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Restoring Blood-Brain Barrier P-glycoprotein Reduces Brain A β in a Mouse Model of Alzheimer's Disease

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Running title: P-glycoprotein in Alzheimer's disease

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Text pages: 32

Tables: 0

Figures: 7 (plus 4 Supplemental Figures)

References: 42

Abstract: 197

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Abbreviations: A β , amyloid- β ; AD, Alzheimer's disease; BCRP, breast cancer resistance protein; FTC, fumitremorgin C; hAPP, human amyloid precursor protein; LTC₄, leukotriene C₄; LRP1, low-density lipoprotein receptor-related protein 1; MRP, multidrug resistance-associated protein; NBD-CSA, [N- ϵ (4-nitrobenzofurazan-7-yl)-D-Lys⁸]-cyclosporine A; PCN, pregnenolone-16 α -carbonitrile; PSC833, 3'-oxo-4-butenyl-4-methyl-threonine(1),(valine(2)) cyclosporine; PXR, pregnane X receptor; RAGE, receptor for advanced glycation endproducts; RAP, receptor-associated protein

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ABSTRACT

Reduced clearance of amyloid- β ($A\beta$) from brain partly underlies increased $A\beta$ brain accumulation in Alzheimer's disease (AD). The mechanistic basis for this pathology is unknown, but recent evidence suggests a neurovascular component in AD etiology. We show here that the ATP-driven pump, P-glycoprotein, specifically mediates efflux transport of $A\beta$ from mouse brain capillaries into the vascular space, thus identifying a critical component of the $A\beta$ brain efflux mechanism. We demonstrate in a transgenic mouse model of AD (human amyloid precursor protein (hAPP)-overexpressing mice; Tg2576 strain) that brain capillary P-glycoprotein expression and transport activity are substantially reduced compared to wild type control mice, suggesting a mechanism by which $A\beta$ accumulates in the brain in AD. Importantly, we show that dosing 12-week old, asymptomatic hAPP mice over 7 days with pregnenolone-16 α -carbonitrile (PCN) to activate the nuclear receptor, pregnane X receptor (PXR), restores P-glycoprotein expression and transport activity in brain capillaries and significantly reduces brain $A\beta$ levels compared to untreated control mice. Thus, targeting intracellular signals that up-regulate blood-brain barrier P-glycoprotein in early stages of AD has the potential to increase $A\beta$ clearance from the brain and reduce $A\beta$ brain accumulation. This mechanism suggests a new therapeutic strategy in AD.

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INTRODUCTION

A hallmark of Alzheimer's disease (AD) is the accumulation of neurotoxic amyloid- β ($A\beta$) peptide within the brain. The $A\beta$ transport-clearance hypothesis of AD proposed by Zlokovic and coworkers states that reduced $A\beta$ clearance (reduced $A\beta$ efflux transport) from the brain underlies $A\beta$ brain accumulation (Deane et al., 2004b; Mooradian et al., 1997; Zlokovic, 2005; Zlokovic and Frangione, 2003). This hypothesis suggests that the mechanism responsible for $A\beta$ brain clearance itself could be a therapeutic target in AD.

$A\beta$ clearance from brain to blood has to be a two-step process. $A\beta$ must first pass through the abluminal (brain-side) and then the luminal (blood-side) plasma membranes of the brain capillary endothelial cells that comprise the blood-brain barrier. Given that $A\beta$ is a peptide, both steps must be facilitated, involving receptors or transporters. At the abluminal membrane, the receptor, low-density lipoprotein receptor-related protein 1 (LRP1), seems to be the major protein responsible for $A\beta$ uptake from brain into capillary endothelial cells (Deane et al., 2004a; Deane et al., 2004b; Shibata et al., 2000). However, the luminal membrane protein mediating the critical second step, $A\beta$ efflux from the endothelial cells into the blood, has not been identified.

One candidate is P-glycoprotein, an ATP-driven efflux transporter that under normal, physiological conditions is highly expressed at the luminal membrane of the brain capillary endothelium. This transporter handles a wide spectrum of non-polar, therapeutic drugs, some of which are small polypeptide derivatives (Miller et al., 2008). Limited data with artificial model systems such as transporter-overexpressing cell lines of non-brain endothelial origin and membrane vesicles from these cell lines suggest that P-glycoprotein can transport $A\beta$ (Cirrito et al., 2005; Kuhnke et al., 2007; Lam et al., 2001) and one report suggests reduced $A\beta$ efflux from the brain in P-glycoprotein-null mice (Cirrito et al., 2005). However, those mice also exhibit

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substantially reduced expression of LPR1 in cerebral vessels, so the involvement of blood-brain barrier P-glycoprotein in A β efflux in AD remains unclear.

Here, we establish a role for blood-brain barrier P-glycoprotein in the efflux transport of A β from the brain into capillary lumen. We use a transgenic mouse model of AD (human amyloid precursor protein (hAPP)-overexpressing mice; Tg2576 strain) to test the hypotheses that defective transport mediated by P-glycoprotein contributes to A β accumulation within the brain and that restoring such transport can reduce brain A β levels. We demonstrate here for the first time that P-glycoprotein mediates efflux transport of A β in intact brain capillaries. We show that P-glycoprotein expression and transport activity are substantially reduced in brain capillaries from hAPP mice. These experiments were done with 12-week old transgenic mice, at a time when brain hAPP and human A β (hA β) levels are substantial, but when there is not yet evidence of cognitive impairment (Hsiao et al., 1996; Kawarabayashi et al., 2001). Finally, and most importantly, using this AD model we show that *in vivo* activation of the nuclear receptor, pregnane X receptor (PXR) over a period of 7 days both restores P-glycoprotein expression and activity at the blood-brain barrier and significantly reduces A β brain levels.

These results suggest that up-regulating blood-brain barrier P-glycoprotein through PXR activation is a therapeutic strategy that could lower A β brain levels and delay onset and progression of AD.

MATERIALS and METHODS

Chemicals. Antibodies against RAGE, human APP, human A β 40, human A β 42, and β -actin were purchased from Abcam (Cambridge, MA, USA). Note that in Western blots using the antibodies to detect human APP, human A β 40, and human A β 42 we observed a single band at the right molecular weight for each of these proteins, indicating that the antibodies used did not cross-react with other amyloid species and are thus, specific for the corresponding amyloid isoform. C219 antibody against P-glycoprotein was from Signet Laboratories (Dedham, MA, USA), LRP1 antibody was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA), and PXR antibody was from BioLegend (San Diego, CA, USA). Fluorescein-hA β 42 (fluorescein- β A(1-42)) was purchased from rPeptide (Bogart, GA, USA), funitremorgin C (FTC) was from Alexis Axxora (San Diego, CA, USA), receptor-associated protein (RAP) was from Calbiochem-Novabiochem (La Jolla, CA, USA), and safflower oil was from MP Biomedicals (Solon, OH, USA). [N- ϵ (4-nitrobenzofurazan-7-yl)-D-Lys⁸]-cyclosporine A (NBD-CSA) was custom-synthesized by R. Wenger (Basel, Switzerland) (Wenger, 1986). PSC833 (valsopodar, PSC833: 3'-oxo-4-butenyl-4-methyl-threonine(1),(valine(2)) cyclosporine) was a kind gift from Novartis (Basel, Switzerland). Sulforhodamine 101 free acid, NaCN, mannitol, LTC₄, and pregnenolone-16 α -carbonitrile (PCN), and all other chemicals were of highest grade and were purchased from Sigma (St. Louis, MO, USA).

Animals. Male transgenic human APP-overexpressing mice (Tg2576 strain; 129S6.Cg-Tg(APP_{SWE})₂₅₇₆Kha) and corresponding male wild-type mice were purchased from Taconic Farms (Germantown, NY, USA). Mice were 12 weeks old with an average body weight of 29 g for wild-type mice and 31 g for transgenic hAPP mice. Animals were kept under controlled environmental conditions (23°C, 35% relative humidity, 12 hour dark-light cycle) with free

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access to tap water and standard rodent feed. After shipping, animals were allowed to adapt to the new environment for at least one week before experiments. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (IACUC Protocol #0801A25321, PI: Bjoern Bauer) and NIH/NIEHS (IACUC Protocol #LPC99-14; PI: David Miller) and were in accordance with the Guides to Animal Use of the University of Minnesota, NIH guidelines, AAALAC regulations, and the USDA Animal Welfare Act.

PCN Dosing and Facial Vein Bleeding. 20 hAPP transgenic mice received daily one i.p. injection of 25 mg/kg PCN for 7 days. 20 hAPP transgenic controls and 20 wild-type mice received vehicle (safflower oil). Blood samples were taken by facial vein bleeding on days 0, 1, 3 and 5 from 7-8 animals per group. Twenty-four hours after the last injection, mice were euthanized by CO₂ inhalation, decapitated, and trunk blood was collected. Brains from each animal were collected for A β determination and isolation of brain capillaries.

Brain Capillary Isolation. Brain capillaries were isolated as described previously (Hartz et al., 2008). Briefly, mice were euthanized by CO₂ inhalation and then decapitated. Brains were removed, dissected, and homogenized in ice cold PBS 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). Ficoll[®] was added to a final concentration of 15% and the homogenate was centrifuged at 5,800g for 20 min at 4°C. After resuspending the pellet in 1% BSA/PBS, the capillary suspension was passed over a glass bead column. Capillaries adhering to the glass beads were collected by gentle agitation in 1% BSA/PBS. Capillaries were washed with BSA-free PBS and used for experiments.

P-glycoprotein Transport Assay. To determine P-glycoprotein transport activity, freshly isolated brain capillaries were incubated for 1 h at room temperature with the fluorescent P-

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glycoprotein-specific substrate, NBD-CSA (2 μM in PBS buffer) (Hartz et al., 2008). For A β -transport studies, capillaries were incubated for 1 h at room temperature with 5 μM fluorescein-hA β 42 in PBS buffer. For each treatment, images of 10 capillaries were acquired by confocal microscopy (Nikon C1 LSC microscope unit, Nikon TE2000 inverted microscope, 40x oil immersion objective, NA 1.3, 488 nm line of an argon laser; Nikon Instruments Inc., Melville, NY, USA, or Zeiss LSM 510 META inverted confocal microscope, 40x water immersion objective, NA 1.2; 488 nm line of argon laser; Carl Zeiss Inc., Thornwood, NY, USA). Images were analyzed by quantitating luminal NBD-CSA fluorescence using Zeiss Image Examiner and Image J 1.41 software (Research Services Branch, NIMH/NIH, Bethesda, MD, USA). As before, specific, luminal NBD-CSA fluorescence was taken as the difference between total luminal fluorescence and fluorescence in the presence of the P-glycoprotein-specific inhibitor, PSC833 (5 μM) (Hartz et al., 2008).

Sulforhodamine 101 Accumulation Assay. Freshly isolated mouse brain capillaries were incubated with 2 μM sulforhodamine 101 for 1 h alone or with transporter inhibitors as indicated. For each treatment group, confocal images (Nikon C1 LSC microscope unit, Nikon TE2000 inverted microscope, 40x oil immersion objective, NA 1.3, 488 nm line of an argon laser; Nikon Instruments Inc., Melville, NY, USA) were obtained from 10 capillaries. Luminal sulforhodamine 101 fluorescence intensity was measured using Image J 1.41 software (Research Services Branch, NIMH/NIH, Bethesda, MD, USA) as described previously (Bauer et al., 2008).

A β Immunostaining of Mouse Brain Capillaries. Isolated mouse brain capillaries were fixed with 3% paraformaldehyde/0.25% glutaraldehyde for 15 min at room temperature. After washing with PBS, capillaries were permeabilized with 0.5% Triton X-100 for 30 min and washed with PBS. Capillaries were blocked with 1% BSA for 60 min and incubated overnight at

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4°C with a 1:500 dilution of monoclonal antibody against hA β 40 or polyclonal antibody against hA β 42. Capillaries were washed and incubated with Alexa-Fluor 488-conjugated secondary IgG (1:750, Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C. Nuclei were counterstained with 2 μ g/ml propidium iodide. Negative controls for each treatment that were processed without primary antibody showed negligible background fluorescence (data not shown). A β immunofluorescence was visualized by confocal microscopy (Nikon C1 LSC microscope unit, Nikon TE2000 inverted microscope, 40x oil immersion objective, NA 1.3, 488 nm line of an argon laser, 543 nm line of a HeNe laser; Nikon Instruments Inc., Melville, NY, USA). For each treatment, confocal images of 10 capillaries were acquired. A β membrane immunofluorescence for each capillary was quantitated using Image J 1.41 software (Research Services Branch, NIMH/NIH, Bethesda, MD, USA). A 10x10 grid was superimposed on each image and fluorescence measurements of capillary membranes were taken between intersecting grid lines. Fluorescence intensity for each capillary was the mean of 6 measurements per capillary.

Western Blotting. Protein expression levels from different tissues were analyzed by Western blotting as described previously (Hartz et al., 2008). Isolated capillaries were homogenized in lysis buffer (Sigma, St. Louis, MO, USA) containing Complete[®] protease inhibitor (Roche, Mannheim, Germany). Homogenized samples were centrifuged at 10,000g for 15 min and denucleated supernatants were centrifuged at 100,000g for 90 min to obtain brain capillary membranes. Brain capillary membranes were resuspended and stored at -80°C.

Western blots were performed using the Invitrogen NuPage[™] Bis-Tris electrophoresis and blotting system (Invitrogen, Carlsbad, CA, USA). After protein electrophoresis and transfer, blotting membranes were blocked and incubated overnight with the primary antibody as indicated. Membranes were washed and incubated with horseradish peroxidase-conjugated

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ImmunoPure[®] secondary IgG (1:15,000; Pierce, Rockford, IL, USA) for 1 h. Proteins were detected using SuperSignal[®] West Pico Chemoluminescent Substrate (Pierce, Rockford, IL, USA). Protein bands were visualized and recorded using a BioRad Gel Doc 2000[™] gel documentation system (BioRad, Hercules, CA, USA). QuantityOne[®] 1-D v4.6.5 software (BioRad, Hercules, CA, USA) was used for densitometric analyses of band intensities and digital molecular weight analyses; molecular weight markers used were RPN800 (GE Healthcare, Piscataway, NJ, USA) and NOVEX Sharp PS (Invitrogen, Carlsbad, CA, USA). Linear adjustments of contrast and brightness were applied to entire Western blot images. None of the Western blots shown were modified by nonlinear adjustments.

ELISA for hA β 40 and hA β 42. hA β 40 and hA β 42 was quantitated in plasma and brain samples by ELISA (KHB3482 and KHB3442 kits; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Plasma was obtained from blood samples by centrifugation at 5,000g for 5 min at 4°C. To determine hA β 40 plasma levels, samples were diluted 1:20 before analysis; to determine hA β 42 levels, samples were diluted 1:4 before analysis. hA β 40 and hA β 42 were extracted from brain tissue from APP and APP-PCN mice by homogenization with Tris-HCl buffer containing 5 M guanidine-HCl. Samples were diluted 1:20 in DPBS buffer containing 5% BSA and 0.03% Tween-20 and centrifuged at 16,000g for 20 min at 4°C. Supernatant was collected and diluted 1:1 with buffer before ELISA analysis.

Statistical Analysis. Data are presented as mean \pm SEM. Two-tailed unpaired Student's t-test was used to evaluate differences between controls and treated groups; differences were considered to be statistically significant when $P < 0.05$.

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RESULTS

Blood-brain barrier P-glycoprotein transports hA β 42

To investigate the role of P-glycoprotein in A β efflux at the blood-brain barrier, we used freshly isolated brain capillaries from normal, wild-type mice. Our previous studies have shown that isolated brain capillaries from both mouse and rat are morphologically intact and remain metabolically active and functionally capable of ATP-driven transport for up to 12 h ((Bauer et al., 2006; Hartz et al., 2008), data not shown). Thus, isolated brain capillaries closely mimic the blood-brain barrier *in vivo* and are well-suited to study transport processes across the brain capillary endothelium.

In the present study, we incubated isolated mouse brain capillaries with fluorescein-hA β 42 and measured its accumulation in capillary lumens using confocal microscopy and quantitative imaging. Fluorescein-hA β 42 accumulated to high levels in the lumens of control capillaries, indicating active transport from bath to vascular space (Figure 1, A and B; Supplemental Figure 1). Luminal fluorescence was significantly ($P < 0.001$) reduced by the P-glycoprotein-specific inhibitors, PSC833 (valsopodar), XR9576 (tariquidar), ivermectin, cyclosporin A, and verapamil (Fellner et al., 2002; Mayer et al., 1997), the metabolic inhibitor, NaCN, and the LRP1-specific inhibitor, receptor-associated protein (RAP). Fumitremorgin C (FTC), Ko143, leukotriene C₄ (LTC₄), and probenecid, inhibitors of the ATP-driven efflux transporters, breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRPs), were without effect. These results are consistent with blood-brain barrier A β efflux being an active and ATP-dependent two-step process, involving LRP1-mediated A β uptake from brain into capillary endothelial cells followed by P-glycoprotein-mediated A β efflux from endothelium into blood (Figure 1C).

P-glycoprotein is compromised at the blood-brain barrier of hAPP transgenic mice

To test the hypothesis that P-glycoprotein is compromised at the blood-brain barrier in AD, we measured P-glycoprotein expression and transport activity in brain capillaries isolated from 12-week old, male wild-type and hAPP transgenic mice (Tg2576; overexpressing human amyloid precursor protein, hAPP with the Swedish mutation), a well-established animal model of AD (Hsiao et al., 1996). At 12 weeks of age, hAPP transgenic mice exhibit accumulation of human A β in brain, but show no evidence of cognitive impairment, which does not start until about 6 months of age and then progressively worsens (Hsiao et al., 1996; Kawarabayashi et al., 2001). Figure 2A shows representative images of isolated brain capillaries from wild-type and hAPP mice that were incubated to steady state with the fluorescent dye, NBD-cyclosporin A (NBD-CSA), a P-glycoprotein substrate. Using isolated brain capillaries from rats and mice we previously demonstrated that PSC833-sensitive luminal accumulation of this dye is a specific measure of P-glycoprotein transport activity (Hartz et al., 2008). In capillaries from wild-type mice, luminal NBD-CSA fluorescence was high and sensitive to P-glycoprotein inhibition with PSC833. Comparison of luminal NBD-CSA fluorescence in capillaries from wild-type and hAPP mice indicated a 70% decrease in P-glycoprotein transport activity for the latter (Figure 2B). Consistent with A β being a P-glycoprotein substrate, isolated brain capillaries from hAPP mice exhibited substantially reduced fluorescein-hA β 42 transport (Figure 2C).

Reduced luminal accumulation of NBD-CSA and fluorescein-hA β 42 in brain capillary lumens from hAPP mice could reflect reduced P-glycoprotein expression or increased permeability of the tight junctions that separate endothelial cells within the endothelium (Hartz et al., 2004). If tight junctional permeability were altered, one would expect to see similar effects for all transporters that are capable of driving concentrative luminal substrate accumulation. In

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this regard, we recently demonstrated that transport of a fluorescent organic anion, sulforhodamine 101, into brain capillary lumens is mediated by another ATP-driven efflux pump, Mrp2 (multidrug resistance-associated protein isoform 2) (Bauer et al., 2008) . When we compared the ability of brain capillaries from wild-type and hAPP mice to transport sulforhodamine 101, we found no difference (Figure 2D, Supplemental Figure 2). Unaltered luminal accumulation of sulforhodamine 101 indicates intact tight junctions in brain capillaries from hAPP mice. Thus, reduced luminal NBD-CSA and fluorescein-hA β 42 accumulation found in brain capillaries from hAPP mice is due to reduced P-glycoprotein transport activity at the blood-brain barrier of these mice.

Consistent with reduced transport activity, we found a marked decrease of P-glycoprotein protein in brain capillary plasma membranes isolated from hAPP mice when compared to membranes from wild-type controls (Figure 2E). Western blot density measurements showed that P-glycoprotein levels were significantly decreased by about 60% (P-glycoprotein in hAPP mice: $39 \pm 6\%$ (SEM, $P < 0.01$) of wild-type control, P-glycoprotein levels normalized to β -actin). Thus, despite the absence of cognitive impairment, 12-week old hAPP mice display early biochemical and physiological changes in expression and transport activity of P-glycoprotein at the blood-brain barrier. In agreement with previous reports (Hooijmans et al., 2007; Mooradian et al., 1997), we also detected reduced protein expression of the glucose transporter, GLUT-1, in brain capillaries from hAPP mice. In contrast, in capillaries from hAPP mice, expression of the abluminal A β -uptake receptor, LRP1, was slightly increased and that of the luminal A β -uptake receptor, RAGE, was unchanged (Figure 2F). This indicates that decreased A β efflux transport in brain capillaries from hAPP mice was not due to altered LRP1 or RAGE expression. Thus, these

data suggest that the critical, limiting step in A β brain clearance in AD seems to be A β transport across the luminal membrane into the vascular space.

PXR activation restores blood-brain barrier P-glycoprotein in hAPP mice

We previously demonstrated that blood-brain barrier P-glycoprotein can be up-regulated by *in vivo* exposure to ligands that activate PXR (pregnane X receptor), a nuclear receptor that controls expression of drug metabolizing enzymes and efflux transporters (Bauer et al., 2004; Bauer et al., 2006; Geick et al., 2001; Kliewer et al., 1998). In preliminary experiments we found that PXR protein was expressed in brain capillaries from wild-type and hAPP mice, but that expression was slightly lower in capillaries from hAPP mice (Supplemental Figure 3). To determine whether PXR activation *in vivo* could restore P-glycoprotein expression and transport activity at the blood-brain barrier, we dosed hAPP mice with the prototypical rodent PXR ligand, pregnenolone-16 α -carbonitrile (PCN) at 25 mg/kg i.p. once a day for 7 days (Kliewer et al., 1998). PCN dosing of hAPP mice increased P-glycoprotein expression in brain capillary membranes to levels observed in untreated wild-type mice (Figure 3A; hAPP: 56 \pm 8% (SEM; P <0.03) of control; hAPP+PCN: 92 \pm 9% (SEM; not statistically significant, P =0.49) of control as determined by optical density measurements; P-glycoprotein levels normalized to β -actin). Consistent with increased protein expression, specific (PSC833-sensitive) P-glycoprotein transport activity in brain capillaries from PCN-treated hAPP mice was restored to levels in wild-type mice for both NBD-CSA and fluorescein-hA β 42 (Figure 3, B and C; Figure 4, A and B). PXR protein expression in brain capillaries was slightly increased by PCN dosing as previously reported (Pascussi et al., 2000); LRP1, RAGE, and GLUT-1 protein levels were unaffected (Figure 5). Thus, PCN dosing of hAPP mice selectively up-regulated P-glycoprotein at the blood-brain barrier and the newly synthesized transporter protein was functionally active.

Restoring blood-brain barrier P-glycoprotein reduces A β brain levels

We next determined the consequences of restoring blood-brain barrier P-glycoprotein on human A β (hA β) levels in hAPP mice. We initially measured hA β 40 and hA β 42 in plasma by ELISA but found no difference between untreated hAPP mice and PCN-treated hAPP mice over the 7-day period of PCN dosing (Supplemental Figure 4, A and B). This finding is consistent with rapid A β clearance from blood by filtration at the kidney.

An early sign of AD is cerebral amyloid angiopathy (CAA), characterized by A β deposition in brain capillary membranes (Greenberg et al., 2004; Iadecola, 2004; Zlokovic, 2008). To measure capillary-associated A β , we isolated brain capillaries from hAPP control and PCN-dosed mice and immunostained for hA β 40 and hA β 42. Capillaries from untreated hAPP mice stained positive for both A β peptides (Figure 6, A-D). Seven days of PCN treatment significantly decreased membrane immunofluorescence of both peptides in brain capillaries from hAPP mice by 28% for hA β 40 ($P < 0.05$) and 31% for hA β 42 ($P < 0.01$) compared to capillaries from untreated hAPP mice (Figure 6, A-D). Western blotting of brain capillary membranes also showed reduced levels of hA β 40 and hA β 42 in hAPP mice treated with PCN compared to untreated hAPP mice (Figure 6E; hA β 40: $82 \pm 1.4\%$ (SEM; $P < 0.05$) of control, hA β 42: $45 \pm 4.4\%$ (SEM; $P < 0.001$) of control as determined by optical density measurements; hA β levels normalized to β -actin). Thus, PCN dosing clearly reduced capillary-associated A β , suggesting reduced angiopathy.

Finally, Western blots and ELISA analysis showed significantly ($P < 0.001$) reduced hA β 40 and hA β 42 levels in brain tissue of hAPP mice dosed with PCN compared to untreated hAPP control mice (Figure 7, A-C). Optical density measurements of Western blots revealed that PCN dosing reduced brain levels of hA β 40 and hA β 42 by about 50% and 60%, respectively

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(Figure 7A; hA β 40: $50 \pm 4.5\%$ (SEM, $P < 0.001$) of control; hA β 42: $36 \pm 1.0\%$ (SEM, $P < 0.001$) of control; hA β levels normalized to β -actin). This was confirmed by ELISA measurements which demonstrated that brain levels of hA β 40 and hA β 42 were reduced by about 35% and 60%, respectively (Figure 7, B and C; hA β 40: $66 \pm 3\%$ (SEM, $P < 0.001$) of control; hA β 42: $38 \pm 2\%$ (SEM, $P < 0.001$) of control). Importantly, hAPP brain levels were not affected (Figure 7A), indicating that PCN-induced PXR activation did not change A β precursor levels. Thus, activation of PXR increased blood-brain barrier P-glycoprotein expression and transport activity, which acutely reduced hA β 40 and hA β 42 brain levels in hAPP mice.

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DISCUSSION

The present report reconciles three disparate observations each of which suggests that P-glycoprotein is a critical component of the A β brain clearance mechanism: 1) cell lines that overexpress human P-glycoprotein transport A β (Kuhnke et al., 2007; Lam et al., 2001; Tai et al., 2009); 2) injecting a P-glycoprotein inhibitor into hAPP transgenic mice increases A β brain levels (Cirrito et al., 2005); and 3) brain deposition of A β in elderly, non-demented patients is inversely correlated with brain capillary expression of P-glycoprotein (Vogelgesang et al., 2002; Vogelgesang et al., 2004). Indeed, we provide here compelling evidence for both P-glycoprotein contributing significantly to A β efflux across the luminal membrane of the brain capillary endothelium and for defective P-glycoprotein-mediated A β efflux transport in a mouse model of AD.

Recent studies and our current report argue for an important neurovascular component in AD etiology. They show that at least 3 plasma membrane proteins facilitate A β movement across the brain capillary endothelium: RAGE (receptor for advanced glycation endproducts), LRP1 and P-glycoprotein (Figure 1C; present study (Deane et al., 2003; Deane et al., 2004b)). Note that a recent publication suggests that the blood-brain barrier ATP-driven efflux pump, BCRP, could affect brain levels of A β (Xiong et al., 2009). This work indicates that BCRP transports A β in BCRP-overexpressing cell lines, that A β accumulates in the brain of BCRP null mice, and that BCRP expression is increased in brain samples from AD patients and in brains from two AD mouse models. However, the latter results are contrary to what one would expect if BCRP contributed to A β efflux from the brain and if reduced BCRP-mediated efflux contributed to A β accumulation. In addition, the findings by Xiong et al. in patients were made after AD diagnosis, which is based on cognitive symptoms. Likewise, based on the age of the AD mice used in the

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study by Xiong et al. (3XTg and Tg-SwDI models), cognitive symptoms were already evident in those animals. In the present study, we used hAPP mice at 12-weeks of age, at a time well before cognitive symptoms and presumably complex neuropathology set in (Hsiao et al., 1996; Kawarabayashi et al., 2001). Since we found no evidence for BCRP mediated A β efflux in intact mouse brain capillaries, increased BCRP expression may appear at later stages of the disease, be model-dependent, or both. Thus, at this time the role of BCRP in AD etiology remains unclear.

On the other hand, the A β receptors, RAGE and LRP1, have been shown to contribute to A β trafficking across the brain capillary endothelium, but not at early stages of the disease (Deane et al., 2003; Deane et al., 2004b). In both AD patients and AD mouse models where cognitive symptoms are evident, RAGE has been shown to be increased and LRP1 to be decreased (Deane et al., 2003; Deane et al., 2004a; Donahue et al., 2006). In the present study with 12-week old, asymptomatic hAPP mice, unchanged protein expression of the luminal A β uptake receptor, RAGE, in brain capillaries suggests that A β uptake from blood into the endothelium may not be increased before the onset of cognitive impairment in AD. Slightly increased expression of the abluminal A β uptake receptor, LRP1, suggests increased A β uptake from brain into the endothelium, again in very early stages of the disease, which may contribute to A β accumulation within the capillary endothelium in CAA. However, such changes in expression of these receptors cannot underlie reduced brain efflux of A β in the early stage AD mouse model used in the present study. Thus, our results point to P-glycoprotein as the final step in clearing A β from the brain. They indicate that efflux mediated by P-glycoprotein may well be the limiting factor in A β brain clearance and the critical step that is defective in AD. Thus, reduced blood-brain barrier P-glycoprotein expression in AD would be a major contributor to A β brain accumulation. These results have two important implications for how we view the disease.

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First, the present findings indicate that reduced P-glycoprotein expression at the blood-brain barrier is an early biochemical manifestation of AD pathology that occurs before cognitive symptoms are evident. The mechanism by which the disease signals the loss of P-glycoprotein is unknown. In the early stages of the disease, accumulation of A β 42 levels in the brain capillary plasma membrane could directly impair P-glycoprotein function, lead to A β accumulation, and reduce P-glycoprotein expression. In this regard, our unpublished experiments show that exposure of rat or mouse brain capillaries to low levels of A β 40 selectively removes P-glycoprotein from the plasma membrane and sends it to the proteasome, which implies that a particularly pernicious positive feedback loop drives the loss of efflux transporter expression leading to a further increase in A β brain levels. We posit that this loop contributes to the progressive nature of AD, at least in the early stages. Second, P-glycoprotein is one transporter for which few endogenous substrates have been identified (King et al., 2001). Our results indicate that A β is one such substrate and that P-glycoprotein is responsible for the final, critical step in A β efflux across the blood-brain barrier (Figure 1C). P-glycoprotein is also a major protective element of the blood-brain barrier, limiting a large number of xenobiotics, including many therapeutic drugs, from entering into the CNS (Fellner et al., 2002; Schinkel et al., 1996). Thus, substantially reduced protein expression and efflux transport activity of blood-brain barrier P-glycoprotein in AD could have significant consequences for AD patients. These include increased brain uptake of xenobiotics, altered dose response relationships for therapeutic drugs and increased neurotoxicity for those drugs that exhibit a narrow therapeutic index and for neurotoxicants that are P-glycoprotein substrates. All of these could aggravate the progression of AD.

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It is clear from the present experiments with hAPP transgenic mice that targeting PXR, a ligand-activated nuclear receptor that modulates blood-brain barrier P-glycoprotein (Bauer et al., 2004; Bauer et al., 2006), restores transporter expression and function, which in turn reduces brain A β burden. PXR is activated by a number of drugs and dietary constituents, and potent ligands for human PXR include the antibiotic, rifampicin, and the St. John's Wort constituent, hyperforin (Jones et al., 2000; Watkins et al., 2001). We previously showed that rifampicin dosing of transgenic mice expressing human PXR increased expression and transport activity of P-glycoprotein at the blood-brain barrier (Bauer et al., 2006). In these mice, the rifampicin dose was adjusted so that free plasma levels of the drug were comparable to those in patients receiving rifampicin treatment. In this regard, a recent clinical trial showed that rifampicin dosing lessened cognitive decline in AD patients over the 12-month treatment period (Loeb et al., 2004). The mechanistic basis for this observation is not known, but rifampicin activation of PXR leading to induction of blood-brain barrier P-glycoprotein is a likely possibility. These findings and the present results indicate that PXR activation to increase blood-brain barrier P-glycoprotein could be used in the clinic to increase A β brain efflux transport and lower A β brain burden. This therapeutic strategy implies PXR protein expression in human brain capillaries. While PXR mRNA has previously been detected in human whole brain and brain capillaries (Dauchy et al., 2009; Lamba et al., 2004), PXR protein expression has not yet been demonstrated. However, our study provides proof-of-principle and we anticipate that up-regulation of P-glycoprotein expression through PXR or other signaling pathways has the potential to increase A β brain efflux transport and lower A β brain burden.

Finally, although brain accumulation of A β is not the only major contributor to cognitive impairment in AD (Blennow et al., 2006; Iadecola, 2004), reducing A β accumulation in the

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transgenic hAPP (Tg2576) mouse model does delay pathology (Karlinski et al., 2009). It remains to be seen to what extent a general, long-term strategy of targeting signals that up-regulate blood-brain barrier P-glycoprotein will reduce brain A β burden over the long-term and will thus prove to be a useful therapeutic strategy for delaying the onset of AD and slowing the progression of the disease.

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ACKNOWLEDGEMENTS

We thank Destiny Sykes, Jonathan Lucking, Sylvia Notenboom, and Anton Pekcec for technical assistance, David Armstrong and James Putney for valuable comments, and Emily Madole for editorial assistance.

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FOOTNOTES

This research was supported in part by a Duluth Medical Research Institute Grant, by the Intramural Research Program of the National Institutes of Health, National Institute of Environmental Health Sciences [Z01 ES080048], and by University of Minnesota College of Pharmacy startup funds.

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LEGENDS FOR FIGURES

Figure 1. P-glycoprotein mediates hA β 42 transport in mouse brain capillaries. (A)

Representative confocal images of brain capillaries isolated from wild-type mice; capillaries were incubated with 5 μ M fluorescein-hA β 42 for 1 h alone (control) or with 5 μ M fluorescein-hA β 42 plus PSC833 (P-glycoprotein inhibitor), NaCN (metabolic inhibitor), RAP (LRP1 inhibitor), FTC (BCRP inhibitor), or LTC₄ (MRP inhibitor). (B) Capillary luminal fluorescein-hA β 42 fluorescence after image analysis; residual fluorescence is due to non-specific binding (13). Data represent mean \pm SEM for 10 capillaries from one preparation (pooled tissue from 10 wild-type mice); shown are arbitrary fluorescence units (scale 0-255). Statistics: ***significantly lower than control, $P < 0.001$. (C) Proposed two-step mechanism of blood-brain barrier A β efflux involving the A β receptor, LRP1, on the abluminal membrane and the efflux transporter, P-glycoprotein, on the luminal membrane of brain capillaries.

Figure 2. P-glycoprotein expression and transport activity are reduced at the blood-brain barrier

of hAPP mice. (A) Representative images of brain capillaries isolated from 12-week old wild-type and hAPP mice; capillaries were incubated with 2 μ M NBD-CSA, a fluorescent P-glycoprotein-specific substrate, for 1 h alone or with PSC833. (B) Specific (PSC833-sensitive) luminal NBD-CSA fluorescence after image analysis of brain capillaries. (C) Luminal fluorescein-hA β 42 fluorescence in brain capillaries from wild-type and hAPP mice. (D) Luminal fluorescence of the MRP-specific, fluorescent substrate, sulforhodamine 101, in brain capillaries alone (control), with mannitol (osmotic tight junction disruptor), LTC₄ (MRP inhibitor), NaCN (metabolic inhibitor), PSC833 (P-glycoprotein inhibitor), RAP (LRP1 inhibitor), or FTC (BCRP inhibitor). Data in B-D are mean \pm SEM for 10 capillaries from one preparation (pooled tissue

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from 10-20 mice per group); shown are arbitrary fluorescence units (scale 0-255). Statistics: ***significantly lower than control, $P < 0.001$. Western blots for (E) P-glycoprotein (P-gp), (F) LRP1, RAGE, and GLUT-1 of brain capillary membranes from wild-type and hAPP mice; β -actin was used as protein loading control (pooled tissue from 20 mice per group).

Figure 3. PXR activation in hAPP mice restores P-glycoprotein expression and transport of NBD-CSA. (A) P-glycoprotein (P-gp) Western blot of brain capillary membranes isolated from vehicle-treated wild-type and hAPP mice, and from hAPP mice dosed with 25 mg/kg PCN once a day for 7 days; β -actin was used as protein loading control (pooled tissue from 20 mice per group). (B) Accumulation of NBD-CSA in brain capillaries from vehicle-treated wild-type and hAPP mice, and from PCN-treated hAPP mice; capillaries were incubated with 2 μ M NBD-CSA for 1 h alone or with PSC833. (C) Data after capillary image analysis. Data are mean \pm SEM for 10 capillaries from one preparation (pooled tissue from 20 mice per group); shown are arbitrary fluorescence units (scale 0-255). Statistics: ***significantly lower than control, $P < 0.001$.

Figure 4. PXR activation in hAPP mice restores P-glycoprotein-mediated hA β 42 transport. (A) Representative images of brain capillaries isolated from wild-type, hAPP, and PCN-treated hAPP mice; capillaries were incubated with 5 μ M fluorescein-hA β 42 for 1 h. (B) Data after capillary digital image analysis. Data are mean \pm SEM for 10 capillaries from one preparation (pooled tissue from 20 mice per group); shown are arbitrary fluorescence units (scale 0-255). Statistics: ***significantly lower than control, $P < 0.001$.

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Figure 5. Western blots for indicated proteins of capillary membranes from vehicle- and PCN-treated hAPP mice (pooled tissue from 20 mice per group).

Figure 6. Restoring P-glycoprotein in hAPP mice reduces A β levels in brain capillaries. Representative images of (A) hA β 40- and (B) hA β 42-immunostained brain capillaries from vehicle- and PCN-treated hAPP mice. (C, D) Data from membrane hA β -immunofluorescence analysis; data are mean \pm SEM for 10 capillaries (pooled tissue from 20 mice per group); shown are arbitrary fluorescence units (scale 0-255). Statistics: *significantly lower than control, $P < 0.05$, **significantly lower than control, $P < 0.01$. (E) hA β 40 and hA β 42 Western blots of brain capillary membranes.

Figure 7. Restoring P-glycoprotein in hAPP mice reduces A β brain levels. (A) hA β 40, hA β 42, and hAPP Western blots of total brain from vehicle- and PCN-treated hAPP mice. (B) hA β 40 and (C) hA β 42 ELISA analysis of brain tissue from vehicle- and PCN-treated hAPP mice (pooled tissue from 19 vehicle-treated hAPP mice and 20 PCN-treated hAPP mice). Statistics: ***significantly lower than control, $P < 0.001$.

Figure 1

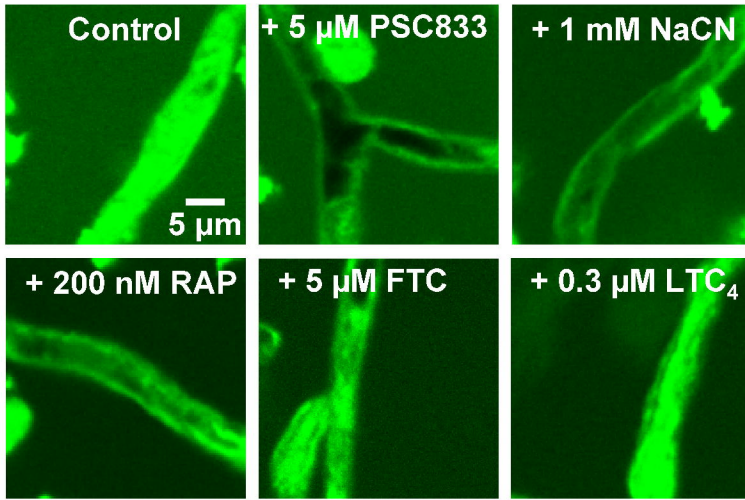
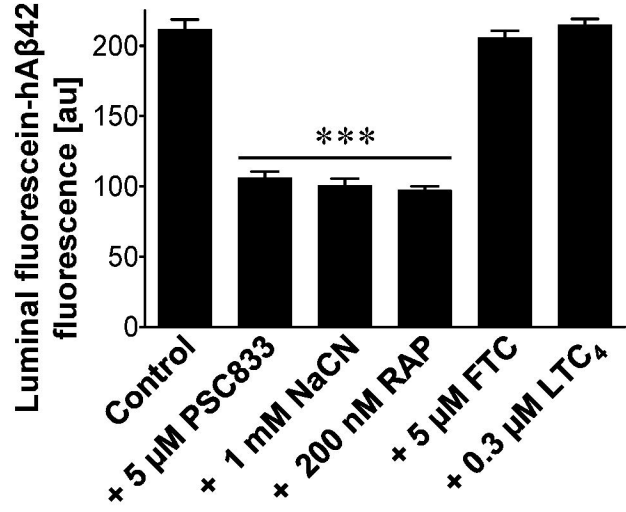
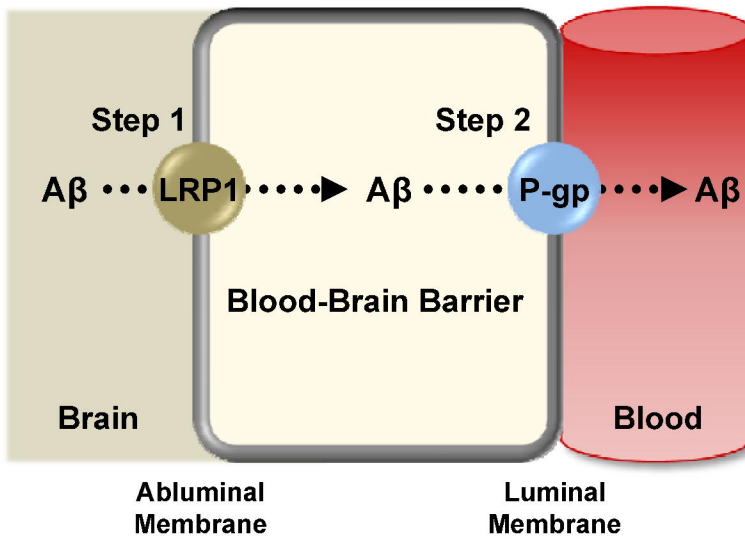
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Figure 2

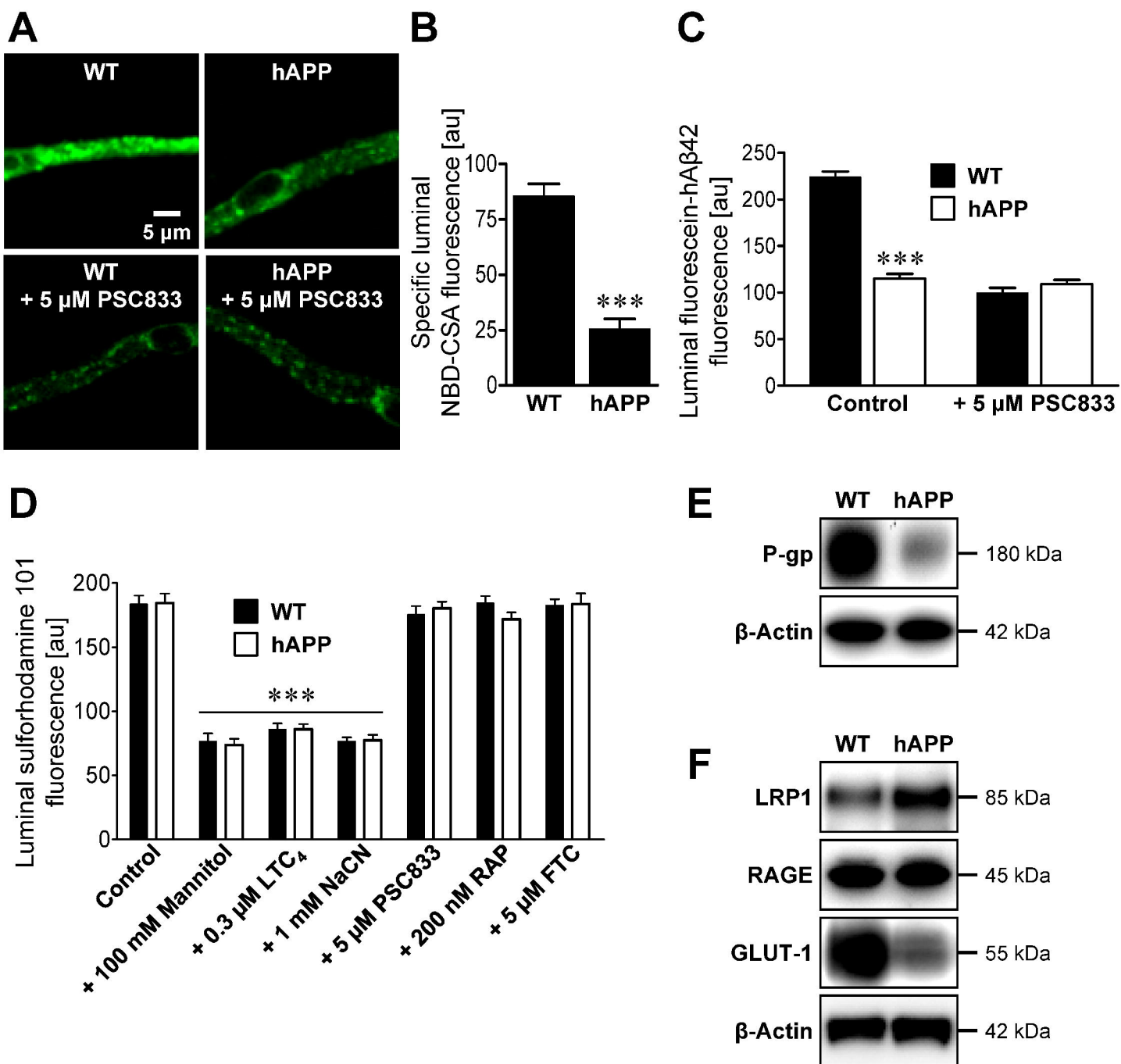


Figure 3

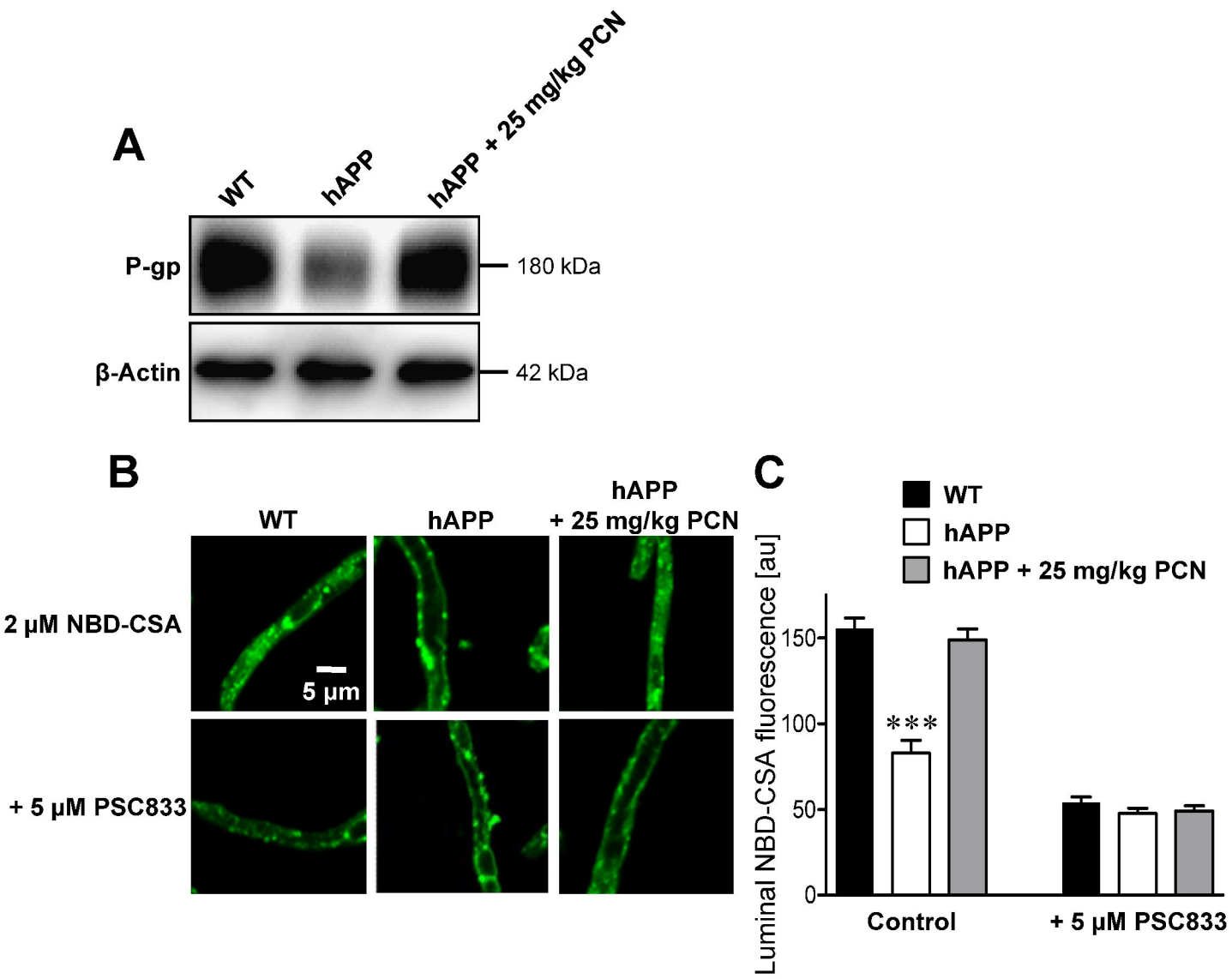


Figure 5

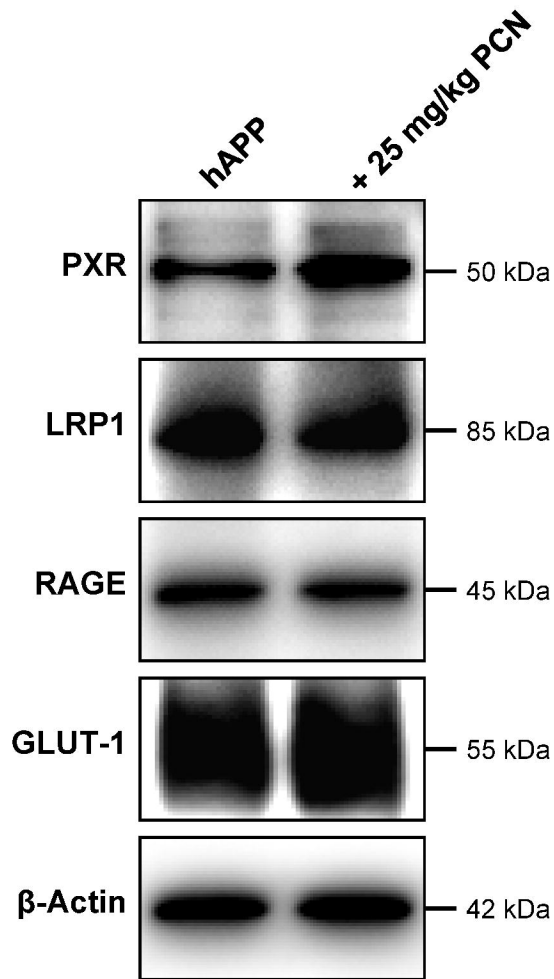


Figure 6

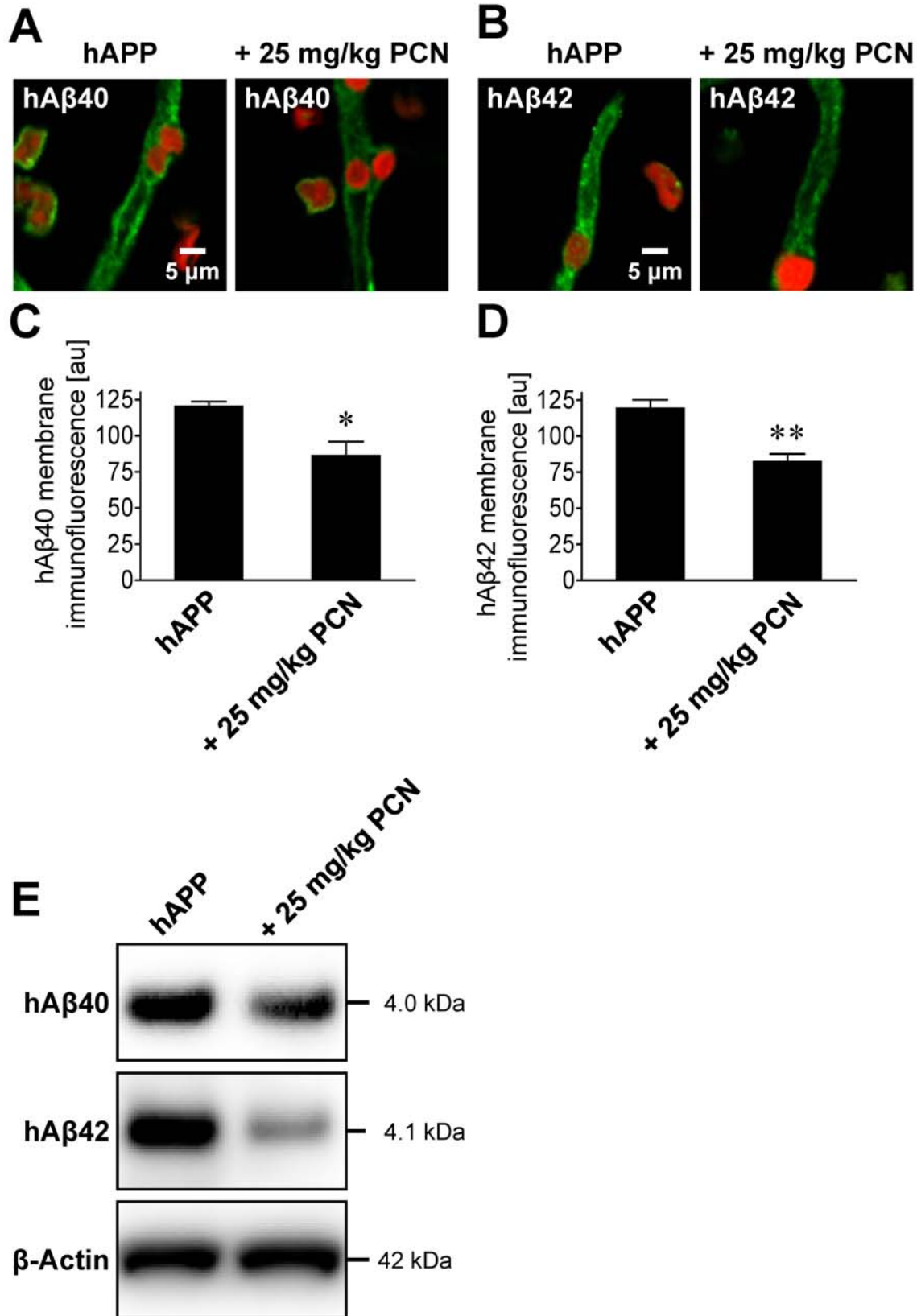
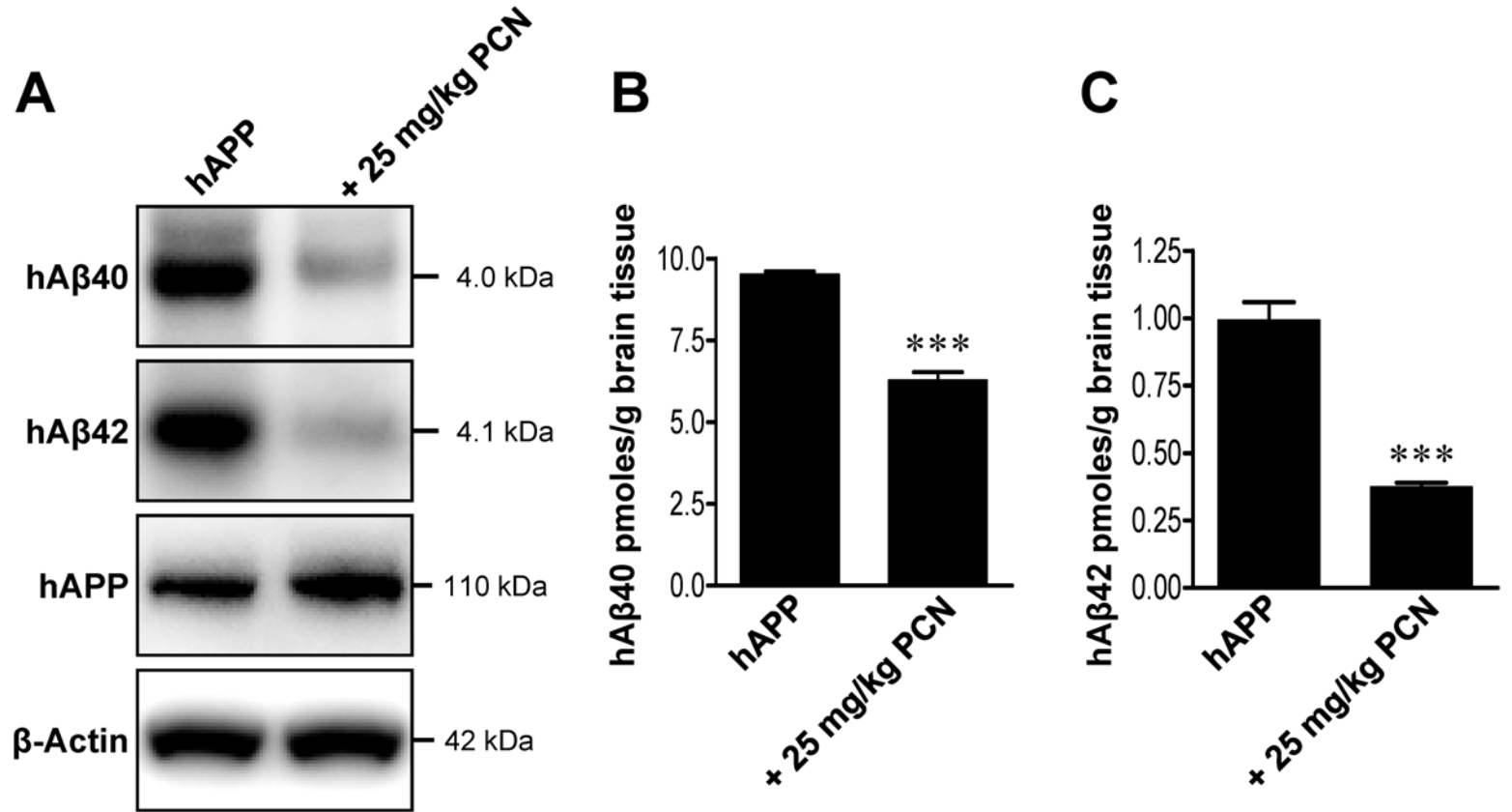


Figure 7

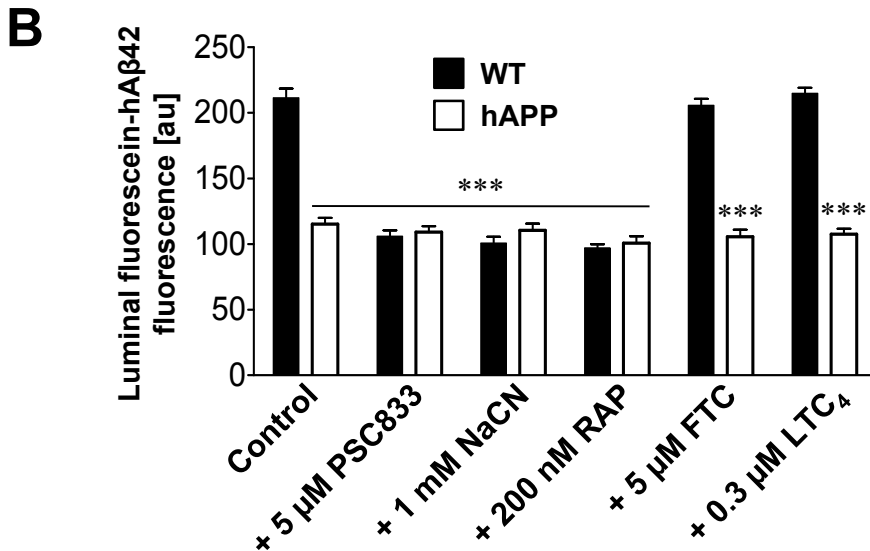
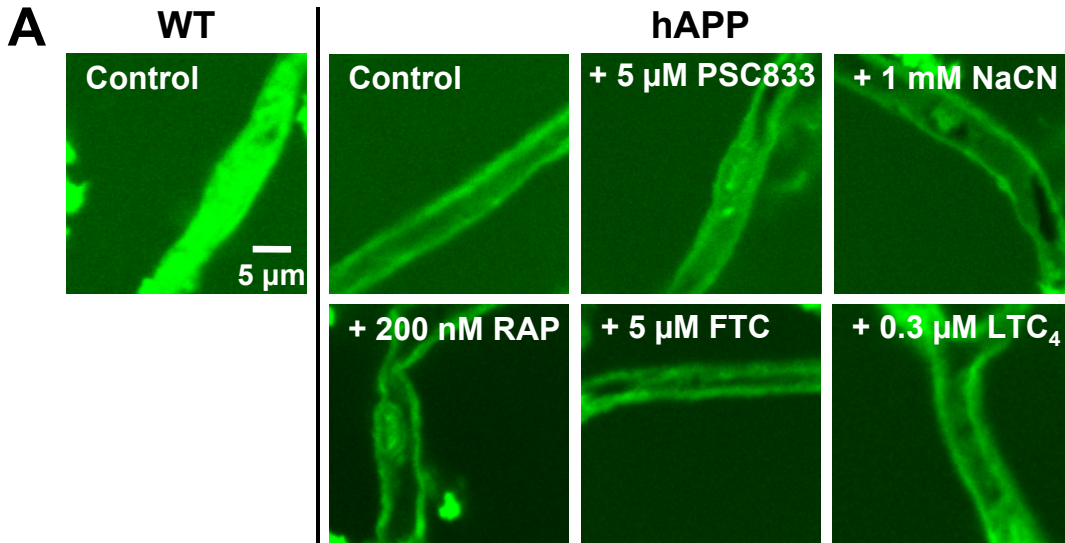


Supplemental Figure 1

Hartz AMS, Miller DS, Bauer B

Restoring Blood-Brain Barrier P-glycoprotein Reduces Brain A β in a Mouse Model of Alzheimer's Disease

Molecular Pharmacology



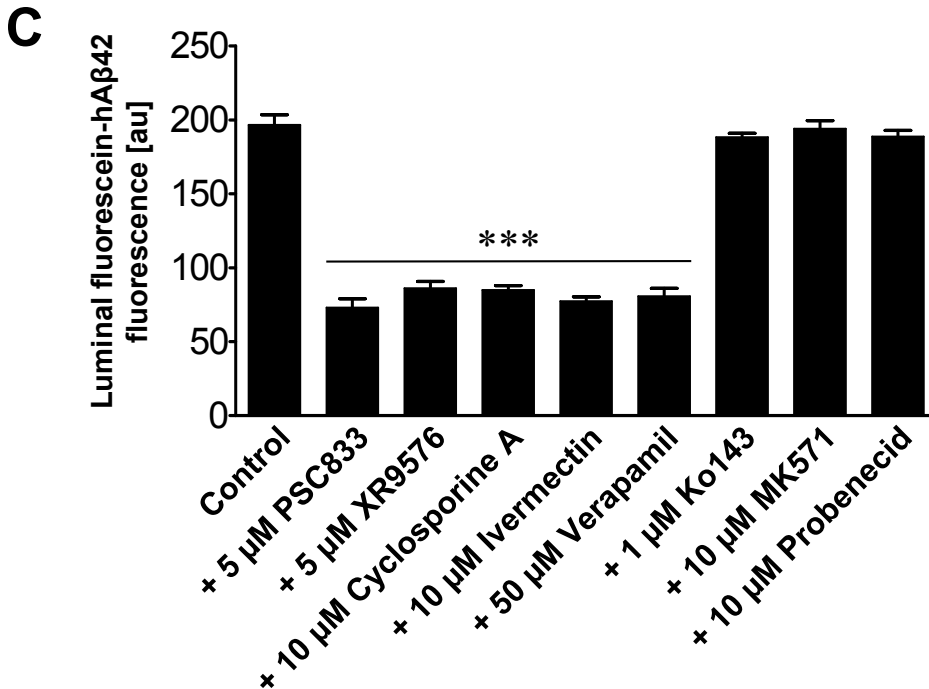
Supplemental Figure 1. P-glycoprotein mediates hA β 42 transport in brain capillaries from wild-type mice but such transport is substantially reduced in brain capillaries from hAPP transgenic mice. **(A)** Representative images of brain capillaries isolated from wild-type and hAPP transgenic mice; capillaries were incubated with 5 μ M fluorescein-hA β 42 for 1 h alone (control) or with 5 μ M fluorescein-hA β 42 plus PSC833 (P-glycoprotein inhibitor), NaCN (metabolic inhibitor), RAP (receptor-associated protein, LRP1 inhibitor), FTC (fumitremorgin C, BCRP inhibitor), or LTC₄ (leukotriene C₄, MRP inhibitor). **(B)** shows luminal fluorescein-hA β 42 fluorescence in brain capillaries after digital image analysis; residual fluorescence is due to non-specific binding (14). Data represent mean \pm SEM for 10 capillaries from one preparation (pooled tissue from 10 wild-type and 10 hAPP transgenic mice); shown are arbitrary fluorescence units (scale 0-255). Statistics: ***significantly lower than control, $P < 0.001$.

Supplemental Figure 1

Hartz AMS, Miller DS, Bauer B

Restoring Blood-Brain Barrier P-glycoprotein Reduces
Brain A β in a Mouse Model of Alzheimer's Disease

Molecular Pharmacology



Supplemental Figure 1. P-glycoprotein mediates hA β 42 transport in brain capillaries from wild-type mice. (C) shows luminal fluorescein-hA β 42 fluorescence in brain capillaries after inhibition with the P-glycoprotein-specific inhibitors, PSC833 (valsopodar), XR9576 (tariquidar), cyclosporine A, ivermectin, and verapamil. Luminal capillary fluorescein-hA β 42 fluorescence was not affected by the BCRP inhibitor, Ko143, and the MRP inhibitors, MK571 and probenecid.

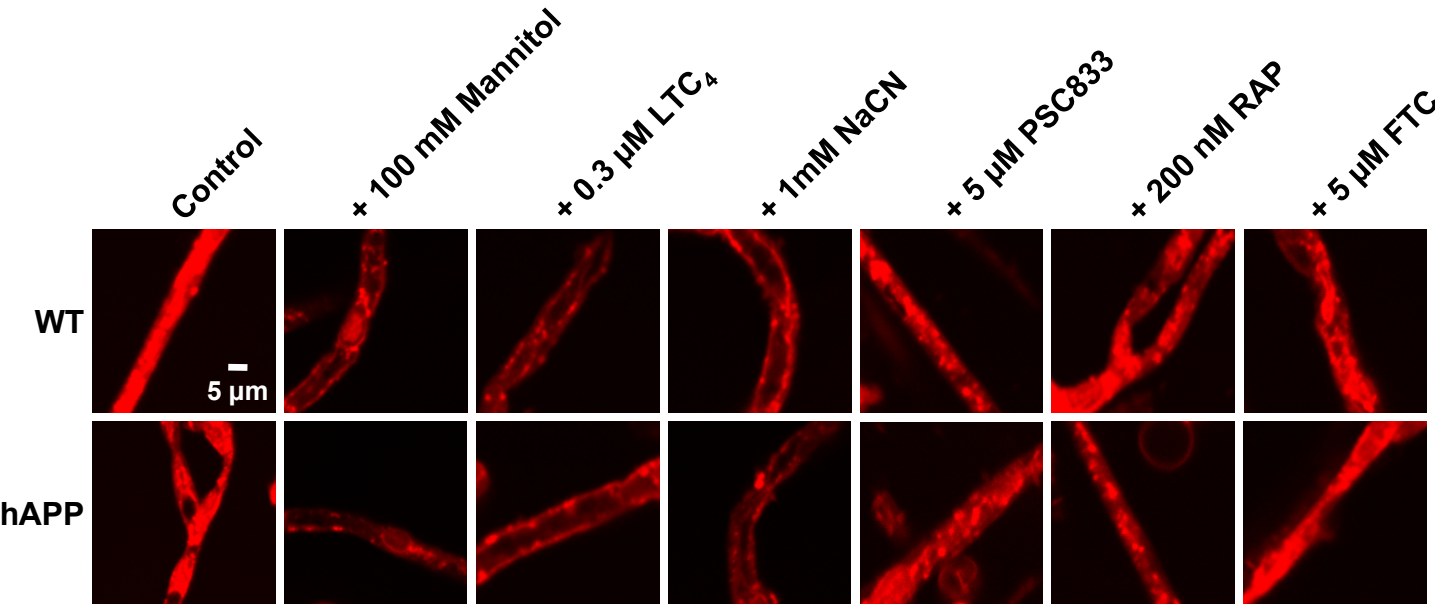
Data represent mean \pm SEM for 10 capillaries from one preparation (pooled tissue from 10 wild-type mice); shown are arbitrary fluorescence units (scale 0-255). Statistics: ***significantly lower than control, $P < 0.001$.

Supplemental Figure 2

Hartz AMS, Miller DS, Bauer B

Restoring Blood-Brain Barrier P-glycoprotein Reduces
Brain A β in a Mouse Model of Alzheimer's Disease

Molecular Pharmacology



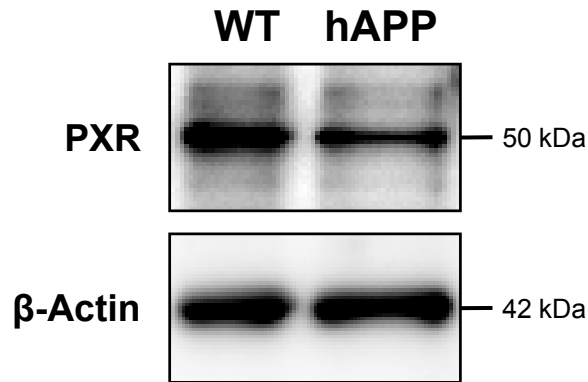
Supplemental Figure 2. Concentrative transport of sulforhodamine 101 into the brain capillary luminal space. Representative images of brain capillaries isolated from wild-type and hAPP mice; capillaries were incubated with 2 μ M sulforhodamine 101 for 1 h alone (control) or with 2 μ M sulforhodamine 101 plus mannitol (osmotic tight junction disruptor), LTC₄ (MRP inhibitor), NaCN (metabolic inhibitor), PSC833 (P-glycoprotein inhibitor), RAP (LRP1 inhibitor), or FTC (BCRP inhibitor). Note that sulforhodamine 101 is transported by Mrp2, another ATP-driven efflux pump (15). Consistent with this, concentrative transport of sulforhodamine 101 into the luminal space was reduced by mannitol, LTC₄, and NaCN, but remained unaltered by inhibitors of other transporters (PSC833, RAP, FTC). Unaltered luminal accumulation of sulforhodamine 101 in control capillaries from hAPP mice indicates that reduced transport of NBD-CSA and fluorescein-hA β 42 found in brain capillaries from hAPP mice was not due to disruption of tight junctions, but rather to reduced P-glycoprotein-mediated transport.

Supplemental Figure 3

Hartz AMS, Miller DS, Bauer B

Restoring Blood-Brain Barrier P-glycoprotein Reduces
Brain A β in a Mouse Model of Alzheimer's Disease

Molecular Pharmacology



Supplemental Figure 3. Western blot of the ligand-activated nuclear receptor, pregnane X receptor (PXR) in brain capillaries from wild-type and hAPP transgenic mice; β -actin was used as protein loading control.

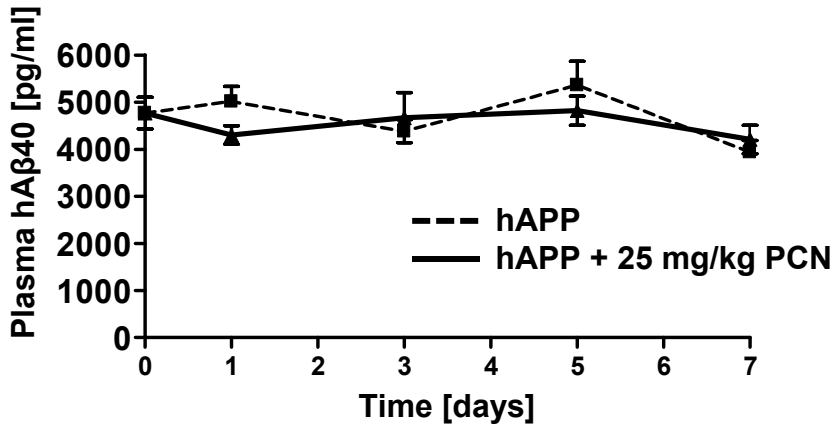
Supplemental Figure 4

Hartz AMS, Miller DS, Bauer B

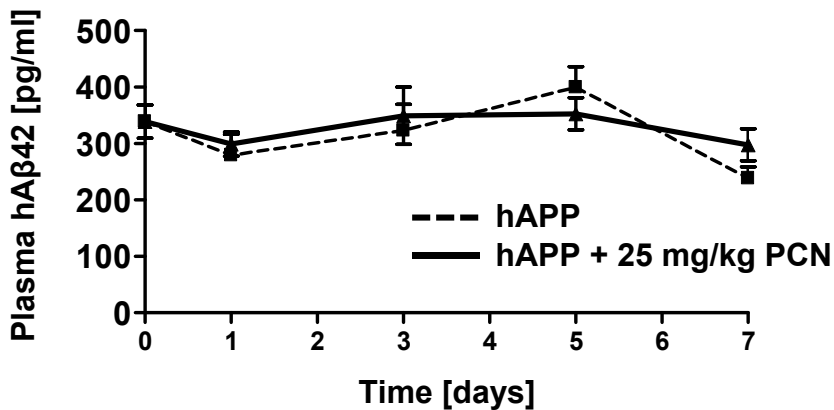
Restoring Blood-Brain Barrier P-glycoprotein Reduces Brain A β in a Mouse Model of Alzheimer's Disease

Molecular Pharmacology

A



B



Supplemental Figure 4. ELISA analysis of (A) hA β 40 and (B) hA β 42 in plasma from vehicle- and PCN-treated hAPP mice. At time point 0 days, n = 14 animals for each group, at time points 1, 3, and 5 days, n = 7-8, at time point 7 days, n = 15-19. Data are mean \pm SEM. Over the entire time course no statistically significant differences in hA β 40 and hA β 42 plasma levels were observed between hAPP and PCN-treated hAPP mice.