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Restoring Blood-Brain Barrier P-glycoprotein Reduces

Brain Aβ in a Mouse Model of Alzheimer's Disease

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Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; BCRP, breast cancer resistance protein; FTC, fumitremorgin C; hAPP, human amyloid precursor protein; LTC_4, leukotriene C_4; LRP1, low-density lipoprotein receptor-related protein 1; MRP, multidrug resistance-associated protein; NBD-CSA, [N-ε (4-nitrobenzofurazan-7-yl)-D-Lys[8]]-cyclosporine A; PCN, pregnenolone-16α-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

ABSTRACT

Reduced clearance of amyloid-β (Aβ) from brain partly underlies increased Aβ 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 Aβ from mouse brain capillaries into the vascular space, thus identifying a critical component of the Aβ 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 Aβ accumulates in the brain in AD. Importantly, we show that dosing 12-week old, asymptomatic hAPP mice over 7 days with pregnenolone-16α-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 Aβ 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 Aβ clearance from the brain and reduce Aβ brain accumulation. This mechanism suggests a new therapeutic strategy in AD.

INTRODUCTION

A hallmark of Alzheimer's disease (AD) is the accumulation of neurotoxic amyloid-β

(Aβ) peptide within the brain. The Aβ transport-clearance hypothesis of AD proposed by

Zlokovic and coworkers states that reduced Aβ clearance (reduced Aβ efflux transport) from the

brain underlies Aβ brain accumulation (Deane et al., 2004b; Mooradian et al., 1997; Zlokovic,

2005; Zlokovic and Frangione, 2003). This hypothesis suggests that the mechanism responsible

for Aβ brain clearance itself could be a therapeutic target in AD.

Aβ clearance from brain to blood has to be a two-step process. Aβ 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 Aβ 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 Aβ 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, Aβ 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 Aβ (Cirrito et

al., 2005; Kuhnke et al., 2007; Lam et al., 2001) and one report suggests reduced Aβ efflux from

the brain in P-glycoprotein-null mice (Cirrito et al., 2005). However, those mice also exhibit

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

Here, we establish a role for blood-brain barrier P-glycoprotein in the efflux transport of Aβ 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 Aβ accumulation within the brain and that restoring such transport can reduce brain Aβ levels. We demonstrate here for the first time that P-glycoprotein mediates efflux transport of Aβ 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 Aβ (hAβ) 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 Aβ brain levels.

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

MATERIALS and METHODS

Chemicals. Antibodies against RAGE, human APP, human Aβ40, human Aβ42, and β-actin were purchased from Abcam (Cambridge, MA, USA). Note that in Western blots using the antibodies to detect human APP, human Aβ40, and human Aβ42 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-hAβ42 (fluorescein-βA(1-42)) was purchased from rPeptide (Bogart, GA, USA), fumitremorgin 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-Lys^8]-cyclosporine A (NBD-CSA) was custom-synthesized by R. Wenger (Basel, Switzerland) (Wenger, 1986). PSC833 (valspodar, 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, LTC$_4$, and pregnenolone-16α-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(APPSWE)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

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 CO_2 inhalation, decapitated, and trunk blood was collected. Brains from each animal were collected for $A\beta$ 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 CO_2 inhalation and then decapitated. Brains were removed, dissected, and homogenized in ice cold PBS buffer (2.7 mM KCl, 1.46 mM KH_2PO_4, 136.9 mM NaCl, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$ 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-

glycoprotein-specific substrate, NBD-CSA (2 µM in PBS buffer) (Hartz et al., 2008). For Aβ-transport studies, capillaries were incubated for 1 h at room temperature with 5 µM fluorescein-hAβ42 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) .

Aβ 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

4°C with a 1:500 dilution of monoclonal antibody against hAβ40 or polyclonal antibody against hAβ42. 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 μg/ml propidium iodide. Negative controls for each treatment that were processed without primary antibody showed negligible background fluorescence (data not shown). Aβ 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. Aβ 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 resuspendend 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

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 hAβ40 and hAβ42. hAβ40 and hAβ42 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 hAβ40 plasma levels, samples were diluted 1:20 before analysis; to determine hAβ42 levels, samples were diluted 1:4 before analysis. hAβ40 and hAβ42 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 ± 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 $P<0.05$.

RESULTS

Blood-brain barrier P-glycoprotein transports hAβ42

To investigate the role of P-glycoprotein in Aβ 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-hAβ42 and measured its accumulation in capillary lumens using confocal microscopy and quantitative imaging. Fluorescein-hAβ42 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 (valspodar), 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 C_4 (LTC_4), 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 Aβ efflux being an active and ATP-dependent two-step process, involving LRP1-mediated Aβ uptake from brain into capillary endothelial cells followed by P-glycoprotein-mediated Aβ 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 Aβ 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 Aβ being a P-glycoprotein substrate, isolated brain capillaries from hAPP mice exhibited substantially reduced fluorescein-hAβ42 transport (Figure 2C).

Reduced luminal accumulation of NBD-CSA and fluorescein-hAβ42 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

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-hAβ42 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 Aβ-uptake receptor, LRP1, was slightly increased and that of the luminal Aβ-uptake receptor, RAGE, was unchanged (Figure 2F). This indicates that decreased Aβ 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 Aβ brain clearance in AD seems to be Aβ 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-16α-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-hAβ42 (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 Aβ brain levels

We next determined the consequences of restoring blood-brain barrier P-glycoprotein on human Aβ (hAβ) levels in hAPP mice. We initially measured hAβ40 and hAβ42 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 Aβ clearance from blood by filtration at the kidney.

An early sign of AD is cerebral amyloid angiopathy (CAA), characterized by Aβ deposition in brain capillary membranes (Greenberg et al., 2004; Iadecola, 2004; Zlokovic, 2008). To measure capillary-associated Aβ, we isolated brain capillaries from hAPP control and PCN-dosed mice and immunostained for hAβ40 and hAβ42. Capillaries from untreated hAPP mice stained positive for both Aβ 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 hAβ40 ($P<0.05$) and 31% for hAβ42 ($P<0.01$) compared to capillaries from untreated hAPP mice (Figure 6, A-D). Western blotting of brain capillary membranes also showed reduced levels of hAβ40 and hAβ42 in hAPP mice treated with PCN compared to untreated hAPP mice (Figure 6E; hAβ40: $82 \pm 1.4\%$ (SEM; $P<0.05$) of control, hAβ42: $45 \pm 4.4\%$ (SEM; $P<0.001$) of control as determined by optical density measurements; hAβ levels normalized to β-actin). Thus, PCN dosing clearly reduced capillary-associated Aβ, suggesting reduced angiopathy.

Finally, Western blots and ELISA analysis showed significantly ($P<0.001$) reduced hAβ40 and hAβ42 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 hAβ40 and hAβ42 by about 50% and 60%, respectively

(Figure 7A; hAβ40: 50 ± 4.5% (SEM, *P*<0.001) of control; hAβ42: 36 ± 1.0% (SEM, *P*<0.001)

of control; hAβ levels normalized to β-actin). This was confirmed by ELISA measurements

which demonstrated that brain levels of hAβ40 and hAβ42 were reduced by about 35% and 60%,

respectively (Figure 7, B and C; hAβ40: 66 ± 3% (SEM, *P*<0.001) of control; hAβ42: 38 ± 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 Aβ precursor levels. Thus, activation

of PXR increased blood-brain barrier P-glycoprotein expression and transport activity, which

acutely reduced hAβ40 and hAβ42 brain levels in hAPP mice.

DISCUSSION

The present report reconciles three disparate observations each of which suggests that P-glycoprotein is a critical component of the Aβ brain clearance mechanism: 1) cell lines that overexpress human P-glycoprotein transport Aβ (Kuhnke et al., 2007; Lam et al., 2001; Tai et al., 2009); 2) injecting a P-glycoprotein inhibitor into hAPP transgenic mice increases Aβ brain levels (Cirrito et al., 2005); and 3) brain deposition of Aβ 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 Aβ efflux across the luminal membrane of the brain capillary endothelium and for defective P-glycoprotein-mediated Aβ 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 Aβ 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 Aβ (Xiong et al., 2009). This work indicates that BCRP transports Aβ in BCRP-overexpressing cell lines, that Aβ 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 Aβ efflux from the brain and if reduced BCRP-mediated efflux contributed to Aβ 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

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 Aβ 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 Aβ receptors, RAGE and LRP1, have been shown to contribute to Aβ 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 Aβ uptake receptor, RAGE, in brain capillaries suggests that Aβ uptake from blood into the endothelium may not be increased before the onset of cognitive impairment in AD. Slightly increased expression of the abluminal Aβ uptake receptor, LRP1, suggests increased Aβ uptake from brain into the endothelium, again in very early stages of the disease, which may contribute to Aβ accumulation within the capillary endothelium in CAA. However, such changes in expression of these receptors cannot underlie reduced brain efflux of Aβ 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 Aβ from the brain. They indicate that efflux mediated by P-glycoprotein may well be the limiting factor in Aβ 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 Aβ brain accumulation. These results have two important implications for how we view the disease.

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 Aβ42 levels in the brain capillary plasma membrane could directly impair P-glycoprotein function, lead to Aβ 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 Aβ40 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 Aβ 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 Aβ is one such substrate and that P-glycoprotein is responsible for the final, critical step in Aβ 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.

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 Aβ 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 Aβ brain efflux transport and lower Aβ 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 Aβ brain efflux transport and lower Aβ brain burden.

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

transgenic hAPP (Tg2576) mouse model does delay pathology (Karlnoski 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 Aβ 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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FOOTNOTES

LEGENDS FOR FIGURES

Figure 1. P-glycoprotein mediates hAβ42 transport in mouse brain capillaries. (**A**) Representative confocal images of brain capillaries isolated from wild-type mice; capillaries were incubated with 5 µM fluorescein-hAβ42 for 1 h alone (control) or with 5 µM fluorescein-hAβ42 plus PSC833 (P-glycoprotein inhibitor), NaCN (metabolic inhibitor), RAP (LRP1 inhibitor), FTC (BCRP inhibitor), or LTC$_4$ (MRP inhibitor). (**B**) Capillary luminal fluorescein-hAβ42 fluorescence after image analysis; residual fluorescence is due to non-specific binding (13). Data represent mean ± 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 Aβ efflux involving the Aβ 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 µM 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-hAβ42 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), LTC$_4$ (MRP inhibitor), NaCN (metabolic inhibitor), PSC833 (P-glycoprotein inhibitor), RAP (LRP1 inhibitor), or FTC (BCRP inhibitor). Data in **B-D** are mean ± SEM for 10 capillaries from one preparation (pooled tissue

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 μM NBD-CSA

for 1 h alone or with PSC833. (**C**) Data after capillary image analysis. Data are mean ± 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 hAβ42 transport. (**A**)

Representative images of brain capillaries isolated from wild-type, hAPP, and PCN-treated

hAPP mice; capillaries were incubated with 5 μM fluorescein-hAβ42 for 1 h. (**B**) Data after

capillary digital image analysis. Data are mean ± 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, $P<0.001$.
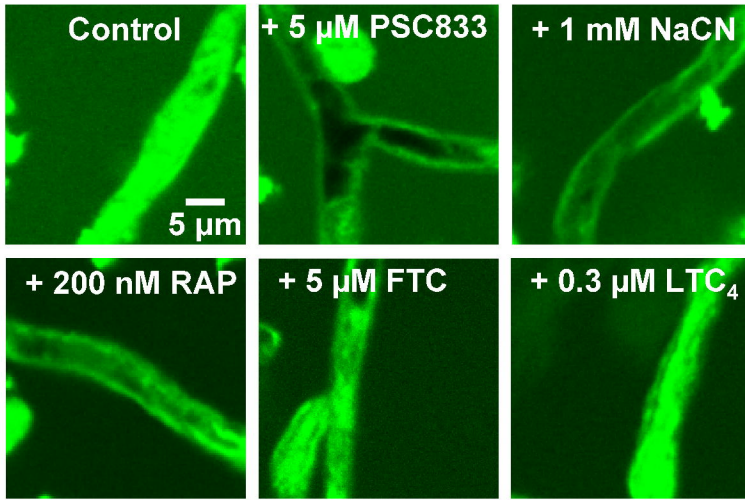
Figure 5. Western blots for indicated proteins of capillary membranes from vehicle- and PCN-treated hAPP mice (pooled tissue from 20 mice per group).

Figure 6. Restoring P-glycoprotein in hAPP mice reduces Aβ levels in brain capillaries. Representative images of (**A**) hAβ40- and (**B**) hAβ42-immunostained brain capillaries from vehicle- and PCN-treated hAPP mice. (**C, D**) Data from membrane hAβ-immunofluorescence analysis; data are mean ± 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**) hAβ40 and hAβ42 Western blots of brain capillary membranes.
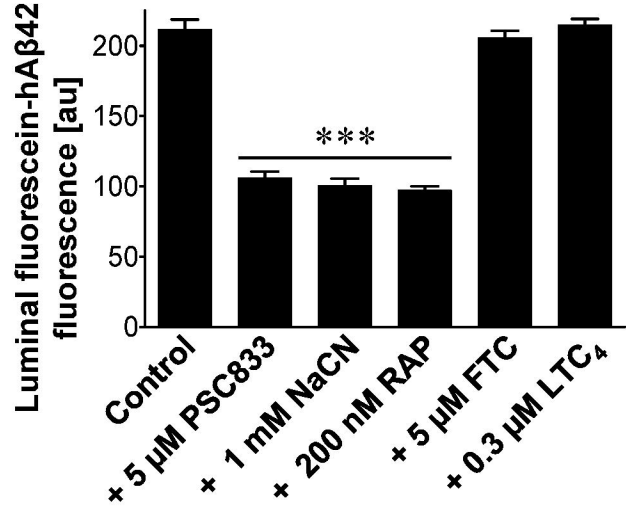
Figure 7. Restoring P-glycoprotein in hAPP mice reduces Aβ brain levels. (**A**) hAβ40, hAβ42, and hAPP Western blots of total brain from vehicle- and PCN-treated hAPP mice. (**B**) hAβ40 and (**C**) hAβ42 ELISA analysis of brain tissue from vehicle- and PCN-treated hAPP mice (pooled tissue from 19 vehicle-treated hAPP mice and 20 PCN-treated hAPP mice). Statistics: ***significantly lower than control, $P<0.001$.

Figure 1

A
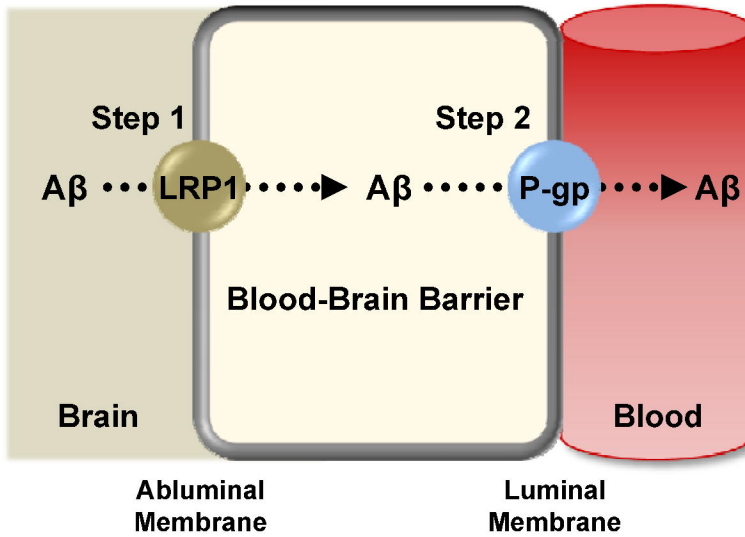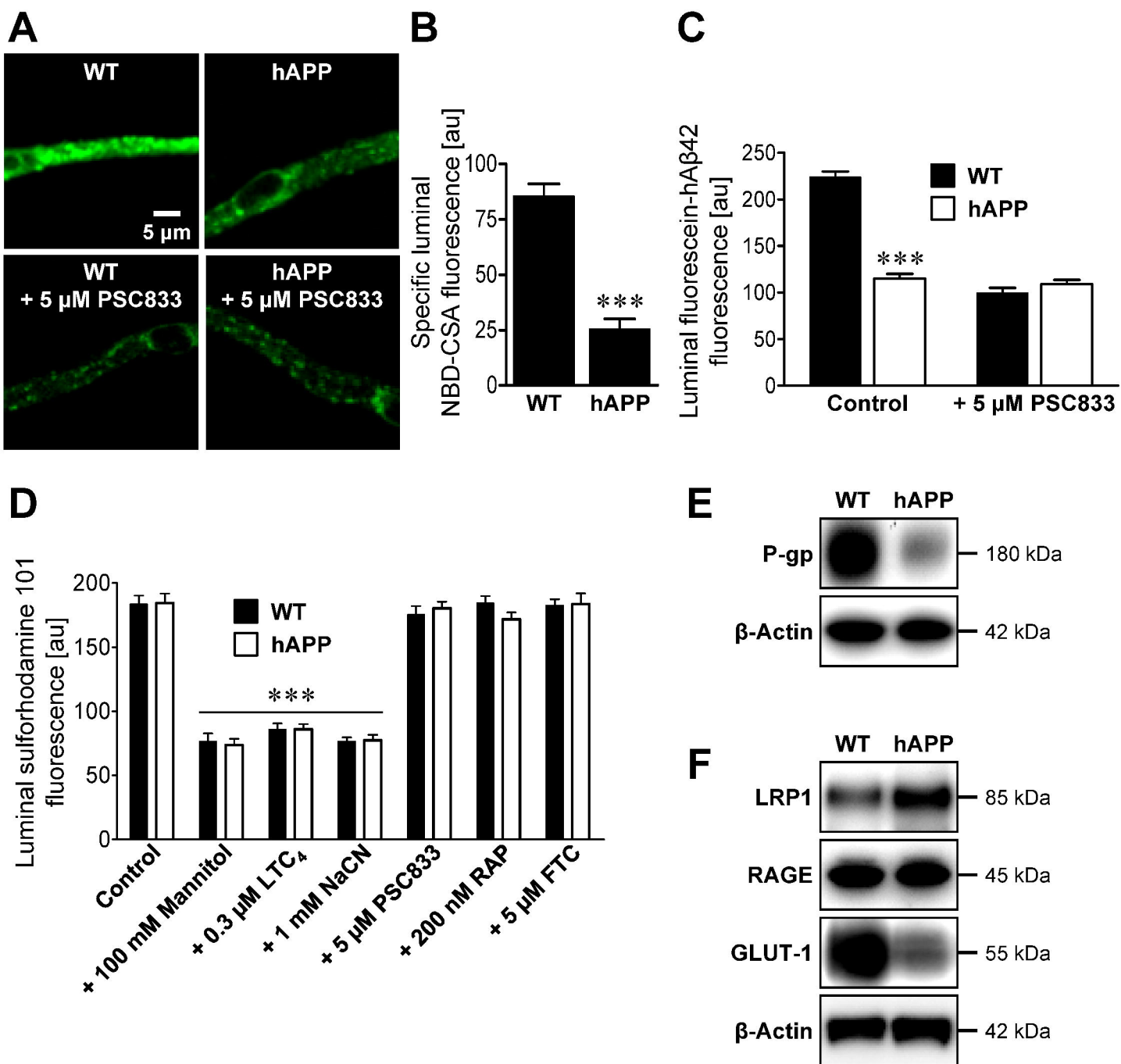

Control

+ 5 µM PSC833

+ 1 mM NaCN

5 µm

+ 200 nM RAP

+ 5 µM FTC

+ 0.3 µM LTC$_4$

B

Luminal fluorescein-hAβ42 fluorescence [au]

***

Control
+ 5 µM PSC833
+ 1 mM NaCN
+ 200 nM RAP
+ 5 µM FTC
+ 0.3 µM LTC$_4$

C

Step 1

Step 2

Aβ ··· LRP1 ····▶ Aβ ····· P-gp ···▶ Aβ

Blood-Brain Barrier

Brain

Blood

Abluminal Membrane

Luminal Membrane

Figure 2

A

WT | hAPP

5 µm

WT + 5 µM PSC833 | hAPP + 5 µM PSC833

B

Specific luminal NBD-CSA fluorescence [au]

***

WT | hAPP

C

Luminal fluorescein-hAβ42 fluorescence [au]

■ WT
□ hAPP

***

Control | + 5 µM PSC833

D

Luminal sulforhodamine 101 fluorescence [au]

■ WT
□ hAPP

***

Control | + 100 mM Mannitol | + 0.3 µM LTC₄ | + 1 mM NaCN | + 5 µM PSC833 | + 200 nM RAP | + 5 µM FTC

E

WT | hAPP

P-gp — 180 kDa

β-Actin — 42 kDa

F

WT | hAPP

LRP1 — 85 kDa

RAGE — 45 kDa

GLUT-1 — 55 kDa

β-Actin — 42 kDa

Figure 3

A

P-gp

β-Actin

WT   hAPP   hAPP + 25 mg/kg PCN

— 180 kDa

— 42 kDa

B

2 µM NBD-CSA

+ 5 µM PSC833

WT   hAPP   hAPP + 25 mg/kg PCN

5 µm

C

■ WT
□ hAPP
■ hAPP + 25 mg/kg PCN

Luminal NBD-CSA fluorescence [au]

***

Control   + 5 µM PSC833

Figure 4

A

WT   hAPP   hAPP + 25 mg/kg PCN

5 µm

B

Luminal fluorescein-hAβ42 fluorescence [au]

■ WT
□ hAPP
■ hAPP + 25 mg/kg PCN

***

Control   + 5 µM PSC833

Figure 5

Figure 6

Figure 7