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PII: S0022-3549(21)00414-7
DOI: https://doi.org/10.1016/j.xphs.2021.08.013
Reference: XPHS 2486

To appear in: *Journal of Pharmaceutical Sciences*

Received date: 31 March 2021
Revised date: 11 August 2021
Accepted date: 11 August 2021

Please cite this article as: Elena Puris , Seppo Auriola , Paula Korhonen , Sanna Loppi , Katja M. Kanninen , Tarja Malm , Jari Koistinaho , Mikko Gynther , Systemic inflammation induced changes in protein expression of ABC transporters and ionotropic glutamate receptor subunit 1 in the cerebral cortex of familial Alzheimer's disease mouse model, *Journal of Pharmaceutical Sciences* (2021), doi: https://doi.org/10.1016/j.xphs.2021.08.013

Systemic inflammation induced changes in protein expression of ABC transporters and ionotropic glutamate receptor subunit 1 in the cerebral cortex of familial Alzheimer`s disease mouse model

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Keywords: Alzheimer's disease; animal models; lipopolysaccharide; proteomics; APdE9 mouse

Abbreviations

Aβ - amyloid-β, ABC - ATP-binding cassette transporters, AD - Alzheimer's disease; BBB - blood-brain barrier, APP - β-amyloid precursor protein, ATP – adenosine triphosphate, BCRP - breast cancer resistance protein, CNS - central nervous system, COX-2 - cyclooxygenase 2; CytC - cytochrome c; GluN1 - ionotropic glutamate receptor subunit 1, GLUT1 - glucose transporter 1, IL-1β - interleukin 1β, IL-6 - interleukin 6, LAT1 - L-type amino acid transporter 1, LC-MS/MS - liquid chromatography tandem mass spectrometry, LPS - lipopolysaccharide, MGLL - monoacylglycerol lipase, MRM - multiple reaction monitoring, MRP - multidrug resistance-associated protein, NFTs - neurofibrillary tangles, NMDA - *N*-Methyl-D-aspartate, PGE_2 - prostaglandin E_2, prostaglandin D_2 - prostaglandin D_2, P-gp - P-glycoprotein, PSEN1 - presenilin 1, sAD - sporadic Alzheimer's disease, SLC - solute carrier, TNFα - tumor necrosis factor alpha, QTAP - quantitative targeted absolute proteomics WT – wild type

Abstract

Alzheimer's disease (AD) is an incurable disease, with complex pathophysiology and a myriad of proteins involved in its development. In this study, we applied quantitative targeted absolute proteomic analysis for investigation of changes in potential AD drug targets, biomarkers, and transporters in cerebral cortices of lipopolysaccharide (LPS)-induced neuroinflammation mouse model, familial AD mice (APdE9) with and without LPS treatment as compared to age-matched wild type (WT) mice. The ABCB1, ABCG2 and GluN1 protein expression ratios between LPS treated APdE9 and WT control mice were 0.58 (95% CI 0.44 – 0.72), 0.65 (95% CI 0.53 – 0.77) and 0.61 (95% CI 0.52 – 0.69), respectively. The protein expression levels of other proteins such as MGLL, COX-2, CytC, ABCC1, ABCC4, SLC2A1 and SLC7A5 did not differ between the study groups. Overall, the study revealed that systemic inflammation can alter ABCB1 and ABCG2 protein expression in brain in AD, which can affect intra-brain drug distribution and play a role in AD development. Moreover, the inflammatory insult caused by peripheral infection in AD may be important factor triggering changes in GluN1 protein expression. However, more studies need to be performed in order to confirm these findings. The quantitative information about the expression of selected proteins provides important knowledge, which may help in the optimal use of the mouse models in AD drug development and better translation of preclinical data to humans.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the primary cause of neurocognitive disorders in the elderly. It is an incurable disease with unmet medical needs, which poses a huge socio-economic burden to the society. [1] The primary causes of sporadic AD (sAD) are still unknown. The major neuropathological hallmarks of AD include extracellular deposits of fibrillar and amorphous amyloid-β peptide (Aβ) aggregates and neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau. [2] The Aβ and NFT accumulation in the brain is associated with neurodegeneration and synaptic loss, which lead to impaired learning and memory functions. Moreover, inflammation observed in the brains of AD patients is considered to be important factor in the pathogenesis of the disease, and is not only associated with neurodegeneration but also facilitates and exacerbates Aβ and NFT pathologies. [3] Neuroinflammation can result from systemic inflammation triggered by peripheral infections or chronic conditions; thereby, systemic inflammation is thought to contribute to the onset of sAD. [4,5]

The majority of the AD drug candidates have failed at the early phase of clinical trials, which raised questions about the use of therapeutic strategies and the relevance of animal models to reproduce cellular and molecular mechanisms underlying AD. The preclinical models mimicking the disease can facilitate drug development by improving the predictability of drug candidate pharmacodynamics and pharmacokinetics in AD patients from preclinical data. One of the commonly used models for AD drug candidate testing is a transgenic APP_{swe}/PS1dE9 (APdE9) mouse overexpressing the Swedish mutation of β-amyloid precursor protein (APP) and presenilin 1 (PSEN1) deleted in exon 9. [7] The APdE9 mouse reproduces several AD-related features, i.e., formation of Aβ plaques, deficits in neuronal activity, mild neuritic abnormalities, impairment of pre- and postsynaptic cholinergic

transmission, and elevated mortality, and therefore can be useful in the development of drug candidates targeting Aβ plagues. [8-11] In our recent study, we demonstrated that inflammation induced by an endotoxin lipopolysaccharide (LPS), a Toll-like receptor 4 (TLR-4) ligand, in APdE9 mice lead to higher number of significant changes in brain metabolic pathways as compared to saline-treated APdE9 mice, and did not affect the brain metabolome and lipidome of wild type (WT) mice. [12] In APdE9 mice treated with LPS, similarly to AD patients, the levels of lysine, myo-inositol, spermine, phosphocreatine, acylcarnitines and diacylglycerols were significantly altered, while these changes were not observed in the saline-treated APdE9 mice or LPS-treated WT mice. These results indicate that infection-induced systemic inflammation may accelerate the changes in biochemical pathways in AD. Moreover, we hypothesised that infection-induced systemic inflammation can lead to alterations not only in metabolome and lipidome, but also affect the protein expression of several potential drug targets and AD-related proteins. Therefore, the investigation of the changes in the protein expression of drug targets and AD-related proteins in these models can provide valuable information about the role of inflammation in AD pathogenesis as well as aid to understand the relevance of the mouse models to mimic the alterations observed in AD patients.

The pathophysiology of AD has a complex and heterogeneous nature with a myriad of proteins involved in the development of the disease, which can be considered as promising drug targets for the treatment of AD. One group of these proteins are the *N*-Methyl-D-aspartate (NMDA) receptors. [16] The NMDA receptors are formed by seven subunits (GluN1, GluN2A-D, and GluN3A-B) into tetrameric receptor complexes. As the GluN1 subunit is an obligatory subunit in all functional NMDA receptors it is widely expressed in the brain. [17] The GluN1 receptor has an important role in the normal function of the central nervous system (CNS) and is critical for synaptic plasticity and survival of neurons. Therefore, it is

not surprising that the GluN1 receptor antagonists are used in order to prevent glutamate-induced excitotoxicity related neuronal loss and in AD. [16,18,19]

Other highly abundant proteins which have been shown to be involved in neuroinflammation and are considered as possible drug targets for the treatment of AD are enzymes monoacylglycerol lipase (MGLL) and cyclooxygenase 2 (COX-2). [20,21] MGLL is a serine hydrolase which is responsible for the hydrolytic conversion of the endocannabinoid 2-arachidonoyl-*sn*-glycerol to arachidonic acid, a precursor for the COX mediated production of neuroinflammatory prostaglandins. [20,21] In the CNS, COX-2 takes part in the synthesis of prostaglandins associated with AD, such as prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2). [22] The binding of PGE_2 to its receptors results in the production of inflammatory mediators such as interleukin 1β, (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα). [23] The altered levels of cytokines were found in the brains of AD patients supporting the notion that cytokines and neuroinflammation takes part in the AD pathogenesis. [24] Another interesting protein, which can serve as a biomarker is cytochrome c (CytC), a small protein primarily found in the inner mitochondrial membrane. [25] During cellular stress CytC is released into the cytosol where it acts as a key initiator of apoptosis and has been connected to the pathogenesis of neurodegenerative diseases such as AD. [25] In addition, CytC may also contribute to the progression of neurodegenerative diseases by inducing the levels of proinflammatory cytokines, including IL-6 and TNFα. [25]

There is growing evidence that ATP-binding cassette transporters (ABC) and solute carrier (SLC) transporters expressed at the blood-brain barrier (BBB) and brain parenchymal cells play a key role in AD pathogenesis. [26,27] For instance, several studies demonstrated that P-glycoprotein (P-gp, ABCB1) and possibly breast cancer resistance protein (BCRP, ABCG2) and multidrug resistance-associated protein 1 (MRP1, ABCC1) are responsible for the clearance of Aβ from the brain and the reduced function of these transporters can be one of

the reasons of Aβ accumulation. [26,27] Another transporter, MRP4 (ABCC4), was shown to be involved in neuroinflammation. [28] In terms of targeted drug delivery across the BBB and into the brain intracellular space, the glucose transporter 1 (GLUT1, SLC2A1) and L-type amino acid transporter 1 (LAT1, SLC7A5) have shown promise in experiments done in mice and rats. [29,30] In AD patients, reduced expression of GLUT1 at the BBB was found, which supports the findings of reduced production of ATP (by 50%) via glucose metabolism in the brain. [31-33] All the mentioned ABC and SLC transporters play a crucial role in passage of drugs and other xenobiotics to the brain and within the brain by restricting their entry across the BBB and cellular barrier of the brain parenchyma in order to protect the brain from harmful molecules. The limited entry of drugs across the brain cellular barriers can result in diminished concentrations at the target site within the brain, thereby contributing to consequent clinical outcome. Currently, the studies focused on quantitative evaluation of changes in transporter expression in AD brains include only two reports of alterations in the transporter expression in the isolated brain microvessels of AD patients, while there is no quantitative information about the transporter expression in the brain parenchymal cells. [34,35] Moreover, none of the studies have focused on investigation of possible alterations in brain parenchymal transporter expression in the animal AD models. Moreover, the effect of systemic inflammation on the protein expression of ABC and SLC transporters in AD has not been investigated.

Quantitative targeted absolute proteomics (QTAP) approach has been extensively used to measure the expression of transporters, receptors, enzymes and other proteins in health and disease. [34,36-39] The present QTAP study was aimed to investigate the effects of systemic inflammation in AD on absolute protein expression of the mentioned above AD-relevant biomarkers, potential drug targets and drug transporters. To achieve the goal, we compared the absolute protein expressions of the selected proteins in the brain cortices of LPS-treated

APdE9 mice, LPS-treated WT mice and saline-treated APdE9 mice to saline-treated WT mice. All these models were recently characterized in terms of alterations in pro-inflammatory cytokines, behavioural changes as well as metabolic and lipidomic perturbations. [12] The changes in protein expressions were compared to those reported in AD patients in order to evaluate the capability of the mouse models to mimic AD. For the absolute quantification of the proteins, we developed a targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) based proteomics method, which allows the accurate, selective quantitative analysis of the proteins of interest.

Materials and methods

Materials

Acetonitrile, ethylenediaminetetraacetic acid (EDTA), dithiothreitol, urea, guanidine hydrochloride, Tris-HCl, formic acid, Tribromoethanol (#75-80-9, Avertin), LPS (#L2880) and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Proteome Extraction Kit was purchased from Merck KGaA, Darmstadt, Germany. The absolute quantified stable isotope-labeled peptides were purchased from JPT Peptide Technologies GmbH (Berlin, Germany). Protease-Max surfactant, tosylphenylalanylchloromethyl ketone-treated trypsin and lysyl endopeptidase (LysC) were purchased from Promega (Madison, WI, USA). BioRad Protein Assay was purchased from EnVision (PerkinElmer, Inc., Waltham, MA, USA).

Study design and animals

The animals and treatments were used from our previous study. [12] All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. All procedures with the animal use were approved by the Finnish National Animal Experimental Board (ESAVI-2015-000744). All

the animals were housed under standard laboratory conditions such as: housing of four to six animals per cage, 12-12 h light-dark day cycle, food (Lactamin R36; Lactamin AB, Södertälje, Sweden), and water consumption *ad libitum*, 60% relative humidity. The inclusion/exclusion criteria were based on the health state of animals. The animals that were healthy and showed no sign of illness as evaluated by the body weight and visual observations were used in the analysis. None of the animals died during the study. Only female C57BL/6J mice were used due to the higher prevalence of AD among women compared to men. The age of 16-17 months was chosen because at this age the mice show learning and memory deficits. [40] The mice referred to one of the four study groups ("Control", "WT plus LPS", "APdE9" and "APdE9 plus LPS", which detailed characteristic and applied treatments are presented in Fig. 1. The "WT plus LPS" group consisted of wild type mice (n = 4) administered i.p. LPS according to the schedule presented in Fig. 1. APdE9 mice were treated with either saline solution i.p. (referred to "APdE9", n = 4) or LPS i.p. ("APdE9 plus LPS", n = 5) according to the same schedule described in Fig. 1. The "Control" group consisted of age-matched wild type mice (n = 5) treated with saline solution i.p. (Fig. 1). The sample size (n = 4-5) selection in the present study was based on previously reported QTAP studies in mice (n = 3). [37,38]

The mice were anaesthetized by tribromoethanol, which use was approved by Finnish National Animal Experimental Board (ESAVI-2015-000744). The mouse brains were perfused using 3-min transcardial perfusion with heparinized saline (2500 IU/L). The brain cortex was separated, snap-frozen in liquid nitrogen, and stored at −80 °C until the LC-MS/MS proteomics analysis.

Quantitative targeted absolute proteomic analysis

The absolute protein expression levels of membrane transporters P-gp (ABCB1), MRP1 (ABCC1), MRP4 (ABCC4), BCRP (ABCG2), GLUT1 (SLC2A1), L-type amino acid transporter 1 (LAT1, SLC7A5), as well as membrane bound enzymes COX-2, MGLL and receptor GluN1 were measured in isolated crude membrane fraction of the mouse brain cortices. The protein expression of CytC and proinflammatory cytokines, IL-1β, IL-6 and TNFα was measured in cytosolic fraction of the brain cortical tissue of mice. In addition, membrane marker Na^+/K^+-ATPase was quantified in both crude membrane and cytosolic fractions of mouse brain cortices.

The cytosolic and crude membrane fractions were isolated from the mouse brain cortical tissue (26.5 ± 1.52 mg tissue per animal) using ProteoExtract Subcellular Proteome Extraction Kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The total protein concentrations in the obtained fractions were measured by the BioRad Protein Assay. The total crude membrane protein levels obtained from the brain cortical tissues were 0.013 ± 0.00092 mg of protein/mg tissue. The samples were prepared according to previously published protocol. [36] For preparation of crude membrane fractions, the aliquots (50 µg of protein) were solubilized in 7 M guanidine hydrochloride, 500 mM Tris–HCl (pH 8.5) and 10 mM EDTA. The cytosolic fraction aliquots (50 µg of protein) were evaporated under nitrogen stream in cold water bath followed by solubilization in 9 µl of 100 mM Tris–HCl (pH 8.5) and 6 M urea. The proteins in both fractions were reduced with dithiothreitol and *S*-carbamoylmethylated with iodoacetamide and precipitated with methanol and chloroform. The precipitates were dissolved by addition of 6 M urea in 0.1 M Tris–HCl (pH 8.5) followed by a 5-fold dilution with 0.1 M Tris–HCl (pH 8.5), which was spiked with a mixture of internal standard peptides (Table 1). This step was followed by the addition of Lys-C and Protease-Max, and incubation at room temperature for 3 h. Finally, tosylphenylalanyl chloromethyl ketone-treated trypsin was added for tryptic digestion of the

samples (enzyme/substrate ratio of 1:100), which were incubated at 37 °C for 16 h. Formic acid in water 20% (v/v) was used to acidify the samples, followed by centrifugation at 14000 × g for 5 min at 4 °C. The supernatants were analyzed using LC-MS/MS methods described below.

The LC-MS/MS analysis was conducted by coupling using an Agilent 1290 Infinity LC (Agilent Technologies, Waldbronn, Germany) system to an Agilent 6495 Triple Quadrupole Mass Spectrometer equipped with an ESI source (Agilent Technologies, Palo Alto, CA, USA). The HPLC method using Advance Bio Peptide Map column (2.1 × 250 mm, 2.7 µm) was applied for separation and elution of peptides as described previously. [41-43] The eluted peptides were simultaneously detected using the positive ion multiple reaction monitoring (MRM) mode. The dwell time was 20 ms per transition. The source temperature was 210 °C with drying gas at a flow rate of 16 L/min. The nebulizer pressure was 45 psi and MS capillary voltage was 3 kV. The quantitation of the target protein was based on one unique peptide (Table 1) selected according to the *in silico* peptide selection criteria [36] and previous reports. [41-45] Data were acquired using the Agilent MassHunter Workstation Acquisition software (Agilent Technologies, Data Acquisition for Triple Quad., version B.03.01) and processed with Skyline software (version 4.1). Three or four MRM transitions for each specific peptide related to high intensity fragment ions were selected for quantification of a stable isotope-labelled peptide and the unlabelled investigated peptide (Table 1). The protein expression, quantified using the peptides for which signal peaks were obtained at only two or one transition(s), was considered as below the lower limit of quantification (LLOQ). The limit of quantification was determined as the lowest concentration of a stable isotope-labelled peptide (spiked into the sample containing 50 µg of total protein followed by processing in similar way as the study samples) for which signal peaks were obtained at three or four transitions (Table 1). The mean of the ratios of light to heavy peaks for each MRM transition

were used for quantification with a dot-product value between the peak areas equal to 1 (exact match). The expression levels of target proteins in crude membrane and cytosolic fractions of mouse brains were expressed as absolute values.

Statistical analysis

The data are presented as mean ± standard deviation (SD) for the absolute protein expression levels and as the ratios of change of the protein expression levels in the study group to controls with the 95% confidence interval (CI) range. Statistical significance between Control group and other investigated groups ("WT plus LPS", "APdE9" and "APdE9 plus LPS") was analysed by One-way ANOVA followed by Dunnett's multiple comparisons test. A *p*-value less than 0.05 was considered statistically significant. Data analysis was done using GraphPad Prism, version 5.03 (GraphPad Software, San Diego, CA).

Results

The protein expression of the drug transporters (P-gp, MRP1, MRP4, BCRP, GLUT1, LAT1), membrane bound enzymes (COX-2, MGLL), receptor GluN1, and cytosolic CytC and proinflammatory cytokines (IL-1β, IL-6 and TNFα) was measured using a QTAP methods in crude membrane or cytosolic fractions, respectively. The absolute levels of expression of the investigated proteins from the cerebral cortical crude membrane and cytosolic fractions for each mouse model and WT control group are provided in Table 2 and presented in Fig. 2 and 3. The results demonstrated that the separation of cytosolic and crude membrane fractions was successful as the concentration of membrane marker protein Na^+/K^+-ATPase was significantly higher in crude membrane fraction of all study groups (range 150.0 – 174.8 fmol/µg protein) compared to that of cytosolic fraction (range 1.38 – 2.97 fmol/µg protein) between these two fractions. In addition, there were no statistically significant differences in the measured levels of Na^+/K^+-ATPase between the investigated groups, and

thus the quantitated differences in the protein levels are not due to artifacts caused by inaccurate sample preparation.

The protein expression of GluN1 was significantly decreased in LPS-treated APdE9 ($p < 0.01$) mice compared to age-matched wild type control mice (Table 2. Fig. 2, 3), while no significant changes were observed in GluN1 expression in LPS-untreated APdE9 mice compared to "control" group. The decreasing trend in the mean protein expression levels of the receptor (not statistically significant, $p = 0.06$) was found in LPS-treated wild type mice vs. "control" group. There were no statistically significant differences in the protein expression of MGLL and COX-2 in all study groups (Table 2. Fig. 2, 3).

The mean P-gp (ABCB1) and BCRP (ABCG2) protein expression levels in cerebral cortices of APdE9 mice treated with LPS were significantly lower ($p = 0.02$ and $p = 0.03$, respectively) compared to those in wild type controls (Table 2, Fig. 2, 3). The changes in the mean protein expression of these transporters in wild type mice treated with LPS and LPS-untreated APdE9 mice were not statistically significant compared to wild type control mice (Table 2, Fig. 2, 3). There were not statistically significant differences in the mean protein expression of MRP1 and MRP4 between the groups (Table 2, Fig. 2, 3). The differences in the mean protein expression of the two investigated SLC transporters, GLUT1 (SLC2A1) and LAT1 (SLC7A5) (Table 2, Fig. 2, 3) between all study groups were not statistically significant.

In cytosolic fraction, the mean protein expression of CytC was not statistically significantly different in all study groups. However, the mean expression of the protein was more than two times higher (not statistically significant, $p = 0.14$) in LPS-treated APdE9 mice compared to "control" group (Table 2. Fig. 2, 3). None of the analysed cytokines (IL-1β, IL-6 and TNFα) were detected in cytosolic fraction of the brain cortices of the studied groups.

Discussion

There is a large unmet medical need for the patients with AD, as most of the drug candidates have failed at the early phase of clinical trials (ca. 99%) due to limitations of current knowledge about the pathogenesis of the disease, lack of relevant animal models and restricted drug delivery to the brain. In the present study, we applied state-of-the-art QTAP method for characterizing the mouse models widely used in AD research, i.e. APdE9 mice and LPS-induced inflammation mouse model, in terms of the changes in expression of several AD-relevant proteins in the cerebral cortex. In addition, we investigated the inflammation-driven changes in the expression of selected proteins in APdE9 mice after systemic LPS administration. [14] As the total volume of the brain endothelial cells in the brain is ca. 0.1%, [46] we assumed that the changes in protein expression quantified in the brain cerebral cortices represent the changes in the brain parenchymal cells and not at the BBB.

The present study showed, that GluN1 expression significantly decreased in the APdE9 mice treated with LPS compared to the wild type control mice, while no changes were observed in APdE9 mice without LPS treatment or LPS treated wild-type mice (Fig. 2, 3). Previously Snyder et al. (2005) reported that the Aβ-mediated increase in glutamate concentration leads to endocytosis and decreased plasma membrane but not total expression of GluN1 in neurons derived from APP_{swe} mice. [47] In addition, Chen et al. (2012) found no changes in GluN1 expression in the hippocampus of 6-month-old 5×FAD transgenic mice compared to wild type. [48] In AD, the reduced expression of the GluN1 mRNA and protein has been reported in the brain and the GluN1 loss has been associated with the severity of AD pathology. [16,19] Although there are data available from APdE9 mice providing evidence of altered glutamatergic signalling compared to wild type mice, this is the first report on quantitative changes in GluN1 expression in cerebral cortex of this model. All together these results

suggest that decreased GluN1 receptor expression can be resulted from systemic inflammation in AD and future studies should focus on investigation of this hypothesis.

Interestingly, COX-2 expression did not show differences between the wild type control group and the investigated animal models. This may at first be surprising finding as COX-2 expression was induced by Aβ, LPS and proinflammatory cytokines in cultured astrocytes and glial cells. [49] However, Guan et al. (2019) showed with immunohistochemistry that COX-2 expression is upregulated in three- and six-month-old APdE9 mice compared to controls, but similar to the results of the present study, only small and not statistically significant differences were found in the 18-month-old APdE9 mice compared to control mice. [50] In addition, several studies performed using human post-mortem brain samples demonstrated that COX-2 expression is initially increased and then reduced in the late stages of AD compared to non-demented controls. [51] Here, we report for the first time the absolute protein expression of COX-2 in the cerebral cortex of the investigated models and wild type mice.

MGLL controls the abundance of arachidonic acid and prostaglandins in the brain by hydrolysing the anti-inflammatory and neuroprotective endocannabinoid 2-arachidonoyl-*sn*-glycerol. [52] Mulder et al. 2011 reported that elevated expression of MGLL correlates with the progression of AD. [53] Therefore, MGLL has been considered as a potential drug target for the alleviation of neuroinflammation and treatment of AD. [48] Surprisingly, we found relatively high MGLL protein expression in the mouse cerebral cortical tissue (5.0 – 6.1 fmol/µg protein), which was not significantly changed in the investigated models compared to the wild type control mice (Fig. 2, 3). Due to the lack of AD mimicking changes in MGLL protein expression in all the investigated groups, other murine AD models should be considered when investigating potential AD drugs affecting the endocannabinoid system in the brain.

CytC release to cytosol is a key step in the Aβ deposit triggered cascade leading to apoptotic neuronal cell death in AD. [54] Therefore, cytosolic CytC can be used as a biomarker for apoptosis in AD. We quantitated relatively high cytosolic CytC (Table 2) protein expression in all investigated cortices. Interestingly, the mean protein expression of cytosolic CytC was more than two times higher in LPS-treated APdE9 mice compared to wild type control group. However, the difference was not statistically significant ($p = 0.14$), therefore these results do not provide conclusive evidence of induced apoptosis in the cerebral cortical tissues of the investigated mouse models.

In the present study, we investigated changes in the brain cortical protein expression of ABC and SLC transporters, which previously demonstrated potential involvement into AD pathogenesis. The QTAP analysis revealed that mean P-gp (ABCB1) protein expression was decreased in all three models with significant reduction observed only in the APdE9 mice treated with LPS compared to the wild type control mice (Fig. 2, 3). P-gp (ABCB1) is the main efflux pump expressed not only at the BBB, but in the brain parenchymal cells such as pericytes, astrocytes and neurons, thus limiting the entry of drugs to the brain and into the brain parenchymal cells. [26] The transporter plays a key role in AD pathogenesis due to its contribution to Aβ clearance across the BBB. [55] The expression levels of P-gp (ABCB1) in the brain parenchyma of AD patients are unknown as the P-gp expression in the currently available studies has been quantitated only at the BBB. In a study utilizing immunofluorescence analysis, P-gp (ABCB1) protein expression levels in the brain vasculature of hippocampus were significantly lower in AD subjects (51 – 83 years old) compared to age-matched non-demented ones [56]. In contrast, the QTAP study by Al-Majdoub et al. (2019) showed that absolute protein expression of P-gp (ABCB1) in isolated brain capillaries from five AD patients (70 – 85 years old) were similar to those from twelve non-demented subjects (53 – 90 years old). [34] Similarly, no significant changes in the P-gp

(ABCB1) expression at the BBB of AD patients and age matched controls were found in the QTAP study by Storelli et al (2020). [35] The results of our study demonstrate that changes in P-gp (ABCB1) expression in the brain cortical tissue may be induced by systemic inflammation in AD, which may affect intra-brain distribution of drugs. Therefore, future studies should focus on investigation of the transporter expression and function in the brain parenchyma in AD and the potential role of systemic inflammation in regulation of transporter expression in AD brain.

Similar to P-gp (ABCB1), the expression of BCRP (ABCG2), another important efflux transporter expressed at the BBB and brain parenchymal cells, [26] was reduced significantly only in APdE9 mice treated with LPS compared to wild type control animals demonstrating that the combination of AD phenotype and inflammatory insult results in diminished protein expression of the transporter in the brain cortical tissue. Several studies showed that BCRP (ABCG2) plays a role in AD via elimination of Aβ from the brain extracellular fluid to the circulating blood across the BBB [57,58] and preventing generation of reactive-oxygen species (ROS) with consequent activation of the ROS responsive transcription factor NF-κB, which results in decreased expression of inflammatory genes. [26] Similar to P-gp, the BCRP (ABCG2) expression in the brain parenchyma of AD patients has not been reported and only BBB expression levels are available. In the study of Xiong et al. (2009) using immunohistochemical and western blot analysis, significantly higher BCRP (ABCG2) protein expression was reported in the brain microvessels of AD patients with cerebral amyloid angiopathy compared to age-matched nondemented control brains; and in the 3×Tg mouse model of AD compared to wild type control. [58] In contrast, Al-Majdoub et al. (2019) and Storelli et al (2020) showed that absolute protein expression of BCRP (ABCG2) was the same in isolated brain capillaries of AD patients and non-demented subjects. [34,35] Our current

findings demonstrate that there is a need for further investigation of BCRP (ABCG2) expression in the brain parenchyma in AD.

In the cerebral cortex of the investigated mouse models, the protein expression of MRP1 (ABCC1) and MRP4 (ABCC4) transporters was not altered significantly. Both MRP1 (ABCC1) and MRP4 (ABCC4) are expressed at the BBB and brain parenchymal cells. [26] There is an evidence that MRP1 (ABCC1) is responsible for Aβ clearance from the brain across the BBB, [59,60] while the role of MRP4 (ABCC4) in AD is unknown. MRP4 (ABCC4) was shown to be involved in elimination of PGD_2 across the BBB, the most abundant prostaglandin in the brain, which is involved in the progression and exacerbation of neuroinflammation. [28] Furthermore, MRP4 (ABCC4) can take part in the transport of PGE_2 and PGD_2 into the brain extracellular space where they bind to their corresponding receptors and mediate their proinflammatory effects. [61] Neither MRP1 (ABCC1) nor MRP4 (ABCC4) brain parenchymal expression in AD patients has been reported. The absolute expression of MRP1 (ABCC1) was not altered in isolated brain microvessels of AD patients compared to non-demented controls. [34] In addition, the mRNA and protein expression of MRP4 (ABCC4) measured with western blot and qRT-PCR analyses, respectively, was reported to be unaffected at the BBB of the hippocampal sections of AD patients compared to non-demented subjects, although significantly higher MRP4 (ABCC4) levels were detected in the hippocampal tissue homogenates from AD patients. [56]

In the present study, the GLUT1 (SLC2A1) expression did not differ between the mouse models and the wild type control mice. The lack of significant reduction of GLUT1 (SLC2A1) expression was surprising, as previously we have shown that the GLUT1 (SLC2A1) protein expression was significantly decreased in LPS treated astrocyte primary cultures derived from wild type and APdE9 mice. [43] However, caution should be used when comparing results from brain samples of adult mice and cultured cells from neonatal mice.

The protein level of the endothelial GLUT1 (SLC2A1) expression is reduced in the AD brains while the expression in the brain parenchyma has been shown to be unaltered. [62] Therefore, the lack of changes in the GLUT1 (SLC2A1) expression in the brain cortical tissue in all the investigated models resembles the findings from the brain parenchyma of AD patients.

No differences in expression of another SLC transporter, LAT1 (SLC7A5), expressed at the BBB and brain parenchymal cells (astrocytes, neurons and microglia) were found in this study. In our previous study we demonstrated that LAT1 (SLC7A5) protein expression and function were the same in wild type astrocytes with and without LPS treatment as well as in transgenic APP/PS1 astrocytes treated with LPS. [43] In the same study, we showed that LAT1 (SLC7A5) function was not altered at the BBB of LPS-induced inflammation mouse model and transgenic APP/PS1 model. [43] Similar findings were observed in isolated microvessels from AD brains, absolute protein expression was similar to that in non-demented subjects. [34] Together, these data demonstrate that the brain protein expression of LAT1, a transporter playing important role in amino acid homeostasis and delivery of several CNS drugs, is unlikely to be altered in AD.

The limitations of the study need to be acknowledged. First, although the aim of the study was to give first insight about the altered expression levels of the selected proteins, we must highlight that future studies with the larger sample size of animals than used in this study (n = 4-5) need to be conducted. Second, in the QTAP analysis performed in the present study, one standard peptide unique for each target protein was used to quantitate the investigated proteins, while the use of two or three standard peptides would increase the reliability of the acquired results. None of the investigated cytokines, IL-1β, IL-6 and TNFα, were detected from the brain cytosolic samples. Therefore, the developed method was not sensitive enough for the quantification of the cytokines in the brain samples. However, 0.22 nmol/l of TNFα

was quantitated from growth media of LPS and interferon gamma (INF-γ) treated immortalised murine microglial BV-2 cells (unpublished data), providing evidence of usability of the method for *in vitro* sample analysis. It has to be noted that even IL-1β could have been quantitated it would provide the combined concentration of the IL-1β precursor and mature forms. This limitation is particular for IL-1β as there are no mature form selective peptide sequences fulfilling the properties of a good surrogate peptide for LC-MS/MS quantification. The cytokine analysis with the QTAP method would likely be more useful in cell cultures where the quantification can be made from the growth media where the activated mature IL-1β is secreted.

Conclusions

In conclusion, we developed QTAP method and quantified the expression of several proteins involved in inflammation and/or AD pathophysiology in cerebral cortices of three mouse models commonly used for AD drug candidate testing, such as APdE9 mice, LPS-induced inflammation wild-type and APdE9 mice. Importantly, we report for the first time quantitative absolute protein expression of the potential drug targets GluN1, COX-2 and MGLL as well as cytosolic CytC, a biomarker of apoptosis, in the mouse cerebral cortical tissue. The results indicated that inflammatory insult may take part in the induction of the changes in GluN1 protein expression in AD brain. In addition, we suggest that the systemic inflammation triggered by infection can alter P-gp (ABCB1) and BCRP (ABCG2) expression in the brain in AD, which may potentially affect intra-brain distribution of CNS drugs and Aβ processing within the brain. These results highlight the importance of investigation of the transporter expression not only at the BBB, but in the brain parenchymal cells for evaluation of possible changes in drug neuropharmacokinetics in AD, in particular in the patients with chronic inflammatory conditions. Moreover, the comparison of the results to published

human data, demonstrated that treatment with LPS in the transgenic APdE9 mice can be important factor providing additional benefits for this model to mimic advanced AD. Ultimately, the study provides important quantitative information about the changes in expression of several important proteins in AD and may help in the translation of preclinical data to human in terms of target validation and drug delivery during the AD drug development.

References

1. Alzheimer's A 2016. 2016 Alzheimer's disease facts and figures. Alzheimers Dement 12(4):459-509.
2. DeTure MA, Dickson DW 2019. The neuropathological diagnosis of Alzheimer's disease. Mol Neurodegener 14(1):32.
3. Kinney JW, Bemiller SM, Murtishaw AS, Leisgang AM, Salazar AM, Lamb BT 2018. Inflammation as a central mechanism in Alzheimer's disease. Alzheimers Dement (N Y) 4:575-590.
4. Calsolaro V, Edison P 2016. Neuroinflammation in Alzheimer's disease: Current evidence and future directions. Alzheimers Dement 12(6):719-732.
5. Walker KA, Ficek BN, Westbrook R 2019. Understanding the Role of Systemic Inflammation in Alzheimer's Disease. ACS Chem Neurosci 10(8):3340-3342.
6. Windisch M 2014. We can treat Alzheimer's disease successfully in mice but not in men: failure in translation? A perspective. Neurodegener Dis 13(2-3):147-150.
7. Jankowsky JL, Fadale DJ, Anderson J, Xu GM, Gonzales V, Jenkins NA, Copeland NG, Lee MK, Younkin LH, Wagner SL, Younkin SG, Borchelt DR 2004. Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. Hum Mol Genet 13(2):159-170.
8. Garcia-Alloza M, Borrelli LA, Hyman BT, Bacskai BJ 2010. Antioxidants have a rapid and long-lasting effect on neuritic abnormalities in APP:PS1 mice. Neurobiol Aging 31(12):2058-2068.
9. Machova E, Rudajev V, Smyckova H, Koivisto H, Tanila H, Dolezal V 2010. Functional cholinergic damage develops with amyloid accumulation in young adult APPswe/PS1dE9 transgenic mice. Neurobiol Dis 38(1):27-35.
10. Meyer-Luehmann M, Mielke M, Spires-Jones TL, Stoothoff W, Jones P, Bacskai BJ, Hyman BT 2009. A reporter of local dendritic translocation shows plaque- related loss of neural system function in APP-transgenic mice. J Neurosci 29(40):12636-12640.
11. Xiong H, Callaghan D, Wodzinska J, Xu J, Premyslova M, Liu QY, Connelly J, Zhang W 2011. Biochemical and behavioral characterization of the double transgenic mouse model (APPswe/PS1dE9) of Alzheimer's disease. Neurosci Bull 27(4):221-232.
12. Puris E, Kouřil S, Najdekr L, Loppi S, Korhonen P, Kanninen KM, Malm T, Koistinaho J, Friedecký D, Gynther M in press. Metabolomic and lipidomic changes triggered by lipopolysaccharide-induced systemic inflammation in transgenic APdE9 mice Scientific Reports.
13. Krstic D, Knuesel I 2013. Deciphering the mechanism underlying late-onset Alzheimer disease. Nat Rev Neurol 9(1):25-34.
14. Catorce MN, Gevorkian G 2016. LPS-induced Murine Neuroinflammation Model: Main Features and Suitability for Pre-clinical Assessment of Nutraceuticals. Curr Neuropharmacol 14(2):155-164.

15. Sheng JG, Bora SH, Xu G, Borchelt DR, Price DL, Koliatsos VE 2003. Lipopolysaccharide-induced-neuroinflammation increases intracellular accumulation of amyloid precursor protein and amyloid beta peptide in APPswe transgenic mice. Neurobiol Dis 14(1):133-145.
16. Liu J, Chang L, Song Y, Li H, Wu Y 2019. The Role of NMDA Receptors in Alzheimer's Disease. Front Neurosci 13:43.
17. Hansen KB, Yi F, Perszyk RE, Furukawa H, Wollmuth LP, Gibb AJ, Traynelis SF 2018. Structure, function, and allosteric modulation of NMDA receptors. J Gen Physiol 150(8):1081-1105.
18. Wang R, Reddy PH 2017. Role of Glutamate and NMDA Receptors in Alzheimer's Disease. J Alzheimers Dis 57(4):1041-1048.
19. Mohamed NE, Lee JH, Francis PT, Esiri MM, Chen CP, Lai MK 2015. Differential alterations of neocortical GluN receptor subunits in patients with mixed subcortical ischemic vascular dementia and Alzheimer's disease. J Alzheimers Dis 44(2):431-437.
20. Berry AJ, Zubko O, Reeves SJ, Howard RJ 2020. Endocannabinoid system alterations in Alzheimer's disease: A systematic review of human studies. Brain Res 1749:147135.
21. Guan PP, Wang P 2019. Integrated communications between cyclooxygenase-2 and Alzheimer's disease. FASEB J 33(1):13-33.
22. Cudaback E, Jorstad NL, Yang Y, Montine TJ, Keene CD 2014. Therapeutic implications of the prostaglandin pathway in Alzheimer's disease. Biochem Pharmacol 88(4):565-572.
23. Sil S, Ghosh T 2016. Role of cox-2 mediated neuroinflammation on the neurodegeneration and cognitive impairments in colchicine induced rat model of Alzheimer's Disease. J Neuroimmunol 291:115-124.
24. Su F, Bai F, Zhang Z 2016. Inflammatory Cytokines and Alzheimer's Disease: A Review from the Perspective of Genetic Polymorphisms. Neurosci Bull 32(5):469-480.
25. Bajwa E, Pointer CB, Klegeris A 2019. The Role of Mitochondrial Damage-Associated Molecular Patterns in Chronic Neuroinflammation. Mediators Inflamm 2019:4050796.
26. Pereira CD, Martins F, Wiltfang J, da Cruz ESOAB, Rebelo S 2018. ABC Transporters Are Key Players in Alzheimer's Disease. J Alzheimers Dis 61(2):463-485.
27. Jia Y, Wang N, Zhang Y, Xue D, Lou H, Liu X 2020. Alteration in the Function and Expression of SLC and ABC Transporters in the Neurovascular Unit in Alzheimer's Disease and the Clinical Significance. Aging Dis 11(2):390-404.
28. Akanuma SI, Hashimoto K, Yoshida Y, Kubo Y, Hosoya KI 2020. Inflammation-Induced Attenuation of Prostaglandin D2 Elimination across Rat Blood-Brain Barrier: Involvement of the Downregulation of Organic Anion Transporter 3 and Multidrug Resistance-Associated Protein 4. Biol Pharm Bull 43(11):1669-1677.
29. Puris E, Gynther M, Auriola S, Huttunen KM 2020. L-Type amino acid transporter 1 as a target for drug delivery. Pharm Res 37(5):88.
30. Gynther M, Ropponen J, Laine K, Leppanen J, Haapakoski P, Peura L, Jarvinen T, Rautio J 2009. Glucose promoiety enables glucose transporter mediated brain uptake of ketoprofen and indomethacin prodrugs in rats. J Med Chem 52(10):3348-3353.
31. Hoyer S 2002. The brain insulin signal transduction system and sporadic (type II) Alzheimer disease: an update. J Neural Transm (Vienna) 109(3):341-360.
32. Lyros E, Bakogiannis C, Liu Y, Fassbender K 2014. Molecular links between endothelial dysfunction and neurodegeneration in Alzheimer's disease. Curr Alzheimer Res 11(1):18-26.
33. Furst AJ, Lal RA 2011. Amyloid-beta and glucose metabolism in Alzheimer's disease. J Alzheimers Dis 26 Suppl 3:105-116.
34. Al-Majdoub ZM, Al Feteisi H, Achour B, Warwood S, Neuhoff S, Rostami-Hodjegan A, Barber J 2019. Proteomic Quantification of Human Blood-Brain Barrier SLC and ABC Transporters in Healthy Individuals and Dementia Patients. Mol Pharm 16(3):1220-1233.

35. Storelli F, Billington S, Kumar AR, Unadkat JD 2021. Abundance of P-Glycoprotein and Other Drug Transporters at the Human Blood-Brain Barrier in Alzheimer's Disease: A Quantitative Targeted Proteomic Study. Clin Pharmacol Ther 109(3):667-675.
36. Uchida Y, Tachikawa M, Obuchi W, Hoshi Y, Tomioka Y, Ohtsuki S, Terasaki T 2013. A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood-brain barrier in ddY, FVB, and C57BL/6J mice. Fluids Barriers CNS 10(1):21.
37. Pan Y, Omori K, Ali I, Tachikawa M, Terasaki T, Brouwer KLR, Nicolazzo JA 2018. Altered Expression of Small Intestinal Drug Transporters and Hepatic Metabolic Enzymes in a Mouse Model of Familial Alzheimer's Disease. Mol Pharm 15(9):4073-4083.
38. Pan Y, Omori K, Ali I, Tachikawa M, Terasaki T, Brouwer KLR, Nicolazzo JA 2019. Increased Expression of Renal Drug Transporters in a Mouse Model of Familial Alzheimer's Disease. J Pharm Sci 108(7):2484-2489.
39. Ohtsuki S, Uchida Y, Kubo Y, Terasaki T 2011. Quantitative targeted absolute proteomics-based ADME research as a new path to drug discovery and development: methodology, advantages, strategy, and prospects. J Pharm Sci 100(9):3547-3559.
40. Minkeviciene R, Ihalainen J, Malm T, Matilainen O, Keksa-Goldsteine V, Goldsteins G, Iivonen H, Leguit N, Glennon J, Koistinaho J, Banerjee P, Tanila H 2008. Age-related decrease in stimulated glutamate release and vesicular glutamate transporters in APP/PS1 transgenic and wild-type mice. J Neurochem 105(3):584-594.
41. Gynther M, Proietti Silvestri I, Hansen JC, Hansen KB, Malm T, Ishchenko Y, Larsen Y, Han L, Kayser S, Auriola S, Petsalo A, Nielsen B, Pickering DS, Bunch L 2017. Augmentation of Anticancer Drug Efficacy in Murine Hepatocellular Carcinoma Cells by a Peripherally Acting Competitive N-Methyl-d-aspartate (NMDA) Receptor Antagonist. J Med Chem 60(23):9885-9904.
42. Puris E, Gynther M, de Lange ECM, Auriola S, Hammarlund-Udenaes M, Huttunen KM, Loryan I 2019. Mechanistic Study on the Use of the l-Type Amino Acid Transporter 1 for Brain Intracellular Delivery of Ketoprofen via Prodrug: A Novel Approach Supporting the Development of Prodrugs for Intracellular Targets. Mol Pharm 16(7):3261-3274.
43. Gynther M, Puris E, Peltokangas S, Auriola S, Kanninen KM, Koistinaho J, Huttunen KM, Ruponen M, Vellonen KS 2018. Alzheimer's Disease Phenotype or Inflammatory Insult Does Not Alter Function of L-Type Amino Acid Transporter 1 in Mouse Blood-Brain Barrier and Primary Astrocytes. Pharm Res 36(1):17.
44. Kamiie J, Ohtsuki S, Iwase R, Ohmine K, Katsukura Y, Yanai K, Sekine Y, Uchida Y, Ito S, Terasaki T 2008. Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. Pharm Res 25(6):1469-1483.
45. Uchida Y, Ohtsuki S, Katsukura Y, Ikeda C, Suzuki T, Kamiie J, Terasaki T 2011. Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. J Neurochem 117(2):333-345.
46. Pardridge WM 2020. The Isolated Brain Microvessel: A Versatile Experimental Model of the Blood-Brain Barrier. Front Physiol 11:398.
47. Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, Greengard P 2005. Regulation of NMDA receptor trafficking by amyloid-beta. Nat Neurosci 8(8):1051-1058.
48. Chen R, Zhang J, Wu Y, Wang D, Feng G, Tang YP, Teng Z, Chen C 2012. Monoacylglycerol lipase is a therapeutic target for Alzheimer's disease. Cell Rep 2(5):1329-1339.
49. Yermakova AV, O'Banion MK 2001. Downregulation of neuronal cyclooxygenase-2 expression in end stage Alzheimer's disease. Neurobiol Aging 22(6):823-836.
50. Guan PP, Liang YY, Cao LL, Yu X, Wang P 2019. Cyclooxygenase-2 Induced the beta-Amyloid Protein Deposition and Neuronal Apoptosis Via Upregulating the Synthesis of Prostaglandin E2 and 15-Deoxy-Delta(12,14)-prostaglandin J2. Neurotherapeutics 16(4):1255-1268.

51. Minghetti L 2004. Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. J Neuropathol Exp Neurol 63(9):901-910.
52. Nomura DK, Morrison BE, Blankman JL, Long JZ, Kinsey SG, Marcondes MC, Ward AM, Hahn YK, Lichtman AH, Conti B, Cravatt BF 2011. Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation. Science 334(6057):809-813.
53. Mulder J, Zilberter M, Pasquare SJ, Alpar A, Schulte G, Ferreira SG, Kofalvi A, Martin-Moreno AM, Keimpema E, Tanila H, Watanabe M, Mackie K, Hortobagyi T, de Ceballos ML, Harkany T 2011. Molecular reorganization of endocannabinoid signalling in Alzheimer's disease. Brain 134(Pt 4):1041-1060.
54. Kim J, Yang Y, Song SS, Na JH, Oh KJ, Jeong C, Yu YG, Shin YK 2014. Beta-amyloid oligomers activate apoptotic BAK pore for cytochrome c release. Biophys J 107(7):1601-1608.
55. Bernstein HG, Holzl G, Dobrowolny H, Hildebrandt J, Trubner K, Krohn M, Bogerts B, Pahnke J 2014. Vascular and extravascular distribution of the ATP-binding cassette transporters ABCB1 and ABCC1 in aged human brain and pituitary. Mech Ageing Dev 141-142:12-21.
56. Wijesuriya HC, Bullock JY, Faull RL, Hladky SB, Barrand MA 2010. ABC efflux transporters in brain vasculature of Alzheimer's subjects. Brain Res 1358:228-238.
57. Do TM, Noel-Hudson MS, Ribes S, Besengez C, Smirnova M, Cisternino S, Buyse M, Calon F, Chimini G, Chacun H, Scherrmann JM, Farinotti R, Bourasset F 2012. ABCG2- and ABCG4-mediated efflux of amyloid-beta peptide 1-40 at the mouse blood-brain barrier. J Alzheimers Dis 30(1):155-166.
58. Xiong H, Callaghan D, Jones A, Bai J, Rasquinha I, Smith C, Pei K, Walker D, Lue LF, Stanimirovic D, Zhang W 2009. ABCG2 is upregulated in Alzheimer's brain with cerebral amyloid angiopathy and may act as a gatekeeper at the blood-brain barrier for Abeta(1-40) peptides. J Neurosci 29(17):5463-5475.
59. Krohn M, Lange C, Hofrichter J, Scheffler K, Stenzel J, Steffen J, Schumacher T, Bruning T, Plath AS, Alfen F, Schmidt A, Winter F, Rateitschak K, Wree A, Gsponer J, Walker LC, Pahnke J 2011. Cerebral amyloid-beta proteostasis is regulated by the membrane transport protein ABCC1 in mice. J Clin Invest 121(10):3924-3931.
60. Hofrichter J, Krohn M, Schumacher T, Lange C, Feistel B, Walbroel B, Heinze HJ, Crockett S, Sharbel TF, Pahnke J 2013. Reduced Alzheimer's disease pathology by St. John's Wort treatment is independent of hyperforin and facilitated by ABCC1 and microglia activation in mice. Curr Alzheimer Res 10(10):1057-1069.
61. Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J, Borst P 2003. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci U S A 100(16):9244-9249.
62. Koepsell H 2020. Glucose transporters in brain in health and disease. Pflugers Arch 472(9):1299-1343.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgements

The authors would like to thank Luca Giudice for the assistance in statistical analysis. This study was supported by the Finnish Cultural Foundation (North Savo Regional Fund), the Emil Aaltonen Foundation, the Biocenter Finland and Biocenter Kuopio.

Figure legends

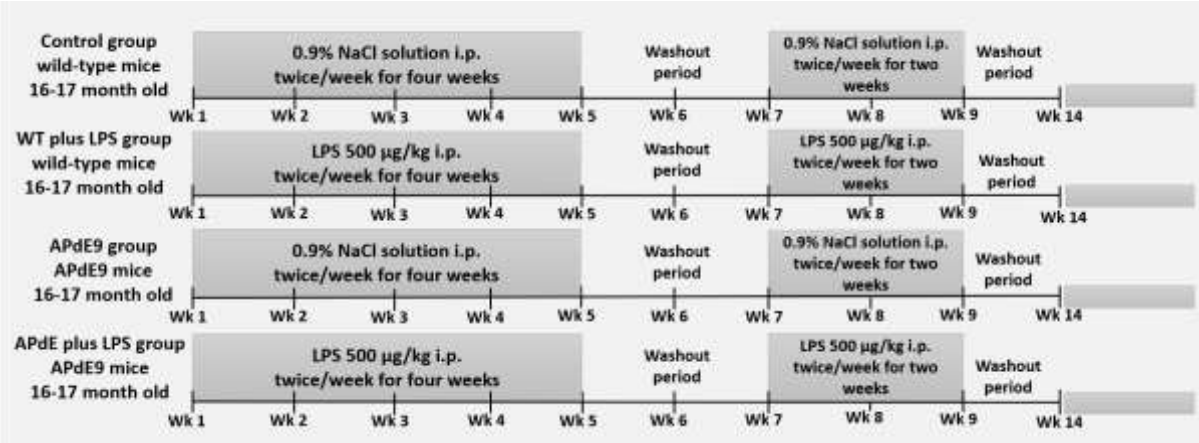

Figure 1. Characteristics of the study groups and treatments.

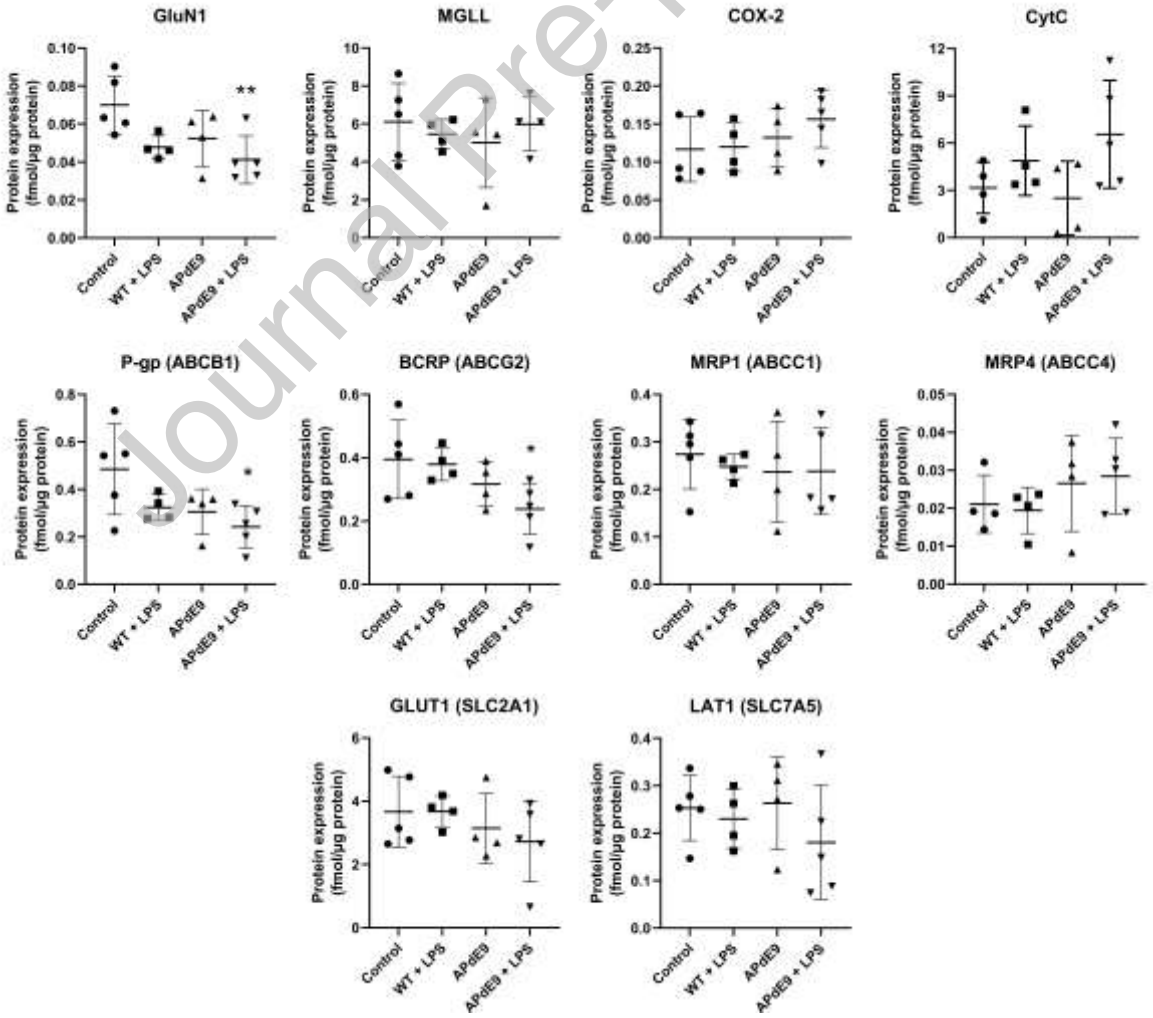

Figure 2. Absolute protein expression of the investigated proteins from the crude membrane and cytosol fractions of the mouse cerebral cortices in "Control" (n = 5), "WT plus LPS" (n = 4), "APdE9" (n = 4), and "APdE9 plus LPS" (n = 5). The data is presented for each study group as a value from individual animal. Statistical significance of changes in protein expression between control group and "WT plus LPS", "APdE9", and "APdE9 plus LPS" were analysed by One-way ANOVA followed by Dunnett's multiple comparisons test. An asterisk denotes statistical significance between groups (*$p < 0.05$; **$p < 0.01$).

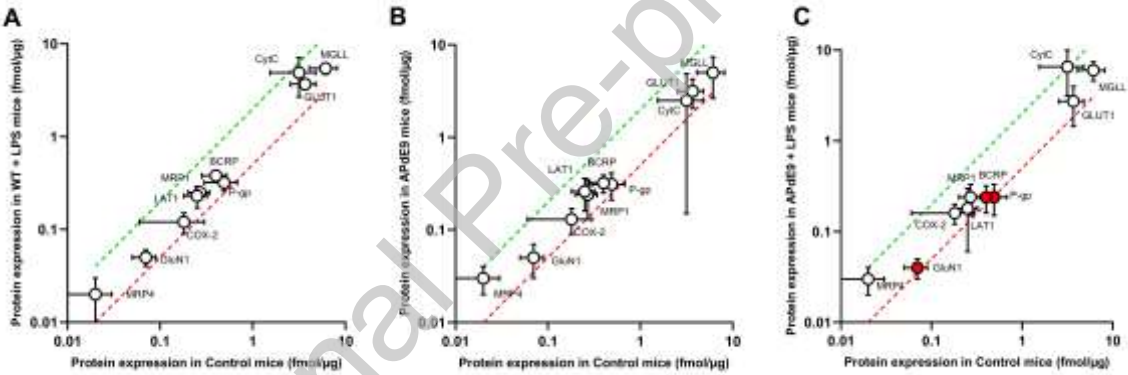

Figure 3. Comparison of protein expression levels of the investigated proteins in the crude membrane fraction of brain cortices of wild type (WT) control (n = 5) versus (A) WT plus LPS (n = 4), (B) APdE9 (n = 4) and (C) APdE9 plus LPS (n=5) mice. The top (green) and bottom (red) dashed lines represent a 2-fold upregulation or downregulation in protein expression, respectively, in the WT plus LPS, APdE9 and APdE9 plus LPS mice compared to control mice. Data are presented as mean ± SD, with statistically significant downregulation in WT plus LPS and APdE9 plus LPS mice denoted by a red data point, with $p < 0.05$ (One-way ANOVA followed by Dunnett's multiple comparisons test).

Table 1. Probe peptide amino acid sequences, MRM transitions for the LC-MS/MS analysis of target proteins and lower limits of quantification (LLOQ).

Protein	St/IS	Unique amino acid sequence	Retention time (min)	MRM transitions (m/z)					LLOQ fmol/ug protein
				Q1	Q3.1	Q3.2	Q3.3	Q3.4	
GluN1	St	IPVLGLTTR	22.4	485.3	856.5	660.4	547.3		0.001
	IS	IPVLGLTT**R***	22.4	490.3	866.5	670.4	557.3		
MGLL	St	LTLPFLLLQGSADR	34.0	722.4	1216.6	746.3	633.2	505.2	0.02
	IS	LTLPFLLLQGSAD**R***	34.0	777.4	1226.6	756.3	643.3	515.2	
COX-2	St	YQVIGGEVYPPTVK	20.8	775.4	989.5	803.4	541.3	629.8	0.05
	IS	YQVIGGEVYPPTV**K***	20.8	779.4	997.5	811.4	549.3	633.8	
P-gp	St	NTTGALTTR	8.7	467.7	719.4	618.3	561.3	490.2	0.1
(ABCB1)	IS	NTTGALTT**R***	8.7	472.7	729.4	628.3	517.3	500.3	
BCRP	St	SSLLDVLAAR	27.5	522.8	757.4	644.3	529.3		0.02
(ABCG2)	IS	SSLLDVLAA**R***	27.5	527.8	767.4	654.3	539.3		
MRP1	St	TPSGNLVNR	9.7	479.2	759.4	672.3	501.3	388.2	0.01
(ABCC1)	IS	TPSGNLVN**R***	9.7	484.2	769.4	682.3	511.3	398.2	
MRP4	St	APVLFFDR	24.6	482.7	796.4	697.3	584.2		0.01
(ABCC4)	IS	APVLFFD**R***	24.6	487.7	806.4	707.3	594.2		
GLUT1	St	TFDEIASGFR	21.0	571.7	894.4	779.4	650.4	537.3	0.1
(SLC2A1)	IS	TFDEIASGF**R***	21.0	576.7	904.4	789.4	660.4	547.3	
LAT1	St	VQDAFAAAK	13.7	460.7	821.4	578.3	507.3		0.02
(SLC7A5)	IS	VQDAFAAA**K***	13.7	464.8	829.4	586.3	515.3		
CytC	St	TGQAAGFSYTDANK	13.1	715.8	1002.4	798.4	332.2		0.05
	IS	TGQAAGFSYTDAN**K***	13.1	719.8	1010.4	806.4	340.2		
IL-1β	St	IPVALGLK	20.9	405.7	697.4	600.4	501.3		0.02
	IS	IPVALGL**K***	20.9	409.7	705.4	608.4	509.3		
IL-6	St	IVLPTPISNALLTDK	29.4	797.9	1269.7	1071.6	861.4		0.1
	IS	IVLPTPISNALLTD**K***	29.4	801.9	1277.7	1079.6	869.4		
TNF-α	St	GDQLSAEVNLPK	18.5	635.8	970.5	857.4	770.4	471.2	0.05
	IS	GDQLSAEVNLP**K***	18.5	639.8	639.8	978.5	778.4	479.3	
Na^+/K^+–	St	AAVPDAVGK	12.2	414.3	685.4	586.3	489.3	204.1	0.15
ATPase	IS	AAVPDAVG**K***	12.2	418.3	693.4	594.3	497.3	212.1	

St – standard, IS – internal standard

Bold letter with* denotes labeled arginine (R) or lysine (K) with a stable isotope ^{13}C and ^{15}N

Table 2. Comparison of cerebral cortical expression (fmol/µg crude membrane protein) of selected proteins in the wild type control group (n = 5) vs. wild type mice treated with LPS i.p. (n = 4), APdE9 mice without (n = 4) and with i.p. LPS administration (n = 5); and APdE9 without LPS vs. APdE9 mice treated with LPS.

Protein	**Control**		**WT plus LPS**		**APdE9**			**APdE9 plus LPS**		
	Mean ± SD fmol/µg crude membrane protein	Mean ± SD fmol/µg crude membrane protein	Ratio of change to control (95% CI)	*p* value	Mean ± SD fmol/µg crude membrane protein	Ratio of change to control (95% CI)	*p* value	Mean ± SD fmol/µg crude membrane protein	Ratio of change to control (95% CI)	*p* value
GluN1	0.070 ± 0.015	0.048 ± 0.010	0.71 (0.63-0.78)	0.1	0.052 ± 0.015	0.77 (0.66-0.89)	0.1	0.041 ± 0.013	0.61* (0.52-0.69)	<0.01
MGLL	6.1 ± 2.0	5.5 ± 0.78	0.98 (0.82-1.1)	0.9	5.0 ± 2.4	0.90 (0.67-1.1)	0.7	6.0 ± 1.4	1.0 (0.88-1.2)	1.0
COX-2	0.12 ± 0.043	0.12 ± 0.032	1.1 (0.92-1.3)	1.0	0.13 ± 0.039	1.2 (1.0-1.4)	0.9	0.16 ± 0.037	1.4 (1.2-1.7)	0.3
P-gp (ABCB1)	0.49 ± 0.19	0.32 ± 0.055	0.78 (0.60-0.96)	0.2	0.31 ± 0.10	0.74 (0.54-0.93)	0.1	0.24 ± 0.090	0.58* (0.44-0.72)	0.02
BCRP (ABCG2)	0.40 ± 0.12	0.38 ± 0.052	1.04 (0.89-1.2)	01.0	0.32 ± 0.069	0.86 (0.70-1.0)	0.4	0.24 ± 0.081	0.65* (0.53-0.77)	0.03
MRP1 (ABCC1)	0.27 ± 0.073	0.25 ± 0.027	0.98 (0.81-1.14)	0.9	0.24 ± 0.11	0.93 (0.69-1.1)	0.8	0.24 ± 0.091	0.94 (0.74-1.1)	0.8
MRP4 (ABCC4)	0.021 ± 0.0077	0.019 ± 0.0061	1.0 (0.79-1.2)	1.0	0.027 ± 0.013	1.3 (0.99-1.7)	0.8	0.028 ± 0.010	1.4 (1.1-1.7)	0.5
GLUT1 (SLC2A1)	3.7 ± 1.1	3.7 ± 0.49	1.0 (0.93-1.2)	>1.0	3.2 ± 1.1	0.92 (0.74-1.1)	0.8	2.7 ± 1.3	0.76 (0.54-0.99)	0.4
LAT1 (SLC7A5)	0.25 ± 0.069	0.23 ± 0.062	0.98 (0.79-1.1)	1.0	0.26 ± 0.098	1.0 (0.87-1.3)	1.0	0.18 ± 0.12	0.76 (0.54-0.99)	0.5
CytC	3.2 ± 1.6	4.9 ± 2.2	2.1 (1.1-3.0)	0.7	2.5 ± 2.4	1.07 (0.39-1.76)	1.0	6.6 ± 3.4	2.8 (1.7-3.9)	0.1

* indicates a significant difference in expression between WT control group vs. APdE9 plus LPS group ($p < 0.05$).
Data are mean ± standard deviation (SD) for absolute protein expression levels, and the ratio of change of the protein expression levels in the study group to controls with the 95% confidence interval (CI) range.