinical studies suggested EVOO and its phenolic constituent, oleocanthal, have several health promoting effects that could protect and decrease the risk of AD.\textsuperscript{8,9,24,25} Understanding the mechanisms by which oleocan-
that, as a natural small molecule, protects against AD will be of great importance particularly due to the dramatic rise in AD prevalence and the lack of options for its effective treatment. Several mechanisms have been postulated for oleocanthal protective effect against AD such as inhibition of Aβ and tau aggregation supported by in vitro results and enhancement of stereotaxically microinjected Aβ clearance across the BBB of wild-type mice. However, none of these studies tested the protective effect of oleocanthal in a model of AD. In the present study, we investigated the effects of oleocanthal treatment on Aβ- and tau-related pathological alterations that are associated with the progression of AD in TgSwDI mice. Moreover, we studied the effect of oleocanthal treatment on Aβ production and clearance in a representative human-based in vitro model.

In AD, memory deficits associated with disease progression are likely to result from pathological changes in the entorhinal cortex and hippocampus; both regions are critical for formation of new memories and among the most severely affected in AD. In addition, it has been suggested that hippocampal Aβ deposition reflects the selective vulnerability of this region for AD pathogenesis. Therefore, reduction of Aβ load in the hippocampus is considered one of the main therapeutic aims in the treatment of AD; hence, in the current study, we monitored changes in Aβ load in the hippocampus region of TgSwDI mice. At an early age, these mice exhibit low levels of soluble Aβ1–40 and Aβ1–42 in their brains, and the levels increase significantly by 12 months. However, the soluble fraction continues to represent a very small fraction of the insoluble Aβ peptides. Due to this low level of soluble Aβ peptides in the mouse brain at the age of 5 months (at end of treatment), total Aβ levels were determined by immunostaining. Immunohistochemical analyses of Aβ load in the hippocampi of TgSwDI mice treated with oleocanthal for 4 weeks (5 months alive at the end of treatment, 5 mg/kg/day administered intraperitoneally) showed a significant reduction in total Aβ levels (detected by 6E10 antibody) and in Aβ plaques (detected by ThS).

Semi quantitative analysis for total Aβ demonstrated a significant 30% reduction in the area covered by Aβ in the hippocampus of oleocanthal-treated mice compared to normal saline treated mice (P < 0.01; Figure 1A). Moreover, staining analysis of Aβ plaques by ThS assay showed a significant reduction in the load of Aβ plaques by 28% (P < 0.01; Figure 1B). TgSwDI mouse model is also characterized by the accumulation of Aβ in the brain blood vessels, which results in the development of cerebral amyloid angiopathy (CAA). CAA is a pathological feature present concomitantly with AD at high frequency. To test the effect of oleocanthal on the vascular deposition of Aβ, immunohistochemical analysis of Aβ colocalization with collagen-IV (a marker of microvessels) in the hippocampus was performed. Findings revealed a significant reduction in Aβ-immunoreactive microvessels in oleocanthal-treated group compared to the control group (Figure 1C) with average of Aβ and collagen-IV Pearson’s colocalization coefficient were significantly reduced from 0.197 (control group) to 0.046 (oleocanthal group). This data support a protective effect of oleocanthal against both CAA and AD.

To explain this reduction in Aβ deposition, the effect of oleocanthal on Aβ production and clearance in the brain of TgSwDI mice was evaluated. Oleocanthal effect on Aβ production was assessed by monitoring levels of full length and shorter forms of amyloid precursor proteins (APP) in the mice brains. Aβ is derived from sequential proteolytic processing of APP. Two principal APP processing pathways have been identified: the amyloidogenic pathway, which leads to Aβ generation; and the nonamyloidogenic pathway, which prevents Aβ generation. In the amyloidogenic pathway, the β-secretase activity initiates Aβ generation by shedding a large part of the ectodomain of APP (sAPPβ) generating an APP carboxy-terminal fragment (βCTF or C99), which is then cleaved by γ-secretase to liberate Aβ to the brain interstitial fluid (ISF). In the nonamyloidogenic pathway, APP is cleaved approximately in the middle of the Aβ region by α-secretase. This processing step generates a large part of the ectodomain of APP (sAPPα) and a truncated APP CTF (αCTF or C83), which lacks the amino-terminal portion of the Aβ domain. Therefore, increase in sAPPα or decrease in full length APP (APP-FL) or sAPPβ would be associated with a reduction in brain Aβ production. In this study, we measured the brain levels of APP-FL, sAPPα, and sAPPβ, by Western blot, to assess the effect of oleocanthal on APP processing and Aβ production. The results showed that oleocanthal treatment did not change the levels of APP-FL, sAPPα, or sAPPβ (Figure 2) and, therefore, did not affect APP processing. These results suggest that reduction in hippocampal Aβ load is caused by a mechanism other than Aβ production.

Although production of Aβ increases significantly in early onset familial AD, increasing evidence suggests that Aβ accumulation in the brain of late-onset AD patients is related to its impaired clearance from the brain. Moreover, mutations in Aβ sequence, such as the Dutch and Iowa mutations, increase Aβ propensity to aggregate specifically on microvessels and decrease its cerebral clearance in patients with familial CAA. Therefore, reduction in cerebral clearance of Aβ is considered one of the major factors contributing to Aβ accumulation and subsequent development of late-onset CAA and AD. Clearance of Aβ from the brain takes place by three pathways, transport across the BBB, brain degradation, and bulk flow of cerebrospinal fluid. Transport across the BBB significantly contributes to the total clearance of Aβ and was estimated to be 62% in mice. Given this major contribution of the BBB to Aβ clearance, we next evaluated oleocanthal effect on Aβ clearance across the BBB using the brain clearance index method (BCI) where 125I-Aβ40 was stereotaxically micro-injected into the mice brains. TgSwDI mouse express Aβ peptides with Dutch and Iowa (D1) mutations, however, in the BCI study, wild type Aβ40 not mutated Aβ, was used to evaluate the modulatory effect of oleocanthal on Aβ clearance for the following reasons: the slow clearance rate of DI-mutated Aβ peptides and their high propensity to aggregate in buffer solutions, which make their use in the clearance studies difficult and impractical when compared to Aβ40. Yet, as wild type Aβ and DI-Aβ peptides use the same clearance proteins expressed at the BBB, it is expected that any alteration in the expression of Aβ transport proteins expressed at the BBB to modulate the clearance of both wild type and DI-mutated Aβ peptides. Findings from the BCI experiments demonstrated a significant ~18% increase in total brain clearance (BCI(total)) of 125I-Aβ40 from 38±4.2% in normal saline treated mice to 56.7 ± 5.6% in oleocanthal treated mice (p = 0.013; Figure 3A), a value that is similar to that observed previously in young aged wild type mice. According to our results, this increase in total Aβ clearance was in part due to the enhanced removal of Aβ across the BBB where oleocanthal treatment significantly increased the BBB clearance (BCI(BBB)) of 125I-Aβ40 by ~13% from 26.2 ± 4.6% in normal saline treated mice to 39.5 ± 2.7% in
BBB, brain microvessels were isolated for analysis. A study by approaching the normal value observed in wild type mice.36

Levels of full length APP, sAPPα, and sAPPβ did not modulate the processing of APP as demonstrated by comparable densities in oleocanthal treatment for 4 weeks starting at the age of 4 months and liver X receptors (LXRs).42 The mechanism by which oleocanthal enhances Aβ clearance is another well-established pathway known for its high efficiency in cerebral Aβ removal.40,41 This pathway includes ApoE and ABCA1 that are transcriptionally regulated by the nuclear receptors peroxisome proliferator–activated receptor gamma (PPARγ) and liver X receptors (LXRs).42 The mechanism by which oleocanthal enhances Aβ clearance could be via enhancing Aβ phagocytosis by macrophages and microglial cells and/or other clearance pathways.43,44 To evaluate the effect of oleocanthal on this pathway, Western blot analysis for ABCA1, ApoE, and LXR was performed in brains' homogenates. Oleocanthal was able to increase the expression of ABCA1, ApoE, and PPARγ but not LXR. Densitometry analysis showed a significant ∼20% increase in ABCA1 expression (P < 0.01; Figure 4A), and a moderate but significant ∼12% increase in ApoE levels (P < 0.05; Figure 4B). This increase in the expression of ABCA1 and ApoE, was associated with increased expression of PPARγ (∼26%, Figure 4C; P < 0.01) but not LXR (Figure 4D). PPARγ and LXR act in a feed-forward manner to induce the expression of ABCA1 and ApoE.42

The significance of inducing this pathway was previously reported in findings in wild type mice,16 oleocanthal treatment significantly increased the expression of Aβ degrading enzymes NEP and IDE in mice brains' homogenates (Figure 3B). These findings are significant because they emphasize the beneficial effect of oleocanthal to reduce brain levels of not only wild type Aβ peptides but to include Dutch- and Iowa-mutated Aβ, found in familial cases, that have been recognized for their slow brain clearance,28,34 therefore extending its effect to, beside the sporadic type, to familial AD.

ApoE-dependent Aβ clearance is another well-established pathway known for its high efficiency in cerebral Aβ removal.40,41 This pathway includes ApoE and ABCA1 that are transcriptionally regulated by the nuclear receptors peroxisome proliferator–activated receptor gamma (PPARγ) and liver X receptors (LXRs).42 The mechanism by which oleocanthal enhances Aβ clearance could be via enhancing Aβ phagocytosis by macrophages and microglial cells and/or other clearance pathways.43,44 To evaluate the effect of oleocanthal on this pathway, Western blot analysis for ABCA1, ApoE, PPARγ and LXR was performed in brains' homogenates. Oleocanthal was able to increase the expression of ABCA1, ApoE, and PPARγ but not LXR. Densitometry analysis showed a significant ∼20% increase in ABCA1 expression (P < 0.01; Figure 4A), and a moderate but significant ∼12% increase in ApoE levels (P < 0.05; Figure 4B). This increase in the expression of ABCA1 and ApoE, was associated with increased expression of PPARγ (∼26%, Figure 4C; P < 0.01) but not LXR (Figure 4D). PPARγ and LXR act in a feed-forward manner to induce the expression of ABCA1 and ApoE.42

The significance of inducing this pathway was previously reported in
several studies; for example, Zolezzi et al. reported that activation of PPARγ reduces Aβ levels and improves cognitive function in mouse models of AD,43 and Cramer et al., who reported that enhanced expression of PPARγ to stimulate ApoE and ABCA1 expression and thus Aβ clearance.44 Interestingly, PPARγ has also shown to regulate LRP1 and P-gp expressions.45,46 In vivo and in vitro studies demonstrated treatment with the PPARγ activators cilostazol, a selective phosphodiesterase 3 inhibitor, and Thunbergia laurifolia, widely used as an antidote, to upregulate hepatic LRP1 protein expression in vivo45 and P-gp activity in vitro,46 respectively. Thus, enhanced LRP1 and P-gp efflux function by oleocanthal could also be mediated, at least in part, by activating PPARγ. The effect of oleocanthal on modulating expression of the nuclear receptor retinoid-X receptor (RXR), that forms obligate heterodimers with PPARγ and LXR,47 was also assessed by Western blot, however findings demonstrated oleocanthal does not alter RXR expression (Figure 4D). Collectively, these findings provide an additional mechanism by which oleocanthal decreases levels of Aβ in the brains of TgSwDI mice by enhancing Aβ clearance across the BBB and ApoE-dependent pathway.

Besides Aβ pathology, in the current work, we investigated the effect of oleocanthal treatment on tau expression and phosphorylation in the brains of TgSwDI mice by Western blot. Antibodies against total tau and different phosphorylation sites of tau, including serine-214, serine-262, threonine-212, and threonine-231 were used. Tau is a microtubule-associated protein that accumulates in an abnormally hyper-phosphorylated state forming intracellular filamentous deposits in AD.48 Tau promotes the assembly of tubulin into microtubules and stabilizes the microtubule structure that supports axoplasmic transport.48 In AD, hyper-phosphorylation of tau at serine and threonine residues such as serine-214, serine-262, threonine-212, and threonine-231 inhibits significantly the binding of tau to microtubules and making tau more cytotoxic.48,49 In contrast to Aβ pathology, our results showed comparable expression level and phosphorylation in all studied epitopes between oleocanthal and normal saline treated groups (Figure 5). This lack of oleocanthal effect against tau could be related to the limited treatment time (4 weeks). Previous studies demonstrated a hierarchical relationship between Aβ and tau pathologies, with Aβ causing tau to accumulate and to undergo phosphorylation.50 Therefore, it is convincible that longer administration time of oleocanthal could alter tau pathogenesis because of reduction in Aβ load.
The anti-inflammatory effect of olecanthol in mice brains was also evaluated. Dysfunctional astrocytes have been recognized as an additional pathological alteration observed in AD. Astrocytes are essential in the control of brain homeostasis and support neurons to function. Astrocytes contribute to synaptogenesis and dynamically modulate information processing and signal transmission, regulate neural and synaptic plasticity, and provide trophic and metabolic support to neurons as well as the BBB. Available evidence showed that Aβ deposition could modify astrocytes physiological function and acquire a reactive phenotype. Activation of astrocytes is basically a protective response to remove unwanted stimuli. However, when this activation is prolonged, astrocytes will have damaging effects where they could foster neuroinflammatory response and secret different cytokines and proinflammatory mediators that are neurotoxic. Reducing astrocytes activation has been suggested as an additional therapeutic approach to restore supportive astrocytes functions and prevent further inflammatory mediated cell death by Aβ and oxidative stress. While the anti-inflammatory effect of olecanthol has been reported previously, its anti-inflammatory effect on astrocytes activation caused by Aβ and on brain levels of cytokines is not yet known. One of the earliest markers of astrocyte inflammatory activation is the increased levels of glial fibrillary acidic protein (GFAP), and the long shape with thick branches. GFAP is rapidly induced by different inflammatory mediators and brain stressful events including Aβ deposits. Our results demonstrated the ability of olecanthol to reduce astrocytes activation (Figure 6A, B) and attenuate GFAP intensity by ∼41% (P < 0.01, Figure 6B).

Figure 6. Olecanthol (Oleo) treatment reduces astrocytes activation in the hippocampus of TgSwDI and brain IL-1β. (A) Representative hippocampus sections double-stained with GFAP antibody and anti-Aβ 6E10 to detect activated astrocytes and Aβ load, respectively. Arrows in normal saline group (NS) indicate activated astrocytes with long and thick branches (seen at higher magnification in the closed inserts). (B) Quantitative analysis of GFAP optical density showed a significant reduction in astrocytes activation associated with reduced Aβ levels. Data is presented as mean ± SEM of 4–6 mice in each group. Scale bar, 50 μm. (C) Oleo treatment reduced brain levels of IL-1β (n = 4/group). (∗P < 0.05 and ∗∗P < 0.01). TgSwDI mice were treated with olecanthol (5 mg/kg/day) or NS for 4 weeks beginning at age 4 months.

In addition, this reduced activation ameliorated the astrocytes shape when compared to the control group (Figure 6A). This reduction in astrocytes activation could be attributed to the reduced levels of Aβ (Figure 1). Moreover, the anti-inflammatory response of olecanthol was further supported by the significant reduction in brains’ levels of the cytokine interleukin-1β (IL-1β) by ∼39% (P < 0.05, Figure 6C). This anti-inflammatory effect of olecanthol is consistent with that of a previously reported in vitro effect on IL-1β levels in murine chondrocyte cells.

Next, to evaluate whether the observed effect of olecanthol on Aβ clearance in the brains of AD mouse model could be extended to humans, a series of in vitro experiments with hCMEC/D3 and SH-SYSY-APP cells were performed (Figure 7A). hCMEC/D3 cells were used as a representative model of human BBB endothelial cells, and SH-SYSY-APP cells as a model for human neuronal cells which secret Aβ. Transport studies showed that treatment of cultured cells with 0, 1, 5, and 10 μM olecanthol caused a concentration-dependent increase, up to 50%, in the basolateral to apical transport of Aβ (TQa→l) secreted from SH-SYSY-APP cells (Figure 7B). This increase was associated with a concentration dependent increase in P-gp and LRP1 expression in hCMEC/D3 cells after 72 h treatment (Figure 7C), which is consistent with the above in vivo results and our previous study. In addition, olecanthol effect on the production and secretion of Aβ from SH-SYSY-APP cells was evaluated by measuring Aβ40, Aβ42, and the different forms of APP levels by Western blot. As shown in Figure 7D, the expression of Aβ40 and Aβ42 peptides secreted in the media of SH-SYSY-APP cells, separately treated with olecanthol for 72 h, were comparable to those without treatment (P > 0.05). Similarly, olecanthol treatment did not modulate the levels of sAPPα and sAPPβ (Figure 7E), confirming that olecanthol has no effect on the production and secretion of Aβ from SH-SYSY-APP. In addition, these constant and comparable levels of Aβ peptides as well as sAPP forms suggest that olecanthol has no γ-secretase modulatory properties. Together, these findings suggest that the positive effect of olecanthol could be extended to humans, and that olecanthol is a promising therapeutic molecule against AD.

Collectively, findings from this study showed that olecanthol treatment has significantly reduced the total Aβ levels in TgSwDI brains hippocampi and decreased Aβ deposits on brain microvessels. The reduction in Aβ levels could be explained, at least in part, to the enhanced Aβ clearance across the BBB via the up-regulation of P-gp and LR1P1, and up-regulation of the ApoE-dependent pathway. In addition, olecanthol demonstrated an anti-inflammatory effect where it reduced astrocytes activation and IL-1β brain levels. To account for species differences, we were able to recapitulate the protective effect of olecanthol against amyloid pathology in a human-based in vitro model. Therefore, olecanthol could have effect on human similar to that observed in transgenic mice. These effects were statistically significant, suggesting olecanthol treatment for longer, and possibly at earlier time, that is, before Aβ brain accumulation starts, could be therapeutically beneficial for AD and CAA prevention and/or treatment. In conclusion, olecanthol is a novel natural molecule possesses several beneficial effects via targeting multiple pathological aspects of AD, and holds a promise for development as a potentially effective AD drug. Studies investigating olecanthol systemic availability and dosage requirements for translational application are currently in progress.
METHODS

Oleocanthal Extraction. Oleocanthal was extracted as previously reported. Oleocanthal was first eluted from a lipophilic Sephadex LH20 packed column (bead size 25–100 μm; Sigma-Aldrich, MO) with n-hexane:CH₂Cl₂ (1:9) system. Next, oleocanthal was purified from its rich fraction on a C-18 reversed phase Bakerbond octadecyl packed column (bead size 40 μm; Mallinckrodt Baker) using isocratic CH₃CN:H₂O (40:60). A purity of >90% was established for oleocanthal as assessed by TLC, ¹H NMR spectroscopy, and HPLC analysis.

Animals Treatment. TgSwDI mice were housed in plastic cages under standard conditions, 12 h light/dark cycle, 22 °C, 55% relative humidity, and ad libitum access to water and food. The TgSwDI mice express human APP under control of Thy 1.2 neuronal promoter harboring double Swedish mutations and the Dutch and Iowa vasculotropic Aβ mutations. Two groups were assigned to test the effect of oleocanthal treatment, one group received daily intraperitoneal injection with 5 mg/kg oleocanthal (Oleo group, n = 6 mice) dissolved in normal saline containing 5% DMSO and the second group received daily intraperitoneal injection of normal saline containing 5% DMSO (NS group, n = 6 mice). To minimize confounding, all experiments were performed using male age-matched mice. Oleocanthal and normal saline treatments were initiated at age of Figure 7. Oleocanthal (Oleo) enhances Aβ transport across brain endothelial monolayer in an in vitro model of neurovascular unit. (A) Schematic presentation for in vitro model of the neurovascular unit that has been used to assess the effect of oleocanthal on Aβ transport across brain endothelium. (B) Effect of increasing concentration of oleocanthal on the transport of Aβ secreted from SH-SY5Y-APP cells across hCMEC/D3 monolayer. Aβ transport is determined as transport quotient (Aβ-TQb→a). (C) Effect of increasing concentration of oleocanthal on the expression of P-gp and LRP1 in hCMEC/D3 cells as measured by Western bolt. Densitometry analysis shows a concentration dependent increase in P-gp and LRP1 after treatment with oleocanthal. (D) ELISA quantitative analysis of the levels of human Aβ40 and Aβ42 in the media of SH-SY5Y-APP cells separately treated with increasing concentrations of oleocanthal shows that oleocanthal has no effect on Aβ production and secretion. (E) Effect of increasing concentration of oleocanthal on APP processing. Representative blots and densitometry analysis of sAPPα and sAPPβ in the media of SH-SY5Y-APP cells confirmed that oleocanthal has no effect on APP processing. Data is presented as mean ± SEM of three independent experiments (ns, not significant; *P < 0.05 and **P < 0.01).
4 months and continued for 4 weeks. The Institutional Animal Care and Use Committee of the University of Louisiana at Monroe approved all procedures with National Institutes of Health guidelines.

**Immunohistochemical Analyses.** For detection of total Aβ load, formaldehyde-fixed cryostat brain slices were immunostained with 6E10 human-specific anti-Aβ antibody (Biologend, CA) at 1:200 dilution followed by fluorescein-conjugated donkey anti-mouse IgG (Santa Cruz, TX). For detection of Aβ-plaque load in hippocampus, the brain tissue sections were stained with a 1% Thioflavin-S (ThS; Sigma-Aldrich, MO) solution in 80% ethanol for 15 min, as described previously.59 Double immunostaining of astrocytes with Aβ and capillaries with Aβ was performed using rabbit polyclonal GFAP antibody (Santa Cruz) at 1:200 dilution for astrocytes detection, rabbit polyclonal collagen IV antibody (Millipore, CA) at 1:200 dilution for capillaries detection and Alexa Fluor® 488 conjugated anti-Aβ antibody (6E10) for Aβ detection followed by IgG-CFL 594 conjugated donkey antimouse (Santa Cruz) as a secondary antibody for astrocytes and capillaries detection. Images were captured using Nikon Eclipse Ti-S inverted fluorescence microscope (Nikon, USA) at a total magnification of 40x for Aβ plaque load detection, and 200x for astrocytes/Aβ and capillaries/Aβ double immunostaining. For each treatment, images acquisition was performed in 6 groups of tissue sections spanning the hippocampus, each separated by 150 μm and each containing three 15 μm sections (total of 18 sections per mouse). Quantification of total Aβ load, Aβ-plaque load and GFAP optical density in the hippocampus was performed using ImageJ version 1.44 software after adjusting for threshold (Research Support Branch, NIMH/NIH, Bethesda, MD). Total Aβ load in the hippocampus was measured as a percentage of Aβ-covered area and Aβ-plaque load was expressed as the total number of Aβ-plaque. Pearson’s correlation coefficient was calculated to describe the colocalization correlation between Aβ and collagen-IV as previously described49 using ImageJ.

**Brain Clearance of 125I-Aβ40.** In vivo Aβ40 clearance was investigated using the BCI method as described previously.60 Animals were anesthetized followed by the insertion of a stainless steel guide cannula into the right caudate nucleus of mice brains. A tracer fluid (0.5 μL) containing 125I-Aβ40 (30 nM, PerkinElmer, MA) and 14C-inulin (0.02 mCi, American Radiolabeled Chemicals, MO) prepared in extracellular fluid buffer (ECF) was microinjected. Thirty minutes later, brains were rapidly collected. One hemisphere of the brain was used for 125I-Aβ40 analysis and the second hemisphere was used for microvessels isolation as described below. Calculations of 125I-Aβ40 clearance were performed as described previously.50 Using trichloroacetic acid (TCA) precipitation intact (precipitate) and degraded (supernatant) 125I-Aβ40 were determined in brain tissue using a Wallac 1420 Wizard Gamma Counter (PerkinElmer, MA). 14C-Inulin in the precipitate and supernatant were also determined using a Wallac 1141 WinSpectral Counter (PerkinElmer). The 125I-Aβ40 brain clearance index (BCI, %) and clearance of 125I-Aβ40 across BBB (BBB%-%) were determined as described previously50.

**Brain MicrovesSEL Isolation.** Brain microvessels were isolated as described previously.57 Each brain hemisphere was homogenized in ice-cold DPBS followed by the addition of one volume of 30% Ficoll 400 (Sigma-Aldrich). Homogenates were centrifuged at 8000g for 10 min, and the resulting pellets were suspended in ice-cold DPBS containing 1% BSA and passed over a glass bead column to collect microvessels adhering to the glass beads. Isolated microvessels were used for determine P-gp and LRP1 expression by Western blot.

**IL-1β ELISA.** For detection of IL-1β levels in brain homogenate, anti-mouse IL-1 beta Quantikine ELISA kit (R&D Systems, MN) was used according to the manufacturer’s instructions. IL-1β concentrations (pg/mL) were calculated from standard curves. All samples were run at least in triplicate.

**Cell Culture.** Human brain endothelial cells (hCMEC/D3; kindly provided by Dr. P. O. Couraud, Institut Cochin, Paris, France), passages 25–35, were used as a representative model for human BBB. hCMEC/D3 cells were cultured in EBM-2 medium (Lonza, MD) supplemented with 1 ng/mL basic fibroblast growth factor (Sigma-Aldrich), 10 mM HEPES, 1% chemically defined lipid concentrate (Gibco, NY), 5 μg/mL ascorbic acid, 1.4 μM hydrocortisone, 1% penicillin–streptomycin, and 5% of heat-inactivated FBS gold (GE Healthcare Life Sciences, PA). Human neuroblastoma cells (SH-SY5Y) stably expressing wild-type human APP695 (kindly provided by Dr. Elizabeth A. Eckman, Biomedical Research Institute of New Jersey, NJ) were maintained in DMEM supplemented with 10% FBS, glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL) and the selective glycosidase Geneticin® (Gibco). Cultures were maintained in a humidified atmosphere (5%CO2/95% air) at 37°C and media was changed every other day.

**Effect of Oleocanthal on Aβ Transport across hCMEC/D3 Monolayer.** The transport of Aβ40 and 125I-inulin, as a marker for paracellular diffusion, was measured across hCMEC/D3 monolayer after treatment with different oleocanthal concentrations. To prepare hCMEC/D3 cell monolayers, transwell polyester membrane inserts, 6.5 mm diameter with 0.4 μm pores (Corning, NY), were coated with rat tail collagen-IV (150 μg/mL) for 90 min at 37°C. Cells were plated onto coated inserts at a seeding density of 50,000 cells/cm²; medium was changed every other day. Transepithelial electrical resistance (TEER) was measured using an EVOM epithelial voltohmeter with STX2 electrodes (World Precision Instruments, FL). hCMEC/D3 cell monolayers were used for Aβ transport experiments on day 6 of culture. On day 6, the TEER value was measured and ranged from 35 to 40 Ω × cm², consistently with previously reported values for this cell line. Cells were treated for 72 h starting from third day of seeding with increasing concentration of oleocanthal (0, 1, 5, and 10 μM) added to the apical side of the model (Figure 7A). At the end of treatment, transwells were transferred to 24-plate well that contains APP695-trasfected SH-SY5Y cells at 70–80% confluence. Basolateral to apical (B → A) transport studies of Aβ secreted from SH-SY5Y-APP transfected cells were initiated by addition of 800 μL of fresh prewarmed media that contains 0.05 mM 125I-inulin to basolateral chamber and 200 μL of fresh media was added to the apical chambers. Cells were maintained in a humidified atmosphere (5%CO2/95% air) at 37°C for the time course of the transport experiments (up to 12 h). At the end of incubation period (12 h), media from both chambers were separately collected for Aβ analysis and 125I-inulin measurements. Aβ concentrations in basolateral and apical chambers were measured using a highly specific sandwich ELISA. Rabbit anti-Aβ40 (Millipore, CA, Cat#: AB5373) and rabbit anti-Aβ42 (Calbiochem, CA, Cat#: PC150) monoclonal antibodies specific against the C-termini of human Aβ40 and Aβ42, respectively, were used as capturing antibodies. Antibodies were coated at 5 μg/mL (100 ng/well) on a Maxisorp ELISA plate (Thermo Scientific, IL) to capture Aβ. Detection was achieved with HRP-conjugated 6E10 (Covance Research Products, MA) monoclonal antibody specific against the N-terminus of human Aβ (aa. 3-8 human Aβ sequence) at 1 μg/mL. For 125I-inulin measurements, samples were mixed with 5 mL of scintillation cocktail then dpm was measured using a Wallac 1414 Liquid Scintillation Counter. Amount of Aβ and 125I-inulin in basolateral and apical chambers were calculated as ng and dpm, respectively. The transport quotients of B → A (Aβ/T-Q) were calculated as described previously.50

To study the effect of oleocanthal on Aβ transporters/receptors expression in hCMEC/D3 cells, Western blot analysis was used to measure the expression of P-gp and LRP1 after treatment of hCMEC/D3 cells with oleocanthal. hCMEC/D3 cells were seeded in 100 mm cell culture dishes (Corning) at a density of 1 × 10⁶ cells per dish. The cells were allowed to grow to 70% confluence before treatment with different concentrations of oleocanthal in a humidified atmosphere (5% CO2/95% air) at 37°C. Cells were treated for 72 h with control media or oleocanthal (0, 1, 5, and 10 μM). At the end of treatment period, cells were washed with ice cold PBS, scraped, and centrifuged at 500 rpm for 10 min, and then the pellet was dissolved in 100 μL of RIPA buffer containing complete mammalian protease inhibitor mixture (Sigma-Aldrich).

**Effect of Oleocanthal on Aβ Production by SH-SY5Y-APP Transfected Cells.** To study the effect of oleocanthal on the production of Aβ, SH-SY5Y-APP cells were seeded in 24-well plates and maintained as described above. At 30–40% confluency, SH-SY5Y-APP cells were treated with oleocanthal at 0, 1, 5, and 10 μM for 72 h.
At the end of the treatment period, media containing oleocanthal was removed, cells were washed with prewarmed media, and then fresh prewarmed 300 μL media was added to the cells. After 12 h, media was collected for Aβ and sAPP analyses. Aβ42 and Aβ40 levels in medium were measured by ELISA. The levels of sAPPα and sAPPβ in the medium from cultured SH-SYSY-APP cells were determined by Western blot analysis.

**Western Blot Analysis.** Protein extracts were prepared from cell lysates, brain microvessels, or brain tissues with RIPA buffer containing 1X complete mammalian protease inhibitor mixture followed by centrifugation at 21,000g for 1 h at 4 °C. The supernatant was collected as protein extract and stored at −80 °C until the time of analysis. Protein concentrations were determined by the BCA method. For Western blot analysis, 25 μg of protein was resolved on 8% bis-tris gels in 3-(N-morpholino)propanesulfonic acid buffer system and electrotransferred onto a 0.45 μm nitrocellulose membrane. Membranes were blocked with 2% BSA and incubated overnight with monoclonal antibodies for P-gp (C-219; Covance Research Products), LRP1 (Calbiochem), ABCA1, ApoE, LXR, RXR, PPARγ, or GAPDH (Santa Cruz). Specific antibodies against sAPPα and sAPPβ were obtained from Immuno-Biological laboratories (IBL). Total tau protein was detected by a phosphate-independent antitau monoclonal antibody (clone Tau-5; Thermo Scientific). Specific antibodies recognizing tau protein phosphorylated at serine residues 214 and 262 and at threonine residues 202 and 212 were used (Sigma). For detection, the membranes were washed free of primary antibody and incubated with HRP-labeled secondary IgG antimouse antibody for P-gp, ABCA1, sAPPα, tau Ab-2, and GAPDH (Santa Cruz); anti-rabbit antibody for LRP1, LXR, RXR, PPARγ, and phosphorylated tau (Santa Cruz); and anti-goat antibody for ApoE (Santa Cruz). The bands were visualized using a Pierce chemiluminescence detection kit (Thermo Scientific). Quantitative analysis of the immunoreactive bands was performed using Li-Cor luminescence image analyzer (LI-COR Biotechnology), and band intensity was measured by densitometric analysis. Three independent Western blotting experiments were carried out for each treatment group.

**Statistical Analysis.** Unless otherwise indicated, the data were expressed as mean ± SEM. The experimental results were statistically analyzed for significant difference using two-tailed Student’s t test for two groups. Values of P < 0.05 were considered statistically significant.

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