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RESEARCH ARTICLE

St. John's Wort Reduces Beta-Amyloid Accumulation in a Double Transgenic Alzheimer's Disease Mouse Model—Role of P-Glycoprotein

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Keywords
Alzheimer's disease, beta-amyloid, blood–brain barrier, P-glycoprotein, St. John's Wort.

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Abstract

The adenosine triphosphate-binding cassette transport protein P-glycoprotein (ABCB1) is involved in the export of beta-amyloid from the brain into the blood, and there is evidence that age-associated deficits in cerebral P-glycoprotein content may be involved in Alzheimer's disease pathogenesis. P-glycoprotein function and expression can be pharmacologically induced by a variety of compounds including extracts of *Hypericum perforatum* (St. John's Wort). To clarify the effect of St. John's Wort on the accumulation of beta-amyloid and P-glycoprotein expression in the brain, St. John's Wort extract (final hyperforin concentration 5%) was fed to 30-day-old male C57BL/6J-*APP/PS1*$^{+/-}$ mice over a period of 60 or 120 days, respectively. Age-matched male C57BL/6J-*APP/PS1*$^{+/-}$ mice receiving a St. John's Wort-free diet served as controls. Mice receiving St. John's Wort extract showed (i) significant reductions of parenchymal beta-amyloid 1–40 and 1–42 accumulation; and (ii) moderate, but statistically significant increases in cerebrovascular P-glycoprotein expression. Thus, the induction of cerebrovascular P-glycoprotein may be a novel therapeutic strategy to protect the brain from beta-amyloid accumulation, and thereby impede the progression of Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) is a common, progressive neurodegenerative disorder that begins as mild short-term memory disturbances and ends in total loss of cognition and executive functions. Histopathologically, AD is characterized by the so-called "core" pathologies such as accumulation of amyloid-β-peptides (Aβ) and abnormal tau-protein (9, 17). It has been proposed that the underlying cause of Aβ accrual in the sporadic, age-related form of AD is a reduced clearance of Aβ from the brain via the blood–brain barrier (BBB) (4, 27, 30).

The adenosine triphosphate-binding cassette (ABC) transporter P-glycoprotein (ABCB1, P-gp), which is encoded by the *multidrug resistance-1 (MDR1/ABCB1)* gene, constitutes a critical component of the BBB (7, 19) and has been shown to mediate the transport of Aβ peptides (15, 16). *In vivo* and *in vitro* experiments as well as autopsy studies support the view that P-gp is essential for the elimination of brain-derived Aβ, thus playing a critical role in the pathogenesis of AD (3, 10, 28, 29).

To date, despite considerable scientific effort, there are no substantially effective treatments or preventives for AD. Given the fact that the activity of P-gp can be modulated pharmacologically by a range of different compounds, including frequently used agents such as St. John's Wort, the antibiotic rifampin, or flavonoids (11, 20, 24), the upregulation of cerebral P-gp could be a new therapeutic strategy for reducing the buildup of Aβ in the brain.

St. John's Wort (SJW; *Hypericum perforatum*), with its biologically active compound hyperforin, is a widely used herbal drug in patients with different types of depression (12). Recently, beneficial effects in animal models of AD have also been reported (2, 5). Because hyperforin has been shown to increase the expression of P-gp (6, 8, 26), the aim of the present study was to investigate the effect of SJW treatment on cerebral Aβ deposition in a double transgenic AD mouse model. We hypothesized that, by enhancing the activity of P-gp, SJW would promote the efflux of Aβ from the brain. Here we report that the administration of SJW significantly reduced soluble and aggregated Aβ levels in the brains of transgenic mice, indicating that upregulation of P-gp may be a valuable approach to diminishing cerebral Aβ accumulation.

MATERIALS AND METHODS

Chemicals

SJW extract containing 5% hyperforin was kindly donated by Dr. Wilmar Schwabe Pharmaceuticals (Karlsruhe, Germany). All other chemicals were purchased from commercial sources and were of analytical grade.

Animals

C57BL/6J-*APP/PS*$^{+/-}$ mice (23, 25) were purchased from KOESLER (Rothenburg, Germany). Mice were housed in a 12-h light/12-h dark cycle at 23°C with free access to water.

Based on established protocols (18). mice were treated with SJW extract (1250 mg/kg body weight daily) containing 5% hyperforin. To avoid excessive stress on the animals because of the long feeding duration, SJW was administered with the food (SNIFF, Soest, Germany). Given a minimal food intake of 3 g/day per mouse, 4.166 mg SWJ were mixed with 1 g of food (Altromin, Lage, Germany). Mice were administered 3 g of the prepared food each day, and intake was monitored to ensure the required dosage.

Treatment started at the age of 30 days. Because, in this mouse model, sufficient plaques can be seen in the brain by the age of 90 days, the animals received SJW for either 60 or 120 days, respectively. C57BL/6J-*APP/PS1*$^{+/-}$ mice receiving food without added SJW served as controls.

Tissue preparation

At the age of 90 or 150 days, respectively, mice were sacrificed by cervical dislocation. The brains were removed and one hemisphere was snap-frozen in liquid nitrogen and stored at −80°C for biochemical analysis. The other hemisphere was fixed in buffered 4% formalin and then paraffin-embedded for immunohistochemistry.

Western blot analysis

For Western blot analysis, a crude membrane fraction was isolated by centrifugation of the brain homogenate at 100.000 × g at 4°C for 30 min. Thereafter, the pellet was resuspended in Tris/HCl buffer (5 mM, pH 7.4, supplemented with protease inhibitors) and protein concentration was determined by the bicinchoninic acid method. Next, 50 µg of membrane protein were loaded on a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) gel. Immunoblotting was performed using a tank blotting system (Bio-Rad, Hercules, CA, USA). Primary antibodies were diluted in Tris-buffered saline containing 0.05% Tween 20 to the following final concentrations: P-gp (C219, Calbiochem, Darmstadt, Germany) 1:1000 and desmin (Sigma-Aldrich, Munich, Germany) 1:500. The secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Bio-Rad) was used at a final concentration of 1:500. Signals were detected using an enhanced chemiluminescence detection system (ECL Plus Western Blotting Substrate, Pierce, Rockford, IL USA) and the ChemiDoc XRS system (Bio-Rad) and then analysed by the QuantityOne 4.6.7 software using the mean intensity values of each band (Bio-Rad). In the case of P-gp detection the C219 antibody showed some cross reactivity, e.g. with a protein which migrates in the same position as myosin (about 200 kDa) resulting in additional unspecific bands. Here, only the P–gp-specific signals (at about 180 kDa) were used for quantification.

Immunohistochemical analyses

For immunohistochemical quantification of Aβ accumulation and P-gp expression 1-µm thick sagittal sections were mounted onto slides and dried for 1 h at 58°C. Brain sections of all mice were processed simultaneously to obtain comparable staining intensity. Automated immunohistochemical staining was performed using the BOND-MAX Autostainer (Leica Microsystems GmbH, Wetzlar, Germany).

For Aβ staining, serial brain sections were pretreated for 3 minutes with concentrated formic acid, and incubated with antibodies directed to Aβ 1–40 and Aβ 1–42, respectively (AB5074P, dilution 1:50, and AB5078P, dilution 1:50, both antibodies by Merck Millipore, Schwalbach, Germany). After antigen retrieval by incubating the slides for 30 minutes at 100°C, nonspecific binding sites were blocked with serum-free Protein Block (DakoCytomation, Glostrup, Denmark). Cerebrovascular P-gp was detected using the mouse monoclonal antibody C219 (dilution 1:25, pH 9.0, Enzo Life Sciences, Lörrach, Germany). Immunoreactivities were detected by appropriate secondary antibodies and the BOND-MAX Bond Polymer Refine Detection Kit (Leica Microsystems GmbH).

To quantitate cortical Aβ accumulation and P-gp expression, hematoxylin/3,3′-diaminobenzidine (DAB)-stained slides were digitized using the Mirax Desk slide scanner (Carl Zeiss MicroImaging GmbH, Göttingen, Germany; Plan-Apochromat 20×/0.8, pixel resolution: 0.36 µm) and saved as 24-bit RGB-tagged image format (tif) files. Image analysis was performed by Image J (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) on high-power image sections that were treated as ImageJ stacks. Hematoxylin/DAB color spaces were extracted by the color deconvolution plugin with predefined H DAB vectors. The individual background was subtracted with a rolling circle diameter of 64. To identify capillaries, the stack of DAB-stained images was blurred via Gaussian Blur (sigma = 2). Aβ plaques and P–gp-stained capillaries were identified as regions of interest (ROI) by thresholding the DAB channel. For each region of interest, DAB staining intensity and mean area were measured.

Quantification of soluble Aβ species

For quantification of soluble Aβ 1–40 and Aβ 1–42 peptides, human amyloid beta 1–40 or 1–42 brain enzyme-linked immunosorbent assay (ELISA) kits (Merck Millipore) were used. Sample preparation was performed according to the manusfacturer's protocol. In brief, brain tissue was disrupted under frozen conditions using a ball mill. Tissue powder was scaled and 10× volume of lysis buffer was added. The resulting lysate was rotated for 2 h at 4°C and cleared by centrifugation at 13 000 rpm for 10 min at 4°C. Quantification of soluble Aβ 1–40 and Aβ 1–42 peptides by ELISA was done according to the manufacturer's instructions. The results were normalized to the amount of brain tissue used for the analysis.

Statistics

Mathematical calculations, statistical tests as well as data presentation were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). For calculation of the ELISA data, standard curve data were fitted using a non-linear regression model and Aβ concentrations within the samples were interpolated. When data are presented using box and whisker blots, the whiskers represent the 10–90 percentile. If not otherwise stated, the Mann–Whitney test was used for statistical analysis.

The experiments were approved by local authorities: Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischereiwesen (LALLF; LALLF M-V/TSD/7221.3-1.1-008/08).

RESULTS

Quantification of cerebral Aβ

The number of Aβ 1–40- and Aβ 1–42-positive plaques was assessed using immunohistochemistry to demonstrate the amount of fibrillar and (in the case of diffuse plaques) protofibrillar Aβ. We found a significant reduction in plaque load after SJW administration for 60 or 120 days, respectively, for both Aβ 1–40 ($P = 0.011$ and $P = 0.003$) and Aβ 1–42 ($P = 0.026$ and $P = 0.001$) in comparison to controls (Figure 1). Furthermore, the size of Aβ 1–40 positive plaques was significantly smaller in SJW treated mice ($P = 0.005$ and $P = 0.013$). For Aβ 1–42 positive plaques there were no differences in plaque size after 60 days of SJW treatment but a highly significant decrease in plaque size after 120 days ($P = 0.006$) (Figure 2). Figure 3 shows representative examples of treated and control mice. In accordance with the characterization of the mouse model by Radde *et al* (23), no vascular Aβ accumulation was observed.

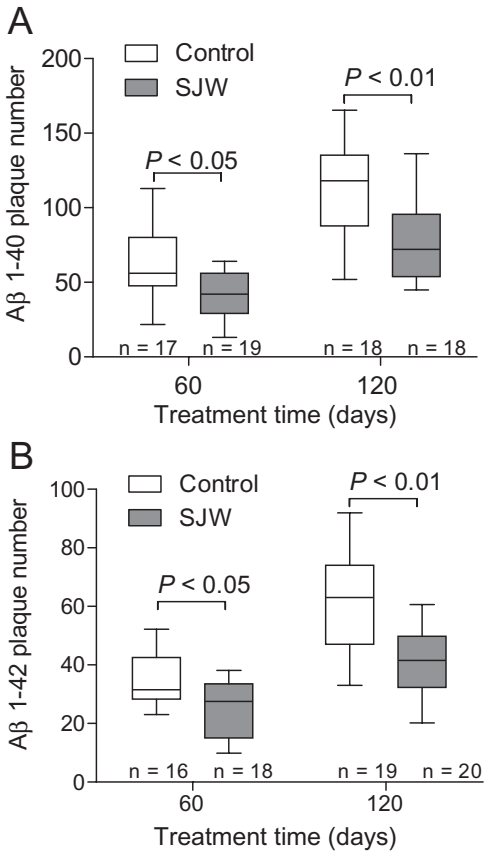

Figure 1. *Aβ 1–40 (**A**) and Aβ 1–42 (**B**) Plaque number is significantly reduced after treatment with St. John's Wort (SJW).*

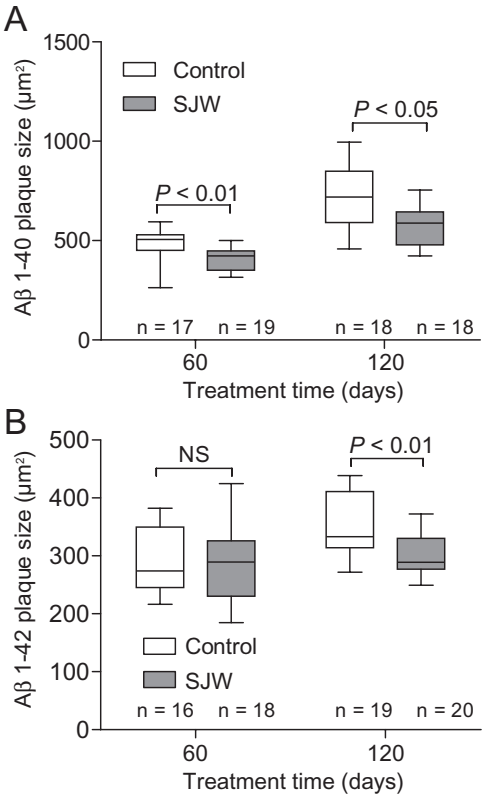

Figure 2. *St. John's Wort (SJW) treatment leads to a marked decrease in the size of Aβ 1–40 immunopositive plaques (**A**).* The size of Aβ 1–42 (**B**) positive plaques was unchanged after 60 days of SJW administration, but significantly reduced after a feeding duration of 120 days.

The observed reduction of the plaque load is in line with the finding of reduced levels of cerebral soluble Aβ in SJW-treated animals measured by ELISA. Specifically, after 60 d of treatment no significant changes in Aβ 1–40 levels (10% reduction compared with control, $P = 0.615$) were detected, while Aβ 1–42 levels were significantly decreased (30% reduction, $P = 0.020$). After 120 days of SJW treatment, the effect was more pronounced for both peptides. For Aβ 1–40, we found a 47% reduction compared with controls; however, this was not statistically significant ($P = 0.206$). Soluble Aβ 1–42 showed a significant reduction of 53% ($P = 0.006$; Figure 4). There was no effect of treatment on food consumption and the mice did not show obvious differences in behavior during the treatment period.

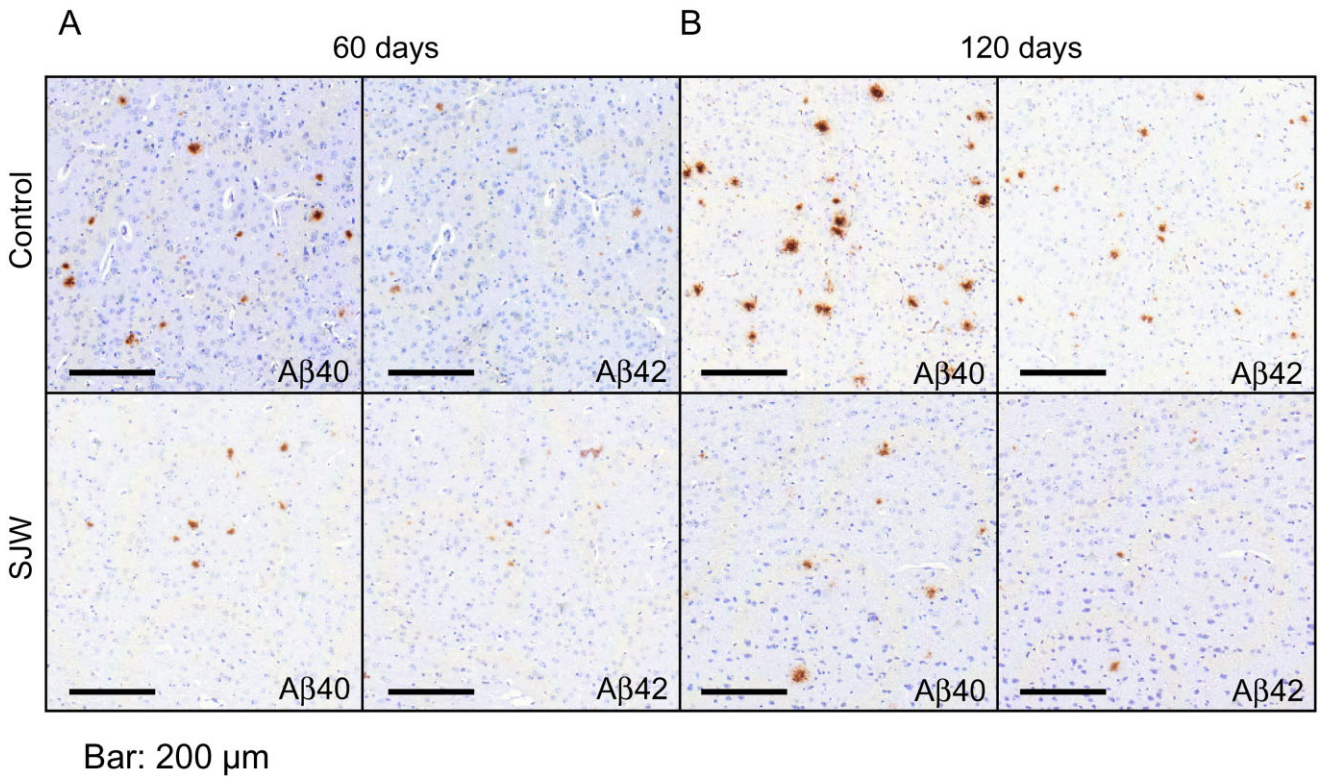

Figure 3. *Representative images of brain sections immunostained for Aβ 1–40 and Aβ 1–42 in St. John's Wort (SJW)-treated C57BL/6J-APP/PS1$^{+/-}$ mice compared with controls (**A**: 60 days, **B**: 120 days).* Original magnification: 100×, bar: 200 µm.

Quantification of cerebrovascular P-gp expression

According to our hypothesis, we also studied the inducing effect of SJW on P-gp expression immunohistochemically. We found that cerebrovascular endothelial P-gp expression was significantly augmented after 60 days ($P = 0.0006$) and 120 days ($P = 0.0001$), respectively, in SJW-treated animals compared with controls (Figure 5A). In addition, when P-gp expression was quantified by Western blot analysis after 120 days, P-gp expression in the SJW-treated animals was moderately, but significantly enhanced ($P = 0.017$; Figure 5B). Figure 6 shows representative examples of P-gp immunostaining of treated and control mice.

DISCUSSION

Our results show that SJW treatment leads to a significant reduction of soluble Aβ 1–42 (representing mainly small oligomers and monomers) as well as Aβ 1–40 and Aβ 1–42 positive plaque number and size (representing mainly fibrillar and protofibrillar Aβ), while vascular P-gp expression was increased in the brains of C57BL/6J-*APP/PS1*$^{+/-}$ mice. These findings support the view that the induction of P-gp might augment the clearance of Aβ from the brain.

SJW, with its active component hyperforin, is a widely used herbal antidepressant that is thought to have potential beneficial effects in AD. Injection of Aβ fibrils together with hyperforin into the hippocampus of male Sprague-Dawley rats resulted in a decrease of Aβ deposit formation, neuronal death and glial reaction as compared with controls (5). Cognitive testing of hyperforin-injected animals revealed significant protection from the spatial memory loss induced by Aβ deposits. Furthermore, hyperforin protected against Aβ neurotoxicity *in vitro* using hippocampal neuronal cultures (5). Interestingly, incubation of amyloid fibrils with increasing concentrations of hyperforin led to almost complete disassembly of Aβ fibrils (5). Other *in vitro* experiments with a microglial cell line showed that pre-treatment with SJW extract reduced the toxic influence of Aβ on microglial activation, proliferation and cell death in a dose-dependent manner, suggesting that SJW might attenuate Aβ-mediated toxicity in AD (14).

In addition to its several neurobiological effects (including neurotransmitter re-uptake inhibition, the ability to increase intracellular sodium and calcium levels, canonical transient receptor potential 6 activation, or N-methyl-D-aspartic acid receptor antagonism), SJW has antioxidant and anti-inflammatory properties (1, 13) that might be beneficial in slowing the pathogenesis of AD. Furthermore, treatment of APP/PS1 double transgenic mice with the hyperforin derivate IDN5706 alleviated cognitive decline and affected the turnover of Aβ plaques (2).

In contrast to our results, Matheny *et al* did not find a significant induction of P-gp in the brains of male FVB mice after SJW treatment for 5 days (18). Functionally, SJW and particularly hyperforin has been shown to be a potent inducer of P-gp in a

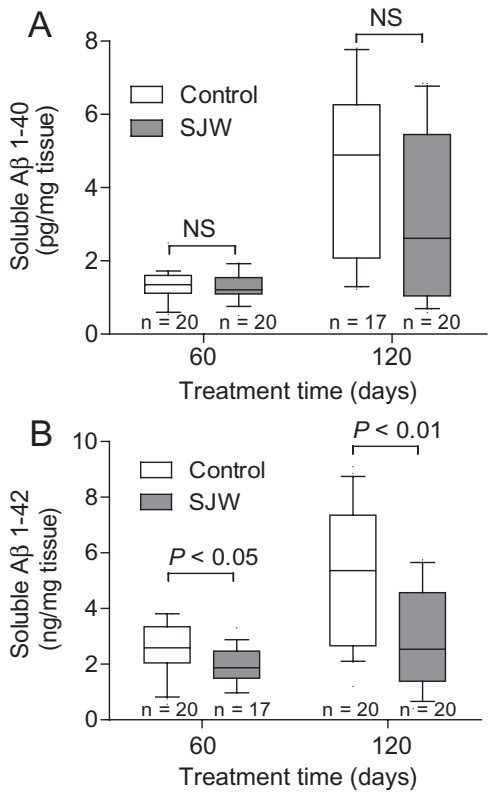

Figure 4. *Quantification of soluble Aβ using enzyme-linked immunosorbent assay.* The levels of Aβ 1–40 did not differ significantly between the St. John's Wort (SJW)-treated group and controls (**A**). Aβ 1–42 was significantly reduced in the SJW-treated mice compared with controls (**B**).

dose-dependent manner (11, 22, 32). While some reports indicate that acute exposure to SJW results in a moderate inhibition of P-gp function (21, 31), long-term administration leads to a significant induction of P-gp (31). SJW is known to induce P-gp through pregnane X-receptor (PXR) activation in several human tissues (6, 26). However, PXR exhibits considerable differences among mammalian species with respect to ligand binding leading to differing degrees of induction (33). In our mouse model, we found a moderate, but significant induction of cerebrovascular P-gp at the protein level. This increase in protein might result from the long period of SJW administration in our experiments, during that P-gp expression and/or function might be upregulated by indirect mechanisms rather than direct PXR interaction. Because SJW is a potent inducer of human PXR (33), one could speculate that the inductive effect of SJW on P-gp would be stronger in humans than in mice.

P-gp is a major constituent of the BBB and is able to transport Aβ 1–40 as well as Aβ 1–42 actively (15, 16). Several lines of evidence suggest that P-gp is involved in the clearance of Aβ from the brain, indicating that the age-associated decline in P-gp function could play a critical part in the pathogenesis of idiopathic AD (3, 10, 28, 29). The clearance of Aβ from the brain into the blood at the BBB comprises a two-step mechanism involving the low-densitiy lipoprotein receptor as a putative transcytosis mediator at the abluminal site of endothelial cells (27), and P-gp and possibly other ABC transporters at the luminal side. However, the mechanism of transportation of Aβ from the brain is still not fully understood; thus, further studies are required to determine possible transporter/receptor interactions.

In conclusion, our study shows that long-term administration of SJW results in a potent decrease of Aβ accumulation in the brains of double transgenic mice, and a moderate but significant upregulation of cerebrovascular P-gp expression. Besides many neuroprotective effects of SJW, the induction of P-gp by SJW thus might enhance Aβ clearance from the brain and thereby reduce the risk of developing AD. To test this hypothesis further studies are warranted to investigate the effects of long term SJW treatment on animal behaviour and memory. Our finding that SJW reduces Aβ load in a mouse model indicates that the induction of P-gp is a promising therapeutic approach to the treatment and/or prevention of neurodegenerative diseases such as AD.

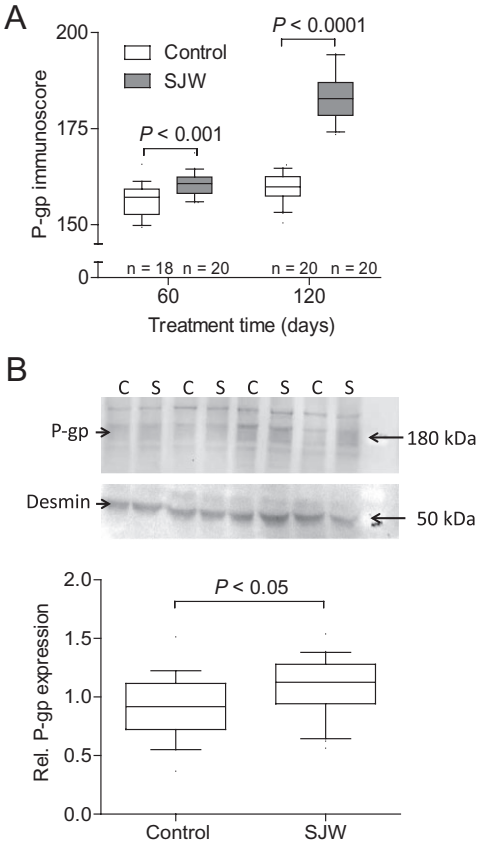

Figure 5. *Immunohistochemical quantification of vascular P-gp expression shows a significant induction of P-gp in the St. John's Wort (SJW)-treated group that was pronounced after administration of 120 days* (***A***). Western blot analysis of P-gp indicates a moderate, but significant upregulation of P-gp in the brain of SJW-treated (120 days) animals. The P-gp-specific band at about 180 kDa (marked with the arrow) was analyzed densitometrically and normalized to desmin (n = 20). An image of a representative Western blot of four controls (C) and four SJW (S)-treated animals is shown in the insert. (**B**).

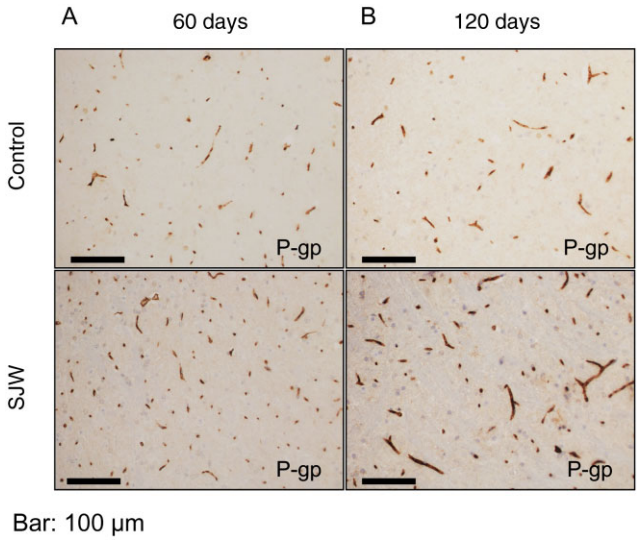

Figure 6. *Representative images of brain sections immunostained for P-gp in St. John's Wort (SJW)-treated mice compared with controls (**A**: 60 days, **B**: 120 days).* Original magnification: 200×, bar: 100 µm.

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AUTHOR CONTRIBUTIONS

AB and SV designed the study. AB, MG, GJ, AF, BS, ME, MK, MHG and SV performed the experiments and analyzed the data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

CONFLICT OF INTEREST

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