# Age-Related Decline in Brain and Hepatic Clearance of Amyloid-Beta is Rectified by the Cholinesterase Inhibitors Donepezil and Rivastigmine in Rats

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ABSTRACT: In Alzheimer's disease (AD), accumulation of brain amyloid-β (Aβ) depends on imbalance between production and clearance of Aβ. Several pathways for Aβ clearance have been reported including transport across the blood-brain barrier (BBB) and hepatic clearance. The incidence of AD increases with age and failure of Aβ clearance correlates with AD. The cholinesterase inhibitors (ChEIs) donepezil and rivastigmine are used to ease the symptoms of dementia associated with AD. Besides, both drugs have been reported to provide neuroprotective and disease-modifying effects. Here, we investigated the effect of ChEIs on age-related reduced Aβ clearance. Findings from in vitro and in vivo studies demonstrated donepezil and rivastigmine to enhance ^{125}I-Aβ_{40} clearance. Also, the increase in brain and hepatic clearance of ^{125}I-Aβ_{40} was more pronounced in aged compared to young rats, and was associated with significant reduction in brain Aβ endogenous levels determined by ELISA. Furthermore, the enhanced clearance was concomitant with up-regulation in the expression of Aβ major transport proteins P-glycoprotein and LRP1. Collectively, our findings that donepezil and rivastigmine enhance Aβ clearance across the BBB and liver are novel and introduce an additional mechanism by which both drugs could affect AD pathology. Thus, optimizing their clinical use could help future drug development by providing new drug targets and possible mechanisms involved in AD pathology.

KEYWORDS: Donepezil, rivastigmine, Alzheimer's disease, amyloid-β clearance, BBB, liver
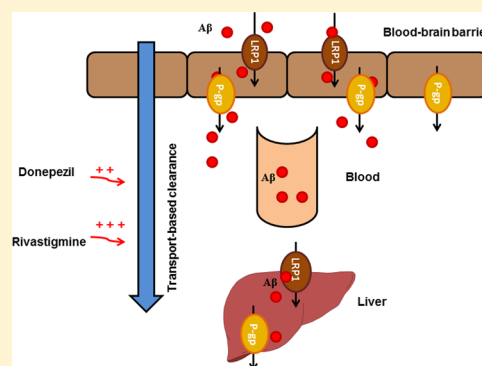
Alzheimer's disease (AD) is the most common cause of dementia and most prevalent in the older population.[1,2] The etiopathology of AD is uncertain with about 90−95% of AD cases being sporadic, while less than 2% of cases are familial with genetic origin.[3] AD is characterized by the presence of amyloid deposits in brain parenchyma (senile plaques) and blood vessels (cerebral amyloid angiopathy), and intraneuronal accumulations of abnormally phosphorelated tau protein (neurofibrilary tangles).[4,5] The plaques consist mainly of amyloid-β peptides (Aβ_{40} and Aβ_{42}) produced by sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases; the majority of these plaques' content is the more hydrophobic Aβ_{42} that has more propensity to aggregate.[6,7] In AD, failure in Aβ clearance and/or its overproduction in the brain are considered major factors that contribute to Aβ brain accumulation.[8−10] Clearance of Aβ across the blood-brain barrier (BBB) is mediated mainly by low density lipoprotein receptor related protein-1 (LRP1) and P-glycoprotein (P-gp).[11,12] Several studies have targeted clearance pathways of Aβ efflux across the BBB and/or enzymatic degradation as a possible therapeutic approach to reduce Aβ brain burden.[13−15] For instance, the administration of oleocanthal extracted from extra-virgin olive oil to C57BL/6 wild-type mice enhanced the clearance of exogenously introduced Aβ_{40} from the brain across the BBB and increased its brain efflux by 29% compared to

control mice, which was associated with up-regulation of P-gp and LRP1 expressions at the BBB.[16] In addition, Aβ brain homeostasis is maintained by the peripheral sink condition that is created by the liver. Hepatic LRP1 and P-gp were also found to play role in Aβ clearance from the periphery.[17,18]

In addition to the major pathological features of AD, amyloid plaques and neurofibrilary tangles, disruption in the neurotransmitter levels of acetylcholine (ACh) and cholinergic dysfunction are observed in AD that are associated with early cognitive impairments, correlate with cognitive decline, and are the basis of the cholinergic hypothesis of AD.[19] Based on the cholinergic dysfunction and associated reduction in brain levels of ACh, inhibitors of enzymes that degrade Ach, including the acetylcholinesterase and butyrylcholinesterase, have been developed in attempt to increase ACh levels and improve the cognitive function of AD patients. Some of these cholinesterase inhibitors (ChEIs) are United States Food and Drug Administration (FDA) approved drugs including tacrine, donepezil, rivastigmine, and galantamine, with a limited clinical use of tacrine due to its reported hepatotoxicity.[20] In addition to ChEIs, N-methyl-D-aspartate (NMDA) receptor antagonists
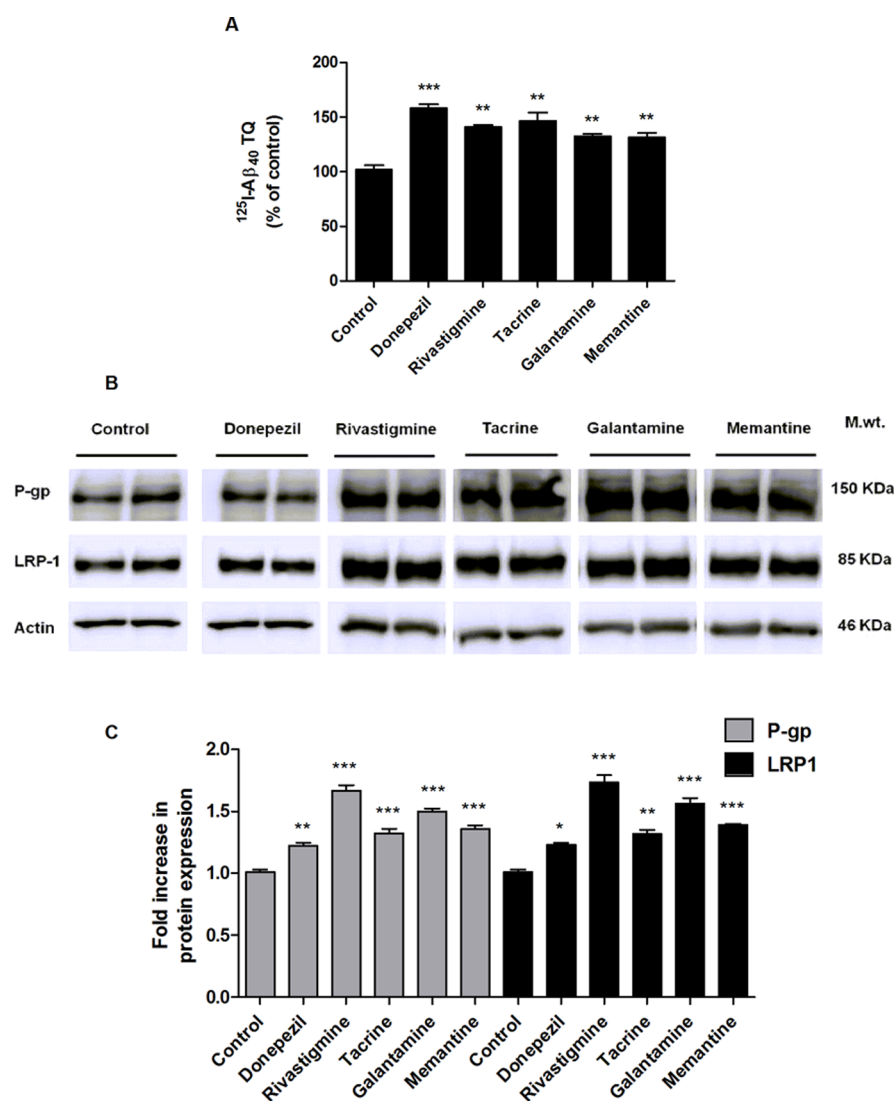
Figure 1. Effect of AD medications on transport of ^{125}I-Aβ_{40} across the BBB model, and on Aβ transport proteins expression in RBE4 cells. (A) Transport quotient (TQ) of ^{125}I-Aβ_{40}, as percent of control, in response to 48 h treatment of 5 μM of each drug. (B) Representative Western blots of P-gp and LRP1 following treatment with AD medications. (C) Graphical representation of normalized protein expression. Data presented as mean $\pm$ SEM ($n = 4$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns = not significant, compared to control untreated cells).

have been developed to inhibit neurotoxicity caused by NMDA receptors hypersensitivity and over activation mediated by various toxins including Aβ oligomers.[21] Memantine is an AD marketed drug that inhibits NMDA receptors and protects neurons from the excitatory effect of excessive glutamate stimulation.[22,23]

In addition to their established mechanisms, these AD medications (ChEIs and NMDA antagonist) are reported to provide neuroprotective and disease-modifying effects,[24−26] and to improve hippocampal-dependent memory in rodents;[27] however, the mechanism by which these drugs produce this effect is not fully understood. Recently, researchers have started to explore possible mechanisms by which ChEIs could influence AD pathology other than of being merely inhibitors of ChEs. For example, available in vitro studies have shown that rivastigmine and donepezil were able to reduce Aβ production by shifting APP processing to α-secretase pathway precluding the formation of toxic Aβ.[28,29] In addition, treatment of rat microglia with galantamine significantly enhanced microglial Aβ phagocytosis by inducing the influx of Ca^{2+} into microglia.[30]

Several mechanisms have been proposed for donepezil neuroprotective effect including down-regulation of NMDA receptors following stimulation of alpha7 nAChRs,[24] increase production of insulin-like growth factor-I in the hippocampus,[27] or inhibition of microglial activation through blocking MAPK and NF-kB signaling pathways.[25]

In this study, we investigated the AD drugs tacrine, donepezil, rivastigmine, galantamine, and the NMDA receptor antagonist memantine for their effect on Aβ brain and hepatic clearance in vitro and in vivo. For the in vitro studies, rat brain endothelial cells (RBE4) cultured on filters as a cell-based model of the BBB, and sandwich-cultured primary rat hepatocytes (SCHs) as a liver model were used to study the effect of the AD medications on ^{125}I-Aβ_{40} transport across the BBB, and biliary clearance, respectively. Subsequently, we chose donepezil and rivastigmine to in vivo investigate their effect on Aβ clearance in rats as both drugs demonstrated the highest significant effect to enhance ^{125}I-Aβ_{40} clearance across both in vitro models. For this, we examined the ability of the chosen drugs to rectify the reduced clearance of Aβ from the brain and
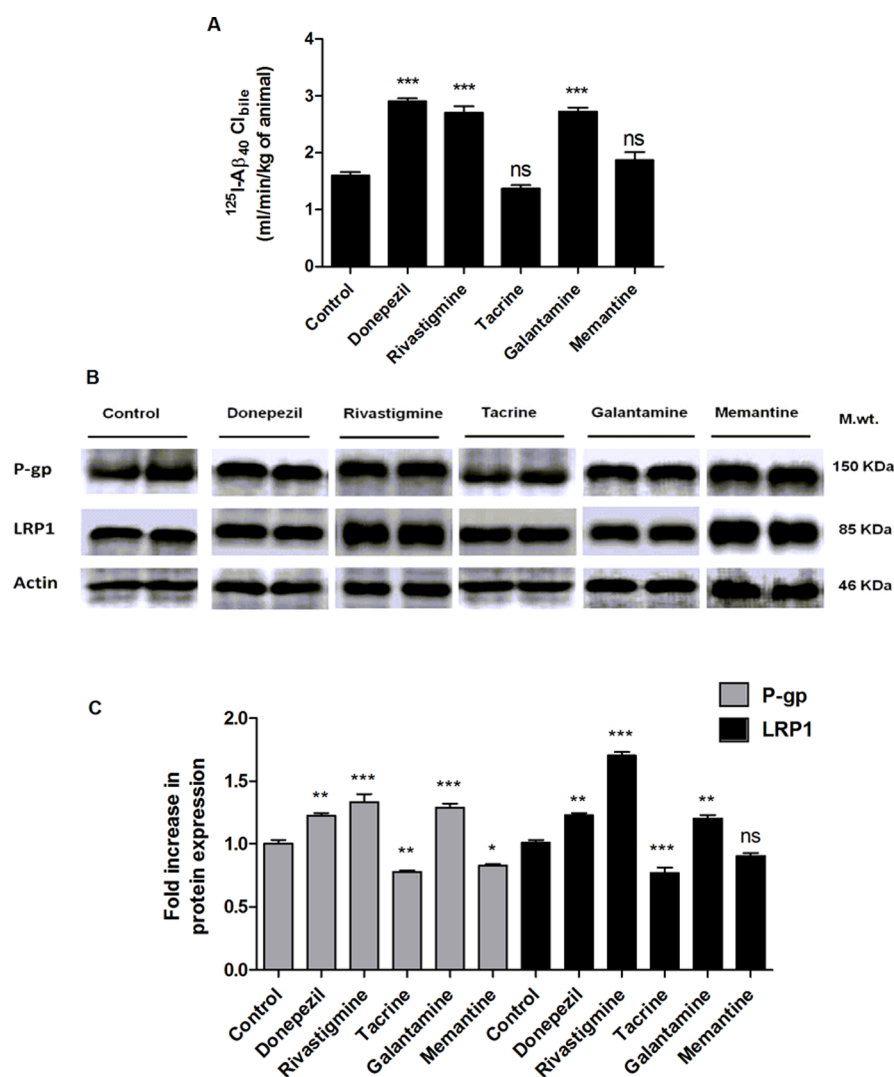
Figure 2. Effect of AD medications on hepatic clearance of ^{125}I-Aβ_{40}, and on Aβ transport proteins expression in SCHs. (A) Biliary clearance (Cl$_{bile}$) of ^{125}I-Aβ_{40} in response to 48 h treatment of 10 μM of each drug. (B) Representative Western blots of P-gp and LRP1 following treatment with AD medications. (C) Graphical representation of normalized protein expression. Data presented as mean ± SEM (n = 4, *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant, compared to control untreated cells).

the liver caused by aging[17,31] in aged rats (24 months of age), and was compared to young rats (4 months of age). Understanding the influence of ChEIs on the major pathways that contribute to AD pathology could optimize their clinical potential and help future drug development endeavor by providing new drug targets and possible mechanisms involved in AD pathology.

■ RESULTS AND DISCUSSION

Currently, one NMDA receptor antagonist, memantine, and four ChEIs, tacrine, donepezil, rivastigmine, and galantamine, are approved for use in the treatment of AD. Although these AD medications do not stop the progression of the disease, they provide symptomatic relief and improve memory in patients with AD. Despite the fact that ChEIs are developed based on their inhibition of ChEs, thereby increasing synaptic ACh level and improving cognitive function, recent studies showed that ChEIs have noncholinergic mechanisms by which they influence Aβ brain level and show neuroprotective effects.[25,26,28,30] In sporadic AD, failure in Aβ clearance from the brain has been suggested as a major mechanism

contributing to brain accumulation of Aβ.[8−10] Aβ clearance from the brain across the BBB is mediated by two major transport proteins, P-gp and LRP1.[11,32] Zlokovic and co-workers[12,33,34] proposed that reduced Aβ clearance across the BBB underlies Aβ brain accumulation, which suggest targeting mechanisms involved in Aβ brain clearance across the BBB as a therapeutic approach for AD treatment.[13−15] Thus, in the present study, we assessed the effect of AD drugs on brain and hepatic clearance of Aβ as an additional mechanism by which they provide neuroprotective effect.

Effect of AD Medications on Aβ Clearance in Vitro. Although Aβ_{42} is more prone to aggregation compared to Aβ_{40}, both Aβ peptides have been implicated in the pathogenesis of AD and participate in the formation of senile plaques with neurotoxicity potentials.[35] Both Aβ_{40} and Aβ_{42} are substrates for P-gp and LRP1;[32,33] however, due to the propensity of Aβ_{42} to rapidly aggregate and its slow clearance compared to Aβ_{40},[36,37] in all our in vitro and in vivo studies we used ^{125}I-Aβ_{40} to evaluate the effect of AD drugs on the clearance of Aβ.

In vitro studies were first performed to screen for the effect of memantine, tacrine, donepezil, rivastigmine and galantamine
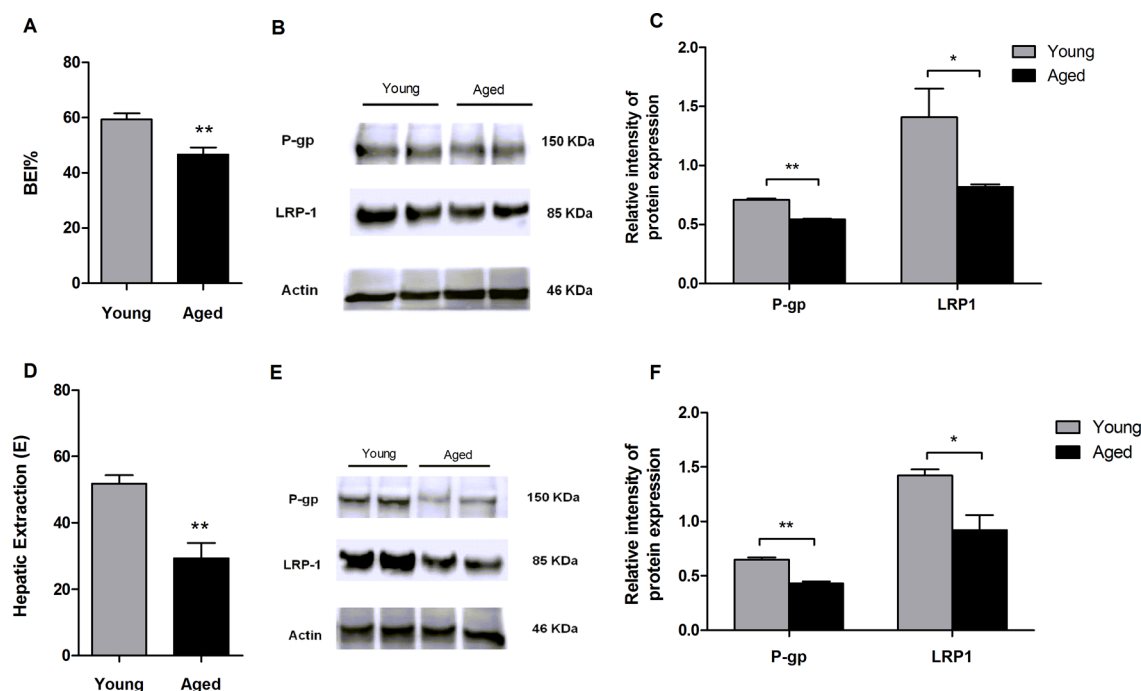
Figure 3. Effect of age on brain clearance and hepatic extraction of ^{125}I-Aβ_{40} and on Aβ transport proteins expression in young (4 months old) and aged (24 months old) rats. (A) Brain efflux index (BEI%) of ^{125}I-Aβ_{40} in young and aged rats. (B) Representative Western blots of brain P-gp and LRP1 proteins expression. (C) Graphical representation of normalized brain proteins expression. (D) Hepatic extraction, E, of ^{125}I-Aβ_{40} in young and aged rats. (E) Representative Western blots of hepatic P-gp and LRP1. (F) Graphical representation of normalized hepatic proteins expression. Data presented as mean ± SEM (n = 5, *P < 0.05, **P < 0.01).

on ^{125}I-Aβ_{40} transport across RBE4 cells, as a cell-based model for the BBB, and ^{125}I-Aβ_{40} biliary clearance in SCHs, a liver model. Transport of ^{125}I-Aβ_{40} from the basolateral side (B; representative of brain) to the apical side (A; representative of blood) of RBE4 monolayer is mediated mainly by active transport processes. To examine the possible effect of AD medications on these processes, 48 h treatment with 5 μM of each AD medications were performed followed by 4 h washout period to avoid any possible interaction between Aβ and the drugs used. As shown in Figure 1A, ^{125}I-Aβ_{40} transport quotient (^{125}I-Aβ_{40} TQ) was significantly enhanced by all drugs with most pronounced effect observed following donepezil, which increased TQ by 57% (P < 0.001). The increase in ^{125}I-Aβ_{40} TQ by the other drugs was also significant and ranged from 40 to 45% (P < 0.01). To examine the effect of the drugs on the expression of Aβ transport proteins, P-gp and LRP1, Western blotting was performed. As shown in Figure 1B and C, all the AD medications caused significant increase in the expressions of P-gp and LRP1 with the highest effect observed with rivastigmine by approximately 1.7-fold for both P-gp and LRP1 (P < 0.001). The effect of other drugs (donepezil, tacrine, galantamine, and memantine) on P-gp and LRP1 expressions was also significant and ranged from 1.2- to 1.5-fold (P < 0.05). These findings could explain, at least in part, the increase in ^{125}I-Aβ_{40} TQ across RBE4 monolayer.

We have previously shown SCHs as a useful model to study Aβ hepatic disposition and its biliary clearance, which was also mediated by P-gp and LRP1.[18] Hence, in this study, SCHs model was used to study the effect of AD medications on ^{125}I-Aβ_{40} hepatic clearance as well as their effect on Aβ transport proteins expression. The 48 h treatment with AD medications, at 10 μM concentration, resulted in a differential effect between the drugs. Cells treated with donepezil, rivastigmine, and

galantamine significantly increased the biliary clearance of ^{125}I-Aβ_{40} by 64%, 55%, and 57%, respectively, compared to control untreated cells (1.6 ± 0.06 mL/min/kg; P < 0.001, Figure 2A). On the other hand, tacrine and memantine showed no significant effect on ^{125}I-Aβ_{40} biliary clearance (P > 0.05; Figure 2A). Consistent with their effect on ^{125}I-Aβ_{40} biliary clearance, SCHs treated with donepezil, rivastigmine, and galantamine upregulated the protein expression of Aβ transporters, P-gp and LRP1 (Figure 2B and C). The three drugs similarly increased P-gp expression by 1.3-fold compared to control cells while for LRP1, its expression was increased by 1.2-fold when SCHs was treated with donepezil and galantamine and 1.7 fold with rivastigmine. Conversely, tacrine downregulated the expression of both LRP1 and P-gp by 20%, while memantine down-regulated P-gp expression and showed no effect on LRP1 expression, which was consistent with their lack of effect on ^{125}I-Aβ_{40} biliary clearance. This correlation between the effect of the investigated drugs on ^{125}I-Aβ_{40} biliary clearance and transport across the cell-based BBB model and their modulatory effect on the expression of Aβ transport proteins LRP1 and P-gp suggests the mechanism by which Aβ clearance is enhanced is due to, at least in part, up-regulation of P-gp and LRP1 expressions.

Treatments of RBE4 cells and SCHs for 48 h with AD medications showed no significant effect on degradation of ^{125}I-Aβ_{40} as determined by the TCA assay (data not shown). From this initial in vitro screening, donepezil, and rivastigmine were selected for in vivo evaluation due to their pronounced effect on ^{125}I-Aβ_{40} clearance when compared to the other tested drugs.

Effect of Donepezil and Rivastigmine on Clearance of Aβ in Vivo. Several studies reported a progressive decline in the BBB expressions of P-gp and LRP1 during normal aging,

and this decline was positively correlated with accumulation of $A\beta$ in the brains of aged nondemented subjects and AD patients.[38,39] Similarly, in rats the expression of P-gp and LRP1 are decreased with aging.[17,31] Hence, we sought to study the effect of aging on brain and hepatic clearance of ^{125}I-$A\beta_{40}$ in rats, and whether donepezil and rivastigmine were able to rectify age-related reduction in $A\beta$ clearance in vivo. First, we examined the effect of aging on the two pathways that regulate $A\beta$ brain and plasma homeostasis where their dysfunction could contribute largely to $A\beta$ brain accumulation and AD pathology. These pathways included the efflux of $A\beta$ across the BBB (from brain to blood) and extraction of $A\beta$ from the general circulation by the liver, which were determined by the brain efflux index (BEI%) and liver uptake index (LUI) methods, respectively. In addition, the effect of aging on the expression of $A\beta$ transport proteins P-gp and LRP1 at the brain and liver was also evaluated. As shown in Figure 3A, the brain efflux index of ^{125}I-$A\beta_{40}$ in aged rats was significantly lower by 22% compared to young rats ($P < 0.01$). Age effect on extravascular extraction (E) of ^{125}I-$A\beta_{40}$ was more pronounced, and when compared to young animals, E was reduced by 44% in aged rats (Figure 3D; $P < 0.01$). These reductions in ^{125}I-$A\beta_{40}$ brain and hepatic clearances were consistent with downregulation of P-gp and LRP1 protein expressions in both brain and liver tissues of aged rats (Figure 3B, C, E, F). P-gp expression was reduced in aged as compared to young rats by 23% and 33% in brain and liver, respectively ($P < 0.01$). For LRP1, the reduction in its protein expression due to aging was also more pronounced in the liver compared to brain with 45% and 20%, respectively (Figure 3C and F; $P < 0.05$).

Next step was to examine the effect of rivastigmine and donepezil on brain clearance and hepatic uptake of ^{125}I-$A\beta_{40}$ in young (4 months) and aged rats (24 months). To evaluate the ability of donepezil and rivastigmine to rectify the age-associated decline in ^{125}I-$A\beta_{40}$ clearance, young and aged rats were treated with clinically relevant doses of either donepezil (2 mg/kg/day) or rivastigmine (0.3 mg/kg/day) using Alzet pump to provide sustained release for 26 days.[40] These doses were selected from previous studies that showed chronic treatment of rats with 2 mg/kg/day of donepezil or 0.3 mg/kg/day of rivastigmine mitigated cholinergic neuronal degeneration, and improved cognitive performance.[41,42] The results are shown in Figure 4. In young animals, BEI studies with both drugs demonstrated a significant increase in the efflux of stereotaxically microinjected ^{125}I-$A\beta_{40}$ across the BBB by 13 ± 2.9% for donepezil ($P < 0.05$) and 31 ± 3.7% for rivastigmine ($P < 0.01$; Figure 4A). In aged animals, donepezil treatment was able to enhance the BEI% of ^{125}I-$A\beta_{40}$ by 30 ± 3.3% compared to vehicle treated control ($P < 0.05$; Figure 4B). Similar to the effect on young rats, rivastigmine effect on aged rats was higher than donepezil and increased the BEI% of ^{125}I-$A\beta_{40}$ by 44 ± 4.3% compared to vehicle treated control ($P < 0.001$; Figure 4B). Interestingly, in aged rats, donepezil increased ^{125}I-$A\beta_{40}$ BEI% from 47 to 61%, a value that was comparable to that of vehicle-treated young rats (59%); and with rivastigmine the increase in ^{125}I-$A\beta_{40}$ BEI% was even higher reaching 67%. These results demonstrated that both drugs were able to rectify age-dependent reduction in $A\beta$ brain clearance. In addition, these findings were consistent with those obtained from clinical trials that reported treating AD patients with rivastigmine for 2 weeks increased plasma level of $A\beta_{42}$, which was associated with positive response to treatment after 6 months and 2 years of follow up.[43,44] This effect could be
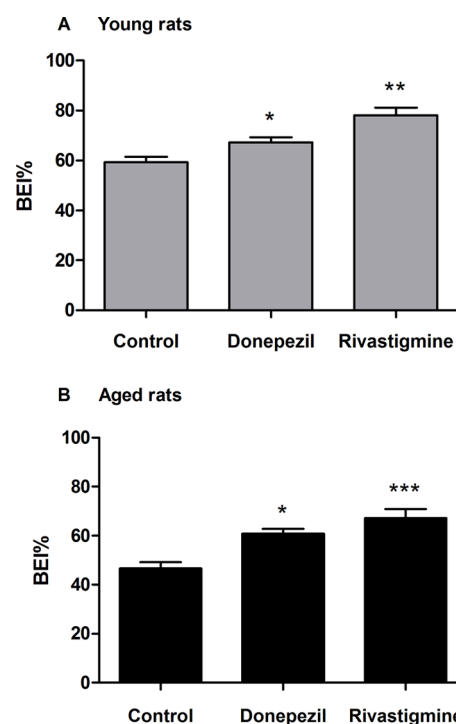

Figure 4. Effect of 26 day treatment of donepezil (2.0 mg/kg/day) or rivastigmine (0.3 mg/kg/day) on brain clearance of exogenous ^{125}I-$A\beta_{40}$ across the BBB in young and aged rats. Brain efflux index (BEI%) of ^{125}I-$A\beta_{40}$ in young (A), and aged (B) rats treated with donepezil or rivastigmine. Data presented as mean ± SEM ($n = 5$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

related to enhanced brain to blood efflux of $A\beta$ and reduced $A\beta$ brain load due to rivastigmine treatment.

In addition to the BBB, targeting the hepatic clearance of $A\beta$ may provide a unique mechanism for rapid and efficient elimination of $A\beta$ from the general circulation, which could in turn reduce $A\beta$ brain load.[17,45] In our studies, the extravascular extraction or hepatic uptake of ^{125}I-$A\beta_{40}$ by a single pass into the portal vein was 51.9 ± 2.4% in vehicle-treated young rats (Figure 5A). The hepatic uptake of ^{125}I-$A\beta_{40}$ was significantly enhanced by 27% in donepezil-treated rats (from 51.9 ± 2.4% to 66 ± 3.2%; $P < 0.05$), and by 23% in rivastigmine-treated rats (from 51.9 ± 2.4% to 64 ± 3.7%; $P < 0.05$, Figure 5A) compared to vehicle-treated rats. In aged rats, the effect was more pronounced and reached values comparable to those of control young animals (Figure 5B), where donepezil treatment increased the hepatic uptake of ^{125}I-$A\beta_{40}$ by 67% (from 29.2 ± 4.6% to 48.7 ± 3.2%; $P < 0.05$) compared to vehicle-treated rats of the same age. For rivastigmine treated rats, the hepatic uptake of ^{125}I-$A\beta_{40}$ was greatly enhanced by 119% (from 29.2 ± 4.6% to 64.0 ± 4.9%; $P < 0.001$), reaching values greater than those of control young rats (Figure 5).

Western blot analysis of P-gp and LRP1 expressions in the brains and livers of treated and untreated animals of both ages showed consistent correlation with ^{125}I-$A\beta_{40}$ clearance. As shown in Figure 6A and B, in the brain, donepezil treatment of young rats enhanced the protein expression of both P-gp and LRP1 by 31% and 22%, respectively. Higher effect was observed with rivastigmine, which up-regulated the expression of both P-gp and LRP1 by 36% and 42%, respectively. For aged animals, donepezil was able to increase the expression of P-gp and LRP1 by 25% and 20%, respectively. Similarly, rivastigmine
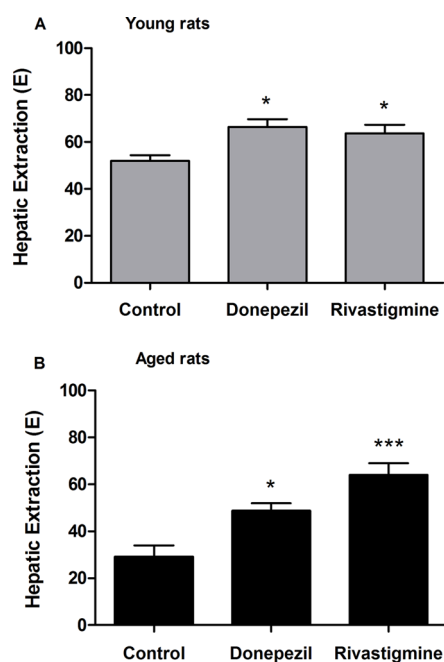
Figure 5. Effect of 26 day treatment of donepezil (2.0 mg/kg/day) or rivastigmine (0.3 mg/kg/day) on hepatic extraction of exogenous ^{125}I-$A\beta_{40}$ in young and aged rats. Hepatic extraction (E) of ^{125}I-$A\beta_{40}$ in young (A) and aged (B) rats treated with donepezil or rivastigmine. Data presented as mean $\pm$ SEM ($n = 5$, *$P < 0.05$, ***$P < 0.001$).

donepezil was able to increase the protein expression level of P-gp and LRP1 by 49% and 31%, respectively. Rivastigmine treated animals up-regulated P-gp and LRP1 by 81% and 88%, respectively, compared to young animals, which was greater than the effect of donepezil by almost two times (Figure 7C and D). These findings suggested that the mechanism by which donepezil and rivastigmine ameliorated $A\beta$ clearance was mediated, at least in part, by their ability to upregulate P-gp and LRP1, whereas both drugs had no effect on $A\beta$ degradation as confirmed by the TCA assay (data not shown). Several studies reported that induction of LRP1, a major receptor involved in $A\beta$ hepatic elimination, would enhance $A\beta$ uptake by the liver. Ravindranath and colleagues reported that a 30 day course of oral administration of *Withania somnifera* root extract reversed behavioral deficits, plaque pathology, and accumulation of $A\beta$ and oligomers in the brains of middle-aged and old APP/PS1 AD transgenic mice, and this effect was mainly attributed to the induction of peripheral "sink condition" via up-regulation of liver LRP1.[46] In addition, we have shown previously, using SCHs, the induction of LRP1 and P-gp by rifampicin significantly increased the biliary clearance of ^{125}I-$A\beta$.[47] Interestingly, treatment of animals with donepezil and rivastigmine showed a significantly higher effect on the hepatic extraction of $A\beta$ by the liver compared to their effect on $A\beta$ efflux across the BBB. This observation could be related to differences in donepezil and rivastigmine accessibility to these tissues influencing their local concentrations thus their effect on transport proteins expression and consequently on $A\beta$ clearance. In addition, this observed effect with donepezil and rivastigmine was more pronounced in aged compared to young rats, which could be attributed to pharmacokinetic changes with age that influenced plasma levels of the two drugs.[48] It has been reported that the blood and brain concentrations of ChEIs are higher in aged than young rats.[48]
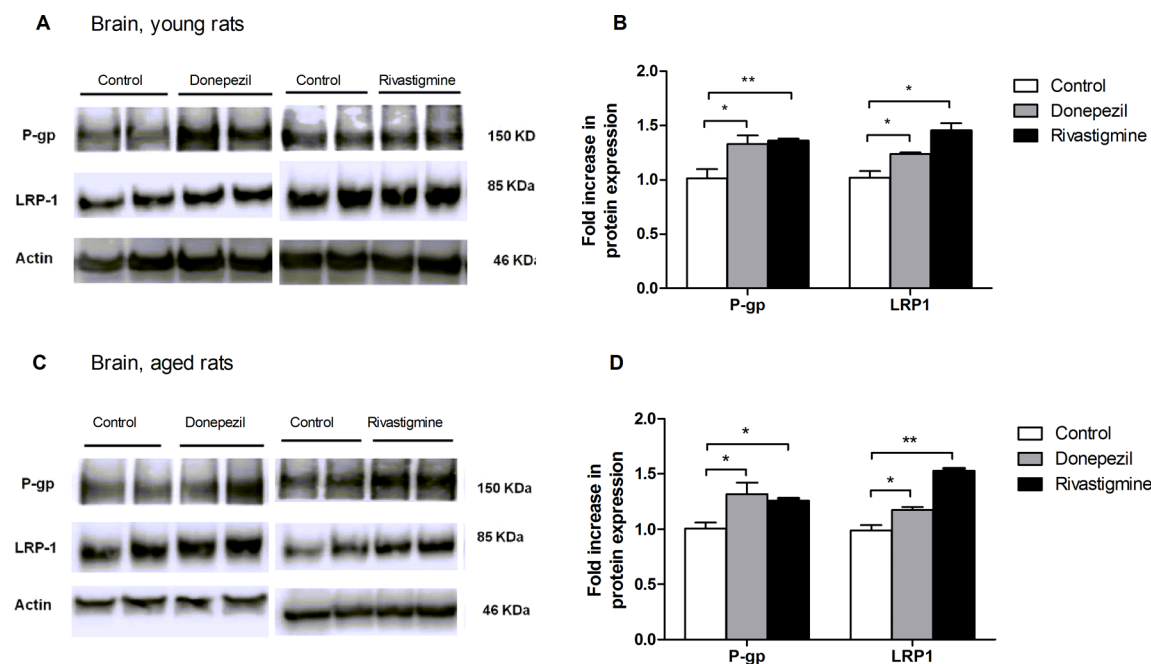
increased P-gp and LRP1 protein expression by 20% and 56%, respectively (Figure 6C and D). The inductive effect of both drugs on P-gp and LRP1 was also seen in the liver. In young animals, both drugs enhanced P-gp and LRP1 expressions by 47% and 41% for rivastigmine, and by 40% and 46% for donepezil, respectively (Figure 7A and B). For aged animals,

Figure 6. Effect of 26 day treatment of donepezil (2.0 mg/kg/day) or rivastigmine (0.3 mg/kg/day) on $A\beta$ transport proteins expression in brains of young and aged rats. (A) Representative Western blots of brain P-gp and LRP1 of young rats. (B) Graphical representation of normalized proteins expression of young rats. (C) Representative Western blots of brain P-gp and LRP1 of aged rats. (D) Graphical representation of normalized proteins expression of aged rats. Data presented as mean $\pm$ SEM ($n = 5$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
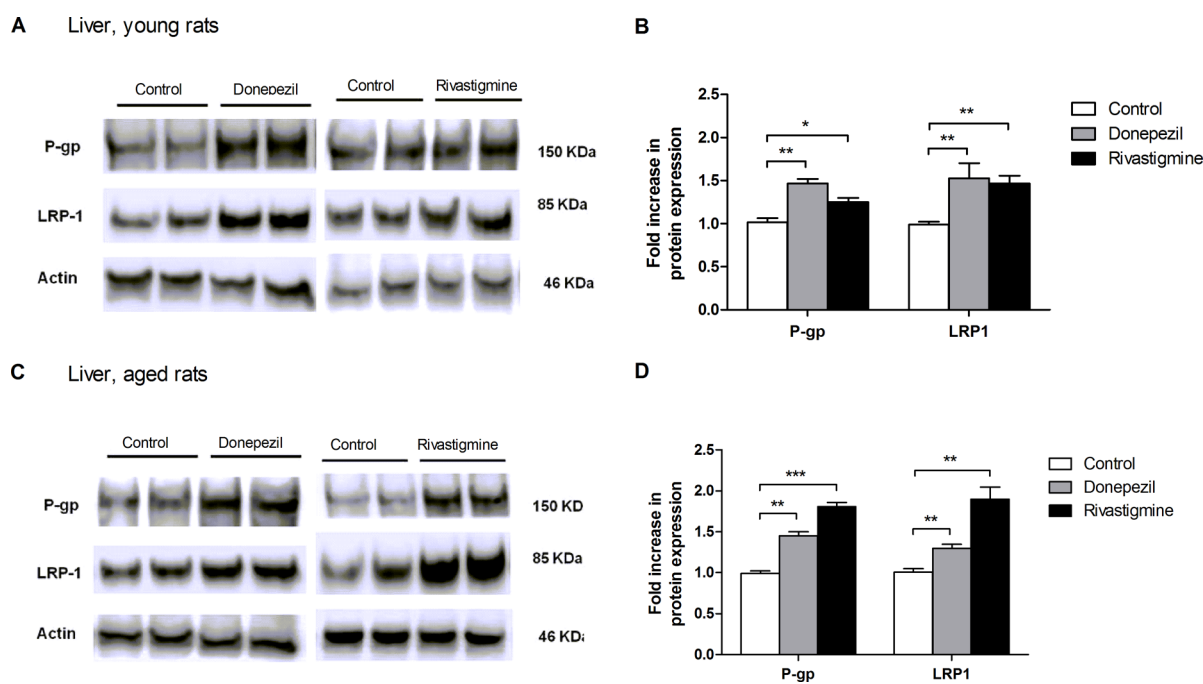
Figure 7. Effect of 26 day treatment of donepezil (2.0 mg/kg/day) or rivastigmine (0.3 mg/kg/day) on $A\beta$ transport proteins expression in livers of young and aged rats. (A) Representative Western blots of hepatic P-gp and LRP1 in young rats. (B) Graphical representation of normalized proteins expression of young rats. (C) Representative Western blots of hepatic P-gp and LRP1 of aged rats. (D) Graphical representation of normalized proteins expression of aged rats. Data presented as mean $\pm$ SEM ($n = 5$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

As both donepezil and rivastigmine were able to enhance the clearance of exogenously administered ^{125}I-$A\beta_{40}$ from the brain and liver, we hypothesized that chronic treatment with both drugs could reduce endogenous $A\beta$ brain load. To determine the overall effect of donepezil and rivastigmine on endogenous levels of both forms of $A\beta$, $A\beta_{40}$ and $A\beta_{42}$, were extracted from the brain and their levels were determined in young and aged rats after 26 days of treatment with either drug. As expected, endogenous levels of both $A\beta$ isoforms, $A\beta_{40}$ and $A\beta_{42}$, were significantly reduced by both drugs in the brains of aged rats, though this reduction was not observed in the brains of young rats (Figure 8). In aged rats, compared to vehicle-treated controls, donepezil reduced total $A\beta_{40}$ levels by 29% from 3520 $\pm$ 383 to 2497 $\pm$ 142 pmol/g brain tissue, and reduced $A\beta_{42}$ level by 41% from 991 $\pm$ 182 to 586 $\pm$ 31 pmol/g brain tissue (Figure 8). Rivastigmine, generated a much greater decline compared to donepezil with comparable reduction in the levels of both $A\beta_{40}$ and $A\beta_{42}$ by approximately 52%, from 3520 $\pm$ 383 to 1713 $\pm$ 260, and from 991 $\pm$ 182 to 466 $\pm$ 67 pmol/g brain tissue, for $A\beta_{40}$ and $A\beta_{42}$, respectively (Figure 8). These reduced levels of $A\beta_{40}$ and $A\beta_{42}$ in the brains of aged rats could be attributed to their enhanced brain and hepatic clearances mediated by increased expression of P-gp and LRP1. Although donepezil and rivastigmine showed a reduction trend in $A\beta_{42}$ levels in young rats, it was not statistically significant ($P = 0.07$). In young rats, brain $A\beta$ levels were already low and close to ELISA quantification limits of both peptides, making further decline difficult to be accurately captured. This, in addition to $A\beta$ efficient clearance machinery in young compared to aged rats, could contribute to the limited effect.

CONCLUSION

$A\beta$ plays a central role in the degenerative process of neurons that lead to neuronal dysfunction in AD.[49] Our findings here
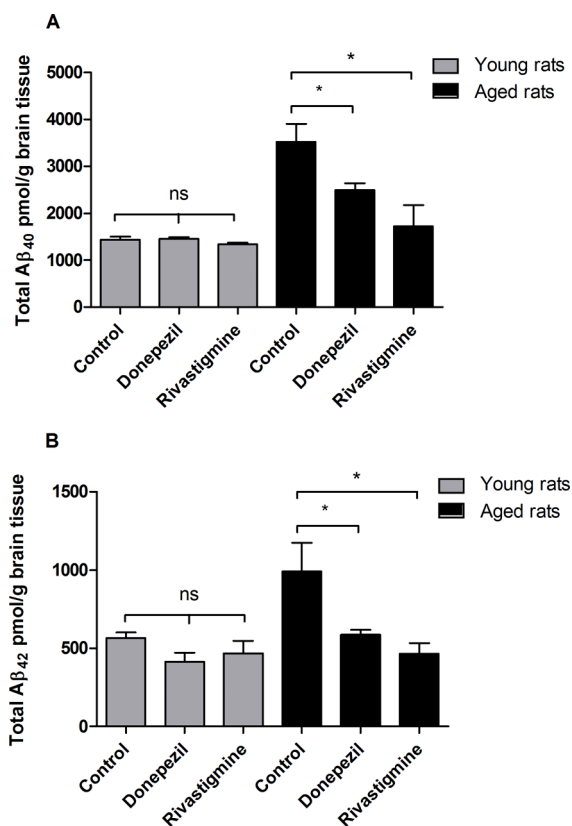

Figure 8. Effect of 26 day treatment of donepezil (2.0 mg/kg/day) or rivastigmine (0.3 mg/kg/day) on endogenous levels of total $A\beta_{40}$ and $A\beta_{42}$ in brains of young and aged rats determined using specific ELISA. (A) Levels of $A\beta_{40}$ in young and aged rats. (B) Levels of $A\beta_{42}$ in young and aged rats. Data presented as mean $\pm$ SEM ($n = 5$, *$P < 0.05$).

demonstrated that the ChEIs donepezil and rivastigmine are able to enhance both brain and hepatic Aβ clearance in young and aged rats, presenting an additional mechanism by which these drugs could implicate AD. Upregulation of P-gp and LRP1 by donepezil and rivastigmine could explain, at least in part, the improved clearance of Aβ across the BBB and the liver. The age-dependent attenuation of Aβ elimination from the brain and the liver confirms aging as a risk factor for AD, and according to our results ChEIs could reduce Aβ brain levels and thus may ameliorate neurodegeneration and memory impairment observed in AD. We thus support the notion that the clinical efficacy of donepezil and rivastigmine in AD are due not only to activation of cholinergic pathway, but also attenuation of Aβ-mediated neurotoxicity. Despite this effect of ChEIs on Aβ and their activation of cholinergic transmission, these agents are not capable of decreasing or altering the progression of AD. Donepezil was investigated in patients with mild to moderate AD in a placebo-controlled double-blind trial for its effect on the cognitive function; the results showed no difference between the treatment and placebo groups,[50] which might indicate that ChEIs do not alter the course of AD. However, in most of the cases, ChEIs are prescribed to patients who already developed AD or at late stages of the disease, where irreversible neuronal damage and potential Aβ deposition and plaques are evident, and therapeutic benefits are expected to be very limited. Thus, we suggest giving donepezil and rivastigmine therapy to patients at very early stages of AD, or patients who are at high risk of developing the disease, which necessitate early diagnostic tools for early therapeutic intervention and better curative effect. In addition, since ChEIs therapeutic doses are determined based on their cholinergic activity, the doses for their noncholinergic effect may need to be adjusted for better therapeutic efficacy.

■ METHODS

Reagents and Antibodies. Synthetic monoiodinated and non-oxidized Aβ$_{40}$ (^{125}I-Aβ$_{40}$; human, 2200 Ci/mmol) was purchased from PerkinElmer (Boston, MA). Fetal bovine serum (FBS) was purchased from ATLANTA biological (Flowery Branch, GA). Ham's F12, MEM alpha medium (α-MEM), HEPES, and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY). Geneticin (G418) was purchased from Calbiochem (Gibbstown, NJ). Tacrine hydrochloride was obtained from Cayman Chemical Company (Ann Arbor, MI). Donepezil hydrochloride and rivastigmine L-tartrate were obtained from Tokyo Chemical Industry Co., LTD (TCI; Portland, OR). RBE4 cells were kindly provided by Dr. P. O. Couraud (Institut Cochin, Paris, France). Collagenase (type I, class I), rat tail collagen (type I), Dulbecco's modified Eagle's medium (DMEM), and insulin were purchased from Invitrogen (Carlsbad, CA). Galantamine hydrochloride, dexamethasone, bovine serum albumin (BSA), basic fibroblast growth factor (bFGF), and soybean trypsin inhibitor were purchased from Sigma-Aldrich (St. Louis, MO). ITS culture supplement (10 μg/mL insulin, 10 μg/mL transferrin, 10 ng/mL selenous acid) was obtained from BD Biosciences (San Jose, CA). Total protein measurement reagents with the bicinchoninic acid (BCA) method were obtained from Pierce (Rockford, IL). For Western blot, the mouse monoclonal antibody C-219 against P-gp was obtained from Covance Research Products (Dedham, MA); mouse monoclonal antibody against light chain LRP1 was obtained from Calbiochem; goat polyclonal antibodies against actin (C-11) and HRP-labeled secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

RBE4 Cell Culture. The immortalized rat brain endothelial RBE4 cells were cultured in dishes or 24-well plates precoated with 150 μg/

mL rat-tail collagen solution. RBE4 cell media consisted of Ham's F10 and α-MEM (1:1, v/v) supplemented with 10% FCS, 1 ng/mL bFGF, 300 mg/mL G418, and 20 mM HEPES. The RBE4 cells were used at a passage number between 26 and 36, and were incubated at 37 °C, air/CO₂ (95%/5%).

Treatment of RBE4 Cells with AD Medications. Cells were seeded in 10 mm cell culture dishes (Corning, NY) at a density of 1 × 10⁶ cells per dish and allowed to grow in a humidified incubator (5% CO₂/95% air) at 37 °C. At 50% confluency, cells were treated with 5 μM of tacrine, donepezil, rivastigmine, galantamine, memantine or vehicle (media contain 0.05% DMSO) for 48 h and replaced with fresh treatment after 24 h. At the end of treatment period, cells were washed with ice-cold phosphate buffer saline (PBS; Gibco) twice, scraped, collected, and lysed in RIPA buffer containing 1% protease inhibitors cocktail. Total protein was determined using the BCA protein assay. Samples were stored at −80 °C until used for Western blot analysis.

Effect of AD Drugs on ^{125}I-Aβ$_{40}$ Transport across the BBB Model. First, RBE4 cells cultured on inserts were treated for 48 h with 5 μM of tacrine, donepezil, rivastigmine, galantamine, memantine, or vehicle. Treatments were then removed and replaced with fresh media for 4 h, as a washout period. The basolateral to apical transport (B → A) was initiated by addition of media containing 0.1 nM ^{125}I-Aβ$_{40}$ and ^{14}C-inulin (0.1 μCi/ml) to B side (lower chamber) and media alone to A side (upper chamber) for 30 min. Aliquots from both A and B sides were taken for radioactivity analysis using Wallac 1470 Wizard Gamma and Wallac 1414 WinSpectral liquid scintillation counters (PerkinElmer Inc. Waltham, MA). Degradation of ^{125}I-Aβ$_{40}$ was determined by the trichloroacetic acid (TCA) precipitation assay.[32] Transport quotient (TQ) of ^{125}I-Aβ$_{40}$ was calculated using the following equation.[51]

$$A\beta_{40}\ TQ = \frac{(A\beta_{40(apical)})/(A\beta_{40(total)})}{(inulin_{(apical)})/(inulin_{(total)})} \tag{1}$$

where Aβ$_{40(total)}$ is the sum of counts per minute (cpm) in the apical and basolateral compartments and inulin$_{(total)}$ is the sum of dpm in the apical and basolateral compartments.

Animals. Sprague–Dawley male rats were purchased from Harlan Laboratories (Houston, TX). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Louisiana at Monroe, and all surgical procedures were consistent with the IACUC policies and procedures. One set of male rats with 2–3 months of age were used for the isolation of hepatocytes. Another set of male rats were used for the in vivo studies, and were divided into two groups (n = 15 rat/group). Group 1 consists of young rats at 4 months of age, and group 2 consists of aged rats at 24 months of age. Both groups were used for intracerebral brain efflux and liver uptake index studies following treatment. Animals were maintained at 22 °C, 35% relative humidity and 12 h dark/light cycle and supplied with water and standard food.

Isolation of Primary Hepatocytes and Preparation of Sandwich Culture (SCHs). Hepatocytes were isolated from rats' livers by the two steps collagenase perfusion method,[52] with slight modification as previously described by us.[18] Hepatocytes were purified from other liver cell populations by a series of centrifugations and resuspended in serum-free medium. Using trypan blue assay, only cells with ≥85% viability were used in the preparation of SCHs. Collagen, 50 μg/mL, coated plates were seeded with 0.7 × 10⁶ cells/mL hepatocytes in serum-free medium supplemented with 1% ITS, 100 U/mL penicillin, 100 g/mL streptomycin, and 0.1 μM dexamethasone. On the second day after seeding, a second layer of 0.25 mg/mL matrigel in ice-cold medium was added over the cells and incubated at 37 °C for 24 h to form a sandwich configuration. The media was replaced with fresh media every day up to day 4 where hepatocytes form extensive bile canaliculi.

Treatment of SCHs with AD Medications. SCHs prepared in 6-well plates were treated with 10 or 25 μM of tacrine, donepezil, rivastigmine, galantamine, memantine, or vehicle for 48 h starting from day 2 of sandwich culture. Treatments were removed and replaced with fresh media for 4 h, as a washout period, followed by biliary

clearance experiments. For protein expression studies, after 48 h of treatment cells were collected and processed as explained under Western blot analysis.

Determination of ^{125}I-Aβ_{40} Biliary Clearance (Cl$_{bile}$) in SCHs Model. The biliary clearance of Aβ in SCHs was determined by the cumulative uptake method as previously described.[47] Treated SCHs were first gently washed with standard HBSS containing calcium or HBSS without calcium (to maintain or break tight junctions that reserve bile canaliculi in culture, respectively) and then incubated with 0.1 nM ^{125}I-Aβ_{40} in respective HBSS buffers containing 2% FBS. After 30 min of cumulative uptake, treatment was removed and cells were washed twice with ice-cold HBSS and once with HBSS containing 2% BSA to reduce Aβ nonspecific binding, and once again with HBSS alone to remove BSA residues. Cells were lysed by adding 300 μL of 1% triton buffer containing 1% protease inhibitors cocktail. Aliquots were taken to determine ^{125}I-Aβ_{40} intracellular accumulation using the gamma counter. Degradation of ^{125}I-Aβ_{40} was determined by TCA precipitation assay.[32] Cell lysate aliquots were mixed with TCA (final concentration 10%) in 1:1 volume and centrifuged at 14 000g at 4 °C for 10 min. Data were normalized to total protein in each well using the BCA protein assay kit. Biliary clearance (Cl$_{bile}$) of Aβ was calculated using the B-CLEAR technology (Qualyst, Inc., Research Triangle Park, NC) according to eq 2.[53]

$$Cl_{bile} = [(\text{accumulation into cells} + \text{canaliculi} (+Ca^{++}))$$
$$- \text{accumulation into cells} (-Ca^{++})]/AUC_{medium} \qquad (2)$$

AUC$_{medium}$ = incubation time × substrate initial concentration in the incubation medium. Biliary clearance values have a unit of mL/min/kg of body weight, based on 200 mg of protein/g of liver and 40 g of liver/kg of rat body weight.[52]

Treatment of Animals. Young, 4 months, and aged, 24 months, Sprague Dawely rats were treated chronically with donepezil (2.0 mg/kg/day), rivastigmine (0.3 mg/kg/day) or vehicle (sterile distilled water) for 26 days using Alzet (DURECT Corporation, Cupertino, CA) osmotic minipumps (model 2ML4); n = 5 rats/treatment. Pumps were implanted subcutaneously by making a small cut in the midscapulary region, inserting the pump, and closing the wound with clips. Pumps delivered 2 mL of solution at a rate of 2.5 μL/h for 26 days. During surgery, rats were anesthetized via isofluorane inhalation. The implanted pumps were removed 1 day before the BEI/LUI surgeries as a washout period to avoid any possible interaction between the drugs used and the injected Aβ inside the brain or the liver as described below.

Brain Efflux Index (BEI) Method. The brain clearance of Aβ was performed using the intracerebral microinjection technique as reported previously.[54] In brief, rats were anaesthetized by an intramuscular injection with xylazine hydrochloride (1.25 mg/kg) and ketamine hydrochloride (125 mg/kg). Then, the coordinates that coincide with the parietal cortex area 2 were determined using stereotaxic apparatus (Stoelting Co., Wood Dale, IL). A small hole was made 5.5 mm lateral and 0.2 mm anterior to the bregma in which a 1 μL microinjection needle was inserted to a 4.5 mm depth. After that, a 0.5 μL of 0.02 μCi/mL ^{125}I-Aβ_{40} and 0.01 μCi/mL inulin mixture prepared in extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM NaHCO$_3$, 3 mM KCl, 1.4 mM CaCl$_2$, 1.2 mM MgSO$_4$, 0.4 mM K$_2$HPO$_4$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4) was administered into Par 2 region over 30 s and the microinjection was left in place for 4 min to avoid any backflow of the administered solution. At 60 min after microinjection, the left and right cerebrum and cerebellum were excised and homogenized in two volume of brain tissue in DPBS buffer (PBS, 5 mM glucose, 1 mM Na-pyruvate). Aliquots were taken for gamma radioactivity measurement using the Wallac gamma counter. Another set of samples was mixed with liquid scintillation mixture (1:10 ratio) for beta radioactivity measurement using the Wallac beta counter. Degradation of ^{125}I-Aβ_{40} was determined by the TCA precipitation assay.[32] The BEI and percentage of substrate remaining in the ipsilateral cerebrum (100-BEI) were determined using eqs 3 and 4, respectively.[54]

$$BEI (\%) = \frac{\text{test substrate undergoing efflux at the BBB}}{\text{test substrate injected into the brain}} \times 100 \qquad (3)$$

$$100 - BEI (\%) = \frac{(^{125}\text{I-A}\beta_{40}/^{14}\text{C inulin}) \text{ counts in the brain}}{(^{125}\text{I-A}\beta_{40}/^{14}\text{C inulin}) \text{ counts in injectate}} \times 100 \qquad (4)$$

Liver Uptake Index (LUI) Method. The hepatic extraction of ^{125}I-Aβ_{40} was determined by the unidirectional uptake of a single injection into the portal vein.[55] Before performing the liver uptake index (LUI) studies, we tested the livers of a separate group of animals (n = 3 rats) that only underwent BEI experiments with ^{125}I-Aβ_{40}, and no radioactivity was detected. Thus, LUI studies were performed on the same rats that underwent BEI experiments. In brief, before the end of the BEI study, a midline incision into the rat abdomen was made to expose the portal vein. The hepatic artery was ligated, followed by a bolus injection of 200 μL of Ringer's HEPES buffer, pH 7.4, containing 0.2 μCi of ^{125}I-Aβ_{40} and 4.0 μCi of ^{3}H water, internal reference, into the portal vein. After 18 s, the major lobe of the liver was excised and homogenized and dissolved for radioactivity analysis. The hepatic extraction (E) of ^{125}I-Aβ_{40} was determined by measuring LUI that was defined by eq 5 and calculated using eq 6.[17]

$$LUI = \frac{E_T}{E_R} \qquad (5)$$

$$LUI (\%) = \frac{(^{125}\text{I-A}\beta_{40}/^3\text{H water}) \text{ counts in liver}}{(^{125}\text{I-A}\beta_{40}/^3\text{H water}) \text{ counts in injectate}} \qquad (6)$$

E_T and E_R are the fractions of ^{125}I-Aβ_{40} and ^{3}H water, respectively, extracted by the liver on a single pass. By applying the E_R value of ^{3}H water, 65 ± 4 in rats as reported previously,[55] in eq 5, and determining the value of LUI experimentally by eq 6, the E_T value can be estimated. The extravascular extraction (E), represents intracellular uptake of ^{125}I-Aβ_{40}, can be determined using eq 7:

$$E = \frac{(E_T - E_{ns})}{(100 - E_{ns})} \qquad (7)$$

E_{ns} is the fractional extraction for distribution in the vascular and extracellular space. Since uptake of ^{125}I-Aβ_{40} upon a single injection into the portal vein represents the apparent extraction by the liver (intracellular uptake, distribution to the interstitial space, and retention in the vascular space), to calculate E, E_{ns} was applied to eq 7. Here, we used an E_{ns} value of 13 ± 3 as reported previously.[55]

Western Blot Analysis. SCHs and RBE4 cells were collected in 1.5 mL eppendorf tubes and centrifuged at 2000g for 10 min at 4 °C. Cells were then lysed in RIPA buffer containing 1% protease inhibitors cocktail and centrifuged at 15 000g for 15 min at 4 °C to remove cell debris. Supernatants were stored at −80 °C for subsequent Western blot analyses. For animals, samples from brain and liver tissues were homogenized and lysed in RIPA buffer containing protease inhibitor, followed by centrifugation at 15 000g for 15 min at 4 °C to collect supernatants for Western blot analysis. Next, 25 μg of protein samples was loaded and resolved using 7.5% SDS-polyacrylamide gel at 140 V for 1 h and transferred electrophoretically onto nitrocellulose membranes at 300 mA for 3 h. After that, membranes were incubated in 2% BSA blocking solution with rocking for 1 h at room temperature followed by overnight incubation at 4 °C with the primary antibodies for P-gp (C-219), LRP1 (light chain), or β-actin (C-11). Secondary antibodies used were anti-mouse IgG antibody for P-gp, anti-rabbit IgG antibody for LRP1, and anti-goat IgG antibody for β-actin, all labeled with horseradish peroxidase (HRP). Protein blots were developed using a chemiluminescence detection kit (SuperSignal West Femto substrate; Thermo Scientific, Waltham, MA). Bands were visualized using a C-DiGit Blot scanner (LI-COR Biosciences; Lincoln, NE), and band intensities were measured by densitometric analysis.

Aβ Enzyme-Linked Immunosorbent Assay (ELISA). The levels of endogenous Aβ_{40} and Aβ_{42} in the homogenates of brains from

young and aged rats treated with donepezil, rivastigmine, or vehicle (n = 5/treatment) were determined using sandwich ELISA. Total Aβ (soluble and insoluble) was extracted from brain tissues by ploytron homogenization in 70% formic acid (FA) and centrifuged for 2 h at 15 000g at 4 °C. Supernatant was collected and diluted 1:20 with TB buffer (1 M Tris base, 0.5 M NaHPO$_4$) for neutralization, followed by further dilution 1:1 with antigen capture buffer provided by the ELISA kit. For Aβ_{40} detection, we used the Sensolyte anti-mouse/rat Aβ_{40} quantitative ELISA kit (AnaSpec, Inc., Fremont, CA), and for Aβ_{42} we used the high sensitivity ELISA kit from Wako Chemicals (Richmond, VA), both according to the manufacturers' instructions. All antibodies used were provided by the kits. For specific detection of Aβ_{40}, a mouse monoclonal antibody-coated plates and a rabbit HRP-conjugated anti-Aβ_{40} were used as capture and detection antibodies, respectively. For specific detection of Aβ_{42} antibody, (BNT77)-coated plates and HRP-conjugated antibody (BC05) were used as capture and detection antibodies, respectively. Total Aβ concentrations were expressed as pg/g brain tissue weight.

Statistical Analysis. All data were expressed as mean ± SEM. The experimental results were statistically analyzed for significant difference using two-tailed Student's t test. A P-value less than 0.05 was considered statistically significant. All statistical analyses were done using GraphPad Prism, version 5.03.

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Author Contributions

All experiments were executed by L.A.M. and H.Q. L.A.M and A.K. designed the experiments and wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

APP, amyloid precursor protein; ACh, acetylcholine; AD, Alzheimer's disease; Aβ, amyloid-β; BEI%, biliary excretion index; BCA, bicinchoninic acid; BBB, blood-brain barrier; BSA, bovine serum albumin; ChEIs, cholinesterase inhibitors; Cl$_{bile}$, biliary clearance; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; E, hepatic extraction; ECE, endothelin-converting enzyme-1; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; LUI, liver uptake index; LRP1, low density lipoprotein receptor related protein 1; NMDA, N-methyl-D-aspartate; P-gp, P-glycoprotein; RBE4, rat brain endothelial cells; SCHs, sandwich cultured primary rat hepatocytes; TQ, transport quotient; TCA, trichloroacetic acid

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