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Short-term treatment with tolfenamic acid improves cognitive functions in Alzheimer's disease mice

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ABSTRACT

Tolfenamic acid lowers the levels of the amyloid precursor protein (APP) and amyloid beta (Aβ) when administered to C57BL/6 mice by lowering their transcriptional regulator specificity protein 1 (SP1). To determine whether changes upstream in the amyloidogenic pathway that forms Aβ plaques would improve cognitive outcomes, we administered tolfenamic acid for 34 days to hemizygous R1.40 transgenic mice. After the characterization of cognitive deficits in these mice, assessment of spatial learning and memory functions revealed that treatment with tolfenamic acid attenuated long-term memory and working memory deficits, determined using Morris water maze and the Y-maze. These improvements occurred within a shorter period of exposure than that seen with clinically approved drugs. Cognitive enhancement was accompanied by reduction in the levels of the SP1 protein (but not messenger RNA [mRNA]), followed by lowering both the mRNA and the protein levels of APP and subsequent Aβ levels. These findings provide evidence that tolfenamic acid can disrupt the pathologic processes associated with Alzheimer's disease (AD) and are relevant to its scheduled biomarker study in AD patients.

1. Introduction

Alzheimer's disease (AD) represents the most prevalent neurodegenerative disease in the elderly. During the course of the disease, memory, cognitive performance, and other daily activities are all impaired as a result of extensive neuronal loss (Berg et al., 1993; Braak and Braak, 1997; Nelson et al., 2012). AD is characterized by the presence of neuropathologic deposits consisting of extracellular senile plaques of amyloid beta (Aβ) core and intraneuronal neurofibrillary tangles, especially in the cerebral cortex, hippocampus, subcortical nuclei, and amygdala (Ballard et al., 2011; Harrington, 2012; Reddy et al., 2010; Selkoe, 2001).

The amyloid precursor protein (APP) is processed by the beta-site APP-cleaving enzyme 1 (BACE1) and γ-secretase to generate various Aβ peptide isoforms that can accumulate resulting in the formation of the insoluble aggregates of amyloid plaques (Querfurth and LaFerla, 2010; Shoji et al., 1992; Urbanc et al., 1999; Zhang et al., 2012). $A\beta_{1-40}$ and $A\beta_{1-42}$ are the major generated isoforms with $A\beta_{1-42}$ found to be more aggregative triggering amyloid plaque formation (Finder and Glockshuber, 2007; Naslund et al., 2000). Accumulation of Aβ into amyloid plaques initiates a pathologic cascade resulting in synaptic dysfunction and neuronal death that contribute to the neurodegeneration observed in AD according to the amyloid hypothesis (Hardy and Higgins, 1992; Selkoe, 2001). However, other studies suggest that Aβ-soluble oligomers and aggregates are the toxic species and that in AD patients soluble Aβ levels highly correlate with disease severity markers (Kroth et al., 2012; McLean et al., 1999).

Food and drug administration (FDA)—approved drugs for treatment of AD include 4 cholinesterase inhibitors and 1 *N*-methyl-D-aspartate receptor antagonist. However, these medications are not disease modifying, and they do not stop the progression of AD (Ozudogru and Lippa, 2012). Current research focuses on interventions that target Aβ production and aggregation and the production of hyperphosphorylated tau (Gotz et al., 2012; Ozudogru and Lippa, 2012; Roberson and Mucke, 2006); however, no therapeutic strategy has explored more upstream interventions at the transcriptional level. Specificity protein 1 (SP1) coactivates the transcription of APP, BACE1, and tau genes (Christensen et al., 2004; Docagne et al., 2004; Heicklen-Klein and Ginzburg, 2000), and consequently, changes in its levels can alter the downstream pathways related to amyloidogensis (Adwan et al., 2011; Basha et al., 2005) and tau pathology (unpublished data). Tolfenamic acid, a nonsteroidal anti-inflammatory drug (NSAID), induces the degradation of SP1 protein (Abdelrahim et al., 2006), and data from our

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laboratory have shown that the treatment of wild-type C57BL/6 mice with tolfenamic acid lowered the levels of cerebral SP1 and the expression of AD-related Sp1 target genes such as APP (Adwan et al., 2011).

Transgenic mouse models of AD are useful for elaborating mechanisms involved in the development and progression of AD. In addition, they allow for testing of new therapies in vivo to provide more accurate data for testing in human clinical trials (Duff and Suleman, 2004; Hock and Lamb, 2001). Our previous studies were conducted in wild-type C57BL/6 mice that do not exhibit AD pathology; so, we could not assess for cognitive function improvement associated with an amyloidogenic pathway. Thus, we decided to examine the ability of tolfenamic acid to lower AD proteins, including SP1, APP, and Aβ, and to examine whether such reductions are commensurate with improvements in cognitive functions in a mouse model of AD. Because the endogenous APP promoter is largely driven by SP1 (Docagne et al., 2004), we chose to study the effects of tolfenamic acid (which degrades SP1) in the hemizygous R1.40 mice, a genomic-base transgenic mouse model that harbors the Swedish mutation APPK670N/M671L, driven by the human APP promoter (Lamb et al., 1993).

Therefore, after the characterization of learning and memory impairment in female hemizygous R1.40 transgenic mice, 5 and 50 mg/kg/d tolfenamic acid was administered to female R1.40 mice aging between 14 and 21 months via oral gavage for 34 days and learning and memory functions were assessed in the Morris water maze (MWM) and the Y-maze. On day 35, mice were euthanized and AD-associated proteins including SP1, APP, and soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were assessed in the frontal cortex, which displays extensive AD pathology in this animal model (Kulnane and Lamb, 2001; Lehman et al., 2003).

2. Methods

2.1. Animal model

The transgenic mouse model R1.40 was used for this study. The rationale for choosing this transgenic line relies on the fact that the mutant human APP gene is driven by its endogenous human promoter unlike most of other transgenic lines, which use hamster PrP or murine Thy-1 promoters (Hock and Lamb, 2001). The human promoter region has numerous CpG boxes that SP1 binds to whilst activating gene expression. Thus, R1.40 is an ideal mouse model to conduct experiments on, as tolfenamic acid lowers SP1 and thus alters upstream transcriptional pathways of APP. These transgenic mice, B6.129-Tg(APPSw)40btla/J, were obtained from the Jackson laboratory (Bar Harbor, ME, USA), and colonies of hemizygous and homozygous strains were established in-house. R1.40 is a genomic-based transgenic mouse model that was developed by Bruce T. Lamb; it uses a yeast artificial chromosome that contains the full 400-kb human APP gene and flanking sequence of approximately 250 kb to harbor the Swedish mutation APPK670N/M671L, including the human transcriptional regulatory elements needed for proper spatial and temporal expression (Hock and Lamb, 2001; Lamb et al., 1997; Reaume et al., 1996). The developed hemizygous R1.40 line shows a significant increase in APP and Aβ production as early as 3 months of age with Aβ plaque deposition occurring at 24–26 months of age compared with the wild-type mice. Furthermore, the mnemonic deficits in R1.40 were similar to those observed in AD (Hock et al., 2009; Lamb et al., 1999).

To establish R1.40 transgenic mice colonies, mice were bred and genotyped in-house at the University of Rhode Island (URI). To ensure the accuracy of genotyping results, 2 genotyping techniques were performed: standard polymerase chain reaction (PCR) followed by gel electrophoresis on 1.5% agarose gel and the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA; for details, see Supplementary data). Animals of mixed genotypes were housed in standard mouse cages in the URI animal quarter rooms with a 12:12 hour light-dark cycle (light on at 6:00 AM, light off at 6:00 PM). Temperature was maintained at 22 ± 2 °C with humidity levels of 55% ± 5%, and food and water were available for mice ad libitum. The University of Rhode Island Institutional Animal Care and Use Committee approved all protocols including the breeding and genotyping methods. Animals were under continuous supervision by a URI veterinarian during the entire study and during drug administration.

2.2. Assessment of cognitive deficits in hemizygous R1.40 transgenic mice

To characterize memory and cognitive deficits in the hemizygous, R1.40, transgenic mouse model, behavioral testing in mazes that are reliant on the integrity of the hippocampus and brain cortex was conducted using the MWM and spontaneous alternations in the Y-maze. Preliminary studies showed cognitive deficits in both male and female mice, and the drug under study was active in both genders. In this study, we decided to use a single gender to minimize any possible influence or interference that may be created because of the use of mixed genders in the experiments. Thus, female, hemizygous, APP transgenic ($n = 19$) and female, control wild-type ($n = 18$) groups of ages ranging between 9 and 20 months were used.

2.2.1. Morris water maze

We have tested the mice in the hidden version of the MWM. In this task, the mice had to locate the hidden platform by learning multiple spatial relationships between the platform and the distal extramaze cues (Gulinello et al., 2009; Laczo et al., 2009; Vorhees and Williams, 2006). The apparatus consisted of a white 48″ diameter pool that is 30″ in height and was filled with water to a depth of 14″. The water was kept opaque by the addition of white, nontoxic liquid washable paint. The pool was surrounded by distinct fixed visual cues that the animals used to navigate to reach the escape platform. A clear Plexiglas platform 10 cm^2 was kept submerged 0.5 cm below the surface of the water. The temperature of the water was maintained at 25 ± 2 °C during all experiments in the water maze. On day 15 of tolfenamic acid administration, mice received a habituation trial in which they were allowed to swim freely for 60 seconds. On the following day and for a total of 8 days, mice received training sessions of 3 trials daily. The starting position for each trial was randomly assigned between the 4 possible positions (1 per quadrant), whereas the platform position was fixed in each trial. Each animal was allowed to swim until they found the immersed hidden platform or for a maximum duration of 60 seconds. If the mouse failed to locate the platform, it would be gently guided to sit on the platform for a maximum duration of 30 seconds. Mice were also left to sit on the platform for a maximum of 10 seconds on successful trial. After completion of the 8 acquisition sessions, probe trials for up to 60 seconds on day 1 and day 11 after the last day of training were performed to assess long-term memory retention by studying the preference of the mice for the correct quadrant that previously contained the hidden platform. The swim paths and latencies to locate the platform and time spent in quadrants were videotaped and tracked with a computerized video-tracking system (ObjectScan; Clever Sys., Inc, Reston, VA, USA), and the resultant data were analyzed.

2.2.2. Spontaneous alternation in the Y-maze

The spontaneous alternation ratio, defined as the percentage of the number of arm entries different from the previous 2 entries

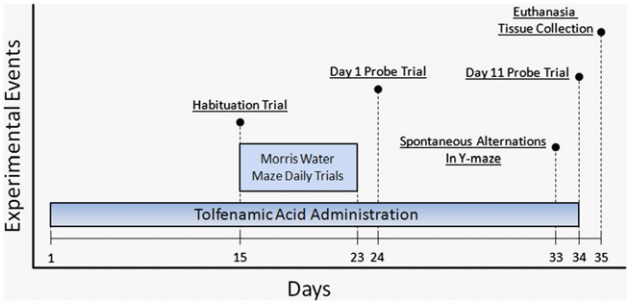

Fig. 1. A timeline of tolfenamic acid administration and behavioral assessment. Mice were administered tolfenamic acid daily for 34 days. Behavioral testing in the Morris water maze began on Day 15 of dosing with daily training sessions that lasted until Day 23 of the study. We conducted the first session of probe trials (Day 1) on Day 24 of dosing and a second session of probe trials (Day 11) on Day 34 of dosing, to assess for long-term memory retention. Trials for the spontaneous alternations in the Y-maze were conducted on Day 33 of dosing as well. Animals were euthanized on Day 35 of the study, and brain tissue was dissected, collected, and stored in the −80 °C freezer.

divided by the total arm entries minus 2 (Hock et al., 2009; King et al., 1999), was measured. Testing was conducted in a white Y-maze with arms that were 12″ (long) by 3″ (wide) with 8″ high walls. Mice were placed into 1 of the Y-maze arms and were left to explore the maze freely for 5 minutes. After each trial, the maze was cleaned with 70% ethanol. Y-maze trials were videotaped and tracked with a computerized video-tracking system (ObjectScan; Clever Sys., Inc), and the data were analyzed.

2.3. Animal exposure to tolfenamic acid and behavioral testing

Tolfenamic acid was obtained from Sigma Aldrich (St Louis, MO, USA), and female hemizygous APP yeast artificial chromosome, R1.40 transgenic mice aged between 14 and 21 months were used to examine the ability of tolfenamic acid to reduce the amyloidogenic load and to improve the learning and memory deficits of the mice. From our initial published studies with tolfenamic acid (Adwan et al., 2011), we found that 5 and 50 mg/kg/d were the optimum low and high doses capable to produce reductions in amyloidogenic proteins. We also found that a treatment duration of 15 days was the minimum period required to show significant reductions in APP and Aβ. Thus, on day 15 of drug administration, we started conducting behavioral experiments and tolfenamic acid administration continued until the last day of the behavioral studies. The animals were divided into 3 groups of similar age variation and were administered tolfenamic acid daily via oral gavage for 34 days: first group was administered 5 mg/kg/d tolfenamic acid in corn oil ($n = 6$), the second group was administered 50 mg/kg/d tolfenamic acid in corn oil ($n = 7$), and the third group was administered the corn oil vehicle ($n = 6$). After 14 days of dosing, mice were tested in the MWM as described in Section 2.2.1. On day 33, mice were tested for spontaneous alternation in the Y-maze as described in Section 2.2.2. Finally, on day 35, mice were euthanized and brain tissue was extracted, dissected, and stored at −80 °C. Fig. 1 provides a timeline for the experimental events involved in drug administration and behavioral assessment.

2.4. Protein extraction and Western blot analysis

Brain cortices were homogenized with radioimmunoprecipitation assay (RIPA) lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, and 0.1% protease inhibitor cocktail. The homogenates were centrifuged at 10,000 × *g* for 10 minutes at 4 °C, and supernatants were collected. Protein levels were determined using the BCA kit (Pierce Biotechnology, Inc, Rockford, IL, USA). Forty micrograms of total protein was separated on 8% sodium dodecyl sulfate polyacrylamide gel and then transferred onto polyvinylidiene diflouride membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked using 5% nonfat milk in Tris-buffered saline + 0.5% Tween-20 (TBST, pH 7.4) for 1 hour. Membranes were then incubated overnight at 4 °C with the specific antibody diluted in TBST for 1 hour (1:2000 dilution of 6E10 for APP [Covance Research Products, Inc, Dedham, MA, USA] and 1:500 dilution of 1C6 for SP1 [Santa Cruz Biotechnology, Santa Cruz, CA, USA]). After washing membranes 4 times with TBST, membranes were incubated for 1 hour with the anti-mouse IRDye 680 (Li-Cor Bioscience, Lincoln, NE, USA) (1:5000) at room temperature. After washing the membranes twice with TBST and once with TBS, the images were developed using the Li-Cor Odyssey infrared imaging system (Li-Cor Bioscience). The membranes were also reprobed for β-actin (Sigma Aldrich) at a dilution of 1:2500 in TBST to obtain the APP/β-actin ratio. The intensities of the obtained Western blot bands were determined using Odyssey V1.2 software (Li-Cor Bioscience).

2.5. RNA isolation, synthesis of complementary DNA, and quantitative real-time PCR

RNA was isolated from cortical tissue after the TRIzol Reagent method (Invitrogen, Carlsbad, CA, USA), checked for integrity using NanoDrop 2000 Micro-Volume UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and gel electrophoresis. RNA was reverse transcribed to complementary DNA (cDNA) using the iScript Select cDNA Synthesis kit following manufacturer's instructions (Bio-Rad, Hercules, CA, USA). About 1000 ng of RNA was diluted to 19.5 μL with nuclease-free water, and 3 μL oligo (dT) mix, 6 μL 5× iScript select reaction mix, and 1.5 μL of iScript reverse transcriptase were added. Samples were incubated at 42 °C for 90 minutes and then at 85 °C for 5 minutes to terminate the reaction. All incubations were conducted using MJ Research MiniCycler PTC-150 (Bio-Rad). Primer pairs for Sp1, APP, and β-actin were obtained from Invitrogen as follows: Sp1 sense 5′-CAA GCC CAA ACA ATC ACC TT-3′ and antisense 5′-CAA TGG GTG TGA GAG TGG TG-3′, APP sense 5′-TGC AGC AGA ACG GAT ATG AG-3′ and antisense 5′-ACA CCG ATG GGT AGT GAA GC-3′, β-actin sense 5′-TGT TAC CAA CTG GGA CGA CA-3′, and antisense 5′-TCT CAG CTG TGG TGG TGA AG-3′. Each real-time PCR reaction mix contained 2 μL of cDNA, 1 μL of each primer, 8.5 μL nuclease-free water, and 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR was conducted using 7500 Real-Time PCR System (Applied Biosystems) following the standard protocol: 50 °C for 2 minutes followed by 95 °C for 10 minutes and then 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Results were analyzed using SDS software, and expression data were reported relative to β-actin messenger RNA (mRNA) using the $2^{-\Delta\Delta Ct}$ method.

2.6. Enzyme-linked immunosorbent assay using $A\beta_{1-40}$ and $A\beta_{1-42}$ assay kits

Enzyme-linked immunosorbent assay (ELISA) using human $A\beta_{1-40}$ and $A\beta_{1-42}$ assay IBL kits JP27713 and JP27711 determined Aβ levels, both soluble and insoluble fractions (Immuno-Biological Laboratories, Gunma, Japan). These kits are solid-phase sandwich ELISA with 2 types of highly specific antibodies that are 100% reactive with mouse $A\beta_{1-40}$ with a sensitivity of 5.00 pg/mL (JP27713) and 70% reactive with mouse $A\beta_{1-42}$ with a sensitivity of 4.03 pg/mL (JP27711). The assay conditions were followed according to a method described in the literature (Morishima-Kawashima et al., 2000; Wu et al., 2008). The levels of Aβ in the test samples

were calculated relative to the standard curve generated on each plate.

2.7. Statistical analysis

Biochemical data are expressed as the mean ± the standard error of the mean (SEM). Assessment of performance in MWM daily training sessions between the hemizygous R1.40 and the wild-type groups was determined using repeated-measure analysis of variance (ANOVA), whereas the significance of probe trials differences between both groups was determined by a 2-tailed Student *t* test. The significance of differences among various treatment groups was determined by repeated-measure ANOVA and Tukey-Kramer multiple comparison a posteriori analysis. All statistical analyses were conducted using GraphPad InStat 3 software (GraphPad Software, La Jolla, CA, USA); p value of <0.05 was considered statistically significant.

3. Results

3.1. Overall safety and toxicity observations

Overt toxic effects of tolfenamic acid administration were not observed in these animals. No changes in body weight were observed, and treatment with tolfenamic acid did not result in any abnormal behavior compared with nonexposed mice. Although the safety of tolfenamic acid has been previously established, as it has been used in Europe for decades for rheumatoid arthritis and for migraine, recent toxicity studies conducted by one of our collaborators showed that chronic administration of tolfenamic acid of 50 mg/kg 3 times a week for 6 weeks did not cause changes in hematocrit or hemoglobin in tolfenamic acid exposed mice. In addition, histopathologic analysis showed that acute and chronic administration of tolfenamic acid did not produce adverse changes in gastric and intestinal epithelia (unpublished data).

3.2. Hemizygous R1.40 mice exhibit cognitive impairments when tested in the MWM and in the Y-maze

Spatial navigation in the MWM is one type of tests used to assess long-term memory functions, which are usually impaired in AD. In the daily sessions that consisted of 3 trials per day for a total of 8 days, repeated-measure ANOVA indicated no significant difference in the performance, assessed by measuring escape latency, between the transgenic group and the control wild-type mice in this task ($F(7,245) = 0.924$, $p > 0.05$) (Fig. 2A). However, probe trials that assessed the percent time in the correct quadrant that contained the hidden platform showed that as the retention delay increased from day 1 after the last day of the daily training sessions to day 11, the transgenic mice exhibited memory impairment as they failed to remember the location of the hidden platform. As shown in Fig. 2B, probe trials in day 1 after the last day of daily training sessions showed no significant difference between both groups ($p > 0.1$). However, after a longer delay on day 11 after the last day of daily trainings sessions, the hemizygous R1.40 transgenic mice significantly spent less time (mean = 23%, SEM = 1.99%) compared with the control wild-type mice (mean = 29.8%, SEM = 2.58%), $p = 0.045$ (Fig. 2B). In addition, ANOVA of target preference showed that both WT and R1.40 mice spent significantly more time in the correct quadrant in the first probe trial. However, in the second probe trial, only the WT group retained such significant target preference. The R1.40 mice group failed to maintain target preference indicating memory impairment (see Supplementary data).

Working memory function was assessed by measuring the spontaneous alternations in the Y-maze. This task is based on the innate nature of the mice to visit arms that have not been recently visited (Hock et al., 2009; King et al., 1999). Results revealed that hemizygous transgenic R1.40 mice exhibited impaired working memory function as they had a significantly lower spontaneous alternation ratio (mean = 0.413, SEM = 0.021) than the control wild-type mice (mean = 0.496, SEM = 0.019), $p = 0.0047$ (Fig. 3).

3.3. Treatment with tolfenamic acid reverses the cognitive deficits in hemizygous R1.40 mouse model

We determined previously the presence of impaired behavioral performance in the hemizygous R1.40 mice. This was manifested by a decline in the long-term memory and the working memory functions as identified by poor retention of the spatial location of the platform in the MWM and spontaneous alternations in the Y-maze, respectively.

We further examined the ability of a tolfenamic acid treatment of 5 and 50 mg/kg/d to attenuate these learning and memory deficits in groups of female hemizygous R1.40 transgenic mice aging between 14 and 21 months. In the MWM task, there was a significant effect of training as ANOVA showed that the difference in escape latency between the first and the last daily training sessions was statistically significant ($F(7,144) = 7.833$, $p < 0.0001$). Escape latency between the treatment and the vehicle groups showed no significant differences in daily learning acquisition (Fig. 4A). However, ANOVA indicated an improvement in long-term memory retention in day 11 probe trials ($F(2,15) = 4.145$, $p = 0.0369$) (Fig. 4B). The Tukey-Kramer HSD post hoc test showed that the mice group

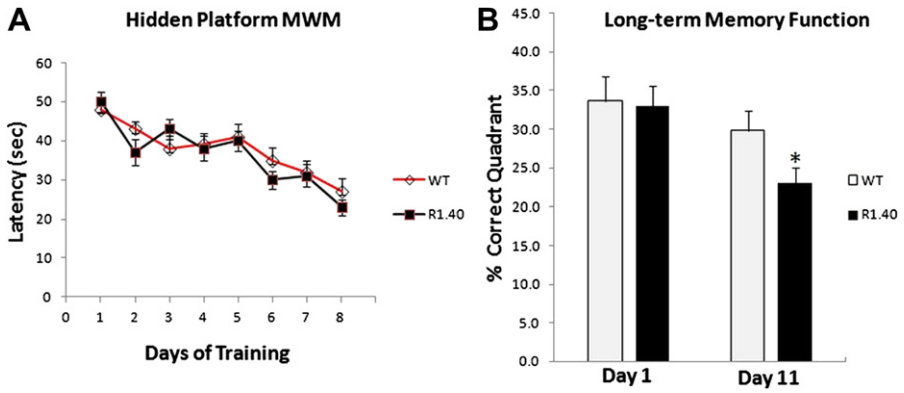

Fig. 2. Memory impairments in hemizygous amyloid precursor protein (APP) yeast artificial chromosome transgenic mice, line R1.40, assessed by the swimming task in the Morris water maze (MWM). (A) Acquisition patterns during daily training. (B) Probe trials for long-term memory retention on Day 1 and Day 11 after acquisition-training trials. Asterisk indicates that the values are significantly different from control, as determined by a Student *t* test ($p < 0.05$). Abbreviation: WT, wild type.

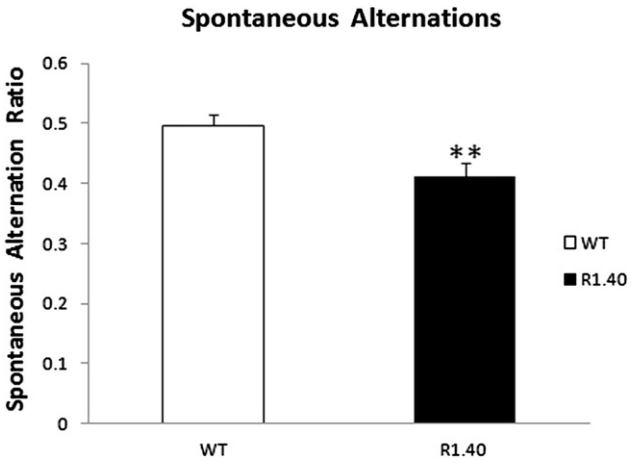

Fig. 3. Working memory impairments in hemizygous APP yeast artificial chromosome transgenic, line R1.40 mice. Mice were examined for working memory function assessed by measuring the spontaneous alternation ratio in the Y-maze. Double asterisks indicate that the values are significantly different from control, as determined by a Student *t* test ($p < 0.01$). Abbreviation: WT, wild type.

treated with 50 mg/kg/d tolfenamic acid exhibited a significant improvement (HSD = 3.12, $p < 0.05$), whereas the treatment with 5 mg/kg/d failed to reach significance (HSD = 0.71 $p > 0.5$) (Fig. 4B). Furthermore, ANOVA of target preference indicated that mice groups treated with 50 mg/kg/d spent significantly more time in the correct quadrant in both probe trials compared with other groups indicating memory improvement (for details, see Supplementary data).

Results from the ANOVA of the spontaneous alternation ratio in the Y-maze showed a significant improvement in working memory ($F(2,15) = 4.479$, $p = 0.0298$) (Fig. 5). The Tukey-Kramer HSD post hoc test revealed a significant improvement with tolfenamic acid treatment of 50 mg/kg/d (HSD = 4.139, $p < 0.05$); however, treatment with 5 mg/kg/d did not reach significance (HSD = 1.30, $p > 0.05$) (Fig. 5).

3.4. Tolfenamic acid treatment lowers SP1 protein without altering Sp1 gene expression

The protein levels of cortical SP1 normalized to β-actin were analyzed by Western blot after treatment with 0, 5, and 50 mg/kg/d tolfenamic acid for 34 days. ANOVA revealed a significant difference in SP1 protein levels ($F(2,11) = 8.747$, $p = 0.0053$) (Fig. 6A). The Tukey-Kramer HSD post hoc test indicated a significant decrease of SP1 after treatment with 5 mg/kg/d (HSD = 4.145, $p < 0.05$, percent change = −43%) and 50 mg/kg/d (HSD = 5.712, $p < 0.01$, percent change = −52%) tolfenamic acid (Fig. 6A).

Sp1 mRNA expression was evaluated for the different treatment groups. Analysis of Sp1 mRNA levels showed that treatment with tolfenamic acid did not alter the gene expression of Sp1 ($F(2,16) = 0.082$, $p = 0.922$) (Fig. 6B).

3.5. Reduction of APP protein and mRNA after treatment with tolfenamic acid

Cortical APP normalized to β-actin was analyzed by Western blot after treatment with 5 and 50 mg/kg/d tolfenamic acid for 34 days or with vehicle. ANOVA revealed that APP levels were lowered significantly ($F(2,9) = 8.377$, $p = 0.009$) (Fig. 7A). The Tukey-Kramer HSD post hoc test indicated a significant decrease in APP after treatment with 5 mg/kg/d (HSD = 4.877, $p < 0.05$, percent change = −30.3%) and 50 mg/kg/d (HSD = 5.139, $p < 0.05$, percent change = −32.5%) tolfenamic acid (Fig. 7A).

In addition to analysis of APP protein levels, APP gene expression was evaluated for the different treatment groups by real-time PCR. ANOVA of APP mRNA levels revealed a significant decrease in APP gene expression ($F(2,15) = 5.238$, $p = 0.019$) (Fig. 7B). The Tukey-Kramer HSD post hoc test indicated a significant decrease of APP mRNA after treatment with 5 mg/kg/d (HSD = 3.885, $p < 0.05$, percent change = −50.3%) and 50 mg/kg/d (HSD = 4.039, $p < 0.05$, percent change = −52.3%) tolfenamic acid (Fig. 7B). The lowering of APP was not associated with adverse effects (Adwan et al., 2011).

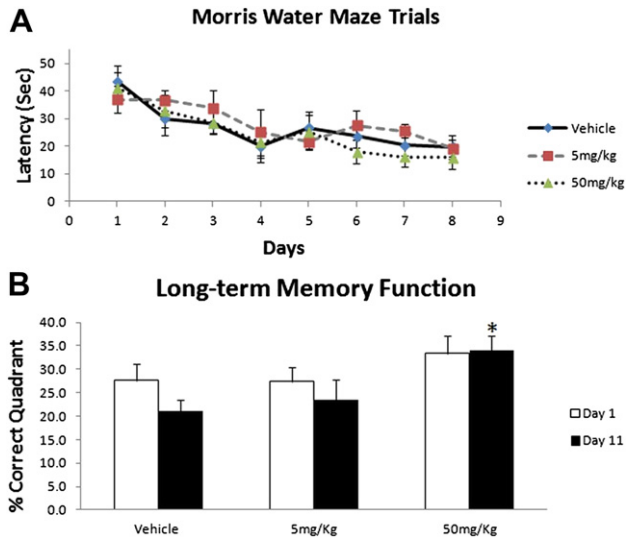

Fig. 4. The administration of tolfenamic acid to hemizygous APP yeast artificial chromosome R1.40 mice improves spatial memory. Tolfenamic acid was administered by oral gavage daily for 34 days. For details, see Section 2. (A) Acquisition patterns during daily training trials of 5 and 50 mg/kg/d and vehicle groups. (B) Probe trials assessing the long-term memory retention on Day 1 and Day 11 after acquisition-training trials. Asterisk indicates that values are significantly different from vehicle, as determined by 1-way analysis of variance with Tukey-Kramer post hoc test to compare all pairs of columns ($p < 0.05$), obtained using GraphPad InStat 3 software. Vehicle $n = 6$, 5 mg/kg/d $n = 6$, and 50 mg/kg/d $n = 7$.

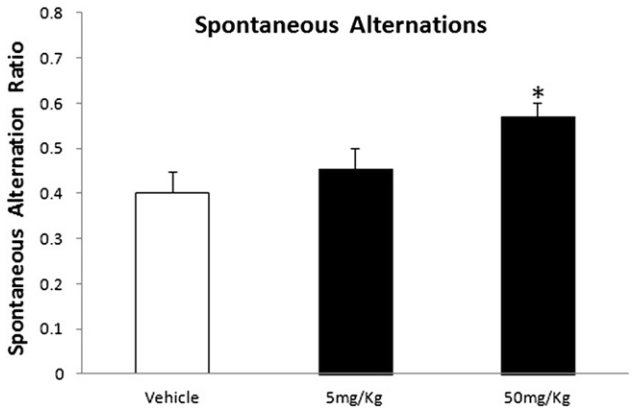

Fig. 5. Working memory improvement in hemizygous APP yeast artificial chromosome transgenic mice, line R1.40, after administration of tolfenamic acid assessed by spontaneous alternations in the Y-maze. Tolfenamic acid was administered by oral gavage daily for 34 days. For details, see Section 2. Asterisk indicates that values are significantly different from vehicle control, as determined by 1-way analysis of variance with Tukey-Kramer post hoc test to compare all pairs of columns ($p < 0.05$), obtained using GraphPad InStat 3 software. Vehicle $n = 6$, 5 mg/kg/d $n = 6$, and 50 mg/kg/d $n = 7$.

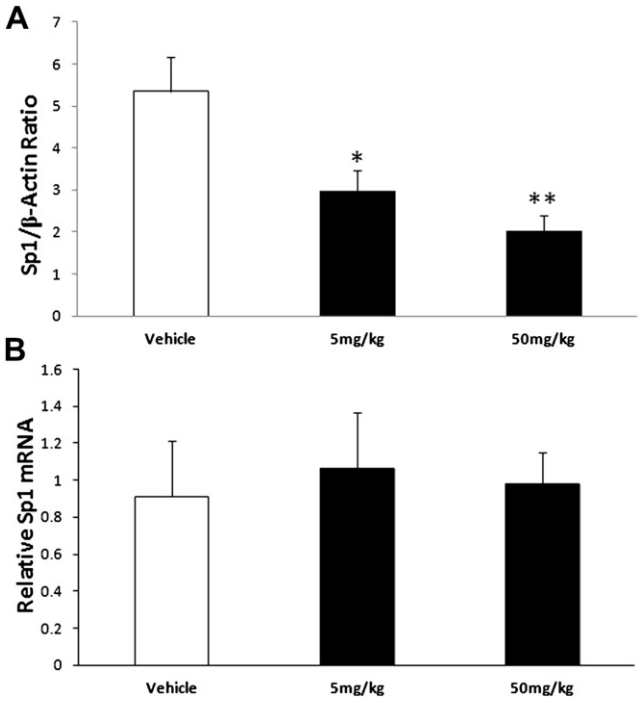

Fig. 6. Reduction of specificity protein (SP1) levels in the brain after treatment with tolfenamic acid. Tolfenamic acid was administered by oral gavage daily for 34 days. For details, see Section 2. (A) Western blot analysis of cortical SP1 levels as a ratio of the housekeeping protein β-actin after treatment with 5 and 50 mg/kg/d of tolfenamic acid for 34 days. (B) Cortical Sp1 messenger RNA (mRNA) levels determined by real-time polymerase chain reaction. Asterisks indicate that values are significantly different from vehicle, as determined by 1-way analysis of variance with Tukey-Kramer post hoc test to compare all pairs of columns (* $p < 0.05$, ** $p < 0.01$), obtained using GraphPad InStat 3 software.

3.6. Tolfenamic acid lowers both soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels

We measured the levels of cortical $A\beta_{1-40}$ and $A\beta_{1-42}$ in the soluble and insoluble fractions of Tris-saline using IBL ELISA kits as described in Section 2 (Morishima-Kawashima et al., 2000). ANOVA showed that there was a significant reduction in the soluble ($F(2,14) = 3.89$, $p = 0.04$) (Fig. 8A) and the insoluble ($F(2,14) = 4.95$, $p = 0.02$) (Fig. 8B) $A\beta_{1-40}$ fractions. The Tukey-Kramer HSD post hoc test indicated a significant decrease in soluble $A\beta_{1-40}$ after treatment with 50 mg/kg/d tolfenamic acid (HSD = 3.91, $p < 0.05$, percent change = −30.1%); however, the reduction in soluble $A\beta_{1-40}$ after treatment with 5 mg/kg/d did not reach significance (HSD = 2.55, $p > 0.05$, percent change = −21.3%) (Fig. 8A). Multiple-group comparison showed a significant decrease in the insoluble $A\beta_{1-40}$ fraction after treatment with both 5 mg/kg/d (HSD = 3.73, $p < 0.05$, percent change = −35.2%) and 50 mg/kg/d (HSD = 3.97, $p < 0.05$, percent change = −37.4%) tolfenamic acid (Fig. 8B).

Also, ANOVA showed a significant decrease in the soluble ($F(2,14) = 6.174$, $p = 0.012$) (Fig. 8C) and the insoluble ($F(2,14) = 6.343$, $p = 0.011$) (Fig. 8D) $A\beta_{1-42}$ fractions. The Tukey-Kramer HSD post hoc test indicated a significant decrease in soluble $A\beta_{1-42}$ after treatment with 5 mg/kg/d (HSD = 4.18, $p < 0.05$, percent change = −22.3%) and 50 mg/kg/d (HSD = 4.518, $p < 0.05$, percent change = −24.9%) tolfenamic acid (Fig. 8C). Similarly, multiple-group comparison showed a significant decrease in the insoluble $A\beta_{1-42}$ fraction after treatment with 5 mg/kg/d (HSD = 4.72, $p < 0.05$, percent change = −31.8%) and 50 mg/kg/d (HSD = 4.79, $p < 0.05$, percent change = −37.7%) tolfenamic acid (Fig. 8D).

We also observed that 5 and 50 mg/kg/d tolfenamic acid treatment reduced total $A\beta_{1-42}/A\beta_{1-40}$ ratio to −16.3% and −19.9%, respectively. However, this reduction in $A\beta_{1-42}/A\beta_{1-40}$ did not reach statistical significance ($F(2,14) = 1.788$, $p = 0.203$).

4. Discussion

Over the last 2 decades, more evidence has been garnered regarding the association between chronic NSAIDs intake and up to 80% reduction in risk of AD incidence, slowing of disease progression, and reduction of microglial activation (Andersen et al., 1995; Cote et al., 2012; Etminan et al., 2003; in t' Veld et al., 2001; Lindsay et al., 2002; McGeer et al., 1996; Stewart et al., 1997; Vlad et al., 2008; Zandi et al., 2002). Several mechanisms have been proposed to explain how NSAIDs could exert their effects on AD independent of their cycolooxygenase (COX) inhibitory pathways. Certain NSAIDs including indomethacin, flufenamic acid, ibuprofen, and fenoprofen are agonists for the nuclear transcriptional regulator peroxisome proliferator-activated receptor γ (PPARγ) (Heneka et al., 2011; Lehmann et al., 1997) that has been reported to be reduced in AD brains (Sastre et al., 2006). PPARγ activation results in a reduction in the levels of Aβ-induced proinflammatory response of microglia and monocytes and promotes Aβ clearance by astrocytes and microglia (Combs et al., 2000; Daynes and Jones, 2002; Mandrekar-Colucci et al., 2012; Matsuo et al., 1996; Yue and Mazzone, 2009). In addition, a PPARγ responsive element has been identified in the BACE1 gene promoter that is repressed by PPARγ binding resulting in a decrease in BACE1 expression (Sastre

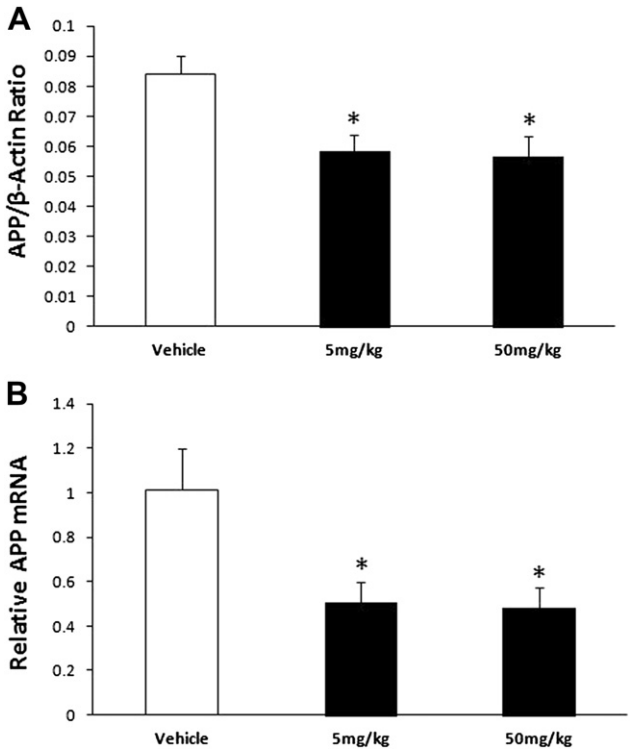

Fig. 7. Reduction of amyloid precursor protein (APP) levels in the brain after treatment with tolfenamic acid. Tolfenamic acid was administered by oral gavage daily for 34 days. For details, see Section 2. (A) Western blot analysis of cortical APP levels as a ratio of the housekeeping protein β-actin after treatment with 5 and 50 mg/kg/d of tolfenamic acid for 34 days. (B) Cortical APP messenger RNA (mRNA) determined by real-time polymerase chain reaction. Asterisk indicates that values are significantly different from vehicle, as determined by 1-way analysis of variance with Tukey-Kramer post hoc test to compare all pairs of columns ($p < 0.05$), obtained using GraphPad InStat 3 software.

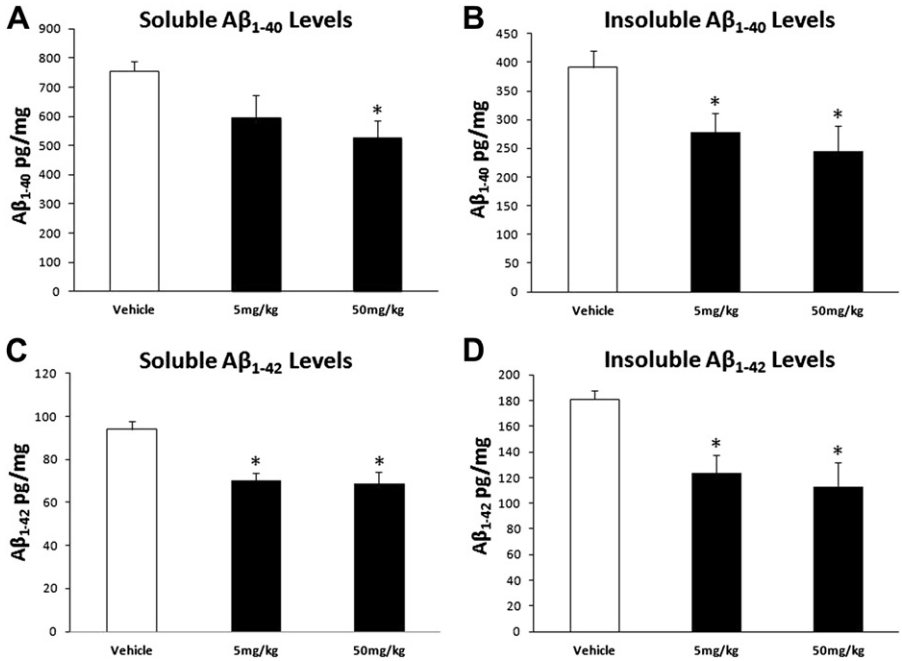

Fig. 8. Reduction of soluble and insoluble amyloid beta $(A\beta)_{1-40}$ and $A\beta_{1-42}$ levels in the brain after treatment with tolfenamic acid. Tolfenamic acid was administered by oral gavage daily for 34 days. For details, see Section 2. Enzyme-linked immunosorbent assay quantified Aβ levels in the cortices of mice treated with 5 and 50 mg/kg/d of tolfenamic acid for 34 days. (A) Soluble $A\beta_{1-40}$, (B) insoluble $A\beta_{1-40}$, (C) soluble $A\beta_{1-42}$, and (D) insoluble $A\beta_{1-42}$ levels. Asterisk indicates that values are significantly different from vehicle, as determined 1-way analysis of variance with Tukey-Kramer post hoc test to compare all pairs of columns ($p < 0.05$), obtained using GraphPad InStat 3 software.

et al., 2003, 2006). Weggen et al. (2001) reported that a subset of NSAIDs specifically lower $A\beta_{1-42}$ that was accompanied by a parallel increase in $A\beta_{1-38}$ suggesting a minor γ-secretase modulatory effect without alteration of the Notch pathway. The specific $A\beta_{1-42}$ inhibition by that subset of NSAIDs was further studied by Zhou et al. (2003), who concluded that the involvement of Rho-Rock pathway in regulating APP processing and inhibition of Rho activity by specific NSAIDs could preferentially reduce $A\beta_{1-42}$.

Tolfenamic acid, of all other NSAIDs, has the unique mechanism of inducing the proteasome-dependent degradation of SP transcription factors including SP1, SP3, and SP4 and thereby decreasing the expression of vascular endothelial growth factor (Abdelrahim et al., 2006), which is involved in tumor progression and metastases. Because of its SP degradation effects, tolfenamic acid has been studied as a therapeutic agent for cancer and research has confirmed its antitumor effects (Abdelrahim et al., 2006; Basha et al., 2011; Colon et al., 2011; Konduri et al., 2009).

Sp1 involvement in AD has been researched, and it was found that its expression was elevated in the frontal cortex of AD brains and in the brains of transgenic mouse models of AD (Citron et al., 2008; Santpere et al., 2006). The elevation of Sp1 was accompanied by an increase in COX-2, another Sp1 target gene, suggesting interplay between inflammatory processes and Sp1–driven gene expression (Citron et al., 2008). Sp1 binds to 5′-GGGGCGGGGC–rich promoter genes such as APP and BACE1 genes, which are involved in AD pathology, and Sp1 overexpression leads to induction of their protein expression (Cai et al., 2001; Christensen et al., 2004; Citron et al., 2008). We have reported that silencing of Sp1 gene by small interfering RNA resulted in 70% decrease in the responsiveness of the human APP promoter (Basha et al., 2005). Also, we have demonstrated the colocalization of APP, Sp1, and Aβ in various regions of the brain (Brock et al., 2008). Thus, we proposed the hypothesis that tolfenamic acid could interrupt the de novo synthesis of APP and, consequently, alter the downstream levels of Aβ. This hypothesis was confirmed when oral treatment with tolfenamic acid resulted in degradation of SP1 that was accompanied by a reduction in cortical and hippocampal APP gene expression and protein and Aβ peptide levels (Adwan et al., 2011). This was further supported by measurements that demonstrated the presence of tolfenamic acid in the brain after intravenous administration (Subaiea et al., 2011).

In the present study, we demonstrate that hemizygous, R1.40 transgenic mice exhibit cognitive deficits on assessment of their working memory and long-term memory retention even before the reported age of 24–26 months at which hemizygous R1.40 start to suffer extensive Aβ deposition (Lamb et al., 1999). Hemizygous R1.40 mice start to exhibit 7–8 times more $A\beta_{1-42}$ levels compared with wild types as early as 3–4 months of age (Lamb et al., 1999). Thus, mice were studied at ages where there is a great increase in Aβ levels in the brain before plaque depositions. Spatial learning was assessed in the hidden version of the MWM, and we found that the transgenic mice were able to learn to escape to the hidden platform in the daily training sessions (Fig. 2A). However, they exhibited memory retention impairments in the probe trials as they failed to retain the spatial location of the hidden platform with increased delay (Fig. 2B, day 11). Working memory assessed in the Y-maze was impaired in these mice as demonstrated by numerous alternation errors and a lower alternation ratio compared with the wild-type control group (Fig. 3). It has been proposed that recent spatial memory is hippocampal dependent, whereas remote spatial memory is dependent on the integrity of the medial prefrontal cortex (Frankland and Bontempi, 2005; King et al., 1999; Teixeira et al., 2006). In turn, working memory assessed in the Y-maze is hippocampal and cortical dependent (Lalonde, 2002). This, together with the findings that the R1.40 mouse model exhibits extensive amyloid deposition in the cortical region compared with that observed in the hippocampal formation (Hock et al., 2009), may explain the observed results.

We report here that short-term tolfenamic acid administration was able to improve the mnemonic deficits observed in hemizygous

R1.40. We found that 50 mg/kg/d tolfenamic acid was able to reverse the long-term memory retention deficits seen in delayed probe trials of the MWM experiment (Fig. 4B). In addition, the 50 mg/kg/d treatment group showed significant improvement in the Y-maze with less error in spontaneous alternations (Fig. 5). However, the short-term treatment with 5 mg/kg/d group showed only modest effects in reversing the memory deficits in these mice. Analysis of amyloid pathology markers indicated that both treatment regimens, 5 and 50 mg/kg/d, were able to significantly lower the protein level of SP1 without altering its gene expression indicating that the decrease in SP1 protein levels is not because of a change in its de novo synthesis but is rather at the post-translational level (Fig. 6A and B). Those results were similar to our previous findings in C57BL/6 treated with tolfenamic acid (Adwan et al., 2011). In addition, both tolfenamic acid doses significantly reduced the protein levels of APP and its gene expression (Fig. 7A and B). Furthermore, levels of the soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were reduced by tolfenamic acid with a trend toward lowering the insoluble fraction of Aβ more than that of the soluble fraction (Fig. 8). It is possible that tolfenamic acid may aid in clearance of insoluble Aβ by a PPARγ activation mechanism; yet, it is not clear if tolfenamic acid activates PPARγ as certain other NSAIDs do.

Although tolfenamic acid was effective in lowering the amyloidogenic proteins with both doses, we observed dose-dependent improvement in the cognitive deficits. Whether longer period of treatment with low-dose tolfenamic acid could result in significant improvements in cognitive function is yet to be studied. However, it is essential to mention that tolfenamic acid produced behavioral improvements after a rather short-term treatment period compared with other FDA-approved AD drugs and NSAIDs. For example, APP23 transgenic mice treated with 0.58 mg/kg/d donepezil, an FDA-approved anticholinesterase drug for AD, showed improved performance in MWM after 2 months of treatment (Van Dam et al., 2008). In addition, memantine, another FDA-approved drug for use in moderate to severe AD, has shown to improve cognition in 3xTG-AD mice after 3 months of treatment (Martinez-Coria et al., 2010). Also, triple transgenic mice (APPswe, PS1M146V, and tauP301L) exhibited improved cognitive functions after treatment with ibuprofen for 6 months (McKee et al., 2008).

Many of the anti-amyloid drugs that failed in clinical trials were anti-Aβ monoclonal antibodies, γ-secretase inhibitors, BACE1 inhibitors, or certain NSAIDs that do not lower SP1 (Herrmann et al., 2011; Ozudogru and Lippa, 2012). This might be because of either lack of efficacy or ensuing adverse effects. The main targets of these drugs were proteins involved at the end stage of the amyloidogenic pathway. Tolfenamic acid interferes with transcriptional pathways associated with AD-related genes; thus, it acts at upstream pathways that can have impacts on downstream events and is not dependent on protein events at the end of the amyloidogenic pathways. Thus, it may hold better promise for success.

In conclusion, tolfenamic acid is unique among other NSAIDs in its ability to inhibit SP1 and thus interrupt APP de novo synthesis and its Aβ products. In this study, we demonstrated that administration of 50 mg/kg/d tolfenamic acid for 34 days reversed the cognitive deficits in hemizygous, R1.40 transgenic mice. Consistent with our previous published findings in C57BL/6 mice, SP1, the amyloidogenic levels of APP and soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were markedly lowered by tolfenamic acid in AD transgenic mice. These data provide further evidence that tolfenamic acid is a promising therapeutic agent as a repurposed AD drug acting through an alternative mechanism and should benefit the scheduled clinical trials of this drug.

Disclosure statement

The authors hereby declare that they have no disclosures or conflicts of interest.

The University of Rhode Island Institutional Animal Care and Use Committee has approved all animal studies conducted in this research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2013.04.002.

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