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# Reduced Alzheimer's disease pathology by St. John's wort treatment is independent of hyperforin and facilitated by ABCC1 and microglia activation in mice

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## Abstract

Soluble β-amyloid peptides (Aβ) and small Aβ oligomers represent the most toxic peptide moieties recognized in brains affected by Alzheimer's disease (AD). Here we provide the first evidence that specific St. John's wort (SJW) extracts both attenuate Aβ-induced histopathology and alleviate memory impairments in APP-transgenic mice. Importantly, these effects are attained independently of hyperforin. Specifically, two extracts characterized by low hyperforin content (i) significantly decrease intracerebral Aβ42 levels, (ii) decrease the number and size of amyloid plaques, (iii) rescue neocortical neurons, (iv) restore cognition to normal levels, and (iv) activate microglia in vitro and in vivo. Mechanistically, we reveal that the reduction of soluble Aβ42 species is the consequence of a highly increased export activity in the blood-brain barrier ABCC1 transporter, which was found to play a fundamental role in Aβ excretion into the bloodstream. These data (i) support the significant beneficial potential of SJW extracts on AD proteopathy, and (ii) demonstrate for the first time that hyperforin concentration does not necessarily correlate with their therapeutic effects. Hence, by activating ABC transporters, specific extracts of SJW may be used to treat AD and other diseases involving peptide accumulation and cognition impairment. We propose that the anti-depressant and anti-dementia effects of these hyperforin-reduced phytoextracts could be combined for treatment of the elderly, with a concomitant reduction in deleterious hyperforin-related side effects.

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**DISCLOSURE STATEMENT**

Björn Feistel and Bernd Walbroel are employees of Finzelberg GmbH & Co. KG, Koblenzer Str. 48, 56626 Andernach, Germany. All experiments were approved by the local animal ethics committee and administration and carried out according to the state law of the government of Mecklenburg-Western Pomerania.

**Keywords**

Alzheimer's disease; ABC transporters; ABCC1; blood-brain barrier; β-amyloid clearance; hyperforin; Hypericum perforatum; St. John's wort

## INTRODUCTION

Alzheimer´s disease (AD) is the most frequent cause of dementing cerebral cortex pathology with protein deposition. Currently, aging is regarded as the major risk factor for this neurodegenerative disorder, and the number of affected people is increasing rapidly (1). Proteolytic processing of the β-amyloid (Aβ) precursor protein (APP) by β- and γ-secretases results in the production of Aβ, which is prone to aggregate, thereby giving rise to a number of toxic Aβ species (2–4). Aggregated Aβ forms amyloid fibrils, which make up the core of senile plaques, a major pathologic hallmark of AD (5–7). Monomeric and oligomeric species of Aβ are toxic to neurons independent of plaque formation; thus, the absolute number of senile plaques is weakly correlated with the degree of clinical dementia in patients. The level of intracerebral Aβ42, however, is strongly correlated with clinical signs of dementia, supporting the hypothesis that the amyloid plaque number alone may not be the critical entity (8–13). Brains of AD patients become atrophic as a result of degenerating neurons and synaptic dysfunction triggered by neurotoxic Aβ, ultimately leading to cognitive impairment (2, 14–16). Currently, the most prominent targets for therapeutic intervention in the Aβ cascade include the inhibition of APP processing and Aβ production (BACE and γ-secretase inhibition), blocking Aβ aggregation and the resulting inflammatory response, and inhibiting Aβ-induced neurotoxicity (17–21). These strategies, however, share at least two major shortcomings: (i) to take action, a sufficient amount of the inhibitory agents must pass the blood-brain barrier (BBB), and (ii) none of these strategies targets the causative event or mechanism that is still controversially discussed, and initially leads to the accumulation of Aβ in the brains of patients with sporadic AD.

A growing number of natural substances are under consideration as potential treatments for AD, including colupulone, an agent of Humulus lupulus (hops), and hyperforin, the main active constituent of Hypericum perforatum (Saint John's wort, SJW) (22–24). SJW and its extracts have a long history of therapeutic use in several disorders. One current application of SJW is in the treatment of mild-to-moderate depression (16, 17, 25–28). The specific role of hyperforin for the treatment of AD, however, is a matter of ongoing discussion. Some studies have found that hyperforin modulates the phagocytic activity of microglia (29, 30). Other investigations indicate that hyperforin effects memory-enhancing properties in rodents (31, 32).

Similarly to colupulone, which indirectly increases ABCB1 expression (22, 24), hyperforin is a potent nuclear receptor ligand for PXR that leads to increased expression of the ATP-binding cassette (ABC) transporter P-glycoprotein, ABCB1, which is an ATP-dependent efflux pump with broad substrate specificity at the human BBB (33, 34). Additionally, Ott et al. showed in vitro that hyperforin inhibited P-glycoprotein transport activity (35), whereas Kuhnke et al. assumed that extracts of SJW with high hyperforin content enhance the export activity of ABCB1 at the blood-brain barrier, and thus, reduce the concentration of intracerebral monomeric Aβ (36). These findings were of particular interest due to indications that insufficient Aβ clearance from the brain may be the main reason for its progressive accumulation over years (37–39). Several studies have shown that ABCB1 contributes to Aβ clearance in mouse models and cell culture studies (24, 31, 36, 40, 41). We have recently demonstrated that another ABC transporter (ABCC1) has a strong influence on Aβ pathology in different mouse models, and thus presents a promising novel

therapeutic target (41). Taken together, these data support the hypothesis that impaired clearance contributes to long-term Aβ accumulation in sporadic AD patients.

In this study, we tested five extracts of SJW with variable hyperforin concentrations, to elucidate their effects on AD hallmarks and cognition in mice. We demonstrate that extracts with low to negligible levels of hyperforin significantly improve memory performance and counteract neurodegeneration in vivo in a murine AD model. Oral administration of these extracts is highly effective at decreasing intracerebral Aβ42 levels and reducing Aβ plaques. These extracts do not only activate cerebral macrophages to improve phagocytosis rates by microglia, as confirmed in vitro and in vivo, but they additionally enhance ABCC1 transporter excretion of Aβ. Thus, SJW represents a viable treatment option for AD when special attention is paid to the extraction procedure of the plant material. Our findings strongly suggest that the focus of attention needs to be shifted away from hyperforin as the 'only' active substance of SJW extracts.

## METHODS

### Mice and treatment paradigms

Mice expressing mutated human amyloid precursor protein (APP) and mutated preseniline 1 (PS1) transgenes under control of the Thy1-promoter (APPKM670/671NL, PSL166P) were generously provided by the University of Tübingen (Germany) and are hereafter referred to 'APP-tg' mice (42). Two experimental strategies using five different extracts of Hypericum perforatum were employed. First, starting with an age of 40 days at the onset of amyloidosis, mice were treated daily with a human-adapted dose of 400 mg extract/kg body weight for 60 days to restrict initiation of Aβ pathology and plaque deposition (AD initiation). Second, to delay ongoing Aβ pathology and to stimulate Aβ degrading or clearing processes, a post-onset treatment strategy (post AD onset) was investigated using a dosage of 4 g extract/kg body weight from 50 to 100 days of age.

Dry extract powders re-suspended in water in addition to a 'water only' vehicle control were applied by daily gavage up to the age of 100 days. The weight of treated mice was measured daily for reasons of dosage and health monitoring. All mice were housed in 12-hour day/night cycles at 21–22°C with free access to food and water. All experiments were approved by the local animal ethics committee (LALLF) and carried out according to the state law of the government of Mecklenburg-Western Pomerania.

### St. John's wort extracts characteristics

Extracts of SJW, and quantifications of hyperforin (Hf) and hypericin (Hi), were provided by Finzelberg GmbH & Co KG (Andernach, Germany) in order to ensure quality and purity at pharmacological standards. Briefly, SJW was extracted exhaustively using water, 60% ethanol, and 80% ethanol. The amount of extractable substances was strongly influenced by the selected polarity strength of the solvent (see Table 1). 60% ethanol has been demonstrated in previous studies to be an optimal extraction solvent for both Hi and Hf, while water has been shown to be a very inefficient extraction solvent for these compounds. Ethanol was removed from the extracts via evaporation in vacuo, then aqueous extracts were freeze-dried to yield dry powder extracts with different contents of Hf and Hi. For the purpose of administration, extracts were fully re-dissolved in drinking water. Extracts with a relative content of hyperforin (Hf) less than 1.5% are denoted as 'low', and with more than 2.5% as 'high'.

### Tissue preparation

For tissue preparation, mice were sacrificed by cervical dislocation and transcardially perfused with PBS. The brain was removed from each mouse, and one hemisphere was stored in buffered 4% paraformaldehyde for paraffin-embedding and immunohistochemistry, while the other hemisphere was snap-frozen in liquid nitrogen and stored at −80°C for biochemical analysis. Frozen hemispheres were thawed in 500 µl RNAlater® (Ambion, USA) for one hour on ice, and then were homogenized for 12 s at 6000 rpm with a homogenizer (PreCellys®24, Peqlab, Germany) for immediate biochemical analysis.

### Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as previously described (41). In brief, using an ELISA Kit (TK42HS, The Genetics Company, Switzerland) Aβ42 was quantified in whole brain hemispheres from which the cerebellum and brain stem had been removed at the level of the midbrain. Hemispheres were homogenized using a PreCellys24 (15 seconds, 6,500 rpm). After addition of carbonate buffer (pH 8.0), homogenates were mixed using the PreCellys24® (5 seconds, 5,000 rpm) and centrifuged for 90 minutes (4°C) at 24,000 g to separate insoluble from soluble Aβ species. The resulting supernatant (buffer-soluble fraction) was mixed with 8 M guanidine hydrochloride at a ratio of 1:1.6. To extract aggregated Aβ species, the pellet was dissolved in 8 volumes of 5 M guanidine hydrochloride, shaken at room temperature for 3 hours, and centrifuged at 24,000 g for 20 minutes at 4°C. The resulting supernatant represented the guanidine-soluble fraction. Protein content of the samples was measured in triplicate using a NanoDrop 1000 (Thermo Fisher Scientific, USA). ELISAs were performed according to the manufacturer's instructions using appropriate dilutions.

### Immunohistochemistry

Brains were prepared and labeled as previously described (41, 43–45). Briefly, brain hemispheres were post-fixed for at least 48 hours in 4% paraformaldehyde prior to embedding in paraffin. For immunohistochemistry, 4 µm-thick coronal sections were stained using a BondMaxx™ (Menarini/Leica, Germany) automated immunostaining system. Sections were pretreated with 98% formic acid for 5 min and immunostained for Aβ using the anti-human Aβ clone 6F3D (1:200, Dako) and the Bond™ Polymer Refine Detection kit (Menarini/Leica, Germany). For double-stained slides, microglias were immunostained on the same sections using anti-Iba1 in a second step (1:1,000, Wako) and the Bond™ Polymer AP-Red Detection kit (Menarini/Leica, Germany). Neurons were stained with anti-NeuN antibody (1:1,000, Millipore). Whole tissue sections were digitized at 230 nm resolution using the MiraxMidi Slide Scanner (ZeissMicroImaging GmbH, Germany) and finally semi-automatically analyzed using the AxioVision software package (ZeissMicroImaging GmbH, Germany) (41, 44, 45).

### Morris water maze

Experiments were carried out according to the protocol of the Johns Hopkins Neurogenetics and Behavior Center with minor adaptations. At an age of 95–100 days, the animals' ability to learn and remember the spatial location of a hidden platform in a circular pool of water (diameter 130 cm, maintained at 19–21°C) was tested. The water was made opaque and the platform was hidden 1 cm below the water surface. Several landmarks were placed on the walls surrounding the pool. The water maze was conceptually divided into 4 quadrants, and movements of each animal (traces) were recorded by a camera during the experiment. Mice were trained and tested in 2 sessions per day over a period of 4 consecutive days. The 2 sessions were separated by 4 hours and consisted of 4 trials per session. The platform was centrally located in the southwest quadrant. At the beginning of every training day, trials

without the platform were given for free swimming, lasting 30 sec, followed by 4 platform trials. The start position of the first trial of each session was south, while the start position was changed counter-clockwise in the subsequent trials to east, north and west. The animals were allowed to move freely until they reached the platform or until 1 min had elapsed. In case the animals did not reach the platform, they were guided to the platform to stay there for 15 sec before returning them to the home cage until the next trial.

### Aβ-phagocytosis assay

In vitro microglial activity was analyzed using an Aβ-phagocytosis assay according to Floden et al. (46), whereby a pure microglia cell culture line was prepared as described by Moussaud et al. (47). Briefly, neonatal animals (day 3) were killed by decapitation and the brains were removed under sterile conditions. Cortices were mechanically dissociated using a scalpel, followed by chemical dissociation in trypsin/EDTA (Merck KGaA, Germany) for 15 min at 37°C. Cells were placed on ice in 25 ml DMEM for 15 min to allow larger tissue chunks to sink. The supernatant was subsequently transferred into fresh medium. This step was repeated 3 times. After centrifugation (315 g, 15 min), the pellet was re-suspended in 1 ml medium and cells seeded in 75 $cm^2$ flasks ($2 \times 10^6$). Cells were incubated at 37°C and 5% CO2 for 21 days. Afterwards, cultures were shaken at RT for 45 min at 300 rpm to detach the microglial cells from glial feeder layer, resulting in a pure microglial cell population. Medium was transferred into a 50 ml tube, centrifuged (315 g, 15 min) and the cells seeded in 100 µl medium into wells of a 96 well plate (30,000 per well). After 1 hour of incubation at 37°C, cells were incubated with water-dissolved SJW extracts (400 µg/ml) for 18 hours at 37°C, and later incubated with 500 nM of FITC-labeled Aβ42 (rPeptide, USA) for an additional 6 hours. Cells were then washed twice with PBS and trypsinized, centrifuged at 340 g for 15 min and resuspended in PBS. Intracellular fluorescence was measured with the PARADIGM spectrophotometer (Beckman Coulter, USA) at appropriate wave length (485 nm excitation / 535 nm emission). The experiments were performed in five replicates.

### ABC-transporter activity assay

In vitro ABCB1 and ABCC1 activities, respectively, were measured using the SB MDR1 and MRP1 PREDEASYTM ATPase Kits (Solvo Biotechnology, Hungary) according to the manufacturer's instructions. SJW extracts were diluted in DMSO (Sigma-Aldrich, Germany) to a final DMSO concentration of 0.05 µg/ml and activity was measured in 96-well plates using a Paradigm spectrophotometer (Beckman Coulter, Germany) at 610 nm.

### Western Blot

For Western blotting, whole cerebrum tissue homogenates were fractionated into three protein fractions as described by Lesné et al. (48). For ABCC1 (MRP1) detection, samples from the choroid plexus were homogenized in RIPA buffer. Protein concentrations were determined using a BCA™ protein assay kit (Pierce, part of Thermo Fisher Scientific, Rockford, USA). After SDS-PAGE using 10 µg total protein per lane, proteins were blotted onto a PVDF membrane. Blots were probed for ABCB1 (MDR1, clone D11, 1:500, Santa Cruz, USA), ABCC1 (MRP1, 1:200; Alexis Biochemicals, USA), ADAM10 (1:1,000, Calbiochem, Germany), BACE1 (1:1,000, Abcam, USA), or β-actin (1:20,000, Sigma-Aldrich, Germany) dissolved in Odyssey® blocking buffer (LI-COR, USA). As detection antibodies, IRDye® secondary anti-mouse, anti-rat and anti-rabbit antibodies of LI-COR (all diluted 1:10,000) were used. The Odyssey® two-channel IR direct detection system (LI-COR, USA) was used for visualization and for relative quantification of the target proteins.

### Statistical analysis

Results are presented as means + standard error of the mean (SEM). The observations were made without any prior knowledge of the experimental group. The corresponding numbers of laboratory animals can be found in table 1. A non-parametric test (Mann-Whitney U-Test) was utilized for significance calculations. Values with a probability level of less than 0.05 ($p<0.05$) were regarded as significant. All statistical calculations were performed using the statistics program Graph Pad Prism 5.

## RESULTS

To determine the treatment effects of SJW extracts containing varying levels of hyperforin on pathological hallmarks in APP-transgenic mice, we investigated two experimental time points: (i) at the beginning of treatment with initiation of plaque deposition, and (ii) after the onset of the β-amyloid pathology (see Materials and Methods section for details).

### Extracts of SJW with low levels of hyperforin strongly decrease intracerebral Aβ42

To investigate the extent to which the extracts are able to lower Aβ42 burden, we analyzed brain homogenates from buffer-soluble (small Aβ aggregates and monomers) and guanidine-soluble Aβ (higher molecular weight aggregates) fractions with ELISA.

Both 80%-ethanol extracts of SJW, which varied in their content of hyperforin (SJW80low, SJW80high), significantly reduced buffer-soluble Aβ42 by −39% (SJW80low) and −38% (SJW80high) in the AD initiation treatment (Fig. 1A). The post-onset strategy led to a reduction of buffer-soluble Aβ42 by −53% (SJW80low) and −49% (SJW80high), respectively (Fig. 1B). Additionally, guanidine-soluble Aβ42 was significantly reduced by −50% (SJW80low) and −31% (SJW80high), whereas the extract characterized by the lowest hyperforin content (SJW80low) showed a significantly better effect (Fig. 1D). In contrast to the 80%-ethanol extracts, the water extract without hyperforin and both 60%-ethanol extracts with low (SJW60low) and high (SJW60high) hyperforin levels exhibited no effects on the buffer-soluble Aβ42 fraction in both treatments (Fig. 1A, B). Also, the guanidine-soluble Aβ42 fraction was neither altered after AD initiation nor post AD onset treatment with the water extract or either of the 60%-ethanol extracts (Fig. 1C, D).

### Extracts of SJW with low levels of hyperforin strongly decrease Aβ plaque content

The comparison of Aβ plaques between controls (Fig. 2A) and treatment groups (Fig. 2B-F) revealed a significant reduction of plaque number and cortical coverage in different treatment groups (Fig. 2G-J). Only treatment with the 80%-ethanol extracts led to a strong decrease in plaque number, by −29% (SJW80low) and −40% (SJW80high) with the AD initiation treatment strategy, and −36% (SJW80low) and −39% (SJW80high) in the post-onset treatment groups, respectively (Fig. 2G, H). This effect did not correlate with hyperforin content, indicating that another compound in the extract may contribute to the effect. The SJW60low extract, however, also showed a significant reduction by −20% in the post-onset treatment strategy (Fig. 2H). In addition to plaque number, plaque size was reduced in both 80%-ethanol extract treatment groups, resulting in significantly decreased cortical plaque coverage in the AD initiation (−47% SJW80low, −51% SJW80high) and post AD onset treatment (−52% SJW80low, −60% SJW80high) conditions. Of the water and 60%-ethanol extracts, only the SJW60low extract showed an effect, with −51% in the AD initiation group and −43% in the post-onset treatment group (Fig. 2I, J). Thus, the Aβ plaque size effects of SJW60low, SJ80low and SJW80high are independent of the hyperforin content.

Subsequent analyses of plaque size categories (<400 μm$^2$, 400–700 μm$^2$, and >700 μm$^2$) showed a consistent reduction of large Aβ plaques independent of the hyperforin content (Fig. 2K). While the proportion of plaques larger than 700 μm$^2$ represented 33% of the total plaque number in the vehicle-treated group, this plaque category was significantly reduced to only 15% (SJW60low), 13% (SJW80low), and 9% (SJW80high) after AD initiation treatment. Conversely, the proportion of small plaques increased significantly (37% controls, 49% SJW60low, 54% SJW80low, 60% SJW80high).The effect was even more pronounced in the post-onset treatment condition (Fig. 2L). Here, we detected highly reduced numbers of large plaques in all extract groups, independent of the hyperforin content, as compared to controls (33% controls, 5% SJWwater, 2.7% SJW60low, 3.1% SJW60high, 3.3% SJW80low, 1.7% SJW80high). As seen in the AD initiation treatment groups, the reduction of the large plaques resulted in a substantial increase in small plaques (37% controls, 74% SJWwater, 71% SJW60low, 72% SJW60high, 70% SJW80low, and 76% SJW80high), indicative of reduced Aβ aggregation due to SJW treatment.

## SJW extracts exhibit neuroprotective properties and enhance cognitive performance

Based on the biochemical and morphological results, we wanted to determine the effects of the SJW extracts on neuronal cell loss and cognition. First, we analyzed the effects on neuronal density histologically (Fig. 3A, B). Intriguingly, 80%-ethanol extracts fully rescued neuronal cell loss in the initiation (+24% SJW80low, +21% SJW80high) and post-onset treatment groups (+17% SJW80low, +22% SJW80high), independent of their hyperforin content, even to the levels of non-transgenic controls (Fig. 3C, D). In addition, SJW60low exhibited neuroprotective effects presented in an increased NeuN-stained area by +20% after early extract application (Fig. 3C). We further assessed whether the biochemical and morphological benefits of 80%-ethanol SJW extract treatment, which led to reduced cerebral Aβ deposits and protection against neuronal loss, also resulted in an improvement of cognitive performance. To determine effects on spatial memory, we analyzed performance of mice in the Morris water maze. A strong memory improvement, as evidenced by significantly reduced escape latencies, was shown after treatment with both 80%-ethanol extracts in both the initiation (−50% SJW80low, −53% SJW80high) and post-onset groups (−41% SJW80low, −46% SJW80high) in comparison to vehicle-treated littermates (Fig. 3E, F). In contrast, there were no significant differences detectable after treatment with the SJWwater, SJW60high, or SJW60low extracts in comparison to controls, indicating that these extracts had no effect on cognitive performance.

## SJW extracts enhance microglial phagocytosis function in vivo and in vitro

Recent reports stated that hyperforin could act as modulator of the phagocytic activity of microglia (29). We investigated the microglial-activating properties of the SJW extracts in vitro using a pure microglia cell culture to determine whether enhanced microglia activity is responsible for Aβ clearance. Primary microglia cells were treated with the extracts and co-incubated with fluorescent-labeled Aβ42 (see methods section for details, Fig. 4A, B). Our measurements detected significantly increased phagocytic activity independent of hyperforin content for all extracts (+60% SJWwater, +63% SJW60low, +46% SJW60high), with the strongest effect after 80%-ethanol extract treatment (+78% SJW80low, +78% SJW80high) (Fig. 4C). To analyze the effect of SJW extracts on microglia in vivo, we analyzed co-staining against Aβ plaques and corresponding microglia cells as published by Scheffler et al. (44) (Fig. 4D-I). In contrast to the in vitro results, quantification of microphotographs detected an augmentation of the cortical microglial area by +23% (SJW80low) and +42% (SJW80high), only after treatment with either of the 80%-ethanol extracts (Fig. 4D). Furthermore, the augmenting effect of both SJW80 extracts on microglial activity was only observable in the post-onset treatment group, when the first Aβ peptides are already deposited.

### SJW does not affect expression of α- and β-secretases

Two major APP-cleavage enzymes that are responsible for either the amyloidogenic (β-secretase) or the non-amyloidogenic (α-secretase) pathway have been recognized to be modulated by natural plant substances, e.g., epigallocatechin-3-gallate (EGCG) extracted from Camellia sinensis (21, 49). To determine whether the Aβ-lowering effect of SJW extracts with variable levels of hyperforin results from altered APP cleavage, we performed Western blot analyses to quantify α-secretase (ADAM10) and β-secretase (BACE1) expression. While BACE1 is involved in Aβ generation, active ADAM10 prevents formation of toxic Aβ peptides. Quantification analyses by Western blot, however, indicated that oral application of any of the SJW extracts did not alter the cerebral expression of either enzyme in both treatment paradigms compared to the vehicle-treated mice (Fig. 5).

### ABCC1 transporter facilitates SJW extract-related improvements independent of hyperforin content

The effect of the induction of microglial phagocytosis in vivo was only detectable in the post-onset treatment groups. Thus, we propose additional factors to must account for (i) the effects seen in the AD initiation treatment strategy, and (ii) the higher efficacy of the 80%-ethanol extracts. Importantly, these factors hypothetically act independently of the hyperforin content present in the phytoextracts.

SJW has been shown to modulate ABC transporter expression mediated by the pregnane-X-receptor (PXR) not in rodent but in porcine brain capillary (22). Therefore, we examined the expression of ABCB1 and ABCC1 as main contributors to Aβ clearance within the ABC transporter family in APP-tg mice (38, 39, 41). We found no changes in protein expression, neither for ABCB1 nor ABCC1, associated with differences in hyperforin content (Fig. 6A, B).

Natural substances as well as synthetic drugs, however, can interact with ABC transporters in several ways, leading to functional effects rather than changes in expression. Therefore, protein abundance is not necessarily representative of the effective transport activity/kinetics. For the determination of the functional transport differences, we used an in vitro assay system that specifically detects ABCB1 or ABCC1 transporter activity while incubated with known substrates or SJW extracts. The assays revealed that, independent of their hyperforin content, the SJW60 and SJW80 extracts contained substance(s) that enhance ABCB1 activity by +12% and +19%, respectively (Fig. 6C). Nevertheless, for both groups the extract substance(s) are typical substrates for ABCB1 per se, shown by an activation of +20% in the absence of the known substrate verapamil. Taken together, the higher ABCB1 activity is entirely consumed (at least) for the transport of the extract's active compounds themselves. In contrast, ABCC1 activity was significantly elevated by +70% with the low-hyperforin SJW80 extract only (Fig. 6D). The high-hyperforin, SJW60 extract did not change the transport capacity significantly. To summarize, the assay implies that the SJW80 extracts contain substance(s) that directly enhance transport efficacy of ABCC1 because they are not being transported themselves but rather improve the efficacy of ATP utilization (as indicated by a reduced baseline activity). Interestingly, this effect of increased action is similar to that which we reported recently for thiethylperazine, a drug that substantially lowers Aβ burden in the brains of APPtg mice (41).

## DISCUSSION

Through the use of several molecular biological, immunohistochemical and behavioral methods, this study detected a significant impact of *Hypericum perforatum* extracts (Saint John's wort, SJW) on the main pathological hallmarks characterizing Alzheimer's disease

(AD) in an APP-tg mouse model. The focus of this study was to determine the efficacy of SJW extracts characterized by different hyperforin contents when used to treat AD-related β-amyloidosis in an APP-tg mouse model. We studied the effects of different daily orally administered extracts in two dosages chosen accordingly to literature whereby dosages commonly vary between 300–600 mg extract/kg body weight, although doses as high as 2–4g/kg body weight have been reported (50–52). According to Radde *et al*. (42), initial Aβ-depositions are detectable at the age of six weeks. To restrict the onset of AD histopathology, our first treatment regime started with low dosages at the age of 40 days during the initiation phase of Aβ deposition. The second treatment regime started after the development of first Aβ plaques at the age of 50 days, with an application of a 10-fold dosage to maximize the extract effectiveness on existing disease processes.

Saint John's wort (SJW) has played an important role in the treatment of mild-to-moderate depression for decades (27, 28). Hyperforin is one of the most well-known active constituents of SJW and is believed to be responsible for the efficacy of the extract in depression treatment (28, 53). It is also reputed to affect cognitive performance, which has brought it into the focus of AD research (16, 17, 54). In recent years, reports have indicated beneficial effects of hyperforin on Aβ oligomerization and neuronal survival *in vitro*, while *in vivo* studies have only showed minimal positive effects after intrathecal or intraperitoneal injection of either the stabilized hyperforin sodium salt or hyperforin-derivatives (i.e., tetrahydrohyperforin (IDN5607)) (17, 55, 56). Interpretation of *in vivo* studies of the effects of hyperforin on AD pathology is quite challenging. The latest studies reported a very low bioavailability of hyperforin after oral administration (25). Only minute amounts (∼0.19%) could be detected intracerebrally after oral administration of a high hyperforin dosage (15 mg/kg) (25, 57). Such data has encouraged the development of alternative approaches, for example, the injection of hyperforin derivatives directly into the brain (or at least intraperitoneally) to circumvent the orders of magnitude lower bioavailability after oral administration (55). It is important to note that the delivery of this compound and its derivatives still presents significant difficulties, while all *in vivo* trials have only demonstrated limited efficacy.

The goal of our study was to investigate the ability of different SJW extracts to lower AD pathology in order to possibly reveal novel and less intrusive treatment options, and to elucidate the functional significance of the hyperforin content in these extracts. Our current data support (i) the significant beneficial potential of SJW extracts on AD proteopathy, and indicate that (ii) hyperforin concentration does not necessarily correlate with the therapeutic effects of such extracts. Importantly, based on a selection of four ethanolic SJW extracts with hyperforin concentrations ranging from 0.32% - 6.08% in addition to a water extract without any detectable amounts of hyperforin, our data present strong evidence that hyperforin does not play a direct role in enhancing the therapeutic potential of SJW extracts for AD.

The appearance and aggregation of Aβ are pathological hallmarks of AD, and several studies have documented the greater importance of soluble Aβ species, i.e., peptides and small oligomers (8, 48, 58). Hence, we analyzed the concentration of soluble and insoluble (guanidine soluble) $A\beta_{42}$ fractions separately. *In vivo* studies showed that the intracerebral injection of hyperforin prevented Aβ mediated neurotoxicity after coinjection of Aβ peptides (17). Conversely, this study did not reveal any effect after oral therapy with the extract containing the highest hyperforin level (SJW60high, 6.08%), even in the high-dose application post-onset group. Nonetheless, we discovered that SJW80 extracts (hyperforin concentration: 0.32% in SJW80low, 2.88% in SJW80high) were consistently able to significantly reduce soluble $A\beta_{42}$ levels by at least 38%, starting early in AD pathogenesis and 50% after AD onset, resulting in a significantly lowered amyloid burden including

insoluble fractions and deposits without developing a cerebral amyloid angiopathy (data not shown). SJW60 extracts, with higher levels of hyperforin, were in contrast not effective in activating Aβ clearance. These results clearly demonstrate the differential effects of SJW extracts, independent of their hyperforin content.

Several studies have indicated an effect of hyperforin and different hyperforin analogues on Aβ deposits, which results in a disaggregation of plaque structure at least *in vitro* (17, 32, 54). Therefore, we analyzed the guanidine-soluble Aβ fraction containing fibril formations and smaller aggregates, and again found significant effects in the SJW80 groups. In these groups, the insoluble levels of Aβ were significantly reduced, although this effect was only observed in the post-onset group.

Hyperforin analogues have been demonstrated to affect Aβ deposits to result in a disaggregation of plaques (17, 32, 54). With immunhistochemical plaque staining, we observed that extracts reduced plaque number as well as plaque size independently of hyperforin content (i.e., both SJW80 and SJW60low, but not SJWhigh and SJWwater extracts), resulting in a decreased coverage of the cortex by senile plaques by at least −29%, as compared to vehicle treated littermates. In contrast, treatment with the SJW60high extract which contains the highest amount of hyperforin, as well as the no hyperforin containing SJWwater extract, changed neither the number nor cortex coverage in APPtg mice. Along with the analyses of plaque size (<400 $\mu m^2$, 400–700 $\mu m^2$, and >700 $\mu m^2$), we subsequently found a significant and consistent reduction of large Aβ plaques that did not correlate with the hyperforin content. In other words, effect of treatment with SJW extracts on Aβ aggregation rather depends on the method of extraction than on hyperforin, since highest hyperforin content did not lead to significant effects. Of notion, only application of the SJW80 extracts consistently diminished the Aβ burden and plaque pathology in APPtg mice in both the AD initiation and post-onset treatment paradigms. Taken together, these findings support the hypothesis that one or more constituents of SJW extracts other than hyperforin is contributing to the different treatment efficacies.

With the reduction of both super groups of Aβ species by SJW extract treatment; we simultaneously observed a decrease of characteristically Aβ-dependent neuropathology (neuronal area and cognitive decline). Lesné *et al*. and Lord *et al*. showed that the oligomeric Aβ species, in particular, are the cause of memory dysfunction (14, 58). Importantly, in addition we observed that the amount of soluble Aβ correlates more strongly with the decline of spatial memory than deposited Aβ. Measuring the neuronal density of the cortex, again we observed significant effects in animals treated with SJW60low and both SJW80 extracts. Interestingly, this effect was even more pronounced in the post-onset paradigm, whereby only SJW80 extracts showed a significant attenuating effect on neuronal decline. Hence, this effect seems not to be directly related to hyperforin, but is rather induced by as-yet unidentified substances in our extracts.

The Morris water maze experiments showed that SJW extracts affect spatial memory. In both treatment paradigms, we observed strong cognitive improvement in the APPtg mice after the application of both SJW80 extracts, while water- and both SJW60 extracts did not improve cognitive performance. Escape latencies were decreased by −50% (SJW80low) and −53% (SJW80high) in the AD initiation paradigm, and by −41% (SJW80low) and −46% (SJW80high) in the post-onset treatment group, which is a performance comparable to the level of non-transgenic mice. This finding is consistent with results of the $A\beta_{42}$ ELISA measurements, assuming a higher toxicity for soluble $A\beta_{42}$ oligomers (14, 58). While Trofimiuk *et al*. observed the ability of SJW to enhance spatial working memory (59, 60), we show that all SJW extracts *per se* are not equal, and that in particular, the extraction conditions strongly influence the efficacy of the extract. This fact has been recognized for

some time in the field of depression research, but has received little attention from the AD research community thus far.

In order to examine possible mechanisms by which constituents in specific SJW extracts could act, leading to the observed positive effects of these extracts, we searched for effects on microglial activation, APP processing pathways, and ABC transporter-mediated export.

Microglia have been recognized to clear Aβ (16, 29). Kraus *et al*. described that treatment with complex SJW extracts may restore or improve microglial viability, thereby attenuating Aβ-mediated toxicity *in vitro* (29). Similarly, our *in vitro* analyses revealed significant activation of microglia, and thus $A\beta_{42}$ phagocytosis, after treatment with all extracts. In contrast, we observed increased microglial activity in the SJW80 treated groups only, in subsequent *in vivo* analyses of brain sections immunohistochemically double-stained for amyloid plaques and microglia. These data suggest that constituents accounting for positive effects are not able to enter the brain in sufficient amounts when given orally and/or in insufficient dosages; therefore, *in vitro* experiments using crude SJW extracts or pure hyperforin must be critically reviewed. We were able to demonstrate that the effects we observed are not hyperforin dependent, since SJW60 extracts containing comparable to higher hyperforin concentrations performed more poorly than SJW80 extracts. We hypothesize that the microglia activating effect of SJW extracts *in vivo* is dose-dependent, because only animals of the post-onset treatment group receiving a 10-fold dosage showed significant effects.

The inverse correlation between low buffer-soluble $A\beta_{42}$ and better cognition supports the previously proposed relevance of soluble Aβ species (9, 58), and implies that Aβ clearance mechanisms leading to a reduction of soluble Aβ species could be beneficial in AD treatment. In 2011, Abuznait *et al*. showed that up-regulation of ABCB1 by hyperforin reduces the intracellular accumulation of Aβ in a human cell line (61). Additionally, Ott *et al*. showed that the human PXR ligand hyperforin don't activates rodent but pig PXR at the BBB, and induces mRNA and protein expression of P-glycoprotein (33), while furthermore providing contrasting *in vitro/ex vivo* evidence that hyperforin directly inhibits its transport activity (35). Consistent with these observations, we measured no effects of the tested extracts, especially with regard to the hyperforin rich SJW60high extract on ABCB1 activity in a cell-free *in vitro* assay. In contrast, our experiments showed *in vivo* effects independent of the hyperforin content and ABC transporter expression changes, a difference that is probably due to multiple substances within crude plant extracts with competitive effects and the reported insensitivity of the murine PXR-receptors to hyperforin (33). ABCC1 transporter activity assays, however, revealed differences between the SJW60high and the SJW80low extracts. We demonstrated a significant increase in activity by using the SJW80low extract, which could account for the significantly reduced Aβ burden in the correspondent treatment groups. We have recently reported that ABCC1 has the strongest influence on cerebral Aβ levels and that activation of ABCC1 by thiethylperazine leads to a marked reduction in cerebral Aβ concentration (41). Therefore, we hypothesize that the prominent effects of SJW treatment results from the activation of microglial phagocytosis and the enhanced clearance of Aβ by an increased ABCC1 transport activity.

Herbal extracts are complex mixtures composed of numerous organic compounds. The presence and tissue-specific concentrations of these compounds likely vary among populations of *H. perforatum*, which are characterized by high levels of genetic variability (62). Our biochemical knowledge is currently limited to the concentrations of hyperforin and hypericin in the extracts, but work is currently ongoing in our laboratories to characterize the targeted extracts and identify bioactive constituents.

## CONCLUSIONS

Currently there is no cure for a multifactorial dementia such as Alzheimer's disease. In this study using a transgenic amyloidosis mouse model, we have described the impact of extracts of the medicinal herb *Hypericum perforatum* (SJW) on the main pathological hallmarks characterizing AD. This work supports a functional context between specified extracts and the level of cerebral β-amyloid species, as well as its consequential pathogenesis. Plaque reduction, microglial activation and cognitive improvement are the main positive effects of gavage with specific extracts tested in this study. More importantly, the activation of the main intracerebral Aβ exporter, ABCC1, by a SJW extract with only minimal amounts of hyperforin suggests that other constituents in the extract are responsible for effects observed and that this activation leads to the observed attenuating effects on AD pathogenesis. Our data further support the hypothesis that SJW extracts are of therapeutic value for AD treatment.

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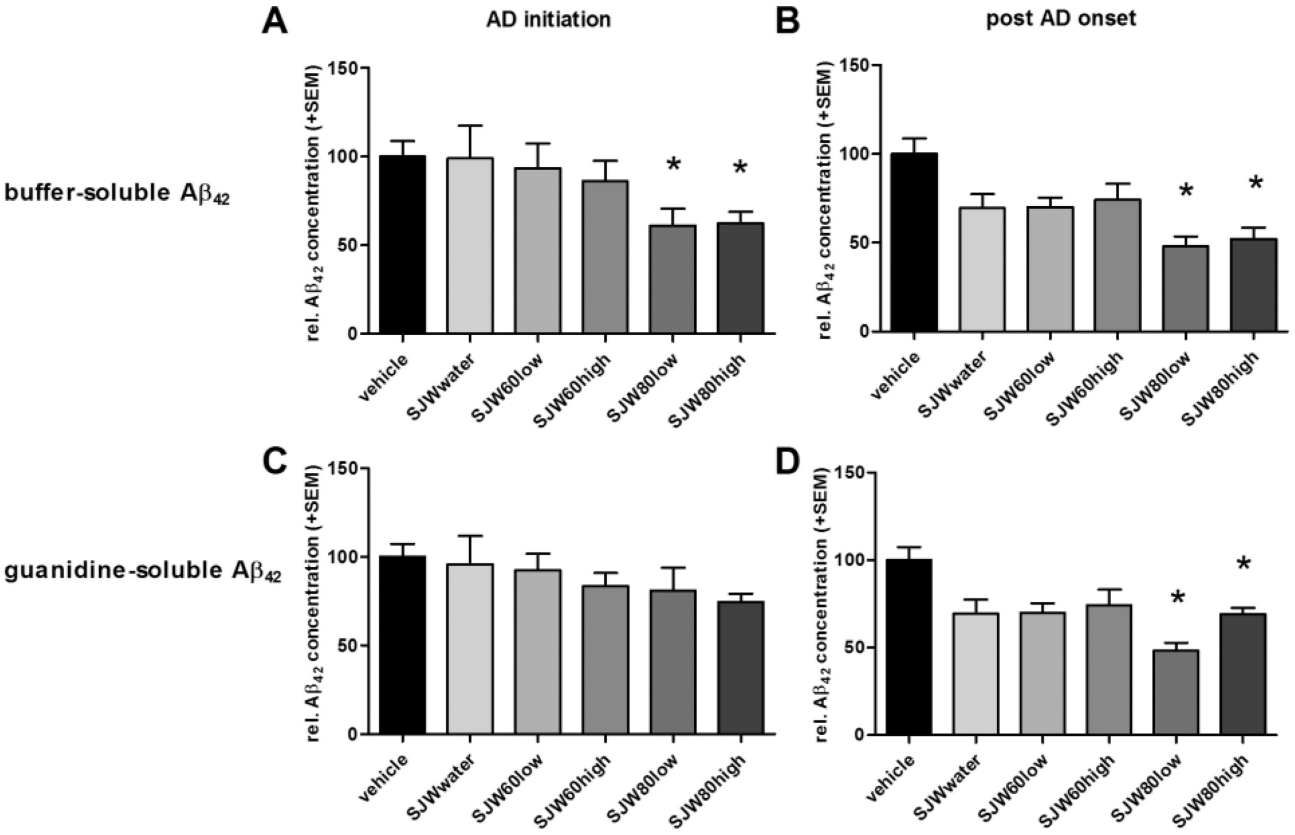


**Figure 1.**

80%-ethanol St. John's wort (SJW) extracts reduce brain Aβ42 levels independently of the hyperforin content. (A, B) Both SJW80 extracts reduced buffer-soluble Aβ42 significantly by −39% (SJW80low) and −38% (SJW80high) in the 'AD initiation' paradigm, and by −53% (SJW80low) and −49% (SJW80high) in the 'post AD onset' treatment paradigm, respectively. (C, D) Guanidine-soluble Aβ42 fraction characterized by insoluble oligomers and small fibril aggregates were significantly reduced only in the post-onset treatment. Here, the SJW80low extract showed a reduction by −50% and was even more potent than the SJW80high extract with a reduction by −31% (mean + SEM, *p≤0.05).

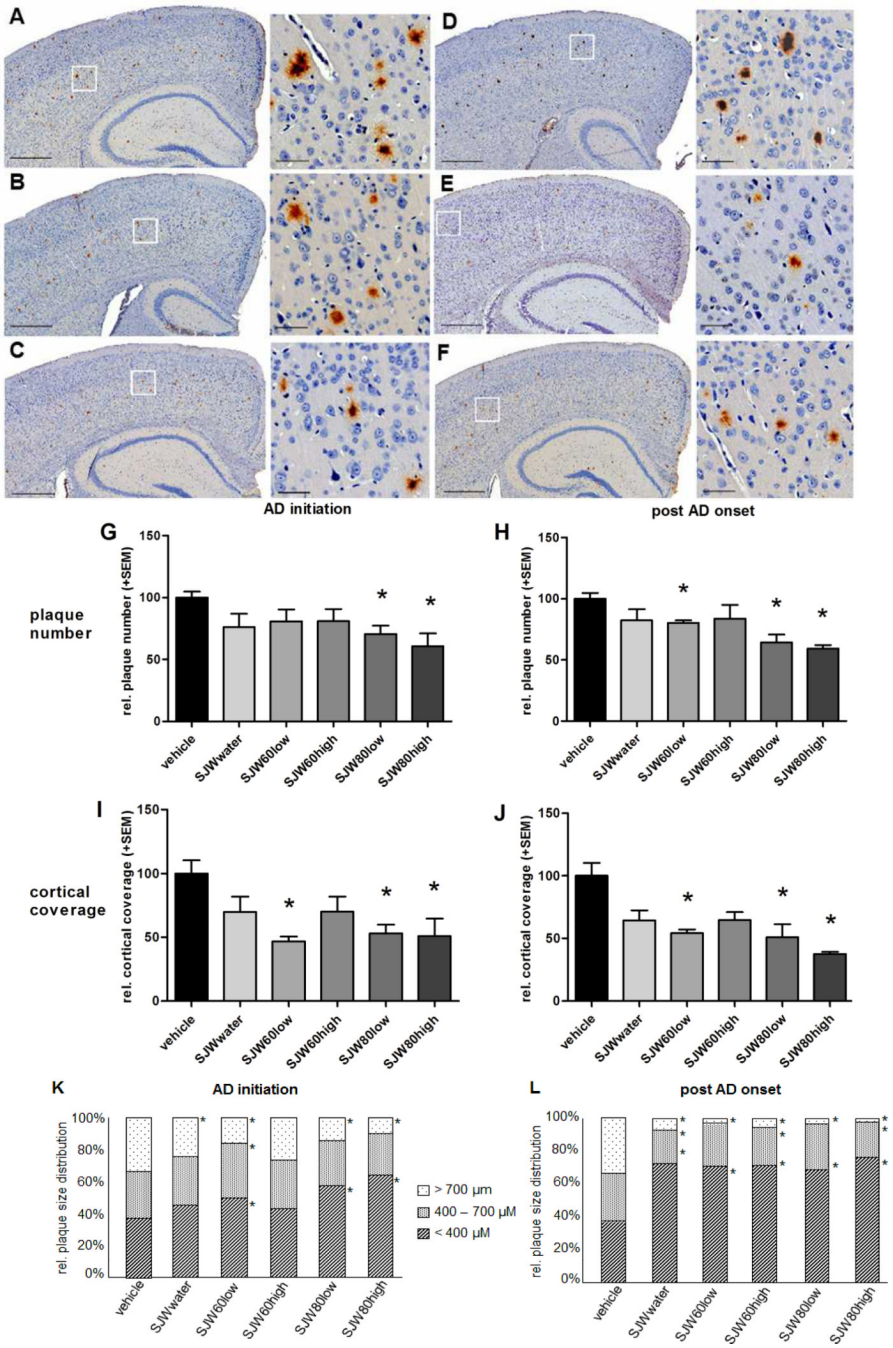


**Figure 2.**

80%-ethanol SJW extracts reduced Aβ plaque number and size. (A-F) Histological presentation of plaque number and size reduction: (A) vehicle treatment, (B) SJWwater, (C) SJW60low, (D) SJW60high, (E) SJW80low, (F) SJW80high (scale bars: 500 µm, 50 µm). (G) 80%-ethanol extracts elicited the strongest reduction of plaque number by −29% (SJW80low) and −40% (SJW80high) in the 'AD initiation' group; and −36% (SJW80low) and −39% (SJW80high) in the 'post AD onset' treatment group. (H) Due to a strong plaque size reduction, the cortical coverage by plaques was finally significantly decreased by −47% (SJW80low) and −51% (SJW80high) after AD initiation and −52% (SJW80low) and −60% (SJW80high) after post-onset treatment, independently of the hyperforin content. (K, L)

SJW treatment reduced plaque growth resulting in a significant size shift from large (>700μm$^2$), via medium (400–700 μm$^2$) to small plaques (<400 μm$^2$). All SJW extracts significantly reduced the development of large plaques in the 'post AD onset' treatment paradigm (mean + SEM, *p≤0.05)

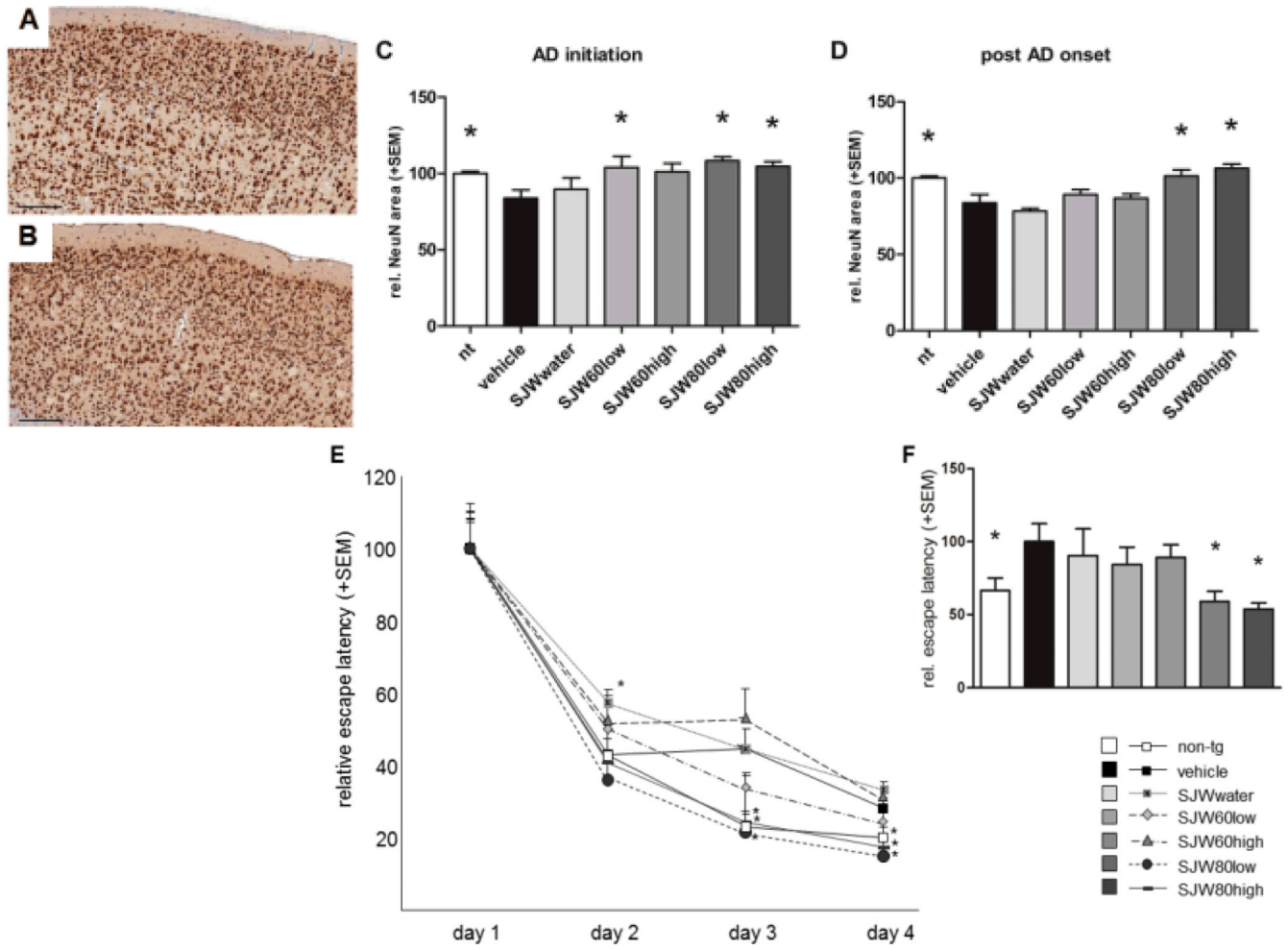


**Figure 3.**

80%-ethanol SJW extract treatment protects against spatial memory impairment and neuronal loss. (A, B) Immunhistochemical NeuN labelings (neurons) of the neuronal layers I-V in the neocortex: (A) vehicle treatment, (B) SJW80low treatment (scale bars: 50 μm). (C, D) Quantification reveals strong neuroprotective effects in the 'AD initiation' group (+24% SJW80low, +21% SJW80high), and the 'post AD onset' treatment group (+17% SJW80low, +22% SJW80high) for both 80%-ethanol SJW extracts. The recovery of the NeuN-labeled area was even similar to levels in non-transgenic controls. (E, F) Spatial memory improvement was evaluated by Morris water maze. 80%-ethanol SJW extracts protect from cognitive decline as shown by the reduced escape latency by −50% (SJW80low) and −53% (SJW80high) in the 'AD initiation' group (E, all days are shown) and by −41% (SJW80low) and −46% (SJW80high) in the 'post AD onset' treatment group (F, day 4 shown). Memory performance was comparable to non-transgenic controls (mean + SEM, *p≤0.05).

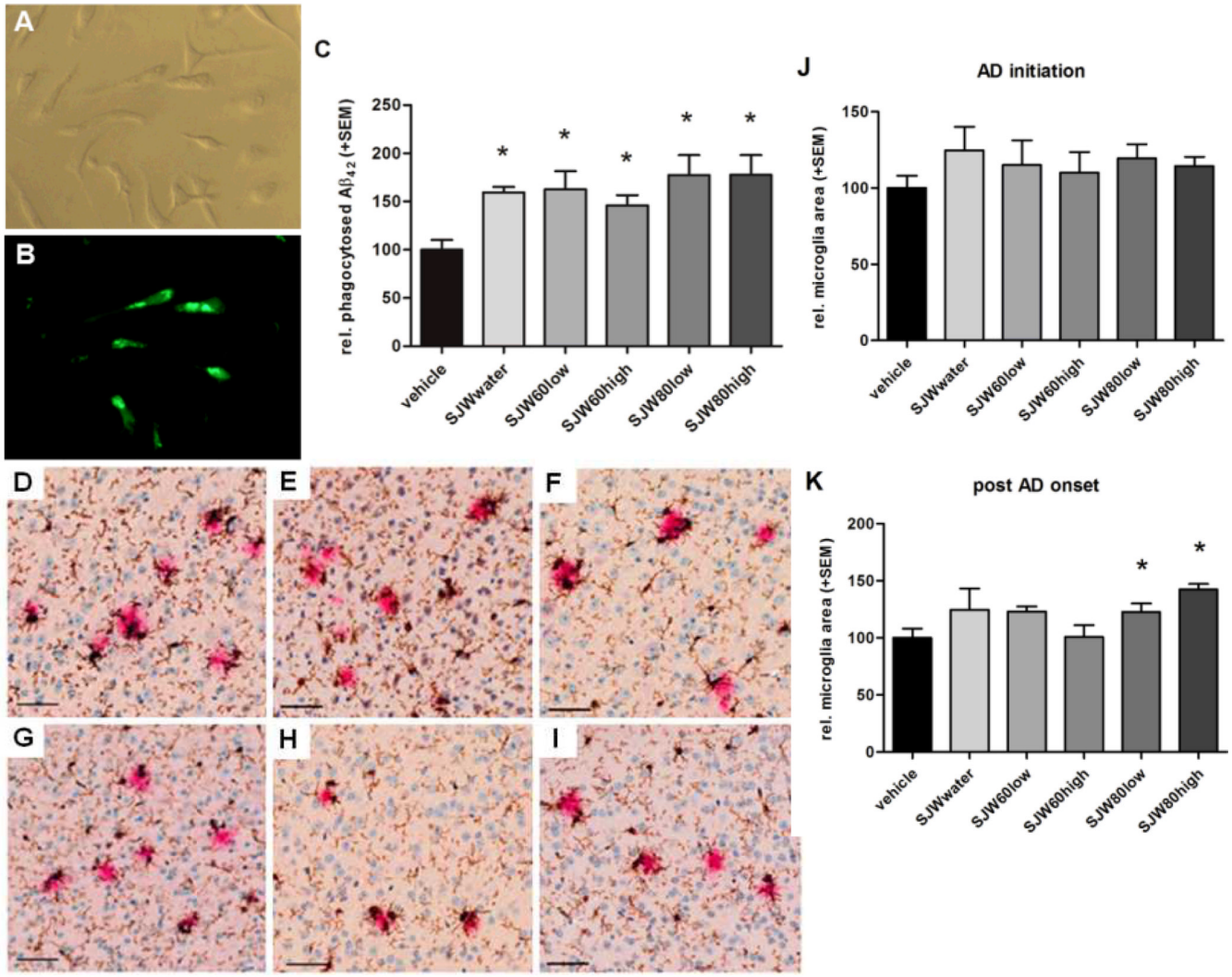


**Figure 4.**

80%-ethanol SJW extracts enhance anti-Aβ activity of microglia in vivo and in vitro. (A-C) In vitro treatment of a primary microglia cell culture with SJW extracts and FITC-labeled Aβ42 (A, bright-field; B, fluorescence micrographs) revealed activating effects by crude SJW extracts independent of hyperforin content. Quantification of phagocytized Aβ42 by microglia resulted in enhanced fluorescence: +60% SJWwater, +63% SJW60low, +46% SJW60high, +78% SJW80low, +78% SJW80high. (D-I) Microphotographs of cortical microglia in 100-days-old mice with 'post AD onset' treatment: (D) vehicle, (E) SJWwater, (F) SJW60low, (G) SJW60high, (H) SJW80low, (I) SJW80high indicated stimulating properties of SJW (scale bars: 50 μm). (J; D) Quantification of microglial area in the vicinity of Aβ-plaques revealed an activation of microglia with SJW80 in the 'post AD onset' treatment group by +23% (SJW80low) and +42% (SJW80high) independently of the hyperforin content (mean + SEM, *p≤0.05).

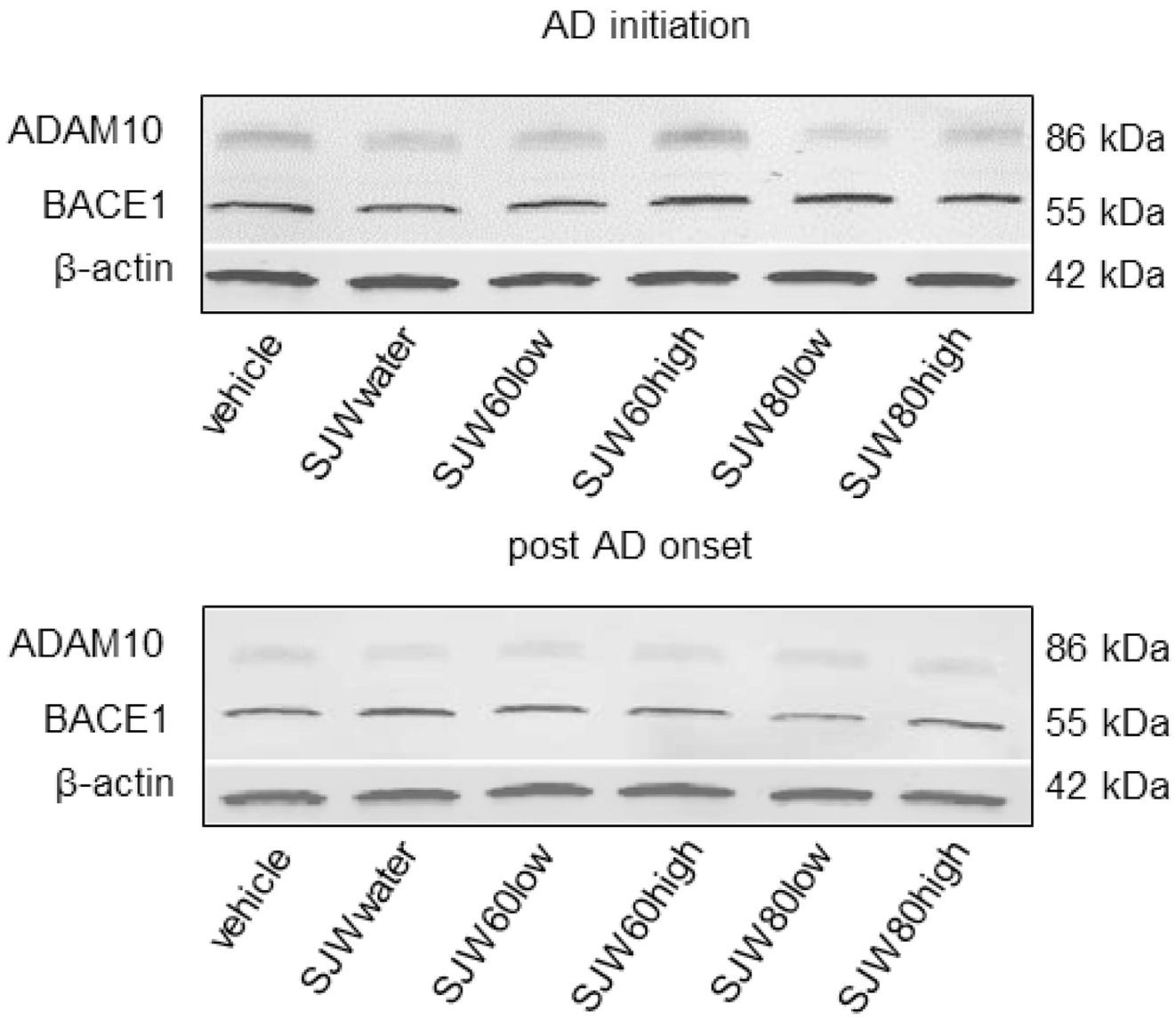


**Figure 5.**
SJW extracts do not affect BACE1 and ADAM10 expression, as shown by Western blot quantification of neocortical homogenates of 100-day-old female APP-tg mice. Neither ’AD initiation’ nor ’post AD onset’ treatment affected expression as compared to vehicle-treated littermates.

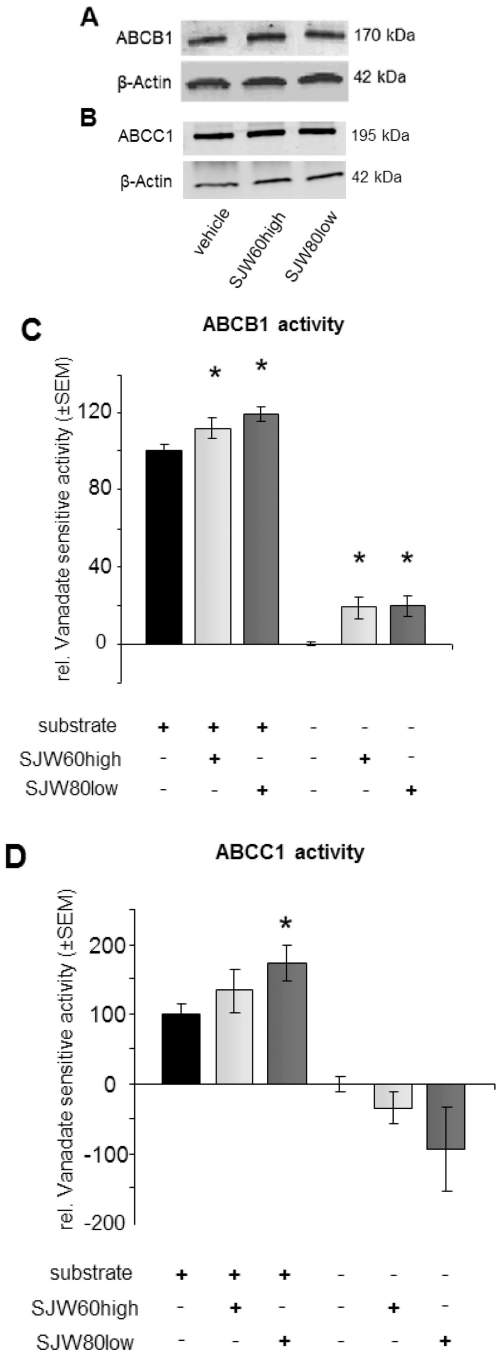


**Figure 6.**

80%-ethanol, low hyperforin SJW extracts activate the Aβ exporter ABCC1. Western blot analyses of neocortical homogenates revealed no changes in the expression of (A) ABCB1, nor (B) ABCC1 after treatment with high or low hyperforin containing extracts. (C) SJW extracts enhance ABCB1 transporter activity (substrate: verapamil), but the elevated activity is consumed by the active extract substance itself as indicated by activity elevation in the absence of verapamil. (D) Only the SJW80low extract significantly enhances ABCC1 transporter activity (substrate: NEM-GS) independently of the hyperforin content without being an ABCC1 substrate itself (mean ± SEM, *p≤0.05).

**Table 1**

St. John's wort (SJW) extracts, solvents, and hyperforin/hypericin content as assessed by HPLC (Finzelberg GmbH & Co KG, Andernach, Germany) used for the investigations. Extracts with a relative content of hyperforin less than 1.5% are denoted as 'low', and with more than 1.5% as 'high'. Number of animals in the treatment groups (vehicle controls N=14).

| Extract Name | Extraction medium | Hyperforin content (Hf) | Hypericin content (Hi) | drug extract ratio (DER) native | number of animals AD initiation group / post AD onset group |
|---|---|---|---|---|---|
| SJWwater | water | 0.00% (−) | 0.002% | 8–12:1 | 6 / 6 |
| SJW60low | 60% EtOH | 1.45% | 0.215% | 3–6:1 | 7 / 5 |
| SJW60high | 60% EtOH | 6.08% | 0.191% | 3–6:1 | 6 / 5 |
| SJW80low | 80% EtOH | 0.32% | 0.090% | 3–6:1 | 7 / 11 |
| SJW80high | 80 % EtOH | 2.88% | 0.165% | 3–6:1 | 5 / 6 |