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Role of P-glycoprotein in mediating rivastigmine effect on amyloid- β brain load and related pathology in Alzheimer's disease mouse model

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Abstract

Recently, we showed that rivastigmine decreased amyloid- β (A β) brain load in aged rats by enhancing its clearance across the blood-brain barrier (BBB) via upregulation of P-glycoprotein (P-gp) and low-density lipoprotein receptor-related protein 1 (LRP1). Here, we extend our previous work to clarify P-gp role in mediating rivastigmine effect on A β brain levels and neuroprotection in a mouse model of Alzheimer's disease (AD) that expresses different levels of P-gp. APPSWE mice were bred with *mdr1a/b* knockout mice to produce littermates that were divided into three groups; APP⁺/*mdr1*^{+/+}, APP⁺/*mdr1*^{+/-} and APP⁺/*mdr1*^{-/-}. Animals received rivastigmine treatment (0.3 mg/kg/day) or vehicle for 8 weeks using Alzet osmotic mini-pumps. ELISA analysis of brain homogenates for A β showed rivastigmine treatment to significantly decrease A β brain load in APP⁺/*mdr1*^{+/+} by 25% and in APP⁺/*mdr1*^{+/-} mice by 21% compared to their vehicle treated littermates, but not in APP⁺/*mdr1*^{-/-} mice. In addition, rivastigmine reduced GFAP immunostaining of astrocytes by 50% and IL-1 β brain level by 43% in APP⁺/*mdr1*^{+/+} mice, however its effect was less pronounced in P-gp knockout mice. Moreover, rivastigmine demonstrated a P-gp expression dependent neuroprotective effect that was highest in APP⁺/*mdr1*^{+/+} > APP⁺/*mdr1*^{+/-} > APP⁺/*mdr1*^{-/-} as determined by expression of synaptic markers PSD-95 and SNAP-25 using Western blot analysis. Collectively, our results suggest that P-gp plays important role in mediating rivastigmine non-cholinergic beneficial effects, including A β brain load reduction, neuroprotective and anti-inflammatory effects in the AD mouse models.

Keywords

Rivastigmine; P-glycoprotein; Alzheimer's disease; amyloid-beta; neuroprotection; astrogliosis

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Conflict of interest

The authors declare no competing financial interest.

1. Introduction

Rivastigmine (Exelon[®], Novartis) is a dual acetyl- and butyryl cholinesterase inhibitor used to alleviate symptoms of Alzheimer's disease (AD). AD is a progressive neurodegenerative disorder mainly characterized by accumulation of amyloid- β ($A\beta$) peptides in brain parenchyma and neurofibrillary tangles of hyperphosphorylated tau protein [1, 2]. In addition, many neuropathological changes in AD are reported including synaptic failure, astrogliosis at sites of $A\beta$ deposition, disturbance of glutamate excitotoxicity, and blood-brain barrier (BBB) dysfunction [3].

According to the cholinergic hypothesis of AD, loss of cognitive function is largely attributed to depletion of cholinergic neurotransmitters such as acetylcholine (ACh) mainly in the cerebral cortex and caudate nucleus [4, 5]. Rivastigmine is designed to correct loss of presynaptic cholinergic function by inhibiting cholinesterases and thus decreases the degradation of acetylcholine in synaptic cleft and allow for increased cholinergic signaling which improves cognition and memory [6]. However, previous preclinical *in vitro* and *in vivo* studies reported that rivastigmine could provide neuroprotective and disease-modifying effects through multiple mechanisms that are not related to its cholinergic effect [7–9]. In one study, rivastigmine has shown to induce presynaptic proteins, preserve neuronal viability and regulate APP processing and $A\beta$ production *in vitro* [10–12]. Recently, we have demonstrated a novel mechanism for rivastigmine to reduce $A\beta$ brain load by enhancing its clearance through induction of $A\beta$ major transport proteins, low-density lipoprotein receptor related protein-1 (LRP1) and P-glycoprotein (P-gp), at the BBB and liver [13]. These findings suggest that rivastigmine therapeutic effect is exerted through multiple mechanisms that interfere with $A\beta$ pathogenesis and not limited to cholinesterase inhibition. In fact, some studies indicated that cholinesterase inhibition is not enough to alter the course of AD, and clinical efficacy seen with some cholinesterase inhibitors are attributed to other mechanisms that are yet to be identified [14, 15].

The amyloid cascade hypothesis suggests that $A\beta$ is the primary player in the development of AD, and failure of $A\beta$ clearance is a major event that contributes to $A\beta$ brain accumulation [16–19]. Human studies suggest that $A\beta$ accumulation in the brain correlates inversely with the degree of P-gp expression at the BBB [20]. In addition, aging, a high risk factor for AD, is associated with a decline in the expression of P-gp that is correlated with the accumulation of $A\beta$ in the brains of aged non-demented subjects and AD patients [21, 22]. This observation was also reported in rats, where P-gp and LRP1 expressions were decreased with aging and inversely correlated with $A\beta$ brain deposition [23]. Therefore, proteins involved in the transport of $A\beta$ are important in regulating $A\beta$ brain steady state levels and their dysfunction may contribute to $A\beta$ -mediated pathogenesis in conditions such as AD.

Following to our previous findings [13], this study aims to investigate the question “how changes in P-gp expression would affect rivastigmine therapeutic effect, including $A\beta$ lowering effect, neuroprotection and anti-inflammatory effect using APPSW mice that carry APP transgene and P-gp WT (APP/mdr1^{+/+}), P-gp one copy (APP/mdr1^{+/-}), or P-gp knockout (APP/mdr1^{-/-}) mice. Overall findings suggest that P-gp mediates the non-

cholinergic effects of rivastigmine, and that understanding various mechanisms of action of rivastigmine is important to optimize its clinical utility for AD patients.

2. Materials and methods

2.1 Materials

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO). Rivastigmine L-tartrate was obtained from Tokyo Chemical Industry Co., LTD (TCI; Portland, OR). Complete mammalian protease inhibitor, donkey serum, Ficoll 400, and sodium dodecylphosphate (SDS) were obtained from (Sigma-Aldrich, MO). Reagents for total protein analysis with the bicinchoninic acid method were obtained from Pierce (Rockford, IL). For Western blot and immunohistochemistry, 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 Abcam (Cambridge, MA); goat polyclonal antibodies against actin (C-11), HRP-labeled secondary antibodies, rabbit polyclonal anti-GFAP antibody, rabbit polyclonal anti-EAAT (GLT-1) antibody, and CFL594-conjugated donkey anti-rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal anti-PSD-95 antibody and rabbit polyclonal anti-SNAP-25 antibody were obtained from GeneTex, Inc (Irvine, CA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

2.2 Animals

All animals used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Louisiana at Monroe, and animal sacrifice procedures were consistent with the IACUC policies and procedures. The transgenic mouse model used is B6;SJL-Tg(APP^{SWE})2576Kha or APP^{SWE} with a mixed B6;SJL background (Line 1349). This AD model expresses the human APP⁶⁹⁵ with double mutations (K670N, M671L) that results in amyloid plaque deposition in the brain starting at the age of 9 months [24]. In addition, *mdr1a/b* knockout mice on FVB background were used. This mouse model carries a disruption in the multi-drug resistance genes *Abcb1a* (ATP-binding cassette, sub-family B (MDR/TAP), member 1A, a.k.a. *Mdr1a*) and *Abcb1b* (ATP-binding cassette, sub-family B (MDR/TAP), member 1B, a.k.a. *Mdr1b*) encoding p-glycoprotein 3 and p-glycoprotein 1, respectively. Both models were purchased from Taconic Biosciences, Inc. (Hudson, NY, USA). APP^{SWE} mice were bred with *mdr1a/b* knockout mice as reported previously [25], and the offspring of both sexes were distributed equally among the experimental groups. Tail biopsies were sampled for DNA analysis to confirm the presence of the hAPP DNA sequence in offspring, along with either of three different genotypes for *mdr1*; homozygous (*mdr1^{+/+}*), heterozygous (*mdr1^{+/-}*), or double knockout (*mdr1^{-/-}*), as reported previously [25]. Littermates negative for hAPP transgene were used as controls for PCR analyses, which were performed using primers for APP and *mdr1* genes as reported previously [25, 26]. Animals were maintained at 22°C, 35% relative humidity and 12 h dark/light cycle and received water and standard food.

2.3 Animal experimental conditions

When reached 8 months, animals were divided into three groups; APP⁺/mdr1^{+/+} (n =10 mice), APP⁺/mdr1^{+/-} (n=12 mice) and APP⁺/mdr1^{-/-} (n=6 mice), and equal number from each group were randomly assigned for rivastigmine treatment (0.3 mg/kg/day) or vehicle (sterile distilled water) for 8 weeks using Alzet osmotic mini-pumps (model 1004; DURECT Corporation, Cupertino, CA). Briefly, mice were anesthetized via isoflurane inhalation and pumps were implanted subcutaneously on the back of mice by making a small cut in the mid-scapular region, incision was closed with wound clips. Pumps delivered 100 μ l of solution at a flow rate of 0.1 μ l/h for 4 weeks. Pumps were replaced every four weeks by freshly filled pumps to maintain treatment for a period of 8 weeks. At the end of the study, animals were sacrificed, blood samples were collected, and brains were cut into two half for immediate capillary isolation, or snap frozen for subsequent microsectioning and immunohistochemistry experiments. Another set of animals (n =5/group) that underwent similar abovementioned treatments were sacrificed, blood samples were collected, and brains were cut into two half for western blot analysis of protein expression and extraction of A β and subsequent analysis with ELISA as shown below.

2.4 Isolation of brain microvessels

Freshly isolated brain hemispheres were immediately homogenized in ice-cold DPBS, phosphate-buffered saline buffer (2.7 mM KCl, 1.46 mM KH₂PO₄, 136.9 mM NaCl, 8.1 mM Na₂HPO₄, 0.9 mM CaCl₂, and 0.5 mM MgCl₂ supplemented with 5 mM D-glucose, 1 mM sodium pyruvate, pH 7.4), and microvessels were isolated as described previously [27]. Briefly, Brain homogenate was added to 30% Ficoll in 1:1 volume (final Ficoll concentration was 15%), mixed thoroughly and centrifuged at 5800 \times g for 15 min at 4°C. Supernatant was discarded and pellet containing microvessels was gently resuspended in ice-cold DPBS containing 1% bovine serum albumin (BSA) and collected by separation over glass beads containing column. Microvessels adhered to glass beads were collected by gentle shaking followed by centrifugation. Pelleted microvessels were lysed in radio-immunoprecipitation assay (RIPA) buffer for subsequent Western blot analysis.

2.5 Western blot analysis

Brain hemispheres were homogenized in RIPA buffer containing 1% protease inhibitor cocktail and centrifuged at 15,000 \times g for 10 min at 4°C. Supernatants were stored at -80°C for subsequent Western blot analysis. Twenty five micrograms of protein samples were 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 blocked in 2% BSA in PBS 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), PSD-95, GLT-1, SNAP-25, and β -actin (C-11). Secondary antibodies used were: anti-mouse IgG antibody (1:2000 dilutions) for P-gp and PSD-95, anti-rabbit IgG antibody (1:2000 dilutions) for LRP1, GLT-1 and SNAP-25, and anti-goat IgG antibody (1:1500 dilutions) for β -actin, all labeled with horseradish peroxidase (HRP). Proteins' blots were developed using a chemiluminescence detection kit (SuperSignal West Femto substrate;

Thermo Scientific, Waltham, MA). Bands were visualized using C-DiGit® Blot Scanner (LI-COR Biosciences; Lincoln, NE) and quantified by densitometric analysis.

2.6 Extraction of total A β from brain

The extraction of A β from the brain tissues was performed as described previously [28]. The SDS fraction of brain samples was used to quantify A β oligomers (A β -SDS fraction) and the formic acid (FA) extract was collected to quantify insoluble deposited A β (FA fraction). Both fractions were neutralized by dilution 1:20 with TB buffer (1 M Tris base, 0.5 M Na₂HPO₄), followed by further 1:1 dilution with antigen capture buffer provided by the ELISA kit. All fractions were stored at -80°C until the time of analysis. The total amount of A β in the brain was calculated by summing the amount of A β in all fractions.

2.7 A β Enzyme-linked immunosorbent assay (ELISA)

Endogenous A β in brain homogenates and plasma samples of mice treated with rivastigmine or vehicle (n=5) were determined using sandwich ELISA. For A β detection Sensolyte Anti-human A β ₄₀ Quantitative ELISA Kit (AnaSpec, Inc., Fremont, CA), and Ultrasensitive anti-human A β ₄₂ ELISA kit (Novex, Frederick, MD) were used, according to the manufacturers' instructions. All antibodies used were provided by the kits. For specific detection of A β ₄₀, a mouse monoclonal antibody-coated plates and a Rabbit HRP-conjugated anti A β ₄₀ were used as capture and detection antibodies, respectively. For specific detection of A β ₄₂, a rabbit monoclonal antibody-coated plates and HRP-conjugated anti-rabbit IgG were used as capture and detection antibodies, respectively. The assessment of A β levels in in SDS and formic acid fractions by ELISA assay was used as described by us previously [28]. A β concentrations were expressed as pg/g brain tissue weight.

2.8 IL-1 β Enzyme-linked immunosorbent assay

Brain and plasma levels of IL-1 β were determined in mice treated with rivastigmine or vehicle (n = 5) using sandwich ELISA. Quantikine ELISA kit was obtained from R&D Systems (Minneapolis, MN) and used according to the manufacturers' instructions. Antibodies provided by the kit include, a monoclonal capture antibody specific for mouse IL-1 β , and a horseradish peroxidase-linked polyclonal detection antibody specific for mouse IL-1 β .

2.9 Immunohistochemistry

Brain sections of 15 μm -thick were prepared using Vibratome UltraPro 5000 Cryostat installed with 5040 rotary retracting microtome (Cambridgeshire, UK), sections were then fixed on glass slides, labeled and stored at -80°C . Subsequently, sections were fixed by incubation in acetone for 10 min at -20°C . Sections were washed 3 times in PBS and blocked in TBST containing 10% donkey serum for 1 h at room temperature. For detection of GFAP, a rabbit anti-GFAP polyclonal IgG was used at 1:100 dilution in blocking solution and incubated for 4 h at room temperature. Then, sections were incubated with anti-rabbit IgG-CFL594 secondary antibody for 1 h at room temperature. After washing 4 times with PBS, GFAP immunoreactivity was visualized using Nikon Eclipse Ti-S inverted fluorescence microscope (Norcross, GA) at magnification of 200 \times . GFAP staining was

analyzed using the image J version 1.44 software provided by NIH (<http://rsbweb.nih.gov/ij/>). After adjusting for threshold, Image J was used to determine the optical density of GFAP in the hippocampus region. Number of sections analyzed were 8 sections/animal for treated and untreated mice (n= 4 mice/group). Data were presented as area fraction of GFAP immunoreactive cells to area of hippocampus region.

2.10 Analysis of acetylcholinesterase activity

We determined AChE activity in plasma and brain homogenate from animals treated with rivastigmine or vehicle indirectly by measuring the level of ACh. For this we used choline/acetylcholine assay kit from Abcam. Samples from plasma and brain homogenates were processed according to protocol provided by the kit and detection of choline was performed fluorometrically at excitation/emission of 535/590 nm, respectively. This assay measures either free choline or total choline, which includes free choline and ACh. Thus, ACh level can be determined as follows; ACh = total choline – free choline.

2.11 Statistical analysis

Data were expressed as mean±SEM. The experimental results were statistically analyzed for significant difference using two-tailed unpaired Student's t-test for two groups and using ANOVA with post hoc test for more than two groups. A P-value of less than 0.05 was considered statistically significant. Data analyses were done using GraphPad Prism, version 5.03.

3. Results

3.1 Effect of rivastigmine treatment on brain and plasma levels of acetylcholine

As expected, APP/mdr1^{+/+} mice treated with rivastigmine significantly increased ACh brain and plasma levels by 2- to 3-fold compared to control group (P<0.001, Fig. 1). Similarly, rivastigmine was able to enhance brain ACh levels, but to a much lesser extent, in APP/mdr1^{+/-} (by 42%) and APP/mdr1^{-/-} (by 33%) (Fig. 1A), which is consistent with rivastigmine mechanism of action as an acetylcholinesterase inhibitor. Interestingly, however, ACh levels in the brains of vehicle treated APP/mdr1^{+/-} and APP/mdr1^{-/-} mice were significantly less by 52 and 32%, respectively, when compared to APP/mdr1^{+/+} vehicle treated mice (Fig. 1A). Conversely, ACh plasma levels in vehicle-treated mice showed a trend toward increase in APP/mdr1^{-/-} and APP/mdr1^{+/-} mice compared to APP/mdr1^{+/+} mice, but was not significant (Fig. 1B).

3.2 Effect of rivastigmine treatment on Aβ major transport proteins' expression at the BBB

As shown in Figure 2, 8 weeks treatment with rivastigmine resulted in the up-regulation of both P-gp and LRP1 by 1.5 and 1.34 fold in APP/mdr1^{+/+} mice and by 1.4 and 1.25 fold in APP/mdr1^{+/-} mice, respectively. No significant effect of rivastigmine was observed on LRP1 expression in APP/mdr1^{-/-} mice. We have noticed that the expression of LRP1 in control mice decreased by 40% in mdr1 double knockout mice compared to mdr1 WT, while LRP1 expression in APP/mdr1^{+/-} was not changed.

3.3 Effect of rivastigmine treatment on A β brain level

In vehicle treated mice, the effect of P-gp deletion on A β oligomers levels was not significantly different between the 3 mice models ($P>0.05$, Fig. 3A). Treatment with rivastigmine reduced A β oligomers only in APP/mdr1^{+/+} mice by 33%, but not in APP/mdr1^{+/-} or APP/mdr1^{-/-} mice (Fig. 3A). Data from the FA-fraction showed that P-gp deletion resulted in a significant accumulation of A β ₄₀, but not A β ₄₂, in the brains of vehicle treated animals, and the effect was higher in P-gp double knockout mice by 33%, and P-gp heterozygous mice by 22% when compared to P-gp WT mice (Fig. 3B & C). For animals treated with rivastigmine, A β ₄₀ brain load was significantly reduced by 25% and 21% in APP/mdr1^{+/+} and APP/mdr1^{+/-} mice, respectively, compared to their vehicle treated controls ($P>0.05$, Fig. 3B). For APP/mdr1^{-/-} mice, rivastigmine treatment showed a trend toward reduction in A β ₄₀ level, but it was not statistically significant. For A β ₄₂ brain level, rivastigmine effect was only seen in APP transgenic, P-gp WT mice (reduced by 30%, $P>0.05$), while no effect was observed in APP transgenic, P-gp deficient mice (Fig. 3B).

3.4 Effect of rivastigmine treatment on plasma levels of A β

Rivastigmine treatment was only able to significantly reduce plasma levels of A β ₄₀ in APP/mdr1^{+/+} mice (43%, Fig. 4A; $P<0.05$); for A β ₄₂ there was no significant change in response to rivastigmine treatment in APP/mdr1^{+/+} mice (Fig. 4B). Rivastigmine treatment had no effect on A β ₄₀ and A β ₄₂ levels in +/- 42 APP/mdr1 or APP/mdr1^{-/-} (Fig. 4). The effect of P-gp deletion on A β plasma levels in control treated mice was differential where A β ₄₀ levels increased with reduced number of mdr1 copies reaching up to 2-fold in P-gp double knockout mice (Fig. 4A, $P<0.05$), while A β ₄₂ levels were not affected by the reduced expression of P-gp (Fig. 4B).

3.5 Effect of rivastigmine treatment on the expression of synaptic markers

Two synaptic markers were evaluated, the pre-synaptic marker synaptosomal-associated protein (SNAP-25), and the post-synaptic marker postsynaptic density protein 95 (PSD-95). As shown in Figure 5, all mice groups treated with rivastigmine showed significant increase in PSD-95 and SNAP-25 protein expression. However, the effect was highest in the APP/mdr1^{+/+} mouse model with 1.7- and 2-fold increase in PSD-95 and SNAP-25, respectively (Fig. 5A, $P<0.01$). The effect of rivastigmine on synaptic marker expression was reduced to 1.25- and 1.34-fold in APP/mdr1^{+/-} and 1.2- and 1.3-fold in APP/mdr1^{-/-} for PSD-95 and SNAP-25, respectively (Fig. 5 B and C). This result suggests a possible role for P-gp in mediating rivastigmine effect to preserve synapses through reducing A β brain load. In parallel to rivastigmine induction of synaptic markers, rivastigmine significantly enhanced the Na⁺-dependent glutamate transporter (GLT-1) protein expression by 2-fold in APP/mdr1^{+/+} mice, and less pronounced, but significant, in APP/mdr1^{+/-} and APP/mdr1^{-/-} by 1.3 and 1.1 fold, respectively.

3.6 Effect of rivastigmine on hippocampal astrogliosis

Two months treatment with rivastigmine resulted in a significant reduction in astrogliosis in all mice groups, which was determined by measuring the astrocyte reactive marker glial fibrillary acidic protein (GFAP) intensity by immunostaining. Rivastigmine reduced GFAP

immunoreactive cells by 45–50% in APP/mdr1^{+/+} and APP/mdr1^{+/-} mice when compared to control mice (Fig. 6 A–D, G, P<0.001). For APP/mdr1^{-/-}, the effect of rivastigmine was less pronounced but significant with 34% reduction in GFAP intensity compared to controls (Fig 6 E–G, P<0.001). In control treated groups, the effect of P-gp deletion was obvious and was associated with a significant increase in GFAP activation by approximately 30% in both APP/mdr1^{+/-} and APP/mdr1^{-/-} mice when compared to APP/mdr1^{+/+}.

3.7 Effect of rivastigmine on brain and plasma levels of the pro-inflammatory cytokine interleukin-1 β

Rivastigmine treatment significantly reduced brain levels of interleukin-1 (IL1- β) by 43% in APP/mdr1^{+/+} mice and by 33% in APP/mdr1^{+/-} mice, however, this effect was not observed in P-gp double knockout mice (Fig. 7A). Similarly, plasma levels of IL1- β were significantly reduced by rivastigmine by 51 and 31% in APP/mdr1^{+/+} and APP/mdr1^{+/-} mice, respectively, while APP/mdr1^{-/-} showed no response to the treatment (Fig. 7B). In control treated mice, P-gp deletion caused a significant increase in the brain and plasma levels of the pro-inflammatory mediator IL1- β . IL1- β brain levels were increased by 26 and 51% in APP/mdr1^{+/-} and APP/mdr1^{-/-} mice, respectively, compared to APP/mdr1^{+/+} (Fig. 7A). The increase in plasma levels of IL1- β was even higher due to P-gp deletion by 75 and 160% in APP/mdr1^{+/-} and APP/mdr1^{-/-} mice, respectively, compared to APP/mdr1^{+/+} (Fig. 7B).

4. Discussion

Currently, AD has no treatment and available medications provide some cognitive improvements and symptomatic relief, but they do not stop the progression of AD. Rivastigmine is one of the FDA-approved drugs for AD and dementia [28, 29]. Rivastigmine is a dual cholinesterase inhibitor which acts by inhibiting degradation of ACh at synapses and improving cholinergic transmission that is known to be impaired in AD. Recently, we have shown that FDA-approved drugs for AD modulate A β transport by up-regulating the expression of P-gp and LRP1 at the BBB and hepatocytes [13]. Among those drugs, we found that rivastigmine was most efficient to induce the expression of P-gp and LRP1 in the brains of aged rats and were associated with reduced levels of endogenous A β ₄₀ and A β ₄₂ [13].

With aging and in AD, A β accumulation has been reported and was associated with decline in A β clearance [30]. This observation suggests that enhancing the clearance of A β may protect the brain from A β -mediated neuropathology [31, 32]. We have shown previously that upregulation of P-gp and LRP1 induces A β clearance across the BBB *in vitro* and *in vivo* [33]. P-gp is a primary transporter involved in brain clearance of A β across the BBB [13, 34]. Vogelgesang and colleagues reported that accumulation of A β in the brain correlates inversely with the degree of P-gp expression at the BBB [22]. These findings suggest that high levels of P-gp at the BBB may be protective from A β deposition by eliminating A β out of the brain and preventing re-entry of A β from peripheral circulation. Previous findings from human studies suggested that peripheral A β can cross a defective BBB and accumulates in the brain [35]. Thus, a healthy and functional BBB has an important role in preserving A β brain homeostasis via expression and activity of proteins involved in the transport of A β .

The aim of the current study was to investigate the role of P-gp in mediating effect of rivastigmine treatment on A β brain level and related pathology in AD mouse model express different levels of P-gp. This model was previously used and characterized by Cirrito and colleagues [25] who reported that P-gp deletion in APP mouse model did not alter the expression of receptor for advanced glycation endproducts (RAGE), an important receptor for influx of A β from blood to brain, but increased multidrug resistance associated protein1 (MRP1) expression as a compensatory mechanism for P-gp deletion in APP/mdr1^{-/-} mice [25]. Role of MRP1 in A β clearance was previously reported in Abcc1, gene encode for Mrp1, deficient AD mouse model where its complete knockout increased A β brain deposit suggesting, like P-gp, its role in controlling A β brain levels [36]. Yet, findings from our study showed that Mrp1 couldn't rescue the absence of P-gp as evident by A β increased load and related pathology with or without rivastigmine treatment. At the BBB, compared to other ABC transporters the expression of P-gp is reported to be significantly higher than MRP1 and ABCG2 [37, 38], demonstrating P-gp important function at the BBB. With regard to ABCG2, it is reported that in mdr1^{-/-} mice the expression of Abcg2 was not altered by the deletion of P-gp [38], thus its expression in our model is not expected to change. In addition, further characterization showed P-gp deletion has no effect on the expression of ABCA1, ApoE and LXR, important proteins involved in A β lipidation and degradation pathway (data not shown).

Our findings support P-gp as important component at the BBB to regulate A β brain levels and its related pathology, and in modulating the neuroprotective effect of rivastigmine in a P-gp dependent manner. As expected, rivastigmine increased ACh brain level similarly (by 1.7- to 2-fold) in all treated APP transgenic mice expressing different levels of P-gp. Also, in plasma, rivastigmine was able to increase ACh level in all mice and this increase, while was higher than that observed in brain, was comparable between the groups, approximately 2- to 2.5-fold increase. These results indicate that P-gp deletion did not interfere with rivastigmine cholinergic stimulation. However, in vehicle treated mice, P-gp deletion significantly reduced brain ACh compared to P-gp WT mice. This decrease in brain level of ACh could be indirectly attributed to P-gp deletion through increasing A β brain accumulation (Fig. 3). The degree of P-gp expression in the brain correlated inversely with A β brain levels, which was reflected on Ach levels where less P-gp was associated with more A β , due to reduced efflux across the BBB, and less Ach levels. This finding is consistent with previous reports indicated that A β inhibits ACh synthesis and release in rat hippocampal slices and *in vivo* in young and aged rats thus causing cholinergic hypo-function [39–41]. This result supports role for P-gp in the development of AD and cognitive impairment by depleting Ach levels that is important for learning and memory [4, 5]. Interestingly, in plasma, among the 3 models rivastigmine effect on ACh level was highest in P-gp deleted mice, an observation that may link rivastigmine to its peripheral cholinergic adverse effects in AD patients. As the expression of P-gp declines at the BBB and A β brain burden increases, this could induce rivastigmine peripheral cholinergic stimulation and exacerbate its peripheral adverse events [42].

Consistent with our previous study [13], rivastigmine induced the expression of P-gp and LRP1 in isolated brain capillaries of APP/mdr1^{+/+} and APP/mdr1^{+/-} mice, and this increase

was equivalent for both models. However, in P-gp double knockout mice, rivastigmine could not induce LRP1 expression. In control group, complete deletion of P-gp in APP transgenic mice reduced LRP1 expression by 40% when compared to APP/mdr1^{+/+} group, which is consistent with previously reported 51% reduction in the same mouse model [25]. In addition, the effect of P-gp levels on A β peptides was differential where P-gp deletion caused a significant increase in parenchymal accumulation of A β ₄₀, but not A β ₄₂, when compared to APP/P-gp WT mice. This inverse correlation between A β ₄₀ deposition and amount of P-gp expression was expected and suggest the important role of P-gp in the removal of A β across the BBB to maintain A β brain homeostasis. The isoform specific accumulation of A β ₄₀ could be attributed to higher brain levels of A β ₄₀ than A β ₄₂, and the clearance rate of A β across the BBB is higher for A β ₄₀ than A β ₄₂ [43, 44]. Therefore, interruption of A β clearance across the BBB by P-gp deletion could be reflected more likely on A β ₄₀ than A β ₄₂. Nonetheless, higher A β ₄₂ depositions might be observed at a later age where aggressive A β deposition occurs. Similar pattern was observed in the plasma where P-gp deletion increased A β ₄₀ levels, but not A β ₄₂. This observation is consistent with P-gp function in the biliary excretion of A β ₄₀ as we reported previously [45]. P-gp deletion in the liver could thus lead to A β increased levels in the plasma due to reduced A β hepatic clearance, which in turn could further contribute to A β accumulation in the brain as a result of decreased extrusion at the BBB. This plasma to brain influx of A β possibly hindered the cholinergic and non-cholinergic effects of rivastigmine in APP/mdr1^{-/-} mice. On the other hand, treatment with rivastigmine decreased both A β ₄₀ and A β ₄₂, and SDS stable oligomers in P-gp WT mice. It is convincing that rivastigmine inductive effect on P-gp and LRP1 stimulates the removal of A β from the brain and reduces its accumulation, and the deletion of P-gp impairs this mechanism, suggesting that rivastigmine lowering effect on A β ₄₀ is proportional to the degree of P-gp expression.

The effect of P-gp in controlling the neuroprotective effect of rivastigmine was also observed. Rivastigmine increased the expression of synaptic proteins including SNAP-25, a pre-synaptic marker, and PSD-95, a post-synaptic marker, in a P-gp dependent manner. This increase in synaptic proteins expression could be attributed indirectly to rivastigmine effect on reducing A β brain load, thereby, decreasing A β -mediated synaptotoxicity. High brain levels of A β could interfere with synaptic plasticity and induce endocytosis of synaptic transporters and receptors and cause synaptic loss [3]. Supportive to rivastigmine induction of synaptic markers and protection of synapses, rivastigmine significantly enhanced GLT-1 protein expression in brain. GLT-1 is a Na⁺-dependent glutamate transporter (EAATs) expressed on the plasma membrane of neurons and glial cells and has important role in regulating neurotransmission and intersynaptic cross-talk [46], and dysfunction in this transporter is reported in neurological diseases including AD [47]. This neuroprotective effect of rivastigmine, while attenuated, was significant in P-gp deleted mice, which suggest the importance of functional BBB to mediate rivastigmine beneficial effects. Nonetheless, rivastigmine partial neuroprotective effect in the P-gp deficient mice could be attributed to its classical effect in maintaining ACh level at synapses and improving synaptic cholinergic function [6].

Astrocytes are major cell type involved in degradation of A β and maintenance of synaptic glutamate concentrations [48]. It is suggested that accumulation of A β inside the brain

causes a cascade of inflammatory events that lead to astrogliosis that localize to A β deposits [49]. Astrocytes activation enhances degradation of A β ; however, their chronic activation has shown to initiate the release of damaging cytokines including, tumor necrosis factor α , IL-1 and IL-6. The release of such cytokines could stimulate neuronal dysfunction and death, and thus contributing to cognitive dysfunction and behavioral abnormalities seen in AD [50, 51]. These A β -mediated inflammatory events concurrently could contribute to cerebrovascular alterations and lead to BBB dysfunction observed in AD. In our AD mouse model, gliosis is evident at early age (5 months of age) and deletion of P-gp caused a significant increase in GFAP immunoreactivity by approximately 30% compared to APP transgenic, P-gp WT mice. As part of rivastigmine neuroprotective effect, we demonstrated that chronic treatment with rivastigmine significantly reduced GFAP immunoreactivity in P-gp WT and P-gp heterozygous mice (by ~50%) compared to P-gp deleted mice (by 36%). Concomitantly, deletion of P-gp resulted in a significant increase in the brain and plasma levels of the pro-inflammatory mediator IL1- β , and rivastigmine treatment decreased IL1- β brain and plasma levels, which was consistent with its anti-astrogliosis effect and support a role for P-gp in this effect. The significant increase in IL1- β brain and plasma level in P-gp deleted mice could be attributed to the higher A β brain and plasma levels which stimulate inflammation and secretion of inflammatory markers. This increased level of IL1- β was significantly reduced by rivastigmine in P-gp expression dependent manner, however, this effect was not observed in the P-gp knockout mice. While an explanation for this observation is not clear, it could be related to the increased A β levels in this mouse model that was not altered by rivastigmine treatment (Fig. 3).

Taken together, despite the fact that rivastigmine was able to increase brain and plasma levels of ACh, and showed some anti-inflammatory effects in P-gp deleted mice, it could not decrease A β brain levels. The reduced neuroprotective and anti-inflammatory effects of rivastigmine in P-gp deleted mice highlight the significant contribution of P-gp to the neuroprotective mechanism of rivastigmine. A β plays a central role in the degenerative process of neurons that lead to neuronal dysfunction in AD [52]. Also, A β decreases the release of ACh from presynaptic terminals and impairs nicotinic ACh receptor (nAChR) signaling [3]. This suggests that the cholinergic and amyloid hypotheses are not independent and proposes a possible cross-talk between the two pathways. Accordingly, it is possible that rivastigmine could boost its cholinergic effect and increase ACh level at synapses via two mechanisms; cholinesterase inhibition and decreasing A β brain load.

5. Conclusion

Collectively, our findings suggest that rivastigmine lowering effect on A β and its neuroprotective effect depend largely on P-gp function at the BBB. In AD, inflammation and oxidative stress alter the expression of BBB transporters which in turn promotes accumulation of A β inside the brain [53, 54]. P-gp is downregulated in AD and with aging and is associated with higher accumulation of brain A β [13, 20–22]. Malfunction in major transporters of A β , like P-gp, at the BBB could thus influence the therapeutic efficiency of drugs that depend largely on those molecules to clear A β out of the brain. This could be true for rivastigmine, where the deletion of P-gp associated with reduction in LRP1 expression deteriorated the ability of rivastigmine to lower A β and negatively affected its anti-

inflammatory and neuroprotective effects. Besides, our findings showed rivastigmine to up-regulate P-gp at the BBB, thus further stimulating A β clearance. Simultaneously, however, it is expected that such increase in P-gp expression to limit brain access of concomitantly administered CNS drugs that are P-gp substrates and may affect their therapeutic outcome.

Acknowledgments

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Abbreviations

ABCA1	ATP-binding cassette sub-family A, member 1
APP	amyloid precursor protein
ACh	acetylcholine
AD	Alzheimer's disease
Aβ	amyloid- β
BBB	blood-brain barrier
BSA	bovine serum albumin
ChEIs	Cholinesterase inhibitors
ELISA	enzyme-linked immunosorbent assay
FA	formic acid
GFAP	glial fibrillary acidic protein
GLT-1	Na ⁺ -dependent glutamate transporter
IL1	interleukin-1
LRP1	low density lipoprotein receptor related protein 1
LTP	long term potentiation
P-gp	P-glycoprotein
PSD-95	postsynaptic density protein 95
SDS	sodium dodecylsulfate
SNAP-25	synaptosomal-associated protein

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Highlights

P-gp could play role in the development of AD by depleting Ach levels

Rivastigmine lowers A β brain load, at least in part, by upregulating P-gp and LRP1 at the BBB.

P-gp deletion attenuates rivastigmine effect on A β and its related pathology.

Rivastigmine's neuroprotective effect is P-gp expression dependent (APP/mdr1^{+/+} > APP/mdr1^{+/-} > APP/mdr1^{-/-})

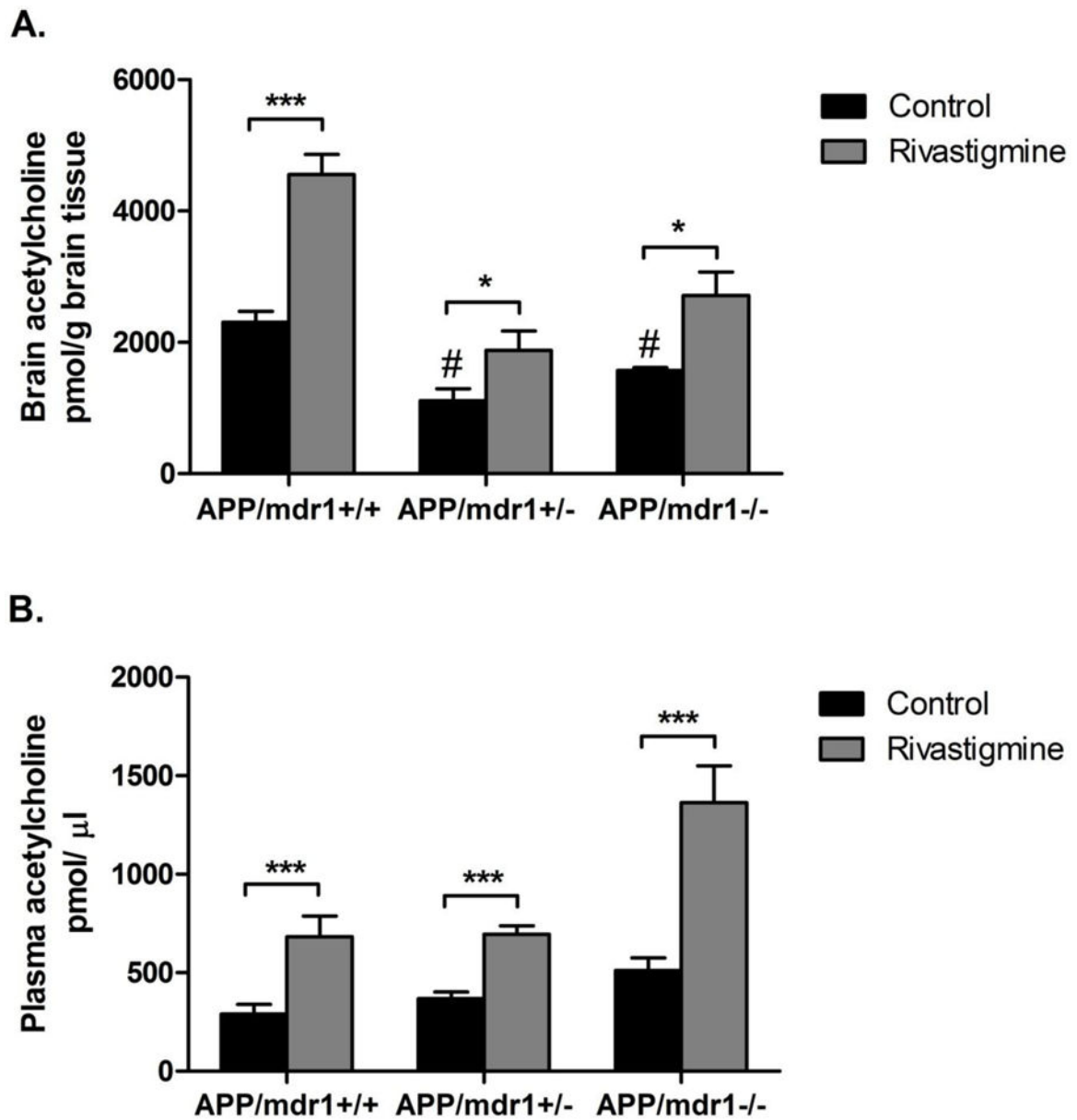


Figure 1. Effect of rivastigmine treatment on brain and plasma levels of acetylcholine (ACh). Levels of ACh after 8 weeks of rivastigmine treatment in APP/mdr1^{+/+}, APP/mdr1^{+/-} and APP/mdr1^{-/-} in brain (A), and plasma (B). Data presented as mean \pm SEM (n = 3–6, * P<0.05, *** P<0.001 compared to vehicle-treated controls, # P<0.05 compared to vehicle-treated control of APP/mdr1^{+/+}).

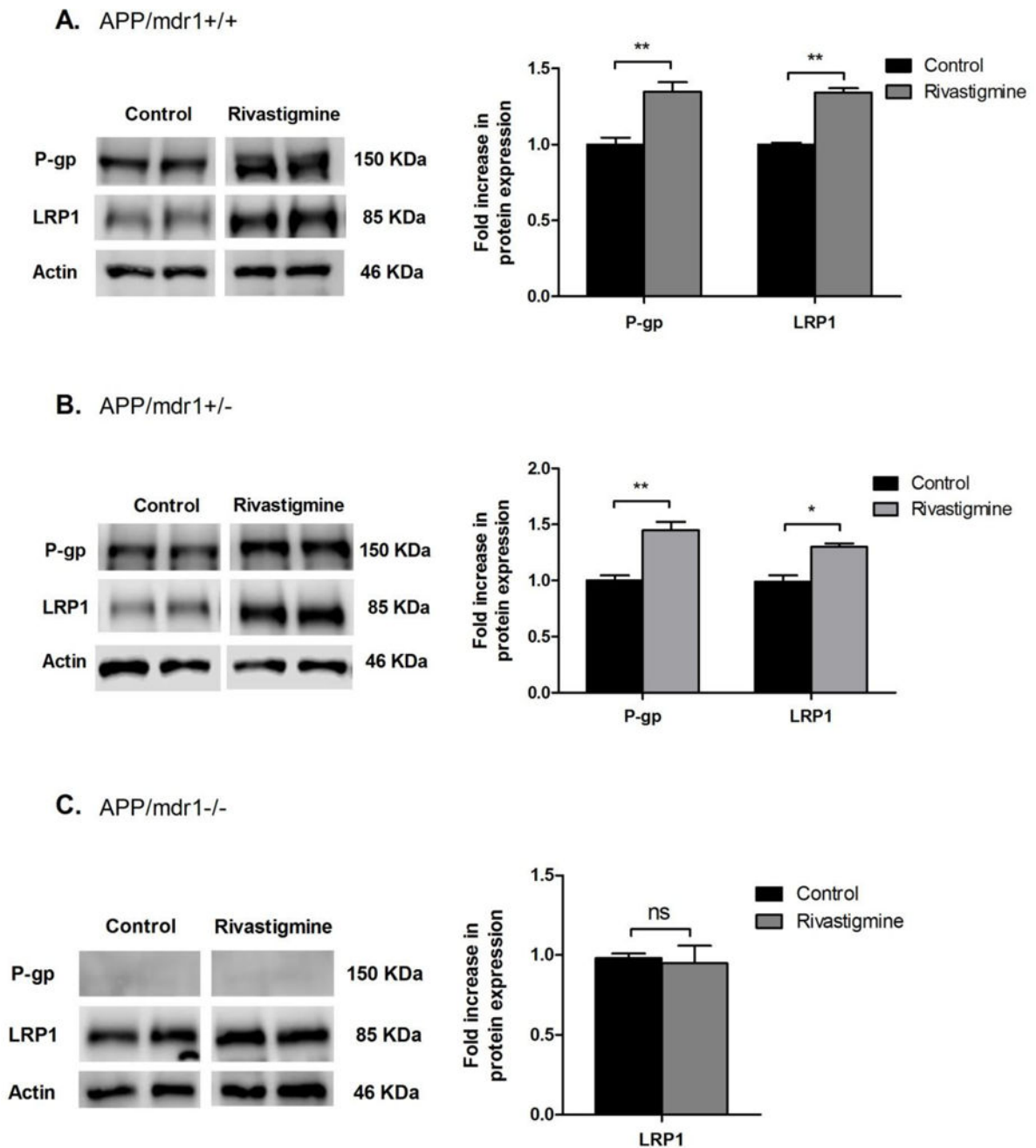
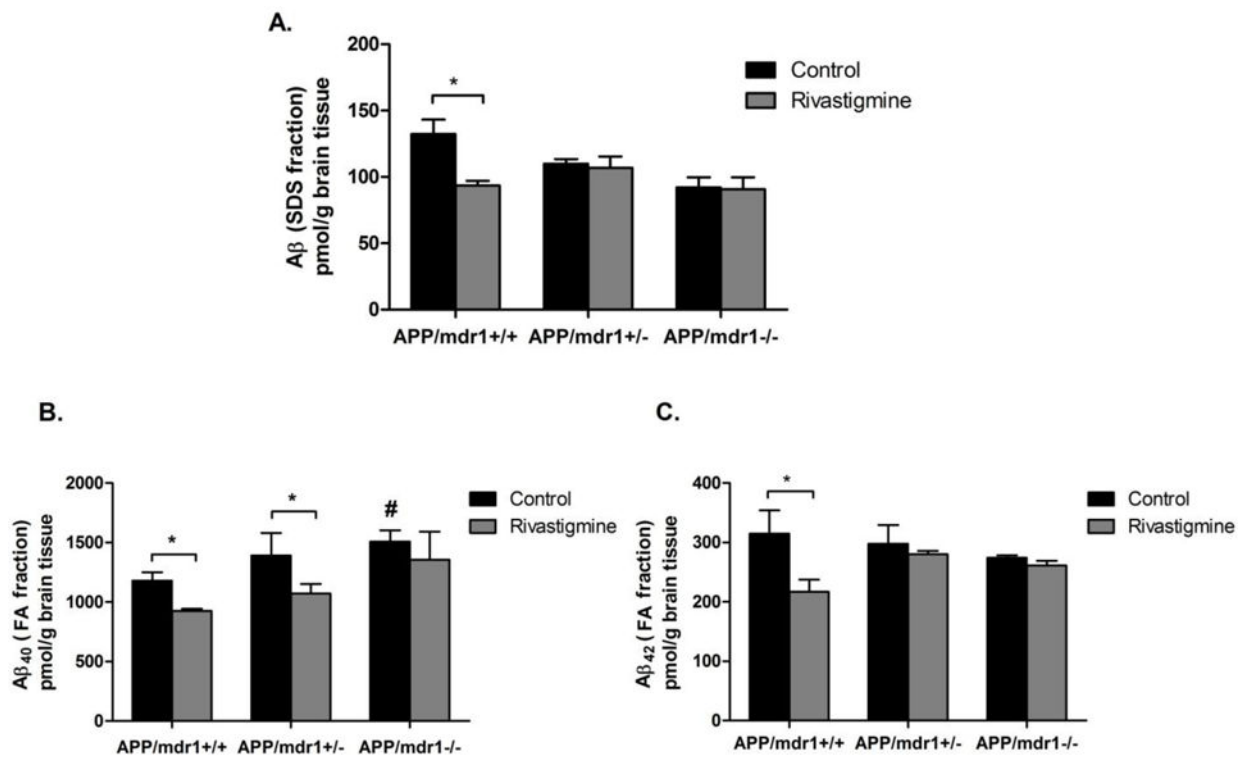


Figure 2.

Effect of rivastigmine treatment on expression of A β major transport proteins at the BBB. Representative Western blots and densitometry analysis of P-gp and LRP1 from total protein of brain microvessels isolated from APP/mdr1^{+/+} (A), APP/mdr1^{+/-} (B), and APP/mdr1^{-/-} (C) mice following treatment with rivastigmine or vehicle. Data presented as mean \pm SEM (n = 3–6, * P<0.05, ** P<0.01, ns = not significant, compared to vehicle-treated controls).

**Figure 3.**

Effect of rivastigmine treatment on brain levels of A β . Quantitative analysis of A β brain levels using specific ELISA for A β -SDS fraction (A), A β_{40} -FA fraction (B), and A β_{42} -FA fraction (C). Data presented as mean \pm SEM (n = 3–6, * P<0.05, compared to vehicle-treated controls; #P<0.05 compared to vehicle-treated control of APP/mdr1^{+/+} mice).

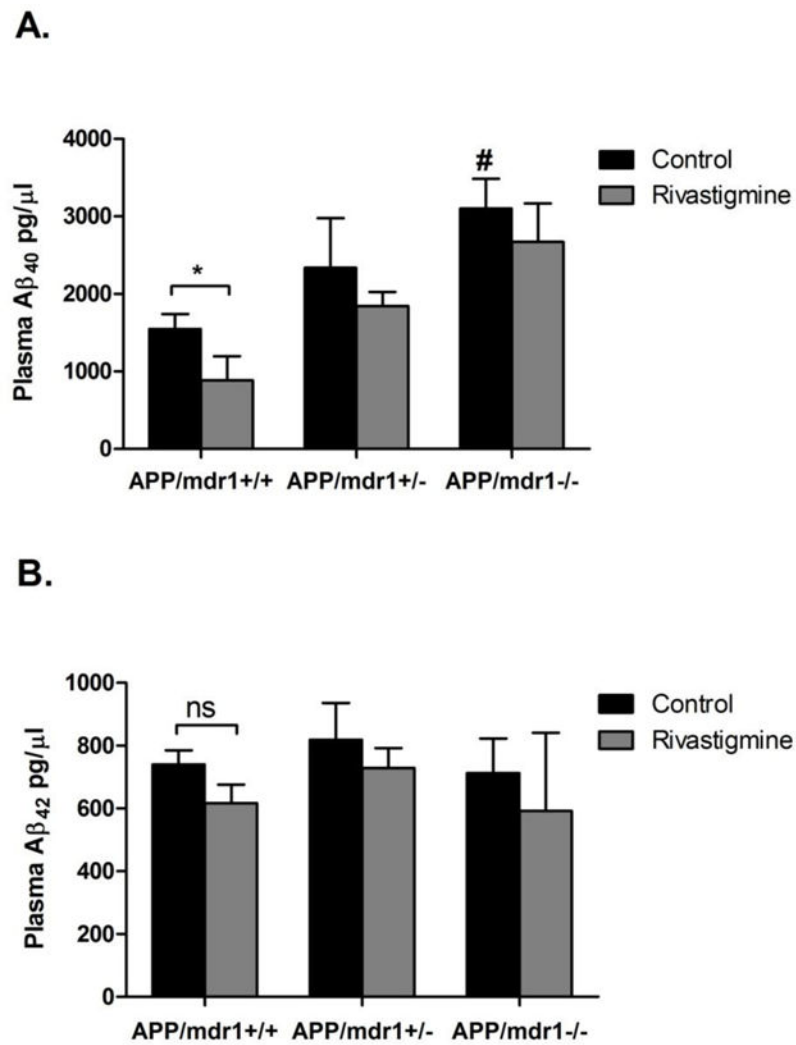


Figure 4. Effect of rivastigmine treatment on plasma levels of A β . Quantitative analysis of total A β plasma levels using specific ELISA for A β ₄₀ (**A**), and for A β ₄₂ (**B**). Data presented as mean \pm SEM (n = 3–6, * P<0.05, ns = not significant, compared to vehicle-treated controls, # P<0.05 compared to vehicle-treated control of APP/mdr1^{+/+}).

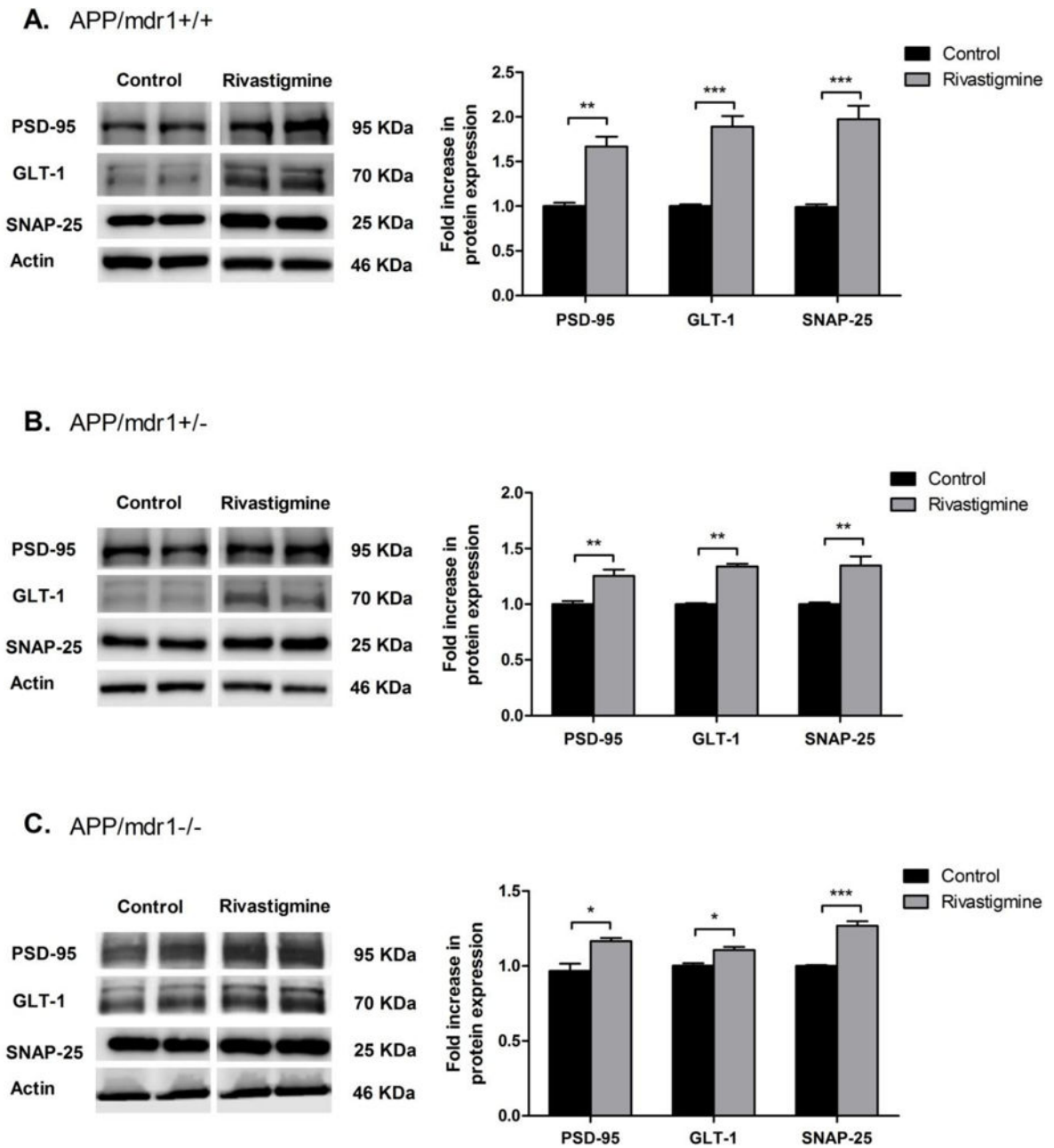


Figure 5.

Effect of rivastigmine treatment on protein expression of synaptic markers. Representative Western blots and densitometry analysis of pre-synaptic marker (SNAP-25), postsynaptic marker (PSD-95) and glutamate transporter 1 (GLT1) from brain homogenates of APP/mdr1^{+/+} (A), APP/mdr1^{+/-} (B) and APP/mdr1^{-/-} (C) mice following treatment with rivastigmine or vehicle. Data presented as mean±SEM (n = 3–6, * P<0.05, ** P<0.01, *** P<0.001 compared to vehicle-treated controls).

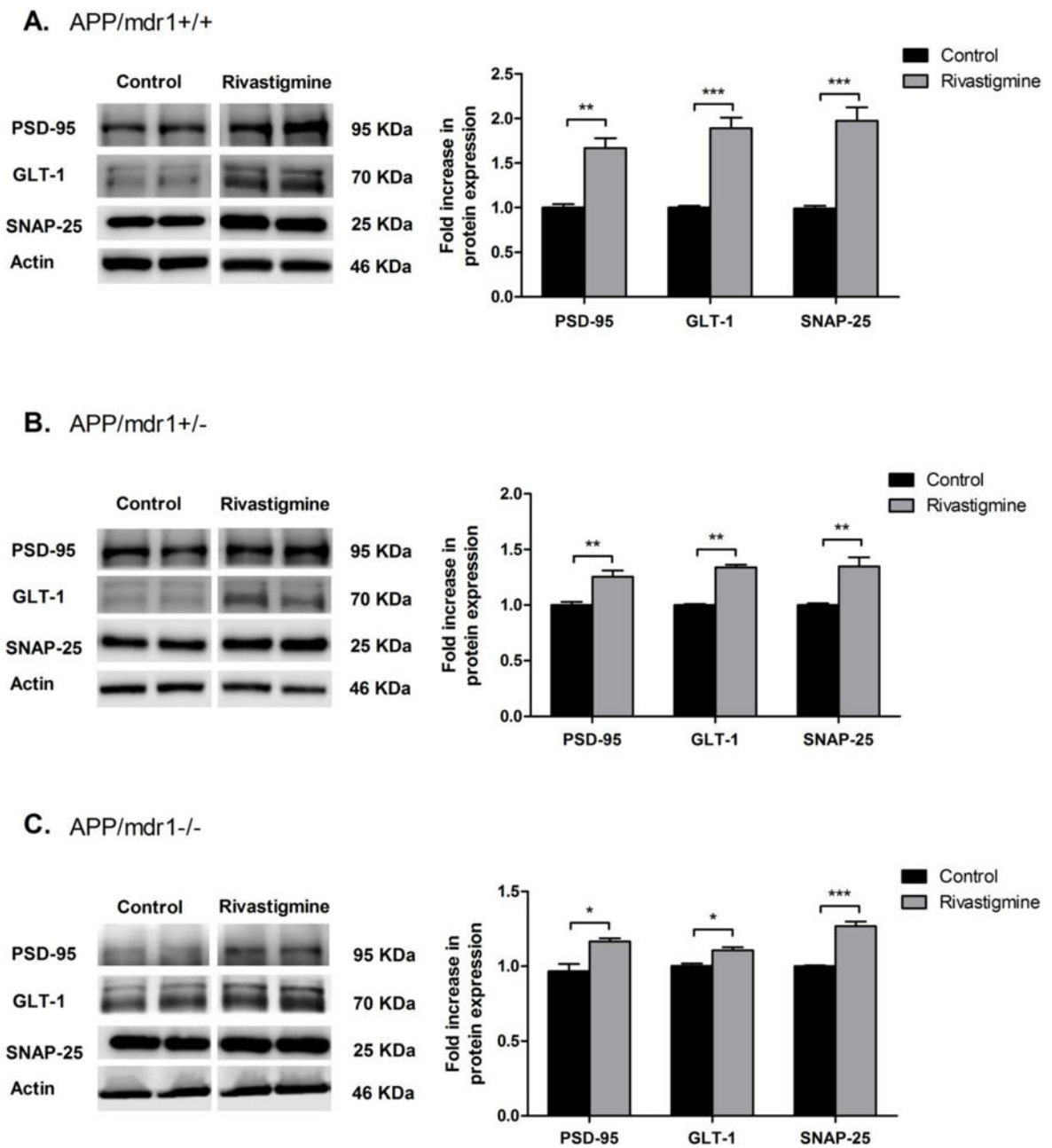


Figure 6.

Effect of rivastigmine on hippocampal astrogliosis. Representative hippocampus sections stained with glial fibrillary acidic protein (GFAP) antibody to detect astrocytes in APP/mdr1^{+/+} (A, B), APP/mdr1^{+/-} (C, D) and APP/mdr1^{-/-} (E, F). Quantification of GFAP staining in hippocampus of animals treated with rivastigmine or vehicle (G). Data presented as mean±SEM (n = 3–6, *** P<0.001 compared to vehicle-treated controls, # P<0.05 compared to vehicle-treated control of APP/mdr1^{+/+}).

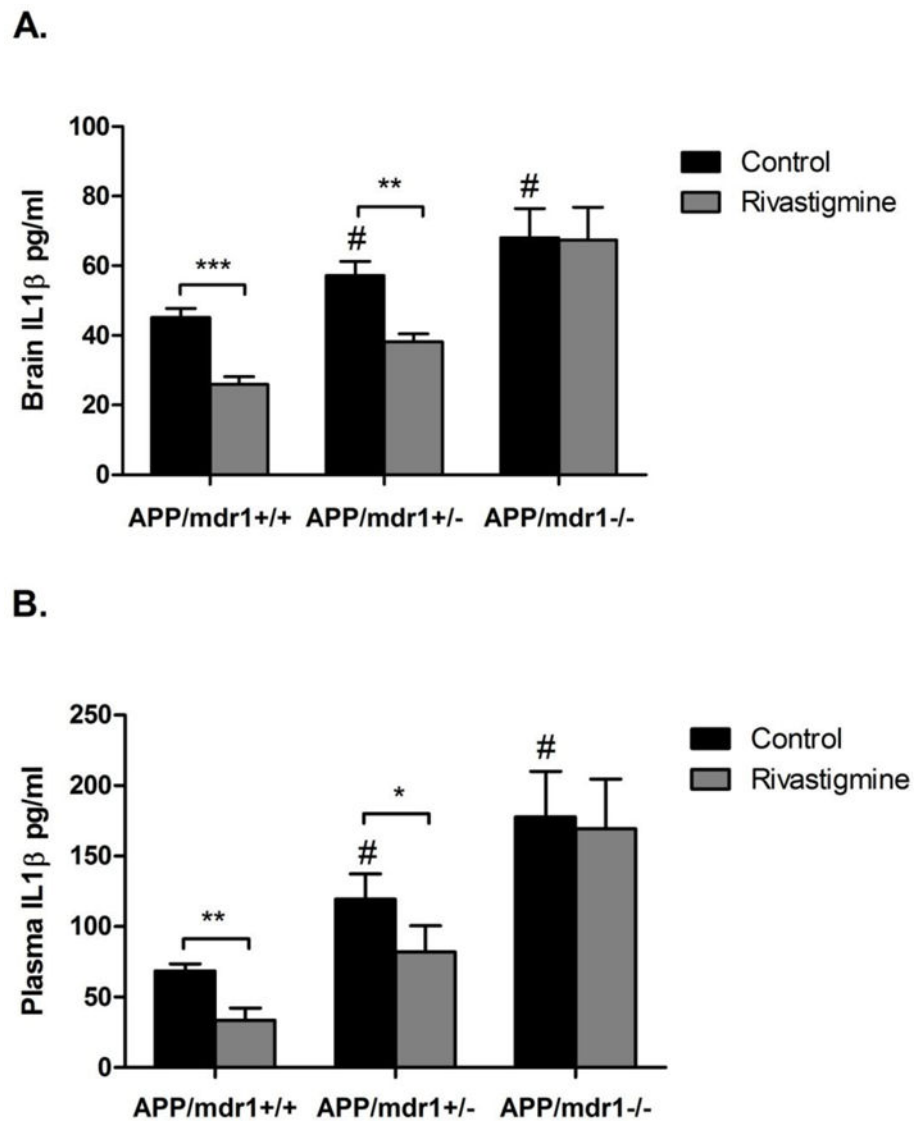


Figure 7. Effect of rivastigmine on brain and plasma levels of the pro-inflammatory cytokine interleukin 1- β (IL1- β). Levels of IL1- β after 8 weeks of rivastigmine treatment in APP/mdr1^{+/+}, APP/mdr1^{+/-} and APP/mdr1^{-/-} brain (A), and plasma (B). Data presented as mean \pm SEM (n = 3–6, * P<0.05, ** P<0.01, *** P<0.001 compared to vehicle-treated controls, # P<0.05, compared to vehicle-treated control of APP/mdr1^{+/+}).