Research Article

Beta-Amyloid Downregulates MDR1-P-Glycoprotein (Abcb1) Expression at the Blood-Brain Barrier in Mice

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Neurovascular dysfunction is an important component of Alzheimer's disease, leading to reduced clearance across the blood-brain barrier and accumulation of neurotoxic β-amyloid (Aβ) peptides in the brain. It has been shown that the ABC transport protein P-glycoprotein (P-gp, ABCB1) is involved in the export of Aβ from the brain into the blood. To determine whether Aβ influences the expression of key Aβ transporters, we studied the effects of 1-day subcutaneous Aβ1-40 and Aβ1-42 administration via Alzet mini-osmotic pumps on P-gp, BCRP, LRP1, and RAGE expression in the brain of 90-day-old male FVB mice. Our results demonstrate significantly reduced P-gp, LRP1, and RAGE mRNA expression in mice treated with Aβ1-42 compared to controls, while BCRP expression was not affected. The expression of the four proteins was unchanged in mice treated with Aβ1-40 or reverse-sequence peptides. These findings indicate that, in addition to the age-related decrease of P-gp expression, Aβ1-42 itself downregulates the expression of P-gp and other Aβ-transporters, which could exacerbate the intracerebral accumulation of Aβ and thereby accelerate neurodegeneration in Alzheimer's disease and cerebral β-amyloid angiopathy.

1. Introduction

Burgeoning evidence implicates cerebrovascular dysfunction in the cognitive decline and neurodegeneration that characterize Alzheimer's disease (AD) [1, 2]. Impaired clearance of β-amyloid (Aβ) across the blood-brain barrier (BBB) and the senescence of the cerebrovascular system are thought to initiate processes that contribute to neurodegeneration [1, 3]. The transport of Aβ across the BBB comprises a two-step process involving transport through the abluminal (brain side) and then through the luminal (blood side) plasma membranes of the endothelial cells. The low-density lipoprotein receptor-related protein-1 (LRP1) mediates the incorporation of brain-derived Aβ peptides into the endothelial cells at the abluminal membrane [4, 5]. On the other hand, the receptor for advanced glycation end products (RAGE) is considered to be a major influx transporter of peripherally derived (blood-borne) Aβ into the endothelial cells of the cerebral vasculature [6, 7]. The ABC transporter

P-glycoprotein (P-gp, ABCB1) is highly expressed at the luminal side of the endothelial cells that form the BBB, and may play a key role in the transport of Aβ peptides (either brain or blood derived) into the blood [8]. *In vitro* and *in vivo* experiments have shown that P-gp is an active transport protein for Aβ1-40 and Aβ1-42 [9–14]. These observations suggest that diminished P-gp expression due to increasing age, genetic, or environmental factors may lead to impaired Aβ clearance, followed by the accelerated accumulation of intracerebral Aβ and eventually the development of AD.

An autopsy study of nondemented elderly humans found that vessels with Aβ accumulation within their walls (cerebral amyloid angiopathy, CAA) showed little or no endothelial P-gp expression whereas unaffected capillaries had high P-gp expression, leading to the hypothesis that Aβ leads to a downregulation of P-gp expression whereas P-gp might be upregulated in the endothelium of capillaries lacking Aβ accumulation in an attempt to compensate for the loss of P-gp in amyloid-bearing vessels [11]. Thus, the aim of

the present study is to investigate the effect of Aβ administration on the expression of P-gp and other Aβ transporters in endothelial cells of the cerebral vasculature.

2. Material and Methods

2.1. Laboratory Animals. 90 day-old male FVB wildtype mice (purchased from Taconic, Hudson, NY, USA) weighing approximately 25 g were used. Animal care and experimental conditions were conducted in compliance with protocols approved by the local Animal Care and Use Committee.

2.2. Preparation and Subcutaneous Implantation of ALZET Mini-Osmotic Pumps. 10–12 animals per group were used for the systemic administration of Aβ1-40 (DAEFRHDS-GYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV), Aβ1-42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVG-GVVIA), or reverse Aβ40-1 (VVGGVMLGIIAGKNSGVD-EAFFVLKQHHVEYGSDHRFEAD) or reverse Aβ42-1 (AIV-VGGVMLGIIAGKNSGVDEAFFVLKQHHVEYGSDHRF-EAD). All peptides were purchased from AnaSpec Inc. (Fremont, CA, USA).

1 mg of each peptide was dissolved in 4 µL DMSO and mixed with 2 mL isotonic NaCl solution, yielding a final Aβ concentration of 0.5 µg/µL. 200 µL (=100 µg peptide) of this solution were used to fill the Alzet mini-osmotic pumps. 200 µL of isotonic NaCl solution served as vehicle control.

ALZET mini-osmotic pumps (model 2001D; DURECT Corporation, Cupertino, USA) were implanted subcutaneously using a midscapular incision. Pumps delivered synthetic, human-type sequence Aβ1-40, Aβ1-42, reverse Aβ40-1, Aβ42-1 peptide, or isotonic NaCl solution, respectively. After a 2-hour start-up transient, the pumps infuse at a constant pumping rate of 8 µLh^{-1}.

After 26 h mice were sacrificed by cervical dislocation and the brains were immediately removed. One hemisphere was shock-frozen in liquid nitrogen and stored at −80°C until use. The other hemisphere was fixed with neutral-buffered 4.5% formalin and was embedded in paraffin.

2.3. Real-Time PCR Analyses. For real-time PCR analysis, RNA was isolated from deep-frozen brain tissue using the *RNeasy Mini* kit form Qiagen according to the manufacturer's instructions (Qiagen, Hilden, Germany). Reverse transcription of total-RNA was performed by the *TaqMan reverse transcription* kit using random hexamer primers (Applied Biosystems, Darmstadt, Germany). Quantitation of RNA (cDNA) levels for Abcb1a, Abcg2 (Mm00496364_m1), LRP1 (Mm00464608_m1), as well as RAGE (Mm00545815_m1) and 18SrRNA (part # 4319413E) for normalization was performed by real-time PCR on a 7900HT TaqMan system (Applied Biosystems) using predeveloped TaqMan assays (Assays on Demand, Applied Biosystems). All PCR reactions were performed in 10 µL reactions using a PCR master mix (2x concentrated) containing 45 mmol/L Tris-HCl (pH 8.4), 115 mmol/L KCl, 7 mmol/L MgCl₂, 460-µmol/L dNTPs, 9% glycerol, 2.3% ROX reference dye (Invitrogen, Paisley, Scotland) and 0.035 U/mL Platinum Taq DNA polymerase (Invitrogen). Thermal cycler conditions were as follows: (50°C, 2 min; 95°C, 10 min followed by 40 cycles of 95°C, 15 s and 60°C, 1 min).

Expression analysis was performed by the comparative ΔΔCt-method using the expression of 18SrRNA as reference gene.

2.4. Immunohistochemistry. For immunohistochemistry, brain tissues were fixed in neutral-buffered, isotonic 4.5% formalin and embedded in paraffin. 1 µm-thick tissue sections were cut, mounted on slides and dried overnight at 60°C. For staining, the biotin-streptavidin immunoperoxidase method with polymer detection system-HRP was used. Automated immunohistochemical staining was performed using the BOND-MAX (Leica) staining systems using pre-diluted antibodies to murine P-gp (clone C219, dilution 1 : 25, pH 9.0, Enzo Life Sciences) and BCRP (clone BxP53, dilution 1 : 50, pH 9.0, Enzo Life Sciences).

Negative controls for each treatment were processed without primary antibody.

2.5. Image Acquisition and Analysis. Digital images were captured using the Zeiss Mirax Scan Desk slide scanner (Carl Zeiss MicroImaging GmbH, Göttingen, Germany; Plan-Apochromat 20x/0.8, pixel resolution: 0.37 µm). To quantitate P-gp expression, image analysis algorithms were developed using ImageJ (version 1.43 q, Research Services Branch, National Institute of Mental Health/National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/), which allowed the identification and analysis of immunolabeled cerebral capillaries in digital images. The amount of P-gp-specific labelling in each animal was determined in ten high-power fields (x40) of the brain cortex, the staining intensity for cortical P-gp of a single animal was the mean of all measurements.

2.6. Statistical Analysis. Statistical analysis was performed using Microsoft Excel and GraphPad Prism 5.01 (GraphPad, San Diego, USA) software using the ΔCt expression values. The statistical tests employed are indicated in the respective sections/figure legends. If the data are represented as boxplots, the whiskers indicate the 10th and 90th percentiles.

3. Results

First, mRNA expression for P-gp (Abcb1a), BCRP (Abcg2), LRP1, and RAGE was measured by real-time PCR in mice treated with Aβ1-42, vehicle control, or the reverse protein (Aβ42-1) for 24 h using subcutaneously transplanted ALZET pumps. Transporter expression was detected in all samples for Abcb1a and Abcb2 while analysis of LRP1, and RAGE failed in one Aβ1-42-treated mouse. Significant changes in the expression of Abcb1a, LRP1 and RAGE were detected in mice given Aβ1-42. While transporter expression was not significantly altered between the control perfusion group and the mice treated with equal amounts of the reverse Aβ protein, animals treated with Aβ1-42 had significantly reduced Abcb1a levels in brain (reduced by 63 ± 24% [mean ± SD]) compared to mice given the vehicle control, and
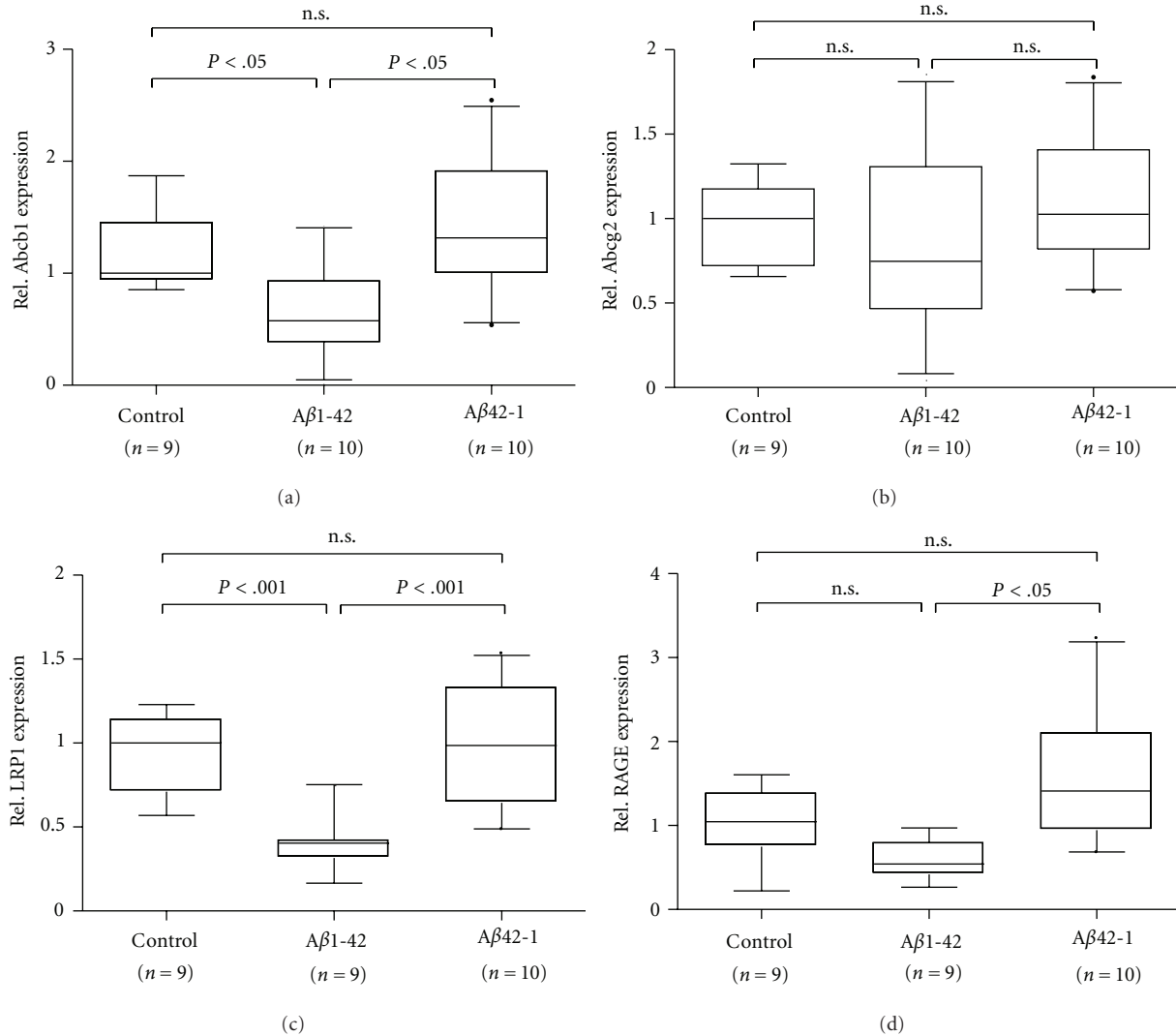
(a)

(b)

(c)

(d)

FIGURE 1: mRNA expression of Abcb1a (a), Abcg2 (b), LRP1 (c), and RAGE (d) in murine brain. Mice were treated with β-amyloid 1-42 (Aβ1-42), reverse β-amyloid 42-1 (Aβ42-1) and vehicle control (control) using ALZET pumps for 24 h (4 μg/h). mRNA expression of each transporter was normalized to 18S rRNA expression and values are depicted in relation to the median expression in vehicle perfused mice. Statistical analysis was performed by one-way ANOVA followed by Newmann-Keuls Multiple Comparison test.

by 54 ± 30% compared to mice given the reverse-sequence protein), LRP1 levels (reduced by 57 ± 17% compared to vehicle controls and by 60 ± 17% compared to the reverse protein controls); and RAGE levels (reduced by 65 ± 15% compared to the reverse protein controls, no significant difference relative to vehicle controls Figures 1(a), 1(c), and 1(d)). In contrast, expression of Abcg2 remained unchanged in these samples (Figure 1(b)).

In a second experiment, mice were treated with Aβ1-40 in a similar manner. Here, no significant changes were detected in any of the four proteins; however, the expression of RAGE tended to be lower compared to the vehicle control samples (Figures 2(a)–2(d)).

At the protein level, P-gp and BCRP were detectable in endothelial cells of brain vessels in all animals using immunohistochemistry (Figures 3(a) and 3(b)). However, quantitatively no changes of P-gp or BCRP could be detected

after administration of Aβ1-42 or Aβ1-40 in comparison to the reverse Aβ peptides or vehicle control, respectively (data not shown).

4. Discussion

There is growing evidence that an alteration of the transport of Aβ peptides across the BBB plays a crucial role in the pathogenesis of AD and CAA. In addition to other transport proteins such as LRP1 and RAGE, the multidrug resistance transport protein P-gp is known to eliminate Aβ1-40 and Aβ1-42 from the brain [12–14]. In nondemented elderly humans, Aβ deposition is inversely correlated with brain capillary P-gp expression [10]. Interestingly, investigations of the correlation between CAA and P-gp expression indicate a loss of P-gp in vessels with abundant cerebrovascular Aβ accumulation. In this study, CAA was
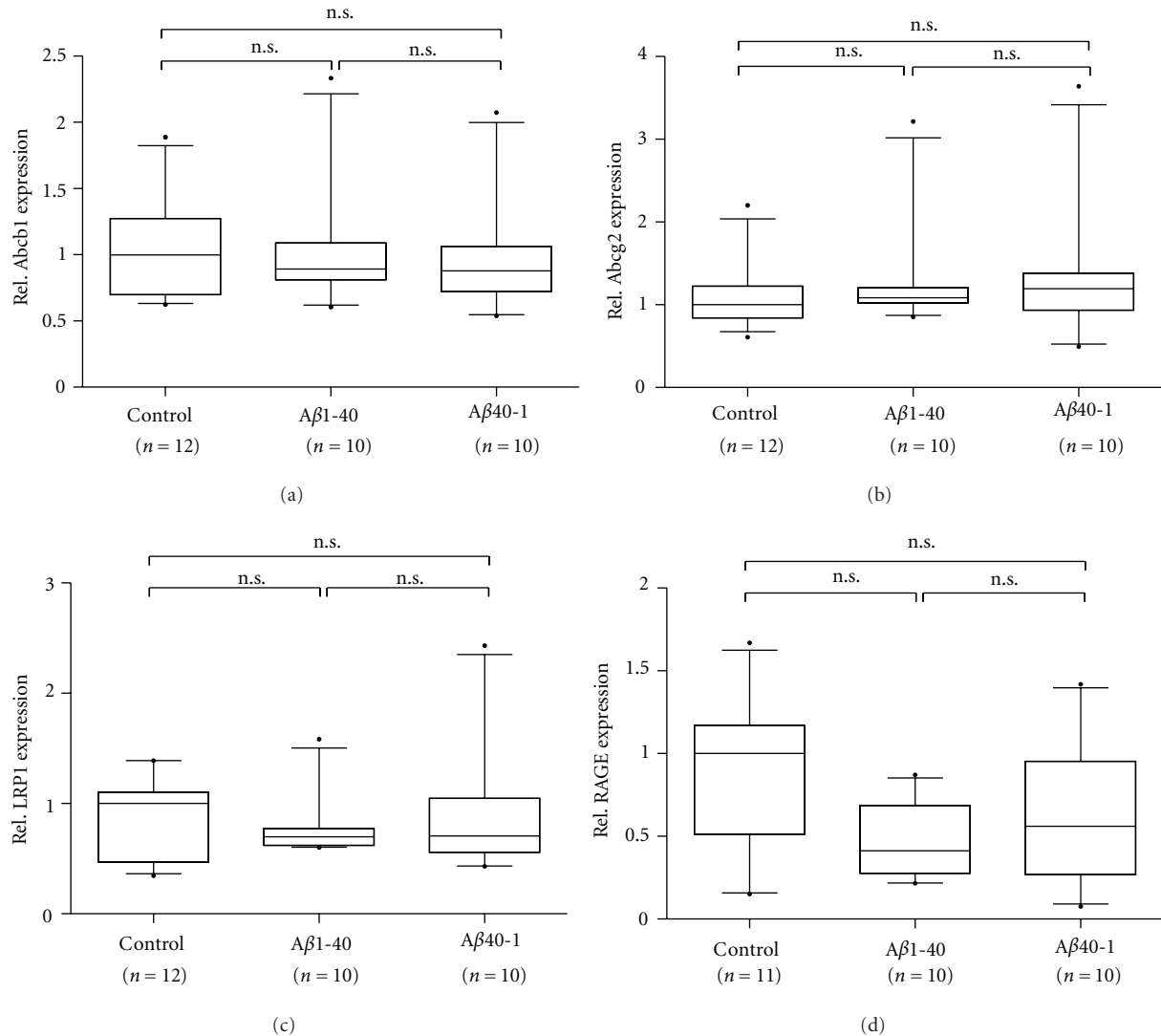
FIGURE 2: mRNA expression of Abcb1a (a), Abcg2 (b), LRP1 (c) and RAGE (d) in murine brain. Mice were treated with β-amyloid 1-40 (Aβ1-40), reverse-sequence β-amyloid 40-1 (Aβ40-1) and vehicle control (control) using ALZET pumps for 24 h (4 μg/h). mRNA expression of each transporter was normalized to 18S rRNA expression, and values are depicted in relation to the median expression in vehicle-perfused mice. Statistical analysis was performed by one-way ANOVA followed by Newmann-Keuls Multiple Comparison test.

primarily found in small arteries, but in the advanced stages of CAA, the capillaries also were affected [11], leading to the question of how Aβ itself affects P-gp expression in the endothelial cells that are a key component of the BBB.

The present study found that peripherally administered Aβ1-42 leads to a decrease of P-gp expression in vivo in mouse brains at the mRNA level. This finding is in concordance with the results of Hartz et al., who reported that P-gp expression and transport activity are compromised in brain capillaries isolated from hAPP transgenic mice that exhibit accumulation of human-type Aβ within the brain [14]. Thus, it can be hypothesized that the compromising effect of Aβ on P-gp expression leads to a further increase of Aβ accumulation, thus initiating a vicious circle that exacerbates the progression of the disease.

LRP1 is localized at the brain-side membrane of capillaries; as a receptor for Aβ, LRP1 mediates the transport of the peptide from the brain into the endothelial cells. LRP1 expression was reported to be reduced during normal aging in animals and in AD patients associated with accumulation of Aβ1-40 and Aβ1-42 within the cerebral vessels [4, 15, 16]. Our results revealed significantly reduced levels of LRP1 mRNA in mice treated with Aβ1-42, confirming the lowering effect of Aβ on LRP1 expression. However, 12-week-old hAPP mice revealed slightly increased LRP1 expression whereas RAGE was unchanged, suggesting the critical limiting step in Aβ brain clearance could be P-gp-mediated Aβ transport across the luminal endothelial membrane into the blood [14].

RAGE is normally expressed at the luminal side of the endothelial cells, and mediates the transport of Aβ1-40 and
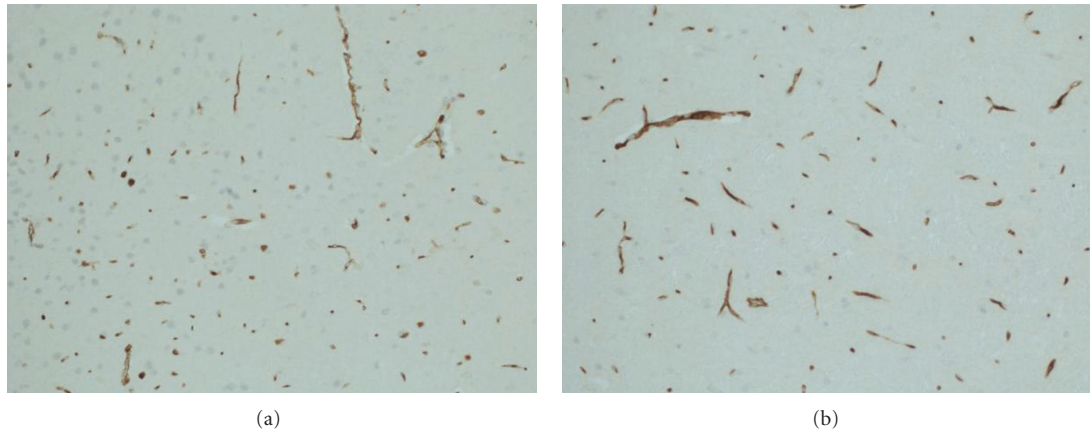
Figure 3: Immunohistochemical expression of P-gp (a) and BCRP (b) in endothelial cells of mice brain capillaries (original magnification ×200).

Aβ1-42 from blood into the endothelium [6]. In AD and AD models, cerebrovascular RAGE was shown to be increased [17]. Our results show that mRNA levels were significantly reduced in mice treated with Aβ1-42 in comparison to the reverse-sequence peptide. Relative to the vehicle control, RAGE mRNA expression showed a trend to be reduced by Aβ1-42, but did not reach statistical significance. However, our findings are in concordance with the observation that the expression of RAGE in Brown-Norway/Fischer rats decreases in early stages of Aβ accumulation and increases with advanced age [18]. This suggests that an increase of RAGE expression plays a role in the later stages but not in the initiation of AD.

Our results show that Aβ1-42, but not Aβ1-40, inhibits P-gp, LRP1 and RAGE expression at the BBB. Under physiological conditions, the ratio of Aβ1-42 to Aβ1-40 in the brain is about $1:10$ [19]. Thus, the amount of Aβ1-40 in our pumps might have been too low to achieve sufficient effects on P-gp expression at the capillary endothelial cells. However, since the aggregative ability and the neurotoxicity of Aβ1-42 are much greater than those of Aβ1-40, Aβ1-42 plays a more important role in the pathogenesis of AD [19, 20]. In this regard, Aβ1-42 was reported to induce free radical-related oxidative stress and neurodegeneration in AD brains [21]. Interestingly, in contrast to Aβ1-40, the concentration of free Aβ1-42 in cerebrospinal fluid is lower in patients with AD, suggesting that Aβ1-42 might be preferentially incorporated into growing plaques [20].

In our study, the decrease of P-gp expression was observed at the mRNA level but not at the protein level using immunohistochemistry. This might be due to the short period during which Aβ is present within the blood, leading to acute effects of Aβ on transcription processes that were not reflected in changes of protein expression within this timeframe. Possibly, a longer treatment period is necessary to address this question.

Like P-gp, BCRP is localized at the luminal site of vascular endothelial cells and contributes to the integrity of the blood brain barrier (BBB), thus protecting the brain parenchyma from the damaging effects of xenobiotics [22]. P-gp and BCRP share extensive similarities regarding their substrate specificity, and the list of substrates of BCRP has been steadily expanding since its discovery [23]. Using a human brain endothelial cell line (hCMEC/D3) Aβ1-40 has been shown to be a substrate of BCRP [24]. Xiong et al. reported that BCRP mediates the transport of Aβ in BCRP-overexpressing cell lines, and that Aβ accumulates in the brains of BCRP-knockout mice. On the other hand, BCRP expression was increased in brain tissue of AD cases [25]. However, in the present study we found BCRP expression to be unchanged by circulating Aβ. This is in agreement with the findings on young hAPP mice, where no evidence was found for BCRP-mediated Aβ transport in brain capillaries [14]. Thus, it can be hypothesised that BCRP, like RAGE, might not be involved in the early stages of AD, but rather may play a role in more advanced stages of the disease.

5. Conclusions

These findings lend further support for the involvement of the ABC transport protein P-gp in the transport of Aβ across the BBB. Specifically, circulating Aβ itself lowers P-gp expression at the BBB, leading to a circulus vitiosus that triggers further Aβ accumulation. The activity of P-gp is well known to be modulated pharmacologically by a range of commonly used drugs [26–28]. Thus, restoring P-gp function could be a novel therapeutic strategy to protect the brain from Aβ accumulation, and thus could be a tool to prevent and/or slow the progression of CNS disorders such as Alzheimer's disease.

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